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REVIEW ARTICLE

Lipophilic Character and Biological Activity of Drugs II: The Parabolic Case

CORWIN HANSCH▲ and JOHN M. CLAYTON

Keyphrases ☐ Lipophilicity–biological activity nonlinear relationships—review ☐ Partition coefficients—determination of nonlinear relationships between lipophilicity and biological activity ☐ Hydrophobic binding—effect on drug activity ☐ Structure–activity relationships—lipophilicity and biological activity, review

In the first part of this review (1), examples were considered in which there was a linear relationship between the response to drug action and the relative lipophilic character of the drug. Relative drug response was defined in terms of log $1/C$ (pC), where C is the molar concentration of drug producing a standard response; relative lipophilic character was defined by log P, where P is the octanol–water partition coefficient. The present review extends this survey to the more general problem of the nonlinear relationship between pC and log P.

The term "drug" is difficult to define. In these discussions it is employed in the widest sense possible. A drug is considered to be any chemical capable of causing a biochemical or biological response. A better term might be pharmacon, which has been employed by Ariëns (2).

Ever since Meyer (3) and Overton (4) discovered that the narcotic potency of the members of a set of congeners tends to increase as their oil–water partition coefficients increase, there has been interest in defining "lipophilic character" and its role in the activity of drugs. The analyses in this review are all based on the operational definition of lipophilic character by log P from the octanol–water system. There is, of course, great advantage in using a single reference system. The reasons behind the choice of octanol–water were discussed previously (1, 5). It is possible to compare work in other solvent systems with that obtained in octanol–water *via* Eq. 1:

$$\log P_1 = a \log P_2 + b \qquad \text{(Eq. 1)}$$

In Eq. 1, first formulated by Collander and recently (6) applied to a variety of systems, P_1 represents the partition coefficient of a solute between one solvent and water, and P_2 is that for the solute between a second solvent and water. Equation 1 holds well when P_1 and P_2 are from similar apolar solvents such as alcohols, esters, and ethers. It fails completely when comparisons are between hydrocarbons (such as heptane or benzene) and solvents with hydrogen-bonding ability such as alcohols, esters, and ethers.

Much of the early work seeking correlations with partition coefficients was concerned with nonspecific narcotic effects. Recently, it has become clear that by using log P to define hydrophobic character operationally, one can correlate the binding of organic compounds (drugs) to proteins (7–20), enzymes (7, 21–31), and membranes (32). Equations 2–4 are typical examples:

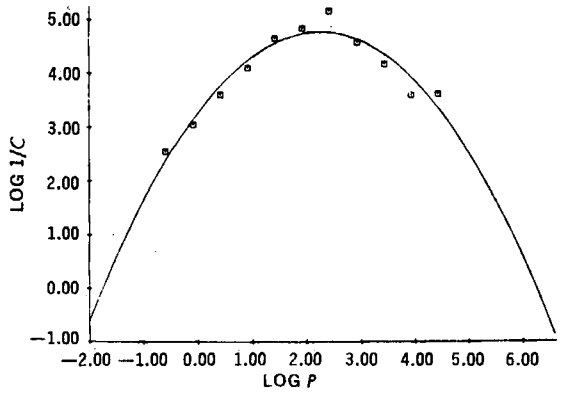


Figure 1—*Bactericidal activity of benzyldimethylalkylammonium chlorides against* Candida albicans [*log 1/C = −0.30 (log P)² + 1.34 log P + 3.25*].

binding of organic compounds by serum albumin (10)

	n	*r*	*s*	
$pC = 0.75 \log P + 2.30$	42	0.960	0.159	(Eq. 2)

hemolysis of red cells by alcohols and esters (32)

	n	*r*	*s*	
$pC = 0.90 \log P - 0.24$	19	0.993	0.096	(Eq. 3)

binding of barbiturates by liver homogenate (5)

	n	*r*	*s*	
$\log (B/F) = 0.52 \log P - 1.14$	5	0.973	0.124	(Eq. 4)

In Eqs. 2 and 3, C is the molar concentration of drug that produces a 1:1 complex *via* equilibrium dialysis. In Eq. 4, B is the percent of barbiturates bound and F is the percent free. For the equations throughout this report, n represents the number of data points used in deriving the equation, r is the correlation coefficient, and s is the standard deviation. Equations 2–4 and hundreds of others like them (1, 5, 33) establish the fact that drugs are bound in varying degrees by a large percentage of the macromolecules they encounter in living tissue. Moreover, this is a partitioning-like process, which is well modeled by the way the drugs partition between octanol and water. This partitioning has a profound effect on the random walk process drugs follow in finding their sites of action.

Under equilibrium conditions as in Eqs. 2–4, one expects and finds linear relations between pC and log P. The higher the value of log P, the tighter is the binding. As log P values become large or the time of the experiment becomes short, linearity is not the rule and one finds a much better correlation by a second-order

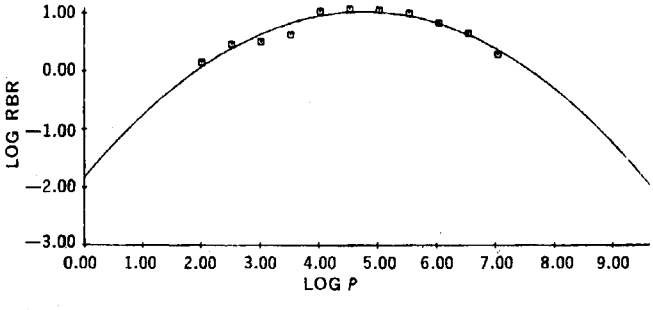


Figure 2—*Mycelia inhibition of 5-alkyl-8-hydroxyquinolines against* Aspergillus niger [*log RBR = −0.13 (log P)² + 1.20 log P − 1.84*].

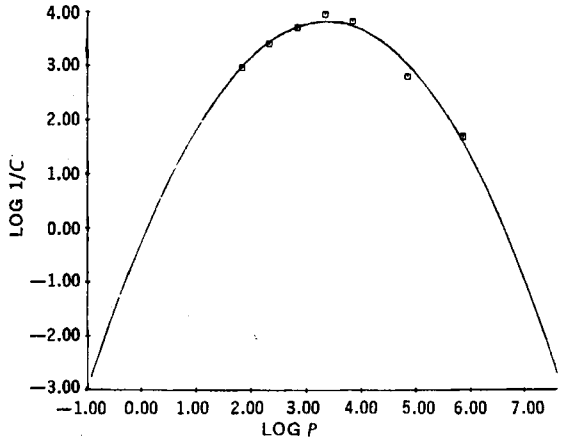


Figure 3—*Hemolytic activity of α-monoglycerides against dove red blood cells* [*log 1/C = −0.36 (log P)² + 2.43 log P − 0.27*].

equation as in Eq. 5:

concentration of X—$C_6H_4B(OH)_2$ localized in mouse brain in 15 min. (34)

	n	*r*	*s*	
$\log C = -0.54(\log P)^2 + 2.47 \log P - 1.05$	14	0.915	0.214	(Eq. 5)

This equation, based on the work of Soloway *et al.* (35), correlates the localization of benzeneboronic acids (injected interperitoneally) in mouse brain. In this time-dependent process, the parabola of Eq. 5 correlates the data much better than linear relations such as Eqs. 2–4. Equations 2–4, from *in vitro* studies, actually lead one to expect nonlinear relationships such as Eq. 5 from living systems. Since one finds tighter and tighter binding between organic compounds and macromolecules as the log P values of the former are increased, it is clear that eventually a point is reached where this restriction of movement is rate controlling. The length of time allowed for attainment of equilibrium is, of course, important in setting the degree of linearity found in any given case.

In early structure–activity studies, a departure from linearity in response and lipophilic character was often observed and was termed the "cutoff" point. Ferguson (36–38) was one of the first to assume that there might be a general "rational" explanation for this phenomenon. He attempted to explain it by arguing that the higher members of a homologous series would become so insoluble that concentrations high enough to cause a standard response could not be obtained. While this might explain certain special situations, in the light of Eqs. 2–4 it is hard to know what one is talking about in terms of solubility when the drug is injected into an animal or added to a complex medium of bacteria and nutrient. Depending on the lipophilic character of the drug, it will be more or less bound to all kinds of macromolecules present.

It is probably best to abandon any traditional ideas of solubility of drugs in an aqueous phase when considering the kind of data presented in this report. One is faced with a highly complex set of equilibria of drugs in an aqueous phase and drugs bound with varying degrees of firmness by a large variety of macromolecules which make up living cells and tissue. For this

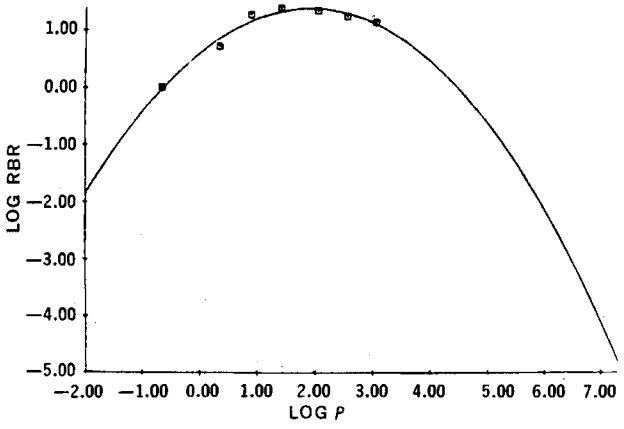


Figure 4—*Survival time of* Calliphora erythrocephala *blowfly larva in aliphatic alcohols [log RBR = −0.21 (log P)² + 0.80 log P + 0.59].*

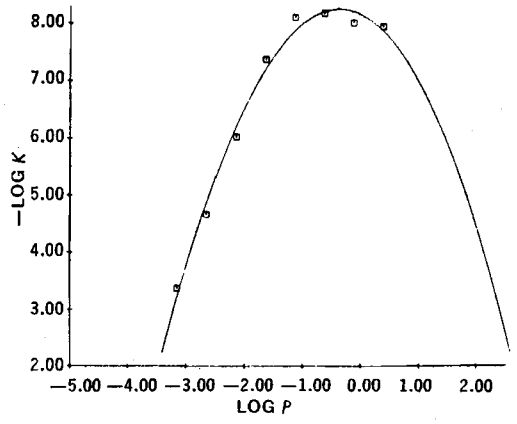


Figure 5—*Inhibitory activity of aliphatic amines against human liver mitochondrial MAO [log K_i' = −0.67 (log P)² − 0.53 log P + 8.14].*

reason, the authors have stressed the advantage of considering in probabilistic terms (34, 39, 40) the movement of drug from the point of introduction to the active sites.

For some time this laboratory has been collecting examples of what can be loosely termed "parabolic" relationships between log $1/C$ and log P. A large amount of evidence is now in hand which clearly shows that the "break" in the linear relation between log $1/C$ and log P is not precipitous (see examples in Figs. 1–6) and that the term "cutoff" is not well suited to describe the phenomenon. In Figs. 1–6, the solid line is the least-squares parabola drawn through the experimental points. These six examples are representative of the cases in Table I. In the present survey, about 230 examples were plotted (by computer). From a study of these plots it was not possible to visualize any kind of curve that would fit the data better than a parabolic expression such as Eq. 6:

$$pC(k) = -a(\log P)^2 + b \log P + \text{constant} \qquad \text{(Eq. 6)}$$

where $pC = \log 1/C$, and C is the molar concentration of drug producing a standard response in constant time. Other rate or equilibrium constants (k) may also be used. Not all of these examples have been included in the present data base, partly for reasons of space but also because it seemed important to select the best examples for study.

For Table I, sets were selected having five or more data points and where the F test (41) indicated that the addition of the $(\log P)^2$ term to the linear equation in log P is significant at the 0.99 level of significance or higher. With a few exceptions, the equations of Table I have correlation coefficients of 0.95 or higher. Some sets meeting these standards were rejected because, from an inspection of the plotted curve, it could be seen that the log P values of the most active compounds (also most hydrophobic compounds) were considerably below log P_0 (the apex of the parabola). In these examples the confidence intervals on log P_0 were very wide or could not be established (40). Ideally, one would want to include only examples where the data points covered the complete parabola from zero activity with a low partition coefficient to zero activity with a high partition coefficient. Such data are rare indeed and sorely needed. For practical reasons it is not very interesting to test the higher members of a homologous series once activity begins to decline. For this reason, many investigators stop studying the more lipophilic homologs once activity is found to drop. Moreover, the very lipophilic members of a series are often extremely difficult to study because of their limited aqueous solubility. Some of the best data this study has uncovered were obtained with carboxylate anions and quaternary salts where the difficulties of solubility can be circumvented.

For the present review, only those data sets were selected where the single variable log P in Eq. 6 gave a high correlation of the data. While about 230 of these sets are now in hand, several hundred others, where an additional term such as one in σ or E_s is necessary for high correlation, support the general importance of the parabolic relationship between the logarithm of a biological rate or equilibrium constants and log P. The data for the results in Table I are contained in Table III. In Table II a set of equations is given for which the limitations are not as severe. In these examples, the $(\log P)^2$ term is significant at the 0.95 level. To conserve space, the experimental data are not included for these examples. However, most of the log P values are in Table III and the pC values can be found in the cited references.

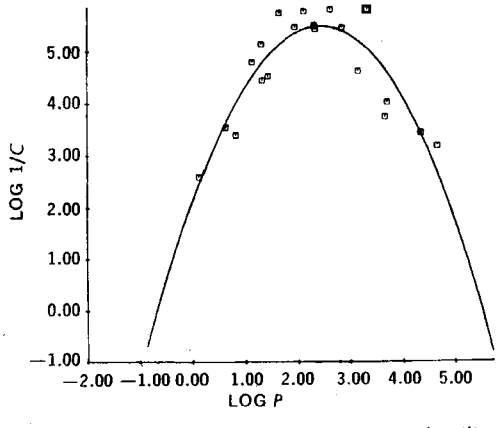


Figure 6—*Inhibitory activity of aminopyridines and anilines against* Mycobacterium tuberculosis *[log 1/C = −0.57 (log P)² + 2.73 log P + 2.22].*

Table I—$pC = a(\log P)^2 + b \log P + c$

Equation Number	b	a	c	$\log P_0$	Confidence Interval	n	r	s	Compound	Type Biological Activity[a]	References 1	References 2
					Part A							
7	−0.53 ± 0.50	−0.67 ± 0.17	8.14 ± 0.32	−0.40	(−0.63, −0.03)	8	0.995	0.213	$R\overset{+}{N}H_3$	MAO, K_i'	III-1	42
8	−0.51 ± 0.71	−0.68 ± 0.24	6.33 ± 0.57	−0.38	(−0.69, 0.22)	6	0.995	0.235	$R\overset{+}{N}H_3$	MAO, K_i	III-2	42
9	0.07 ± 0.15	−0.16 ± 0.07	4.48 ± 0.45	0.23	(−0.28, 0.72)	11	0.883	0.437	$RCOO^-$	*M. audouinii*, MIC	III-5	43
10	0.21 ± 0.10	−0.41 ± 0.08	3.75 ± 0.21	0.26	(0.13, 0.43)	5	0.999	0.069	$RCHOHCOO^-$	*V. cholerae*, pH 6.5, MKC	III-7	44
11	0.28 ± 0.24	−0.10 ± 0.06	2.77 ± 0.27	1.44	(0.14, 7.19)	11	0.982	0.239	Alkylguanidines	Rat liver mitochondria, I_{50}	III-12	45
12	1.11 ± 0.58	−0.77 ± 0.61	4.48 ± 0.46	0.72	(0.21, 4.70)	6	0.993	0.233	$RCONH_2$	ADH, binding ($K_{ER\cdot I}$)	III-13	46
13	1.19 ± 0.34	−0.40 ± 0.14	4.43 ± 0.17	1.49	(1.32, 1.78)	6	0.990	0.097	Isonicotinic acid hydrazides	MAO, I_{50}	III-14	47
14	1.23 ± 0.17	−0.56 ± 0.08	3.96 ± 0.20	1.10	(1.01, 1.22)	5	0.999	0.073	$RCHOHCOO^-$	*S. aureus*, pH 6.5, MKC	III-8	44
					Part B							
15	0.31 ± 0.10	−0.09 ± 0.04	4.32 ± 0.21	1.82	(1.32, 3.00)	7	0.974	0.141	$RCOO^-$	*M. tuberculosis*, MIC	III-6	48
16	0.44 ± 0.17	−0.11 ± 0.05	3.03 ± 0.34	2.03	(1.49, 3.06)	7	0.963	0.245	$RCHOHCOO^-$	*B. lepisepticus*, pH 7.5, MKC	III-9	44
17	0.44 ± 0.16	−0.11 ± 0.03	4.89 ± 0.16	1.93	(1.68, 2.10)	6	0.994	0.030	Alkylguanidine acetates	*M. fructicola*, ED_{50}	III-15	49
18	0.51 ± 0.17	−0.13 ± 0.07	6.33 ± 0.47	1.94	(1.10, 4.26)	10	0.953	0.430	$(CH_3)_3\overset{+}{N}(CH_2)_n\overset{+}{N}(CH_3)_3$	Rabbit, head drop	III-17	50
19	0.55 ± 0.30	−0.13 ± 0.07	2.85 ± 0.26	2.13	(1.70, 2.70)	9	0.880	0.208	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*P. aeruginosa*, MIC	III-19	51
20	0.57 ± 0.18	−0.12 ± 0.05	4.08 ± 0.17	2.38	(1.99, 3.09)	10	0.949	0.183	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*S. typhosa*, MIC	III-20	51
21	0.58 ± 0.09	−0.16 ± 0.06	3.06 ± 0.21	1.85	(1.38, 2.80)	6	0.997	0.108	$RCHOHCOO^-$	*B. lepisepticus*, pH 8.5, MKC	III-10	44
22	0.60 ± 0.22	−0.18 ± 0.09	1.89 ± 0.13	1.69	(1.47, 2.21)	13	0.918	0.079	*N,N'*-Diacylureas	Mouse, MHD	40	52
23	0.61 ± 0.25	−0.17 ± 0.08	2.61 ± 0.38	1.76	(1.36, 2.40)	7	0.958	0.264	$RCHBrCOO^-$	*V. cholerae*, pH 7.5, MKC	III-30	53
24	0.61 ± 0.23	−0.19 ± 0.08	1.78 ± 0.15	1.64	(1.42, 2.00)	5	0.993	0.043	Acetals	Rat, MHD	III-41	54
25	0.64 ± 0.07	−0.16 ± 0.03	3.07 ± 0.12	2.02	(1.82, 2.26)	8	0.995	0.097	$C_5H_5\overset{+}{N}R$	*V. inaequales*, KS	55	56
26	0.65 ± 0.11	−0.13 ± 0.04	2.55 ± 0.19	2.44	(2.03, 3.15)	8	0.989	0.156	$C_5H_5\overset{+}{N}R$	*P. infestans*, KS	55	56
27	0.72 ± 0.24	−0.17 ± 0.09	2.58 ± 0.44	2.16	(1.61, 3.63)	7	0.973	0.295	$C_5H_5\overset{+}{N}R$	*A. oleracea*, KS	55	56
28	0.75 ± 0.18	−0.15 ± 0.08	2.08 ± 0.11	2.46	(1.90, 4.26)	10	0.983	0.110	Misc.	Chick embryo, MED Inh hatching	57, 58	59
29	0.77 ± 0.42	−0.19 ± 0.11	2.17 ± 0.39	1.97	(1.54, 2.54)	6	0.959	0.206	$RCHBrCOO^-$	*V. cholerae*, pH 8.5, MKC	III-31	53
30	0.78 ± 0.16	−0.18 ± 0.04	2.93 ± 0.15	2.21	(2.04, 2.43)	10	0.974	0.123	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*P. vulgaris*, MIC	III-21	51
31	0.78 ± 0.17	−0.16 ± 0.05	2.38 ± 0.16	2.50	(2.19, 3.00)	7	0.990	0.115	$C_5H_5\overset{+}{N}R$	*M. fructicola*, KS	55	56
32	0.80 ± 0.21	−0.21 ± 0.08	0.59 ± 0.16	1.90	(1.63, 2.40)	7	0.985	0.102	ROH	Blowfly, survival time (RBR)	III-42	60
33	0.80 ± 0.35	−0.20 ± 0.07	4.81 ± 0.35	1.99	(1.67, 2.18)	6	0.990	0.065	Alkylguanidine acetates	*S. pastorianus*, ED_{50}	III-16	49
34	0.82 ± 0.14	−0.15 ± 0.03	2.89 ± 0.15	2.71	(2.52, 2.95)	46	0.878	0.306	$XC_6H_4CH_2\overset{+}{N}(R)(CH_3)_2$	*S. typhosa*, MKC	III-45	61
35	0.84 ± 0.47	−0.23 ± 0.12	2.42 ± 0.43	1.78	(1.48, 1.99)	12	0.841	0.100	Barbiturates	Rabbit, MLD	III-48	62
36	0.84 ± 0.23	−0.21 ± 0.06	2.94 ± 0.20	1.97	(1.77, 2.17)	10	0.960	0.172	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*P. vulgaris*, MKC	III-22	51
37	0.85 ± 0.11	−0.22 ± 0.04	4.69 ± 0.17	1.93	(1.71, 2.22)	7	0.995	0.119	$(CH_3)_3\overset{+}{N}(CH_2)_n\overset{+}{N}(CH_3)_3$	ChE Rabbit, I_{50}	III-18	50
38	0.86 ± 0.12	−0.14 ± 0.02	4.48 ± 0.18	3.00	(2.80, 3.22)	6	0.997	0.100	$R_3SnOCOCH_3$	*A. niger*, MIC	III-53	63
39	0.87 ± 0.14	−0.17 ± 0.03	2.93 ± 0.16	2.62	(2.45, 2.82)	45	0.884	0.306	$XC_6H_4CH_2\overset{+}{N}(R)(CH_3)_2$	*S. aureus*, MKC	III-46	61

Table I—(*Continued*)

Equation Number	*b*	*a*	*c*	log P_0	Confidence Interval	*n*	*r*	*s*	Compound	Type Biological Activity[a]	References 1	References 2
40	0.89 ± 0.24	−0.17 ± 0.10	2.84 ± 0.53	2.62	(1.91, 4.97)	7	0.986	0.305	$R\overset{+}{N}(CH_3)_3$	*E. typhosa*, MKC	III-55	64
41	0.89 ± 0.25	−0.19 ± 0.08	2.60 ± 0.24	2.40	(1.96, 3.43)	8	0.978	0.214	$RCOO^-$	Red cell dove, MHC	32	65
42	0.90 ± 0.14	−0.21 ± 0.04	4.80 ± 0.14	2.18	(2.02, 2.38)	12	0.979	0.158	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*S. aureus*, MIC	III-23	51
43	0.91 ± 0.21	−0.17 ± 0.06	3.87 ± 0.21	2.68	(2.34, 3.28)	12	0.966	0.230	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*Cl. welchii*, MIC	III-24	51
44	0.91 ± 0.47	−0.18 ± 0.09	4.67 ± 0.66	2.61	(1.98, 3.31)	5	0.988	0.271	$R_3SnOCOCH_3$	*R. nigricans*, MIC	III-54	63
45	0.93 ± 0.20	−0.23 ± 0.05	2.76 ± 0.20	1.99	(1.81, 2.21)	12	0.962	0.220	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*P. aeruginosa*, MKC	III-25	51
46	0.95 ± 0.45	−0.25 ± 0.11	3.11 ± 0.42	1.93	(1.57, 2.34)	6	0.971	0.219	$RCHBrCOO^-$	*V. cholerae*, pH 6.0, MKC	III-32	53
47	0.98 ± 0.35	−0.27 ± 0.09	3.03 ± 0.33	1.79	(1.53, 2.04)	6	0.985	0.171	$RCHBrCOO^-$	*B. lepisepticus*, pH 6.0, MKC	III-33	53
48	0.99 ± 0.17	−0.19 ± 0.05	3.80 ± 0.17	2.65	(2.39, 3.05)	12	0.980	0.190	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*Cl. welchii*, MKC	III-26	51
49	1.00 ± 0.57	−0.26 ± 0.14	2.54 ± 0.53	1.89	(1.43, 2.39)	6	0.959	0.277	$RCHBrCOO^-$	*B. lepisepticus*, pH 7.5, MKC	III-34	53
50	1.02 ± 0.16	−0.29 ± 0.06	−0.44 ± 0.14	1.78	(1.60, 2.02)	8	0.992	0.105	4-R-Lincomycin	*S. lutea*, curve assay (RBR)	III-57	66
51	1.04 ± 0.29	−0.24 ± 0.07	3.11 ± 0.26	2.19	(1.98, 2.45)	11	0.947	0.224	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*A. niger*, MIC	55	51
52	1.12 ± 0.44	−0.29 ± 0.18	1.85 ± 0.23	1.91	(1.57, 3.23)	25	0.864	0.208	Ethers	Mouse, LD_{50}	III-58	67
53	1.21 ± 0.70	−0.21 ± 0.12	3.50 ± 0.91	2.91	(2.26, 3.64)	8	0.898	0.593	$RCHBrCOO^-$	*D. pneumoniae*, pH 6.5, MKC	III-35	53
54	1.23 ± 0.74	−0.22 ± 0.12	3.12 ± 0.95	2.82	(2.14, 3.50)	8	0.898	0.620	$RCHBrCOO^-$	*S. haemolyticus*, pH 6.0, MKC	III-36	53
55	1.26 ± 0.35	−0.24 ± 0.11	3.31 ± 0.37	2.59	(2.13, 3.68)	6	0.991	0.213	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	Red cell sheep, $C_{H_{50}}$	III-27	68
56	1.28 ± 0.45	−0.37 ± 0.15	0.70 ± 0.30	1.72	(1.56, 1.97)	10	0.936	0.124	ROH	Rabbit, excretion (RBR)	III-43	69
57	1.33 ± 0.42	−0.25 ± 0.08	3.97 ± 0.48	2.71	(2.49, 2.97)	5	0.995	0.089	$R\overset{+}{N}H_3$	*S. viridans*, MIC	III-3	70
58	1.34 ± 0.31	−0.30 ± 0.08	3.25 ± 0.28	2.25	(2.06, 2.48)	11	0.961	0.243	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*C. albicans*, MKC	III-28	51
59	1.36 ± 0.26	−0.26 ± 0.06	3.24 ± 0.23	2.57	(2.37, 2.84)	11	0.978	0.199	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*C. albicans*, MIC	55	51
60	1.37 ± 0.49	−0.35 ± 0.15	2.32 ± 0.38	1.96	(1.78, 2.30)	10	0.951	0.071	Barbiturates	Rabbit, MHD	III-49	62
61	1.50 ± 0.63	−0.26 ± 0.12	2.50 ± 0.72	2.86	(2.55, 3.31)	5	0.991	0.133	$R\overset{+}{N}H_3$	*S. aureus*, MIC	III-4	70
62	1.55 ± 0.42	−0.42 ± 0.17	1.92 ± 0.24	1.84	(1.64, 2.27)	12	0.973	0.096	Barbiturates	Mouse, AD_{50}	III-50	71, 72
63	1.57 ± 0.55	−0.45 ± 0.16	2.25 ± 0.44	1.73	(1.61, 1.88)	14	0.884	0.115	Barbiturates	Rat, MHD	III-51	73
64	1.79 ± 0.42	−0.33 ± 0.09	0.90 ± 0.44	2.69	(2.57, 2.89)	7	0.994	0.034	Thiobarbiturates	Mouse, AD_{50}	40	71
65	1.96 ± 0.64	−0.49 ± 0.26	4.61 ± 0.33	2.02	(1.67, 3.05)	13	0.951	0.289	1,2,4-Triazine-4-amino-5-one	Chloroplast, I_{50}	74	74
66	2.10 ± 0.87	−0.67 ± 0.30	1.66 ± 0.57	1.56	(1.45, 1.73)	8	0.947	0.082	1,1-Dialkyl-propynyl carbamates	Mouse, HD_{50}	40	75
67	2.16 ± 1.11	−0.52 ± 0.32	0.84 ± 0.91	2.09	(1.90, 2.72)	9	0.944	0.096	1,1-Dialkyl-1-propynols	Mouse, HD_{50}	III-59	76
68	2.25 ± 0.59	−0.37 ± 0.17	0.03 ± 0.47	3.01	(2.56, 4.18)	10	0.991	0.096	Barbiturates	Brain rat, I_{50}	III-52	77
69	2.45 ± 0.81	−0.69 ± 0.24	0.72 ± 0.64	1.79	(1.69, 1.92)	8	0.965	0.058	1,1-Dialkyl-1-propynols	Mouse, HD_{50}	40	76
70	2.73 ± 0.70	−0.57 ± 0.14	2.22 ± 0.75	2.38	(2.22, 2.55)	20	0.898	0.474	Aminopyridines and anilines	*M. tuberculosis*, MIC	III-60	78
71	3.05 ± 1.37	−0.63 ± 0.33	−1.78 ± 1.37	2.43	(2.24, 2.91)	8	0.958	0.134	$RC_6H_4COO^-$	Rabbit, excretion (RBR)	III-61	79
72	3.72 ± 2.39	−0.68 ± 0.41	−0.42 ± 3.14	2.74	(2.32, 2.98)	8	0.900	0.359	β-Carbolines	MAO, I_{50} M	80	81
					Part C							
73	0.71 ± 0.29	−0.09 ± 0.06	3.22 ± 0.28	4.07	(3.25, 8.63)	12	0.955	0.174	$XC_6H_4CH_2\overset{+}{N}(R)(CH_3)_2$	Red cell rabbit, $C_{H_{100}}$	III-47	82
74	0.76 ± 0.18	−0.11 ± 0.06	3.32 ± 0.35	3.38	(2.54, 5.59)	7	0.987	0.259	$RCHOHCOO^-$	*D. pneumoniae*, pH 7.5, MKC	III-11	44
75	0.88 ± 0.52	−0.14 ± 0.09	2.58 ± 0.67	3.21	(2.56, 4.38)	8	0.891	0.436	$RCHBrCOO^-$	Red cell sheep, $C_{H_{100}}$	32	53

(*continued*)

Table I—(*Continued*)

Equation Number	*b*	*a*	*c*	log P_0	Confidence Interval	*n*	*r*	*s*	Compound	Type Biological Activity[a]	References 1	References 2
76	1.04 ± 0.14	−0.16 ± 0.04	3.05 ± 0.15	3.15	(2.74, 3.82)	6	0.998	0.084	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	Red cell sheep, $C_{H_{50}}$	III-29	68
77	1.06 ± 0.15	−0.16 ± 0.04	3.29 ± 0.14	3.29	(2.99, 3.75)	11	0.992	0.119	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*T. mentagrophytes*, MIC	55	51
78	1.06 ± 0.21	−0.16 ± 0.05	3.23 ± 0.19	3.23	(2.86, 3.90)	11	0.983	0.166	$C_6H_5CH_2\overset{+}{N}(R)(CH_3)_2$	*T. mentagrophytes*, MKC	55	51
79	1.19 ± 0.34	−0.19 ± 0.10	2.40 ± 0.40	3.06	(2.44, 4.66)	6	0.991	0.200	$C_5H_5\overset{+}{N}R$	*E. typhosa*, MKC	III-62	83
80	1.20 ± 0.31	−0.13 ± 0.03	−1.84 ± 0.64	4.74	(4.56, 4.96)	11	0.955	0.106	5-Alkyl-8-hydroxy-quinolines	*A. niger*, Inh (RBR)	III-64	84
81	1.20 ± 0.18	−0.16 ± 0.05	0.32 ± 0.14	3.64	(3.14, 4.57)	9	0.996	0.097	9-(1-Hydroxy-2-alkyl)adenines	HAD, Inh $[I/S]_{0.5}$	III-66	85
82	1.21 ± 0.71	−0.20 ± 0.12	2.88 ± 0.92	3.05	(2.40, 3.94)	8	0.893	0.596	$RCHBrCOO^-$	*D. pneumoniae*, pH 7.5, MKC	III-37	53
83	1.22 ± 0.27	−0.18 ± 0.08	2.36 ± 0.33	3.35	(2.68, 5.09)	6	0.996	0.160	$R\overset{+}{N}(CH_3)_3$	*S. aureus*, MKC	III-56	64
84	1.22 ± 0.64	−0.19 ± 0.11	2.60 ± 0.83	3.15	(2.58, 4.03)	8	0.909	0.542	$RCHBrCOO^-$	*D. pneumoniae* pH 8.5, MKC	III-38	53
85	1.23 ± 0.24	−0.17 ± 0.06	2.59 ± 0.19	3.56	(3.07, 4.56)	9	0.993	0.139	$C_5H_5\overset{+}{N}R$	Red cell dove, MHC	32	86
86	1.24 ± 0.31	−0.17 ± 0.07	2.33 ± 0.30	3.72	(3.18, 4.98)	6	0.996	0.135	$ROSO_3^-Na^+$	Red cell sheep, $C_{H_{50}}$	32	68
87	1.24 ± 0.81	−0.21 ± 0.14	2.51 ± 1.05	3.02	(2.26, 4.08)	8	0.872	0.685	$RCHBrCOO^-$	*S. haemolyticus*, pH 7.5, MKC	III-39	53
88	1.25 ± 0.81	−0.21 ± 0.14	2.20 ± 1.05	3.02	(2.26, 4.08)	8	0.872	0.686	$RCHBrCOO^-$	*S. haemolyticus*, pH 8.5, MKC	III-40	53
89	1.31 ± 0.26	−0.18 ± 0.08	2.78 ± 0.26	3.63	(2.87, 5.56)	6	0.997	0.152	$R\overset{+}{N}(CH_3)_3$	Red cell sheep, $C_{H_{50}}$	32	87
90	1.37 ± 0.32	−0.23 ± 0.09	2.37 ± 0.38	3.01	(2.49, 4.08)	6	0.994	0.190	$C_5H_5\overset{+}{N}R$	*S. aureus*, MKC	III-63	83
91	1.52 ± 0.25	−0.18 ± 0.04	1.01 ± 0.30	4.25	(3.92, 4.78)	11	0.992	0.141	*N*-Alkylpiperidinium$^+$	Red cell dove, MHC	32	86
92	1.71 ± 0.34	−0.21 ± 0.07	−1.57 ± 0.41	4.03	(3.62, 4.78)	8	0.995	0.086	4-Alkylresorcinol	*S. typhosa*, *PC'*	III-67	88
93	1.74 ± 0.13	−0.27 ± 0.04	3.84 ± 0.11	3.18	(2.97, 3.44)	8	0.999	0.087	ROH	Blowfly, ED_{50}	III-44	89
94	1.91 ± 1.11	−0.19 ± 0.13	0.55 ± 2.33	4.97	(4.61, 6.49)	10	0.936	0.104	XC_6H_4NCS	*C. albicans*, I_{50}	55	90
95	1.98 ± 0.37	−0.24 ± 0.04	0.17 ± 0.72	4.16	(4.03, 4.29)	8	0.989	0.107	α-Monoglycerides	Red cell dove, MHC	III-68	91
96	2.34 ± 1.27	−0.27 ± 0.17	−1.81 ± 2.33	4.33	(4.03, 5.33)	9	0.944	0.118	4-(5*H*)-Imidazolones	Mouse, HD_{50}	III-69	92
97	2.43 ± 0.80	−0.36 ± 0.10	−0.27 ± 1.40	3.36	(3.09, 3.55)	7	0.987	0.152	α-Monoglycerides	Red cell dove, MHC	32	91
98	2.88 ± 1.02	−0.48 ± 0.19	−0.15 ± 1.32	3.01	(2.86, 3.28)	7	0.989	0.062	Thiobarbiturates	Rat, MAD	III-70	93
99	2.95 ± 1.55	−0.46 ± 0.25	−0.76 ± 2.26	3.20	(2.93, 3.64)	9	0.890	0.233	Benzoxathiols	*C. albicans*, MIC	III-71	94
					Part D							
100	0.80 ± 0.25	−0.06 ± 0.02	0.36 ± 0.66	6.26	(5.95, 6.77)	6	0.991	0.044	RSCN	Aphid, LD_{50}	III-72	95
101	1.07 ± 0.38	−0.10 ± 0.04	1.06 ± 0.77	5.27	(4.88, 6.08)	8	0.973	0.104	X-Phenylmethacrylates	*S. faecalis*, MIC	III-73	96
102	1.13 ± 0.54	−0.10 ± 0.05	1.37 ± 1.24	5.87	(5.36, 7.12)	7	0.962	0.176	RSCN	Aphid, % kill (RBR)	58, 97	95
103	1.36 ± 0.24	−0.14 ± 0.03	−2.35 ± 0.53	5.03	(4.86, 5.22)	9	0.986	0.076	5-Alkyl-8-hydroxy-quinolines	*A. niger*, Inh (RBR)	III-65	84
104	1.43 ± 0.32	−0.12 ± 0.03	1.16 ± 0.72	5.81	(5.50, 6.20)	17	0.936	0.300	Hydrocupreines	*B. diphtheriae*, MIC	98	99
105	2.30 ± 0.51	−0.23 ± 0.06	−3.47 ± 1.02	5.10	(4.85, 5.51)	17	0.966	0.178	Phenols	*E. dysenteriae*, *PC'*	III-74	100
106	2.45 ± 1.15	−0.24 ± 0.11	−2.03 ± 2.61	5.15	(4.72, 5.66)	7	0.948	0.370	RSCN	Thrip, percent kill (RBR)	5, 97	95

[a] Abbreviations for biological systems and activities include: MAO = monoamine oxidase; K_i' = pH-independent, enzyme inhibitory rate constant; K_i = apparent enzyme inhibitor constant; MIC = minimum inhibitory concentration, either bacteriostatic or fungistatic; I_{50} = concentration for 50% inhibition; MKC = minimum killing concentration, either bactericidal or fungicidal; ADH = alcohol dehydrogenase; $K_{ER\cdot I}$ = enzyme binding constant; ED_{50} = effective dose for 50% of the population; MED = minimum effective dose; KS = dose necessary to kill spores (a relative biological response); MLD = minimum lethal dose; ChE = plasma hydrolase cholinesterase; MHC = minimum concentration for hemolysis; LD_{50} = lethal dose for 50% of the population; $C_{H_{50}}$ = minimum concentration for 50% hemolysis; AD_{50} = minimum anesthetic dose for 50% of the population; HD_{50} = minimum hypnotic dose for 50% of the population; HAD = hydrolase adenosine deaminase; $C_{H_{100}}$ = minimum concentration for 100% hemolysis; MAD = minimum anesthetic dose; RBR = relative biological response rather than p*C*; MHD = minimum hypnotic dose; Inh = inhibition; *K* = binding constant; $K_{E\cdot I}$ = dissociation constant for effect of inhibitor on enzyme; MDD_{50} = minimum dose causing 50% reduction in motor activity; DFR = dihydrofolate reductase; *PC'* = molar phenol coefficient; and $[I/S]_{0.5}$ = ratio of the millimolar concentration of inhibitor for 50% inhibition to the millimolar concentration of substrate.

Table II—$pC = a(\log P)^2 + b \log P + c$

Equation Number	b	a	c	$\log P_0$	Confidence Interval	n	r	s	Compound	Type Biological Activity[a]	Reference
					Part A						
107	−0.34 ± 0.20	−0.12 ± 0.05	1.81 ± 0.20	−1.37	(−1.65, −0.87)	12	0.950	0.073	$R\overset{+}{N}H_3$	Liver cat, deamination (RBR)	101
108	−0.03 ± 0.25	−0.08 ± 0.07	3.35 ± 0.27	−0.18	(−1.06, 10.26)	15	0.845	0.351	$RCOO^-$	*P. omnivorum*, MIC (100%)	55, 102
109	0.13 ± 0.05	−0.05 ± 0.02	0.10 ± 0.03	1.32	(1.17, 1.50)	6	0.983	0.016	ROH	Blowfly, survival time (RBR)	60
110	0.19 ± 0.25	−0.32 ± 0.20	2.97 ± 0.51	0.29	(−0.08, 1.26)	5	0.989	0.171	$RCHOHCOO^-$	*B. melitensis*, pH 7.5, MKC	44
111	0.21 ± 0.66	−0.12 ± 0.11	3.03 ± 0.67	0.88	(−21.66, 1.93)	5	0.997	0.102	RSCN	Mouse, MLD	103
112	0.27 ± 0.20	−0.35 ± 0.12	2.55 ± 0.22	0.38	(0.10, 0.75)	23	0.826	0.437	$XC_6H_4COO(CH_2)_nR$	Cornea rabbit, MAD	104
113	0.29 ± 0.12	−0.10 ± 0.05	3.53 ± 0.13	1.42	(0.62, 3.39)	13	0.979	0.178	$RCOO^-$	*T. interdigitale*, pH 6.5, MIC	105
114	0.31 ± 0.42	−0.20 ± 0.16	7.06 ± 0.64	0.78	(−0.94, 1.66)	7	0.870	0.440	$(CH_3)_3\overset{+}{N}(CH_2)_n\overset{+}{N}(CH_3)_3$	Cat muscle, 95% paralysis, MED	50
115	0.33 ± 0.27	−0.14 ± 0.12	2.03 ± 0.13	1.20	(0.12, 15.47)	7	0.994	0.087	$RCOO^-$	*A. niger*, pH 6.5, KS	105
116	0.56 ± 0.28	−0.31 ± 0.18	4.23 ± 0.67	0.90	(0.47, 1.92)	6	0.973	0.339	$RCHOHCOO^-$	*B. lepisepticus*, pH 6.5, MKC	44
117	0.64 ± 0.33	−0.24 ± 0.17	2.81 ± 0.40	1.35	(0.92, 2.93)	5	0.986	0.146	$RCHOHCOO^-$	*S. haemolyticus*, pH 8.5, MKC	44
118	0.66 ± 0.45	−0.35 ± 0.23	2.95 ± 0.55	0.95	(0.52, 1.67)	5	0.978	0.202	$RCHOHCOO^-$	*V. cholerae*, pH 8.5, MKC	44
119	0.66 ± 0.45	−0.35 ± 0.23	2.95 ± 0.55	0.95	(0.52, 1.67)	5	0.978	0.202	$RCHOHCOO^-$	*V. cholerae*, pH 7.5, MKC	44
120	0.72 ± 0.47	−0.34 ± 0.24	2.90 ± 0.56	1.04	(0.62, 1.94)	5	0.979	0.207	$RCHOHCOO^-$	*B. typhosa*, pH 8.5, MKC	44
121	1.01 ± 0.91	−0.42 ± 0.39	4.79 ± 0.46	1.21	(0.63, 3.08)	6	0.899	0.259	Isonicotinic acid hydrazides	MAO, I_{50} ammonia	47
122	1.03 ± 0.46	−0.55 ± 0.48	3.24 ± 0.37	0.94	(0.30, 11.17)	6	0.994	0.186	$RCONH_2$	ADH, binding ($K_{E.I}$)	46
123	1.06 ± 0.79	−0.47 ± 0.39	3.39 ± 0.93	1.12	(0.60, 3.06)	5	0.972	0.344	$RCOO^-$	*B. lepisepticus*, pH 7.5, MKC	44
					Part B						
124	0.13 ± 0.04	−0.02 ± 0.01	1.59 ± 0.10	2.70	(2.08, 3.90)	13	0.927	0.111	$RCOO^-$	Gut turtle, percent adsorption (RBR)	106
125	0.22 ± 0.27	−0.06 ± 0.05	0.42 ± 0.30	1.94	(−3.43, 2.46)	8	0.889	0.072	Misc.	Ant, alarm release (RBR)	107
126	0.43 ± 0.13	−0.07 ± 0.04	5.58 ± 0.37	2.98	(1.48, 8.63)	12	0.961	0.390	$(CH_3)_3\overset{+}{N}(CH_2)_n\overset{+}{N}(CH_3)_3$	Mouse, LD_{50}	50
127	0.49 ± 0.15	−0.13 ± 0.12	2.69 ± 0.31	1.90	(0.80, 36.36)	5	0.997	0.105	$RCHOHCOO^-$	*B. melitensis*, pH 8.5, MKC	44
128	0.60 ± 0.30	−0.15 ± 0.11	1.25 ± 0.18	2.00	(1.56, 4.50)	8	0.948	0.117	*sec*-Amines$^+$	Amine oxidase, deamination (RBR)	101
129	0.70 ± 0.20	−0.12 ± 0.09	2.47 ± 0.32	2.85	(1.89, 8.66)	6	0.991	0.174	$RCHBrCOO^-$	*B. melitensis*, pH 7.5, MKC	53
130	0.79 ± 0.39	−0.21 ± 0.15	1.33 ± 0.27	1.90	(1.48, 3.75)	8	0.935	0.210	$R\overset{+}{N}H_3$	Amine oxidase, deamination (RBR)	101
131	0.88 ± 0.24	−0.18 ± 0.10	−1.57 ± 0.12	2.48	(1.99, 4.09)	5	0.999	0.034	4-Alkylsulfadiazines	RNA yeast, binding (K)	17
132	0.94 ± 0.58	−0.24 ± 0.15	2.22 ± 0.54	1.98	(1.48, 2.70)	6	0.949	0.285	$RCHBrCOO^-$	*B. lepisepticus*, pH 8.5, MKC	53

(continued)

Table II—(*Continued*)

Equation Number	*b*	*a*	*c*	log P_0	Confidence Interval	*n*	*r*	*s*	Compound	Type Biological Activity[a]	Reference
133	0.95 ± 0.31	−0.30 ± 0.16	2.85 ± 0.37	1.58	(1.20, 2.56)	5	0.995	0.136	$RCHOHCOO^-$	*B. diphtheriae*, pH 8.5, MKC	44
134	1.01 ± 0.20	−0.22 ± 0.07	2.19 ± 0.13	2.34	(2.07, 2.86)	27	0.960	0.107	Ethers	Mouse, AD_{50}	67
135	1.02 ± 0.49	−0.27 ± 0.19	1.86 ± 0.28	1.93	(1.59, 3.76)	13	0.923	0.113	Barbiturates	Mouse, LD_{50}	71, 72
136	1.53 ± 0.08	−0.39 ± 0.11	1.69 ± 0.06	1.94	(1.56, 2.62)	5	0.999	0.020	Carbamates	Tadpole, Inh movement (100%), MED	108
137	1.59 ± 0.95	−0.41 ± 0.27	1.32 ± 0.79	1.92	(1.72, 2.42)	12	0.805	0.130	ROH	Guinea pig, HD_{50}	109
138[b]	2.53 ± 1.17	−0.56 ± 0.34	−1.07 ± 0.94	2.25	(1.98, 3.21)	14	0.912	0.217	Benzeneboronic acids	Mouse brain, localization (RBR)	34, 35
139	3.68 ± 1.16	−0.72 ± 0.33	−0.07 ± 0.77	2.56	(2.26, 3.31)	5	0.998	0.118	ROH	MAO, K_i	42
					Part C						
140	0.45 ± 0.06	−0.05 ± 0.03	3.75 ± 0.09	4.15	(2.45, 9.27)	13	0.993	0.117	$RCOO^-$	*T. purpureum*, pH 6.5, MIC	55, 105
141	0.59 ± 0.51	−0.08 ± 0.08	3.10 ± 0.67	3.57	(3.10, 11.14)	6	0.925	0.094	2-Alkyl-*N*-dodecylpyridinium	*V. inaequales*, KS	55, 56
142	0.66 ± 0.32	−0.10 ± 0.08	2.38 ± 0.31	3.44	(2.80, 8.33)	34	0.885	0.106	Thioureas	Mouse, MHD	110
143	0.96 ± 0.15	−0.16 ± 0.09	4.16 ± 0.36	3.07	(1.96, 7.47)	6	0.996	0.181	$RCHOHCOO^-$	*D. pneumoniae*, pH 6.5, MKC	44
144	1.03 ± 0.38	−0.15 ± 0.09	2.93 ± 0.36	3.47	(2.86, 5.41)	6	0.989	0.164	$ROSO_3^-Na^+$	Red cell sheep, $C_{H_{50}}$	68
145	1.19 ± 0.80	−0.19 ± 0.15	2.91 ± 0.91	3.19	(2.63, 5.90)	5	0.981	0.169	$R\overset{+}{N}H_3$	*Cl. welchii*, MIC	70
146	1.25 ± 0.78	−0.19 ± 0.14	2.83 ± 0.89	3.29	(2.74, 5.80)	5	0.985	0.164	$R\overset{+}{N}H_3$	*Cl. sporogenes*, MIC	70
147	1.36 ± 0.49	−0.16 ± 0.07	2.64 ± 0.77	4.17	(3.75, 5.11)	15	0.912	0.190	1-R-$(CH_2)_n$-3-(*m*-X-C_6H_4)-Imidazolin-2-ones	Mouse, MDD_{50}	111, 112
148	1.47 ± 0.75	−0.23 ± 0.21	1.82 ± 0.56	3.17	(2.41, 14.33)	12	0.927	0.385	2-Amino-5-alkyl-6-methylpyrimidine-4-ol	DFR, I_{50}	113
149	1.55 ± 0.58	−0.17 ± 0.11	−1.71 ± 0.71	4.68	(3.84, 8.39)	11	0.980	0.185	Phenols	*S. typhosa*, *PC'*	114
150	2.18 ± 1.62	−0.34 ± 0.31	−0.29 ± 1.95	3.22	(2.82, 9.76)	6	0.970	0.148	Thioureas	*A. solani*, I_{50} spores	55, 115
151	2.22 ± 1.29	−0.33 ± 0.25	0.63 ± 1.63	3.40	(3.01, 5.96)	10	0.960	0.097	Thiobarbiturates	Rabbit, MHD	116
152	3.22 ± 0.70	−0.37 ± 0.09	−4.87 ± 1.29	4.36	(4.21, 4.58)	25	0.945	0.173	Phenols	*S. typhosa*, *PC'*	100
153	5.98 ± 4.13	−0.65 ± 0.47	−9.33 ± 8.81	4.60	(4.34, 5.36)	6	0.948	0.226	3-Alkylpyrazoles	*T. interdigitale*, MIC	55, 117
154	8.07 ± 4.99	−0.88 ± 0.57	−13.32 ± 10.65	4.60	(4.36, 5.15)	6	0.957	0.272	3-Alkylpyrazoles	*S. aureus*, MIC	117
					Part D						
155	0.69 ± 0.06	−0.04 ± 0.03	2.51 ± 0.08	9.25	(4.96, 36.05)	13	0.997	0.098	$RCOO^-$	*T. interdigitale*, pH 6.5, KS	105
156	0.90 ± 0.25	−0.07 ± 0.02	2.09 ± 0.70	6.11	(5.60, 6.77)	15	0.913	0.339	Glyoxalidines	*M. sarcinaeforme*, LD_{50}	55, 118
157	0.94 ± 0.27	−0.08 ± 0.02	1.89 ± 0.75	6.16	(5.63, 6.86)	15	0.910	0.363	Glyoxalidines	*A. solani*, LD_{50}	55, 118
158	0.96 ± 0.37	−0.07 ± 0.03	1.44 ± 1.03	6.95	(6.09, 8.72)	15	0.875	0.500	Glyoxalidines	*G. cingulata*, LD_{50}	55, 118
159	1.05 ± 0.24	−0.09 ± 0.03	1.05 ± 0.45	5.64	(5.19, 6.45)	23	0.960	0.141	Phenols	*A. niger*, I_{50}	119
160	1.07 ± 0.24	−0.09 ± 0.02	2.17 ± 0.68	6.14	(5.70, 6.68)	14	0.946	0.324	Glyoxalidines	*M. fructicola*, LD_{50}	55, 118
161	1.22 ± 0.84	−0.11 ± 0.10	0.66 ± 1.68	5.64	(4.84, 15.81)	9	0.915	0.243	Phenylmethacrylates	*B. cereus*, MIC	96
162	1.24 ± 0.26	−0.07 ± 0.04	−0.77 ± 0.44	8.41	(6.85, 12.79)	13	0.997	0.078	Phenols	*C. albicans*, MKC	120
163	1.26 ± 0.23	−0.05 ± 0.05	0.49 ± 0.23	12.03	(7.53, 76.65)	10	0.998	0.143	ROH	Tadpole, reflex response, MED	121
164	1.33 ± 0.40	−0.11 ± 0.07	0.49 ± 0.41	6.25	(4.68, 14.21)	13	0.973	0.445	Misc.	Ventricle frog, I_{50}	122

Table II—(*Continued*)

Equation Number	b	a	c	log P_0	Confidence Interval	n	r	s	Compound	Type Biological Activity[a]	Reference
165	1.35 ± 0.78	−0.10 ± 0.07	−0.13 ± 1.95	6.56	(5.79, 9.18)	7	0.944	0.325	RSCN	Aphid, percent killed (RBR)	95
166	1.38 ± 0.34	−0.08 ± 0.07	0.52 ± 0.34	8.69	(5.78, 43.43)	10	0.995	0.210	ROH	Tadpole, narcosis, MED	123
167	1.42 ± 0.47	−0.07 ± 0.06	−2.28 ± 0.92	9.65	(7.20, 28.61)	32	0.983	0.161	Phenols	*S. haemolyticus, PC'*	100
168	1.57 ± 0.36	−0.10 ± 0.04	−2.45 ± 0.75	8.17	(6.95, 11.28)	34	0.983	0.157	Phenols	*S. aureus, PC'*	100
169	1.67 ± 0.29	−0.11 ± 0.03	−2.68 ± 0.58	7.55	(6.74, 9.07)	21	0.996	0.070	Phenols	*T. rosaceum, PC'*	100
170	1.74 ± 0.46	−0.12 ± 0.06	−2.79 ± 0.90	7.33	(6.27, 10.25)	33	0.981	0.160	Phenols	*M. smegmatis, PC'*	100
171	1.78 ± 0.45	−0.15 ± 0.07	−2.27 ± 0.68	5.76	(4.92, 8.02)	12	0.994	0.098	4-RS-Phenols	*S. aureus, PC'*	88
172	2.25 ± 0.63	−0.17 ± 0.05	−5.47 ± 1.83	6.55	(6.29, 6.93)	14	0.938	0.199	Testosterone esters	Red cell rat, percent hemolysis (RBR)	124
173	2.32 ± 0.91	−0.21 ± 0.13	−5.61 ± 1.55	5.52	(4.72, 8.84)	17	0.983	0.117	Benzene derivatives	Red cell rabbit, acceleration of 100% hemolysis by saponin	32, 125

[a] See *Footnote a*, Table I. [b] This equation varies slightly from that previously reported (34) due to minor refinements in the values of log P (6).

Most of the equations in Tables I and II are based on homologous series. This is not simply a chance occurrence. When elements other than CH_2 units are incorporated into a parent molecule, important electronic and possibly steric effects are brought into the structure–activity relationship. These must be accounted for by the addition of other terms to Eq. 6.

One main reason that the strong and often quantitatively definable dependence of drug activity on lipophilic character has been so slow in coming into focus has been the shortage of partition coefficients from a suitable reference system. This is still a handicap. Values are not available for all of the data used for the correlations of Tables I and II. Unknown values have been calculated from additivity principles (6).

From the values in the section on data, the equations in Tables I and II were derived by the standard nonweighted least-squares method (41). In addition to fitting the data to Eq. 6, each set was fit to the third-order equation in which a term in $(\log P)^3$ was added to Eq. 6. Out of 233 cases tested, the cubic term yielded an improved correlation (significance at >0.95 in F test) in 28 examples. Twelve of these examples were with equations in Table I. Examination of the plots of the cubic equations did not uncover any general pattern of correlation. The results do not appear to warrant further consideration at present.

RESULTS

The resulting equations selected for this study are listed in Tables I and II. In these tables, a represents the coefficient of the parabolic $(\log P)^2$ term, b is the coefficient of the linear $(\log P)$ term, and c is the regression constant generated by the least-squares analysis. The 95% confidence interval for each of these values is also given. The calculated ideal value of log P, log P_0, is also listed along with its 95% confidence limits.

For convenience in analysis, the equations in Tables I and II have been factored into four sets based on the range of their log P_0 values, namely, equations (Table I, Part A and Table II, Part A) with log P_0 less than 1.5, those (Table I, Part B and Table II, Part B) with log P_0 between 1.5 and 3.0, those (Table I, Part C and Table II, Part C) with log P_0 varying between 3.0 and 5.0, and those (Table I, Part D and Table II, Part D) with log P_0 greater than 5.0. Within each set the equations have been ordered by increasing values of the coefficient (b) of the linear (log P) term. Most of the equations in Table I were derived from the data listed in Table III. *Reference 1* in Table I refers to the location in Table III of the corresponding data or to the appropriate literature reference if the equation was previously reported in the literature. *Reference 2* indicates the original literature source of the biological activity data used in the equation.

A summary of the distribution and ranges of the values of log P_0, b, a, c, and log P for the 100 equations in Table I is given in Table IV. From these results, it is interesting to note that while the ranges of the coefficients (b) of the linear term within each group (Table I, Part A–Table I, Part D) vary from group to group, the range of the coefficients (a) of the parabolic term within

Table III—Data for Table I

R	log *P*	III-1 $1/K_i'$ Obs.	III-2 $1/K_i$ Obs.	III-3 p*C* Obs.	III-4 p*C* Obs.
C_2H_5	−3.15	3.37	1.44	—	—
C_3H_7	−2.65	4.67	2.70	—	—
C_4H_9	−2.15	6.02	4.14	—	—
C_5H_{11}	−1.65	7.37	5.46	—	—
C_6H_{13}	−1.15	8.10	6.17	—	—
C_7H_{15}	−0.65	8.17	—	—	—
C_8H_{17}	−0.15	8.01	—	—	—
C_9H_{19}	0.35	7.95	6.02	4.43	2.95
$C_{12}H_{25}$	1.85[a]	—	—	5.52	4.52
$C_{14}H_{29}$	2.85	—	—	5.87	4.57
$C_{16}H_{33}$	3.85	—	—	5.44	4.32
$C_{18}H_{37}$	4.85	—	—	4.66	3.66

R	log *P*	III-5 p*C* Obs.	III-6 p*C* Obs.
C_3H_7	−3.20	3.04	—
C_4H_9	−2.70	3.01	—
C_5H_{11}	−2.20[a]	2.77	3.24
C_6H_{13}	−1.70	4.41	—
C_7H_{15}	−1.20	3.86	3.76
C_8H_{17}	−0.70	4.60	—
C_9H_{19}	−0.20	4.24	4.31
$C_{10}H_{21}$	0.30	4.67	—
$C_{11}H_{23}$	0.80	—	4.51
$C_{12}H_{25}$	1.30	4.64	—
$C_{13}H_{27}$	1.80	—	4.74
$C_{15}H_{31}$	2.80	3.11	4.31
$C_{17}H_{35}$	3.80	2.55	4.36

R	log *P*[b]	III-7 p*C* Obs.	III-8 p*C* Obs.	III-9 p*C* Obs.	III-10 p*C* Obs.	III-11 p*C* Obs.
C_6H_{13}	−2.22	1.30	—	1.30	1.00	1.30
C_8H_{17}	−1.22	2.81	1.60	2.50	2.20	1.90
$C_{10}H_{21}$	−0.22	3.71	3.71	3.11	2.81	3.11
$C_{12}H_{25}$	0.78	3.71	4.61	3.41	3.41	3.71
$C_{14}H_{29}$	1.78	2.81	4.31	3.41	3.71	4.61
$C_{16}H_{33}$	2.78	—	3.11	3.11	3.41	4.61
$C_{20}H_{41}$	4.78	—	—	2.81	—	4.31

R	log *P*	III-12 RBR Obs.
Methyl	−4.38	0.04
Ethyl	−3.88	0.23
Propyl	−3.38	0.65
Isopropyl	−3.58	0.08
Butyl	−2.88	1.03
Amyl	−2.38	1.50
Hexyl	−1.88	1.90
Heptyl	−1.38	2.38
Octyl	−0.88[a]	2.70
Decyl	0.12	2.79
Dodecyl	1.12	2.85

R	log *P*	III-13 $1/K_{ER,I}$ Obs.
H	−1.46[a]	0.99
CH_3	−1.21	2.30
C_2H_5	−0.71	3.30
C_3H_7	−0.21[a]	4.19
C_4H_9	0.29	4.60
C_6H_{11}	0.79	4.96

R	log P^c	III-14 p*C* Obs.
Methyl	−0.43	3.80
Ethyl	0.07	4.60
Propyl	0.57	4.90
Butyl	1.07	5.30
Hexyl	2.07	5.10
Heptyl	2.57	4.90

R	log *P*	III-15 p*C* Obs.	III-16 p*C* Obs.
$C_{11}H_{23}$	0.65	5.15	5.30
$C_{12}H_{25}$	1.15[a]	5.22	5.40
$C_{13}H_{27}$	1.65	5.30	5.60
$C_{14}H_{29}$	2.15	5.30	5.58
$C_{16}H_{33}$	3.15	5.18	5.40
$C_{18}H_{37}$	4.15	4.74	4.64

n	log *P*	III-17 p*C* Obs.	III-18 p*C* Obs.
4	−3.34	3.42	—
5	−2.84	3.73	—
6	−2.34	4.03	—
7	−1.84	4.54	2.46
8	−1.34	5.72	3.00
9	−0.84	6.35	3.92
10	−0.34[a]	6.53	4.35
11	0.16	6.35	4.74
12	0.66	6.01	5.25
18	3.66	6.50	4.85

R	log *P*	III-19 p*C* Obs.	III-20 p*C* Obs.	III-21 p*C* Obs.	III-22 p*C* Obs.	III-23 p*C* Obs.	III-24 p*C* Obs.	III-25 p*C* Obs.	III-26 p*C* Obs.	III-27 p*C* Obs.	III-28 p*C* Obs.	III-29 p*C* Obs.
C_8H_{17}	−1.08	—	3.52	—	—	3.69	3.00	1.68	2.69	1.52	—	1.76
C_9H_{19}	−0.58	2.54	3.72	2.54	2.54	4.02	3.02	2.08	3.02	—	2.54	—
$C_{10}H_{21}$	−0.08[a]	2.57	3.74	2.74	2.74	4.74	3.57	2.41	3.57	3.45	3.04	2.95
$C_{11}H_{23}$	0.42	3.06	4.06	3.06	3.06	5.06	4.06	2.92	4.06	—	3.59	—
$C_{12}H_{25}$	0.92	3.48	4.61	3.61	3.61	5.61	4.61	3.45	4.61	4.34	4.09	3.82
$C_{13}H_{27}$	1.42	3.50	4.80	3.80	3.80	5.80	5.10	3.85	5.10	—	4.63	—
$C_{14}H_{29}$	1.92	3.52	4.82	3.65	3.65	5.82	5.12	3.96	5.12	4.63	4.82	4.40
$C_{15}H_{31}$	2.42	3.14	4.84	3.84	3.84	5.84	5.14	3.74	5.14	—	5.14	—
$C_{16}H_{33}$	2.92	—	4.68	3.68	3.68	5.56	5.16	3.30	5.16	4.88	4.56	4.79
$C_{17}H_{35}$	3.42	—	—	3.57	—	5.18	4.70	3.06	4.70	—	4.16	—
$C_{18}H_{37}$	3.92	2.89	—	—	2.71	5.19	4.71	2.63	4.71	4.60	3.59	4.52
$C_{19}H_{39}$	4.42	2.91	4.21	2.91	2.60	4.91	4.73	2.52	4.73	—	3.61	—

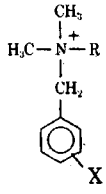
R	log P^d	III-30 p*C* Obs.	III-31 p*C* Obs.	III-32 p*C* Obs.	III-33 p*C* Obs.	III-34 p*C* Obs.	III-35 p*C* Obs.	III-36 p*C* Obs.	III-37 p*C* Obs.	III-38 p*C* Obs.	III-39 p*C* Obs.	III-40 p*C* Obs.
C_4H_9	−1.68	1.30	—	—	—	—	—	—	—	—	—	—
C_6H_{13}	−0.68	1.90	1.60	2.50	2.20	1.90	2.81	2.50	2.20	1.90	1.90	1.60
C_8H_{17}	0.32	2.50	2.20	3.11	3.41	2.50	3.71	3.41	3.11	2.81	2.81	2.50

Table III—(*Continued*)

R	log P^d	III-30 pC Obs.	III-31 pC Obs.	III-32 pC Obs.	III-33 pC Obs.	III-34 pC Obs.	III-35 pC Obs.	III-36 pC Obs.	III-37 pC Obs.	III-38 pC Obs.	III-39 pC Obs.	III-40 pC Obs.
					RCHBrCOO$^-$							
$C_{10}H_{21}$	1.32	3.41	3.11	4.01	3.71	3.41	4.31	3.71	3.71	3.41	3.11	2.81
$C_{12}H_{25}$	2.32	3.11	2.81	4.01	4.01	3.71	5.21	4.61	4.61	4.61	4.01	3.71
$C_{14}H_{29}$	3.32	2.81	2.50	3.71	3.11	2.81	5.52	5.21	4.91	4.61	4.91	4.61
$C_{16}H_{33}$	4.32	1.90	1.90	2.51	2.20	1.90	5.52	5.21	5.21	4.91	4.91	4.61
$C_{18}H_{37}$	5.32	—	—	—	—	—	3.11	2.81	2.81	2.81	2.50	2.20
$C_{20}H_{41}$	6.32	—	—	—	—	—	3.11	2.20	2.81	2.81	2.20	1.90

R	log *P*	III-41 pC Obs.
	$CH_3CH(OR)_2$	
Methyl	−0.15	1.70
Ethyl	0.85[a]	2.12
Propyl	1.85	2.30
Isopropyl	1.45	2.30
Butyl	2.85	2.00

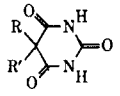
Alcohol	log *P*	III-42 RBR Obs.	III-43 RBR Obs.	III-44 pC Obs.
Methanol	−0.66[a]	0.00	—	2.57
Ethanol	−0.16	—	—	3.62
Propanol	0.34[a]	0.70	—	4.27
Isopropanol	0.14	—	1.01	—
Butanol	0.88[a]	1.25	—	5.24
sec-Butanol	0.61[a]	—	1.16	—
Pentanol	1.40[a]	1.36	—	5.66
Hexanol	2.03[a]	1.32	—	6.24
Heptanol	2.53	1.22	—	6.55
Octanol	3.03	1.12	—	—
Decanol	4.03	—	—	6.38
Dipropylcarbinol	2.18	—	1.83	—
Diisopropylcarbinol	1.75	—	1.82	—
Ethylbutylcarbinol	2.20	—	1.79	—
Methylamylcarbinol	2.33	—	1.74	—
2-Hexanol	1.83	—	1.74	—
2-Pentanol	1.11	—	1.65	—
3-Pentanol	1.11	—	1.59	—
Methylhexylcarbinol	2.83	—	1.19	—



X	R	log *P*	III-45 pC Obs.	III-46 pC Obs.	III-47 pC Obs.
H	$C_{10}H_{21}$	−0.08[a]	2.70	2.79	3.11
H	$C_{12}H_{25}$	0.92	3.79	3.74	4.04
H	$C_{14}H_{29}$	1.92	4.07	4.17	4.52
H	$C_{16}H_{33}$	2.92	3.92	3.92	4.54
H	$C_{18}H_{37}$	3.92	3.23	3.34	4.62
2-Cl	C_8H_{17}	−0.32	1.92	2.02	—
2-Cl	$C_{10}H_{21}$	0.68	3.11	3.41	3.47
2-Cl	$C_{12}H_{25}$	1.68	3.85	4.14	—
2-Cl	$C_{14}H_{29}$	2.68	4.18	4.14	—
2-Cl	$C_{16}H_{33}$	3.68	3.65	3.74	4.66
2-Cl	$C_{18}H_{37}$	4.68	3.46	3.43	4.74
4-Cl	C_8H_{17}	−0.38	2.88	2.60	—
4-Cl	$C_{10}H_{21}$	0.62	3.53	3.60	3.70
4-Cl	$C_{12}H_{25}$	1.62	4.23	4.04	—
4-Cl	$C_{14}H_{29}$	2.62	—	4.34	—
4-Cl	$C_{16}H_{33}$	3.62	3.54	3.85	—
4-Cl	$C_{18}H_{37}$	4.62	3.53	3.17	—
4-NO_2	C_8H_{17}	−0.84	1.92	2.11	—
4-NO_2	$C_{10}H_{21}$	0.16	3.11	3.11	—
4-NO_2	$C_{12}H_{25}$	1.16	3.65	4.20	—
4-NO_2	$C_{14}H_{29}$	2.16	4.25	4.23	—
4-NO_2	$C_{16}H_{33}$	3.16	3.82	4.00	—
4-NO_2	$C_{18}H_{37}$	4.16	3.54	3.46	—
2-Cl, 4-Cl	C_8H_{17}	0.38	2.50	2.63	—
2-Cl, 4-Cl	$C_{10}H_{21}$	1.38	3.58	3.65	3.85
2-Cl, 4-Cl	$C_{12}H_{25}$	2.38	4.14	4.25	4.43
2-Cl, 4-Cl	$C_{14}H_{29}$	3.38	4.13	4.28	4.39
2-Cl, 4-Cl	$C_{16}H_{33}$	4.38	3.46	3.41	—
2-Cl, 4-Cl	$C_{18}H_{37}$	5.38	3.49	3.30	—
2-OH, 5-NO_2	$C_{10}H_{21}$	−0.51	2.79	—	—
2-OH, 5-NO_2	$C_{12}H_{25}$	0.49	2.58	2.70	—
3-Cl, 4-Cl	C_8H_{17}	0.38	2.92	2.92	—
3-Cl, 4-Cl	$C_{10}H_{21}$	1.38	3.85	3.79	—
3-Cl, 4-Cl	$C_{12}H_{25}$	2.38	4.25	4.36	—
3-Cl, 4-Cl	$C_{14}H_{29}$	3.38	4.11	4.04	—
3-Cl, 4-Cl	$C_{16}H_{33}$	4.38	3.39	3.39	—
3-Cl, 4-Cl	$C_{18}H_{37}$	5.38	3.08	3.11	—
3-OCH_3, 4-OCH_3	$C_{12}H_{25}$	1.00	3.67	3.71	—
3-OCH_3, 4-OCH_3	$C_{14}H_{29}$	2.00	4.20	4.11	—
3-OCH_3, 4-OCH_3	$C_{16}H_{33}$	3.00	3.95	3.41	—
3-OCH_3, 4-OCH_3	$C_{18}H_{37}$	4.00	3.47	—	—
3,4-(OCH_2O)	C_8H_{17}	−1.13	2.04	2.04	—
3,4-(OCH_2O)	$C_{10}H_{21}$	−0.13	2.92	3.08	—
3,4-(OCH_2O)	$C_{12}H_{25}$	0.87	3.85	4.00	—
3,4-(OCH_2O)	$C_{14}H_{29}$	1.87	4.17	4.23	—
3,4-(OCH_2O)	$C_{16}H_{33}$	2.87	4.00	4.20	—
3,4-(OCH_2O)	$C_{18}H_{37}$	3.87	3.36	3.23	—

(*continued*)

Table III—(*Continued*)



R	R′	log *P*	III-48 pC Obs.	III-49 pC Obs.	III-50 pC Obs.	III-51 pC Obs.	III-52 pC Obs.
Methyl	$CH_3CHC(CH_3)$	0.65	—	—	2.64	—	—
Methyl	$CH_2C(CH_3)$	0.15	—	—	2.12	—	—
Ethyl	Ethyl	0.65[a]	2.87	3.09	—	2.91	1.32
Ethyl	Isopropyl	0.95[a]	3.00	3.30	—	3.34	1.89
Ethyl	Butyl	1.89[a]	3.33	3.72	—	3.53	2.80
Ethyl	Isobutyl	1.69[a]	3.25	3.63	—	—	—
Ethyl	*sec*-Butyl	1.69[a]	3.03	3.63	—	—	—
Ethyl	*sec*-Pentyl	2.07	—	—	—	—	3.07
Ethyl	Isoamyl	2.07[a]	3.12	3.75	—	3.59	3.12
Ethyl	Hexyl	2.77	—	—	—	—	3.40
Ethyl	Allyl	0.85	—	—	—	3.28	—
Ethyl	$CH_2C(CH_3)$	0.65	—	—	2.91	—	—
Ethyl	$CH_3CHC(CH_3)$	1.15	—	—	3.15	—	—
Ethyl	Phenyl	1.42[a]	3.19	3.46	—	—	2.36
Propyl	Propyl	1.65	2.98	3.55	—	—	—
Propyl	Isopropyl	1.45	3.15	3.63	—	—	—
Propyl	Isoamyl	2.57	3.04	3.48	—	—	—
Propyl	$CH_3CHC(CH_3)$	1.65	—	—	3.29	—	—
Propyl	$CH_2C(CH_3)$	1.15	—	—	3.04	—	—
Propyl	Allyl	1.35	—	—	—	3.47	—
Propyl	Benzyl	3.08	2.76	—	—	—	—
Isopropyl	Butyl	2.07	—	—	—	3.49	—
Isopropyl	Allyl	1.15	—	—	—	3.60	—
Isopropyl	Benzyl	2.88	2.87	—	—	—	—
Butyl	Butyl	2.89	—	—	—	3.08	—
Butyl	Allyl	1.85	—	—	—	3.47	—
Butyl	$CH_3CHC(CH_3)$	2.15	—	—	3.36	—	—
Butyl	$CH_2C(CH_3)$	1.65	—	—	3.33	—	—
Isobutyl	Allyl	1.65	—	—	—	3.63	2.80
Isobutyl	$CH_2C(CH_3)$	1.45	—	—	3.27	—	—
sec-Butyl	Allyl	1.65	—	—	—	3.78	—
Amyl	$CH_2C(CH_3)$	2.15	—	—	3.32	—	—
Isoamyl	Allyl	2.15	—	—	—	3.45	—
Isoamyl	$CH_2C(CH_3)$	1.95	—	—	3.26	—	—
Allyl	*sec*-Pentyl	2.15[a]	—	—	—	—	3.19
Allyl	Cyclopentenyl	1.69	—	—	—	—	2.90
Allyl	Allyl	1.05	—	—	—	3.54	—
Allyl	$CH_3CHC(CH_3)$	1.35	—	—	3.39	—	—

$R_3SnOCOCH_3$			
R	log *P*	III-53 pC Obs.	III-54 pC Obs.
Methyl	−1.32	3.05	3.05
Ethyl	0.18	4.72	5.12
Propyl	1.68[a]	5.49	5.49
Butyl	3.18	5.84	5.84
Hexyl	6.18	4.34	3.64
Phenyl	3.57	5.61	—

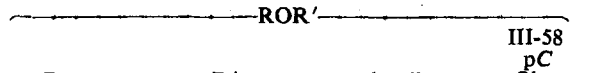
$R\overset{+}{N}(CH_3)_3$			
R	log *P*	III-55 pC Obs.	III-56 pC Obs.
C_6H_{13}	−2.07	0.53	—
C_8H_{17}	−1.07[a]	1.28	0.88
$C_{12}H_{25}$	0.84[a]	3.44	3.09
$C_{14}H_{29}$	1.84	4.08	4.11
$C_{16}H_{33}$	2.84	4.16	4.46
$C_{17}H_{35}$	3.54	3.97	4.25
$C_{17}H_{37}$	3.84	3.50	4.40

4-R-Lincomycin		
R	log *P*	III-57 RBR Obs.
H	−0.95	−1.60
Ethyl	0.05	−0.52
Propyl	0.55[a]	0.00
Butyl	1.05	0.32
Amyl	1.55	0.53
Hexyl	2.05	0.56

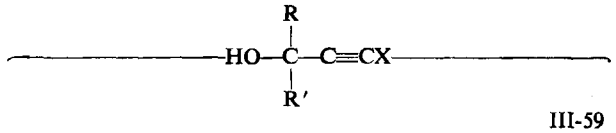
4-R-Lincomycin		
R	log *P*	III-57 RBR Obs.
Heptyl	2.55	0.18
Octyl	3.05	0.00

ROR′			
R	R′	log *P*	III-58 pC Obs.
Methyl	Methyl	−0.23	1.43
Methyl	Ethyl	0.27	1.74
Methyl	Propyl	0.77	2.45
Methyl	Isopropyl	0.57	2.26
Methyl	Cyclopropyl	0.48	2.75
Methyl	Butyl	1.27	2.70
Methyl	Isobutyl	1.08	2.79
Methyl	*sec*-Butyl	1.04	2.79
Methyl	*tert*-Butyl	0.80	2.79
Methyl	Amyl	2.03	2.88
Ethyl	Ethyl	0.77[a]	2.22
Ethyl	Propyl	1.27	2.60
Ethyl	Isopropyl	1.07	2.60
Ethyl	Cyclopropyl	0.98	3.00
Ethyl	Butyl	2.03[a]	2.82
Ethyl	Isobutyl	1.83	2.82
Ethyl	*sec*-Butyl	1.80	2.85
Ethyl	*tert*-Butyl	1.56	2.92
Ethyl	Isoamyl	2.35	3.00
Ethyl	*tert*-Amyl	2.08	3.15
Ethyl	Vinyl	0.47	2.34
Propyl	Propyl	2.03[a]	2.79
Propyl	Isopropyl	1.83	2.82

Table III—(*Continued*)

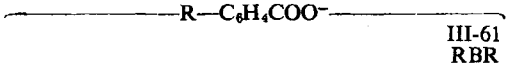


R	R′	log *P*	III-58 p*C* Obs.
Isopropyl	Isopropyl	1.63	2.82
Vinyl	Vinyl	0.17	2.33



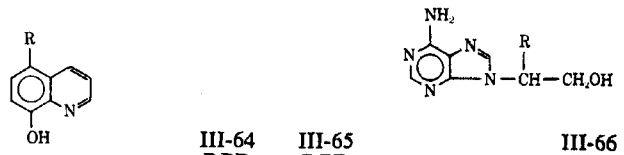
R	R′	X	log *P*	III-59 p*C* Obs.
Methyl	Ethyl	H	1.18	2.59
Methyl	Ethyl	Cl	1.51	2.94
Methyl	Vinyl	H	0.88	2.41
Methyl	ClCH=CH	H	1.50	2.94
Ethyl	Vinyl	H	1.38	2.79
Ethyl	ClCH=CH	H	2.00	3.20
Propyl	ClCH=CH	H	2.50	2.90
Isopropyl	Vinyl	H	1.68	2.92
Isopropyl	ClCH=CH	H	2.30	3.17

Compound	log *P*	III-60 p*C* Obs.
4-OCH_3-Aniline	0.78	3.39
4-OC_2H_5-Aniline	1.28	4.44
4-OC_4H_9-Aniline	2.28	5.42
4-OC_5H_{11}-Aniline	2.78	5.45
4-OC_6H_{13}-Aniline	3.28	5.80
4-OC_8H_{17}-Aniline	4.28	3.44
2-OCH_3-3-NH_2-Pyridine	0.09	2.59
2-OC_2H_5-3-NH_2-Pyridine	0.59	3.54
2-OC_3H_7-3-NH_2-Pyridine	1.09	4.79
2-*iso*-OC_4H_9-3-NH_2-Pyridine	1.39	4.52
2-OC_5H_{11}-3-NH_2-Pyridine	2.09	5.76
2-(3-OC_5H_{11})-3-NH_2-Pyridine	1.89	5.46
2-OC_7H_{15}-3-NH_2-Pyridine	3.09	4.62
2-OC_8H_{17}-3-NH_2-Pyridine	3.59	3.75
2-$OC_{10}H_{21}$-3-NH_2-Pyridine	4.59	3.19
4-OC_4H_9-3-NH_2-Pyridine	1.59	5.73
4-OC_6H_{13}-3-NH_2-Pyridine	2.59	5.80
2-OC_4H_9-5-NH_2-Pyrimidine	1.26	5.13
2-OC_6H_{13}-5-NH_2-Pyrimidine	2.26	5.49
4-OC_4H_9-1-Naphthylamine	3.63	4.03

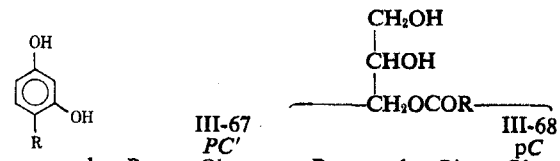


R	log *P*	III-61 RBR Obs.
H	1.87[a]	1.92
4-Cl	2.65[a]	1.93
4-F	2.06[a]	1.90
4-CH_3	2.29[a]	1.79
4-I	3.01[a]	1.76
4-OCH_3	1.95[a]	1.63
4-CN	1.56[a]	1.54
4-$NHCOCH_3$	1.08	0.74

R	log *P*	III-62 p*C* Obs.	III-63 p*C* Obs.
C_8H_{17}	−0.95[a]	1.14	0.84
$C_{12}H_{25}$	1.05	3.15	3.73
$C_{14}H_{29}$	2.05	4.12	4.33
$C_{16}H_{33}$	3.05	4.21	4.43
$C_{18}H_{37}$	4.05	3.98	4.20
3-$OC_{12}H_{25}$-3-OH-Propyl	1.66	4.00	3.76

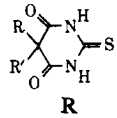


R	log *P*	III-64 RBR Obs.	III-65 RBR Obs.	R	log *P*	III-66 $[I/S]_{0.5}$ Obs.
H	2.02[a]	0.14	−0.12	CH_3	−0.37	−0.08
CH_3	2.52	0.45	—	C_2H_5	0.14[a]	0.31
C_2H_5	3.02	0.49	—	C_3H_7	0.66[a]	1.15
C_3H_7	3.52	0.61	0.62	C_4H_9	1.16[a]	1.48
C_4H_9	4.02	1.01	0.99	C_5H_{11}	1.67	1.82
C_5H_{11}	4.52	1.05	1.07	C_6H_{13}	2.18	2.21
C_6H_{13}	5.02	1.03	1.07	C_7H_{15}	2.69	2.33
C_7H_{15}	5.52	0.98	1.06	C_8H_{17}	3.20	2.41
C_8H_{17}	6.02	0.81	0.90	C_9H_{19}	3.71	2.52
C_9H_{19}	6.52	0.63	0.83			
$C_{10}H_{21}$	7.02	0.27	0.48			

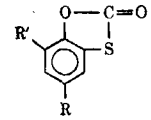


R	log *P*	III-67 *PC′* Obs.	R	log *P′*	III-68 p*C* Obs.
H	0.80[a]	−0.33	C_8H_{17}	1.83	3.00
C_4H_9	2.80	1.59	C_9H_{19}	2.33	3.48
iso-C_4H_9	2.60	1.42	$C_{10}H_{21}$	2.83	3.84
C_5H_{11}	3.30	1.80	$C_{11}H_{23}$	3.33	4.18
iso-C_5H_{11}	3.10	1.66	$C_{12}H_{25}$	3.83	4.34
C_6H_{13}	3.80	2.02	$C_{14}H_{29}$	4.83	4.25
iso-C_6H_{13}	3.60	1.75	$C_{16}H_{33}$	5.83	3.45
C_7H_{15}	4.30	1.82	$C_{18}H_{37}$	6.83	2.70

R	R′	log *P*	III-69 p*C* Obs.
Cyclohexylidine	Ethyl	3.10	2.68
Isopropylidine	Propyl	2.50	2.46
1-Methylhexylidine	Propyl	4.50	3.38
1-Methylheptylidine	Propyl	5.00	3.06
1-Phenylethylidine	Propyl	4.13	3.18
1,2-Methylpropylidine	Propyl	3.30	3.00
1,3-Methylbutylidine	Propyl	3.80	3.28
1-Methyl-4-pentenylidine	Propyl	3.70	3.18
Isopropylidine	Butyl	3.00	2.71



R	R′	log *P*	III-70 p*C* Obs.
Ethyl	Ethyl	1.70	3.35
Ethyl	Isoamyl	3.00[a]	4.12
Ethyl	1-Methylbutyl	3.00	4.25
Ethyl	Hexyl	3.70	3.97
Isopropyl	Allyl	2.20	3.92
sec-Butyl	Allyl	2.70	4.12



R	R′	log *P*	III-71 p*C* Obs.
CH_3	H	2.75	4.04
OCH_3	H	2.21	3.77

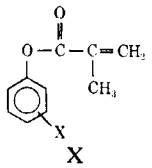
(*continued*)

Table III—(*Continued*)

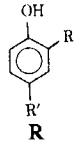
R	R′	log *P*	III-71 p*C* Obs.
Cl	H	3.01	3.78
Br	H	3.19	3.88
tert-C_4H_9	H	4.43	3.23
OH	H	1.64	2.84
COC_2H_5	H	2.20	3.22
CH_3	CH_3	3.25[a]	3.77
CH_3	Br	3.50	4.20

RSCN

R	log *P*[a]	III-72 p*C* Obs.
C_6H_{13}	3.03	2.17
C_8H_{17}	4.03	2.60
$C_{10}H_{21}$	5.03	2.75
$C_{12}H_{25}$	6.03	2.82
$C_{14}H_{29}$	7.03	2.84
$C_{16}H_{33}$	8.03	2.66



X	log *P*	III-73 p*C* Obs.
H	1.99	2.89
2-Cl	2.75	3.08
4-Cl	2.69	3.25
2-Cl, 4-Cl	3.45	3.49
2-Cl, 4-Cl, 5-Cl	4.21	3.84
2-Cl, 4-Cl, 6-Cl	4.21	3.76
2-Cl, 4-Cl, 5-Cl, 6-Cl	4.97	3.97
Pentabromo	6.74	3.64



R	R′	log *P*	III-74 p*C*′ Obs.
H	Cl	2.39[a]	0.81
Methyl	Cl	2.89	1.34
Ethyl	Cl	3.39	1.73
Propyl	Cl	3.89	2.26
Butyl	Cl	4.39	2.52
Amyl	Cl	4.89	2.63
sec-Amyl	Cl	4.69	2.23
Cyclohexyl	Cl	4.90	2.25
Heptyl	Cl	5.89	2.51
Octyl	Cl	6.39	1.83
Cl	H	2.15[a]	0.50
Cl	Methyl	2.65	0.91
Cl	Ethyl	3.15	1.35
Cl	Propyl	3.65	1.86
Cl	Butyl	4.15	2.20
Cl	Amyl	4.65	2.23
Cl	*tert*-Amyl	4.33	2.00

[a] Experimentally determined value of log *P*. All other values of log *P* were calculated according to additivity principles outlined in *References 1* and *6*. [b] Based on measured value of −0.62 for α-hydroxypropionic acid. [c] Based on measured value of 0.37 for iproniazid. [d] Based on measured value of −3.18 for α-bromopropionic acid. [e] Based on measured value of −0.16 for *N,N*-dimethyldecylammonium bromide. [f] Based on measured value of −0.17 for monobutyrin. [g] Based on measured value of 2.03 for butylthiocyanate.

each grouping is essentially constant (−0.50 to −0.10) for all groups. In addition, there is significant variation in the ranges of the constants of regression (*c*) within each group when compared between groups. Also of interest is the observation that the ranges of the individual log *P* values represented in the data used to derive the equations vary noticeably between groups.

Of the eight equations in Table I, Part A, five describe enzyme systems. Of the 58 equations in Table I, Part B, 36 are derived from data involving bacterial systems. In addition, 14 of the 27 equations in Table I, Part C, were derived from bacterial systems while nine describe hemolysis data. From the entire series of 100 equations, it appears that the coefficient of the linear term in the equation describing a bacterial system most often lies between 0.50 and 1.50. In addition, most of the equations involving hemolysis data contain coefficients of the linear term between 0.75 and 1.75.

Of the compounds used to derive these equations, the largest single grouping consists of those that are ionic. Of the eight equations in Table I, Part A, six were derived from ionic compounds. Also, 35 of the 58 equations in Table I, Part B, consist of ionic compounds, while 17 of the 27 equations in Table I, Part C, were derived from data composed of ionic compounds. From these data, it appears that most of the equations derived from these compounds contain coefficients of the linear term between 0.5 and 1.5.

The second best set of 67 equations (listed in Table II) was selected according to less stringent statistical criteria and might, therefore, be considered a slightly less reliable basis set than those given in Table I. Specifically, requirements for inclusion in this set included an *F* ratio showing the parabolic equation to be more significant than the corresponding linear equation at the 95–99% level and an *r* value greater than 0.80. In addition, the plot of log *P* values *versus* biological activity for each equation appeared unquestionably parabolic.

A summary of the results of the equations listed in Table II is given in Table IV. Of the 67 equations in Table II, the distribution among the four subsets was markedly different from that for the 100 equations in Table I. In this set the equations are essentially equally distributed among the four groups. The range of log P_0 varies in this set from −1.37 to 12.03, a range double that for the data in Table I.

However, the ranges of the coefficients of the linear term for the data in Table II are similar to those for Table I. Also very similar to the data in Table I are the ranges of the values of the coefficients of the parabolic terms in Table II. On the other hand, the ranges of the constants of regression for the equations in Table II vary drastically between groups, very much like the data in Table I.

Again similar to the data in Table I, the most common biological system type appearing in Table II is bacterial in nature. Of the 67 equations, 37 (55%) describe bacterial systems. Of the 16 equations in Table II, Part A, 10 equations (63%) involve bacterial systems, as do four of the 17 equations (23%) in Table II, Part B, 10 of the 15 equations (67%) in Table II, Part C, and 13 of the 19 equations (68%) in Table II, Part D. In addition, 40% of the equations contained in Table II were derived from ionic compounds. Of the 16 equations in Table II, Part A, 12 involve ionic compounds while eight of the 17 equations in Table II, Part B, six of the 15 equations in Table II, Part C, and one of the

Table IV—Summary of Comparison of Results

Values Compared	Equations in Table I	Equations in Table II
A. Distribution of log P_0 values		
<1.5	8%	25%
1.5–3.0	58%	24%
3.0–5.0	27%	22%
>5.0	7%	29%
Range	−0.40–6.26	−1.37–12.03
B. Range of values of b		
Group A	−0.53–1.23	−0.34–1.06
Group B	0.33–3.72	0.13–3.68
Group C	0.71–2.95	0.45–8.07
Group D	0.80–2.45	0.69–2.32
Average	0.33–2.59	0.23–3.78
C. Range of values of a		
Group A	−0.77–−0.10	−0.55–−0.05
Group B	−0.69–−0.09	−0.72–−0.02
Group C	−0.48–−0.09	−0.88–−0.05
Group D	−0.24–−0.06	−0.21–−0.04
Average	−0.54–−0.08	−0.59–−0.04
D. Range of values of c		
Group A	2.77–8.14	0.10–7.06
Group B	−1.78–6.33	−1.57–5.58
Group C	−1.84–3.84	−13.32–4.16
Group D	−3.47–1.37	−5.61–2.51
Average	−1.08–4.92	−5.10–4.82
E. Range of values of log P		
Group A	−4.38–3.80	−4.70–5.03
Group B	−3.34–6.32	−4.34–4.40
Group C	−2.22–6.32	−3.70–5.63
Group D	1.53–9.53	−3.70–10.07
Average	−2.10–6.49	−4.11–6.28

19 equations in Table II, Part D, also describe data from ionic compounds.

DISCUSSION

By what ways can the parabolic relationship between log $1/C$ and log P be explained? There are, of course, a variety of possible explanations, any one or a combination of which might be involved in a given problem. If the partition coefficient is defined as P = concentration in fatty phase/concentration in aqueous phase, one can reason that if P for a drug approaches zero, the drug will be so insoluble in fatty phases that it will not cross a lipid membrane and will remain localized in the first aqueous phase it contacts. Conversely, as P approaches infinity, the drug will be so insoluble in water that it will remain localized in fatty tissue. Somewhere between the value of zero and infinity there will be an optimum P value (termed P_0) such that those drugs possessing this value will be least inhibited in their movement through the aqueous and lipophilic phases of living tissue. Intuitively, it was felt that a parabola would approximate the relationship between the concentration of drug administered and the concentration at the active site (after a certain fixed time interval) under nonequilibrium conditions (39, 126). By definition, it is impossible to attain true equilibrium with a living system. Under certain conditions, with cells or isolated tissue, it may be possible to reach a pseudoequilibrium.

The finding of active sites by drugs can be regarded as a random walk process in which drug molecules must cross many membranes. This partitioning process is much like that of the drug's partitioning on and off of lipophilic macromolecules (Eqs. 2–4). An astronomical number of such events must occur with each drug molecule before it ultimately hits its final target. The progress a drug molecule makes in running this gantlet of aqueous and lipophilic phases is heavily dependent on its hydrophilic–lipophilic balance.

After the drug reaches the active site, it must partition onto it. This may be a much more specific kind of partitioning in which the steric and electronic characteristics of the drug may play rate-limiting roles. The rate of response can be formulated as:

$$\frac{d\ \text{response}}{dt} = Ak_XC \qquad \text{(Eq. 174)}$$

where A is the probability a drug molecule will reach the active site in the time Δt allotted for the test, C is the molar concentration of applied drug, and k_X is a rate or equilibrium constant for the combination of drug and receptor. In the first attempt to treat the problem mathematically (127), the assumption was made that A would be normally distributed with respect to log P:

$$A = ae^{-(\log P - \log P_0)^2/b} \qquad \text{(Eq. 175)}$$

For a fixed time interval of testing, d response/dt is constant so that Eq. 174 can be written as:

$$k_1 = ae^{-(\log P - \log P_0)^2/b} \cdot k_X \cdot C \qquad \text{(Eq. 176)}$$

Taking the logarithm of Eq. 176, collecting constants (bearing in mind that P_0 is a constant), and rearranging give:

$$\log 1/C = -k_2(\log P)^2 + k_3 \log P + k_4k_X + k_5 \qquad \text{(Eq. 177)}$$

In general, one might expect to correlate k_X *via* the linear combination of steric, electronic, and hydrophobic terms as in Eq. 178:

$$k_X = a \log P + b\sigma + cE_s + d \qquad \text{(Eq. 178)}$$

However, for the present review, data were selected to avoid cases where significant electronic and steric effects were involved so that it is assumed that log k_X is linearly related to log P. Substituting this into Eq. 177 yields:

$$\log 1/C = -k_2(\log P)^2 + k_3 \log P + k_6 \log P + k_7 \qquad \text{(Eq. 179)}$$

or:

$$\log 1/C = -k_2(\log P)^2 + k_8 \log P + k_7 \qquad \text{(Eq. 180)}$$

Four important parameters are associated with Eq. 180: k_2, k_8, k_7, and log P_0. The first three are obtained by fitting experimental data to Eq. 180. Log P_0 is found by setting $(d \log 1/C)/d \log P$ equal to zero and solving for log P. Since k_8 is the sum of k_3 and k_6, its value depends in part on the random walk process and in part on the hydrophobic interaction of the drug and the active site. The value of log P_0 also depends on the resultant sum of these two processes. This can be better visualized (40) by taking the derivative of Eq. 179, setting it equal to zero, and solving for log P:

$$\log P_0 = \frac{k_3}{2k_2} + \frac{k_6}{2k_2} \qquad \text{(Eq. 181)}$$

The first term on the left of Eq. 181 relates the localization rate at the sites of action to log P. Since there is reason to believe that this might be rather constant for certain types of systems (*e.g.*, mammalian), it could be defined as log P_i in the following equation:

$$\log P_0 = \log P_i + \frac{k_6}{2k_2} \qquad \text{(Eq. 182)}$$

The above equations, of course, rest on the Meyer–Overton assumption that partition coefficients between a fatty solvent and water serve to model partitioning between the lipophilic and aqueous phases of biological material; that is, Eq. 1 must hold where P_2 is from a reference system such as octanol-water and P_1 is a kind of average partition coefficient for the heterogeneous phases of biological tissue. Equation 1, first suggested by Collander (128), was shown to have certain but not unlimited generality (129). Not every solvent system serves as a suitable reference system. Recently, Seeman *et al.* (130) measured the partition coefficients for a series of alcohols between red cell ghosts and water. These were correlated (1) with octanol–water values in Eq. 183:

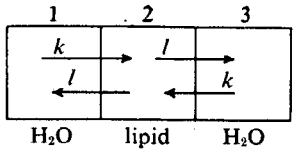
$$\log P_{\text{ghosts}} = 1.00 \log P - 0.88 \qquad \begin{matrix} n & r & s \\ 5 & 0.998 & 0.082 \end{matrix} \qquad \text{(Eq. 183)}$$

The slope of 1 in Eq. 183 indicates a 1:1 correspondence in the two processes. The negative intercept indicates that it is about seven times more difficult for an alcohol molecule to move into the ghost membrane than into octanol. Equation 183 does suggest that octanol–water is a good reference system to model partitioning in and out of membranes, while Eq. 2 and others of its kind (1, 5) show that octanol–water serves to model partitioning between an aqueous phase and proteins.

The many excellent linear correlations between log P values and various equilibrium and rate constants that are heavily dependent on partitioning processes (1, 5) emphasize that membranes and proteins in an aqueous environment are much more fluid than was indicated by the ideas developed up to 1960. The fluid mosaic model (131) of membranes developed by Singer suggests an ever changing, loose association of the lipids in which other large molecules may be rather loosely held. Branton (132) aptly described this model as: "a sea of lipid in which other molecules swim." Many enzymes and proteins must have a similar fluidity; otherwise, the kind of structure–activity correlations obtained using log P or π would not be possible. There are now over 1000 such correlations in the authors' data base alone.

The "parabolic" relationship between log $1/C$ and log P can be rationalized in a number of mechanistic ways. The following nine seem most important.

1. The kinetic model (34) is possibly the general explanation for truly complex systems such as whole animals. To formulate this model, assume a simple fluid membrane as depicted in Scheme I, where k is the rate constant for passage from the aqueous to the lipid phase, and l is the rate constant for the reverse passage. Compartment 1 has a given volume, V_1, and a given concentration of solute, A_1, at zero time. The other compartments have corresponding values. The



Scheme I

surface area between compartments is assumed to be the same for all. It is assumed that in living tissue, one is considering a "stirred" solution. The differential equations governing solute concentrations in the three compartments are:

$$\frac{dA_1}{dt} = \frac{S}{V_1}(lA_2 - kA_1) \qquad \text{(Eq. 184}a\text{)}$$

$$\frac{dA_2}{dt} = \frac{S}{V_2}(kA_1 - 2lA_2 + kA_3) \qquad \text{(Eq. 184}b\text{)}$$

$$\frac{dA_3}{dt} = \frac{S}{V_3}(lA_2 - kA_3) \qquad \text{(Eq. 184}c\text{)}$$

For cells of uniform volume and surface, $S/V_1 = S/V_2 = S/V_3 =$ constant.

In the general model, it was assumed that the solute was bound in the final phase with a rate constant m. The general set of differential equations is then:

$$\frac{dA_1}{dt} = -kA_1 + lA_2 \qquad \text{(Eq. 185}a\text{)}$$

$$\frac{dA_{2i}}{dt} = -2lA_{2i} + k(A_{2i-1} + A_{2i+1}) \qquad \text{(Eq. 185}b\text{)}$$

$$\frac{dA_{2i+1}}{dt} = -2kA_{2i+1} + l(A_{2i} + A_{2i+2}) \qquad \text{(Eq. 185}c\text{)}$$

$$\frac{dA_{n-1}}{dt} = -(l + m)A_{n-1} + kA_{n-2} \qquad n = \text{odd} \qquad \text{(Eq. 185}d\text{)}$$

$$= -(k + m)A_{n-1} + lA_{n-2} \qquad n = \text{even} \qquad \text{(Eq. 185}e\text{)}$$

$$\frac{dA_n}{dt} = mA_{n-1} \qquad \text{(Eq. 185}f\text{)}$$

In these equations, A_i represents the concentration in the ith phase and A_n that in the last phase. Since A_1/A_n does not depend on A_1^0, an arbitrary initial concentration such as 1.0 can be used. For a specific value of n, the partition coefficient ($P = k/l$) can be varied over an interval to obtain a series of solutions to the set of equations by integrating over time t. Values of k and l were chosen so that $k \times l = 1$; that is, it is assumed that there is a reciprocal relation between hydrophobic

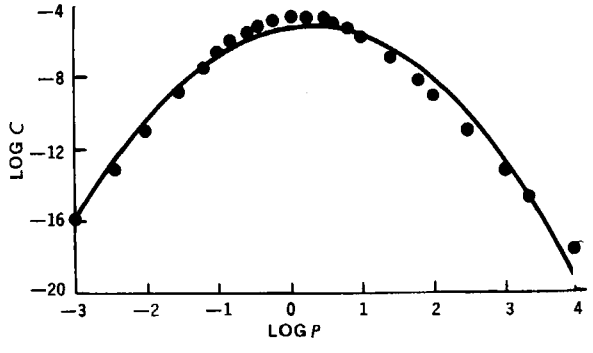


Figure 7—*Concentration in 20th compartment as a function of log* P *when* t = *10 and* m = *1. The curve is a parabola fitted to the calculated points by the method of least squares.*

character and hydrophilic character. The points in Fig. 7 show the concentration in the last compartment as a function of log P for a 20-barrier model when $t = 10$ and $m = 1$. The least-squares line in Fig. 7 results from fitting these points to Eq. 186:

$$\log A_n = a(\log P)^2 + b \log P + c \qquad \text{(Eq. 186)}$$

The fact that the points fit the line quite well justifies the postulate of Eq. 175.

The use of Eq. 180 for structure-activity correlations was also justified by McFarland (133) using a strictly probabilistic approach, which is, in effect, a kinetic justification.

As already considered, the parabolic relationship between log $1/C$ and log P has been rationalized in kinetic terms for systems not at or near equilibrium. The observed biological response is quite time dependent, and the parameters of Eq. 180, including log P_0, are at least in part determined by the time span allotted between the introduction of the drug and the "reading" of the biological response. For practical studies that one hopes to correlate *via* regression analysis, it is most important to achieve a sharp definition of Δt for the biological test.

2. The thermodynamic model (134) considers the case where, under certain conditions (for example, in a closed system of isolated tissue *in vitro*), one may approach rather close to equilibrium between drug in solution and drug on the sites of action. Generally, under such conditions (1) one can expect to find a high log P_0 (4–6) with a "linear" relationship between log $1/C$ and log P. However, Higuchi and Davis (134) showed that even under equilibrium conditions, one can expect to find "parabolic" relationships between log $1/C$ and log P. This time-independent model assumes that equilibrium or better quasiequilibrium conditions obtain (by definition, living systems are never at equilibrium). Their model is developed as follows:

a. The test system can be represented by the following compartments: w, 1, 2, 3, ... t, and r, where w represents the water phase and r the receptor. All the phases except w (1, 2, 3, *etc.*) are lipophilic. The effective volume of each compartment is V_w, V_1, V_2, *etc.*

b. Thermodynamic equilibrium is essentially reached so that, for all practical purposes, the activity of drug, inhibitor, or substrate is the same in each phase and all can be related to a standard reference state. The drug is distributed to all compartments according to Nernst's distribution law.

c. Biological or biochemical response is proportional to the fraction of active sites occupied by the substrates or inhibitors.

d. A relatively small amount (S) of the applied drug is attached to the receptor, the rest being in phases w, 1, 2, 3, ... t; that is:

$$S = C_wV_w + C_1V_1 + C_2V_2 + \ldots + C_iV_i \qquad \text{(Eq. 187)}$$

In Eq. 187, the C's refer to the effective concentration in each accessible phase. By assuming that drug distribution between the aqueous phase and each biophase follows a linear partition isotherm, the partition coefficient can be defined as:

$$K_i = \frac{C_i}{C_w} \qquad \text{(Eq. 188}a\text{)}$$

and:

$$S = C_w\left(V_w + \sum_{i=1}^{i=t} K_iV_i\right) \qquad \text{(Eq. 188}b\text{)}$$

The effective concentration of small molecules on the receptor is:

$$C_r = K_rC_w = \frac{SK_r}{V_w + \sum_{i=1}^{i=t} K_iV_i} \qquad \text{(Eq. 189)}$$

or:

$$E = \frac{C_r}{S} = \frac{K_r}{V_w + \sum_{i=1}^{i=t} K_iV_i} \qquad \text{(Eq. 190)}$$

It is assumed that relative biochemical response is proportional to E.

In a system where $V_w \gg \Sigma_{i=1}^{i=t}K_iV_i$, Eq. 190 reduces to:

$$E = \frac{K_r}{\text{constant}} \qquad \text{(Eq. 191)}$$

Increasing the partition coefficient results in increased activity up to the point set by bulk tolerance or micelle formation or to the point where $\Sigma_{i=1}^{i=t}K_iV_i > V_w$. This eventually occurs if there are compartments whose K_i's are much greater than K_r.

For the case where $V_w < \Sigma_{i=1}^{i=t}K_iV_i$, an increase in lipophilic character can yield a less active congener since now:

$$E \cong \frac{K_r}{K_iV_i} \qquad \text{(Eq. 192)}$$

For the comparison of relative activity of derivatives with a parent compound, Higuchi and Davis (134) defined the function R:

$$R = \frac{E}{E^*} = \frac{K_r\left(V_w + \sum_{i=1}^{i=t} K_i^*V_i\right)}{K_r^*\left(V_w + \sum_{i=1}^{i=t} K_iV_i\right)} \qquad \text{(Eq. 193)}$$

By the proper choice of parameters in Eq. 193, one can calculate various sets of R values, which Higuchi and Davis plotted against increasing numbers of carbon atoms in, for example, side chains. In this way, one obtains whole families of curves varying all the way from linear relations that level off at a limiting value to symmetrical parabolas.

3. The principle of bulk tolerance (135) could also lead to a nonlinear relationship between log $1/C$ and log P. In general, an increase in log P means an increase in the size of the drug. In going to larger members of a series of congeners, a point is reached where it becomes more and more difficult for each successively larger derivative to fit into or onto the active site.

4. Conformational distortion of the active site can also result in nonlinear relationships which may be

"parabolic" in certain instances. As members of a congeneric series become more hydrophobic, they produce greater distortions in a critical enzyme or membrane. This effect could account for the gradual change from agonist to antagonist often observed in the study of homologous series (136). Such distortions could be considered as overinduced fits. Koshland (137) showed that conformational changes in enzymes caused by the substrate induce the proper arrangement of enzyme components for catalytic activity. No doubt the hydrophobic portions of the substrate play an important role in inducing the proper fit. Such an induction could be overdone, with the resultant mismatching of parts producing less than optimal activity.

5. Metabolism could also be responsible for a biphasic relationship between log $1/C$ and log P. Since Brodie *et al.* (138) pointed out that there appears to be a direct relationship between the rate of microsomal metabolism and the lipophilic character of drugs, evidence has been found that this phenomenon can be quantitatively correlated using the log P scale (139, 140). As the members of a congeneric series become more lipophilic, other factors being equal, they are more rapidly destroyed by microsomal metabolism.

6. Micelle formation may, under certain conditions, account for a break in the linear relationship between activity and log P. Micelles can trap drug molecules (141). It is hard to imagine that drugs injected into whole animals could remain in micellar form when Eqs. 2–4 and many others of this type (1) indicate that organic compounds bind hydrophobically with so many of the macromolecules of living systems. However, in simpler systems such as isolated enzymes, micelle formation could be important. Even when working with drugs at concentrations below the CMC, it is possible that micelle-like clumps of molecules could form on enzyme (27) surfaces. These islands could function as a second compartment and produce a parabolic relationship *via* mechanism 2 above.

7. The limited solubility of the higher members of a congeneric series can, in principle, cause a "cutoff" in linear correlation between activity and lipophilic character. Ferguson (36, 37) demonstrated this, but it seems unlikely that this is a generally important mechanism because of the reasons discussed in connection with Eqs. 2–4.

8. Poisoning of an enzyme by a reaction product could also result in a biphasic relationship between log $1/C$ and log P. For example, consider a hydrolytic process in which an increase in log P results in better binding between enzyme and substrate. As log $1/K_m$ increases, overall hydrolysis goes more rapidly; but as log P for one of the hydrolysis products increases, desorption of this from the enzyme may become increasingly more difficult to the point where this step becomes rate controlling. It was shown (7) that the coefficient with π for enzyme–substrate complex formation is positive; but for the catalytic step, the coefficient for this term is negative.

9. Finally, the linear relation between log k and log P cannot prevail past the point where insufficient drug molecules are present to activate the minimum number of sites necessary to produce the standard biological response.

There are such a variety of reasons to expect parabolic relationships that it is extremely difficult or impossible to deduce in any given situation which mechanism or combination of mechanisms is responsible for the final result. What is most important to establish at this time is whether or not Eq. 180 can be employed to delineate the role of hydrophobic forces in the structure–activity relationship for a set of congeneric drugs. Even though, because of the variety of the discussed mechanisms, one cannot expect Eq. 180 to describe lipophilic effects perfectly and invariably, it will be enormously helpful in regression analysis if it can account for most of the variance in the hydrophobic effects. Only after these effects have been more or less separated can one begin to assign electronic and steric roles to the structural modifications present in a set of congeners.

The examples in Figs. 1–6 were selected to show the variation in types of parabolas as well as the variation in types of systems and drugs. While these examples and all of the others in Tables I and II are very well fit by symmetrical parabolas, this does not mean that other functions of log P would not give as good or even better correlations. For example, in Fig. 5 the results could also be interpreted to imply that activity increases linearly and levels off in a rather flat fashion. One might want to interpret Figs. 1 and 2 as being best described by two straight lines. Figure 2 is a very broad parabola, while Fig. 5 is more pointed. Plotting the data is helpful in understanding the variation in the linear terms (b) in Tables I and II. Since the constraint that the "best" symmetrical curve be drawn through the points is employed, a single very bad point on the right-hand side of the parabola can have a large influence on the value of b. Since these points are the most difficult to determine experimentally, caution must be used in interpreting the value of b. Only when a good spread in data points on both sides of the apex is present can one make significant comparisons with other equations.

The equations in Table I have first been categorized by log P_0, and within these sets they have been ordered on the slope of the linear term (b). One of the first points of interest is that the sets having the highest log P_0 values are composed of neutral compounds. Of the seven sets in Table I, Part D, only number 104 contains drugs ionized at pH 7. In this example, log P values were used for the neutral amines because log P ion is not available. Since these compounds are almost completely ionized at pH 7, log P_0 should be 3–4 units lower than the listed value of 5.8. This set might better be placed in Table I, Part B.

The largest number of log P_0 values in Table I falls in the 1.5–3.0 range. Many of those in Table I, Part C, are near 3 or have confidence intervals considerably below 3.

It is harder to generalize about log P_0 from Table II because of the wider confidence limits on the values of log P_0 in this set. In Table II the distribution of log P_0 values is more evenly spread. The most general statement that can be made about log P_0 is that values below 1 and above 4 are less common. Negative log P_0

values are rare. Out of 167 examples in Tables I and II, only four having negative signs occur. In this connection, it is interesting to point out that anticancer alkylating agents have negative log P_0 values (142).

It is clear from Tables I and II that the charge on a set of congeners has an important part in setting the value of log P_0. As mentioned previously, properly there are no uncharged molecules in Table I, Part D, and only one set in Table II, Part D. Since the apex of the parabola occurs at a lower value when either a positive or negative charge is present on a set of congeners, this suggests that the apolar portion of a molecule may exert a considerable drag on the molecule in its movement to the site of action, regardless of the fact that overall the molecule is relatively hydrophilic. On the other hand, it may be that the charge or a combination of the charge and the apolar moiety acting together causes the drag effect which results in a lower log P_0 for sets of ions.

In Table I there are 27 data sets where cationic drugs (the positive charge being on the organic ion) are acting on microorganisms. The mean value and standard deviation for log P_0 for these are 2.51 ± 0.43. Considering the great variety of organisms (Gram positive, Gram negative, and fungi) and the variety of drugs employed, this is a relatively sharp constant.

The results with anionic drugs are not as sharp. Omitting Eqs. 9 and 10, there are 16 examples that have a mean value of 2.34 ± 0.68. While the mean value is close to that of the cationic drugs, the standard deviation is much larger and would be even greater if the two data sets omitted were included. There are relatively few sets of cationic drugs acting on microorganisms among the less good correlations of Table II, while there are a good many sets of anionic compounds. Cationic drugs apparently show a more limited range of specificity and give more precise correlations with log P.

The log P_0 for charged compounds acting on microorganisms can be compared with log P_0 for neutral compounds. It was shown (58) that the log P_0 for neutral compounds acting *in vitro* on Gram-negative organisms is about 4, and for Gram-positive organisms it is about 6. The fact that it is possible to go to higher log P values in a congeneric series before reaching log P_0 may mean that these compounds are less hindered in their movement and that one can approach equilibrium in a shorter time.

In Table I there are very few examples of log P_0 in whole animals outside of the 1.5–3.0 range. For a wide variety of hypnotics acting in various whole animals, log P_0 of about 2 was observed (40, 143). No doubt, in these systems nothing approaching an equilibrium between drug in the open system (whole animal) and drug on the receptor sites occurs. The mean log P_0 of about 2.5 for charged drugs acting in closed *in vitro* systems against microorganisms may result from equilibrium not being reached because of the additional drag placed on drug movement by the positive or negative charge. Localization of the drug in the first lipophilic material it encounters appears to become severe as log P approaches 2–2.5.

Most equations in Tables I and II are based on log $1/C$ data; one can, therefore, compare intrinsic activities by comparing intercepts (listed under c). The value of the intercept is determined by the sensitivity of the system and the intrinsic activity of the pharmacophoric function in the set of congeners (32, 55). Comparing intercepts (other factors being equal) means comparing sets of congeners under isolipophilic conditions (log P = 0). If the systems have the same sensitivity, then differences in intercept represent differences in the stereoelectronic character of the pharmacophoric function common to the members of the set. The diversity of systems is so great in Tables I and II that not much in the way of useful generalization is possible. The mean intercept of cationic drugs (except guanidines) acting on microorganisms is about 3; this is close to the 3.2 value previously found for fungi (55). Neutral nonspecific compounds such as phenols and alcohols have low values (<1); RSCN, for example (Eq. 111), has a value of 3, showing a specificity at least two orders of magnitude above phenols, alcohols, and thioureas (Eq. 150).

In summary, it can be said that the present review provides a large amount of support for the thought embodied in Eq. 177; that is, if one can assume that the relationship between log $1/C$ and log P is well approximated by a parabola, then the role of the hydrophobic character of drugs can be at least roughly separated from the electronic and steric characteristics of drugs. This should be of great help in drug design. The many good correlations and the general agreement among log P_0 and intercept values for sets of charged congeners provide further support for the utility of log P values from the octanol–water system as an operational definition of relative lipophilic character.

Finally, it is hoped that this summary of equations will prove useful for comparison with the results of future work.

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Toxicity of Methyl- and Halogen-Substituted Alcohols in Tissue Culture Relative to Structure–Activity Models and Acute Toxicity in Mice

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Abstract □ The relationship of *in vitro* and *in vivo* toxicity of a series of methyl- and halogen-substituted alcohols was examined with respect to their octanol–water partition coefficients (P), charge (Q), and steric (E_s) parameters. A high correlation ($r = 0.98$) was found between tissue culture toxicity (ID_{50}, the concentration required to produce 50% inhibition of growth) and hemolytic activity (H_{50}, the concentration required to produce 50% hemolysis in saline, 37°). The product of intrinsic toxicity (T_i, the slope of the dose–response curve in tissue culture) and $1/P$ for aliphatic alcohols was found to have a uniform relationship to acute *in vivo* toxicity (LD_{50}, the single intraperitoneal dose required to kill 50% of the mice in 7 days). The T_i/P to LD_{50} ratio showed a fourfold variation as compared to a 164-fold variation for the T_i/LD_{50} ratio. This relationship supported the conclusions that T_i from tissue culture was a valid, time-independent estimate of the toxicant–receptor interaction and that $1/P$ was directly related to the effective aqueous concentration of the toxicants *in vivo*. The T_i/P to LD_{50} ratio was higher for the halogenated than for the aliphatic alcohols, which suggested a difference in mechanism of the toxicant–receptor interaction. Hansch analysis showed both Q and E_s parameters to be important to the differential intrinsic toxicities of the two groups. Both Free–Wilson and Hansch–Fujita analyses supported the conclusion that the tissue culture system conforms closely to the Higuchi–Davis equilibrium model. The usefulness of the tissue culture assay in conjunction with P for the analysis and prediction of *in vivo* toxicity was demonstrated.

Keyphrases □ Alcohols, methyl and halogen substituted—*in vitro–in vivo* toxicity, octanol–water partition coefficients, tissue culture toxicity concentration–hemolytic activity correlated, toxicant–receptor interaction estimates, structure–activity relationships discussed using models □ Tissue culture assay—predictive value *in vivo*, tissue culture toxicity concentration–hemolytic activity correlated, toxicant–receptor interaction estimates, structure–activity relationships of methyl- and halogen-substituted alcohols □ Structure–activity relationships, methyl- and halogen-substituted alcohols—discussed in terms of models, tissue culture assay, toxicant–receptor interaction estimates □ Partition coefficients, methyl- and halogen-substituted alcohols—octanol–water system, correlated with biological response, tissue culture assay studies □ Lipophilicity, alcohols, methyl and halogen substituted—relationship to *in vivo* aqueous toxicant concentration, tissue culture assay

Models of structure–toxicity relationships directed toward the rationalization of *in vivo* toxicity or other pharmacological responses are necessarily simplistic in approach and are limited in applicability by the constraints of simplicity and by the availability of data consistent with those constraints. A significant limitation of current models based on correlation of physical parameters of toxicant molecules with gross biological response *in vivo* is the simultaneous dependency of response on secondary time-dependent mechanisms (*e.g.*, transport, excretion, and biotransformation) and the primary time-independent toxicant–receptor interaction, both of which are affected by the physicochemical parameters of the toxicant. Meaningful interpretation of the correlation of any given physicochemical parameter with biological response depends upon independent knowledge of the relative importance of the parameter to these two basic processes.

The linear free energy model of Hansch and Fujita (1) and Fujita *et al.* (2), the thermodynamic equilibrium model of Higuchi and Davis (3), and the purely mathematical model of Free and Wilson (4) confound time-dependent and time-independent processes, and their predictive value is limited by the validity of necessary assumptions with respect to time-dependent processes, particularly in prediction of *in vivo* response where lipophilicity of the toxicant significantly influences the effective aqueous concentration through partitioning of the toxicant to hydrophobic compartments. An independent estimate of the primary structure-dependent, time-independent toxicant–receptor interaction would, therefore, be useful in the analysis and prediction of gross biological response. The objective of the present investigation was to assess the relevance of tissue culture assay of biological activity as a means of obtaining time-independent estimates of intrinsic toxicity (T_i, the slope of the dose–response curve)[1] and its predictive value with respect to gross biological response. A series of methyl- and halogen-substituted alcohols was examined, and comparative analyses were carried out with the Free–Wilson and Hansch–Fujita models.

TISSUE CULTURE ASSAY SYSTEM

The tissue culture system conforms closely to the constraints of the equilibrium (time-independent) model of structure–activity proposed by Higuchi and Davis (3). Their conditions and fundamental assumptions with respect to the equilibrium model are discussed here with respect to the tissue culture system:

1. "A biological test system can be represented by t number of accessible compartments, w, 1, 2, 3 . . . $t + r$, where compartment w is the aqueous phase; 1, 2, 3, *etc.*, are tissue, lipoidal, protein, *etc.*, phases; and r is the receptor [specific or nonspecific]." In the mouse fibroblast monolayer tissue culture system, the predominant compartment is w, containing a quantitatively defined toxicant concentration. The r compartment is extremely small compared to

[1] "Intrinsic toxicity" is related to intrinsic activity as discussed by Ariens (5).

w, and the lipoidal, protein, *etc.*, compartments (components of the culture medium) are small and uniform relative to w. The tissue compartment (the fibroblast monolayer), also very small, is initially uniform in volume with respect to w. A maximum fivefold change in the tissue compartment occurs during the test, a very small percentage change relative to w.

2. "Thermodynamic equilibrium or quasiequilibrium is reached in all accessible phases, and the thermodynamic activity of the drug in the rth compartment is the same as that in the aqueous, first, second, *etc.*, all with reference to a common standard state. If a drug is added to the aqueous compartment, it will be distributed to all the other available compartments according to Nernst's distribution law." The tissue culture system maximizes the opportunity for such conditions to prevail during the test. Higuchi and Davis (3) concluded that the same relationships would apply to systems in which the toxicant activities are governed by rates of transport across membrane barriers, since transport rate is directly influenced by equilibrium concentration on the surface of the barrier.

3. "For a series of drugs of closely related structures, biological activity is proportional to the fraction of the active sites occupied. If the fractions occupied are made the same, then equal biological response will be elicited." The linear dose–response relationships found in the tissue culture system and the hyperbolic relationship between ID_{50}, the dose required to produce 50% inhibition of growth, and T_i are consistent with this constraint.

4. "Essentially all of the administered drug will be distributed to the various accessible body compartments, and only an insignificant amount will actually be attached to the receptor site." If the previous assumptions are valid for the tissue culture system, it is reasonable to assume that this condition also prevails.

Potentially significant factors (time dependent) such as specific (enzymatic) or nonspecific reactions that produce tangible changes in activity or concentration of the toxicant are neglected in the equilibrium model and are uncontrolled in the tissue culture system. The tissue culture system does, however, conform to a high degree to the constraints of a time-independent, quasiequilibrium test system.

These considerations apply equally well to hemolysis as a time-independent test system for the estimation of toxicity. The real time (1 hr.) of the hemolysis assay is less than 2% of the real time (72 hr.) of the tissue culture assay, and a comparison of the results with the two assays should indicate any significant differences in time dependency. If equilibrium conditions prevail in the assay systems and secondary partitioning is negligible, time dependencies of the systems would be limited to those associated directly with the kinetics of the toxicant–receptor interaction. Therefore, if the experimental assay time is invariant, the quantitative response of the system to the toxicant should provide a time-independent estimate of the intrinsic activity.

EXPERIMENTAL

Tissue Culture Assay—A method similar to that of Rosenbluth *et al.* (6, 7) was employed to evaluate the growth inhibitory response of mouse fibroblast L-cells, NCTC clone 929 strain L (Earle) (8), to the alcohol series. The medium used for maintenance of stock cultures was modified Basal Medium Eagle[2] (BME) (9) supplemented with 5% newborn calf serum, 1% L-glutamine, and 50 mcg./ml. of streptomycin sulfate. Stock cultures were subcultured every 72 hr., and cells for inoculum were harvested using 0.05% trypsin in phosphate-buffered saline. The cells for inoculum, propagated as monolayers in 900-ml. (32-oz.) prescription bottles, after trypsin treatment, were suspended in BME containing 10% serum and 2% L-glutamine by repeated forceful jetting of a 20-ml. aliquot of the BME by syringe against the monolayer. The suspended cells were then transferred to a 250-ml. conical flask containing 80 ml. of the same BME preparation and a Teflon magnetic stirring bar. A gentle rate of stirring was used to keep the cells in suspension, and cell density (cells/ml.) was estimated by hemocytometer counts. Cell density was adjusted to 2×10^6 cells/ml. with the BME preparation.

Toxicants were dissolved and diluted with BME, which contained no serum or L-glutamine, immediately prior to use. A 1-ml. aliquot of the toxicant–BME solution was added to each growth tube followed by the addition of 1 ml. of cell suspension (2.0×10^5 cells/tube). Controls (72 hr.) received the identical treatment except for the absence of toxicant. Toxicant concentrations were employed to provide growth inhibition between 10 and 90%. Four to eight toxicant concentrations were used with fivefold replication. Zero-time and 72-hr. controls were run with 10-fold replication. Zero-time controls were harvested and washed by centrifugation, $540 \times g$ for 15 min., using phosphate-buffered saline (Dulbecco solution) (10), stored at 2–4° and assayed at 72 hr.

Both treated and 72-hr. control cultures were incubated at a 10° angle in a humidified 5% CO_2–air atmosphere at 37°. The protein content of each tube was determined after decantation of the medium and washing the monolayers with phosphate-buffered saline three times. The colorimetric method of Oyama and Eagle (11) was employed for the protein determination. Percent growth was calculated as follows:

$$\% \text{ growth} = \frac{(A - B)}{(A_0 - B)} \times 100 \qquad \text{(Eq. 1)}$$

where A is the mean absorbance for cultures receiving a specific dose of toxicant, A_0 is the mean absorbance for the untreated 72-hr. control cultures, and B is the mean absorbance of the initial zero-time controls. The ID_{50} and T_i were obtained from a least-squares analysis of the dose–response data (absorbance values from the protein determinations). The adjusted ID_{50} (Adj ID_{50}) values were obtained by substituting 100% growth (for the Y intercept) into the linear equation resulting from the least-squares fit of the dose–response data.

***In Vivo* Toxicity**[3]—Male albino mice[4], ICR strain, were used to determine the LD_{50} of each compound, *i.e.*, the dose required to kill 50% of the test animals within 7 days after a single intraperitoneal injection. The experimental procedure and method of calculation were described elsewhere (12).

Partition Coefficients—Partition coefficients (Table I) were determined by the method of Hansch and Muir (13), employing equal octanol–water volumes and analyzing the concentration of the solute in the water phase or octanol phase, depending on the equilibrium concentrations in the two phases. Partitioning was carried out at four concentrations of the solute and analyzed with the system developed by Bluestein and Posmanter (14) for aqueous solutions of alcohols using a chromatograph[5].

Charge Distribution—Charge parameters (Table I) were calculated by the method of Del Re (15) for each compound in the series, employing a computer[6]. The computer program and various parameters needed for the calculations were taken from the work of Bass (16).

The charge values reported (*i.e.*, Q_0, the net charge on the hydroxyl oxygen) are in terms of electrons and were not converted to electrostatic units.

Taft's E_s Steric Parameter—The E_s values (Table I) were taken from the work of Taft (17) and were based on hydrolysis of aliphatic esters as the model reaction or the hydrolysis of *ortho*-substituted benzoic acid esters in aromatic systems. The methyl group had an assigned value of 0.0.

Free–Wilson Analysis—The method of Free and Wilson (4) was employed to analyze the structure–activity relationships in the alcohol series with three independent sets of biological response data: ID_{50}, H_{50}, and LD_{50} (Table II). One derived set of data, Adj ID_{50}, was also analyzed, the negative logarithm of the biological response being used in all analyses. The Free–Wilson substituent model for the alcohols is given in Table III, along with the substituent matrix and symmetry equations employed in the analyses.

Hansch Analysis—Utilizing the approach of Hansch and Fujita (1) and Fujita *et al.* (2), the generalized equation given in Table IV was employed in the analysis of biological response data for the alcohol series. The series was analyzed in subsets as indicated in Table IV, and the substituent model employed is given in Table V. The regression analyses were carried out with a computer[6], em-

[2] Magnesium added as magnesium chloride, 177 mg./l.

[3] Data on the *in vivo* toxicity of the alcohols employed were obtained from W. H. Lawrence, Materials Science Toxicology Laboratories, University of Tennessee Medical Units, Memphis, Tenn.

[4] Harlan Industries, Cumberland, Ind.

[5] Beckman GC45.

[6] IBM 1620[II].

Table I—Parameters Used in Hansch Analysis of Biological Response Data

Compound[a]	Parameters[b]						
	P	Q_c	Q_0	$Q(R_1)$	$Q(R_2)$	$E_s(R_1)$	$E_s(R_2)$
1. Ethanol	0.50	0.0355	−0.4582	0.0026	0.0157	1.24	0.00
2. 1-Propanol	2.00	0.0617	−0.6411	0.0045	0.0364	1.24	−0.07
3. 2-Propanol	1.13	0.0825	−0.4615	0.0162	0.0162	0.00	0.00
4. 2-Methyl-1-propanol	5.71	0.0331	−0.4586	0.0026	0.0378	1.24	−0.47
5. 2,2-Dimethyl-1-propanol	20.78	0.0324	−0.4558	0.0026	0.0547	1.24	−0.93
6. 2-Butanol	4.07[c]	0.0812	−0.4616	0.0162	0.0248	0.00	−0.07
7. 3-Methyl-2-butanol	19.12	0.0802	−0.4618	0.0162	0.0381	0.00	−0.47
8. 3,3-Dimethyl-2-butanol	30.08	0.0794	−0.4619	0.0162	0.0549	0.00	−1.54
9. 2-Chloroethanol	1.06	0.0638	−0.4533	0.0030	0.0393	1.24	−0.24
10. 2,2-Dichloroethanol	2.34	0.0889	−0.4488	0.0034	0.0543	1.24	−1.54
11. 2,2,2-Trichloroethanol	22.30	0.1111	−0.4449	0.0038	0.0608	1.24	−2.06
12. 2-Bromoethanol	1.68	0.0586	−0.4542	0.0030	0.0400	1.24	−0.27
13. 2-Fluoroethanol	0.12	0.0522	−0.4553	0.0029	0.0688	1.24	−0.24
14. 1,1,1-Trifluoro-2-propanol	5.07	0.1227	−0.4555	0.0153	0.3836	0.00	−1.16

[a] 1-Bromo-2-propanol showed significant spontaneous chemical degradation at the time of determination of P and was eliminated from analyses involving P. Biological response data were obtained on the newly prepared compound (Table II). [b] P = octanol–water partition coefficient. The following symbols are based on the structural model (Table V): Q, charge parameters are in terms of electrons; E_s, Taft's steric parameter, see text; Q_c = charge on hydroxyl carbon; Q_0 = charge on hydroxyl oxygen; R_1 and R_2 = substituent for which the indicated parameter was calculated. [c] From *Reference 27*.

Table II—Biological Response Data for Alcohol Series

Compound	ID_{50}, M	CI[a]	Adj ID_{50}, M	T_i, %/mole	Intercept, % Growth	H_{50}[b], M	LD_{50}[c], mole/kg.
1. Ethanol	0.15540	0.00674	0.1641	−304.6	97.3	2.160	0.01208
2. 1-Propanol	0.05581	0.00197	0.0525	−950.9	103.1	0.698	0.00363
3. 2-Propanol	0.09843	0.00297	0.0983	−508.3	100.0	1.178	0.00608
4. 2-Methyl-1-propanol	0.02025	0.00129	0.0211	−2366.9	97.9	0.270	0.004208
5. 2,2-Dimethyl-1-propanol	0.01078	0.00022	0.0104	−4770.7	101.5	0.128	0.00505
6. 2-Butanol[d]	0.04300	0.00215	0.0360	−1130.0	110.0	——	0.008345
7. 3-Methyl-2-butanol	0.01439	0.00026	0.0072	−6857.2	148.7	0.168	0.00519
8. 3,3-Dimethyl-2-butanol	0.00329	0.00008	0.00255	−19566.5	114.4	0.073	0.00477
9. 2-Chloroethanol	0.03193	0.00115	0.0303	−1649.8	102.7	0.593	0.00168
10. 2,2-Dichloroethanol	0.00345	0.00015	0.0054	−9116.5	81.5	——	0.000746
11. 2,2,2-Trichloroethanol	0.00084	0.00003	0.000716	−69789.8	109.1	0.0072	0.000536
12. 2-Bromoethanol	0.00253	0.00003	0.00121	−41174.6	154.5	0.048	0.0009416
13. 2-Fluoroethanol	0.02522	0.00073	0.0250	−1997.3	100.4	——	0.000321
14. 1,1,1-Trifluoro-2-propanol	0.01492	0.00065	0.0167	−2986.1	94.6	——	0.0032877
15. 1-Bromo-2-propanol	0.00223	0.00004	0.002169	−23051.6	101.6	0.086	0.000319

[a] 95% confidence interval of $ID_{50} = ID_{50} \pm CI$. [b] Molar concentration effecting 50% hemolysis of rabbit erythrocytes; isotonic saline, 1 hr. at 37°. [c] Obtained from W. L. Lawrence, Materials Science Toxicology Laboratories (unpublished data, see text). [d] Graphical approximation of tissue culture parameters.

ploying a program based on IBM Program 06.0.148[7], "Single and Multiple Linear Regression Analyses."

RESULTS AND DISCUSSION

Biological Response Data—The primary biological response data are given in Table II. An illustrative set of dose–response curves obtained by tissue culture assay is given in Fig. 1, along with the indicated standard deviation of the primary data points. The standard deviation of the experimental data was of the order of ±5% for the 15 compounds investigated. The 95% confidence limits of the calculated ID_{50} values were also of the order of ±5%. The internal consistency of ID_{50} and T_i (hyperbolic relationship, Fig. 2) is an indication of the precision and accuracy of the tissue culture assays. It was concluded that differential evaporative loss of alcohols from the culture medium was not a significant factor in the tissue culture assay. The high correlation (r = 0.98) between the 1-hr. hemolysis assays (H_{50}) and the 72-hr. tissue culture assay (ID_{50}) was consistent with that conclusion. The precision of the H_{50} data was slightly greater than the ID_{50} data and may partly account for the higher explained variance (EV) with H_{50} data in the Hansch analysis. The higher lipophilicity of the erythrocyte membrane probably plays a significant role with respect to the higher absolute magnitude of toxicant required to produce an equivalent response in the hemolysis assay. The LD_{50} data had 95% confidence limits of ±10% or less.

[7] Provided by the Department of Molecular and Quantum Biology, University of Tennessee Medical Units, Memphis, Tenn.

Free–Wilson Analysis—A high degree of explained variance was obtained with ID_{50} data (92%) and H_{50} data (94%) with the Free–Wilson mathematical model (Table VI). Since the absolute times of the H_{50} assay and ID_{50} assay were significantly different, the consistency of results and the high degree of explained variance with the two sets strongly support the time independence of those assay systems. The poor fit of the Free–Wilson model with LD_{50} data was not surprising and indicated significant secondary interactions, shown by Hansch analysis to be associated with P, charge parameters, and steric parameters. This is consistent with the conclusion of Singer and Purcell (18) as to when the Free–Wilson model would be expected to fail.

The substituent group activities, as calculated from the Free–Wilson analysis of the ID_{50} data, are given in Table VII in order of decreasing activity. As expected from receptor theories, the parent portion of the molecule contributes the major portion of the biological activity. Calculated ID_{50} values are compared to observed values in Fig. 2. The only compound falling off the regression significantly was 2-fluoroethanol; this finding is consistent with its known conversion to a more active intermediate. The fact that the other compounds were very close to the calculated regression suggested that metabolic conversion did not play a major role in the toxicity of those compounds. The decrease in explained variance with Adj ID_{50} data is not consistent with the hypothesis of significant secondary partitioning of toxicant in the tissue culture system and supports the conclusion of time independence of the system. The theoretical ID_{50} for 2-butanol was calculated from substituent activities obtained in the analysis of ID_{50} values for the other members of the series prior to experimental determination of the ID_{50}. The values differed by 18%, indicating the predic-

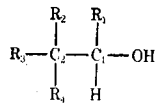


Table III—Substituent Model Employed in Free–Wilson Analysis

Compound	Substituents at C_1[a]		Substituents at C_2[b]				
	H	CH_3	H	CH_3	Cl	Br	F
1. Ethanol	1		3				
2. 1-Propanol	1		2	1			
3. 2-Propanol		1	3				
4. 2-Methyl-1-propanol	1		1	2			
5. 2,2-Dimethyl-1-propanol	1			3			
6. 2-Butanol (excluded)[c]							
7. 3-Methyl-2-butanol		1	1	2			
8. 3,3-Dimethyl-2-butanol		1		3			
9. 2-Chloroethanol	1		2		1		
10. 2,2-Dichloroethanol	1		1		2		
11. 2,2,2-Trichloroethanol	1				3		
12. 2-Bromoethanol	1		2			1	
13. 2-Fluoroethanol	1		2				1
14. 1,1,1-Trifluoro-2-propanol		1					3
15. 1-Bromo-2-propanol		1	2			1	

[a] Symmetry equation: position C_1, $-9H + 5CH_3 = 0$. C_1 = substituent position one. [b] Symmetry equation: position C_2, $-19H + 11CH_3 + 6Cl + 2Br + 4F = 0$. C_2 = substituent position two. [c] The Free–Wilson analysis was used to calculate the activity of this compound.

tive value of the Free–Wilson model for low molecular weight alcohols *in vitro*.

Hansch Analysis—The structural model employed for Hansch analysis is given in Table V. The following physical parameters (Table I) were used in the analysis. The octanol–water partition coefficient, P, was employed as a basic estimate of the relative lipophilicity of the compounds. The charge on the oxygen atom (Q_0), the charge on the hydroxyl carbon (Q_c), the charge on the R_1 substituent [$Q(R_1)$], and the R_1 steric parameter [$E_s(R_1)$] were associated with the hydroxyl end of the molecules. The R_2 steric parameter [$E_s(R_2)$] and the charge parameter [$Q(R_2)$] were associated with the net properties about the C_2 carbon which was included in the R_2 substituent. The results (EV) of selected single and multiple linear correlations of those parameters with biological response are given in Table IV.

The linear dependence of the ID_{50} of aliphatic alcohols on P (Fig. 3) is consistent with the conclusions that the tissue culture system is in quasiequilibrium and that the correlations with physical parameters obtained using the Hansch model relate primarily to the toxicant interactions with the mouse fibroblast L-cell receptor system. The consistency of the correlative results obtained with the ID_{50} and H_{50} lends further support to these conclusions. The regression of $-\log ID_{50}$ and $-\log H_{50}$ on $\log P$, considering all compounds, gave regression coefficients of 0.513 and 0.805 and intercepts of 0.554 and 0.134, respectively, indicating a greater sensitivity (19) of the hemolysis assay than the tissue culture assay to changes in P. The high degree of correlation of ID_{50} and H_{50} data and the general consistency of explained variance between the two sets with respect to physical parameters are consistent with the current concept of nonspecific membrane activity of alcohols (20). A linear dependency of $-\log$ biological response on $\log P$ should be expected when a one-step partitioning process is involved, as with external receptor sites of a cell membrane or in enzymatic studies (21). The linear dependence found for the aliphatic alcohols is consistent with that found by Hansch and Anderson (22). The generally lower explained variance obtained with LD_{50} data and the differential results *in vivo* and *in vitro* with respect to correlation with specific physical parameters are indicative of qualitative differences in the toxicant–receptor interaction *in vivo* and *in vitro*. Although qualitative differences were apparent in the Hansch analysis, the uniformity of the T_i/P to LD_{50} ratios within subhomologies of the alcohol series suggested that the differences were small (Tables VIII and IX).

Considering all *in vitro* data, P gave the highest overall single-parameter explained variance but gave no significant explained variance with *in vivo* data. The $Q(R_2)$ was the least quantitatively reliable parameter, but it was the only single parameter that gave a statistically significant explained variance with *in vitro* and *in vivo* data. However, the explained variance for halogenated alcohols *in vitro* decreased significantly as compared to the explained variance with P.

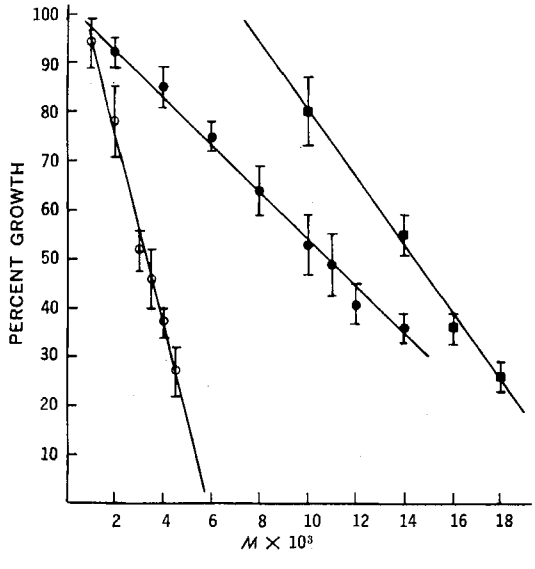


Figure 1—*Illustrative dose–response curve obtained for three alcohols by tissue culture assay. Key:* ●, *2,2-dimethyl-1-propanol;* ■, *3-methyl-2-butanol; and* ○, *3,3-dimethyl-2-butanol.*

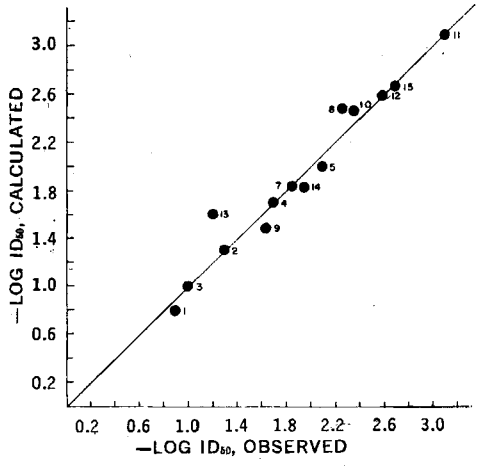


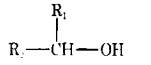
Figure 2—*Correlation of calculated $-\log ID_{50}$ (Free–Wilson analysis) and $-\log$ of experimentally observed ID_{50} (Table II).*

Table IV—Explained Variance of Biological Response Using Selected Equations of the General Form: $-\log BR = k(\log P)^2 = k_1 \log P + k_2 Q_x + k_3(E_s)_x + k_4$

	ID_{50}	Adj ID_{50}	H_{50}	LD_{50}	ID_{50}	Adj ID_{50}	H_{50}	LD_{50}	ID_{50}	Adj ID_{50}	H_{50}	LD_{50}	ID_{50}	Adj ID_{50}	H_{50}	LD_{50}
	Variable: P				Variables: P^2,P				Variables: $Q_c,E_s(R_2)$				Variables: $P^2,P,E_s(R_1)$			
All alcohols	0.25	0.27	0.46	−0.06[a]	0.23	0.26	0.40	−0.15[a]	0.63	0.46	0.66	−0.15[a]	0.30	0.29	0.46	−0.22[a]
Secondary	0.80	0.95	—	−0.22[a]	0.71[a]	0.93	—	−0.81[a]	0.84	0.66[a]	—	0.63	—	—	—	—
All aliphatic	0.91	0.94	0.98	0.16[a]	0.91	0.95	0.98	0.26[a]	0.85	0.79	0.70	−0.17[a]	0.89	0.94	0.97	0.19[a]
Secondary	0.86	0.96	0.96	0.07[a]	0.85	0.98	0.93	0.77[a]	0.998	0.96[a]	0.999	−0.38[a]	—	—	—	—
Primary	0.98	0.99	0.99	0.07[a]	0.98	0.99	0.99	0.78[a]	0.76[a]	0.78[a]	0.80[a]	0.05[a]	—	—	—	—
All halogenated	0.38[a]	0.27[a]	—	−0.20[a]	0.23[a]	0.08[a]	—	0.25[a]	0.33[a]	−0.02[a]	—	−0.11[a]	0.36[a]	0.16[a]	—	−0.05[a]
Primary	0.61	0.50[a]	—	−0.32[a]	0.44[a]	0.26	—	0.05[a]	0.17[a]	−0.25[a]	—	−0.41[a]	−0.11[a]	—	—	—
	Variable: $Q(R_2)$				Variables: P,Q_c				Variables: $Q_0,E_s(R_2)$				Variables: $P^2,P,Q(R_2)$			
All alcohols	−0.07[a]	−0.08[a]	0.68	−0.05[a]	0.24	0.25	0.48	−0.16[a]	0.63	0.47	0.65	−0.16[a]	0.16[a]	0.19[a]	0.61	−0.25[a]
Secondary	−0.27[a]	−0.33[a]	—	0.55	0.81	0.94	—	0.52[a]	0.84	0.66[a]	—	0.62	0.81[a]	0.98	—	0.85
All aliphatic	0.82	0.74	0.81	0.33	0.90	0.94	0.98	−0.01[a]	0.84	0.73	0.68	0.34[a]	0.91	0.93	0.98	0.47[a]
Secondary	0.999	0.96	0.95	0.22	0.86[a]	0.93[a]	0.99	−0.62[a]	0.98	0.997	0.94[a]	−0.36[a]	—	—	—	—
Primary	0.84	0.88	0.88	0.25	0.96[a]	0.97	0.98	0.45[a]	0.77[a]	0.80[a]	0.81[a]	0.14[a]	—	—	—	—
All halogenated	−0.16[a]	−0.13[a]	—	−0.16[a]	0.41[a]	0.46[a]	—	−0.47[a]	0.35[a]	0.05[a]	—	−0.36[a]	0.34[a]	0.16[a]	—	−0.06[a]
Primary	−0.33[a]	−0.33[a]	—	0.67	0.42[a]	0.33[a]	—	0.80	0.17[a]	−0.25[a]	—	−0.42[a]	0.05[a]	−0.47[a]	—	0.03[a]
	Variable: Q_c				Variables: P,Q_0				Variables: $Q(R_1),Q(R_2)$				Variables: $P^2,P,E_s(R_2)$			
All alcohols	0.09[a]	0.06[a]	0.13[a]	−0.08[a]	0.26	0.27	0.43	−0.16[a]	−0.13[a]	−0.16[a]	0.65	−0.07[a]	0.59	0.44	0.73	−0.19[a]
Secondary	−0.33[a]	−0.33[a]	—	0.43[a]	0.81	0.94	—	0.50[a]	0.999	0.95	—	0.59[a]	0.99	0.9999	—	−0.36[a]
All aliphatic	−0.15[a]	−0.12[a]	−0.15[a]	−0.16[a]	0.90	0.93	0.98	0.53	0.92	0.91	0.91	0.20	0.99	0.95	0.998	0.22[a]
Secondary	0.92	0.96	0.99	−0.01[a]	0.92[a]	0.99	0.95[a]	0.39[a]	0.998	—	—	—	—	—	—	—
Primary	−0.34[a]	−0.38[a]	−0.37[a]	−0.21[a]	0.96[a]	0.97	0.98	0.48[a]	0.87[a]	0.89[a]	0.90[a]	0.28[a]	—	—	—	—
All halogenated	−0.08[a]	−0.18[a]	—	−0.25[a]	0.44[a]	0.18[a]	—	0.34[a]	−0.14[a]	−0.11[a]	—	−0.07[a]	−0.13[a]	−0.36[a]	—	0.61[a]
Primary	0.43	0.17[a]	—	−0.19[a]	0.42[a]	0.32	—	0.80	0.33[a]	0.01[a]	—	0.52[a]	−0.07[a]	−0.41[a]	—	0.41[a]
	Variable: Q_0				Variables: $P,Q(R_1)$				Variables: $P,Q_c,Q(R_2)$				Variables: P^2,P,Q_c			
All alcohols	0.02[a]	0.01[a]	−0.02[a]	−0.08[a]	0.32	0.30	0.51	−0.12[a]	0.18[a]	0.19[a]	0.71	−0.25[a]	0.24[a]	0.25[a]	0.40	−0.26[a]
Secondary	−0.33[a]	−0.33[a]	—	0.42[a]	0.81	0.94	—	0.51[a]	0.995	0.98	—	0.12[a]	0.79[a]	0.97[a]	—	0.89[a]
All aliphatic	−0.11[a]	−0.11[a]	−0.11[a]	0.10[a]	0.90	0.93	0.98	0.05[a]	0.91	0.93	0.98	0.15[a]	0.89	0.94	0.97	−0.08[a]
Secondary	0.94	0.997	0.97	0.26[a]	0.71[a]	—	—	—	—	—	—	—	—	—	—	—
Primary	−0.42[a]	−0.44[a]	−0.44[a]	−0.09[a]	0.96	0.97	0.98	0.48[a]	—	—	—	—	—	—	—	—
All halogenated	0.50	0.29	—	−0.02[a]	0.55[a]	0.43[a]	—	−0.48[a]	0.29[a]	0.21[a]	—	0.72[a]	0.28[a]	0.36[a]	—	−0.06[a]
Primary	0.43[a]	0.18[a]	—	−0.18[a]	0.44[a]	0.26[a]	—	0.78[a]	0.29[a]	0.43[a]	—	0.68[a]	−0.10[a]	−0.13[a]	—	0.84[a]
	Variable: $E_s(R_2)$				Variables: $P,Q(R_2)$				Variables: $E_s(R_1),E_s(R_2)$				Variables: $P,Q_c,E_s(R_2)$			
All alcohols	0.65	0.51	0.68	−0.07[a]	0.18[a]	0.20[a]	0.65	−0.14[a]	0.65	0.47	0.65	−0.12[a]	0.59	0.43	0.62	−0.17[a]
Secondary	0.78	0.55	—	0.40[a]	0.82	0.94	—	0.53[a]	—	—	—	—	0.995	0.996	—	0.27[a]
All aliphatic	0.86	0.78	0.74	0.02[a]	0.92	0.93	0.98	0.22[a]	0.85	0.78	0.70	−0.14[a]	0.97	0.97	0.98	−0.24[a]
Secondary	0.87	0.73	0.70[a]	0.29[a]	0.998	0.995	0.98	−0.49[a]	—	—	—	—	—	—	—	—
Primary	0.81	0.79	0.81	−0.32[a]	0.97	0.97	0.98	−0.28[a]	—	—	—	—	—	—	—	—
All halogenated	0.34[a]	0.09[a]	—	−0.11[a]	0.52[a]	0.42[a]	—	−0.52[a]	0.36[a]	0.03[a]	—	−0.22[a]	0.56[a]	0.37[a]	—	0.36[a]
Primary	0.45[a]	−0.15[a]	—	−0.11[a]	0.50[a]	0.26[a]	—	0.51[a]	0.17[a]	—	—	—	0.84[a]	0.62[a]	—	0.83[a]
	Variables: $Q_0,E_s(R_1)$				Variables: $P,E_s(R_2)$				Variables: $Q(R_2),E_s(R_2)$				Variables: $P^2,P,Q(R_1)$			
All alcohols	−0.03[a]	−0.06[a]	−0.11[a]	−0.12[a]	0.63	0.48	0.65	−0.08[a]	0.65	0.50	0.71	0.14	0.29	0.29	0.45	−0.22[a]
Secondary	—	—	—	—	0.99	0.994	—	0.29[a]	0.82	0.62[a]	—	0.63[a]	0.80[a]	0.97[a]	—	0.88[a]
All aliphatic	−0.29[a]	−0.24[a]	−0.29[a]	−0.07[a]	0.98	0.96	0.99	0.00[a]	0.89	0.78	0.80	0.39[a]	0.89	0.94	0.97	0.16[a]
Secondary	—	—	—	—	0.995	0.996	0.98	−0.41[a]	0.998	0.99	0.99	−0.42[a]	—	—	—	—
Primary	—	—	—	—	0.96[a]	0.98	0.98	0.99	0.83[a]	0.86[a]	0.87[a]	0.48[a]	—	—	—	—
All halogenated	0.34[a]	0.05[a]	—	−0.31[a]	0.23[a]	0.03[a]	—	0.55[a]	0.38[a]	0.06[a]	—	−0.29[a]	0.36[a]	0.17[a]	—	−0.06[a]
Primary	0.14[a]	—	—	—	0.46[a]	0.26[a]	—	0.48[a]	0.28[a]	−0.11[a]	—	0.53[a]	−0.11[a]	−0.32[a]	—	0.60[a]

[a] Less than 90% statistical confidence by F test.

Table V—Substituent Model Employed for Hansch Analyses



Compound	R_1	R_2
1. Ethanol	H	CH_3
2. 1-Propanol	H	C_2H_5
3. 2-Propanol	CH_3	CH_3
4. 2-Methyl-1-propanol	H	iso-C_3H_7
5. 2,2-Dimethyl-1-propanol	H	*tert*-C_4H_9
6. 2-Butanol	CH_3	C_2H_5
7. 3-Methyl-2-butanol	CH_3	iso-C_3H_7
8. 3,3-Dimethyl-2-butanol	CH_3	*tert*-C_4H_9
9. 2-Chloroethanol	H	$ClCH_2$
10. 2,2-Dichloroethanol	H	Cl_2CH
11. 2,2,2-Trichloroethanol	H	Cl_3C
12. 2-Bromoethanol	H	$BrCH_2$
13. 2-Fluoroethanol	H	FCH_2
14. 1,1,1-Trifluoro-2-propanol	CH_3	F_3C

The importance of charge on the hydroxyl end of the molecule can be seen in the correlations with Q_0 and P,Q_0, the latter giving the highest explained variance "overall" *in vitro* and *in vivo*. The two-parameter fit with $P,Q(R_2)$ gave a slightly increased overall explained variance *in vitro*, particularly for the halogenated alcohols as compared to $Q(R_2)$ alone. The importance of substituents at C_2 is apparent in the correlation with $P,E_s(R_2)$, which gave significant explained variance for all compounds *in vitro*, an excellent fit *in vivo* for primary aliphatic alcohols (99.5%), and a substantial explained variance (although of low statistical confidence) for the halogenated alcohols. The significance of the methyl group in the C_1-position was reflected in the $P,E_s(R_2)$ statistics for the primary and secondary aliphatic alcohols and suggested that the steric aspects of the receptor site favor the hydrogen substituent at C_1. The possible significance of steric parameters was suggested by Hansch *et al.* (23). It is significant that $P,E_s(R_2)$ gave a substantially higher explained variance *in vivo* for the aliphatic primary alcohols than the P^2,P correlation. This suggests that toxicant–receptor interactions are of primary importance *in vivo* and the explained variance is of the same high order of magnitude as for the *in vitro* correlations where there are nonstatistical reasons to believe that the biological response is essentially dependent on the toxicant–receptor interaction. Although P^2,P correlations for both primary and secondary aliphatic alcohols *in vivo* gave 78 and 77% explained variance, respectively, the partial regression coefficients for P^2 were −0.495 and 0.342, respectively, which raises a question as to the physical significance of the P^2 term (24). The difference in sign of the coefficients and the highly significant difference in explained variance between the primary and secondary aliphatic alcohol with $P,E_s(R_2)$ *in vivo* indicated a significant qualitative difference between the *in vitro* and *in vivo* systems. However, the magnitude of this difference cannot be greater than that responsible for the fourfold variation observed in the T_i/P to LD_{50} ratios for those compounds (discussed below). The correlation with $E_s(R_2)$ alone gave a slightly lower overall explained variance *in vitro* than $P,E_s(R_2)$ but gave a significantly lower explained variance with *in vivo* data, indicating a qualitative difference *in vitro* and *in vivo*.

The high, statistically significant explained variance (94–99.7%) *in vitro* for the secondary aliphatic alcohols was associated with relatively high positive values of Q_c as compared to from −42 to −44% explained variance for the primary aliphatic alcohols associated with significantly lower Q_c values. Halogenation of the primary aliphatic alcohols induces a higher positive charge on the hydroxyl carbon, and the explained variance for that set increased to 43% (statistically significant). Although the absolute values of Q_0 do not vary as much as Q_c, the same statistical relationship exists for that parameter and, as pointed out previously, contributes significantly to the P,Q_0 correlation. The correlations with $Q_c,E_s(R_2)$ and $Q_0,E_s(R_2)$ gave a generally lower explained variance than $P,E_s(R_2)$. This was not surprising because P depends upon the overall physical properties of the molecules and has greater predictive value than single parameters associated with specific or localized molecular properties. Low order correlations with specific parameters, although not expected to give high explained variances, serve to isolate those factors of importance to net biological response.

For any significant improvement in overall explained variance for the halogenated alcohols, a three-parameter fit using P, Q_c, and $E_s(R_2)$ was required. This suggested the importance of both charge and steric interactions; however, the statistical confidence of the correlations was less than 90%. The generally lower explained variance obtained for the halogen-substituted alcohols can be rationalized in terms of the heterogeneity of that set of compounds. Except for mono-, di-, and trichloroethanol, there is no systematic homology of substitution; each of the other members of the set represents single members of different subhomologies with different mechanisms of action, as indicated by the T_i/P to LD_{50} ratios.

The Adj ID_{50} values were calculated on the assumption that the variation in the percent growth intercept reflected a variation in the effective concentration of toxicant as a result of partitioning of the toxicant to nonaqueous compartments (the cell monolayer and components of the medium). Although the Free–Wilson results tended to support the assumption of nonsignificant partitioning in the cell culture system, the results of the Hansch analysis on the Adj ID_{50} data indicated that there may have been a small amount of toxicant loss due to partitioning for the secondary (high partitioning) aliphatic alcohols. In general, there is a slight decrease in the Adj ID_{50} explained variance or no change at all as compared with the ID_{50} explained variance. This is probably a result of the random statistical error in the intercept being greater than the real, but small, partitioning effect *in vitro*.

Linear equations relating biological response and physical parameters, which give a high degree of explained variance, have value for the prediction of biological response of untested con-

Table VI—Statistical Results of Free–Wilson Analyses

Statistical Parameters	Biological Response: ID_{50}	Adj ID_{50}	H_{50}[a]	LD_{50}
r^{2}[b]	0.95	0.93	0.97	0.54
Explained variance (*EV*), %	92	88	94	25
Significance of *F* ratio, %	99.9	99.9	99.9	90

[a] Biological response for 11 compounds. 2-Butanol, 2,2-dichloroethanol, 2-fluoroethanol, 1,1,1-fluoro-2-propanol, and 1-bromo-2-propanol were not included. [b] The square of the coefficient of multiple correlation, *r*.

Table VII—Substituent Group Contribution to ($-\log ID_{50}$)

Substituent	Activity ($-\log ID_{50}$) Dependent Variables, Methyl[a] and Bromine[b]
Substituent position C_1	
Methyl	0.085323
Hydrogen	−0.047402
Substituent position C_2	
Bromine	1.330441
Chlorine	0.409717
Methyl	0.089450
Fluorine	−0.014229
Hydrogen	−0.318222
Overall average, μ	1.911543

[a] At substituent position C_1. [b] At substituent position C_2.

Table VIII—Relationship of T_i, P, and LD_{50}: Aliphatic Alcohols

Compound	$[T_i/LD_{50}] \times 10^{-4}$	$[(T_i/P)/LD_{50}] \times 10^{-4}$
1. Ethanol	2.5	5.0
2. 1-Propanol	26.2	13.1
3. 2-Propanol	8.4	7.4
4. 2-Methyl-1-propanol	56.3	9.9
5. 2,2-Dimethyl-1-propanol	94.5	4.6
6. 2-Butanol	13.5	3.3
7. 3-Methyl-2-butanol	132.2	6.9
8. 3,3-Dimethyl-2-butanol	410.2	13.6

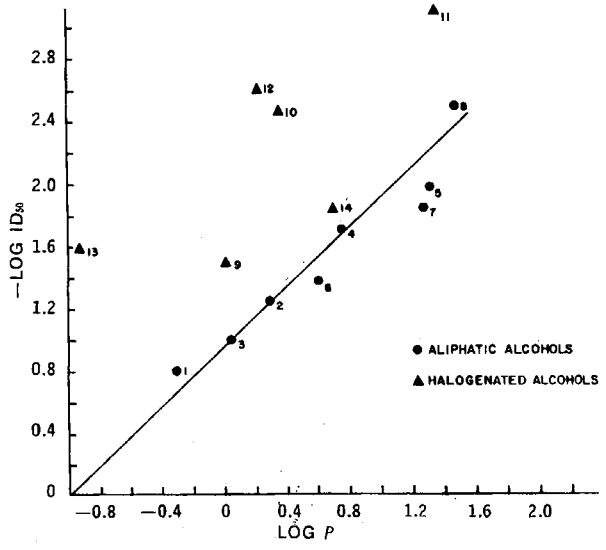


Figure 3—*Correlation of −log ID_{50} and log* P. (*For key, see Table II.*)

geners. Selected equations giving the highest explained variance for the alcohol series investigated are given in Table X.

Time Independence of T_i and ID_{50}—The essentially perfect hyperbolic relationship between T_i and ID_{50} (*i.e.*, linear relationship between ID_{50} and $1/T_i$) (Fig. 4) gives strong support for the conclusion that equilibrium conditions prevail in the tissue culture system. That T_i bears this direct relationship to cellular toxicity, the ID_{50}, is consistent with the conclusion that it provides an estimate of the time-independent interaction or affinity of the toxicant with the receptor system of the cell. The overlap of the intrinsic toxicities of the methyl-substituted and halogen-substituted members of the series, divergent in chemical and physical attributes, within the hyperbolic distribution is further indication that T_i is an estimate of the toxicant–receptor interaction rather than secondary factors such as diffusion. The linearity of the dose–response relationship in the tissue culture was consistent with the assumptions of equilibrium and that the assay conditions were such that a first-order relationship prevailed in the toxicant–receptor interaction. The findings of Fink and Bender (25) regarding the kinetics and mechanism of inhibition of papain-catalyzed

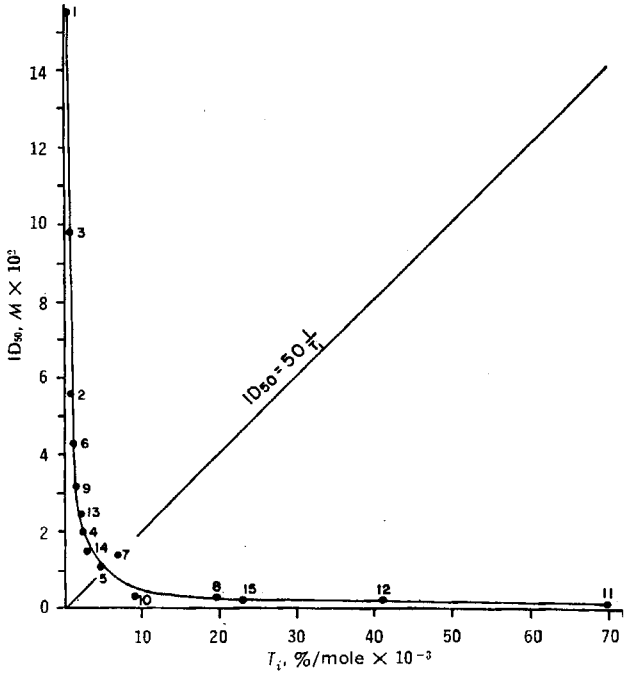


Figure 4—*Correlation of ID_{50} and* T_i. (*For key, see Table II.*)

Table IX—Relationship of T_i, P, and LD_{50}: Halogenated Alcohols

Compound	$[T_i/LD_{50}] \times 10^{-4}$	$[(T_i/P)/LD_{50}] \times 10^{-4}$
9. 2-Chloroethanol	98.2	92.7
10. 2,2-Dichloroethanol	1222.1	522.2
11. 2,2,2-Trichloroethanol	13020.4	583.9
12. 2-Bromoethanol	437.3	260.3
13. 2-Fluoroethanol	622.2	5185.0
14. 1,1,1-Trifluoro-2-propanol	90.8	17.9

hydrolyses by a series of straight-chain, primary alcohols is consistent with the observed tissue culture response (T_i and ID_{50}) obtained with the alcohol series in the present investigation. The shift in the kinetics observed in the enzyme system in the presence of an increasing molecular weight of the alcohols coincided with the hyperbolic break in ID_{50} values obtained in tissue culture in this investigation, which gives further indirect support to the conclusion that the tissue culture system reflects a toxicant–receptor interaction.

Relationship of Tissue Culture Intrinsic Toxicity (T_i) and Acute Toxicity (LD_{50}) in the Mouse—The hyperbolic relationship of T_i and ID_{50} (Fig. 4) relates to the degree of inhibition or saturation of the receptor system and not to the mechanism of the inactivation. If the receptor system of the mouse fibroblast cell in tissue culture is an adequate model for the receptor system in the mouse and if quasiequilibrium conditions attain in the 7-day LD_{50} determination, the product of T_i and the equilibrium concentration of the toxicant in the aqueous phase (proportional to $1/P$) should have a direct and constant relationship:

$$\frac{T_i/P}{LD_{50}} = \text{constant } (C) \qquad \text{(Eq. 2)}$$

where:

T_i = time-independent intrinsic toxicity of the toxicant
P = time-independent octanol–water partition coefficient of the toxicant (assuming the octanol–water system to be an adequate model of the partitioning system *in vivo*)
LD_{50} = single intraperitoneal dose required to kill 50% of the test animals in 7 days (which assumes percent kill to be directly proportional to the number of receptor sites inactivated)

If T_i is a valid time-independent estimate of intrinsic toxicity, experimental variations in C reflect the time-dependent reactions or processes *in vivo* that affect equilibrium concentration of toxicant, intrinsic toxicity of the toxicant (through biotransformation), and constancy of the receptor system.

The ratio of T_i to LD_{50} varied 164-fold and 143-fold for the aliphatic and halogenated alcohols, respectively (Tables VIII and IX). The inclusion of P in the T_i/P to LD_{50} ratio reduced the variation to fourfold for the aliphatic alcohols, indicating that the T_i/P relationship largely accounted for the *in vivo* toxicity of that series. Involved in the fourfold variation was the statistical accuracy of the parameters, the fact that T_i and LD_{50} were expected to vary inversely, and the fact that the ratio was the result of dividing large numbers by small numbers. It was, therefore, not possible to ascribe the observed variations to time dependencies, but the magnitude of time dependencies that exist must fall within the observed variation in the ratio.

The ratios obtained with the halogenated alcohols were higher than for the aliphatic alcohols and had a significantly higher range of variation overall. This was consistent with the greater heterogeneity of structural substituents within that set. It also suggested a qualitative difference in the toxicant–receptor interaction as compared to the aliphatic alcohols or a qualitative difference in the receptor systems affected by the two sets. The qualitative differences between the sets observed in the Hansch analysis, particularly with respect to charge parameters, and the consistency of the hyperbolic relationship of T_i and ID_{50} suggested that the primary receptor system was the same for the two sets. It was significant that the magnitude of the deviations of the halogenated set from the linear regression of $-\log ID_{50}$ on $\log P$ for aliphatic alcohols (Fig. 3) generally followed the shift in magnitude of the T_i/P to LD_{50} ratio, which was consistent with the conclusion that the variation

Table X—Linear Regression of Biological Activity on Physical Parameters: Selected Equations

	EV, %
All compounds	
$-\log ID_{50} = -0.787E_s(R_2) + 0.310$	65
$-\log$ Adj $ID_{50} = -0.751E_s(R_2) + 0.380$	51
$-\log H_{50} = 0.805 \log P + 0.134$	46
$= -0.875E_s(R_2) + 0.166$	68
All secondary alcohols	
$-\log ID_{50} = 0.904 \log P - 0.042$	80
$= 0.549 \log P - 0.432E_s(R_2) - 0.028$	99
$-\log$ Adj $ID_{50} = 1.06 \log P - 0.084$	95
$= 0.860 \log P - 0.242E_s(R_2) - 0.076$	99
$-\log LD_{50} = 0.342 \log P^2 - 0.409 \log P + 9.25Q_c - 1.53$	89
$= 0.350 \log P^2 - 0.421 \log P + 62.56Q_0 + 28.10$	88
All aliphatic alcohols	
$-\log ID_{50} = 0.820 \log P - 0.011$	91
$= 0.501 \log P - 0.445E_s(R_2) + 0.01$	98
$-\log$ Adj $ID_{50} = 0.917 \log P - 0.018$	94
$-\log H_{50} = 0.772 \log P - 0.093$	98
$-\log LD_{50} = 0.178 \log P - 1.69Q_0 - 1.70$	53
All primary aliphatic alcohols	
$-\log ID_{50} = 0.735 \log P + 0.049$	98
$-\log$ Adj $ID_{50} = 0.751 \log P + 0.042$	99
$-\log H_{50} = 0.770 \log P - 0.078$	99
$-\log LD_{50} = -0.495 \log P^2 + 0.712 \log P - 0.803$	78
$= 1.04 \log P + 1.40E_s(R_2) - 0.768$	99
All secondary aliphatic alcohols	
$-\log ID_{50} = 0.932 \log P - 0.122$	86
$= 0.497 \log P - 0.484E_s(R_2) - 0.003$	99
$-\log$ Adj $ID_{50} = 1.07 \log P - 0.124$	96
$= 0.809 \log P - 0.294E_s(R_2) - 0.052$	99
$-\log H_{50} = 0.778 \log P - 0.121$	96
$= -388.22Q_c + 31.93$	99
$-\log LD_{50} = 0.342 \log P^2 - 0.427 \log P - 0.769$	77
All halogenated alcohols	
$-\log ID_{50} = 0.592 \log P + 1.00$	38
$= 115.07Q_0 + 53.19$	50
$-\log LD_{50} = 0.292 \log P^2 - 1.40 \log P + 43.86Q_c - 5.50Q(R_2) - 2.93$	89
All primary halogenated alcohols	
$-\log ID_{50} = 0.704 \log P + 1.10$	61
$= 1.57 \log P - 701.65Q_0 - 2.85E_s(R_2) - 318.22$	90
$= 1.52 \log P - 114.26Q_c - 2.61E_s(R_2) + 7.22$	84
$-\log$ Adj $ID_{50} = 0.721 \log P + 1.14$	50
$= 1.97 \log P - 136.18Q_c - 2.79E_s(R_2) + 8.65$	62
$= 2.04 \log P - 836.34Q_0 - 3.08E_s(R_2) - 379.25$	62
$-\log LD_{50} = -1.38 \log P + 48.27Q_c - 3.39$	80
$= -1.36 \log P + 269.82Q_0 + 121.99$	80

in the ratio reflects a variation in the toxicant–receptor interaction rather than secondary time dependencies. The variation in ratios of T_i to LD_{50} for the halogenated set was of the same order of magnitude as for the aliphatic set and, excluding the fluorine-substituted compounds, the sixfold variation of the T_i/P to LD_{50} ratios was of the same order of magnitude as for the aliphatic set, indicating again the significance of T_i and P in the rationalization of *in vivo* response. The significantly higher ratio for 2-fluoroethanol is consistent with its known conversion to an intrinsically more toxic intermediate (26). The ratio for 1,1,1-trifluoro-2-propanol was only slightly higher than for the aliphatic series. Structurally, it resembles more closely its homolog, 2-propanol, in the aliphatic series than the other halogenated alcohols resemble their respective homologs because of the distinctly larger size of the chlorine and bromine substituents. The significance of substituent size can be seen in the mono-, di-, and trichloroethanol ratios and in the fact that the bromoethanol ratio falls between mono- and dichloroethanol. This is consistent with the significant contribution of $E_s(R_2)$ in the Hansch analysis.

The T_i/P to LD_{50} ratio reflects qualitative similarities or differences in mechanism and has value in the prediction of *in vivo* toxicity when the magnitude of the ratio is established for a well-defined series of congeners, as in the aliphatic series of alcohols. In this study, the ID_{50} of 2-butanol was predicted prior to the experimental determination of the LD_{50}. By using the theoretically calculated ID_{50} from the Free-Wilson analysis, T_i was estimated from the hyperbolic relationship of ID_{50} and T_i (Fig. 4) and was used in conjunction with P and the average value of the T_i/P to LD_{50} ratio for secondary aliphatic alcohols to predict the LD_{50} of 2-butanol. The predicted value was a factor of 2 lower than the experimentally determined LD_{50} but was within the 95% confidence limits of the comparison and well within the fourfold variation of the aliphatic set.

When LD_{50} is plotted against T_i, the basic hyperbolic relationship is observed (Fig. 5), with the high partitioning secondary alcohols and primary alcohols showing significantly lower toxicity than expected, resulting from active partitioning into the hydrophobic compartments of the animal. The position of 1,1,1-trifluoro-2-propanol falls between the secondary aliphatic alcohols and the lower segment of the hyperbolic curve made up primarily of the halogenated compounds. This relationship was reflected in the T_i/P to LD_{50} ratio already discussed. 1,1,1-Trifluoro-2-propanol is a single member of a subhomology, and mono- and difluoro-2-propanol would be expected to fall on a curve between 2-propanol and 1,1,1-trifluoro-2-propanol; significant deviation from this relationship would indicate an alteration in mechanism by metabolic conversion or other specific secondary reactions affecting T_i, as found in the case of 2-fluoroethanol.

The "hyperbolic" relationship of LD_{50} and T_i is not as precise as that found in the ID_{50} and T_i relationship. The LD_{50} values through which the curve is drawn are high with respect to a true hyperbolic relationship. The magnitude of the deviations for the high partitioning halogenated alcohols was less than those found in the aliphatic set, which is consistent with the suggested qualitative

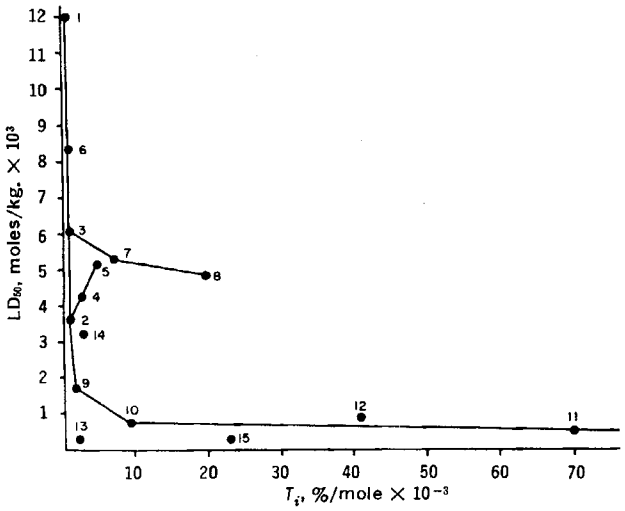


Figure 5—*Correlation of* LD_{50} *and* T_i. (*For key, see Table II.*)

difference in mechanism for the halogenated compounds. The general hyperbolic relationship of LD_{50} and T_i and the fact that the octanol–water partition coefficient serves as a basis for the rationalization of *in vivo* response with respect to T_i indicate a significant relationship of *in vitro* T_i to *in vivo* response. They also indicate the pertinence of the Higuchi equilibrium model for the rationalization of *in vivo* response where estimates of effective aqueous concentration of toxicant are available. The significant correlation of *in vitro* and *in vivo* toxicity through the T_i/P to LD_{50} relationship strongly supports the assumption that P is the predominant factor in determining the time course of toxicant effect *in vivo* and that the LD_{50} based on mortality data at 7 days provides adequate time for expression of dose-related primary toxicity. This seems reasonable in view of the relatively rapid assimilation and excretion of low molecular weight alcohols. Variations in the time dependencies of these processes no doubt contribute to the four- to sixfold variation in the T_i/P to LD_{50} ratios.

CONCLUSIONS

The results of this investigation support the conclusion that intrinsic cellular toxicities obtained by tissue culture assay of methyl- and halogen-substituted alcohols are directly related to the intrinsic *in vivo* toxicities of those compounds. This conclusion rests on the relative uniformity of the T_i/P to LD_{50} ratio within subhomologies of the series and supports the conclusion that $1/P$ is directly related to the effective aqueous concentration of toxicant *in vivo*. This relationship also supports the conclusion that the tissue culture system is in quasiequilibrium and provides valid estimates of T_i. Major differences in the T_i/P to LD_{50} ratios between the aliphatic and halogenated alcohols are attributed primarily to differences in toxicant–receptor interactions rather than secondary time-dependent processes. Qualitative differences in *in vivo* and *in vitro* toxicity are detectable by Hansch analysis, but the quantitative contributions of those differences cannot exceed that responsible for the relatively small variation in the T_i/P to LD_{50} ratios observed within the subhomologies.

Hansch analysis of the biological response data leads to the conclusion that P and $E_s(R_2)$ are the most significant physical parameters associated with biological response of primary aliphatic alcohols and that $E_s(R_1)$ is of primary importance to the biological response of the secondary aliphatic alcohols. A higher positive charge at C_1, associated with the methyl substituent at C_1 and with halogenation at C_2, is a significant factor in the toxicant–receptor interaction, and a qualitative difference exists between *in vitro* and *in vivo* response relative to that parameter. Charge on the hydroxyl oxygen, Q_o, is related to that phenomenon and is of primary importance in the toxicant–receptor interaction.

The excellent fit of *in vitro* data to the Free–Wilson model and the results of Hansch analysis support the conclusion that the tissue culture system is a quasiequilibrium, time-independent system.

The usefulness of tissue culture intrinsic toxicity, T_i, and the octanol–water partition coefficient, P, for the analysis and prediction of *in vivo* response is demonstrated.

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Improved Method for Determining Dibenzepine and Its *N*-Demethylated Metabolites in Human Urine

A. De LEENHEER▲ and A. HEYNDRICKX

Abstract □ Urine samples taken from two psychiatric patients who ingested known amounts of the drug dibenzepine hydrochloride were extracted by a two-phase procedure with ether. A first screening by UV spectrophotometry showed the presence of compound(s) with a dibenzepine structure. The TLC separation of aliquots of extracts revealed four spots: two upper spots, each containing two substances, and two lower spots corresponding to single *N*-demethylated metabolites. GLC on the acetylated extracts by use of a polar column enabled the qualitative and quantitative (with prochlorperazine as internal standard) determinations of free dibenzepine and its five *N*-demethylated metabolites. IR and mass spectroscopic analyses of these compounds, isolated by micropreparative GLC of an aliquot of the acetylated extract, confirmed the specificity of results and gave detailed structural information.

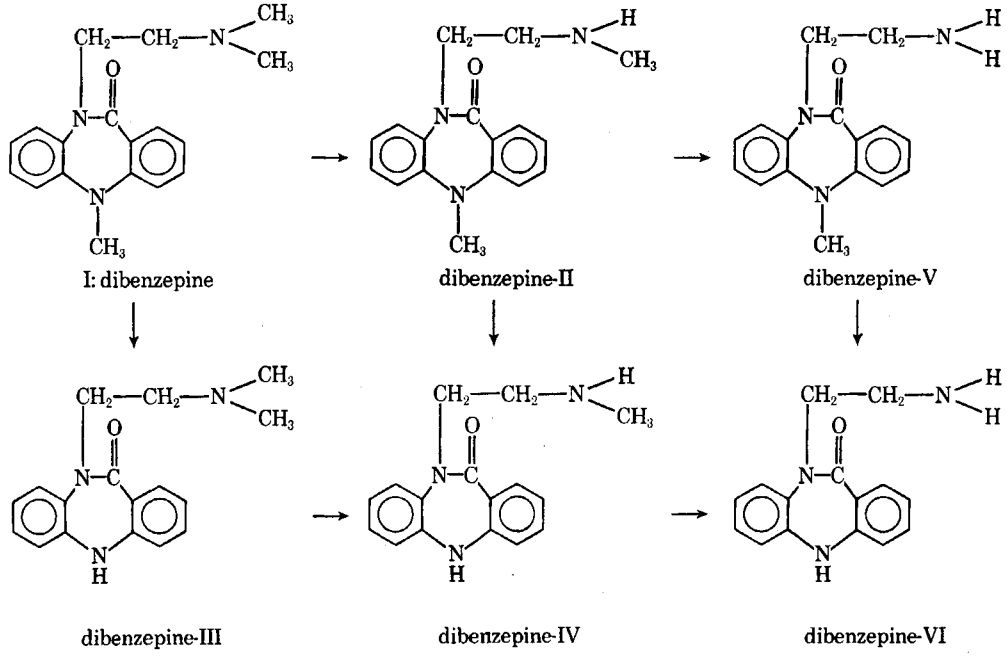
Keyphrases □ Dibenzepine and *N*-demethylated metabolites—characterization, analysis, human urine □ GLC—analysis, dibenzepine and *N*-demethylated metabolites, human urine

Dibenzepine (I) is a dibenzodiazepine derivative used as a drug with antidepressive properties. From the structural formula it may be deduced that the compound has a basic character due to the presence of an aliphatic tertiary amine function. Dibenzepine has been described to undergo *N*-demethylation and phenolic hydroxylation, followed by glucuronide formation in man, mouse, rat, rabbit, and dog (1–3). These studies were performed using UV spectrophotometry, paper chromatography, TLC, GLC, and radiochromatography and yielded insight into dibenzepine metabolism. However, a valuable method for the quantitative determination of dibenzepine and its *N*-demethylated metabolites was not developed.

Current interest in these laboratories in the metabolism of I in man led to the identification of the previously described (1–3) five *N*-demethylated products (II–VI) as important metabolites (Scheme I). By a different experimental approach, a procedure of improved specificity was developed for qualitative and quantitative determinations of free dibenzepine and its five *N*-demethylated metabolites (II–VI) in urine of man. Special attention was paid to the complete characterization of free basic compounds and their assay by GLC using an internal standard.

EXPERIMENTAL

Over periods of 4 successive days, the total urine outputs of two male patients who ingested, for the indicated period, a known amount of I (orally administered as chlorhydrate) together with a



I: 5-methyl-10-β-dimethylaminoethyl-10,11-dihydro-11-oxo-5*H*-dibenzo[*b*,*e*][1,4]diazepine
II: 5-methyl-10-β-methylaminoethyl-10,11-dihydro-11-oxo-5*H*-dibenzo[*b*,*e*][1,4]diazepine
III: 10-β-dimethylaminoethyl-10,11-dihydro-11-oxo-5*H*-dibenzo[*b*,*e*][1,4]diazepine
IV: 10-β-methylaminoethyl-10,11-dihydro-11-oxo-5*H*-dibenzo[*b*,*e*][1,4]diazepine
V: 5-methyl-10-β-aminoethyl-10,11-dihydro-11-oxo-5*H*-dibenzo[*b*,*e*][1,4]diazepine
VI: 10-β-aminoethyl-10,11-dihydro-11-oxo-5*H*-dibenzo[*b*,*e*][1,4]diazepine

Scheme I—N-*Demethylation metabolism of dibenzepine occurring in man*

Table I—Case Histories of Psychiatric Patients under Major Dibenzepine Treatment

Patient	Age, years	Daily Dose (in mg.) of Orally Administered: Dibenzepine (I) (Chlorhydrate)	Methotrimeprazine (Maleate)	Diazepam	Time Medication Began before First Urine Collection
1	62	6 × 80	25	—	6.5 months
2	36	5 × 80	25	3 × 10	3 weeks

much lower dose of methotrimeprazine[1] and diazepam (only for the second patient) were collected separately (Table I). Each sample was refrigerated immediately. Each 24-hr. urine collection was homogenized, and a 200-ml. aliquot was extracted prior to analysis without delay.

Screening Test—As a first indication of the presence of compounds, a ferrichloride, perchloric acid, and nitric acid (FPN) test (4, 5) and a chromium^{+6} test (6) were performed on each urine sample. The FPN reagent (4, 5) was prepared by combining 5 ml. of 5% (w/v) $FeCl_3$, 45 ml. of 20% (concentration 1/5) $HClO_4$, and 50 ml. of 50% (concentration 1/2) HNO_3. The chromium^{+6} reagent (6) consisted of 200 mg. of $K_2Cr_2O_7$, 75 ml. of concentrated H_2SO_4, 125 ml. of concentrated HNO_3, 50 ml. of concentrated $HClO_4$, and distilled water to 1000 ml.

Extraction Procedure—A 200-ml. aliquot of each urine sample was acidified with 2 *N* HCl and extracted twice under nitrogen atmosphere with 400 and 200 ml. of peroxide-free ether (freshly distilled over hydroquinone, reagent grade); the ether layers were discarded. The aqueous phase was alkalized with 10 *N* NaOH and again extracted twice under nitrogen atmosphere with 200 ml. of ether. The ether layers, which contained I and its five *N*-demethylated metabolites (II–VI) were washed with 5-ml. portions of 0.001 *N* NaOH until the aqueous layer remained colorless. The washed ether was evaporated in a rotary evaporator to a final volume of about 100 ml.; it was then saturated with nitrogen and extracted twice with 10 ml. of 0.1 *N* HCl. The total acid extracts were made basic with 1 ml. of 10 *N* NaOH, saturated with nitrogen, and extracted twice with 40 ml. of peroxide-free ether. The combined ether phases were dried over anhydrous sodium sulfate, filtered, and evaporated on a water bath at 40° under a slow stream of nitrogen. The residue was dissolved in 10 ml. of ethanol and kept in a well-closed tube at −15° for further analysis ("alcoholic extraction liquid").

UV Spectrophotometry—The UV spectra of the acidic extracts (20 ml. 0.1 *N* HCl) were automatically recorded with a double-beam spectrophotometer[2] using 1-cm. silica cells in the 450–190-nm. wavelength range.

TLC—Aliquots (0.1 ml.) of the alcoholic extracts were evaporated to a small volume on a water bath at 40° with the aid of a nitrogen stream. These, together with 20 mcg. of the reference compounds dibenzepine hydrochloride and its demethylated products II[3], III, IV[3], V[3], and VI[4] and 10 mcg. of methotrimeprazine maleate and methotrimeprazine sulfoxide, were spotted in a 2-cm. line at 3 cm. from the lower edge of 250-μ thin-layer plates, prepared with equal parts of silica gel H_{254} and cellulose MN 300. Chloroform, acetone, and ammonia (25%) (50:50:1) were used as the solvent system. After development the plates were consecutively sprayed with ferric reagent (7), vanadium^{+5} reagent, and chromium^{+6} reagent (6) for visualization.

For the ferric reagent (7), 500 mg. of $Fe(NO_3)_3$ is dissolved in 80 ml. of concentrated H_2SO_4 and the solution is diluted to 1000 ml. For the vanadium^{+5} reagent, 500 mg. of V_2O_5 is dissolved in 80 ml. of concentrated H_2SO_4 and the solution is diluted to 1000 ml.

GLC—GLC analysis was performed with a research gas chromatograph[5] equipped with dual flame-ionization detectors. The column was 1% FFAP[6] coated on Diatoport S[7], 80–100 mesh, and was used for single-column operation.

The operating conditions were: glass spiral column, 1.80-m. length, 4-mm. i.d.; carrier gas, nitrogen, 70 ml. min.$^{-1}$ (bubble flowmeter); oven temperature, 245°; temperature of injector block, 250°; temperature of detector block (flame-ionization detector), 248°; and air and hydrogen, calibrated for optimum sensitivity.

Qualitative Determination—Qualitative assays of urinary dibenzepine and its *N*-demethylated metabolites were performed with the acetylated derivatives. For this purpose, 0.5-ml. aliquots of the individual alcoholic extracts were transferred to conical siliconized tubes of 15-ml. capacity and evaporated on a water bath at 40° under a slow stream of nitrogen. To the final residues, 0.2 ml. of pyridine (refluxed and distilled over potassium hydroxide) and 0.2 ml. acetic acid anhydride (refluxed and distilled over calcium carbide) were added. After a 1-hr. reaction time in a phosphorus pentoxide desiccator, the reaction mixtures were evaporated again under the above-mentioned conditions. The residues obtained were dissolved in 40 μl. of ethyl acetate with the aid of a whirlmixer.

From the standards of I, its five *N*-demethylated compounds (II–VI), methotrimeprazine maleate, and methotrimeprazine sulfoxide, 10-mg. amounts were treated in the same way, but the residues obtained were taken up in 2 ml. of ethyl acetate (0.5% solutions). Volumes varying from 1 to 2 μl. were separately injected on top of the column with a syringe (Hamilton) of 10-μl. capacity.

Quantitative Determination—The same treatment was applied as for the qualitative determination, except that a known amount (200 mcg., but 160 mcg. for recovery experiments) of prochlorperazine was added as an internal standard before the first evaporation.

Calibration was made with a standard mixture. This was prepared by combining 50-μl. volumes of the 0.5% ethyl acetate solutions of the acetylated active compounds and a 1% ethyl acetate solution of prochlorperazine-(āc.)[8]. After evaporation in a water bath at 40° with the aid of a nitrogen stream, the residue was dissolved in 50 μl. of ethyl acetate. The composition of the standard solution was: 250 mcg. of I-(āc.), 250 mcg. of II-ac.[9], 250 mcg. of III-(āc.), 250 mcg. of IV-ac., 250 mcg. of V-ac., 250 mcg. of VI-ac., and 500 mcg. of prochlorperazine-(āc.) in 50 μl. of ethyl acetate.

For each active compound, a calibration factor k_i was calculated:

$$k_i = 2 \times \frac{S_x}{S_s} \qquad \text{(Eq. 1)}$$

where S_x/S_s is the quotient of peak areas[10] on chromatograms of the compound to be determined and the internal standard prochlorperazine-(āc.), and 2 is the reciprocal of the weight proportion of the compound to be quantitated (250 mcg.) w.r.t. internal standard prochlorperazine-(āc.) (500 mcg.).

The arithmetic mean $\bar{k}$ of all calibration factors k_i for a given compound was determined from three chromatograms according to:

$$\bar{k} = \left(\sum_1^3 k_i\right) \Big/ 3 \qquad \text{(Eq. 2)}$$

Results of C_u (mcg./100 ml. urine) were obtained with the equation:

$$C_u = \frac{10}{\bar{k}} \times \frac{S_x'}{S_s'} \times m_s \qquad \text{(Eq. 3)}$$

where S_x'/S_s' is the quotient of peak areas of the compound to be determined as present in the extract and the internal standard prochlorperazine-(āc.), m_s is total amount in micrograms of the internally added standard prochlorperazine-(āc.), and 10 is the conversion factor because 0.5 ml. alcoholic extract corresponds to a 10-ml. urine sample.

Recovery Experiments—To 200 ml. of urine, collected as a pool of samples from subjects under no drug medication, 400 mcg. dibenzepine hydrochloride was added. The sample was taken through the whole experimental procedure as described.

Trapping of GLC Effluents—A splitting system[11] was installed at the exit of each column, yielding a 10:1 ratio. As a collection device,

[1] Known in Europe as levomepromazine.
[2] Unicam SP800.
[3] As the chlorhydrate.
[4] As the oxalate.
[5] Hewlett-Packard 5 750.
[6] Reaction product of Carbowax 20M and *m*-dinitroterephthalic acid.
[7] Acid and silane-treated, Hewlett-Packard.

[8] (āc.) indicates that the compound underwent the acetylation treatment without being changed (no acetylable function present).
[9] ac. indicates that the compound was transformed to the acetamide.
[10] Measured using the method of peak height times peak width at half height ($h_{max} \times W_{0.5h}$).
[11] Hewlett-Packard.

Table II—Volume, pH, FPN, and Chromium^{+6} Test of Urine Samples Collected from Psychiatric Patients

Patient	Sample	Volume of Urine, ml./24 hr.)	pH	FPN Test	Chromium^{+6} Test
1	1 (1st day)	1000	6.0	—	—
	2 (2nd day)	800	5.5	—	—
	3 (3rd day)	1050	6.0	—	—
	4 (4th day)	750	5.5	—	—
2	1 (1st day)	800	5.0	± (blue)	± (blue)
	2 (2nd day)	900	5.5	± (blue)	± (blue)
	3 (3rd day)	600	6.0	± (blue)	± (blue)
	4 (4th day)	1200	5.0	—	± (blue)

a Pyrex capillary (12.5-cm. length and 1.5-mm. i.d.) fixed with two Teflon O-rings in a brass tube (10.5-cm. length, 7-mm. o.d., and 4-mm. i.d.) provided with an 1/8 swagelok nut, was used. A 1.0-ml. aliquot of the alcoholic extract was acetylated, and the residue was dissolved in 25 μl. of ethyl acetate. This solution was at once preparatively chromatographed on the 1% FFAP column at 245° for the trapping of separated compounds.

IR and Mass Spectrometry—After collection, each compound was rinsed out from its capillary with 2 × 10 μl. of ethyl acetate. This solution was poured on 25 or 7 mg. of KBr in an agate mortar, and the solvent was allowed to evaporate. A micropellet was prepared by means of a punched disk (2 × 10 or 1 × 4-mm. hole), and the IR spectrum was recorded from 625 to 4000 cm.$^{-1}$ with a spectrophotometer[12] equipped with a microbeam condenser[13]. The mass spectrum was recorded with an instrument[14], using the direct-insertion probe system loaded with a small pulverized piece of the KBr pellet and running the spectra in standard conditions (70 ev., 150°).

RESULTS AND DISCUSSION

The volume, pH, FPN test (4, 5), and chromium^{+6} test (6) of the urine samples were determined just after each 24-hr. collection. These preliminary data are summarized in Table II.

Only for Patient 2 were slightly positive FPN and chromium^{+6} tests noticed. In fact, the administered doses of methotrimeprazine were too low for good color reactions. Dibenzepine and diazepam did not develop any color under these experimental conditions.

The UV spectra of all extracts showed a striking correspondence with the reference spectrum of I (λ_{max}: 207 and 221 nm. with ϵ 28,652 and 28,419, 283 Nm.[15]; λ_{min}: 215 nm.). UV spectra of the five *N*-demethylated metabolites were qualitatively almost identical. UV spectrophotometry, being essentially an additive method, gave an overall pattern of all UV absorbing compounds as present in the acidic extract and no differentiation could be made at this stage of the analysis.

TLC of reference compounds in the described system gave: V, R_f 0.59 yellow; I, R_f 0.54 yellow; VI, R_f 0.50 blue; III, R_f 0.47 blue; II, R_f 0.24 yellow; and IV, R_f 0.19 blue. Notwithstanding the presence of six substances, four spots were obtained for all extracts: V and I yielded one yellow spot, VI and III appeared as one blue spot, and only the two lower spots (II yellow and IV blue) were single. *N*-Demethylation of the ring nitrogen resulted in formation of a blue color with the spray reagents used. This may be explained on the basis that essentially the entire ring system is responsible for the colors given, with the aminoalkyl side chain having no influence.

GLC analysis of a 0.5-ml. acetylated fraction of the alcoholic extracts on the 1% FFAP column (245°) showed the presence of free I-(āc.) (R_t 3 min. 40 sec.), III-(āc.) (R_t 7 min. 46 sec.), II-ac. (R_t 25 min. 5 sec.), V-ac. (R_t 33 min. 11 sec.), IV-ac. (R_t 58 min. 4 sec.), and VI-ac. (R_t 76 min. 54 sec.) in all extracts. Inspection of qualitative chromatograms permitted the choice of prochlorperazine as a suitable standard (R_t 11 min. 5 sec.) which did not overlap with any peak from the extracts. Calibration factors $\bar{k}$, obtained as previously described, are: I-(āc.), 2.14; II-ac., 4.74; III-(āc.), 1.28; IV-ac., 4.34; V-ac., 4.43; and VI-ac., 2.42.

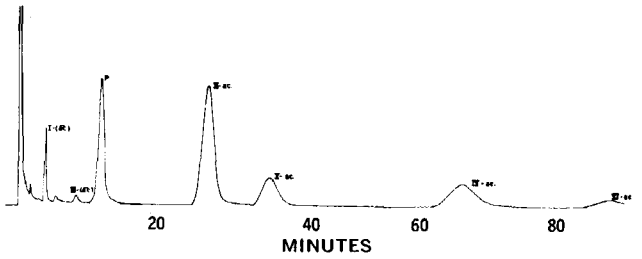


Figure 1—*Quantitative GLC on the 1% FFAP column from urinary extract 4 of Patient 2. Sample size was: 200 ml. urine, 0.5 ml. alcoholic extraction liquid evaporated and residue redissolved in 30 μl. of ethyl acetate, and 1.1 μl. injected on top of the column (corresponding to 0.37 ml. urine). Attenuation settings were: range, × 10; and attenuation, × 16. Key (from left to right): I-(āc.); III-(āc.); P, prochlorperazine-(āc.) internal standard; II-ac.; V-ac.; IV-ac.; and VI-ac.*

As seen from Table III, monodemethylation of the aminoalkyl side chain is predominant. *N*-Demethylation of the ring nitrogen is only a minor pathway. Patients 1 and 2 show differences as to their preferences for described *N*-demethylation processes. An example of the GLC quantitation of free I and its five *N*-demethylated metabolites in a single run on the 1% FFAP column is shown in Fig. 1.

Recovery experiments for I yielded 78% for the first urine sample examined and 86% for the second. These results indicate a reasonable extraction recovery, which may be extrapolated for the *N*-demethylated compounds as well. Previous results obtained by UV, TLC, and GLC did not reveal any methotrimeprazine or some of its metabolites. This result is certainly due to the major intake of I *versus* a minor intake of methotrimeprazine (about 20 times lower doses) and the small sample aliquots taken for analysis.

For confirmation of the specificity of the GLC results, a 1-ml. alcoholic extract (Sample 2 of Patient 1 and Sample 1 of Patient 2, Tables II and III) was separately evaporated under a nitrogen stream; the residue was acetylated and chromatographed on the 1% FFAP column installed for trapping. Under identical conditions, 20-μl. aliquots of 0.5% standard solutions of acetylated reference substances were chromatographed and separately collected. IR and mass spectra obtained on the purified I, II-ac., IV-ac., V-ac., and VI-ac., as isolated from mentioned urine samples (Figs. 2, 3, and 5–7), agreed completely with those obtained from reference compounds. III-(āc.) could not be isolated in a sufficient amount, but its IR and mass spectra (Fig. 4) were obtained from a reference substance[16].

IR spectroscopy enabled easy differentiation of each compound on the basis of its fingerprint characteristics. An N—H absorption around 3300 cm.$^{-1}$ showed up in all spectra except for I-(āc.) and II-ac. Structures containing the aliphatic tertiary amine function N—$(CH_3)_2$, such as I and III-(āc.), gave a 2770-cm.$^{-1}$ band which was of weak intensity but easily distinguishable. The C=O amide frequency around 1600 cm.$^{-1}$ permitted a classification in three groups, each including two compounds: I-(āc.) and III-(āc.) had the sharpest band, II-ac. and IV-ac. showed a medium broad band (tertiary amide functions), and II-ac. and VI-ac. gave the broadest absorption (secondary amide function). The 1458-cm.$^{-1}$ absorption had a definite lower intensity for III-(āc.), IV-ac., and VI-ac. (CH_3 at the seven ring substituted by H). This feature was opposite to the sharp band identified for I, II-ac., and V-ac. (*N*-ring CH_3). The 1250-cm.$^{-1}$ strong band, which was present only in I, II-ac., and V-ac., could be assigned to the *N*-ring CH_3 function. As a general phenomenon, substitution of N—CH_3 by N—H in the diazepine ring resulted in spectra with broader bands and less fine structure.

Mass spectroscopic results gave the right molecular ion for each compound: I-(āc.) at *m/e* 295 ($C_{18}H_{21}N_3O$), II-ac. at *m/e* 323($C_{19}H_{21}N_3O_2$), III-(āc.) at *m/e* 281 ($C_{17}H_{19}N_3O$), IV-ac. at *m/e* 309

[12] Perkin-Elmer 257.
[13] RIIC C-41.
[14] LKB 9000.
[15] Shoulder.
[16] With identical treatment by acetylation and GLC.

Table III—Quantitative Results of GLC Determination of Dibenzepine and Its Five *N*-Demethylated Metabolites

Patient	Sample	Concentrations, C_u, mcg./100 ml. Urine						Percent of Dose Recovered as Metabolites
		I	II	III	IV	V	VI	
1	1	42.0 (0.09)[a]	901 (1.97)	Traces	289 (0.66)	626 (1.43)	442 (1.08)	5.14
	2	75.9 (0.13)	1356 (2.37)	63.6 (0.11)	410 (0.75)	779 (1.43)	480 (0.94)	5.60
	3	12.3 (0.03)	462 (1.06)	Traces	205 (0.49)	394 (0.95)	347 (0.89)	3.39
	4	32.4 (0.05)	1356 (2.22)	Traces	403 (0.69)	763 (1.31)	525 (0.96)	5.18
2	1	224 (0.45)	1201 (2.52)	98.4 (0.21)	507 (1.11)	412 (0.91)	384 (0.90)	5.65
	2	601 (1.35)	1039 (2.45)	106.0 (0.25)	323 (0.80)	272 (0.67)	289 (0.76)	4.93
	3	258 (0.39)	799 (1.26)	67.2 (0.11)	307 (0.51)	162 (0.27)	172 (0.30)	2.45
	4	173 (0.52)	683 (2.15)	63.1 (0.20)	326 (1.08)	226 (0.75)	189 (0.66)	4.84

[a] Percent of administered dose recovered as unchanged drug.

($C_{18}H_{19}N_3O_2$), V-ac. at *m/e* 309 ($C_{18}H_{19}N_3O_2$), and VI-ac. at *m/e* 295 ($C_{17}H_{17}N_3O_2$). A low relative intensity was obtained for the molecular ion of the aliphatic tertiary amine structures I-(ac.) and III-(ac.) *versus* the high relative intensities for the aliphatic secondary and tertiary amides. Side chains were identified by three typical mass spectroscopic fragmentation patterns. Indeed, the six structures examined could be subdivided in three groups of two compounds each, just differing by one methylene group (14 a.m.u.) located at the ring nitrogen (not interfering with the side-chain fragmentation). The tertiary aliphatic amines I-(ac.) and III-(ac.) showed peaks due to α-cleavage at *m/e* 251 and 237 (M − 44 or M − C_2H_6N), α-cleavage and hydrogen-ion transfer to the eliminated fragment at *m/e* 250 and 236 (M − 45 or M − C_2H_7N), β-cleavage at *m/e* 237 and 223 (M − 58 or M − C_3H_8N), and a major γ-cleavage with hydrogen-ion transfer to the radical ion at *m/e* 224 and 210 (M − 71 or M − C_4H_9N) (base peak). In the low mass range, an intense ion was observed at *m/e* 58 [$H_2C{=}N^+(CH_3)_2$] which was due to simple β-cleavage.

The aliphatic secondary amide structures V-ac. and VI-ac. gave peaks due to fission β to the carbonyl at *m/e* 251 and 237 (M − 58 or M − C_2H_4NO), fission γ to the carbonyl with hydrogen-ion transfer (resulting in elimination of acetamide CH_3CONH_2) at *m/e* 250 and 236 (M − 59 or M − C_2H_5NO) (base peak), fission δ to the carbonyl at *m/e* 237 and 223 (M − 72 or M − C_3H_6NO), and γ-cleavage with hydrogen-ion transfer to the radical ion at *m/e* 224 and 210 (M − 85 or M − C_4H_7NO). The major fragmentation M − C_2H_5NO was further proven by occurrence of metastable ions at 202.3 for V-ac. and 188.8 for VI-ac. The aliphatic tertiary amide structures II-ac. and IV-ac. produced peaks due to fission β to the carbonyl at *m/e* 251 and 237 (M − 72 or M − C_3H_6NO), fission β to the carbonyl with hydrogen-ion transfer (resulting in elimination of methylacetamide $CH_3CONHCH_3$) at *m/e* 250 and 236 (M − 73 or M − C_3H_7NO), fission γ to the carbonyl at *m/e* 237 and 223 (M − 86 or M − C_4H_8NO), and fission δ to the carbonyl with hydrogen-ion transfer to the radical ion at *m/e* 224 and 210 (M − 99 or M − C_5H_9NO). The predominant fragmentation M − C_3H_7NO was also indicated by the metastable ions at 193.5 for II-ac. and at 180.2 for IV-ac.

I-(ac.)—ν^{KBr}: 3065 (phenyl), 2960 (CH_3), 2820 (N—CH_3), 2775 [$N(CH_3)_2$], 1640 (C=O amide), 1593 and 1505 (phenyl), 1478 (CH_3 and CH_2), 1458 and 1392 (CH_3), 1325 (Ar—N), 1252 (N—CH_3), 1167 and 1152 (C—N), and 768 (*o*-substituted phenyl) cm.$^{-1}$. *m/e* (relative intensity): 295(16) $C_{18}H_{21}N_3O$, 251(4) $C_{16}H_{15}N_2O$, 250(11) $C_{16}H_{14}N_2O$, 237(10) $C_{15}H_{13}N_2O$, 236(14) $C_{15}H_{12}N_2O$, 224 (1000) $C_{14}H_{12}N_2O$, 210(16) $C_{13}H_{10}N_2O$, 209(10) $C_{13}H_9N_2O$, 195(9) $C_{13}H_9NO$, 177(16) $C_{13}H_7N$, 149(48) $C_{12}H_5$, 59(18) C_3H_9N, and 58 (524) C_3H_8N.

II-ac.—ν^{KBr}: 3065 (phenyl), 2955 (CH_3), 2815 (N—CH_3), 1640 (C=O amide), 1590 and 1503 (phenyl), 1477 (CH_3 and CH_2), 1452 and 1392 (CH_3), 1322 (Ar—N), 1248 (N—CH_3), 1167 and 1140

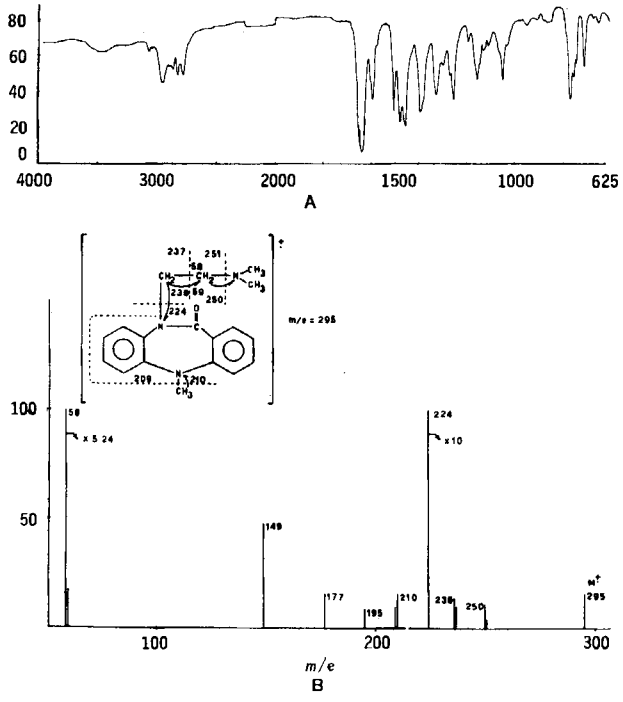


Figure 2—*IR spectrum (A) and mass spectrum (B) of trapped I-(ac.) isolated from urinary extract 2 of Patient 1.*

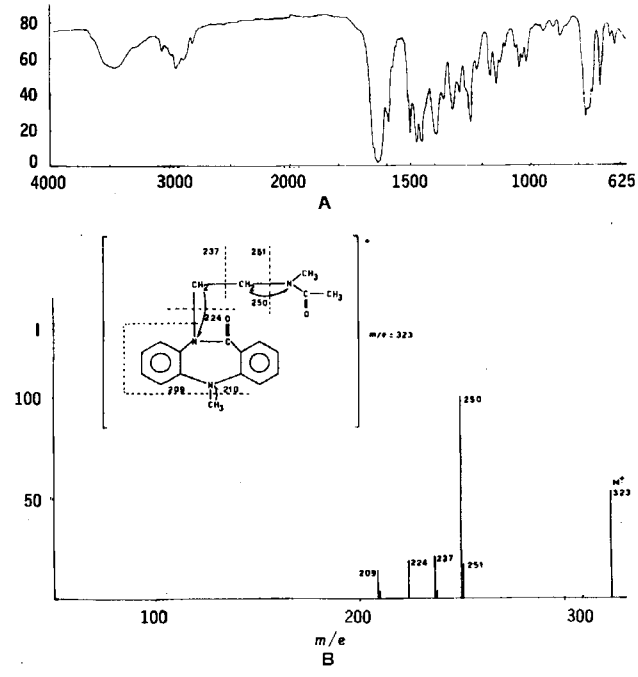


Figure 3—*IR spectrum (A) and mass spectrum (B) of trapped II-ac. isolated from urinary extract 2 of Patient 1.*

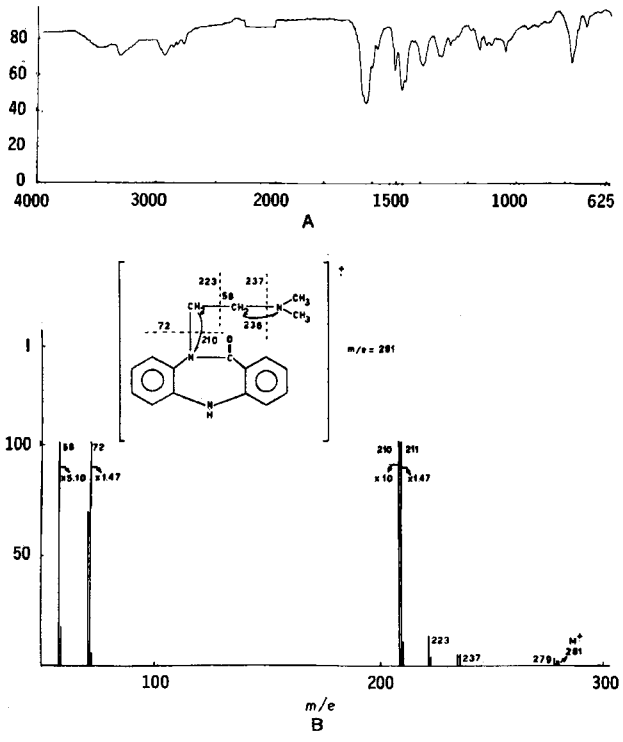


Figure 4—*IR spectrum (A) and mass spectrum (B) of trapped III-(ac.) reference substance.*

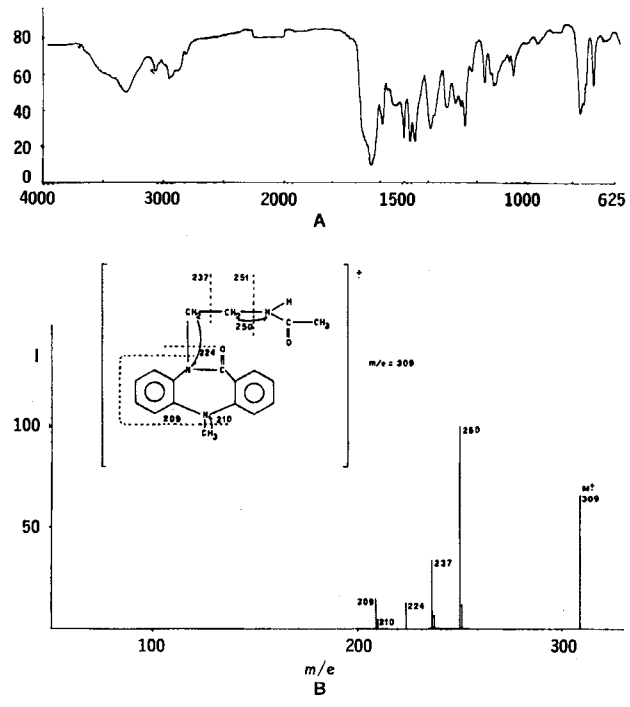


Figure 6—*IR spectrum (A) and mass spectrum (B) of trapped V-ac. from urinary extract 2 of Patient 1.*

(C—N), and 770 (*o*-substituted phenyl) cm.$^{-1}$. *m/e* (relative intensity): 323(53) $C_{19}H_{21}N_3O_2$, 309(1) $C_{18}H_{19}N_3O_2$, 251(17) $C_{16}H_{15}N_2O$, 250(100) $C_{16}H_{14}N_2O$, 238(4) $C_{15}H_{14}N_2O$, 237(21) $C_{15}H_{13}N_2O$, 224 (19) $C_{14}H_{12}N_2O$, 210(4) $C_{13}H_{10}N_2O$, and 209(14) $C_{13}H_9N_2O$; m^* = 193.5 [323(M^+) → 250].

III-(ac.)—ν^{KBr}: 3300 (N—H), 2930 (CH_3), 2820 (N—CH_3), 2775 [N(CH_3)$_2$], 1625 (C=O amide), 1598 and 1505 (phenyl), 1476 (CH_3 and CH_2), 1460 and 1388 (CH_3), 1318 (Ar—N), 1308 (*N*-ring H), 1150(C—N), and 767 (*o*-substituted phenyl) cm.$^{-1}$. *m/e* (relative intensity): 281(2) $C_{17}H_{19}N_3O$, 280(2) $C_{17}H_{18}N_3O$, 279(3) $C_{17}H_{17}N_3O$, 237(5) $C_{15}H_{13}N_2O$, 236(5) $C_{15}H_{12}N_2O$, 224(4) $C_{14}H_{12}N_2O$, 223(13) $C_{14}H_{11}N_2O$, 212(11) $C_{13}H_{12}N_2O$, 211(147) $C_{13}H_{11}N_2O$, 210(1000) $C_{13}H_{10}N_2O$, 73(6) $C_4H_{11}N$, 72(147) $C_4H_{10}N$, 71(69) C_4H_9N, 59(18) C_3H_9N, and 58(510) C_3H_8N.

IV-ac.—ν^{KBr}: 3295 (N—H), 3060 (phenyl), 2930 (CH_3), 1630 (C=O amide), 1598 and 1502 (phenyl), 1476 (CH_3 and CH_2), 1454 and 1392 (CH_3), 1300 (*N*-ring H), 1249 (N—CH_3), 1162 and 1142 (C—N), and 762 (*o*-substituted phenyl) cm.$^{-1}$. *m/e* (relative intensity): 309(63)

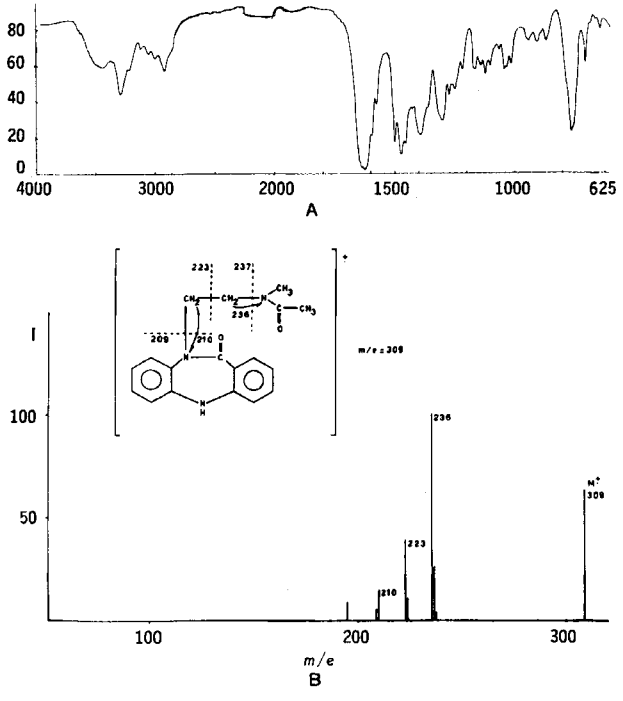


Figure 5—*IR spectrum (A) and mass spectrum (B) of trapped IV-ac. from urinary extract 2 of Patient 1.*

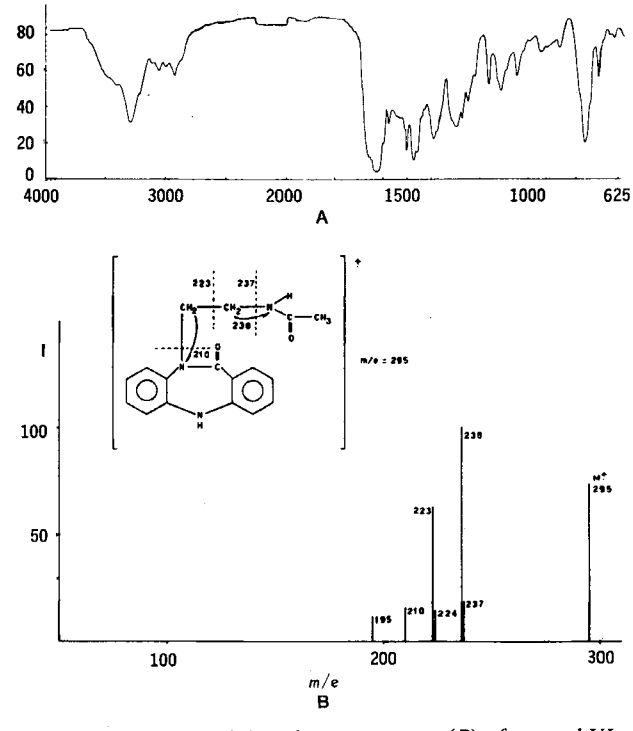


Figure 7—*IR spectrum (A) and mass spectrum (B) of trapped VI-ac. from urinary extract 2 of Patent 1.*

$C_{18}H_{19}N_3O_2$, 238(4) $C_{15}H_{14}N_2O$, 237(26) $C_{15}H_{13}N_2O$, 236(100) $C_{15}H_{12}N_2O$, 224(11) $C_{14}H_{12}N_2O$, 223(39) $C_{14}H_{11}N_2O$, 210(15) $C_{13}H_{10}N_2O$, 209(6) $C_{13}H_9N_2O$, and 195(9) $C_{13}H_9NO$; m^* = 180.2 [309(M^+) → 236].

V-ac.—ν^{KBr}: 3315 (N—H), 3070 (phenyl), 2960 (CH_3), 2820 (N—CH_3), 1640 (C=O amide), 1592 and 1506 (phenyl), 1478 (CH_3 and CH_2), 1457 and 1392 (CH_3), 1327 (Ar—N), 1250 (N—CH_3), 1168 and 1142 (C—N), and 770 (*o*-substituted phenyl) $cm.^{-1}$. *m/e* (relative intensity): 309(66) $C_{18}H_{19}N_3O_2$, 251(12) $C_{16}H_{15}N_2O$, 250(100) $C_{16}H_{14}N_2O$, 238(7) $C_{15}H_{14}N_2O$, 237(34) $C_{15}H_{13}N_2O$, 224(13) $C_{14}H_{12}N_2O$, 210(5) $C_{13}H_{10}N_2O$, and 209(15) $C_{13}H_9N_2O$; m^* = 202.3 [309(M^+) → 250].

VI-ac.—ν^{KBr}: 3295 (N—H), 3060 (phenyl), 2930 (CH_3), 1630 (C=O amide), 1597 and 1502 (phenyl), 1475 (CH_3 and CH_2), 1458 and 1390 (CH_3), 1298 (*N*-ring H), 1249 (N—CH_2), 1160 (C—N), and 762 (*o*-substituted phenyl) $cm.^{-1}$. *m/e* (relative intensity): 295(74) $C_{17}H_{17}N_3O_2$, 237(19) $C_{15}H_{13}N_2O$, 236(100) $C_{15}H_{12}N_2O$, 224(15) $C_{14}H_{12}N_2O$, 223(63) $C_{14}H_{11}N_2O$, 210(16) $C_{13}H_{10}N_2O$, and 195(12) $C_{13}H_9NO$; m^* = 188.8 [295(M^+) → 236].

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Keyphrases □ MAO inhibitors, potential—synthesis of 2,9-disubstituted β-carbolinium halides □ β-Carbolinium halides, 2,9-disubstituted—synthesis, inhibition of MAO

Accumulated evidence for multiple forms of MAO in various tissues suggests differences in the metabolism of biogenic amines in the central and peripheral nervous systems (1–8). Many compounds, including those of the potent irreversible type such as iproniazid, pargyline, and tranylcypromine, do not exert selective inhibition of MAO of either the central or peripheral nervous system. An inhibitor whose action is specific for MAO of peripheral tissues may be useful in determining the origin of hypertension and the mechanism of reserpine action and would prove valuable in the therapy of angina pectoris because of the absence of central behavioral effects. In addition, selective inhibition of centrally localized MAO could be achieved with the combination of a nonspecific irreversible inhibitor and a specific peripheral reversible inhibitor; this would involve pretreatment with the latter to saturate the peripheral enzyme binding site, thus allowing exposure of only central MAO to the irreversible inactivation by the former. The effects of peripheral inhibition would be short lived, while the central effects from the irreversible inhibitor would be manifested until the enzyme could be regenerated (9).

One approach to designing an inhibitor with these characteristics involves exploiting the impermeability of cerebral tissue to highly polar compounds. Quater-

$C_{18}H_{19}N_3O_2$, 238(4) $C_{15}H_{14}N_2O$, 237(26) $C_{15}H_{13}N_2O$, 236(100) $C_{15}H_{12}N_2O$, 224(11) $C_{14}H_{12}N_2O$, 223(39) $C_{14}H_{11}N_2O$, 210(15) $C_{13}H_{10}N_2O$, 209(6) $C_{13}H_9N_2O$, and 195(9) $C_{13}H_9NO$; m^* = 180.2 [309(M^+) → 236].

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One approach to designing an inhibitor with these characteristics involves exploiting the impermeability of cerebral tissue to highly polar compounds. Quater-

Table I—Physical Constants of 2,9-Disubstituted β-Carbolinium Halides

Compound	Melting Point	Yield, %	Empirical Formula	Analysis, % Calc.	Found
I	270–274°	66	$C_{13}H_{13}IN_2$	C 48.17	48.03
				H 4.04	4.07
				N 8.64	8.64
II	281–284°	70	$C_{13}H_{13}ClN_2 \cdot {}^1/_2H_2O$	C 64.59	64.67
				H 5.84	5.96
				N 11.59	11.48
III	279–279.5°	75	$C_{14}H_{15}IN_2$	C 49.72	49.50
				H 4.47	4.41
				N 8.29	8.43
IV	256–257°	50	$C_{15}H_{17}IN_2$	C 51.15	51.02
				H 4.87	4.83
				N 7.95	7.74
V	265–266°	60	$C_{14}H_{17}IN_2O$	C 47.47	47.37
				H 4.27	4.13
				N 7.91	7.73
VI	237–238°	70	$C_{13}H_{13}IN_2O$	C 45.90	45.78
				H 3.85	3.68
				N 8.24	8.21
VII	219–220°	53	$C_{15}H_{13}BrN_2$	C 59.82	59.70
				H 4.35	4.78
				N 9.30	9.40

nary ammonium compounds, for instance, are well documented as exhibiting difficulty in penetrating the brain (10). In this laboratory, a number of 2,9-disubstituted β-carbolinium compounds (Table I) were synthesized with this potential.

EXPERIMENTAL[1]

2,9-Dimethyl-β-carbolinium Iodide (I)—To a solution of 0.7 g. (3.8 mmoles) of 9-methyl-β-carboline (11) in 5 ml. of ethanol was added 0.56 g. (4.0 mmoles) of methyl iodide. After standing at room temperature for 48 hr., the product was filtered, yielding 0.8 g. (66%), m.p. 274–276°. Compounds III–VI were prepared by this procedure. See Table I for their physical constants.

2-Propynyl-9-methyl-β-carbolinium Bromide (VII)—To a solution of 0.88 g. (5 mmoles) of 9-methyl-β-carboline in 3 ml. of ethanol was added gradually 0.66 g. (5.5 mmoles) of propargyl bromide. After standing at room temperature for 24 hr., the product was filtered and twice recrystallized from isopropanol–water, yielding 0.80 g. (53.4%), m.p. 219–220° dec. See Table I for its physical constants.

2,9-Dimethyl-β-carbolinium Chloride (II)—A mixture of 1.8 g. (10 mmoles) of 9-methyl-β-carboline, 2 g. (20 mmoles) of methyl chloride, and 15 ml. of ethanol was heated in a Parr bomb at 50° for 8 hr. After cooling, the product was filtered to yield 1.6 g. (70%), m.p. 281–284° dec. See Table I for its physical constants.

Tissues—Human heart, liver, and brain were obtained at autopsy, within 12 hr. after death, from individuals devoid of pathological factors; these organs were stored at −25°. Bovine liver was obtained from a commercial source[2]. Rat heart, liver, and brain were taken from male Sprague-Dawley rats, 200–250 g., and mouse liver was taken from male Yale Swiss mice, 25–30 g.

Purification of Enzyme—Mitochondrial MAO from human and rat liver was prepared by the method of Sordahl *et al.* (12) with the following modifications: (*a*) the tissue was homogenized in 10 volumes of 0.25 *M* sucrose; and (*b*) the mitochondrial pellet was suspended in 1.6 times the original tissue weight of 0.01 *N* sodium phosphate buffer, pH 7.6, and sonified for 30 min. while being maintained at 4°. The suspension was then centrifuged at 100,000×*g* for 1 hr., and the resulting supernate was stored at −25° until assay.

In the preparation of mitochondrial MAO from human and rat heart, to ensure complete homogenization the heart tissue was ground through a small chilled tissue press before homogenization in 12 volumes of 0.25 *M* sucrose. The procedure described for liver MAO was then followed, and the sonified suspension was used in the assay without further centrifugation.

Since purification of mitochondrial MAO from human and rat brain using the procedure for liver and heart yielded preparations of low specific activity, a modified method of Nagatsu *et al.* (13) was used. The mitochondrial pellet obtained from the whole rat brain or occipital cortex of human brain was suspended in five times the original tissue weight of 0.01 *M* potassium phosphate buffer, pH 7.4. The suspension was heated at 40° for 10 min. and then rapidly cooled in an ice bath. To solubilize the mitochondrial enzyme, a nonionic detergent[3] was added to a final concentration of 1% with constant stirring. The mixture was stored overnight at 0° and then centrifuged for 1 hr. at 107,000×*g*. The supernate was stored at −25° until assay.

Mitochondrial MAO from bovine and mouse liver was isolated and purified according to a published procedure (11). This procedure was similar to that used for human and rat liver.

Enzyme Assay—All stock solutions of the compounds were prepared in water, except IV which was dissolved in 50% aqueous dimethyl sulfoxide. Incubation using tryptamine-2-^{14}C hydrochloride as the substrate was carried out as previously described (11). When tyramine-1-^{14}C was used as the substrate, the product of the enzyme reaction was extracted with ethyl acetate from a highly acidic medium. In the case of IV, an equal amount of dimethyl sulfoxide was present in both the controls and the samples containing varying concentrations of the inhibitor. From a plot of the percent inhibition *versus* the molar concentration of the inhibitor, the concentration of the compound producing 50% inhibition (I_{50}) was then determined.

K_i and K_m Determination—The approximate concentration of I producing 50% inhibition was used in experiments in which tryptamine-2-^{14}C concentration was varied from 6 to 60 μ*M*. Plots of the reciprocal of the substrate concentration *versus* the reciprocal of the reaction velocity were constructed using the method of least squares. The K_i value, where competitive inhibition was encountered, was calculated according to the method of Dixon and Webb (14). When mixed-type inhibition was encountered, the method of Krupka (15) was used to calculate K_i values.

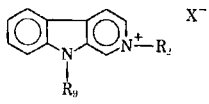
Determination of Reversible and Irreversible Inhibition—Using the I_{50} concentrations of I, VII, or pargyline hydrochloride found with tryptamine deamination by human liver MAO, the inhibitors were preincubated with the corresponding enzyme preparations in the reaction mixture (without substrate) as described previously. After designated time intervals, tryptamine-2-^{14}C was added and the residual MAO activity was determined after an additional 30 min. of incubation. In another experiment with human liver MAO, I and pargyline were combined in the same concentrations as when they were preincubated separately. A control was prepared by

[1] Melting points were taken on a Mel-Temp apparatus and are corrected. Analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. IR spectra were taken with a Perkin-Elmer spectrophotometer, model 237B, and were compatible with the assigned structures.

[2] Burton Brothers, Houston, Tex.

[3] Triton X-100.

Table II—Inhibition of Human and Bovine Liver MAO by 2,9-Disubstituted β-Carbolinium Halides



Compound	R_2	R_9	X	I_{50}, μM[a,b,c] Human	Bovine
I	CH_3	CH_3	I	3.0	16.0
II	CH_3	CH_3	Cl	2.0	19.0
III	C_2H_5	CH_3	I	21.0	100.0
IV	C_3H_7	CH_3	I	23.5	112.0
V	CH_3	CH_2OCH_3	I	18.0	180.0
VI	CH_3	CH_2OH	I	5.5	178.0
VII	$CH_2C{\equiv}CH$	CH_3	Br	4.0	3.5

[a] Concentration of an inhibitor required to produce 50% inhibition of the enzyme. [b] Each value represents the mean obtained from at least two experiments. [c] Substrate: tryptamine-2-^{14}C hydrochloride.

preincubating the enzyme alone for the same time intervals before the addition of substrate. From a semilog plot of nanomoles of product formed *versus* time of preincubation, reversibility or irreversibility of the inhibitors was estimated according to the method of Zeller *et al.* (16).

RESULTS AND DISCUSSION

The inhibitory activities of several 2,9-disubstituted β-carbolinium halides with MAO from human and bovine liver mitochondria are shown in Table II. With the exception of VII, these β-carbolines exhibited greater inhibition with the enzyme from human liver than that from bovine liver. While MAO from both sources was equally inhibited by VII, VI was 30 times more active with the human MAO. Further differentiation could be made by comparing V and VI; Compound VI inhibited human MAO three times more than V, but its ability to inhibit bovine MAO did not differ from that of V. It is possible that a group on the human enzyme capable of forming hydrogen bonding with the hydrogen of the 9-CH_2OH group on VI is lacking in the bovine enzyme. The six- and 11-fold losses of activity when comparing the I_{50} values of I with V observed for human and bovine MAO, respectively, could indicate the lack of tolerance of the enzyme for the bulk of the OCH_3 group.

The degree of inhibition decreased by six- to eightfold when the alkyl chain at the N-2 position of β-carbolines was lengthened beyond the methyl. This decrease in the inhibitory activities of III and IV does not appear to be due to the steric interaction between the 2-ethyl or 2-propyl group and the enzyme, since VII with a 2-propargyl group comparable in size to the propyl was six times more active than IV. Compound VII was 4.5 times more active than I with bovine MAO. It is most likely that the 2-propargyl group further facilitated the binding of VII to the enzyme, although the nature of binding of this group is yet to be determined.

The inhibition of I was further studied with additional sources of MAO and in the presence of an additional substrate, tyramine (Table III). Unlike pargyline, which exhibited greater inhibitory action toward tyramine oxidation than toward tryptamine oxidation, selective inhibition of tyramine oxidation by I was limited only to the enzyme from rat heart and liver as well as human brain and liver. Tissue selectivity was observed for I with human MAO, where it was 20–70 times more effective in inhibiting the heart and liver enzyme than MAO from the brain. A similar pattern was detected with rat MAO, but only when tyramine was used as substrate. Pargyline, on the other hand, was found to be more active with human brain MAO than with the heart and liver enzyme; with rat MAO, the liver enzyme was affected to a greater extent than that from the heart and brain. The selective inhibition of the deamination of tyramine, as opposed to tryptamine, by mouse and bovine liver MAO agreed with data reported by Gorkin *et al.* (17) and Fuller (18). The inhibitory activity of I with mouse or bovine liver MAO was not substrate dependent.

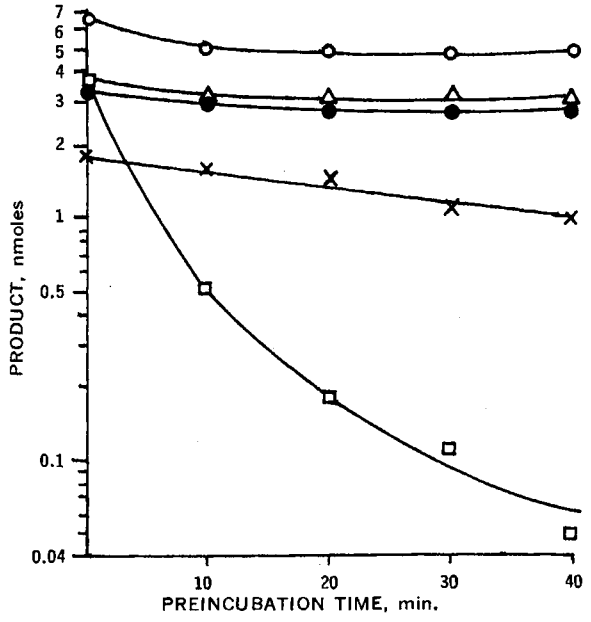


Figure 1—*Effect of I, VII, and pargyline preincubation on tryptamine oxidation catalyzed by human liver MAO. Key:* ○, *enzyme control;* △, *3 μM I;* ●, *5 μM VII;* ×, *3 μM I and 10 μM pargyline; and* □, *10 μM pargyline.*

When the activities of both inhibitors were compared, pargyline was found to be more potent than I in inhibiting tyramine oxidation by MAO from human tissues; I, on the other hand, was more effective than pargyline in inhibiting tryptamine oxidation by human MAO, with the exception of the brain enzyme. With mouse and bovine enzyme, I was a less effective inhibitor than pargyline regardless of the substrate. The comparative effects of the two inhibitors with rat MAO varied with the source of the enzyme and the substrate used.

The K_i values of I obtained with various enzyme preparations in the presence of tryptamine-2-^{14}C are shown in Table IV. Mixed-type inhibition (combination of competitive and noncompetitive) was observed with all sources of human MAO; in all cases the K_i value for competitive inhibition was smaller than that for noncompetitive inhibition, indicating a preference of the compound to bind more competitively. In view of recent evidence of MAO isozymes in human and rat liver (3) and human brain (8), it is possible that at least two forms of MAO are present in a given tissue, one being susceptible to competitive inhibition by I and the other being noncompetitively inhibited. In the rat, mixed-type inhibition was observed with only liver MAO and was also of a more competitive nature. The enzyme from rat heart, rat brain, and bovine liver was

Table III—Concentrations of I and Pargyline Required to Produce 50% Inhibition of MAO from Various Tissues (I_{50})

Inhibitor	Substrate	I_{50}, μM[a] Human Liver	Human Heart	Human Brain	Rat Liver	Rat Heart	Rat Brain	Mouse Liver	Bovine Liver
I	Tryptamine	3.0	2.3	143.0	7.0	1.4	4.0	24.0	16.0
	Tyramine	1.7	3.5	75.0	0.60	0.15	9.8	23.0	11.0
Pargyline	Tryptamine	13.0	9.8	0.87	7.5	27.2	23.0	0.20	0.10
	Tyramine	0.66	0.69	0.15	0.83	1.40	0.95	0.059	0.070

[a] Each value represents the mean obtained from at least two experiments.

Table IV—Inhibition Constants for I with MAO from Various Sources

Enzyme Source	K_i, μM[a,b] Competitive	K_i, μM[a,b] Noncompetitive	K_m, μM[a,b]
Human			
Liver	0.83	3.2	12.0
Heart	0.92	4.8	20.3
Brain	66.7	125.0	31.3
Rat			
Liver	2.3	8.8	23.6
Heart	0.21	—	13.8
Brain	3.4	—	62.9
Bovine			
Liver	8.5	—	83.4

[a] Each value represents the mean obtained with two separate preparations of the enzyme. [b] Substrate: tryptamine-2-^{14}C hydrochloride.

competitively inhibited by I. This finding does not preclude the possible presence of isozymes in these tissues; several forms of the enzyme have been reported in the rat brain (5).

The larger K_m value for tryptamine with the bovine liver MAO indicated a lesser affinity of this enzyme for the substrate as compared with the liver MAO from both humans and rats (Table IV). Tryptamine also demonstrated a weaker affinity for brain MAO than for the enzyme from all other tissues.

The nature of binding of I and VII, in terms of reversibility or irreversibility, was studied during tryptamine oxidation. Although the structure of VII bears a propargyl ($CH_2C{\equiv}CH$) group, this compound, unlike pargyline, did not exert irreversible inhibition of human liver MAO, since preincubation of VII (and also I) with the enzyme preparation followed by titration of residual enzyme with the substrate did not show progressive inactivation with time (Fig. 1). In another experiment, when the combined effect of pargyline and I preincubation on MAO from human liver was studied, the rate of irreversible inactivation of MAO by pargyline was decreased by the presence of I (Fig. 1). This suggests either a common binding site for the two compounds or, alternatively, the binding of I and/or the conformational changes it produced in the enzyme sterically hindered the binding of pargyline.

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Theoretical Treatment of Diffusional Transport into and through an Oil-Water Emulsion with an Interfacial Barrier at the Oil-Water Interface

TOSHIHISA YOTSUYANAGI, WILLIAM I. HIGUCHI[▲], and ABDEL-HALIM GHANEM

Abstract □ The transport of solute into and through a heterogeneous system involving interfacial barriers was theoretically investigated. The system consisted of a "donor" bulk aqueous phase, the aqueous diffusion layer, and a matrix in which oil droplets of uniform size were dispersed. The theoretical method employed was able to account quantitatively for the barriers existing at the oil droplet-water interface, and the technique should have general applicability to all situations involving a heterogeneous medium in which the local interface equilibrium is achieved slowly. Computations were carried out using the interfacial barrier permeability coefficient, the volume fractions of the phases, and the particle size of the oil droplet as the parameters.

Keyphrases □ Diffusion through oil-water emulsions—theoretical treatment of interfacial barriers, calculation of influence of barrier permeability coefficient, phase volume fraction, and particle size □ Interfacial barriers, oil-water emulsions—theoretical treatment of solute transport rates □ Heterogeneous systems—theoretical treatment of transport rates involving interfacial barriers □ Transport of solutes through heterogeneous system—theoretical treatment of interfacial barriers

When mass transfer occurs into and through polyphase media, especially those involving finely dispersed heterogeneous phases, various factors influence the transport behavior of solutes. Among these factors, the transport resistances existing at the dispersed phase–continuous phase boundaries would be expected to be very important in certain situations (1–3). Yet little is understood about the problem.

The purpose of this study is to describe a procedure for the computations of the transport rate of a solute through a matrix containing a dispersion of oil droplets and for which the solute partitioning rate between the local bulk aqueous and the oil droplet phases might be slow and for which the diffusion through the oil droplets might also be slow (1). The general technique should be applicable to any situation involving a heterogeneous medium in which the local interface equilibrium is achieved slowly. Thus, for example, this approach should also be applicable to the treatment of transport and distribution rate data of drugs in various biological tissues in which the interfacial transport resistance of the cell membrane is appreciable.

THEORY AND GENERAL DESCRIPTION OF MODEL

The system consists of three compartments (Fig. 1). The first compartment is the donor aqueous phase and the second is the aqueous diffusion layer. The third is the matrix in which oil droplets of uniform size are assumed to be dispersed. The donor phase is assumed to be well stirred, and the nature and intensity of the agitation determine the thickness, h, of the diffusion layer. It is assumed that steady-state conditions exist in the diffusion layer, but the distribution of diffusing solute in the matrix compartment follows nonsteady-state diffusion.

In using the finite difference method (4), the matrix compartment is divided into appropriate small segments of equal width. The assumptions on which the model is based are: (*a*) diffusion is one dimensional within the diffusion layer and the matrix, (*b*) the solute begins to penetrate the continuous phase at $t = 0$, (*c*) the number of particles in each segment is constant, and (*d*) the particle size of the oil phase is small compared to the overall dimensions of the transport matrix so that equilibrium inside each oil phase is fast. Then, the solute that enters each segment may be divided into two parts: the portion that goes into the continuous phase and the portion that goes into the oil phase.

The concentration changes with time, t, of the donor phase may be expressed as follows:

$$\frac{dC_b}{dt} = -\frac{D_w A}{V_w h}(C_b - C_{ai}) \qquad \text{(Eq. 1)}$$

where C_b is the concentration of the solute in Compartment I, D_w is the diffusion coefficient of the solute in the diffusion layer, V_w is the volume of Compartment I, A is the surface area exposed, and C_{ai} is the solute concentration at the diffusion layer–matrix interface. The fluxes passing through the interface between the donor phase and the first segment of the matrix are assumed to be equal and may be expressed by:

$$\frac{D_w}{h}(C_b - C_{ai}) = \frac{D_m}{(TH/2)}[C_{ai} - C_{a(1)}] \qquad \text{(Eq. 2)}$$

where D_m is the effective solute diffusion coefficient in the matrix, TH is the thickness of the segment, and $C_{a(1)}$ is the average aqueous solute concentration in the first segment.

The mass balance for each segment is given by:

$$V_t \frac{dC_{t(i)}}{dt} = V_a \frac{dC_{a(i)}}{dt} + V_o \frac{dC_{o(i)}}{dt} \qquad \text{(Eq. 3)}$$

where V_t, V_a, and V_o are the total volume, the volume of the aqueous portion, and the volume of the oil phase, respectively, of a segment;

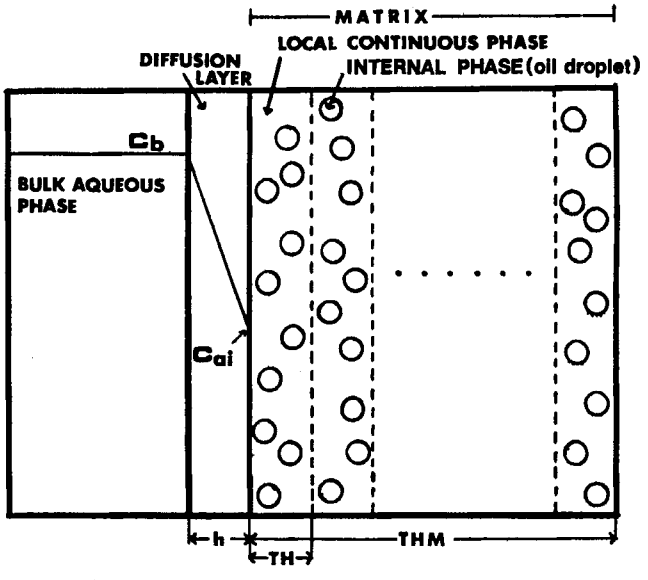


Figure 1—*Schematic model describing the uptake of the solute from the donor bulk aqueous phase to the matrix.* THM *is the thickness of the matrix,* TH *is the thickness of the segment, and* h *is the thickness of the diffusion layer.*

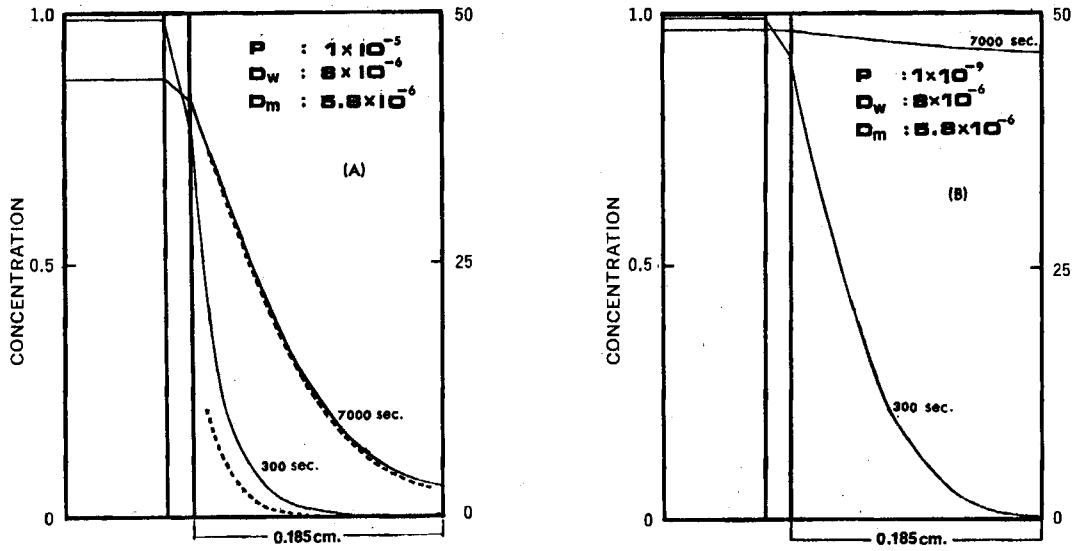


Figure 2—*The concentration–distance distribution profiles in the local bulk aqueous phase (solid line, which corresponds to the left scale) and in the oil phase (dotted line, which corresponds to the right scale). Permeability coefficients are* $P = 1 \times 10^{-5}$ *cm. sec.*$^{-1}$ *(A) and* 1×10^{-9} *cm. sec.*$^{-1}$ *(B). The diffusion coefficient of the donor aqueous phase is* $D_w = 8 \times 10^{-6}$ *cm.*^{2} *sec.*$^{-1}$. *The diffusion coefficient in the matrix is* $D_m = 5.8 \times 10^{-6}$ *cm.*^{2} *sec.*$^{-1}$. *Volume fractions of the aqueous and the oil phases are 0.8 and 0.2, respectively. Other parameters remain fixed.*

$C_{a(i)}$ and $C_{o(i)}$ are the aqueous solute concentration and the oil phase concentration of the solute in segment i, respectively. One may also write the following relations involving the first segment:

$$\frac{dC_{t(1)}}{dt} = \frac{2AD_m}{THV_t}[C_{ai} - C_{a(1)}] - \frac{AD_m}{THV_t}[C_{a(1)} - C_{a(2)}] \quad \text{(Eq. 4)}$$

$$\frac{dC_{o(1)}}{dt} = \frac{n4\pi a^2 P}{V_o}\left[C_{a(1)} - \frac{C_{o(1)}}{K_{ao}}\right] \quad \text{(Eq. 5)}$$

where $C_{a(2)}$ is the aqueous concentration of segment 2, a is the radius of the oil droplet, n is the number of droplets in a segment, P is the permeability coefficient for the oil–water interfacial barrier, and K_{ao} is the oil–water partition coefficient.

The following equations may be written where segment i is neither the first nor the last in the matrix:

$$\frac{dC_{t(i)}}{dt} = \frac{AD_m}{THV_t}[C_{a(i-1)} - 2C_{a(i)} + C_{a(i+1)}] \quad \text{(Eq. 6)}$$

$$\frac{dC_{o(i)}}{dt} = \frac{n4\pi a^2 P}{V_o}\left[C_{a(i)} - \frac{C_{o(i)}}{K_{ao}}\right] \quad \text{(Eq. 7)}$$

The rate expressions for the last segment, segment n, are:

$$\frac{dC_{t(n)}}{dt} = \frac{AD_m}{THV_t}[C_{a(n-1)} - C_{a(n)}] \quad \text{(Eq. 8)}$$

Table I—Parameters Describing the System and Their Numerical Values and Dimensions

Parameter	Values
D_w	8×10^{-6} (cm.^{2} sec.$^{-1}$)
D_m	5.8×10^{-6} (cm.^{2} sec.$^{-1}$)
h	0.085 (cm.)
V_w	50 (cm.^{3})
THM	0.185 (cm.)
P	$0 \rightarrow \infty$ (cm. sec.$^{-1}$)
a	2.3×10^{-4}, 0.5×10^{-4} (cm.)
V_o	0.8, 0.5
V_a	0.2, 0.5
CONCN1[a]	1 (mole)
A	0.37 (cm.)
K_{ao}	50
NOSEG[b]	10

[a] CONCN1 = initial concentration in the donor phase. [b] NOSEG = number of segments divided.

$$\frac{dC_{o(n)}}{dt} = \frac{n4\pi a^2 P}{V_o}\left[C_{a(n)} - \frac{C_{o(n)}}{K_{ao}}\right] \quad \text{(Eq. 9)}$$

Accordingly, the concentrations of drug in the bulk aqueous phase and in each segment in the matrix can be calculated as a function of time by solving the set of differential equations, Eqs. 1–9.

The transport behavior for a typical situation was simulated by the IBM 360/67 digital computer. The parameters selected for the calculations are shown in Table I along with the other input data. The calculations were then carried out for several sets of values of the interfacial barrier permeability coefficient, the volume fraction of the phases, and the oil phase particle size. The detailed outline of the method of computation is given in the *Appendix*.

RESULTS AND DISCUSSION

Typical profiles of the solute concentration–distance distribution in the local bulk aqueous phase and in the oil phase with time are shown in Figs. 2A and 2B. These were simulated under the conditions where the interfacial barrier permeability coefficient, P, was 10^{-5} and 10^{-9} cm. sec.$^{-1}$, respectively. The total penetration into the oil phase is shown in Fig. 3 for three P values. These results show that a strong effect of the permeability coefficient is evident in this range of P values. When the permeability coefficient is 10^{-5}

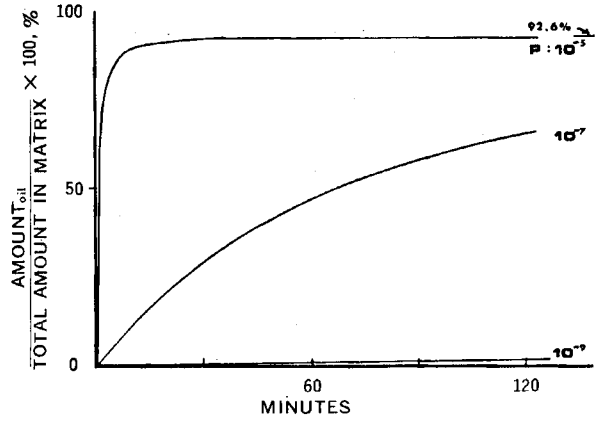


Figure 3—*Time dependence of solute penetration into the total oil phase of the matrix. At equilibrium, the amount in the oil phase is 92.6% of the total amount in the entire matrix.*

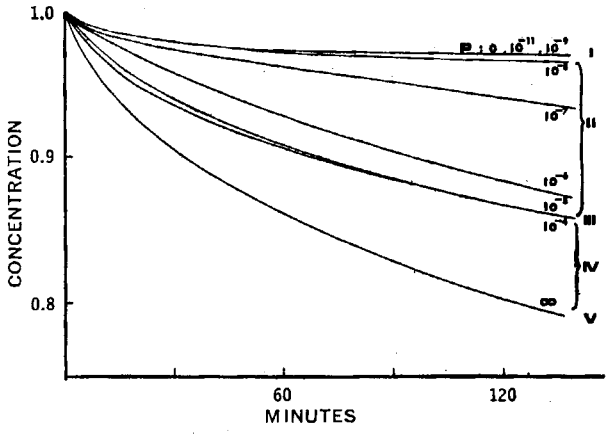


Figure 4—*The concentration change of the donor phase for different oil–water permeability coefficients.* $D_w = 8 \times 10^{-6}$ *cm.*^{2} *sec.*$^{-1}$, $D_m = 5.8 \times 10^{-6}$ *cm.*^{2} *sec.*$^{-1}$, $V_a = 0.8$, $V_o = 0.2$, *and* $a = 2.3 \times 10^{-4}$ *cm. The calculations for* $0 < P \leqq 10^{-4}$ *are carried out with the present procedure. The calculation for* $P = \infty$ *is based upon a previous procedure (5) using the Bruggeman mixture relationship (6) for* D_m.

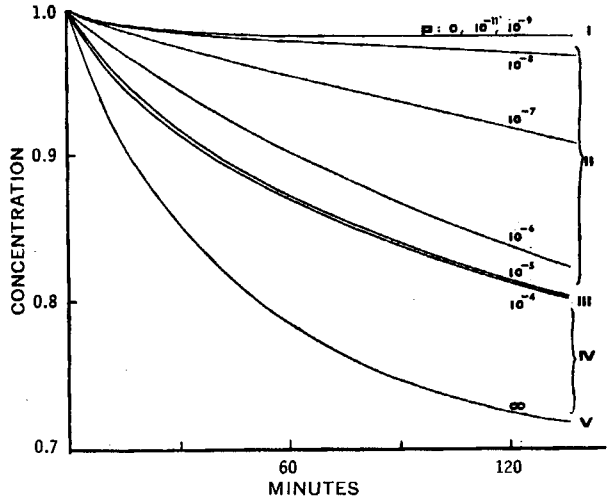


Figure 6—*The solute concentration changes in the donor phase for different permeability coefficients.* $D_w = 8 \times 10^{-6}$ *cm.*^{2} *sec.*$^{-1}$, $D_m = 5.8 \times 10^{-6}$ *cm.*^{2} *sec.*$^{-1}$, $V_a = 0.5$, $V_o = 0.5$, *and* $a = 2.3 \times 10^{-4}$ *cm.*

cm. sec.$^{-1}$, the distribution rate between the local aqueous phase and the oil phase is very fast, and the solute distribution between the aqueous and the oil phases is nearly at equilibrium about 30 min. later. On the other hand, in the case of $P = 10^{-9}$ cm. sec.$^{-1}$, the amount that enters the oil phase is only 2% of the total amount even at $t = 2$ hr. Figure 4 gives the results of the present calculations and those for additional situations expressed in terms of the time changes in the donor phase solute concentration. The only parameter that was varied in these calculations was P from zero to infinity. The other parameters, shown in Table I, remained fixed.

These results may be summarized in the following manner. For the case of extremely low P values, *i.e.*, from zero to 10^{-9} cm. sec.$^{-1}$, the uptake rates for these situations were nearly the same and are classified in the first group. For the range of P values of $10^{-8} \simeq P \simeq 10^{-5}$, the uptake rate was rather sensitive to P and are classified in the second group. The third groups' results correspond to $10^{-5} \simeq P \simeq 10^{-6}$, where again the uptake behavior is somewhat insensitive to P. When the P values are larger than 10^{-4} cm. sec.$^{-1}$ but not very large, the uptake rates again increase with increasing P. This region is classified as the fourth group. When the P is very large, the maximum rate is approached and these results are classified as the fifth group. For this last situation, the present method of calculation was not used. Instead a previous procedure (5) and a D_m value were used based upon the Bruggeman mixture equation (6).

The physical description of these results may be conveniently presented with the help of Fig. 5, in which the effects of changing the permeability coefficient are schematically illustrated. The first situation (Group I results) corresponds to that in which P is so small that oil phase droplets act as impermeable spheres (*e.g.*, glass beads) and only a little solute enters the internal phase. The second situation is that in which the solute distribution rate between the continuous phase and the internal one is still relatively slow but begins to influence significantly the uptake kinetics. For this situation, the partition coefficient, K_{ao}, may not be so influential when it is large and when P is in the lower end of this range. In the third situation (III), the solute distribution is sufficiently fast and the aqueous–oil equilibrium is essentially maintained throughout the matrix. In the fourth situation, the P values are sufficiently great so that the oil droplets are not only locally equilibrated with the aqueous phase but are contributing as significant pathways to the transport of solute. Finally, when P approaches infinity the oil droplets are both fully equilibrated with the aqueous phase and maximally contributing to the effective diffusion coefficient of the heterogeneous system (5).

Figure 6 shows the effect of changing the volume fraction ratio from 0.8/0.2 to 0.5/0.5. As can be seen, there are no significant qualitative differences with the results in Fig. 4. However, the effects are much larger as might be expected.

The effect of particle size is shown in Fig. 7. The particle-size effect is largely restricted to Group II results, where the larger the size of the oil droplets the faster is the uptake rate. However, for much larger and for much smaller P values, the particle size should have no effect on the uptake kinetics.

This study has provided a quantitative method for analyzing the transport behavior of solutes through a heterogeneous system in which local interfacial barriers play important roles in determining: (*a*) the extent to which the internal phase is in equilibrium with the

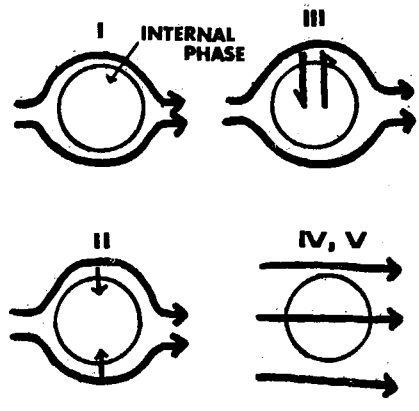


Figure 5—*Schematic illustrations of drug transport through the continuous phase (aqueous bulk phase) and involving the internal phase (oil phase), corresponding to different situations dependent upon the magnitude of the interfacial barrier permeability coefficient.*

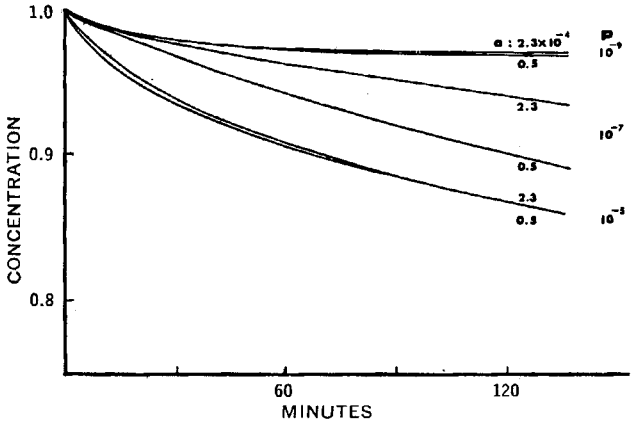
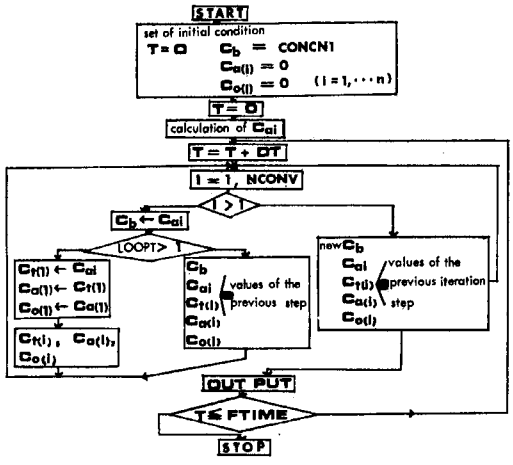


Figure 7—*Effect of particle size on the solute uptake rate. Particle radius* $= 2.3 \times 10^{-6}$ *cm. and* 0.5×10^{-4} *cm.,* $V_a = 0.8$, *and* $V_o = 0.2$.

local continuous phase, and (*b*) the extent to which the internal phase acts as a pathway for transport of the solute. The theoretical procedure permits the calculation of the influences of the interfacial barrier permeability coefficient, the particle size, the volume fractions of the phases, and the partition coefficients in the phases upon the solute transport rates through the heterogeneous media.

APPENDIX: NUMERICAL COMPUTATION PROCEDURE

The flow diagram of the calculating procedure is briefly shown in Scheme I. After $t = 0$, a series of calculation procedures undergo



Scheme I—Flow diagram for computation of concentration distributions

successive approximation for each time increment, Δt. On the calculation of C_b, $C_{t(i)}$, $C_{a(i)}$, and then $C_{o(i)}$ at $t = \Delta t$, the first approximation of ΔC_b is obtained from Eq. 1, assuming $(C_{ai})_{t=\Delta t} = (C_{ai})_{t=0}$. Then under the assumption of both $C_{a(i)}$ and $C_{a(i+1)} = 0$, the first approximation of $\Delta C_{t(i)}$ is calculated by Eqs. 4, 6, and 8, respectively, and then $C_{t(i)}$ at $t = \Delta t$ is assumed $\Delta C_{t(i)}$ for $0 < t \leqq \Delta t$. Next, by assuming $\Delta C_{o(i)} = 0$, $\Delta C_{t(i)} = \Delta C_{a(i)}$ according to Eq. 3 and then $C_{a(i)}$ is also assumed $0.5 \times \Delta C_{a(i)}$ for $0 < t \leqq \Delta t$. Accordingly, $\Delta C_{o(i)}$ is calculated by Eqs. 5, 7, and 9, respectively, under the assumption of $C_{o(i)} = 0$, and then $C_{o(i)}$ is assumed $0.5 \times \Delta C_{o(i)}$ for $0 < t \leqq \Delta t$. The second approximations of C_{ai}, C_b, $C_{t(i)}$, $C_{a(i)}$, and $C_{o(i)}$ are calculated by using the first approximations successively. These procedures are repeated until respective values converge. The calculation of the next step (added Δt) starts from the values of one step before in the same manner mentioned above.

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A New Tablet Disintegrating Agent: Cross-Linked Polyvinylpyrrolidone

SAUL S. KORNBLUM▲ and SAMUEL B. STOOPAK

Abstract □ Cross-linked polyvinylpyrrolidone was studied for its disintegration property in comparison to starch USP and alginic acid. Certain physical parameters of the disintegrants (maximum moisture sorption, hydration capacity, bulk density, and specific surface area) were determined for the purpose of differentiating their relative efficiency. A linear relationship was found to exist when the maximum moisture sorption was plotted *versus* the specific surface area for each disintegrant. It was postulated that capillary activity of the cross-linked polyvinylpyrrolidone for water appears responsible for its tablet disintegration property. Cross-linked polyvinylpyrrolidone demonstrated superiority over starch USP and alginic acid in most of the experimental tablet formulations made by either dry or wet granulation. A quinazolinone compound was formulated into tablets employing each disintegrant to provide identical disintegration times, and these tablets were submitted to dissolution rate analysis. The dissolution results showed some differences for those made by direct compression but no variation for wet granulated tablets.

Keyphrases □ Polyvinylpyrrolidone, cross linked—evaluated as tablet disintegrant, compared to starch USP and alginic acid □ Tablet disintegrants—cross-linked polyvinylpyrrolidone evaluated, compared to starch USP and alginic acid □ Disintegration properties—cross-linked polyvinylpyrrolidone, compared to starch USP and alginic acid

Today's emphasis on the availability of drugs highlights the importance of the relatively rapid disintegration of a tablet as a criterion for ensuring uninhibited drug dissolution behavior (1–3). Only a few acceptable tablet disintegrating agents are available to the research pharmacist. The starches (corn, potato, wheat, rice, and arrowroot) have been extensively studied (4–7) as to their varying properties as disintegrants (8). The mechanism of action of the starches is still undergoing study, although at one point it was wrongly felt that grain swelling was responsible (7). Experiments with compressed starch, both dry and wetted, observed with

local continuous phase, and (*b*) the extent to which the internal phase acts as a pathway for transport of the solute. The theoretical procedure permits the calculation of the influences of the interfacial barrier permeability coefficient, the particle size, the volume fractions of the phases, and the partition coefficients in the phases upon the solute transport rates through the heterogeneous media.

APPENDIX: NUMERICAL COMPUTATION PROCEDURE

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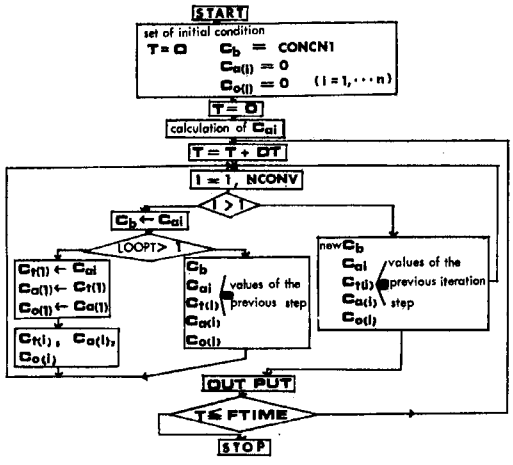

Scheme I—Flow diagram for computation of concentration distributions

successive approximation for each time increment, Δt. On the calculation of C_b, $C_{t(i)}$, $C_{a(i)}$, and then $C_{o(i)}$ at $t = \Delta t$, the first approximation of ΔC_b is obtained from Eq. 1, assuming $(C_{ai})_{t=\Delta t} = (C_{ai})_{t=0}$. Then under the assumption of both $C_{a(i)}$ and $C_{a(i+1)} = 0$, the first approximation of $\Delta C_{t(i)}$ is calculated by Eqs. 4, 6, and 8, respectively, and then $C_{t(i)}$ at $t = \Delta t$ is assumed $\Delta C_{t(i)}$ for $0 < t \leqq \Delta t$. Next, by assuming $\Delta C_{o(i)} = 0$, $\Delta C_{t(i)} = \Delta C_{a(i)}$ according to Eq. 3 and then $C_{a(i)}$ is also assumed $0.5 \times \Delta C_{a(i)}$ for $0 < t \leqq \Delta t$. Accordingly, $\Delta C_{o(i)}$ is calculated by Eqs. 5, 7, and 9, respectively, under the assumption of $C_{o(i)} = 0$, and then $C_{o(i)}$ is assumed $0.5 \times \Delta C_{o(i)}$ for $0 < t \leqq \Delta t$. The second approximations of C_{ai}, C_b, $C_{t(i)}$, $C_{a(i)}$, and $C_{o(i)}$ are calculated by using the first approximations successively. These procedures are repeated until respective values converge. The calculation of the next step (added Δt) starts from the values of one step before in the same manner mentioned above.

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A New Tablet Disintegrating Agent: Cross-Linked Polyvinylpyrrolidone

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Abstract □ Cross-linked polyvinylpyrrolidone was studied for its disintegration property in comparison to starch USP and alginic acid. Certain physical parameters of the disintegrants (maximum moisture sorption, hydration capacity, bulk density, and specific surface area) were determined for the purpose of differentiating their relative efficiency. A linear relationship was found to exist when the maximum moisture sorption was plotted *versus* the specific surface area for each disintegrant. It was postulated that capillary activity of the cross-linked polyvinylpyrrolidone for water appears responsible for its tablet disintegration property. Cross-linked polyvinylpyrrolidone demonstrated superiority over starch USP and alginic acid in most of the experimental tablet formulations made by either dry or wet granulation. A quinazolinone compound was formulated into tablets employing each disintegrant to provide identical disintegration times, and these tablets were submitted to dissolution rate analysis. The dissolution results showed some differences for those made by direct compression but no variation for wet granulated tablets.

Keyphrases □ Polyvinylpyrrolidone, cross linked—evaluated as tablet disintegrant, compared to starch USP and alginic acid □ Tablet disintegrants—cross-linked polyvinylpyrrolidone evaluated, compared to starch USP and alginic acid □ Disintegration properties—cross-linked polyvinylpyrrolidone, compared to starch USP and alginic acid

Today's emphasis on the availability of drugs highlights the importance of the relatively rapid disintegration of a tablet as a criterion for ensuring uninhibited drug dissolution behavior (1–3). Only a few acceptable tablet disintegrating agents are available to the research pharmacist. The starches (corn, potato, wheat, rice, and arrowroot) have been extensively studied (4–7) as to their varying properties as disintegrants (8). The mechanism of action of the starches is still undergoing study, although at one point it was wrongly felt that grain swelling was responsible (7). Experiments with compressed starch, both dry and wetted, observed with

a scanning electron microscope resulted in this conclusion.

The purpose of the current research was to evaluate cross-linked polyvinylpyrrolidone as a disintegrant in comparison with starch (*i.e.*, corn starch) and alginic acid. Alginic acid possesses good disintegrating properties at reasonably low concentrations and provides other desirable physical characteristics to the tablet structure. Certain physical parameters of the disintegrants were evaluated to obtain an insight as to their potential disintegrating efficiency. Tablets prepared by dry and wet granulations using a poorly water-soluble drug contained sufficient quantities of each disintegrant to give identical disintegration times. The effects of the various disintegrating agents were then evaluated utilizing dissolution studies as a measure of *in vitro* availability.

EXPERIMENTAL

Materials—Three substances were studied for their comparative tablet disintegrating property: starch, alginic acid[1], and cross-linked polyvinylpyrrolidone[2]. Starch USP was used, and the alginic acid and cross-linked polyvinylpyrrolidone were accepted on the basis of manufacturer's specifications. The same lot of disintegrants and excipients were utilized for all experimental trials.

All other tablet excipients were USP or NF grade. The drug substances used in the preparation of the various tablet formulations were: 1-isopropyl-7-methyl-4-phenylquinazolin-2(1*H*)-one[3], butalbital, and 6-(6,10-dihydroxyundecyl)-β-resorcylic acid μ-lactone[4].

Selection of a Tablet Disintegration Agent—Starch and alginic acid have been well accepted as disintegrating agents and are used extensively in tablet formulations. Cross-linked polyvinylpyrrolidone was submitted to testing to justify its ranking with the other disintegrants available to the research pharmacist. The criteria by which the authors selected this new disintegrant stemmed from the ability of cross-linked polyvinylpyrrolidone to sorb a large quantity of moisture under isothermal, constant relative humidity conditions. Cross-linked polyvinylpyrrolidone is a white, free flowing, high molecular weight, cross-linked polymer of vinylpyrrolidone formed under the influence of a special catalytic environment. Cross-linked polyvinylpyrrolidone is highly insoluble in water, strong mineral acids, and alkali, so consequently there is a lack of information relating to its molecular weight. Its main commercial use, thus far, has been as a filtering aid for the wine and vinegar industries.

Determination of Maximum Moisture Sorption of Disintegrants—Ten grams of starch, alginic acid, or cross-linked polyvinylpyrrolidone was accurately weighed and evenly distributed over the surface of an 89-mm. tared petri dish. The sample was then placed in a large desiccator containing distilled water in its reservoir. The desiccator was then stored in a laboratory oven and the temperature was maintained constant at 37 ± 0.5°. At various time intervals, the weight gained by the exposed samples was recorded and the amount of water sorbed was calculated from the weight difference.

Determination of Hydration Capacity of Disintegrants—The procedure utilized (9) was developed for materials that do not contain appreciable (over 25%) water-soluble constituents. A 2-g. sample of disintegrant was placed into a 100-ml. centrifuge tube tared with stopper. Forty milliliters of water (pH 6–7) was added, and the tube was stoppered and shaken vigorously to suspend the sample thoroughly. The suspension was allowed to stand for 10 min. During this time, it was mixed by inverting three times at the end of 5 and 10 min. The stopper was then removed and the tube was centrifuged for 15 min. at 1000×*g*. The centrifuge was allowed to stop without braking. The supernate was carefully decanted and the tube was inverted to allow draining. The tube was then restoppered and the contents were weighed. The hydration capacity was calculated in the following manner:

$$\text{hydration capacity} = \frac{(\text{weight of tube} + \text{sediment}) - (\text{weight of tube})}{\text{sample weight (dry basis)}} \quad \text{(Eq. 1)}$$

Determination of Specific Surface Area of Disintegrants—In an attempt to establish the relationship that existed between the maximum moisture sorbed by the disintegrants and their specific surface area, the Brunauer, Emmett, and Teller (B.E.T.) technique[5] was performed.

The B.E.T. method involved low-temperature nitrogen adsorption by the powder sample. Samples were pretreated by evacuation at 0.001 mm. Hg under elevated temperature to outgas any adsorbed gases from the solid surface. The surface area analysis involved incremental gas volume adsorption measurements, which were made by accurately determining the equilibrium pressure following the introduction of gas quantities into the system.

Determination of Bulk Density of Disintegrants—The procedure utilized (10) is often referred to as the cylinder-drop technique. An accurately weighed sample of each disintegrant, of about 50-ml. volume, was introduced into a 100-ml. graduated cylinder. The cylinder was dropped onto a hard wood surface six times from a height of 2.54 cm. (1 in.) at 2-sec. intervals. The bulk density was then calculated by dividing the weight of the sample (grams) by the final tapped volume (milliliters) of the sample contained in the cylinder.

Direct Compression and Wet Granulation Tablet Formulation—Various tablet formulations exemplifying both direct compression and wet granulation techniques were selected to compare the disintegration property of the three disintegrants under investigation. The material compositions of these tablets are presented in Table I, with the categorizing of each tablet type into six series. Three drugs of varying water solubility were chosen for this segment of the experiments. In Series 1–6, wherever the concentration of disintegrant was varied, lactose powder was substituted to maintain the same total tablet weight.

Direct Compression Tablet Preparation for Disintegration Time Studies—The required amounts of drug, tablet diluent, and disintegrant were accurately weighed and passed through a No. 20-mesh U. S. Standard sieve. The materials were then transferred to a twin-shell blender[6] and tumbled for 10 min. The required amount of lubricant was accurately weighed, passed through a No. 60-mesh U. S. Standard sieve, and added to the blender. The materials were tumbled for an additional 5 min. The materials were then compressed into tablets of the appropriate weight and hardness, utilizing a single-punch tablet press[7] equipped with selected size tablet punches.

Wet Granulation Tablet Preparation for Disintegration Time Studies—The required amounts of drug, tablet diluent, disintegrant, and lubricant were accurately weighed and passed through a No. 20-mesh U. S. Standard sieve. The materials were then transferred to a twin-shell blender, equipped with an intensifier bar[6], and mixed for 5 min. The required amount of tablet binder (polyvinylpyrrolidone) was accurately weighed and dissolved in a suitable quantity of equal volumes of granulating solvents. This solution was added to the blender through the intensifier bar, and mixing was continued for 5 min. after the addition of the granulating solution. The wet granulation was placed on a paper-lined tray in an oven at 40° and dried to 1–3% moisture. The dry granulation was passed through a No. 18-mesh U. S. Standard sieve and compressed into tablets of the appropriate weight and hardness, utilizing a single-punch tablet press equipped with selected size tablet punches.

Tablet Preparation for Dissolution Rate Studies—Granulations were prepared utilizing both the direct compression and wet granulation techniques previously outlined. Tablets were prepared by compression in a hydraulic press[8] equipped with a gauge measuring forces from 0 to 909 kg. (0 to 2000 lb.) and calibrated in 22.7-kg. (50-lb.) increments. A special holder held the bottom punch and die in a fixed position during compression. The required amount of

[1] Kelacid, Kelco Corp., San Diego, Calif.
[2] Polyclar AT, GAF Corp., New York, N. Y.
[3] Chemical Development Dept., Sandoz-Wander, Inc., East Hanover, N. J.
[4] Commercial Solvents Corp., New York, N. Y.
[5] Model 400 surface-area analyzer, Micromeritics Instrument Corp., Norcross, Ga.
[6] Patterson-Kelley, Inc., East Stroudsburg, Pa.
[7] Model E, Stokes Equipment Corp., Warminster, Pa.
[8] Model C, Fred S. Carver, Inc., Summit, N. J.

Table I—Tablet Composition Used to Compare Disintegration Property for Cross-Linked Polyvinylpyrrolidone, Alginic Acid, and Starch

	Quantity per Tablet, mg.			
Series 1 (water-insoluble drug)	A	B	C	D
Resorcylic acid compound	100.0	100.0	100.0	100.0
Lactose powder	65.4	65.4	65.4	49.0
Cross-linked polyvinylpyrrolidone	3.6 (2%)	—	—	—
Starch	—	3.6 (2%)	—	20.0 (11%)
Alginic acid	—	—	3.6 (2%)	—
Polyvinylpyrrolidone	10.0	10.0	10.0	10.0
Magnesium stearate	1.0	1.0	1.0	1.0
Total tablet weight	180	180	180	180
Granulating solvents				
S.D. No. 30 alcohol				
Purified water				
Wet granulation tablets: 9-mm. deep concavity, 4–6 SCH[a]				
Series 2 (moderately water-soluble drug)	E	F	G	
Butalbital acid	50	50	50	
Lactose, spray dried	62	62	62	
Cross-linked polyvinylpyrrolidone	6 (5%)	—	—	
Starch	—	6 (5%)	—	
Alginic acid	—	—	6 (5%)	
Magnesium stearate	2	2	2	
Total tablet weight	120	120	120	
Direct compression tablet: 7-mm. standard concavity, 5–7 SCH				
Series 3 (placebo)	H	I	J	
Lactose, spray dried	200	200	200	
Calcium sulfate, dihydrate	83	83	83	
Cross-linked polyvinylpyrrolidone	15 (5%)	—	—	
Starch	—	15 (5%)	—	
Alginic acid	—	—	15 (5%)	
Stearic acid	1	1	1	
Magnesium stearate	1	1	1	
Total tablet weight	300	300	300	
Direct compression tablets: 8-mm. standard concavity, 11–13 SCH				
Series 4 (gastric fluid-soluble drug)	K	L	M	
Quinazolinone compound	75.0	75.0	75.0	
Lactose, spray dried	134.4	134.4	134.4	
Cross-linked polyvinylpyrrolidone	6.6 (3%)	—	—	
Alginic acid	—	6.6 (3%)	—	
Starch	—	—	6.6 (3%)	
Magnesium stearate	4.0	4.0	4.0	
Total tablet weight	220.0	220.0	220.0	
Direct compression tablets: 9-mm. flat-face, beveled-edge concavity, 9–11 SCH				
Series 5 (gastric fluid-soluble drug)	N	O	P	
Quinazolinone compound	25.0	25.0	25.0	
Lactose, spray dried	184.0	177.4	102.6	
Cross-linked polyvinylpyrrolidone	6.6 (3%)	—	—	
Alginic acid	—	13.2 (6%)	—	
Starch	—	—	88.0 (40%)	
Stearic acid	4.4	4.4	4.4	
Total tablet weight	220.0	220.0	220.0	
Direct compression tablets: 9-mm. flat-face, beveled-edge, 4–5 SCH				
Series 6 (gastric fluid-soluble drug)	Q	R	S	
Quinazolinone compound	25.0	25.0	25.0	
Lactose powder	185.5	182.2	120.6	
Cross-linked polyvinylpyrrolidone	1.1 (0.5%)	—	—	
Alginic acid	—	4.4 (2%)	—	
Starch	—	—	66.0 (30%)	
Polyvinylpyrrolidone	4.0	4.0	4.0	
Stearic acid	4.4	4.4	4.4	
Total tablet weight	220.0	220.0	220.0	
Granulating solvents				
S.D. No. 30 alcohol				
Purified water				
Wet granulation tablets: 9-mm. flat-face, beveled-edge concavity, 4–5 SCH				

[a] SCH, Strong–Cobb hardness, was determined with a Heberlein hydraulic drive tablet hardness tester.

Table II—Pertinent Physical Parameters[a] for Evaluating Relative Disintegrant Efficiency

Physical Property	Tablet Disintegrant—Cross-Linked Polyvinylpyrrolidone	Alginic Acid	Starch
Bulk density, g./ml.	0.26	0.68	0.63
Specific surface area, $m.^2/g.$	1.03	0.75	0.59
Maximum moisture sorption, % w/w	58.5	33.2	19.8
Hydration capacity	5.6	7.0	1.8

[a] Each determination is an average of four individual experiments.

material to make a tablet was weighed and transferred into the die cavity. The top punch was then gently inserted into the die cavity, and the granulation was compressed to a predetermined force.

The tablet thickness for each tablet was measured using a micrometer with vernier. Each tablet employed in the dissolution studies had to adhere to a tolerance of 0.02 mm. This physical measurement assured compliance of each tablet to a similar disintegration time for consecutive tablets.

The slow compression method was useful in controlling reproducibility of the physical character of each tablet. However, it provided substantially different stresses than those normally obtained with a high speed rotary tablet machine. Therefore, tablets prepared under production conditions may differ considerably from the test results.

Disintegration Test for Tablets Prepared to Evaluate Disintegrants—The USP XVIII (11) test procedure for uncoated tablets was utilized and modified to the extent that no disks were employed.

Friability Test for Tablets Prepared to Evaluate Disintegrants—The friability of all tablets studied was determined in a test apparatus consisting of a 27-cm. diameter, Plexiglas hollow chamber, 6 cm. in width. A curved arm in the chamber extends from a point on the circumference to slightly above the center of the apparatus. The apparatus is rotated at 25 r.p.m. by use of a shaft and motor at its center. The tablets were dropped a distance of 14 cm. from the arm at each rotation.

Twenty previously weighed tablets were placed in the apparatus, and the apparatus was then rotated for 4 min. to subject the tablets to 100 drops. The tablets were then weighed and the weight loss was calculated in terms of "percent friability."

Dissolution Rate Analysis of Tablets—The apparatus utilized is described in USP XVIII (13). A volume of 900 ml. of aqueous hydrochloric acid (pH 1.2, 37 ± 0.5°) was used as the dissolution medium, and a stirring speed of 50 r.p.m. was maintained.

Dissolution of the drug from the tablets was conducted through a 30-min. interval, where at least three half-lives were encompassed. Apparent sink conditions were adhered to in this study, as calculated by the solubility of the pure drug at 37° and pH 1.2.

The analytical details for following dissolution involved the withdrawal of 10-ml. aliquots at specified time intervals using a pipet with a filter tip. Then 10 ml. of aqueous pH 1.2 fluid was added to the beaker to replace the withdrawn volume. The aliquot was diluted to 20 ml. with aqueous pH 1.2 fluid. The absorption of the diluted solution was measured on a recording spectrophotometer[9] over the UV spectrum, and the maximum absorbance at 232 nm. was used to calculate the concentration of the quinazolinone compound. A correction factor was calculated for each sequential sampling as a result of the amount of dissolved drug removed for each sampling.

RESULTS AND DISCUSSION

Physical Parameters Employed for Evaluation of Disintegrants—Certain physical parameters were determined for the three materials studied in an attempt to predict their relative efficiency as tablet disintegrants (Table II). In comparing the various properties of cross-linked polyvinylpyrrolidone with the other two disintegrants, the most obvious large difference exists for the maximum moisture

[9] Cary model 14.

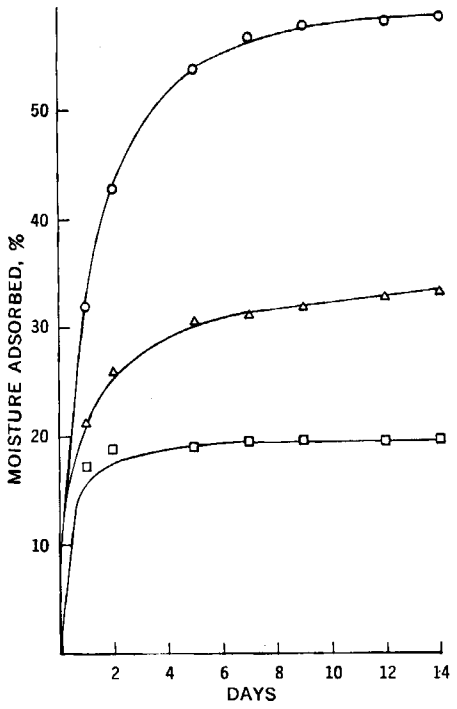

Figure 1—*Moisture sorption profile for the three disintegrants evaluated at 47 mm. aqueous tension and exposed to 37°. Key: ○, cross-linked polyvinylpyrrolidone; △, alginic acid; and □, starch.*

sorption. Figure 1 shows this parameter to be significantly greater for cross-linked polyvinylpyrrolidone than for either of the other compounds studied, resulting in the sorption of more than 50% of its own weight of water. This magnitude of difference between cross-linked polyvinylpyrrolidone and alginic acid is quite significant; however, the hydration capacity wherein water actually wets the material reveals about the same liquid–solid interactions or swelling power. Both parameters suggest that starch has substantially lower efficiency, in keeping with its poor action as a disintegrant in low concentrations. The lower bulk density of cross-linked polyvinylpyrrolidone acts as a positive factor for more adequate distribution within a tablet matrix.

The large specific surface area observed for cross-linked polyvinylpyrrolidone confirms the authors' theory that the greater capacity for water sorption under the conditions utilized in conducting the maximum moisture sorption experiments is a surface-related phenomenon. Figure 2 depicts the linear relationship existing between maximum moisture sorption and specific surface area for the three disintegrants. Under the normal environmental conditions to which the cross-linked polyvinylpyrrolidone and its tablets were exposed during storage, moisture sorption was determined to be negligible.

The four physical parameters discussed may possibly predict the relative usefulness and efficiency of new materials as disintegrants. The major emphasis apparently must be placed upon maximum moisture sorption and hydration capacity. Starch may be employed to establish a baseline for comparison of these properties with a new material.

Mechanism of Action Postulated for Disintegrants—Researchers have reported that starch's mechanism of action as a disintegrant involves only a limited amount of swelling of the grains (7) and that pores and porosity (12) of the tablet generally could not be correlated with disintegration times. Therefore, these phenomena are not the major mechanisms by which starch brings about tablet disintegration.

The authors believe that the mechanism of action of cross-linked polyvinylpyrrolidone depends greatly upon capillary effect in the presence of water. Maximum moisture sorption results obtained for cross-linked polyvinylpyrrolidone, alginic acid, and starch strongly suggest that the affinity of water for the disintegrant would permit distinction between their relative efficiencies. Hydration capacity would also support the thesis proposed inasmuch as it is related to swelling capacity. Cross-linked polyvinylpyrrolidone demonstrated

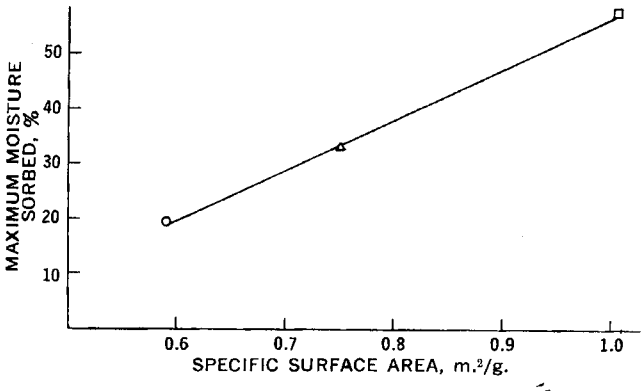

Figure 2—*Relationship of the maximum moisture sorption* versus *the specific surface area for the three disintegrants. Key:* □, *cross-linked polyvinylpyrrolidone;* △, *alginic acid; and* ○, *starch.*

similar behavior when compared with alginic acid. Since the data in Table III show cross-linked polyvinylpyrrolidone to be a superior disintegrant to alginic acid, swelling does not, in itself, appear to be the major mechanism of action.

The specific surface area results correlate well with the ranking of maximum moisture sorption for each disintegrant. The greater the surface area of the disintegrant, the more numerous are the sites for capillary attraction of water to its surface. The bulk density and maximum moisture sorption differences for cross-linked polyvinylpyrrolidone and alginic acid very possibly predict the differences that were observed for their relative disintegrant efficiency (discussed in the next section).

The mechanism of action of disintegrants is very complex and cannot be readily simplified into one or more categories. While it seems apparent that cross-linked polyvinylpyrrolidone acts through capillary activity with a secondary swelling effect, it is difficult to provide a conclusive statement as to the overall mechanism of action.

Relative Efficiency of Disintegrants in Various Tablet Formulations—The ultimate manner in which disintegrants can be properly evaluated is to prepare tablets for evaluation. Tablet formulations of various excipient composition and method of manufacture were employed to evaluate the relative efficiency of cross-linked polyvinylpyrrolidone, alginic acid, and starch as disintegrants. The tablet formulations manufactured for each series maintained the same physical parameters as shown in Table III for Series 1–4. The quantity of disintegrant was kept the same within each series for the purpose of material comparison. The disintegrant concentrations used were deliberately selected to illustrate the superiority of cross-linked polyvinylpyrrolidone. In all instances, this resulted in lower concentrations of starch than are normally employed.

Table III summarizes the disintegration time and friability as determined for all of the tablets studied. The outstanding difference between cross-linked polyvinylpyrrolidone and the other two disintegrants is its ability to bring about rapid disintegration with relatively low concentrations.

Cross-linked polyvinylpyrrolidone performed better than alginic acid and starch as a disintegrant in Series 1, 2, and 4. Each of these series contained drug, whereas in Series 3, which showed some similarity of disintegration times for cross-linked polyvinylpyrrolidone and alginic acid, no drug was incorporated.

Series 1 formulations represent a tablet containing about 55% drug which is almost totally water insoluble. These tablets were manufactured by a conventional wet granulation technique employing polyvinylpyrrolidone as the binder. Cross-linked polyvinylpyrrolidone was shown to surpass the alginic acid by threefold and the starch by 15-fold as a disintegrant. When 11% starch was employed, a reasonable disintegration time of 10 min. resulted; however, this resulted in a significant increase in tablet friability. The tablet formulation from Series 1, without the addition of a disintegrant, resulted in a lack of disintegration after 2 hr. of exposure. An important asset of cross-linked polyvinylpyrrolidone, which was pointed out during these studies, was that the wet granulation technique did not interfere with the desired tablet distintegration properties.

Series 2 shows cross-linked polyvinylpyrrolidone to be superior to alginic acid and starch from both a disintegration time and friability standpoint. Cross-linked polyvinylpyrrolidone disintegrated three times as fast as alginic acid, while with starch the tablet failed to disintegrate after 2 hr. of exposure. Cross-linked polyvinylpyrrolidone has been proven to be directly compressible in pure form, and this phenomenon relates to the low percent friability exhibited with its tablet formulations. In fact, cross-linked polyvinylpyrrolidone is presently being evaluated in higher concentrations in tablets as a direct compression binder–diluent–disintegrant.

Table III—Disintegration Time and Friability for Various Tablet Formulations Containing Cross-Linked Polyvinylpyrrolidone, Alginic Acid, and Starch

	Disintegration Time, min.[a]	Friability, %
Series 1 (water-insoluble drug)		
Formula A (2% cross-linked polyvinylpyrrolidone)	4	0.17
Formula B (2% starch)	60	0.20
Formula C (2% alginic acid)	12	0.17
Formula D (11% starch)	10	0.54
Series 2 (moderately water-soluble drug)		
Formula E (5% cross-linked polyvinylpyrrolidone)	3	0.20
Formula F (5% starch)	>120	0.37
Formula G (5% alginic acid)	9	0.30
Series 3 (placebo)		
Formula H (5% cross-linked polyvinylpyrrolidone)	0.67	0.20
Formula I (5% starch)	10	0.34
Formula J (5% alginic acid)	0.67	0.16
Series 4 (gastic fluid-soluble drug)		
Formula K (3% cross-linked polyvinylpyrrolidone)	1	0.46
Formula M (3% starch)	120	2.10
Formula L (3% alginic acid)	3	0.80

[a] Represents average of six individual tablets.

Series 3 represents a placebo tablet formulation using 5% disintegrant because starch was not operative below this level. The lower friability values for cross-linked polyvinylpyrrolidone and alginic acid indicate the presence of a tablet-binding property, whereas the starch renders a tablet considerably more friable. When the starch content was increased to 10% to reduce the disintegration time, an unacceptable friability was obtained which is typical for this material.

Series 4 demonstrated that a 3% level of cross-linked polyvinylpyrrolidone surpassed both alginic acid and starch in disintegration time. Furthermore, the friability was extremly poor for alginic acid and starch, whereas cross-linked polyvinylpyrrolidone bordered on an acceptable range. Of course, Series 4 tablets contained no auxiliary binder; by increasing the concentration of cross-linked polyvinylpyrrolidone to 5%, the friability was reduced by 65%. The same change with the other two disintegrants did not reduce the friability satisfactorily.

The authors have employed cross-linked polyvinylpyrrolidone and alginic acid as tablet disintegrating agents in an unconventional tablet matrix, and the information will be published at a later date. However, it should be noted that 20% cross-linked polyvinylpyrrolidone was necessary to obtain a 4-min. disintegration; when alginic acid was substituted at the same concentration, it required 60 min. to accomplish disintegration. When the alginic acid content was increased to 30%, no reduction of disintegration time was demonstrated.

The interesting properties of cross-linked polyvinylpyrrolidone stem from its ability at low concentrations (2–5%) to bring about acceptable tablet disintegration as well as its inherent ability to function as a tablet binder. The tablets resulting from its use possess low percent friability characteristics.

Effect of Disintegrants on Dissolution Behavior of Tablets—To ascertain the overall acceptability of a new disintegrant, it must be shown that no interference with the dissolution behavior of the drug from the tablet is involved. To investigate this parameter for cross-linked polyvinylpyrrolidone, tablets were formulated using the

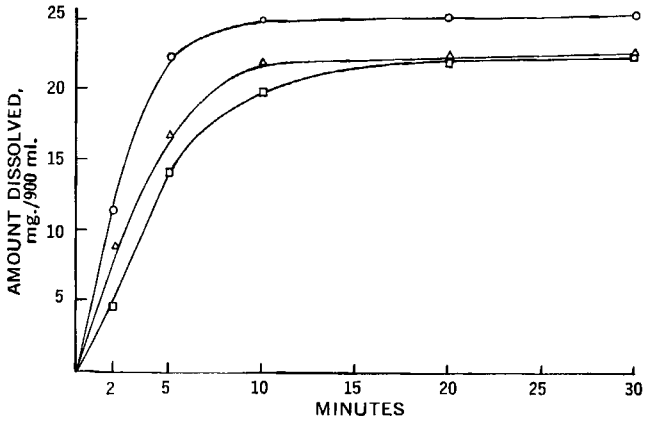

Figure 3—*Dissolution of quinazolinone compound in 900 ml. aqueous solution at pH 1.2 from directly compressed tablets of identical disintegration time. Key: ○, 3% cross-linked polyvinylpyrrolidone; △, 6% alginic acid; and □, 40% starch. Each data point plotted is the average of four experimental runs.*

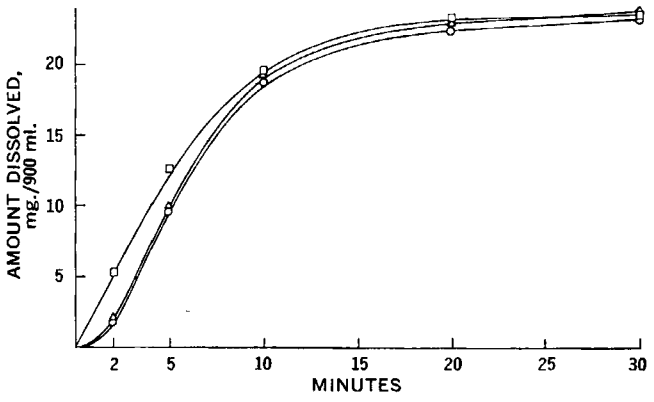

Figure 4—*Dissolution of quinazolinone compound in 900 ml. aqueous solution at pH 1.2 from wet granulated compressed tablets of identical disintegration time. Key: △, 0.5% cross-linked polyvinylpyrrolidone; ○, 2% alginic acid; and □, 30% starch. Each data point plotted is the average of four experimental runs.*

direct compression and wet granulation methods. In this instance, cross-linked polyvinylpyrrolidone was again compared to alginic acid and starch. This required the formulation of tablets providing identical disintegration times so that the only parameter dissimilar would be the concentration of disintegrant.

Series 5 represents the formulation manufactured by direct compaction. To obtain meaningful dissolution rate data, uninhibited by disintegration, all of these tablets were formulated to give a 1-min. disintegration; 3% cross-linked polyvinylpyrrolidone, 6% alginic acid, and 40% starch were required. Friability of these tablets was not performed since the starch formulation would have been unsatisfactory. Figure 3 depicts the dissolution rate profile obtained with the Series 5 tablets. The cross-linked polyvinylpyrrolidone definitely enhanced the dissolution rate for the isoquinazolinone tablets; this parameter can be readily compared by studying Table IV where $t_{50\%}$, $t_{75\%}$, and $t_{90\%}$ values for each disintegrant studied are tabulated. The data suggest that the dissolution behavior is not solely dependent upon the tablet's disintegration time but involves a more complex phenomenon. Perhaps a surface interaction of the disintegrant with the dissolved or undissolved drug resulted in influence upon its dissolution behavior. The data indicate incomplete dissolution with the alginic acid and starch tablets for the time period studied.

Figure 4 depicts the dissolution behavior for the tablets manufactured from Series 6 by a wet granulation method. The tablets contained 25 mg. of the isoquinazolinone compound and were formulated to disintegrate within 2 min. They required the following quantities of disintegrant: 0.5% cross-linked polyvinylpyrrolidone, 2% alginic acid, and 30% starch. Polyvinylpyrrolidone was employed as the binder for Series 6 tablets. The dissolution rate profiles for the three disintegrants in this case did not differ significantly. The important factor that can be emphasized here results from the low quantity (0.5%) of cross-linked polyvinylpyrrolidone that accomplished this acceptable disintegration time and dissolution behavior, even though the tablets were prepared by a wet process.

The results obtained demonstrate that tablets with the same disintegration times do not necessarily imply the attainment of similar drug dissolution rate profiles. In the case of Series 5, identical disintegration times yielded different dissolution rates, while this did not occur for Series 6. In any event, both series demonstrated the superiority of cross-linked polyvinylpyrrolidone due to the lower concentrations needed for rapid disintegration. At no point was there an indication of any interaction between drug and cross-linked polyvinylpyrrolidone that would result in an inhibition of availability.

Table IV—Dissolution Rates[a] of a Quinazolinone Compound in 900 ml. Aqueous Solution at pH 1.2 for Tablets Containing Cross-Linked Polyvinylpyrrolidone, Alginic Acid, and Starch Prepared by Direct Compression

Disintegrant	$t_{50\%}$, min.	$t_{75\%}$, min.	$t_{90\%}$, min.
Cross-linked polyvinylpyrrolidone	2.2	3.8	5
Alginic acid	3.4	6.8	11.3
Starch	4.5	8.5	17.5

[a] Each time interval was obtained from Fig. 3.

SUMMARY

Cross-linked polyvinylpyrrolidone was found to possess very acceptable properties for use as a tablet disintegrant. It appears that the parameters of maximum moisture sorption and hydration capacity collectively can be employed as a guideline for the selection of new materials as potential tablet disintegrants. A concentration of from 0.5 to 5% cross-linked polyvinylpyrrolidone was adequate as a disintegrant for the tablet formulations screened in this research. The apparent binding property exhibited by cross-linked polyvinylpyrrolidone resulted in low percent tablet friability when it was employed as a disintegrant even in low concentrations. The lack of effect on drug dissolution behavior by cross-linked polyvinylpyrrolidone verified that it did not interfere with the dissolution of a quinazolinone compound utilized in both the dry and wet granulated tablets studied.

Cross-linked polyvinylpyrrolidone may very well find its ultimate use in tablet formulations as a binder–disintegrant. Cross-linked polyvinylpyrrolidone surpasses, in this usage, microcrystalline cellulose since larger quantities of microcrystalline cellulose are usually required in combination with spray-dried lactose. Series 3, with 5% cross-linked polyvinylpyrrolidone in a placebo tablet, demonstrated the excellent properties of both rapid disintegration and low percent tablet friability. Cross-linked polyvinylpyrrolidone in most of the tablets studied excelled in performance over alginic acid. The inert nature of cross-linked polyvinylpyrrolidone justifies its use as a tablet disintegrant of choice when an acid, such as alginic acid, would interfere with the chemical stability of the drug.

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Abstract □ The effects of L-lysine, L-arginine, L-ornithine, and glycine on acute ethanol intoxication, and the degree of ethanol-induced CNS depression, were investigated. Amino acid treatment was shown to prolong the onset of ataxia, reduce the duration of sleeping time, and decrease the number of rats losing the righting reflex, but it did not alter the LD_{50} values in response to ethanol. The protective actions of the amino acids are attributed to the formation of an amino acid–acetaldehyde complex, although other possible interactions are discussed.

Keyphrases □ Ethanol toxicity—effect of amino acids, rats □ Amino acids—effect on ethanol toxicity, rats □ CNS depression, ethanol induced—effect of amino acids, rats □ Toxicity, ethanol—effect of amino acids, rats

The effect of amino acids on the concentration and rate of disappearance of ethanol from the blood was the subject of the investigations of Schiller *et al.* (1, 2). After simultaneous administration of 120 ml. p.o. of ethanol (50% v/v) together with an amino acid–dextrose mixture (protein hydrolysates[1]), given intravenously to chronic alcoholics without hepatic dysfunction or nutritional deficiency, ethanol utilization curves showed that the amino acid mixture significantly decreased the maximum blood ethanol levels obtained and accelerated the rate of disappearance of ethanol from the blood. The activity of the amino acid–dextrose mixture was confirmed in an *in vitro* preparation of rat liver slices incubated with ethanol; while the specific amino acids responsible for these effects could not be identified, these experiments indicated that they contain four carbons or less.

Alanine has been shown to follow the pattern reported for pyruvate in increasing the disappearance of ethanol from the blood (3, 4). This is presumed to be the result of transformation of alanine to pyruvate, a Kreb's cycle intermediate which is thought to accelerate the disappearance of ethanol by increasing its aerobic metabolism (5).

When glycine or alanine was administered orally simultaneously with 15 g. of ethanol to dogs, the maximum concentration of ethanol appearing in the blood was lower than the control group (6, 7). The author concluded that the disappearance of ethanol must take place during absorption (6) and that a stable compound of ethanol and the amino acids may be formed to account for the decrease in the concentration of ethanol in the blood (7).

The nature of dietary protein fed to rats which were given a constant volume (4.0–6.6 g./kg.) of 40% ethanol four times weekly for 16 months was found to be related to the ability to reduce the degree of inebriation and the numbers of deaths due to ethanol toxicity (8). In this study, egg protein was the most effective while a mixture of peanut meal and soy protein was the least effective in reducing the toxic and CNS depressant effects of ethanol. It was concluded that the protective effects of the dietary proteins were not related to an effect on the rate of ethanol absorption or the emptying time of the stomach but were due to differences in ethanol utilization once it was absorbed.

Jarowski and Ward (9) reported that 30 min. of pretreatment with L-tryptophan (165 mg. i.p.) before a 2-g./kg. i.p. dose of 95% ethanol in unfasted rats produced a significant potentiation of the acute depressant effects of ethanol as measured by changes in the LD_{50}, sleeping, and immobility times. The fasting essential amino acid profile of blood plasma, which has been useful in predicting the relative dietary value of proteins (10), was determined in that study and served as a basis for the use of L-tryptophan supplementation (9). In the fasting essential amino acid profile of blood plasma, the concentrations of amino acids in the plasma

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of fasting rats indicate which essential amino acid is the limiting amino acid (present in lowest concentration) and also which amino acid would be most appropriate to supplement in an attempt to affect drug toxicity (10).

Other data originating in this laboratory showed that there was a slight elevation of the LD_{50} of ethanol after rats had been fed a diet supplemented with L-lysine and/or L-tryptophan.

Mueller *et al.* (11), using a model based on the performance of a trained rat, were able to quantify and differentiate ethanol-induced changes in the behavioral performance of rats. The effect of ethanol on the "number of shocks not terminated" was inhibited by D,L-threonine, L-methionine, and D,L-glutamine.

Hakkinen and Kulonen (12) found that simultaneous administration of 1.67 g./kg. p.o. of L-glutamine abolished the symptoms of intoxication produced by a 4.3-g./kg. dose of ethanol (33% v/v) in fasted rats. In a similar study, Forney *et al.* (13) demonstrated a significant potentiation of the depressant action of ethanol (4 g./kg. p.o.) following a 1- or 4-hr. pretreatment with 1 g./kg. s.c. of L-asparagine, as indicated by prolonged sleeping and immobility times.

Significant reduction in the depressant action of ethanol (30% v/v) after a 30-min. pretreatment with L-lysine (2.5 g./kg. i.p. or p.o.) was reported by Ward *et al.* (14). When ethanol, 3.6 g./kg., and L-lysine were given orally, there was a complete abolition of the hypnotic effect of ethanol; when both drugs were administered intraperitoneally, there was a 65% reduction in sleeping time. Furthermore, the LD_{50} of ethanol (30% v/v, intraperitoneally or orally) was slightly elevated by pretreatment with a 2.5-g./kg. dose of L-lysine.

Acetaldehyde, the toxic metabolite of ethanol, was shown (15) chromatographically to combine rapidly with the amino group of certain amino acids. The rate and extent of this reaction were dependent on the nature of the amino acid and the pH of the reaction medium. Arginine reacted most rapidly, followed by glycine, lysine, and ornithine. Reactive carbonyl groups, in compounds such as acetaldehyde, are thought to combine with the undissociated amino groups of the amino acids (16). Of the essential amino acids, lysine has the greatest ratio of amino groups in undissociated form available for reaction with acetaldehyde (16).

The purpose of this investigation was to determine whether the oral administration of selected amino acids, before or concurrently with ethanol, has a protective action against the acute toxicity and CNS depression produced by ethanol in rats.

EXPERIMENTAL

Adult male and female (125–150 g.) Sprague-Dawley rats, approximately 2 months old, were employed for all experimental procedures. The animals were kept in the animal quarters for 1 week after shipment to allow them to become acclimated. During this period the animals were fed a standard laboratory animal diet and tap water *ad libitum*. On the day prior to use, the animals were inspected for disease and diseased animals were discarded.

Aqueous solutions of the following amino acids[2] were prepared just prior to use: L-lysine hydrochloride, L-arginine hydrochloride, L-ornithine hydrochloride, glycine hydrochloride (10 and 20% w/v), sodium chloride USP (0.9%), and alcohol USP (ethanol 95%). Stock solutions of ethanol in distilled water (37.8 and 50.4% by volume) were prepared and standardized by specific gravity determination according to the method of Thor *et al.* (17). The choice and use of these particular percentages of ethanol were based on two considerations: to parallel human consumption and to keep the injected volume below 6 ml. According to Gillespie and Lucas (18), administration of ethanol (60% by volume) to rats resulted in a negligible amount of damage to the gastric and intestinal mucosa. All injections, both of ethanol and amino acid solutions, were made by intubation with curved animal feeding needles. A modified "rotarod" was constructed to determine the forced coordinated motor ability of rats (19).

Twenty-four hours before all experimentation, animals were randomly chosen, caged separately (to prevent cannibalism), and deprived of food (tap water *ad libitum*). To ensure a uniform rate of absorption of the test preparations, animals were fasted for 24 hr. Each animal was used only once, and each experiment was intiated at the same time every day throughout the testing period.

The effect of selected amino acids on ethanol toxicity was ascertained by two distinct procedures, with all treatments given orally.

Seventy-Two-Hour LD_{50} as a Measure of Acute Toxicity—Acute toxicity measurements by LD_{50} determinations, for all treatments described here, were carried out with 10 fasted Sprague-Dawley rats (five males and five females per group) and a total of six groups per LD_{50} determination. Ethanol dose levels ranged from 6 to 16 g./kg. where applicable. The number dead at 72 hr. was counted and analyzed by the method of Litchfield and Wilcoxon (20). Ethanol (50.5% by volume) was used throughout this procedure.

The treatments given can be divided into three distinct series as follows.

Pretreatment with Amino Acid before Ethanol Administration—The amino acid solution was administered at two dose levels 30 min. before ethanol. The dosages of the amino acids employed were: L-lysine, 120 mg./kg. and 2.5 g./kg.; L-arginine, 140 mg./kg. and 2.31 g./kg.; glycine, 73 mg./kg. and 1.53 g./kg.; and L-ornithine, 111 mg./kg. and 2.31 g./kg. The L-arginine, L-ornithine, and glycine dose levels are equimolar to the low and high dose levels of L-lysine to which they correspond. The dose of 120 mg./kg. of L-lysine was equal to two times the minimum daily requirement for the adult rat (21); the dose of 2.5 g./kg. was recommended by results from previous work (14). Saline controls of 1.3 ml./kg., corresponding to the volume of the low doses of the amino acids, and 13.3 ml./kg., corresponding to the volume of the high doses of the amino acids, were tested simultaneously. The concentrations of the amino acid solutions (in distilled water) used for low and high dosages were 10 and 20% (w/v), respectively, to keep the volume of solution to a minimum.

Administration of Amino Acid–Ethanol Solution—The amino acid–ethanol solution was formulated to deliver the dose of amino acid and ethanol required simultaneously. The doses of the amino acids employed were equal to the low doses already described. Administration of ethanol alone served as the control for this series of experiments.

Administration of Amino Acids Alone for LD_{50} Determination—Amino acid solutions (in distilled water) were administered to demonstrate the acute toxicity of the individual amino acids. The concentrations were varied to keep the injected volume below 6 ml.

Evaluation of Degree of Intoxication after Ethanol Treatment—The degree of intoxication for all treatment groups, described below, was evaluated using 10 fasted rats (five males and five females) per group. Ethanol (37.8% by volume) was given at a dose level of 6 g./kg. p. o. either as a single or a divided dose.

The statistical analysis, Student's *t* test, of intoxication was based on the following neurological parameters:

1. *Ataxia*—This was defined as the inability to maintain position by using four-leg motor coordination for one revolution of the modified "rotarod." Each animal was subjected to this test individually throughout the experimental period, unless unconscious (19). A normal rat can maintain its equilibrium for an indefinite period (19), and a short training period of three to four trials was added to ensure uniform response (22).

2. *Sleeping Time*—This was defined as the total elapsed time from the initial loss of righting reflex to its return without remission (13). The righting reflex was said to be lost when the animal was placed on its back and did not immediately right itself fully.

The treatments given can be divided into four distinct series as follows.

[2] All amino acids were water-soluble monohydrochloride salts, Sigma grade, Sigma Chemical Co., St. Louis, Mo.

Single-Dose Administration of Ethanol with Amino Acid Pretreatment—Amino acid solutions were administered at two dose levels 30 min. before the ethanol dose of 6 g./kg. The doses of amino acids and associated controls were equal to the low and high doses previously described.

Single-Dose Administration of Amino Acid–Ethanol Solution—The amino acid–ethanol solution was formulated to deliver the required dose of amino acid and 6 g./kg. of ethanol simultaneously. The dosages of amino acids employed were equal to the low doses already described. The administration of 6 g./kg. ethanol alone served as the control group for these experiments.

Serial Administration of Ethanol with Amino Acid Pretreatment—Ethanol was administered in three equally divided doses, 2.0 g./kg. per dose (total of 6 g./kg.), at 15-min. intervals. The amino acid solutions were administered at two dose levels 30 min. before the first ethanol dose. The doses of amino acids and associated controls were equal to the low and high doses previously described.

Serial Administration of Amino Acid–Ethanol Solution—The amino acid–ethanol solution used was formulated to deliver the required dose of amino acid and ethanol simultaneously (6 g./kg. total) in three equally divided doses at 15-min. intervals. The doses of amino acids employed were equal to the low doses already described. The administration of ethanol in divided doses served as the control for these experiments.

Evaluation of Toxicity of Amino Acids—Amino acid solutions were administered at two dose levels, as previously described, with no ethanol treatment. The concentrations of the amino acid solutions used for the low and high dosages were 10 and 20% (w/v), respectively. The rats were then subjected to the same neurological tests for signs of pharmacological effect.

pH Controls for Amino Acid Pretreatment—In this series of experiments, hydrochloric acid buffer solution[3] (pH 1.2) and phosphate buffer solution[3] (pH 5.8), which were equivalent to the pH of the corresponding amino acid solutions (20% w/v), were given at the same dose level as the saline (volume) controls (13.3 ml./kg.) for the high dose amino acid pretreatment before the single and divided ethanol doses. Amino acid solutions (20% w/v) were found to have the following pH values: glycine hydrochloride, 1.2; L-lysine hydrochloride, 5.8; L-arginine hydrochloride, 5.7; and L-ornithine, 5.8. The buffer solutions were administered 30 min. prior to the single or divided ethanol dose (6 g./kg.), after which the degree of intoxication was determined as previously described.

RESULTS

Determinations of Acute Toxicity—There was no significant alteration of the LD_{50} for ethanol (50.4% by volume) with either pretreatment or simultaneous administration of the amino acids. For the concentration and route of administration of ethanol used in this study, the LD_{50} values obtained (Tables I and II) are in agreement with a previously published LD_{50} range following ethanol administration (23). In chronological order (earliest first), the following signs of ethanol intoxication were observed in all groups after ethanol administration: ataxia, decreased muscle tonus, bradypnea, decreased spontaneous activity, hind-leg paralysis, loss of righting reflex, loss of corneal reflex, loss of pinna reflex, and death. The rapidity, intensity, and number of these signs of acute intoxication to which the animals responded were dose related. When an animal recovered, the process was very gradual with the animal reversing the above-mentioned sequence. The sequence described is similar to observations made by other investigators (8, 24).

When rats were treated with amino acids alone, all of the amino acids were found to be without pharmacological effect at the doses employed for pretreatment or simultaneous administration in the ethanol LD_{50} determinations. At high dosages of the amino acids, some signs of toxicity did appear such as ataxia, dyspnea, decreased muscle tonus, and loss of righting reflex. Toxicity signs were first observed for each amino acid at the following doses: glycine hydrochloride 2.5 g./kg.; L-lysine hydrochloride, 8 g./kg.; L-ornithine hydrochloride, 9 g./kg.; and L-arginine hydrochloride, 11 g./kg. When the LD_{50} values for the four amino acids used in this study were calculated by the method of Litchfield and Wilcoxon (20), the results were as follows (g./kg.): L-lysine hydrochloride, 10.13 ± 0.97; L-arginine hydrochloride, 12.40 ± 0.61; L-ornithine hydrochloride, 10.27 ± 0.070; and glycine hydrochloride, 3.34 ± 0.65.

[3] See "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 913.

Table I—Effect on Amino Acid Pretreatment on Acute Ethanol Toxicity in Rats

Drug Treatment	Dose	LD_{50} of Ethanol[a], g./kg.
L-Lysine hydrochloride	120 mg./kg.	9.50 ± 0.78
L-Lysine hydrochloride	2.50 g./kg.	11.20 ± 1.86
L-Arginine hydrochloride	140 mg./kg.	9.70 ± 0.61
L-Arginine hydrochloride	2.88 g./kg.	10.00 ± 1.67
L-Ornithine hydrochloride	111 mg./kg.	10.21 ± 1.30
L-Ornithine hydrochloride	2.31 g./kg.	10.05 ± 1.41
Glycine hydrochloride	73 mg./kg.	8.40 ± 1.12
Glycine hydrochloride	1.53 g./kg.	9.20 ± 0.80
Saline control	1.3 ml./kg.	10.00 ± 0.83
Saline control	13.3 ml./kg.	10.15 ± 1.16

[a] Dead animals per group counted at 72 hr. after drug administration. No difference in mortality between males and females was noted.

Degree of Intoxication—Any delaying of the onset of ataxia and/or shortening of the subsequent sleeping time by an agent can be seen as a protective action from the effects of acute ethanol intoxication (14, 22). The term used to express the delay or prolongation of the onset of ataxia was "protection factor," and that used to express the shortening of sleeping time was "percentage of reduction of sleeping time."

The mean time for onset of ataxia for different groups (Tables III–VI) was compared by the number of times the elapsed time for the onset of ataxia of the test group was greater than the elapsed time for the onset of ataxia of the control group (protection factor) (Tables VII and VIII). For the purpose of this comparison, only significant ($p < 0.05$) changes due to amino acid treatment were considered.

Pretreatment with all of the amino acids at the higher dosage level, when ethanol was administered as a single dose, resulted in significant prolongation of the onset of ataxia, with L-lysine prolonging it most, followed by glycine, L-arginine, and L-ornithine. The single-dose administration of the L-arginine–ethanol solution showed a sevenfold increase in the onset of ataxia. The responses for all amino acid treatments with serial administration were similar, with the exception of the glycine–ethanol solution treatment. The protection factors were higher for the high dose pretreatment than for the low dose pretreatment of amino acids, but protective factors for the low dose simultaneous administration of L-lysine and L-arginine were higher than either the low or high pretreatment dose of L-lysine or L-arginine (Table VIII). The protective factor was usually indicative of the subsequent degree of depression due to acute ethanol intoxication.

Significant reduction in the sleeping time and a decrease in the number of rats responding characterized the groups receiving the high dose pretreatment with amino acids when ethanol was administered as a single or divided dose (Tables IX–XII). There was almost a complete abolition of the sleeping time after glycine (1.53 g./kg.) pretreatment with single-dose administration of ethanol. The other high dose pretreatments with amino acids showed large and uniform responses in the reduction of sleeping time

Table II—Effect of Simultaneous Administration of Amino Acid–Ethanol Solution on Acute Ethanol Toxicity

Drug Treatment	Dose of Amino Acids, mg./kg.	LD_{50} of Ethanol[a], g./kg.
L-Lysine hydrochloride	120	10.15 ± 2.05
L-Arginine hydrochloride	140	10.05 ± 1.32
L-Ornithine hydrochloride	111	10.05 ± 0.96
Glycine hydrochloride	73	10.10 ± 1.42
Ethanol control	—	10.10 ± 1.09

[a] Dead animals per group counted at 72 hr. after drug administration. No difference in mortality between males and females was noted.

Table III—Onset of Ataxia with Amino Acid Pretreatment before Single-Dose Administration of Ethanol

Treatment	Dose	Mean Onset[a] of Ataxia, min.	*p* Value[b]
L-Lysine hydrochloride	120 mg./kg.	5.05 ± 0.18	0.5
L-Lysine hydrochloride	2.50 g./kg.	74.76 ± 7.85	0.001
L-Arginine hydrochloride	140 mg./kg.	8.23 ± 3.27	0.2
L-Arginine hydrochloride	2.88 g./kg.	30.98 ± 5.29	0.0001
L-Ornithine hydrochloride	111 mg./kg.	13.25 ± 6.05	0.2
L-Ornithine hydrochloride	2.31 g./kg.	23.49 ± 6.44	0.02
Glycine hydrochloride	73 mg./kg.	3.56 ± 0.23	0.3
Glycine hydrochloride	1.53 g./kg.	68.61 ± 10.06	0.001
Saline	1.3 ml./kg.	3.06 ± 0.32	—
Saline	13.3 ml./kg.	6.47 ± 1.50	—

[a] Onset of ataxia values (min.) ± *SE*. [b] *p* value < 0.05 was significant.

Table V—Onset of Ataxia with Amino Acid Pretreatment before Serial Administration of Ethanol

Treatment	Dose	Mean Onset[a] of Ataxia, min.	*p* Value[b]
L-Lysine hydrochloride	120 mg./kg.	25.10 ± 4.89	0.05
L-Lysine hydrochloride	2.50 g./kg.	56.40 ± 4.81	0.001
L-Arginine hydrochloride	140 mg./kg.	26.76 ± 1.70	0.001
L-Arginine hydrochloride	2.88 g./kg.	66.32 ± 9.46	0.001
L-Ornithine hydrochloride	111 mg./kg.	19.06 ± 1.58	0.01
L-Ornithine hydrochloride	2.31 g./kg.	57.50 ± 11.20	0.01
Glycine hydrochloride	73 mg./kg.	34.67 ± 3.78	0.001
Glycine hydrochloride	1.53 g./kg.	89.82 ± 6.71	0.001
Saline	1.3 ml./kg.	12.34 ± 1.54	—
Saline	13.3 ml./kg.	19.13 ± 1.90	—

[a] Onset of ataxia values (min.) ± *SE*. [b] *p* value < 0.05 was significant.

whether ethanol was administered as a single or divided dose. L-Arginine was the only amino acid which, when given (140 mg./kg.) before a single dose of ethanol as a low dose, resulted in a significant reduction of sleeping time. However, this reduction was not apparent in the low dose pretreatment of arginine when the ethanol was administered serially. L-Lysine and L-arginine were the only amino acids that had a significant reduction in sleeping time when given simultaneously with serial administration of ethanol (Table VIII). Thus, significant antagonism of the intoxicating effects was found upon treatment with L-lysine (120 mg./kg.) and L-arginine (140 mg./kg.), but these results were more variable than the results obtained with the respective high doses of these amino acids.

The amino acid controls for each amino acid administered demonstrated that no acute toxicological effects in this experimentation were due to the administration of amino acids alone.

Saline (volume) controls were used as the basis for comparison for amino acid pretreatment in all tables. The pH controls were found not to be significantly different from the saline (volume) controls in the degree of intoxication produced by 6 g./kg. of ethanol.

DISCUSSION

In the LD_{50} determinations, amino acid treatment did not demonstrate any antagonism to the acute toxic effects of ethanol. This lack of protective action of the amino acids could be due to the inhibition of the absorption of amino acids with an increasing concentration of ethanol present in the GI tract (25) or to the rapid CNS effects of ethanol on vital cortical centers due to rapid absorption (26). Since amino acid pretreatment was not effective in elevating the LD_{50} of ethanol, it seems apparent that the absence of antagonism was probably due to the rapid absorption of large doses of ethanol which could mask any detoxicant actions of the amino acids.

Ward *et al.* (14) reported a slight elevation of the oral LD_{50} of ethanol (30% by volume) after pretreatment with L-lysine hydrochloride (2.5 g./kg. orally). In the present investigation, the percentage of ethanol used for all LD_{50} determinations was 50.4% by volume, which is known to be more toxic (27) than ethanol (30% by volume) used in the former report (14). Thus, amino acid treatment appears to be somewhat limited in preventing the acute toxic effects of ethanol.

After experimentation with dogs using simultaneous oral administration of either glycine (8.0 g./kg.) or alanine (3.0 g./kg.) and ethanol (5.5 g./kg.), Widmark (6) concluded that an amino acid–ethanol complex is formed with the subsequent reduction of ethanol absorption. The formation of this complex would explain the low peak of ethanol blood levels and the rapid disappearance of ethanol from the blood after amino acid treatment observed in his study.

After simultaneous oral administration of glycine (0.5 g./kg.) and ethanol (1.0 g./kg.) to rats, Haggard and Greenberg (28) also reported the same reductions in blood ethanol concentration (6). These authors concluded that glycine caused a prolonged retention of ethanol in the stomach and that this delay in absorption resulted in the metabolism of ethanol almost as quickly as it was absorbed.

In these investigations (6, 28), no determinations of the degree of ethanol intoxication were made; but because the depressant effects of ethanol are related to blood ethanol levels (26), a reduction in CNS depression would be an expected result with the alterations in blood ethanol concentration described.

In the present investigation, pretreatment with L-lysine (2.50 g./kg.), L-arginine (2.88 g./kg.), L-ornithine (2.31 g./kg.), and

Table IV—Onset of Ataxia after Singe-Dose Administration of Amino Acid–Ethanol Solution

Treatment	Dose of Amino Acid, mg./kg.	Mean Onset[a] of Ataxia, min.	*p* Value[b]
L-Lysine hydrochloride	120	4.92 ± 0.72	0.2
L-Arginine hydrochloride	140	23.16 ± 7.60	0.02
L-Ornithine hydrochloride	111	3.77 ± 0.34	0.2
Glycine hydrochloride	73	2.62 ± 0.22	0.1
Ethanol control	—	3.19 ± 0.20	—

[a] Onset of ataxia values (min.) ± *SE*. [b] *p* value < 0.05 was significant.

Table VI—Onset of Ataxia after Serial Administration of Amino Acid–Ethanol Solution

Treatment	Dose of Amino Acid, mg./kg.	Mean Onset[a] of Ataxia, min.	*p* Value[b]
L-Lysine hydrochloride	120	39.07 ± 3.85	0.001
L-Arginine hydrochloride	140	52.63 ± 7.21	0.001
L-Ornithine hydrochloride	111	24.14 ± 2.54	0.02
Glycine hydrochloride	73	18.47 ± 3.04	0.2
Ethanol control	—	11.98 ± 3.49	—

[a] Onset of ataxia values (min.) ± *SE*. [b] *p* value < 0.05 was significant.

Table VII—Effect of Amino Acid on Ethanol-Induced Ataxia and Sleeping Time with Single-Dose Administration of Ethanol

Amino Acid	Dose	Protection Factor[a]	Percentage Reduction of Sleeping Time[b]
L-Lysine hydrochloride	120 mg./kg.[c]	NS[d]	NS[d]
L-Lysine hydrochloride	2.50 g./kg.[c]	11.55	60.05
L-Lysine hydrochloride	120 mg./kg.[e]	NS	NS
L-Arginine hydrochloride	140 mg./kg.[c]	NS	58.27
L-Arginine hydrochloride	2.88 g./kg.[c]	4.79	43.72
L-Arginine hydrochloride	140 mg./kg.[e]	7.26	NS
L-Ornithine hydrochloride	111 mg./kg.[c]	NS	NS
L-Ornithine hydrochloride	2.31 g./kg.[c]	3.63	51.51
L-Ornithine hydrochloride	111 mg./kg.[e]	NS	NS
Glycine hydrochloride	73 mg./kg.[c]	NS	NS
Glycine hydrochloride	1.53 g./kg.[c]	10.62	98.96
Glycine hydrochloride	73 mg./kg.[e]	NS	NS

[a] Protection factor is the number of times onset of ataxia time for test group is greater than onset of ataxia for control group. [b] Percentage reduction of sleeping time = 100% − percentage of control sleeping time. [c] Amino acid pretreatment. [d] NS = not significant because p value > 0.05. [e] Simultaneous administration.

Table VIII—Effect of Amino Acids on Ethanol-Induced Ataxia and Sleeping Time with Serial-Dose Administration of Ethanol

Amino Acid	Dose	Protection Factor[a]	Percentage Reduction of Sleeping Time[b]
L-Lysine hydrochloride	120 mg./kg.[c]	2.03	NS[d]
L-Lysine hydrochloride	2.50 g./kg.[c]	2.95	55.38
L-Lysine hydrochloride	120 mg./kg.[e]	3.26	11.08
L-Arginine hydrochloride	140 mg./kg.[c]	2.17	NS
L-Arginine hydrochloride	2.88 g./kg.[c]	3.47	56.17
L-Arginine hydrochloride	140 mg./kg.[e]	4.39	39.22
L-Ornithine hydrochloride	111 mg./kg.[c]	1.54	NS
L-Ornithine hydrochloride	2.31 g./kg.[c]	3.01	61.15
L-Ornithine hydrochloride	111 mg./kg.[e]	2.02	NS
Glycine hydrochloride	73 mg./kg.[c]	2.81	NS
Glycine hydrochloride	1.53 g./kg.[c]	4.70	64.57
Glycine hydrochloride	73 mg./kg.[e]	NS	NS

[a] Protection factor is the number of times onset of ataxia time for test group is greater than onset of ataxia for control group. [b] Percentage reduction of sleeping time = 100% − percentage of control sleeping time. [c] Amino acid pretreatment. [d] NS = not significant because p value > 0.05. [e] Simultaneous administration.

glycine (1.53 g./kg.) before the single and serial administration of an ethanol dose (6 g./kg.) resulted in a significant reduction in sleeping time, a prolongation of the onset of ataxia, and a decrease in the number of animals losing the righting reflex. Glycine demonstrated the greatest reduction in sleeping time (98%), while the other three amino acids reduced the sleeping time 50–60%. Ward *et al.* (14), on the other hand, demonstrated a complete prevention of sleeping time with L-lysine pretreatment (2.5 g./kg. p.o.) before a single intoxicating dose of ethanol (4.3 g./kg. p.o.). If a comparison were made between their results and those reported in this investigation, it would seem that there was less antagonism to ethanol intoxication in the latter report. But this might not be so, because the toxicity of ethanol is known to increase with an increase in the concentration and/or dose of ethanol (27).

Schiller *et al.* (1) demonstrated, in humans, that there is a decrease in maximum ethanol blood levels and an increase in the disappearance of ethanol from the blood after simultaneous administration of ethanol orally and an amino acid mixture intravenously. Thus, by separation of the routes of administration of amino acids and ethanol, it becomes apparent that there might be a direct systemic reduction of ethanol blood levels rather than a reduced absorption of ethanol in response to amino acids. This direct antagonism could take the form of either an increase in the metabolism of ethanol by amino acids or a complex formation between amino acids and ethanol or one of its metabolites. Several investigations (1, 6, 28) showed that ethanol metabolism is unaffected by amino acids, so the latter explanation seems most plausible.

Westerfeld and Schulman (29) suggested that: ". . . the chemically reactive acetaldehyde could combine with amino groups (of amino acids, *in vivo*) and alter some biochemical function." Beck *et al.* (30) also suggested that acetaldehyde may react (*in vivo*) with amino acids and demonstrated that these reaction products produce cortical stimulant activity in rabbits.

Robert and Penaranda (15) demonstrated that acetaldehyde, the primary metabolite of ethanol, combines rapidly *in vitro* with amino acids. The combination of acetaldehyde and arginine is the most rapid, followed by glycine, lysine, and ornithine.

It is unknown whether acetaldehyde contributes to the CNS depression due to ethanol intoxication, but there are indications that acetaldehyde potentiates the CNS actions of ethanol (31). Therefore, the combination of acetaldehyde with amino acids could reduce the degree of intoxication produced by ethanol.

Pretreatment with relatively high doses of amino acids, as done in the present investigation, would be expected to increase the concentration of circulating free amino acids available for combination with acetaldehyde and would be the reason why all the amino acids (in this investigation) were effective agents in reducing the depressant

Table IX—Sleeping Time with Amino Acid Pretreatment before Single-Dose Administration of Ethanol

Treatment	Dose	Mean Sleeping Time[a], min.	p Value[b]	Percentage of Rats Responding
L-Lysine hydrochloride	120 mg./kg.	179.32 ± 53.7	0.3	80
L-Lysine hydrochloride	2.50 g./kg.	159.87 ± 40.8	0.001	80
L-Arginine hydrochloride	140 mg./kg.	116.56 ± 48.3	0.05	50
L-Arginine hydrochloride	2.88 g./kg.	224.10 ± 50.3	0.01	70
L-Ornithine hydrochloride	111 mg./kg.	118.20 ± 58.7	0.1	40
L-Ornithine hydrochloride	2.31 g./kg.	193.77 ± 46.1	0.001	70
Glycine hydrochloride	73 mg./kg.	293.15 ± 16.9	0.9	90
Glycine hydrochloride	1.53 g./kg.	4.12 ± 3.2	0.001	20
Saline	1.3 ml./kg.	278.03 ± 58.7	—	80
Saline	13.3 ml./kg.	398.98 ± 17.0	—	100

[a] Sleeping time values (min.) ± *SE*. [b] p value < 0.05 was significant.

Table X—Effect of Amino Acid Pretreatment on Sleeping Times Produced by Serial Administration of Ethanol

Treatment	Dose	Mean Sleeping Time[a], min.	*p* Value[b]	Percentage of Rats Responding
L-Lysine hydrochloride	120 mg./kg.	299.51 ± 42.2	0.7	90
L-Lysine hydrochloride	2.50 g./kg.	170.05 ± 42.5	0.001	70
L-Arginine hydrochloride	140 mg./kg.	293.39 ± 30.9	0.6	100
L-Arginine hydrochloride	2.88 g./kg.	167.89 ± 51.6	0.01	80
L-Ornithine hydrochloride	111 mg./kg.	284.26 ± 51.0	0.6	90
L-Ornithine hydrochloride	2.31 g./kg.	148.29 ± 43.2	0.001	70
Glycine hydrochloride	73 mg./kg.	301.35 ± 56.2	0.8	90
Glycine hydrochloride	1.53 g./kg.	135.39 ± 39.1	0.001	80
Saline	1.3 ml./kg.	322.7 ± 37.3	—	100
Saline	13.3 ml./kg.	381.32 ± 30.3	—	100

[a] Mean sleeping time (min.) ± *SE*. [b] *p* value < 0.05 was significant.

effects of ethanol. L-Arginine, when given at its low dose (140 mg./kg.) by either mode of administration (pretreatment or simultaneous), was the only amino acid used in the present investigation that demonstrated significant protective activity against ethanol intoxication. In the studies of Robert and Penaranda (15), arginine reacted most rapidly with acetaldehyde. Thus, the protective effect of L-arginine may well be dependent on its reaction rate with acetaldehyde. This dependency of amino acids for their protective activity on the rate of reaction with acetaldehyde is not reflected when the high doses of amino acids are used for pretreatment and may be due to a change in the relative reaction rates of the amino acids with acetaldehyde produced by the increased concentrations of circulating free amino acids.

The chronic toxicity of ethanol has also been shown to be ameliorated by the administration of amino acids. Clinically, amino acids have been used, with much success, to treat the following conditions related to chronic ethanol ingestion: delirium tremens (32), nutritional deficiencies (33), and cirrhosis of the liver (34).

Although no toxicity was observed at the amino acid doses used in this study, an excess of amino acids may be toxic; the accumulation of ammonia, urea, and other nitrogen acceptors follows excess amino acid ingestion, especially glycine (35, 36).

Table XI—Sleeping Time after Serial Administration of Amino Acid–Ethanol Solution

Treatment	Dose of Amino Acid, mg./kg.	Mean Sleeping Time[a], min.	*p* Value[b]	Percentage of Rats Responding
L-Lysine hydrochloride	120	297.02 ± 49.9	0.05	90
L-Arginine hydrochloride	140	203.92 ± 35.2	0.01	100
L-Ornithine hydrochloride	111	336.2 ± 34.0	0.9	100
Glycine hydrochloride	73	325.09 ± 33.0	0.9	100
Ethanol control	—	334.01 ± 24.3	—	100

[a] Mean sleeping time values (min.) ± *SE*. [b] *p* value < 0.05 was significant.

Table XII—Sleeping Time after Single-Dose Administration of Amino Acid–Ethanol Solution

Treatment	Dose of Amino Acid, mg./kg.	Mean Sleeping Time[a], min.	*p* Value[b]	Percentage of Rats Responding
L-Lysine hydrochloride	120	223.88 ± 37.7	0.3	90
L-Arginine hydrochloride	140	315.79 ± 58.1	0.9	90
L-Ornithine hydrochloride	111	320.94 ± 41.4	0.8	90
Glycine hydrochloride	73	283.76 ± 54.3	0.9	100
Ethanol control	—	300.95 ± 48.0	—	90

[a] Mean sleeping time values (min.) ± *SE*. [b] *p* value < 0.05 was significant.

Small volumes of saline or buffer solutions used as controls for amino acid pretreatment had no effect on acute ethanol toxicity; this was expected because the absorption of ethanol takes place by simple diffusion with no active processes involved (37).

CONCLUSIONS

Within the framework of this experimental design, amino acid treatment demonstrates a definite antagonism of the intoxicating effects of ethanol. The nature of the interaction between amino acids and ethanol intoxication appears to be related to alterations in ethanol blood levels. Although there is no proof for the formation of an amino acid–acetaldehyde complex *in vivo*, the present study indicates that this may be the possible mechanism by which pretreatment or simultaneous oral administration of amino acids reduces the toxic CNS depressant effects of ethanol.

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Caffeine Complexes with Low Water Solubility: Synthesis and Dissolution Rates of 1:1 and 1:2 Caffeine–Gentisic Acid Complexes

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Abstract □ The syntheses of 1:1 and 1:2 molecular complexes of caffeine with gentisic acid are described, and their rates of dissolution are reported and compared with that of caffeine. Both complexes were less soluble in water than caffeine, and their rates of dissolution in 0.1 *N* hydrochloric acid and in a phosphate buffer at pH 7.5 were less than that of caffeine. These complexes thus present a potentially useful way of formulating caffeine in dosage forms such as chewable tablets that are intended to linger in the mouth. Such dosage forms would only release caffeine slowly and should, consequently, have an improved taste factor over ones containing pure caffeine. The rates of dissolution of the complexes were close to those predicted by equations that take into account both the diffusional and chemical equilibrium processes occurring. These equations are shown to be useful in the selection of a complex to achieve a specific dissolution rate.

Keyphrases □ Caffeine complexes with gentisic acid—synthesis, characterization, solubility, dissolution rate, compared to caffeine dissolution rate □ Dissolution rates of caffeine–gentisic acid complexes—determination, compared to caffeine dissolution rate □ Complexes, caffeine–gentisic acid—synthesis, characterization, solubility, dissolution rate □ Tablets, chewable, potential—caffeine–gentisic acid complexes

Molecular complexes of drugs with other chemicals have frequently been proposed[1] for inclusion in dosage forms to enhance the solubility, chemical stability, and absorption characteristics of the drugs. The present report describes the results of a search for molecular complexes of caffeine that would dissolve less rapidly in aqueous solutions than caffeine. The complexes that were prepared and studied had the stoichiometry of 1 : 1 and 1 : 2 caffeine–gentisic acid. It is believed that caffeine complexes that dissolve less rapidly in water than caffeine provide a useful alternative means of formulating caffeine in chewable tablets and other dosage forms which linger in the mouth. Such dosage forms should have an enhanced taste factor over ones containing pure caffeine, because their caffeine would be released more slowly and, consequently, the intensity of the extremely bitter taste produced by caffeine should be reduced.

The principles involved in this mechanism of taste masking are similar to those involved in the use of ion-exchange absorbates (2) to mask taste.

It has long been recognized that caffeine and other xanthines form molecular complexes with organic acids and organic acid anions (3). The latter type of complex is generally more soluble than the xanthine (soluble complex), while the former is commonly less soluble (insoluble complex). The present report concerns the properties of two insoluble complexes.

EXPERIMENTAL

Chemicals—Caffeine was recrystallized from water, dried under vacuum at 80°, and stored in a desiccator (m.p. 238–238.5°). Gentisic acid was boiled in an aqueous suspension of charcoal and

[1] This subject is reviewed in *Reference 1*.

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Caffeine Complexes with Low Water Solubility: Synthesis and Dissolution Rates of 1:1 and 1:2 Caffeine–Gentisic Acid Complexes

T. HIGUCHI and IAN H. PITMAN▲

Abstract □ The syntheses of 1:1 and 1:2 molecular complexes of caffeine with gentisic acid are described, and their rates of dissolution are reported and compared with that of caffeine. Both complexes were less soluble in water than caffeine, and their rates of dissolution in 0.1 *N* hydrochloric acid and in a phosphate buffer at pH 7.5 were less than that of caffeine. These complexes thus present a potentially useful way of formulating caffeine in dosage forms such as chewable tablets that are intended to linger in the mouth. Such dosage forms would only release caffeine slowly and should, consequently, have an improved taste factor over ones containing pure caffeine. The rates of dissolution of the complexes were close to those predicted by equations that take into account both the diffusional and chemical equilibrium processes occurring. These equations are shown to be useful in the selection of a complex to achieve a specific dissolution rate.

Keyphrases □ Caffeine complexes with gentisic acid—synthesis, characterization, solubility, dissolution rate, compared to caffeine dissolution rate □ Dissolution rates of caffeine–gentisic acid complexes—determination, compared to caffeine dissolution rate □ Complexes, caffeine–gentisic acid—synthesis, characterization, solubility, dissolution rate □ Tablets, chewable, potential—caffeine–gentisic acid complexes

Molecular complexes of drugs with other chemicals have frequently been proposed[1] for inclusion in dosage forms to enhance the solubility, chemical stability, and absorption characteristics of the drugs. The present report describes the results of a search for molecular complexes of caffeine that would dissolve less rapidly in aqueous solutions than caffeine. The complexes that were prepared and studied had the stoichiometry of 1 : 1 and 1 : 2 caffeine–gentisic acid. It is believed that caffeine complexes that dissolve less rapidly in water than caffeine provide a useful alternative means of formulating caffeine in chewable tablets and other dosage forms which linger in the mouth. Such dosage forms should have an enhanced taste factor over ones containing pure caffeine, because their caffeine would be released more slowly and, consequently, the intensity of the extremely bitter taste produced by caffeine should be reduced.

The principles involved in this mechanism of taste masking are similar to those involved in the use of ion-exchange absorbates (2) to mask taste.

It has long been recognized that caffeine and other xanthines form molecular complexes with organic acids and organic acid anions (3). The latter type of complex is generally more soluble than the xanthine (soluble complex), while the former is commonly less soluble (insoluble complex). The present report concerns the properties of two insoluble complexes.

EXPERIMENTAL

Chemicals—Caffeine was recrystallized from water, dried under vacuum at 80°, and stored in a desiccator (m.p. 238–238.5°). Gentisic acid was boiled in an aqueous suspension of charcoal and

[1] This subject is reviewed in *Reference 1*.

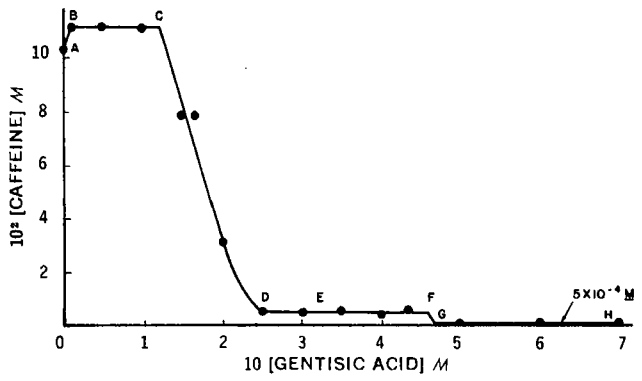

Figure 1—*Plot against* $[gentisic\ acid]_{added}$ *of the total concentration of caffeine (free and complexed).*

then recrystallized from water (m.p. 205°). All other chemicals were of analytical reagent grade and were used without further purification. The water used in all experiments was distilled from aqueous acidified potassium permanganate solutions and stored in Pyrex glass containers.

Preparation of Caffeine Complexes—*1:1 Caffeine–Gentisic Acid Complex*—Gentisic acid, 15.41 g. (0.11 mole), dissolved in 100 ml. of hot water was mixed with 19.42 g. (0.1 mole) of caffeine dissolved in 100 ml. of hot water and the mixture cooled rapidly. The complex precipitated as a microcrystalline powder which, after washing with water and drying under vacuum at 80°, had a melting point of 209.5–210°. Titration of a solution of the powder with standard sodium hydroxide to the phenolphthalein end-point showed that it had a molecular weight of 348 ± 1 g. This powder thus corresponded to the 1:1 caffeine–gentisic acid complex which has a molecular weight of 348.31 g.

1:2 Caffeine–Gentisic Acid Complex—Caffeine, 19.4 g. (0.1 mole), dissolved in 600 ml. of hot water and 33.9 (0.22 mole) of gentisic acid dissolved in 600 ml. of hot 3×10^{-3} *M* hydrochloric acid were mixed, allowed to cool slowly, and shaken at room temperature for 12 hr. After filtration, washing with water, and drying at 80° under vacuum, the microcrystalline precipitate had a melting point of 194–196°. The molecular weight of the complex was shown by titration to be 502 ± 1 g. The molecular weight of the 1:2 complex would be 502.43 g.

Phase Solubility Study—Caffeine, 0.45 g. (2.317×10^{-3} mole), and 10 ml. of water or a standardized gentisic acid solution were added to screw-cap vials, which were then sealed and rotated in a water bath at 25 ± 0.1° for 24 hr. Preliminary experiments had shown that the solutions reached equilibrium after this time. The total amount of caffeine in each equilibrated solution was determined by filtering the solution, measuring out 1-ml. aliquots, adding sufficient sodium bicarbonate to bring them to pH 7, extracting with 4×10 ml. of chloroform, and measuring the absorbance of a solution made by combining the chloroform extracts and making them up to an appropriate volume with chloroform. Absorbance values were converted to concentrations of dissolved caffeine using a previously constructed Beer's law plot (Fig. 1).

Dissolution Rate Studies—The solid whose dissolution rate was to be measured was reduced to a uniform particle size by sifting it through a 40-mesh sieve and collecting it in a 60-mesh sieve. It was then compressed into tablets in an evacuable KBr die[2] at 5000 p.s.i. for 2 min. using a laboratory press[3]. A vacuum was applied during the compression. The tablets had a diameter of 13 mm. and a thickness of 3 mm. (400-mg. tablet).

The dissolution medium was either a 0.1 *N* solution of hydrochloric acid or a pH 7.5 buffer solution, which was made by mixing 50 ml. 0.1 *M* potassium dihydrogen phosphate with 40.8 ml. 0.1 *M* sodium hydroxide and 9.2 ml. of distilled water.

The method of obtaining absorbance against time data during the dissolution process was essentially the same as that described in an earlier publication from this laboratory (4). The stirring rate was maintained at 45 ± 1 r.p.m. The dissolution of caffeine was followed at either 290 or 297 nm. and that of the complexes was followed at wavelengths (330, 347, 350, and 355 nm.) where the gentisic acid was the main absorbing species.

Plots of absorbance against time during the dissolution were linear initially, but their gradients then decreased continually with time. A typical plot is displayed in Fig. 2. Initial rates of change in absorbance at the analytical wavelength $(dD/dt)_0$ were taken to be the gradient of the initial linear portion of the curve. Values of $(dD/dt)_0$ were converted to rates of increase in concentration by using the identity:

$$\left(\frac{dD}{dt}\right)_0 = E_C\left(\frac{d[C]}{dt}\right)_0 + E_G\left(\frac{d[G]}{dt}\right)_0 \qquad \text{(Eq. 1)}$$

where E_C and E_G were the molar absorptivities of caffeine and gentisic acid, respectively, at the particular wavelengths; and $[C]$ and $[G]$ were the corresponding concentrations of these species, respectively. Preliminary experiments had shown that the dissolved complex was essentially completely dissociated into caffeine and gentisic acid during the initial period of the dissolution process. Because $E_C \simeq 0$ at the wavelengths at which the dissolution of the complexes was followed, the term $E_C(d[C]/dt)_0$ could be excluded from Eq. 1 in these experiments and $(d[G]/dt)_0$ could be calculated directly from the absorbance changes. The absorptivities of each species at the appropriate wavelength were calculated from independent determinations and are believed to be accurate to within ±5%. Results are included in Table I.

RESULTS AND DISCUSSION

Selection of a Molecular Complex of a Drug that Has the Desired Dissolution Characteristics—The rate at which the total *A* component in a molecular complex AB_n will dissolve is given by the flux of all forms of *A* (*i.e.*, AB_n, AB_{n-1}, . . . , *A*) from the solid–liquid interface into the bulk of the solution. In the following discussion this flux will be referred to as the rate of dissolution of the complex AB_n.

To select rationally a particular molecular complex of a drug which will dissolve either faster, slower, or at the same rate as the uncomplexed drug, it is useful to be able to predict the relative rates of dissolution of these two species. This type of prediction can be satisfactorily made in the following manner by using a film theory of mass transport.

Rate of Dissolution of Pure *A*—The Noyes–Whitney (5) equation, which is based on a film theory of mass transport, states that in a nonreacting medium that is being agitated in a constant manner:

$$\text{rate of dissolution of pure } A \text{ at time } t = kD_A(S_A - [A]_t) \qquad \text{(Eq. 2)}$$

In this equation, D_A is the diffusion coefficient of *A* in the dissolu-

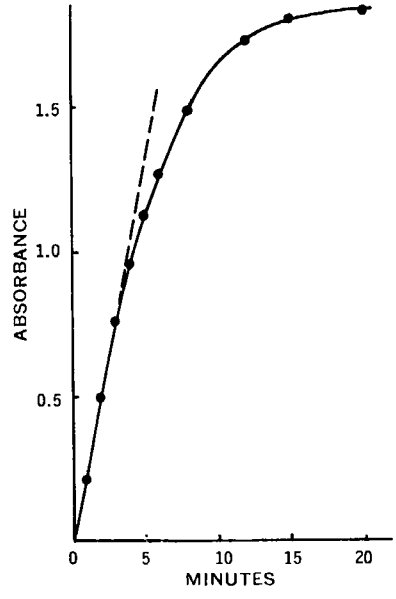

Figure 2—*Plot against time of the absorbance changes at 297 nm. which accompanied the dissolution of caffeine (400-mg. tablet) in 100 ml. of phosphate buffer at pH 7.5. Stirring speed = 45 r.p.m.*

[2] Research and Industrial Instruments Co., London, England.
[3] Fred S. Carver Inc., Summit, N. J.

Table I—Rates[a] of Appearance of Caffeine $(d[C]/dt)_0$ and Gentisic Acid $(d[G]/dt)_0$ in Solution during the Dissolution at 25° of Caffeine and Its 1:1 and 1:2 Complexes with Gentisic Acid

Compound	pH	$\lambda_{analytical}$, nm.	$\left(\frac{dD}{dt}\right)_0$, min.$^{-1}$	$\left(\frac{dG}{dt}\right)_0$, M min.$^{-1}$	Rate of Dissolution of the Solid, M min.$^{-1}$
Caffeine (400 mg.)	7.5	297[b]	1.12	—	2.1×10^{-3}
1:1 complex (400 mg.)	7.5	347[c]	0.83	8.8×10^{-4}	8.8×10^{-4}
1:2 complex (400 mg.)	7.5	350[d]	0.61	9.4×10^{-4}	4.7×10^{-4}
Caffeine (400 mg.)	1.0	297[b]	1.05	—	2.0×10^{-3}
1:1 complex (400 mg.)	1.0	350[e]	0.43	2.1×10^{-4}	2.1×10^{-4}
1:2 complex (400 mg.)	1.0	355[f]	0.21	1.7×10^{-4}	8.5×10^{-5}

[a] Values represent the mean of three to four experiments under each condition. [b] $E_C = 527$. [c] $E_G = 945$. [d] $E_G = 647$. [e] $E_G = 2018$. [f] $E_G = 1252$.

tion medium, S_A is the solubility of A in the dissolution medium and is the concentration of A in solution at the solid–liquid interface, and $[A]_t$ is the concentration of A in the bulk of the solution at time t. The constant k is proportional to the surface area of the solid and inversely proportional to the thickness of a diffusional layer or film of liquid surrounding the solid. This equation has been well tested and yields acceptable estimates of the rate of dissolution of pure solids.

Rate of Dissolution of a Molecular Complex AB_n—If a molecular complex of a drug can dissociate in solution according to reactions such as:

$$AB_n \overset{K_{1:n}}{\rightleftharpoons} AB_{n-1} + B;\ \ldots;\ AB \overset{k_{1:1}}{\rightleftharpoons} A + B \qquad \text{(Eq. 3)}$$

it is necessary to use a rate equation that takes into account the effects of these dissociation reactions on the flux of AB_n. General equations of this type were developed by Olander (6) and he established that, for such a system, the flux of the total A component is equal to the sum of the fluxes of all species containing A. Hence:

$$\text{rate of dissolution of } AB_n \text{ at time } t = k' \{D_{AB_n}([AB_n]_0 - [AB_n]_t) + D_{AB_{n-1}}([AB_{n-1}]_0 - [AB_{n-1}]_t) + \ldots + D_A([A]_0 - [A]_t)\} \qquad \text{(Eq. 4)}$$

The symbols in this equation have similar meanings to those in Eq. 2, except that $[i]_0$ terms refer to the concentrations of the i species at the solid–liquid interface. If the restriction is imposed that the solid phase is composed entirely of AB_n and this is the only species that can reenter the interface, the value of $[AB_n]_0$ is the intrinsic solubility of AB_n in the dissolution medium, S_{AB_n}, and values of all other $[i]_0$ terms are less than the solubilities of these species.

In discussing the relative rates of dissolution of uncomplexed and complexed drugs, it is convenient to consider the initial rates of dissolution. These will be the rates of dissolution at times when the $[i]_t$ terms are very much smaller than the $[i]_0$ terms. Thus, if solid samples of species A and AB_n with equal surface areas are separately dissolving in identical solvents that are being agitated in the same manner (and hence the k and k' values in Eqs. 2 and 4 are equal), the ratios of their initial rates of dissolution are given by:

$$R = \frac{\text{initial rate of dissolution of } AB_n}{\text{initial rate of dissolution of } A} = \frac{D_{AB_n}S_{AB_n} + D_{AB_{n-1}}[AB_{n-1}]_0 + \ldots + D_A[A]_0}{D_AS_A} = \frac{(D_{AB_n}/D_A)S_{AB_n} + (D_{AB_{n-1}}/D_A)[AB_{n-1}]_0 + \ldots + [A]_0}{S_A} \qquad \text{(Eq. 5)}$$

In the special situation where the rates of the association and dissociation reactions are very fast compared to the diffusional processes, it can be assumed that equilibrium between all of the species containing A is maintained throughout the diffusional layer. This situation is believed to apply to the dissolution of caffeine–gentisic acid complexes because UV spectral observations suggest that they form and dissociate extremely rapidly. Hence, in the dissolution of these complexes, the total concentration at the solid–liquid interface of all species containing A would be the same as the total concentration of all species containing A that would pertain in a solution made by saturating the solvent used in the dissolution experiment with AB_n. This total concentration (A_T) would be given by:

$$A_T = S_{AB_n} - [AB_{n-1}]_0 + \ldots + [A]_0 \qquad \text{(Eq. 6)}$$

and is a property that can be rapidly determined for any complex.

Substitution of Eq. 6 in Eq. 5 leads to:

$$R = \frac{A_T + S_{AB_n}[(D_{AB_n}/D_A) - 1] + [AB_{n-1}]_0 + [(D_{AB_{n-1}}/D_A) - 1] + \ldots}{S_A} \qquad \text{(Eq. 7)}$$

Hence, it can be seen that in situations where the diffusion coefficients of all species have similar values, the relative rates of dissolution of complexed and uncomplexed drugs are simply given by:

$$R = \frac{A_T}{S_A} \qquad \text{(Eq. 8)}$$

Equation 8 is expected to give a good estimate of relative dissolution rates of caffeine–gentisic acid complexes and caffeine because the molecular weights of these species differ by less than a factor of 3 (molecular weights: 1:2 complex, 502; 1:1 complex, 384; and caffeine, 196). Hence, the diffusion coefficients of these substances are expected to be comparable because the diffusion coefficient is proportional to only the $^1/_2$ or $^1/_3$ power of the molecular weight (4).

Equation 8 is quite general for dissolution of substances that can undergo any rapid reversible reaction in the dissolution medium. Hence, as well as taking into account dissociation reactions, it also takes into account ionization reactions in the dissolution medium of any or all of the species.

Because the value of A_T is dependent on both the intrinsic solubility of the complex and the extent to which it dissociates or ionizes in solution, these factors will also affect the dissolution rate of the complex relative to the uncomplexed drug. Hence, the value of R for a particular complex can be increased or decreased by using a solvent that increases or represses, respectively, the intrinsic solubilities of the complex or uncomplexed drug, the dissociation reactions, or ionization reactions.

Synthesis and Characterization of Caffeine–Gentisic Acid Complexes—The initial evidence for formation of 1:1 and 1:2 caffeine–gentisic acid complexes was provided by the phase solubility diagram shown in Fig. 1. The portion A–D of this diagram is a typical B_s-type diagram (7). Based on arguments similar to those proposed by Higuchi and Connors (7), this portion of the diagram provides evidence for the formation of a 1:1 caffeine–gentisic acid complex with an approximate association constant of 100 M^{-1} and a solubility of the complex of 5×10^{-3} M. Insufficient data points were acquired for an accurate determination of the value of the association constant. Further evidence that a 1:1 complex was precipitating out in the C–D region was provided by the isolation of a pure solid 1:1 complex when 100 ml. of a 0.011 M solution of gentisic acid was mixed with 100 ml. of a 0.01 M solution of caffeine.

The D–H portion of this diagram is interpreted as providing evidence for the formation of a 1:2 caffeine–gentisic acid complex, because this portion is typical of the B_i diagram described by Higuchi and Connors (7). The solid phase in the D–F segment would be a mixture of 1:1 and 1:2 complexes and that in the G–H segment would be the 1:2 complex. Again, confirmation that this

Table II—Experimentally Determined and Predicted[a] Rates of Dissolution of 1:1 and 1:2 Caffeine-Gentisic Acid Complexes Relative to that of Caffeine

Solid	pH	A_T[b], M	R_{expt}[c]	R_{calc}[a]
1:1 complex	7.5	4.8×10^{-2}	4.2×10^{-1}	4.6×10^{-1}
1:2 complex	7.5	1.13×10^{-2}	2.2×10^{-1}	1.1×10^{-1}
1:1 complex	1.0	8.6×10^{-3}	1.1×10^{-1}	8.2×10^{-2}
1:2 complex	1.0	3.5×10^{-3}	4.3×10^{-2}	3.3×10^{-2}
Caffeine	1.0 or 7.5	1.05×10^{-1}	—	—

[a] Using Eq. 8. [b] As defined in Eq. 6. [c] Data from Table I.

latter conclusion was correct came from the fact that an essentially pure solid sample of the 1:2 complex precipitated when solutions of gentisic acid and caffeine with the molar concentration ratio of 2.2:1 were mixed. Although it was not possible to estimate the value of the 1:2 association constant because of the lack of precise data points, this portion of the diagram does provide an estimate of the solubility of the 1:2 complex. Thus, the maximum value of the solubility of the 1:2 complex can be seen from the value of the G–H plateau region to be 5×10^{-4} M. The caffeine in solution in this region could come from the dissolved 1:2 complex and any dissolved 1:1 complex or free caffeine that resulted from its dissociation. However, the fact that the concentration of caffeine in solution does not decrease with increasing gentisic acid concentration in this region suggests that the excess gentisic acid is effectively repressing the dissociation of the complex, and 5×10^{-4} M is consequently its solubility. This argument would not be valid if a 1:3 or higher order caffeine–gentisic acid complex was precipitating in this region but no evidence was found for this occurrence.

The total amounts (in M units) of the 1:1 and 1:2 complexes that would dissolve in aqueous 0.1 M HCl and a phosphate buffer at pH 7.5 were calculated from the amount of caffeine that could be extracted from aliquots of the supernatant liquids over their saturated solutions. The results are the A_T values referred to in Eq. 8, and they are included in Table II.

Comparison of Measured and Predicted Initial Dissolution Rates of 1:1 and 1:2 Caffeine–Gentisic Acid Complexes and Caffeine—The initial rates of dissolution of caffeine and its 1:1 and 1:2 gentisic acid complexes were calculated from the results of experiments carried out as described in the *Experimental* section. The initial rates of appearance of caffeine or gentisic acid in the bulk of the solution are listed in Table I.

During the initial stages of the dissolution of the 1:1 and 1:2 complexes (*i.e.*, when the concentrations of solute in the bulk of the solution were extremely small), the complexes were essentially totally dissociated in the bulk of the solution. Hence, the rate of dissolution of the complex was the same as the rate of appearance of gentisic acid in the solution in the case of the 1:1 complex and was half the rate of appearance of the gentisic acid in the case of the 1:2 complex. In all experiments, the dissolving tablets had the same dimensions (diameter, thickness, and particle size of the powder) and it was assumed that the surface area of the dissolving solid was identical. Stirring rate and temperature were also kept constant in each experiment. The rates of dissolution of the complexes relative to that of caffeine are listed in Table II together with the values of these ratios that were calculated using Eq. 8. It can be seen that Eq. 8 provides a useful way of predicting the rate of dissolution of a molecular complex of caffeine relative to pure caffeine. The fact that the complexes dissolve much faster in a buffer at pH 7.5 than in 0.1 M HCl is almost certainly because both the complexes and gentisic acid ionize in this medium. It is important to remember that while the bulk solvent in the experiments in phosphate buffer initially has a pH of 7.5, the pH at the solid–liquid interface, where the solvent is saturated with acidic species such as gentisic acid and its complexes, is much less.

The rates of dissolution of the complexes in saliva (pH 6.7–7.3) were not measured, but they are expected to approach the rates of dissolution in the phosphate buffer. Hence, they would just be marginally less than the rate of dissolution of pure caffeine. However, in an actual formulation it should be possible to reduce the dissolution rate of the complexes in saliva by including excess gentisic acid in the formulation. Dissolution of the excess gentisic acid would have the effects of both lowering the pH of the dissolution medium (addition of 1 ml. 0.1 M gentisic acid to 3 ml. of saliva lowers its pH from 6.7 to 3.2) and repressing the dissociation of the complexes. Both these effects would be expected, from Eq. 8, to lower the rate of dissolution. Evidence that this approach is valid comes from the observation that when 200 mg. of powdered 1:1 complex was stirred in water, it took 8 min. for the concentration of dissolved caffeine to reach 1.15×10^{-3} M, whereas the same concentration of caffeine was only attained after 113 min. when the dissolution medium contained 1×10^{-2} M gentisic acid.

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Deuterium Isotope Effect in Enzymatic Transamination of L-Deuterio-phenylalanine to Deuterio-phenylpyruvic Acid

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Abstract □ L-Deuterio-phenylalanine was isolated by gradient elution ion-exchange chromatography of the ionic fraction obtained from the cell wall hydrolysate of algae grown in culture medium containing 99.6% deuterium oxide. The enzyme system present in rat brain extract was used for determining the rate of transamination of L-deuterio-phenylalanine and its protio analog. The phenylpyruvate formed was determined spectrophotometrically at 300 nm. using the arsenate-catalyzed enol–borate assay procedure. Transamination rates were determined in media containing varying concentrations of amino acid and α-ketoglutarate. Kinetic determination of maximum velocity was conducted through graphical analysis of the data. The deuterium isotope effect, expressed as the V_H/V_D value, was 1.64.

Keyphrases □ L-Deuterio- and L-protio-phenylalanine—isolation, identification, enzyme transamination to phenylpyruvates, deuterium isotope effect □ Deuterio-phenylpyruvic acid—enzymatic transamination product from L-deuterio-phenylalanine, deuterium isotope effect □ Deuterium isotope effect—L-deuterio-phenylalanine enzyme transamination to deuterio-phenylpyruvic acid □ Transamination rates—L-deuterio- and L-protio-phenylalanine, deuterium isotope effect □ Ion-exchange chromatography—isolation, L-deuterio-phenylalanine □ UV spectrophotometry—monitoring, L-deuterio-phenylalanine transamination

The large-scale cultivation of algae grown in a culture medium containing 99.6% deuterium oxide has made available a source of fully deuterated chlorophyll, carbohydrates, and proteins (1). Hydrolysis of the cell walls of *Scenedesmus obliquus*, followed by ion-exchange chromatography, was reported by Blake *et al.* (2) to yield L-amino acids that are fully deuterated in nonexchangeable positions. L-Deuterio-phenylalanine was shown to be present in the algae hydrolysate in sufficient amounts to permit isolation and purification.

In living systems the transamination reaction is an important biological process involving the transfer of an amino group between certain amino and keto acids. The mechanism of enzymatic and nonenzymatic transamination was reviewed by Snell and Jenkins (3). Deuterium isotope effects in the transamination reaction of L-alanine and L-deuterio-alanine with pyridoxal were studied by Blake *et al.* (4). The effect of the nature and concentration of added buffer, the pH, and the effect of metal ion were included in the study. Lin *et al.* (5) reported the deuterium isotope effect in the nonenzymatic transamination of L-deuterio-glutamic acid. Fang *et al.* (6) performed a kinetic study on enzymatic transamination using four analogs of L-glutamic acid (fully deuterated glutamic acid, two partially deuterated forms of glutamic acid, and protio-glutamic acid) as substrates. The deuterium isotope effect reflected in the transamination rate was determined by maximum velocity analysis.

In the present study, L-deuterio-phenylalanine was isolated from the ionic fraction of the algae cell wall hydrolysate by gradient elution ion-exchange chromatography. The compound was recrystallized from acetone–water and was characterized by TLC; NMR, IR, and mass spectrometry; polarimetric analysis; and elemental analysis. Transamination of the protio and deuterio analogs of L-phenylalanine to the corresponding analogs of phenylpyruvic acid was performed with rat brain extract as the enzyme system. The rate of the reaction was followed spectrophotometrically at 300 nm., using the arsenate-catalyzed enol–borate assay procedure for phenylpyruvate described by George *et al.* (7). The deuterium isotope effect was determined from a comparison of maximum velocity analysis based on the binary mechanism of enzymatic transamination involving L-protio-phenylalanine and L-deuterio-phenylalanine.

EXPERIMENTAL

Isolation and Purification of L-Deuterio-phenylalanine—The ionic fraction obtained from the hydrolysate of the residual cell wall of *Scenedesmus obliquus*, grown autotrophically in a nutrient medium containing 99.6% deuterium oxide (8), was chromatographed in a two-step procedure utilizing the following ion-exchange resins: (*a*) Dowex 50-X8 for concentrating and desalting, and (*b*) Dowex 1-X8 for concentrating and isolating the deuterated dicarboxylic acids, L-deuterio-glutamic and L-deuterio-aspartic acids. A detailed description of this two-step chromatographic procedure was reported earlier (9). Effluent fractions from the second step that were shown to contain L-deuterio-phenylalanine by TLC were combined and evaporated *in vacuo* at room temperature until the residue was free of acetic acid. Since other deuterated amino acids were present in each of the fractions pooled, the isolation of L-deuterio-phenylalanine from this mixture was effected by means of gradient elution chromatography on a column of polystyrene sulfonic acid resin. For this step, Rexyn 101 (453 g.), in the sodium salt form, was hydrated, freed of fines, and packed into a chromatographic column (2.7 × 869 cm.). The resin was subsequently converted to its acid form by washing with 1 *N* HCl. Excess acid was then removed by washing the resin with distilled water until the effluent was neutral.

The performance and efficiency of the separation procedure were determined by conducting preliminary runs using samples containing L-protio-phenylalanine, L-protio-leucine, and a mixture containing equal amounts of L-protio-phenylalanine and L-protio-leucine. The gradient of hydrochloric acid needed to effect complete separation, as well as the elution volumes of the respective amino acids, were determined. The column was then washed with distilled water until the effluent was neutral.

The residue containing the deuterated amino acids was dissolved in 180 ml. of distilled water and then loaded on the column. Elution of the amino acids was started with 1 *N* HCl. The effluent was collected in 27-ml. fractions on an automatic fraction collector[1] at a rate of 55 ml./hr. After elution with 300 ml. of 1 *N* HCl, the hydrochloric acid concentration was increased gradually by feeding 4 *N* HCl dropwise into a 1000-ml. mixing chamber containing the remaining 1 *N* HCl (600 ml.). Elution with this gradient was continued until 5000 ml. of total effluent was collected. Fractions whose elution volumes corresponded with those determined previously for L-protio-phenylalanine and L-protio-leucine were evaporated to dryness at room temperature with the aid of an electric fan, and a small portion of each was subsequently tested with ninhydrin reagent.

[1] Rinco.

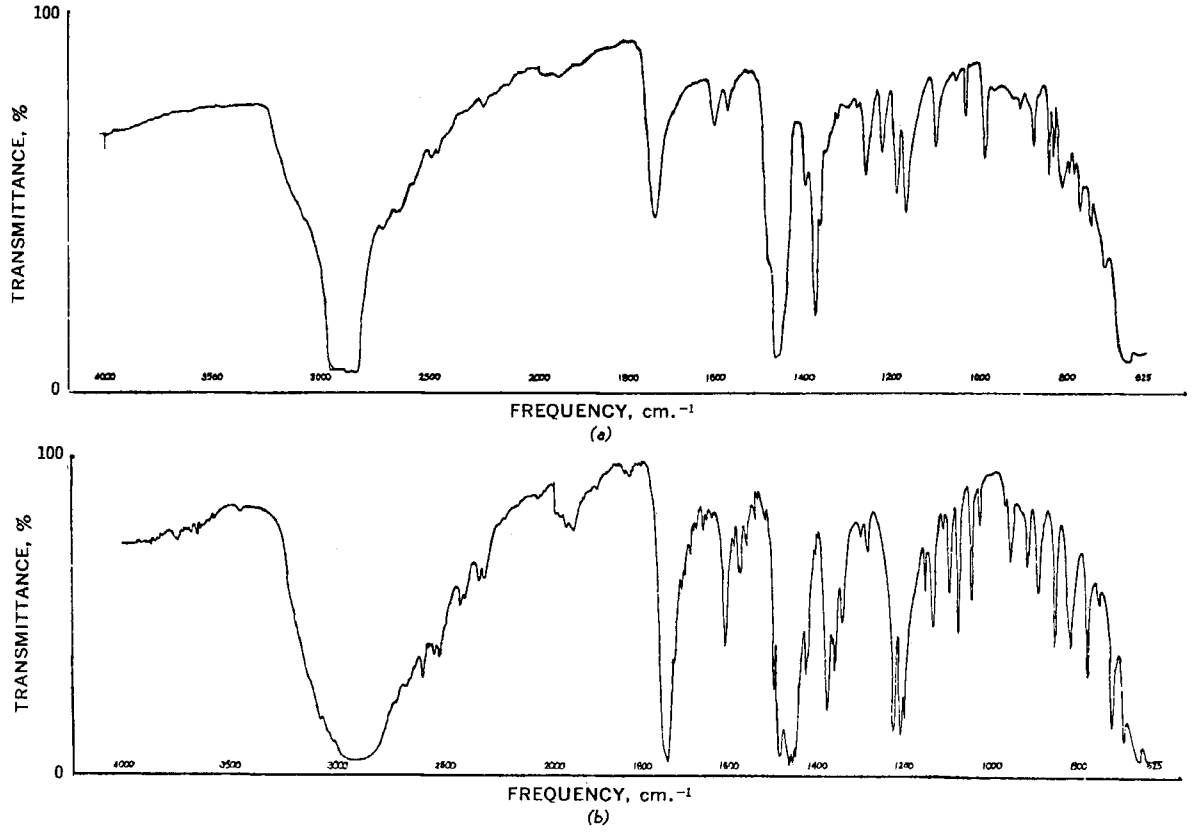

Figure 1—*IR spectra of:* (a) L-*protio-phenylalanine hydrochloride, and* (b) L-*deuterio-phenylalanine hydrochloride.*

The residues from each of the fractions representing L-deuterio-phenylalanine hydrochloride were dissolved in a minimum volume of water, combined, and reevaporated to dryness. A small quantity of the residue was employed for IR analysis. The remainder was converted to the free amino acid by the technique of Hirs *et al.* (10) as described below.

A chromatographic column containing Dowex 1-X8 (110 g.) was washed with 3 *M* sodium acetate solution until the effluent was chloride free, then with distilled water until the effluent left no residue upon evaporation, and finally with 0.5 *M* acetic acid until the effluent was acidic to litmus paper. The residue of L-deuterio-phenylalanine hydrochloride was dissolved in 5 ml. of 0.5 *M* acetic acid and loaded on the column. The amino acid was eluted from the column with 0.5 *M* acetic acid, and the amino acid, contained in the first 20 ml. of effluent, was freed of solvent *in vacuo* at room temperature. The white-gray residue, 420 mg., was then crystallized twice from acetone–water. The yield of purified L-deuterio-phenylalanine was 210 mg., m.p. 262° [lit. (11) m.p. 260–262°]. Using the same melting-point apparatus[2], a known sample of L-protio-phenylalanine melted at 283° [lit. (12) m.p. 283°].

Anal.—Calc. for $C_9H_3{}^2H_8NO_2$: C, 62.39; H + ^{2}H, 11.06; N, 8.08. Found: C, 61.67; H + ^{2}H, 10.27; N, 8.33.

Optical activity of the protio and deuterio forms of L-phenylalanine was determined[3]. Water was employed as a blank in these determinations. The observed specific rotation for L-protio-phenylalanine, $[\alpha]_D^{32}$, was −35.4° (c, 1.94 in water) [lit. (12) $[\alpha]_D^{29}$ −35.5°]. The observed specific rotation for L-deuterio-phenylalanine, $[\alpha]_D^{32}$, was −35.1° (c, 1.94 in water) [lit. (11) $[\alpha]_D^{25}$ −34.0° (c, 1.00)].

Both the protio and deuterio forms of the amino acid were subjected to TLC on silica gel G plates (5 × 20 cm., 250 μ in thickness) with two different solvent systems. Solvent System A contained isopropyl alcohol–water (19:1) and Solvent System B contained phenol–water (3:1) to which was added 20 mg. of sodium cyanide. The chromatograms were developed to a height of 10 cm. in saturated chromatographic chambers. The chromatograms were air dried after being developed, sprayed with ninhydrin reagent, and then placed in a drying oven at 100° for 10 min. The positions where the first ninhydrin-positive color appeared were marked for determining the R_f values for each form of the amino acid. With Solvent System A, the R_f values for the protio and deuterio species were 0.30 and 0.28, respectively; for Solvent System B, the values were 0.62 and 0.59, respectively.

The IR spectra of L-protio-phenylalanine hydrochloride and L-deuterio-phenylalanine hydrochloride were determined with an IR spectrophotometer[4] as mineral oil mulls between two plates[5]. Spectra of the protio and deuterio analogs of the amino acid hydrochloride were compared with a standard reference spectrum of the protio compound. All three spectra showed peaks at 2900 (—NH_3^+-Cl^-), 1750 (—C=O), 1450 (aromatic nucleus), and 1600 (—NH_3^+) cm.$^{-1}$. At 1380 cm.$^{-1}$, the peak pattern and intensity differed for the protio and deuterio species. The spectrum of the deuterio form showed a multiple signal at 1150–1200 cm.$^{-1}$ and a single sharp peak at 990 cm.$^{-1}$, both of which were absent in the spectrum of the protio form. The spectra are shown in Fig. 1.

The NMR spectra of both forms were obtained on an NMR spectrometer[6] as saturated solutions in deuterium oxide, using an internal tetramethylsilane standard. The spectrum for L-protio-phenylalanine showed a multiplet at δ 3.15–3.35, a quadruplet at δ 3.95–4.20, a single large peak at δ 7.50, and a peak at δ 4.85. The spectrum of L-deuterio-phenylalanine showed a single peak at δ 4.85 only.

The mass spectra for the protio and deuterio analogs were obtained with a mass spectrometer[7]. The spectrum of L-protio-phenylalanine showed the largest fragment at *m/e* 165 and three high percentage fragments at *m/e* 120, 91, and 74. The spectrum of L-deuterio-phenylalanine showed the largest fragment at *m/e* 173 and three high percentage fragments at *m/e* 128, 98, and 75.

Transamination Study—The following reagents were used in this study: potassium phosphate buffer, 0.10 *M*, pH 7.0; potassium phosphate buffer, 0.40 *M*, pH 8.0; arsenate–borate reagent solution, 1.00 *M*, pH adjusted to 6.5 with concentrated hydrochloric acid; L-protio-phenylalanine stock solution, 0.10 *M* in potassium phosphate buffer, 0.40 *M*, pH 8.0; L-deuterio-phenylalanine stock solu-

[2] Thiele.
[3] In 1.6 × 100-mm. Koehler polarimeter cells with a Rudolph polarimeter, model 80.
[4] Perkin-Elmer model 257.
[5] Irtran 2.
[6] Varian A-60.
[7] Hitachi-Perkin-Elmer RMU-6D.

Table I—Reaction Mixtures for the Enzymatic Transamination between L-Protio-phenylalanine and α-Ketoglutarate[a]

Set	Reaction	0.10 *M* L-Protio-phenylalanine, ml.	0.02 *M* Pyridoxal-5-phosphate, ml.	0.20 *M* α-Keto-glutarate, ml.	Enzyme Solution[b], ml.	Buffer Solution[c], ml.
A	Blank	0.45	0.01	—	1.0	1.55
	1	0.45	0.01	0.25	1.0	1.30
	2	0.45	0.01	0.15	1.0	1.40
	3	0.45	0.01	0.10	1.0	1.45
	4	0.45	0.01	0.05	1.0	1.50
B	Blank	0.25	0.01	—	1.0	1.75
	5	0.25	0.01	0.25	1.0	1.50
	6	0.25	0.01	0.15	1.0	1.60
	7	0.25	0.01	0.10	1.0	1.65
	8	0.25	0.01	0.05	1.0	1.70
C	Blank	0.20	0.01	—	1.0	1.80
	9	0.20	0.01	0.25	1.0	1.55
	10	0.20	0.01	0.15	1.0	1.65
	11	0.20	0.01	0.10	1.0	1.70
	12	0.20	0.01	0.05	1.0	1.75
D	Blank	0.10	0.01	—	1.0	1.90
	13	0.10	0.01	0.25	1.0	1.65
	14	0.10	0.01	0.15	1.0	1.75
	15	0.10	0.01	0.05	1.0	1.85

[a] A total of 12 measurements was performed upon four different concentrations of L-deuterio-phenylalanine in combination with three different concentrations of α-ketoglutarate. (The lowest concentration was excluded from Sets A, B, and C.) [b] One milliliter equivalent to 300 mcg. of protein. [c] Potassium phosphate, 0.40 *M*, pH 8.0.

tion, 0.10 *M*, in potassium phosphate buffer, 0.40 *M*, pH 8.0; α-ketoglutarate stock solution, 0.20 *M* in potassium phosphate buffer, 0.40 *M*, pH 8.0; pyridoxal-5-phosphate stock solution, 0.02 *M*, stored in amber bottle; sodium phenylpyruvate stock solution, freshly prepared, 0.05 *M* in potassium phosphate buffer, 0.40 *M*, pH 8.0; and transamination enzyme stock solution[8], purified rat brain extract (acetone powder, dried) (used as the source of the enzyme system for these studies).

The protein concentration of the rat brain extract was analyzed according to the method of Lowry *et al.* (13), using protein standard solution[9] as a reference standard. The stable enzyme solution preparation was obtained in the following manner. One gram of purified rat brain extract was added to 60 ml. of 0.10 *M* potassium phosphate (pH 7.0) previously cooled to 4°. The mixture was then stirred at this temperature for 45 min. The clear supernate obtained by decantation and subsequent filtration was used.

With the exception of the arsenate–borate stock solution (stored at 0°) and sodium pyruvate stock solution (freshly prepared), all other stock solutions were stored at −20°.

A standard calibration curve for determining the pyruvate concentration was prepared by using the sodium phenylpyruvate stock solution (0.05 *M*) as a reference standard. Varying amounts (50–500 nmoles) were added to a basic reaction mixture containing α-ketoglutarate (50 μ*M*, pH 8.0), pyridoxal-5-phosphate (200 n*M*), potassium phosphate buffer (555 μ*M*, pH 8.0), and enzyme (equivalent to 300 mcg. of protein) in a final volume of 3.1 ml. The mixture was incubated at 37° for 15 min. The rate of transamination was followed by measuring the absorbance of the enol–borate complex formed from the phenylpyruvate generated during the incubation (14) at 300 nm., using a spectrophotometer[10]. The arsenate-catalyzed modification described by George *et al.* (7) was applied to the assay procedure.

The standard reaction mixture contained: potassium phosphate buffer (555 μ*M*, pH 8.0), α-ketoglutarate (10–50 μ*M*, added last), pyridoxal-5-phosphate (200 n*M*), L-protio- or L-deuterio-phenylalanine (10–45 μ*M*), and enzyme (rat brain extract, equivalent to 300 mcg. of protein) in a final volume of 3.1 ml. The α-ketoglutarate was omitted in the blank. The reaction mixture minus the α-ketoglutarate was preincubated at 37° in a constant-temperature bath for 10 min. Transamination was then generated by the addition of the α-ketoglutarate to the reaction mixture. An amount of potassium phosphate buffer (pH 8.0) equivalent to the amount of α-ketoglutarate added to the reaction mixture was then added to the blank.

An aliquot (0.5 ml.) was withdrawn from both the reaction mixture and reaction blank (free of α-ketoglutarate), and each was added to separate solutions of trichloroacetic acid (0.25 ml., 25% w/v) at appropriate time intervals (1–10 min.).

The precipitated enzyme protein was removed by centrifugation, after which a 0.5-ml. aliquot of the reaction mixture supernate was removed and added to 2.5 ml. of arsenate–borate reagent. The thoroughly mixed solution was then allowed to stand at room temperature for 20 min. A sample of the reaction blank was treated similarly. The absorbance of the sample relative to the blank was measured at 300 nm.

Kinetic Measurements—A total of 15 measurements was performed on four different concentrations of L-protio-phenylalanine in combination with three to four different concentrations of α-ketoglutarate. A similar series of 12 measurements was conducted with the deuterio analog in combination with three concentrations

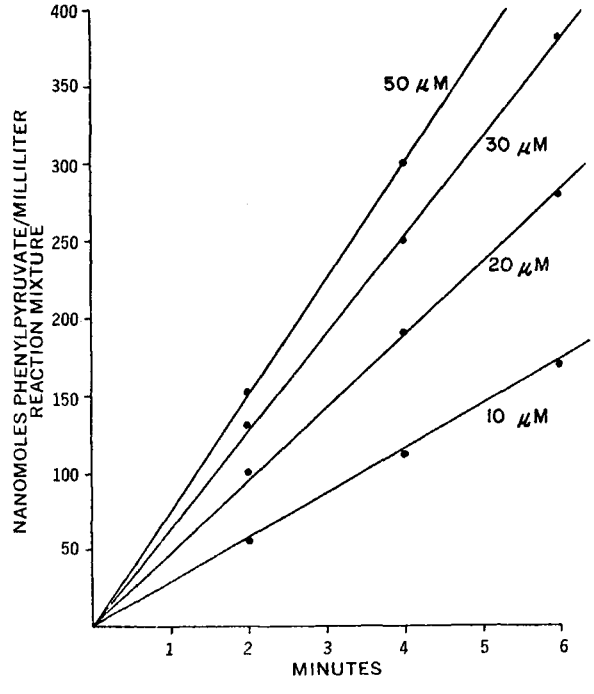

Figure 2—*Rate of transamination between* L-*protio-phenylalanine and* α-*ketoglutarate* (L-*protio-phenylalanine, 45* μM; *0.40* M *phosphate buffer, pH 8.0; 37°; pyridoxal-5-phosphate, 200* n*M; enzyme concentration, 300 mcg./ml.).*

[8] Obtained from Schwarz-Mann Research Laboratories, Inc.
[9] Bovine serum albumin, Armour & Co.
[10] Zeiss PMQ II.

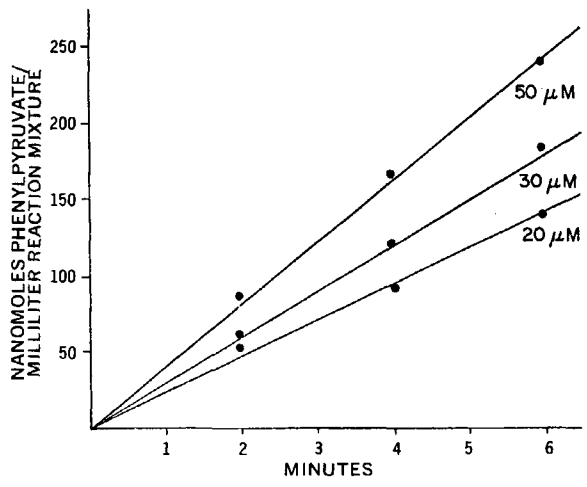

Figure 3—*Rate of transamination between* L-*deuterio-phenylalanine and α-ketoglutarate* (L-*deuterio-phenylalanine, 45 μM; 0.40* M *phosphate buffer, pH 8.0; 37°; pyridoxal-5-phosphate, 200 n*M; *enzyme concentration, 300 mcg./ml.*).

of α-ketoglutarate. The quantities of all reactants employed in these determinations are given in Table I. Graphical presentations of the initial velocities obtained are given in Figs. 2 and 3 for the protio and deuterio analogs, respectively, at a concentration level of 45 μ*M*. The initial velocities obtained from these determinations and their respective reciprocal values are given in Tables II and III.

DISCUSSION

Isolation of L-**Deuterio-phenylalanine**—The technique of continuous gradient elution was applied to the final step of the procedure to isolate L-deuterio-phenylalanine. Since the mixture containing the desired amino acid had been stripped of the deuterated acidic amino acids, L-aspartic and L-glutamic acids, a polystyrene sulfonic acid resin was chosen because of its ability to separate the basic amino acids from the neutral amino acids. The use of conventional elution techniques to separate the individual neutral amino acids by ion-exchange chromatography is not possible. For example, phenylalanine is usually contaminated with leucine. Although the technique of continuous gradient elution can be effectively used for separating compounds with similar chromatographic properties, it is successful only if the proper gradient increase in the concentration of the eluting agent is used.

L-Protio-leucine was selected as a contaminant of L-protio-phenylalanine for determining the gradient change in hydrochloric acid concentration required for effecting separation because it is

Table II—Initial Velocities of Transamination Reactions between L-Phenylalanine and α-Ketoglutarate (Enzyme Concentration, 300 mcg./ml.; 37°; pH 8.0; Pyridoxal-5-phosphate, 200 n*M*; Phosphate Buffer, 0.4 *M*)

Reaction	L-Phenylalanine, μ*M*	α-Ketoglutarate, μ*M*	Initial Velocity (V_i), n*M*/ml./min.	$1/V_i$
1	45.00	50.00	75.00	0.0133
2	45.00	30.00	63.00	0.0159
3	45.00	20.00	46.75	0.0214
4	45.00	10.00	29.25	0.0342
5	25.00	50.00	63.75	0.0157
6	25.00	30.00	50.75	0.0197
7	25.00	20.00	41.25	0.0242
8	25.00	10.00	27.50	0.0364
9	20.00	50.00	56.25	0.0177
10	20.00	30.00	45.00	0.0222
11	20.00	20.00	37.50	0.0266
12	20.00	10.00	26.25	0.0381
13	10.00	50.00	45.75	0.0219
14	10.00	30.00	38.75	0.0258
15	10.00	10.00	22.00	0.0454

Table III—Initial Velocities of Transamination Reactions between L-Deuterio-phenylalanine and α-Ketoglutarate (Enzyme Concentration, 300 mcg./ml.; 37°; pH 8.0; Pyridoxal-5-phosphate, 200 n*M*; Phosphate Buffer, 0.4 *M*)

Reaction	L-Deuterio-phenylalanine, μ*M*	α-Ketoglutarate, μ*M*	Initial Velocity (V_i), n*M*/ml./min.	$1/V_i$
1	45.00	50.00	40.00	0.0250
2	45.00	30.00	30.00	0.0333
3	45.00	20.00	23.00	0.0435
4	25.00	50.00	35.75	0.0280
5	25.00	30.00	27.00	0.0370
6	25.00	20.00	21.25	0.0471
7	20.00	50.00	29.25	0.0342
8	20.00	30.00	23.25	0.0430
9	20.00	20.00	20.00	0.0500
10	10.00	50.00	23.25	0.0430
11	10.00	30.00	18.75	0.0533
12	10.00	10.00	11.25	0.0899

the amino acid most difficult to separate from L-phenylalanine. When using a gradient of 1–4 *N* HCl, the elution volumes for the protio forms of L-leucine and L-phenylalanine were 1655–1990 and 3235–3750 ml., respectively. With the deuterio forms, the elution volumes for L-leucine and L-phenylalanine were 1550–1850 and 2880–3600 ml., respectively.

Identification and Characterization—L-Deuterio-phenylalanine was identified on the basis of its melting point, elemental analysis, specific rotation, and a comparison of its R_f value with L-protio-phenylalanine in two solvent systems using TLC. For the last, lower R_f values were obtained for the deuterio compound. This finding is in agreement with earlier studies (2, 9) involving other deuterio amino acids.

The IR spectrum of the deuterio compound showed excellent correlation with the protio amino acid, particularly in the higher regions (3500–1450 $cm.^{-1}$). Marked differences were noted in the "fingerprint" region of the spectrum (1400–900 $cm.^{-1}$). The spectrum for the deuterio amino acid showed a multiple signal at 1200–1150 $cm.^{-1}$ and a single sharp peak at 990 $cm.^{-1}$. These peaks were absent in the spectrum of the protio derivative. Generally, the peaks in the fingerprint region resulted from bending and stretching vibrations of C—O, C—N, and C—H bonds.

The NMR spectra were compared in a spectrometer[6]. The spectrum for the protio compound revealed the nonequivalence of the protons on the α- and β-carbons and the equivalency of the protons of the aromatic nucleus. The peak observed at δ 4.85 is due to water formed by exchange at the amino and carboxyl functional groups. Integration of the spectral peaks confirmed the peak assignments. The spectrum for L-deuterio-phenylalanine revealed the presence of a lone water peak. The absence of proton signals in the spectrum indicated that nonexchangeable sites in the molecule were fully deuterated.

The mass spectra of L-protio- and L-deuterio-phenylalanine showed the largest fragments at *m/e* 165 and 173, respectively, which correspond to their respective molecular weights. The low percentage of the two fragments can be attributed to the low volatility of the amino acids in their zwitterion form (15). Cleavage of the carboxyl group from the protio species yields $C_6H_5—CH_2—{}^+CH—NH_2$ as a fragment, with a molecular weight of 120. The same type of cleavage from the deuterio species yields $C_6D_5—CD_2—{}^+CD—NH_2$ as a fragment, with a molecular weight of 128.

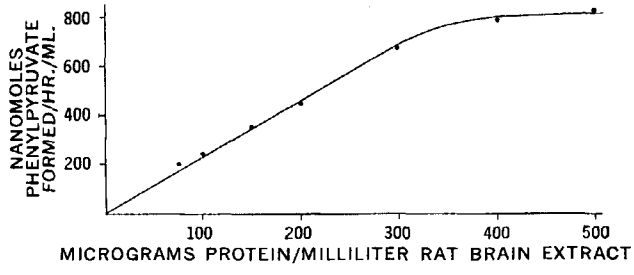

Figure 4—*Effect of enzyme concentration on the* in vitro *formation of phenylpyruvate.*

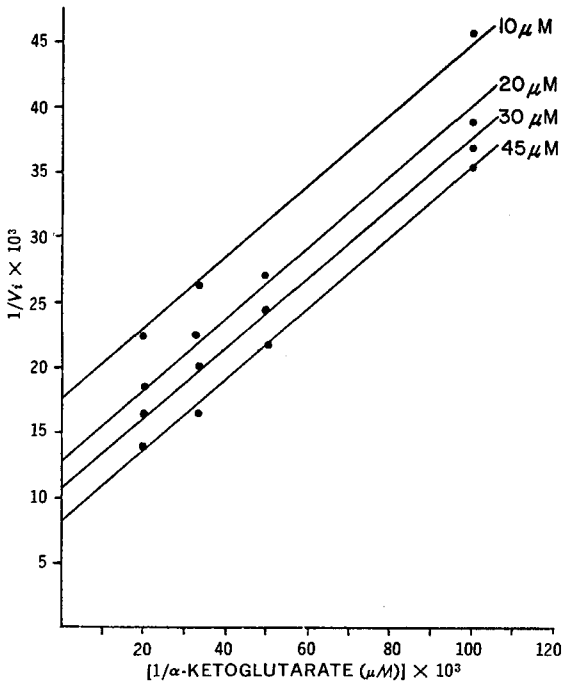

Figure 5—*Double reciprocal plot of initial velocities* versus *α-keto-glutarate concentrations at a series of fixed concenirations of* L-*protio-phenylalanine.*

The high percentage fragments appearing at *m/e* 74 and 91 in the protio species spectrum are attributed to bond cleavage between the α- and β-carbons, *e.g.*, C_6H_5—$^+C_\beta H_2$ (mol. wt. 91) and $^+CH(NH_2)$-COOH (mol. wt. 74). Similar cleavage, as observed in the deuterio spectrum, gives rise to the fragmentation peaks observed at *m/e* 98 and 75, respectively, which correspond to the molecular weights of fragments C_6D_5—$^+C_\beta D_2$ (mol. wt. 98) and $^+CD(NH_2)COOH$ (mol. wt. 75).

Transamination Studies—The rate of L-protio-phenylalanine metabolism was determined by analysis of the reaction for phenyl-pyruvate at regular time intervals while samples were incubated at 37° for 2 hr. The reaction was found to proceed linearly over the first 6–8 min., after which the rate of formation of phenylpyruvate decreased. This decrease in phenylpyruvate formation may be attributed to inactivation of the enzyme and/or breakdown of the phenylpyruvate formed to phenylacetaldehyde or phenylacetate.

To establish the enzyme concentration range over which the observed initial velocity is directly proportional to the enzyme concentration, L-protio-phenylalanine (45 μ*M*) was incubated in the standard reaction mixture (containing 30 μ*M* of α-ketoglutarate) with varied amounts of rat brain extract. The results are shown in Fig. 4.

The steady-state kinetics of transamination may be represented by the following equation:

$$\frac{1}{V_i} = \frac{1}{V_{max}\cdot[E]}\left(1 + \frac{K_{Am}}{[Am]} + \frac{K_{Ket}}{[Ket]}\right) \qquad \text{(Eq. 1)}$$

where V_i = initial velocity, V_{max} = maximum velocity, $[E]$ = enzyme concentration, K_{Am} = Michaelis constant for the amino acid, K_{Ket} = Michaelis constant for the keto acid, $[Am]$ = amino acid concentration, and $[Ket]$ = keto acid concentration.

Under the assumptions of steady-state kinetics, the initial velocity, V_i, of an enzymatic reaction will increase with an increase in the concentration of substrates.

Double reciprocal plots of determined initial velocities, $1/V_i$ (Tables II and III), *versus* α-ketoglutarate concentrations at a series of fixed concentrations of L-protio-phenylalanine or L-deuterio-

Table IV—Michaelis Constants and Maximum Velocities Obtained from the Transamination Reactions between α-Ketoglutarate and L-Phenylalanine Analogs (0.4 *M* Phosphate Buffer; pH 8.0; 37°; Pyridoxal-5-phosphate, 200 n*M;* Enzyme Concentration, 300 mcg./ml.)

Amino Acid Substrate	K_{Am}[a], μ*M*	K_{Ket}[b], μ*M*	$V_{max}\cdot[E]$[c]	V_H/V_D
L-Protio-phenylalanine	21.33	45.77	0.1742	1.00
L-Deuterio-phenylalanine	26.19	65.23	0.1062	1.64

[a] Michaelis constants of the amino acids. [b] Michaelis constants of α-ketoglutarate. [c] Maximum velocities at the enzyme concentration (300 mcg./ml.).

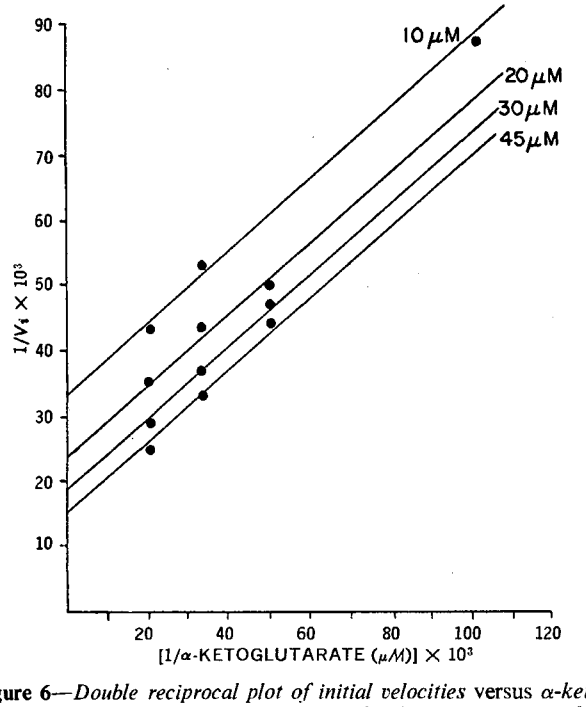

Figure 6—*Double reciprocal plot of initial velocities* versus *α-keto-glutarate concentrations at a series of fixed concentrations of* L-*deuterio-phenylalanine.*

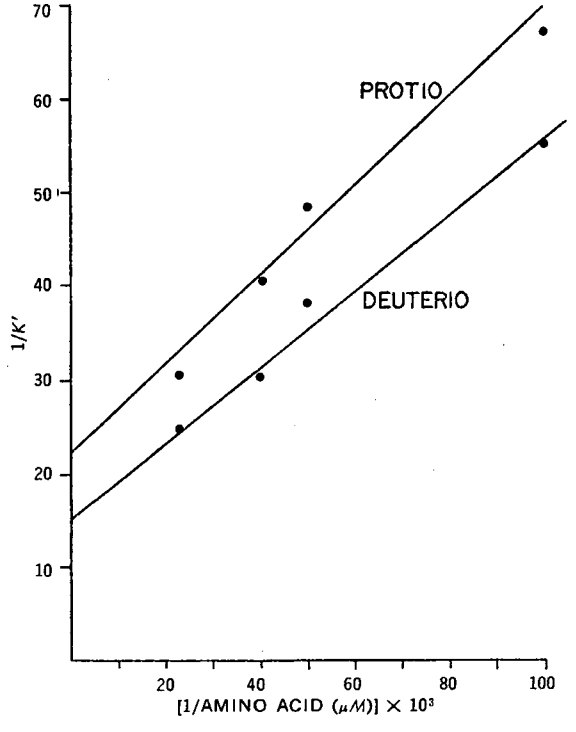

Figure 7—*Double reciprocal plot of apparent Michaelis constants* versus *amino acid concentrations of* L-*phenylalanine analogs.*

Table V—Intermediate Values of the Statistical Analyses on the Kinetic Studies of the Transamination Reactions between α-Ketoglutarate and L-Phenylalanine Analogs

Amino Acid Substrate	S_1[a], min./mM/ml.	I_1, min./nM/ml.	$1/K'$, μM^{-1}	S_2	I_2, μM^{-1}	S_3, min./mM/ml.	I_3, min./nM/ml.
L-Protio-phenylalanine				0.4660	21.85	0.1225	5.74
45 μM	0.2628	7.86	29.91				
25 μM	0.2628	10.66	40.56				
20 μM	0.2628	12.81	48.74				
10 μM	0.2628	17.60	66.97				
L-Deuterio-phenylalanine				0.4015	15.33	0.2344	9.42
45 μM	0.5837	13.88	23.78				
25 μM	0.5837	17.28	29.60				
20 μM	0.5837	22.38	38.34				
10 μM	0.5837	31.93	54.70				

[a] The slope given here is the average value.

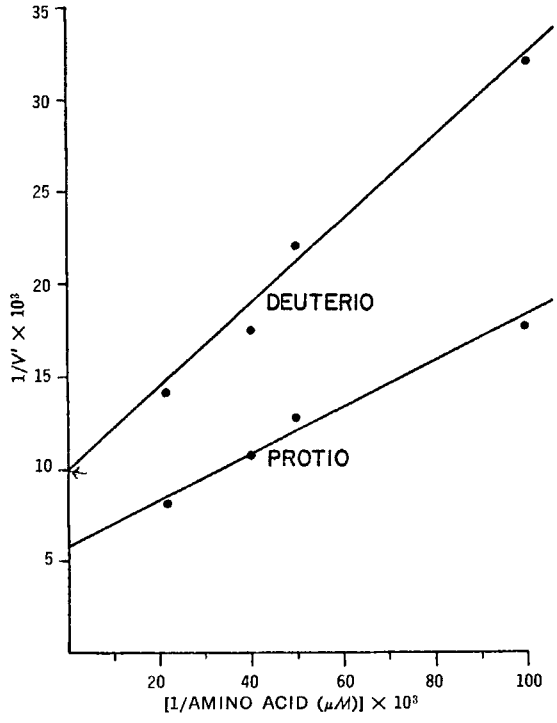

Figure 8—*Double reciprocal plot of apparent maximum velocities* versus *amino acid concentrations of* L-*phenylalanine analogs.*

phenylalanine are shown in Figs. 5 and 6, respectively. The y-intercept, I_1, of each plot yields the reciprocal of the apparent maximum velocity, $1/V'$. The corresponding slope, S_1, yields the ratio of the Michaelis constant for the keto acid, K_{Ket}, to the product of the maximum velocity and enzyme concentration, $V_{max}[E]$. The ratio of the slope of the plot, S_1, to the corresponding y-intercept, I_1, is defined as the apparent Michaelis constant, K'.

Double reciprocal plots of apparent Michaelis constants, $1/K'$, *versus* amino acid concentrations, $1/[Am]$, of the protio and deuterio analogs of L-phenylalanine are given in Fig. 7. The y-intercept of these plots, I_2, yields the reciprocal of the Michaelis constant of the keto acid, $1/K_{Ket}$. The corresponding slope, S_2, of these plots is equal to the ratio of the Michaelis constant of the amino acid (protio or deuterio analog) to the Michaelis constant of the keto acid, K_{Am}/K_{Ket}.

The maximum velocity, V_{max}, for the protio and deuterio forms of L-phenylalanine was determined, as shown in Fig. 8, by means of a double reciprocal plot of apparent maximum velocities, $1/V'$, *versus* protio and deuterio amino acid concentration, $1/[Am]$. The y-intercept of these plots yields the reciprocal of the maximum velocity, $1/V_{max}$, for the corresponding analogs.

The values of K_{Am}, K_{Ket}, and $V_{max}[E]$ (Table IV) were obtained by statistical analysis of the experimental data. Least-squares regression analysis was employed to obtain the equations representing the best-fit straight lines in Figs. 5 and 6. The slopes, S_1, and the intercepts, I_1, obtained by statistical derivation were subsequently used to determine the best-fit straight lines shown in Fig. 7. The best-fit straight lines for those lines shown in Fig. 8 were also obtained by statistical analysis. Table V shows the intermediate values obtained from the statistical analysis.

The maximum velocity for L-deuterio-phenylalanine was found to be 61% of the value obtained for the protio analog.

The deuterium isotope effect, expressed as the V_H/V_D value (the ratio of the maximum velocity of L-protio-phenylalanine to that of L-deuterio-phenylalanine), was found to be 1.64.

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In Vitro Dissolution and *In Vivo* Absorption of Nitrofurantoin from Deoxycholic Acid Coprecipitates

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Abstract □ The *in vitro* dissolution and *in vivo* absorption characteristics of various nitrofurantoin and nitrofurantoin–deoxycholic acid preparations were studied. *In vitro* particulate dissolution studies compared the dissolution of a 1:5 molar ratio nitrofurantoin–deoxycholic acid coprecipitate to that of a 1:5 physical mixture of nitrofurantoin and deoxycholic acid, precipitated nitrofurantoin, and pure nitrofurantoin. In pH 7.4 buffer, the dissolution rate of the coprecipitate system was approximately six times greater than the dissolution rate of the 1:5 physical mixture. However, no statistical differences could be shown among the dissolution rates of the 1:5 physical mixture, the pure nitrofurantoin, and the precipitated form of nitrofurantoin. In pH 1.2 buffer solution, the 1:5 coprecipitate showed a faster initial dissolution rate than the pure nitrofurantoin but became slower than the pure compound after 35 min. The *in vivo* absorption of the 1:5 coprecipitate, the 1:5 physical mixture, and pure nitrofurantoin, as determined by urinary excretion of unchanged nitrofurantoin, was carried out in four subjects in a crossover study. The 1:5 nitrofurantoin–deoxycholic acid coprecipitate showed significant increases in both the initial urinary excretion and the total cumulative amount of unchanged nitrofurantoin excreted when compared to either the 1:5 physical mixture or the pure nitrofurantoin. The study demonstrated the potential usefulness of drug–bile acid coprecipitate systems in increasing the *in vivo* absorption of dissolution rate-limited drugs.

Keyphrases □ Nitrofurantoin dissolution and absorption—deoxycholic acid coprecipitates, compared to nitrofurantoin alone and mixtures with deoxycholic acid □ Dissolution, nitrofurantoin–deoxycholic acid coprecipitates—compared to nitrofurantoin alone and mixtures with deoxycholic acid □ Absorption, nitrofurantoin–deoxycholic acid coprecipitates—compared to nitrofurantoin alone and mixtures with deoxycholic acid □ Bile acid–drug coprecipitate systems (nitrofurantoin–deoxycholic acid)—effect on absorption of dissolution rate-limited drugs □ Drug–bile acid coprecipitate systems (nitrofurantoin–deoxycholic acid)—effect on absorption of dissolution rate-limited drugs

Since Sekiguchi and Obi (1) first reported the principle of using solid solutions and eutectic mixtures of drugs and inert carriers (*e.g.*, urea and succinic acid) to enhance the rate of dissolution and oral absorption of poorly water-soluble drugs, reports dealing with the *in vitro* dissolution characteristics of these systems have appeared in the literature (2, 3). More recently, reports using other inert carriers such as polyethylene glycol (4) and polyvinylpyrrolidone (5, 6) to form solid dispersions of insoluble drugs have been made.

A previous article (7) reported that the increased dissolution rate noted for bile acid–reserpine coprecipitate systems was due most likely to a reduction in the particle size of reserpine, which occurred during the preparation of the coprecipitates. As a result, the *in vivo* blepharoptotic activity of reserpine was promoted. It was natural, therefore, to apply this approach to a drug whose dissolution is known to be particle-size dependent and whose rate of absorption and bioavailability have been shown to be influenced by the particle size of the drug. Such studies are unique in that a naturally occurring physiological material is used to prepare such solid dispersion or coprecipitate systems.

Nitrofurantoin, 1-[(5-nitrofurfurylidene)amino]hydantoin, is such a drug, since the available data (8, 9) show that the absorption and excretion of nitrofurantoin in man and the dog are more efficient when microcrystalline drug is administered than when the same dose of macrocrystalline material is given. Consequently, this drug appears to be suitable for study as a coprecipitate of a bile acid. We wish to report studies of the *in vitro* dissolution and *in vivo* absorption characteristics of several nitrofurantoin and nitrofurantoin–deoxycholic acid systems; deoxycholic acid was the agent with which nitrofurantoin was coprecipitated.

EXPERIMENTAL

Materials—Nitrofurantoin[1] was prepared from an aqueous solution of sodium nitrofurantoin by slowly acidifying the solution with hydrochloric acid until the free acid form of the drug precipitated from the system. The precipitate was washed with several portions of deionized water and was dried to constant weight at 60° in a vacuum oven. Deoxycholic acid[2], hyamine 10X monohydrate[3], *N,N*-dimethylformamide[4], absolute methanol[5], acetone[5], and all other chemicals were of reagent grade and used as received.

Preparation of Coprecipitate Systems—Coprecipitates of nitrofurantoin and deoxycholic acid, in a 1:5 molar ratio, were prepared by dissolving simultaneously the constituents in a minimum quantity of acetone and subsequently removing the solvent *in vacuo*. The coprecipitates were then dried *in vacuo* to constant weight. Precipitated nitrofurantoin was prepared by treating pure nitrofurantoin in a similar manner. A physical mixture composed of a 1:5 molar ratio of nitrofurantoin and deoxycholic acid was also prepared by mechanically mixing the two substances in a mortar and pestle. Unless otherwise specified, 40–50-mesh particles of the test systems were employed in *in vitro* dissolution rate and *in vivo* absorption experiments. The chemical uniformity of all dispersions was confirmed by assaying for nitrofurantoin (see *Assay Procedures*).

Dissolution Studies—The dissolution characteristics of 40–50-mesh particles of nitrofurantoin in the form of a 1:5 coprecipitate and a physical mixture with deoxycholic acid, nitrofurantoin alone, and precipitated nitrofurantoin were studied in a Clark-Lubs pH 7.4 phosphate buffer system. In all cases, the amount used contained 25 mg. of nitrofurantoin. The studies were carried out under sink conditions using 400 ml. of buffer in a 500-ml., three-necked, round-bottom flask maintained at 37° in a constant-temperature water bath. A three-bladed polyethylene propeller was immersed into the dissolution medium to a depth of 23 mm., and the stirring speed was maintained at 150 r.p.m. by a constant-speed stirring apparatus[6]. Five-milliliter samples were taken for analysis at specified time periods and replaced with fresh solvent. The effect of particle size on the dissolution behavior of nitrofurantoin was determined by studying the dissolution of 40–50-mesh (297–420-

[1] Supplied as sodium nitrofurantoin by Eaton Laboratories, Norwich Pharmacal Co., Norwich, N. Y.
[2] Nutritional Biochemical Corp., Cleveland, Ohio.
[3] Rohm and Haas, Philadelphia, Pa.
[4] Reagent grade, Fisher Chemical Co., Medford, Mass.
[5] Reagent grade, Allied Chemical Co., Morristown, N. J.
[6] Standard Servodyne power drive system, Cole-Parmer Instrument Co., Chicago, Ill.

Table I—*In Vitro* Dissolution Rates of Nitrofurantoin Systems in pH 7.4 Buffer at 37°

System	Mesh Size Range[a]	Dissolution Rate Constant[b], min.$^{-1}$ × 10^2
Nitrofurantoin	100–120 (125–149)	14.4 (14.4; 14.4)
Nitrofurantoin	70–80 (177–210)	9.15 (9.63; 8.66)
Nitrofurantoin	40–50 (297–420)	3.55 (3.43; 3.66)
Precipitated nitrofurantoin	40–50 (297–420)	3.36 (3.06; 3.66)
1:5 Mixture of nitrofurantoin–deoxycholic acid	40–50 (297–420)	3.20 (3.06; 3.34)
1:5 Coprecipitate of nitrofurantoin–deoxycholic acid	40–50 (297–420)	17.6 (17.0; 18.1)

[a] Particle-size range in microns in parentheses. [b] Mean of duplicate runs; individual runs shown in parentheses.

μ), 70–80-mesh (177–210-μ), and 100–120-mesh (125–149-μ) particles of nitrofurantoin at pH 7.4. The dissolution of 40–50-mesh particles of nitrofurantoin and the 1:5 coprecipitate was also studied in the presence of a pH 1.2, 0.1 *M* HCl buffer. All dissolution media contained 0.005% polysorbate 80[7] to ensure proper wetting of the various nitrofurantoin systems (10).

Absorption of Nitrofurantoin in Man—The absorption characteristics of nitrofurantoin from the test systems were evaluated in four healthy, adult male subjects using the urinary excretion method (8). The subjects were fasted overnight (*i.e.*, for not less than 8 hr.) and, on arising, voided their bladders of overnight urine. They then consumed a light standard breakfast consisting of 240 ml. (8 oz.) of milk and 30 g. (1 oz.) of breakfast cereal to minimize the possibility of drug-induced GI upset. One hour later, urine blanks were collected. Subsequently, a single dose of 100 mg. of nitrofurantoin in the form of a 1:5 coprecipitate, 1:5 physical mixture, or nitrofurantoin alone was orally administered to the subjects as a suspension of 40–50-mesh particles in 150 ml. of distilled water. All subjects were allowed free access to fluids following drug administration.

Each subject received all test systems, using a completely balanced crossover design and allowing at least a 1-week interval between each preparation. Urine samples were collected at hourly intervals for the first 8 hr. and then at convenient periods for 24 hr. after drug administration. The pH of each urine specimen was measured, and an aliquot was stored in 60-ml. (2-oz.) amber glass bottles at 4° until assayed for drug content. The amount of drug excreted in the urine at each time period was determined spectrophotometrically (see *Assay Procedures*) and appropriately corrected for blank urine values.

Assay Procedures—Nitrofurantoin was assayed spectrophotometrically using a spectrophotometer[8] at 383 nm. for the pH 7.4 dissolution studies and at 369 nm. for the pH 1.2 dissolution studies.

The urine samples were assayed spectrophotometrically at 397 nm. using the method of Conklin and Hollifield (11), which is specific for unchanged nitrofurantoin in urine. Deoxycholic acid was shown not to interfere with this assay. All samples containing nitrofurantoin were protected from light at all times.

RESULTS AND DISCUSSION

Dissolution Rate Studies—Dissolution was found to follow first-order kinetics. Semilogarithmic plots of percent drug undissolved *versus* time had excellent linearity, the correlation coefficient being 0.98 or greater in all cases. The dissolution rate constants obtained (Table I) are the means of duplicate runs on each system. It is obvious that the dissolution rate of nitrofurantoin at pH 7.4 is essentially the same as for nitrofurantoin alone, when precipitated, and when present as a 1:5 mixture with deoxycholic acid. However, there is almost a sixfold increase in the dissolution rate of nitrofurantoin when present in the 1:5 deoxycholic acid coprecipitate system.

These data permit the exclusion of certain factors as being responsible for the enhanced dissolution rate of nitrofurantoin from the coprecipitate system. Thus, since the precipitated form of nitrofurantoin does not dissolve more rapidly than the pure, nonprecipitated form, the enhanced dissolution of the coprecipitate is not due to the formation of a more soluble solvate or polymorph during its preparation. Additionally, bulk effects due to the presence of deoxycholic acid in solution are not responsible for the enhanced rate observed, since there is no significant difference between nitrofurantoin alone and when in the 1:5 mixture with deoxycholic acid. Therefore, factors such as complex formation, micelle formation, and surface tension lowering are not responsible for the enhanced nitrofurantoin dissolution from the coprecipitate system presented in Table I.

The various drug systems were also studied at pH 1.2 to simulate gastric pH conditions. As seen in Fig. 1, nitrofurantoin in the coprecipitate system dissolves at a rate faster than the pure drug during the first 30 min. Thereafter, the pure drug continues to dissolve at a steady rate, while the nitrofurantoin in the coprecipitate system shows a decreased rate with respect to its initial dissolution rate. Thus, the $T_{50\%}$ for nitrofurantoin in the coprecipitate is approximately twice that for the pure nitrofurantoin alone. Such behavior may be due to the poor solubility of deoxycholic acid at this acid pH. This would slow down the rate of dissolution of the bile acid matrix which, in turn, would retard the dissolution of nitrofurantoin. The enhanced dissolution of nitrofurantoin seen initially may be due to dissolution of the drug from the surface of the coprecipitated particles. As nitrofurantoin dissolves from the surface, the poor dissolution properties of deoxycholic acid at pH 1.2 leads to a decrease in the rate of formation of new surfaces in which the drug appears. Since the drug remains embedded inside the coprecipitate particle for a longer period of time before erosion exposes it, dissolution of nitrofurantoin is slowed.

To demonstrate that the dissolution rate of nitrofurantoin is affected by particle size, dissolution runs were carried out at pH 7.4 using three different size fractions. The results (Table I) show that as particle size decreases the rate of dissolution increases. The significance of this effect in terms of the *in vivo* absorption of the various nitrofurantoin systems is discussed in the following sections.

Absorption Studies—McGilveray *et al.* (12) demonstrated that a relationship exists in man between the blood levels of nitrofurantoin and its rate of urinary excretion. Hence, it is possible to determine the rate and extent of absorption of this drug by measuring the rate of appearance of unchanged drug in the urine. Individual data for the four subjects are presented in Table II.

Nitrofurantoin is a weakly acidic drug (pKa 7.2) so its urinary excretion rate in man can be significantly affected by variations in urinary pH (13). Since, in the present investigation, the absorption characteristics of nitrofurantoin from the three test systems were assessed by the urinary excretion rate method, it was important to monitor the pH of all urine specimens. In this connection, the mean pH (±*SD*) of the urine specimens over the experimental time

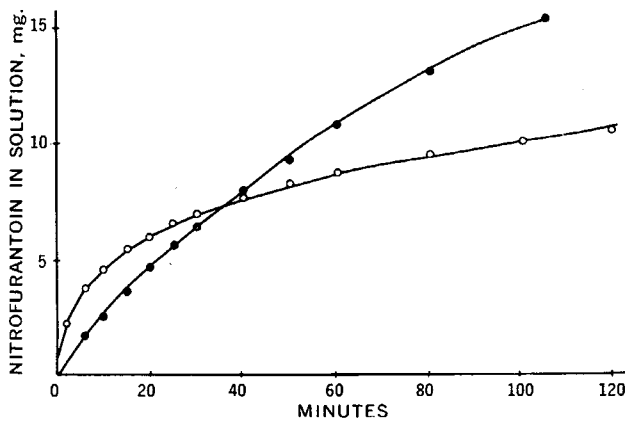

Figure 1—*Dissolution of nitrofurantoin* (●) *and a 1:5 molar ratio coprecipitate of nitrofurantoin deoxycholic acid* (○) *in pH 1.2 HCl buffer at 37°.*

[7] Tween 80.
[8] Beckman DB-G.

period was 5.77 (±0.52), 5.80 (±0.49), and 5.72 (±0.47) following the oral administration to the four subjects of nitrofurantoin alone, as a 1:5 physical mixture with deoxycholic acid, and as a 1:5 coprecipitate with deoxycholic acid, respectively. The similarity of these values for all test systems indicates that the slight variation in urinary pH would not affect the interpretation of results of the *in vivo* absorption studies. This is supported by the fact that the elimination rate constant ($hr.^{-1}$ $\pm SD$) for the drug, determined from the least-squares slope of the terminal portion of a plot of the logarithm of the excretion rate *versus* time, was found to be independent of the system used, *i.e.*, for the drug alone, 1.14 (±0.27); as a physical mixture with deoxycholic acid, 1.14 (±0.25); and as a coprecipitate with deoxycholic acid, 1.15 (±0.26).

The average amount of unchanged nitrofurantoin excreted by the four subjects during the experimental time intervals for each nitrofurantoin test system is summarized in Table III, and plots of the cumulative amount of drug excreted *versus* time are shown in Fig. 2. Figure 2 reveals that all of the plots tend to reach a plateau between 6 and 8 hr. after drug administration, indicating that the excretion of nitrofurantoin is essentially over by 8 hr. and that the cumulative amount of nitrofurantoin excreted in the urine in 24 hr. is a valid estimate of the extent of absorption or availability of the drug from the test preparations.

An examination of the excretion data presented in Table III indicates that nitrofurantoin from the coprecipitate material was excreted in the urine, and therefore absorbed, at both a faster rate and to a greater extent than either the drug alone or the 1:5 physical mixture. Approximately 2.5–4.5 times as much nitrofurantoin was excreted from the coprecipitate system during the first 3 hr. of the study as compared with the other two systems. Overall, the 24-hr. excretion of unchanged drug was increased between 50 and 80% for the coprecipitate system. Analysis of variance of the treatment averages shows that there are significant differences at the 99% level of confidence during the first 3 hr. postadministration of the three systems. There is also a significant difference for the cumulative amount excreted between 0 and 24 hr. No significant differences in excretion rate were found during the intermediate time periods. Analysis by paired comparison of the test systems verifies that there are no statistical differences at any time interval between the amounts excreted following administration of pure nitrofurantoin and the 1:5 physical mixture of nitrofurantoin and deoxycholic acid. The similarity between the percent of the dose of drug excreted in 24 hr. from the nitrofurantoin–deoxycholic acid coprecipitate (approximately 28%) and the value of 33% obtained by Reckendorf *et al.* (14), following the intravenous administration of 180 mg. of drug to 16 patients, suggests that nitrofurantoin is nearly maximally absorbed from the coprecipitate system.

Table IV lists the time of occurrence of the peak excretion rate for the various nitrofurantoin systems administered orally to each individual subject, and it illustrates that the drug was absorbed more rapidly from the nitrofurantoin coprecipitate than from the other two test systems. The average time of peak excretion was 1.6 hr. when the coprecipitate was administered, while the average peak excretion time was 3.6 hr. following the oral administration of the drug alone or as a physical mixture with deoxycholic acid. Paul *et al.* (8) reported values of 4.9–3.6 hr. for the time of occurrence of the peak excretion rate for nitrofurantoin administered to human subjects in various particle-size fractions of the pure drug (*i.e.*, 50–60 to 200–400 mesh and 10 μ). The coprecipitate used in the present study gave an earlier peak excretion rate than the micronized (10 μ) drug studied by Paul *et al.* Also, none of the subjects in the present studies gave any evidence of nausea or GI distress.

A comparison of the dissolution and absorption rates of nitrofurantoin, as the pure compound and as a 1:5 molar ratio physical mixture with deoxycholic acid, shows them to be almost identical. This observation eliminates the possibility that deoxycholic acid functions in the coprecipitate to increase the bulk solubility of nitrofurantoin in the fluids of the GI tract or to increase its rate of dissolution by lowering the interfacial tension between the hydrophobic drug and these biological fluids. In addition, the potential influence of deoxycholic acid on such physiological processes as gastric emptying, GI motility, and GI membrane permeability can be ruled out, since it is reasonable to assume that these changes would be the same for both the physical mixture and the coprecipitate.

The similarity of the *in vitro* dissolution rates of pure and precipitated nitrofurantoin at pH 7.4 (Table I) precludes the possibility

Table II—Amount of Unchanged Nitrofurantoin Excreted in Urine following a Single Dose of 100 mg. Nitrofurantoin in the Form of Three Different 40–50-Mesh Dosage Systems

Time Interval, hr.	Subject Number	Nitrofurantoin	1:5 Mixture of Nitrofurantoin–Deoxycholic Acid	1:5 Coprecipitate of Nitrofurantoin–Deoxycholic Acid
0	1	0.0 (5.97)[a]	0.0 (5.72)	0.0 (5.25)
	2	0.0 (5.60)	0.0 (6.40)	0.0 (6.00)
	3	0.0 (5.30)	0.0 (5.15)	0.0 (5.35)
	4	0.0 (5.40)	0.0 (5.20)	0.0 (5.30)
0–1	1	0.25 (5.20)	0.49 (5.65)	1.49 (5.30)
	2	1.03 (6.15)	0.72 (6.30)	1.73 (6.00)
	3	0.36 (5.35)	0.58 (5.85)	2.36 (5.40)
	4	0.06 (5.40)	0.52 (5.30)	2.10 (5.35)
1–2	1	1.19 (5.65)	2.48 (6.50)	6.13 (5.20)
	2	2.15 (6.60)	2.10 (6.20)	12.93 (6.70)
	3	2.52 (5.70)	2.51 (6.20)	12.92 (5.75)
	4	2.29 (5.40)	1.58 (5.25)	4.91 (5.25)
2–3	1	2.36 (5.37)	1.53 (5.95)	6.51 (5.20)
	2	4.66 (6.70)	3.27 (6.70)	8.41 (6.70)
	3	5.94 (6.30)	4.16 (6.80)	9.18 (6.10)
	4	2.46 (5.20)	2.61 (5.40)	4.88 (5.10)
3–4	1	2.96 (5.80)	2.30 (5.40)	1.24 (5.25)
	2	2.68 (6.40)	3.45 (6.50)	8.02 (6.40)
	3	4.67 (6.30)	5.18 (6.85)	6.18 (5.90)
	4	2.42 (5.15)	2.42 (5.50)	3.22 (5.10)
4–5	1	2.76 (5.60)	2.38 (6.25)	0.08 (5.78)
	2	4.51 (6.35)	2.04 (5.80)	4.59 (6.00)
	3	2.74 (5.90)	5.25 (6.15)	1.28 (6.10)
	4	2.51 (5.25)	2.18 (5.40)	3.97 (5.10)
5–6	1	0.88 (5.80)	1.31 (5.85)	0.22 (6.20)
	2	2.97 (7.35)	1.13 (6.20)	2.59 (6.10)
	3	0.76[b] (—)[c]	3.63 (5.50)	0.38 (5.50)
	4	0.46 (6.00)	0.81 (5.20)	2.71 (5.15)
6–7	1	0.23 (5.90)	0.90 (5.28)	0.18 (6.20)
	2	1.83 (7.35)	0.69 (5.60)	0.40 (6.10)
	3	0.48 (6.15)	2.61 (5.50)	0.18 (6.15)
	4	0.77 (5.80)	0.72 (5.30)	0.59 (5.20)
7–8	1	0.20 (5.70)	0.27 (5.75)	0.25 (6.28)
	2	0.13 (6.80)	1.34 (5.70)	0.23 (5.70)
	3	0.00 (6.00)	0.63 (6.05)	0.19 (6.45)
	4	0.09 (5.30)	0.48 (5.15)	0.71 (5.30)
0–24	1	11.29	12.43	18.86
	2	19.97	24.20	38.86
	3	17.56	25.65	33.11
	4	10.39	11.67	23.14

[a] Urine pH values. [b] Estimated graphically from cumulative excretion curve since no 6-hr. sample was taken. [c] No sample was taken.

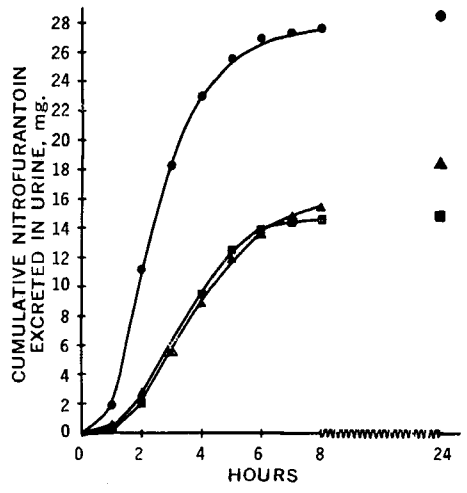

Figure 2—*Mean cumulative amount of nitrofurantoin excreted in the urine following oral administration of 100-mg. doses of nitrofurantoin. Key:* ●, *nitrofurantoin as a 1:5 molar ratio coprecipitate with deoxycholic acid;* ▲, *nitrofurantoin as a 1:5 molar ratio mixture with deoxycholic acid; and* ■, *nitrofurantoin alone.*

Table III—Average Amount of Unchanged Nitrofurantoin Excreted in Urine for Various 40–50-Mesh Nitrofurantoin Systems

Time Interval, hr.	Amount of Nitrofurantoin Excreted, mg.[a] Nitrofurantoin	1:5 Mixture	1:5 Coprecipitate	Differences among Systems, Average from ANOVA	Paired Comparison by Paired *t* Test: Nitrofurantoin *versus* Coprecipitate	Mixture *versus* Coprecipitate	Mixture *versus* Nitrofurantoin
0–1	0.43 (0.21)	0.58 (0.05)	1.92 (0.19)	Significant ($0.001 < p < 0.005$)	Significant ($0.01 < p < 0.02$)	Significant ($0.001 < p < 0.01$)	n.s. ($p \gg 0.10$)
1–2	2.04 (0.29)	2.16 (0.22)	9.22 (2.51)	Significant ($0.005 < p < 0.010$)	Significant ($0.02 < p < 0.05$)	Significant ($0.02 < p < 0.05$)	n.s. ($p \gg 0.10$)
2–3	3.10 (0.96)	2.89 (0.56)	7.24 (0.96)	Significant ($0.001 < p < 0.005$)	Significant ($0.02 < p < 0.05$)	Significant ($0.001 < p < 0.01$)	n.s. ($p \gg 0.10$)
3–4	3.94 (0.76)	3.34 (0.67)	4.67 (1.51)	n.s. ($p > 0.25$)	n.s. ($p > 0.10$)	n.s. ($p > 0.10$)	n.s. ($p > 0.10$)
4–5	3.13 (0.47)	2.96 (0.77)	2.48 (1.08)	n.s. ($p > 0.25$)	n.s. ($p > 0.10$)	n.s. ($p > 0.10$)	n.s. ($p > 0.10$)
5–6	1.23 (0.58)	1.72 (0.64)	1.47 (0.68)	n.s. ($p > 0.25$)	n.s. ($p > 0.10$)	n.s. ($p > 0.10$)	n.s. ($p > 0.10$)
6–7	0.69 (0.40)	1.23 (0.46)	0.33 (0.10)	n.s. ($p > 0.25$)	n.s. ($p > 0.10$)	n.s. ($p > 0.10$)	n.s. ($p > 0.10$)
7–8	0.11 (0.03)	0.68 (0.23)	0.35 (0.12)	n.s. ($0.10 < p < 0.25$)	n.s. ($p > 0.10$)	n.s. ($p > 0.10$)	n.s. ($0.05 < p < 0.10$)
Total amount excreted, 0–24 hr.	14.80 (2.35)	18.48 (3.73)	28.49 (4.57)	Significant ($0.001 < p < 0.005$)	Significant ($0.01 < p < 0.02$)	Significant ($0.01 < p < 0.02$)	n.s. ($p > 0.10$)

[a] Amount reported as mean of four subjects. Standard errors are in parentheses.

of the formation of a drug–solvate during the preparation of the coprecipitate system. However, two plausible explanations do exist. First, the two components of the coprecipitate system are probably more intimately associated with one another than they are in the physical mixture. As a result, only in the case of the coprecipitate can the concentration of deoxycholic acid in the diffusion layer surrounding the dissolving particles increase to such an extent so as to influence the solubility and/or wetting of nitrofurantoin in this region and, hence, increase its rate of dissolution and absorption. The second, more probable, explanation involves an examination of the procedure used to prepare the coprecipitate system. Initially both drug and bile acid are in solution. As the solvent system is stripped from the solution, a supersaturated solution is formed. This is apparent since the total quantity of material is still in solution even though the quantity of solvent has been reduced about 10-fold from the initial quantity necessary to effect solution of the drug and bile acid. Finally, the supersaturated solution loses enough solvent so that a very rapid precipitation of solute occurs. Such a rapid crystallization is commonly known to cause the formation of very fine particles, and herein seems to lie the effectiveness of the coprecipitate system. The smaller particles of drug evolved during precipitation are retained by the matrix of bile acid formed around it. When the coprecipitate is administered orally, the bile acid component readily goes into solution in the fluid of the small intestine because of its ability to dissociate under these pH conditions and/or be solubilized by endogenous conjugated bile salts and other physiological surfactants. Both the reduction in the particle size of nitrofurantoin and the ability of deoxycholic acid to be solubilized readily in the small intestine most likely contribute to the enhanced dissolution and absorption of the drug.

The present investigation has shown that the drug–bile acid coprecipitate system can be employed to potentiate the absorption characteristics of a drug that possesses a dissolution rate-limited absorption mechanism, such as nitrofurantoin. This approach may be applicable to other drugs whose absorption is also dissolution rate limited.

Table IV—Time (Hours) of Occurrence of Peak Excretion Rate

Subject Number	Nitrofurantoin	1:5 Mixture	1:5 Coprecipitate
1	4.0	3.5	1.5
2	3.5	3.5	2.0
3	2.5	4.5	1.5
4	4.5	3.0	1.5
Mean	3.6	3.6	1.6
SD	0.9	0.6	0.3

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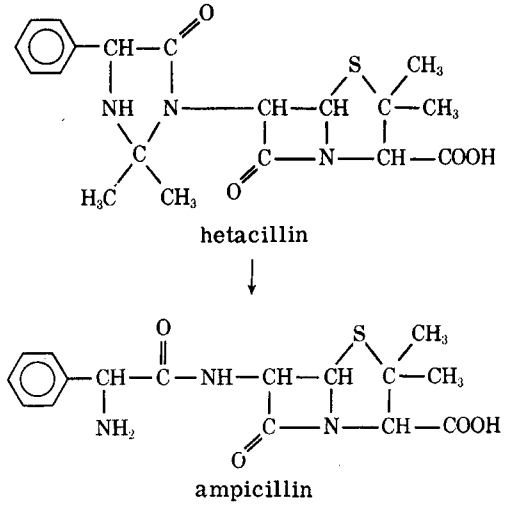
Comparison of Ampicillin and Hetacillin Pharmacokinetics in Man

WILLIAM J. JUSKO▲ and GEORGE P. LEWIS

Abstract □ The pharmacokinetics of oral and intravenous doses of ampicillin were studied when ampicillin was given directly and as its inactive precursor, hetacillin. The two compounds were assayed separately in plasma using an electrophoresis–bioautography method. Plasma concentration and urinary excretion rates of ampicillin after direct intravenous injection and after hetacillin administration were computer fitted using a two-compartment open model. Hetacillin hydrolyzed rapidly and completely to ampicillin with an *in vivo* half-life of 11 ± 2 min. in eight human subjects. No differences were found in the distribution rate and volume constants, elimination rates and clearance parameters, and urinary recovery of ampicillin when it was given either as intravenous ampicillin or intravenous hetacillin. The major effect of intravenous hetacillin is that early plasma, urine, and peripheral compartment levels of ampicillin resemble those from a rapid absorption process, with a peak plasma concentration found at about 0.5 hr. Limited bioavailability studies in eight fasting subjects showed 32% absorption from ampicillin capsules, while 38 and 42% absorption values from hetacillin capsules were found in four fasting and four nonfasting subjects, respectively. The primary rationale for clinical use of ampicillin precursors is, in general, the improvement of the limited intestinal absorption of the antibiotic as well as increasing the stability of ampicillin in aqueous solutions.

Keyphrases □ Ampicillin pharmacokinetics—oral and intravenous, compared to intravenous hetacillin, man □ Hetacillin pharmacokinetics—intravenous, compared to oral and intravenous ampicillin, man □ Pharmacokinetics—ampicillin compared to hetacillin, man □ Drug precursors—oral and intravenous ampicillin compared to intravenous hetacillin □ Absorption, drug—ampicillin and hetacillin, man □ Elimination, drug—ampicillin and hetacillin, man

Hetacillin is representative of an unusual and somewhat controversial class of chemotherapeutic agents. It is a condensation product of ampicillin and acetone, which is stable in the dry state but susceptible to rapid conversion to ampicillin (Scheme I) in aqueous solution (1, 2). A recent study employing a short-duration bacterial lysis technique showed that unchanged hetacillin has little or no bactericidal activity (3). However, because of its hydrolysis, microbiological assays that require prolonged incubation reveal hetacillin and ampicillin to be eventually equipotent on a molar basis (4). Hetacillin is thus an inactive precursor of ampicillin, but its slightly different physicochemical properties and the fact that it must hydrolyze to ampicillin can be expected to modify the pharmacokinetics and possibly the chemotherapeutic effect of ampicillin. In fact, apparent increases in blood levels and decreases in renal clearance of ampicillin have been reported after hetacillin administration (5–7). However, most of these studies did not involve separation of unchanged hetacillin from ampicillin in plasma specimens prior to microbiological assay and are, therefore, misleading.



Scheme I

The present investigation was performed to elucidate and compare the pharmacokinetic properties of ampicillin when administered directly and as hetacillin to human subjects. These studies are of general interest because hetacillin represents the first of several attempts to enhance the chemotherapeutic usefulness of ampicillin by the use of precursors of the antibiotic.

METHODS

Eight normal subjects, four males and four females, participated in the study; their ages and relevant descriptions are listed in Table I. Each volunteer received doses of intravenous ampicillin[1], intravenous hetacillin[1], and oral ampicillin[1], with the latter following an overnight fast. The female subjects also received oral doses of hetacillin[1] after fasting and immediately following a standard breakfast consisting of 30 g. cornflakes, 250 ml. whole milk, and 5 g. sucrose. The oral dosage forms were loosely packed commercial capsules, which were ingested with 100–200 ml. of tap water. The sequences of drugs and dosage forms were randomized for the female subjects, except that determination of the effect of food on hetacillin absorption was performed after completion of all other studies.

The dosages were determined by analysis of several capsules and vials of injectable material. The ampicillin and hetacillin capsules contained 550 and 483 mg. ampicillin equivalent, respectively. All intravenous solutions were prepared within 3 min. of injection, and the residual drug in the vial was analyzed to allow calculation of the actual dose administered. These doses are listed in Table I.

Urine specimens and, in most instances, blood samples were obtained following each dose of antibiotic. Blood was collected from either the cubital vein or forearm cephalic vein using an indwelling needle and cannula and heparinized vacuum tubes. Clotting in the tubing was prevented with a dilute solution of heparin in saline. Blood samples were drawn prior to drug administration and at seven intervals during the subsequent 8 hr. A blank urine specimen was obtained and hourly urine collections were made for 12 hr. followed by less frequent samples for an additional 12 hr. All specimens were refrigerated until drug analyses were performed on the day after drug administration. However, during the studies with hetacillin, the first four blood samples were rapidly centrifuged, diluted with the blank plasma from the same subject when appropriate, and quickly frozen to −60°, in which state hetacillin is

[1] Bristol Laboratories, Syracuse, N. Y.

Table I—Drug Dosages and Physiological Characteristics of the Subjects

Subject	Sex	Age, Years	Surface Area[a], m.²	Intravenous Doses, mg.[b] Ampicillin	Intravenous Doses, mg.[b] Hetacillin	Plasma[c] Proteins, g. % Total	Plasma[c] Proteins, g. % Albumin	Creatinine[d] Clearances, ml./min.
1	F	26	1.73	647	533	7.0	3.1	104
2	F	23	1.65	608	550	7.2	3.2	94
3	F	26	1.82	639	532	7.2	2.9	128
4	F	23	1.57	633	533	6.9	2.8	101
5	M	28	2.09	570	554	7.2	2.8	174
6	M	43	2.01	570	538	7.1	2.9	127
7	M	28	1.92	570	558	6.6	3.0	92
8	M	24	2.03	570	561	6.9	3.4	109

[a] Determined from height and weight by the method of Dubois and Dubois (20). [b] Ampicillin equivalent. [c] Mean of two determinations. [d] Mean of four and two determinations for the F and M subjects, respectively.

stable. These specimens were subjected to electrophoresis to separate ampicillin and hetacillin immediately following collection of the 2.5-hr. sample.

Assays—All samples were analyzed for ampicillin using a fluorometric method described previously (8) as well as the agar-well diffusion assay of Bennett *et al.* (9), with *Bacillus subtilis* as the microorganism. Both assays involved use of reference standards prepared with blank plasma from the individual subject as well as nonproteinaceous standards. Hetacillin was separated from ampicillin using duplicate electrophoretic agar gels buffered with tris-maleate at pH 5.6 (10). After electrophoresis at approximately 240 v. and 60 ma. for 45 min., the gels were overlayed with 1.5% nutrient agar containing *B. subtilis* spores. Following overnight incubation, the relative fractions of hetacillin and ampicillin were determined by comparison of the zone sizes with a graph of the mean inhibition diameter plotted *versus* the logarithm of ampicillin concentration of standards. All measurements were made with analytical grade sodium ampicillin[1] (potency 865 mcg./mg.) as the reference material.

One set of urine and plasma samples obtained after each dose of drug was analyzed for endogenous creatinine, using the alkaline picrate method (11) to calculate creatinine clearances. Plasma samples collected after each intravenous dose of antibiotic also were analyzed to determine total protein by the biuret method (12) and the albumin fraction using cellulose acetate electrophoresis.

Protein Binding—The degree of protein binding of ampicillin and α-aminobenzylpenicilloic acid was determined at 23° using a centrifugal ultrafiltration procedure described previously (13). Fractional binding of both compounds to 4 g.% human serum albumin was measured with the fluorometric assay procedure (8).

Dissolution Tests—Dissolution rates of ampicillin and hetacillin from capsules were determined by a method similar to that of Levy and Hayes (14). The dissolution medium was 1000 ml. of 0.1 *N* HCl maintained at 37°. The capsule was restrained near the bottom of the beaker by means of an aluminum screen. The concentration of ampicillin or hetacillin in Millipore-filtered dissolution medium was measured at 5–10-min. intervals by spectrophotometric analysis at 258 nm.

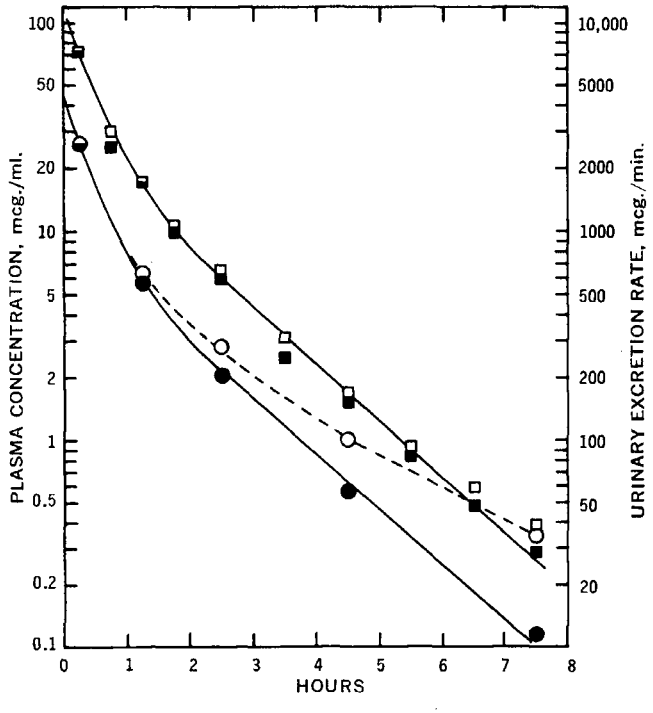


Figure 1—*Plasma concentrations (●,○) and urinary excretion rates (■,□) of ampicillin as a function of time after intravenous administration of 570 mg. to Subject 5. Closed symbols are results of microbiological assay, and open symbols are measurements by fluorometry. The solid lines represent a least-squares fit of the microbiological data according to Eqs. 1 and 2.*

RESULTS

Ampicillin Disposition—Typical plasma concentration and urinary excretion rate data obtained after intravenous injection of ampicillin are shown in Fig. 1. The data obtained by microbiological assay represent unchanged ampicillin, while the fluorometric procedure also measures a metabolite in plasma (8). The two assays produced essentially identical results when ampicillin was analyzed in urine, which indicates that little of the apparent metabolite is excreted by the kidneys. Similar assay differences which reflect the presence of the metabolite were found whether ampicillin or hetacillin was administered. The urinary recovery of unchanged ampicillin averaged 90 ± 7.5% after intravenous administration, which indicates that only a minor portion of the dose was eliminated by metabolism and/or biliary excretion.

The decline in plasma concentrations (C_p) and urinary excretion rates (ER) of unchanged ampicillin as a function of time (t) after intravenous injection is curvilinear, as shown in Fig. 1. The experimental data were fit to the biexponential equations:

$$C_p = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} \qquad \text{(Eq. 1)}$$

and:

$$ER = Cl_R(A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}) \qquad \text{(Eq. 2)}$$

where A and B represent zero-time intercepts on the ordinate, α and β are disposition slope constants, and Cl_R is the renal clearance. The data were fit to these equations simultaneously by digital computer least-squares iteration using a time-share adaptation of the program NONLIN (15). The data employed were the microbiologically assayed plasma concentrations of ampicillin and the average of the microbiological and fluorometric assay results for the urinary excretion rates. After the plasma concentration data were included twice to compensate for their fewer number but greater intrinsic reliability, all data values were weighted numerically equal. Within the computer program, this involves multiplying the squared deviations: $(Y_{observed} - Y_{calculated})^2$ by a weight factor, which was calculated for individual data values as: weight =

Table II—Biexponential Least-Squares Regression Parameters of Total Plasma Antibiotic after Intravenous Injection of Ampicillin and Hetacillin

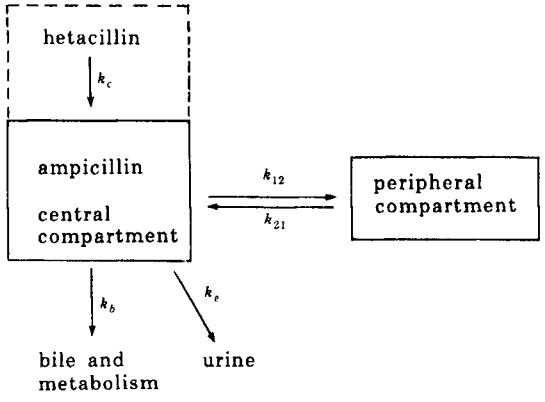
Parameter[a]	Ampicillin (*SD*)	Ampicillin with Hetacillin (*SD*)[b]
A, mcg./ml.	33.7 (7.9)	35.4 (9.1)
α, hr.$^{-1}$	2.30 (0.60)	1.81 (0.35)
B, mcg./ml.	4.04 (1.78)	7.66 (4.56)
β, hr.$^{-1}$	0.541 (0.048)	0.501 (0.090)
Cl_R, ml./min.	341 (91)	224 (50)
area, mcg. hr./ml.	22.7 (5.2)	34.7 (10.4)

[a] According to Eqs. 1 and 2 with *A*, *B*, Cl_R, and area normalized for a 500-mg. dose of ampicillin and 1.73 m.^{2} body surface area. [b] For curve-fitting purposes only.

($Y_{mean}/Y_{observed}$). The use of Eqs. 1 and 2 together permits all of the experimental data to be used simultaneously in calculation of the least-squares parameters. The averages of the latter values, as well as the plasma level area, are listed in Table II.

Hetacillin Disposition—Plasma concentration and urinary excretion rate data obtained after intravenous injection of both ampicillin and hetacillin are shown in Fig. 2. The disappearance of unchanged hetacillin was extremely rapid in both subjects, and the half-life of hetacillin averaged about 11 min. in the eight subjects. Hetacillin could be detected only in the first two plasma samples due to the limited sensitivity of the bioautography procedure. Figure 2 also shows the plasma concentrations of total antibiotic (ampicillin + hetacillin) after injection of both compounds. If hetacillin is not separated from ampicillin prior to microbiological or fluorometric analysis, the plasma antibiotic levels after hetacillin injection appear to exceed those obtained after ampicillin. For the purpose of estimating the total plasma area and obtaining a general impression of the behavior of both compounds after hetacillin injection, the total antibiotic data were also fit with Eqs. 1 and 2. These results are shown in Table II along with the intravenous ampicillin data for comparative purposes. It can be noted that the apparent β-values are very similar after administration of both ampicillin and hetacillin, but the apparent renal clearances are much less after hetacillin. The latter is due to the elevation of ampicillin plasma levels by the inclusion of hetacillin and suggests that little unchanged hetacillin appears in the urine.

Pharmacokinetic Analysis—The general pharmacokinetic model used to describe the distribution and elimination behavior of ampicillin after intravenous injection and with hydrolysis from hetacillin is shown in Scheme II. The rate constant for hydrolysis of hetacillin to ampicillin is k_c. The distribution rate constants of ampicillin are k_{12} and k_{21}, and elimination of ampicillin (k_{el}) occurs by renal (k_e) and biliary metabolic (k_b) pathways. These distribution and elimination constants for ampicillin were calculated from the least-squares



Scheme II—Multiple-compartment pharmacokinetic model used to characterize hetacillin conversion to ampicillin (k_c), *ampicillin distribution* (k_{12}, k_{21}), *and ampicillin renal* (k_e) *and extrarenal* (k_b) *elimination*

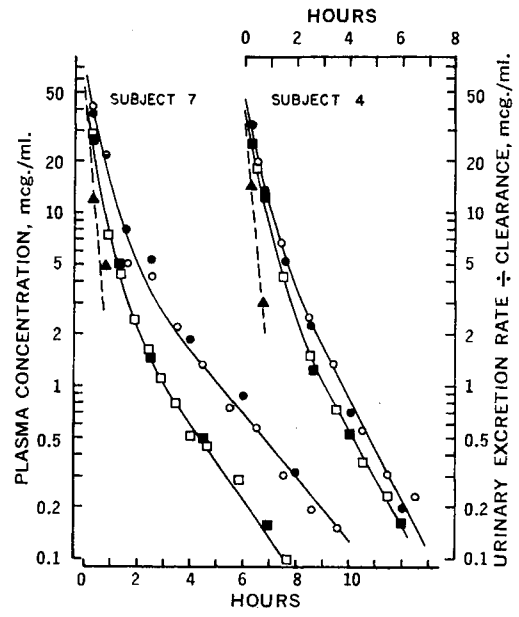


Figure 2—*Plasma concentrations of total apparent ampicillin as a function of time after intravenous administration of ampicillin* (■,□) *and hetacillin* (●,○) *to Subjects 7 and 4. Triangles and dashed lines show disappearance of unchanged hetacillin. The data are normalized for a 500-mg. dose of ampicillin. Solid symbols are actual plasma concentrations, and open symbols are urinary excretion rates divided by the least-squares renal clearance. Solid lines were obtained by computer least-squares regression according to Eqs. 1 and 2.*

parameters of Eqs. 1 and 2 using methods described previously (16–19), except that k_e and k_b were calculated from:

$$k_e = f_e \cdot k_{el} \qquad \text{(Eq. 3)}$$

and:

$$k_b = (1 - f_e) \cdot k_{el} \qquad \text{(Eq. 4)}$$

where f_e is the fraction of the intravenous dose (D_o) excreted in the urine unchanged. Other conventional parameters of a two-compartment open model (16–19) that were also calculated included the central compartment volume (V_c), steady-state distribution volume (V_{DSS}), and body clearance (Cl_B). The data obtained after hetacillin injection were quantitated with a more direct approach. A general solution to the model shown in Scheme II was generated using the methods recently proposed by Benet (18). The expression used to describe the time course of unchanged hetacillin (C_H) in the plasma was:

$$C_H = \frac{D_o}{V_c} \cdot e^{-k_c \cdot t} \qquad \text{(Eq. 5)}$$

Simultaneously, the time course of ampicillin concentrations (C_A) in plasma can be described by:

$$C_A = \frac{k_c \cdot (k_{21} - \alpha) \cdot D_o}{(\beta - \alpha) \cdot (k_c - \alpha)} \cdot e^{-\alpha \cdot t} + \frac{k_c \cdot (k_{21} - \beta) \cdot D_o}{(\alpha - \beta) \cdot (k_c - \beta)} \cdot e^{-\beta \cdot t} + \frac{k_c \cdot (k_{21} - k_c)}{(\alpha - k_c) \cdot (\beta - k_c)} \cdot e^{-k_c \cdot t} \qquad \text{(Eq. 6)}$$

where:

$$\alpha = \tfrac{1}{2} \cdot (b + \sqrt{b^2 - 4 \cdot k_{el} \cdot k_{21}}) \qquad \text{(Eq. 7)}$$

and:

$$\beta = \tfrac{1}{2} \cdot (b - \sqrt{b^2 - 4 \cdot k_{el} \cdot k_{21}}) \qquad \text{(Eq. 8)}$$

where $b = k_{12} + k_{21} + k_{el}$.

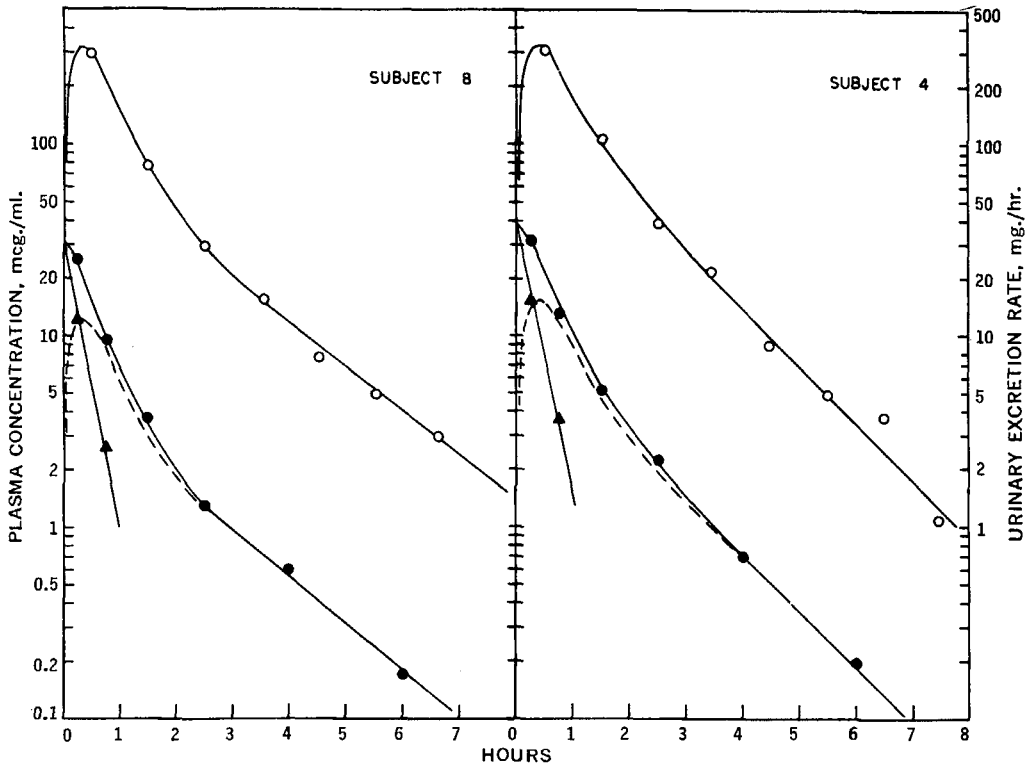


Figure 3—*Plasma concentrations of hetacillin* (▲), *ampicillin* (- - -), *and total antibiotic* (●) *as a function of time after intravenous administration of hetacillin to Subjects 4 and 8. The open circles are urinary excretion rates, and data are normalized for a 500-mg. dose. All lines were obtained by computer least-squares regression with the following constants:*

Subject	V_c, *l.*	k_e, *hr.*$^{-1}$	k_{12}, *hr.*$^{-1}$	k_{21}, *hr.*$^{-1}$	k_{el}, *hr.*$^{-1}$	Cl_R, *l./hr.*
4	*13.3*	*3.18*	*0.441*	*1.00*	*1.81*	*21.4*
8	*16.5*	*3.23*	*0.405*	*0.704*	*2.05*	*26.5*

The type of data fit by computer least-square iteration is shown in Fig. 3. Plasma levels of unchanged hetacillin were described with Eq. 5, while plasma levels of total antibiotic (C_T) were equal to:

$$C_T = C_H + C_A \qquad \text{(Eq. 9)}$$

where C_H and C_A were used as defined by Eqs. 5 and 6, respectively. Also, urinary excretion rates (ER_A) of unchanged ampicillin were quantitated with the enlargement of the expression:

$$ER_A = Cl_R \cdot C_A \qquad \text{(Eq. 10)}$$

where Eq. 6 was substituted into Eq. 10 for C_A. In essence, three detailed equations were used simultaneously to characterize hetacillin hydrolysis to ampicillin, ampicillin distribution, and ampicillin elimination, where the experimental data were C_H, C_T, and ER_A. However, the model was simplified by setting the distribution volume of hetacillin identical to that of ampicillin so that V_c was used as the same parameter for both compounds. The one-compartment assumption for hetacillin distribution was made because the hydrolysis of hetacillin to ampicillin was so rapid that hetacillin distribution rates could not be adequately characterized, because initial graphical estimation of V_c for both compounds yielded identical values, and because of the limited number of data points for hetacillin. The six least-squares constants thus generated from the hetacillin-ampicillin data were: V_c, k_e, k_{12}, k_{21}, k_{el}, and Cl_R. The excellent fit of the experimental data from two subjects using this approach is demonstrated by the results shown in Fig. 3. After these least-squares constants were obtained, the plasma levels of ampicillin were calculated for each subject using Eq. 6. The maximum level of ampicillin was attained at about 30 min. At a later time, ampicillin levels essentially converged with those of total antibiotic when most of the hetacillin was converted to ampicillin.

The computer-derived distribution and elimination constants of ampicillin that were obtained after injection of hetacillin are listed in Table III along with the parameters obtained after intravenous ampicillin. The plasma level area of ampicillin formed from hetacillin ($area_A$) was calculated from the integral of Eq. 6 or:

$$\text{area}_A = \int_0^\infty C_A \cdot dt \qquad \text{(Eq. 11)}$$

The method of paired comparisons was used to evaluate statistically the effect of the two methods of ampicillin administration on its distribution and elimination parameters. As shown by the mean data values for the eight subjects in Table III, the use of hetacillin produced essentially no change in the distribution volumes, clearances, distribution rate constants, renal excretion fractions, and plasma level areas of ampicillin.

Integral Coefficients—The use of integral coefficients to summarize the interaction of the major factors that control body levels of drugs, including protein binding, was recently introduced (19). An integral coefficient represents the fractional dose–time area for nonprotein-bound drug located in each pharmacokinetic compartment. For ampicillin, the integral coefficients for the central (D_{1a}) and peripheral (D_2) compartments are defined as:

$$D_{1a} = \frac{\int_0^\infty X_1^* \cdot dt}{D_o} = \frac{1}{k_{el}} \cdot \left(1 - \frac{V_p \cdot F_b}{V_c}\right) \qquad \text{(Eq. 12)}$$

and:

$$D_2 = \frac{\int_0^\infty X_2 \cdot dt}{D_o} = \frac{k_{12}}{k_{21} \cdot k_{el}} \qquad \text{(Eq. 13)}$$

where X_1^* is the amount of free drug in Compartment 1, V_p is the actual plasma volume, and F_b is the mean fractional binding of the

Table III—Distribution and Elimination Parameters of the Two-Compartment Open Model for Ampicillin

Parameter[a]	Intravenous Ampicillin (*SD*)	Ampicillin from Hetacillin (*SD*)
Distribution volumes, l.		
V_c	12.0 (1.9)	12.5 (2.8)
V_D^{ss}	17.9 (1.5)	19.3 (2.9)
Clearances, ml./min.		
Cl_R	341 (91)	296 (91)
Cl_B	335 (56)	350 (102)
Rate constants, hr.$^{-1}$		
k_{12}	0.384 (0.185)	0.419 (0.180)
k_{21}	0.733 (0.163)	0.728 (0.161)
k_{el}	1.73 (0.49)	1.68 (0.30)
k_e	1.55 (0.47)	1.58 (0.31)
k_b	0.17 (0.12)	0.10 (0.13)
Slow $t_{1/2}$, hr.	1.29 (0.11)	1.34 (0.20)
Fraction (f_e) excreted in urine	0.899 (0.075)	0.939 (0.076)
Plasma level area, mcg. hr. ml.$^{-1}$	22.7 (5.2)	22.9 (7.6)
Integral coefficients, hr.		
D_{1a}	0.581 (0.133)	0.581 (0.118)
D_2	0.297 (0.081)	0.355 (0.102)
D_T	0.878 (0.151)	0.921 (0.141)

[a] V_c, V_D^{ss}, Cl_B, Cl_R, and area are normalized for 1.73 m.^{2} body surface area.

drug to plasma proteins. The plasma volume was estimated by the method of Dagher (20), using the age and body weight of each subject. Over the concentration range of 1–10 mcg./ml., the fractional binding of ampicillin to 4 g.% human serum albumin was 0.29 ± 0.01, which yielded an association constant (k) of 704 l./mole, assuming one ampicillin binding site per protein molecule. This constant, along with the plasma albumin concentration (P) of each subject, was used to estimate F_b according to (13):

$$F_b = \frac{k \cdot P}{1 + k \cdot P} \qquad \text{(Eq. 14)}$$

A summary of the central and peripheral compartment integral coefficients for the two studies in each of the eight subjects is shown in Fig. 4. The total integral coefficient (D_T), which is equal to:

$$D_T = D_{1a} + D_2 \qquad \text{(Eq. 15)}$$

is a model-independent parameter which best reflects the overall fractional dose-time area between subjects and among various drugs (19). As shown in Table III and Fig. 4, these were essentially identical for ampicillin (0.878 hr.) and ampicillin formed from hetacillin (0.921 hr.). These values are also very similar to the total integral coefficient previously generated for ampicillin (1.01 ± 0.07 hr.) based on data of Jusko *et al.* (19) and Dittert *et al.* (21). The peripheral compartment integral contributes, on the average, about one-third of the whole body integral. There was greater variation in both D_{1a} and D_2 among subjects than within each subject for the studies with ampicillin and hetacillin. This was primarily due to the intrasubject variation in ampicillin elimination which, as can be noted from Eqs. 12 and 13, is the major factor controlling the value of both integral coefficients.

Rate of Hetacillin Hydrolysis—The least-squares rate constant for the conversion of hetacillin to ampicillin (k_c) was relatively constant among the eight subjects, with an arithmetic mean of 3.83 hr.$^{-1}$ and a standard deviation of 0.78 hr.$^{-1}$. The range of half-lives for this process was 8–13 min., with an average of 11.2 min. The narrow range of k_c values is consistent with a chemical, rather than biochemical, mechanism of hetacillin hydrolysis (1, 2). The listed rate constants for hetacillin degradation are probably slight overestimates because of hydrolysis occurring during the centrifugation process after collection of the blood samples. The blood samples were cooled during centrifugation to minimize such an effect.

Absorption Properties—The time course of plasma concentrations of ampicillin in a representative subject after administration of ampicillin and hetacillin during fasting (both compounds) and following ingestion of food (hetacillin) is shown in Fig. 5. Electrophoresis of the early plasma samples revealed no detectable hetacillin when it was given. The rapid absorption and apparent monoexponential decline of plasma levels of ampicillin made it difficult to characterize the data using a two-compartment open model, even by applying computer least-squares techniques. The plasma concentration (C_p) and urinary excretion rate (ER) data were, therefore, analyzed semiempirically by appropriate use of a zero-order input function for a one-compartment open model (18) where:

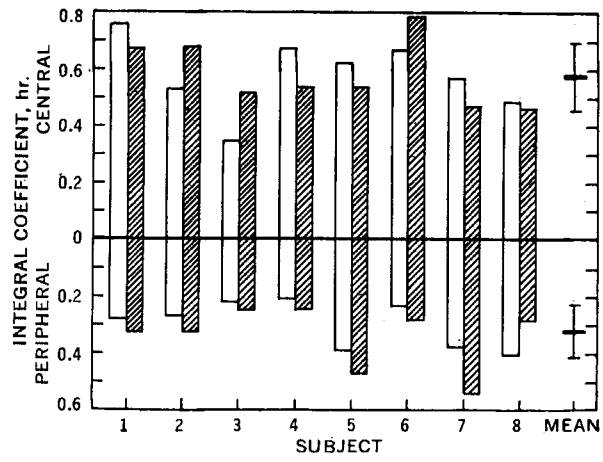


Figure 4—*Integral coefficients of ampicillin in the central and peripheral compartments after administration of ampicillin (□) and hetacillin (▨) to eight normal subjects. Standard deviations are shown as vertical lines with the mean values.*

$$C_p = \frac{R_a}{\delta} \cdot (e^{\delta \cdot t_o} - e^{\delta \cdot t_d}) \cdot e^{-\delta \cdot t} \qquad \text{(Eq. 16)}$$

and:

$$ER = C_p \cdot Cl_R \qquad \text{(Eq. 17)}$$

with the conditions that $t_o = t$ at $t < t_o$ and $t_d = t$ at $t_d < t$ and where R_a is a relative absorption rate constant that includes a volume of

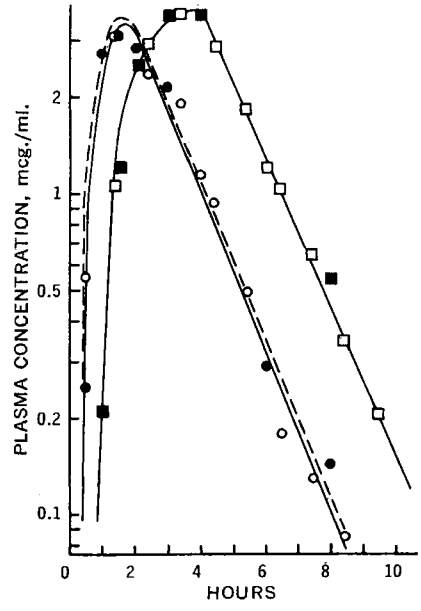


Figure 5—*Plasma concentrations of ampicillin in Subject 1 as a function of time after oral administration of ampicillin after fasting (●,○), hetacillin with food (■,□), and hetacillin after fasting (- - -, no symbols). Ampicillin data are corrected to the 483-mg. dose of hetacillin. Solid symbols are actual plasma concentrations, and open symbols are urinary excretion rates divided by the least-squares renal clearance. All lines were obtained by computer least-squares regression according to Eqs. 16 and 17.*

Table IV—Comparison of Ampicillin and Hetacillin Absorption Parameters[a] after Ingestion of Oral Capsules

Parameter[b]	Ampicillin Fasting (*SD*)	Hetacillin Fasting (*SD*)	Hetacillin With Food (*SD*)
Dose[c], mg.	550	483	483
Onset of absorption (t_o), hr.	0.32 (0.08)	0.65 (0.09)	0.64 (0.12)
Duration of absorption (t_d), hr.	1.70 (0.89)	1.74 (0.59)	2.22 (0.66)
Relative absorption rate (R_a), mg./hr. l.	4.05 (1.97)	3.45 (0.77)	2.99 (0.51)
Disposition half-life, 0.693/δ, hr.	1.08 (0.13)	1.08 (0.15)	1.12 (0.20)
Renal clearance, ml./min.	298 (71)	337 (59)	328 (115)
Plasma level area, mcg. hr./ml.	8.73 (1.43)	8.89 (1.79)	10.53 (3.71)

[a] Least-squares parameters calculated with Eqs. 16 and 17. [b] Mean and standard deviation for four female subjects. [c] Ampicillin equivalent.

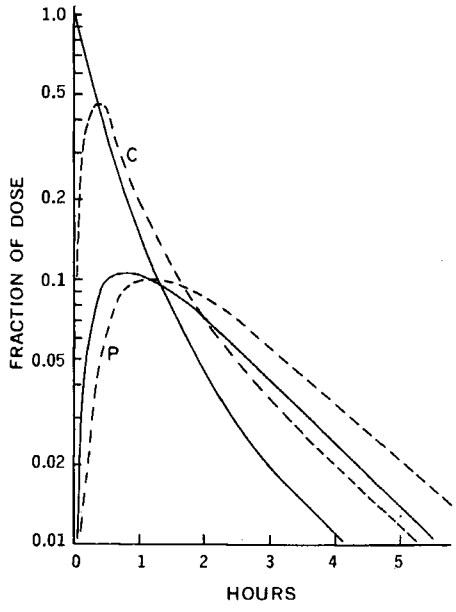


Figure 6—*Mean fractional dosage levels (semilogarithmic scale) of ampicillin in the central (C) and peripheral (P) compartments as a function of time after intravenous administration of ampicillin* (——) *and hetacillin* (- - - -). *The linear areas under the respective curves are the approximate integral coefficients.*

distribution, δ is the disposition rate constant, t_d is the duration of the absorption process, and t_o is the lag time before absorption starts. This function is useful primarily for curve fitting, comparison of crossover data, and numerical estimation of plasma level areas (trapezoidal rule). The plasma concentration and urinary excretion data were used simultaneously with the weighting method described previously to obtain least-squares estimates of the parameters: R_a, δ, t_d, t_o, and Cl_R.

As shown in Fig. 5, an excellent fit of the experimental data was obtained by using Eqs. 16 and 17. The average values of the least-squares parameters are listed in Table IV for the four female subjects. The t_o values show that the onset of absorption of hetacillin is slightly delayed relative to ampicillin. When food is coadministered with hetacillin, the onset of absorption is unaffected, but the duration of absorption is increased by about 0.5 hr. Partially offsetting this effect, food also produces an apparent decrease in the relative absorption rate of hetacillin. The apparent disposition half-life of oral ampicillin is slightly less than that found after intravenous ampicillin or hetacillin. As will be considered further, the plasma level areas listed in Table IV indicate that ampicillin is somewhat better absorbed when administered as hetacillin than as ampicillin.

Bioavailability—Since both plasma level areas and urinary excretion data were obtained, the bioavailability of ampicillin was calculated using two methods. The urinary recovery ratios after oral (po) and parenteral (iv) doses were used to calculate the fractional absorption (F_U) according to:

$$F_U = \frac{\%\ \text{recovery}_{po}}{\%\ \text{recovery}_{iv}} \qquad \text{(Eq. 18)}$$

The plasma level areas were similarly used to calculate bioavailability (F_A) from:

$$F_A = \frac{\text{area}_{po} \cdot \text{dose}_{iv}}{\text{area}_{iv} \cdot \text{dose}_{po}} \qquad \text{(Eq. 19)}$$

In both equations, the average results from the studies with intravenous ampicillin and ampicillin formed from hetacillin were used as denominator data for each subject. Numerator data were obtained by the measurement of total antibiotic in plasma or urine after oral doses of the two compounds. The bioavailability estimates are summarized in Table V for all studies performed. In general, there is good agreement between the F_U and F_A values, with an average difference of 1.7% absorption. The fact that the F_A values do not exceed the F_U values is in agreement with the electrophoretic absence of hetacillin in the plasma. If hetacillin itself was absorbed unchanged, the use of total antibiotic measurements would elevate the F_A estimate relative to the F_U value due to the addition of hetacillin plasma concentrations to those of ampicillin.

The average bioavailability estimates from the crossover studies in the female subjects can be used for comparison of the relative absorption of ampicillin and hetacillin. These data suggest that ampicillin is better absorbed when given as hetacillin (38%) than as ampicillin (29%). Coadministration of food with hetacillin appears to enhance ampicillin absorption slightly (42%). The individual data values are somewhat variable, and Subject 4 showed essentially constant absorption of ampicillin in the three studies. This tended to offset the increases in absorption found with the other three female subjects. No difference can be seen in the limited data reflecting bioavailability in male *versus* female subjects, and overall the eight subjects absorbed an average of 32 ± 8% of the dose of ampicillin.

Dissolution Rates—The small differences in absorption from ampicillin and hetacillin capsules may be caused partly by dissolution rate differences. The time for 50% dissolution of ampicillin in 0.1 *N* HCl was 13.4 ± 0.4 min., while that of hetacillin was 19.3 ± 2.0 min. (five and three determinations, respectively). This may account for the slight delay in onset of hetacillin absorption compared to that of ampicillin (Table IV).

Table V—Bioavailability of Ampicillin and Hetacillin from Gelatin Capsules[a]

Subject	Ampicillin: Fasting F_U	Ampicillin: Fasting F_A	Hetacillin: Fasting F_U	Hetacillin: Fasting F_A	Hetacillin: With Food F_U	Hetacillin: With Food F_A	Subject	Ampicillin: Fasting F_U
1	0.320	0.303	0.396	0.318	0.516	0.476	5	0.281
2	0.235	0.274	0.468	0.442	0.485	0.510	6	0.210
3	0.306	0.317	0.433	0.382	0.518	0.394	7	0.405
4	0.306	0.285	0.299	0.289	0.195	0.281	8	0.462
Mean	0.293		0.378		0.422		5–8	0.340
							1–8	0.316

[a] Calculation methods: F_U = urinary recovery ratio (Eq. 18), and F_A = plasma area ratio (Eq. 19).

DISCUSSION

Ampicillin Elimination—The elimination of ampicillin from the body primarily involves renal excretion, since about 90% of an intravenous dose of ampicillin or hetacillin appears in the urine unchanged. A similar value was found previously by Dittert *et al.* (21) in normal subjects. The remaining portion of the dose is eliminated by metabolism and biliary excretion. The occurrence of biliary excretion of ampicillin in man was confirmed by Mortimer *et al.* (22), who detected the antibiotic in the bile of patients. Although the fraction of the dose excreted in the bile was not determined, enterohepatic cycling of a small amount of ampicillin is thus possible.

The susceptibility of ampicillin to *in vivo* metabolism or degradation was demonstrated in the rat by Kind *et al.* (23) using ^{35}S-labeled ampicillin, but the apparent metabolite of ampicillin in man has not been identified. It was previously suggested on the basis of indirect evidence (8) that α-aminobenzylpenicilloic acid could be the degradation product. β-Lactamase enzymes are normally present in bacteria of the GI tract (24), and at least three other penicillins are known to yield their penicilloic acid derivatives in urine (25). However, the time course of plasma metabolite levels shown in Fig. 1 indicates that the apparent metabolite is eliminated more slowly than is ampicillin. Since the degree of human serum albumin (4 g.%) binding of α-aminobenzylpenicilloic acid was found to be only 9 ± 2%, slow elimination caused by strong protein binding is ruled out. Additional studies (unpublished) in rats have shown that ampicillin and α-aminobenzylpenicilloic acid have very similar elimination half-lives, and it therefore appears unlikely that the latter is the metabolite. Studies of the transformation product of ampicillin in the rat were carried out recently by Nishida *et al.* (26). In agreement with the results of the present study, the transformation product was found in greatest relative concentration in the serum of the rat and was retained in the body longer than ampicillin. Further identification of this metabolite would be of interest since its slow elimination may lead to accumulation during multiple dosing.

Hetacillin Elimination—Since hetacillin itself is not bactericidal, an essential consideration in its clinical use is whether all of the dose is converted to ampicillin *in vivo*. Because of its rapid rate of hydrolysis, it was not feasible to assay unchanged hetacillin in the urine. However, the evidence indicates that most, if not all, of the hetacillin is converted to ampicillin in the central compartment. Extrarenal elimination of appreciable hetacillin is ruled out by the urinary recovery of the same fraction of the dose whether ampicillin or hetacillin is injected (Table III). Negligible renal excretion of hetacillin is suggested by the renal clearances of total antibiotic (ampicillin + hetacillin), which are substantially less than renal clearances of ampicillin alone (Table II). The strongest evidence is the excellent fit of the urinary excretion rate data to the pharmacokinetic model when it was assumed that renal excretion of only ampicillin occurs (Scheme II and Fig. 3). If renal excretion of both compounds occurred, then it would not be possible to fit both the early and the late urinary excretion rate data with the devised pharmacokinetic model.

The rate at which hetacillin converts to ampicillin is somewhat greater *in vivo* than has been found *in vitro*. A conversion half-life for hetacillin hydrolysis *in vitro* has been found to be about 25 min. both in pH 7 buffered solution (1) and in plasma (2) at 37°. At 4°, the half-life in plasma increases to more than 4 hr. and thus freezing freshly drawn plasma samples stabilizes hetacillin somewhat (2). The *in vivo* half-life estimates for hetacillin conversion found in the present study (average of 11.2 min.) are more rapid than, but consistent with, the previously determined *in vitro* values. This suggests that the *in vivo* mechanism for hetacillin conversion to ampicillin does not necessarily require the presence of drug-metabolizing enzymes.

Effect of Hetacillin on Ampicillin Pharmacokinetics—The distribution and elimination parameters of ampicillin formed from hetacillin were essentially identical to those for intravenous ampicillin (Table III). The integral coefficients of the antibiotic were not changed (Fig. 4), and all of the dose of hetacillin appeared to be converted *in vivo* to ampicillin. Based on these characteristics, the use of equimolar parenteral doses of ampicillin and hetacillin should be chemotherapeutically equivalent (19). This, in fact, appears to be the case since clinical studies with the two compounds have produced essentially identical results (3, 27).

The primary effect of parenteral injection of hetacillin, therefore, is to alter slightly the time course of the central and peripheral compartment levels of ampicillin. The average values of the constants (Table III) were used with appropriate equations (18) to obtain the fractional dose levels shown in Fig. 6 for each compartment after injection of the two compounds. It can be seen that intravenous injection of hetacillin is analogous to administration of ampicillin by a very rapid absorption process. Maximum levels of ampicillin in the central compartment are reached at about 30 min. after hetacillin injection. The time course of ampicillin in the peripheral compartment is similar after both compounds, except that occurrence of the peak level is delayed by about 30 min. after hetacillin is given. As shown previously by the individual values of the integral coefficients, the linear areas under the curves in the corresponding compartments do not differ significantly. The areas for the central compartment in the graph are slightly greater than the calculated $D_{1\alpha}$ values because graphical correction is not made for the small fraction of protein-bound ampicillin (Eq. 12).

Claims were made in earlier investigations (6, 7) that parenteral hetacillin produces an enhancement of ampicillin plasma levels by virtue of a decreased renal clearance. However, as shown by the data in Fig. 2 and Table II, this conclusion can be reached by misinterpretation of the results of nonspecifically assaying hetacillin along with ampicillin. When ampicillin and hetacillin are separated electrophoretically prior to the prolonged microbiological incubation, this technical artifact is resolved and the behavior of hetacillin and ampicillin formed from hetacillin can be described as shown in Fig. 3. Similar precaution must be made in pharmacokinetic evaluation of other ampicillin precursors when the commonly employed microbiological assay is involved.

Absorption of Ampicillin and Hetacillin—An average of 32% (ranging from 21 to 46%) of the dose of ampicillin was absorbed from the GI tract by the subjects of the present study. Although the data are limited due to the small number of subjects, there does not seem to be any difference in ampicillin absorption between the male and female subjects. This finding agrees with data of Poole (28) who found that in beagle dogs the amphoteric penicillins, unlike others such as dicloxacillin and nafcillin, do not exhibit sex differences in intestinal absorption.

The rapid rate and only partial absorption of ampicillin made pharmacokinetic characterization of its absorption properties difficult. The limited number of data points did not permit direct utilization of a two-compartment model, while the intrasubject variation in distribution parameters (Table III and Fig. 4) precluded accurate use of the methods of Loo and Riegelman (29). The day-to-day change in elimination also contributed to variation in plasma level areas, which probably caused the differences in bioavailability estimates when comparing the results from the two methods of calculation (Table V). The zero-order input function for a one-compartment model was, therefore, chosen primarily for curve-fitting purposes and to evaluate differences in the general time course of absorption. However, the intestinal absorption of many penicillins does not occur by simple diffusion (30) and the zero-order input function is thus partly consistent with a specialized absorption process.

The results of the present study suggest that ampicillin absorption is somewhat better when the antibiotic is administered as hetacillin. Of practical importance, the presence of food appeared to enhance absorption of the antibiotic in three of the four subjects. This finding agrees with the results of Sutherland and Robinson (4), who found that the 6-hr. urinary recovery of ampicillin after a 500-mg. dose of hetacillin increased from 32 to 48% of the dose in fasting and nonfasting subjects, respectively.

The literature concerning the comparative absorption properties of hetacillin and ampicillin has been conflicting. Sutherland and Robinson (4) as well as Modr and Dvoracek (7) found greater urinary recovery of the antibiotic after giving oral ampicillin in comparison with oral hetacillin. Another study by Magni *et al.* (1) showed plasma level areas and urinary recoveries of ampicillin to be almost twice as great after ampicillin capsules as compared to hetacillin capsules. On the other hand, two additional studies demonstrated similar absorption of the antibiotic from ampicillin and hetacillin capsules. Kirby and Kind (31) found essentially equal plasma level areas of ampicillin after oral doses of ampicillin or hetacillin, as did Smith and Hamilton-Miller (3). It thus appears that the comparison of the relative degree of absorption from ampicillin and hetacillin capsules depends on the particular investigation and the commercial products employed.

Several additional factors may account for differences in ampi-

cillin bioavailability among subjects and commercial products. Intersubject variability (32), the age of the subjects (33), the presence of liver disease (34), and the hydrate (35) and salt forms (36) of the antibiotic are several factors shown to affect ampicillin absorption. In general, the bioavailability of different pharmaceutical formulations of ampicillin should be considered on an individual basis and variation of between 20 and 50% absorption can be expected among subjects and commercial products.

Design and Use of Ampicillin Precursors—The rationale for designing precursors to ampicillin is partially for enhancement of GI absorption of the antibiotic. Since only 20–50% of an oral dose of ampicillin is absorbed from the GI tract, the potential for improvement of absorption of the antibiotic is considerable. Hetacillin appears to offer a small improvement in absorption of ampicillin when compared with the ampicillin product tested in the present study.

The parenteral administration of an inactive precursor is not an *in vivo* therapeutic advantage since a maximum of an equimolar amount of active product can be produced and, if the conversion rate is not sufficiently rapid, there exists the possibility that elimination of the precursor will result in loss of part of the intended dose of antibiotic. This appears to occur to a substantial degree with metampicillin, a condensation product of ampicillin and formaldehyde, which is converted to ampicillin during renal excretion (37). On the other hand, if the precursor has some degree of antibiotic activity, then its use could considerably enhance the effectiveness of the dose of drug. This is of greatest potential importance for antibiotics such as ampicillin which are excreted very rapidly. A major pharmaceutical or *in vitro* advantage to use of parenteral precursors is that, like hetacillin, they may provide greater chemical stability in infusion solutions which are used over extended time periods. For example, using the criterion of loss of 10% of microbiological activity as an end-point, ampicillin is "stable" for only 1 hr. while hetacillin can be used over a period of 6 hr. in infusion solutions (38).

The assay procedure must be carefully considered in pharmacological evaluation of a precursor of an antibiotic. The nonspecific type of microbiological procedure which is commonly used for most antibiotics requires several hours of incubation of the drug with the microorganism (9). This can result in hydrolysis of the inactive precursor to active compound, and the assay procedure will reveal bactericidal activity *in vitro* that exceeds that actually present *in vivo*. Since hetacillin appears to be completely converted to ampicillin in the GI tract prior to absorption, reports of relative absorption of the two compounds where the nonspecific assay was used are probably accurate. This does not necessarily apply to other ampicillin precursors. A separation method of analysis, along with employment of the pharmacokinetic approach used in the present study, is necessary for elucidation of the actual chemotherapeutic value of antibiotic precursors.

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Misconceptions and Thermodynamic Untenability of Deviations from pH–Partition Hypothesis

VICTOR F. SMOLEN

Abstract □ Elementary thermodynamic principles are applied to the analysis of concepts associated with pH-partitioning of weakly electrolytic solutes across proton-impermeable barriers. Several common misconceptions concerning the permeability of ionized species as well as the postulated occurrence and significance of a "virtual pH" on biomembrane surfaces are demonstrated.

Keyphrases □ pH–partition hypothesis—thermodynamic discussion of deviations and misinterpretations □ Virtual pH—theoretical aspects of relevant thermodynamic principles, misconceptions concerning the pH–partition theory discussed □ Thermodynamics—application to pH–partition theory and concept of virtual pH, misconceptions discussed, equations

The theory of nonionic diffusion, also known as the pH–partition hypothesis, was described in quantitative terms by Jacobs (1) in 1940 and was widely applied by some researchers to explain the distribution and transport of drugs across biological membranes. The principles and results were reviewed by Schanker (2) and, more recently, by Brodie (3).

The purpose of the present report is the application of elementary thermodynamic principles to the clarification of certain points concerning the pH–partitioning of weak electrolytes across biological and other barriers capable of maintaining a pH gradient. It will be demonstrated that:

1. The thermodynamic partitioning equilibrium ratio of total solute concentrations in the bulk phases is identical and independent of whether or not the barrier is permeant to the ionized species. It depends only upon the magnitude of the pH gradient and the ionization constant of the solute; it is also entirely independent of the existence of any "virtual pH" at the surfaces of the barrier which may differ from the pH of their respective bulk phases.
2. Equilibrium pH-partitioning is thermodynamically identical to the Gibbs–Donnan equilibrium.
3. It is invalid to compute estimates of the virtual pH from thermodynamic equilibrium or steady-state partitioning ratios (3) of total concentrations of solute in the bulk phases.
4. Observed deviations of total bulk phase concentration ratios from predicted equilibrium values are thermodynamically untenable, irrespective of the permeability of the barriers to ions and attributable only to the occurrence of nonthermodynamic equilibrium partitioning conditions. This applies even if the bulk phases are each simultaneously maintained in steady states in which no net transfer of total solute occurs across the barrier.
5. The occurrence of a steady-state partitioning condition is inconsistent with an assumption of an equality in the concentrations of the unionized species in the bulk phases; any computations of virtual pH and unionized–ionized species permeability coefficient ratios that at any stage involve equations utilizing this assumption are erroneous.

The ability of both uncharged and ionic solute species to permeate biological barriers is well established with the observation that ionic solutes generally permeate at slower rates. Hydrogen ion, however, is unique in that biomembranes, such as the GI barrier, are capable of maintaining large pH gradients. In this laboratory a similar capability was observed for the maintenance of pH gradients by a synthetic membrane constructed of a highly polar polysalt complex containing approximately 70% water (4, 5) and another described by Levy and Mroczak (6) in which a lipid is definitely the barrier to transport. Kavanau (7) described a mechanism that can be responsible for such observed impermeability of biological and other membranes to hydrogen ions. He considers surfaces, such as those associated with living cells and tissues, to structure water; such water exists in the immediate vicinity of the surface due to hydration of fixed ions and hydrophobic hydration of nonpolar groups. At a distance further from the surface, he postulates that considerable structure breaking of the water occurs. In this region, hydrogen bonding between water molecules is poor relative to ordinary bulk water. This unstructured region is postulated to act as an insulator with regard to proton transfer between the membrane and bulk aqueous phases. Convincing evidence that the apparent lipoidal properties of biological barriers are, in fact, also primarily a consequence of their content of structured water (proteins and lipids are primarily responsible for the structuring) was described by Smolen and Hagman (4), Horowitz and Fenichel (8), and Ling[1] (9).

The obvious pharmaceutical and general biological significance of pH–partitioning with regard to the transfer and distribution of solutes emphasizes the importance of gaining a rigorous understanding of this phenomenon. Three cases will be considered: (*a*) equilibrium pH–partitioning across a barrier permeable to the unionized species of the weakly electrolytic solute (*b*) equilibrium partitioning across a barrier permeable to both unionized and ionized species, and (*c*) steady-state partitioning across a barrier permeable to both species.

THEORETICAL

Irrespective of the manner in which the total concentrations of solute and pH in bulk phases separated by a proton-impermeable membrane are maintained, an experimentally observed or assumed net zero transfer of total solute across the barrier is indicative of either a steady-state or thermodynamic partitioning equilibrium

[1] G. N. Ling, Molecular Biology Laboratory, Pennsylvania Hospital, Philadelphia, Pa., personal communication.

(10); a basis for the distinction between these two possible conditions will be discussed here.

The assumption of a thermodynamic partitioning equilibrium requires an equality of chemical potential, μ_i (or electrochemical potential, $\bar{\mu}_i$, for charged species) for each species in all phases into which it can enter. This includes the two membrane-separated bulk phases, I and II, as well as any interphase or transition phase, T, that may exist at the surfaces or within the membranous barrier, *i.e.*, $\mu_i^{I} = \mu_i^{T} = \mu_i^{II}$. When the barrier is impermeable to the ith species, it can only be stated that $\mu_i^{I} < \mu_i^{T} > \mu_i^{II}$; *i.e.*, the chemical or electrochemical potential of the species in the barrier from which the species is excluded is greater and obviously unequal to that in the two bulk phases. In this case, it is not necessary that $\mu_i^{I} = \mu_i^{II}$. However, if the species is in equilibrium with other solute species to which the barrier is permeant, it is necessary that the chemical potential of the solute treated as a component, c, be equal in every phase into which the species can enter; *i.e.*, $\mu_c^{I} = \mu_c^{T} = \mu_c^{II}$, irrespective of the nature of the intervening barriers through which it passes.

This can readily be exemplified for the weakly acidic electrolyte, $HA \rightleftharpoons A^- + H^+$. For the first case when the barrier is permeant only to HA species, Eq. 1 must be satisfied:

$$\mu_{HA}^{I} = \mu_{HA}^{II} \qquad \text{(Eq. 1)}$$

In each bulk phase, the following ionization equilibrium relations also hold:

$$\mu_{HA}^{I} = \mu_{A^-}^{I} + \mu_{H^+}^{I} \qquad \text{(Eq. 2)}$$

$$\mu_{HA}^{II} = \mu_{A^-}^{II} + \mu_{H^+}^{II} \qquad \text{(Eq. 3)}$$

The chemical potential of the solute treated as a component is the sum of the chemical potentials of the species:

$$\mu_A^{I} = \mu_{HA}^{I} + \mu_{A^-}^{I} + \mu_{H^+}^{I} \qquad \text{(Eq. 4)}$$

$$\mu_A^{II} = \mu_{HA}^{II} + \mu_{A^-}^{II} + \mu_{H^+}^{II} \qquad \text{(Eq. 5)}$$

It follows that:

$$\mu_A^{I} = \mu_A^{II} \qquad \text{(Eq. 6)}$$

By expanding Eq. 6 in terms of the activities [A], [HA], $[H^+]$ and $[A^-]$ and the standard chemical potentials, μ_i^0, it can be rewritten as Eqs. 7 and 8:

$$(\mu_A^0)_I + RT \ln [A]_I = (\mu_A^0)_{II} + RT \ln [A]_{II} \qquad \text{(Eq. 7)}$$

$$\mu_{HA}^0 + \mu_{A^-}^0 + \mu_{H^+}^0 + RT \ln [HA]_I[A^-]_I[H^+]_I = \mu_{HA}^0 + \mu_{A^-}^0 + \mu_{H^+}^0 + RT \ln [HA]_{II}[A^-]_{II}[H^+]_{II} \qquad \text{(Eq. 8)}$$

The often justifiable assumption that the bulk aqueous phases are similar enough in composition that the partition coefficient of each species distributed between them is unity permits the removal of the μ_i^0's from Eq. 8, which can then be rewritten as Eq. 9:

$$\frac{[A^-]_{II}[H^+]_{II}}{[A^-]_I[H^+]_I} = \frac{[HA]_I}{[HA]_{II}} \qquad \text{(Eq. 9)}$$

Setting $[HA]_I = [HA]_{II}$ in effect provides one with the pH–partition hypothesis (1–3, 10); simplifying the treatment by assuming, where convenient, that activities are equivalent to concentrations yields the ratio of the total concentrations of the weak electrolyte equilibrated across a hydrogen-ion and A^--ion-impermeable barrier, as given by the familiar Eqs. 10 and 11. Equation 11 is obtained by substituting $[A^-] = [HA]\,K_a/[H^+]$ into Eq. 10:

$$\frac{C_{I,\,total}}{C_{II,\,total}} = \frac{[HA]_I + [A^-]_I}{[HA]_{II} + [A^-]_{II}} \qquad \text{(Eq. 10)}$$

$$= \frac{(1 + (K_a/[H^+]_I)}{1 + (K_a/[H^+]_{II})} \qquad \text{(Eq. 11)}$$

It will be shown that the form of Eq. 11 is identical for Case (*b*) in which the barrier is assumed permeant to the ionized species, as well as the unionized form. For this case, thermodynamic equilibrium will, in addition to Eqs. 1 and 6, require an equality of electrochemical potentials, $\bar{\mu}_{A^-}$, of A^- in the bulk phases as expressed by Eq. 12(12):

$$\bar{\mu}_{A^-}^{I} = \bar{\mu}_{A^-}^{II} \qquad \text{(Eq. 12)}$$

Expanding each side of Eq. 12 into chemical and electrical contributions yields Eq. 13, in which ψ_I and ψ_{II} are the Galvani potentials of bulk phase I and II, respectively, and F is the Faraday constant:

$$\mu_{A^-}^{0}{}_I + RT \ln [A^-]_I - F(\psi_I) = \mu_{A^-}^{0}{}_{II} + RT \ln [A^-]_{II} - F(\psi_{II}) \qquad \text{(Eq. 13)}$$

Equating standard chemical potentials and rearranging Eq. 13 yield Eq. 14, which is identical to an expression relating the equilibrium distribution ratio of ions (distributed in accordance with an ideal Donnan equilibrium, *i.e.*, when $\Delta\mu_i^0 = 0$) to the Donnan potential, E_D, given by $E_D = \psi_I - \psi_{II}$:

$$\frac{RT}{F} \ln \frac{[A^-]_I}{[A^-]_{II}} = E_D \qquad \text{(Eq. 14)}$$

Use of Eqs. 1–3 provides Eq. 15, which can be expanded and rearranged to obtain Eqs. 16–18 where r is analogous to a Donnan ratio (11):

$$\mu_{A^-}^{I} + \mu_{H^+}^{I} = \mu_{A^-}^{II} + \mu_{H^+}^{II} \qquad \text{(Eq. 15)}$$

$$RT \ln \frac{[A^-]_I}{[A^-]_{II}} = RT \ln \frac{[H^+]_{II}}{[H^+]_I} \qquad \text{(Eq. 16)}$$

$$E_D = RT \ln r \qquad \text{(Eq. 17)}$$

$$r = \frac{[A^-]_I}{[A^-]_{II}} = \frac{[H^+]_{II}}{[H^+]_I} \qquad \text{(Eq. 18)}$$

The resemblance of Eqs. 14–18 for equilibrium pH–partitioning to identical expressions appropriate to the Gibbs–Donnan membrane equilibrium (11) is not fortuitous or unexpected when it is considered that the Gibbs–Donnan equilibrium is generally applicable to describing the distribution of mobile charged species in systems containing immobile, or relatively immobile, charges. In the present case, the immobilization of charge is a consequence of the existence of a pH gradient across a relatively H^+-ion-impermeable barrier. In compliance with this analysis, existence of electrical potentials across the intestine and the movement of anions down an electrochemical potential gradient were reported previously (12, 13).

Using Eq. 18 to express $[A^-]_I = [A^-]_{II}[H^+]_{II}/[H^+]_I$ and substituting this expression into Eq. 10 for $[A^-]_I$ yield Eq. 19:

$$\frac{C_{I,\,total}}{C_{II,\,total}} = \frac{[HA]_I + ([H^+]_I/[H^+]_{II})\,[A^-]_{II}}{[HA]_{II} + [A^-]_{II}} \qquad \text{(Eq. 19)}$$

Substituting $[A^-]_{II} = [HA]_I K_a/[H^+]_{II}$ for $[A^-]_{II}$ in Eq. 19 yields Eq. 11 upon cancellation of $[H^+]_I$ from the numerator and $[HA]_I = [HA]_{II}$ between the numerator and denominator. This result is not unexpected, since the derivation of Eq. 11 in the first place requires no assumption concerning the permeability of the barrier to unionized species.

DISCUSSION

It should be quite apparent from the analysis that the equilibrium total concentration ratio of solute predicted by Eq. 11 must always be realized irrespective of whether or not the barrier is permeable to the ionized species. Conversely, the observation of the predicted ratio does not justify any assumptions concerning the permeability of the barrier to either species.

Attributing deviations from predicted equilibrium total solute distributions to the permeability of the barrier to unionized species or to the virtual pH of the absorbing surface (3, 10) is thermodynamically untenable. It is quite conceivable that a virtual pH at the mucosal surface exists which is different from that of the bulk phase in the lumen. Its origin may, as suggested (3, 10), result from the secretion of H^+ ions by the mucosal cells. In addition, a Donnan accumulation or exclusion of H^+ due to the occurrence of fixed ionic sites on the tissue surface can be operative (14). The sign and density of the surface fixed charge and, therefore, its contribution to the virtual pH may be expected to vary with the bulk phase pH.

However, the implementation of an *in vivo* bioelectrometric method (*e.g.*, 14–16) in our laboratory has revealed the variation of the fixed charge density of the lower two-thirds of the duodenal mucosal surface of the rabbit to be reversible over a pH range of approximately 3.5–10.8 and quite anomalous relative to the observed behavior of less physiologically active surfaces studied previously (14–16). The pH dependency of the fixed charge density was found to be U-shaped, with extremes at pH. 3.5 and 10.8 of −28 and −52 meq./l., respectively, and a minimum at pH 7.0 of +1.7 meq./l. The virtual pH values attributable to the fixed charge for the bulk phase pH values of 3.5, 7.0, and 10.8 were calculated as 3.46, 7.02, and 10.72, respectively. These virtual pH values, as well as a value of 5.89 corresponding to a fixed charge density of 3.0 meq./l. at a bulk phase pH of 5.9, are not significantly different, for an ionic strength of bulk solution corresponding to isotonic 0.15 *M* NaCl, from the pH of the lumen. Based on these results the existence of a postulated virtual pH of 5.3, which is insensitive to the pH of the luminal solution (3, 10), would have to arise from another source such as the secretion of H^+ ions as suggested by Brodie (3) and Hogben *et al.* (10).

That this may, however, not be the case either was suggested by the results of preliminary experiments performed to measure the pH of the *in vivo* intestinal mucosal surface relative to the bulk solution, using a flat bulb pH electrode. Placing the electrode in a pool of isotonic phosphate buffered solution in the bulk phase at a distance from the surface provided a value of pH 7.0. Lowering the electrode against the surface yielded a value of 7.4. Assuming a verity of such measurements in reflecting the surface virtual pH, the relative alkalinity of the surface is contrary to the predictions of the pH-partition hypothesis but is qualitatively consistent with our observed net positive charge density for the mucosal surface at a bulk pH of 7.0. The details of these and further experiments will be presented in a subsequent report.

The above thermodynamic analysis has demonstrated that deviations, other than those attributable to complexation and nonideal solution behavior, in the expected total solute concentration ratio, as given by Eq. 11, are impossible for a true thermodynamic equilibrium state; the observation of a C_{total} ratio predicted by Eq. 11 is a necessary condition for its occurrence. It can be further surmised that the many observed deviations from Eq. 11 (17) must be the conseqeuences of nonthermodynamic equilibrium conditions despite an observed zero transfer of total solute between the bulk phases (10). This latter condition is a special case of nonthermodynamic equilibrium which has been described as a steady state (10). For the thermodynamic equilibrium state, the equality of chemical and electrochemical potentials of all permeant species in all phases requires that the total solute flux across the barrier, J_{TOT}, as well as the flux of each species, J_{A^-} and J_{HA}, are identically zero; *i.e.*, $J_{TOT} = J_{HA} = J_{A^-} = 0$. The steady-state partitioning condition is, according to Brodie (3) and Hogben *et al.* (10), characterized by a zero net flux of total solute, which is in this manner the same as the thermodynamic equilibrium state but differs in that the flux of the individual species is nonzero, equal in magnitude but opposite in direction. The steady-state flux conditions are expressed by Eqs. 20 and 21, which can be inferred from the relations given by Hogben *et al.* (10):

$$J_{TOT} = J_{A^-} + J_{HA} = 0 \qquad \text{(Eq. 20)}$$

$$J_{A^-} = -J_{HA} \neq 0 \qquad \text{(Eq. 21)}$$

It is important to note that the postulation of a steady state in which $J_{A^-} \neq 0$ requires a permeability of the barrier to one or more cations, which must be simultaneously transferred to maintain at all times the necessary condition of electroneutrality (18); *i.e.*, the condition $\Sigma_{i=1}^{n} Z_i J_{C_i^+} - J_{A^-} = 0$, where C_{i^+} and Z_i refer to the cations and their valence, respectively, must be satisfied for a transfer of A^- to occur. If the accompanying cations are other than H^+ (*e.g.*, Na^+, K^+, or organic), a change in the pH of unbuffered bulk phases is to be expected due to shifts in the $HA \rightleftharpoons A^- + H^+$ equilibrium. This phenomenon may contribute to the observed differences in the pH of the inflowing and effluxing fluids of intestinal perfusion streams (10) and pH changes noted in everted sac experiments (19); such observations may be simply interpretable as steady-state manifestations attributable to the relative impenetrability of the barrier to protons.

Equation 11 is derived on the assumption of $[HA]_I = [HA]_{II}$, which is correct under thermodynamic equilibrium conditions; but, as shown above, it is not the case for the steady state. (When HA is the only species present, the steady state and thermodynamic equilibrium conditions of net zero flux are identical.) The use of Eq. 11 to compute a virtual pH for a system in thermodynamic equilibrium is invalid, because the pH variables appearing in Eq. 11 refer only to the bulk phases. In addition, since the postulation of a thermodynamic equilibrium requires adherence to Eq. 11 in the first place, there would be no occasion to compute a virtual pH to account for theoretically nonexistent deviations. It should, therefore, be apparent that Eq. 11 does not apply to a steady state (*i.e.*, unless the steady-state and thermodynamic equilibrium partitioning states are the same) and its use to compute a virtual pH (4) is erroneous. The subsequent use of such virtual pH values to calculate unionized–ionized permeability coefficient ratios, p_u/p_i, propagates the error. This error may be surmised to contribute to the large discrepancy between the p_u/p_i values of 4500 reported by Hogben *et al.* (10) for salicylic acid and the value of 6 more correctly obtained by Nogami and Matsuzawa (19).

SUMMARY AND CONCLUSIONS

In brief, it can be concluded that although the pH-partition hypothesis may often provide a good approximation of the solute transport and distribution behavior of biological barriers and is an excellent rule of thumb, it cannot be considered so seriously as to postulate that biological barriers are generally impermeable to ions and to attribute observed deviations from its predictions to thermodynamically untenable mechanisms which invoke a virtual pH.

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Resistance of Large-Volume Parenteral Containers to Forced Microbial Contamination

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Abstract □ The hypothesis that contamination of large-volume parenteral containers may be due to improper handling technique was examined. Extensive studies were implemented involving carefully controlled conditions for both test (900) and control (450) units. Test containers were immersed in bacterial broth preparations in intact and "partially uncapped" conditions and were not swabbed; control containers were handled under "ideal" conditions except that they were not swabbed. A new administration set was used for each container examined and was opened only at the time of examination. Statistical evaluation of the results indicated that certain procedures may indeed result in contamination in many cases, whereas there was no significant contamination of any of the three systems tested when recommended procedures, even without swabbing, were followed.

Keyphrases □ Large-volume parenteral containers—resistance to forced microbial contamination □ Contamination, microbial—resistance of large-volume parenteral containers □ Sterility, large-volume parenteral containers—resistance to forced microbial contamination □ Parenteral containers, large volume—resistance to forced microbial contamination

The ability of large-volume parenterals and their companion administration sets to serve as sustaining and transmitting media of serious infection has been the subject of recent controversy. Various manufacturers have claimed that the proper use of their intravenous fluids and administration sets would forestall all contamination. However, several studies dealing with contamination of these fluids under conditions of normal use have raised questions both for and against such claims (1–5).

The purpose of this study was to determine whether large-volume parenteral containers (marketed by three different manufacturers[1]), used with their recommended administration sets, could resist bacterial contamination under adverse conditions.

EXPERIMENTAL

Systems Used—In this investigation, three types of containers were examined: A, nonfiltered air system, vacuum packed; B, filtered air system, vacuum packed; and C, open air system.

The administration set used for a particular container was designed by the manufacturer for that container. This condition was important since the study was designed to investigate microbial contamination of the intravenous liquid before it enters the venous system and since each set is designed by the manufacturer to maintain sterility for its specific system.

All liquids used in this study were lactated Ringer's solution, and all containers were of the 250-ml. size. One lot number for each type of container was used for both the test and controls. Analysis of preliminary results was used to determine a statistically valid sampling for the following studies.

Staphylococcus aureus was used as the test organism, since preliminary studies showed that this organism would propagate in lactated Ringer's solution. Propagation in lactated Ringer's solution was ensured by inoculation with microliter quantities of a known culture followed by both plating and filtration of the samples. In both cases, growth curves were found with extended lag times. Broths were prepared by inoculation from pure *S. aureus* (S-209) colonies and consisted of: trypticase soy broth (B.B.L.), 3.0 ml.; and distilled water, 100.0 ml.

A 24-hr. *S. aureus* broth was used for immersion of all test bottles. To determine the amount of contamination in the broth, a viable count method (6) was used to count the number of organisms. The counts taken ensured that the broth contained at least 1.7 billion organisms/ml.

Procedure—These forced contamination studies were divided into two categories: I, fully capped bottles (intact closures); and II, partially uncapped bottles (first closure removed). For example, in Category II, Type A bottles were uncapped to the rubber diaphragm, Type B bottles were uncapped to the rubber bung, and Type C containers were stripped to the screw cap.

Since no method was available to measure forced contamination of large-volume containers, the following techniques were developed and found to yield reproducible results.

Each bottle was immersed, mouth down, to about one-fourth of its length into the bacterial broth solution. The bottles were dipped in alternating order (Types A, B, and C), and the broth was continuously stirred to eliminate the possibility of bacterial sedimentation. After dipping in the broth, the bottles were placed in cases which were closed and spot taped. Each group of 24 test bottles and 12 control bottles was designated as a "run." The test bottles for several runs were dipped at the same time. Dipping was followed by a drying period of up to 10 days, after which the infusion sets were attached to the bottles one run at a time. Under a laminar flow hood, each set was attached as recommended by the manufacturer except that no wiping or swabbing procedure was used.

Bottles with attached infusion sets were shaken and inverted several times before being incubated[2] at 36 ± 1° for 15–24 hr. The control groups, in unopened cases, were placed in the incubator along with the tests.

Detection of bacteria in intravenous fluids was carried out by modifying several known techniques (7–12). After incubation, the contents of each bottle were passed through a Millipore filter apparatus which consisted of a Swinnex-25 filter assembly equipped with MF type HA black Millipore filter disks with grid, 0.45-μ openings, 25-mm. diameter, and a gasket. The entire apparatus was prepared and sterilized preceding filtration. Twenty-six filter assemblies were sterilized as a batch. One filter from each batch was removed under aseptic conditions, cultured, and examined for bacterial contamination. In a laminar flow hood, the effluent end of the Millipore filter assembly was connected to a vacuum line with a liquid trap. A vacuum was pulled, and the fluid was drawn from the intravenous bottle through the filter assembly into a conical flask. The organisms were collected on the filter disk located within the Swinnex-25 assembly. During each run and after filtration, each intact filter assembly was placed in a predetermined area prepared for it under the hood. The filter disk representing each bottle was then removed from the assembly and cultured using sterile technique (7–9).

Bacterial colony counts were taken from each filter on each of the first 3 days following filtration. The bacterial counting method (6) ensured accurate counts up to 300 organisms or colonies, beyond which estimates should be reasonably accurate to about 600 organisms. Although counts were made, they are not presented in this report since results were considered only as contaminated or not contaminated—an all or none approach to contamination.

Each test run consisted of eight of each type container, and each control run consisted of four of each type container. The control

[1] Abbott Laboratories, Baxter Laboratories, and Cutter Laboratories.

[2] In a Precision Thelco model 60 oven.

Table I—Fraction Contaminated[a] of Fully Capped Containers

	Type of Test Container			Type of Control Container		
	A	B	C	A	B	C
Series I	21/104	0/104	1/103	1/52	0/52	0/52
Series II	20/96	0/96	1/96	3/48	0/48	0/48
Total	41/200	0/200	2/199	4/100	0/100	0/100

[a] Containers yielding one or more colonies were considered contaminated.

Table II—Fraction Contaminated[a] of Partially Uncapped Containers

Type of Container	Test Units	Control Units
A	64/104	0/52
B	67/104	1/52
C	1/104	0/52

[a] Containers yielding one or more colonies were considered contaminated.

containers remained in their cartons as received from the manufacturer and were separated from the test groups. These bottles were exposed to normal room conditions and to a minimal amount of handling.

To ensure the possibility of potential contamination of intravenous bottles, the cap area of 36 bottles, after being immersed in the broth and dried for 7- and 8-day intervals, was washed with a known quantity of sterile water. These bottles yielded a calculated minimum count of 50,000 viable organisms when an aliquot was plated.

RESULTS AND DISCUSSION

Fully Capped Study—This portion of the experimentation was carried out in two segments (Series I and Series II) separated in time by 1 month. Each series and each test group were of approximately equal size, and control and test units were examined in an identical manner.

Two hundred each of the three types of containers were initially used as test units, and 100 of each type container were used as controls. In Table I, it can be seen that Types B and C test containers resisted contamination to a greater degree than A. Table I also shows that there was a similarity in the contamination rate of the 52 controls used in each case.

Table II was constructed from data collected from partially uncapped, stressed containers. From this table, it can be seen that containers of Types A and B were equally susceptible to contamination. The controls yielded almost identical results to those found in Table I. The results of colony counts made daily for 3 days after filtration are not reported here; only contamination and noncontamination are compared.

From these results, a statistical analysis of the data was carried out. The data from Table I consist of a set of proportions from bottles (both test and control) that were contaminated. An appropriate significance test for the comparison of several proportions when all or a part of the fractions are quite small (*e.g.*, 1/103 and 0/52 for Type C in the fully capped Series I) is the arc sin or $\sin^{-1}$ transformation (13, 14). If the actual rates of contamination are identical, the Brownlee (13) equation may be augmented so that:

$$Z = \frac{\sin^{-1}[(2n_1/N_1) - 1] - \sin^{-1}[(2n_2/N_2) - 1]}{\sqrt{(1/N_1) + (1/N_2)}} \quad \text{(Eq. 1)}$$

Table III—*Z* Values from Results in Table I[a]

Type of Test Container			Type of Control Container		
A	B	C	A	B	C
−0.10[b]	0.00[b]	0.05[b]	1.14[c]	0.00[c]	0.00[c]

[a] Comparison of Sections I and II. [b] No significant difference. [c] No difference.

Table IV—Pairwise Comparison of Fractions Contaminated[a]

Type Compared	Probability of Differences	
	In Test Units	In Control Units
C *versus* A	1×10^{-12}[b]	4.4×10^{-3}[b]
C *versus* B	4.55×10^{-2}[c]	No difference
A *versus* B	1×10^{-13}[b]	4.4×10^{-3}[b]

[a] Treatment of data from Table I. [b] Significant difference. [c] Possible significant difference.

Table V—Comparison of Test *versus* Control Containers[a]

Type of Container	Probability of Differences between Test and Controls	
	Probability of Difference	Significance of Difference
A	5.9×10^{-6}	Definite significance
B	5.0×10^{-1}	No significance
C	5.15×10^{-2}	Possible significance

[a] Treatment of data from Table I.

The quantity will have, to a good approximation, *normal probability distribution* with a mean value of zero and a standard deviation of one. Since no differences (Table III) were found between Sections I and II for both tests and controls of all types, the remainder of the analysis on this data can be done on the combined sections. Thus, fractions of tests contaminated on a pairwise basis (Table IV) indicate a tendency, under controlled conditions, for Type A containers to be more susceptible to contamination than those of either Type B or C.

Table V shows that there is a significant difference in the contamination of test and control units in the case of Type A containers, whereas there is no significance in Type C and only a possibility of significance for Type B. The finding of "possibly significant" for Type B containers is appropriate since federal regulations permit no contamination and commonly used statistical calculations would have, indeed, shown no significance.

A similar analysis of the data in Table II, for partially uncapped containers, shows that there is a definite increase in contaminated test containers compared to controls for Types A and B, whereas there is no significant difference between tests and controls for Type C.

A pairwise comparison of the three types of containers indicates that there is no difference in relative contamination between Types A and B and that Type C is less apt to be contaminated than either Type A or B under the test conditions.

From a practical point of view, one may extrapolate the findings of this experimentation to support the contention that contamination can occur if containers are improperly managed during preadministration. For example, to allow containers to remain in a partially opened state in a nonsterile area should significantly contribute to the loss of sterility. The study also shows that in an aseptic environment such as a laminar flow hood, there is little chance for contamination of the types of containers tested (Tables I and IV).

CONCLUSIONS

It was found that all three types of containers were well protected from bacterial contamination when procedures recommended by the manufacturers were used. The Type C open air system was least susceptible to contamination under stress conditions.

Table VI—Summary of 99% Confidence Intervals for True Contamination Rates under Experimental Conditions

	Type A		Type B		Type C	
	Test	Control	Test	Control	Test	Control
Capped	14–28%	1–12%	0–3%	0–5%	0–5%	0–5%
Partially uncapped	48–73%	0–10%	51–76%	0–14%	0–7%	0–10%

On the basis of the results reported here, one may speculate that contamination, emanating from outside the containers, can be the result of poor handling techniques. It may further be concluded that under the stress conditions studied, fully capped Type A containers were contaminated to a greater extent than those of Types B and C. It was also observed that with the containers partially uncapped, Types A and B were contaminated more frequently than was Type C. Furthermore, there was no difference between contamination rates for the control units.

It is the recommendation of the authors that regardless of the type of container used, or the precautions and engineering by the manufacturer, it is imperative that aseptic techniques be employed from the time the container is removed from the carton to the time infusion is completed.

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2-(Aminoethanesulfonylamino)thiazole and Related Compounds I: Stability, Absorption, Excretion, and Some Pharmacological Activities

SHUN-ICHI NAITO▲ and SAKAE SHIMIZU

Abstract □ Decomposition rate constants of nicotinoyltauraminothiazole in aqueous solution at high temperatures were determined. Unchanged nicotinoyltauraminothiazole and three decomposition products (tauraminothiazole, aminothiazole, and an unknown substance) were detected by TLC. Nicotinoyltauraminothiazole has both analgesic and anti-inflammatory activities. Blood levels, tissue distribution, and excretion of nicotinoyltauraminothiazole in animals were also investigated. In urine of rabbits after the administration of the chemical, unchanged compound, tauraminothiazole, and a metabolite of undetermined structure were isolated.

Keyphrases □ Nicotinoyltauraminothiazole—stability and biopharmaceutical studies □ 2-(Aminoethanesulfonylamino)thiazoles—stability and biopharmaceutical studies, nicotinoyltauraminothiazole □ Anti-inflammatory activity—nicotinoyltauraminothiazole

Only a few thiazole derivatives are marketed drugs. These include dithiazanine iodide (anthelmintic) (1), succinylsulfathiazole (sulfonamide) (2), sulfathiazole (sulfonamide) (3), thiabendazole (anthelmintic) (4), and thiamine derivatives (vitamin).

Although tauraminothiazole [2-(aminoethanesulfonylamino)thiazole] was synthesized by Winterbottom *et al.* (5), nicotinoyltauraminothiazole is a new compound and was synthesized in a series of 2-aminoethanesulfonic acid derivatives (6–8). If taurine is liberated partially from nicotinoyltauraminothiazole *in vivo*, the surface activity of taurine must have some effect on the activity of the remaining components (2-aminothiazole and/or unchanged nicotinoyltauraminothiazole). It was established that the bond between the amino group and the 2-aminoethanesulfonyl group cannot be ruptured easily in rabbits; liberation of taurine from the nicotinoyltauraminothiazole will be examined later in man.

In the present work, the stability, acute toxicity, and some pharmacological activities of nicotinoyltauraminothiazole were investigated. Its absorption, excretion, distribution, and metabolic pathway were also studied.

EXPERIMENTAL

Sample for Kinetic Study of Stability—About 1 ml. of a 0.8% nicotinoyltauraminothiazole hydrochloride solution was placed in a colorless ampul (1-ml. capacity). The samples were stored at 50, 60, and 70 ± 1°, and the solutions were assayed at various times.

On the basis of the results reported here, one may speculate that contamination, emanating from outside the containers, can be the result of poor handling techniques. It may further be concluded that under the stress conditions studied, fully capped Type A containers were contaminated to a greater extent than those of Types B and C. It was also observed that with the containers partially uncapped, Types A and B were contaminated more frequently than was Type C. Furthermore, there was no difference between contamination rates for the control units.

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In the present work, the stability, acute toxicity, and some pharmacological activities of nicotinoyltauraminothiazole were investigated. Its absorption, excretion, distribution, and metabolic pathway were also studied.

EXPERIMENTAL

Sample for Kinetic Study of Stability—About 1 ml. of a 0.8% nicotinoyltauraminothiazole hydrochloride solution was placed in a colorless ampul (1-ml. capacity). The samples were stored at 50, 60, and 70 ± 1°, and the solutions were assayed at various times.

Determination of Nicotinoyltauraminothiazole in Aqueous Solution—Five microliters of the sample solution was spotted on TLC plates, and the plates were developed for a 15-cm. distance using a mixture of butanol–dioxane–chloroform–28% ammonia (8:2:1:1) as a developing solvent and diatomite (Kieselgel G) as the adsorbent. Zones were scraped off the glass plate 1 cm. above and below the original point and were extracted with 3 ml. of water on a boiling water bath for 3 min. After centrifugation, a mixture of 2 ml. of the supernate and 1 ml. of 10% cyanogen bromide solution was allowed to stand for 20 min. at room temperature and then 0.2 ml. of 10% *p*-aminoacetophenone solution was added. After 10 min., 4 ml. of *n*-butanol was added to the mixture and this mixture was shaken for 5 min. The mixture was kept in a dark place for 10 min., and the absorbance of the butanol layer (the supernate) after centrifugation was measured at 450 nm. The absorbances were corrected by subtracting a blank value.

Acute Toxicity in Mice—The experiment was designed in the same way as described previously (6). Nicotinoyltauraminothiazole, its hydrochloride, 2-aminothiazole, or tauraminothiazole hydrochloride as aqueous solutions was administered to mice. The volume of the test sample solutions was adjusted to keep it constant at 0.1 ml./10 g. body weight. The LD_{50} of the chemical was calculated from the number of animals that died during the next 72 hr., according to the method of Litchfield and Wilcoxon (9).

Effect of Nicotinoyltauraminothiazole on Squirming and Capillary Permeability—Male dd strain mice, weighing 20 ± 1 g., were given the test compound orally. The experiment was carried out using the method described previously (6).

Tail Withdrawal Reflex in Mice—The same method as the analgesimetry described in the paper of Ben-Bassat *et al.* (10) was used. Each group consisted of 10 male mice (dd strain, average weight 16 g.).

Anti-Inflammatory Activity—Inflammation was produced by injection of carrageenin (0.1 ml. of 2% suspension) into the plantar surface of the hind paw of male rats (Wistar strain, average weight 230 g.). The method described in a previous paper (7) was used.

Inflammation was also produced by injection of serotonin (0.1 ml. of 0.0025% aqueous solution) into the plantar surface of the hind paw of male rats (Wistar strain, average weight 150 g.). Normal saline solution was injected into the other hind paw as a control. The test compound was administered subcutaneously in an aqueous solution or suspension whose volume was kept constant at 0.5 ml./100 g. body weight. The test compound was administered 30 min. before injection of the phlogistic agent. Control animals received an injection of the vehicle, and each group consisted of five rats. The effect of the test compound on the edema was determined volumetrically 1 hr. after injection of the phlogistic agent. The experimental conditions reported by Theobald and Domenjoz (11) were used.

Anaphylactic Shock in Guinea Pigs—Female guinea pigs (Hartley strain), with a body weight of about 320 g., were used in the experiment following the procedure of Labelle and Tislow (12). Guinea pigs were challenged exactly 0.5 or 1 hr. after drug administration by rapid injection of 1 ml. of horse serum into the saphenous vein.

Antihistamine Activity—Female guinea pigs (Hartley strain, average weight 360 g.) were used in the histamine tests. The compound was administered subcutaneously 0.5 and 1 hr. before histamine injection.

Histamine dihydrochloride was injected rapidly into the saphenous vein in a dosage of 1.1 mg./kg. The test was made in fasted animals.

Antipyretic Activity—To male rabbits weighing 2.7 kg., 15 mcg./kg. of nonanaphylactogenic polysaccharides obtained from *Pseudomonas fluorescens*[1] was administered intravenously, and nicotinoyltauraminothiazole solution was given subcutaneously 2 hr. after the administration of the pyrogen solution. Body temperature was recorded every 15 min. for 6 hr. Each group consisted of three rabbits.

Detection of Metabolites of Nicotinoyltauraminothiazole in Blood—A mixture of 1 ml. of water, 0.8 g. of sodium chloride, and 7 ml. of ethyl acetate was added to 1 ml. of plasma taken 1 hr. (time of peak blood level) after ingestion of 350 mg./kg. of nicotinoyltauraminothiazole. This mixture was shaken vigorously for 30 min. After centrifugation for 5 min., 4 ml. of the ethyl acetate layer was evaporated to dryness on a boiling water bath, and the residue was dissolved in 50 μl. of water. Ten microliters of this solution was spotted on a chromatographic plate for TLC. A blood sample gathered prior to ingestion of the chemical was treated by the same procedure as a control.

For TLC, the solvent system was ethanol–chloroform–28% ammonia (8:2:1), the adsorbent was diatomite (Kieselgel G), and the color developer was modified Folin reagent (13). One spot having an R_f value of 0.76 (blue) was observed in treated plasma; no spots were observed in control plasma. The R_f value of nicotinoyltauraminothiazole was 0.76 (blue).

Determination of Nicotinoyltauraminothiazole in Plasma—A mixture of 1 ml. of plasma and 1 ml. of 10% cyanogen bromide solution was treated by the procedure described under *Determination of Nicotinoyltauraminothiazole in Aqueous Solution.* A mixture of 0.9 ml. of untreated (normal) animal plasma and 0.1 ml. of nicotinoyltauraminothiazole solution of known concentration was treated as above to prepare a calibration curve. The procedure described is a modification of the method reported by Harris and Raymond (14).

Determination of Nicotinoyltauraminothiazole in Rat Bile—Male rats (Wistar strain, body weight 150–170 g.) were treated by the method described previously (15). Each group consisted of five rats.

Following oral administration of 200 mg./kg. of nicotinoyltauraminothiazole by a stomach tube, 1 ml. of bile (instead of plasma) was treated according to the method for determination of nicotinoyltauraminothiazole in blood.

Distribution of Nicotinoyltauraminothiazole in Rat Organs—To male rats (Wistar strain, body weight 150 ± 10 g.), 200 mg./kg. of nicotinoyltauraminothiazole was administered orally and the animals were killed either 1 hr. (time of peak blood level) or 24 hr. following administration. Each group consisted of five rats. Six organs (heart, lungs, spleen, kidneys, brain, and liver) were assayed for nicotinoyltauraminothiazole. After bleeding each rat, each organ was separated and homogenized with 4 ml. of water for the brain, 10 ml. of water for the liver, and 3 ml. of water for the heart, lungs, spleen, or kidneys. After centrifugation, 1 ml. of the supernate of each homogenate was used for the assay, which was performed as described under *Determination of Nicotinoyltauraminothiazole in Aqueous Solution.*

Separation of Metabolites of Nicotinoyltauraminothiazole in Rabbits—Urine from three female rabbits (average weight 3.0 kg.), following ingestion of 200 mg./kg. of nicotinoyltauraminothiazole, was submitted to lyophilization. This residue was designated as A. The residue of urine obtained from three rabbits who did not receive the drug was designated as B and served as a control.

A mixture of 100 mg. of Residue A or B and 1 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1 hr. After the addition of 1 ml. of 5 *N* Na_2CO_3 solution, the mixture A′ from A or B′ from B was submitted to TLC. Also, a mixture of 2 ml. of water and 100 mg. of Residue A or B was submitted to TLC. No difference was observed in the number of spots between Residue A and A′ or between Residue B and B′.

Assay of Metabolites of Nicotinoyltauraminothiazole in Rabbit Urine—*Determination of Nicotinoyltauraminothiazole*—A mixture of 1 ml. of water and 20 mg. of the lyophilized residue of urine from rabbits (average weight 3.0 kg.) who had received 200 mg./kg. of nicotinoyltauraminothiazole was centrifuged, and 10 μl. of the supernate was submitted to TLC, using a mixed solvent of butanol–dioxane–methylene chloride–28% ammonia (8:2:1:2). The spot corresponding to nicotinoyltauraminothiazole was scraped off the TLC plate and extracted for 3 min. with 3 ml. of water on a boiling water bath. Two milliliters of the centrifugation supernate was treated by the same procedure as described under *Determination of Nicotinoyltauraminothiazole in Aqueous Solution.*

Determination of Tauraminothiazole—Ten microliters of the supernate obtained by the same procedure as was used above was submitted to paper chromatography (PC), using a mixed solvent of ethanol–chloroform–28% ammonia (5:1:1) and filter paper[2]. The spot corresponding to tauraminothiazole was cut out and extracted for 3 min. with 3 ml. of water on a boiling water bath. To determine the concentration of tauraminothiazole, 1 ml. of the centrifugation supernate was treated with 0.4% ferricyanide and 1% $Fe(NO_3)_3$ in 0.7 *N* HNO_3 (13).

Determination of Unknown Metabolite (Metabolite I)—A mixture

[1] T. T. G. No. 1, Fujisawa Yakuhin Kogyo Co., Osaka, Japan.

[2] No. 50, Toyo Roshi Co., Osaka, Japan.

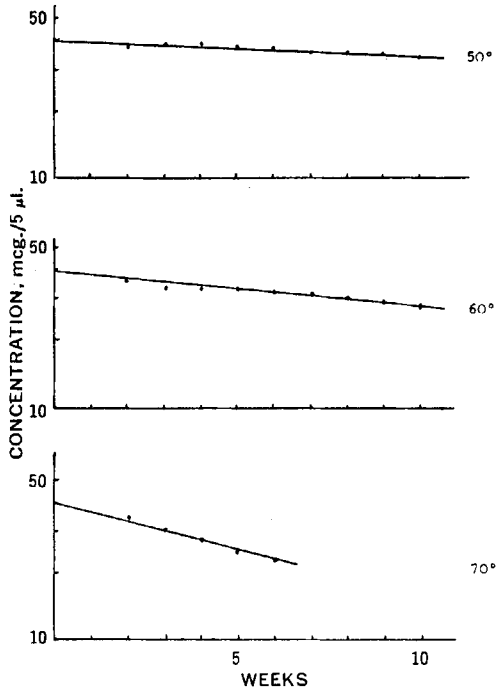

Figure 1—*Concentration of nicotinoyltauraminothiazole in aqueous solution as its hydrochloride.*

of 50 mg. of the lyophilized residue of urine and 0.5 ml. of 1 *N* NaOH was heated for 100 min. on a boiling water bath. This mixture was combined with 0.5 ml. of 1 *N* HCl and was then made alkaline with 0.1 ml. of 5 *N* Na_2CO_3 solution. The mixture thus obtained was made up to 1 ml. with water and analyzed according to the method for tauraminothiazole. Tauraminothiazole must be liberated from nicotinoyltauraminothiazole and Metabolite 1 in urine by alkali hydrolyzation. Therefore, tauraminothiazole liberated from Metabolite 1 can be calculated by subtracting both the amount of tauraminothiazole that existed originally and the amount of tauraminothiazole equivalent to nicotinoyltauraminothiazole

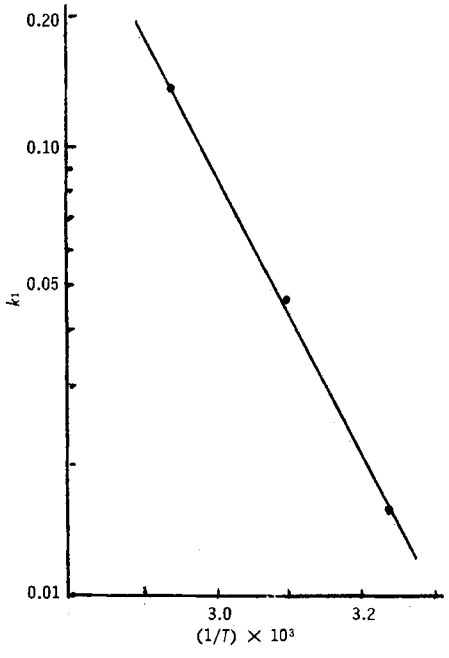

Figure 2—*Arrhenius plot of decomposition rate constants of nicotinoyltauraminothiazole hydrochloride in aqueous solution. Key:* T, *absolute temperature; and* k_1, *decomposition rate constant in reciprocal days.*

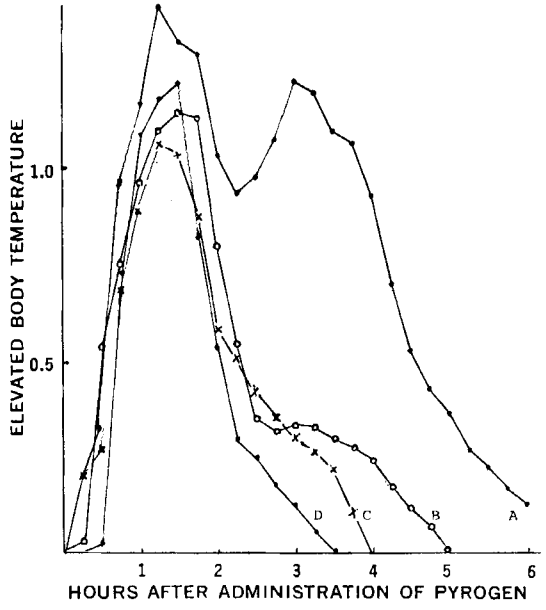

Figure 3—*Mean curve for the fall of elevated body temperature of male rabbits receiving pyrogen. Each group consisted of three rabbits. Key: A, control group, mean basal body temperature (MBBT) 39.1°; B, 75 mg./kg. of nicotinoyltauraminothiazole, MBBT 39.2°; C, 100 mg./kg. of nicotinoyltauraminothiazole, MBBT 39.2°; and D, 150 mg./kg. of nicotinoyltauraminothiazole, MBBT 39.3°.*

in urine from the total tauraminothiazole obtained by alkali hydrolyzation of the lyophilized residue of urine.

Identification of Tauraminothiazole Obtained by Hydrolyzation of Metabolite 1—In the procedure under *Determination of Nicotinoyltauraminothiazole in Rabbit Urine*, 10 spots corresponding to Metabolite 1 were scraped off the TLC plate and extracted for 1 min. with 10 ml. of ethyl acetate on a boiling water bath. Following evaporation of the solvent, the residue was mixed with 0.5 ml. of 1 *N* NaOH and heated for 100 min. on a boiling water bath. The mixture was mixed with 0.5 ml. of 1 *N* HCl and then made alkaline with 0.1 ml. of 5 *N* Na_2CO_3 solution. This mixture was extracted with 3 ml. of ethyl acetate, and the residue obtained after evaporation of the ethyl acetate layer was dissolved in a small amount of ethyl acetate and chromatographed (TLC), using a mixed solvent of butanol–dioxane–methylene chloride–28% ammonia (8:2:1:2). The spot corresponding to tauraminothiazole was scraped off the TLC plate and extracted with a small amount of ethyl acetate. The solvent was removed and the residue was submitted to a mixed micromelting-point determination with the authentic sample.

RESULTS AND DISCUSSION

TLC of the extract from stability samples of nicotinoyltauraminothiazole hydrochloride (10 weeks at 70 ± 1°) produced four spots when a mixed solvent of butanol–dioxane–chloroform–28% ammonia (8:2:1:1) was used. The R_f values of the four spots corresponding to 2-aminothiazole, tauraminothiazole, unchanged nicotinoyltauraminothiazole, and an unknown decomposition product were 0.78, 0.23, 0, and 0.41, respectively. These spots were detected as a blue color by use of modified Folin reagent (13). The assay results (Fig. 1) of the unchanged nicotinoyltauraminothiazole indicate that degradation of nicotinoyltauraminothiazole hydrochloride obeyed first-order kinetics. Constants (16) concerning the stability of nicotinoyltauraminothiazole hydrochloride were as follows: decomposition rate constant (k_1 in reciprocal days) at 50°, 0.00154; k_1 at 60°, 0.00473; k_1 at 70°, 0.01337; heat of activation, 23.6 kcal./mole/°; and log frequency factor, 13.7. The Arrhenius plot of the rate constants was linear (Fig. 2). The predicted concentration after 2 years at 25° is 83.3%. Therefore, it is assumed that nicotinoyltauraminothiazole is fairly stable in aqueous solution at room temperature.

The LD_{50}'s of 2-aminothiazole, tauraminothiazole hydrochloride, nicotinoyltauraminothiazole, and its hydrochloride by intraperitoneal administration to mice were 940 (866–1020), 780 (719–846),

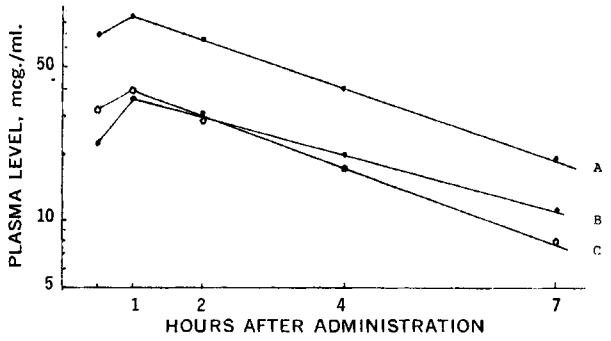

Figure 4—*Mean plasma level of nicotinoyltauraminothiazole after oral administration to three male rabbits (average weight 3.0 kg.) at different doses. Key: A, 200 mg./kg.; B, 150 mg./kg.; and C, 100 mg./kg.*

1280 (1143–1534), and 940 (797–1109) mg./kg., respectively (with 95% confidence limits). No significant difference was observed in the LD_{50}'s between male and female mice. Although the animals receiving intraperitoneal doses below 800 mg./kg. of nicotinoyltauraminothiazole exhibited no behavioral abnormality, the intraperitoneal dose of 4000 mg./kg. produced a transient ataxic walk with extended hind limbs. After a longer period, the animal showed lacrimation, piloerection, and convulsions; all of the animals died from respiratory failure at the 4000-mg./kg. dose.

The purpose of squirming and permeability tests was to obtain information concerning the concurrent changes in the peritoneal capillary permeability during squirming through the method of Whittle (17). The ED_{50} of nicotinoyltauraminothiazole for inhibition of squirming was 200 (165–242) mg./kg., and this chemical did not change the permeability response compared with that of the control group.

With respect to the tail withdrawal reflex in mice (10), computation of the ED_{50} and its confidence limits was made by probit analysis. This was done by dichotomizing the reactions after 30 min. into two groups: "refractory," *i.e.*, pain reaction time of 2.0 sec. or more, and "nonrefractory" for aminopyrine and nicotinoyltauraminothiazole. The ED_{50}'s and 95% confidence limits were 48 (35–66) mg./kg. for aminopyrine and 225 (186–272) mg./kg. for nicotinoyltauraminothiazole. The analgesic activity of nicotinoyltauraminothiazole seems to be less than that of aminopyrine, according to the ED_{50}'s of the tail withdrawal reflex in mice.

The ED_{50}'s (with 95% confidence limits) of nicotinoyltauraminothiazole on edema induced by carrageenin and serotonin were 185 (112–305) and 43.5 (27.2–69.6) mg./kg., respectively. The ED_{50} of sodium salicylate for edema induced by serotonin was 370 (203–666) mg./kg., and this drug had no effect on carrageenin-induced edema at doses lower than 500 mg./kg. These results show that nicotinoyltauraminothiazole has fairly potent anti-inflammatory activity which compares with that of sodium salicylate.

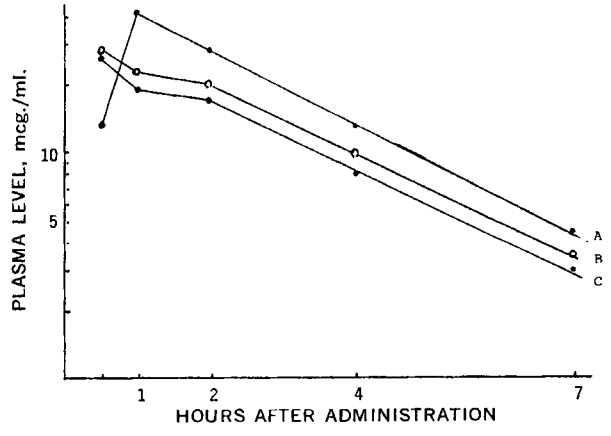

Figure 5—*Mean plasma level of nicotinoyltauraminothiazole after oral administration to male rats (Wistar strain, average weight 130 g.) at different doses. Each group consisted of five rats. Key: A, 200 mg./kg.; B, 150 mg./kg.; and C, 100 mg./kg.*

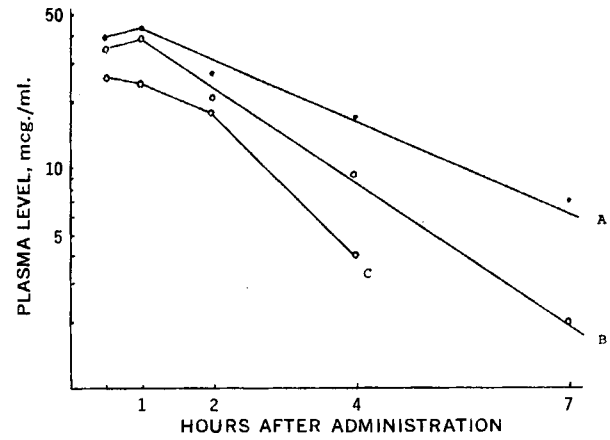

Figure 6—*Mean plasma level of nicotinoyltauraminothiazole after oral administration to male mice (dd strain, average weight 17 g.) at different doses. Each group consisted of 10 mice. Key: A, 200 mg./kg.; B, 150 mg./kg.; and C, 100 mg./kg.*

Antihistaminic and antianaphylactic shock activities of nicotinoyltauraminothiazole were evaluated by the method of Labelle and Tislow (12). No difference in antihistaminic activity was observed between 200- and 300-mg./kg. doses of nicotinoyltauraminothiazole when the compound was administered subcutaneously 0.5 hr. before histamine injection. Forty percent of 10 guinea pigs survived in each group. When the compound was given subcutaneously 1 hr. before histamine injection, only 20% of 10 guinea pigs survived. Therefore, the antihistaminic activity seems to be greater when the chemical is given 30 min. rather than 1 hr. before histamine injection. Antihistaminic activity of the chemical was not investigated in detail because of its low activity.

Three out of 10 guinea pigs, who received 200 mg./kg. of nicotinoyltauraminothiazole, survived the first 10 min. following the horse serum injection. When 100 or 300 mg./kg. of nicotinoyltauraminothiazole was given, two out of 10 guinea pigs survived. These results indicate that the antianaphylactic activity of this compound is weak.

Results of antipyretic activity tests are presented in Fig. 3. Nicotinoyltauraminothiazole, at doses of 100 and 150 mg./kg., gradually decreased the elevated body temperature of all rabbits used.

Before performing quantitative determinations of nicotinoyltauraminothiazole in blood, it was ascertained by TLC that there are no metabolites of the chemical detectable in blood. Semilogarithmic plots of blood level *versus* time are shown in Figs. 4–6. A semilogarithmic plot of mean plasma level of nicotinoyltauraminothiazole after intravenous administration of 33 mg./kg. to rabbits is presented in Fig. 7. For the latter experiment, the compound was dissolved in a mixed solution of 0.5 ml. of 5 *N* HCl and 15.5 ml. of 0.9% normal saline solution to make a 2% solution of the compound (pH 2.0).

Collection of bile from rats under continuous urethan anesthesia indicated that nicotinoyltauraminothiazole has no effect upon bile

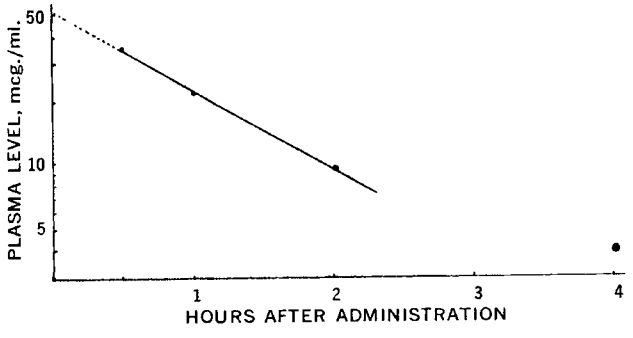

Figure 7—*Mean plasma level of nicotinoyltauraminothiazole after intravenous administration to male rabbits (average weight 3.0 kg.) at 33-mg./kg. dose.*

Table I—TLC of Lyophilized Urine of Rabbits and Rat Bile[a]

Sample[b]	Solvent[c]	R_f[d] (Substance Assumed)
2-Aminothiazole (I)	A	0.85
Tauraminothiazole (II)	A	0.10
Nicotinoyltaura-minothiazole (III)	A	0.41
Residue A	A	0.47
		0.41 (III)
		0.36 (Metabolite 1)
		0.17
		0.10 (II)
Residue B	A	0.47
		0.17
Mixture A′	A	0.47
		0.41 (III)
		0.36 (Metabolite 1)
		0.17
		0.10 (II)
2-Aminothiazole	B	0.81
Tauramino-thiazole	B	0.29
Nicotinoyltaura-minothiazole	B	0.53
Residue A	B	0.53 (III)
		0.43 (Metabolite 1)
		0.29 (II)
		0.23
Residue B	B	0.23
Tauramino-thiazole	C	0.24
Nicotinoyltaura-minothiazole	C	0.54
Hydrolyzed Residue A	C	0.54 (III)
		0.24 (II)
		0.18
Hydrolyzed Residue B	C	0.18
Residue A	C	0.54 (III)
		0.43 (Metabolite 1)
		0.24 (II)
		0.18
Residue B	C	0.18
Rat bile A	C	0.54 (III)
Rat bile B	C	Nil
Nicotinoyltaura-minothiazole	D	0.87
Tauramino-thiazole	D	0.43
Hydrolyzed Residue A	D	0.87 (III)
		0.55
		0.43 (II)
		0.35
		0.15
Hydrolyzed Residue B	D	0.55
		0.35
		0.15
Residue A	D	0.87 (III)
		0.80 (Metabolite 1)
		0.55
		0.43 (II)
		0.35
		0.15
Residue B	D	0.55
		0.35
		0.15
Rat bile A	D	0.87 (III)
Rat bile B	D	Nil

[a] The adsorbent was diatomite (Kieselgel G), 0.25 mm. in thickness. The color developer was modified Folin reagent, and the developed distance was 15 cm. [b] Residues A and B were described in the *Experimental* section. Hydrolyzed Residues A and B were composed of mixtures of 50 mg. of Residue A or B and 0.5 ml. of 1 *N* NaOH solution heated for 100 min. on a boiling water bath. The reaction mixture was neutralized with 1 *N* HCl and made slightly alkaline with sodium carbonate. This mixture was used for TLC. Mixtures A′ and B′ were described in the *Experimental* section. Rat bile A was bile collected for 7.5 hr. following oral administration of 200 mg./kg. of nicotinoyltauraminothiazole to rats. Three milliliters of bile was extracted with 7 ml. of ethyl acetate, the solvent was removed, and the residue was dissolved in 50 μl. of ethyl acetate. Ten microliters of the ethyl acetate solution was spotted on a TLC plate. Rat bile B was bile collected from rats receiving no compound and was treated by the same procedure as in the case of rat bile A. [c] Solvent systems were: A, butanol–dioxane–methylene chloride–28% ammonia (8:2:1:1); B, butanol–dioxane–methylene chloride–28% ammonia (8:2:1:3); C, butanol–dioxane–methylene chloride–28% ammonia (8:2:1:2); and D, ethanol–chloroform–28% ammonia (4:1:1). [d] Color of all spots was blue or light blue.

flow and total bile volume excreted in 7.5 hr., as compared with untreated control rats. The residue from ethyl acetate extraction of rat bile contained no metabolites when nicotinoyltauraminothiazole was administered orally. This fact was supported by the TLC data shown in Table I (Solvents C and D). The concentration of nicotinoyltauraminothiazole in rat bile, excreted for 7.5 hr. following oral administration of a 200-mg./kg. dose, was 375 ± 48 mcg./kg. (mean value of five rats and standard error).

Tissue levels of nicotinoyltauraminothiazole in rat organs 1 hr. after oral administration to five rats were 5.6 ± 0.9 in the brain, 23.1 ± 2.3 in the heart, 30.3 ± 5.5 in the lungs, 20.4 ± 3.3 in the liver, 36.3 ± 3.2 in the spleen, and 44.3 ± 7.2 mcg./g. in the kidneys (mean values ± standard errors). Nicotinoyltauraminothiazole concentrations in all rat organs were below the assay sensitivity 24 hr. following administration.

Metabolites of nicotinoyltauraminothiazole were examined in the lyophilized residue of rabbit urine after oral administration of 200 mg./kg. of the chemical to three rabbits. When the lyophilized residue was submitted to TLC, three spots [unchanged nicotinoyltauraminothiazole, tauraminothiazole, and an unknown metabolite (Metabolite 1)] were detected. Two of the three metabolites, nicotinoyltauraminothiazole and tauraminothiazole, were identified by mixed micromelting-point determination with the respective authentic samples. The chemical structure of Metabolite 1 has not been established, but tauraminothiazole was obtained when Metabolite 1 was treated with 1 *N* NaOH on a boiling water bath. On the other hand, it was known by TLC that tauraminothiazole cannot be liberated from Metabolite 1 after incubation with 5 *N* HCl at 37 ± 1° for 1 hr. The structure of Metabolite 1 will be investigated.

The results of quantitative determination of the metabolites in rabbit urine suggested that 8.7% of the compound administered orally is excreted as nicotinoyltauraminothiazole, 64.1% is excreted as tauraminothiazole, and 17.1% is excreted as Metabolite 1. Total recovery was 89.9% of the dose, indicating excellent absorption of nicotinoyltauraminothiazole in rabbits. It was also ascertained by TLC that all of the detectable metabolites were excreted within 48 hr. The ring system of sulfathiazole is known to be stable *in vivo*, but 2-aminothiazole, an antithyroid agent, was reported to be rapidly destroyed in the body (18). In the case of nicotinoyltauraminothiazole, the ring system seems to be fairly stable *in vivo*.

SUMMARY

1. Nicotinoyltauraminothiazole in aqueous solution is fairly stable at room temperature.

2. Analgesic, antihistaminic, antianaphylactic, and antipyretic activities of nicotinoyltauraminothiazole seem to be relatively low. However, the activity of this compound is about eight times higher than that of sodium salicylate on edema induced by serotonin.

3. About 90% of the dose was excreted in urine as drug plus metabolites after oral administration of nicotinoyltauraminothiazole to rabbits.

4. Nicotinoyltauraminothiazole may be an interesting compound for clinical trials because of its low toxicity, good absorbability, and potent anti-inflammatory activity.

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▲ To whom inquiries should be directed.

Evaluation of Interactions between Polymers and Low Molecular Weight Compounds by GLC I: Methodology and Interaction Evaluation

JACOB L. VARSANO*▲ and SEYMOUR G. GILBERT†

Abstract □ Sorption–desorption thermodynamics were evaluated for two polymers and 13 low molecular weight compounds. The polymers, polyethylene and polyvinyl chloride, are of major use in packaging. The sorbents were bacteriostatic agents of pharmaceutical utility and organic solvents employed in package fabrication. GLC procedures were used where the polymers constituted the stationary phases and the sorbents constituted the mobile phases of the columns. Auxiliary methods were adapted for obtaining the data necessary for the thermodynamic computations. The measurement of interaction affinities was based on Raoult's law activity coefficients. The GLC methodology was fast and convenient to perform and could be a valuable tool for applied and theoretical investigations in this area.

Keyphrases □ Polymer–low molecular weight compound interactions—sorption–desorption thermodynamic methodology, GLC □ Sorption–desorption thermodynamic methodology—polyethylene and polyvinyl chloride, 13 low molecular weight compounds, GLC □ Package–content interactions—polymers and low molecular weight compounds, GLC method for evaluating sorption–desorption thermodynamics □ Plastics—interactions of packaging materials with low molecular weight compounds evaluated by GLC, sorption–desorption thermodynamics □ GLC—evaluation of polymer–low molecular weight compound interactions, sorption–desorption thermodynamic methodology

The use of polymers in packaging may result in sorption of ingredients from the contents or desorption of compounds used in package fabrication. These processes may render a product unsuited for use. The phenomena result from interactions between the polymer and the low molecular weight compound and diffusion. The thermodynamics of the interaction determine the equilibrium amount of sorbent in the polymer, and diffusion affects the rate for attaining the equilibrium. The available information on such interactions is insufficient for optimum use of polymers in packaging; thus the objectives of this work were to evaluate the interaction thermodynamics and to use the quantities obtained for relative grading of affinities and toward a better understanding of the systems. Since conventional methods for these objectives are cumbersome and time consuming, GLC procedures were adapted by using the polymers as the stationary phases and the low molecular weight compounds as the mobile phases of the columns.

GLC BACKGROUND

The retention of a compound in a GLC column is a function of its saturation vapor pressure at the column temperature and the energy of interaction with the stationary phase. The specific retention volume, V_g^0, is characteristic for a given mobile–stationary phase system and is derived from experimental quantities through (1):

$$V_g^0 = \frac{(t_r - t_a)\dot{V}j}{W_1}\frac{273}{T} \qquad \text{(Eq. 1)}$$

where t_r is the retention time for the compound, t_a is the injection pulse, $\dot{V}$ is the corrected inert gas flow rate, j is the compressibility

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Table I—Solute-Solvent Systems

Low Molecular Weight Compounds Employed as Solutes	
a. Organic Solvents[a]	b. Bacteriostatic Agents
n-Butanol	Chlorobutanol anhydrous USP[b]
tert-Butanol	Benzyl alcohol NF[c]
Methyl isobutyl ketone	Phenol USP[d]
2-Nitropropane	Methylparaben USP[b]
Butyl acetate	Propylparaben USP[b]
Hexane	
Octane	
Toluene	
Polymers Employed as Solvents	
Polyethylene[e]	
Polyvinyl chloride[f]	

[a] Matheson, Coleman and Bell, E. Rutherford, N. J. [b] Fisher Scientific Co., Fair Lawn, N. J. [c] Eastman Chemical Products, Inc., Kingsport, Tenn. [d] J. T. Baker Chemical Co., Phillipsburg, N. J. [e] Marlex EMN TR-885, Phillips Petroleum Co., Bartlesville, Okla. [f] Allied Chemical Co., Morristown, N. J.

correction, W_1 is the weight of the stationary phase, and T is the absolute temperature. The specific retention volume is unaffected by operational variables, such as column length and amount of stationary phase, but depends exclusively on the thermodynamic variables of the system. Raoult's law activity coefficient, γ, is derived from the specific retention volume through (2):

$$\gamma = \frac{273R}{V_g^0 P_2^0 M_1} = \frac{17.06 \times 10^6}{V_g^0 P_2^0 M_1} \qquad \text{(Eq. 2)}$$

where P_2^0 is the vapor pressure of the pure compound at the column temperature, M_1 is the molecular weight of the stationary phase, and R is the gas constant. The activity coefficient reflects only the interaction energy between the compound and the stationary phase; values decreasing from unity result from interactions favorable to mixing and those increasing from unity reflect interactions unfavorable to mixing. The coefficients derived from GLC are identical to those obtained from solutions where the polymers are the solvents and the low molecular weight compounds are the solutes. Since the coefficients are concentration dependent, their values are constants at infinite dilution. Rigorously, Eq. 2 requires fugacities; however, such values are rarely available and vapor pressures have been used instead, resulting in the apparent activity coefficients, γ_p.

Experimental Background—The solute-solvent systems used are presented in Table I. The solvents were polyethylene and polyvinyl chloride. The solutes consisted of organic solvents used in package fabrication and bacteriostatic agents employed in pharmaceuticals. The systems represent varying polarities, functional groups, and structures.

For the computations from Eq. 2, the number average molecular weight of the polymers, $\bar{M}_n$, was used. The vapor pressures for most of the organic solvents were computed by means of the Antoine equation (3) using empirical constants from *Reference 4*. For several of the solutes, the vapor pressures were determined experimentally.

The densities at the experimental temperatures for the thermodynamic computations (5) were derived as reasonable approximations through (6):

$$\rho_T = \rho\{1 - (T - T_\rho)10^{-3}\} \qquad \text{(Eq. 3)}$$

where ρ_T is the density at the experimental temperature T, and ρ is the literature density at temperature T_ρ. The error incurred in the computed values by this approximation is negligible. The melt densities for polyethylene were derived from Terry and Yang (7) as recommended by the supplier. Melt densities for polyvinyl chloride, chlorobutanol, phenol, methylparaben, and propylparaben were determined experimentally.

The elevated temperatures at which the experiments were conducted posed the question of chemical stability of the solutes and the polymers. The group of organic solvents was considered stable from the general knowledge of their properties. The literature contains reports on GLC of benzyl alcohol and phenol (8) and of methylparaben and propylparaben (9) without degradation. Chlorobutanol is known to be stable at the lower temperatures used. For the higher temperatures, the stability of the solutes was monitored from the chromatograms, since degradation products would exhibit markedly different retention characteristics, and also from plots of retention values against temperature where deviation from linearity would indicate changes in the compound. This self-controlling capability of GLC obviated the need for further identification of an eluting compound as the temperature was raised, once a retention value was associated with it at a lower temperature. Preliminary experiments revealed degradation only for chlorobutanol starting from 210°, so experiments with this compound were conducted up to 200°.

Table II—Column Characteristics and Applications

Stationary Phase	Column Length, m.	Stationary Phase Content, g.	Solutes for Which Employed
Polyethylene	3.0	6.5825	Organic solvents, chlorobutanol, phenol, benzyl alcohol
Polyethylene	1.5	2.4445	Methylparaben, propylparaben
Polyvinyl chloride	5.0	9.9674	Organic sorbents
Polyvinyl chloride	2.5	3.0226	Chlorobutanol, phenol, benzyl alcohol
Polyvinyl chloride	1.2	0.9640–0.8480	Methylparaben, propylparaben

The stability of the polymers was monitored by means of column performance experiments at the initiation of the experiments and periodically thereafter, using compounds of varying polarities. The stability of polyethylene did not present difficulties; however, for polyvinyl chloride, 3% stabilizer was incorporated. Retention attributable to the stabilizer was not observed during preliminary experiments. Although the temperature regimens for the two polymers were different because of stability reasons, overlapping at least at one temperature was always assured. The temperature ranges for polyethylene and polyvinyl chloride were 140–230 and 110–210°, respectively. For the polyvinyl chloride–parabens systems, which required high temperatures, a new column was used at each experimental temperature and only single runs were performed since replicates would have incurred greater error due to degradation of the polymer.

The loading of the column with stationary phase has usually been high in work of this kind in order to minimize adsorption effects on the solid support. Since the support used in this work was a silanized grade, the amount of loading was determined by feasibility. Polyvinyl chloride did not lend itself to heavy loading, so a lower level was used compared to polyethylene and compensation was achieved by a longer column.

The least amount of solute usable was determined by preliminary experiments and was found to be below any concentration effect on the retention. The solutes were applied as appropriate mixtures to save time and column deterioration.

Instrumentation—The dual-column gas chromatograph[1], equipped with flame-ionization detectors, was used. The temperature was measured with a millivolt potentiometer[2] and a set of four copper-constantan thermocouples.

Materials—The polyethylene was a narrow molecular weight distribution, high density homopolymer used for container molding. Its characteristics were: density, 0.965; molecular weight, $\bar{M}_n$ = 15,000; and antioxidant content, 0.015% 2,6-di(*tert*-butyl)-4-methylphenol[3]. The polyvinyl chloride was a blow molding grade homopolymer of $\bar{M}_n$ = 40,000 and $\bar{M}_n/\bar{M}_w \sim 2$. The organic solvents were chromato or spectral quality. The bacteriostatic agents were USP or NF grade. The stabilizer for polyvinyl chloride was di(*n*-butyl)tin β-mercaptopropionate[4] used at 3% (w/w) of polyvinyl chloride. The solid support for the columns was diatomaceous earth (acid and base washed and silanized)[5], 70–80 mesh.

[1] Model 5750B, Hewlett-Packard Analytical Instruments, Pasadena, Calif.
[2] Model 8690, Leeds and Northrup Co., Philadelphia, Pa.
[3] Ionol, Shell Chemical Co.
[4] Advastub, Carlisle Chemical Works, Inc., New Brunswick, N. J.
[5] Anakron ABS, Analabs, Inc., North Haven, Conn.

Tale III—Experimentally Determined Vapor Pressures

Compound	Temperature	Vapor Pressure, mm. Hg—Experimental	Vapor Pressure, mm. Hg—Literature	Experimental / Literature × 100	Reference
Decane	119.8°	153.0	150	102	4
	140.9°	296.8	300	99	—
	150.5°	398.7	400	99.5	—
	158.4°	524.6	500	105	—
tert-Butanol	60.9°	309.0	300	103	4
	86.9°	921.0	900	102	—
Benzyl alcohol	141.7°	101.8	100	102	12
	183.0°	399.5	400	100	—
Butyl acetate	110°	528.6	—	—	—
	120°	666.0	—	—	—
	125°	749.0	760	98.5	12
	130°	841.5	—	—	—
	140°	1108.0	—	—	—
Chlorobutanol	120°	136.4	—	—	13
	135°	—	246	—	—
	140°	295	—	—	—
	150°	595	—	—	—
Methylparaben	190°	19.8	—	—	—
	200°	26.9	—	—	—
	210°	36.4	—	—	—
	220°	49.3	—	—	—
Propylparaben	190°	6.21	—	—	—
	200°	8.97	—	—	—
	210°	12.53	—	—	—
	220°	16.53	—	—	—

Preparation of Chromatographic Columns and Column Characteristics—The weighed polymer was dissolved in a suitable solvent in a refluxing apparatus. For polyethylene, the solvent was toluene; for polyvinyl chloride, a mixture of equal parts of toluene and methyl isobutyl ketone was employed. The stabilizer for the latter polymer was also dissolved in the solvent. The weighed amount of solid support was stirred into the solution, and the solvent was removed until dryness in a rotating vacuum evaporator. When preparing the polyvinyl chloride column material, glass beads were added as a stirring aid. The coated solid support was dried at 70° in a vacuum oven to constant weight, and the 50–70-mesh fraction was used for column packing. The stationary phase content of the packing was determined by extraction of a sample in a soxhlet apparatus, followed by gravimetric procedures. A correction was made for the amount of stabilizer in a polyvinyl chloride packing material. The prepared material was packed in 0.63-cm. (0.25-in.) stainless steel tubing. The amount of packing in the column was determined by weighing. The column characteristics are shown in Table II.

Chromatographic Procedures—The chromatographic columns were attached to the gas chromatograph through 6.4-cm. (2.5-in.) extension tubes to prevent the column ends from heating above the experimental temperature, since the blocks were maintained at 250°. The temperature of the column was measured by means of four thermocouples attached at various positions along the length and was controlled within 0.5°. The column was equilibrated for at least 10 min. before charging the solutes. The carrier gas was helium. Its flow rate was measured through the detector block at the experimental temperatures with a 50-ml. soap bubble flowmeter, and corrected values accounting for the water vapor pressure were employed in the computations. The inlet pressure was measured with a mercury manometer, and the outlet pressure was assumed to be 760 mm. Hg. The retention time was measured from the recorder chart using the front profiles of the elution peaks as recommended (10) for better consistency.

The main portion of experiments were run in triplicates, and the mean V_g^0 was used for further computations. Only those with methylparaben and propylparaben on polyvinyl chloride were performed individually. Every series of experiments was bracketed by column performance experiments to verify the column stability. The data were considered valid if the column performance parameters were within the experimental error.

Auxiliary Experiments—Vapor pressure and density data for several substances were unavailable and were determined within the framework of this investigation.

Vapor Pressure Determination by Means of GC—The method of Frank (11) using GC was employed. An apparatus suited for the compounds in this work was developed and placed in a gas chromatograph[6] equipped with a thermal conductivity detector[7]. The validity of the procedures and the performance of the apparatus were determined with substances of known vapor pressures. The experimental pressures are shown in Table III and appear to be within 5% of those determined by other means.

Table IV—Experimentally Determined Densities (g./ml.) at Varying Temperatures

Temperature	Chlorobutanol	Phenol	Methylparaben	Propylparaben	Polyvinylchloride
95°	—	1.0400	—	—	—
100°	—	1.0310	—	—	—
110°	1.2960	1.0170	—	—	—
120°	1.2720	1.0050	1.1575	1.0610	1.1834
130°	1.2490	0.9860	—	1.0520	1.1730
135°	—	—	1.1420	—	—
140°	1.1750	—	—	1.0380	1.1655
145°	—	—	1.1310	—	—
150°	—	—	—	1.0250	1.1590
155°	—	—	1.1200	—	—
160°	—	—	—	—	1.1490
170°	—	—	—	—	1.1425

Density Determinations—These were performed at several temperatures with a modified Russell (14) pycnometer immersed in an oil bath, since the substances are solids at ambient conditions. The densities were plotted against temperature and the required values were read from the linear plots. The experimentally determined densities are shown in Table IV.

RESULTS AND DISCUSSION

The specific retention volumes computed from Eq. 1 depend on the vapor pressures of the solutes at the respective temperatures and, consequently, are not a reliable measure of the solute–solvent interaction energies. They are shown in the *Appendix* tables since they may

[6] Model 90 P-3, Varian-Aerograph, Walnut Creek, Calif.
[7] For particulars on the apparatus and the procedures, see the doctoral dissertation.

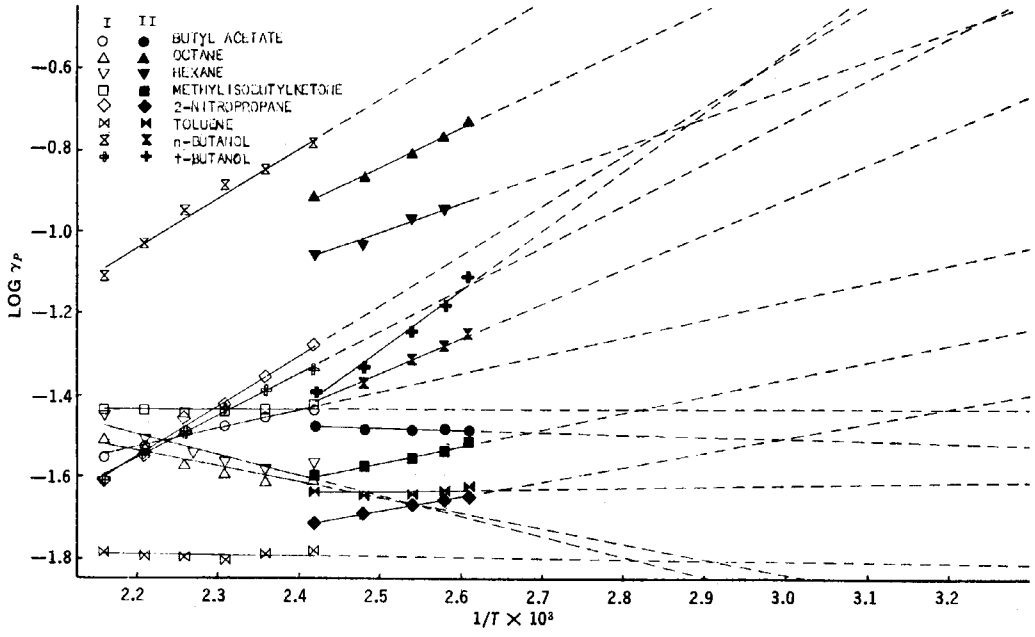

Figure 1—*Apparent activity coefficients at varying temperatures for the organic solvents. Key: I, polyethylene; and II, polyvinyl chloride.*

be of chromatographic utility. Their use in this work is in the computation of Raoult's law activity coefficients by means of Eq. 2. The coefficients depend exclusively on the interaction energies in the solute-solvent systems and were, therefore, used for relative grading of interaction affinities. The apparent activity coefficients are presented in Fig. 1 for the organic solvents and in Fig. 2 for the bacteriostatic agents. From the slopes of these plots, it may be seen that their temperature dependence varies considerably from solute to solute; therefore, the relative grading of the interaction affinities would depend on the temperature at which they are compared. To obtain the coefficients at ambient temperature and the temperatures used for accelerated stability testing, the plots were extrapolated as shown by the dashed lines. The theoretical premises and good linear fit of the data permitted this extrapolation which employed the regression points to reduce the error. Since the polymers contain crystalline regions at these temperatures, the extrapolated activity coefficients are pertinent only to the amorphous portion of the polymer (15).

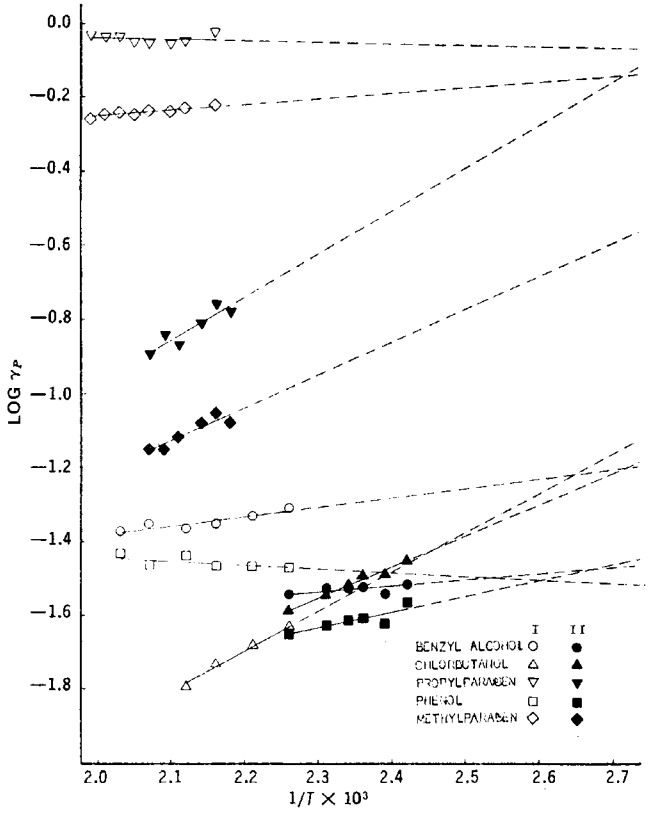

Figure 2—*Apparent activity coefficients at varying temperatures for the bacteriostatic agents. Key: I, polyethylene; and II, polyvinyl chloride.*

The extrapolated activity coefficients are presented in Table V. The solutes are arranged in the order of decreasing activity coefficient values, *i.e.*, in increasing order of interaction affinity, within each group. These values reveal that, for the most part, the sorption of the solutes by the polymers is favored even at ambient temperatures, as evidenced by the values smaller than unity. Referring to the organic solvents in respect to polyethylene, it may be seen that the highest activity coefficients were exhibited by *n*-butanol, 2-nitropropane, and *tert*-butanol. The lowest values were observed for toluene, hexane, and octane, and this finding agrees with the general knowledge on miscibility and sorption for these systems. Comparing the values in relation to the polymers reveals a general trend for reversal of the interaction affinity grading. Thus, octane and hexane were among the least miscible with polyvinyl chloride, and 2-nitropropane showed a relatively strong mixing tendency with that polymer. The relative ordering of normal and *tert*-butanol was reversed, and the same was observed with methyl isobutyl ketone and butyl acetate.

Concerning the bacteriostatic agents, the least interaction affinity with both polymers was exhibited by the parabens. Propylparaben was more miscible with polyethylene than methylparaben and vice versa in respect to polyvinyl chloride. The miscibility of the

Table V—Apparent Activity Coefficients at 25° [a]

On Polyethylene		On Polyvinyl Chloride	
n-Butanol	3.800	Octane	1.000
2-Nitropropane	0.813	*tert*-Butanol	0.902
tert-Butanol	0.432	Hexane	0.393
Butyl acetate	0.097	*n*-Butanol	0.237
Methyl isobutyl ketone	0.040	Methyl isobutyl ketone	0.062
Toluene	0.017	2-Nitropropane	0.042
Hexane	0.009	Butyl acetate	0.030
Octane	0.009	Toluene	0.026
Methylparaben	1.070	Propylparaben	1.335
Propylparaben	0.776	Methylparaben	0.891
Chlorobutanol	0.367	Chlorobutanol	0.182
Benzyl alcohol	0.089	Phenol	0.048
Phenol	0.038	Benzyl alcohol	0.039

[a] The coefficients at 30, 40, and 60° are presented in the dissertation. The relative grading remained the same at these temperatures.

others was in the following increasing order: with polyethylene—chlorobutanol < benzyl alcohol < phenol, and with polyvinyl chloride—chlorobutanol < phenol < benzyl alcohol. It may also be seen that at ambient conditions the miscibility of chlorobutanol and benzyl alcohol was less with polyethylene than with polyvinyl-chloride; for phenol, it was slightly less with polyvinyl chloride than with polyethylene.

The experimental error incurred during the GLC procedures did not exceed 3%, as evidenced by the fluctuations of replicate runs and the linear fit in Figs. 1 and 2. The errors arising from gas imperfections, approximations of vapor pressures, and other similar data are estimated to be within 5%.

CONCLUSIONS

This work has shown that GLC techniques may be applied successfully for evaluating and grading interaction affinities between some polymers employed in packaging and low molecular weight compounds contained in food and pharmaceuticals or used for package fabrication. The methodology is applicable for low molecular weight compounds which are volatile and chemically stable at the temperatures employed for GLC. The chemical stability of the polymers at these temperatures is also a necessary requirement. The procedures were fast and relatively simple to perform. The required instrumentation is widely available. The proposed auxiliary techniques were accessible and adequate for generating the data needed for the computations, and values not previously reported were obtained by this means. The methodology employed may, therefore, be considered a useful tool for evaluating and grading sorption-desorption propensities for polymer–low molecular weight compound systems.

The thermodynamics of the interactions and their elucidation are presented in the second part of this work (5).

APPENDIX

Table VI—Specific Retention Volumes at Varying Temperatures for the Organic Solvents

Solute	Polymer	V_g^0, ml. 383°K.	413°K.	443°K.
n-Butanol	Polyethylene	—	4.398	2.812
2-Nitropropane	Polyethylene	—	15.350	8.643
tert-Butanol	Polyethylene	—	5.117	3.556
Methyl isobutyl ketone	Polyethylene	—	21.050	11.206
Butyl acetate	Polyethylene	—	28.186	15.300
Hexane	Polyethylene	—	9.119	4.876
Octane	Polyethylene	—	41.650	18.857
Toluene	Polyethylene	—	41.740	22.206
Octane	Polyvinyl chloride	4.781	3.178	—
Hexane	Polyvinyl chloride	—	1.059	—
tert-Butanol	Polyvinyl chloride	2.737	2.174	—
n-Butanol	Polyvinyl chloride	13.202	6.887	—
Butyl acetate	Polyvinyl chloride	25.069	11.626	—
Methyl isobutyl ketone	Polyvinyl chloride	22.063	11.763	—
Toluene	Polyvinyl chloride	24.079	11.262	—
2-Nitropropane	Polyvinyl chloride	34.448	15.778	—

Table VII—Specific Retention Volumes at Varying Temperatures for the Bacteriostatic Agents

Solute	Polymer	V_g^0, ml. 413°K.	443°K.	463°K.
Benzyl alcohol	Polyethylene	—	85.02	51.64
Phenol	Polyethylene	—	59.38	30.08
Chlorobutanol	Polyethylene	—	56.45	35.13
Benzyl alcohol	Polyvinyl chloride	141.95	54.46	—
Chlorobutanol	Polyvinyl chloride	40.01	19.15	—
Phenol	Polyvinyl chloride	83.78	33.64	—
		V_g^0, ml. 463°K.	483°K.	503°K.
Propylparaben	Polyethylene	193.86	102.53	55.90
Methylparaben	Polyethylene	94.59	53.13	30.87
Propylparaben	Polyvinyl chloride	389.42	263.65	—
Methylparaben	Polyvinyl chloride	240.57	164.70	—

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Evaluation of Interactions between Polymers and Low Molecular Weight Compounds by GLC II: Thermodynamics and Elucidation

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Abstract □ Raoult's law activity coefficients, obtained by GLC procedures for systems consisting of polymers used in packaging as solvents, bacteriostatic agents, and organic solvents as solutes, were related to the Gibbs free energies, enthalpies, and entropies of mixing. Solution theories were employed for elucidating the factors and mechanisms operative in the polymer–low molecular weight compound interactions. The main factor favoring miscibility was the configurational entropy arising from molecular size difference of the components. For the major part of the systems, the contact interactions were unfavorable to mixing in varying degrees. The data strongly indicated that the parabens interacted as aggregates, most probably pairs, participating in dispersion-type interactions; thus, the contact interactions were more favorable to mixing with polyethylene than with polyvinyl chloride. Alternatively, the retention values obtained from GLC were related to the partition coefficients which were used to compare the sorption–desorption propensities of the low molecular weight compounds with respect to the polymers. Heats of solution and vaporization not previously reported were also derived from the data. Agreement with results from other sources is presented.

Keyphrases □ Polymer–low molecular weight compound interactions—sorption–desorption thermodynamic equations, GLC □ Sorption–desorption thermodynamic equations—polyethylene and polyvinyl chloride, 13 low molecular weight compounds, GLC □ Package–content interactions—polymers and low molecular weight compounds, GLC method for evaluating sorption–desorption thermodynamics □ Plastics—interactions of packaging materials with low molecular weight compounds evaluated by GLC, sorption–desorption thermodynamic equations □ GLC—evaluation of polymer–low molecular weight compound interactions, sorption–desorption thermodynamic equations

Raoult's law activity coefficients for systems comprised of polymers used in packaging and low molecular weight compounds were obtained by GLC and employed for relative grading of interaction affinities as described previously (1). These coefficients were further applied in this work for evaluating and elucidating the interaction thermodynamics. Thus, they were linked to the thermodynamics of binary solutions where the polymers, polyethylene and polyvinyl chloride, were the solvents and the low molecular weight compounds, including organic solvents and bacteriostatic agents, were the solutes.

The equations employed for evaluating the excess partial molar functions of mixing were (2–5):

$$\overline{\Delta G}^E = RT \ln \gamma \qquad \text{(Eq. 1)}$$

$$\overline{\Delta H}^E = \left[\frac{d(\overline{\Delta G}^E/T)}{d(1/T)}\right]_p = R\left[\frac{d(\ln \gamma)}{d(1/T)}\right]_p \qquad \text{(Eq. 2)}$$

$$\overline{\Delta S}^E = \frac{\overline{\Delta H}^E - \overline{\Delta G}^E}{T} \qquad \text{(Eq. 3)}$$

where $\overline{\Delta G}^E$ is the Gibbs free energy, $\overline{\Delta H}^E$ is the enthalpy, $\overline{\Delta S}^E$ is the entropy, γ is the activity coefficient, and T is the absolute temperature. These experimentally determined quantities were interpreted by applying solution theory. The enthalpy was attributed to the balance of contact intermolecular interactions between the "like" and the "unlike" molecules from the Hildebrand–Scatchard "regular solution" theory (6, 7). The Flory–Huggins (8, 9) theory was used to compute the configurational entropy arising from molecular size difference of the system components. The theory of Prigogine *et al.* (10), which considers the free volume dissimilarity between the low molecular weight compound and the polymer as a factor contributing a negative enthalpy and more significantly a negative entropy to the free energy, was also employed.

RESULTS AND DISCUSSION

The excess free energies derived through Eq. 1 from the activity coefficients in Part I (1) express the interaction affinities in energy units[1]. Their main utility in the work was for revealing the factors contributing to the interactions through the enthalpy and entropy terms of the Gibbs equation. The excess enthalpies were derived from Eq. 2 using the regression plots of the activity coefficients *versus* temperature. The enthalpies and entropies are shown in Table I. Figures 1 and 2 present the interrelation among the thermodynamic quantities within the Gibbs equation. Since the criterion for miscibility is a negative free energy change, the $-T\,\Delta S$ term is the main factor favoring mixing for most systems. The enthalpies that are mostly positive reduce the negative quantity for the free energy. The balance between these opposing energy contributions determines the level of the Gibbs free energy. Negative enthalpies were obtained only for the alkane–polyethylene and the butyl acetate–polyvinyl chloride systems; thus, the enthalpy and entropy terms are additive toward a negative free energy. For the propylparaben–polyethylene system, the enthalpy and the entropy are both negative. The negative free energy in this case is due to the negative enthalpy, while the positive entropy term reduces the energy favorable to mixing.

According to theory, the entropy receives contributions from the configurational factor due to molecular size difference (8, 9) and from the free volume dissimilarity (10). The configurational entropy was calculated on the basis of the Flory–Huggins theory (8, 9), using the derivations of Ashworth and Everett (11) and of Martire (12), since the literature does not provide indications for preference between them. The entropies obtained according to *Reference 11* were somewhat larger than the ones from *Reference 12*, but the difference did not alter the rationale for interpreting the interactions. The difference between the experimental and the configurational entropies contained the free volume dissimilarity contribution. The configurational entropies were also used to compute the "size-corrected" activity coefficients (4), which reflect the true intermolecular interactions since they contain only the contact interactions and the volume dissimilarity contributions. The values derived are shown in Table II. Comparing the size-corrected coefficients with the experimental ones (1), at the same temperature, reveals a large increase in values for the former. Therefore, the contact interactions and volume dissimilarity contributed

[1] The free energies at varying temperatures are presented in the dissertation.

Table I—Excess Partial Molar Enthalpies and Entropies of Mixing

	On Polyethylene		On Polyvinyl Chloride	
	$\overline{\Delta H}^{Ea}$	$\overline{\Delta S}^{Eb}$	$\overline{\Delta H}^{E}$	$\overline{\Delta S}^{E}$
n-Butanol	5.6	17.2	3.9	15.8
2-Nitropropane	5.7	19.7	1.7	11.9
tert-Butanol	4.6	17.2	6.7	22.7
Butyl acetate	2.1	11.6	−0.2	6.2
Methyl isobutyl ketone	0.2	6.9	1.9	11.9
Toluene	0.1	8.3	0.3	8.1
Octane	−1.8	3.0	4.5	15.0
Hexane	−2.0	2.3	3.4	13.1
Chlorobutanol	5.2	19.1	3.7	15.6
Benzyl alcohol	1.1	8.5	0.5	8.3
Methylparaben	0.9	2.9	4.1	13.7
Phenol	0.1	7.0	1.3	10.5
Propylparaben	−0.3	−0.4	5.2	14.9

[a] $\overline{\Delta H}^{E}$ in kilocalories per mole. [b] ΔS^{E} in calories per mole degree.

energy unfavorable to mixing in varying degrees. The major factor favoring miscibility was the configurational entropy due to molecular size difference. Only for the hydrocarbon–polyethylene systems are the size-corrected coefficients smaller than unity. These are due to the negative enthalpies which, in view of the contact similarity of the components of these systems, result most probably from negative volume changes. This is in agreement with Flory *et al.* (13) who worked with similar systems.

The entropies containing the free volume dissimilarity show a number of negative values which conform with the concepts of Prigogine *et al.* (10). The agreement is clearly illustrated by the alkane–polyethylene systems since hexane would be expected to exhibit a greater free volume dissimilarity with polyethylene than octane. With the polyvinyl chloride systems, for the most part, there is a parallel reduction of values for the size-corrected activity coefficients and the entropies containing the free volume dissimilarity effect, the latter reaching into the negative range. Therefore, as the contact interaction energy increases, the solutes undergo a greater reduction in free volume to fit the intersegmental distances of the polymer. Systems that do not conform to this pattern will be discussed further.

The contact interactions may be considered through the characteristics of the polymers and their relation to the individual solutes. Polyethylene participates exclusively in dispersion-type interactions. Since these are weak, mixing would be favored only when the forces between the like molecules of the solute are also dispersion type. The size-corrected activity coefficients, the entropies containing the free volume dissimilarity, and the enthalpies of the solutes with polyethylene reveal four distinguishable patterns.

Activity Coefficients Greater than Unity and Large Positive Enthalpies—This pattern is typical of solute–solvent interactions unfavorable to mixing because of the greater interaction energy among the solute molecules. The entropy containing the free volume dissimilarity is positive because the solute–solvent interactions are not strong enough to be conducive to volume shrinkage. In this category are *n*-butanol, 2-nitropropane, *tert*-butanol, and chlorobutanol. The *n*-butanol molecules associated by means of hydrogen bonds exhibit the largest activity coefficients since this type of interaction between the "like" molecules is energetically superior to the others in this work. The 2-nitropropane molecules are strong permanent dipoles (14) due to resonance in the nitro group; therefore, the size-corrected activity coefficient is also large but not as large as that for the *n*-butanol. The *tert*-butanol exhibits a considerably smaller coefficient compared to *n*-butanol due to steric shielding of the hydroxyl group. Since this value and the positive enthalpy are smaller than the respective ones for 2-nitropropane, it appears that the interactions between the like molecules may be attributed to dipole forces rather than hydrogen bonding. The dipoles seem to be "buried" in the molecule and the interactions are, therefore, weaker than those for 2-nitropropane. Chlorobutanol appears to behave in a similar manner.

Activity Coefficients Smaller than Unity, Negative Enthalpies and Negative Entropies with Free Volume Dissimilarity—This pattern is characteristic of solute–solvent interactions favorable to mixing. It is observed with hexane and octane. The behavior of this system conforms with the observations of Desmyer and Van DerWaals (15) concerning negative volume changes during mixing of alkanes that were proportional to the chain length difference.

Activity Coefficients Greater than Unity but Negative Entropies Containing Free Volume Dissimilarity—This pattern is observed with methylparaben, propylparaben, phenol, and methyl isobutyl ketone. The parabens appear to behave as low number molecular aggregates, most probably dimers, where a hydroxyl group of one molecule participates in hydrogen bonding with the carboxyl group of the other. The hydrogen bonding and polar forces are thus balanced within the molecular pairs which then participate predominantly in dispersion-type interactions. In agreement with this, the excess enthalpy for the methylparaben is a small positive value; for propylparaben, it is negative approaching those for the alkanes. If a polymeric self-associated structure had been assumed for these compounds, the enthalpies would have been large positive values as observed for *n*-butanol. The size-corrected coefficients for the parabens are large; therefore, the condition unfavorable to mixing arises from the large free volume dissimilarity between the aggregates and the polymer chains as evidenced by the large negative entropies resulting from it. The participation of molecular aggregates in the solute–solvent interactions of phenol and methyl isobutyl ketone with polyethylene rationalizes the thermodynamic quantities observed with these compounds. To a lesser degree, the same appears to be true for benzyl alcohol and butyl acetate, which may be considered as borderline cases. For these, the hydrogen bonding and polar energy are balanced within the aggregates to a lesser degree; therefore, some energy is required to break the bonding with a polymeric structure, as evidenced by the enthalpies.

Activity Coefficients Approaching Unity—This is a special case and is observed with toluene. The enthalpy indicates an almost athermal mixing; thus the main factor favoring miscibility is the configurational entropy. The volume change for this system appears

Table II—Size-Corrected Activity Coefficient[a], γ_S, at 25° and Entropies Containing the Free Volume Dissimilarity, $\overline{\Delta S_V}^{E}$

On Polyethylene			On Polyvinyl Chloride		
Solute	γ_S	$\overline{\Delta S_V}^{Eb}$	Solute	γ_S	$\overline{\Delta S_V}^{E}$
n-Butanol	146.0	8.9	*tert*-Butanol	112.2	13.1
2-Nitropropane	49.8	11.3	Octane	67.7	6.6
tert-Butanol	25.0	8.9	Hexane	36.3	4.2
Butyl acetate	4.35	3.9	*n*-Butanol	29.4	6.3
Methyl isobutyl ketone	1.98	−0.8	Methyl isobutyl ketone	5.55	3.0
Toluene	0.98	0.3	2-Nitropropane	5.41	2.3
Hexane	0.49	−5.3	Toluene	2.74	−1.1
Octane	0.39	−4.3	Butyl acetate	2.55	−2.6
Methylparaben	53.0	−4.9	Propylparaben	251.0	6.4
Propylparaben	30.6	−7.7	Methylparaben	92.5	4.6
Chlorobutanol	17.4	11.6	Chlorobutanol	17.3	6.8
Benzyl alcohol	5.27	0.5	Phenol	5.96	0.9
Phenol	2.47	−1.4	Benzyl alcohol	4.19	−1.0

[a] Computed from *Reference 10*. [b] $\overline{\Delta S}^{E}$ in calories per mole degree.

Table III—Partial Molar Heats of Solution, $\overline{\Delta H^S}$, and Heats of Vaporization, $\overline{\Delta H^V}$

Solute	$\overline{\Delta H^S}$[a] Polyethylene	$\overline{\Delta H^S}$[a] Polyvinyl Chloride	$\overline{\Delta H^V}$[a] Experimental[b]	$\overline{\Delta H^V}$[a] Literature	Reference
n-Butanol	4.5	6.8	10.4	10.3	21
				10.0	3
tert-Butanol	4.1	2.5	8.9	9.3	21
2-Nitropropane	6.7	8.2	11.2	—	
Methyl isobutyl ketone	8.0	6.6	8.4	—	
Butyl acetate	7.3	8.0	8.6	—	
Hexane	8.8	3.6	6.9	6.9	21
				6.4	3
Octane	10.3	4.3	8.6	8.6	3
				8.2	21
Toluene	7.9	7.9	8.1	8.1	2
				8.1	3
				7.9	21
Chlorobutanol	9.6	9.1	13.8	—	
Phenol	11.2	11.4	10.9	10.9	21
Benzyl alcohol	11.8	12.0	12.7	—	
Methylparaben	13.1	10.3	14.2	—	
Propylparaben	14.5	10.6	15.2	—	

[a] In kilocalories per mole. [b] Means of values obtained on polyethylene and on polyvinyl chloride.

to be negligible, as evidenced by the entropy containing the free volume dissimilarity.

The interactions with polyvinyl chloride were shown to involve hydrogen bonding and polar forces (16, 17). Adelman and Klein (18) observed that compounds exhibiting only dispersion forces and those with strong self-associative capacity showed poor interaction affinity for this polymer. Among the compounds capable of hydrogen bonding, the interaction affinity increased with the electron donor capacity, provided steric hindrance did not interfere. This finding is substantiated by the size-corrected activity coefficients and enthalpies in this work. Hexane, octane, and *n*-butanol exhibited a poor interaction affinity, while methyl isobutyl ketone and butyl acetate exhibited a considerably stronger one because of their electron donor capacity. Crowley *et al.* (19) and Hansen and Skaarup (20) assigned higher hydrogen-bonding values to butyl acetate (8.8 and 3.1) than for methyl isobutyl ketone (7.7 and 2.0), and this relative grading of the hydrogen-bonding capacity is substantiated by their enthalpies and size-corrected coefficients in this work. Benzyl alcohol and phenol appear to participate in strong hydrogen bonding with polyvinyl chloride. Most probably, these compounds exhibit less self-association compared to *n*-butanol and thus participate more readily in hydrogen bonding. The large activity coeffi-

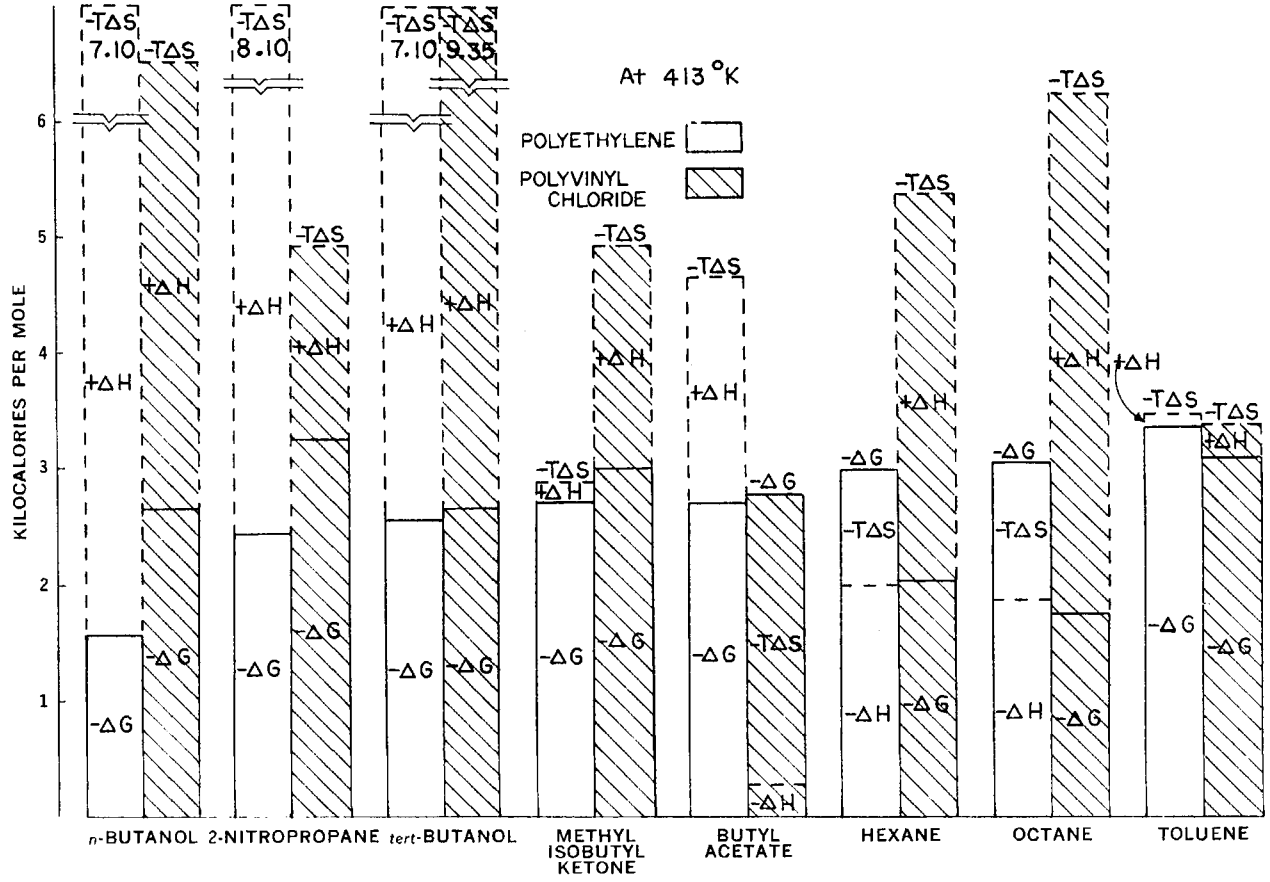

Figure 1—*Enthalpy and entropy contributions to the Gibbs free energy of mixing for the organic solvents group.*

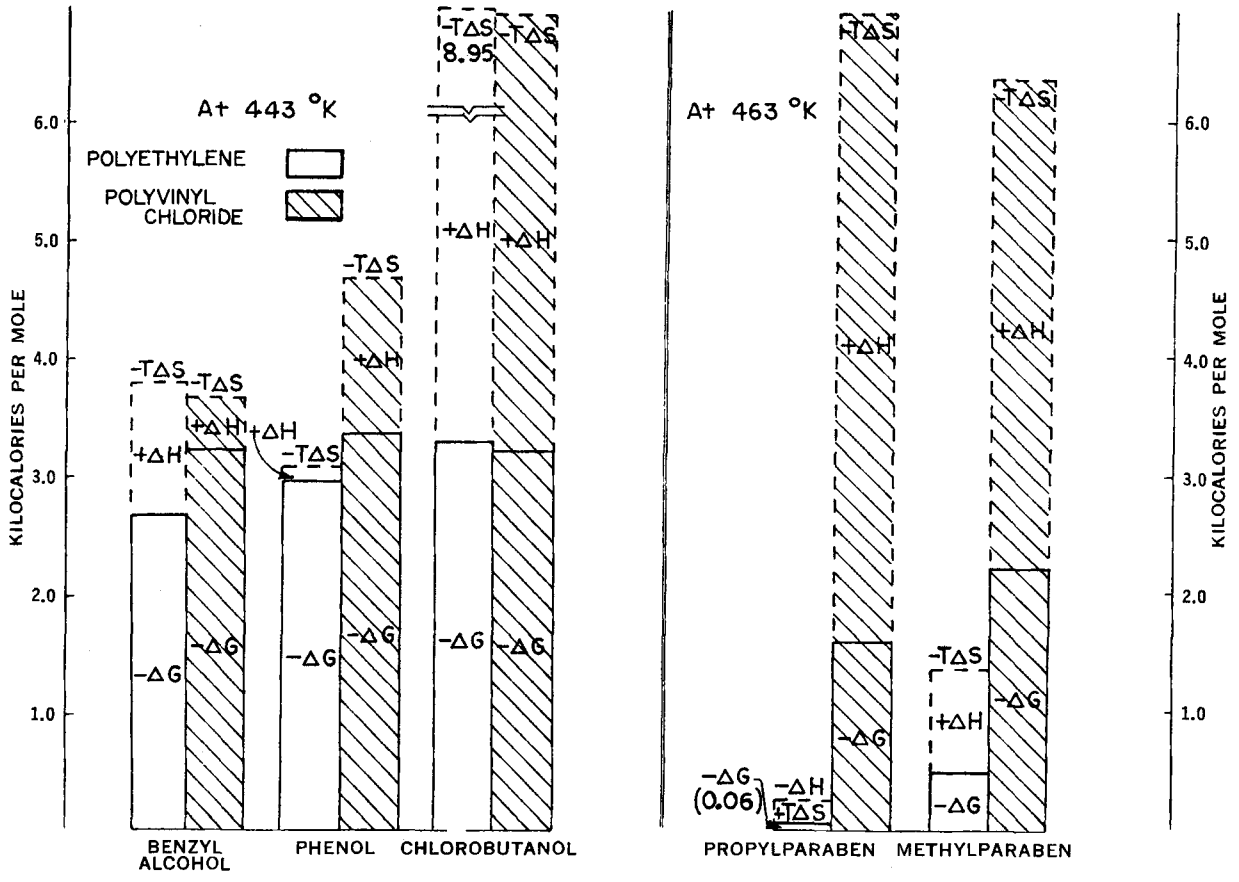

Figure 2—*Enthalpy and entropy contributions to the Gibbs free energy of mixing for the bacteriostatic agents.*

cients and large positive enthalpies for *tert*-butanol are most probably due to steric factors. The interaction affinity for chlorobutanol is considerably greater than for *tert*-butanol, indicating that hydrogen bonding involving a chlorine atom of the solute may be occurring. The parabens show large positive enthalpies, which approach those of octane and hexane, and also large size-corrected activity coefficients. These quantities indicate contact interactions considerably more resistant to mixing of the parabens with polyvinyl chloride than the respective values with polyethylene. The rationalization that the parabens interact as aggregates exhibiting predominantly dispersion forces is, therefore, further substantiated. The interaction affinity of toluene with polyvinyl chloride is strong, as may be seen from the small positive enthalpy and the small activity coefficient, although these values are somewhat less favorable to mixing than the respective ones for polyethylene. Crowley *et al.* (19) and Hansen and Skaarup (20) assigned smaller hydrogen-bonding values for toluene (4.5 and 1.0) than those for methyl isobutyl ketone and butyl acetate; therefore, the hydrogen-bonding capacity of toluene does not completely rationalize the observed interaction affinity. The behavior of this system supports the observation (18) that cyclic or bulky molecules exhibit enhanced interaction affinity compared to flexible straight-chain aliphatic molecules because they act to separate the polymer chains and thus reduce the interchain forces.

Supplementary Data Obtained by GLC—In addition to serving the main objectives of this work, the methodology employed provides a means for obtaining data that are closely related to the main purpose or are of general interest.

Heats of Solution (Sorption) and Heats of Vaporization—Littlewood *et al.* (2) demonstrated that the partial molar heat of solution, $\overline{\Delta H}^S$, for a solute may be obtained from the slope of the log V_g^0 *versus* $1/T$ plots and sign inversion. This quantity is identical to the heat of sorption. The heat of vaporization, $\overline{\Delta H}^V$, may be derived from the excess heat of mixing and the heat of solution through (4):

$$\overline{\Delta H}^V = \overline{\Delta H}^E - \overline{\Delta H}^S \qquad \text{(Eq. 4)}$$

Table III presents the values obtained in this manner. The comparison with data from other sources is reasonably good.

Partition Coefficients—The partition coefficients, K, of the solutes at the experimental temperatures were derived using the specific retention volumes from (22):

$$K = \frac{V_g^0 \rho_1 T}{273} \frac{\text{weight/milliliter of liquid phase}}{\text{weight/milliliter of gaseous phase}} \qquad \text{(Eq. 5)}$$

where ρ_1 is the density of the solvent at the column temperature. The heats of solution were applied to compute the partition coefficients at varying temperatures from (22):

$$\log \frac{K}{T\rho_1} = \frac{\overline{\Delta H}^S}{2.303RT} + C \qquad \text{(Eq. 6)}$$

where C is a constant. The values computed from Eq. 6 for the experimental temperatures and those found experimentally coincided, indicating that the heats of solution employed in the computations were valid within that temperature range. The partition coefficients are meaningful for comparing the sorption propensities of a solute in respect to different polymers at identical temperature and concentration of the solute in the surrounding medium, since they depend both on the vapor pressure of the solute and the solute–solvent interaction energy. The comparison is in weight of solute per volume of polymer rather than in energy quantities or activity coefficients and may thus be more directly pertinent to applied sorption–desorption considerations. Further convenience in comparing the partitioning of a solute with respect to the two polymers may be gained by means of the ratios of the partition coefficients (Table IV)[2]. It may be noted that a unit volume of polyethylene contained almost twice the mole quantity compared to polyvinyl chloride; consequently, the partition coefficients do not always follow the

[2] The partition coefficients at 25, 30, 40, 60, and 140° are presented in the dissertation.

Table IV—Ratios of Partition Coefficients

	$K_{polyethylene}/K_{polyvinyl\ chloride}$ 25°	40°	60°
n-Butanol	0.154	0.185	0.230
2-Nitropropane	0.328	0.367	0.426
tert-Butanol	3.50	3.07	2 62
Butyl acetate	1.20	1.27	1.36
Methyl isobutyl ketone	2.32	2.08	1.82
Toluene	2.48	2.49	2.50
Hexane	69.0	45.1	27.2
Octane	149	102	51.6
Methylparaben	1.40	1.11	0.845
Propylparaben	3.18	2.39	1.65
Chlorobutanol	2.60	2.51	2.49
Benzyl alcohol	0.096	0.980	0.995
Phenol	1.08	1.10	1.12

relative grading obtained from the activity coefficients presented in Part I (1).

Comparison with Data from Other Sources—The results obtained in this work concerning the organic solvents correlate with the general knowledge of solvent–polymer miscibility; however, there is a scarcity of values available for direct comparison. Therefore, some additional evidence is presented which has a bearing in substantiating these results.

Martire (12) determined the activity coefficients of several hydrocarbons on *n*-eicosane which is expected to exhibit interaction characteristics similar to polyethylene. To make the analogy more valid, the values from this work used for comparison are the size-corrected activity coefficients:

hexane/*n*-eicosane: $\gamma = 0.877$ from *Reference 12*
hexane/polyethylene: $\gamma = 0.643$ from this work
toluene/*n*-eicosane: $\gamma = 0.888$ from *Reference 12*
toluene/polyethylene: $\gamma = 0.982$ from this work

It may be seen that, considering the assumptions made, the activity coefficients are in good agreement.

It was shown that the activity coefficients for the toluene–polyethylene system may be attributed predominantly to configurational entropy and could, therefore, be computed from theory. The comparison is presented below:

experimental	0.0167
calculated from *Reference 11*	0.0172
calculated from *Reference 12*	0.0188

The observed agreement substantiates the results in this work and its theoretical premises. For this system *Reference 11* yielded better agreement with experimental values than *Reference 12*.

The pharmaceutical literature offers very few possibilities for comparison. Autian (23) reported sorption of bacteriostatic agents by polyethylene determined by static methodology at 50°. The grading of sorption propensities appears to be phenol > benzyl alcohol > propylparaben > methylparaben, and it coincides with the grading of the activity coefficients in Part I (1).

CONCLUSIONS

The thermodynamic quantities obtained from GLC were meaningful toward revealing the nature of the interactions in the investigated systems. The values obtained from theory are approximations which should be regarded as indicators for elucidating the interactions—not as rigorous quantities. This, however, does not detract from the value of the presented approach. Its utility for evaluation and elucidation of polymer–sorbent interactions is substantiated by the evidence from other sources and the consistency with solution theory.

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Effect of Trichloroacetate Anion on Partition Behavior of Tetracyclines

PAUL R. KLINK and JOHN L. COLAIZZI▲

Abstract □ The influence of the trichloroacetate anion on the *n*-octyl alcohol–aqueous phosphate buffer apparent partition coefficients of various tetracyclines was determined. At acidic pH values, the presence of trichloroacetate caused a significant increase in the apparent partition coefficients of most of the tetracycline analogs studied, presumably through intermolecular ion-pairing between the positively charged tetracycline moiety and the trichloroacetate anion. From intermediate to mildly alkaline pH values, trichloroacetate had essentially no effect on partitioning. The transfer of compounds such as minocycline and 9-dimethylamino-6-demethyl-6-deoxytetracycline to the *n*-octyl alcohol phase was not as significantly altered by the addition of trichloroacetate, apparently because the effect of two charged moieties in each of these analogs was not as easily overcome by ion-pair formation. The alteration of the apparent partition coefficients of the tetracycline antibiotics by intermolecular ion-pair formation is discussed in terms of its relationship to the absorption of these compounds.

Keyphrases □ Trichloroacetate—effect on tetracycline partition coefficients, ion-pair formation □ Tetracycline partition coefficients —trichloroacetate effect, ion-pair formation □ Ion-pair formation —effect of trichloroacetate on tetracycline partition behavior

Irwin *et al.* (1) defined ion-pairs as neutral species formed by electrostatic attraction between oppositely charged ions in solution, which are often sufficiently lipophilic to dissolve in nonaqueous solvents. The concept of ion-pairs was introduced in 1926 to account for the low conductance of strong electrolytes in nonaqueous solutions (2). Kraus (3) elaborated on the ion-pair concept and discussed in mathematical terms the electrostatic forces holding ions together in a pair. Ions that unite in some fashion to form a pair essentially cancel all or part of the charge of each ion, and thus there are fewer species in the solution having an exposed charge.

Since the pH–partition hypothesis does not readily explain the absorption of highly ionized compounds, such as quaternary ammonium compounds, sulfonic acids, dextromethorphan, and the tetracyclines, Irwin *et al.* (1) postulated that ion-pair formation and subsequent transport across the lipid GI barrier may constitute an important mechanism for drug absorption. By increasing the lipophilic character of isopropamide through ion-pair formation, they showed increased mydriatic activity in mice. Through the selection of appropriate ion-pair formers, the efficiency, rate, and uniformity of absorption from the GI tract of other highly ionized drugs such as the tetracyclines may also be significantly enhanced. The objectives of this investigation were: (*a*) to study the effect of the trichloroacetate anion on the apparent partition coefficients of the various tetracyclines, and (*b*) to consider the possibility of ion-pair formation between trichloroacetate anion and the various ionic forms of the tetracyclines that exist at different pH values.

EXPERIMENTAL

The experimental design was similar to that used in determining the relative lipophilicity of the different ionic forms of the tetracyclines (4). The tetracycline analogs were obtained from the same sources and were of the same purity as those used previously. Phosphate buffers containing trichloroacetate anion were freshly prepared. Trichloroacetate was prepared by neutralizing a solution of trichloroacetic acid[1] with a solution of sodium hydroxide[2]. To force the equilibrium to the direction favoring ion-pair formation, the concentration of trichloroacetate anion in the buffer solutions was 50 times the molar concentration of the tetracycline analog in the study, unless specified otherwise. The ionic strength was maintained at 0.1 for all buffers by the addition of sodium chloride[3], if necessary. The total molar buffer concentration was 0.050 for buffers of pH 2.1, 3.0, 3.9, and5.6, but it was reduced for buffers of pH 6.6, 7.5, and 8.5 to 0.042, 0.027, and 0.025, respectively, so as not to exceed the ionic strength of 0.1. The ion content of the water used in these experiments was below 0.1 p.p.m. (as sodium chloride) as determined by a conductivity meter[4].

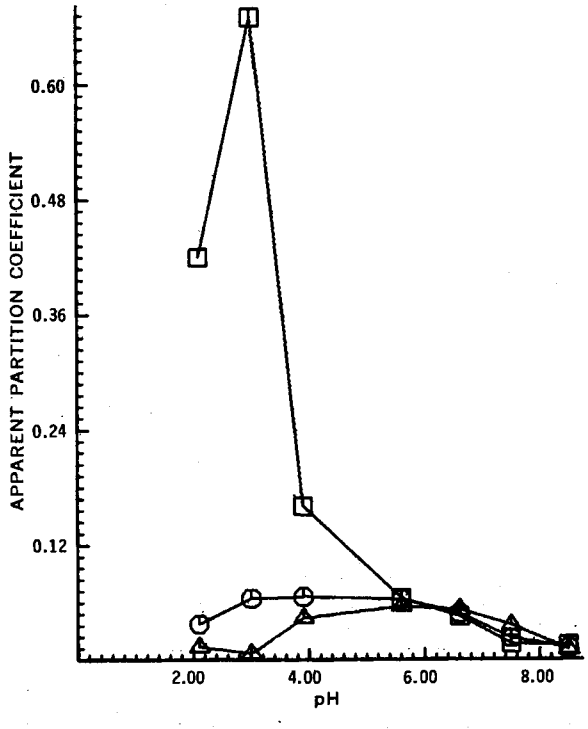

Figure 1—*Comparison of the effect of pH and various concentrations of trichloroacetate anion on the* n*-octyl alcohol–aqueous phosphate buffer apparent partition coefficients for 0.00052* M *tetracycline hydrochloride at 25°. Key:* □, *tetracycline hydrochloride and 0.02600* M *trichloroacetate;* ○, *tetracycline hydrochloride and 0.00520* M *trichloroacetate; and* △, *tetracycline hydrochloride (no trichloroacetate).*

[1] Analytical reagent grade, Mallinckrodt Chemical Works, St. Louis, Mo.
[2] Analytical reagent grade, Baker Chemical Co., Phillipsburg, N. J.
[3] Certified reagent grade, Fisher Scientific Co., Fair Lawn, N. J.
[4] Barnstead Still and Sterilizer Co., Boston, Mass.

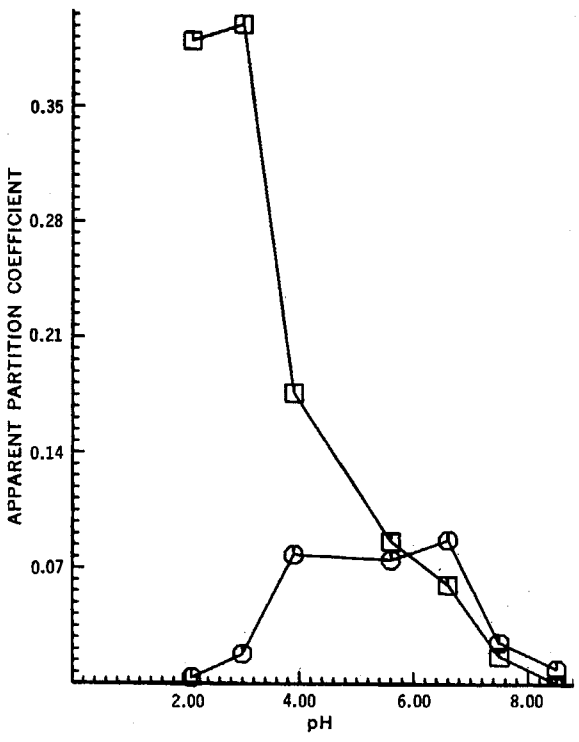

Figure 2—*Plot showing the effect at 25° of 0.02600* M *trichloroacetate on the* n*-octyl alcohol–phosphate buffer apparent partition coefficients of 0.00052* M *oxytetracycline hydrochloride at various pH values. Key:* □, *oxytetracycline hydrochloride and trichloroacetate; and* ○, *oxytetracycline hydrochloride.*

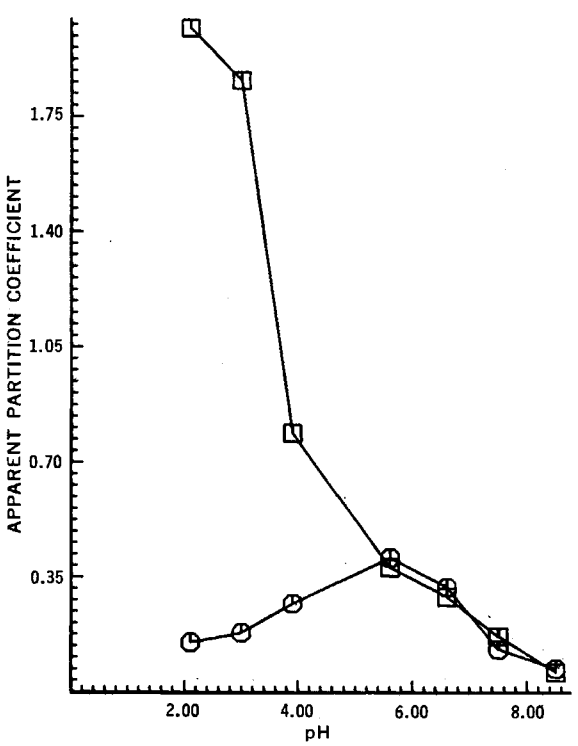

Figure 3—*Plot showing the effect at 25° of 0.02600* M *trichloroacetate on the* n*-octyl alcohol–phosphate buffer apparent partition coefficients of 0.00052* M *chlortetracycline hydrochloride at various pH values. Key:* □, *chlortetracycline hydrochloride and trichloroacetate; and* ○, *chlortetracycline hydrochloride.*

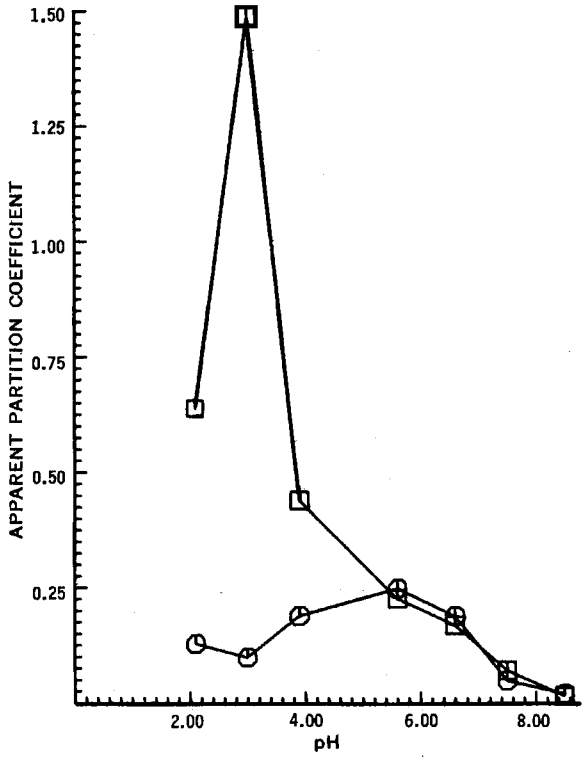

Figure 4—*Plot showing the effect at 25° of 0.02600* M *trichloroacetate on the* n*-octyl alcohol–phosphate buffer apparent partition coefficients of 0.00052* M *demeclocycline hydrochloride at various pH values. Key:* □, *demeclocycline hydrochloride and trichloroacetate; and* ○, *demeclocycline hydrochloride.*

The apparent partition coefficients of the tetracycline analogs in the presence of trichloroacetate anion were determined as previously described (4) for the tetracycline analogs. The procedure was carried out for each of four test flasks containing 10 ml. *n*-octyl alcohol saturated buffer solution, which was 5.20×10^{-4} *M* in tetracycline analog and 2.60×10^{-2} *M* in trichloroacetate anion. The blank consisted of *n*-octyl alcohol saturated buffer solution containing trichloroacetate anion.

RESULTS AND DISCUSSION

Partitioning Experiments—Figures 1–8 illustrate the effects of trichloroacetate on the apparent partition coefficients of selected tetracycline analogs. At the acidic experimental pH values of 2.1 and 3.0, tetracycline hydrochloride, oxytetracycline hydrochloride, chlortetracycline hydrochloride, demeclocycline (demethylchlortetracycline) hydrochloride, methacycline hydrochloride, and doxycycline hyclate exhibited a marked increase in partitioning in the presence of a 50 *M* excess of trichloroacetate anion (Figs. 1–6). On the other hand, minocycline (7-dimethylamino-6-demethyl-6-deoxytetracycline hydrochloride) and 9-dimethylamino-6-demethyl-6-deoxytetracycline were not greatly affected at any of the experimental pH values (except possibly for minocycline at pH 3.9), as Figs. 7 and 8 illustrate.

The very significant increase in the apparent partition coefficients at the acidic pH values of 2.1 and 3.0 (Figs. 1–6) can be rationalized on the basis of the percent of the cationic form of tetracycline analog present. As shown in Fig. 8 of *Reference 4*, the tetracycline molecule exists predominantly as a cation at the experimental pH values of 2.1 and 3.0. Due to the larger concentration of cations, there is greater potential for interaction with trichloroacetate. More cations would be participating in an ion-pair formation, thus imparting greater lipophilicity to the tetracycline system.

The possible role of intermolecular ion-pair formation in enhancing lipophilicity or membrane transfer of tetracycline analogs, as reflected by changes in the magnitude of apparent partition coefficients, depends upon the molar concentration of the anion available for ion-pair formation, as illustrated in Fig. 1 for tetracycline, and

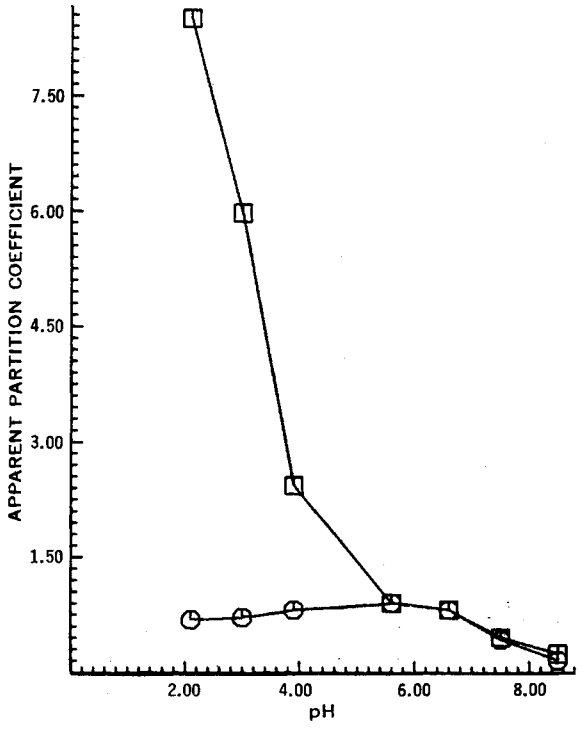

Figure 5—*Plot showing the effect at 25° of 0.02600* M *trichloroacetate on the* n*-octyl alcohol–phosphate buffer apparent partition coefficients of 0.00052* M *methacycline hydrochloride at various pH values. Key:* ⊡, *methacycline hydrochloride and trichloroacetate; and* ○, *methacycline hydrochloride.*

upon the ability or tendency of the analog to pair with other ions. In the case of minocycline and 9-dimethylamino-6-demethyl-6-deoxytetracycline, it appears that the presence of the two dimethylamino moieties in each of these analogs apparently limits the effectiveness of intermolecular ion-pair formation in producing a more lipophilic compound (Figs. 7 and 8).

In the intermediate pH region (beginning at pH 5.6), the trichloroacetate anion appears to have essentially no effect upon the apparent octyl alcohol–aqueous buffer partition coefficients of tetracycline analogs. As noted previously (4), in the absence of the trichloroacetate anion, the maximum apparent partition coefficients of the various tetracycline analogs occurred at the experimental pH values of 5.6 and 6.6, where the existence of isoelectric species of the tetracyclines is favored.

In the absence of trichloroacetate anion, tetracycline methiodide exhibited essentially no transfer into the *n*-octyl alcohol phase (4). In the presence of trichloroacetate anion, however, transfer was exhibited at all pH values studied except at pH 7.5 and 8.5[5].

Biological Implications—Hogben *et al.* (5) and Schanker (6) indicated that the rate of absorption of various weak electrolytes parallels the lipid–aqueous partition coefficients of the unionized form of a drug. However, a drug must be in the solution state to be absorbed. If a significant concentration of dissolved drug in the aqueous contents of the GI tract cannot be achieved, absorption may be negligible in spite of a favorable partition coefficient. For optimum absorption of dissolved drug, the partition coefficient should not be so high that the drug accumulates in lipophilic phases and is denied access to aqueous barriers, nor should it be so low that the drug cannot enter a lipophilic membrane such as the intestinal wall at a sufficient rate to provide an adequate flux. In the case of the tetracyclines, it was suggested (4) that there may be a direct relationship between apparent octyl alcohol–aqueous buffer partition coefficients and quantity of each analog absorbed or quantity of each analog necessary to obtain comparable blood levels. The authors also noted that the pH values of highest apparent partition coefficient for the various analogs correspond to the pH values at which optimum antimicrobial activity has been reported (7). This seems to indicate that it might be possible to enhance absorption and/or activity by increasing the apparent partition coefficients of the various analogs.

A low gastric pH is essential for rapid dissolution of tetracycline. The aqueous solubility of tetracycline is about 100 times greater at pH 1–3 than at pH 5–6 (8). The oral administration of a 2-g. dose of sodium bicarbonate has been shown to reduce significantly the rate and the cumulative amount of tetracycline excreted in the urine of humans. This observation has been attributed to the fact that the gastric pH increased above pH 4, which is less favorable for the dissolution of tetracycline (8).

By maintaining normal gastric pH, a more rapid and complete dissolution of tetracycline may be achieved. However, at low pH values the apparent partition coefficients of many of the tetracycline analogs are markedly lower and this could cause absorption to be significantly lower. Thus, it would seem that if the apparent partition coefficients of the tetracyclines could be increased at lower pH values without greatly affecting dissolution, absorption of the analogs from the stomach would be enhanced beyond that suggested by the work of Pindell *et al.* (9) in dogs and of Perrin and Vallner (10) in rats.

Certain anions, such as perfluorobutyrate, perfluoropropionate, trichloroacetate, and citrate (11, 12) have been shown to promote the absorption of the tetracyclines, although the mechanism has not been completely elucidated. Irwin *et al.* (1) showed that trichloroacetate anion increases both the lipid solubility and the absorption of a quaternary ammonium compound, isopropamide, presumably through ion-pair formation. This same phenomenon likely accounts for the increased partition coefficients of the tetracyclines observed in the present study at lower pH values in the presence of trichloroacetate anions. The negative charge of the trichloroacetate anion combines with the positive charge of the cationic form of the tetra-

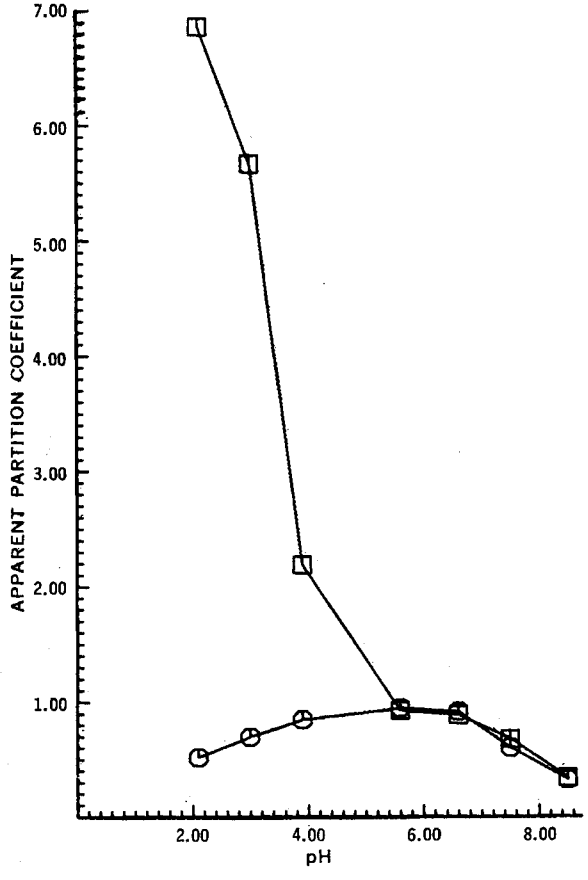

Figure 6—*Plot showing the effect at 25° of 0.02600* M *trichloroacetate on the* n*-octyl alcohol–phosphate buffer apparent partition coefficients of 0.00052* M *doxycycline hyclate at various pH values. Key:* ⊡, *doxycycline hyclate and trichloroacetate; and* ○, *doxycycline hyclate.*

[5] Apparent partition coefficients for tetracycline methiodide in the presence of trichloroacetate anion were 0.47 at pH 2.1, 6.23 at pH 3.0, 0.17 at pH 3.9, 0.02 at pH 5.6, 0.01 at pH 6.6, and zero at the remaining two pH values.

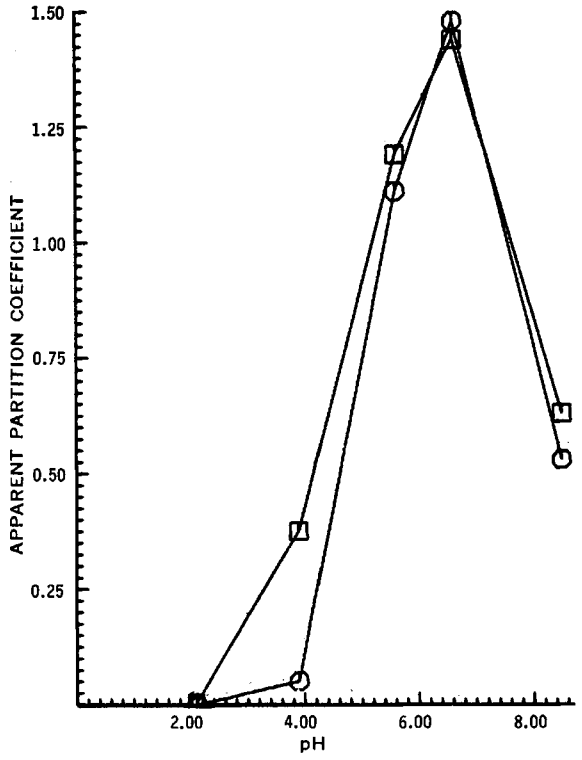

Figure 7—*Plot showing the effect at 25° of 0.02600* M *trichloroacetate on the* n*-octyl alcohol–phosphate buffer apparent partition coefficients of 0.00052* M *minocycline hydrochloride at various pH values. Key:* □, *minocycline hydrochloride and trichloroacetate; and* ○, *minocycline hydrochloride.*

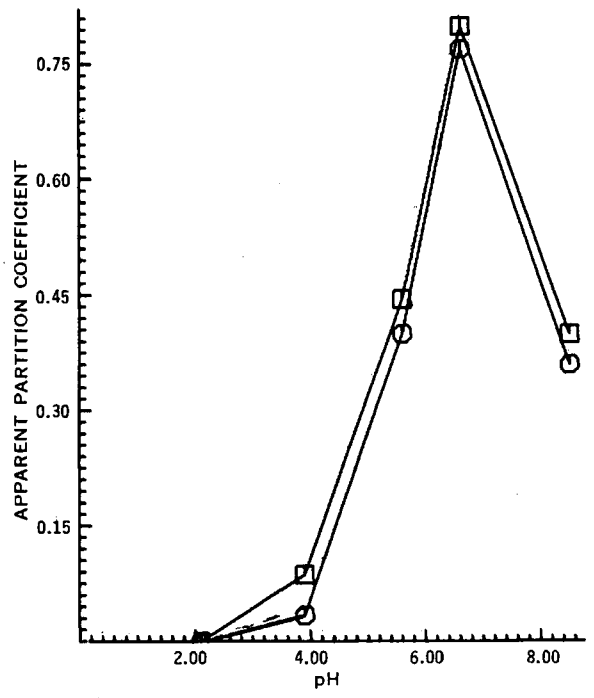

Figure 8—*Plot showing the effect at 25° of 0.02600* M *trichloroacetate on the* n*-octyl alcohol–phosphate buffer apparent partition coefficients of 0.00052* M *9-dimethylamino-6-demethyl-6-deoxytetracycline at various pH values. Key:* □, *9-dimethylamino-6-demethyl-6-deoxytetracycline and trichloroacetate; and* ○, *9-dimethylamino-6-demethyl-6-deoxytetracycline.*

cycline molecule, thus forming an intermolecular type of ion-pair which is more lipid soluble by reason of being more thermodynamically stable in nonaqueous environments.

Perrin and Vallner (10) utilized ^{14}C-labeled trichloroacetate to study the absorption of tetracycline from various buffer systems. These authors showed that less trichloroacetate was absorbed into the phosphate buffer from the stomach pouch of the rat when tetracycline was added to the mucosal fluid, even though both the extent and rate of tetracycline absorption were increased by trichloroacetate. They concluded that tetracycline was not being absorbed in conjunction with trichloroacetate.

In the presence of trichloroacetate, one can expect increased partitioning of tetracycline at the lower pH values in the biological system due to ion-pair formation. One cannot, on the other hand, account for the general effects of the formed complex on the integrity of the barrier. If partitioning is increased by a factor of 10 but permeability is decreased by 25%, there is a net 7.5-fold increase in tetracycline flux. Since the permeability of the free acid species would experience a similar decrease due to changes in general membrane integrity but, unlike tetracycline, the membrane concentration would not be substantially increased by ion-pair formation due to the huge molar excess at which trichloroacetate is present in the lumen, there would be a decrease in trichloroacetate flux. That such general effects on membranes occur is well documented for cholesterol, anti-inflammatory steroids, *etc.* Therefore, Perrin and Vallner's experiment does not rule out partitioning as the dominant effect and, based on other considerations, their surface tension theory is questionable. One should point out here that surface excesses resulting in lowered surface tensions are determined by the same free energy factors that determine partitioning between aqueous and lipid phases.

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GLC Determination of Strychnine and Brucine in Pharmaceutical Preparations

DAVID L. SONDACK▲ and WILLIAM L. KOCH

Abstract □ The quantitative analysis of strychnine and brucine in pharmaceutical preparations is described. Samples are made alkaline and extracted with chloroform. The organic layer is collected and evaporated. The residue is dissolved in an aliquot of the internal standard solution in chloroform. Favorable quantitation has been achieved using papaverine as the internal standard for samples containing both strychnine and brucine. For samples containing strychnine only, brucine may be used. The rapidity and precision of this method represent an improvement over previous assays.

Keyphrases □ Strychnine and brucine—GLC analysis in liquid and tablet formulations □ Brucine and strychnine—GLC analysis in liquid and tablet formulations □ GLC—analysis, strychnine and brucine formulations

Strychnine and brucine are the most common tertiary *Strychnos* alkaloids (1). Many methods for their estimation have been described, including gravimetric and titrimetric analyses (2), paper chromatography (2–4), TLC (5), ion-exchange chromatography (6), spectrophotometry (7), and NMR spectroscopy (8). The two alkaloids have been separated by GLC (9, 10). This article describes the application of GLC to the estimation of strychnine and brucine in some liquid and tablet formulations.

EXPERIMENTAL[1]

Materials—Helium was used as a carrier gas, and electrolytic hydrogen and oxygen were used in the detector. The stationary phase was 3.0% OV-1 on Gas Chrom Q[2], 80–100 mesh, and was packed in borosilicate glass columns, 0.91 m. × 0.64 cm. o.d. All chemicals used were reagent grade or the best quality available.

Operating Conditions—The column was operated isothermally at 280° with the detector block and injection port at 295°. The helium flow rate was 55 ml./min. with an inlet pressure of 40 p.s.i. The electrometer range was 10 with an attenuation of 128. Sample injections of between 1 and 5 μl. were made.

Internal Standard—Papaverine hydrochloride, 1 mg./ml. in chloroform, was used as an internal standard for the formulation containing both strychnine and brucine. The quinine-containing elixirs displayed a large peak in a position to interfere with the use of papaverine. Since brucine was absent from the formulations, it was used. Either brucine or papaverine could be used for samples containing strychnine alone.

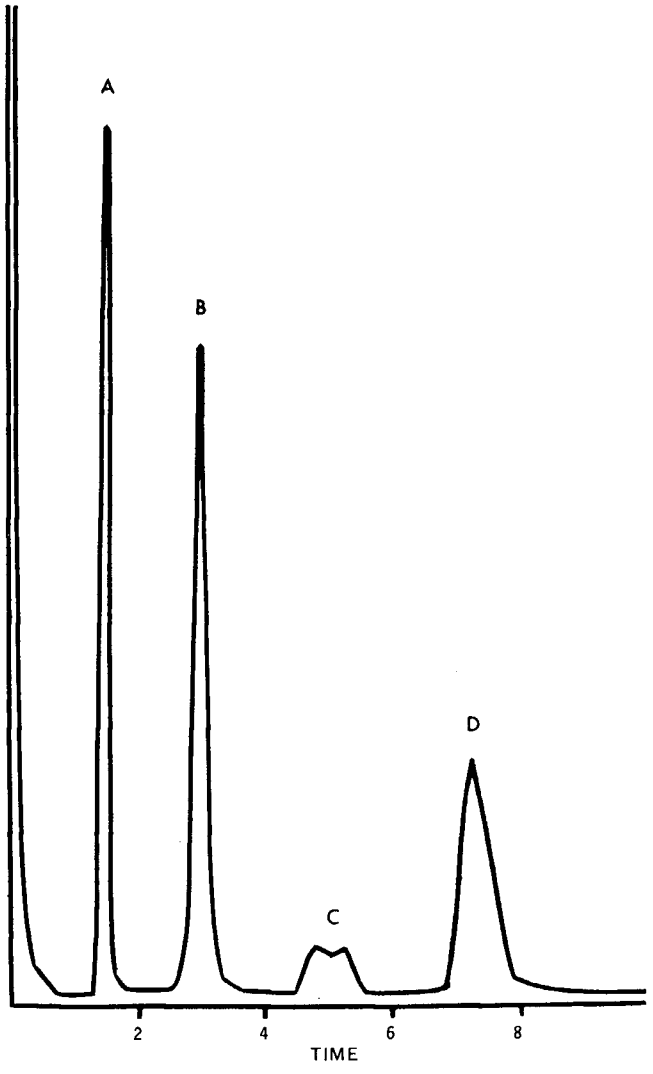

Figure 1—*GLC of nux vomica tincture with internal standard. Key: A, papaverine (internal standard); B, strychnine; C, probably α- and β-colubrine and icajine (10); and D, brucine.*

Standard Reference Solutions—*For Nux Vomica Tincture*—Accurately weigh 75 mg. of strychnine sulfate and 35 mg. of brucine. Quantitatively transfer the material to a 50-ml. volumetric flask. Dilute to the mark with deionized water. Pipet 10 ml. of the solution into a 60-ml. separator. Add 2 ml. of 10% sodium hydroxide solution and extract three times with 10-ml. portions of chloroform. Filter the organic phase through anhydrous sodium sulfate. Collect the filtrate in a small beaker and evaporate to dryness without heat. Redissolve the residue in 10 ml. of the papaverine hydro-

[1] A gas chromatograph (Hewlett-Packard model 402) equipped with a flame-ionization detector was used. The detector signal was fed to an IBM 1800 computer for peak integration and to a 1-mv. recorder (Honeywell Electronik 16) with a chart speed of 38.1 cm. (15 in.)/hr. and a 1-sec. full-scale response. Samples were injected with a 10-μl. syringe (Hamilton No. 701).

[2] Applied Science Laboratories, State College, Pa.

Table I—Precision and Accuracy

Formulation	RSD		RE	
	Strychnine	Brucine	Strychnine	Brucine
Nux vomica tincture	+2.0%	±3%	+3%	+1%
Iron quinine and strychnine tonic	±1.0%	—	−1%	—
Iron quinine and strychnine phosphates	±0.5%	—	+2%	—
Strychnine sulfate tablets	±2%	—	+2%	—

chloride internal standard solution. Inject 1-μl. quantities for chromatography.

For Elixirs and Tablets—Accurately weigh 50 mg. of strychnine sulfate and quantitatively transfer the material to a 250-ml. volumetric flask. Dilute to the mark with deionized water. Pipet 10 ml. into a 60-ml. separator. Add 2 ml. of 10% sodium hydroxide solution and extract as above. Redissolve the residue in 1 ml. of the brucine internal standard solution. Inject 1-μl. quantities for chromatography.

Formulations—*Nux Vomica Tincture*—Pipet 10 ml. of the tincture into a 60-ml. separator. Direct a stream of filtered dry nitrogen at the surface for 1 hr. to evaporate most of the alcohol. Add 5 ml. of 10% sodium hydroxide and treat as described for the reference standard.

Elixirs: Iron Quinine and Strychnine Tonic and Iron Quinine and Strychnine Phosphates—Pipet 10 ml. of the elixir into a 60-ml. separator. Direct a stream of filtered dry nitrogen at the surface for 1 hr. to drive off most of the alcohol. Add 5 ml. of 10% sodium hydroxide (the pH should be greater than 11) and proceed as described for the reference standard.

Tablets: Strychnine Sulfate—Obtain an average tablet weight using 10 tablets. Grind them to a fine powder. Accurately weigh and quantitatively transfer the equivalent of 1 mg. of strychnine sulfate to a 60-ml. separator. Add 10 ml. of deionized water and 2 ml. of 10% sodium hydroxide. Proceed as described for the reference standard.

Calculations—For each chromatogram, determine the ratio of the area of the strychnine peak to that of the internal standard. For the nux vomica tincture, determine the ratio of the areas for brucine and the internal standard also. The concentration of strychnine in liquid formulations is then given by:

$$\frac{R\ \text{sample}}{R\ \text{standard}} \times \frac{\text{mg. standard}}{\text{volume standard stock solution}} \times \text{standard purity} \times \frac{10\ \text{ml. standard}}{10\ \text{ml. sample}} \times 0.7804^{3} = \text{mg. strychnine (free base)/ml.} \quad \text{(Eq. 1)}$$

The concentration of brucine is obtained by using the appropriate ratios and standard weights in a similar equation. The strychnine content in tablets is obtained from:

$$\frac{R\ \text{sample}}{R\ \text{standard}} \times \frac{\text{mg. standard}}{\text{volume standard stock solution}} \times \text{standard purity} \times \frac{\text{average tablet weight}}{\text{weight of sample}} \times 10\ \text{ml.} \times 0.8949^{4} = \text{mg. strychnine sulfate/tablet} \quad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

The linearity of response of the chromatographic system to 1-μl. injections made from solutions containing 0.2–4 mg./ml. was ascertained. Five replicate samples of each formulation were analyzed to determine the precision of the method. Known amounts of reference standard were added to samples prior to extraction to obtain accuracy data. The results are shown in Table I. A typical chromatogram is shown in Fig. 1.

Samples of nux vomica tincture were assayed by this method and by the method described in NF XI (11). The results are compared in Table II. The NF method requires oxidative destruction of brucine and other interfering alkaloids prior to titration of the excess acid used to neutralize extracted strychnine. Typically, a standard deviation of ±5% is obtained, and as much as 2 days may be used in analyzing many samples.

Table II also lists the results obtained for samples of the elixirs and tablets. NF XI does not describe a quantitative assay for strychnine in such formulations. Thus, the present assay compares favorably in its rapidity, precision, and accuracy. No sacrifice in these parameters is experienced even with such a mixture as the nux vomica tincture.

Table II—Assay of Some *Strychnos* Alkaloid Formulations

Sample	NF Method, mg. Strychnine/ml.	GLC	
		mg. Strychnine/ml.	mg. Brucine/ml.
Nux vomica			
1	1.32	1.10	0.46
2	1.22	1.20	0.45
3	1.22	1.26	0.55
4	1.22	1.16	0.48
Iron quinine and strychnine tonic	—	0.144	—
Iron quinine and strychnine phosphates	—	0.171	—
Tablets	—	0.819[a]	—

[a] Milligrams strychnine sulfate (anhydrous) per tablet.

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[3] 0.7804 is the fraction of strychnine free base in the pentahydrated sulfate salt.

[4] 0.8949 is the fraction of strychnine sulfate in the pentahydrate salt.

Absorbance–pH Relationship in the Steroid–Tetrazolium Reaction

ROBERT E. GRAHAM* and CHARLES T. KENNER†▲

Abstract □ The absorbance produced by a given weight of corticosteroid in the blue tetrazolium method is shown to be a function of the apparent pH as measured by a glass and saturated calomel electrode. In general, the absorbance decreases as the apparent pH decreases, irrespective of the cause of the apparent pH change. An equation is derived which relates the absorbance to the apparent pH for hydrocortisone solutions, and the relationship is shown to be similar for several representative corticosteroids. It is recommended that the apparent pH be determined on all samples that are run without a prior acidic or basic extraction to determine if low readings are due to interference or to lower than expected quantities.

Keyphrases □ Corticosteroid–blue tetrazolium reaction—absorbance–pH relationship □ Steroid–blue tetrazolium reaction—absorbance–pH relationship □ Blue tetrazolium–steroid reaction—absorbance–pH relationship □ Colorimetry—steroid–blue tetrazolium reaction, absorbance–pH relationship

The blue tetrazolium {3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-bis[2,5-diphenyl-2*H*-tetrazolium chloride]} reaction of Mader and Buck (1) is, with slight modification, the method for corticosteroid analysis in both USP XVII (2) and NF XIII (3). The reaction involves the oxidation–reduction reaction between the C_{17} side chain of the corticosteroid and the blue tetrazolium (I) in alkaline solution to form a colored formazan (4). Since the reaction is not specific and is affected by a large number of interfering compounds (5), extreme care must be exercised in cleanup procedures for pharmaceutical products that include corticosteroids in the formulation. In some cases, interfering substances which are not removed in the cleanup procedures can be detected by the variation of absorbance with time method of Graham *et al.* (6).

The reaction rate and the extent of completion of the formation of the formazan are influenced by numerous factors, including the structural features of the corticosteroid as well as the temperature and solvent used together with the concentrations of base, water, and I. Temperatures ranging from room temperature to 90° with color development times from 2 to 120 min. have been used (1–3, 7–9). Alkalies utilized include alcoholic sodium hydroxide (9, 10), tetramethylammonium hydroxide (II) (1, 11), trimethylbenzylammonium hydroxide[1] (12), and choline (10, 12) in various concentrations.

Rechnagel and Litteria (9) found that water inhibits the reaction and that the extent of inhibition increases as the concentration of water in the final reaction mixture increases. They recommended that reagents be prepared with minimal amounts of water. These investigators also studied the effect of the concentration of I and found that the absorbance due to 9 mcg. of 11-desoxycorticosterone in 3 ml. of reaction mixture increased to a constant value of approximately 120 mcg./ml. and utilized 167 mcg./ml. in their procedure. Concurrent blank, sample, and standards are usually run to offset the effect of these variables as much as possible.

[1] Triton B.

Table I—Effect of Water upon Absorbance and Apparent pH

Sample Number	Water Added, ml.	Water in Reaction Mixture[a,b], % v/v	Net Absorbance[c]	Apparent pH[c]
1	0.00	1.63	0.592	13.75
2	1.00	3.29	0.589	13.79
3	2.00	4.96	0.588	13.75
4	3.00	6.63	0.580	13.72
5	4.00	8.29	0.572	13.70
6	5.00	9.96	0.555	13.67
7	7.00	13.3	0.540	13.58
8	10.00	18.3	0.460	13.40
9	12.00	21.6	0.383	13.31
10	15.00	26.6	0.256	13.23
11	20.00	35.0	0.084	13.03
12	25.00	43.3	0.034	12.83

[a] Each reaction mixture contains 0.200 mg. of hydrocortisone and 0.22 mmole of II. [b] The water in the reaction mixture includes the added water plus the water present in the I and II reagents. [c] Average of duplicates.

This paper reports a study of the relationship between the absorbance and the apparent pH (as measured with a glass and saturated calomel electrode) for the blue tetrazolium reaction with selected corticosteroids. The effect of water, acids, and the concentration of II upon the absorbance and apparent pH of several corticosteroids was investigated.

EXPERIMENTAL[2]

Reagents—The following were used:

1. Tetramethylammonium hydroxide[3], pentahydrate (II).
2. Tetramethylammonium hydroxide[3], 10% aqueous.
3. Tetramethylammonium hydroxide, 10% alcoholic. Dissolve 5.00 g. of II and dilute to 25.0 ml. with absolute ethanol. The resulting solution is 10% anhydrous II and 10% water.
4. Tetramethylammonium hydroxide, 1%. Dilute 5.00 ml. of II, 10% aqueous, to 50.0 ml. with alcohol USP (95% ethanol).
5. Blue tetrazolium, 5 mg./ml. Dissolve 50.0 mg. of blue tetrazolium[4] in 10.0 ml. of alcohol USP.
6. Corticosteroid standards, 0.100 mg./ml. Dissolve 10.0 mg. of the steroid (USP or NF reference standard) and dilute to 100.0 ml.

[2] A Cary model 15 ratio recording spectrophotometer was used. Absorbances were measured in matched 1-cm. quartz cells by scanning from 700 to 490 nm. A Corning model 10 pH meter with a Corning model 476050 combination electrode was standardized with pH 10 standard buffer. Usually 5–10 min. of equilibration was required before the readings became constant.

[3] Eastman Organic Chemicals.

[4] Dajac Laboratories.

Table II—Effect of Salicylamide upon Absorbance and Apparent pH

Solution	Salicylamide in Reaction Mixture[a], mmole	Absorbance[b]	Apparent pH[b]
Reagent blank	0.000	ND[c]	14.40
Standard	0.000	0.584	14.09
1	0.041	0.578	13.70
2	0.082	0.564	13.60
3	0.123	0.515	13.54
4	0.164	0.428	13.50
5	0.180	0.356	13.30
6	0.197	0.232	13.20
7	0.213	0.130	12.86
8	0.230	0.022	12.01
9	0.246	0	11.64
10	0.262	0	11.36
11	0.328	0	10.92
12	0.410	0	10.70
13	0.820	0	10.33

[a] Each reaction mixture contains 0.200 mg. of hydrocortisone and 0.22 mmole of II. [b] Average of duplicates. [c] Not determined.

with alcohol USP or absolute ethanol as required. Lower concentrations are prepared by quantitative dilution of these standards. Many of the 0.01-mg./ml. standards were prepared by dissolving 1.000 mg. of the steroid in 100.0 ml. of alcohol.

All other reagents and solvents were ACS, USP, NF, or analytical reagent grade.

General Procedure - The procedure, unless otherwise specified, is the official procedure given in NF XIII (2) in which a 20.00-ml. volume of standard or sample corticosteroid in alcohol USP is treated with 2.00 ml. of blue tetrazolium reagent followed by 2.00 ml. of II (1%). The absorbance is measured against a reagent blank 90 min. after the addition of II. In this study, the apparent pH of each solution was determined after completion of the absorbance measurements.

Effect of Water Concentration upon Absorbance and Apparent pH—Five-milliliter aliquots of standard hydrocortisone solution (0.10 mg./ml.) in absolute ethanol were transferred to 50.0-ml. volumetric flasks, containing the amounts of water shown in Table I, and diluted to volume with absolute ethanol. Blanks were prepared similarly with the substitution of absolute ethanol for the standard. Duplicate 20.00-ml. aliquots of each standard and a single 20.00-ml. aliquot of each blank were transferred to separate 50-ml. glass-stoppered conical flasks, and the blue tetrazolium reagent and II (1%) were added in the order of standard, standard, and blank for each different amount of water. The time of addition of II was counted as zero time for each set. The flasks were placed in the dark at room temperature (24 ± 1°) for 90 min. and then scanned against absolute ethanol in the same order. The absorbances of the blanks were subtracted from the absorbances of the standards to obtain the net absorbances.

Effect of Salicylamide upon Absorbance and Apparent pH—Solutions of hydrocortisone and salicylamide were prepared in alcohol USP so that 20.00-ml. aliquots contained 0.200 mg. (0.552 μmole) of hydrocortisone and the amounts of salicylamide shown in Table II. These solutions were run in duplicate according to the general procedure by addition of blue tetrazolium reagent (13.7 μmoles) and II (1%, 0.22 mmole) and were scanned after 90 min. against a reagent blank containing no salicylamide.

Effect of Increased Tetramethylammonium Hydroxide Concentration upon Absorbance and Apparent pH—Twenty-milliliter aliquots of a series of hydrocortisone solutions (0.01 mg./ml.) in absolute ethanol were run in duplicate according to the general procedure, except that different concentrations of II in absolute ethanol were used and both the blanks and solutions were scanned against absolute ethanol. The solutions of II were prepared by dilution of II (10% alcoholic) with absolute ethanol. The study was repeated using hydrocortisone solutions in absolute alcohol with conditions adjusted so that the percentage of water in the reaction mixture increased from 4.67 to 6.42% (average 4.96%) and from 5.67 to 6.42% (average 6.00%). (The percentage of water in the reaction mixture in the general procedure is 5.8%.) The study was also repeated using hydrocortisone solutions in alcohol USP and solutions of II prepared by dilution of II (10% aqueous) with alcohol USP.

Effect of Added Acid and Increased Tetramethylammonium Hydroxide Concentration upon Absorbance and Apparent pH—A series of solutions was prepared to contain 0.200 mg. of hydrocortisone and various amounts of salicylic acid per 20.00 ml. These solutions were run in duplicate according to the general procedure, except that increased concentrations of II were used in some determinative steps and all blanks and solutions were scanned against alcohol USP. The study was repeated using stearic acid in place of salicylic acid.

Effect of Various Acids upon Absorbance and Apparent pH—A series of solutions was prepared so that 20.00-ml. aliquots contained 0.200 mg. of hydrocortisone and either 0.100 or 0.200 meq. of one of the following acidic substances: citric acid, tartaric acid, stearic acid, *p*-aminobenzoic acid, salicylic acid, phenobarbital, niacin, or niacinamide. These solutions were run according to the general procedure.

Effect of Added Water and Added Acid upon Absorbance and Apparent pH of Several Corticosteroids—Solutions of several representative corticosteroids were prepared in alcohol USP to contain 0.200 mg. of the steroid in 20.00 ml. For the esters, the weights were adjusted so that the solutions contained the equivalent of 0.200 mg. of the unesterified steroid. These solutions were run according to the

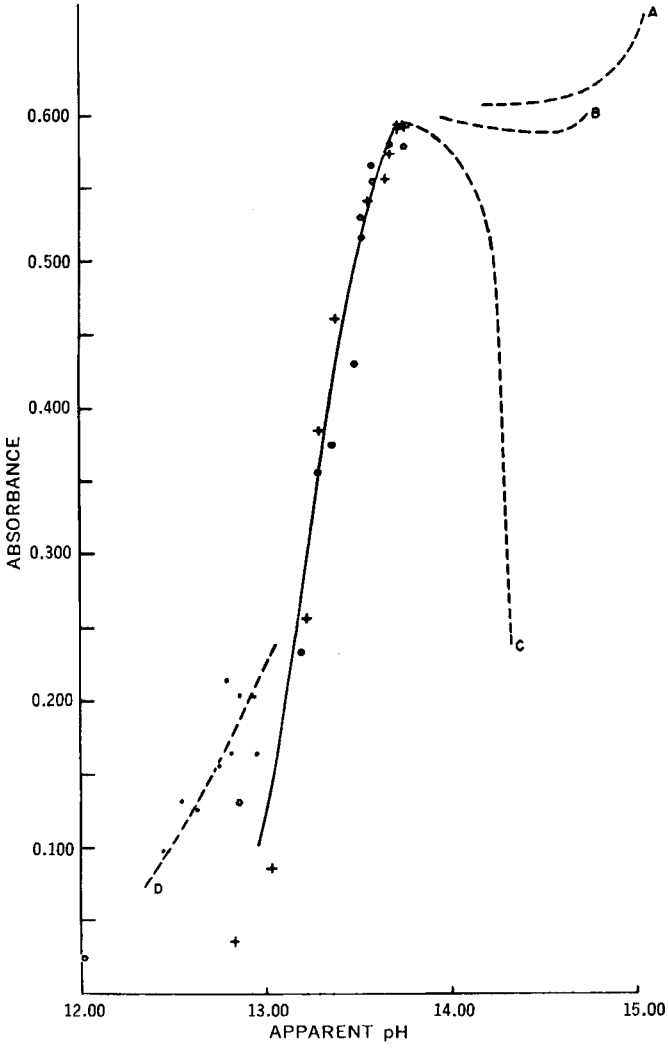

Figure 1—*Variation of absorbance produced by 0.200 mg. of hydrocortisone with apparent pH. Key: ——, derived by calculation using Eq. 1; ○, experimental points from salicylamide study; and +, experimental points from water study. Dashed lines A, B, and C represent the effect of increasing concentrations of II at different water percentage levels: A, low water (0.50–1.24, average 0.84%); B, official amount of water (5.67–6.42, average 6.00%); and C, above official amount of water (5.79–12.92, average 9.09%). Dashed line D represents the effect of adding 0.200 meq. of various acids.*

Table III—Effect of Increased Tetramethylammonium Hydroxide (II) Concentration upon Absorbance and Apparent pH

Percent II Added	Alcoholic II[a]			Alcoholic II[b]		
	Water in Reaction Mixture, %	Net Absorbance[c]	Apparent pH[c]	Water in Reaction Mixture, %	Net Absorbance[c]	Apparent pH[c]
1	0.50	0.605	14.24	4.63	0.599	13.91
2	0.58	0.606	14.50	4.71	0.598	14.24
4	0.75	0.614	14.80	4.88	0.601	14.47
6	0.91	0.628	14.94	5.05	0.604	14.61
8	1.08	0.645	15.02	5.21	0.606	14.70
10	1.24	0.666	15.09	5.38	0.617	14.76
Percent II Added	Alcoholic II[b]			Alcoholic II[d]		
	Water in Reaction Mixture, %	Net Absorbance[c]	Apparent pH[c]	Water in Reaction Mixture, %	Net Absorbance[c]	Apparent pH[c]
1	5.67	0.596	13.99	5.79	0.592	13.76
2	5.75	0.590	14.27	6.58	0.576	13.99
4	5.92	0.588	14.50	8.17	0.548	14.16
6	6.09	0.587	14.64	9.75	0.476	14.24
8	6.25	0.593	14.73	11.33	0.425	14.29
10	6.42	0.598	14.76	12.92	0.243	14.33

[a] Hydrocortisone, 0.200 mg., in absolute ethanol. II prepared by diluting II, 10% alcoholic, with absolute ethanol. [b] Hydrocortisone, 0.200 mg., in diluted ethanol. II prepared as in *Footnote a*. [c] Average of duplicates. [d] Hydrocortisone, 0.200 mg., in alcohol USP. II prepared by diluting II (10% aqueous) with alcohol USP.

general method and also with the general method modified by the use of II (1%) prepared by dilution of II (10% aqueous) with water. Use of water as the diluent in place of alcohol USP raised the percentage of water in the reaction mixture from 5.8 to 12.9%. Solutions containing the same weights of steroids and 0.20 mmole of stearic acid in 20.00 ml. were prepared using alcohol USP. These solutions were run according to the general method.

RESULTS AND DISCUSSION

The results of the study of the effect of water upon the absorbance and apparent pH in the blue tetrazolium determination of hydrocortisone are shown in Table I and Fig. 1. The effect is essentially negligible if the percentage of water is below approximately 6 and the apparent pH is not below 13.75. Above 6% water, both the absorbance and the apparent pH decrease significantly with an increase in the amount of water. In this region, the relatively small changes in apparent pH due to the increase in water are associated with large changes in the absorbance produced by 0.200 mg. of hydrocortisone. The percentage of water in the general or official method [due to the use of alcohol USP and II (10% aqueous) in the preparation of reagents and samples] is 5.8, which is close to, but below, the critical value at which the effect becomes significant.

The results of the study of the effect of salicylamide on the absorbance and apparent pH are shown in Table II and Fig. 1. Both the absorbance due to a given amount of hydrocortisone and the apparent pH decrease with an increase in salicylamide, with as little as 0.041 mmole of salicylamide having a significant effect. There is no development of color in solutions that contain more millimoles of salicylamide than II and in which the apparent pH is less than 12. As in the water study, relatively small changes in the apparent pH below 13.70 are associated with large changes in absorbance.

The values for the net absorbance (A) and apparent pH for these two studies were combined and used to derive Eqs. 1 and 2, which relate absorbance to apparent pH irrespective of the cause of decrease in apparent pH:

$$pH = 6.265A^3 - 5.446A^2 + 2.725A + 12.74 \quad \text{(Eq. 1)}$$

$$A = -1.76679pH^3 + 70.5814pH^2 - 939.004pH + 4160.56 \quad \text{(Eq. 2)}$$

The solid line in Fig. 1 represents values calculated by Eq. 1. The agreement between experimental and calculated points is shown on the figure and by the fact that the coefficient of multiple correlation, R^2, is 0.946 for the combined data from the water study and the salicylamide study.

To determine whether or not the absorbance is affected by the apparent pH only or by both the water and the apparent pH, several studies were made on the effect of an increased concentration of II (Table III). For percentages of water well below the official method amount of 5.8%, both the apparent pH and absorbance increase with an increase in concentration of II. For percentages of water close to the 5.8% of the official method, the apparent pH increases with increase in II but the absorbance is not affected greatly by up to a sixfold increase. Above a sixfold increase in II, the absorbance increases significantly as the apparent pH rises above the 14.60–14.70 range. However, the increase in the percentage of water from the range of 4.63–5.38 (average 4.96) to the range of 5.67–6.42 (average 6.00) as the concentration of II is increased causes an average decrease in absorbance of approximately 0.012 unit per percent water increase for identical concentrations of II. Evidently, an increase in the amount of water above that specified in the official method cannot be offset by increases in the concentration of II alone. This conclusion is further substantiated by the study in which the water was increased from 5.79 to 12.92% as the concentration of II added was increased from 1 to 10% since the absorbance decreased markedly even though the apparent pH increased. The more rapid decrease with increased water concentrations also indicates that the variation in the amount of water has a greater effect upon

Table IV—Effect of Added Acid and Increased Tetramethylammonium Hydroxide (II) Concentration upon Absorbance and Apparent pH

Solution[a]	Acid Added, mmole	II Added, mmole	Net[b] II, mmole	Stearic Acid		Salicylic Acid	
				Net Absorbance[c]	Apparent pH[c]	Net Absorbance[c]	Apparent pH[c]
Blank	0.00	0.22	0.22	0.037	13.70	0.030	13.75
Standard	0.00	0.22	0.22	0.584	13.81	0.584	13.77
Acid blank 1	0.20	0.22	0.02	0.013	ND[d]	0.025	12.90
Sample 1	0.20	0.22	0.02	0.130	12.55	0.203	12.95
Acid blank 2	0.20	0.33	0.13	0.032	13.64	0.044	13.70
Sample 2	0.20	0.33	0.13	0.567	13.65	0.551	13.70
Acid blank 3	0.20	0.44	0.24	0.039	13.85	0.054	13.91
Sample 3	0.20	0.44	0.24	0.579	13.85	0.564	13.93

[a] Each solution contained 0.200 mg. hydrocortisone. [b] Calculated as difference between millimoles of acid and II. [c] Average of duplicates. [d] Not determined.

Table V—Effect of Various Acidic Substances upon Absorbance and Apparent pH

Added Acid[a]	—0.100 meq. Added— Net Absorbance[b]	Apparent pH[b]	—0.200 meq. Added— Net Absorbance[b]	Apparent pH[b]
Standard (no added acid)	0.584	13.77	0.584	13.77
Citric acid	0.567	13.68	0.125	12.63
Tartaric acid	0.573	13.65	0.155	12.75
Salicylic acid	0.554	13.70	0.163	12.96
Niacin	0.573	13.72	0.163	12.82
p-Aminobenzoic acid	0.585	13.72	0.203	12.87
Stearic acid	0.574	13.65	0.097	12.45
Phenobarbital	0.576	13.68	0.214	12.80
Niacinamide	0.637	13.95	0.637	13.95

[a] Each solution contained 0.200 mg. hydrocortisone. [b] Average of duplicates.

absorbance than an increase in the apparent pH or concentration of II. The variations of absorbance with apparent pH for these studies are shown as dashed lines in the upper portion of Fig. 1.

The decrease in absorbance and apparent pH caused by the neutralization of II by acids can be offset, at least in part, by an increase in the concentration of II provided the water concentration does not exceed the amount prescribed by the official method (Table IV). However, the effect of equivalent amounts of various acids is not offset to the same extent by equivalent increases in the concentration of II.

The effect of different acids upon the absorbance and apparent pH is shown further in Table V, which lists the apparent pH and absorbance obtained by the addition of 0.1 and 0.2 meq. of various acids to hydrocortisone solutions. The larger amounts of acid neutralize essentially all of the II present, while the smaller amounts neutralize approximately half. The extent of the effect upon the apparent pH and the absorbance may be essentially negligible for 0.1 meq. of some acids such as *p*-aminobenzoic, while absorbance and apparent pH are actually raised by niacinamide which can act as a strong base in nonaqueous media. The variation of the extent of the effect of various acids is shown more markedly in the solutions containing 0.2 meq. of the acids. In each case, there is a great decrease in absorbance as the apparent pH is lowered to values below 13.00, but the actual absorbances vary from 0.097 to 0.214 and the apparent pH values range from 12.45 to 12.96. The results are shown by the dashed line and experimental points in the lower portion of Fig. 1.

Table VII—Normal Variation of Apparent pH and Absorbance of Hydrocortisone Standards due to Reagents

Date	Apparent pH	Absorbance[a]
10–16–69	13.88	0.581
10–22–69	14.11	0.577
8–14–70	14.15	0.580[b]
9–8–70	14.11	0.578
8–19–71	13.85	0.584
8–19–71	13.87	0.587
8–30–71	13.77	0.586
8–30–71	13.76	0.586
9–2–71	13.80	0.578
10–14–71	13.76	0.587
10–14–71	13.76	0.585
10–22–71	14.06	0.599
10–22–71	14.07	0.598
11–2–71	13.99	0.585[b]
11–2–71	13.99	0.587[b]
11–9–71	13.90	0.598[b]
11–9–71	13.91	0.598[b]

[a] Each standard contained 0.200 mg. of hydrocortisone. [b] The solution of II was prepared from the pentahydrate rather than the 10% aqueous solution.

The results of the study to determine the effect of water and acids upon the absorbance and apparent pH of several representative corticosteroids are summarized in Table VI. For the water study, the average percent change in absorbance per unit change in apparent pH is 47.7, with a range of 40.4–52.9 and with a relative standard deviation of 2.95%. These values indicate that the relative effect of water is essentially the same for all of the corticosteroids studied. The average decrease in absorbance in this study is 0.0477 unit or 0.0067 unit for each unit percent change in water from 5.8 to 12.9%. This value agrees satisfactorily with the average decrease of 0.0060 unit per percent increase in water from 6.6 to 13.3% shown in Table I for hydrocortisone solutions. Desoxycorticosterone

Table VI—Effect of Water and Acid upon Absorbance and Apparent pH of Several Corticosteroids

Corticosteroid[a]	—Official Method— Net Absorbance	Apparent pH	—Water Study[b]— Net Absorbance	Apparent pH	ΔA, %	ΔpH	$\frac{\Delta A}{\Delta pH}$	—Acid Study[c]— Net Absorbance	Apparent pH	ΔA, %	ΔpH	$\frac{\Delta A}{\Delta pH}$
Hydrocortisone	0.600	14.06	0.546	13.89	9.00	0.17	52.9	0.015	11.04	97.5	3.02	32.3
Hydrocortisone	0.599	14.07	0.546	13.89	8.85	0.18	49.2	0.038	11.60	93.7	2.47	37.9
Hydrocortisone acetate	0.598	14.06	0.550	13.89	8.03	0.17	47.2	0.004	11.71	99.3	2.35	42.3
Hydrocortisone acetate	0.596	14.05	0.549	13.89	7.89	0.16	49.3	0.003	11.61	99.5	2.44	40.8
Desoxycorticosterone acetate	0.673	14.05	0.613	13.88	8.92	0.17	52.5	0.005	11.60	99.3	2.45	40.5
Desoxycorticosterone acetate	0.668	14.05	0.614	13.89	8.08	0.16	50.5	0.011	11.65	98.4	2.40	41.0
Prednisone	0.576	14.05	0.533	13.89	7.47	0.16	46.7	0.125	12.04	78.3	2.01	39.0
Prednisone	0.576	14.05	0.530	13.88	7.99	0.17	47.0	0.088	11.75	84.7	2.30	36.8
Dexamethasone	0.535	14.05	0.490	13.89	8.41	0.16	52.6	0.039	11.89	92.7	2.16	42.9
Dexamethasone	0.533	14.05	0.490	13.89	8.07	0.16	50.4	0.021	11.60	96.1	2.45	39.2
Prednisolone	0.589	14.05	0.545	13.88	7.47	0.17	43.9	0.042	12.09	91.9	1.96	46.9
Prednisolone	0.589	14.05	0.544	13.88	7.64	0.17	44.9	0.028	11.81	95.2	2.24	42.5
Prednisolone acetate	0.591	14.06	0.548	13.88	7.28	0.18	40.4	0.013	12.20	97.8	1.86	52.6
Prednisolone acetate	0.591	14.06	0.548	13.88	7.28	0.18	40.4	0.006	12.00	99.0	2.06	48.1
Average					8.03		47.7			94.5		41.6
Standard deviation[d]					0.29		1.41			2.39		2.56
Standard deviation, % of average					3.59		2.95			2.53		6.15

[a] Each solution contained 0.200 mg. of the steroid or the equivalent weight for the esters. [b] General method except that II (1%) was prepared by dilution of II (10% aqueous) with water in place of alcohol USP to give 12.9% water in the reaction mixture. [c] Each solution contains 0.20 mmole of stearic acid in addition to the steroid. [d] Calculated by the difference in duplicate method in which the standard deviation equals the square root of the sum of the squares of the differences between duplicates divided by twice the number of duplicate pairs.

Table VIII—Interference in Samples by Acidic Components

Sample Number	Corticosteroid Present	Blue Tetrazolium Method: Before Extraction — Found, % of Declared	Blue Tetrazolium Method: Before Extraction — Apparent pH	Blue Tetrazolium Method: After Extraction — Found, % of Declared	Blue Tetrazolium Method: After Extraction — Apparent pH: Sample	Blue Tetrazolium Method: After Extraction — Apparent pH: Standard	INH[a] Method, Found, % of Declared
1	Prednisolone[b]	NC[c]	6.74	107.4	14.20	14.14	103.5
2	Prednisolone[b]	NC[c]	6.76	21.2	14.22	14.20	26.9
3	Dexamethasone[d]	NC[c]	5.76	90.0	14.20	14.20	88.5

[a] INH = isonicotinic acid hydrazide. [b] Samples 1 and 2 were declared to contain 0.75 mg. prednisolone, 325 mg. aspirin, 120 mg. salicylamide, and 100 mg. aluminum hydroxide per tablet. [c] NC means no color was developed by the sample. [d] Sample 3 was declared to contain 0.25 mg. dexamethasone, 500 mg. aspirin, and 75 mg. aluminum hydroxide per tablet.

acetate gives unusually high values of absorbance compared to the other compounds since it has a lower molecular weight and a larger molar absorptivity. Omission of the values for desoxycorticosterone acetate and for hydrocortisone from the values of Table VI changes the rate of decrease to 0.0063 absorbance unit, which agrees more closely with the value obtained from Table I.

For the acid study with several corticosteroids, which covers much larger changes in both absorbance and apparent pH, the average percent change in absorbance per unit change in apparent pH is 41.7, with a range of 32.3–52.6 and with a relative standard deviation of 6.15%. These values indicate that the relative effect of 0.200 meq. of stearic acid upon the absorbance and apparent pH is approximately the same for the representative corticosteroids studied. The average decrease in absorbance for all of the steroids is 0.563 unit. This value compares satisfactorily with: (*a*) the average value of 0.424 for the effect of 0.200 meq. of several different acids upon the absorbance of hydrocortisone solutions shown in Table V, (*b*) the average value of 0.418 due to the addition of stearic acid and salicylic acid to hydrocortisone solutions shown in Table IV, and (*c*) the value of 0.454 caused by the addition of 0.213 meq. of salicylamide shown in Table II. Evidently, the almost complete neutralization of II by acids does not affect all of the corticosteroids equally but does cause a great change in both absorbance and apparent pH.

Equations 1 and 2 cannot be used to calculate the absorbance quantitatively from the apparent pH or the apparent pH from the absorbance for a single measurement. This is due to the variation in the response of the various corticosteroids and to the variation in absorbance and apparent pH of standard solutions of the same corticosteroids caused by variations in reagents from batch to batch. The normal variations in the apparent pH and the absorbance of hydrocortisone standards over 24 months are shown in Table VII.

Equations 1 and 2 can be used as a qualitative check upon the absorbance associated with a given apparent pH to determine whether or not a lower than expected absorbance reading is due to decomposition, to less than the stated amounts of the steroid, or to pH variation. It is better, however, to utilize the differences in the absorbance and apparent pH of the standard and the sample or the differences between the actual absorbance of the sample and the expected absorbance of the sample. If the difference between the actual absorbance and the expected absorbance is greater than 0.03 and the apparent pH is below 13.75, the low results may be due to water, acid, or other interference rather than low concentrations of the undecomposed steroid. If, however, the actual and expected absorbances agree and the apparent pH is above 13.75, it is possible that positive interference due to basic components is offsetting lower than expected quantities of the steroid. These checks are possible since the sample size is usually adjusted so that the 20-ml. aliquot used in the color-development step contains approximately 0.200 mg. of the steroid and the absorbance is compared to a standard containing 0.200 mg. of the steroid. As a consequence, apparent pH measurements should be made as a routine procedure in the blue tetrazolium method for corticosteroids.

This study originated due to discrepancies in the analysis of a partially decomposed prednisone capsule preparation before and after basic extraction. The capsule originally contained 2 mg. of prednisone, 225 mg. of salicylamide, 20 mg. of ascorbic acid, and other neutral components. The results by the blue tetrazolium method when run directly by dissolving and adding I and II to a 20-ml. aliquot were zero, but the results were 34.3% of the declared amount when determined after a basic extraction of the capsule contents. Typical examples of samples run since that time in which the apparent pH measurement indicated acidic interference are shown in Table VIII. The results were checked by the isonicotinic acid hydrazide method of Umberger (13), which involves reaction with a different portion of the corticosteroid molecule and which is not affected by acidic components. Due to the low apparent pH, no color was developed during the blue tetrazolium method when the tablet contents were dissolved directly in alcohol USP. After solution of the corticosteroid and acidic components by repeated treatment with acetone followed by evaporation and solution in chloroform, the acidic components were extracted into 0.2 *N* NaOH and the chloroform was evaporated. The residue was dissolved in alcohol USP and gave results by the blue tetrazolium method that agree satisfactorily with the results by the isonicotinic acid hydrazide method.

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Characterization of Crystalline Calcium Pantothenate by Physicochemical Assay Methods

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Abstract □ Crystalline calcium pantothenate was characterized by application of analytical techniques including: (*a*) TLC separation of intact calcium pantothenate from its degradation product β-alanine, (*b*) electrochemical methods using a calcium-specific ion electrode, (*c*) spectropolarimetry, and (*d*) colorimetric determination of pantothenate according to Schmall and Wollish. These measurements complement one another to ensure the specificity and stability of crystalline calcium pantothenate. Analysis of calcium pantothenate materials having varying degrees of purity was accomplished by these physicochemical methods as well as the prescribed microbiological procedure. Data indicative of good agreement between the two procedures, within the precision of the microbiological assay, are shown. The spectropolarimetric technique is not applicable to materials that contain optically active species other than calcium pantothenate. Stability data for the solid-state degradation of calcium pantothenate in the presence of ascorbic acid, benzoic acid, barbituric acid, and succinic acid are presented. The stability results are interpreted in terms of general-acid catalysis.

Keyphrases □ Calcium pantothenate, crystalline—characterization by TLC, potentiometric titrimetry, spectropolarimetry, and colorimetry, compared to microbiological method □ Pantothenates, calcium pantothenate—characterization by combination of physicochemical methods, compared to microbiological method □ TLC—separation, calcium pantothenate from β-alanine □ Potentiometric titrimetry—calcium assay, calcium pantothenate □ Spectropolarimetry—determination, optical rotation of calcium pantothenate □ Colorimetry—determination, pantothenate, ninhydrin reagent

The analysis of pantothenates, in the pure state and in dosage forms, has been the subject of numerous publications in the past 3 decades. Several established methodologies, particularly chemical colorimetry and microbiological methods, have been used to determine pantothenates. The colorimetric methods are based on the color reactions of the products which result from the hydrolytic cleavage of the vitamin in acidic or basic media. Acid hydrolysis of pantothenates produces β-alanine (3-aminopropionic acid) and pantolactone (2,4-dihydroxy-3,3-dimethylbutyric acid γ-lactone); with alkali treatment, pantothenates split into β-alanine and pantoic acid (2,4-dihydroxy-3,3-dimethylbutyric acid) sodium salt.

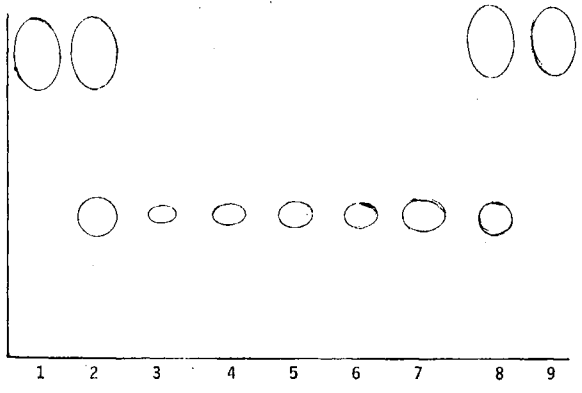

Figure 1—*Thin-layer chromatogram of USP and 80% "stabilized" forms of calcium pantothenate and of its degradation product β-alanine. Key: 1, stabilized form, 250-mcg. experimental sample; 2, stabilized form, 250-mcg. commercial sample; 3, β-alanine, 0.5 mcg.; 4, β-alanine, 1.0 mcg.; 5, β-alanine, 3.0 mcg.; 6, β-alanine, 5.0 mcg.; 7, β-alanine, 10.0 mcg.; 8, USP form, 250-mcg. commercial sample; and 9, USP form, 250-mcg. experimental sample. Note: All samples are initial.*

Colorimetric assay methods described in the literature include the reactions of β-alanine with 2,4-dinitrophenylhydrazine (1), 1,2-naphthoquinone-4-sulfonate (2), or ninhydrin (3); the reaction of 2,7-naphthalenediol with pantolactone (4); the reaction of pantolactone with hydroxylamine and the final development of a purple color with ferric chloride (5); and the chlorination of β-alanine followed by the liberation of iodine on the addition of an iodide solution (6). The colorimetric methods have been modified further by the elimination of interfering substances by solvent extraction and column chromatography prior to colorimetry (7–9), as well as by improvements in the reagents employed in the color-development step (10–12).

The microbiological assay procedures are based on the reaction of a suitable test organism with pantothenate. The microbiological methods are superior to the colorimetric techniques in that the former are specific for the physiologically active *d*-form of the vitamin, whereas colorimetry fails to differentiate between the enantiomorphs of pantothenate. Several microbiological assay methods employing a variety of test organisms have been documented (13–15); the presently recommended procedure described in USP XVIII utilizes *Lactobacillus plantarum* as the test organism. Less publicized analytical methods used to determine pantothenate include the complexometric titration of calcium in calcium pantothenate with disodium ethylenediaminetetraacetate (16), GLC (17, 18), optical rotatory dispersion, and circular dichroism (19).

There appears to be no report in the literature in which a combination of physicochemical assay methods has been applied to characterize crystalline calcium pantothenate [calcium D(+)-pantothenate] completely. The present investigation was initiated to apply existing physicochemical techniques and to develop new methods for the analysis of calcium pantothenate materials having varying degrees of purity. The physicochemical techniques utilized include: (*a*) TLC separation of intact calcium pantothenate from its degradation product β-alanine, (*b*) electrochemical methods using a calcium-specific ion electrode, (*c*) spectropolarimetry, and (*d*) colorimetric determination of pantothenate according to Schmall and Wollish (7). These methods

complement each other to ensure specificity in the analysis of crystalline calcium pantothenate, as evidenced by the excellent agreement of assay results obtained with the physicochemical techniques and the prescribed microbiological procedure (within the limits of reproducibility of the latter).

In addition to the analytical investigation, a study was initiated to determine the kinetics and mechanism of the solid-state degradation of calcium pantothenate, particularly in the presence of such organic acids as ascorbic acid, barbituric acid, benzoic acid, and succinic acid. Due to the complexity of the interactions involved, only preliminary stability data are presented; the entire kinetic study will be published later.

EXPERIMENTAL

Apparatus—*TLC*—Thin-layer plates were prepared by coating 20 × 20-cm. glass plates with 0.5 mm. of a cellulose MN 300 G/UV[1] slurry and were dried overnight at 40°. A commercially available spreader[2] was used to prepare all plates.

Potentiometric Titrimetry—Saturated calomel and calcium-specific ion electrodes[3] were employed as the reference electrode and auxiliary electrode, respectively. The potential changes during the titration were measured with a digital pH meter[4], and all titrations were performed with an automatic constant-rate buret[5] in synchronization with a linear recorder[6].

Spectropolarimetry—Optical rotations were measured with a single-beam digital polarimeter[7] using a cell with a 100-mm. path length.

Colorimetry—Spectra and spectrophotometric assays were determined with a double-beam UV/visible recording spectrophotometer[8] using matched 1-cm. quartz cells.

Reagents—Specific reagents are described under the individual analytical procedures. All other common reagents were ACS certified grade. Distilled, deionized water was used for the preparation and dilution of all solutions unless otherwise specified.

Analytical Procedures—*TLC*—Calcium pantothenate (250 mg.) was dissolved in 10 ml. of water, and 10 μl. (250 mcg.) was applied to the plate. Then 1.000 g. of β-alanine[9] was dissolved in 100 ml. of water, and solutions containing 0.125, 0.25, 0.50, 1.0, 3.0, 5.0, and 10.0 mcg./5 μl. of solution were prepared. Five microliters of each solution was applied to the plate to serve as a comparison standard. The chromatogram was developed in a solvent system consisting of *n*-butanol–ethanol–water (40:30:30), and the solvent front was allowed to ascend 15 cm. above the point of application of the sample.

The detection reagent was prepared by dissolving 300 mg. of ninhydrin[10] in 100 ml. of *n*-butanol and adding 3 ml. of glacial acetic acid. The plate was then sprayed with the ninhydrin reagent and heated for 30 min at 105°. The β-alanine and calcium pantothenate appear as violet bands at R_f 0.40 and 0.65, respectively. Since 2.67 mg. of calcium pantothenate degrades to form 1.0 mg. of β-alanine, the β-alanine standard that corresponds to the β-alanine found in the sample must be multiplied by 2.67 to obtain the amount of calcium pantothenate that has degraded. A typical chromatogram is presented in Fig. 1.

Although the analytical procedure for determining the percent intact drug retained may have a precision of ±2% and the estimation of degradation by TLC may have a precision of only ±20%, it is more accurate to gauge degradation by the amount of decomposition product formed when the amount of degradation lies in the 0–5% range. The direct determination of small amounts of decomposition products by TLC is certainly more meaningful than ascertaining degradation based on small variations in the assay for the intact drug.

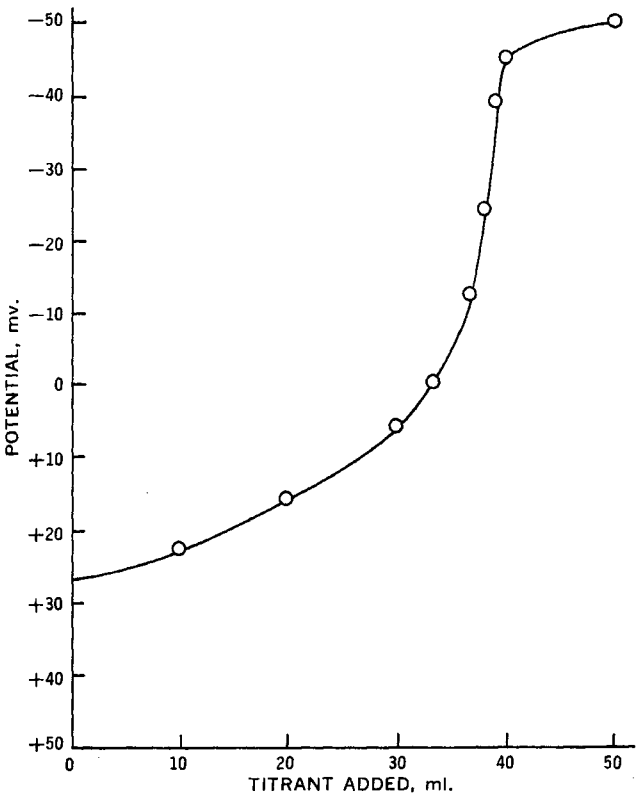

Figure 2—*Titration curve of calcium pantothenate against 0.05* N *disodium ethylenediaminetetraacetate.*

Calcium Assay—*Reagent: pH 10 Buffer*—Ammonium chloride (4.3 g.) and 26.6 ml. of concentrated ammonium hydroxide were dissolved and diluted to 1 l. with water.

Reagent: 0.05 N *Disodium Ethylenediaminetetraacetate, Dihydrate*—Disodium ethylenediaminetetraacetate, dihydrate, 18.61 g., was dissolved in 1 l. of water.

Procedure—Approximately 4.0 g. of calcium pantothenate was weighed accurately and transferred to a 250-ml. volumetric flask. The sample was dissolved in 100 ml. of water and diluted to volume with water. In the event the solution appeared cloudy, Whatman 2^V filter paper was used to filter the solution. A 50-ml. aliquot of the clear solution was pipeted into a 250-ml. beaker, and 100 ml. of the buffer was added. The solution then was titrated with 0.05 *N* disodium ethylenediaminetetraacetate employing the apparatus already described. The titration was repeated on a second 50-ml. aliquot of the stock solution. A characteristic titration profile is illustrated in Fig. 2.

Optical Rotation—The equivalent of 400 mg. of calcium pantothenate was weighed into a 25-ml. volumetric flask and dissolved in 20 ml. of water. The sample was diluted to volume with water and filtered with Whatman 2^V filter paper. If cloudiness persisted, the previously filtered solution was refiltered with 0.45-μ filter paper[11]. The rotation was measured at a suitable wavelength, usually 589 nm., and the specific rotation was calculated. The optical purity was determined by comparing the specific rotation of the sample to that obtained with calcium pantothenate, USP reference standard. Table I shows the specific rotation of calcium pantothenate and pantolactone as a function of wavelength at 20°, using water as the solvent. The specific rotation will vary with the solution composition; *i.e.*, the presence of succinic acid or some other optically inactive solute will affect the specific rotation of the optically active species. The optical rotatory dispersion curves of calcium pantothenate and pantolactone were reported previously in the literature (19). Calcium pantothenate was found to exhibit a positive Cotton

[1] Macherey, Nagel & Co., Duren, West Germany.
[2] Quickfield and Quartz, Ltd., Stone, England.
[3] Orion model 92-20.
[4] Orion Ionalyzer model 801.
[5] Sargent model C.
[6] Sargent model SRL.
[7] Perkin-Elmer model 141.
[8] Beckman Acta III.
[9] Matheson, Coleman & Bell.
[10] Eastman.
[11] Millipore Filter Corp.

Table I –Specific Rotation of Calcium Pantothenate and Pantolactone as a Function of Wavelength at 20°

Wavelength, nm.	Specific Rotation of Calcium Pantothenate	Specific Rotation of Pantolactone
589	+27.4	−50.9
578	+28.8	−53.6
546	+33.0	−62.1
436	+58.8	−121.2
365	+99.1	−227.6

Table II—Precision of Spectropolarimetric Method

Sample	Specific Rotation	Deviation from Mean
1	26.7	−0.4
2	27.4	+0.3
3	26.9	−0.2
4	27.4	+0.3
5	27.4	+0.3
6	26.8	−0.3
7	27.3	+0.2
8	27.1	0.0
9	27.1	0.0
10	27.0	−0.1
Mean	27.1	
SD	0.26	

effect with a peak at 227 nm. (α = 1066), while pantolactone showed a negative Cotton effect with a trough at 233 nm. (α = −6081). The positive Cotton effect in calcium pantothenate and the negative Cotton effect in pantolactone are presumably due to the carbonyl chromophore found in the amide group and the γ-lactone of the two species, respectively.

The precision obtained with the spectropolarimetric method is illustrated in Table II. A standard deviation (*SD*) of 0.26 was obtained in 10 replicate experiments, when the specific rotation was determined at 589 nm. The precision of the spectropolarimetric technique employed in the present study compares favorably to the precision obtained by Schmall and Wollish (7) with the colorimetric methods.

Colorimetry– Calcium pantothenate was determined according to the method of Schmall and Wollish (7), using ninhydrin as the coloring reagent. The procedure and reagents, with the exception of the preliminary column chromatography, are exactly as described in *Reference 7*. The assay consists of the basic hydrolysis of calcium pantothenate under reflux conditions to yield β-alanine and pantoic acid. On heating a dilute solution containing β-alanine and ninhydrin, oxidative deamination results in the formation of a purple species having an absorbance maximum at 570 nm. A known amount of calcium pantothenate is also hydrolyzed and serves as a comparison standard. A Beer's law plot for the β-alanine–ninhydrin complex is presented in Fig. 3.

Microbiological Assay—The microbiological assay was performed as described in USP XVIII (p. 864). When conducted with extreme care, the precision of the assay was ±3%.

Stability Studies—The solid-state stability study was prepared by combining calcium pantothenate with ascorbic, barbituric, benzoic, or succinic acid in a mole ratio of 1:16. This ratio was based

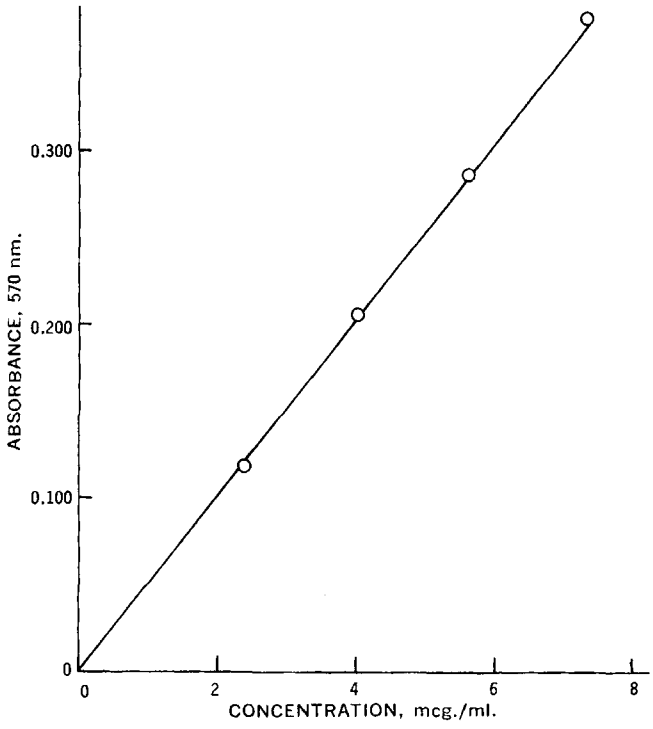

Figure 3—*Beer's law plot for the β-alanine–ninhydrin complex.*

on the recommended dietary allowance of calcium pantothenate and ascorbic acid established by the Food and Nutrition Board of the National Research Council (20). The samples were stored in 10-ml. rubber-stoppered vials at various temperatures and were withdrawn at appropriate times for assay. Whenever possible, the stability was followed spectropolarimetrically at 365 nm., using the specific rotation of calcium pantothenate and that of the degradation product, pantolactone, to determine the amount of intact calcium pantothenate. The microbiological assay method was utilized to assay samples containing ascorbic acid.

The percent of intact calcium pantothenate in a solution originally containing 10 mg./ml., as determined by spectropolarimetry, is given by the following equation:

$$\% \text{ calcium pantothenate} = \frac{A - 0.00545(B)}{0.01(C) - 0.00545(B)} \times 100 \quad \text{(Eq. 1)}$$

where:

A = observed rotation at wavelength λ
B = specific rotation of pantolactone at wavelength λ
C = specific rotation of calcium pantothenate at wavelength λ

RESULTS AND DISCUSSION

The results of the assay of calcium pantothenate obtained with the various analytical methods are summarized in Table III. The percent loss on drying is listed for samples that are theoretically 100% calcium pantothenate in order to provide a better estimation of the accuracy of each method. The data indicate that the precision

Table III—Results of Calcium Pantothenate Assays

Sample	Theoretical Percent Calcium Pantothenate	Percent Loss on Drying[a]	Percent β-Alanine	Assay for Calcium Pantothenate: Titrimetry	Spectropolarimetry	Colorimetry	Microbiological Method
A	80	—	<0.1	82.6	81.1	81.5	82
B	100	2.8	3.8	95.4	92.3	96.4	93
C	100	0.32	<0.05	99.8	98.9	99.5	99
D	80	—	0.05	81.3	81.9	81.7	82
E	100	2.7	3.9	95.0	94.5	96.8	94
F	100	0.78	<0.05	99.7	99.6	99.0	99

[a] Three hours at 105°.

Table IV—Solid-State Stability of Calcium Pantothenate in the Presence of Several Acids[a,b]

Sample	Condition	TLC	Spectropolarimetry	Microbiological Method
Calcium pantothenate + ascorbic acid	4 days, 70°	—	—	10
Calcium pantothenate + barbituric acid	4 days, 70°	25	25	23
Calcium pantothenate + benzoic acid	4 days, 70°	75	73	71
Calcium pantothenate + succinic acid	4 days, 70°	75	76	72
Calcium pantothenate + succinic acid[c]	16 hr., 55°	20	18	20
Calcium pantothenate + ascorbic acid[c]	16 hr., 55°	—	—	6

[a] Mole ratio of calcium pantothenate–acid of 1:16. [b] The pK_1a of each acid is about 4.2. [c] Three percent water.

of the assay for a single sample using the various analytical techniques is extremely good. When considerable amounts of β-alanine are present, the colorimetric method yields consistently high results because the technique is based on the β-alanine–ninhydrin color reaction. This difficulty may be eliminated by separating any preformed β-alanine on a Dowex 50-X4(H) column (7), thus rendering the method rather specific for intact pantothenate.

In view of the excellent agreement between the spectropolarimetric and microbiological methods, particularly in the presence of excess β-alanine, the optical purity of the vitamin appears to be closely approximated by the spectropolarimetric technique. The complexometric titration for calcium complements the assay results obtained with the other methods, as evidenced by the data presented in Table III. The titrimetric method is only for the calcium content and does not reflect the stability of the pantothenate moiety *per se*. The analytical results suggest that these physicochemical techniques, when applied in combination, assure a specificity and precision of assay that allow the accurate determination of the purity, identity, and stability of crystalline calcium pantothenate materials having varying degrees of purity. The spectropolarimetric and TLC methods, however, are not applicable to the assay of calcium pantothenate in multivitamin preparations and in the presence of certain materials, such as ascorbic acid.

To test the applicability of the spectropolarimetric and TLC procedures to stability studies, a limited solid-state compatibility study was initiated. The results of this investigation are tabulated in Table IV. Where spectropolarimetry and TLC are applicable, the agreement between the physicochemical and microbiological assays are excellent. The results are noteworthy in that the amount of degradation observed in the presence of benzoic and succinic acids is much greater than when barbituric acid or ascorbic acid is utilized, although the pK_1a for all the acids is about 4.2. This phenomenon may possibly be due to differences in the nature of the acidic moiety of the acids in question. Benzoic acid and succinic acid are both carboxylic acids, whereas ascorbic acid is a lactone which owes its acidic properties to the presence of an enediol grouping; barbituric acid derives its acidity from acid amide groups. The data suggest that, in the solid state, the effective acidity of benzoic and succinic acids is greater than that of ascorbic and barbituric acids, despite the fact that all exhibit comparable acidity in solution. An extended investigation is presently underway to explain more clearly the above observations as well as to ascertain the fundamental factors affecting the rate and kinetics of the reaction in the solid state.

The data in Table V summarize the stability results obtained on a commercially available "stabilized" calcium pantothenate and an experimental stabilized form tested under a variety of conditions. The stability of Sample A (experimental sample) is clearly superior to that of Sample B under the stress conditions employed; in addition, the amount of β-alanine present initially in Sample B is significant, suggesting that the vitamin should be analyzed for β-alanine by TLC prior to its incorporation into dosage forms.

Table V—Stability of Stabilized Calcium Pantothenate Forms

Condition	Percent β-Alanine A	Percent β-Alanine B
Initial	0.05	5.8
24 hr., 43% relative humidity	0.08	6.0
24 hr., 63% relative humidity	0.11	7.0
24 hr., 75% relative humidity	0.15	8.0
1 week, 55°, as is	0.08	6.0
1 week, 55°, 3% water	0.4	7.0
1 week, 55° + succinic acid[a]	2.0	10.0
1 month, 45° + ascorbic acid[b], 3% water	17	54

[a] Calcium pantothenate + succinic acid (1:4 w/w). [b] Calcium pantothenate + ascorbic acid (1:7.5 w/w).

Although the stability of pantothenates in solution has been well studied (21–23), a detailed stability of the solid state has not been reported. Frost (21) stated that the rate of destruction of calcium pantothenate in the solid state, just as in solution, is dependent upon the effective pH, but he did not define the meaning of this term. The present limited study suggests that the rate of degradation is dependent on the nature of the acid involved and may be related to the concept of general-acid catalysis. Further elucidation of the observed phenomena will be possible upon completion of the detailed investigation in progress.

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Separation and Quantitation of Mixtures of the Most Commonly Abused Drugs

NICOLAS H. CHOULIS

Abstract □ TLC has been used for the separation and semiquantitative estimation of various mixtures of narcotics, barbiturates, amphetamines, and tranquilizers appearing in the illicit market. Of the solvent systems employed, chloroform–dioxane–ethyl acetate–concentrated ammonium hydroxide (25:60:10:5 v/v) gave complete separation for all 17 compounds examined. Potassium permanganate (0.1%) and iodine fumes were used as detection reagents. Twelve different mixtures of two, three, and four compounds, randomly selected, were separated and the spots were quantitated using a UV absorption–concentration relationship. The spots were eluted from the plates, and their absorption was measured and compared to standard curves. Recoveries were found to be between 93 and 95% for quantities as small as 0.1 mg./ml., while quantities as small as 1 mcg. could be detected.

Keyphrases □ Narcotic mixtures with barbiturates, amphetamines, tranquilizers—separation, analysis □ Barbiturate mixtures with narcotics, amphetamines, tranquilizers—separation, analysis □ Amphetamine mixtures with narcotics, barbiturates, tranquilizers—separation, analysis □ Tranquilizer mixtures with narcotics, barbiturates, amphetamines—separation, analysis □ Abuse drugs (narcotic, barbiturate, amphetamine, tranquilizer mixtures)—separation, analysis □ TLC—separation, mixtures of narcotics, barbiturates, amphetamines, tranquilizers □ UV spectrophotometry—analysis, narcotics, barbiturates, amphetamines, tranquilizers

Capsules, tablets, powders, solutions, and other forms in various sizes have appeared on the illicit market, identified under various names and consisting of mixtures of drugs. Some examples are: LSD–amphetamine (in various forms), heroin hydrochloride containing methapyrilene, marijuana cigarettes dipped in opium (better known as O. J., "opium joints"), phencyclidine hydrochloride, LSD (in capsules), amphetamine with cannabis (most commonly used), amphetamine with barbiturates (known as drimaryl, particularly in England), strychnine with LSD and methamphetamine (tablets, known as "blue cheer," particularly in Australia), LSD on marijuana, heroin with methamphetamine and cocaine, and barbital with heroin. Numerous methods have been used to investigate all of these compounds (1–32), but combinations of these drugs have not always been thoroughly examined.

The aim of the present investigation was to provide a simple, *in vitro*, routine TLC method for the separation and quantitative determination of various mixtures of the most commonly abused drugs.

EXPERIMENTAL

Materials—The following were used: sodium amobarbital[1], m.p. 288° [lit. (33) m.p. 287–289°]; dextroamphetamine, puriss.[2], b.p. 204° [lit. (33) b.p. 203–204°]; cocaine hydrochloride[3], m.p. 195° [lit. (33) m.p. 195°]; codeine[3], m.p. 154° [lit. (33) m.p. 154–156°]; ephedrine hydrochloride[4], m.p. 219° [lit. (33) m.p. 216–220°]; methadone hydrochloride[4], m.p. 235° [lit. (33) m.p. 235°]; methamphetamine[5], m.p. 173.5° [lit. (33) m.p. 170–175°]; morphine[4], m.p. 255° dec. [lit. (33) m.p. 254–256°]; sodium pentobarbital[1], m.p. 313°; phenobarbital[1], m.p. 175.5° [lit. (33) m.p. 174–178°]; and sodium secobarbital[1], m.p. 293° [lit. (34) m.p. 293°]. Cannabidiol, hashish, LSD, marijuana, and opium were supplied by the National Institute of Mental Health and were used without further testing.

Table I—λ_{max} of All Drugs Used for UV Studies

Drug	λ_{max}
Dextroamphetamine	258
Cannabidiol	280
Cocaine hydrochloride	231
Codeine	211
Ephedrine hydrochloride	250
LSD	237
Marijuana	280
Methamphetamine	257
Morphine	250
Opium	250
Phenobarbital	253
Sodium amobarbital	238
Sodium pentobarbital	239
Sodium secobarbital	240

Developing Systems—The following solvent systems were used: (1) chloroform–acetone (9:1 v/v), (2) ethanol–methanol–concentrated ammonium hydroxide (85:10:5 v/v), (3) methanol–concentrated ammonium hydroxide (100:2 v/v); and (4) chloroform–dioxane–ethyl acetate–concentrated ammonium hydroxide (25:60:10:5 v/v).

Detection of TLC Spots—The following detection reagents were used: (1) UV light (long wavelength), (2) iodine fumes, (3) Dragendorff, and (4) potassium permanganate, 0.1% solution. The Dragendorff reagent is prepared as follows. Dissolve 2.125 g. bismuth subnitrate in 100 ml. of distilled water (Solution A) and 50 g. of potassium iodide in 125 ml. of distilled water (Solution B). Combine 10 ml. of Solution A, 10 ml. of Solution B, 20 ml. of glacial acetic acid, and 100 ml. of distilled water. The minimum concentration of drug detectable by Reagents 2, 3, and 4 was 1 mcg., while 2–3 mcg. of compound was the sensitivity limit for UV light detections.

Preparation of Plates—The plates (20 × 20 cm.) were coated with silica gel G[6], 250 nm. thick, according to Stahl (35). Silica gel precoated plates[7] (250 nm. thick) were also used.

General Procedure—Solutions of the compounds were prepared by dissolving 3 mg. of the pure compounds in 1 ml. of the appropriate solvent, namely: (*a*) water for sodium amobarbital, cocaine hydrochloride, ephedrine hydrochloride, methadone hydrochloride, sodium pentobarbital, and sodium secobarbital; (*b*) ethanol for dextroamphetamine, codeine, methamphetamine, phenobarbital, and LSD; and (*c*) chloroform for opium, marijuana, hashish, cannabidiol, and morphine.

Using accurately calibrated micropipets, samples of the solutions were applied to the plates 1.5 cm. from the bottom edges, and ascending chromatograms were run at room temperature (about 23°). All chromatograms were carried out in chromatographic chambers

[1] Ruger Chemical Co.
[2] Aldrich.
[3] Merck & Co.
[4] Mallinckrodt.
[5] City Chemical Corp.
[6] Scientific Manufacturing Industries.
[7] Analtech, Inc.

Table II—R_f Values (in Increasing Order) of the Various Compounds Using Solvent System 4

Compounds	R_f Values	Detection Systems[a] 1	2	3	4
Marijuana	0.17[b], 0.95[c]	+	+	+	+
Hashish	0.17[b], 0.41[d], 0.95[c]	+	+	+	+
Opium	0.25[e], 0.44[f], 0.75[g]	−	+	+	+
Morphine	0.25[e]	−	+	+	+
Ephedrine hydrochloride	0.32	−	+	+	+
Methamphetamine	0.40	−	+	+	+
Codeine	0.44	−	+	+	+
Dextroamphetamine	0.50	−	+	−	+
LSD	0.70	+	+	+	+
Methadone hydrochloride	0.73	−	+	+	+
Amobarbital	0.76	+	+	−	+
Cocaine	0.78	−	+	+	+
Phenobarbital	0.80	−	+	−	+
Sodium pentobarbital	0.83	−	+	−	+
Sodium secobarbital	0.87	−	+	−	+
Cannabidiol	0.95	+	+	+	+

[a] 1 = UV light, 2 = iodine fumes, 3 = Dragendorff, and 4 = potassium permanganate, 0.1%. [b] Δ-9-Tetrahydrocannabinol. [c] Cannabidiol. [d] Cannabinol. [e] Morphine. [f] Codeine. [g] Papaverine.

saturated in solvent vapors. The solvent front was allowed to travel 10 cm.; the plates were removed, air dried, and sprayed with the appropriate reagent.

Mixtures of two, three, and four compounds, selected at random, were prepared for qualitative analysis. The same mixtures, formed by using accurately measured amounts, were prepared for separation and quantitative determination of the components.

For commercially available or counterfeit types of dosage forms, an extraction procedure was followed. The tablet (pulverized) or the capsule form was dissolved in water (10 ml.) and extracted with chloroform (3 × 10 ml.). The layers were then separated, and the chloroform layer was rendered alkaline with 0.5 *N* NaOH. The two formed layers were again separated, and the aqueous layer was treated with dilute hydrochloric acid and extracted with chloroform (3 × 10 ml.). The chloroform extract was washed twice with water (2 × 10 ml.), dried over anhydrous magnesium sulfate, filtered, concentrated, and chromatographed for the detection of acidic drugs.

For basic drugs the initial aqueous layer was treated with sodium hydroxide (0.5 *N*) and extracted with chloroform (3 × 10 ml.); the chloroform phase was extracted with dilute hydrochloric acid, and the aqueous acidic phase was rendered alkaline with sodium hydroxide (0.5 *N*), and extracted with chloroform (3 × 10 ml.). The chloroform extracts were combined, washed with water (2 × 10 ml.), dried, concentrated, and chromatographed.

Method—The relationship between UV absorption and concentration of the various compounds was established by preparing solutions of the compounds at various concentrations (0.5–2.5 mg./ml. at 0.5-mg. intervals) and measuring their absorption at λ_{max} (Table I) (33, 36), using a spectrophotometer[8] with 1-cm. cells. Standard curves of absorbance *versus* concentration of the various compounds were then constructed.

Accurately measured solutions of the various compounds were applied on thin layers; duplicates were run parallel. The chromatograms were developed and the parallel running spots were detected, using one of the spray reagents, and marked. The areas corresponding to the marked spots were carefully scraped off, and the layers were quantitatively transferred to 10-ml. conical flasks. The compounds were eluted from the coating material by adding about 7 ml. of the appropriate solvent; the flasks were shaken well for about 10 min., centrifuged, and filtered into 10-ml. volumetric flasks. Solvent was added to the volume. A similar elution process was followed for a blank sample of the coating material alone. Spectrophotometric measurements were carried out, at λ_{max}, using the spectrophotometer and the same cells. The absorbance values obtained (after reducing the absorbance due to the blank) were compared to the standard curves.

[8] Beckman DU.

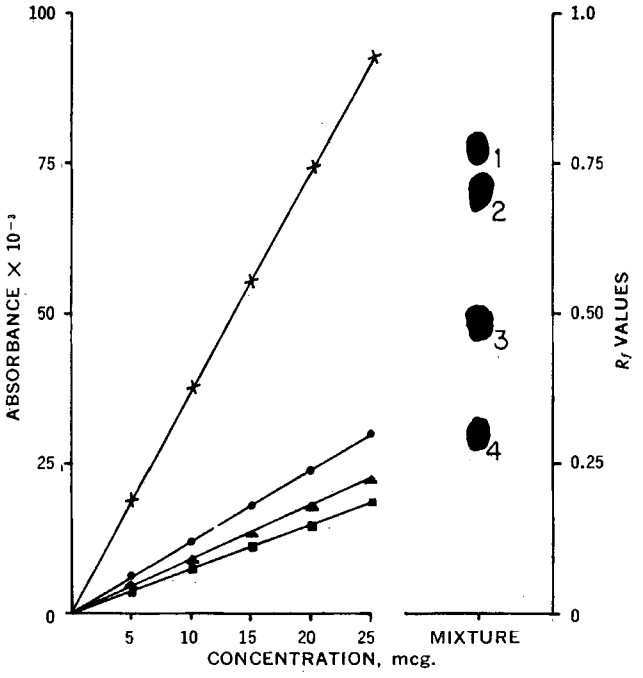

Figure 1—*UV absorption–concentration relationship (left) and* R_f *values (right) of the following compounds: 1,* ×, *phenobarbital; 2,* ●, *methadone hydrochloride; 3,* ▲, *dextroamphetamine; and 4,* ■, *ephedrine hydrochloride.*

RESULTS AND DISCUSSION

Only Solvent System 4 [chloroform–dioxane–ethyl acetate–concentrated ammonium hydroxide (25:60:10:5)] gave complete separation of all 17 compounds used; potassium permanganate, 0.1%, and iodine fumes detected all compounds. (See Table II, where the sensitivities of the detection reagents are also depicted.)

When a mixture of four compounds, namely ephedrine hydrochloride, dextroamphetamine, methadone hydrochloride, and phenobarbital was used, the chromatographic results (Fig. 1) indicated that the R_f values obtained agreed with the ones in Table II. Following the procedure described in the *Experimental* section (UV absorption–concentration relationship), quantitation of the four spots was carried out. The results obtained were compared to the standard curves of absorbance *versus* concentration of the four compounds (Fig. 1).

All experiments were performed at least in triplicate, and the mean values were recorded. No significant difference in these values was observed; 93–95% recoveries of the compounds from the spots were achieved. The results are tabulated in Table III, where the relationships of these compounds in concentration ranges between 0.5 and 2.5 mg./ml. were established.

The procedure was repeated using 12 different mixtures of two, three, and four compounds, randomly selected. The results were very similar to those already indicated.

Although the method is considered sufficiently accurate for the routine analysis of a wide variety of compounds commonly abused, some technical limitations may be imposed, particularly for compounds whose R_f values differ only between 2 and 3%, such as amobarbital, cocaine, and phenobarbital (R_f 0.76, 0.78, and 0.80, respectively), or for compounds for which more than one R_f value appears such as marijuana and opium (R_f 0.17, 0.95, and 0.25 and 0.44 and 0.75, respectively).

The R_f values are not absolute physical constants and may vary for many predictable and valid reasons. For example, the temperature of the experiments must be kept constant, and the purity of the solvents used must be examined so the R_f values will be reproducible. During the preparation of the plates, the variation of the thickness should be considered, since solvent vapor can be preadsorbed by the adsorbent easier on a thinner layer while a thick layer will not have time to reach a good equilibration. A lower proportion of adsorbed solvent will result with a thick layer and, therefore, a greater volume of solvent will be needed to wet a given

Table III—Absorbances of Amphetamine, Ephedrine Hydrochloride, Methadone Hydrochloride, and Phenobarbital at Various Concentrations

Concentrations, mg./ml.	Absorbances[a]							
	Amphetamine		Ephedrine Hydrochloride		Methadone Hydrochloride		Phenobarbital	
	1	2	1	2	1	2	1	2
0.5	0.004	0.003	0.003	0.002	0.006	0.004	0.018	0.012
1.0	0.009	0.008	0.007	0.006	0.012	0.011	0.037	0.035
1.5	0.014	0.014	0.011	0.0105	0.0175	0.017	0.056	0.052
2.0	0.0185	0.0175	0.015	0.014	0.024	0.023	0.075	0.072
2.5	0.0225	0.022	0.019	0.0185	0.030	0.029	0.092	0.089
Percent recovery		95		93		95		93.5

[a] 1 = pure compound, and 2 = TLC recovery.

area of the plate, resulting in higher R_f values on thicker plates. On the other hand, dry solvent vapor can displace some moisture from a partially activated plate, thus increasing its activity; and since it is likely that this will occur more rapidly and efficiently with a thin rather than a thick layer of silica gel, this effect could lead to an increase in R_f value with layer thickness. Readymade plates, such as the ones used in the present experiment, are often more reliable, provided that they all come from the same source.

The developing tank must be equilibrated with solvent prior to its use for at least 1 hr. at room temperature. The solvent system should not be reused more than five times, and freshly prepared solvents should not be kept for more than 48 hr.; otherwise, the R_f values obtained are not completely reproducible.

For quantitative determinations, if the compound is not detectable under UV light, parallel spots should be run simultaneously and should be detected first, separately, so that the positions of the spots to be quantitated can be determined without spraying over their area. Spray reagents interfere during spectrophotometric measurements, giving exaggerated results. Using this method, quantities as small as 1 mcg. can be detected, while quantities as small as 0.1 mg./ml. can be quantitated using UV spectrophotometry.

The described technique can be considered rapid and accurate for routine analysis. With certain modifications, it also can be used for the detection and quantitation of these compounds excreted from biological systems. For the latter, however, the presence of metabolites and urine contaminants should be taken into consideration.

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NMR Analysis of Pharmaceuticals VIII: Determination of Trimethadione in Various Dosage Forms

BRUCE A. GOLDWITZ▲ and JOHN W. TURCZAN

Abstract □ An NMR procedure is described for the determination of trimethadione in its various pharmaceutical dosage forms. The method is both accurate and precise, with a standard deviation of ±0.3%. The NMR results of synthetic mixtures and commercial preparations were compared to those obtained by USP XVIII and NF XIII procedures. The NMR technique offers the advantages of speed, simplicity, and specificity.

Keyphrases □ Trimethadione dosage forms—NMR analysis, compared to compendial methods □ NMR spectroscopy—analysis, trimethadione dosage forms

Trimethadione (3,5,5-trimethyl-2,4-oxazolidinedione) (I) is an anticonvulsant drug which is used primarily in the treatment of petit mal epilepsies.

Approaches to the quantitative determination of I vary. Gallelli and Kostenbauder (*1*) reported an IR assay method in which I is determined in the presence of its hydrolysis products. A manometric procedure based upon the decomposition of I by alkali and the subsequent measurement of the carbon dioxide formed was described by Taylor and Bertcher (2). Booker and Darcey (3) developed a GC method for the quantitation of I and its metabolite dimethadione in blood serum. Other techniques used for the assay of I in the pure form and in combination with other drugs include spectrophotometry (4), volumetric analysis (5), and the measurement of alkali consumed during the hydrolysis of I in strong base (6).

The method adopted by USP XVIII (7) for the analysis of I in the capsule and solution dosage forms, and by NF XIII (8) for the analysis of I in tablets, is based upon the hydrolysis of I with sodium hydroxide. The reaction products formed in the hydrolysis are *N*-methylcarbamylo-α-hydroxyisobutyric acid and *N*-methyl-α-hydroxyisobutyramide. The volatile amines produced during the total degradation of the amide (9) are refluxed and distilled into a boric acid solution and are subsequently measured by direct titration with standard sulfuric acid.

By virtue of its relatively high unit dosage (150 and 300 mg.) and characteristic resonance pattern, a quantitative NMR procedure was investigated for the determination of I. With the addition of an internal standard followed by extraction with a suitable solvent, a rapid and accurate quantitation can be accomplished.

An NMR procedure is described here for the analysis of I in its official pharmaceutical dosage forms. *tert*-Butyl alcohol was employed as the internal standard and water as the solvent. Besides providing quantitative results, the recorded NMR spectrum furnishes an identification of the active ingredient, thereby contributing to the specificity of the technique. The method was used to analyze both known mixtures and commercial preparations.

EXPERIMENTAL

Apparatus—An NMR spectrometer[1] equipped with a variable temperature probe[2], having a six-turn insert, was used.

Standard—Trimethadione reference standard was obtained from a commercial source[3].

Internal Standard—*tert*-Butyl alcohol[4], 99+ mol. %, was used.

Samples—Trimethadione capsules, solutions, and tablets were used.

Procedure—*Tablet Preparation*—Weigh and finely powder not less than 20 tablets.

Capsule Preparation—Weigh and mix thoroughly the contents of not less than 20 capsules.

Weigh accurately a portion of the powder equivalent to about 150 mg. of I into a glass-stoppered centrifuge tube. In the case of I solution, pipet an equivalent portion into the tube. Add about 50 mg., accurately weighed, of the *tert*-butyl alcohol internal standard and fill the tube to approximately the 4-ml. mark with water. Stopper the tube and shake thoroughly for about 2 min. The tablet and capsule sample should be centrifuged to separate the solution from the insoluble excipients.

Transfer about 0.4 ml. of the sample solution to an analytical NMR tube. Place in an NMR spectrometer and obtain the spectrum. Care should be taken to adjust the spin rate so that no spinning side band occurs in the region of interest between 1.0 and 1.8 p.p.m., using the delta scale to express magnetic field strength. All peak field positions are referenced to sodium 2,2-dimethyl-2-silapentanesulfonate (III) at 0 p.p.m. Integrate the peaks of interest at least five times.

The amount of I may be calculated as follows:

$$\frac{\text{mg. I}}{\text{tablet}} = \frac{A_{spl}}{A_{std}} \times \frac{\text{E.W.}_{spl}}{\text{E.W.}_{std}} \times \frac{\text{mg. } tert\text{-butyl alcohol}}{\text{mg. sample}} \times \text{average tablet weight} \quad \text{(Eq. 1}a\text{)}$$

$$\frac{\text{mg. I}}{\text{capsule}} = \frac{A_{spl}}{A_{std}} \times \frac{\text{E.W.}_{spl}}{\text{E.W.}_{std}} \times \frac{\text{mg. } tert\text{-butyl alcohol}}{\text{mg. sample}} \times \text{average capsule contents} \quad \text{(Eq. 1}b\text{)}$$

$$\frac{\text{mg. I}}{\text{ml. solution}} = \frac{A_{spl}}{A_{std}} \times \frac{\text{E.W.}_{spl}}{\text{E.W.}_{std}} \times \frac{\text{mg. } tert\text{-butyl alcohol}}{\text{ml. solution used}} \quad \text{(Eq. 1}c\text{)}$$

where:

A_{spl} = integral value of the signal representing I
A_{std} = integral value of the signal representing *tert*-butyl alcohol
E.W._{spl} = formula weight of I/6 = 23.86
E.W._{std} = formula weight of *tert*-butyl alcohol/9 = 8.236

Although no evidence of the instability of I was encountered in the dosage forms studied, the use of the described NMR procedure in those cases where decomposition products are present is discussed later.

[1] Varian A-60.
[2] V-6031.
[3] Abbott Laboratories, North Chicago, IL 60064
[4] Matheson, Coleman and Bell, East Rutherford, N. J.

Table I—Determination of Trimethadione in Standard Mixtures by NMR[a]

Standard Mixture	*tert*-Butyl Alcohol Internal Standard Added, mg.	Trimethadione Added, mg.	Trimethadione Found, mg.	Recovery, %
1	54.2	158.9	159.7	100.5
2	58.1	154.0	155.2	100.8
3	51.2	150.7	151.6	100.6
4	52.4	157.2	157.2	100.0
5	78.4	155.5	156.0	100.3
6	29.1	148.3	148.4	100.1
7	51.9	154.1	154.9	100.5

[a] *SD* ±0.3%.

RESULTS AND DISCUSSION

The selection of water as the solvent was a relatively straightforward choice. Compound I is soluble in water to about 5%. Furthermore, I solution USP contains about 40 mg./ml. in water. Utilizing this solvent obviates the necessity for a required extraction procedure and thus provides for a more simplified analysis. Equally important is the fact that the broad resonance signal of the solvent is sufficiently downfield from the I resonance pattern to allow for interference-free quantitation and distinct identification of the active ingredient.

tert-Butyl alcohol proved to be a suitable internal standard because it is very soluble in water and provides a single strong spectral signal at a useful upfield position.

Figure 1 represents a spectrum of trimethadione under the prescribed analytical conditions. The amount of I is calculated from the area integration of the singlet at about 1.55 p.p.m. due to the six methyl protons on the oxazolidine ring at position 5 on the ring, and the singlet at 1.25 p.p.m. is attributed to the nine methyl protons of the *tert*-butyl alcohol internal standard. The additional resonance peaks present in the spectrum are due to the methyl group of I at position 3 on the ring at about 3.02 p.p.m. and the broad solvent signal at approximately 4.6 p.p.m. In the actual analysis, it is preferred not to add the III reference standard since the methylene group beta to the sulfonyl group of III exhibits a multiplet that would overlap the sample signal in the analytical region of interest between 1.3 and 2.2 p.p.m. Even though the concentration of III in Fig. 1 is reduced to the point where no observable integration error would occur, the absence of III in the described analytical procedure provides for truer results and a more simplified analysis.

A group of known standard I mixtures was analyzed by this NMR technique, and the results are summarized in Table I. The method was both accurate and precise, with a standard deviation of ±0.3%. The relative proportions of I to *tert*-butyl alcohol had no significant bearing on the accuracy of the determination.

Approximately 10 commercial preparations of I in its various dosage forms were assayed by the described NMR procedure. Six of the results were also compared to results obtained using the official USP and NF procedure, and the findings are listed in Table II. Although the results are in good agreement, the NMR procedure has several distinct advantages over the official method. Banes (9) pointed out that the quantitative analysis of I as performed in the official procedure would be more conclusive if the drug constituents

Table II—Determination of Trimethadione in Commercial Preparations by NMR

Dosage Form	Declared, mg./Unit[a]	Found (NMR) mg./Unit[a]	Found (NMR) %	Found (Official Method) mg./Unit[a]	Found (Official Method) %
Tablet	150	141.9	94.6	141.3	94.2
Tablet	150	146.3	97.5	144.6	96.4
Capsule	300	305.1	101.7	298.5	99.5
Capsule	300	294.0	98.0	296.7	98.9
Solution[b]	1200	1175	97.9	1165	97.1
Solution	1200	1150	95.8	1156	96.3

[a] Single dosage form analysis. [b] For solution (mg./oz.).

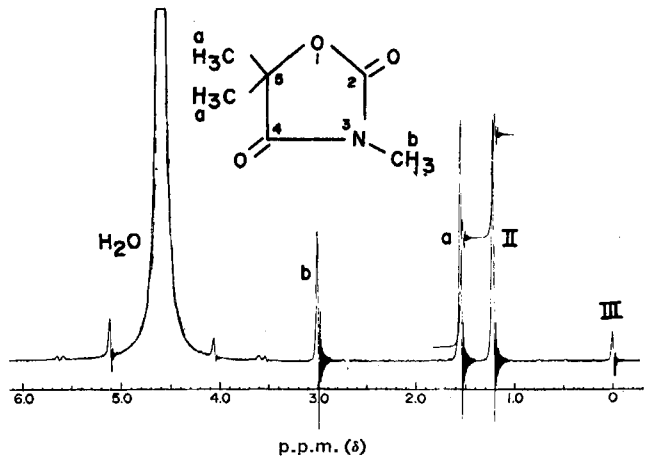

Figure 1—*NMR spectrum of trimethadione in water. Key: II, tert-butyl alcohol; and III, sodium 2,2-dimethyl-2-silapentanesulfonate.*

were first separated to ensure identity of the compound being hydrolyzed. The specificity drawback is not applicable to the NMR procedure as a result of the distinguishing resonance pattern ascribable to I. Speed and simplicity are additional characteristic traits of the NMR technique when applied to the quantitative analysis of pharmaceuticals. A complete assay can be performed in approximately 30 min. in the case of I, thus avoiding the tedious and time-consuming refluxing and distillation called for by the official method.

No interferences from any of the capsule, solution, or tablet excipients were observed in any of the analyses.

The ability of an analytical method to determine a drug in the presence of its decomposition products is a desirable attribute. Since I is stable under the analytical conditions described, no decomposition will take place as a result of the NMR procedure. However, the history of the drug could include conditions leading to decomposition. The kinetics study of Gallelli and Kostenbauder (1) demonstrated that I was hydrolyzed primarily in alkaline solution. As a result, the possibility of using this procedure in those instances where decomposition products are present was investigated, even though no evidence of decomposition was evident from the dosage forms analyzed.

The spectrum of the partially hydrolyzed drug provides an opportunity to analyze the extent of decomposition as well as the intact drug. The presence of the hydrolysis products can be detected by the appearance of two singlets at about 7 and 13 cycles upfield from the singlet due to the two methyl groups at position 5 on the oxazolidine ring and the appearance of two singlets at about 17 and 22 cycles upfield from the singlet due to the methyl group at position 3 on the ring. The total drug may be calculated by comparing the area of the three-peak system ascribable to the methyl groups on position 5 of the drug and the corresponding groups of the decomposed compounds with the area of the internal standard signal. The extent of decomposition may be measured by first comparing the area of the methyl group singlet for the intact drug at 3.02 p.p.m. (noting the correct equivalent weight) with the internal standard area and then dividing this weight by the weight of the total drug found.

The choice of the latter procedure is dictated by the fact that the differential integration is possible without difficulty since the peaks are well separated. On the other hand, although decomposition is manifest in the first three-peak system mentioned, the chemical shifts within the system do not allow for satisfactory integration and subsequent calculation of decomposition. It is possible to determine the extent of decomposition to about 2%.

In conclusion, the NMR technique, when applied to the analysis of trimethadione in its various pharmaceutical dosage forms, can provide an assay with an accuracy of about 1–2% while offering the benefits of speed, simplicity, and specificity.

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PHARMACEUTICAL TECHNOLOGY

Analog Computer Program for Simulating Variable Dosing Regimens

L. KIRSCHNER▲, T. H. SIMON, and C. E. RASMUSSEN

Abstract □ Based on a single-compartment model, an analog computer program was developed which utilizes both analog and logic components of a general-purpose hybrid computer. The program permits the simulation of the blood and urine levels that would be obtained following the administration of an initial dose followed by a series of additional doses. The magnitude of the doses, as well as the time period between doses, may be varied independently. The program can be used to aid the formulator in establishing the amount of drug that should be released during selected time periods from a timed-release dosage form to provide uniform blood levels.

Keyphrases □ Computer program, analog—simulation of variable dosing regimens □ Simulation of variable dosing regimens—analog computer program □ Timed-release dosage formulation—use of computer simulated blood and urine levels □ Dosing regimens, variable—simulation of blood and urine levels

The use of the analog computer for simulating drug distribution in the body has been widely accepted (1–5). Many of the reported analog computer programs have been limited to simulating drug levels after single-dose administration (6–14). Several authors have reported simulations based on multiple-dose administrations that were achieved by either digital computer techniques (15–17) or manual manipulation of analog computer components (18–21). At best, the manual methods are cumbersome and there is a possible loss of accuracy due to time-dependent manual manipulation. In addition, digital computer simulation does not permit the instantaneous observation of the effect of parameter modification on the drug level in a particular compartment.

This paper discusses how digital logic components (22), which have recently been made available as an integral part of general-purpose analog computers, can be used in the simulation of variable dose sequencing regimens. Such components permit manipulation, in microseconds, of operations that were previously performed manually. With the aid of the logic components, programs can be prepared that change parameter values automatically and make decisions based on either timing considerations or other arbitrary conditions inherent in the problem to be simulated.

The flexible analog computer program described here permits the simulation of a variety of dosing regimens and has many applications in pharmacokinetic research. It has the feature of simulating the administration of an initial dose followed by a sequence of doses of variable size where the time periods between the administration of doses may be varied independently.

EXPERIMENTAL

The program is based on the single-compartment model:

$$G \xrightarrow{k_a} B \xrightarrow{k_{el}} U \qquad \text{(Eq. 1)}$$

where G = amount of drug in the gut, B = amount of drug in the blood, U = cumulative urinary excretion level, k_a = first-order rate constant of absorption, and k_{el} = first-order rate constant of excretion.

The model is described by the following differential equations:

$$\frac{dG}{dt} = -k_a G \qquad \text{(Eq. 2a)}$$

$$\frac{dB}{dt} = k_a G - k_{el} B \qquad \text{(Eq. 2b)}$$

$$\frac{dU}{dt} = k_{el} B \qquad \text{(Eq. 2c)}$$

Although the single-compartment model was chosen for demonstration purposes, the program can be easily modified to represent almost any pharmacokinetic model.

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The flexible analog computer program described here permits the simulation of a variety of dosing regimens and has many applications in pharmacokinetic research. It has the feature of simulating the administration of an initial dose followed by a sequence of doses of variable size where the time periods between the administration of doses may be varied independently.

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Although the single-compartment model was chosen for demonstration purposes, the program can be easily modified to represent almost any pharmacokinetic model.

Table I—Key to Analog Computation Symbology[a]

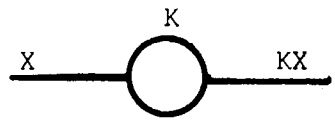
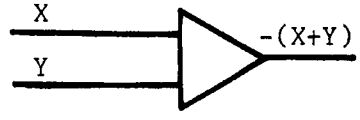
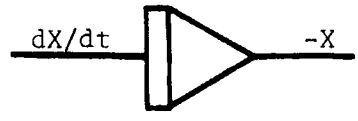
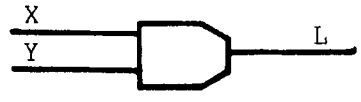
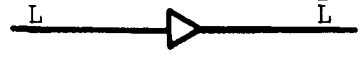
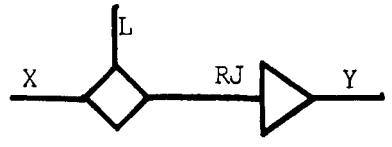
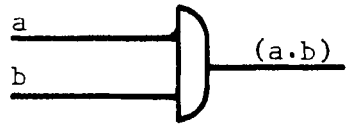
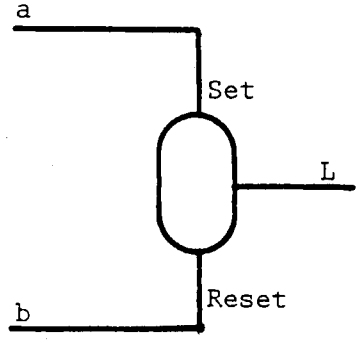
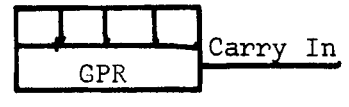
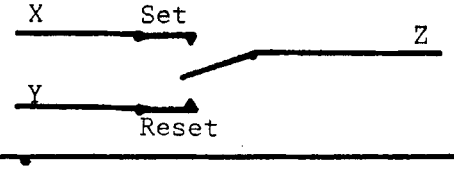
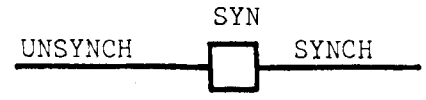
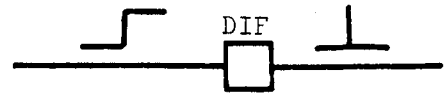
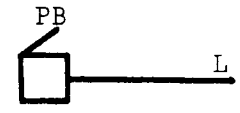
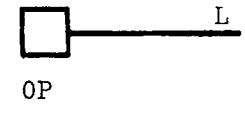
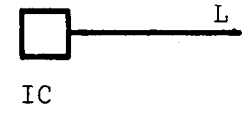
Component	Symbol	Remarks
Potentiometer		
Inverter		
Summer		
Integrator		
Comparator		L = high if (X + Y) = positive low if (X + Y) = negative
Logic inverter		
Electronic switch		Y = X if L = high 0 if L = low
AND gate		
Flip-flop		
General-purpose register		
Function relay		Z = X if switch is Set Y if switch is Reset

Table I—(*Continued*)

Component	Symbol	Remarks
Synch		Synchronizes asynchronous events in accordance with an internal timer
Differentiator		Output is a pulse of 1-μsec. duration when input changes from low to high
Logic push button		L = high if PB is Set low if PB is Reset
Operate bus line		L = high if computer is operating low if computer is in IC
IC bus line		L = low if computer is operating high if computer is in IC

[a] X, Y, and Z are analog signals; a, b, and L are logic signals.

The analog computer[1] program (Fig. 1 and Table I) can be visualized as being divided into three interdependent subprograms: timing, switching, and analog simulation. The basic portion is the analog simulation subprogram, which solves the differential equations describing the pharmacokinetic model. For the analog computer program to simulate the administration of a variable dose regimen, doses must be introduced into the analog simulation subprogram at preselected time intervals. The program section that causes doses to be entered into the analog simulation portion will be called the switching subprogram, and the program portion that controls the time at which these doses are switched into the analog subprogram will be called the timing subprogram. The key aspect of the program is the simulation of drug disposition in the gut compartment after the administration of repetitive doses. Since doses may only be added to integrators in the initial conditions (IC) mode, the simulation is accomplished by using two integrators operating in tandem to follow the amount of drug present in the gut. The modes of the two integrators are complementary when the program is operating; when a new dose is being added to one integrator, which is in the IC mode, the other integrator is integrating the previous dose. A more detailed description of the operations of the three subprograms follows.

Timing Subprogram—The timing subprogram is used to control the time at which the individual doses are switched into the analog simulation and to synchronize the operations of the switching and analog subprograms. To accomplish these tasks, the timing subprogram generates repetitive logic signals of variable frequency.

The timing subprogram functions as a triangular and square wave oscillator where the comparator, by switching, controls its own bias voltage. The triangular wave is the output of integrator 14, and the square wave is the output of the comparator. The frequency of oscillation is controlled by the gain of integrator 14. Examples of the outputs of the comparator and integrator 14 are illustrated in Fig. 2.

At time zero, the program is in the IC mode and the logic signal from the IC bus line is high[2], while the logic signal from the operate (OP) bus line and the output of AND gate 9 are low and the electronic digital/analog (D/A) switch 1 is open. The outputs of summer 13 and integrator 14 have values of −1.0 machine unit[3], so the output of the comparator is low.

When the program is put into OP, the logic level of the OP bus line goes high, the logic level of the IC bus line goes low, and the states of the comparator, AND gate 9, and D/A switch 1 remain unchanged. The output of summer 13 remains at −1.0 mu while integrator 14 integrates from −1.0 to +1.0 mu in time $2T$ at the rate of $1/T$, where T is the time required for integrator 14 to integrate from 0.0 to +1.0 mu. When the output of integrator 14 attains a value of +1.0 mu, the negative bias on the comparator is overcome, forcing the output of the comparator high. The change of state of the comparator drives the output of AND gate 9 high, which closes D/A switch 1. The output of summer 13 changes from −1.0 to +1.0 mu; therefore, integrator 14 integrates from +1.0 to −1.0 mu during the time period from $2T$ to $4T$. At time $4T$, the cycle repeats itself. The output of integrator 14 is a triangular wave of period $4T$, and the output of the comparator is a square wave which is low from $0T$ to $2T$ and is high from $2T$ to $4T$. The square wave output of the comparator is routed to the differentiator in the switching subprogram and to AND gates 10 and 11, which control the modes of integrators 1 and 2 in the analog subprogram.

Switching Subprogram—The switching subprogram controls the administration of the doses so that only the dose desired is switched into the analog subprogram. In operation, it accepts timing pulses from the timing subprogram and switches the selected dose, which is set on a potentiometer, so that the dose is routed to integrator 1 in the analog subprogram.

The values of the doses are set on potentiometers (pots) 7–11, which are connected to amplifier 12 through D/A switches controlled by logic signals. A D/A switch is open when the gate is logic low and is closed when the gate is logic high. To control the switching of the doses by the timing subprogram, the output of the comparator is put through a differentiator (DIF). The output of the DIF is a pulse of 1-μsec. duration when its input signal changes from low to high. Therefore, for each period of the comparator, one pulse is generated by the DIF.

The general-purpose register (GPR) counts pulses and its combined output, in this case a grouping of four logic levels, can be used to designate a number in binary form. The AND gates 2–7 are used to decode specific binary numbers, and their outputs go high when the number they are programmed to detect is loaded in the

[1] An Electronic Associates Inc., fully expanded, 380 analog/hybrid computing system, equipped with a series 1140 Variplotter recorder, was used throughout.

[2] "High" denotes a logic signal of +5 v. "Low" denotes a logic signal of 0 v.

[3] One machine unit equals 10 v. and is designated mu.

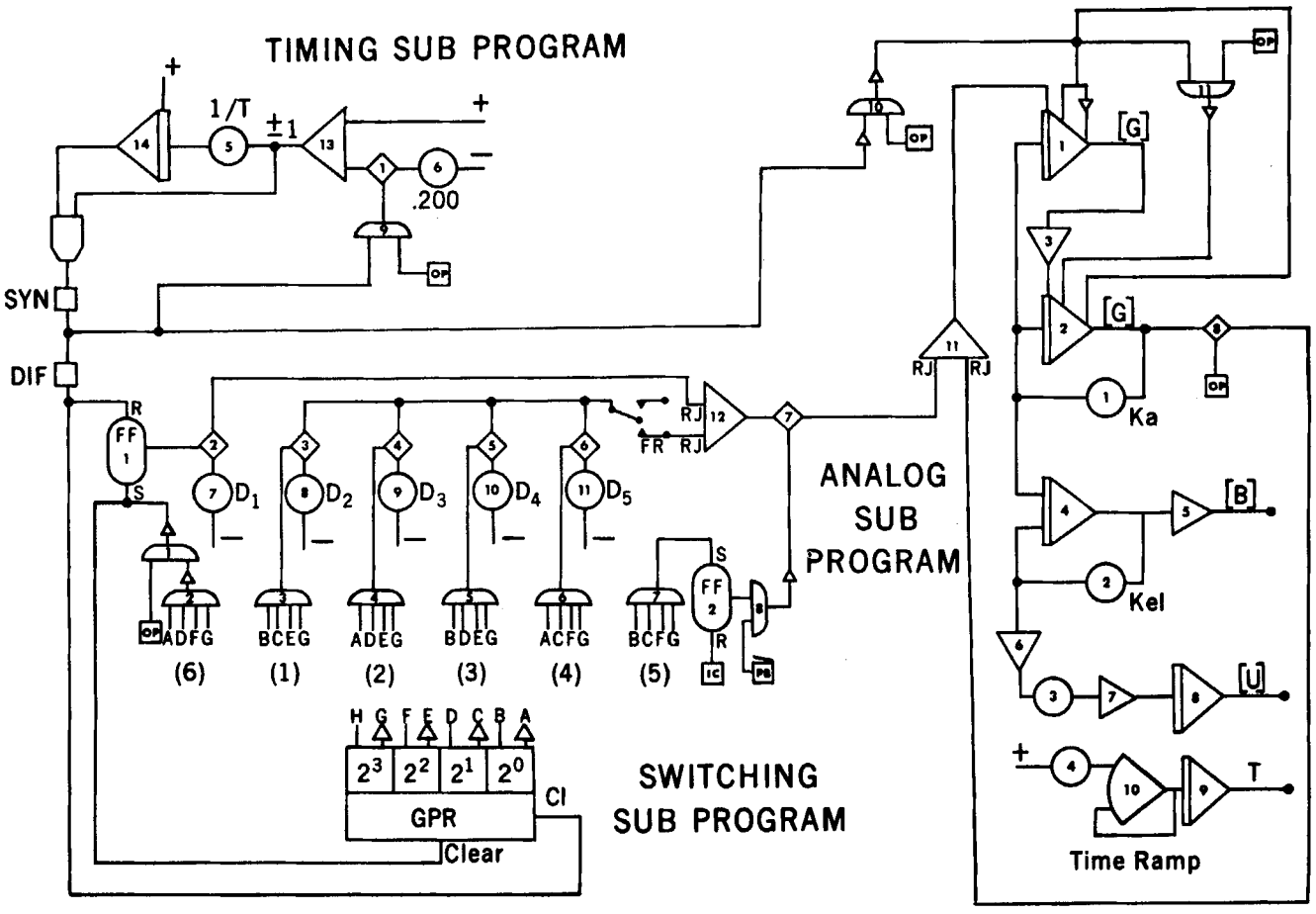


Figure 1—*Analog computer program.*

GPR. When the output of one of these AND gates goes high, the appropriate D/A switch is closed and the corresponding dose is routed through amplifiers 12 and 11 to the IC input of integrator 1 in the analog subprogram. Dose 1 (D1), which is set on pot 7, is connected when either the computer is in the IC mode or the output of AND gate 2 is high.

The GPR is programmed to clear whenever the computer is in the IC mode or AND gate 2 is high. Thus, the cycle of dose administrations is automatically repeated. The repetitive dosing feature of the program can be eliminated by manually depressing a push button (PB) causing a logic level high to be transmitted to AND gate 8. If the PB is high, D/A switch 7 opens after dose 5 is administered, disconnecting all of the doses from the analog portion of the program. However, if the output of the PB is low, the sequence of doses is repeated when the number loaded into the GPR equals the value set on AND gate 2. For single-dose administration, the function relay (FR) switch is opened, disconnecting doses 2–5.

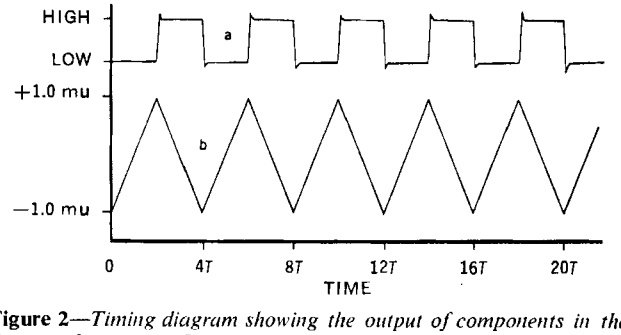


Figure 2—*Timing diagram showing the output of components in the timing subprogram. Curves a and b represent the outputs of the comparator and integrator 14 with time. The value of* T *is variable and is equal to the time needed for integrator 14 to integrate from 0 to +1.0 mu.*

Analog Subprogram—The differential equations, which describe the pharmacokinetic model, are solved by the analog subprogram. Values for initial conditions can only be set on integrators when they are in the IC mode. To simulate the administration of multiple doses, the gut integrator is returned to the IC mode each time a dose is given while the remaining integrators are operating. If only a single integrator was used to simulate the disposition of the drug in the gut, and if the new dose was entered into the gut integrator before the previous dose had been completely dissipated, then the amount of drug remaining in the gut would be lost from the simulation at the instant the integrator went into the IC mode. To prevent such losses, a two-integrator system was developed, where integrators 1 and 2 simulate the disposition of the drug in the gut. The integrators act in tandem and are in complementary modes (when one integrator is in the IC mode, the other integrator is in the OP

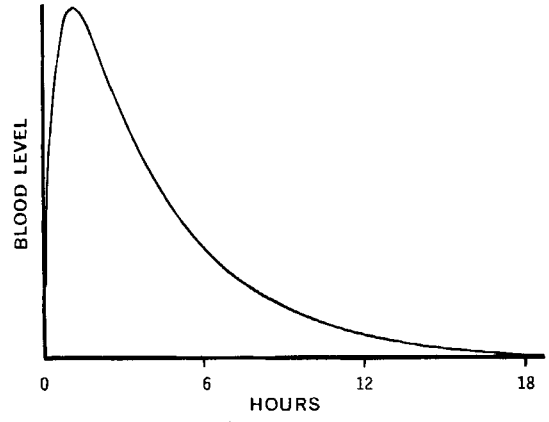


Figure 3—*Projected blood level curve from a single 50.0-mg. administration of the hypothetical drug* (k_a = *2.0 hr.*$^{-1}$, k_{el} = *0.30 hr.*$^{-1}$).

Table II—Pharmacokinetic Parameters

Parameter	Value
Dose	50.0 mg.
k_a	2.00 hr.$^{-1}$
k_{el}	0.30 hr.$^{-1}$

mode) at all times, except when the computer is in the IC mode.

The two integrators are interconnected so that the output of one integrator is the IC input of the other. When an integrator is in the IC mode, its output is equal to its IC input; therefore, if its IC input is a changing value, the integrator tracks this value. Amplifier 11 sums the new dose and any residual drug that is left in the gut and its output is connected to the IC input of integrator 1. When integrator 1 is in the IC mode, its output is equal to the sum of [G] plus the new dose; when it goes into the OP mode, it integrates from that value. When integrator 1 goes into the OP mode, integrator 2 goes into the IC mode and tracks the output of integrator 1. When either integrator is in the IC mode, the value of the amount of drug remaining in the gut is being tracked, so this value is never lost.

The modes of integrators 1 and 2 are controlled by AND gates 10 and 11 which, in turn, are controlled by the output of the comparator. When the program is in the IC mode, both integrators are in IC. At this time the outputs of both integrators equal the value of dose 1. When the program is put into the OP mode, integrator 1 goes into OP and integrator 2 remains in IC. The output of integrator 1 is the value of the amount of drug in the gut, [G], and since integrator 2 tracks the output of integrator 1, its output is also equal to [G]. At time $2T$, integrator 1 goes into IC and integrator 2 goes into OP. Integrator 2 integrates from the last value of [G], while the output of integrator 1 becomes [G] plus the value of dose 2. At time $4T$, integrator 1 goes into OP and integrator 2 into IC and the cycle is repeated. The value of the output of integrator 2 is always equal to [G], the amount of drug in the gut.

As stated previously, the single-compartment model was used only for demonstration purposes. Almost any pharmacokinetic model can be programmed utilizing the "two integrator" system.

RESULTS AND DISCUSSION

Once kinetic parameters have been determined from an immediate-release dosage form, the variable dose program can be used to project blood and urine levels for various pharmacokinetic problems. Additionally, it would not be difficult to proceed to a more complex model since the subprograms that control the administration of doses, namely the timing and switching subprograms, are model independent.

For demonstration purposes, a hypothetical situation based on the single-compartment model will be used to illustrate one application of this program. The situation selected is one where it is desired to formulate a timed-release dosage form and where the single-compartment pharmacokinetic constants have been previously determined (in this case they will be arbitrarily assigned). The goal is to determine what the release pattern of the active from the dosage form should be so that the most uniform blood level above the therapeutic level is produced. The assumption made is that the drug is released in bursts (at periodic intervals, portions of the drug are released instantaneously).

The arbitrarily selected pharmacokinetic parameters (Table II) describe the distribution of a 50-mg. immediate-release dose of a drug with rapid absorption and moderately fast elimination. As illustrated in Fig. 3, the drug is eliminated rapidly from the body, making it a good candidate for a timed-release preparation.

Assuming it is desired to formulate a 150-mg. product that would maintain therapeutic and uniform blood levels for 12 hr., the variable dose program can be used to predict the amount of drug that should be released from a timed-release formulation at given periods. For this situation, one can simulate bursts of drug being released at the beginning of each time interval. Since both the amount of drug administered and the interval between administrations can be controlled, the optimized blood level curve can be rapidly obtained. The effect of manipulating the sizes of the doses and the time interval between doses may be visualized directly on either an oscilloscope or a graphic plotter. To predict the "stacking" effect, the program was designed so that the sequence of dose

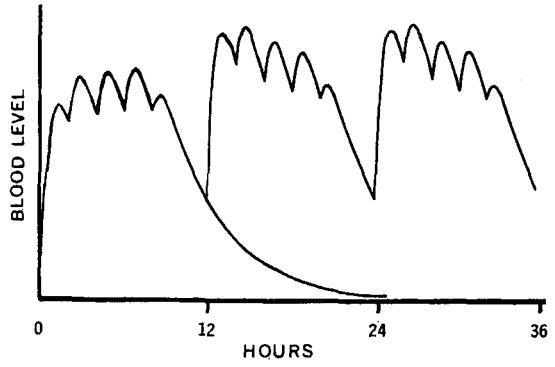


Figure 4—*Projected blood level curve depicting the timing sequence that produced the most uniform blood level curve* (k_a = *2.0 hr.*$^{-1}$, k_{el} = *0.30 hr.*$^{-1}$). *The administration of doses is as follows: 60.0, 25.0, 25.0, 25.0, and 15.0 mg. at 0, 2, 4, 6, and 8 hr., respectively. The regimen was repeated every 12 hr.*

administrations may be repeated continually. The results of these trials, including the repeated dose sequences, and the selected dose regimen are shown in Fig. 4. Since the basic assumption in this case was that the dose is released in bursts at these time intervals, it would be suggested that the formulator prepare an oral dosage form that would provide an *in vitro* release pattern in accordance with this regimen.

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Nitroglycerin Sublingual Tablets I: Stability of Conventional Tablets

SALVATORE A. FUSARI

Abstract □ Nitroglycerin in molded sublingual tablets volatilizes and undergoes intertablet migration. Consequently, tablets that are uniform when manufactured develop enough intertablet dose variation that they frequently fail the USP XVIII content uniformity test. Factors influencing volatilization and migration are discussed.

Keyphrases □ Nitroglycerin tablets—stability, volatilization, and intertablet migration □ Tablets, nitroglycerin—stability, volatilization, and intertablet migration □ Content uniformity, nitroglycerin tablets—volatilization and intertablet migration

In recent years, a growing body of evidence has accumulated which shows that conventional nitroglycerin tablets lose potency if stored improperly (1–5). A little-known fact is that the stability of nitroglycerin tablets is complicated by the development of intertablet dose variation caused by the volatilization and subsequent intertablet migration of nitroglycerin[1]. Tablets that are uniform when manufactured develop increased individual tablet variation upon storage, even when little potency loss is observed and even if the tablets are refrigerated. This seeming anomaly was observed in this laboratory, and supporting data are given here.

Banes (2) reported an almost total loss of nitroglycerin from tablets stored in blister packs; most of this nitroglycerin was recovered from the adhesive used to cement the blister pack laminate together. Earlier, at least one manufacturer[2] found that nitroglycerin tablets, dried on brown Kraft paper, transferred substantial amounts of nitroglycerin to the paper. The present author found that stuffing materials in contact with nitroglycerin tablets readily absorb varying amounts of nitroglycerin from the tablets.

Bell (6) examined 55 lots of nitroglycerin tablets from four different manufacturers. Ten individual tablets were assayed per lot. His results showed that 101 of the 550 tablets assayed fell outside of the USP XVI limits of 80–112%. Although Bell made no mention of instability, it is now clear that his data reinforce the finding in this article.

The observation that nitroglycerin readily migrates through the bottle space into cotton stuffing offered a possible explanation for the instability of sublingual nitroglycerin tablets. Consequently, this study was undertaken to define the nature of the problems associated with the instability of nitroglycerin tablets. This information would be fundamental to the development of methods for stabilizing the tablets through either modification of the immediate container or reformulation.

[1] During the remainder of this article, the term stability will encompass both potency loss and lack of content uniformity.

[2] Personal communication, Warner-Chilcott Laboratories.

Table I—Assay of Placebos and Tablets[a] in Contact with Each Other at 25° for 1 Month

	Average Assay in Percent —of Label Claim— Placebos	Tablets
Placebos mixed with nitroglycerin tablets	93.3	34.7
Placebos above nitroglycerin tablets	12.4	91.1
Placebos below nitroglycerin tablets	29.1	90.1

[a] 600 mcg. (1/100 gr.).

EXPERIMENTAL

Assay Procedure—The assay procedure was an automated adaptation of the method of Bell (6). Previous experience showed that this method is stability indicating[3]. Tablets (0.3 mg.) were dissolved in 10 ml. of water in a glass-stoppered 50-ml. centrifuge tube; 0.4- and 0.6-mg. tablets required 15 and 20 ml. of water, respectively. The tubes were shaken vigorously initially and after two 5-min. intervals to ensure solution of the nitroglycerin. After solution was accomplished, the samples were placed in sample cups for the Sampler II using an Auto Analyzer[4] set at a rate of 30 samples/hr. At least 30 individual tablets were assayed per lot of product. Standards preceded and followed every 10 samples to allow close monitoring of the assay procedure. Calculations for each set of assays included the average, the range, and the standard deviation in order to facilitate comparisons among lots. The standard deviation was calculated, although normal distributions were probably not obtained due to large, irregular losses of nitroglycerin from the tablets. However, it serves as a convenient method for comparison.

Transfer of Nitroglycerin from Sublingual Tablets to Placebo Tablets—Placebo tablets were prepared using a rotary press[5]. The physical specifications for these tablets were as follows: 55–60-mg. weight, 0.48-cm. (0.19-in.) diameter, 0.29-cm. (0.115-in.) thickness, flat-faced, and beveled-edge punches. These tablets were purposely made larger than commercial nitroglycerin tablets to permit ease of sorting for assay purposes.

Three separate experiments were performed to evaluate if drug migration would occur, and whether the position of the placebo relative to the nitroglycerin tablets in the container had any effect.

In each experiment, 25 placebo tablets and 50 600-mcg. (1/100-gr.) nitroglycerin tablets were placed in normal commercial bottles[6] and allowed to stand at room temperature for 1 month. Four bottles were set up for each experiment. The results, which were obtained by one analyst and confirmed independently by another on a separate set of bottles, are shown in Table I.

Comparison of Content Uniformity at Time of Release and after Aging—All samples checked were in commercial bottles containing 100 tablets, and all had been stored at ambient room temperature. Sixty lots of tablets of various ages and dosage sizes were assayed. Fifty-six of these lots had already been marketed and four were new lots pending release. Comparisons of the "release" assays and aged assays are given in Tables II–IV.

Content Uniformity of Freshly Manufactured Lots—Four lots of newly manufactured 400-mcg. (1/150-gr.) tablets were assayed to

[3] To be submitted for publication.

[4] Technicon.

[5] Stokes B-2.

[6] Six-milliliter, round, amber bottle with screw cap containing an Excelloseal liner.

Table II—Comparison of "Release" and "Aged" Assays of Nitroglycerin Tablets [600 mcg. (1/100 gr.)] with Ambient Room Temperature Storage

Average Assay at Time of Manufacture	Range of Assay	σ[a]	Elapsed Time for "Aged" Assay, Months	Average Assay of 30 "Aged" Tablets	Range	σ
			Passes[b]			
101.8 (10)	93.3–109.0	4.5	14	95.0	75.5–122.1	9.8
101.1 (10)	96.6–109.2	3.5	14	100.3	78.5–116.1	11.0
102.4 (20)	96.7–109.3	3.8	13	98.2	77.0–121.1	13.4
103.1 (20)	89.4–115.0	5.1	12	97.9	83.2–123.9	8.7
111.4 (10)	101.1–116.8	4.2	11	96.3	84.1–114.9	6.7
106.1 (10)	98.3–111.9	3.9	8	98.0	78.7–120.3	9.0
103.6 (10)	96.6–109.8	3.6	8	95.0	81.1–116.6	8.4
105.0 (10)	98.9–112.7	4.9	7	96.0	81.7–113.8	9.3
98.0 (10)	90.2–111.0	5.9	6	98.0	77.7–117.5	11.3
104.1 (10)	96.1–112.2	4.9	6	98.1	78.9–121.0	9.9
93.5 (10)	90.7–101.2	3.0	5	94.2	78.0–110.5	8.3
96.2 (10)	91.7–102.4	3.5	5	99.8	79.5–115.6	10.0
			Fails[b]			
100.6 (10)	92.2–106.3	3.6	10	87.3	66.6–106.7	8.9
104.9 (10)	101.6–110.0	2.4	7	94.2	72.7–110.4	8.5

[a] Standard deviation. [b] Refers to passing or failing the content uniformity test in USP XVIII prior to the allowance of one "flyer" in 30 tablets.

Table III—Comparison of "Release" and "Aged" Assays of Nitroglycerin Tablets [400 mcg. (1/150 gr.)] with Ambient Room Temperature Storage

Average Assay at Time of Manufacture	Range of Assay	σ	Elapsed Time for "Aged" Assay, Months	Average Assay of 30 "Aged" Tablets	Range	σ
			Passes			
108.4 (40)	95.4–126.5	6.3	11	99.6	88.8–117.6	7.4
106.8 (10)	99.9–113.1	4.8	10	96.1	81.0–116.1	8.6
101.7 (10)	94.2–112.1	5.5	10	96.2	87.0–111.7	6.0
105.9 (10)	100.2–114.0	5.0	8	103.9	83.7–122.8	7.5
100.1 (10)	94.6–107.9	3.9	8	101.2	75.6–130.0	10.5
101.9 (10)	95.7–106.5	3.5	8	96.4	86.5–107.1	5.8
109.0 (10)	102.3–114.9	4.3	7	95.8	78.3–115.2	9.3
93.8 (10)	83.8–104.6	7.8	7	99.5	84.9–115.6	7.4
99.0 (10)	78.6–110.1	8.5	7	97.3	84.3–107.7	5.6
107.0 (10)	90.9–108.0	6.2	5	99.1	82.8–115.5	9.3
102.3	96.5–113.0	4.7	3	102.1	88.7–119.1	8.5
103.1 (10)	99.3–109.4	3.5	2	103.9	83.7–135.2	10.3
104.4 (10)	99.5–108.8	3.6	2	98.6	75.9–131.7	15.6
105.4 (10)	97.4–110.5	3.8	1	102.0	82.5–120.6	9.5
103.9 (10)	95.5–111.2	5.2	1	102.0	76.2–129.2	10.7
101.4 (10)	95.1–107.8	4.1	1	98.2	80.9–120.6	9.0
98.9 (30)	87.2–109.8	5.3				
103.6 (30)	93.7–114.8	4.9				
102.8 (30)	95.5–111.1	4.0				
102.7 (30)	92.2–112.7	4.6				
			Fails			
95.9 (10)	90.3–110.8	6.6	11	97.3	72.5–124.7	9.7
105.3 (10)	99.3–109.6	4.2	8	101.1	74.3–120.4	10.7
108.9 (40)	99.5–116.9	4.5	7	102.0	72.2–121.9	13.5
104.7 (10)	96.1–108.2	3.8	7	100.1	69.0–123.8	11.5
105.4 (10)	98.6–111.9	3.6	5	95.0	53.1–121.4	13.9
105.4 (10)	100.1–109.5	3.3	5	96.6	71.4–119.3	13.5
102.8 (10)	97.9–112.8	4.4	5	91.6	65.9–113.9	11.2
102.9 (20)	96.5–113.0	3.8	4	95.3	68.7–131.9	16.6
103.1 (10)	96.7–107.1	3.4	3	99.5	62.5–131.0	15.0
103.8 (10)	103.7–109.8	3.8	3	97.7	59.4–135.3	15.8
99.3 (10)	90.3–109.5	4.9	2	92.1	66.3–115.2	11.7
101.7 (10)	98.2–104.8	2.4	1	101.9	72.3–113.6	11.2

show the manufacturing process capability and to establish a baseline for additional experimental work. The results are shown in Table V.

Effect of Room Temperature Storage for 1 Month with and without Rayon Stuffing and Wax Seals—The four new lots of nitroglycerin tablets described above were placed in containers for this experiment while the assays described in Table V were being performed. Tablets were put into 120 commercial bottles[6]. Sixty of the bottles were "stuffed" with rayon and the other half were not. After storage for 1 month at room temperature, 30 tablets from each set were reassayed (Table VI).

Effect of Stuffing and Cap Liners at 37 and 45° for 1 Month—Tablets from Lot 345906 (Table V) were placed in amber glass bottles with screw caps. Seven different cap liners were used, both with and without a wax seal and without stuffing. Rayon, tissue, or cotton stuffing was used with each of the different cap liners in bottles without wax seals. The 136 bottles were stored at 37 and 45° for 1 month. At the end of this time, a bottle of each type was removed from storage and 30 tablets from each bottle were assayed individually (Table VII).

Comparison of Storage at Room and Refrigerator Temperatures—

Table IV—Comparison of "Release" and "Aged" Assays of Nitroglycerin Tablets [300 mcg. (1/200 gr.)] with Ambient Room Temperature Storage

Average Assay at Time of Manufacture	Range of Assay	σ	Elapsed Time for "Aged" Assay, Months	Average Assay of 30 "Aged" Tablets	Range	σ
			Passes			
100.7 (10)	92.0–105.1	5.0	9	93.3	77.6–108.6	6.6
105.9 (10)	98.5–113.8	5.1	8	94.2	77.7–129.2	11.4
102.8 (10)	97.3–111.7	5.0	7	95.6	76.9–112.2	8.3
103.1 (10)	98.5–108.8	4.7	7	94.7	81.8–120.1	11.1
104.5 (10)	100.6–109.4	2.4	7	99.4	79.9–128.4	12.5
106.3 (10)	102.5–112.0	3.2	4	104.1	80.2–128.2	13.0
102.6 (10)	96.4–107.7	3.8	3	95.3	84.9–104.9	6.3
			Fails			
100.9 (20)	89.1–111.2	6.2	11	75.2	39.6–91.0	11.8
93.8 (20)	83.3–102.5	3.6	10	88.5	71.3–105.3	8.1
103.6 (10)	94.1–113.0	6.4	7	102.1	63.5–132.9	13.6
101.9 (10)	98.5–105.8	3.2	7	95.3	67.4–121.8	13.8
103.8 (10)	100.8–108.4	2.3	4	88.3	69.2–125.0	14.3
104.8 (10)	97.7–110.8	4.1	4	95.5	63.3–152.6	17.0
98.0 (10)	78.6–105.2	8.2	4	89.4	56.6–131.8	18.7

Table V—Assay of Freshly Manufactured Nitroglycerin Tablets [400 mcg. (1/150 gr.)]

Lot Number	Assay in Percent of Label Claim: Average of 30 Tablets	Range of Assays	Standard Deviation
345903	98.9	87.2–109.8	5.3
345904	103.6	93.7–114.8	4.9
345905	102.8	95.5–111.1	4.0
345906	102.7	92.2–112.7	4.6

Tablets in commercial packages were stored at 4, 25, 37, and 45° and at 37° and 85% relative humidity. Data accumulated for 600-, 400-, and 300-mcg. (1/100-, 1/150-, and 1/200-gr.) tablets at 4 and 25° (room temperature) are given in Table VIII for comparison.

Stability of Nitroglycerin Tablets [400 mcg. (1/150 gr.)] at 25, 37, and 45° at Monthly Intervals—After preliminary experiments showed that polyethylene terephthalate[7] was a better cap liner than Excelloseal for nitroglycerin tablets, two lots of tablets were placed in commercial bottles with screw caps containing polyethylene terephthalate[7] liners. Initial assay values were obtained and the bottles were then stored at 25, 37, and 45°. At monthly intervals, one bottle of each lot stored under each of the three conditions was removed and 30 tablets were assayed (Table IX).

RESULTS AND DISCUSSION

Until recently, analytical techniques did not lend themselves to large numbers of individual tablet assays of low drug dosage forms. This situation improved dramatically with Bell's (6) publication of an individual tablet assay and the advent of automated analytical methodology. A combination of these two techniques has enabled the present author to assay many individual tablets, both rapidly and accurately, thus avoiding the "leveling" effect obtained from composite assays. As a result, greater insight was gained concerning the causes of the instability of nitroglycerin tablets. The tremendous amount of work involved in both stability and formulation studies on nitroglycerin tablets would have been physically impossible without the use of an automated analytical system.

The results shown in Table I clearly demonstrate that nitroglycerin can and does migrate from tablet to tablet. The migration occurs because of the inherent volatility of nitroglycerin[8] and must occur through the vapor phase. This migration is mediated by the stresses applied to the tablet by its immediate environment. Placebos that were physically intermixed with the nitroglycerin tablets received the most transfer of nitroglycerin, while those striated either above or below the nitroglycerin tablets received the least (Table I and Figs. 1 and 2). Many of the placebos that were intermixed with the nitroglycerin tablets contained more nitroglycerin than a tablet containing 100% of the label claim; this is explained on the basis that there were one-half as many placebos as nitroglycerin tablets and that the placebos were approximately twice the size of the nitroglycerin tablets.

The fact that the placebos intermixed with the nitroglycerin tablets received the most nitroglycerin by migration indicates that soon after volatilization the nitroglycerin must be absorbed by some nearby receptor surface. If this were not so, it would be expected that all of the placebo tablets, regardless of position in the bottle, would reach the same nitroglycerin content. The fact that the placebos both above and below the nitroglycerin tablets received less nitroglycerin than the placebos intermixed with the nitroglycerin tablets is indicative of the small amount of travel that occurs once the nitroglycerin volatilizes. In some respects, this is analogous to the process of molecular distillation.

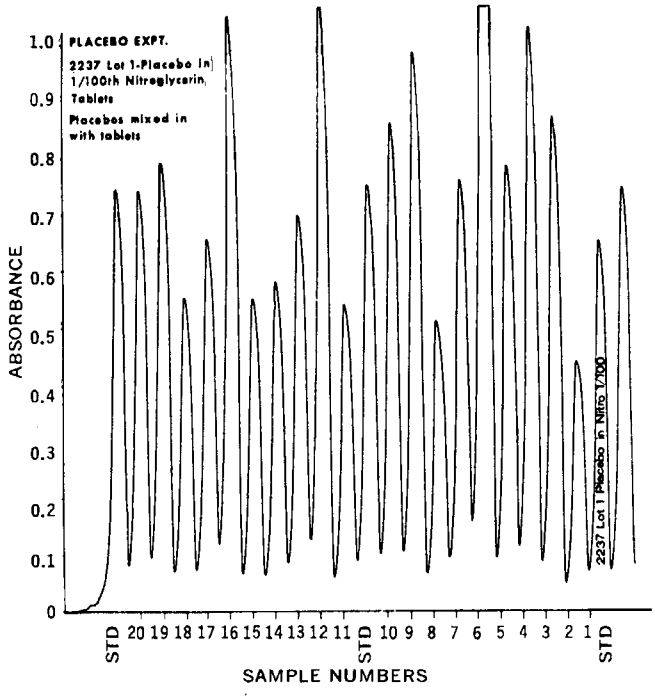

Figure 1—*Curves from automated analysis of placebo tablets intermixed with 600-mcg. (1/100-gr.) nitroglycerin tablets.*

[7] Mylar.
[8] The vapor pressure of nitroglycerin is 0.00026 mm. Hg at 20° (Merck Index).

Table VI—Nitroglycerin Tablets [400 mcg. (1/150 gr.)] with Room Temperature Storage for 1 Month in Commercial Bottles with Excelloseal Liner

Lot Number	Description	Average Assay of 30 Tablets	Range	Standard Deviation	Stuffing Tablet Equivalent
345903	No wax, no rayon	91.0	65.6–115.7	13.0	—
345903	No wax, with rayon[a]	100.6	75.2–126.5	12.2	0.36
345903	With wax, no rayon	91.6	61.4–123.3	16.2	—
345903	With wax, with rayon	96.3	76.9–111.3	9.5	0.22
345904	No wax, no rayon	97.5	67.6–129.4	15.3	—
345904	No wax, with rayon	94.9	69.5–127.3	12.8	0.73
345904	With wax, no rayon	98.2	85.6–118.1	9.3	—
345904	With wax, with rayon	99.2	83.1–120.5	10.9	0.70
345905	No wax, no rayon	94.8	52.5–133.1	19.9	—
345905	No wax, with rayon	96.9	62.2–115.6	13.9	0.76
345905	With wax, no rayon	97.4	64.3–123.2	14.5	—
345905	With wax, with rayon	96.3	61.5–123.9	13.3	0.95
345906	No wax, no rayon	95.3	61.1–124.9	16.4	—
345906	No wax, with rayon	99.7	65.8–138.1	16.6	0.56
345906	With wax, no rayon	97.4	73.8–120.1	11.4	—
345906	With wax, with rayon	97.5	75.8–118.9	10.1	0.55

[a] This is the normal commercial package for the conventional tablets.

It could appear that migration occurred only because of the high concentration gradient that existed between the nitroglycerin and placebo tablets, and that migration would not occur if all tablets in a bottle contained nitroglycerin. However, the evidence presented in Tables II–IV on 60 lots of nitroglycerin tablets demonstrates that the latter premise is untenable. The data show that all lots passed the content uniformity test easily when they were first manufactured, but that the assay range spread considerably as these lots aged. It may be argued that when the lots were first assayed, only 10 tablets were assayed instead of 30, thus lessening the chances of noticing variability. However, since this occurred in about 60 lots, the chances of not seeing this variability should be slim indeed. It is to be noted that the trend is toward higher variability in the reassayed tablets. In spite of a general net loss in potency, the high values are frequently considerably higher than the high values in the starting tablets; the frequency of this occurrence

Table VII—Effect of Stuffing and Cap Liners on Nitroglycerin Tablets [400 mcg. (1/150 gr.)][a] with 1 Month of Storage at 37 and 45°

	37°				45°			
Description	Average Assay of 30 Tablets	Range	Standard Deviation	Stuffing Tablet Equivalent	Average Assay of 30 Tablets	Range	Standard Deviation	Stuffing Tablet Equivalent
No wax, Esterfoil	101.7	89.8–122.1	7.4	—	93.4	77.9–109.7	8.3	—
With wax, Esterfoil	101.5	89.4–119.6	8.2	—	95.3	77.2–116.3	9.8	—
Rayon, Esterfoil	97.9	80.5–113.6	8.5	2.3	101.5	79.5–129.9	11.4	1.8
Tissue, Esterfoil	99.6	80.3–116.2	8.7	2.1	102.0	60.7–126.5	13.9	5.3
Cotton, Esterfoil	98.3	83.7–117.4	8.8	6.8	97.9	59.6–124.8	16.1	12.3
No wax, Tinfoil	101.0	92.0–114.1	6.7	—	101.0	86.8–120.3	7.0	—
With wax, Tinfoil	102.3	90.2–123.6	8.5	—	97.5	82.6–123.5	8.0	—
Rayon, Tinfoil	100.4	64.4–138.4	17.3	3.3	94.4	70.9–112.2	11.4	4.9
Tissue, Tinfoil	103.6	83.5–131.9	10.6	2.8	93.9	39.2–128.7	20.5	4.9
Cotton, Tinfoil	99.0	75.7–126.7	10.6	9.4	90.4	52.1–123.9	15.2	7.6
No wax, wax vinyl	81.6	28.8–104.1	22.0	—	88.8	68.6–107.0	10.9	—
With wax, wax vinyl	102.2	83.4–116.7	7.9	—	87.7	39.6–114.8	19.2	—
Rayon, wax vinyl	93.6	54.6–128.4	17.2	2.8	92.0	72.6–115.3	11.1	3.0
Tissue, wax vinyl	97.1	57.5–142.7	18.9	1.9	94.6	31.5–136.7	24.2	2.5
Cotton, wax vinyl	94.4	65.6–114.7	13.2	10.1	88.2	56.5–109.6	16.1	28.9
No wax, with Mylar[b]	102.1	74.4–117.0	10.5	—	102.3	92.0–123.4	6.5	—
With wax, Mylar	99.9	84.6–115.7	8.7	—	99.8	82.3–110.8	6.9	—
Rayon, Mylar	94.0	64.6–113.0	14.9	2.5	102.0	63.1–134.6	17.2	2.6
Tissue, Mylar	101.6	53.8–146.4	21.1	2.4	97.5	45.1–126.7	19.5	3.6
Cotton, Mylar	—	—	—	—	96.0	50.0–118.0	14.1	10.5
No wax, lubricated vinyl	91.7	58.7–117.1	13.8	—	77.2	29.6–108.7	25.8	—
With wax, lubricated vinyl	90.1	46.5–114.8	17.8	—	72.0	19.9–108.3	25.5	—
Rayon, lubricated vinyl	97.4	51.1–131.3	21.1	1.7	90.2	55.2–132.9	17.9	2.3
Tissue, lubricated vinyl	100.6	48.5–147.4	20.7	1.4	91.6	50.1–118.0	17.9	2.5
Cotton, lubricated vinyl	91.8	55.5–113.0	15.3	2.3	86.0	40.4–134.0	22.5	7.5
No wax, Excelloseal	90.0	50.5–122.9	18.0	—	91.6	40.4–117.6	16.4	—
With wax, Excelloseal	90.0	52.6–129.8	18.5	—	89.6	57.4–116.5	15.7	—
Rayon, Excelloseal	93.9	54.6–132.9	18.0	1.8	104.1	80.5–129.5	14.6	2.8
Tissue, Excelloseal	104.8	74.9–144.1	18.8	2.5	97.7	45.3–140.1	18.8	3.3
Cotton, Excelloseal	96.4	60.2–131.5	15.6	5.9	96.7	61.9–121.6	14.8	11.1
No wax, Aclar[c]	101.0	80.3–116.5	7.9	—	99.2	87.7–115.0	7.7	—
With wax, Aclar	103.2	88.7–119.7	8.2	—	100.9	86.4–117.6	7.7	—
Rayon, Aclar	101.7	68.7–149.3	17.6	18.7	94.7	64.3–126.6	13.9	14.7
Tissue, Aclar	101.8	48.4–129.8	19.4	7.5	97.2	70.7–127.5	15.0	14.1
Cotton, Aclar	104.0	74.3–132.2	16.0	18.7	99.0	68.4–127.0	16.5	33.6

[a] Lot 345906, initial assays: average, 102.7% label claim; range, 92.2–112.7% label claim; and σ, 4.6. [b] Polyethylene terephthalate. [c] Aclar is a fluorohalocarbon. The results above may be weighted too heavily toward failure when stuffing is introduced. The Aclar seals were available only on bottles larger than the bottles used for the other studies so that more stuffing was used in the Aclar sealed bottles.

Table VIII—Comparison at Room Temperature and 4° of Conventional Nitroglycerin Tablets[a]

Release Date	Size of Tablet, mcg. (gr.)	Initial Assay: Average Assay, Percent of Labeled Claim	Initial Assay: Range of Assay, Percent of Labeled Claim	Initial Assay: Standard Deviation, σ	Room Temperature: Average Assay, Percent of Labeled Claim	Room Temperature: Range of Assay, Percent of Labeled Claim	Room Temperature: Standard Deviation, σ	4°: Average Assay, Percent of Labeled Claim	4°: Range of Assay, Percent of Labeled Claim	4°: Standard Deviation, σ
5-67	600 (1/100)	103.2	—[b]	—[b]	100.5	79.0–122.9	11.7	100.9	81.4–125.7	11.4
9-68	600 (1/100)	102.0	—	—	111.5	90.2–145.0	11.5	108.9	90.3–128.3	10.0
1-70	600 (1/100)	99.3	86.1–109.0	6.9	98.3	80.4–118.9	9.2	95.3	65.5–123.7	16.8
2-70	600 (1/100)	101.9	96.9–106.6	3.2	95.6	64.0–118.5	12.5	97.3	74.4–114.5	10.1
6-67	400 (1/150)	103.9	—	—	98.5	76.4–116.5	9.8	106.9	91.5–125.8	8.9
9-68	400 (1/150)	99.7	—	—	99.9	76.7–121.0	10.1	109.1	94.9–143.0	11.0
5-69	400 (1/150)	103.5	—	—	101.1	90.0–123.7	7.7	100.6	84.9–133.7	8.5
12-69	400 (1/150)	97.5	93.0–106.0	4.0	92.6	56.8–130.5	15.2	99.8	64.8–135.0	18.0
1-70	400 (1/150)	100.1	96.0–104.0	2.1	94.6	74.3–120.0	10.3	95.9	77.2–116.7	8.2
9-68	300 (1/200)	101.0	—	—	95.1	72.2–137.1	12.7	104.0	67.7–164.3	19.8
12-69	300 (1/200)	100.0	94.6–104.1	3.0	96.0	66.9–138.1	14.9	97.0	63.2–120.5	13.4
1-70	300 (1/200)	98.1	94.1–102.9	2.8	95.0	63.8–140.3	14.5	100.9	80.3–129.3	12.3

[a] Assays performed 2/17–2/18, 1972. [b] Only average assay is given; individual tablet assays were not being performed until 8-69. However, the ranges shown are typical of our manufacturing capability so that they may be assumed for those lots where ranges are not given. All assays shown that do not indicate assay ranges for individual tablets were done by either the IR or phenoldisulfonic acid procedures of USP XVII and USP XVIII. The automated version of Bell's (6) assay was used for all other samples.

Table IX—Stability of Nitroglycerin Tablets [400 mcg. (1/150 gr.)] in Glass Bottles with Screw Cap and Polyethylene Terephthalate Liner at 25, 37, and 45°

Temperature	Elapsed Time, Months	Lot 1: Average Assay, Percent of Labeled Claim	Lot 1: Range of Assays, Percent of Labeled Claim	Lot 1: Relative Standard Deviation, %	Lot 2: Average Assay, Percent of Labeled Claim	Lot 2: Range of Assays, Percent of Labeled Claim	Lot 2: Relative Standard Deviation, %
25°	0	103.8	94.9–113.5	3.9	104.7	98.5–111.2	3.1
	1	102.0	76.6–122.1	12.7	103.3	64.7–129.8	14.1
	2	102.1	84.9–131.2	10.6	102.2	83.7–119.6	7.9
	3	100.3	74.5–120.7	12.4	102.9	84.9–121.0	9.4
	4	101.2	79.5–128.6	12.6	99.9	80.5–131.0	11.7
	5	101.4	85.9–123.2	10.7	103.1	79.2–127.9	10.7
	6	101.1	81.6–127.0	12.5	104.9	79.7–132.7	14.3
37°	0	103.8	94.9–113.5	3.9	104.7	98.5–111.2	3.1
	1	107.6	72.0–147.2	15.6	101.8	69.5–143.3	17.4
	2	100.0	73.1–130.7	12.9	101.8	86.1–117.0	8.3
	3	99.8	76.7–137.6	13.9	103.7	86.7–113.5	7.2
	4	96.4	76.2–127.6	10.0	99.9	73.5–124.7	12.9
	5	100.1	76.5–142.4	16.7	104.6	73.8–137.1	16.4
45°	0	103.8	74.9–113.5	3.9	104.7	98.5–111.2	3.1
	1	102.3	71.8–142.9	13.9	101.6	74.6–124.3	12.7
	2	97.2	77.5–116.1	10.7	101.8	86.1–117.0	8.3
	3	100.4	76.5–119.4	11.7	97.7	73.7–140.9	12.4
	4	97.3	69.1–120.4	10.4	96.1	62.7–113.9	13.7
	5	98.2	79.0–133.1	10.9	99.7	78.7–119.6	10.0

does not appear to be based on chance. The assays performed on the freshly manufactured lots show one value at 126.5 and all other values (about 800) are below 116.9 (one at 116.9, another at 116.8, and the next high at 115). This finding is in solid contrast with the values obtained after the tablets were manufactured, packaged, and on the market; the high value is 152.6 and there are many tablets assaying greater than 130. Thus, it appears that these higher tablets are picking up nitroglycerin from other tablets.

The absolute age of these tablets is not the sole factor involved in determining whether a product passes or fails content uniformity. The passing tablets ranged in age from 1 to 14 months, whereas some of the tablets that failed were 1–2 months old.

The behavior of the tablets on reassay led to the belief that the problem in this laboratory with nitroglycerin tablets was probably industry wide. Assay of other commercial nitroglycerin tablets revealed that this was indeed true. Analysis of Bell's data (6) on 55 lots of tablets reinforces these observations. These lots encompassed tablets of 600, 400, 300, 250, and 150 mcg. (1/100, 1/150, 1/200, 1/250, and 1/400 gr.) made by four different manufacturers. Bell performed 10 individual tablet assays per lot. Calculation of the percent relative standard deviation for each lot[9] (Table X) gave values that were certainly much higher than the manufacturing process capability. Our manufacturing process usually gives a percent relative standard deviation below 4.5; the values on Bell's samples ranged from 5.4 to 21.8. Forty-one of the 55 values are greater than 7 and 29 are greater than 10. Of the 550 tablets assayed by Bell, 101 fell outside of the USP XVI limits of an average value of 80–112%; 78 of these showed excessive dosage and 23 insufficient nitroglycerin. The high percent relative standard deviations, coupled with the large number of tablets showing excess dosage, give presumptive evidence[10] that migration of nitroglycerin has occurred.

Table V shows the assay results obtained on four newly manufactured lots of nitroglycerin tablets. The close similarity of these lots indicates a pretty uniform manufacturing procedure. Of the

[9] Ten tablets (a small number of tablets for this calculation, but it does give a basis for comparison).

[10] Presumptive only because the range and relative standard deviations of these lots when manufactured were not known. However, since these tablets had to meet weight variation and average potency requirements, it would be unlikely that 78 of 550 tablets would be out of range on the high side.

Table X—Range and Relative Standard Deviation Calculated from Bell's (6) Data

Range of Lot, mg. × 100	Average Assay, mg.	Range of Lot, Percent of Labeled Claim	Average Assay, Percent of Labeled Claim	Relative Standard Deviation, Percent	Range of Lot, mg. × 100	Average Assay, mg.	Range of Lot, Percent of Labeled Claim	Average Assay, Percent of Labeled Claim	Relative Standard Deviation, Percent
600-mcg. (1/100-gr.) Tablets					**300-mcg. (1/200-gr.) Tablets**				
55–80	0.64	85–123	98	11.7	25–40	0.32	78–125	100	13.6
51–75	0.62	78–115	95	12.1	23–36	0.29	72–113	91	13.9
50–70	0.62	77–108	95	11.3	22–39	0.30	69–122	94	20.2
54–76	0.62	83–117	95	11.3	21–41	0.30	66–128	94	21.2
50–69	0.61	77–106	94	12.7	24–48	0.33	75–150	103	21.8
50–75	0.65	77–115	100	11.1	25–29	0.27	78–91	84	5.7
58–86	0.69	89–132	106	12.2	25–29	0.27	78–91	84	5.7
50–87	0.69	77–134	106	14.7	25–30	0.27	78–94	84	6.7
57–78	0.65	88–120	100	10.4	25–31	0.28	78–97	88	5.4
48–73	0.66	74–112	102	11.0	25–30	0.28	78–94	88	5.6
52–74	0.66	80–114	102	9.4	26–39	0.33	81–122	103	12.6
63–78	0.68	97–120	105	7.4	26–41	0.37	81–128	116	11.2
58–71	0.66	89–109	102	6.0	26–41	0.34	81–128	106	17.1
59–71	0.66	91–109	102	5.7	26–39	0.34	81–122	106	11.9
66–75	0.70	102–115	108	5.4	29–41	0.35	91–128	109	13.7
400-mcg. (1/150-gr.) Tablets					**250-mcg. (1/250-gr.) Tablets**				
34–45	0.38	79–105	88	8.5					
38–46	0.41	88–107	95	6.9	21–27	0.24	81–104	92	7.0
31–45	0.39	72–105	91	10.9	21–26	0.24	81–100	92	5.8
35–49	0.42	81–114	98	10.0	21–25	0.24	81–96	92	6.4
32–49	0.39	74–114	91	12.0	22–27	0.24	85–104	92	7.2
38–50	0.45	88–116	105	7.9	21–28	0.24	81–108	92	9.9
35–50	0.43	81–116	100	11.8					
37–47	0.43	86–109	100	8.0					
42–50	0.46	98–116	107	7.4	**150-mcg. (1/400-gr.) Tablets**				
39–47	0.43	91–109	100	7.4					
36–51	0.44	84–119	102	10.4	12–21	0.18	75–131	113	14.5
39–49	0.46	91–114	107	7.2	16–21	0.18	100–131	113	10.9
42–50	0.46	98–117	107	5.8	14–19	0.17	88–119	106	9.5
33–49	0.43	77–114	100	10.5	14–21	0.18	88–131	113	13.6
40–49	0.46	93–114	107	6.0	13–21	0.18	81–131	113	15.7

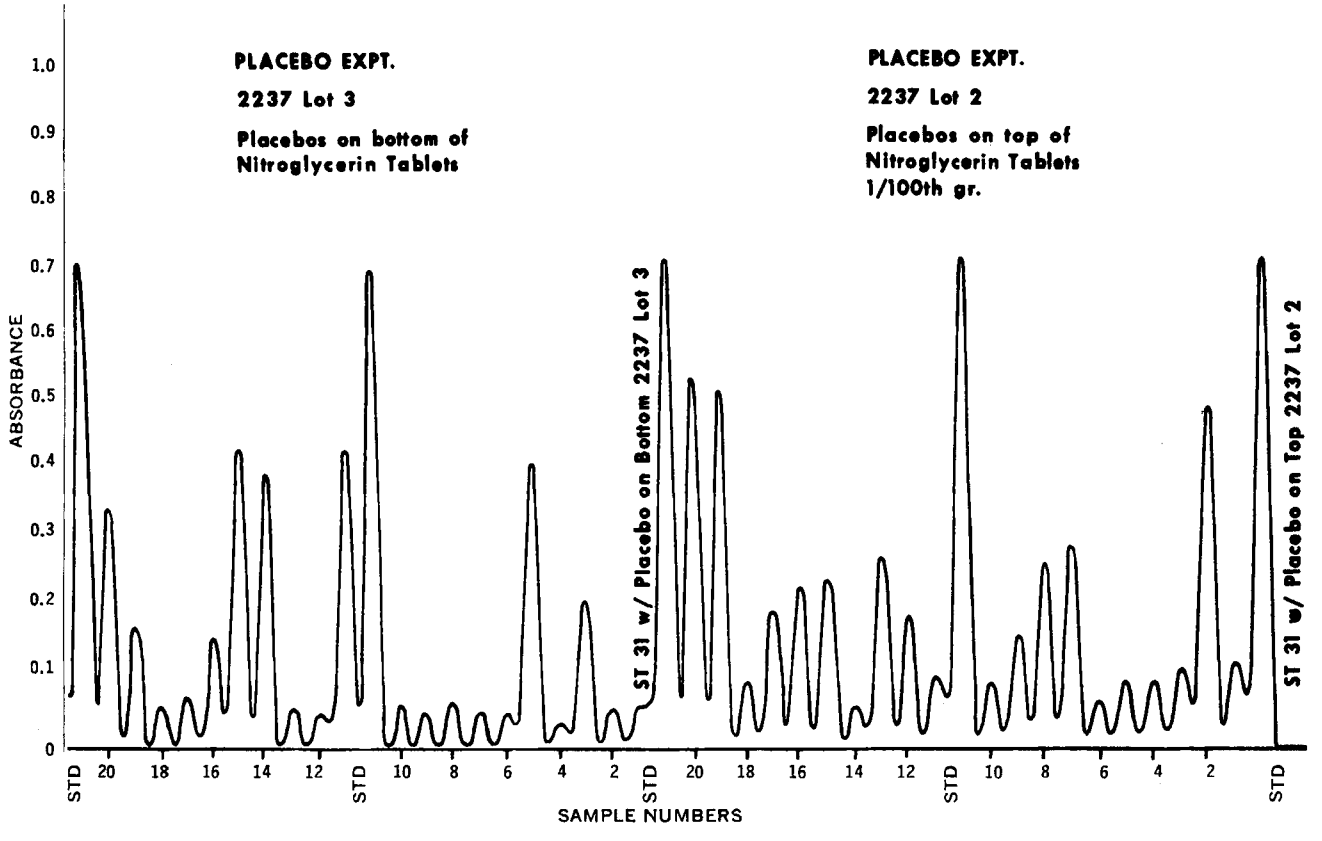

Figure 2—*Curves from automated analysis of placebo tablets. Key: left, placebos on bottom of nitroglycerin tablets; and right, placebos on top of nitroglycerin tablets.*

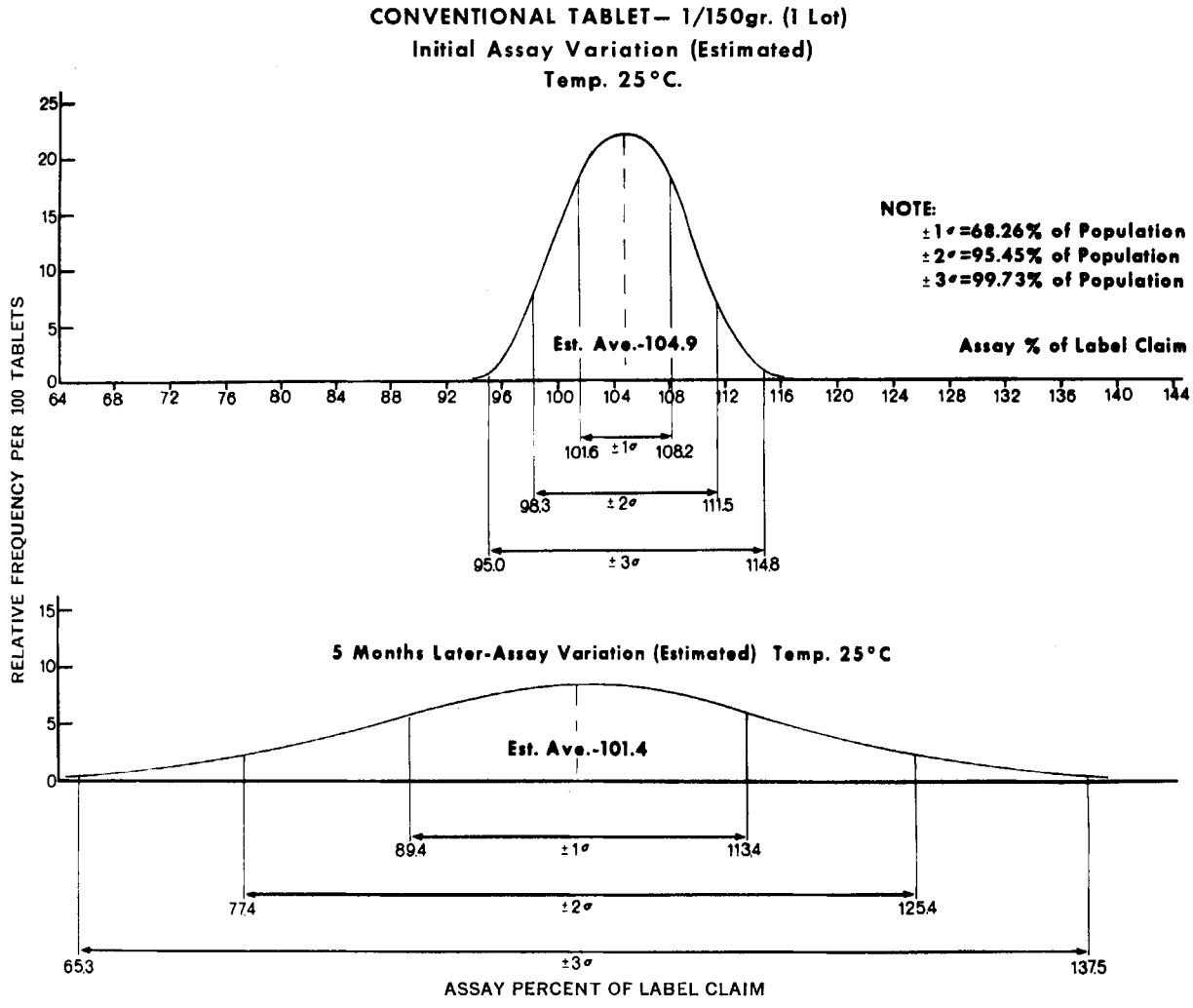

Figure 3—*Distribution curves for conventional sublingual tablets at the time of manufacture and after being packaged for 5–7 months.*

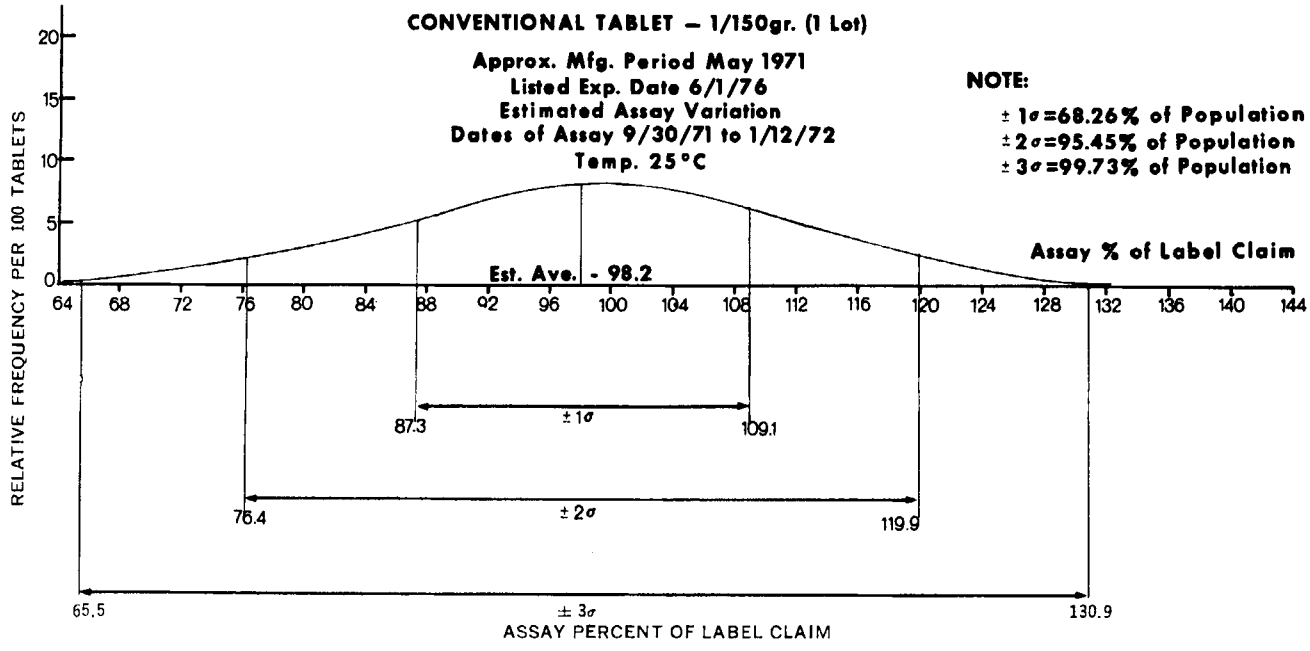

Figure 4—*Distribution curves for conventional sublingual tablets at the time of manufacture and after being packaged for 5–7 months.*

120 tablets tested, no tablet assayed lower than 87.2 nor greater than 114.8% of labeled claim. Since the lots had just been manufactured at the time the tablets (Tables II–IV) were assayed, it was decided to test the effects of certain variables (*e.g.*, time, temperature, stuffing materials, and cap liners) on the stability of nitroglycerin tablets.

Table VI shows the effect of storing these tablets at room temperature for 1 month in commercial bottles[6] using the modifications shown in the table. In all cases, the range of assays for the tablets widened considerably, the lows being lower and the highs being considerably higher in spite of a general net loss in potency. This result can only be explained on the basis that nitroglycerin volatilized and migrated from tablet to tablet. No other explanation is possible based on the average assay, range, and standard deviation of the original tablets.

The effects of using different cap liners and stuffing materials at 37 and 45° for 1 month are shown in Table VII. These accelerated temperature conditions were chosen to screen quickly the effectiveness of cap liners and stuffing materials. Once again, the range of assays widened considerably in spite of a general net loss in potency. The high values are frequently considerably higher than the high value obtained on the initial assay of Lot 345906 or, for that matter, on any of the three other lots made at the same time (Table V). In fact, many of the samples fail content uniformity requirements.

A large amount of nitroglycerin was absorbed by the cotton stuffing. Cotton is still used by pharmacists in repackaging operations; this practice must be discouraged for nitroglycerin tablets. Additionally, it should be noted that of the seven liners evaluated, only four (Esterfoil, tinfoil, polyethylene terephthalate[7], and a fluorohalo-carbon film[11]) offered any protection to the nitroglycerin tablets for even short periods of time. Although these liners are imperfect, they certainly are superior to the wax vinyl, lubricated vinyl, and Excelloseal liners. The latter cap liners seemed to accelerate the instability; it should be emphasized that the stability of no other known drug dosage form seems to be so susceptible to the proper choice of cap liner and stuffing material.

With most unstable drugs, the stability of refrigerated samples is usually better than for samples stored at room or higher temperatures. This is not true with nitroglycerin tablets (Table VIII). The data show that refrigeration does not prevent the development of intertablet variation in conventional sublingual nitroglycerin tablets. This is additional evidence for the complex nature of the instability of sublingual nitroglycerin tablets.

Table IX shows the stability of two additional newly manufactured lots of nitroglycerin tablets at 25, 37, and 45° assayed at monthly intervals. Marked intertablet variability devloped within 1 month after the samples were stored. The loss of content uniformity in these tablets is important, because it means a patient cannot be assured a uniform, predictable dose with this type of tablet unless something can be done to stabilize the nitroglycerin content. Figures 3 and 4 show distribution curves for conventional sublingual tablets at the time of manufacture and after being packaged for 5–7 months. The spread in range of assay results is dramatic when one considers the close range of assay values in the freshly manufactured lots.

The foregoing data and discussion demonstrate that sublingual nitroglycerin tablets are unstable due to volatilization and intertablet migration of nitroglycerin. The inherent low volatility of nitroglycerin and the manufacturing process for making molded sublingual tablets are the predisposing factors leading to instability. During the manufacture of molded sublingual tablets, a 10% nitroglycerin β-lactose adsorbate[12] is blended with a lactose–sucrose mixture. This mixture is then moistened and blended with a mixture of alcohol and water. The resultant tablet mixture is then molded and the tablets so prepared are air dried. While the tablets are air drying, the nitroglycerin is brought to the surface of the tablet by the evaporation of the alcohol and water. This situation is analogous to the development and elution of a solute zone during chromatographic development. Since the tablets are air dried in batches and in contact with each other, it is quite conceivable that the surface concentrations of nitroglycerin are erratic, creating concentration gradients among the tablets. These gradients are in addition to all other stresses inherent in the immediate tablet environment including the stuffing, cap liner, imperfect vapor seal, and temperature.

When the nitroglycerin is brought close to, or at the surface of, the tablet, the stage is set for loss and/or migration, depending on the character of the immediate tablet environment. Once a driving force is present which causes nitroglycerin to volatilize and leave the tablet, the process leading to the development of intertablet dose variation is set in motion. In an attempt to establish an equilibrium condition not only among the tablets but with the stuffing, the cap liner, and an imperfect vapor seal as well, the small amount of volatilized nitroglycerin encounters other tablets. The latter, due to differences in surface concentration of nitroglycerin, porosity, hardness, and proximity to the tablets losing the nitroglycerin, may very well act as partial condensers in the same manner as the packing in a distillation column. Thus, one sees the development of high and low content tablets. Many lots that are uniform when manufactured develop enough intertablet variation within a few months to fail the USP XVIII content uniformity test. This tendency toward failure seems to be an intrinsic property of molded tablets using present manufacturing methods. Our experience with this tablet indicates that stabilization can best be achieved by reformulation.

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[11] Aclar, Allied Chemical Corp.

[12] Supplied by E. I. duPont de Nemours & Co., Inc., Wilmington Del.

Variation on the USP–NF Rotating-Basket Dissolution Apparatus and a New Device for Dissolution Rate Studies of Solid Dosage Forms

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Abstract □ Tablet dissolution studies were conducted comparing three devices: the USP–NF rotating basket, a modification of this called the bent basket, and a newly designed L-shaped Teflon holder/stirrer. In these studies, the latter two devices were superior to the official device.

Keyphrases □ Dissolution equipment—modified compendial rotating basket, new rotating assembly □ Tablet dissolution—modified compendial rotating basket, new rotating assembly

The USP XVIII and NF XIII include a description of a rotating-basket dissolution apparatus, the purpose of which is to provide: "an objective means of determining the dissolution characteristics of a solid dosage form" (1). The intent of this apparatus is to determine compliance with the limits on dissolution where stated in the individual (USP) monograph for a tablet or capsule form (1). The USP monograph for prednisolone tablets, for instance, states that the time required for 60% of the labeled amount of $C_{21}H_{28}O_5$ to dissolve is not more than 20 min., deaerated water being used as the dissolution medium and the basket being rotated at 100 r.p.m. (2). Similar time limits are cited for five other tablets in USP XVIII. While the designated use of the USP–NF dissolution apparatus is quite limited, its official status ensures that it will be extensively used for many solid drug products, both new and old. To date, only a very limited quantity of published data exists relating to the use of the USP–NF rotating-basket apparatus. In short, its utility for characterizing the dissolution properties of the large number of drugs marketed as tablets or capsules remains unproven. This preliminary report describes some difficulties encountered in our laboratories with the official apparatus and describes a variation on this apparatus that subsequently led to the design of a new rotating device which may offer certain advantages.

EXPERIMENTAL

Dissolution studies, using the USP–NF rotating-basket device (Fig. 1*b*), were conducted on two batches of an experimental tablet formulation. The active ingredient was a steroid (chlormadinone acetate) having a water solubility of 1.7 mcg./ml. at 37°. Pertinent physical characteristics of two lots of the same formulation, differing appreciably in their dissolution behavior, are shown in Table I. A volume of boiled (deaerated) water sufficient to provide sink conditions (1500 ml.) was employed, and all experiments were conducted at 37°. The basket was rotated at 80 r.p.m. Samples were taken by filtering the entire volume of dissolution fluid through a 0.45-μ membrane[1] and using 250 ml. of the filtrate for chloroform extraction. The extract was evaporated to dryness, and the residue was redissolved in an accurately measured volume of chloroform. The absorbance of this solution was recorded at 286 nm. on a spectrophotometer[2], and the quantity of steroid dissolved at various time points was determined. At a later date, USP–NF rotating baskets made with 24-mesh stainless steel screen, rather than the 40-mesh screen, were used in an attempt to get better dispersion of the tablet particles into the dissolution fluid as disintegration occurred.

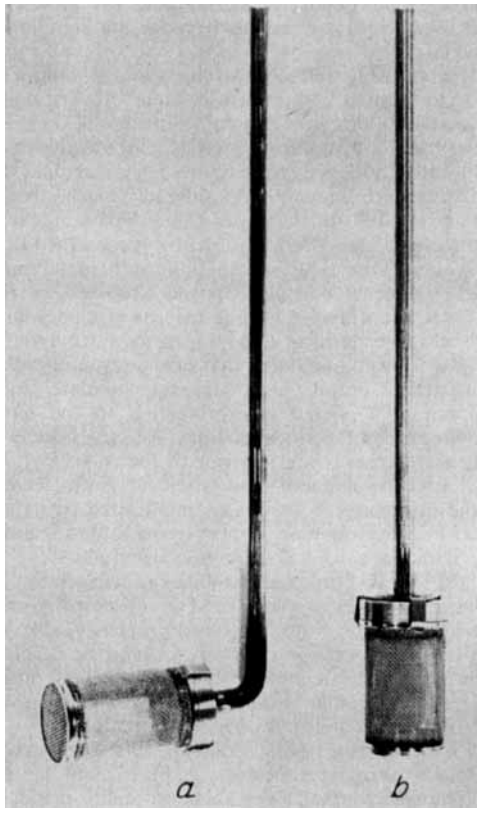

Figure 1—(a) *Bent basket, and* (b) *USP NF rotating basket.*

The USP–NF basket, with 40-mesh screen, was further modified by bending the stainless steel stirring rod of the apparatus at a 90° angle just above the basket to yield an L-shaped configuration (Fig. 1*a*). This device provided a greater flow of dissolution fluid over the tablet.

As a consequence of the experience with the bent basket, a third device was designed and constructed in this laboratory. The tablet holder/stirrer (Fig. 2) consisted of a piece of Teflon rod which was attached at a 90° angle to a stainless steel stirring rod, and a cylinder of stainless steel screen (24 mesh) was seam-welded to fit tightly over the Teflon holder. This device ensured that the tablet (or capsule) was held at a fixed distance from the center of rotation during the disintegration phase of the dissolution experiment. The agitation intensity could be varied by changing either the stirring rate or the length of the Teflon rod.

[1] Metricel.

[2] Beckman DB.

Table I—Drug and Tablet Characteristics

Active ingredient	Chlormadinone acetate, 0.5 mg./tablet	
Total tablet weight	80 mg.	
Drug solubility	1.7 mcg./ml. (37°)	
	—Batch A—	—Batch B—
Hardness (S–C units)	3–4	5–6
Disintegration time:		
With disk	3–5 min.	4–5 min.
Without disk	11–14 min.	11–13 min.

When highly acidic solutions, such as artificial gastric fluid (pH 1.2), are employed as the dissolution medium, the stainless steel used in components of the apparatus may be subject to corrosion. For such experiments the stainless steel rod and screen can be replaced with a glass rod and nylon mesh.

RESULTS AND DISCUSSION

Dissolution data obtained for the two tablet batches with the USP–NF rotating basket are shown in Fig. 3*a*. These data indicate that significant *in vitro* differences exist between the dissolution rates of the two different batches. With Batch A, the substitution of 24-mesh screen for 40-mesh screen had no effect on the dissolution except at the 15-min. time point. With Batch B, the dissolution rate did increase with the 24-mesh screen but not as much as expected.

It was obvious from subsequent observation of tablet remains that the slow dissolution rates shown in Fig. 3*a* were the result of slow and incomplete *disintegration* of the tablets using the USP–NF apparatus. Tablets from both batches disintegrated poorly in the official dissolution device, and disintegration was incomplete after several hours at stirring speeds up to 100 r.p.m. Whether the difference between batches was primarily due to different disintegration characteristics was uncertain at this point. Disintegration tests conducted with the official disintegration apparatus, both with and without disks (Table I), indicated no significant differences between Batches A and B.

The use of deaerated water was essential for all of the dissolution studies. If there is dissolved air in the dissolution fluid, bubbles collect on the screen and hamper the flow of fluid over the tablet. This can lead to low values and extensive scatter in the data.

The purpose of the basket in the USP–NF apparatus presumably is to hold the tablet or capsule in a fixed position during the disintegration process. In essence, the distance of the tablet from the center of rotation is fixed within small limits. However, the position and orientation of a tablet in the bottom of the basket may vary somewhat from experiment to experiment unless the stirring speed is sufficient to hold the tablet against the outer perimeter of the screen by centrifugal force.

As a tablet disintegrates during the dissolution test, the flow of solvent through the basket must be sufficient to disperse the tablet components and sweep them through the openings in the basket screen. If not, disintegration may be impeded and unusually slow dissolution of the active ingredient will result. Experiments conducted during these studies suggested that the basket of the USP–NF apparatus provided what might be described as a protective envelope around the tablet. This was indicated by the relatively minor effect on dissolution rates produced by changes in the stirring rate or the mesh size of the screen. In addition, installation of a 12.7-cm. (5-in.) stirring blade on the stirring shaft directly above the basket produced no appreciable effect on the dissolution rate, although a considerable increase in the agitation in the bulk dissolution medium was observed. Beyer and Smith (3) indicated that the degree of vibration in the USP–NF dissolution apparatus has a marked effect on the dissolution rate of tolbutamide tablets at rotation speeds below 150 r.p.m. They noted that vibration at lower stirring speeds caused particles of disintegrating tablets to escape from the basket, producing a higher rate of dissolution.

A configurational change in the standard USP–NF rotating-basket apparatus, which ensured better flow of the solvent through the basket, radically changed the results obtained with this device in the present study. The stainless steel stirring rod of the apparatus was bent at a 90° angle just above the basket to yield an L-shaped configuration (Fig. 1*a*). This modification gave a

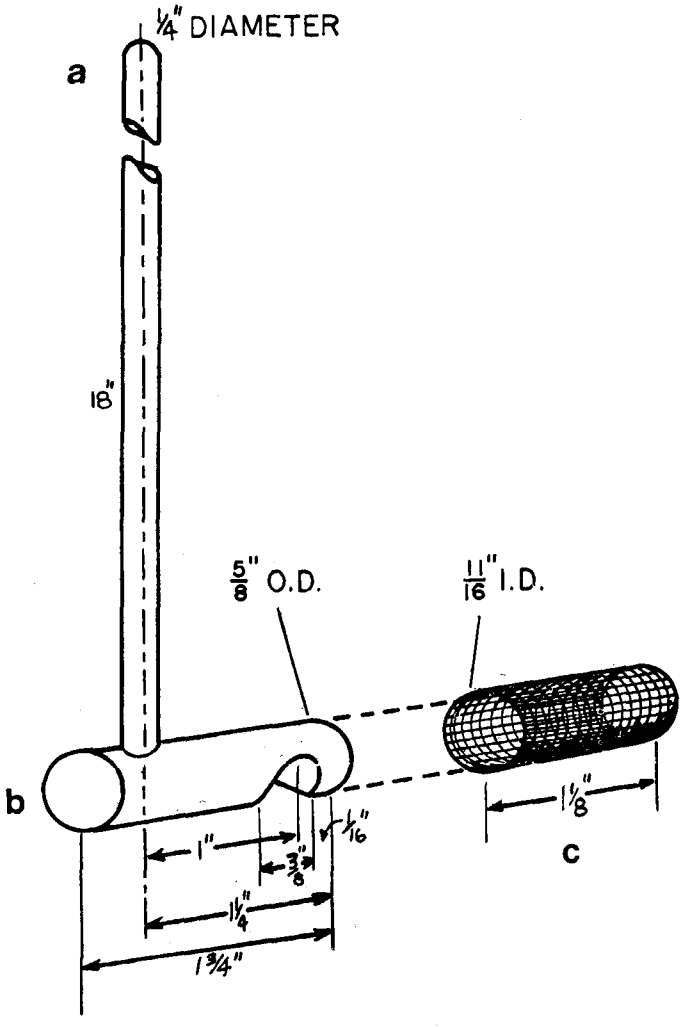

Figure 2—*Diagram of Teflon stirrer/holder. Key: a, stainless steel rod; b, Teflon tablet holder; and c, stainless steel screen.*

maximum stirring radius of 6.1 cm. (2.4 in.) for the tablet. The dissolution data for Batches A and B obtained with this bent basket are shown in Fig. 3*b*. With the bent basket the dissolution increased considerably over that obtained with the straight USP–NF rotating basket—from 30 to 55% for Batch B and from 75 to 95% for Batch A at 60 min. For Batch A the bent basket with 40-mesh screen gave a better dissolution rate than that with 24-mesh screen. The authors have no explanation for this[3]. There was essentially no difference for Batch B.

Dissolution data obtained using the L-shaped Teflon device are shown in Fig. 3*c*. With this device, as with the bent basket, complete disintegration of both batches occurred in approximately 30 min. Nonetheless, a distinct difference in dissolution rates between the two batches was found, as noted previously with both the bent and straight baskets. The data obtained with the Teflon device were lower—by 1–20%—than those obtained with the bent basket. This was undoubtedly due to the much greater stirring radius obtained with the bent basket, 6.1 cm. (2.4 in.) *versus* 2.54 cm. (1.0 in.). A disadvantage of the modified or bent USP–NF basket is that at low stirring speeds (<60 r.p.m.) a tablet tends to wander within the basket, so greater variability in the data may be obtained.

As recently noted by Mattok *et al.* (4), highly acidic media can be very detrimental to the stainless steel rotating-basket device. The new device described here can be constructed entirely of non-

[3] The reviewer observed in his laboratory, in a similar situation, "heavy excipients with the drug settling to the bottom of the vessel and remaining there undisturbed." In his opinion, the smaller mesh basket prevents this.

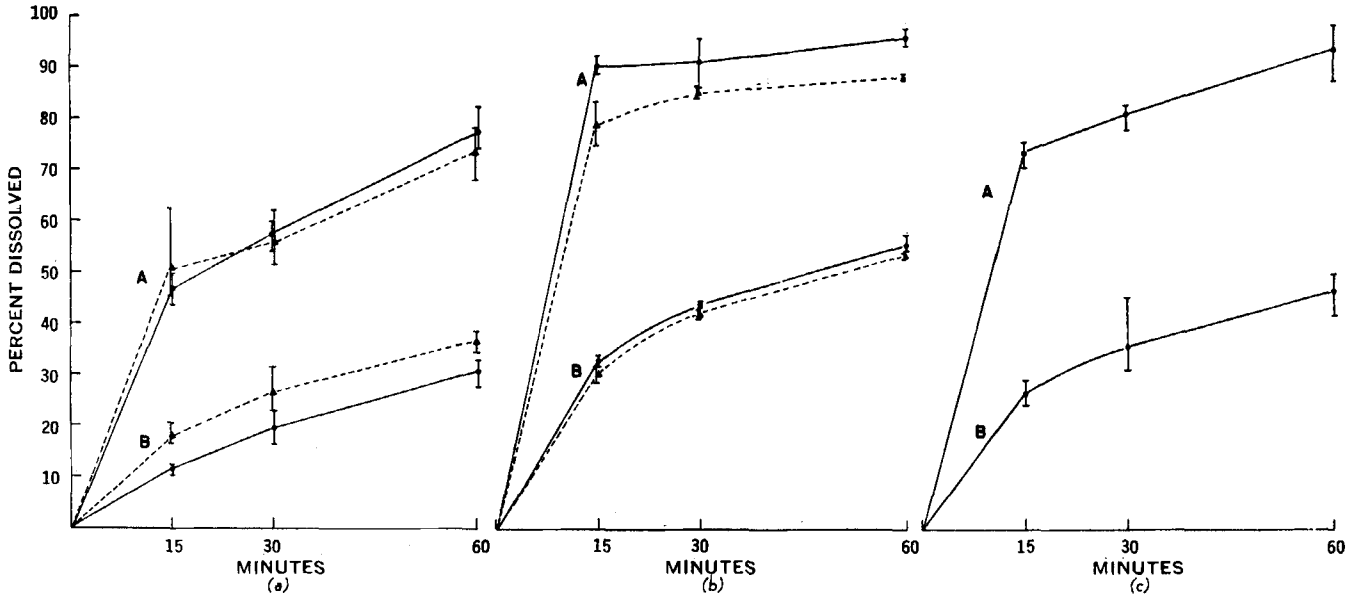

Figure 3—*Dissolution profiles of chlormadinone acetate (0.5-mg.) tablets (Batches A and B) determined at 80 r.p.m. Key:* (a), *USP–NF rotating basket;* (b), *bent basket (modified USP–NF basket);* (c), *Teflon device;* ——, *40-mesh screen; and* - - -, *24-mesh screen.*

corroding materials: a glass rod, a Teflon tablet holder, and nylon screening.

Although the Teflon device was not originally designed for capsule studies, it will accommodate up to a number 3 hard gelatin capsule. For larger sizes, the cavity of the device can be appropriately enlarged.

The Teflon holder/stirrer described in this report has the following features:

1. The tablet is held at a fixed distance from the center of rotation regardless of the stirring speed.
2. The configuration of the device ensures that particles of the disintegrating tablet are swept from the tablet holder into the bulk dissolution medium. The holder also functions effectively as a stirring blade to provide sufficient agitation to keep tablet particles dispersed in the dissolution medium.
3. The stainless steel components can be replaced with glass and nylon for use in highly acidic media. In addition, the device is simple in design and inexpensive to construct.

As a final point, it must be emphasized that *in vitro* dissolution rate differences observed in the *in vitro* test are of limited value in the estimation of relative bioavailability. Such conclusions can be arrived at only following appropriate *in vivo* experiments.

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Design and Use of a Laboratory Extruder for Pharmaceutical Granulations

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Abstract □ The purposes of this study were to design and build a laboratory extruder and to evaluate various factors relating to its use for the preparation of both wet and hot fusion granulations. The extruder used is a specially designed, single screw type, equipped with removable anvils, liquid addition ports, a heating jacket, and a thermocouple probe. A study of the extrusion of a typical antacid granulation was made. The variables studied were: (*a*) type of granulating fluid, (*b*) type of endplate, (*c*) number of mixing anvils, and (*d*) screw speed. Measurements were made on torque, powder throughput, liquid required, and whether or not a uniform consistency was obtained. Mesh sizes of both milled and unmilled granulations were measured along with bulk densities of 30–40-mesh fractions. A series of wax fusion granulations was also made with varying jacket temperatures, and drug release rates for 1 and 5 hr. were tested.

Keyphrases □ Extruder for pharmaceutical granulations—evaluation of granulating fluid, endplate, mixing anvils, screw speed □ Granulation extruder—evaluation of granulating fluid, endplate, mixing anvils, screw speed

Because of the large volume needed, conventional batch techniques of manufacture are no longer economically or practically feasible for a number of solid pharmaceutical products. Modern advances in tablet compression machinery have contributed to high production rates. Advances in the processing of pharmaceutical granulations have also been made but to a lesser extent. Use of fluid bed granulation and drying and of special mixers has become common. However, research and development groups have given relatively little attention to the continuous processing of solid dosage forms.

Several examples of continuous tableting processes were reported (1, 2) and others are certain to exist within the industry. The continuous processing of aspirin tablets was described (1), and this method includes: mixing, compaction in a Chilsonator, size reduction, conveying, and compression. A continuous method of producing antacid tablets using the extrusion method of granulation was described (2). In this process the dry ingredients are mixed in a ribbon blender and conveyed automatically to a hopper from which the material is metered into a Rietz extruder. Liquid is added to the dry material in the extruder where the granulation is mixed and blended to the proper consistency for extrusion. After extrusion, the wet granulation is dried automatically in Witte fluid bed driers and pneumatically conveyed to a hopper and mill. Following size reduction, the flavor and tablet lubricant are metered into a constant stream of dried granulation. The material is transported to a mezzanine and dumped into large overhead hoppers above tablet machines. In this last example, the continuity of the process is broken since the finished granulation is not transported automatically to the tablet machines.

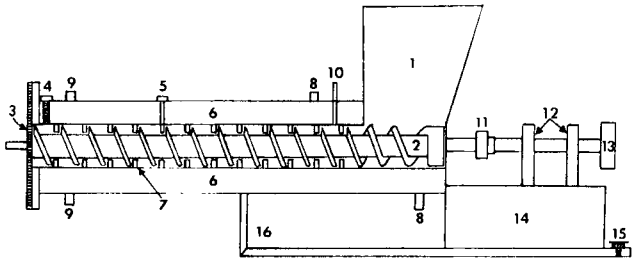

Figure 1—*Schematic diagram of laboratory extruder. Key: 1, feed hopper; 2, screw; 3, endplate; 4, pressure transducer well; 5, thermocouple well; 6, heating and cooling jacket; 7, anvils; 8, jacket inlet ports; 9, jacket outlet ports; 10, liquid feed ports; 11, screw coupling; 12, bearings; 13, drive coupling; 14, base; 15, attaching bolt; and 16, brace.*

The continuous operation of a tableting process consists of simply linking various parts of the basic procedures which have customarily been used. The most critical aspect of the continuous processing described above was the mixing extruder step. While this process was well adapted to antacid granulations made currently, it is not always possible to process other formulations by the extrusion method. Since it was desired to use extrusion processes for other products, smaller scale research and development equipment was required. However, no equipment was available on a laboratory or pilot scale that could be used for formulation or development work. Therefore, a special extruder-mixer was designed and built to serve this function.

The equipment desired was to be multipurpose, since both standard wet granulation formulations and hot fusions were to be made on a continuous basis. For wet granulations, a premixed dry powder would be fed to the extruder where liquid could be added and mixed thoroughly with the powder. A mass of the proper consistency would be extruded at the end of the mixing chamber. Wetting of the material in the extruder itself was deemed necessary so that a separate mixer need not be used prior to the extrusion step. The achievement of uniform, reproducible mixing in the extruder was an important and difficult objective. It was also envisioned that the same extruder could be used for fusion and extrusion of waxy granulations commonly used to produce timed-release tablets.

EXPERIMENTAL

Design Requirements—Considerable experience in extrusion was obtained over the years using the Rietz equipment[1]; therefore, the design of a new extruder should consider the deficiencies of existing equipment and correct them accordingly. One problem with the Rietz equipment was the cycling from an overwet to an overdry condition. This problem was caused by inadequate wetting or mix-

[1] Rietz Manufacturing Co., West Chester, PA 19380

Table I—List of Screw Configurations

Pitch, cm.	Number of Anvils	Root Diameter[a], cm.	Special Remarks
2.54 (1.0 in.)	3	1.91 (0.75 in.)	—
3.81 (1.5 in.)	3	2.54 (1.0 in.)	—
4.08 (2.0 in.)	3	2.54 (1.0 in.)	—
3.81–4.08 (1.5–2.0 in.) combination	8	2.54 (1.0 in.)	Double flight at end[b]
3.81–4.08 (1.5–2.0 in.) combination	11	2.54 (1.0 in.)	Double flight at end[b]
3.81–4.08 (1.5–2.0 in.) combination	12	2.54 (1.0 in.)	Double flight at end[b]

[a] Thickness of screw exclusive of flight portion. [b] Flight is the length of screw required for one helix.

ing and periodic surges. The adequacy of the screw pitch and its root diameter was unknown. To make a general pharmaceutical extruder, it was believed that a longer barrel would be required and that better mixing must be obtained. With this in mind, the general requirements were provision for a long mixing chamber and an adequate impediment of material flow. A split barrel allowed easy cleaning and maintenance as well as quick interchange of screws and endplates. The extruder was jacketed, which provided a method of producing wax fusion granulations or other granulation types that require temperature control. A well and hole were made for the placement of a thermocouple for measurement of granulation temperature. The drive unit provided excess power, since it was envisioned that a larger extruder might someday be coupled to the same drive system. The instruments on the drive unit were a tachometer and a recording dynamometer for torque measurement.

Construction—With the above requirements in mind, the general design of the extruder was made and a drive unit was selected. Figure 1 illustrates the design, and Fig. 2 shows the extruder coupled to the drive unit[2]. The extruder is of the single screw type, 50.8 cm. (20 in.) long and 5.4 cm. (2.125 in.) wide. The closed section or barrel is 40.64 cm. (16 in.) long and the hopper portion is 10.16 cm. (4 in.). Two ports near the hopper end are used for addition of liquid. A spraying attachment was also made for wetting powders in the hopper. The barrel is made in two sections and is hinged along the long axis so that it may be opened readily and cleaned easily. The barrel contains two permanent steel anvils and 10 removable

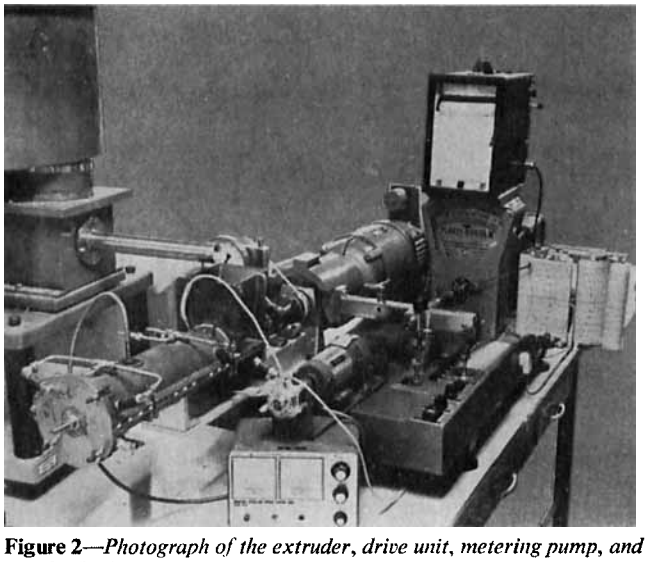

Figure 2—*Photograph of the extruder, drive unit, metering pump, and powder feeder.*

[2] C. W. Brabender Plasticorder model PL-V34D, C. W. Brabender Instruments, Inc., South Hackensack, NJ 07606

Table II—List of Endplates

Hole Diameter, mm.	Plate Thickness, mm.	Open Area, %
1.0	3.18	—
1.2	3.18	—
1.4	3.18	—
2.0	3.18	—
4.0	1.59	63
4.2	1.59	51

Table III—Amounts of Powder and Liquid Used at Various Extrusion Conditions

Extruder Speed, r.p.m.	Powder Delivery, g./min.			
40	260			
50	320			
60	365			
	Liquid Delivery, ml./min.			
	Water		Water, 75%–Isopropanol, 25%	
Speed, r.p.m.	Endplate Open Area 51%	63%	Endplate Open Area 51%	63%
	Eight Interruptions			
40	55	70	80	95
50	75	85	100	110
60	85	95	125	—
	12 Interruptions			
40	50	60	80	80
50	70	75	95	95
60	—	85	105	105

anvils made of an acetal resin[3]. Blank anvils can be used in any of the 10 positions, so it is possible to experiment with various anvil arrangements. Each side of the barrel is jacketed and contains two inlet and two outlet ports for the circulation of heated liquid when fusion granulations are made. The top section contains a well for a thermocouple probe. Construction is of type 304 stainless steel, except for the extruder support which is aluminum.

The drive unit[2] consists of a 3.4-hp. adjustable speed d.c. motor, a recording dynamometer, a tachometer, and a two-point temperature recorder. A high and low sensitivity speed adjustment permits screw revolutions per minute to be adjusted from 0 to 240, and constant speed can be maintained regardless of the torque on the screw.

Accessory Equipment—A heater-circulator[4] was used to provide heat to the extruder jacket, and a 2-cu. ft. live bin feeder[5] was used to feed powder at a fixed rate. The feeder has a variable-speed drive and tachometer so powder feed rate can be closely controlled. It is mounted on an adjustable hydraulic platform for easy height adjustment. A metering pump and controller[6] were used to feed liquids.

Startup—Initial experimentation was concerned with such factors as screw pitch, screw root diameter, number of anvils, and endplate configuration. Screw root diameter and pitches were made in a number of combinations (Table I).

A screw of 2.54-cm. pitch conveyed material too fast for good mixing and extruding. Both the 2.54- and 3.81-cm. screws gave considerable back-pressure during extrusion. A series of three screws was made; they were all alike except for the number of interruptions. These screws are the last three listed in Table I. The screws start out having a 3.81-cm. pitch, which conveys material from the hopper into the barrel. Inside the barrel, the screw pitch is 4.08 cm., which allows more slippage and mixing of material. A twin flight at the end of the screw improves the rate of output and prevents, to some degree, high back-pressures.

Endplates were made having the dimensions shown in Table II.

[3] Delrin, E. I. Dupont de Nemours & Co., Inc., Wilmington, DE 19898
[4] C. W. Brabender Instruments, Inc., South Hackensack, NJ 07606
[5] Vibra Screw Inc., Totawa, NJ 07512
[6] Model 7561, Cole-Parmer, Chicago, IL 60648

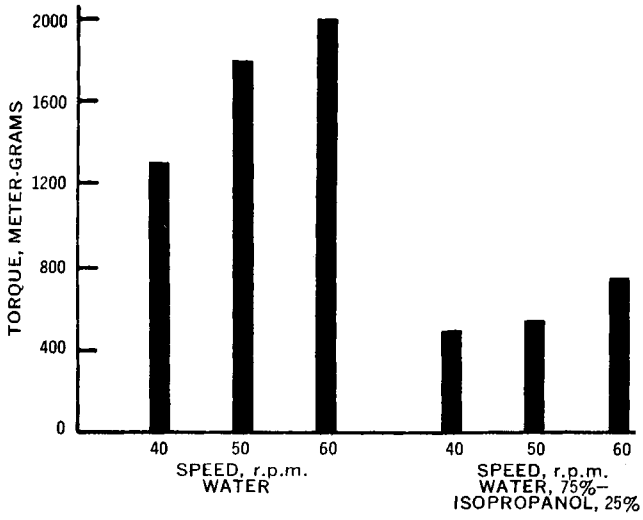

Figure 3—*Average torque values for an antacid granulation as a function of extruder factors and kind of granulating fluid.*

Endplates of 3.18-mm. thickness and hole size 2 mm. or less were satisfactory for hot fusion granulations. However, a 1.59-mm. endplate with hole sizes listed in Table II was needed for extruding the antacid granulation. Thicker plates and plates with too little open area gave too much back-pressure.

These factors were studied during various trials. Some of the initial problems were to obtain good fits of the screw in the extruder, to obtain a fixed position for the screw, and to avoid rubbing the inside of the endplate and barrel. A spray system for the addition of liquid to the powder in the hopper was unsatisfactory for granulation because of agglomeration and sticking in the hopper and on the screw. Therefore, the two barrel ports were used to add liquid for all wet granulations. Circulation of hot liquid through the extruder barrel was very good and heat exchange was excellent for wax fusion granulations.

Extrusion of Antacid Granulation—The extrusion of a typical antacid granulation was studied. The variables were: (*a*) granulating fluid, *i.e.* water alone *versus* water–isopropanol (75:25); (*b*) endplates with 4.0-mm. hole diameter and 63% open area or 4.2-mm. hole diameter and 51% open area; (*c*) number of anvils, either 8 or 12; and (*d*) screw speeds of 40, 50, and 60 r.p.m.

A powder blend was made containing two antacids and powdered sugar. The powder blend was fed to the extruder by means of the feeder[5], which had previously been calibrated in grams of antacid powder delivered per minute. Powder throughput was adjusted according to the screw speed to maintain a constant extruder hopper level. Rate of delivery was 260, 320, and 365 g./min. at 40, 50, and 60 r.p.m., respectively (Table III). Liquid feed was adjusted using the metering pump to give the most consistent granulation at each experimental condition (Table III). Granulations were tray dried at 38° to 4.8–6.3% loss on drying.

Experimental observations and measurements were: (*a*) torque produced during extrusion, (*b*) extent of cycling, (*c*) bulk density,

Table IV—Extent of Cycling[a] with Various Extrusion Conditions

Speed, r.p.m.	Water, Endplate Open Area 51%[b]	Water, Endplate Open Area 63%[c]	Water, 75%–Isopropanol, 25%, Endplate Open Area 51%	Water, 75%–Isopropanol, 25%, Endplate Open Area 63%
Eight Interruptions				
40	1	1	1	3
50	1	1	2	3
60	2	2	3	—
12 Interruptions				
40	2	2	2	3
50	3	2	2	2
60	—	1	2	3

[a] 1, least cycling; 2, moderate cycling; and 3, heavy cycling. [b] Hole diameter = 4.2 cm. [c] Hole diameter = 4.0 cm.

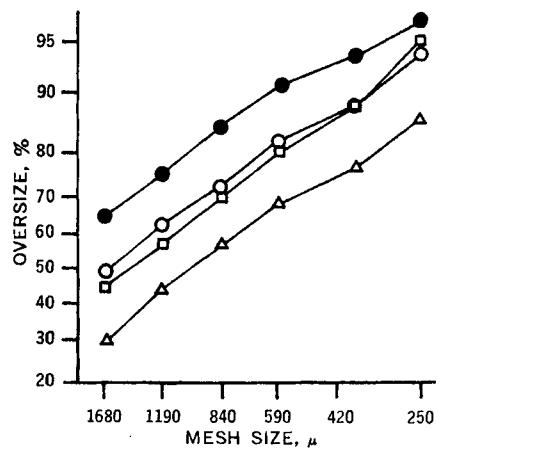

Figure 4—*Mesh sizes of dried unmilled antacid granulations. Key: ●, water, 12 interruptions; ○, water, eight interruptions; □, water–isopropanol (75:25), 12 interruptions; and △, water–isopropanol (75:25), eight interruptions.*

(*d*) mesh pattern of unmilled material obtained by sieve analysis, (*e*) tablet texture, and (*f*) mesh pattern of milled and unmilled material.

RESULTS AND DISCUSSION

Torque measurement is in meter-grams, a unit directly related to viscosity measurement. The torque can be recorded or read directly on a scale on the drive unit. This principle of operation is based on a weighted arm connected to the drive unit. The drive unit is mounted to swing freely as force is applied by the driving screw. The arm can be loaded with various weights, which offset the driving torque, and the torque working range during an experimental run can be measured. The practical meaning of torque is that higher readings relate to a thicker, stiffer granulation which requires more work for extrusion. A granulation that is less agglomerated and softer is extruded, giving a lower torque reading. This also relates to a softer textured granulation and tablet. A summary of the average torque reading during various runs is shown in Fig. 3. The main effect on

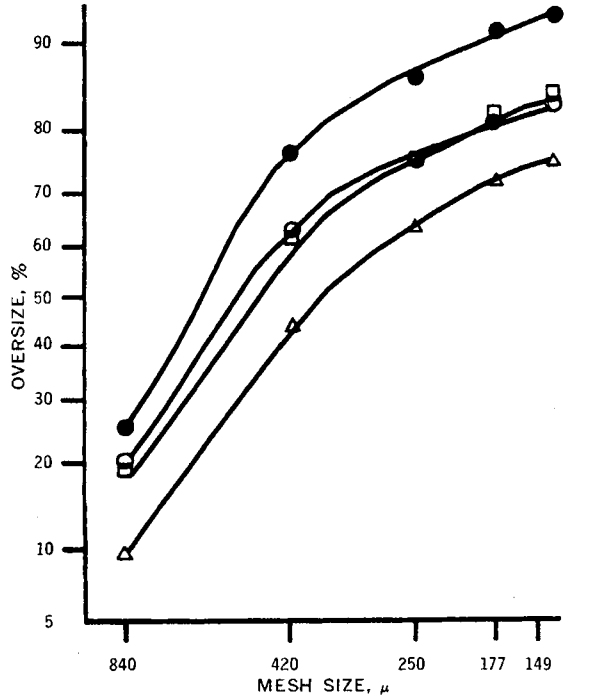

Figure 5—*Mesh sizes of dried and milled antacid granulations. Key: ●, water, 12 interruptions; ○, water, eight interruptions; □, water–isopropanol (75:25), 12 interruptions; and △, water–isopropanol (75:25), eight interruptions.*

Table V—Composition of Wax Fusion Granulation Used in Extruder Experiments

	Percent by Weight
Spray congealed fat-wax mixture (1:4)	25
Drug-diluent blend	75

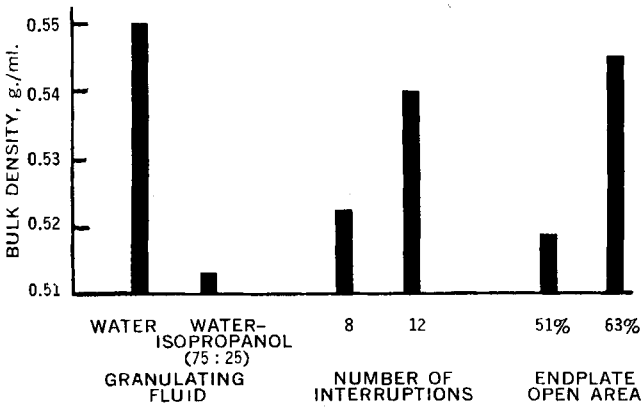

Figure 6—*Bulk densities of antacid granulations as a function of the kind of granulating fluid and various extruder factors.*

torque is the granulating fluid. In these experiments the average torque is 1360 for water and 580 for water–isopropanol. This result is not unexpected since water–isopropanol produces a lighter mass than water for this granulation. Probably less sugar is dissolved in the water–alcohol solution, thus providing less binding of ingredients. Higher speed rates also increase torque because more material is being processed in a given period of time.

Generally, more granulating fluid is used for the 63% open area endplate than the 51% open area endplate. This occurs because of less back-pressure and mixing with the 63% open area endplate compared to the plate with the lesser open area.

The property of the extrudate to change from a relatively wet condition to a relatively dry condition (referred to as cycling) was graded by observation and given ratings of 1, 2, 3. Class 1 was very little cycling, Class 2 was moderate cycling, and Class 3 was heavy cycling (Table IV). As Table IV indicates, the least cycling occurs with water as the granulating fluid, but low cycling was obtained for one condition using water–isopropanol as the granulating fluid. Speed, number of interruptions, and endplate open area are relatively insignificant factors. However, experiments using less than eight interruptions indicate that more and more cycling will occur as additional anvils are removed. A minimum number of interruptions and thus a minimum L/D ratio (extruder length to diameter ratio) are required for good extrusion.

Mesh patterns of dried unmilled granulations are shown in Fig. 4. In general, the mesh analysis is similar for all granulations[7], but water–isopropanol granulations show a significantly lower mesh size. Significant mesh size variations also occurred between the milled granulations made with water alone compared to water–isopropanol granulating fluid (Fig. 5). Fewer coarse particles and more fines are made with the water–isopropanol granulating fluid, probably due to the fact that an all aqueous granulating fluid binds particles more strongly than an alcoholic solution.

The bulk densities of 30–40-mesh granulation were measured using an automatic tapping device (600 taps). The trends shown in Fig. 6 indicate that bulk density differences exist for various extrusion conditions. The largest significant factor is the granulating fluid, closely followed by endplate open area and the number of interruptions. On the average, a more dense particle is obtained from water as opposed to water–isopropanol. Denser granulations are also obtained from more interruptions and a more open endplate area.

Tablet Texture—All the machine and formulation variables previously discussed have an effect on the ultimate texture of the tablet. The single most significant variable is the type of granulating fluid (water *versus* water–isopropanol).

Tablets made from granulations using the water–isopropanol mixture were considerably less gritty and smoother than tablets made from granulations using water alone. The addition of alcohol as a cosolvent with water reduces the solubility of sugar in that fluid as opposed to using water by itself. This reduction of sugar solubility causes less binding of the powders, thus giving a softer and less dense granulation with improved texture properties.

Based on the data collected on endplate opening, speed of extruder, number of interruptions, torque reading, densities, and mesh patterns, a general statement can be made concerning conditions that give a tablet of improved texture: A water–isopropanol granulating fluid, eight interruptions, and a 51% open area endplate tend to give granulations having lower bulk densities.

Wax Fusion Granulations—Experimental runs made during startup indicated that the extruder held promise for making fused granulations. A series of experiments was run to determine the optimum extrusion condition for meeting the release specifications of an existing formulation. To obtain a good physical mix prior to fusion and extrusion, a modification was made in the existing formula. The fat and wax ingredients were used as a fine uniform spray-congealed mixture. The formulation used is shown in Table V.

A uniform blend of the mixture was made and fed to the extruder by hand. Three extrusion runs were made under the conditions given in Table VI. Twelve anvils were used, and the throughput was 150 g./min. In general, low extrusion temperatures give slightly fused granulations whereas temperatures that are too high give an overfused and sticky extrusion. The smaller sized endplates, 2 and 1.4 mm., are preferable to the 3-mm. endplate for producing a uniform extrudate.

The drug release rates listed in Table VI show that about the same amount is released at the 1-hr. interval regardless of the fusion temperature. However, at the 5-hr. interval, drug release is affected by fusion temperature.

Table VI—Effect of Fusion Temperature on Drug Release[a]

Jacket Temperature	Material Temperature	Drug Release, %— 1 hr.	5 hr.
70°	60°	26	81
90°	78°	26	86
95°	82°	28	90

[a] Method of Souder and Ellenbogen, *Drug Stand.*, **26**, 77(1958).

CONCLUSIONS

The design and construction of a laboratory extruder for the preparation of a wide range of tablet granulations are described. The machine conditions for successful preparation of antacid granulations and wax fusion granulations are discussed. The special features included in this extruder seem to warrant consideration of extrusion on a larger scale, using the principles developed with the laboratory model.

Future uses will include the preparation of extrusions for pelletizing using the Marumerizer. The extruder will be used in new product development, in testing of presently marketed products, and for feasibility of preparing effervescent granulations.

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▲ To whom inquiries should be directed.

[7] All granulations were milled using the Fitzmill comminuting machine, with a No. 2A round hole screen, knives forward, at medium speed. The Fitzpatrick Co., Elmhurst, IL 60126

Chemical Constituents of Gentianaceae IV: New Xanthones of *Canscora decussata*

S. GHOSAL[A], R. K. CHAUDHURI, and A. NATH

Abstract □ Three new naturally occurring xanthones—*viz.*, 1-hydroxy-3,5,6-trimethoxyxanthone, 1,6-dihydroxy-3,5-dimethoxyxanthone, and 1,3,6-trihydroxy-5-methoxyxanthone, were isolated from the roots of *Canscora decussata* Schult (family Gentianaceae). The identity of these xanthones was established by chemical reactions and spectral (UV, IR, NMR, and mass spectra) evidence. Phylogenetic significance of the cooccurrence of these and other polyoxygenated xanthones in *C. decussata* is discussed.

Keyphrases □ Xanthones, 1-hydroxy-3,5,6-trimethoxy-, 1,6-dihydroxy-3,5-dimethoxy-, and 1,3,6-trihydroxy-5-methoxy—isolation and identification from *Canscora decussata* □ *Canscora decussata* Schult (Gentianaceae)—isolation and identification of three new xanthones □ Gentianaceae—chemical constituents, isolation and identification of three new xanthones from *Canscora decussata* □ Medicinal plants—*Canscora decussata*, isolation and identification of three new xanthones

The occurrence of polyoxygenated xanthones in the different parts of *Canscora decussata* Schult (family Gentianaceae) was recently reported from this laboratory (1, 2). Further chemical investigation with the roots of this plant resulted in the isolation of three more new xanthones. Characterization of these xanthones and the chemotaxonomic significance of the cooccurrence of several polyoxygenated xanthones and a xanthone C-glucoside are reported in this paper. *C. decussata* is used in the Ayurvedic system of medicine for a variety of purposes (3). Since xanthones are the major constituents of this plant, pharmacological screening of these compounds was also performed to rationalize the uses of the plant extracts in the indigenous system of medicine. The findings of pharmacological studies were previously reported (4).

EXPERIMENTAL[1]

Extraction of *C. decussata* Roots[2]—Dried and milled roots (2.1 kg.) were continuously extracted (Soxhlet) (16 hr.) with petroleum ether (60–80°). The solvent was removed from the petroleum ether extract under reduced pressure when an amorphous residue (161 g.) was obtained. The residue dissolved in diethyl ether (100 ml.), and the solution was extracted with aqueous sodium hydroxide (5%, four 25-ml. portions). The aqueous layer was cooled and acidified with concentrated hydrochloric acid, and the acidic solution was extracted with chloroform (four 30-ml. portions). The combined chloroform extract was washed with water, dried (anhydrous calcium chloride), and concentrated to a small volume (about 15 ml.). The chloroform concentrate was chromatographed over silica gel (200 g.). Elution was done with petroleum ether (60–80°, 2 l.) and benzene (5 l.). The eluates, upon evaporation, gave light-yellow amorphous solids (0.51 and 1.42 g., respectively).

The solid obtained from the petroleum ether fraction showed several spots on TLC plates, but repeated column chromatography failed to separate any individual xanthone. The mixture of xanthones remained unchanged upon treatment with dimethyl sulfate and potassium carbonate, indicating that they are permethylated.

The solid obtained from the benzene fraction showed several spots on TLC plates. It was dissolved in chloroform (8 ml.) and chromatographed over silica gel (100 g.). Benzene, chloroform, and different proportions of mixtures thereof were used as eluents.

Xanthone A (1-Hydroxy-3,5,6-trimethoxyxanthone)—Early benzene eluates afforded 1-hydroxy-3,5-dimethoxyxanthone (300 mg.), m.p. 178–179° (mixed melting point, co-TLC, and superimposable IR) (1). Later benzene eluates gave a mixture of xanthone A (major component) and 1-hydroxy-3,5-dimethoxyxanthone. The major component was purified by rechromatography. It crystallized from ethanol as light-yellow needles (92 mg.), m.p. 179–181°; mixed melting point with an authentic synthetic sample, m.p. 179–181° [prepared according to the method of Shah and Shah (5) and also from maclurin] remained undepressed.

Table I—Mass Spectral Data of the Xanthones of *C. decussata* Roots

Xanthone	Molecular Ion, *m/e* (%)	Significant Peaks, *m/e* (%)	Metastable Peaks, *m/e* Calc.	Found
A	302 (100)	287 (22); 273 (22); 272 (11); 259 (65)	302 → 287	272.7
			302 → 273	246.4
B	288 (100)	273 (9); 259 (13); 258 (12); 245 (22)	288 → 273	258.4
			288 → 259	232.9
C	274 (100)	259 (10); 245 (38); 244 (12); 231 (7)	274 → 259	244.8
			274 → 245	219.0

Table II—UV Absorption Maxima of the Xanthones of *C. decussata* Roots

Xanthone	λ_{max}, nm. (log ϵ) in Ethanol
A	245 (4.67), 282[a] (4.03), 314 (4.37), 338 (3.97)
B	225 (4.50), 240 (4.47), 280[a] (3.92), 315 (4.30)
C	205 (4.39), 220 (3.94), 248 (4.72), 280[a] (4.01), 315 (4.24), 332 (4.08)

[a] Inflection.

[1] All melting points were determined on a Toshniwal melting-point apparatus in open capillaries and are uncorrected. UV spectra were determined in aldehyde-free ethanol on a Carl-Zeiss spectrophotometer. IR spectra were determined on a Perkin-Elmer 237 instrument in KBr pellets, unless otherwise stated.

NMR spectra were run in $CDCl_3$ or dimethyl sulfoxide-d_6 on a Varian A-60D instrument. Mass spectra were recorded on a A.E.I. MS-9 double-focusing spectrometer with an ionizing potential of 70 ev.; samples were directly inserted on a probe. Separation by column chromatography was carried out using silica gel (British Drug Houses, 60–120 mesh). TLC experiments were done with Kiesel-G (E. Merck).

[2] The plant material was supplied by Mr. B. Singh, Varanasi, India, and a herbarium specimen has been preserved at the Botany Department, Banaras Hindu University, Varanasi, India.

Table III—NMR Data[a] of the Xanthones of *C. decussata* Roots

Xanthone	Methoxyl Protons	H-2	H-4	H-7	H-8
A[b]	3.95–4.0 (9H)	6.35/ 6.31	6.58/ 6.53	7.03/ 6.87	8.08/ 7.92
B[c]	3.89–3.95 (6H)	6.34/ 6.30	6.62/ 6.58	7.06/ 6.90	8.04/ 7.88
C[c]	3.85 (3H)	6.28/ 6.23	6.53/ 6.48	6.98/ 6.82	7.63/ 7.47

[a] The signals (in parts per million) were recorded from tetramethylsilane. [b] Deuteriochloroform as solvent. [c] Dimethyl sulfoxide-d_6 as solvent.

The 1-acetyl derivative of xanthone A crystallized from acetone as colorless needles, m.p. and mixed m.p. 147–148°.

The 1-methyl ether was prepared with dimethyl sulfate and potassium carbonate in dry acetone under reflux conditions (46 hr.). It crystallized from ether–petroleum ether (1:1) as colorless needles, melting point and mixed melting point with an authentic synthetic specimen 146–148°.

Xanthone B (1,6-Dihydroxy-3,5-dimethoxyxanthone)—Early benzene–chloroform (1:1) eluates afforded a mixture of 1,5-dihydroxy-3-methoxyxanthone, m.p. 272° (mixed melting point, co-TLC, and superimposable IR) (1) and xanthone B. These were separated by fractional crystallization from ethanol, in which 1,5-dihydroxy-3-methoxyxanthone was sparingly soluble. Xanthone B, obtained from the alcoholic mother liquor, recrystallized from alcohol–petroleum ether (1:1) as pale-yellow needles, m.p. 192–193°.

The 6-*O*-methyl ether was prepared by treatment of xanthone B with ethereal diazomethane. The derivative crystallized from ethanol as yellow needles, m.p. 179–181°. Mixed melting point with 1-hydroxy-3,5,6-trimethoxyxanthone remained undepressed.

Xanthone C (1,3,6-Trihydroxy-5-methoxyxanthone)—The chloroform eluates afforded xanthone C as a brown microcrystalline solid (32 mg.), m.p. 285–289°. Xanthone C crystallized from methanol as light-yellow needles, m.p. 290–291°.

The 3,6-di-*O*-methyl ether, prepared with ethereal diazomethane, crystallized from ethanol as yellow needles, m.p. 181°. It was identical with xanthone A.

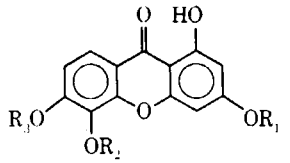
The mass, UV, and NMR spectral data of the three xanthones (A–C) are recorded in Tables I–III, respectively.

RESULTS AND DISCUSSION

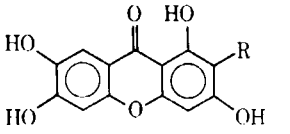
Three previously unreported tetraoxygenated xanthones (xanthones A–C) were isolated from the petroleum ether extract of the roots of *C. decussata*, and their identity was established by chemical reactions and spectral evidence (Tables I–III). In addition to the three new naturally occurring xanthones, two previously reported xanthones—*viz.*, 1-hydroxy-3,5-dimethoxyxanthone and 1,5-dihydroxy-3-methoxyxanthone (1), together with a number of permethylated unidentified xanthones were isolated from the petroleum ether extract. The characterization of the new xanthones is described here in the order of their isolation.

Xanthone A, $C_{16}H_{14}O_6$ (M^+, 302), m.p. 179–181°, formed a monoacetate and a monomethyl ether (with dimethyl sulfate and alkali). It remained unchanged upon treatment with ethereal diazomethane. The UV absorption spectrum of the compound indicated its close similarity with 1,3,5,6-tetraoxygenated xanthones (1). Xanthone A showed three methoxyl groups and four aromatic protons in its NMR spectrum. The aromatic protons appeared as *meta* and *ortho* split doublets associated with H-2, H-4, and H-7, H-8 protons, respectively. A one-proton singlet appeared at δ 12.97 and was ascribed to the strongly chelated 1-OH. The signal remained unchanged upon treatment of the xanthone with deuterium oxide. On the basis of these observations, xanthone A was identified as 1-hydroxy-3,5,6-trimethoxyxanthone. This conclusion was further confirmed by a direct comparison of the xanthone with an authentic synthetic specimen of 1-hydroxy-3,5,6-trimethoxyxanthone (5).

Xanthone B, $C_{15}H_{12}O_6$ (M^+, 288), m.p. 192–193°, is a dihydroxydimethoxyxanthone; it formed a diacetate and a monomethyl ether (diazomethane). The methyl ether was identical with 1-hydroxy-3,5,6-trimethoxyxanthone. The UV absorption and NMR spectra of xanthone B also showed its close similarity with 1,3,5,6-tetraoxygenated xanthones. Its insolubility in aqueous sodium carbonate and unchanged UV maxima in ethanolic sodium acetate (6) indicated that a methoxyl group is at C-3. The abundance of the M − 15 peak (fragment ion at *m/e* 273) in its mass spectrum locates the methoxyl group at C-5. Xanthones with a 5-methoxy substituent are known (1, 7, 8) to produce abundant fragment ions corresponding to the loss of a methyl radical, while for *m*-methoxyphenols (equivalent to a C-6 methoxyl in xanthones) virtually no M − 15 peak has been found (9). Similar anticipated peaks were observed in the current studies with other methoxylated xanthones.



xanthone A: $R_1 = R_2 = R_3 = CH_3$
xanthone B: $R_1 = R_2 = CH_3$, $R_3 = H$
xanthone C: $R_1 = R_3 = H$, $R_2 = CH_3$



mangiferin
(R = β-D-glucoside)

Xanthone C, $C_{14}H_{10}O_6$ (M^+, 274), m.p. 290–291°, was previously obtained (1) from the alcoholic extracts of *C. decussata* in appreciable quantities but could not be completely characterized at that time. It formed a dimethyl ether with diazomethane and a trimethyl ether with dimethyl sulfate and alkali. The UV and NMR spectra of the xanthone are characteristic of a 1,3,5,6-tetraoxygenated xanthone. The dimethyl ether was identical with 1-hydroxy-3,5,6-trimethoxyxanthone. The position of the only methoxyl group in xanthone C was determined on the basis of: (*a*) its solubility in aqueous sodium carbonate, (*b*) its failure to give Tollen's test, (*c*) its unchanged UV absorption maxima in ethanolic sodium acetate–boric acid, and (*d*) the shift in its major UV absorption peaks in the presence of a trace of sodium acetate. These observations locate the methoxyl group of xanthone C at either C-5 or C-6 but not at C-3. The facile loss of 15 mass units from its molecular ion peak (M^+) indicated that the methoxyl group is at C-5.

This is the first report of the occurrence of 1,3,5,6-tetraoxygenated xanthones in the family Gentianaceae and of xanthones A–C in nature. Also, the cooccurrence of 1,3,5,6-tetraoxygenated xanthones with the free 1,3,6,7-tetrahydroxyxanthone (8) and 1,3,6,7-tetrahydroxyxanthone C_2-β-D-glucoside, mangiferin, in a single plant species (*C. decussata*), may have phylogenetic and biogenetic significance, since these two oxygenated patterns (1,3,5,6 and 1,3,6,7) are reported to be derived from a common benzophenone intermediate, maclurin, by phenolic oxidation (10).

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Abstract □ The chloroform extract of *Alnus oregona* showed antitumor activity against the Walker 256 (5WA16) tumor system. Lupeol and betulin were identified as the two constituents responsible for this activity.

Keyphrases □ *Alnus oregona* (Betulaceae)—isolation and identification of two antitumor constituents, lupeol and betulin □ Lupeol—antitumor agent identified from *Alnus oregona* □ Betulin—antitumor agent identified from *Alnus oregona* □ Antitumor activity—isolation and identification of lupeol and betulin as antitumor constituents from *Alnus oregona*

During the routine screening of Southwestern plants for potential antitumor activity, the chloroform extract of the stembark of *Alnus oregona* Nutt. showed significant antitumor activity in Sprague rats against the Walker 256 intramuscular tumor system (5WA16)[1]. Activity in this system is defined as a percent T/C value of less than 60 in a satisfactory dose–response test (1). The plant was collected in California[2].

Triterpenes belonging mainly to the taraxane and lupane series have been isolated from various species of *Alnus*. Taraxerol, taraxerone, lupeol, lupenone, betulin, and betulinic acid as well as other triterpene compounds have been isolated from *A. glutinosa* (2, 3), *A. incana* (4, 5), *A. virdis* (6), *A. barbata* (7), and *A. subcordata* (8). However, a search of the literature failed to reveal any chemical investigation of *A. oregona*.

RESULTS AND DISCUSSION

Because of its chemical complexity, the chloroform extract was separated into six fractions by column chromatography using partially deactivated alumina (Table I). Fraction E, which consisted essentially of β-sitosterol, was not screened further since the Cancer Chemotherapy National Service Center has indicated that this compound showed marginal activity in the 5WA16 tumor system. Only Fractions C and F showed significant activity (Table II).

Fractions C and F contained essentially single components. Fraction C, upon recrystallization from chloroform–methanol, yielded a crystalline compound, m.p. 210–212°. Its mass spectrum showed an M^+ peak at 426 with major fragments at *m/e* 218, 207, and 189. These were indicative of the lupene skeleton (9). The NMR spectrum of the compound indicated vinyl protons at δ 4.66 and 4.75 (d) as well as 3α-H at δ 3.28 (m), further proof that the compound was probably lupeol. Identification was confirmed by melting point, optical rotation, and IR of the compound as well as of the acetate and benzoate. The IR of the latter was superimposable with the IR of an authentic sample of lupeol benzoate[3].

The crystalline compound isolated from Fraction F (m.p. 252–254°) was identified as betulin. The mass spectrum showed an M^+ peak at 442, and the NMR spectrum indicated the appropriate signal for vinyl protons. The IR spectra of the compound and its diacetate were superimposable with authentic samples[4].

Table I—Alumina Chromatography of Crude Extract

Fraction	Eluent		Components
A	Hexane to hexane–benzene	(3:1)	—
B	Hexane–benzene	(6:4)	—
C	Hexane–benzene	(1:1)	Lupeol
D	Hexane–benzene	(1:1)	—
E	Benzene		β-Sitosterol
F	Benzene–chloroforn	(3:1)	Betulin

Table II—Biological Activity against 5WM Tumor System

Compound	Dose, mg./kg.	Survivors	Percent T/C (1)
Crude extract	200	4/4	28
Fraction C	400	4/4	22
	200	4/4	46
Fraction F	400	4/4	39
Lupeol	200	4/4	39
Betulin	600	4/4	13
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EXPERIMENTAL

Extraction—Twelve kilograms of the dried stembark of *A. oregona* was extracted with 21 l. of chloroform in an extractor (Lloyd). The extract, after filtration and removal of the chloroform, weighed 382 g.

Column Chromatography—Neutral alumina (3.8 kg., activity III) was packed in a glass column (64 × 10.5 cm.), using *n*-hexane

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Isolation of Lupeol—Fraction C yielded a large precipitate of crystals. After repeated crystallizations from chloroform–methanol, 14 g. of a colorless crystalline compound was obtained. Its melting point was 210–212°, $[\alpha]_D$ +25°, compared with the values for lupeol of 215° and $[\alpha]_D$ +27° (10).

Preparation of Lupeol Acetate—Lupeol (0.138 g.) was dissolved in 2.0 ml. of pyridine, and 2.0 ml. of acetic anhydride was added to the mixture. The mixture was left standing overnight at room temperature. It was then poured over crushed ice. The precipitated compound was collected and washed thoroughly with water until free of acetic acid and pyridine. After drying under vacuum, the compound was recrystallized twice from chloroform–methanol. Solid needles of crystals, m.p. 211–212° and $[\alpha]_D$ +33°, were obtained. The reported values for lupeol acetate were m.p. 218° and $[\alpha]_D$ +47° (10).

Preparation of Lupeol Benzoate—A mixture of isolated lupeol (200 mg.), pyridine (2.0 ml.), and benzoyl chloride (2.0 ml.) was refluxed for 1 hr. It was then cooled and poured over crushed ice. The mixture was refrigerated for 1 day. The precipitated compound was filtered off and then redissolved in ether. The ethereal solution was successively washed three times each with 5% sodium bicarbonate, 5% hydrochloric acid, and water. After drying the washed ethereal layer over anhydrous sodium sulfate, it was filtered and the ether was allowed to evaporate at room temperature. The residue, upon repeated crystallization from chloroform–ethanol, gave long platelike crystals, m.p. 258–260°, $[\alpha]_D$ +56° [lit. (10) m.p. 273–274°, $[\alpha]_D$ +61°]. The IR spectrum of this compound (KBr pellet) and that of an authentic sample of lupeol benzoate were superimposable.

Isolation of Betulin—Fraction F yielded a large quantity of a crystalline compound. This material was purified by recrystallization from chloroform and then from ethanol. Solid, thin, rodlike crystals, m.p. and mixed m.p. 253–254°, $[\alpha]_D$ + 18° [lit. (11) m.p. 251–252°, $[\alpha]_D$ +20°] were obtained. The IR spectrum of the isolated betulin (KBr pellet) and that of an authentic sample of betulin were in complete agreement.

Preparation of Betulin Diacetate—Betulin (0.3 g.), pyridine (0.3 ml.), and acetic anhydride (3.0 ml.) were refluxed for 1 hr. After cooling the reaction mixture, a precipitate was obtained. The material, after repeated crystallization from ethanol, gave bladelike crystals, m.p. 222–225°, $[\alpha]_D$ +24° [lit. (10) m.p. 223–224°, $[\alpha]_D$ +22°]. An authentic sample of this compound gave an identical IR spectrum (KBr pellets).

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Antibacterial Activity of Certain 3-Substituted Benzothiazoline-2-thiones

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EXPERIMENTAL

The agar medium was inoculated with 1 ml. of 24-hr.-old culture of the test organism. Filter paper disks (5-mm. diameter) saturated with the solution of the test compound (20 mg./ml. in ethanol) were

as the chromatographic solvent. Then 200 g. of the chloroform extract of *A. oregona* was adsorbed onto 500 g. of alumina III, and the dried pulverized mixture was placed on top of the column. The column was eluted first with hexane and then with solvents of increasing polarity. Each fraction was examined by TLC, silica gel G, using chloroform–benzene–ethyl acetate (4:8:1) as the solvent system and ceric sulfate as the developing reagent. The fractions showing the same materials on TLC were combined into six groups.

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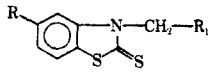
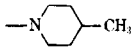
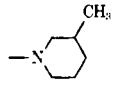
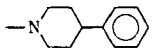
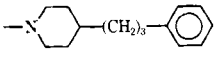
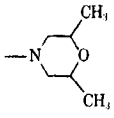
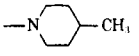
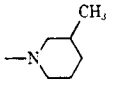
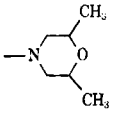


Compound	R	R_1	Microbial Spectrum[a] *Escherichia coli*	*Salmonella typhosa*	*Aerobacter aerogenes*	*Staphylococcus aureus*
1	H		−	−	+	−
2	H		++	++	+	+
3	H		−	—[b]	−	−
4	H		+	+	+++	−
5	H		+	++	+	−
6	Cl		+	−	—[b]	+++
7	Cl		+++	−	+	+++
8	Cl		++	+	+	+++
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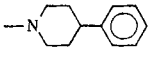
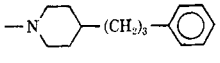
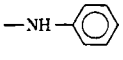
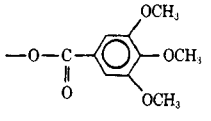
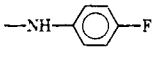
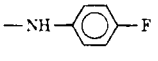
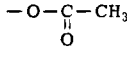
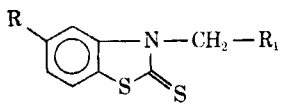
10	Cl		+	++	++	++
11	Cl		++	+	−	+++
12	Cl		+	−	+	−
13	H		−	−	−	−
14	Cl		+	−	−	−
15	H		−	−	−	+
16	Cl		−	−	−	+
17	H	−OH	++	++	+	+
18	Cl	−OH	+	+	+	+++

[a] Cultures maintained at Central Drug Research Institute, Lucknow, India, were used. Zone size: 6–10 mm. (+), 11–15 mm. (++), and greater than 15 mm. (+++). [b] No study was made in this case.



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LUCY S. C. WAN

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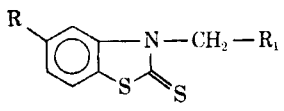

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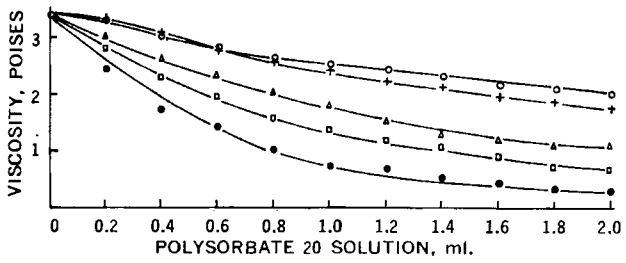

Figure 1—*Effect of various concentrations of polysorbate 20 on the viscosity of the salicylic acid–cetrimide system. Key (percent polysorbate 20 concentration): O, 1; +, 2; △, 4; □, 6; and ●, 10.*

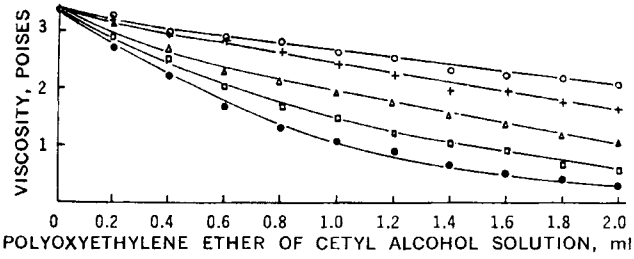

Figure 2—*Effect of various concentrations of polyoxyethylene ether of cetyl alcohol on the viscosity of the salicylic acid–cetrimide system. Key (percent polyoxyethylene ether of cetyl alcohol concentration): O, 1; +, 2; △, 4; □, 6; and ●, 10.*

measured[5] at a shear rate of 78.56 sec.$^{-1}$ in the same manner as described before (5). The additive solution was delivered from a graduated pipet to a sample of 50 g. (about 51 ml.) of the salicylic acid–cetrimide system and dispersed in the sample without removing the viscometer cylinders (6).

RESULTS AND DISCUSSION

Figures 1 and 2 show that the viscosity of the salicylic acid–cetrimide system decreased in the presence of solutions of some nonionic surfactants. The viscosity became lower when a higher concentration and a larger volume of the additive solution were added. This was also true with the other surfactants, polysorbate 40, polysorbate 60, polysorbate 80, and polysorbate 85. However, the amount of viscosity lowering due to the addition of a solution of greater additive concentration was slightly less than that produced by a larger volume of more dilute concentration such that both volumes contained an equivalent quantity of additive. This may be attributed to the effect of the increase in volume of the total system. Evidence in support of this can be seen in the small decrease in viscosity resulting from the addition of water to such a system (6).

It is believed that the interaction of cetrimide and salicylic acid leads to the formation of macromolecules which are responsible for the high viscosity achieved in the system. Molecules are considered to move more or less individually as in an ordinary liquid. Macromolecules, however, may be dispersed in the medium in the form of a network. The greater the degree of proximity of the macromolecules, the higher is the viscosity. The surfactant additives incorporated in this system are likely to penetrate the mesh structure. Since the high viscosity of the system is essentially due to the presence of the macromolecules produced by the interaction of the acid with cetrimide molecules and to the compactness of the network, the intermacromolecular penetration by the nonionic surfactant additives causes the macromolecules to move further apart from neighboring macromolecules, thereby reducing the viscosity. It was found previously (5) that cetomacrogol 1000 did not interact with salicylic acid in the absence of cetrimide.

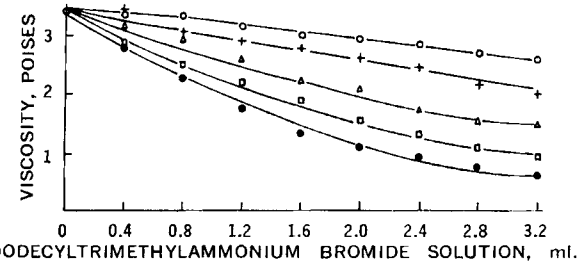

Figure 3—*Effect of dodecyltrimethylammonium bromide of varying concentration on the viscosity of the salicylic acid–cetrimide system. Key (percent dodecyltrimethylammonium bromide concentration): O, 1; +, 2; △, 4; □, 6; and ●, 10.*

[5] Using a portable Ferranti viscometer, model VL, Ferranti Ltd., Moston, Manchester 10, England.

Table I—Viscosity of Salicylic Acid–Cetrimide Systems Containing 5% Cetrimide and Varying Amounts of Salicylic Acid at 25° (Shear Rate is 78.56 sec.$^{-1}$)

Salicylic Acid Concentration, %	Viscosity, poises
1.2	0.71
1.3	1.82
1.4	3.41
1.5	4.59
1.6	6.26
1.8	7.52
1.9	7.92

The effect produced by the inclusion of cationic surfactants as additives to the system (Figs. 3 and 4) was the same as that produced by the nonionic surfactants. The viscosity reduction is probably due to the fact that the system has become diluted with the same type of surfactant. In addition, since the acid content remains the same, there is a decrease in saturation. This would lower the viscosity; it was found that when the amount of salicylic acid present was less and the system was not saturated to the same degree, there was a fall in viscosity (Table I). The penetration of the added cationic additive into the mesh structure exerts negligible effect because of the immediate dilution effect by a surfactant of a similar charge.

When salicylic acid was added to sodium lauryl sulfate solutions, no change in viscosity was observed. Even after adding graded

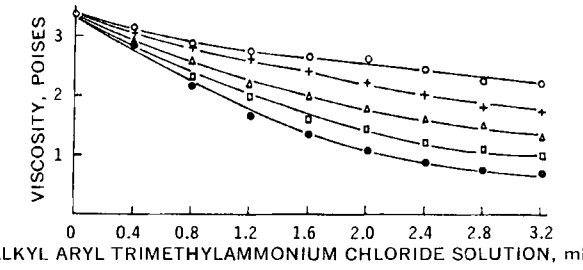

Figure 4—*Effect of alkyl aryl trimethylammonium chloride of varying concentration on the viscosity of the salicylic acid–cetrimide system. Key (percent alkyl aryl trimethylammonium chloride concentration): O, 1; +, 2; △, 4; □, 6; and ●, 10.*

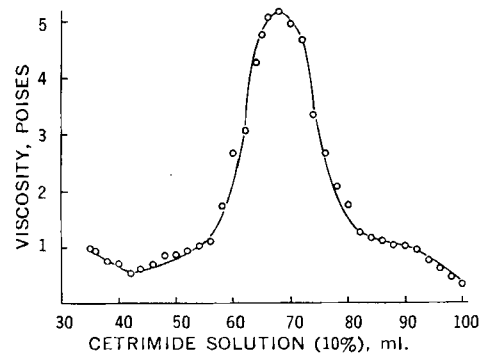

Figure 5—*Viscosity of salicylic acid sodium lauryl sulfate system on addition of a 10% cetrimide solution.*

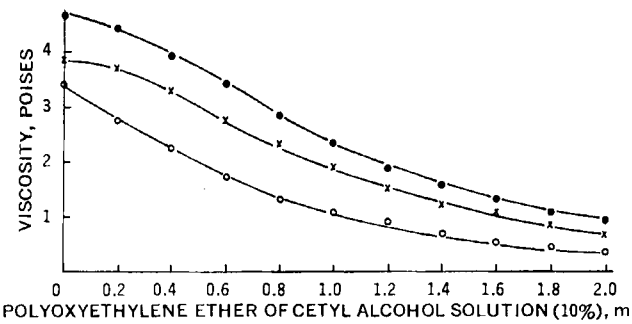

Figure 6—*Viscosities of systems containing fixed salicylic acid concentration (1.4%) and varying cetrimide concentration on addition of a solution of polyoxyethylene ether of cetyl alcohol. Key (percent cetrimide concentration): ×, 3; ●, 4; and ○, 5.*

volumes of a 10% cetrimide solution to an aqueous system containing 4% sodium lauryl sulfate and 2% salicylic acid, there was still no change. Eventually, when a much larger amount was incorporated, the viscosity decreased slightly and then increased markedly, rising to a maximum after which it decreased again (Fig. 5). The viscosity values could not be determined accurately in the presence of less than 35 ml. of a 10% cetrimide solution that was added to the system, because the precipitate formed by the neutralization of sodium lauryl sulfate with cetrimide interfered with the rotation of the viscometer cylinders.

The results can be explained in terms of neutralization occurring in the system during the continuous addition of the cetrimide solution as the additive. The initial incorporation of the cetrimide solution brings about the precipitation of a complex between the anionic and cationic surface-active ions. When an adequate amount of cetrimide solution is present (*i.e.*, about 35 ml.), most of the sodium lauryl sulfate ions are attracted to the positively charged molecules of the additive. It was observed that at about this stage the precipitate became more uniformly dispersed and the viscosity values could be measured. Subsequent additions of cetrimide solutions allow the cationic surfactant to interact with the salicylic acid and thus produce a rise in viscosity. This viscosity increase continues until most of the acid molecules interact with the cetrimide molecules and then the viscosity begins to fall. Then the effect is similar to that obtained by the addition of a cationic surfactant solution directly to the salicylic acid–cetrimide systems (Figs. 3 and 4).

When the concentration of salicylic acid was fixed (1.4%) and the concentration of cetrimide was varied, the viscosity was reduced by solutions (10%) of polyoxyethylene ether of cetyl alcohol (Fig. 6). This also occurred with the other nonionic surfactants investigated. Figure 7 shows that similar results were obtained with systems in which the acid content was different and the cetrimide content was kept constant (5%). This was true when the cetrimide solution was employed as the additive. The system containing 3% cetrimide and 1.4% salicylic acid is above saturation because excess acid is found after the system is allowed to equilibrate in a thermostatically controlled water bath for 24 hr. (1). In such a system, there will be a minimal amount of free cetrimide molecules; in the other two systems containing 4 and 5% cetrimide, a relatively larger number of free cetrimide molecules will be present (Fig. 6). It appears, therefore, that irrespective of whether free cetrimide molecules were present or absent, the added solution of polyoxyethylene ether of cetyl alcohol did not increase the viscosity but decreased it. This finding is in contrast to that obtained with the anionic surfactants as additives (1). There is no neutralization taking place in these systems since the added surfactant is compatible with cetrimide.

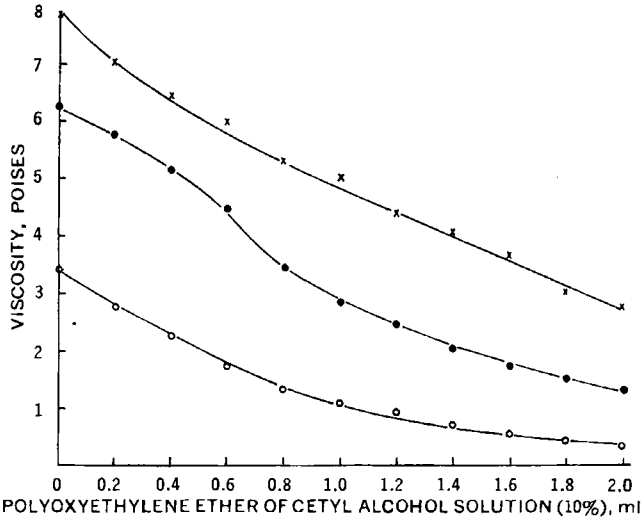

Figure 7—*Viscosities of systems containing fixed cetrimide concentration (5%) and varying salicylic acid concentration on addition of a solution of polyoxyethylene ether of cetyl alcohol. Key (percent salicylic acid concentration): ×, 1.9; ●, 1.6; and ○, 1.4.*

These findings, together with those described previously (1), demonstrate the effects of different types of surfactant additives and provide knowledge that is useful when attempting to combine surfactants of the quaternary ammonium type in the formulation of products involving salicylic acid. This is particularly so in those preparations where the viscous property influences the physical stability of the presentation form of the preparation.

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In Vitro and *In Vivo* Hydrolysis of 4-Benzoylphenyl *N*-Methylcarbamate

LEO W. BROWN and ARLINGTON A. FORIST▲

Abstract □ The kinetics of hydrolysis of the carbamoyl group of 4-benzoylphenyl *N*-methylcarbamate in 50% aqueous ethanol showed that the reaction was first order with respect to both hydroxide ion and carbamate. The calculated half-life at pH 7.3, 37°, was 73 min. Following oral administration in the dog, urinary excretion products in separate experiments were (percent of the dose): the intact compound (<0.1%), 4-benzoylphenol (0.3; 0.4%), and conjugated 4-benzoylphenol (17.0; 8.3%). Taken together, these results are consistent with considerable hydrolysis of the drug in the intestine, erratic absorption, and rapid hydrolysis following absorption.

Keyphrases □ 4-Benzoylphenyl *N*-methylcarbamate—*in vitro* and *in vivo* hydrolysis, half-life, urinary excretion products in dog □ *N*-Methylcarbamate, 4-benzoylphenyl—*in vitro* and *in vivo* hydrolysis, half-life, urinary excretion products in dog □ Hydrolysis, *in vitro* and *in vivo*—4-benzoylphenyl *N*-methylcarbamate

A number of aryl *N*-methylcarbamates have shown anti-inflammatory activity (1, 2). 4-Benzoylphenyl *N*-methylcarbamate (I) possesses similar biological activity (3). Earlier investigations with salicylanilide *N*-methylcarbamate and 4-biphenylyl *N*-methylcarbamate demonstrated marked differences in hydrolytic stability and indicated that carbamate hydrolysis at pH's encountered in the intestine could have a significant effect on absorption and resultant activity (2). Accordingly, the hydrolytic stability of I was determined and is the subject of this report. Included also are data on the oral absorption and *in vivo* hydrolysis of I in the dog based on the urinary excretion of I and its metabolites.

EXPERIMENTAL

***In Vitro* Hydrolysis Studies**—Solutions of I (5.98×10^{-6} *M*) were prepared in 50% aqueous ethanolic phosphate (0.025 *M*) buffers at the apparent pH values shown in Table I. Absorbances (A_t) were determined at appropriate times at 295 nm., the absorption maximum of 4-benzoylphenol (II), at 37° until no further absorbance increases occurred (A_∞). Pseudo-first-order reaction rate constants, k', were obtained from the slopes of plots of log ($A_\infty - A_t$) *versus* time.

***In Vivo* Studies**—In examining the absorption and excretion of I, a fasting 10-kg. beagle dog received 200 mg. of I orally, followed 14 days later by 220 mg. orally. A 24-hr. urine sample (blank) was collected prior to drug administration, and urine samples (acidified on collection) were obtained following drug administration (Table II).

Table I—Kinetic Constants for 4-Benzoylphenyl *N*-Methylcarbamate Hydrolysis in 50% Ethanol at 37°

Apparent pH	k', min.$^{-1}$	$t_{1/2}$, min.	k_{OH^-}, l. moles^{-1} min.$^{-1}$
7.4	1.14×10^{-2}	61	1.90×10^4
8.0	5.30×10^{-2}	13	2.22×10^4
8.6	1.84×10^{-1}	4	1.89×10^4
		Mean	2.00×10^4

Table II—Urinary Excretion of Free and Conjugated 4-Benzoylphenol (II) in the Dog following Oral Administration of 4-Benzoylphenyl *N*-Methylcarbamate

Dose, mg.	Interval, hr.	Percent of Dose Excreted— II	Conjugated II
200	0–24	0.27	16.3
	24–55	0.05	0.7
220	0–8	0.20	5.8
	8–24	0.20	0.6
	24–48	0.04	1.9

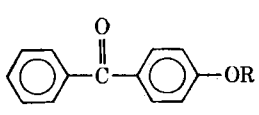
Free and conjugated drug-related materials were determined by a quantitative TLC procedure. For free materials, 10 ml. of urine, adjusted to pH 2, was extracted with 25 ml. of chloroform. A 20-ml. portion of the chloroform phase was concentrated to dryness in a nitrogen stream, and the residue was dissolved in a few drops of chloroform saturated with 0.1 *N* HCl. The resulting solution was quantitatively transferred to a silica gel G TLC plate containing phosphors and developed with chloroform–ethanol–formic acid (98:3:2) in parallel with standard I (R_f 0.7) and II (R_f 0.5). Zones corresponding to I and II were detected with a UV lamp, scraped from the plate, and eluted with 5 ml. of 0.1 *N* NaOH. Compound II was determined from the absorbance of the basic eluate at 345 nm. Interfering materials from the urine absorbing at 340 nm. made estimation of I at 345 nm., based on its hydrolysis to II in the eluting solvent, impossible. Therefore, the basic eluate of zone I was acidified and extracted with three 5-ml. portions of chloroform, and the extract was analyzed by the quantitative TLC procedure for II.

To determine conjugated materials, 15 ml. of urine was adjusted to pH 5.0, mixed with 3 ml. of 0.5 *M* pH 5.0 acetate buffer and 0.3 ml. of an enzyme solution[1] containing 30,000 units of β-glucuronidase and 15,000 units of sulfatase, and incubated at 37° for 48 hr. The sample was then acidified and carried through the extraction and TLC determination for II. All calculations were based on the responses (A_{345}) obtained for standard samples of I or II added to urine and carried through the same procedures, with appropriate corrections for urine blanks.

RESULTS AND DISCUSSION

Previous reports (1, 2, 4) showed that the hydrolysis of aryl carbamates is first order in both hydroxide ion and carbamate. In the present kinetic studies, plots of log ($A_\infty - A_t$) *versus* time were strictly linear, confirming the first-order dependence on I. Pseudo-first-order reaction rate constants, k', are shown in Table I. Moreover, a plot of log k' *versus* apparent pH was linear with a slope of unity, establishing first-order dependence on hydroxide ion and permitting calculation of the specific reaction rate constants, k_{OH^-}, presented in Table I.

Use of 50% ethanol as solvent was dictated by the low solubility of I in water. As a consequence, a direct calculation of hydrolysis rates in aqueous systems cannot be made. However, earlier studies



I: R = —NHCH$_3$
II: R = H

[1] Glusulase, Endo Laboratories, Richmond Hill, N. Y.

with the structurally similar 4-biphenylyl *N*-methylcarbamate (1, 2) indicated that k_{OH^-} in water was six times that of the value in 50% ethanol. Thus, it may reasonably be predicted from the data in Table I that considerable hydrolysis of I would occur over the pH range encountered in the small intestine and that absorption of the intact drug could be variable from dose to dose. In addition, nonenzymatic hydrolysis at the pH of the blood, following absorption, would be rapid. The half-life of I at pH 7.3, 37°, calculated from the data in Table I is 73 min. Comparison of these results with those obtained earlier with 4-biphenylyl *N*-methylcarbamate (1) indicates that substitution of the 4-benzoyl group for the 4-phenyl group increases the hydrolysis rate nearly 200-fold.

Following oral administration of I to the dog, traces of intact I were detected in the urine but were below the level of reasonable quantification (<0.1% of the dose). The only drug-related materials detected in the urine were the hydrolysis product, II, and glucuronide and/or sulfate conjugates of II (Table II). Overall excretion ranged from 8.7 to 17.3% of the dose in the two experiments. These results are consistent with erratic absorption and the predicted extensive hydrolysis of I based on the *in vitro* kinetic studies.

Urinary excretion of 47–92% of orally administered II as a glucuronide conjugate in the rabbit was reported by Robinson (5). The present results for II (produced from I) in the dog are qualitatively similar.

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▲ To whom inquiries should be directed.

Mass Fragmentographic Detection of Normorphine in Urine of Man after Codeine Intake

WILHELM O. R. EBBIGHAUSEN▲, JOHN MOWAT, and PER VESTERGAARD

Abstract □ The biological disposition of codeine in urine was studied after intake of a single therapeutic dose of 20 mg. of codeine phosphate. Human urine after ingestion of codeine was previously reported to contain codeine as well as two of its metabolites, morphine and norcodeine. The presence of an additional metabolite, normorphine, was detectable after acid hydrolysis of an aliquot of human urine collected for 10 hr. after intake. Analysis was performed using a combined GC–mass spectrometer, with an additional accelerating voltage alternator unit as a specific detector. Presence of normorphine was indicated by mass fragmentography. A partial mass spectrum for identification was obtained by repeated scanning across the normorphine peak area.

Keyphrases □ Normorphine—mass spectroscopic detection as a metabolite of codeine in human urine □ Codeine—mass spectroscopic detection of normorphine as a metabolite, human urine □ Mass spectroscopy—detection of normorphine as a metabolite of codeine in human urine

Researchers have investigated the biological disposition of codeine in man, monkey, dog, rat, mouse, rabbit, and guinea pig (1, 2). The metabolic pathway of codeine in different species includes conjugation, *O*- and *N*-dealkylation to morphine and norcodeine, and conjugation of these metabolites.

In 1962, Way and Adler (3) reported: "Thus far there has been no direct evidence to indicate that the same codeine molecule is demethylated at both the *O*- and the *N*-positions to yield normorphine." However, during the same year, Kuhn and Friebel (4) identified traces of normorphine in urine of rats after treatment with codeine. In 1970, Yeh and Woods (5) reported the detection of normorphine by paper and thin-layer chromatography in urine and bile of rats after subcutaneous injection of large amounts of codeine phosphate. In the present investigation, traces of normorphine were detected, by means of mass fragmentography, in addition to the already known metabolites in urine of man after intake of a single therapeutic dose of either 10 or 20 mg. of codeine phosphate.

MASS FRAGMENTOGRAPHY

In 1968, mass fragmentography was introduced and its application has been demonstrated for the identification of chlorpromazine and its metabolites in human blood (6). With mass fragmentography, it is possible to take advantage of certain physicochemical properties of compounds or a group of compounds with similar chemical structures. The mass spectrometer is used as a GC detector. Compounds are detected by the presence of characteristic mass numbers (their molecular ion or fragments).

The accelerating voltage alternator[1] allows the detection and simultaneous recording of up to three mass numbers of a compound by keeping the magnetic field constant while switching the accelerating voltage. With this technique, amounts as small as 5 pg. can be detected. Another advantage is the high selectivity of mass fragmentography. There is very little chance that two different compounds have the same retention time and correspond at the same time to preselected fragments with identical peak ratios. The information for identification is, however, less than that obtained when a complete mass spectrum is recorded.

[1] LKB Produkter AB, Bromma, Sweden.

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Table I—The 10 Strongest Peaks above *m/e* 200 of the Mass Spectra of Morphine, Codeine, Normorphine, and Norcodeine as Trifluoroacetyl Derivatives

Trifluoroacetyl-Morphine, mol. wt. 477			Trifluoroacetyl-Codeine, mol. wt. 395			Trifluoroacetyl-Normorphine, mol. wt. 559			Trifluoroacetyl-Norcodeine, mol. wt. 577		
	Relative Intensity, %			Relative Intensity, %			Relative Intensity, %			Relative Intensity, %	
m/e	a[a]	b[b]	*m/e*	a	b	*m/e*	a	b	*m/e*	a	b
364	100(28)[c]	100	395	100(20)	100	305	100(11)	100	477	100(15)	100
477	57(16)	55	282	94(19)	98	559	47(5)	45[d]	305	48(7)	52
365	22(6)	33	281	25(5)	26	306	44(5)	50	223	38(6)	40
478	12	13	396	23	26	319	30	32	478	26	25
311	10	9	283	21	23	332	27	28[d]	351	21	22
380	8	9	225	10	19	317	22	22	237	19	23
363	6	7	229	10	16	433	18	17[d]	224	18	25
307	6	6	266	10	10	333	14	13[d]	337	15	16
476	5	5	223	8	13	560	13	13	235	11	16
362	3	2	280	8	9	235	13	—	306	10	15

[a] Reference compounds. [b] Extract of urine. [c] Number in parentheses is relative total ionization. [d] Recorded but not shown in Fig. 2.

EXPERIMENTAL

Twenty milligrams of codeine phosphate (14.1 mg. base) was given to a healthy male of 83 kg. body weight. The subject did not take any drugs previous to these studies. A total of 400 ml. of urine was collected during the 10 hr. after intake. Urine used in all experiments as a blank control was collected before intake of the codeine phosphate. Since most of the administered codeine and its main metabolite, morphine, are excreted in urine as glucuronides, all samples were hydrolyzed before analysis. One milliliter of urine was transferred to a 10-ml. culture test tube, 1 ml. of 6 *N* HCl was added, and the sample was hydrolyzed in a paraffin bath for 30 min. at 100–110°. After hydrolysis, 1 ml. of borax–hydrochloric acid buffer (0.04 *M*, pH 8.85) was added and the mixture was titrated by careful addition of 8 *N* NaOH to pH 8.85. It was then extracted for 20 min. with 4 ml. of a 3:1 mixture of chloroform–isoamyl alcohol, using a mechanical shaker. After centrifugation at 3000 r.p.m. for 5 min., 3 ml. of the organic layer was transferred to a glass-stoppered 3-ml. centrifuge tube and evaporated to dryness in a paraffin bath at 80–90° under a stream of nitrogen. The dry residue was reacted with approximately 100 μl. of trifluoroacetic acid anhydride for 30 min. After evaporating to dryness, the dried residue was taken up in 100 μl. of chloroform and samples of this solution were injected into the GC column.

GC–mass spectroscopy analysis was performed with a gas chromatograph–mass spectrometer[2] with an accelerating voltage alternator. GC was done on a silanized 1.8-m. (6-ft.) × 1.5-mm. i.d. glass column, packed with 3% SE-30 on 100–120-mesh Gas Chrom Q. Temperatures were: column, 210°; flash heater, 250°; separator, 255°; and ion source, 290°. The carrier gas was helium, with a 20-ml./min. flow rate. All mass spectra and accelerating voltage alternator recordings were registered at an ionizing potential of 70 ev. and a basic accelerating voltage of 3.5 kv.

Table I represents, in a tabulated form, the relative abundance of the 10 strongest peaks above *m/e* 200 in the mass spectra of the trifluoroacetyl derivatives from: (*a*) the reference compounds and (*b*) hydrolyzed urine after codeine intake. The peaks are tabulated in order of decreasing abundance.

Figure 1 displays the recordings of the trifluoroacetyl derivatives. The accelerating voltage alternator recording was performed at 70 ev. The analyzer/collector slit setting was 0.1/0.3. The multiplier gain setting was at position 8 (range 1–11 = 1.2–3.5 kv.).

The switching frequency of the accelerating voltage alternator unit was 2 Hz. The range of the accelerating voltage alternator unit is relatively small. By adjusting the magnetic field to *m/e* 282 (at a given basic accelerating voltage of 3.5 kv.), the accelerating voltage alternator unit allows a maximum simultaneous recording of *m/e* 310 (282 + 10%). Since we had to have a registering range from *m/e* 282 to *m/e* 560, we had to readjust the magnetic field (and, subsequently, the accelerating voltage) to obtain at least three or four characteristic mass fragments of all four derivatives.

[2] LKB 9000.

The accelerating voltage alternator recordings in Fig. 1B indicate, by retention time and peak ratio, the presence of the trifluoroacetyl derivatives of morphine, codeine, normorphine, and norcodeine.

For further identification, the complete mass spectra of codeine, morphine, and norcodeine as their trifluoroacetyl derivatives could be recorded. Since the amount of normorphine present was very small, a 30-ml. sample of urine was prepurified by filtering off the

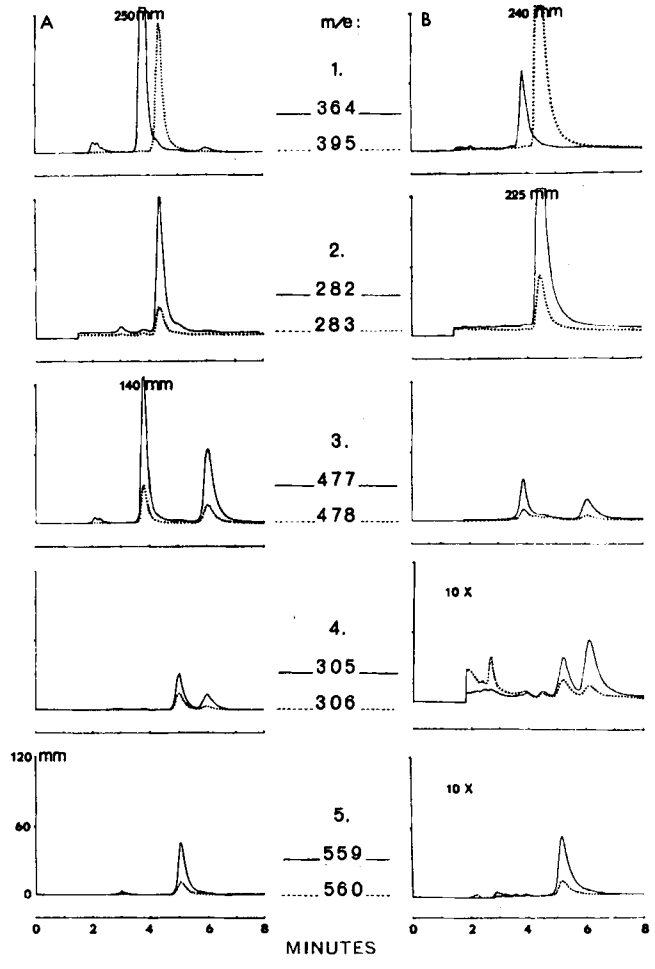

Figure 1—*Accelerating voltage alternator recording of indicated mass units (trifluoroacetyl derivatives, 200 ng. each) of: A1, morphine (364) and codeine (395); A2, codeine (282/283); A3, morphine and norcodeine (477/478); A4, normorphine and norcodeine (305/306); A5, normorphine (559/560); and B1–5, corresponding compounds after urine extraction.*

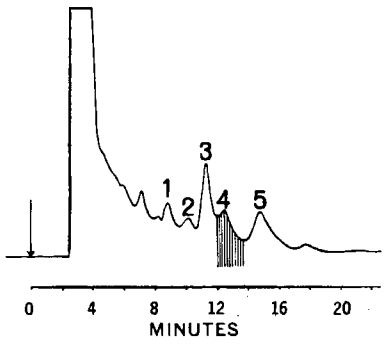

Figure 2—*Gas chromatogram of urine extract after codeine intake. For GC details, see text. Key: trifluoroacetyl derivatives of 1, morphine; 2, codeine; 3, unknown impurity; 4, normorphine; and 5, norcodeine. Strokes under 4 indicate the area where the repetitive scanning was performed.*

sediments and extracting it three times with an equal volume of ethyl ether. No normorphine was detected in the ether extract. The aqueous layer was then extracted twice with 25 ml. of the chloroform-isoamyl alcohol (3:1) mixture. The residue, after evaporation of the solvent and derivative formation, was taken up in 50 μl. of chloroform.

The column temperature was decreased to 188° and 5 μl. of the solution was injected into the GC column. The retention times of the trifluoroacetyl derivatives of morphine, codeine, normorphine, and norcodeine were found to be 8.8, 10.1, 12.4, and 14.7 min., respectively (Fig. 2, peaks 1, 2, 4, and 5, respectively). Small amounts of morphine and codeine were left in this prepurified mixture. By focusing the accelerating voltage alternator on mass units 559 and 560, the M^+ and $M^+ + 1$ ions of the trifluoroacetyl derivative of normorphine, the compound appeared as a shoulder on the tailing side of a somewhat larger peak due to impurities (Fig. 2, peaks 2 and 4).

In subsequent studies, repetitive scans were taken at intervals of 10 sec. during a retention time period from 12 to 13.67 min. The mass range encompassed in one scan was *m/e* 250–600, and a fast scan speed was chosen. Figure 3 shows the relative abundance of the most important and characteristic fragments of the trifluoroacetyl derivative of normorphine plotted *versus* elution time. It shows, furthermore, that *m/e* 277 and 386, which decrease in relative abundance with time, originate from the concurrently eluting unknown impurity peak, tailing all over the trifluoroacetyl derivative of normorphine peak area (Fig. 3, peak 3). The recording of *m/e* 355, derived from the SE-30 column coating, keeps essentially constant over the whole area. It is evident that the peak ratios of the different mass units are nearly constant during the whole elution from the GC column.

RESULTS AND DISCUSSION

The results demonstrate the detection and identification of normorphine in human urine after intake of codeine phosphate. The detection of codeine and morphine has been reported in the literature. The presence of normorphine as a metabolite of codeine has been confirmed by analyzing the urine of five individuals after intake of 20 or 10 mg. of codeine phosphate.

A corresponding sample of urine, to which 20 mg. of codeine phosphate was added, was allowed to stand for 12 hr. at room temperature and then subjected to the above-mentioned hydrolysis, extraction, and analysis procedures. Except for codeine, none of the identified metabolites was detected.

To estimate the quantities of excreted metabolites, the peak heights of identified compounds in urine (Fig. 1B) were compared with those of the known standards (200 ng. each) in Fig. 1A. An excretion of approximately 70% total codeine, approximately 9% total norcodeine, and approximately 10% total morphine was thus calculated in a sample of urine (400 ml. collected for 10 hr. after intake of 20 mg. codeine phosphate). Although we are presently unable to do exact quantitative determinations, these findings are very close to those reported by Adler *et al.* (7). The excretion of normorphine was calculated to be less than 4% of the administered dose of codeine.

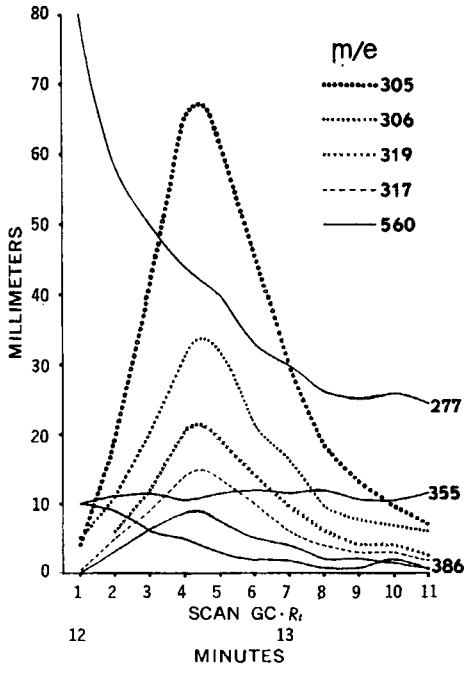

Figure 3—*Response of peaks* m/e: *305, 306, 317, 319, and 560 of the trifluoroacetyl derivative of normorphine plotted* versus *elution time from GC column;* m/e *277 and 386 indicate tailing of previous eluting impurity peak (see Fig. 2, peak 3). The SE-30 column background is shown at* m/e *355. For more details, see text.*

A more quantitative investigation of the excretion of these metabolites with these techniques remains to be done. As can be seen from Fig. 1A, the response of the base peaks of each compound (morphine, codeine, normorphine, and norcodeine as trifluoroacetyl derivatives) to the accelerating voltage alternator recorder is very different since the contribution of the base peaks to the total ion current differs for each compound. A slight drifting of the focusing and variations in the valve opening between the separator and the ion source may adversely affect quantitative determinations. It would, therefore, be preferable to use an internal standard in the test mixture. The ideal internal standard would be an isotopically labeled compound with the same chemical structure (8). For mass fragmentography, no chemical differences are necessary and the mass difference alone is sufficient to discriminate between the substance under investigation and the internal standard. No variations concerning extraction, derivative formation, column adsorption, *etc.*, will be obtained.

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Partial Characterization of Antitumor Tannin Isolated from *Calycogonium squamulosum* (Melastomataceae)

W. D. LOUB*, H. H. S. FONG, M. THEINER†, and N. R. FARNSWORTH▲

Abstract □ The active antitumor tannin from *Calycogonium squamulosum* was isolated and determined to be a tri-*O*-galloyl-D-glucose.

Keyphrases □ Tannins, antitumor—isolated from *Calycogonium squamulosum*, identified as a tri-*O*-galloyl-D-glucose □ *Calycogonium squamulosum*—isolation of antitumor tannin, partial characterization as a tri-*O*-galloyl-D-glucose □ Tri-*O*-galloyl-D-glucose—isolated from *Calycogonium squamulosum*, determined to be the antitumor agent □ Antitumor tannins—isolated from *Calycogonium squamulosum*, characterized as a tri-*O*-galloyl-D-glucose

Previously it was reported that the antitumor activity elicited by extracts of *Calycogonium squamulosum* against the Walker 256 (IM) carcinosarcoma could be attributed to the tannin present in the plant (1). Hartwell and Abbott (2) pointed out that, in their experience, all antitumor tannins have been shown either to be highly toxic or to possess a low therapeutic index. Thus, for the most part, work on tannin antitumor agents has not progressed in the programs sponsored by the Drug Research and Development Branch of the National Cancer Institute.

To understand better the mechanism of tannin antitumor activity and the toxicity of this class of compounds, the structures of the active entities should be elaborated. To date, the only antitumor tannin studied from a structure point-of-view has been that derived from *Rumex hymenosepalus*, which was shown to be a polymeric leucoanthocyanidin (3).

The purpose of the present investigation was to characterize the antitumor tannin isolated from *C. squamulosum*.

EXPERIMENTAL

Plant Material—The aerial parts of *C. squamulosum* (Melastomataceae) were used in this study[1].

Isolation of Tannin—A sample of milled plant material was fractionated as previously reported (1), and the tannin was isolated according to the method of Wall *et al.* (4).

Antitumor Activity of Tannin—A sample of the isolated tannin gave a T/C of 32% at 100 mg./kg. against the Walker 256 (IM) carcinosarcoma using established protocols (5). A T/C of ≤42% is considered active in this system.

Hydrolysis of Tannin Fraction—A sample (250 mg.) of the tannin was dissolved in 25 ml. of aqueous sulfuric acid (5% v/v), forming a clear, amber solution, which was heated under reflux for 18 hr. After cooling to room temperature, the mixture was filtered to yield a clear, light-yellow solution. This solution showed the properties of a hydrolyzable tannin, since phlobaphenes, which form under these conditions with condensed tannins, were not apparent (6). The acidic hydrolysate was made neutral to litmus paper with ammonium hydroxide and diluted to 200 ml. with 95% ethanol to precipitate the ammonium sulfate formed during the neutralization procedure. The precipitate was removed by filtration and washed with hot ethanol, and the filtrate was heated *in vacuo* at 50° to remove the ethanol. The residue was then frozen and lyophilized.

TLC of Tannin Fraction—Samples of unhydrolyzed tannin, hydrolyzed tannin, gallic acid, pyrogallol, and ferulic acid were subjected to TLC analysis on replicate silica gel G plates, using a solvent mixture of chloroform–ethyl acetate–formic acid (5:4:1) for phenols and *n*-butanol–acetic acid–water (4:1:1) for sugars. The chromatograms were sprayed with 1% $FeCl_3$ in methanol to reveal the phenolic components and with *p*-anisidine to reveal the sugars.

GLC of Hydrolyzed Tannin—A sample (23.6 mg.) of the tannin was hydrolyzed as previously described and the hydrolysate was dissolved in 3.0 ml. of anhydrous, freshly distilled pyridine, followed by the addition of 0.60 ml. of hexamethyldisilazane and 0.30 ml. of trimethylchlorosilane. The mixture was shaken for 30 sec., followed by centrifugation. The supernate was taken for analysis. A 1.0-μl. (2.94 mcg. of hydrolyzed tannin) sample of the supernate was injected into the gas chromatograph[2], which was equipped with a U-shaped glass column, 0.64 cm. o.d. × 1.83 m. (0.25 in. o.d. × 6 ft.), containing 3% SE-30 on Chromosorb W (80–100 mesh) as the stationary phase. The injection port was maintained at 250°, and the column temperature was programmed to hold at 85° for 5 min. after injection of the sample and then to give a linear rise in temperature of 10°/min. up to 260°. Nitrogen was used as the mobile phase at a flow rate of 60 ml./min. (25 p.s.i.), and the effluent gases were monitored by a hydrogen flame-ionization detector operating at 200°.

In addition to the tannin hydrolysate, standard solutions of gallic acid and glucose were similarly prepared for GLC determination.

DISCUSSION

Hydrolysis of the antitumor tannin from *C. squamulosum*, followed by TLC examination, revealed the presence of gallic acid and glucose. Subsequent GLC examination of the hydrolysate, in comparison with standard solutions of gallic acid and glucose, revealed that a ratio of 3 moles of gallic acid was present for each mole of D-glucose. This suggests a partial characterization of the tannin present in *C. squamulosum* to be a tri-*O*-galloyl-D-glucose. The complete structure of this tannin will be the subject of a future investigation.

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[1] The plant material was collected at Pico del Oeste, Puerto Rico, under the supervision of Dr. R. A. Howard, Arnold Arboretum, Harvard University. A voucher specimen of the plant material collected was identified by Dr. Howard and deposited at the Herbarium of the Arnold Arboretum.

[2] Biomedical gas chromatograph, model 400, F & M Scientific Corp., Avondale, Pa.

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Synthesis of Site-Directed Chelating Agents I: Pteridine Carboxaldehyde Thiosemicarbazones

HOWARD I. HOCHMAN, KRISHNA C. AGRAWAL, CHARLES W. SHANSKY, and ALAN C. SARTORELLI▲

Abstract □ Several 6-formylpteridine thiosemicarbazones and semicarbazones were synthesized as potential inhibitors of the enzyme dihydrofolate reductase. The most active derivative in this series was 2,4-diaminopteridine-6-carboxaldehyde semicarbazone, which caused 50% inhibition of dihydrofolate reductase of human liver at a concentration of 1.4×10^{-7} *M*.

Keyphrases □ Pteridine carboxaldehyde thiosemicarbazones—synthesized and tested as site-directed chelating agents of dihydrofolate reductase □ 6-Formylpteridine thiosemicarbazones and semicarbazones—synthesized and tested as site-directed chelating agents of dihydrofolate reductase □ Dihydrofolate reductase—synthesis and testing of pteridine carboxaldehyde thiosemicarbazones as potential inhibitors

Thiosemicarbazones of heterocyclic carboxaldehydes with the formyl group alpha to a heteroaromatic ring nitrogen have been shown to possess both antineoplastic and antiviral activities (1–5); several different heteroaromatic ring systems are active carcinostatic agents with such potency correlating with the inhibition of the synthesis of DNA (6–8). The site of the metabolic lesion on the DNA biosynthetic pathways, as elucidated with 1-formylisoquinoline thiosemicarbazone and 2-formylpyridine thiosemicarbazone, two members of the most potent heterocyclic ring systems, is at the level of the conversion of ribonucleotides to deoxyribonucleotides (9–11). A study of the mechanism of action of 1-formylisoquinoline thiosemicarbazone and 2-formylpyridine thiosemicarbazone indicated that they inhibit the enzyme ribonucleoside diphosphate reductase either by binding to the target enzyme through chelation of iron in a metal-containing form of the enzyme or by initially forming an iron chelate of the inhibitors which interacts with the enzyme (9, 11).

In an effort to orient the chelating potential of the formyl thiosemicarbazone side chain positioned alpha to a heteroaromatic ring nitrogen atom to a vulnerable enzymatic site of neoplastic cells other than ribonucleoside diphosphate reductase, several pteridine thiosemicarbazones were synthesized. The pteridine nucleus was selected to direct the formyl thiosemicarbazone portion of the molecule to the enzyme dihydrofolate reductase. Although dihydrofolate reductase has not been shown to be a metal-containing enzyme, Hakala and Suolinna (12) showed that the enzyme from neoplastic cells is inactivated by the metal-chelating agents *o*-phenanthroline and ethylenediaminetetraacetate.

EXPERIMENTAL

Enzymatic Studies—Dihydrofolate reductase was prepared to homogeneity from human liver[1]. Enzymatic activity was assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm. which results from the conversion of NADPH and dihydrofolate to $NADP^+$ and tetrahydrofolate (13).

2,4-Diamino-6-formylpteridine Semicarbazone—2,4-Diaminopteridine-6-carboxaldehyde (0.19 g.) was dissolved in 50 ml. of water containing 1 ml. of concentrated hydrochloric acid. Semicarbazide (0.1 g.) was dissolved separately in 10 ml. of water, and the two solutions were mixed and warmed for 15 min. Sodium acetate (1 g.) was added and the mixture was stirred. The resulting compound was filtered, washed with hot water and ethanol, and dried. The compound did not melt but slowly decomposed above 300°.

For analytical purposes, the compound was isolated as its hydrochloride salt.

Anal.—Calc. for $C_8H_9N_9O \cdot HCl$: C, 33.86; H, 3.17; N, 44.44. Found: C, 33.36; H, 3.12; N, 43.95.

Thiosemicarbazone—The thiosemicarbazone derivative of 2,4-diaminopteridine-6-carboxaldehyde was synthesized as described above except that thiosemicarbazide replaced semicarbazide and was analyzed as its hydrochloride. The compound did not melt but slowly decomposed above 300°.

Anal.—Calc. for $C_8H_9N_9S \cdot HCl$: C, 32.05; H, 3.34; N, 42.07; S, 10.68. Found: C, 31.65; H, 3.30; N, 41.35; S, 10.49.

2-Amino-4-hydroxy-6-formylpteridine Semicarbazone—2-Amino-4-hydroxypteridine-6-carboxaldehyde (0.19 g.) was dissolved in 5 ml. of water by the addition of the minimum amount of 1 *N* sodium

[1] Performed by Dr. David R. Makulu and Dr. Joseph R. Bertino, who donated the enzyme for these studies.

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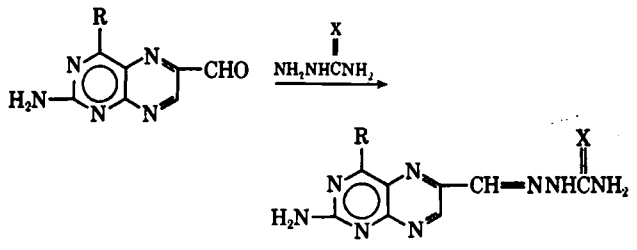

I: R = NH_2, X = O
II: R = NH_2, X = S
III: R = OH, X = O
IV: R = OH, X = S

Scheme I

hydroxide. This solution was added dropwise to a hot solution of semicarbazide (0.1 g.) in 10 ml. of water containing a few drops of acetic acid, and the mixture was heated for a few minutes. The resulting compound was filtered, washed with hot water and ethanol, and then dried. It was purified by dissolving the compound in 1 *N* sodium hydroxide and reprecipitating with dilute acetic acid. The compound did not melt but slowly decomposed above 300°.

Anal.—Calc. for $C_8H_8N_8O_2 \cdot H_2O$: C, 36.09; H, 3.76; N, 42.10. Found: C, 35.71; H, 3.87; N, 42.17.

Thiosemicarbazone—The thiosemicarbazone derivative of 2-amino-4-hydroxypteridine-6-carboxaldehyde was synthesized as described above except that thiosemicarbazide replaced semicarbazide. The compound did not melt but slowly decomposed above 300°.

Anal.—Calc. for $C_8H_8N_8OS$: C, 36.36; H, 3.03; N, 42.42; S, 12.12. Found: C, 36.12; H, 3.21; N, 41.92; S, 11.90.

This paper reports the synthesis of the semicarbazones and thiosemicarbazones of 2,4-diaminopteridine-6-carboxaldehyde and 2-amino-4-hydroxypteridine-6-carboxaldehyde and the measurement of the potency of these compounds as inhibitors of dihydrofolate reductase from human liver. The pteridine aldehydes were synthesized by oxidative cleavage of folic acid (14) and aminopterin[2]; the resultant products were then reacted with semicarbazide or thiosemicarbazide (Scheme I).

RESULTS AND DISCUSSION

A comparison of the relative effectiveness of the pteridine carboxaldehyde thiosemicarbazones as inhibitors of the enzyme dihydrofolate reductase is shown in Table I. The results indicate that the 2,4-diaminopteridine derivatives are more potent than the corresponding 2-amino-4-hydroxypteridine compounds; these findings are in agreement with previous data indicating the requirement for 2,4-diamino groups for optimal inhibition of dihydrofolate reductase by pteridines. The best inhibitor of dihydrofolate reductase in this series was 2,4-diaminopteridine-6-carboxaldehyde semicarbazone (I), which caused 50% inhibition at a concentration of 1.4×10^{-7} *M*. Previous results from this laboratory (8, 15) indicated that the chelating potential of α-*N*-heterocyclic carboxaldehyde thiosemicarbazones is considerably greater than that of the corresponding semicarbazones; therefore, the findings indicate that chelating potential does not significantly enhance the activity of inhibitory pteridines.

[2] S. Kushner, Lederle Laboratories, Pearl River, N. Y., personal communication.

Table I—Effect of Pteridine Carboxaldehyde Thiosemicarbazones on Dihydrofolate Reductase Activity of Human Liver[a]

Compound	ID_{50}, *M*
2,4-Diaminopteridine-6-carboxaldehyde semicarbazone (I)	1.4×10^{-7}
2,4-Diaminopteridine-6-carboxaldehyde thiosemicarbazone (II)	7.5×10^{-7}
2-Amino-4-hydroxypteridine-6-carboxaldehyde semicarbazone (III)	1×10^{-6}
2-Amino-4-hydroxypteridine-6-carboxaldehyde thiosemicarbazone (IV)	1×10^{-6}

[a] Inhibitors were added to the enzymic reaction mixture in 10% dimethyl sulfoxide. Enzyme activity of controls as measured by the decrease in absorbance at 340 nm. was 0.187 absorbance unit/5 min. A concentration of 1.8×10^{-5} *M* methotrexate was required to inhibit enzyme activity by 50%.

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Isoxazoles I: Protonation of Isoxazole Derivatives in Aqueous Sulfuric Acid

RUBEN H. MANZO* and M. MARTINEZ de BERTORELLO▲

Abstract □ The protonation of 3-amino-5-methylisoxazole, 5-amino-3,4-dimethylisoxazole, sulfamethoxazole (*N*-sulfanilamido-5-methyl-3-isoxazole), and sulfisoxazole (*N'*-sulfanilamido-3,4-dimethyl-5-isoxazole) in aqueous sulfuric acid was studied. The pK_{SH^+} and $\log SH^+/S$ or $\log SH_2^{+2}/SH^+$ values were determined by UV spectrophotometry. The data were treated by the acidity function method and by the method of linear free energy relationships. The comparison of the correlations obtained and the basicity and the UV spectra of the four compounds indicate that the process of protonation is the same in the amino and sulfonamide derivatives.

Keyphrases □ Isoxazole derivatives—protonation in sulfuric acid, ionization ratios, log *I* correlated with Hammett's *Ho* function, UV spectrophotometry □ Sulfisoxazole—protonation in sulfuric acid, ionization ratios, log *I* correlated with Hammett's *Ho* function, UV spectrophotometry □ Sulfamethoxazole—protonation in sulfuric acid, ionization ratios, log *I* correlated with Hammett's *Ho* function, UV spectrophotometry

It is well known that the derivatives of 3- and 5-aminoisoxazoles are found in their protonated form in mineral acid solutions. After the pKa values for these compounds were determined, the protonation site of the heterocycle was found to be the nitrogen atom, according to UV spectroscopic studies (1).

Similar studies on the sulfonamide derivatives have not been done, nor are there any data about the behavior of the ionization ratio *I* (SH^+/S or SH_2^{+2}/SH^+) in various conditions of acidity in the medium for the four compounds studied.

This paper reports studies of the basicity and behavior of the ionization ratio *I* of these derivatives in connection with the study of the isoxazole ring cleavage in mineral acid solutions (2).

The fact that amino- and sulfonamidoisoxazoles are cleaved at the oxygen–nitrogen bond in hydrolytic reactions catalyzed by acids (3) and the importance in this type of reaction in determining the equilibrium existing between the basic substrate and the hydrogen ions of the medium led to a comparative investigation of the protonation equilibrium for these substrates. The equilibria considered were $S + H^+ \rightleftharpoons SH^+$ for the amino derivatives and $SH^+ + H^+ \rightleftharpoons SH_2^{+2}$ for the sulfonamido derivatives.

The determinations of the ionization ratio *I* were done using UV spectrophotometry by the indicator method (4), utilizing the difference in the spectra of the basic forms and their respective protonated forms. The data were evaluated by the Davis and Geissman (5) method for 5-amino-3,4-dimethylisoxazole (II) and the Alder *et al.* (6) method for the remaining compounds. The acidity function method (7) was used to correlate the data of log *I* with Hammett's *Ho* function (plots of log *I* against $-Ho$). Correlations by the linear free energy method (8) were carried out for the sulfonamide derivative {plots of $-(\log I + Ho)$ or $-(\log I - \log [H^+])$ against $-(\log [H^+] + Ho)$}.

EXPERIMENTAL[1]

Materials and Apparatus—Sulfisoxazole[2] (IV) was a commercial USP product recrystallized from ethanol, m.p. 193–194°. Sulfamethoxazole[3] (III) was a commercial NF product recrystallized from ethanol–water (1:1), m.p. 165.5–166.5°. 5-Amino-3,4-dimethylisoxazole (II) was prepared according to the method described (9) for other sulfonamides. Ten grams of sulfisoxazole was dissolved in 200 ml. glacial acetic acid and 25 ml. of acetic anhydride. The solution was boiled for 5 min., cooled, and diluted with 500 ml. of distilled water. A precipitate was formed, which was separated, dried, and subsequently solubilized in 25 ml. of 40% (w/v) sulfuric acid and boiled for 3 min.; the solution was then cooled and alkalinized with a sufficient amount of 10% sodium hydroxide solution. This solution was extracted three times with 40 ml. of ether; the ethereal extracts, once combined and evaporated, yielded 3.2 g. of II, which was recrystallized first from benzene and then from water, m.p. 123.5–124.5°.

3-Amino-5-methylisoxazole (I) was prepared in the same way and purified by successive vacuum sublimations up to a constant melting point of 60–61°[4].

Preparation of Solutions—The solutions of the purified compounds were prepared in distilled water (I and II) and in 1 *N* sulfuric acid (III and IV). The concentrations were designed so that when 1.0 ml. of the solution was added to a 50-ml. volumetric flask filled to the mark with sulfuric acid of known concentration, the final absorbance would be 0.2–0.8.

The blank solutions were prepared in the same way as the sample solutions; but 1 ml. of water was added instead of I or II, and 1 ml. of 1 *N* sulfuric acid was added instead of III or IV.

Complete absorption spectra of III and IV were recorded over 205–300 nm. Table I shows acidity conditions and characteristics of the spectra of these compounds. In all cases a substantial change in absorption occurred when acidity conditions were modified. After diluting the 22 *N* and 24 *N* acidic solutions of III and IV with distilled water, new spectra were obtained which were similar to those corresponding to lower acidity conditions (8 *N*).

The wavelengths used for quantitative measurements were selected from comparative analysis of the spectra taken in extreme acidity conditions. For II, wavelengths corresponding to a maximum of the basic and protonated forms were selected; Table I shows the respective absorptivities. For the remaining compounds, five wavelengths were selected, taken symmetrically at the maximum band of the protonated form for I and the biprotonated form for III and IV (Table I).

Processing of Data—The Davis and Geissman (5) correction treatment for effects of the medium on II was used to assess the values of *I* obtained by the indicator method (4). The method[5] of Alder *et al.* (6) was applied to the remaining compounds.

[1] Melting points were determined in a melting-point apparatus devised by Dr. Tottoli. The values given are uncorrected. Spectrophotometric readings (UV) were made in a Hilger & Watts photoelectric spectrophotometer. The complete UV absorption spectra were taken in a Cary 14 recording spectrophotometer.

[2] Preferred USAN chemical name is N^1-(3,4-dimethyl-5-isoxazolyl)-sulfanilamide.

[3] Preferred USAN chemical name is N^1-(5-methyl-3-isoxazolyl)sulfanilamide.

[4] Sulfuric acid (Carlo Erba G.R.) (96%, d 1.84) product was used.

[5] In every case, experimental data that were correlated were adjusted by the method of least squares, giving them all the same statistical weight.

Table I—UV Spectra of Isoxazole Derivatives in Water and in Sulfuric Acid

Substrate	Water λ	Water ε	Sulfuric Acid 2 N λ	Sulfuric Acid 2 N ε	Sulfuric Acid 8 N λ	Sulfuric Acid 8 N ε	Sulfuric Acid 24 N λ	Sulfuric Acid 24 N ε	Sulfuric Acid 22 N λ	Sulfuric Acid 22 N ε	λ Used[a], nm.
I[b]	209[c]	6820	230[c]	8040	—	—	—	—	—	—	228.5, 231, 233.5, 236, 238.5
II[b]	244[c]	8230	263[c]	13,800	—	—	—	—	—	—	224, 263[d]
	263[e]	5230	244[e]	3990	—	—	—	—	—	—	—
III	—	—	—	—	218[c]	13,600	238[c]	18,700	—	—	233, 235.5 238,
	—	—	—	—	238[e]	5950	—	—	—	—	240.5, 243
IV	—	—	—	—	220[c]	—	—	—	220[c]	—	—
	—	—	—	—	264[e]	2690	—	—	264[e]	11,400	257, 260.5, 264, 267.5, 271

[a] Wavelengths used for ionization ratio *I* determination. [b] λ_{max} and absorptivity were taken from *Reference 1*. [c] λ_{max}. [d] Ionization ratio determined by the Davis and Geissman (5) method. [e] Absorptivity at λ_{max} for the protonated form.

Table II—Correlations of log *I* *versus* $-Ho$ (Acidity Function) and (log *I* $-$ log [H^+]) *versus* $-$(log [H^+] + *Ho*) (Linear Free Energy Relationship)

Substrate	Acidity Function Plots: Slope	Half-Protonated	*r*[a]	Linear Free Energy Relationship: Slope ϕ	pK_{SH^+}	*r*	pK_{SH^+}[b]
I	0.86	0.46	0.995	—	—	—	00.47
II	1.02	1.03	0.998	—	—	—	1.16
III	0.90	−3.81	0.997	0.12	−3.52	0.996	—
IV	1.07	−3.33	0.992	−0.08	−3.50	0.990	—

[a] *r* is the regression coefficient. [b] The pK_{SH^+} determined by the spectrophotometric method (1).

Log *I* values obtained in this way were correlated with Hammett's *Ho* acidity function using values given by Bascome and Bell[9] (10) for I and II and Ryabova *et al.* (11) for III and IV. Table II gives parameters obtained from all correlations made. The value of *Ho* corresponding to the protonation of half of the compound is given in all cases.

RESULTS AND DISCUSSION

The UV absorption spectra of the protonated forms of I, II, and III show a bathochromic shift of 20 nm. and an increase of the absorptivity with respect to the spectrum of the basic forms. With IV, the shift cannot be seen due to the existence of a strong absorption band with λ_{max} 220 nm.; however, the appearance of a band with λ_{max} 264 nm. in 22 *N* sulfuric acid can be observed, similar to that of the corresponding aminoisoxazole II.

The plot of log *I* against $-Ho$ for I is linear with a 0.86 slope. Consequently, the protonation process does not follow Hammett's acidity function, and the value of the intercept is not representative of the thermodynamic pK_{SH^+} (12). The *Ho* value corresponding to the protonation halfway through the titration as a measure of the basicity of the compound is given in Table II. Similar behavior was observed with III.

For II, log *I* against $-Ho$ is a linear plot with unitary slope (1.02). The compound behaves like a Hammett base, and it is approximately accurate to take log *I* value when *Ho* is extrapolated to zero as the pK_{SH^+}, because it satisfies the acidity function method postulates. Compound IV behaved in a similar manner, although the value of the slope (1.07) was somewhat above one.

Compounds III and IV both possess an aromatic primary amino group which, in the acidity conditions of these determinations, are completely in the protonated form; thus log *I* measurements correspond to a second protonation. Some research has been done on protonation of positively charged bases (13–17). On the whole, the behavior of this type of compound in media of considerable ionic strength does not differ appreciably from the behavior of neutral bases of a similar structure; nevertheless, the correlations made with *Ho* by the acidity function method are subject to the same limitations as the correlations having unitary slope. The correlation process by the linear free energy relationship method avoids these limitations, supplying a treatment that is applicable to any type of base (8, 18).

The linear free energy relationship method was applied to III and IV; Table II lists the pK_{SH^+} values obtained. Protonation of I and II cannot be determined by this method.

The similarities existing in the changes in the UV spectra as a consequence of protonation when I is compared with III and when II is compared with IV might indicate that the nature of the protonation process is the same in amino and sulfonamido derivatives. This similarity in behavior can also be observed when comparing slopes obtained by the acidity function method between I (0.86) and III (0.90) and between II (1.02) and IV (1.07).

On the other hand, ΔHo between half-protonation of I and II is 0.57, a comparable value to ΔHo between half-protonation of III and IV (0.48); ΔHo between I and III (4.27) is comparable to ΔHo between II and IV (4.36).

If the 3-amino and 3-sulfonamido derivatives are compared to the 5-amino and 5-sulfonamido derivatives, differences between slope values are outstanding (0.86, 0.90, and 1.02, 1.07, respectively). At present, considering the small number of derivatives studied, it would be rash to conclude that structural differences between 3-sulfonamido and 5-sulfonamido derivatives are the factors conducive to different relationships between activity coefficients ($\gamma SH^+/\gamma S$ or $\gamma SH_3^{+2}/\gamma SH^+$) for both types of derivatives.

[9] To interpolate percent w/w values of sulfuric acid used in the determinations and to obtain the respective *Ho* values given by Bascome and Bell, given the curvature that presents *Ho* functions in that zone, this function was transformed into *ho* (*Ho* = − log *ho*); it was proven that a more linear relationship exists between percent w/w sulfuric acid and *ho* up to a concentration of 5–10% (w/w) than with *Ho*.

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Isoxazoles II: Kinetics and Mechanism of Degradation of Sulfisoxazole in Moderately Concentrated Acids

RUBEN H. MANZO* and M. MARTINEZ de BERTORELLO▲

Abstract □ The acid degradation of sulfisoxazole (*N*-sulfanilamido-3,4-dimethyl-5-isoxazole) in hydrochloric acid solutions in concentrations from 10 to 24% and at 108 ± 0.1° was studied. Two parallel pathways of molecular degradation which lead simultaneously to the formation of sulfanilic acid (pathway A) and sulfanilamide (pathway B) as final products were determined. The isoxazole ring is degraded as much in the sulfisoxazole molecule (pathway B) as in the 5-amino-3,4-dimethylisoxazole (pathway A), leading to formation of 2-butanone and hydroxylamine (and ammonia in the latter case) as final products. Both degradative pathways show pseudo-first-order kinetics. A study was also made of how the reaction rates are affected with the acid concentration. Degradative pathway B prevails at low acid concentrations and can be associated with a mechanism in which water participates in the rate-determining step; degradative pathway A prevails at higher acid concentrations and the mechanism implies nonparticipation of water in the rate-determining step.

Keyphrases □ Sulfisoxazole—kinetics and mechanism of acid degradation, ionization ratios □ Isoxazole derivatives—kinetics and mechanism of acid degradation of sulfisoxazole, ionization ratios □ Degradation—sulfisoxazole in hydrochloric acid solutions, rates, mechanisms, ionization ratios

The isoxazoles form a group of heterocycles that may be characterized by their chemical behavior; they exhibit properties that are typical of aromatic systems and also, under certain conditions, show ring lability, particularly at the oxygen–nitrogen bond (1).

At present, more attention is paid to the investigation of the mechanism of chemical reactions under extreme conditions (high and low temperatures, high and low pressures, effects of radiation, *etc.*); available data show that under such conditions the course of reactions often becomes uncommon.

Based on these findings and on some experimental data obtained in this laboratory, it appeared interesting to perform a thorough study of the cleavage reactions of the isoxazole ring in strong acid solutions and at high temperatures. These studies may contribute more basic information on the stability of the ring, especially since data related to the problem are quite scarce (1). Moreover, since numerous derivatives of the isoxazole ring have pharmacological properties (2), the importance of the conditions and mechanism by which these heterocycles are degraded is greatly increased.

This paper presents the results of a study of the influence of hydrochloric acid concentrations and high temperatures on the hydrolytic degradation of one isoxazole derivative: sulfisoxazole[1] (*N*-sulfanilamido-3,4-dimethyl-5-isoxazole).

EXPERIMENTAL[2]

Materials—All of the solvents used were commercial products, purified by the usual techniques. Hydrochloric acid, sulfuric acid, and sodium β-naphthoquinone-4-sulfonate were analytical[3] reagent grade. 2,4-Dinitrophenylhydrazine[4] was recrystallized from methanol. Sulfisoxazole USP[5] was recrystallized from ethanol, m.p. 193–194°. 5-Amino-3,4-dimethylisoxazole was prepared according to the method described (3) for other sulfonamides: 10 g. of sulfisoxazole was dissolved in 200 ml. of glacial acetic acid and 25 ml. of acetic anhydride. The solution was boiled for 5 min., cooled, and diluted with 500 ml. of distilled water. A precipitate

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Abstract □ The acid degradation of sulfisoxazole (*N*-sulfanilamido-3,4-dimethyl-5-isoxazole) in hydrochloric acid solutions in concentrations from 10 to 24% and at 108 ± 0.1° was studied. Two parallel pathways of molecular degradation which lead simultaneously to the formation of sulfanilic acid (pathway A) and sulfanilamide (pathway B) as final products were determined. The isoxazole ring is degraded as much in the sulfisoxazole molecule (pathway B) as in the 5-amino-3,4-dimethylisoxazole (pathway A), leading to formation of 2-butanone and hydroxylamine (and ammonia in the latter case) as final products. Both degradative pathways show pseudo-first-order kinetics. A study was also made of how the reaction rates are affected with the acid concentration. Degradative pathway B prevails at low acid concentrations and can be associated with a mechanism in which water participates in the rate-determining step; degradative pathway A prevails at higher acid concentrations and the mechanism implies nonparticipation of water in the rate-determining step.

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was formed, which was separated, dried, and subsequently solubilized in 25 ml. of 40% (w/v) sulfuric acid and boiled for 3 min. The solution was cooled, alkalinized with 10% sodium hydroxide solution, and extracted with ether (3 × 40 ml.). The ethereal extracts yielded 3.2 g. of 3,4-dimethyl-5-aminoisoxazole, which was recrystallized first from benzene and then from water, m.p. 123.5–124.5°.

Qualitative Identification of Degradation Products of Sulfisoxazole—*Sulfanilic Acid, Sulfanilamide, 5-Amino-3,4-dimethylisoxazole, and 2-Butanone*—A solution of 0.045 mole (1.200 g.) of sulfisoxazole in 100 ml. of hydrochloric acid (24%) was boiled at reflux for 100 min. Aliquots were taken every 10 min. of the reaction and were immediately cooled in an ice bath.

Sulfanilic Acid and Sulfanilamide—Approximately 5 μl. of each reaction sample was spotted on a 20 × 20-cm. silica gel[6] TLC plate. The chromatogram was developed with heptane–chloroform–ethanol–glacial acetic acid (2:2:3:1). The spots were detected by spraying with a 1% sodium nitrite in 0.5 *N* hydrochloric acid solution and coupling the diazo compounds formed with a 0.5% *N*-naphthylethylenediamine dihydrochloride[7] aqueous solution. The spot corresponding to sulfisoxazole (R_f 0.87) faded progressively and was not detectable in approximately 80 min. Two spots (R_f 0.72 and 0.42) appeared simultaneously, and their intensities increased progressively with reaction time. They were identified by comparison with known standard samples and were found to be sulfanilamide and sulfanilic acid, respectively.

5-Amino-3,4-dimethylisoxazole—Twenty-five milliliters of the solution hydrolyzed during 20 min. was alkalinized with 20% sodium hydroxide solution; three ether extractions (20 ml. each) were performed on the alkaline solution. The ethereal extracts were combined and dried with anhydrous sodium sulfate. Evaporation of the solvent yielded a solid, which was recrystallized from water and identified as 5-amino-3,4-dimethylisoxazole by a comparison of its melting point and mixed melting point with those of a known pure sample. Comparison of its R_f value (0.83) in TLC on a silica gel G plate, using heptane–chloroform–ethanol (2:2:3) as a developing solvent and a hydrochloric acid solution of *p*-dimethylaminobenzaldehyde as a spray reagent, resulted in further identification.

2-Butanone—To the rest of the solution, hydrolyzed during 100 min., a sodium bicarbonate solution was carefully added until it was neutralized. This solution was distilled, collecting approximately one-third of the original volume in a conical flask which contained a cooled solution of 2,4-dinitrophenylhydrazine in hydrochloric acid. A yellow precipitate was formed, which was separated, dried, and recrystallized from ethanol. It was identified as the 2,4-dinitrophenylhydrazone of the 2-butanone by comparison of its melting point, mixed melting point, and IR spectra with those of an authentic sample.

Qualitative Identification of Degradation Products of 5-Amino-3,4-dimethylisoxazole—*2-Butanone, Ammonia, and Hydroxylamine*—A solution of 7.5 mmoles (0.840 g.) of 5-amino-3,4-dimethylisoxazole in 150 ml. of 24% hydrochloric acid was refluxed for 60 min.

2-Butanone—One hundred milliliters of this solution was neutralized carefully by addition of a 20% solution of sodium bicarbonate. The neutralized solution was distilled, and one-third of the original volume was collected (this volume ensured the complete distillation of the 2-butanone formed) in a cooled 2,4-dinitrophenylhydrazine solution in hydrochloric acid. The precipitate was separated and recrystallized from ethanol and identified as the 2,4-dinitrophenylhydrazone of the 2-butanone by a comparison of its melting point, mixed melting point, and IR spectra with those of an authentic sample.

Ammonia—Thirty milliliters of the hydrolyzed solution, alkalinized with 20% sodium hydroxide solution and heated in a water bath, gave off vapors which changed the color of red litmus paper and had a typical ammonia odor.

Hydroxylamine—One milliliter of 40% potassium hydroxide solution was added to 1 ml. of the hydrolyzed solution together with 3 drops of ethyl acetate and then 0.4 ml. of the same potassium hydroxide solution. After standing for 10 min., 1 ml. of a 7.5% hydrochloric acid solution and 2 drops of ferric chloride solution were added. This produced a reddish-violet color typical of ferric hydroxamate complexes. The test proved negative when the ester was not added. These results indicate the formation of hydroxylamine during hydrolysis.

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[7] Carlo Erba R.P.

Table I—Pseudo-First-Order Rate Constants for Sulfisoxazole Hydrolysis in 24% (6.47 *M*) Hydrochloric Acid Solution and at 108 ± 0.1°

Reaction Time, min.	*A*(Average)	$\log A_0 - \log A$	$K_\psi \cdot 10^{-3}$ sec.$^{-1}$
0	0.355	0.000	
5	0.232	0.184	1.41
10	0.158	0.351	1.35
15	0.098	0.559	1.43
25	0.042	0.927	1.42
		Average K_ψ: $14.0 \cdot 10^{-4}$ seg.$^{-1}$	

Quantitative Determinations and Kinetic Study—Hydrolysis rates in hydrochloric acid solutions of 24, 15, and 10% (w/v) concentrations at 108 ± 0.1° were studied.

Sulfisoxazole solutions were prepared by quantitatively transferring 5-mmole quantities to a volumetric flask of 100-ml. capacity and adding the hydrochloric acid solution until the volume was completed. The same procedure was followed with each acid solution.

Once the solutions were prepared, equal aliquots of 3 ml. were introduced in a series of 10-ml. ampuls. The ampuls were sealed and immersed in a constant-temperature bath. They were then withdrawn at appropriate intervals and quickly cooled in an ice bath for analysis at a later time.

Degradation of Sulfisoxazole—*Determination of Sulfisoxazole, Sulfanilamide, and Sulfanilic Acid*—All three compounds were separated by TLC using 20 × 20-cm. silica gel F_{254} PL chromatofolios[8]. Upon development the samples were evaluated using the Bratton–Marshall colorimetric technique (4) adapted to the present operating conditions. Determinations were performed in triplicate.

Three aliquots of 5 μl. each were taken from each ampul, using a 10-μl. graduated micropipet. The chromatogram was developed with a solvent system of heptane–chloroform–ethanol–glacial acetic acid (2:2:3:1) to 12 cm. The spots were localized by observing the plate under UV light and delineating the respective areas with a stylet, taking care to preserve a constant surface for each compound.

The areas were separated independently. The sulfisoxazole fraction was extracted with 10% hydrochloric acid (3 × 2 ml.), suspending and centrifuging the material each time. Sulfanilic acid was extracted in the same way but hot. Sulfanilamide was extracted at room temperature using 1 ml. of hydrochloric acid solution in each extraction[9].

For each component, the elution liquids were transferred and collected in a test tube where the Bratton–Marshall colorimetric technique was carried out as follows. Three milliliters[10] of 0.1% sodium nitrite aqueous solution was added to 6 ml. of eluate, mixing both solutions thoroughly. After 3 min. a further 3 ml. of 0.5% sulfamic acid aqueous solution and 4 ml. of 0.1% *N*-naphthylethylenediamine dihydrochloride aqueous solution were added. The absorbance of the colored solution that formed was determined

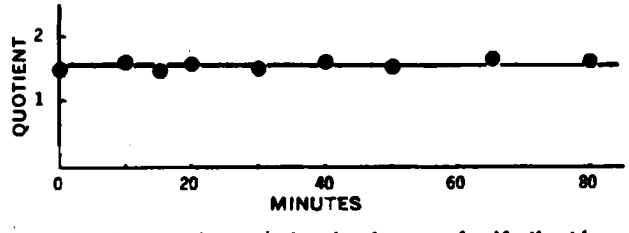
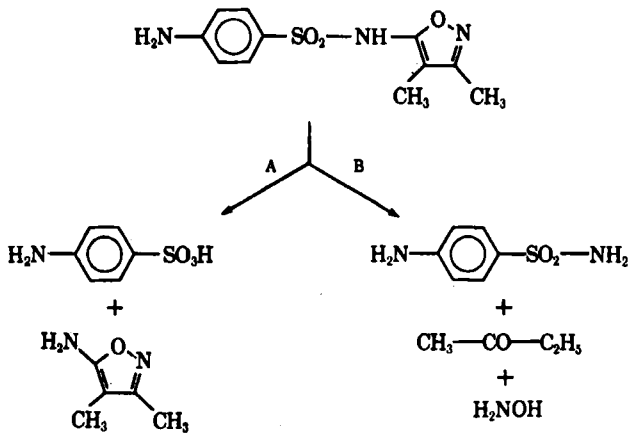


Figure 1—*Quotient between the absorbances of sulfanilamide and sulfanilic acid during sulfisoxazole hydrolysis.*

[8] Merck.
[9] It was determined through a previous test with pure samples that the extraction of the three components in the way shown is quantitative.
[10] Half of each reagent was added in the determination of sulfanilamide.



Scheme I

in a spectrophotometer at 545 nm. against a blank with all of the reagents. The final result for each compound at a definite time was taken from the average of the three determinations.

From the values obtained it was possible to calculate the pseudo-first-order constant K_ψ for the degradation of sulfisoxazole, which was calculated according to Eq. 1:

$$K_\psi = \frac{(\log A_0 - \log A) \cdot 2.303}{t} \qquad \text{(Eq. 1)}$$

where A_0 is the absorbance corresponding to the solution at $t = 0$, and A is the absorbance of a hydrolyzed solution during time t; $t = 0$ was the absorbance of a solution contained in an ampul which was submerged in the thermostatically controlled bath for 2 min. Table I shows the procedure used to calculate K_ψ for the degradation of sulfisoxazole in 24.5% hydrochloric acid. The values of K_ψ in 15 and 10% hydrochloric acid solutions were calculated using the same procedure.

Sulfanilamide and sulfanilic acid determinations made possible the calculation of the quotient between the absorbance of both compounds during hydrolysis of sulfisoxazole in 24.5% hydrochloric acid (Fig. 1). Sulfanilamide and sulfanilic acid concentrations were calculated in relation to a standard solution prepared as follows:

1. Sulfanilic acid, 191.9 mg. (1.107 mmoles), was introduced quantitatively in a volumetric flask of 50-ml. capacity and diluted to volume with a 24.5% hydrochloric acid solution.

2. A solution was prepared in the same way with 109.5 mg. (0.631 mmole) of sulfanilamide.

Both solutions were mixed in a 1:1 proportion and three aliquots were made with this solution on a TLC plate. Separation and determination were carried out exactly the same as when the problem samples were determined. The results were the following: microliters spotted, 5.0; sulfanilic acid absorbance, 0.170, 0.174, and 0.174; and sulfanilamide absorbance, 0.207, 0.202, and 0.207.

Calculations of the proportions of sulfanilamide and sulfanilic acid formed during hydrolysis were made relating the absorbances of the same samples to the absorbances determined with the standard solution of known concentration.

The fact that the absorbances produced by both compounds remained constant throughout the whole process of hydrolysis made possible the determinations of their final percentages without requiring the reaction to be carried out completely.

The proportions of sulfanilic acid and sulfanilamide were calculated through the relationship:

$$\frac{A_T}{C_T} = \frac{A_P}{C_P} \qquad \text{(Eq. 2)}$$

where A_T and A_P are the absorbances of the standard solution and the problem, respectively; and C_P and C_T are the problem and the reference concentrations, respectively. The results were 29 moles% sulfanilamide and 71 moles% sulfanilic acid. These results permitted the estimation of the pseudo-first-order rate constants for the formation of sulfanilamide and sulfanilic acid through the following relationships:

$$K_\psi \text{ sulfisoxazole} \cong K_\psi \text{ sulfanilamide} + K_\psi \text{ sulfanilic acid} \qquad \text{(Eq. 3)}$$

$$\frac{K_\psi \text{ sulfanilamide}}{K_\psi \text{ sulfanilic acid}} = \frac{29}{71} \qquad \text{(Eq. 4)}$$

Table II—Pseudo-First-Order Rate Constants for Sulfisoxazole Hydrolysis and the Formation of Sulfanilamide and Sulfanilic Acid at Three Different Hydrochloric Acid Concentrations

HCl, moles/l.	$K_\psi \cdot 10^{-4}$ seg.$^{-1}$, Sulfisoxazole Degradation	Proportions, —moles%— Sulfanilamide	Sulfanilic Acid	—$K_\psi \cdot 10^{-4}$ seg.$^{-1}$— Sulfanilamide	Sulfanilic Acid
6.47	14.0	29	71	4.1	9.9
4.10	2.4	49	51	1.2	1.2
2.74	1.1	59	41	0.65	0.45

Determination of 5-Amino-3,4-dimethylisoxazole during Sulfisoxazole Hydrolysis—Folin and Wu's colorimetric technique (5), adapted to the reaction system, was used. Samples were taken at adequate reaction times as described previously; accurately measured aliquots of these samples were diluted with distilled water in volumetric flasks of adequate capacity at concentrations of 1/50, 1/100, and 1/150. Then 1 ml. of each diluted sample was neutralized to phenolphthalein with potassium hydroxide solution[11] (a parallel tube was used to calculate the necessary quantity so as not to introduce phenolphthalein into the system). Then 2 ml. of 0.1 *M* phosphate buffer solution of pH 11.6 was added to the neutralized solutions, as well as 2 ml. of 0.13% sodium β-naphthoquinone-4-sulfonate; the solutions were shaken and allowed to stand for 2 min. Subsequently, 1.2 ml. of 7.5% hydrochloric acid solution was added and the colored substance that formed was extracted with 5.0 ml. *n*-hexane. The absorbances were determined at 450 nm. against a pure solvent blank.

Concentrations of 5-amino-3,4-dimethylisoxazole were calculated from a standard solution prepared with pure sample in adequate concentration in 24.5% hydrochloric acid solution, applying the procedure described for the problem. The system obeyed Beer's law in the concentration range used. The results of these determinations are shown in Fig. 2.

The extractions with *n*-hexane offer a specific method for the determination of 5-amino-3,4-dimethylisoxazole in the presence of hydroxylamine, ammonia, sulfisoxazole, sulfanilamide, and sulfanilic acid; these compounds also react with the reagent, but the compounds they form cannot be extracted by the solvent, which selectively extracts the colored substance formed between the reagent and 5-amino-3,4-dimethylisoxazole.

Determination of Carbonyl Groups—The opening of the isoxazole ring in aqueous solutions implies the formation of compounds having carbonyl groups. They were determined using a colorimetric method adapted from the one proposed by Clarck and Lappin (6), which consists of allowing 2,4-dinitrophenylhydrazine to react with the compound to be determined and measuring the color yielded by the 2,4-dinitrophenylhydrazones in an alkaline medium.

The procedure used was as follows. One milliliter of distilled water and 0.1–1 ml. of hydrolyzed acid solution containing the compound to be determined were placed in a test tube, and this

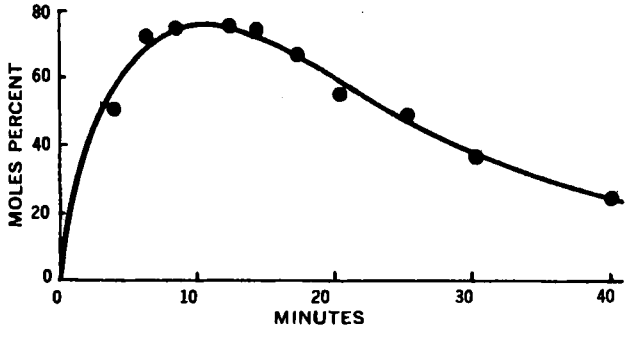
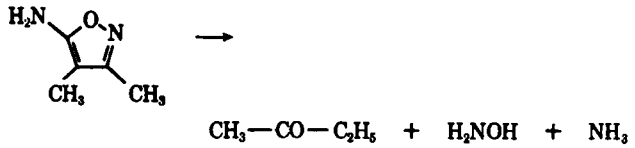
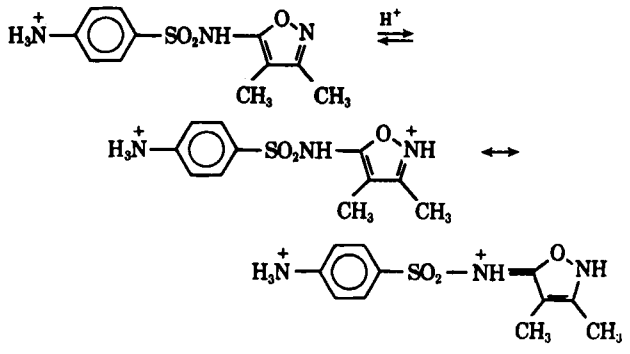


Figure 2—*Formation and degradation of 5-amino-3,4-dimethylisoxazole during sulfisoxazole hydrolysis.*

[11] Prepared by diluting in the same way as the sample from the 40% potassium hydroxide solution.



Scheme II



Scheme III

solution was completed to 2 ml. with 24.5% hydrochloric acid solution where necessary. Later, 5.0 ml. of methanolic solution saturated with 2,4-dinitrophenylhydrazine was added and the tubes were heated to 50° for 30 min. in a water bath. After cooling, 10 ml. of 10% sodium hydroxide solution was added and the resulting solution was quantitatively transferred to a 50-ml. volumetric flask, diluting to volume with methanol. The absorbance was determined at 480 nm. against a blank with all of the reagents; these were prepared according to the recommendations already indicated (6).

The concentration of the carbonyl groups formed was calculated from a standard curve prepared by means of a series of determinations made on a solution of 44.76 mmoles/l. of pure 2-butanone.

The determination of the carbonyl groups in a sulfisoxazole solution hydrolyzed during 140 min. yielded the following results: initial concentration of sulfisoxazole (moles per liter), 4.47×10^{-3}; and concentration of carbonyl groups in hydrolyzed solution, 4.42×10^{-3}. The results indicate that 1 mole of sulfisoxazole leads to the formation of 1 mole of the carbonyl groups.

Influence of Acid Concentration on Reaction Rates—A determination was made of the pseudo-first-order rate constants for the degradation of sulfisoxazole and the proportions of the final products, sulfanilamide and sulfanilic acid, at three different acid concentrations. Table II shows these results.

DISCUSSION

Experimental results indicate that, in the degradation of sulfisoxazole by the action of moderately concentrated hydrochloric

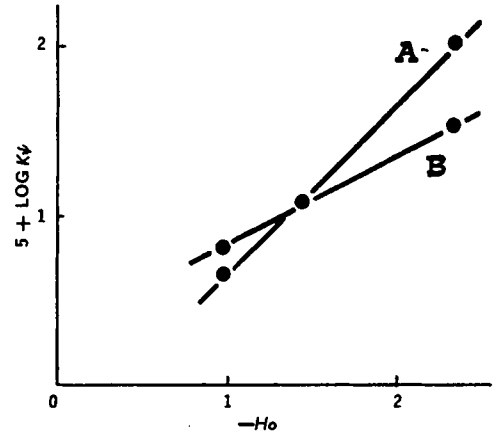


Figure 3—*Correlation between log* K_ψ *and* −Ho *for degradative pathways A and B.*

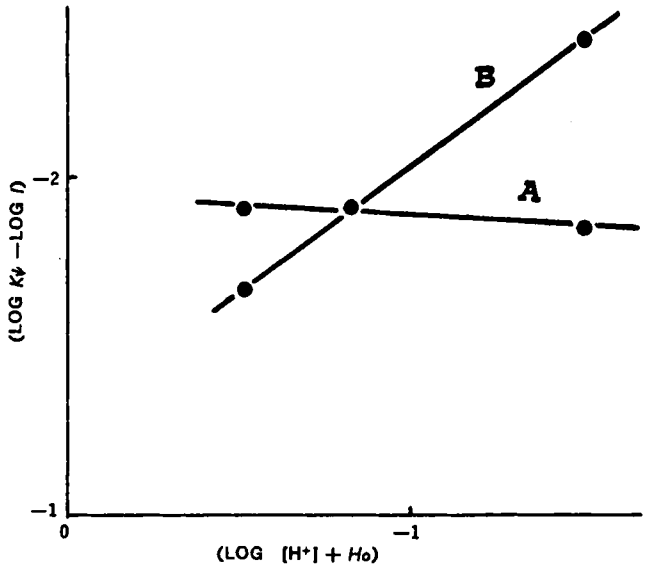


Figure 4—*Correlations between log* K_ψ − *log* I *and* (Ho + *log*[H^+]) *or degradative pathways A and B.*

acid at 108°, two degradative pathways (Scheme I) coexist in the concentration range studied. Degradative pathway A implies hydrolytic cleavage of the molecule at the sulfonamide group bond and is common to all sulfonamides. Degradative pathway B implies the opening of the isoxazole ring and leads to the formation of sulfanilamide as one of the final products.

Von Ernst and Seydel (7) studied the hydrolysis of sulfisoxazole in 1 *N* hydrochloric acid within the 50–90° temperature range and postulated one single degradative way, which agrees with pathway A proposed in the present paper.

An opening of the ring also takes place in the 5-amino-3,4-dimethylisoxazole, formed in degradative pathway A, similar to that which takes place in the sulfisoxazole molecule in degradative pathway B (Scheme II).

These degradative processes can be associated with a mechanism of acid catalysis which implies a preequilibrium between the substrates and protonated substrates (Scheme III). Sulfisoxazole possesses an aromatic primary amino group which, under reaction conditions, is in its complete protonated form; the respective activated states for the two degradative pathways can be associated with a second protonation in the isoxazole ring (8).

To study the influence of acidity changes on the velocity of the two degradative pathways, the ionization ratio *I* between diprotonated and monoprotonated forms of sulfisoxazole for the three concentrations of hydrochloric acid used was determined. Calculations were made starting from the regression line of the linear free energy relationships correlation for the second sulfisoxazole protonation (8) using values of *Ho*[12,13]. This implies taking the behavior of sulfisoxazole ionization in sulfuric acid as a reference.

To recognize how the rates of reaction for pathways A and B are affected when acidity conditions vary, velocity coefficients were correlated by the classical Zucker and Hammett method (9) (log K_ψ against $-Ho$) and by the treatment proposed by Bunnett and Olsen (10) which correlates (log K_ψ − log *I*) with (log [H^+] + *Ho*).

Both treatments supply linear correlations for the two degradative pathways. Correlation slopes between log K_ψ and $-Ho$ were 1.01 and 0.52 for A and B, respectively (Fig. 3). The values of ϕ parameter which appear from correlations between (log K_ψ − log *I*) and (log [H^+] + *Ho*) were 0.05 and 0.74 for A and B, respectively (Fig. 4).

The behavior of pathway B with the variations in acidity conditions, interpreted according to the Bunnett and Olsen (10) criterion, suggests the participation of water in the rate-determining step;

[12] *Ho* values for hydrochloric acid solutions were taken from the scale given by Paul and Long, *Chem. Rev.*, 57, 1(1957).

[13] Part I (8) discusses the utilization of *Ho* in the treatment of the equilibrium of a biprotonated compound.

in the A degradative process with ϕ value near zero and a 1.01 slope in plots of (log K_ψ against $-Ho$), water would not participate in the rate-determining step, displaying a typical A-1 mechanism according to Zucker and Hammett (9). Therefore, degradative pathway B prevails at low acid concentrations and A prevails at high acid concentrations.

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(5) *Ibid.*, pp. 423–425.

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▲ To whom inquiries should be directed.

New Compounds: *N,N*-Dimethyl-[3-(1-alkylpiperidyl)]carbamates, Potential Cholinesterase Inhibitors

IAN W. MATHISON▲, KATHLEEN C. FOWLER, and ELIZABETH R. PETERS

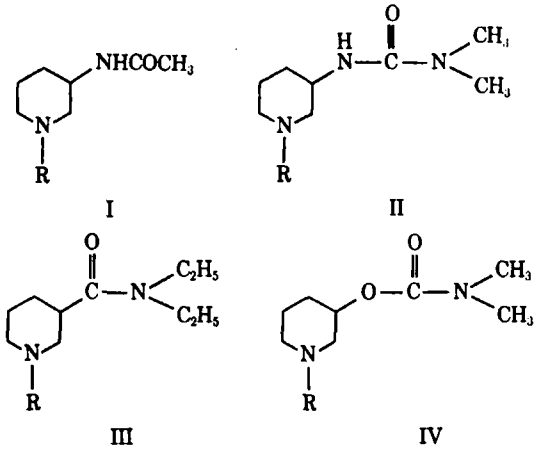
Abstract □ The synthesis of 10 piperidine carbamates was carried out by condensation of various 3-hydroxypiperidines with dimethylcarbamyl chloride or, alternatively, by treatment of the hydroxy compound with phosgene followed by dimethylamine. Changes were made in the length of the alkyl chain substituted on the heterocyclic nitrogen. The compounds were developed as potential cholinesterase inhibitors as a continuation of previous studies.

Keyphrases □ *N,N*-Dimethyl-[3-(1-alkylpiperidyl)]carbamates—synthesized as potential cholinesterase inhibitors □ Cholinesterase inhibitors, potential—synthesis of *N,N*-dimethyl-[3-(1-alkylpiperidyl)]carbamates □ Piperidine carbamates—synthesized as potential cholinesterase inhibitors

The established interest of these laboratories in the synthesis and evaluation of potential cholinesterase inhibitors (1, 2) led recently to the completion of the synthesis of some compounds intimated in earlier work (1). The studies related to the effects on inhibitory potency of a series of compounds showing isosteric replacements of known cholinesterase inhibitors. The compounds possessed the general structural formulas indicated for I and II and were correlated with compounds of general formula III which were synthesized earlier (3) and shown to be active inhibitors. The data suggested (1) that the compounds having the urea moiety (II) provided a better "fit" in the area of the esteratic site than those possessing the acetamide function (I). To complete the series of isosteric modifications of the acetylcholine skeleton, the synthesis of carbamate derivatives of piperidines was considered appropriate. This approach was further substantiated by the presence of the carbamate moiety in physostigmine—a potent cholinesterase inhibitor. The compounds reported in this paper possess the general formula shown for IV and can be readily related to compounds of the general structures I–III.

The synthetic route adopted involved a preliminary quaternization of 3-hydroxypyridine followed by catalytic hydrogenation to the corresponding *N*-alkyl-3-hydroxypiperidine (Scheme I).

Two methods were chosen to introduce the carbamate function: treatment of the 3-hydroxypiperidine with di-



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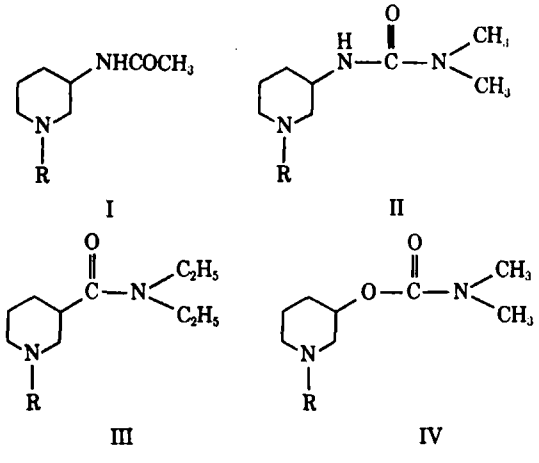
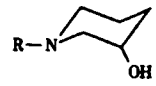


Table I—*N*-Alkyl-3-hydroxypiperidines



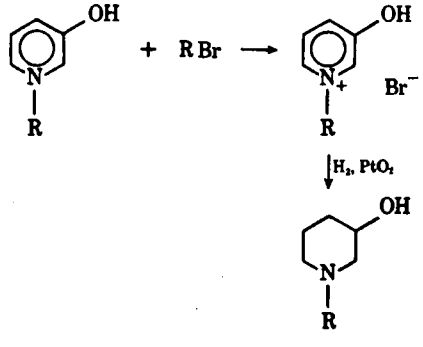
R	Boiling Point (mm. Hg)	Yield, %
CH_3	30–34° (0.1)	35.4
C_2H_5	32–34° (0.05)	30.4
C_3H_7	26–46° (0.05)	40.0
C_4H_9	26–60° (0.15)	43.7
C_5H_{11}	58–68° (0.1)	57.8
C_6H_{13}	60–76° (0.1)	21.0
C_7H_{15}	72–82° (0.05)	47.6
C_8H_{17}	85–96° (0.05)	56.6
C_9H_{19}	66–98° (0.05)	48.9
$C_{10}H_{21}$	110–112° (0.05)	32.0

methylcarbamyl chloride (4) (Scheme II) or treatment of the hydroxy compound with phosgene followed by dimethylamine (5) (Scheme III). Neither procedure showed superiority over the other and the yields varied considerably. Particular difficulty was noted with Compound 2, and only after a prolonged effort was the product obtained and in low yield.

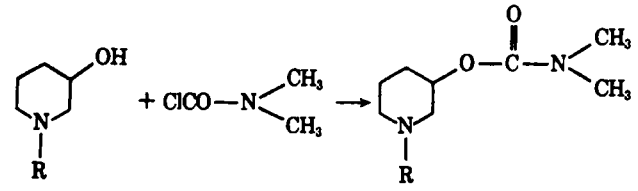
EXPERIMENTAL[1]

***N*-Alkyl-3-hydroxypiperidines**—3-Hydroxypyridine (1.0 mole) and the appropriate *N*-alkyl bromide (1.1 moles) were refluxed in absolute ethanol for 4 days. The ethanol was removed *in vacuo*, leaving the crude *N*-alkyl-3-hydroxypyridinium bromide as a yellow, viscous oil which, in some of the higher molecular weight compounds, solidified on standing at room temperature. The crude product was dissolved in absolute ethanol and hydrogenated at 45 p.s.i. over platinum oxide catalyst (2–5% ratio of catalyst to compound by weight). After 20 hr., the hydrogenation was stopped, the catalyst was removed by filtration, and the solvent was removed *in vacuo*, leaving an oily or a semisolid residue. The residue was dissolved in water, made strongly alkaline with sodium hydroxide pellets, and extracted with ether. The dried ether extract was concentrated to yield a brown oil, which was purified by vacuum distillation to yield the desired compound; in all cases, the compound was a colorless oil. The physical constants for the compounds prepared are shown in Table I. This method was used in the synthesis of Compounds 2–10.

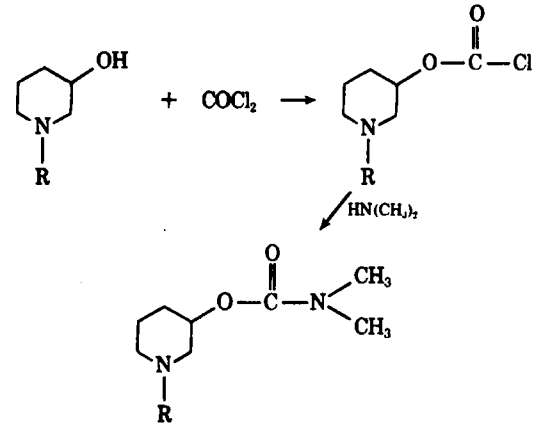
***N*-Alkyl-3-piperidyl Dimethylcarbamate Hydrohalide**—*Method I*—*N*-Alkyl-3-hydroxypiperidine (1.0 mole) was dissolved in benzene, and the solution was cooled in an ice bath. Then a solution of



Scheme I



Scheme II



Scheme III

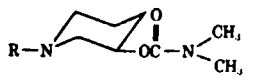
12.5% phosgene in benzene (1.1 moles) was added dropwise with stirring. At the end of the phosgene addition, the resulting bright-yellow solution was left stirring in an ice bath for 0.5 hr. and was then allowed to stand at room temperature with stirring for a further 2 hr. The mixture turned into an opaque, yellow, jelly-like mass. The benzene was removed *in vacuo*, maintaining the temperature below 60°, leaving a yellow tacky residue which was the crude *N*-alkyl-3-piperidyl chlorocarbonate hydrochloride. The crude product was dissolved in chloroform and the solution cooled in an ice bath. A large excess of dimethylamine (5 moles excess) in chloroform was added slowly through an addition funnel, and the reaction mixture (fitted with a condenser) was left stirring in the ice bath and allowed to warm gradually to room temperature as the mixture stirred overnight. The chloroform was then removed *in vacuo*, leaving a pale-yellow granular precipitate suspended in an amber-colored viscous oil. The oil was dissolved in anhydrous ether and the ether was decanted, separating it from the solid precipitate of dimethylamine hydrochloride. The decanted ether solution was reduced in volume, and the resulting reddish-brown oil was purified by vacuum distillation to yield *N*-alkyl-3-piperidyl dimethylcarbamate as a colorless oil. The oil was converted to the appropriate hydrohalide salt which was, in all cases, a white powder. The physical constants for the compounds synthesized together with the recrystallization solvent can be found in Table II. This method was used for the syntheses of Compounds 3, 4, 6, 7, 9, and 10.

***N*-Alkyl-3-piperidyl Dimethylcarbamate Hydrohalide**—*Method II*—*N*-Alkyl-3-hydroxypiperidine (1.0 mole) was dissolved in anhydrous ether, and the solution was cooled in an ice bath. Dimethylcarbamyl chloride (3 moles) was added slowly to the solution fitted with a reflux condenser, The mixture was allowed to warm slowly to room temperature and was left stirring overnight. The ether was then removed *in vacuo*, leaving a brown oil which solidified on standing at room temperature. The residue was suspended in 10% sodium carbonate solution and the mixture was extracted with ether. The ether was dried over anhydrous sodium sulfate and distilled off to leave a brown oil, which was purified by vacuum distillation to yield the *N*-alkyl-3-piperidyl dimethylcarbamate as a colorless oil. The oil was converted to the appropriate hydrohalide salt which was, in all cases, a white powder. The physical constants for the compounds prepared are shown in Table II. This method was used in the syntheses of Compounds 2, 5, and 8.

***N*-Methyl-3-piperidyl Dimethylcarbamate Hydrochloride (Compound 1)**—3-Hydroxypyridine (25.0 g.) was dissolved in methanol (250 ml.), and the solution was cooled in an acetone–dry ice bath. Methyl bromide was bubbled through the cooled solution for 0.5 hr. The mixture was allowed to warm to room temperature, methyl

[1] All melting points were determined using a Swissco melting-point apparatus and are uncorrected. Elemental analyses were carried out by Galbraith Laboratories, Inc., Knoxville, Tenn. IR spectra were recorded on a Perkin-Elmer model 137B Infracord spectrophotometer, and examination of the IR spectra of all compounds showed them to be consistent with the proposed structures [OH absorption 3350 cm.$^{-1}$ (thin film); C=O of carbamate 1725 cm.$^{-1}$ (thin film)].

Table II—Physical Data



Number	R	Boiling Point (mm. Hg)[a]	Melting Point[b]	Yield, %[c]	Solvent of Recrystallization[d]	Analysis, % Calc.	Found
1	CH_3	—	201–202°	50.0	Ethanol	C 48.54	48.38
						H 8.60	8.48
						Cl 15.92	16.05
						N 12.58	12.37
2	C_2H_5	62–64° (0.04)	160–162° (sealed tube)	26.0	Ethyl acetate–ethanol	C 50.73	50.93
						H 8.94	9.20
						Cl 14.98	—[e]
						N 11.83	11.57
3	C_3H_7	66–76° (0.1)	161–162.5°	41.5	Ethyl acetate–ethanol	C 44.75	44.89
						H 7.85	7.79
						Br 27.07	27.21
						N 9.49	9.36
4	C_4H_9	50–68° (0.05)	141–146°	41.5	Ethyl acetate–ethanol	C 46.60	46.34
						H 8.15	8.05
						Br 25.84	25.54
						N 9.06	8.77
5	C_5H_{11}	82–88° (0.05)	132.5–134°	40.5	Ethyl acetate–ether	C 56.00	55.92
						H 9.76	9.64
						Cl 12.72	12.89
						N 10.05	9.95
6	C_6H_{13}	90–100° (0.1)	123–125°	90.0	Ethyl acetate	C 49.85	49.71
						H 8.67	8.70
						Br 23.69	23.75
						N 8.30	—[f]
7	C_7H_{15}	98–114° (0.11)	133–134.5°	50.0	Ethyl acetate	C 51.28	51.10
						H 8.89	8.97
						Br 22.74	22.94
						N 7.97	7.91
8	C_8H_{17}	100–118° (0.05)	128.5–130°	48.3	Ethyl acetate	C 52.60	52.45
						H 9.10	9.23
						Br 21.87	22.07
						N 7.67	7.46
9	C_9H_{19}	100–118° (0.05)	111–113°	59.5	Ethyl acetate	C 53.82	53.78
						H 9.30	9.08
						Br 21.06	20.93
						N 7.38	7.16
10	$C_{10}H_{21}$	140–144° (0.15)	117–119°	55.7	Ethyl acetate	C 54.95	55.11
						H 7.12	6.91
						Br 20.31	20.32
						N 9.48	9.26

[a] For the free bases. [b] For the corresponding hydrohalide salts. [c] Calculated for the free base from *N*-alkyl-3-hydroxypiperidine. [d] For hydrohalide salt. [e] No satisfactory analysis for chlorine could be obtained. [f] No satisfactory analysis for nitrogen could be obtained.

bromide was bubbled through for an additional 0.5 hr., and the mixture was allowed to stir at room temperature for 3 days. The methanol was removed *in vacuo*, leaving *N*-methyl-3-hydroxypyridinium bromide (41.0 g.) as a light-tan solid which was recrystallized from ethanol–water. This compound was dissolved in water and hydrogenated over platinum oxide catalyst (2% ratio of catalyst to compound) at 50 p.s.i. The resulting solution was filtered to remove the catalyst, made alkaline with sodium hydroxide pellets, and extracted with chloroform. This extract was dried over sodium sulfate, and the solvent was distilled off to leave a dark oil, which was purified by vacuum distillation to yield *N*-methyl-3-hydroxypiperidine as a clear colorless oil (10.7 g.), b.p. 34–36° (100 μ). The *N*-methyl-3-hydroxypiperidine (10.7 g., 0.093 mole) thus obtained was dissolved in dry ether and the solution was cooled in an ice bath. Dimethylcarbamyl chloride (21.6 g., 0.2 mole) was added to the hydroxy compound solution and allowed to warm slowly to room temperature; it was left stirring overnight during which time a precipitate formed. The ether was evaporated, leaving a yellow powdery precipitate which was recrystallized from ethanol–ether to yield the hydrochloride salt of *N*-methyl-3-piperidyl dimethylcarbamate as off-white granular crystals (10.3 g. crude weight). The physical data for this compound are shown in Table II.

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Effect of an Inhibitor of Glucuronide Formation on Elimination Kinetics of Diphenylhydantoin in Rats

Keyphrases □ Diphenylhydantoin elimination kinetics—effect of inhibition of glucuronide formation, rats □ Elimination kinetics, diphenylhydantoin—effect of inhibition of glucuronide formation, rats □ Salicylamide inhibition of glucuronide formation—used to study diphenylhydantoin elimination kinetics, rats □ Glucuronide formation, inhibition using salicylamide—effect on diphenylhydantoin elimination kinetics, rats

Sir:

The elimination of diphenylhydantoin (I) is dose dependent in mice (1), rats (2), dogs (3), and man (4). We recently showed (5) that the elimination of I in rats cannot be described by either first-order or simple Michaelis–Menten kinetics but that it is qualitatively consistent with product inhibition of the metabolism of I. This hypothesis was supported by observations that the major metabolite of I, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (II), does indeed inhibit the biotransformation of I *in vitro* (6) and *in vivo* (7). These experiments involved the addition of II to a supernate of rat liver homogenate containing I (6) or the repetitive administration of relatively large doses of II to rats who received a single injection of I (7).

For a more definitive assessment of the role of product inhibition in the elimination of I, it was desirable to determine the effect of endogenous II (*i.e.*, II derived entirely from the biotransformation of I) on the *in vivo* elimination of I. Since II is eliminated primarily by conjugation with glucuronic acid (8), it should be possible to increase the concentration of II at sites of biotransformation of I by blocking the formation of the glucuronide of II. This, in turn, should result in the inhibition of the elimination of I if product inhibition is operative as hypothesized.

We determined the effect of salicylamide, a potent inhibitor of glucuronide formation (9), on the elimination of I. Male Sprague–Dawley rats, weighing 310–380 g., received single intravenous injections of I, 10 mg./kg. body weight. Each injection included 2-μc. ^{14}C-I (specific activity 4.65 mc./mmole). Six of these animals were also given intraperitoneal injections of salicylamide, 200 mg./kg. 30 min. before I and 100 mg./kg. every hour thereafter for a total of four doses. Another five animals received injections of 0.9% sodium chloride solution adjusted to pH 10 with sodium hydroxide. The salicylamide solution contained 10% of the drug and was prepared fresh with sufficient sodium hydroxide to yield a final pH of about 10. Blood samples were obtained every 10–30 min. for 210 min. and were assayed specifically for I by liquid scintillation spectrometry as described elsewhere (2, 7).

The results, presented as the geometric mean concentrations of I in the blood of the salicylamide-treated and control animals, are shown in Fig. 1. The elimination of I was much slower in the salicylamide-treated rats than in the control animals. Additional experiments with one-half and one-quarter of the doses of salicylamide yielded definite evidence of inhibition of I elimination with the higher doses and none with the lower doses. Single experiments (due to limited material) to determine the effect of salicylamide on the elimination of ^{14}C-II, using the experimental methods described previously (7), indicated appreciable inhibition of II elimination (terminal half-life 2.2 hr. *versus* 1.0 hr. for the control).

The results of this study provide additional support for the suggested role of product inhibition in the elimination of I. If the inhibitory effect of salicylamide on the elimination of I is due to accumulation of II, as would appear on the basis of the preliminary data presented here, then a new type of drug interaction has been demonstrated. This type of interaction consists of a primary inhibitory effect on the elimination of a drug metabolite, resulting in a decreased rate of elimination of the drug due to inhibition of the biotransformation process by the accumulated metabolite. Hydroxylated metabolites of drugs other than I are also able to inhibit the biotransformation of their precursors (10), and other drugs with dose-dependent elimination characteristics, notably dicumarol, are also biotransformed to hydroxylated metabolites. It is important, therefore, to explore

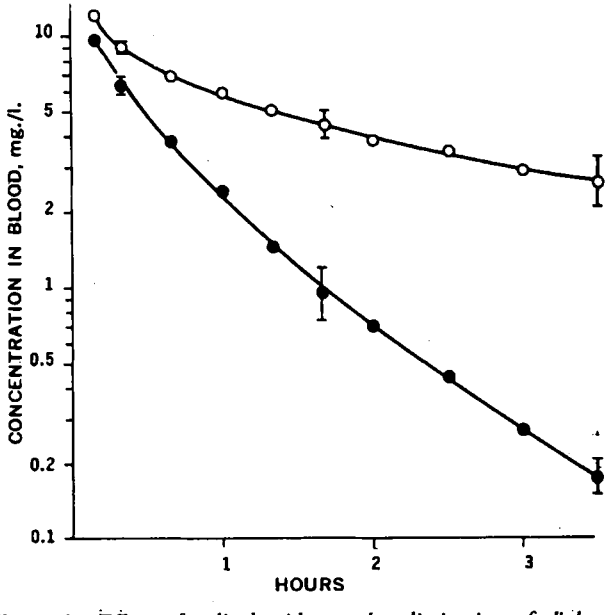

Figure 1—*Effect of salicylamide on the elimination of diphenylhydantoin, 10 mg./kg. i.v., by adult male Sprague–Dawley rats. Key: ●, control rats; and ○, rats treated with salicylamide, 200 mg./kg. 30 min. before diphenylhydantoin injection and 100 mg./kg. every hour thereafter. Vertical bars indicate standard errors of the mean of the logarithmically transformed data for five control and six salicylamide-treated animals.*

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Further Comments on the Existence of Spherical Micelles

Keyphrases ☐ Micelles—spherical *versus* nonspherical shape ☐ Surfactants—spherical *versus* nonspherical micelles

Sir:

A study of the shape of micelles (1), based on the combination of their measured size with geometric considerations of the extended length of the normal paraffin moiety, indicated that few surfactants having a single normal alkyl chain form spherical micelles. In Fig. 1 of a recent communication (2), Zografi and Yalkowsky superimposed the results of their calculations, based on similar geometric considerations, on Fig. 1 of *Reference 1*. The figure is a plot of z, the aggregation number or the number of surfactant molecules per micelle, *versus* the number, n, of carbon atoms in the linear hydrocarbon chain of the single-chain surfactants. Micelles with (z, n) coordinates above the curves are unlikely to be spherical. Of the 32 surfactants considered and plotted in Fig. 1, only one fulfilled the geometric prerequisites for spherical shape according to the solid line embodying the results of our original computations (1). Only 26 separate points were shown in Fig. 1 because the coordinates of another six surfactants overlapped points already plotted.

The values of z calculated according to Eq. 5 and listed in the last column of Table I of *Reference 2*[1] have the largest differences from our maximum z values consistent with spherical shape. They are represented by the uppermost of the three curves of Fig. 1. There are 18 surfactants with coordinates above that curve and 14 with coordinates below or on it, which shows that even according to the more divergent of the two analyses of Zografi and Yalkowsky (2), the majority of the surfactants under consideration are still unlikely to form spherical micelles. Moreover, the vertical axis was compressed by using a logarithmic scale for z, so that even points apparently lying close to the curve correspond to distinctly higher aggregation numbers than are consistent with spherical shape.

Of the two simplifications introduced in the original geometric considerations, the first is expressed in Eq. 4 of *Reference 1*, which correlates the molecular weight, m, of the normal alkyl chain of a single surfactant molecule with the number, n, of carbon atoms in that chain:

$$m = 14.03n + 1.01 \cong 14.03n \qquad \text{(Eq. 1)}$$

This is equivalent to neglecting the length of the carbon–hydrogen bond and the van der Waals radius of the farthest hydrogen atom of the terminal methyl group. The second approximation, designed to compensate for the previous one, is to consider n carbon–carbon bond lengths in the hydrocarbon chain instead of the actual number $n - 1$.

With the aid of Fig. 1, the length of the n-paraffin chain is now calculated rigorously and without approximations. The chain is fully extended and in the all-*trans* conformation. Segment a, representing the contribution of the farthest hydrogen atom of the terminal methyl group, consists of two parts. The first is the van der Waals radius of this hydrogen atom, 1.2 Å (3). The second part is the length of the corresponding hydrogen–carbon bond projected in the direction of the longitudinal axis of the extended chain, namely, 1.09 Å times the sine of one-half of the hydrogen–carbon–hydrogen bond angle of 111.5° (3–5), or 1.09 Å sin (111.5°/2) = 1.09 Å × 0.8266 = 0.90 Å. Adding the two parts gives a = 2.10 Å.

Segment b in Fig. 1 represents the contribution of a single carbon–carbon bond to the length of the extended chain. It equals the length of the carbon–carbon bond, namely, 1.541 Å (4, 5), multiplied by the sine of one-half of the tetrahedral angle of 109.5° (3–5), or 1.541 Å sin (109.5°/2) = 1.541 Å × 0.8166 = 1.258 Å. Alternatively, it can be calculated as 1.541 Å cos 35.25° = 1.258 Å. There are $n - 1$ such carbon–carbon bonds in a hydrocarbon chain of n carbon atoms, contributing ($n - 1$) 1.258 Å to the length of the extended chain.

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Segment b in Fig. 1 represents the contribution of a single carbon–carbon bond to the length of the extended chain. It equals the length of the carbon–carbon bond, namely, 1.541 Å (4, 5), multiplied by the sine of one-half of the tetrahedral angle of 109.5° (3–5), or 1.541 Å sin (109.5°/2) = 1.541 Å × 0.8166 = 1.258 Å. Alternatively, it can be calculated as 1.541 Å cos 35.25° = 1.258 Å. There are $n - 1$ such carbon–carbon bonds in a hydrocarbon chain of n carbon atoms, contributing ($n - 1$) 1.258 Å to the length of the extended chain.

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Table I—Maximum Values for the Aggregation Number of Micelles of *n*-Alkyl Surfactants Consistent with Spherical Shape

n	Extended Chain Length[a], Å	$d_{35}°/_{35}°$[b]	*m*[c], Å	z_{max}[d]
10	14.05	0.791	141.31	39.2
12	16.57	0.802	169.37	54.3
14	19.08	0.811	197.43	72.0
16	21.60	0.818	225.49	92.2
18	24.12	0.823	253.55	114.9

[a] From Eq. 2. [b] Specific gravity of the hydrocarbon moiety. [c] From Eq. 5. [d] From Eq. 4.

The contribution *e* of the bond between the α-carbon atom and the oxygen or nitrogen heteroatom is calculated as the covalent radius of the carbon atom, 0.77 Å (3), multiplied by the sine of one-half of the tetrahedral bond angle. The presence of a heteroatom does not significantly distort that angle (4, 5). Therefore, $e = 0.77$ Å $\sin(109.5°/2) = 0.63$ Å. The heteroatom may be an ether or an ester oxygen belonging to a polyoxyethylated nonionic surfactant or an alkyl sulfate, respectively. Either oxygen atom has two pairs of unshared electrons with which it binds two water molecules by hydrogen bonds (6). If the heteroatom is a nitrogen cation, it is also hydrated. Therefore, the boundary between the hydrocarbon core and the shell of hydrated headgroups of the micelle lies between the α-carbon and the heteroatom. It could even be argued that the α-carbon atom bound to an ether or ester oxygen, being at the positive end of a permanent dipole of medium strength, attracts the oxygen atom of a water molecule through dipole–dipole interaction.

The extended chain length of the hydrocarbon moiety, *L*, is thus represented by the equation:

$$L = 2.10 + (n - 1)\,1.258 + 0.63 = 2.73 + (n - 1)\,1.258 \quad \text{(Eq. 2)}$$

This is the maximum value for the radius of the hydrocarbon core of a spherical micelle that does not have a hole in its center. The approximate Eq. 6 of the original calculations (1) is now replaced by the exact expressions:

$$0.7346\,(zm/d)^{1/3} \leqslant L \quad \text{(Eq. 3)}$$

and:

$$z \leqslant \left(\frac{L}{0.7346}\right)^3 \frac{d}{m} \quad \text{(Eq. 4)}$$

where *d* is the specific gravity of the hydrocarbon core. Use of the true values for *m* given by:

$$m = 14.03n + 1.01 \quad \text{(Eq. 5)}$$

results in the exact values for z_{max}, the highest aggregation number consistent with spherical shape, listed in the last column of Table I. According to these values, of the 32 surfactants considered, 23, or 72%, have aggregation numbers in excess of z_{max} and are, therefore, unlikely to form spherical micelles. Only nine surfactants have aggregation numbers equal to or below z_{max} and may form spherical micelles.

The geometric considerations of Zografi and Yalkowsky (2) have two shortcomings. First, the contribution of a single bond to *L* does not equal the length of that bond because the bond is not parallel to the chain axis but follows a zigzag course conforming with the tetrahedral angle. The contribution is the projected length of that bond onto the chain axis, or the bond length times the sine of one-half the tetrahedral bond angle. The second point, already discussed, is that the heteroatom, which is either an oxygen atom or a nitrogen cation, is associated with water and cannot be considered part of the hydrocarbon core of the micelle. As Zografi and Yalkowsky pointed out, Tartar (7) solved this point by taking one-half of the carbon–oxygen bond[2] distance as contribution to the hydrocarbon core. Therefore, one C—C bond length in their Eq. 4 should be replaced by one-half of a C—O bond length multiplied by the sine of one-half of the tetrahedral bond angle. Applying these considerations to Eq. 4 of *Reference 2* changes it to:

$$R \leqslant 1.27\,(n - 1) + 2 + (1.42/2)\sin(109.5°/2) \quad \text{(Eq. 6)}$$

which brings it into agreement with Eq. 2 in this article.

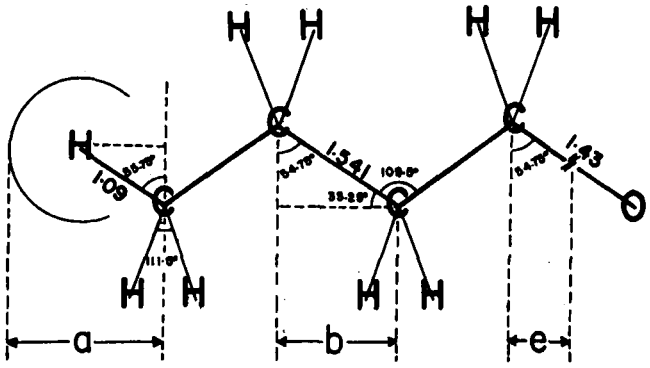


Figure 1—*Normal alkyl chain in the fully extended all-*trans *conformation.*

When assuming a spherical shape for micelles, the radii of their hydrocarbon cores cannot exceed the length of the extended alkyl chains as long as the cores have uniform density. Combining the values for *L* with those of the hydrocarbon density results in the revised values of z_{max}, the maximum aggregation number consistent with spherical micelles, listed in Table I. Comparison of z_{max} with the experimentally determined *z* values for 32 surfactants having a single *n*-alkyl chain shows that 23, or 72%, have higher *z* values and are unlikely to form spherical micelles.

The experimental data refer to surfactant solutions in water. The addition of electrolytes generally produces moderate increases in *z* of ionic surfactants in concentrations not exceeding 0.5 *M* for univalent counterions; higher electrolyte concentrations produce considerable increases (8), especially on approaching salting-out conditions. Therefore, the percentage of systems forming nonspherical micelles would have been even greater if aggregation numbers of ionic surfactants in dilute salt solutions had been included. Micelles in solu-

[2] Zografi and Yalkowsky (2) referred to a C—S bond in the alkyl sulfate molecule but, since there is only a C—O—S bond, they evidently had the C—O bond in mind.

Table II—Areas at Saturation Adsorption of Single-Chain Surfactant Molecules at 20–25°

Surfactant	System	Method	Area/ Molecule, Å²	Reference
Sodium lauryl sulfate	Air–water	Radiotracer	52.0	13
Sodium lauryl sulfate	Air–0.115 *M* NaCl	Radiotracer	38.4	14
Sodium lauryl sulfate	Air–water	Radiotracer	35	15
Sodium lauryl sulfate	Air–0.1 *M* Na^+[a]	Radiotracer	33	15
Sodium lauryl sulfate	Air–water	Foaming	49.9	16
Sodium lauryl sulfate	Air–water	Surface tension	47	16
Sodium lauryl sulfate	Air–water	Foaming	34	17
Sodium lauryl sulfate	Air–water	Foaming	40	18
Sodium lauryl sulfate	Air–water	Surface tension	47	16
Sodium lauryl sulfate	Air–water	Surface tension	43.9	19
Sodium lauryl sulfate	Air–sodium chloride[b]	Surface tension	52	20
Sodium lauryl sulfate	Air–water	Surface tension	37.7	21
Sodium lauryl sulfate	*n*-Hexane–water	Interfacial tension	45.2	19
Sodium lauryl sulfate	*n*-Nonane–water	Interfacial tension	47.6	19
Sodium lauryl sulfate	*n*-Heptadecane–water	Interfacial tension	51.0	19
Sodium lauryl sulfate	*n*-Heptane–water[c]	Interfacial tension	49	22
Sodium lauryl sulfate	*n*-Heptane–sodium chloride[b]	Interfacial tension	53.4	20
Sodium lauryl sulfate	*n*-Heptane–water ± sodium chloride	Interfacial tension	50.0	23
Sodium lauryl sulfate	Petroleum ether–water ± sodium chloride	Interfacial tension	35.9	23
Sodium lauryl sulfate	Petroleum ether–0.05 *M* NaCl	Interfacial tension	43.0	24[d]
Sodium lauryl sulfate	Petroleum ether–0.10 *M* NaCl	Interfacial tension	45.0	24
Sodium lauryl sulfate	Petroleum ether–0.25 *M* NaCl	Interfacial tension	41.2	24
Sodium lauryl sulfate	Petroleum ether–0.50 *M* NaCl	Interfacial tension	41.0	24
Sodium decyl sulfate	*n*-Heptane–water[c]	Interfacial tension	50.0	22
Sodium tetradecyl sulfate	*n*-Heptane–water[c]	Interfacial tension	50.0	22
Sodium octadecyl sulfate	*n*-Heptane–water	Interfacial tension	45.6	23
Sodium undecanesulfonate	Air–sodium chloride[b]	Surface tension	53	20
Sodium undecanesulfonate	*n*-Heptane–sodium chloride[b]	Interfacial tension	54	20
Sodium dodecanesulfonate	Air–water	Foaming	50.7	16
Sodium dodecanesulfonate	Air–water	Suface tension	53	16
Sodium dodecanesulfonate	Air–water	Surface tension	48	25
Sodium pelargonate	Air–water	Surface tension	39.0	23
Sodium laurate	Air–water	Surface tension	39.0	23
Sodium laurate	Air–sodium chloride[b]	Surface tension	47	20
Potassium laurate	Air–potassium chloride[e]	Surface tension	43	20
Potassium laurate	Air–water	Surface tension	39.2	21
Potassium palmitate	Air–water	Surface tension	35.9	21
Potassium palmitate	Air–water	Radiotracer	37.7	21
Sodium laurate	*n*-Heptane–sodium chloride[b]	Interfacial tension	45	20
Potassium laurate	*n*-Heptane–potassium chloride[e]	Interfacial tension	44	20
Dodecylammonium chloride	Air–water	Interfacial tension	31.9	21
Dodecylammonium chloride	Air–water	Foaming	24	17

[a] Na_2HPO_4 + NaH_2PO_4 buffer + enough NaCl to make the total Na^+ concentration 0.1 *M*. [b] *q.s.* for constant Na^+ concentration. [c] Measured at 50°. [d] *Reference 24* is the same as *Reference 6* of Zografi and Yalkowsky (2), who mistakenly quote the journal as *Proc. Roy. Soc.* [e] *q.s.* for constant K^+ concentration.

tions of ionic surfactants of concentrations not far above the CMC and at moderate salt concentrations, up to about 0.5 *M* but well below salting-out concentrations, are believed to maintain their spherical shape (9, 10). The effect of 0.2 *M* NaCl on the micellar size of sodium lauryl sulfate is smaller than the difference between the micellar sizes measured by various methods at a given sodium chloride concentration (8, 11).

By assuming spherical shape and using the values for *L*, hydrocarbon density, and z_{max} of Table I, the surface area per hydrocarbon chain on the spherical boundary enclosing the hydrocarbon core ranges from 63.3 Å² for decyl derivatives to 63.6 Å² for octadecyl derivatives. This is twice the limiting area per molecule of sodium lauryl sulfate and three times that of dodecylamine hydrochloride and of fatty acid soaps at the air–water interface, indicating that an ionic headgroup shields its hydrocarbon chain only incompletely from contact with water in spherical micelles. An alternative model for small micelles, with a smaller external area per hydrocarbon chain, would be energetically more favorable (1).

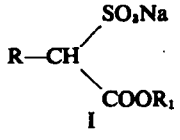
It is argued that limiting surface areas are only attainable under the influence of significant external forces capable of overcoming the electrostatic repulsion and hydration of polar headgroups and that a more appropriate area for the purpose of comparison would be the area per molecule at the point of maximum adsorption at the air–water or oil–water interface (2). The first point does not apply to the 18 nonionic surfactants used in the study nor to ionic surfactants in solutions containing 1:1 electrolytes at concentrations below 0.5 *M*, where the area per hydrocarbon chain on the periphery of the core is still 63–64 Å² if they formed spherical micelles. For the 14 ionic surfactants in water, this argument was answered by Bury and Browning (12), who showed that the CMC value of a nonionic surfactant was lower by a factor of 10 than that of an ionic surfactant having the same hydrocarbon moiety. The difference results precisely from the need of the ionic surfactant to overcome the electrostatic repulsion of the polar headgroups to form micelles. Moreover, being water soluble, the surfactant molecules adsorbed at the interfaces will be de-

sorbed and enter the bulk aqueous solution to form micelles when the compression at the interface becomes excessive.

Concerning the second point, even the cross-sectional areas per surfactant molecules at the interfaces observed at saturation adsorption exceed the 63–64 Å² area per alkyl chain on the core periphery of spherical micelles by significant amounts. This is shown in Table II, where the area for sodium lauryl sulfate averaged from the 23 reported values is 44 Å² per molecule, 30% below the peripheral area calculated for a spherical micelle. Saturation adsorption was usually reached considerably below the CMC (20).

The agreement between the areas measured directly, using radiotagged surfactants or foam analysis, and the areas calculated from surface and interfacial tension data by means of the Gibbs adsorption equation is excellent. For sodium lauryl sulfate at the air–water interface, the average value was 42.2 Å² per molecule according to direct measurements and 43.9 Å² from the Gibbs adsorption equation. This vindicates the unit activity coefficient customarily used in that equation, as well as the factor "2" employed for ionic surfactants (16, 23).

Zografi and Yalkowsky (2) quoted a range of 50–70 Å² per molecule at saturation adsorption, which is considerably higher than the range of 24–54 Å² in Table II. Their high values stem from the inclusion of two series of surfactants which have more than one alkyl chain. The data of Boucher *et al.* (26) refer to α-sulfo fatty esters having Structure I. Each molecule



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contains two hydrophilic groups and two alkyl chains, which obviously increase its cross-sectional area compared to surfactants containing a single alkyl chain and a single hydrophilic group. The interfacial orientation of the ester–sulfonate surfactants is also unusual (27). The data of Weiner and Zografi (28) refer to the chlorides of dodecylpyridinium, dodecyltrimethylammonium, and dodecylethyldimethylammonium which, at saturation adsorption, occupied an area of 69 Å² per molecule. The nitrogen cation of the first surfactant is part of a ring with five carbon atoms. In the other two, it is buried among four alkyl groups. The presence of these extra hydrocarbon moieties results in headgroups of large sizes in these and similar cationic surfactants, causing them to form micelles with more loosely packed hydrocarbon cores than surfactants containing single alkyl chains. This is shown by their superior solubilizing capacity when compared with micelles of anionic and cationic surfactants having a single alkyl chain (29, 30).

Since the micelles of tetraalkylammonium halides have hydrocarbon cores with comparatively low densities, the area of the long hydrocarbon chain on the envelope of the micellar core is higher than the 63–64 Å² value calculated for spherical micelles having the densities listed in Table I. These two classes of surfactants should not be included in a study (1, 2) on whether surfactants containing a single normal alkyl chain form spherical micelles. The interfacial area per molecule of a primary amine hydrochloride at saturation adsorption was less than one-half of the area per hydrocarbon chain on the periphery of the micellar hydrocarbon core if the micelle were spherical (17, 21). For mono-*n*-alkyl sulfates and alkali metal soaps, the interfacial areas per molecule were 30 and 35% below the micellar areas, respectively (Table II).

Another way of expressing the incomplete shielding of the hydrocarbon core from contact with water by the hydrophilic headgroups in spherical micelles is as follows (31). The cross-sectional area of the sulfate headgroup in an alkyl sulfate, measured on atomic models or estimated from atomic radii (3), is 25 Å², identical with the limiting area per molecule in an interface (24). This is the area actually occupied by the headgroup. The difference between 25 Å² and the interfacial area at saturation adsorption (44 Å²) or the peripheral area on a spherical micelle (63.3 Å²) per molecule of sodium lauryl sulfate is due chiefly to repulsion among the negatively charged headgroups. The void between the headgroups is filled with water. For an *n*-dodecyl surfactant forming spherical micelles, $z_{max} = 54$ Å and $L = R = 16.57$ Å. The area on the spherical envelope of the hydrocarbon core is $4\pi R^2 = 4\pi (16.57 \text{ Å})^2 = 3450$ Å². Of this peripheral area, 54×25 Å² = 1350 Å² or 39% is covered by sulfate headgroups, leaving 61% of the hydrocarbon surface exposed to water. Alkyl sulfates have comparatively bulky headgroups. Fatty acid soaps and primary amine hydrochlorides have considerably smaller headgroups, so that an even greater percentage of the periphery of their hydrocarbon core, approximately 75%, is in contact with water. A different model for micelles, exposing less of the hydrocarbon portion to water, would be energetically more favorable.

It is argued that the determination of surface potentials of micelles by acid–base titration and electrophoresis gives charge densities and, hence, molecular areas that are similar to areas at saturation adsorption (2). Potentiometric titrations are applicable only to surfactants like trialkylamine oxides (32), which are nonionic but become cationic in the presence of strong acids, or to amphoteric surfactants like *N*-dodecyl-β-aminopropionic acid, *N*-dodecyl-β-iminodipropionic acid (33), and long-chain fatty acylcarnitines (34). The headgroups of these surfactants are bulky because they contain two to three hydrophilic moieties and/or multiple alkyl groups. This produces micelles with hydrocarbon cores of lower density than those formed by surfactants with single *n*-alkyl chains and with headgroups consisting of single ions, as already discussed. Therefore, even if the molecular areas in micelles of the complex, amphoteric surfactants approach or exceed 64 Å², this does not corroborate the existence of spherical micelles of single-chain surfactants.

Electrophoresis determines the ζ-potential from measurements of the electrophoretic mobility, U, according to the equation:

$$\zeta = \frac{f(\kappa R)\pi\eta U}{D} \qquad \text{(Eq. 7)}$$

where κ is the Debye–Hückel parameter, and η is the viscosity and D the dielectric constant of the water in the electric double layer. Lacking the exact values of η and D and their distribution across the double layer, it is common practice to use the corresponding values for bulk water, although D is somewhat lower and η considerably higher, especially in double layers associated with the high surface potentials of ionic micelles (35). The ζ-potentials are usually not corrected for surface conductance despite the pronounced effect of the latter on the mobility of particles with charge densities as high as those of ionic micelles in aqueous media of low ionic strength (36). These are the chief experimental difficulties in determining ζ-potentials. The main theoretical difficulties are to calculate the charge density at the micellar surface from the value of the ζ-potential measured at the plane of shear within the double layer and to correct for counterion binding. The exact solution to these complex problems remains to be found (35). Molecular surface areas in ionic micelles derived from electrokinetic measurements are subject to uncertainties of the same order of magnitude as the areas themselves and, therefore, do not lend themselves to distinguishing between spherical micelles and other models.

It is argued that independent evidence, obtained by light-scattering or hydrodynamic methods, is consistent with spherical shape (2). Light scattering only provides information on the shape of those particles whose largest dimension exceeds one-twentieth of the wavelength of light, or about 250 Å. In that case, dissymmetry develops, and it is possible to distinguish between a sphere, rod, random coil, ellipsoid, or cylinder from the angular intensity distribution of the scattered light (37, 38). A cylindrical micelle of sodium lauryl sulfate having an aggregation number of 200 (which is larger than that observed in 0.5 M NaCl solutions) would have a length of only 65 Å at a density of 1.14. It is too small by a factor of 4 to give measurable dissymmetry. Therefore, light scattering is not suitable for determining the shape of the small micelles formed at concentrations that are low multiples of the CMC.

To deduce the shape of a particle from hydrodynamic data requires a knowledge of the extent of its hydration. For instance, by assuming that the particle is ellipsoidal, the frictional ratio, f/f_o, the ratio of translational diffusion coefficients, D/D_o, or the intrinsic viscosity can be used to determine its asymmetry or axial ratio, provided its degree of hydration is known. The subscript o refers to the equivalent unhydrated sphere. An added complication is that the relationship between hydration, asymmetry, and frictional ratio or ratio of diffusion coefficients is different for micelles shaped like a sphere, a prolate ellipsoid, an oblate ellipsoid, a rod, and a lamella (38). Thus, hydrodynamic methods do not provide sufficient information to discern between the combined effects of micellar shape, asymmetry, and hydration on the data.

In conclusion, exact geometric considerations combined with experimentally determined aggregation numbers for surfactants containing a single normal alkyl chain are inconsistent with spherical shape for 72% of the micelles for which data are available. Spherical shape would also result in incomplete shielding of the hydrocarbon portion of the surfactants from contact with water by the hydrophilic headgroups.

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Proposed Mechanism for Gentamicin Transport

Keyphrases □ Gentamicin—proposed mechanism for transport across biological membranes □ Transport across biological membranes—gentamicin, proposed mechanism □ Stearyl aldehyde—interaction with gentamicin, used to formulate mechanism for gentamicin transport, effect of sodium bisulfite

Sir:

This communication reports our preliminary work dealing with the biological transport of the water-soluble aminoglycoside antibiotic, gentamicin[1]. This antibiotic does not transport to any significant degree across the membranes of the GI tract, as evidenced by the facts that measurable blood levels of gentamicin are not observed following oral administration to humans and animals and that nearly 98% of the antibiotic is excreted unchanged in the feces[2]. On the other hand, gentamicin has been shown to cross the otic (1) and placental (2) membranes and, in the case of sensitive bacteria, it has been demonstrated that gentamicin crosses the cell membrane and binds to bacterial ribosomes (3).

In view of the variability of gentamicin transport, it seems reasonable to assume that the transport of this antibiotic is dependent, at least in part, upon the physical-chemical properties of the biological interface (*viz.*—membrane). On the basis that the primary amino moieties of gentamicin are capable of interacting with aldehyde compounds *via* Schiff base formation, we hypothesized that biological membrane fatty aldehydes might also react with and subsequently bind gentamicin to the particular membrane, thus constituting a possible major step in the pathway for the transport of this antibiotic across biological membranes. Until recently, cellular lipid aldehydes were assumed to be present *in vivo* only in their bound, cryptic forms (*viz.*—plasmologens) (4). However, more recent work has shown that free fatty aldehydes are normal components of a variety of living systems, including bacteria (5), human placenta (6), mouse muscle (7), human collagen (8), and rat, dog, and bovine heart (9).

To test the validity of the gentamicin aldehyde Schiff base hypothesis, we studied the interaction of gentamicin with stearyl aldehyde, using previously described monolayer techniques (10).

Figure 1 shows the interfacial activity of gentamicin in the presence of insoluble films of stearyl aldehyde as a function of pH. The development of surface pressure, at areas per molecule where stearyl aldehyde alone exhibits no surface pressure, indicates penetration of the aldehyde film by gentamicin in the subphase. It can be seen that an enhancement of gentamicin penetration occurs with increasing pH. At a pH of 6.0, where gentamicin exists essentially in the protonated form, no significant penetration was observed. This enhancement may be explained by the fact that at higher pH values there is a concomitant increase in the fraction of nonprotonated primary amino groups. Furthermore, only these nonprotonated primary amino groups are capable of participating in Schiff base formation (11). Earlier experiments in these laboratories established that gentamicin (1×10^{-3} *M*) exhibited no surface activity at the air–water interface and that gentamicin did not interact with stearyl alcohol and only interacted slightly with stearic acid.

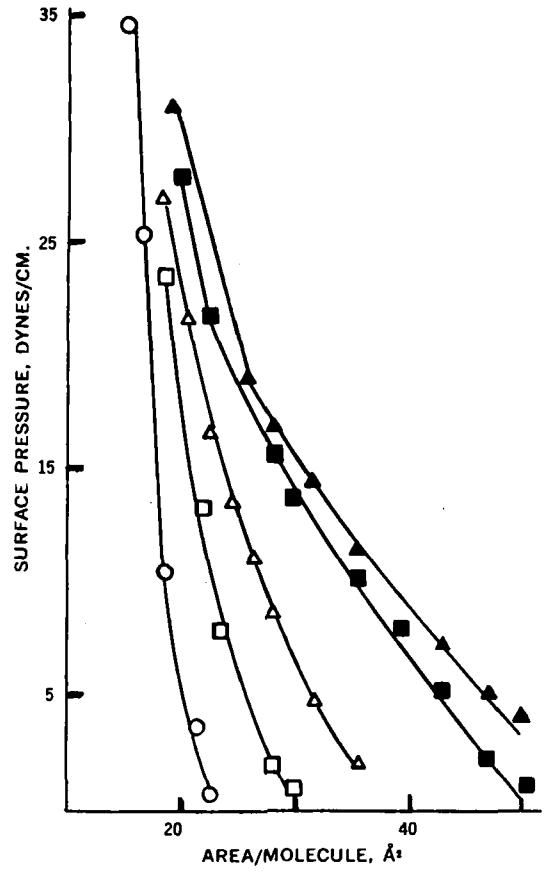

Figure 1—*Surface pressure–surface area curves of stearyl aldehyde alone at pH values of 7.2–8.4* (○) *and in the presence of* 1×10^{-3} M *gentamicin at pH values of 6.0* (○), *7.2* (□), *7.6* (△), *8.0* (■), *and 8.4* (▲).

Figure 2 demonstrates the uptake of gentamicin into an insoluble film of stearyl aldehyde, with and without the addition of 0.05% sodium bisulfite to the subphase. Gentamicin was injected under a film of stearyl aldehyde, and the rate of surface pressure development was followed. A lag period of approximately 4 min. was

Table I—Effect of Sodium Bisulfite on Antibacterial Activity of Gentamicin against *E. coli*

Sodium Bisulfite Concentration, %[a]	—Gentamicin, mcg./Disk— 0.12	0.60
	Zone of Inhibition, mm.[b]	
0	1.5	3.0
0.05	0.2	1.5

[a] Incorporated into agar medium. [b] Measured from edge of disk to edge of zone of inhibition.

[1] Received as a mixture of components (C_1, C_{1a}, and C_2); see D. Cooper *et al.*, *J. Chem. Soc., C*, 1971, 3126.

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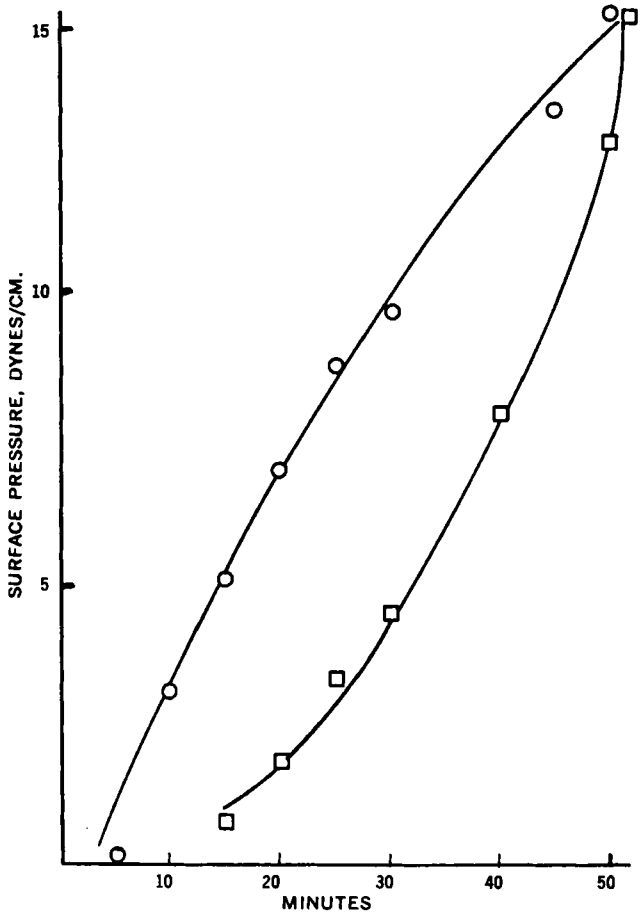

Figure 2—*Rate of surface pressure increase of a stearyl aldehyde film at pH 8.0 in the presence of 1 × 10^{-3} M gentamicin* (○) *and 1 × 10^{-3} M gentamicin with 0.05% sodium bisulfite* (□).

observed when gentamicin was studied in the absence of bisulfite ion.

In the case of a similar experiment conducted with 0.05% sodium bisulfite in the subphase, the previously observed lag period was increased to 14 min. The data clearly show that bisulfite significantly diminishes the interaction of gentamicin with stearyl aldehyde. This may be explained by the fact that bisulfite reacts with aldehydes to form α-hydroxysulfonic acid derivatives (12) which do not form Schiff bases.

The implications of the monolayer studies were extended by studying the effect of the bisulfite ion on the antibacterial activity of gentamicin. A disk-plate method (13), utilizing *Escherichia coli* as the test organism, was employed. Gentamicin-containing filter paper disks were placed on seeded nutrient agar plates containing various concentrations of sodium bisulfite. Following incubation at 37° for 18 hr., the plates were examined and the diameters of the observable zones of inhibition were measured (Table I).

The data indicate that bisulfite ion at 0.05% inhibits the antibacterial activity of gentamicin against *E. coli*. If the mechanisms of interaction of gentamicin at the monolayer and the bacterial membrane are the same, then it appears that bisulfite inhibits gentamicin activity by blocking membrane aldehyde sites which may be necessary for the transport of gentamicin into the bacterial cell.

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Self-Association of Theophylline in Aqueous Solution

Keyphrases □ Theophylline, association in aqueous solution—determination, ultracentrifugation, molecular weights □ Association, theophylline—aqueous solution □ Ultracentrifugation—determination, theophylline self-association, molecular weights

Sir:

In 1957, Guttman and Higuchi (1) concluded that theophylline did not self-associate in water at concentrations from 2.3 × 10^{-3} to 28 × 10^{-3} *M*; partitioned with chloroform–isooctane (9:1) where the theophylline concentration was 1 × 10^{-4} to 12 × 10^{-4} *M*.

In 1971, Ng (2) presented IR evidence for the self-association of theophylline by hydrogen bonding in nonaqueous (deuterochloroform) solutions. Thakkar *et al.* (3) showed, from NMR spectra of aqueous solutions (5 × 10^{-3} to 42 × 10^{-3} *M*), that theophylline self-associates by hydrophobic interactions in water. The present communication presents data, obtained

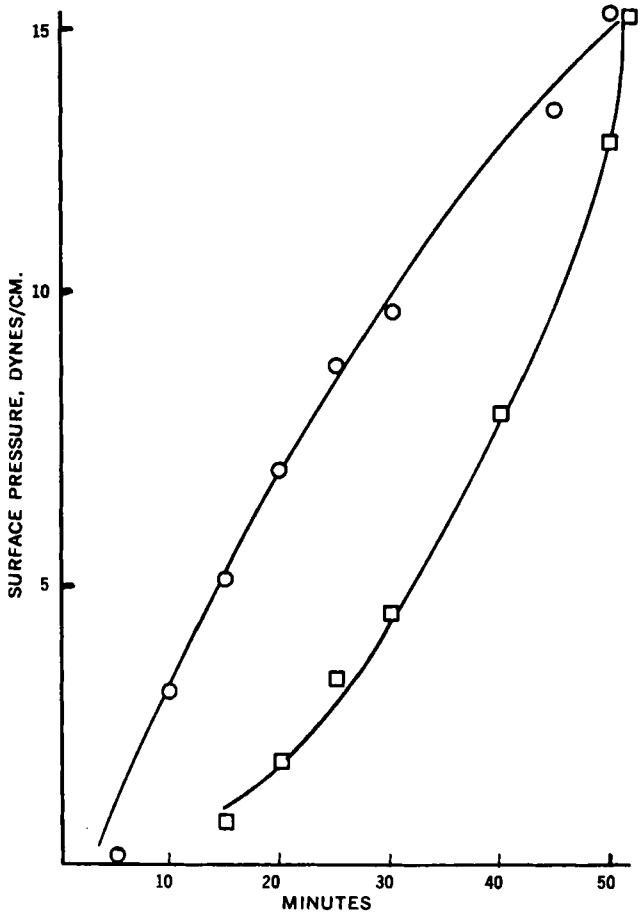

Figure 2—*Rate of surface pressure increase of a stearyl aldehyde film at pH 8.0 in the presence of 1 × 10^{-3} M gentamicin* (○) *and 1 × 10^{-3} M gentamicin with 0.05% sodium bisulfite* (□).

observed when gentamicin was studied in the absence of bisulfite ion.

In the case of a similar experiment conducted with 0.05% sodium bisulfite in the subphase, the previously observed lag period was increased to 14 min. The data clearly show that bisulfite significantly diminishes the interaction of gentamicin with stearyl aldehyde. This may be explained by the fact that bisulfite reacts with aldehydes to form α-hydroxysulfonic acid derivatives (12) which do not form Schiff bases.

The implications of the monolayer studies were extended by studying the effect of the bisulfite ion on the antibacterial activity of gentamicin. A disk-plate method (13), utilizing *Escherichia coli* as the test organism, was employed. Gentamicin-containing filter paper disks were placed on seeded nutrient agar plates containing various concentrations of sodium bisulfite. Following incubation at 37° for 18 hr., the plates were examined and the diameters of the observable zones of inhibition were measured (Table I).

The data indicate that bisulfite ion at 0.05% inhibits the antibacterial activity of gentamicin against *E. coli*. If the mechanisms of interaction of gentamicin at the monolayer and the bacterial membrane are the same, then it appears that bisulfite inhibits gentamicin activity by blocking membrane aldehyde sites which may be necessary for the transport of gentamicin into the bacterial cell.

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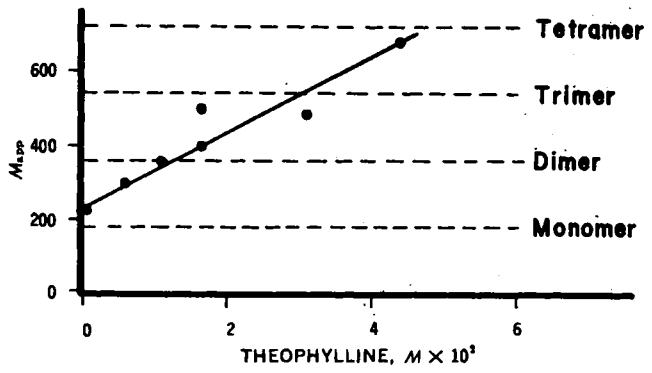

Figure 1—*Plot of apparent molecular weight* (M_{app}) *against concentration of theophylline (mol. wt. 180). Hatched horizontal lines indicate the calculated molecular weight of the monomer, dimer, trimer, and tetramer.*

with the aid of an analytical ultracentrifuge, that confirm the formation in aqueous solution of species of theophylline with molecular weights higher than the formula molecular weight of 180.

Theophylline[1] was authenticated by its IR spectrum using a mull and a spectrophotometer[2]. The molecular weight was determined in an analytical ultracentrifuge[3] by the Archibald approach-to-sedimentation equilibrium method, using the schlieren optical system and solutions with concentrations from 1 mg. (5.5×10^{-3} *M*) to 8 mg. (4.4×10^{-2} *M*) solute/ml. solvent. The solvent was water or 0.075 *M* NaCl or 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer, pH 6.95. Attempts also were made to determine the molecular weight of 1 mg. (5.5×10^{-3} *M*) and 0.1 mg. (0.55×10^{-3} *M*)/ml. theophylline solutions using the UV scanner attachment to the ultracentrifuge.

Apparent molecular weights (M_{app}) were determined using Eq. 1:

$$M_{app} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2 Cr} \times \frac{dc}{dr} \qquad \text{(Eq. 1)}$$

where R is the gas constant, $\bar{v}$ is the partial specific volume, T is the absolute temperature, ρ is the density of the solvent, ω is the angular speed of rotation, dc/dr is the concentration gradient at either the meniscus or cell bottom, C is the total solute concentration at either the meniscus or cell bottom, and r is the distance from the center of rotation at either the meniscus or cell bottom.

The concentration gradients at the meniscus and cell bottom were measured from schlieren patterns recorded on metallographic plates[4] and enlarged 10-fold on a magnifier[5]. Total concentration was measured by use of a capillary centerpiece in an interference cell. The integral of the concentration gradient was evaluated by the procedure of Engelberg (5). The partial specific volume, $\bar{v}$, of theophylline was calculated to be 0.72 cm.^{3}/g. from density data, using methodology described by Schachman (6).

[1] Calbiochem.
[2] Perkin-Elmer model 237.
[3] Spinco model E.
[4] Kodak.
[5] Nikon.

Figure 1 summarizes the apparent molecular weights (M_{app}) obtained at various theophylline concentrations. The data indicate the existence of monomers, dimers, trimers, and tetramers, a property similar to some other xanthines (1). The average deviation of 11% between the experimentally determined multimer molecular weight and the nearest integral multimer molecular weight can be explained best by the presence of at least two self-associating species at most concentrations. Indeed, at a concentration of 3 mg./ml. (1.65×10^{-2} *M*), the M_{app} at the meniscus was 400 daltons and the M_{app} at the bottom of the ultracentrifuge cell was 500 daltons, indicating some separation by the ultracentrifuge of dimer from trimer. Another possible source of error, especially at the lower concentrations where small gradients had to be measured, could have been caused by difficulty in measuring the concentration gradients. This latter possibility is less likely than the first explanation since two different ultracentrifugal techniques yielded M_{app} between monomer and dimer at the lower concentrations.

The conclusion by Thakkar *et al.* (3) of self-association of theophylline at concentrations between 5×10^{-3} and 44×10^{-3} *M* was verified by our direct evidence, although their NMR techniques apparently could not differentiate dimers from the higher molecular weight species existing under their experimental conditions[6]. Our data also demonstrate the well-known dissociation of a self-associating system with decreasing solute concentration (11, 12), since monomer predominated at a concentration of 0.55×10^{-3} *M*, dimer predominated at a concentration of 1.1×10^{-2} *M*, and higher molecular weights were found at higher concentrations.

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[6] See also the discussion by Guttman and Higuchi (10).

Factors Affecting Release of Medicaments from Hard Gelatin Capsules

Keyphrases ☐ Drug release from hard gelatin capsules—effect of temperature and pH ☐ Capsules, hard gelatin—effect of temperature and pH on drug release ☐ Dissolution, hard gelatin capsules—effect of temperature and pH

Sir:

In a recent communication, it was suggested that decreased absorption of encapsulated tetracycline when administered concurrently with antacids was due to the insolubility of the gelatin capsules (1). The authors investigated the dissolution of tetracycline capsules at pH 1–9 and found that the capsules disintegrated in acidic media but remained intact in basic media. Although no mention was made of temperature, the study was apparently conducted at room temperature. Because it is not unusual to administer antacids with a number of encapsulated drugs, this finding gives rise to a number of unfavorable implications.

An attempt was made to reproduce the authors' results in a similar experiment in which size 0 gelatin capsules were filled with amaranth and added to solutions whose pH ranged from 1.2 to 9.0. Prolonged release times were obtained at pH greater than 1.2. The capsules did not dissolve or disintegrate to release their contents; instead they swelled and the two halves of the capsules pulled apart.

It was decided to do more closely controlled experiments using 250-mg. chloramphenicol capsules made with a gelatin band around the center[1]. This band prevented the halves of the capsule from separating. For the drug to be released, the capsules would have to disintegrate and/or dissolve.

The capsules were added to a 600-ml. beaker containing 300 ml. of 0.05 *M* phosphate buffer at varying pH. The solutions were stirred at 60 r.p.m. with a Plexiglas paddle, 4.6 × 2.6 × 0.1 cm. The time required for the capsules to break open was recorded at 22.5 and 37.0°.

At body temperature, varying the pH (1.2–9.0) did not affect the average release time (120.7 ± 6.5 sec.) of the capsules. However, at room temperature, pH did affect the release times. The average release times at pH 1.2, 3.0, and 9.0 were 51, 38, and 320 min., respectively. At pH 5.0 and 7.0, the capsules remained intact after 24 hr.

From these data it appears that temperature is the major determinant in the disintegration and/or dissolution of gelatin capsules. The temperature of the stomach is normally about 36°. This temperature can be lowered by eating such things as ice cream, but the temperature returns to normal within 30 min. (2). It is unlikely that this variation in stomach temperature would frequently interfere with the release of encapsulated medications. Also, it is clear that raising the gastric pH does not, as the authors suggest, affect the disintegration and/or solubility of hard gelatin capsules unless accompanied by a lowering of the temperature of the stomach's contents.

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Batch Production of Pharmaceutical Granulations in a Fluidized Bed III: Binder Dilution Effects on Granulation

Keyphrases ☐ Granulation, fluidized bed—effects of binder dilution on granule properties ☐ Binder dilution effect—fluidized-bed granulation

Sir:

Previous reports (1, 2) were concerned with process variables in the operation of a fluid-bed spray granulator[1] and the effects of various binders and their concentrations on the physical properties of fluidized-bed granulations and tablets compressed from these granulations. The present communication describes the effects of two aqueous dilutions of various formula weights of gelatin binder on the physical properties of fluidized-bed granulations.

Successful granulation in a fluidized bed results primarily from a balance of material input and output (3, 4) which is determined by operational factors such as the rate of binder addition, fluidizing air temperature, and volume and nozzle height with respect to the fluidized solids (1). In addition, further studies showed that the quantity of binder solvent used for fluidized-bed granulating may be equally influential. Since the quantity of solvent used in preparing a binder solution may often be arbitrary, depending upon solution viscosity, binder solubility, desired granulating cycle time, *etc.*, the influence of the solvent quantity on the physical

[1] Glatt model WSG-15, Fa. W. Glatt, Haltingen, West Germany.

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Table I—Physical Properties of Fluidized-Bed Granulations Prepared from Various Formula Weights and Aqueous Dilutions of Gelatin Binder[a]

Physical Properties	Formula Weight					
	2.75% w/w		3.50% w/w		4.25% w/w	
	2.0[b]	4.0[b]	2.0[b]	4.0[b]	2.0[b]	4.0[b]
Average granule size, μ	299	287	328	317	367	351
Friability, %	11.3	6.6	10.2	7.2	8.2	6.9
Bulk density, g./ml.	0.39	0.45	0.36	0.42	0.34	0.39
Granule density, g./ml.	1.492	1.480	1.487	1.472	1.479	1.471
Interparticulate porosity, %	74.3	69.6	75.8	71.5	77.0	73.5
Flow rate, g./min.	127.5	156.8	105.9	129.6	93.5	116.5

[a] For comparison purposes, results of the physical properties of granulations prepared with 2.0 kg. of distilled water as the binder dilution factor are reprinted from *Reference 2*. [b] Aqueous dilution factor in kilograms.

properties of the final granulation should be investigated. The following formulation was manufactured in 10-kg. batches in the fluid-bed granulator:

	% w/w
lactose USP	85.25–86.75
starch USP	10.00
gelatin USP	2.75–4.25
magnesium stearate USP	0.50
distilled water	—

Three formula concentrations (2.75, 3.50, and 4.25% w/w) of gelatin binder were selected, and two aqueous dilution factors (2.0 and 4.0 kg. distilled water) were utilized in preparing the binder solution at each concentration. Preparation of the binder solutions, granulation and drying procedures, and the fluid-bed spray granulator were extensively discussed previously (1, 2) along with the methods employed in determining average granule size, granule friability, loose bulk density, granule density, interparticulate porosity, and flow rate.

The effects on the granulation's physical properties of various formula weights of gelatin binder dissolved in 2.0 and 4.0 kg. of distilled water are illustrated in Table I. Since the effects of the formula binder weight, *i.e.*, 2.75–4.25% (w/w), on the physical properties of fluidized-bed granulations were discussed in a previous paper (2), only the effects of the quantity of water utilized in binder solution preparation are now interpreted. Those binder solutions prepared with 4.0 kg. of distilled water were approximately one-half the concentrations of their respective solutions made with 2.0 kg. of water. Since this dilution doubles the binder solvent employed in the granulation operation, the lack of any significant influence on the average granule size implies that water, alone, would be a poor granulating agent for these particular formulations. In fact, a slight decrease in the average granule size was noted on dilution of each of the three formula binder concentrations. Presumably, this may be attributed to a decrease in the binder solution's tackiness or adhesiveness.

The most significant changes in the physical properties affected by binder dilution were found in granule friability and bulk density. It is obvious from the data presented in Table I that the granule size was most affected by the formula binder concentration, *i.e.*, 2.75–4.25% (w/w), while granule friability was most influenced by aqueous binder dilution, *i.e.*, 2.0–4.0 kg. water. This development is significant since weak granulations may be made less friable simply by further diluting the binder solution prior to its addition in the granulating cycle. This effect of strengthening granules may be somewhat correlated with previous investigations (1), where similar results were observed by increasing the penetration and wetting of the fluidized solids by the binder solution.

Dilution of the binder solution with an additional 2.0 kg. of water (4.0-kg. total) increased the bulk density of the granulation similarly by prolonging the exposure of the fluidized solids to the binder solution. This method of increasing granule strength and density by further diluting the binder system has one noted distinction from previously reported methods (1), *i.e.*, increasing binder solution addition rate, decreasing binary nozzle atomization pressure, decreasing inlet air temperature, *etc.* These latter process changes all resulted in significant increases in the average granule size; this particle growth was not observed when granulating with the less adhesive diluted binder solutions.

The differences observed in the granule densities were small. Since granule density is a measure of the entrapped air within the interstices of the granules, it may be concluded that the intraparticulate void spaces of the granules were unaffected by binder dilution. The increased flow rates of granulations manufactured from equal but diluted formula binder concentrations are associated with the decreased porosities of these more dense granulations.

In summary, while binder dilution has insignificant effects on the average granule size and granule density of granulations manufactured in a fluid-bed spray granulator, considerable influence was observed on granule friability, bulk density, interparticulate porosity, and, thus, on flow rate.

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Orthokinetic Coagulation in Pharmaceutical Suspensions

Keyphrases ☐ Suspensions, pharmaceutical—orthokinetic coagulation due to sedimentation and laminar shear ☐ Coagulation (orthokinetic), suspensions—based on sedimentation and laminar shear

Sir:

The classical theory of perikinetic coagulation of lyophobic colloids was developed by Smoluchowski (1) for spherical uniformly sized particles undergoing diffusion as a result of Brownian motion. Attempts to apply the Smoluchowski theory to pharmaceutical suspensions are of limited success for the following reasons.

Particle Size—In pharmaceutical suspensions, most of the particles are of dimensions greater than the upper limit for lyophobic colloids, which is usually put at 0.5-μm. diameter (2). The basic equations derived by Smoluchowski for the coagulation rate of particles in these systems (3) have no term for particle size, but it was previously reported (4) that a 1.1-μm. diameter polystyrene latex has a slower maximum coagulation rate than one of 0.7-μm., whose rate was also slower than the Smoluchowski theory. Such deviations from theory were explained by Higuchi *et al.* (5) in terms of hydrodynamic effects. From the Smoluchowski theory, it may be calculated that the 2.5% (w/v) suspensions of the drug griseofulvin, described earlier (6), would have a coagulation time (*i.e.*, time to reduce the original particle concentration by one-half) of 6 sec. if the particles were 1.0 μm. in diameter and of 100 min. if they were 10 μm. in diameter. In the latter case the particles would be subject to rapid sedimentation and to only relatively little Brownian motion. It has been shown (7) that for griseofulvin suspensions, Brownian motion is theoretically a greater source of displacement than sedimentation up to a diameter of 1.7 μm. Above this figure the reverse applies.

Polydispersity—The Smoluchowski theory was extended by Müller (8) to allow for polydispersity. This theory was shown by Matthews and Rhodes (9) to apply qualitatively to particles in the size range of 0.5–2.0 μm.

Dimensional Anisotropy—Müller (10) also extended the Smoluchowski theory to allow for nonspherical particles. He showed that these should coagulate more rapidly than spherical ones and this was confirmed by Wiegener and Marshall (11).

Nature of the Interface—Many powdered drugs that are formulated as aqueous suspensions are truly hydrophobic and must be wetted prior to dispersion. The use of amphipathic adjuvants for this purpose is likely to effect steric stabilization of the particles (12, 13).

The question of whether or not drug particles may be flocculated or coagulated in an analogous way to lyophobic colloids to prevent impaction on storage was discussed extensively in the literature (6, 13–18). Because of the previously mentioned differences between lyophobic colloids and drug suspensions, it can be misleading to apply criteria derived from colloid chemistry to differentiate between aggregation mechanisms in coarse systems. For example, coagulation in a lyophobic colloid produces a compact sediment whereas flocculation yields a porous compressible structure. However, when a suspension of highly dispersed drug particles is allowed to sediment, a compact structure usually is produced. An evaluation of such a suspension on the basis of criteria taken from colloid chemistry would suggest that it had coagulated. A state of a high degree of dispersion or peptization is, however, the exact opposite of coagulation in true colloids. When the forces causing particle dispersion in a suspension are removed, coagulation occurs. Since Brownian motion cannot be considered as the main cause of coagulation in the case of pharmaceutical suspensions, it has been tentatively suggested (13) that differential sedimentation rates could cause the necessary particle collisions. Theories of orthokinetic coagulation, where the causative agent is systematic movements within the system (*e.g.*, sedimentation) were formulated by von Smoluchowski (1), Tuorila (19), and Müller (10). Hiestand (20), in his comprehensive review of coarse suspensions, differentiated between the effects of sedimentation on peptized and aggregated systems and described cases in the literature where agitation was shown to accelerate flocculation. As far as is known, however, no attempt has been made to interpret aggregation processes in pharmaceutical systems using these early theories (1, 10, 19). This is the purpose of the present communication. Two types of orthokinetic coagulation that could occur in pharmaceutical suspensions will be considered.

Coagulation Due to Sedimentation—Whereas the theory for perikinetic coagulation was formulated for a monodispersed suspension, coagulation due to sedimentation can only occur if the system is polydispersed. Müller (10) considered a large spherical particle of radius R sedimenting under gravity with a speed V_R in a rapidly coagulating colloid containing, at time t_0, N_0 spherical particles of radius r. Brownian motion of the large particle and sedimentation of the small ones are initially ignored. He derived the following equation to express the number of small particles, dN, that coagulate with the large one in time dt:

$$\frac{dN}{dt} = 4\pi N_0 D x \frac{\sinh C}{C} \qquad \text{(Eq. 1)}$$

where D is the diffusion constant of the small particles and x is the collision radius of the large and small particles $= r + R$. The value of C is defined by the equation:

$$C = \frac{V_R}{D}\left[x - R\left(\frac{3}{2}\ln\frac{x}{R} + \frac{R^2}{4x^2} + \frac{3}{4}\right)\right] \qquad \text{(Eq. 2)}$$

Since the rate at which the small particles would be expected to coagulate with large ones is given according to the Smoluchowski theory by the equation:

$$\frac{dN}{dt} = 4\pi N_0 D x \qquad \text{(Eq. 3)}$$

the probability of coagulation when one of the particles

Table I—Dependence of the Value of the Müller Constant C on the Radii of the Coagulating Particles in a Suspension of Fine-Particle Griseofulvin

r, μm.	R, μm.	(α)	R^4	$f(\alpha)$[a]	C
0.5	10	0.05	10^{-12}	2.4×10^{-6}	0.107
1.0	10	0.1	10^{-12}	3.6×10^{-6}	1.601
5.0	10	0.5	10^{-12}	7.7×10^{-3}	342.4
8.0	10	0.8	10^{-12}	1.46×10^{-2}	649.3
9.0	10	0.9	10^{-12}	1.10×10^{-2}	493.6
0.5	5	0.1	6.25×10^{-14}	3.6×10^{-5}	0.100
1.0	5	0.2	6.25×10^{-14}	4.5×10^{-4}	1.25
2.0	5	0.4	6.25×10^{-14}	4.25×10^{-3}	11.81
4.0	5	0.8	6.25×10^{-14}	1.46×10^{-2}	40.57
4.5	5	0.9	6.25×10^{-14}	1.10×10^{-2}	30.57
0.5	2.5	0.2	3.90×10^{-15}	4.5×10^{-4}	0.078
1.0	2.5	0.4	3.90×10^{-15}	4.25×10^{-3}	0.737
1.5	2.5	0.6	3.90×10^{-15}	1.13×10^{-2}	1.959
2.0	2.5	0.8	3.90×10^{-15}	1.46×10^{-2}	2.53
2.25	2.5	0.9	3.90×10^{-15}	1.10×10^{-2}	1.910

[a] Data taken from Müller (10).

is sedimenting with a speed of V_R is increased by the factor sinh C/C.

When the ratio of the radii r/R is not very small, the Brownian motion of the large particle and sedimentation of the small ones have to be considered by the modified equation for the diffusion constant:

$$D = \frac{kT}{6\pi\eta}\left(\frac{1}{R} + \frac{1}{r}\right) \qquad \text{(Eq. 4)}$$

V_R is replaced by the relative sedimentation velocity, v, in Eq. 2 and the modified Stokes' law:

$$v = (V_R - V_r) = \frac{2}{9}\frac{\rho g(R^2 - r^2)}{\eta} \qquad \text{(Eq. 5)}$$

where ρ is the difference in density between the dispersed and continuous phases. The other symbols have their usual meanings.

By combining Eqs. 2, 4, and 5, Müller (10) derived the following expression for the value of C:

$$C = \frac{4\pi\rho g R^4}{3kT} f(\alpha) \qquad \text{(Eq. 6)}$$

where:

$$f(\alpha) = \alpha(1 - \alpha)\left[\alpha + \frac{\alpha(2 + \alpha)}{4(1 + \alpha)^2} - \frac{3}{2}\ln(1 + \alpha)\right] \qquad \text{(Eq. 7)}$$

and:

$$\alpha = \frac{r}{R} \qquad \text{(Eq. 8)}$$

Müller showed that for the sedimentation effect to be significant, the sedimenting particle should be larger than:

$$R \geq \sqrt[4]{\frac{40kT}{\pi\rho g}} \qquad \text{(Eq. 9)}$$

and the particles that are caught by the falling one should be larger than:

$$r \geq \sqrt[4]{\frac{1{\cdot}2\,kT}{\pi\rho g}} \qquad \text{(Eq. 10)}$$

It is now possible to consider the application of the Müller theory to the fine-particle griseofulvin suspensions described earlier (21). Using a value for ρ of 0.445 g./ml., the critical values for r and R are 0.77 and 1.85 μm., respectively. Measurement of the particle size of the drug by means of the Coulter counter (22) showed that most of the particles were between 0.5- and 10.0-μm. equivalent spherical radius. Therefore, values of C were calculated using values of R of 2.5, 5, and 10 μm. and of r within the range of 0.5–9.0 μm. The results are shown in Table I. The dependence of the ratio (sinh C/C) upon C is shown in Table II. When C = 2.1, the speed of coagulation is doubled; when $C > 5$, the sedimentation effect is so great that coagulation due to Brownian diffusion can be ignored. An inspection of Table I shows that for large particle radii, R of 10 and 5 μm., very pronounced effects due to sedimentation can be expected. Even for particles of R = 2.5 μm., an approximate twofold increase in coagulation rate can be expected for r values of about 1.5 μm. and above.

Table II—Relationship between the Müller Constant C and sinh C/C[a]

C	$\frac{\sinh C}{C}$	C	$\frac{\sinh C}{C}$
0	1	2	1.81
0.2	1.006	3	3.84
0.4	1.027	5	14.83
0.6	1.066	7	78.4
0.8	1.110	9	450
1	1.175	10	1100

[a] Data taken from Müller (10).

Similar calculations can be performed for other drugs and for different vehicles; for example, the suspension of fine-particle griseofulvin in 70% glycerol described earlier by Matthews and Rhodes (7) has a phase density difference of 0.272 g./ml., very similar to that of a suspension of hydrocortisone in water. For such systems the critical radii increase to r = 0.88 μm. and R = 2.11 μm. and a similar sedimentation effect would only be experienced with larger particles.

The use of a slow speed centrifuge was advocated by Jones (23) as a means of accelerating physical changes in suspensions. The Müller theory provides a possible method of quantizing the effect of centrifuging on particle coagulation since the centrifugal force can be substituted for g in Eqs. 6, 9, and 10. However, it is important to bear in mind that centrifuging may also cause the breakup of the loose coagulum at the base of the container, causing an impaction which may not occur under conditions of normal gravity.

Coagulation Due to Laminar Shear—The von Smoluchowski (1)–Tuorila (19) theory of laminar shear coagulation was conveniently summarized by Overbeek (24) and was used to interpret the flocculation of agitated suspensions of ferric oxide by Reich and Vold (25). The equation derived was:

$$\frac{J}{I} = \frac{\eta R^3(du/dz)}{2kT} \qquad \text{(Eq. 11)}$$

where J is the probability of collision due to motion in a laminar flow field of shear gradient du/dz, I is the probability of collision due to Brownian motion, η is

the viscosity of the medium, and R is the particle radius. Since the effect of laminar shear is independent of the particle density, the conclusions of Reich and Vold are also applicable to pharmaceutical suspensions. They calculated that for a shear gradient of 100 sec.$^{-1}$, J/I is 10^{-2} for 0.1-μm. diameter particles, 10 for 1.0-μm. diameter, and 10^{4} for 10-μm. diameter. This shows again that, at around a diameter of 1 μm., orthokinetic effects assume a greater importance in comparison with perikinetic.

Discussion—To produce a pharmaceutical suspension that will sediment to give an open-structured redispersible coagulum, two processes are necessary: (*a*) particle collisions in the suspension, and (*b*) controlled particle adhesion on contact.

This communication has indicated that, in the absence of Brownian motion, sedimentation and shear effects can cause the former and the magnitude of the latter can be estimated by calculating energy of interaction curves (13).

Coagulation could be produced in a pharmaceutical suspension by the application of controlled shear, but care would be necessary since shear can also reverse the process and cause a breakdown in the structure. The formulator must design the suspension so that shear forces induced by shaking the container achieve this purpose. The sedimentation effect is inherent in the nature of the system, provided that there is a density difference between the phases and the suspension is polydispersed. Most pharmaceutical suspensions containing finely milled powders may be expected to have a proportion of particles in the region where Brownian motion is operative, and this proportion can be significant if calculated by number. Brownian coagulation eventually produces aggregates which are large enough to take part in sedimentation coagulation.

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Compendial Dissolution Tests: Merits of Sequential over Standard Inspection Plans

Keyphrases ☐ Dissolution tests, compendial—statistical analysis, proposed sequential analysis inspection of dosage units ☐ Sequential analysis inspection—application in dissolution testing ☐ Inspection of tablet and capsule lots—sequential analysis plan for dissolution rate testing ☐ Sampling of tablet and capsule lots—sequential analysis inspection plan.

Sir:

Dissolution rate tests for tablets and capsules are destructive tests, so any acceptance inspection plan for this property that is included in a compendial monograph must be based on the results of the complete analysis of one or more randomly selected samples. All sampling inspection plans carry two inherent risks because the quality of the chosen sample or samples may not truly reflect the absolute quality of the lot. The first risk is that a lot whose absolute quality is acceptable will have to be rejected. This risk, designated α, has its greatest influence on the economics of production. The second risk is that a lot whose absolute quality is unacceptable will pass inspection. This risk, designated β, has its greatest effect on the therapeutic effectiveness of the lot and, hence, on the consumer.

This communication is concerned with computing the values of α and β that are inherent in the dissolution tests in USP XVIII (1) and NF XIII (2) and with proposing the adoption of an alternative test based on a sequential analysis plan (3). No attempt is made to address the equally important question concerning the meaningfulness, in terms of the bioavailability, of the presently defined criterion of good and bad dissolution behavior which is based on the time it takes for 60% of the drug to dissolve from its dosage form. Regardless of what criteria may be laid down to ensure bioavailability, it is essential that the inspection plan used is one that carries values of risks α and β that are consistent with production economy and therapeutic effectiveness.

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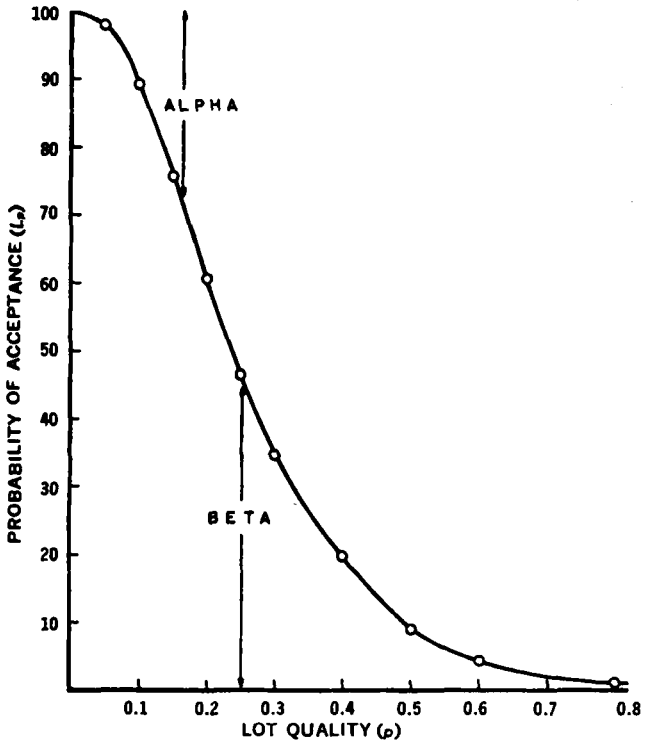

Figure 1—*Operating characteristic curve for the current compendial dissolution test.*

The current compendial dissolution tests (1, 2) are based on a standard double-sampling inspection plan. They require that an initial sample of six dosage units be individually subjected to a standardized dissolution test. The lot can then be accepted if all six units meet specified requirements but must be rejected if three or more units do not meet these requirements. If one or two units fail to meet the requirements, a further sample of six units must be examined. The lot can then only be accepted if no fewer than 10 of the 12 units meet the requirements. The overall probability, L_p, that a lot will be accepted under this plan is given by the sum of the probabilities that: (*a*) there are no defective units in the first sample of six, (*b*) there is one defective unit in the first sample of six and one or none in the second sample, and (*c*) there are two defective units in the first sample and none in the second.

The value of L_p is, in turn, related to the absolute quality of the lot, p, that is presented for inspection (quality being expressed as the fraction of defective units in the lot). Since the individual monograph in the USP specifies a single criterion for a good or bad product (*i.e.*, the time required for 60% of the drug to dissolve), the sample appears to follow a binomial distribution. By employing the Poisson distribution as its approximation, L_p can be readily computed for any value of p. (Molina's table was used in this report. See *Reference 4* for more details.) A plot of L_p against p (operational characteristic curve) for the current compendial tests is shown in Fig. 1. It can be seen that these tests involve a probability or risk of 47% of accepting a lot of quality 3/12. This is the maximum value of the risk β of a lot that is really of unacceptable quality passing inspection. Similarly, it can be seen that the probability

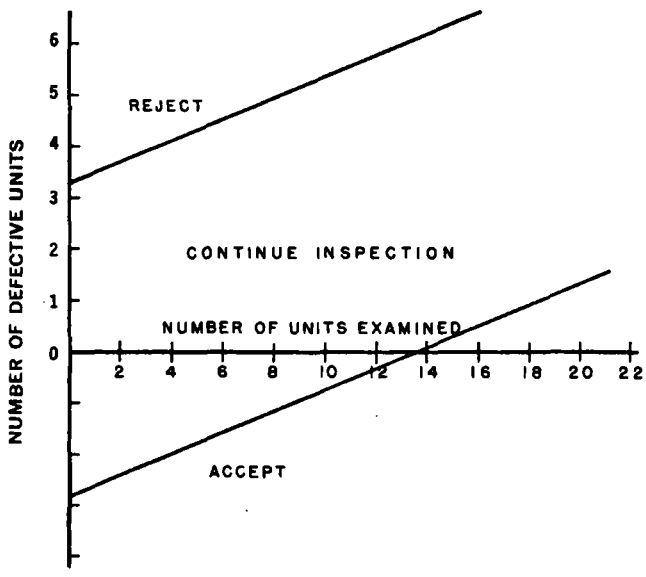

Figure 2—*Criteria for acceptance or rejection of a lot when the risks α and β are assigned to 15 and 20% and acceptable and unacceptable quality are specified as two or less defective units out of 12 and three or more defective units out of 12, respectively.*

of accepting a lot of absolute quality 2/12 is only 70%. Hence, the risk of rejecting an acceptable lot, α, using this plan runs as high as 30%. These risks seem extraordinarily high for a compendial test.

An alternative inspection plan based on sequential analysis appears to offer the possibility of a more meaningful test. A sequential analysis plan involves continuous analysis of units (or groups of units) from a lot until the inspector accumulates sufficient evidence to decide that, within certain predetermined values of α and β, the lot is of acceptable or unacceptable quality.

The criteria for acceptance or rejection of a lot under this type of plan are specified by two parallel oblique lines on a plot of number of defective units found against the total number of units tested. Such a figure is shown in Fig. 2 for a plan where α and β are assigned values of 15 and 20%, respectively, and acceptable and unaccept-

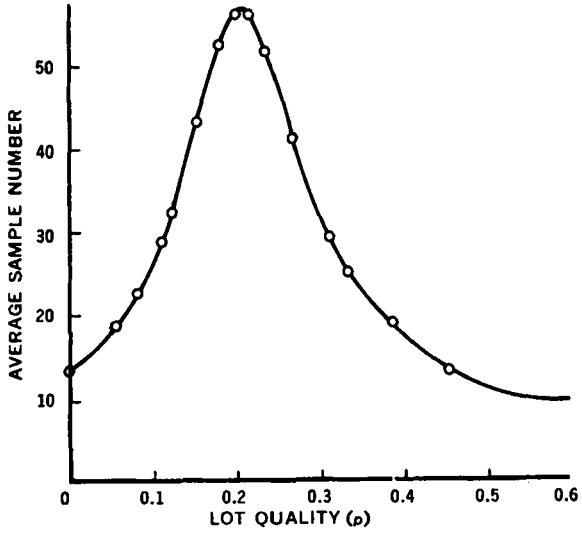

Figure 3—*Average sample of number as a function of the submitted lot quality under the same conditions as in Fig. 2.*

able quality are specified as two or less defective units out of 12 and three or more defective units out of 12. As the inspector tests units selected at random from the lot, he constructs a plot of number of defectives against number of units tested. If the plot intersects or crosses the lower line, the lot is accepted. Conversely, if the plot intersects the upper line, the lot is rejected. Inspection must, however, continue as long as the plot remains within the parallel lines.

The equations defining the parallel lines will not be developed here as they are well established in the literature (3). Suffice it to say that the slopes of both lines are uniquely defined by the limits set for acceptable or unacceptable quality, and the intercepts of the lines on the y axis are a function of both the quality limits and the predetermined values of risks α and β.

Some advantages of such an inspection plan over standard inspection plans are:

Flexibility—While it is anticipated that compendia would specify common quality criteria for all tablets and capsules, a sequential analysis inspection plan makes it possible to assign different values of the tolerable risks α and β to meet specific situations. For example, if it were known that the therapeutic effectiveness of Drug A was very much more dependent on the dissolution rate than that of Drug B, it may be advantageous to have a lower tolerable value of β for Drug A than for Drug B. This flexibility is not possible in a standard plan where the number of tests to be carried out is specified and, consequently, the values of α and β are fixed.

Number of Tests Required—To reach a decision on whether to accept or reject a lot within specified limits of risks α and β, a sequential analysis plan will always involve less testing than a standard plan (3). Consequently, such a plan will result in a production economy. Also, the number of tests required before a lot can be accepted can be minimized by a manufacturer who uses good quality control and only submits high quality material for inspection. This can be seen from the plot in Fig. 3 of the average number of tests required to reach a decision against the absolute quality of the submitted lot (computed as described in *Reference 3*).

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REVIEWS

USAN 10 and the USP Dictionary of Drug Names. Published for the USAN Council by the U. S. Pharmacopeial Convention, Inc., Rockville, MD 20852, 1972. xii + 200 pp. 50 × 60 cm. Price $15.00.

If the USP officials had resisted the temptation to project a bit of chauvinism in their new title to this publication, it would be hard to fault the new edition. Indeed, it has much more information, presented in a larger and more convenient page size, than ever before. Moreover, the thought and editorial care devoted to organizing and providing a considerable amount of diverse factual information in a concise yet readily comprehensible format are immediately apparent to the reader.

There also is every reason to expect that the accuracy of the technical content will merit the reader's confidence. Although we are aware that past USAN booklets published by the USPC have taken certain liberties with the "official" USAN lists as they have emanated from the USAN office, the extent of such editorial license in the present volume can only be assessed after considerable use and close review. Furthermore, the matter of compiling completely accurate information of this nature in a uniform format presents so many difficulties and complications that few if any other drug name dictionaries could even approach the apparent completeness and reliability of this current volume.

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Reviewed by H. Latham Breunig
Eli Lilly and Company
Indianapolis, IN 46206 ■

Ups and Downs, Drugging and Duping. By J. Rice, M.D. Macmillan, 866 Third Ave., New York, NY 10022, 1972. 214 pp. 13.5 × 21 cm. Price $5.95.

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Journal of Pharmaceutical Sciences

FEBRUARY 1973
VOLUME 62 NUMBER 2

REVIEW ARTICLE

Oral Contraceptives: Therapeutics *versus* Adverse Reactions, with an Outlook for the Future I*

A. S. BINGEL▲ and P. S. BENOIT

Keyphrases ☐ Oral contraceptives—mechanisms of action, adverse effects, review ☐ Contraceptives, oral—mechanisms of action, adverse effects, review ☐ Thromboembolic disorders—relationship to oral contraceptives, review ☐ Cancer and hyperplasia—relationship to oral contraceptives, review ☐ Hypertension—relationship to oral contraceptives, review ☐ Carbohydrate metabolism—effect of oral contraceptives, review ☐ Diabetes—relationship to oral contraceptives, review ☐ Lipid metabolism—effect of oral contraceptives, review ☐ Protein metabolism—effect of oral contraceptives, review ☐ Liver dysfunction and jaundice—relationship to oral contraceptives, review ☐ Amenorrhea and galactorrhea—relationship to oral contraceptives, review

Oral contraceptives have been in general use for only slightly more than a decade, but during this time a large volume of literature has accumulated concerning primarily the mechanisms of action and the adverse reactions of the estrogenic and progestagenic components of these drugs. In addition, numerous reports have been published concerning clinical studies in which these synthetic hormones have been employed for the purpose of contraception, but in dosage forms and on dosage schedules differing, sometimes markedly, from those of the commercially available oral contraceptives. The purposes of this review are to discuss and to summarize the more recent developments concerning these various aspects of hormonal contraception. However, since the mechanisms of action of the oral contraceptives involve modifications of the hormonal and hormone-related events occurring during the normal ovulatory menstrual cycle, such a review would be incomplete unless prefaced by a summary of these events.

GENERAL CONSIDERATIONS

Menstrual Cycle—The recently increased accumulation of knowledge concerning hypothalamic–pituitary–ovarian interrelationships in the human have been made possible as a result of the discovery of the hypothalamic-releasing hormones (factors) (1) and the development of competitive binding techniques (2) that permit the accurate measurement of the minute amounts of gonadotropins and gonadal steroid hormones present in as little as 1 ml. of serum.

A number of recent reviews (1–15) summarized the observations made in numerous studies since the late 1960's. Briefly, gonadotropin [follicle-stimulating hormone (FSH) and luteinizing hormone (LH)] secretion by the anterior pituitary is under the control of the hypothalamus which secretes small polypeptides into the portal system connecting the median eminence of the hypothalamus with the anterior pituitary. These hormone-specific releasing factors (FSH-RF and LH-RF) [designated by some investigators as releasing hormones (FSH-RH and LH-RH) (1)] act on the pituitary to stimulate the release and, perhaps, also the synthesis of FSH and LH. Furthermore, evidence indicates that two hypothalamic centers may be responsible for LH release: a "tonic" center concerned with the regulation of basal LH secretion and a "clonic" ("cyclic") center responsible for triggering the ovulatory surge in LH secretion. The hypothalamus, in turn, is influenced by the estrogen and progesterone secreted by the ovary

* *Editor's note:* Part II of this article will appear in the March 1973 issue of the *Journal of Pharmaceutical Sciences*.

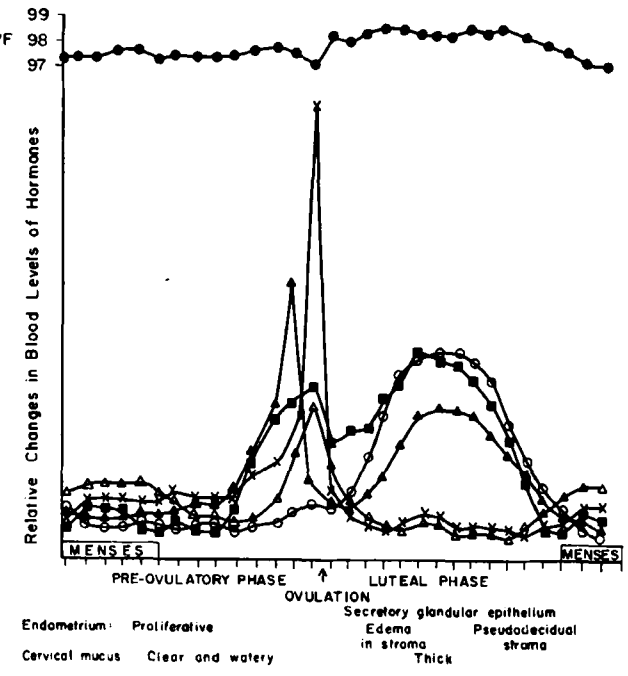


Figure 1—*An idealized composite of the hormonal and hormone-related events occurring during the normal menstrual cycle (2–15). Key: basal body temperature (●—●), FSH (△—△), LH (×—×), estrogen (▲—▲), 17α-hydroxyprogesterone (■—■), and progesterone (○—○).*

in response to gonadotropic hormone stimulation. Depending on conditions, their effects may be either inhibitory or facilitative; that is, they may exert a negative or positive feedback on the hypothalamus. In addition, the tropic hormones themselves may exert a negative feedback on the hypothalamus (3). Finally, emotional and external environmental factors, mediated by various areas of the CNS, can also influence the hypothalamic control of the menstrual cycle (induction of psychogenic amenorrhea, for example) (4).

The various hormonal and hormone-related events occurring during the normal menstrual cycle are depicted in Fig. 1. Since the units of measure vary for the individual hormones and since absolute concentrations vary among individual women, only relative, qualitative changes during the cycle have been indicated. Indeed, the possibility was suggested (5) that an occasional woman may be sufficiently stressed by daily blood sampling procedures that an adverse effect on hormonal events during the cycle may be produced; hence, the differences (*vide infra*) observed among various studies may or may not represent true physiological variability.

As shown in Fig. 1, toward the end of the luteal phase of the preceding cycle, concentrations of estrogen and progesterone decline; in response, the previously suppressed FSH and LH levels begin to increase, initiating another cycle of follicular maturation (6). Estrogen secretion increases markedly, culminating in a sharp peak followed by a drop prior to ovulation; then there is a subsequent smaller rise during the luteal phase.

The preovulatory rise in estrogen secretion exerts a negative feedback on the secretion of FSH which, therefore, declines. LH levels continue to rise, however, culminating in a sharp preovulatory surge of LH; following ovulation, LH levels decrease to values comparable to or less than those seen during the preovulatory phase. The cyclic (clonic, ovulatory) release of LH is thought to be due to a positive feedback effect of rising estrogen levels (7) and/or a negative feedback effect due to the dip in estrogen secretion that may precede the peak LH level (3).

The possible role that may be played by preovulatory progesterone secretion by the follicle and/or the adrenal (8) in the triggering of LH release in the human is a matter of controversy (9). 17α-Hydroxyprogesterone has been shown to increase prior to the LH peak, but this compound is not believed to be of physiological significance (10). The small periovulatory increase in progesterone secretion, on the other hand, appears to be a consequence of LH secretion rather than a stimulus for it, although the possibility that it may act centrally in a facilitatory manner to maintain and prolong the surge of LH has not been ruled out (7).

The low postovulatory secretion of LH appears to be responsible for stimulating the newly formed corpus luteum to continue secreting increased amounts of progesterone and estrogen. Nevertheless, despite continuous exogenous administration of LH, it has been shown that the secretory capacity of the corpus luteum declines (spontaneously or perhaps due to some unknown luteolytic factor) at 9–11 days after ovulation (7).

Most investigators also find a midcycle peak in FSH secretion, usually coincident with the LH peak (2, 3, 6, 7), although sometimes preceding (3) or following it (9). Its physiological significance is unknown, and the physiological stimulus responsible for it is merely speculative (7).

Concurrent with the preovulatory peak in estrogen secretion, a drop in basal body temperature is usually seen (3, 11); following ovulation, there is a rise in basal body temperature which parallels the rise in progesterone levels. Sometimes the rise in temperature is abrupt, as shown in Fig. 1, but at other times it is slow, requiring several days before preovulatory levels are exceeded. Results of a study (12) comparing these two types of temperature rise suggest that ovulation may not bear a similar temporal relationship to the low temperature point in these two conditions, *i.e.*, ovulation may occur later when there is a slow ("staircase") rise.

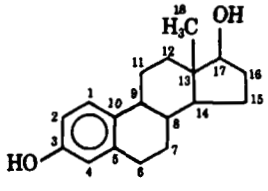
In addition to the negative and positive feedback effects that ovarian steroids have on the hypothalamus, they also produce cyclic changes in the endometrium and the cervical mucus. Specifically, prior to ovulation the endometrium is of a proliferative type due to the action of estrogen alone; following ovulation, progesterone and estrogen combine to produce secretory changes in the glandular epithelium and edema in the stromal tissue. Toward the latter portion of the cycle, pseudodecidual changes occur in the endometrial stroma (13). Very late in the cycle, as a result of the decline in estrogen and progesterone production, menstruation occurs; the endometrium responds to the withdrawal of the hormonal support by desquamating (14).

During the preovulatory phase and especially just prior to ovulation, the cervical mucus becomes increasingly more favorable, in physicochemical char-

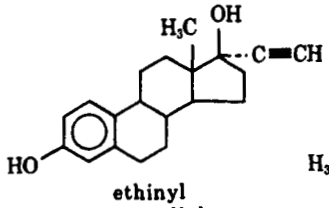
acteristics, to the migration of sperm (15). It forms a thin, clear, watery liquid which can be stretched into threads (spinnbarkeit). It is rich in sodium chloride, a decisive factor in determining consistency, and when dried on a slide, crystals of the salt form fern-like patterns (arborization); viscosity is minimal and cellular debris virtually absent. Finally, the micelles comprising the mucus are thought to be present in long chains arranged approximately in parallel order. In contrast, the mucus becomes thick under the influence of progesterone in the luteal phase. There is coiling of the mucoid molecules, and the micelles form a dense network containing much cellular debris; spinnbarkeit and arborization (ferning) disappear.

Chemistry—The chemical structures of the two estrogens and six progestagens present in the commercially available oral contraceptives are shown here. The structures of naturally occurring estradiol and testosterone, to which the synthetic compounds, respectively, are chemically related, are included for comparison.

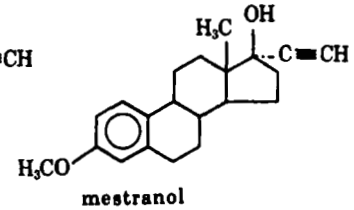
estrogens



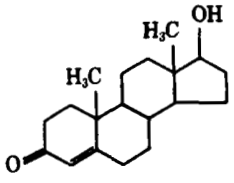
estradiol



ethinyl estradiol

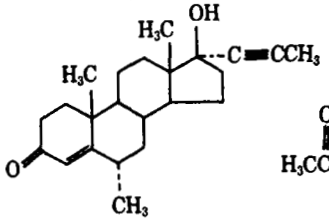


mestranol (ethinyl estradiol 3-methyl ether)

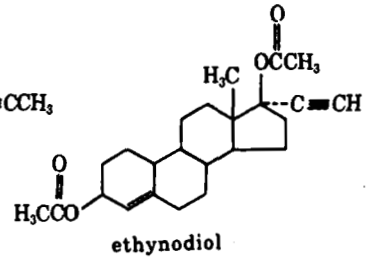


testosterone

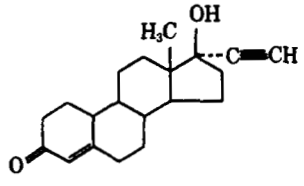
progestagens



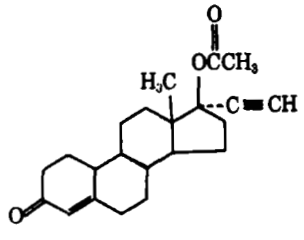
dimethisterone



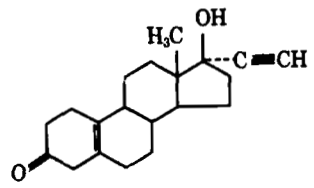
ethynodiol diacetate



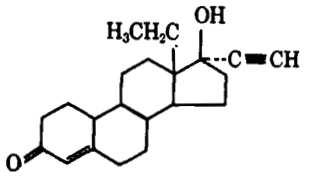
norethindrone (norethisterone)



norethindrone acetate



norethynodrel



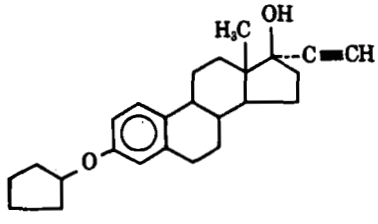
norgestrel

Removal of the 19-methyl group from testosterone, yielding 19-nortestosterone, markedly reduces the androgenicity of the molecule (16). It is actually the latter compound to which the progestagens, excluding dimethisterone, are more closely related. Addition of the 17α-ethinyl group confers progestational activity on 19-nortestosterone (17), and further modifications have resulted in compounds which, on the basis of data obtained from studies in lower animals (16), appear to differ slightly among their other biological properties (18).

Dimethisterone, a potent antiandrogen, for example, is without estrogenic or androgenic properties in contrast to ethynodiol diacetate, which has some estrogenicity and slight androgenicity. Norethindrone, norethindrone acetate, and norgestrel all possess some androgenicity, while norethynodrel has estrogenic activity thought to be due to the double-bond configuration between carbon positions 5 and 10 in the latter compound (19).

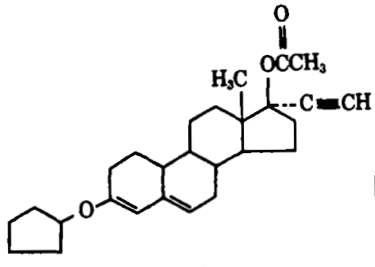
The chemical structures of a number of other steroids with contraceptive properties are also shown here.

estrogen

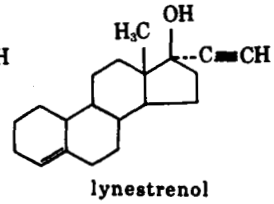


quinestrol

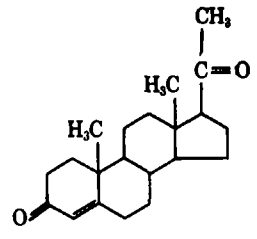
progestagens



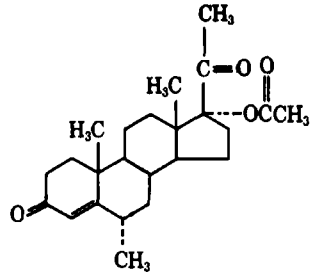
quingestanol acetate



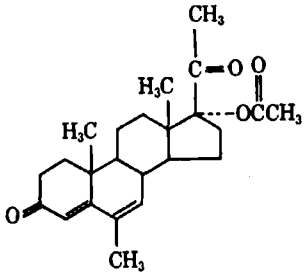
lynestrenol



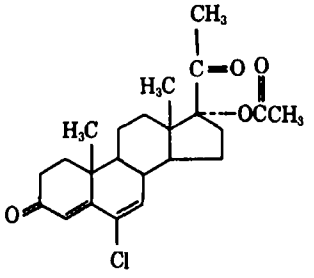
progesterone



medroxyprogesterone acetate



megestrol acetate



chlormadinone acetate

These compounds have been and/or are being used alone or in combined estrogen–progestagen preparations, largely in investigational studies, in the United States and elsewhere (*vide infra*).

Estradiol is not active orally; addition of the 17α-ethinyl radical, however, as in ethinyl estradiol and its 3-methyl ether, mestranol, renders the molecule orally active (17). The 3-cyclopentyl ether derivative quinestrol is not only active orally but has an extremely prolonged duration of action due to its slow release from fat stores (20). In contrast, quingestanol acetate, the 3-cyclopentyl enol ether derivative of norethindrone acetate, is not stored in body fat and does not have prolonged activity (21). Results of a study (22) of other 19-nortestosterone 3-cyclopentyl enol ethers suggest that the nature of the parent ketone, rather than enol etherification *per se*, governs the biological behavior of such compounds.

Lynestrenol is another 19-nortestosterone derivative present in oral contraceptive preparations that are commercially available outside the United States. Like norethindrone acetate and its derivative, quingestanol acetate, lynestrenol is a progestagen which has some androgenicity (18).

Finally, there are three progestagenic steroids shown that are structurally related to progesterone, the structure of which is given also. Neither progesterone nor 17α-hydroxyprogesterone has activity when administered orally; the progesterone molecule, furthermore, does not lend itself to useful derivatives (17). Esterification of the 17α-hydroxyl group of the second compound, however, gives this molecule progestational properties, and further modifications enhance its contraceptive effects (23). Specifically, a major pathway of 17α-hydroxyprogesterone catabolism involves hydroxylation at C-6. Addition in this position of substituents such as chlorine, as in chlormadinone acetate, or a methyl group, as in medroxyprogesterone acetate and megestrol acetate, hinders the catabolism of the compound, thereby enhancing its biological effects. Finally, medroxyprogesterone acetate has slight androgenicity while chlormadinone acetate and megestrol acetate are potent antiandrogens (18).

Contraceptive Preparations—The commercially available oral contraceptives are of two types, the combined products and the sequential preparations. The various doses and combinations available in the United States are listed in Table I. It can be seen that doses vary not only among the different progestagens but also for the same progestagen used either with the same or a different dose of estrogen. The reasons for the variability are multiple and are related to the factors of potency, dosage regimens and therapeutic uses other than contraception (see footnotes to Table I), and side effects.

Although much valuable preliminary information can be obtained by studying the effects of oral contraceptive components in experimental animals, the potency of these compounds, as assessed by animal studies, may not always be relevant to their effectiveness in the human. Consequently, Swyer and Little (24) compared a number of progestagens, with and without estrogen, with respect to their ability to postpone menstruation in normal women given 20-day courses of progestagen from Day 20 of the cycle. The respective doses (and variations available for given progestagens) listed in Table I for norethindrone, norethindrone acetate, norethynodrel, ethynodiol diacetate, and norgestrel are consistent with their respective potencies as determined by the menstruation postponement assay; dimethisterone was not studied in this manner.

The combined preparations containing the highest doses of norethindrone and norethynodrel have been used for the treatment of endometriosis as well as for contraception. The rationale behind this use is that the higher the dose of progestagen the greater are the degree and probability of occurrence of endometrial suppression (25).

The occurrence of side effects, such as lack of withdrawal bleeding on the one hand and breakthrough

Table I—Oral Contraceptive Preparations

Estrogen	Dose, mcg.	Progestagen	Dose, mg.
Ethinyl estradiol	100	Dimethisterone	25.0[a]
	50	Ethynodiol diacetate	1.0[b]
	50	Norethindrone acetate	1.0[b]
	50	Norethindrone acetate	2.5[b]
	50	Norgestrel	0.5[b]
Mestranol	100	Ethynodiol diacetate	1.0[c]
	50	Norethindrone	1.0[c]
	60	Norethindrone	10.0[d,e]
	80	Norethindrone	1.0[b]
	80	Norethindrone	2.0[f]
	100	Norethindrone	2.0[d,g]
	75	Norethynodrel	5.0[d,e]
	100	Norethynodrel	2.5[d]

[a] Sequential preparation: 16 tablets of ethinyl estradiol followed by six tablets of ethinyl estradiol plus dimethisterone. [b] Combined preparation: 21-day dosage schedule. [c] Combined preparation: 20- and 21-day dosage schedules. [d] Twenty-day dosage schedule. [e] Used also for treatment of hypermenorrhea and endometriosis. [f] Sequential preparation: 14 tablets of mestranol followed by six tablets of mestranol plus norethindrone. [g] Used also for treatment of hypermenorrhea.

bleeding on the other, may also be dose related (26); higher doses of progestagen may be associated with scanty withdrawal bleeding, while lower doses may be associated with breakthough bleeding. It has also been suggested that a higher dose of estrogen (*i.e.*, 80 mcg. mestranol instead of 50 mcg.) may be needed by some women to avoid spotting and breakthrough bleeding. Nevertheless, it was shown that by the sixth cycle the differences between the two doses were negligible with respect to the occurrence of breakthrough bleeding (27). In view of this observation and of the possible association between more serious adverse reactions and estrogen dosage (*vide infra*), it would seem wiser to rely on higher progestagen dosage preparations to control breakthrough bleeding in patients for whom this effect is a significant problem.

The variations in estrogen dosage are also of interest from the point of view of contraceptive effectiveness. A number of investigators (28–36) studied the effectiveness (pregnancy rate) and/or the ovulation-blocking effects (pregnanediol measurements and/or endometrial biopsy) of various doses of both ethinyl estradiol and mestranol, alone as in the initial portion of a sequential regimen or in combination with a progestagen. The results of one study (28) seem to imply that a dose of 80 mcg. mestranol is necessary to produce the same (and acceptable, although less than complete) degree of ovulation blockade as 50 mcg. ethinyl estradiol; mestranol, however, apparently was not studied at a dose of 50 mcg. A subsequent study (29) showed that a daily dose of 100 mcg. ethinyl estradiol was necessary to inhibit ovulation consistently. Furthermore, six of eight patients ovulated during treatment with 80 mcg. mestranol following a nontreatment cycle. The results of a more recent study (30) support the data of Jackson *et al.* (29) concerning ethinyl estradiol; three pregnancies, two of which were attributed to method failure, occurred among 80 women treated for 695 cycles with 70-mcg. doses of ethinyl estradiol, yielding a pregnancy rate of 3.5/100 woman-years of use.

In larger scale studies (31–33) in which the contraceptive effectiveness of 80 mcg. mestranol was investigated, pregnancy rates were low. In one report (33) the pregnancy rates for the 27 centers in which the study was carried out showed a distribution comparable to that for published data concerning various combination products; the data from the 27 centers averaged out to a rate of 1.12/100 woman-years. The authors pointed out, however, that factors such as the reluctance of women to admit not having taken the preparation correctly until they were assured that they were not pregnant made it difficult to determine the true effectiveness of any contraceptive.

Finally, two investigations were carried out to compare the effectiveness of various contraceptive preparations. Results of a 4-year study (34) yielded pregnancy rates of 0.1/100 woman-years for combined preparations and of 0.5/100 for the sequential preparations; these values increased to 0.7 and 1.4, respectively, when patient failures were included. Unfortunately, the doses of estrogen and progestagen used were not given. The other study (35) compared nine preparations, four of which were sequentials. All preparations contained 80 mcg. mestranol except for two combined preparations that contained 75 and 125 mcg. mestranol, respectively. The preparations were used for total numbers of cycles ranging from 71 to 6721. The seven pregnancies attributed to method failure occurred in patients using three of the four sequentials; however, since the directions given were to wait until the 5th day of withdrawal bleeding before beginning a new 20- or 21-day course of medication, 11 days of no medication elapsed for three of the patients in whom withdrawal bleeding had been delayed. Resumption of medication on the 6th or 7th day after the last tablet of the previous cycle, regardless of whether or not withdrawal bleeding had occurred, became the retrospective and, probably, safer recommendation.

There have been reports of women omitting tablets and becoming pregnant, of women omitting tablets and not becoming pregnant, of women ovulating during treatment but not becoming pregnant, and of women not omitting tablets but becoming pregnant (30–36). Some data also suggest a lesser effectiveness with use of the sequentials (34, 35). These phenomena can be explained at least partly on the basis of the mechanisms of action of the contraceptive preparations (*vide infra*). However, very few studies have taken into consideration the absolute frequency of coitus. Since both ova and sperm have a finite viability, pregnancy would not be expected to occur if coitus had not taken place close to the day(s) of missed medication and/or breakthrough ovulation. As will be shown later, the combined preparations provide secondary, backup mechanisms in the event a breakthrough ovulation occurs. However, data are not available to indicate whether coincidental lack of coital exposure took place at the time that tablets of either type were missed by patients who did not subsequently become pregnant. At best, an occasional study (30) reported the average coital exposure per week or that patients were required to have frequent (no numerical value given) intercourse as a prerequisite for receiving the test medication (35).

Mechanisms of Action—Both the combined and sequential preparations are believed to prevent ovulation by inhibiting the release of the gonadotropin-releasing factor(s) from the hypothalamus (1, 16); that is, the synthetic hormone(s) exerts a negative feedback on the hypothalamus in a manner similar to that by which the naturally occurring hormones act. The major difference, however, is that the oral contraceptives provide a constant (inhibitory) dosage, a steady-state level, of synthetic ovarian hormone(s) in contrast to the changing (and, hence, sometimes stimulatory) levels of naturally occurring steroids provided during the normal, untreated menstrual cycle (*vide supra*).

As a result of hypothalamic suppression by an oral contraceptive, gonadotropin output from the pituitary is decreased. However, results of studies (37–41) of serum and urine samples obtained from women taking various oral contraceptives have revealed both qualitative and quantitative differences between the effects of the combined and sequential preparations. Such differences are to be expected in view of the normal hormonal interrelationships already discussed. Specifically, the sharp midcycle peak in LH secretion was

almost always abolished by both the combined (37–41) and sequential preparations (37, 38), as was the midcycle peak of FSH (37, 38, 41). In addition, basal, tonic secretion of FSH was suppressed to about 70% of control values by both types of preparation. In contrast, whereas the combined preparations decreased tonic LH secretion to about 20–30% of the control value, sequential preparations decreased LH values to only about 50% of the control (37). Furthermore, large, multiple fluctuations in LH levels, "inappropriately timed LH peaks," were found in serum samples obtained from several women during the estrogen phase of their sequential medication (38). An additional peak in LH secretion occurred in five of eight patients following addition of the progestagen on the 16th day of medication.

In summary, if FSH is sufficiently suppressed by the estrogenic compound of both the combined and sequential preparations, follicular growth will be minimal (16). Therefore, the occurrence of ovulation would be unlikely even in the presence of the higher LH levels found during sequential therapy since there would be no ripe follicle mature enough to ovulate. Nevertheless, modest fluctuations in FSH secretion have been observed, even in women receiving combined preparations (37, 41), suggesting that follicular growth might proceed further in some women than in others.

The possibility of ovulation occurring is increased when a tablet of either type of preparation is missed, although one preparation containing 0.5 mg. norgestrel and 50 mcg. ethinyl estradiol was shown to be effective when administered on alternate days (42). The possibility of a pregnancy occurring is increased, especially when a tablet of the sequential type is missed. These possibilities are further increased when more than one tablet is omitted. Finally, although taking a missed tablet as soon as it is remembered or doubling the dosage when more than one is missed will serve to end the cycle of medication at the appropriate time, such measures are not likely to provide adequate contraception in the case of the sequentials nor even in the case of the combined preparations if more than one tablet of the latter type is omitted. Consequently, use of an additional method of contraception for the remainder of the cycle is advisable whenever oral contraceptive tablets have been omitted. Explanations for taking this precaution lie partly in the results of studies (43–45) in which various doses of estrogens and/or progestagens were administered daily to women.

Briefly, FSH concentrations in the urine were found to increase with daily subtherapeutic doses of ethinyl estradiol (20 mcg.) (43) and mestranol (10 mcg.) (44), whereas therapeutic doses [70 and 100 mcg. (43) and 50 and 100 mcg. (44), respectively] usually depressed FSH excretion. Furthermore, high daily doses (2 mg.) of one progestagen, ethynodiol diacetate, suppressed FSH (44) and LH (45) secretion while lower doses [0.25 and 0.50 mg. (44) and 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg. (45)] often resulted in high levels of FSH and LH, respectively.

Although the progestagen–estrogen ratio in combination products apparently influences the degree of FSH and LH suppression (44), the important point to be realized from these studies is that low doses of these contraceptive steroids do not suppress, and actually may stimulate, FSH and/or LH secretion. Hence, when tablets are missed, blood levels of the steroids decrease, resulting in an increased output of FSH and/or LH by the pituitary. Follicular growth or, possibly, ovulation may then occur if sufficient FSH secretion has been occurring in spite of the medication. Assuming the lowering of steroid levels has merely resulted in a subsequently increased secretion of gonadotropin, which has in turn merely resulted in follicular maturation, doubling the dosage upon remembering the omission would increase blood steroid levels and possibly trigger an ovulatory surge of LH, just as rising steroid levels appear to do during the normal, untreated cycle. Indeed, changing the dosage of estrogen during the combined portion of a sequential regimen (75 mcg. ethinyl estradiol for 16 days followed by 50 mcg. ethinyl estradiol plus a progestagen for 5 days) was associated with a pregnancy rate of 15.4/100 woman-years in comparison with the rate of 6.3 obtained when the dosage of ethinyl estradiol remained 75 mcg. throughout the 21-day sequential cycle (46). Furthermore, low doses of conjugated estrogen (0.1 mg. daily on Days 9–14 of the cycle) were used to induce ovulation at a predictable time (97% of 336 ovulations occurred no later than Day 16) in patients wishing to use the rhythm method of birth control (47).

Pituitary suppression tends to be more complete with the combined preparations, as indicated by a marked decrease usually in both FSH and LH secretion, with the probable consequence being a more marked lack of follicular development. However, these preparations also provide secondary contraceptive mechanisms that can be of importance in the event of a breakthrough ovulation. One such mechanism involves the effect of the progestagen on the cervical mucus. In contrast to the watery mucus facilitatory to sperm penetration (15) present during the preovulatory phase of the untreated cycle and throughout the estrogenic phase of the sequential regimen, the mucus present during treatment with the combined preparations is similar to that found during the normal luteal phase; that is, the cervical mucus is thick, scanty, and cellular, providing a physical barrier to penetration by sperm (13). The physical barrier hypothesis, moreover, is supported by electron microscopic evidence (48) which indicates a close approximation of, and significant cross-linkages between, the main fibers comprising the cervical mucus obtained from women taking combined preparations. This mechanism, furthermore, is thought to be the one primarily responsible for the contraceptive effectiveness of low-dose progestagen-only preparations (*vide infra*).

Endometrial changes occurring under the influence of combined preparations differ from those observed during the normal, untreated cycle. As a result of the presence of progestagen along with estrogen early in the cycle, glandular secretion begins promptly and reaches a peak within 4 days of the start of medication (13). The glands then involute and may acquire an atrophic appearance. The stroma, after passing through a phase of edema, reaches a pseudodecidual pattern earlier and of greater intensity than in the untreated

cycle. It has been suggested that such an endometrium may be unfavorable in some manner for the implantation of a fertilized ovum (23, 49).

Investigational Steroid Contraceptives—A number of orally administered progestagens have been or are being investigated for their ability to control fertility in the absence of exogenous estrogen. Most studies involved chlormadinone (50–55) and, more recently, norgestrel (55–60), although results obtained with continuous administration of low doses of norethindrone (51, 61, 62), norethindrone acetate (55), and megestrol acetate (55) were reported as well. A discussion of the observations made in women taking chlormadinone acetate is virtually of academic interest only, since this drug was withdrawn from the market due to the finding of mammary gland nodules in dogs treated with it (63). Nevertheless, the results of clinical studies with this drug provide much valuable information concerning the mechanism(s) by which low dose progestagen-only preparations may exert their contraceptive effects.

It was indicated above that the primary antifertility effect of low dose progestagen-only preparations was thought to be the result of their production of a cervical mucus that was "hostile" to sperm. Indeed, studies (50) with doses lower than 0.25 mg. chlormadinone acetate revealed a failure to produce inhibitory changes consistently in the cervical mucus; these doses also did not afford completely effective contraception. Higher doses produced greater inhibitory effects on the cervical mucus and greater contraceptive effectiveness. When doses higher than 0.3 mg. were used, suppressive effects on the endometrium were obtained; these effects presumably contributed to the contraceptive effectiveness of the drug.

Subsequent studies of volunteers taking daily doses of 0.5 mg. chlormadinone acetate (51–54) or 0.5 mg. norethindrone (61) revealed discrepancies among factors normally associated with the occurrence of ovulation and, hence, with one another. These factors include the midcycle LH (and FSH) peak(s), biphasic basal body temperature, and increased serum progesterone and increased urinary pregnanediol levels during the second half of the cycle. The fact that sometimes one, although not always all, of these factors might be negative in patients taking chlormadinone acetate suggested the possibility of a central effect (52) of the dose to which some patients might be more sensitive than others (53). In addition, these observations raised the question as to whether there might be some minimal levels of LH and pregnanediol compatible with normal ovulation and corpus luteum function (51). In other words, would the gonadotropin levels found during chlormadinone acetate therapy be sufficient merely to cause luteinization of one or more follicles but not ovulation (53) or, if ovulation did occur, would it be followed by an "inadequate luteal phase" (54, 61)?

Similar observations were made in studies employing low doses of norgestrel. Specifically, the results suggested that in addition to producing inhibitory effects on the cervical mucus and on the endometrium, norgestrel in doses of 0.03 (56), 0.05 (57), and 0.075 (58) mg. appeared also to interfere with the formation and/or functioning of the corpus luteum. The progestagens, norethindrone, norgestrel, chlormadinone acetate, and medroxyprogesterone acetate, administered orally for several days following ovulation, have, in fact, been shown (64) to decrease progesterone production by the corpus luteum. However, the doses used for norethindrone (5–100 mg.), norgestrel (2–5 mg.), and chlormadinone acetate (50 mg.) were higher than those used clinically for progestagen-only contraception. The mechanism by which hormone production was decreased was not determined, but it was not likely to have involved luteolysis since the administration of human chorionic gonadotropin (HCG) resulted in increased production of progesterone despite continued administration of the synthetic progestagens.

It seems apparent that the mechanism(s) by which daily administered oral preparations of low dose progestagens exert their contraceptive effects is (are) complex and still somewhat obscure. By means of culdoscopic examinations (51) and ovarian biopsies (52), for example, it was shown that ovulation does occur in some, but not all, women taking these preparations. Moreover, inconsistencies among parameters normally indicative of ovulation have been found in some women. Furthermore, although it is generally agreed that the production of a cervical mucus that prevents the entrance of sperm into the uterine cavity is the primary mechanism by which these preparations act, postcoital tests, although usually poor, are not always so, in women using these contraceptives (55, 60). Finally, the "hostility" may not be entirely due to a physical barrier but may also be biochemical in nature (65). Trypsin, for example, is thought to affect sperm penetration of the zona pellucida of the ovum, and it has been postulated that one mechanism might involve antitrypsin in the cervical mucus (60).

Whether due to a physical and/or a biochemical effect, the production of cervical mucus that is nonpermeable to sperm formed the basis on which clinical trials of precoital progestagen-only contraceptives were undertaken (66). Megestrol acetate, at a single oral dose of 0.5 mg., was shown to be effective when coitus was limited to the period between 4 and 14 hr. after dosing; 17 women were studied for a total of 187 cycles, and coital frequency was estimated to be once every 3 days on the average. A previous group of 26 women studied for a total of 468 cycles also achieved contraceptive protection with this preparation, although there was one patient failure. In these patients, coital activity had been restricted to between 5 and 10 hr. following dosing. Protection was not achieved, however, in a third group of patients in whom coitus took place largely between 20 and 21 hr. after dosing. This preparation appears to have advantages for women exposed only occasionally to intercourse; however, frequent coitus requires frequent medication, with the result that cycle irregularity may occur, similar to that observed with continuous progestagen administration (*vide infra*).

It should be obvious that women using oral contraceptives of the combined type have an advantage over those using sequentials since additional contraceptive mechanisms are provided by the progestagen. Unfortunately, in the absence of exogenous estrogen (*i.e.*, in patients using the continuously administered progesta-

gen-only oral preparations), high pregnancy rates were reported in some studies. Rates for 0.05 mg. norgestrel vary from 0.8 (59) to 6/100 woman-years (62); for 0.5 mg. chlormadinone acetate, the rates vary from 0.2 to 9/100 woman-years (62). In his own study using 0.35 mg. norethindrone, Board (62) obtained a rate of 0.6/100 woman-years based on 154 women observed for a total of 1888 months. For norethindrone acetate, on the other hand, a rate of 4/100 was obtained by Mears *et al.* (55); however, the group in this study consisted merely of 41 women, only 19 of whom completed an entire year's treatment. In retrospect, the influence that factors such as ethnic group (67), especially if a marked difference in body size is involved, and coital frequency might have had on these results cannot be determined. In view of the high pregnancy rates reported in some of these studies, it would seem unwise for women to use progestagen-only oral contraceptives unless they wish merely to space their families rather than to eliminate pregnancy completely.

In addition to their drawback of having a variable degree of effectiveness, progestagen-only preparations also produce a high incidence of menstrual irregularity manifested by breakthrough bleeding and prolonged cycles (15, 26, 50, 59, 62). Better cycle control can be obtained by administering estrogen in addition from Day 19 or 21 to Day 25 (68), the so-called reverse sequential regimen (26). Although contraceptive effectiveness was reported for this regimen (68), the advisability of giving estrogen to women in whom ovulation but not necessarily follicular development may have been delayed is questionable in view of the stimulatory effect that estrogen may have on the hypothalamus (*vide supra*).

Progestagen-only contraception has been achieved by administering the compounds by other than the oral route. A depot form of medroxyprogesterone acetate, for example, has been shown to be effective when injected intramuscularly at a dose of 150 mg. every 3 months (15). Inhibition of LH release, increased viscosity of the cervical mucus, and thinning of the endometrium are all believed to contribute to the contraceptive effect. Unlike the oral progestagens, the injectables appear to be as effective as the commercially available combined oral preparations (26). Like the oral progestagens, however, the injectables have erratic bleeding as a major side effect. Nevertheless, amenorrhea eventually develops in many patients and may persist for up to 12 months after the last injection.

Other means of delivering contraceptive doses of progestagens in a continuous manner that have been tried recently include: (*a*) the subcutaneous implantation of silastic capsules containing progestagens such as medroxyprogesterone acetate and megestrol acetate (26), (*b*) the use of a silicone rubber ring impregnated with medroxyprogesterone acetate and placed around the cervix (26), and (*c*) the intrauterine administration of progesterone contained in a silastic capsule attached to an intrauterine contraceptive device (IUD) (15). However, insufficient studies have been published concerning these newer methods to permit their critical evaluation.

Clinical studies have been carried out also with long-acting, intramuscularly injected preparations containing an estrogen as well as a progestagen. These preparations share with the progestagen-only injectables the advantage that effective hormonal contraception can be achieved without requiring the patient to remember to take daily medication. In addition, it was suggested (69) that cycle control may be slightly better with the combined injectables; the latter, however, have usually been administered once a month in comparison to once every 3 months for the progestagens.

Examples of combinations that have been shown to be clinically effective include estradiol undecylate plus norethindrone enantnate [5 and 50 mg., respectively, or 10 and 70 mg., respectively (69)], 5 mg. estradiol cypionate plus 25 mg. medroxyprogesterone acetate (70), and 10 mg. estradiol enanthate plus 150 mg. 16α,17α-dihydroxyprogesterone acetophenide (71). The last preparation, studied in 385 patients during 4512 cycles, produced continued or irregular bleeding in 7.5% of the patients.

The depot concept was also applied to orally administered formulations in which the long-acting estrogen, quinestrol, was combined with one of several short-acting progestagens (15). In one study (20), for example, a dose of 2 mg. quinestrol alone was given on Day 1 of the first cycle; on Day 22 and then subsequently at 4-week intervals, doses of 2 mg. quinestrol plus 5 mg. quingestanol acetate were given. The effect of the progestagen was primarily to induce withdrawal bleeding, although the latter began at variable times following dosing: 8–14 days after administration in 65% of 7441 cycles observed in 719 patients and 5–20 days later in 92% of the cycles. The effect of the estrogen, on the other hand, was to prevent ovulation during the subsequent 4 weeks. However, the progestagen may have contributed to the contraceptive effect as well; six patients, four of whom did receive the quinestrol later than Day 1, became pregnant during the first cycle in which they received the estrogen alone, yielding a pregnancy rate of 4.0/100 woman-years. When the first cycle and subsequent patient failures were eliminated from the total data, the pregnancy rate was calculated to be 2.1/100.

Estrogens alone have been used postcoitally to prevent implantation in the event that fertilization may have taken place, as in rape cases (26) and condom failures (72) for example. Several possible mechanisms of action have been suggested (73), including disturbances in endometrial histology and histochemistry, tubal locking that would prevent the ovum from reaching the uterus, and rapid expulsion of the ovum from the tube and uterus. Various dosage regimens also have been suggested: 1 mg. ethinyl estradiol b.i.d. for 5 days following coitus (15), 0.5 mg. ethinyl estradiol or 5–50 mg. diethylstilbestrol daily for 4–6 days following coitus (74), 5 mg. diethylstilbestrol five times per day for 5 days beginning within 48–72 hr. after coitus (72), 75 mg. diethylstilbestrol for 3 days following coitus (75), and 25 mg. diethylstilbestrol b.i.d. for 5 days beginning immediately after coitus (73). The larger doses especially are likely to produce nausea and vomiting; consequently, one investigator (72) recommended administering an antiemetic along with the estrogen. Because of its

tendency to produce these symptoms, this method of contraception is not likely to be used by patients on a regular basis. Furthermore, its effectiveness is difficult to evaluate since a large number of patients in whom the method has been tested have been rape cases, and the certainty of pregnancy following rape is not thought to be very great (74).

ADVERSE REACTIONS: THROMBOEMBOLISM

The manifestations of adverse reactions (side effects) to oral contraceptives are not limited to abnormalities produced in the reproductive system, but rather they involve almost all systems of the body. Oral contraceptive therapy is rather unique since an abnormal, nonphysiological response in the normal reproductive system of the female is the therapeutic result. In addition to or perhaps as a consequence of this effect, other abnormalities, both mild and severe, may occur. In the following discussion, the term "side effect" will be used in its broadest form and will represent any change from normal in systems other than those specifically involved in the production of contraception.

Side effects may be produced in three ways: (*a*) an abnormality may be produced directly by an effect of the drug on the tissue, (*b*) the effect may occur indirectly as a result of a change in endogenous hormonal balance produced by the drug, and (*c*) the abnormality may be the consequence of a combination of (*a*) and (*b*). Unfortunately, little concern has been given to determining which possibilities may be correct.

Problems in Assessment of Side Effects—One major problem encountered in the assessment of the side effects of oral contraceptives is that the literature concerning this subject is controversial (76, 77). This enigma seemingly has resulted from controversial data that have been published, and the problem has been compounded by biases from several sources.

There are biases that are inherent in a particular type of investigation or reporting system. Such biases as are seen in retrospective epidemiologic studies, wherein patient records are searched to obtain data, have been identified in studies (78) concerned with the evaluation of side effects of oral contraceptives. They are usually considered normal and expected, and allowances can be made for them.

However, certain other biases have had a marked effect on the investigations and on the conclusions drawn from them. These biases are not usual, measurable, or accountable for, and they arise from various sources. Some seem to have originated from personal prejudices on the part of certain investigators who allowed their prejudices concerning the "pill" to affect the design of their experiments, the reporting of their results, and the conclusions derived. Such prejudices are not surprising when one considers the polemic attitudes prompted by religious and social influences (79, 80).

Other biases, often termed diagnostic biases, are more insidious and may have contributed to the problem of assessing the side effects of oral contraceptives. Seigel (81) pointed out that the evaluation of data from case-control studies may be biased because the proportion of women hospitalized who take oral contraceptives may be larger than a corresponding group who do not. He ascribes this observation to the fact that oral contraceptive users probably see their physicians more often. In addition, since both the physician and patient are likely to be aware of some of the undesirable effects of these agents, the women in this group may be diagnosed and hospitalized more readily than those in the general population. Therefore, the cases that come under scrutiny in these studies may yield a disproportionate number of incidences of adverse effects in oral contraceptive users. Seigel concluded that when analyzing studies of conditions such as thromboembolic disorders, particularly when they are not severe, the potential bias must be carefully assessed.

The controversies and biases have been polarized further by influences from political arenas, various antipill factions, representatives of government agencies, and people of the news media seeking sensationalism (79, 82). The influence of these factors on the reported side effects of the pill and on the use of the pill itself was discussed by Kistner (79). Jeffcoate (83) stated: "At times, there has appeared to be almost a campaign to discredit the pill, and the views of prejudiced if well-meaning individuals have been given disproportionate emphasis and allowed to outweigh scientific evidence."

Another major problem particularly pertinent to the assessment of the common, minor side effects has been the paucity of double-blind clinical trails. Such testing procedures are essential to determine whether an observed effect is causally related to the drug or due to other factors such as psychological effects. In this type of procedure, the tablets containing drug and those containing an inert material, as well as the packages in which they are contained, are identical in appearance; the packages are identified by a code, the key to which is kept under seal. The obvious problem with this type of study is finding women who will agree to subject themselves to this randomized dosing, the consequence of which might be pregnancy. It is also difficult to find investigators who are willing to charge themselves with the responsibliity of conducting such a study. However, it is possible to find populations of women who are not at risk of pregnancy, such as prison inmates (84); alternatively, other contraceptive devices such as intrauterine devices (85) or condoms can be provided. Obviously, with the danger of pregnancy obviated, investigators are less reluctant to conduct the experiment, and subjects can be found more easily. These double-blind clinical trials should be expanded to correlate the incidence of side effects with various dosages of the estrogens and progestagens, as well as with dosages of various combinations thereof.

Another difficulty encountered in evaluating the occurrence of side effects, which has made comparisons among data from different studies unreliable (86), is the variability in the protocols and reporting systems of these studies. No unified system or protocol exists; as a result, different variables are present in the various studies and often they are not fully described. These variables include: (*a*) a wide range of populations in terms of ethnic, cultural, and socioeconomic background; (*b*) variations in patient management; and (*c*)

variations in the methods used for collecting, recording, and evaluating data (84). For example, in clinical trials with oral contraceptives, the "open-ended" approach is often utilized to obtain information from the patient, wherein the patient is requested to volunteer any information concerning changes in her health status that she might have noticed. On the other hand, specific questioning about certain symptoms can be used; this probing type of question yields a higher incidence of side effects (85).

In the area of rare, major side effects such as thromboembolism, retrospective epidemiologic studies have been somewhat productive but few in number. Their reliability is open to question because of a lack of information concerning the incidence of many of the reported side effects in the general population (87), the small number of patients studied, and the lack of appropriate controls. Any disease that occurs in a normal population may also occur in a population that uses oral contraceptives. The important question is whether or not it occurs more frequently in women using oral contraceptives (84). The above-mentioned problems were described at some length by Tietze (84).

Although often used, the division of side effects of oral contraceptives into major and minor categories is arbitrary. Since the status of many side effects may change rapidly as new information is uncovered, no further designations of this type will be used.

Thromboembolic Disorders—The first association between oral contraceptives and thromboembolism was reported in 1961 when Jordan (88) described the development of bilateral pulmonary embolism in a woman following the use of norethynodrel (10 mg.) with mestranol for the treatment of endometriosis. Symptoms developed in the right lung 10 days following the termination of treatment and in the left lung at 17 days posttreatment. The patient also had been taking cyclizine and perphenazine to control the nausea produced by the steroid drugs. Jordan stated that the norethynodrel with mestranol might have provoked the pulmonary embolism and infarction. He made no reference to the possible effects of the other drugs. Later in the same year, two cases of fatal pulmonary embolism occurred in two young women taking oral contraceptives (89).

Subsequently, many cases such as these were reported in the literature, as well as to various government agencies both here and abroad. These thromboembolic episodes were reported most frequently in the lungs and the large leg veins, as well as in the coronary, cerebral, and mesenteric vessels. In 1963, Tyler (89) commented on 347 instances of thrombophlebitis that had been reported since 1961 in women using norethynodrel with mestranol for contraception. These isolated cases had been reported to the manufacturer of the contraceptive product. Thirty-five instances of fatal pulmonary embolism occurred during the same period. In his discussion of the latter, Tyler pointed out that other etiologic factors might have been involved. He recognized the fact that thrombophlebitis and pulmonary embolism could occur spontaneously in the general population but that no control figures for this spontaneity were available.

Since thromboembolic disorders are known to occur in young women who do not use oral contraceptives (90), these isolated case reports cannot serve to establish a relationship between oral contraceptives and thromboembolism.

One of the first organized efforts to determine whether a relationship might exist between thromboembolism and the use of oral contraceptives was a conference sponsored in 1962 by G. D. Searle and Co. (90). The participants' comparisons of the incidence of thromboembolism in oral contraceptive users and in the general population yielded no evidence to suggest a causal relationship between oral contraceptive therapy and thromboembolism.

In 1963, a committee of nine medical experts, assembled by the Food and Drug Administration (FDA), reported a study of 350 cases of thromboembolism in women using norethynodrel with mestranol. These cases included those reported both to FDA and to the manufacturer. The members of the committee reviewed the pertinent literature and conducted experiments in their own laboratories pertaining to hypercoagulability. They concluded that there was no evidence that this contraceptive preparation produced hypercoagulability and that there was no significant increase of thromboembolic deaths in the oral contraceptive users (91).

In 1965, Cahal (92, 93) reported that in the 12 months ending August 31, 1965, 16 thromboembolic deaths in oral contraceptive users had been reported to the Committee on Safety of Drugs in England; only 13 would have been expected, based both on the estimated occurrence in the general population and also on a round, theoretical figure of 400,000 oral contraceptive users. Cahal admitted that the accuracy of the latter figure was open to question and that the incidence of deaths might have been underreported. Cahal went on to point out that when the deaths due to thromboembolism were further subdivided according to site of the embolism, eight were found to have occurred in the lung in contrast to the two that would have been expected on the basis of mortality statistics found in the Registrar General's Statistical Review. Cahal himself concluded that the numbers involved were too few to state that the differences were meaningful.

Weatherall (94) was critical of Cahal's estimate of two expected instances of death due to pulmonary embolism in the general population, pointing out that pulmonary embolism commonly was not reported as an underlying cause of death when it occurred during some other disease. She stated that because of the method of reporting, only approximately 13% of the instances in which pulmonary embolism was the underlying cause of death were being reported as such; therefore, the estimate of death due to pulmonary embolism given by Cahal was seven times too low. However, in 1969, Vessey (90) quoted without qualification the fourfold difference in pulmonary embolism occurrence between users and nonusers that had been reported by Cahal.

In 1966, the Advisory Committee on Obstetrics and Gynecology of FDA issued its first report (95). Using similar basic data and procedures as the earlier *ad hoc*

committee (91), this group calculated that on the basis of the estimate of users in 1962, 1,187,000, the expected mortality rate from pulmonary embolism would be 12. They adjusted the latter figure to 10 since they felt the estimated number of users might have been inflated. The actual number of such deaths reported to FDA was 13; this difference was not statistically significant. On the basis of these figures, 13 reported and 10 expected, a relationship very similar to that found by the earlier *ad hoc* committee was evident.

This group also pointed out that since the number of oral contraceptive users had increased from 1,187,000 in 1962 to 5,000,000 in 1966, the number of deaths from pulmonary embolism should have increased to 50. However, the reported number was 13, exactly the same as in 1962. This discrepancy was thought to be due to gross underreporting, which they attributed to physicians becoming increasingly fearful of reporting adverse reactions and deaths because of possible legal reprisals. It was further pointed out that mortality statistics probably were unreliable, since the reporting of the underlying causes of death was left to the judgment and conscientiousness of the certifying physician, and these might vary with the physician. The discrepancy also could be due partly to a greater awareness of adverse reactions leading to more rapid diagnosis and treatment.

The Advisory Committee concluded that the data derived from mortality statistics were inadequate to determine an association between oral contraceptives and thromboembolic disease. However, they indicated that if the oral contraceptives were involved in the pathogenesis of thromboembolic disease, the incidences were very infrequent. The group suggested a need for well-controlled epidemiologic studies.

All of the foregoing studies were of the retrospective type. Vessey (90) pointed out that such studies suffer from an absence of comparable control data and also depend upon reported episodes of thromboembolism. He agreed with the Advisory Committee (95) that gross underreporting was a problem.

In 1961, the Records Unit and Research Advisory Service of the Royal College of General Practitioners in England (96) organized the recording of diseases by a number of general practitioners. Each of 29 practitioners made note of every woman in his practice, age 15–49, who had suffered a new episode of various thromboembolic disorders. The practitioner also categorized the thromboembolic disorder into one of 14 groups. Some of these categories took into account the pregnant and puerperal states.

Two control patients were selected for each thromboembolic patient. The controls were matched for age (within 5 years), parity, and marital status. At the interviews, the patients were questioned as to the type of contraceptive used prior to and at the time of the episode, as well as to the length of time that the form(s) of contraception had been used. There were 147 "affected" patients as well as 294 controls. It was found that a statistically significant excess of oral contraceptive users existed in the affected group as compared with the control group. The excess was present for superficial thrombophlebitis and generally for conditions on the venous side of the circulation. Data for the arterial side of the circulation were insufficient to give significant results. The data also indicated an increase in thromboembolic disorders for women in the pregnant and puerperal states. The investigators concluded that the latter conditions were associated with a fourfold increase in thromboembolic disorders and that oral contraceptive use was associated with a twofold increase. In addition, the study pointed out that the incidence of venous thromboembolic disease increased with age and parity. The authors acknowledged that the number of patients in their study was small and pointed out that prospective studies might provide more reliable data.

In 1968, Inman and Vessey (97) reported on their studies of thromboembolic deaths occurring during 1966. Four hundred ninety-nine death certificates of 20–44-year-old women were obtained in which thrombosis or embolism of the pulmonary, cerebral, or coronary vessels or peripheral venous thrombosis was mentioned. If thromboembolism was a terminal event in the course of another fatal disease, the case was not used. Many other cases were omitted for a variety of reasons, such as: (*a*) the attending physicians were not available, (*b*) some patients had not been registered with a doctor or hospital, and (*c*) some individuals were not at any risk of pregnancy. The number remaining after all exclusions, which formed the basis of the report, was 334. These were subdivided as follows: 95 attributed to pulmonary thrombosis or embolism, 209 to coronary thrombosis or myocardial infarction, and 30 to cerebral thrombosis or embolism.

Each death was investigated by a medical officer who completed a questionnaire with the assistance of the physician who had attended the patient during her terminal illness. The information obtained in the questionnaire was supplemented by hospital case notes and postmortem reports. In addition to data concerning the fatal case, the physician also supplied information concerning four to six controls who were of similar age and parity. In all, 998 controls were used in the analysis.

Since many conditions were believed to be predisposing toward thromboembolic disorders, the questionnaires were assessed and divided into three categories: Class A, patients with no predisposing conditions; Class B, patients with known predisposing conditions who were neither pregnant nor puerperal; and Class C, patients who were pregnant or who had been delivered during the month before the onset of the episode; this last class was not discussed in this paper. An important fallacy of this paper was that no information was obtained about the health of the control women.

The results showed that 77 deaths occurred from pulmonary embolism. Of these, 26 were in Class A, 16 of whom had been taking oral contraceptives; 4.2 would have been expected from the control group data. The difference between the two was stated to be highly significant ($p < 0.001$). Fifty-one deaths were noted in Class B; however, since there was uncertainty concerning the use of oral contraceptives by two of the women, only 49 were used for tabulation. Of the 49, nine had been using oral contraceptives whereas 6.8 would have been expected. This difference was not significant.

Coronary thrombosis was implicated in 205 deaths, 89 of which were in Class A. The authors reduced the number to 84 since the history of oral contraceptive use was doubtful in five patients. Of the 84, 18 deaths occurred in users while 11.4 would have been expected. This difference was not statistically significant. The number of 116 patients in Class B was reduced to 110 because of uncertainty of oral contraceptive use. Only five of the 110 had been using oral contraceptives whereas 12.6 would have been expected.

Twenty-seven deaths were attributed to cerebral thrombosis, 10 in Class A and 17 in Class B. Five of the 10 patients in Class A had been using oral contraceptives whereas 1.5 would have been expected; this difference was statistically significant. Of the 17 patients in Class B, one was excluded because of a poor history of oral contraceptive usage. None of the other 16 had used oral contraceptives.

In summary, the authors stated that a strong relationship existed between the use of oral contraceptives and death from pulmonary embolism or cerebral thrombosis, but not from coronary thrombosis, in the absence of predisposing conditions (Class A). In the presence of predisposing conditions (Class B), no significant relationship was found to indicate an increased number of deaths from any type of thromboembolism, although one might theoretically have been expected. In fact, for coronary thrombosis a statistically significant difference was found that indicated a decrease in the number of deaths associated with this type. As a possible explanation, the authors suggested that physicians might have been reluctant to prescribe oral contraceptives for women suffering from serious chronic diseases. However, since the health status of the controls was not known, this group was not divided into classes as was the thromboembolic group. Therefore, a more legitimate summary statement would involve a comparison of Class A plus Class B *versus* the controls. When this comparison was made, a statistically significant difference between oral contraceptive use and thromboembolism was seen only where pulmonary embolism was involved.

In the same issue of the *British Medical Journal*, Vessey and Doll (98) reported the results of another study concerning women admitted to 19 general hospitals for deep vein thrombosis and pulmonary embolism. The nosological index of each hospital was searched and case notes were reviewed relating to women who: (*a*) had been hospitalized in 1964–1966, (*b*) were 16 to 40 years old, and (*c*) had been diagnosed as having suffered from thromboembolic diseases in any vein except the cerebral, coronary, hepatic, or mesenteric. Patients having had pulmonary embolism or infarction were included. Women were excluded if they: (*a*) were single or widowed, (*b*) had a predisposing condition, (*c*) were pregnant, postmenopausal, or sterilized, (*d*) had suffered only superficial thrombophlebitis, or (*e*) had died. Two case controls were obtained for each affected patient. Those selected were suffering from an acute medical or surgical ailment and matched the affected patient with regard to hospital, date of admission, age, parity, and absence of attributes that had been used to exclude patients from the affected group. Patients were interviewed in their homes by a medical social worker.

For the 3-year period, 399 patients were found who had suffered pulmonary embolism or venous thrombosis. After exclusions were made for the reasons described, only 61 patients remained in the study; 122 controls were selected. Three sets of patients were lost for various reasons, so the study group contained 58 affected patients and 116 controls.

The results showed that 26 of 58 (45%) patients admitted for deep vein thrombosis or pulmonary embolism had been taking oral contraceptives during the month before the start of the episode; in the control group admitted for other conditions, 10 of 116 (9%) had been taking them. The difference was highly significant ($p < 0.001$). Further comparison of the data suggested that in the presence of a history of previous thromboembolic disease, use of oral contraceptives did not lead to further thromboembolic episodes.

The type of preparation used was known for 23 affected patients and for eight controls; none had used the sequential type. Without giving specific data, the authors stated that there was no indication that any one preparation was more likely to produce thromboembolism than another.

Finally, based on hospital admission records, Vessey and Doll (98) estimated the incidence of venous thromboembolic disease to be approximately 5/100,000 in nonusers of oral contraceptives and 47/100,000 in users; in other words, the calculated risk of hospital admission for this disease was nine times greater in oral contraceptive users.

The investigation was amplified further to study the incidence of cerebral thrombosis and coronary thrombosis, using the same criteria and protocols already described. Only nine patients were available who had had a confirmed cerebral thrombosis. Five of the nine had been using oral contraceptives for periods of from 1 week to 4 years immediately prior to becoming ill. The expected number from the control group data was one. Despite these few patients, the authors stated that the difference between the two groups was statistically significant.

Thirteen women were available who had suffered a coronary thrombosis. None of these had taken oral contraceptives during the month before the onset of the disease, a figure not statistically different from that of 0.7 obtained from the control data.

In a later article, Vessey (90) stated that these three British studies (96–98) were subject to the usual problems of case-control studies, but that their statistical inadequacies were less than those of earlier studies.

A "leading article" (99) published simultaneously in the same journal also reviewed the three British studies (96–98). Three conclusions were presented that had been drawn from the results of those studies as well as from data from studies (100, 101) in which estrogens had been used for purposes other than contraception: (*a*) sequential preparations might be more dangerous since they contained more estrogen than the combined type, (*b*) the continuous low dose progestagen technique might afford a means of avoiding the thromboembolic effects, and (*c*) the administration of estrogens for any

purpose, particularly when given in high doses or for long periods, carried a definite risk.

Strong objection to these conclusions was voiced by Reid (102) who claimed that this article led to a minor panic in Australia. In particular, he objected to the conclusion that the estrogenic component primarily was responsible for thrombosis, since Vessey and Doll (98) had not been able to show such a difference. In addition, the other evidence referred to was concerned with ethinyl estradiol given to men in doses higher than those used in women for contraception (100) and with an estrogenic substance other than those used in oral contraceptives and which had been used in higher than equivalent contraceptive doses (101).

Vessey and Weatherall (103) estimated the mortality rates due to venous thromboembolism for the years 1963–1967 based on known mortality rates per million per annum for the years 1953–1962. Separate data for males and for females were further subdivided for four age groups. The actual mortality rates were higher than predicted in three groups: 35–44-year-old males, 20–34-year-old females, and 35–44-year-old females. The authors acknowledged that the slopes of the regression lines they fitted to the data were subject to considerable standard errors, especially at the lower ages, although they did not include their values. They also suggested that similar factors could have been operating in all three groups to increase their mortality rates. Nevertheless, they felt that the increased mortality in the two groups of females was sufficiently high to be accounted for at least partly by the use of oral contraceptives.

In 1968, Drill and Calhoun (104) summarized data from a number of sources in an effort to determine the incidence of thromboembolic disease (thrombophlebitis and thromboembolism) during pregnancy, the puerperium, and oral contraceptive use, as well as in nonpregnant nonusers of oral contraceptives. They found that in nonpregnant women, 20–44 years of age, the natural incidence of thrombophlebitis, based on hospital admissions, was 0.91 (range 0.65–1.08)/1000 women/year. This mean was derived from averages of data for 1947–1962 obtained from three independent sources.

Drill and Calhoun were critical of the figure given by Vessey and Doll (98). The latter investigators had derived a figure of 0.05 case/1000 women/year in nonpregnant women, 16–40 years old. Drill and Calhoun stated that this rate was not comparable with results obtained in other studies. They concluded that the most satisfactory estimate was approximately 1/1000/year based on data from hospital admissions. When the incidence was based on patients' visits to the physician, the mean of the averages from five independent sources for 1955–1966 was 2.2 (range 1.2–3.0) cases/1000 women/year.

The incidence of thromboembolism in the puerperium reported for European studies was 10.4 (range 4.0–21.5) cases/1000 deliveries, while in the United States the incidence was 3.1 (2.6–6.4). Drill and Calhoun (104) pointed out that if these figures were extrapolated to a yearly basis, they would be even higher than the figure for nonpregnant women.

During the antepartum period, the incidence was found to be considerably below that in the postpartum period. The incidence of thrombophlebitis in the antepartum period, based on 379,766 pregnancies reported in 15 studies, was 0.56 ± 0.15 case. When this figure was extrapolated from the 9-month period to 1 year, the rate became 0.74/1000 woman-pregnancies/year. The authors pointed out that the incidence in the antepartum period was approximately one-half that found in nonpregnant females of childbearing age when based on data from hospital admissions and approximately one-fourth that found in similar women when based on data from visits to physicians.

Drill and Calhoun pointed out that in early reports published between 1956–1959, norethynodrel with mestranol was used in doses of 20–30 mg. (norethynodrel)/day for various menstrual disorders but that no thrombophlebitis was reported. A compilation of 29 of the early (1959–1966) major studies with norethynodrel and mestranol revealed one case of thrombophlebitis in 4271 woman-years of use, yielding a calculated incidence of 0.23 case/1000 women/year. In these studies the doses of norethynodrel ranged from 2.5 to 10.0 mg. In 16 studies in which norethindrone (2.0–10.0 mg.) with mestranol was used, three instances of thrombophlebitis were found during 4427 woman-years of use, yielding a calculated figure of 0.68 case/1000 women/year.

Drill and Calhoun also compiled the data from six major studies, reported between 1962 and 1965, which employed eight different contraceptive preparations and encompassed a total of 50,781 woman-years of use. A total of 28 cases of thrombophlebitis occurred in the entire group, providing an incidence rate of 0.55 ± 0.37 (range 0–3.7) case/1000 women/year. They stated that taken singly or totally the data from these studies did not provide any evidence of a relationship between oral contraceptives and thrombophlebitis.

The authors were critical of the 1967 study conducted by the Royal College of General Practitioners (96) because the matching of affected patients with controls had not been exact. Also, if the two affected patients who had stopped taking their oral contraceptives more than a month prior to the episode were dropped from the list, and if a figure of 10% were estimated for use of oral contraceptives in the general population, an incidence rate of 2.4 would be obtained. This overall rate would be well within the normal range.

Drill and Calhoun (104) also pointed out that the final figure arrived at by Vessey and Doll (98) for the estimate of risk of thromboembolic disease in oral contraceptive users, as judged from data on hospital admissions, was 0.5 case/1000 women/year in comparison with their figure of 0.55.

Studies involving the use of oral contraceptives for the treatment of endometriosis, in doses greater than those used for contraception, also were described by Drill and Calhoun (104); 456 patients were reported to have been treated on a continuous basis without a single report of thrombophlebitis.

Mortality statistics were presented for deaths due to thromboembolic disease and to pulmonary embolism in 20–44-year-old men and women in England and Wales for 1956–1966. The apparently slight increase over the 10-year period existed for both men and

women; as a result, there was no significant change in the ratio of deaths in women to those in men.

Data concerning the incidence of pulmonary embolism, both idiopathic and with other factors present, in young adults at a London Hospital for 1954–1964 also were presented by Drill and Calhoun. There was a significant increase over that time period. However, they pointed out that similar numbers of males and females had been involved, and that none of the females had been taking oral contraceptives.

Seigel and Markush (105) reviewed U. S. mortality statistics in which various thromboembolic diseases were involved. They stated that the mortality trends in this country were consistent with those shown by the British studies, which indicated an association between oral contraceptives and venous thrombosis. As in the British studies, an association with coronary thrombosis was not shown. However, the U. S. trends, unlike the British ones, did not suggest a relationship with cerebral embolism and thrombosis either. The relative risk of death from venous and pulmonary embolism estimated by Seigel and Markush was of the same order of magnitude as indicated in the British studies.

In 1969, Vessey and Doll (106) published a subsequent report of their findings which was a continuation of the study described previously (98); the same procedures were used throughout and similar results were obtained. Data obtained on 26 additional patients were added in this study to those obtained from the original 58 patients; in addition, several other factors were examined. For example, a comparison was made of the number of patients affected with thromboembolism and the number that would have been expected to be affected with regard to the type of oral contraceptive combination used. Seven combinations of two estrogens and seven progestagens had been used by the group. None had used sequential products, and no doses for the combined products were given. The authors concluded that the data did not provide any evidence that the risk of thromboembolism might be greater with preparations that they believed to be more inherently estrogenic. The data were reviewed in an attempt to determine whether the duration of use of the oral contraceptives at the time of onset of the attack might be a determining factor. The data did not suggest to them that either short-term or long-term use might carry a greater risk of producing thromboembolism.

In their earlier report, Vessey and Doll (98) noted that the affected patients, on the average, were heavier smokers than were the controls. In this study, they found no evidence to verify the earlier findings. Frederiksen and Ravenholt (107) retabulated the data of Vessey and Doll (98, 106) and concluded that these data could not be used as evidence against the possibility that cigarette smoking could potentiate the role that oral contraceptives might play in the pathogenesis of thromboembolism.

Crombie and Cross (108) were critical of Vessey and Doll's report (106). They suggested that most of the difference between the higher estimate of risk of thromboembolism in oral contraceptive users supplied by Vessey and Doll and their own lower estimate could be attributable to an increased hospital admission rate, on the order of 2.5 :1, for users as opposed to nonusers.

Hougie (109) also was critical of the estimated ninefold increase in thromboembolic disease in oral contraceptive users given by Vessey and Doll. He quoted a study in which a methodical examination of one lung resulted in the finding of pulmonary emboli in 52% of 263 autopsy cases while routine autopsy procedures on the other lung revealed pulmonary emboli in only 12%. Since the possibility that oral contraceptives might be implicated in thromboembolism had been recognized by the medical profession since 1962, Hougie suggested that more extensive physical examinations and autopsy procedures might have been carried out on users as opposed to nonusers in an effort to find evidence of thromboembolism.

Sartwell *et al.* (110) reported in 1969 on an epidemiologic, retrospective, case-control study conducted in five large eastern cities in the United States. This study involved 15–44-year-old women who had been discharged from 43 hospitals within the previous 3 years following a thromboembolic episode of any type. From their hospital histories, cases were excluded on the following grounds: a chronic condition thought to be predisposing to thromboembolism, recent trauma or surgery, pregnancy, sterility, and conditions that might be considered as contraindications to oral contraceptives. Some cases were excluded later because of uncertainty of diagnosis, prior attack of thrombophlebitis, or evidence of some predisposing condition. Of 2648 records of cases of thromboembolism abstracted at the hospitals, 2387 were rejected because of the reasons given above; only 175 cases with an unequivocal diagnosis of idiopathic thromboembolism were finally accepted.

Married control patients were individually matched with the cases for age within 5 years, residence, race, parity, hospital room pay status (private, semiprivate, or ward), and discharge from the same hospital within the same 6-month period. The same exclusions, on the basis of infertility or chronic disease, were also applied to the controls. During the study, however, in three of the cities, unmarried cases and controls were included because: "it had become evident that idiopathic thromboembolism was relatively frequent among younger, unmarried women, and it seemed unwise to disregard this source of case material."

The most common thromboembolic problem was thrombophlebitis of the lower extremity (115 cases) followed by thrombophlebitis with pulmonary embolism (19 cases), pulmonary embolism (18 cases), intracranial or carotid vascular lesion (13 cases), retinal vascular lesion (six cases), and thrombophlebitis at other sites (four cases). Coronary artery disease was not represented.

The relative risk of thromboembolism was determined by dividing the number of instances, 57, in which only the case had used oral contraceptives by the number of instances, 13, in which only the control had used them. By using this formula, a relative risk of 4.4 was determined. Sartwell *et al.* (110) pointed out that 26 of the cases were student nurses, all of whom had thrombophlebitis of the lower extremity and only two of whom had used oral contraceptives. They suggested that some

hospital jobs that involved standing on one's feet for considerable periods might be conducive to thrombophlebitis of the lower extremities.

A comparison of the period of use of the oral contraceptive preparations ranged from between less than 1 week to 2 years before admission. There was no evidence to indicate an increased risk with use up to 2 years.

The oral contraceptive preparations used in this study included three sequential products and nine combination products. The doses of estrogen ranged from 50 to 150 mcg. (mestranol) or 50 to 100 mcg. (ethinyl estradiol), and doses of seven different progestagens ranged from 1.0 to 25.0 mg. To determine whether either the combined or sequential products might show a greater propensity toward producing thromboembolism, a comparison of these two types of products was made. Fifteen cases and no controls had used the sequential type; the corresponding numbers for the combination products were 48 and 23, respectively. It was concluded that these ratios displayed a statistically significant difference. Furthermore, in the group of sequential users, seven of 15 developed pulmonary embolism. However, the doses of mestranol were higher in some of the combined preparations than in the two sequential products containing this estrogen. No additional information was given to indicate an association, or a lack thereof, between any of the other disorders and any of the preparations.

Inman *et al.* (111) analyzed reports of thromboembolism, following the use of oral contraceptives, received by drug safety committees in the United Kingdom, Sweden, and Denmark. The purpose was to determine whether the risk of thromboembolism might be related to the nature and/or doses of the steroids in the various preparations. The numbers of reports attributed to each product were compared with the distribution that would have been expected based on market research estimates of sales, assuming that all products carried the same risk.

In comparing sequential *versus* combined preparations containing corresponding doses of ethinyl estradiol or mestranol, Inman *et al.* (111) found no evidence to indicate that either type of preparation might have had a greater propensity to produce venous or arterial thromboembolism. In contrast, a comparison of the number of reports of thromboembolism with various doses of the two estrogens showed a consistently higher number of reports in which preparations containing higher doses of the estrogens were used. This trend was observed for cases of pulmonary embolism, cerebral thrombosis, and coronary thrombosis.

Observed and expected reports of thromboembolic disorders in patients using 12 different combinations of estrogen and progestagen were presented. These involved doses of mestranol of 50, 75, 100, and 150 mcg. and doses of ethinyl estradiol of 50 and 100 mcg., in combination with one of six different progestagens. No two preparations contained the same dose of any progestagen and, therefore, no direct comparisons could be made. Furthermore, the total number of patients using the preparations varied from less than 15 up to 76; as a result, when thromboembolism was categorized into four different types, there were often less than six affected patients per preparation. Nevertheless, the authors pointed out a "significant" deficit of reports in all categories of thromboembolism for the combination of mestranol (100 mcg.) and norethynodrel (2.5 mg.), in comparison with other combinations of progestagens with this dose of mestranol. Also, a "significantly higher" number of reports of venous thromboembolism was found for the combination of ethinyl estradiol (50 mcg.) and megestrol acetate (4 mg.) when the latter was compared with other combinations of progestagens with the same dose of ethinyl estradiol. The authors suggested that such inconsistencies might represent an influence of the progestagenic components.

It was concluded in this study that there was a positive correlation between the risk of thromboembolism and the dose of estrogen but that there was no difference between the two estrogens, nor between the combined and sequential products when these products contained the same type and dose of estrogen.

Nanni (112) criticized this study because it had been based on voluntary reports from physicians and because no patient control data had been used. He also objected to the use of a market research estimate of sales as a substitute for control data.

Anderson (113) compared mortality rates for women, 15–44 years old, in Ontario, Canada, for 1959–1961, when oral contraceptives had not been used, and for 1966–1968, the most recent period of contraceptive use for which mortality statistics were available. He estimated that 20–30% of the women in the age group studied during the second time period had used oral contraceptive preparations.

According to Anderson, the death rate from venous thromboembolic disease had increased from 0.32/100,000 women/year in 1959–1961 to 0.97 in 1966–1968 (+200%); the corresponding increase for men was from 0.36 to 0.45/100,000 men/year (+25%). The increase in mortality attributed to cerebral thrombosis was from 0.24 to 0.38 (+58%) for women and from 0.29 to 0.34 (+17%) for men. Increased mortality due to thromboembolic disease in other vascular beds was not evident.

This retrospective study did not utilize case controls of any type. Furthermore, the author stated that it was well recognized that the validity of diagnostic information on death certificates often was doubtful. However, he felt that if a causal relationship did exist between oral contraceptives and thromboembolism, "it would be surprising if the new treatment did not eventually result in a change in the death rate."

Anderson pointed out that although the oral contraceptives seemingly had caused a large percentage increase in venous thromboembolism and cerebral thrombosis, these causes of death represented a very small fraction of the total mortality for that age group. Furthermore, the decrease in mortality rate associated with pregnancy and childbirth was much greater than the increase in mortality supposedly produced by oral contraceptives.

In contrast to previous studies that had merely determined the incidence of thromboembolic disease, Irey *et al.* (114) conducted a study concerning the type of

pathological lesion found in the blood vessels of women who had suffered from thromboembolism. Blood vessels from 20 women who had succumbed to thromboembolism while taking oral contraceptives were studied histologically and compared with similar preparations from 22 women in the same age category who had not been taking oral contraceptives but who had died from diseases associated with thrombosis and thromboembolism. The oral contraceptive users ranged in age from 18 to 41 years. Fifteen were white and five were black, and none had conditions thought to be predisposing to thromboembolism. The duration of use of the oral contraceptives ranged from 5 weeks to 13 months; two patients had been taking sequential preparations, and 15 others had been taking the combined type. Nine of the patients had also been taking other drugs including tranquilizers, anorectics, thyroid extract, and cortisone.

For the oral contraceptive users, distinctive vascular lesions in association with thrombosis were widely distributed in the arteries and veins. These lesions were characterized by the following intrinsic vascular changes: (*a*) three-layered thrombi of at least 1 week's duration, with underlying structural and microscopic changes (19 instances); (*b*) endothelial proliferation and intimal thickening, with no changes in the media and adventitia (four instances); and (*c*) focal nodular thickening of the intima, media, and adventitia (one instance). Vascular lesions without occlusive thrombi were present in four patients, indicating that the changes in the blood vessels were primary.

In the control group, intrinsic vascular lesions resembling those found in the oral contraceptive users were found in only one patient. This woman had suffered from pulmonary hypertension associated with congenital heart disease. Further inquiries concerning this patient revealed that she had been taking an oral contraceptive for 6 weeks prior to her death.

It was concluded from this study that although further studies were necessary to establish a causal relationship between the ingestion of steroid hormones and intimal hyperplasia, the consistent finding of these vascular changes in oral contraceptive users, as opposed to a lack of such changes in nonusers, suggested that such a relationship might indeed exist.

Opposed to the findings of the retrospective, epidemiologic British studies, results from other studies have been in agreement with the conclusions of Drill and Calhoun (104) that there is no causal relationship between oral contraceptive use and thromboembolism. For example, in 1970, Fuertes-de la Haba *et al.* (115) reported the results of an investigation of the causes of death in a group of women in Puerto Rico. From July 1961 to May 1969, 9633 women participated in the study (Project Population).

The women were distributed randomly into an oral contraceptive group (4846) and a vaginal group (4787). The oral group received norethynodrel 5 mg. with mestranol, and those in the vaginal group were provided with a vaginal contraceptive method excluding any form of intracervical or intrauterine device; pertinent instructions were given. The women also were given a comprehensive medical survey which included: (*a*) medical and socioeconomic history; (*b*) complete physical examination; (*c*) urinalysis; (*d*) cervical cytologic study; and (*e*) determination of hematocrit, 17-ketosteroids, and sulfobromophthalein (BSP) retention. The women returned at 2 month intervals, following the initial survey, to receive more contraceptive material and a health questionnaire. At yearly intervals they received a physical examination, urinalysis, and blood study, unless some abnormality had developed which demanded more frequent attention.

As of May 1969, 87% of the patients were under active supervision. Of the remainder, some had moved to other areas of Puerto Rico or the United States, some had been contacted but had not returned for the followup examination, and others had died from a variety of causes. Of the 30 verified deaths, 12 had occurred in the oral group and 18 in the vaginal group. When the death rates were averaged over the 8-year period of the study, the rate for the oral group was 4.0/10,000 and that for the vaginal group was 7.5/10,000. Therefore, the authors concluded that the data did not support the contention that mortality increases among oral contraceptive users.

Several months later, Fuertes-de la Haba *et al.* (116) published a subsequent paper involving the same individuals discussed previously (115), but data from 265 additional women were included. This report concerned the occurrence of thrombophlebitis in users and nonusers of oral contraceptives. Lists of patients diagnosed as having thrombophlebitis since the start of the experiment were obtained from all hospitals where the clinics were located and matched against the patient list of the study. Forty-eight cases of thrombophlebitis were found, and these patients were interviewed and examined by an internist. Of these, 31 were excluded for various reasons, including: (*a*) having no verification of the disease, (*b*) having had the disease before entering the study, and (*c*) being postpartum. Of the remaining 17, eight were from the control (vaginal) group and nine from the oral group.

The incidences of thrombophlebitis in the oral group and in the control group were 1.8/1000 and 1.6/1000, respectively; the difference was not statistically significant. The relative risk also was calculated after controlling for variables such as age and number of cycles of treatment; again no significant difference was found between the oral and control groups with respect to the risk of developing thrombophlebitis.

To determine the effect, if any, that oral contraceptives might have on the occurrence of pulmonary embolism in supposedly healthy persons, Zimmerman *et al.* (117) reviewed the records concerning this condition, of the Cuyahoga County Coroner's Office, for two periods of time: January 1, 1951, to July 31, 1962, before oral contraceptive use was introduced, and August 1, 1962, to December 31, 1969, during which period these drugs were used extensively. Persons included in the study were of either sex and between the ages of 15 and 45 years at the time of death; persons who had had a condition that might have led to pulmonary embolism were excluded.

During the first period, there were 28 cases of unexpected death due to pulmonary embolism: 10 in men,

13 in nonpregnant women, and five in pregnant women; figures for the second period were five, 10, and two, respectively. The crude death rate based on the 1960 census figures were as follows: for the first period, 2.55/1,000,000 for men, 3.04 for nonpregnant women, and 4.20 for all women; for the second period the figures were 2.16/1,000,000, 3.96, and 4.76, respectively. No significant difference existed between the frequency in men and nonpregnant women during either period, nor between the death rates of nonpregnant women when the two periods were compared. The authors concluded that this study did not reveal a significant increase in death due to pulmonary embolism in nonpregnant women since the time of introduction and widespread use of oral contraceptives.

Goldzieher (118) examined 23,217 subjects who were using a sequential type of oral contraceptive. The subjects were required to make monthly visits to clinics or Planned Parenthood Centers to obtain their contraceptive supplies and were followed intensively through 363,469 cycles, an average of 16 months per patient. Seventeen instances of thrombophlebitis occurred, producing an incidence of 0.56/1000 women/year. As the author pointed out, this figure was strikingly similar to the figure given by Drill and Calhoun (104) for the control population and for the incidence found in users of the combination type of oral contraceptives.

In 1970, Goldzieher (80) critically reviewed the evidence associating oral contraceptives with thromboembolism. He stated that statistical studies of the type presented in the British and American retrospective, epidemiologic studies did not establish causal relationships but simply established strengths of correlation, a fact generally recognized by statisticians and clinical investigators.

In 1972, Drill (119) reported a study which included data in addition to those reported previously (104). This report summarized data concerning the incidence of superficial and deep vein thromboembolic disease of the lower extremity in untreated (normal incidence), postpartum, and antepartum women and in users of oral contraceptives. Women with and without predisposing conditions were included.

Based on data concerning hospital admissions obtained from several countries, the average normal incidence of the disease in women of childbearing age was 0.9 (range 0.71–1.08)/1000 women/year. When the incidence was based on patients' visits to the physician, the rate was 2.2 cases/1000 women/year. For idiopathic cases alone, the rate was 0.65/1000 women/year.

In postpartum patients the frequency of these diseases was elevated. Based on 199,730 pregnancies, the total incidence was 9.8 cases/1000 women; this figure was divided between 5.3 for superficial vein involvement and 4.5 for deep vein involvement.

During the antepartum period, the incidence was reduced. Based on 350,661 pregnancies, the incidence of superficial and deep vein disease was 0.46 case/1000 women/9-month pregnancy. When this figure was extrapolated to 12 months, it became 0.61: 0.36 for deep vein disease and 0.31 for superficial vein involvement. The rate of 0.61 was not above that found for nonpregnant women, suggesting that the increased secretion of estrogen during the antepartum period did not result in an increased incidence of thromboembolism.

The incidences of thromboembolic disease reported between 1963 and 1970 in 15 large-scale and 53 small-scale prospective studies of oral contraceptive users were compiled. In the large-scale studies involving 66,915 women treated for 68,616 woman-years, the average rate was 0.92 case/1000 women/year. The difference between this figure and that for normals (2.2) was not statistically significant. The small-scale studies totaled 13,162 woman-years of experience. The average rate was 1.22 cases/1000 women/year, and again the difference between it and the nonuser figure was not statistically significant. When the two types of studies were totaled, 81,778 woman-years of experience were represented, and the average rate was 0.97 case/1000 women/year.

The large-scale prospective studies also were utilized to determine the incidences of deep vein and superficial involvements separately. Based on 32,668 woman-years of use, the rate for deep vein disease was 0.21 case/1000 women/year and it was 1.04 for superficial involvement. These rates were not above the control values.

It was also determined from this extensive study that five cases of pulmonary embolism were associated with oral contraceptive use during 65,281 woman-years, yielding a rate of 7.7/100,000. Drill (119) also quoted from the unpublished data of Dr. R. L. Burket that the incidence of pulmonary embolism in 15–44-year-old nonpregnant women in 12 Cincinnati hospitals during 1959 was 15.9/100,000 and that the rate for idiopathic cases alone was 10.8.

Drill summarized his review by listing six salient points:

1. The increased output of hormones during pregnancy was not associated with an increase in the incidence of superficial and deep vein thromboembolic disease.

2. Neither large-scale nor small-scale studies of oral contraceptive users demonstrated an increased incidence of the diseases.

3. If a relationship between oral contraceptives and thromboembolism existed, a high proportion of cases should occur during the first few months of use; this point would not be true if the relationship depended on a cumulative effect of the drugs. However, studies such as those of Vessey and Doll (106) and Sartwell *et al.* (110) suggested that duration of use, at least up to 2 years, had no effect on the incidence of thromboembolism.

4. If a dose–response relationship existed between oral contraceptive use and thromboembolism, increased evidence for the relationship should have been obtained when higher doses of the compounds were administered. Drill cited evidence that large doses of oral contraceptive preparations had been given without adverse effects occurring. Some inconsistencies in this respect do exist, however (*vide supra*); the answer might be related to whether the large doses refer to estrogenic and/or progestagenic activity.

5. If a relationship existed between oral contraceptives and thromboembolic disease, patients with a

history of the disease might be expected to be sensitive to these drugs. Drill pointed out that the data available, although limited in amount, did not demonstrate an increased recurrence of thrombophlebitis when oral contraceptives were used.

6. Women who have developed thrombophlebitis have responded to therapeutic measures without stopping the use of oral contraceptives. Again, however, the data are quite limited.

Drill concluded that the extensive prospective studies he had reviewed demonstrated that oral contraceptive use did not increase the incidence of venous thromboembolic disease of the lower extremities.

At the same time, Drill and Calhoun (120) reported a review of data from prospective studies concerning the relationship between the dose of estrogen in oral contraceptive preparations and thromboembolic disease of the lower extremities. This study had two goals: (*a*) to determine whether the disease rate might be above normal at any dose of either mestranol or ethinyl estradiol and (*b*) to determine whether a statistically significant dose-response curve could be found between the estrogen dose and the incidence of thromboembolic disease.

The prospective studies included for analysis were those in which a definite statement had been made regarding the presence or absence of superficial and deep vein thromboembolic disease, associated with the use of a specific oral contraceptive product for which the number of woman-years of drug use had been stated. Data from these studies then were analyzed in three groups: (*a*) large-scale studies involving 1000 or more woman-years of use, (*b*) small-scale studies involving less than 1000 woman-years of use, and (*c*) a group wherein the data from the large- and small-scale studies were combined after it had been shown that there was no difference between the two sets of data.

The data collected involved a study of 72,075 women for 902,446 cycles during a total of 69,502 woman-years of use. The oral contraceptive preparations studied contained one of the following six doses of estrogen: 50 or 100 mcg. ethinyl estradiol or 75, 80, 100, or 150 mcg. mestranol. No information was given concerning the progestagen(s) in the preparations.

There was no significant difference between the thromboembolic disease rates associated with the two doses of ethinyl estradiol, and these incidences were not significantly different from the normal rate of 2.2/1000 women/year. Neither did the disease rates differ among the four doses of mestranol, nor did they differ significantly from the normal rate.

The authors critically discussed the study of Inman *et al.* (111), which initiated the idea that a relationship might exist between the risk of thrombophlebitis and the dose of estrogen. They offered criticisms of the study similar to those discussed previously by Nanni (112). Because of these fallacies, Drill and Calhoun (120) questioned the conclusions drawn by Inman *et al.* (111). Nevertheless, the latter workers at least attempted to consider the possible effects of the progestagenic components, a point apparently ignored by Drill and Calhoun.

Meanwhile, additional articles, each containing a small number of case reports concerning thromboembolic phenomena in oral contraceptive users, appeared in the recent literature. The phenomena observed include: aorto-iliac occlusion (121), dural sinus and cerebral venous thrombosis (122–124), cerebral arterial occlusion (125–128), cerebral ischemia (129), vascular occlusion of the colon (130), Budd–Chiari syndrome (131), mesenteric vascular thrombosis (132, 133), myocardial infarction (134–137), and pulmonary embolism (138–140). As mentioned previously, the usefulness of these case reports in establishing a cause-and-effect relationship is nil. However, their importance is manifested in the fact that by uncovering and reporting such information, others can be made aware of their possible occurrence. This awareness can lead to similar observations which, when numerous enough, may become meaningful and deserving of case-control epidemiologic and prospective studies. For example, some investigators (128, 130, 133, 141) found that certain thromboembolic disorders originated at the time that oral contraceptive therapy started but that the symptoms regressed spontaneously a short time after terminating their use. This type of observation suggests a degree of involvement between drug and effect.

In summary, the evidence for the existence of a cause-and-effect relationship between thromboembolism and oral contraceptive use is tenuous. It consists for the most part of data from retrospective, epidemiologic, survey-type studies which, as previously stated, cannot prove such a relationship. On the other hand, the large volume of data from prospective studies from which one is better able to establish such a relationship indicates that such a relationship may be nonexistent.

While retrospective and prospective studies have been, and continue to be, carried out in an effort to determine conclusively whether or not there is a cause-and-effect relationship between oral contraceptives and thromboembolism, concurrent investigations have attempted to elucidate factors that might be involved in the development of thromboembolism in certain users of oral contraceptives. Specifically, these investigations have involved blood types and clotting factors.

In 1969, Jick *et al.* (142) reported the results of a cooperative study involving three countries. Initially, data collected for a prospective drug surveillance program in Boston had revealed that among male and female patients receiving anticoagulants for venous thromboembolism, there was a deficit of patients with type O blood. These results stimulated the cooperation of interested groups in Sweden and the United Kingdom. The three groups then conducted retrospective studies to determine the blood types of young white women who had developed thromboembolism: (*a*) while taking oral contraceptives, (*b*) during pregnancy or the puerperium, or (*c*) while neither pregnant nor taking oral contraceptives. In addition, the proportions of blood types A, B, AB, and O in control populations were determined. The percentages for the three countries ranged from 37 to 44% for type A, from 8 to 12% for B, from 3 to 6% for AB, and from 40 to 47% for O.

The numbers of patients representing blood groups B and AB were too small to permit valid comparisons with the control population. However, a trend could be

seen when comparisons were made between the proportions of thromboembolic patients and controls having blood types A and O. Specifically, the percentage of patients having type A ranged from 47 to 61; for type O, the percentage ranged from 16 to 38. These observations, which applied to all three groups of young white women, suggested that individuals with type A blood were more likely to develop thromboembolism and/or that those with type O were less likely to do so.

In 1971, Mourant *et al.* (143) recalculated the data of Jick *et al.* (142) after including some additional data from other studies. They determined the relative incidence of blood group A by dividing [(number of A patients)/(number of O patients)]/[(number of A controls)/(number of O controls)]. In women taking oral contraceptives, the estimated mean incidence of group A was 3.12; in pregnant and puerperal women, it was 1.85. For both conditions, the difference between cases and controls was statistically significant; however, no conclusion could be drawn as to relative liability to thrombosis of women taking oral contraceptives as opposed to those who were pregnant or puerperal.

Additional supportive data were supplied later in 1971 by Westerholm *et al.* (144) and Allan (145). In addition, Allan offered the hypothesis that hormonal factors might intensify what appeared to be a basic association between thromboembolism and blood group A. He listed some indirect evidence that could lend support to his hypothesis, including: (*a*) oral contraception raises the serum cholesterol level, which is already higher on the average for blood group A_1 than for O; and (*b*) oral contraception lowers the antithrombin-III level, which is already lower on the average for A_1 than for O. Furthermore, Fagerhol *et al.* (146) found that pulmonary embolism and diffuse intravascular coagulation also are associated with lowered levels of antithrombin-III; in contrast, levels of the latter appear to be increased during oral anticoagulant therapy.

Although precipitating and contributory factors have long been identified, the basic mechanism of thrombosis is unknown (147). Nevertheless, studies of moieties, such as antithrombin-III, involved in the blood coagulation or fibrinolytic systems have been undertaken because of the possible relationship between oral contraceptives and thromboembolism. It has been presumed that a balance is normally maintained in the blood between coagulative and fibrinolytic activities (148). It has been suggested, furthermore, that oral contraceptives might upset this balance by causing an increase and/or decrease in one or more of the components of these systems.

Many factors of the coagulation and fibrinolytic systems have indeed been shown to be affected by oral contraceptives. For example, electrophoretic behavior of blood platelets from women taking combined or sequential oral contraceptives, but not progestagen-only oral contraceptives, became abnormal due to increased sensitivity to adenosine diphosphate; the sensitivity to noradrenaline, however, remained normal (149). This pattern of platelet behavior was similar to that shown by platelets from patients with arterial disease.

Factors II (prothrombin) (150, 151), VII (150, 152, 153), X (150–153), and fibrinogen (151), all components of the coagulation system, have been shown to increase in women taking oral contraceptives of the combined type. Similar increases in Factors VII and X have been seen in women using the sequential type (152). In contrast, other investigators reported no significant change in fibrinogen levels in women using oral contraceptives of the combined (148, 154), sequential (148), and progestagen-only (154) types, nor in those using combined and progestagen-only injectable contraceptives (154). Nevertheless, blood viscosity, which is partly a function of fibrinogen concentration, and hematocrit were shown to be increased in eight of 47 women using combined and sequential oral contraceptives (155). Furthermore, cryofibrinogen, a cold-precipitable fibrinogen found in disease states such as those associated with intravascular coagulation, was found in the plasma of 17 of 68 users of combined and sequential oral contraceptives, seven of 55 users of an intrauterine contraceptive device, and five of 59 untreated women (156).

Antithrombin levels (*vide supra*) have also been shown to be depressed in women taking oral contraceptives of unspecified and combined types (157–160). Moreover, it has been suggested (159) that of all the clotting changes reported to occur in women using oral contraceptives, the loss of antithrombin-III activity might be the one most significant from a clinical point of view. On the other hand, Hedlin and Monkhouse (148) found no significant changes in antithrombin levels in women using combined and sequential preparations.

Reports concerning fibrinolytic activity and plasminogen levels, as well as interpretations of increases in the latter, in users of steroid contraceptives have been even more conflicting. Most workers (148, 150, 154) reported an increase in fibrinolytic activity in women using oral preparations, suggesting the possibility of a tendency toward hypocoagulability. Brakman *et al.* (154), however, found no change in this parameter in women given a parenteral estrogen–progestagen preparation and decreased fibrinolytic activity in women given an injectable progestagen preparation.

Plasminogen levels also have been reported by most workers (150, 151, 154, 158) to be increased in women using oral contraceptives. However, Brakman *et al.* (154) reported that in earlier studies they had found either no change or decreased levels of plasminogen in oral contraceptive users; these workers also reported no change in plasminogen levels in women given parenteral preparations. The ranges of values presented by these workers did overlap among the controls and various treatment groups, however, and, unfortunately, no levels of statistical significance were given to support the conclusions drawn.

With respect to the clinical significance of increased plasminogen levels, Ambrus *et al.* (151) interpreted this phenomenon as an indication of increased activity in the fibrinolytic system. On the other hand, Peterson *et al.* (158) interpreted low plasminogen levels as evidence of increased fibrinolytic activity. Conversely, high levels suggested to them the possibility of an increased risk of thrombosis. Presumably, they felt that

increased levels signified that the plasminogen was not being converted to plasmin (fibrinolysin). Indeed, Ygge *et al.* (150) reported high plasminogen levels and low spontaneous fibrinolytic activity in patients 1–2 weeks postoperatively, *i.e.*, during a period in which there is a high incidence of thromboembolism.

The question remains, however, as to what significance these changes in clotting and fibrinolytic factors may have with respect to thromboembolism and the use of oral contraceptives. First of all, results from different studies obviously are in disagreement. Brakman *et al.* (154) suggested that these discrepancies could have been caused by differences: (*a*) among assay procedures, (*b*) in the composition of the various hormone preparations used, (*c*) among the dosages of the various hormones, and/or (*d*) in the duration of treatment. Finally, individual treatment and control groups in some studies occasionally contained five or fewer women.

More importantly, it is still necessary to determine whether a certain pattern in blood coagulation and fibrinolysis may really be related to an increased incidence of thromboembolism (150). Nilsson and Astedt (161) maintained that there are no common characteristic changes in the coagulation factors, fibrinolytic system, and platelet aggregation or adhesiveness in patients with thrombosis, although they agreed that a normal fibrinolytic activity in vessel walls is important for counteracting thrombosis. Wolf (162) also questioned the association of clotting factor changes with thrombosis, maintaining that there is no definite distribution pattern of antithrombin-III in patients with thrombosis and that measurement of Factor VII activity, for example, shows much overlap between normal controls and patients with thrombosis.

At any rate, it has been stated (147) that increased levels of clotting factors in the nonactivated form could possibly predispose to thrombosis, but that they could not *per se* initiate intravascular coagulation. However, if together with a loss in antithrombin-III activity, for example, the homeostatic coagulation equilibrium is further unbalanced by events such as vascular endothelial damage, vasculitis, prolonged stasis, increased platelet adhesiveness, or postsurgical hypercoagulability, intravascular clotting could possibly be precipitated (159). Finally, Peterson *et al.* (158) suggested that perhaps only an occasional individual may be unable to handle the changes that appear to be induced by the oral contraceptives. Whether or not such patients could be screened out prior to or during oral contraceptive therapy is not known, but the measurement of clotting factors in users and potential users of these agents would seem to be worthwhile.

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RESEARCH ARTICLES

Drug Transfer across Intact Rat Intestinal Mucosa following Surgical Removal of Serosa and Muscularis Externa

DONALD L. WOLFE*, STEVEN C. FORLAND*, and LESLIE Z. BENET▲

Abstract □ The *in vitro* absorption kinetics for salicylate ion were followed through rat intestinal membranes from which the serosa and longitudinal and transverse layers of the muscularis externa had been removed. Techniques are described where up to 15 cm. of intestine may be stripped of musculature so that absorption studies may be carried out utilizing commonly employed *in vitro* methods such as the everted sac, the Crane–Wilson technique, and a perfusion apparatus. A histological examination of the stripped intestinal mucosa showed that the section consisted of epithelium, underlying lamina propria, muscularis mucosa, and submucosa. The latter two layers do not remain when mucosa is isolated by scraping off the mucosal surface with a glass slide, but they are necessary for maintaining an intact epithelial membrane during *in vitro* transport studies. Transfer rates were measured using 10 cm. of everted intestine in a perfusion apparatus and 5 cm. of everted intestine made into a Crane–Wilson sac. Absorption rates for salicylate through stripped intestinal segments were found to be 1.5–1.8 times greater than those found with nonstripped segments.

Keyphrases □ Drug transfer—*in vitro–in vivo* correlations improved, serosa and muscularis externa removed from intact rat intestinal mucosa □ Transfer rates—procedure for removal of serosa and muscularis externa from intact rat intestinal mucosa preparations, improved *in vitro–in vivo* drug transfer correlations □ Absorption kinetics, *in vitro*—salicylate ion through rat intestinal membrane stripped of serosa and muscularis externa, improved correlation with *in vivo* data □ Intestinal mucosa preparation, rat—procedure for stripping serosa and muscularis externa to improve *in vitro–in vivo* drug transfer correlations

Since many intestinal absorption studies in the pharmaceutical sciences are done employing *in vitro* techniques, the obvious question arises: What correlation can be attached to observations made from *in vitro* studies and events occurring *in vivo*? Ideally, one would hope that the information gained from studying absorption mechanisms can be applied ultimately to the design of more predictable and more effective oral drug dosage forms.

As pointed out by Turner *et al.* (1), a number of studies in the literature showed large differences between *in vitro* results and those expected upon the basis of *in vivo* hypotheses. While one cannot expect *in vitro* preparations to provide a direct reflection of *in vivo* events, one can hope to gain insight into mechanisms that are difficult or impossible to elucidate *in vivo*. However, the reliability of *in vitro* observations in providing meaningful information depends upon experimental conditions conducive to optimum physiological activity. Up to the present time, three commonly employed *in vitro* techniques have been utilized in studying drug absorption: the everted sac method of Wilson and Wiseman (2), the Crane–Wilson method (3), and a perfusion apparatus (4, 5) where transmembrane potentials are neither measured nor short circuited. Some of the problems inherent to these *in vitro* methods that may be contributory to *in vitro versus in vivo* absorption discrepancies are: (*a*) maintenance of biological viability, (*b*) maintenance of structural integrity, and (*c*) artifactual influences due to unnatural absorption barriers.

A number of workers have investigated the extent of viability of excised gut preparations. Under controlled conditions, rather constant metabolic activity for 2–3 hr. was demonstrated with the rat intestine by Bramford (6) and Jordana and Ponz (7) through oxygen consumption studies and by Robinson and Felber (8) with L-methionine and L-phenylalanine uptake. Duration of viability during drug absorption studies would be affected by drugs that are toxic to, or interfere with, metabolic mechanisms and/or the use of bathing solutions (especially buffered solutions) that are not of optimum physiological compatibility.

The problem of structural integrity has been considered by some (9, 10) to be even more crucial to the absorption processes than viability, especially where passive mechanisms seem to predominate. Alteration in structural relationships certainly would have a major influence upon the passive absorption of drugs and, consequently, the ques-

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A number of workers have investigated the extent of viability of excised gut preparations. Under controlled conditions, rather constant metabolic activity for 2–3 hr. was demonstrated with the rat intestine by Bramford (6) and Jordana and Ponz (7) through oxygen consumption studies and by Robinson and Felber (8) with L-methionine and L-phenylalanine uptake. Duration of viability during drug absorption studies would be affected by drugs that are toxic to, or interfere with, metabolic mechanisms and/or the use of bathing solutions (especially buffered solutions) that are not of optimum physiological compatibility.

The problem of structural integrity has been considered by some (9, 10) to be even more crucial to the absorption processes than viability, especially where passive mechanisms seem to predominate. Alteration in structural relationships certainly would have a major influence upon the passive absorption of drugs and, consequently, the ques-

tion of discriminating between absorption mechanisms becomes a moot point. Levine *et al.* (9) made histological studies of everted rat gut preparations and found that although the intestinal sacs were morphologically intact after eversion, they began to lose structural integrity rapidly after 10–15 min. of incubation under conditions normally employed (oxygenated Krebs–Henseleit buffer at 37°), even in the absence of drug. This phenomenon has been attributed to fluid uptake into the tissue (water follows mucosal to serosal sodium flux), with the resultant distension due to the loss of a functional vascular system into which the accumulated water and solutes are normally dissipated.

Benet *et al.* (11), Mayersohn *et al.* (12), Mayersohn and Gibaldi (13), and Morishita *et al.* (14) demonstrated that the presence of various cations and solutes in the bathing buffer solution can affect the transfer rates of certain drugs across intestinal membranes. The degree of inhibition is believed to be correlated to the degree of fluid uptake by the tissue under the influence of the presence or absence of these cations and solutes. Benet *et al.* (11) suggested that the determination of the constancy of transfer rate as a function of time might be a valid approach to assessing the functional integrity (*i.e.*, both biological and structural) of the everted gut preparation. These workers found that a Krebs–Henseleit, pH 7.4 buffer maintained constant transport rates over 2 hr. Although Mayersohn *et al.* (12) demonstrated decreased transfer rates for polar compounds in the presence of glucose, Taraszka (15) made a gross observation (without histological examination) that inclusion of 10 m*M* glucose in the buffer appeared to enhance the structural durability of her cannulated rat gut preparations.

Finally, there is the consideration of overcoming the artifactual influences due to unnatural absorption barriers. One difficulty in extrapolating *in vitro* flux data to the *in vivo* transport of drugs is the fact that the *in vitro* fluxes are measured through the columnar epithelium plus the underlying connective and muscle tissue. Therefore, the rate-limiting step for *in vitro* transport might well be the intestinal musculature, a barrier that would not be encountered during *in vivo* transport. Nayak and Benet (16) attempted to measure the transport rate through rat intestinal musculature after the columnar epithelium was removed by edetic acid treatment. Their findings—high transfer rates through the muscle layer and no difference in directional transport—were in accord with earlier observations (17, 18) that the selective and absorptive elements in the absorption of various drugs are epithelial. Currently, drug transport across the short-circuited epithelial membrane is being followed using radioactive tracers, following the removal of the intestinal musculature by surgical techniques. Field *et al.* (19) observed that the transmural potential and sodium transport capacity of the intestinal mucosa are better maintained *in vitro* if the muscularis is removed. A possible explanation for this observation may be that the unstripped intestine is relatively hypoxic due to an interference by the muscularis externa with oxygen diffusion from the serosal bathing solution to the basal surface of the epithelium.

Considering this information, it would be advantageous to carry out *in vitro* mucosal absorption studies across gut preparations stripped of musculature. The purpose of this study was to develop techniques for the removal of the muscularis externa from large segments of rat intestine so that transport studies across the mucosa could be carried out using any of the three commonly employed *in vitro* techniques. Since the problems of biological and structural integrity involved with these methods have been investigated and defined, greater control over those parameters is possible.

EXPERIMENTAL

Materials—The following USP, NF, or reagent grade chemicals were employed: sodium salicylate, monobasic sodium phosphate with one water of hydration, dibasic sodium phosphate anhydrous, sodium chloride, calcium chloride, magnesium sulfate with seven waters of hydration, sodium bicarbonate, oxygen–carbon dioxide gas (95:5), and air–carbon dioxide gas (95:5).

Surgical Procedures—Male Sprague–Dawley rats, weighing 250–300 g., were allowed free access to both food and water prior to the experiment. The rats were sacrificed by a sharp blow at the base of the skull. The small intestine was exposed *via* a midline abdominal incision, and the gut was wetted with a pH 7.4 Krebs bicarbonate buffer (11), which was used throughout the experiment. The first 25 or 65 cm. of gut distal to the pylorus was discarded, depending upon which section of intestine was to be examined. The next 30 cm. of the intestine was flushed with approximately 20 ml. of the pH 7.4 buffer at room temperature. The 30-cm. segment was then cut into appropriate lengths, depending on the procedure to be followed as discussed below. Sections of intestine were then slid onto glass rods with tapered points that had been prewetted in the pH 7.4 buffer at room temperature, and then the intestine and rod were placed in buffer chilled on an ice bath (3–4°).

The following procedures were carried out on only one-half of the segments placed onto the rods. The fat and mesentery were pulled loose along the mesentery reflection with a pair of tweezers. The serosa and longitudinal and transverse layers of the muscularis externa were loosened, starting at the point of the mesentery reflection and working around the intestine in both directions. This was done until about 2.54 cm. (1 in.) of the intestine was free of these layers at the mesentery reflection and the reverse side had about 0.32 cm. (0.12 in.) free. The remaining length of intestine was stripped by loosening the layers of the mesentery reflection and progressively moving the stripped front down the length of the section. (Tissue layers are diagrammatically shown in Fig. 1.)

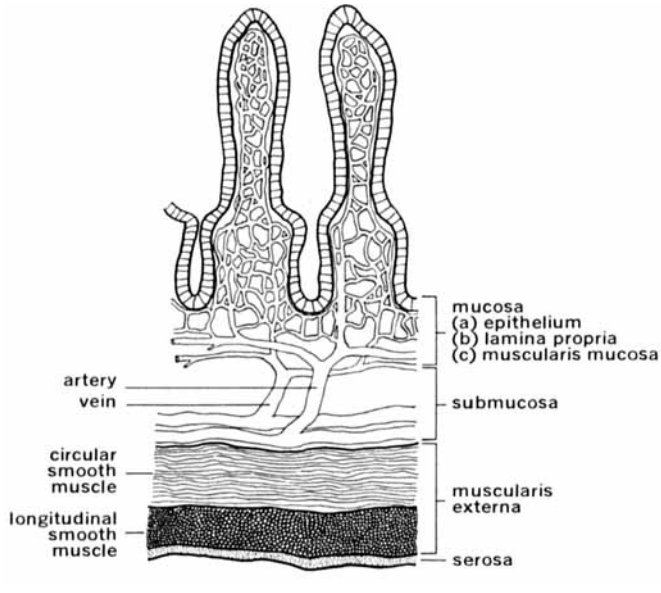


Figure 1—*Diagrammatic representation of the small intestine in cross section.*

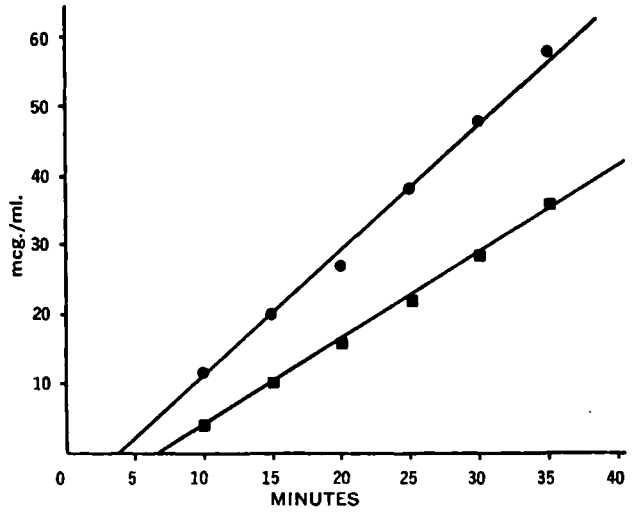


Figure 2—*Sample data plot for the absorption of salicylate through stripped (●) and nonstripped (■) everted rat intestine 25 cm. distal to the pyloric junction, using a perfusion apparatus. Outside solution = 1 mg./ml. drug in isotonic pH 7.4 Krebs bicarbonate buffer. Initial absorption rates through stripped segment = 1.77 mcg./ml./min. and through nonstripped segment = 1.20 mcg./ml./min. Ratio of rates stripped/nonstripped = 1.48.*

All sections of the intestine (stripped and nonstripped) were then slipped off the glass rods into the bathing solution at 3–4°. The sections were placed into pH 7.4 buffer at room temperature and then everted.

Perfusion Experiments—Adjacent 15-cm. everted intestinal segments, one from which the muscularis externa had been removed and one intact, were placed on perfusion devices and absorption studies were carried out following the techniques previously described (1). The inside compartment of each apparatus was filled with pH 7.4 buffer, and the outside compartments were filled with pH 7.4 buffer containing 1000 mcg./ml. salicylate as the sodium salt. Six 0.1-ml. samples were removed from the inner chamber of each apparatus at the following times after the perfusion experiment was begun: 10, 15, 20, 25, 30, and 35 min.

Crane–Wilson Studies—Four successive 6.5-cm. sections of intestine were obtained; two were stripped of the external musculature, and the remaining two were reserved as nonstripped controls. In successive runs, the order of the segments used for stripping was alternated. Following eversion, cannulated sac preparations were made from the gut segments in the manner described by Crane and Wilson (3). The reservoir of each gut segment was filled with 3 ml. of pH 7.4 buffer, and the gut was immersed in 15 ml. of pH 7.4 buffer containing 1000 mcg./ml. salicylate as the sodium salt. Six 0.1-ml. samples were withdrawn at 5-min. intervals beginning with t_1 = 10 min.

Assay Procedures—The 0.1-ml. samples were diluted with 5.0 ml. of 0.1 *N* sodium hydroxide, and drug concentrations were determined spectrophotofluorometrically as described previously (1). Blank values were sufficiently low at all times during the sampling period and could be ignored. Plots were made of the concentration in the inner chamber *versus* time, as shown in Fig. 2 for a typical perfusion study. Rates of transfer, intercepts, and ratios were obtained by the use of a weighted least-squares CPS program[1]. Weights were assigned as the inverse of the concentration squared; thus, theoretically, all data points are assumed to be equally accurate. All statistical comparisons were tested at 95% or greater confidence levels using Student's *t* test.

Histological Examination—A histological examination was also made to compare an untreated, uneverted intestinal segment with stripped and control everted preparations to confirm which anatomical portions of the membrane were removed and to assess the extent of mucosal trauma induced by the procedures. The tissues were fixed in formalin solution and stained with hematoxylin and eosin.

Table I—Salicylate Transfer across Stripped and Nonstripped Segments of Everted Rat Intestine with Perfusion Apparatus

	Rat Number				
	1	2	3	4	5
			25 cm.		
Stripped rate, mcg./ml./min.	1.69	2.21	2.73	1.77	2.24
Nonstripped rate	1.06	1.25	1.96	1.20	1.53
Ratio of rates stripped/nonstripped	1.59	1.77	1.39	1.48	1.46
Stripped clearance, ml./hr./cm.	0.152	0.199	0.246	0.159	0.201
Nonstripped clearance	0.096	0.112	0.176	0.108	0.137
Lag time, min.					
Stripped	6.2	5.7	4.0	3.7	2.9
Nonstripped	7.5	7.4	5.4	6.6	5.7
			65 cm.		
Stripped rate, mcg./ml./min.	1.94	1.84	1.96	2.13	2.09
Nonstripped rate	1.30	1.22	1.31	1.32	1.23
Ratio	1.49	1.51	1.50	1.61	1.70
Stripped clearance, ml./hr./cm.	0.175	0.166	0.176	0.192	0.188
Nonstripped clearance	0.117	0.110	0.118	0.119	0.111
Lag time, min.					
Stripped	2.8	2.4	2.7	4.2	3.7
Nonstripped	4.2	4.7	6.2	6.0	6.3

Table II—Salicylate Transfer across Stripped and Nonstripped Segments of Everted Rat Intestine with Crane–Wilson Apparatus

	Rat Number				
	1	2	3	4	5
			25 cm.		
Stripped rate, mcg./ml./min.	4.37	4.55	4.35	4.61	4.50
Nonstripped rate	2.24	2.68	2.39	2.56	2.22
Ratio of rates stripped/nonstripped	1.95	1.70	1.82	1.80	2.03
Stripped clearance, ml./hr./cm.	0.158	0.164	0.157	0.166	0.162
Nonstripped clearance	0.081	0.096	0.087	0.092	0.080
Lag time, min.					
Stripped	5.5	7.1	4.6	2.9	4.7
Nonstripped	6.8	8.5	7.2	6.8	6.4
			65 cm.		
Stripped rate, mcg./ml./min.	5.22	3.73	4.44	5.71	4.66
Nonstripped rate	3.24	2.74	3.35	3.43	3.23
Ratio	1.61	1.36	1.33	1.66	1.44
Stripped clearance, ml./hr./cm.	0.188	0.134	0.162	0.206	0.168
Nonstripped clearance	0.118	0.099	0.121	0.123	0.117
Lag time, min.					
Stripped	5.4	4.1	6.0	5.3	2.7
Nonstripped	5.6	7.0	5.1	5.9	3.7

RESULTS AND DISCUSSION

The rate data were determined as described previously (1) and are reported in Tables I and II. Clearance values, in units of milliliters per hour per centimeter of intestinal length, were calculated by dividing the rate of drug transport across the intestine by the product of the drug concentration in the outside compartment and the length of the intestinal segment. Equation 1 defines clearance in terms of Fick's law parameters:

$$\text{clearance} = \frac{dQ_i/dt}{(C_o)(\text{length})} = \frac{D_m R_{m/s} A_m}{(\Delta x_m)(\text{length})} \qquad \text{(Eq. 1)}$$

where:

Q_i = amount of salicylate in the inside compartment at any time, t

[1] Executed on an IBM 360/50 computer through a 2741 remote terminal.

Table III—Summary of Data Presented in Tables I and II (Averages and Standard Deviations)

	Perfusion		Crane–Wilson	
	25 cm. (±*SD*)	65 cm. (±*SD*)	25 cm. (±*SD*)	65 cm. (±*SD*)
Stripped rate, mcg./ml./min.	2.13 (0.42)	1.99 (0.18)	4.48 (0.11)	4.75 (0.76)
Stripped clearance, ml./hr./cm.	0.191 (0.037)	0.179 (0.011)	1.161 (0.004)	0.172 (0.027)
Nonstripped rate	1.40 (0.36)	1.28 (0.05)	2.42 (0.20)	3.20 (0.27)
Nonstripped clearance	0.126 (0.032)	0.115 (0.004)	0.087 (0.007)	0.116 (0.010)
Ratio of rates (or clearances), stripped/nonstripped:	1.54 (0.15)	1.56 (0.09)	1.86 (0.13)	1.48 (0.15)
Lag time, min.				
Stripped	4.5 (1.4)	3.2 (0.8)	5 (1.5)	4.7 (1.3)
Nonstripped	6.5 (1.0)	5.5 (1.0)	7.1 (0.8)	5.5 (1.2)

D_m = effective diffusivity of salicylate in intestinal membrane
A_m = total area of the membrane available for salicylate diffusion
$R_{m/s}$ = partition coefficient for salicylate between the membrane and solvent
Δx_m = thickness of the membrane
C_o = concentration of salicylate in the outside compartment at any time, t

Since salicylate was used in all runs at the same outside concentration (1000 mcg./ml.) and at the same pH, it seems reasonable to conclude that D_m and $R_{m/s}$ are constant throughout all experiments. Assuming that there is no significant difference in the circumference (A_m/length) of the intestinal segments used, clearance should equal a constant divided by the thickness of the intestinal strip. In Tables I and II the ratios reported are the rates through the stripped segment divided by the rates through the nonstripped segment, both from the same rat. This ratio should exhibit an inverse relation with respect to the thickness of the intestine, *i.e.*, the greater the ratio the thinner the intestine.

Lag times (Tables I and II) should be directly related to the thickness of the intestinal wall. That is, the thinner the intestinal wall, the shorter is the time required for a steady state to be reached in the membrane. This is reflected in the shorter lag times seen for the stripped intestine as compared to those for the nonstripped intestine (Table III).

The average ratios of the clearance through the stripped segment to clearance through the nonstripped segment, as well as average lag times, are given in Table III. The inverse relation of the ratios and the direct relation of lag time to thickness were not found to be related by an absolute proportionality constant. This result is certainly due to the heterogeneity of the cell types and tissues densities encountered in transport. For example, the cellular composition of the submucosa is less dense than that of the muscularis externa, which is surgically removed from the stripped segment. Although an absolute proportionality cannot be made between lag time or rate of absorption and thickness, it should be noted that the average ratios for perfusion at 65 cm. (Table III) and for perfusion at 25 cm. have values close to the ratios of the average lag times (*i.e.*, at 65 cm., the lag time ratio is 1.72; at 25 cm., the lag time ratio is 1.44).

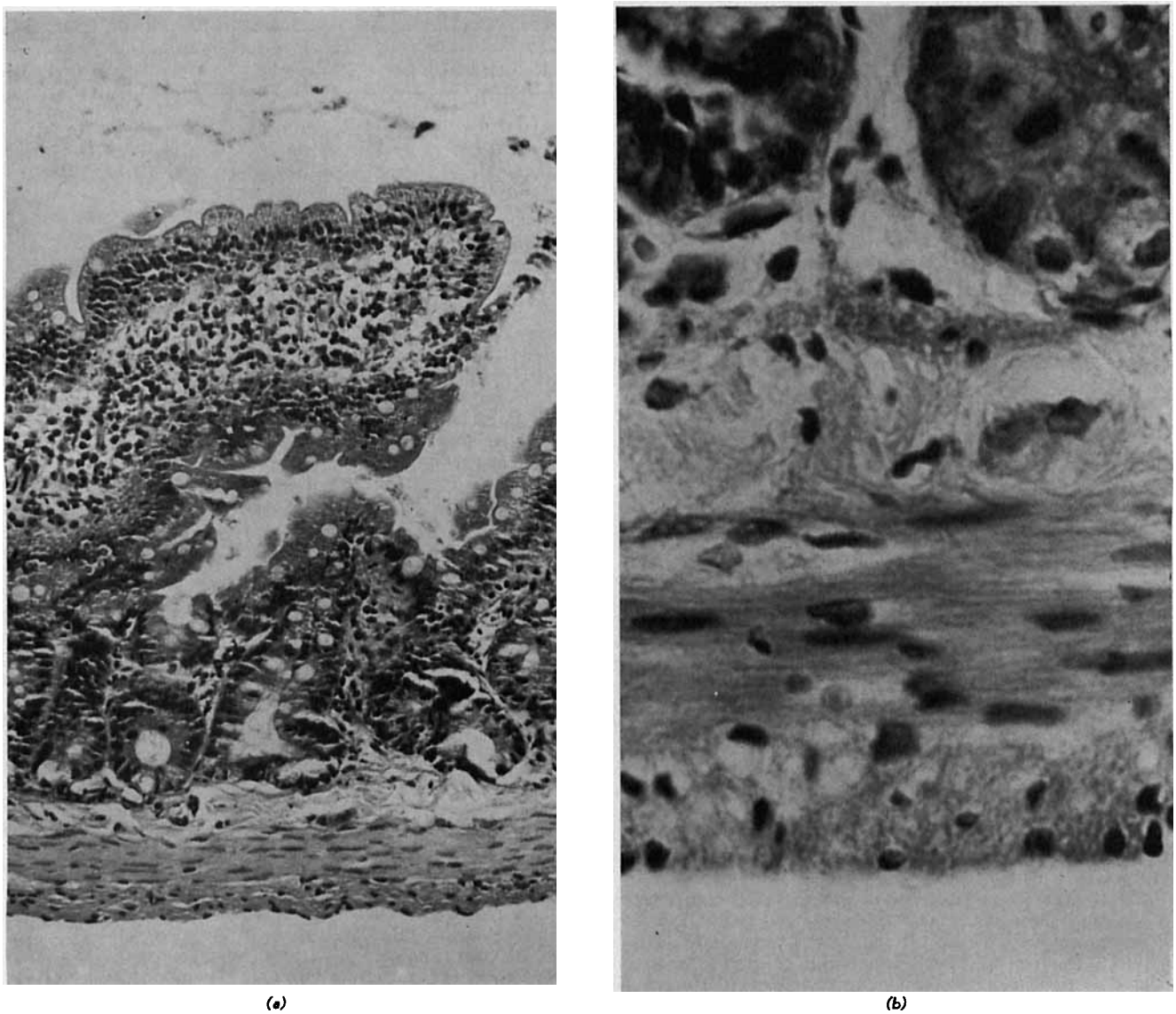
During this work, several other experiments were undertaken to determine the significance of: (*a*) the effect of using 95% air with 5% CO_2 *versus* the effect of using 95% O_2 with 5% CO_2 to oxygenate the preparations during each run, (*b*) the effect on the rate of transport of aerating the inside compartment in the Crane–Wilson studies *versus* nonaeration, and (*c*) differences between rates of absorption 25 and 65 cm. distal to the pylorus. In the trials undertaken to determine the effect of 95% O_2 *versus* 95% air, no significant difference was found either in the rates of absorption or in lag times. In the studies to determine the effect of aerating the inside compartment in the Crane–Wilson method, no advantage in total oxygenation was found over the method in which only the outside of the compartment was oxygenated. However, problems arose when the inside compartment was oxygenated, since air bubbles occasionally became lodged in the intestine, thereby separating the fluid at the bottom of the cannulated sac from that at the top and, thus, preventing uniform mixing of the inside fluids. For this reason, oxygenation of the inside compartment was discontinued and all reported Crane–Wilson data were obtained with outside aeration only. Experiments were also run to determine if any significant difference in absorption rates would be obtained through intestinal segments 25 and 65 cm. distal to the pylorus. In Table III it can be seen that the ratios of rates or clearances (stripped to nonstripped) for perfusion studies at 25 and 65 cm. and for Crane–Wilson studies at 65 cm. are not significantly different. The nonstripped Crane–Wilson studies at 25 cm. are significantly different ($p < 0.05$) than the other three ratios, but no explanation for this difference can be offered at this time.

It would appear that an artifact could be introduced as a function of the design of the apparatus itself. Normally, in carrying out studies using the Crane–Wilson type of sac, a small weight is attached to the bottom end of the intestinal segment so that the segment remains taut during the absorption run. However, weights were not used in this experiment since once the muscularis externa is removed the stripped segments tend to stretch to a length of about 8 cm., even when no weight is attached. This extension is due to the effect of the pressure head of water in the internal reservior. This phenomenon was not noted in the perfusion apparatus where there is a continuous flow of fluid through the tissue segment.

This work was designed to develop a well-founded and easily utilized *in vitro* procedure for studying drug absorption under conditions simulating *in vivo* transport. Therefore, a technique was developed for removing the serosa and the two layers of smooth muscle that form the muscularis externa, so that these anatomical portions of the rat intestine cannot serve as absorption barriers to drug transport. Up to the present time, all *in vitro* intestinal absorption studies with drugs have measured transport across the total thickness of the intestine, including the muscularis externa and serosa as shown in Fig. 1. However, as can be seen in Fig. 1, the capillary supply to the intestine is in the base of the submucosa, at a point in the membrane proximal to the muscularis externa when transport occurs from the intestinal lumen to blood.

To ascertain which anatomical portions of the intestine had been removed by the surgical procedures and to make sure that the structural integrity of the mucosal border had been maintained, histological studies of the everted rat gut preparations were undertaken. Four photographs, representative of the observations made, were taken of the intestine of a single rat. Figures 3*a* and 3*b* are of a cross section of rat jejunum on which no mechanical manipulations have been performed. That is, this section was neither placed on glass rods nor everted. The portions of the intestine in the lower half of Fig. 3*b* are those layers of muscle removed by the surgical procedure; Fig. 3*c* was made from a section of rat jejunum that would serve as the nonstripped control segment discussed with respect to Tables I and II. This section was placed on the glass rod, subjected to ice bath temperatures, removed from the rod, brought back to room temperature, and everted. Figure 3*d* was made using a section of rat jejunum that had undergone the surgical procedure to remove the serosa and muscularis externa, as described in the *Experimental* section, as well as the procedures listed for the control above.

At the start of this experiment, it was feared that the integrity of the epithelium and underlying submucosa would be damaged by the stripping and eversion process. As can be seen by comparing Fig. 3*a* with Fig. 3*d*, there was no damage to the epithelium or to the integrity of the submucosa of the stripped segment. Figure 3*c*, depicting an intestinal section subjected to control procedures, indicated that the control process and eversion of the intestine cause some disruption of the muscularis externa, in comparison to the untreated section in Figs. 3*a* and 3*b*. The control procedure could account for the fact that the clearance values obtained from the control (unstripped) runs for a 10-cm. segment ranged from 0.87 to 1.16 ml./hr. for salicylate, while the clearance for salicylate reported by Mayersohn *et al.* (12) using the Crane–Wilson method



(a) (b)

Figure 3—(a) *Cross section of rat jejunum on which no mechanical manipulations have been performed* (×*150*). (b) *High power magnification of the muscularis externa and submucosal portions of Fig.* 3a. *Note cellular composition and density of serosa and muscularis externa* (×*625*).

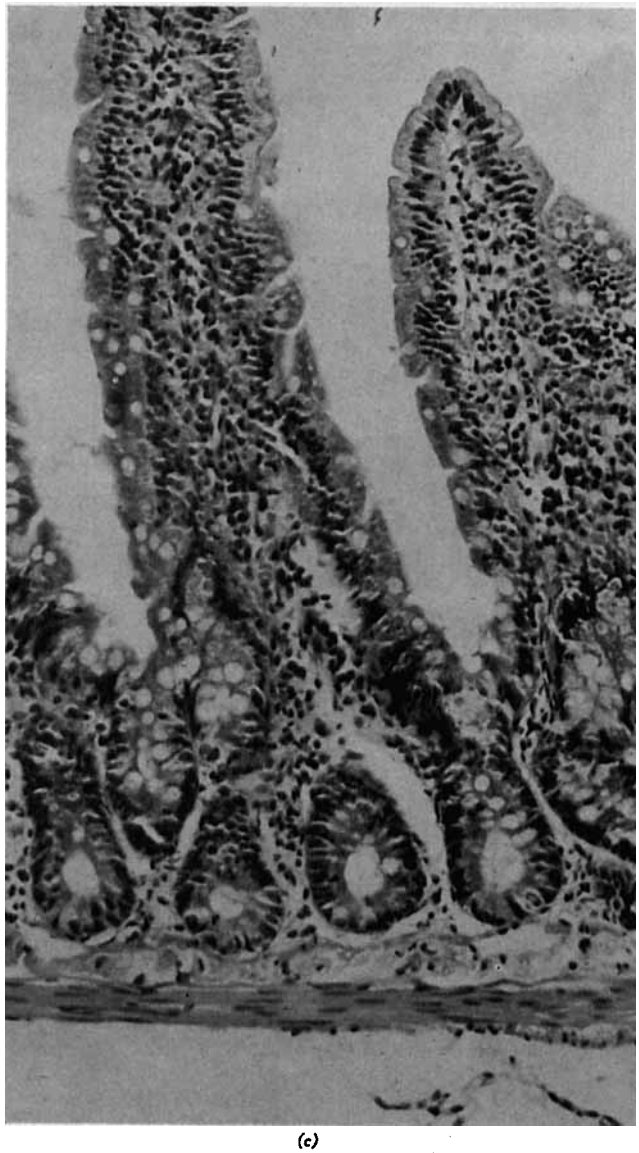
was 0.8 ml./hr. However, statistically there is no significant difference between the average values found in this study and the previously reported studies at the 95% level of confidence.

By comparing Figs. 3*a* and 3*c* with Fig. 3*d*, the relative thickness of the muscularis externa barrier to *in vitro* transport can be estimated. The significance of this barrier is clearly shown by the average ratios reported in Table III. The ratios of clearance values (stripped/unstripped) range from 1.54 to 1.86 in the four studies described, indicating that removal of this artifactual barrier to salicylate transport in *in vitro* studies brought about a 50–85% increase in transport rates. With the *in vitro* techniques previously reported in the literature (2–4), drug has to diffuse through several layers of tissue beyond the capillary bed, the point where the drug would enter into the blood circulation during *in vivo* transport. Using the techniques reported in this work, the serosal solution would be in contact with the submucosa of the gut preparation at the bottom of Fig. 3*d*.

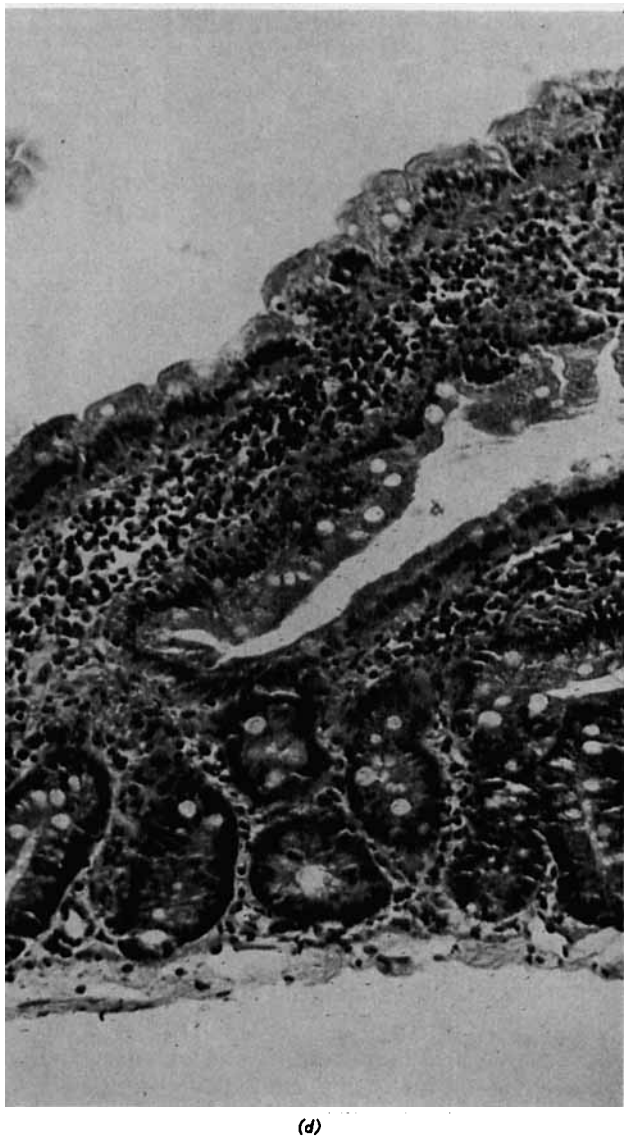
SUMMARY AND CONCLUSIONS

The *in vitro* absorption kinetics for salicylate ion were followed through rat intestinal membranes from which the serosa and longitudinal and transverse layers of the muscularis externa had been removed. It was found that the serosa and muscularis externa can be removed from an intestinal segment in one intact sheet and that there is no resulting damage to the absorptive tissue as a consequence of the surgical procedure. Furthermore, it was established that when the stripping procedure is incorporated with the presently used *in vitro* techniques for studying drug absorption, there is a substantial increase in salicylate transport rates and a corresponding decrease in lag times. This is clearly shown by the data in Table III. For example, Crane–Wilson studies at 25 cm. through unstripped intestinal membranes yielded salicylate clearance values of 0.087 ml./hr./cm. [Mayersohn *et al.* (12) reported a value of 0.080 ml./hr./cm.], while salicylate clearance through stripped segments was found to be 0.161 ml./hr./cm. Since the great majority of the capillaries in the small intestine are found in the lamina propria at the base of the epithelial cells, it is highly improbable that *in vivo* drug absorption is retarded by any physical barrier comparable to that of the serosa and muscularis externa.

In summary, the procedure for surgically removing the serosa and muscularis externa did not produce any change in the integrity of the epithelium, lamina propria, or submucosa. The procedure can be easily incorporated into the three commonly employed *in vitro* techniques utilized in studying drug absorption. The procedure allows the removal of a significant artifactual influence to the comparison of *in vitro* and *in vivo* transport studies. In light of these findings, it is the opinion of the authors that the use of stripped intestinal segments for *in vitro* drug transport studies will provide the investigator with a well-controlled, reproducible technique for elucidating the mechanisms involved in the *in vivo* absorption of drugs from the intestinal lumen.



(c)



(d)

Figure 3—(c) *Cross section of everted rat jejunum that has been subjected to the control procedure (before incubation)* (×*150*). (d) *Cross section of everted rat jejunum from which the serosa and muscularis externa have been removed. Note the integrity of the epithelium and submucosa* (×*150*).

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Improved Microdetermination for Diphenylhydantoin in Blood by UV Spectrophotometry

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Abstract □ A modification of the UV spectrophotometric method proposed by Wallace for the determination of diphenylhydantoin in blood samples is described. Improvement of the Wallace method was made possible by changing various reaction conditions, including oxidation time, and by determining the diphenylhydantoin oxidation product using an appropriately expanded scale of a recording spectrophotometer. The improved method is simple, reproducible, and capable of screening large numbers of samples in a short time, since the tedious procedure of collecting the oxidation product by continuous extraction (as in the original method) is not required to oxidize diphenylhydantoin. The improved method can be used to determine blood and plasma levels after a single injection of 50 mg. of sodium diphenylhydantoin in a human subject. Plasma levels of diphenylhydantoin and phenobarbital were determined after a simultaneous administration of 5 mg./kg. of these compounds in rabbits using a combination of the present method with the Williams method for analysis of barbiturates.

Keyphrases □ Diphenylhydantoin in blood—UV spectrophotometric analysis, in presence of phenobarbital □ Blood levels, diphenylhydantoin—UV spectrophotometric analysis, in presence of phenobarbital □ UV spectrophotometry—analysis, diphenylhydantoin in blood

In the clinical management of patients during long-term treatment with diphenylhydantoin, it is desirable to check frequently its blood levels so that the adequacy of dosage and the possibility of toxicity may be appraised. For such purposes, a rapid, simple, and accurate method for determining diphenylhydantoin in small amounts of blood is necessary. It has been found that the frequent therapeutic use of diphenylhydantoin and phenobarbital in combination presents a considerable problem in the analysis of the two drugs, since they interfere with each other in most colorimetric (1–3) and spectrophotometric methods (4–8). The presence of interfering substances, particularly phenobarbital, in biological specimens requires preliminary tedious separation by extraction procedures (1, 4–6) or TLC (2, 3, 7, 8). Recently, GLC methods for the analysis of diphenylhydantoin became available (9–17), but they are time consuming and technically difficult for routine clinical situations.

The UV spectrophotometric assay described by Wallace and his coworkers (18–21) is based on permanganate oxidation of diphenylhydantoin to benzophenone, which is subsequently extracted into *n*-heptane. The reaction product, benzophenone, which has a well-defined UV absorption spectrum and high molar absorptivity, is determined spectrophotometrically at 247 nm. in *n*-heptane. This method does not require the preliminary separation of diphenylhydantoin from other drugs. However, the procedure requires a relatively large volume (5–10 ml.) of whole blood or plasma for a single determination. Morselli (22) and Bock and Sherwin (23) employed improved equipment and a modified extraction method to reduce the blood samples to 1–2 ml. Lee and Bass (24) described a micromodification of the Wallace method, in which a micro-Kjeldahl steam distillation apparatus and microcells were used. However, variations in the blood sample blanks in the modified methods as well as in the original Wallace method contribute to the inaccuracy in determining diphenylhydantoin at relatively low concentrations.

The present study describes a modification that improves the diphenylhydantoin assay reported by Wallace. The improvement was made possible by changing various reaction conditions, including oxidation time, in the oxidation of diphenylhydantoin with alkaline permanganate and by determining the oxidation product on an appropriately expanded scale of a recording spectrophotometer using microcells. The conversion of diphenylhydantoin to benzophenone was found to be quantitative, and the blank value was negligibly small. This modified determination, employing only 0.1–0.4 ml. of whole blood or plasma, is simple, reproducible, and capable of determining large numbers of diphenylhydantoin samples in a short time, since the refluxing of the reaction mixture with stirring has been eliminated.

EXPERIMENTAL

Reagents and Apparatus—A phosphate buffer solution at pH 6.8 was prepared by mixing equal volumes of 1 *M* Na_2HPO_4 and 1 *M* KH_2PO_4. All chemicals used in this study were reagent grade except *n*-heptane which was spectrophotometric grade. A double-beam recording spectrophotometer[1] was used for UV absorption measurements. Absorbances were recorded on an appropriately expanded scale (×5 or ×10) using standard cells (10 mm. in path length and 5-ml. capacity) or microcells (10 mm. in path length and 0.8-ml. capacity).

Examination of Blank Absorbance—In the procedure described by Wallace (20), 20 ml. of 1% $KMnO_4$ in 7 *N* NaOH and 5 ml. of *n*-heptane are added to diphenylhydantoin extracted from biological specimens, and the mixture is then refluxed for 30 min. with constant magnetic stirring. After cooling, the *n*-heptane layer is separated from the aqueous permanganate layer and its absorbance is determined at 247 nm. against pure *n*-heptane. The blank values (less than 0.04 in absorbance) from 10 ml. of oxalated whole blood containing no diphenylhydantoin were shown to be less than 0.5 mcg./ml. diphenylhydantoin (20). Reexaminations of the blank values from 5 ml. of plasma and water showed them to be about 0.020 and 0.010 in absorbance, respectively.

However, small deviations in the blank absorbance may be responsible for inaccuracy in estimating diphenylhydantoin using an expanded scale of the recording spectrophotometer. Therefore, the reaction conditions in the oxidation of diphenylhydantoin with permanganate were examined to minimize the blank value. Ten milliliters of the alkaline permanganate solution was added to 5 ml. of *n*-heptane and to 5 ml. of 3.97×10^{-6} *M* benzophenone[2] in *n*-heptane in separate centrifuge tubes. The mixtures were refluxed for 30 min. with constant magnetic stirring. After cooling and cen-

[1] Hitachi model 124.

[2] If converted quantitatively, 5 mcg. of diphenylhydantoin corresponds to the amount of benzophenone in 5 ml. of this solution.

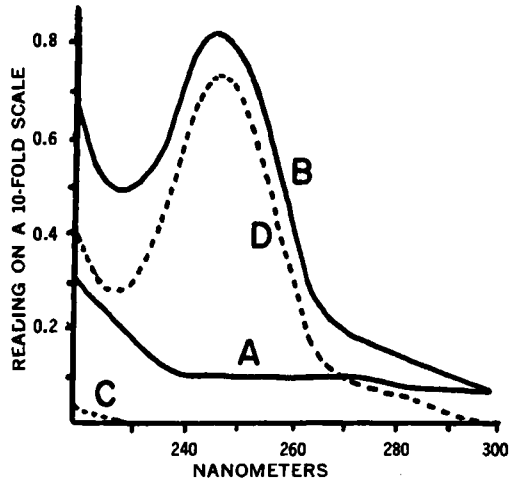

Figure 1—*UV absorption spectra of* n-*heptane and* n-*heptane containing benzophenone with and without the permanganate oxidation procedure. Solid lines A and B are UV absorption spectra of* n-*heptane and* n-*heptane containing benzophenone after the permanganate oxidation, respectively. Dotted lines C and D are UV absorption spectra of* n-*heptane and* n-*heptane containing benzophenone shaken with the alkaline permanganate solution, respectively. The benzophenone concentration in* n-*heptane is 3.97 × 10⁻⁶* M.

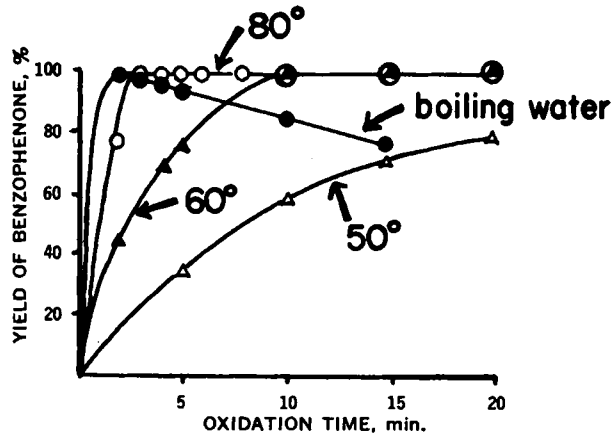

Figure 2—*Relationship between oxidation time and benzophenone yield. Curves show benzophenone yields against time in a constant-temperature bath (50, 60, and 80°) and in boiling water. Curves at 70 and 90° are omitted from the figure, since they almost coincide with the curve at 80°, with the exception of a gradual decrease in the yield at 90° after 10 min.*

trifuging, the *n*-heptane layers were separated from the aqueous permanganate layers, and the absorbance values were recorded on a 10-fold scale of the recording spectrophotometer from 220 to 300 nm. against pure *n*-heptane. The UV absorption spectra of *n*-heptane and *n*-heptane containing benzophenone after the permanganate oxidation are shown in Fig. 1. The spectrum of the *n*-heptane containing no benzophenone exhibits a considerably high absorption at 247 nm. On the other hand, no absorption of the *n*-heptane layer at 247 nm. was observed (Fig. 1) when a mixture of 5 ml. of the alkaline permanganate solution and *n*-heptane containing no benzophenone was shaken mechanically for 10 min. at room temperature. From these results it can be concluded that the blank reading from plasma containing no diphenylhydantoin was attributed to unknown reaction products derived from *n*-heptane during heating with the alkaline permanganate.

Effect of Reaction Conditions upon Yield of Benzophenone—The procedure of oxidation under refluxing and stirring in the Wallace (20) method makes it difficult to determine diphenylhydantoin in a number of samples during a short time. Ten milliliters of the alkaline permanganate solution was added to 1 ml. of 10 mcg./ml. diphenylhydantoin in 0.1 *N* NaOH to examine the effect of reaction conditions upon yields of benzophenone from diphenylhydantoin. The reaction mixtures in centrifuge tubes, closed with ground-glass stoppers, were immersed in a constant-temperature bath (50, 60, 70, 80, and 90°) and in boiling water. The centrifuge tubes were withdrawn at various time intervals, and 5 ml. of *n*-heptane was added to the reaction mixtures after cooling. After the mixtures were shaken for 10 min., the *n*-heptane layer was separated from the aqueous layer, and its absorbance was recorded on a fivefold scale from 220 to 300 nm. against pure *n*-heptane. Figure 2 shows the yield of benzophenone as a function of immersing time. At higher temperatures, the reading (fivefold absorbance) rapidly reached a value[3] of 0.750 based on the quantitative yield of benzophenone. The readings obtained at temperatures lower than 80° remained constant, while the readings obtained at 90° and in boiling water decreased gradually after 10 and 3 min., respectively. Therefore, the oxidation of diphenylhydantoin with alkaline permanganate should be performed at temperatures lower than 80°. The decrease in the readings was presumed to be caused by leakage of the benzophenone formed by oxidation through minute openings between the centrifuge tube and its ground-glass stopper. The results described here show that the refluxing for 30 min. with constant magnetic stirring proposed by Wallace (20) is unnecessary and that the benzophenone formed quantitatively by the permanganate oxidation at 80° for 5 min. is completely extracted by *n*-heptane.

Improved Analytical Method—*Procedure A*—In the absence of phenobarbital, the following procedure was used for determining diphenylhydantoin. Ten milliliters of water is added to 0.1 ml. of plasma or blood in a centrifuge tube, followed by 2 ml. of 1 *M* phosphate buffer solution (pH 6.8) and 30 ml. of chloroform. The mixture is shaken for 10 min. and centrifuged at 3000 r.p.m. for 5 min. After removal of the aqueous layer by aspirating, 27 ml. of the chloroform layer is transferred into another centrifuge tube containing 10 ml. of 1 *N* NaOH. This mixture is shaken for 10 min. and also centrifuged. Nine milliliters of the sodium hydroxide layer is then transferred into a third centrifuge tube, which is connected to a vacuum rotary evaporator assembly, and this tube is immersed in a water bath of 50–60°. The tube is rotated until the volume within it is reduced to approximately 0.5 ml. It is removed from the assembly, 10 ml. of 1% $KMnO_4$ in 7 *N* NaOH is added, and the tube is immersed in a water bath of 80° for 5 min. After cooling, 2 ml. of *n*-heptane is added to the solution, and the mixture is shaken for 10 min. and centrifuged for 5 min. The *n*-heptane layer is then separated from the aqueous permanganate layer and transferred into a microcell. The absorbance is recorded on a 10-fold scale of the recording spectrophotometer over the range of 220–270 nm. against pure *n*-heptane. The peak reading at 247 nm. is read on the recording paper[4].

Procedure B—Wallace (21) demonstrated that high levels of diphenylhydantoin do not affect the determination of phenobarbital by the Williams and Zak (25) method, nor do large amounts of phenobarbital influence the absorption curve of the diphenylhydantoin oxidation product. In the presence of phenobarbital, the following combination procedure with the Williams and Zak (25) method for analyzing barbiturates was used for determining diphenylhydantoin and phenobarbital.

Ten milliliters of water is added to 0.4 ml. of plasma or blood in a centrifuge tube, followed by 2 ml. of 1 *M* phosphate buffer solution (pH 6.8) and 30 ml. of chloroform. The mixture is shaken for 10 min. and centrifuged for 30 min. After removal of the aqueous layer, 27 ml. of the chloroform layer is transferred into another centrifuge tube containing 2 ml. of 0.45 *N* NaOH. This mixture is shaken for 10 min. and centrifuged for 5 min. An upper layer of 0.4 ml. of the sodium hydroxide solution is transferred into a microcell, and its absorbance is recorded on a 10-fold scale of the recording spectrophotometer over the 230–280-nm. range against 0.45 *N* NaOH. To the sodium hydroxide solutions in the microcells is added 0.1 ml. of 16% NH_4Cl, and again the absorbance is recorded over the 230–280-nm. range. The difference (ΔA) in absorbance at 260 nm. is used for

[3] Calculated from the amount of benzophenone corresponding to 10 mcg. of diphenylhydantoin and the molar absorptivity (1.985×10^4) of benzophenone at 247 nm.

[4] Analysis using an absorption spectrum is desirable, since interfering absorption can be checked.

Table I—Standard Curve Data of Diphenylhydantoin Reaction Product by Procedure A

Diphenylhydantoin Added, mcg./ml.	Reading[a] of Reaction Product, Average ± SD[b]	Reading[a] / Concentration	Percent Recovery[c], Average ± SD[b]
5	0.153 ± 0.003	0.031	100.9 ± 2.1
10	0.303 ± 0.002	0.030	99.7 ± 0.7
15	0.456 ± 0.005	0.030	100.1 ± 1.1
20	0.611 ± 0.001	0.031	100.6 ± 0.2
25	0.760 ± 0.005	0.030	99.8 ± 0.6
30	0.909 ± 0.002	0.030	99.6 ± 0.3
Plasma blank	0.006 ± 0.002	—	—

[a] Absorbance determined on a 10-fold expanded scale of the recording spectrophotometer. [b] Standard deviation calculated from three determinations at each plasma level. [c] Recovery (%) = $100(A \times B)/(C \times D \times E \times F \times G)$, where A = expanded reading determined by Procedure A; B = volume (2/1000 l.) of *n*-heptane; C = molar absorptivity of benzophenone (1.895×10^4); D = molar quantity of diphenylhydantoin added to the sample; E and F = fractions (27/30 and 9/10) of $CHCl_3$ layer and 1 *N* NaOH layer used for the subsequent procedure, respectively; and G = expanded scale (10) of the spectrophotometer.

determining phenobarbital and is given by:

$$\Delta A = A_1 - 1.25A_2 \qquad \text{(Eq. 1)}$$

where A_1 and A_2 are the first and second readings at 260 nm.

One milliliter of the remaining sodium hydroxide layer is transferred into a centrifuge tube, which is connected to a vacuum rotary evaporator assembly, and the tube is immersed in a water bath of 50–60°. The tube is rotated until the volume is reduced to approximately 0.5 ml. Procedure A is then carried out using a fivefold scale.

Efficiency of Extraction of Diphenylhydantoin—Ten milliliters of water, 2 ml. of 1 *M* phosphate buffer solution (pH 6.8), and 30 ml. of chloroform were successively added to 0.4 ml. of plasma at a diphenylhydantoin concentration of 20 mcg./ml. The mixture was centrifuged after shaking, and 15 ml. of the chloroform layer was evaporated at 50–60°. The diphenylhydantoin in the residue was determined by Procedure A using a fivefold scale, and the average of six determinations was found to be 0.738 ± 0.002 (*SD*). If diphenylhydantoin is completely extracted from plasma with 30 ml. of chloroform, the extracted amount of diphenylhydantoin in 15 ml. of the chloroform layer would be 4 mcg. Fifteen milliliters of chloroform containing 4 mcg. of diphenylhydantoin was evaporated at 50–60° to obtain a standard value, and the diphenylhydantoin in the residue was determined by the same method. The average of six determinations was found to be 0.739 ± 0.003. Furthermore, 2 ml. of 0.45 *N* NaOH was added to 30 ml. of chloroform containing 8 mcg. of diphenylhydantoin, and the mixture was centrifuged after shaking. One milliliter of the 0.45 *N* NaOH layer was evaporated at 50–60°. If diphenylhydantoin is completely extracted from the chloroform layer with 2 ml. of 0.45 *N* NaOH, the extracted amount of diphenylhydantoin in 1 ml. of the 0.45 *N* NaOH layer would be 4 mcg. The diphenylhydantoin in the residue was determined by the same method, and the average of six determinations was found to be 0.735 ± 0.006. These results show that the extraction of diphenylhydantoin from plasma with chloroform and the extraction of diphenylhydantoin from the chloroform layer with 0.45 *N* NaOH are quantitatively performed, since there was no significant difference between the values determined.

Clinical Study—A healthy male subject was given a single intravenous dose of 50 mg. of sodium diphenylhydantoin[5] dissolved in 1 ml. of a vehicle consisting of 40% propylene glycol and 10.5% ethanol in water. The drug was injected into an antecubital vein at a rate of 50 mg./min. Heparinized blood samples (about 1.5 ml.) from the opposite arm were taken 1, 15, 30, and 45 min. and 1, 2, 3, 6, 8, and 10 hr. after administration. The hematocrit values were determined by centrifuging at 3000 r.p.m. for 10 min. and the resulting plasma samples were assayed for diphenylhydantoin using Procedure A. A blood sample was taken as the blank before injection.

Animal Study—Plasma concentrations of diphenylhydantoin and phenobarbital at various times after intravenous administration were determined in three male white rabbits (2.5–3.0 kg.). Sodium diphenylhydantoin was dissolved in 0.9% NaCl to contain 50 mg. of diphenylhydantoin/ml., and phenobarbital was dissolved in an appropriately small volume of equimolar sodium hydroxide and diluted with 0.9% NaCl to contain 50 mg./ml. Both solutions were mixed in equal volume. Diphenylhydantoin and phenobarbital at a dose of 5 mg./kg. were simultaneously administered to rabbits through an ear vein. Heparinized blood samples (about 1 ml.) from the opposite ear were taken 1, 15, and 30 min. and 1, 2, 3, 4, 5, 6, 7, 8, 9, 24, and 32 hr. after administration. A blood sample was taken as the blank before injection. The plasma samples were assayed for diphenylhydantoin and phenobarbital using Procedure B.

[5] Aleviatin sodium for injection, Dainippon Pharmaceutical Co. Ltd., Osaka, Japan.

RESULTS AND DISCUSSION

In the determination of diphenylhydantoin (Procedure A) there is a linear relationship between the absorbance readings and the concentrations up to 30 mcg./ml. (Table I). A linear capability of the analysis for diphenylhydantoin in the presence of phenobarbital (Procedure B) over the 0–20-mcg./ml. range is illustrated in Table II. The standard curve data of phenobarbital in combination with diphenylhydantoin are also shown in Table II. The averages of blank values were 0.006 and 0.003 for 0.1 ml. (Procedure A) and 0.4 ml. (Procedure B) of plasma or whole blood, which were equivalent to 0.2 and 0.1 mcg./ml. diphenylhydantoin, respectively. The standard curves of diphenylhydantoin and phenobarbital in whole blood were the same as those in plasma. It has been shown (see *Experimental*) that diphenylhydantoin is quantitatively converted to benzophenone in permanganate reagent at 80° within 5 min. The recoveries calculated from the molar absorptivity of benzophenone and the amounts of diphenylhydantoin added to plasma are shown in Tables I and II. The average recoveries (percent) of all the determinations by Procedures A and B were 100.1 ± 1.0 and 101.9 ± 1.6, respectively.

The higher sensitivity and reliability of the proposed method can be illustrated by comparing the recoveries of diphenylhydantoin associated with several published procedures. Wallace and coworkers (18–21) reported that the average recovery of diphenylhydantoin added in known amounts to whole blood was 87.5–95.9% by reference to a standard curve prepared from aqueous solutions of diphenylhydantoin (oxidized with the permanganate reagent in the presence of *n*-heptane). Morselli (22) obtained an average recovery of 86% for the determinations of diphenylhydantoin added to plasma.

It was observed in the *Experimental* section that the reading of the diphenylhydantoin reaction product decreased with an increase in the oxidation time at temperatures higher than 90°. One milliliter of 25 mcg./ml. benzophenone solution, which was prepared by diluting

Table II—Standard Curve Data of Diphenylhydantoin Reaction Product and Phenobarbital by Procedure B

Diphenylhydantoin and Phenobarbital Added, mcg./ml.	Reading[a] of Reaction Product, Average ± SD[b]	Reading[a] / Concentration	Percent Recovery[c], Average ± SD[b]
4	0.139 ± 0.004 (0.167 ± 0.002)[d]	0.035[e] (0.042)[d]	103.0 ± 2.7
8	0.277 ± 0.003 (0.326 ± 0.004)	0.035 (0.041)	102.7 ± 1.0
12	0.409 ± 0.005 (0.494 ± 0.004)	0.034 (0.041)	100.8 ± 1.1
16	0.553 ± 0.005 (0.651 ± 0.002)	0.035 (0.041)	102.1 ± 0.9
20	0.685 ± 0.007 (0.818 ± 0.005)	0.034 (0.041)	101.0 ± 1.0
Plasma blank	0.003 ± 0.003 (−0.001 ± 0.002)	— —	— —

[a] Absorbance determined on a fivefold (diphenylhydantoin) or a 10-fold (phenobarbital) expanded scale of the recording spectrophotometer. [b] Standard deviation calculated from three determinations at each plasma level. [c] Calculated in the same way as in Table I, using $F = 1/2$ and $G = 5$. [d] Calculated from differential absorption of phenobarbital at 260 nm. based on Eq. 1.

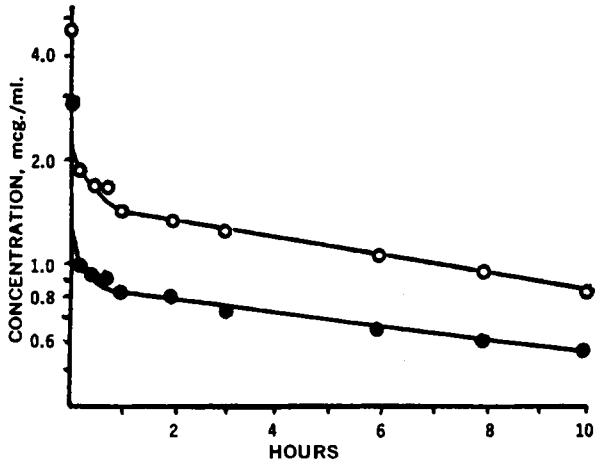

Figure 3—*Diphenylhydantoin concentration as a function of time after intravenous administration of 50 mg. of sodium diphenylhydantoin in a human subject. Key: ●, whole blood concentration; and ○, plasma concentration.*

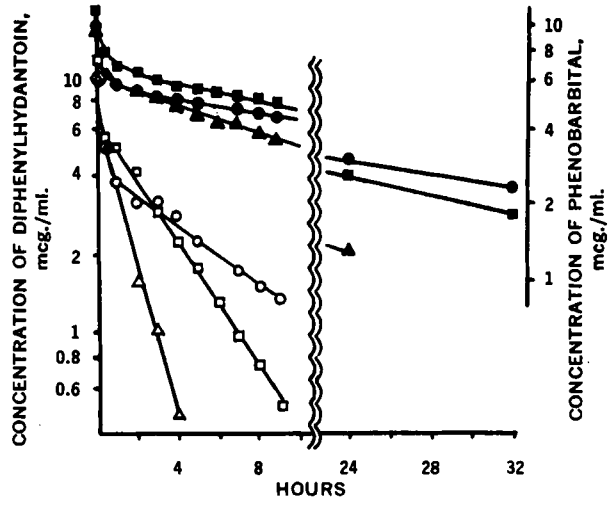

Figure 4—*Semilogarithmic plots of diphenylhydantoin and phenobarbital plasma levels after simultaneous intravenous administration of diphenylhydantoin (5 mg./kg.) and phenobarbital (5 mg./kg.) in rabbits. Open and closed symbols show the concentrations of diphenylhydantoin and phenobarbital, respectively. Key: ○,●, Rabbit B; □,■, Rabbit D; and △,▲, Rabbit E.*

1 mg./ml. benzophenone in ethanol with water, and 9 ml. of 7 *N* NaOH were mixed in a centrifuge tube. The centrifuge tube, closed with a ground-glass stopper, was immersed in a constant-temperature bath (70, 80, and 90°) and in boiling water for various lengths of time. After cooling, 2 ml. of *n*-heptane was added, and the mixture was shaken for 10 min. The reading of the *n*-heptane layer, separated from the aqueous layer by centrifugation, was recorded on a fivefold scale from 220 to 270 nm. against pure *n*-heptane. The peak reading at 247 nm. gradually decreased at 90° and in boiling water, while it remained constant over 60 min. at temperatures below 80°. A 20% decrease in the reading was observed at 90° and in boiling water after 30 min., and 25 and 34% decreases were observed at 90° and in boiling water after 1 hr., respectively. These results show that a decrease in benzophenone concentration results from vaporization through minute openings between the centrifuge tube and its ground-glass stopper during heating. The oxidation of diphenylhydantoin must be performed at temperatures below 80° to suppress vaporization of volatile benzophenone.

Various volumes (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 ml.) of a plasma sample at a diphenylhydantoin concentration of 2 mcg./ml. were used to examine the effect of appropriate alterations of a sample volume upon the determination of diphenylhydantoin at relatively lower concentrations by Procedure A. These same volumes of a plasma sample at an equal concentration (8 mcg./ml.) of diphenylhydantoin and phenobarbital were also used for the determination of diphenylhydantoin and phenobarbital by Procedure B. The results were plotted using the amount of diphenylhydantoin or phenobarbital as the abscissa and the absorbance reading as the ordinate. The slopes of the straight lines so obtained were found to be equal to those of standard curves prepared from the data in Tables I and II, using the amount of diphenylhydantoin or phenobarbital in the sample solution as the abscissa. Consequently, appropriate sample volumes were used to obtain a reading higher than 0.2 in the following determination of diphenylhydantoin in a human subject and rabbits.

A normal subject was given a single intravenous dose of 50 mg. of sodium diphenylhydantoin. The diphenylhydantoin concentrations in whole blood and plasma after intravenous administration are shown in Fig. 3. The average of apparent diphenylhydantoin concentrations (micrograms per milliliter)[6] in the red cells was calculated to be 0.30 ± 0.05 over a concentration range in plasma diphenylhydantoin of 1.87–0.86 mcg./ml. except immediately after injection. However, higher apparent concentrations (4–11 mcg./ml.) were obtained in this laboratory for 12–20 mcg./ml. in plasma diphenylhydantoin level in patients during long-term treatment. The average ratio of whole blood concentration to plasma concentration for the samples shown in Fig. 3 was 0.58 ± 0.06. This was shown to be 0.50 ± 0.11 for 20 samples with an average plasma concentration of 13.8 mcg./ml. by Bock and Sherwin (23). The diphenylhydantoin concentration in plasma as a function of time could be described by $C_p = 3.6e^{-10.4t} + 1.7e^{-0.06t}$, yielding a half-life of 10.5 hr. for the terminal exponential phase. This value is comparable to 9.5 hr. (125 mg.) and 11.2 hr. (250 mg.) reported by Suzuki *et al.* (26) for higher doses. The concentrations extrapolated to time zero were proportional to the doses. The plasma levels of diphenylhydantoin and phenobarbital after simultaneous intravenous administration of diphenylhydantoin (5 mg./kg.) and phenobarbital (5 mg./kg.) in rabbits were determined by Procedure B. The plots of the logarithms of the plasma concentrations of diphenylhydantoin and phenobarbital against time are shown in Fig. 4. Rabbits exhibiting faster elimination rates for phenobarbital were found to possess increased rates of loss for diphenylhydantoin.

The method utilizing the UV absorption of benzophenone formed by permanganate oxidation is simple and specific. No interference with the assay occurred in the presence of many commonly used drugs (18–20, 22, 24). The present study adds acetylpheneturide, 4-amino-3-hydroxybutyric acid, carbamazepine, chlordiazepoxide, chlorpromazine hydrochloride, cyclandelate, diazepam, ethosuximide, mephobarbital, metharbital, nitrazepam, perphenazine, and thioridazine hydrochloride to the list of noninterfering substances. The primary metabolite, 5-(*p*-hydroxy)-5-phenylhydantoin, also did not interfere as reported by Wallace (20). The oxidation product of this metabolite was identified as benzoic acid by its UV and IR absorption spectra and by the results of the elemental analysis.

[6] Calculated from the whole blood concentration (C_w), plasma concentration (C_p), and hematocrit value (Ht) using the formula: $[C_w - C_p(1 - Ht)]/Ht$.

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Transport of Alkyl Homologs across Synthetic and Biological Membranes: A New Model for Chain Length-Activity Relationships

S. H. YALKOWSKY▲ and G. L. FLYNN

Abstract □ The ability of each member of a biologically active homologous series to reach the receptor site can often be equated with its relative ability to permeate biological barriers. This paper presents a structure–activity model based entirely on firmly established diffusional theories as well as solubility relationships generally applicable to aqueous systems. The equations derived and presented here were previously experimentally verified using synthetic membranes. These equations are generally applicable for transport across membranes and can be of use in describing a variety of passive absorption or permeation phenomena. Since true equilibrium is rarely attained in biological systems, a kinetic model provides an appropriate description of the physiological situation. Furthermore, a kinetic model offers considerable practical utility, because it can readily be incorporated into the overall pharmacokinetic treatment.

Keyphrases □ Membrane permeability, alkyl homologs—model for chain length–activity relationships, equations □ Transport across synthetic and biological membranes—alkyl homologs, model for chain length–activity relationships, equations □ Structure–activity relationships—model for transport of alkyl homologs across synthetic and biological membranes based on diffusion theories □ Chain length–activity relationships—alkyl homolog transport across membranes

Many theories have been advanced to explain relative biological activity of the members of a series of structurally related compounds (1–5). These theories usually relate the affinity of a particular congener for the receptor site to some property such as its molecular weight, its solubility, its surface tension, its partition coefficient between water and an organic solvent, or some other related physicochemical parameter. The structure–activity models can be categorized operationally as being either of kinetic or equilibrium nature. Current mathematical models for the former are generally of empirical design; quantitative relationships for the latter are complex and difficult to apply to actual data.

The present paper is concerned with a new kinetic approach applicable to many structure–activity profiles. The ability of each member of a homologous series to reach the receptor site is equated with its ability to permeate biological barriers, including the biomembrane(s) and contiguous aqueous layers. This is accomplished by modification of firmly established diffusional theories. Virtually all aspects of the theory and equations derived and presented here were experimentally verified (6–11) using synthetic membranes. It will be shown that much of the current biological data are interpretable based on these simple diffusional relationships.

These equations are general for transport across any membrane and can be of use in describing a variety of passive absorption or permeation phenomena. Furthermore, since true equilibrium is rarely attained in biological systems, a kinetic model may provide a more appropriate description for the general physiological situation, facilitating incorporation of passive transport processes into the total pharmacokinetic picture.

THEORETICAL

Importance of Diffusion Layers in Membrane Transport—The resistance to the transport of a solute across a membrane, which separates two aqueous compartments, lies not only within the membrane but also within the regions of unstirred water adjacent to its surfaces (6–11). The resistances of these aqueous regions of diffusion layers can be treated mathematically as additive resistances in series with the membrane (8–10). The flux of a solute across a membrane system (membrane and diffusion layers) is proportional to the concentration difference, C, across the barrier and is inversely proportional to the sum of the resistances of the laminates. Thus:

$$F^c = \frac{C}{R_{aq} + (R_m/PC)} \qquad \text{(Eq. 1)}$$

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Transport of Alkyl Homologs across Synthetic and Biological Membranes: A New Model for Chain Length-Activity Relationships

S. H. YALKOWSKY▲ and G. L. FLYNN

Abstract □ The ability of each member of a biologically active homologous series to reach the receptor site can often be equated with its relative ability to permeate biological barriers. This paper presents a structure–activity model based entirely on firmly established diffusional theories as well as solubility relationships generally applicable to aqueous systems. The equations derived and presented here were previously experimentally verified using synthetic membranes. These equations are generally applicable for transport across membranes and can be of use in describing a variety of passive absorption or permeation phenomena. Since true equilibrium is rarely attained in biological systems, a kinetic model provides an appropriate description of the physiological situation. Furthermore, a kinetic model offers considerable practical utility, because it can readily be incorporated into the overall pharmacokinetic treatment.

Keyphrases □ Membrane permeability, alkyl homologs—model for chain length–activity relationships, equations □ Transport across synthetic and biological membranes—alkyl homologs, model for chain length–activity relationships, equations □ Structure–activity relationships—model for transport of alkyl homologs across synthetic and biological membranes based on diffusion theories □ Chain length–activity relationships—alkyl homolog transport across membranes

Many theories have been advanced to explain relative biological activity of the members of a series of structurally related compounds (1–5). These theories usually relate the affinity of a particular congener for the receptor site to some property such as its molecular weight, its solubility, its surface tension, its partition coefficient between water and an organic solvent, or some other related physicochemical parameter. The structure–activity models can be categorized operationally as being either of kinetic or equilibrium nature. Current mathematical models for the former are generally of empirical design; quantitative relationships for the latter are complex and difficult to apply to actual data.

The present paper is concerned with a new kinetic approach applicable to many structure–activity profiles. The ability of each member of a homologous series to reach the receptor site is equated with its ability to permeate biological barriers, including the biomembrane(s) and contiguous aqueous layers. This is accomplished by modification of firmly established diffusional theories. Virtually all aspects of the theory and equations derived and presented here were experimentally verified (6–11) using synthetic membranes. It will be shown that much of the current biological data are interpretable based on these simple diffusional relationships.

These equations are general for transport across any membrane and can be of use in describing a variety of passive absorption or permeation phenomena. Furthermore, since true equilibrium is rarely attained in biological systems, a kinetic model may provide a more appropriate description for the general physiological situation, facilitating incorporation of passive transport processes into the total pharmacokinetic picture.

THEORETICAL

Importance of Diffusion Layers in Membrane Transport—The resistance to the transport of a solute across a membrane, which separates two aqueous compartments, lies not only within the membrane but also within the regions of unstirred water adjacent to its surfaces (6–11). The resistances of these aqueous regions of diffusion layers can be treated mathematically as additive resistances in series with the membrane (8–10). The flux of a solute across a membrane system (membrane and diffusion layers) is proportional to the concentration difference, C, across the barrier and is inversely proportional to the sum of the resistances of the laminates. Thus:

$$F^c = \frac{C}{R_{aq} + (R_m/PC)} \qquad \text{(Eq. 1)}$$

where F^c is the steady-state flux across the barrier at a concentration gradient of C; R_m and R_{aq} are the resistances of the membrane and aqueous regions, respectively; and PC is the membrane-water partition coefficient of the diffusant. For a single membrane of unit area, R_m and R_{aq} are equal to h_m/D_m and $(h_{aq}^d + h_{aq}^r)/D_{aq}$, respectively, where h is the laminate thickness. The superscripts d and r designate the high (donor) and low (receptor) concentration sites of the membrane, and D_m and D_{aq} are the membrane and aqueous diffusion coefficients of the solute, respectively. For a sequential arrangement of j membranes, the resistances can be defined by:

$$R_m = \sum_{i=1}^{j} \frac{h_m^i}{D_m} \qquad \text{(Eq. 2a)}$$

$$R_{aq} = \sum_{k=2}^{2j} \frac{h_{aq}^k}{D_{aq}} \qquad \text{(Eq. 2b)}$$

where h_{aq}^k and h_m^i are the thicknesses of the individual diffusion layers and membranes that are traversed; *i.e.*, the resistance of thick tissues can be viewed in terms of the sum of the resistances of the tissue's component strata.

Equation 1 shows that for diffusants of low partition coefficient the flux is approximated by $(C)(PC)/R_m$; *i.e.*, the membrane resistance controls the transport rate of the permeant. On the other hand, when the permeant's partition coefficient becomes large, $R_m/(PC)$ becomes negligible and, thus, the flux is determined primarily by the diffusion layer resistance, R_{aq}. These conditions are referred to as membrane control of flux and diffusion layer control of flux, respectively.

Transport of Homologs at Equimolar Concentration—In a homologous series the respective diffusivities, D_m and D_{aq}, for all practical purposes, remain constant as the series is ascended. Some workers (12, 13) argued that the change in molecular weight (MW) or, more realistically, molecular volume with increasing chain length should be accounted for by multiplying the flux $(MW)^{1/2}$ for small molecules and $(MW)^{1/3}$ for larger molecules. This refinement is unnecessary for alkyl derivatives of large molecules, because the factor $(MW)^{1/3}$ is essentially constant over a reasonable alteration of chain length. Furthermore, other flux-determining factors will be shown to be exponentially dependent on chain length, and these changes completely mask any small changes in D_m or D_{aq}. A relevant literature example illustrative of the lack of dependency of D on alkyl chain length is found in the work of Scheuplein and Blank (14), who showed that there is less than a 30% decrease in diffusion coefficient for normal alcohols (methanol through octanol) within the stratum corneum. Thus, for a homologous series of compounds the values of R_{aq} and R_m can be treated as being essentially invarient.

As previously stated, the membrane-water partition coefficient (PC) changes exponentially with chain length and can be related to the number of methylene and methyl groups in excess of those in a reference homolog, n, by:

$$\log (PC)_n = \log (PC)_o + \pi n \qquad \text{(Eq. 3)}$$

where $(PC)_o$ and $(PC)_n$ are the partition coefficients of the reference homolog and the higher homolog, respectively; π is a constant which is characteristic of the membrane and its bathing solvent, which for biological systems is essentially aqueous. For a simple homologous series, it is convenient to define $(PC)_o$ as the partition coefficient of the hypothetical zero chain length congener (the Y intercept of a plot of log PC *versus* n); n, in this circumstance, becomes the alkyl chain length. The relevance of this partitioning relationship with respect to biological transport can be found by examining Eq. 1; for any given concentration differential, C, the steady-state flux of a particular homolog, F_n^c, can be related to alkyl chain length by substituting Eq. 3 into the logarithmic form of Eq. 1:

$$\log F_n^c = \log C + \log (PC)_o + \pi n - \log [R_{aq}(PC)_o 10^{\pi n} + R_m] \qquad \text{(Eq. 4)}$$

For small values of n, R_m is usually much greater than $R_{aq}(PC)_o 10^{\pi n}$. This is the condition for membrane control of flux and is described by reducing Eq. 4 to:

$$\log F_n^c = \log\left(\frac{C(PC)_o}{R_m}\right) + \pi n \qquad \text{(Eq. 5)}$$

Thus, for the shorter homologs the log [flux] at fixed concentration differential grows by the value π per methylene unit added. On the other hand, for large values of n, where $R_{aq}(PC)_o 10^{\pi n}$ dominates the last term of Eq. 4, the system is under diffusion layer control of flux and:

$$\log F_n^c = \log\left(\frac{C}{R_{aq}}\right) \qquad \text{(Eq. 6)}$$

In other words, a plot of the logarithm of the flux across a given membrane for equimolar concentrations of a series of homologs usually has an initial slope of π and ultimately levels off as n becomes large. Since π is a property of the membrane and the contiguous solvent and the solvent is assumed to be water, all series of homologs should have the same initial slope for a given membrane.

Obviously, the critical parameter determining flux and mechanism is not alkyl chain length *per se* but the membrane-water partition coefficient. Unfortunately, in biological systems partition coefficients are not easily measurable. Because of the relationship between chain length and partition ceofficient (Eq. 3), chain length is not only a convenient reference parameter but also of equivalent utility to partition coefficients obtained in arbitrary partitioning systems such as ether-water and octanol-water. The parallelism between *in vitro* partitioning and partitioning in biological systems is not clearly known. Since the present discussion is primarily limited to homologs, n is a parameter chosen for the mathematical model.

Limitations Based upon Solubility—For any given diffusant and membrane system, R_m, R_{aq}, and (PC) are presumed constant; therefore, the transport rate is dependent only upon the effective concentration gradient. Regardless of the distribution of the gradient in the various laminae, the gradient can be maximized by saturating the donor solution and maintaining the receptor compartment at zero concentration, *i.e.*, in a sink condition. The steady-state flux from a maximized gradient system, F^s, is described by Eq. 1 in which C has been replaced by the solubility of the permeant, S:

$$F^s = \frac{S}{R_{aq} + (R_m/PC)} \qquad \text{(Eq. 7)}$$

Several workers (15–18) demonstrated that the aqueous solubility of each member of a homologous series can usually be related to alkyl chain length by:

$$\log S_n = \log S_o - \delta n \qquad \text{(Eq. 8)}$$

where S_o and S_n are the solubilities of the reference congener and the nth linear alkyl homolog of the series, respectively, and δ is a constant. The value of δ varies somewhat from series to series but usually lies between 0.5 and 0.7. These values represent a decrease in solubility by a factor of about 3.2–5.0 for each methylene unit.

By substituting Eq. 8 into Eq. 4, an expression for the saturation-limited flux as a function of chain length is obtained:

$$\log F_n^s = \log S_o + \log (PC)_o + (\pi - \delta)n - \log[R_{aq}(PC)_o 10^{\pi n} + R_m] \qquad \text{(Eq. 9)}$$

For small values of n, Eq. 9 is approximated by:

$$\log F_n^s = \log \frac{S_o(PC)_o}{R_m} + (\pi - \delta)n \qquad \text{(Eq. 10)}$$

For large values of n, Eq. 9 becomes:

$$\log F_n^s = \log \frac{S_o}{R_{aq}} - \delta n \qquad \text{(Eq. 11)}$$

Therefore, a semilogarithmic plot of flux obtained from saturated donor solutions (into a receptor sink) against alkyl chain length has an initial slope of $(\pi - \delta)$. This slope can be positive, negative, or zero, depending upon the relative magnitudes of π and δ. As chain length is increased, the flux from saturated solutions becomes a direct function of the solubility (Eq. 11) and decreases by

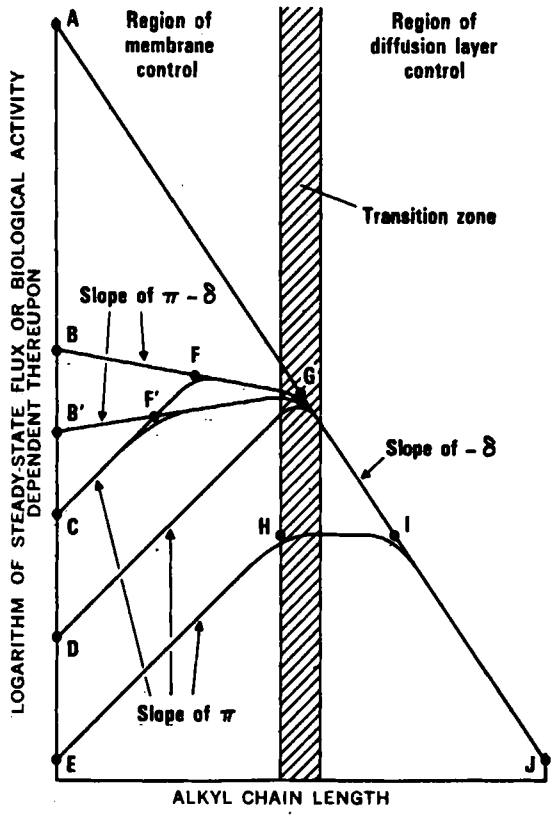

Figure 1—*Hypothetical plot illustrating the various possible flux dependencies for a homologous series as a function of concentration in a system fixed with regard to diffusional parameters. Where biological activity parallels flux, the plot can be regarded as a series of structure-activity profiles.*

the antilogarithm of δ for each methylene unit. Even if the members of a series are studied at equimolar concentrations, the solubility becomes determinative, because any arbitrary concentration must eventually exceed $S_o 10^{-\delta n}$ for some value of n, *i.e.*, for some long chain derivative.

The importance of this solubility restriction on the transport rate profile of a homologous series can be seen in Fig. 1, which is a pictorial summary of the equations presented. The curves drawn place the steady-state flux on an arbitrary logarithmic scale and represent some of the expected relationships between transport rate, concentration, and solubility. Line AJ is the solubility restriction under diffusion layer control as described by Eq. 11. It has a slope of $-\delta$ and an intercept at A of log (S_o/R_{aq}). It can be thought of as representing the flux from a saturated solution into a sink receptor solution across an infinitely thin membrane, where the resistance to transport lies only within the aqueous diffusion layers.

Line BFG is for saturated solution flux under membrane control and is drawn for the case where δ exceeds π, which would be the general situation. Since the line is described by Eq. 10, the intercept at B is equal to log $[S_o(PC_o/R_m)]$. Curve EHI is the equimolar case described by Eq. 4. The initial portion EH has a slope of π and an intercept at E of log $[C_E(PC)_o/R_m]$ (see Eq. 5). The plateau occurring at subsaturation conditions is described by Eq. 6; but as chain length is increased, the concentration C_E becomes greater than the solubility and curve EHIJ is formed. Line DG is also initially equimolar, but at a higher concentration than the previously described case. There is no plateau because of its early intersection with line AJ to form curve DGJ. Line CF is also for the equimolar case but at a still higher concentration. The solubility is exceeded while the system is still under membrane control to form curve CFG. Its ultimate intersection with diffusion layer control produces curve CFGJ. This type of dependence is likely to occur when R_{aq} is small compared to $R_m(PC)$.

Molecular Modification other than Increasing Chain Length—The effects of adding a polar substituent to each member of a homologous series to make a new series will be twofold. In the first place, the aqueous solubility of each member of the new series will be higher than the corresponding member of the reference series. If the two series have similar δ values, the solubilities of corresponding homologs will differ by a constant. Therefore, all curves in Fig. 1 dependent upon solubility will show a parallel or nearly parallel upward displacement. Secondly, the membrane-water partition coefficient of each member of the new series will be lower than the corresponding homolog of the reference series. Consequently, the curves of Fig. 1 that are dependent upon the partition coefficient will be parallel to, but lower than, those of the reference series.

Figure 2 illustrates this point for curve DGJ of Fig. 1. Other curves found in Fig. 1 are omitted from Fig. 2 for clarity only; they would experience similar shifts. When the increase in log [solubility] is less than the decrease in log [partition coefficient], curve D'G'J' is formed. The maximum flux occurs at a longer chain length and is less in magnitude than the maximum of the original series. When the factor by which solubility increases is greater than the factor for the partition coefficient decrease (D'G" J"), the maximum flux exceeds that of the parent series.

Applications to Biological Systems—Stehle and Higuchi (19, 20) and Barrie *et al.* (21) showed the *in vitro* applicability of the equimolar expressions (Eqs. 4–6), and Flynn and Yalkowsky (6) experimentally verified the equations for both equimolar and saturated cases using synthetic membranes. Of the utmost significance is the fact that this approach to membrane transport has broad applicability; it is equally suited to biological membranes and to synthetics. This means the π value for biological membranes can be determined by studying the relationship between alkyl chain length and membrane permeability or a directly related response under the condition of membrane control of flux. Differences between biological π values, π^B, reflect differences in polarities of barriers. All of this can be accomplished even when the values of R_m, R_{aq}, PC_o, and S_o are unknown.

The inclusion of diffusion layers or, more generally, aqueous resistances as part of the total barrier is likely of great importance in biological systems, because agitation *in vivo* would be expected to be inefficient and because biological membranes, particularly cell membranes, are much thinner than synthetic barriers commonly chosen to mimic biological systems. It should also be realized that cytoplasm contributes to the "unstirred" regions. In characterizing

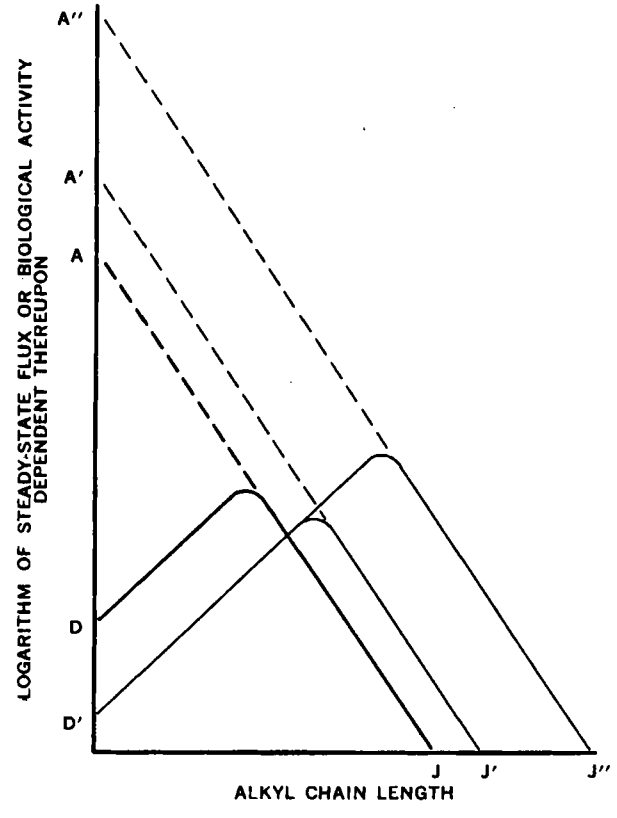

Figure 2—*Expected effect of increasing polarity on the flux profile of a homologous series. See text for explanation of curves.*

Table I—Values of $\pi^{B}_{CH_2}$ for Alcohols

Source[a]	π_{CH_2}	Correlation Coefficient	Number of Alcohols	Biological System Studied
V7	0.436	0.993	5	10-mv. change in rest potential, lobster axon
V8	0.419	0.996	5	5-mv. change in rest potential, lobster axon
V9	0.652	0.999	7	Stabilization against hypotonic hemolysis, red cell
V12	0.522	0.994	4	Narcosis, *A. faetida*
V13	0.503	0.999	5	I_{100}, rabbit gut
V14	0.588	0.987	4	Narcosis, goldfish, 37°
V15	0.470	0.965	4	I_{50}, tortoise heart
V16	0.478	0.990	4	I_{20}, paramecium mobility
V17	0.558	0.998	8	Narcosis, larvae barnacle
V18	0.653	0.997	7	I_{100}, movement, tadpoles, 18°, 2.5 days old
V19	0.617	0.998	7	I_{100}, movement, tadpoles, 1°, 12 days old
V20	0.496	0.997	8	I_{50}, ileum, guinea pig
V21	0.598	0.998	7	I_{100}, movement, tadpoles, −18°, 85 days old
V22	0.505	0.998	6	MIC, fish
V23	0.460	0.985	5	MLD, fish, carp
V25	0.471	0.993	5	MLD, fish, goby
V26	0.471	0.993	5	MLD, fish, roach
V27	0.488	0.986	7	T_{50}, pancreatic lipase, rat
V28	0.543	0.980	5	LD_{100}, cat
V29	0.418	0.997	4	Inhibition oxygen consumption, kidney, rabbit
V33	0.421	0.994	7	I_{50} oxygen consumption, lung, guinea pig
V34	0.403	0.998	4	Toxicity, paramecium
V35	0.381	0.999	5	I_{50}, generation time, *L. aerogenes*
V37	0.397	0.970	5	MLD, eel
V38	0.403	0.998	4	MLD, fish
V44	0.428	0.996	4	I_{50}, indophenol oxidation, kidney, rabbit
V45	0.510	0.999	8	MLD, *S. typhosa*
V46	0.498	0.998	7	Cytolysis, yeast cells
V55	0.497	0.999	6	Narcosis, larvae arenicola
V98	0.454	0.996	4	MLD, South African toad

[a] Table and column number of *Reference 24* from which data were abstracted.

diffusional resistance within whole tissues or isolated cells, cognizance must be given to the thickness and diffusivity in cytoplasmic regions. Nothing is sacrificed by the inclusion of the aqueous resistance term, R_{aq}, as part of the overall resistance because, if aqueous resistance is negligible, the term vanishes from the equation.

In applying this type of treatment to biological systems, it is important that the biological end-point be a reflection of permeability only. If a biological parameter other than permeability is measured, it must be assumed that the variances in the intrinsic activity of the homologs are negligible and overshadowed by the expected variations in permeability. Care must be taken not to apply these equations to studies in which intrinsic activities span a range as wide as that produced in permeability by the molecular modifications. (The activity of a series of homologs in an isolated enzyme system is an example of the kind of data that cannot be treated by the above equations.)

With the above-mentioned limitations in mind, the authors attempted to organize some literature data for diverse biological systems and homologous series and to interpret these data in terms of transport concepts. By not including modifications other than chain length, the introduction of new specific interactions with biological receptors is avoided, as are the needs to define and to assign additional substituent constants. Also, since the effect of polar substituents on diffusion coefficients in biological membranes can be quite large (22, 23), inclusion of polar moieties in the treatments would undermine our basic premise: that the values of R_m and R_{aq} are constant.

Equimolar Studies—Two very different types of end-points are generally chosen for structure–activity studies, namely the relative activity of a substrate at a fixed concentration and the reciprocal of the substrate concentration required to produce a given response. In either case, the data can be reported in terms of activity per mole. It has already been shown in this presentation that, at constant concentration and under membrane control, the slope of log (response) *versus* n is equal to the biological π value of the organism or tissue studied. Data from a large number of equimolar or concentration-normalized structure–activity studies on homologous series are given in Tables I–III. These data were excerpted largely from the existing compilations of Hansch and his coworkers (24–28), who analyzed them in terms of the octanol–water partition coefficient. To keep these tables representative and reasonably concise, only systems containing four or more homologs and having correlation coefficients greater than 0.980 were treated. These arbitrary restrictions were chosen for convenience and in no way affect the conclusions to be drawn. The biological π values reported in the tables give the increase in activity with respect to chain length rather than with respect to the octanol–water partition coefficient. Since the change in log (*PC* octanol–water) per methylene unit is about 0.50, the π value of the biological systems studied by Hansch and his coworkers can be approximated simply by dividing the slopes reported by 0.50.

The intercepts at $n = 0$ of these plots are equal to the product of the intrinsic activity (α_{int}) of the members of the series (which is assumed to be invarient) and $[C(PC)_o/R_m]$ (Eq. 5). Because the intercept is dependent upon three parameters (R_m, PC_o, and α_{int}) that cannot be evaluated independently, as well as on the concentration C which differed from study to study, no attempt will be made to interpret intercept data.

In Table I, biological π values obtained by least-squares analysis of data for linear aliphatic alcohols are presented. As mentioned previously, the validity of the biological π values rests upon the assumption that all of the alkanols have at least similar intrinsic activities, an assumption that is probably valid for most of the nonspecific activities cited. Data collected and processed by Hansch and his coworkers that involve isolated enzymes were deliberately excluded in compiling Table I. Such data are essentially independent of a transport step. The π^B values of Table I are all around 0.45 ± 0.1 and appear to be independent of the nature of the system studied.

In contrast to Table I, Table II contains data for a fixed biological response, the hemolysis of red blood cells. As can be seen, hemolysis data are available for many different types of homologous series. This response was selected not only because it has been studied extensively but also because the phenomenon is clearly physically produced and involves no specific cellular interactions. Thus, the activities observed are, of necessity, directly dependent upon permeabilities.

Because of the general interest that exists in the relative antibacterial activities of homologs on Gram-positive and Gram-negative organisms, it was decided to determine and compare π values obtained with differing homologous series tested on a representative organism of each type. These data are given in Table III.

About half of the π^B values in Tables II and III were determined

Table II—Values of $\pi^B_{CH_2}$ Determined from Hemolysis of Erythrocytes

Source[a]	π_{CH_2}	Correlation Coefficient	Number of Homologs	Homologous Series	Type of Erythrocyte
1A	0.429	0.985	5[b]	*n*-Alcohols	Rabbit
1B	0.477	0.999	7[b]	*n*-Alcohols	Bovine
1C	0.464	0.998	4[b]	Alkyl acetates	Bovine
1D	0.473	0.999	6	*n*-Alcohols	Rabbit
1G	0.528	0.989	6	*n*-Alcohols	Rabbit
1H	0.330	0.990	4[c]	α-Monoglycerides	Dove
1L	0.485	0.997	6[c]	*N*-Alkyl pyridinium bromides	Dove
1M	0.445	0.997	7[c]	*N*-Alkyl piperidine hydrochlorides	Dove
1N	0.439	0.988	4[c]	Alkyl dimethylbenzyl-ammonium chlorides	Sheep
1O	0.487	0.978	5[c]	Alkyl trimethylammonium bromides	Sheep
1P	0.585	0.979	4	Alkyl trimethylammonium bromides	Human
1Q	0.386	0.995	4	Alkyl trimethylammonium bromides	Bovine
1S	0.477	0.984	5	Alkyl amine hydrochlorides	Dog
1T	0.440	0.988	5	*N*-Alkyl pyridinium iodides	Dog
1V	0.536	0.997	4	Sodium alkyl sulfates	Human
1W	0.458[d]	0.990	6[b]	Sodium alkyl sulfates	Dog
1X	0.272	0.988	5[c]	*n*-Carboxylic acids	Sheep
1Y	0.447	0.992	5[c]	*n*-Carboxylic acids	Dove

[a] Table and column number of *Reference 26* from which data were abstracted. [b] Only data for straight-chain compounds used. [c] Only data for the ascending portion of the parabola used. [d] Two data points were assumed to be high by a factor of 10.0.

Table III—Values of $\pi^B_{CH_2}$ for Gram-Positive and Gram-Negative Organisms[a]

Homologous Series	Gram-Positive, *M. pyrogines* var. *aureus*: π_{CH_2}	Correlation Coefficient	Number of Homologs	Gram-Negative, *S. typhosa*: π_{CH_2}	Correlation Coefficient	Number of Homologs
3-Alkyloxy phenols	0.434	0.997	7	0.409	0.997	5
4-Alkyloxy phenols	0.455	0.997	7	0.431	0.992	5
4-Alkyl phenols	0.456	0.999	7	0.486	1.00	4
2-Alkyl 4-chlorophenols	0.432	0.998	6	0.403	0.987	4
4-Alkyl 2-chlorophenols	0.438	0.997	6	0.412	0.998	4
4-Thioalkyl phenols	0.423	0.997	4	0.432	0.997	4
2-Alkyl 4-bromophenols	0.479	0.991	5	0.440	0.993	5
Alkyl amines	0.486	0.997	4	0.228	1.00	5
Alkyl diguanidines	0.478	0.997	5	0.331	0.985	7
Alcohols	0.450	0.993	5	0.511	0.999	8

[a] Data abstracted from *Reference 25*.

from the linear ascending portion of so-called "parabolic"[1] structure–activity curves. These data are indicated by the superscript *c*. From the data, it can be seen that the π^B values obtained from linear and parabolic structure–activity data are equivalent and that these values are independent of the nature of the series studied. Furthermore, they all fall within the range of the values given in Table I. This independence of π^B values on the nature of the homologous series and the biological response studied has been repeatedly noted in the literature (1–6) and can be explained on the basis of both equilibrium and kinetic structure–activity theories, including the one presented here.

Many workers (1–5, 12–14) have noted (although in different terminologies) that the π values for a methylene unit in several common organic solvents (Table IV) are not appreciably different from the biological π^B values given above. Therefore, if, in lieu of plotting *n*, one plots the logarithms of the partition coefficients of the compounds obtained in a solvent for which all the substituent π values are the same as the biological system against the logarithms of their respective biological responses, the slope will be equal to unity. This method has the convenience of enabling substituents other than methylene units to be analyzed. However, because of the scatter and nonlinearity frequently introduced into such plots by nonhydrocarbon substituents, this type of data must be analyzed cautiously, especially when the range of the data is less than one to two orders of magnitude. The presumed scatter introduced by hetero groups is an expected consequence of predictable differences in *in vitro* and *in vivo* π values for the hetero moieties. To illustrate, consider the π and π_{OH} values obtained for partitioning between water and red blood cell ghosts on one hand and several commonly used reference solvents on the other. The π values in Table IV for most of the solvents listed are fairly close to the value for erythrocyte ghosts. There is a marked lack of agreement in the π_{OH} values between the solvents and the biophase as well as among the solvents themselves. This shows that a particular organic solvent may be a good model for the red blood cell ghosts as far as methylene groups are concerned and, at the same time, not accurately reflect the partitioning influence of polar substituents. In view of the fact that it is necessary to use separate equations to relate octanol–water and ether–water partition coefficients when dealing with hydrogen donor and hydrogen acceptor solutes (29), it is unlikely that a single *in vitro* partitioning system will accurately mimic biological membranes, which themselves may differ significantly. Thus, if truly meaningful substituent constants are to be acquired, *they must be obtained for the biological systems themselves*. Seeman *et al.* (30) already demonstrated the feasibility of the approach using erythrocyte ghosts. Substituent constants could also be obtained from experimental transport studies using biological membranes and the equations described in the first portion of this report.

[1] The term parabolic is used in a general sense to describe any curve that has an ascending portion and either a descending or plateauing region rather than in its strict mathematical sense which describes a curve of constantly changing slope.

Table IV—Values of π_{CH_2} and π_{OH} for Some Common Solvents and for Red Blood Cell Ghosts

Solvent	π_{CH_2}	π_{OH}	Reference
Ether	0.573	−1.51	45
Ether	0.612	−1.86	46
Octanol	0.500	−1.18	29
Chloroform	0.609	−2.01	46
Olive oil	0.525	−0.530	47
Castor oil	0.545	−2.48	48
Red blood cell ghosts	0.526	−2.08	30

The process of analyzing certain biological data has led to some conclusions in conflict with previously reported biological trends suggested by Hansch and his coworkers (24–28). Using the octanol–water partition coefficient as a reference parameter, they found the slopes obtained in both Gram-positive and Gram-negative bacterial systems to be significantly lower than the slopes found for hemolysis, narcosis, and most other parameters. The authors believe that this discrepancy has several explanations. Since Hansch and his coworkers were primarily interested in overall structure–activity relationships, they analyzed a much wider variety of data, including many sets of data for which it is questionable whether the activities were primarily dependent upon transport to the receptor site. Some of their slopes, particularly those obtained with isolated enzymes, reflect changes in intrinsic activity rather than transportability with chain length, or log (*PC*). Moreover, since Hansch and his coworkers did not restrict themselves only to homologs and since the biological π values for some hetero substituents found in some analogs are not the same as those for octanol–water partitioning, the slopes would be affected. Finally, if there is a slight plateauing of activity at high partition coefficients (see next section) and all of the data are fitted to a linear equation, the slopes obtained will be artifactually low.

The average π values obtained from the analysis of structure–activity studies of homologous series on several biological endpoints, including those already discussed, are shown in Table V. For all studies involving simple membranes, the value of π does not differ significantly from 0.45. However, the π values for studies involving skin or mucosa of mammals are consistently lower than 0.45. The average value determined from the data of seven series, in which homologs were tested on mammalian epithelial tissue, is 0.25. The reason for this low value is not entirely clear but is probably related to the high degree of hydration of these tissues.

Many equimolar structure–activity curves show, in addition to an initial slope of π, a leveling off of activity with increasing chain length beyond some critical value. This plateauing effect was observed by several workers for both steady-state flux (19, 20) and biological activity (31–35) and can be explained as being a consequence of the transition from membrane control to diffusion layer control of transport (see Eqs. 4–6 and curve EHI of Fig. 1). A plateau is only to be expected in systems where considerations dictate the existence of a significant diffusion layer. It is not generally observed in the analysis of antimicrobial or hemolysis data because small particles (*i.e.*, the cells) have extremely thin diffusion layers associated with them.

An apparent case demonstrating the plateauing effect is found in the work of Rosen *et al.* (31) on the permeability of the toad bladder to nonionic aliphatic acids at pH 4 and 6. Their data (plotted semilogarithmically in Fig. 3) indicate that there is a linear increase in

Table V—Comparison of $\pi^{B}_{CH_2}$ Values Obtained for Several Homologous Series and Various Biological End-Points

Series	End-Point	Subject	Number of Studies	Mean	*SD*
Alcohols	Many	Many	32	0.483	0.079
Many	Hemolysis	Erythrocyte	17	0.465	0.057
Many	Antibacterial	Gram positive	10	0.453	0.021
Many	Antibacterial	Gram negative	9	0.428	0.051
Many	Permeation	Mammalian epidermal	7	0.250	0.047

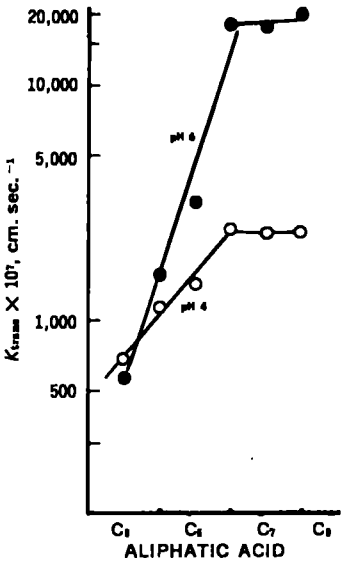

Figure 3—*Permeation rate of unionized aliphatic acids across toad bladder (31). Key: ○, pH 4; and ●, pH 6.*

transport rate constant from propanoic to hexanoic acid and a plateau at higher chain lengths. The differences in the initial slopes are explained by Rosen *et al.* The results of Buchi and Perlia (35) on local anesthetics applied to rabbit cornea likely provide an additional example of a transition from membrane to diffusion layer control of transport. These workers determined the minimum effective concentration and the duration of activity (Fig. 4) of several alkyl derivatives of cinchocaine. The reciprocal of the minimum effective concentration of each homolog is a measure of activity and is a direct reflection of its ability to penetrate the skin and reach the site of action. The duration of activity is a measure of the time it takes for all of the drug applied to pass through the region in which it is active (where the receptor sites are located)

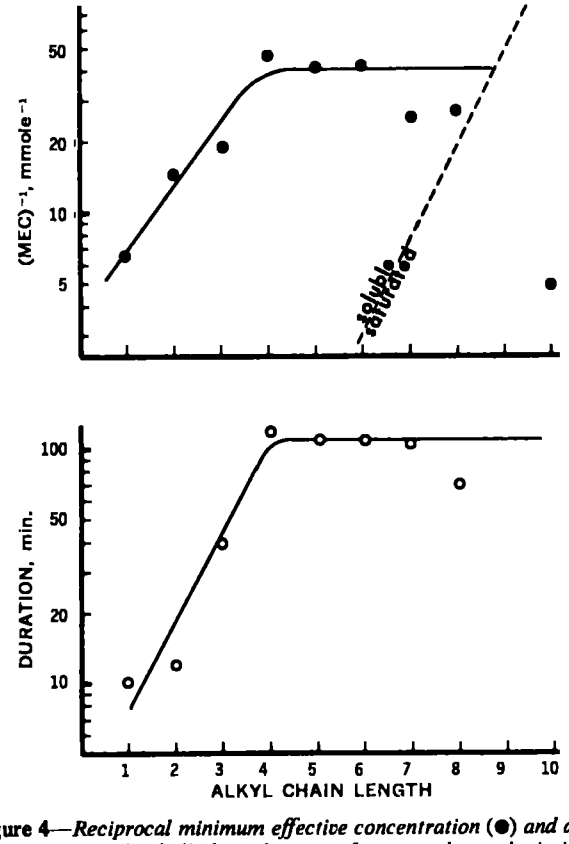

Figure 4—*Reciprocal minimum effective concentration (●) and duration of action (○) of alkyl cinchocaines for corneal anesthesia in the rabbit as a function of chain length (35).*

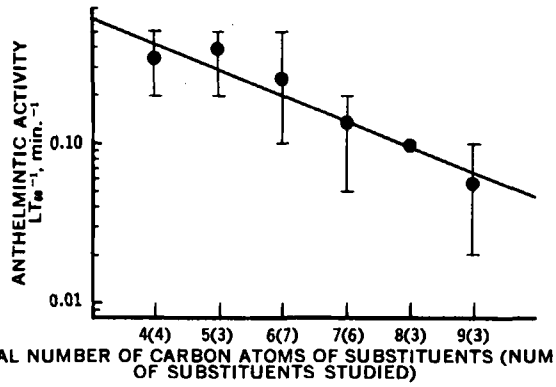

Figure 5—*Anthelmintic activity of 26 mono- and disubstituted alkylphenols from saturated solution (36).*

and be carried away by the circulating blood. If a substance is transported rapidly, it has a rapid onset of activity but a short duration. Conversely, if it is transported more slowly, the onset time is longer but so is the time required for all of the applied drug to pass through the receptor-containing region.

Since both curves of Fig. 4 are dependent upon transport across the same membranes and diffusion layers, it is not surprising that both showed the transition to diffusion layer control for the same chain length. As chain length is increased beyond six carbons, the activity begins to diminish and falls precipitously for the decyl derivative. This decline in activity is probably due to the fact that the concentrations of the higher homologs used in the study are very close to the solubility limit. In fact, the concentration reported for the decyl derivative greatly exceeds the solubility of that compound. The dotted line in Fig. 4 is an extrapolation of solubility data (35) at pH 7.4 for the first six derivatives. The π values obtained from the two sets of data appear to differ significantly (0.28 for duration data and 0.385 for minimum effective concentration data). There are many possible causes of this discrepancy, including differences in the ability to reach and leave the receptor region and differences in intrinsic activity of the substrates. In any case, these data should not be used to determine π because of their degree of scatter and their proximity to the transition region.

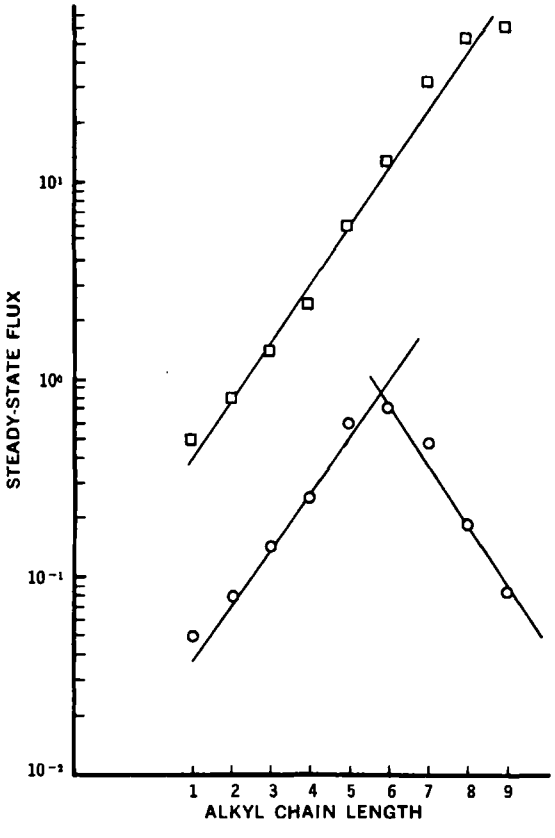

Figure 6—*Data of Scheuplein and Blank (49) for permeation of normal aliphatic alcohols across human stratum corneum* in vitro. *Key: □, concentration-normalized flux; and ○, flux from 0.10* M *solution (last four points represent saturated solutions).*

Many sets of biological data that have been treated as parabolic could alternatively be treated by Eq. 9. Similarly, studies for which no chain length dependency has been observed might be examples in which all activities of the compounds studied are on the plateau. Other examples of the plateauing effect are found in *References 6* and *33*.

Saturated Solution Studies—Returning to the arguments built around Fig. 1, one can find an alternative explanation of the plateauing effect which is also built entirely on simple diffusional theory. Since π is usually around 0.45 ± 0.10 and δ is about 0.60 ± 0.05, the value of $\pi - \delta$ is normally between 0.0 and −0.03. This being the case, curve CFG of Fig. 1 can also serve to explain plateauing data. In fact, if $\pi - \delta$ is near zero, it is not possible to distinguish between curves CFG and EHI from biological data unless the extent of saturation of each solution is known.

The data of Lamson *et al.* (36) provide a clearcut example of the relative activity of saturated solutions of alkyl homologs under membrane control of flux (curve BG of Fig. 1). Figure 5, taken from their data, shows the time required to kill at least 50% (LT_{50}) of pig ascaris *in vitro* by saturated solutions of various alkyl phenols. Their LT_{50} data, which represent 26 mono- and disubstituted alkyl phenols, give a slope of −0.16 when plotted semilogarithmically *versus* total chain length. This value falls in the midrange of the expected values for $\pi - \delta$. Significantly, the LT_{50} data of Lamson *et al.* (36) for shorter homologs, which are in true solution, fall below the line, as do the data for $n = 11$ and $n = 12$, suggesting the total curve CFGJ of Fig. 1. These results are clearly contrary to the predictions, based upon the Ferguson principal, *i.e.*, that all saturated solutions of a homologous series have the same biological activity (37, 38).

For almost all examples of "parabolic" structure–antibacterial activity relationships, the concentrations reported on the descending portion of the curve are greater than the solubility. Furthermore, the reproducibility from worker to worker of the data is good on the ascending portion and is usually quite poor above the solubility limit. As pointed out by several authors (2, 37–39), the meaningfulness of the data on this portion of the curve is questionable. If the experiments are performed properly, an exponential increase in activity is observed with increasing chain length until the required concentration for a particular derivative exceeds its solubility and that compound will have little or no activity (37). This is because it is impossible to get sufficient drug in solution to produce the desired biological effect. To get reliable data on the descending portion of the curve, the experiment must be designed to measure the magnitude of the effect produced by a given concentration (saturated or not) of material, as was done in the example just discussed, rather than the concentration required to produce a given effect. The following examples, pointed out by Ferguson (39) in 1939, illustrate the questionable nature of measurements of concentration required to produce a constant effect.

1. The concentrations of a series of alcohols required to produce a particular degree of surface tension lowering appear to go through a maximum, and the position of that maximum with regard to chain length depends upon the degree of surface tension lowering chosen.

2. The adsorption of homologs on charcoal increases with chain length; but as the solubility is exceeded, the adsorption appears to go through a maximum and then decreases with chain length. Thus, the maximum observed is dependent upon the experimental conditions chosen.

The transition from equimolar to saturated solutions under membrane control is nicely illustrated in Fig. 6. The value of π is determined from the concentration-normalized data (open squares) for the permeation of nine aliphatic alcohols across human stratum corneum to be 0.30. This low value is not unusual for epithelial tissue (Table V). The open circles for methyl through pentyl alcohol represent the flux from 0.1 *M* solution. The solubility of the higher alcohols was below this value, so the last four open circles represent the flux from saturated solutions. The slope of this line (0.30) is in excellent agreement with the expected value of $\pi - \delta$ of 0.30.

SUMMARY

According to this model in its present form, the only explanation for a decrease in activity with chain length is the reduced solubility of the higher alkyl homologs. Other physical or biological phenomena, such as micelle formation (40), incorporation into mixed micelles (41, 42), adsorption onto inert surfaces, competing receptors or absorption into fatty compartments (43), and enzyme specificity, all of which usually show an exponential dependence upon chain length, could be responsible for the descending portion of the curve. Any of these phenomena can be incorporated into the model to extend its range of applicability. Membrane pores have not been discussed in this report. However, the existence of such pores also causes no great difficulty, because they can be readily accounted for by a straightforward extension of this treatment to include parallel pathways (44).

It is interesting (and reassuring) to note that the equimolar curves obtained in this kinetic analysis, which have a slope of $\pi = 0.35$ to 0.55, are in accordance with the predictions of the equilibrium theories of Ferguson (1, 37–39) and others (1–5), even though the physical-chemical foundations differ significantly. The solubility restrictions as described are actually a kinetic facsimile and a quantitation of Ferguson's explanation of the "cutoff" phenomena (37). Nevertheless, unlike other models, this theory predicts, for certain circumstances, a plateau in the activity *versus* chain length profile of a series of drugs under subsaturated conditions. It also predicts a region of slight slope for saturated solutions for reasonably expected conditions. These solubility relationships are presently being investigated in goldfish. These experimental studies strongly support the membrane-diffusion layer model as the operative mechanism in the absorption of homologous anesthetics by the fish.

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Biological and Phytochemical Evaluation of Plants XI: Isolation of Aspidospermine, Quebrachidine, Rhazinilam, (−)-Pyrifolidine, and Akuammidine from *Aspidosperma quebracho-blanco* (Apocynaceae)

R. L. LYON, H. H. S. FONG[▲], N. R. FARNSWORTH, and G. H. SVOBODA*

Abstract □ A phytochemical investigation of the leaves of *Aspidosperma quebracho-blanco* (Apocynaceae) resulted in the isolation of rhazinilam, a new nonindole lactam alkaloid, in addition to the known bases, aspidospermine, quebrachidine, (−)-pyrifolidine, and akuammidine. Aspidospermine and quebrachidine were the major leaf alkaloids. Akuammidine and (−)-pyrifolidine were isolated from the leaves for the first time, and the isolation of the latter compound confirms its natural occurrence in this plant.

Key phrases □ *Aspidosperma quebracho-blanco* leaves—isolation, identification of aspidospermine, quebrachidine, rhazinilam, (−)-pyrifolidine, akuammidine □ Rhazinilam—new nonindole lactam alkaloid isolated from *Aspidosperma quebracho-blanco* □ Akuammidine—isolated from leaves of *Aspidosperma quebracho-blanco* □ (−)-Pyrifolidine—isolated from leaves of *Aspidosperma quebracho-blanco* □ Quebrachidine—major leaf alkaloid in *Aspidosperma quebracho-blanco* □ Medicinal plants—isolation, identification of constituents from *Aspidosperma quebracho-blanco*

Aspidosperma or quebracho, a drug composed of the bark of *Aspidosperma quebracho-blanco* Schlecht. (Apocynaceae), has been employed in Argentine and Chilean folk medicine for the treatment of fever, especially as an antiperiodic (1–3). The drug was included in USP VII and IX as well as in NF III, and the powdered drug, as well as the fluidextract and other extractives, was employed at the turn of the century as a remedy in cardiac and asthmatic dyspnea. It is said to be valuable as a respiratory stimulant in cases of emphysema, bronchitis, chronic pneumonia, and other pulmonary dysfunctions (3). The fluidextract of this drug is still commercially available[1].

DISCUSSION

Since the isolation of aspidospermine from *A. quebracho-blanco* by Fraude (1), in 1878, more than 25 alkaloids have been isolated or detected as constituents of the bark of this plant (1, 2, 4–11). The compounds so identified to date include: aspidospermine (1, 2, 4, 7, 8), aspidosamine (4), aspidospermatine (4, 7, 8), hypoquebrachine (4), quebrachamine (4, 7, 8), quebrachine (yohimbine) (5, 7, 8), quebrachacidine (6), deacetylaspidospermine (7, 8), N_a-methyldeacetylaspidospermine (7, 8), (−)-pyrifolidine (7, 8), N_a-acetylaspidospermatidine (8), aspidospermatidine (8), aspidospermidine (8), deacetylaspidospermatine (8), deacetylpyrifolidine (8), 1,2-dehydroaspidospermidine (8), 14,19-dihydroaspidospermatine (8), eburnamenine (8), N_a-methylaspidospermatidine (8), 1-methylaspidospermidine (8), 12-methoxyaspidospermatidine (8), eburnamonine (9), rhazidigenine N_b-oxide (10), rhazidine (11), and akuammidine (11). Additional nonalkaloid constituents of the bark have been identified as the triterpenes lupeol and α-amyrin (12).

The pharmacological actions of the quebracho alkaloids have been reported by a number of investigators. Aspidospermine, aspidosamine, quebrachine, and quebrachamine have been shown to be hypotensive (13). Quebrachamine and yohimbine have also been found to be sympatholytic agents (14). Aspidospermine has been reported to possess diuretic, peripheral vasoconstrictor, arterial hypertensive (15), respiratory stimulant (15, 16), and uterine sedative (16) actions, as well as having an inhibitory effect on the growth and mitosis of chicken heart fibroblasts (17). Pharmacological actions of akuammidine include local anesthetic (18), intestinal spasmogenic (19), hypotensive (20, 21), skeletal muscle relaxant, and sedative (21).

Although phytochemical and pharmacological studies on the bark of *A. quebracho-blanco* have been extensive, investigations of the leaves are limited to a single report describing the isolation of aspidospermine and quebrachidine (22). The latter alkaloid has been reported to be a psychosedative and adrenergic blocking agent (23).

In view of the limited studies on the alkaloids of the leaves and the large number of pharmacologically interesting alkaloids found in the bark of this plant, the current study was initiated in an attempt to isolate new biologically active alkaloids.

EXPERIMENTAL[2]

Extraction and Separation of Alkaloid Fractions—Fifty-five kilograms of dried, milled leaves of *A. quebracho-blanco* was extracted by percolation, using 350 l. of methanol. *In vacuo* concentration of the percolate resulted in a separation of 1.14 kg. of nonalkaloid precipitate, which was removed by filtration; the filtrate was then mixed with 14 l. of 5% acetic acid. Removal of the methanol from the mixture was followed by vacuum filtration, and the filtrate was set aside. The residue was redissolved in methanol and again mixed with acetic acid (6 l.); then the methanol was removed, and the resulting solution was filtered. This process was repeated twice to obtain a total volume of 32 l. of acid filtrate and 2.6 kg. of insoluble residue. The acid solution was made alkaline with 28% ammonium hydroxide and extracted with a total of 125 l. of chloroform. The combined chloroform extracts were dried over anhydrous magnesium sulfate, filtered, and taken to dryness *in vacuo* to yield 952 g. of Fraction A.

The post-Fraction A aqueous alkaline solution was extracted with 22 l. of *n*-butanol. This solution was dried over anhydrous magnesium sulfate and evaporated *in vacuo* to yield 370 g. of resinous alkaloid, Fraction B.

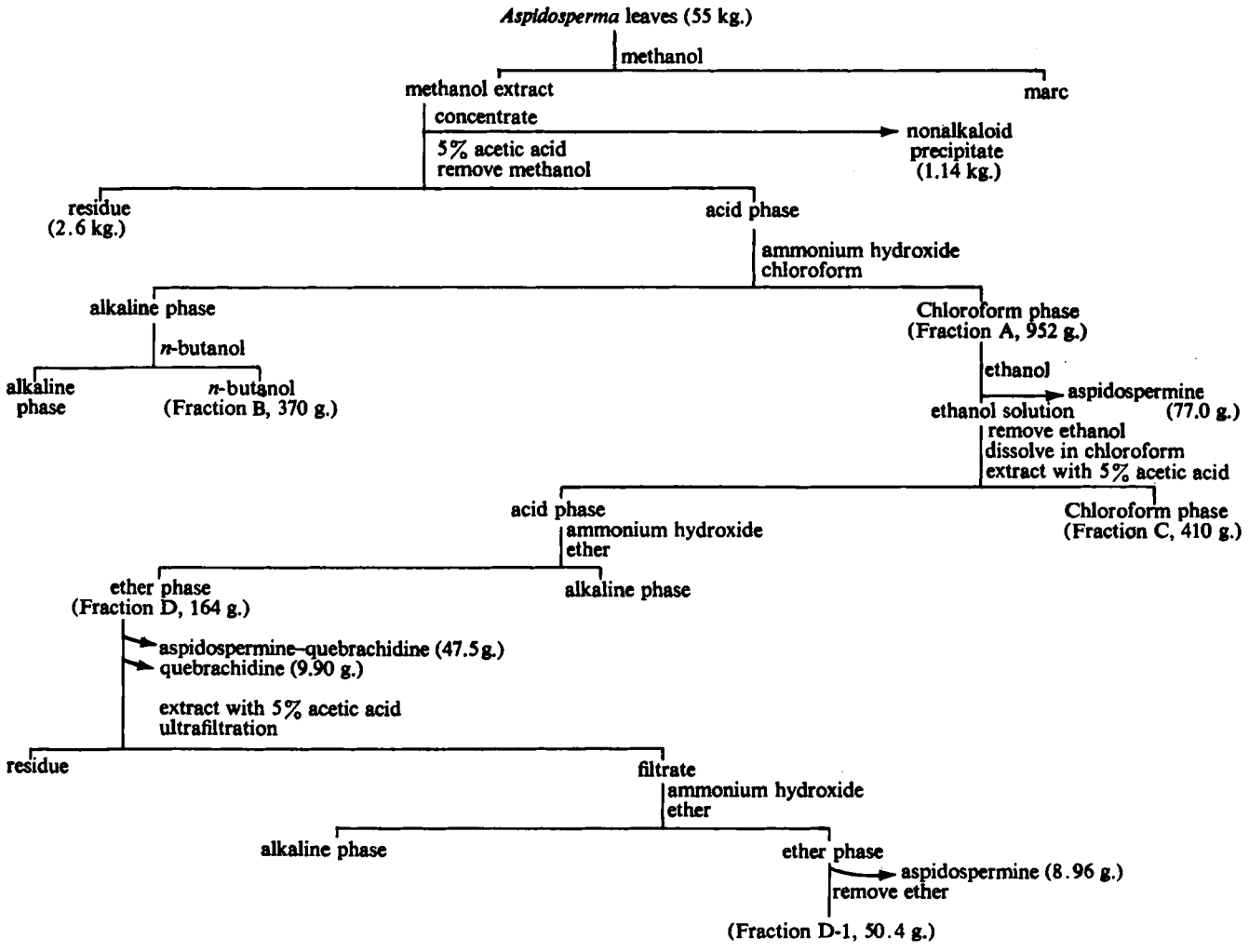
Treatment of Fraction A (952 g.) with hot ethanol, followed by filtration and cooling to room temperature, yielded 77.0 g. of a crystalline alkaloid, which was subsequently identified as aspidospermine. The ethanolic mother liquor was taken to dryness, dissolved in chloroform (3 l.), and extracted four times with 1.5-l. portions of 5% acetic acid solution. *In vacuo* evaporation of the chloroform phase yielded 410 g. of alkaloid Fraction C. The acid phase was rendered alkaline to pHydrion paper and extracted with 30 l. of anhydrous ether. The ethereal extracts were dried over anhydrous magnesium sulfate, filtered, and taken to dryness to yield 164 g. of alkaloid Fraction D. The alkaloid-free alkaline mother liquor was discarded.

Direct crystallization of Fraction D from anhydrous ether yielded two crops of an alkaloid mixture, A (47.5 g), and 9.9 g. of crude quebrachidine. Removal of ether from the mother liquor was followed by the dissolution of the residue in 900 ml. of 5% acetic acid and subsequent purification by ultrafiltration. This was accomplished by passing the solution through a 0.22-μ membrane[3]

[1] Fluidextract quebracho bark USP IX (aspidospermatis), Sherwood Laboratories, Cleveland, Ohio.

[2] The leaves of *A. quebracho-blanco* Schlecht. used in this investigation were obtained from the Meer Corp. Voucher samples of the plant material are deposited at the Meer Corp., North Bergen, NJ 07047

[3] GSWP 142000, Millipore Corp., Bedford, Mass.



Scheme I—Flow diagram for the extraction and fractionation of A. quebracho-blanco *leaf alkaloids*

with the aid of nitrogen delivered at a pressure of 20 p.s.i. to remove resinous and fine particulate matter. The filtrate was then passed through a membrane[4] at 40 p.s.i. nitrogen to remove high molecular weight, nonalkaloidal compounds. Basification of the final filtrate with ammonium hydroxide was followed by extraction with five 900-ml. portions of ether. Concentration of the pooled, magnesium sulfate-dried, ether extract afforded two crops of crude aspidospermine in yields of 3.8 and 5.78 g., respectively. Complete removal of ether resulted in 50.4 g. of alkaloid residue, designated as Fraction D-1.

A flow diagram for this extraction and fractionation scheme is presented in Scheme I.

Identification of Aspidospermine—A 2.0-g. sample of the crude crystalline isolate obtained from the hot ethanol treatment of Fraction A was repeatedly recrystallized from ethanol to yield 0.86 g. of fine, colorless needles having m.p. 206–209° (Kofler hot stage, uncorr.); $[\alpha]_D^{30}$ −96° (concentration 1.0 ethanol); λ_{max}^{MeOH}: 208 (log ϵ 4.59), 256 (4.09), and a shoulder at 290 (3.42) nm., indicating a dihydroindole alkaloid. A molecular weight of 354 was obtained by mass spectrometry; the fragmentation pattern, showing other significant peaks at *m/e* 339, 326, 311, 174, 160, 152, and 124, was identical with that reported in the literature for aspidospermine (8). The IR spectra of the isolate and reference aspidospermine were superimposable, and a mixture melting-point determination showed no depression, thus confirming the identity of the alkaloid.

Identification of Quebrachidine—The first two crops of alkaloid crystals (47.5 g.) from the ether treatment of Fraction D proved to be a mixture (A) of the alkaloids aspidospermine and quebrachidine, as shown by TLC. Silica gel G thin-layer chromatograms were developed with four solvent systems: A, ethyl acetate–absolute ethanol (3:1); B, *n*-butanol–acetic acid–water (4:1:1); C, methanol; and D, benzene–ethyl acetate–95% ethanol (4:4:1). The alkaloid mixture was revealed by chromogenic reactions with the ceric ammonium sulfate and modified Dragendorff's reagents (24) to be composed of two alkaloids. One of these compounds gave R_f values of 0.67, 0.17, 0.65, and 0.77, which corresponded with those values obtained for aspidospermine when developed in Solvent Systems A–D, respectively. The second alkaloid in the mixture gave R_f values identical with those found for quebrachidine (0.46, 0.34, 0.55, and 0.58 in Solvent Systems A–D, respectively). Furthermore, fractional crystallization from methanol afforded 0.78 g. of an alkaloid whose physical constants were identical with those cited for aspidospermine.

The third crop of alkaloid crystals (9.9 g.) obtained from the ether solution was found to be predominantly that of the lower R_f alkaloid in the TLC solvent systems. It was readily recrystallized from methanol, yielding an analytical sample of alkaloid prisms having m.p. 271–273° dec.; $[\alpha]_D^{28}$ + 38° (concentration 0.5 in chloroform); λ_{max}^{MeOH}: 207 (log ϵ 4.62), 242 (3.90), and 291 (3.58) nm. The IR and mass spectral data for the isolate were identical with those obtained for reference quebrachidine, and no depression was found with a mixture melting-point determination of the isolate and reference quebrachidine.

Chromatographic Separation of Fraction D-1 Alkaloids—The alkaloids of this fraction were found to be best resolved on silica gel G TLC plates developed using a solvent mixture of benzene–ethyl acetate–95% ethanol (4:4:1). Consequently, the alkaloids were separated over a column of silica gel PF_{254}[5], employing the same developing solvent system according to a detailed procedure to be described elsewhere. Two kilograms of the adsorbent was

[4] Millipore PSAC 14205.

[5] E. Merck, Darmstadt, Germany.

Table I—Chromatographic Data for *A. quebracho-blanco* Leaf (D-1) Fraction (50.4 g.)

Fraction[a]	Fraction Weight, g.	Alkaloid Isolated	Isolate Weight, g.
1–37	—	—	—
38–42	1.34	Rhazinilam	0.188
43–46	1.93	Aspidospermine	0.574
47–49	7.36	Aspidospermine	1.394
50–55	16.64	Aspidospermine	2.424
56–60	5.00	(−)-Pyrifolidine	0.619
61–66	2.62	Akuammidine	0.052
67–69	1.61	Quebrachidine	0.252
70–84	11.55	Quebrachidine	2.163
85–91	0.88	—	—
92–99	1.50	—	—
100–115	1.11	—	—
116–121	1.19	—	—
122–123	0.77	Rhazinilam	0.128
124–142	1.98	—	—

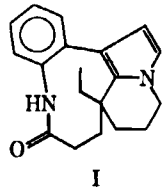
[a] Each fraction was 100 ml. with the eluting solvent being a mixture of benzene–ethyl acetate–ethanol (4:4:1).

first dried at 110° for 24 hr., followed by deactivation with 200 ml. of distilled water. A 25% slurry of the deactivated adsorbent in the solvent mixture of benzene–ethyl acetate–ethanol was prepared; after standing for 24 hr., the slurry was poured into a flat-bed column (8 × 150 cm.). The adsorbent was allowed to settle over 36 hr., with the solvent being passed through the column at a rate of 60 ml./hr. The alkaloid fraction (50.4 g.) was dissolved in 250 ml. of the developing solvent and added to the top of the packed column. Fractions of 100 ml. each were collected and monitored by means of silica gel G TLC and the ceric ammonium sulfate and modified Dragendorff's spray reagents. Those consecutive fractions containing alkaloids of similar R_f value and chromogenic response were combined for further workup. The results are presented in Table I.

Isolation of Rhazinilam—The residue from the combined fractions 38–42 from the column was dissolved in 100 ml. of ether and extracted with four 30-ml. portions of 5% acetic acid to effect the separation of an ether-soluble weak base. The acid extracts were combined, made alkaline with ammonium hydroxide, and extracted with benzene, and the benzene-soluble alkaloids were set aside. The ethereal mother liquor was dried over anhydrous magnesium sulfate, filtered, and concentrated, yielding 0.188 g. of crystalline alkaloid. Repeated recrystallization from ether afforded large prisms, m.p. 214–216°; λ_{max}^{EtOH} 224 (log ϵ 4.68) with a shoulder at 264 nm. (4.13); $[\alpha]_D^{29}$ −432 (concentration 1.25 in methanol). An IR spectrum showed the presence of an amide NH (3230 cm.$^{-1}$), an amide carbonyl (1672 cm.$^{-1}$), and four adjacent hydrogens on an aromatic nucleus (755 cm.$^{-1}$). The NMR spectrum showed vinyl protons at 5.9 and 6.65 δ, four aromatic protons as a multiplet from 7.36 to 7.69 δ, terminal methyl protons as a triplet at 0.46 to 0.89 δ, CH_2 protons adjacent to a carbonyl as a triplet at 3.75 to 4.20 δ, and an NH proton at 6.84 δ. The compound gave an M^+ ion at *m/e* 294.1727 (calc. 294.1732) for a molecular formula of $C_{19}H_{22}N_2O$ by high-resolution mass spectrometry. This alkaloid exhibited a mass spectral fragmentation pattern similar to that of eburnamonine, showing significant peaks at *m/e* 265, 237, 224, and 209. Structure I was finally deduced for the isolate by X-ray analysis (25), and it was identified as rhazinilam, an alkaloid first isolated from *Rhazya stricta* by Banerji *et al.* (26).

A second crop of crystalline rhazinilam (0.128 g.) was obtained from later column fractions, 122–123, by similar workup.

Isolation of Aspidospermine—The pooled fractions 43–46, 47–49, and 50–55 from the column were dissolved in methanol. Concentration of the filtered methanolic solution yielded 0.574, 1.394, and 2.424 g. of aspidospermine from the respective fractions. Identification of the isolates was based on a comparison of the melting points, R_f values, and IR spectra with reference aspidospermine.



I

Isolation of (−)-Pyrifolidine—By using ether as the crystallizing solvent, 0.619 g. of a crystalline alkaloid was realized from the residues of fractions 56–60 from the column. Recrystallizations from ethanol and a 1:1 mixture of acetone–water gave 0.085 g. of an analytical sample of fine needles, m.p. 144–146°; $[\alpha]_D^{20}$ − 98° (concentration 1.0 in chloroform). Literature (8) values of m.p. 148–150° and $[\alpha]_D$ − 93° have been reported for (−)-pyrifolidine. The UV spectrum (methanol) gave absorptions at λ 224 (log ϵ 4.61), 253 (4.06), and 289 (3.46) nm., indicating a dihydroindole alkaloid. The IR spectrum (KBr) showed absorption bands at ν_{max} 1655 [N—C(=O)CH_3], 2850 (aromatic —OCH_3), and 802 (two adjacent aromatic hydrogen) cm.$^{-1}$. The NMR ($CDCl_3$) spectrum revealed the presence of two aromatic protons (7.02 δ) (*AB* quartet), six methoxy protons (4.08 and 4.00 δ), and three protons [N—C(=O)-CH_3] (2.33 δ). Mass spectrometric analysis of the isolate indicated a molecular weight of 386, and its fragmentation pattern was found to be identical with that reported in the literature for (−)-pyrifolidine (8). Identification of the isolate was made on the basis of interpretation of the physical data obtained for the isolate and a comparison of the data reported in the literature for (−)-pyrifolidine.

Isolation of Akuammidine—Pooled fractions 61–66 from the column were reduced to dryness and dissolved in a mixture of methanol–ether (1:1), resulting in a yield of 0.052 g. of prismatic crystals. Recrystallization from methanol afforded an analytical sample, m.p. 243–245°. Identification of the isolate as akuammidine was made on the basis of mixture melting-point determination and superimposable IR, UV, and mass spectra with reference akuammidine.

Isolation of Quebrachidine—Pooled fractions 67–69 and 70–84 from the column were reduced to dryness, and each was taken up separately in minimum volumes of hot methanol. From the cooled solutions, 0.252 and 2.163 g. of crystalline prismatic crystals were obtained from the respective fractions. A comparison of the physical data for these isolates with those obtained earlier for quebrachidine revealed the compounds to be identical.

SUMMARY

The isolation of rhazinilam in this study represents the first example of this new class of alkaloids to be found in the genus *Aspidosperma*. The attempt to elucidate the structure of this alkaloid by spectral means proved to be a formidable task. The IR and mass spectral data of this isolate resembled very closely those of eburnamonine, an alkaloid that Biemann and Watson (9) reported as being present in the bark of this plant on the basis of GC–mass spectrometric data. The UV absorptions, however, ruled out the isolate as belonging to the eburnamonine group of alkaloids. The structure (I) elucidated for rhazinilam by X-ray analysis was confirmed by the independent elucidation of this structure by DeSilva *et al.* (27) using chemical means.

(−)-Pyrifolidine was reported to occur in this plant by Biemann *et al.* (7, 8). Their isolation of the compound, however, was achieved by hydrolysis of an alkaloid separated from an acetylated alkaloid fraction of the bark. The isolation of this alkaloid from a chemically untreated leaf fraction in the present study confirms (−)-pyrifolidine to be a naturally occurring base.

The identification of akuammidine as one of the leaf alkaloids presented no difficulty, and the copious yields of aspidospermine and quebrachidine established them to be the major alkaloid constituents of *A. quebracho-blanco* leaves.

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Model Transport Studies Utilizing Lecithin Spherules II: Transport of 3-*O*-Methyl-^{14}C-D-glucose in D-Glucose Solution

ZAKA-UD-DIN T. CHOWHAN, TOSHIHISA YOTSUYANAGI, and WILLIAM I. HIGUCHI▲

Abstract □ Recently, quantitative methods were developed for determining the permeability coefficient of solutes in lecithin spherules. The technique involved following (*a*) the direct release of solutes from the dispersions, (*b*) the release after prior dilution, and (*c*) the release from dispersions partly equilibrated with the solutes for a predetermined period. A quantitative evaluation of several physical models indicated that the models that assume that the spherules are equally spaced, multiconcentric bilayers of lecithin were in satisfactory agreement with the experimental release data. In the present study, this technique was applied to the transport of 3-*O*-methyl-D-glucose in liposome dispersions prepared from lecithin–dicetyl phosphate (10:1) and lecithin–dicetyl phosphate–cholesterol (10:1:1). The transport results for 3-*O*-methyl-D-glucose yielded a permeability coefficient that was 50 times larger than that for D-glucose. The dispersions prepared from lecithin–dicetyl phosphate containing 10% cholesterol yielded a permeability coefficient that was 2.4 times smaller than the dispersions prepared without cholesterol. The analysis of the results indicated that, for relatively large permeability coefficients as obtained in these studies, the dilution-release experiments show greater sensitivity in the determination of this parameter compared to the direct-release experiments.

Keyphrases □ Permeability coefficients, 3-*O*-methyl-D-glucose, radiolabeled—liposome dispersions, comparison of three methods □ D-Glucose solution—transport of 3-*O*-methyl-^{14}C-D-glucose, lecithin spherule dispersions, permeability coefficients □ 3-*O*-Methyl-D-glucose, radiolabeled—transport in D-glucose solution, lecithin spherule dispersions, permeability coefficients □ Lecithin spherules—model transport studies, 3-*O*-methyl-^{14}C-D-glucose in D-glucose solution, comparison of permeability coefficients determined by three methods □ Transport studies, model using lecithin spherules—3-*O*-methyl-^{14}C-D-glucose in D-glucose solution, comparison of permeability coefficients determined by three methods

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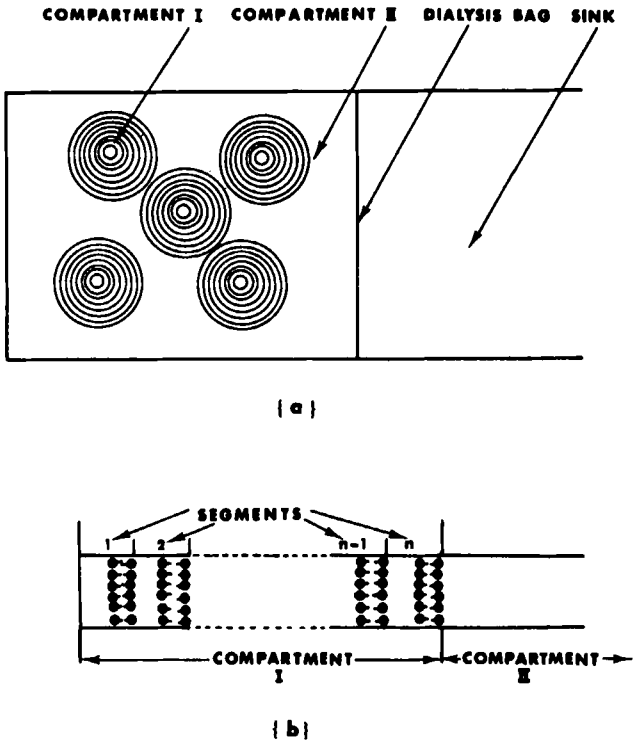

Figure 1—(a) *Schematic representation of the monosize, multiconcentric layer Model 2.* (b) *Schematic cross section of the multiconcentric layer Model 2 showing the division of Compartment I into* i = *1 to* i = n *number of segments of equal thickness.*

particle-size distribution into the theoretical analysis did not significantly alter the solute release curves. As a result of these findings, it was suggested that the monosize, multiconcentric models may be used in the determination of effective bulk permeability coefficients.

In the present studies the same procedures were used in determining the effective bulk permeability coefficients for 3-*O*-methyl-D-glucose.

EXPERIMENTAL

Materials—Egg yolk lecithin was purified using the procedures reported previously (1). Dicetyl phosphate[1] was used without further purification. Cholesterol[2] was recrystallized twice from ethanol. Anhydrous D-glucose[3] was analytical reagent grade. The dialysis bags[4] were used without pretreatment. The 3-*O*-methyl-^{14}C-D-glucose[5] was obtained in the crystalline solid form.

Procedures—The liposome dispersions were prepared from 5.0% egg yolk lecithin and 0.5% dicetyl phosphate, with or without 0.5% cholesterol. The procedures used in the preparation of dispersions and for the transport of solute were the same as reported earlier (1). The dispersions were prepared in 5.1% anhydrous D-glucose solution, which also served as a sink solution. Five milliliters of the dispersion was added to the dialysis bag, which was held by a square framework in a dialysis bag holder-stirring assembly. This assembly also served as a cover plate for the water-jacketed beaker containing 100 ml. sink solution. At the end of each sampling time, this assembly was transferred to another water-jacketed beaker containing fresh sink solution. The overall rate of the solute transport represented both the movement of solute out of the spherules and the transfer of solute across the dialysis bag. The procedures for the determination of radioactivity and for the particle-size distribution determinations by means of the Coulter counter were reported earlier (1).

[1] Sigma Chemical Co., St Louis, Mo.
[2] Fisher Scientific Co., Fair Lawn, N. J.
[3] J. T. Baker Chemical Co., Phillipsburg, N. J.
[4] Union Carbide Corp., Chicago, Ill.
[5] New England Nuclear, Boston, Mass.

THEORY

The monosize, multiconcentric layer models, Model 1 and Model 2, were previously investigated in the determination of the permeability coefficient for D-glucose (1). For molecules like D-glucose or 3-*O*-methyl-D-glucose which have relatively low partition coefficients, either model could be used in the determination of permeability coefficients. Theoretical analysis was, therefore, carried out in the present investigation using the monosize, multiconcentric layer Model 2.

A schematic representation of this model is presented in Fig. 1*a*. In Fig. 1*b*, a schematic cross section of this model, showing the division of Compartment I into $i = 1$ to $i = n$ segments, is shown. This model assumes that all of the spherules are of the same size and consist of multiconcentric layers (segments) of equal thickness. Each of these concentric layers consists of a lipid bilayer separating aqueous compartments. It is assumed that the solute instantaneously partitions between the inner aqueous–lipid interface and that the only barrier to solute transport is located at the external lipid–aqueous interface. It is also assumed that there is good mixing in both phases, so that there is no concentration gradient in each segment, neither in the lipid phase nor in the aqueous phase.

Equation 1 relates the average solution concentration to the aqueous and lipid contributions for the $i = 1$ to $i = n$th segment:

$$\begin{aligned} C_{1,t} &= (v_a + v_l \cdot k)C_{1,a} \\ &\vdots \\ C_{n,t} &= (v_a + v_l \cdot k)C_{n,a} \end{aligned} \qquad \text{(Eq. 1)}$$

Here, $C_{1,t}$ and $C_{1,a}$ are the average and aqueous solution concentrations in segment 1, respectively; v_a and v_l are the aqueous and lipid volume fractions, respectively; and k is the lipid–aqueous partition coefficient.

The rates at which solute leaves segments $i = 1$ to $i = n$ are given by a set of equations:

$$-V_1(v_a + v_l \cdot k)\frac{dC_{1,a}}{dt} = S_1 \cdot p(C_{1,a} - C_{2,a}) \qquad \text{(Eq. 2a)}$$

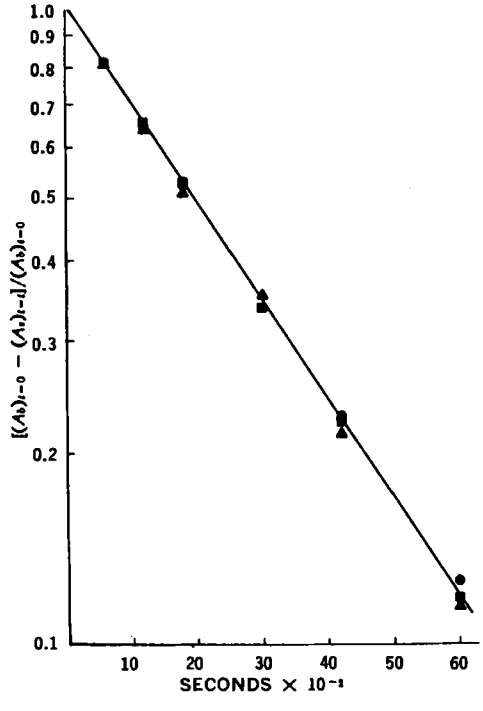

Figure 2—*First-order plots used for the determination of the bag constant for the transport of 3-O-methyl-D-glucose through the dialysis bag membrane. Different symbols represent data from the three different experiments.*

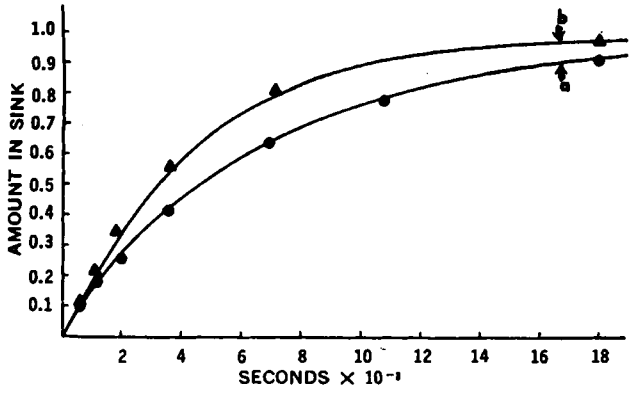

Figure 3—*Comparisons of the experimental data and results of computations using the monosize, multiconcentric layer Model 2 for the direct-release and dilution-release experiments, using a lecithin–dicetyl phosphate dispersion system. Key (symbols represent experimental data): ●, direct-release experiment; and ▲, dilution-release experiment. Curves represent results of computations. Curves a and b represent theory for the direct-release and dilution-release cases, respectively, and are based on the parameter values given in Table I. The ordinate represents A_s, the normalized amount of solute in the sink.*

$$V_2(v_a + v_1 \cdot k)\frac{dC_{2,a}}{dt} = S_1 \cdot p(C_{1,a} - C_{2,a}) - S_2 \cdot p(C_{2,a} - C_{3,a}) \quad \text{(Eq. 2b)}$$

$$V_n(v_a + v_1 \cdot k)\frac{dC_{n,a}}{dt} = S_{n-1} \cdot p(C_{n-1,a} - C_{n,a}) - S_n \cdot p(C_{n,a} - C_{II}) \quad \text{(Eq. 2c)}$$

Here, V_1 is the volume of segment 1, S_1 is the surface area of segment 1, p is the permeability coefficient per segment, and C_{II} is the concentration of solute in Compartment II, which is the external phase of the dispersion. The rate expression for solute in Compartment II is given by Eq. 3:

$$V_{II} \cdot \frac{dC_{II}}{dt} = S_n \cdot p(C_{n,a} - C_{II}) - S_b \cdot p_b \cdot C_b \quad \text{(Eq. 3)}$$

Here, V_{II} is the volume of Compartment II; S_b and p_b are the surface area and effective permeability coefficient of the bag, respectively; and C_b is the concentration of solute in the bag.

The rate of appearance of solute in the sink is given by Eq. 4:

$$\frac{dA_s}{dt} = p_b \cdot S_b \cdot C_b \quad \text{(Eq. 4)}$$

Here, A_s is the normalized amount of solute in the sink. These equations were solved by a digital computer[6] using the procedure described elsewhere (1).

RESULTS AND DISCUSSION

The results of the bag constant experiments for 3-*O*-methyl-D-glucose carried out by the procedure identical to that already reported (1) are presented in Fig. 2. The slope of the first-order plots represented the bag constant divided by the volume of the solution inside the bag. The bag constant was defined as the product of the surface area of the bag and the effective permeability coefficient of the bag.

The results of the direct-release and dilution-release experiments for 3-*O*-methyl-D-glucose in lecithin–dicetyl phosphate dispersions are given in Fig. 3. In Fig. 4 the results of the uptake release after 0 and 2 hr. prior to uptake through the same dispersion system are presented. In the monosize, multiconcentric Model 2, the partition coefficient is mainly a function of the initial slope of the direct-release experiment and was determined by the procedure outlined recently (1). Different values of p, the permeability coefficient per

[6] IBM 360.

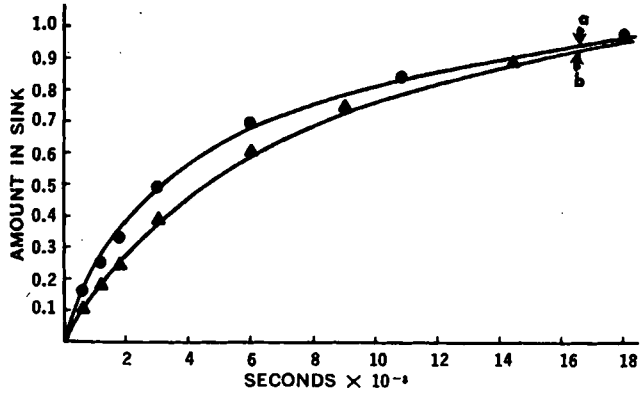

Figure 4—*Comparisons of the experimental data and results of computations using the monosize, multiconcentric layer Model 2 for the uptake-release experiments, using a lecithin–dicetyl phosphate dispersion system. Key (symbols represent experimental data): ●, 0-hr. uptake-release experiment; and ▲, 2-hr. uptake-release experiment. Curves represent results of computations. Curves a and b represent theory for the 0- and 2-hr. uptake-release cases, respectively, and are based on the parameter values given in Table I.*

segment, were then tried to obtain agreement between the experiment and the theory for the three types of experiments. The calculation results using a "best" set of parameter values, which are given in Table I, are compared with the experimental data in Figs. 3 and 4. The excellent agreement between the experimental data and the calculation results shows the usefulness of the technique in obtaining reliable values of the permeability coefficients. These results indicate that by substituting a hydroxyl group at the 3-position by a methoxy group in D-glucose the effective bulk permeability coefficient increased 50-fold. It is important to point out that this group substitution does not significantly change the partition coefficient of the solute in the liposome dispersions.

An important result of this study is revealed in Fig. 3. While significant differences between the direct-release and dilution-release experiments were not observed in the study of D-glucose transport (1), the shapes of the normalized direct-release and dilution-release curves for 3-*O*-methyl-D-glucose were significantly different. This resulted from the differences in the relative permeability coefficients of the two compounds (for D-glucose $p/n = 2.666 \times 10^{-10}$ cm. sec.$^{-1}$ and for 3-*O*-methyl-D-glucose $p/n = 1.33 \times 10^{-8}$ cm. sec.$^{-1}$). The dilution-release experiments showed greater sensitivity in the determination of permeability coefficients than the direct-release experiments when the permeability coefficient was relatively large. For example, it was found that in the direct-release

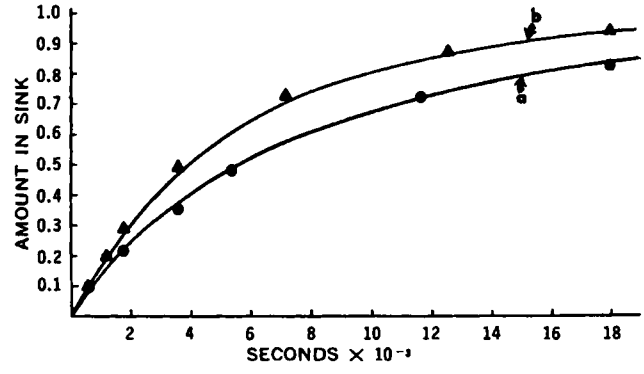

Figure 5—*Comparisons of the experimental data and results of computations using the monosize, multiconcentric layer Model 2 for the direct-release and dilution-release experiments, using a lecithin–dicetyl phosphate–cholesterol dispersion system. Key (symbols represent experimental data): ●, direct-release experiment; and ▲, dilution-release experiment. Curves represent results of computations. Curves a and b represent theory for the direct-release and dilution-release cases, respectively. The parameter values are given in Table I.*

Table I—Input Data Used in Calculations for a Lecithin–Dicetyl Phosphate and a Lecithin–Dicetyl Phosphate–Cholesterol Dispersion System Used in the 3-*O*-Methyl-D-glucose Transport Studies

Solute	Dispersion System	V_I, ml.	n	$p \times 10^7$, cm. sec.$^{-1}$	$p_b \cdot S_b \times 10^3$, cm.^{3} sec.$^{-1}$	Partition Coefficient, k	Mean Radius, μ
3-*O*-Methyl-D-glucose	Lecithin–dicetyl phosphate	1.05	45	6.0	1.764	20	0.685
3-*O*-Methyl-D-glucose	Lecithin–dicetyl phosphate–cholesterol	1.03	45	2.5	1.764	20	0.673
D-Glucose[a]	Lecithin–dicetyl phosphate	1.05	45	0.12	1.919	21	0.685

[a] Data taken from *Reference 1.*

curve a 20% change in the permeability coefficient was difficult to detect, while in the dilution-release case a 20% change in the permeability coefficient significantly changed the theoretical curve.

Several workers utilized liposome dispersions prepared from a lecithin–cholesterol–dicetyl phosphate mixture for solute transport studies (2–8). There is evidence in the literature (9) that dispersions prepared from lecithin–cholesterol are morphologically similar to those prepared without cholesterol. Papahadjopoulos and Miller (8) indicated that cholesterol in molar proportions of up to 50% could be incorporated without apparent change in the general morphology of the spherules. Although permeability coefficients have never been determined in such dispersion systems, a decrease in the permeability of glycerol (10) was observed when liposome dispersions were prepared from egg yolk lecithin and cholesterol compared to the dispersions prepared from lecithin alone. This decrease was proportional to the concentration of cholesterol. Similar observations were made (11) on chloride permeability. In the light of such studies, the results of the transport of 3-*O*-methyl-D-glucose in liposome dispersions prepared from lecithin–dicetyl phosphate containing cholesterol were found interesting. The experimental data of the direct-release and dilution-release cases are given in Fig. 5 and are compared with the computation results utilizing a best set of parameter values (Table I). In Fig. 6 the experimental results of 0- and 2-hr. uptake-release experiments are compared with the theory, utilizing the same set of parameter values. The effective bulk permeability coefficient obtained for 3-*O*-methyl-D-glucose in this dispersion system was smaller by a factor of 2.4 compared to the dispersion system without cholesterol.

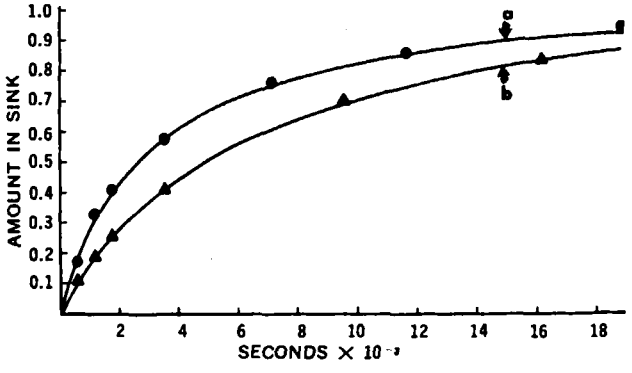

Figure 6—*Comparisons of experimental data and results of computations using the monosize, multiconcentric layer Model 2 for the uptake-release experiments, using a lecithin–dicetyl phosphate–cholesterol dispersion system. Key (symbols represent experimental data): ●, 0-hr. uptake-release experiment; and ▲, 2-hr. uptake-release experiment. Curves represent results of computations. Curves a and b represent theory for the 0- and 2-hr. uptake-release cases, respectively. The parameter values are given in Table I.*

CONCLUSION

The present study has demonstrated the effect of a group substitution on the permeability coefficient. A 50-fold increase in the permeability coefficient is obtained by the substitution of a hydroxyl group at the 3-position in D-glucose by a methoxy group.

This work has also shown that the dispersions prepared from a lecithin–cholesterol–dicetyl phosphate mixture yielded a smaller permeability coefficient than dispersions prepared without cholesterol.

In the development of the method (1), three different initial boundary conditions which led to three types of experimental procedures (direct release, dilution release, and uptake release) were selected. The usefulness of the choice of these procedures is supported by the present investigation. For solutes having relatively large permeability coefficients, the dilution-release experiments become more sensitive than the direct-release experiments in the determination of permeability coefficients.

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Calculation of Absorption Rate Constants for Drugs with Incomplete Availability

DONALD PERRIER▲ and MILO GIBALDI

Abstract □ The absorption rate constant of a drug as determined by the Wagner–Nelson or Loo–Riegelman method, by nonlinear least-squares regression analysis, or by the method of residuals is in error when drug removal from the absorption site is due not only to absorption but also to other competing processes resulting in reduced availability, regardless of the kinetics of such processes. These methods overestimate the true absorption rate constant, the degree of overestimation increasing with decreasing bioavailability. Accordingly, calculation of absorption rate constants from plasma drug concentration–time data by commonly employed methods cannot be performed with any degree of confidence unless the drug is known to be completely absorbed as such.

Keyphrases □ Absorption rate constants, true, apparent—drugs with incomplete availability, calculation □ Drug absorption rate constants, true, apparent—calculation, incomplete drug availability □ Bioavailability—absorption rate constants for drugs with reduced availability, unreliability of plasma drug concentration–time data □ Plasma drug concentration–time profiles—unreliability for use in calculating absorption rate constants of drugs with reduced availability

Several methods have been commonly employed in pharmacokinetics for the calculation of drug absorption rate constants from plasma drug concentration–time data. Probably the most commonly used method for the determination of absorption rate constants is that of Wagner and Nelson (1, 2). The following relationship was developed to calculate the percent of an oral dose absorbed at various times after administration (2):

$$\text{percent absorbed} = \frac{A_T}{A_\infty} \cdot 100 = \frac{C_T + K_E \int_{t=0}^{t=T} C\,dt}{K_E \int_{t=0}^{t=\infty} C\,dt} \cdot 100 \qquad \text{(Eq. 1)}$$

where A_T is the cumulative amount of drug absorbed at any time T, A_∞ is the total amount of drug eventually absorbed, C_T is the drug concentration in the plasma at time T, K_E is the apparent first-order elimination rate constant for the drug, and $\int_{t=0}^{t=T} C\,dt$ and $\int_{t=0}^{t=\infty} C\,dt$ are the respective areas under the plasma concentration *versus* time curves from time $t=0$ to $t=T$ and $t=0$ to $t=\infty$. The utilization of this relationship is dependent upon the drug in question being distributed in the body according to a one-compartment model.

A similar approach was employed by Loo and Riegelman (3) for calculation of absorption rate constants for drugs distributed according to a two-compartment model. An equation analogous to Eq. 1 for a drug obeying two-compartment kinetics is:

$$\text{percent absorbed} = \frac{A_T}{A_\infty} \cdot 100 = \frac{C_T + \beta \int_{t=0}^{t=T} C\,dt + P_T}{\beta \int_{t=0}^{t=\infty} C\,dt} \qquad \text{(Eq. 2)}$$

where A_T, A_∞, C_T, $\int_{t=0}^{t=T} C\,dt$, and $\int_{t=0}^{t=\infty} C\,dt$ are as defined previously; β is the apparent first-order rate constant associated with the terminal exponential phase of drug elimination; and P_T represents the drug concentration in the tissue as defined in the original report (3). From Eqs. 1 and 2, percent drug absorbed–time data can be generated. These methods place no limitation on the order of the absorption rate process. However, semilogarithmic plots of percent drug remaining unabsorbed *versus* time are frequently linear and yield an apparent first-order absorption rate constant.

Nonlinear least-squares regression analysis has also been used to determine absorption rate constants from plasma drug concentration–time curves (4). Such curves are generally described by a one- or two-compartment model with first-order elimination and an apparent first-order absorption process. This method involves fitting a theoretical equation to the plasma drug concentration–time curves with the aid of a digital computer.

Employment of the method of residuals to calculate absorption rate constants from plasma drug concentration–time data was illustrated by Wagner (5). The residuals are generally plotted on semilogarithmic coordinates, with the slope of this plot yielding the apparent first-order rate constant.

A recent report by Notari *et al.* (6) considered the application of the Wagner–Nelson and Loo–Riegelman methods to a situation where there is parallel first-order absorption and loss of a drug from the absorption depot. It was shown that the apparent first-order absorption rate constant obtained is the sum of the true absorption rate constant and the rate constant for the parallel first-order loss of drug from the absorption site. These authors pointed out that simultaneous first-order "nonabsorption" processes, such as chemical degradation, biotransformation by enzymes or intestinal bacteria, or the transfer of a drug to a compartment other than the blood, which compete for a drug at the absorption site may influence the calculated value of the absorption rate constant. These findings are contrary to the prevailing view that employment of the Wagner–Nelson method to calculate absorption rate constants requires no knowledge of the fraction of a dose absorbed (1, 2). Therefore, it was of interest to consider from a general point of view the influence of incomplete availability,

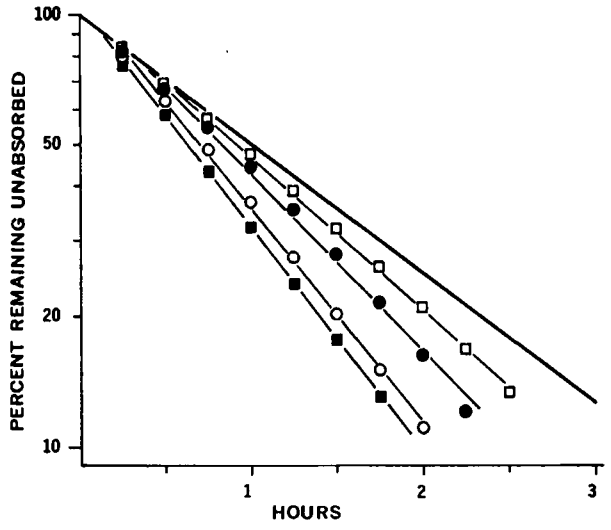


Figure 1—*Semilogarithmic plots of percent drug remaining unabsorbed* versus *time. Data were simulated according to Scheme I for a competing first-order process* ($K = 0.5\ hr.^{-1}$) *with various lag times. Slope* $= -K_A/2.3$. *Key:* ——, *control,* $K_A = 0.693\ hr.^{-1}$; ■, *lag time = 0.5 hr., 70% available,* $K_A = 1.195\ hr.^{-1}$; ○, *lag time = 1.0 hr., 79% available,* $K_A = 1.136\ hr.^{-1}$; ●, *lag time = 2.0 hr., 90% available,* $K_A = 0.936\ hr.^{-1}$; *and* □, *lag time = 3.0 hr., 95% available,* $K_A = 0.806\ hr.^{-1}$.

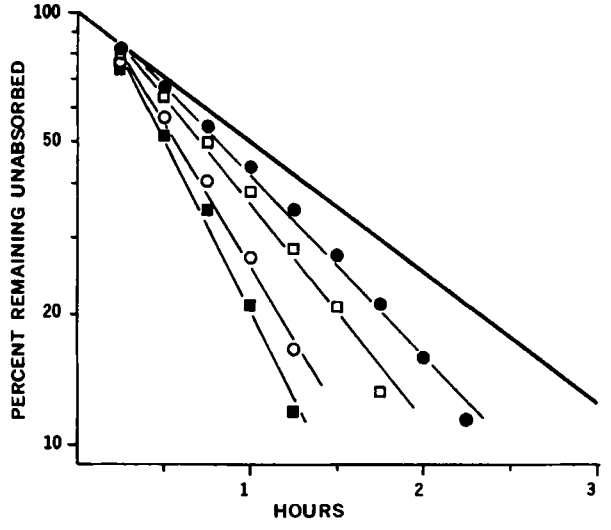


Figure 3—*Semilogarithmic plots of percent drug remaining unabsorbed* versus *time. Data were simulated according to Scheme I for a competing zero-order process* ($K_0 = 25.0$ *weight units/hr.*) *with various lag times. Slope* $= -K_A/2.3$. *Key:* ——, *control,* $K_A = 0.693\ hr.^{-1}$; ■, *lag time = 0.5 hr., 61% available,* $K_A = 1.824\ hr.^{-1}$; ○, *lag time = 1.0 hr., 69% available,* $K_A = 1.540\ hr.^{-1}$; □, *lag time = 2.0 hr., 81% available,* $K_A = 1.155\ hr.^{-1}$; *and* ●, *lag time = 3.0 hr., 89% available,* $K_A = 0.949\ hr.^{-1}$.

irrespective of whether this is due to competing processes or to limited duration of drug at the absorption site, on the estimation of apparent first-order absorption rate constants.

EXPERIMENTAL

A drug that is being removed from the site of absorption in the GI tract by an apparent first-order absorption process and by a second process that renders it unavailable for absorption may be described by Scheme I, where X_u and X_G are the respective amounts of drug unabsorbed and at the absorption site; X_A, the total amount of drug absorbed, is the sum of the amounts of drug in all "body" compartments plus drug that has been eliminated from the body; and K_A is the apparent first-order rate constant associated with the absorption process.

A drug is usually incompletely absorbed due to poor permeability characteristics of the drug itself and/or incomplete release of drug from the formulation. When either of these conditions is operative, a portion of the administered dose is removed from the absorption site by some process, resulting in the appearance of drug in the feces. Although the exact kinetic process by which a drug leaves an absorption site and is thereby rendered unavailable does not appear to be known, several possibilities exist. Grevsten *et al.* (7) administered a test solution of radioactive material by gavage and suggested that the movement of radioactivity down the GI tract could be described

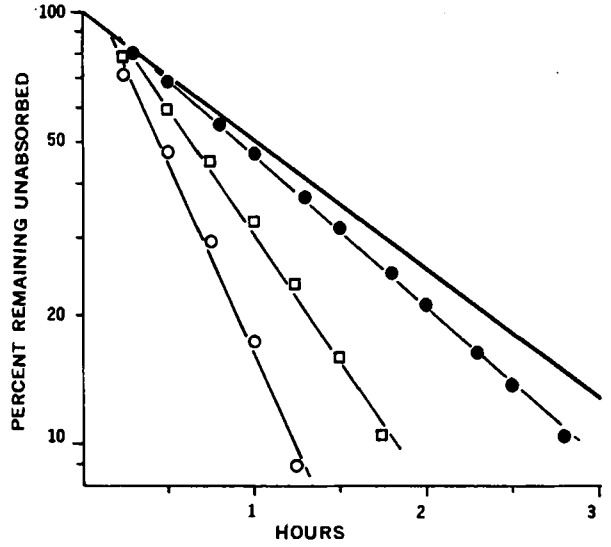


Figure 2—*Semilogarithmic plots of percent drug remaining unabsorbed* versus *time. Data were simulated according to Scheme I for competing simultaneous parallel zero-order processes* (K_0). *Slope* $= -K_A/2.3$. *Key:* ——, *control,* $K_A = 0.693\ hr.^{-1}$; ○, $K_0 = 25.0$ *weight units/hr., 52% available,* $K_A = 2.038\ hr.^{-1}$; □, $K_0 = 10.0$ *weight units/hr., 70% available,* $K_A = 1.359\ hr.^{-1}$; *and* ●, $K_0 = 1.0$ *weight unit/hr., 94% available,* $K_A = 0.806\ hr.^{-1}$.

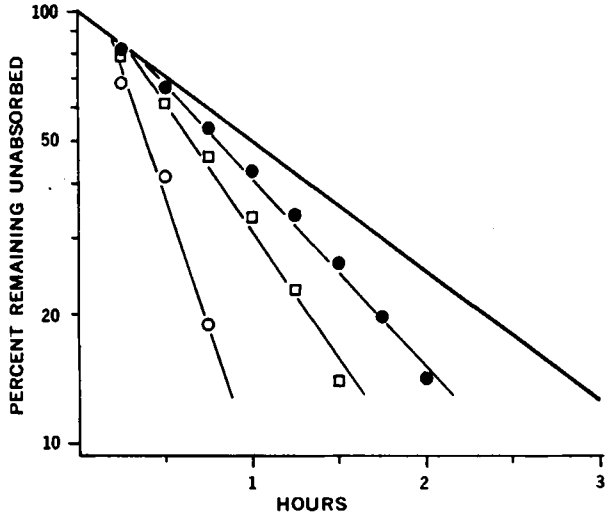


Figure 4—*Semilogarithmic plots of percent drug remaining unabsorbed* versus *time. Data were simulated according to Scheme I for an all or none process (see text) with various lag times. Slope* $= -K_A/2.3$. *Key:* ——, *control,* $K_A = 0.693\ hr.^{-1}$; ○, *lag time = 1.0 hr., 50% available,* $K_A = 2.888\ hr.^{-1}$; □, *lag time = 2.0 hr., 75% available,* $K_A = 1.386\ hr.^{-1}$; *and* ●, *lag time = 3.0 hr., 88% available,* $K_A = 0.990\ hr.^{-1}$.

Table I—Nonlinear Least-Squares Regression Analysis of Plasma Concentration–Time Data According to Scheme III (Data for Regression Analysis Obtained from Simulations According to Scheme II)

Condition Imposed on Drug Being Absorbed[a]	K_A	K_E	Coefficient of Determination[b]
Complete absorption	0.693 (0.000)[c]	0.0693 (0.000)[c]	1.0000
Parallel first-order loss (K = 0.5 hr.$^{-1}$) after 1-hr. lag	0.980 (0.066)	0.0716 (0.0016)	0.9997
Absorption terminated at 2 hr.	1.075 (0.090)	0.0755 (0.0048)	0.9979
Parallel zero-order loss (K_0 = 25 units/hr.)	1.576 (0.087)	0.0731 (0.0027)	0.9992
Parallel zero-order loss (K_0 = 25 units/hr.) after 1-hr. lag	1.221 (0.216)	0.0750 (0.0105)	0.9985

[a] Dose = 100 units. [b] $(\Sigma obs^2 - \Sigma dev^2)/\Sigma obs^2$. [c] Parenthetic values denote the standard error of the estimate.

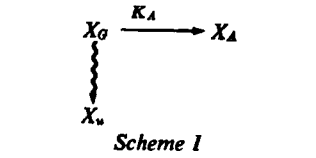
by an exponential function. If this were the case, removal of the drug from the absorption site (which can be considered as a finite length of the GI tract) could probably be approximated by an apparent first-order process. Since a certain period of time would be expected to elapse before this finite length of GI tract is traversed by the drug, a number of simulations were performed by initiating a competing first-order process at various time intervals after administration. These time intervals are referred to as lag times.

Another possibility for the removal of drug is by a zero-order process. Sikov *et al.* (8) suggested that the velocity at which a material passes through any one segment of the intestine is constant throughout that segment. Therefore, several simulations were undertaken employing simultaneous, competing, parallel, zero-order processes as well as a competing zero-order process with various lag times.

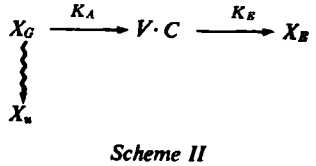
There may also be instances where a drug passes down the GI tract as a nondisintegrating unit. An example of this may be a timed-release dosage form where the drug is dispersed in an insoluble matrix. In such a case the drug is continually released until it passes out of the absorption site, at which time the drug is no longer absorbed even though it is still being released from the dosage form. This type of process is referred to as an "all or none" phenomenon. To simulate this pattern, the absorption process was halted at various times after administration.

Initially, the effect of incomplete availability (due to the various competing processes mentioned above) on the apparent first-order absorption rate constant of a drug was determined using the Wagner–Nelson and Loo–Riegelman methods. Simulations of the processes were obtained by generating amounts of drug absorbed as a function of time after oral administration using the appropriate differential equations and rate constants, according to Scheme I, as input data for the "MIMED" digital computer analog simulation program (9). Semilogarithmic plots of percent drug remaining unabsorbed *versus* time were prepared.

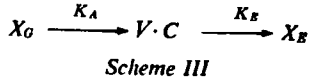
Apparent first-order absorption rate constants were also calculated for representative examples of each type of competing process simulated according to Scheme II, where X_G, X_u, and K_A are as defined previously; V is the apparent volume of distribution; C is the plasma drug concentration, the product of V and C gives the amount of drug in the body; and X_E and K_E are the amount of drug eliminated and the apparent first-order rate constant for elimination, respectively. The plasma drug concentration data simulated according to Scheme II were given equal weight and used as input for the digital computer program of Marquardt (10) to obtain a nonlinear least-squares regression fit of these data to Scheme III, where all terms are as defined previously. This treatment yielded estimates of the apparent first-order absorption rate constants for the models studied.



Scheme I



Scheme II



Scheme III

Data generated according to Scheme II were also plotted on semilogarithmic coordinates, and the apparent first-order absorption rate constants were determined by the method of residuals. This method involves extrapolating the linear elimination phase of the semilogarithmic plasma concentration–time plot to $t=0$ and subtracting the plasma drug concentrations in the absorption phase from the corresponding time–concentration values on the extrapolated line. The apparent first-order absorption rate constant was determined from the slope of a semilogarithmic plot of the values obtained by subtraction *versus* time.

RESULTS AND DISCUSSION

An overestimate in the apparent first-order absorption rate constant, as illustrated by the increase in the slopes of the percent drug remaining unabsorbed *versus* time plots in Figs. 1–4, occurred in all instances where there was a lack of availability due to a competing

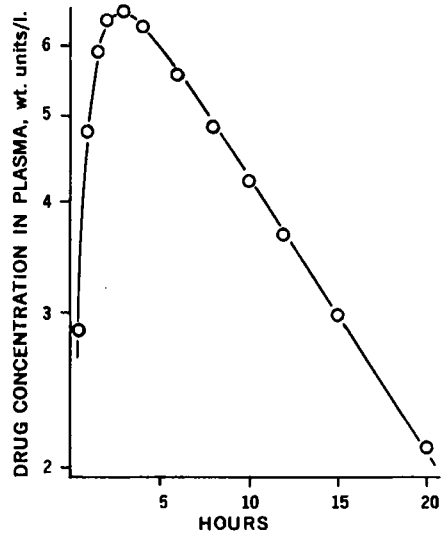


Figure 5—*Semilogarithmic plot of plasma drug concentration* versus *time after oral administration of 100 weight units of a drug. Key:* O, *data simulated according to Scheme II for a first-order absorption process* (K_A = *0.693 hr.*$^{-1}$) *and a competing first-order process* (K = *0.5 hr.*$^{-1}$) *with a lag time of 1 hr.;* ——, *biexponential least-squares fit of the simulated plasma drug concentration–time data to Scheme III,* K_A = *0.980 hr.*$^{-1}$.

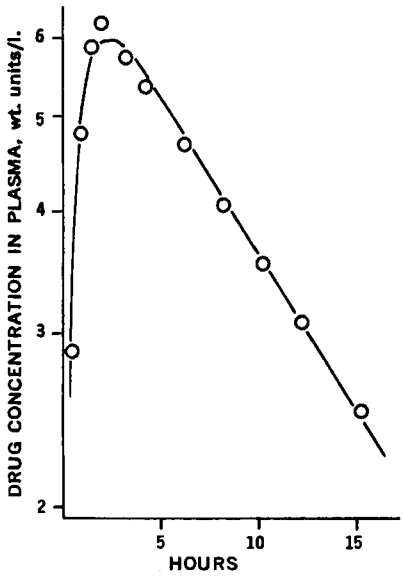


Figure 6—*Semilogarithmic plot of plasma drug concentration* versus *time after oral administration of 100 weight units of a drug. Key:* O, *data simulated according to Scheme II for a first-order absorption process* (K_A = *0.693 hr.*$^{-1}$) *and a competing zero-order process* (K_0 = *25.0 weight units/hr.*) *with a lag time of 1 hr.;* ——, *biexponential least-squares fit of the simulated plasma drug concentration–time data to Scheme III,* K_A = *1.221 hr.*$^{-1}$.

process at the absorption site. Employment of the Wagner–Nelson or Loo–Riegelman method results in an incorrect estimate of the true absorption rate constant whenever a drug is less than 100% available. While the percent remaining unabsorbed–time plots are actually slightly curvilinear rather than linear (Figs. 1–4), it is quite likely in our view that such data would normally be fit to a straight line with a lag time, considering the normal scatter and limited number of data points generally available in the absorption phase of a plasma concentration–time curve, the lag time being associated perhaps with gastric emptying.

When data simulated according to Scheme II were fit to Scheme III by a nonlinear least-squares method, a substantial overestimate of the apparent first-order absorption rate constant was again observed whenever a decrease in the availability occurred (Table I). Reasonable fits to the simulated plasma concentration–time data were obtained in all cases, as shown by the high coefficient of determination and low standard error of the estimate and as illustrated by Figs. 5 and 6. Similar results were arrived at when the absorption rate constants were calculated by the method of residuals (Fig. 7). It is quite clear that the commonly employed methods for the estimation of first-order absorption rate constants from plasma concentration *versus* time data all overestimate the true first-order absorption rate constant when there is a decrease in drug availability.

In two instances, corrections may be made for the lack of availability when determining absorption rate constants. As discussed by Notari *et al.* (6), if a drug is removed from the site of absorption by a mechanism that is first-order and occurs parallel to the absorption process, the true first-order absorption rate constant may be obtained by multiplying the apparent first-order absorption rate constant, as arrived at by the Wagner–Nelson or Loo–Riegelman method, by the fraction of the orally administered dose absorbed. Also, if the drug is less than fully available solely due to an all or none phenomenon, the percent of drug absorbed can be calculated relative to the total dose administered rather than relative to the total amount of drug eventually absorbed, A_∞. Theoretically, such corrections are feasible but practically it would be virtually impossible to determine the kinetic characteristics of the process responsible for the competitive removal of a drug from the site of absorption.

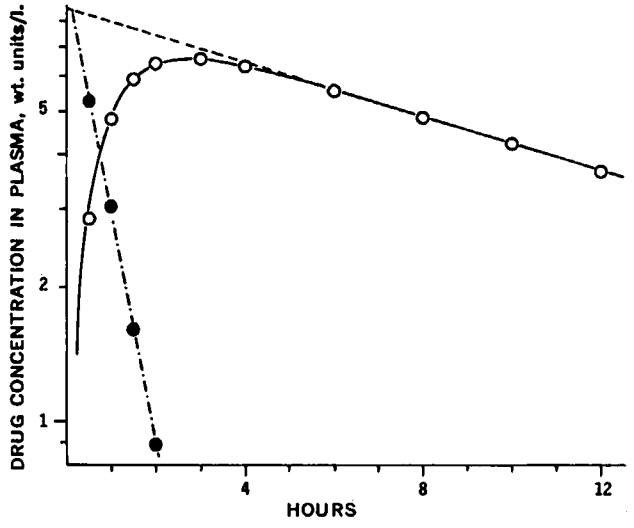


Figure 7—*Semilogarithmic plot of plasma drug concentration* versus *time after oral administration of 100 weight units of a drug. Key:* O, *data simulated according to Scheme II for a first-order absorption process* (K_A = *0.693 hr.*$^{-1}$) *and a competing first-order process* (K = *0.5 hr.*$^{-1}$) *with a lag time of 1 hr.; and* ●, *semilogarithmic plot of residuals, the slope of which yields the apparent first-order absorption rate constant* (K_A = *1.260 hr.*$^{-1}$).

Therefore, it appears that in all cases where a drug is less than completely absorbed as such, true absorption rate constants cannot be calculated. Apparent absorption rate constants that are calculated where there is less than complete availability may be used to describe the time course of drug in the plasma after single or multiple doses, but they cannot be used for quantitating the absorption characteristics of a drug.

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Absorption, Distribution, and Metabolic Fate of Seclazone [7-Chloro-3,3a-dihydro-2*H*,9*H*-isoxazolo(3,2-*b*)(1,3)benzoxazin-9-one]

JEROME EDELSON[A], J. F. DOUGLAS, and B. J. LUDWIG

Abstract □ The absorption, distribution, and metabolic fate of seclazone was studied in the rat, dog, and rhesus monkey. Seclazone is readily absorbed by all species. The half-life of blood radioactivity following oral administration of seclazone-9-^{14}C was 10, 8.5, and 6 hr. in the rat, beagle hound, and rhesus monkey, respectively. Studies in the rat, using seclazone-9-^{14}C, indicated that about one-half of the radioactivity was excreted in the urine within 20 hr. Significant amounts of ^{14}C were also found in the carcass, liver, and GI tract. Only trace amounts of unchanged drug were voided. Seclazone undergoes metabolic cleavage of the oxazine ring, yielding 5-chlorosalicylic acid which is excreted in the free form and as conjugates with glucuronic acid and glycine. Similar studies using seclazone-2-^{14}C showed that the carbon chain of the isoxazoline ring is cleaved to malonic acid and carbon dioxide.

Keyphrases □ Seclazone, radiolabeled—absorption, distribution, metabolism in rats, dogs, rhesus monkeys □ 7-Chloro-3,3a-dihydro-2*H*,9*H*-isoxazolo(3,2-*b*)(1,3)benzoxazin-9-one, radiolabeled—absorption, distribution, metabolism in rats, dogs, rhesus monkeys □ 5-Chlorosalicylic acid—major metabolite of seclazone

Seclazone[1] [7-chloro-3,3a-dihydro-2*H*,9*H*-isoxazolo-(3,2-*b*)(1,3)benzoxazin-9-one] is a new compound which possesses uricosuric and anti-inflammatory activity in animals and man (1, 2). This report describes studies on the absorption, distribution, and metabolic fate of seclazone in the rat, dog, and rhesus monkey.

EXPERIMENTAL

Preparation of 7-Chloro-3,3a-dihydro-2*H*,9*H*-isoxazolo(3,2-*b*)(1,3)-benzoxazin-9-one-9-^{14}C (Seclazone-9-^{14}C)—A mixture of 1.0 g. (3.0 mc.) of 5-chlorosalicylic acid-carboxyl-^{14}C, 2.0 ml. of methanol, and 0.25 ml. of concentrated sulfuric acid was stirred in a centrifuge tube fitted with a condenser and heated in an oil bath at 70° for 18 hr. Ice water was added to the reaction mixture, and the solid which formed was sedimented by centrifugation. This solid was sequentially washed with cold water, cold saturated sodium bicarbonate solution, and cold water. Ten milliliters of an aqueous solution containing 750 mg. hydroxylamine hydrochloride and 865 mg. sodium hydroxide was added, and the mixture was stirred at room temperature until solution occurred (about 2 hr.). The resulting hydroxamic acid was precipitated by the addition of 10 ml. of 12 *M* hydrochloric acid. The solid obtained by centrifugation was washed with cold water and dried *in vacuo* over phosphorus pentoxide.

A solution containing 540 mg. anhydrous hydrogen chloride dissolved in 5 ml. of glacial acetic acid was added to the dried hydroxamic acid in the reaction tube, followed by the addition of 0.45 ml. of freshly distilled acrolein. This reaction mixture was stirred for 3 hr. in an oil bath at 50°. Ice water was added and the solid which formed was separated by centrifugation, washed with cold water, and dried *in vacuo*. The solid in the centrifuge tube was reacted with 10 ml. of cold 1 *M* sodium hydroxide, and the mixture was agitated in ice for 30 min. The product was sedimented by centrifugation and sequentially washed with cold water, cold 5% (w/v) sodium bicarbonate solution, and cold water. The dried residue yielded 600 mg. of seclazone-9-^{14}C, m.p. 149–150°, specific activity 4.61 × 10^6 d.p.m./mg.

This product gave a single radioactive zone by TLC in chloroform–methanol (97:3), and its IR spectrum was identical to that of authentic seclazone.

Preparation of 7-Chloro-3,3a-dihydro-2*H*,9*H*-isoxazolo(3,2-*b*)-(1,3)-benzoxazin-9-one-2-^{14}C (Seclazone-2-^{14}C)—A solution of 540 mg. of anhydrous hydrogen chloride in 5 ml. of glacial acetic acid was placed in a centrifuge tube, and 1.05 g. of 5-chlorosalicylohydroxamic acid was added. A solution of 829 mg. (3.0 mc.) of the diethyl acetal of 3-chloropropionaldehyde-3-^{14}C in 5 ml. of benzene was transferred to the centrifuge tube. The reaction mixture was vigorously stirred in a water bath at 54° for 2.5 hr. and allowed to stand overnight at room temperature. The benzene was removed *in vacuo*, and a solid was precipitated by the addition of ice water. After centrifugation, the solid pellet was washed with cold water, dried, and suspended in 20 ml. of cold 1 *M* ammonia, and the suspension was stirred for 1.5 hr. in an ice water bath. The product, seclazone-2-^{14}C, was sedimented by centrifugation in the cold, washed with water, and dried. The yield was 250 mg. of seclazone-2-^{14}C, m.p. 145–146°, specific activity 5.25 × 10^6 d.p.m./mg.

The product was homogeneous as determined by TLC in two different solvent systems.

Preparation of 5-Chlorosalicyluric Acid—A mixture of 69 g. of 5-chlorosalicylic acid, 100 g. of thionyl chloride, 4 ml. of *N,N*-dimethylformamide, and 400 ml. of benzene was warmed to 50° for 1.5 hr. and then heated to 80° for an additional 1.5 hr. The solvent was removed *in vacuo*, and the residue was added to a solution of 30 g. of glycine and 18 g. of sodium hydroxide in 100 ml. of water. Additional sodium hydroxide was added to maintain alkaline conditions, pH 9–10. After stirring the mixture overnight, the oily residue was removed and discarded. The supernatant solution was acidified with concentrated hydrochloric acid, which precipitated the 5-chlorosalicyluric acid. The purified compound was obtained by recrystallization from ethyl acetate–hexane. The yield was 7.5 g., m.p. 203–204°.

Anal.—Calc. for $C_9H_8ClNO_3$: C, 47.02; H, 3.52; N, 6.13. Found: C, 47.12; H, 3.55; N, 6.10.

Absorption and Blood Concentration—Six male Sprague–Dawley rats, weighing between 150 and 200 g., each received by stomach tube a solution of 4.08 mg. of seclazone-9-^{14}C dissolved in 0.5 ml. of polyethylene glycol 400. Blood samples were taken from the tail vein at appropriate intervals and were assayed for radioactivity by liquid scintillation counting as previously described (3).

A female rhesus monkey, weighing 3.9 kg., was given a capsule orally containing 105.6 mg. of seclazone-9-^{14}C. Blood samples taken from the jugular vein at suitable intervals were assayed for radioactivity.

Table I—Blood Radioactivity after Oral Administration of Seclazone-9-^{14}C to Six Rats

Time after Administration, hr.	Mean Radioactivity[a], d.p.m./ml. × 10^4
0.25	3.88 ± 0.30
0.50	6.09 ± 0.55
1.0	7.51 ± 0.71
1.5	7.17 ± 0.37
2.0	7.60 ± 0.50
3.0	7.63 ± 0.48
4.0	6.67 ± 0.47
5.0	6.00 ± 0.44
6.0	5.85 ± 0.39
7.0	5.12 ± 0.40
24.0	1.51 ± 0.14
26.0	1.41 ± 0.15
28.0	1.38 ± 0.16
30.0	1.18 ± 0.19
48.0	0.30 ± 0.05

[a] Values are given with their standard error.

[1] Seclazone previously has been referred to in the literature as W-2354.

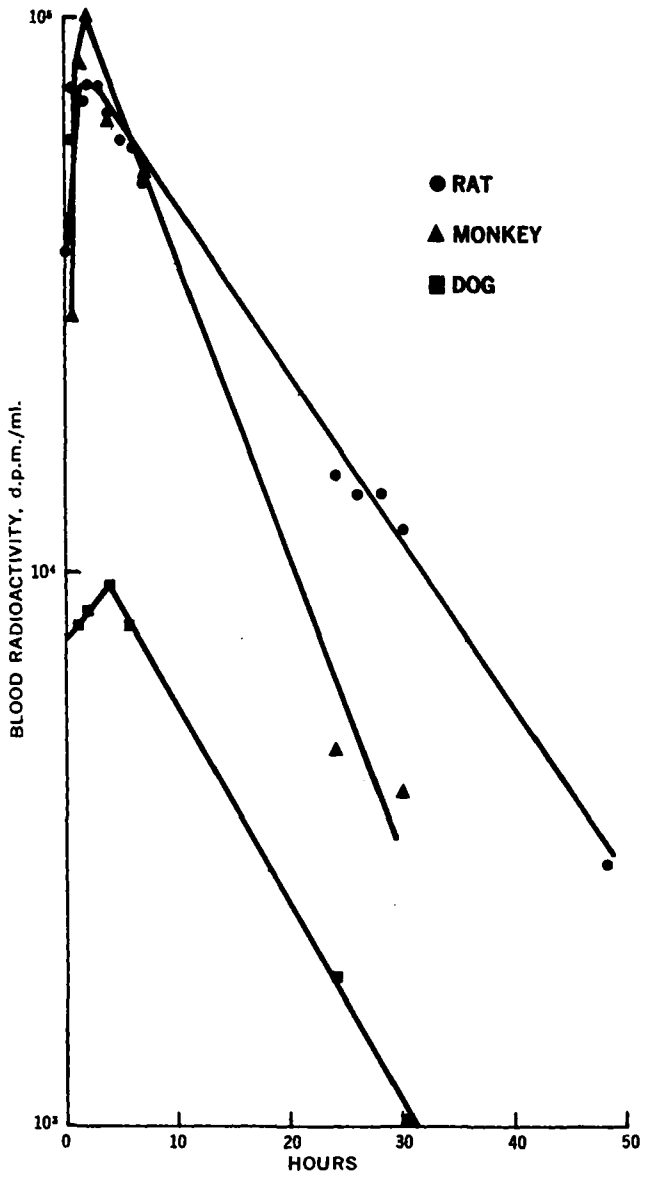


Figure 1—*Blood radioactivity after oral administration of seclazone-9-^{14}C to three species. Dose: rat, 1.07 × 10^{8} d.p.m./kg., 23.4 mg./kg.; monkey, 3.54 × 10^{7} d.p.m./kg., 27.1 mg./kg.; and dog, 1.95 × 10^{7} d.p.m./kg., 42.8 mg./kg.*

A male beagle hound, weighing 12 kg., was given a capsule containing 515.4 mg. of seclazone-9-^{14}C. At appropriate time periods, blood samples were taken from the vein in the toe nail and their plasma radioactivity was measured.

Tissue Distribution in the Rat—A male Sprague–Dawley rat, weighing about 200 g., received by stomach tube 1.7 mg. of seclazone-9-^{14}C dissolved in 1.0 ml. of polyethylene glycol 400. The animal was placed in a metabolism chamber for 20 hr. and then sacrificed. The excreta, soft tissue organs, and the remainder of the carcass were assayed for radioactivity as previously reported (3).

A second rat, weighing about 200 g., received orally a solution containing 2.35 mg. of seclazone-2-^{14}C in 1.0 ml. of polyethylene glycol 400. After 24 hr., the animal was sacrificed and its tissues were processed and analyzed for radioactivity. The carbon dioxide of respired air was trapped in 2.5 *M* sodium hydroxide, and the radioactivity of an aliquot of this solution was measured.

Isolation of Metabolites—Pooled 24-hr. dog urine (1300 ml.) from three male beagles, who had each been given 50 mg./kg. of nonradioactive seclazone by capsule, was continuously extracted with carbon tetrachloride for 18 hr. The organic phase was separated, washed with 50 ml. of water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The residue was crystallized from ethyl acetate, m.p. 149–150°. This solid was identified as seclazone.

The urine remaining from the initial extraction was acidified to pH 1 with concentrated hydrochloric acid and continuously extracted with carbon tetrachloride for an additional 18 hr. The organic phase was separated, washed with 50 ml. of acidified water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The resulting solid, recrystallized from methanol and then from chloroform, was identified as 5-chlorosalicylic acid, m.p. 171–172°.

Inverse Isotope Dilutions—Inverse isotope dilution experiments were carried out on the urine of animals that had received labeled seclazone. A typical procedure is described below:

1. A male Sprague–Dawley rat, weighing about 200 g., received by stomach tube 1.7 mg. of seclazone-9-^{14}C dissolved in 1.0 ml. of polyethylene glycol 400. Urine was collected for 24 hr. and its radioactivity was measured. Isotope dilutions were carried out on separate aliquots of urine as follows:

(*a*) Nonradioactive 5-chlorosalicylic acid (1000 mg.) was added to the first 1.0-ml. aliquot, followed by sufficient methanol to give a clear solution. Upon the addition of water, 5-chlorosalicylic acid precipitated. The solid was removed and repeatedly recrystallized from methanol–water to constant specific activity, m.p. 174–176°.

(*b*) Nonradioactive 5-chlorosalicyluric acid (1000 mg.) was added to a second 1.0-ml. aliquot of urine, and the mixture was diluted with water and heated until homogeneous. Upon cooling, crystalline 5-chlorosalicyluric acid separated and was removed by filtration. It was recrystallized from ethyl acetate–hexane to constant specific activity, m.p. 202–204°.

(*c*) Nonradioactive seclazone was added to another aliquot of urine. Sodium carbonate solution was added to adjust the pH to 9–10, and the mixture was extracted three times with 10 volumes of chloroform. The chloroform phase was evaporated to dryness. The residue, consisting of crude seclazone, was recrystallized from ethyl acetate to constant specific activity, m.p. 149–150°.

(*d*) The metabolites occurring in the urine in a combined form were quantitated following conversion to the free form by hydrolysis. An aliquot of urine was treated with an equal volume of concentrated hydrochloric acid, and the mixture was heated on a steam bath for 10 min. Nonradioactive carrier was then added, and the inverse isotope dilution was carried out as described for the free metabolites. Conjugated urinary end-products were calculated by difference.

Similar studies were performed on the urine of a dog and monkey that had received 515 and 106 mg. of seclazone-9-^{14}C, respectively.

2. A male Sprague–Dawley rat, weighing about 200 g., received by stomach tube 2.35 mg. of seclazone-2-^{14}C dissolved in 1.0 ml. of polyethylene glycol 400. Urine was collected for 24 hr. and assayed for radioactivity.

(*a*) Nonradioactive malonic acid (1000 mg.) was added to a 3.0-ml. aliquot of urine, and the mixture was warmed gently until a clear solution was obtained. A few drops of concentrated hydrochloric acid were added, and the mixture was extracted twice with 15 ml. of ethyl acetate. The organic phase was dried

Table II—Tissue Distribution of ^{14}C in the Rat after Oral Administration of Seclazone

Specimen	Radioactivity Recovered, —Percent of Dose— Seclazone-9-^{14}C[a]	Seclazone-2-^{14}C[b]
Urine	52.1	58.9
Carcass	34.6	5.98
Liver	4.9	1.24
GI tract, including feces	3.4	4.52
Kidney	0.8	0.21
Lung	0.6	0.07
Heart	0.3	0.04
Spleen	0.1	0.03
Carbon dioxide	0.0	27.2
Recovery	96.8	98.2

[a] Animal sacrificed 20 hr. after drug administration. [b] Animal sacrificed 24 hr. after drug administration.

Table III—TLC[a] R_f Values of Urinary End-Products of Seclazone

Compound	R_f Solvent System A[b]	R_f Solvent System B[c]
Seclazone	0.60	0.68
5-Chlorosalicylic acid	0.47	0.47
5-Chlorosalicyluric acid	0.35	0.16
5-Chlorosalicylic acid glucosyluronide	—	0.06

[a] Silica gel. [b] Benzene–methanol–glacial acetic acid (180:32:16). [c] *n*-Butanol saturated with 2 *N* ammonia.

Table IV—Quantitation of Seclazone-9-^{14}C Urinary End-Products in the Rat, Dog, and Monkey

Urinary End-Product	Percent of Urinary Radioactivity[a] Rat 1	Rat 2	Dog	Monkey
5-Chlorosalicylic acid				
Free	70.5	63.2	75.4	66.0
Bound[b]	25.7	25.6	22.3	21.2
5-Chlorosalicyluric acid				
Free	1.3	0.2	0.3	6.0
Bound[b]	n.d.[c]	n.d.	0.4	0.6
Seclazone				
Free	n.d.	0.97	0.6	0.03
Bound[b]	n.d.	n.d.	n.d.	n.d.
Total radioactivity recovered	97.5	90.0	99.0	93.8

[a] Values obtained by inverse isotope dilution. Animals received the following oral doses of drug: rat 1, 1.7 mg.; rat 2, 55 mg.; dog, 515 mg.; and monkey, 106 mg. [b] Values obtained by difference, before and after acid hydrolysis. [c] None detected.

over anhydrous sodium sulfate, and hexane was added to the ethyl acetate solution to precipitate malonic acid. The solid was separated and recrystallized from ethyl acetate–hexane to constant specific activity, m.p. 133–134°.

(*b*) Nonradioactive succinic acid (1000 mg.) was added to a 2.0-ml. aliquot of urine, and the mixture was diluted with a few milliliters of water and heated until homogeneous. After concentrated hydrochloric acid was added to adjust the pH to 1–2, the solution was cooled and allowed to stand. The crystals of succinic acid which separated were removed by filtration and recrystallized from ethyl acetate–hexane to constant specific activity, m.p. 185–186°.

RESULTS AND DISCUSSION

Absorption and Blood Concentration—Plasma radioactivity was observed within 1 hr. following oral administration of 9-labeled seclazone to the rat, dog, or rhesus monkey, indicating that the drug was readily absorbed in each of these three species. The elimination of ^{14}C from the bloodstream appeared to follow first-order kinetics. The half-lives of radioactivity in the blood of the rat, beagle hound, and rhesus monkey were approximately 10, 8.5, and 6 hr., respectively (Fig. 1). The means plus the standard errors obtained for six rats are given in Table I.

Tissue Distribution in the Rat—A distribution study of radioactivity following oral administration of seclazone-9-^{14}C showed that the ^{14}C was principally excreted in the urine within 20 hr. Significant amounts of ^{14}C were also found in the carcass, liver, and GI tract at that time (Table II).

A similar study with seclazone-2-^{14}C showed that radioactivity from the 2-carbon atom was also largely eliminated in the urine, since almost 60% of the radioactivity of the administered dose was voided in the urine within 24 hr. Moreover, over one-fourth of the ^{14}C was exhaled as respiratory carbon dioxide, indicating that the carbon chain of the isoxazoline ring was cleaved and oxidized in the body. The distribution of the residual radioactivity is shown in Table I.

Additional experiments with rats showed that these animals excreted similar levels of radioactivity during the 24-hr. period after drug administration. The ^{14}C voided, as percent of dose, for two animals given seclazone-9-^{14}C was 52.0 and 62.1%, while the urinary content of two rats given 2-labeled drug was 52.0 and 53.3%.

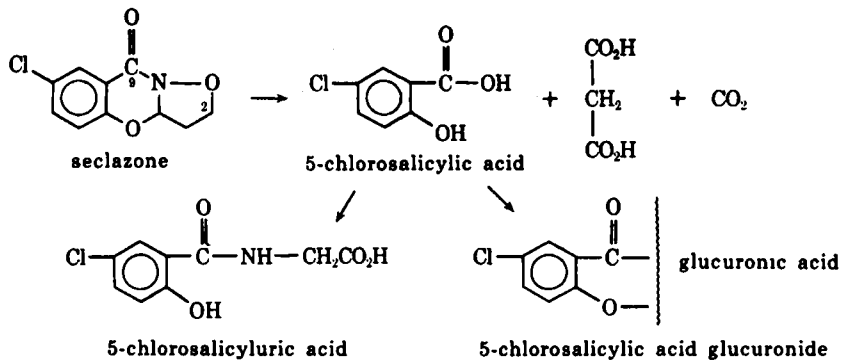
Identification, Isolation, and Quantitation of Urinary End-Products—Four radioactive substances were detected by TLC in the urine of animals that had received seclazone-9-^{14}C. Three of these compounds were identified by isolation and/or isotope dilution as seclazone, 5-chlorosalicylic acid, and 5-chlorosalicyluric acid. In addition, evidence was obtained indicating that 5-chlorosalicylic acid was also present in the urine in the form of a glucuronide conjugate. The R_f's of these compounds in two different solvent systems are shown in Table III.

Unchanged seclazone and 5-chlorosalicylic acid, isolated from the urine of three beagle hounds, were identified by their TLC properties, melting points, and IR spectra.

Quantitation of the various metabolites of seclazone-9-^{14}C occurring in the urine of the three animal species is summarized in Table IV.

The major urinary end-product in the three species examined—rat, dog, and monkey—is 5-chlorosalicylic acid, resulting from hydrolysis of the ingested compound. Seclazone is almost completely broken down in the animal body since only insignificant amounts of unchanged drug were excreted in the urine. Essentially all of the urinary radioactivity could be accounted for as 5-chlorosalicylic acid, either free or in the form of its conjugates with glycine and glucuronic acid.

In a single experiment on radioactive dog urine, evidence was obtained which indicated that the "bound" 5-chlorosalicylic acid occurs chiefly as the glucuronide. In this study the glucuronides were separated using the basic lead salt method of Kamil *et al.* (4). These were found to contain the same amount of radioactivity as that present in the bound 5-chlorosalicylic acid (approximately 20% of the urinary ^{14}C). The glucuronide was hydrolyzed by hydrochloric acid and β-glucuronidase to yield 5-chlorosalicylic acid, but it was



Scheme I—Metabolic fate of seclazone

not hydrolyzable by aryl sulfatase. The identity of the hydrolysis product was confirmed by inverse isotope dilution and TLC. The nature of the glucuronide bonding has not been determined.

Inverse isotope dilutions on the urine of three rats that had received seclazone-2-^{14}C indicated the presence of malonic acid in an amount that accounted for 2–14% of the urinary radioactivity. In addition, succinic acid, a known metabolite of malonic acid, was present and represented 1–3% of the urinary radioactivity. The presence of these two compounds suggests that the carbon dioxide-^{14}C present in respired air (Table I) originates either from the direct decarboxylation of malonic acid or from succinic acid through the tricarboxylic acid cycle. In any event, the radioactive carbon dioxide arises through metabolic cleavage of the isoxazoline ring of seclazone.

The metabolic fate of seclazone is summarized in Scheme I.

SUMMARY

Seclazone-9-^{14}C is readily absorbed after oral administration to the rat, beagle hound, and rhesus monkey. The half-lives of blood radioactivity in the three species were found to be 10, 8.5, and 6 hr., respectively. In the rat, about half of the ^{14}C given orally was excreted in the urine within 24 hr. Significant amounts of radioactivity were also found in the carcass, liver, and GI tract. Seclazone is almost completely metabolized to 5-chlorosalicylic acid in each of these three species. The latter compound was excreted either uncombined or conjugated with glucuronic acid or glycine. Studies with seclazone-2-^{14}C suggested that carbons 2, 3, and 3A of seclazone are metabolized to malonic acid.

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Displacement of Sulfonylureas from Human Serum Proteins by Coumarin Derivatives and Cortical Steroids

JOSEPH JUDIS

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Reports in the recent literature have cited interactions of sulfonylureas and various drugs including coumarin derivatives (1–9). Although there have been apparently no reports of displacement of cortical steroids by sulfonylureas, compounds such as phenylbutazone have been shown to displace cortical steroids bound to human serum proteins; since competition has been demonstrated between phenylbutazone and sulfonylureas for binding sites on human serum proteins, one could conclude that similar competition should exist between cortical steroids and sulfonylureas in serum protein bindings (10). Sellers and Koch-Weser (11) recently pointed out that, although the clinical importance of metabolic interactions between drugs has become increasingly clear, not enough work has been done to demonstrate the mechanisms of drug interactions by *in vitro* laboratory experiments including studies of displacement of drugs bound to plasma proteins. Such data would make it possible to predict clinically important drug interactions.

not hydrolyzable by aryl sulfatase. The identity of the hydrolysis product was confirmed by inverse isotope dilution and TLC. The nature of the glucuronide bonding has not been determined.

Inverse isotope dilutions on the urine of three rats that had received seclazone-2-^{14}C indicated the presence of malonic acid in an amount that accounted for 2–14% of the urinary radioactivity. In addition, succinic acid, a known metabolite of malonic acid, was present and represented 1–3% of the urinary radioactivity. The presence of these two compounds suggests that the carbon dioxide-^{14}C present in respired air (Table I) originates either from the direct decarboxylation of malonic acid or from succinic acid through the tricarboxylic acid cycle. In any event, the radioactive carbon dioxide arises through metabolic cleavage of the isoxazoline ring of seclazone.

The metabolic fate of seclazone is summarized in Scheme I.

SUMMARY

Seclazone-9-^{14}C is readily absorbed after oral administration to the rat, beagle hound, and rhesus monkey. The half-lives of blood radioactivity in the three species were found to be 10, 8.5, and 6 hr., respectively. In the rat, about half of the ^{14}C given orally was excreted in the urine within 24 hr. Significant amounts of radioactivity were also found in the carcass, liver, and GI tract. Seclazone is almost completely metabolized to 5-chlorosalicylic acid in each of these three species. The latter compound was excreted either uncombined or conjugated with glucuronic acid or glycine. Studies with seclazone-2-^{14}C suggested that carbons 2, 3, and 3A of seclazone are metabolized to malonic acid.

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Reports in the recent literature have cited interactions of sulfonylureas and various drugs including coumarin derivatives (1–9). Although there have been apparently no reports of displacement of cortical steroids by sulfonylureas, compounds such as phenylbutazone have been shown to displace cortical steroids bound to human serum proteins; since competition has been demonstrated between phenylbutazone and sulfonylureas for binding sites on human serum proteins, one could conclude that similar competition should exist between cortical steroids and sulfonylureas in serum protein bindings (10). Sellers and Koch-Weser (11) recently pointed out that, although the clinical importance of metabolic interactions between drugs has become increasingly clear, not enough work has been done to demonstrate the mechanisms of drug interactions by *in vitro* laboratory experiments including studies of displacement of drugs bound to plasma proteins. Such data would make it possible to predict clinically important drug interactions.

Table I—Characteristics of Inhibition of Albumin Binding of Sulfonylureas by Ethyl Biscoumacetate

Sulfonylurea	Concentration of Ethyl Biscoumacetate ($\times 10^{-5}$ mole)	Percent Reduction of r at pH: 6.5	Coefficient of Correlation[a]	7.4	Coefficient of Correlation	8.4	Coefficient of Correlation
Acetohexamide[b]	0	—		—		—	
	12	6.08		0.19		−4.93	
	24	20.01		9.10		2.09	
	48	21.53		32.07		25.57	
	216	80.11	0.9751	77.93	0.9607	72.95	0.9704
Chlorpropamide[c]	0	—		—		—	
	12	30.15		12.00		22.84	
	24	39.27		24.67		42.30	
	48	63.89		65.12		64.86	
	216	91.32	0.8343	88.10	0.8849	93.19	0.8250
Tolbutamide[c]	0	—		—		—	
	12	11.51		11.23		8.09	
	24	19.27		18.20		13.97	
	48	46.00		38.36		52.04	
	216	86.29	0.9225	89.22	0.9415	89.01	0.9022

[a] Coefficient of correlation (Pearson's) calculated for plot of r values *versus* concentration of sulfonylurea. [b] A total of 11.88 $\times 10^{-5}$ mole present in system in all experiments involving coumarin derivatives. [c] A total of 12 $\times 10^{-5}$ mole present in system in all experiments involving coumarin derivatives.

Table II—Characteristics of Inhibition of Albumin Binding of Sulfonylureas by Phenprocoumon

Sulfonylurea	Concentration of Phenprocoumon ($\times 10^{-5}$ mole)	Percent Reduction of r at pH: 6.5	Coefficient of Correlation	7.4	Coefficient of Correlation	8.4	Coefficient of Correlation
Acetohexamide	0	—		—		—	
	12	5.31		1.97		−0.09	
	24	6.73		7.60		8.87	
	48	15.29		16.65		14.24	
	216	48.53	0.9928	44.71	0.9918	51.86	0.9903
Chlorpropamide	0	—		—		—	
	12	6.15		7.61		2.45	
	24	8.74		27.74		16.41	
	48	32.36		40.42		54.08	
	216	82.69	0.9612	74.77	0.9372	78.51	0.9343
Tolbutamide	0	—		—		—	
	12	3.91		1.36		12.69	
	24	7.91		4.56		23.70	
	48	15.74		13.81		34.99	
	216	61.96	0.9912	66.33	0.9928	61.26	0.9638

Within this context, it seemed valuable to study: (*a*) the ability of several coumarin derivatives to displace the three major sulfonylurea compounds in clinical use from human serum albumin and (*b*) the ability of acetohexamide to displace several cortical steroids from human serum proteins.

EXPERIMENTAL[1]

Stock solutions of the coumarin derivatives were made in pH 8.4 tromethamine buffer (0.067 *M*) except diphenadione which was dissolved in pH 9.5 tromethamine buffer. The stock solutions of the cortical steroids were made with absolute ethanol as the solvent. Binding and displacement of bound drugs were studied utilizing equilibrium dialysis as described previously (10). The specific activities of the sulfonylurea derivatives and concentrations of stock solutions were also as previously described. The specific activities of the cortical steroids used were as follows: corticosterone-1,2-^{3}H, 36 c./mmole; prednisone-T (G), 2.5 c./mmole; cortisone-1,2-^{3}H, 500 mc./mmole; prednisolone-^{3}H (G), 2.5 c./mmole; and tritium-labeled cortisol-1,2, 2.0 c./mmole. Serum proteins were dissolved in tromethamine buffer of appropriate pH and placed in one side of the dialysis cell. The compound, the binding of which was being studied, and the competitor, if any, were placed in the other side of the cell. A total of 1.0 ml. was contained in each side of the cell. Other details of the procedure were identical with those previously published (10).

The amounts of proteins in the systems were as follows. Albumin, either as the sole protein or together with α-globulin fraction IV-4, was present in the amount of 5.797 $\times 10^{-7}$ mole. α-Globulin fraction IV-4 as the sole protein was present in the amount of 5.714 $\times 10^{-8}$ mole (assuming an average molecular weight of 70,000); when in combination with albumin, 7.15 $\times 10^{-8}$ mole of fraction IV-4 was present.

Although data relevant to possible clinical implications of com-

[1] Acetohexamide-^{14}C was a gift of Eli Lilly and Co., Indianapolis, Ind. Tolbutamide-^{35}S, chlorpropamide-^{35}S, and the tritium-labeled cortical steroids were obtained from Amersham/Searle. Diphenadione was obtained from The Upjohn Co. Ethyl biscoumacetate and acenocoumarol were gifts from Geigy Pharmaceuticals, phenprocoumon was a gift from Organon Inc., and anisindione was a gift from Schering Corp. The liquid scintillation phosphor solution used in the determination of radioactivity was Aquasol, supplied by New England Nuclear Corp. All serum proteins were obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

Table III—Characteristics of Inhibition of Albumin Binding of Sulfonylureas by Anisindione

Sulfonylurea	Concentration of Anisindione ($\times 10^{-6}$ mole)	Percent Reduction of r at pH: 6.5	Coefficient of Correlation	7.4	Coefficient of Correlation	8.4	Coefficient of Correlation
Acetohexamide	0	—		—		—	
	12	10.23		6.99		6.29	
	24	18.89		11.94		10.42	
	48	38.03		36.79		39.81	
	216	77.32	0.9502	75.62	0.9571	80.66	0.9468
Chlorpropamide	0	—		—		—	
	12	22.24		10.85		13.22	
	24	22.78		16.78		24.79	
	48	53.44		38.79		51.62	
	216	87.72	0.8982	80.60	0.9475	78.06	0.9288
Tolbutamide	0	—		—		—	
	12	10.77		7.22		5.00	
	24	21.42		16.45		14.49	
	48	41.41		39.64		45.41	
	216	83.87	0.9297	79.79	0.9458	88.59	0.9519

Table IV—Characteristics of Inhibition of Albumin Binding of Sulfonylureas by Acenocoumarol

Sulfonylurea	Concentration of Acenocoumarol ($\times 10^{-6}$ mole)	Percent Reduction of r at pH: 7.4	Coefficient of Correlation	8.4	Coefficient of Correlation
Acetohexamide	0	—		—	
	12	8.24		9.50	
	24	4.74		8.08	
	48	20.88		32.03	
	216	49.37	0.9950	70.92	0.9683
Chlorpropamide	0	—		—	
	12	13.39		25.03	
	24	19.49		29.62	
	48	30.62		67.47	
	216	71.38	0.9634	88.10	0.8749
Tolbutamide	0	—		—	
	12	3.05		−2.41	
	24	11.03		4.58	
	48	31.07		25.87	
	216	71.78	0.9674	76.72	0.9713

Table V—Characteristics of Inhibition of Albumin Binding of Sulfonylureas by Diphenadione at pH 8.4

Sulfonylurea	Concentration of Diphenadione ($\times 10^{-6}$ mole)	Percent Reduction of r	Coefficient of Correlation
Acetohexamide	0	—	
	12	9.43	
	24	22.40	
	48	60.89	
	216	97.93	0.8385
Chlorpropamide	0	—	
	12	19.79	
	24	34.83	
	48	76.13	
	216	97.63	0.7659
Tolbutamide	0	—	
	12	7.94	
	24	27.74	
	48	63.84	
	216	91.33	0.8577

petitive protein binding could be obtained at pH 7.4, determinations were made also at pH 6.5 and 8.4. One objective of accumulating data at two pH values outside of the physiological range was to determine from a more basic viewpoint whether pH affects the binding process and competition between bound molecules. Furthermore, in the case of several compounds with solubilities limited to certain pH ranges, it was necessary to work at a nonphysiological pH. This was true, for example, with diphenadione which was insoluble at pH values below 8.4.

RESULTS AND DISCUSSION

Over the range of concentrations of the various coumarin compounds used, substantial reduction in binding of the three sulfonylureas resulted (Tables I–V). Diphenadione was studied at pH 8.4 only because of solubility problems at values below 8.4. For the same reason, acenocoumarol could not be tested at pH 6.5. The other derivatives were examined at the three pH values indicated, and the final concentrations were adjusted to be comparable to that found in plasma when dicumarol is used in therapy (12–17). Although the abilities of the various coumarin compounds to displace sulfonylureas from human serum albumin varied with the type of coumarin compound as well as the pH, there appeared to be no discernible clearcut pattern in terms of the effect of pH on displacement.

Table VI—Comparison of Highest Percent Reduction in r Values of Sulfonylureas Caused by Coumarin Derivatives

Coumarin Derivative in System	Acetohexamide pH			Chlorpropamide pH			Tolbutamide pH		
	6.5	7.4	8.4	6.5	7.4	8.4	6.5	7.4	8.4
Ethyl biscoumacetate	80.11	77.93	72.95	91.32	88.10	93.10	86.29	89.22	89.01
Phenprocoumon	48.53	44.71	51.86	82.69	74.77	78.51	61.96	66.33	61.26
Anisindione	77.32	75.62	80.66	87.72	80.60	78.06	83.87	79.79	88.59
Acenocoumarol	—	49.37	70.92	—	71.38	88.10	—	71.78	76.72
Diphenadione	—	—	97.93	—	—	97.63	—	—	91.33

Table VII—Potency of Coumarin Derivatives to Displace Sulfonylureas from Human Serum Albumin

Sulfonylurea	pH	Decreasing Reduction in r Values of Sulfonylureas
Acetohexamide	6.5	Ethyl biscoumacetate > anisindione > phenprocoumon
	7.4	Ethyl biscoumacetate > anisindione > acenocoumarol > phenprocoumon
	8.4	Diphenadione > anisindione > ethyl biscoumacetate > acenocoumarol > phenprocoumon
Chlorpropamide	6.5	Ethyl biscoumacetate > anisindione > phenprocoumon
	7.4	Ethyl biscoumacetate > anisindione > phenprocoumon > acenocoumarol
	8.4	Diphenadione > ethyl biscoumacetate > acenocoumarol > phenprocoumon > anisindione
Tolbutamide	6.5	Ethyl biscoumacetate > anisindione > phenprocoumon
	7.4	Ethyl biscoumacetate > anisindione > acenocoumarol > phenprocoumon
	8.4	Diphenadione > ethyl biscoumacetate > anisindione > acenocoumarol > phenprocoumon

Table VIII—Characteristics of Inhibition of Serum Protein (Albumin) Binding of Steroids by Acetohexamide

Steroid[a]	Concentration of Acetohexamide ($\times 10^{-3}$ mole)	r ($\times 10^{-4}$)	Percent Reduction of r	Coefficient of Correlation
Prednisone	0	5.11	—	
	9.88	5.08	0.59	
	19.76	4.69	8.22	
	39.52	4.24	17.03	
	197.6	3.35	34.44	0.9423
Prednisolone	0	4.47	—	
	9.88	4.48	0	
	19.76	3.81	14.77	
	39.52	3.51	21.48	
	197.6	3.02	32.44	0.8257
Cortisone	0	6.45	—	
	9.88	5.72	11.32	
	19.76	6.19	4.03	
	39.52	5.00	22.48	
	197.6	4.01	37.83	0.9018
Cortisol	0	5.13	—	
	9.88	4.07	20.66	
	19.76	4.71	8.19	
	39.52	4.43	13.65	
	197.6	2.58	49.71	0.9364
Corticosterone	0	9.41	—	
	9.88	9.49	0	
	19.76	8.98	4.57	
	39.52	8.37	11.05	
	197.6	7.19	23.59	0.9421

[a] Total steroid concentration was 10^{-9} mole in the experimental system throughout.

Table IX—Characteristics of Inhibition of Serum Protein (α-Globulin Fraction IV-4) Binding of Steroids by Acetohexamide

Steroid	Concentration of Acetohexamide ($\times 10^{-3}$ mole)	r	Percent Reduction of r	Coefficient of Correlation
Prednisone	0	1.02×10^{-3}	—	
	9.88	1.35×10^{-3}	0	
	19.76	1.20×10^{-3}	0	
	39.52	4.73×10^{-4}	53.63	
	197.6	1.28×10^{-4}	87.45	0.8372
Prednisolone	0	9.34×10^{-4}	—	
	9.88	9.49×10^{-4}	0	
	19.76	9.84×10^{-4}	0	
	39.52	7.15×10^{-4}	23.45	
	197.6	4.01×10^{-4}	57.07	0.9429
Cortisone	0	1.45×10^{-3}	—	
	9.88	1.31×10^{-3}	9.66	
	19.76	1.26×10^{-3}	13.10	
	39.52	8.91×10^{-4}	38.55	
	197.6	3.17×10^{-4}	78.14	0.9597
Cortisol	0	1.64×10^{-3}	—	
	9.88	1.54×10^{-3}	6.10	
	19.76	1.28×10^{-3}	21.95	
	39.52	1.15×10^{-3}	29.88	
	197.8	3.77×10^{-4}	77.01	0.9788
Corticosterone	0	3.21×10^{-3}	—	
	9.88	3.56×10^{-3}	0	
	19.76	3.12×10^{-3}	2.80	
	39.52	2.54×10^{-3}	20.87	
	197.6	1.50×10^{-3}	53.27	0.9397

As estimated by percent reduction in r value[1], phenprocoumon showed less displacement of acetohexamide at all three pH values and acenocoumarol showed similar lower displacement of acetohexamide at pH 7.4. The other coumarin derivatives produced a 70–80% reduction of r values in all other instances. A comparison of the percent reduction in r values of the sulfonylureas is tabulated in Table VI. In Table VII, the coumarin derivatives are ranked in decreasing order of reduction in r values obtained. At the two lower

[1] The r value refers to moles of steroid bound per mole of protein at the concentrations of steroid and protein present in the system.

Table X—Characteristics of Inhibition of Serum Protein (Albumin + α-Globulin Fraction IV-4) Binding of Steroids by Acetohexamide

Steroid	Concentration of Acetohexamide ($\times 10^{-3}$ mole)	r ($\times 10^{-4}$)	Percent Reduction of r	Coefficient of Correlation
Prednisone	0	5.03	—	
	9.88	5.06	0	
	19.76	4.66	7.36	
	39.52	4.52	10.14	
	197.6	3.06	39.17	0.9884
Prednisolone	0	3.91	—	
	9.88	4.22	0	
	19.76	4.03	0	
	39.52	3.32	15.09	
	197.6	2.42	39.11	0.9373
Cortisone	0	5.08	—	
	9.88	5.19	0	
	19.76	5.36	0	
	39.52	4.90	3.54	
	197.6	3.04	40.16	0.9933
Cortisol	0	4.73	—	
	9.88	4.81	0	
	19.76	4.23	10.57	
	39.52	4.53	4.23	
	197.6	2.92	38.27	0.9583
Corticosterone	0	9.20	—	
	9.88	8.72	5.22	
	19.76	8.53	7.28	
	39.52	8.43	8.37	
	197.6	6.61	28.15	0.9938

pH values studied, ethyl biscoumacetate was the most potent derivative in terms of ability to displace sulfonylureas from human serum albumin; but at the higher pH value (8.4), diphenadione was the most potent displacing agent with phenprocoumon the least potent except in the case of chlorpropamide. At physiological pH (7.4), ethyl biscoumacetate would appear to be the coumarin derivative most likely to potentiate the action of a sulfonylurea compound. These results clearly indicate that the coumarin derivatives studied do displace sulfonylureas from human serum albumin, and this could easily be the mechanism by which coumarin derivatives could cause hypoglycemia when administered concurrently with sulfonylureas. A clinical report of this interaction was published by Kristensen and Hansen (3), involving potentiation of the effects of tolbutamide by dicumarol.

Cortical steroids have been shown to bind primarily to a specific blood protein, with corticosteroid binding globulin (18) or transcortin until the latter is saturated and then the excess steroid is is bound to albumin. Albumin is known to bind a variety of compounds, and the binding of cortical steroids to albumin is not specific as it is to corticosteroid binding globulin. Since both of these blood proteins bind cortical steroids, they were both included in the study. Cohn fraction IV-4 α-globulin contains the cortical binding globulin (18). The results obtained are tabulated in Tables VIII–X. The r values are rather low, but these are not to be considered as true r values. For an estimation of true r values, it would be necessary to study a variety of concentrations of the steroids up to saturation of the protein; in these studies, the concentrations of steroids were adjusted to that normally found in plasma when cortical steroids are used in therapy (19–25).

Only one sulfonylurea compound, acetohexamide, was chosen for this study as a representative of the group. Acetohexamide, as well as chlorpropamide and tolbutamide, was added in concentrations typical of blood levels attained in therapy (26–29). Acetohexamide caused a greater reduction in the r value when α-globulin fraction IV-4 was the protein studied than in the case of albumin or a combination of albumin and fraction IV-4. The greater displacement from fraction IV-4 is more significant clinically probably in the sense that binding of cortical steroids would be greatest to the α-globulin fraction with normal plasma steroid concentrations. Acetohexamide caused the greatest reduction in binding to human serum albumin in the case of cortisol and the least with corticosterone. With human serum α-globulin fraction IV-4, the greatest reduction in binding was seen with prednisone and least with corticosterone. When both proteins were present in the system, the binding of cortisone was the most reduced and the binding of corticosterone was the least reduced. On the basis of the data in Tables VIII–X, specifically the percent reduction in r values, it would seem that with human serum albumin as the protein in the system, the several steroids showed a decreasing percent reduction in r value in the presence of acetohexamide as follows: cortisol, cortisone, prednisone, prednisolone, and corticosterone. Apparently, one cannot consider the r value obtained in the absence of acetohexamide as an indication of binding capacity, because one does not observe an inverse relationship between binding capacity and percent reduction in r value in the presence of a displacing agent.

Table XI indicates r values in the absence of acetohexamide and this inverse relationship does not seem to hold except in the case of corticosterone. This was equally true when human serum α-globulin fraction IV-4 was the protein in the system, once again with the exception of corticosterone. The lack of an inverse relationship between binding capacity and ease of displacement by acetohexamide could very well be due to the lack of a proper index of binding capacity. The r values in Table XI do not represent true r values in the sense that saturation of the protein was not nearly achieved with the very low concentrations of steroids employed. It probably would be more realistic to use such values as n (number of binding sites) or k_a (binding constant). In future work, it is planned to make a comparison utilizing the latter indexes of binding capacity and binding strength.

For the data in Tables I–V and VIII–X, coefficients of correlation were calculated for each set of concentrations of binding inhibitors and resulting r values. In almost all cases, the coefficients of correlation were significant to a level of confidence of 90%, although a somewhat lower level of confidence is indicated in a very few cases. The statistical values suggest that there is a reasonable quantitative relationship between concentrations of the inhibitors and amounts of organic molecule bound.

These results indicate that acetohexamide is capable of displacing cortical steroids from plasma proteins and potentially could result in the equivalent of an overdosage of steroid if sulfonylureas and cortical steroids are administered concurrently. It is interesting that there are apparently no reports in the clinical literature of this sort of drug interaction and perhaps it is of no clinical consequence. It would be interesting to note whether diabetics with rheumatic or arthritic conditions experience an improvement in the latter conditions upon introduction of sulfonylurea therapy. If such cases do exist, they may be examples of a beneficial drug interaction.

Table XI—Comparison of Steroids in Terms of Moles Bound per Mole of Human Serum Albumin and α-Globulin Fraction IV-4

Protein	Steroid[a] and r Value[b]				
	Prednisone	Prednisolone	Cortisone	Cortisol	Corticosterone
Human serum albumin[c]	5.11×10^{-4}	4.47×10^{-4}	6.45×10^{-4}	5.13×10^{-4}	9.41×10^{-4}
Human serum α-globulin fraction IV-4[d]	1.02×10^{-3}	9.34×10^{-4}	1.45×10^{-3}	1.64×10^{-3}	3.21×10^{-3}

[a] The amount of steroid in the system in all cases was 10^{-3} mole, and thus r does not represent a true r value indicating total binding capacity of the protein. [b] The r value refers to moles of steroid bound per mole of protein at the concentrations of steroid and protein present in the system. [c] Steroids ranked in order of decreasing r values: corticosterone > cortisone > cortisol > prednisone > prednisolone. [d] Steroids ranked in order of decreasing r values: corticosterone > cortisol > cortisone > prednisone > prednisolone.

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KENNETH WHITE*, J. V. BRUCKNER†, and W. L. GUESS▲

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Keyphrases □ 2-Mercaptoethanol—toxicity profile □ Toxicity profie—2-mercaptoethanol □ Sterilization residues, toxic—profile of 2-mercaptoethanol as potential reaction product between ethylene oxide and sulfides □ Ethylene oxide sterilization—toxicity profile of 2-mercaptoethanol as potential residue

The contemporary procedure of sterilization by ethylene oxide of rubber and plastic medical devices has provoked a number of pertinent questions as to potential health hazards such devices might propound. Bronsted *et al.* (1) demonstrated that ethylene oxide will react with a wide variety of nucleophilic agents, thus introducing the likelihood of production of reaction products from chemical contaminants in the sterilized articles. One such reaction product, the extremely toxic chlorohydrin 2-chloroethanol, was investigated by Guess (2). Guess and O'Leary (3) found another reaction product of ethylene oxide sterilization, 2-(2-hydroxyethylmercapto)benzothiazole, to be quite toxic to cells in culture and to mice.

A number of rubber devices, including tracheotomy tubes, in-dwelling catheters, multidose vial stoppers, bottle cap liners, and gloves are commonly sterilized with ethylene oxide. During the vulcanization of rubber with sulfur, it is likely that sulfides, including hydrogen sulfide, are formed. The formation of 2-mercaptoethanol by the mechanism proposed by Bronsted *et al.* (1) is then possible. The aforementioned medical materials come into both direct and indirect contact with various body tissues, including mucous membranes, epithelial and endothelial cells, muscle, and ocular tissues. Therefore, if these devices are to be used with safety, the toxic potential of any possible contaminants must be determined.

A search of the literature revealed a paucity of information on the systemic and specific tissue toxicity of 2-mercaptoethanol. Finley and Carlson (4) speculated that the LD_{50} by intraperitoneal injection in mice was approximately 195 mg./kg. A patent authored by Utrumi

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Keyphrases □ 2-Mercaptoethanol—toxicity profile □ Toxicity profie—2-mercaptoethanol □ Sterilization residues, toxic—profile of 2-mercaptoethanol as potential reaction product between ethylene oxide and sulfides □ Ethylene oxide sterilization—toxicity profile of 2-mercaptoethanol as potential residue

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A search of the literature revealed a paucity of information on the systemic and specific tissue toxicity of 2-mercaptoethanol. Finley and Carlson (4) speculated that the LD_{50} by intraperitoneal injection in mice was approximately 195 mg./kg. A patent authored by Utrumi

Table I—LD_{50} Values for 2-Mercaptoethanol in Mice

Route	LD_{50}, mg./kg.	95% Confidence Interval
Intraperitoneal	322.0	296.5–350.9
Oral	344.8	317.7–374.3

et al. (5) included an LD_{50} value of 0.48 mg./g. on intravenous administration to mice. Smyth and Carpenter (6) investigated the toxicity of 2-mercaptoethanol, although the studies were intended only as rapid, range-finding assessments of toxicity potential. These investigators determined the oral range-finding LD_{50} for rats to be 300 mg./kg., the topical range-finding LD_{50} for guinea pigs to be 0.3 ml./kg., a rabbit epidermal irritation similar to morpholine, and a rabbit corneal toxicity equivalent to butyl alcohol.

2-Mercaptoethanol has enjoyed widespread academic interest in the biological and biochemical fields. The presence of a sulfhydryl group in the molecule portends a number of potential *in vivo* reactivities. According to Coombs *et al.* (7), 2-mercaptoethanol may inhibit zinc carboxypeptidase activity *via* complexation with zinc ions vital to enzymatic functionality. Erdös and Wohler (8) postulated a potentiation of action of bradykinin and kallidin by such a complexation mechanism, with resultant inhibition of the specific catabolizing carboxypeptidase. A number of investigators (9–11) reported the activation of enzymes *via* reduction of enzymatic sulfhydryl groups by 2-mercaptoethanol. However, as suggested by Edman *et al.* (9), higher concentrations of 2-mercaptoethanol may inhibit these same enzymes by disruption of their tertiary protein structure.

In view of the lack of concrete data concerning animal toxicity of 2-mercaptoethanol, an evaluation of various toxicity parameters was carried out. When pharmacological effects were observed, efforts were made to assess the cause through tests designed to block specific actions or symptoms. These various tests were intended to elucidate the toxicity profile of the compound and to determine its mechanism of toxic action.

MATERIALS AND METHODS

Purity Determination—2-Mercaptoethanol, with a boiling range of 69.5–70.5° (157–159°F.), was obtained from a commercial source[1]. Purity of the compound was ascertained by IR analysis and GC. The pH of aqueous solutions of the chemical was monitored with a pH meter[2]. All dilutions were prepared with physiological saline immediately prior to administration.

Animal Care—Rabbits (New Zealand albino males) and mice (Swiss-Webster albino males, 20–25 g.) were the animals of choice for this series of experiments. All subjects were maintained under constant environmental conditions, with laboratory chow[3] and water available *ad libitum*, except as noted in subsequent sections of this paper.

LD_{50} Determination—Both oral and intraperitoneal LD_{50} values for mice were determined by use of the Cornfield–Mantel (12) modification of the Karber method. Food was withheld from the oral intubation group for approximately 24 hr. before testing. The mice were divided into groups of 10 per dosage level, each animal receiving the specified dose in a constant volume of 0.5 ml./20 g. body weight. All subjects were observed closely during the post-injection period of 24 hr. for toxic symptoms and for 14 days for fatalities. Survivors were also observed periodically during this time for delayed or progressive toxic manifestations.

Subacute Studies—To study the effects of repeated exposure to sublethal doses of 2-mercaptoethanol, three groups of 15 mice each were administered the chemical intraperitoneally daily for 5 consecutive days in each of 4 weeks. The first group received one-half the LD_{50} dose, the second one-fourth the LD_{50} dose, and the third normal saline as a control. A daily record was kept of toxic symptoms and body weights. Several subjects from each group exhibiting extreme toxic manifestations were sacrificed by cervical fracture, and the kidneys, spleen, liver, lungs, heart, adrenals, pancreas, stomach, intestine, and brain of each were removed and weighed to yield organ–body weight ratios. Specimens of each organ were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathological examination.

Primary Skin Irritation—The method of Draize *et al.* (13), with certain modifications by Guess (2), was employed to evaluate irritation by 2-mercaptoethanol upon topical application. Both abraded and unabraded sites of exposure on the shaved rabbit back were scored for extent of erythema and edema at 24 and 72 hr. after exposure.

Intradermal Irritation—Evaluation of the intradermal irritation potential of 2-mercaptoethanol was accomplished by the technique utilized by Guess (2). The test solution (0.2 ml.) was injected intracutaneously into two sites on the shaved backs of two rabbits. Ethanol (20%) was used as a positive control, while normal saline served as a negative control. Toxic reactions were graded after 1 hr. and at 24-hr. intervals thereafter on the basis of a system ranging from a value of +4 for extreme irritation with necrosis to a value of 0 for no reaction.

Ocular Irritation—Ocular irritation was evaluated according to the modification of the Draize technique published in the Federal Register (14), utilizing the regulations and color charts included therein. The irritation scores of six subjects per concentration level were averaged to yield a primary irritation number. Evaluations of reaction severity were performed 1, 24, 48, and 72 hr. following exposure to the test solution.

Mucosal Irritation—The irritation potential of 2-mercaptoethanol to penile mucosa was evaluated by the method outlined by Guess (2), using penile tissue of anesthetized rabbits. The test solution (0.2 ml.) was instilled into a well formed with peripenile tissue and was allowed to remain in contact with the mucosal tissue for 1 min. Adhering solution was not removed upon release of the well.

RESULTS AND DISCUSSION

The results of the LD_{50} determination by oral and intraperitoneal routes are given in Table I. Previous determinations of LD_{50} values by other workers resulted in the publication of varying estimates. Smyth and Carpenter (6), as previously stated, found the oral (range-finding) LD_{50} for rats to be 0.3 g./kg. However, no mention was made of whether or not the test animals were fasted prior to treatment. Finley and Carlson (4), estimating the LD_{50} by intraperitoneal injection in mice to be approximately 195 mg./kg., used a peanut oil vehicle with a standard injection volume of 0.2–0.3 ml. The disparity in procedures of intraperitoneal administration between the present investigation and that conducted by Finley and Carlson (4) may account for the difference in reported LD_{50} values. The technique utilized for the present study dictated a standard injection volume of 0.5 ml./20 g. body weight. It was felt that this larger injection volume would minimize errors in accurately measuring amounts of active principle to be administered. Also, the influence a larger volume should have in increasing the effective area of drug absorption must be considered. One would expect that such an enlarged area would enhance both time of onset and degree of toxic manifestations. Normal saline was selected instead of an oil to rule out inhibition of systemic absorption of toxicant by the vehicle.

Both routes of administration yielded essentially the same LD_{50} values, but the nature and onset of symptomatology varied with the dosage administered. Doses of 480 mg./kg. and greater killed within 1–2 hr. Within 30 min. of such treatment, the subjects exhibited intermittent tremors and difficulty in coordinated muscular

[1] J. T. Baker Chemical Co.
[2] Corning.
[3] Purina.

Table II—Average Scores for Primary Irritation in the Rabbit

Compound	Dilution	Hour[b]	Rabbit Skin[a] Unabraded Erythema	Unabraded Edema	Abraded Erythema	Abraded Edema
2-Mercaptoethanol	Undiluted	24	3.3	2.0	3.3	1.0
		72	0.3	0.0	3.3	0.7
	1:5	24	1.0	0.3	2.7	1.0
		72	0.3	0.0	1.0	0.0
	1:10	24	1.0	0.0	1.7	0.3
		72	0.7	0.0	1.0	0.0
Alcohol USP	1:5	24	0.0	0.0	1.0	0.3
		72	0.0	0.0	0.0	0.0
Saline control	—	24	0.0	0.0	0.3	0.0
		72	0.0	0.0	0.0	0.0

[a] Score (each score represents an average of six animals): 1, barely perceptible erythema or edema; 2, well-defined erythema or edema; 3, moderate erythema or edema; and 4, severe (beet red) erythema or edema. [b] The time after treatment at which the evaluation was made.

activities. Severe clonic convulsions of long duration, accompanied by salivation and urination, were experienced by all subjects immediately preceding death. Death apparently resulted from convulsive seizures with subsequent respiratory failure. Histopathological examination of specimens of brain, lung, heart, liver, kidney, spleen, adrenals, pancreas, intestine, and stomach from these mice revealed no degenerative alterations.

With doses of less than 480 mg./kg., death was not so rapid. Rather, the mice slowly entered a state of deepening depression, terminating in coma and death in 1–3 days. During this progressive state, there were few voluntary movements, respiration was slowed and deepened, and response to externally applied pain stimuli was minimal. Histopathological studies of organs removed 24 hr. after dosing mice intraperitoneally with 322 mg./kg. revealed minimal changes in the liver and kidneys, with no alteration of other tissues. Slight vacuolation of scattered hepatocytes and deposition of eosinophilic, amorphous material in some renal tubular lumens was observed. Specimens excised after 48 hr. from mice receiving 322 mg./kg. i.p. exhibited similar toxic manifestations, although vacuolation of hepatocytes was slightly more pronounced. An additional finding was a marked increase in hyperchromacity and mitotic activity of hepatocyte nuclei.

Judging from the rapid onset and the nature of toxic responses elicited by higher doses of 2-mercaptoethanol, there appeared to be important involvement of the CNS. Since salivation and urination were commonly observed at the higher dosage levels, investigation of a possible cholinergic reaction mediated by the autonomic nervous system was initiated. Three groups of 10 mice each were injected intraperitoneally with 0.5 mg./kg. of atropine sulfate. Thirty minutes later, the first group received 500 mg./kg. of 2-mercaptoethanol intraperitoneally, the second 322 mg./kg. of 2-mercaptoethanol intraperitoneally, and the third 500 mg./kg. of sodium chloride intraperitoneally. No alteration in toxicity was evident in the first group, because each mouse exhibited extreme CNS stimulation with salivation, followed by convulsions terminating in death within 1.5 hr. Six mice in the second group died within 24 hr., while all animals in the third group lived and remained symptom free. These results suggested that some toxic mechanism other than a cholinergic action be suspect.

The possibility of mediation of action *via* the cervical sympathetic ganglion was investigated. The mice were given 20 mg./kg. of sodium pentobarbital intraperitoneally, 15 min. prior to administration of 500 mg./kg. of 2-mercaptoethanol by the same route. A second group received 500 mg./kg. of 2-mercaptoethanol but no pentobarbital, while the third group received only 20 mg./kg. pentobarbital. All subjects receiving either pentobarbital-mercaptoethanol or pentobarbital alone exhibited a decreased level of activity, with some members appearing markedly depressed. No subject in either of these groups experienced enhanced salivation and urination or convulsions or died within 12 hr. However, seven of the mice receiving pentobarbital-mercaptoethanol died within 24 hr. In contrast, mice receiving only 2-mercaptoethanol experienced the typical CNS stimulatory effects, each member dying within 2 hr.

Since several investigators (7, 8) proposed that 2-mercaptoethanol complexes with metal ions critical to various enzyme systems, an attempt was made to block toxic effects mediated by such a mechanism. Twenty mice were each administered intraperitoneally the following dose of salts in 0.5 ml. distilled water: 4.0 mg. sodium chloride, 0.1 mg. potassium chloride, 0.1 mg. calcium chloride, 0.1 mg. zinc chloride, 0.05 mg. magnesium chloride, and 0.1 mg. ferrous sulfate. Thirty minutes later, 10 of the mice were administered 322 mg./kg. of 2-mercaptoethanol intraperitoneally. A third group of 10 mice received only 322 mg./kg. of 2-mercaptoethanol intraperitoneally. All subjects were then observed for 24 hr. No mice receiving only the solution of metal ions exhibited toxic symptoms or died. However, members of each group treated with 2-mercaptoethanol showed marked depression within an hour. Six subjects pretreated with metal ions died, while five animals in the group given only 2-mercaptoethanol died. These results suggest that the toxic effects of an LD_{50} dose of 2-mercaptoethanol in mice are not dependent upon complexation of sodium, potassium, calcium, zinc, magnesium, or iron ions.

The likelihood of metabolism of 2-mercaptoethanol to toxic metabolites must be considered. From a study of the metabolism of 2-chloroethanol, an analogous compound to 2-mercaptoethanol, Johnson (15) suggested that the toxicity of 2-chloroethanol was due to oxidation to chloroacetaldehyde in target organs. Peterson *et al.* (16) reported that ethanol exerted a protective action against the lethal effects of 2-chloroethanol and 2-fluoroethanol in rats and monkeys. The LD_{50} of 2-chloroethanol in unprotected rats was found to be four times less than that for a group dosed previously with ethanol. These investigators concluded that, at present, there is no reason to propose the existence of more than one alcohol dehydrogenase in liver.

Based on the results of such studies, it was decided to investigate the protection potential of ethanol in 2-mercaptoethanol poisoning. One group of 10 mice was given 322 mg./kg. of 2-mercaptoethanol intraperitoneally. Ten other mice were pretreated with 500 mg./kg. of ethanol, 15 min. before administration of the 322 mg./kg. of 2-mercaptoethanol. Animals exhibiting excessive depression in the latter group were given a subsequent intraperitoneal injection of 500 mg./kg. of ethanol during the next 12 hr. Twenty-four hours after dosing with 2-mercaptoethanol, six mice had died in the unprotected group while all but one animal given ethanol-mercapto-

Table III—Intradermal Irritation in the Rabbit[a]

Chemical	Dilution	1 hr.	24 hr.	48 hr.	72 hr.
2-Mercapto-ethanol	Undiluted	4[b]	4[b]	4[b]	4[b]
	1:5	4	4[b]	4[b]	4[b]
	1:10	4	4[b]	4[b]	4[b]
	1:50	4	4[b]	3.7[c]	3.7[c]
	1:100	0	2	1	0
	1:200	0	0	0	0
Alcohol USP	Undiluted	4	4[b]	4[b]	4[b]
	1:5	0	4	4	4[b]
	1:10	0	2	1	0
Saline control	—	0	0	0	0

[a] Score (each score represents an average of two injection sites in two rabbits): 0, no visible reaction; 1, barely perceptible erythema; 2, well-defined erythema; 3, moderate erythema; and 4, severe (beet red) erythema with a blanched center. [b] Necrosis at the site of injection. [c] Necrosis at the site of injection in only one animal.

Table IV—Average Scores for Ocular Irritation in the Rabbit[a]

Compound	Dilution	Hours after Administration	Cornea	Iris	Conjunctiva	
					Chemosis	Redness
2-Mercapto-ethanol	Undiluted	1	2	3	3	3
		24	1	2	3	3
		48	1	2	3	3
		72	1	0	0	1
	1:5	1	0	1	0.4	0.6
		24	0	0.4	0	0.4
		48	0	0	0	0.2
		72	0	0	0	0
Alcohol USP[b]	Undiluted		—	—	—	—
		24	0	1	1.5	1.1
		48	0	0.6	1.5	0.6
		72	0	0.5	0.8	0.6
	1:5	1	—	—	—	—
		24	0	0	0	0
		48	0	0	0	0
		72	0	0	0	0
Saline control		1	0	0	0	0
		24	0	0	0	0

[a] Score (each score represents an average from six animals); scoring was graded according to photographs and description in the Federal Register (14). [b] Results previously reported by Guess (2) were used here since the series of compounds was evaluated at the same time.

ethanol survived. Such results imply that ethanol may competitively inhibit the oxidation of 2-mercaptoethanol to toxic metabolites.

The dependence of symptomatology upon dosage level lends support to the premise of a dual mechanism of toxicity of 2-mercaptoethanol. Toxic responses to higher concentrations of the compound are indicative of a strong CNS stimulation, possibly initiated by the irritating effect of the nonmetabolized alcohol. The onset of toxic reactions following injection of a high concentration of the toxicant was too rapid to allow for its significant metabolism. Doses of 2-mercaptoethanol near and below its LD_{50} value produced strikingly different symptomatology, characteristic of CNS depression. Such toxicity appeared cumulative, evidenced in almost all subjects by deepening depression with eventual coma. These phenomena suggest that as long as a critically high dose of 2-mercaptoethanol is not administered acutely, the characteristic CNS stimulation manifestations will not occur. Rather, the subject will steadily degrade the alcohol to toxic metabolites which elicit depression of bodily functions. This premise is supported by the finding that sodium pentobarbital blocked the rapidly fatal convulsive effects of 2-mercaptoethanol but not the delayed lethal actions.

Subacute toxicity studies, as outlined previously, were conducted to ascertain the effect of prolonged exposure to 2-mercaptoethanol. Seven animals died after 8 days of treatment in the group receiving one-half the LD_{50} dose, while only two subjects receiving one-fourth the LD_{50} dose died. All mice in each group, however, succumbed within the 4-week period. Only one death was recorded among the saline control animals. Symptomatology was similar to that noted during the low dose, acute experiments. The mice exhibited progressive weakness and depression with curtailed activity, decreased response to pain stimuli, and loss of body weight. Gross observation of several vital organs of these animals revealed no significant alterations. Histopathological examination of specimens of brain, heart, lung, liver, kidney, spleen, adrenals, pancreas, intestine, and stomach revealed only small, scattered foci of hepatic necrosis. Organ–body weight ratios of control and test animals showed no significant differences.

It can be seen from Table II that undiluted 2-mercaptoethanol was moderately irritating on topical application to both abraded and normal skin of rabbits. Although the irritation to normal skin was transient, that to abraded areas was quite long lasting. Furthermore, primary reactions of a similar magnitude to ethanol healed much more quickly than those to 2-mercaptoethanol. Dilutions of 1:5 and 1:10 of 2-mercaptoethanol produced a moderate, transitory irritation in abraded skin and only a very slight reaction in normal skin. Edema was of little significance at any concentration level below 100%. It is apparent from Table II that 2-mercaptoethanol is considerably more toxic when applied topically than is its congener, ethanol.

As may be noted in Table III, a very severe reaction resulted from intracutaneous injection of undiluted 2-mercaptoethanol. Within 5 min. following such treatment, it became evident that a marked necrotic reaction would ensue. Therefore, only one rabbit was administered this concentration. Injection of dilutions of the toxicant of 1:5, 1:10, and 1:50 also produced marked inflammation with subsequent necrosis. After 72 hr., the slightest tendency of recovery was present only with the 1:50 dilution. A concentration of 1% 2-mercaptoethanol produced a well-defined irritation only after 24 hr. of exposure. At 72 hr. postinjection, the reaction was essentially negative. All higher dilutions of 2-mercaptoethanol elicited no inflammation. No systemic toxic effects were observed from this route of administration, although the test animal given the pure compound received a total dose of approximately 800 mg./kg.

Only in undiluted form was 2-mercaptoethanol markedly toxic to ocular tissue and surrounding mucosa. Iritis, redness, and chemosis were tabulated in Table IV as severe, while corneal opacity was moderate in severity although prolonged in duration. Scars and opacity of the cornea were apparent 4 months after treatment of the single rabbit with the pure chemical. In addition to those toxic manifestations included in Table IV, enhanced ocular secretory activity and depilation were manifest. Two days after treatment with undiluted 2-mercaptoethanol, there was complete loss of hair in the periocular area.

Pure 2-mercaptoethanol was observed to be at least twice as potent an ocular toxicant as 95% ethanol. However, upon dilution, the toxic potential of each compound decreased drastically. A dilution of 1:5 of 2-mercaptoethanol was only slightly irritating, while the same concentration of ethanol elicited no inflammatory response. Further dilutions of 2-mercaptoethanol produced no observable injurious effects.

Reaction of penile mucosal tissue to 100% 2-mercaptoethanol was intense. Severe erythema, moderate edema, and eschar formation were manifest 24 hr. after exposure. Due to the application technique, some of the pure chemical contacted the scrotum and was hindered from evaporating normally. Necrotic lesions developed in these regions within 24 hr. As in the ocular mucosal study, a 1:5 dilution produced only transitory erythema and edema; lesser concentrations caused no apparent irritation. 2-Mercaptoethanol-induced injuries to penile mucosal and scrotal epithelium healed more promptly than analogous lesions in intradermal and ocular tissues. No manifestations of toxicity were visible 10 days following application of 100% 2-mercaptoethanol to penile and scrotal tissue.

2-Mercaptoethanol, when present in a high enough concentration, actively denatures cellular protein and solvates lipid. Such actions of alcohols have a profound effect on the structural and functional integrity of both tissues and cellular organelles (17, 18). Necrotic foci caused by 2-mercaptoethanol in intradermal and in ocular tissues were slow to heal. Complete repair, as ascertained by visual examination, generally took over 1 month. This phenomenon of delayed healing might be anticipated because several investiga-

tors reported (19–21) mitotic inhibition by 2-mercaptoethanol. 2-Mercaptoethanol is believed to exert this inhibitory effect by interference with the formation of intermolecular protein linkages involving sulfhydryl moieties, *via* either reduction of disulfide bonds and/or competition with protein —SH for sites at which intermolecular bonds are formed.

SUMMARY

This study delineates the toxicity profile of 2-mercaptoethanol in a variety of exposure situations. The major findings include:

1. LD_{50} values for mice by both oral and intraperitoneal routes of administration were determined and found to be 322.0 mg./kg. (i.p.) and 344.8 mg./kg. (oral).

2. 2-Mercaptoethanol appeared to exert its toxic effects *via* a dual mechanism, dependent upon dosage level. Higher doses produced severe toxic effects within minutes, characteristic of CNS stimulation. However, doses near and below the LD_{50} elicited marked depression, possibly resulting from metabolism to toxic metabolites.

3. Although atropine sulfate did not inhibit the CNS stimulatory effects of 2-mercaptoethanol, sodium pentobarbital was quite effective in this respect.

4. The systemic toxicity of 2-mercaptoethanol did not appear to result from complexation of potassium, calcium, zinc, magnesium, or iron ions.

5. Concurrent administration of ethanol and 2-mercaptoethanol significantly reduced the toxicity of the latter, lending credence to the concepts of metabolism of each *via* the same enzymatic pathway and to metabolism of 2-mercaptoethanol to toxic metabolites.

6. Exposure to low levels of 2-mercaptoethanol for 4 weeks produced progressive loss of body weight and deepening depression followed by death.

7. 2-Mercaptoethanol was only slightly irritating upon topical application to normal skin but elicited quite severe reactions on exposure to abraded and intracutaneous tissues.

8. Mucous membranes were extremely sensitive to the irritant effects of undiluted 2-mercaptoethanol, although a rapid decline in toxicity occurred upon dilution of the alcohol.

9. 2-Mercaptoethanol was, in every instance, more toxic to tissues than was ethanol.

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Salicylate Release Characteristics of Selected Polyethylene Glycol Suppositories

HAROLD W. PUFFER▲ and WILFRED J. CROWELL

Abstract □ Salicylate suppositories were prepared using polyethylene glycol 1540 and polyethylene glycol 6000 in various proportions. The *in vitro* release of salicylates from each suppository combination was studied using a closed system apparatus designed to circulate fluid about the suppository at 37 ± 0.2°. At scheduled time intervals, samples of the circulating fluid were assayed spectrophotometrically for salicylate content. Suppositories were also removed from the apparatus, their length and diameter were measured with a vernier caliper, and the change in their surface area was calculated. These surface area changes were then correlated with the salicylate release data. Release of salicylate from the polyethylene glycol suppositories simulated zero-order kinetics, and the time for 50% release of salicylate ($t_{1/2}$) from each polyethylene glycol combination did not differ significantly for salicylic acid or its sodium salt. Salicylate release differed markedly for bases composed solely of polyethylene glycol 1540 or 6000; however, when suppositories contained a mixture of the two polyethylene glycols, the rate of release of salicylate approximated that observed for a base composed solely of polyethylene glycol 6000.

Keyphrases □ Suppositories, polyethylene glycol—salicylate release characteristics □ Polyethylene glycol suppositories—salicylate release characteristics □ Salicylate release characteristics—polyethylene glycol suppositories □ Release characteristics, salicylate—polyethylene glycol suppositories

The polyethylene glycols are water-soluble polymers of ethylene glycol; they are widely used in pharmaceuticals as solvents, as tablet binders and coatings, as cosmetic bases, and as ointment and suppository vehicles. When used as suppository vehicles, they release incorporated medicament by dissolving in the rectal fluids. Several investigators compared the liquefaction (1) and disintegration times (2, 3) and the absorption of drugs from polyethylene glycol bases with other suppository bases such as theobroma oil and glycerinated gelatin (4–10). However, studies on the effects of varying polyethylene glycol base composition on release kinetics have not been reported. Therefore, the purpose of this study was to investigate the drug release characteristics of various polyethylene glycol suppository bases.

EXPERIMENTAL

Preparation of Suppositories—The suppositories were formulated to contain 60 mg. of salicylic acid or 70 mg. of sodium salicylate by melting the polyethylene glycols[1] at 64 ± 2° on a hot plate and then dissolving the salicylates[2] into the melt. The molten mass was poured into a polytetrafluoroethylene-coated (11) mold[3] and allowed to solidify at 23 ± 3°. The suppositories were removed from the mold and stored in well-closed containers at 8°. Prior to use, all suppositories were allowed to stand for 4–6 hr. at room temperature.

Five lots, of 20 suppositories each, were prepared for each formulation (Table I). Each suppository was cylindrical in shape, measured 17.8 mm. in length and 8 mm. in diameter, and weighed approximately 1 g.

All chemicals used were USP grade.

Dissolution Apparatus—The dissolution apparatus consisted of a flow chamber, constant flow pump, and solution reservoir. The flow chamber was constructed from a glass tube with an inside diameter of 2.2 cm. and a length of 7.5 cm. A stainless steel screen, resting on a fiber washer, was used to support the suppository inside the chamber. Each end of the chamber was fitted with a Büchner filter holder. The upper filter holder was modified to accommodate a calibrated thermometer having a range of from −2 to 68°, graduated in intervals of 0.2°. A vial filler[4] was used to circulate distilled water from a sampling reservoir and past the suppository in the flow chamber at a rate of 1 ml./sec.

The volume of the circulating fluid was kept constant at 500 ml. by adding an equal volume of water each time a sample was withdrawn. The fluid was maintained at 37 ± 0.2°, and evaporation from the system was prevented by placing a Mylar closure around the opening of the sampling flask.

Salicylate Release Determination—Suppositories from the various lots were chosen at random and placed individually in the flow chamber of the suppository dissolution apparatus. Half-milliliter samples of the circulating fluid were withdrawn at 100-sec. intervals and assayed for salicylate content. Reference solutions for the assay of the salicylate released were prepared by adding 0.5 ml. of dilute salicylate solution to 4.5 ml. of 0.1 *N* HCl. The absorbance of each reference solution was read at 304 nm. on a spectrophotometer[5].

Suppository Surface Area Determination—Suppositories were removed from the flow chamber of the dissolution apparatus, and the length and diameter of the suppositories were measured using a vernier caliper. The measurements obtained were then used to calculate the surface area, A, of each suppository using the equation:

$$A = \frac{\pi D^2}{2} + \pi DL \qquad \text{(Eq. 1)}$$

where π is 3.142, D is the diameter of the suppository, and L is the length of the suppository in millimeters.

RESULTS AND DISCUSSION

In general, the release of salicylates from the polyethylene glycol bases shows deviation from linearity for the first 6 mg. and for the last 12 mg. from each base (Figs. 1–4). The deviation from linearity for the last 12 mg. of drug released, that is, for the last 20% of the data, may be explained in terms of crystal formation by the polyethylene glycol matrix during cooling. Although the suppositories retained a cylindrical shape remarkably well, a core of harder material was observed during the latter stages of dissolution. The appearance of this slower dissolving core correlated approximately with the last 20% of the dissolution period. This harder core most likely arises from a slower cooling of the inner core producing more crystallinity to the mass than the quicker cooling of the outer areas of the cylinder and the resulting lower degree of crystallinity in the polyethylene glycol. The greater the degree of crystallinity, the slower is the dissolution. The degree of crystallinity of these polymers is known to be related to their thermal history, particularly the time element[6].

[1] Union Carbide Corp., New York, NY 10017
[2] Mallinckrodt Chemical Works, St. Louis, MO 63147
[3] The mold, constructed from aluminum plate and having a capacity of 30 cavities, 0.9 ml. each, was polytetrafluoroethylene coated by Tetrafluor, Division of Amerco Inc., Inglewood, CA 90301

[4] The model A B Filamatic vial filler, manufactured by National Instruments Co., Baltimore, Md., was equipped with a 5-ml. syringe.
[5] A Beckman model DUR recording quartz spectrophotometer equipped with a Gilford modification.
[6] The authors are grateful to the reviewer for suggesting crystal formation as an explanation for the slower dissolving core.

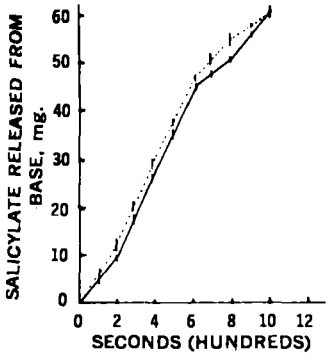

Figure 1—In vitro *release o salicylates from a suppository base consisting of polyethylene glycol 1540 (100%). Key: ——, salicylic acid, 60 mg., in base; and . . ., sodium salicylate, 70 mg., in base. Each point represents the mean value of five suppositories (vertical bars ± SE).*

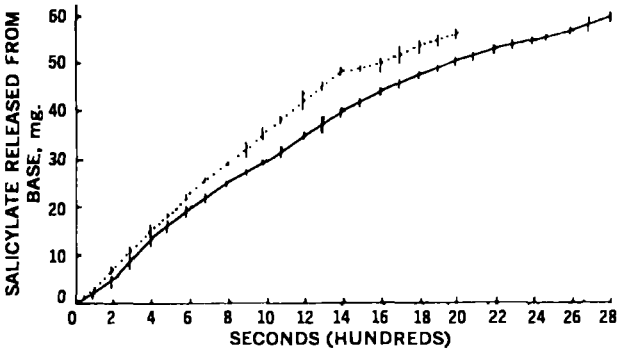

Figure 2—In vitro *release of salicylates from a suppository base consisting of polyethylene glycol 6000 (100%). Key: ——, salicylic acid, 60 mg., in base; and . . ., sodium salicylate, 70 mg., in base. Each point represents the mean value of five suppositories (vertical bars ± SE).*

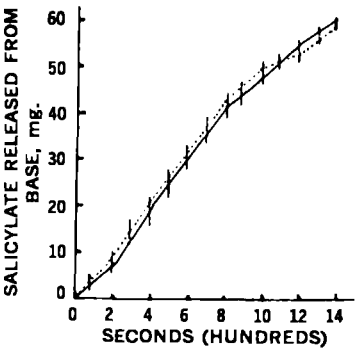

Figure 3—In vitro *release of salicylates from a suppository base consisting of polyethylene glycol 1540 (80%) and polyethylene glycol 6000 (20%). Key: ——, salicylic acid, 60 mg., in base; and . . ., sodium salicylate, 70 mg., in base. Each point represents the mean value of five suppositories (vertical bars ± SE).*

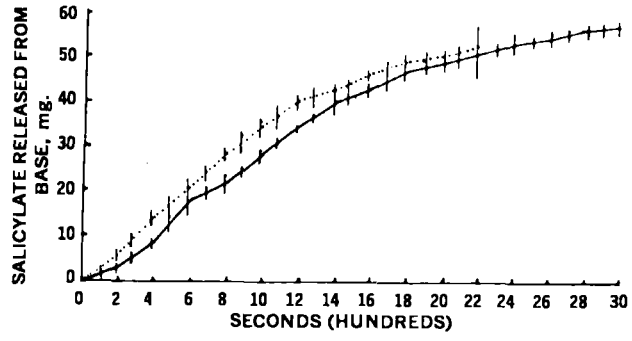

Figure 4—In vitro *release of salicylates from a suppository base consisting of polyethylene glycol 1540 (20%) and polyethylene glycol 6000 (80%). Key: ——, salicylic acid, 60 mg., in base; and . . ., sodium salicylate, 70 mg., in base. Each point represents the mean value of five suppositories (vertical bars ± SE).*

From the data presented, it would seem that the drug itself has little effect on the rate of dissolution. Rather, it is the character of the polyethylene glycol matrix that is most influential. However, it is known that salicylates form complexes with the polyethylene glycols (12–14); this phenomenon probably occurs and may have some minor influence.

The deviation from linearity for the first 10% of salicylate release is attributed to incomplete mixing of the circulating test solution and correlates with the time required for fresh solution to be pumped from the reservoir past the suppository.

Plots of the linear portion of the salicylate release data are corrected to pass through the origin using the equation:

$$Sr' = k''t \qquad \text{(Eq. 2)}$$

where Sr' represents the corrected value for the milligrams of salicylate released at time t, and k'' is the slope of the linear portion of the plotted data. Plots of the corrected salicylate release data are shown in Figs. 5 and 6.

Although it was apparent that the release of salicylate from polyethylene glycol bases simulated zero-order kinetics, the existent deviation from linearity suggested the necessity of further studies, regarding the change in the surface area of the suppositories with time. Therefore, suppositories were removed from the dissolution apparatus at selected time intervals, and the length and diameter of each suppository were measured and its area was calculated as previously described (see *Experimental: Suppository Surface Area Determination*) using Eq. 1. Plots of the calculated area *versus* time are shown in Fig. 7.

In addition, the rate constants for the change in surface area of the suppository with respect to time were calculated by differentiating Eq. 1 to give:

$$\frac{dA}{dt} = \frac{d}{dt}\left(\pi \frac{D^2}{2}\right) + \frac{d}{dt}(\pi DL) \qquad \text{(Eq. 3)}$$

which reduces to:

$$\frac{dA}{dt} = \pi D\frac{dD}{dt} + \pi \frac{d(DL)}{dt} \qquad \text{(Eq. 4)}$$

which for a small change in time may be expressed as shown in Eq. 5:

$$\frac{\Delta A}{\Delta t} = \pi D\frac{\Delta D}{\Delta t} + \pi \frac{\Delta(DL)}{\Delta t} \qquad \text{(Eq. 5)}$$

Table I—Suppository Formulations

Number	Formula: Component	g.	PEG[a] Expressed as Percent of Base	Assayed Salicylate Content[b], mg./Suppository
1	Polyethylene glycol 1540	18.80	100	63
	Salicylic acid	1.20	—	
2	Polyethylene glycol 1540	18.61	100	62
	Sodium salicylate	1.39	—	
3	Polyethylene glycol 6000	18.80	100	63
	Salicylic acid	1.20	—	
4	Polyethylene glycol 6000	18.61	100	61
	Sodium salicylate	1.39	—	
5	Polyethylene glycol 1540	15.04	80	61
	Polyethylene glycol 6000	3.76	20	
	Salicylic acid	1.20	—	
6	Polyethylene glycol 1540	14.89	80	59
	Polyethylene glycol 6000	3.72	20	
	Sodium salicylate	1.39	—	
7	Polyethylene glycol 1540	3.76	20	59
	Polyethylene glycol 6000	15.04	80	
	Salicylic acid	1.20	—	
8	Polyethylene glycol 1540	3.72	20	55
	Polyethylene glycol 6000	14.89	80	
	Sodium salicylate	1.39	—	

[a] Abbreviation for polyethylene glycol. [b] See *Experimental: Salicylate Release Determination.*

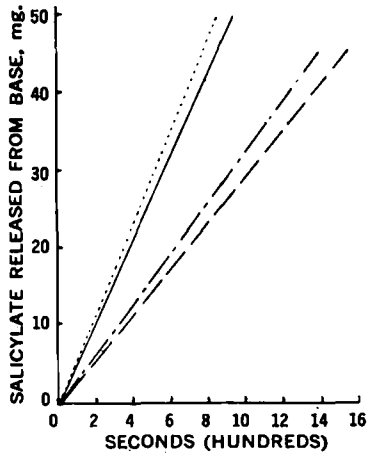

Figure 5—*Plots of corrected data for* in vitro *release of salicylates from various suppository bases. Key:* ——, *salicylic acid, 60 mg., in polyethylene glycol 1540 (80%) and polyethylene glycol 6000 (20%) (Formulation No. 5);* . . ., *sodium salicylate, 70 mg., in polyethylene glycol 1540 (80%) and polyethylene glycol 6000 (20%) (Formulation No. 6);* — —, *salicylic acid, 60 mg., in polyethylene glycol 1540 (20%) and polyethylene glycol 6000 (80%) (Formulation No. 7); and* — · —, *sodium salicylate, 70 mg., in polyethylene glycol 1540 (20%) and polyethylene glycol 6000 (80%) (Formulation No. 8). The average value of salicylate released from five suppositories at each time interval was used to determine the slope of each plot.*

Taking $D = D_1$ at $t = t_1$ and $D = D_2$ at $t = t_2$:

$$\Delta A = A_2 - A_1 \qquad \text{(Eq. 6)}$$

and:

$$\frac{\Delta A}{\Delta t} = \frac{\pi D(D_2 - D_1) + \pi[(DL)_2 - (DL)_1]}{t_2 - t_1} \qquad \text{(Eq. 7)}$$

where:

$$D = \frac{D_1 + D_2}{2} \qquad \text{(Eq. 8)}$$

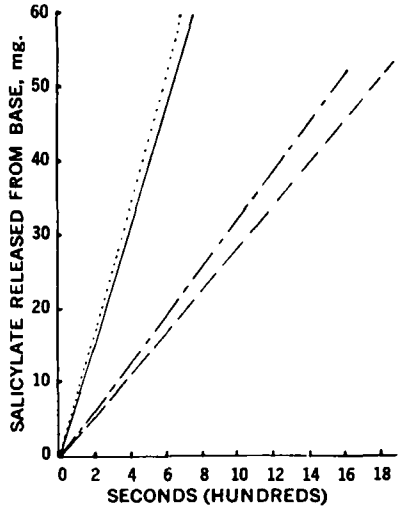

Figure 6—*Plots of corrected data for* in vitro *release of salicylates from various suppository bases. Key:* ——, *salicylic acid, 60 mg., in polyethylene glycol 1540 (100%) (Formulation No. 1);* . . ., *sodium salicylate, 70 mg., in polyethylene glycol 1540 (100%) (Formulation No. 2);* — —, *salicylic acid, 60 mg., in polyethylene glycol 6000 (100%) (Formulation No. 3); and* — · —, *sodium salicylate, 70 mg., in polyethylene glycol 6000 (100%) (Formulation No. 4). The average value of salicylate released from five suppositories at each time interval was used to determine the slope of each plot.*

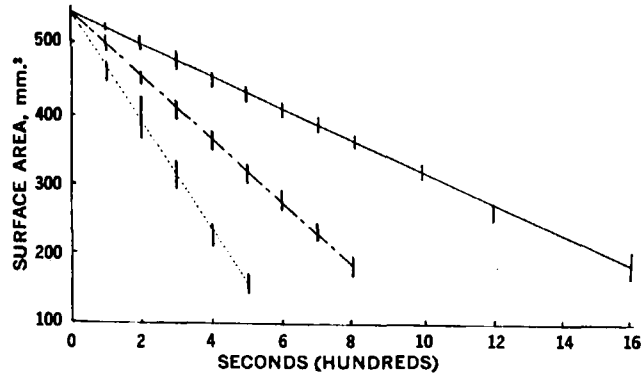

Figure 7—*Change in surface area of various suppositories with respect to time. Key:* . . ., *sodium salicylate, 70 mg., in polyethylene glycol 1540 (100%) (Formulation 2);* — · —, *sodium salicylate, 70 mg., in polyethylene glycol 1540 (80%) and polyethylene glycol 6000 (20%) (Formulation No. 6); and* ——, *salicylic acid, 60 mg., in polyethylene glycol 6000 (100%) (Formulation No. 3). Each point represents the mean value of five suppositories (vertical bars ± SE).*

If A decreases uniformly with time, one can say:

$$k = \frac{dA}{dt} \qquad \text{(Eq. 9)}$$

where k is the rate constant for a pseudo-zero-order reaction. For a small change in time, Eq. 9 may be expressed as:

$$k = \frac{\Delta A}{\Delta t} \qquad \text{(Eq. 10)}$$

Substituting Eq. 10 in Eq. 7, one has:

$$k = \frac{\pi D(D_2 - D_1) + \pi[(DL)_2 - (DL)_1]}{t_2 - t_1} \qquad \text{(Eq. 11)}$$

Taking a typical set of experimental results, one can calculate the rate constant, k, for the change in surface area of the suppository with respect to time. For example, consider the means (Fig. 7) for the suppository base containing polyethylene glycol 1540 (80%) and polyethylene glycol 6000 (20%), where:

$$D_1 = 8.0 \text{ mm.}$$
$$D_2 = 3.8 \text{ mm.}$$
$$L_1 = 17.8 \text{ mm.}$$
$$L_2 = 9.6 \text{ mm.}$$
$$t_1 = 0.0 \text{ sec.}$$
$$t_2 = 800.0 \text{ sec.}$$

Substituting these values into Eq. 11, one has:

$$k = \frac{3.142\left(\frac{8.0 + 3.8}{2}\right)(3.8 - 8.0)}{800 - 0} + \frac{3.142 \times [(3.8 \times 9.6) - (8.0 \times 17.8)]}{800 - 0} \qquad \text{(Eq. 12a)}$$

or:

$$k = -0.513 \qquad \text{(Eq. 12b)}$$

The value calculated for k (−0.513) approximates the value obtained by graphical analysis of the data (Fig. 3) and indicates that the change in surface area simulates zero-order kinetics. Therefore, one can assume that the release of salicylates from the polyethylene glycol suppositories also simulates zero-order kinetics. This assumption is in accord with findings reported by Wagner (15).

Table II—Average[a] Half-Lives and Rate Constants for Release of Salicylate from Various Polyethylene Glycol Suppository Formulations

Number	Suppository Formulation[b]	Average Half-Lives ($t_{1/2}$) ± *SE*, sec.	Average Rate Constants (k') ± *SE*, mg./sec.
1	Salicylic acid Polyethylene glycol 1540 (100%)	426 ± 31	0.07483 ± 0.00356
2	Sodium salicylate Polyethylene glycol 1540 (100%)	385 ± 9	0.08119 ± 0.00191
3	Salicylic acid Polyethylene glycol 6000 (100%)	1118 ± 5	0.02827 ± 0.00041
4	Sodium salicylate Polyethylene glycol 6000 (100%)	834 ± 14	0.03320 ± 0.00041
5	Salicylic acid Polyethylene glycol 1540 (80%) Polyethylene glycol 6000 (20%)	586 ± 11	0.05244 ± 0.00074
6	Sodium salicylate Polyethylene glycol 1540 (80%) Polyethylene glycol 6000 (20%)	546 ± 17	0.05411 ± 0.00161
7	Salicylic acid Polyethylene glycol 1540 (20%) Polyethylene glycol 6000 (80%)	1090 ± 12	0.02966 ± 0.00063
8	Sodium salicylate Polyethylene glycol 1540 (20%) Polyethylene glycol 6000 (80%)	805 ± 7	0.03446 ± 0.00112

[a] Represents the average of five suppositories. [b] Each suppository was formulated to contain either 60 mg. of salicylic acid or 70 mg. of sodium salicylate.

The time for 50% release of salicylate from each suppository, $t_{1/2}$, was calculated as follows:

$$t_{1/2} = \frac{Sro}{2k'} \qquad \text{(Eq. 13)}$$

where *Sro* is the total milligrams of salicylate released from the suppository (Table I), and k' is the release rate constant expressed in milligrams per second. The release rate constant, k', was also calculated using the expression:

$$k' = \frac{Sr}{t} \qquad \text{(Eq. 14)}$$

where t is the time when 80% of the salicylate, *Sr*, is released.

Taking a typical set of experimental results, that is, where t = 1800 sec. and *Sr* = 51 mg. (see Fig. 6, Formulation No. 3), one has:

$$k' = \frac{51}{1800} = 0.0283 \text{ mg./sec.} \qquad \text{(Eq. 15)}$$

and:

$$t_{1/2} = \frac{63}{(2)(0.0283)} = 1113 \text{ sec.} \qquad \text{(Eq. 16)}$$

Plots of the corrected salicylate release data (Figs. 5 and 6) and the half-lives for salicylate release from each suppository base (Table II) indicate that sodium salicylate is released at a slightly faster rate than is salicylic acid. The data also show that the composition of the polyethylene glycol base can affect markedly the time for 50% salicylate release. For example, the half-life of salicylic acid in polyethylene glycol 1540 is 426 sec., whereas in polyethylene glycol 6000 it is 1118 sec.

The appreciable differences between the half-lives for salicylates in the various polyethylene glycol bases may be predicted from a knowledge of the physical-chemical properties of the polyethylene glycols. For example, polyethylene glycol 1540 is approximately 1.5 times more soluble in water than polyethylene glycol 6000 (16).

The data also show that when mixtures of the polyethylene glycols are used in the suppository bases (Figs. 5 and 6), the rate of release of salicylate tends to approximate that observed for the higher molecular weight polyethylene glycol component. For example, the base consisting of 80% polyethylene glycol 6000 has a salicylate release half-life (Table II) almost identical with that observed for the base composed solely of polyethylene glycol 6000. In contrast, the base containing 80% polyethylene glycol 1540 has a salicylate release half-life that differs appreciably from that for the base consisting solely of polyethylene glycol 1540.

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Structural Requirements for Centrally Acting Drugs I

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Abstract □ Various CNS activities, such as antielectroshock, antipentylenetetrazol-induced seizures, muscle relaxant, and acute lethal toxicity, of different types of compounds were correlated with three physicochemical parameters, namely lipophilicity (log P), dipole moment, and steric considerations (E_s). The relatively narrow range of log P_0 (2.0 ± 0.7) for compounds possessing a polar group, *e.g.*, amido, thioamido, keto, or alcoholic function, strongly suggests that the rate-limiting step for these different CNS-acting drugs is probably the same, namely the penetration of the tightly packed neuroglial cells (the so-called blood–brain barrier). Negative dependence of the anticonvulsant or CNS depressant activity on the dipole moment and positive dependence of the stimulatory CNS toxicity on the same parameter were found to be statistically significant. Entirely different sites and mechanisms of action appear to be evident from the very low estimated log P_0 values of lactones and 2-sulfamoylbenzoates for their CNS depressant activities. The CNS activity of five lactones was studied in mice. While γ-butyrolactone caused dosage-dependent sedation and sleep at 100–1000 mg./kg., no loss of the righting reflex was observed for α-methyl-γ-butyrolactone (1000 mg./kg.), γ-valerolactone (2000 mg./kg.), δ-valerolactone (750 mg./kg.), and γ-heptalactone (1000 mg./kg.). After intraperitoneal injection of γ-valerolactone and γ-heptalactone, paralysis of the hind legs was observed, indicating local anesthetic activity on the peripheral nervous system.

Keyphrases □ CNS activity—investigation of structural requirements □ Structure–activity relationships—structural requirements for CNS activity □ γ-Butyrolactone—CNS depressant activity, effect of lipophilicity □ Lipophilicity—relationship to CNS activity

Due to the highly intricate circuitry of the CNS and the very complex interplay of various neurotransmitters, the exact mechanisms of actions of many centrally acting drugs are poorly understood. Since the CNS depressant activity and the degree of depression vary according to the part of the nervous system affected, the overall action may be a summation of several effects. Furthermore, most CNS stimulants or depressants have an action that spreads to other parts of the nervous system, even though the initial action affects only a certain site.

To develop safer and more specific agents for various therapeutic applications, it would be desirable to have some quantitative and qualitative guidelines to assist medicinal chemists in drug design. For example, Hansch *et al.* (1) reported that the ideal log P_0 (octanol–water) for the penetration of the brain by benzeneboronic acids and the ED_{50} of barbiturates is 2.3–2.4.

The sedative–hypnotic effects of γ-butyrolactone and its corresponding open-chain compound, γ-hydroxybutyrate, have been demonstrated in various laboratory animals (2–4). Relatively high dosage levels (100–1000 mg./kg.) of γ-butyrolactone are required to cause dosage-dependent sedation and sleep (4). Although many elegant pharmacological studies have been reported on γ-butyrolactone, few systematic studies have been reported on the structure–activity relationship of its congeners or bioisosteres (5). It has been shown that in many cases the CNS activity of congeneric members increases as the lipophilic character increases until the apex is reached, and then further increase in lipophilic character causes a decrease in activity (6–10). This has been attributed to the ease of random walk of organic molecules. Since γ-butyrolactone has a very low octanol–water partition coefficient (log $P < 0.8$), it is conceivable that its relatively low potency is due to its poor lipoid solubility and limited ability to penetrate through the blood–brain barrier.

The purposes of this paper are to report a number of quantitative structure–activity correlations using various physicochemical parameters, to examine the structural requirements for various types of CNS activity, and to investigate whether the CNS depressant activity of γ-butyrolactone could be enhanced by increasing its lipophilicity.

Table I—Biological Data and Physicochemical Constants Used in the Regression Analysis

Antielectroshock, −log 1/C, moles/kg.		Muscle Relaxant, log 1/C		Acute Lethal Toxicity, log 1/C				
Obs.[a]	Calc.[b]	Obs.[a]	Calc.[c]	Obs.[a]	Calc.[d]	log P	μ[e]	Compound
2.33	2.46	2.04	2.14	1.75	1.88	0.81[f]	3.08	Cyclohexanone
3.02	3.05	2.74	2.68	2.20	2.40	3.62	3.31	2-(2-Tolyl)cyclohexanone
3.24	3.05	2.74	2.81	2.47	2.38	1.71	(3.31)[g]	2-(*p*-Aminophenyl)cyclohexanone
2.95	3.17	2.76	2.94	2.38	2.49	2.62	3.64	2-(α-Hydroxy-*p*-chlorobenzyl)cyclohexanone
3.00	2.71	2.48	2.25	2.48	2.13	4.14	3.31	2-(*p*-Chlorobenzyl)-cyclohexanone
2.92	3.18	2.31	2.52	2.31	2.48	4.31	1.67	α-Cyclohexyl-*p*-chlorobenzyl alcohol
2.92	2.87	—	—	—	—	4.63	1.67	α-Cyclohexyl-*p*-bromobenzyl alcohol
3.90	3.78	3.38	3.30	3.03	2.93	1.42[h]	0.87[h]	Sodium phenobarbital
—	—	2.96	2.79	2.48	2.41	1.41	(2.68)[g]	Mephenesin

Table I—(*Continued*)

Antielectroshock, log 1/C, moles/kg. Obs.	Calc.[i]	log P	μ	Compound
2.16[i]	1.90	−0.37[i]	1.74[i]	Trimethadione
2.59	2.52	0.13	1.69	Paramethadione
3.72	3.60	1.53	1.74	5-Ethyl-5-phenylhydantoin
4.40	3.84	2.47	1.74	Diphenylhydantoin
3.05	3.05	0.70	1.74	5-Phenylhydantoin
3.28	3.32	0.98	1.61	3-Methyl-3-phenylsuccinimide
3.00	3.23	0.65[h]	1.13	Barbital
3.54	3.62	1.48	1.61	3-Ethyl-3-phenylsuccinimide
3.74	3.87	2.25	1.61	3,3-Diphenylsuccinimide
2.77[i]	2.90	0.48[i]	1.61[i]	3-Phenylsuccinimide
2.33[a]	2.65	0.81[f]	3.08[e]	Cyclohexanone
3.02	3.03	3.62	3.31	2-(2-Tolyl)cyclohexanone
3.24	3.10	1.71	(3.31)[g]	2-(*p*-Aminophenyl)-cyclohexanone
2.95	3.15	2.62	3.64	2-(α-Hydroxy-*p*-chlorobenzyl)cyclohexanone
3.00	2.73	4.14	3.31	2-(*p*-Chlorobenzyl)-cyclohexanone
2.92	3.22	4.31	1.67	α-Cyclohexyl-*p*-chlorobenzyl alcohol
2.92	2.95	4.63	1.67	α-Cyclohexyl-*p*-bromobenzyl alcohol
3.90[a]	3.86	1.42[h]	0.87[h]	Sodium phenobarbital

Antipentylenetetrazol-Induced Seizures, —log 1/C, moles/kg.— Obs.[o]	Calc.[p]	log P	μ[q]	Compound
1.07	0.80	1.98	0.87	Mephobarbital
0.97	0.91	2.09	1.74	Mephenytoin
0.01	0.21	1.40	1.61	Phensuximide
0.38	0.35	1.54	1.61	Methsuximide
−1.17	−1.21	0.01	1.47	Ethosuximide

Antipentylenetetrazol-Induced Seizures, —log 1/C, moles/kg.— Obs.[o]	Calc.[q]	Calc.[r]	log P	μ	Compound
0.93	0.59	0.89	1.42[h,i]	0.87[h]	Phenobarbital
0.99	0.71	0.99	1.98	0.87[h]	Mephobarbital
0.69	0.52	0.68	1.21	1.13	Metharbital
0.76	0.72	0.84	2.78	1.13	Phetharbital
0.47	0.72	—	2.10	—	Primidone
0.61	0.72	0.48	2.09	1.74	Mephenytoin
0.73	0.74	—	2.51	—	Albutoin
0.15	0.24	—	0.57	—	Phenacemide
−0.65	−0.39	−0.45	−0.37	1.74	Trimethadione
−0.76	−0.88	−0.87	−0.93	1.74	Dimethadione
0.36	0.58	0.45	1.40	1.61	Phensuximide
0.49	0.62	0.49	1.54	1.61	Methsuximide
0.02	−0.10	−0.05	0.01	1.47	Ethosuximide

Acute Lethal Toxicity, —log 1/C, moles/kg.— Obs.[o]	Calc.[s]	Calc.[t]	log P	μ	Compound
0.61	0.28	0.54	1.42[h,i]	0.87[h]	Phenobarbital
0.58	0.36	0.56	1.98	0.87	Mephobarbital
0.43	0.22	0.40	1.21	1.13	Metharbital
0.14	0.25	0.24	2.78	1.13	Phetharbital
0.35	0.36	0.23	2.09	1.74	Mephenytoin
0.10	0.32	—	2.51	—	Albutoin
−0.56	−0.09	—	0.57	—	Phenacemide
−0.89	−0.86	−0.78	−0.37	1.74	Trimethadione
−1.27	−1.49	−1.39	−0.93	1.74	Dimethadione
0.20	0.28	0.27	1.40	1.61	Phensuximide
0.31	0.31	0.29	1.54	1.61	Methsuximide
−0.45	−0.50	−0.35	0.01	1.47	Ethosuximide
−0.95	−0.75	—	−0.26	—	Acetazolamide
0.43	0.33	—	2.44	—	Chlordiazepoxide
−0.71	—	—	2.10	—	Primidone

Antielectroshock — In Mice — log 1/C, moles/kg. Obs.[k]	Calc.[l]	In Rats — log 1/C, moles/kg. Obs.[k]	Calc.[m]	log P	E_s[n] (R_1)	Compound
2.76	2.57	2.86	2.87	0.58	0.00	1-Methyl-5-α-thienylhydantoin
2.91	2.78	3.13	3.12	1.08	−0.07	1-Ethyl-5-α-thienylhydantoin
2.72	2.87	3.14	3.09	1.58	−0.36	1-*n*-Propyl-5-α-thienylhydantoin
2.94	2.72	3.01	3.00	1.38	−0.47	1-Isopropyl-5-α-thienylhydantoin
2.90	2.72	3.19	3.03	1.28	−0.36	1-Allyl-5-α-thienylhydantoin
2.59	3.10	2.81	3.03	2.08	−0.39	1-*n*-Butyl-5-α-thienylhydantoin
3.04	3.10	3.36	3.34	0.34	1.24	5-Phenylhydantoin
4.44	4.15	3.95	3.76	2.47[f]	1.24	Diphenylhydantoin
2.75	2.95	2.99	3.03	0.03	1.24	5-α-Thienylhydantoin
3.82	3.84	3.84	3.95	1.84	1.24	5,5-Di-α-thienylhydantoin
3.92	3.99	3.83	3.89	2.15	1.24	5-Phenyl-5-α-thienylhydantoin

Acute Lethal Toxicity, —log 1/C, moles/kg. Obs.[u]	Calc.[v]	log P	Compound
2.55	2.51	0.14	*N*-Dimethylaminoethyl-γ-butyrolactam
2.59	2.57	0.32	*N*-Diethylaminoethyl-δ-valerolactam
2.61	2.70	0.74	*N*-Diethylaminoethyl-ε-caprolactam
3.10	3.11	2.06	*N*-(3-Diethylaminopropyl)-*o*-phenyleneurea
3.12	3.08	1.97	4-(2-Keto-1-benzimidazolinyl)-piperidine[w]

[a] From *Reference 14*. [b] Calculated from Eq. 3. [c] Calculated from Eq. 5. [d] Calculated from Eq. 7. [e] From *Reference 21*. [f] From C. Hansch, private communication. [g] Estimated value. [h] For the undissociated form. [i] From *Reference 8*. [j] From *Reference 22*. [k] Calculated from Eq. 9. [l] Calculated from Eq. 11. [m] From *Reference 23*. [n] From *Reference 24*. [o] Calculated from Eq. 12. [p] Calculated from Eq. 14. [q] Calculated from Eq. 16. [r] Calculated from Eq. 18. [s] Calculated from Eq. 20. [t] From this study. [u] Calculated from Eq. 23. [v] This compound, m.p. 185–187°, was purchased from Aldrich Chemical Co.

EXPERIMENTAL[1]

The mathematical model used in the regression analysis was first developed by Hansch *et al.* (1, 6), and used fairly extensively by our group as well as many other investigators (7–11). The equations correlating the biological activity with the physicochemical constants were derived *via* the method of least squares using a computer[2]. The biological data and the physicochemical constants used in the study are assembled in Table I. The log *P* values were calculated from the log *P* of the parent molecule and the π values of the substituent, unless otherwise stated. Octanol-water was the solvent system used in measuring *P*. The dipole moment values were either taken from the literature or estimated from the moment of the homolog.

Materials—The *N*-diethylaminoethyl lactams were synthesized by treating the corresponding lactam with equivalent amounts of sodium hydride and diethylaminoethyl chloride, using dioxane as the solvent (12), and were then purified on a cationic ion-exchange resin and alumina column.

N-Diethylaminoethyl-δ-valerolactam—Boiling point 148° (6.5 mm. Hg), yield 41%.

Anal.—Calc. for $C_{11}H_{22}N_2O$: C, 66.62; H, 11.18; N, 14.12. Found: C, 66.85; H, 11.05; N, 14.20.

[1] The elemental analyses were performed by C. F. Geiger of Ontario, Calif.

[2] IBM 360/65.

Table II—Equations Correlating CNS Activity with Physicochemical Constants

Animal	Test	Compounds	Number	Equation	n	r	s	log P_0 (±95% CL)
Mice	Antielectroshock	Cyclohexanones, *etc.*	1	$\log 1/C = -0.138 (\log P)^2 + 0.736 \log P + 2.323$	8	0.43	0.47	2.66 (± ∞)[a]
			2	$\log 1/C = -0.212 \mu + 3.588$	8	0.51	0.41	—
			3	$\log 1/C = -0.267 (\log P)^2 + 1.423 \log P - 0.368 \mu + 2.619$	8	0.89	0.26	2.66 (1.64–3.17)
Mice	Muscle relaxant	Cyclohexanones, *etc.*	4	$\log 1/C = -0.237 (\log P)^2 + 1.181 \log P + 1.591$	8	0.68	0.36	2.49 (± ∞)
			5	$\log 1/C = -0.313 (\log P)^2 + 1.601 \log P - 0.276 \mu + 1.895$	8	0.92	0.21	2.56 (2.08–2.85)
Mice	Acute lethal toxicity	Cyclohexanones, *etc.*	6	$\log 1/C = -0.138 (\log P)^2 + 0.729 \log P + 1.649$	8	0.42	0.38	2.65 (± ∞)
			7	$\log 1/C = -0.217 (\log P)^2 + 1.166 \log P - 0.286 \mu + 1.964$	8	0.85	0.25	2.69 (± ∞)
Mice	Antielectroshock	Miscellaneous	8	$\log 1/C = -0.222 (\log P)^2 + 1.153 \log P - 0.368 \mu + 2.994$	18	0.92	0.24	2.59 (2.39–2.84)
Mice	Antielectroshock	Hydantoins	9	$\log 1/C = 0.490 \log P + 0.525 E_s(R_1) + 2.286$	11	0.92	0.26	—
Rats	Antielectroshock	Hydantoins	10	$\log 1/C = 0.298 \log P + 0.373 E_s(R_1) + 2.727$	11	0.91	0.19	—
			11	$\log 1/C = -0.330 (\log P)^2 + 1.124 \log P + 0.540 E_s(R_1) + 2.330$	11	0.96	0.14	1.70 (1.45–3.88)
Mice	Antipentylenetetrazol-induced seizures	Barbiturates, hydantoins, imides	12	$\log 1/C = 1.023 \log P - 1.224$	5	0.98	0.21	—
			13	$\log 1/C = 0.998 \log P - 0.482 \mu - 0.486$	5	0.996	0.116	—
Mice	Antipentylenetetrazol-induced seizures	Barbiturates, hydantoins, imides	14	$\log 1/C = -0.142 (\log P)^2 + 0.695 \log P - 0.111$	13	0.93	0.22	2.44 (1.68–9.57)
			15	$\log 1/C = -0.145 (\log P)^2 + 0.716 \log P - 0.093$	10	0.94	0.24	2.47 (± ∞)
			16	$\log 1/C = -0.123 (\log P)^2 + 0.588 \log P - 0.597 \mu + 0.825$	10	0.99	0.12	2.39 (1.72–5.39)
Mice	Acute lethal toxicity	Barbiturates, hydantoins, imides	17	$\log 1/C = -0.207 (\log P)^2 + 0.802 \log P - 0.523$	15	0.84	0.37	1.94 (1.36–12.6)
			18	$\log 1/C = -0.210 (\log P)^2 + 0.856 \log P - 0.510$	14	0.94	0.23	2.04 (1.59–3.56)
			19	$\log 1/C = -0.236 (\log P)^2 + 0.877 \log P - 0.381$	10	0.98	0.16	1.83 (1.48–2.64)
			20	$\log 1/C = -0.226 (\log P)^2 + 0.800 \log P - 0.361 \mu + 0.175$	10	0.99	0.11	1.77 (1.49–2.26)
Mice	Acute lethal toxicity	Lactams, thiolactams, ureas, thioureas	21	$\log 1/C = -0.373 (\log P)^2 + 1.010 \log P + 0.227 \mu + 1.405$	22	0.83	0.31	1.35 (1.11–1.78)
			22	$\log 1/C = -0.364 (\log P)^2 + 1.005 \log P + 0.247 \mu + 1.298$	20	0.89	0.24	1.38 (1.17–1.71)
Mice	Acute lethal toxicity	*N*-Substituted lactams, *o*-phenyleneureas	23	$\log 1/C = 0.312 \log P + 2.468$	5	0.98	0.06	>2.0

[a] *CL* = confidence limits.

N-Diethylaminoethyl-ε-caprolactam—Boiling point 131–134° (6 mm. Hg), yield 34%.

Anal.—Calc. for $C_{12}H_{24}N_2O$: C, 67.88; H, 11.39; N, 13.19. Found: C, 67.62; H, 11.07; N, 12.97.

N-Diethylaminoethyl-γ-butyrolactam—Boiling point 120–124° (6.5 mm. Hg), yield 38%.

Anal.—Calc. for $C_{10}H_{20}N_2O$: C, 65.17; H, 10.93; N, 15.19. Found: C, 64.96; H, 10.78; N, 15.00.

Similar procedures were used to prepare the substituted *o*-phenyleneurea using *N*-dimethylformamide as the solvent.

N-(3-Dimethylaminopropyl)-o-phenyleneurea—Melting point 69–72°, yield 4%.

Anal.—Calc. for $C_{12}H_{17}N_3O$: C, 65.71; H, 7.82; N, 19.15. Found: C, 65.22; H, 7.67; N, 19.10.

The structures of these lactam and urea derivatives were confirmed by IR and NMR.

γ-Butyrolactone (n^{25}_D = 1.4354), α-methyl-γ-butyrolactone (n^{25}_D = 1.4318), γ-valerolactone (n^{25}_D = 1.4324), and δ-valerolactone (n^{25}_D = 1.4564) were purchased[3] and used without further purification. γ-Heptalactone was obtained from a commercial source[4] and used directly.

[3] Aldrich Chemical Co.
[4] K & K Laboratories.

CNS Activity and General Signs—Groups of six mice, three males and three females weighing 17–27 g., were injected intraperitoneally with the aqueous solution of the compound (0.3–1.2 ml.). Signs of CNS activity or other changes were observed continuously for 2 hr. and then at regular intervals for 2 days.

Preliminary Test for LD_{50}—The previously reported method (13) was used to determine the LD_{50} of the *N*-substituted lactams and the *o*-phenyleneurea derivative.

RESULTS AND DISCUSSION

The equations correlating the biological activity with the physicochemical parameters are summarized in Table II. The optimum lipophilic character (log P_0) for maximum CNS activity was obtained by setting (log $1/C$)/log P = 0. The log P_0 values with well-defined 95% confidence intervals are assembled in Table IV according to the descending order of log P_0.

Equations 1–7 were derived from three different tests of series of cyclohexanones and cyclohexyl benzyl alcohols reported by Brodie *et al.* (14). Equation 8 was obtained from these compounds plus imides, barbiturates, hydantoins, *etc.* One should note the negative dependence of the activity on the dipole moment (μ) in Eqs. 3, 5, 7, and 8 and the very narrow log P_0 values for maximum activity (2.56–2.66, Eqs. 3 and 5 of Table II, with well-defined 95% confidence

Table III—Preliminary Screening of Neuropharmacological Activities of Lactones in Mice

Compound	Dosage, mg./kg. i.p	Observation
γ-Butyrolactone	750	6/6[a] loss of righting reflex, ataxia in 2 min., eyes bulged, 1/6 died, average sleeping time 1.5 hr.
α-Methyl-γ-butyrolactone	1000	3/6 normal, 3/6 mild sedation in 5 min.
γ-Valerolactone	1000	4/6 with both hind legs stretched out, 6/6 mild sedation in 5 min.
	2000	2/2 moderate sedation, irregular respiration, hind legs paralyzed
δ-Valerolactone	750	6/6 mild sedation, 3/6 with bulging eyes
γ-Heptalactone	100	4/6 with sluggish hind-leg movement
	300	2/6 with sluggish hind-leg movement
	1000	3/6 with one hind leg paralyzed, 2/6 with both hind legs paralyzed, no apparent CNS activity
γ-Thiobutyrolactone	100	5/6 clonic and tonic convulsions[b]

[a] Ratio of number of animals responding to number of animals tested. [b] From *Reference 13*.

intervals). Equations 9–11 correlate the antielectroshock activity of 11 substituted hydantoins with log P and the Taft's steric constant E_s of the substituent at the N_1 position. The positive dependence on E_s indicates that a bulky substituent at the N_1 position will retard the anticonvulsant activity. This is in agreement with the well-known fact that alkylation of both nitrogens of a barbiturate tends to confer convulsant activity. The $(\log P)^2$ term was not justifiable in Eq. 9, where mice were the test animals. In rats (Eq. 11) the log P_0 value is very close to the previously reported value of 1.75 (8).

For anticonvulsant activity against pentylenetetrazol[4]-induced seizures, Eqs. 12–16 were obtained. Again there is negative dependence of activity on the dipole moment (Eqs. 13 and 16). The μ term in Eq. 13 is not significant at the 90 percentile level, since only five data points are available. The μ term in Eq. 16 is significant at the 97.5 percentile level, as indicated by an F test ($F_{1,6} = 13.8$, $F_{1,6_{0.975}} = 8.8$). Equations 17 and 18 were derived from the LD_{50} of series of barbiturates, hydantoins, amides, imides, *etc.* Since the log P_0 of 2.04 is only slightly below the log P_0 of 2.4–2.6 for antielectroshock or hypnotic activity, one should not expect complete separation of the acute lethal toxicity from the therapeutic effect by merely changing the lipophilic character of these drugs.

Equations 19 and 20 correlate the acute lethal toxicity of 10 CNS depressants among barbiturates, hydantoins, and imides. Equation 21 was obtained from the acute lethal toxicity data of 22 CNS stimulants belonging to different chemical groups and reported in two separate papers previously (9, 10). The dependence of the dipole moment (μ) is positive in contrast with that of CNS depressants or anticonvulsants. The correlation coefficient (r) increased from 0.83 to 0.89 when two poorly predicted compounds were deleted (Eq. 22). The μ term is significant at the 95 percentile level in Eq. 21, and it is significant at the 99.5 percentile level in Eq. 22. The log P_0 for the acute lethal toxicity of these convulsants is about 1.4.

Equation 23 was derived from the LD_{50} of N-substituted lactams and o-phenyleneureas, studied for the first time in these laboratories. Since the highest log P value of these five compounds was 2.06, a linear rather than a parabolic equation of log P was obtained.

Results of the preliminary screening for the CNS activity and other neuropharmacological activities of lactones are shown in Table III. From Table III, it is evident that the CNS depressant activity of γ-butyrolactone is decreased rather than increased when a methyl group is attached to the α- or γ-position. No loss of righting reflex was observed for these lactones at the 1000-mg./kg. level. The six-membered δ-valerolactone also showed lower CNS depressant activity as compared with γ-butyrolactone. For the most lipophilic, γ-heptalactone, no CNS activity was apparent even at the 1000-mg./kg. level; however, partial paralysis of the hind legs was observed even at 100-mg./kg. level, indicating local anesthetic activity on the peripheral nervous system. A similar phenomenon was observed for γ-valerolactone (1000 mg./kg.). Bulging eyes were observed for the lactones without a side chain (γ-butyrolactone and δ-valerolactone). Many lactones are known to have depression action on earthworms, frog gastrocnemius, rabbit small intestine, and frog heart (15).

It is known that γ-hydroxybutyric acid, when administered to animals or man, is converted to γ-butyrolactone and causes sleep. It was also reported (3) that the induced sleep in rats is related to the concentration of the lactone in the brain when the acid or the lactone is administered. Giarman and Schmidt (2) demonstrated that γ-butyrolactone alters the levels of endogenous acetylcholine in the brains of mice and rats. Concentration of acetylcholine in the cerebral cortex is increased by γ-butyrolactone, with a time course parallel to the degree of depression of the animal.

The results of this structure–activity analysis reflect the steric and hydrophilic aspects of the binding site for acetylcholine and γ-butyrolactone. An additional alkyl group at the α- or γ-position decreases the CNS activity even though the lipophilic character is increased. Since an alkyl group on the lactone ring decreases the activity and the most lipophilic member (γ-heptalactone) does not give any apparent CNS activity at 1000 mg./kg., the binding site of acetylcholine conceivably may be surrounded by a hydrophilic rather than a lipophilic region. It is well established that the active site of acetylcholinesterase is composed of imidazole nitrogen, a serine hydroxy group, and an anionic site (16). A similar decrease in acute toxicity was reported (5) for a series of γ-methyl-α-alkyl butyrolactones as the α-alkyl group is lengthened from C_2 to C_6.

On the other hand, Roth and Suhr (17) reported that γ-butyrolactone increases brain dopamine by blocking the release of this amine from dopamine-containing neurons. It is also suggested that the CNS depressant properties of γ-hydroxybutyrate may be related to this block in the release of brain dopamine.

While γ-butyrolactone is a CNS depressant, its sulfur analog γ-thiobutyrolactone has been shown to be a convulsant (13). Ito (18) also reported that running fits are induced in mice by intraperitoneal injection of 150–200 mg./kg. of γ-mercaptobutyric acid after a latent period of 4–8 min. It is quite possible that γ-mercaptobutyric acid also cyclizes *in vivo* to produce the fairly potent CNS stimulant γ-thiobutyrolactone. It is not clear at present why the CNS activity is changed from a depressant to a stimulant by replacing —COO— with —COS—. It also remains to be clarified whether γ-thiobutyrolactone acts at the same site as γ-butyrolactone *via* the same mechanism.

It was reported (19) that lactones are not hydrolyzed by esterases that attack the ordinary open-chain esters. Butyrolactone, γ-valerolactone, *etc.*, in alkaline medium showed no appreciable hydrolysis by liver esterase preparations that were reactive toward methyl butyrate. On the contrary, lactones inhibited the cleavage of the ester, presumably due to the formation of a stable complex with the enzyme (19). These findings suggest that lactones and γ-thiobutyrolactones are probably the pharmacologically active species rather than the hydrolyzed products.

Table IV summarizes the ideal lipophilic character (log P_0) for maximum CNS activity under various testing conditions. As reported (1, 20) previously, the log P_0 for many centrally acting drugs centered around 2.0 ± 0.7, with two distinct exceptions at the end of Table IV. γ-Butyrolactone appeared to be the most potent CNS depressant among the several lactone derivatives examined.

γ-Butyrolactone should have a log P less than 0.71, the calculated log P value of the open-chain methyl propionate. In the case of 2-sulfamoylbenzoates, the most potent member has a log P of -0.13 and the dependence on log P is negative, therefore, a log P_0 of < -0.13 is expected for this series of compounds.

The relatively narrow range of log P_0 (2.0 ± 0.7) for the many compounds containing a polar amido, thioamido, keto, or alcoholic group strongly suggests that the rate-limiting step for the CNS action caused by these compounds is probably the same, namely the penetration of the tightly packed neuroglial cells by these compounds.

[4] Metrazole.

Table IV—Optimum Lipophilic Character (log P_0) for Maximum CNS Activity

Animal	Compounds	Number of Data Points	CNS Activity	log P_0 (±95% CL)	Reference
Mice	Cyclohexanones, *etc.*	8	Antielectroshock	2.66 (1.64–3.17)	14
Mice	Barbiturates, hydantoins, imides, cyclohexanones, *etc.*	18	Antielectroshock	2.59 (2.39–2.84)	This study, 8, 14
Mice	Cyclohexanones, *etc.*	8	Muscle relaxant	2.56 (2.08–2.85)	14
Mice	Barbiturates	10	Hypnotic	2.40	1
Mice	Barbiturates, hydantoins, imides	10	Antipentylenetetrazole-induced seizures	2.39 (1.72–5.39)	24
Mice	Barbiturates, hydantoins, imides	14	Acute lethal toxicity (CNS depression)	2.04 (1.59–3.56)	24
Mice	Barbiturates, hydantoins, imides	10	Acute lethal toxicity (CNS depression)	1.77 (1.49–2.26)	24
Rats	Hydantoins	11	Antielectroshock	1.70 (1.45–3.88)	22
Mice	Ureas, thioureas, lactams, thiolactams	20	Acute lethal toxicity (CNS stimulation)	1.38 (1.17–1.71)	This study, 7, 10
Mice	γ-Butyrolactones	5	CNS depression	<0.71	This study
Mice	2-Sulfamoylbenzoates	9, 12	Antistrychnine Antielectroshock	<0.13	11

The finding of the dependence on the dipole moment also suggests that in the development of new anticonvulsants or sedative–hypnotics of comparable structures, it would be highly desirable to have a minimum resultant dipole moment on the polar moiety, while for a potent CNS stimulant or analeptic agent, high dipole moment enhances the activity.

Entirely different sites and mechanisms of action appear to be evident from the very low log P values of γ-butyrolactone and series of 2-sulfamoylbenzoates.

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Constituents of *Cannabis sativa* L. II: Absence of Cannabidiol in an African Variant

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Abstract □ Cannabidiol is shown to be absent in an African variant of *Cannabis sativa* L. (marijuana) grown in Mississippi. TLC, GC, and GC–mass spectrometry were used for identification. The absence of cannabidiol in a variant of African Cannabis questions the validity of published biosyntheses of the cannabinoids.

Keyphrases □ *Cannabis sativa* L., African variant grown in Mississippi—absence of cannabidiol, cannabinoid biosynthesis questioned □ Cannabidiol absence in African *C. sativa* grown in Mississippi—role in cannabinoid biosynthesis questioned □ Biosynthesis of cannabinoids—questioned due to absence of cannabidiol in African *C. sativa* grown in Mississippi

De Faubert Maunder (1) reported that cannabidiol is definitely absent in some samples of Cannabis and put forth the hypothesis that Cannabis plants grown south of the Sahara may have a different biogenesis from plants grown in the Middle East. Moreover, Korte *et al.* (2) reported an absence of cannabidiol in extracts of hashish from Cyprus, Nigeria, and Brazil. These hashish samples were obtained from plants grown using seed obtained from the United Nations and coded as UNC-33, 59, and 61, respectively. Additionally, Schou and Nielsen (3) reported zero for the extinction maximum (nm.) for the azo-dye derivative of cannabidiol from extracts of Indian, Brazilian, and UNC-135 Cannabis. Schou and Nielsen also reported that South African Cannabis from UNC-134 seed stock grown in Denmark contained only traces of cannabidiol regardless of the plant part analyzed. Although the aforementioned Cannabis preparations were analyzed primarily by TLC, Vree *et al.* (4) used combined GC–mass spectrometry to ascertain that a Brazilian sample of Cannabis contained no cannabidiol.

Samples analyzed by De Faubert Maunder (1), Korte *et al.* (2), Schou and Nielsen (3), and Vree *et al.* (4) contained measurable amounts of (−)-Δ^9-*trans*-tetrahydrocannabinol and cannabinol. Korte *et al.* also reported that UNC-33, 59, and 61 contained measurable amounts of tetrahydrocannabinol II, which has since been identified as cannabichromene (5, 6). Vree *et al.* noted that the propyl derivative of cannabinol and cannabichromene could not be easily separated by GC but that the propyl derivative of cannabinol could be easily identified because of its characteristic mass fragments at 282 and 267. Although one published report (6) gives slightly different retention times for cannabidiol and cannabichromene, this laboratory and others (7) have been unsuccessful in obtaining a neat separation of cannabidiol and cannabichromene using only GC. Therefore, three cannabinoids (cannabichromene, cannabidiol, and cannabivarin[1]) are practically impossible to identify using relative retention times only. This fact, as well as the apparent absence of cannabidiol but presence of cannabichromene in certain samples of Cannabis, prompted an investigation of this problem using TLC, GC, and combined GC–mass spectrometry.

Additionally, it seemed worthwhile to examine De Faubert Maunder's (1) proposal that a "different biogenesis" than that originally proposed by Todd (8) and Simonsen and Todd (9) and modified by Mechoulam (10) and Mechoulam and Gaoni (11) may be operative in certain "sub-species of cannabis." Although Schou and Nielsen (3) did not suggest that a different biogenesis may be operative in certain variants of Cannabis, they did state that "the insignificant content of cannabidiol seems to be a characteristic feature" of UNC-134 from South Africa. To ascertain if, indeed, cannabidiol is absent in a particular variant of Cannabis, several variants grown in Mississippi from known origin were investigated.

METHODS

The samples used were grown in Mississippi from seed obtained through the National Institute of Mental Health (NIMH), the U. S. Department of Agriculture (USDA), and the United Nations (UN). The plant material was grown in Mississippi in 1970, 1971, and 1972. Samples were taken of various aged plant material. Each sample, containing aboveground plant parts, was taken from four different plants and dried at 22 ± 2° for 4 days. They were manicured by passing the material through a 14-mesh sieve; this process removed all large stems and fruits.

GC Analyses—The extraction procedure was basically that described by Lerner (12) and Fetterman *et al.* (13) with minor modifications. Three 1-g. samples were extracted simultaneously with 40 ml. of spectrograde chloroform. The resulting solutions were refrigerated at 6° and shaken at 10-min. intervals for 1 hr.; the plant material then was removed by filtration, and the mother liquor was concentrated *in vacuo* at ambient temperature to a greenish paste void of solvent. This concentrate was then dissolved in approximately 1 ml. of ethanol. The ethanolic solution was subjected to continuous vibration from an ultrasonic vibrator until all resin was in solution. At this point, an additional 0.5 ml. of an ethanolic solution of 4-androstene-3,17-dione was added as the internal standard. The concentration of the internal standard required will vary depending upon the age of the plant material. Usually from 0.05 to 0.5 μl. of this solution was injected into the chromatograph.

Analyses were performed using gas chromatographs[2] equipped with hydrogen flame-ionization detectors and operated isothermally at 210°. The inlet temperature and detector temperature were 240 and 260°, respectively. Glass columns, 0.63 cm. (0.25 in.) o.d. and 2 mm. i.d. × 2.43 m. (8 ft.), were packed with 2% OV-17 (high purity polar phenyl methyl silicone; approximately 30,000 mol. wt.) on 100–120-mesh Chromosorb "WHP." Nitrogen was used as the carrier gas at a flow rate of 10–30 ml./min., depending upon separation requirements, *etc.* Peak area measurements were made using the method of peak height times width at half height. The peak area of each cannabinoid was compared with the peak area of the internal standard, and the cannabinoid concentration was

[1] *n*-Propyl homolog of cannabinol. (See *References 15, 22,* and *23.*)

[2] Beckman GC-45 and GC-72-5.

Table I—Analyses[a] of South African (A-A) *C. sativa* L.

Generation	Sex	Age, weeks	V and XI	VII	XIII	VI	VIII
First	Mixture[b]	16	0.17	0.11	Trace	1.60	0.03
Second	Mixture	16	0.05	0.27	0.02	1.18	0.03
Third	Young[c]	9	0.12	0.06	0.15	0.84	0.05

[a] Key: V, tetrahydrocannabivarin; XI, cannabicyclol; VII, cannabichromene; XIII, cannabigerol monomethyl ether; VI, Δ^9-tetrahydrocannabinol; and VIII, cannabigerol. [b] Contains mature male and female plants. [c] Plant material analyzed prior to sexual differentiation.

determined by using the appropriate relative response factor. Synthetic cannabinoids used in determining the relative response factors were obtained through NIMH.

TLC (Qualitative)—Extracts for TLC were prepared as described for GC with the exception of adding the internal standard. Plates were precoated with 0.25 mm. of silica gel G[3] without fluorescent indicator. Plates were then impregnated according to the procedure described by Korte and Sieper (14), using dimethylformamide in carbon tetrachloride at a ratio of 6:4. Development was in saturated chambers to a height of 15 cm. above the starting point. The temperature of the room used for thin-layer work was maintained at 20 ± 1°. Relative humidity was maintained at 50–60% by a dehumidifier[4]. The solvent systems were: (1) cyclohexane (14), using the procedure described by Merkus (15); (2) hexane–toluene (3:2); (3) hexane–xylene (3:1); (4) hexane–xylene–acetone at various ratios; (5) ethyl acetate–hexane (1:1) (16); and (6) petroleum ether–ether (4:1) as described by Machata (17). Fast Blue Salt B[5] was the spray reagent (0.5% aqueous solution).

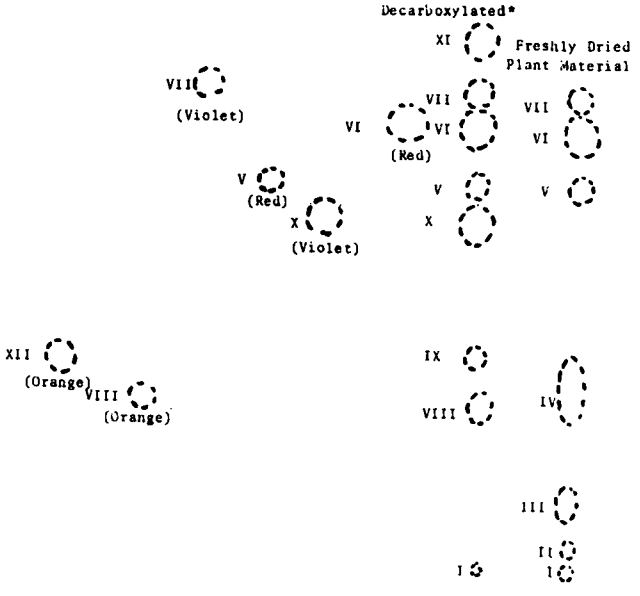

Figure 1—*Thin-layer chromatogram of South African (A-A) C. sativa L. plate was impregnated with dimethylformamide and developed twice with petroleum ether–ether (4:1). Spray reagent was Fast Blue Salt B, 0.5% aqueous solution. Key: I, origin; II, cannabigerolic acid (orange); III, (−)-Δ^9-trans-tetrahydrocannabivarinic acid (red); IV, (−)-Δ^9-trans-tetrahydrocannabinolic acid (red); V, (−)-Δ^9-trans-tetrahydrocannabivarin (red); VI, (−)-Δ^9-trans-tetrahydrocannabinol (red); VII, cannabichromene (violet); VIII, cannabigerol (orange); IX, cannabivarin (violet); X, cannabinol (violet); XI, cannabicyclol (red); and XII, cannabidiol (orange). (* Material was prepared by heating the plant material at 100° for 1 hr.)*

Table II—Relative Retention Times of Cannabinoids[a]

	Relative Retention Time[b]
Olivetol	0.04
Cannabidivarin	0.18
Tetrahydrocannabivarin	0.26
Cannabicyclol	0.26
Cannabichromene	0.34
Cannabivarin	0.34
Cannabidiol	0.34
Cannabigerol monomethyl ether	0.38
$\Delta^{9,11}$-Tetrahydrocannabinol (exocyclic)	0.41
Δ^8-Tetrahydrocannabinol	0.44
Δ^9-Tetrahydrocannabinol	0.49
Cannabigerol	0.57
Cannabinol	0.63
4-Androstene-3,17-dione	1.00
Unknown	1.18
Unknown	1.29
Unknown	1.38

[a] 4-Androstene-3,17-dione was used as the internal standard. [b] Relative retention times were calculated using synthetic cannabinoids and Cannabis plant material. Column conditions were described previously under *Methods* section.

GC–Mass Spectrometry—A gas chromatograph[6], equipped with a hydrogen flame-ionization detector, in combination with a high-resolution mass spectrometer[7], equipped with a jet separator for sample enrichment, was utilized. A stainless steel column, 0.31 cm. (0.125 in.) o.d. × 1.52 m. (5 ft.) packed with 2% OV-17 on Supelcoport was used as previously described. Helium was the carrier gas, and the mass spectrometer was operated at 70 ev.

RESULTS AND DISCUSSION

Cannabichromene, referred to as tetrahydrocannabinol II by Korte *et al.* (2), has been isolated and characterized by several researchers (5, 18–20). The biological activity has also been studied. Gaoni and Mechoulam (19) originally reported that cannabichromene was active in the dog assay. Later Isbell *et al.* (20) reported that they observed no activity in humans. Mechoulam *et al.* (21) concluded that, except for (−)-Δ^9-*trans*-tetrahydrocannabinol, there was no active constituent in a sample of hashish analyzed to contain 0.1% cannabichromene. To minimize conflicts in this report, only variant(s) of Cannabis from known geographical origin were investigated. In each case the three investigative methods previously described were employed.

Since most previous reports pertaining to the absence of cannabidiol and/or the presence of cannabichromene (1–4) were from parts of Africa or Brazil, it was decided that a variant of Cannabis from South Africa would best serve as a vehicle for: (*a*) ascertaining if there is a total absence of cannabidiol in a certain variant of Cannabis and (*b*) exploring the possibility of a potential "different biogenesis" for certain variants of Cannabis.

Three successive generations of South African Cannabis (UNC-255), coded as A-A, were grown in Mississippi in 1970, 1971, and 1972. Samples of the young material were taken in 1970 and 1971 prior to and after sexual maturity; in 1972, samples were taken each week. Each sample was prepared for analysis as previously described.

TLC was carried out routinely, as was GC, on fresh plant material from the 1972 plantings. The thin-layer systems previously mentioned were utilized; however, the system of petroleum ether–ether at a ratio of 4:1 gave best results in these laboratories. Plates impregnated with dimethylformamide in carbon tetrachloride at a ratio of 6:4, as described by Korte and Sieper (14), were removed from the storage tank and dried approximately 1 min. with an air blower[8] set on cool. The plate was spotted 1.5 cm. from the bottom and was then placed in the developing tank. Usually the time period from removal of the plate to immersion in the developing tank was

[3] Merck.
[4] Sears Coldspot.
[5] Merck.
[6] Varian series 1400.
[7] Du Pont 21-492.
[8] Heat-Blo.

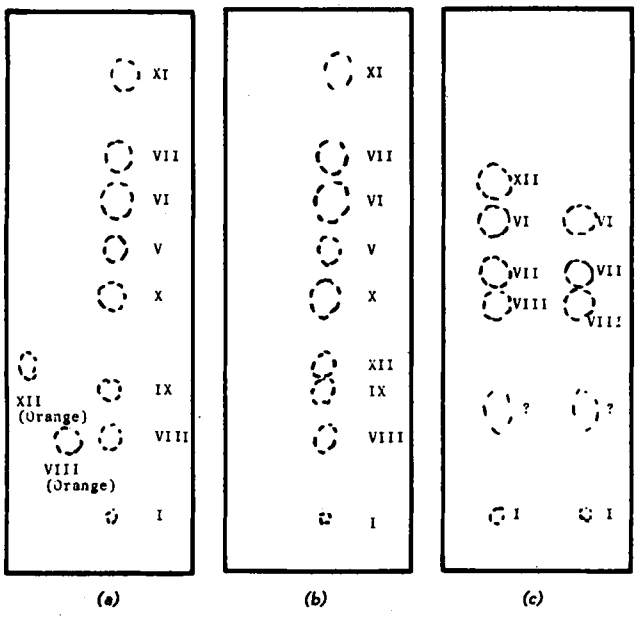

Figure 2—(a) *Thin-layer chromatogram of A-A decarboxylated plant material. Plate was impregnated with* N,N-*dimethylformamide. Solvent system was the same as described for Fig. 1.* (b) *Thin-layer chromatogram of A-A decarboxylated with synthetic cannabidiol added. Plate was developed according to procedures described for Figs. 1 and 2a.* (c) *Thin-layer chromatogram of A-A decarboxylated plant material (right) and decarboxylated plant material with synthetic cannabidiol (left). Plate was developed as described for preceding figures. Plate was not impregnated with* N,N-*dimethylformamide.*

no more than 10 min. The plates were developed one time, air dried for 1 min., replaced in the chamber, and developed a second time with subsequent air drying for 1 min. The plate was then sprayed with a 0.5% solution of Fast Blue Salt B in water.

Merkus (15, 22, 23) used cyclohexane as the developing solvent and developed each plate three times to obtain excellent separation. However, in our hands the petroleum ether–ether system afforded comparable separation at approximately half the time. In addition, impregnated plates in these laboratories showed no evidence to indicate destruction of the impregnation as was previously observed (3).

Evidence was found to support the contention by Merkus (15) that hydrogen bonding between the stationary phase, dimethylformamide, and the phenolic group of the cannabinoids was stronger in the *n*-propyl homologs than the *n*-amyl. Moreover, when hexane–toluene in a ratio of 55:45 was used to separate the acids of (−)-Δ^9-*trans*-tetrahydrocannabinol and (−)-Δ^9-*trans*-tetrahydrocannabivarin[9], hydrogen bonding was more pronounced in the propyl homolog. Tentative evidence indicates that the *n*-propyl homologs undergo decarboxylation slower than the *n*-amyl; however, the *n*-propyl homologs tend to oxidize easier. More data will be forthcoming on this subject.

The thin-layer chromatogram of African Cannabis, using impregnated plates with petroleum ether–ether as described by Merkus (15) and De Zeeuw *et al.* (24, 25), shows a distinct, elongated spot for (−)-Δ^9-*trans*-tetrahydrocannabinolic acid and (−)-Δ^9-*trans*-tetrahydrocannabivarinic acid. Other acids of the cannabinoids are near the origin (Fig. 1). Additionally there are spots for the neutral decarboxylated cannabinoids. Cannabichromene is located near the solvent front followed by (−)-Δ^9-*trans*-tetrahydrocannabinol and (−)-Δ^9-*trans*-tetrahydrocannabivarin. No cannabidiol is observed. Additionally, when all cannabinoids are decarboxylated to their neutral derivatives (Figs. 1 and 2*a*), cannabidiol is still undetectable. However, when cannabidiol is added to the sample, a corresponding spot is observed (Fig. 2*b*).

When utilizing the same solvent system with unimpregnated plates, cannabidiol is near the solvent front (24, 25). In Fig. 2*c*, a sample enriched with cannabidiol is compared with a sample of regular plant material. Although thin-layer evidence strongly suggests that cannabidiol is absent in African Cannabis grown in Mississippi, the sample was then subjected to further analysis by GC.

GC analyses of the first, second, and third generations of African (A-A) Cannabis showed some fluctuation in the cannabinoid content (Table I). The possibility that hybridization caused some juxtaposition of the cannabinoids cannot be overlooked, since there were no major attempts to prevent cross-pollination of the growing area. However, similar variants were grown in the same area taking advantage of the prevailing winds. Certain variants were planted prior to others to minimize further cross-pollination. Figure 3, a chromatogram of the second generation, is typical of the African variant and contains a peak corresponding to cannabidiol. The dotted overlay represents the increase in intensity of the peak normally attributed to cannabidiol when spiked with synthetic cannabidiol. At first, this would seem to indicate that cannabidiol is, indeed, present in plant material from Africa; however, Table II of the relative retention times shows cannabidiol, cannabichromene, and cannabivarin are, for all practical purposes, inseparable under the conditions stated previously. Therefore, the sample was subjected to combined GC–mass spectrometry analysis.

The mass spectra data obtained from A-A plant material is given in Table III. Spectra of synthetic cannabidiol and cannabichromene were used as standards. The absence of a significant *m/e* at 286, 260, 259, 246, 229, and 230 from the plant material is indicative of an absence of cannabidiol. Cannabidiol has an *m/e* 246 of strong intensity, which was very hard to distinguish from background noise in A-A. Present in the plant material were *m/e* 267, 257, and 243; all were found in synthetic cannabichromene but were absent or

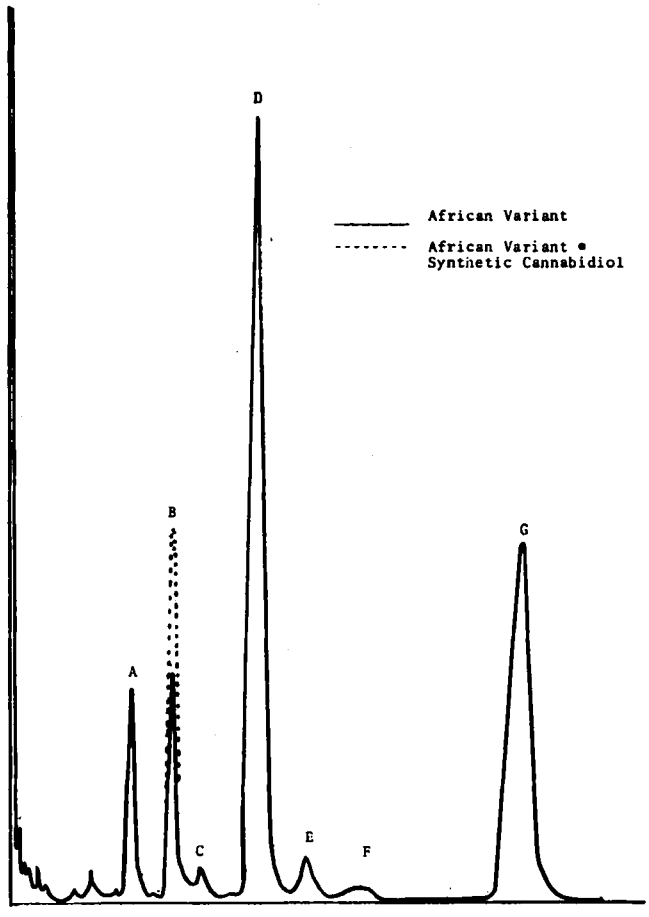

Figure 3—*Chromatogram of African* C. sativa *L. coded A-A (second generation). Key: A,* (−)-Δ^9-trans-*tetrahydrocannabivarin and/or cannabicyclol; B, cannabidiol, cannabichromene, cannabivarin, or any combination of these cannabinoids (in this variant, only cannabichromene is present); C, cannabigerol monomethyl ether; D,* (−)-Δ^9-trans-*tetrahydrocannabinol; E, cannabigerol; F, cannabinol; and G, 4-androstene-3,17-dione (internal standard).*

[9] *n*-Propyl homolog of (−)-Δ^9-*trans*-tetrahydrocannabinol.

Table III—Mass Spectra Data[a]

Compound[b]	m/e														
XII	314	299	286	271	—	260	259	258	—	246	—	232	231	230	229
VII	314	299	—	271	267	—	—	258	257	—	243	232	231	—	—
A-A	314	299	—	271	267	—	—	258	257		243	232	231	—	—

[a] Data obtained from combined GC–mass spectrometry as described under *Methods* section. [b] Key: XII, cannabidiol; and VII, cannabichromene.

insignificant in cannabidiol. The molecular ion for both cannabidiol and cannabichromene was at *m/e* 314 and the base peak was at *m/e* 231. These data correspond to published data for cannabidiol (26) and cannabichromene (5). Additional mass spectra data on the cannabinoids can be found in *References 27–31*.

Thus, TLC and GC–mass spectrometry indicate that each generation of the African variant (A-A) grown in Mississippi does not contain cannabidiol but rather contains cannabichromene, which can easily be erroneously reported as cannabidiol using only conventional GC methods. Moreover, from past experiences, TLC methods, even with pure reference standards, cannot be used independently to identify cannabinoids positively. For positive identification, it is best to use a combination of the three investigative tools described.

In continuation of the investigation of the cannabinoids in A-A, several interesting observations were made. Table I contains the percent of cannabinoids analyzed from A-A using GC after the constituents were identified by GC–mass spectrometry. As previously mentioned, the cannabinoid content does fluctuate from the first through the third generation when grown in Mississippi.

Analyses reported for the first and second generations of A-A were at 16 weeks, and the one for the third generation was at 9 weeks. Published (32) and unpublished reports from these laboratories showed that the cannabinoid content of different variants of Cannabis varies with age; thus, the fluctuation of major cannabinoids can be explained as a factor of age and/or possible hybridization. However, the increase in cannabigerol monomethyl ether from the first through the third generation cannot be adequately explained. Mass spectral data of the peak in Fig. 3 labeled cannabigerol monomethyl ether do match the data published by Yamauchi *et al.* (33). The molecular ion at *m/e* 330 corresponds with the fragmentation described by Yamauchi *et al.*

Additionally, the peak (Fig. 3) labeled (−)-Δ^9-*trans*-tetrahydrocannabivarin and cannabicyclol in this variant (A-A) was a mixture. Cannabicyclol is formed from cannabichromene (34) and could possibly be the product of cyclization in the injector port of the gas chromatograph. Moreover, cannabicyclol is formed when a fresh sample of Cannabis is heated (Fig. 1). Tentative data seem to indicate that samples containing cannabichromene and cannabidiol can be qualitatively analyzed by cyclization of the cannabichromene to cannabicyclol while cannabidiol, in these laboratories, remains unchanged.

(−)-Δ^9-*trans*-Tetrahydrocannabivarin was originally reported by Gill *et al.* (35) to be a constituent in the tincture of Cannabis. Later, Fetterman and Turner (30) reported its presence in a variant of Indian origin grown in Mississippi. (−)-Δ^9-*trans*-Tetrahydrocannabivarin has been observed in several variants grown in Mississippi and is probably present in most *C. sativa* L. samples in varying amounts. Additionally, as with most cannabinoids, the *n*-propyl homologs exist in fresh plant material as their carboxylic acid derivatives. Decarboxylation of fresh plant material containing (−)-Δ^9-*trans*-tetrahydrocannabivarinic acid, by heating at 100° for 1 hr., produced (−)-Δ^9-*trans*-tetrahydrocannabivarin and cannabivarin.

According to the published biogenesis of Cannabis (8–11), it is very improbable that cannabidiol could be absent in any plant sample; moreover, by using the three investigative tools described herein, it is highly unlikely that cannabidiol could be present and remain undetected. Thus, until adequate biogenesis experiments using tagged materials are reported, it is the contention of these authors that three possibilities exist:

1. A different biogenesis is operative in the African (A-A) and possibly other variants of Cannabis.
2. An error exists in the accepted structure of cannabichromene.
3. An enzymatic system is operative in certain variants that facilitates a concomitant allylic rearrangement of hydroxycannabigerol and cyclization of the rearranged intermediate to (−)-Δ^9-*trans*-tetrahydrocannabinol, bypassing or preventing the detection of cannabidiol. Numbers one and three are the most logical, whereas number two appears to be very remote.

SUMMARY

Cannabidiol is absent in a South African (A-A) variant of *C. sativa* L. obtained from seed stock UNC-225 furnished by the UN and grown in Mississippi. Three investigative tools were employed in a concerted effort to obtain physical data on the presence of cannabidiol without any positive results. Cannabichromene was found to be present in A-A and was cyclized to cannabicyclol by heating at 100° for 1 hr. When analyzed only by GC, cannabichromene, cannabidiol, and cannabivarin can easily be erroneously reported since they have the same relative retention time.

The absence of cannabidiol in a variant of Cannabis indicates the need for biogenesis experiments using tagged intermediates. The biogenesis currently accepted is not adequate without cannabidiol serving as an intermediate in the synthesis of (−)-Δ^9-*trans*-tetrahydrocannabinol.

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Production of Hypertension with Desoxycorticosterone Acetate-Impregnated Silicone Rubber Implants

H. S. ORMSBEE, III, and C. F. RYAN▲

Abstract □ A simple method for the production of drug-impregnated silicone rubber implants for sustained-release drug administration is described. This method involves incorporating the drug in unpolymerized silicone rubber, adding catalyst, and casting the drug–silicone rubber mixture in a hemicylindrical shape in a methacrylate mold. The utility of this method was investigated with desoxycorticosterone acetate. Desoxycorticosterone acetate-impregnated silicone rubber implants were inserted subcutaneously in rats, unilateral nephrectomy was performed, and the rats were maintained on 1% sodium chloride solution in place of drinking water. Systolic blood pressure was determined by an indirect tail cuff method. Rats receiving doses of desoxycorticosterone acetate, ranging from 50 to 500 mg./kg., developed hypertension within 3 weeks. Desoxycorticosterone acetate in a dose of 100 mg./kg. appeared to be most suitable for inducing sustained hypertension. The advantages of this method for the production of experimental hypertension and the general applicability of this method for sustained-release drug administration are discussed.

Keyphrases □ Desoxycorticosterone acetate-impregnated silicone rubber implants—preparation, used to induce hypertension, rats □ Silicone rubber implants, desoxycorticosterone acetate impregnated—preparation, used to induce hypertension, rats □ Hypertension—induced by desoxycorticosterone-impregnated silicone rubber implants, rats □ Implants, desoxycorticosterone acetate-impregnated silicone rubber—preparation, used to induce hypertension, rats

Dimethylpolysiloxane has been shown to be inert in the presence of many chemicals and to be well tolerated by biological tissues following intradermal or subcutaneous administration (1). For these reasons, dimethylpolysiloxane and other types of silicone rubber have found wide application as tissue prostheses and, more recently, as an experimental means of sustained-release drug administration. In 1965, Bass *et al.* (2) demonstrated a prolonged pharmacological response following the subcutaneous administration of atropine encapsulated in segments of silicone rubber tubing. Dziuk and Cook (3) reported that subcutaneous administration of melengestrol acetate encapsulated in silicone rubber tubing or sheeting was a suitable method for the inhibition of estrous in ewes. Numerous *in vitro* studies (3–5) demonstrated that a variety of steroids are able to diffuse through silicone rubber barriers.

Chang and Kincl (6) compared the effectiveness of megestrol acetate as a function of the route of administration. They observed that subcutaneous implantation of segments of silicone rubber tubing containing megestrol acetate was the most efficacious mode of administration; from 6 to 25 times less steroid was needed to produce comparable biological effects. Later, Chang and Kincl (7) presented further evidence indicating that the effectiveness of subcutaneous administration of steroids encapsulated in silicone rubber is much greater than conventional subcutaneous administration of steroids in oil.

A review of this literature suggested that a modification of these procedures might be a convenient method of drug administration for the production of experimental hypertension in rats. Studies are currently in progress in this laboratory to evaluate the antihypertensive activity of a series of grayanotoxin analogs. To evaluate antihypertensive activity properly, it is necessary to conduct the studies in hypertensive animals. Hypertension may be produced in rats by a variety of procedures. One convenient procedure involves unilateral nephrectomy, chronic administration of desoxycorticosterone acetate, and maintenance of

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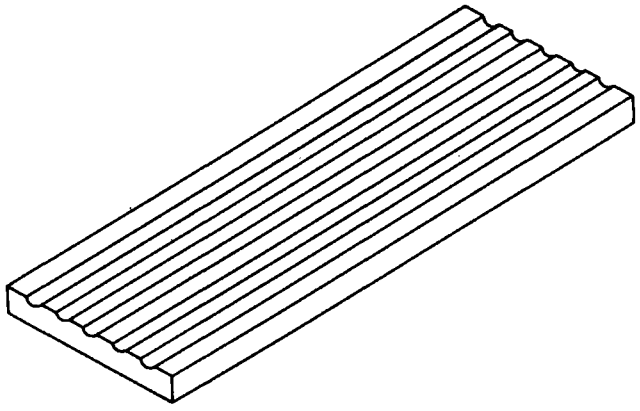

Figure 1—*Plexiglas mold used to cast silicone rubber implants. The mold is constructed of methacrylate plastic, with five hemicylindrical grooves routed in it. The number of grooves and the dimensions of the mold are not critical.*

the rats on 1% sodium chloride solution in place of drinking water. A significant degree of hypertension usually develops within 4–8 weeks. Daily administration of desoxycorticosterone acetate over this period becomes quite time consuming. For this reason, a variety of sustained-release preparations of desoxycorticosterone acetate have been evaluated, but these preparations have the disadvantage of being time consuming to prepare and/or are expensive.

This study describes a simple and convenient method for the preparation of drug-impregnated silicone rubber implants. This preparation was used to produce hypertension in rats.

EXPERIMENTAL

Construction of Mold—A silicone rubber implant of constant geometry and constant drug content was prepared by curing a silicone rubber fluid in a suitable mold. The mold was constructed from a 1.27-cm. thick sheet of methacrylate[1] plastic, 10.32 cm. wide and 31.00 cm. long (0.5 × 4.0 × 12.0 in.). The dimensions of this mold are not critical (Fig. 1). The plastic sheet was routed lengthwise to form five hemicylindrical grooves with a radius of 0.322 cm. each. The interior surfaces of these grooves were smoothly polished to facilitate removal of the cured silicone rubber. Sticking was further decreased by coating the mold with a thin film of silicone mold releasing agent[2] before each use.

Preparation of Implants—Preliminary experiments had suggested that a convenient concentration of desoxycorticosterone acetate[3] was approximately 10–100 mg./implant. Five doses of desoxycorticosterone acetate were tested, and a multiple of each dose was carefully weighed and added to a quantity of uncured silicone rubber[4] sufficient to produce a desired number of implants. The drug was thoroughly incorporated into the silicone rubber in geometric dilution by stirring with a spatula on a watch glass (15-cm. diameter).

After the drug was incorporated into the silicone rubber, the catalyst, stannous octoate[5], was added and thoroughly mixed. The addition of 48 drops of catalyst/454 g. of drug–silicone mixture provided an adequate working time of about 10 min. The mixture was then transferred into the grooves of the mold. The edge of a spatula or a glass microscope slide was used to smooth the fluid mixture into the grooves and ensure that the surface of the hemicylinder being formed was even with the surface of the mold. The silicone was allowed to cure at room temperature for 24 hr. After curing, the silicone rubber hemicylinder was stripped from the mold. The appropriate dosage of desoxycorticosterone acetate could then be easily obtained by laying the silicone rubber hemicylinder beside a ruler and cutting off a given length with a razor blade.

Table I—Desoxycorticosterone Acetate Treatment Schedule

Treatment Group	Number of Animals Treated	Number of Animals Alive at Week 11	Dose, mg./kg.
Control	7	5	0
1	9	8	1
2	11	10	10
3	8	7	50
4	10	8	100
5	8	0	500

Production of Hypertension—Male Rolfsmeyer rats, with an average weight of 144 g., were assigned to six treatment groups of a completely random design with unequal replication. Systolic blood pressure of each rat was indirectly determined at weekly intervals using a tail cuff plethysmograph apparatus[6]. After control blood pressure parameters were established, the rats were anesthetized with methoxyflurane and unilaterally nephrectomized using aseptic technique. A 1-cm. transverse incision was made through the skin just anterior to the scapulae, and a 5-cm. silicone rubber implant was inserted subcutaneously. The implant was positioned with its flat surface parallel to the spine on the dorsal musculature of the rat, and the incisions were closed with wound clips. The treatment schedule is presented in Table I.

The rats were randomly housed in individual cages, with free access to commercial rat food and to 1% sodium chloride solution in place of drinking water. It was necessary to add tetracycline (5 g./l.) to the sodium chloride solution at periodic intervals to prevent the development of chronic respiratory disease. The rats received the tetracycline–sodium chloride solution during Weeks 2, 4, 6, and 8. During Weeks 0, 1, 3, 5, 7, 9, and 10, the rats received sodium chloride drinking water only.

The data were analyzed by an analysis of variance for a completely random design with unequal replication (8). Statistical significance was determined using Waller–Duncan's (9) multiple-comparison procedure for a "k" ratio of 100:1, which corresponds to the conventional 5% level of significance. This method was used to test the data within each week.

Rats with systolic blood pressures greater than 160 mm. of mercury were considered to be hypertensive.

RESULTS

Figure 1 illustrates a convenient mold design for producing silicone implants of the type used in this study. The results of various desoxycorticosterone acetate implant treatments are presented graphically in Fig. 2. Figure 2 presents the mean systolic blood pressure of the various treatment groups as a function of time in weeks. The major findings illustrated by Fig. 2 are:

1. Doses of desoxycorticosterone acetate ranging from 50 to 500 mg./kg. produced significant ($p < 0.05$) hypertension by Week 3 (2 weeks postimplantation).
2. Doses of desoxycorticosterone acetate of 1 and 10 mg./kg. did not produce any significant ($p > 0.05$) change in systolic blood pressure.
3. The initial rate of development of hypertension was similar after desoxycorticosterone acetate implants of 50, 100, and 500 mg./kg.

A desoxycorticosterone acetate–silicone implant containing a 100-mg./kg. dose of steroid appears to be most suitable for inducing hypertension. This dose (100 mg./kg.) produced hypertension significantly greater ($p < 0.05$) than any other dose after Week 3, except for the 500-mg./kg. dose. The hypertension produced by 100 mg./kg. desoxycorticosterone acetate was sustained throughout the entire study. The 500-mg./kg. dose level of desoxycorticosterone acetate produced hypertension which was not significantly different

[1] Plexiglas.
[2] IMS silicone spray parting agent.
[3] 21-Hydroxypregn-4-ene-3,20-dione acetate.
[4] Dow Corning Silastic 382.
[5] Dow Corning Catalyst M.
[6] Narco Biosystems, Inc.

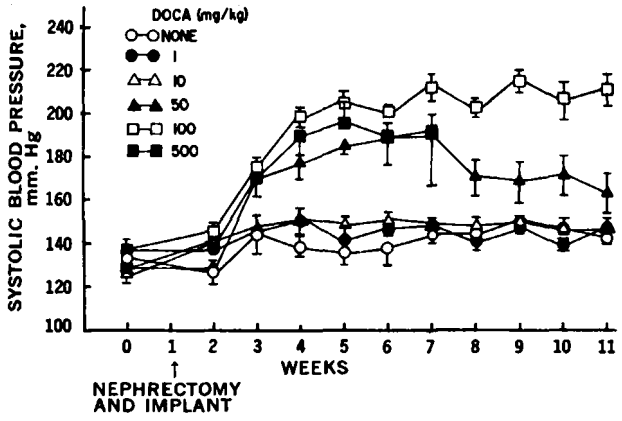

Figure 2—*Production of desoxycorticosterone acetate (DOCA)–sodium chloride hypertension in rats. The systolic blood pressure was determined indirectly at weekly intervals by tail cuff plethysmography. At Week 1, all animals were unilaterally nephrectomized and a 5-cm. silicone rubber implant was inserted subcutaneously. Five of the six treatment groups received implants containing various doses of desoxycorticosterone acetate. All animals were maintained on 1% NaCl solution in place of drinking water. The data are expressed as the mean systolic blood pressure in millimeters of mercury* ± SEM versus *time in weeks.*

($p > 0.05$) from that produced by the 100-mg./kg. dose; however, all of the rats that received the 500-mg./kg. dose were dead by Week 8 of the study.

DISCUSSION

The subcutaneous insertion of desoxycorticosterone acetate-impregnated silicone rubber implants combined with unilateral nephrectomy and maintenance on 1% sodium chloride solution is an effective means of producing hypertension in the rat. Desoxycorticosterone acetate in doses of 50-, 100-, and 500-mg./kg. produced statistically significant elevations of systolic blood pressure. Apparently, the dose of 50 mg./kg. is approximately the threshold dose for producing sustained hypertension, since some animals within this treatment group remained hypertensive while others returned to control blood pressure levels. The 100-mg./kg. dose appears to be the most suitable, because apparent metacorticoid hypertension was produced with this dose of desoxycorticosterone acetate. The highest dose was clearly unsuitable, since all of the animals treated with desoxycorticosterone acetate in doses of 500 mg./kg. died within 8 weeks. The total dose of desoxycorticosterone acetate necessary to produce a statistically significant degree of hypertension is much less than that employed in other desoxycorticosterone acetate–sodium chloride methods (10, 11).

The method described here appears to have several advantages over previously described methods of inducing desoxycorticosterone acetate–salt hypertension. The desoxycorticosterone acetate-impregnated silicone rubber implants take only minutes to prepare, are ready for implantation within 24 hr., and do not require any specialized, expensive equipment for their preparation. The molds may be made easily and inexpensively in practically any desired size or shape. Dosage adjustment is easy and reproducible.

The results presented here suggest that desoxycorticosterone acetate slowly diffuses from the silicone rubber implant to produce sustained blood levels of desoxycorticosterone acetate. The kinetics of this release are currently under investigation. The possibility of altering the kinetics of release with various adsorbents and changes in implant geometry and drug concentration are also being studied.

It is apparent that this method may have general applicability for the chronic administration of a variety of drugs. Some possibilities include the chronic administration of histamine or nicotine to induce gastric ulceration, the administration of antibiotics, use in chronic toxicity studies, and the induction of experimental drug dependence.

SUMMARY

1. A simple and inexpensive method of producing drug-impregnated silicone rubber implants was described.

2. The implants were used to produce hypertension in the rat. The method involves unilateral nephrectomy, subcutaneous insertion of a desoxycorticosterone acetate-impregnated silicone rubber implant, and maintenance of the rats on 1% sodium chloride solution in place of drinking water.

3. The general utility, advantages, and potential uses of this method were discussed.

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Tetracycline Epimerization Kinetics Utilizing NMR Spectrometry

K. D. SCHLECHT▲ and C. W. FRANK

Abstract □ NMR was used to monitor the epimerization of tetracycline since the dimethylamino resonance of tetracycline and its C-4 epimer differ by 0.1 p.p.m. This method eliminated the need for acidification in the previously reported spectrophotometric method, thereby lessening the possibility of anhydro formation which occurs in the presence of mineral acids. Anhydro formation would be readily detected *via* the NMR method.

Keyphrases □ Tetracycline epimerization—kinetics, monitored using NMR □ Epimerization, tetracycline—kinetics, monitored using NMR □ NMR spectroscopy—monitoring tetracycline epimerization kinetics

Tetracyclines were first reported to epimerize in 1955 when Doerschuk *et al.* (1) reported a reversible "isomerization" for chlortetracycline, bromotetracycline, oxytetracycline, and tetracycline[1] (I). The epimer of I was named 4-epitetracycline (II) when the reaction was shown to take place at the C-4 position (2, 3). McCormick *et al.* (4) reported that epimerization occurred in aqueous solutions of pH 2–6 in the presence of anions such as phosphate, acetate, and citrate. The reactions reached equilibrium when 20–55% of the epimer was produced. The epimers were found to have substantially lower antibiotic activity against a large variety of tetracycline-susceptible microorganisms.

In 1963, Remmers *et al.* (5) reported additional kinetic data on the epimerization in the presence of phosphate and citrate. In 1968, Hussar *et al.* (6) published the results of their studies in the presence of acetate. Both these reports made use of a spectrophotometric absorbance ratio technique very similar to the one first reported by McCormick *et al.* (4). The technique is based on the difference in molar absorptivity (ϵ) of I (approximately 19,000) and II (approximately 15,000) at 267 nm., as well as the presence of an isosbestic point at 254 nm. Aliquots of the reaction mixtures are acidified and then absorbances are measured. The results of some previous work (7) indicate that small amounts of anhydrotetracycline (III) may form when solutions of I are acidified. Compound III has a very high ϵ (approximately 40,000) at 272 nm., so even small amounts of III could seriously affect the spectrophotometric method of assay.

NMR spectra of various tetracyclines were obtained and studied to determine if NMR would be a useful method of monitoring the kinetics of epimerization. Von Wittenau and Blackwood (8) first reported a difference in the chemical shifts of the dimethylamino group in I and II of about 0.1 p.p.m. in pyridine solvent. The NMR method could eliminate the need for acidification of reaction aliquots. Small amounts of III should be easily detected due to its significantly different structure, yet this should not significantly influence the kinetic monitoring.

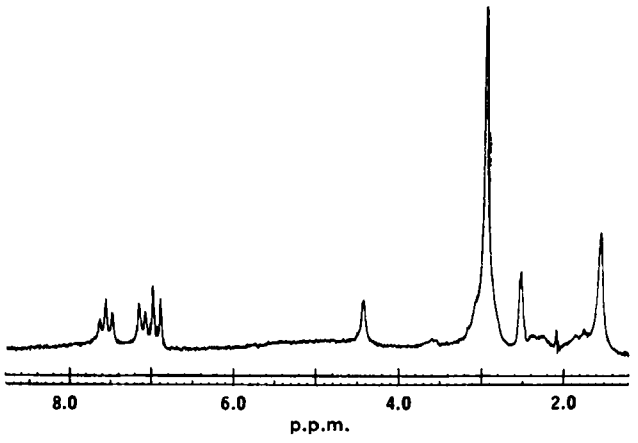

Figure 1—*NMR spectrum of tetracycline hydrochloride in dimethyl sulfoxide-*d_6.

EXPERIMENTAL

The I-HCl and dedimethylaminotetracycline (IV) were obtained from a commercial source[2]. Compound II was prepared by a method very similar to that reported by McCormick *et al.* (4); 4.0 g. of I-HCl was dissolved in isopropanol–methanol–hydrochloric acid (4:1:2), heated to 70° for at least 0.5 hr., cooled, and filtered. Recrystallization from methanol–hydrochloric acid (30:1) yielded yellow needlelike crystals which were dried under vacuum for 2 days, yielding 1.91 g. of crystals (m.p. 219–222° dec.). The product was found to be pure by TLC. The TLC method used acid-washed Kieselguhr (MN) slurried with 5% calcium disodium edetate (EDTA) at pH 7.5, as reported by Fernandez *et al.*(9). Compound I (base) was prepared by dissolving I-HCl in water near saturation and

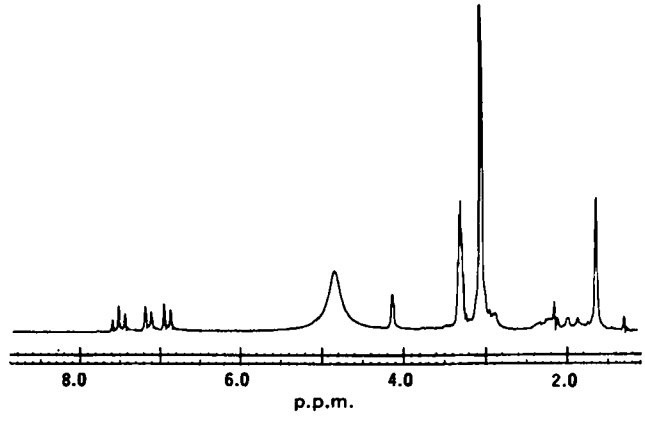

Figure 2—*NMR spectrum of tetracycline hydrochloride in methanol-*d_4.

[1] 4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide.

[2] Chas. Pfizer and Co.

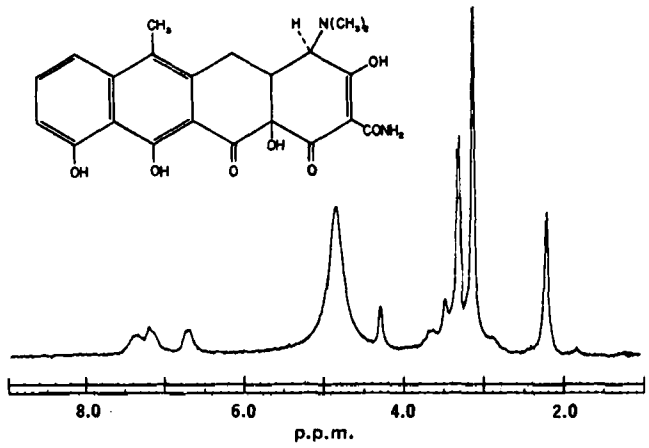

Figure 3—*NMR spectrum of tetracycline (base) in acetone-*d_6.

then adjusting to pH 5.5 with aqueous ammonia. The resulting precipitate was filtered, washed three times with water, and dried under vacuum at 60° (m.p. 175° dec.).

NMR spectra were obtained on a spectrometer[3]. Deuterated solvents were obtained from a commercial source[4]. Tetramethylsilane was used as an internal reference and lock. The solvent used for epimerization studies was a mixture of methanol-d_4 and D_2O, approximately 0.5 *M* in sodium dihydrogen phosphate. More specifically, 1 *M* NaH_2PO_4 was prepared in D_2O and then 1 ml. of this solution was mixed with 0.884 g. methanol-d_4. Approximately 30 mg. of I-HCl was dissolved in 1 ml. of this solvent. If the solvent composition differed significantly from that described here, either the desired concentration could not be achieved or a I-phosphate complex would precipitate (2). The apparent pH's of the reaction mixtures were determined using a semimicro combination electrode[5] and a pH meter[6] standardized with pH 4.00 buffer. They ranged from 4.05 to 4.10.

The NMR spectra during epimerization were obtained and integrated on the spectrometer. Tetramethylsilane sealed in a drawnout melting-point capillary was used as an external lock signal. Reproducible lock signals were obtained with such an external reference; however, each sealed capillary provided different lock signals relative to each other. The chemical shifts of benzene and chloroform were determined with each tetramethylsilane capillary, and each was corrected to the literature values for these compounds.

Prepared reaction mixtures were placed in an NMR sample tube and left in the probe of the instrument until equilibrium was approached. The temperature was maintained by a variable temperature probe unit[7]. The actual temperature was determined by measuring the difference in chemical shifts of the hydroxyl and methylene protons in ethylene glycol. Recent literature (10–12) indicated

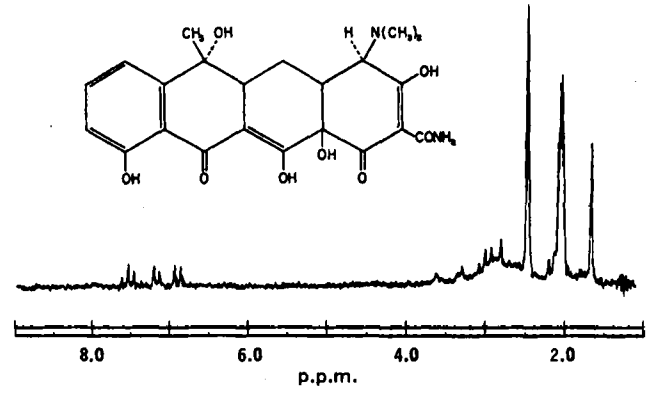

Figure 4—*NMR spectrum of anhydrotetracycline in methanol-*d_4.

[3] Varian HA-100 spectrometer.
[4] Stohler Isotopes.
[5] Corning.
[6] Fisher Accumet model 210.
[7] Varian.

Table I—Chemical Shifts (p.p.m.) of Some Protons of Tetracyclines

Compound	Solvent	CH_3 (C-6)	$N(CH_3)_2$ (C-4)	H (C-7)	H (C-8)	H (C-9)
I-HCl	Dimethyl sulfoxide-d_6	1.55	2.94	7.11	7.55	6.93
I-HCl	Methanol-d_4	1.62	3.05	7.13	7.50	6.89
I (base)	Acetone-d_6	1.67	2.49	7.14	7.52	6.88
IV	Acetone-d_6	1.62	—	7.13	7.51	6.88

Table II—Chemical Shifts (p.p.m.) of Anhydrotetracycline Hydrochloride (III)

Relative Concentration	CH_3 (C-6)	$N(CH_3)_2$ (C-4)
1	2.23	3.15
One-half	2.33	3.11
One-eighth	2.42	3.05

some discrepancy in the [illegible] equat[illegible] that have [illegible] for converting the temperature was used.

$$T°K. = 411.0 - 1.694\ \Delta c.p.s.\ (\text{for 60-MHz. values}) \quad (\text{Eq. 1})$$

RESULTS DISCUSSION

The NMR spectra of I-HCl were obtained in dimethyl sulfoxide-d_6 and methanol-d_4 as shown in Figs. 1 and 2, respectively. The spectrum of I (base) was obtained in acetone-d_6 as shown in Fig. 3. The data are summarized in Table I. Assignments were made using cataloged protons of similar classification (13). The difference in the chemical shifts of the two methyl groups of the C-4 nitrogen in I-HCl and I (base) can be readily explained since the methyl groups in I (base) are attached to a tertiary nitrogen in contrast to a quaternary ammonium nitrogen. The chemical shifts of IV in acetone-

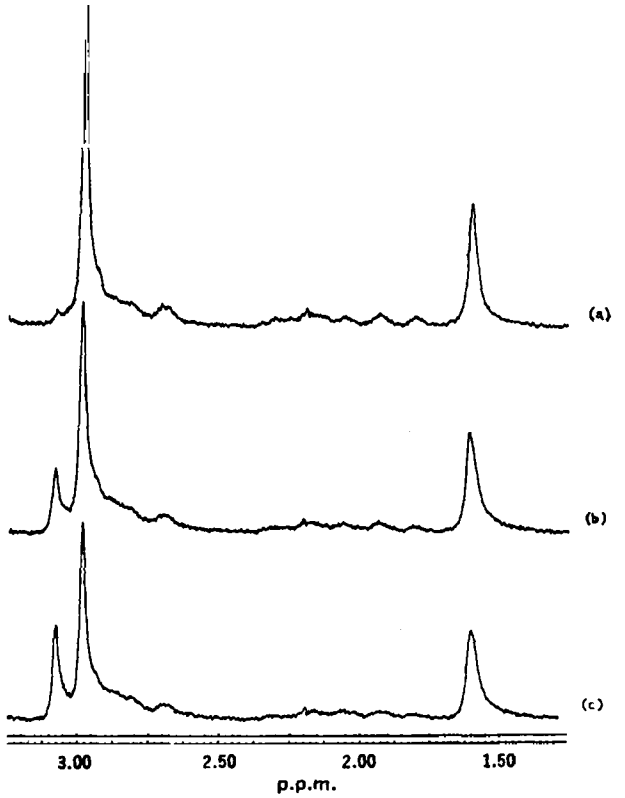

Figure 5—*NMR spectrum of tetracycline epimerization reaction mixture after* (a) *0.5,* (b) *10, and* (c) *24 hr. at 35.9°.*

Table III—Reversible First-Order Rate Constants for Epimerization of Tetracycline at Various Temperatures

Temperature	Rate Constants ($hr.^{-1}$) × 10^2		
	$k_1 + k_{-1} \pm$ Estimated *SD*	k_1	k_{-1}
52.0°	50 ± 1.1	20	30
46.2°	25 ± 0.5	10	15
35.9°	9.8 ± 0.3	3.9	5.9

d_6 are also reported in Table I. The D-ring splitting constants of all these compounds were 8 Hz.

The NMR spectrum of III-HCl in methanol-d_4 was also obtained as shown in Fig. 4. The chemical shift of the C-6 methyl appeared at approximately 2.3 p.p.m., and the two methyl groups on the C-4 nitrogen were at approximately 3.1 p.p.m. The D-ring was not well resolved; the bands were relatively broad and were not distinct doublets and triplets. It was not possible to report chemical shifts accurately for any of the protons of III since they were found to be concentration dependent. Table II lists the observed chemical shifts at various concentrations. The D-ring protons were also shifted approximately 0.3 p.p.m. downfield as the concentration was decreased, but they were the first to be lost in the background noise at low concentrations.

It is concluded that the concentration-dependent chemical shifts in III are due to an intermolecular complex, since the degree of intermolecular complexation should decrease with a decrease in concentration. Compound I was subjected to similar concentration experiments, and no significant shifts were observed. All shifts changed less than 0.05 p.p.m. over the same relative concentration range of Table II.

The NMR spectra of tetracyclines indicated that the formation of III during epimerization would be easy to detect due to the resonances of the C-6 methyl groups. Therefore, NMR was used to detect the progress of epimerization reactions in methanol-d_6–D_2O solutions in the presence of phosphate. Three typical spectra obtained at various times during a reaction are shown in Fig. 5. The C-6 methyl group signal at 1.6 p.p.m. appears to broaden slightly with time; however, this is probably due to the C-6 methyl of II which is about 1–2 Hz. upfield of the one for I. The intense signal at 2.97 p.p.m. is due to the two methyl groups of the C-4 nitrogen of I and the signal at 3.07 p.p.m. is due to the same group on II. There is no observable signal formed in the vicinity of 2.3 p.p.m., indicating no III formation.

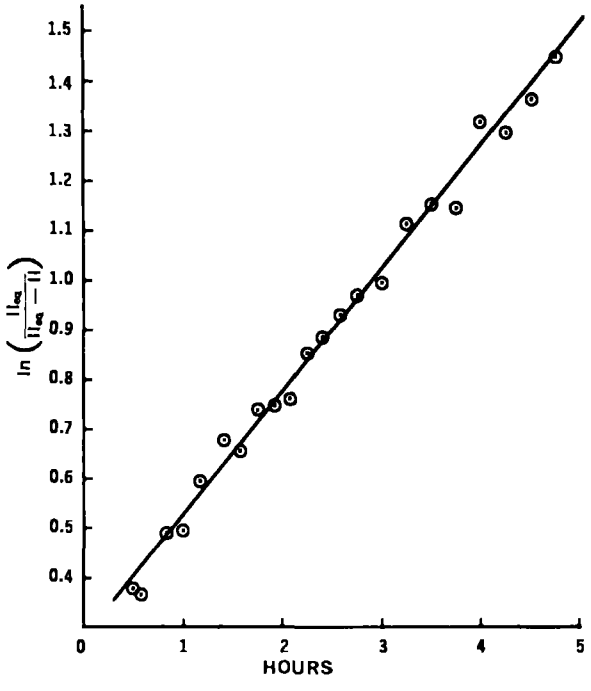

Figure 6—*Reversible first-order kinetic plot of tetracycline epimerization at 46.2°*

The various spectra were electronically integrated on the spectrometer. The area of the dimethylamino group of II was compared to the total area of the C-6 methyl groups of I and II. This would compensate for small differences in spectrum output or slight changes in tuning from spectrum to spectrum.

Reversible first-order kinetics were found to fit the data, in agreement with earlier workers (1, 4–6); a typical fit is shown in Fig. 6. A derivation of the equations used for fitting the kinetics may be found in the *Appendix*.

The equilibrium mixtures were found to contain 40% II under these experimental conditions. No significant deviations were observed with a change in temperature. This is in agreement with Remmers *et al.* (5), who reported approximately 38% epimer in 0.1 *M* phosphate at pH 4.0 in aqueous solutions. Hussar *et al.* (6) found some variation in the percent epimer at equilibrium as the temperature was varied in 0.1 *M* acetate solutions. However, in 1.0 *M* acetate, no such variations were reported.

Values obtained for k_1 and k_{-1} are listed in Table III. Interpolating the data presented by Remmers *et al.* (5) yielded reasonable correlation with these data.

An Arrhenius plot of the data in Table III yields an activation energy of 19 kcal./mole (*SD* 0.9). Remmers *et al.* (5) reported a value of 20.4 kcal./mole in 0.1 *M* phosphate at pH 4.0 in aqueous solution. Hussar *et al.* (6) also reported activation energies in aqueous solutions in this vicinity.

The values reported here using the NMR techniques compare quite favorably with the values previously reported using the absorbance ratio technique. The NMR method of monitoring the epimerization of I does not require an acidification step prior to the analysis of reaction mixtures, thereby minimizing the possibility of anhydro formation; in fact, no anhydro formation was detected. The reaction mixture can conveniently be monitored more frequently than by the previously reported methods.

APPENDIX

The following describe the first-order reversible reaction involved in the epimerization of tetracycline:

$$I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} II$$

Scheme I

$$\ln\left(\frac{II_{eq}}{II_{eq}-II}\right) = (k_1 + k_{-1})t \qquad \text{(Eq. A1}a\text{)}$$

$$\ln II_{eq} - \ln(II_{eq} - II) = (k_1 + k_{-1})t \qquad \text{(Eq. A1}b\text{)}$$

A computer program for nonreversible first-order kinetics will fit data to the following form:

$$\ln c_o - \ln c = kt \qquad \text{(Eq. A2)}$$

where c_o is a constant.

The term $\ln II_{eq}$ of Eqs. A1*a* and A1*b* is a constant like $\ln c_o$ in Eq. A2. If the term $(II_{eq} - II)$ is used in place of c in Eq. A2, the k obtained from the computer program will equal $k_1 + k_{-1}$.

The following derivation shows how to obtain k_1 and k_{-1}:

$$\frac{-d[I]}{dt} = k_1[I] - k_{-1}[II] \qquad [II] = [I]_o - [I] \qquad \text{(Eq. A3}a\text{)}$$

$$= k_1[I] - k_{-1}([I]_o - [I]) \qquad \text{(Eq. A3}b\text{)}$$

$$\frac{-d[I]}{dt} = k_1[I] - k_{-1}[I]_o + k_{-1}[I] \qquad \text{(Eq. A3}c\text{)}$$

$$= (k_1 + k_{-1})[I] - k_{-1}[I]_o \qquad \text{(Eq. A3}d\text{)}$$

$$= 0 \quad \text{at equilibrium} \qquad \text{(Eq. A3}e\text{)}$$

Therefore:

$$(k_1 + k_{-1})[I]_{eq} = k_{-1}[I]_o \qquad [I]_o = 100\%$$
$$[I]_{eq} = 100 - \%II_{eq} \qquad \text{(Eq. A4)}$$

$$\frac{k_{-1}}{(k_1 + k_{-1})} = \frac{100 - \%\Pi_{eq}}{100} \quad \text{(Eq. A5)}$$

$$k_{-1} = \frac{100 - \%\Pi_{eq}}{100}(k_1 + k_{-1}) \quad \text{(Eq. A6)}$$

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Multiple-Dose Kinetics of Oral Anticoagulants: Methods of Analysis and Optimized Dosing

THEOFANIS G. THEOFANOUS▲ and RONALD G. BARILE

Abstract □ A mathematical technique for estimating the kinetic parameters that control patient response to oral anticoagulant administration is presented. The technique utilizes routinely obtained and recorded data such as anticoagulant dose regimen and prothrombin times. It is possible to apply during any stage of therapy and to gain predictive capability from data covering the measured response over a minimum period of 2 days. The applicability of the technique is demonstrated by direct comparison with two actual patient records.

Keyphrases □ Anticoagulant therapy—estimation of kinetic response parameters using routine clinical data, equations, compared to patient records □ Multiple-dosing kinetics, oral anticoagulants—estimation of response parameters using routine clinical data, equations, compared to patient records □ Prothrombin time data—used to estimate kinetic response parameters to oral anticoagulant therapy, equations □ Dosing regimens, oral anticoagulants—determined using estimated kinetic response parameters based on routine clinical data, equations

The pharmacological effects of hypoprothrombinemic anticoagulant drugs vary widely among individuals and preclude a universal response to a fixed dose of these agents. A given dosage schedule may be totally inadequate to prevent thrombosis in one individual but may cause hemorrhage in another (1). This fact, as well as a need sometimes to readjust therapeutic levels of activity *during* therapy (1), obviously necessitates patient individualization of dosing regimens for these drugs and clearly emphasizes the need for predictive relationships between dosage regimens and the magnitude of drug response they produce.

The first and basic steps toward such an individualization are the elucidation of the basic biochemical mechanisms involved in the synthesis of prothrombin complex activity and an assessment of the relevant intrinsic kinetics. The excellent works of other researchers (2–9) cover the whole range of development of this stage, from the pioneering level up to a complete and extensive verification of the proposed kinetic model.

Following these fundamental works, the "engineering aspects" of the process remain to be developed in detail to close the gap between laboratory (controlled) studies and health care applications and thus to arrive at a reliable and convenient aid which could be used by the doctor in prescribing anticoagulant dose regimens. This study reports the results from the authors' initial efforts in this direction. In particular, the following may be recognized as two major engineering aspects:

1. *Parameter estimation.* A recent article (10) showed how the response to a *single* dose may be utilized to evaluate all of the kinetic constants of a given patient needed to *predict* his or her future behavior. In the present study, a method is developed that meets *all conditions* for practical application by allowing implementation at *any time* after the initiation of typical hos-

$$\frac{k_{-1}}{(k_1 + k_{-1})} = \frac{100 - \%\Pi_{eq}}{100} \quad \text{(Eq. A5)}$$

$$k_{-1} = \frac{100 - \%\Pi_{eq}}{100}(k_1 + k_{-1}) \quad \text{(Eq. A6)}$$

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THEOFANIS G. THEOFANOUS▲ and RONALD G. BARILE

Abstract □ A mathematical technique for estimating the kinetic parameters that control patient response to oral anticoagulant administration is presented. The technique utilizes routinely obtained and recorded data such as anticoagulant dose regimen and prothrombin times. It is possible to apply during any stage of therapy and to gain predictive capability from data covering the measured response over a minimum period of 2 days. The applicability of the technique is demonstrated by direct comparison with two actual patient records.

Keyphrases □ Anticoagulant therapy—estimation of kinetic response parameters using routine clinical data, equations, compared to patient records □ Multiple-dosing kinetics, oral anticoagulants—estimation of response parameters using routine clinical data, equations, compared to patient records □ Prothrombin time data—used to estimate kinetic response parameters to oral anticoagulant therapy, equations □ Dosing regimens, oral anticoagulants—determined using estimated kinetic response parameters based on routine clinical data, equations

The pharmacological effects of hypoprothrombinemic anticoagulant drugs vary widely among individuals and preclude a universal response to a fixed dose of these agents. A given dosage schedule may be totally inadequate to prevent thrombosis in one individual but may cause hemorrhage in another (1). This fact, as well as a need sometimes to readjust therapeutic levels of activity *during* therapy (1), obviously necessitates patient individualization of dosing regimens for these drugs and clearly emphasizes the need for predictive relationships between dosage regimens and the magnitude of drug response they produce.

The first and basic steps toward such an individualization are the elucidation of the basic biochemical mechanisms involved in the synthesis of prothrombin complex activity and an assessment of the relevant intrinsic kinetics. The excellent works of other researchers (2–9) cover the whole range of development of this stage, from the pioneering level up to a complete and extensive verification of the proposed kinetic model.

Following these fundamental works, the "engineering aspects" of the process remain to be developed in detail to close the gap between laboratory (controlled) studies and health care applications and thus to arrive at a reliable and convenient aid which could be used by the doctor in prescribing anticoagulant dose regimens. This study reports the results from the authors' initial efforts in this direction. In particular, the following may be recognized as two major engineering aspects:

1. *Parameter estimation.* A recent article (10) showed how the response to a *single* dose may be utilized to evaluate all of the kinetic constants of a given patient needed to *predict* his or her future behavior. In the present study, a method is developed that meets *all conditions* for practical application by allowing implementation at *any time* after the initiation of typical hos-

pital therapy (daily and variable dosing) and by utilizing only the *routinely obtained and recorded* dose and prothrombin history data.

2. *Multiple-dose kinetics.* After obtaining estimates of the kinetic constants for a given patient, the doctor must estimate his or her response not only to the next few doses and, therefore, appropriately adjust their level, but also to longer range dose–effect relationships. General formulas that allow such estimates are then particularly convenient for quick computations. In the present study, such general formulas are derived for two classes of dosing schedules, namely, uniform (fixed dosing at fixed frequency); and quasiuniform (a single initial loading dose followed by a uniform dosing schedule). The results for some case computations are also presented for illustration.

THEORETICAL

Formulation—The differential equation that governs the time variation of prothrombin complex activity (P) is (2):

$$\frac{dP}{dt} = -m' \ln \frac{c_p(t)}{c_{pmax}} - k_d P \qquad \text{(Eq. 1)}$$

In this equation, k_d is the degradation rate constant of P, m' characterizes the synthesis rate of P, and c_{pmax} is the concentration of anticoagulant in plasma for which the synthesis rate of P is reduced to zero. Finally, $c_p(t)$ denotes the concentration of anticoagulant in the plasma at time t. Physical logarithms (ln) are utilized so that $m' = m/2.3$, with m as introduced previously (2). Following the standard procedure, P is expressed as percent of normal throughout this paper.

If c_{pmin} is defined as the minimum effective concentration, the steady-state [$(dP/dt) = 0$] value of P, P^0, in the absence of drug is given by:

$$P^0 = \frac{m'}{k_d} \ln \frac{c_{pmax}}{c_{pmin}} \qquad \text{(Eq. 2)}$$

To a good approximation (2), the absorption of oral anticoagulant may be considered instantaneous, while its removal (*i.e.*, elimination) follows first-order kinetics, *i.e.*:

$$\frac{dc_p}{dt} = -k_r c_p \qquad \text{(Eq. 3)}$$

If at some given time, say $t = 0$, the values of c_p and P are known, say $c_p(0)$ and $P(0)$, then the values of $c_p(t)$ and $P(t)$ for any later time may be obtained by integration of Eqs. 1 and 3:

$$c_p(t) = c_p(0)e^{-k_r t} \qquad \text{(Eq. 4)}$$

$$P(t) = P(0)e^{-k_d t} - \frac{m'k_r}{k_d^2}\{1 - e^{-k_d t} - k_d t\} - \frac{m'}{k_d}\{1 - e^{-k_d t}\} \ln \frac{c_p(0)}{c_{pmax}} \qquad \text{(Eq. 5)}$$

Equation 5 applies only for as long as c_p is less than c_{pmax} and more than c_{pmin}, *i.e.*:

$$c_{pmin} \leq c_p(0)\, e^{-k_r t} \leq c_{pmax} \qquad \text{(Eq. 6)}$$

In case the lower bound is met, the integration of Eq. 1 should continue with $c_p = c_{pmin}$. In case the upper bound is met, the integration of Eq. 1 should continue after setting the first term on the right-hand side equal to zero [for as long as $c_p(t) \geq c_{pmax}$].

Estimation of Parameters—The kinetic constants, k_d, k_r, m', and c_{pmax}, that appear in Eq. 5 must be identified for the particular patient if this equation is to give predictive capability. In a typical hospital situation, the *daily* values of prothrombin complex activity are available, as well as the correspondingly administered doses of anticoagulant. Let P_i denote the value of P at the end of the ith day and c_{pi}^0 denote the increase in concentration (in milligrams per liter) corresponding to the amount of anticoagulant given at the end of the ith day. The following two points should be noted:

1. Day zero may be taken as any day within the therapy period. However, if the choice of the start of anticoagulant therapy is made, it would lead more quickly to reliable results. In any case, c_{p0}^0 should be taken as the *total* concentration (*i.e.*, drug initially present plus amount given at time zero).

2. A patient's blood sample for the determination of P is usually obtained in the morning while the anticoagulant is administered at about 1:00 p.m. However, the required value in the derivation is P_i, *i.e.*, the value of P at the same time that c_{pi}^0 is administered. This may easily be obtained by graphical interpolation (see *Results* section).

The method of parameter estimation is as follows. By applying Eq. 5 for the end of the 1st day ($t = 1$ day), an estimate of P_1 is given by:

$$P_1 \text{ (estimated)} = P_0 e^{-k_d} - E - F \ln \frac{c_{p0}^0}{c_{pmax}} \qquad \text{(Eq. 7)}$$

where, for brevity, we have set:

$$E = \frac{m'k_r}{k_d^2}\{1 - e^{-k_d} - k_d\} \qquad \text{(Eq. 8)}$$

$$F = \frac{m'}{k_d}\{1 - e^{-k_d}\} \qquad \text{(Eq. 9)}$$

This estimate must be compared with the experimental value P_1, the "error" given by:

$$\epsilon_1 = P_0 e^{-k_d} - E - F \ln \frac{c_{p0}^0}{c_{pmax}} - P_1 \qquad \text{(Eq. 10)}$$

By applying Eq. 5 over the 2nd day, together with the help of Eq. 4, an estimate of P_2 is given by:

$$P_2 \text{ (estimated)} = P_1 e^{-k_d} - E - F \ln \frac{c_{p0}^0 e^{-k_r} + c_{p1}^0}{c_{pmax}} \qquad \text{(Eq. 11)}$$

The error over the 2nd day is given by:

$$\epsilon_2 = P_1 e^{-k_d} - E - F \ln \frac{c_{p0}^0 e^{-k_r} + c_{p1}^0}{c_{pmax}} - P_2 \qquad \text{(Eq. 12)}$$

This procedure can be continued for any number, n, of days for which data P_i and c_{pi}^0 are available; the values of k_d, k_r, m', and c_{pmax} for which the overall error is minimized are then sought. One choice of a measure of the overall error is the sum of the squares of the error committed in each day. Clearly, other choices are possible. The problem is then to find values for k_r, k_d, m', and c_{pmax} that minimize:

$$\Phi \equiv \sum_{i=1}^{n} \epsilon_i^2 \qquad \text{(Eq. 13}a\text{)}$$

or:

$$\Phi \equiv \sum_{i=1}^{n} (P_{i-1}^2 e^{-2k_d} + P_i^2) + nE^2 + F^2 \sum_{g=0}^{n-1} \ln^2 \sum_{g=i+j}^{i,j\geq 0} (c_{pi}^0/c_{pmax})e^{-ik_r} + 2F \sum_{g=0}^{n-1} [E - P_g e^{-k_d} + P_{g+1}] \ln \sum_{g=i+j}^{i,j\geq 0} (c_{pi}^0/c_{pmax})e^{-ik_r} + 2E \sum_{i=1}^{n} (P_i - P_{i-1}e^{-k_d}) - 2e^{-k_d} \sum_{i=1}^{n} P_i P_{i-1} \qquad \text{(Eq. 13}b\text{)}$$

The notation $\Sigma_{g=i+j}^{i,j\geq 0}$ means to form the indicated sum with all possible combinations of positive or zero i and j but such that $i + j$ equals the current value of g. As an illustration, the expression for Φ is given in the *Appendix*, without the summation notation, for the case of $n = 3$.

A large number of search methods may be applied to find the set of constants that minimizes Φ. However, since upper and lower

bounds on the possible values of them are available (*i.e.*, for the commonly utilized warfarin, $0 < k_r < 1, 0 < k_d < 5, 10 < m' < 100$, and $1 < c_{p\max} < 20$ constitute ranges within which almost certainly the constants of any individual must lie; as more experience is gained, revision of these bounds might become evident), the complete scan by evaluating Φ for all possible combinations of these parameters within the range of their expected values provides the most straightforward procedure for choosing the set that minimizes the overall error (Φ). This particular technique was utilized for the calculations presented in this paper. Some operational details for its implementation are given in the *Results and Discussion* section.

Naturally, a larger value of n yields a more reliable and precise evaluation of the kinetic constants. In principle, a minimum value of $n = 2$ seems adequate for a tentative first estimate. As more data become available during therapy, the minimization of Φ should be repeated for continuously improving all previous estimates.

General Solutions—First consider a quasiuniform dosing schedule. An initial ($t = 0$) loading dose corresponding to an increase in concentration of $c_{p0}^{(l)}$ is given and is followed by maintenance doses of fixed magnitude equivalent to $c_{p0}^{(m)}$. The time interval between dosing is kept fixed at τ days. Usually $\tau = 1$ day for anticoagulant therapy, but the more general case is considered where τ is arbitrary. No drug is present in the plasma previous to the administration of the loading dose, and the initial value of P (steady-state value) is P^0. By successive application of Eqs. 4 and 5 and generalization, the value of P is obtained at the end of the nth period (each of length τ). This value is denoted by $P_n(\tau)$ and is given by:

$$P_n(\tau) = P^0 e^{-nk_d\tau} - \frac{m'k_r}{k_d^2}(1 - e^{-k_d\tau} - k_d\tau)S_d^{(n)} - \frac{m'}{k_d}(1 - e^{-k_d\tau})R_n(\tau) \quad \text{(Eq. 14)}$$

where:

$$R_n(\tau) = \ln\left\{\left[\frac{c_{p0}^{(m)}}{c_{p\max}}\right]^{S_d^{(n)}} \lambda_r^{(n)}[\lambda_r^{(n-1)}]^{e^{-k_d\tau}}[\lambda_r^{(n-2)}]^{e^{-2k_d\tau}} \ldots [\lambda_r^{(1)}]^{e^{-(n-1)k_d\tau}}\right\} \quad \text{(Eq. 15)}$$

and:

$$\lambda_r^{(n)} = S_r^{(n)} + \left(\frac{c_{p0}^{(l)}}{c_{p0}^{(m)}} - 1\right)e^{-(n-1)k_r\tau} \quad \text{(Eq. 16)}$$

$$S_r^{(n)} = \sum_{j=0}^{n-1} e^{-jk_r\tau} \quad \text{(Eq. 17a)}$$

$$S_d^{(n)} = \sum_{j=0}^{n-1} e^{-jk_d\tau} \quad \text{(Eq. 17b)}$$

The drug concentration at the end of the nth period and just after the administration of the $(n + 1)$st dose is given by:

$$c_{pn}(\tau) = (c_{p0}^{(l)} - c_{p0}^{(m)})e^{-k_r n\tau} + c_{p0}^{(m)}S_r^{(n+1)} \quad \text{(Eq. 18)}$$

The variation of P within the $(n + 1)$st period is denoted by $P_{n+1}(t)$ and may be calculated from:

$$P_{n+1}(t) = P_n(\tau)e^{-k_d t} - \frac{m'k_r}{k_d^2}(1 - e^{-k_d t} - k_d t) - \frac{m'}{k_d}(1 - e^{-k_d t})\ln\frac{c_{p0}^{(m)}S_r^{(n+1)} + (c_{p0}^{(l)} - c_{p0}^{(m)})e^{-nk_r\tau}}{c_{p\max}} \quad \text{(Eq. 19)}$$

In Eq. 19, t measures time *within* a period, so $0 < t < \tau$.

Finally, with the use of Eq. 2, Eq. 14 may be written in a normalized form:

$$\frac{P_n(\tau)}{P^0} = e^{-nk_d\tau} - \frac{1}{\ln(c_{p\max}/c_{p\min})}\left\{\frac{k_r}{k_d}(1 - e^{-k_d\tau} - k_d\tau)S_d^{(n)} + (1 - e^{-k_d\tau})R_n(\tau)\right\} \quad \text{(Eq. 20)}$$

From this last equation, it is seen that the decrease in P/P^0 is independent of m' and depends only on the dimensionless groups: τk_d, τk_r, $c_{p\max}/c_{p\min}$, $c_{p0}^{(m)}/c_{p\max}$, and $c_{p0}^{(l)}/c_{p\max}$. This observation might prove important not only for convenience of data (or calculation)

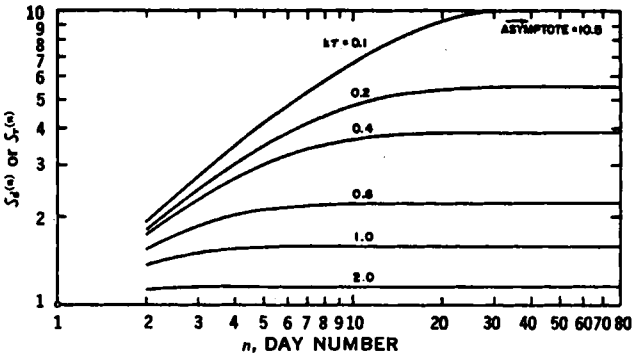

Figure 1—*Values of* $S_d^{(n)}$ *or* $S_r^{(n)}$ *for different values of* $k\tau$ *and* n.

presentation but also for achieving a more generalized approach to actual data analysis. This topic is under current investigation.

Clearly, the results for a uniform dosing schedule ($c_{p0}^{(m)}$) are contained in Eqs. 14–19 and may explicitly be obtained from them by simply letting $c_{p0}^{(l)} = c_{p0}^{(m)}$. This point is not further elaborated here because these results were also given explicitly previously (10).

It might appear that these equations are cumbersome to use for predictions over extensive periods of time (large n). Fortunately, however, they may be considerably simplified for large n to their asymptotic form:

$$P_\infty(\tau) = -\frac{m'k_r}{k_d^2}\left\{1 - \frac{k_d\tau}{1 - e^{-k_d\tau}}\right\} - \frac{m'}{k_d}\ln\frac{c_{p0}^{(m)}}{c_{p\max}(1 - e^{-k_r\tau})} \quad \text{(Eq. 21)}$$

Or, in normalized form:

$$\frac{P_\infty(\tau)}{P^0} = \frac{-1}{\ln(c_{p\max}/c_{p\min})}\left\{\frac{k_r}{k_d}\left[1 - \frac{k_d\tau}{1 - e^{-k_d\tau}}\right] + \ln\frac{c_{p0}^{(m)}}{c_{p\max}(1 - e^{-k_r\tau})}\right\} \quad \text{(Eq. 22)}$$

In this asymptotic region, the variation of P within any period is given by:

$$P_\infty(t) = P_\infty(\tau)e^{-k_d t} - \frac{m'k_r}{k_d^2}(1 - e^{-k_d t} - k_d t) - \frac{m'}{k_d}(1 - e^{-k_d t})\ln\frac{c_{p0}^{(m)}}{c_{p\max}(1 - e^{-k_r\tau})} \quad \text{(Eq. 23)}$$

Clearly, the asymptotic results apply for both uniform or quasiuniform dosing or for any case in which a constant dosing schedule is maintained for an extensive time. For convenience of computations, the values of the S's for different values of n and for the common values of $k_r\tau$ and $k_d\tau$ are given in graphical form in Fig. 1. Interpolation may easily be used for intermediate values of the arguments.

Provided the values of the kinetic constants for a particular individual have been determined, the equations presented in this sec-

Table I—Data for Patient W.F., Male, Age 58, Weight 88.9 kg. (196 lb.)

Date	Prothrombin Time, sec.	P, %	Sodium Warfarin Dose, mg.
8–13–71 (admitted)	—	—	20 (10:50 p.m.)
8–14–71	13.0	83 (9:00 a.m.)	10 (1:00 p.m.)
8–15–71	20.5	32 (9:00 a.m.)	5 (1:00 p.m.)
8–16–71	24.1	25 (9:00 a.m.)	5 (1:00 p.m.)
8–17–71	27.0	22 (9:00 a.m.)	3 (1:00 p.m.)
8–18–71	30.3	21 (9:00 a.m.)	0 (1:00 p.m.)
8–19–71	23.6	26 (9:00 a.m.)	5 (1:00 p.m.)
8–20–71	20.6	36 (9:00 a.m.)	5 (1:00 p.m.)
8–21–71 (discharged)	23.5	26 (9:00 a.m.)	4 (1:00 p.m.)

Table II—Data for Patient E.F., Female, Age 81, Weight 59.1 kg. (130 lb.)

Date	Prothrombin Time, sec.	P, %	Sodium Warfarin Dose, mg.
8–11–71 (admitted)	12.5	100 (9:00 a.m.)	0
8–12–71	12.1	97 (9:00 a.m.)	15 (1:00 p.m.)
8–13–71	14.1	62 (9:00 a.m.)	15 (1:00 p.m.)
8–14–71	28.8	22 (9:00 a.m.)	5 (1:00 p.m.)
8–15–71	32.3	16 (9:00 a.m.)	0 (1:00 p.m.)
8–16–71	27.1	22 (9:00 a.m.)	2 (1:00 p.m.)
8–17–71	25.5	23 (9:00 a.m.)	2 (1:00 p.m.)
8–18–71	24.0	28 (9:00 a.m.)	2 (1:00 p.m.)
8–19–71	21.0	31 (9:00 a.m.)	5 (1:00 p.m.)
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.	.	.	.
9–4–71 (discharged)	25.0	24 (9:00 a.m.)	5 (9:00 a.m.)

tion enable one to *predict* the complete response [*i.e.*, variation of prothrombin complex activity (P) and drug plasma concentration (c_p) with time] to any choice of a loading dose ($c_{p0}^{(l)}$), maintenance dose ($c_{p0}^{(m)}$), and dosing interval (τ). Such capability would greatly facilitate the choice of an appropriate dosing regimen in accordance with the response sought.

RESULTS AND DISCUSSION

Application of Theory to Actual Hospital Cases—Recent, routinely taken, patient records from a Midwestern hospital were utilized for assessing the applicability of the theoretical results. The pertinent data arising from sodium warfarin therapy for two randomly selected cases are given in Tables I and II. For brevity, only the first 9 days of data are shown for Patient E.F. (Table II). As remarked later, the evaluations were found equally applicable to those data omitted from Table II (8–20 to 9–4). The dosing is illustrative of that presently utilized in practice (trial-and-error procedure). Data became available after the patient discharge so as to assure no feedback influence to patient care from the results, which are still to be considered experimental in nature. Spontaneous changes in P and interaction effects with other drugs are not unknown (11). From the data examined, such apparent effects were easily recognized and were not infrequently encountered. Presently, only qualitative information exists on such not-well-behaved cases. Those cases in which such effects were not pronounced enough to be recognizable by a qualitative examination of the data were characterized as well behaved. The two case studies reported here represent a random selection from the class of well-behaved cases.

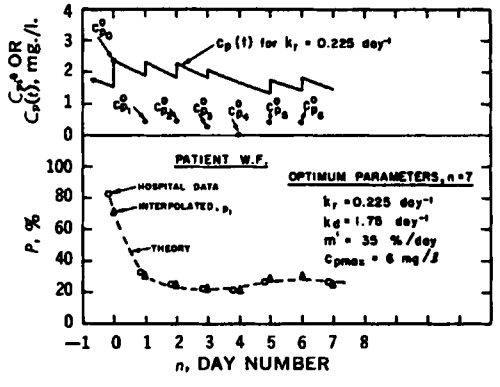

Figure 2—*Comparison of theoretical and experimental variation of* P *with time for Patient W.F. Upper half: magnitude of jumps in plasma level concentrations following each dose administration* (●). *The continuous line represents the resulting plasma concentration due to the combination of indicated drug administration and elimination (with a rate constant* k_r*). Lower half: comparison between experimental and predicted (with the kinetic constants as indicated) "prothrombin complex activity" variation with time.*

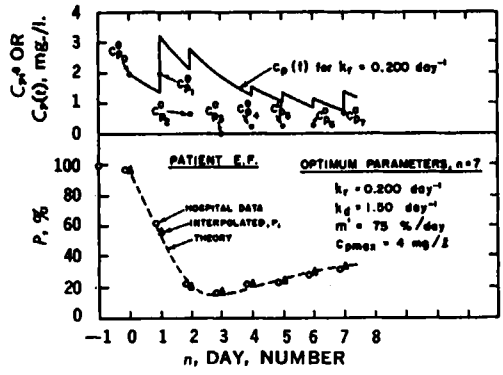

Figure 3—*Comparison of theoretical and experimental variation of* P *with time for Patient E.F.*

By taking the apparent volume of distribution as 0.13 l./kg. body weight (2), the values of $c_{p_i}^0$ were deduced and are shown in Figs. 2 and 3. Thus, c_{p1}^0, c_{p2}^0, c_{p3}^0, *etc.*, represent *increases* in plasma concentration due to dosing. On the same figures the actual data obtained for P are represented by circles. By drawing a smooth curve (not shown on figures) between these circles, the values of P_i were obtained by interpolation (triangles). Notice that for the case of Patient W.F., the absence of a P reading upon admission (P^0) necessitated taking the zero time for the evaluations as shown. The experimental point corresponding to the date 8–20 (n = 6) was omitted from Fig. 2 since it was abnormally high, and a check with other analyses recorded on this particular date indicated a systematic deviation in the P determination of an approximate magnitude of +10% of normal.

Minimizations of Φ for different values of n were performed (all having the same zero time), and the resulting best estimates of k_r, k_d, m', and c_{pmax} are summarized in Tables III and IV. The arithmetic for evaluating Φ as given by Eq. 13 was programmed for a digital computer. For each n, a sequential computation of Φ is afforded for all possible combinations of values of the parameters (k_r, k_d, m', and c_{pmax}) as they obtain values at prescribed increments within prescribed ranges. The expected ranges are given in the *Estimation of Parameters* section and were found to be indeed realistic. The increments utilized were as follows: 0.025 for k_r, 0.25 for k_d, 5 for m', and 1 for c_{pmax}. Clearly, a more refined mesh is possible but, in view

Table III—Optimum Kinetic Constants for Patient W.F.

n[a]	$\sqrt{\frac{\Phi}{n}}$[b]	k_r, day^{-1}[c]	k_d, day^{-1}[d]	m', %/Day[e]	c_{pmax}, mg./l.[f]
2	0.090	0.30	1.75	25	9
3	0.098	0.025	2.25	35	12
4	0.24	0.075	2.25	45	8
5	0.92	0.175	1.75	45	5
6	1.22	0.225	1.50	45	9
7	1.56	0.225	1.75	35	6

[a] Time length (in days) for which data were utilized in the computation. [b] Average error between theory and experiment. [c] Drug elimination rate constant. [d] Prothrombin complex activity degradation rate constant. [e] Constant characterizing synthesis rate of P, introduced in Eq. 1. [f] Concentration of anticoagulant in plasma for which the synthesis rate of P is zero.

Table IV—Optimum Kinetic Constants for Patient E.F.

n[a]	$\sqrt{\frac{\Phi}{n}}$[b]	k_r, day^{-1}[c]	k_d, day^{-1}[d]	m', %/Day[e]	c_{pmax}, mg./l.[f]
2	0.0028	0.075	1.25	65	4
3	1.03	0.375	1.25	75	3
4	1.83	0.200	1.50	80	4
5	2.08	0.200	1.50	75	4
6	1.88	0.200	1.50	75	4
7	1.76	0.200	1.50	75	4

[a–f] See footnotes in Table III.

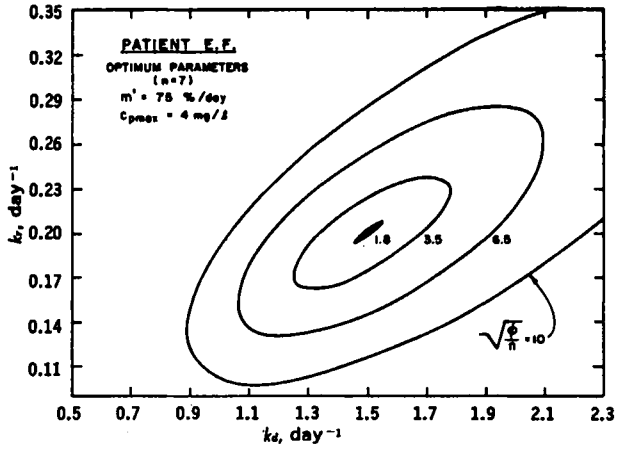

Figure 4—*Sensitivity plot for the determination of kinetic constants. The number on each ellipse indicates the maximum mean error expected between theory and experiment for* any *combination of* k_r *and* k_d *within the same ellipse and values of* m' *and* c_{pmax} *as shown on the figure.*

of the good comparisons with the experimental data thus obtained and the sensitivity properties discussed below, it appears unwarranted.

From the values of Φ thus obtained, the minimum is selected and the corresponding values of the k_r, k_d, m', and c_{pmax} then constitute the "optimum parameters." In Tables III and IV the value of $\sqrt{\Phi/n}$ is also shown since, in view of the manner in which Φ is being calculated, the former gives a measure of the "average" error between theory and experiment for the indicated choice of the kinetic constants. The value of $\sqrt{\Phi/n}$ reflects experimental (random) errors in the determination of P, as well as intrinsic variations in time of the kinetic constants that are not accounted for in the kinetic model. The detailed variation of $\sqrt{\Phi/n}$ with n seen in these tables is thus not significant. It is significant, however, that $\sqrt{\Phi/n}$ remains consistently small in all cases, a fact that testifies to both small errors in experimental data as well as to the suitability of the analytical technique and the accuracy of the kinetic model utilized. A minimal fluctuation (with increasing n) on the optimum values of the parameters is also observed. In view of the maintained smallness of the average error, this fluctuation is insignificant and the fit is always extremely good.

The comparisons between experimental data and prediction (dashed line), utilizing the optimum parameters obtained for $n = 7$, are shown in graphical form in Figs. 2 and 3. The theoretically obtained time variation of anticoagulant concentration for each case is also shown on the upper half of the figures with a continuous line. The see-saw appearance of these lines results from the consecutive

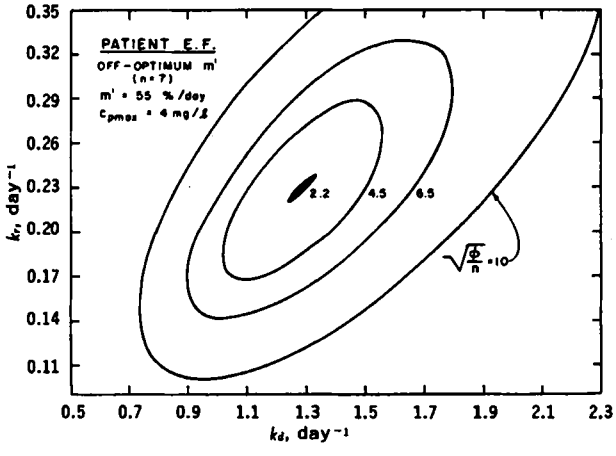

Figure 5—*Change in sensitivity and in "best" values of* k_r *and* k_d *for Patient E.F. resulting from a decrease of the optimum value of* m' *by 25%.*

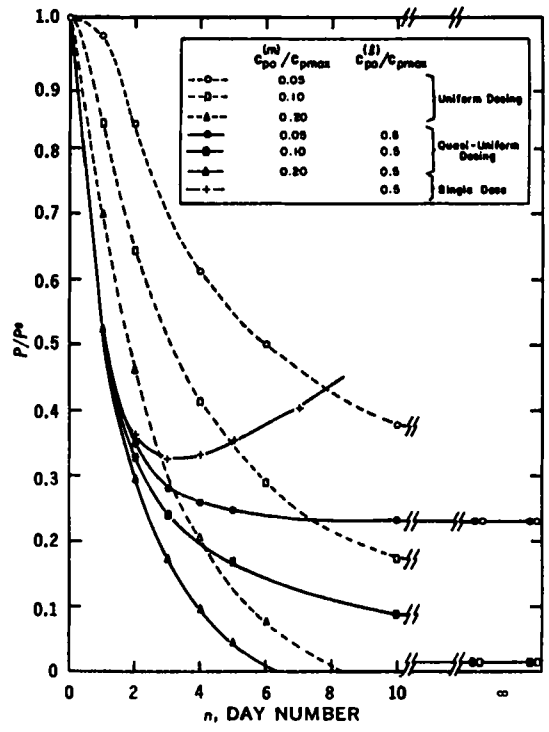

Figure 6—*Comparison in variation of* P/P^0 *with time for uniform and quasiuniform dosing and different dosing regimens.* $c_{po}^{(1)}$ *and* $c_{po}^{(m)}$ *represent the drug concentration jumps due to loading and maintenance dosing, respectively.*

application of drug elimination (with rate constant k_r) and a jump corresponding to an increase in concentration due to dosing (the magnitude of the jump is given by c_{pi}^0 as already discussed). With the exception of c_{pmax}, the values of the kinetic constants determined for these two cases are in the expected range as determined from previous laboratory studies (2–9). The values of c_{pmax} incorporate the variation of volume of distribution utilized from that of its actual value. In fact, it is significant that no precise knowledge of the volume of distribution is needed for application of this method, due to the fact that the formulas contain only the ratio c_{pi}^0/c_{pmax}. The suggested estimate of 0.13 l./kg. body weight (2) allows an evaluation of the c_{pi}^0's that is an approximation to the actual jump in concentration due to drug administration, and any variation of the utilized volume of distribution from the actual one of the particular individual is simply compensated for by a corresponding variation of the calculated c_{pmax} from its actual value. This interrelationship between volume of distribution and c_{pmax} is made more clear by means of Eq. 24:

$$\frac{c_{pi}^0}{c_{pmax}} = \frac{g_i/V}{c_{pmax}} = \frac{g_i}{(Vc_{pmax})} \qquad \text{(Eq. 24)}$$

where g_i is the dose (in milligrams) given the ith day.

The sensitivity of the fit between theoretical and experimental results is reflected in the variation of the value of $\sqrt{\Phi/n}$ relative to the minimum obtained for a corresponding variation in the parameters from their optimum values. The most comprehensive sensitivity illustration would require a five-dimensional diagram and would be more confusing than informative. However, the sensitivity to k_r and k_d is easily shown for fixed-optimum values of m' and c_{pmax} by plotting the values of $\sqrt{\Phi/n}$ for each combination of k_r and k_d as in Fig. 4. This diagram is termed a *contour* plot and is similar to those in cartography. Any ellipse is a locus of pairs of values (k_d,k_r) which give equal error as measured by $\sqrt{\Phi/n}$. The general shape of both figures indicates that the equations are not hypersensitive to small changes in these parameters. As a result, determined values of k_d and k_r are unique and significant. On the other hand, grossly horizontal or vertical contours would indicate a poorly behaved parameter evaluation; for example, a horizontal contour would signify that given a k_r, many possible values of k_d would yield an equally good

data fit. It is easily seen that any value of $0.19 < k_r < 0.21$ day^{-1} and $1.4 < k_d < 1.6$ day^{-1} would give essentially the same quality fit as in Fig. 3. Similar evaluation may be made for the sensitivity to m' and $c_{p\max}$ by plotting similar diagrams for fixed values of k_r and k_d. Although the sensitivity, in principle, changes from one patient to another, some broad conclusions may be drawn from case results as to which parameter is *usually* more important and needs the most precise evaluation.

Finally, to show the combined sensitivity to variations in m' and k_r and k_d, the value of m' was decreased by 25% from its optimum value and again the average error, $\sqrt{\Phi/n}$, was evaluated for various combinations of k_r and k_d. The results plotted in Fig. 5 indicate that this variation in m' from its optimum value resulted in a *new* "optimum" set of k_r and k_d as indicated. However, the error at this partial optimum is slightly larger than that at the true optimum. The overall relative sensitivity to k_r and k_d variations, reflected by the relative size of the areas enclosed by the lines of $\sqrt{\Phi/n} = 6.5$ for example, remained about the same.

Illustrative Calculations for Uniform and Quasiuniform Multiple Dosing—Computations of the normalized multiple-dose response were performed utilizing Eq. 20, and the results are presented in Fig. 6. These calculations are meant to be only illustrative and not typical or exhaustive. The following values of the parameters were utilized: $k_r\tau = 0.1$; $k_d\tau = 1.0$; $c_{p\max}/c_{p\min} = 26$; and $c_{p0}^{(m)}/c_{p\max} = 0.05$, 0.1, and 0.2 as indicated. If τ is selected different from 1 day, the "day number" should be interpreted as "period number" (each of length τ). For the quasiuniform dosing, the value of $c_{p0}^{(1)}/c_{p\max} = 0.5$ was utilized as a loading dose and was followed by uniform dosing with the parameters utilized before. The response to a single dose equivalent to $c_{p0}^{(1)}/c_{p\max} = 0.5$ is also included for comparison. The asymptotic values were obtained employing the asymptotic formula (Eq. 22). From these plots, the faster approach of a loading dose to asymptotic behavior is clearly illustrated, together with its dependence on the kinetic parameters. As more experience is gained with the practical applications and after a more definitive bracketing of the expected values of the kinetic parameters is established, accumulating graphical results such as those in Fig. 6 for an exhaustive number of cases might be a worthwhile undertaking. From those plots the response, $P_n(\tau)$, may be evaluated if the value of m' is known (for then P^0 may be calculated from Eq. 2).

SUMMARY AND CONCLUSIONS

A mathematical technique was presented for estimating the kinetic parameters that control patient response to oral anticoagulant administration. The technique utilizes only routinely obtained and recorded data such as dose magnitude and prothrombin times. It is possible to apply during any stage of therapy and to gain predictive capability from data covering the measured response over a minimum period of 2 days. The applicability of the technique was demonstrated by direct comparison with two actual patient records. The numerical evaluation required is only minimal, and the sensitivity of the results was illustrated by two examples.

Spontaneous changes and interaction effects are outside the scope of the presently proposed analytical technique. However, it does provide the means to exploit a large volume of hospital records and thus attempt to correlate the variation of the kinetic constants with disease states or to establish the onset (and hence the cause) of other drug interactions and spontaneous changes.

Finally, formulas were included for convenient computations of long-term responses to uniform or quasiuniform dosing.

APPENDIX

From Eq. 13, with $n = 3$, the following explicit form of Φ is obtained:

$$\Phi = e^{-2k_d}(P_0^2 + P_1^2 + P_2^2) + (P_1^2 + P_2^2 + P_3^2) + 3E^2 + F^2\{\ln^2[c_{p0}^0/c_{p\max}] + \ln^2[(c_{p0}^0/c_{p\max})e^{-k_r} + (c_{p1}^0/c_{p\max})] + \ln^2[(c_{p0}^0/c_{p\max})e^{-2k_r} + (c_{p1}^0/c_{p\max})e^{-k_r} + (c_{p2}^0/c_{p\max})]\} + 2F\{(E - P_0e^{-k_d} + P_1)\ln[(c_{p0}^0/c_{p\max})] + (E - P_1e^{-k_d} + P_2)\ln[(c_{p0}^0/c_{p\max})e^{-k_r} + (c_{p1}^0/c_{p\max})] + (E - P_2e^{-k_d} + P_3)\ln[(c_{p0}^0/c_{p\max})e^{-2k_r} + (c_{p1}^0/c_{p\max})e^{-k_r} + (c_{p2}^0/c_{p\max})]\} + 2E(P_1 + P_2 + P_3) - 2Ee^{-k_d}(P_0 + P_1 + P_2) - 2e^{-k_d}(P_1P_0 + P_2P_1 + P_3P_2) \quad \text{(Eq. A1)}$$

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Kinetic Analysis of Complex Formation between Penicillin and Sucrose

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Abstract □ The formation of 1:1 molar complexes between sucrose and the following penicillins was studied: potassium penicillin G USP; potassium phenoxymethyl penicillin USP; sodium dicloxacillin, monohydrate; and ampicillin, anhydrous. Kinetic parameters, based on a model system, are presented for each interaction. The degree of complexation was greatest with sodium dicloxacillin and least with ampicillin. The rate of degradation of complexed penicillin was 5–6 times the rate for the uncomplexed penicillin. The relatively high equilibrium constant obtained for sodium dicloxacillin was consistent with the strong effect of sucrose on the observed rate constant. Ampicillin, whose observed rate constant was only slightly affected, had a very low equilibrium constant. Complexation involves the intact penicillin and accelerates the degradation of penicillin but does not appear to change the degradation pathway.

Keyphrases □ Potassium penicillin G—complexation with sucrose, effect on degradation rate □ Potassium phenoxymethyl penicillin—complexation with sucrose, effect on degradation rate □ Sodium dicloxacillin, monohydrate—complexation with sucrose, effect on degradation rate □ Ampicillin, anhydrous—complexation with sucrose, effect on degradation rate □ Penicillins—complexation with sucrose, effect on degradation rate □ Sucrose—complex formation, effect on degradation rate of penicillins □ Complex formation—sucrose with penicillins, effect on penicillin stability

A number of reports (1–6) indicated that dextrose causes the inactivation of penicillin, although other workers (7, 8) concluded that dextrose has little effect on the chemical stability of potassium penicillin G USP. Moss and Cole (9) found that the degradation of 6-aminopenicillanic acid was accelerated in the presence of dextrose, maltose, or lactose. In that study, the *N*-glycosyl, *N*-maltosyl, and *N*-lactosyl derivatives of 6-aminopenicillanic acid were isolated by column chromatography and were found to contain approximately 1 mole of sugar/mole of 6-aminopenicillanic acid. The authors concluded that the amino group of 6-aminopenicillanic acid and the reducing group of the sugar were involved in the interaction, because they observed no reaction when benzylpenicillin or a nonreducing sugar was present.

Schneider and de Weck (10) recently reported a reaction between benzylpenicillin and a number of carbohydrates, including reducing sugars, nonreducing sugars, dextran, and simple glycols. They speculated that the reaction resulted in the formation of an α-ester of penicilloic acid. The reaction product of benzylpenicillin and raffinose was separated by Sephadex chromatography and contained equimolar amounts of raffinose and benzylpenicillin.

Preliminary experiments in these laboratories also indicated an interaction between sucrose and penicillin. The investigation reported here was undertaken to investigate this interaction through a determination of its kinetic parameters.

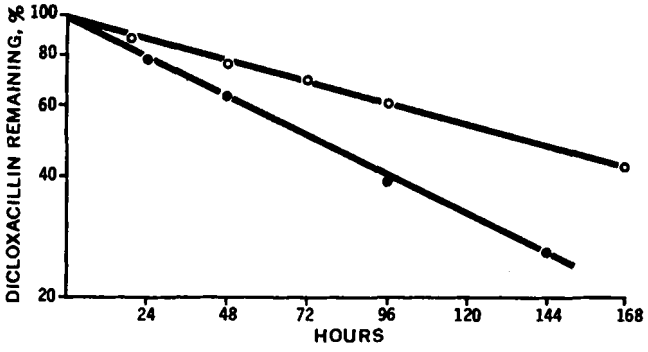


Figure 1—*Change in potency of sodium dicloxacillin, monohydrate, during aging in a 0.01* M *solution at pH 7, 45°. Key:* ○, *in absence of sucrose; and* ●, *in presence of 0.0175* M *sucrose.*

EXPERIMENTAL

Materials—Four penicillins were studied: potassium penicillin G USP; potassium phenoxymethyl penicillin USP; sodium dicloxacillin, monohydrate; and ampicillin, anhydrous. Each was taken from one lot of commercially prepared material. The sucrose[1] and other chemicals used were reagent grade.

Procedure—Solutions for the kinetic studies were prepared at pH 7.0 nsing a 0.06 *M* citrate buffer. The buffer solution containing the appropriate amount of sucrose was brought to the desired temperature in a constant-temperature bath before the penicillin was added. The pH of each solution was determined initially and at the end of the experiment. No significant changes in pH occurred.

Samples were withdrawn during the study and assayed for intact penicillin by a modified iodometric titration procedure (11) or by the microbiological cylinder-plate assay, with *Staphylococcus aureus*, ATCC 6538P, as the test organism.

The rate of hydrolysis of penicillin was also determined by following the acid produced with the aid of a Radiometer setup, consisting of a PHM 26 pH meter, a TTT-1 automatic titrator, a TTA3 titrator assembly, an SBR2 titragraph, and an ABU 12 autoburet.

Ampicillin solutions (0.01 *M*), buffered to pH 7.0 with 0.06 *M* citrate buffer, were examined by TLC after storage for 6 days at 45° both in the presence and absence of 0.029 *M* sucrose. Samples containing 20 mg. of ampicillin were diluted 1:1 with *n*-propanol, and 10 μl. was applied to a 250-μ silica gel G-HR/UV plate[2]. The plate was activated by heating at 100° for 15 min. and was developed in a saturated chamber containing *n*-propanol–water (70:30 v/v). The solvent front was allowed to travel 15 cm. Visualization was effected by: (*a*) exposing the plate to iodine vapors for 60 min., and (*b*) spraying the plate with 0.25% ninhydrin in ethanol and heating at 100° for 10 min.

RESULTS AND DISCUSSION

The four penicillins were found to degrade according to apparent first-order kinetics. Plots similar to Fig. 1 were obtained throughout the study when the logarithm of the penicillin concentration *versus* time was plotted. As seen in Fig. 2, at pH 7.0 and 45°, sucrose accelerated the degradation of 0.01 *M* penicillin solutions in 0.06

[1] Analyzed reagent, J. T. Baker Chemical Co., Phillipsburg, N. J.
[2] Analtech, Inc., Wilmington, Del.

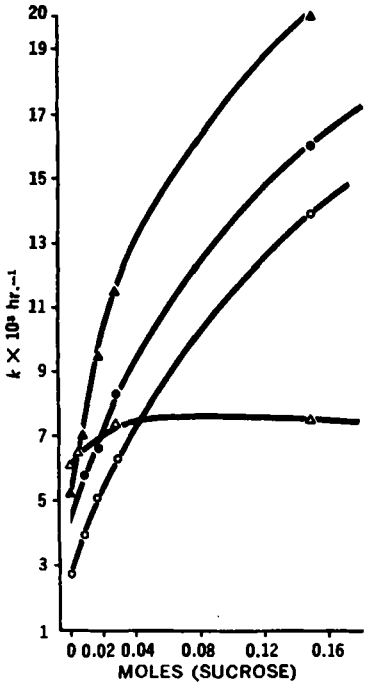


Figure 2—*Effect of sucrose on the apparent first-order rate constant for various penicillins at pH 7.0, 45°, in 0.06 M citrate buffer. Key: ○, potassium phenoxymethyl penicillin USP; ●, potassium penicillin G USP; △, ampicillin, anhydrous; ▲, sodium dicloxacillin, monohydrate.*

M citrate buffer. Potassium penicillin G, potassium phenoxymethyl penicillin, and sodium dicloxacillin showed a 3–5-fold increase in apparent first-order rate constant as the sucrose concentration was increased to 0.15 *M*. A significant but much smaller increase in the rate of degradation of ampicillin occurred in the presence of sucrose. The overall relationship between apparent first-order rate constant and sucrose concentration is nonlinear, although it approximates linearity for sucrose concentrations up to 0.03 *M* or 3 times the molar concentration of sucrose.

The observed effect of sucrose was not due to trace metal catalysis caused by impurities. Highly purified sucrose was used in this study, and its effect on the rate of degradation of sodium dicloxacillin was identical in the presence or absence of 0.05% disodium edetate USP.

TLC analysis of ampicillin solutions degraded in the presence and absence of sucrose (Fig. 3) indicated that sucrose has no effect on the degradation products. Several degradation products were observed in each solution. The R_f values of these products were unchanged in the presence of sucrose.

Schneider and de Weck (10) speculated that the reaction between benzylpenicillin and a number of carbohydrates occurred by esterification of the potential acid group of the β-lactam. They noted that the penicilloyl–dextran conjugate separated by Sephadex chromatography showed no antibiotic activity by the Oxford cup assay. This observation supports their esterification hypothesis since biological activity is only present in penicillins when the β-lactam is intact (12). To determine if esterification was responsible for the observed effect of sucrose on penicillin (Fig. 2), an experiment was run comparing biological activity to the iodometric assay results for a series of buffered sodium dicloxacillin solutions aged for 7 days at 25°. Three identical solutions (A, B, and C) were prepared using 0.025 *M* sodium dicloxacillin and citrate buffer. Three similar solutions (D, E, and F) which also contained 2 *M* sucrose were prepared. The biological and iodometric assay results for these solutions are presented in Table I. In all cases the biological activity agrees well with the iodometric assay results. The retention of biological activity in the presence of sucrose indicates that a different interaction is occurring than the probable esterification observed by Schneider and de Weck (10).

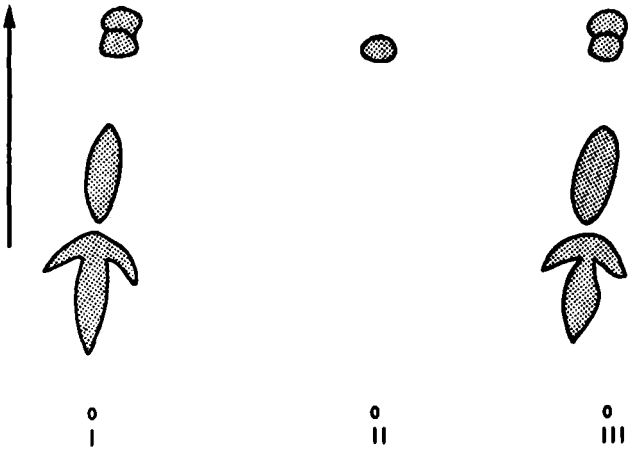


Figure 3—*TLC analysis of 0.01 M solutions of ampicillin, anhydrous, buffered at pH 7.0 with 0.06 M citrate. Key: I, preparation aged 6 days at 45°; II, fresh preparation; and III, preparation with 0.029 M sucrose, aged 6 days at 45°.*

Table I—Comparison of Potency of Buffered Sodium Dicloxacillin Solutions Determined by Biological and Iodometric Assay

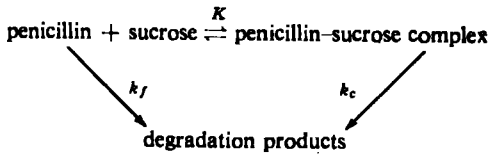
	Initial *M*		Aged 7 Days at 25°, % Initial	
	Iodometric	Biological	Iodometric	Biological
No sucrose				
A	0.0253	0.0244	95	105
B	0.0245	0.0250	96	101
C	0.0249	0.0244	96	98
Average			96	101
With 2 *M* sucrose				
D	0.0247	0.0243	90	89
E	0.0258	0.0236	87	84
F	0.0263	0.0253	91	83
Average			89	85

In addition, the rate of degradation of a pH 10 solution of 0.02 *M* potassium penicillin G at 45° in the presence of 0.29 *M* sucrose was determined by the pH-stat and iodometric methods. The results obtained show good agreement between these two methods (Table II). At pH 10, where the possibility of ester formation would be most likely to occur, there is good agreement for the degradation of sodium dicloxacillin between the pH-stat and iodometric methods both in the presence and absence of sucrose. The agreement in the rate of degradation as determined by pH-stat titration, which measures the release of protons upon cleavage of the β-lactam, and the iodometric assay, which is based on the consumption of iodine by the cleaved β-lactam, indicates that penicillin in the presence of sucrose does not degrade through the formation of an intermediate ester. The presence of any significant amount of ester would give differences in the observed penicillin degradation rates between the pH-stat and iodometric methods. These results, therefore, further confirm the postulation that esterification of the penicillins is insignificant in these systems.

As seen in Fig. 1, no induction period or change in slope was noted during the kinetic studies. This observation, in addition to the nonlinear dependence of the apparent first-order rate constant on sucrose concentration (Fig. 2), suggests that the interaction is based upon an equilibrium such as occurs in complexation. The

Table II—Comparison of Rates of Penicillin Degradation by pH-Stat and Iodometric Assay Techniques

System	Rate of Degradation, min.⁻¹ pH-Stat	Iodometric
0.02 *M* sodium dicloxacillin monohydrate, pH 10 and 45°	8.9×10^{-2}	8.8×10^{-2}
0.02 *M* sodium dicloxacillin monohydrate with 0.29 *M* sucrose, pH 10 and 45°	4.5×10^{-1}	4.9×10^{-1}



Scheme I

retention of biological activity indicates that the β-lactam is intact in the presence of sucrose. The R_f values of the degradation products were identical in the presence or absence of sucrose. Based on these observations and the conclusion of Moss and Cole (9) and Schneider and de Weck (10) that a 1:1 reaction occurs between carbohydrates and penicillin, the model shown in Scheme I was selected as the simplest system that completely satisfies the experimental observations; K is the equilibrium constant for the penicillin–sucrose complex, and k_f and k_c are the apparent first-order rate constants for the degradation of uncomplexed and complexed penicillin, respectively.

The formation of a penicillin–sucrose complex as proposed by the kinetic model is supported by a recent study of the interaction of penicillin and guanosine (13). NMR studies showed that penicillin G interacts with guanosine in dimethyl sulfoxide by forming a binary hydrogen-bonded complex. The binding sites were shown to be the carboxylate and carbonyl groups in penicillin G and the NH and NH_2 groups of guanosine. Although no attempts were made in the present study to elucidate the structure of the proposed penicillin–sucrose complex, the hydroxyl groups of sucrose may be able to act as hydrogen donors and form a binary hydrogen-bonded complex analogous to the penicillin–guanosine complex.

The complexing behavior of pharmaceuticals with various other materials has been reported by numerous workers. In most previous reports discovered by the authors, complexation contributed favorably to the stability of the drug. The rate of hydrolysis of benzocaine was significantly reduced in the presence of caffeine (14). Chelate formation by boric acid stabilized epinephrine (15). Guttman (16) and Wadke and Guttman (17, 18) demonstrated that molecular interactions can improve the stability of riboflavin and related compounds.

Although the effect of complex formation has been to inhibit the rate of reaction in pharmaceuticals, acceleration of the apparent rate also is possible and has been observed in the cyclohepta-amylose-catalyzed hydrolysis of penicillin (19), the acetolysis of

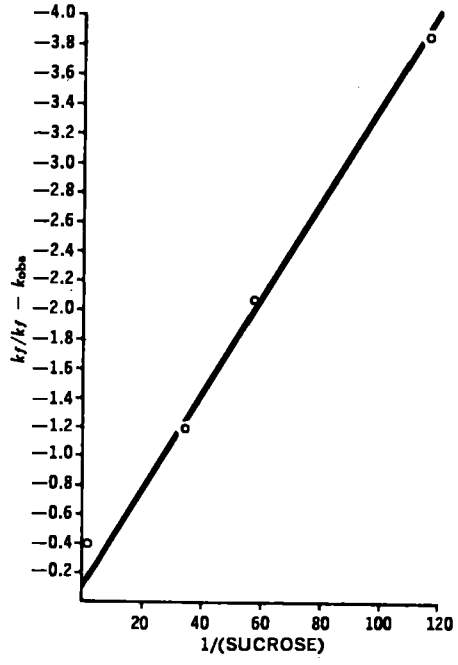


Figure 4—*Interaction between potassium penicillin G USP and sucrose. Plot based on equation of Connors and Mollica (25).*

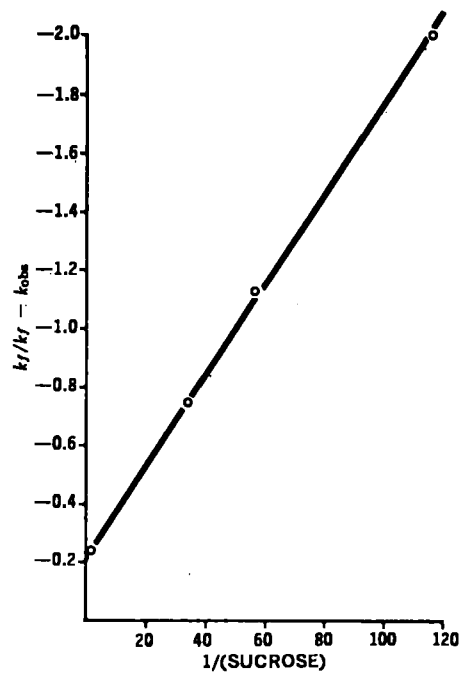


Figure 5—*Interaction between potassium phenoxymethyl penicillin USP and sucrose. Plot based on equation of Connors and Mollica (25).*

certain toluenesulfonates (20–23), and the racemization of optically active binaphthyl donors (24).

In the present study, the interaction between sucrose and penicillin was studied using the kinetic analysis for a 1:1 molar complex developed by Connors and Mollica (25). This analysis makes no assumptions regarding the relative magnitude of k_f and k_c. They derived the following equation to describe the effect of the formation of a 1:1 molar complex on the observed reaction rate:

$$\frac{k_f}{k_f - k_{obs}} = \frac{1}{q_{11}K(\text{sucrose})} + \frac{1}{q_{11}} \qquad \text{(Eq. 1)}$$

where k_{obs} is the observed rate constant at any sucrose concentration, and $q_{11} = [1 - (k_c/k_f)]$.

The rate of degradation of uncomplexed penicillin was determined in the absence of sucrose and confirmed by extrapolating

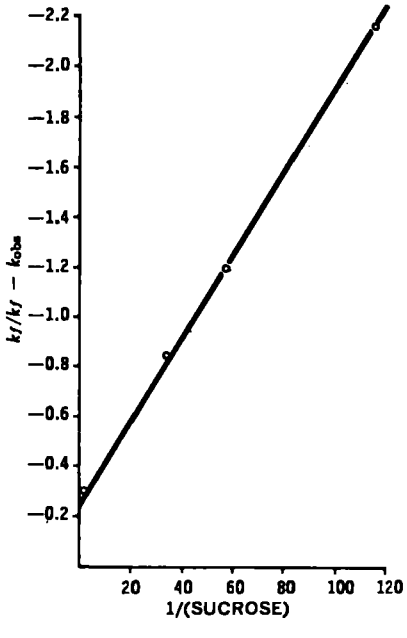


Figure 6—*Interaction between sodium dicloxacillin, monohydrate, and sucrose. Plot based on equation of Connors and Mollica (25).*

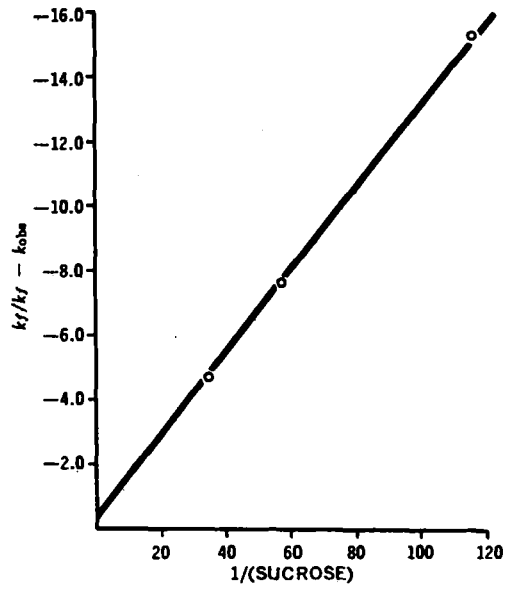


Figure 7—*Interaction between ampicillin, anhydrous, and sucrose. Plot based on equation of Connors and Mollica (25).*

the effect of sucrose on the observed rate constant to zero sucrose concentration.

The rate of degradation of complexed penicillin was obtained from the intercept of a plot of $[k_f/(k_f - k_{obs})]$ *versus* [1/(sucrose)] and the relationship between q_{11} and k_c/k_f. The equilibrium constant was calculated by dividing the intercept of this plot by the slope.

The data in Fig. 2 are plotted in Figs. 4–7 for each penicillin according to the variables in Eq. 1. An excellent linear relationship is obtained for potassium penicillin G (Fig. 4), potassium phenoxymethyl penicillin (Fig. 5), sodium dicloxacillin (Fig. 6), and ampicillin (Fig. 7). The excellent agreement between the experimental data and the behavior predicted by Eq. 1 also supports the kinetic model based upon the formation of a 1:1 molar complex. The equilibrium constant, k_f, and k_c were calculated from Figs. 4–7 and are given in Table III.

The data indicate that the complexed penicillin degrades 5–6 times as fast as the uncomplexed penicillin and results in an increased overall rate of degradation. These results indicate that the sucrose–penicillin complex that forms is degraded by the same mechanism, regardless of the penicillin tested. The differences in overall rate constants, however, are due to the differences in the equilibrium constants for the penicillins. The relatively high equilibrium constant obtained for sodium dicloxacillin was consistent with the strong effect of sucrose on the observed rate constant. Ampicillin, whose observed rate constant was only slightly affected by sucrose, had a very low equilibrium constant, indicating a much lesser degree of complexation. If the penicillin–sucrose complex has a structure analogous to the penicillin–guanosine complex, then the presence of the amine group in the ampicillin side chain, which is unique with ampicillin, may interfere with complex formation due to stearic or charge factors. Tutt and Schwartz (19) also observed that the effect of cycloheptaamylose on the hydrolysis of penicillin was dependent on the structure of the penicillin side chain.

The present study offers a unique example of a drug that is less stable in the complexed form. The complexes between penicillin and sucrose appear to form instantly. No induction period or change in the rate of degradation was observed in any case. Complexation thus appears to occur between the intact penicillin molecule and sucrose. Degradation of the complexed and uncomplexed penicillin then occurs by the usual pathway and results in an increased rate of degradation.

Table III—Kinetic Parameters for Penicillin–Sucrose Complexes at pH 7.0, 45°, in 0.06 *M* Citrate Buffer

	K, l./mole	$k_f \times 10^3$ hr.$^{-1}$	$k_c \times 10^3$ hr.$^{-1}$	k_c/k_f
Potassium phenoxymethyl penicillin USP	11.5	2.7	17.0	6.3
Potassium penicillin G USP	6.6	4.6	26.6	5.9
Ampicillin, anhydrous	1.5	6.1	38.1	6.2
Sodium dicloxacillin, monohydrate	15.5	5.2	25.4	4.9

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Determination of Dantrolene in Biological Specimens Containing Drug-Related Metabolites

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Abstract □ A sensitive and specific method is described for the determination of dantrolene, a new skeletal muscle relaxant. Method specificity for dantrolene in the presence of dantrolene-related metabolites is based on a combination of solvent extractions, chromatography, and the fluorescence characteristics of dantrolene. The method is satisfactory for use with plasma, blood, urine, bile, tissues, and feces. Modifications of the dantrolene method are also provided for estimating two dantrolene-related metabolites in biological fluids.

Keyphrases □ Dantrolene—analysis, biological fluids containing metabolites □ Spectrophotofluorometry—analysis, dantrolene in biological fluids containing metabolites

Dantrolene sodium[1], 1-[[5-(*p*-nitrophenyl)furfurylidene]amino]hydantoin sodium hydrate, is a new muscle relaxant which apparently acts directly and selectively on skeletal muscle (1–3). During the early development of this drug, a spectrophotofluorometric procedure was developed for the determination of dantrolene, 1-[[5-(*p*-nitrophenyl)furfurylidene]amino]-hydantoin, in blood and urine (4). Subsequent investigation revealed that dantrolene is metabolized by both nonreductive and reductive pathways, and a polarographic method was developed which determines the total nonreduced or reduced metabolites (5). The drug is reduced to 1-[[5-(*p*-aminophenyl)furfurylidene]amino]-hydantoin and subsequently acetylated to 1-[[5-(*p*-acetamidophenyl)furfurylidene]amino]hydantoin (5). A major nonreduced metabolite also was isolated which is currently designated as Metabolite A (5). Recently, it was established that Metabolite A interferes in the determination of dantrolene by the original fluorometric procedure.

A modification of the original procedure is now presented for determining dantrolene in biological specimens containing known drug-related metabolites. The method is satisfactory for use with plasma, blood, urine, bile, tissues, and feces. Methods for estimating two of the major metabolites, acetylated dantrolene and Metabolite A, in plasma, blood, and urine are also described.

EXPERIMENTAL

Reagents and Instrument—The reagents include *N,N*-dimethylformamide, reagent grade[2]; ammonium sulfate, special enzyme grade[3]; sodium hydroxide, analytical reagent[4]; hydrochloric acid, analytical reagent[5]; 1-hexanol, practical grade[6]; heptane, practical grade[7]; 1-nitropropane, practical grade[8]; ethyl acetate, spectroquality[9]; methanol, anhydrous, analytical reagent grade[10]; and Sephadex G-10[11].

A spectrophotofluorometer[12] with the following instrumental parameters was used to measure fluorescence. Slit arrangement for both excitation and fluorescence was the No. 3 position, the light path was adjusted with a No. 3 spacer, and sensitivity was No. 8. For dantrolene and Metabolite A measurements, the excitation wavelength was 395 nm. and the fluorescence wavelength was 530 nm. For acetylated dantrolene measurements, the excitation wavelength was 365 nm. and the fluorescence wavelength was 405 nm.

Standard Solutions—Fifty milligrams of crystalline dantrolene or Metabolite A is dissolved in 50 ml. of *N,N*-dimethylformamide. Ten milliliters of this solution is placed in a 100-ml. volumetric flask, 40 ml. of *N,N*-dimethylformamide is added, and the solution is diluted to volume with water to obtain a drug concentration of 100 mcg./ml. This solution is then diluted with water to obtain the required drug concentrations. Fifty milligrams of crystalline acetylated dantrolene is dissolved in 50 ml. of *N,N*-dimethylformamide to obtain a 1000-mcg./ml. solution. This solution is then diluted with water to obtain the required drug concentrations.

Analysis of Dantrolene in Plasma, Blood, and Urine—To 0.5 ml. of biological fluid, add 2.0 ml. of water and then 2.0 ml. of a saturated ammonium sulfate solution and mix thoroughly. Mix the solution with 5.0 ml. of a hexanol–heptane admixture (4:1) by shaking the tube vigorously for 1 min. and then centrifuging the sample at 2000 r.p.m. for 10 min. Transfer 4.5 ml. of the hexanol–heptane extract (top layer) to a test tube and add 2.5 ml. of 0.2 *N* sodium hydroxide. Shake the tube for 2 min. and centrifuge at 2000 r.p.m. for 10 min. Remove 2.0 ml. of the sodium hydroxide extract (bottom layer) and place on a hydrated Sephadex G-10 column (0.6 cm. wide × 3.5 cm. long). The column is prepared with previously hydrated Sephadex. After the extract passes through the Sephadex, wash the column with 4.0 ml. of 0.2 *N* sodium hydroxide and discard the eluate. Dantrolene is eluted with 4.0 ml. of a 5% *N,N*-dimethylformamide water solution. Addition of 1.0 ml. of 0.3 *N* hydrochloric acid to the *N,N*-dimethylformamide eluate adjusts the pH to 1–2. Mix the solution with 4.0 ml. of a nitropropane–heptane admixture (1:1) by shaking the tube vigorously for 1 min. and centrifuging at 2000 r.p.m. for 10 min.

Then remove a 3.0-ml. aliquot of the nitropropane–heptane extract (top layer) for fluorometric measurement. Set the spectrophotofluorometer at 30 fluorescence units with a 4-mcg. aqueous dantrolene standard subjected to the analytical procedure. The fluorescences of the solvent extracts from the biological specimens are then measured, and the amount of dantrolene is calculated from aqueous dantrolene standards. When the fluorescence obtained with dantrolene exceeds 30 fluorescence units, quenching occurs. Under these conditions the solvent extract is diluted with additional nitropropane–heptane (1:1) containing 2.7% hexanol until a value of 30 fluorescence units or less is obtained.

Estimation of Metabolite A in Plasma, Blood, and Urine—To 0.5 ml. of biological fluid, add 2.0 ml. of water and then 2.0 ml. of a saturated ammonium sulfate solution and mix thoroughly. Mix the solution with 5.0 ml. of a hexanol–heptane admixture (4:1) by shaking the tube vigorously for 1 min. and then centrifuging the sample at 2000 r.p.m. for 10 min. Transfer 4.5 ml. of the solvent extract (top layer) to a test tube and add 2.5 ml. of 0.2 *N* sodium hydroxide. Shake the tube vigorously for 2 min. and centrifuge at 2000 r.p.m. for 10 min. Remove 2.0 ml. of the sodium hydroxide extract and transfer to a test tube. Addition of 3.0 ml. of 0.3 *N* hydrochloric acid adjusts the pH to 1–2. Mix the solution with 4.0

[1] Dantrium, Eaton Laboratories.
[2] Matheson, Coleman and Bell (DX 1730).
[3] Schwarz/Mann (1946).
[4] Mallinckrodt Chemical Works (7708).
[5] Mallinckrodt Chemical Works (2612).
[6] J. T. Baker Chemical Co. (N-229).
[7] Matheson, Coleman and Bell (HX-80).
[8] Matheson, Coleman and Bell (NX-705).
[9] Matheson, Coleman and Bell (EX-245).
[10] Mallinckrodt Chemical Works (3016).
[11] Pharmacia Fine Chemicals.
[12] Baird Atomic Fluorispec, model SF-1.

Table I—Recovery of Dantrolene from Biological Fluids

Fluid	Fluorescence Units[a] — Dantrolene, mcg. — Control	0.05	0.10	0.50	1.0	2.0	4.0	Percent Recovery[b], Mean ± SD
Water	0.2	0.3	0.7	3.4	7.4	14.8	29.8	—
Dog blood	0.2	0.3	0.7	3.4	7.3	14.8	29.8	99.7 ± 0.6
Dog plasma	0.2	0.3	0.7	3.3	7.2	14.5	30.2	98.8 ± 1.7
Dog urine	0.2	0.3	0.7	3.5	6.7	15.4	29.8	99.5 ± 4.7
Human blood	0.2	0.3	0.7	3.4	7.1	14.4	29.8	98.8 ± 1.8
Human plasma	0.2	0.3	0.6	3.4	6.9	14.4	29.8	96.0 ± 5.6
Human urine	0.2	0.3	0.6	3.3	6.7	14.4	29.8	95.1 ± 5.7

[a] Control-corrected data based on a mean from at least three samples. [b] Based on aqueous dantrolene standards.

ml. of a nitropropane–heptane admixture (1:1) by shaking the tube vigorously for 1 min. and then centrifuging at 2000 r.p.m. for 10 min.

Then remove a 3.0-ml. aliquot of the nitropropane–heptane extract (top layer) for fluorometric measurement. The spectrophotofluorometer is set at 30 fluorescence units using a 4-mcg. aqueous dantrolene standard subjected to the analytical procedure. The fluorescences of the solvent extracts from the biological samples are then measured. If a fluorescence greater than 30 units is encountered, quenching occurs. Then the solvent extract is diluted with nitropropane–heptane (1:1) containing 2.7% hexanol until 30 fluorescence units or less is obtained.

To obtain an estimate of Metabolite A in biological fluids, aliquots of each sample must be analyzed by this procedure and by the procedure described for dantrolene. The amount of fluorescence attributed to Metabolite A is based on the difference in fluorescence units found between the two methods. The estimated amount of Metabolite A present is calculated from aqueous Metabolite A standards.

Analysis of Dantrolene in Bile, Tissues, and Feces—The dantrolene method was slightly modified since large amounts of dantrolene and drug-related metabolites were anticipated in bile, tissues, and feces. Homogenize 0.5 g. of tissue or feces in 2.0 ml. of a saturated ammonium sulfate solution in a Potter-Elvehjem glass homogenizer[13]. Add 1.0 ml. of water to the homogenate, mix, and transfer the homogenate to a test tube. Then rinse the homogenizer tube and pestle with 3.5 ml. of water which is added to the homogenate. Bile samples are processed by mixing 0.25 ml. of bile with 2.0 ml. of a saturated ammonium sulfate solution and then adding 4.5 ml. of distilled water.

Mix the homogenate or bile solution with 10.0 ml. of a hexanol–heptane admixture (4:1) by shaking the tube vigorously for 1 min. and then centrifuging at 2000 r.p.m. for 10 min. Remove 9 ml. of the solvent extract (top layer) and place in a test tube. Add 5 ml. of 0.2 *N* sodium hydroxide, shake the tube vigorously for 2 min., and centrifuge at 2000 r.p.m. for 10 min. Remove 4.0 ml. of the sodium hydroxide extract (bottom layer) and place on a hydrated Sephadex G-10 column (0.8 cm. wide × 9.0 cm. long), prepared as described earlier. After the extract passes through the Sephadex, wash the column with succeeding 0.2 *N* sodium hydroxide aliquots of 2.0, 5.0, and 4.0 ml. Then wash the column with 5.0 ml. of a 20% methanol–water solution and discard the eluate. Dantrolene is eluted from the column with 8.0 ml. of a 10% *N,N*-dimethylformamide–water solution. Addition of 2.0 ml. of 0.3 *N* hydrochloric acid to the *N,N*-dimethylformamide eluate adjusts the pH to 1–2. Mix the solution with 4.0 ml. of a nitropropane–heptane admixture (1:1) by shaking the tube vigorously for 1 min. and centrifuging at 2000 r.p.m. for 10 min.

Then remove 3 ml. of the nitropropane–heptane extract (top layer) for fluorometric measurement. Fluorometric measurement is handled exactly as described previously for dantrolene, with the exception that necessary dilutions to avoid quenching are made with nitropropane–heptane (1:1) containing 4.3% hexanol.

Analysis of Acetylated Dantrolene in Plasma, Blood, and Urine—Acetylated dantrolene is determined in biological fluids by slightly modifying the procedure used to determine dantrolene in tissues. Samples are processed by mixing 0.5 ml. of biological fluid with 2.0 ml. of a saturated ammonium sulfate solution and then with 4.5 ml. of distilled water. The dantrolene tissue procedure is then followed as described through the step involving acidification of the *N,N*-dimethylformamide eluate.

The acidified *N,N*-dimethylformamide eluate from the Sephadex column is mixed with 3.5 ml. of an ethyl acetate–nitropropane admixture (9:1) by shaking the tube vigorously for 1 min. and centrifuging at 2000 r.p.m. for 10 min. A 2.5-ml. aliquot of the ethyl acetate–nitropropane extract (top layer) is then removed for fluorometric measurement. The spectrophotofluorometer is set at 3 fluorescence units with a 3-mcg. aqueous acetylated dantrolene standard subjected to the analytical procedure. The fluorescences of the solvent extracts from biological samples are then measured, and the amount of acetylated dantrolene is calculated from aqueous acetylated dantrolene standards. When the fluorescence obtained with acetylated dantrolene exceeds 3 fluorescence units, quenching occurs. Under such conditions the solvent extract is diluted with ethyl acetate–nitropropane (9:1) until a value of 3 fluorescence units or less is obtained.

RESULTS

Analysis of Dantrolene—When subjected to the procedure, dantrolene standards in water, biological fluids, and tissues exhibit linear standard curves from 0.1 to 4.0 mcg. The recoveries of added dantrolene from dog and human biological fluids (plasma, blood, and urine) are presented in Table I. Average recoveries of added dantrolene from rat and monkey biological fluids (plasma, blood, and urine) were 100.9% ± 4.3 *SD* and 99.8% ± 0.4 *SD*, respectively, over a range of 0.1–4.0 mcg. The recoveries of added dantrolene from dog bile and selected tissues are presented in Table II. An average recovery of added dantrolene from selected monkey tissues of 97.1% ± 5.5 *SD* was obtained over a range of 0.1–4.0 mcg. When 4.0 mcg. of dantrolene was added to dog feces, a recovery of 100.2% ± 2.8 *SD* was obtained. Agreement between the aqueous and reference (fluids, tissues, and feces) dantrolene standards makes it unnecessary to analyze reference dantrolene standards with each set of biological samples.

As shown by the results in Table I, the lowest dantrolene level detectable in biological fluids with accuracy is 0.1 mcg./ml., using a 0.5-ml. specimen. However, recent investigation revealed that a

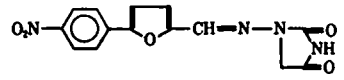
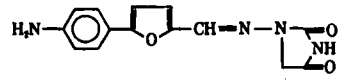
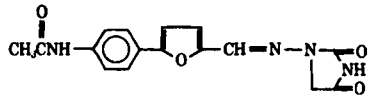
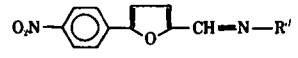
Table II—Recovery of Dantrolene from Dog Bile and Tissues

Fluid or Tissue	Fluorescence Units[a] — Dantrolene, mcg. — Control	0.1	1.0	4.0	Percent Recovery[b], Mean ± SD
Water	0.2	0.7	6.6	29.6	—
Bile	0.3	0.7	6.3	29.4	98.0 ± 2.6
Liver	0.2	0.7	6.7	29.4	100.5 ± 1.7
Kidney	0.2	0.6	6.6	28.8	97.5 ± 4.7
Lung	0.2	0.7	6.7	29.8	100.9 ± 1.2
Muscle	0.2	0.6	6.2	29.4	93.2 ± 6.2
Heart	0.2	0.6	6.7	29.4	98.7 ± 5.1
Spleen	0.2	0.7	6.5	29.4	99.7 ± 1.1
Fat	0.2	0.6	6.5	29.1	95.9 ± 5.9
Brain	0.2	0.6	6.3	28.8	94.7 ± 5.3
				Mean	98.0 ± 3.7

[a] Control-corrected data based on a mean from at least three samples. [b] Based on aqueous dantrolene standards.

[13] Kontes, size c, K-88550.

Table III—Fluorescence of Dantrolene and Related Metabolites

Compound[a]	Structure	Fluorescence Units[b]
Dantrolene		74.8
Amino-dantrolene		2.4
Acetylated dantrolene		ND[c]
Metabolite A		2.3

[a] Each compound (10 mcg.) was subjected to the method specific for dantrolene. [b] Control-corrected fluorescence units. [c] ND = none detectable. [d] *Reference 5.*

Table IV—Recovery of Dantrolene or Metabolite A from Biological Fluids

Fluid	Fluorescence Units[a] — Dantrolene, mcg. — Control	0.05	0.10	0.50	1.0	2.0	4.0	Percent Recovery[b], Mean ± *SD*	Fluorescence Units[a] — Metabolite A, mcg. — 0.10	0.50	1.0	4.0	Percent Recovery[b], Mean ± *SD*
Water	0.2	0.3	0.7	3.5	7.4	14.8	29.8	—	0.4	2.1	4.4	19.8	—
Dog blood	0.2	0.3	0.6	3.4	7.1	15.1	29.7	96.6 ± 5.6	0.4	2.2	4.3	19.8	100.7 ± 3.0
Dog plasma	0.2	0.3	0.7	3.6	7.2	15.5	30.5	101.1 ± 2.8	0.4	2.1	4.3	19.8	99.5 ± 1.0
Dog urine	0.3	0.3	0.7	3.4	6.8	15.0	29.7	98.1 ± 3.3	0.4	2.2	4.2	20.7	101.0 ± 4.5
Human blood	0.2	0.3	0.7	3.4	7.4	15.1	29.8	99.8 ± 1.6	0.4	2.0	3.9	19.8	95.7 ± 5.7
Human plasma	0.2	0.3	0.7	3.4	7.0	14.7	29.8	98.3 ± 2.4	0.4	2.2	3.9	20.8	99.5 ± 8.0
Human urine	0.2	0.3	0.6	3.3	7.0	14.7	29.1	95.0 ± 5.0	0.4	2.2	4.2	19.8	100.0 ± 4.1

[a] Control-corrected data based on a mean from at least three samples. [b] Based on aqueous dantrolene or Metabolite A standards.

concentration as low as 0.02 mcg./ml. is also detectable. Method sensitivity for dantrolene is 0.2 mcg./g. in tissues (0.5-g. specimen) and 0.4 mcg./ml. in bile (0.25-ml. specimen).

Method Specificity—The specificity of the method for the determination of dantrolene is based on a combination of solvent extractions, chromatography, and the fluorescence characteristics of dantrolene in the nitropropane–heptane admixture (1:1). As shown by the results in Table III, appreciable fluorescence is not obtained when known dantrolene-related metabolites are subjected to the method specific for the determination of dantrolene.

Analysis of Metabolite A—The dantrolene metabolite A is estimated in biological fluids by using a combination of the method specific for dantrolene and a method capable of measuring both dantrolene and Metabolite A simultaneously. Subjection of either dantrolene or Metabolite A standards in water, plasma, blood, and urine to this procedure yields linear curves for both dantrolene and Metabolite A from 0.1 to 4.0 mcg. Recoveries of either added dantrolene or Metabolite A from biological fluids as determined by this procedure are presented in Table IV. The procedure has a sensitivity of 0.2 mcg./ml. (0.5-ml. specimen) for Metabolite A.

Analysis of Acetylated Dantrolene—The acetylated dantrolene metabolite is determined in biological fluids by slightly modifying the method specific for the determination of dantrolene in tissues. When acetylated dantrolene standards in water, plasma, blood, and urine are subjected to this modified method, linear curves are obtained from 0.2 to 3.0 mcg. Recoveries of added acetylated dantrolene from biological fluids as determined by this method are shown in Table V. Method sensitivity is 0.4 mcg./ml. for a 0.5-ml. specimen. Neither dantrolene nor the dantrolene-related metabolites, amino-dantrolene and Metabolite A, interfere in the analysis of acetylated dantrolene by this procedure.

Table V—Recovery of Acetylated Dantrolene from Biological Fluids

Fluid	Fluorescence Units[a] — Acetylated Dantrolene, mcg. — Control	0.2	0.5	1.0	3.0	Percent Recovery[b], Mean ± *SD*
Water	0.4	0.2	0.5	0.8	2.6	—
Human blood	0.5	0.2	0.5	0.8	2.6	100.0 ± 0.0
Human plasma	0.4	0.2	0.5	0.8	2.6	103.0 ± 6.0
Human urine	0.4	0.2	0.5	0.8	2.6	100.0 ± 0.0

[a] Control-corrected data based on a mean from at least three samples. [b] Based on aqueous acetylated dantrolene standards.

DISCUSSION

The described method is sensitive and specific for the determination of dantrolene in biological fluids and tissues. Essentially, the procedure consists of the extraction of dantrolene and two major dantrolene-related metabolites, Metabolite A and acetylated dantrolene, from biological specimens into a solvent admixture and then into a dilute alkaline solution. Metabolite A is then separated and removed from dantrolene and acetylated dantrolene by column chromatography. After both dantrolene and acetylated dantrolene are eluted, dantrolene is extracted into a solvent admixture and measured fluorometrically.

Apparently, blood dantrolene concentrations are related to the resultant skeletal muscle relaxation observed in man after oral administration of dantrolene sodium (6). Since dantrolene-related metabolites also are present in the blood under these conditions (5), a method specific for dantrolene is required to evaluate accurately the drug-induced pharmacological effect in relation to the concurrent blood dantrolene concentration. This method specificity is also necessary for investigating urinary dantrolene excretion since greater amounts of both Metabolite A and acetylated dantrolene than of dantrolene are found in human urine after oral administration of dantrolene sodium (5).

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DRUG STANDARDS

Determination of Liothyronine and Thyroxine in Dried Thyroid by GLC

ROMAN BILOUS▲ and JOHN J. WINDHEUSER*

Abstract—The analysis reported here was successful in the quantitative determination of liothyronine and thyroxine in driedthyroid. Three basic steps were employed: (*a*) hydrolysis of the dried thyroid to release the iodoamino acids from their protein linkage, (*b*) separation of liothyronine and thyroxine from the interfering substances by extraction and column chromatography, and (*c*) detection and quantitation by GLC.

Keyphrases □ Thyroid, dried—separation, GLC analysis, liothyronine and thyroxine □ Liothyronine and thyroxine—separation, GLC analysis in dried thyroid □ Thyroxine and liothyronine—separation, GLC analysis in dried thyroid □ GLC—analysis, liothyronine and thyroxine in dried thyroid

The thyroid gland contains a number of iodoamino acids, of which the principal hormones secreted are thyroxine and liothyronine. Small amounts of 3,3′,5′-triiodothyronine, diiodotyrosine, and monoiodotyrosine are also present (1). However, biological activity appears to be present only in thyroxine and liothyronine, with little or no activity in 3,3′,5′-triiodothyronine (2, 3). The original method of standardization was done by the determination of total organic iodine (4) or by the "thyronine" iodine content determination of Harington and Randall (5). Although this method is highly nonspecific, it is still being utilized, with slight modifications, as the official USP XVIII method.

GLC appeared to hold a great deal of promise in the determination of thyroid hormones. The results would be specific and would differentiate between the compounds found useful in therapy, namely liothyronine and thyroxine. Other compounds of lesser activity such as 3,3′,5′-triiodothyronine would also be separated and quantitatively determined.

The high molecular weight and the lack of volatility necessitated the formation of a volatile derivative. Derivatives prepared by Stouffer *et al.* (6) and Richards and Mason (7) required a two-step reaction. Zimmerer and Grady (8) used a one-step method to form methyl derivatives by means of diazomethane, but their derivatives eluted as a pair of peaks for both liothyronine and thyroxine and this did not appear suitable for our approach. A one-step derivatization method using a silylating agent appeared to be the method of choice. Although Zimmerer and Grady (8) noted the difficulty in obtaining reproducible silylation, this was not noticed in our work when suitable precautions were taken. Since thyroxine and liothyronine are found bound in a peptide linkage to thyroglobulin, which is present in the colloid material in the follicles of the thyroid gland (1), it is necessary to break this peptide linkage either chemically or enzymatically to obtain the free iodoamino acids prior to GC.

Some authors used alkaline hydrolysis to break the peptide linkage (9–11) while others used various enzymes to achieve the same result (12–15). Generally, the method involved the refluxing of dried thyroid with various bases or the utilization of a number of proteolytic enzymes. The hydrolysates, obtained by either method, were extracted with 1-butanol.

However, the quantitation of liothyronine and thyroxine in the 1-butanol extract by GLC was not feasible due to the presence of interfering substances

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DRUG STANDARDS

Determination of Liothyronine and Thyroxine in Dried Thyroid by GLC

ROMAN BILOUS▲ and JOHN J. WINDHEUSER*

Abstract—The analysis reported here was successful in the quantitative determination of liothyronine and thyroxine in driedthyroid. Three basic steps were employed: (*a*) hydrolysis of the dried thyroid to release the iodoamino acids from their protein linkage, (*b*) separation of liothyronine and thyroxine from the interfering substances by extraction and column chromatography, and (*c*) detection and quantitation by GLC.

Keyphrases □ Thyroid, dried—separation, GLC analysis, liothyronine and thyroxine □ Liothyronine and thyroxine—separation, GLC analysis in dried thyroid □ Thyroxine and liothyronine—separation, GLC analysis in dried thyroid □ GLC—analysis, liothyronine and thyroxine in dried thyroid

The thyroid gland contains a number of iodoamino acids, of which the principal hormones secreted are thyroxine and liothyronine. Small amounts of 3,3′,5′-triiodothyronine, diiodotyrosine, and monoiodotyrosine are also present (1). However, biological activity appears to be present only in thyroxine and liothyronine, with little or no activity in 3,3′,5′-triiodothyronine (2, 3). The original method of standardization was done by the determination of total organic iodine (4) or by the "thyronine" iodine content determination of Harington and Randall (5). Although this method is highly nonspecific, it is still being utilized, with slight modifications, as the official USP XVIII method.

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Some authors used alkaline hydrolysis to break the peptide linkage (9–11) while others used various enzymes to achieve the same result (12–15). Generally, the method involved the refluxing of dried thyroid with various bases or the utilization of a number of proteolytic enzymes. The hydrolysates, obtained by either method, were extracted with 1-butanol.

However, the quantitation of liothyronine and thyroxine in the 1-butanol extract by GLC was not feasible due to the presence of interfering substances

which necessitated further purification of the extract. It was reported in a previous article (16) that the interference was probably due to charring of nonvolatile material on the GLC column.

To accomplish this purification, a number of different methods were used: column chromatography (14, 17–22), paper chromatography (10, 12, 13, 15), TLC (11, 23), and gel filtration (24). Although all methods achieved limited success, they were still not totally specific for liothyronine and thyroxine. Quantitation was based on the determination of iodine from the separated fractions. This approach suffered from the obvious disadvantage of being only partially specific, because iodine could come from physiologically active and nonactive iodoamino acids.

The clean separation of the physiologically active thyroid hormones from the other constituents, present after hydrolysis and extraction, is the main problem that has defied many workers in this field. Without this separation, the specific determination of the amounts of liothyronine and thyroxine in dried thyroid is difficult to achieve.

Although GLC has been successfully used for synthetic mixtures of iodoamino acids, it has not been successfully applied to dried thyroid (6, 8, 24–30). This article reports the successful separation and quantitation of liothyronine and thyroxine from dried thyroid by GLC.

EXPERIMENTAL[1]

Reagents and Chemicals—The following were used: thyroxine (T_4, 3,5,3′,5′-tetraiodothyronine) recrystallized from 80% (v/v) aqueous ethanol and 5 ml. 2 *N* hydrochloric acid (25); liothyronine (T_3, 3,5,3′-triiodothyronine) USP reference standard; 3,3′,5′-triiodothyronine[2] (T_3', reverse T_3, or T_r-iso); Sephadex G-10[3]; dimethyl silicone[4]; diatomaceous earth[5], silane treated, 60–80 mesh; and *N,O*-bis(trimethylsilyl)acetamide[6]. All other chemicals were reagent grade.

Solvents and Solutions—All solvents were of reagent grade or as noted. The following were used: pyridine, anhydrous, reagent grade, distilled and stored over potassium hydroxide, b.p. 113–115°; 1-butanol, redistilled, b.p. 114°; ammonia–methanol solution, 5% v/v: ammonium hydroxide in anhydrous methanol; aqueous ammonia–methanol solution, 10% v/v: ammonium hydroxide and water in anhydrous methanol (10:40:50); acid–methanol solution, 5% v/v: hydrochloric acid in anhydrous methanol; and 1-butanol equilibrated with 0.1 *N* sodium thiosulfate.

Preparation of Sephadex Column—Thirty-three grams of Sephadex G-10 was mixed with 100 ml. of aqueous ammonia–methanol solution, 10% v/v, and allowed to solvate overnight. A column (27 × 2.1 cm.) was then prepared and allowed to stand overnight. It was foil covered.

[1] The instrumentation consisted of a Nuclear-Chicago Research gas chromatograph, series 5000, with hydrogen-flame ionization detector and U-shaped column oven; a Barber-Colman strip chart recorder, series 8000, equipped with a disk chart integrator, model 205; a Beckman Expandomatic pH meter, and a Mettler Micro Gram-atic balance, high-speed M-5 S/A. The apparatus used were a Buchler portable flash evaporator; a Burrell wrist-action shaker (Burrell Corp., Pittsburgh, Pa.); Hamilton microliter syringes, #701-N (10 μl.) and #701-NCH (500 μl.); U-shaped borosilicate glass columns, 1.83-m. (6-ft.) heavy wall 3-mm. i.d.; and liquid chromatography columns, 27 × 2.1 cm., with Teflon stopcocks.

[2] Warner-Lambert Research Institute, Lot 9, Feb. 1970, through Dr. Daniel Banes, Director, Office of Pharmaceutical Research and Testing, Bureau of Drugs.

[3] Pharmacia Fine Chemicals, Piscataway, N. J.

[4] OV-1, Applied Science Laboratories, Inc., State College, Pa.

[5] Gas-Chrom Q, Applied Science Laboratories, State College, Pa.

[6] Pierce Chemical Co., Rockford, Ill.

PROCEDURE

Preparation of Sample—Mix approximately 0.3 g. of dried thyroid, accurately weighed, with 3 g. of barium hydroxide [$Ba(OH)_2 \cdot 8H_2O$] and 6 ml. of water in a foil-covered round-bottom flask. Flush with nitrogen and air flux for 6 hr. at 96°. When reflux temperature is reached, flush again with nitrogen and lightly seal the apparatus. Cool and acidify with approximately 3.5 ml. of dilute hydrochloric acid (1:1) to pH 0.8–0.9 as determined by a pH meter. Extract the acidified solution with 10, 5, 5, and 5 ml. of 1-butanol saturated with 0.1 *N* sodium thiosulfate while protecting from light. Wash the combined 1-butanol extracts with three 25-ml. quantities of distilled water saturated with 1-butanol. The final washing is to be neutral to pH indicator paper. Filter through a medium sintered-glass filter. Rinse the separator with 1-butanol and, after passing the rinsings through the filter, combine with the original extract. Evaporate to dryness under reduced pressure with a rotary evaporator in foil-covered flasks. Add ethanol to aid in removal of moisture and evaporate to dryness.

Transfer the residue quantitatively, using a total of 5 ml. of aqueous ammonia–methanol solution, 10% v/v, to a Sephadex G-10, light-protected, 27 × 2.1-cm. column. Elute with the same solvent. Discard the first 35 ml. of eluate but collect the second fraction of 80 ml. Evaporate the eluate to dryness under reduced pressure in a light-protected flask. Add 5 ml. of acid–methanol solution, 5% v/v, and evaporate to dryness under reduced pressure. Add 5 ml. of ethanol, evaporate to dryness, and flush with nitrogen.

Add 100 μl. of anhydrous pyridine and 300 μl. of *N,O*-bis(trimethylsilyl)acetamide and heat for 2 hr. at 50°. Refrigerate overnight.

Preparation of Standard Derivative—Accurately weigh on a microbalance approximately 1 mg. of liothyronine and 1.5 mg. of thyroxine into a 4-ml. (1-dram) vial with a foil-lined screw cap. Wrap the vial with foil. Add 100 μl. of anhydrous pyridine and 300 μl. of *N,O*-bis(trimethylsilyl)acetamide and heat for 2 hr. at 50°. Store overnight in a refrigerator. Inject 3 μl. onto the GLC column.

GLC—For analysis, use GLC under the following conditions: 1.83-m. (6-ft.) × 3-mm. i.d. heavy wall borosilicate glass U-shaped column, packed with 1% dimethyl silicone coated onto 60–80-mesh diatomaceous earth, silane treated, and conditioned at 300° overnight; initial column temperature, 165°; final column temperature, 285°; programmed rate, 5°/min.; chart speed, 1.3 cm. (0.5 in.)/min.; nitrogen carrier gas flow, 50 ml./min.; injector temperature, 240°; detector temperature, 330°; attenuation for thyroxine, liothyronine, and 3,3′,5′-triiodothyronine, 16×10^{-10} amp.; and attenuation for dried thyroid sample, 16×10^{-10} amp., changed to 2×10^{-10} amp. at 235° (13 min.). Inject 3 μl. of standard derivative and obtain the areas for liothyronine and thyroxine. Then inject 3 μl. of the dried thyroid sample derivative to obtain areas of liothyronine and thyroxine.

Quantitation of Results—Calculate the amount of liothyronine and thyroxine from the integration areas:

$$\frac{A_u}{A_s} \times \frac{C_s}{W} = \text{mg./g.} \qquad \text{(Eq. 1)}$$

where A_u = integrated area of unknown, A_s = integrated area of standard, C_s = milligrams of standard in 400 μl., and W = weight of dried thyroid sample in grams.

RESULTS AND DISCUSSION

Effect of Hydrolysis on Synthetic Mixtures of Liothyronine and Thyroxine and Completeness of Extraction—This portion of the study was undertaken to determine if the hydrolytic conditions caused degradation of the iodoamino acids and if the extraction procedure gave quantitative results. To determine these points, synthetic mixtures of liothyronine and thyroxine were subjected to a 6-hr. hydrolysis as described previously. The hydrolysis flask fitted with an air condenser was protected from light and flooded with nitrogen. The recovery of liothyronine and thyroxine was 100.6% (1.2σ) and 98.92% (2.22σ), respectively, using the described analytical procedure. The results are recorded in Table I.

The study indicated that liothyronine and thyroxine were stable under the conditions of hydrolysis; extraction was complete and could be determined by GLC.

Table I—Recovery of Liothyronine (T_3) and Thyroxine (T_4) after Hydrolysis

Liothyronine (T_3)			Thyroxine (T_4)		
Added, mg.	Found, mg.	Percent Recovery	Added, mg.	Found, mg.	Percent Recovery
0.883	0.885	100.2	1.529	1.450	94.83
0.812	0.792	97.54	1.645	1.585	96.35
0.905	0.932	103.0	1.610	1.604	99.63
1.040	1.058	101.7	1.486	1.559	104.9
	Mean	100.6		Mean	98.92
	σ	1.2		σ	2.22

Table II—Recovery of Liothyronine (T_3) and Thyroxine (T_4) from Sephadex G-10 Columns

Liothyronine (T_3)			Thyroxine (T_4)		
Added, mg.	Found, mg.	Percent Recovery	Added, mg.	Found, mg.	Percent Recovery
1.086	1.111	102.3	1.527	1.619	106.0
1.086	1.084	99.82	1.527	1.569	102.8
1.005	1.041	103.6	1.507	1.534	101.8
	Mean	101.9		Mean	103.5
	σ	1.1		σ	1.3

Direct extraction of the synthetic mixtures without prior hydrolysis was also found to be quantitative.

Purification—Several techniques were attempted to separate the iodoamino acids from interfering substances in the 1-butanol extract. Column chromatography appeared to offer the greatest probability of separation; therefore, columns of aluminum oxide, diatomaceous earth[7], and Sephadex G-10, G-15, and LH-20 were tried using a number of solvents. Recoveries ranged from zero to 95%. The low recoveries could have been due to adsorption on the columns or the failure to remove the charring impurities.

However, when liothyronine and thyroxine were eluted with aqueous ammonia–methanol solution, 10% v/v, from a Sephadex G-10 column, previously prepared with the same solvent, the first fraction of 35 ml. contained the interfering material. The second fraction of 80 ml. contained the liothyronine and thyroxine. The results are contained in Table II.

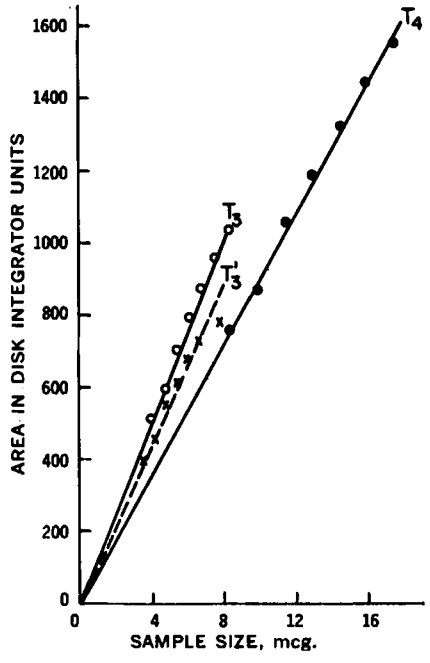

Figure 1—*Detector response for varying amounts of liothyronine (T_3), 3,3',5'-triiodothyronine (T_3'), and thyroxine (T_4).*

[7] Celite 535, Johns-Manville, New York, N. Y.

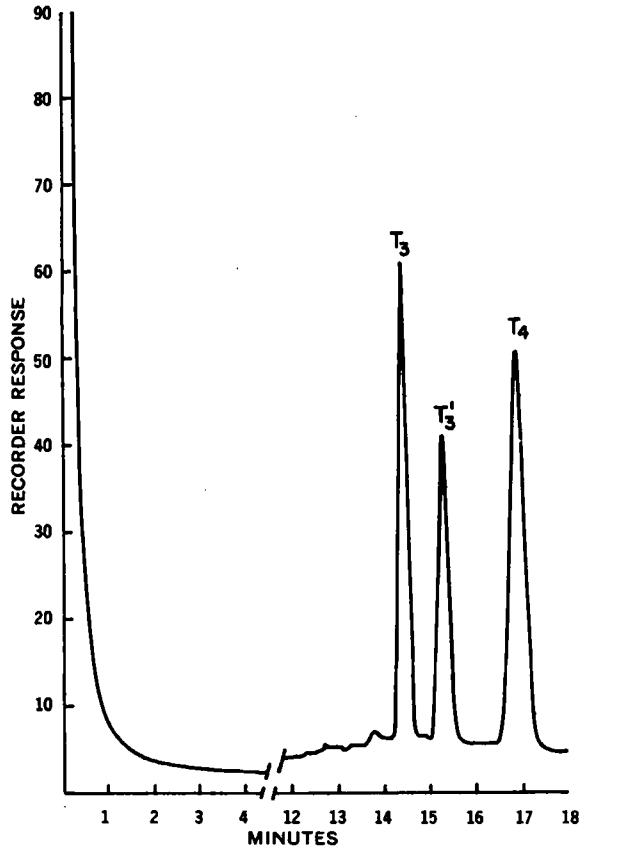

Figure 2—*Temperature-programmed chromatogram of a standard derivative (programmed temperature = 165–285°, programmed rate = 10°/min., and attenuation = 16 × 10^{-10} amp.).*

The success of separation depends on the preparation and packing of the column. Reproducible elution patterns were obtained only if the Sephadex G-10 was solvated overnight and then the packed column was allowed to stand overnight to ensure uniform density. Directly before using, 200 ml. of eluting solvent is passed through the column.

After elution and removal of the solvent from the fraction containing the iodoamino acids, 5 ml. of 5% v/v acid–methanol solution is added to the flask and removed under reduced pressure. This step is vital to the successful preparation of silyl derivatives. The conversion of liothyronine and thyroxine to the acid form is necessary since the corresponding salts will not readily form these derivatives.

Effect of Light—Initially, attempts to elute quantitatively liothyronine and thyroxine from columns gave low results. It was suspected that the low results might have been due to light-catalyzed degradation. Experiments were then carried out where all operations, from elution to the preparation of the trimethylsilyl derivative, were protected from light. Recoveries were reproducible and quantitative. Based on these findings, all other facets of the procedure, from hydrolysis to derivatization, were protected from light and treated with nitrogen gas. The exact nature of the degradation was not studied further.

Preparation of a Trimethylsilyl Derivative—To prepare this derivative, a method similar to that of Alexander and Scheig (25) was used. Pyridine was used to facilitate silylation of the iodoamino acids. The temperature was kept at 50°, because signs of possible degradation appeared when higher temperatures were employed. Greater and more reproducible responses were obtained if the derivatives were refrigerated overnight. As is the case with most trimethylsilyl derivatives, they are prone to hydrolytic degradation, so care must be exercised to prevent contact with moisture. After overnight refrigeration, the sample must be allowed to come to room temperature before being opened.

Table III—Determination of Liothyronine (T_3), 3,3′,5′-Triiodothyronine (T_3'), and Thyroxine (T_4) in Dried Thyroid

Porcine Dried Thyroid[a]		T_3, mg./g.	T_3', mg./g.	T_4, mg./g.
Lot 263 containing 0.89% w/w iodine		0.344	—	1.571
		0.355	—	1.352
		0.331	—	1.249
		0.345	—	1.432
		0.392	0.082	1.421
		0.383	0.073	1.326
		0.360	0.077	1.265
	Mean	0.358	0.077	1.374
	σ	0.008	0.003	0.042
Lot 102		0.298	—	1.348
		0.326	0.090	1.187
	Mean	0.312	0.090	1.267
	σ	0.014	—	0.081

[a] Armour.

Quantitative Results—It was found that there was a linear relationship between the sample size and recorder response for liothyronine, 3,3′,5′-triiodothyronine, and thyroxine as shown in Fig. 1. A typical chromatogram of a standard derivative is shown in Fig. 2.

When the described method, including the purification step, was applied to dried thyroid, the results given in Table III were obtained.

Data are presented on two lots of dried thyroid. These results appear reasonable based on estimates of iodine content. However, it is not possible to state with absolute certainty the total percentage recovery of liothyronine and thyroxine in these samples since the absolute analysis is not known. However, when liothyronine and thyroxine were added to dried thyroid (Lot 263) and assayed according to the described method, quantitative recovery of the added material was obtained when calculated by difference. Although this is not an absolute proof of quantitation, it does lend support to the data.

A chromatogram of the 1-butanol extract of a dried thyroid hydrolysate is shown in Fig. 3. In addition to liothyronine and thyroxine, 3,3′,5′-triiodothyronine and an unknown were separated. The unknown compound is probably an iodoamino acid whose identity has still to be verified.

Other iodoamino acids were not quantitatively determined because it was found in previous work that 3-monoiodotyrosine, 3,5-diiodotyrosine, and 3,5-diiodothyronine were not quantitatively extracted by this procedure; therefore, correlation with the total iodine content of the dried thyroid powder could not be done. Further work to substantiate absolute quantitation must be carried out as well as the correlation of this method with biological activity measurements.

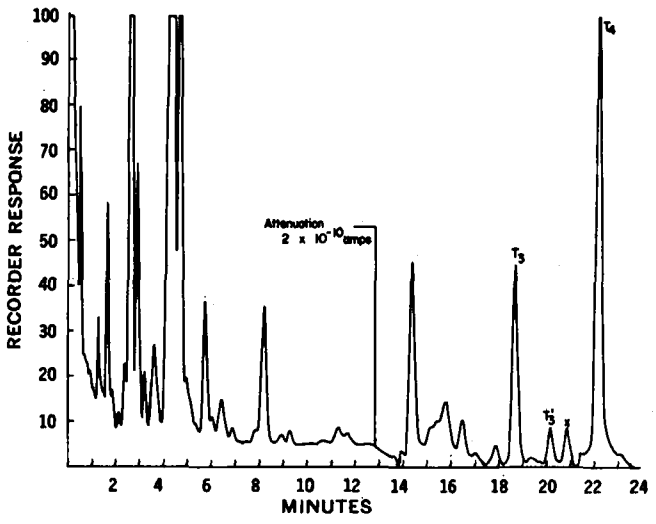

Figure 3—*Chromatogram of dried thyroid hydrolysate. The thyroid hormones were liothyronine (T_3), 3,3′,5′-triiodothyronine (T_3'), thyroxine (T_4), and an unknown, X.*

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Steroid Determination from Complex Mixtures by High Pressure Chromatographic Techniques

W. C. LANDGRAF▲ and E. C. JENNINGS

Abstract □ The concepts described in this work emphasize the fabrication of components, the importance of partitioning supports, and an interesting ternary mobile phase system for high pressure liquid chromatography. The particular application of its utility as an analytical tool relates to quantitative determination of fluocinolone acetonide and fluocinonide from cream- or ointment-like formulations. Sensitivity of detection and resolution capability of the partitioning system for a number of related steroids are discussed in some detail.

Keyphrases □ Steroids in complex mixtures—analysis, high pressure liquid chromatography, ternary mobile phase system □ Fluocinolone acetonide cream, ointment—analysis, high pressure liquid chromatography □ Fluocinonide cream, ointment—analysis, high pressure liquid chromatography □ High pressure liquid chromatography—analysis, steroids in complex mixtures, ternary mobile phase system

The determination of specific steroids or other "active" components from their related degradation products in pharmaceutical preparations is a common problem (1). This requirement of specificity has frequently caused concern for quantitative measurements. As techniques in liquid chromatography have developed, the ability to resolve closely related components has improved to the extent that good quantitation at low concentration levels (2–4) is possible. To further this objective, a high pressure unit has been designed, built, and operated. This system permits efficient separation of a number of steroids from complex pharmaceutical preparations. The intent of this effort was to provide a rapid steroid analysis where problems of sensitivity and separation are simultaneously present. The nature of the substrate has proved to be an important factor in the development of this assay procedure.

EXPERIMENTAL

Instrument Design—Insofar as possible, the components fabricated into the system were acquired from commercial sources. The overall concept is depicted in Fig. 1. The majority of reciprocating pumps, as the one used here, is associated with cyclic pressure changes. Therefore, a surge attenuator is used in this system, and it is responsible for the reduction in cyclic pressure surges. The pump[1] employed (5) provides the pressurization of the mobile phase throughout the system. Upon leaving the pump, the fluid passes through a 10-μ fritted stainless steel filter prior to entering the surge attenuator[2] (6) operating at 3000 psi. This unit performs two functions: (*a*) it initially serves as a sump for the mobile phase, and (*b*) it efficiently dampens the pump surges which develop within the system. After leaving the surge attenuator, the fluid travels through narrow bore tubing (0.23 mm.) to the reference side of the differential UV detector and then to the sampling injector valve[3] (7). This loop injector valve permits varying the injection volume between 2 and 20 μl. Furthermore, this system can be continuously operated under high pressures while introducing samples. Upon exiting this valve, the mobile phase is carried through the chromatographic column to the sample side of the UV detector. The effluent material from the cell can be recovered for additional analyses.

The initial choice for a detector was a high pressure UV absorption unit[4]. This detector unit has an internal volume of 12 μl. and is capable of operating up to 2000 p.s.i. It normally operates at 254 nm. but has the capability of operating at 280 and 360 nm. with appropriate filtering. The 10-mv. output from the differential detector can be then applied to a recorder.

In addition to the UV detector described previously, a nondifferential UV detector[5] was employed. This detector provides a convenient alternative where desired wavelengths, other than those previously mentioned, should be employed. A 250-μl. cell is used in the current configuration; however, smaller cells have been found useful. No special fabrication is required to employ this model. Usually 100-mcg. samples are used where strong UV chromophores exist. Furthermore, the detector was utilized in preliminary preparative level work where milligram quantities of sample were employed. If one accepts the current limits of sensitivity for this UV detector, it provides an acceptable alternative to existing detectors. In addition, this detector has a wide range of wavelength utility, *i.e.*, ~200–~700 nm.

Perhaps the most important aspect of the total concept relates to the stationary support of this system. These materials consist of spherically shaped particles of silica, approximately 40–70 μ in size, having an effective surface area of 300–400 m.^{2}/g. (8–12) (Spherosil or Porasil particles). The selection of uniformly spherical particles is critical because the developed flow and pressure patterns reflect the homogeneity of the substrate's surface. Since the purpose of this effort was to achieve separation of relatively similar types of steroids, it was considered best to establish some degree of partitioning capability with the solid support. The "surface" of the solid support was, therefore, viewed as requiring partitioning between it and the mobile phase. A program for chemical modification of the surface was then developed.

Silica surfaces were activated with either trichloro or similar silanes (13, 14) containing the desired substituted polar functional groups (*e.g.*, cyano) used in the reaction. When groups other than cyano were thought appropriate [*i.e.*, glycidoxy (15, 16)], they were substituted for the nitrile silanes. Glycidoxy groups could later be modified with other reagents. Approximately 15% of the composite weight of the treated beads was found to represent the increase in weight caused by the reactive silanes. To ensure complete removal of unreacted materials, the treated beads were extracted using a soxhlet extractor for varying lengths of time. In all cases, this was followed by appropriate oven drying. For a column of 100 cm. × 2 mm. i.d., about 3 ml. of this packing material was required. One normally obtains about 52 ml. of treated beads from 20 g. of the uncoated Porasil.

Packing of Columns—Steel columns (100 cm. × 2 mm. i.d.) are equipped with a single fritted steel plug (2-μ porosity) at one end. A small funnel is secured with Tygon tubing to the open end of the steel tube, and the packing material is introduced, 0.5 ml. at a time, into the column. To ensure uniform distribution of the material, the column is continuously tapped on a solid support. When the packing is within 5 mm. of the top of the steel tube, a glass wool plug is inserted securely into place to minimize support losses while handling. Each column is inserted into the system such that the

[1] Milton Ray D HBD1-30R.
[2] Robert Shaw hydropad.
[3] Valco.
[4] Nester/Faust model 250.
[5] Model 350 Turner spectrophotometer.

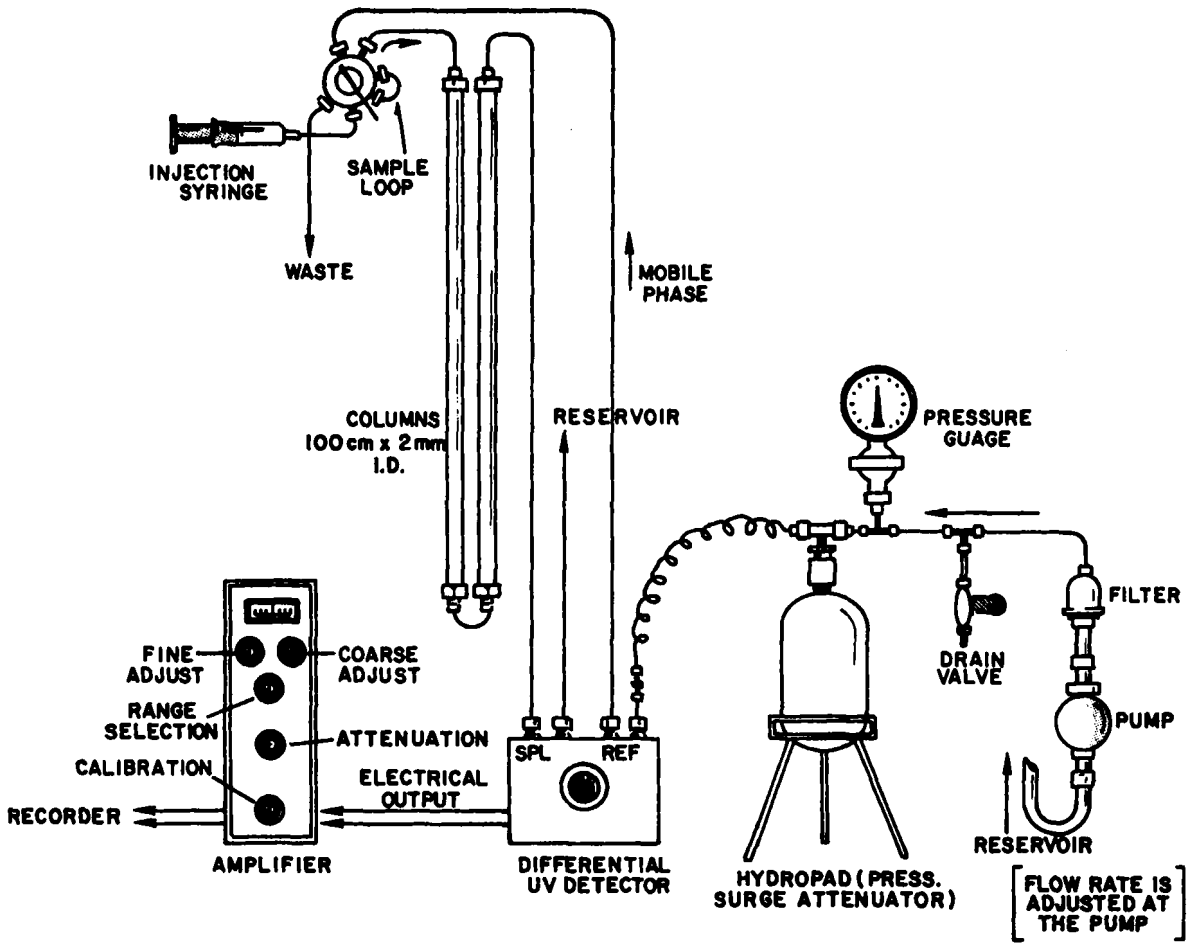


Figure 1—*Schematic layout of high pressure liquid chromatographic system.*

glass plug faces the incoming solvents. This practice tends to minimize the plugging of the fine bore tubing. In a normal exchange of, or addition to, the columns in the system, about 10–15 min. is required.

Analytical Procedure—A 10-ml. disposable syringe is filled with the ointment or cream sample, and the weight is noted to the nearest milligram. A sample weight of 5 g., determined by differential weighings, is then transferred into a 250-ml. separator. Fifty milliliters of an 80% methanol–water mixture is added to 50 ml. of cyclohexane in the separator. Total mixing of the phases is completed by vigorous shaking; subsequently the two layers are permitted to separate and the lower aqueous methanol layer is removed. Repeated extraction of the remaining cyclohexane is completed with portions of a methanol–10% aqueous salt solution (4:1). The latter extract is added to the first methanol extract. About 100 ml. of a 5% potassium alum sulfate solution is added to the methanol phase. This solution is then extracted with 15-ml. portions of chloroform. The latter volumes are dried by pouring the solution through anhydrous desiccant (sodium sulfate). These combined extracts are reduced in volume under nitrogen to a final volume of about 4 ml. The remaining volume is transferred quantitatively to a 10-ml. volumetric flask for a final 10-ml. dilution using chloroform.

The final transfer of this diluted volume of extracted sample is achieved using a 1-ml. gas-tight glass syringe[6] using a Luer lock fitting. It is connected to the hub fitting of the injection valve described in Fig. 1. The sample is transferred to the loop and thereafter injected onto the chromatographic column. This loop has a variable volume ranging from 10 μl. to 1 ml. For much of the current work, three 100-cm. columns were connected in series using the treated Porasil beads. Flow rates varied from 2 to 5 ml./min., and the usual pressure was about 1500 p.s.i. at room temperature with the following mobile phase:

2,2,4-trimethylpentane	69.0% (v/v)
isopropanol	18.5%
acetonitrile	12.5%

Sensitivity ranges most frequently used for the UV detector were about 0.05 × 1 for fullscale absorbance. In all determinations, two or more samples were examined. These values were compared areawise (mm.²) with those peaks of the standard solutions or internal standards used for the comparison. Peak height and half height widths were used in these comparisons.

DISCUSSION

Calibration Data—The data shown in Table I were obtained with a solution of fluocinonide in chloroform by dilution of a

Table I—Calibration Data Using Fluocinonide in Chloroform[a]

Concentration, mg./ml.	Retention Time, min.	Peak Area, mm.² (Sensitivity = 0.05 × 1)	mcg./mm.² × 10^{-3}
0.134	18.8	210	6.38
0.134	18.5	255	5.25
0.267	18.5	465	5.75
0.267	18.5	440	6.07
0.400	18.5	700	5.73
0.400	18.5	670	5.98
0.534	18.0	910	5.87
0.534	18.3	890	6.00

[6] Hamilton 101.

[a] Average response ± *SD* = (5.88 ± 0.33) × 10^{-3} mcg./mm².

Table II—Analytical Data for Fluocinonide Ointment

Sample Weight, g.	Peak Area, mm.²	Fluocinonide, mg. Found	Theory	Percent Recovery
4.930	405	2.38	2.47	96.5
	395	2.32		94.3
			Average	95.4
5.436	480	2.82	2.72	103.5
	476	2.80		103.0
			Average	103.3

g.	mm.²	Found	Theory	Recovery
2.324	344	0.572	0.581	98.4
	348	0.578		99.5
2.509	368	0.612	0.627	97.8
	376	0.608		97.2
			Average	98.2
			$SD = \pm$	0.98

standard stock solution (2.67 mg./ml.) of the steroid in methanol–chloroform (4:1).

Figure 2 illustrates a typical separation using the current column-partitioning system.

Assay data for a fluocinonide ointment preparation (0.05%) is displayed in Table II. In comparison, the assay data relating to a typical cream formulation containing 0.025% fluocinolone acetonide are contained in Table III.

Mobile Phase System—The use of a multiple-component mobile phase system permits wider flexibility in the choice of operating parameters (17). From an inspection of the ternary phase diagram (Fig. 3), the operating range of mutual solubilities of trimethylpentane, isopropanol, and acetonitrile can be seen. The composition of this three-component system that is most frequently used is 10–15% acetonitrile, 15–30% isopropanol, and the remainder trimethylpentane. To a limited extent, the use of the ternary mobile phase permits modification of the retention times of the solutes. These types of correlations are under continued study. If one operates the ternary system in the region of two phases, the problem of light scattering by the second phase becomes a serious source of noise. Therefore, one should select those concentrations of mobile phases that will not lead to phase separation.

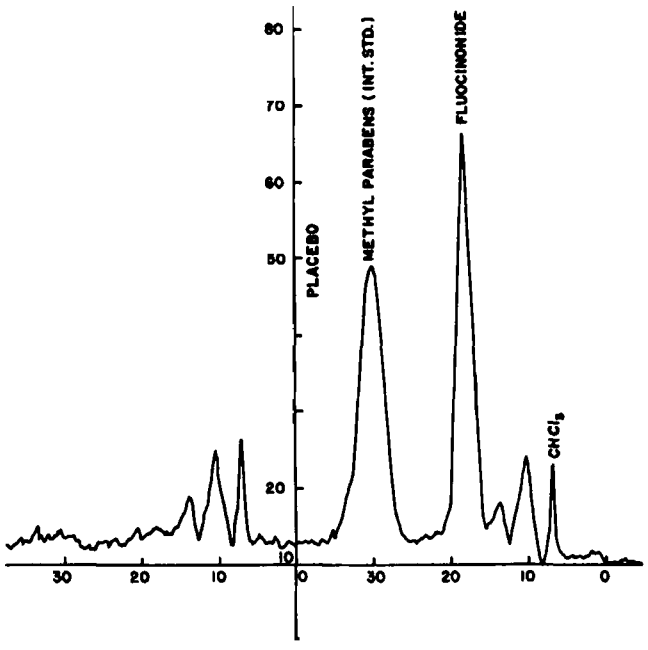


Figure 2—*High pressure liquid chromatogram of fluocinonide* versus *placebo separation.*

Retention Times of Various Steroids—Examination of Table IV illustrates the relative retention times of a number of steroids examined during the present work. By using progesterone (I) as the reference, the data were tabulated (Table V) for several steroids where specific types of substitution were made, and the differences in the retention times are shown.

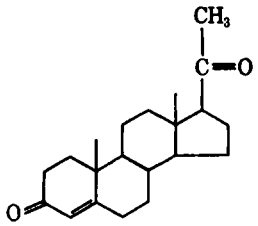
Since the work is only preliminary in nature, claims cannot be made for unambiguous incremental changes due to specific substitution. However, the development of this preliminary work suggests that further efforts to secure possible correlations are desirable.

SUMMARY

In the present paper the utility of employing chemically treated, spherically shaped particles for chromatographic purposes has been established. In particular, partitioning of various steroids between the mobile phase and the solid phase supporting system has been demonstrated. The mechanism for such a partitioning appears to arise from the polar interactions of the solid support with the solute

Table IV—Retention Times of Various Steroids

Number	Compound	Retention Time, min.
	17-acetal with acetone)	18.5
4	Desoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione)	20.4
5	Flurandrenolide (6α-fluoro-11β,16α,17,21-tetrahydroxypregn-4-ene-3,20-dione, cyclic 16,17-acetal with acetone)	22.0
6	Paramethasone acetate (6α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione 21-acetate)	22.0
7	11-Deoxycortisol (17α,21-dihydroxy-4-pregnene-3,20-dione)	23.2
8	Corticosterone (11β,21-dihydroxy-pregn-4-ene-3,20-dione)	26.0
9	Cortisone acetate (17,21-dihydroxypregn-4-ene-3,11,20-trione 21-acetate)	28.2
10	Fluocinolone acetonide (6α,9α-difluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione, cyclic acetal with acetone)	27.6
11	Lactone from Compound 10 (6α,9α-difluoro-11β,16ξ,20ξ-trihydroxypregna-1,4-diene-3-one-21-oic-16-lactone)	64.0
12	21-Aldehyde from Compound 10 (6α,9α-difluoro-11β,16α,17α-trihydroxypregna-1,4-diene-3,20-dione-21-al-16,17-acetonide)	66.0
13	21-Acid from Compound 10 (6α,9α-difluoro-11β,16α,17α,20-tetrahydroxypregna-1,4-diene-3-one-21-carboxy-16,17-acetonide)	>60.0



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molecules (18). The desire to employ chemically bound polar phases was initiated by the inability to obtain satisfactory results from commercially available products. In general, these commercial materials were found to degrade rather quickly and to be of lower overall performance for the present work.

In comparison to other methods for extraction of steroids from various formulations, the single method alluded to in the *Experimental* section was the most satisfying and, therefore, was used exclusively in the present work. In attempting to quantitate the method, internal and external standards have been used with success. At present, the use of internal standards has been found to be more acceptable since smaller intrinsic errors are involved with their use.

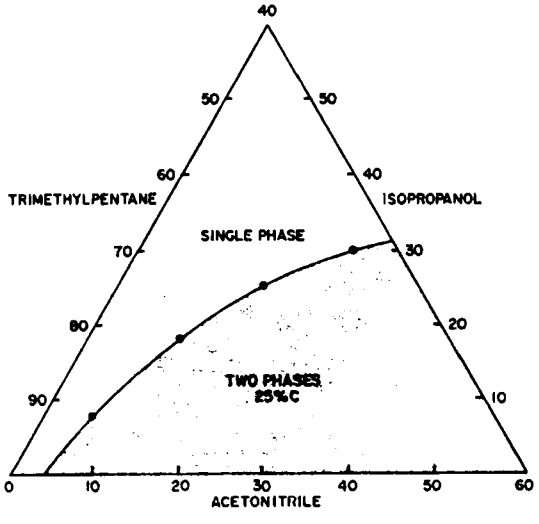

Figure 3—*Ternary phase diagram for mutual solubilities of trimethylpentane, isopropanol, and acetonitrile.*

Table V—Steroid Retention Times

15.8 min., Room Temperature, Progesterone Substitution	Retention Time Increment, Δ_{time}		
	21-OH	11-OH, 17-OH	11-one
21-OH	4.6	—	—
17α-OH, 21-OH	4.6	2.8	—
11β-OH, 21-OH	4.6	5.6	—
11-one, 17α-OH, 21-OH	4.6	2.8	4.0

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Chemical Determination of Component Ratio and Potency of Gentamicin Complex

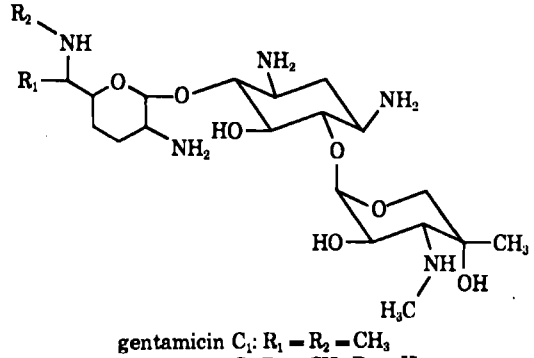
WILLIAM L. WILSON[A], GINETTE RICHARD, and DAVID W. HUGHES

Abstract □ A rapid chemical method for estimating the total gentamicin potency as well as for quantitating the individual components in one step by direct densitometry after resolution on TLC is described. Because the amount of each component is read directly from the plate after separation of impurities and the values are interpolated from standards treated in an identical manner, this method is more accurate for estimating total potency than the official microbiological assay in which biologically active impurities may interfere.

Keyphrases □ Gentamicin—simultaneous component variation analysis and potency determination, TLC–direct densitometry □ Microbiological potency, gentamicin—simultaneous determination with component variation analysis, TLC–direct densitometry □ TLC–direct densitometry—simultaneous component variation analysis and potency determination, gentamicin □ Direct densitometry and TLC—simultaneous component variation analysis and potency determination, gentamicin □ Antibiotics—method for simultaneous component variation analysis and potency determination

Gentamicin is a broad spectrum oligosaccharide antibiotic produced by *Micromonospora purpurea*. It has been shown by paper chromatography (1) to consist of three active components, C_1, C_2, and C_{1a} (Structure I). These have also been separated by column (1) and ion-exchange (2) chromatography. The drawbacks of the column method are the comparative difficulty of column packing and equilibration, the large eluant volume, and the high adsorbent to antibiotic ratio required for resolution. This method was recently improved on a preparative scale by Cooper *et al.* (3).

The present USP (4) and BP (5) requirements for this antibiotic consist of a microbiological estimation of potency. In addition, the USP has set limits on the allowable percentage of the individual components in the gentamicin complex and prescribes a differential paper chromatographic bioassay for their control. Wagman *et al.* (6) also published a chemical method for quantitating the various components by direct densitometry after paper chromatography. This method, however, involves an 18-hr. development of the paper chromatogram.



gentamicin C_1: $R_1 = R_2 = CH_3$
C_2: $R_1 = CH_3$, $R_2 = H$
C_{1a}: $R_1 = R_2 = H$

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This paper reports a rapid chemical method for estimating the total gentamicin potency as well as for quantitating the individual components, all in one step by direct densitometry after resolution on TLC. This method, which has already been applied successfully to the analysis of the tetracycline (7) and erythromycin (8) antibiotics, is much faster than the official microbiological procedures.

EXPERIMENTAL

Standard Solutions—Gentamicin sulfate working standard (potency 586 mcg./mg.) was assayed in two independent laboratories by the differential microbiological assay and was found to contain, on a weight basis, 164 mcg. C_1-base, 217 mcg. C_2-base, and 204 mcg. C_{1a}-base/mg. gentamicin sulfate. It was dissolved in distilled water to a concentration of 15.00 mg. gentamicin sulfate/ml., giving a standard solution containing 2.46 mg. C_1-base, 3.26 mg. C_2-base, and 3.06 mg. C_{1a}-base/ml.

Assay Solutions—Accurately weighed samples of gentamicin sulfate were dissolved in water to a concentration of about 30 mg. gentamicin sulfate/ml. In the case of the injection preparation, a volume (1 μl.) equivalent to about 30 mcg. gentamicin sulfate was applied directly to the TLC plate.

TLC—Precoated analytical silica gel 60[1] plates (20 × 20 cm., 0.25-mm. thickness) were heated for 1 hr. at 135° prior to use. The solvent system was composed of the lower layer (150 ml.) of a mixture of methanol–chloroform–28% ammonium hydroxide

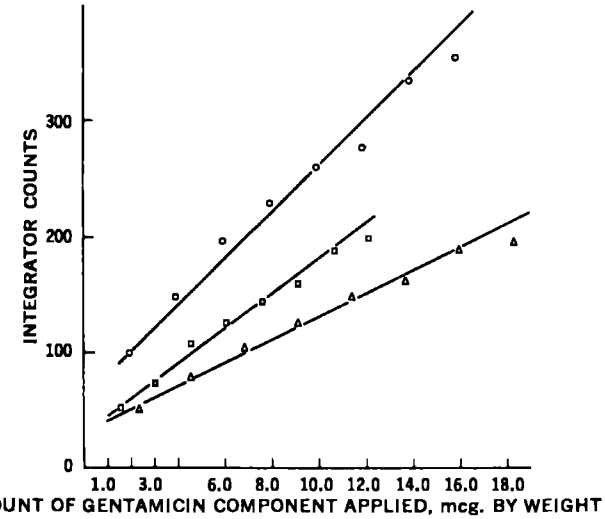

Figure 1—*Representative calibration curves for the gentamicin components in the gentamicin sulfate standard. Key:* □, *gentamicin* C_1; △, *gentamicin* C_2; *and* ○, *gentamicin* C_{1a}.

[1] E.M. Reagents, E.M. Laboratories Inc., Elmsford, NY 10523

Table I—Determination of Components in Bulk Samples by the Densitometric Method

Sample	Percent by Weight of Components C_1	C_2	C_{1a}
Bulk A	25.2	44.5	30.2
Bulk B	34.8	40.1	25.1
Bulk C	25.3	44.9	29.8
Bulk D	27.7	41.6	30.7
Bulk D[a]	29.1	40.5	30.5
Injection E	35.1	44.6	20.3

[a] Results obtained by differential bioassay method.

(1:1:1). This was passed through 100 g. of anhydrous sodium sulfate before being placed in a chromatographic chamber lined with Whatman 3-mm. filter paper. The chamber was saturated with solvent for 1 hr. prior to use. Samples were applied to the plates using 1-μl. micropipets[2]. One- and 3-μl. aliquots of the reference solution and a 1-μl. aliquot of the sample to be assayed, with a concentration lying within those of the two reference amounts, were applied across the plate 2 cm. from the lower edge as alternate spots, giving a total of eight measurements, four for the standard and four for the unknown.

The plate was developed to a height of 15 cm. (80 min.), removed from the tank, dried at 135° for 20 min., and allowed to cool. Uniform spraying with 10 ml. of a freshly prepared solution of ninhydrin (1.0 g.) in a mixture of 95% ethanol (50 ml.) and glacial acetic acid (10 ml.), followed by heating for 15 min. at 135°, revealed the gentamicins as magenta spots on a white background. These were analyzed the same day by direct densitometry.

Densitometry—Measurements were performed on a double-beam chromatogram analyzer[3] with the following settings: monochromator, 500 nm.; spectral resolution, 10 nm.; double-beam mode; motor drive speed, 2.03 cm. (0.8 in.)/min.; and recorder chart speed, 2.54 cm. (1.0 in.)/min. Spots were scanned in the direction of solvent flow and the areas were quantitated by means of the digital integrator.

A plot of peak area (integrator counts) *versus* amount of each component (micrograms) yielded linear calibration curves for amounts between 2 and 12 mcg. of each component per spot (Fig. 1). In practice, duplicate spots of two reference concentrations, within the values of which the unknown concentration will be located, are applied to the plate; curves are prepared for each individual plate by plotting the integrals of the observed peaks for the four reference spots *versus* the amount (micrograms) of each component present in the reference solution. The values of each component in the four unknown samples are found by interpolation from the respective curve (Fig. 2). The results from each of the three components are added and expressed as a percentage by weight of the total. The individual values of each component (in micrograms) are then converted into micrograms of gentamicin base activity

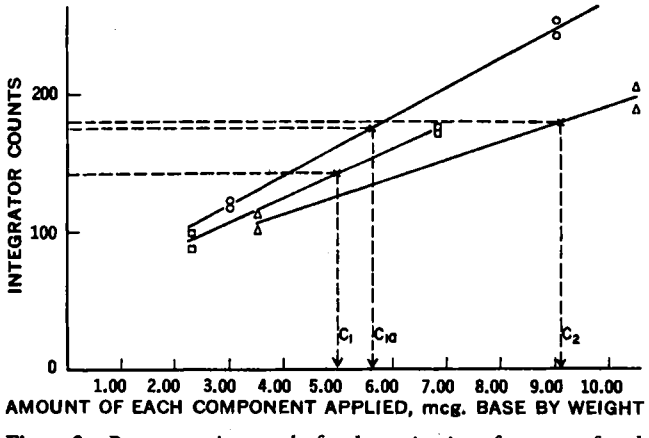

Figure 2—*Representative graphs for determination of amount of each component in a gentamicin sample. Values of components are found by interpolation from the respective plot. Key: □, gentamicin C_1; △, gentamicin C_2; and ○, gentamicin C_{1a}.*

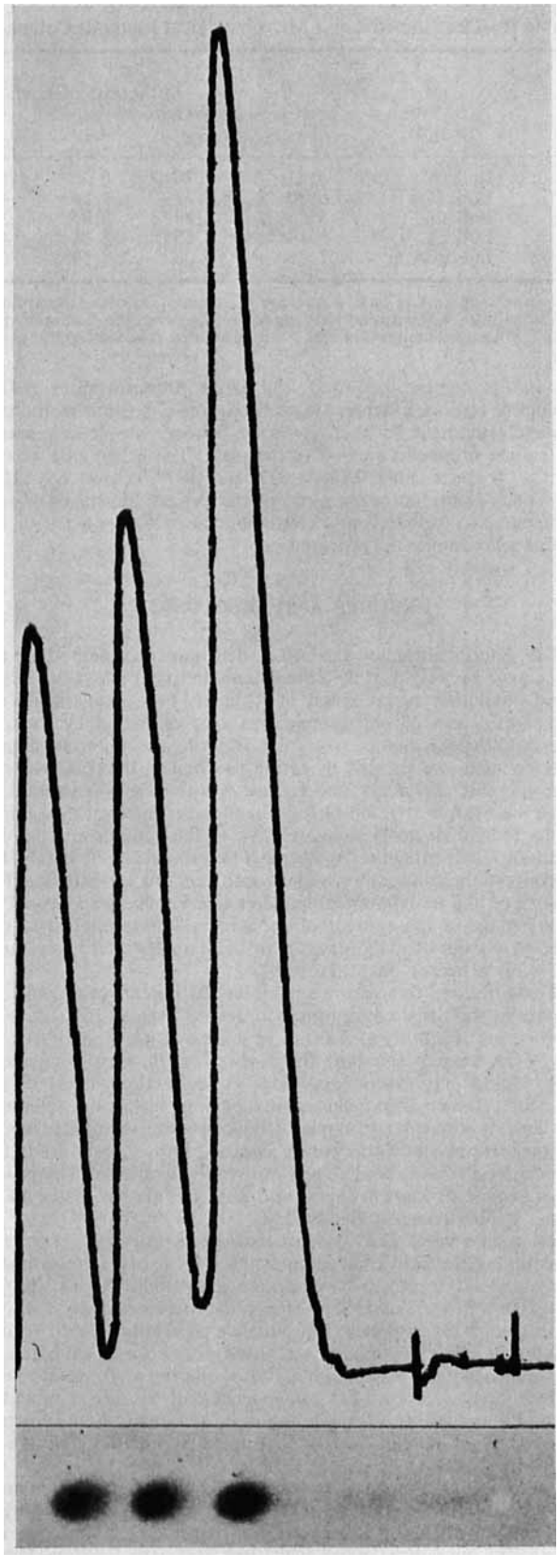

Figure 3—*TLC separation and densitometer scan of gentamicin complex. Starting at origin on the right, the spots are gentamicin C_{1a}, C_2, and C_1, respectively.*

[2] Drummond Scientific Co.
[3] Vis-UV, Farrand Optical Co., Mount Vernon, NY 10550

Table II—Densitometric and Microbiological Methods Comparison

Sample	Amount[a] of Each Component — By Weight C_1	By Weight C_2	By Weight C_{1a}	By Activity C_1	By Activity C_2	By Activity C_{1a}	Densitometric Assay[b] — Potency[c]	Coefficient of Variation	Microbiological Assay Potency[c]
Bulk A	5.21	9.20	6.25	4.10	9.42	6.12	655	4.4	641[d]
Bulk B	6.30	7.27	4.54	4.95	7.44	4.44	561	9.8	561
Bulk C	5.05	8.98	5.95	3.97	9.19	5.81	632	2.8	641
Bulk D	5.10	7.65	5.65	4.00	7.82	5.52	578	5.7	577
Injection E	7.95	10.08	4.59	6.25	10.31	4.48	1052[e]	6.3	1000[f]

[a] In micrograms of base. [b] Average of a minimum of six determinations for each value reported. [c] In micrograms of base per milligram of gentamicin sulfate. [d] Determinations made by Bio-Research Laboratories, Pointe Claire, Quebec, Canada, with required pharmacopeial precision of ±5%. [e] Assayed contents of vial. [f] Amount present based on label claim.

by multiplying by the Food and Drug Administration (FDA) biological response factors (4), totaled, and expressed as the percentage gentamicin base activity in the 30-mcg. sample of gentamicin sulfate originally applied to the plate. These response factors for C_1, C_2, and C_{1a} are 0.786, 1.023, and 0.977, respectively. Thus from each plate, four estimations of the percent by weight of each component as well as four determinations of the potency of the gentamicin complex are provided.

RESULTS AND DISCUSSION

The determination by the direct densitometric method of the percentage by weight of the components in four bulk samples and one formulation is presented in Table I. For comparison, the weight variation of one sample was also estimated by the differential bioassay method (4). Table II shows the amounts of each component by weight and by activity as well as the total potency assayed by densitometry and by the microbiological method. By using the FDA correction factors for the microbiological response of the individual gentamicins relative to the gentamicin complex standard (assayed against the same FDA standard), it is possible to convert the amount by weight into amount by activity. The activity of the individual components when totaled and taken as a percentage of the amount of gentamicin sulfate applied to the plate gave values for the potency of the complex that agree quite well with the microbiological results.

It was decided to use amount by weight instead of amount by activity in the densitometry method and to convert the values to micrograms of activity at the end. In this way the eventual use of a new FDA master standard (of perhaps a different component ratio) would only involve applying new correction factors in the last step. Rather than using pure components as the reference standard, a commercial sample of bulk gentamicin sulfate, whose composition and potency were accurately known, was used; the pure materials are expensive and not readily available. Any gentamicin sample of known weight and potency relative to the FDA master standard may be employed.

Although several TLC systems have been proposed (1) for the resolution of the individual gentamicins, the use of the lower phase of methanol–chloroform–concentrated ammonium hydroxide (1:1:1) gave the best separation. This system was originally used by Cooper *et al.* (3); however, reproducible separations could be obtained only when the solvent was dried prior to use. Commercial precoated silica gel plates, although not offering any improvement in resolution, were used for convenience and because they had a more uniform surface for quantitation by reflectance densitometry. An example of the TLC separation and densitometric quantitation is shown in Fig. 3.

The use of ninhydrin for detection of the spots after chromatography also required improvement. Kantor and Selzer (9) reported that ninhydrin was unsatisfactory for densitometry since the intensities of the colors varied with time and with fraction. However, the inclusion of acetic acid ensures an optimum pH of about 5, as required for the reaction (10), and gives bright-red spots on a white background, which were shown by densitometry to be stable for up to 6 hr. (repetitive scanning gave a variation of less than 1%). Other potential problems due to different responses of the gentamicin components to the spray and unevenness of spraying are easily overcome when standards and unknowns are compared under identical conditions on the same plate.

This TLC–direct densitometric assay technique offers several improvements over the methods presently available for the gentamicin component variation analysis and potency determination. In the first place both tests are combined in one experiment. Second, it accomplishes these tests in a much faster time and with the same precision as the microbiological assay. It is well known that antibiotics of the deoxystreptamine group may be mixtures, and FDA has set limits on allowable percentages. Maehr and Schaffner (2) showed this also to be true for the gentamicin complex. Because the amount of each component is read directly from the plate after separation of impurities and the values interpolated from standards are treated in an identical manner, the densitometric method is more accurate for estimating total potency than the official microbiological assay in which biologically active impurities may interfere.

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Determination of Trace Amounts of Glycidol in Milk of Magnesia

EUGENE IVASHKIV and JOHN M. DUNHAM▲

Abstract □ A very sensitive procedure is described to determine traces of glycidol in milk of magnesia formulated with 1% of this sterilant. Glycidol is separated from a milk of magnesia–diatomaceous earth column by continuous extraction with 1,2-dichloroethane and reacted with 2,4-dinitrobenzenesulfonic acid under anhydrous conditions. The resulting ester gives a yellow color with piperazine which is measured in a spectrophotometer. Even traces of water present in the reaction mixture interfere with esterification. Water is eliminated completely by the addition of 2,2-dimethoxypropane. In the presence of 2,4-dinitrobenzenesulfonic acid, it reacts with water to form methanol and acetone. No glycidol was found in milk of magnesia samples after 14 weeks of storage at room temperature. Quantitative recoveries were made for 1–27 p.p.m. glycidol added to milk of magnesia.

Keyphrases □ Milk of magnesia formulations—separation, analysis, glycidol sterilant □ Glycidol sterilant—separation, analysis, milk of magnesia formulations □ Colorimetry—analysis, glycidol in milk of magnesia formulations

A method for sterilizing aqueous pharmaceutical formulations with glycidol (2,3-epoxy-1-propanol) was described by Riffkin and Marcus (1). Because of the toxicity of this sterilant, a very sensitive procedure for determining traces of glycidol was needed. Such a procedure has been developed to monitor milk of magnesia formulated with 1% glycidol.

The colorimetric method described by Urbanski and Kainz (2) was selected for investigation because it appeared to be sensitive, simple, and selective. Glycidol reacts with 2,4-dinitrobenzenesulfonic acid under anhydrous conditions. When the resulting dinitrobenzenesulfonic ester is reacted with piperazine, it gives a yellow color which is measured spectrophotometrically. Even traces of water present in the reaction mixture interfere with the esterification of glycidol. Urbanski (3) also showed that the color intensity depended strongly on the water content. It was found in the present investigation that water can be eliminated completely during esterification by the addition of 2,2-dimethoxypropane. In the presence of acid, it reacts with water to form methanol and acetone (4). Glycidol is separated from milk of magnesia on a diatomaceous earth column by continuous extraction with 1,2-dichloroethane prior to its colorimetric analysis.

EXPERIMENTAL

Apparatus—Spectral measurements were made in a spectrophotometer[1]. The chromatographic column was 20 cm. long × 1.5 cm. i.d., equipped with a Teflon stopcock and a 200-ml. reservoir.

Reagents and Chemicals—Diatomaceous earth[2] (acid washed), 2,2-dimethoxypropane, anhydrous sodium sulfate, standard 0.1 *N* iodine, standard 0.1 *N* sodium thiosulfate, 0.25 *N* acetic acid, starch indicator solution[3], 0.5% 2,4-dinitrobenzenesulfonic acid in redistilled dioxane (prepared fresh daily), 2.5% piperazine in dimethylformamide, and redistilled and washed 1,2-dichloroethane were used. The dichloroethane is distilled at atmospheric pressure and washed successively with 1 *N* hydrochloric acid, 1 *N* sodium hydroxide, and distilled water until the final wash is neutral to phenolphthalein. The dichloroethane is washed the day it is used.

Preparation of Standard—*Assay*—Weigh about 60 mg. of glycidol into a 125-ml. conical flask. Pipet 25 ml. of standard 0.1 *N* sodium thiosulfate solution and 5.0 ml. of 0.25 *N* acetic acid into the flask and mix. Incubate the solution in an oven at 70° for 15 min. Cool the sample in an ice bath. Add 5 ml. of starch indicator solution, and titrate with standard 0.1 *N* iodine solution to a blue end-point. Calculate purity from the amount of thiosulfate consumed by the glycidol.

Dilution—Weigh about 60 mg. of glycidol of known purity into a 100-ml. volumetric flask. Dilute to volume with redistilled and freshly washed 1,2-dichloroethane.

Assay Procedure—*Separation*—Place a glass-wool plug at the bottom of the chromatographic column, and pack with 20 g. of anhydrous sodium sulfate. Accurately weigh about 5.5 g. of milk of magnesia into a 100-ml. beaker. Add 5 g. of diatomaceous earth and mix with a spatula. Transfer in portions to the column, gently compressing each time with a tamping rod.

Wash the beaker with 20 ml. of distilled and freshly washed dichloroethane and transfer to the column. Allow the solvent to pass by gravity into a 250-ml. separator. Add more distilled and freshly washed dichloroethane, and elute glycidol from the column by a positive pressure of about 2 psig. until 135 ml. of total eluate is collected.

Pipet 2 ml. of the diluted glycidol standard solution into a second 250-ml. separator containing 133 ml. of the distilled and freshly washed dichloroethane. Add 135 ml. of the dichloroethane to a third 250-ml. separator to serve as a blank.

Esterification—To each separator, add 5 ml. of 2,2-dimethoxypropane and mix. Add 25 ml. of 2,4-dinitrobenzenesulfonic acid solution and again mix. After 5 min., add 50 ml. of 1 *N* hydrochloric acid and shake for 1 min. Transfer the lower dichloroethane layer into another 250-ml. separator. Wash the acid aqueous layer with 25 ml. of dichloroethane for 30 sec., and transfer the lower layer into the separator containing the original dichloroethane solution. Wash the combined dichloroethane with 50 ml. of 2% sodium bicarbonate solution for 1 min.

Filter the bottom dichloroethane layer through anhydrous sodium sulfate into a 200-ml. volumetric flask. Wash the sodium bicarbonate solution with 25 ml. of dichloroethane and filter into the volumetric flask. Wash the sodium sulfate with dichloroethane, dilute to volume, and mix.

Color Development—Pipet 5 ml. of sample, 5 ml. of standard, and 5 ml. of the blank into 175-mm. test tubes. Pipet 2 ml. of the 2.5% piperazine solution into each tube and mix. After 10 min., measure the absorbance of the sample and standard in 1-cm. cells at the 390-nm. maximum with the blank in the reference cell.

If the sample absorbance reads over 1.0, dilute aliquots of both the sample and the blank from the 200-ml. flasks with dichloroethane. If the reading is low or if no reading is obtained, transfer the sample and blank solutions to 500-ml. round-bottom flasks and evaporate to dryness. Use a rotating vacuum evaporator and a water bath with a temperature up to 70° to speed the evaporation. Add dichloroethane to produce a concentration of about 3 mcg. glycidol/ml.

React 5.0-ml. aliquots of both blanks (diluted or concentrated and undiluted), the adjusted sample, and the standard with 2.0 ml. of the piperazine solution. After 10 min., measure the absorbance of

[1] Beckman DU.
[2] Celite 545.
[3] Fisher.

Table I—Effect of Piperazine Concentration on Color Intensity

Piperazine Concentration in Dimethylformamide, %	Absorbance
5	0.980
4	0.920
3	0.800
2.5	0.800
1	0.310
0.5	0.055

Table II—Elution of Glycidol from Diatomaceous Earth (5.5 g. of Milk of Magnesia plus 5.0 g. of Diatomaceous Earth)

25-ml. Dichloroethane Fractions	Glycidol Eluted, %
1	39
2	26
3	17
4	11
5	7
6	0

these four solutions *versus* dichloroethane in the reference cell. Subtract the absorbance of the adjusted blank from that of the adjusted sample and the reading of the undiluted blank from the standard reading.

Calculate the quantity (micrograms of glycidol per gram of milk of magnesia) by the formula $(A_u - B_u/A_s - B_s)(W_s \times P/W_u)(V_F/V_I)20$, where A_u and A_s are the absorbances of the sample and standard solutions, respectively, and B_u and B_s are the absorbances of their respective blanks (if the sample was diluted or concentrated); W_s is the weight, in milligrams of the standard glycidol, and P is the purity of the standard (%/100); W_u is the weight, in grams of the milk of magnesia sample; V_F is the final volume, in milliliters of the diluted or concentrated sample; and V_I is the volume of sample, in milliliters taken initially for dilution or concentration.

RESULTS AND DISCUSSION

Glycidol Purity—Leary's (5) procedure for determining oxiranes with sodium thiosulfate was investigated for assaying the purity of glycidol. Reaction between glycidol and thiosulfate is quantitative under the conditions described in the procedure under *Preparation of Standard*. To verify this, glycidol was purified by solvent extraction from an aqueous solution into ether (6). The purity of this sample was 99.0% or greater, with a two- or threefold equivalent excess of sodium thiosulfate to glycidol. With a 1.5-fold excess of thiosulfate, only 95% of the glycidol reacted. Reaction of glycidol and thiosulfate was dependent on both time and temperature. Samples were heated in a 70° oven for 10, 15, and 30 min.; 15 min. was needed for completion of the reaction. Four replicate assays of one glycidol sample, using the recommended procedure, gave results varying from 91.0 to 91.4%.

Chemistry of Method—Reaction of glycidol with 2,4-dinitrobenzenesulfonic acid to yield an ester proceeds in dioxane, methylene chloride, and chloroform, as well as dichloroethane. Very little ester is formed in ether or carbon tetrachloride. The reaction does not proceed in butanol.

Table III—Recovery of Glycidol from Milk of Magnesia

Added Glycidol, mcg.[a]	Dichloroethane Final Volume, ml.	Absorbance	Recovery, %
146.2	200	0.940	99.5
97.4	200	0.624	99.1
48.7	200	0.320	101.6
24.4	200	0.156	99.2
4.87	20	0.324	102.8
0	20	0	—
Standard (97.4)	200	0.630	—

[a] Added to 5.5 g. of milk of magnesia.

Table IV—Decomposition of Glycidol in Milk of Magnesia

Storage, days	Glycidol, mg./g. Room Temperature (about 25°)	Glycidol, mg./g. 40°
Initial	9.91	10.02
1	8.56	8.54
4	7.00	4.88
7	5.92	2.37
14	4.68	0.62
21	3.27	0.20
28	2.13	0.034
35	1.62	0.000
42	1.08	0.000
49	0.74	—
56	0.58	—
63	0.44	—
70	0.27	—
77	0.153	—
84	0.024	—
93	0.000	—

Even traces of water interfere with the esterification. Drying dichloroethane extracts of glycidol with anhydrous sodium sulfate, calcium chloride, or magnesium sulfate before adding the dinitrobenzenesulfonic acid gave slightly higher absorbance values than those for undried solutions. But results were low compared with 2,2-dimethoxypropane-treated samples and varied from run to run. Previous investigators (4) used hydrochloric acid to catalyze the reaction of water with dimethoxypropane. In this procedure, the 2,4-dinitrobenzenesulfonic acid serves as both catalyst and colorimetric reagent.

The concentration of 2,4-dinitrobenzenesulfonic acid is important. About 150 mcg. of glycidol was reacted with various concentrations of the reagent in dioxane. A maximum absorbance was found between 0.1 and 0.5%. The higher concentration of reagent was selected to compensate for its consumption by impurities in solvents or samples. Colored products formed from impurities encountered in this laboratory were removed from the dichloroethane solution by later acidic and basic washes.

Absorbance readings of an unwashed blank solution increase with time. Fortunately, the absorbance of the blank is nearly eliminated by successive washings with 1 *N* hydrochloric acid and 2% sodium bicarbonate solutions. The reaction product of glycidol and 2,4-dinitrobenzenesulfonic acid is not extracted with these washes. After the aqueous washes, sample volumes can be reduced to increase the sensitivity of the method. The reaction product is a nonvolatile solid, and its dichloroethane solutions are stable at room temperature for at least 1 month. Because of this stability, the standard and blank solutions can be kept at room temperature and used as needed.

The concentration of piperazine for color development is important. Color intensity increases with the concentration of piperazine, as shown in Table I. Because the intensity of color depends on the concentration of piperazine, the 2 ml. of piperazine solution is added by pipet in the *Color Development* step of the procedure.

Solutions of the reaction product of glycidol with 2,4-dinitrobenzenesulfonic acid and piperazine follow Beer's law. A solution containing 1 mcg. of glycidol/ml. had an absorbance reading of 0.142.

Separation from Milk of Magnesia—Glycidol can be extracted from milk of magnesia with methylene chloride, dichloroethane, chloroform, ether, and other solvents. About eight 25-ml. portions of these solvents are needed to extract glycidol completely from 5 ml. of milk of magnesia in a separator. Since this extraction is tedious, continuous elution of glycidol from a diatomaceous earth column is used. As shown in Table II, 125 ml. of dichloroethane is needed for complete elution of glycidol.

Recovery—Various amounts of glycidol were added to milk of magnesia and subsequently assayed. Results are summarized in Table III.

Stability—Samples of milk of magnesia were sterilized with 1% glycidol and stored at room temperature (about 25°) and 40°. The decrease in the glycidol concentration is shown in Table IV.

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PHARMACEUTICAL TECHNOLOGY

Mechanism of Action of Starch as a Tablet Disintegrant VI: Location and Structure of Starch in Tablets

WERNER LOWENTHAL▲ and JOHN H. WOOD

Abstract □ By using a scanning electron microscope, the location and structure of starch grains in experimental and commercial aspirin tablets and aspirin–phenacetin–caffeine tablets were studied. By scrutinizing tablet faces and cross sections before and after the addition of water, it was observed that rupture of the surfaces occurred where starch agglomerates were found. It was postulated that water hydrates the hydroxy groups of the starch molecules, causing them to move apart. The slight swelling that occurs is due to the rapid hydration step and a slower sorption of addition water step. Channels or pores lined with starch were not evident. The conditions for rapid tablet disintegration are sufficient starch agglomerates, low pressure, and presence of water.

Keyphrases □ Starch as tablet disintegrant—location, structure in aspirin and aspirin–phenacetin–caffeine tablets, scanning electron microscope □ Aspirin, aspirin–phenacetin–caffeine tablets—location and structure of starch, scanning electron microscope □ Tablets, aspirin, aspirin–phenacetin–caffeine—location and structure of starch, scanning electron microscope □ Scanning electron microscopy—location, structure of starch in aspirin and aspirin–phenacetin–caffeine tablets □ Disintegrants—mechanism of action of starch in aspirin and aspirin–phenacetin–caffeine tablets, location and structure of starch agglomerates, conditions for rapid disintegration

Previous study has shown that corn and waxy maise starch grains plastically deform and that this deformation increases with increasing pressure. The addition of water has no apparent effect on this deformation (1, 2). Potato starch grains also have been shown to deform (3). It was also previously observed that when a small amount of moisture was added to corn and waxy maise starch tablets, a "blistering" effect and disruption of the moistened area occurred. Moisture did not cause the individual grains to regain their shape (1).

It is not practical to attempt to measure a change in volume due to the addition of moisture by the scanning electron microscope, because sample preparation for the scanning electron microscope involves the use of a vacuum which would remove the moisture and the sample is coated with a thin layer of a conductor of carbon and gold. When conductor layering was compared to an electrostatic method of sample preparation, similar results were obtained so that the scanning electron photomicrographs show a true picture of the surfaces (4).

The purpose of this investigation is to show the location of and the structure formed by the starch grains in experimental and commercial aspirin tablets and aspirin–phenacetin–caffeine tablets. The effect of moisture on these tablets is also shown.

EXPERIMENTAL

Tablets weighing 0.5 g. were compressed using 1.27-cm. (0.5-in.) diameter flat-face punches and die. Aspirin[1] was sieved to obtain 40–50- and 70–100-mesh crystal fractions. These fractions were mixed with starch[2] to give 2.5, 5, 7.5, 10, 12.5, and 15% (w/w) starch concentrations. The ingredients were mixed by tumbling. Tablets were made by compression at 10,000 psig. on a hydraulic laboratory press[3] with special holders for the punches and die. An aspirin–10% starch granulation[4], 12–50 mesh, was also used. Tablets from the commercial granulation were made by compression at 2500 psig. At higher pressures the tablets appeared to be impervious to water and did not disintegrate. For the photomicrographs only, the 40–50-mesh aspirin with 2.5 and 10%

[1] Catalog No. A-42, lot 752285, Fisher Scientific Co.
[2] STR-Rx, A. E. Staley Manufacturing Co.
[3] Model B, Fred S. Carver, Inc., Hydraulic Equipment.
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Table I—Disintegration Times

Tablet		Disintegration Time, sec. Average	Range
Aspirin (40–50 mesh)	2.5%	530[a]	378–685
	5	16[a]	9–40
	10	6[a]	5–6
	15	5[b]	4–6
Aspirin–starch granulation		173	45–490
Product B		10	8–13
Product S		11	9–12
Product N		13	10–14
Aspirin–phenacetin–caffeine		23	15–31

[a] Five tablets. [b] Three tablets.

starch was used. There was no apparent difference in disintegration times observed between the two particle-size ranges of aspirin, and very little difference was seen between the 5 and 15% concentration ranges of starch.

The USP apparatus (5) with distilled water was used to determine disintegration times.

Three commercially made aspirin tablets (designated as Product B[4], Product S[5], and Product N[7]) and aspirin–phenacetin–caffeine[8] tablets were observed.

The scanning electron microscope and technique previously described were used (1). Both the "as is" face and the cross sections by breaking the tablets were examined. The effect of moisture was determined after a small drop of water was added to the surfaces.

RESULTS

In this study, aspirin mixed with varying concentrations of starch showed only a marked decrease in disintegration times when concentration of starch increased from 2.5 to 5%. This occurred with both 40–50- and 70–100-mesh aspirin. Tablets made with 5% or more starch all had similar disintegration times. As a result, only 40–50-mesh aspirin with 2.5 and 10% starch were examined microscopically. Table I gives the results of the disintegration times of the tablets used.

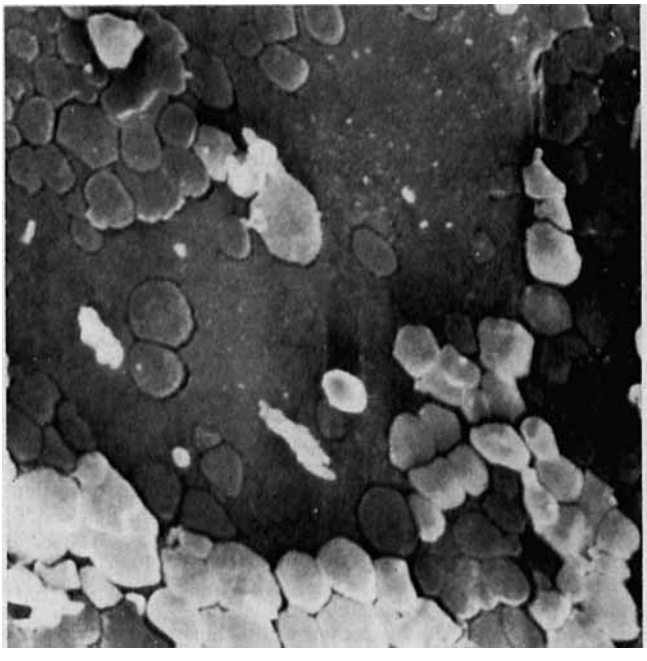

Figure 1—*Face view of aspirin plus 10% starch tablets, dry (about 600×).*

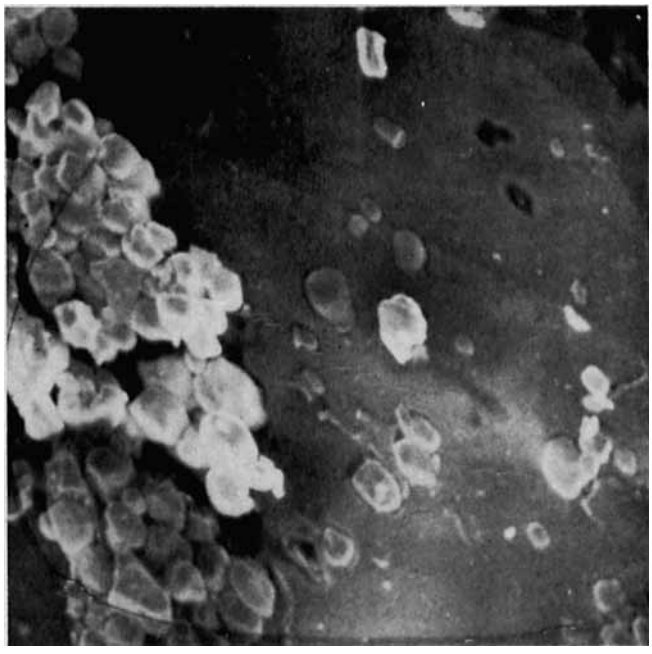

Figure 2—*Face view of aspirin plus 10% starch tablets, moistened (about 600×).*

Analyzing the surfaces of aspirin plus 2.5% starch showed a few widely scattered chains of starch grains. These appeared to be only one layer thick. The starch appeared in scattered clusters and, because starch is poorly compressible, the grains stood out. The aspirin crystals were fused. The face of the tablets looked smooth except where the grains occurred. The starch was not fused to the aspirin. On moistening, aspirin crystals were recognizable, the surface was disrupted, and cracks arose. These cracks usually had starch grains in and around them. A few grains with dimples or depressions were observed, as was reported previously (1). The markings are due to the grains growing next to a protein (zein) membrane in the kernel, are due to enzyme action during storage, or are produced during refining.

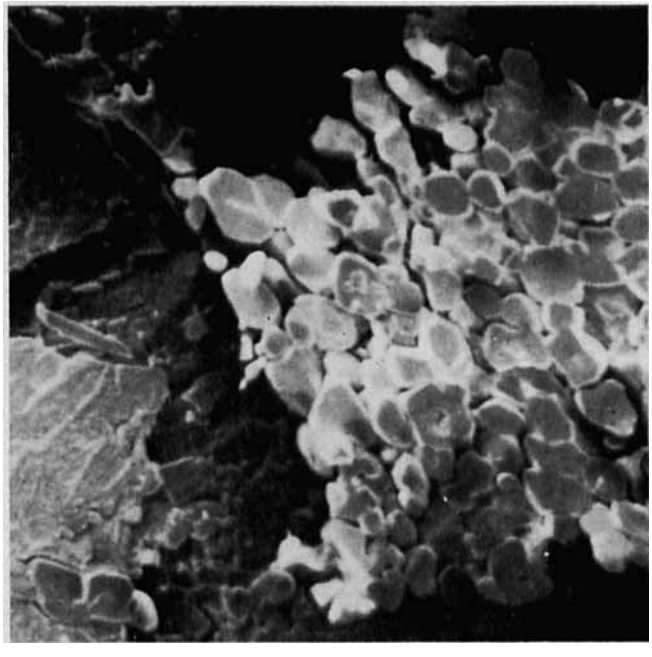

Figure 3—*Cross-sectional view of aspirin plus 10% starch tablets, dry (about 600×). Darkened, irregular shaped spots in starch grains are hilum canals.*

[4] Lots 1L196A and 1B029, Bayer Aspirin, Glenbrook Laboratories, New York, N. Y.
[5] Lot 9N465, St. Joseph Aspirin, Plough, Inc., Memphis, Tenn.
[7] Lot 671885, Norwich Pharmacal Co., Norwich, N. Y.
[8] Lot 8739, Barre Drug Co., Baltimore, Md.

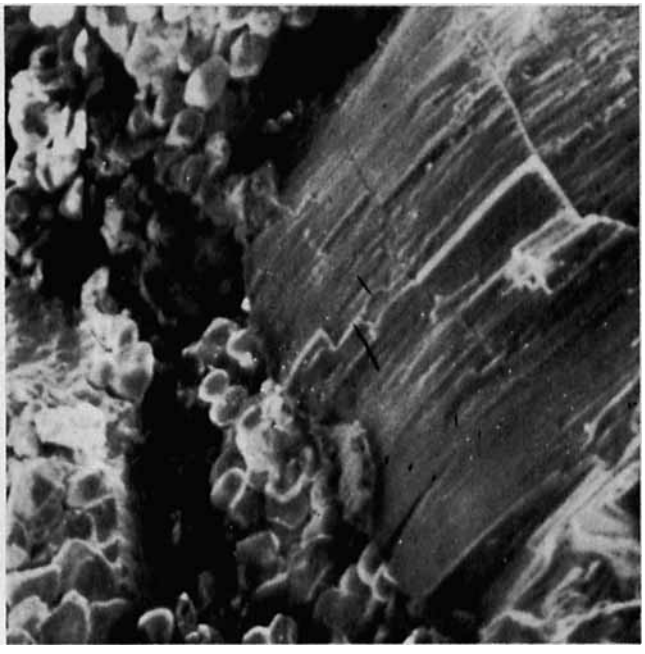

Figure 4—*Cross-sectional view of aspirin plus 10% starch tablets, moistened (about 600×). The large smooth surface is fused aspirin crystals.*

Examination of the surfaces of aspirin with 10% starch showed large agglomerations of starch grains. These masses seem to have created cracks and what could be interpreted as stresses in the tablet. The aspirin was fused to itself but not to the starch. A few short, dispersed chains were noted. Disruption of the surfaces occurred in the moistened area, with the rest of the area still smooth. The grains appeared prominent, and the subsurface also may have been loosened because of movement of the starch grains. Figure 1 shows clumps of flattened grains on the surface, and Fig. 2 shows how the grains seem to have become raised above the surface after moistening. The darker spots could have been places where the grains popped out. Figure 3 is part of a cross section illustrating clumping, two short chains, aspirin fusion, and hilum channels. Figure 4 gives a view of a crack with the characteristic clusters of starch grains.

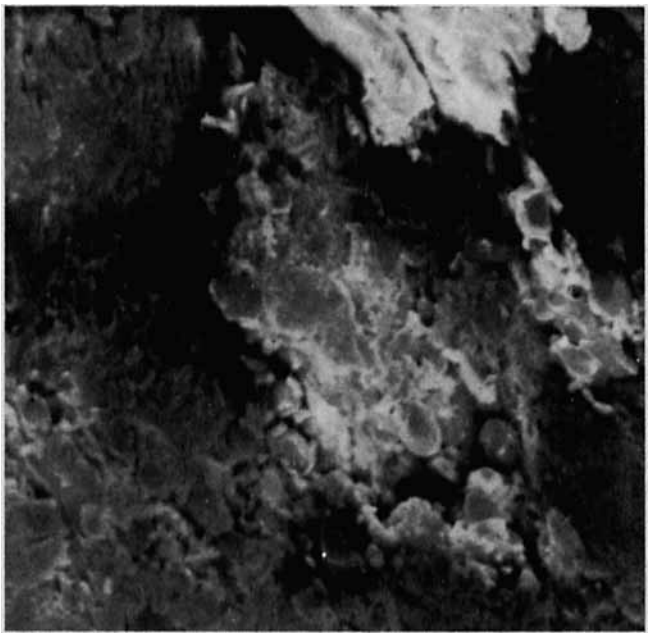

Figure 6—*Face view of tablet made from the commercial aspirin granulation, moistened (about 600×).*

In the tablets made with the commercial aspirin granulation, the starch grains appeared to be scattered and isolated with only a few agglomerates. When viewing the surface, the grains often seemed hidden under a thin layer of aspirin. The faces of the tablets appeared rough and grainy. Both the faces and cross sections appeared similar (Fig. 5). When water was added, there was only a minimal surface disruption and a minimal increase in roughness; starch grains became more visible. In cross sections, grains with hilum canals were seen. Figures 6 and 7 illustrate these surfaces after they were moistened; cracks appeared and the agglomerates became more pronounced.

Analysis of the surfaces of commercial aspirin tablets (Product B) showed that there were agglomerates of starch grains. There was no evidence of channels or pores lined with starch. Aspirin was mostly fused, but aspirin crystals and granules were often visible.

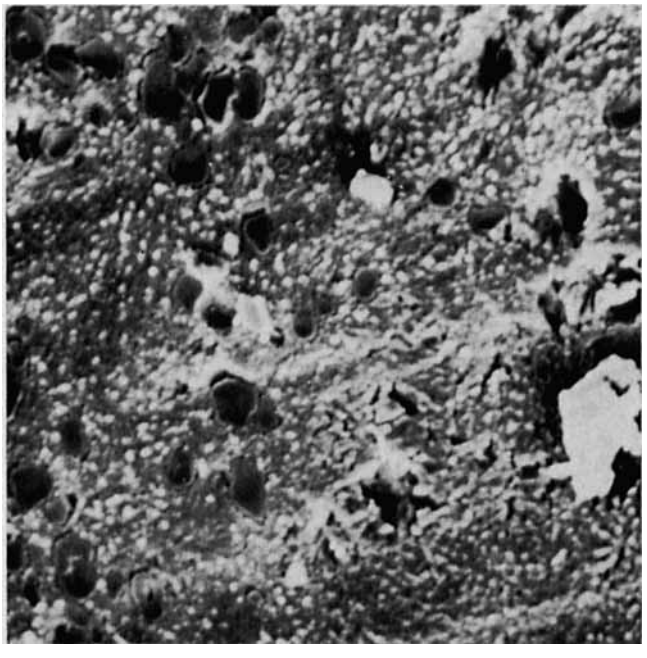

Figure 5—*Cross-sectional view of tablet made from the commercial aspirin granulation, dry (about 600×).*

Figure 7—*Cross-sectional view of tablet made from the commercial aspirin granulation, moistened (about 600×).*

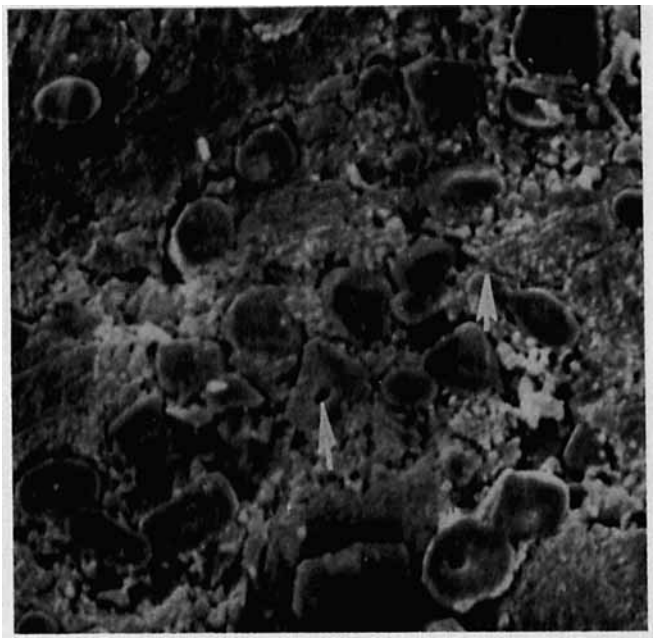

Figure 8—*Cross-sectional view of Product B, dry (about 720×). Hilum canals are readily apparent. The arrows point to hilum canals and cracks.*

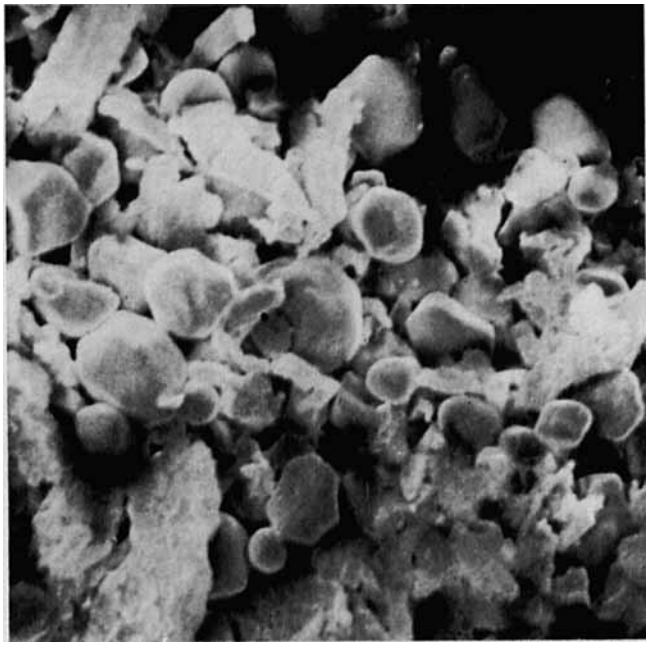

Figure 10—*Face view of Product B, moistened (about 720×).*

There was very little difference between the face and cross sections of the tablet. Figures 8 and 9 show clumping of starch, cracks in the surfaces, some loose aspirin crystals, and the rough surface. Examination of embossed letters showed less fusion of the aspirin. When moistened, the water soaked in very rapidly and did not spread on the surface. The moistened surfaces became raised and flaky in appearance. Figure 10 shows the disruption of the face of the tablets when moisture was added, and cracks, aspirin crystals, and starch are plainly visible. Figures 11 and 12 give evidence of disruption and clumps of starch grains but only an occasional chain, one grain thick. Hilum canals are again observed in the cross section.

Examination of a second commercial aspirin tablet (Product N) demonstrated a rough surface, with aspirin crystals and starch grains readily visible (Fig. 13). The agglomerates did not seem as plentiful as was observed in Product B. Figure 14 shows a cross section of the tablet with hilum canals discernible, and Fig. 15 verifies surface disruption when moisture was added. The aspirin crystals and starch grains are readily apparent. Figure 16 shows the edge of the moistened area. The degree of breakup of the brighter two-thirds compared to the relative smoothness of the darker one-third is evident. The water readily soaked into the tablet surfaces, leaving no sharp boundary visible.

The third brand of aspirin tablets (Product S) studied again showed relatively rough surfaces, with aspirin crystals and starch grains discernible (Fig. 17). Figure 18 illustrates fused aspirin and a large clump of starch grains. When the surfaces were moistened, the aspirin crystals and starch grains were more conspicuous. Compare Figs. 17 and 19. The area moistened was only just visible to the naked eye. Fine cracks appeared where there was a local concentration of starch grains. The layer of plate-like crystals seen

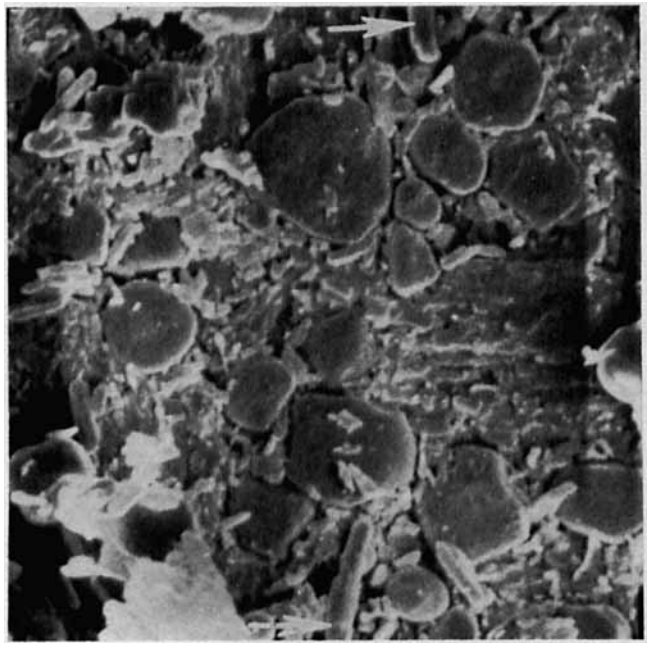

Figure 9—*Face view of Product B, dry (about 720×). The arrows point to loose aspirin crystals.*

Figure 11—*Cross-sectional view of Product B, moistened (about 720×).*

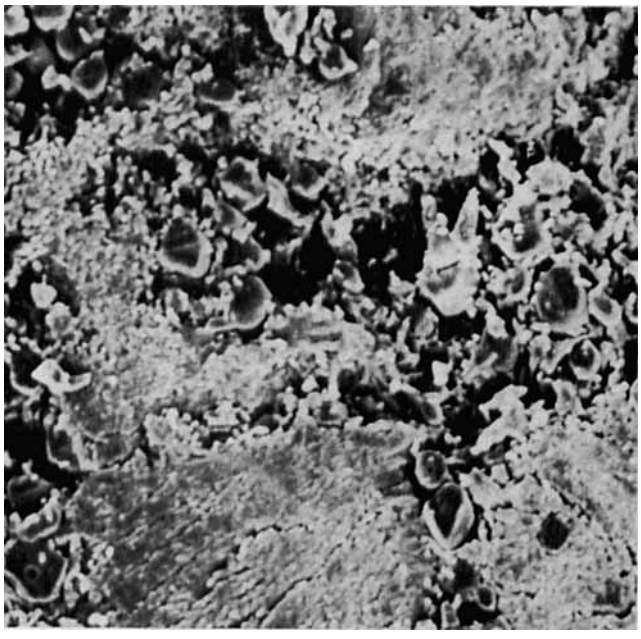

Figure 12—*Cross-sectional view of Product B, moistened (about 450×).*

in Fig. 20, a view of the moistened cross section showing disorganization of the area, was not observed in other tablets.

Finally, aspirin–phenacetin–caffeine tablets were examined. The surfaces were smooth, with only occasional starch grains and crystals evident. Water did not readily penetrate the surfaces and no discernible disruption, occurred. The faces exhibited more discontinuity than the cross sections where only occasional starch grains were observed. No new additional structures not previously mentioned were seen in these tablets.

DISCUSSION

The scanning electron photomicrographs reveal that the surface of the starch grains is smooth. The hilum normally seen in light microscopy appears to be an internal phenomenon since it is not visible in the scanning electron photomicrographs (6). Cross sectioning of tablets also results in the cross sectioning of starch grains,

Figure 13—*Face view of Product N, dry (about 720×).*

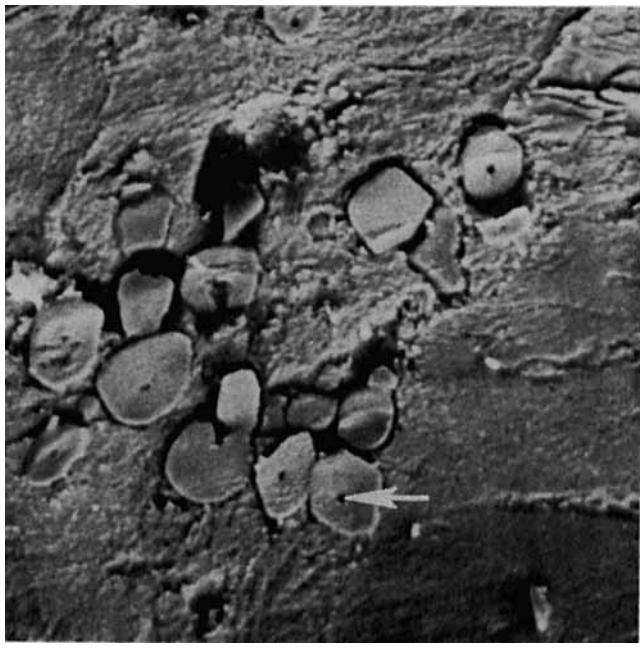

Figure 14—*Cross-sectional view of Product N, dry (about 720×). The arrow points to the hilum canals.*

so hilum channels are clearly visible, indicating a hollow starch grain. These channels appear normal, indicating no damage due to heat or other processing treatments[9], but drying may increase the number of the canals (7, 8). Not all grain cross sections showed this channel, since the channel may not cover the whole length of the grain. Since birefringence is present, the canals apparently do not affect molecular orientation (7).

There was no indication of starch-lined pores, but occasional chains of starch grains were observed. These were only one grain thick and of finite length (from six to eight grains). The macroscopic and microscopic observations of Patel and Hopponen (9) and Commons *et al.* (10) that the starch formed chains and that starch lined pores could not be confirmed after study of a large

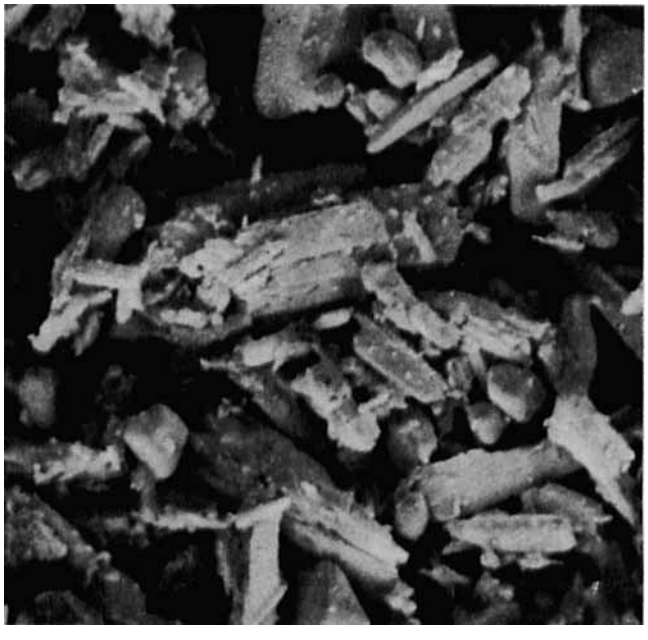

Figure 15—*Face view of Product N, moistened (about 720×).*

[9] H. Zobel, CPC International Inc., Moffett Technical Center, Argo, IL 60501

Figure 16—*Face view of Product N at edge of moistened area (about 720×).*

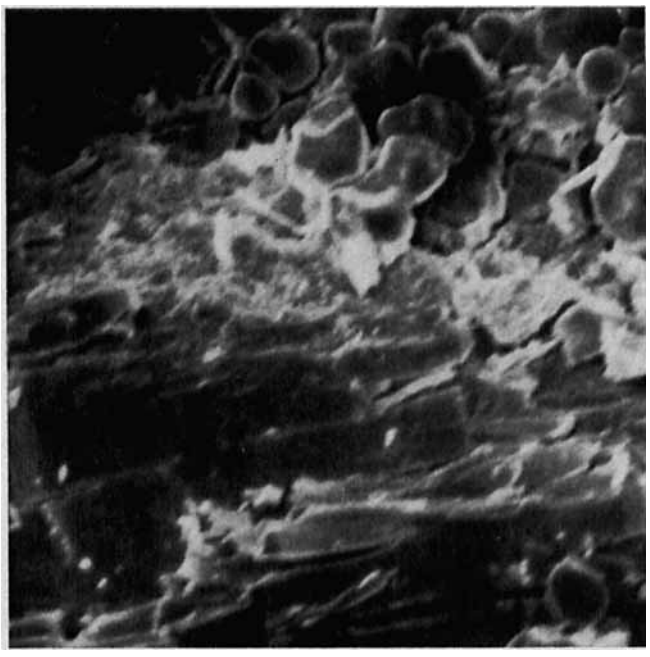

Figure 18—*Cross-sectional view of Product S, dry (about 720×). Large, smooth area appears to be aspirin crystals.*

number of tablet cross sections. The occasional isolated chains observed (Figs. 3 and 12) would not have a significant effect on tablet disintegration. Starch-lined pores or starch surrounding individual granules or crystals were absent. If they did occur, there would have been evidence of starch chains at least two grains thick on a cross section. These were never seen.

Starch is poorly compressible and does not appear to adhere to itself or to other materials in the tablet, and it does not fuse. The starch was not uniformly distributed in the tablets and often appeared as agglomerates in the tablets examined. These result in weak points within the tablet and in fine cracks around the agglomerates.

Isolated and embedded grains cannot cause disruption of the tablet that would result in disintegration. When moisture is added, the surface is generally disrupted. The degree of breakup of the surface depends on the number and size of the starch grain agglomerates (total starch concentration in the tablet) and the pressure used. The greater the number and size of agglomerates, the greater is the disruption. There could be a critical concentration, as suggested by Commons *et al.* (10). The greater the pressure used to form the tablet, the greater is the fusion of the tablet ingredients (except starch) and the greater is the force needed to disrupt the tablet.

Drying starch grains may cause the surface layer of molecules to become more tightly bonded intermolecularly (7). It was postulated (12) that microcrystalline cellulose in tablets is a special form of cellulose fibril in which the individual crystallites are held together mainly by hydrogen bonding. Water causes these bonds to break, producing tablet disintegration. When moisture is added, the degree of association of the starch molecules is reduced because the hydrogen bonds between the hydroxy groups are broken. The hydrated molecules could reduce surface attraction and even cause the grains to repel each other. Thus, the moisture releases stress built up in the starch grains due to their relatively low moisture

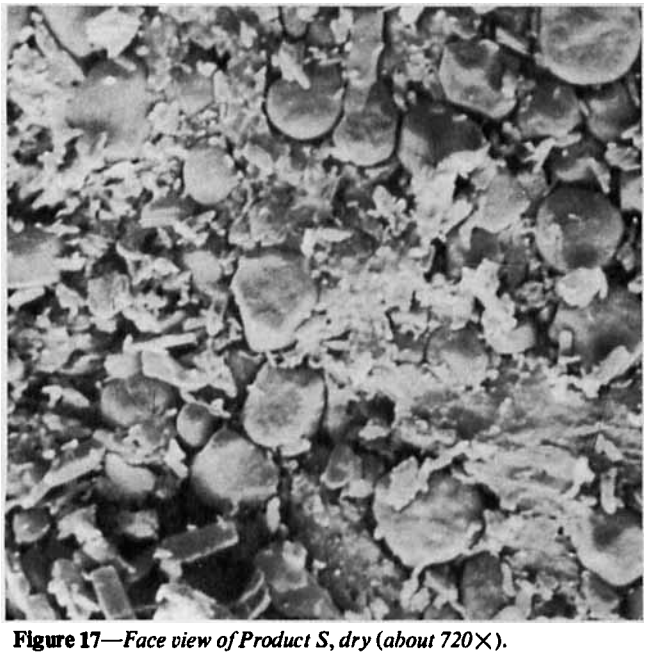

Figure 17—*Face view of Product S, dry (about 720×).*

Figure 19—*Face view of Product S, moistened (about 720×).*

Figure 20—*Cross-sectional view of Product S, moistened (about 720×).*

content and distortion caused by pressure. Fuhrer (3) mentioned that pressure distorts potato starch grains and theorized that energy-rich grains are formed, so that no additional energy is needed for swelling. Close scrutiny of the grains before and after water was added seemed to indicate that the grains are less angular, supporting the theory that there is a degree of restoration to the original grain shape due to hydration of the starch molecules. The hydration and resulting effects occur rapidly (*i.e.*, a fraction of a second). The hydration of the hydroxy groups and sorption of water could account for the slight swelling (5–10%) of the starch (9, 11). This amount of swelling could result in about a 70% increase in volume, and the swelling would take a few seconds. These two combined effects of the attachment of water molecules and the increase in volume of the grains cause sufficient force to break up the tablet. Channels or pores lined with starch grains were not manifest in any of the surfaces examined. Therefore, the thesis that starch grains act as as a "wicking agent" and draw water into the tablet should be layed aside. The conditions for rapid disintegration are sufficient starch agglomerates, low pressure, and presence of water.

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▲ To whom inquiries should be directed.

Dissolution Rate Studies III: Effect of Type and Intensity of Agitation on Dissolution Rate

J. TINGSTAD▲, E. GROPPER, L. LACHMAN, and E. SHAMI

Abstract □ The effect of flow rates on dissolution rates using a continuous flow, column-type apparatus was determined. The flow method of agitation is compared to three other types used with the static beaker procedure. The advantages of the column-type apparatus in attaining low intensities of agitation without sacrificing accuracy or homogeneity are discussed.

Keyphrases □ Dissolution rates—effect of flow agitation, compared to compendial methods, column-type equipment □ Agitation, column flow type—effect on dissolution rates, compared to compendial methods

The importance of low agitation intensities in detecting real differences in dissolution rates and in obtaining good *in vitro–in vivo* correlations in dissolution rate experiments has been established (1, 2). In addition, the importance of the accurate control of the degree of agitation has been pointed out (2). However, the use of low agitation intensities and the accurate control of variables associated with agitation are severely limited with the static beaker method but not with the continuous flow, column-type procedure (3,

Figure 20—*Cross-sectional view of Product S, moistened (about 720×).*

content and distortion caused by pressure. Fuhrer (3) mentioned that pressure distorts potato starch grains and theorized that energy-rich grains are formed, so that no additional energy is needed for swelling. Close scrutiny of the grains before and after water was added seemed to indicate that the grains are less angular, supporting the theory that there is a degree of restoration to the original grain shape due to hydration of the starch molecules. The hydration and resulting effects occur rapidly (*i.e.*, a fraction of a second). The hydration of the hydroxy groups and sorption of water could account for the slight swelling (5–10%) of the starch (9, 11). This amount of swelling could result in about a 70% increase in volume, and the swelling would take a few seconds. These two combined effects of the attachment of water molecules and the increase in volume of the grains cause sufficient force to break up the tablet. Channels or pores lined with starch grains were not manifest in any of the surfaces examined. Therefore, the thesis that starch grains act as as a "wicking agent" and draw water into the tablet should be layed aside. The conditions for rapid disintegration are sufficient starch agglomerates, low pressure, and presence of water.

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The importance of low agitation intensities in detecting real differences in dissolution rates and in obtaining good *in vitro–in vivo* correlations in dissolution rate experiments has been established (1, 2). In addition, the importance of the accurate control of the degree of agitation has been pointed out (2). However, the use of low agitation intensities and the accurate control of variables associated with agitation are severely limited with the static beaker method but not with the continuous flow, column-type procedure (3,

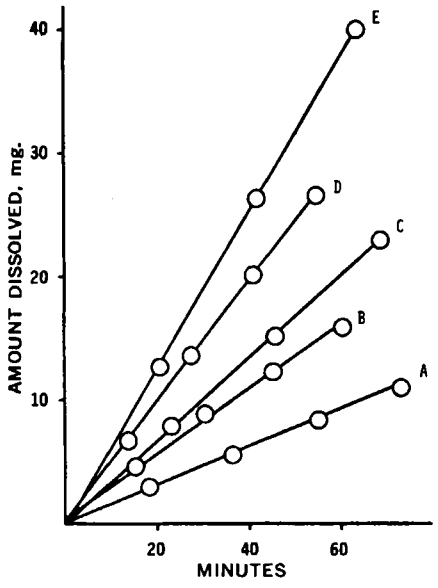

Figure 1—*Pseudo-zero-order plot showing the dissolution of salicylic acid constant-surface pellets in the 13-mm. dissolution chamber (flow method) at various flow rates. Key: A, 5.48 ml./min.; B, 13.3 ml./min.; C, 21.8 ml./min.; D, 36.6 ml./min.; and E, 47.2 ml./min. The plot for 45.1 ml./min. is too close to that for 47.2 ml./min. to be depicted here.*

4). This article reports the effect of flow rates (*i.e.*, liquid velocity) on dissolution rates using a column-type apparatus and compares the columnar flow type of agitation with that provided by: (*a*) the USP XVIII disintegration apparatus, (*b*) a three-bladed stirrer, and (*c*) the USP XVIII–NF XIII rotating basket in the static beaker method.

EXPERIMENTAL

Preparation of Constant-Surface Pellets—Constant-surface pellets of analytical grade salicylic acid (without lubricant) were prepared in the usual manner, using a 0.56-cm. (0.22-in.) circular punch and die and compressing at 2724-kg. (6000-lb.) pressure. The finished pellets weighed 200 mg. and varied less than 0.2% in weight.

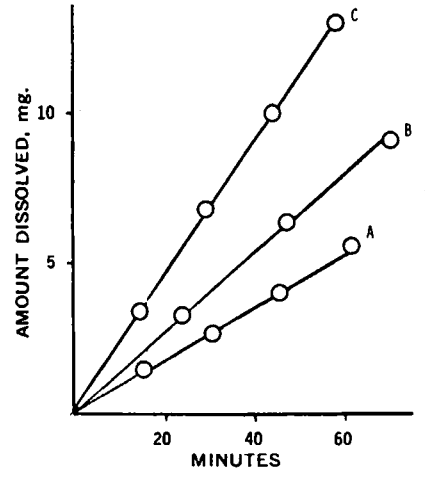

Figure 2—*Pseudo-zero-order plot showing the dissolution of salicylic acid constant-surface pellets in the 25-mm. dissolution chamber (flow method) at various flow rates. Key: A, 13.1 ml./min.; B, 21.3 ml./min.; and C, 34.1 ml./min. The plot for 36.4 ml./min. is too close to that for 34.1 ml./min. to be depicted here.*

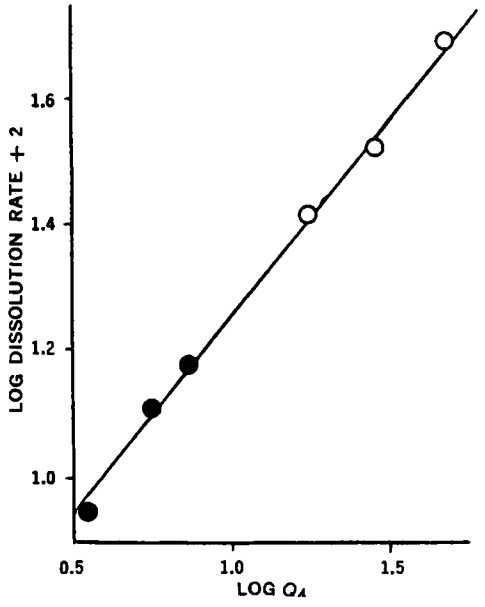

Figure 3—*Log-log plot of* Q_A *(liquid velocity)* versus *dissolution rate. Key:* ○, *13-mm. dissolution chamber; and* ●, *25-mm. dissolution chamber. See text for further explanation.*

Procedure for Flow Method—The column-type apparatus used in these experiments was described in detail previously (5). The effluent solution was routed through a 1-cm. spectrophotometric flow cell and then into a receptacle. The temperature was 25.0 ± 0.1°, and the solvent system was 0.1 *N* HCl with 0.05% polysorbate 80 added to ensure uniform wetting of the solid. The flow rate was held constant for each experiment but was varied among experiments by adjusting the setting on the solid-state speed control.

Between determinations, the dissolution chamber (13- and 25-mm. size) was cleaned and the system was flushed until the UV absorbance receded to a constant value. A blank was taken, the pump was turned off, and the dissolution chamber was disassembled. The pellet was placed in a small beaker and cleansed of loose powder with solvent; then it was placed in the dissolution chamber at the center of the lower support screen. The chamber was reassembled

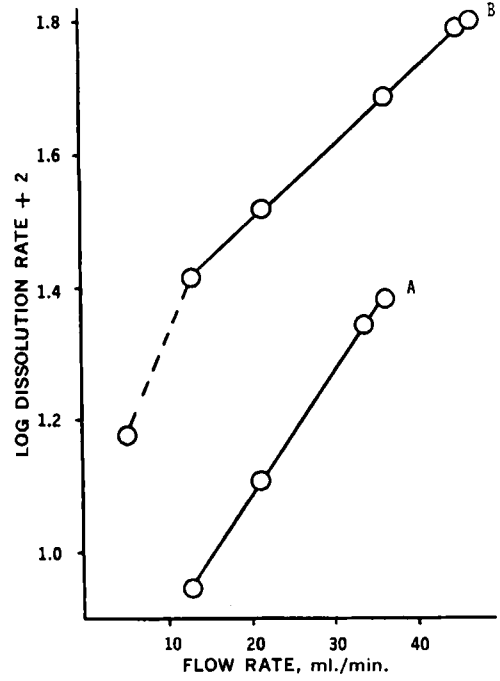

Figure 4—*Semilog plot of flow rate* versus *dissolution rate. Key: A, 25-mm. dissolution chamber; and B, 13-mm. dissolution chamber.*

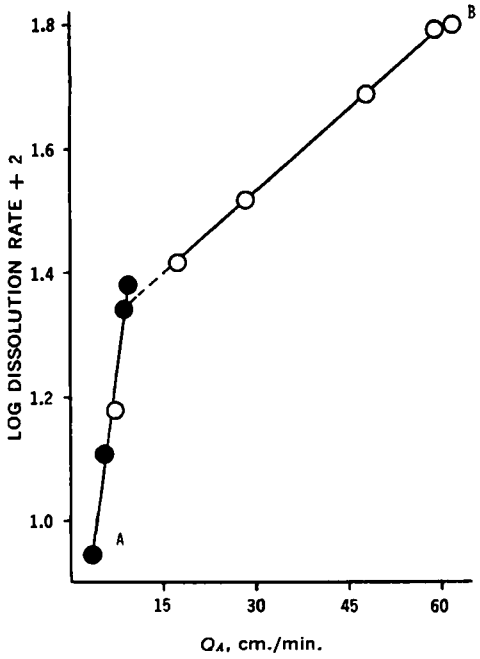

Figure 5—*Semilog plot of* Q_A *(liquid velocity)* versus *dissolution rate. Key: A,* ●, *25-mm. dissolution chamber; and B,* ○, *13-mm. cell. See text for further explanation.*

and placed in the constant-temperature bath containing the solvent reservoir. The pump was activated and the system was allowed to flush for exactly 5 min.; this time was sufficient to stabilize the dissolution rate as determined by the continuous tracing on the strip chart. The effluent solution was then collected in three to five volumetric flasks of appropriate size for about 60 min. The absorbance of each fraction was determined at 303 nm., and the amount dissolved (along with the flow rate) was calculated from absorbance-volume-time measurements.

Procedure for Beaker Method—The temperature, solvent system, and pellet treatment were identical to those used in the flow method. Exactly 900.0 ml. of solvent in a 1-l. jacketed beaker was used in each case, and the volume lost through sampling was immediately replaced. Sampling was begun 5 min. after introduction of the pellet to correspond to the procedure used in the flow method. Samples were taken every 15 min. thereafter for 60 min.

When the USP-NF rotating basket was used for agitation, the pellet was placed inside it and the basket was stationed in the center

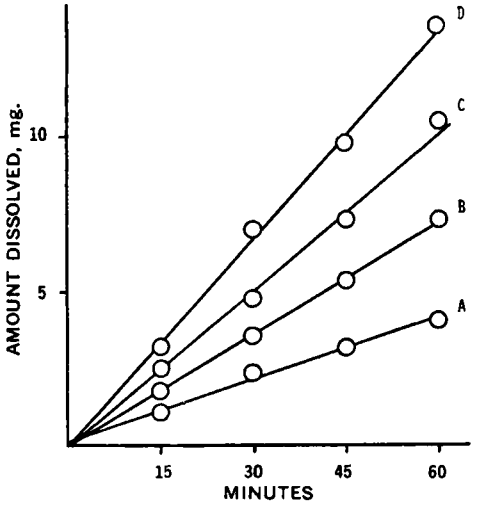

Figure 6—*Pseudo-zero-order plot showing the dissolution of salicylic acid constant-surface pellets in the beaker method using a three-bladed stirrer at various rotational speeds for agitation. Key: A, 50 r.p.m.; B, 100 r.p.m.; C, 150 r.p.m.; and D, 200 r.p.m.*

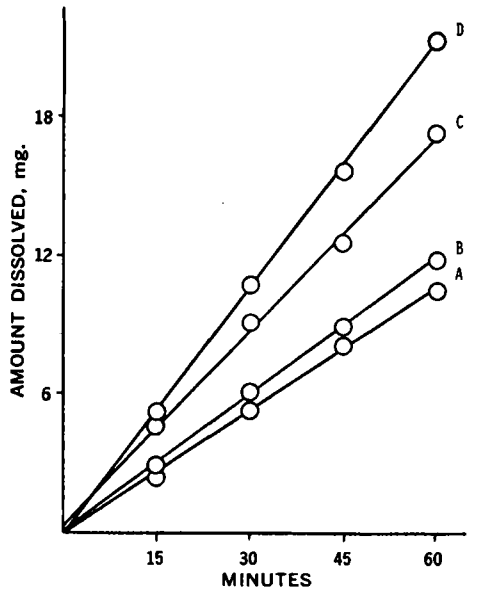

Figure 7—*Pseudo-zero-order plot showing the dissolution of salicylic acid constant-surface pellets in the beaker method using the USP disintegration apparatus (A) and the USP-NF rotating basket (B, 50 r.p.m.; C, 100 r.p.m.; and D, 150 r.p.m.) for agitation.*

of the liquid 2.0 cm. from the bottom. Samples were taken from the same position in the beaker each time. Rotational speed was varied among experiments using solid-state speed control.

With the three-bladed stirrer (1.5-cm. blades positioned in the center of the liquid 2.0 cm. from the bottom), the pellet was placed in the USP-NF basket and held stationary at the side of the beaker halfway between the top and the bottom of the liquid. Rotational speed was varied as before.

The USP disintegration apparatus was used at the official speed only. The pellet was not placed on the screen but on the bottom of the beaker to avoid physical abrasion by the vertical motion of the screen. Experiments with both the flow and beaker methods were conducted in duplicate.

RESULTS

Flow Method—The dissolution of the salicylic acid pellets at various flow rates using the 13- and 25-mm. dissolution chambers

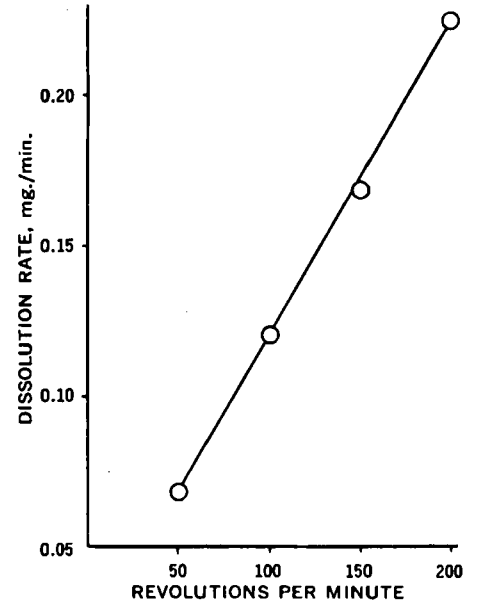

Figure 8—*Plot showing the effect of rotational speed on dissolution rate using the three-bladed stirrer.*

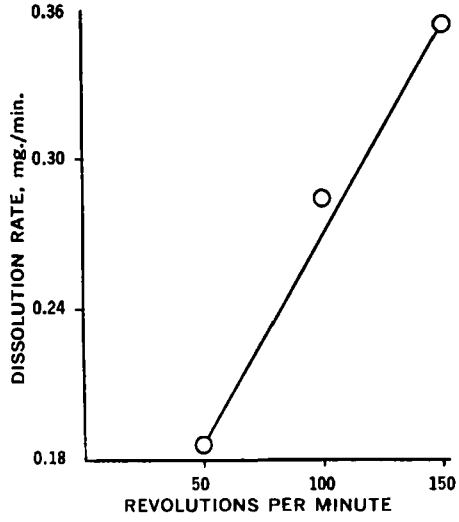

Figure 9—*Plot showing the effect of rotational speed on dissolution rate using the USP–NF rotating basket.*

is shown in Figs. 1 and 2, respectively. In this type of system the usual correlation between dissolution rate and flow rate takes the form:

$$\text{dissolution rate} \propto (Q_A)^{0.2-0.5} \qquad \text{(Eq. 1)}$$

where Q_A is the liquid velocity (centimeters per minute) and equals the flow rate (milliliters per minute) divided by the cross-sectional area of the dissolution chamber (0.765 and 3.87 cm.^{2} for the 13- and 25-mm. units, respectively). Thus, a plot of log dissolution rate *versus* log liquid velocity yields a straight line with a slope of 0.2–0.5. The slope of the line in Fig. 3 is 0.6.

For each individual dissolution chamber, the dissolution rate was found to vary logarithmically with the flow rate within a certain range (Fig. 4), and a plot of liquid velocity *versus* log dissolution rate yields the two-slope linear relationship shown in Fig. 5.

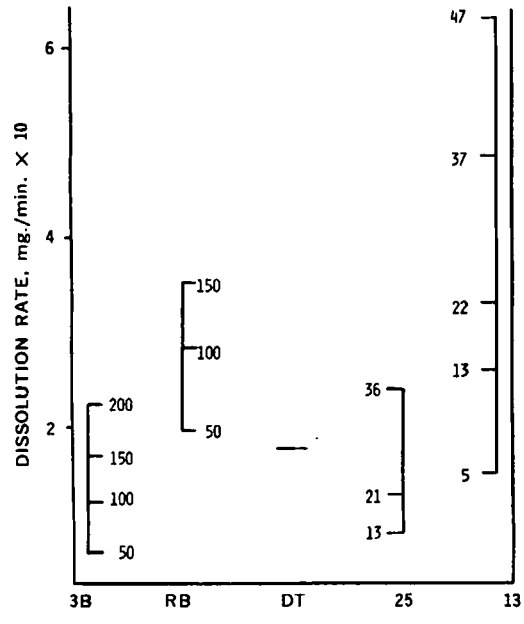

Figure 10—*Nomogram showing the relative effect of agitation on dissolution rate for the flow method (13- and 25-mm. chambers at various flow rates in milliliters per minute) and the beaker method (DT, USP disintegration apparatus at official speed; RB, USP–NF rotating basket at various rotational speeds in revolutions per minute; and 3B, three-bladed stirrer at various rotational speeds in revolutions per minute).*

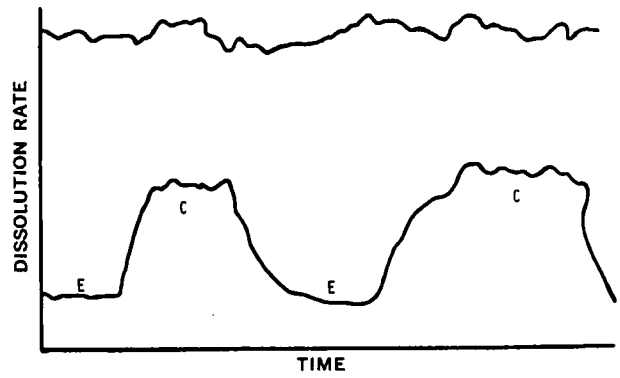

Figure 11—*Continuous UV tracings of dissolution rate* versus *time for salicylic acid constant-surface pellets. Lower curve (25-mm. chamber at flow rate of 80 ml./min.) shows sharp rise in dissolution rate when pellet is moved from edge (E) of lower support screen to center (C). Upper curve is standard tracing at nonturbulent flow rate (25-mm. chamber at 13.1 ml./min.).*

Beaker Method—The dissolution behavior of the pellets in the beaker experiments is shown in Figs. 6 and 7. A linear relationship was found between dissolution rate and agitation intensity for the three-bladed stirrer for the 50–200-r.p.m. range (Fig. 8). A similar relationship was found for the USP–NF rotating basket between 50 and 150 r.p.m. (Fig. 9). These results are similar to those obtained by Levy *et al.* (6).

Comparison of Methods—An estimate of the equivalence of agitation intensity from one method to the next, under the conditions of these experiments, can be made quickly using the nomogram in Fig. 10.

DISCUSSION

Flow Method—The discrepancy between the slope of the line in Fig. 3 and the "theoretical" range of 0.2–0.5 is probably because the dissolving solid was a single pellet rather than a packed bed of nondisintegrating granules (3). The data points obtained at 34 and 36 ml./min. with the 25-mm. chamber and those determined at 45 and 47 ml./min. with the 13-mm. unit were not included in this plot. Although these points exhibited a reasonable degree of correlation (and their exclusion did not result in an appreciable change in the value of the slope), their deviation from the straight line and the semilog nature of the plots in Fig. 4 made their exclusion seem appropriate.

At present, we have no theoretical explanation for the deviations nor for the apparent semilog relationship between dissolution rate and flow rate/liquid velocity. A partial explanation could involve: (*a*) the geometry of the dissolution chambers, (*b*) the drag associated with solvent flowing past the cylindrical pellets, and (*c*) the involvement of more "actual" surface area of the pellet due to increased fluid velocity. The validity of the semilog relationship and of the dual slope nature of the plots (under the conditions of these experiments) is strengthened by the fact that the nonlinear point (5.48 ml./min. flow rate) for the 13-mm. cell in Fig. 4 falls on the line for the 25-mm. unit in Fig. 5 (open circle on plot A).

The plots in Figs. 4 and 5 were not extended beyond 36 ml./min. for the 25-mm. chamber or beyond 47 ml./min. for the 13-mm. unit because results were too erratic with faster flow rates. Apparent dissolution rates above those limits were lower than those for lower flow rates. The cause of this anomaly is the greatly increased turbulence at higher flow rates. The pellets did not remain in the center of the lower support screen but tended to slide along the screen until they reached an eddy (usually close to the edge) and then stopped. At these higher flow rates (up to 80 ml./min.), the dissolution rate varied noticeably, depending upon where the pellet was located on the screen. This can be seen clearly in Fig. 11 (lower curve) where the continuous differential plot of dissolution rate *versus* time, obtained from the recording spectrophotometer, shows a marked increase when the pellet was moved from the edge to the center of the screen.

As expected, this phenomenon is especially apparent with the larger chamber. The problem does not seem to occur to any ap-

preciable extent at lower flow rates. This finding gives rise to the interesting observation that, for constant-surface pellets, the system is, in a sense, more "homogeneous" at lower agitation intensities. Determination of solvent flow patterns in the dissolution chambers at different flow rates would aid in the selection of proper flow rates for any particular system. Such studies, using the method described by Withey and Bowker (7), are in progress in these laboratories.

Beaker Method—In a previous publication (5) dealing with tablet formulations, the three-bladed stirrer (50 r.p.m.) method of agitation resulted in a faster dissolution rate than the rotating basket method (100 r.p.m.). Yet the nomogram in Fig. 10 shows that the three-bladed stirrer, even at 200 r.p.m., produced a slower dissolution rate than the rotating basket at 100 r.p.m. The following explanation emphasizes the inherent difficulties associated with the beaker system. When working with disintegrating tablets, the granules fall out of the basket and are then subject to lower (actual) agitation intensities beause they are not in close proximity to the basket. This problem is compounded by the fact that the basic design of the basket makes it a poor stirring device for the solvent. The three-bladed stirrer was designed to agitate and therefore provided greater agitation than did the rotating basket in the tablet formulation experiments, especially after disintegration took place. However, with the nondisintegrating pellet the solid remains in the basket and is continuously subjected to the recorded rotational speed. Furthermore, in these experiments, the pellet was in the stationary basket well removed from the three-bladed stirrer; thus the agitation intensity near the pellet was considerably less than that near the blades. With the USP disintegration apparatus, the dissolution of the pellet was undoubtedly slowed by positioning it on the bottom of the beaker.

These results indicate that, with the beaker method, unwanted variations are likely, depending on the nature of the dissolving solid (disintegrating or nondisintegrating) and the particular conditions of the experiment (placement of the solid in relation to the stirrer, *etc.*). In addition, the "constant" stirring does not yield a constant (effective) agitation intensity throughout experiments with disintegrating tablets; the agitation near the intact tablet is quite different from that near disintegrated particles dispersed throughout the medium.

Comparison of Methods—The inherent deficiencies of the beaker method are essentially absent with the flow method. First, after defining the cell size, the only variable under nonturbulent flow conditions is the flow rate. Even under turbulent flow conditions, the problems seen with the pellet are reduced with disintegrating tablets, since the granules are usually small enough to circulate throughout the dissolution chamber.

Second, in light of the importance of low agitation intensities, it is encouraging to see (Fig. 10) that flow rates of less than 10 ml./min. with the 25-mm. cell yield agitation intensities lower than those provided by the three-bladed stirrer at 50 r.p.m. The inherent advantages of the flow method are emphasized by the fact that the lower flow rates needed for more laminar flow are most likely to yield meaningful *in vitro–in vivo* correlations.

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Evaluation of Tablet Breaking Strength Testers

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Abstract □ The objective of this study was to determine the relative merits of various tablet hardness testers now used along with one new tester, the Heberlein (Tester A). The Instron Tension-Compression machine (Tester B) was used as the standard machine to which all comparisons were made. A force washer was calibrated using the Instron machine, and the identical force washer anvil setup was used on various air-operated testers for comparative measurements. The air-operated testers studied were the Strong-Cobb (Tester C1) and three modified Strong-Cobb testers (Testers C2, C3, and C4). At an Instron breaking load of 6.00 kg., Testers C2, C3, and C4 gave scale readings (kilograms per square inch) of 9.60, 9.53, and 10.2, while Tester C1 gave a reading of 10.8. Tester A results were 10–15% higher than those of Tester C1 when the units of comparison were Strong-Cobb units. The kilogram scale on Tester A gave values about 10% higher than those of Tester B. A review of the current literature on this subject is given together with a theoretical analysis of tablet breaking. From the results obtained, it is apparent that there are distinct advantages to using a hardness tester with a mechanical drive rather than a pneumatic type, because more uniform force application rates may be achieved and there is less maintenance work and less need for calibration checks.

Keyphrases □ Tablets, strength—evaluation and comparison of six testers, theoretical aspects of tablet breaking □ Strength, tablets—evaluation and comparison of six testers, theoretical aspects of tablet breaking □ Hardness testers for tablets—mechanical *versus* pneumatic driven, theoretical aspects of tablet breaking, calibration method for force response

The determination of tablet breaking strength (tablet hardness test) has become an important measurement in the formulation and manufacture of compressed tablets. During formulation, breaking strength is determined along with friability and disintegration–dissolution measurements since these factors are often inter-

preciable extent at lower flow rates. This finding gives rise to the interesting observation that, for constant-surface pellets, the system is, in a sense, more "homogeneous" at lower agitation intensities. Determination of solvent flow patterns in the dissolution chambers at different flow rates would aid in the selection of proper flow rates for any particular system. Such studies, using the method described by Withey and Bowker (7), are in progress in these laboratories.

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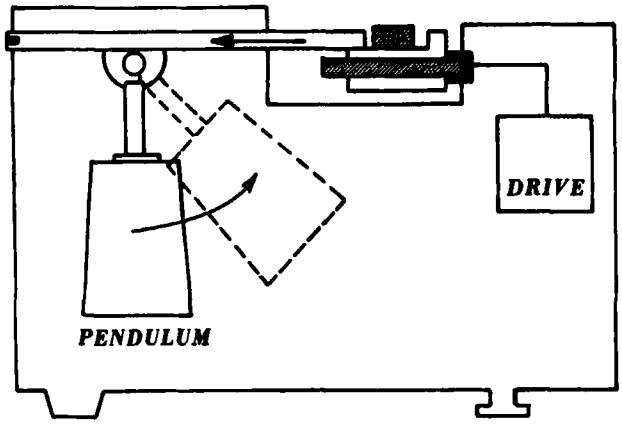

Figure 1—*Operating principle of the Heberlein (Tester A) hardness tester. (See text for description.)*

related. Generally, a manufacturing formula contains a breaking strength specification. A specified range of breaking strength indicates that factors such as disintegration, chewability, and friability (which, in turn, is related to packaging and handling properties) are all within acceptable limits. Since measurement of breaking strength is simpler and less time consuming than testing friability and disintegration, it has become a common control procedure in tablet manufacturing.

DISCUSSION

Various instruments have been used over the years to measure tablet breaking strength. The most popular ones are the Strong-Cobb, hand and air operated, and the Monsanto. The Pfizer and Erweka testers are also frequently used in this country. The fact that various instruments are used attests to some dissatisfaction among users. One problem with the use of hardness testers is the question of operator variability. On those testers where mechanical force is applied by hand, operator variability can be a significant factor in the results obtained. Another factor that causes suspicion on the part of users is the lack of adequate or even recommended calibration procedures for ensuring the accuracy of testers. However, Brook and Marshall (1) described a calibration procedure for various testers using a piezo-electric force transducer.

Specific deficiencies in the Strong-Cobb, Monsanto, Pfizer, and Erweka testers were pointed out by Brook and Marshall (1) and Ritschel *et al.* (2). The Strong-Cobb tester records breaking strength as air pressure in kilograms per square inch rather than as a compressional load. Therefore, its scale values cannot be directly compared to other testers that have force scales. The Strong-Cobb instrument needs frequent checking and calibration, because the air cylinder gets dirty easily and this interferes with plunger movement. Also, the needle valve sometimes needs adjustment so that the load is applied at a uniform rate. Ritschel *et al.* (2) reported an initial delay in the registration of pressure for this tester. Brook and Marshall (1) demonstrated that three new Monsanto testers agreed fairly well in reproducibility among instruments, but two older instruments gave significantly higher readings and this was attributed to fatigued springs. However, Ritschel *et al.* reported that two older instruments of the same kind gave lower readings and questioned the initial uniformity of springs in testers when they are produced. The Pfizer tester was tested by both groups of researchers, and Ritschel *et al.* reported that their results did not conform to those of Brook and Marshall. Spring fatigue and manufacturing variations were again mentioned as factors. Significant operation variability was found when using the Pfizer tester and was believed to be caused by a variable rate of load application. The Erweka tester presumably eliminates operator effects, since it is mechanically driven; but it has been shown that significant large zero errors can occur (1). Ritschel *et al.* (2) found a surprisingly large operator variation for the Erweka tester.

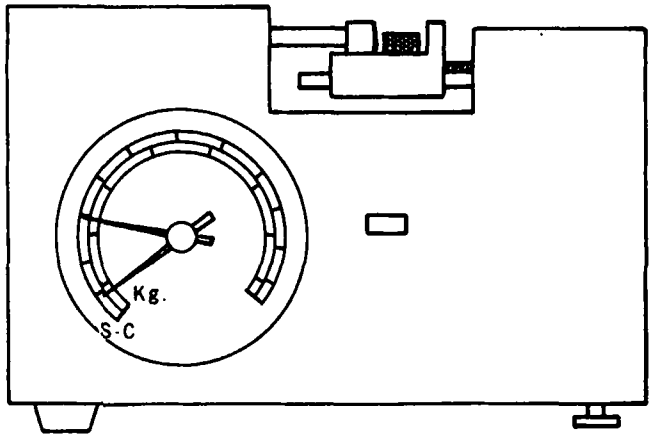

Figure 2—*Schematic diagram of Tester A hardness tester. (See text for description.)*

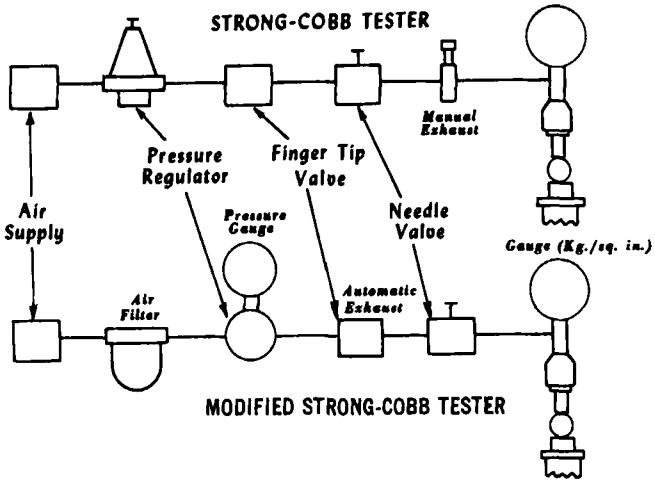

Figure 3—*Diagrammatic representation of air-operated hardness testers. (See text for description.)*

A number of other testers have been employed to a lesser extent; these are the Dynstat (3), the DBT (2), and a Dillon force gauge setup (4). A new instrument, the Heberlein (Figs. 1 and 2) became available in this country in 1970. Both hand- and motor-operated versions are available. This instrument provides a horizontal mounting surface for the tablet. The right side of the instrument contains a horizontal shaft and rectangular anvil which contacts the tablet during operation. The left-hand side of the instrument also has an anvil and shaft. The shaft is connected by a gear to a pendulum weight inside the instrument housing. When in operation, the pendulum is displaced until the tablet breaks. The degree of displacement of the pendulum is recorded on a scaled dial on the front of the instrument. This dial is calibrated in kilograms and Strong-Cobb units. The instrument calibration can be simply checked by mount-

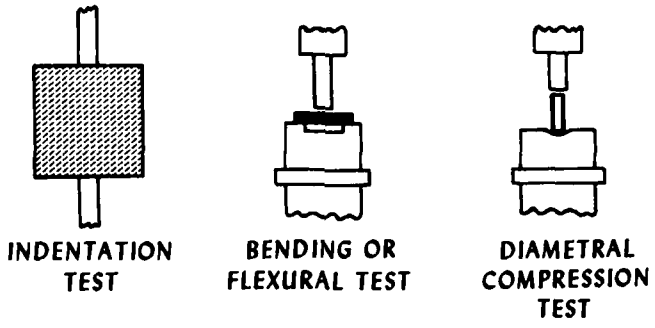

Figure 4—*Methods of determining the breaking strength of brittle materials. (See text for description.)*

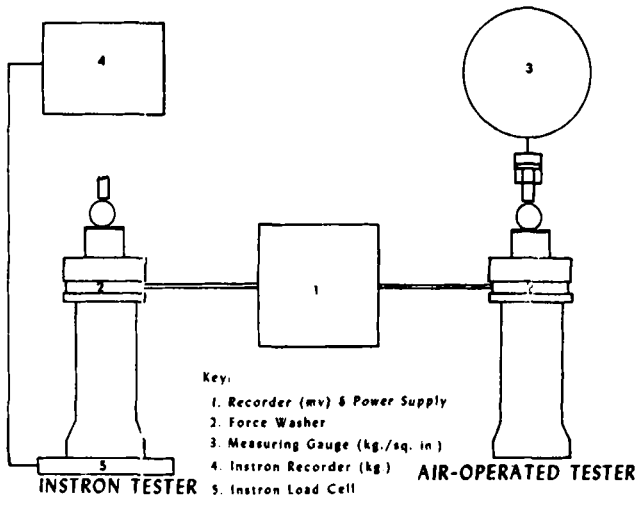

Figure 5—*Schematic view of the method for comparing force washer response to Tester B and air-operated testers. (See text for description.)*

ing a weight over a pulley and attaching it to a screw which fits into the left-hand shaft connected to the pendulum. The results obtained from the hand-operated and motor-driven units do not agree (2).

The Strong-Cobb and modified Strong-Cobb testers operate on the same mechanical principle and resemble each other in appearance. However, the modified Strong-Cobb tester has several features not supplied with the Strong-Cobb tester (Fig. 3). It includes an air filter in the apparatus to ensure clean air to the instrument. Both instruments have pressure regulators, but the modified Strong-Cobb tester has a pressure gauge to allow for adjustment of air pressure to the tester. Another difference is the kind of needle valve used on the two testers. The Strong-Cobb tester is adjustable by means of a handle at the back of the instrument. The modified Strong-Cobb tester has a more sensitive needle valve which is adjusted with a screwdriver during calibration. The function of these valves is to provide a certain rate of pressure application. Moreover, the Strong-Cobb tester has a manual exhaust while the modified Strong-Cobb tester has an automatic exhaust. An automatic exhaust releases the air pressure when the tablet breaks. The piston of the Strong-Cobb tester plunges to the anvil when a tablet breaks and the pressure must be relieved manually.

Instructions for the Strong-Cobb tester indicate that an in-line air filter should be used. Also, the pressure regulator is set by the factory to supply 27.2 kg. (60 lb.) of pressure. The instructions suggest that the needle valve be adjusted for various-sized tablets, giving a slow rate of load application for soft tablets and a higher rate to the harder tablets.

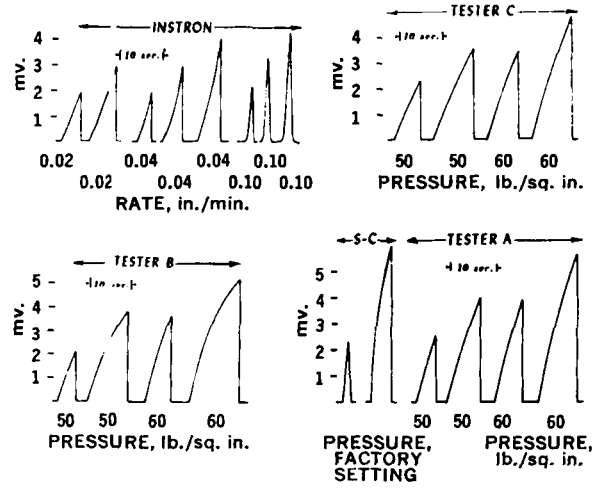

Figure 6—*Response of various hardness testers to 1.59-cm. steel slugs. (See text for description.)*

Table I—Calibration of the Force Washer Using the Tester B Machine

Run	Response Curve[a]	Correlation Coefficient	Predicted Load at 2.0 mv.
Day 1	kg. = 0.059 + 2.94 mv.	1.00	5.94
Day 2	kg. = 0.059 + 2.94 mv.	1.00	5.94
Day 3	kg. = −0.053 + 3.10 mv.	1.00	6.15
Day 4	kg. = −0.018 + 3.10 mv.	1.00	6.00

[a] Includes data for three sizes of steel slugs and three rates of load application.

The modified Strong-Cobb tester is checked for calibration routinely by the maintenance shop. Calibrations are made using a spring tester and gauge, which is checked for accuracy using a deadweight tester. The tester is adjusted for rate using the needle valve to give a scale reading of 15 in 10 sec. at 22.7 kg. (50 lb.) of air pressure. Before this calibration, the freedom of piston movement must be checked. Dust particles that enter the cylinder body on exhausting will retard free movement of the plunger or cause leakage around the neoprene cylinder washer. Testers should periodically be dismantled and cleaned. The plunger should be lubricated periodically to ensure free movement.

THEORY OF TABLET BREAKING STRENGTH

Many papers have appeared on the evaluation of tablet hardness testers, but only a few papers have treated the theory of tablet breaking strength tests (5, 6). The following discussion is included to give a basic view of tablet breaking strength.

The testing of tablet crushing strength is more fundamentally known as the determination of *breaking load*. Breaking load is one means of measuring the *tensile strength* of brittle materials. Various methods of tensile strength testing were described in detail by Berenbaum and Brodie (7) and by Earnshaw and Smith (8). The three kinds of tensile strength testing described by these authors are the *indentation test*, the *bending* or *flexural test*, and the *diametral compression test* (Fig. 4).

The principle of the indentation test is to compress a cubic specimen on opposite sides using a pair of flat-ended indenters (Fig. 4). When a material fails under tension, a vertical crack appears, whereas failure of shear strength results in a large number of irregular broken pieces. The values for tensile strength and shear strength can be determined using the indentation test by altering the thickness of the sample and the diameter of the indenter. For the indentation test to measure breaking load under tension, the shear strength of a material should be at least four times the tensile strength.

An alternative to direct tensile strength measurement is the bending or flexural test (Fig. 4). In this test the specimen is supported at several points, and a load is applied from above which causes the sample to break. This test was criticized by Rudnick *et al.* (9) because of nonuniform stress distribution, which in practice gives results that are considerably in excess of true tensile strength. The bending test was adapted to the Strong-Cobb tester by Endicott *et al.* (10) and to the Erweka tester by Delonca *et al.* (11). This type of measurement was shown to give smaller and more precise results.

A third method, the diametral compression test (Fig. 4), is the most accepted method of measuring tensile strength. In this test a

Table II—Summary of Correlation Results Obtained from Running Tablets and Steel Slugs on Tester B and Monitoring with the Force Washer[a]

Specimen	Response Curve
Tablets	mv. = 0.047 + 0.354 kg.
Slugs	mv. = −0.007 + 0.330 kg.
Tablets	kg. = −0.117 + 2.816 mv.
Slugs	kg. = −0.012 + 3.024 mv.

[a] Includes data for three sizes of steel slugs and three tablet sizes, each made at two compression force levels, and tested at three rates of load application on Tester B.

Table III—Response of the C Type Testers Using a Steel Slug and Tablet and Monitoring with the Force Washer[a]

Tester[b]	Specimen	Response Curve[c]	Correlation Coefficient	Predicted Scale Reading at 2.5 mv.[d]
C1	Steel slug	S.R. = 0.77 + 4.05 mv.	1.00	10.90
C1	Tablet	S.R. = 0.88 + 4.11 mv.	0.96	11.16
C2	Steel slug	S.R. = 0.16 + 3.76 mv.	1.00	9.56
C2	Tablet	S.R. = 0.01 + 4.22 mv.	0.98	10.56
C3	Steel slug	S.R. = 1.11 + 3.75 mv.	1.00	10.48
C3	Tablet	S.R. = 1.12 + 3.57 mv.	0.99	10.05
C4	Steel slug	S.R. = 0.92 + 3.89 mv.	1.00	10.65
C4	Tablet	S.R. = 1.96 + 3.50 mv.	1.00	10.71

[a] Response curves of the 1.59-cm. slug were generated from 32 measurements on each tester. Response curves of tablets were generated from 20 or 30 measurements on each tester. [b] See text for designation of tester. [c] S.R. = scale reading in kilograms per square inch. [d] Calculated from equation under response curve column.

right circular cylinder, disk, or tablet is compressed between platens in contact with the edges of the sample. A uniform tensile stress occurs across the diametral plane joining the two lines of contact of the specimens and platens. The tensile strength is directly proportional to load, and its magnitude is given by the equation (5):

$$\sigma_t = \frac{2P}{\pi DT} \qquad \text{(Eq. 1)}$$

where σ_t is the tensile strength, P is the applied load, D is the diameter, and T is the thickness of the specimen.

Both tensile and compressive stresses exist along the loaded diameter of a tablet under test. Compressive and shear stresses vary along the loaded diameter and are maximal at the points of contact. Use of Eq. 1 is contingent principally on a vertical break as previously described. Another precaution in the determination of tensile strength by the diametral compression test is to minimize the area of contact between the platen and the specimen. The smaller contact area reduces the compressive and shear stresses in regions near the applied load where they are greatest and where the specimen will most likely break in tension. Fell and Newton (5) demonstrated the use of soft padding material between the tablet and the platen in testing the strength of various lactose tablets. This was necessary to obtain breakage of tablets in tension only and thus provide more reproducible results.

A multiobjective study of three tablet hardness testers was undertaken to determine their relative merits and shortcomings as routine instruments. The testers studied were: (*a*) the air-operated Strong-Cobb tester, (*b*) three modified Strong-Cobb testers containing some upgraded components to improve their performance[1], and (*c*) the electrically operated Heberlein tester which is a constant-speed motorized unit. The objectives were: (*a*) to determine if all three kinds of instruments give the same crushing strength readings, (*b*) to determine the accuracy and precision of the testers, and (*c*) to obtain a method of calibration for the testers.

EXPERIMENTAL

Equipment—The Instron Tension-Compression machine, model TM (Tester B), was used as the standard machine to which comparisons of the three testers were made. The anvils of the Strong-Cobb tester (Tester C1) and the modified Strong-Cobb testers (Testers C2, C3, and C4) are easily removed and can be used as mounting supports for the specimens being tested on Tester B. A force

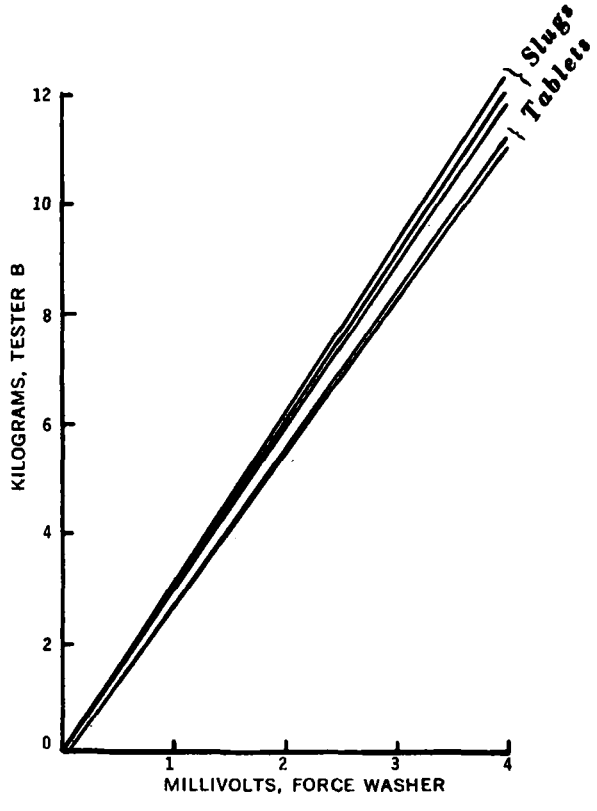

Figure 7—*Comparison of Tester B force response to force washer, 1.59-cm. steel slug and 1.59-cm. tablet.*

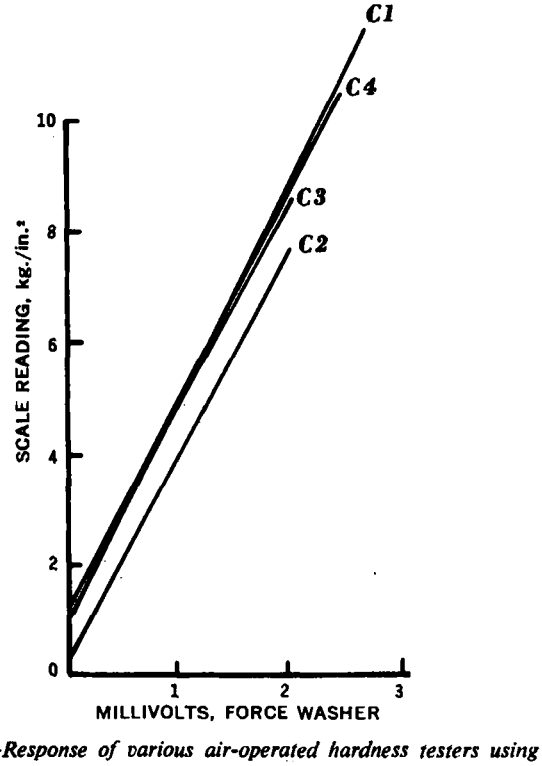

Figure 8—*Response of various air-operated hardness testers using steel slugs.*

[1] These testers are made by the maintenance shop, Warner-Lambert Co., Morris Plains, N. J.

Table IV—Comparison of Breaking Strength Obtained from Tester B to the C Type Testers

Instrument[a]	Correlation Analysis	Breaking Load, kg., at Scale Reading of 10.0	Scale Reading at Breaking Load of 6.00 kg.
C1	kg. (Tester B) = −0.08 + 0.562 × (scale reading)	5.54	10.8
C2	kg. (Tester B) = 0.28 + 0.596 × (scale reading)	6.24	9.60
C3	kg. (Tester B) = −0.32 + 0.663 × (scale reading)	6.31	9.53
C4	kg. (Tester B) = −0.55 + 0.644 × (scale reading)	5.89	10.2

[a] See text for instrument designations. [b] Data illustrated in Fig. 9.

washer[2] was obtained and mounted on the anvil as illustrated in Fig. 5. The force washer was the full wheatstone bridge type, having a maximum load capacity of 27.2 kg. (60 lb.). It was activated by a 6-v. power supply[3], and its output was recorded in millivolts on a strip-chart recorder[4]. A steel plunger having the same diameter as is used on Testers C1, C2, C3, and C4 was made for use with the Tester B machine. Specimens were mounted as illustrated in Fig. 5, and an Instron force reading as well as a force washer reading was obtained simultaneously. In this way, the force washer was calibrated and could be used as a secondary standard when the entire anvil assembly was used with the type C testers.

Materials—Stainless steel slugs, having 1.59-cm. (0.63-in.), 1.11-cm. (0.43-in.), and 0.71-cm. (0.28-in.) diameters, were made to use as artificial tablets for the comparison of instruments. Tablets of the same diameter were also made, consisting of 64.5% spray-dried lactose, 35% microcrystalline cellulose, and 0.5% magnesium stearate. A number of tablets were made as uniform as possible by weighing the correct quantity of granulation and compressing tablets on an Instron tester (model TT) using standard 1.91-cm. (0.75-in.) barrel diameter punches and a 3.02-cm. (1.19-in.) die. These tools were used in a special jig which serves as a holder for compression of a single tablet. Tablets of 1.59-cm. (0.63-in.) diameter weighed 1.15 g. and were compressed at 907, 1134, and 1361 kg. (2000, 2500, and 3000 lb.); tablets of 1.11-cm. (0.43-in.) diameter weighed 500 mg. and were compressed at 726 and 930 kg. (1600 and 2050 lb.); and tablets of 0.71-cm. (0.28-in.) diameter weighed 150 mg. and were compressed at 454 and 680 kg. (1000 and 1500 lb.). Tablets were stored in well-sealed glass bottles until ready for testing.

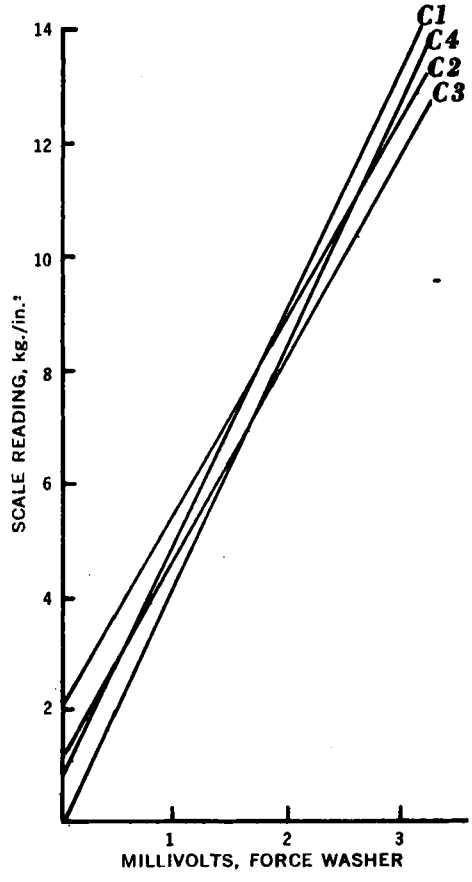

Figure 9—*Response of various air-operated hardness testers using tablets.*

RESULTS

Response of Force Washer—The steel slugs were mounted on the anvil of type C testers and the assembly was then placed into position on the load cell of Tester B (Fig. 5). The assembly was centered under the plunger, which was attached to the upper platen of Tester B. Tester B and the strain-gauge recorder were zeroed before each run. Three rates were tested: 0.051, 0.102, and 0.254 cm./min. (0.02, 0.04, and 0.10 in./min.), and runs were made on 4 separate days.

The results of typical runs are illustrated in Fig. 6, which shows recordings obtained from the force washer. A series of correlation analyses was made from the data compiled from various force washer peak heights *versus* the load value from Tester B. No significant differences were found for the three sized slugs or the three rates used. Table I summarizes the calibration curve data for 4 separate days, in which each day includes all slug sizes and all rates of load application. At a 2.0-mv. (about 6.01-kg.) reading, the predicted load on various days varied by no more than 3.5% with a high degree of correlation.

The same experiment was repeated using tablets of different sizes and made with various compression forces. Tester B load readings were again compared to the millivolt output of the force washer, and the results of the correlation analysis indicate that the rate of load application, tablet size, and compression force used to make the tablets were not significant factors in the results obtained. The pooled results from both tablets and slugs are given in Table II.

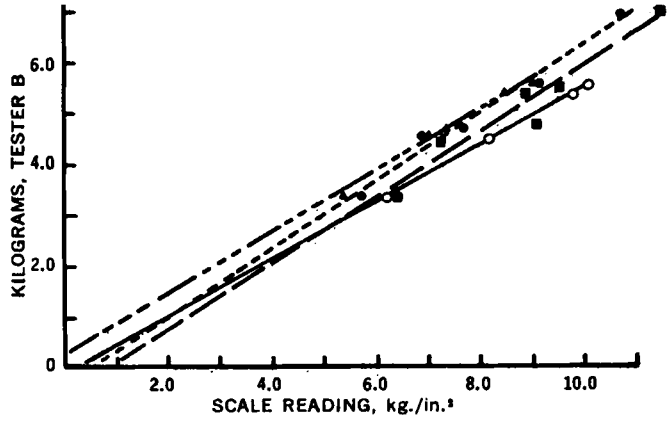

Figure 10—*Comparison of air-operated tester response to Tester B response. Key: ○, Tester C1; ▲, Tester C2; ●, Tester C3; and ■, Tester C4.*

[2] Model 1200B, Houston Scientific Industries, Inc., Houston, TX 77018
[3] Model 801C, Hewlett-Packard, Paramus, NJ 07652
[4] Electronic 19, Honeywell Industrial Division, Fort Washington, PA 19034

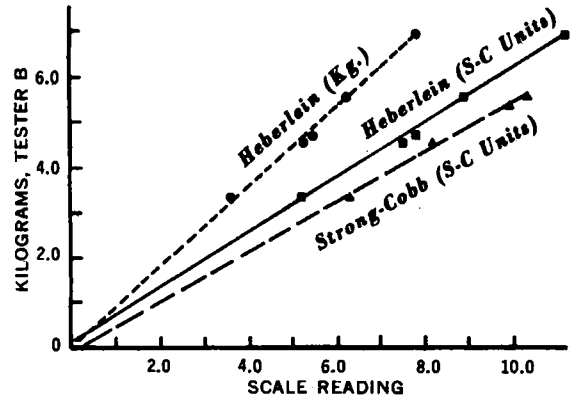

Figure 11—*Comparison of breaking strength of Testers B, C1, and A.*

The results for both steel slugs and tablets are illustrated in Fig. 7. Although the results from each experiment were linear, the force washer response for these two different materials was somewhat divergent. A possible explanation for this result is that the tablets were more resilient than the steel slugs. However, the main objective of this part of the experimentation was to prove good linearity and reproducibility in the force washer response. Having achieved excellent working characteristics on the anvil-force washer combination, the same setup could be used to evaluate the operating characteristics of the C type hardness testers.

Response of the C Type Testers Using a Steel Slug and Tablets—Testers C1, C2, C3, and C4 were tested for response using a 1.59-cm. steel slug and the various tablets described in the *Experimental* section. The same anvil-force washer assembly was used as described for the Tester B machine. The hardness testers were used in the normal fashion, and both the force washer readings and the instrument gauge readings were recorded. These results are illustrated in Figs. 8 and 9; the correlation analysis is given in Table III.

These results indicate good linearity in response for both specimens on all testers. For the slug, similar slopes were obtained for Testers C1, C2, and C3, but Tester C1 gave a somewhat higher slope. Positive intercepts were obtained for all testers, but Tester C2 had a much lower intercept than the other testers. These intercept data contrast with the results for Tester B, which were nearly zero for both tablets and slugs.

The results obtained for tablets were similar to those obtained for slugs, but there was less agreement between slopes of the various testers. Tester C2 had a lower intercept for tablets than the other three testers and this compares to the results obtained for slugs. For testers having intercepts which were significantly different from zero, no simple conversion factor to a kilogram force measurement can be given. For Tester C2, whose intercept was practically zero, a conversion factor of scale reading to kilograms is about 0.66. This factor is derived from the results of the Tester B response curve for tablets in Table II. Brook and Marshall (1) reported a conversion factor of 0.728 for the Strong-Cobb hardness tester, and Strong-Cobb claims a theoretical factor of 0.78 based on a 2.54-cm. (1-in.) cylinder area of the piston drive. The high positive intercepts for three of the testers indicate that a higher than expected loading is occurring early in the run or that significant curvature exists in the early portion of the response curve. No readings were obtained

Table V—Comparison of Breaking Strength Obtained from Tester B to Testers C1 and A

Instrument	Correlation Analysis
Tester A	kg. (Tester B) = 0.05 + 0.624 × (scale reading in Strong-Cobb units)
Tester A	kg. (Tester B) = 0.01 + 0.901 × (scale reading in kg.)
Tester C1	kg. (Tester B) = −0.08 + 0.562 × (scale reading)

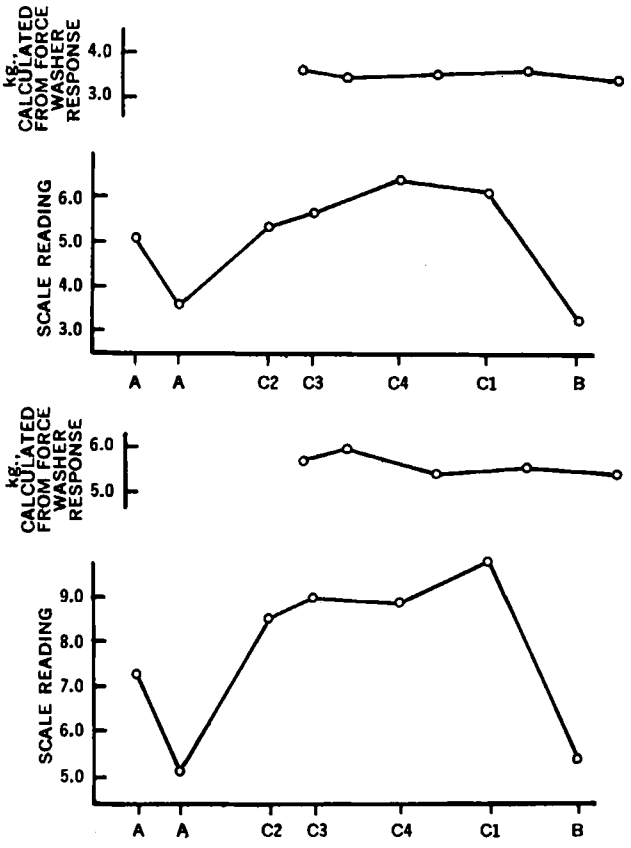

Figure 12—*Overall comparison of tester response, 0.71-cm. tablet. Upper curve is for 454-kg. compression force, and lower curve is for 680-kg. compression force. Units for Tester A are kilograms per square inch and kilograms (left to right); units for Testers C1, C2, C3, and C4 (air-operated testers) are kilograms per square inch; and units for Tester B are kilograms.*

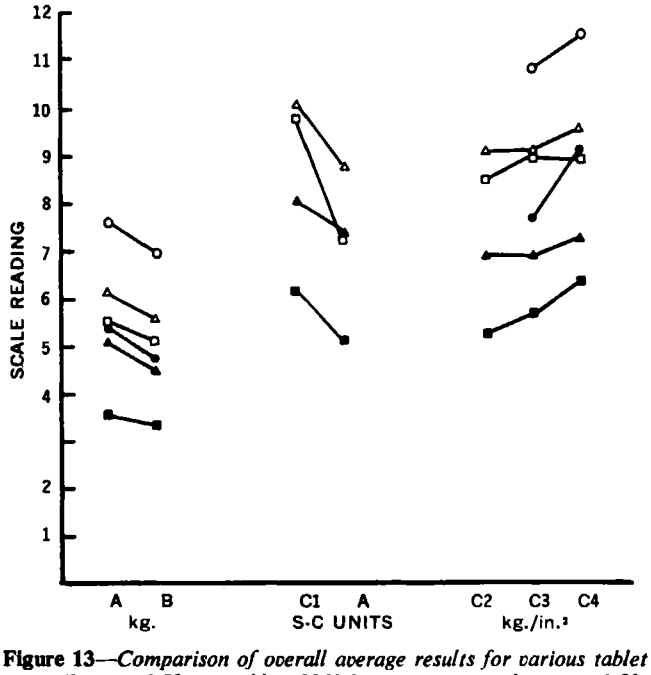

Figure 13—*Comparison of overall average results for various tablet sizes. Key: ○, 1.59-cm. tablet, 1361-kg. compression force; ●, 1.59-cm. tablet, 907-kg. compression force; △, 1.11-cm. tablet, 930-kg. compression force; ▲, 1.11-cm. tablet, 726-kg. compression force; □, 0.71-cm. tablet, 680-kg. compression force; and ■, 0.71-cm. tablet, 454-kg. compression force. (Tester A = Heberlein, Tester B = Instron, and Testers C1, C2, C3, and C4 = air-operated testers. See text for description.)*

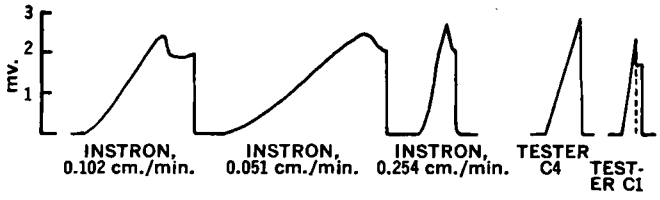

Figure 14—*Comparison of the rates of force application for tablets. Tester C4 = a modified Strong-Cobb tester, and Tester C1 = Strong-Cobb tester.*

below a scale reading of 4, which would have indicated whether initial loading or a curvature existed. A high initial loading would indicate some frictional forces in the instrument must be overcome before reaching linearity.

Calculated tester scale readings from the response curves listed in Table III indicate that a 2.5-mv. force washer loading (6.92 kg. equivalent from Tester B tablet response curve) gives readings of 11.2, 10.6, 10.1, and 10.7 for Testers C1, C2, C3, and C4, respectively. These data indicate that significant variations can occur between the same instruments when actually breaking tablets. Figure 3 indicates that the response slopes are more uniform with slugs than with tablets (Fig. 9). The individual tester response to tablets is dependent on both its intercept and slope.

Comparison of C Type Tester Response to Tester B Response—The results obtained from breaking random samples of the various, specially prepared tablets on the Tester B machine and the C type testers were compared as illustrated in Fig. 10. From this curve, the correlation of breaking strength in kilograms to scale readings can also be determined, and these comparisons are given in Table IV. A similar comparison could be made by combining response equations for the Tester B machine and the C type testers since a millivolt response was obtained for each.

The instruments are variable in response, as noted by the different slopes and intercepts for each tester. The data in general indicate that one conversion factor is not valid for conversion of scale reading to breaking load in kilograms. Table IV gives an example of the calculated load for a scale reading of 10.0 for each tester. The calculated scale reading for a 6.00-kg. breaking load is also given in the same table. Among the modified C type testers, a calculated range of 9.53–10.2 is obtained in scale readings. The Tester C1 calculated scale reading for a 6.00-kg. load is 10.8, which is significantly higher than the modified testers. The modified testers were calibrated by the previously mentioned procedure just prior to this work, and Tester C1 was new and used according to the instructions supplied. The reason for nonagreement between testers was not readily apparent during the testing.

Comparison of Tester C1 and Tester A Response to Tester B Response—Figure 11 illustrates the results obtained from breaking the specially prepared tablets on the Tester B machine *versus* the scale reading obtained from tablets broken on Testers C1 and A. The correlation analysis of Tester B breaking strength to the tester scale readings is given in Table V.

In comparing breaking strengths of tablets between Testers A and C1, it was found that the former tester gives significantly higher results and that these could be 10–15% greater than Tester C1, depending on the hardness range considered. The usually assumed "scale factor" of 0.78 (scale reading to kilograms) for Tester C1 does not compare favorably with the slope of 0.624 obtained for Tester A.

The kilogram scale on Tester A is about 10% higher than the actual breaking load determined on Tester B. A very small intercept was obtained for this correlation, indicating that a scale factor of 0.90 would be fairly accurate in converting Tester A breaking load in kilograms to Tester B breaking load in kilograms.

Overall Comparison of Tester Response—Overall comparisons between testers are illustrated in Fig. 12 for 0.71-cm. diameter tablets. The lower curve presents the scale reading from each instrument and illustrates the variability that can be expected by breaking tablets that are prepared in the most uniform method possible. In general, the results for Testers A and B in kilograms breaking load are significantly lower than the readings of other testers whose scales are in kilograms per square inch. Modified Testers C2 and C3 usually gave somewhat lower readings than Tester C4. Also, results of Tester C1 were higher than those of Testers C2, C3, and C4.

The upper curves in Fig. 12 illustrate the actual breaking load.

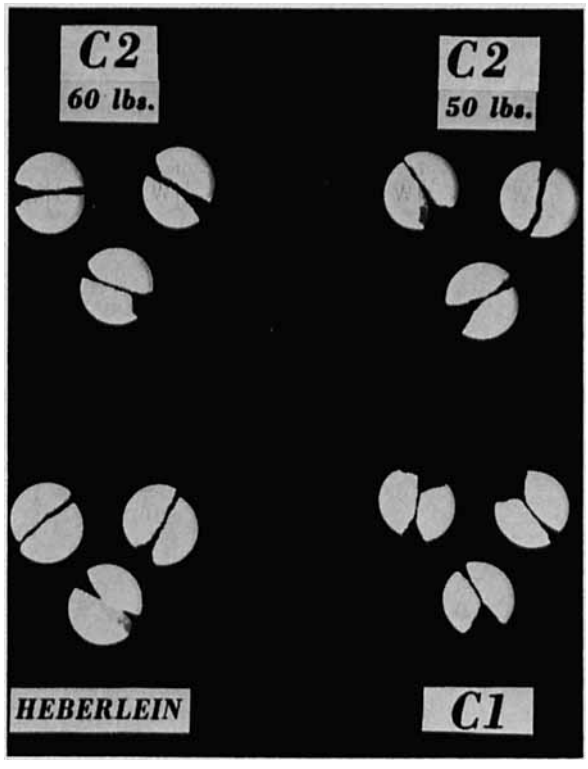

Figure 15—*Typical broken tablet fragments. Key: top, modified Tester C2 at 27.2 kg. (60 lb.) (left) and 22.7 kg. (50 lb.) (right) operating pressure; bottom, Tester A and Tester C1.*

The data for the air-operated testers were obtained by measurement of force washer response. The equation used for this calculation was given in Table II and is repeated here:

$$\text{kg.} = -0.117 + 2.816 \text{ mv.} \qquad \text{(Eq. 2)}$$

Under ideal working conditions for the testers, a relatively flat curve for breaking load would be expected. It has been shown that the force washer response was not significantly affected by using different sizes of tablets made at two compression force levels.

To illustrate the trends in the various testers, overall average scale readings were plotted for all tablets in Fig. 13. These data show that on the average the Tester A results in a kilogram breaking load consistently higher than Tester B. A comparison of Testers C1 and A indicates that higher values are obtained from Tester C1 and that these differences range from 0.8 to 1.6 units. Inspection of the results of modified Testers C2, C3, and C4 indicates that Tester C4 is 0.3–1.5 units higher in most instances.

Rate of Force Application—The force–response curve for the various testers was examined to determine whether this factor affected the breaking strength obtained. Figure 14 illustrates the type of force curves obtained for Tester B at three rates, for Tester C4, and for Tester C1. Tester B results indicate that a piston travel rate of 0.254 cm./min. approximates the rate of force application rate of Tester C4. Tester C1, however, applies force at a much faster rate, almost twice the rate of Tester C4 and Tester B. The rates of 0.102 and 0.051 cm./min. for Tester B are considerably slower than the rates obtained for any of the air-operated testers. The peak responses on the Tester B curve indicate the point of diametral cracking. Force level drops after cracking, but if the tablet remains in place the force level again rises, as seen for the 0.102-cm./min. rate (Fig. 14). The sharp drop is obtained on releasing the Tester B drive. The modified C type testers, in general, produce a diametral crack similar to Tester B, but several fragments may be formed. Typical broken tablet fragments are shown in Fig. 15. Of particular interest is the fact that the relatively fast rate of Tester C1 results in the shattering of the tablet. This fast rate is responsible for the more complex breakage of a tablet; *i.e.*, tablets are not broken in tension and this results in the higher scale reading results obtained for Tester C1.

A better understanding of the mode of force application can be seen in Fig. 6, which depicts curves obtained from the various testers with steel slugs. These slugs are rigid and are not penetrated

by the plunger. This results in an increasing rate of force application when Tester B is used. The air-operated testers, on the other hand, give a decreasing rate of load application as load is increased. This occurs because of the buildup of pressure within the tester air cylinder to the point where it begins to approach the pressure level supplied to the tester. Sometimes hard tablets cannot be broken by using the air-operated testers, because of the limitation of force applied by these testers. Because of the change in the rate of load application of the air-operated testers at higher load levels, the constant-speed mechanical tester would be preferred. Figure 6 also points out the fact that variable rates are obtained for different air-operated testers and that this rate is not easily adjusted or controlled.

SUMMARY AND CONCLUSIONS

A suitable method of calibrating the force response of air-operated hardness testers was developed. The results obtained from various type C testers were variable and could be traced to inconsistencies between instruments such as variable rate of load application and variable friction in the piston. The Tester A instrument load scale (kilograms) gave values about 10% higher than were obtained in Tester B.

There are distinct advantages for using a mechanical tester such as Tester A:

1. More uniform force application may be achieved.
2. Less maintenance work is required.
3. There is less need for calibration checks.

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New *In Vitro* Disintegration and Dissolution Test Method for Tablets and Capsules

FRANK W. GOODHART▲, ROBERT H. McCOY, and FRED C. NINGER

Abstract □ An *in vitro* technique for testing the disintegration and dissolution of tablets and capsules was developed and evaluated. The apparatus consists of a beaker with a cylindrical well in the bottom into which is placed a platform containing the dosage form to be tested. Shallow cylindrical depressions in the platform are used to hold capsules snugly in a vertical position for testing while variously shaped depressions are used for tablets, depending on their size and shape. Comparisons between the official and the new method indicated that the official test does not differentiate between capsule formulations containing a hydrophobic lubricant. A phenylpropanolamine hydrochloride capsule formulated with a high level of magnesium stearate was shown to release drug more slowly *in vitro* and *in vivo*. The effects of capsule formulation factors such as type and level of lubricant and disintegrant as well as the presence of a surfactant were determined. It was found that the use of magnesium stearate and hydrogenated vegetable oil as lubricants significantly prolonged the *in vitro* disintegration time of hard gelatin capsules. Hard gelatin capsules also disintegrated more rapidly in artificial gastric fluid as compared to distilled water, and machine-filled capsules generally disintegrated more slowly than hand-filled capsules. Studies on tablets containing a slightly water-soluble drug indicated that the method of preparing the granulation has an important effect on the *in vitro* release of the drug.

Keyphrases □ Dissolution—method and equipment for tablets and capsules, compared to compendial method □ Tablet dissolution—method and equipment, compared to compendial method □ Capsule dissolution—method and equipment, effect of lubricant and disintegrant characteristics, surfactants, compared to compendial method □ Surfactant effect—dissolution of capsules, method, equipment □ Phenylpropanolamine hydrochloride capsule—dissolution characteristics, effect of formulation

Progress in *in vitro* dissolution technology of solid dosage forms resulted in the adoption of a specific apparatus and methodology by NF XIII and USP XVIII for testing drug availability from tablets and capsules. In addition, the basket-rack assembly is still recognized by the official compendia as a test method for the disintegration of tablets. No test method has ever been adopted for testing the disintegration of capsules. *In*

by the plunger. This results in an increasing rate of force application when Tester B is used. The air-operated testers, on the other hand, give a decreasing rate of load application as load is increased. This occurs because of the buildup of pressure within the tester air cylinder to the point where it begins to approach the pressure level supplied to the tester. Sometimes hard tablets cannot be broken by using the air-operated testers, because of the limitation of force applied by these testers. Because of the change in the rate of load application of the air-operated testers at higher load levels, the constant-speed mechanical tester would be preferred. Figure 6 also points out the fact that variable rates are obtained for different air-operated testers and that this rate is not easily adjusted or controlled.

SUMMARY AND CONCLUSIONS

A suitable method of calibrating the force response of air-operated hardness testers was developed. The results obtained from various type C testers were variable and could be traced to inconsistencies between instruments such as variable rate of load application and variable friction in the piston. The Tester A instrument load scale (kilograms) gave values about 10% higher than were obtained in Tester B.

There are distinct advantages for using a mechanical tester such as Tester A:

1. More uniform force application may be achieved.
2. Less maintenance work is required.
3. There is less need for calibration checks.

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New *In Vitro* Disintegration and Dissolution Test Method for Tablets and Capsules

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Abstract □ An *in vitro* technique for testing the disintegration and dissolution of tablets and capsules was developed and evaluated. The apparatus consists of a beaker with a cylindrical well in the bottom into which is placed a platform containing the dosage form to be tested. Shallow cylindrical depressions in the platform are used to hold capsules snugly in a vertical position for testing while variously shaped depressions are used for tablets, depending on their size and shape. Comparisons between the official and the new method indicated that the official test does not differentiate between capsule formulations containing a hydrophobic lubricant. A phenylpropanolamine hydrochloride capsule formulated with a high level of magnesium stearate was shown to release drug more slowly *in vitro* and *in vivo*. The effects of capsule formulation factors such as type and level of lubricant and disintegrant as well as the presence of a surfactant were determined. It was found that the use of magnesium stearate and hydrogenated vegetable oil as lubricants significantly prolonged the *in vitro* disintegration time of hard gelatin capsules. Hard gelatin capsules also disintegrated more rapidly in artificial gastric fluid as compared to distilled water, and machine-filled capsules generally disintegrated more slowly than hand-filled capsules. Studies on tablets containing a slightly water-soluble drug indicated that the method of preparing the granulation has an important effect on the *in vitro* release of the drug.

Keyphrases □ Dissolution—method and equipment for tablets and capsules, compared to compendial method □ Tablet dissolution—method and equipment, compared to compendial method □ Capsule dissolution—method and equipment, effect of lubricant and disintegrant characteristics, surfactants, compared to compendial method □ Surfactant effect—dissolution of capsules, method, equipment □ Phenylpropanolamine hydrochloride capsule—dissolution characteristics, effect of formulation

Progress in *in vitro* dissolution technology of solid dosage forms resulted in the adoption of a specific apparatus and methodology by NF XIII and USP XVIII for testing drug availability from tablets and capsules. In addition, the basket-rack assembly is still recognized by the official compendia as a test method for the disintegration of tablets. No test method has ever been adopted for testing the disintegration of capsules. *In*

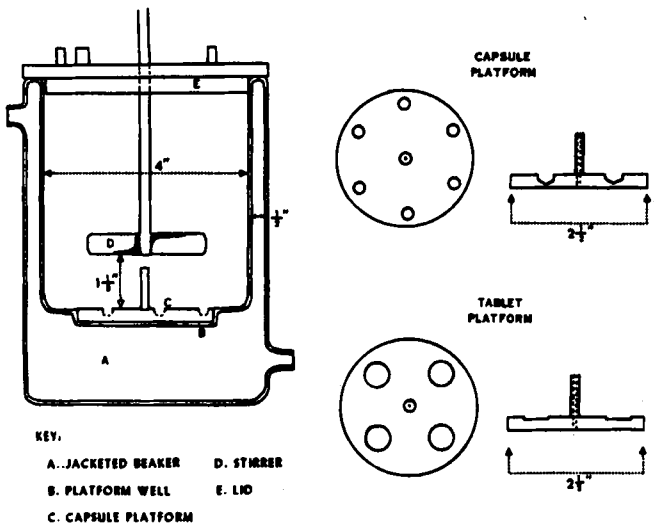

Figure 1—*Diagram of the tablet and capsule disintegration and dissolution apparatus.*

vitro test methods for gelatin capsules received little attention until Wood (1) pointed out that a standard test for this dosage form was needed. The difference in behavior between capsules and tablets in a specific dissolution apparatus has prevented the development of a standard test method suitable for both products.

Some specific faults of hard gelatin capsule formulations were pinpointed by Samyn and Jung (2). Their studies showed prolonged disintegration/dissolution times with an increase in the level of the lubricant, magnesium stearate. In these formulations, liquid penetration was increasingly retarded with increased levels of magnesium stearate and the percent of moisture in the capsule plug was inversely proportional to the amount of this lubricant. Magnesium stearate was, in effect, waterproofing the capsule contents, and it was theorized that the *in vivo* drug availability of these formulations must also be affected.

Little specific information regarding drug availability from capsules as a function of formulation is available. However, Poole (3) reported that the lubricant system in capsules significantly affected the degree of drug absorption in dogs and humans. The *in vivo* results were in rank order with *in vitro* slow dissolution results.

Samyn and Jung (2) pointed out that the official disintegration method with disks probably disguises the true disintegration time of capsules. The pharmaceutical formulator must, therefore, develop some method for testing drug availability from capsules. This is particularly necessary during early formulation when *in vivo* studies would be premature and costly. While slow dissolution methods give useful data which are readily correlatable to *in vivo* performance, a disintegration method would also be very useful for quick screening of capsule formulations.

Lin *et al.* (4) screened seven dissolution methods for release of drugs from hard gelatin capsules. They pointed out that the official method has several disadvantages. The capsule cannot be visually observed during disintegration, so a slow wetting plug cannot be seen. Also, the 40-mesh screen used in this method is prone to becoming clogged with many formulations; therefore, an 8-mesh screen was suggested by Lin *et al.* (4). Poole (3) described the difficulties of testing capsule formulations; these included the tendency of most capsules to float in the dissolution media and to become tacky and to stick to the stirrer or vessel. Also, the use of the present NF XIII and USP XVIII methods is not to be recommended since the wire mesh of the basket is easily fouled by the gelatin or other insoluble or gumming components, resulting in a nonuniform solvent flow around the capsule. Because of the seeming lack of a satisfactory disintegration testing method, one was developed in these laboratories. It allows visual observation and, hence, a quick method of screening; it is also suitable for disintegration testing of tablets as well as the dissolution testing of all solid dosage forms.

EXPERIMENTAL

Description of Apparatus—The tablet and capsule disintegration/dissolution apparatus[1] is shown in Fig. 1. It consists of a jacketed 800-ml. chamber with a centrally located well in the bottom measuring 6.35 cm. (2.5 in.) in diameter and 0.64 cm. (0.25 in.) deep. Various platforms for testing either capsules or tablets are placed into the well.

The capsule platforms are made of Plexiglas and have six shallow wells symmetrically located on the platform for the insertion of six capsules. These platforms can also be made of Teflon with Plexiglas inserts for holding the capsules. The capsule wells are drilled every 60° on a 2.54-cm. (1-in.) radius around the platform. A post, 2.54 cm. high, is placed in the center of each platform to facilitate removal of the platform from the beaker well. A small hole is drilled vertically through the post to the bottom side of the platform to allow air to escape from the underside of the platform at the beginning of a study.

The tablet platforms are similar in appearance to the capsule platforms, but they are made of Teflon. There are four shallow wells drilled on the surface of these platforms to restrain nondisintegrated tablets from changing position in the beaker. The tablet wells are drilled with an end-mill every 90° and are centered 2.54 cm. (1 in.) from the platform midpoint. Capsule platforms made solely of Teflon were impractical because of poor adhesion between the slippery Teflon surface and gelatin capsule. Platforms for capsules and tablets can be made having other configurations to hold various numbers of tablets and capsules. Well depths may be varied for tablets, depending on their size and shape.

A Plexiglas lid was made for the top of the beaker to retard evaporation of the testing fluid. The top contains holes for a stirrer, thermometer, and inlet and outlet sampling ports.

A two-bladed glass stirrer, 5.50 cm. (2.165 in.) in diameter with a 45° blade pitch, is centered and operated 2.86 cm. (1.125 in.) above the surface of the platform. The stirring speed is controlled and monitored with a constant-speed control unit[2]. The temperature of the bath was kept at 37 ± 0.5° with a constant-temperature circulating bath. An adjustable jack[3] was used to remove the stirrer and lid from the beaker after each run.

Materials—The following were used: starch USP; lactose USP hydrous, improved flow; magnesium stearate USP; microcrystalline cellulose NF[4]; tranquilizing drug powder; phenylpropanolamine hydrochloride NF (powder); sodium lauryl sulfate USP, washed and dried; stearic acid USP[5]; and hydrogenated vegetable oil[6].

[1] The apparatus was built by Mr. William Campbell with the help of Mr. Walter Lugrin, Warner-Lambert Research Institute. A forerunner apparatus was previously used in these laboratories; it consisted of a platform which was lowered into a beaker of dissolution fluid and had a centrally located well in the platform directly under the stirrer. This device, known as the "fixed geometry apparatus," was developed by Mr. Larry Kirschner, Dr. Thomas Simon, and Mr. William Campbell. Patents are pending.

[2] Standard Servodyne Controller, Cole-Parmer, Catalog No. 4420.

[3] "Quick-Action," Scientific Glass Apparatus, Catalog No. S-9014.

[4] Avicel PH 101, FMC Corp., Marcus Hook, PA 19061

[5] Groco 55-F powder, A. Gross & Co., New York, N. Y.

[6] Sterotex HM, The Capital City Products Co., Columbus, Ohio.

Table I—Disintegration Times of Capsule Formulas Tested in the New Apparatus under Various Test Conditions[a]

Disintegration Media:	No Lubricant				1.0% Magnesium Stearate			
	Distilled Water		Artificial Gastric Fluid		Distilled Water		Artificial Gastric Fluid	
Stirring Speed, r.p.m.: Formula	40	75	40	75	40	75	40	75
25% Microcrystalline cellulose	5	5	3	3	>30	>30	>30	>30
25% Microcrystalline cellulose with 0.2% sodium lauryl sulfate	5	4	3	2	>30	>30	3	5
50% Microcrystalline cellulose	5	3	3	2	>30	>30	>30	>30
50% Microcrystalline cellulose with 0.2% sodium lauryl sulfate	4	4	4	2	>30	>30	4	4
25% Starch	15	7	9	4	>30	>30	>30	>30
25% Starch with 0.2% sodium lauryl sulfate	14	9	7	4	>30	>30	>30	15
50% Starch	6	8	7	10	>30	12	15	8
50% Starch with 0.2% sodium lauryl sulfate	6	8	9	9	24	14	12	10

[a] Time in minutes.

The two-piece hard gelatin capsules[7] used were: No. 0 green opaque, No. 1 clear, No. 1 pink, No. 1 orange opaque, No. 1 two-tone blue opaque, No. 2 pink opaque, No. 3 yellow and gray opaque, and No. 4 pink.

General Procedure for Capsule Disintegration and Dissolution Studies—The proper size platform is selected for the capsule size, and the capsules are inserted into the wells, caps down, with a slight twisting action. After all capsules are in place, the platform is inverted to determine that the capsules are secured. The platform is slowly lowered into the apparatus containing 600 ml. of preheated test fluid and guided into the beaker well. The stirrer, premeasured to the proper height, is lowered and stirring is initiated. The capsule disintegration test is run until the capsule has substantially disintegrated or for a maximum of 30 min.

General Procedure for Tablet Disintegration and Dissolution Studies—The appropriate platform is fixed in the beaker well, 600 ml. of preheated test fluid is added, and the tablets are dropped into the bath and positioned, one in each well.

Preliminary Disintegration Testing in Tablet and Capsule Disintegration and Dissolution Apparatus—A formula containing 1.3% D&C Red No. 2 in lactose was hand filled into No. 1 clear gelatin capsules. Six capsules were placed in the apparatus and disintegrated in distilled water (37°) at 40 r.p.m. The medium was observed for a uniform distribution of color.

To test positional effects, a formula containing equal amounts of microcrystalline cellulose and lactose was encapsulated by hand into No. 1 red opaque capsules. The fill weight was 300 mg., and six capsules were disintegrated in distilled water (37°) using a 40-r.p.m. stirring speed.

Formulation Variables—Disintegration tests on capsules having explicit formula variations were run in the new apparatus. The factors studied were: (*a*) level of microcrystalline cellulose, 25 and 50%; (*b*) level of starch, 25 and 50%; (*c*) level of magnesium stearate, 0 and 1%; and (*d*) level of sodium lauryl sulfate, 0 and 0.2%.

In each formula, the disintegrant was dry blended with lactose. Sodium lauryl sulfate and magnesium stearate were added through No. 60 bolting cloth. The powders were mixed thoroughly in a tumbling bottle and hand filled into No. 1 red opaque capsules. Duplicate samples of each formula were randomly tested in the new apparatus.

Test Condition Variables—The capsules employed in the formula variation study were tested in the new apparatus in distilled water and artificial gastric fluid without pepsin at 40 and 75 r.p.m. These conditions, together with the formulation variables, constituted a 2^6 factorial design.

Reproducibility of Apparatus—Duplicate runs were carried out on some capsules, described under formulation variables, to demonstrate the reproducibility of the apparatus at the various test conditions. Two formulas were randomly positioned in the platform for simultaneous testing.

Comparision of New Method to USP Method—The hand-filled capsules prepared for the formula variation study were tested in the USP basket-rack assembly, with and without disks and using both distilled water and artificial gastric fluid.

Disintegration Comparison of Machine-Filled Capsules *versus* Hand-Filled Capsules—Nine formulations were filled by hand and on a semiautomatic capsule filling machine[8]. These contained stearic acid, hydrogenated vegetable oil, or magnesium stearate at three levels: 0.5, 1.0, and 2.0%. In each case, the lubricant was added to a lactose diluent through a No. 60 bolting cloth and mixed well. A No. 1 pink capsule was used, and a fill weight of 450 mg. was used for all of these experiments. The capsules were disintegrated in the new apparatus and by the USP method with and without disks.

***In Vitro* Capsule and Tablet Disintegration/Dissolution Study**—Four capsules and two tablets, each containing 10 mg. of a slightly water-soluble tranquilizer drug, were tested in the new apparatus. Variations in the capsule formulas were: (*a*) lubricant, magnesium stearate or stearic acid; (*b*) micronized *versus* unmicronized drug; and (*c*) wet process (*i.e.*, adding drug from solution) *versus* dry process. The method of preparation of the four different formulas was as follows:

Capsule A—The drug was passed through a No. 30 mesh screen and blended well with lactose. Three and one-half percent magnesium stearate was added to the formulation through a No. 60 bolting cloth and mixed well. The capsules (No. 1 orange opaque) were filled on the semiautomatic machine at 480 mg.

Capsule B—The drug was passed through a No. 30 mesh screen and blended with lactose. After sufficient mixing, the formulation was passed through a No. 40 mesh screen. An equivalent of 1.5% stearic acid was added to the blend through a No. 60 bolting cloth and mixed well. The formulation was hand filled into No. 1 two-tone blue opaque, hard gelatin capsules to 420 mg. fill weight.

Capsule C—The drug and 0.2% sodium lauryl sulfate (based on final granulation weight) were dissolved in an appropriate amount of warm isopropanol (50°) to allow for proper distribution on the lactose. The solution was added to the lactose and mixed well. The granulation was dried at 25° overnight and then passed through a No. 40 mesh screen. Two percent stearic acid was added through a No. 60 mesh bolting cloth and mixed well. The powders were hand filled into No. 1 two-tone blue opaque, hard gelatin capsules to a 400 mg. fill.

Capsule D—This formulation was prepared in the same way as Capsule B, except that micronized drug was used.

The tranquilizer tablets were randomly sampled from two different batches of the same formula. The method of tablet preparation was as follows.

Initially, the drug was milled in a Fitzpatrick comminuting machine using a 0.79-mm. round hole screen, impact forward at high speed. A color blend was prepared by micropulverizing 20% of the lactose with the starch and magnesium stearate. This blend was

[7] All manufactured by Parke-Davis, Division of Warner-Lambert Co.

[8] Parke-Davis.

Table II—Reproducibility of Capsule Disintegration Times in the New Apparatus[a]

Formula	Disintegration Time, min., in Distilled Water, 40 r.p.m. $\bar{X}$[b]	40 r.p.m. R[c]	75 r.p.m. $\bar{X}$[b]	75 r.p.m. R[c]
25% Starch	15.0	0	7.0	2
25% Starch, 0.2% sodium lauryl sulfate, 1.0% magnesium stearate	>30	—	>30	—
50% Starch, 0.2% sodium lauryl sulfate	5.5	1	8.0	2
50% Starch, 0.2% sodium lauryl sulfate, 1.0% magnesium stearate	24.5	2	16.5	2

[a] All capsules contain lactose, hydrous, improved flow. [b] $\bar{X}$, average of four capsules. [c] R, range of four capsules.

passed through a 0.99-mm. round hole screen, impact forward at high speed. The drug was then mixed with microcrystalline cellulose, the remainder of the lactose, and the color blend, and was mixed for 30 min. The final powder blend used in Tablet B was milled a second time through the same screen to obtain a better color distribution. Tablets A and B were the same weight, gauge, and hardness.

The *in vitro* test utilized only one capsule or tablet per study. The dosage form was tested in the new apparatus, using 400 ml. of artificial gastric fluid without enzymes at 37° and stirred at 75 r.p.m. During the dissolution study, samples were taken periodically, filtered, diluted, and observed spectrophotometrically at 238 nm. Placebo formulations were tested in the same way and used to correct the assay for interference caused by gelatin and excipients. The amount of drug dissolved at various times was calculated from the differential spectrophotometric values.

***In Vitro* and *In Vivo* Correlation of Phenylpropanolamine Hydrochloride Capsules**—A fast disintegrating capsule and a poor disintegrating capsule, each containing 75 mg. of phenylpropanolamine hydrochloride, were compared by testing disintegration, dissolution, and bioavailability.

In vitro testing of the two formulations was performed in the new apparatus with 600 ml. of artificial gastric fluid without enzymes at 75 r.p.m. Six capsules were used for each test. The solution was sampled periodically, filtered, and assayed by the method of Chafetz (5); the details are given below.

The preparations and an aqueous solution containing 75 mg. of phenylpropanolamine hydrochloride were administered to two healthy, fasting male subjects. These three formulations were each given, allowing 1 week between tests. The bladder was emptied approximately 1 hr. prior to taking the dosage form. The solution and capsules were taken with 120 ml. water. Immediately after administration, a urine blank was collected. Urine was collected for the 0–0.5, 0.5–1, 1–1.5, 1.5–2, 2–4, 4–6, and 6–24-hr. intervals and was refrigerated until assayed.

Assay Procedure for Phenylpropanolamine Hydrochloride—To a 60-ml. separator, add 2 ml. of urine, 1 ml. of saturated sodium carbonate solution, and 5 ml. of 5% sodium metaperiodate; mix well and let stand for 15 min. Extract the aqueous mixture with 15 ml. of hexane for 1 min. and discard the aqueous phase. Transfer the hexane to a 50-ml. stoppered centrifuge tube containing 1 g. of anhydrous sodium sulfate and shake. Determine the absorbance of the hexane phase spectrophotometrically at 241 nm. against a reagent blank.

Reagent Blank—Use 2 ml. of water to replace the 2 ml. of urine and proceed as above.

Preparation of Standard—Accurately weigh 1 g. of phenylpropanolamine hydrochloride into a 50.0-ml. volumetric flask and bring to volume with distilled water. Pipet 1 ml. of the solution to another 100-ml. volumetric flask and bring to volume with distilled water. Use 2 ml. of this solution instead of 2 ml. of urine and proceed as above.

RESULTS AND DISCUSSION

The new apparatus has been used successfully for disintegration and dissolution testing of capsules and tablets. The apparatus is

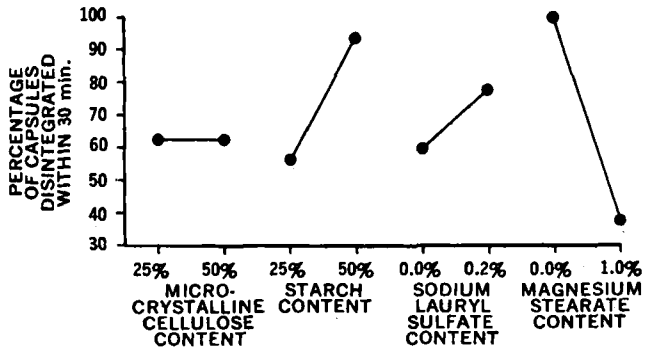

Figure 2—*Relative effects of formula variations tested in the tablet and capsule disintegration and dissolution apparatus.*

easily adapted for any size soft or hard gelatin capsule or tablet by changing the platform to accommodate the size and shape of the dosage form. The observation of either six capsules or four tablets statically positioned under mild agitation allows for disintegration comparisons to be made rapidly and accurately. Dissolution tests are run by removing fluid manually or automatically through the sampling ports.

During capsule disintegration, the top of the gelatin capsule often opens in about 2 or 3 min. and air is released from the capsule. A fast release of air bubbles is usually indicative of an immediate wetting and, consequently, is a first indication of a good disintegrating formula. If the capsule contents are wetted, the gelatin capsule itself is weakened by the test medium and the capsule will collapse. Occasionally, it is difficult to determine when the disintegration is complete. In this work, when the top half of the capsule had disintegrated, the test was judged to be complete.

Some gelatin capsule shells dissolve completely without collapse of the formulation because the capsule contents are not wetted. This capsule-shaped "plug" is often seen when testing formulas containing magnesium stearate. The disintegration test can be run for any length of time; however, in this work each study was run for a maximum of 30 min. Generally, any formula not disintegrating in 15 min. is considered unsatisfactory.

Preliminary Evaluation of Tablet and Capsule Disintegration and Dissolution Apparatus—Six capsules incorporating a dye showed a quick and uniform distribution of color in the new apparatus at a stirring speed of 40 r.p.m. This uniform flow pattern indicated that sampling can be made from any position in the beaker above the stirrer.

Capsules containing 50% microcrystalline cellulose and 50% lactose were used to test the reproducibility of all six capsule positions. All capsules disintegrated between 3 and 5 min. The wetting of the capsule formula was delayed for about 2 min. until the top of the capsule erupted. After the capsules were wetted, they collapsed within 2 min. and disintegrated to a mound within the next minute. All six positions of the sampling platform yielded similar results. The disintegration differences observed were due to individual capsule variations.

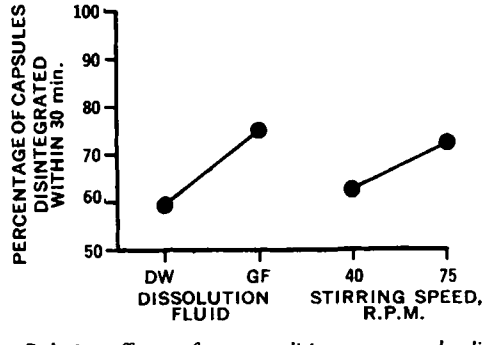

Figure 3—*Relative effects of test conditions on capsule disintegration in the tablet and capsule disintegration and dissolution apparatus. Key: DW, distilled water, and GF, artificial gastric fluid without enzymes.*

Table III—Comparative Disintegrations of Capsule Formulas Tested in the New Apparatus and the USP Basket-Rack Assembly[a,b]

Formula	Disintegration Time, min. — No Lubricant — New Apparatus	No Lubricant — USP — With Disks	No Lubricant — USP — Without Disks	1.0% Magnesium Stearate — New Apparatus	1.0% Magnesium Stearate — USP — With Disks	1.0% Magnesium Stearate — USP — Without Disks
25% Microcrystalline cellulose	5	2	2	>30	4	45
25% Microcrystalline cellulose with 0.2% sodium lauryl sulfate	4	2	2	>30	4	25
50% Microcrystalline cellulose	3	2	3	>30	4	>35
50% Microcrystalline cellulose with 0.2% sodium lauryl sulfate	4	2	3	>30	5	15
25% Starch	7	4	5	>30	8	>40
25% Starch with 0.2% sodium lauryl sulfate	9	3	4	>30	8	>40
50% Starch	8	3	7	12	5	11
50% Starch with 0.2% sodium lauryl sulfate	8	3	5	14	5	10

[a] Fluid was distilled water. [b] New apparatus stirring speed was 75 r.p.m.

Table IV—Comparative Disintegrations of Hand-Filled and Machine-Filled Capsules in the New Apparatus and USP Basket-Rack Assembly

Lubricant	Disintegration Time, min.[a] — Hand-Filled Capsules — New Apparatus	Hand-Filled Capsules — USP — With Disks	Hand-Filled Capsules — USP — Without Disks	Machine-Filled Capsules — New Apparatus	Machine-Filled Capsules — USP — With Disks	Machine-Filled Capsules — USP — Without Disks
0.5% Stearic acid	4	2	3	4	2	3
1.0% Stearic acid	4	2	3	4	2	3
2.0% Stearic acid	4	2	3	4	3	4
0.5% Hydrogenated vegetable oil[b]	3	2	3	4	2	3
1.0% Hydrogenated vegetable oil[b]	3	2	3	4	2	4
2.0% Hydrogenated vegetable oil[b]	3	2	3	>30	3	16
0.5% Magnesium stearate	>30	3	18	>30	7	16
1.0% Magnesium stearate	>30	5	>30	>30	11	19
2.0% Magnesium stearate	>30	6	>30	>30	22	25

[a] Average of four determinations. [b] Sterotex HM.

Duplicate runs were made on selected capsules of the formulation variables study shown in Table I. The disintegration times of the capsules retested were observed throughout the 30-min. test period. The average disintegration times and the range of four observations are shown in Table II. The disintegration times were rounded off to the nearest minute. The results can vary ±0.5 min. from the average for any observation. In Table II the largest range for any average disintegration time is 2 min. Factors such as the condition of the gelatin capsules, the composition of the capsule formula, the compaction forces during filling, or the testing conditions can affect the reproducibility of the test.

Formulation Variables—The effects of these factors are illustrated in Table I and Fig. 2. The formulation effects illustrated in Fig. 2 indicate that the major factor affecting capsule disintegration is the absence or presence of magnesium stearate. All formulations without 1% magnesium stearate disintegrated within 16 min., while only 38% of those with the lubricant disintegrated within 30 min. Capsule studies by Samyn and Jung (2) in the USP apparatus without disks also showed retarded disintegration and dissolution rates in formulations containing magnesium stearate. This finding was attributed to poor wetting of the formulation due to the hydrophobic lubricant. The addition of a wetting agent, namely sodium lauryl sulfate, improved the disintegration of the capsules when tested in artificial gastric fluid without enzymes. There was an overall increase from 59 to 78% in the capsule disintegrations due to the presence of sodium lauryl sulfate. It is notable that this surfactant allowed disintegration in gastric fluid of magnesium stearate-containing formulas, but disintegration in water was still slow.

The disintegrants used in this study, that is, starch and microcrystalline cellulose, provide fast disintegration of formulations containing no lubricant. The capsules containing microcrystalline cellulose disintegrated, on the average, in 4 min., while those containing starch disintegrated in 8 min.

A further effect of each disintegrant can be seen when capsules containing magnesium stearate are considered. Sixty-two percent of all the capsules containing 25% microcrystalline cellulose disintegrated within 30 min. The same percentage disintegration was obtained for capsules containing 50% microcrystalline cellulose. An increase in the level of starch from 25 to 50% provided better disintegration; the percentage of disintegrations increased from 56 to 93%. In formulas containing 1% magnesium stearate, starch has been shown to be a better disintegrant than microcrystalline cellulose.

Test Condition Variables—The comparison of distilled water *versus* artificial gastric fluid without enzymes and 40 r.p.m. *versus* 75 r.p.m. is shown in Table I and Fig. 3. Seventy-five percent of the capsule formulations disintegrated in artificial gastric fluid, with only 59% disintegrating in distilled water. Seventy-two percent of the capsules disintegrated at 75 r.p.m., whereas only 63% disintegrated at 40 r.p.m. There is a mound formation following the capsule disintegration at 40 r.p.m. that is not recommended for dissolution studies. However, at 75 r.p.m. the capsule disintegration test is satisfactory and the agitation is sufficient to disperse the disintegrated portion of the capsule in the bath.

Comparison of New Method to USP Method—According to the data reported in Table III, the USP method with disks yielded disintegration times that were much faster and less discriminating than those obtained by the new method or the USP method without disks. This was especially true for formulas containing magnesium stearate. Dissolution studies by Withey and Mainville (6) on chloramphenicol capsules in a modified USP apparatus were too rapid for proper differentiation between the various formulations. Using the USP method with disks, all capsule formulas without magnesium stearate disintegrated within 4 min., while those with magnesium stearate disintegrated between 4 and 8 min. The USP method without disks provides the longer disintegration times

Table V—Disintegration and Dissolution of Capsules and Tablets Containing a Water-Insoluble Tranquilizer Drug[a]

Formula	Method of Preparation	Disintegration Time, min.	50% Dissolution, min.
Capsule A	Dry blend, 2.5% magnesium stearate	>180	>180
Capsule B	Dry blend, 1.5% stearic acid	3	103
Capsule C	Drug added from alcoholic solution, 1.5% stearic acid	3	29
Capsule D	Dry blend of micronized drug, 1.5% stearic acid	3	20
Tablet A	Drug milled once	1	29
Tablet B	Drug milled twice	20	12

[a] Data obtained using the tablet and capsule disintegration and dissolution apparatus.

that differentiate between the fast and slow disintegrating formulations. However, a larger variation in disintegration times was obtained in testing some capsule formulas using this method.

Hand-Filled Capsules *versus* Machine-Filled Capsules—Three methods of disintegration testing showed major differences between uniformly hand-filled and machine-filled capsules (Table IV and Fig. 4). The results were expressed as the percentage of formulations that disintegrated within 30 min. The new apparatus was the most sensitive to the difference in filling method, while the USP method with disks was the least sensitive. Use of the USP method without disks gave results more closely aligned to the new apparatus. The shift toward longer disintegration for machine-filled capsules cannot be explained by bulk density differences since all capsules were prepared at a fill weight of 450 mg. If the new method or the USP method without disks is used as a criterion for the disintegration of machine-filled capsules, it can be noted in Table IV that 2% stearic acid and 1% hydrogenated vegetable oil are satisfactory lubricant levels. These formulations disintegrate in 4 min., while those containing 2% hydrogenated vegetable oil or magnesium stearate all exceed 30 min.

***In Vitro* Capsule and Tablet Disintegration/Dissolution Study**—The disintegration and dissolution comparison of capsule and tablet formulations tested in the tablet and capsule disintegration and dissolution apparatus is shown in Table V. The presence of 3.5% magnesium stearate in Capsule A retarded both the disintegration and dissolution of the water-insoluble tranquilizer from the formulation. Magnesium stearate, being a strong hydrophobic lubricant, does not allow the formulation to become wetted. After 3 hr. of testing, only 6% of the dose was released from the non-disintegrated capsule plug. The 3.5% magnesium stearate used in Capsule A was replaced with 1.5% stearic acid in Capsule B. The dry-blend Capsule B disintegrates quickly (3 min.) and has a dissolution half-life of 103 min.

Efforts to improve the dissolution of the tranquilizer capsules resulted in the formulation of Capsules C and D. In Capsule C, both the drug and sodium lauryl sulfate were added to the lactose diluent from alcoholic solution. This method of formulation allows the drug to be finely distributed onto the surface of the water-soluble lactose particles. Capsule C had a dissolution half-life of 29 min. This value is 3.5 times faster than the dissolution of Capsule B. The improved dissolution rate is probably due to the presence of a smaller drug particle size and the improved wetting of the drug by the addition of 0.2% sodium lauryl sulfate.

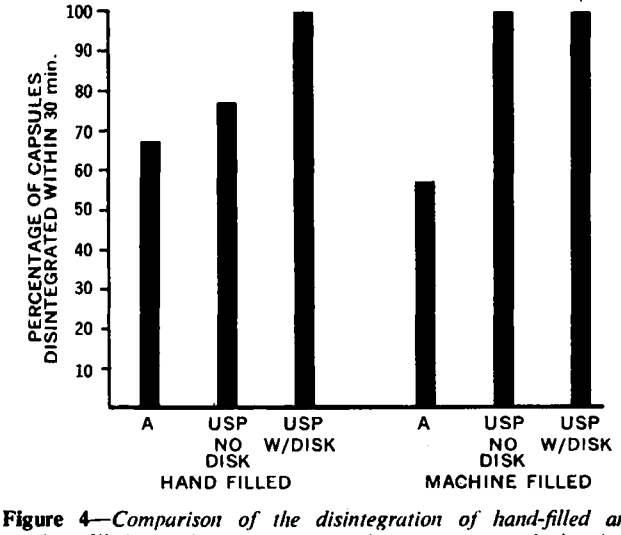

Figure 4—*Comparison of the disintegration of hand-filled and machine-filled capsules using various disintegration methods. A = new apparatus.*

Because of the low solubility of the drug in water (0.14 mg./ml.), the drug was micronized to increase the drug surface area available for dissolution. The micronized powder was then dry blended with lactose and stearic acid as in Capsule B. The only difference between Capsules B and D is that Capsule D contained finer drug particles. Capsule D showed a dissolution half-life of 20 min., which was five times faster than that of Capsule B but only slightly faster than that of Capsule C.

The tablet disintegrations are not in rank order with the 50% dissolution results. Tablet A showed an immediate disintegration with a dissolution half-life of 29 min. Tablet B was the same formula as Tablet A, but in the preparation of Tablet B the powder blend was milled a second time to obtain better color distribution. Tablet B disintegrated much slower than Tablet A but, nevertheless, dissolved much faster. Tablet B had a 50% dissolution of 12 min., which was 2.5 times faster than that of Tablet A. The improved dissolution rate in Tablet B is again attributed to the smaller drug particle size. The unexpectedly long disintegration time of Tablet B can only be explained by an improvement in the binding capacity of smaller and more homogeneously mixed excipients. The lack of a rank order correlation between the disintegration and dissolution data contradicts the general assumption that fast disintegration is necessary for fast dissolution. Since disintegration is easy to test, the formulator usually makes this the first objective and runs dissolution studies afterward. It is logical to assume that fast disintegrations can improve dissolutions by making the drug available for dissolution quickly, but this is not always true. Satisfactory dissolution rates can be obtained from dosage forms with slow disintegrations and, furthermore, improved disintegrations may or may not improve dissolution.

***In Vitro* and *In Vivo* Correlation of Phenylpropanolamine Hydrochloride Capsules**—The disintegration and dissolution data shown in Table VI demonstrate the *in vitro* differences obtained with the new apparatus for the two capsules. Capsule B disintegrated in 3 min., and 94% of the phenylpropanolamine hydrochloride was dissolved in 30 min. Capsule A, containing 5% magnesium stearate,

Table VI—Dissolution of Phenylpropanolamine Hydrochloride Capsules in the New Apparatus[a,b]

Time, min.	Cumulative Percentages of Drug Release Capsule A	Capsule B
3	4.6	11.3
5	8.7	27.0
10	21.1	67.6
15	28.2	84.5
30	44.9	94.0
60	66.6	—
90	80.9	—
120	87.9	—

[a] Dissolution fluid was 600 ml. of artificial gastric fluid without enzymes, and stirring speed was 75 r.p.m. [b] Disintegration times were: Capsule A, >2 hr.; and Capsule B, 3 min.

Table VII—Comparison of Cumulative Percentages of Phenylpropanolamine Hydrochloride Excreted in the Urine[a]

Dosage Form	Subject	Time, hr. 0.5	1	1.5	2	4	6	24
Solution	1	1.6	7.7	—	17.8	38.0	52.0	93.9
Solution	2	0.5	8.7	—	20.0	38.3	49.9	93.4
Solution	Average	1.1	8.2	—	18.9	38.2	51.0	93.6
Capsule A	1	0.0	1.3	4.4	11.4	35.9	50.5	90.5
Capsule A	2	0.0	2.6	5.5	12.5	30.7	46.4	89.4
Capsule A	Average	0.0	2.0	5.0	12.0	33.3	48.4	90.0
Capsule B	1	0.2	6.3	15.2	22.4	43.3	58.7	94.8
Capsule B	2	0.0	4.1	8.4	20.5	36.5	49.1	86.1
Capsule B	Average	0.1	5.2	11.8	21.4	39.9	53.9	90.4

[a] All dosages contained 75 mg. of drug.

did not disintegrate but released 88% of the drug in 2 hr. Capsule B had a dissolution half-life about 4.5 times faster than Capsule A. This illustrates the large difference between the capsule dissolution rates, even for a highly water-soluble drug.

Bioavailability data are shown in Table VII as the cumulative percent of phenylpropanolamine hydrochloride excreted in the urine *versus* time. A solution containing the same dose was used as a standard. The urine data showed a lag time in the excretion from both capsules in the 30-min. sampling. However, after the first interval, significant differences in the amounts of phenylpropanolamine hydrochloride excreted were apparent. There was a delay in the urinary excretion of Capsule A through the first 6 hr. of the study. The largest difference in the excretion data of the two capsules occurred in the 1.5–2-hr. interval, where 21.4% was excreted from Capsule B and only 12.0% from Capsule A. After 24 hr., the average cumulative percent of drug excreted from both Capsules A and B was similar, that is about 90% of the dose was excreted.

The rank order correlation between the *in vitro* and *in vivo* data demonstrates how the new apparatus can be used effectively in the evaluation of capsule formulations. In this situation with phenylpropanolamine hydrochloride capsules, the extent of the *in vitro* dissolution differences was evaluated through *in vivo* testing and was found to be significant.

CONCLUSION

The tablet and capsule disintegration and dissolution apparatus has been shown to be a useful tool for characterizing the disintegration and dissolution properties of capsule and tablet formulations. Although the explicit formula variation gave noticeable *in vitro* differences, biological testing is necessary to determine the extent of these differences. The phenylpropanolamine hydrochloride *in vivo* studies clearly demonstrate that careless capsule formulation can result in retarded *in vivo* drug availability, and the new apparatus can provide data that will lead the researcher to improving the formulation for better *in vivo* performance.

Wagner (7) proposed three requirements for an apparatus that qualifies it for both research and quality control purposes. The tablet and capsule disintegration and dissolution apparatus satisfies these requirements in that it is inexpensive, scientifically realistic, versatile, and has good precision. Wagner (8) also pointed out that a universal dissolution test is desirable but largely impractical, since each drug must be handled on an individual basis. However, the new apparatus offers flexibility which enables the formulator to alter the test conditions for any drug in a solid dosage form. After the *in vitro* test conditions are correlated with *in vivo* data, the new apparatus with its established testing specifications can be subsequently used in quality control laboratories for testing batches of either tablets or capsules.

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Selection of Best pH Range for Extraction of Amine–Bromthymol Blue Complexes

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Abstract □ This paper describes the determination of the best pH range for the extraction of amine–bromthymol blue complexes with chloroform. The experimental data on eight amine salts indicate that the best pH range is about 5.2–6.4 for a greater sensitivity in the analytical technique. A theoretical equation was derived and solved using a computer. The experimental results are in fairly good agreement with the theoretical results.

Keyphrases □ Amine–bromthymol blue complexes, extraction—theoretical and experimental determination of optimum pH range □ Bromthymol blue complexes with amines, extraction—theoretical and experimental determination of optimum pH range □ Extraction of amine–bromthymol blue complexes—determination of optimum pH range, theoretical, experimental □ pH range, extraction of amine–bromthymol blue complexes—theoretical and experimental determination

The variation in the amount of amine–dye complexes extracted from an aqueous solution by an organic solvent is dependent on the pH of the aqueous phase (1, 2). The selection of pH for the quantitative determination of amines has been arbitrary (3). Eight amine salts, atropine sulfate (I), codeine phosphate (II), emetine hydrochloride (III), ephedrine hydrochloride (IV), pilocarpine nitrate (V), procaine hydrochloride (VI), quinine hydrochloride (VII), and strychnine sulfate (VIII), were investigated both theoretically and experimentally to determine the best pH range for the extraction of amine–dye complexes. Bromthymol blue (IX) was selected as the dye and chloroform as the organic solvent for the extraction of amine–bromthymol blue complexes since they have been reported to be the best for the analysis of amines (4). The purpose of this paper is to report these studies.

EXPERIMENTAL

Reagents and Materials—All chemicals and reagents used were USP, NF, or ACS grade. Bromthymol blue[1] was used as supplied without further purification. Procaine hydrochloride was purchased from one source[2], and all other amine salts (I–V, VII, and VIII) were purchased from another[3].

Preparation of Solutions—Buffered (0.05 *M* phosphate) aqueous solutions of bromthymol blue (1×10^{-4} *M*) of various pH values (3.0, 4.0, 4.6, 5.2, 5.8, 6.4, 7.0, 7.6, and 8.4) were prepared according to the procedure reported earlier (5). Aqueous solutions of various amine salts of various concentrations were prepared using a simple solution method.

Determination of Best pH Ranges—A 5.0-ml. quantity of the amine salt solution (10 mcg./ml.) was mixed with 5.0 ml. of the dye solution of appropriate pH value in a 125-ml. separator. A 10.0-ml. quantity of chloroform was added, and the mixture was shaken for 1 min. The polar and nonpolar phases were allowed to separate, and the organic phase was collected. The absorbance of the amine–IX complex in the organic phase was measured[4] at 420 nm., the wavelength of maximum absorbance. A blank was prepared by substituting 5.0 ml. of distilled water for 5.0 ml. of the amine salt solution. All extraction curves were similar to the ones reported earlier (1, 6–9) under different experimental conditions. The best pH ranges (maximum absorbance ±5%) are presented in Table I.

Table I—Best pH Ranges for Extraction of Various Amine–Bromthymol Blue Complexes

Amine Salt	Best pH Range, Experimental	Best pH Range, Theoretical
I	5.2–6.4	5.6–6.8
II	5.2–5.8	5.8–6.0
III	4.0–5.8	5.8–6.2
IV	5.2–6.4	6.0–6.6
V	5.2[a]	5.2–5.8
VI	5.2–5.8	5.8–6.0
VII	3.0–4.6	4.2–6.4
VIII	3.0–4.6	4.4–6.0

[a] No range; this appears to be the optimum pH.

Determination of Partition Coefficients of Various Amine Salts—A 5.0-ml. quantity of the aqueous solution of the amine salt of appropriate concentration (I, 2.0 mg./ml.; II, 0.2 mg./ml.; III, 0.1 mg./ml.; IV, 0.6 mg./ml.; V, 0.75 mg./ml.; VI, 0.01 mg./ml.; VII, 0.06 mg./ml.; and VIII, 0.02 mg./ml.) was mixed with 5.0 ml. of pH 7.0 phosphate buffer (0.05 *M*) in a 125-ml. separator. A 10.0-ml. quantity of chloroform was then added, and the mixture was shaken for 1 min. (same time as was given for the extraction of amine–IX complexes). The clear aqueous layer was collected and measured at the wavelength of maximum absorbance (I, 257.5 nm.; II, 284 nm.; III, 282 nm.; IV, 257 nm.; V, 239 nm.[5]; VI, 290 nm.; VII, 330 nm.; and VIII, 254 nm.) using a spectrophotometer[6]. A blank was prepared by mixing 5 ml. of distilled water with 5.0 ml. of the buffer solution. The initial reading of each mixture (equal volumes of buffer and the aqueous solution of the amine salt) was also recorded. The partition coefficients of various amines were then calculated according to the procedure reported (1) earlier for thiamine, except that the undissociated fractions of four amines (III, V, VII, and VIII) were determined using Eq. 1 instead of the Henderson equation since two pKb values for these amines were reported (10, 11) previously. The pKb values and the results are presented in Table II. Equation 1 is:

$$(F)_{Aua} = \frac{(OH^-)^3}{(OH^-)^3 + (OH^-)K_{b1} + K_{b1}K_{b2}} \qquad \text{(Eq. 1)}$$

where $(F)_{Aua}$ is the fraction of the undissociated amine in the aqueous phase.

[1] Eastman Organic Chemicals.
[2] Mallinckrodt Chemical Works.
[3] Merck & Co.
[4] Using a Bausch & Lomb Spectronic 20.
[5] Not the wavelength of maximum absorbance. No sharp peak was recorded.
[6] Beckman DK.

Table II—Partition Coefficients of Various Amine Salts

Amine Salt	Partition Coefficient	pKb Values Used (10, 11)
I	154.0	4.35
II	57.3	6.10
III	1252.0	5.73, 6.74
IV	2.7	4.64
V	2.1	7.2, 12.5
VI	262.7	5.2
VII	116.8	6.0, 9.89
VIII	145.3	6.0, 11.7

Determination of Equilibrium Constant (K)—The equilibrium constants, K, of the amine–IX complexes were determined using Eqs. 2 and 3. Equation 2 was used for 1:1 complexes and Eq. 3 for 1:2 (amine–dye) complexes. The K values of four amines (III, V, VII, and VIII) were determined using Eq. 3. The assumption of 1:2 (amine–dye) complexes for these amines appears to be reasonable and was reported (12) earlier. For the determination of the K values, the experimental data at pH 7.6, as recorded under the *Determination of Best pH Ranges*, were used. A pH value of 7.6 was preferred since at lower pH values the concentration of free dye in the organic phase could not be assumed to be zero. At a pH value higher than 7.6, the extraction of amine complexes was either very poor or nil. The equations are:

$$K = \frac{(C)_{co}}{(A)_{ua}(D)_{ua}} \qquad \text{(Eq. 2)}$$

$$K = \frac{(C)_{co}}{(A)_{ua}(D)_{ua}^2} \qquad \text{(Eq. 3)}$$

where $(C)_{co}$ is the concentration of the amine–IX complex in the organic phase in moles per liter, $(A)_{ua}$ is the concentration of the undissociated amine in the aqueous phase in moles per liter, and $(D)_{ua}$ is the concentration of the undissociated dye in the aqueous phase in moles per liter.

To determine the concentration of the complex in the organic phase, an absorbance value of 0.285 was shown to represent a 1.6×10^{-5} M concentration of the 1:1 complex (12) and a 8×10^{-6} M concentration of the 1:2 (amine–dye) complex (12). The concentrations of the amine and dye in water were calculated by difference. The undissociated fractions of the amines in the aqueous phase were determined according to the procedure reported above (Eq. 1 and the Henderson equation). The undissociated fraction of IX in the aqueous phase was determined according to the procedure reported earlier (5). The calculated equilibrium constant values are presented in Table III.

THEORETICAL

By assuming the following reaction, an equation was derived to calculate the amount of amine–IX complexes extracted by the organic solvent:

$$n(A)_{ua} + n(D)_{ua} = (A^{+n} - D^{-n})_o \qquad \text{(Eq. 4)}$$

where n represents the number of moles of each reacting. Assuming the above reaction and using the symbol $(C)_{co}$ for the concentration of the complex in the organic phase:

$$K = \frac{(C)_{co}}{(A)_{ua}^n(D)_{ua}^n} \qquad \text{(Eq. 5)}$$

since in both types of complexes the n value for amines is 1; therefore, the equation simplifies to:

$$K = \frac{(C)_{co}}{(A)_{ua}(D)_{ua}^n} \qquad \text{(Eq. 6)}$$

In Eq. 6, the value of n is 1 for 1:1 complexes (I, II, IV, and VI) and 2 for the other four amines (III, V, VII, and VIII).

By a process reported earlier (1), it can be shown that:

$$(A)_{ua} = \frac{(C)_A - (C)_{co}}{(P)_A + [1/(F)_{Aua}]} \qquad \text{(Eq. 7)}$$

Table III—Equilibrium Constant (K) Values of Various Amine–Bromthymol Blue (IX) Complexes

Amine–IX Complex	K Value
I–IX	2.32×10^{16}
II–IX	3.16×10^{13}
III–IX	2.87×10^{27}
IV–IX	1.22×10^{16}
V–IX	8.83×10^{24}
VI–IX	2.4×10^{14}
VII–IX	3.21×10^{27}
VIII–IX	1.10×10^{27}

where $(C)_A$ is the total concentration of the amine in both phases (free as well as in the complex), $(P)_A$ is the partition coefficient of the amine, and $(F)_{Aua}$ is the undissociated fraction of the amine in the aqueous phase and equals:

$$(F)_{Aua} = \frac{(OH^-)^2}{(OH^-)^2 + (OH^-)K_{b1} + K_{b1}K_{b2}} \qquad \text{(Eq. 8)}$$

If the amine has only one K_b value, K_{b2} equals zero and:

$$(D)_{ua} = \frac{(C)_D - n(C)_{co}}{(P)_D + [1/(F)_{Dua}]} \qquad \text{(Eq. 9)}$$

where $(C)_D$ is the total concentration of the dye in both phases (free as well as in the complex), $(P)_D$ is the partition coefficient of the dye, and $(F)_{Dua}$ is the undissociated fraction of the dye in the aqueous phase and equals:

$$(F)_{Dua} = \frac{(H^+)^2}{(H^+)^2 + (H^+)K_{a1} + K_{a1}K_{a2}} \qquad \text{(Eq. 10)}$$

With the values for $(A)_{ua}$ and $(D)_{ua}$ derived, the values of $(C)_{co}$ in Eq. 6 were determined at various pH values (3–9 at intervals of 0.2) using a computer[7].

To solve Eq. 6, the following information was used:

K_{a1} and K_{a2} = first and second acid dissociation constants of IX, respectively (5)
K_{b1} and K_{b2} = first and second base dissociation constants of the amines, respectively (Table II) (If the amine has only one K_b value, K_{b2} equals zero)
$(P)_A$ = partition coefficient value of the amine (Table II)
$(P)_D$ = partition coefficient value of the dye (5)
K = equilibrium constant (Table III)
$(OH^-) = \frac{10^{-14}}{(H^+)}$

All curves, as drawn by the computer[8], were similar to the one reported earlier (1) which was drawn using a slightly different equation. The best pH range (maximum extraction ±2%) is presented in Table I.

In the derivation of Eq. 5, all of the complex in the aqueous phase was assumed to be completely dissociated since calculations using a typical value of +1.0 for the dissociation constant of the complex gave negligible concentrations of the undissociated complex in water as compared with the concentrations in the organic phase. Also, to keep the model as simple as possible, the use of an average value for the partition coefficient of bromthymol blue was preferred in these calculations. The incorporation of the effect of pH on the partition coefficient into the model will shift the extraction curves slightly but will not affect the general conclusions of this study. For example, at pH 5.5, the average $(P)_D$ value is 1.27 times the experimental value (5). In the case of codeine phosphate, it will cause an error of about 8% in the concentration of $(C)_{co}$ extracted. At higher pH values, the error is of a smaller magnitude but opposite in sign. The net effect of this correction will shift the theoretical

[7] IBM 360/75.
[8] Using RANGEF and PLOTF plotting subroutines as described in memorandum number LS-30-1 from Triangle Universities Computation Center, Research Triangle Park, N. C. A complete computer program is available on request.

optimum to lower pH values and this will result in increased overlap of the theoretical and experimental optimum ranges.

DISCUSSION

Many authors have recommended (3) a pH range of 7–7.5 for the extraction of amine–IX complexes with chloroform or other organic solvents. The experimental results of these studies (Table I) indicate that the best pH range for these extractions is from 5.2 to 6.4 for 1:1 complexes (I, II, IV, and VI) and from 3.0 to 5.8 for 1:2 (amine–dye) complexes (III, V, VII, and VIII). The theoretical results (Table IV) indicate a best pH range of 5.6–6.8 for 1:1 complexes and of 4.2–6.4 for 1:2 complexes. The best pH range was determined from the maximum absorbance value ±5% for the experimental data and from the maximum theoretical extraction ±2% for the computer data. Since experimental data are not as precise as theoretical data, a wider range was allowed on experimental values. Both the experimental and the theoretical data (Tables I and IV) show that the arbitrarily reported pH range of 7–7.5 (3) is not the optimum for a maximum sensitivity in the assay technique. Nevertheless, sometimes it may be preferable to sacrifice the sensitivity in favor of eliminating the interferences from other ingredients such as in the biological systems. At too low pH values (about 3–4.6), the chances of forming hard-to-break emulsions are greater than at higher pH values. The blank absorbance value against pure chloroform is zero above a pH value of 6.4. At lower pH values, the blank increases with a decrease in the pH, becoming significantly high (0.04–0.05) below pH 5.

It appears that a pH range of about 5.2–6.4 is the best for the extraction of amine–IX complexes. For 1:2 (amine–dye) complexes, the lower value (5.2) should be preferred.

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Mississippi-Grown *Cannabis sativa* L. III: Cannabinoid and Cannabinoid Acid Content

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Abstract □ A procedure for the assay of acidic and nonacidic cannabinoids qualitatively and quantitatively by chemical fractionation prior to TLC and GLC analyses is reported. Various samples of foreign and domestic, wild and cultivated marijuana were analyzed. It was demonstrated that cannabinoids occur largely as acids, that these acids undergo decarboxylation upon storage, and that plants vary significantly in their cannabinoid composition due to heredity.

Keyphrases □ *Cannabis sativa* L.—analysis, acidic and nonacidic cannabinoids by chemical fractionation prior to GLC and TLC, hereditary plant composition and effect of storage on cannabinoid content □ Cannabinoids, acidic, nonacidic—analysis, chemical fractionation prior to GLC and TLC, hereditary plant composition, decarboxylation during storage □ Marijuana—analysis, acidic and nonacidic cannabinoids by chemical fractionation prior to GLC and TLC, hereditary plant composition, and effect of storage on cannabinoid content

The major cannabinoids and cannabinoid acids that occur naturally in the various strains of *Cannabis sativa* L. (marijuana) plants included in this investigation are Δ^9-tetrahydrocannabinol (I), its two corresponding acids Δ^9-tetrahydrocannabinoic acid A (II) and Δ^9-tetrahydrocannabinoic acid B (III), cannabidiol (IV), and its corresponding acid cannabidiolic acid (V) (1–3). It has been established that I is the principal psychotomimetic compound in marijuana (4). When marijuana is smoked, II and III undergo decarboxylation and are inhaled as I (5, 6). This is one reason why marijuana exerts higher activity when smoked than when eaten.

GLC is being used for the quantitative analysis of marijuana (7–9), but these procedures do not distinguish between I and its respective acids. The acids decarboxylate rapidly to I in the injection port. Thus, the data determined by GLC analysis represent I plus its two respective acids.

The Department of Pharmacognosy at the University of Mississippi is growing *C. sativa* L. in order to supply analyzed marijuana and marijuana extracts to the National Institute of Mental Health (NIMH) for

optimum to lower pH values and this will result in increased overlap of the theoretical and experimental optimum ranges.

DISCUSSION

Many authors have recommended (3) a pH range of 7–7.5 for the extraction of amine–IX complexes with chloroform or other organic solvents. The experimental results of these studies (Table I) indicate that the best pH range for these extractions is from 5.2 to 6.4 for 1:1 complexes (I, II, IV, and VI) and from 3.0 to 5.8 for 1:2 (amine–dye) complexes (III, V, VII, and VIII). The theoretical results (Table IV) indicate a best pH range of 5.6–6.8 for 1:1 complexes and of 4.2–6.4 for 1:2 complexes. The best pH range was determined from the maximum absorbance value ±5% for the experimental data and from the maximum theoretical extraction ±2% for the computer data. Since experimental data are not as precise as theoretical data, a wider range was allowed on experimental values. Both the experimental and the theoretical data (Tables I and IV) show that the arbitrarily reported pH range of 7–7.5 (3) is not the optimum for a maximum sensitivity in the assay technique. Nevertheless, sometimes it may be preferable to sacrifice the sensitivity in favor of eliminating the interferences from other ingredients such as in the biological systems. At too low pH values (about 3–4.6), the chances of forming hard-to-break emulsions are greater than at higher pH values. The blank absorbance value against pure chloroform is zero above a pH value of 6.4. At lower pH values, the blank increases with a decrease in the pH, becoming significantly high (0.04–0.05) below pH 5.

It appears that a pH range of about 5.2–6.4 is the best for the extraction of amine–IX complexes. For 1:2 (amine–dye) complexes, the lower value (5.2) should be preferred.

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Mississippi-Grown *Cannabis sativa* L. III: Cannabinoid and Cannabinoid Acid Content

ASAAD N. MASOUD▲ and NORMAN J. DOORENBOS*

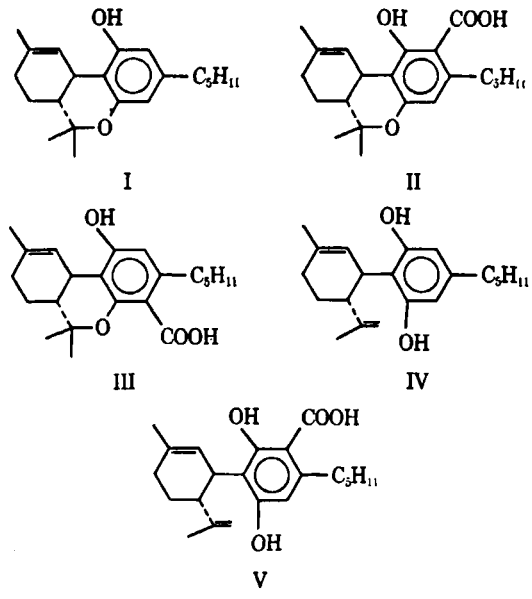
Abstract □ A procedure for the assay of acidic and nonacidic cannabinoids qualitatively and quantitatively by chemical fractionation prior to TLC and GLC analyses is reported. Various samples of foreign and domestic, wild and cultivated marijuana were analyzed. It was demonstrated that cannabinoids occur largely as acids, that these acids undergo decarboxylation upon storage, and that plants vary significantly in their cannabinoid composition due to heredity.

Keyphrases □ *Cannabis sativa* L.—analysis, acidic and nonacidic cannabinoids by chemical fractionation prior to GLC and TLC, hereditary plant composition and effect of storage on cannabinoid content □ Cannabinoids, acidic, nonacidic—analysis, chemical fractionation prior to GLC and TLC, hereditary plant composition, decarboxylation during storage □ Marijuana—analysis, acidic and nonacidic cannabinoids by chemical fractionation prior to GLC and TLC, hereditary plant composition, and effect of storage on cannabinoid content

The major cannabinoids and cannabinoid acids that occur naturally in the various strains of *Cannabis sativa* L. (marijuana) plants included in this investigation are Δ^9-tetrahydrocannabinol (I), its two corresponding acids Δ^9-tetrahydrocannabinoic acid A (II) and Δ^9-tetrahydrocannabinoic acid B (III), cannabidiol (IV), and its corresponding acid cannabidiolic acid (V) (1–3). It has been established that I is the principal psychotomimetic compound in marijuana (4). When marijuana is smoked, II and III undergo decarboxylation and are inhaled as I (5, 6). This is one reason why marijuana exerts higher activity when smoked than when eaten.

GLC is being used for the quantitative analysis of marijuana (7–9), but these procedures do not distinguish between I and its respective acids. The acids decarboxylate rapidly to I in the injection port. Thus, the data determined by GLC analysis represent I plus its two respective acids.

The Department of Pharmacognosy at the University of Mississippi is growing *C. sativa* L. in order to supply analyzed marijuana and marijuana extracts to the National Institute of Mental Health (NIMH) for



distribution to researchers who, in turn, will study the effects of these materials on animals and humans. It is essential that analytical methods distinguish between cannabinoids and cannabinoid acids in these research materials. This report includes the description of the first such procedures developed and the results of a comparative study of marijuana produced from several strains of *C. sativa* L.

EXPERIMENTAL

Materials—Mexican, Turkish, Swedish, Italian (Carmagnola, Fibranova), and French (Fibriman) *C. sativa* L. seeds were obtained in the spring of 1968 through NIMH. Marijuana produced from the plants grown from these seeds in the drug plant garden of the University of Mississippi in 1968 and 1969 was utilized in this study, along with samples of marijuana collected in September 1968 from wild plants growing in Iowa and Minnesota.

Extraction and Fractionation—One gram of plant material, finely manicured by passing through a 1-mm. sieve to remove seeds and stems and to reduce leaves, flowers, and bracts to small particles, was extracted by shaking overnight with 10 ml. of 95% ethanol. The ethanol was removed *in vacuo* at room temperature. The residue was dissolved in 20 ml. of a 1:1 mixture of petroleum ether and an aqueous solution of 2% sodium hydroxide and 2% sodium bisulfite. Cannabinoid acids dissolve in sodium hydroxide but not in sodium bicarbonate or sodium carbonate solutions. The sodium hydroxide solution does not extract the nonacid cannabinoids under these conditions, and they remain in the petroleum ether. Other workers (2, 10) reported similar results and attributed these observations to steric hinderance or to chelation between the carboxyl and neighboring oxygen functional groups. The very low water solubility of phenolic salts of the cannabinoids probably contributes to these observations. Sodium bisulfite was added to minimize oxidation. The petroleum ether layer was separated, washed with water, dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo* to obtain the nonacidic cannabinoids. The sodium hydroxide extract was acidified with cold 10% sulfuric acid, extracted with ether, washed with water, dried over anhydrous sodium sulfate, and evaporated *in vacuo* at room temperature to obtain the cannabinoid acids.

Table I—Δ^9-Tetrahydrocannabinol and Its Acids in Mexican Strain Marijuana

Percent of Dry Weight[a]	Immature 1969	Female 1969	Female 1968	Male 1969	Male 1968
I	0.22	0.16	0.57	0.45	0.50
II and III	1.00	1.00	0.43	0.71	0.10

[a] Each figure represents the average of two determinations that were within 10% variance.

Table II—Cannabinoids and Cannabinoid Acids in Foreign Strains of Fiber-Type Marijuana

Percent of Dry Weight[a]	Turkish Male	Turkish Female	Swedish	Carmagnola (Italian)	Fibriman (French)	Fibranova (Italian)
I	0.01	0.04	Trace	0.06	Trace	Trace
II and III	0.06	0.02	Trace	0.15	0.05	0.13
IV	0.52	0.44	0.03	0.08	0.08	0.21
V	0.60	0.80	0.04	0.10	0.12	0.45

[a] Each figure represents the average of two determinations that were within 10% variance.

GLC—Both acidic and nonacidic fractions were analyzed using an instrument[1] equipped with flame-ionization detectors and a 0.31-cm. × 3.1-m.(0.125- in. × 10-ft.) stainless steel column packed with 2% OV-17 on 100–120-mesh Gas Chrom Q. Helium was used as carrier gas at 40 ml./min. The oven temperature was kept at 210°, while the inlet and detector temperatures were 250 and 280°, respectively. A steroid internal standard (4-androstene-3,17-dione) was added to each extract. The instruments were calibrated with reference samples of I and IV. The areas under the peaks were directly correlated with the concentration of the compounds (11). The retention time of I was used for the analysis of I and its two respective acids. Similarly, the retention time of IV was used in the analysis of IV and V.

TLC—Each sample was examined by TLC to determine if the separation of acids and nonacids was complete.

Plates—Silica gel GF was coated[2] at a thickness of 0.25 mm. and was activated by warming at 105° for 1 hr.

Solvent Systems—Both acidic and nonacidic fractions were chromatographed in two solvent systems: System A, benzene; and System B, hexane–ether–acetic acid (87:12:1). In System A, the cannabinoids migrated and separated but the cannabinoid acids remained at the starting point. In System B, the acids migrated and separated. Thus, it was possible to tell the presence or absence of the acids as well as the number of acids in the acid fractions. Authentic reference samples of I or IV were always used on each plate.

Detection of Spots—All plates were viewed under a short wavelength UV lamp. All of the cannabinoid acids, as well as IV, quenched the fluorescent background of the plate and were easily detected. After locations of these spots were outlined, the plates were sprayed with freshly prepared diazotized benzedine reagent (12). This reagent yielded a characteristic yellow-orange color with IV, a red-orange color with I, and a brown color with the cannabinoid acids. The R_f values of I in Solvent Systems A and B averaged as follows: A = 0.42 and B = 0.36. The R_x values (x = I) of the other compounds in both systems were: II, A = 0.00 and B = 0.91; III, A = 0.00 and B = 0.86; IV, A = 1.19 and B = 1.11; and V, A = 0.00 and B = 0.64.

RESULTS AND DISCUSSION

Only one strain of the marijuana utilized for this study, the Mexican strain, is of the drug type (high in Δ^9-tetrahydrocannabinol and its acids). Data obtained from the analysis of five samples of this strain are presented in Table I.

The 1969 samples were 6 months old and the 1968 samples were approximately 18 months old at the time of analysis. The lower proportion of acid content in the 1968 samples compared with the 1969 samples suggests that the acids undergo decarboxylation upon storage.

The acidic fraction in System B showed two spots, indicating the presence of both II and III. This was further confirmed by the IR spectra of the acidic fraction and the methyl ester derivative prepared by reaction with diazomethane (2).

[1] Beckman GC-5 or GC-45.
[2] By a Desaga apparatus.

Table III—Cannabinoids and Cannabinoid Acids in Domestic[a] Marijuana

Percent of Dry Weight	Iowa I	Iowa II	Des Moines	Minnesota I	Minnesota II	Minnesota III
I	0.34	Trace	Trace	0.04	0.03	0.08
II and III	0.20	Trace	Trace	Trace	Trace	0.04
IV	0.14	0.38	0.60	0.11	0.07	0.63
V	0.25	0.33	0.33	0.27	0.33	0.11

[a] These samples, with the exception of Minnesota III, were collected in the wild in September 1968.

The other foreign strains of marijuana produced in the drug plant garden in 1969 were of the fiber type (low in Δ^9-tetrahydrocannabinol and high in cannabidiol). The results of the analyses of plants produced in 1969 from these strains are illustrated in Table II.

The cannabinoid acid content of each of these samples was higher than the corresponding cannabinoids. Although each of these marijuana samples was prepared from plants at about the same stage of maturity (flowering stage), the cannabinoid content varied considerably, the lowest being the Swedish strain which is almost devoid of cannabinoids.

Three samples of marijuana collected in Iowa and two samples collected in Minnesota (near Minneapolis) were compared with a sample of marijuana grown on campus in 1969 from seed found in the Minnesota I marijuana (Table III). With the exception of the Iowa I sample, which contained a moderately high quantity of the active I, II, and III, all other domestic varieties investigated contained mainly IV and V and were almost void of the active entities. The change of growing conditions provided to the Minnesota III sample did not change this pattern, *i.e.*, did not trigger the plant to produce active entities in any significant quantities.

SUMMARY AND CONCLUSIONS

A procedure was developed for the assay of the acidic and nonacidic cannabinoids, qualitatively and quantitatively, by chemical fractionation prior to GLC and TLC analyses. Various samples of foreign and domestic, wild and cultivated marijuana were analyzed. From the data obtained, the following were demonstrated.

1. Cannabinoids occur in nature as acids or nonacids with comparable abundance.
2. Tetrahydrocannabinoic acids undergo decarboxylation upon storage or exposure to heat.
3. Plants vary significantly in their cannabinoid composition due to heredity.
4. Change in environment does not change the cannabinoid pattern in plants.

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Mississippi-Grown *Cannabis sativa* L. IV: Effects of Gibberellic Acid and Indoleacetic Acid

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Abstract □ Four-week-old *Cannabis sativa* L. plants were treated with 1 ml. of gibberellic acid or indoleacetic acid at concentrations up to 250 p.p.m. The treatment was repeated at weekly intervals for 3 weeks. These plants were harvested 1 week after the last treatment. Gibberellic acid caused a significant increase in height and a decrease in the weight of leaves and Δ^9-tetrahydrocannabinol content. Indoleacetic acid produced no significant changes.

Keyphrases □ *Cannabis sativa* L.—effects of gibberellic and indoleacetic acids on plant growth and Δ^9-tetrahydrocannabinol content □ Δ^9-Tetrahydrocannabinol content in *Cannabis sativa* L.—effects of gibberellic and indoleacetic acids □ Gibberellic acid—effect on plant growth and Δ^9-tetrahydrocannabinol content of *Cannabis sativa* L. □ Indoleacetic acid—effect on plant growth and Δ^9-tetrahydrocannabinol content of *Cannabis sativa* L. □ Plant growth regulators—effects of gibberellic and indoleacetic acids on *Cannabis sativa* L.

Many interesting effects upon plants have been demonstrated in recent years with the gibberellins and other plant hormones. The general effects on growth are increased internodal elongation, resulting in taller and spindlier plants. Apical dominance, a growth feature attributed to auxins, is strongly enhanced by the gibberellins (1–3). Gibberellins are reported to decrease alkaloid concentrations in some species (4–6) and increase the concentration of sugars and glycosides in *Digitalis lanata* (7).

Few studies on the cultivation of *Cannabis sativa* L., especially of the drug type, have been reported. At the time this investigation was initiated, there were no reports of the effects of plant hormones on this species. During this study, a group reported (8) that gibberellic acid increased stem length, the percentage of male plants, and the length of the growing season and that a combination of gibberellic acid and colchicine delayed flowering about 20 days. No data on the effects upon yield or concentration of Δ^9-tetrahydrocannabinol (III) were presented, and data on the quantities of gibberellic acid (I) and colchicine used were not included in the abstract.

The objective of this investigation was to determine the effects of gibberellic acid (I) upon the growth and Δ^9-tetrahydrocannabinol (III) content of *C. sativa* L. Since it has been postulated that gibberellins act by increasing the diffusible auxin in plants (9), indoleacetic acid (II) was included in the study.

EXPERIMENTAL

Procedure—Plants employed in this study were of a Mexican strain[1] which is high in III and hence of the drug type (10). Seeds were cultivated in rows 1 m. in width. When the plants were 4 weeks old, they were thinned, allowing 50 cm. between plants.

The plants were divided into nine groups at random. Each group contained 20 plants. These groups included one control group, four kinds of treatments with I[2], and four kinds of treatments with II[2]. The four treatments with each growth substance were 25 p.p.m., 25 p.p.m. × *F*, 250 p.p.m., and 250 p.p.m. × *F*, in which *F* is a factor correlating the height of the plant at the time of application and its height at the time of the first application. One milliliter of the designated concentration of growth substance was applied by a pipet on the uppermost leaves of the treated plants. In the case of the *F* treatments, *F* equaled the number of milliliters applied. Height measurements were taken prior to the application of growth substances and then at weekly intervals. Plants were harvested 1 week after the third application (fourth measurement) which occurred before flowering.

At harvesttime, each plant was uprooted and the roots were thoroughly washed. Plants were divided into leaf, stem, and root portions. Fresh weights were determined immediately in the field. The respective plant parts were then wrapped separately in cheesecloth, marked, and placed in a warm air (50°) circulating dryer. Dry weights were determined. The dried leaves were pooled according to treatment, manicured through a 1-mm. sieve, and thoroughly mixed to a homogeneous mixture.

Analysis—Samples of the pooled manicured leaf samples were assayed for III. One gram of the leaf material was extracted by shaking overnight with 95% ethanol. The ethanolic extract was evaporated under reduced pressure and redissolved in 1 ml. of 95% ethanol containing 10 mg. of the internal standard, 4-androstene-3,17-dione. GLC analysis was carried out using an instrument[3] equipped with a flame-ionization detector and a stainless steel column 0.31 cm. × 3.1 m. (0.125 in. × 10 ft.) packed with 2% OV-17 on 100–200-mesh Gas Chrom Q. Helium was used as a carrier gas at 40 ml./min. The oven temperature was kept at 210°, while the inlet and detector temperatures were 250 and 280°, respectively. The areas under the peaks were directly correlated with the concentration of the compound using a calculated correction factor (11).

The samples were then spotted on silica gel GF plates, developed in two solvent systems [benzene and benzene–hexane–diethylamine (25:10:1)], and then sprayed with diazotized benzedine spray reagent for qualitative comparison of the cannabinoids present (12).

RESULTS

Remarkable changes in branching habit and height were observed in the plants treated with I (Fig. 1). In general, the branching characteristics of the Mexican strain, which is usually bushy with two or more main branches close to the base, demonstrated apical dominance. The plants acquired one main stem with longer internodes and fewer leaves. At the 25-p.p.m. concentration, the plants were healthy and the stems were strong enough for adequate support. At the higher concentrations of 25*F*, 250, and 250*F*, the plants were tall and spindly and had poor support. Furthermore, necroses occurred in the leaves of many of these latter plants.

Treatment with II did not produce any noticeable changes in growth pattern. *T* tests on uncorrelated data were carried out on the height measurements (Table I) and indicated that there was no difference between the groups before treatment, no difference after

[1] Second generation seed harvested in one of the marijuana gardens of the University of Mississippi were used. Original seed was collected near Acapulco, Mexico, and furnished by Dr. John Scigliano, National Institute of Mental Health.

[2] Gibberellic acid (10% potassium salt) and indoleacetic acid (highest purity) were purchased from LaPine Scientific Co., Chicago, Ill., Irwington, N. Y., and Berkeley, Calif.

[3] Beckman GC-5 or GC-45.

Figure 1—*Effect of indoleacetic acid and gibberellic acid on the height and branching habit of marijuana plants.*

treatment with various concentrations of II, and a significant increase in treatments with I utilizing the 0.05 significance level.

Weight data of all plant parts were analyzed statistically using the same test (Table II). There was no effect due to the treatments with II. Treatments with I showed a significant decrease in the fresh and dry weights of the leaves at the 0.05 significance level.

It was unexpected not to find an increase in the stem weights. This indicated that there was no actual increase in the stem growth, but that rather the habit of growth was changed from bushy to tall nonbranched.

The concentration of 1.04% of dry weight of III in the control was considered as the population mean (Table III). The standard deviation of this population was found to be 0.1% of dry weight, since this represented the limitations of the sensitivity of the procedure and sampling techniques. Utilizing the above parameters and the significance level of 0.05, the concentration of III of the treated plants was tested for significance. There was a significant decrease in the two factorial treatments of I. These data agree also with the dry weight and height data. The *F* treatments caused a more pronounced effect than expected. This could be interpreted as due to the larger volume of applied solution which is more evenly distributed and better absorbed in the plant and, consequently, acts on more sites producing more pronounced effects.

TLC revealed no qualitative changes in the cannabinoids present in the plants.

DISCUSSION AND CONCLUSIONS

A typical gibberellin effect characterized by increased internodal elongation, apical dominance, and taller plants was noted. Compound I also caused a decrease in the fresh and dry weights of the

Table I—Height[a] of Plants

Elapsed Days between Measurements	Control	I 25	I 25*F*	I 250	I 250*F*	II 25	II 25*F*	II 250	II 250*F*
0	45	46	45	39	38	38	38	42	39
7	64	75	77	67	65	56	52	59	58
14	83	116	128	99	106	76	72	77	74
21	125	167	185	156	176	124	116	118	119

[a] Average in centimeters per group of 20 plants.

Table II—Weights[a] of *C. sativa* Plant Parts[b]

Treatment	Leaves		Stems		Roots		Total Weight/Plant	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Control	303.8	89.0	255.5	62.8	71.8	15.4	631.1	165.5
I 25	176.6	55.8	258.0	67.8	58.4	12.4	497.0	135.7
I 25*F*	133.9	44.4	261.4	66.3	53.7	11.2	448.9	119.3
I 250	139.3	47.1	216.8	50.35	52.3	9.5	408.0	106.9
I 250*F*	113.5	41.1	268.0	61.1	50.6	11.0	436.2	111.4
II 25	270.6	82.6	236.9	60.8	73.0	14.1	574.2	157.6
II 25*F*	237.5	74.6	207.3	47.98	55.8	10.92	496.3	134.6
II 250	286.8	87.8	247.7	58.8	66.0	13.7	600.1	160.4
II 250*F*	291.5	87.0	247.2	60.8	63.8	13.7	601.2	161.8

[a] In grams. [b] Average of 20 plants.

Table III—Concentration[a] of Δ^9-Tetrahydrocannabinol

	Gibberellic Acid				Indoleacetic Acid			
Control	25	25F	250	250F	25	25F	250	250F
1.04	0.90	0.60	0.89	0.65	0.78	0.96	0.90	0.91

[a] Average percent dry weight of two samples.

leaves and in the content of III. There was no effect on the stem weight, which suggests that the significant increase in height was due to a change in the growing habit from a branching plant to one main stem.

The concentrations of II used were not high enough to induce any effect on the plants. Similar results were observed on *Hyoscyamus niger* L., where concentrations of 10 and 50 p.p.m. of I induced significant effects on rosette plants while concentrations up to 500 p.p.m. of II applied in the same manner failed to induce any stem elongation.

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Kinetics of Hydrolysis of Hypoglycemic 1-Acyl 3,5-Dimethylpyrazoles

ARLINGTON A. FORIST▲ and DENNIS J. WEBER

Abstract □ Other investigators suggested that various 1-acyl 3,5-dimethylpyrazoles might owe their hypoglycemic activity to a nonenzymatic hydrolysis *in vivo* to the potent compound 3,5-dimethylpyrazole. As a test of this hypothesis, relative rates of hydrolysis at pH 2.0 and 6.7 (37.6°) were determined for a representative series of compounds covering a wide range of hypoglycemic potencies. No correlation between hydrolysis rate and activity was observed. 3,5-Dimethylpyrazole-1-carboxamide and 3,5-dimethylpyrazole-1-*N*,*N*-dimethylcarboxamide possess equivalent biological activity; the former was rapidly hydrolyzed (half-life about 1 hr. at pH 2.0 and 6.7), whereas the latter was totally stable. Differences in biological activity reflect intrinsic potencies of the various compounds or differences in their absorption and/or metabolism.

Keyphrases □ 3,5-Dimethylpyrazoles, 1-acyl series—kinetics of hydrolysis, hydrolysis rate–hypoglycemic activity correlation □ Hypoglycemic activity–hydrolysis rate correlation—1-acyl 3,5-dimethylpyrazoles, kinetics of hydrolysis □ Hydrolysis rates—1-acyl 3,5-dimethylpyrazole series, correlated with hypoglycemic activity, kinetics

Wright *et al.* (1) reported the hypoglycemic activities of a large number of pyrazoles in the intact, fasted, glucose-primed rat. The most active compounds were 3,5-dimethylpyrazole (I) (2) and various 1-acyl derivatives. Preliminary experiments showed that 3,5-dimethylpyrazole-1-carboxamide (II) was readily hydrolyzed to I at pH's encountered *in vivo* in the GI tract and the blood. As a consequence, it was suggested that the various 1-acyl derivatives of I might owe their activities either to nonenzymatic hydrolysis *in vivo* or to metabolic transformation to I (1). To test the hypothesis of nonenzymatic conversion, relative rates of

Table III—Concentration[a] of Δ^9-Tetrahydrocannabinol

	Gibberellic Acid				Indoleacetic Acid			
Control	25	25F	250	250F	25	25F	250	250F
1.04	0.90	0.60	0.89	0.65	0.78	0.96	0.90	0.91

[a] Average percent dry weight of two samples.

leaves and in the content of III. There was no effect on the stem weight, which suggests that the significant increase in height was due to a change in the growing habit from a branching plant to one main stem.

The concentrations of II used were not high enough to induce any effect on the plants. Similar results were observed on *Hyoscyamus niger* L., where concentrations of 10 and 50 p.p.m. of I induced significant effects on rosette plants while concentrations up to 500 p.p.m. of II applied in the same manner failed to induce any stem elongation.

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Kinetics of Hydrolysis of Hypoglycemic 1-Acyl 3,5-Dimethylpyrazoles

ARLINGTON A. FORIST▲ and DENNIS J. WEBER

Abstract □ Other investigators suggested that various 1-acyl 3,5-dimethylpyrazoles might owe their hypoglycemic activity to a nonenzymatic hydrolysis *in vivo* to the potent compound 3,5-dimethylpyrazole. As a test of this hypothesis, relative rates of hydrolysis at pH 2.0 and 6.7 (37.6°) were determined for a representative series of compounds covering a wide range of hypoglycemic potencies. No correlation between hydrolysis rate and activity was observed. 3,5-Dimethylpyrazole-1-carboxamide and 3,5-dimethylpyrazole-1-*N*,*N*-dimethylcarboxamide possess equivalent biological activity; the former was rapidly hydrolyzed (half-life about 1 hr. at pH 2.0 and 6.7), whereas the latter was totally stable. Differences in biological activity reflect intrinsic potencies of the various compounds or differences in their absorption and/or metabolism.

Keyphrases □ 3,5-Dimethylpyrazoles, 1-acyl series—kinetics of hydrolysis, hydrolysis rate–hypoglycemic activity correlation □ Hypoglycemic activity–hydrolysis rate correlation—1-acyl 3,5-dimethylpyrazoles, kinetics of hydrolysis □ Hydrolysis rates—1-acyl 3,5-dimethylpyrazole series, correlated with hypoglycemic activity, kinetics

Wright *et al.* (1) reported the hypoglycemic activities of a large number of pyrazoles in the intact, fasted, glucose-primed rat. The most active compounds were 3,5-dimethylpyrazole (I) (2) and various 1-acyl derivatives. Preliminary experiments showed that 3,5-dimethylpyrazole-1-carboxamide (II) was readily hydrolyzed to I at pH's encountered *in vivo* in the GI tract and the blood. As a consequence, it was suggested that the various 1-acyl derivatives of I might owe their activities either to nonenzymatic hydrolysis *in vivo* or to metabolic transformation to I (1). To test the hypothesis of nonenzymatic conversion, relative rates of

Table I—Reaction Conditions and Kinetic Constants for the Hydrolysis of 1-Acyl 3,5-Dimethylpyrazoles at 37.6°

Compound	pH 2.0 Concentration, $M \times 10^5$	λ, nm.	k', min.$^{-1}$	$t_{1/2}$	pH 6.7 Concentration, $M \times 10^5$	λ, nm.	k', min.$^{-1}$	$t_{1/2}$	Hypoglycemic Activity[a]
II	7.83	235	1.09×10^{-2}	63 min.	7.23	235	1.12×10^{-2}	62 min.	25
III	6.21	239	1.18×10^{-4}	4.1 days	6.89	238	7.57×10^{-5}	6.4 days	4–8
IV	10.64	236	—[b]	—	9.92	234	—[b]	—	20–30
V	7.39	237	1.54×10^{-2}	45 min.	8.55	237	1.79×10^{-2}	6.5 hr.	16
VI	6.10	255	6.78×10^{-3}	102 min.	6.10	254	1.22×10^{-3}	9.5 hr.	100

[a] *Reference 1*, tolbutamide = 1. [b] No reaction.

hydrolysis were determined (pH 2.0 and 6.7, 37.6°) for a series of representative compounds (II–VI) covering a wide range of hypoglycemic potencies (Table I).

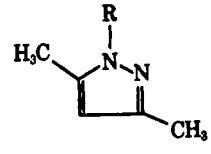
EXPERIMENTAL

Samples of II–VI were dissolved in aqueous ethanol and diluted with 0.01 *N* HCl or 0.08 *M* phosphate buffer, preequilibrated at 37.6°, to yield the final concentrations and pH values shown in Table I. The final ethanol concentration was 0.4% (v/v). Each solution was placed in a UV spectrophotometer equipped with a constant-temperature cell compartment maintained at 37.6°. For the rapidly hydrolyzed compounds (II, V, and VI), absorbances (A_t) were determined at appropriate times at the characteristic absorption maxima (Table I) until no further absorbance decreases occurred (A_∞). Pseudo-first-order reaction rate constants, k', were obtained from the slopes of plots of log $(A_t - A_\infty)$ *versus* time. For the slowly hydrolyzed III, k' was estimated from the initial slope of log A_t *versus* time.

RESULTS AND DISCUSSION

The method utilized for the determination of the kinetics of hydrolysis of Compounds II–VI is based on the differences in their UV spectra from the spectrum of the product, I (λ_{max}: 218 nm. at pH 2.0, 214 nm. at pH 6.7). In each case, first-order kinetics were observed and permitted calculation of the pseudo-first-order reaction rate constants, k', and corresponding half-lives, $t_{1/2}$, shown in Table I.

Examination of the data in Table I shows no correlation between rates of nonenzymatic hydrolysis and hypoglycemic activity. Compounds II and IV possess the same hypoglycemic activity; nevertheless, II was rapidly hydrolyzed in both acid and neutral solutions



I: R = —H

II: R = —C(=O)—NH$_2$

III: R = —C(=O)—NHC$_4$H$_9$

IV: R = —C(=O)—N(CH$_3$)$_2$

V: R = —C(=O)—NH—NH$_2$

VI: R = —C(=O)—C$_6$H$_5$

while IV was totally stable under the conditions studied. Thus, differences in biological activities of these compounds appear to reflect differences in their intrinsic potencies, in their absorption, or in their metabolism. The last is considered the most likely. Metabolism to I *via* *N*-dealkylation and/or hydrolysis and the oxidation of the 3-methyl group to a 3-carboxylic acid are possible. 5-Methylpyrazole-3-carboxylic acid, a metabolite of I in the rat (3, 4), is also a potent hypoglycemic agent (5). Efficient absorption of these compounds from the GI tract would be expected based on the complete absorption after oral administration found for I in the rat (4) and for the highly polar 5-methylpyrazole-3-carboxylic acid in the rat, dog, and man (6).

Scott (7) reported the kinetics of ethanolysis of a number of 1-acyl 3,5-dimethylpyrazoles, including II; Staab (8) and Hüttel and Kratzer (9) determined the kinetics of neutral hydrolysis and of aminolysis of 1-acetyl-3,5-dimethylpyrazole. However, the nature of the catalysis involved in the hydrolysis reaction has not been elucidated. Data in Table I for Compounds III, V, and VI indicate that hydronium-ion catalysis is involved. In addition, the magnitude of the pseudo-first-order reaction rate constants at pH 6.7 relative to those at pH 2.0 implicates hydroxide-ion and/or direct water catalysis. Results for Compounds II–IV indicate a correlation with the degree of steric hindrance by substituents on the carboxamide nitrogen. A similar relationship was observed by Scott (7) in the ethanolysis reaction and is consistent with the mechanism involving attack on the acyl group proposed by Meloche and Laidler for the hydrolysis of aromatic amides (10).

Compound II was unique in that its hydrolysis rates at the pH's studied were essentially identical. The kinetics and mechanism of the facile hydrolysis of II will be the subject of a separate report.

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Chemical Synthesis of 1-^{14}C-Octadecyldihydroxyacetone Phosphate and 1-^{14}C-Octadecyldihydroxyacetone

CLAUDE PIANTADOSI*▲, KUN CHAE*, KHALID S. ISHAQ*, and FRED SNYDER†

Abstract □ The synthesis of 1-^{14}C-octadecyldihydroxyacetone phosphate of high specific activity was accomplished *via* the cyclohexylammonium salt of 1-*O*-(1-^{14}C-octadecyl)-2,2-dimethoxypropane-3-phosphate.

Keyphrases □ 1-^{14}C-Octadecyldihydroxyacetone and phosphate—synthesis □ 1-*O*-(1-^{14}C-Octadecyl)-2,2-dimethoxypropane-3-phosphate, cyclohexylammonium salt—used in synthesis of high specific activity 1-^{14}C-octadecyldihydroxyacetone and phosphate

Piantadosi *et al.* (1, 2) previously reported the total chemical synthesis of unlabeled *O*-alkyl dihydroxyacetone phosphate, *O*-alkyl dihydroxyacetone, and the derivatives. However, to study the metabolism of these compounds, it became necessary to synthesize them with radioactive labels. In biological systems, the *O*-alkyl dihydroxyacetone phosphate is a key intermediate in the biosynthesis of complex ether-linked lipids (3–11). This report describes the preparation of 1-^{14}C-octadecyldihydroxyacetone and its phosphate derivative having high radiopurities and high specific activities.

The sequence of reactions used to synthesize the labeled compounds was based on the previous procedure for the synthesis of unlabeled *O*-alkyl dihydroxyacetone phosphate and derivatives (2). In the present synthetic procedure (Scheme I), 3-*O*-benzylglycerol (I) was esterified with *p*-nitrobenzoyl chloride in the presence of pyridine, resulting in a crude mixture of mono- and diesters (II); this mixture contained over 70% of the monoester derivative as determined by TLC. The Compound II mixture was oxidized to III with dimethyl sulfoxide and dicyclohexylcarbodiimide in the presence of trifluoroacetic acid. Compound III was ketalized to IV, which was hydrolyzed to V with sodium hydroxide. Compound V was then reacted with 1-^{14}C-octadecyl bromide in the presence of powdered potassium hydroxide in benzene, resulting in VI. After purification by silica gel column chromatography, Compound VI was debenzylated with palladium black to VII, which was phosphorylated to VIII. Compound VIII was hydrogenated with platinum oxide to remove the phenyl groups, and finally the cyclohexylammonium salt (X) was isolated. Compounds X and VII were treated with dilute hydrochloric acid to release XI and XII, respectively.

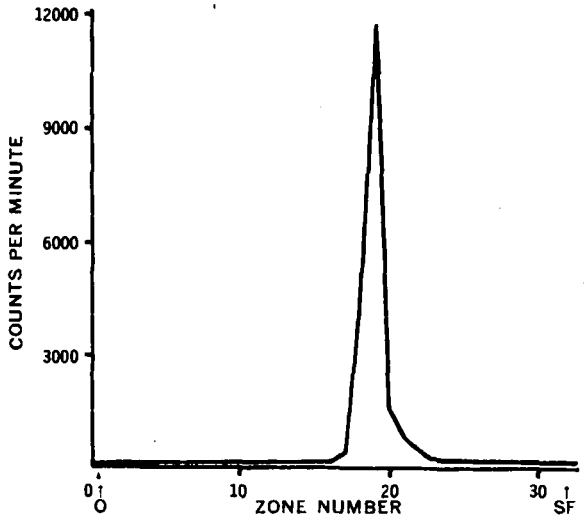


Figure 1—*TLC ^{14}C-zonal profile scans of 1-^{14}C-octadecyldihydroxyacetone phosphate. The solvent system used was chloroform–methanol–acetic acid–water (50:25:8:4 v/v). O = origin, and SF = solvent front.*

EXPERIMENTAL[1]

3-*O*-Benzylglycerol (I)—3-*O*-Benzylglycerol was prepared according to the procedures of Sowden and Fischer (13) and Howe and Malkin (14).

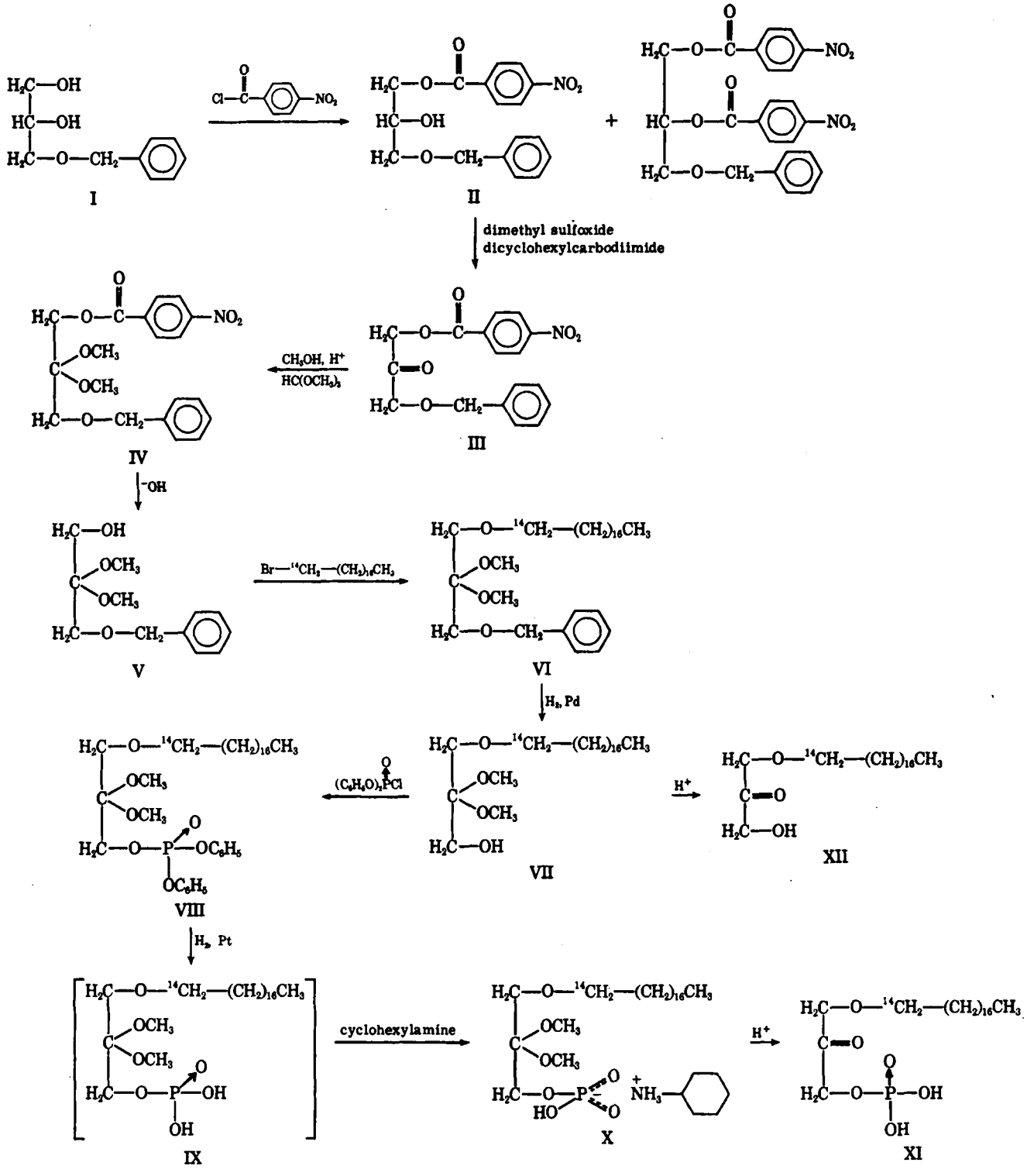
1-*O*-(*p*-Nitrobenzoyl)-3-*O*-benzyl-2-propanone (III)—Compound I was esterified with *p*-nitrobenzoyl chloride in the usual manner to give Compound II. TLC, using a solvent system of chloroform–diethyl ether (60:40 v/v), revealed a 70% yield of the mono-*p*-nitrobenzoyl derivative. Compound II was subjected to the Pfitzner–Moffatt oxidation for a period of 5 hr. (15, 16). The filtrate was extracted three times with 200-ml. portions of an ice-cold saturated sodium bicarbonate solution, two 200-ml. portions of water, and finally dried over anhydrous sodium sulfate. The ether was removed under reduced pressure, and the residue was taken up in 300 ml. of warm ether and left overnight at 5°. The resulting 25 g. of product was recrystallized from 200 ml. of ether at 5° and yielded 21 g. (32% based on the reacted 3-*O*-benzylglycerol ether), m.p. 92–93°. TLC in chloroform–ether (60:40 v/v) revealed one spot having R_f 0.68.

Anal.—Calc. for $C_{17}H_{15}NO_6$: C, 62.00; H, 4.59; N, 4.25. Found: C, 62.01; H, 4.68; N, 4.28.

1-*O*-(*p*-Nitrobenzoyl)-2,2-dimethoxy-3-*O*-benzylpropane (IV)—Compound III was ketalized in the usual manner in the presence of absolute methanol, trimethylorthoformate, and a catalytic amount of concentrated sulfuric acid. TLC in *n*-hexane–ether (80:20 v/v) revealed a single component with R_f 0.51, indicating that complete ketalization of Compound III had occurred to give Compound IV.

1-Hydroxy-2,2-dimethoxy-3-*O*-benzylpropane (V)—Seven grams of Compound IV was dissolved in 100 ml. of methanol containing 10 ml. of 4 *N* sodium hydroxide solution, and the mixture was stirred at room temperature for 24 hr. The methanol was evaporated and the remaining aqueous mixture was extracted three times with 150-ml. portions of ether. The combined ethereal extracts were washed once with 100 ml. of water and dried over anhydrous sodium sulfate. After removing the ether under reduced pressure, 3.2 g. (80% yield) of the syrupy product was obtained. TLC in *n*-hexane–ether (50:50 v/v) revealed one spot with R_f 0.25.

[1] The 1-^{14}C-octadecyl bromide (2.5 mc./mmole), which was purchased from International Chemical and Nuclear Corp., Irvine, Calif., had a radiopurity >99%. Radioactive intermediates were purified on columns (1.9 × 12 cm.) containing 15–20 g. silica gel (70–325 mesh, Brinkmann). Melting points were taken with a Mel-Temp melting-point apparatus and are corrected. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga. The specific radioactivities were determined with a Packard Tri-Carb scintillation spectrometer (model 3320) which had a ^{14}C counting efficiency of 79%. The radiopurity of the labeled compounds was determined by a zonal profile scanning method (12).



Scheme I

Anal.—Calc. for $C_{12}H_{18}O_4$: C, 63.69; H, 8.01. Found: C, 63.60; H, 7.98.

1-*O*-(1-^{14}C-Octadecyl)-2,2-dimethoxy-3-*O*-benzylpropane (VI)—In a 30-ml. reaction filter flask, equipped with a magnetic stirrer, Dean-Stark receiver, reflux condenser, and anhydrous calcium sulfate[2] drying tube, were placed 200 mg. of powdered potassium hydroxide and 340 mg. (1.5 mmoles) of Compound V dissolved in 10 ml. of dry benzene. Then 333 mg. of 1-^{14}C-octadecyl bromide (1.0 mmole) in 15 ml. benzene was added, and the mixture was refluxed in a sand bath at 83–87° for 24 hr. The cooled mixture was filtered under nitrogen to remove the potassium bromide, and the filtrate was collected in a 100-ml. round-bottom flask. After the precipitate was thoroughly washed with chloroform, the combined filtrate was evaporated under reduced pressure. The oily product (750 mg.) was dissolved in 5 ml. of warm *n*-hexane and was applied on a column (1.9 × 12 cm.) containing 15 g. of silica gel. The column was eluted with the following sequence of solvents: Solvent I, 100 ml. *n*-hexane; Solvent II, 100 ml. *n*-hexane–ether (7:3 v/v); and Solvent III, 100 ml. *n*-hexane–ether (1:1 v/v); 20-ml. fractions were collected.

[2] Drierite.

Fractions 1–5 contained the alkyl halide and hydrocarbon breakdown products. The desired product, Compound VI, appeared in fractions 7 and 8 (425 mg., 89% yield).

Anal.—Calc. for $C_{30}H_{54}O_4$: C, 75.26; H, 11.37. Found: C, 75.23; H, 11.53.

1-*O*-(1-^{14}C-Octadecyl)-2,2-dimethoxy-3-hydroxypropane (VII)—Compound VI (425 mg.) in 50 ml. of *n*-hexane was debenzylated with 250 mg. of palladium black in a Parr hydrogenator at 20 p.s.i. at room temperature for 5 hr. The catalyst was filtered and washed thoroughly with chloroform. The combined filtrate was evaporated under reduced pressure. Then the white solid product (350 mg.) was dissolved in about 5 ml. of warm *n*-hexane and applied on a column (1.9 × 12 cm.) containing 20 g. of silica gel. The column was eluted with the following solvents: fraction 1, 150 ml. *n*-hexane; fraction 2, 100 ml. *n*-hexane–ether (9:1 v/v); fraction 3, 100 ml. *n*-hexane–ether (8:2 v/v); and fraction 4, 100 ml. *n*-hexane–ether (7:3 v/v). Fractions 1–3 contained some of the hydrocarbon breakdown products, and fraction 4 contained Compound VII (230 mg., 66% yield).

Anal.—Calc. for $C_{23}H_{48}O_4$: C, 71.08; H, 12.44. Found: C, 71.21, H, 12.30.

Cyclohexylammonium Salt of 1-*O*-(1-^{14}C-Octadecyl)-2,2-dimethoxypropane-3-phosphate (X)—A solution of 200 mg. of Compound VII (0.51 mmole) in 10 ml. of dry pyridine was placed in a 30-ml. reaction filter flask and cooled to 0° in an ice bath. Over 5 min., 250 mg. of diphenyl chlorophosphate was added with stirring. The flask was stoppered and allowed to stand at 5° for 24 hr. The precipitated pyridine hydrochloride was removed by filtration under nitrogen pressure and the filtrate was collected. After the precipitate was thoroughly washed with benzene, the combined filtrate was evaporated to dryness under reduced pressure. The oily product (320 mg.) was dissolved in 5 ml. of benzene and was applied to a column (1.9 × 12 cm.) containing 15 g. of silica gel. The column was eluted with 150-ml. portions of benzene, benzene–chloroform (3:1 v/v), and benzene–chloroform (1:1 v/v); 50-ml. fractions were collected. Fractions 5–8 contained 255 mg. (80%) of the phosphorylated intermediate, VIII. Compound VIII was dissolved in 50 ml. of absolute ethanol containing 150 mg. of platinum oxide and hydrogenated at 15 p.s.i. at room temperature for 3 hr. to remove the phenyl groups. The catalyst was removed by filtration and washed with absolute ethanol. Subsequently, 100 mg. of cyclohexylamine was added to the filtrate, and the mixture was left overnight at room temperature. After the solvent was removed under reduced pressure, a white solid product (325 mg.) appeared which was dissolved in 15 ml. of chloroform and stored at −15°.

1-*O*-(1-^{14}C-Octadecyl)dihydroxyacetone Phosphate (XI)—1-*O*-(1-^{14}C-Octadecyl)dihydroxyacetone phosphate was prepared from Compound X by the method described previously (2). About 10 mg. of Compound X was dissolved in 5 ml. of 0.1*N* HCl and shaken at 37° for 2 hr. The mixture was immediately extracted by a modification of the Bligh and Dyer (17) procedure, the solvent was removed under reduced pressure, and the product was dissolved in chloroform. TLC in chloroform–methanol–acetic acid–water (50:25:8:4 v/v) showed the product to be over 99% pure; the specific radioactivity was 2.46 mc./mmole. Figure 1 shows the ^{14}C-zonal profile scan of 1-*O*-(1-^{14}C-octadecyl)dihydroxyacetone phosphate after treatment of the cyclohexylammonium salt with dilute hydrochloric acid.

1-*O*-(1-^{14}C-Octadecyl)dihydroxyacetone (XII)—1-*O*-(1-^{14}C-Octadecyl)dihydroxyacetone was prepared from Compound VII by the same method used to prepare Compound XI. The radiopurity was 98–99%, as determined by TLC using a solvent system of hexane–ether–methanol–acetic acid (70:30:5:1 v/v).

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Structure and Activity in Molluscicides IV: New Substituted Tetralins with Potential Molluscicidal Effect

I. NABIH▲ and J. METRI

Abstract □ Upon a rational biochemical and biological basis, a series of new compounds related to the tetralin system was synthesized. Biological testing showed their molluscicidal activity at very high dilutions.

Keyphrases □ Tetralins, substituted—synthesized and tested as potential molluscicides, structure–activity relationships □ Molluscicides, potential—synthesis and biological testing of new substituted tetralins, structure–activity relationships □ Structure–activity relationships—substituted tetralins and molluscicidal effect

In a previous article (1), the structure–activity relationship in molluscicides of the phenolic type was discussed. In this group of compounds, certain specific structural features regarding the nature of substituents on the aromatic ring govern the biological activity of the candidate compound. The presence of an enzyme system of the peroxidase type within the snail body was identified through specific systemic reactions and assay measurements for activity in some species of economic importance (2). The biological transformation of the active phenols to the corresponding quinones through this enzymatic system could explain the molluscicidal effectiveness of the former. The latter are highly toxic (3).

DISCUSSION

The main source of energy in snails, required for the metabolism of living cells, is liberated due to oxidation of many compounds. The most important energy-yielding reactions are those of the oxidation–reduction type. Snails, schistosomiasis intermediate hosts, appear to respire aerobically. In typical aerobic respiration, the hydrogen and electrons of substrate are transferred to molecular oxygen through enzymatic protein catalysts (4, 5). Quinones uncouple phosphorylation by diverting electrons from the enzyme to molecular oxygen; *e.g.*, 2,4-dichloronaphthoquinone reacts by binding the enzyme to the quinone molecule through substitution or addition at the double bond, an oxidative effect on sulfhydryl enzymes, or changing the redox potential in some systems.

Pentachlorophenol, a widely used molluscicidal agent, could undergo biological transformation through enzymatic peroxidase catalysis to furnish 2,2′,3,3′,5,5′,6,6′-octachlorobiphenylquinone, a polysubstituted quinone of potential molluscicidal activity (6).

Generally, the molluscicides of the phenolic and salicylanilide types that are now in common use are still unsatisfactory. The phenolics are toxic during applications, not easily handled, and unstable during storage. The salicylanilides exhibit a water-insolubility problem (a major one in molluscicides), and they are immiscible with water during application. Thus, the rate of distribution is not satisfactory.

Because of these considerations, new synthetic work is necessary to obtain efficient molluscicides of higher activity and fewer disadvantages. In this work the synthesis of new compounds that may undergo biological transformations to quinoidal systems is emphasized. Substituted compounds related to tetralin were chosen to be common structural moieties in these agents. These types of compounds could be oxidized to give products related to naphthoquinones, which are known to possess potent molluscicidal activity (3). In addition, it was of concern to include the salicylanilide type of structure, since these compounds exert their molluscicidal activity through a different mechanism. They act through chelation with the important metals (*e.g.*, iron and magnesium) present and necessary for the biological functions within the snail body[1].

Compounds of Structures I–V were desired.

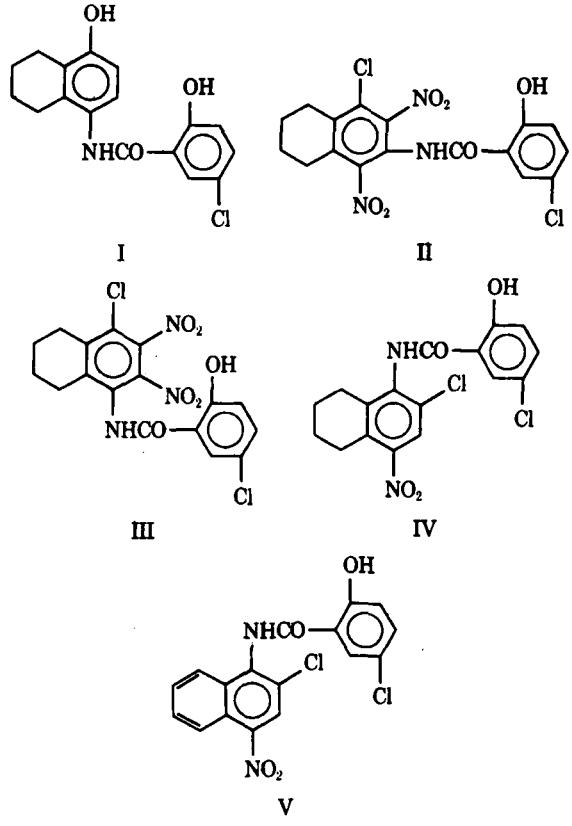
Through the condensation of 5-hydroxy-8-aminotetralin (VI) with 5-chlorosalicylic acid (VII) in the presence of phosphorus pentachloride, 5-hydroxy-8-*N*-(5′-chlorosalicylanilido)tetralin (I) was obtained.

Diazotization of 5-amino-7-nitrotetralin (VIII), followed by subsequent halogenation at the 5-position, reductive acetylation at the 7-position, and nitration at both the 6- and 8-positions, gave 5-chloro-7-amino-6,8-dinitrotetralin (IX). Condensation of VII with IX gave 5-chloro-6,8-dinitro-7-*N*-(5′-chlorosalicylanilido)-tetralin (II).

Chlorination of 8-acetaminotetralin in chloroform gave the 5-chloro derivative (7). Upon nitration (sulfuric acid–nitric acid at −10°), this gave 5-chloro-6,7-dinitro-8-acetaminotetralin (X). Hydrolysis and condensation with VII gave 5-chloro-6,7-dinitro-8-*N*-(5′-chlorosalicylanilido)tetralin (III).

Chlorination of 5-acetamino-8-nitrotetralin (8), followed by hydrolysis, gave 5-amino-6-chloro-8-nitrotetralin (XI). Upon condensation with VII, this gave 5-*N*-(5′-chlorosalicylanilido)-6-chloro-8-nitrotetralin (IV).

Amination of α-nitronaphthalene with hydroxylamine hydrochloride and methanolic potassium hydroxide at 50° gave 4-nitro-1-



[1] This laboratory, to be published.

naphthylamine. Upon acetylation, chlorination, and hydrolysis, this gave 1-amino-2-chloro-4-nitronaphthalene (9). Upon condensation of this amine with VII, the product 1-*N*-(5'-chlorosalicylanilido)-2-chloro-5-nitronaphthalene (V) was obtained.

BIOLOGICAL TESTING

In all tests, the fresh water snails, *Biomphalaria alexandrina*, vector of *Schistosoma mansoni*, were the test animals. Young adult snails (8–11-mm. diameter) were used. Water, freshly boiled to avoid any interference by contaminated chlorine present in tap water (pH 6–6.5), was used as the test medium. Each aquarium was fitted with a glass cover to prevent the accidental dropping of foreign material into test medium. Compounds I–V were tested; they were diluted in volumetric flasks with water to the desired parts per million (p.p.m.). In each test, five snails were used in 200 ml. of the test chemical at each concentration (10, 5, 3, and 1 p.p.m.) and five snails were used as the control in the same volume of water. The exposure period was 24 hr. At the end of each contact period, the snails were removed from the chemically treated water, washed thoroughly, and transferred to fresh water. Food (lettuce) was made available to the snails. On the following day, the mortality rate was determined. Snails that were not fully recovered at the time of the examination were held for future daily observations until death or recovery occurred. Determination of recovery was based on the ability of the snails to move about or cling to the bottom and sides of the glass container.

The concentration effects of different compounds upon *B. alexandrina* (within the 24-hr. exposure period at 25°) are recorded in Table I.

Table I—Effects of Compounds I–V upon Snails of *B. alexandrina* Type

Compound	Concentration, p.p.m. 10	5	3	2	1
I	++[a]	++	++	++	++
II	++	++	++	++	++
III	++	++	++	++	++
IV	++	++	++	++	++
V	++	++	++	++	+

[a] + + = average mortality of 100% and + = average mortality of 80%.

EXPERIMENTAL[1]

5-Hydroxy-8-*N*-(5'-chlorosalicylanilido)tetralin (I)—A mixture of 5-chlorosalicylic acid (VII) (1.06 g.) and 5-hydroxy-8-aminotetralin (VI) (1 g.) (10) in 10 ml. of dry chlorobenzene was refluxed for 10 min. at 160°; then 0.2 g. of phosphorus pentachloride was added to the reaction mixture. Reflux continued for 3 hr., and then the chlorobenzene was steam distilled. The product formed upon cooling was filtered, washed with hot diluted hydrochloric acid (to remove traces of unreacted amine), and then washed with water to give 1.5 g. (79% yield) of I. This compound was purified by dissolving in dilute sodium hydroxide solution and filtering; it was then neutralized with dilute hydrochloric acid, and a dark-brown product formed. Upon recrystallization from ethanol, it melted at 230–232°; IR: 1390 (—OH), 1650 (—CONH—), and 720 (Ar—Cl) cm.$^{-1}$.

Anal.—Calc. for $C_{17}H_{16}ClNO_3$: Cl, 11.16; N, 4.40. Found: Cl, 11.28; N, 4.32.

5-Chloro-7-amino-6,8-dinitrotetralin (IX)—To 8 ml. concentrated sulfuric acid was added 8 g. of 5-chloro-7-acetaminotetralin (11). The mixture was cooled to 0° and nitrated, dropwise, with a nitrating mixture of nitric acid (4 ml.) and concentrated sulfuric acid (12 ml.). The mixture was allowed to stand overnight at room temperature; it was then poured over ice water and the crude dinitro product precipitated. This product was filtered and washed thoroughly with cold water. After drying, it gave 5.2 g. (72% yield) of IX. Upon recrystallization from alcohol, it gave white flakes, m.p. 300°; IR: 1540 (—NO_2), 1680 (—NHCOCH$_3$), and 725 (Ar—Cl) cm.$^{-1}$.

Anal.—Calc. for $C_{12}H_{12}ClN_3O_5$: C, 45.94; H, 3.83; Cl, 11.32; N, 13.39. Found: C, 45.94; H, 4.05; Cl, 11.41; N, 13.41.

The acetamino product was then submitted to acid hydrolysis (hydrochloric acid–ethanol) to give the free amine IX. Due to difficulties encountered in the preparation of an analytical sample, the crude amine, m.p. 162–165°, was used for the condensation reaction in the following step.

5-Chloro-6,8-dinitro-7-*N*-(5'-chlorosalicylanilido)tetralin (II)—A mixture of VII (1.27 g.) and the amine IX (2 g.) was dissolved in 20 ml. of chlorobenzene and heated to 160°. Then 0.4 g. of phosphorus pentachloride was added during reflux, which continued for 2 hr. The solvent was removed through steam distillation. The product was filtered, washed with sodium carbonate solution and then with water, and dried to give 2.2 g. of II (yield 70%). After recrystallization from alcohol, it melted at 194–196°.

Anal.—Calc. for $C_{17}H_{13}Cl_2N_3O_6$: C, 48.00; H, 3.06; Cl, 16.47; N, 9.88. Found: C, 48.46; H, 3.13; Cl, 16.67; N, 9.50.

5-Chloro-6,7-dinitro-8-acetaminotetralin (X)—A mixture of 5-chloro-8-acetaminotetralin (8) (2.36 g.) and 4 ml. of concentrated sulfuric acid was cooled to −10° and then nitrated by the addition of a cold mixture of nitric acid (2 ml.) and concentrated sulfuric acid (6 ml.). The mixture was allowed to stand overnight at room temperature and was then poured over ice. The formed yellow precipitate was filtered and washed with water. After drying, it gave 2.68 g. (82% yield) of X. Upon recrystallization from ethanol, it gave yellow needles, m.p. 250–251°; IR: 1550 (—NO_2), 1685 (—NHCOCH$_3$), and 725 (Ar—Cl) cm.$^{-1}$.

Anal.—Calc. for $C_{12}H_{12}ClN_3O_5$: C, 45.93; H, 3.83; Cl, 11.32; N, 13.39. Found: C, 45.97; H, 3.92; Cl, 11.18; N, 13.19.

5-Chloro-6,7-dinitro-8-aminotetralin—A mixture of X (1.5 g)., ethanol (30 ml.), and concentrated hydrochloric acid (8 ml.) was refluxed for 5 hr., cooled, and poured over ice water. The precipitate was collected, washed with water, and dried to give 1.06 g. of the free amine. After recrystallization from dilute ethanol, it melted at 140–142°.

Anal.—Calc. for $C_{10}H_{10}ClN_3O_4$: C, 44.28; H, 3.69; Cl, 13.10; N, 15.44. Found: C, 44.38; H, 3.73; Cl, 13.20; N, 15.23.

5-Chloro-6,7-dinitro-8-*N*-(5'-chlorosalicylanilido)tetralin (III)—The condensation between the above-mentioned free amine (1 g.) and the acid VII (0.64 g.) was carried out as described for II. The yield (75%) was 1.2 g. After recrystallization from benzene–*n*-hexane, it melted at 187–190°.

Anal.—Calc. for $C_{17}H_{13}Cl_2N_3O_6$: C, 48.00; H, 3.06; N, 9.88. Found: C, 48.26; H, 3.13; N, 9.86.

5-Amino-6-chloro-8-nitrotetralin (XI)—In glacial acetic acid (35 ml.) was chlorinated 9 g. of 5-acetamino-8-nitrotetralin (8) at 100° with a dry stream of chlorine gas for about 15 min. After cooling, the formed yellow precipitate was collected, washed with water, and dried. It weighed 6 g. (90% yield). Recrystallization from ethanol gave white needles, m.p. 181–183°.

Anal.—Calc. for $C_{12}H_{13}ClN_2O_2$: C, 53.53; H, 4.84; Cl, 13.22; N, 10.43. Found: C, 52.97; H, 4.83; Cl, 13.27; N, 10.44.

The free amine XI was obtained through acid hydrolysis of this product. It was recrystallized as yellow crystals (from ethanol), m.p. 110–112°.

Anal.—Calc. for $C_{10}H_{11}ClN_2O_5$: C, 52.98; H, 4.86; Cl, 15.67; N, 12.36. Found: C, 53.11; H, 4.92; Cl, 16.05; N, 12.22.

5-*N*-5'-(Chlorosalicylanilido)-6-chloro-8-nitrotetralin (IV)—The same method of condensation between the free amine (XI) (1 g.) and the acid VII (0.76 g.) was followed as described for II. The product IV, 1.2 g. (70% yield), was recrystallized from benzene–petroleum ether, m.p. 156–158°.

Anal.—Calc. for $C_{17}H_{14}Cl_2N_2O_4$: Cl, 18.42; N, 7.37. Found: Cl, 18.43; N, 7.43.

1-*N*-(5'-Chlorosalicylanilido)-2-chloro-5-nitronaphthalene (V)—The condensation described for II was followed for the acid VII (0.78 g.) and 1-amino-2-chloro-4-nitronaphthalene (1 g.) (9). The product V, 1.3 g. (75% yield), was recrystallized from benzene–petroleum ether, m.p. 210–212°.

Anal.—Calc. for $C_{17}H_{10}Cl_2N_2O_4$: C, 54.26; H, 2.66; Cl, 18.62; N, 7.45. Found: C, 53.98; H, 2.68; Cl, 18.51; N, 7.41.

[1] Melting points were taken in open capillary tubes with a Gallenkamp melting-point apparatus and are uncorrected. The IR spectra were recorded with a Carl-Zeiss Infracord spectrophotometer, model UR 10. Microanalyses were performed by the Spang Microanalytical Laboratory, Ann Arbor, Mich., and the Microanalytical Laboratory, National Research Center, Cairo, Egypt.

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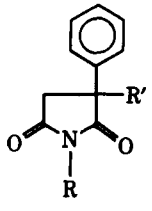
Anticonvulsant Properties of Mannich Base Derivatives of 2-Phenylsuccinimide III

EDWARD O. MAGARIAN▲, GERALD W. BECKER, and LOUIS DIAMOND

Abstract □ Several Mannich base derivatives of 2-phenylsuccinimide were synthesized and screened for anticonvulsant activity. All products were evaluated by maximal electroshock seizure and pentylenetetrazol seizure threshold tests.

Keyphrases □ 2-Phenylsuccinimide, Mannich base derivatives—synthesis, screened for anticonvulsant properties □ Anticonvulsants, potential—synthesis and screening of Mannich base derivatives of 2-phenylsuccinimide

The preceding papers (1, 2) of this series reported the preparation and anticonvulsant properties of several derivatives of phensuximide[1], *N*-methyl-2-phenylsuccinimide (I). One series of compounds (II), consisting of C-Mannich base derivatives of I, proved interesting since several members were more effective than the parent molecule in eliminating the tonic extensor component of electroshock seizures.



I: R = CH_3, R′ = H
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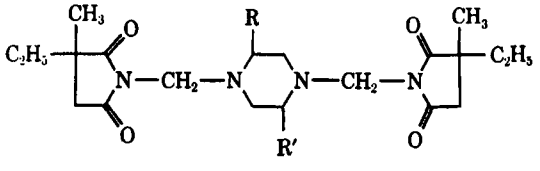

IV: R = CH_3, R′ = H
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Some researchers (3, 4) reported the preparation of mono- and bis-Mannich bases of succinimides (III–V). These compounds were shown to possess significant anticonvulsant properties.

In view of the foregoing, it was of interest to prepare for evaluation the *N*,2-bis-Mannich bases of 2-phenylsuccinimide (Compounds 1–6 in Table I). The corresponding mono bases (Compounds 7–12 in Table I) were also prepared for comparison purposes. All products were subjected to preliminary pharmacological screening, and the results are presented in Tables II and III.

EXPERIMENTAL[2]

2-Phenylsuccinimide—The title compound was prepared from 2-phenylsuccinic acid and concentrated ammonium hydroxide according to Miller and Long's (5) procedure. It was obtained as a white solid in a yield of 78%, m.p. 79–81° [lit. (6) m.p. 90°].

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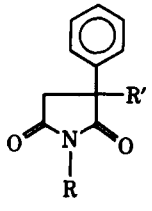

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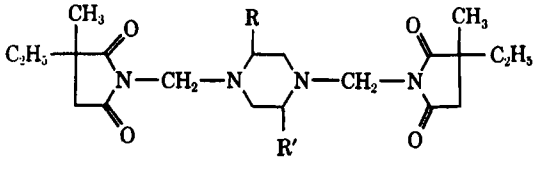

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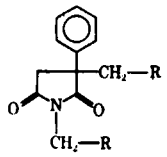
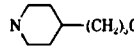
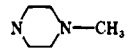
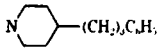
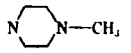


Table I—Mannich Bases of 2-Phenylsuccinimide

Compound Number	R	Recrystallization Solvent	Melting Point	Formula	Analysis, % Calc.	Analysis, % Found	Yield, %	Reflux Time, hr.
1		Ethanol (95%)	104.5–105.5°	$C_{40}H_{51}N_3O_2$	C 79.30 H 8.48 N 6.94	79.27 8.24 6.98	46	19
2		Hexane	112.5–115.5°	$C_{22}H_{31}N_5O_2$	C 66.14 H 8.32 N 17.53	66.27 8.39 17.44	24	93
3		Isopropanol	83.5– 84.5°	$C_{22}H_{27}N_3O_2$	C 72.30 H 7.45 N 11.50	72.47 7.51 11.43	48	21
4		Isopropanol–chloroform (1:1)	178.5–181°	$C_{28}H_{39}N_3O_2$	C 74.80 H 8.74 N 9.35	74.50 8.89 9.60	64	21
5		Isopropanol	118–119°	$C_{20}H_{27}N_3O_4$	C 64.32 H 7.29 N 11.25	64.36 7.42 11.10	18	65
6		Isopropanol	97–98°	$C_{22}H_{31}N_3O_2$	C 71.52 H 8.46 N 11.37	71.26 8.36 11.18	24	21
7		Ethanol (95%)	121–123°	$C_{26}H_{30}N_2O_2$	C 76.89 H 7.74 N 7.17	76.99 7.54 7.27	86	
8		Isopropanol	127–129°	$C_{16}H_{21}N_3O_2$	C 66.88 H 7.37 N 14.62	66.95 7.45 14.52	67	
9		Isopropanol	100.5–105°	$C_{16}H_{18}N_2O_2$	C 71.09 H 6.71 N 10.36	71.17 6.84 10.31	84	
10		Isopropanol	105–106.5°	$C_{19}H_{24}N_2O_2$	C 73.05 H 7.74 N 8.97	72.96 7.47 8.93	94	
11		Isopropanol	113–114.5°[a]	$C_{15}H_{18}N_2O_2$	—	—	86	
12		Ethanol (95%)	110–111°[b]	$C_{16}H_{20}N_2O_2$	—	—	69	

[a] Lit. (3) m.p. = 111–112°. [b] Lit. (3) m.p. = 107–108°.

Preparation of Mono-Mannich Bases (Table I)—To 0.05 mole of the appropriate amine, 5.0 ml. of 40% formalin (2.0 g., 0.067 mole formaldehyde) was added slowly with cooling. This mixture was added immediately to a solution of 8.8 g. (0.05 mole) of 2-phenylsuccinimide in 20 ml. of 95% ethanol. The reaction mixture was stirred at room temperature for 15–30 min. and cooled in an ice bath, and the product was removed by filtration.

PHARMACOLOGY

The anticonvulsant activity of each compound was evaluated by maximal electroshock seizure and pentylenetetrazol seizure threshold tests (7). A group of five male albino mice[3] was used for each test, and the results were compared with vehicle-treated control groups.

For the maximal electroshock seizure tests, animals were stimulated with 60-cycle alternating current for 200 msec. *via* a pair of earclip electrodes. A variable transformer was used to regulate the applied voltage. The desired duration of stimulus was obtained by the inclusion of a relay in the circuit, which was operated by a square-wave stimulator[4]. A cathode ray oscilloscope was used to monitor the voltage drop across a 10-ohm resistor coupled in series with the animal. This enabled the calculation of the corresponding current. The sweep on the cathode ray oscilloscope screen was triggered by the stimulator, so accurate readings could be obtained with each shock. Preliminary studies showed that 12–16 ma. was required to produce a convulsive seizure pattern having a tonic flexor phase, a tonic extensor phase, and a clonic phase.

After adequate control data were obtained, each group of animals received 10–600 mg./kg. i.p. of the drug under study. The maximal electroshock seizure tests were repeated 1, 2, and 3 hr. after drug administration. All drugs were administered as suspensions in 0.5% methylcellulose (4000 cps.).

An animal was considered to be protected if the tonic extensor phase of the seizure pattern was absent.

[3] Swiss-Webster mice obtained from the Laboratory Supply Co., Inc., Indianapolis, IN 46241. Food and water were allowed *ad libitum*.

[4] Grass S-4.

Table II—Maximal Electroshock Seizure Tests

Compound Number	Dose, mg./kg.	Number of Animals Protected[a] 1 hr.	2 hr.	3 hr.	Number of Animals Showing Toxicity[a] 1 hr.	2 hr.	3 hr.
1	600	0/5	0/4	0/2	3/5	1/4	1/2
2	10	1/5	0/5	0/5	0/5	0/5	0/5
	100	3/5	2/3	0/3	0/5	0/3	0/3
	600[b]	—	—	—	—	—	—
3	100	1/5	0/4	1/4	1/5	0/4	0/4
	600[b]	—	—	—	—	—	—
4	600	0/5	0/5	0/5	4/5	4/5	5/5
5	100	0/5	0/5	0/5	0/5	0/5	0/5
	600	5/5	5/5	3/4	5/5	5/5	3/4
6	100	0/5	0/3	0/2	1/5	1/3	0/2
	600	3/4	1/2	0/1	3/4	2/2	1/1
7	10	0/5	0/5	0/5	0/5	1/5	1/5
	100	2/5	1/5	3/5	2/5	2/5	1/5
	600	2/5	3/4	3/3	1/5	1/4	1/3
8	100	1/5	0/5	0/5	0/5	0/5	0/5
	600	3/3	1/1	0/0	3/3	1/1	0/0
9	100	2/5	1/5	0/5	0/5	0/5	0/5
	600	2/2	2/2	2/2	2/2	2/2	2/2
10	100	0/5	0/3	0/3	0/5	0/3	0/3
	600[b]	—	—	—	—	—	—
11[c]	10	1/5	1/5	0/5	1/5	1/5	1/5
	100	2/5	1/5	1/5	0/5	0/5	0/5
	600	5/5	5/5	5/5	5/5	5/5	5/5
12[c]	100	2/5	0/5	1/5	2/5	0/5	0/5
	600[b]	—	—	—	—	—	—
Diphenylhydantoin	25	5/5	5/5	5/5	0/5	0/5	0/5
Phenobarbital	25	5/5	5/5	5/5	0/5	0/5	0/5
Methylcellulose, 0.5%	0.5 ml.	0/5	0/5	0/5	0/5	0/5	0/5

[a] Animals not surviving are excluded from the ratios. [b] Drug was 100% lethal at this dosage level. [c] See *Reference 3*.

A similar experimental design was used for conducting the pentylenetetrazol seizure threshold tests. Animals received pentylenetetrazol, 85 mg./kg. s.c., 1 or 2 hr. after the administration of a test compound. Animals that showed no convulsive activity of any kind during a 30-min. observation period were considered protected.

The ability of the selected experimental testing procedures to detect anticonvulsant activity was assessed by including three agents known to provide significant protection against either electrically or chemically induced seizures—*viz.*, diphenylhydantoin, phenobarbital, and phensuximide.

The neurological status of each animal was evaluated prior to each test for anticonvulsant activity. Toxicity was considered present if the animal failed to exhibit a righting reflex or a hind-limb placing reaction or if the animal's equilibrium, gait, or stance deviated from normal.

It appears from the data in Tables II and III that the mono-Mannich bases were generally more active than the corresponding bis-substituted analogs. The products were less effective in preventing seizures with the maximal electroshock seizure test (Table II) than with the pentylenetetrazol seizure threshold test (Table III). As a rule, protection was obtained in the maximal electroshock seizure test only for dosage levels at which toxic effects were observed. In the pentylenetetrazol seizure threshold test, Compound 9 (Table III) displayed an activity pattern equivalent to that of the standard compound phensuximide. Compounds 8 and 11 (Table III) compared favorably with phensuximide in that they also provided full protection at a dosage level of 300 mg./kg.

Table III—Pentylenetetrazol Seizure Threshold Tests

Compound Number	Dose, mg./kg.	Number of Animals Protected[a] 1 hr.	2 hr.
1	100	1/5	1/5
	600	0/5	2/5
2	100	1/5	1/5
	300[b]	—	—
3	100	0/5	1/5
	600	0/2	0/0
4	100	1/5	2/5
	600	1/5	2/5
5	100	1/5	0/5
	300	0/5	0/5
6	100	1/5	0/5
	400	1/5	2/5
7	100	2/5	3/5
	300	0/5	1/5
8	100	0/5	3/5
	300	5/5	5/5
9	100	4/5	1/5
	300	5/5	5/5
10	100	0/5	2/5
11[c]	100	1/5	0/5
	300	5/5	5/5
12[c]	100	3/5	0/5
	600[b]	—	—
Phensuximide	100	4/5	1/5
	300	5/5	5/5
Methylcellulose, 0.5%	0.5 ml.	0/5	0/5

[a] Animals not surviving are excluded from the ratios. [b] Drug was 100% lethal at this dosage level. [c] See *Reference 3*.

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Calculating Rate Constants in Forward Drug Transfer Reactions

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Abstract □ A simple method for obtaining and processing drug transfer data derived from *in vitro* model cells such as the Schulman cell is presented. Simplified calculation procedures are developed for determining the rate constants that describe the transfer of the drug through the compartments. Transfer of the acidic drug salicylic acid from a pH 2.0 compartment through a lipid phase to a pH 7.4 compartment was utilized as the test system and example for the method. Theoretical *versus* experimental transfer curves are presented, along with statistical considerations for data of this type.

Keyphrases □ Rate constants—calculations and equations for forward drug transfer reactions, Schulman cell, salicylic acid □ Drug transfer (forward) reactions—calculations and equations for rate constants, Schulman cell, salicylic acid □ Schulman cell—calculations and equations for rate constants in forward drug transfer reactions, salicylic acid

In the area of pharmacokinetics and in the study of drug absorption, the use of *in vitro* or idealized model systems has become increasingly popular for simulation of *in vivo* transport, even with the deficiencies and cautions pointed out by Higuchi *et al.* (1) in extrapolating this type of data to *in vivo* situations. There are many situations arising in which an understanding of the factors influencing solute transport rates is of great importance in the design of new drugs with improved therapeutic properties. These studies appear to have particularly useful implications when one is dealing with a diffusion-limited, weakly acidic or weakly basic drug in which the transport can be regarded as two consecutive first-order reactions.

An investigator using these techniques would find useful a simplified and rapid method of calculation, particularly when measurement output is a continuous graph of concentration *versus* time such as is produced by continuous monitoring by means of flow cells in a recording spectrophotometer. In approaching this problem, we were surprised to find a general lack of statistical evaluation of the various kinetic parameters associated with the diffusion-limited transport investigated using the so-called Schulman cell (2, 3) or similar device (4). The general absence of these data very likely reflects the long periods of time required for a single experiment to be completed, the wide variability of constants derived from a given set of experiments, and the difficulty of obtaining the constants. The purposes of this study were: (*a*) to develop a simple method of calculation for the rate parameters from a plot of concentration *versus* time generated from a continuous monitoring system, and (*b*) to demonstrate the method with an example.

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

Scheme I

Table I—Statistical Evaluation of Rate Constants for the Salicylic Acid System

Run Number	Calculated k_1	Calculated k_2
1	0.9815	0.6617
2	0.8322	0.5958
3	0.9547	0.6497
4	1.0102	0.5856
5	0.8804	0.5242
6	0.9176	0.4707
Arithmetic mean	0.9294	0.5812
Standard deviation	0.0603	0.0669
95% Confidence interval	$0.8659 < k_1 < 0.9929$	$0.5177 < k_2 < 0.6447$

THEORETICAL

A drug transfer process that can be regarded as two consecutive first-order reactions may be described as shown in Scheme I, where A, B, and C are used for $A(t)$, $B(t)$, and $C(t)$, the concentrations of salicylic acid in compartments A, B, and C, respectively, at time t, as shown in Scheme II. Let V_A, V_B, and V_C equal the fluid volume in each of the three compartments. The three differential equations governing these first-order reactions are:

$$\frac{dA}{dt} = -k_1A \qquad \text{(Eq. 1)}$$

$$V_B\frac{dB}{dt} = k_1AV_A - k_2BV_B \qquad \text{(Eq. 2)}$$

$$V_C\frac{dC}{dt} = k_2BV_B \qquad \text{(Eq. 3)}$$

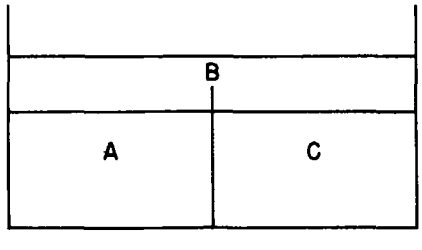
Initial conditions are set so that salicylic acid is only found in compartment A; thus, at time $t = 0$, $A(0) = A_0$ and $B(0) = C(0) = 0$. The solutions to Eqs. 1–3 as a function of time are as follows:

$$A(t) = A_0e^{-k_1t} \qquad \text{(Eq. 4)}$$

$$B(t) = \frac{V_AA_0k_1}{V_B(k_2 - k_1)}(e^{-k_1t} - e^{-k_2t}) \qquad \text{(Eq. 5)}$$

$$C(t) = \frac{V_AA_0}{V_C(k_2 - k_1)}(k_2 - k_1 - k_2e^{-k_1t} + k_1e^{-k_2t}) \qquad \text{(Eq. 6)}$$

Since the data output from the system consists of a continuous plot of the drug concentration in the A and C chambers as a function of time, as for example in Fig. 1, a convenient point for cal-



Scheme II—Sketch of Schulman-type in vitro *model cell*

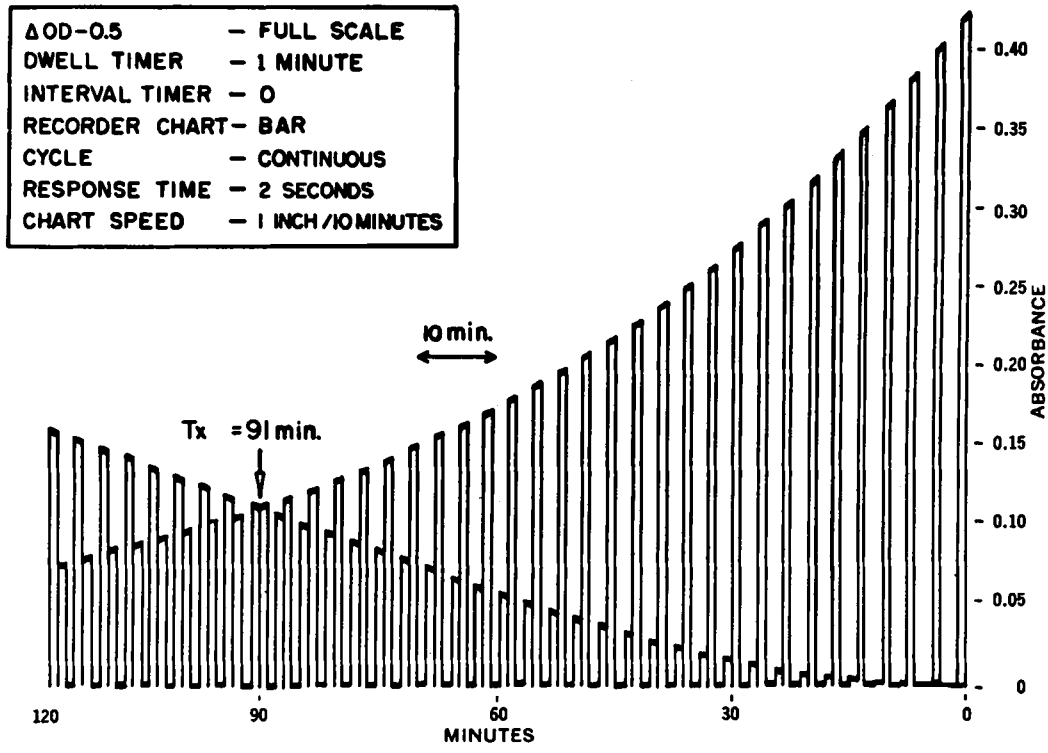


Figure 1—*Actual recorded data for a typical transfer run. Conditions are indicated in the figure insert.* T_x *indicates the time at which the salicylic acid concentrations in the* A *and* C *chambers are equal. The heavy line segment on the time axis represents the blank flow cell containing pH 2.0 buffer only. The starting salicylic acid concentration was 18.3 mcg./ml. in the* A *chamber.*

culation purposes is the point at which the concentration of drug in the A and C chambers is equal. If one designates the time at which this equilibrium occurs as the crossing time (t_x), then at t_x, $A(t_x) = C(t_x) = a$, where a is a constant. It then follows from Eq. 4 that:

$$k_1 = \frac{\ln A_0 - \ln a}{t_x} \qquad \text{(Eq. 7)}$$

where $\ln A_0$ is the natural (base e) logarithm function.

Also, since $A(t_x) = C(t_x)$, equating Eqs. 4 and 6 at time t_x yields:

$$e^{-k_1 t_x} = \frac{V_A}{V_C(k_2 - k_1)}(k_2 - k_1 - k_2 e^{-k_1 t_x} + k_1 e^{-k_2 t_x}) \qquad \text{(Eq. 8)}$$

Substituting for k_1 the solution from Eq. 7 into Eq. 8 and solving in terms of k_2, one has:

$$\alpha k_2 + \beta e^{-k_2 t_x} + \gamma = 0 \qquad \text{(Eq. 9)}$$

where:

$$\alpha = \frac{a(V_A + V_C)}{A_0} - V_A \qquad \text{(Eq. 10)}$$

$$\beta = \frac{V_A(\ln a - \ln A_0)}{t_x} = -k_1 V_A \qquad \text{(Eq. 11)}$$

and:

$$\gamma = V_A k_1 - [V_C k_1 a / A_0] \qquad \text{(Eq. 12)}$$

Equation 9 may be easily solved iteratively since the left-hand side, when considered as a function of k_2, *i.e.*, $f(k_2)$, is strictly monotonically increasing for all $k_2 \geq 0$. Furthermore, at $k_2 = 0$:

$$f(0) = \beta + \gamma = -V_C k_1 a / A_0 < 0 \qquad \text{(Eq. 13)}$$

Therefore, the function $f(k_2)$ is always negative at zero. Thus, if a particular trial value k_2^* produces $f(k_2^*) < 0$, k_2^* must be increased; if $f(k_2^*) > 0$, k_2^* must be decreased. For programming on a digital computer, the initial k_2 can be taken to be zero and then successively increased until $f(k_2)$ changes sign.

DISCUSSION

The solutions to Eqs. 7 and 13 for k_1 and k_2 were programmed on a laboratory-type digital computer[1]. Output from the computer was obtained both as a printout of k_1 and k_2 and as a plot of drug concentration in chambers A, B, and C *versus* time. This allows a direct comparison of the calculated (or theoretical) concentrations in A and C as a function of time and the concentration in A and C determined experimentally. This method has the added advantage of allowing the investigator to see quickly how close the experimental system approximates simple first-order kinetics.

As an example of the method, salicylic acid was transported from the A chamber (pH 2.0) through a layer of 20% decanol in cyclohexane (v/v) saturated with pH 2.0 buffer to the C chamber (pH 7.4). A continuous recording of the concentration in the A and C chambers was done with flow cells read by a recording spectrophotometer. A photograph of the data appears in Fig. 1. Points taken from the graph in Fig. 1 were plotted on Fig. 2, and the smooth lines were generated by a computer using the constants derived from the data of Fig. 1. As is evident from Fig. 2, transport seems to follow the model of two consecutive first-order reactions very well.

The concentration of salicylic acid in the B layer as a function of time is included in the figure, although this layer was not monitored.

After establishing the validity and convenience of the method, six independent runs were made on six different days to evaluate the statistical day by day variability of the rate constants. The results of these runs are shown in Table I. On each individual run, theoretical and empirical curves matched at least as well as those in Fig. 2, so the variabilty is between day to day determinations. Since errors are eliminated on a particular day once this system is in progress, it must be concluded that the between run variability is caused by random errors which are other than the more usual measurement variations. Variability in such systems becomes critical when one is comparing the transport properties of a closely related homologous series of compounds; for this reason, these data are presented. It is evident that comparisons from single determinations on different compounds with closely related rate constants must be made with extreme caution, especially if measurement variability is also introduced. The estimates of variability in these data can be used to provide future experimenters with a means of establishing appropriate sample sizes to detect specified differences with statistical confidence. The correlation coefficient $r = 0.6124$ between k_1 and k_2 is significant with 80% confidence on only six runs, which suggests a future area of research.

[1] Digital Equipment PDP-12.

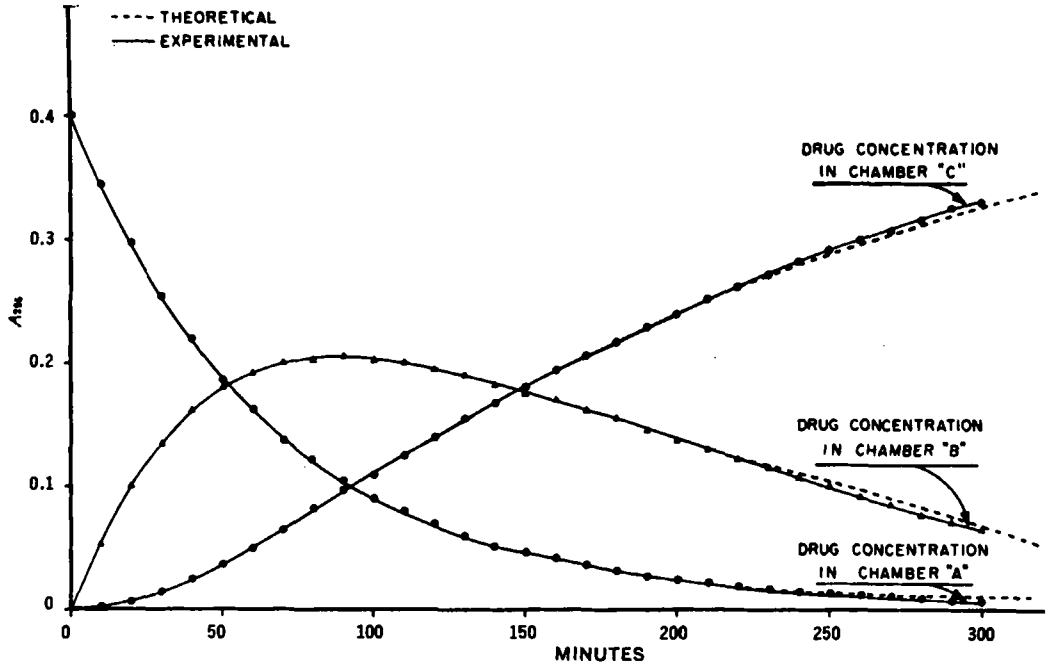


Figure 2—*Theoretical* (- - -) versus *experimental* (——) *salicylic acid transfer curves. Even though the organic layer was not monitored, the concentration–time curve was calculated from input data for comparison* (▲). *Theoretical curves were generated from Eqs. 1–3. The starting salicylic acid concentration for this experiment was 18 mcg./ml.*

The original intention was to utilize methods of calculation already present in the literature (5). However, by using the integrated rate equations which were reported as describing the forward transfer of an acidic drug, it was found that these equations did not describe the actual transfer process. It was then determined that the equations (5) do not satisfy the differential equations for the transfer process and hence are invalid.

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Synthesis and Pharmacology of a Pargyline Analog, *N*-3-(1-Butynyl)-benzylamine

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Abstract □ The synthesis of a structural isomer of pargyline was undertaken to test certain structure–activity relationships. *In vivo*, the new compound, *N*-3-(1-butynyl)-benzylamine, exhibited slight central stimulant effects but showed no MAO inhibitory activity *in vitro*.

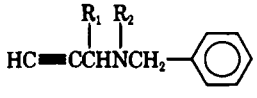
Keyphrases □ *N*-3-(1-Butynyl)-benzylamine (pargyline analog)—synthesis and pharmacology, structure–activity relationships □ Pargyline analog—synthesis and pharmacology of *N*-3-(1-butynyl)-benzylamine, structure–activity relationships □ MAO inhibitors—synthesis and pharmacology of pargyline analog, *N*-3-(1-butynyl)-benzylamine, structure–activity relationships

Pargyline (I*a*) is an MAO inhibitor which demonstrates antidepressant action and also serves as a clinically effective antihypertensive agent. Structure–activity relationships for the propynylamine series (I) indicate that when the R groups are larger than methyl, a decrease in both MAO inhibition and antidepressant activity is observed (1). The synthesis of *N*-3-(1-butynyl)-benzylamine (I*b*), a structural isomer of pargyline, was undertaken to determine whether the shift of the methyl group from the nitrogen to the adjacent carbon in the propynyl chain would greatly affect pharmacological activity. Compound I*b* was synthesized in two steps, beginning with the conversion of 1-butyn-3-ol to 3-chloro-1-butyne (in low yield) followed by condensation of the latter with benzylamine.

DISCUSSION

As an *in vivo* testing procedure, a method similar to that described by Everett and Wiegand (2) was employed. This procedure involves injection of the potential drug into mice followed by injection of dopa. The degree of increase in central activity is considered related to the effectiveness of MAO inhibition. The method has been utilized to determine indirectly MAO inhibition. Since both I*a* and I*b* have identical molecular weights, comparisons were made at equal weight dose levels. Although I*b* produced a slight increase in central activity, this increase was much less than that caused by pargyline. Even at higher toxic dose levels, this effect on the central activity was much less intense than that of pargyline. The overall results indicate that I*b* is at best a weak MAO inhibitor compared to pargyline.

Because I*b* was questionable as an MAO inhibitor, it was tested *in vitro*. Pargyline exhibited a straight-line log dose–response relationship with an ID_{50} (50% inhibition) of MAO at approximately 0.7 mcg./ml. Compound I*b* at concentrations up to 20 times the ID_{50} of pargyline demonstrated no MAO inhibitory activity. It is possible that the slight increase in central activity exhibited by I*b* was not due to MAO inhibition but rather some other unknown factor. However, the antidepressant encyprate was shown to be inactive *in vitro* but active *in vivo*, probably due to its metabolism to an active metabolite *in vivo* (3). Nevertheless, based on these results, it was concluded, in agreement with Swett *et al.* (1), that optimum activity is apparently associated with a small atom or groups of atoms, *i.e.*, hydrogen or methyl connected to the nitrogen atom, and that further *N*-alkylation or further substitution on the aliphatic chain tends to decrease activity.



I*a*: $R_1 = H$, $R_2 = CH_3$
I*b*: $R_1 = CH_3$, $R_2 = H$

EXPERIMENTAL

Pharmacology—The hydrochloride salts of both I*a* and I*b* were dissolved in distilled water. For testing *in vivo*, 152 male, albino mice (Swiss-Webster strain), weighing 20–32 g., were initially divided into groups of eight animals each. Each group was further separated into two subgroups of four mice each from which data were collected and pooled. According to a preset dosing schedule, all four mice in each group were administered various doses of the test drug intraperitoneally and placed in Plexiglas boxes, 12.7 × 12.7 × 12.7 cm. (5 × 5 × 5 in.) with adequate ventilation. At time intervals ranging from 15 min. to 4 hr. following drug administration, mice were injected with 200 mg./kg. i.p. of DL-dopa suspended in 0.25% agar-agar. Immediately following dopa administration and for the ensuing 30 min., the mice were observed as a group and behavioral changes were observed and rated similar to a procedure described by Everett and Wiegand (2). Each parameter received a numerical score: Straub tail = 1, salivation = 2, jumping = 3, and fighting = 4. The maximum total score for any group would be 10 (4 + 3 + 2 + 1), which would be related to a very high level of MAO inhibition.

Pretreatment of mice with I*b* at 40–160 mg./kg. 1 hr. prior to DL-dopa did not produce symptoms suggesting significant MAO inhibitory activity. A dose of 320 mg./kg. of I*b* resulted in a 100% mortality within 5 min.; I*a* resulted in a dose-dependent relationship, with the maximal effect occurring at the 80-mg./kg. dose. When administered by themselves, neither drug caused any of the behavioral characteristics being measured.

When DL-dopa was administered at various time intervals following intraperitoneal injection of 240 mg./kg. of I*b*, pronounced effects were observed. At the 15-min. time interval, when the increase in central activity was maximal, I*b* appeared to be one-third to one-sixth as potent as pargyline. However, the lethal effects of I*b* were evident within 30 min. and all of the animals were dead within the 4-hr. period.

For *in vitro* testing, the procedure of Lovenberg *et al.* (4) was employed, using tryptamine as a substrate. The MAO enzyme was obtained fresh from rat liver. Pargyline was tested at concentrations from 0.1 to 1.6 mcg./ml., and I*b* was tested at concentrations of 0.1 to 13 mcg./ml.

Chemistry[1]—*3-Chloro-1-butyne*—A procedure similar to that described by Hennion and coworkers (5, 6) was employed. A mixture of 1-butyn-3-ol (14.0 g., 0.20 mole), pyridine (0.3 g.), and freshly distilled thionyl chloride (23.8 g., 0.20 mole) was gently refluxed for 30 min., cooled, and poured onto 60 g. of ice water. The mixture was extracted with 30 ml. of ether, and the organic phase was washed successively with water (2 × 5 ml.), 5% aqueous sodium bicarbonate (2 × 5 ml.), and finally again with water (2 × 5 ml.). After drying the ether solution for 2 days over calcium chloride, it was distilled through a 10 × 200-mm. Vigreaux column and 3.15 g. of the frac-

[1] Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer Infracord spectrophotometer. NMR spectra were recorded on a Varian Associates A-60 spectrometer using spectroquality carbon tetrachloride as a solvent; chemical shifts are reported in parts per million (δ) relative to tetramethylsilane as an internal standard. Elemental analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich.

tion distilling at 60–61.5° was collected. Longer or shorter reaction times and higher or lower temperatures failed to produce a yield in excess of 18%. Vapor phase chromatographic analysis of the products from various experiments indicated a purity of 70–90%. Redistillation of the 60–61.5° fraction afforded little additional purity. This material was used in the reaction with benzylamine. The purest sample was employed in spectral studies; IR (neat): 2150 (—C≡CH) cm.$^{-1}$; NMR (CCl_4): 1.65 (d, 3, C—CH_3), 2.43 (d, 1, —C≡CH), and 4.50 (quartet of doublets, 1, Cl—CH).

N-3-(1-Butynyl)-benzylamine Hydrochloride—Similar to a procedure described by Hennion and Nelson (7), a mixture of the above chloro compound (2.5 g.), benzylamine (13.1 g.), and water (3.2 ml.) was stirred at room temperature for 60 hr. At the end of this time, an equal volume of water (15 ml.) was added and two phases formed. The upper layer (organic phase) was separated, and the bottom layer (aqueous phase) was diluted again with an equal volume of water. Again two phases formed and the upper layer was combined with the first. Ether (25 ml.) was added, this solution was washed with water (2 × 5 ml.) and dried over potassium hydroxide, and the ether was removed *in vacuo*. The residue was distilled through a Vigreaux column, and the fraction distilling at 168–172°/17 mm. Hg was collected; IR (neat): 3400 (>NH) and 2150 (—C≡CH) cm.$^{-1}$; NMR (CCl_4): 1.31 (d, 3, C—CH_3), 2.16 (d, 1, —C≡CH), 3.38 (quartet of doublets, 1, N—CH), 3.88 (d, 2, $ArCH_2$), and 7.22 (s, 5, ArH). This fraction was dissolved in 50 ml. of anhydrous ether, and hydrogen chloride gas was passed through the solution, giving 1.2 g. of a white solid (22% yield based on pure 3-chloro-1-butyne). Several crystallizations from benzene afforded an analytically pure sample, m.p. 210.5–211.5°.

Anal.—Calc. for $C_{11}H_{11}ClN$: C, 67.52; H, 7.16; N, 7.16. Found: C, 67.37; H, 6.97; N, 7.00.

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Synthesis and Antifungal Activity of Polyhalophenyl Esters of Pyridyl- and 4-Quinolylcarbamic Acids IV

I. LALEZARI▲, H. GOLGOLAB, A. SHAFIEE, and M. WOSSOUGHI

Abstract □ Polyhalophenyl esters of 2-, 3-, and 4-pyridyl- and 2-phenyl-4-quinolylcarbamic acids were synthesized. All prepared compounds inhibited the growth of *Candida albicans* at 50-mcg./ml. concentration.

Keyphrases □ Pyridylcarbamic acid, polyhalophenyl esters—synthesis, antifungal activity □ 2-Phenyl-4-quinolylcarbamic acid, polyhalophenyl esters—synthesis, antifungal activity □ Carbamic acid esters—synthesis, antifungal activity □ Antifungal agents, potential—polyhalophenyl esters of pyridyl- and 2-phenyl-4-quinolylcarbamic acids

In a continuation of the studies on the chemistry and antifungal activity of substituted carbamic acid esters (1–3), polyhalophenyl esters of 2-, 3-, and 4-pyridyl- and 2-phenyl-4-quinolylcarbamic acids were synthesized by interaction of the appropriate azide and polyhalophenol (Scheme I).

$$RCOCl \rightarrow RCON_3 \rightarrow RNHCO_2Ar$$

R = 2-, 3-, or 4-pyridyl or 2-phenyl-4-quinolyl
Ar = 2,4,6-tribromophenyl, 2,4,6-trichlorophenyl, 2,4,6-triiodophenyl, or pentachlorophenyl

Scheme I

EXPERIMENTAL[1]

Picolinyl azide was prepared according to Meyer and Mally (4). Nicotinyl azide was prepared by the method of Curtius and Mohr (5). Isonicotinyl azide was synthesized according to Yoshikowa (6). 2-Phenyl-4-quinolylcarboxazide was obtained by the method of John *et al.* (7).

2-Pyridylcarbamic Acid Pentachlorophenyl Ester—2-Pyridyl azide, 0.74 g. (5 mmoles), and 1.33 g. (5 mmoles) of pentachlorophenol in 30 ml. of dry toluene were gently refluxed for 30 min. After evaporation of the solvent under reduced pressure, the residue was recrystallized from 80% ethanol to give 1.16 g. (60%), m.p. 135°; *m/e* 384, 386, 388, and 390; ν_{max}: 2900, 1710, 1620, 1540, 1470, 1440, 1410, 1360, 1320, 1250, 1150, 995, 980, 880, 778, and 715 cm.$^{-1}$.

3-Pyridylcarbamic Acid 2,4,6-Triiodophenyl Ester—This compound was prepared in a similar manner to its pentachlorophenyl analog; *m/e* 592; ν_{max}: 3300, 1750, 1580, 1550, 1480, 1420, 1200, 1060, 1020, 850, 810, 780, 740, and 703 cm.$^{-1}$.

4-Pyridylcarbamic Acid 2,4,6-Triiodophenyl Ester—This compound was prepared in a similar manner to its pentachlorophenyl analog; *m/e* 592; NMR (dimethyl sulfoxide): τ 7.9–8.1 (d, 2H,

[1] Melting points were taken on a Kofler hot-stage microscope and are uncorrected. The IR spectra were determined with a Leitz model III spectrograph. NMR spectra were obtained on a Varian A60A instrument. Mass spectra were determined with a Varian Mat 111 instrument.

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Synthesis and Antifungal Activity of Polyhalophenyl Esters of Pyridyl- and 4-Quinolylcarbamic Acids IV

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Table I—Polyhalophenyl Esters of Pyridyl- and 4-Quinolylcarbamic Acids

R	Ar	Melting Point	Yield, %	Formula[a]	RNHCO$_2$Ar Calc.	RNHCO$_2$Ar Found
2-Pyridyl	2,4,6-Trichlorophenyl	142°	25	$C_{12}H_7Cl_3N_2O_2$	C 45.35	45.30
					H 2.20	2.22
2-Pyridyl	2,4,6-Tribromophenyl	115°	20	$C_{12}H_7Br_3N_2O_2$	C 31.92	32.04
					H 1.55	1.60
2-Pyridyl	2,4,6-Triiodophenyl	140°	25	$C_{12}H_7I_3N_2O_2$	C 24.30	24.29
					H 1.18	1.11
2-Pyridyl	Pentachlorophenyl	135°	60	$C_{12}H_7Cl_5N_2O_2$	C 37.25	37.30
					H 1.29	1.28
3-Pyridyl	2,4,6-Trichlorophenyl	225°	25	$C_{12}H_7Cl_3N_2O_2$	C 45.35	45.33
					H 2.20	2.19
3-Pyridyl	2,4,6-Tribromophenyl	145°	30	$C_{12}H_7Br_3N_2O_2$	C 31.92	31.88
					H 1.55	1.62
3-Pyridyl	2,4,6-Triiodophenyl	165°	45	$C_{12}H_7I_3N_2O_2$	C 24.30	24.32
					H 1.18	1.20
3-Pyridyl	Pentachlorophenyl	90°	30	$C_{12}H_5Cl_5N_2O_2$	C 37.25	37.25
					H 1.29	1.31
4-Pyridyl	2,4,6-Trichlorophenyl	146°	60	$C_{12}H_7Cl_3N_2O_2$	C 45.35	45.38
					H 2.20	2.27
4-Pyridyl	2,4,6-Tribromophenyl	160°	20	$C_{12}H_7Br_3N_2O_2$	C 31.92	31.99
					H 1.55	1.48
4-Pyridyl	2,4,6-Triiodophenyl	145°	50	$C_{12}H_7I_3N_2O_2$	C 24.30	24.33
					H 1.18	2.01
4-Pyridyl	Pentachlorophenyl	130°	35	$C_{12}H_5Cl_5N_2O_2$	C 37.25	37.37
					H 1.29	1.21
2-Phenyl-4-quinolyl	2,4,6-Trichlorophenyl	167°	80	$C_{22}H_{13}Cl_3N_2O_2$	C 59.52	60.01
					H 2.93	3.03
2-Phenyl-4-quinolyl	2,4,6-Tribromophenyl	175°	65	$C_{22}H_{13}Br_3N_2O_2$	C 45.75	45.60
					H 2.25	2.26
2-Phenyl-4-quinolyl	2,4,6-Triiodophenyl	180°	45	$C_{22}H_{13}I_3N_2O_2$	C 36.76	36.70
					H 1.81	1.86
2-Phenyl-4-quinolyl	Pentachlorophenyl	135°	60	$C_{22}H_{11}Cl_5N_2O_2$	C 51.51	51.40
					H 2.14	2.22

[a] IR, NMR, and mass spectra of all compounds were as expected.

α-hydrogens), 7.69 (s, 2H, aromatic H), 7.35 (s, 1H, NH), and 6.65–6.85 (d, 2H, β-hydrogens).

2-Phenyl-4-quinolylcarbamic Acid 2,4,6-Tribromophenyl Ester—This compound was prepared in a similar manner to the pyridyl analog; ν_{max}: 3330, 3050, 1755, 1615, 1560, 1538, 1445, 1380, 1280, 1200, 995, 860, 810, 775, 765, 745, and 690 cm.$^{-1}$.

All other compounds were prepared similarly. The physical data of prepared compounds are listed in Table I.

All prepared compounds were tested against *Candida albicans*[1] *in vitro* using BBL Sabouraud dextrose agar medium. Concentrations of 10 and 50 mcg./ml. of each compound were used. Compounds were dissolved in acetone, diluted with hot culture medium to the desired concentration, and autoclaved at 120° for 1 hr.

Pentachlorophenyl esters of 2-pyridyl- and 2-phenyl-4-quinolylcarbamic acids inhibited the growth at 10 mcg./ml. concentrations against the blank. Slight growth inhibition was observed with other compounds at the same concentrations.

After 5 days at 28°, the growth was entirely inhibited with 50-mcg./ml. concentrations of all compounds prepared.

[1] This microorganism was obtained from the Department of Parasitology, Public Health Institute, Tehran, Iran.

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Labile Triazinoindole Hydrates

H. C. CALDWELL

Abstract □ Aqueous topical suspensions of two triazinoindoles in fine particle form produced crystals even when the protective colloid methylcellulose was added. These crystals were found to be monohydrates that readily lost water of hydration under mild heat, but they could be air milled with no water loss. One hydrate was formulated in suspension and found to be free of crystal growth for 2 years.

Keyphrases □ Triazinoindole hydrates, labile—formation, isolation from topical suspensions, effects of air milling and methylcellulose on crystal growth □ Hydrates, labile—formation, isolation from topical triazinoindole suspensions, effects of air milling and methylcellulose on crystal growth □ Suspensions of labile triazinoindole hydrates—formation, isolation, effects of air milling and methylcellulose on crystal growth

Over 90 hydrates are included in USP XVIII and NF XIII. Four of these that readily lose water of hydration in warm dry air are atropine sulfate monohydrate, morphine sulfate pentahydrate, scopolamine hydrobromide trihydrate, and sodium acetate trihydrate. These, like other official hydrates, are usually stored in tight, light-resistant containers.

According to the USP and NF, a tight container protects the contents from contamination by extraneous liquids, solids, or vapors; from loss of the drug; and from efflorescence, deliquescence, or evaporation under ordinary or customary conditions of handling, shipment, storage, and distribution. It is also capable of tight reclosure. Thus, official hydrates properly packaged and stored should not lose moisture. Further, it is reasonable to assume that these hydrates exist as such in formulations.

It is important to recognize that many official compounds form hydrates that readily lose water when dried under mild conditions. The review (1) on hydrates of alcohols and glycols is illustrative. These labile hydrates become particularly important when aqueous suspensions of slightly soluble, anhydrous chemicals are formulated, because these hydrates can produce large particles as they form (2). These large particles would have less surface area for a given dose, and the rate and extent of absorption could be markedly decreased (3). Protective colloids might be expected to retard but not stop this transformation (2, 4).

This report presents a case history of suspension work on two triazinoindoles (I[1] and II[2]) and their labile hydrates. Ravin *et al.* (4) recently described the physical-chemical evaluation of I. They reported that it is practically insoluble (0.008 mg./ml.), that it exists as two polymorphs, and that it forms a hydrate.

The present tasks were to prepare and to evaluate formulations for oral and nasal use. The most stable polymorph of I was used, but it was not known then that I or II formed hydrates.

EXPERIMENTAL

Particle-Size Reduction—The purified compounds were passed through a fluid energy mill[3]. The products were sized with the Coulter counter[4].

Suspensions—The milled chemicals were suspended in buffered vehicles containing a preservative and a protective colloid.

Hydrate Preparation—Small batches of I-hydrate and II-hydrate were made by suspending 5 g. of air-milled I and II in 750 ml. of distilled water and heating the suspensions overnight on the steam bath. The small anhydrous crystals were converted to long hydrate needles. Larger batches of I-hydrate were made by dissolving I in dilute hydrochloric acid and precipitating it with dilute sodium hydroxide solution. The suspension was stirred overnight at room temperature. Samples were collected and air dried at room temperature.

X-Ray Diffraction Procedure—The procedure employed was described (4) previously but was modified so that intensities were not measured by recording the time necessary to count a fixed number of particles.

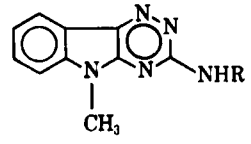
Thermal Work—A differential thermal analyzer[5] and a thermogravimetric analyzer[6] were used. Heat rates were 20 and 5°/min., respectively. A thermobalance[7] was used to follow weight loss as a function of temperature.

Isolation of Hydrates from Topical Suspensions—Suspensions made with I and II were followed for chemical and physical changes at various storage conditions. Samples that appeared, by microscopic examination, to have completely changed from small to large particles were diluted with water, filtered, washed, and dried at room temperature.

RESULTS AND DISCUSSION

Suspensions of air-milled anhydrous I and II were found to produce large crystals even in the presence of classical crystal growth inhibitors such as methylcellulose. To determine if hydrate formation was responsible, it was necessary to isolate large crystals from I and II suspensions and compare them to crystals of authentic hydrates of I and II.

These data are reported in Tables I and II. Both hydrates of I and both hydrates of II (Table II) analyze as monohydrates. In the thermal gravimetric analysis, all hydrates start to lose weight at relatively low temperature and each loses weight equivalent to 1 mole of water. As expected, the anhydrous forms do not lose weight. In the differential thermal analysis, the hydrate of I shows transitions at about 108–117°, which are not seen with the anhy-



I: R = $(CH_2)_2$—$C(CH_3)_2$—OH
II: R = $(CH_2)_3$—OH

[1] SK&F 30097.
[2] SK&F 21687.

[3] Trost jet mill, Helme Products, Inc., Helmetta, N. J.
[4] Model B.
[5] Du Pont 900.
[6] Du Pont 950.
[7] Perkin-Elmer TGS-1.

Table I—Thermal Gravimetric Analysis (TGA) and Differential Thermal Analysis (DTA) Studies on I and II and Their Hydrates

Number	Compound	Description	—Weight Loss by TGA— Onset of Loss	End of Loss	Loss, %	DTA, Transitions				
1	I	Air-milled base	—	—	0	—	—	171°	—	192.5°
2	I-hydrate	Isolated from topical suspension, air dried at room temperature	34°	115°	5.9[a]	117°	—	168°	—	190°
3	I-hydrate	Air-milled No. 2	52°	96°	5.7	108°	—	167.5°	—	189°
4	I-hydrate	Prepared in warm water, air dried at room temperature	50°	78°	5.9	112.5°	—	169°	—	191°
5	I-hydrate	Air-milled No. 4	57°	88°	5.9	111.5°	—	170°	—	192°
6	II	Air-milled base	—	—	0	23[b]	—	—	—	164.5°
7	II-hydrate	Isolated from topical suspension, air dried at room temperature	32°	118°	6.35[c]	23[b]	—	105°	125°	163°
8	II-hydrate	Prepared in warm water, air dried at room temperature	32°	104°	6.75	23[b]	100°	107.5°	126°	163°

[a] 5.95% theoretical for monohydrate. [b] Exotherm—others are endotherms. [c] 6.54% theoretical for monohydrate.

drous form, and the hydrate of II shows transitions at about 105 and 125°, which are not seen with the anhydrous form. Thus, these data show that suspensions of I and II form hydrates even in the presence of protective colloids. X-ray diffraction patterns for II and II-hydrate are distinctly different, as Fig. 1 shows. X-ray diffraction patterns for I and I-hydrate were reported (4) to be different previously. Weight loss as a function of temperature was determined. Samples were placed in the thermobalance, and percent weight loss was recorded after 15 min. at various temperatures. I-hydrate (air milled) lost no weight at 45°, 1% at 50°, 4% at 55°, and all (5.95%) at 60°. II-hydrate lost no weight at 25°, 0.8% at 28°, 1.5% at 33°, 6.2% at 45°, and all (6.54%) at 60°. Hydrous lactose USP lost no weight at 45° and 1% at 60°, while hydrous sodium citrate lost no weight at 60°.

These data show that the hydrates of I and II are heat labile, and they explain why hydrate samples dried at 60° would be rapidly converted to anhydrous forms.

Because the anhydrous forms of I and II were converted to hydrates in suspension, it was reasonable to think that suspensions of hydrates would be stable with respect to crystal growth. But one other problem had to be solved before suspension work could begin. It had to be established that the hydrates of I and II could be air milled with no water of hydration loss. Three batches of I-hydrate were passed through the air mill with no water loss, as determined by Karl Fischer titration and weight loss on drying. Two batches of II-hydrate treated in the same manner also did not lose water.

Once it had been established that the large crystals formed in suspensions of I and II were the respective hydrates and that the

Table II—Elemental Analysis of Hydrates

Number[a]	Compound	Description		—Analysis, %— Calc.	Found
2	I-hydrate	Isolated from topical suspension, air dried at room temperature	C	59.39	59.45
			H	6.98	6.92
			N	23.09[b]	23.02
4	I-hydrate	Prepared in warm water, air dried at room temperature	C	59.39	59.25
			H	6.98	6.89
			N	23.09[b]	23.09
7	II-hydrate	Isolated from topical suspension, air dried at room temperature	C	56.72	56.82
			H	6.22	6.26
			N	25.44[c]	25.44
8	II-hydrate	Prepared in warm water, air dried at room temperature	C	56.72	56.92
			H	6.22	6.28
			N	25.44[c]	25.61

[a] Numbers are from Table I. [b] Calculated for $C_{15}H_{19}N_5O_2 \cdot H_2O$. [c] Calculated for $C_{13}H_{15}N_5O \cdot H_2O$.

Table III—I-Hydrate Suspension

Ingredients	% w/w
I-hydrate (air milled)	2.658[a]
Methylcellulose[b]	1.000
Sodium citrate USP	0.200
Potassium biphthalate, reagent	0.130
Eucalyptol	0.020 v/v
Thimerosal NF	0.001
Sodium chloride USP	0.810
Water for injection, *q.s.* ad	100.000
pH	5.4
Freezing-point depression	0.61°

[a] Equivalent to 2.500% anhydrous I. [b] Methocel, Type MC Premium 15 cps.

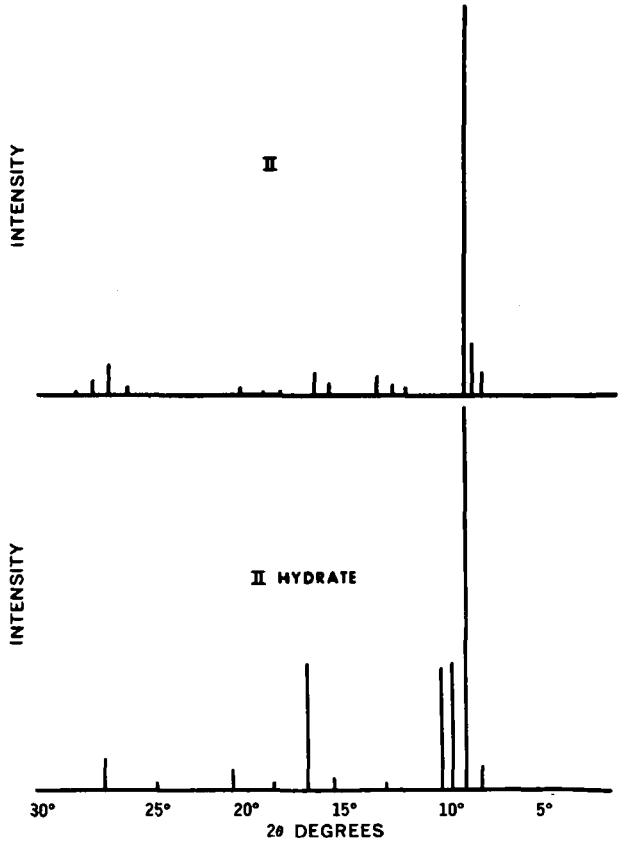


Figure 1—*X-ray diffraction patterns for anhydrous and hydrate forms of II.*

hydrates could be air milled, the author proceeded to prepare suspensions with air-milled hydrate to see if crystal growth still occurred. Work was done with I-hydrate, because at that time development work on II had been discontinued. A 2.5% suspension of air-milled I-hydrate was made (Table III) and observed for 2 years by microscopic examination and particle-size analysis. No evidence for crystal growth was observed.

This case history shows that suspensions are dynamic systems and that it is important to establish which forms are present at equilibrium. It shows that careful work is needed to identify these forms and prove that they can be processed to specifications prior to suspension formulation work. In addition, this report shows that satisfactory suspensions can be made with these equilibrium forms.

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Keyphrases □ Thiadiazoles, bicyclic—synthesis of 2-(2-thienyl)-5-phenylthiazolo[2,3-*b*]-1,3,4-thiadiazole, evaluated as a potential diuretic □ 2-(2-Thienyl)-5-phenylthiazolo[2,3-*b*]-1,3,4-thiadiazole—synthesis, screened as potential diuretic □ Diuretics, potential—synthesis and screening of 2-(2-thienyl)-5-phenylthiazolo[2,3-*b*]-1,3,4-thiadiazole

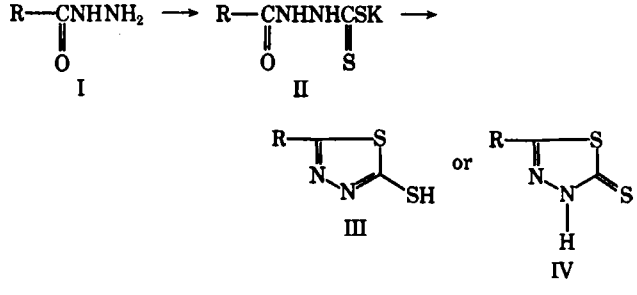
Thiazole and thiadiazole derivatives have been used successfully as diuretics (1–4). Synthesis of 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (acetazolamide) by Roblin and Clapp (1) led to many acetazolamide derivatives as potential diuretics (5, 6). Heterocyclic mono- and disulfonamides, particularly thiophene and benzothiophene analogs, thiazole and benzothiazole analogs, and thiazides have proved useful as diuretics (7).

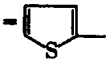
These facts prompted us to synthesize a bicyclic thiadiazole to see the effect of the condensed thiadiazole and thiazole nucleus on diuretic activity. The compound synthesized exhibited significant diuretic activity.

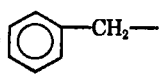
DISCUSSION

Treatment of 2-thiophenecarboxylic acid hydrazide (I*a*) and phenylacetic acid hydrazide (I*b*) with carbon disulfide and potassium hydroxide gave the desired unstable potassium dithiocarbazates II*a* and II*b* (8). Without further purification, these salts were cyclized with boron trifluoride etherate (8) to give 2-(2-thienyl)-5-thio-1,3,4-thiadiazole (III*a*) and 2-benzyl-5-thio-1,3,4-thiadiazole (III*b*). The IR of Compounds III*a* and III*b* showed the presence of the (C=S) band at 7.55 μ, the (C—NH) band at 7.05 μ, and (NH) vibration at 3.5 μ, which is only compatible with thione Structures IV*a* and/or IV*b* (Scheme I).

Reaction of 1,3,4-thiadiazole-5-thiol with 2-halogeno ketones was found to be an effective route for the synthesis of a bicyclic system (V). 2-Substitution of the 1,3,4-thiadiazole nucleus had a pronounced effect on the ease of ring closure. Compound III*a* with phenacyl bromide gave 2-(2-thienyl)-5-phenylthiazolo[2,3-*b*]-1,3,4-thiadiazole (V) using a 6-hr. reaction period (Scheme II). Under the same conditions, 2-benzyl-5-thio-1,3,4-thiadiazole (III*b*) gave an intermediate 2-benzyl-5-(phenacylthio)-1,3,4-thiadiazole (VI) (Scheme III). Increasing the reaction time to 24, 36, and 48 hr. resulted in the same product (VI). This observation can also be substantiated by the reaction of 2-furyl-5-thiol-1,3,4-thiadiazole with phenacyl bromide, which is not reported in this paper but was observed in this laboratory. This reaction also resulted in a compound



I*a*, II*a*, III*a*, and IV*a*: R = 

I*b*, II*b*, III*b*, and IV*b*: R = 

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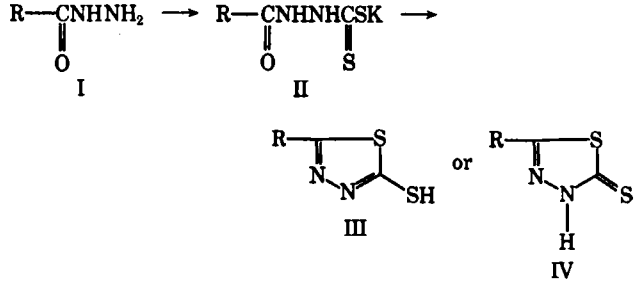
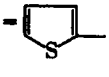
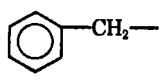


I*a*, II*a*, III*a*, and IV*a*: R = 

I*b*, II*b*, III*b*, and IV*b*: R = 

Scheme I

Table I—Urine Volume, Sodium Output, and Potassium Output at 6 Hr. following Intramuscular Injection of Compound V

Treatment	Mean Urine Volume (ml.) for Group of Five Rats	Mean Urine Electrolyte Concentration, meq./l.		Paired *t* Probabilities		
		Sodium	Potassium	Volume	Sodium	Potassium
Control	3.9	24	3.4	<0.0025	>0.15	<0.025
10 mg./kg.	5.4	31	5.0	—	—	—

having a phenacylthio side chain. Attempts to cyclize VI with phosphoryl chloride in xylene were unsuccessful in this system (*9*, *10*). The identity of Compound V was established by NMR. The chemical shifts of C_6 and C_5 protons are δ 7.10 (s) and 2.50 (s), respectively.

PHARMACOLOGICAL EVALUATION

The diuretic activity of 2-(2-thienyl)-5-phenylthiazolo[2,3-*b*]-1,3,4-thiadiazole (V) was studied in male Sprague-Dawley rats (165–190 g.) by measurement of the effect on the volume of urine and the urinary output of sodium and potassium. The test compound was suspended in 5% acacia and administered intramuscularly to five rats at a dose of 10 mg./kg. in a volume not exceeding 0.2 ml. Animal subjects were fasted for 18 hr. prior to use and were given a 5-ml. injection of 0.9% saline intraperitoneally immediately before the drug injection. They were housed individually in metabolism cages, and urine was collected in volumetric vessels. Levels were recorded at 60-min. intervals, and at the end of 6 hr. the final urine volumes were recorded and the sodium and potassium concentrations were determined by flame photometer. Control levels of urine volume and electrolyte output were recorded for each experimental rat prior to use in the evaluation of the experimental compound. Table I presents data from the studies described above.

Interval measurements of urine volume indicated that diuretic action began at approximately 4 hr. All mean values (volume, sodium, and potassium) were above control levels at 6 hr. Paired *t* tests, however, did not indicate statistical significance in the sodium output change.

EXPERIMENTAL[1]

2-(2-Thienyl)-5-thio-1,3,4-thiadiazole (IIIa)—To a solution of 2-thiophenecarboxylic acid hydrazide (*Ia*) (11.4 g., 0.08 mole) and potassium hydroxide (9.8 g.) in methanol (160 ml.) was added carbon disulfide (10 ml.). The solution was stirred at room temperature for 5 hr., diluted with cold ether (800 ml.), and cooled in the refrigerator for 2 hr. It was then filtered. The precipitate was washed with cold ether and dried. Without further purification, the potassium dithiocarbazate (*IIa*) [yield 15.2 g. (75%), m.p. 284–285°] was suspended in methylene chloride (750 ml.) under a nitrogen atmosphere. After 80 ml. of boron fluoride ethyl ether was added, the mixture was stirred for 18 hr., poured onto a mixture of ice and water, and extracted with ether. The ether layers were combined and extracted with potassium hydroxide (10%). The alkaline solution was acidified with cold hydrochloric acid (10%). A white precipitate formed which was filtered, washed with water, and dried. The crude product, on recrystallization from benzene, yielded 2.1 g. (17%) of analytical sample, m.p. 193–194°. The IR spectrum (KBr) showed absorptions at 6.50 and 6.70 (typical of thiadiazole), 7.55 (C=S), 7.05 (C—NH), and 3.50 (NH) μ.

Anal.—Calc. for $C_6H_4N_2S_2$: C, 35.97; H, 2.01; N, 13.98. Found: C, 36.02; H, 2.12; N, 13.83.

Similarly, 2-benzyl-5-thio-1,3,4-thiadiazole (*IIIb*) was obtained in 30% yield. Recrystallization from benzene afforded needles, m.p. 118–119°; IR (KBr): 6.40, 6.60, 7.60, and 7.00 μ.

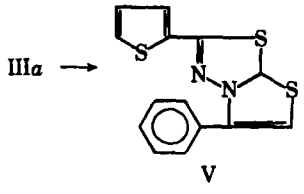
Anal.—Calc. for $C_9H_8N_2S_2$: C, 51.89; H, 3.87; N, 13.45. Found: C, 51.80; H, 3.87; N, 13.62.

2-(2-Thienyl)-5-phenylthiazolo[2,3-*b*]-1,3,4-thiadiazole (V)—A solution of *IIIa* (1.0 g., 0.005 mole) and phenacyl bromide (1.0 g., 0.005 mole) in absolute ethanol (40 ml.) was refluxed for 6 hr. and left at room temperature for 3 days. Light-pink needles separated. Recrystallization from benzene afforded an analytical sample, m.p. 134–135°, yield 0.5 g. (32%); IR (KBr): 6.00, 6.30, 6.80, and 6.95 μ.

Anal.—Calc. for $C_{14}H_{10}N_2S_2$: C, 55.59; H, 3.33; N, 9.26. Found: C, 55.30; H, 3.23; N, 9.01.

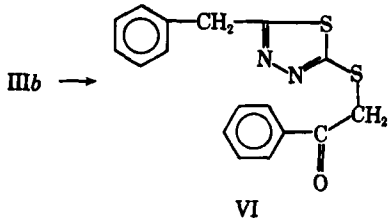
2-Benzyl-5-(phenacylthio)-1,3,4-thiadiazole (VI)—A procedure similar to the one used for Compound V was followed. The reaction time was increased to 36 hr. The crude material, on recrystallization from benzene, gave a pure sample, m.p. 105–106°, yield 80%; IR (KBr): 5.80 (C=O), 6.30 (C=N), 6.40, and 6.75 μ.

Anal.—Calc. for $C_{17}H_{14}N_2OS_2$: C, 62.54; H, 4.32; N, 8.58. Found: C, 62.48; H, 4.10; N, 8.56.



V

Scheme II



VI

Scheme III

[1] Reported melting points are uncorrected. A Thomas-Hoover Uni-Melt apparatus was used for melting-point determinations. Galbraith Laboratories, Inc., Knoxville, Tenn., conducted the elemental analyses. IR spectra were taken on Perkin-Elmer model 137-B Infracord spectrophotometer. The NMR spectra were taken on Varian XL-100 NMR spectrometer.

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▲ To whom inquiries should be directed.

Influence of Dosage Form on Antipyretic Activity of Acetaminophen Administered Intraperitoneally in the Rat

MICHAEL H. STOLAR▲, FREDERICK SIEGEL, and RALPH W. MORRIS

Abstract □ The relatively insoluble drug, acetaminophen, administered as a solution and injected intraperitoneally in rats, has been shown to produce an antipyretic response approximately twice that of the same dose administered as a suspension. This experiment indicates that ED_{50}, LD_{50}, and relative potency calculations may be inaccurately determined if dosage form influences on biological availability of insoluble drugs are not considered in the design and evaluation of animal drug testing procedures.

Keyphrases □ Acetaminophen—influence of dosage form on antipyretic activity, bioavailability, and potency calculations, intraperitoneal administration, rat □ Dosage forms—influence on antipyretic activity, bioavailability, and potency calculations, intraperitoneal acetaminophen administration, rat □ Antipyretic activity, acetaminophen—influence of dosage form, related potency calculations and bioavailability considerations, intraperitoneal administration, rat

Failure to maximize absorption of insoluble drugs by proper dosage form design may result in inaccurate determinations of drug potency and activity. The influence of dosage form design on drug activity has been extensively investigated in animals and man (1). The time course of a drug response has been shown to be influenced by factors other than the magnitude of the dose administered. Particle size, for example, has been demonstrated to have a striking effect on biological activity. Tablets prepared from micronized particles of spironolactone are approximately four times as potent as tablets made from coarser drug powders, illustrating that biological activity is generally inversely related to particle size (2).

Thus, it is apparent that improper dosage form design may produce significant alterations in the amount of drug available for transport to the site of action, *i.e.*, the bioavailability of the drug is altered. Drugs can only diffuse through membrane systems as single molecules[1]. A drug suspension consisting of an insoluble powder dispersed in a liquid vehicle (usually aqueous) represents not single molecular units but comparatively large aggregates of drug molecules held together by surface forces or crystalline bridges forming a particle. These particles are too large to diffuse through body membrane systems and must dissolve before absorption can occur. At a given time, the amount of drug in solution below the concentration at saturation is a function of the surface area of the particles, surface area being inversely related to particle size (3).

Such considerations are of importance primarily for drugs of limited aqueous solubility. Ingestion of a sugar cube would probably result in about the same blood level profile as a comparable dose of sugar syrup simply because the cube rapidly and completely dissolves in the GI fluids. With insoluble drugs, however, a significant amount of time is required for the particles to dissolve. The rate at which the primary drug particles dissolve, dissolution rate, is often the rate-limiting step for insoluble drugs in the absorption process. It is well established that the dissolution rate and, hence, the rate of absorption are directly related to particle surface area, which in turn is inversely related to particle size.

Administration of an insoluble drug as a solution rather than as a suspension increases, in effect, the surface area toward infinity and should theoretically produce faster absorption and higher blood levels of drug even though the total amount of drug ultimately absorbed may be the same for both dosage forms[2].

Investigations of the influence of dosage form in dose–response data have centered upon the oral and, to a lesser extent, the intramuscular and subcutaneous routes of administration. This is to be expected because these are the most common methods, along with the intravenous route, of administering drugs to humans. However, in many drug testing procedures involving animals, the preferred route of administration is the intraperitoneal route.

Inspection of the literature pertaining to drug screening procedures and bioassay shows that dosage form considerations are generally neglected. Typically, the testing of a group of drugs entails dissolving those drugs

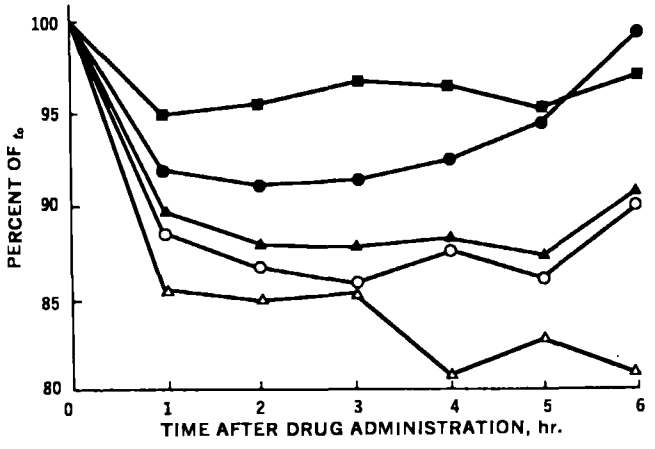

Figure 1—*Mean rectal temperature of rats expressed as percent of* t_0. *Key:* ○, *200 mg./kg. of solution;* △, *400 mg./kg. of solution;* ●, *200 mg./kg. of suspension;* ▲, *400 mg./kg. of suspension; and* ■, *control vehicle.*

[1] With the exception of special situations such as oral polio vaccine which is probably absorbed through the gut wall by a phagocytic process.

[2] Blood levels of drug are determined by a complex interrelationship between absorption, biotransformation, and excretion mechanisms; the level of drug in the body increases when the latter two are exceeded by the rate of absorption.

Table I—Analysis of Variance Table

Variation	*df*	*SS*	*MS*	*F*	*p*
Between formulations	4	0.05605	0.01401	55.4	<0.001
Group A *versus* Group C	1	0.00775	0.00775	30.6	<0.001
Group B *versus* Group D	1	0.00634	0.00634	25.1	<0.001
Between times	5	0.00265	0.000530	2.10	0.10
Interaction (formulation × time)	20	0.00586	0.000293	1.15	—
Between rats (error)	90	0.02276	0.000253	—	—
Total	119	0.08732	—	—	—

that are soluble and preparing simple tragacanth or methylcellulose suspensions of the insoluble drugs. Thus, the possibility exists that intraperitoneal injection of slightly soluble drugs as suspensions rather than solutions will result in reduced pharmacological activity by virtue of the fact that the rate of absorption and, therefore, the amount of drug absorbed during the test period is quite low, even though the peritoneal membranes present a large absorbing surface. This is a result of the particle-size considerations already discussed. It is conceivable then that a drug might be discarded as inactive due to the dosage form used in its evaluation rather than because of any inherent lack of biological activity. At the very least, the determination of parameters such as LD_{50} and ED_{50} will be in error. In this report, data are presented demonstrating the misleading results that may be obtained from a bioassay procedure if precautions to ensure optimum drug absorption are not observed.

EXPERIMENTAL

Acetaminophen[3] was used as a test drug since it is of limited aqueous solubility and possesses good antipyretic activity, a convenient response to measure. Twenty female, albino rats (approximately 300 g.) were marked and randomly divided (*via* a table of random numbers) into five groups of four animals each. Fever was produced in the rats by the subcutaneous injection of 15% brewer's yeast suspension at a dose of 1 ml./100 g. body weight (4). Three test formulations were prepared: a solution of acetaminophen in 75% propylene glycol, a 0.5% tragacanth suspension of acetaminophen in saline, and a control formulation containing the solution vehicle plus tragacanth but without acetaminophen.

Ten hours after the yeast injection, intraperitoneal injections were given to the rats as follows: Group A, acetaminophen solution, 200 mg./kg.; Group B, acetaminophen solution, 400 mg./kg.; Group C, acetaminophen suspension, 200 mg./kg.; Group D, acetaminophen suspension, 400 mg./kg.; and Group E, control vehicle at dosage volume equivalent to that of the 400-mg./kg. groups on a body weight basis.

Rectal temperatures of the animals were recorded[4] at 1-hr. intervals for 6 hr. Room temperature during the experiment was 24 ± 0.5°.

RESULTS AND DISCUSSION

Analysis of variance was used to analyze the results. The experimental differences tested were: (*a*) differences in response between all drug formulations and between Groups A and C and Groups B and D specifically (the primary objective of the experiment was to determine whether a dose of an insoluble drug administered intraperitoneally as a suspension would produce the same response when administered intraperitoneally as a solution), (*b*) differences in response between times after administration, *i.e.*, the change in antipyretic effect with time, and (*c*) interaction of formulation and time, *i.e.*, to determine if the differences in response between formulations would remain constant over the test period. If the interaction term was found to be significant, the specific interaction for formulations A *versus* C and B *versus* D would be tested.

Analysis of variance methods assume that the data are normally distributed and that the variances are homogeneous. Since dose–response data generally follow a log–normal curve, the dosage data were transformed to $\log_{10}$ values. Bartlett's test was used to test the data for homogeneity of variances. Chi-square was calculated to be 0.0718, a value that is not statistically significant ($\chi^2_{0.99(4)} = 0.297$). The variances are thus assumed to be equally distributed.

The temperature measurements were converted to percent of t_0, where t_0 = mean temperature of the rats at the time of drug injection. This transformation was corrected for the variation in fevers produced by the yeast. Figure 1 shows that injection of acetaminophen as a solution produces a much larger percent reduction in temperature than the corresponding dose administered as a suspension. A dose of 200 mg./kg. of solution produced approximately the same response as 400 mg./kg. given as a suspension.

The analysis of variance data are shown in Table I. The interaction term, although suggestive, is not statistically significant. Therefore, the variation between rats was used as the error term to test the other differences. Differences in antipyretic response between times after administration were found to be significant at the 0.10 level. This was to be expected, although the differences were somewhat less than anticipated. Formulation differences (the primary interest of this study) were highly significant at less than the 0.001 level.

The results demonstrate that an insoluble drug, upon intraperitoneal injection, will produce a greater biological response if administered as a solution rather than as a simple aqueous suspension. One may speculate that this is due to the large particle size of the suspended drug powder acting to decrease the amount of drug available for absorption during the test period. Calculation of ED_{50}, LD_{50}, and relative potency values may be adversely affected by neglect of dosage form design. Drug testing procedures involving the intraperitoneal injection of insoluble or slightly soluble drugs may thus be significantly improved by the simple procedure of altering the formulation vehicle.

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[3] Lot 702-1, Eastman.

[4] Using a Yellow Springs Telerecorder 43TD with a thermistor probe 402.

New Compounds: Synthesis of 1-Aryl Substituted Hydantoins

JAY NEMATOLLAHI[▲], ZARINE MEHTA, and JOHN LANGSTON

Abstract □ A series of 1-aryl substituted hydantoins was synthesized by condensation of monosubstituted ureas and glyoxal. The method is facile and applicable for the preparation of 1-alkyl substituted hydantoins as well as analogs of the compounds.

Keyphrases □ Hydantoins, 1-aryl substituted—synthesis, condensation of monosubstituted ureas and glyoxal □ 1-Aryl-substituted hydantoins—synthesis, condensation of monosubstituted ureas and glyoxal

Because hydantoins manifest a variety of biological activities and function as intermediates in the preparation of some important organic compounds, their chemistry has been investigated extensively. Few reports of the chemistry of 1-aryl substituted hydantoins[1] (1), however, are available. This fact may be ascribed to the difficulty in synthesizing these compounds by the usual procedure involving reaction of an *N*-substituted glycine and an isocyanate.

This report describes a synthetic method for the synthesis of a few representative 1-aryl substituted hydantoins. The method is facile, unequivocal, and applicable to the synthesis of alkyl or aryl analogs of the compounds listed in Table I. It consists of an acid-catalyzed condensation of a substituted urea with glyoxal in relatively polar medium. Some small quantities (less than 5%) of a 1,3-disubstituted tetrahydroimidazo[4,5-*d*]imidazole-2,5-dione (2) and a 3-substituted hydantoin which were formed along with the 1-substituted isomer, were separated simply by fractional crystallization.

The structures of the compounds were elucidated by elemental analysis and spectral data. The NMR spectra were particularly useful both for determining the relative quantity of 1- and 3-substituted isomers present in the product of the reaction and for determining their structures. The peak positions at δ 4.6 and 4.4 (using trifluoroacetic acid as a solvent and sodium 2,2-dimethyl-2-silapentane-5-sulfonate as a reference) for CH_2 were assigned to 1- and 3-substituted hydantoins, respectively, based on comparison with the spectra of authentic samples of 3-substituted hydantoins (3).

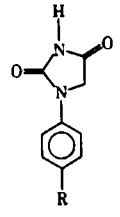
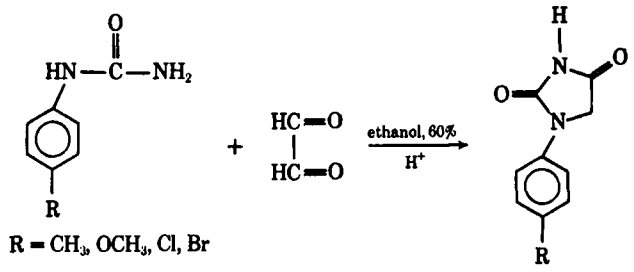


Table I—Data for 1-Substituted Hydantoins

Number	R	Yield, %	Melting Point	Formula	Analysis, % Calc.	Found
I	CH_3	43	212–214°	$C_{10}H_{10}N_2O_2$	C 63.15	63.29
					H 5.30	5.26
					N 14.47	14.73
II	OCH_3	30	197–198°	$C_{10}H_{10}N_2O_3$	C 58.25	58.30
					H 4.85	5.10
					N 13.59	13.21
III	Cl	30	231–233°	$C_9H_7ClN_2O_2$	C 51.32	51.22
					H 3.35	3.36
					N 13.30	13.66
IV	Br	35	235–237°	$C_9H_7BrN_2O_2$	C 42.35	42.27
					H 2.74	3.01
					N 10.98	10.76

Four *para*-substituted 1-phenylureas, varying in electronic and steric properties, were prepared as representatives for the synthesis of 1-substituted hydantoins. The substituents are CH_3, OCH_3, Br, and Cl (Scheme I). The Hammett σ values for the first two groups are about the same; for Cl and Br, they are identical. The van der Waals' radii for CH_3 and Br are about the same. An analysis of the experimental results using reactants containing such electronic and steric factors is envisaged to contribute both to the synthetic and mechanistic aspects of the urea–glyoxal condensation reaction. So far, the data obtained from this particular series of reactions seem to indicate that

[1] The report by A. Hill *et al.*, *J. Amer. Chem. Soc.*, **44**, 2357(1922), of the synthesis of 3-substituted hydantoins, erroneously designated them as 1-substituted hydantoins.



Scheme I

the nature or the yield of the reaction products is not influenced significantly by either factor.

EXPERIMENTAL[3]

The preparation of compounds listed in Table I is exemplified by the following method described for 1-*p*-methylphenylhydantoin (I).

To a hot solution of 3.0 g. (0.02 mole) of *p*-tolylurea in 50 ml. of 50% ethanol was added 1.56 g. (0.02 mole) of 80% glyoxal, followed by 10 ml. of a 10% solution of hydrochloric acid in ethanol. The solution was heated under reflux for 6–10 hr. A solid that formed was separated from the hot reaction medium to give 1.05 g. of first crop, whose NMR spectrum indicated that it was a mixture of I containing less than 5% 1,4-di-*p*-tolyltetrahydroimidazo[4,5-*d*]imidazole-2,5-dione. Crystallization of this crop from methanol gave 0.9 g. of pure I. The filtrate from the reaction mixture was allowed to remain at room temperature for about 2 hr., at which time an additional 0.4 g. of solid was separated. This crop was identified to be I. A third crop, 0.5 g., obtained after complete evaporation of filtrate, contained about 10% of the 3-substituted isomer of I. Crystallization of this crop from methanol gave 0.3 g. of pure I. The combined yield of I was 1.6 g. (43%), m.p. 212–214°. Microanalysis is recorded in Table I.

[3] Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. The NMR spectra were determined on a Perkin-Elmer R-12. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was used as a reference; trifluoroacetic acid was used as a solvent except for Compound II for which dimethyl sulfoxide was used. The IR spectra were determined in KBr disk with a Beckman IR-8 instrument.

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COMMUNICATIONS

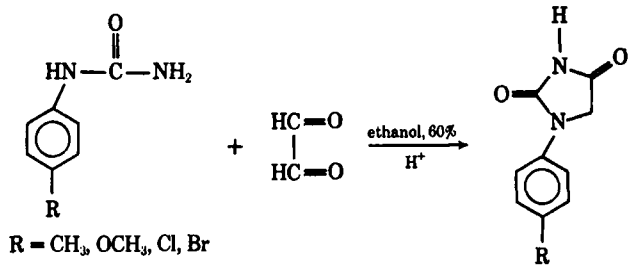
Effect of Chain Length in Homologous Series of Anionic Surfactants on Irritant Action and Toxicity

Keyphrases □ Chain length effect—irritant action and toxicity of homologous series of anionic surfactants □ Surfactants, anionic—effect of chain length in homologous series on irritant action and toxicity □ Toxicity, anionic surfactants—effect of chain length in homologous series □ Irritant action, anionic surfactants—effect of chain length in homologous series □ Skin—effect of chain length of anionic surfactants on swelling and irritant action

Sir:

Among the investigations into the irritant action and toxicity of homologous series of anionic surfactants containing a single normal alkyl chain are the following. Choman investigated the swelling of human skin in solutions of sodium alkyl sulfates (1) and the swelling of dermal collagen of calf in sodium soaps (2). Edwards (3) studied the effect of sodium soaps on the lysis of red blood cells and on segments of earthworms, while Emery and Edwards (4) studied their irritant action on human skin. Gale and Scott (5) investigated the intraperitoneal and oral toxicity of sodium alkyl sulfates as well as their effects on various types of muscles. In these and other investigations, the greatest effects among the sodium alkyl sulfates ranging from octyl to octadecyl were shown by the dodecyl sulfate. In the series of soaps ranging from sodium octanoate to stearate, sodium laurate displayed the highest activity. Representative curves are shown in Figs. 1 and 2. Figure 1 refers to the net swelling, *i.e.*, swelling in the surfactant solutions minus swelling in pure water, measured as increase in thickness. The surfactant solutions were at or slightly above the CMC.

The purpose of this communication is to present a hypothesis to explain the maximum effectiveness observed for intermediate members of the homologous series. Maxima or minima in the relationship between two variables often arise from the effect of two opposing factors on these variables, one of which tends to enhance the relationship while the other tends to diminish it. In the present case, one of the two factors affecting the relationship between the length of the alkyl chain of



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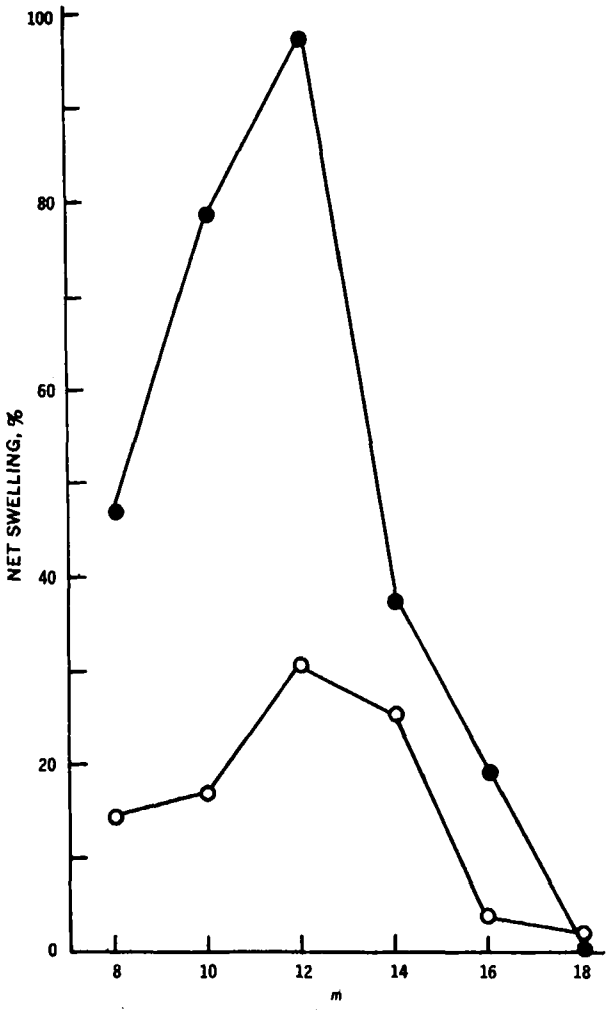

Figure 1—*Net swelling of calf skin collagen in sodium soap solutions and of human skin in sodium alkyl sulfate solutions as a function of the number,* m, *of carbon atoms per surfactant molecule. Key:* ○, *human skin in sodium alkyl sulfate solutions (from data of* Reference 1*);* ***and*** ●, *calf skin collagen in sodium soap solutions (from data of* Reference 2*).*

anionic surfactants and their interaction with proteins is the oil–water partition coefficient P. The other factor is the upper limit of the concentration of nonassociated surfactant molecules attainable in the aqueous phase, given by the surfactant solubility below the Krafft point and by the CMC above it.

Oil–Water Partition Coefficients—Partition coefficients of ionic surfactants between octanol and water increase monotonically within a given homologous series. Augmenting the chain length by an ethylene group increased log P by about 0.8 (6). According to:

$$\Delta G = -RT \ln P \qquad \text{(Eq. 1)}$$

the free energy of transfer of an ethylene group from water to octanol at room temperature is −1090 cal./mole. This estimate assumes that the ionic surfactants are equally ionized in water and in octanol. Despite the fact that the water concentration in octanol saturated with water is 2.3 M (more than 25 mole-%) (6), which should lead to considerable ionization of the surfactant molecules in the oil phase, the true P values are probably lower than the apparent partition coefficients obtained from analytical data. Nevertheless, the ΔG value is in reasonable agreement with the value of −1280 cal./mole corresponding to the transfer of an ethylene group from water to the hydrocarbon interior of a micelle (7). Part of the difference is probably due to the fact that the transition from water to octanol saturated with water is less abrupt than that from water to a liquid paraffinic hydrocarbon. Similarly, it is seen from Table I that the CMC decreases approximately by a factor of 4 in a homologous series for each increase in the chain length by an ethylene group; log 4 = 0.6 is reasonably close to the corresponding increase of 0.8 in log P.

According to the hypothesis of this communication, the increase in the oil–water partition coefficient with increasing chain length n is responsible for the ascending branches of the curves in Figs. 1 and 2. In the physiological situations, the oil phase in contact with the aqueous surfactant solutions consists of collagen, skin, muscle tissue, erythrocyte membrane, *etc.*

Limiting Monomer Activity—In all likelihood, only monomeric or nonmicellar surfactant molecules enter the "oil" (collagen, skin, or muscle tissue) phase or interact with erythrocyte membranes. The upper limit

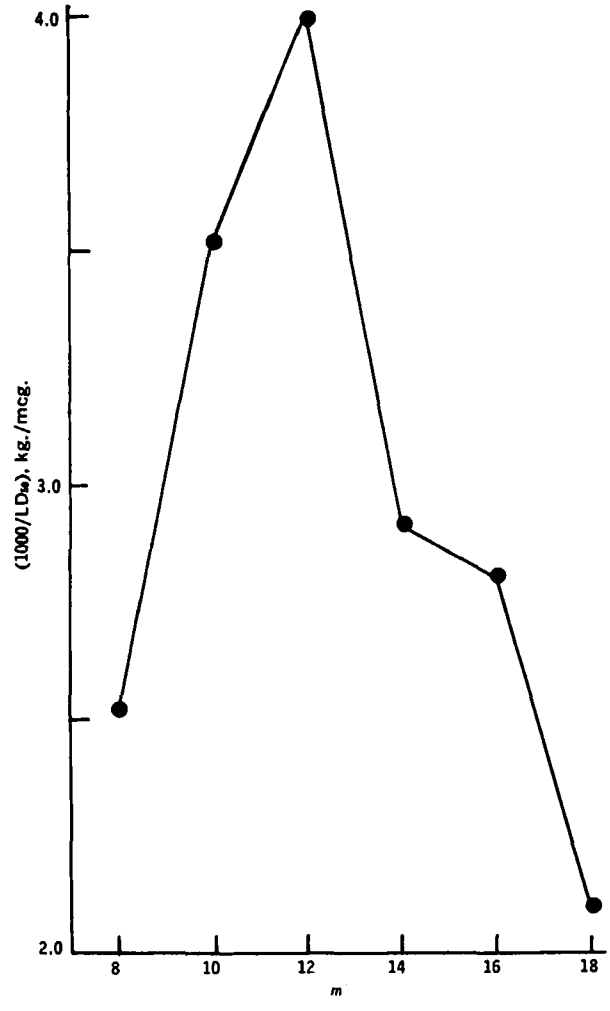

Figure 2—*Reciprocal of intraperitoneal* LD_{50} *values of sodium alkyl sulfates for mice* versus *the number,* m, *of carbon atoms per surfactant molecule (from data of* Reference 5*).*

Table I—Values of Krafft Point and CMC for Sodium Soaps and Sodium Alkyl Sulfates in Water

n^a	Krafft Point[b]	CMC[c], moles/l. × 10^3
	$C_nH_{2n+1}OSO_3Na$	
10	8°	33.1 (25°)
12	20°	8.1 (25°)
14	33°	2.2 (40°)
16	46°	0.5 (40°)
18	58°	—
	$C_nH_{2n+1}COONa$	
7	—	340 (25°)
9	—	94 (25°)
11	36°	24.4 (25°)
13	53°	7.1 (50°)
15	62°	—
17	71°	—

[a] Number of carbon atoms in the normal alkyl chain. [b] From *Reference 8.* [c] From *Reference 9.*

for the concentration of nonassociated surfactant molecules is the solubility limit at temperatures below the Krafft point and the CMC above it. The solubility at the Krafft point is equal to the CMC (7). The monomer activity of aqueous surfactant solutions increases only slightly after the overall concentration has been raised above the CMC because the bulk of the surfactant added in excess of the CMC forms micelles. CMC values and Krafft points for two homologous series of anionic surfactants are assembled in Table I.

As the alkyl chain length increases on ascending a homologous series, there is a monotonic decrease in the maximum concentration of single or nonmicellar surfactant species owing to a decrease in solubility or in the CMC. This effect is responsible for the downturn in the curves of Figs. 1 and 2. This reasoning is only valid if, at temperatures above the Krafft point, the surfactant concentrations in the aqueous phase are equal to or exceed the CMC values.

Conclusion—With the oil–water partition coefficient increasing and the limiting monomer concentration decreasing on ascending a homologous series, a maximum surfactant concentration in the "oil" (collagen, skin, muscle tissue, or erythrocyte membrane) phase is found at an intermediate chain length. As a result, a surfactant of intermediate chain length has the greatest irritant, toxic, or hemolytic effectiveness.

It is to be expected that the chain length for maximum effectiveness within each homologous series depends on the hydrophilicity of the polar headgroup, being greater for the more hydrophilic groups. The $—O—SO_3^-\ Na^+$ group is more hydrophilic than the $—COO^-\ Na^+$ group. The most active alkyl sulfate was found to be the dodecyl ester ($m = n = 12$), whereas sodium laurate ($m = 12$ but $n = 11$) was the most active soap. Unfortunately, only surfactants with even numbers m of carbon atoms were investigated in the two homologous series, making the most effective member among the alkyl sulfates the one with $n = 12 \pm 1$ and among the soaps the one with $n = 11 \pm 1$; n is the number of carbon atoms in the alkyl chain.

Since the increase in P and the decrease in the CMC as a function of n are of comparable magnitude, the ascending and descending branches of curves like the ones of Figs. 1 and 2 should have slopes of comparable absolute values though of opposite signs, as long as the experimental temperatures exceed the Krafft points.

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Enhancement of Intestinal Absorption of a Quaternary Ammonium Compound by Salicylate and Trichloroacetate

Keyphrases ☐ Quaternary ammonium compounds—enhancement of intestinal absorption by salicylate and trichloroacetate ☐ Ammonium compounds, quaternary—enhancement of intestinal absorption by salicylate and trichloroacetate ☐ Absorption, intestinal—effect of salicylate and trichloroacetate on *N,N*-bis(phenylcarbamoylmethyl)dimethylammonium chloride absorption ☐ Salicylate effect—intestinal absorption of a quaternary ammonium compound ☐ Trichloroacetate effect—intestinal absorption of a quaternary ammonium compound

Sir:

The possibility of increasing the GI absorption of poorly permeable, charged drug molecules by formation of lipoid-soluble ion-pairs with various counterions has received a good deal of attention (1–7). The present report concerns the influence of certain anions on the intestinal absorption of *N,N*-bis(phenylcarbamoylmethyl)dimethylammonium chloride (I), a quaternary ammonium compound with antiarrythmic activity[1].

The purity of the drug[2], tritium-labeled on an *N*-methyl group (specific activity 14.5 μc./mg.) as well as

[1] Personal communication, Astra Pharmaceutical Products, Inc., Worcester, Mass.
[2] Provided by Astra Pharmaceutical Products, Inc.

Table I—Values of Krafft Point and CMC for Sodium Soaps and Sodium Alkyl Sulfates in Water

n^a	Krafft Point[b]	CMC[c], moles/l. × 10^3
	$C_nH_{2n+1}OSO_3Na$	
10	8°	33.1 (25°)
12	20°	8.1 (25°)
14	33°	2.2 (40°)
16	46°	0.5 (40°)
18	58°	—
	$C_nH_{2n+1}COONa$	
7	—	340 (25°)
9	—	94 (25°)
11	36°	24.4 (25°)
13	53°	7.1 (50°)
15	62°	—
17	71°	—

[a] Number of carbon atoms in the normal alkyl chain. [b] From *Reference 8.* [c] From *Reference 9.*

for the concentration of nonassociated surfactant molecules is the solubility limit at temperatures below the Krafft point and the CMC above it. The solubility at the Krafft point is equal to the CMC (7). The monomer activity of aqueous surfactant solutions increases only slightly after the overall concentration has been raised above the CMC because the bulk of the surfactant added in excess of the CMC forms micelles. CMC values and Krafft points for two homologous series of anionic surfactants are assembled in Table I.

As the alkyl chain length increases on ascending a homologous series, there is a monotonic decrease in the maximum concentration of single or nonmicellar surfactant species owing to a decrease in solubility or in the CMC. This effect is responsible for the downturn in the curves of Figs. 1 and 2. This reasoning is only valid if, at temperatures above the Krafft point, the surfactant concentrations in the aqueous phase are equal to or exceed the CMC values.

Conclusion—With the oil–water partition coefficient increasing and the limiting monomer concentration decreasing on ascending a homologous series, a maximum surfactant concentration in the "oil" (collagen, skin, muscle tissue, or erythrocyte membrane) phase is found at an intermediate chain length. As a result, a surfactant of intermediate chain length has the greatest irritant, toxic, or hemolytic effectiveness.

It is to be expected that the chain length for maximum effectiveness within each homologous series depends on the hydrophilicity of the polar headgroup, being greater for the more hydrophilic groups. The $—O—SO_3^-\ Na^+$ group is more hydrophilic than the $—COO^-\ Na^+$ group. The most active alkyl sulfate was found to be the dodecyl ester ($m = n = 12$), whereas sodium laurate ($m = 12$ but $n = 11$) was the most active soap. Unfortunately, only surfactants with even numbers m of carbon atoms were investigated in the two homologous series, making the most effective member among the alkyl sulfates the one with $n = 12 \pm 1$ and among the soaps the one with $n = 11 \pm 1$; n is the number of carbon atoms in the alkyl chain.

Since the increase in P and the decrease in the CMC as a function of n are of comparable magnitude, the ascending and descending branches of curves like the ones of Figs. 1 and 2 should have slopes of comparable absolute values though of opposite signs, as long as the experimental temperatures exceed the Krafft points.

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The purity of the drug[2], tritium-labeled on an *N*-methyl group (specific activity 14.5 μc./mg.) as well as

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Table I—Effect of Salicylate and Trichloroacetate on the Absorption of I from the Rat Intestine

Minutes	Percent Remaining[a] Control	Salicylate	Trichloroacetate
5	93.5 ± 12.9	75.5 ± 13.1	70.8 ± 8.6
10	84.4 ± 7.3	54.9 ± 5.0	48.5 ± 4.4
15	81.0 ± 8.4	48.7 ± 3.6	39.6 ± 4.0

[a] Mean of five experiments ± one standard deviation.

Table II—Effect of Anions on the Plasma Level and Apparent Partition Coefficient of I

	Plasma Concentration at 30 min.[a], mcg./ml.	Butanol–Water Partition Coefficient
Control	0.24 ± 0.13	23
Salicylate	1.37 ± 0.67	58
Trichloroacetate	—	97

[a] Mean of five rats ± one standard deviation.

unlabeled, was verified by high-voltage electrophoresis. All other chemicals were reagent or scintillation grade. Tritium-labeled I and sufficient unlabeled drug were dissolved in isotonic buffer to give a concentration of 0.4 mg./ml. The buffer (pH 7.4) contained sodium bicarbonate (26 m*M*), potassium chloride (5 m*M*), potassium acid phosphate (1 m*M*), and either sodium chloride, sodium salicylate, or sodium trichloroacetate (122 m*M*). Trichloroacetate solutions were prepared by dissolving equimolar quantities of sodium hydroxide and trichloroacetic acid in water. The molar ratio of anion (*i.e.*, salicylate or trichloroacetate) to drug was about 90:1.

Male Sprague–Dawley rats, weighing about 300 g. and fasted for 24 hr., were anesthetized with ethyl carbamate (1.3 g./kg. i.p.) and prepared as described by Doluisio *et al.* (8) for studying drug absorption from the *in situ* small intestine. Seven milliliters of drug solution was placed in the intestine, and 0.1-ml. samples were removed at 5-min. intervals for 15 min. The volume of the luminal solution was maintained constant by adding buffer immediately prior to sample removal. In a second series of experiments, the renal pedicles as well as the bile duct were ligated, and loss of drug from the intestinal lumen was followed for 30 min. At the end of the absorption period, blood was collected by cardiac puncture and centrifuged for 10 min. at 575 × *g* to obtain the plasma.

Tritium levels were determined by liquid scintillation spectrometry[3]. Scintillation fluid was prepared by dissolving 0.28 g. 1,4-bis[2-(5-phenyloxazolyl)]benzene, 9.46 g. 2,5-diphenyloxazole, and 142 g. naphthalene in 946 ml. dioxane and 50 ml. water. Two-tenths-milliliter samples of plasma were added to 15 ml. scintillation fluid and counted for 100 min.; raw counts were 500–2000 above background. Half-milliliter samples of diluted intestinal perfusate were counted in 15 ml. of scintillation fluid to 10^5 counts. Tritiated water was used as an internal standard; the efficiency of the system was 32% for tritium in buffer, plasma, and intestinal solution.

The results of the absorption studies are summarized in Table I. Both salicylate and trichloroacetate significantly enhance the disappearance of I from the intestinal lumen. Both anionic agents exert their greatest influence on the initial absorption rate of the drug and, thereafter, the effect tends to decline with time. Throughout the experiment, trichloroacetate was slightly more effective than salicylate in enhancing the absorption of I. Determination of tritium levels in the plasma immediately after the 30-min. absorption studies (Table II) clearly showed that the enhanced loss of I from the intestinal perfusate containing salicylate results in substantially higher apparent drug levels in the body. Salicylate concentration in the plasma at the same time averaged about 1.1 mg./ml. Since most of the apparent drug in whole blood is associated with the nonserum fraction, there was some concern that the elevated levels of I in plasma containing salicylate might be due to displacement of the quaternary ammonium compound from the formed elements of the blood. This possibility was ruled out by demonstrating equal concentrations of I in the plasma of whole blood spiked with drug and of whole blood spiked with both drug and salicylate.

The absorption and plasma level data are in general agreement with equilibrium partitioning data (Table II). Both salicylate and trichloroacetate significantly increase the apparent butanol–water partition coefficient of I, with trichloroacetate being somewhat more effective. The results of our investigations are quite encouraging and offer the possibility of substantially improving the bioavailability of quaternary ammonium compounds and other highly ionized drugs.

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[3] Packard Tricarb model 2111.

Tumor Inhibitory Agent from *Magnolia grandiflora* (Magnoliaceae) I: Parthenolide

Keyphrases ☐ *Magnolia grandiflora* L.—isolation, identification of parthenolide, antitumor activity ☐ Parthenolide—isolation, identification from *Magnolia grandiflora*, antitumor activity ☐ Antitumor activity—evaluation of parthenolide, isolated and identified from *Magnolia grandiflora*

Sir:

As a result of the continuing search for plants having tumor-inhibiting constituents, it was found that the petroleum ether extract of the leaves and stems of *Magnolia grandiflora* L.[1] showed inhibitory activity toward the human epidermoid carcinoma of the nasopharynx test system (9KB cell culture)[2].

A preliminary examination of the petroleum ether extract revealed one major component. This component was isolated and shown to be the active constituent. The isolation was effected by solvent extraction followed by silica gel G, dry column chromatography. The compound was identified as parthenolide by means of its melting point; mixed melting point; IR, mass spectrometry, NMR, and elemental analyses; and comparison with an authentic sample[3]. The compound demonstrated activity at a dilution level of 2.3×10^1 mcg./ml. Activity in the 9KB cell culture is defined as ED_{50} less than or equal to a dilution of 20 mcg./ml. for plant extracts. The results are expressed as a dose that inhibits growth to 50% of the control growth 3 days after drug addition (1).

The dried leaves and stems (7 kg.) were ground, placed in a Lloyd-type extractor, and exhaustively extracted with petroleum ether (b.p. 40–60°). After removal of the solvent, the residue (160 g.) was treated several times with petroleum ether (b.p. 40–60°). The material obtained from the solution was inactive and, therefore, was discarded. The crystalline insoluble portion (30 g.) was further purified by use of silica gel G, dry column chromatography. Seven grams of the crystalline petroleum ether-insoluble material was placed on a silica gel G column and eluted with dichloromethane–benzene–ethyl acetate (12:24:3). Forty 10-ml. fractions were collected. On the basis of TLC (silica gel G, dichloromethane–benzene–ethyl acetate, 12:24:3), fractions 20–39, which showed a single spot, were combined. The solvent was removed, the residue was dissolved in chloroform and treated with activated charcoal[4], and excess ether was added after evaporation of most of the solvent. A crystalline precipitate (2.9 g.) occurred which was identified as parthenolide (2).

[1] The plant was collected on the campus of the University of Arizona, Tucson, Ariz., in January 1969. Identification was confirmed by Robert J. Barr, College of Pharmacy, and Dr. Charles Mason, Botany Department, University of Arizona. A reference specimen was also deposited in the University of Arizona Herbarium.

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Simple Method for Portal Vein Infusion in the Rat

Keyphrases ☐ Portal vein infusion method—used to study first-pass effect in rats ☐ First-pass effect—studied using simple method for portal vein infusion, rats ☐ Absorption, first-pass effect—studied using simple method for portal vein infusion, rats

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The rate and extent of drug absorption into the systemic circulation have been estimated by pharmacokinetic analysis of plasma concentration–time data or urinary excretion data (1, 2). The percent of absorption can be assessed by comparison of the relative areas under the plasma concentration–time curves after oral and intravenous administration. This method is based on the presumptions that the distribution and elimination of a drug may be expressed in terms of first-order kinetics within the dose range studied and that the parameters of the distribution and elimination remain constant after administering the same quantity of drug by different routes. Thus, the resultant areas are independent of the route of administration and proportional to the dose even when given by different routes. However, it was recently shown that the areas under the blood level–time curves for aspirin (3) and lidocaine (4) after infusion into a peripheral vein were considerably greater as compared with results observed after infusion of an equal dose into the portal vein in dogs. The reduction in area under the blood level–time curves after portal vein infusion has been attributed to a significant degree of metabolism of the drugs during the first passage through the liver.

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In this communication, we report a simple method for portal vein infusion in the rat to study the first-pass

effect, using propranolol as a test compound. The method consists of cannulation into the pyloric vein. A rat weighing 200–240 g. was anesthetized lightly with ether at suitable intervals, and the abdomen was opened through a midline incision. The pyloric vein was exposed by pushing aside the duodenum of the intestine, and a minimal amount of connective tissue was removed from the pyloric vein to facilitate ligation. The pyloric vein was then ligated approximately 0.7 cm. distal to the junction of the pyloric vein and hepatic portal vein. A cannula (polyethylene tubing, 0.04-cm. i.d., 0.06-cm. o.d., 7-cm. length), filled with heparin solution (200 units/ml.) and closed with an arterial clamp at the other end, was inserted into the pyloric vein, approximately 0.2 cm. proximal to the ligature, after careful venous puncture using a needle point. The cannula was gently guided upward into the portal vein so that its tip projected several millimeters into the vein (Fig. 1). The cannula was secured by ligation with nylon suture. A syringe was attached to the end of the cannula, and it was then confirmed that the portal blood could be detected in the cannula with a slight suction. The abdominal incision was closed, and the syringe was replaced by another syringe containing infused solution. After the end of the infusion experiment, it is desirable to inject methylene blue solution at an appropriate concentration into the cannula and to observe the liver stained with the dye in order to check for lack of leakage during infusion.

Propranolol was administered to rats by rapid infusion into the femoral vein or by constant infusion into the hepatic portal vein during 50 min. using an infusion pump. Blood samples (0.1–0.2 ml.) were taken at various intervals through a cannula inserted into the femoral artery and were diluted with 1.0 ml. of water prior to extraction. The propranolol concentrations in whole blood were determined spectrophotofluorometrically by a minor modification of the method of Shand *et al.* (5). The fluorescence of propranolol in 0.1 *N* HCl was measured in a spectrophotofluorometer[1] (maximum excitation 295 nm., maximum emission 360 nm.). An equal volume of blood to sampled blood was transfused each time by an intravenous infusion. The blood for transfusion was taken from another rat of the same strain.

The blood concentration–time curve after intravenous administration was described in all rats by a biexponential equation of the form $C = Ae^{-\alpha t} + Be^{-\beta t}$. Table I lists the mean areas under the blood concentration–time curves after intravenous and portal vein infusion. The data show that the mean areas under the blood concentration–time curves after portal vein infusion were found to be 7.8 (2.5 mg./kg.) and 90.9% (12.5 mg./kg.) of the mean areas of the corresponding intravenous infusion curves. Although the area under the blood concentration–time curve after intravenous administration was directly proportional to the dose, the mean area obtained after portal vein infusion at a dose of 2.5 mg./kg. was less than 10% of the mean area of intravenous infusion at an equal dose. Shand *et al.* (5) reported that, in the same human subjects, propranolol reaching the systemic circulation was estimated to be 16–60% of an oral dose from the ratio of the areas under the plasma concentration curves after oral and intravenous administration. After oral administration of doses less than 30 mg., only trace amounts of propranolol were detected in the systemic circulation in six human subjects, while the area under the blood concentration–time curve was linearly related to dose with oral doses exceeding 40 mg. (6). The results in rats show that the clearance of propranolol from the portal circulation during its first passage through the liver can explain the significant reduction of the area under the curve at the lower dose.

Table I—Mean Areas under Blood Concentration–Time Curves after Rapid Intravenous and Constant Portal Vein Infusion of Propranolol

Administration Route[a]	Dose, mg./kg.	Number of Rats	Area Mean ± *SE*, ng. min./ml.	Relative Area
Intravenous	2.5	3	52746 ± 3937[b]	1.0
	12.5	3	260546 ± 19245[b]	4.9[c]
Intraportal	2.5	5	4129 ± 750[d]	0.078[e]
	12.5	5	236837 ± 21946[d]	0.909[e]

[a] Intravenous doses were given within 30 sec. into the femoral vein, and intraportal doses were given at a constant rate during 50 min. into the hepatic portal vein. [b] Calculated as $(A/\alpha + B/\beta)$. [c] Compared with the area for an intravenous dose of 2.5 mg./kg. [d] Calculated using the trapezoidal rule. The area for the tail end after the last sampling at a dose of 12.5 mg./kg. was calculated by C_t/β, where C_t is the blood concentration at time t, and the rate constant β was estimated from the terminal slope of a semilogarithmic plot of the blood concentrations of propranolol after the end of infusion. [e] Compared with the areas for the corresponding intravenous doses. Significantly different at a dose of 2.5 mg./kg. ($p < 0.001$) and not significantly different at a dose of 12.5 mg./kg. ($p < 0.4$).

Administration of a drug into the portal vein blood has been attempted only in the dog to study differences in areas under blood level–time curves as a function of route of administration (3, 4). In these reports, a cannula was introduced into the hepatic portal vein through a branch of the splenic vein (3) or a tributary of the superior mesenteric vein (4). The method for sampling portal vein blood presented by Pelzmann and Havemeyer (7) could be used for administering a drug into

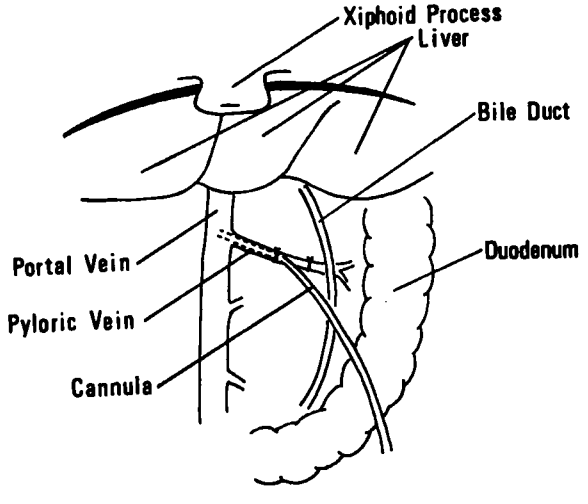

Figure 1—*Representation of pyloric vein cannulation. Mesenteries were retracted to show the cannulation.*

[1] Hitachi model 203.

the portal vein of the rat. However, the portal vein infusion using the pyloric vein cannulation overcomes several disadvantages inherent in the use of direct cannulation into the portal vein, since the latter surgery is delicate, requiring speed and accuracy to prevent excessive engorgement of the intestinal veins and acute loss of blood in the liver. The pyloric vein was selected because of its accessibility and a suitable branch of the hepatic portal vein to facilitate cannulation. The method for portal vein infusion in this communication is simple and practical as compared with that in the dog. This procedure in the rat has proven useful in drug absorption and metabolism studies. Details of these studies will be reported elsewhere.

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BOOKS

REVIEWS

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REVIEW ARTICLE

Oral Contraceptives: Therapeutics *versus* Adverse Reactions, with an Outlook for the Future II*

A. S. BINGEL▲ and P. S. BENOIT

ADVERSE REACTIONS: OTHER EFFECTS

Cancer and Hyperplasia—The historical developments that led to the theory that steroid hormones might play a role in the etiology and pathogenesis of cancer were reviewed in 1967 by Hertz (163). He discussed the pertinent animal and human studies prior to that time. It was evident from this review that the steroid hormones were able to induce cancer in various tissues of some nonhuman species. However, at that time, data from clinical observations were inadequate to determine whether or not similar effects were produced in humans. The inadequacy of the data resulted from several factors: (*a*) most of the studies had been uncontrolled, (*b*) the populations involved were lacking in sufficient numbers to provide statistically adequate samples, and (*c*) the periods of observation had been too short to allow for the usually long latent period for carcinogenesis in humans. Subsequent studies (*vide infra*), including those reviewed by Goldzieher (80) in 1970, have served to demonstrate further that no simple relationship exists between estrogens, with or without a progestagen, and breast or genital cancer in the human.

Hyperplasia of the uterine cervix was reported in a total of 35 oral contraceptive users (164–166) and in five pregnant patients (166). The distinctive lesion, characterized by atypical polypoid endocervical hyperplasia, had not been recognized previously (164). Of importance was the fact that this lesion resembled adenocarcinoma (164), and an erroneous diagnosis of the latter had been made initially in some instances (165).

The incidence of general atypical cytology of the genital tract has been reported. Kline *et al.* (167) compared cytological smears from the genital tracts of 2296 oral contraceptive users with smears from the genital tracts of a comparable group of 17,724 nonusers. The duration of oral contraceptive use ranged from 2 months to 7 years. A 2.0% incidence of atypical cells was found in the oral contraceptive group and a 1.0% incidence was found in the controls. A significant difference ($p < 0.01$) between the two groups was found for patients less than 30 years of age. The possibility that these atypical manifestations might be precancerous was discussed; observations that their course is unpredictable argues in favor of continuous followup of patients exhibiting them.

Dougherty (168) reported the results of a study of 1983 women who had used sequential oral contraceptives for 0–92 menstrual cycles. He found that the occurrence of atypical cytology in treated patients was twice as high as expected but that the length of time of exposure to the oral contraceptives did not affect the incidence rate of atypical cytology. No patients developed adenocarcinoma during the study.

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Melamed *et al.* (169) compared the prevalence rate of carcinoma *in situ* between two groups of patients: 27,508 women using oral contraceptives and 6809 using the diaphragm. A small difference was found between the two groups, which suggested a higher rate in oral contraceptive users. This difference, however, could have been due to a decreased prevalence rate for women using the diaphragm (a possible protective barrier effect) and/or to an increased rate in oral contraceptive users. The initial observations had shown this apparent difference also, but prior contraceptive use was not reported.

Interestingly, Stern *et al.* (170) found a significant association of cervical dysplasia with contraceptive choice. The prevalence of dysplasia among women from an apparently homogeneous population, who chose oral contraceptives, was 82/1000; among choosers of intrauterine devices, it was 51/1000; and among choosers of other methods, it was 36/1000. These observations emphasize the importance of making genital tract examinations prior to starting women on oral contraceptives, particularly if an association between the latter and cervical cancer is to be proven or disproven.

The data from a 5-year family planning clinic study of cervical epithelial changes during oral contraceptive use were reported by Chai *et al.* (171). Smears from 14 of 4164 women who had been using various oral contraceptives for 0–3 years were found to be positive, *i.e.*, suggestive of severe dysplasia or carcinoma *in situ*. Of the 14, followup was lacking in four. Of the other 10, there were six cases of carcinoma *in situ*, one of moderate dysplasia, one of severe dysplasia, and two of chronic cervicitis. The incidence rate in the oral contraceptive users was compared with that in a group of 30,834 women who were attending other clinics in the same medical center. The incidence rates of positive findings per 1000 were calculated to be 3.36 for the oral contraceptive users and 6.32 for the other group; the corresponding rates for carcinoma *in situ* were 0.96/1000 and 1.68/1000, respectively. No information was given concerning the health status or drug usage of the women attending the other clinics, but at least cervical abnormalities did not appear to be greater in the family planning clinic oral contraceptive users.

Nichols and Fidler (172) examined 128 consecutive cone biopsies that had been taken from patients because of suspicious or positive cytologic findings. Microglandular hyperplasia was found in 24% of the entire series, and the greater proportion of the patients with this condition had been oral contraceptive users. On the other hand, there was no significant difference between users and nonusers with respect to the degree or duration of cytologic squamous dyskaryosis or malignant change.

In 1972 the results were reported of an 8-year prospective study (173) involving the largest group of oral contraceptive users observed thus far for abnormal cervical cytology. The 9634 women were assigned either to an oral group (4846) or a nonoral group (4788) according to a predetermined random procedure. Those in the oral group took norethynodrel with mestranol; those in the nonoral group used other contraceptive methods, excluding intrauterine devices. Requirements for admission into the study included the following: (*a*) age between 21 and 39 years, (*b*) at least one normal pregnancy prior to admission, and (*c*) no previous use of oral contraceptives or intrauterine devices. Excluded were individuals who had a history of serious illness including neoplastic disease, those who were bedridden, and those who had a life expectancy of less than 5 years. At the initial interview the women were given a general physical and a gynecological examination; thereafter, the women returned every 2 months for interviews and annually for a physical examination.

Changes in cervical cytology, *i.e.*, progression to a higher Papanicolaou classification or regression to a lower Papanicolaou classification, were recorded. During the 8-year period, 43.9% of the oral group and 44.3% of the nonoral experienced one or more changes. The data given for the first three changes suggested that there was no difference between the two groups either with respect to the numbers of women undergoing progressive changes or with respect to the numbers undergoing regressive changes.

Various hormones, particularly estrogens, are known to influence the physiology of the breasts, producing cyclic engorgement, for example. Such changes, which can be accentuated by exogenous estrogens, may appear pathologic in some women, but upon scrutiny they often are found merely to be the result of aberrations in breast physiology (174).

Wiegenstein *et al.* (175), however, reported observing multiple fibroadenomas in 12 patients between 1967 and 1971; 11 of the patients were known to be oral contraceptive users. The condition had not been observed during the prior 7 years in that laboratory and was believed to be relatively uncommon. Unfortunately, no figure was given for the normal incidence of this condition.

Arthes *et al.* (176) described three studies involving a total of 283 cases of breast cancer and 585 controls. The results offered no evidence to indicate that administration of female hormones might be associated with the development of breast cancer; no significant excess of estrogen or oral contraceptive users was found among the cancer patients. In one of the studies, however, the number of oral contraceptive users was small, most of the diagnoses having been made prior to 1963 and the majority of women having been over 45 years of age at the time. In the second study, only 30 cases of breast cancer in patients under 35 years of age were found in a diagnostic index of hospitals in four of five study areas. The third study, however, involved 119 patients with breast carcinoma who had been treated at the Johns Hopkins Hospital from June 1969 to December 1970; these cases were matched with an equal number of controls. Analysis of data concerning prior hormone treatment indicated that neither in the total series nor in only those patients under 50 years of age was there any evidence of greater use of estrogens or oral contraceptives in the cases as opposed to the controls.

Vessey *et al.* (177) similarly reported that in a case-control study of 220 women and 216 matched controls, no evidence was found to associate oral contraceptives with either an increased risk of breast neoplasia or an increased development of benign breast lesions.

Leis (178) reviewed a number of studies involving various estrogens and estrogen–progestagen combinations. He concluded that there was no statistically significant evidence to indicate an increased incidence of breast cancer since the advent of widespread use of such compounds. He pointed out studies that had shown no difference between the degree of papillomatosis, hyperplasia, or other types of fibrocystic disease in the breasts of users and in those of nonusers of oral contraceptives. Other studies had shown no difference between the incidence of fibroadenomas, fibrocystic disease, or breast cancer in otherwise comparable groups of users and nonusers.

Taylor (179) reviewed several studies that had shown: (*a*) no histological differences between normal mammary tissue from oral contraceptive users and that taken from nonusers, and (*b*) no histological differences between fibroadenomas, other fibrocystic diseased mammary tissue, and breast carcinomas taken from users as opposed to nonusers.

Leis (178) further commented on the fallacy of drawing generalizations from isolated reports of abnormal breast findings in patients using oral contraceptives. He added, however, that it would be reasonable to stop use of such medication were abnormal breast lesions to develop while using it; altering the hormonal milieu in which breast cancers develop, for example, can lead to retardation of the cancer growth. Finally, in 1971, Lewison (180) observed that the data available were still insufficient for one to draw definite conclusions concerning the association, or lack thereof, between estrogens and/or contraceptives and breast malignancies. Nevertheless, he recommended caution in prescribing such preparations for high-risk patients. He included in this group patients with: (*a*) a strong family history of cancer, (*b*) a history of cancer of one breast (especially lobular cancer), (*c*) recurrent chronic cystic mastitis, (*d*) lobular carcinoma *in situ* of the breast, and (*e*) abnormal mammograms. He concluded, moreover, that prolonged use of these hormones was contraindicated in these predisposed patients.

Hypertension—In their 1970 review, Russell and Sullivan (181) pointed out that prior to 1967 only two isolated case reports concerning the development of hypertension in oral contraceptive users had appeared in the literature; hypertension, furthermore, was reported to be strikingly similar to that which occurred in toxemia of pregnancy. In 1967, reports began to appear of studies of small groups of women in whom the development or enhancement of hypertension was thought to be related to the use of oral contraceptives. In addition, it was suggested that the hypertensive action might be mediated through the renin–angiotensin–aldosterone system (*vide infra*).

The authors quoted three subsequent studies of oral contraceptive users in which it was reported that seven of 45, 51 of 129, and 40 of 141 women, respectively, had shown an elevation in blood pressure. The degree of elevation and definition of hypertension varied among the studies, however, as did the type of drug administered and the population of women receiving treatment. Moreover, in the one study that employed a control group of intrauterine device users, no significant difference was found between the latter group's blood pressure changes and those of the oral contraceptive users. A large-scale study, however, involving 1575 women did indicate a higher and statistically significant adjusted mean systolic and diastolic blood pressure in users as opposed to nonusers of oral contraceptives. The investigator reported that a blood pressure elevation does occur in oral contraceptive users but that it is not marked and requires sophisticated analysis, such as covariance analysis, to reveal statistically significant differences.

Russell and Sullivan (181) also reported data concerning 411 women who had had their blood pressure measured either prior to or during contraceptive therapy. Twenty-three percent of 34 patients and 22% of 26 were classified as hypertensive (b.p. $\geq$ 140/90 mm. Hg) prior to and during, respectively, their use of an intrauterine device or diaphragm. Nine percent of 225 patients and 16% of 126 were classified as hypertensive prior to and during, respectively, their use of various oral contraceptives. These observations might be of interest if it were known that the "before" and "during" users of the nonoral and oral contraceptives, respectively, had been comparable prior to treatment.

During 1970 and 1971, a number of other reports appeared that also associated the use of oral contraceptives with induced or aggravated hypertension. Lansing (182), for example, presented four case reports of oral contraceptive users with hypertension (b.p. $\geq$ 160/100 mm. Hg). In each instance the pressure returned to normal levels ($\leq$ 130/70) when the medication was discontinued.

Within a 10-month period, Clezy (183) observed 11 patients who had developed hypertension while taking oral contraceptives. Guanethidine appeared to produce an unsatisfactory response in the presence of oral contraceptives while blood pressure appeared to decrease when the oral contraceptives were withdrawn. An accurate evaluation is difficult, however, since other drugs, including amobarbital, bendroflumethiazide, chlorothiazide, phenobarbital, and/or methyldopa, had been used concurrently in some patients.

Spellacy and Birk (184) reported a study of 57 women whose blood pressure was measured during pregnancy (45 women only), during labor, at 4 weeks or more postpartum (prior to contraceptive treatment), and during the sixth cycle of oral contraceptive therapy. The women randomly were given a combined (100 mcg. mestranol plus 1 mg. ethynodiol diacetate, 24 women) or sequential (100 mcg. mestranol, 100 mcg. mestranol plus 1.5 mg. chlormadinone acetate, 33 women) preparation. Eight of the combined preparation users demonstrated hypertension (systolic pressure $\geq$ 140 mm. Hg and/or diastolic pressure $\geq$ 90 mm. Hg) while only one of the sequential preparation users did. However, four of the eight in the former group previously had had high blood pressure reading(s) during pregnancy; a prenatal value for the sequential product user was not given.

Chidell (185) reported the results of a study in which 99 of 9000 oral contraceptive users were found to be hypertensive (b.p. > 140°/90 mm. Hg). Approximately one-third previously had been normotensive. One-third

previously had had a higher than average starting blood pressure; 50% of the latter patients showed a further rise while using oral contraceptives. The remaining third already were taking oral contraceptives when their first blood pressure measurements showed them to be hypertensive. Withdrawal of the medication was followed by a reduction in pressure in 27 of 32 women, by no change in four, and by a rise in one. Continuing the medication in 34 other women was accompanied by a continued elevation of pressure in 29, a fall in three, and a further rise in two.

Similar observations were made by Saruta *et al.* (186). Fifty-six of 62 postpartum patients were classified initially as normotensive, and the remaining six were classified as hypertensive; the dividing line was a blood pressure of 140/90 mm. Hg. The initially normotensive patients were classified as becoming hypertensive if their diastolic pressure increased by at least 10 mm. Hg to a level of 90 or more. For those initially hypertensive, a rise of the diastolic pressure by at least 10 mm. Hg was interpreted as their having developed increased hypertension. During the use of 50 mcg. mestranol plus 1 mg. norethindrone, 10 of the 56 became hypertensive under these criteria while of the other six, blood pressure became normal in one, remained unchanged in four, and increased further in the sixth. Among those who became hypertensive, there had been a higher incidence of hypertension during pregnancy.

These workers also found that blood pressure returned to normal in three women in whom oral contraceptive therapy had been discontinued for 6 weeks. Finally, they commented on 18 additional patients, four of whom also became hypertensive during oral contraceptive treatment. The total incidence of hypertension development or enhancement in their series, therefore, became 19%.

Wallace (187) observed retrospectively that 15 of 26 (58%) 20–45-year-old women who had been admitted to one hospital over a 22-month period had been using oral contraceptives for an average of 5.5 years (range 18 months–9 years) at the time of being diagnosed hypertensive (mean diastolic blood pressure for the group: 133 mm. Hg, range 120–170 mm. Hg). He compared this figure (58%) with that of an estimated 32% usage of oral contraceptives in the general population. However, it cannot be assumed that these 26 patients necessarily were a representative sample of New Zealand women. Furthermore, precontraceptive blood pressure values were not known for eight of the 15 and were known to have been high in two others.

A much smaller proportion (17%) of a much larger group (325) of hypertensive women referred to a California medical center was reported by Crane *et al.* (188) to have been taking oral contraceptives or conjugated estrogen. For their study, these workers defined hypertension as a persistent blood pressure $\geq$ 160 mm. Hg systolic or $\geq$ 100 mm. Hg diastolic, and they defined normotensive as a blood pressure $\leq$ 145/95 mm. Hg. Twenty-two of the 36 oral contraceptive users observed by them and five of 20 users of conjugated estrogen had been normotensive prior to therapy. Withdrawal of the medication resulted in a return of the blood pressure in most of the women to normotensive levels.

Weir *et al.* (189), in their 1-year prospective study, found a significant increase in mean systolic pressure in 50 of 66 oral contraceptive users, but in no case did the blood pressure rise as high as 140/90. No change in mean systolic pressure occurred in a control group of 21 women using diaphragms or intrauterine devices; neither did mean diastolic pressure change in either group. Of the two women who had a history of hypertension during pregnancy (preeclampsia), systolic pressure rose in one and decreased in the other after 1 year of oral contraceptive use.

Investigations also have been carried out in an effort to determine the mechanism by which blood pressure may be increased in some women during the use of oral contraceptives. Walters and Lim (190), for example, studied a number of hemodynamic parameters including cardiac output, stroke volume, blood pressure, and heart rate in a group of 30 healthy young women using oral contraceptives of the combined type. Although the absolute numerical value of the mean obtained for the group showed a slight increase for most variables and a slight decrease for a few during therapy, in contrast to before or following therapy, the values of the standard deviations overlapped to a large extent.

Most investigations, however, have been concerned with changes in the renin–angiotensin–aldosterone system. As a result of such studies, it has been postulated that the pressor effects of estrogen and/or progestagen preparations may be mediated by derangements in this system and by associated induced changes in sodium and water balance (191, 192). Indeed, a significant correlation between diastolic pressure and blood angiotensin-II concentration has been found in three forms of hypertension (severe essential, renal, and malignant) while prominent increases in blood levels of this substance have been observed also during oral contraceptive therapy (193, 194).

Actually, a number of parameters have been studied including: (*a*) aldosterone excretion rate (188), (*b*) renin activity (the ability of the plasma to form angiotensin on incubation) (181, 186, 188), (*c*) blood angiotensin concentration (determined by radioimmunoassay) (193, 194), (*d*) renin concentration (renin activity at a constant substrate concentration) (181, 186, 194), and (*e*) renin substrate (angiotensin released by excess renin) (181, 186, 188, 194). A review of the works cited reveals some inconsistencies among the results. Increases usually were reported for blood levels of angiotensin-II, plasma renin activity, renin substrate, and aldosterone excretion, while reports concerning plasma renin concentration tend to be conflicting. Russell and Sullivan (181), for example, quoted a study which had shown that renin concentration tended to fall while renin substrate increased, resulting in no change in the rate of angiotensin formation. Cain *et al.* (194) also found a decrease in renin concentration. However, this decrease was inversely correlated with a marked rise in blood angiotensin-II. Saruta *et al.* (186), on the other hand, found essentially no change in renin concentration in their oral contraceptive users whose blood pressure had remained unchanged; they did find a slight increase in the former parameter in those whose blood pressure had increased. The latter workers suggested that such data

support the hypothesis that hypertension induced by oral contraceptives may be related to a diminished suppression of renin release.

Further complicating an attempt to explain these observations, however, is the fact that derangements in the renin–angiotensin–aldosterone system were found by other workers in oral contraceptive users who had not developed hypertension (181, 191). Nevertheless, it does appear that the oral contraceptives may aggravate preexisting hypertension or precipitate its onset in some patients and that the effect may be mediated by changes in the renin–angiotensin–aldosterone system. However, as Stokes (191) pointed out, it is necessary to postulate the possible presence of other determinants of hypertension, such as an hereditary predisposition, or a previous history of toxemia of pregnancy or renal disease in those patients in whom the blood pressure rises. In conclusion, caution in prescribing oral contraceptives to patients suspected of being at risk is certainly warranted (191), and regular blood pressure determinations in all oral contraceptive users obviously are advisable (181, 186).

Effects on Carbohydrate Metabolism—In 1969, Spellacy (195) reviewed the literature concerning the effects of oral contraceptives on carbohydrate metabolism. Abnormalities that had appeared in some women using these preparations included: (*a*) abnormal response to the oral or cortisone-stimulated oral glucose tolerance test; (*b*) abnormal response to the intravenous glucose tolerance test; (*c*) elevated levels of growth hormone; and (*d*) elevated levels of circulating insulin. Greater numbers of abnormal responses had been associated with oral glucose tolerance tests than with intravenous tests. Greater numbers of abnormal responses also had been associated with the use of combination-type preparations than with the sequential type. Fewer reports were available concerning the sequential type, however, and the duration of use of the latter tended to be shorter. Spellacy suggested a number of factors that might be related to the production of such abnormalities in women using oral contraceptives: (*a*) type(s) of steroid in the preparations, (*b*) duration of treatment, (*c*) age, (*d*) family history of diabetes mellitus, and (*e*) body weight, weight gain while taking the medication, and birth weight of previous infants. He also speculated on the possible mechanisms by which the oral contraceptives might alter carbohydrate metabolism by: (*a*) production of excess cortisol and/or excess growth hormone, (*b*) alteration of liver function, (*c*) alteration of the GI tract (absorption and/or gut-insulin release factors), and/or (*d*) alteration of peripheral tissue glucose utilization. Finally, Spellacy concluded that the presence of diabetes mellitus need not be considered a contraindication to the use of oral contraceptives, but that close supervision of such patients using these drugs was advisable. Subsequent studies (*vide infra*) have provided additional information concerning many of the above points.

A number of studies (196–200), in which a variety of oral contraceptives were employed for varying periods of time, have shown no difference between fasting blood glucose levels in normal women prior to and during therapy with these drugs. In contrast, fasting blood glucose was increased during oral contraceptive use in women with subclinical diabetes (201). Furthermore, 33 of 37 women using an injectable contraceptive, medroxyprogesterone acetate, developed increased fasting blood glucose levels after 1 year of use. Although the remaining four showed a decrease in fasting levels, the increase for the group as a whole was statistically significant (202). Fasting insulin levels, on the other hand, usually have been reported as increased during steroid contraceptive therapy (197, 199, 200, 202), although evidence to the contrary has been reported (196, 198). In general, it would appear that normal glucose levels may be maintained in oral contraceptive users at the expense of increased insulin levels (197). Such observations suggest an increased peripheral resistance to the actions of insulin during oral contraceptive therapy (196).

Oral (196, 199, 200, 202, 203), prednisone-stimulated oral (204), and intravenous (196, 197, 205–207) glucose tolerance tests, as well as intravenous arginine-stimulation tests (198) have been performed in steroid contraceptive users prior to and during therapy. The arginine-stimulation test in 26 women revealed no abnormalities in blood glucose and insulin responses after 3 months of therapy with daily doses of 0.35 mg. ethynodiol diacetate.

The different types of glucose tolerance tests have shown some variation in the results produced, apparently at least partly due to the different contraceptive preparations used as well as to variations in duration of use. Also, more insulin has been reported to be released in the oral test than in the intravenous, probably because gut factors may be stimulated to act at the β-cells, in addition to the glucose (200), in the oral test.

Wynn and Doar (196) reported a deterioration of oral glucose tolerance, as measured by the area under the plasma glucose curve, in 78% of 91 women after they had been treated for 6 months (range 3–42) with a variety of combination and sequential oral contraceptives. Intravenous glucose tolerance similarly was impaired in 70% of these women. Plasma insulin levels also rose significantly. Thirty-nine other women were tested after oral contraceptive therapy was discontinued; 90% of these showed improved oral glucose tolerance and 85% showed improved intravenous glucose tolerance.

One study (197) employed intravenous glucose tolerance tests in users of combined (50 mcg. ethinyl estradiol plus 4.0 mg. megestrol acetate), sequential (100 mcg. mestranol, 100 mcg. mestranol plus 2.0 mg. anagestone acetate), and progestagen-only (0.5 mg. chlormadinone acetate) oral contraceptives. Insulin levels increased during the test after 12–16 months of therapy, but no changes from pretreatment values were observed in the glucose tolerance curves. In a second study (207), no absolute glucose values were given. However, the investigator concluded that one of the three oral contraceptives tested (100 mcg. mestranol plus 1.0 mg. ethynodiol diacetate, but not 50 mcg. ethinyl estradiol plus 4.0 mg. norethindrone acetate or 50 mcg. ethinyl estradiol plus 4.0 mg. megestrol acetate) had a transient diabetogenic effect, as indicated by a slower rate of glucose disappearance from the blood following intra-

venous administration. The effect was statistically significant after 6 months of therapy with this oral contraceptive but almost negligible after 1 year. The results of a study (204) in which patients were given prednisone-stimulated oral glucose tolerance tests also suggested that mestranol (80 mcg.) might have a transient diabetogenic effect; the percentage of abnormal tests increased during the 1st and 3rd months of therapy but had decreased by the 9th month. These investigators had concluded previously that progestagens had no effect on glucose tolerance. Consequently, they presented combined data for women using preparations containing two different progestagens: 2.0 mg. chlormadinone acetate in a sequential preparation and 5.0 mg. norethindrone acetate in a combination product. Moreover, not all women were tested at each time period.

Clinch *et al.* (205) suggested that there might be an improved intravenous glucose tolerance in patients using 50 mcg. mestranol with 1.0 mg. norethindrone, as indicated by a faster decrease in blood glucose levels. The absolute mean values attained during testing were higher, however, after 4 months of therapy in comparison with pretreatment levels.

Spellacy (206) compared the results of intravenous glucose tolerance tests in patients using a combined oral contraceptive (80 mcg. mestranol plus 5.0 mg. norethynodrel) or a sequential product (80 mcg. mestranol, 80 mcg. mestranol plus 2.0 mg. chlormadinone acetate). All women were tested prior to therapy, but only some were tested after 6 months and others after 12 months. During the 6-month test, plasma insulin values, but not glucose values, were elevated significantly in both groups; at the 12-month test, both insulin and glucose values increased above pretreatment test levels in the combination drug-treated group but not in the other. Since the mestranol doses were the same, an influence of the progestagens was suggested. However, the group results tended to return to normal after 2–3 years of oral contraceptive therapy, although some individuals developed abnormal glucose tolerance curves.

Somewhat different responses to oral glucose tolerance tests were reported (199, 200) for patients using a sequential preparation containing 100 mcg. mestranol followed by 100 mcg. mestranol plus 1.5 mg. chlormadinone acetate. In these studies, blood glucose levels during testing were significantly elevated after 6 months of treatment, while plasma insulin levels were increased above pretreatment test values at both 6 and 12 months. Oral glucose tolerance tests in patients using injectable medroxyprogesterone acetate for 12 months produced higher mean blood glucose levels and higher mean plasma insulin levels as compared with pretreatment values of these substances (202).

In the studies just described, levels of blood constituents were measured before and following a period of oral contraceptive use (usually 1 year or less). In other studies (208, 209), these same constituents were measured in women following long-term (5 or more years) use of oral contraceptives. These studies have the disadvantage that no information prior to drug use is available. Abnormal glucose curves were obtained in response to standard oral glucose tolerance tests in 36.8% of 19 women who had used a combination-type (100 mcg. mestranol plus 1.0 mg. ethynodiol diacetate) oral contraceptive for 72–85 cycles. Abnormal responses occurred in 24.5% of 45 women who had used a sequential preparation (80 mcg. mestranol, 80 mcg. mestranol plus 2.0 mg. chlormadinone acetate) for 65–97 cycles. The difference between the two groups was not significant (208). However, a significant difference between the incidence of abnormal oral glucose tolerance curves was observed in the other study (209); the incidence of abnormal responses in this case was 77.4 and 25.8% among 59 combination product users and 43 sequential-type users, respectively. The sequential preparation used in both studies was the same and the duration of use was similar. The parameters differed, however, for the combination product users. The latter in this study (209) had used a preparation containing 60 mcg. mestranol and 10.0 mg. norethindrone for 6 years (approximately 72 cycles), followed by a preparation containing 100 mcg. mestranol and 2.0 mg. norethindrone, which provided this group with a total minimum of 100 cycles of oral contraceptive use. In both studies (208, 209), women who exhibited abnormal glucose curves also had delayed insulin peaks. Moreover, the insulin levels reached at the peaks were higher than normal.

No significant relationship was found in one of these retrospective studies (208) between the occurrence of an abnormal glucose tolerance test and: (*a*) family history of diabetes mellitus; (*b*) the delivery of an excessively large, anomalous, or stillborn infant; or (*c*) the subject's age, parity, or weight. A similar lack of correlation between abnormal glucose tolerance and the patient's: (*a*) age, (*b*) degree of obesity or change of weight during therapy, (*c*) parity, or (*d*) family history of diabetes also was found by Wynn and Doar (196). On the other hand, Spellacy (206) and Spellacy *et al.* (200), in reporting their short-term prospective-type studies, suggested that glucose and insulin changes might be related to age, parity, weight changes, delivery of excessively large infants, and family history of diabetes. Duration of treatment, if sufficiently long, may obscure any influence these other factors might have had initially.

Some patients indeed may be at risk (203). Fifteen patients showed abnormal oral glucose tolerance in their third trimester but normal tolerance following delivery. Five of five who subsequently received a combination-type oral contraceptive (50 mcg. mestranol plus 1.0 mg. norethindrone) had a recurrence of abnormal glucose tolerance during therapy. The other 10 practiced other methods of birth control, and three of them showed a deterioration of glucose tolerance. Although tolbutamide has been shown to correct oral contraceptive-induced glucose tolerance disturbances (201), some investigators have advised against the use of hormonal contraception in women exhibiting definite carbohydrate metabolic derangement (201, 204) or in those with latent diabetes (203, 204) or a family history of diabetes (204). On the other hand, one investigator (207) suggested that such women merely should receive special study. The available evidence, at the very least, seems to argue for close monitoring of carbohydrate metabolism in oral contraceptive users (206, 209).

The mechanism(s) by which oral contraceptives may alter carbohydrate metabolism in some women is(are) still unclear. Growth hormone has been reported to be diabetogenic (195, 204, 208). However, most recent studies of this hormone during contraceptive therapy have not provided evidence to support its having an etiologic effect. For example, growth hormone levels have remained normal in spite of the development of impaired glucose tolerance (196, 202). Furthermore, the normal suppressibility of growth hormone by increased glucose levels has been reported to be intact in oral contraceptive users (208, 209). A second suggested mechanism, involving impaired liver function, still seems tenable (*vide infra*). That the effect may be mediated at least partly by cortisol also seems plausible. Indeed, plasma cortisol levels have been shown to increase in some patients during oral contraceptive therapy with ethinyl estradiol and dimethisterone (210). More importantly perhaps, it has been pointed out (196) that several metabolic patterns are shared by nonobese women using oral contraceptives, nonobese women receiving glucocorticoid therapy, and obese, nondiabetic women. For example, fasting blood pyruvate levels, as well as the increment of the latter following a glucose load, have been reported to be elevated in these three conditions. It has been suggested that these changes might result from glucocorticoid excess in all three classes of women. Finally, although Seng *et al.* (211) did find significant elevations of plasma levels of a number of metabolites, including pyruvate, in women using 75 mcg. mestranol with 2.5 mg. lynestrenol, all of the values found were within their respective normal ranges.

Effects on Lipid Metabolism—Plasma triglyceride levels have been found to be elevated in women using a number of different oral contraceptives (211–218). Although statistically significant, the average elevation usually did not exceed the normal upper limit of 150 mg./100 ml. (215). Evidence does suggest that severe endogenous hypertriglyceridemia might develop in response to the use of contraceptive steroids in individuals who already have high triglyceride levels and in whom an excessive insulin response occurs following a glucose load (219). The increased triglyceride levels have been reported by most workers (212, 213, 215, 217) to be associated with concomitant increases in plasma levels of very low density, low density, and/or high density lipoproteins. Seng *et al.* (211), however, found increased triglyceride levels in the absence of increased lipoprotein levels.

Slight, but significant, increases in cholesterol levels also have been reported in some oral contraceptive users (212, 215–217), although not for eight of nine women using a quinestrol–quingestanol acetate preparation (218). The increases were associated with increases in low (215) and very low (212, 215) density lipoproteins, and no consistent changes occurred in high density lipoprotein cholesterol (215). One study reported a significant decrease in cholesterol esterification (213).

Fewer data are available concerning other parameters of lipid metabolism in oral contraceptive users. Phospholipids have been reported to be significantly elevated in association with a rise in high density lipoproteins (215, 218), although Rössner *et al.* (212) found an increase in phospholipids associated with very low density lipoproteins. Free fatty acids were reported to be significantly increased in one study (213) and unchanged in another (212).

Changes in lipid metabolism parameters can be seen within a few weeks after initiation of oral contraceptive therapy (214, 215). They increase progressively in magnitude for several weeks; within 2–3 months the values may reach a plateau which then is maintained throughout therapy (215). Levels of these metabolites may continue to rise during treatment with some preparations, although they can be expected to return almost to pretreatment values within 6 months after cessation of therapy (216). Virtually no reversal of effects was observed, however, within 1 month after withdrawal (214).

Although the evidence is not conclusive, it has been suggested that the estrogenic component of the oral contraceptives may be responsible for the lipid metabolism alterations. Increases in serum cholesterol and triglycerides were seen in women using combination-type preparations (100 mcg. mestranol plus 2.0 mg. norethindrone and 50 mcg. mestranol plus 1.0 mg. norethindrone) but not in those using a progestagen-only preparation (216). However, since the latter contained 0.5 mg. chlormadinone acetate, these data merely indicate that one particular progestagen, but not necessarily any other, appears to have no effect on lipid metabolism. Triglyceride levels, which had been elevated markedly in patients using 100 mcg. mestranol with 3.0 mg. chlormadinone acetate, did indeed decrease, although not to pretreatment levels, when the medication was changed to 3.0 mg. chlormadinone acetate alone (212). Dosage also might be of some importance. Barton *et al.* (216) pointed out that mean serum cholesterol and triglyceride levels were higher in those patients using the higher dose combined preparation. However, no information was given to indicate the degree of variability among patients.

Studies designed to elucidate the mechanism(s) by which oral contraceptives may alter lipid metabolism have focused primarily on the changes produced in triglyceride levels. That impaired removal of triglycerides might be involved was suggested by studies (212, 220–223) in which a decrease in postheparin lipoprotein lipase activity during oral contraceptive use was found. However, neither oral (223) nor intravenous (212) fat tolerance was shown to be impaired in oral contraceptive users, suggesting that increased triglyceride levels in these patients might not be the result of impaired removal of these metabolites.

An alternative explanation for the increased triglyceride levels found during oral contraceptive use is an increase in triglyceride production rate (turnover) (212, 214) due to increased secretion of these compounds by the liver (212). A study (214) of the transport kinetics of plasma triglycerides showed that triglyceride production rate was almost twice as high in a group of oral contraceptive users as in a control group. Furthermore, there actually was a simultaneous increase in the efficiency of triglyceride removal in the experimental

group which, however, only partly compensated for the large increase in triglyceride production.

Oral contraceptive use, therefore, has been associated with both increased insulin levels (223) (*vide supra*) and increased triglyceride levels. Consequently, the hypothesis has been suggested (206, 223) that the increase in triglyceride levels may be secondary to the hyperinsulinemia; that is, the elevated insulin levels might stimulate increased hepatic lipogenesis. What relationship, if any, might exist between such changes in lipid (and carbohydrate) metabolism and the occurrence of vascular lesions in oral contraceptive users (*vide supra*) remains to be determined (15).

Effects on Protein Metabolism—The plasma levels of a number of proteins have been reported to be increased, and for others decreased, in users of a variety of oral contraceptives. However, the relevance, if any, of such changes to the overall health status of the patients is unknown in most cases. Furthermore, protocols of the various studies discussed below have differed markedly, and in some cases the reports concerning specific proteins have been conflicting.

Plasma levels of transferrin, the β-globulin responsible for iron transport, have been shown to increase in oral contraceptive users (224–227). The total iron-binding capacity of the serum, a measure of transferrin levels, also was shown to increase in women given 1.0- or 3.0-mg. doses of norethindrone acetate with 50-mcg. doses of ethinyl estradiol (228); it did not increase, however, when doses of 10–75 mcg. of ethinyl estradiol alone were given.

Plasma levels of copper (229, 230) and ceruloplasm, a copper-binding globulin (224, 225, 229), also have been shown to increase during oral contraceptive therapy. Norethindrone acetate (0.3 mg.) did not have these effects when given alone (229). However, Schenker *et al.* (230) showed that a different progestagen, chlormadinone acetate (2.0 mg.), did cause an increase in serum copper.

Studies of α_2-macroglobulin levels during oral contraceptive use have revealed either insignificant changes (225, 226) or significant increases (224, 231). Since this substance has antiplasmin activity *in vitro*, it has been speculated (226, 231) that increases in its plasma levels might lead to an increased clotting tendency and, hence, play a role in the production of thromboembolism (*vide supra*).

C-Reactive protein, a complex glycoprotein synthesized in the liver, is an abnormal, nonspecific, acute-phase protein seen in pregnancy and with tissue injury and necrosis. This protein also was found in the serums of greater numbers of oral contraceptive (combined and sequential types) users and of women who had been using an intrauterine device for longer than 3 years than of control women (232).

Transcortin (cortisol-binding globulin) is increased during oral contraceptive therapy (233), with the result that increased plasma levels of cortisol can be found in women using these drugs (210, 224, 233) (*vide supra*). Some progestagen-only preparations do not appear to have this effect (210). Furthermore, it appears that the free (active) fraction of cortisol may be increased as well as the bound (inactive) fraction (233).

Thyroxine-binding globulin also is increased during oral contraceptive therapy (224, 234). As a result, the serum protein-bound iodine (PBI) is increased and the triiodothyronine (T_3) resin uptake is decreased (234, 235). Therefore, although the functional status of the thyroid is believed to remain normal during oral contraceptive therapy (234), thyroid function tests in patients using these drugs are likely to yield abnormal results (235). These effects are believed to be due primarily to estrogenic components of the oral contraceptives; except for norethynodrel, administration of oral progestagens did not alter PBI levels or T_3 uptake (234).

Plasma levels of a number of other proteins in oral contraceptive users have been studied less extensively. Haptoglobulins (224, 225, 228), orosomucoid (224, 228), and α_1-acid glycoprotein (225) have been shown to decrease during oral contraceptive therapy, whereas α_1-antitrypsin (224, 225) and β_{1A}-globulin (225) have been found to increase. Albumin levels have been unchanged (225, 226) or decreased (224, 228). Some investigators (224, 225) found no change in immunoglobulin levels in women using oral contraceptives, while others (226, 231) found increases in serum levels of γG-globulin.

Many of the proteins mentioned here are synthesized in the liver. Consequently, it has been suggested (225) that changes in plasma levels of these proteins occurring during oral contraceptive therapy might be the result of actions of the estrogens and/or progestagens on the liver.

Liver Dysfunction and Jaundice—A 1967 review (236) of the hepatic effects of oral contraceptives revealed that a total of 40 cases of jaundice, in patients from five different countries, had been attributed to five different oral contraceptives. This side effect was considered to be rare (236, 237), and an incidence rate of 1/10,000 or lower was estimated (238).

The 40 case histories reviewed (236) displayed a marked similarity. The early symptoms of pruritus, malaise, anorexia, and nausea usually appeared within the first 4 weeks of oral contraceptive therapy. Subsequently, the urine darkened and jaundice appeared. Bilirubin and transaminase levels were elevated, with marked increases in serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) being found occasionally. Histological examination of liver biopsy revealed: (*a*) canalicular and hepatocellular bile stasis; (*b*) generally slight, but variable, degrees of hepatocellular degeneration and necrosis; and (*c*) minimal or absent inflammatory reaction. Electron microscopy revealed dilatation of the canaliculi, shortening or disappearance of the microvilli, intracytoplasmic dense bodies, myelin figures, and dilation of the smooth endoplasmic reticulum. Cessation of oral contraceptive treatment usually was associated with complete clinical and chemical remission within a few weeks or months. Jaundice recurred, however, in two patients following readministration of oral contraceptives. Of significance is the fact that 13 of these women previously had experienced recurrent jaundice or severe pruritus of pregnancy.

It appeared to the reviewer (236) that, in a small number of women, oral contraceptives could produce jaundice associated with reversible, noninflammatory intrahepatic cholestasis. The finding of other abnormalities of liver function tests in oral contraceptive users was more common, however. Sulfobromophthalein retention, for example, was mildly increased in 21% of the women in a Los Angeles study who had been using norethindrone (2.0 mg.) with mestranol. The same product was associated with mildly increased sulfobromophthalein retention in 42% of the women in a Swedish study. Other oral contraceptive products were reported to have produced an increase in sulfobromophthalein retention in 19–48% of users in Finland and in 7–29% of users in the United States. Various studies demonstrated elevations in transaminase levels in 0–18% of oral contraceptive users and slight elevations in alkaline phosphatase in 2%, or fewer, of the subjects. Minimal elevations of serum bilirubin occurred only rarely or not at all.

Observations that greater numbers of oral contraceptive users in Scandinavia and in Chile than in other parts of the world appear to develop jaundice (234) and/or show abnormalities in liver function tests (234) have suggested to a number of workers (234, 237, 239, 240) that some type of predisposition, possibly genetic, might be involved.

Results of studies reported since Ockner and Davidson's review (236) have tended to support earlier findings. There have been isolated case reports (241, 242) of women developing jaundice and pruritus during use of norethindrone (2.0 mg.) with mestranol. One woman (241) had a history of jaundice during pregnancy. Neither woman developed symptoms when treated subsequently with 0.5 mg. chlormadinone acetate (241) or 80 mcg. mestranol followed by 80 mcg. mestranol plus 2.0 mg. chlormadinone acetate (242).

Liver biopsies from six jaundiced and/or pruritic and six nonjaundiced, nonpruritic oral contraceptive users were compared histologically and found to be similar (243); no samples from untreated patients were studied. The authors did point out, however, that the type of mitochondria-like organelles found were similar to those found in livers of patients with hyperbilirubinemia induced by anabolic steroids. Except for one case of elevated serum glutamic pyruvic transaminase, liver function tests were normal in the asymptomatic patients. Bilirubin, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, and alkaline phosphatase, but not thymol turbidity, were elevated in most cases in the symptomatic group. Four of the five parous, symptomatic patients, but none of the five parous women in the asymptomatic group, had a history of pruritus during pregnancy.

Two other studies (244, 245) also attempted to correlate liver function test results with liver biopsy findings. In one study (244), data were compared from women with gallbladder disease who were either users or nonusers of oral contraceptives. Histological appearance of the livers from the two groups was similar, but again no healthy, untreated control livers were examined. Levels of bilirubin, serum glutamic oxaloacetic transaminase, and serum glutamic pyruvic transaminase, but not of thymol turbidity or alkaline phosphatase, were elevated in the users as compared with the nonusers. The second study (245) found filamentous mitochondrial inclusions in a small group of oral contraceptive users. However, they were present both in jaundiced and nonjaundiced patients, and in those with extremely or only moderately elevated levels of serum glutamic oxaloacetic transaminase and/or serum glutamic pyruvic transaminase. Although these workers did not find such inclusions in liver biopsies from their one normal female and three normal male patients, they pointed out that others had reported such observations. They suggested that individuals with such inclusions could be the ones more likely to develop toxic hepatitis when using oral contraceptives.

Liver function tests have been performed on other asymptomatic oral contraceptive users. The results of one study (246) suggested that oral contraceptive use was associated with increased numbers of abnormal sulfobromophthalein tests, that the increase was dose dependent, and that the proportion of women with abnormal sulfobromophthalein tests might decrease as the duration of steroid administration increased. Somewhat different observations were made in another study (247). In the latter study, the percentage of abnormal sulfobromophthalein tests increased during the first 3 months of oral contraceptive therapy and then maintained a plateau throughout the remaining nine cycles of observation; however, the criteria for abnormal sulfobromophthalein retention differed between the two studies. Larsson-Cohn (247) also studied serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase levels in his patients; the frequency of elevated transaminases was found to be maximum after 1 month of oral contraceptive use and then was observed to decrease.

It appears that morphological and at least temporary functional changes may take place in the livers of some oral contraceptive users. Such findings lend support to the hypothesis (*vide supra*) that at least some of the changes seen in carbohydrate, fat, and protein metabolism in oral contraceptive users might be mediated by an effect(s) of the medication on the liver. Whether the estrogenic and/or progestagenic component(s) of the oral contraceptives may be responsible for the liver changes has not been resolved. It has been shown, for example, that 17α-alkyl-substituted derivatives of testosterone (anabolic steroids) may interfere with bile excretion at the biliary canalicular level and cause cholestatic jaundice (234), as well as reduce the biliary transport maximum for sulfobromophthalein (239). Moreover, most progestagens in the commercially available contraceptive preparations are chemically very closely related to the former (*vide supra*). However, the estrogenic components, ethinyl estradiol and mestranol, also have unsaturated hydrocarbon groups in the 17α-position (234) and have been shown (241) to impair liver function tests when given to women with a history of jaundice of pregnancy. Although the 19-nortestosterone-derived progestagens and the commercially available synthetic estrogens all may have the potential of affecting the liver, the former are administered in doses of 1.0 mg. or greater while the latter are used in

doses of 100 mcg. or less (247). The details of one case report (242) described previously serve to emphasize this point. The woman became jaundiced while using 2.0 mg. norethindrone with 100 mcg. mestranol but remained asymptomatic while using 80 mcg. mestranol followed by 80 mcg. mestranol plus 2.0 mg. chlormadinone acetate. This latter progestagen, furthermore, is a 17α-hydroxyprogesterone derivative (*vide supra*).

Amenorrhea and Galactorrhea—It was noted that use of injectable progestagen-only contraceptives often results in prolonged amenorrhea (26). However, this phenomenon also has been reported to occur following termination of the use of oral contraceptive preparations of both the combined and sequential types (248–251). Its occurrence obviously would be of concern to women who were using these compounds merely to delay the start of their families or to space their children.

The actual incidence of this phenomenon, variously termed "post-pill amenorrhea" (251) and the "oversuppression syndrome" (252, 253), appears to be low. Arrata and de Alvarez (253), for example, pointed out that various workers estimated the postcontraceptive incidence to be 1–10%. On the other hand, little is known about the incidence of spontaneous secondary amenorrhea in the general population, making it difficult to determine the significance of its incidence in women who have discontinued the use of oral contraceptives. As a possible comparison, it can be stated that reports indicated a 2–7% (15) and 6–22% (254) incidence of secondary amenorrhea in young women exposed to minor life-stresses such as leaving home to live in a new environment. Postcontraceptive amenorrhea has occurred in women who had regular menstrual cycles prior to treatment as well as in those who previously exhibited irregular cycles (250–253, 255). Consequently, inquiries into the true incidence of oral contraceptive-induced temporary amenorrhea following termination of use of the latter should take into consideration the patient's menstrual history prior to its use.

The phenomenon of postpill amenorrhea, providing other causative factors such as a pituitary tumor can be ruled out, represents an extension of the therapeutic effect of the oral contraceptives (*vide supra*). In some patients, apparently, the hypothalamic blockade continues, to varying degrees (253), following cessation of medication. For example, the first posttreatment cycle merely may be prolonged, 36–80 days in length (248). On the other hand, the spontaneous return of menstruation did not occur in one woman until she had been amenorrheic for 6 years (250), while a second woman was still amenorrheic after 8 years.

That some degree of hypothalamic dysfunction probably is responsible tends to be supported by the results of treatment (*vide infra*) and by laboratory determinations. Specifically, although Arrata and Howard (255) did find that total urinary gonadotropins were within normal limits by bioassay criteria, they pointed out that the bioassay method yields grossly inaccurate and nonspecific results. Subsequent measurements of serum LH by radioimmunoassay revealed normal tonic levels but the absence of an ovulatory surge (253). Gambrell *et al.* (251), furthermore, found low to normal serum FSH and LH levels in their amenorrheic patients. Estrogen levels tended to be low in a number of the patients as well (251, 253, 255).

Galactorrhea accompanies the amenorrhea in some women (250–253, 255). The former may begin while the patient is still taking the contraceptive tablets (251, 255) or, alternatively, it may not become manifest until after cessation of therapy. Presumably, this phenomenon also is the result of excessive hypothalamic blockade. In this case, however, the blockade is thought to release the pituitary from hypothalamic inhibition (253). Specifically, secretion of the pituitary hormone, prolactin, is thought normally to be suppressed by a hypothalamic inhibitory factor, PIF. Furthermore, although the exact function of prolactin in the human is poorly understood (256), its concentration is known to be elevated in lactating women.

A comparison of the works cited revealed no relationship between the duration of contraceptive therapy and the occurrence of prolonged amenorrhea and/or galactorrhea. It may be of significance, however, that of the 105 amenorrheic patients reported (248, 250–253, 255), none had taken the medication for less than three cycles.

It should be emphasized that: (*a*) the incidence of postpill amenorrhea is low (251); (*b*) menstruation was reported in one study (248) to recur within 48 days (somewhat sooner for sequential-type users than for users of the combined type) following cessation of therapy in the vast majority of women and that ovulation did occur in a large number of these women during their first three posttreatment cycles; and (*c*) that among the patients in another study (253), normal menses tended to resume within the first 6 months following cessation of oral contraceptive therapy.

Assuming they wish to become pregnant, at least some of the women with secondary amenorrhea following the use of oral contraceptives may be treated successfully. The true success is difficult to evaluate, however, because the number of women reported in the various studies (250–253, 255) is small. It would appear that about half the women may respond to treatment with clomiphene citrate, again supporting the idea that the phenomenon represents hypothalamic dysfunction and/or a desynchronization of normal cyclic events. For clomiphene to be effective, the woman's pituitary must be capable of secreting gonadotropins and her ovaries must contain follicles capable of maturing and rupturing (257). In addition, there should be adequate estrogen secretion (255). Women not responding to clomiphene may respond to gonadotropins [human pituitary (250), human menopausal (251), FSH (252), and/or human chorionic (251, 252)] administered instead of, or in addition to, clomiphene. Despite spontaneous or induced ovulation in these women, however, pregnancy is not always achieved. One possible explanation was suggested by Maqueo *et al.* (258); these workers found histological evidence indicative of postcontraceptive endometrial refractoriness (inactivity) in some patients, a phenomenon which, theoretically, could impair fertility.

Miscellaneous Reactions and Complaints—Numerous reports concerning a variety of reactions and complaints

that might possibly be associated with the concurrent use of oral contraceptives have been cited in the literature (15, 23, 26, 84, 234, 240, 259–262). It has been pointed out (84), however, that many such reports involve observations made on only one or two patients. Consequently, the use of statistical analysis to determine the probability of a cause-and-effect relationship in such cases is an impossibility. On the other hand, the appearance of unusual symptoms during oral contraceptive therapy (in the absence of other drug use or predisposing conditions), their disappearance following drug withdrawal, and their reappearance upon readministration of the oral contraceptives would seem to suggest that unusual reactions may develop in rare cases as a result of oral contraceptive use. Nevertheless, the lack of placebo trials (*vide infra*) in these isolated cases prevents one from concluding positively that these reactions actually were due to the medication and were not merely coincidental occurrences (26).

Jelinek (260), for example, reviewed the available data concerning a number of cutaneous reactions reported to be associated with oral contraceptive use. These reactions include photosensitivity, herpes gestationis (in women who had previous attacks during pregnancy), and the induction or exacerbation (260, 261) of lupus erythematosus. Melasma, brown facial pigmentation, may be the most common cutaneous complication of oral contraceptive use; its incidence in users in the United States has been estimated at 5–8% (260). After cessation of oral contraceptive therapy, melasma appears to fade more slowly than does that which accompanies pregnancy, and it actually may be permanent. In addition, women who have had melasma during pregnancy appear to be more likely to develop the condition during subsequent oral contraceptive use.

Various neurological manifestations similarly have been associated with oral contraceptive use. In some cases, however, the events cited may have had a vascular etiology. Reported symptoms include chorea (262), migraine (15, 26, 259), and decreased visual acuity (259), as well as other ocular disorders (15, 259).

Goldzieher *et al.* (263) recently pointed out that although numerous reports exist in the literature that suggest an association between subjective complaints, such as nervousness and depression, and the use of oral contraceptives, the frequency of such symptoms actually attributable to these drugs is still unknown. Previous studies lacked an experimental design that incorporated: (*a*) randomization of drug and placebo assignment, (*b*) double-blind administration, and (*c*) appropriate crossover of drugs and placebo. Using such a design, these workers showed that: (*a*) the incidence, during a pretreatment cycle, of complaints including nausea, vomiting, abdominal discomfort, mastalgia, headache, and depression could not be used as a baseline, and all of these symptoms, except for nausea and vomiting, diminished markedly upon initiation of placebo treatment; (*b*) significant increases in nausea, vomiting, headache, and nervousness could be demonstrated statistically only in the first treatment cycle with high estrogen agents; and (*c*) although a weight gain of 2.3 kg. (5 lb.) or more occurred in a substantial number of women over a period of four cycles, the weight gain was the same in the placebo group as in the other four groups which had been using, respectively, two different combination-type preparations, a sequential preparation, and a progestagen-only preparation.

The authors pointed out that the results of their study should not be construed as indicating that these various phenomena were not associated with oral contraceptive use, but rather that the true incidence of drug-related complaints was probably much lower than previously had been assumed. In support of this conclusion, Goldzieher *et al.* (263, 264) cited a study in which changes in the color of contraceptive medication had elicited changes in nausea and headache, as well as a decrease in libido. On the other hand, Goldzieher *et al.* (263) indicated that some degree of weight gain, for example, theoretically could be induced by the oral contraceptives since metabolic studies had shown an anabolic effect of progestagenic steroids when they were given to estrogen-primed women. Kudzma *et al.* (265), however, showed that in four women on a constant caloric intake, metabolic balance changes occurring during oral contraceptive-treated cycles were similar in magnitude to those occurring during normal, untreated menstrual cycles. Nevertheless, the results of a study (266) of adolescent users of oral contraceptives suggested that other factors, nutritional or psychological, for example, might play a role in weight gain during oral contraceptive use. The risk of significant weight gain in this study was found to be related directly to the patient's initial weight percentile at the start of contraceptive treatment.

In spite of the drawbacks of non-double-blind studies, a number of such investigations have been carried out in an effort to determine the incidence of various subjective and semisubjective complaints, particularly depression, in users and nonusers of oral contraceptives. Goldzieher (85), for example, compared the answers given by oral contraceptive users and intrauterine device users to nonleading questions regarding their health. Except during the first three cycles of oral contraceptive use, when the incidence of nausea was found to be high, the incidence of various symptoms including nausea, vomiting, diarrhea, malaise, tiredness, weakness, headache, dizziness, nervousness, depression, and decreased libido was found to be similar in oral contraceptive and intrauterine device users. The results of other studies, however, indicated: (*a*) less depression in oral contraceptive users than in intrauterine device users (267) or (*b*) greater depression in oral contraceptive users than in nonusers, the majority of the latter having been diaphragm users (268). Several explanations for the discrepancies have been suggested. With respect to the diaphragm users, it is Hart's (269) opinion that such a group would be highly selected (nonrandom), *i.e.*, that individuals using the diaphragm were likely to be of a more stable temperament than would be oral contraceptive users. With respect to the other study (267), the intrauterine device users had been significantly more depressed than the oral contraceptive users prior to the start of contraception by these groups. Other studies, furthermore, have shown that women with histories of depression (270–272) and other emotional disorders (272, 273) were the ones most likely to

complain of depression (270–272), to have psychotic reactions (273), and/or to experience tension, nausea, dizziness, forgetfulness, and anger (272) while using oral contraceptives.

Other factors also are likely to have an influence on whether or not side effects such as depression may develop in oral contraceptive users. Reactions such as depression, headache, and loss of libido were conspicuously absent from O'Dwyer's (274) patients. These women were strongly motivated to remain well and nonpregnant; the majority already had three or more children and had experienced unhealthy, unpleasant, and dangerous episodes during their pregnancies. Main (275) also emphasized the psychogenic aspect of oral contraceptive-induced depression. He outlined a number of problems that use of such medication produced in certain women: (*a*) sadness that there would be no chance of a slip-up and pregnancy; (*b*) sadness at removal of an "essential" ingredient for sexual pleasure—the risk; and (*c*) a number of other points, all of which suggested that the women studied had a poor adjustment to their sexuality. In conclusion, it is tempting to speculate that similar psychological factors contribute to the discrepancies found between the theoretical effectiveness and the use effectiveness of the various oral contraceptives (*vide supra*), as well as of various other methods of birth control.

OUTLOOK FOR THE FUTURE

The commercially available oral contraceptives are highly effective agents, although patient failures and even method failures can occur. They are also agents whose use has been associated with serious adverse reactions in some women. Consequently, the contraindication of their use in thromboembolic disorders, markedly impaired liver function, and estrogen-dependent neoplasia seems justified. Nevertheless, there is some evidence to suggest that both their effectiveness and the occurrence of severe adverse reactions in patients using them may be partly related to factors such as size of the individual and ethnic group (67) or genetic predisposition (142–146, 234, 237, 239). Furthermore, in the case of women with thromboembolic disorders, care must be taken to provide equivalently effective alternative method(s) of birth control, since the incidence of postpartum thrombosis has been shown to be higher than that occurring with the use of oral contraceptives (104, 119). With respect to women with liver disease, cautious advice (236) has been to avoid oral contraceptives in the presence of any type of acute or chronic disturbance of liver function; others (240), however, have indicated that the only absolute contraindication need be in patients with any type of acute or chronic cholestatic hepatobiliary illness "since the hepatic abnormalities induced by the pill are basically in the bile-secreting function."

Attempts to develop steroid contraceptives that might have less potential for producing serious adverse reactions, the low dose, progestagen-only preparations, for example, unfortunately have resulted in compounds that also are somewhat less effective than the conventional agents. On the other hand, greater effectiveness from the point of view of eliminating patient failures may be achieved by more extensive use of depot preparations, although method effectiveness in comparison with that of the conventional oral contraceptives does not seem to be altered. Furthermore, irregular bleeding seems to be more common with dosage forms and dosage regimens other than those of the commercially available combined and sequential oral preparations. Perhaps it is for this reason that it has been suggested that the injectable preparations may be abandoned as contraceptives for future use (75).

The intrauterine device, of which many types are available (276), seems to offer a good alternative to steroid contraception in that patient failure due to forgetting to take one's medication obviously is eliminated. Effectiveness varies, however, due to a number of factors, although the addition of a chemical, such as copper, to the intrauterine device may increase its effectiveness (277). Nevertheless, the long-term effects of the presence of an intrauterine device, not to mention the presence of a chemical in addition, cannot be anticipated at this time, particularly since the mechanism of action of the intrauterine device is thought to involve a foreign body-induced low grade inflammation in the endometrium (278).

Perhaps the most promising contraceptive means for the future may prove to be the use of agents with abortifacient, luteolytic, and/or menses-inducing properties. Such agents would be used once a month (15) and perhaps only in the event that menstruation was delayed due to fertilization and implantation having taken place. Menstruation has indeed been induced in women by the intravaginal administration of PGE_2 and $PGF_{2\alpha}$ (279). Bleeding was induced in 24 hr. or less in 11 of 12 women in whom menstruation had been delayed for 2–7 days. Eight of the 11 had a positive test for pregnancy. The 12th woman also was found to be pregnant and was aborted with a 5-mcg./min. PGE_2 infusion.

Prostaglandins are thought to be involved in the natural process of menstruation (279). Nevertheless, the mechanism by which intravaginally administered prostaglandins induce menstruation is not clear. It has been suggested that it may be the result of the increased uterine contractility that is produced, although an inhibitory effect on the corpus luteum has not been ruled out. The ease with which prostaglandins could be self-administered intravaginally within a few days of a missed menstrual period [or, alternatively, just prior to the expected time of menstruation every month, regardless of whether or not fertilization might have occurred (280)] gives these compounds a potentially important place in the future of contraception.

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RESEARCH ARTICLES

Pharmacokinetics of the β-Adrenergic Blocker Sotalol in Dogs

KURT SCHNELLE and EDWARD R. GARRETT▲

Abstract □ The time course of absorption, distribution, and excretion of the β-blocker sotalol was studied in three unanesthetized dogs at three dosage levels of 1, 2, and 4 mg./kg. i.v. and 2, 4, and 8 mg./kg. p.o. The drug was assayed in body fluids and excreta by a spectrophotofluorometric method and by counting of tritiated drug. Unchanged sotalol is excreted up to 90% (±12%) in the urine. There was no protein binding, and the partition coefficient between plasma and red blood cells was unity. The data obtained in the studies were fitted graphically and by analog computer techniques and demonstrated no dose dependence. The graphical fit of the plasma levels following intravenous administration in accordance with a two-compartment open body model revealed a rapid distribution phase with a $t_{1/2}$ of 3.2 ± 1.1 min., which was followed by a disposition phase with a $t_{1/2}$ of 4.8 ± 1.03 hr. The analog computer fittings of the plasma and urine data according to the two-compartment model gave constants similar to those obtained from the graphical fits. The addition of a third tissue compartment that was in slow equilibrium with the central compartment proved necessary for a better fit of the data obtained at the high dosage level and in the ^{3}H-sotalol assays. The 75–90% absorption of sotalol in solution following oral administration was rapid ($t_{1/2}$ = 11–17 min.).

Keyphrases □ Sotalol pharmacokinetics—absorption, distribution, and excretion after intravenous and oral administration to dogs, protein binding and plasma/red blood cell partition studies □ Pharmacokinetics, sotalol—absorption, distribution, and excretion following intravenous and oral administration, dogs □ Absorption, sotalol—following intravenous and oral administration, dogs

Sotalol hydrochloride[1], 4′-[1-hydroxy-2-(isopropylamino)ethyl]methanesulfonanilide monohydrochloride, is a specific and potent β-adrenergic blocking drug. Several studies in both animals and man have demonstrated that the compound reverses isoproterenol- and epinephrine-induced tachycardia (1–3) and reverses the increase in blood levels of free fatty acids, lactic acid, and glucose that follows isoproterenol and epinephrine infusion (4, 5). Since the drug possesses less "quinidine-like" activity, it has been suggested that sotalol therapy may be preferred over other β-blockers with pronounced negative inotropic side effects (6).

It has been indicated that the plasma levels of tritium-labeled drug are related to the drug's pharmacological

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Keyphrases □ Sotalol pharmacokinetics—absorption, distribution, and excretion after intravenous and oral administration to dogs, protein binding and plasma/red blood cell partition studies □ Pharmacokinetics, sotalol—absorption, distribution, and excretion following intravenous and oral administration, dogs □ Absorption, sotalol—following intravenous and oral administration, dogs

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It has been indicated that the plasma levels of tritium-labeled drug are related to the drug's pharmacological

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Table I—Rate Constants[a] (in min.$^{-1}$) and Apparent Half-Lives ($t_{1/2}$) after Intravenous and Oral Administration of Sotalol in Dogs

Dog Number	Weight, kg.	Dose, mg./kg.	α	$10^2\beta$	10^2k_a	$10^2k_{P,T}$	$10^2k_{T,P}$	$10^2k_{P,U}$	$10^2k_{P,T'}$	$10^2k_{T',P}$	$t_{1/2}$[b], hr.
1[c]	18.1	2 (i.v.)	0.273	2.50	—	22.1	3.26	2.08	—	—	4.62
						(14.9)	(2.26)	(2.46)			(3.57)
	16.8	1 (i.v.)	0.196	2.40	—	14.3	1.26	3.70	—	—	4.81
						(9.0)	(1.08)	(2.85)			(3.79)
	17.2	4 (i.v.)	0.416	2.60	—	35.1	2.76	3.91	—	—	4.44
						(23.8)	(2.72)	(2.79)	(6.98)	(2.30)	(5.29)
	20.4	8 (p.o.)		1.80	1.49	—	—	—	—	—	6.58
					(6.33)	(23.7)	(2.71)	(2.47)	(6.99)	(2.30)	(5.97)
	18.6	4 (p.o.)		2.10	6.2	—	—	—	—	—	5.48
					(4.16)	(23.7)	(2.71)	(2.52)	(6.99)	(2.30)	(5.84)
	20.2	2 (p.o.)		1.70	—	—	—	—	—	—	6.8
					(6.50)	(23.7)	(2.71)	(2.27)			(4.96)
2[d]	18.1	1 (i.v.)	0.266	3.20	—	21.0	2.45	3.47	—	—	3.57
	20.0	2 (i.v.)	0.174	1.93	—	14.0	1.65	2.05	—	—	5.99
	19.1	4 (i.v.)	0.125	2.03	—	9.45	1.33	1.90	—	—	5.69
						(12.1)	(1.50)	(1.61)	(7.32)	(5.93)	(7.41)
	21.8	2[e] (i.v.)	0.245	2.32	—	20.0	2.16	2.62	—	—	4.99
						(16.5)	(2.07)	(2.50)	(7.59)	(2.65)	(5.48)
	21.8	2[e] (i.v.)	0.213	1.91	—	17.4	1.65	2.46	—	—	6.05
						(16.5)	(2.07)	(2.50)	(7.54)	(2.65)	(5.48)
	18.6	1[f] (i.v.)	0.301	1.72	—	25.6	3.04	1.71	—	—	6.72
						(17.9)	(2.45)	(2.11)	(5.69)	(2.15)	(6.00)
	18.6	1[f] (i.v.)	0.259	2.86	—	20.4	3.88	1.91	—	—	4.04
						(17.9)	(2.45)	(2.11)	(5.69)	(2.15)	(6.00)
3[g]	19.1	4 (i.v.)	0.235	2.86	—	17.2	1.27	5.29	—	—	4.04
						(20.2)	(1.78)	(4.82)	(8.27)	(3.44)	(3.54)
	18.6	2 (i.v.)	0.145	2.90	—	10.2	3.28	1.28	—	—	3.98
						(15.5)	(2.13)	(2.71)			(3.53)
	18.6	1 (i.v.)	0.203	3.29	—	15.3	2.03	3.83	—	—	3.52
						(15.5)	(2.19)	(2.52)			(3.71)

[a] The values for a cited experiment were obtained from graphical analysis of the data; the parenthetical values were obtained from analog computer fitting of the data. [b] Values obtained from the graphically determined slope, $\beta = k_e$ of ln(plasma levels) against time. The parenthetical values were calculated in accordance with $t_{1/2} = \ln 2/k_e$ where (12):

$$k_e = \frac{k_{P,U}}{1 + \frac{k_{P,T}}{k_{T,P}} + \frac{k_{P,T'}}{k_{T',P}}}$$

and $k_{P,T'}$ and $k_{T',P}$ values were included when significant and needed for a good fit of the data with the analog computer. [c] The studies were conducted in the tabular sequence at 0, 1, 8, 9.5, 11, and 12.5 months from the initial experiment. [d] The studies were conducted in the tabular sequence at 0, 3.5, 6, 13, and 14 months from the initial experiment. [e] The sotalol concentrations in blood and urine were analyzed by spectrophotofluorometric and radioactive assays simultaneously in the same experiment. The upper listing is based on the radioactive assay. All other studies were conducted by spectrophotofluorometric assays. [f] The sotalol concentrations in blood and urine were analyzed by the spectrophotofluorometric and radioactive assays simultaneously in the same experiment. The upper listing is based on the radioactive assay. In this experiment, bile samples were obtained on a biliary-cannulated dog. All other studies were conducted by spectrophotofluorometric assays. [g] The studies were conducted in the tabular sequence at 0, 1.5, and 2.5 months from the initial experiment.

activity in dogs (2). No significant metabolism occurred in dogs where 90% of the unmetabolized drug was excreted in the urine (2). Therefore, knowledge of the pharmacokinetic profile of sotalol should allow the design of an optimum dosage regimen from the plasma levels. The availability of a reasonably sensitive cold assay for the drug (7) is convenient for such studies. This paper describes the pharmacokinetics in the dog at multiple dosage levels of sotalol administered intravenously and orally.

EXPERIMENTAL

Treatment of Animals—The dogs were conditioned to the metabolic cages and trained for laboratory routine prior to experimentation. They were fasted for 24 hr. before each experiment. On the day of the experiment, the animals were placed on a table in a standing position and restrained by two straps which were tied around the fore and hind legs and then fixed to a horizontal bar above the dog. A jugular vein was punctured with a 14-gauge needle, and a vinyl tubing [0.11-cm. (0.044-in.) i.d., 0.16-cm. (0.065-in.) o.d.] was inserted through the needle. After free blood flow through the tubing was established, the needle was removed and 0.9% saline solution was dripped continuously at a rate of 1 ml./min. This infusion was maintained throughout 24 hr. to compensate for blood loss and to establish an adequate urine flow.

The urinary bladder was catheterized routinely through the urethra by inserting a ureteral catheter[2], French size 6 or 8. This catheter was kept in place for 24 hr. for continuous urine collection.

In one dog, Dog 2, the common bile duct was cannulated 1 week prior to an experiment. A laparotomy was performed under pentobarbital anesthesia (30 mg./kg.). After the cystic duct was tied, two 3.8-cm. (1.5-in.) pieces of polyethylene tubing[3] were inserted into the proximal and distal portions of the common bile duct. The two pieces were then connected by a flexible silicone rubber tubing [0.15-cm. (0.059-in.) i.d., 0.35-cm. (0.138-in.) o.d.] of 1.3-m. length to shunt bile. After the abdomen was closed, the loop was pulled under the skin by a wire to an incision on the back of the neck. There the tubing was cut and reconnected through a 14-gauge metal adapter which could be separated at will. Free passage through the tubing was assured by daily flushing of the distal part leading to the duodenum.

Pharmacokinetic Studies—Three male mongrel dogs, approximately 2 years of age and in apparent good health, were used in the balanced study. Hematocrit, white blood cell count, and erythrocyte sedimentation rate were within normal ranges (8). No dirofilarial larvae in the blood were observed microscopically. Body weights and the sequence of the experiments are listed in Table I.

Intravenous Studies—Sotalol[4] was administered intravenously to each of three dogs at three dosage levels: 1, 2, and 4 mg./kg. The

[2] Bard Inc., Murray Hill, N. J.
[3] Intramedic PE 240.
[4] Mead Johnson & Co., Evansville, Ind.

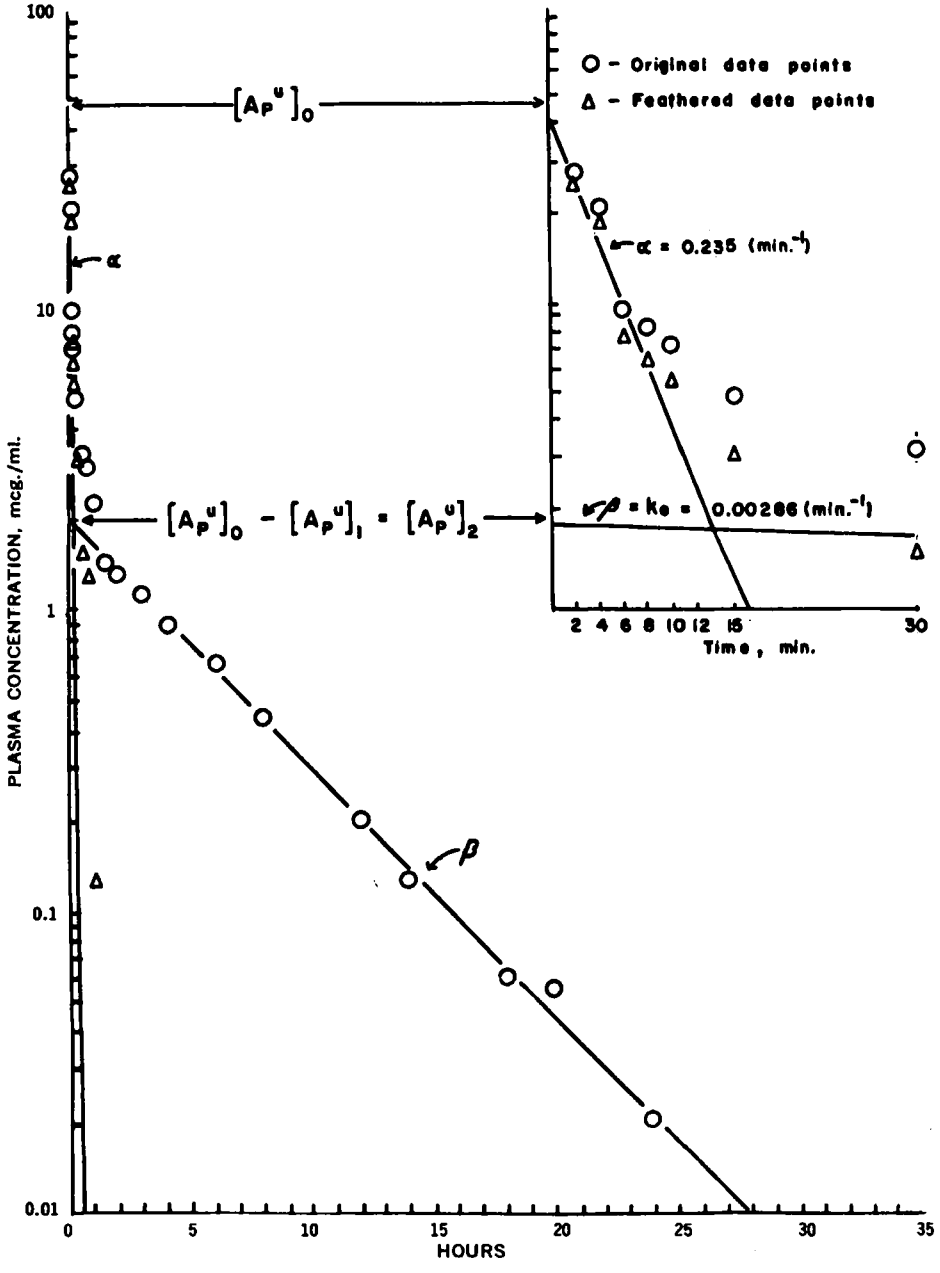


Figure 1—*Semilogarithmic plot of plasma levels following intravenous administration of 4 mg./kg. sotalol to 19.1-kg. Dog 3. The antilogarithmic values of the linear terminal portion with slope β were subtracted from the initial data points, and these feathered points were fitted to a straight line of slope α. $[A_P^u]_0$ is the intercept of the extrapolated line with slope α, i.e., the initial drug concentration in the central compartment, and $[A_P^u]_2$ at zero time is the drug concentration in the equilibrated tissues from the extrapolation of the linear terminal portion of the curve with slope β = k_e in accordance with a two-compartment open body model (Scheme I). The inset is for an expanded time scale. Key: O, original data points; and Δ, feathered values.*

time intervals between experiments in one animal were at least 4 weeks. The drug was injected as its hydrochloride salt in sterile solution containing 10 mg./ml. of the drug through a threeway stopcock into the jugular tubing. The duration of the injection was kept constant at 10 sec. The catheter was flushed with isotonic saline solution after the injection. A distinct reaction due to the rapid administration was only observed after the 4-mg./kg. dose as tachycardia, dyspnea, and increased salivation. Blood samples were withdrawn from the jugular vein at 1, 2, 4, 6, 8, 10, 12, 15, 20, 25, and 30 min. after completion of the injection; then in 15-min. intervals up to 120 min.; in 30-min. intervals up to 4 hr.; hourly up to 12 hr.; and every 2 hr. up to 24 hr. Urine was collected every 30 min. up to 4 hr., hourly up to 12 hr., and every 2 hr. up to 24 hr. The dogs were then transferred to a metabolic cage, and blood and urine were collected at 6-hr. intervals up to 48 hr. and every 12 hr. up to 72 hr. Completeness of urine collection was assured by catheterization. The blood volume withdrawn for each sample was 7 ml. from 0 to 12 hr. and 16 ml. thereafter.

Intravenous Studies with ^{3}H-Sotalol—The tritiated sotalol[4] was prepared as described elsewhere (2). The sterile solution contained 0.5 mg./ml. of the drug with a specific activity of 70 μc./ml. Sterile solutions of cold drug (10 mg./ml.) and labeled material were mixed in a sterile beaker to obtain a drug concentration that was adequate to yield dosages of 1 and 2 mg./kg. of total drug administered to Dog 2. The total activity administered was 1046 μc. at the 2-mg./kg. dose and 1123 μc. at the 1-mg./kg. dose. The schedule for blood and urine sampling was the same as described previously for up to 72 hr. Additional samples were collected every 24 hr. up to 144 hr. Over the same period of time, the feces were collected every 24 hr. in both experiments. Bile samples were obtained in one experiment on Dog 2 (1 mg./kg.) at 2.5, 5, 8, 10, 15, 20, 23, and 30 hr. after injection.

Oral Studies—The same sotalol solution (10 mg./ml.) used in the intravenous study was administered through stomach tubing in doses of 8, 4, and 2 mg./kg. to Dog 1. Blood samples were withdrawn at 2.5, 5, 7.5, 10, and 15 min. after drug administration and at the schedule given previously in the intravenous studies up to 4 hr., every 2 hr. up to 24 hr., and then again as described for the intravenous study. Urine was collected every 30 min. up to 4 hr. and thereafter at the same schedule given for blood samples.

Treatment of Samples—Blood was withdrawn with a disposable syringe and transferred to centrifuge tubes containing 30 USP units of sodium heparin[5] in a volume of 0.1 ml. distilled water to prevent clotting. Blood and urine samples were centrifuged at 3000 r.p.m.

[5] The Upjohn Co., Kalamazoo, Mich.

Table II—Values for First-Order Elimination Rate Constants, k_e (in min.$^{-1}$) as Obtained from Urinary Excretion Data; Estimates of Apparent Volumes of Distribution in Liters Determined by Different Methods for Intravenous and Oral Studies

Dog Number	Dose, mg./kg.	$10^3 k_e$ ln $(\Delta U/\Delta t)$ *versus* Time	$10^3 k_e$ ln $(U_\infty - U)$ *versus* Time	Renal Clearance, ml./min.	Recovery in Urine, % of Dose	V_{P_e}[a]	$V_{P_{true}}$[b]	$V_P{}^u$[c]	V_T[d]	$V_{T'}$[e]	$V_{tot} = (V_P{}^u + V_T + V_{T'})$	$V_{DP}{}^u$[f] from Clearance	$V_{DP}{}^u$[g] from Extrapolated β-Phase
1	2 i.v.	1.70	2.60	50.0	116	2.63	1.04	1.91	12.6	—	14.5	20.0	26.8
	1 i.v.[h]	—	—	—	—	2.53	1.01	1.85	15.4	—	17.2	—	29.9
	4 i.v.	2.04	1.83	45.6	87	2.58	1.03	1.89	16.5	4.3	22.7	17.5	33.8
	8 p.o.	2.31	2.47	56.7	78	—	1.22	1.67	14.6	5.0	21.3	32.2	39.7
	4 p.o.	2.28	2.43	80.0	102	—	1.12	1.97	17.3	5.9	25.2	38.1	34.4
	2 p.o.	1.72	1.63	51.3	88	—	1.21	1.79	15.6	—	17.4	32.0	40.7
2	1 i.v.[i]	2.31	2.98	37.5	108	2.36	1.04	1.77	15.2	—	17.0	11.7	26.1
	2 i.v.[i]	2.42	2.22	67.5	98	3.99	1.20	3.34	28.3	—	31.6	34.9	42.7
	4 i.v.	1.86	1.36	62.0	76	4.48	1.15	3.86	31.1	4.8	39.8	30.5	46.4
	2 i.v.[i]	1.54	1.62	75.0	94	3.29	1.31	2.58	20.6	7.4	30.6	32.4	46.4
	2 i.v.[i]	1.59	1.56	54.4	72	4.41	1.31	3.70	29.5	10.5	43.2	28.5	47.9
	1 i.v.[i]	1.79	2.00	66.7	91	2.90	1.12	2.30	16.8	6.1	25.2	38.8	40.3
	1 i.v.[i]	2.25	1.99	70.0	79	3.20	1.12	2.60	19.0	6.9	28.5	24.5	39.4
3	4 i.v.	2.72	2.87	103.0	97	1.79	1.15	1.03	11.7	2.5	15.2	36.0	39.6
	2 i.v.	2.58	2.64	93.3	82	4.71	1.12	3.97	28.9	—	32.9	32.2	37.0
	1 i.v.	1.92	2.25	145.0	87	4.62	1.12	3.88	27.5	—	31.4	44.1	37.6

[a] V_{P_e} calculated from the quotient, $A_0/[A_P]_0$, of the dose administered intravenously, A_0, and the initial concentration of total drug in the plasma, $[A_P]_0$, obtained from the intercept of the $[A_P]$ plot of the best computer fits against time with zero time. [b] $V_{P_{true}} = (1 - H)V_{B_{true}}$, where $V_{B_{true}}$ = dog weight in kg. × 100 ml./kg. (17), where the hematocrit, H, is taken as 0.40, 0.35, and 0.40 for Dogs 1, 2, and 3, respectively. [c] Calculated in accordance with $V_P{}^u = V_{P_e}{}^u - HV_{P_{true}}D/(1 - H)$ (Eq. 6). The volume of the central compartment, $V_P{}^u$, in the oral studies was calculated in accordance with Eq. 14 after solving for $V_P{}^u = (\gamma A_0/\text{I.C.}) - V_{RBC}$. When there is no protein binding, as in this case, $V_P{}^u$, the volume of the central compartment referenced to the concentration of total drug in the plasma, is the same as V_P, the volume of the central compartment referenced to the concentration of total drug in the plasma. [d] Calculated in accordance with $V_T = V_P{}^u(k_{P,T}/k_{T,P})$ (Eq. 16); the rate constants were taken as the values obtained from the best analog computer fits. [e] Calculated in accordance with $V_{T'} = V_P{}^u(k_{P,T'}/k_{T',P})$ (Eq. 17). [f] Calculated in accordance with the clearance equation (12), where the renal clearance of sotalol in milliliters per minute was divided by k_e in min.$^{-1}$ to yield $V_{DP}{}^u$ in milliliters. The fraction F of drug renally excreted becomes 1 when, as in this case, virtually all drug is excreted through the kidneys. [g] Calculated in accordance with $V_{DP}{}^u = V_{DP_e}{}^u - HDV_{P_{true}}/(1 - H)$ (Eq. 9), where the $V_{DP_e}{}^u$ was obtained in accordance with $V_{DP_e}{}^u$ = dose/$[A_P{}^u]_0$ (Eq. 7), where $[A_P{}^u]_0$ is the intercept of the extrapolated slope of k_e to zero time. [h] No urine data were obtained in this experiment. [i] The values for V_{P_e}, $V_P{}^u$, and V_T were obtained from values derived from the graphical fitting in accordance with the two-compartment body model (Scheme I). [j] The sotalol concentrations in blood and urine were analyzed by the spectrophotofluorometric and radioactive assays simultaneously in the same experiment. The upper listing is based on the radioactive assay.

for 10 min., and the supernatant plasma and urine were then refrigerated. The samples obtained in the radioactive study were split; one portion was deep frozen and sent away for the counting and chromatographic studies[6], and one portion was kept for the chemical assay.

Assay of Samples—The analyses were based on a modified spectrophotofluorometric method which was reported previously (7). The sensitivity was increased from 0.1 to 0.02 mcg./ml. of the drug by extracting from a larger volume (5 ml.) of deproteinized plasma. The lower limit of detection in the urine was 2.5 mcg./ml. Spiked plasma and urine samples were carried through the analytical procedures and assayed at the same time as the samples of unknown concentration. Radioactivity measurements[6] were conducted with a liquid scintillation spectrometer[7] corrected for background and quenching. Duplicate samples of 0.5 ml. plasma were mixed with 1 ml. of a solubilizer[8] and 10 ml. of a counting solution consisting of 5 g. 2,4-diphenyloxazole[9] and 0.5 g. *p*-bis-(*o*-methylstyrylbenzene)[9]/l. of toluene. Duplicate samples of 0.2 or 0.1 ml. of urine and 0.1 ml. of bile were added to 0.6 ml. of water and 15 ml. of solution of 5 g. 2,4-diphenyloxazole, 0.3 g. *p*-bis-(*o*-methylstyrylbenzene), and 120 g. naphthalene/l. of dioxane. Fecal samples were homogenized with nine volumes of water, and aliquots (100–200 mg.) of the homogenates were mixed with 0.6 ml. water and 15 ml. of the same counting solution used for urine.

The presence of possible metabolites of ^{3}H-sotalol was evaluated by paper chromatography of urine, plasma, and bile samples using the following systems: (*a*) strips of 2.54-cm. (1-in.) Whatman No. 1 paper and a solvent mixture of *n*-butanol–acetic acid–water (30:7.5:12.5), and (*b*) Whatman DE20 paper strips and 0.02 *N* phosphate buffer of pH 7.4 as the developing solvent. In each case, 50 µl. of sample was used. The strips were dried, cut into 1.27-cm. (0.5-in.) segments, and placed in counting vials with 0.6 ml. of water and 15 ml. of the dioxane counting solution already described.

Protein Binding Study—Binding of sotalol to plasma proteins was studied by an ultrafiltration method (9). The cone membranes used in these experiments[10] are retentive for plasma protein and permit only passage of ions and compounds of low molecular weight. Thus, only free diffusable drug was found in the filtrate. Possible binding to the membranes is cancelled out when equally spiked buffer–drug and plasma–drug solutions are carried through the entire procedure.

The binding to plasma protein can be estimated by:

$$100f(\%) = \frac{R_B - R_P}{R_B} \times 100 \qquad \text{(Eq. 1)}$$

where f is the fraction bound to plasma protein, R_B is the fluorescence reading of buffer filtrates, and R_P is the reading of plasma filtrates that were spiked with equal concentrations of drug.

Heparinized dog plasma and isotonic phosphate buffer solutions of pH 7.45 were spiked with sotalol hydrochloride to achieve drug concentrations of 0.3, 0.6, 1.0, 5.0, 10.0, 25.0, 50.0, 100.0, and 1000.0 mcg./ml. Aliquots (6.00 ml.) of these solutions and of plasma and buffer blanks were transferred to cone membranes which were placed in plastic supports on top of 40-ml. centrifuge tubes. The solutions were then centrifuged at 1700 r.p.m. The time needed to obtain approximately half-filtration was 2 min. for the buffer solutions and 25 min. for the plasma solutions. The filtrates were carried through the analytical procedure and assayed spectrophotofluorometrically.

Red Blood Cell Partition—The partition of sotalol into the red blood cells was studied as functions of drug concentration and time. Freshly drawn heparinized whole blood and plasma were transferred into 125-ml. conical flasks which were placed in a slowly shaking water bath at 37.5°. The whole blood was then spiked with sotalol to achieve concentrations of 0.5, 1.0, 10.0, and 100.0 mcg./ml. of the drug. Plasma spiked with 1.0 mcg./ml. and whole blood without drug were processed by the experimental procedure and

[6] Performed by Mead Johnson & Co., Evansville, Ind.
[7] Model 3375, Packard Instrument Co., Palo Alto, Calif.
[8] Bio-Solv BBS-3, Beckman Instruments Inc., Palo Alto, Calif.
[9] Eastman Kodak Co., Rochester, NY 14650

[10] Centriflo CF50 membrane filter cone with CSTL support, Amicon Co., Lexington, Mass.

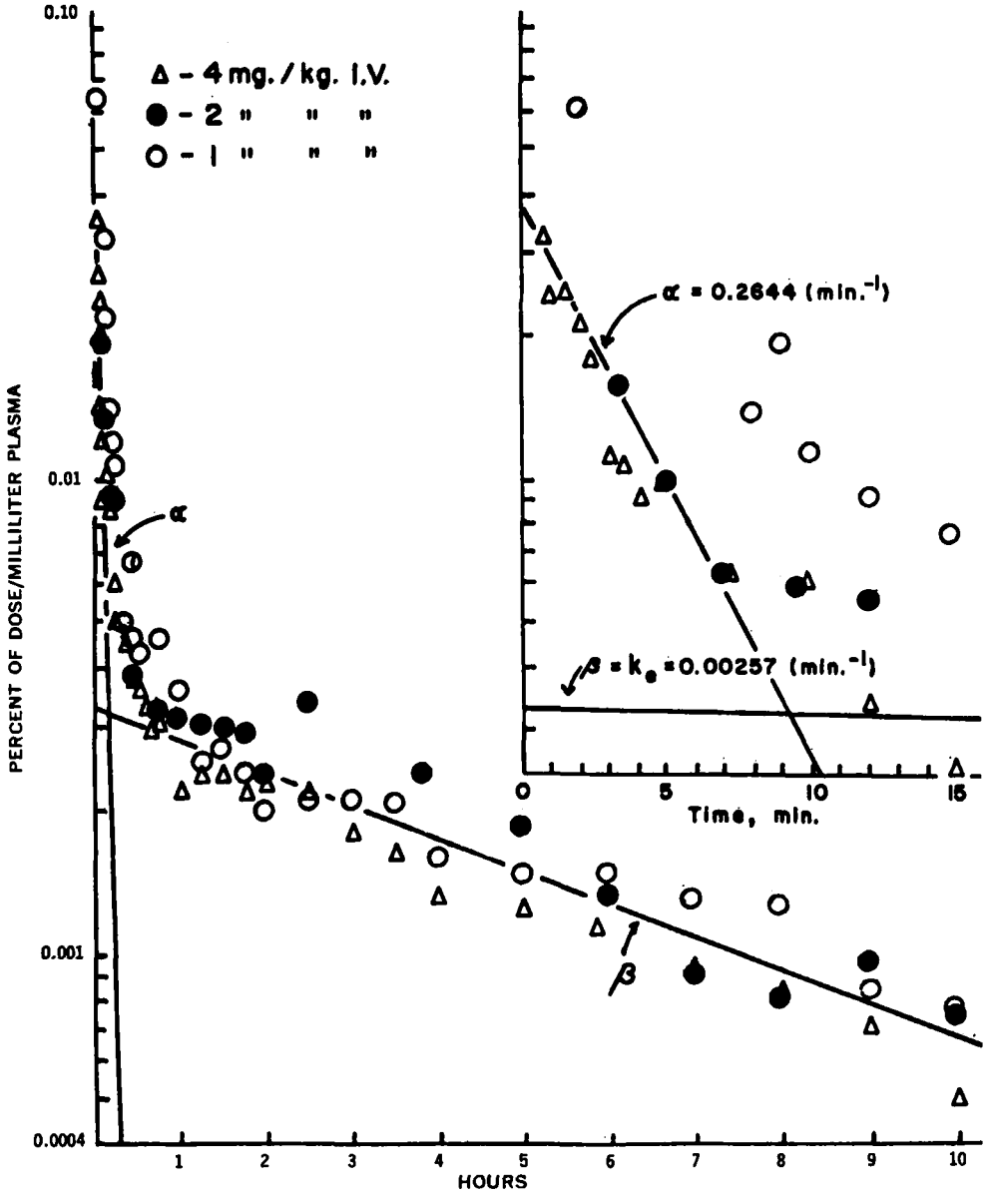


Figure 2—*Semilogarithmic plots of the plasma levels in terms of percent of dose per milliliter plasma against time following intravenous administration of 4 (Δ), 2 (●), and 1 (○) mg./kg. of sotalol to 19-kg. Dog 1. The data points obtained at the three dose levels could be graphically fitted with one common curve in accordance with a two-compartment open body model (Scheme I). The individual values for α and β are given in Table I. The inset is for an expanded time scale.*

the obtained results served as reference standard and blank, respectively. Samples of the blank blood and of the whole blood containing 1.0 mcg./ml. of the drug were withdrawn at 1, 3, 7, 10, 30, 60, and 120 min. after spiking. Samples of the plasma solution of 1.0 mcg./ml. were taken at 1, 30, and 120 min. Samples were withdrawn at 30 min. from all whole blood solutions of the various drug concentrations. Small specimens for the hematocrit determination were taken immediately after sampling, and the blood was centrifuged for 5 min. at 3000 r.p.m. The plasma was stored at 4° until the assay was performed. The fluorescence readings of all samples were compared to a calibration curve in plasma spiked with 0.1, 0.3, 0.6, and 1.0 mcg./ml. sotalol and to the readings of the plasma standard of 1.0 mcg./ml. that was carried through the procedure. The red blood cell/plasma unbound drug partition coefficient could be estimated by (10, 11):

$$D = [A_{RBC}]/[A_P^u] = \{[A_B]/[A_P](1 - f)(1 - H) - [f/(1 - f)] - 1\}(1 - H)/H \quad \text{(Eq. 2)}$$

where $[A_{RBC}]$, $[A_B]$, and $[A_P]$ are the drug concentrations in the red blood cells, whole blood, and plasma, respectively; $[A_P^u]$ is the drug concentration in plasma unbound to proteins; H is the hematocrit; and f is the fraction of drug in plasma bound to plasma proteins. If, as in this specific case, there is no significant protein binding, $f = 0$ and then $[A_P^u] = [A_P]$, which is the total concentration of drug in the plasma, and Eq. 2 reduces to:

$$D = \{[A_B]/[A_P^u](1 - H) - 1\}(1 - H)/H \quad \text{(Eq. 3)}$$

CALCULATIONS AND TREATMENT OF DATA

Plasma—The raw data were plotted in terms of micrograms per milliliter of plasma *versus* time on semilogarithmic paper. In the great majority of cases, the concentrations of unbound drug in the plasma, $[A_P^u]$, on intravenous administration exhibited a biexponential decay of the plasma levels as a function of time (Fig. 1), which can be described as:

$$[A_P^u] = [A_P^u]_1 e^{-\alpha t} + [A_P^u]_2 e^{-\beta t} \quad \text{(Eq. 4)}$$

The value for β was obtained from the slope of the linear terminal portion of the logarithm of the plasma level–time curve where excretion was the rate-determining process. The value for the slope of the rapid distribution phase, α, was estimated by subtracting the corresponding antilogarithmic values of the line of slope β extrapolated to zero time from the antilogarithms of the initial points of the log plasma concentration–time curve (12). Hence, a two-compartment open body model (Scheme I) was assumed, where P_u is amount of drug in the central compartment that is associated with plasma and referenced to the concentration of unbound drug in the

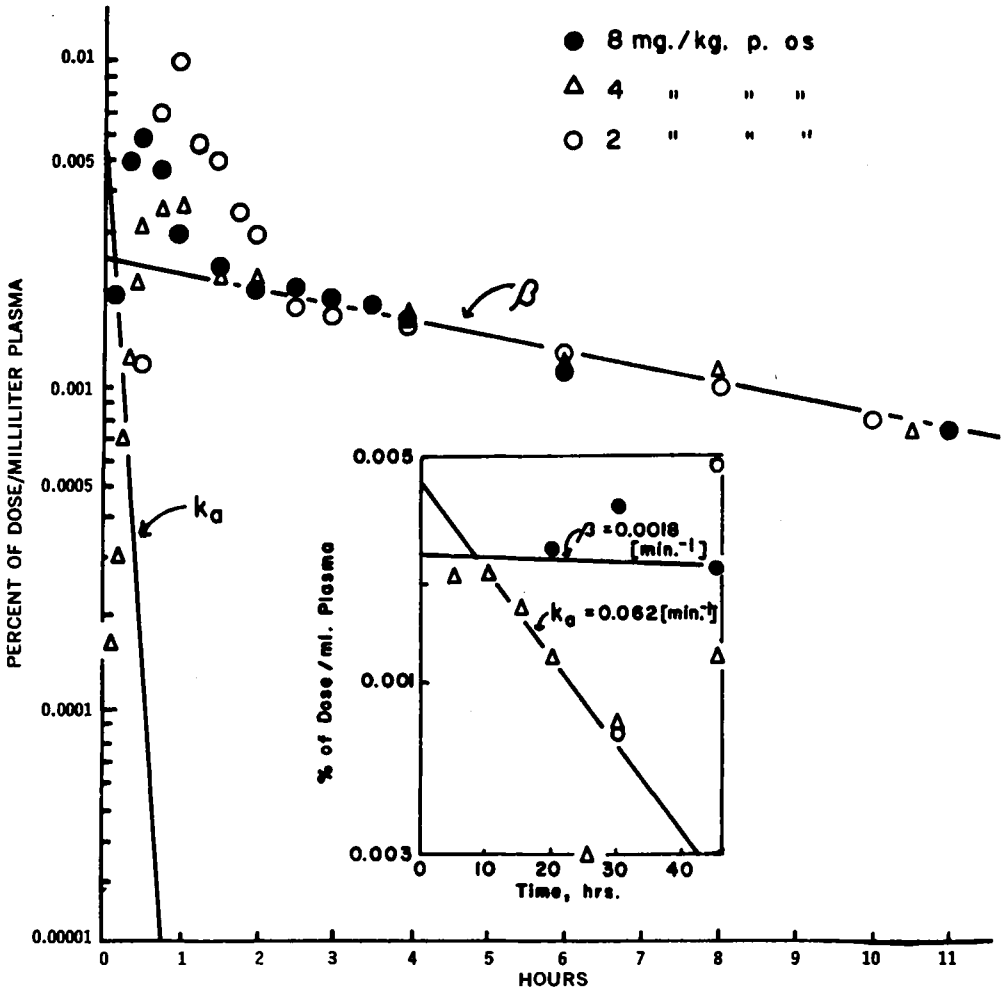


Figure 3—*Semilogarithmic plots of plasma levels in percent of dose per milliliter plasma following oral administration of 8 (●), 4 (△), and 2 (○) mg./kg. of sotalol to 19-kg. Dog 1; the plots show an apparent independence of dose. The extrapolation of the linear terminal portion of the curve cuts off the top of the absorption phase. Therefore, it was not possible to obtain an accurate estimate of k_a by the feathering technique. The individual values for $\beta = k_e$ and the absorption rate constants k_a estimated by graphical and analog computer methods are given in Table I.*

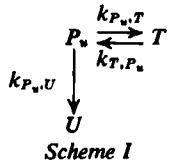
plasma (11, 13), T is the amount of drug in a peripheral tissue that equilibrates readily with P_u, and U is the amount eliminated from the body.

The apparent first-order rates $k_{P_u,T}$, k_{T,P_u}, and $k_{P_u,U}$ were calculated from the α- and β-values obtained (14). Estimates of the half-lives ($t_{1/2}$) of the distribution and disposition–excretion phases were obtained by dividing α and β into ln 2.

The extrapolation of the α-phase to zero time represented the initial concentration, $[A_P{}^u]_0$, of drug in the central compartment that was in the plasma and unbound, where instantaneous mixing of the drug with the plasma and its associated fluids occurred (12). Thus, the apparent volume of distribution of the central compartment, $V_P{}^u$, referenced to the concentration of unbound drug in the plasma would be estimated (11) from:

$$V_P{}^u = \{A_0 - (A_{RBC})_0 - (A_P{}^b)_0\}/[A_P{}^u]_0 = V_{P_s}/(1-f) - HV_{P_{true}}D/(1-H) - V_{P_{true}}f/(1-f) \quad \text{(Eq. 5)}$$

where A_0 is the total amount of drug administered; and $(A_{RBC})_0$ and $(A_P{}^b)_0$ are the initial amounts of drug in the red blood cells and in plasma bound to plasma proteins, respectively. The V_{P_s} is the pseudo-apparent volume of distribution of the central compartment referenced to total drug concentration in plasma, $A_0/[A_P]_0$, where $[A_P]_0$ is the total concentration of drug in the plasma at zero time; D, H, and f are the red blood cell/plasma partition coefficient, the hematocrit, and the fraction of drug in the plasma bound to plasma proteins, respectively. The $V_{P_{true}}$ is the true plasma volume of the animal (11).



Scheme I

If there is no protein binding, $f = 0$ and $(A_P{}^b)_0 = 0$, which is so in this specific case; then $V_P{}^u = V_P$, which is the apparent volume of distribution referenced to total drug concentration in the plasma. Similarly, $V_{P_s}{}^u = V_{P_s}$. Thus, Eq. 5 reduces (11) to:

$$V_P{}^u = V_{P_s}{}^u - HV_{P_{true}}D/(1-H) \quad \text{(Eq. 6)}$$

The sum of the pseudoapparent volumes of distribution of the central compartment and of the readily perfused tissue, $V_{D_{P_s}}{}^u$, referenced to the concentration of unbound drug in the plasma can be estimated (11) from:

$$V_{D_{P_s}}{}^u = V_{P_s}{}^u + V_{T_{P_s}}{}^u = \text{dose}/[A_P{}^u]_2 = A_0/([A_P{}^u]_0 - [A_P{}^u]_1) \quad \text{(Eq. 7)}$$

where the divisor of the last two terms is the concentration of unbound drug in the equilibrated fluids after the rapid equilibration of the α-phase on the assumption of insignificant drug excretion during the time interval of this equilibration.

It has been shown (11) that the sum of the apparent volumes of distribution of the central compartment and of the readily perfused tissues, $V_{D_P}{}^u$, referenced to the total unbound drug concentration in the plasma is actually:

$$V_{D_P}{}^u = V_P{}^u + V_{T_P}{}^u = \frac{\text{dose} - (\text{amounts in red blood cells and bound to plasma protein})}{[A_P{}^u]_2} = V_{D_{P_s}}/(1-f) - HDV_{P_{true}}/(1-H) - V_{P_{true}}f/(1-f) \quad \text{(Eq. 8)}$$

and can be calculated from the equivalent pseudovolume of dis-

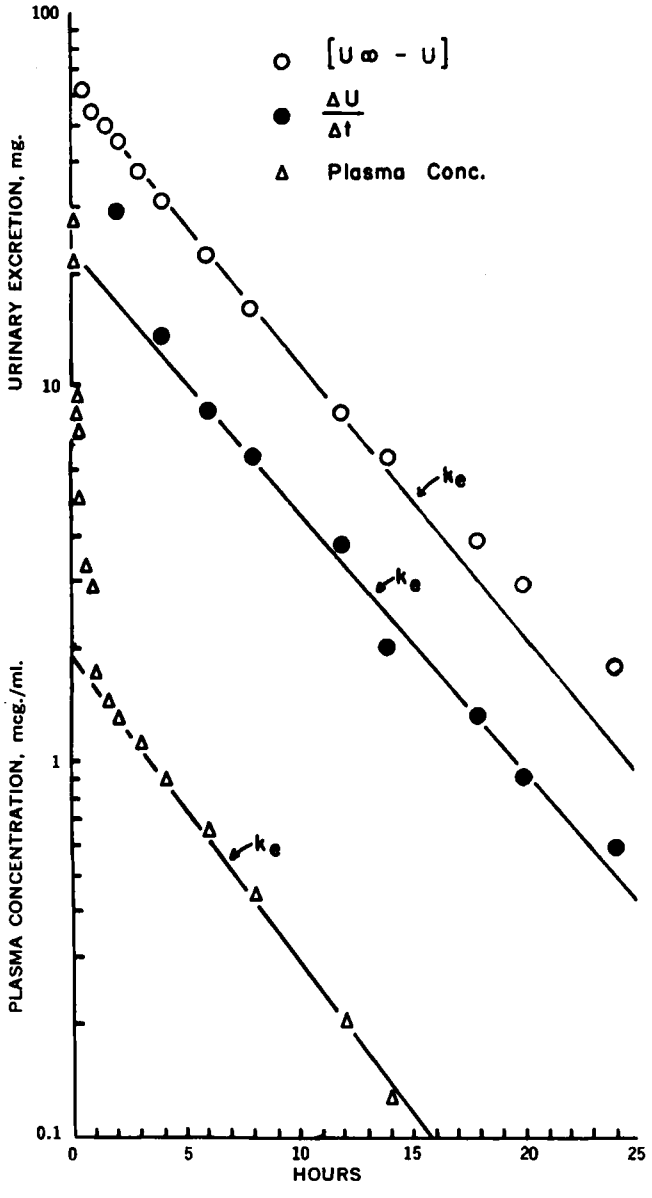


Figure 4—*Semilogarithmic plots of plasma concentrations in micrograms per milliliter (Δ), of the rate of urinary excretion per 2 hr., ΔU/Δt, in milligrams (●), and of the differences between the amount of drug recovered in the urine, U_∞, and the amounts excreted at time t, U, in milligrams (○). Data were obtained following the intravenous administration of 4 mg./kg. sotalol to 19.1-kg. Dog 3.*

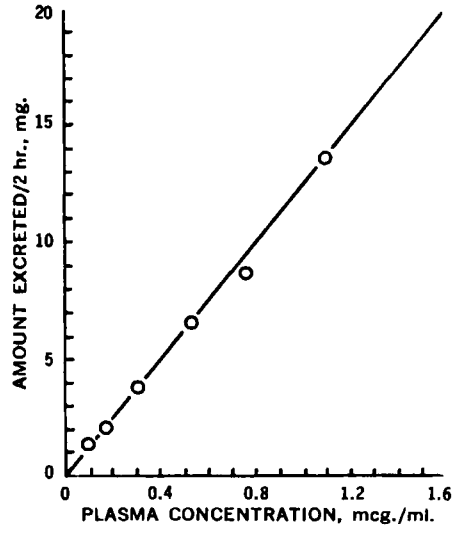


Figure 5—*Linear plot of the rate of urinary excretion per 2 hr., ΔU/Δt, against the plasma concentrations at midpoint of the periods of urine collection. The resulting points were fitted to a straight line through the origin whose slope is the renal clearance in accordance with* $\Delta U/\Delta t = k_{P,U}A_P^u = k_e F V_{D_P}^u [A_P^u]$ *(Eq. 11), where* k_e *is the overall elimination constant,* F *is the drug concentration in the plasma cleared by the kidneys,* $V_{D_P}^u$ *is the apparent volume of distribution, and* $[A_P^u]$ *is the drug concentration at the midpoint of urine collection. Data were obtained following the intravenous administration of 4 mg./kg. sotalol to 19.1-kg. Dog 3.*

tribution (Eq. 7) estimated from the linearly extrapolated intercept, $[A_P^u]_2$, of the β-phase if negligible excretion is assumed in the α-phase.

If $f = 0$, as in this case, the last term of Eq. 8 vanishes and:

$$V_{D_P} = V_{D_P}^u = V_{D_{P_2}}^u - HDV_{P_{true}}/(1 - H) \qquad \text{(Eq. 9)}$$

These estimates of $[A_P^u]_2$ and thus $V_{D_{P_2}}^u$ were obtained from plots such as the typical one given in Fig. 1. The rate constants and the other parameters obtained by graphical procedures are summarized in Tables I and II.

The plasma levels were plotted as percent of dose administered per milliliter of plasma against time for the several doses in the same dog. If those peaks were superimposable, the possibility of dose-dependent pharmacokinetics could be rejected. Such a typical set of plots is given for the intravenous studies in Fig. 2 and for the oral studies in Fig. 3. The β-values, $V_{D_P}^u$, and $t_{1/2}$, in the oral studies were obtained from the linear terminal portion of the semilogarithmic plasma concentration–time plots. A preliminary estimate of the absorption rate constant, k_a, was obtained from feathering the plasma level–time data (15) in accordance with:

$$\ln [A_P^u] = -k_a t + \ln [A_P^u]_0' \qquad \text{(Eq. 10)}$$

where $[A_P^u]_0'$ is an initial estimate of the concentration of the drug in the rapidly equilibrated tissues. It is realized that the validity of this method of k_a and $[A_P^u]_0'$ estimation is based on the premise of negligible excretion during the absorption phase. Since the line extrapolated from the semilogarithmic plot of the terminal data that represented the disposition phase with the slope of β was considerably lower than the actual plasma level data at its maximum values in all three oral experiments (Fig. 3), this feathering procedure could not yield the real k_a. This phenomenon is a consequence of relatively rapid absorption where equilibration of drug among the available tissues is not readily effected during the absorption phase.

Urine—The concentrations determined in the urine were multiplied by the volumes of urine excreted, and these amounts were plotted cumulatively against time to obtain the total recovery of drug, U_∞. Estimates of the overall elimination rate constant, k_e (*i.e.*, β), were also obtained (12) from semilogarithmic plots of the amounts not yet excreted in the urine, $\ln (U_\infty - U)$, *versus* time and the rate of excretion, $\ln \Delta U/\Delta t$, *versus* time (Fig. 4 and Table II). The renal clearance of sotalol was determined by a plot of the rate of excretion, $\Delta U/\Delta t$, against the plasma concentrations taken at the midtime of the interval of the urine collections, where ΔU is the amount of drug excreted in the urine in the time interval, Δt (Fig. 5 and Table II). The slope of this plot, in accordance (12) with:

$$\Delta U/\Delta t = k_{P,U}A_P^u = k_e F V_{D_P}^u [A_P^u] \qquad \text{(Eq. 11)}$$

is the renal clearance, where k_e is the disposition constant β, F is the fraction of the drug in the body that is cleared by the kidneys, $V_{D_P}^u$ is the apparent volume of distribution of the equilibrated tissue referenced to the concentration of unbound drug in the plasma, and $[A_P^u]$ is the concentration of drug in the plasma at the midtime of the interval of urine collection. Since the k_e values are known from other treatments of the data (Figs. 1–4) and F is the ratio of drug renally excreted to the dose administered, this slope also permits the determination of the apparent volume of distribution of the readily equilibrated tissues, $V_{D_P}^u$, referenced to the plasma concentration of unbound drug (Table II).

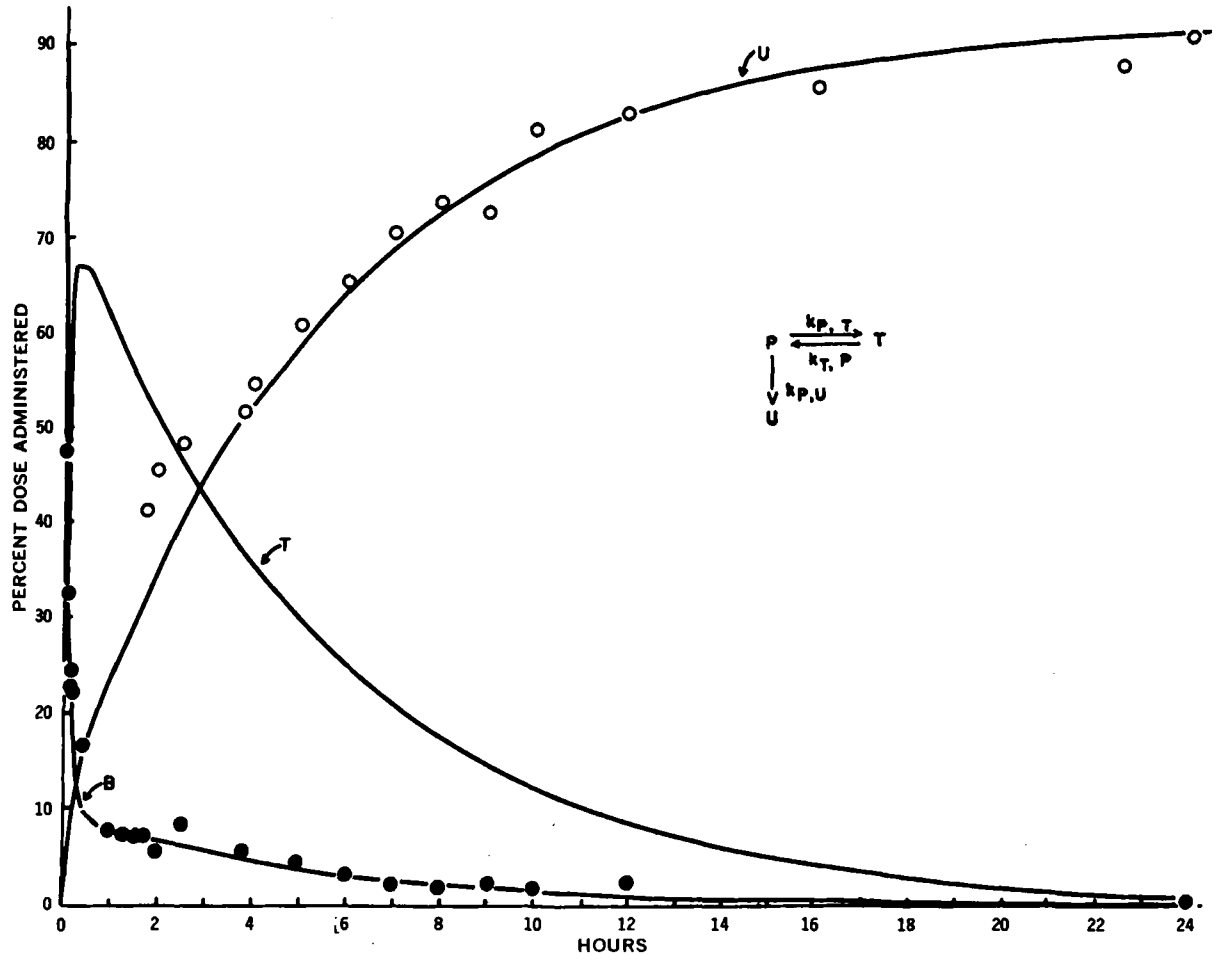


Figure 6—*Analog computer fitting of plasma* (●) *and urine* (○) *data in accordance with a two-compartment body model (Scheme I) following the intravenous administration of 2 mg./kg. to 18.1-kg. Dog. 1.* T *is the generated curve for the tissue compartment.*

Analog Computer Fitting: Programming—The program used in the analog computer[11] fitting of the pharmacokinetic data for a three-compartment body model was given previously (16). The initial conditions that represented the administered dose were fed into the central compartment *P* (plasma) for the data of the intravenous studies and into the GI compartment (gastrointestinal tract) for the data of the oral studies. The time scaling was set with a "ramp function" in such a manner that 0.5 in. on the graph paper equaled 1 hr. of the total 30 hr. of data and consumed 4 sec. of machine time. The recorder speed for the 3- and 6-hr. fits was increased by a factor of 10 and 5, respectively, through different settings of the *x*-axis range switch on the *x*–*y* recorder[12]. The range of the *y*-axis was increased 10-fold when the later portion of the plasma curves was fitted.

Intravenous Administration—The initial drug concentration in the central compartment was obtained from the best analog computer fit of the linear plot of the plasma concentrations against time in accordance with the two-compartment body model (Scheme I) and was considered as 100% of the dose in the plasma and associated fluids. Instantaneous mixing of the drug in the blood was assumed. The other data were represented as fractions of this 100%. The cumulative urinary excretion was plotted in terms of percent of dose administered. The urinary data were plotted over 30 hr.; the plasma data were plotted on a 30-hr. and expanded 6- and 3-hr. time scales and over 30 hr. on a 10 times enlarged ordinate covering 0–10% of dose administered. In this manner, an excellent fitting of the early as well as the later phases of the plasma level–time curves was possible.

As a first approach, the two-compartment body model (Scheme I) was assumed and the rate constants obtained from the graphical fit were used as first estimates for the potentiometer settings. A good fit was obtained with this model for the lower dosages of 1 and 2 mg./kg., and the curves for the latter are shown in Fig. 6. At the high dosage level of 4 mg./kg. and in the radioactive studies where the assay sensitivity was greater, positive deviations of the urinary excretion and terminal plasma data from the best fit with time were observed. A typical fit of such data by the two-compartment body model is given in Fig. 7.

The plateau observed in the terminal plasma data could be assigned to the return of the drug from a deeper compartment. Therefore, to obtain a better fit in these instances, a tissue compartment *T′* with comparatively slow equilibration rate constants, $k_{P,T'}$ and $k_{T',P}$, was added, and the same data were fitted according to the resultant three-compartment body model (Fig. 8). The three-compartment body model fitted the data following administration of lower doses equally well (Fig. 9). The rate constants obtained from the best fits of plasma and urine data following intravenous administration are summarized in Table I.

Oral Administration—The plasma levels after oral administration were plotted in micrograms per milliliter as a function of time. The initial conditions (I.C.) given to the analog computer were placed in the GI compartment. They were estimated on the presumption that they were equal to the initial concentrations, $[A_P^u]_0$, that would appear in the plasma and be unbound to plasma proteins if the fraction γA_0 of the dose were absorbed instantaneously. Such initial conditions, I.C., can be calculated from:

$$\text{I.C.} = [A_P^u]_0 = \frac{\gamma A_0 - (V_{RBC}D + V_{P_{true}})[f/(1-f)][A_P^u]_0}{V_{P^u}} \quad \text{(Eq. 12)}$$

since $[A_{RBC}]_0 = D[A_P^u]_0$ from Eq. 2, and $[A_P^b]_0 = [f/(1-f)]\,[A_P^u]_0$ (11). When there is no protein binding and $D = 1$, as in this case,

[11] Model TR 48, Electronics Associates, Inc., West Long Branch, N. J.

[12] Model 12D2, Hewlett-Packard/Moseley Division, Pasadena, Calif.

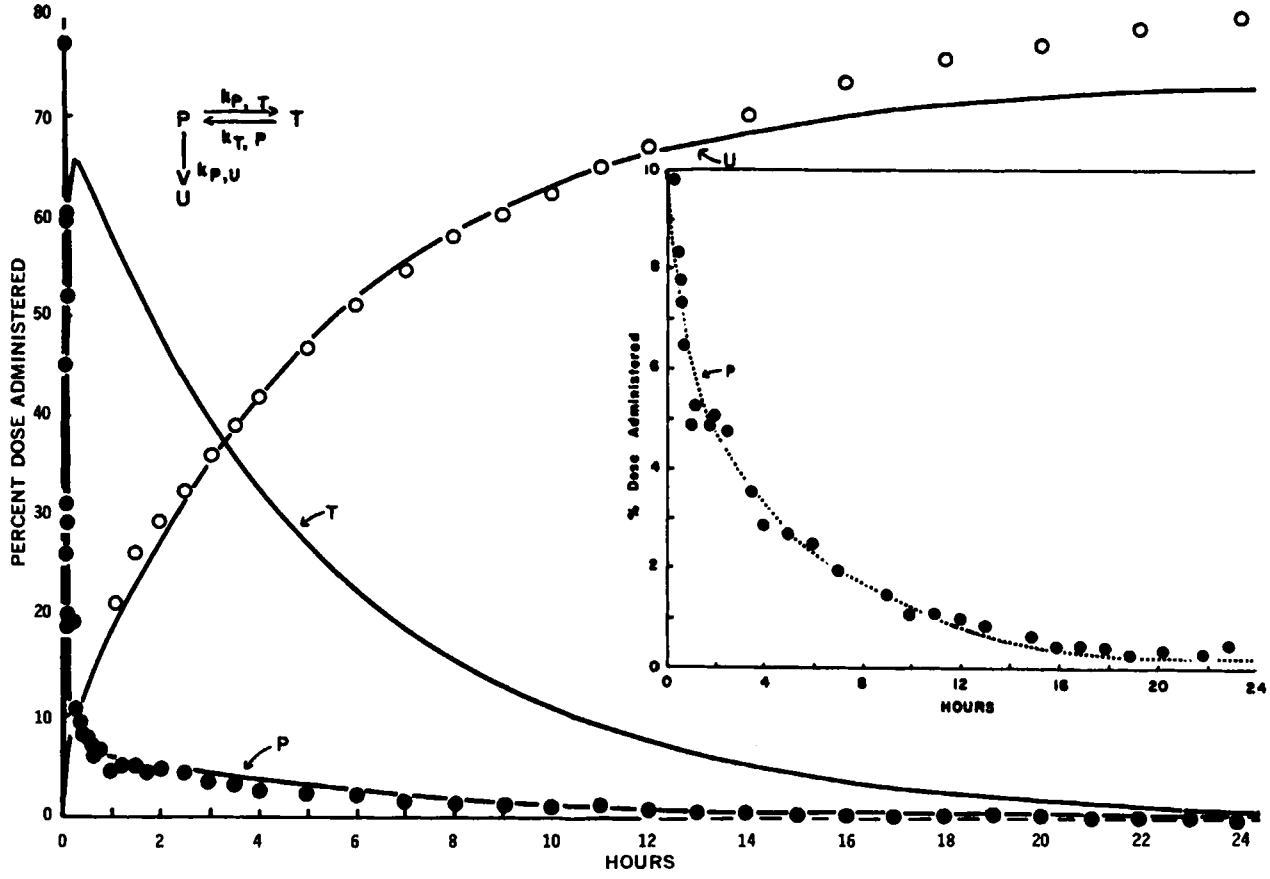


Figure 7—*Analog computer fitting of plasma (●) and urine (○) data following the intravenous administration of 4 mg./kg. sotalol to 17.2-kg. Dog 1 in accordance with a two-compartment body model. The computer curve for urine does not fit the data points in the later time period, indicating a "dribbling out" of drug from a deeper compartment. The inset is for an expanded ordinate scale.*

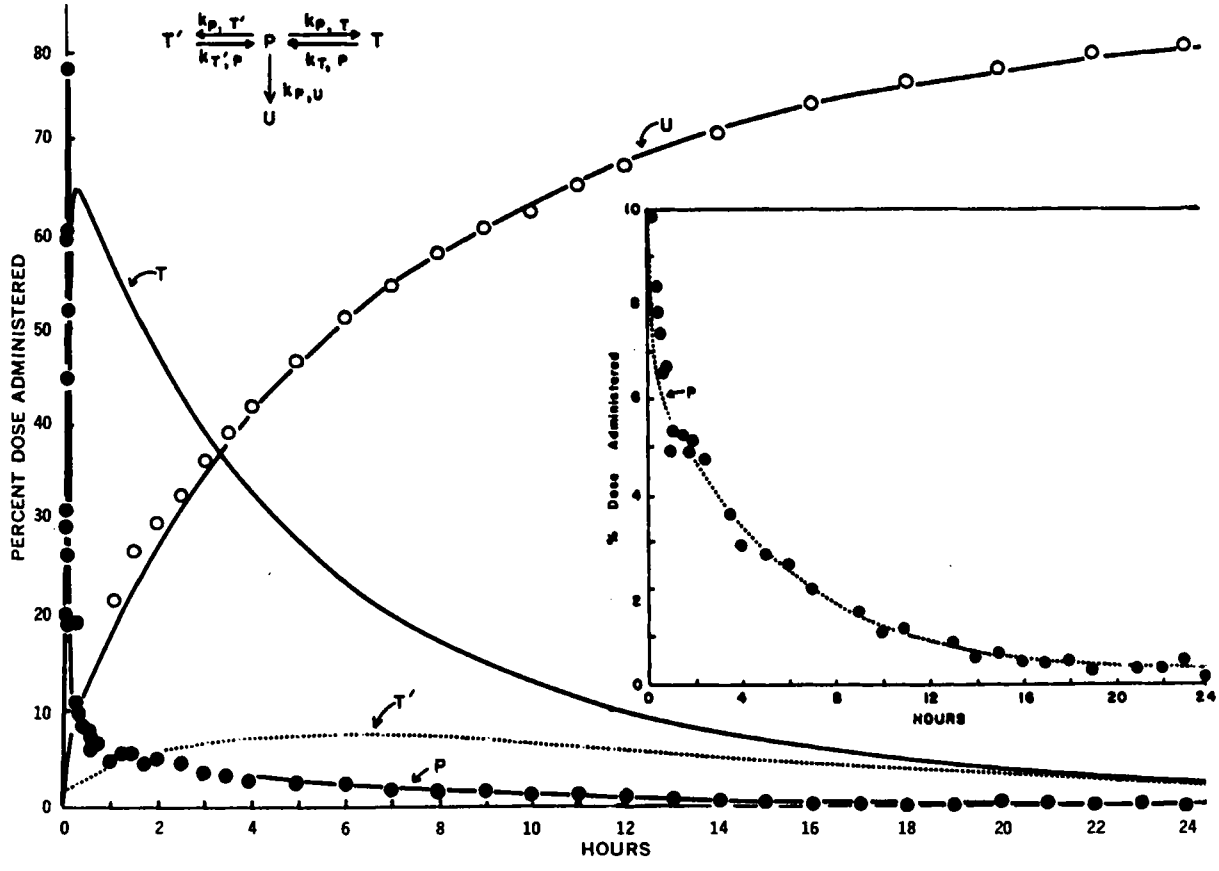


Figure 8—*Analog computer fitting of the same plasma (●) and urine (○) data as in Fig. 7 in accordance with a three-compartment body model. The addition of the tissue compartment, T', represented by the dashed line, that is in slow equilibrium with the central compartment, P, allows good fitting of the terminal portion of the cumulative urinary excretion and the 10-fold enhanced terminal plasma curve (inset).*

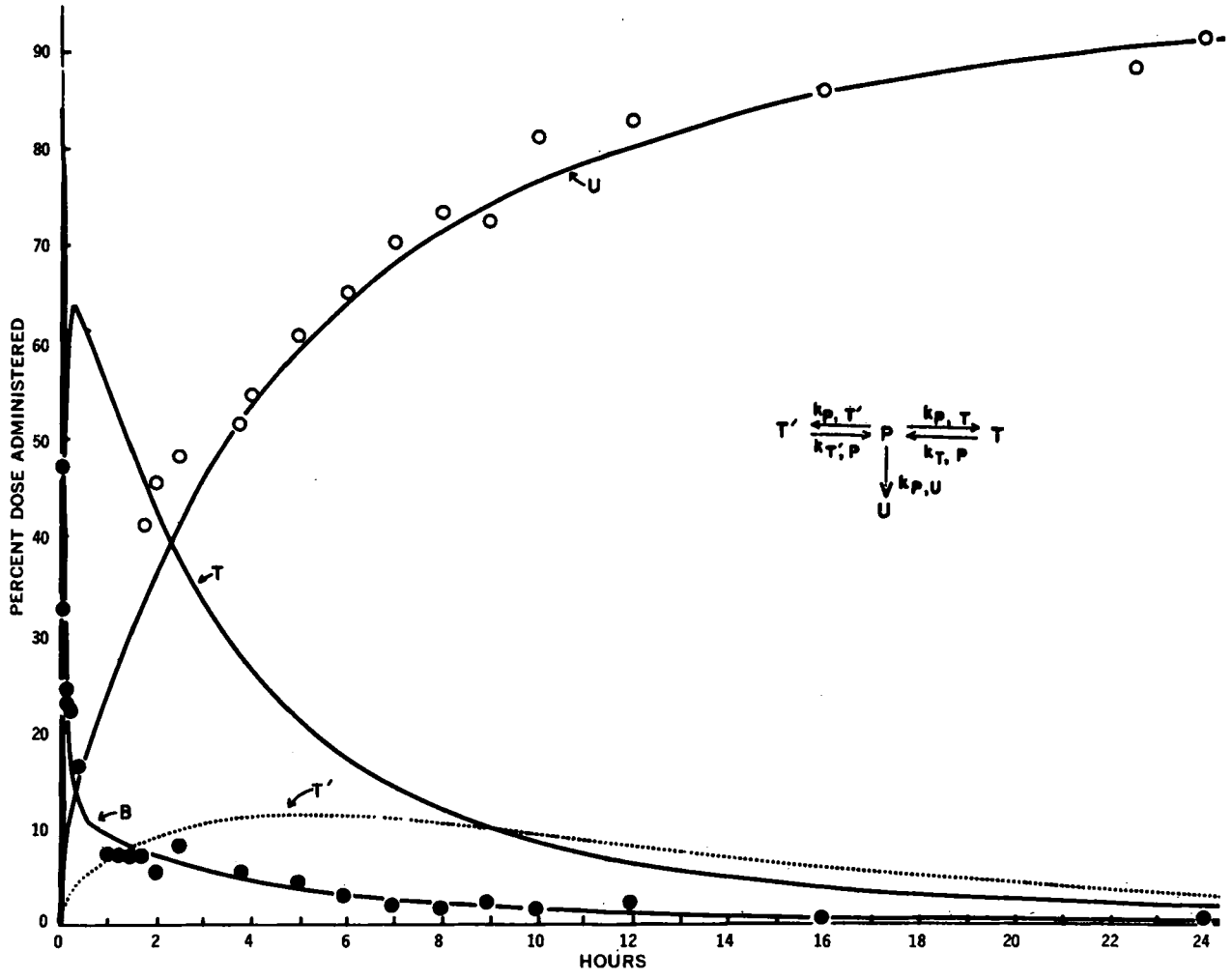


Figure 9—*Analog computer fitting of the same plasma (●) and urine (○) data as in Fig. 6 in accordance with a three-compartment body model (shown in figure). The dashed line represents the generated curve for* T'.

Eq. 12 simplifies to:

$$[A_P{}^u]_0 = \frac{\gamma A_0 - V_{RBC}[A_P{}^u]_0}{V_P{}^u} \qquad \text{(Eq. 13)}$$

Explicit solution of this equation results in:

$$[A_P{}^u]_0 = [A_P]_0 = \text{I.C.} = \frac{\gamma A_0}{V_P{}^u + V_{RBC}} \text{ [mcg./ml.]} \qquad \text{(Eq. 14)}$$

The fraction absorbed, γA_0, may be obtained from the ratio of the areas under the plasma curves of oral to intravenous administration of the same dose in the same dog (15). It may also be estimated from the ratio of the cumulative amounts excreted in the urine on oral administration to the amounts on intravenous administration of equal doses in the same dog. The volumes of distribution of the central compartment, $V_P{}^u$, referenced to unbound drug concentration in plasma were initially postulated to be the same as the corresponding intravenous values (Table II) for the first estimates of the initial conditions, I.C. The volume of the red blood cells was calculated for a blood volume, $V_{B_{true}}$, of 100 ml./kg. (17) and a hematocrit of 40% in this dog as $0.4 \times V_{B_{true}}$.

These I.C. values and the rate constants derived from the best fit of the data obtained from intravenous administration of 4 mg./kg. to the same dog (Table I) were used in the first fittings of the three sets of oral data. The potentiometers representing $k_{P,T'}$ and $k_{T',P}$ were turned to zero, and the data were first simulated according to a two-compartment model (Scheme I) by adjusting only the rate constant for absorption, k_a.

A good fit was obtained with this model for the data of the 2-mg./kg. dose (Fig. 10). The tissue, T', and the pertinent rate constants, $k_{P,T'}$ and $k_{T',P}$, were included for the 4- and 8-mg./kg. dose, and the fit of the oral dose of 8 mg./kg. is given in Fig. 11 as an example in accordance with the three-compartment body model. A lag time for absorption (15–45 min.) had to be assumed to fit the first portion of the data, and a slight decrease in the $k_{P,U}$ setting was needed to give the best fits of the plasma concentrations following oral administration (Table I). Curves for the amounts of drug in terms of the arbitrary microgram per milliliter concentrations for urine and the tissues were generated by the computer from the best fits of the plasma data in accordance with the two-compartment and three-compartment body models.

Since these curves were generated from drug concentrations that were distributed in the volume of the central compartment, $V_P{}^u$, they were all in terms of this $V_P{}^u$, *i.e.*, $T/V_P{}^u$, $T'/V_P{}^u$, and $U/V_P{}^u$, where T, T', and U are the amounts in the tissues and urine, respectively. The capacity of the $V_P{}^u$ in milliliters, *i.e.*, $V^u_{P(est)}$, can be estimated when the experimentally observed amounts excreted in the urine, U in micrograms, are divided by the generated urine curves, $U/V^u_{P(est)}$ in micrograms per milliliter, at the times of urine collection and is related to the true $V_P{}^u$ by:

$$V^u_{P(est)} = V_P{}^u \frac{\gamma A_0}{U_\infty} \qquad \text{(Eq. 15)}$$

If all of the drug absorbed were excreted in the urine as unchanged material, $V_P{}^u = V^u_{P(est)}$. In this case, there was no apparent difference in the percent of the dose recovered in the urine between intravenous and oral studies (Table II). Thus, a plot of U in micrograms divided by $V_{P(est)}$ in milliliters gave the cumulative excretion consistent with the actual data. These plots were only slightly off the computer-generated curves for urine. Small adjustments of the I.C. and of $k_{P,U}$ were sufficient.

The adjusted initial conditions, I.C., *i.e.*, $[A_P{}^u]_0$, were then inserted into Eq. 14, which was solved for the best estimates of $V_P{}^u$ based on the combined fittings of plasma and urine data (Table II).

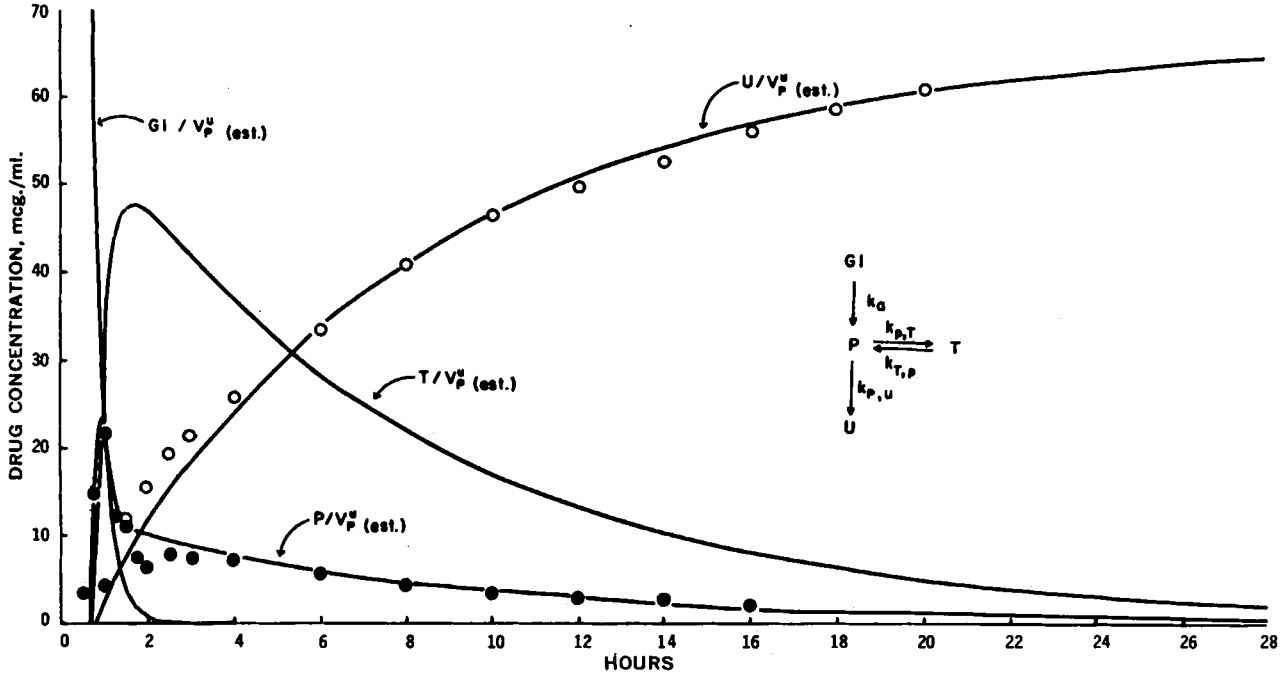


Figure 10—*Analog computer fitting of plasma* (●) *and urine* (○) *data following oral administration of 2 mg./kg. sotalol to 20.2-kg. Dog 1 in accordance with the two-compartment model and addition of a GI compartment. The plasma data in micrograms per milliliter were first fitted with rate constants identical to those of the 4-mg./kg. i.v. dose to the same dog. The I.C.'s were calculated according to Eq. 14 and only the* k_a *was varied. Curves for* $U/V^u_{P(est)}$ *and* $T/V^u_{P(est)}$ *were generated by the computer.* $V^u_{P(est)}$ *was obtained by dividing* U_{exp} *in micrograms by* $U/V^u_{P(est)}$ *in micrograms per milliliter. The final fit was made according to the plot of* $U_{exp}/V^u_{P(est)}$ *to yield the real* $V_P{}^u$ *in accordance with Eq. 14:*

$$V_P{}^u = \frac{\gamma A_0}{I.C.} - V_{RBC}$$

A lag time for gastric emptying of 45 min. had to be assumed to fit the early portion of the plasma curve.

Estimates of Absorption—The plasma concentrations following the intravenous administration of 1, 2, and 4 mg./kg. and oral administration of 2, 4, and 8 mg./kg. were plotted as functions of time, and the area under the curves was determined by a line follower[13] connected to the *x-y* recorder of the computer. The photocell followed the plasma concentration–time curves, and the voltages were accumulated by an integrator. Estimates of the percent of drug absorbed were obtained from the ratio of the area under the plasma level–time curve for oral absorption to that for intravenous administration for the same dosages (15). For the 8-mg./kg. oral dose, the average area under the curves for the three intravenous studies corrected for the different dose was taken as 100%.

RESULTS AND DISCUSSION

Metabolism of Sotalol—The chromatography of plasma and urine samples containing ^{3}H-sotalol revealed spots that were identical to those of the parent compound. Hence, it could be concluded that there was no significant metabolism of the drug. A scattering of spots assignable to radioactive material was observed in the bile. Since this route of excretion contributed less than 1% of the administered dose, it was of negligible significance.

Protein Binding Study—No binding of sotalol to plasma proteins was found in the 0.3–1000.0-mcg./ml. range of drug concentrations, *i.e.*, $f = 0$. The total drug concentrations in plasma assayed spectrophotofluorometrically and radiochemically were identical with the free diffusable drug. Thus, the rate constants and volumes of distribution were directly referenced to unbound drug concentrations in the plasma.

Red Blood Cell/Plasma Partitioning—The partition coefficient, D (Eq. 3), between plasma and red blood cells was found to be unity (1.16 ± 0.21). Equilibrium was achieved within 1 min. and did not change over a period of 2 hr. No drug concentration dependency of the 1:1 distribution between red blood cells and plasma could be detected in the range of 0.5–100.0 mcg. sotalol/ml. of blood.

Fit of Data from Intravenous Studies—The plasma data following intravenous administration were fitted graphically for all experiments in accordance with a two-compartment body model (Scheme I). The rapid initial drop of the drug concentration could be characterized by the α-slope of the first phase of the semilogarithmic plot of the data with time, which was equivalent to a $t_{1/2}$ of 3.2 ± 1.1 min. with extreme values of 1.6 and 5.5 min. and was followed by a comparatively slow disposition phase with the slope β or k_e of the terminal semilogarithmic plot that gave a $t_{1/2}$ of 4.8 ± 1.03 hr. with extreme values of 3.52 and 6.8 hr. (Table I and Figs. 1 and 2).

The k_e values obtained from the slopes of semilogarithmic plots of the rate of excretion, $\Delta U/\Delta t$, and the amounts of drug not yet excreted in the urine, $U_\infty - U$, against time were in good agreement with the estimates from the plasma data (Fig. 4 and Tables I and II). The microscopic rate constants derived from the graphical procedures and from the best analog computer fits were generally in good agreement (Table I). Whereas the plasma data could be fitted to a two-compartment body model by both methods in almost all cases (Figs. 1, 2, 6, and 10), it proved necessary to add a slowly equilibrating tissue compartment to fit the later portion of the urine curves at the high dosage level (Figs. 7 and 8) and in the case of the ^{3}H-sotalol data (Table I). The slow return from this "deep" compartment (12) may be held responsible for the prolonged enhancement of urinary excretion. A maximum of 5–12% of the dose was estimated to be in this compartment on acute administration.

The values for the individual rate constants were consistent within an animal for different doses as well as among the three animals. Dose-independent kinetics could also be demonstrated by the superimposable curves, where the plasma data were plotted in terms of percent of dose administered per milliliter plasma as a function of time (Fig. 2).

Rate Constants and Apparent Volumes of Distribution—The drug distributed instantaneously after bolus injection into the space associated with the central compartment, *i.e.*, $V_P{}^u$. The values in liters (Table II) corresponded generally to the volume of the extracellular space [190–350 ml./kg. (17)].

[13] Autograf Type F-3B, Hewlett-Packard/Moseley Division, Pasadena, Calif.

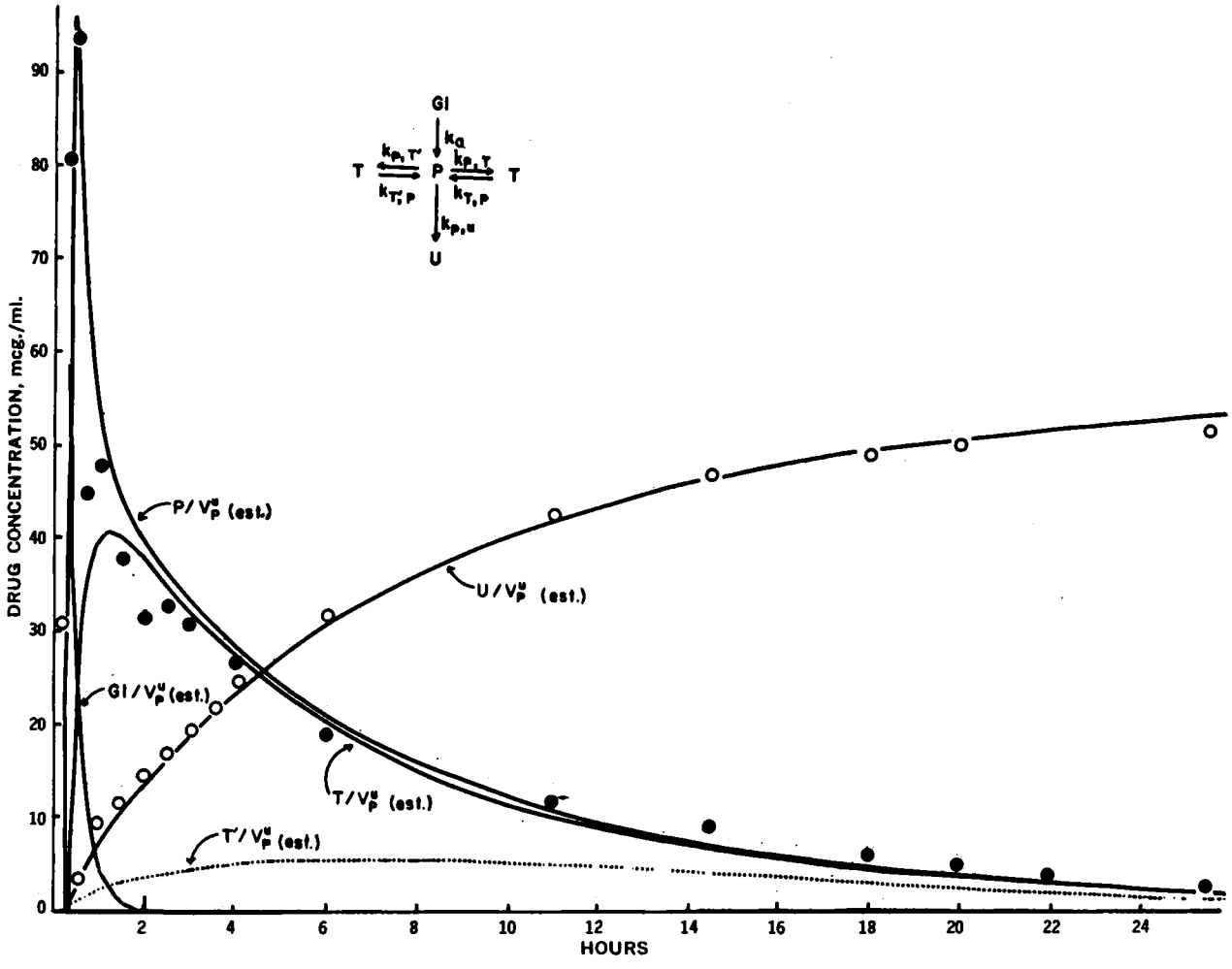


Figure 11—*Analog computer fitting of plasma and urine data following oral administration of 8 mg./kg. sotalol to Dog 1. Here, a lag time of 15 min. was assumed to fit the initial plasma data. For symbols, see Fig. 10.*

The drug diffuses rapidly from the central compartment into the readily equilibrating tissues, T. Since $k_{P,T}$ is 7–10 times greater than $k_{T,P}$, the capacity of this compartment is high. The apparent volume of T can be estimated by (11, 12):

$$V_T = V_P{}^u \frac{k_{P,T}}{k_{T,P}} \qquad \text{(Eq. 16)}$$

referenced to the concentration of unbound drug in the plasma. Similarly, for the apparent volume of the deep compartment, T':

$$V_{T'} = V_P{}^u \frac{k_{P,T'}}{k_{T',P}} \qquad \text{(Eq. 17)}$$

The apparent volumes, V_T, are reasonably consistent with a total body water of 600–800 ml./kg. (17). The fact that the V_T value exceeded this quantity in some cases indicated that the drug may be trapped or bound to the tissue, T, to some extent. It might be speculated that since sotalol is a zwitterion with a narrow pH range allowable for partitioning into fats (7), minute pH differences inside the cells would inhibit back-diffusion to the central compartment. The same phenomenon could rationalize the deeper compartment, T'.

Apparent Volumes of Distribution and Renal Clearance—The renal clearance of sotalol (Table II) was well within the range of a normal inulin clearance of 40–130 ml. for a 20-kg. dog (17). Also, an inulin clearance study in Dog 1 gave a value of 36 ml./min., as estimated from the plasma decay curve. Thus, sotalol can be assumed to be renally excreted essentially by glomerular filtration.

Clearance values of this magnitude affect the estimates of the apparent volumes of distribution that are based on the assumption of pseudosteady state (11) or, as it has also been called, the pseudo-distribution equilibrium (18). These estimated volumes can differ from those calculated for steady-state equilibrium in accordance with Eqs. 16 and 17 (13).

If excretion is significant during the rapid distribution phase, the initial decay in the plasma level of drug will be due to both distribution and excretion. Thus, the extrapolation of the β-phase from the semilogarithmic plots (Figs. 1–4) to zero time will estimate a value for the concentration of drug in the pseudoequilibrated tissues of distribution, $[A_P{}^u]_0$, which leads to values of $V_{D_P}{}^u$ (Eq. 7) and $V_{D_P}{}^u$ (Eq. 9) that are greater than the total apparent volumes of distribution of the central compartment and the rapidly equilibrating tissues T, which are derived from steady-state considerations (Eqs. 8, 16, and 17).

The $V_{D_P}{}^u$ calculated from the quotient of the renal clearance in milliliters per minute and k_e in min.$^{-1}$ (from plasma data, Table I) for the conditions when significant excretion occurs during the α-phase will also be higher than the sum of the steady-state volumes of distribution in Eq. 8 (13).

Estimates of the apparent volumes of distribution based upon these three methods are given in Table II, and it can be seen that the $V_{D_P}{}^u$ estimates based on the pseudosteady-state assumption and the renal clearance/k_e quotient tended to yield larger values than the sum of $V_P{}^u$ and steady-state volumes of the equilibrated tissues (Eq. 16), even when V_T (Eq. 17) was included.

Comparison of Chemical and Radioactive Assays—The semilogarithmic plots of the radioactive data in plasma showed a three-exponential decay and a slow terminal phase with a $t_{1/2}$ of approximately 12 days. This is consistent with the fact that deuterium oxide is cleared from plasma water with a $t_{1/2}$ of 9.3 ± 1.5 days (19). The terminal phase was, therefore, assigned to the clearance of tritiated water, which is a probable result of the exchange between the hydrogens of water and the tritium of labeled sotalol.

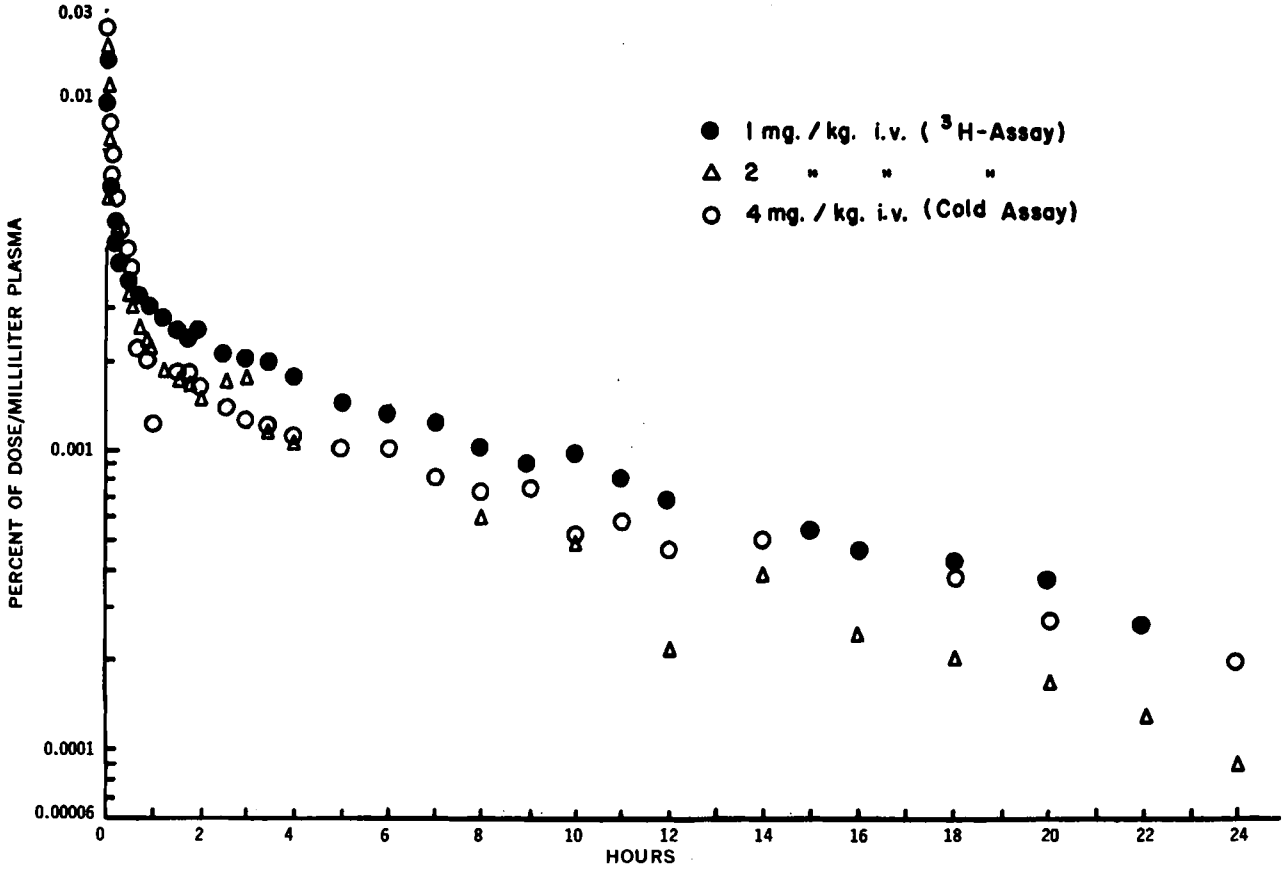


Figure 12—*Semilogarithmic plots of plasma levels in percent of dose per milliliter plasma following intravenous administration of 1 (●) and 2 (△) mg./kg. of ³H-sotalol, assayed by counting procedure, and 4 mg./kg. (○) of sotalol, assayed by spectrophotofluorometric analysis, to 20-kg. Dog 2. The radioactive data were corrected for the longer retention of tritiated water by prior feathering.*

The two assay methods were comparable after the terminal portion of the labeled plasma level–time curves was "feathered" (Fig. 12). The spectrophotofluorometric and radioactive data in plasma and urine could be fitted with identical rate constants in accordance with the three-compartment body model. The estimates for the apparent volumes of distribution of the central compartment, V_P^u, were slightly lower for the radioactive data since the initial concentrations of unbound drug in plasma, $[A_P^u]_0$, upon which these estimates are based (Eqs. 5 and 6) were slightly higher for the radioactive studies.

Unchanged ³H-sotalol, in the amounts of 91 and 93.6% of the dose, was found in the urine by radioactive assay as compared to 90.3 ± 12% (72–116%) from the spectrophotofluorometric data of the other experiments. The extreme overestimates of 108 and 116% recovery (Table II) were obtained in earlier experiments where the assay methodology was not as well established.

The fecal excretion of ³H-sotalol was 0.21 and 3.37% of the total dose in the two radioactive experiments.The former value was due to a constipated dog which permitted few fecal samples. The bile had 0.73% of the radioactive dose given to the biliary-cannulated dog.

Oral Studies—The graphical estimates of the absorption rate constants by feathering of the curves were not accurate since equilibration of drug among the available tissues was not readily effected during the absorption phase (Fig. 3). The $t_{1/2}$'s of absorption obtained from the best analog computer fits were (lag time for absorption in parentheses) 10.9 min. (15 min.) for 8 mg./kg., 16.7 min. (30 min.) for 4 mg./kg., and 10.7 min. (45 min.) for 2 mg./kg.

Such a lag time may be indicative of an intestinal absorption. The differences may be explained by variable gastric motilities in the fasted and achlorhydric dog.

Best fitting was achieved by applying the three-compartment body model, including a GI compartment, for the doses of 4 and 8 mg./kg. (Fig. 11). The data following administration of 2 mg./kg. could be fitted with both two- and three-compartment body models. The two-compartment fit is shown in Fig. 10.

Estimates of the percent of total dose absorbed were made from the total amounts excreted in the urine. They were 87% following a dose of 2 mg./kg., 101.7% for 4 mg./kg., and 78.2% for 8 mg./kg. The estimates from the ratio of the corresponding areas under the plasma level–time curves following intravenous and oral administration were 84.1, 83.0, and 90.5% absorption for the oral dosages of 2, 4, and 8 mg./kg., respectively. Hence, it can be concluded that sotalol is rapidly and rather completely absorbed from the GI tract (probably from the intestine) following oral administration of the drug in solution.

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GLC Determination and Urinary Recovery of Bumetanide in Healthy Volunteers

P. W. FEIT▲, K. ROHOLT, and H. SØRENSEN

Abstract □ A GLC determination of bumetanide (3-*n*-butylamino-4-phenoxy-5-sulfamylbenzoic acid), a potent "high ceiling" diuretic, was developed using flash-heater methylation. A mixture of tetramethylammonium hydroxide and trimethylanilinium hydroxide was advantageously used as the methylation reagent. The flash-heater methylation product proved to be methyl 3-(*N*-*n*-butylanilino)-5-dimethylsulfamyl-4-methoxybenzoate by spectroscopic comparison with authentic material achieved by a corresponding gram scale model experiment. The probable sequence of reactions involving methylation under simultaneous Smiles rearrangement is outlined. The GLC determination was found to be accurate at concentrations as low as 0.1 mcg./ml. human urine. In six healthy volunteers, the urinary recovery of bumetanide, the urinary excretion of sodium, potassium, and chloride ions, and the urine volume were determined after oral administration of 0.5 and 1 mg. of the drug. A parallelism between bumetanide excretion and saluretic action over the total period of response is shown.

Keyphrases □ Bumetanide—urinary excretion, man, relationship to saluretic action, GLC analysis after flash-heater methylation □ Urinary excretion, bumetanide—relationship to saluretic action, man □ Saluretic activity, bumetanide—relationship to urinary excretion, man □ GLC—analysis, bumetanide, after flash-heater methylation

Bumetanide (3-*n*-butylamino-4-phenoxy-5-sulfamylbenzoic acid) (I, Scheme I) was recently described as a new "high ceiling" diuretic in the experimental animal (1, 2) and in man (3, 4). In patients suffering from congestive heart failure, bumetanide has shown a diuretic profile and dose response comparable to that of furosemide, but at doses approximately one-fortieth of the latter drug (4). The aim of the present investigation was to develop a sensitive GLC assay for the determination of bumetanide in biological material. Special interests were to evaluate the urinary recovery after oral administration of the drug to healthy volunteers and to obtain information relating the renal drug excretion with the diuretic–saluretic activity.

Since functional groups of bumetanide make the drug unsuitable for direct GLC determination, it was necessary to find a suitable derivative. A relatively simple way of making derivatives of anionic compounds is by methylation in the injection port of the gas chromatograph, using a solution of tetramethylammonium hydroxide or trimethylanilinium hydroxide as the methylation reagent. The principle of flash-heater methylation was first utilized in preparation of methyl esters of carboxylic acid (5) with the aid of tetramethylammonium hydroxide. The advantage of using trimethylanilinium hydroxide was recently shown for barbiturates, phenolic alkaloids, and xanthine bases (6).

EXPERIMENTAL

GLC Determination—*Instruments and Conditions*—The GLC analyses were performed using a gas chromatograph[1] equipped with a flame-ionization detector and the following experimental conditions: column, 2 m. × 3.3-mm. o.d., stainless steel; packing, 1.5% OV-17 silicone[2] on 100-120-mesh diatomaceous earth[3]; column temperature, 270°; injection port temperature, 370°; detector temperature, 300°; carrier nitrogen flow, 30 ml./min.; and recorder chart speed, 0.3 cm. (0.1 in.)/min.

Reagents—For the methylation reagent, two parts of an approximately 1 *M* aqueous solution of trimethylanilinium hydroxide (6) were mixed with one part of a 10% methanolic solution of tetramethylammonium hydroxide. The latter was obtained by evaporating an aqueous tetramethylammonium hydroxide solution[4] in a rotating evaporator and redissolving the residue in the appropriate amount of methanol.

The internal reference solution consisted of 25 mcg. of 4-benzyl-3-*n*-butylamino-5-sulfamylbenzoic acid (7)/ml. in ether.

Procedure—Urine, 5–10 ml., was adjusted to pH 2 by addition of 1 *N* hydrochloric acid. Then 200 μl. of internal reference solution and 15 ml. of ether were added, and the mixture was shaken in a

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[2] Applied Science.
[3] J.J.'s Chromatography Ltd., Diatomite CQ.
[4] Merck, Darmstadt, Germany.

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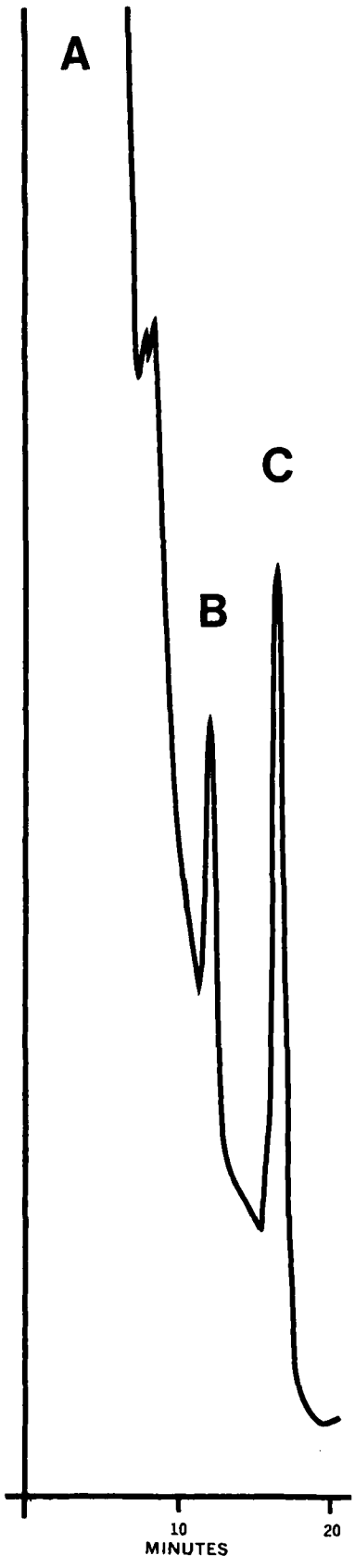
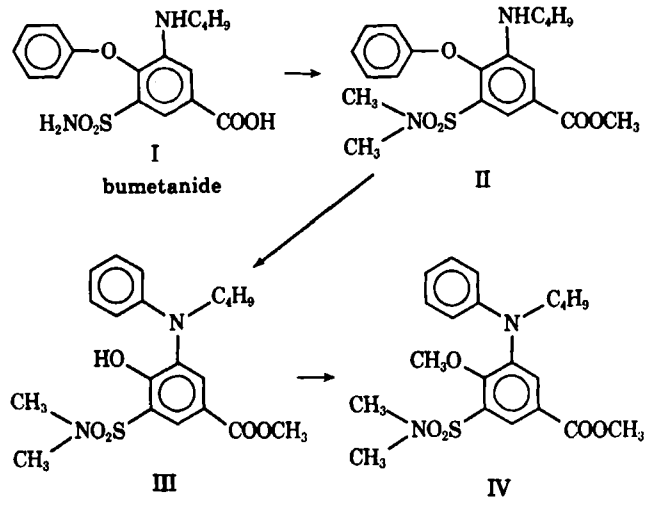


Figure 1—*Gas chromatogram of bumetanide from urine. Key: A, reagent peak; B, peak originated by bumetanide; and C, peak originated by the reference compound.*

separator for 2 min. The organic layer was washed with three 5-ml. portions of water and then filtered into a tapered centrifuge tube (10 ml.) through a small cotton plug, previously washed with ether and covered with a thin layer of anhydrous sodium sulfate. Then 50 μl. of methylation reagent was added, and the tube



Scheme I—Sequence of reactions during flash-heater methylation of bumetanide

was shaken for 2 min. After centrifugation, 1–2 μl. of the aqueous layer was injected into the gas chromatograph. The amount of bumetanide, in micrograms per milliliter of urine, was calculated from the formula:

$$\text{bumetanide} = \frac{A \cdot 2.5}{B \cdot u} \qquad \text{(Eq. 1)}$$

where A is the peak height ratio (bumetanide/internal reference), B is the peak height ratio in a corresponding standard determination in which 2.5 mcg. of bumetanide is added to 5 ml. of blank urine, and u is the quantity of the urine sample in milliliters. The peak heights were measured from a baseline drawn with the aid of a large French curve.

When the apparatus had been in disuse for some time for the determination of bumetanide, the peaks resulting from the first injections tended to be either very small or absent. This could be rectified by saturating the system with an injection of a concentrated solution of bumetanide and the reference compound in methylation reagent.

Synthesis: Methyl 3-*n*-Butylamino-5-dimethylsulfamyl-4-phenoxybenzoic Acid (II, Scheme I)—An alkaline (sodium hydroxide) solution of bumetanide (3-*n*-butylamino-4-phenoxy-5-sulfamylbenzoic acid) (I) was methylated with excess methyl iodide at room temperature while stirring for 24 hr. The excess methyl iodide was distilled off, and 3-*n*-butylamino-5-dimethylsulfamyl-4-phenoxybenzoic acid was precipitated from the reaction mixture by addition of 4 *N* hydrochloric acid until pH 2 (m.p. 187–188° after recrystallization from aqueous ethanol). The esterification was performed in methanolic hydrochloric acid by standing at room temperature for 24 hr. and was worked up as usual to yield II, m.p. 135–136°, after recrystallization from acetone–benzene.

Anal.—Calc. for $C_{20}H_{26}N_2O_6S$ (mol. wt. 406.49): C, 59.09; H, 6.45; N, 6.89. Found: C, 58.92; H, 6.42; N, 6.93.

Model Experiment: High Temperature Reaction of Bumetanide with Tetramethylammonium Hydroxide—A solution of bumetanide (3 g.) in tetramethylammonium hydroxide (45 ml., 10% in water) was introduced into a glass flask placed in an oil bath at 220–235° for 3 hr. while evaporating *in vacuo*. After heating for an additional 15 min., the reaction flask was cooled and the reaction product was dissolved in a mixture of 1 *N* hydrochloric acid (50 ml.) and ether (50 ml.). The organic layer was dried (magnesium sulfate) and evaporated. Two grams of the resulting residue (approximately 3 g.) was redissolved in ether (50 ml.) and extracted twice with 10-ml. portions of 1 *N* sodium hydroxide while ice cooling. The alkaline extract was immediately acidified with 1 *N* hydrochloric acid (30 ml.). The separated oil was reextracted with ether. After washing with water, drying (magnesium sulfate), and evaporating, 1.6 g. of oily material was obtained. After redissolving in ether, the solution was washed with diluted sodium hydrogen carbonate and dried (magnesium sulfate). After evaporation, oily methyl 3-(*N*-*n*-butylanilino)-5-dimethylsulfamyl-4-hydroxybenzoate

Table I—Mean Urinary Excretion in Healthy Volunteers during the Periods of Collection following Oral Administration of 0.5 and 1 mg. Bumetanide

Excretion[a]	Collection following 0–6 hr. Control[b]	0–6 hr. 0.5 mg.	0–6 hr. 1 mg.	6–24 hr. Control[b]	6–24 hr. 0.5 mg.	6–24 hr. 1 mg.	0–24 hr. Control[b]	0–24 hr. 0.5 mg.	0–24 hr. 1 mg.
Bumetanide, mcg.	—	166.7 ±9.9	404.3 ±39.5	—	10.0 ±4.2	23.2 ±6.0	—	176.7 ±8.9	427.5 ±35.1
Sodium, meq.	53.7 ±12.6	131.1 ±23.7	188.5 ±22.7	99.7 ±11.1	62.5 ±11.0	41.7 ±5.7	153.3 ±21.6	193.5 ±34.2	230.2 ±23.0
Potassium, meq.	28.2 ±2.0	45.7 ±3.4	51.0 ±5.1	40.0 ±5.7	41.0 ±7.2	34.0 ±7.4	68.2 ±6.3	86.7 ±8.1	85.0 ±11.3
Chloride, meq.	65.0 ±11.8	159.0 ±20.4	219.7 ±18.8	80.2 ±11.4	49.2 ±10.7	24.7 ±3.4	145.2 ±22.4	208.2 ±28.9	244.3 ±20.0
Volume, ml.	442.8 ±49.5	1469.2 ±90.6	2042.5 ±156.2	834.2 ±93.4	805.0 ±134.7	546.7 ±69.9	1277.0 ±113.1	2274.2 ±135.9	2589.2 ±177.9

[a] Values represent the mean ± *SD* for six volunteers. [b] Mean values calculated from the day before administration.

(III, Scheme I) was obtained. Crystallization was afforded from ethanol by slowly adding water. Recrystallization from ethanol–water yielded the analytically pure compound (0.64 g.), m.p. 75–77°; NMR [10% in $(CD_3)_2SO$, tetramethylsilane]: 0.88 (m, 3H, CH_3C), 1.0–1.8 (m, 4H, CH_2CH_2), 2.80 (s, 6H, CH_3NSO_2), 3.55 (m, 2H, CH_2N), 3.84 (s, 3H, CH_3OOC), 6.5–7.4 (m, 5H, C_6H_3N), 7.91 (d, J = 2.2 Hz., 1H, aromatic H), 8.29 (d, J = 2.2 Hz., 1H, aromatic H), and 10.85 (broad s, 1H, HO) p.p.m.

Anal.—Calc. for $C_{20}H_{26}N_2O_5S$ (mol. wt. 406.49): C, 59.09; H, 6.45; N, 6.89. Found: C, 58.83; H, 6.44; N, 6.89.

The remaining ether solution, after sodium hydroxide extraction, was washed with 1 *N* hydrochloric acid and water. Drying and evaporation resulted in crude methyl 3-(*N*-*n*-butylanilino)-5-dimethylsulfamyl-4-methoxybenzoate (IV, Scheme I) (0.45 g.). Recrystallization from ethanol (4 ml.) yielded the analytically pure compound (0.19 g.), m.p. 97–98°; NMR [10% in $(CD_3)_2SO$, tetramethylsilane]: 0.86 (m, 3H, CH_3C), 1.0–1.9 (m, 4H, CH_2CH_2), 2.78 (s, 6H, CH_3NSO_2), 3.72 (m, 2H, CH_2N), 3.75 (s, 3H, CH_3O), 3.88 (s, 3H, CH_3OOC), 6.7–7.5 (m, 6H, C_6H_3N), 8.01 (d, J = 2.2 Hz., 1H, aromatic H), and 8.23 (d, J = 2.2 Hz., 1H, aromatic H) p.p.m.

Anal.—Calc. for $C_{21}H_{28}N_2O_5S$ (mol. wt. 420.56): C, 59.98; H, 6.71; N, 6.66. Found: C, 59.78; H, 6.66; N, 6.54.

Human Volunteer Studies—The study was performed at dose levels of 0.5 and 1 mg. bumetanide. Scored tablets containing 1 mg. of bumetanide were used.

The trial was comprised of six volunteers who were members of the staff and 30–44 years old. All had normal blood urea and electrolyte levels. They took a normal diet and consumed, as far as possible, the same volume of fluid each day. On the 1st day of trial, each subject emptied his bladder at 7 a.m. and the urine was discarded. On the two pretreatment days, urine was collected over the 0–6- and 6–24-hr. periods. On the day of the test, urine was collected over the 0–1-, 1–2-, 2–4-, 4–6-, 6–8-, and 8–24-hr. periods following administration of the drug. On the day following treatment, urine specimens were collected over a 24-hr. period.

The content of bumetanide in urine was measured using the GLC determination as described. Blank urine samples taken before administration of bumetanide were analyzed to exclude the presence of other drugs that might interfere with the method. The presence of furosemide would interfere with the analysis, because it shows a peak with almost the same retention time as the peak originating from bumetanide. Consequently, it was found that the method could be adapted to furosemide.

The urinary excretion of electrolytes was determined using standard procedures.

RESULTS AND DISCUSSION

GLC Determination—A sensitive method was necessary for measuring bumetanide in urine at the low levels estimated to be present in urine. The described procedure is convenient for the determination of the drug in urine at levels as low as 0.1 mcg./ml. The use of the quaternary ammonium bases for the extraction of bumetanide from the organic solvent phase simplified the extraction procedure. No time-consuming evaporation steps were necessary to achieve the concentration of the drug required for GLC analysis. The accuracy of the analysis was tested by adding known amounts of bumetanide (between 0.1 and 0.5 mcg./ml.) to urine samples. The recovery was between 90 and 105%. A chromatogram from urine containing 0.19 mcg. bumetanide/ml. is shown in Fig. 1. A mixture of tetramethylammonium hydroxide and trimethylanilinium hydroxide (1:2) was used for methylation and extraction. Used alone, trimethylanilinium hydroxide resulted in an additional peak in the chromatogram, with a peak height dependent on the surplus of the reagent. Tetramethylammonium hydroxide gave only one peak; but when the reagent was used in a concentration sufficient for extraction from urine samples, a very broad reagent peak disturbed the analysis.

From a theoretical point of view, the target of the flash-heater methylation of bumetanide should be the carboxyl group and the sulfonamide nitrogen resulting in methyl 3-*n*-butylamino-5-dimethylsulfamyl-4-phenoxybenzoate (II). Compound II was prepared by synthesis and proved, by its different retention time, not to be identical with the flash-heater methylation product of bumetanide. Compound II, however, resulted in the same retention time as the bumetanide derivative in question when injected together with the methylation reagent. This indicated that the flash-heater methylation of bumetanide is rather more complicated. The gram scale investigation of the high temperature reaction of bumetanide with tetramethylammonium hydroxide resulted in the formation of methyl 3-(*N*-*n*-butylanilino)-5-dimethylsulfamyl-4-hydroxybenzoate (III, Scheme I) and methyl 3-(*N*-*n*-butylanilino)-5-dimethylsulfamyl-4-methoxybenzoate (IV, Scheme I).

IR, TLC, and GLC analyses of IV, together with the IR and TLC analyses of the peak collected from the GLC effluent of bumetanide in tetramethylammonium hydroxide, showed the derivative measured in this GLC method to be identical with Compound IV. Formation of IV involves Smiles rearrangement (8) and methylation of the intermediate phenolic hydroxyl of III. The probable sequence of reactions is outlined in Scheme I.

Human Volunteer Studies—The mean urinary recovery of bumetanide, excretion of electrolytes, and urinary volume in six volunteers after oral administration of 0.5 and 1.0 mg. of the drug are presented in Table I. The values during 0–6, 6–24, and 0–24 hr. after administration and control mean values are given. The differences for the 0–6-hr. interval are statistically significant except for potassium.

The histograms in Figs. 2 and 3 depict the excretion of bumetanide and corresponding urinary sodium in the urine samples over a 24-hr. period following 0.5 and 1 mg. of drug administration, respectively.

The present data show that the urinary excretion of bumetanide is rapid and attains its maximum, in the majority of cases, between 1 and 4 hr. after administration. Excretion is virtually complete after 8 hr. Apart from two subjects receiving 1 mg. bumetanide, no drug was detectable in the urine after 24 hr. The total 48-hr. urinary excretion of bumetanide amounted to 35 and 44% for the 0.5-and 1-mg. doses, respectively.

After enzymatic hydrolysis of the 1–2-hr. urine samples using β-glucuronidase-arylsulfatase[5], the concentration of bumetanide

[5] C. F. Boehringer GmbH., Mannheim, Germany, Catalog No. 15247 EGAF.

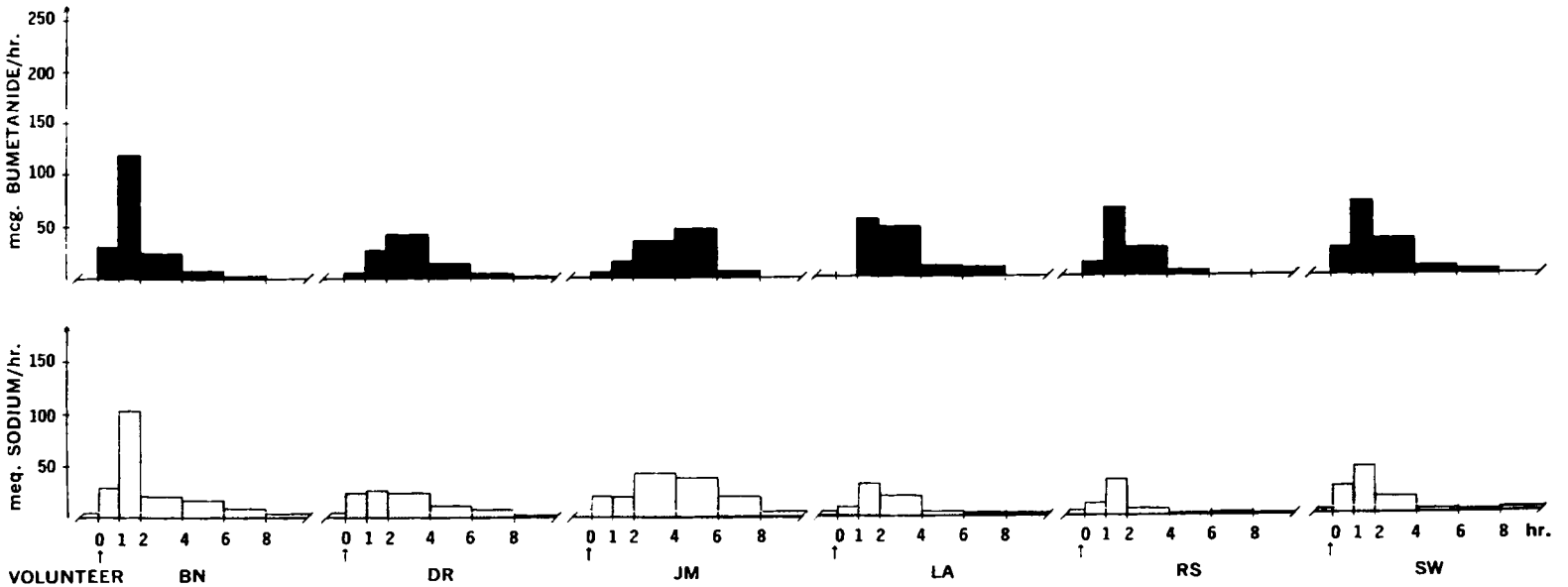


Figure 2—*Urinary excretion of bumetanide (bars upper diagram) and sodium (bars lower diagram) in six healthy volunteers following oral administration of 0.5 mg. of bumetanide.*

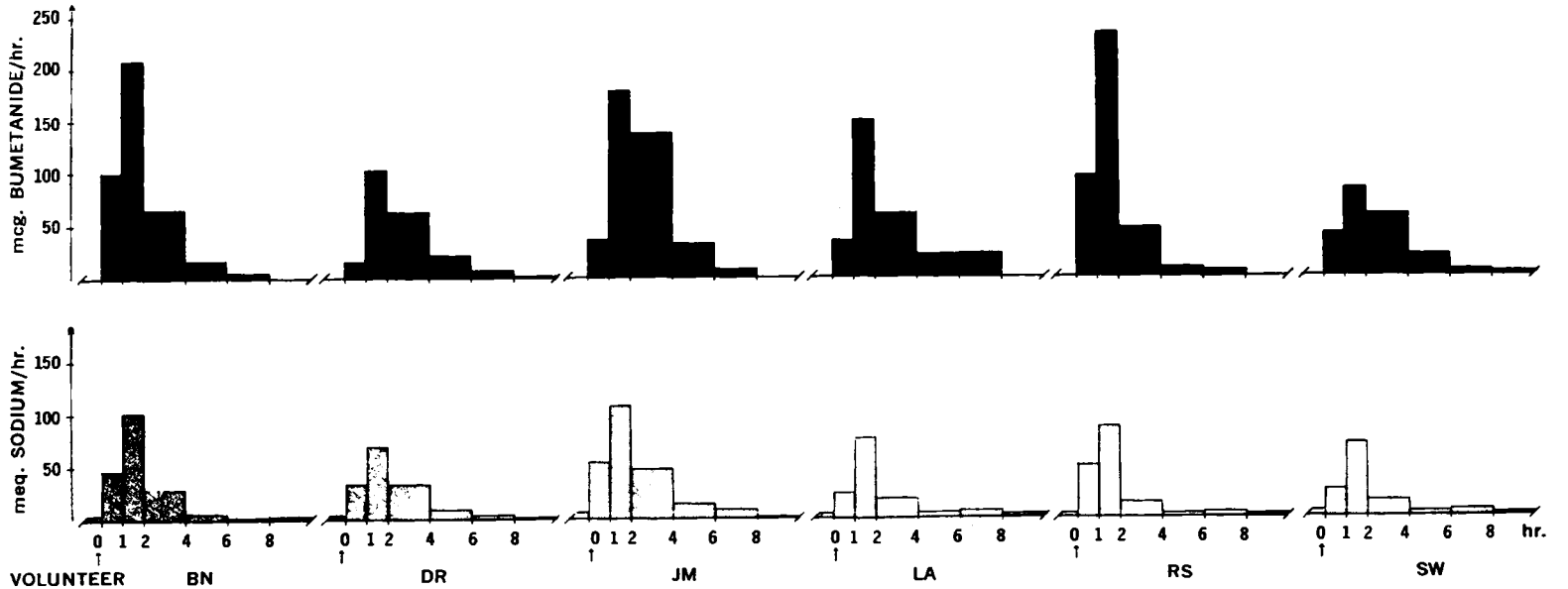


Figure 3—*Urinary excretion of bumetanide (bars upper diagram) and sodium (bars lower diagram) in six healthy volunteers following oral administration of 1 mg. of bumetanide.*

found did not exceed the original level. This indicates that bumetanide is excreted unconjugated in human urine.

However, the urinary excretion of bumetanide cannot be considered as a measure of orally absorbed drug since a biliary excretion of the drug cannot be excluded. Biliary excretion would make the results in accordance with the low dose dog assay, where an almost equal response to the drug was obtained after oral and intravenous administration.

The results confirm the marked saluretic and diuretic effect of bumetanide after oral administration of both 0.5 and 1 mg. to human volunteers and show clearly a parallelism between bumetanide excretion and saluretic action over the total period (Figs. 2 and 3).

SUMMARY

A GLC determination of bumetanide, using flash-heater methylation by means of a mixture of tetramethylammonium hydroxide and trimethylanilinium hydroxide, was developed. The flash-heater methylation product of bumetanide proved to be methyl 3-(*N*-*n*-butylanilino)-5-dimethylsulfamyl-4-methoxybenzoate, formed by methylation under simultaneous rearrangement. The GLC determination was found to be accurate at concentrations as low as 0.1 mcg./ml. human urine. In six healthy volunteers, the urinary excretion of sodium, potassium, and chloride, the urine volume, and the urinary recovery of bumetanide were determined after oral administration of 0.5 and 1 mg. of the drug. A parallelism between bumetanide excretion and saluretic action over the total period of response is shown.

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Influence of Solution Electrolyte Content and Dielectric Constant on Drug Adsorption by Kaolin

N. A. ARMSTRONG▲ and C. D. CLARKE*

Abstract □ The adsorption of benzoic acid and crystal violet on kaolin was investigated to elucidate the influence of the system dielectric constant and the electrolyte content on this example of drug–adjuvant interaction. The differing results obtained with the two adsorbates reflect the dissimilar adsorption sites on the kaolin platelet and the different mechanisms involved in the uptake of acids and bases on clays. The experimental data presented are satisfactorily explained by a consideration of the effect of both the dielectric constant and the electrolyte concentration and valency on adsorbent and adsorbate characteristics.

Keyphrases □ Kaolin, adsorption of benzoic acid and crystal violet—location of adsorption sites, influence of dielectric constant and electrolyte content □ Adsorption of benzoic acid and crystal violet by kaolin—influence of dielectric constant and electrolyte content □ Drug–adjuvant interactions—influence of dielectric constant and electrolyte content, drug adsorption by kaolin □ Dielectric constant, electrolyte content—effect on drug adsorption by kaolin □ Electrolyte content, dielectric constant—effect on drug adsorption by kaolin

Previous papers (1, 2) showed that the Langmuir adsorption isotherm represents the adsorption of benzoic acid and crystal violet on kaolin, and they discussed the effect of environmental pH on this equilibrium process. From the results obtained, the probable adsorption sites for acidic and basic materials on clays were suggested, namely that anionic materials were adsorbed on the edge and cationics on the cleavage surface of the clay. The significance of such an evident drug–adjuvant interaction on preservative efficiency or therapeutic potency was briefly discussed. The presence of soluble material in a suspension formulation, for example as active principle, flavoring, coloring, buffering, or peptizing agents will not only influence the formulation's pH but also contribute to the electrolyte content of the system. This increase in electrolyte concentration will also result in an increase in the dielectric constant of the system (3). Both of these factors influence the characteristics of the electrical double layer of the suspended phase, this double layer constituting the area where adsorption and/or ion exchange takes place. It was, therefore, considered desirable to determine the effect of both the system dielectric constant and the electrolyte content (with respect to concentration and valency) on the problem of drug–adjuvant interaction through investigating the influence of these factors on the uptake of benzoic acid and crystal violet on kaolin.

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The results confirm the marked saluretic and diuretic effect of bumetanide after oral administration of both 0.5 and 1 mg. to human volunteers and show clearly a parallelism between bumetanide excretion and saluretic action over the total period (Figs. 2 and 3).

SUMMARY

A GLC determination of bumetanide, using flash-heater methylation by means of a mixture of tetramethylammonium hydroxide and trimethylanilinium hydroxide, was developed. The flash-heater methylation product of bumetanide proved to be methyl 3-(*N*-*n*-butylanilino)-5-dimethylsulfamyl-4-methoxybenzoate, formed by methylation under simultaneous rearrangement. The GLC determination was found to be accurate at concentrations as low as 0.1 mcg./ml. human urine. In six healthy volunteers, the urinary excretion of sodium, potassium, and chloride, the urine volume, and the urinary recovery of bumetanide were determined after oral administration of 0.5 and 1 mg. of the drug. A parallelism between bumetanide excretion and saluretic action over the total period of response is shown.

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Influence of Solution Electrolyte Content and Dielectric Constant on Drug Adsorption by Kaolin

N. A. ARMSTRONG▲ and C. D. CLARKE*

Abstract □ The adsorption of benzoic acid and crystal violet on kaolin was investigated to elucidate the influence of the system dielectric constant and the electrolyte content on this example of drug–adjuvant interaction. The differing results obtained with the two adsorbates reflect the dissimilar adsorption sites on the kaolin platelet and the different mechanisms involved in the uptake of acids and bases on clays. The experimental data presented are satisfactorily explained by a consideration of the effect of both the dielectric constant and the electrolyte concentration and valency on adsorbent and adsorbate characteristics.

Keyphrases □ Kaolin, adsorption of benzoic acid and crystal violet—location of adsorption sites, influence of dielectric constant and electrolyte content □ Adsorption of benzoic acid and crystal violet by kaolin—influence of dielectric constant and electrolyte content □ Drug–adjuvant interactions—influence of dielectric constant and electrolyte content, drug adsorption by kaolin □ Dielectric constant, electrolyte content—effect on drug adsorption by kaolin □ Electrolyte content, dielectric constant—effect on drug adsorption by kaolin

Previous papers (1, 2) showed that the Langmuir adsorption isotherm represents the adsorption of benzoic acid and crystal violet on kaolin, and they discussed the effect of environmental pH on this equilibrium process. From the results obtained, the probable adsorption sites for acidic and basic materials on clays were suggested, namely that anionic materials were adsorbed on the edge and cationics on the cleavage surface of the clay. The significance of such an evident drug–adjuvant interaction on preservative efficiency or therapeutic potency was briefly discussed. The presence of soluble material in a suspension formulation, for example as active principle, flavoring, coloring, buffering, or peptizing agents will not only influence the formulation's pH but also contribute to the electrolyte content of the system. This increase in electrolyte concentration will also result in an increase in the dielectric constant of the system (3). Both of these factors influence the characteristics of the electrical double layer of the suspended phase, this double layer constituting the area where adsorption and/or ion exchange takes place. It was, therefore, considered desirable to determine the effect of both the system dielectric constant and the electrolyte content (with respect to concentration and valency) on the problem of drug–adjuvant interaction through investigating the influence of these factors on the uptake of benzoic acid and crystal violet on kaolin.

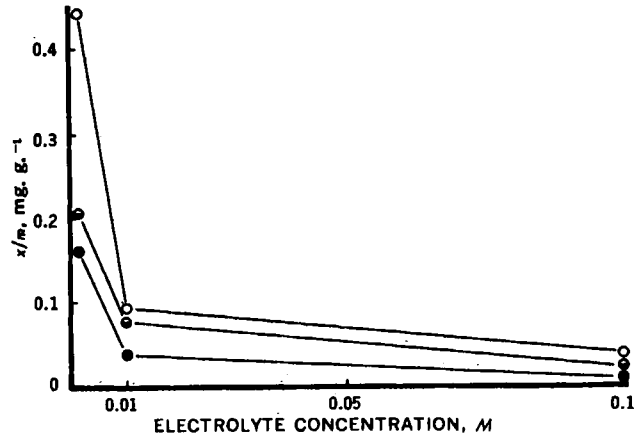

Figure 1—*Effect of electrolyte concentration and valency on the adsorption of benzoic acid on kaolin (pH 4.8). Key: ○, sodium chloride; ◒, sodium sulfate; and ●, sodium citrate.*

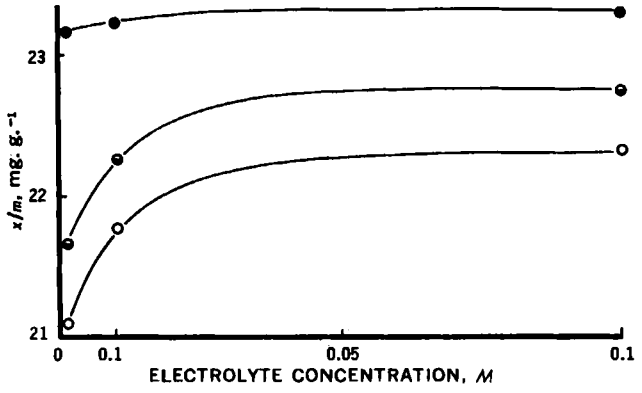

Figure 2—*Effect of electrolyte concentration and valency on the uptake of crystal violet on kaolin (pH 5.6). Key: ○, sodium chloride; ◒, sodium sulfate; and ●, sodium citrate.*

EXPERIMENTAL

Materials—Benzoic acid (Analar grade), crystal violet, sodium salts, and ethanol (reagent grade) were used without further purification. Light kaolin (BP quality) was dried at 100° (3 hr.), blended thoroughly, and stored in sealed containers. The weight mean diameter of the kaolin was 3.3 μm. (sedimentation balance), the surface area was 11.2 $m.^2 g.^{-1}$ (nitrogen adsorption[1]), and the cation-exchange capacity was 10.3 meq./100 g. of clay (ammonium acetate method).

Influence of Electrolyte Concentration on Adsorption—*Benzoic Acid*—One hundred milliliters of a solution containing benzoic acid (40 mg.) in sodium chloride of strengths 0.001, 0.01, and 0.1 *M* was added to flasks containing kaolin (20 g.). Duplicate determinations were carried out in each case. After agitation at 24 ± 0.5° for 24 hr. and centrifugation at 10,000×*g* (2 min.), the supernate was diluted with 0.1 *N* hydrochloric acid and the benzoic acid concentration was determined spectrophotometrically at 275 nm. Solutions of benzoic acid were shown to obey Beer's law, and it was demonstrated that the presence of sodium chloride did not interfere with the assay procedure.

Crystal Violet—To flasks containing kaolin (2 g.) was added 100 ml. of solution containing crystal violet (50 mg.) and 1% w/v disodium ethylenediaminetetraacetate (I) (1 ml.) in sodium chloride solutions of strengths 0.001, 0.01, and 0.1 *M*. The sequestering agent was included to maintain the salts of soil cations in solution, which otherwise have only low water solubility. After agitation at 24 ± 0.5° for 12 hr., a portion of the suspension was centrifuged at 10,000×*g* (2 min.). The supernate was then examined for dye concentration by spectrophotometric assay at 590 nm. It also was examined for magnesium content by atomic absorption spectroscopy, since it was previously shown (1) that cation exchange is the major mechanism for dye uptake by kaolin. Solutions of crystal violet were previously found to obey Beer's law, and it was also shown that the presence of sodium chloride and I did not interfere with the assay methods.

Influence of Electrotype Type on Adsorption—The effect of 0.001, 0.01, and 0.1 *M* concentrations of sodium chloride, sodium sulfate, and sodium citrate on the uptake of benzoic acid and crystal violet on kaolin was investigated. The procedure followed was as described above.

Influence of Dielectric Constant on Adsorption—Solutions of the adsorbate were prepared in solvents of differing water–ethanol proportions, thus providing a range of dielectric constants from 33.6 to 81.7.

Benzoic Acid—Benzoic acid (10 mg.) was dissolved in a water–ethanol mixture (100 ml.), the concentrations of ethanol being 0, 25, 50, 75, and 95% v/v. Kaolin (20 g.) was then added; and after equilibration, centrifugation, and dilution, the benzoic acid concentration of the supernate was spectroscopically determined. Duplicate determinations and appropriate blanks were used.

Crystal Violet—Crystal violet (50 mg.) and I (100 mg.) were dissolved in a water–ethanol mixture (100 ml.), the concentrations of ethanol being identical to those already described. Kaolin (2 g.) was then added; and after equilibration and centrifugation, the supernate was assayed for dye and magnesium content. Higher alcohol concentrations affected the determination of magnesium by atomic absorption spectroscopy, but this was corrected by use of a calibration curve of atomic absorption spectroscopic readings of known magnesium concentrations in the presence of alcohol.

RESULTS AND DISCUSSION

The molecular structure of kaolin, $Al_4Si_4O_{10}(OH)_8$, is composed of two basic units and is well documented (4, 5). One unit is an octahedron of oxygen atoms (or hydroxyl groups) surrounding a central aluminum atom, which forms a continuous sheet through the sharing of oxygen atoms of identical adjacent units. The second unit is composed of four oxygen atoms situated at the corners of a regular tetrahedron, with a silicon atom in the center. The atoms at the base of the tetrahedra are shared by neighboring modules, and the two basic units are joined such that the apexes of the tetrahedron and the top layer of the octahedron form a common plane (6). The origin and nature of the electrostatic charges to which the adsorptive capacity of the clay has been attributed and the variation of these charges with pH were discussed elsewhere (2, 3). It has been pointed out that the kaolin lamella has two distinct areas for adsorption: the kaolin edge surface and the larger cleavage plane surface.

Influence of Electrolyte Concentration and Valency on Adsorption—*Benzoic Acid*—The adsorption of benzoic acid on kaolin is markedly influenced by the electrolyte content of the system (Fig. 1), with sorption of the preservative decreasing with an increase in sodium chloride concentration. A valency increase of the electrolyte anion also further depresses the adsorption of benzoic acid on the clay.

Chloride, fluoride, and oxalate ions are known to be adsorbed on the edges of kaolin particles (7–9) because of their affinity for lattice aluminum. Moreover, phosphate anions are stated to be edge adsorbed on clays (10, 11), such phosphate adsorption being progressively reduced in systems of increasing saline concentrations (12). Citrate ions are also claimed to be edge adsorbed by clay minerals, the affinity of the anion for the adsorption site being greater than that of chloride ions, so that citrate is capable of displacing previously held chloride ions from the positive sites occurring on the edge of the kaolin platelet (13).

The results discussed in a previous article (2) strongly suggest that the adsorption of benzoic acid on kaolin is mediated through the attachment of the negatively charged benzoate anion to sites of positive charge occurring on the edge of the kaolin particles. Since the results of other workers, as briefly reviewed here, also indicate the edge adsorption of other anionic species, it seems reasonable to assume that in systems containing benzoic acid and other anionogenic material, competition will exist between the benzoate and other anions present for the available adsorption sites on the clay. Increasing the concentration of the added salt will favor its preferential sorption and, therefore, reduce the likelihood of vacant sites

[1] Perkin-Elmer, Shell Sorptometer, model 212D.

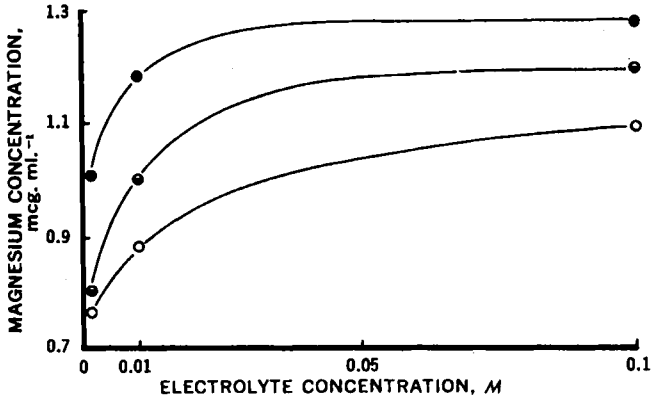

Figure 3—*Effect of electrolyte concentration and valency on the liberation of magnesium from kaolin at pH 5.6. Key:* ○, *sodium chloride;* ◒, *sodium sulfate; and* ●, *sodium citrate.*

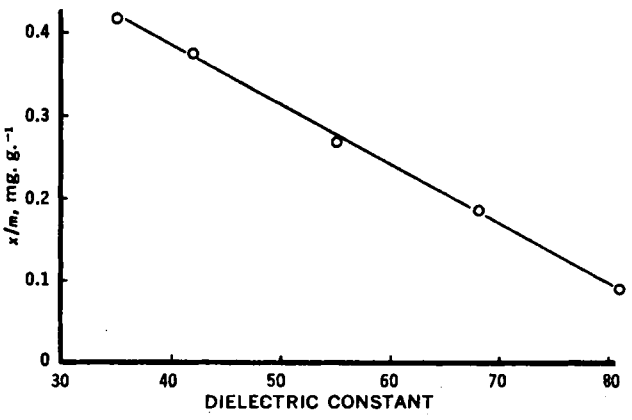

Figure 4—*Variation in the adsorption of benzoic acid on kaolin with the dielectric constant of the suspension medium.*

existing for benzoate anion sorption. The depressive effect of increasing the valency of the added anion is considered to result from its increased affinity for the adsorption sites, the affinity of binding being citrate > sulfate > chloride.

Crystal Violet—Contrary to its effect upon the adsorption of benzoic acid, an increase in the electrolyte content of the system raises the amount of dye taken up by kaolin (Fig. 2). The extent of dye removed from solution is also further increased with an increase in the valency of the added salts (Fig. 2). Blaug and Gross (14) obtained similar results for the adsorption of methantheline bromide by antacids in systems of varying electrolyte conditions.

Since it has been demonstrated that crystal violet uptake on kaolin is mediated through two distinct mechanisms, namely a cation-exchange process and physical adsorption (1), it is necessary to determine the influence of added electrolyte on each of these mechanisms to explain the overall increase in dye uptake.

The distribution of ions in the electrical double layer is a function of the electrolyte content of the bulk solution, the diffuse counterion atmosphere being compressed toward the surface with increasing bulk electrolyte concentration. The effect of the electrolyte addition on the surface potential and the surface charge of the particle depends on the type of double layer. With most hydrophobic colloids, the double layer is created by the adsorption of potential-determining ions when the magnitude of this potential is not affected by the addition of an indifferent electrolyte, providing the presence of the electrolyte does not influence the activity of these potential-determining ions. For this type of double layer, therefore, the surface charge of the particle increases with increasing indifferent electrolyte concentration, and the surface potential remains constant.

The electrical double layer on the cleavage surfaces of kaolin is of a different type, since this surface has a constant charge solely determined by the type and degree of isomorphous replacement of metal cations within the lattice. The charge density of the layer surface is, therefore, independent of the presence of electrolytes in suspension (15). However, an increase in bulk electrolyte concentration decreases the surface potential and the ζ-potential due to the compression of the double layer, which, in turn, favors a decrease in the amount of dye held by physical adsorption. This effect is small, since the added anions are of the same sign as the surface charge. On this basis, the uptake of dye should lessen with an increase in the bulk electrolyte concentration.

The results shown in Fig. 2 indicate an overall increase in dye uptake with increased electrolyte content. It is considered that the influence of the added salts on the cation-exchange process must be responsible for this, the ion-exchange mechanism being responsible for the greater part of the total amount of dye held by kaolin (1). Increasing both electrolyte concentration and valency produces an increase in ion exchange, as indicated by the presence of magnesium ions in the suspending medium (Fig. 3). This increased exchange of sodium for divalent ions produces an increasingly negative charge on the clay, leading to increased dye uptake.

Sorby *et al.* (16) noted the increase of promazine adsorption on kaolin and talc from solutions of increasing sodium chloride concentration. These authors commented that sodium ions, as well as promazine, compete for the anionic sites on the kaolin surface; thus the addition of large amounts of sodium ion would be expected to decrease the extent of promazine adsorption if ion-exchange mechanisms were solely responsible for uptake. They concluded that since adsorption of the phenothiazine derivatives was actually increased in systems of high electrolyte concentration, ion exchange cannot be the only mechanism of adsorption. They suggested that the sodium chloride may be exerting its action through effects on the solubility of the promazine or on the physical properties of the adsorbent. This argument ignores the influence of the exchange of univalent sodium for counterions of higher valency on the imbalance of charge on the clay surface.

The observed increase in uptake on kaolin of positively charged crystal violet from solutions of increasing electrolyte concentration is, therefore, concluded to be due to the increase in the negative charge on the clay surface due to the exchange of divalent compensating cations on the clay surface for the added sodium ions.

Effect of Dielectric Constant on Adsorption—*Benzoic Acid*—The adsorption of benzoic acid on kaolin shows a linear increase in uptake with a decrease in the dielectric constant of the suspension medium (Fig. 4).

Crystal Violet—Contrary to what was observed for benzoic acid adsorption, the overall uptake of crystal violet by kaolin shows a linear decrease with decreasing dielectric constant of the suspending medium (Fig. 5). To determine whether this was due to the effect of the dielectric constant on ion exchange or physical adsorption, the extent of the magnesium replacement under these conditions was determined. The ion-exchange process, as indicated by the appearance of magnesium in the clay supernate, was markedly increased with an increase in the ethanol proportion (Fig. 6); that is, although the total uptake of dye progressively diminished in systems of lowering dielectric constant, the degree of ion exchange occurring increased under these conditions.

The use of ethanol–water mixtures in differing proportions to achieve a range of solvent dielectric constants introduces many

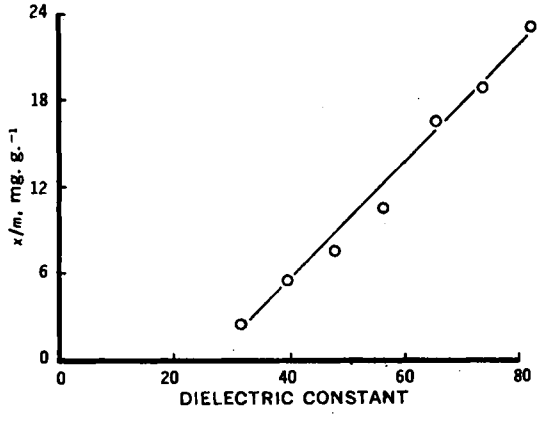

Figure 5—*Variation in the uptake of crystal violet on kaolin with the dielectric constant of the suspension medium.*

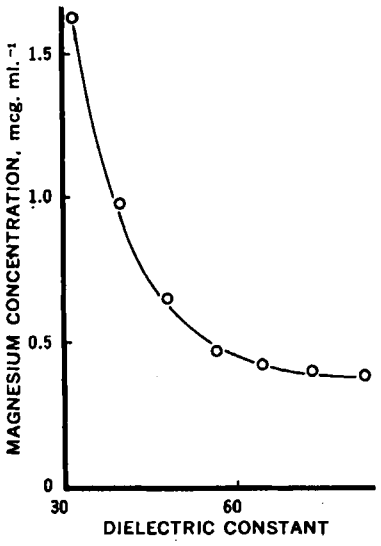

Figure 6—*Influence of the dielectric constant of the suspension medium on the liberation of magnesium from kaolin.*

variable and confusing factors, so the role of the dielectric constant becomes very difficult to identify. Such water–ethanol solutions can influence the characteristics of both adsorbent and adsorbate.

Influence of Water–Ethanol Mixtures on Adsorbent Characteristics—With a decreasing dielectric constant of the system, the diffuse counterion atmosphere surrounding the clay particle is compressed toward the particle surface, thereby reducing the value of the ζ-potential and the surface potential (15). The potential reduction might be expected to diminish the force of attraction between the surface and the adsorbate, leading to a decrease in the amount of drug held by physical adsorption. Indeed, Coulomb's law relates the force of attraction between two charged surfaces, q_1 and q_2, separated by a distance, r, to the medium dielectric constant, D, by the relationship:

$$\text{force of attraction} = \frac{q_1 q_2}{D r^2} \qquad \text{(Eq. 1)}$$

A further effect on the adsorbent is the possible preferential adsorption of alcohol by the clay which may result in displacement of the zero point of charge (15).

Furthermore, increasing concentrations of ethanol alter the viscosity and surface tension of the system when the increased wetting of the solid by solutions of decreasing surface tension might favor increased sorption through enhanced adsorbent–adsorbate contact.

Influence of Water–Ethanol Mixtures on Adsorbate Characteristics—The degree of solvation of the ions in the system (*i.e.*, their effective size) is reduced in solutions of increasing ethanol concentration, this effect favoring better adsorbent–adsorbate contact.

Ionization and dissociation of the adsorbates are depressed in conditions of low medium dielectric constant.

All of these considerations are simultaneously influencing the adsorbent–adsorbate system; hence, pending further investigations, a possible valid explanation can only be tentatively proposed for the results obtained in this study.

On the basis of previously discussed experimental evidence (2), the adsorption of benzoic acid on kaolin is considered to be mediated through the attachment of benzoate anions to the positive adsorption sites located on the edge of the clay particles. However, although dissociation of the acid to benzoate anion is depressed in solutions of low dielectric constant, adsorption is greatest under these conditions (Fig. 4). It is, therefore, to be assumed that these experimental results reflect the increased force of adsorbent–adsorbate attraction under conditions of low dielectric constant as expressed by Coulomb's law, this taking dominance over the other aqueous ethanol effects in the system.

The uptake of dye is more complex, involving as it does the mechanisms of cation exchange and physical adsorption. The total amount of dye taken up by the kaolin is seen to decrease with a decreased medium dielectric constant (Fig. 5), and this may be due to the suppression of dye ionization and dissociation in these solutions. However, a decrease in dye uptake is accompanied by a marked increase in ion exchange, a seemingly contradictory situation. Although ion-exchange mechanisms in aqueous systems have been well documented, little work appears to have been done on exchange in organic or mixed solvent systems, and the data available are often of a conflicting nature.

It is stated (17) that smaller ions will be more tightly held to the exchange surface than larger ions. In solutions of increasing ethanolic concentration, the ionic radius of the exchanging sodium and magnesium ions decrease as the ions become less hydrated. Grim (18) claimed that in alcohol, exchangeable cations are held in the order of their true (nonhydrated) ionic size; magnesium was observed to have a smaller ionic radius than sodium (19) and, therefore, was less likely to be replaced. The results obtained in the study are contrary to this observation.

Moody and Thomas (20) observed that the use of ethanol in a solvent system influences the degree of selection of a cation exchanger toward the available ions in the ambient solution. Wiegner and Jenny (21) studied ion exchange between a calcium zeolite and various alkali chlorides dissolved in aqueous ethanol, and they observed that more sodium ions passed on to the exchanger as the concentration of ethanol was increased. Other workers (22–24), using synthetic cation-exchange resins (hydrogen form) in aqueous ethanol systems, noted an increased exchange of sodium–hydrogen with an increasing ethanol content of the system.

From the results of this study and the observations of previous workers, it seems reasonable to suggest that the selectivity of the clay toward exchangeable cations in the surrounding solution is strongly influenced by the ethanol concentration of the system. The theory is advanced that with an increasing ethanol concentration, the kaolin shows a greater affinity for cation exchange involving sodium ions in preference to those of the dye species. This would produce an increasing negative charge on the clay and, therefore, promote increased crystal violet uptake by physical adsorption. However, cation exchange represents the major mechanism in dye uptake by kaolin; with increased exchange of solution sodium ions, the participation of the dye in this process is suppressed, resulting in the overall decrease in dye uptake in solutions of decreasing dielectric constant.

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Determination of Total Salicylates, Phenacetin and/or Total *N*-Acetyl-*p*-aminophenol, and Caffeine in Biological Samples and Analgesic Formulations

HIRA L. GURTOO▲ and BARRIE M. PHILLIPS

Abstract □ An analytical method was developed for the determination of phenacetin (apparent phenacetin), total salicylate (aspirin, salicylic acid, and its conjugates), total *N*-acetyl-*p*-aminophenol (*N*-acetyl-*p*-aminophenol and its conjugates), and caffeine in biological samples. Two 1-ml. samples are required. The first sample, after being acid hydrolyzed (2 *N* HCl, 2 hr., 121°) to convert total salicylate to free salicylic acid and total *N*-acetyl-*p*-aminophenol to *p*-aminophenol, is extracted with chloroform to remove caffeine and salicylic acid. *p*-Aminophenol in the aqueous phase is estimated by its reaction with phenol and sodium hypobromite. Salicylic acid extracted from the chloroform with sodium bicarbonate solution is estimated by reacting it with either Folin–Ciocalteu reagent or ferric nitrate. The caffeine, remaining in the chloroform, is estimated from its absorbance at 276 nm. In the second sample, phenacetin is hydrolyzed (8 *N* HCl, 6 hr., 121°) to *p*-aminophenol (total *p*-aminophenol), which is estimated as before. Time-dependent plasma and kidney levels of caffeine, phenacetin, total *N*-acetyl-*p*-aminophenol, and total salicylate, following the oral administration of a single dose (500 or 900 mg./kg. body weight) of aspirin–phenacetin–caffeine mixture to rats, were determined. The method also was adapted to the analysis of aspirin–phenacetin–caffeine powders.

Keyphrases □ Analgesic formulations—determination of total salicylates, phenacetin and/or total *N*-acetyl-*p*-aminophenol, and caffeine □ Plasma and tissue levels—determination of total salicylates, phenacetin and/or total *N*-acetyl-*p*-aminophenol, and caffeine □ Salicylate content, biological samples and analgesic formulations—determination, mixtures with phenacetin and caffeine □ Phenacetin and/or *N*-acetyl-*p*-aminophenol content, biological samples and analgesic formulations—determination, mixtures with aspirin and caffeine □ Caffeine content, biological samples and analgesic formulations—determination, mixtures with aspirin and phenacetin □ Aspirin–phenacetin–caffeine mixtures—determination of components in biological samples and analgesic formulations

As a result of continuing interest in the elucidation of the biochemical and toxicological mechanisms of "analgesic abuse nephropathy," different groups of researchers have employed single-entity or combination analgesics in their *in vivo* studies. Aspirin, phenacetin, and caffeine, the common components of analgesic mixtures, and their metabolites (1–6) have been studied and the current consensus points to the involvement of analgesic mixtures rather than of any single component in the production of "analgesic abuse nephropathy" (7).

In the final analysis, the availability of an analytical technique suitable for the analysis of caffeine and total amounts of salicylate and phenacetin in the tissue or biological fluid is more meaningful as injury to the tissue is related to the concentration of the injurious chemical in that tissue. This report describes an analytical method developed for the estimation of phenacetin and total *N*-acetyl-*p*-aminophenol (which includes *N*-acetyl-*p*-aminophenol and its conjugates) as *p*-aminophenol, total salicylates (including aspirin, salicylate, and its conjugates) as free salicylate, and caffeine. The major advantages of this method are its accuracy, its applicability to small size samples, and its feasibility for the analysis of aspirin, phenacetin, and caffeine in compound analgesic tablets and powders.

EXPERIMENTAL

For the GC analysis, a gas chromatograph[1] was used under the following operating parameters: column contents, 3% OV-1 on Anakrom Q, 80–100; column size, 0.42 cm. × 1.83 m. (0.125 in. × 6 ft.); column temperature, 160°; carrier gas, helium; carrier gas flow rate, 33 ml./min.; detector, flame-ionization type; injection port and detector block temperatures, 300°; hydrogen flow rate, 3 ml./min.; and air flow rate, 300 ml./min.

Chemicals—Sodium salicylate[2], *p*-aminophenol[3], phenacetin[3], and caffeine[4] of analytical grade were obtained. Analytical grade dichloroethylene, sodium bicarbonate, sodium hydroxide, dibasic potassium hydrogen phosphate, ferric chloride, and hydrochloric acid were used. Solvent grade ether and chloroform were washed successively with 1 *N* NaOH, 1 *N* HCl, and distilled water (three washings) before use. Double-distilled water was used for all purposes.

Biological Materials—The analyses were performed on rat plasma or rat kidney homogenate prepared by homogenizing (Potter-

[1] Varian 1200.
[2] Matheson, Coleman and Bell.
[3] Aldrich Chemical Co.
[4] Eastman Chemical Co., Inc.

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Determination of Total Salicylates, Phenacetin and/or Total *N*-Acetyl-*p*-aminophenol, and Caffeine in Biological Samples and Analgesic Formulations

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Abstract □ An analytical method was developed for the determination of phenacetin (apparent phenacetin), total salicylate (aspirin, salicylic acid, and its conjugates), total *N*-acetyl-*p*-aminophenol (*N*-acetyl-*p*-aminophenol and its conjugates), and caffeine in biological samples. Two 1-ml. samples are required. The first sample, after being acid hydrolyzed (2 *N* HCl, 2 hr., 121°) to convert total salicylate to free salicylic acid and total *N*-acetyl-*p*-aminophenol to *p*-aminophenol, is extracted with chloroform to remove caffeine and salicylic acid. *p*-Aminophenol in the aqueous phase is estimated by its reaction with phenol and sodium hypobromite. Salicylic acid extracted from the chloroform with sodium bicarbonate solution is estimated by reacting it with either Folin–Ciocalteu reagent or ferric nitrate. The caffeine, remaining in the chloroform, is estimated from its absorbance at 276 nm. In the second sample, phenacetin is hydrolyzed (8 *N* HCl, 6 hr., 121°) to *p*-aminophenol (total *p*-aminophenol), which is estimated as before. Time-dependent plasma and kidney levels of caffeine, phenacetin, total *N*-acetyl-*p*-aminophenol, and total salicylate, following the oral administration of a single dose (500 or 900 mg./kg. body weight) of aspirin–phenacetin–caffeine mixture to rats, were determined. The method also was adapted to the analysis of aspirin–phenacetin–caffeine powders.

Keyphrases □ Analgesic formulations—determination of total salicylates, phenacetin and/or total *N*-acetyl-*p*-aminophenol, and caffeine □ Plasma and tissue levels—determination of total salicylates, phenacetin and/or total *N*-acetyl-*p*-aminophenol, and caffeine □ Salicylate content, biological samples and analgesic formulations—determination, mixtures with phenacetin and caffeine □ Phenacetin and/or *N*-acetyl-*p*-aminophenol content, biological samples and analgesic formulations—determination, mixtures with aspirin and caffeine □ Caffeine content, biological samples and analgesic formulations—determination, mixtures with aspirin and phenacetin □ Aspirin–phenacetin–caffeine mixtures—determination of components in biological samples and analgesic formulations

As a result of continuing interest in the elucidation of the biochemical and toxicological mechanisms of "analgesic abuse nephropathy," different groups of researchers have employed single-entity or combination analgesics in their *in vivo* studies. Aspirin, phenacetin, and caffeine, the common components of analgesic mixtures, and their metabolites (1–6) have been studied and the current consensus points to the involvement of analgesic mixtures rather than of any single component in the production of "analgesic abuse nephropathy" (7).

In the final analysis, the availability of an analytical technique suitable for the analysis of caffeine and total amounts of salicylate and phenacetin in the tissue or biological fluid is more meaningful as injury to the tissue is related to the concentration of the injurious chemical in that tissue. This report describes an analytical method developed for the estimation of phenacetin and total *N*-acetyl-*p*-aminophenol (which includes *N*-acetyl-*p*-aminophenol and its conjugates) as *p*-aminophenol, total salicylates (including aspirin, salicylate, and its conjugates) as free salicylate, and caffeine. The major advantages of this method are its accuracy, its applicability to small size samples, and its feasibility for the analysis of aspirin, phenacetin, and caffeine in compound analgesic tablets and powders.

EXPERIMENTAL

For the GC analysis, a gas chromatograph[1] was used under the following operating parameters: column contents, 3% OV-1 on Anakrom Q, 80–100; column size, 0.42 cm. × 1.83 m. (0.125 in. × 6 ft.); column temperature, 160°; carrier gas, helium; carrier gas flow rate, 33 ml./min.; detector, flame-ionization type; injection port and detector block temperatures, 300°; hydrogen flow rate, 3 ml./min.; and air flow rate, 300 ml./min.

Chemicals—Sodium salicylate[2], *p*-aminophenol[3], phenacetin[3], and caffeine[4] of analytical grade were obtained. Analytical grade dichloroethylene, sodium bicarbonate, sodium hydroxide, dibasic potassium hydrogen phosphate, ferric chloride, and hydrochloric acid were used. Solvent grade ether and chloroform were washed successively with 1 *N* NaOH, 1 *N* HCl, and distilled water (three washings) before use. Double-distilled water was used for all purposes.

Biological Materials—The analyses were performed on rat plasma or rat kidney homogenate prepared by homogenizing (Potter-

[1] Varian 1200.
[2] Matheson, Coleman and Bell.
[3] Aldrich Chemical Co.
[4] Eastman Chemical Co., Inc.

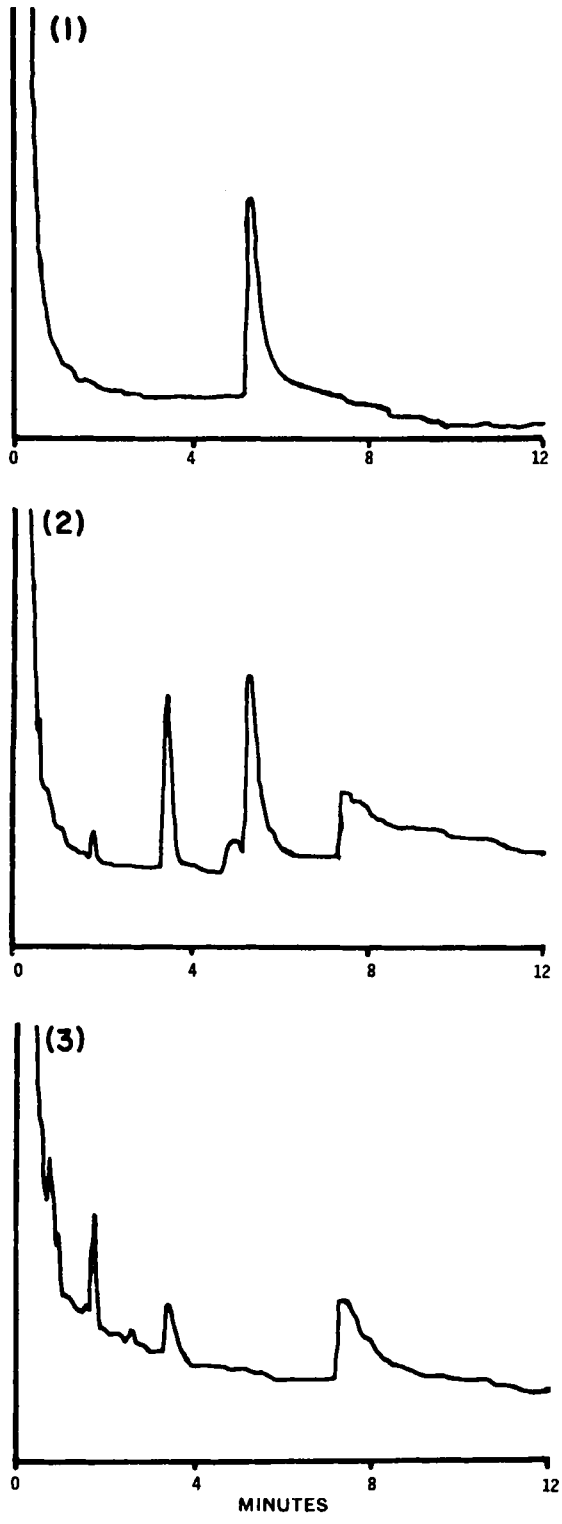

Figure 1—*GC analysis of plasma samples from control and aspirin–phenacetin–caffeine-treated rats. The chromatograms were run for 40 min., and no significant differences were observed between the control and the treated groups beyond 12 min. Key: 1, caffeine in chloroform; 2, chloroform extract of plasma from rats that received up to 900 mg. aspirin–phenacetin–caffeine/kg. (Rat Study III); and 3, chloroform extract of plasma from control group of rats.*

Elvehjem homogenizers) rat kidneys in water or 0.1 *N* HCl (1 g. wet weight/2 ml. liquid).

Analytical Method—Under suitable conditions of acid hydrolysis of biological samples containing aspirin, phenacetin, and caffeine and/or metabolites, aspirin and its metabolic products are converted to free salicylic acid; phenacetin, *N*-acetyl-*p*-aminophenol, and its metabolic products are converted to *p*-aminophenol. However, caffeine and its metabolites (demethylation and oxidation products) are not likely to undergo any change under these conditions. This knowledge, coupled with the information about the differing solubility of *p*-aminophenol, salicylic acid, and caffeine in chloroform and ether under acidic conditions, made it possible to develop the analytical technique described here.

Table I—Computer-Generated Algebraic Formulas for Calibration Curves of Salicylic Acid, *p*-Aminophenol, and Caffeine

Material	Concentration Range, mg./ml. Plasma	Equation for Calibration Curve	r^a
Salicylic acid[b]	0.0–0.52	$Y = 1.435 \times +0.016$	1.000
Salicylic acid[c]	0.0–1.50	$Y = 0.337 \times +0.006$	1.003
p-Aminophenol	0.0–0.20	$Y = 9.355 \times -0.061$	1.036
Caffeine	0.0–0.60	$Y = 0.711 \times +0.00$	1.001

[a] Regression correlation coefficient. [b] The final color reaction was developed with Folin–Ciocalteu reagent (9). [c] The final color reaction was developed with ferric nitrate (10).

When the acid-hydrolyzed sample is shaken with chloroform and the phases are separated, *p*-aminophenol is retained in the salt form in the aqueous phase while caffeine and free salicylic acid are extracted into the chloroform layer. Salicylic acid is subsequently removed with sodium bicarbonate solution; the caffeine remaining in the chloroform layer, after being thoroughly washed with sodium bicarbonate and ether, is estimated from its spectrophotometric absorption at 276 nm.

The hydrolysis is carried out for 2 hr. in 2 *N* hydrochloric acid in an autoclave at 121° under a pressure of 6.8 kg. (15 lb.)/in.^{2}. To 1 ml. of kidney homogenate or plasma in a 50-ml. centrifuge tube is added 0.67 ml. of concentrated (12 *N*) hydrochloric acid, and the volume is adjusted to 4 ml. with distilled water. After autoclaving and cooling, the mixture is shaken (for 5 min. on an automatic shaker) with chloroform (15 ml.); the phases are separated by centrifugation (5 min.) and analyzed as follows for *p*-aminophenol, salicylic acid, and caffeine.

Determination of Total *N*-Acetyl-*p*-aminophenol—To 2 ml. of the aqueous phase, transferred into a 50-ml. centrifuge tube, are added water (2 ml.), potassium hydrogen phosphate (3 g.), and ether (30 ml.) in that order. The mixture is shaken and either centrifuged or allowed to stand for 20 min. (with stopper on to minimize evaporation of ether). Then 25 ml. of the top layer (ether phase) is transferred to another 50-ml. centrifuge tube containing 5 ml. of 0.01 *N* hydrochloric acid; this mixture is shaken and centrifuged, the ether layer is aspirated, and 3 ml. of the aqueous phase is transferred to a test tube to which is added 1% phenol (1 ml.) and 1 *N* sodium hypobromite solution (1 ml.). The solution is mixed well and allowed to stand for 20 min., and its absorbance at 620 nm. is read. This method of analysis is similar to that described by Brodie and Axelrod (8).

Determination of Total Salicylate (Free Salicylic Acid)—The initial extraction with chloroform yields caffeine and salicylic acid in the chloroform phase. Ten milliliters of the chloroform phase is transferred to a 50-ml. centrifuge tube, and 5 ml. of 1% sodium bicarbonate is added. The mixture is shaken and then centrifuged, and this chloroform phase is saved for the analysis of caffeine. The aqueous phase (sodium bicarbonate solution) is used for the analysis of salicylate as follows.

An aliquot, 3.5 ml., of the sodium bicarbonate solution is transferred to a 50-ml. centrifuge tube containing 0.70 ml. concentrated hydrochloric acid, and the mixture is extracted as described below with ethylene dichloride.

After the addition of ethylene dichloride (30 ml.), the mixture is shaken for 5 min. Then it is allowed to settle and the aqueous layer is aspirated. Twenty-five milliliters of the ethylene dichloride phase is transferred to another 50-ml. centrifuge tube containing 10 ml. of 1% sodium bicarbonate or water. If sodium bicarbonate is used, the mixture is shaken and centrifuged as before. Then 3 ml. of the aqueous phase is transferred to a test tube, and 1 ml. of Folin–

Table II—Recoveries[a] of Salicylic Acid, *p*-Aminophenol, or Caffeine Added Individually to Rat Plasma

	Salicylic Acid[b]		*p*-Aminophenol[b]		Caffeine[b]	
	Milligrams Added	Percent Recovered	Milligrams Added	Percent Recovered	Milligrams Added	Percent Recovered
1	0.052	95.91	0.0165	90.91	0.120	98.91
2	0.104	92.00	0.0330	93.30	0.240	106.62
3	0.156	93.87	0.049	92.06	0.360	98.44
4	0.208	92.46	0.066	87.50	0.480	97.11
5	0.312	94.31	0.099	93.83	0.600	102.12
6	0.416	96.87	0.132	98.89	—	—
7	0.520	96.65	0.165	96.69	—	—
8	—	—	0.198	102.72	—	—
Mean ± *SE*[c]		94.58 ±0.74		94.50 ±1.69		100.62 ±1.71

[a] Calibration curves prepared with aqueous solutions. [b] Averages of at least four determinations. [c] SE = standard deviation/$\sqrt{n}$, where n is the number of determinations.

Table III—Recoveries of Salicylic Acid, *p*-Aminophenol, and Caffeine Added as Mixtures to Rat Plasma or Kidney Homogenate

Sample Number	Salicylic Acid[a]		*p*-Aminophenol[a]		Caffeine[a]	
	Milligrams Added	Percent Recovered	Milligrams Added	Percent Recovered	Milligrams Added	Percent Recovered
1	0.180[b]	—[c]	0.0385	100.40	0.072	—[b]
2	0.180[b]	—[c]	0.048	—[c]	0.384	101.5
3	0.52[b]	105.93	0.132	92.50	0.60	99.0
4	0.060	—[c]	0.124	98.71	0.060	—[c]
5	0.156	—[c]	0.0495	96.36	0.180	—[c]
6	0.113	100.00	0.054	—[c]	0.060	—[c]
7	0.180	—[c]	0.048	—[c]	0.384	102.3
8	0.42	108.26	0.100	113.15	0.60	104.3

[a] Averages of four determinations. [b] Recoveries obtained from kidney homogenate. [c] Determinations not carried out.

Table IV—Recoveries of Phenacetin as *p*-Aminophenol from Water, Rat Plasma, or Kidney Homogenate

Phenacetin Added, mg.	*p*-Aminophenol Obtained, mg.	Phenacetin Equivalent Recovered, mg.	Percent Recovery[a]
0.0300	0.0195	0.03249	108.31
0.0600	0.0398	0.06615	110.25
0.0900	0.0535	0.08884	98.70
0.1976	0.1107	0.18510	93.77
0.4200	0.2570	0.42590	101.41

[a] Average of four determinations.

Table V—Effect of Phenacetin and Salicylate on the Analysis of Caffeine in Rat Plasma

Phenacetin Present, mg.	Caffeine Present, mg.	Salicylate Present, mg.	Absorbance[a] at 276 nm.
0.152	0.600	0.000	0.448 ± 0.013[b]
0.000	0.600	0.420	0.452 ± 0.004
0.000	0.600	0.000	0.443 ± 0.003
0.152	0.000	0.000	0.016 ± 0.000
0.000	0.000	0.420	0.018 ± 0.000
0.000	0.000	0.000	0.017 ± 0.000

[a] Average of three determinations. [b] Mean ± *SE*.

Ciocalteu reagent and 1 ml. of 1.5 *N* sodium hydroxide are added in that order. The mixture is mixed well and the absorbance is read at 670 nm. after allowing it to stand for 5 min. (9). In the other method, 0.25 ml. of 1% ferric nitrate is added to the water and ethylene dichloride mixture, which is subsequently shaken and centrifuged. The concentration of salicylic acid is calculated from the absorbance of the colored aqueous phase at 540 nm. (10).

Determination of Caffeine—The 10 ml. of chloroform phase saved from the first extraction is washed (by shaking and centrifuging) twice with sodium bicarbonate solution, first with 30 ml. of 5% solution and then with 30 ml. of 1% solution. The latter is aspirated and 7 ml. of the chloroform phase is transferred to a 50-ml. centrifuge tube, evaporated to near dryness (0.1 ml.), and cooled; then 4 ml. of 2 *N* HCl solution and 30 ml. of ether are added in that order. The mixture is shaken and centrifuged, the ether phase is aspirated, and 2.5 ml. of the aqueous phase is transferred to another 50-ml. centrifuge tube to which 10 ml. of chloroform is added. The mixture is shaken and centrifuged, the aqueous phase is aspirated, and the chloroform phase is read in a spectrophotometer at 276 nm. against chloroform.

Determination of Phenacetin (Apparent Phenacetin[5])—One milliliter of plasma or homogenized kidney tissue is hydrolyzed in the presence of 8 *N* HCl (4 ml. 12 *N* acid, 1 ml. sample, 1 ml. water) by autoclaving for 6 hr. at 121° under a pressure of 6.8 kg. (15 lb.)/in.^{2}. This results in the conversion of phenacetin, *N*-acetyl-*p*-aminophenol, and its conjugates to *p*-aminophenol (total *p*-aminophenol), which is determined by the method already described except that the volume of acid hydrolysate is adjusted to 10 ml. and extracted with chloroform (30–35 ml.), and 2.50 ml. of the aqueous phase (top layer) is transferred to a 50-ml. centrifuge tube containing potassium hydrogen phosphate (4–5 g.) and water (1.5 ml.). Phenacetin in the sample is obtained by subtracting the absolute amount of total *N*-acetyl-*p*-aminophenol (determined earlier) from the absolute amount of total *p*-aminophenol (obtained after 6 hr. of hydrolysis); the resulting difference is multiplied by a factor, mol. wt. phenacetin/mol. wt. *p*-aminophenol, that includes the contribution due to the higher molecular weight of phenacetin.

Analysis of Aspirin–Phenacetin–Caffeine Powders—Three samples of such powders of unknown composition were obtained[6] and analyzed by the methods described for biological samples. However, for each type of analysis, separate calibration curves using aqueous solutions were set up. One hundred milligrams of each powder was dissolved in chloroform (100 ml.), and 1 ml. of the solution after removal of the solvent was hydrolyzed for 2 or 6 hr. under the conditions already described. The hydrolyzed solutions were analyzed for salicylate, *p*-aminophenol, and caffeine. As pro-

[5] Apparent phenacetin obtained as difference. In addition to phenacetin, this should include *N*-acetyl-*p*-aminophenol glucuronide and phenetidin present in the biological sample as a result of biotransformation.

[6] Pharmacy Research and Development Laboratories, Miles Laboratories, Inc., Elkhart, Ind.

Table VI—Plasma and Kidney Levels in Male Rats following Oral Administration of a Single 500-mg./kg. Dose of Aspirin–Phenacetin–Caffeine Powder

Hours[a]	Plasma Levels, mg./l., Mean ± SE[b]			Kidney Tissue Levels, mcg./g., Mean ± SE		
	Total Salicylate	Total APAP[c]	Caffeine	Total Salicylate	Total APAP[c]	Caffeine
0.50	237 ± 12	25 ± 1	45 ± 4	56 ± 0	28 ± 0	50 ± 11
1.00	229 ± 27	29 ± 1	36 ± 8	65 ± 0	32 ± 0	54 ± 10
1.50	285 ± 15	35 ± 1	49 ± 8	88 ± 0	37 ± 0	85 ± 10
2.00	229 ± 23	40 ± 3	41 ± 7	40 ± 0	38 ± 0	48 ± 11
4.00	206 ± 15	25 ± 3	32 ± 5	35 ± 0	28 ± 0	69 ± 16
8.00	194 ± 9	22 ± 1	38 ± 6	33 ± 0	26 ± 0	71 ± 8
24.00	140 ± 21	11 ± 2	29 ± 11	12 ± 0	21 ± 0	38 ± 11

[a] Time of sacrifice after the administration of aspirin–phenacetin–caffeine. [b] Each group comprised of nine rats; most of the values reported are the means for nine rats. [c] APAP denotes *N*-acetyl-*p*-aminophenol.

Table VII—Kidney Levels of Total *N*-Acetyl-*p*-aminophenol and Phenacetin following Acute Oral Doses of Aspirin–Phenacetin–Caffeine Powder

Hours	500 mg./kg., Mean ± SE[a]		900 mg./kg., Mean ± SE[a]	
	Total APAP[b], mcg./g.	Phenacetin, mcg./g.	Total APAP, mcg./g.	Phenacetin, mcg./g.
1.00	27 ± 1	9 ± 4	38 ± 3	26 ± 3
1.50	33 ± 3	11 ± 4	52 ± 3	46 ± 3
2.00	32 ± 1	8 ± 2	44 ± 2	11 ± 4
4.00	29 ± 2	5 ± 3	64 ± 23	26 ± 20
8.00	—	—	29 ± 2	1.5 ± 1.5

[a] Each group had three rats. [b] APAP denotes *N*-acetyl-*p*-aminophenol.

vided by the supplier, the percentage composition of all three powders was 42:42:16 of aspirin–phenacetin–caffeine.

Rat Experiments—Three studies were undertaken in which rats were employed.

Study I—In Study I*a*, male rats[7] weighing 110–150 g. were used. Rats were allowed to acclimatize to the laboratory environment until they reached the desired weight (1 week) and then they were assigned at random to different groups, nine animals in each group. Aspirin–phenacetin–caffeine (42:42:16) powder, prepared as a fine suspension in distilled water containing 0.5% sodium carboxymethylcellulose and 0.4% polysorbate 80 at a constant volume of 10 ml./kg. and at a dose level of 500 mg./kg. body weight in a single dose, was administered orally to all animals in each group. At each specified time interval following the administration of aspirin–phenacetin–caffeine, a group of rats was anesthetized; blood was obtained by cardiac puncture (opened thorax) and both kidneys were removed prior to the sacrifice of the animal. Plasma was separated from blood and stored frozen for future use; in the same way, kidneys were stored frozen after being homogenized in 0.1 *N* HCl (1 g./2 ml.). Plasma and kidney samples were analyzed for total *N*-acetyl-*p*-aminophenol, total salicylate, and caffeine at a later date.

In Study I*b*, male rats of similar weights were used and a similar protocol (three animals in a group) was followed; however, only the kidneys were removed and their phenacetin content was estimated at a later date.

Study II—Protocol was similar to the one described for Study I (three animals in a group). However, the rats, which were of a different weight range (220–260 g.), received 900 mg. aspirin–phenacetin–caffeine/kg. In addition, phenacetin was determined in both kidney and plasma samples.

Study III—About 60 ml. of plasma was obtained from control and experimental groups of male and female rats that had received an aspirin–phenacetin–caffeine mixture (42:42:16) at twice daily oral doses up to 450 mg./kg. 5 days a week for 1 month. The 1-ml. plasma samples were extracted as described for the estimation of caffeine. The final chloroform extracts from these samples were pooled separately for control and experimental groups. The volumes of these pooled samples were reduced to about 1 ml. by evaporation in a water bath, and 2-μl. samples were analyzed by GC for the detection of caffeine and/or its metabolites.

RESULTS AND DISCUSSION

Calibration curves for the estimation of salicylate, *p*-aminophenol, and caffeine in 1-ml. aliquots of rat plasma are described in Table I.

Comparison of extraction between water and plasma showed that about 95% of salicylic acid and *p*-aminophenol is extracted from plasma and quantitative extraction is possible for caffeine. Results of these studies are summarized in Table II. The determinations of either salicylic acid or *p*-aminophenol or caffeine were not interfered with when these chemicals were present either singly or as mixtures. From inspection of the results (Table III), it is apparent that the determinations of salicylic acid, *p*-aminophenol, and caffeine are consistent with the percent recoveries described here. Almost identical results were obtained for similar determinations from kidney homogenates.

After absorption of aspirin–phenacetin–caffeine-containing analgesics, the major chemical species and metabolites in plasma and tissues are likely to be: (*a*) aspirin, salicylic acid, and its conjugates (salicyluric acid and salicylic acid glucuronides); (*b*) phenacetin, *N*-acetyl-*p*-aminophenol, and its conjugates (conjugates of glucuronic acid and sulfate); and (*c*) caffeine and its metabolites (oxidation and demethylation products). Under the hydrolytic conditions described earlier, aspirin, phenacetin, and their major metabolites are converted to free salicylic acid and *p*-aminophenol. If this acid-hydrolyzed mixture is shaken with chloroform and phases are separated, the *p*-aminophenol salt is retained in the aqueous phase, whereas caffeine and salicylic acid are isolated in the lower chloroform phase. Caffeine is soluble in chloroform, but the solubility characteristics (slightly soluble) of salicylic acid in chloroform (11) made it obligatory to estimate the percent isolation of salicylic acid into the chloroform phase over the analytical concentration range. It was found that, on the average, over a wide concentration range (Table I), 95.4 ± 2% of salicylic acid[8] was extracted. Autoclaving for 2 hr. in acidic medium, which is a reported method for the hydrolysis of *N*-acetyl-*p*-aminophenol and conjugated *p*-aminophenol in biological samples to *p*-aminophenol (8), was found to be as good a method for the hydrolysis of aspirin and salicylic acid conjugates to free salicylic acid in biological samples as that described by Bedford *et al.* (5). This concentration of hydrochloric acid (2 *N*) and the duration of hydrolysis (2 hr.) were found to hydrolyze only about 5% of phenacetin. However, phenacetin was quantitatively hydrolyzed to *p*-aminophenol under more intense conditions: autoclaving for 6 hr. in the presence of 8 *N* hydrochloric acid (Table IV). The presence of salicylic acid or caffeine did not interfere with the analysis of phenacetin. Although both salicylic acid and phenacetin have significant absorption at 276 nm., neither is carried over into the final chloroform extract which is used for the estimation of caffeine. Effects of both salicylic acid and phenacetin on the estimation of caffeine were studied, and the results are summarized in Table V.

GC analyses were performed to resolve the question of whether the absorbance at 276 nm. of the final chloroform extract of biological sample obtained from animals that had received aspirin, phenacetin, and caffeine was due only to the presence of intact caffeine or included some contributions from its metabolites. The

[7] Charles River COBS-CD Strain. All animals were maintained in an animal care facility fully accredited by the American Association of Laboratory Animal Care.

[8] Mean ± *SE*.

Table VIII—Plasma and Kidney Levels of Total Salicylate, Total *N*-Acetyl-*p*-aminophenol, Phenacetin, and Caffeine following Acute Oral Administration of 900 mg./kg. Aspirin–Phenacetin–Caffeine Powder

Time[a]	Total Salicylate, Mean ± SE[b]		Caffeine, Mean ± SE		Total APAP[c], Mean ± SE		Phenacetin, Mean ± SE	
	Plasma, mg./l.	Kidney, mcg./g.	Plasma, mg./l.	Kidney, mcg./g.	Plasma, mg./l.	Kidney, mcg./g.	Plasma, mg./l.	Kidney, mcg./g.
20 min.	217 ± 11	65 ± 13	19 ± 4	36 ± 16	22 ± 1	30 ± 1	39 ± 6	31 ± 0
40 min.	270 ± 9	79 ± 6	47 ± 4	55 ± 2	29 ± 1	36 ± 0	36 ± 5	34 ± 5
1.00 hr.	244 ± 12	60 ± 5	39 ± 20	25 ± 18	34 ± 3	38 ± 3	30 ± 7	26 ± 3
1.50 hr.	271 ± 34	136 ± 22	42 ± 10	62 ± 7	38 ± 3	52 ± 4	45 ± 28	46 ± 31
2.00 hr.	256 ± 23	139 ± 20	51 ± 4	70 ± 22	28 ± 2	44 ± 2	24 ± 13	12 ± 4
4.00 hr.	281 ± 80	193 ± 53	41 ± 31	89 ± 25	33 ± 10	64 ± 23	—	—
8.00 hr.	301 ± 41	118 ± 11	83 ± 8	73 ± 7	—	—	—	—
24.00 hr.	278 ± 29	81 ± 11	68 ± 18	34 ± 11	37 ± 5	46 ± 4	6 ± 2	6 ± 2

[a] Time of sacrifice after the administration of aspirin–phenacetin–caffeine. [b] Each group comprised of three rats and the values reported represent the mean for the group. [c] APAP denotes *N*-acetyl-*p*-aminophenol.

Table IX—Analysis of Aspirin–Phenacetin–Caffeine Powders[a]

Powder	Ingredient	Percent Present	Percent Found
A	Phenacetin	42	45.25
	Aspirin	42	42.48
	Caffeine	16	15.70
Total			103.43
B	Phenacetin	42	42.59
	Aspirin	42	43.42
	Caffeine	16	15.49
Total			101.50
C	Phenacetin	42	42.44
	Aspirin	42	43.97
	Caffeine	16	14.83
Total			100.64

[a] One-milligram samples were analyzed.

results are illustrated in Fig. 1. Inspection reveals that the only additional peak in samples from treated rats not present in samples from the control group is that of caffeine. Thus, it seems that the method described is specific for the analysis of caffeine; however, if any metabolites are present in the final extract, their concentration must be negligible.

The biological usefulness of this technique is evident from the studies conducted in rats. The results of Studies I*a*, I*b*, and II are summarized in Tables VI–VIII, respectively. Inspection of Table VII reveals that within 4 hr. after the oral administration of a 500-mg. aspirin–phenacetin–caffeine mixture/kg., phenacetin disappears as a result of its conversion to *N*-acetyl-*p*-aminophenol, which is in conformity with the other reported observations (7). However, at higher dose levels of aspirin–phenacetin–caffeine (900 mg./kg.), higher levels of phenacetin are found in the kidney over a longer time following drug administration. Earlier reports (1) also demonstrated the presence of unchanged phenacetin in the dog kidney. Table VIII summarizes the results obtained at various time intervals following the oral administration of 900 mg./kg. aspirin–phenacetin–caffeine in a single dose to rats. The disappearance of the components of the aspirin–phenacetin–caffeine mixture from plasma and kidney occurs at a much slower rate at this dose level. The phenacetin concentration at 24 hr. was negligible, although significant concentrations of total salicylate, total *N*-acetyl-*p*-aminophenol, and caffeine were found in plasma and kidney.

It should be noted that: (*a*) the gentisic acid formed from salicylic acid (about 1% of the dose) and 2-hydroxyphenacetin, constituting about 1% of the phenacetin dose (4), will not be estimated by the procedure described; and (*b*) the phenacetin concentration calculated from the difference between total *p*-aminophenol (obtained after 6 hr. of hydrolysis) and total *N*-acetyl-*p*-aminophenol (after 2 hr. of hydrolysis) represents only an apparent value, because it might include the minor metabolite, *p*-phenetidin (1) and unexcreted *N*-acetyl-*p*-aminophenol glucuronide, the ether linkage of which might be resistant to 2-hr. hydrolysis. However, observations[9] indicated that *N*-acetyl-*p*-aminophenol glucuronide, if present, is completely hydrolyzed to *p*-aminophenol under the conditions described for the hydrolysis of phenacetin.

Since the method was designed to estimate caffeine, total salicylate, and total *p*-aminophenol, it can be employed for the analysis of aspirin–phenacetin–caffeine formulations. The results of the analyses of three such powders are summarized in Table IX. In comparison to currently employed column and GC techniques for the estimation of the percentage composition of aspirin–phenacetin–caffeine formulations (12), the solvent extraction method is simple and requires no equipment other than a spectrophotometer.

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[9] Under these conditions of hydrolysis, rat plasma and kidney homogenate and human urine samples obtained following the administration of phenacetin were found to be completely hydrolyzed to *p*-aminophenol.

Optimizing Dermatological Formulations of Retinoic Acid

DAVID A. SCHLICHTING▲, WILLIAM M. WOODING, and MICHAEL BROWN

Abstract □ Formulations of retinoic acid for the topical treatment of ichthyosiform and seborrheic dermatoses were developed. They were evaluated for their skin irritation potential on rabbits, as a measure of pharmacological "activity," by studying erythema and scab-formation times. Different types of vehicles and varying concentrations of retinoic acid were studied. It was found that relatively high concentrations of retinoic acid (0.1–0.2%) were necessary in lipophilic vehicles (in which retinoic acid is relatively insoluble) to achieve a desirable irritation level. Such formulations should be useful for treating ichthyosiform dermatoses. Conversely, it was found that relatively low concentrations of retinoic acid (0.001–0.050%) were preferable in hydrophilic vehicles (in which retinoic acid is relatively soluble) to achieve a desirable irritation level. Such formulations should be useful for treating seborrheic dermatoses.

Keyphrases □ Retinoic acid—development of topical formulations for treatment of ichthyosiform and seborrheic dermatoses □ Topical formulations, retinoic acid—developed for treatment of ichthyosiform and seborrheic dermatoses □ Dermatology—retinoic acid formulations developed for treatment of ichthyosiform and seborrheic dermatoses □ Ichthyosis, treatment—development of retinoic acid formulations □ Seborrhea, treatment—development of retinoic acid formulations

The application of vitamin A acid or retinoic acid to therapy of topical diseases was first reported by Stüttgen in 1962 (1). He observed irritation in which vasodilation and slight perivascular infiltration were seen and in which keratolysis of the epidermis occurred with no observable effects on the dermis. He reported some success in treating ichthyosis vulgaris, senile hyperkeratosis, and acanthosis, using lipophilic ointments containing 0.1–1.0% retinoic acid. He observed minimal pharmacological activity without irritation in ichthyosis, using an ointment containing 0.01% retinoic acid. He found neither activity nor irritation using a 0.01% alcoholic solution.

Subsequently, much new information has been added. Possible effectiveness in Darier's disease (2, 3), keratoacanthomas (4), acne (2, 5–8), psoriasis (2, 9), congenital ichthyosiform erythroderma (2, 10), zosteriform keratosis (11), lamellar ichthyosis (12), and wound healing (13) has been reported. Of special interest are the reports on the treatment of seborrheic conditions such as acne, in which hydrophilic vehicles are preferred, and of ichthyosiform conditions, in which hydrophobic vehicles are preferred.

From the early reports that discuss vehicles and retinoic acid concentration in relation to irritation, it was hypothesized that irritation is probably directly related to retinoic acid activity and that a desirable "activity" level would be mild erythema with little or no scab formation. Thus, screening of vehicles and evaluation of concentration effects could be determined effectively in animal skin irritation tests. Ichthyosiform dermatoses can best be treated with lipophilic vehicles that partially occlude the area, render the skin more pliable, and offer physical protection. The skin barrier is not intact due to the disease; to prevent rapid penetration and to achieve a sustained local effect, retinoic acid should be relatively insoluble in the vehicle. However, seborrheic dermatoses (especially acne) can best be treated with hydrophilic vehicles that tend to remove excess sebum, impart a desirable drying effect, and enhance retinoic acid absorption. This can be accomplished using a vehicle that penetrates well and also dissolves retinoic acid well. Thus, two different types of vehicle are required for broad clinical evaluation.

As a result of these considerations, a series of experiments was planned. The objectives were: (*a*) to determine the purity and stability of retinoic acid and its solubility in various materials that could be used in dermatological formulations; (*b*) to choose the materials in which the retinoic acid showed greater solubility for possible use as hydrophilic vehicles and those in which it showed lesser solubility for use as hydrophobic vehicles; (*c*) to formulate o/w and w/o test vehicles; and (*d*) to carry out a pharmacological screening study to assist in selecting optimal formulations containing sufficient retinoic acid to produce a desirable, mild irritation without undesirable edema and scab formation. The solvents and the formulations used are identified below.

EXPERIMENTAL

Chemicals—All of the commercially available isomers of retinoic acid were investigated, and all-*trans*-retinoic acid[1] was chosen for these studies. This is the most readily available form and has been used in some reported clinical studies. Of course, the activity of physical forms of all-*trans*-retinoic acid other than that used and of other isomers and combinations of isomers may be different than that chosen, so these results will not necessarily be representative of all forms.

The purity of all-*trans*-retinoic acid was verified by UV, IR, differential scanning calorimetry, TLC, and column chromatography, and good evidence of at least 95% purity was obtained.

Sherman (14) demonstrated unusually good stability for an all-*trans*-retinoic acid solution in petroleum ether subjected to UV light. The sclution also showed good stability in various other organic solvents when stored in the dark at room temperature for up to 3 months. These results have been verified in the authors' laboratory for some solvents and for the formulations reported here.

Solubility—Retinoic acid solubility in potential ointment vehicles was determined, and it was found that higher molecular weight liquid alkanols and polyethylene glycol ethers of these alkanols were relatively good solvents, but that fatty esters, cholesterol esters, and hydrocarbons were relatively poor. Representative room temperature solubilities (percent w/w) are as follows: lauryl alcohol[2], 2.5; 2-octanol, 1.8; ethoxylated lauryl alcohol[3], 1.5; polyethylene glycol 400, 0.5; almond oil, 0.2; isopropanol, isopropyl myristate, and acetylated cholesterols[4], 0.2; and mineral oil, 0.008. A supersaturated solution (0.04 at room temperature) was obtained by incorporating retinoic acid in liquid petrolatum at 58°.

Formulation—Using this solubility information, an o/w cream (Formula A) in which retinoic acid is relatively soluble was formu-

[1] Eastman Kodak Co.
[2] Loral 5, Du Pont.
[3] Brij 30, Atlas.
[4] Acetulan, American Cholesterol Products, Inc.

Table I—Identification of Formulas

Treatment	Percent Retinoic Acid	Description of Vehicle
1	0.2000	Formula B[a] (w/o, mineral oil)
2	0.2000	Formula B[b] (w/o, mineral oil)
3	0.2000	Formula C[a] (w/o, almond oil)
4	0.2000	Formula C[b] (w/o, almond oil)
5	0.2000	Formula A (o/w, lauryl alcohol)
6	0.0500	Formula A (o/w, lauryl alcohol)
7	0.0100	Formula A (o/w, lauryl alcohol)
8	0.0500	Mineral oil
9	0.2000	Mineral oil
10	0.0100	Almond oil
11	0.0500	Almond oil
12	0.2000	Almond oil
13	0.0100	Lauryl alcohol
14	0.0500	Lauryl alcohol
15	0.2000	Lauryl alcohol
16	0.0500	Formula D (o/w, hexadecyl alcohol)
17	0.0000	Formula A (o/w, lauryl alcohol)
18	0.0001	Formula A (o/w, lauryl alcohol)
19	0.0010	Formula A (o/w, lauryl alcohol)

[a] Retinoic acid incorporated cold. [b] Retinoic acid incorporated hot.

lated. It contained the following ingredients (percent w/w): laury[1] alcohol, 2; ethoxylated lauryl alcohol, 3; propylene glycol, 5; stearyl alcohol, 4; ethoxylated stearic acid[5], 3; glyceryl monostearate, 4; silicone 500 CST[6], 2; and water *qs*. The first three ingredients, based upon the solubility data, apparently contributed most of the solubility characteristics of the cream. For the w/o ointment (Formula B), in which retinoic acid is relatively insoluble, cold cream USP XVII containing spermaceti, white wax, mineral oil, sodium borate, and water were used. None of these ingredients appeared to contribute appreciably to retinoic acid solubility. A w/o cold cream formula, substituting almond oil for mineral oil, was also prepared (Formula C). The ointments were milled so that retinoic acid crystals were reduced to a mean size of about 50 μ. An alternate o/w cream (Formula D), similar to Formula A but containing 5% hexadecyl alcohols[7] instead of lauryl alcohol and ethoxylated lauryl alcohol, was also evaluated. The chemical and physical stabilities of these formulations were determined to be adequate for testing purposes. The formulas are identified in Table I.

Pharmacological Screening—Preliminary work indicated that the albino rabbit would be an acceptable model. For Experiment 1, New Zealand White male rabbits were selected and were equilibrated with plastic collars. It was planned to evaluate 16 formulations (Table I), and 16 rabbits were randomly selected for testing from a larger group. The experimental design was a Youden "square," having six rows and 16 columns, with rows representing positions on each animal's back and columns representing individual rabbits. A unique subset of six of the 16 formulations was to be tested on the six sites on each rabbit's back. This Youden square design has the characteristic that every formulation is tested an equal number of times (six), and every formulation appears together with every other one an equal number of times (twice). The subsequent statistical analysis of the data, described below, ensured that treatment means would be adjusted to isolate the influences of position (if any) and animal differences, so that treatments could be compared in an unbiased fashion.

The dorsal-lateral hair was removed from the backs of the 16 animals by careful clipping on the day before the test began. Tattoos were applied to designate test areas on each back. A template providing six 3.8 × 7.6-cm. (1.5 × 3.0-in.) sites, with 3.8 cm. (1.5 in.) or more between each, was used to locate the six positions. On the 1st day of test, a 100-mg. quantity of test ointment or liquid was applied to each site, using a smooth polyethylene spatula to distribute the test material uniformly. The order of treating the 16 sites on each rabbit's back was randomized, and for each subsequent application, new randomizations were used. Treatments 1–16 were used (Table I).

[5] Myrj 52, Atlas.
[6] Dow.
[7] Enjay.

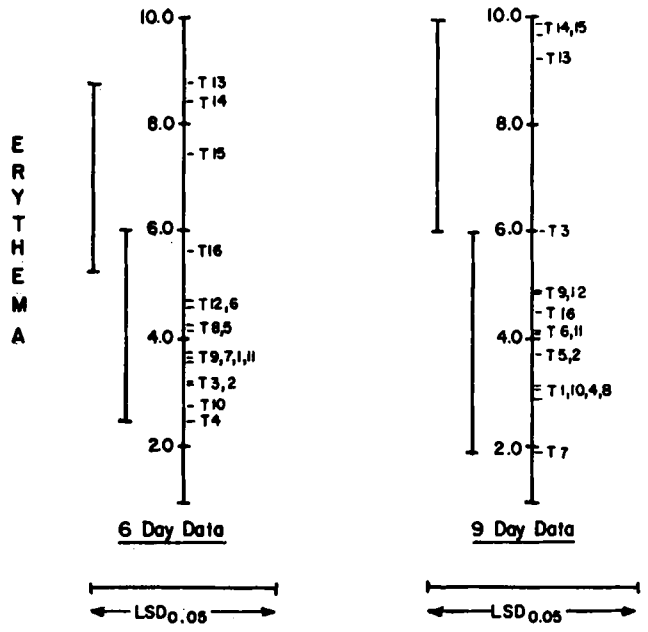

Figure 1—*Tukey multiple-comparison tests for all two-treatment erythema comparisons of the first 16 treatments listed in Table I. Mean scores are arranged in rank order. $LSD_{0.05}$ values are plotted on the same scale.*

The six sites on each back were treated daily for 10 days. Prior to each daily application, each treated area was carefully observed, again in a random order and with the observer having no knowledge of the identity of the formulation used at each site. One observer scored each site for erythema each day in this way, using a scale of 1–12 (1 = "no erythema," 12 = "maximum erythemal response"). Edema was scored similarly and separately by the same observer.

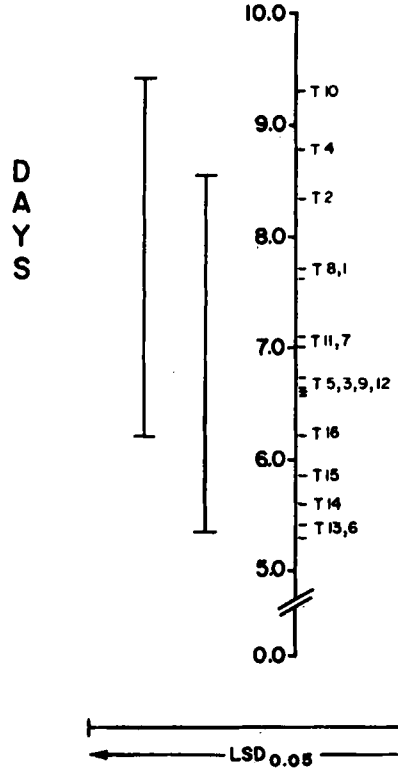

Figure 2—*Tukey multiple-comparison tests for all two-treatment scab formation time comparisons of the first 16 treatments listed in Table I. Mean scores are arranged in rank order. $LSD_{0.05}$ values are plotted on the same scale.*

Table II—Mean Erythema Scores for Formula A (o/w, Lauryl Alcohol)

Treatment	Retinoic Acid Concentration %	Retinoic Acid Concentration p.p.m.	Means[a] Day 3	Means[a] Day 6	Means[a] Day 9
17	0.0000	0	1.81	1.88	1.84
18	0.0001	1	1.91	1.84	1.78
19	0.0010	10	2.56	2.31	2.00
7	0.0100	100	3.06	2.75	2.63

[a] Lowest score (1) = no erythema and highest score (5) = mild erythema.

Table III—Significance Levels (Tukey Tests) for Erythema Scores in Table II

Formula A Concentrations Compared, p.p.m.	Significance Level ($p < 0.10$) Day 3	Day 6	Day 9
(0)—(1)	n.s.	n.s.	n.s.
(0)—(10)	<0.01	<0.01	n.s
(0)—(100)	<0.01	<0.01	<0.05
(1)—(10)	<0.05	<0.01	n.s.
(1)—(100)	<0.01	<0.01	<0.01
(10)—(100)	n.s	<0.05	<0.10

Eschar formation was checked daily for 10 days and recorded so that scabbing formation time could be determined by adopting the convention that there was scab if it was observed in two successive days, in which case the elapsed time to formation was taken as that on the first of the 2 days. Independent statistical analyses of the erythema scores obtained on Days 3, 6, and 9 were done. These three intervals were specified *a priori*. Edema scores did not appear to contribute much information and were not analyzed. Figure 1 shows the results of the comparison of means of erythema score data using computed least-significant difference values[8] for all treatments on Days 6 and 9. Figure 2 shows the results of the comparison of means of scabbing time data using computed least-significant difference values for all treatments.

Experiment 2 was done in a similar manner, but the design provided for the use of all treatments on each animal. In this experiment, four concentrations of Formula A were studied (0, 1, 10, and 100 p.p.m.), and only four sites were used on each rabbit. Since the erythemal scores were expected to occur predominantly at the low end of the original 12-point scale and the observer was sufficiently experienced to score smaller differences, the precision of the measurement was increased by a fourfold expansion of the lower portion of the scale used previously (between 1 and 5). Edema and scabbing time were not considered important to this experiment and were not reported. The same observer scored the results in both experiments. A 4 × 4 Latin square design was used with all four levels of retinoic acid applied to each animal. The Latin square was replicated once; therefore, eight animals were used. The formulas, Treatments 7, 17, 18, and 19, are reported in Table I. The mean erythema scores are reported in Table II. Tukey HSD tests for all possible combinations are reported in Table III.

RESULTS AND DISCUSSION

The data from Experiment 1 were analyzed after replacing missing values by least squares (there were four missing values). Then a residual analysis was done to check the effectiveness of the randomizations and to test the adherence of the data to the assumptions underlying the analysis. Treatment means adjusted for block effects were then computed, and an independent analysis of variance was done on the data from each of the 3 days chosen for analysis. There was a significant effect ($p < 0.05$) for treatments on Day 3 and a highly significant effect ($p < 0.01$) on Days 6 and 9. Scab formation time data were analyzed similarly, showing a highly significant effect for treatments ($p < 0.001$) and a significant effect for positions ($p < 0.05$).

Tukey multiple-comparison tests were done on all two-treatment comparisons for erythema for Days 6 and 9 and are shown in Fig. 1. The means of erythema scores are arranged in rank order, with the Tukey least-significant difference ($p = 0.05$) plotted alongside them on the same scale.

These comparisons show that Treatments 13, 14, and 15 were highest of all treatments on the irritation scale for Experiment 1. Since they contained very different quantities of retinoic acid but used lauryl alcohol exclusively as the vehicle, the data suggest that the alcohol contributed strongly to the irritation observed. In view of the literature, however, it was believed that retinoic acid itself is an irritant; therefore, it was hypothesized that a strong irritating effect of lauryl alcohol was masking differences in irritation due to retinoic acid concentration. The results of Experiment 2 tended to verify this hypothesis.

The poor solvents that were tested (mineral oil and almond oil) either as retinoic acid suspensions or as ointments containing 0.2–0.01% retinoic acid appeared predominantly at the low end of the irritation scale, as desired.

As mentioned, the edema scores were not sufficiently sensitive to give much additional information and are not reported.

Figure 2 presents the means of scabbing times arranged in rank order, with the slowest scabbing time being highest in the order, and the Tukey least-significant difference plotted alongside them on the same scale. These results reinforce the observation that the poor solvents gave low erythema scores.

A Spearman Rank Correlation test, done to investigate the relationship between erythema scores and scab formation time on each of the 3 days, showed rank correlation coefficients of −0.85 and −0.79 for Days 6 and 9, respectively, significant at $p < 0.01$ in both cases. These results show that either response is a reasonably good predictor of the other.

Experiment 2 was designed to investigate the dose–response effect of retinoic acid at lower concentrations (0, 1, 10, and 100 p.p.m.) in Formula A. Residual analyses were carried out, and conventional analyses of variance for Latin squares were done separately for Days 3, 6, and 9 for erythema scores only. Neither edema scores nor scabbing time observations were useful measurements at these low irritation levels. The effect of concentration was strongly significant in all cases—*viz.*, on Days 3 and 6 ($p < 0.001$) and on Day 9 ($p < 0.01$). The mean erythema scores are reported in Table II. Following the ANOVA's, Tukey least-significant difference tests were done for all pairs of concentrations for each of the 3 days. The results are shown in Table III. The table indicates that the concentrations chosen were on an effective portion of the dose–response curve and that a significant difference could be found in comparing all possible combinations except the lowest (0.0001), which was not different from zero.

By assuming that irritation does reflect retinoic acid activity, then the choice of the vehicle and the availability of retinoic acid in that vehicle must be optimized for the intended drug use.

It was concluded that, when extending these results to human clinical work, it would be advisable to treat ichthyosiform dermatoses with w/o emulsions in which retinoic acid is relatively insoluble and preferably at concentrations of 0.10% or more. For treating acneiform or seborrheic dermatoses, the preferable form would be o/w emulsions in which the solubility of retinoic acid is relatively high and preferably at concentrations of 0.05% or less.

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Alterations in Absorption of Dicumarol by Various Excipient Materials

M. J. AKERS*, J. L. LACH*, and L. J. FISCHER†▲

Abstract □ A plasma level study was conducted in dogs to determine the effects of various excipient materials on the absorption of dicumarol. The drug was combined with the excipients by an equilibration process and administered orally. Plasma concentrations of dicumarol after administration with excipients were compared to control levels produced by the drug given alone. Significant differences in the plasma levels of dicumarol were observed with six of the 10 excipients used in the study. Significantly higher plasma levels (up to 180% of control values) were observed when dicumarol was administered with magnesium oxide or hydroxide. This effect may be due to chelate formation because the magnesium chelate of dicumarol produced higher plasma levels of dicumarol than the drug administered alone. Dicumarol administration with talc, colloidal magnesium aluminum silicate, aluminum hydroxide, or starch resulted in significantly lower plasma levels of the drug. It is suggested that these types of interactions may be an explanation for differences in the bioavailability of dicumarol from different dosage formulations.

Keyphrases □ Dicumarol absorption—effect of various excipients on plasma levels, dogs □ Absorption, dicumarol—effect of various excipients on plasma levels, dogs □ Plasma levels, dicumarol—effect of various excipients, dogs □ Excipient effect—dicumarol absorption, plasma levels, dogs □ Bioavailability, dicumarol—effect of various excipients on plasma levels, dogs □ Magnesium oxide, hydroxide excipients—effect on dicumarol absorption, dogs

Substantial evidence is available to show that the absorption characteristics and, ultimately, the therapeutic performance of a drug can be significantly altered by changes in the materials and methods used in its formulation. Wagner (1) recently provided a review of studies involving the determination of generic equivalency or inequivalency of different commercial brands of a single drug. Ten of the 12 drugs studied showed significant differences in the bioavailability of drug when two or more commercial brands of the same drug were compared.

Lach and his coworkers (2–4), through diffuse reflectance spectroscopic studies, demonstrated that some adjuvants used in product formulation interact with certain drugs to form complexes. These combinations exhibited markedly different diffuse reflectance spectroscopic characteristics compared to those of the drug alone. The oral anticoagulant dicumarol was shown to interact with various organic and inorganic formulation materials, and it was suggested that these types of interactions may alter the bioavailability of the drug (3). Dicumarol has been shown to be poorly absorbed (5), and its bioavailability may be particularly susceptible to formulation changes. Losinski (6) reported that formulation changes involving the addition or reduction of inert fillers in tablets of dicumarol resulted in significant therapeutic variations.

The purpose of this study was to investigate whether the absorption of dicumarol could be altered by administration of the drug in combination with various formulation materials. The characteristics of its absorption and elimination, coupled with a pharmacological response dependent on plasma concentration, permitted the use of dicumarol to show that drug–excipient interactions may be an explanation for differences in therapeutic efficacy among different dosage formulations.

EXPERIMENTAL

Materials—Dicumarol[1] was recrystallized from dioxane (m.p. 288–289°). Other materials used were: magnesium oxide, heavy[2]; magnesium hydroxide, reagent grade[2]; magnesium stearate USP; dibasic calcium phosphate NF, hydrous[2]; aluminum hydroxide powder[2]; colloidal magnesium aluminum silicate[4]; silicic acid[5]; starch USP; talc USP; polyvinylpyrrolidone[6]; and polysorbate 80[7].

Elemental analysis of the magnesium chelate of sodium dicumarol[8] showed it to have the form $(dicumarol\text{-}Na)_2Mg$. A method for the preparation of the chelate was reported previously (4).

Animals—Eight healthy mongrel dogs of either sex, weighing 10–21 kg., were utilized in the study. The animals were housed in stainless steel cages and fed dog chow[9] between 8 a.m. and noon daily, unless specified otherwise.

Dicumarol–Excipient Mixtures—Dicumarol was mixed with an excipient in two different weight to weight ratios—6 dicumarol:94 excipient and 1 dicumarol:1 excipient. Dicumarol was dissolved in chloroform to make a solution containing 0.3 g. in 50 ml. An appro-

[1] K and K Laboratories.
[2] Mallinckrodt.
[3] Matheson, Coleman and Bell.
[4] Veegum, regular, Vanderbilt.
[5] Fisher.
[6] Antara Chemicals.
[7] Tween 80, Atlas.
[8] A gift from Dr. L. Bighley, College of Pharmacy, University of Iowa.
[9] Purina.

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Alterations in Absorption of Dicumarol by Various Excipient Materials

M. J. AKERS*, J. L. LACH*, and L. J. FISCHER†▲

Abstract □ A plasma level study was conducted in dogs to determine the effects of various excipient materials on the absorption of dicumarol. The drug was combined with the excipients by an equilibration process and administered orally. Plasma concentrations of dicumarol after administration with excipients were compared to control levels produced by the drug given alone. Significant differences in the plasma levels of dicumarol were observed with six of the 10 excipients used in the study. Significantly higher plasma levels (up to 180% of control values) were observed when dicumarol was administered with magnesium oxide or hydroxide. This effect may be due to chelate formation because the magnesium chelate of dicumarol produced higher plasma levels of dicumarol than the drug administered alone. Dicumarol administration with talc, colloidal magnesium aluminum silicate, aluminum hydroxide, or starch resulted in significantly lower plasma levels of the drug. It is suggested that these types of interactions may be an explanation for differences in the bioavailability of dicumarol from different dosage formulations.

Keyphrases □ Dicumarol absorption—effect of various excipients on plasma levels, dogs □ Absorption, dicumarol—effect of various excipients on plasma levels, dogs □ Plasma levels, dicumarol—effect of various excipients, dogs □ Excipient effect—dicumarol absorption, plasma levels, dogs □ Bioavailability, dicumarol—effect of various excipients on plasma levels, dogs □ Magnesium oxide, hydroxide excipients—effect on dicumarol absorption, dogs

Substantial evidence is available to show that the absorption characteristics and, ultimately, the therapeutic performance of a drug can be significantly altered by changes in the materials and methods used in its formulation. Wagner (1) recently provided a review of studies involving the determination of generic equivalency or inequivalency of different commercial brands of a single drug. Ten of the 12 drugs studied showed significant differences in the bioavailability of drug when two or more commercial brands of the same drug were compared.

Lach and his coworkers (2–4), through diffuse reflectance spectroscopic studies, demonstrated that some adjuvants used in product formulation interact with certain drugs to form complexes. These combinations exhibited markedly different diffuse reflectance spectroscopic characteristics compared to those of the drug alone. The oral anticoagulant dicumarol was shown to interact with various organic and inorganic formulation materials, and it was suggested that these types of interactions may alter the bioavailability of the drug (3). Dicumarol has been shown to be poorly absorbed (5), and its bioavailability may be particularly susceptible to formulation changes. Losinski (6) reported that formulation changes involving the addition or reduction of inert fillers in tablets of dicumarol resulted in significant therapeutic variations.

The purpose of this study was to investigate whether the absorption of dicumarol could be altered by administration of the drug in combination with various formulation materials. The characteristics of its absorption and elimination, coupled with a pharmacological response dependent on plasma concentration, permitted the use of dicumarol to show that drug–excipient interactions may be an explanation for differences in therapeutic efficacy among different dosage formulations.

EXPERIMENTAL

Materials—Dicumarol[1] was recrystallized from dioxane (m.p. 288–289°). Other materials used were: magnesium oxide, heavy[2]; magnesium hydroxide, reagent grade[2]; magnesium stearate USP; dibasic calcium phosphate NF, hydrous[2]; aluminum hydroxide powder[2]; colloidal magnesium aluminum silicate[4]; silicic acid[5]; starch USP; talc USP; polyvinylpyrrolidone[6]; and polysorbate 80[7].

Elemental analysis of the magnesium chelate of sodium dicumarol[8] showed it to have the form $(dicumarol\text{-}Na)_2Mg$. A method for the preparation of the chelate was reported previously (4).

Animals—Eight healthy mongrel dogs of either sex, weighing 10–21 kg., were utilized in the study. The animals were housed in stainless steel cages and fed dog chow[9] between 8 a.m. and noon daily, unless specified otherwise.

Dicumarol–Excipient Mixtures—Dicumarol was mixed with an excipient in two different weight to weight ratios—6 dicumarol:94 excipient and 1 dicumarol:1 excipient. Dicumarol was dissolved in chloroform to make a solution containing 0.3 g. in 50 ml. An appro-

[1] K and K Laboratories.
[2] Mallinckrodt.
[3] Matheson, Coleman and Bell.
[4] Veegum, regular, Vanderbilt.
[5] Fisher.
[6] Antara Chemicals.
[7] Tween 80, Atlas.
[8] A gift from Dr. L. Bighley, College of Pharmacy, University of Iowa.
[9] Purina.

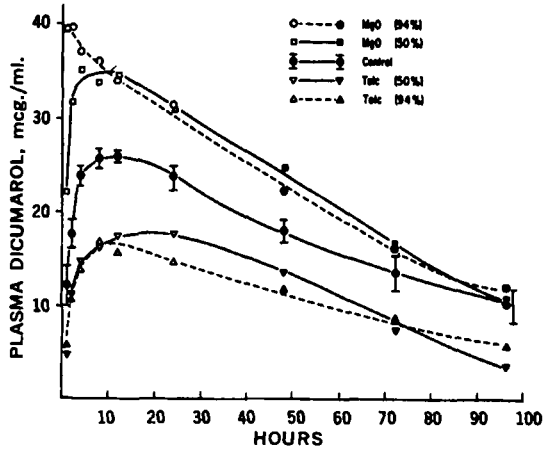

Figure 1—*Plasma concentrations of dicumarol after single oral doses of the drug given alone (control) or with various excipient materials. The values shown are the mean of four animals. Open data points indicate significant differences from control. Variation about the mean values was not statistically different from that of the control values which are shown with* SEM.

priate amount of excipient was added to the solution, and the mixture was stirred briefly by hand and allowed to stand in a glass-stoppered flask for 16 hr. The solvent was removed on a rotary evaporator, and the dry powder was collected after passing through a U. S. Standard No. 100 sieve.

Drug Administration—The dogs were fasted approximately 8 hr. before and 24 hr. after drug administration. Water was allowed at all times during the experiments. Dicumarol alone or the dicumarol–excipient mixtures were administered as aqueous suspensions by gastric intubation using a soft rubber catheter. The suspension was prepared by stirring the required amount of drug with 1–2 ml. of distilled water containing 0.05% polysorbate 80. Additional distilled water was added to bring the volume to 25 ml. and the suspension was immediately administered to the animal. A wash of 50 ml. of distilled water followed the dose to ensure quantitative administration.

The dose of the magnesium chelate of dicumarol was prepared in an identical fashion. In one dog, an intravenous dose of 5 mg./kg. of dicumarol was given after the animal had repeatedly received the same dose of drug orally. The vehicle used was 5 ml. of a dilute sodium hydroxide solution.

Blood Samples—Blood samples (7 ml.) were withdrawn by venipuncture and collected in heparinized Vacutainers[10]. A sample was obtained just prior to dosage administration, and subsequent samples were obtained 1, 2, 4, 8, 12, 24, 48, 72, and 96 hr. after the dose. Starting at 12 hr. after the dose, an additional blood sample (2.7 ml.) was withdrawn at each sampling time in Vacutainers containing sodium citrate for prothrombin time measurements. Heparinized plasma was stored at −20° prior to dicumarol analysis.

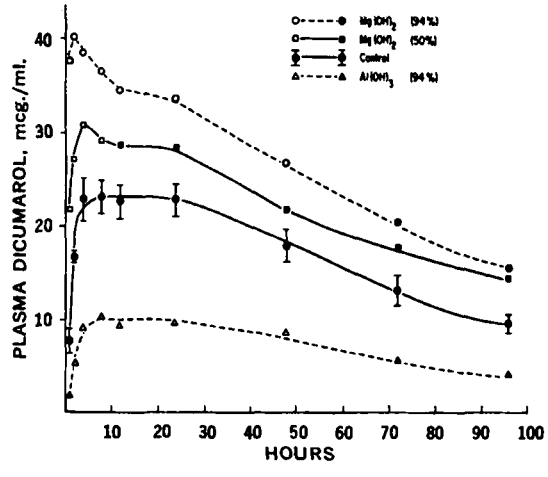

Figure 2—*Same as Fig. 1.*

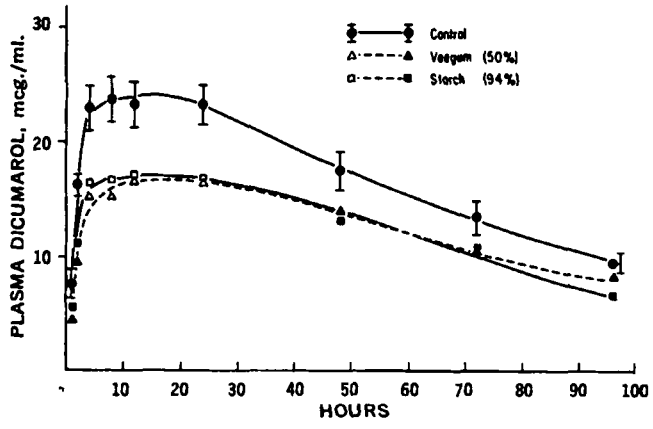

Figure 3—*Same as Fig. 1.*

Analysis of Plasma—Plasma samples were analyzed for dicumarol following the spectrophotometric procedure of Axelrod *et al.* as revised by Nagashima *et al.* (7). Plasma samples were analyzed by the same method after oral administration of the magnesium chelate of dicumarol. The UV absorbance maximum and molar absorptivity of the chelate in 2.5 *N* NaOH were the same as those for dicumarol. Analysis of dog plasma containing known amounts of the magnesium chelate of dicumarol produced an identical standard curve when compared to dicumarol.

Prothrombin Time Measurements—The one-stage method of Quick (8) was used on fresh plasma. Measurements were made on a BBL Fibrometer[11] using rabbit brain thromboplastin[11].

Pharmacokinetic Analysis—Absorption and elimination rate constants were obtained by submitting plasma level data to a nonlinear regression analysis[12]. Areas under the individual animal plasma level curves (0–96 hr.) were calculated using the trapezoidal technique. Areas from 96 hr. to infinity were calculated by the usual method (9), using the elimination rate constant determined by linear regression analysis of the logarithms of the 48-, 72-, and 96-hr. plasma dicumarol concentrations.

Experimental Design—Plasma levels of dicumarol after an oral dose of the drug in a mixture with excipient were compared to those resulting from oral administration of dicumarol powder. To reduce interanimal variation in plasma levels, a recognized characteristic of dicumarol administration (5), the dose of the drug used in each dog was that which produced a peak plasma level of 20–25 mcg./ml. after an oral dose of dicumarol powder. On the basis of the results of a preliminary test dose of dicumarol, the animals received 4, 5, or 7 mg./kg. of the drug. Each of the animals received three control doses (pure dicumarol powder) and from seven to 10 different dicumarol–excipient mixtures. A control dose was given first to each animal, and the subsequent control and dicumarol–excipient doses were given in a completely randomized manner. Blood samples were taken at selected intervals after each dose for plasma dicumarol and prothrombin time analyses. There was at least a 2-week interval between doses of the drug, during which time the drug plasma levels fell to undetectable levels and the prothrombin times returned to normal.

Statistical Analysis—The following parameters were analyzed statistically: plasma concentrations, areas under the plasma level curves, and peak prothrombin responses. The control values for each animal were the averages obtained from three doses of pure dicumarol given at different times. Mean parameters were computed from the data obtained from four dogs. Each dog in a group of four dogs received the same complement of dicumarol–excipient mixtures and three control doses. The means were compared by a one-way analysis of variance randomized complete block design, and statistical

[10] Becton-Dickinson.

[11] Bioquest Laboratories.

[12] Using the IBM-360 computer and the NONLIN program of Dr. Carl Metzler, The Upjohn Co.

Table I—Control Dicumarol Plasma Levels for Each Dog Used in the Study

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 6	Dog 7	Dog 8	Dog 9
Sex	M	M	M	M	M	M	M	F
Weight, kg.	10	11	18	20	17	22	21	20
Dose, mg./kg.	4	7	7	4	5	5	5	5

Hours	Mean (*SE*) Concentration of Plasma Dicumarol, mcg./ml. Dog 1	Dog 2	Dog 3	Dog 4	Dog 6	Dog 7	Dog 8	Dog 9
1	14.5[a]	16.5[a]	10.1 (2.4)	9.8 (0.6)	5.6 —	6.1 (1.5)	5.2 (1.3)	11.3 (4.0)
2	20.2 (1.0)	19.4 (1.5)	17.7 (0.6)	15.2 (0.3)	16.8 (1.8)	13.3 (1.8)	17.2 (4.5)	17.8 (4.0)
4	21.9[a]	23.1[a]	25.8 (1.3)	16.5 (0.8)	26.0 (1.2)	25.4 (1.2)	23.5 (1.0)	24.9 (3.0)
8	24.6 (0.4)	24.2 (1.7)	25.9 (1.8)	17.8 (1.0)	25.2 (0.9)	26.3 (1.1)	23.8 (0.4)	27.4 (4.9)
12	24.9 (0.5)	25.5 (0.8)	25.7 (1.6)	17.1 (0.7)	24.6 (1.5)	27.9 (1.8)	23.2 (0.4)	25.2 (4.6)
24	20.4 (1.0)	22.4 (0.5)	27.3 (1.5)	18.2 (2.7)	18.2 (2.7)	24.4 (1.7)	26.9 (3.0)	24.8 (3.8)
48	17.8 (0.7)	14.6 (0.6)	21.6 (1.2)	12.6 (1.3)	17.7 (0.6)	20.6 (2.3)	19.4 (0.4)	18.8 (1.8)
72	13.4 (0.5)	8.1 (1.5)	16.6 (1.0)	9.4 (1.0)	12.5 (0.4)	17.7 (2.1)	14.5 (0.3)	14.6 (1.4)
96	12.4 (0.4)	4.6 (1.0)	11.6 (1.5)	7.0 (1.7)	8.9 (0.8)	13.4 (1.4)	10.6 (0.0)	10.5 (1.4)

[a] Only one value at this time. The rest of the values are the mean of three experiments.

differences from control values were determined by the least significant difference method (10).

RESULTS

Reproducibility of Dicumarol Plasma Levels—Considerable interanimal variation was observed in dicumarol plasma levels after identical oral doses to dogs. Because of this, the oral dose of dicumarol necessary to produce a standard (control) level from which alterations in drug absorption could be evaluated was determined in preliminary experiments on each dog. Adequate plasma levels and prothrombin time responses were produced with oral doses ranging from 4 to 7 mg./kg. Each dog was given the same dose of dicumarol throughout the experiments. Plasma levels of dicumarol after an oral dose were found to be reproducible in each animal. The mean plasma levels resulting from control doses of dicumarol powder administered to each dog at three different times throughout the study are shown in Table I. Analysis of the control plasma levels in each dog showed that time- or drug-dependent changes in dicumarol absorption and elimination did not occur in these experiments. The reproducibility of the control plasma levels of dicumarol is indicated by the low standard errors of the mean values. These mean values were used for comparison to plasma levels resulting when the drug was administered in combination with various excipient materials.

Dicumarol Plasma Levels after Oral Administration with Various Excipients—The mean plasma levels of dicumarol administered alone or in combination with various excipient materials are shown in Figs. 1–3. Significant increases in dicumarol plasma levels over control resulted when the drug was administered with magnesium oxide (Fig. 1). Slightly higher levels were observed with the mixture containing 94% magnesium oxide compared to the mixture containing equal weights of the drug and magnesium oxide. Significant decreases in dicumarol plasma levels were observed when the drug was administered with talc. Decreasing the proportion of talc in the mixture had little effect on the ability of this material to lower drug plasma levels (Fig. 1).

The results in Fig. 2 show that significant increases in dicumarol plasma levels resulted when the drug was given in a mixture containing either 94 or 50% magnesium hydroxide. In contrast, significant decreases in drug plasma levels were observed after administration of dicumarol in a mixture containing 94% aluminum hydroxide. No

Table II—Plasma Level Parameters and Prothrombin Time Responses following Oral Doses of Dicumarol in Combination with Various Excipient Materials[a]

Excipient	Percent of Excipient in Dose Mixture, % w/w	Peak Plasma Level Time, hr.	Dicumarol Concentration at Peak Level, mcg./ml.	Area under Plasma Level Curve, % Change from Control 0–96 hr.	0–∞ hr.	Maximum Prothrombin Time Response[b], sec.
Group I (Dogs 1, 2, 7, and 9)						
Control	0	8–12	25.8 (0.6)	—	—	16.3 (1.1)
Magnesium oxide	94	1–2	*39.6 (5.0)	*+31.4 (11.9)	+22.8 (17.5)	*23.3 (4.0)
Magnesium oxide	50	4	*35.1 (3.6)	*+30.0 (11.2)	+11.6 (20.4)	18.1 (2.1)
Talc	94	8	*16.9 (1.4)	*−37.0 (5.1)	*−45.7 (6.0)	14.1 (1.6)
Talc	50	12–24	*17.5 (1.0)	*−34.2 (4.7)	*−56.5 (3.0)	18.0 (2.3)
Silicic acid	94	8	26.4 (1.5)	−10.0 (12.2)	−1.3 (22.1)	15.0 (1.9)
Group II (Dogs 3, 4, 6, and 8)						
Control	0	8–12	23.1 (1.8)	—	—	14.7 (1.0)
Magnesium hydroxide	94	2	*40.1 (5.2)	*+56.6 (22.3)	*+71.0 (40.0)	*20.2 (1.8)
Magnesium hydroxide	50	4	*30.8 (3.6)	*+27.7 (18.6)	*+63.6 (33.1)	15.0 (1.3)
Aluminum hydroxide	94	8	*10.1 (1.7)	*−56.6 (5.2)	*−60.0 (5.6)	11.3 (1.1)
Aluminum hydroxide	50	8	25.1 (1.2)	+5.4 (12.5)	+12.2 (23.4)	14.4 (1.6)
Magnesium stearate	94	4	30.1 (5.1)	+10.4 (20.0)	+11.9 (21.3)	15.7 (1.6)
Colloidal magnesium aluminum silicate	50	12–24	*16.7 (3.3)	*−23.7 (13.2)	+1.1 (31.5)	11.7 (0.5)
Calcium phosphate	94	8	22.1 (2.1)	*−26.2 (5.0)	−28.5 (8.3)	14.7 (1.7)
Group III (Dogs 4, 6, 7, and 9)						
Control	0	8–12	24.1 (2.3)	—	—	15.8 (2.9)
Starch	94	4–24	*16.8 (2.5)	*−25.0 (8.2)	−30.9 (4.3)	15.7 (3.3)
Group IV (Dogs 3, 4, 6, and 7)						
Control	0	8–12	24.2 (2.0)	—	—	15.1 (2.4)
Polyvinylpyrrolidone	50	12–24	27.2 (2.5)	−19.0 (4.0)	+12.3 (17.2)	17.3 (2.6)

[a] Values are mean (*SE*) for four dogs; asterisks (*) indicate a significant difference from control, $p < 0.05$. [b] Normal prothrombin times were not subtracted. Range of normal values was 7.3–8.5 sec.

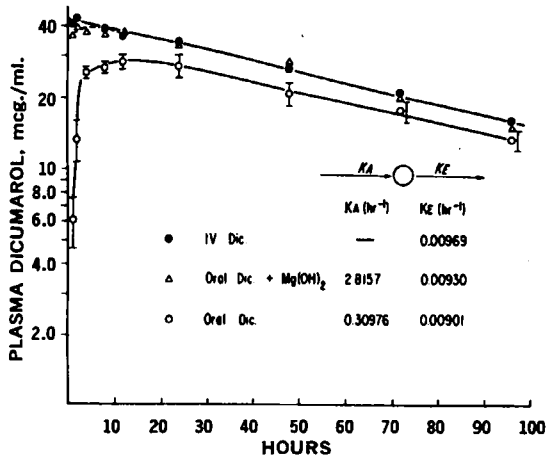

Figure 4—*Semilogarithmic plots of plasma dicumarol concentrations after identical doses of the drug were given intravenously* (●), *orally alone* (○), *or orally with 94% magnesium hydroxide* (△) *to Dog 7. First-order rate constants for absorption and elimination were determined from a nonlinear regression analysis as described in* Methods.

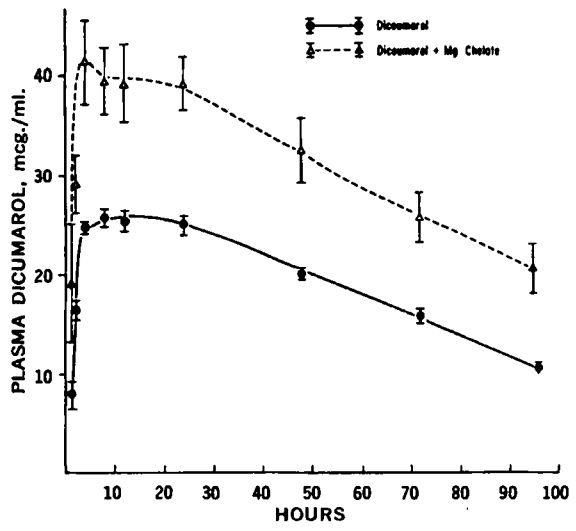

Figure 5—*Mean plasma concentrations of dicumarol after oral administration of the drug or its magnesium chelate to Dogs 3, 4, 8, and 9. Open data points are statistically different from the corresponding values at the same time.*

difference in the dicumarol plasma levels was observed when the drug was administered as a 50% mixture with aluminum hydroxide (Table II). It is evident from the results presented in Fig. 3 that the combination of dicumarol with starch or colloidal magnesium aluminum silicate resulted in lower plasma levels of the drug compared to those resulting from administration of the drug alone.

It can be observed in Figs. 1–3 that the postabsorptive decline in dicumarol plasma levels was similar whether the drug was given alone or with excipients. This suggests that the differences in plasma levels observed were due to absorption of the drug and not to its elimination from the plasma.

Dicumarol administered in combination with several other excipients did not alter the dicumarol plasma levels compared to those produced by the drug given alone. These results are not given in the figures, but the pertinent parameters measured are presented in Table II. The excipients that did not alter dicumarol absorption were silicic acid, magnesium stearate, dibasic calcium phosphate, and polyvinylpyrrolidone.

Comparison of Bioavailability Parameters—Table II presents data commonly used to evaluate bioavailability. Both magnesium oxide and magnesium hydroxide increased the peak plasma concentration of dicumarol in addition to shortening the time necessary to reach the peak levels. The time for peak plasma concentrations to be reached was reduced from 8–12 hr. after administration of the control doses to 1–4 hr. when the magnesium oxide or magnesium hydroxide combinations were administered. Peak plasma levels of dicumarol were increased by as much as 80% compared to controls when the drug was combined with these agents. If it is assumed that these agents do not alter the elimination of the drug from the plasma, these parameters suggest that magnesium oxide or hydroxide increased the absorption rate of dicumarol.

Table II presents the effects of the excipient materials on the areas under the dicumarol plasma level curves. Magnesium oxide and hydroxide, which were observed to increase the peak plasma concentrations of dicumarol compared to control, also increased the area under the 0–96-hr. plasma level curve. A significant increase was observed for magnesium hydroxide but not for magnesium oxide when the 0–∞ areas were compared to control. This indicates that magnesium hydroxide may increase the total amount of drug absorbed.

The rapidity of the absorption of dicumarol when given in a mixture with magnesium oxide was evaluated in one dog by comparing the plasma levels after an oral dose of the mixture to that produced by an identical intravenous dose of the drug. The results (Fig. 4) show that shortly after an oral dose of dicumarol as a mixture containing 94% magnesium oxide, the plasma levels of the drug were nearly identical to those resulting from an intravenous dose. A pharmacokinetic analysis shows that the absorption rate constant (K_a) of dicumarol given with magnesium oxide was 2.8157 hr.$^{-1}$, which is nine times larger than the K_a value of 0.3098 hr.$^{-1}$ obtained when the drug was administered alone. The elimination rate constants (K_e) calculated from oral and intravenous doses to the dog were similar: 0.00901 and 0.00969 hr.$^{-1}$, respectively. Comparison of areas under the plasma level curves indicated that only 70% of a control dose of dicumarol was absorbed and that magnesium oxide increased bioavailability to near 100%.

Prothrombin times were measured after the administration of the anticoagulant either alone or combined with excipient materials. The maximal prothrombin time responses, which usually occurred at 48 hr. after the dose, are also listed in Table II. These values were increased by 50% over the control when dogs were given the drug–excipient combinations containing 94% magnesium oxide or hydroxide, indicating that these agents increased the pharmacological response.

Those excipients, which when combined with dicumarol significantly reduced drug plasma levels, generally delayed the time at which the peak plasma levels were attained. The time for the peak plasma level to be reached was extended from 8–12 hr. after administration of dicumarol alone to 12–24 hr. after a dose of the drug combined with talc (50%) or colloidal magnesium aluminum silicate and to 4–24 hr. after dicumarol was administered with starch. Although aluminum hydroxide did not delay the peak plasma level time, the mixture containing 94% aluminum hydroxide produced the greatest reduction in the concentration of dicumarol at the peak plasma level. This mixture reduced the peak plasma concentration to 44% of that produced by the drug given alone. Both talc and aluminum hydroxide (94%) significantly reduced the areas under the plasma level curves; it is suggested that these materials reduced the total amount of drug absorbed compared to control. Colloidal magnesium aluminum silicate and calcium phosphate showed a small reduction in the area under the 0–96-hr. blood level curve. This reduction was not apparent in the 0–∞ area value.

None of the dicumarol–excipient combinations that exhibited decreased absorption of the drug produced a statistically significant different prothrombin response. A trend existed, however, toward lower prothrombin times when the dicumarol was administered with aluminum hydroxide, talc, or colloidal magnesium aluminum silicate.

Absorption of Magnesium Chelate of Dicumarol—Since dicumarol has been shown to form chelates with metals, including magnesium, the role of chelation in the production of increased dicumarol plasma levels by magnesium oxide and hydroxide was investigated. The magnesium chelate of sodium dicumarol was administered to four dogs. Plasma concentrations of dicumarol resulting from dicumarol–magnesium chelate administration were compared to those produced by oral administration of sodium dicumarol to the same animals. The results (Fig. 5) indicate that the magnesium chelate produced significantly higher plasma levels of dicumarol. The plasma level curve of dicumarol after administration of the chelate was almost identical to that produced by administration of the drug

as a mixture with 94% magnesium hydroxide (Fig. 2). The mean $\pm SE$ peak prothrombin time after administration of the dicumarol–magnesium chelate was 21.7 ± 4.0 sec. This value is statistically larger than that observed after administration of dicumarol alone (15.1 ± 1.0 sec.), showing that the higher plasma levels of the drug resulting from the chelate also increased the biological effect of the drug.

DISCUSSION

The results presented indicate that certain excipient materials can dramatically alter the absorption of dicumarol. Both increases and decreases in the absorption of dicumarol were observed as a result of administering the drug with these materials. The well-recognized adsorbent properties of aluminum hydroxide (11) probably account for the reduced bioavailability of dicumarol when it was administered with this agent. Decreasing the proportion of aluminum hydroxide in the dose mixture to 50% eliminated its inhibiting effects on the absorption of dicumarol. This concentration effect was not seen with talc, which decreased dicumarol absorption at both drug concentrations tested. Thus, it may not be possible to assume in every case that an excipient effect on drug absorption can be reduced by lowering the amount of excipient given with the drug. Colloidal magnesium aluminum silicate and starch were only tested at one concentration, and no assessment of a concentration dependence on their inhibitory effects can be made. It is assumed that the adsorptive properties of these excipients coupled with their water insolubility are the chief characteristics that serve to decrease dicumarol absorption. The lack of effect of polyvinylpyrrolidone on dicumarol absorption was somewhat surprising in view of its recognized ability to complex drugs (12) including dicumarol (13).

Magnesium oxide and hydroxide enhance the absorption rate of dicumarol and also potentiate its anticoagulant action when combined with dicumarol in a mixture containing 94% excipient and 6% drug. Dicumarol is a weak organic acid; as such, it is considerably more soluble in alkaline solutions and is less readily absorbed from the acidic stomach contents (14). When the drug is administered with a basic substance such as magnesium oxide or hydroxide, it is possible that these excipients increase the rate of dissolution and allow a more rapid rate of absorption of the drug from the GI tract.

The administration of the magnesium chelate of dicumarol significantly increased the bioavailability of the drug. Comparison of dicumarol plasma levels produced by the chelate and those produced by the mixture with magnesium oxide or hydroxide show that they are similar except at the earliest sampling time. The mixture produced significantly higher dicumarol levels than the chelate 1 hr. after an oral dose. It is possible that magnesium oxide or hydroxide increases the absorption of dicumarol by a combination of factors: (*a*) increasing the pH of the microenvironment of the drug in the GI tract, thereby increasing the solubility and perhaps the dissolution rate; and (*b*) causing the formation of a dicumarol–magnesium chelate which is more readily absorbed. Administration of magnesium hydroxide 30 min. after a dose of dicumarol significantly increased plasma levels of the drug in dogs (unpublished observations). These results suggest that chelate formation may take place in the GI tract.

It is not known whether the chelate is absorbed as such or whether it releases dicumarol for absorption. The physical characteristics of the magnesium chelate that may explain how it increases plasma levels of dicumarol will be reported separately. Magnesium stearate did not increase the absorption of dicumarol. This material may be so insoluble in the GI fluids that magnesium was not available for chelate formation.

It can be speculated that the interactions between drug and excipient, which were observed in this study to alter the absorption of the therapeutic agent, could account for differences in drug bioavailability from various commercial formulations. The processes involved in tableting may even increase these interactions. Further experimentation is necessary, however, to determine whether the actual physical interactions involved are between drug and excipient or between the excipient and the biological system involved in the absorption of drugs.

Several reviews of drug interactions state that the concurrent administration of antacids reduces the absorption of weakly acidic coumarin anticoagulants (15, 16). The results reported here show that the antacid magnesium hydroxide can increase the absorption of dicumarol. Concurrent administration of milk of magnesia with dicumarol increased plasma levels of the anticoagulant in two dogs (unpublished observations). As a result of this study in dogs, the effects of concurrent administration with antacids on the absorption of dicumarol in man are being investigated.

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Compatibility of ^{14}C-Labeled Phenylephrine Hydrochloride in Polyethylene Nasal Spray Containers

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Abstract □ The interaction of phenylephrine hydrochloride with low density polyethylene containers was investigated using ^{14}C-labeled phenylephrine hydrochloride. In the first part of the study, nasal spray bottles with and without dip tubes containing 0.25 and 0.5% ^{14}C-phenylephrine hydrochloride were evaluated at temperatures of 7, 22, 40, and 50°. No losses of ^{14}C-phenylephrine hydrochloride were observed. Units stored at 40 and 50° showed apparent increases in concentration of the ^{14}C-phenylephrine hydrochloride. This reflected the loss of water from these units, the loss being confirmed by gravimetric data. This demonstrated a technique for the observation of solvent loss by means of a radioisotopic method. In the second part of the study, the influence of two preservatives, benzalkonium chloride (1:5000) and thimerosal (1:5000) on the phenylephrine hydrochloride–polyethylene container interaction was investigated. The data indicated apparent binding of phenylephrine hydrochloride to low density polyethylene by means of a preservative agent. Interaction was most significant at room temperature, with elevated temperature disrupting the apparent weak binding.

Keyphrases □ Phenylephrine hydrochloride, ^{14}C-labeled—interaction with low density polyethylene nasal spray containers, effect of preservatives □ Polyethylene nasal spray containers—interaction with ^{14}C-phenylephrine hydrochloride, effect of preservatives □ Drug–plastic interactions—^{14}C-phenylephrine hydrochloride and polyethylene nasal spray containers □ Nasal spray containers, polyethylene—interaction with ^{14}C-phenylephrine hydrochloride, effect of preservatives □ Preservatives, benzalkonium chloride and thimerosal—effect on binding of phenylephrine hydrochloride to polyethylene containers □ Plastic (polyethylene) nasal spray containers—interaction with phenylephrine hydrochloride, effect of preservatives □ Liquid scintillation spectrometry—used to study interaction of ^{14}C-phenylephrine hydrochloride with polyethylene

Many different products are packaged in plastics today. Among the first to recognize the versatility and practicality of plastics for packaging were the cosmetic and pharmaceutical manufacturers. The use of plastic containers in the pharmaceutical and cosmetic industry has multiplied rapidly to include a wide range of products. The plastic most frequently used in the manufacture of molded pharmaceutical and cosmetic containers is polyethylene.

Despite the widespread and increasing use of polyethylene containers to package drug and cosmetic preparations, limited information is available in the literature as to the compatibility of medicinal agents and solutions with polyethylene containers. One potential problem is that solutions of a drug in contact with a plastic can lead to sorption of the drug by the plastic, resulting in a significant loss of potency. Another major consideration encountered with polyethylene is that it is permeable in varying degrees to liquids, gases, and volatile substances.

Marcus *et al.* (1) and Kim and Autian (2) studied the sorption of certain bacteriostatic agents and several weak organic acids by polyethylene syringes. Using conventional analytical techniques, they determined that no sorption had occurred after 1 week of storage at 5, 30, and 50°. Neuwald and Schmitzek (3) found that preservative solutions stored in low and high density polyethylene containers showed no apparent reduction in preservative concentration resulting from sorption into plastics. Schoenwald and Belcastro (4) demonstrated the sorption of chlorobutanol by polyethylene strips stored in ^{14}C-labeled chlorobutanol solution. At present, there appears to be no satisfactory general method for predicting which plastic material should be used for one drug or another.

Parliman (5–7) was one of the first to study permeability of polyethylene with regard to containers. He developed an empirical method of determining the transmission rates of certain liquids through polyethylene. More recently, Salame and Pinsky (8) developed the Permachor method for estimating the permeabilities of organic liquids in polyethylene. One reason why permeation data often do not agree is that the particular plastic material influences the permeation rate, so great variations can occur in plastics even when manufactured under the same generic name (9).

One popular treatment employed for the symptomatic relief of nasal congestion is the administration of sympathomimetic-containing nasal solutions from polyethylene squeeze bottles. Phenylephrine hydrochloride is

Table I—Sample Grouping, Part A

Temperature	0.25% ^{14}C-Phenylephrine Hydrochloride Solution		0.5% ^{14}C-Phenylephrine Hydrochloride Solution	
	Group 1 Units with Dip Tubes	Group 2 Units without Dip Tubes	Group 3 Units with Dip Tubes	Group 4 Units without Dip Tubes
7°	Subgroup A Units: 1–5, 101–105	Subgroup A Units: 6–10, 106–110	Subgroup A Units: 41–45, 141–145	Subgroup A Units: 46–50, 146–150
22°	Subgroup B Units: 11–15, 111–115	Subgroup B Units: 16–20, 116–120	Subgroup B Units: 51–55, 151–155	Subgroup B Units: 56–60, 156–160
40°	Subgroup C Units: 21–25, 121–125	Subgroup C Units: 26–30, 126–130	Subgroup C Units: 61–65, 161–165	Subgroup C Units: 66–70, 166–170
50°	Subgroup D Units: 31–35, 131–135	Subgroup D Units: 36–40, 136–140	Subgroup D Units: 71–75, 171–175	Subgroup D Units: 76–80, 176–180

Table II—Sample Groupings, Part B

Temperature	0.25% ^{14}C-Phenylephrine Hydrochloride Solution—Benzalkonium Chloride (1:5000)		0.25% ^{14}C-Phenylephrine Hydrochloride Solution—Thimerosal (1:5000)		0.5% ^{14}C-Phenylephrine Hydrochloride Solution—Benzalkonium Chloride (1:5000)		0.5% ^{14}C-Phenylephrine Hydrochloride Solution—Thimerosal (1:5000)	
7°	Units	1–5	Units	101–105	Units	41–45	Units	141–145
22°	Units	6–10	Units	106–110	Units	46–50	Units	146–150
40°	Units	11–15	Units	111–115	Units	51–55	Units	151–155
50°	Units	16–20	Units	116–120	Units	56–60	Units	156–160

Table III—Mean Disintegrations per Minute (d.p.m.) for 0.25% ^{14}C-Phenylephrine Hydrochloride Solution, Part A

Weeks	Mean d.p.m. Units 1–5, 7°, DT[a]	Mean d.p.m. Units 6–10, 7°, NDT[b]	Mean d.p.m. Units 11–15, 22°, DT	Mean d.p.m. Units 16–20, 22°, NDT	Mean d.p.m. Units 21–25, 40°, DT	Mean d.p.m. Units 26–30, 40°, NDT	Mean d.p.m. Units 31–35, 50°, DT	Mean d.p.m. Units 36–40, 50°, NDT
0	19,963	19,963	19,963	19,963	19,963	19,963	19,963	19,963
0.28	20,135	20,079	19,729	19,857	19,876	20,002	20,079	20,315
0.57	19,958	20,059	20,036	19,893	20,136	19,842	20,295	20,215
1.1	19,762	20,125	20,027	19,991	20,090	20,038	20,372	20,157
2.0	20,020	20,132	19,871	19,976	20,073	19,926	20,083	20,337
3.0	19,773	19,871	19,935	19,945	20,032	20,258	20,353	20,522
4.0	19,696	19,997	20,054	19,929	20,307	20,044	20,621	20,730
6.0	20,085	20,245	20,015	19,902	20,185	20,168	20,771	21,090
8.0	20,065	19,807	20,004	20,002	20,289	20,277	20,609	21,473
10.0	19,932	20,018	19,916	19,981	20,387	20,330	21,339	22,329
16.0	—	—	—	—	20,354	20,703	21,848	23,785
20.0	—	—	—	—	20,395	20,456	21,983	23,803

[a] DT = dip tube. [b] NDT = no dip tube.

perhaps the most widely employed agent for this purpose, being the main ingredient in a large number of commercial products available for over-the-counter as well as prescription sales.

The intent of this investigation was to study the behavior of phenylephrine hydrochloride in the complete polyethylene spray bottle unit as it is sold to the consumer. Internal liquid scintillation spectrometry was employed for studying phenylephrine hydrochloride concentrations throughout the study because it is more sensitive to minute changes in concentration than are traditional assay methods.

While previous studies of drug–plastic interactions have been concerned with single-component systems, the use of internal liquid scintillation spectrometry permitted the study of a multicomponent system. One reason for not studying a multicomponent system by traditional methods is that the method of analysis can become quite complex. The ^{14}C-phenylephrine hydrochloride could be studied in the presence of various unlabeled components to see what effect they might have on its interaction with the polyethylene.

EXPERIMENTAL

Polyethylene spraying bottles were used throughout the study[1]. These bottles were made to contain 15 ml. of liquid and were supplied with dip tube, sprayhead, and screw-on cap.

The ^{14}C-phenylephrine hydrochloride[2], with a specific activity of 6.02 mc./mmole, was determined by the manufacturer to have a radiochemical purity greater than 99%. To confirm this value, the upper layer of a butanol–acetic acid–water (5:1:3) solution was used as the developing solvent for the paper chromatographic analysis of the ^{14}C-phenylephrine hydrochloride. Development of the chromatogram was followed by autoradiography on X-ray film, sectioning of the chromatogram, and scintillation counting. A radiochemical purity of greater than 98% was indicated.

Four main groups comprised Part A of the study. Group 1 contained 0.25% ^{14}C-phenylephrine hydrochloride solution in spray containers complete with dip tubes, while Group 2 units had the dip tubes removed. Groups 3 and 4 contained 0.5% ^{14}C-phenylephrine hydrochloride, with Group 3 spray containers being complete and Group 4 containers being without dip tubes.

Each main group was divided into four subgroups of five spray units each. One subgroup was stored at each of the following temperatures: 7, 22, 40, and 50°. Table I depicts the grouping of the units. A duplicate set of units was prepared for all four primary groups. These duplicate units were prepared to study weight loss and to determine the effects, if any, of repeated sample withdrawals on the results obtained with the primary groups.

In Part B of the study, the effects of two preservatives, benzalkonium chloride (1:5000) and thimerosal (1:5000), on the behavior of the ^{14}C-phenylephrine hydrochloride–polyethylene interaction were studied. The units stored at 7 and 22° in Part A (from both the primary and duplicate groups) were used for Part B. No change occurred in these units during the first 10 weeks of Part A of the study.

The preservative solutions were added in such a manner as to yield a final volume of 10.1 ml. in each unit. All units used in Part B were assembled complete with dip tubes. Grouping of the units according to preservative and storage temperature is depicted in Tables II and III.

The spray containers were stored at the respective temperatures in the upright position in both parts of the study. Partitioned cardboard trays, open at the top, were constructed to store the spray units. Each partitioned carton held 20 spray containers.

A 100-μl. Hamilton[3] microliter syringe with a Chaney adapter was used to withdraw 100-μl. samples from the spray containers. A scintillator solution (0.4% 2,5-diphenyloxazole in equal parts of toluene and 2-ethoxyethanol) was used for all samples.

A two-channel, ambient temperature, internal liquid scintillation spectrometer[4], equipped with automatic sample changer and printout, was used throughout the study. Each sample was counted at

[1] Spray bottles No. 25163, N-3/4, Wheaton Plastic, Millville, NJ 08332.
[2] Tracerlab, Waltham, Mass.
[3] Hamilton Co., Whittier, Calif.
[4] Tri-Carb model 2002, Packard Instrument Co., Inc., LaGrange, Ill.

Table IV—Mean Disintegrations per Minute (d.p.m.) for 0.5% ^{14}C-Phenylephrine Hydrochloride Solution, Part A

Weeks	Mean d.p.m. Units 41–45, 7°, DT[a]	Mean d.p.m. Units 46–50, 7°, NDT[b]	Mean d.p.m. Units 51–55, 22°, DT	Mean d.p.m. Units 55–60, 22°, NDT	Mean d.p.m. Units 61–65, 40°, DT	Mean d.p.m. Units 66–70, 40°, DT	Mean d.p.m. Units 71–75, 50°, DT	Mean d.p.m. Units 76–80, 50°, NDT
0	20,680	20,680	20,680	20,680	20,680	20,680	20,680	20,680
0.28	20,825	20,725	20,546	20,809	20,873	20,728	20,808	20,836
0.57	20,618	21,072	20,720	20,716	20,765	20,756	20,892	20,857
1.1	20,617	20,411	20,879	20,669	20,624	20,772	20,856	20,769
2.0	20,689	20,690	20,563	20,543	20,734	20,861	21,057	20,546
3.0	20,826	20,635	20,846	20,644	20,828	20,737	20,761	20,850
4.0	20,653	20,759	20,847	20,660	20,795	20,714	21,197	21,106
6.0	20,552	20,739	20,808	20,635	20,912	20,917	21,231	21,622
8.0	20,441	20,735	20,724	20,547	20,842	21,011	21,622	21,762
10.0	20,623	20,645	20,624	20,611	20,860	20,999	22,177	22,048
16.0	—	—	—	—	21,342	21,366	22,912	23,726
20.0	—	—	—	—	21,199	21,135	22,788	23,410

[a] DT = dip tube. [b] NDT = no dip tube.

optimum gain for a 1-min. interval, with the instrument set to subtract the background count rate automatically. Net counts per minute (c.p.m.) ranged from approximately 14,000 to 16,000. A counting error of less than 1% was obtained, and the counting efficiency was around 78% throughout the investigation. By using a toluene-^{14}C internal standard, the absolute count rate was calculated according to the method described by Wang and Willis (10). All further calculations were based upon the disintegrations per minute (d.p.m.) obtained.

Each container in the duplicate set of units was weighed on an analytical balance at the beginning and at the end of Part A to determine moisture loss. In addition, two units from each subgroup of the duplicate set were weighed periodically. Weighings were conducted each time sampling of the primary group was performed.

RESULTS

Drug–Plastic Interaction: Part A—Table I lists the solution groups investigated in Part A of the study. The mean disintegrations per minute for each five-container group is listed in Tables III and IV. The three major parameters investigated were the effects of solution concentration, temperature, and container components on possible drug–plastic interaction.

The data presented in Table III show that no loss of ^{14}C-phenylephrine hydrochloride occurred in any of the 0.25% solutions. The disintegrations per minute values at the end of 10 weeks were of the same magnitude as the initial values or greater. Table IV shows similar results for the 0.5% ^{14}C-phenylephrine hydrochloride. In all cases, group disintegrations per minute values either remained the same or increased during 10 weeks of storage at 7, 22, 40, or 50°. An increase in concentration of the ^{14}C-phenylephrine hydrochloride had no apparent effect on the behavior of the phenylephrine hydrochloride toward the polyethylene. No sorption was demonstrated, indicating complete compatibility of the drug with the container and its components. There was no difference seen in the units without dip tubes. All units behaved in identical fashion at each temperature regardless of the presence or absence of dip tube components.

Reference to Tables III and IV shows that no loss of ^{14}C-phenylephrine hydrochloride occurred at any of the four temperatures studied. There was no increase or decrease for any of the groups stored at 7 and 22°. Elevated temperatures failed to promote any loss of ^{14}C-phenylephrine hydrochloride through sorption. There was an apparent gain in disintegrations per minute values, however, for each group stored at 40 and 50°. This gain was attributed to the loss of solvent from the spray containers.

Moisture Loss: Part A—Each unit in the duplicate set of units from Part A was weighed at the beginning of the study and at the end of 10 weeks. The units stored at 40 and 50° were also weighed again at the end of 16 and 20 weeks. The weight loss for each unit, represented as percent weight loss after 10 weeks, is listed in Table V. Table VI lists the percent weight loss for each unit stored at 40 and 50° for Weeks 11–16 of the study, while Table VII shows the percent weight loss for these units for Weeks 17–20. Since there was a corresponding increase in disintegrations per minute with weight

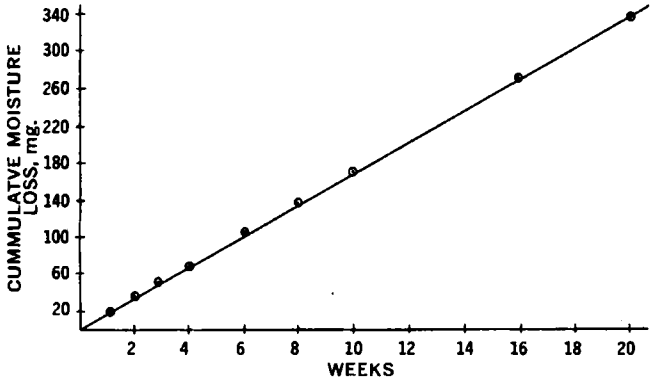

Figure 1—*Example of a typical weight loss curve for a unit stored at 40°.*

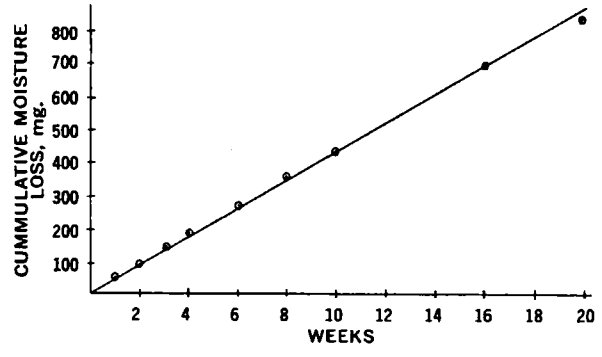

Figure 2—*Example of a typical weight loss curve for a unit stored at 50°.*

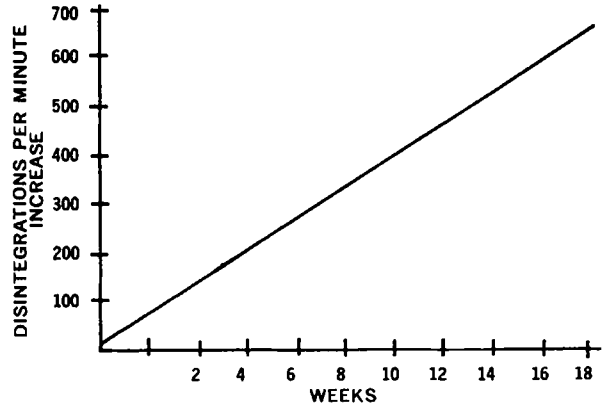

Figure 3—*Average moisture loss as reflected by disintegrations per minute increase for a typical group of units stored at 40°.*

Table V—Weight Loss Tabulation for First 10 Weeks of Study, Part A

Group Description	0.25% ^{14}C-Phenylephrine Hydrochloride Solution		0.5% ^{14}C-Phenylephrine Hydrochloride Solution	
	Unit Number	Percent Weight Loss	Unit Number	Percent Weight Loss
Storage at 7°, units with dip tubes	101	0.030	141	0.044
	102	0.203	142	0.233
	103	0.954[a]	143	0.015
	104	0.034	144	0.492[a]
	105	0.031	145	0.017
Storage at 7°, units without dip tubes	106	0.029	146	0.182
	107	0.029	147	0.023
	108	0.028	148	0.044
	109	0.025	149	0.012
	110	0.033	150	0.082
Storage at 22°, units with dip tubes	111	0.280	151	0.271
	112	0.295	152	0.268
	113	0.280	153	0.263
	114	0.276	154	0.272
	115	0.282	155	0.269
Storage at 22°, units without dip tubes	116	0.993	156	0.540
	117	0.286	157	0.267
	118	0.280	158	0.283
	119	0.258	159	0.275
	120	0.274	160	0.277
Storage at 40°, units with dip tubes	121	2.274	161	2.443
	122	1.794	162	1.767
	123	1.740	163	3.130[a]
	124	1.745	164	1.708
	125	1.726	165	1.738
Storage at 40°, units without dip tubes	126	1.753	166	1.643
	127	1.673	167	1.705
	128	1.819	168	1.747
	129	1.764	169	1.691
	130	2.445	170	1.788
Storage at 50°, units with dip tubes	131	4.512	171	4.158
	132	4.673	172	4.403
	133	5.741	173	4.639
	134	5.111	174	4.666
	135	5.294	175	9.099[a]
Storage at 50°, units without dip tubes	136	4.444	176	4.071
	137	4.636	177	4.277
	138	5.007	178	4.411
	139	5.001	179	4.536
	140	5.232	180	4.706

[a] Denotes unit improperly sealed.

Table VI—Weight Loss Tabulation for Weeks 11–16 and 17–20 of Study, Part A

Group Description	0.25% ^{14}C-Phenylephrine Hydrochloride Solution		0.5% ^{14}C-Phenylephrine Hydrochloride Solution	
	Unit Number	Percent Weight Loss	Unit Number	Percent Weight Loss
Weeks 11–16				
Storage at 40°, units with dip tubes	121	1.076	161	1.440
	122	1.430	162	1.496
	123	1.521	163	1.531
	124	0.972	164	1.079
	125	1.223	165	0.923
Storage at 40°, units without dip tubes	126	1.382	166	1.452
	127	1.350	167	0.990
	128	1.824	168	1.076
	129	1.075	169	0.962
	130	1.290	170	0.982
Storage at 50°, units with dip tubes	131	2.289	171	2.097
	132	2.546	172	2.333
	133	2.823	173	2.528
	134	2.874	174	2.625
	135	2.995	175	2.823
Storage at 50°, units without dip tubes	136	2.398	176	2.062
	137	2.680	177	2.203
	138	4.324	178	3.031
	139	2.880	179	2.881
	140	2.907	180	2.636
Weeks 17–20				
Storage at 40°, units with dip tubes	121	0.638	161	0.836
	122	0.628	162	0.939
	123	0.669	163	0.615
	124	0.689	164	0.607
	125	0.898	165	0.618
Storage at 40°, units without dip tubes	126	0.629	166	1.206
	127	0.816	167	0.614
	128	1.032	168	0.615
	129	0.836	169	0.845
	130	0.635	170	0.601
Storage at 50°, units with dip tubes	131	1.547	171	1.353
	132	1.663	172	1.502
	133	1.808	173	1.635
	134	1.890	174	1.707
	135	1.998	175	1.827
Storage at 50°, units without dip tubes	136	1.493	176	1.327
	137	1.612	177	1.433
	138	2.160	178	1.578
	139	1.825	179	1.647
	140	1.915	180	3.059

loss of the solutions studied, the weight loss was attributed to permeation of the solvent, water, through the polyethylene container. Average percent moisture loss values for all groupings as demonstrated by weight loss are listed in Table VII.

The presence or absence of dip tubes did not affect the moisture loss. Groups having no dip tubes showed moisture losses of the same magnitude as their counterparts with dip tubes. The average 10-week percent moisture loss for all groups stored at 7° was 0.031%, while the average loss was 0.275% for groups stored at 22°. Groups stored at 40 and 50° showed average moisture losses of 1.8 and 4.7%, respectively, during the first 10 weeks. Weight loss data for the second 10-week segment of Part A show an additional average moisture loss of 2.0% for units stored at 40° and of 4.4% for units stored at 50°.

Representative units of each group stored at 40 and 50° were weighed periodically during the first 10 weeks of the study, at the end of 16 weeks, and again at the end of 20 weeks. Figures 1 and 2 show the moisture loss curves for typical units stored at 40 and 50°. Water was lost from the units at a constant rate, the average loss being approximately 19 mg./week for units stored at 40° and 45.5 mg./week for units stored at 50°.

The loss of solvent from the polyethylene spray bottles was also reflected in the increased disintegrations per minute values at the end of 10, 16, and 20 weeks. The percent increase in disintegrations per minute values for both primary (regularly sampled) and duplicate (unsampled until Week 10) groups stored at 40 and 50° is shown in Table VIII. Reference to Table VIII shows that at the end of 10 weeks the percent increase in disintegrations per minute values for both primary and duplicate groups stored at 40° compares favorably with the percent weight loss data. While the percent increase in disintegrations per minute values after 10 weeks for the duplicate units stored at 50° compares favorably with the weight loss data, higher losses were reflected by the primary or regularly sampled units. The higher losses in these units may be attributed to their regular sampling, additional moisture loss occurring each time a unit was opened and a withdrawal made. These data are valuable in projecting what might be observed during normal patient use of these products.

The disintegrations per minute values for all groups stored at 40 and 50° continued to increase during the second 10-week period. The percent increase in disintegrations per minute values for the 20-week period compares favorably with the weight loss data for that period. Figures 3 and 4 show the moisture loss curves for groups stored at 40 and 50°, respectively, as reflected by the disintegrations per minute increase. The solid lines in Figs. 3 and 4 represent the lines of best fit for the data as determined by least-squares calculations.

Container Suitability: Part A—The results of Part A demonstrate that low density polyethylene containers are suitable for the packaging of aqueous solutions of phenylephrine hydrochloride. Phenylephrine hydrochloride showed no interaction with the polyethylene polymer at any of the temperatures studied. Furthermore, phenyl-

Table VII—Mean Moisture Loss in Percentages for All Groups as Determined Gravimetrically, Part A

Group Description	Percent Moisture Loss — After 10 Weeks: 0.25% ^{14}C-Phenylephrine Hydrochloride Solution	After 10 Weeks: 0.5% ^{14}C-Phenylephrine Hydrochloride Solution	After 16 Weeks: 0.25% ^{14}C-Phenylephrine Hydrochloride Solution	After 16 Weeks: 0.5% ^{14}C-Phenylephrine Hydrochloride Solution	After 20 Weeks: 0.25% ^{14}C-Phenylephrine Hydrochloride Solution	After 20 Weeks: 0.5% ^{14}C-Phenylephrine Hydrochloride Solution
Storage at 7°, with dip tubes	0.031	0.025	—	—	—	—
Storage at 7°, without dip tubes	0.028	0.040	—	—	—	—
Storage at 22°, with dip tubes	0.283	0.268	—	—	—	—
Storage at 22°, without dip tubes	0.274	0.276	—	—	—	—
Storage at 40°, with dip tubes	1.856	1.738	3.100	3.031	3.805	3.755
Storage at 40° without dip tubes	1.891	1.713	3.275	2.805	4.068	3.582
Storage at 50° with dip tubes	5.066	4.446	7.771	6.947	9.551	8.552
Storage at 50°, without dip tubes	4.864	4.400	7.902	6.962	9.703	8.772

Table VIII—Mean Moisture Loss in Percentages for Primary and Duplicate Groups Stored at 40 and 50°, Determined Radioactively, Part A

Group Description	After 10 Weeks: 0.25% ^{14}C-Phenylephrine Hydrochloride Solution	After 10 Weeks: 0.5% ^{14}C-Phenylephrine Hydrochloride Solution	After 16 Weeks: 0.25% ^{14}C-Phenylephrine Hydrochloride Solution	After 16 Weeks: 0.5% ^{14}C-Phenylephrine Hydrochloride Solution	After 20 Weeks: 0.25% ^{14}C-Phenylephrine Hydrochloride Solution	After 20 Weeks: 0.5% ^{14}C-Phenylephrine Hydrochloride Solution
Primary group, 40°, with dip tubes	2.1	0.9	4.5	3.2	2.2	2.5
Duplicate group, 40°, with dip tubes	1.6	1.7	—	—	2.1	2.0
Primary group, 40°, without dip tubes	1.8	1.5	3.7	3.3	2.5	2.2
Duplicate group, 40°, without dip tubes	2.1	0.7	—	—	1.7	0.9
Primary group, 50°, with dip tubes	6.9	7.2	9.4	10.7	10.1	10.2
Duplicate group, 50°, with dip tubes	5.4	4.3	—	—	8.1	7.4
Primary group 50°, without dip tubes	11.8	6.6	19.1	14.7	19.2	13.2
Duplicate group, 50°, without dip tubes	4.7	4.8	—	—	9.2	6.6

ephrine hydrochloride demonstrated no visual evidence of decomposition when stored at 7, 22, or 40° in combination with 2% sodium bisulfite. At 50°, no decomposition was evident after 10 weeks had elapsed. By the end of 16 weeks, the characteristic brownish color of degraded phenylephrine hydrochloride was evident.

The polyethylene containers studied presented a good barrier to moisture transmission. Water permeation through the container walls was negligible at 7° and only 0.03%/week at room temperature (22°). At elevated temperatures, the losses were about 0.2%/week at 40° and 0.45%/week at 50°. Overall, low density polyethylene was shown to be an adequate container for packaging of aqueous solutions of phenylephrine hydrochloride utilizing a radioisotopic method of investigation.

Drug–Plastic Interaction: Part B—In Part B, the influence of two preservatives, benzalkonium chloride (1:5000) and thimerosal (1:5000), on the phenylephrine hydrochloride–polyethylene container interaction was investigated. Table II lists the different solution groups investigated in Part B. Mean disintegrations per minute values for each five-container group are listed in Tables IX and X. Table XI shows the net percentage increase or decrease in disintegrations per minute values for each of the groups investigated in Part B.

Both benzalkonium chloride and thimerosal were found to cause a slight interaction of the ^{14}C-phenylephrine hydrochloride with the polyethylene container. At 7°, the average loss for all units was 1.0% after 14 weeks of storage. At 22°, the loss of ^{14}C-phenylephrine hydrochloride averaged 2.6% after 14 weeks, with the average loss values ranging from 1.9 to 3.0%. The decrease in phenylephrine hydrochloride concentration for groups stored at 22° is depicted in Fig. 5.

After 14 weeks at 40°, there was a small increase in the average disintegrations per minute values. The increase averaged 0.23% for 14 weeks. When this value is compared to the average disintegrations per minute increase (1.75% for 14 weeks) for the unpreserved solutions stored at 40°, a difference of approximately 1.5% is indicated. Thus, there was an apparent 1.5% loss of ^{14}C-phenylephrine hydrochloride offsetting the loss of moisture from the container.

The average percent disintegrations per minute increase for units preserved with benzalkonium chloride and stored at 50° was approximately 0.5%/week or about the same magnitude as the increase per week (0.53%) for unpreserved solutions stored at 50° in Part A. The disintegrations per minute increase for units preserved with thimerosal was greater than that of the units preserved with benzalkonium chloride. The average percent disintegrations per minute increase for the thimerosal-preserved units was 0.71%/week. If the percent increase in average disintegrations per minute values for the thimerosal-preserved units is projected over a 20-week period, a gain of 14.2% is indicated. This value compares favorably with the range

Table IX—Mean Disintegrations per Minute (d.p.m.) for 0.25% ^{14}C-Phenylephrine Hydrochloride, Preservative Solutions, Part B

Weeks	Mean d.p.m. Units 1–5, Benzalkonium Chloride, 7°	Mean d.p.m. Units 101–105, Thimerosal, 7°	Mean d.p.m. Units 6–10, Benzalkonium Chloride, 22°	Mean d.p.m. Units 106–110, Thimerosal, 22°	Mean d.p.m. Units 11–15, Benzalkonium Chloride, 40°	Mean d.p.m. Units 111–115, Thimerosal, 40°	Mean d.p.m. Units 16–20, Benzalkonium Chloride, 50°	Mean d.p.m. Units 116–120, Thimerosal, 50°
0	17,794	19,830	17,903	19,746	17,820	19,913	17,934	19,856
1	17,848	20,114	17,934	19,826	18,107	19,701	18,294	20,376
3	17,982	20,028	18,014	19,642	18,025	20,236	18,515	20,490
5	18,358	19,960	17,751	19,612	18,040	20,280	18,613	20,946
7	17,960	19,642	17,632	19,507	17,869	19,700	18,509	20,798
9	17,645	19,549	17,607	19,364	17,937	19,859	18,932	21,044
12	17,981	19,822	17,595	19,592	17,882	20,120	18,734	21,468
14	17,927	19,461	17,485	19,136	17,857	19,774	19,168	21,819

Table X—Mean Disintegrations per Minute (d.p.m.) for 0.5% ^{14}C-Phenylephrine Hydrochloride, Preservative Solutions, Part B

Weeks	Mean d.p.m. Units 41–45, Benzalkonium Chloride, 7°	Mean d.p.m. Units 141–145, Thimerosal, 7°	Mean d.p.m. Units 46–50, Benzalkonium Chloride, 22°	Mean d.p.m. Units 146–150, Thimerosal, 22°	Mean d.p.m. Units 51–55, Benzalkonium Chloride, 40°	Mean d.p.m. Units 151–155, Thimerosal, 40°	Mean d.p.m. Units 56–60, Benzalkonium Chloride, 50°	Mean d.p.m. Units 156–160, Thimerosal, 50°
0	18,455	20,377	18,596	20,404	18,485	20,383	18,555	20,388
1	18,722	20,791	18,567	20,658	18,675	20,630	18,919	20,949
3	18,694	20,510	18,700	20,519	18,870	20,663	19,166	20,990
5	18,848	20,847	18,331	20,468	18,804	20,824	19,172	21,634
7	18,410	20,386	18,216	20,102	18,386	20,382	19,219	21,422
9	18,634	20,181	18,053	20,115	18,529	20,466	19,528	21,702
12	18,395	20,434	18,149	20,042	18,515	20,454	19,610	22,338
14	18,252	20,171	18,043	20,006	18,703	20,434	19,899	22,408

Table XI—Net Percentage Increase or Decrease in Group Disintegrations per Minute Averages for 0.25% and 0.5% ^{14}C-Phenylephrine Hydrochloride, Preservative Solutions, Part B

Group Description			Weeks						
Concentration of ^{14}C-Phenylephrine Hydrochloride, %	Preservative	Storage Temperature	1	3	5	7	9	12	14
0.25	Benzalkonium chloride	7°	+0.3	+1.1	+3.1	−0.6	−0.8	+1.1	+0.7
0.25	Thimerosal	7°	+1.4	+1.0	+0.7	−0.9	−1.4	0	−1.9
0.5	Benzalkonium chloride	7°	+1.4	+1.3	+2.1	−0.2	+1.0	−0.3	−1.0
0.5	Thimerosal	7°	+2.0	+0.6	+2.3	0	−0.9	+0.3	−1.0
0.25	Benzalkonium chloride	22°	+0.2	+0.6	−0.8	−1.5	−1.7	−1.7	−2.3
0.25	Thimerosal	22°	+0.4	−0.5	−0.7	−1.2	−1.9	−0.8	−3.0
0.5	Benzalkonium chloride	22°	+0.2	+0.5	−1.4	−2.0	−2.9	−2.4	−3.0
0.5	Thimerosal	22°	+1.2	+0.6	+0.3	−1.4	−1.4	−1.8	−1.9
0.25	Benzalkonium chloride	40°	+1.6	+1.2	+1.2	+0.3	+0.6	+0.3	+0.3
0.25	Thimerosal	40°	−1.0	+1.6	+1.8	−1.0	−0.3	+1.0	−0.7
0.5	Benzalkonium chloride	40°	+1.0	+2.0	+1.7	−0.5	+0.2	+0.2	+1.2
0.5	Thimerosal	40°	+1.2	+1.4	+2.2	0	+0.4	+0.3	+0.2
0.25	Benzalkonium chloride	50°	+2.0	+3.2	+3.8	+3.2	+5.6	+4.5	+6.9
0.25	Thimerosal	50°	+2.6	+3.2	+5.5	+4.7	+6.0	+8.1	+9.9
0.5	Benzalkonium chloride	50°	+2.0	+3.3	+3.3	+3.6	+5.2	+5.7	+7.2
0.5	Thimerosal	50°	+2.8	+3.0	+6.1	+4.8	+6.4	+9.5	+9.9

of percent increase in disintegrations per minute values for unpreserved solutions from Part A listed in Table X. Overall, the moisture loss (as reflected by increased average disintegrations per minute values) for the preserved solutions stored at 50° was the same as that for the unpreserved solutions studied in Part A.

Study of the data from Part B reveals that there is an apparent interaction between the ^{14}C-phenylephrine hydrochloride and the polyethylene container caused by both preservatives. The interaction was strongest at room temperature (22°), with apparent binding of ^{14}C-phenylephrine hydrochloride as high as 3.0% after 14 weeks An increase in temperature appears to disrupt the interacted system For example, at 40° the net loss of ^{14}C-phenylephrine hydrochloride was 1.5%. At 50°, no loss was shown.

The data further reveal that there is no apparent overall trend visible as to any difference between the solutions containing thimerosal or benzalkonium chloride or between solutions containing 0.25% as opposed to 0.5% ^{14}C-phenylephrine hydrochloride. Since the preservative is the limiting factor in the interaction, a

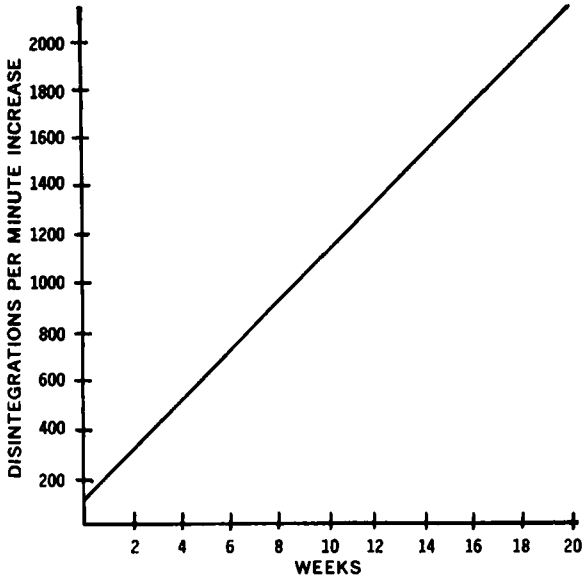

Figure 4—*Average moisture loss as reflected by disintegrations per minute increase for a typical group of units stored at 50°.*

change in preservative concentration would probably alter the percentage lost or bound. Accelerated stability studies at elevated temperatures could not be used to predict the long-term stability of these units at room temperature since the results showed that elevated temperatures disrupt the interaction between container and preservative–drug.

A study of the preservatives as the labeled compound would reveal more about their role in this study and about possible serious loss of preservative due to interaction with the polyethylene container.

SUMMARY

The utility of radioisotopic techniques in evaluating container performance of pharmaceutical packaging was demonstrated by the use of ^{14}C-phenylephrine hydrochloride to investigate the compatibility of phenylephrine hydrochloride in low density polyethylene containers. Phenylephrine hydrochloride in aqueous solution apparently does not bind with low density polyethylene as a blow molded container. Moisture loss from low density polyethylene containers was clearly demonstrated by use of radioisotopic means as well as by conventional gravimetric techniques. It was demonstrated that, at room temperature, phenylephrine hydrochloride binds either to or in combination with a preservative (thimerosal or benzalkonium chloride) to produce an interacted system with low density polyethylene. In this study an elevated temperature disrupted the weak binding. This indicates the necessity of always evaluating polyethylene packaging of liquid pharmaceutical products at room temperature to avoid the possibility of erroneous conclusions based on accelerated testing.

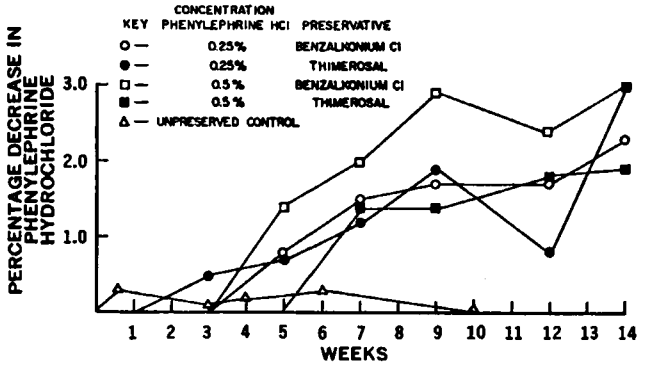

Figure 5—*Average percentage decrease of phenylephrine hydrochloride for groups stored at 22° (Part B).*

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Biosynthetic Production of Aberrant Alkaloids in *Dolichothele sphaerica* (Cactaceae)

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Abstract □ Preliminary studies of the production of aberrant alkaloids were carried out by introducing unnatural precursors of dolichotheline into *Dolichothele sphaerica* in the hope that the corresponding dolichotheline analogs would be formed. Authentic samples of the required dolichotheline analogs were synthesized and served both as standards and as carriers during the extraction procedure. Radioactive analogs were separated from dolichotheline by preparative TLC, recrystallized to constant specific activity, and degraded to localize the label. Using appropriate precursors, the aberrant alkaloids *N*-isocaproylhistamine and 4(5)-[*N*-isovalerylaminomethyl]imidazole were produced by *D. sphaerica*, while the plant appears to be unable to produce *N*-benzoylhistamine, *N*-isobutyrylhistamine, and *N*-isovaleryl-*N*-isopropylhistamine.

Keyphrases □ *Dolichothele sphaerica* (Cactaceae)—biosynthesis of aberrant alkaloids □ Alkaloids, aberrant—biosynthesis of dolichotheline analogs from unnatural precursors in *Dolichothele sphaerica* □ Unnatural alkaloids—biosynthesis of dolichotheline analogs from unnatural precursors in *Dolichothele sphaerica* □ Aberrant alkaloid biosynthesis—*Dolichothele sphaerica* (Cactaceae) □ Biosynthesis of aberrant alkaloids—dolichotheline analogs in *Dolichothele sphaerica*

Studies on the biosynthesis of alkaloids in higher plants have been carried out almost entirely by means of labeled precursor feeding experiments. Such investigations assume that only the natural precursor will be incorporated efficiently into the alkaloid of interest. Yet, there are published accounts that document the incorporation of unnatural precursors into naturally occurring alkaloids (1, 2). In addition, the possibility of the incorporation of unnatural precursors into unnatural or aberrant alkaloids exists. Recently, Leete *et al.* (3) succeeded in inducing the formation of 5-fluoronicotine by feeding 5-fluoronicotinic acid to *Nicotiana tabacum*. Rueppel and Rapoport (4, 5) tested 2- and 3-methyl-substituted pyrrolinium precursors in *Nicotiana glutinosa* and reported their incorporation into the aberrant alkaloids 3′-methylnicotine, 2′-methylnicotine, and 3′,3′-dimethylnicotine.

Dolichotheline (I), a monosubstituted amide alkaloid isolated from the cactus *Dolichothele sphaerica* (Dietrich) Britton and Rose, appears to arise biosynthetically by means of a mechanism involving the condensation of histamine and isovaleryl-CoA (6). Thus, *D. sphaerica* was considered to be suitable for carrying out experiments to determine whether the enzyme(s) responsible for the linkage of histamine and isovaleric acid could also yield aberrant alkaloids following administration of selected precursors to the plant.

The following compounds were tested in the expectation that their corresponding dolichotheline analogs would be formed: isobutyric acid, isocaproic acid, benzoic acid, 4(5)-aminomethylimidazole, and *N*-isopropylhistamine. Authentic samples of the required dolichotheline analogs were synthesized and served both as standards and as carriers during the extraction procedure.

Such investigation into the biosynthesis of unnatural or aberrant alkaloids is of interest for several reasons:

1. There are few reports that deal with the incorporation of an unnatural precursor into an unnatural alkaloid.
2. Such experiments with a series of related unnatural precursors may shed light on the specificity of the enzyme(s) involved.
3. The formation of unnatural alkaloids *in vivo* has broad potential application for the preparation of analogs of biologically active natural products.

EXPERIMENTAL[1]

***N*-Isobutyrylhistamine (II)**—Isobutyric anhydride (2.2 g.) and histamine (1.1 g.) were gently refluxed for 1 hr. Several drops of water were added and the reaction mixture was condensed on a steam bath to a thick, oily residue. The addition of a few drops of acetone caused the crystallization of *N*-isobutyrylhistamine. It was recrystallized from methanol–ether, yielding 1.39 g., m.p. 139–140° [lit. (7) m.p. 123°[2]]; IR (amide) 1640 cm.$^{-1}$. NMR spectral data for all dolichotheline analogs are presented in Table I.

Anal.—Calc. for $C_9H_{15}N_3O$: C, 59.64; H, 8.34; N, 23.19. Found: C, 59.76; H, 8.34; N, 23.25.

***N*-Isocaproylhistamine (III)**—Histamine (0.9 g.) and 4-methylvaleryl chloride (1.7 g.) were refluxed gently for 20 min. The reaction mixture was then cooled, dissolved in 10 ml. of 1 *N* HCl, and extracted three times with equal portions of ether. The aqueous solution was made alkaline with concentrated ammonium hydroxide to pH 9.5 and submitted to extraction with hot chloroform in a liquid–liquid extractor for 24 hr. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to yield the crude *N*-isocaproylhistamine. It was recrystallized from methanol–ether, yielding 0.95 g., m.p. 137–138°; IR (amide) 1635 cm.$^{-1}$.

Anal.—Calc. for $C_{11}H_{19}N_3O$: C, 63.13; H, 9.15; N, 20.08. Found: C, 62.97; H, 9.24; N, 20.14.

***N*-Benzoylhistamine (IV)**—Histamine (1.1 g.) and benzoyl chloride (1.4 g.) were gently refluxed for 15 min. The reaction mixture was then cooled, treated with 10 ml. of 1 *N* HCl, and extracted three times with equal portions of ether. The aqueous solution was made alkaline with concentrated ammonium hydroxide to pH 9.5 and extracted with hot chloroform for 24 hr. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to yield crude *N*-benzoylhistamine. It was recrystallized from methanol–ether, yielding 0.93 g., m.p. 147–149°; IR (amide) 1640 cm.$^{-1}$.

Anal.—Calc. for $C_{12}H_{13}N_3O$: C, 66.96; H, 6.09; N, 19.52. Found: C, 67.13; H, 6.22; N, 19.47.

***N*-Isopropylhistamine Dihydrochloride**—Histamine (1.11 g.), in 25 ml. absolute ethanol and 1.5 ml. of acetone, was hydrogenated over 300 mg. of platinum oxide at atmospheric pressure for 4 hr. After the catalyst was removed, the free base was converted to the

[1] Melting points were determined on a Fisher-Johns melting-point apparatus and are corrected. IR absorption spectra were recorded using KBr pellets and a Perkin-Elmer 337 grating spectrophotometer. NMR spectra were determined in deuterated dimethyl sulfoxide using tetramethylsilane as the reference standard on a Varian A-60 spectrometer. Microanalyses were carried out by Spang Microanalytical Laboratory, Ann Arbor, Mich.

[2] The reported melting point appears to have been interchanged with that of *N*-acetylhistamine, which was also synthesized by the author.

Table I—NMR Spectra of Dolichotheline Analogs (60 Hz. in Dimethyl Sulfoxide using Tetramethylsilane Reference)[a]

Compound		Aromatic 2-CH	Aromatic 4(5)-CH	Imino N-H	—CH_2— Next to Ring	Gem Dimethyl	Miscellaneous
							C-2 and C-3 of isovaleryl group
VI: 4(5)-[*N*-Isovaleryl-aminomethyl]imidazole	p.p.m.	7.52	6.80	8.05	4.37	1.11	2.22
	J, c.p.s.	s	s	s	d 4.0	d 5.0	m
							C-2 isocaproyl group
III: *N*-Isocaproylhistamine	p.p.m.	7.39	6.67	7.75	2.61	0.88	2.02
	J, c.p.s.	s	s	s	t 7.0	d 5.0	t 7.0
							C-2 isobutyryl group
II: *N*-Isobutyrylhistamine	p.p.m.	7.26	6.54	7.53	2.54	0.99	2.20
	J, c.p.s.	s	s	s	t 7.0	d 5.0	m
							Aromatic (benzene)
IV: *N*-Benzoylhistamine	p.p.m.	7.61	6.89	9.33	2.80	—	7.88 and 7.55
	J, c.p.s.	s	s	s	t 7.0	—	m
							N-Isopropyl
V: *N*-Isopropyl-*N*-isovalerylhistamine	p.p.m.	8.96	7.36	4.40	3.04	0.94	1.24
	J, c.p.s.	s	s	s	t 7.0	d 5.0	d 5.0

[a] s = singlet, d = doublet, m = multiplet, and t = triplet.

dihydrochloride by dissolving it in 25 ml. of methanol and adding 5 ml. of 6 *N* HCl. The resulting solution was evaporated to dryness and the residue was recrystallized from absolute ethanol–ethyl acetate, yielding 1.23 g., m.p. 195–197° [lit. m.p. 197–199° (14) and 195–196° (15)].

Anal.—Calc. for $C_8H_{17}Cl_2N_3$: C, 42.49; H, 7.58; N, 18.58. Found: C, 42.71; H, 7.74; N, 18.61.

N-Isopropyl-N-isovalerylhistamine (V)—*N*-Isopropylhistamine (0.82 g.) and isovaleric anhydride (2 g.) were gently refluxed for 4 hr. When cool, several drops of water were added to the reaction mixture, which was then condensed on a steam bath to a syrupy residue. This residue could not be induced to crystallize. As a consequence, the picrate derivative was formed by dissolving the residue in 2 ml. of ethanol and adding 10 ml. of a saturated solution of picric acid in ethanol. The crystalline picrate derivative was filtered and recrystallized from water, yielding 817 mg., m.p. 145°; IR (amide) 1640 $cm.^{-1}$.

Anal.—Calc. for $C_{19}H_{26}N_6O_8$: C, 48.92; H, 5.62; N, 18.02. Found: C, 48.89; H, 5.63; N, 18.25.

Imidazole-4(5)-formaldehyde—Hydroxymethylimidazole (13 g.) (8, 9) and nitric acid (19 ml.) were digested in a covered beaker on a steam bath until the evolution of brown fumes was almost complete. The cover was then removed and the liquid was evaporated to dryness.

This residue was dissolved in a warm concentrated solution of sodium carbonate and kept at room temperature for 24 hr., at which time the insoluble imidazole-4(5)-formaldehyde was filtered and dried. It was recrystallized from water, yielding 5.1 g., m.p. 173–174° [lit. (10) m.p. 170°].

Imidazole-4(5)-formaldoxime—The above aldehyde (2.0 g.), hydroxylamine hydrochloride (1.4 g.), and sodium carbonate (1.0 g.) were dissolved in 10 ml. of water and the resulting solution was allowed to stand at room temperature for 24 hr., at which time the insoluble imidazole-4(5)-formaldoxime was filtered and dried. It was recrystallized from alcohol, yielding 1.8 g., m.p. 183° [lit. (11) m.p. 183–184°].

4(5)-Aminomethylimidazole Dihydrochloride—A solution of 1.11 g. of the above oxime in 80 ml. of methanol containing 0.05 mole of dry hydrogen chloride was hydrogenated at 15 lb. pressure over 5% palladium-on-charcoal. After 90 min., the mixture was filtered and the filtrate was evaporated to dryness. The residue was recrystallized from methanol–water to give 1.20 g. of 4(5)-aminomethylimidazole dihydrochloride, m.p. 238–241° [lit. m.p. 246–247° (12) and 244° (13)].

4(5)-[*N*-Isovalerylaminomethyl]imidazole (VI)—The free base of the above amine (0.63 g.) and isovaleryl chloride (1.1 g.) were gently refluxed for 20 min. The reaction mixture was then cooled, dissolved in 10 ml. of 1 *N* HCl, and extracted three times with equal portions of ether. The aqueous solution was basified with concentrated ammonium hydroxide to pH 9.0 and extracted with hot chloroform in a liquid–liquid extractor for 24 hr. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness. Crude VI was recrystallized from methanol–ether, yielding 0.61 g., m.p. 117°; IR (amide) 1630 $cm.^{-1}$.

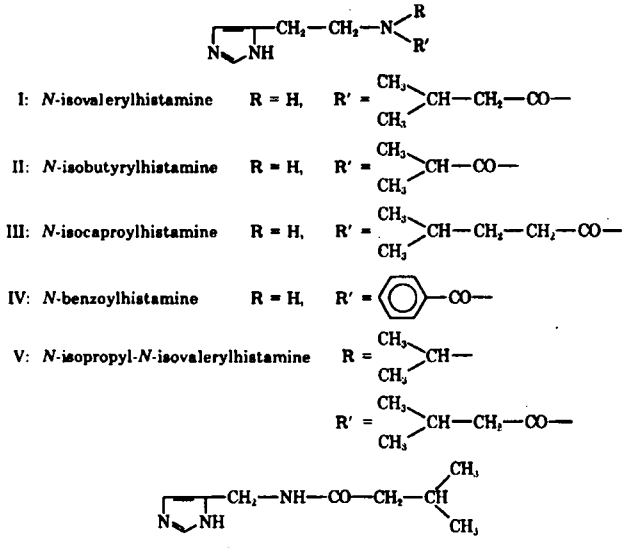
Anal.—Calc. for $C_9H_{15}N_3O$: C, 59.64; H, 8.34; N, 23.19. Found: C, 59.45; H, 8.37; N, 23.23.

Preparation of Labeled Precursors—Isovaleric acid (carboxyl-^{14}C), isocaproic acid (carboxyl-^{14}C), isobutyric acid (carboxyl-^{14}C), and benzoic acid (carboxyl-^{14}C) were purchased from commercial sources.

4(5)-Aminomethylimidazole (ring-2-^{14}C) was prepared by the method of Pyman (13, 16). This synthesis was initially accomplished using nonradioactive material. The product displayed an IR spectrum identical with that of 4(5)-aminomethylimidazole dihydrochloride and showed no melting-point depression when mixed with authentic 4(5)-aminomethylimidazole dihydrochloride. Diaminoacetone dihydrochloride (100 mg.) was added to a hot solution of 66 mg. of potassium thiocyanate-^{14}C in 0.2 ml. of water, and the mixture was heated on a steam bath for 30 min. The resulting crystalline product was filtered, added to 5 ml. of hot water, and filtered again.

The filtrate was added to 900 mg. of ferric chloride in 10 ml. of water, and the mixture was digested for 30 min. on the steam bath. Three milliliters of 10% aqueous sodium carbonate was added, followed by a hot solution of 400 mg. of picric acid in 10 ml. of boiling water. The mixture was boiled with a little charcoal and filtered. On cooling, 96 mg. of 4(5)-aminomethylimidazole-2-^{14}C (ring) dipicrate separated, m.p. 210–212° [lit. (16) m.p. 212°].

The dihydrochloride was prepared by treating the picrate with 5 ml. of 6 *N* HCl, removing the picric acid with four successive 5-ml.



portions of benzene, evaporating the acidic aqueous solution to dryness, and recrystallizing the residue from methanol–water, yielding 22 mg., m.p. 241–243°.

Plants—Living plants of *D. sphaerica*[3] were maintained in a controlled environment lab to provide optimal light, temperature, and humidity (17, 18).

Administration of Test Compounds—All precursors, with the exception of benzoic acid, were dissolved in 1 ml. of sterile water and slowly injected, by means of a 1.0-ml. sterile disposable syringe and a sterile 26-gauge, 1.3-cm. (0.5-in.), hypodermic needle, into the fleshy roots of 10 plants. The benzoic acid was dissolved in 1 ml. of 0.05 *N* NaOH and similarly injected into 10 plants. Nonradioactive *N*-isopropylhistamine was introduced simultaneously with isovaleric acid-1-^{14}C (sodium salt). After each injection, the needle was left in place for a minute to allow the solution to disperse. Following this procedure, the plants were carefully repotted, watered, and returned to the environment lab.

Isolation of Aberrant Alkaloids—Three weeks after the injection of the labeled precursors, the plants were removed from the controlled environment lab and sliced, dried in an oven at 45° for 96 hr., and ground. In each case, 500 mg. of the appropriate, nonradioactive dolichotheline analog was added to the ground material. The method used for the extraction and isolation of dolichotheline and its unnatural analogs was identical to that previously described for the isolation of dolichotheline (19). Any radioactivity residing in the unnatural analog was monitored using TLC and radiochromatogram scanning. When the analog was devoid of activity, no further work was attempted. On the other hand, when the analog did contain radioactivity, it was separated from dolichotheline by preparative TLC.

Separation of III from Dolichotheline—A mixture (600 mg.) of III and dolichotheline was dissolved in a small volume of methanol and applied (100 mg./plate) to a 2-mm. layer of silica gel GF_{254}, 20 × 20-cm. plates. The plates were then subjected to multiple development (three times) using benzene–methanol–chloroform–concentrated ammonium hydroxide (3:1:6:0.1) as the solvent system. The solvent was allowed to migrate to 2 cm. from the top edge of the plate, and the plate was carefully dried each time upon removal from the developing chamber. The R_f value for dolichotheline was 0.20; for III, it was 0.28. The area corresponding to III on each plate was carefully scraped off, and the silica gel was eluted with three 100-ml. portions of methanol. The eluant was filtered and evaporated to dryness. The residue was dissolved in 100 ml. of hot chloroform, which was then dried over anhydrous sodium sulfate, filtered, and reduced to dryness to yield *N*-isocaproylhistamine, 273 mg., m.p. 136–137°.

Separation of VI from Dolichotheline—A mixture (300 mg.) of VI and dolichotheline was separated by a method similar to that employed for the separation of III and dolichotheline. The preparative plates in this case were developed three times using chloroform–ethanol–1 *N* HCl (8:2:0.1) as the solvent system. The R_f for dolichotheline was 0.39; for VI, it was 0.55. The silica gel scraped off the plates was extracted with three 100-ml. portions of methanol. The methanol extract was reduced to dryness and dissolved in 15 ml. of water; the pH was adjusted to 9.5 with concentrated ammonium hydroxide, and the solution was extracted with hot chloroform in a liquid–liquid extractor for 24 hr. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to yield 113 mg. of VI, m.p. 115–116°.

***N*-Isocaproylhistamine Picrate**—III (100 mg.) was dissolved in 1 ml. of ethanol, 1 ml. of a saturated solution of picric acid in ethanol was added, and the mixture was stirred with a glass rod. The resulting yellow crystals were filtered and washed with a few drops of water. The picrate was recrystallized from water, yielding 82 mg., m.p. 138°.

Anal.—Calc. for $C_{17}H_{23}N_6O_8$: C, 46.57; H, 5.06; N, 19.17. Found: C, 46.50; H, 5.11; N, 19.29.

Using an identical procedure, 20 mg. of radioactive III was reacted with picric acid to yield 13 mg. of *N*-isocaproylhistamine picrate, m.p. 137–138°.

4(5)-[*N*-Isovalerylaminomethyl]imidazole Picrate—The amide (100 mg.) was dissolved in 1 ml. of ethanol, and 1 ml. of a saturated solution of picric acid in ethanol was added. The resulting yellow crystals were filtered and rinsed with a few drops of water. This picrate derivative was recrystallized from water, yielding 79 mg., m.p. 171°.

Anal.—Calc. for $C_{15}H_{18}N_6O_8$: C, 43.90; H, 4.42; N, 20.48. Found: C, 43.69; H, 4.38; N, 20.41.

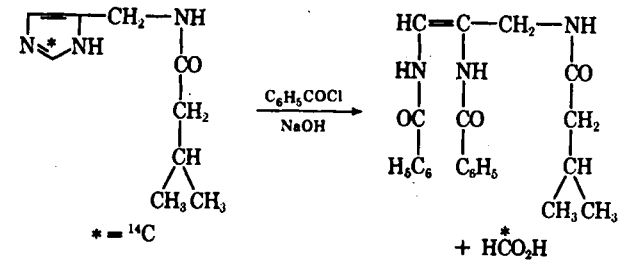
Using the above procedure, 20 mg. of the separated radioactive amide was reacted with picric acid to obtain the picrate derivative, 15 mg., m.p. 170–171°.

Degradation of VI (Scheme I)—The amide (190 mg.) was added to a cold solution of 1 g. of sodium hydroxide in 5 ml. of water. This was followed by the addition of 0.5 ml. of benzoyl chloride and 2 drops of benzene. The mixture was stirred vigorously for 2 hr. with cooling, after which the solid product was filtered, washed with a little water, and dried. The 1,2-dibenzoylamino-3-isovalerylaminopropene-1 was recrystallized from ethanol–water, yielding 170 mg., m.p. 205°.

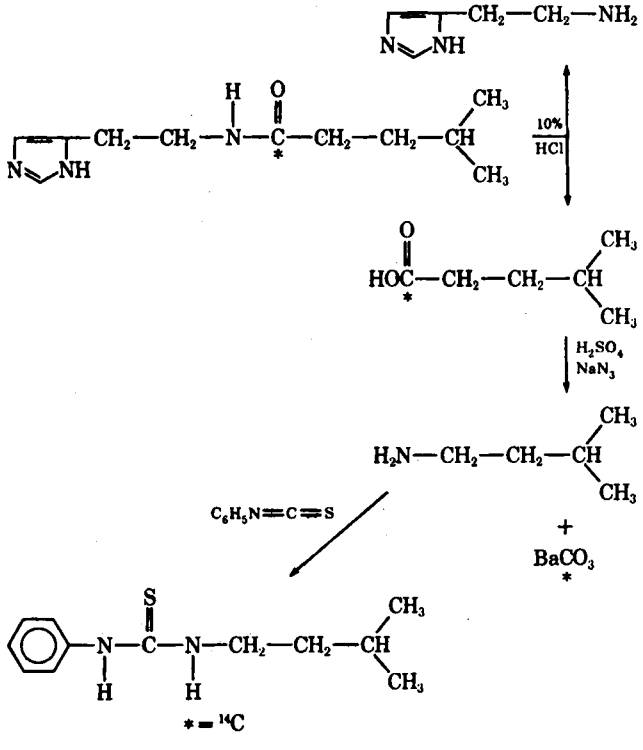
Anal.—Calc. for $C_{22}H_{25}N_3O_3$: C, 69.64; H, 6.64; N, 11.07. Found: C, 69.46; H, 6.58; N, 11.19.

Using an identical procedure, 40 mg. of the separated radioactive amide was reacted with benzoyl chloride to yield 21 mg. of 1,2-dibenzoylamino-3-isovalerylaminopropene-1, m.p. 204–205°.

Degradation of III (Scheme II)—III was hydrolyzed with 10% HCl, and the resulting histamine and isocaproate were recovered as the dihydrochloride and sodium salts, respectively, by identical procedures described for the hydrolysis of dolichotheline (6). The sodium isocaproate was further degraded by means of a Schmidt decarboxylation essentially as described by Phares (20). The sodium isocaproate and sodium chloride mixture (300 mg.) was dissolved



Scheme I—Degradation of VI



Scheme II—Degradation of III

[3] Purchased from El Paso Cactus Gardens, Anthony, N. M.-Tex. Living voucher specimens are on deposit at the University of Michigan Botanical Gardens, Ann Arbor, Mich.

Table II—^{14}C-Labeled Precursors Tested

Precursor	Position of Label	Specific Activity, mc./mM	Amount Injected, μ	Percent Injected Activity Recovered as Dolichotheline Analog
Isocaproic acid (sodium salt)	1-^{14}C	2.1	300	0.81
Isobutyric acid (sodium salt)	1-^{14}C	30.0	212	—
Benzoic acid	Carboxyl-^{14}C	2.65	179	—
4(5)-Aminomethylimidazole dihydrochloride	2-^{14}C (ring)	0.70	75.5	1.27
N-Isopropylhistamine and isovaleric acid (sodium salt)	1-^{14}C	2.0	70.5	—

Table III—Compound III Obtained from Isocaproic Acid-1-^{14}C-Injected Plants

Compound	Yield, mg.	Melting Point	Reference Melting Point	Mixed Melting Point	Specific Activity, d.p.m./mM
III (crude)	273	136–137°	137–138°	—	2.44×10^6
III recrystallized one time	246	137–138°	137–138°	—	2.44×10^6
III recrystallized two times	208	137–138°	137–138°	137–138°	2.45×10^6
III picrate	13	137–138°	138°	137–138°	2.27×10^6
Histamine dihydrochloride	53	239–243°	243–246°	242–244°	0.002×10^6
Isoamylphenylthiourea	19	100–101°	102°	101–102°	0.019×10^6

in cold 100% sulfuric acid (3 ml.) in a 25-ml. pear-shaped flask, and powdered sodium azide (250 mg.) was added in small amounts, with shaking, to the chilled solution. The flask was immediately connected to two bead towers in series, the first containing 10 ml. of 5% potassium permanganate in 1 N H_2SO_4 and the second containing 10 ml. of 1 N carbon dioxide-free sodium hydroxide. A slow stream of carbon dioxide-free air was drawn through the solution while the flask was heated to 60–65° for 7 hr. The bead tower containing the sodium hydroxide was then washed down with carbon dioxide-free water, and 2 ml. of a saturated solution of barium chloride was added. The resulting precipitate was centrifuged and the supernate was decanted. The barium carbonate was resuspended in water and again centrifuged. This process was repeated with ethanol and the carbonate was finally dried in a desiccator.

To recover the isoamylamine, the contents of the reaction flask were basified to pH 12 with 20% sodium hydroxide and the isoamylamine was extracted three times with equal portions of ether. The isoamylamine was then reacted with phenylisothiocyanate to form isoamylphenylthiourea (21).

Radioactive Assay—The method employed for the preparation of samples for scintillation counting was described previously (17, 18). The aberrant products were recrystallized to constant specific activity, and the single derivative of each product was also counted as a check of the radiopurity. The two degradation products were assayed to localize the label. The barium carbonate (0.5 mg.) was assayed as a suspension in scintillation fluid containing a thixotropic gel[4] (5 g./1000 ml. of scintillation cocktail). All samples were counted in a liquid scintillation system[5] at a preset 2σ statistical counting error of 1%. The external standard ratio method was used to determine losses due to quenching. All counts were corrected for counter efficiency.

RESULTS AND DISCUSSION

The data obtained from the *in vivo* studies on the production of aberrant alkaloids are summarized in Tables II–IV. They indicate that isocaproic acid and 4(5)-aminomethylimidazole can serve as precursors of aberrant alkaloids in *D. sphaerica*. The data also show that isobutyric acid, benzoic acid, and N-isopropylhistamine do not serve as precursors of their corresponding dolichotheline analogs.

When III obtained from plants injected with isocaproic acid-1-^{14}C was hydrolyzed, the activity resided in the isocaproate portion of the molecule. Decarboxylation by the Schmidt reaction provided carbonate which contained greater than 98% of the activity present in the sodium isocaproate. The remaining isoamylamine, isolated as isoamylphenylthiourea, was essentially inactive.

The plants injected with 4(5)-aminomethylimidazole (ring-2-^{14}C) yielded VI which, when benzoylated, yielded nearly inactive 1,2-dibenzoylamino-3-isovalerylaminopropene-1. This degradation demonstrated that greater than 98% of the activity resided in the expected position.

Calculations of percent injected activity recovered as dolichotheline analog are based on the 500 mg. of the analog added as carrier. Since the actual amount of analog formed is less than 1 mg., the error made in calculating percent incorporation based on a total yield of 500 mg. is very small.

Of the five unnatural precursors tested, only two, isocaproic acid and 4(5)-aminomethylimidazole, were incorporated into unnatural or aberrant alkaloids. With respect to the specificity of the enzymes involved, it appears that the activating enzyme(s) is capable of activating isocaproic acid, the higher homolog of isovaleric acid, to isocaproyl-CoA. In addition, the condensing enzyme can utilize the isocaproyl-CoA as a substrate and bind it to histamine in amide linkage. Furthermore, this condensing enzyme can form an amide bond between isovaleryl-CoA and a compound with an aminomethyl side chain at the 4(5)-position of the imidazole nucleus in addition to an aminoethyl side chain.

The differences in the incorporation (0.81% in III and 1.27% in VI) are reasonable because 4(5)-aminomethylimidazole occupies a more proximal position to the final biosynthetic step than isocaproic acid, which must first be activated to isocaproyl-CoA. However, the differences may also be due to differences in *in vivo* metabolism of the two precursors, although such metabolic differences are probably of little significance in this case.

The positive result obtained from the isocaproic acid experiment makes it seem surprising that isobutyric acid was not utilized as a precursor of its corresponding dolichotheline analog. This failure may be attributed to the specificity of the enzymes involved. However, a more plausible explanation is that isobutyric acid was activated to isobutyryl-CoA, a naturally occurring intermediate in the catabolism of valine (22), and then was further metabolized along the normal pathway for valine degradation,

In the animal body, benzoic acid is activated to benzoyl-CoA, which is then condensed with glycine to form hippuric acid (23–25). These reactions may be analogous to the activation of isovaleric acid to isovaleryl-CoA and the subsequent condensation of the latter with histamine. It was reasonable to assume that the benzoic acid injected into *D. sphaerica* would result in the benzoylation of

[4] Cab-O-Sil.
[5] Beckman LS-150.

Table IV—Compound VI Obtained from 4(5)-Aminomethylimidazole-2-^{14}C(ring)-Injected Plants

Compound	Yield, mg.	Melting Point	Reference Melting Point	Mixed Melting Point	Specific Activity, d.p.m./mM
VI (crude)	113	115–116°	117°	—	7.72×10^5
VI recrystallized one time	91	116–117°	117°	—	7.70×10^5
VI recrystallized two times	60	116–117°	117°	116–117°	7.79×10^5
VI picrate	15	170–171°	170°	170–171°	7.73×10^5
1,2-Dibenzoylamino-3-isovalerylamino-propene-1	21	204–205°	205°	204–205°	0.10×10^5

histamine. This expected formation of IV did not take place, possibly because of enzyme specificity and/or *in vivo* metabolism of the benzoic acid.

The remaining unnatural precursor, *N*-isopropylhistamine, also did not yield the expected dolichotheline analog. Perhaps the specificity of the condensing enzyme is such that it requires a primary amine rather than a secondary amine to be present in the histamine side chain in order to form the amide linkage.

These investigations with unnatural precursors are attempts to prepare aberrant dolichotheline analogs in the hope that these model techniques can be applied in the preparation of analogs of biologically active natural products that are difficult to synthesize. Furthermore, this work is an *in vivo* approach to the study of the specificity of the enzyme system that catalyzes the condensation of isovaleric acid and histamine. In this respect, the present experiments not only show that this enzyme system is not completely specific but also partially defines the specificity. Finally, the experiments provide additional support for the hypothesized final step in the biosynthesis of dolichotheline.

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Cactus Alkaloids XV: β-Phenethylamine Derivatives from *Coryphantha macromeris* var. *runyonii*

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Abstract □ Eleven β-phenethylamine derivatives were isolated and identified from fresh-frozen specimens of *Coryphantha macromeris* var. *runyonii* (Br. and R.) L. Benson. Isolations were accomplished using preparative TLC, and identifications were based on chromatographic properties, IR and NMR spectra, and comparisons with authentic materials. The data provide initial documentation for: the occurrence of *N*-formylnormacromerine in nature; the first recorded detection of metanephrine and *N*-methylmetanephrine in the plant kingdom; the first occurrence of synephrine and *N*-methyltyramine in this cactus; confirmation of earlier GLC–mass spectral data on the presence of *N*-methyl-4-methoxy-β-phenethylamine, *N*-methyl-3,4-dimethoxy-β-phenethylamine, tyramine, and hordenine in the plant; and verification of previous reports on the isolation of macromerine and normacromerine.

Keyphrases □ Cactus alkaloids—isolation, identification of 11 β-phenethylamine derivatives from *Coryphantha macromeris* var. *runyonii* □ *Coryphantha macromeris* var. *runyonii*—isolation and identification of 11 β-phenethylamine derivatives □ *N*-Formylnormacromerine—isolation, identification from *Coryphantha macromeris* □ Metanephrine and *N*-methylmetanephrine—isolation, identification from *Coryphantha macromeris* □ Synephrine—isolation, identification from *Coryphantha macromeris* □ *N*-Methyltyramine—isolation, identification from *Coryphantha macromeris*

Dona Ana [*Coryphantha macromeris* (Engelm.) Br. and R.] is currently being promoted as a "natural and legal" psychedelic agent (1). The hallucinogenic alkaloid, (−)-macromerine (*N*,*N*-dimethyl-3,4-dimethoxy-β-hydroxy-β-phenethylamine), has been isolated from the cactus (2), and *C. runyonii* Br. and R., which is considered currently to be a variety of the former species (3), yielded the same compound (4). *N*-Methyl-4-methoxy-β-phenethylamine, *N*-methyl-3,4-dimethoxy-β-phenethylamine, tyramine, and hordenine (*N*,*N*-dimethyltyramine) were detected as minor alkaloids of *C. macromeris* var. *runyonii* (Br. and R.) L. Benson by combining GLC with mass spectrometry (5, 6). Recently, (−)-normacromerine (*N*-demethylmacromerine) was observed to be a major nonphenolic alkaloid of this variety (7).

The present phytochemical studies of *C. macromeris* var. *runyonii* were undertaken to isolate and confirm the identities of previously detected alkaloids and to continue an examination of the alkaloid fraction of this cactus.

EXPERIMENTAL[1]

Plant Material—Living specimens of *C. macromeris* var. *runyonii*[2] were purchased from two commercial sources[3]. A portion of each shipment was frozen immediately upon receipt, and the remainder was oven dried at 48° and ground to a coarse powder.

Isolation of Crude Alkaloid Fractions—Fresh-frozen plant material (2.54 kg.) was diced and homogenized with 95% ethanol in a large blender[4]. The extraction was carried out before any appreciable thawing had occurred, thereby effectively chilling the extraction mixture. The filtered ethanolic extract was concentrated to approximately one-fourth volume under reduced pressure at 45°. This aqueous solution was acidified by addition of 600 ml. of 25% acetic acid and was extracted with two successive 500-ml. portions each of ether, ethyl acetate, and chloroform to remove the nonpolar, nonalkaloidal material. The resulting aqueous solution was rendered basic with 28% ammonium hydroxide, and the alkaloids were extracted successively with three 500-ml. volumes of chloroform and one 500-ml. volume of ether. Then the combined chloroform–ether extract was filtered through anhydrous sodium sulfate and concentrated to a syrup; this crude alkaloidal mixture was resolved into phenolic and nonphenolic fractions, using an ion-exchange resin as previously described (8).

TLC—A 0.25-mm. layer of silica gel GF_{254} was used for the analytical TLC plates, and the preparative TLC plates were prepared with a 1-mm. layer of silica gel PF_{254}[5]. Five solvent systems were used for the various analytical and preparative separations: A, benzene–chloroform–methanol–28% ammonium hydroxide (8:6:5:1); B, ether–methanol–28% ammonium hydroxide (20:2:1); C, chloroform–*n*-butanol–methanol–28% ammonium hydroxide (145:3:42:10); D, ether–acetone–methanol–28% ammonium hydroxide (9:8:2:1); and E, ethyl acetate–methanol–28% ammonium hydroxide (17:2:1). Alkaloids on the chromatograms were detected under shortwave UV light followed by use of dansyl chloride (0.05% in acetone) and tetrazotized benzidine spray reagents (8).

Synthesis of Reference Nonphenolic Alkaloids—Established synthetic procedures were used to produce racemic macromerine (2) and normacromerine (7). Reference *N*-methyl-3,4-dimethoxy-β-phenethylamine was synthesized by refluxing equimolar quantities of 3,4-dimethoxyphenethylamine[6] and ethyl formate[7] to give an *N*-formyl intermediate, which was subsequently reduced with lithium aluminum hydride to yield the desired product. After purification by preparative TLC with Solvent A, the product was converted to the hydrochloride and then recrystallized from absolute ethanol–ether (49% yield). A similar procedure was employed for the production of reference *N*-methyl-4-methoxy-β-phenethylamine hydrochloride (46% yield), using 4-methoxyphenethylamine[8] as the reactant.

Resolution of Nonphenolic Alkaloids—Analytical TLC using Solvents A, B, and E revealed the presence in this fraction of seven alkaloid-positive substances, two of which were present only in trace amounts. The alkaloid mixture was dissolved in ethanol–chloroform (1:1) and streaked onto 78 preparative TLC plates using

[1] UV spectra were determined in absolute methanol with a Beckman DB spectrophotometer scanning from 340 to 200 nm. IR spectra were obtained neat or using KBr pellets with a Beckman IR5A. NMR spectra were recorded on a Varian T-60 NMR spectrometer using $CDCl_3$ or D_2O solutions. Specific rotations were determined with a Rudolph polarimeter using a sodium lamp and a 2-dcm. tube. Melting points were taken with a Fisher-Johns melting-point apparatus and are uncorrected.

[2] Identification was confirmed by Dr. E. F. Anderson, Department of Biology, Whitman College, Walla Walla, WA 99362. Representative plants are being maintained as greenhouse specimens.

[3] From Sunderland's Cactus Garden, Alamo, TX 78516, and Texas Cactus Gardens, Canutillo, TX 79835

[4] Waring.

[5] Brinkmann Instruments.

[6] Calbiochem.

[7] Aldrich Chemical Co.

[8] Calbiochem.

a previously described technique (9). The alkaloid material was eluted from the silica gel scrapings with three portions of 95% ethanol; the extracts were filtered, combined, and concentrated to near dryness under reduced pressure. The residue in each case was taken up in 40 ml. of 5% HCl, and the solution was rendered distinctly alkaline with 28% ammonium hydroxide and extracted immediately with three 50-ml. portions of chloroform. The chloroform extracts were filtered through sodium sulfate, combined, and reduced to a 5-ml. volume with a rotary evaporator. At this point, each alkaloidal solution was monitored for indication of impurities using TLC, the chloroform was evaporated from the solutions under a stream of nitrogen, and the residues were dissolved in 5 ml. of 95% ethanol and stored under nitrogen in a freezer.

This initial resolution of the nonphenolic alkaloid fraction resulted in two chromatographically pure alkaloids and a ternary mixture. Cochromatography with reference materials suggested that the pure alkaloids were (−)-normacromerine and (−)-macromerine and that the ternary mixture consisted of *N*-methyl-4-methoxy-β-phenethylamine, *N*-methyl-3,4-dimethoxy-β-phenethylamine, and an unidentified compound.

Resolution of Ternary Nonphenolic Mixture—An equal volume of chloroform was added to the ethanolic solution of the ternary alkaloid mixture, and the material was streaked onto 44 preparative TLC plates. Solvent B resolved the mixture into three components, which were removed and processed as already described. The chromatographic evaluations revealed no impurities in the isolated constituents.

Identification of Known Nonphenolic Alkaloids—The ethanolic solutions of the isolated alkaloids were evaporated under a stream of nitrogen, the residues were taken up in 0.7 ml. of deuterochloroform[9], and the NMR spectra were recorded. Deuterium-exchange experiments were carried out in the cases of macromerine and normacromerine. The deuterochloroform solutions were then evaporated to dryness under nitrogen, the residues were dissolved in small volumes of absolute ethanol, the solutions were acidified with anhydrous hydrochloric acid in absolute ethanol (5% w/w), and anhydrous ether was added slowly to effect the crystallization of the hydrochloride salts. One recrystallization from absolute ethanol assured chemical purity. The yields of crystalline hydrochloride salts, the melting points, and the IR spectra (KBr pellet) were recorded. Various properties of the isolated alkaloids corresponded with those reported for normacromerine (7), macromerine (2, 4, 10), *N*-methyl-3,4-dimethoxy-β-phenethylamine (11, 12), and *N*-methyl-4-methoxy-β-phenethylamine (11). Identifications were confirmed by direct comparisons of corresponding IR and NMR spectra of the isolated constituents and synthetic reference compounds.

Characterization of Unknown Nonphenolic Alkaloid—The isolated material was a chromatographically homogeneous (Solvent A, R_f 0.70; Solvent B, R_f 0.42; Solvent E, R_f 0.47), golden-yellow oil which possessed some distinctive properties. The constituent failed to react or to give visible chromophores with the dansyl chloride and tetrazotized benzidine reagents, but it could be detected with Dragendorff's reagent. It also failed to form a crystalline hydrochloride salt. The UV spectrum (0.0066 mg./ml. absolute methanol) showed indication of a λ_{max} at 201 (ε 32,200) and peaks at 229 (ε 6100) and 278 (ε 1800) nm., absorption features indicative of a 3,4-dimethoxy-β-phenethylamine (12). The IR spectrum (neat) exhibited a broad hydroxyl band near 3333 $cm.^{-1}$ and an intense carbonyl stretching vibration at 1642 $cm.^{-1}$. The remainder of the IR spectrum was typical for a phenethylamine derivative. An interpretation of the NMR spectrum (deuterochloroform) was quite informative regarding the structure of the unknown alkaloid. A skewed pair of singlets at 7.95 δ integrating for one proton was attributed to an *N*-formyl proton, peaks at 6.99 and 6.91 δ were indicative of three aromatic protons, and a coalescent doublet centered at 3.88 δ indicated two methoxyl groups. A benzylic hydroxyl proton (4.2 δ) exhibited shifts with concentration differences and disappeared with deuterium exchange. Complex multiplets centered at 4.8 and 3.4 δ indicated a benzylic proton and a methylene group, respectively, while a three-proton singlet at 2.9 δ characterized an *N*-methyl group. On the basis of these data, it appeared that the new alkaloid was the *N*-formyl analog of normacromerine. Synthetic normacromerine was refluxed with an equimolar quantity of ethyl formate to obtain a reference sample of *N*-formylnormacromerine. Comparison of the isolated and synthetic materials revealed indistinguishable UV, IR, and NMR spectra and chromatographic properties.

Resolution of Phenolic Alkaloids—Five major constituents and traces of three other compounds were detected using analytical TLC with Solvents C and D. The phenolic alkaloid fraction was dissolved in an ethanol–chloroform (1:1) mixture and streaked onto 38 preparative TLC plates. After development with Solvent C, the individual plates and resulting silica gel scrapings were processed in the same manner described for the nonphenolic fraction. This separation process resulted in four fractions, three of which were chromatographically pure; the fourth fraction was a binary mixture. Chromatographic comparisons[10] indicated that the pure alkaloids were synephrine, metanephrine, and hordenine and that the binary mixture contained tyramine and *N*-methyltyramine.

Resolution of Binary Phenolic Mixture—The binary fraction was dissolved in a suitable volume of ethanol–chloroform (1:1) and streaked onto nine preparative TLC plates. The plates were developed with Solvent D, and the two desired bands were processed in the usual manner.

Identification of Phenolic Constituents—Characterization of the five isolated constituents proceeded essentially as described for the nonphenolic alkaloids. The observed melting points and IR spectra of the hydrochloride salts corresponding to hordenine and *N*-methyltyramine agreed with reported properties for these substances (8, 12–14). Identifications were confirmed by direct comparisons of IR and NMR spectra of the isolated and reference materials.

The melting points and the IR spectra of the salts from the fractions chromatographically identified as metanephrine, synephrine, and tyramine were consistent with anticipated and reported properties for the corresponding salts (14–16), and no distinctive features were noted upon direct comparisons of the IR spectra of isolated and reference compounds. The small quantities of these three constituents obtained from the cactus (Table I) were insufficient for the determination of NMR spectra. Since tyramine had been detected previously in the plant (5), the chromatographic, melting-point, and IR spectral data were considered adequate to confirm the identity of this constituent. However, NMR spectra or some additional evidence appeared desirable with the other two compounds since the occurrence of metanephrine in the plant kingdom was unknown, since the presence of synephrine in this cactus was unrecorded, and since the IR spectra of the racemate and levorotatory isomer of synephrine were known to exhibit some differences (16).

Preliminary experiments with oven-dried cactus material indicated that the integrity and concentration of both metanephrine and synephrine were unaltered, even though the heat-drying process appeared to produce some artifacts. Thus, an available supply of dried cactus was processed to obtain sufficient quantities of metanephrine and synephrine for further characterization. The coarsely powdered plant material (1.55 kg.) was extracted exhaustively with methanol in a large blender, and the methanolic solution was removed by filtration. The thick syrup, which was obtained upon removal of the methanol under reduced pressure, was acidified with 700 ml. of 5% HCl and filtered. The acidic solution was extracted twice with approximately 700-ml. volumes of a series of organic solvents (petroleum ether, ether, ethyl acetate, and chloroform). This aqueous solution was then adjusted to pH 10.5 (pH meter) with 7.5 *N* NaOH and again subjected to partitioning with the series of organic solvents. The resulting aqueous solution, which contained relatively water-soluble constituents including metanephrine and synephrine, was placed under reduced pressure in a rotary evaporator to remove traces of the organic solvents and was freeze dried. The residue from lyophilization was extracted several times with an ethanol–chloroform (1:9) mixture, and the combined extract was concentrated to a small volume under reduced pressure and examined chromatographically. The concentrated extract, which contained a number of nonalkaloidal substances, was subjected to ion-exchange chromatography[11] and an established method (8) to give an aqueous phenolic fraction rich in metanephrine and synephrine with only traces of other phenolic alkaloids and contaminants. This

[9] Tetramethylsilane from Stohler Isotope Chemicals was used as the internal standard.

[10] Sources of reference phenolic alkaloids: hordenine sulfate and tyramine hydrochloride, Nutritional Biochemicals Corp.; *N*-methyltyramine hydrobromide, courtesy of Hoffmann-La Roche, Inc.; synephrine tartrate, K & K Laboratories, Inc.; and metanephrine hydrochloride, Sigma Chemical Co.

[11] Using Amberlite IRA-401S C.P., Mallinckrodt Chemical Works.

phenolic fraction, dissolved in a small volume of equal parts of ethanol and chloroform, was streaked onto 48 preparative TLC plates. Solvent C was used to achieve separation, and processing of the desired bands gave chromatographically homogeneous, ethanolic extracts of the two phenolic alkaloids.

The ethanolic extract containing synephrine was evaporated to dryness under nitrogen. A portion of the residue was taken up in 4 ml. of absolute methanol, the solution was heated to boiling, and 12 mg. of dry oxalic acid was added. Crystals of synephrine oxalate formed upon cooling, and recrystallization from aqueous methanol gave 13 mg. of material, m.p. 223–224° dec. [lit. (16) m.p. 221–222° dec.]. The remaining portion of the isolated alkaloid was dissolved in a minimum volume of absolute ethanol and converted to the hydrochloride salt; one recrystallization gave a yield of 33 mg., m.p. 166–167°. A portion of the hydrochloride salt (13 mg.) was placed in an NMR tube, 0.7 ml. of D_2O and a small quantity of an internal standard (sodium 2,2-dimethyl-2-silapentane-5-sulfonate[12]) were added, and the NMR spectrum was recorded. The number of exchangeable protons was determined by the integration difference of the sample and a D_2O–internal standard blank of comparable volume run at the same spectrum and integral amplitudes. The observed NMR spectrum of the salt of the isolated alkaloid followed a predictable chemical-shift pattern and proved to be identical to the spectrum obtained with reference material. The hydrochloride salt of the isolated synephrine was levorotatory: $[\alpha]_D^{27}$ −52.5° (c 0.010 g./ml. in absolute methanol).

The ethanolic extract of the isolated metanephrine was evaporated to dryness under nitrogen, and the entire residue was converted to the hydrochloride salt. One recrystallization from absolute ethanol–ether gave 9 mg. of crystalline material, m.p. 158–159°. The NMR spectral studies were carried out with the hydrochloride in D_2O solution as described for synephrine. The predicted NMR spectrum and an excellent NMR spectral correlation between the isolated and reference compounds provided the desired evidence to confirm the presence of metanephrine in the plant extracts. The small quantity of isolated material did not permit determination of optical rotation.

DISCUSSION AND CONCLUSIONS

Five nonphenolic and five phenolic β-phenethylamine derivatives were isolated and identified from *C. macromeris* var. *runyonii* (Table I). Trace quantities of two nonphenolic and three phenolic constituents[13] were also detected, but the minimal amounts of these substances in the quantity of cactus employed in the investigation precluded their purification and characterization. The use of a cold extraction process and fresh-frozen plant material, except for certain studies on metanephrine and synephrine, circumvented the possibility of artifact formation during slow thermal drying. Thus, the 10 isolated compounds, as well as the five unidentified alkaloid-positive substances which were detected, were considered to be metabolic constituents of the cactus.

Identifications of the 10 isolated constituents were based on chromatographic properties, melting points of salts, IR and NMR spectra, and direct comparisons with reference materials. The only exceptions to this protocol involved *N*-formylnormacromerine, which did not form a salt, and the omission of NMR data for tyramine.

N-Formylnormacromerine, metanephrine, and synephrine were the most interesting of the isolated constituents. The natural occurrence of *N*-formylnormacromerine was previously unrecognized. Alkaloids with amide functions are uncommon, and within the Cactaceae, only peyote [*Lophophora williamsii* (Lem.) Coult.] had been shown to possess *N*-formyl derivatives (17). The presence of metanephrine in a biological system was initially demonstrated by Axelrod *et al.* (18), and various chemical and physiological parameters of this metabolite of epinephrine have been studied (19, 20). However, all prior reports directly or indirectly restrict the known occurrence of metanephrine to zoological entities. The natural occurrence of synephrine was first reported from tangerine leaves (16), and until recently it was restricted to the Rutaceae. The known distribution of synephrine has been expanded by its reported presence in genera from the Amaryllidaceae and Moraceae (21), its detection in seven species of coryphantha (22), and its isolation from *C. ramillosa* (15). Identification of synephrine as a constituent of *C. macromeris* var. *runyonii* provides a further expansion of knowledge on the occurrence of this sympathomimetic compound.

The data provided the first evidence for the occurrence of *N*-methyltyramine in *C. macromeris* var. *runyonii;* however, this constituent was known to be distributed widely in the Cactaceae (5, 8, 12–14, 22) and had been detected in other plant families (21). The isolation of hordenine, *N*-methyl-3,4-dimethoxy-β-phenethylamine, *N*-methyl-4-methoxy-β-phenethylamine, and tyramine confirmed earlier GLC–mass spectral data (5, 6) on the presence of these constituents in this cactus. The identification of macromerine and normacromerine from the plant verified previous reports (2, 4, 7).

Macromerine (2), synephrine (23), and the less potent constituents (19, 23–25), hordenine, metanephrine, *N*-methyltyramine, and tyramine, are known to possess sympathomimetic properties. The occurrence of these constituents in the cactus may provide a scientific basis for the purported use of Dona Ana as a CNS stimulant.

Table I—Quantities of β-Phenethylamine Constituents Isolated from Fresh-Frozen *C. macromeris* var. *runyonii*

	Yield[a], g.	Concentration in Cactus[b], %
Nonphenolic constituents:		
Normacromerine hydrochloride	2.133	0.0710
N-Formylnormacromerine (oil)	0.195	0.0077
Macromerine hydrochloride	0.062	0.0021
N-Methyl-3,4-dimethoxy-β-phenylamine hydrochloride	0.019	0.0006
N-Methyl-4-methoxy-β-phenethylamine hydrochloride	0.014	0.0005
Phenolic constituents:		
N-Methyltyramine hydrochloride	0.059	0.0019
Hordenine hydrochloride	0.012	0.0004
Metanephrine hydrochloride	0.006	0.0002
Synephrine hydrochloride	0.003	0.0001
Tyramine hydrochloride	0.002	0.0001

[a] Reported yields represent the total quantities of the constituents or their salts, as indicated, obtained upon processing 2.54 kg. of fresh-frozen plant material. [b] Calculated as free β-phenethylamine in fresh plant material.

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[13] *Note added in proof:* Further observations have provided tentative identification of one of the trace phenolic alkaloids as *N*-methylmetanephrine. The synthesis of reference *N*-methylmetanephrine involved condensation of *O*-acetylvanillin and nitromethane, catalytic reductive methylation with hydrogen and formaldehyde, hydrolysis, and purification by preparative TLC with Solvent C. A six-proton *N*-methyl peak, rather than a three-proton peak, was the only significant NMR spectral difference between the synthetic product and reference metanephrine. The synthetic *N*-methylmetanephrine and one of the bands isolated (yield approximately 1 mg.) during preparative TLC of the phenolic alkaloid fraction were indistinguishable with TLC Solvents A and C, and both the synthetic and isolated materials were extremely labile. This chromatographic detection provides the first evidence for the occurrence of *N*-methylmetanephrine in the plant kingdom.

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Cactus Alkaloids XVI: Isolation and Identification of Alkaloids in *Coryphantha ramillosa*

P. T. SATO, J. M. NEAL, L. R. BRADY, and J. L. McLAUGHLIN▲

Abstract □ The cactus genus *Coryphantha* has been reported to contain certain β-phenethylamine alkaloids. In this investigation, a Texas species, *C. ramillosa* Cutak, yielded five alkaloids which were isolated chromatographically and crystallized as their hydrochloride salts. The isolated compounds were identified as *N*-methyl-4-methoxy-β-phenethylamine hydrochloride, hordenine hydrochloride, *N*-methyltyramine hydrochloride, synephrine hydrochloride, and β-*O*-methylsynephrine hydrochloride, all of which have been previously identified in other *Coryphantha* species.

Keyphrases □ Cactus alkaloids—isolation, identification of alkaloids in *Coryphantha ramillosa* □ *Coryphantha ramillosa*—isolation, identification of alkaloidal content □ Medicinal plants—isolation, identification of alkaloids in *Coryphantha ramillosa* □ TLC—isolation, identification, alkaloids in *Coryphantha ramillosa*

Folk medicine of various peoples of the world has, of necessity, included many different plant drugs (1). One such product is the peyote cactus, which is still used as an amulet, hallucinogen, and panacea by the Indians of Mexico and the southwestern United States (2, 3). Phytochemical investigations of the peyote cactus, or *Lophophora williamsii* (Lem.) Coult., have shown the presence of mescaline, mescaline analogs (β-phenethylamines), tetrahydroisoquinolines, and other compounds, the presence of which explains some of the claimed effects of this plant (4–6). The hallucinogen, mescaline, has also been found in some other cactus species, especially in members of the South American genus trichocereus (7–9).

Knowledge of the alkaloid content of various cacti is significant since it can help to explain or disprove the claimed physiological effects of a particular species. Unfortunately, knowledge of the phytochemical distribution of even the various known cactus alkaloids is quite incomplete. The genus *Coryphantha* is a good example. This genus contains approximately 60 species (10, 11). *C. palmeri* Br. and R. has been reported to have folkloric use as a "narcotic" (12), and *C. macromeris* (Engelm.) Br. and R. has ostensibly obtained some stature as a "natural and legal" psychedelic (13). The presence of macromerine and normacromerine in this latter species may explain these reputed effects (14). The screening of seven species of *Coryphantha* previously demonstrated the presence of alkaloids in all seven, and six β-phenethylamines have been isolated and/or identified in *C. cornifera* (DC.) Br. and R. var. *echinus* (Engelm.) L. Benson (15). No information is available in the literature regarding the phytochemistry of *C. ramillosa* Cutak, a species from southern Texas and north-central Mexico. Although it has no recorded folkloric uses, it was selected for study in a search for unusual cactus alkaloids that might have psychotropic potential.

EXPERIMENTAL

Plant Material—Specimens of the cactus were purchased[1], and

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EXPERIMENTAL

Plant Material—Specimens of the cactus were purchased[1], and

[1] From Homer A. Jones, Southwest Cactus Co., Alpine, Tex.

identification of the plants was made by comparison with the description of Benson (16)[2].

The cacti were sliced and thoroughly dried in a forced air oven at 48° for several days. The dry weight of the plant material was 1.3 kg., representing a weight loss of 3.3 kg. (72% moisture). The dried plant material was then comminuted in a large Wiley mill to obtain a coarse powder.

Preparation of Crude Alkaloid Fractions—The powdered plant material was defatted, basified, and extracted *via* chloroformic percolation as previously described (17, 18). Condensing the filtered chloroform extract produced a thick syrup, which was extracted with two successive 250-ml. portions of 1 *N* HCl; emulsions were treated essentially as was previously reported (19). After filtration, the aqueous solutions were combined and extracted with two successive 500-ml. volumes each of chloroform and ethyl ether to remove organic-soluble, nonalkaloidal material. The pH of the aqueous solution was then adjusted to 10.5 (pH meter) with 7.5 *N* sodium hydroxide, and alkaloids were extracted from the basic aqueous solution with three successive 500-ml. volumes each of chloroform and ethyl ether. These organic extracts were dried over anhydrous sodium sulfate, filtered, combined, and condensed under rotary vacuum to yield a residue (crude alkaloid Fraction A).

In previous studies it had been observed that the organic-extracted, basic, aqueous solution would still retain some water-soluble β-phenethylamines such as tyramine (19). Consequently, this solution was placed under rotary vacuum to remove traces of organic solvents and was then freeze dried. The residue from the lyophilization, largely sodium chloride, was extracted repeatedly with portions of an absolute ethanol–chloroform (1:9) mixture until the filtrates appeared colorless. The combined filtrates were then condensed under rotary vacuum to a small volume (crude alkaloid Fraction B).

Crude alkaloid Fraction A was separated into phenolic and nonphenolic portions by using an anion-exchange resin. The alkaloid fraction, dissolved in ethanol, was passed down a basic ion-exchange[3] column, and the phenolic and nonphenolic portions were obtained as previously described (17).

TLC—Previously described TLC techniques were used analytically to provide a tentative identification of alkaloids in the extracts and preparatively to separate the components (15, 17, 18, 20). Analytical TLC plates were prepared with a 0.25-mm. layer of silica gel G, and preparative plates were prepared with a 1-mm. layer of silica gel PF_{254}[4]. Analytical and preparative separations were achieved by the use of the following solvent systems: Solvent A, ethyl ether–methanol–28% ammonium hydroxide (20:2:1); Solvent B, ethyl ether–methanol–28% ammonium hydroxide (17:2:1); and Solvent C, chloroform–methanol–28% ammonium hydroxide (80:20:1). Solvent D, ethyl acetate–methanol–28% ammonium hydroxide (17:2:1), and Solvent E, benzene–chloroform–methanol–28% ammonium hydroxide (8:6:2:1), were used analytically only. The alkaloids were detected using dansyl chloride (0.05% in acetone) and tetrazotized benzidine spray reagents (17). The R_f values are not reported since routine chromatography with reference compounds provided a superior type of presumptive identification.

Resolution of Nonphenolic Alkaloids from Fraction A—Analytical TLC revealed that this fraction contained a single predominant alkaloid with only traces of other compounds. The entire extract, dissolved in ethanol, was streaked as a narrow band (21) onto five preparative TLC plates and developed with Solvent A. The compound was readily detected under UV light, and the appropriate bands were removed. The alkaloid was leached from the combined bands of the five plates by washing with 5 ml. of ammoniacal ethanol (28% ammonium hydroxide–ethanol, 1:19), centrifuging, and rewashing the adsorbent with two additional 5-ml. portions of the basic ethanol.

After filtration, the combined washings were reduced to near dryness under rotary vacuum. The residue was redissolved in a small amount of absolute ethanol. Analytical TLC indicated that the alkaloid was chromatographically pure. The solution was then acidified (moist pH paper) with anhydrous hydrochloric acid in absolute ethanol (5% w/w). Ethyl ether was slowly added and the solution was refrigerated to cause crystallization of the hydrochloride salt.

Identification of Major Nonphenolic Alkaloid—Analytical TLC in Solvents A, B, D, and E of the major nonphenolic alkaloid and comparison with reference compounds (15) suggested that the alkaloid was *N*-methyl-4-methoxy-β-phenethylamine. The melting points[5] and mixed melting point were identical to that of authentic *N*-methyl-4-methoxy-β-phenethylamine hydrochloride (182.5–184°). The IR spectra[6] of the isolated and synthesized compounds were essentially the same, with distinctive absorption peaks noted at 1600, 1510, 1475, and 1095 $cm.^{-1}$. An interpretation of the NMR spectrum[7] of the isolated material was also consistent with that expected and observed for *N*-methyl-4-methoxy-β-phenethylamine hydrochloride. A pair of doublets centered at 7.1 δ integrated for four protons (aromatic), a three-proton singlet was noted at 3.8 δ (*O*-methyl), a diffuse doublet integrating for four protons was centered at 3.1 δ (methylene), and a three-proton singlet was seen at 2.7 δ (*N*-methyl). The molecular ion (*m/e* 165) observed upon mass spectrometry[8] of the isolated material was in agreement with the molecular weight of the proposed structure.

Resolution of Phenolic Alkaloids from Fraction A—Analytical TLC of this fraction revealed the presence of four major alkaloids. The extract was streaked as a narrow band onto eight preparative TLC plates. The first plate was developed once with Solvent B, and the remaining seven plates were developed twice with Solvent B, allowing a 15-min. interval of air drying between developments. Visualization under UV light allowed the detection of four major bands, which were removed and eluted with ammoniacal ethanol as already described. The residues of each of the four bands were dissolved in an amount of absolute ethanol suitable for analytical TLC.

Resolution of Alkaloids from Fraction B—Analytical TLC of this fraction indicated that it contained the same major alkaloids as observed in the phenolic portion of crude alkaloid Fraction A. This observation suggested the possibility of obtaining these phenolic alkaloids without passing the fraction through the anion-exchange column.

Upon storage of this concentrated fraction in ethanol and under refrigeration, abundant crystallization occurred. The crystals were filtered, rinsed twice with cold ethanol, and recrystallized from boiling benzene. The crystals were then converted to the hydrochloride salt as already described.

Separation of the alkaloids in the combined mother liquors from the ethanol and benzene crystallizations was achieved by preparative TLC. This material was applied as a narrow band onto 42 preparative plates, which were developed twice with Solvent B. The four major bands (A, B, C, and D, lettering from the top of the plate downward) were recovered, and each was eluted with 30 ml. of the ammoniacal ethanol followed by two elutions of 30 ml. each of ethanol. The eluants for each band were filtered, combined, and concentrated to dryness under rotary vacuum.

Further analytical TLC (appropriate selection of Solvent B, C, D, or E) with portions of these residues and the residues from the four phenolic alkaloid bands of Fraction A, with 10-mcg. quantities of reference compounds, provided tentative identifications of the phenolic alkaloids. Slight contamination of band B was observed; to purify this band, further preparative TLC was employed with Solvent C. Crystalline hydrochloride salts of the four compounds were then prepared as previously described.

Identification of Major Phenolic Alkaloids—The four major phenolic alkaloids, purified from Fractions A and B, had the same chromatographic properties as hordenine, β-*O*-methylsynephrine, *N*-methyltyramine, and synephrine. Since the alkaloids from Fraction B and the phenolic portion of Fraction A were identical, studies for verification of identity were carried out only with the crystalline alkaloids obtained from the respective bands of Fraction B.

Cochromatography of authentic hordenine and the crystals that were obtained prior to preparative TLC of Fraction B showed that these two compounds were identical. Analytical TLC (Solvents B

[2] A freeze-dried specimen was sent to Dr. L. Benson, Director, Herbarium of Pomona College, Claremont, Calif., who confirmed the identification and assigned the specimen herbarium No. 317944. In addition, living plants are being maintained for future reference in the greenhouse of the Drug Plant Laboratory, University of Washington.

[3] Amberlite IRA 401S C.P., Mallinckrodt Chemical Works.

[4] Brinkmann Instruments, Inc.

[5] Fisher-Johns melting-point apparatus, uncorrected.

[6] KBr pellets, Beckman IR5A spectrophotometer.

[7] Varian T-60, using 10 mg. of the hydrochloride in tetradeuterated methanol in a microcell.

[8] Performed at Hoffmann-La Roche Laboratories.

and D) further confirmed that band A corresponded to these crystals. Melting points and mixed melting points of authentic hordenine hydrochloride and the hydrochloride of this alkaloid were identical (180.5–181.5°). The IR spectra of hordenine hydrochloride and the isolated compound were also comparable, with distinctive absorption peaks in both spectra noted at 1610, 1512, 1454, and 1348 cm.$^{-1}$.

Reference β-*O*-methylsynephrine and band B consistently cochromatographed (Solvents B and C); their IR spectra, both as the hydrochlorides, exhibited absorption peaks at 2800, 1610, 1512, 1355, 1265, and 1093 cm.$^{-1}$ and agreed in their characteristic features. Additional analytical TLC revealed the presence of a slight contaminant in the isolated crystals, which was assumed to explain three minor peaks present in the IR spectrum of only the isolated β-*O*-methylsynephrine hydrochloride. The melting point of the isolated material was 182–184°, and no melting-point depression was noted on admixture with synthetic (racemic) material. The isolated compound was optically inactive.

TLC of band C (Solvents B, C, and D) and reference *N*-methyltyramine revealed the same chromatographic properties, and melting points of the isolated *N*-methyltyramine hydrochloride and reference *N*-methyltyramine hydrochloride were identical (150–151°). The IR spectra of both compounds, as the hydrochloride salts, were also comparable, with major absorption peaks noted at 1635, 1555, 1530, 1368, 1232, and 1191 cm.$^{-1}$.

Cochromatography of synthetic (racemic) synephrine and the alkaloid from band D (Solvents B, D, and E) revealed indistinguishable chromatographic properties for the isolated and reference compounds. The major peaks of the IR spectra of the two as hydrochlorides were very similar, with matching adsorption peaks at 1610, 1512, and 1449 cm.$^{-1}$. As previously noted (22), the IR spectra of salts of (−)- and (±)-synephrine present generally comparable absorption patterns but with some differences. The melting point of the hydrochloride of the isolated material was 166–167°, in contrast to racemic synephrine hydrochloride which melted at 156–158°. The salt of the isolated alkaloid was optically active: $[\alpha]_D^{27}$ −52.0°, c 7.6 mg./ml. in absolute methanol.

Cold Extraction Process—To eliminate the possibilities that the alkaloids might have been enzymatically modified during the slow heat drying process, that some thermolabile alkaloids had been destroyed or altered, or that the small amount of basic methanol involved in the maceration and extraction process might have caused β-*O*-methylation of syneprine (23), a second cactus sample was examined using a cold extraction process (24) and freeze-dried plant material.

The freeze-dried plant (45.5 g.) was sliced and ground to a powder in a blender at low speed. Cold ethanol was added and the mixture was blended for 25 min. The extract was filtered with the aid of reduced pressure and concentrated to 50 ml. under rotary vacuum (water bath at 36°). About 50 ml. of 5% hydrochloric acid was added and the mixture was shaken vigorously. This acidic ethanolic solution was extracted twice with 50-ml. portions of chloroform and twice with 50-ml. portions of ethyl ether. The pH of the aqueous fraction was then adjusted to 9.5 (pH meter) with 28% ammonium hydroxide. This extract was then run through a basic anion-exchange column, in the manner previously described, to separate phenolic and nonphenolic portions.

Comparison by analytical TLC of the nonphenolic portion obtained from the two different extraction methods revealed the same alkaloid composition. Resolution of the cold-processed nonphenolic extract to the point of crystallization also yielded the same compound as the procedure involving heat.

Comparison of the cold-processed phenolic extract with the previous phenolic extract from Fraction A substantiated the presence of the same alkaloid components in the phenolic extracts prepared by the two procedures.

DISCUSSION AND CONCLUSIONS

Chromatographic procedures have yielded the isolation of one nonphenolic and four phenolic alkaloids from *C. ramillosa*. Using melting points, spectral properties, and chromatographic similarities in comparisons with authentic compounds, identification of the isolated alkaloids was achieved. The isolated nonphenolic alkaloid was established to be *N*-methyl-4-methoxy-β-phenethylamine, and the identified phenolic alkaloids were hordenine, β-*O*-methylsynephrine, *N*-methyltyramine, and synephrine.

Table I—Quantities of Alkaloids Isolated from Soxhlet-Extracted Sample of *C. ramillosa*

	Yield[a], g.	Proportion of Total Alkaloid Fraction, %	Concentration in Cactus[b], %
Hordenine hydrochloride	11.530	91.8	0.73
N-Methyltyramine hydrochloride	0.692	5.5	0.043
β-*O*-Methylsynephrine hydrochloride	0.241	1.9	0.015
Synephrine hydrochloride	0.090	0.7	0.0057
N-Methyl-4-methoxy-β-phenethylamine hydrochloride	0.015	0.1	0.00092

[a] Reported yields represent the total quantities of alkaloid salts obtained upon processing 1.3 kg. of dried plant material. Phenolic alkaloids were recovered from both Fractions A and B. [b] Calculated as free alkaloid base in dried plant material.

Evidence was also obtained that indicated that the isolated compounds, especially β-*O*-methylsynephrine which is believed to be formed by β-*O*-methylation of synephrine during the extraction of tangerine leaves (23), were not artifacts due to the drying and/or extraction processes. These possibilities were discounted when freeze-dried cactus material processed by a cold extraction procedure with ethanol yielded the same alkaloid composition as the initial extracts. Furthermore, no evidence for β-*O*-methylsynephrine formation was detected when reference synephrine was chromatographed with methanol-containing solvent systems. Presumably, the optical inactivity of the isolated β-*O*-methylsynephrine hydrochloride indicated the occurrence of racemization of the natural material during either natural methylation or extraction and purification.

Analytical TLC evaluation illustrated that the five isolated alkaloids were present in the cactus in concentrations several times those of some unidentified trace alkaloids. The compound identified as hordenine was the major alkaloid; it was present in concentrations greater than 10 times that of any other single alkaloid (Table I). Such a high concentration of hordenine has not been observed in any of our previous studies of the cactus alkaloids.

N-Methyl-4-methoxy-β-phenethylamine was isolated previously from *C. macromeris* (Engelm.) Br. and R. var. *runyonii* L. Benson (24), *Ariocarpus retusus* Scheid. (25), and *C. cornifera* (DC.) Br. and R. var. *echinus* (Engelm.) L. Benson (15). Thus far, the natural occurrence of this compound has only been reported in the Cactaceae. No pharmacological studies concerning any possible psychotropic effects of this compound have been reported.

Hordenine and *N*-methyltyramine were previously isolated from several cactus species as well as from other plant families (8, 15, 17–20, 24, 26 *inter alia*). Both are known to have weak sympathomimetic activity (27–29).

β-*O*-Methylsynephrine was isolated previously from the cactus *C. cornifera* var. *echinus* (15). No reported studies concerning its pharmacological activities were detected in the literature; however, a similar compound, β-*O*-methylepinephrine, produces CNS stimulation (30).

Traces of synephrine itself were detected previously in different coryphantha species, and it was isolated from *C. macromeris* var. *runyonii* (24) as well as from tangerine leaves (22). This compound is a known sympathomimetic (29).

The presence of these sympathomimetic β-phenethylamines in *C. ramillosa* confers physiological activity on the cactus and suggests that consumption of it (minus the abundant spines, of course) might cause some stimulant effects.

SUMMARY

This investigation provides the first reported study of the alkaloid content of the cactus *C. ramillosa*. The isolated and identified β-phenethylamine alkaloids included *N*-methyl-4-methoxy-β-phenethylamine, hordenine, β-*O*-methylsynephrine, *N*-methyltyramine, and synephrine.

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Keyphrases □ Tropanes, bifunctional—simplified NMR spectra induced by tris(dipivalomethanato)europium(III) □ Tris(dipivalomethanato)europium(III)—used as NMR shift reagent to produce simplified spectra for bifunctional tropanes □ NMR spectroscopy—tris(dipivalomethanato)europium(III) shift reagent used to produce simplified bifunctional tropane spectra □ Paramagnetic shifts induced by tris(dipivalomethanato)europium(III)—simplified spectra of bifunctional tropanes

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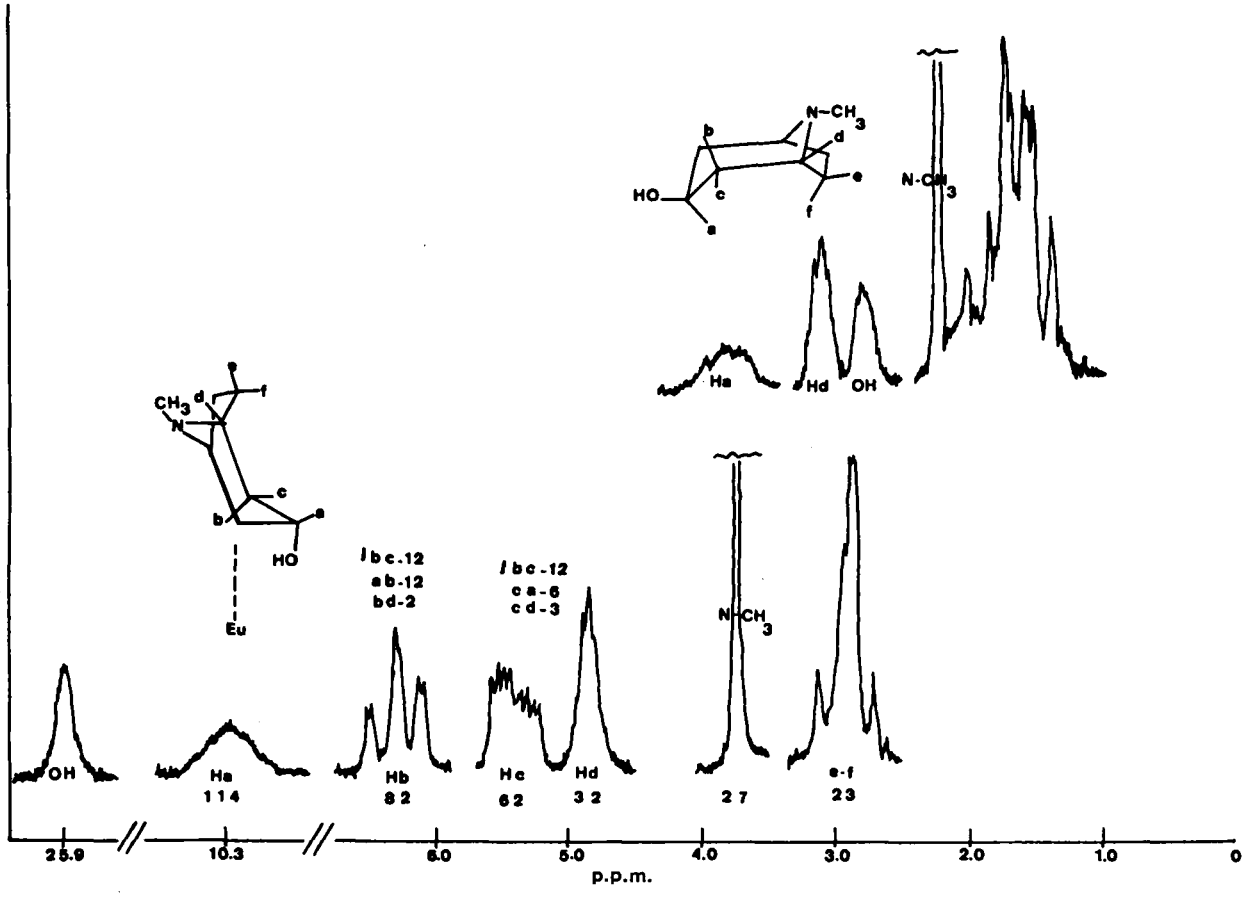


Figure 1—*Pseudotropine (II) at 60 MHz. in deuterochloroform. Key: top, without I treatment; and bottom, shifted spectra resulting at 0.35 mole ratio I/II. Numbers immediately below proton assignments represent the relative rates of proton shift between 0.2 and 0.8 mole ratio* ($\Delta\delta \times 10^1$). *The alignment with the europium complex and the conformation indicated in the molecular diagram are discussed in the text.*

and those associated with the shift reagent through lone-pair electrons. The paramagnetic shifts induced in complexes of europium appear to be the result of a predominately pseudocontact mechanism and do not appreciably broaden peaks or affect the spin-spin coupling constants as proton signals are shifted. The pseudocontact expression given in simplified form is:

$$\frac{\Delta H_i}{H_0} = \left(\frac{3\cos^2\chi_i - 1}{r_i^3}\right)K \qquad \text{(Eq. 1)}$$

where ΔH_i is the average shift defined as the difference in resonance position for the ith substrate proton in the paramagnetic complex and in the diamagnetic ligand. K is a constant incorporating in part the g tensor anisotropy and is a characteristic of the lanthanide under consideration. K is assumed to have the same value for all protons of a substrate molecule. χ_i is the angle between the ith proton and the principal axis, and r_i is the distance from the ith proton to the europium atom[1] (1-9).

Since pseudocontact shifting of NMR spectra offers a simple approach to stereochemical problems, it is useful to assess the effect of such reagents on a wide variety of compounds of biological activity. In conjunction with our interest in the preparation of centrally acting bicyclic amine derivatives (10), investigations were begun on the effect of europium complexes on this class of compounds. Previously, Ohashi *et al.* (11) investigated the effect of small upfield and downfield contact shifts on some tropanes. The current paper reports the effect of I on 3-hydroxy- and 3-ketotropanes (II–VI). This series is attractive in that it (*a*) offers the possibility of much needed simplification while it (*b*) tests the more practical utility of the shift reagent in resolving questions of stereochemistry when the substrate contains dissimilar heterobifunctionality with some conformational mobility present.

The 60-MHz. NMR spectra of 3-substituted tropanes (*e.g.*, Fig. 1, top) show methine (*a*) and bridgehead (*d*) protons as clearly spaced absorptions. The hydroxy proton may be revealed depending on concentration. The *N*-methyl of tropane is seen as a sharp singlet; however, in simple derivatives this signal is found within an uninformative envelope of nearly coincident methylene protons (*b*, *c*, and *e*, *f*). The closely spaced methylene envelope leaves detailed spin decoupling analysis with much to be desired.

Most pseudocontact studies have shown the effect on compounds containing only one type of heteroatom. The tropane series with two different heteroatoms con-

[1] Pseudocontact is defined as the interaction arising from the combined action of an anisotropic g tensor and a through space dipolar hyperfine coupling (as opposed to a through bond spin delocalized hyperfine contact interaction). The lanthanide has a short electron spin lattice relaxation time, contributing to inefficient proton relaxation and little line broadening.

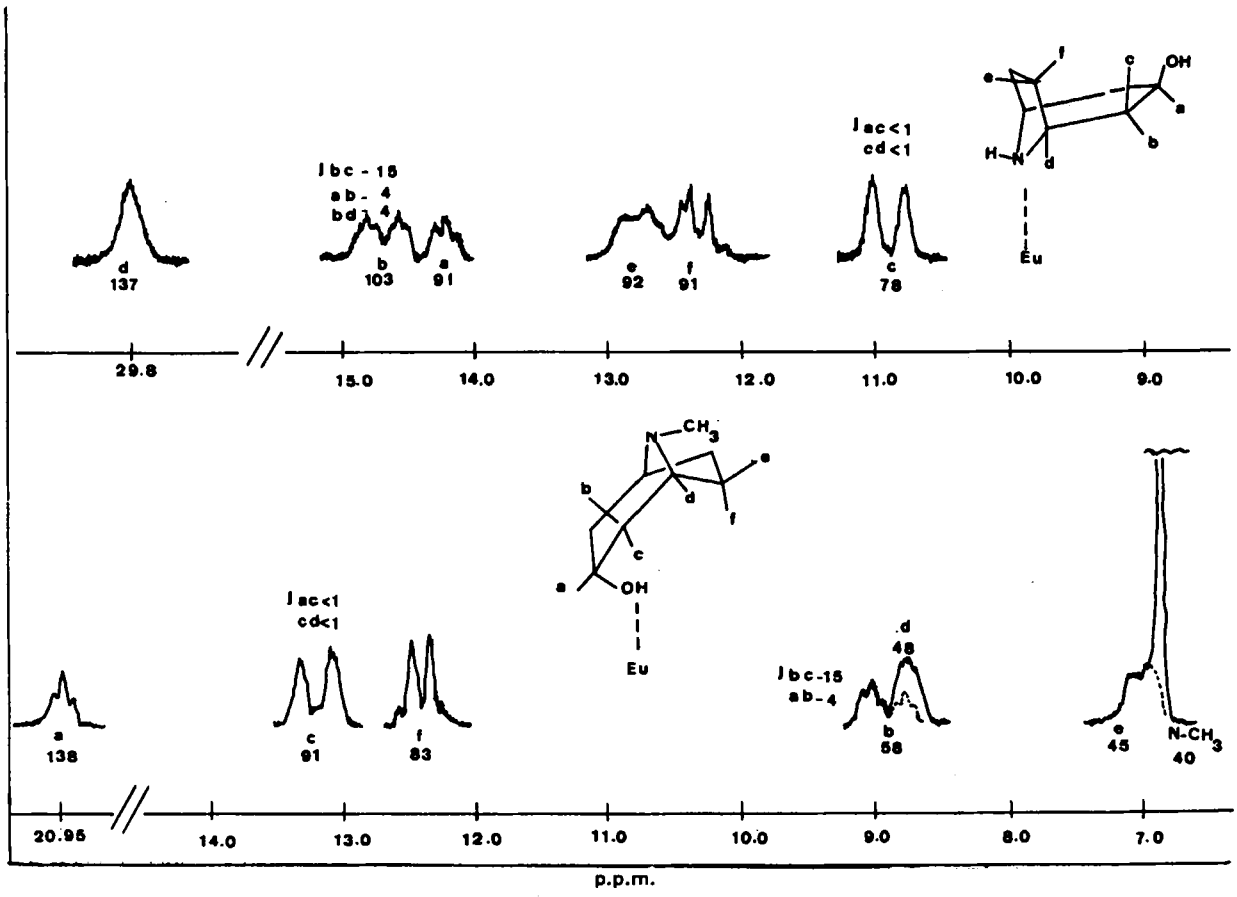
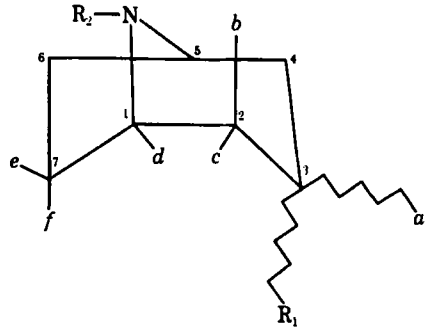


Figure 2—*The 60-MHz. shifted spectra of IV and III as occurring at 0.75 mole ratio I/substrate. Numbers immediately below proton assignments represent* $\Delta\delta$ *as in Fig. 1. The upfield half of resonance for proton* b *(bottom) was taken from the spectrum at a mole ratio where* d *and* b *resonances were independent. Resonance for proton* e *was similarly obtained.*

stitutes a practical "intramolecular competition" experiment[2] and tests the ability of the shift reagent to be selective enough in complexation to yield interpretable NMR spectra. In this regard the tropanes are particularly good models for study. With I, only one deshielded signal would be expected for each type of proton (*e.g.*, grouping for *d* integral = 2) since a plane of symmetry exists through both heteroatoms. An additional practical test of the usefulness of the shift reagent comes about through the conformational mobility at C-2 and C-4 of tropane and the axial–equatorial orientation possible about trivalent nitrogen.

The pseudocontact deshielded spectra should lead to structural information on two levels of sophistication. First, as an "empirical approach," the deshielding process should expose spin-spin coupling constants (*J*) that would allow assignment of predominate conformers in a relative manner, *i.e.*, in comparison to Karplus' (22) interpretations. This advantage, so long as resonance lines are sharp and unperturbed by induced conformational changes, is realized whether the exact mechanism producing the shifts has been correctly understood or not. Secondly, as a "parameter approach," the europium–proton distances could, if theory is correctly interpreted, be calculated from the rate of proton shift for a refined stereochemical outline. Shift data from conformationally immobile substrates having constant average europium–proton distances have been used on numerous occasions for testing the second approach. In these cases, data demonstrate a linear relationship between the rate of proton shift and the concentration ratio



amino alcohols

II: $R_1 = \beta$-OH, $R_2 = CH_3$
III: $R_1 = \alpha$-OH, $R_2 = CH_3$
IV: $R_1 = \alpha$-OH, $R_2 = H$

amino ketones

V: R_1, a = (=O), $R_2 = CH_3$
VI: R_1, a = (=O), $R_2 = H$

[2] Hart and Love (12) pointed out the importance of accumulating information regarding preferred sites of complexation and reported effects of I on morpholine and several other polyfunctional molecules. During the time this research was underway, Fleming *et al.* (13) reported the effect of I on the spectra of hydroxy acids. Other reports that have appeared regarding polyfunctional substrates include: Hinckley *et al.* (14) on testosterones, Tjan and Visser (15) on furanones, Osashi *et al.* (16) on contact shifts in nicotine, Brederode and Huysmans (17) on bis(4-aminocyclohexyl)methane, Taylor and Walters (18) on nitrogen–phosphorus bidentate ligands, Skolik *et al.* (19) on lupanine, Tronchet *et al.* (20) on sugar oximes, and Farid *et al.* (21) on hydroxyoxetans.

of shift reagent to substrate (*e.g.*, 21, 23). Hinckley *et al.* (14) appropriately emphasized the cautions and difficulties in the latter approach for bifunctional substrates. Accordingly, it was desired to test the parameter approach in greater detail and to set the stage for further reports by offering here the results with the "empirical approach."

EXPERIMENTAL

Substrates—Nortropine (IV) was prepared by the method of Willstätter (24) and was found to be identical to a product prepared by the method of Perrine (25). Pseudotropine (II) was prepared by lithium aluminum hydride reduction of tropinone (V) (26) and independently from tropine (III) (27). Purification was by silica gel column chromatography and sublimation. Analysis was within accepted limits. Nortropinone (VI) was prepared from 2,5-dimethoxytetrahydrofuran and acetonedicarboxylic acid (28) and was confirmed as its *N*-benzoyl derivative.

Other compounds used were purchased as authentic[3] and then recrystallized or redistilled to literature purity as required. 1-Methyl-4-piperidone, pseudopelletierine, and 3-quinuclidinone were also treated with I or the more soluble tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedione)europium (VIII), and their shifted spectra were found useful in the interpretation of results presented in this paper.

NMR Investigation—All NMR work work was performed on a 60-MHz. spectrometer[4]. Deuterochloroform, used as solvent, was dried over preheated (120°) 4-A molecular sieves to remove traces of acid which might decompose the reagent complex. Chemical shift values reported are relative to internal tetramethylsilane (VII) and are in parts per million. Coupling constants (J) are reported in hertzes.

Substrate concentrations ranged from 1.4 to 1.9 $\times$ 10^{-4} mole in 0.4 ml. deuterochloroform. Compound V and pseudopelletierine and III and II were run at the same mole concentrations of 1.46 and 1.91 $\times$ 10^{-4} mole, respectively. Study compounds were analyzed at successive additions of 5–15 mg. of I for six to 12 additions, depending on solubility and the need to separate coalescing peaks for spin decoupling dictated by each substrate. Sample tubes were warmed after each addition of I to effect solution. Solutions were allowed to equilibrate to probe temperature before recording shifted spectra. Sample solutions of tertiary amino ketones gave the lowest solubility (shift reagent/substrate mole ratio of 0.53 for V). Solutions of secondary amines and tertiary amino alcohols permitted mole ratios[5] close to 1.0.

Proton assignments were based on integration and spin decoupling of suitably shifted spectra for each compound and aided by comparisons among substrates. Resonance from the tertiary butyl absorption of the europium reagent complex appeared upfield of VII in all cases except VI and pseudopelletierine. In these cases, reagent absorption moved from just downfield of VII to upfield of VII after several additions of reagent complex. VII was easily located in all spectra. In the usual manner to aid in interpretation of results, the data generated from shifted spectra were plotted on coordinate axis, with the shift of each proton in δ (p.p.m.) units as the ordinate and I/substrate mole ratios as the abscissa. The $\Delta\delta$ values reported were abstracted from these plots.

RESULTS AND DISCUSSION

NMR work with deuterium chloride salts has shown that the *N*-substituent in tropanes is predominantly equatorial. On quaternization, the small proportion of *N*-axial conformer present reacts faster to yield a large proportion of product in which the entering group takes up the equatorial position (29). In recent years there has been considerable research regarding the conformation of the tropane piperidine ring.

Early X-ray analysis of II free base and tropine hydrobromide indicated a chair conformation for both of these compounds (26, 30). IR spectral evidence is contradictory with regard to intramolecular hydrogen bonding in II (31, 32). Dipole moment studies in conjunction with an NMR investigation of partially deuterated tropanes led to the conclusion that α-substituted tropanes (*e.g.*, III) probably exist with a major proportion in the deformed chair and a minor proportion in the boat form. 3β-Substituted derivatives such as the 3-cyano derivative were postulated to exist in the chair form. Conclusions were aided by observations of spin-spin coupling of the exposed methine proton as viewed in light of the Karplus rule. Deuteration permitted the approximate assignment of methylene protons, but the protons of the methylene envelope were not used to refine conclusions (26).

More recent X-ray analysis coupled with NMR work indicated a deformed chair for II (33). Because of the replacement of 6β- and 7β-protons by oxygen in scopolamine and scopine, C-2 and C-4 methylene protons are more evident in the NMR and determination of conformation becomes more conclusive. An elegant study of these derivatives, using spin decoupling at 60 MHz., defined J values and fortified the conclusion that α-substituted tropanes exist in the chair form with some distortion (34). Contact shift studies noted the differences in both upfield and downfield shifts induced in 1,4-dimethylpiperidine on the one hand and those occurring in tropinone and 3-tropine esters on the other. Results led to the conclusion that the tropanes exist in a nearly semiplanar form (11). Our results with the effect of I on tropanes are in general agreement with recent findings of distorted chair forms for the α-tropines and tropinones. Compound II also appears to exist predominately in a distorted chair form.

A Lewis acid–base relationship exists between a europium complex and substrate (3). Selective association between I and the two different heteroatoms would be expected (12). Work with molecular models in consideration of the rate of shift found among the protons in the substrates studied and the required relative proton–europium distance[6] indicates that tertiary amino alcohols associate with europium *via* the hydroxy function whereas secondary amino alcohols associate with the nitrogen atom. Amino ketones appear to associate with the amine function. The latter relation was more apparent in VI and 1-methyl-4-piperidone than in V. The results do not conclusively rule out bidentate behavior for the ligands of V at the low mole ratio obtained with I. However, as a result of the complexation encountered, an adequate separation of proton resonances was evident among shifted spectra. All protons were seen as unique absorption for each compound studied (Figs. 1–3), except for the *e* and *f* resonances of II and IV.

Amino ketones exhibited linear plots of mole ratio *versus* chemical shift throughout the range of study permitted by solubility of the europium complex in deuterochloroform. Amino alcohols, however, showed slight curvature for some protons on these plots. This result could be taken as an indication of conformational change induced by the paramagnetic shift reagent or as a slight change in the predominate bonding equilibrium of the paramagnetic shift reagent between the two basic sites in the molecule. There is evidence that slight deviation from linearity is not a result of induced conformational changes. Conformationally free 1-methyl-4-piperidone gave linear results. The conformational mobility of this derivative was clearly evident in shifted spectra wherein, as a result of conformer averaging, no resolution was obtained between axial–

[3] Aldrich Chemical Co. and K & K Laboratories.

[4] Varian Associates T-60.

[5] The low solubility of I–substrate complex in deuterochloroform for the compounds in this study prevented evaluation to the limit of saturation of both functional groups. However, 3-quinuclidinone, tropine, and several other substrates were treated with the more soluble VIII, permitting evaluation through a 2-mole ratio. These results will appear in a future publication.

[6] Alignment of the tropanes with europium was immediately apparent because of variation inherent in the series. Results have usually been interpreted considering the angular factor as invarient with r estimated from $\Delta\delta$ values. The importance of the angular variable has been demonstrated (1). Although it is clear that the induced shift decreases with increasing distance between the proton and coordinating functionality, the exponent of r has been questioned (19). Briggs *et al.* (36) pointed out that, for a potentially bidentate ligand, the geometric factor requires two angular variables. Poly(monodentate) molecules may require weighted means of shifts expected for the various modes of coordination with the lanthanide. Dew Horrocks *et al.* (37) suggested, from X-ray crystallographic results with the eight-coordinate bis(4-picoline) adduct of 2,2,6,6-tetramethylheptane-3,5-dionatoholmium, that the assumption of axial symmetry for shift reagent adducts may not be totally valid and detailed structural inferences should be made cautiously. We have thus avoided reporting empirically derived specific r values and prefer to subject data to careful computer analysis before reporting this aspect.

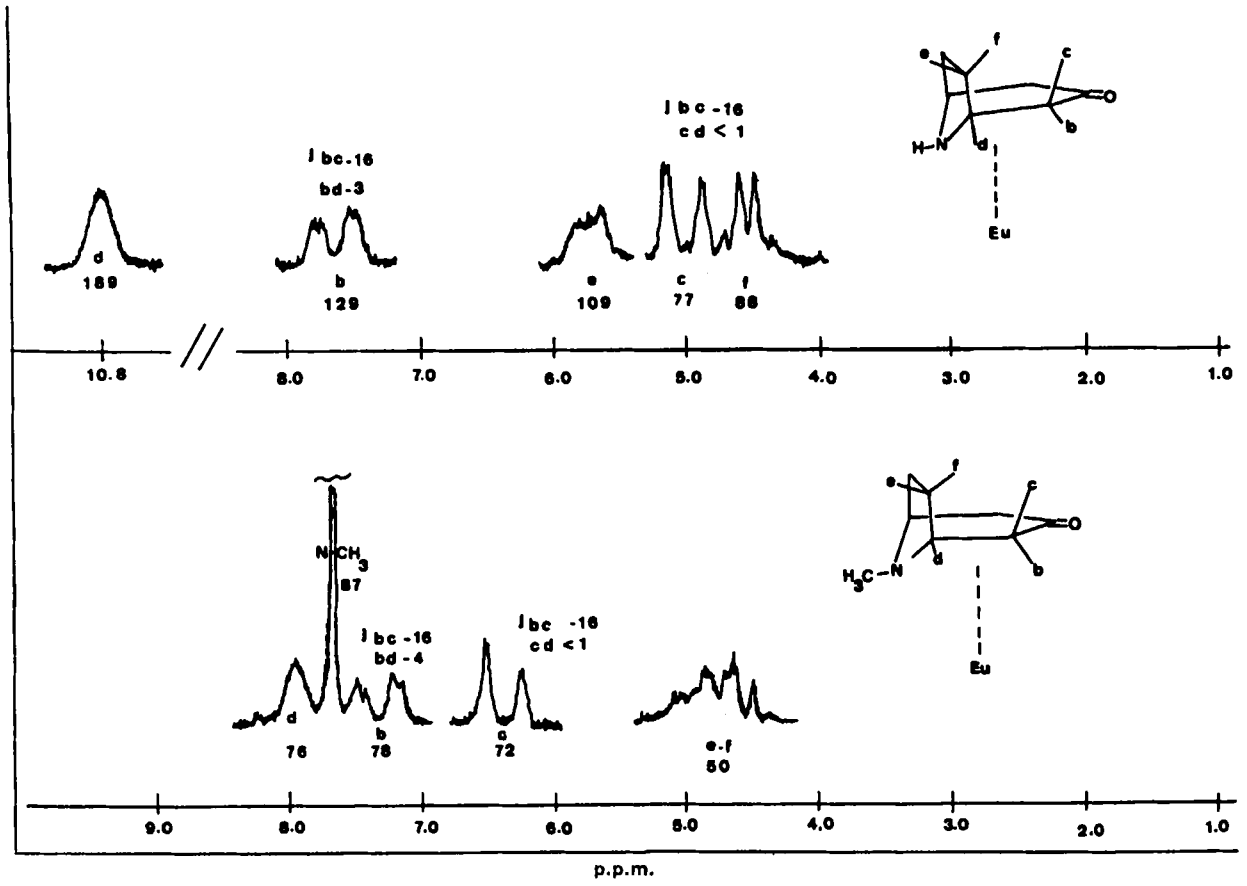


Figure 3—*Protons of VI and V as occurring at 60 MHz. in deuterochloroform solution containing 0.40 mole ratio I/substrate. Numerical data have the same meaning as in Figs. 1 and 2.*

equatorial protons of a methylene set. Moreover, no conformational change was found for the tropanes, as best evidenced by the failure of exposed protons to change significantly[7] in peak width or coupling constant from the normal deuterochloroform spectrum to the final addition of reagent. It has been shown that contact shifts are more important near the point of complex–substrate association (9). Results with the methine protons of III and II, which are geminal to the associating heteroatom, show that contact contribution was insufficient to alter J values significantly. Detailed results in line with empirical approach to data interpretation are presented below.

Amino Alcohols—The NMR of II (Fig. 1) without I revealed the methine resonance as a broad multiplet (W 1/2 = 24 Hz.). On addition of I, the methine remained as a broad multiplet (W 1/2 = 24 Hz.). The coupling constants were found to be $J_{bc} = 12$, $J_{ac} = 6$, $J_{ab} = 12$, $J_{bd} = 2$, and $J_{cd} = 3$. These data suggest a slightly distorted chair conformation. The coupling constant $J_{ac} = 6$ is somewhat larger than is normally observed for axial–equatorial coupling (2–4 Hz.) in chair cyclohexane. This can be explained by a closing of the dihedral angle between Ha and Hc from 60 to 40° which, according to the Karplus rule, would give a larger coupling constant. This closing of the angle leads one to describe the conformation as a slightly distorted chair. The data of the present study are in agreement with crystallography studies (33). Shifts are in the order OH ≫ Ha ≫ Hb > Hc ≫ Hd > N—CH_3 > Hef. A study of molecular models shows that these shift rates require europium–proton distances as indicated in the molecular diagram and evidence association *via* the hydroxy function (Fig. 1).

Both IV and III (Fig. 2) presented the methine proton in deuterochloroform as a simple triplet, $J_{ab} = 4$, $J_{ac} < 1$. These values remained constant in shifted spectra. On deshielding, the following additional J values were determined for the resulting AMXY system of the piperidine ring of the deshielded tropines: $J_{bc} = 15$, J_{ac} and $J_{cd} < 1$, and $J_{bd} = 4$ (J_{bd} was not measurable in III). According to the Karplus curve, J_{ab} and J_{bd} on the order of four or five dictate a 40° dihedral angle between these proton sets. J_{ac} and $J_{cd} < 2.0$ would indicate a dihedral angle on the order of 80° for the two α-substituted derivatives III and IV. For IV, the relative rates of shift found were Hd > Hb > Ha ≃ Hef > Hc, suggesting predominant alignment with the europium ion *via* the nitrogen function during the first mole of reagent addition. A distorted chair form is at least in concert with the observation that the shift rate of Hb is greater than Ha, while the rates for He and Hf are nearly equal and greater than Hc.

The relative rates of shift for tropine suggest the alignment in Fig. 2. The comparative shift rate for tropine protons was found to be Ha > Hc > Hf > Hb > Hd > He > N—CH_3. A boat form for III would require Hc > Ha, while a chair requires Hd > He. Thus, an empirical evaluation of the rate of shift is in relative agreement with the conformation indicated by exposed coupling constants. Measurement of the europium–proton distance and the angle factor would really be required to discriminate further.

Amino Ketones—Spectra of VI and V (Fig. 3) may be interpretated in light of the deshielded ABX coupling revealed on addition of the shift reagent. The following J values were determined: $J_{bc} = 16$, J_{bd} (IV) = 4, J_{bd} (V) = 3, and $J_{cd} < 1$ for both derivatives. These data are consistent with dihedral angles Hd–Hb of 40° and Hd–Hc of 80°, the conditions for a distorted chair form. The rates listed in Fig. 3 appear to be in line with a distorted chair conformation for the amino ketones. The rates for VI evidence alignment of europium ion with the secondary amine function. Rates for V suggest association with the tertiary amine, although both ligands could be simultaneously involved during the first mole ratio of I addition (from the crude data).

CONCLUSIONS

Compound I was found to be a useful NMR shift reagent capable of simplifying the NMR of bifunctional substrates, thus allowing

[7] Compound I is expected to induce a broadening of at least 0.003 Hz./Hz. of shift (38).

for the solution of stereochemical problems from a purely empirical approach. In accordance with the Karplus rule, use of the shift reagent permitted an independent proof of previous indications that a distorted chair form predominates in the conformational equilibrium of α- and β-tropines and tropinones. At mole ratios of I/substrate of less than 1.0, it was possible to observe substrate ligand bonding orders of secondary amine > secondary alcohol > tertiary amine $\geq$ ketone.

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Microbiological Determination of Drug Partitioning II: Functional Dependence of Partition Coefficient on Drug Concentration

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Abstract □ Data were collected pertaining to the partitioning of phenylmercuric nitrate in complex gelatin–acacia coacervate systems. The partition coefficient of the drug could be measured reliably only over a relatively short concentration range using traditional chemical methods. With microbiological analysis the useful range of measurement of partition coefficients was extended significantly by comparing the number of partially inhibited *Escherichia coli* to the amount of drug in the coacervate system. Mathematical relationships were derived which relate the amount of partitioned drug in the complex coacervate to the reduction in growth of *E. coli* B/r.

Keyphrases □ Phenylmercuric nitrate—microbiological determination of partitioning in coacervate systems, partition coefficients, equations □ Microbiological determination of partitioning—phenylmercuric nitrate partition coefficients, equations □ Partition coefficients, phenylmercuric nitrate—microbiological determination, equations □ *Escherichia coli*—used in microbiological determination of phenylmercuric nitrate partitioning □ Gelatin–acacia complex coacervate system—microbiological determination of phenylmercuric nitrate partitioning □ Drug partitioning—microbiological determination, phenylmercuric nitrate in gelatin–acacia coacervate system

In a previous paper, gelatin–acacia complex coacervate systems were developed to serve as the model system for the determination of the partitioning of phenylmercuric nitrate. Partial inhibition in the growth of *Escherichia coli* B/r by the antimicrobial drug was used as the end-point of such investigation. The amount of drug present in an aliquot was determined from reproductive index measurements made with a Coulter counter[1] (1). Coacervate systems have served as models for investigating the partitioning of many types of drug compounds including barbiturates, methylene blue, sugars, phenothiazines, phenol, hexachlorophene, and salicylic acid (2). The partitioning of halothane has been studied in gelatin–acacia, gelatin–benzalkonium chloride (2), and benzalkonium chloride–salicylate or Aerosol OT coacervate systems (3). The results of such investigations have demonstrated the suitability of coacervate model systems for partitioning studies.

In most studies involving various model systems, the partition coefficients of drugs have been determined by traditional chemical analytical techniques. Although the ultimate goal of drug partitioning studies is to obtain a better understanding of the processes by which drug molecules exert their biological activities, chemical measurements do not always seem to serve this goal adequately. For example, drug molecules may undergo physical or chemical alteration to bioinactive forms due to the experimental or analytical process itself. On the other hand, the reliability of biological activity assay procedures has been emphasized extensively, although they are not used as frequently as their applicability may warrant. Apparently one reason that bioassay procedures are not utilized more frequently in drug partitioning studies is that some procedures are not as rapid as the commonly used physical or chemical methods. Since the bioassay is generally accepted as representing an accurate picture of drug activity in various experimental systems, these methods are particularly valuable in analyzing systems that contain drugs specifically used for clinical biological activity, such as the antibiotics and antimicrobial agents. Based on the fact that coacervate systems are increasingly accepted as accurate models of biological systems (4, 5) and the general adaptability of bioassay procedures, the purpose of these studies includes an investigation of the biological determination of drug partitioning in complex coacervate systems.

In this work, phenylmercuric nitrate was incorporated into coacervate and noncoacervate systems. After partitioning was completed between the equilibrium liquid and the coacervate phase, aliquots were withdrawn from the equilibrium liquid and/or the noncoacervate systems and incubated for specific times with rapidly growing microorganisms. Partial inhibition in the growth of *E. coli* was the parameter chosen to determine the amount of drug in each aliquot. The number of organisms was plotted against the total amount of drug in the system, and equations were derived which relate the partition coefficient of phenylmercuric nitrate to its inhibitory effect on the growth of *E. coli*. The general nature of these equations allows the partition coefficient of drugs in any system to be calculated from any biological parameter exhibited by the drug entities.

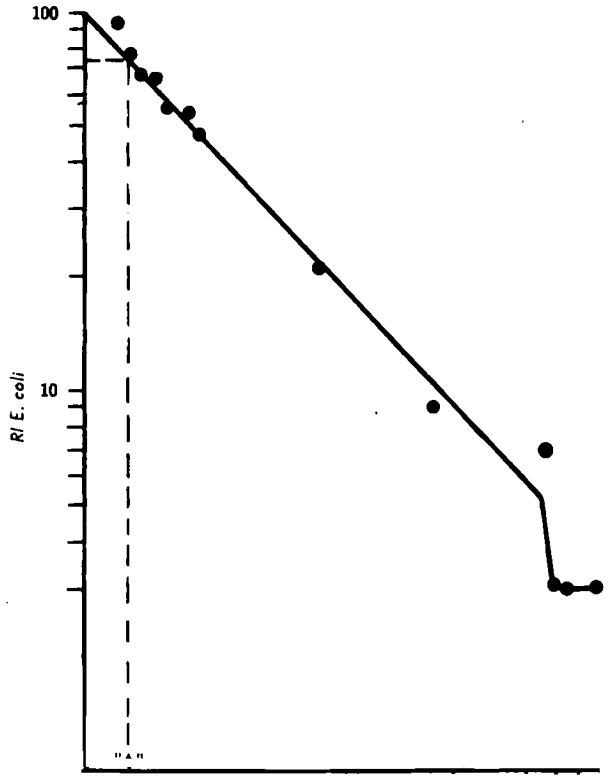
EXPERIMENTAL

Organisms and Reagents—*E. coli* B/r (ATCC 23227) was used as the test organism. Subculturing of the organism and the preparation of culture media were previously reported (1). Phenylmercuric nitrate[2] was used as the antimicrobial agent. Granulated acacia USP[3] and Bacto gelatin[4] were used as colloidal solutes in the coacervate system. Filtered nitric acid[5] (0.1 *N*) was used to form the coacervates.

Preparation of Coacervate Systems—Gelatin–acacia stock solutions containing 5% (w/v) gelatin and 6% (w/v) acacia were prepared. A sterile stock solution of drug was prepared by filtering a solution of phenylmercuric nitrate (600 mcg./ml.). Into each of a series of 30-ml. sterile graduated glass centrifuge tubes was placed 4 ml. of the gelatin–acacia stock solution. After the addition of

[1] Model B, Coulter Electronics, Hialeah, Fla.

[2] Eastman Organic Chemicals, Rochester, N. Y.
[3] Lot No. NHB-283, Penick Co., New York, N. Y.
[4] Lot No. 530946, Difco Laboratories, Detroit, Mich.
[5] Baker Analyzed Reagent.



1.5 ml. of 0.1 *N* nitric acid, aliquots of phenylmercuric nitrate (Table I) were added to each tube. The contents of the tubes were diluted to 30 ml. by the addition of sterile distilled water and mixed. In another series of tubes, the same amounts of reagents were added, except for the omission of the nitric acid. This series did not form a coacervate because of the absence of nitric acid. All tubes were stored at 37° to allow for the completion of the drug partitioning and the separation of the phases.

Growth Conditions—After 24 hr., aliquots were withdrawn from each coacervate and noncoacervate system and inoculated into flasks containing organisms growing in an exponential phase. After 4.5 hr., a sample of the organisms was counted by a colony-count method and by means of the Coulter counter as previously described (1).

Chemical Assay of Phenylmercuric Nitrate—A modification of the procedure used by Gage (6) was adopted for the assay. The following aqueous solutions were prepared in 100-ml. volumetric flasks: hydroxylamine hydrochloride[6] 20% (w/v), urea[7] 10% (w/v), disodium edetate[8] 2.5% (w/v), and sodium acetate trihydrate[9] 50% (w/v). Dithizone[10] stock solution was prepared by dissolving 20 mg. purified dithizone in carbon tetrachloride[11] to make 100 ml. of solution. The solution was refrigerated until used. A working solution was prepared by diluting 1 ml. of stock solution of dithizone to 20 ml. with chloroform[12]. Potasssium permanganate[13] and sulfuric acid[14] 50% (v/v) were used to oxidize the phenylmercuric nitrate. Mercuric chloride[15] was used to make a standard solution containing 67.7 mg./l., with 0.5 *N* HCl as the solvent.

[6] Lot No. 702-1, Eastman Organic Chemicals, Rochester, N. Y.
[7] Fisher Scientific Co.
[8] Lot No. 6354, Eastman Organic Chemicals.
[9] Lot No. 43246, Merck & Co., Rahway, N. J.
[10] Lot No. F42368, Fisher Scientific Co.
[11] Lot No. TNE, Mallinckrodt Chemical Works.
[12] Lot No. YHR, Mallinckrodt Chemical Works.
[13] Lot No. F140, Fisher Scientific Co.
[14] Mallinckrodt Chemical Works.
[15] E. I. du Pont de Nemours & Co.

The chemical method involves the oxidation of phenylmercuric nitrate with acidic permanganate and then titration of the mercury content with dithizone solution. Twenty milliliters of equilibrium liquid from each coacervate system was transferred to 125-ml. conical flasks with stoppers. Two milliliters of 50% (v/v) sulfuric acid was added to the solution; 0.5 g. potassium permanganate and one drop of octanol were then added. The flasks were placed in boiling water for 10 min., and 3 ml. of hydroxylamine hydrochloride solution was added to each flask to decolorize the mixture. The mixture was allowed to cool to room temperature. To the oxidized solution, 2 ml. of urea and 1 ml. of edetate were added. The pH was adjusted to 1.5 with sodium acetate solution, and 0.5 ml. of chloroform was added. Twenty milliliters of the equilibrium phase of a coacervate system containing no drug was treated in the same way as the test solution to prepare a suitable blank.

Diluted dithizone–chloroform was placed in a 25-ml. buret, and small portions were added to each test solution. After each increment, the stopper was replaced and the solution was shaken vigorously. In the presence of mercury, the separating organic layer had an orange color. Increments of dithizone solution were added until a grayish color appeared, which was intermediate between the orange of the mercury complex and the green color of the dithizone solution. The volume of the dithizone was recorded, and the same volume was added to the blank solution. The blank was then titrated to the same grayish color with standard mercuric chloride solution, and the amount of mercury in the original sample was obtained by calculation from the known volume of added standard.

RESULTS AND DISCUSSION

Microbiological Determination of Partition Coefficient—*One-Line Method*—The total number of organisms obtained from Coulter counts were converted into reproductive coefficient (*RC*) and reproductive index (*RI*) values using the following relationships:

$$RC \text{ (reproductive coefficient)} = \frac{\text{total number of organisms in each flask}}{\text{total number of organisms inoculated}} \quad \text{(Eq. 1)}$$

$$RI \text{ (reproductive index)} = \frac{RC \text{ in the presence of drug}}{RC \text{ in the absence of drug}} \times 100 \quad \text{(Eq. 2)}$$

When log *RI* values obtained from noncoacervate systems were plotted *versus* the concentration of drug (micrograms per milliliter) in the noncoacervate system, a straight line was obtained (Fig. 1). The least-squares regression line was calculated using regression models of the univariate normal type, where x = concentration of drug in micrograms per milliliter in the noncoacervate system and y = (log *RI*). The line in Fig. 1 may be used as a standard to predict the concentration of drug corresponding to a measured *RI* value obtained with aliquots of known volume drawn from equilibrium liquids incubated with *E. coli*.

For example, assume that a 1-ml. aliquot was removed from the equilibrium phase of a coacervate system containing some total quantity of phenylmercuric nitrate and that it produced an *RI* value equal to 75 when measured by the technique of the inhibition of *E. coli*. From Fig. 1, a corresponding value on the x-axis equivalent to 30 mcg. of drug is obtained, thereby indicating the actual amount of phenylmercuric nitrate contained in the 1-ml. aliquot drawn from the drug-partitioned equilibrium phase. In this way, any aliquot from a coacervate system equilibrium layer may yield a corresponding concentration of phenylmercuric nitrate that relates to its observed *RI* value.

To derive a mathematical expression for the partition coefficient, let a assume a value that is equal to the actual concentration of drug in the equilibrium liquid expressed in micrograms per milliliter. Considering the total volume of the equilibrium liquid in the coacervate system to V_2 ml., then the amount of drug in the equilibrium liquid is aV_2 mcg. If the total amount of drug added to each system is T mcg., the amount of drug partitioned into the coacervate phase would be $(T - aV_2)$ mcg. Dividing this value by the volume of the coacervate layer itself, V_1 ml., the concentration of the drug in the coacervate phase can be obtained as $(T - aV_2)/V_1$ mcg./ml.

Since the partition coefficient (*PC*) is defined as the ratio of the drug concentration in the coacervate phase over the concentration

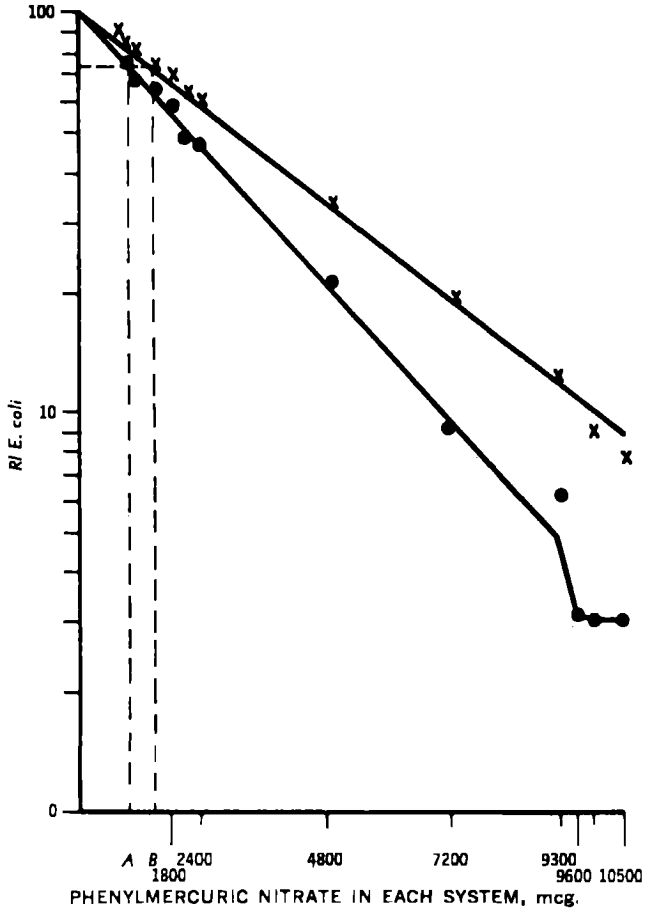


Figure 2—*Effect of phenylmercuric nitrate on the reproductive index* (RI) *of* E. coli *B/r (two-line method). Key:* ×, *aliquots drawn from equilibrium liquids; and* ●, *aliquots drawn from noncoacervate systems.*

of the drug in the equilibrium liquid, an equation may be written:

$$PC = \frac{T - aV_2}{V_1} + a = \frac{T - aV_2}{aV_1} \qquad \text{(Eq. 3a)}$$

or:

$$PC = \frac{T}{aV_1} - \frac{V_2}{V_1} \qquad \text{(Eq. 3b)}$$

Two-Line Method (Derivation I)—Logarithmic *RI* values obtained from both coacervate and noncoacervate systems were plotted against T, the total amount of drug introduced into each system. Figure 2 shows the straight lines obtained when (log *RI*) values are plotted *versus* the microgram quantities of phenylmercuric nitrate present in each system. For any selected value of *RI*, there are two different corresponding values on the x-axis, labeled A and B. These represent the different amounts of drug that must be added to each system (A = noncoacervate, B = coacervate) to yield identical reproductive indexes in the equilibrium liquid of the coacervate system and the noncoacervate system after drug partitioning has taken place. It is assumed that for equivalent *RI* values, the amounts of drug in the aliquots are identical regardless of the total amounts of drug in the system as indicated by A and B. If the concentration of drug in the equilibrium liquid and the noncoacervate system required to produce the same *RI* is designated as C_2, C_1 is the concentration of the drug in the coacervate phase (micrograms per milliliter), and V_1 and V_2 are the volumes of the coacervate phase and the equilibrium liquid in milliliters, respectively, an equation may be written that relates the apparent total amount of drug in the coacervate system (B) as determined from Eq. 4:

$$C_1V_1 + C_2V_2 = B \qquad \text{(Eq. 4)}$$

By dividing Eq. 4 by C_2, the concentration of drug in the noncoacervate system at the same *RI* value (and similarly the concentration of drug in the equilibrium phase), the following equation may be obtained:

$$\frac{C_1}{C_2}V_1 + V_2 = \frac{B}{C_2} \qquad \text{(Eq. 5)}$$

Once again the partition coefficient is defined as the concentration of the drug in the coacervate phase with respect to its concentration in the equilibrium liquid and is expressed as $PC = (C_1/C_2)$. Substitution of PC in Eq. 5 and subsequent rearrangement yield:

$$(PC)V_1 + V_2 = \frac{B}{C_2} \qquad \text{(Eq. 6a)}$$

$$PC = \frac{B}{C_2V_1} - \frac{V_2}{V_1} \qquad \text{(Eq. 6b)}$$

Because the total volume of the noncoacervate systems has been experimentally fixed to equal $V_1 + V_2$ (the volume of the coacervate system) and the amount of drug needed to produce the same *RI* in both systems is equal to C_2, one can write:

$$A = C_2(V_1 + V_2) \qquad \text{(Eq. 7a)}$$

or:

$$C_2 = \frac{A}{V_1 + V_2} \qquad \text{(Eq. 7b)}$$

And by substituting Eq. 7*b* into Eq. 6*b*:

$$PC = \frac{B}{(AV_1/V_1 + V_2)} - \frac{V_2}{V_1} \qquad \text{(Eq. 8a)}$$

or:

$$PC = \frac{B(V_1 + V_2)}{AV_1} - \frac{V_2}{V_1} \qquad \text{(Eq. 8b)}$$

As before, A and B are obtained from straight-line plots such as Fig. 2 and represent amounts of drug needed to give equivalent *RI* values in coacervate and noncoacervate systems. V_1 and V_2 can be measured visually after equilibrium is completed in the coacervate system.

Two-Line Method (Derivation II)—In Fig. 2, B/A represents the ratio of the concentration of drug in noncoacervate systems to that of the equilibrium liquid in the coacervate system needed to produce equivalent values of *RI*. Of course, this relationship holds only when the same amount of drug has been added to both systems. Under these conditions, for any value of *RI* that is selected, the ratio may be expressed as:

$$\frac{B}{A} = \frac{\text{concentration of drug in noncoacervate system}}{\text{concentration of same drug in equilibrium liquid of coacervate}} \qquad \text{(Eq. 9)}$$

Since concentrations in these systems are proportional to the reciprocal volumes, Eq. 9 may be rewritten as:

$$\frac{B}{A} = \frac{\text{ml. equilibrium liquid producing a certain } RI}{\text{ml. noncoacervate system producing the same } RI} \qquad \text{(Eq. 10)}$$

In all instances, 1 ml. was withdrawn from both systems. Therefore, Eq. 10 can be restated as:

$$\frac{B}{A} = \text{ml. equilibrium liquid containing same amount of drug as 1 ml. noncoacervate system} \qquad \text{(Eq. 11)}$$

If T micrograms of drug is added to each system and V is the volume of the noncoacervate system in milliliters, T/V equals the amount present per milliliter of noncoacervate system. These expressions indicate that in B/A ml. of equilibrium liquid, T/V mcg. drug is present. The amount of drug in the total volume of equilibrium liquid is:

$$V_2 = \frac{V_2TA}{VB} \qquad \text{(Eq. 12)}$$

Table I—Partition Coefficient of Phenylmercuric Nitrate (PMN) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *E. coli* B/r and by Chemical Method

PMN (600 mcg./ml.) Stock Solution Added to Each System, ml. (1)	PMN mcg. Present in 1 ml. Noncoacervate System: Each System (2)	PMN mcg. Present in 1 ml. Noncoacervate System: Noncoacervate System (3)	Reproductive Index of Noncoacervate System (RI_n): Colony Count (4)	Reproductive Index of Noncoacervate System (RI_n): Total Count (5)	Reproductive Index of Coacervate System (RI_c) (6)	*a* Value Calculated from Graph, mcg. (7)	*PC* Calculated from Microbiological Method (8)	*PC* Calculated from Chemical Assay (9)
1.25	750	25	82.1	84.51	88.5	13.84	13.1	13.39
1.5	900	30	77.0	76.1	86.2	16.95	12.55	12.67
2.0	1200	40	69.1	68.2	79.3	25.4	9.61	9.7
2.5	1500	50	64.3	65.1	75.0	32.3	9.22	9.10
3.0	1800	60	57.2	56.4	69.91	39.65	8.70	8.89
3.5	2100	70	48.6	49.7	64.7	47.72	8.0	8.30
4.0	2400	80	45.0	47.1	60.7	55.5	7.6	8.0
8.0	4800	160	19.8	20.5	33.7	114.8	6.9	6.51
12.0	7200	240	8.6	9.2	19.2	175.1	6.59	5.92
15.0	9300	310	5.0	6.2	12.3	220.3	5.37	5.11
16.0	9600	320	2.0	3.05	9.02	252.6	5.01	4.62
16.6	9900	330	0	3.01	8.3	260.6	5.00	4.89
17.5	10500	350	0	3.0	6.7	279.11	5.00	4.87

which assumes the units of micrograms. The concentration of drug in the equilibrium liquid can be obtained by dividing the expression for the amount of drug by the volume of the equilbrium liquid:

$$\frac{V_2TA}{VB} \div V_1 = \frac{TA}{VB} \text{ mcg./ml.} \qquad \text{(Eq. 13)}$$

Subtracting Eq. 12 from the total amount of drug in each system (T) gives the amount of drug in the coacervate phase:

$$T - \frac{V_2TA}{VB} = \frac{TVB - V_2TA}{VB} \qquad \text{(Eq. 14)}$$

Similarly, the concentration of the drug in the coacervate phase may be obtained by dividing its amount by the appropriate volume V_1:

$$\frac{TVB - V_2TA}{VB} \div V_1 = \frac{TVB - V_2TA}{V_1VB} \text{ mcg./ml.} \qquad \text{(Eq. 15)}$$

From Eqs. 13 and 15, the definition of the partition coefficient may be written as a ratio of concentrations of drug in coacervate phase to drug in equilibrium phase:

$$PC = \frac{\text{Eq. 15}}{\text{Eq. 13}} = \frac{T(VB - V_2A)}{V_1VB} \div \frac{TA}{VB} \qquad \text{(Eq. 16)}$$

Simplification yields:

$$PC = \frac{VB - V_2A}{V_1A} = \left(\frac{B}{A}\right)\frac{V}{V_1} - \frac{V_2}{V_1} \qquad \text{(Eq. 17)}$$

Since the total volumes of the coacervate and noncoacervate systems were kept equal and $V = V_1 + V_2$, Eq. 17 may be rewritten to give an identity with Eq. 8*b* from the previous derivation. Therefore, it is evident that the expressions obtained for the partition coefficients obtained by the one-line and two-line methods are the same.

Chemical Determination of Partition Coefficient—Each milliliter of the standard mercury solution used in the assay of phenylmercuric nitrate is equivalent to 0.75 mcg. of organic mercury present in the original sample (6). Phenylmercuric nitrate has been reported to contain 63.2% (w/w) mercury (7). Therefore, each milliliter of the standard solution consumed in the titration is equivalent to 375/316 or 1.1867 mcg. of phenylmercuric nitrate. In the experimental studies reported here, the volume of the equilibrium liquid was 20 ml. If n ml. of the standard mercuric chloride solution is used in the chemical assay, then a, the value of the concentration of phenylmercuric nitrate in micrograms per milliliter of the equilibrium liquid, can be calculated from Eq. 18:

$$a = \frac{1.1867n}{20} = 0.0593n \qquad \text{(Eq. 18)}$$

Finally, the partition coefficient of phenylmercuric nitrate in the coacervate system may be determined from Eq. 3*b*. Calculated partition coefficient values from this method are shown in Table I, Column 9.

A comparison of Column 8 in Table I with Column 9 reveals that the partition coefficient for phenylmercuric nitrate in complex coacervate systems as determined by the microbiological method is in agreement with that obtained by the chemical assay procedure for organic mercurial compounds. The deviations at low concentrations of drug are attributed to the limiting sensitivity of the chemical assay method.

The microbiological measurement of drug partitioning in complex coacervate systems need not be confined to antimicrobial agents (8, 9). As long as substances are capable of exhibiting *any type* of biological activity, they are candidates for studies of this type. In nearly every instance, microorganisms may be selected whose growth will be predictably altered by the presence of the drug being studied. In this way, vitamins, cytotoxic agents, nutrient components, *etc.*, could be partitioned in coacervate systems and measured microbiologically by the methods described here.

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Microbiological Determination of Drug Partitioning III: Partition Coefficient Profiles of Several Antimicrobial Agents

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Abstract □ Drug partitioning of four antimicrobial agents in gelatin–acacia complex coacervate systems was studied. Partial inhibition in the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* permitted the determination of partition coefficients for the antimicrobial agents in coacervates over a concentration range of 0.12–2.4 mg./ml. The usefulness of this method is apparent from a comparison of partition coefficients obtained with the same drugs using traditional chemical analysis. The dependence of partition coefficient on drug concentration in the system demonstrates the suitability of the methods for drug binding studies using coacervates as biological models.

Keyphrases □ Antimicrobial agents—microbiological determination of partitioning in coacervate systems, partition coefficients, compared to chemical assay techniques □ Microbiological determination of partitioning—four antimicrobial agents, partition coefficient profiles, compared to chemical assay techniques □ Partition coefficient profiles, four antimicrobial agents—microbiological determination □ Gelatin–acacia complex coacervate system—microbiological determination of partition coefficient profiles for four antimicrobial agents □ Drug partitioning—microbiological determination, four antimicrobial agents in gelatin–acacia coacervate system, compared to chemical assay techniques

Coacervate systems were introduced and described by Kruyt and Bundenberg de Jong in 1929 (1). Later, these systems were utilized as biological model systems by Oparin and coworkers (2–6) and other investigators (7–9). Several investigators (10–13) adapted complex coacervate systems to serve as biological model systems for the study of drug partitioning. The advantages of these systems over other types of models used for partitioning studies of drug molecules are numerous. Coacervate systems have been reported to be the most suitable model for comparative studies with the biological system (14–16). These systems do not suffer from the simplistic shortcomings common to the lipid-like solvent systems such as octanol–water or chloroform–water when used as biomodels (12). Furthermore, partitioning studies with coacervate models do not necessitate the use of specially devised experimental membrane barriers. Investigation of drug partitioning by means of dialysis membranes introduces certain assumptions concerning the membrane and requires several corrections due to the adsorption of drug entities to the membrane. General freedom from these shortcomings invites the coacervate systems to serve increasingly as models for biosystems in comparative drug partitioning studies.

The similarity between the structure of water in the membraneless coacervate system and the biological system has been repeatedly recognized (2–9, 14–16). This affords additional incentive to utilize coacervate systems as models of biological barriers in the study of drug transfer. Judicious use of these systems may offer additional insight into processes of drug distribution in the body.

Garrett and Miller (17) demonstrated the relationship existing between dilute drug solutions and microbial growth below their minimum inhibitory concentrations. Similar results were obtained by these workers (10, 11) using drug-treated complex coacervate systems. Hansch *et al.* (18) described equations which relate the partition coefficient of drugs with their biological activities. Javidan and Mrtek (11) developed equations whereby the partition coefficient of any drug could be calculated from its biological activity using microbiological systems. It seems desirable to measure the partition coefficient of drugs at various concentrations in coacervate systems using biological parameters as the end-point because such assay procedures satisfy the prime requisite for the determination of the biologically active drug, rather than one or more functional groups on drug molecules which may have undergone alterations to bioinactive forms during the experiment itself.

In this investigation, the partition coefficients of four antimicrobials were studied over an extended concentration range using several organisms with various sensitivities to each drug. Partition coefficients were calculated using the microbiological one-line method previously reported (11), and the results were compared with those obtained by traditional chemical assay techniques.

EXPERIMENTAL

Organisms and Culture Media—*Test Organisms*—*Escherichia coli* B/r (ATCC 23227), *Pseudomonas aeruginosa*[1], and *Staphylococcus aureus*[1] were used as the test organisms. The general preparation of cultures and storage conditions were previously described (10).

Culture Media—The following were used:

1. Difco E. C. Medium[2]. Thirty-seven grams of the dried medium was rehydrated in 1 l. of cold distilled water and stirred at room temperature until a clear solution resulted. This solution was filtered through Millipore[3] filters (2 and 0.22 μ) to remove all cellular and particulate debris. Filtered media were distributed in batches of 18 ml. among 125-ml. conical flasks and autoclaved at 15 lb. of pressure (121°) for 15 min. The pH of the sterilized medium was 6.98.
2. Difco Brain Heart Infusion[2]. Thirty-seven grams of the dehydrated material was dissolved in cold distilled water to make 1 l. The solution was boiled for 1 min. and stirred until it was clear. Then the hot solution was filtered and treated in the same manner as the E. C. medium. The infusion was freshly prepared each week and had a pH of 6.9.
3. Defined Medium. The culture medium developed by Anton (19) was used to obtain a slow rate of growth for microorganisms that were not highly sensitive to the particular antibacterial being

[1] Received from the Culture Collection, Department of Microbiology, University of Illinois College of Medicine, Chicago, Ill.
[2] Difco Laboratories, Detroit, Mich.
[3] Millipore Corp., Bedford, Mass.

Table I—Quantities of Antimicrobials Used to Study Effect of Drug Concentration on Coacervation

Antimicrobial Solution[a]		I				II					III				
Milliliters antimicrobial stock solution added to each system	PMN	20	16	8	4	17.5	15	7.5	3.75	1.4	7.0	3.5	1.2	0.6	0
	FCl	12	6	3	1.2	—	—	—	—	—	2.4	12	6	3	0
	CP	24	20	16	12	20	10	6	4	2	2	1.5	1	0.5	0
	BZCl	24	18	12	9	24	18	12	6	3	—	—	—	—	0
Micrograms antimicrobial in each system	PMN	12,000	9600	4800	2400	1050	900	450	225	84	42	21	7.2	3.6	0
	FCl	120,000	6000	3000	12,000	—	—	—	—	—	1440	720	360	180	0
	CP	9000	7500	6000	4500	3000	1500	900	600	300	240	180	120	60	0
	BZCl	72,000	54,000	36,000	2000	23,920	19,440	12,960	6480	3240	—	—	—	—	0

[a] See text for explanation of roman numerals. PMN = phenylmercuric nitrate, FCl = furazolium chloride, CP = chloramphenicol, and BZCl = benzalkonium chloride.

studied or when the concentration of antimicrobial agent was below the minimum inhibitory concentration. The medium was prepared by dissolving all components in cold distilled water, filtering through Millipore filters to remove debris, and autoclaving for 20 min. The pH was 6.9 and did not change throughout the study.

4. Frazier's (20) medium, composed of 4% (w/v) each Bacto tryptone[4] and Soya agar[4] and 0.5% (w/v) Bacto gelatin[4], was prepared by heating all components to about 70° in distilled water while stirring continuously with a glass rod. After all the particulate matter was dispersed, the medium was autoclaved in a 500-ml. flask for 15 min. at 15 lb. of pressure. After the flask had cooled to 45°, the medium was dispensed into sterile plastic Petri dishes under a microbiological hood equipped with a UV light. The solidified medium was used to determine if the organisms used in the study metabolized gelatin, an important component in the coacervate systems.

Reagents—*Drugs*—Phenylmercuric nitrate[5], chloramphenicol USP[6], furazolium chloride[7], and benzalkonium chloride USP[8] were the antimicrobial agents used in the drug partitioning studies.

Biochemicals—The reagents used in the preparation of coacervate systems were described previously (10).

Reagents for Chemical Analysis—Chemicals required for the assay of phenylmercuric nitrate, according to the method of Gage, have been described (11). A mixture of hexanol[5]–heptane[9] (4:1) and 0.1 *N* sodium hydroxide[10] solutions were used in the assay of furazolium chloride. The assay for benzalkonium chloride required orange II[5], which was used to form a salt with the drug. This was subsequently extracted into chloroform[11]. Sodium carbonate[10] and sodium bicarbonate[10] were used to prepare Delory and King's buffer solution.

Preparation of Solutions—*Phenylmercuric Nitrate Stock Solutions*—Sixty milligrams of phenylmercuric nitrate was placed in a sterile 100-ml. volumetric flask wrapped with aluminum foil. Sterile distilled water was the solvent. Dissolution of the drug was accomplished at room temperature with the aid of a magnetic stirrer and a sterilized magnetic stirring bar which was placed in the flask. After dissolution was completed in 4 hr., the solution was filtered through a 0.22-μ Millipore filter. This solution was labeled Phenylmercuric Nitrate Stock Solution I. Ten- and 100-fold dilutions of this solution were prepared in sterile brown bottles using sterile distilled water and labeled Phenylmercuric Nitrate Stock Solutions II and III, respectively. All stock solutions were freshly prepared each week. The concentrations of phenylmercuric nitrate in the Phenylmercuric Nitrate Stock Solutions I, II, and III were 600, 60, and 6 mcg./ml., respectively.

Furazolium Chloride Stock Solutions—By dissolving 1 g. furazolium chloride in 100-ml. sterile distilled water at room temperature, a stock solution labeled Furazolium Chloride Stock Solution I was prepared. The concentration of drug in this solution was 10 mg./ml. Diluting 3 ml. of this solution to 100 ml. with sterile distilled water resulted in a solution labeled Furazolium Chloride Stock Solution II. A third dilution was made by adding 80 ml. of sterile distilled water to 20 ml. Furazolium Chloride Stock Solution II and was labeled Furazolium Chloride Stock Solution III. All solutions were filtered through 0.22-μ Millipore filters and stored at room temperature in brown bottles until used or replenished in 2 weeks. The concentrations of furazolium chloride in Furazolium Chloride Stock Solutions I, II, and III were 10 mg./ml. and 300 and 60 mcg./ml., respectively.

Chloramphenicol Stock Solutions—Chloramphenicol stock solutions were freshly prepared 0.5 hr. prior to use. Four solutions—375, 150, 120, and 60 mcg./ml.—were prepared separately by diluting an accurately weighed amount of chloramphenicol in cold sterile distilled water. The solutions were labeled Chloramphenicol Stock Solutions I, II, III, and IV, respectively, and stored in the refrigerator until used.

Benzalkonium Chloride Stock Solutions—One and one-half milliliters of a 50% (w/v) solution of benzalkonium chloride was diluted to 250 ml. with sterile distilled water. The resulting solution contained 3 mg./ml. drug and was labeled Benzalkonium Chloride Stock Solution I. A second stock solution, prepared by appropriate dilution to contain 1080 mcg./ml. of the antimicrobial agent, was labeled Benzalkonium Chloride Stock Solution II.

Preparation of Coacervate Systems—A stock solution containing 6% acacia and 5% gelatin was prepared according to the method described previously. This solution was labeled A–G Stock Solution and was refrigerated until used. The method of preparation of the coacervate systems from A–G Stock Solution was identical to that described elsewhere (10). The concentration of gelatin in the A–G Stock Solution was increased to 5% (w/v) from that described in an earlier paper to provide a maximum coacervate phase volume.

Effect of Drug Concentration on Coacervation Volume—Into each of a series of 45-ml. graduated centrifuge tubes were placed separately the quantities of different antimicrobials indicated in Table I. Four milliliters of A–G Stock Solution was added. Tubes containing phenylmercuric nitrate were acidified with 1.5 ml. of 0.1 *N* HNO_3 to prevent precipitation of insoluble phenylmercuric chloride. All other drug-containing tubes were acidified with 1.5 ml. of 0.1 *N* HCl. The volume of each tube was adjusted to 30 ml. by the addition of sterile distilled water. Clouding of liquid indicated the formation of a coacervate phase.

After the tubes were capped and secured with a rubberband, they were shaken horizontally in a reciprocating water bath at 37° for 6 hr. to permit partitioning of the drug between the coacervate droplets and the equilibrium liquid. Then the capped tubes were stored undisturbed in a cabinet at 37° for 24 hr. to permit phase separation. The volume of the coacervate layer was recorded visually at the end of this period. The sequence of addition of the different components in the formation of the coacervate or the duration of the shaking period was altered from 0.5 to 6 hr. to study any possible effect on the final volume of the coacervate phase.

Utilization of Gelatin by Microorganisms—Inocula from rapidly growing cultures of *E. coli*, *S. aureus*, and *P. aeruginosa* were streaked onto the surface of three replicate plates of a specially prepared solidified medium to test the utilization of gelatin by these organisms (20). The plates were covered, inverted, and incubated for 24 hr. at 37°. At the end of this time, the surface of each plate was flooded with about 2 ml. of 2% acidic solution of mercuric chloride. The difference in the intensity of the background color was compared with the color of the areas where the organisms had grown. This visual comparison represented the criterion for the determination of gelatin utilization by the microorganisms (20).

[4] Difco Laboratories, Detroit, Mich.
[5] Eastman Organic Chemicals, Rochester, N. Y.
[6] Courtesy of Parke-Davis & Co., Detroit, Mich.
[7] Courtesy of Norwich Pharmacal Co., Norwich, N. Y.
[8] Lot No. S107RC, Winthrop Laboratories, New York, N. Y.
[9] Lot No. 701-1, Eastman Organic Chemicals.
[10] Fisher Scientific Co.
[11] Lot No. YHR, Mallinckrodt Chemical Works.

Table II—Determination of Possible Inactivation of Antimicrobials in Coacervation Process

Micrograms added to each system / Group	Furazolium Chloride 180 A	Furazolium Chloride 180 B	Furazolium Chloride 360 A	Furazolium Chloride 360 B	Furazolium Chloride 800 A	Furazolium Chloride 800 B	Phenylmercuric Nitrate 900 A	Phenylmercuric Nitrate 900 B	Phenylmercuric Nitrate 2400 A	Phenylmercuric Nitrate 2400 B	Phenylmercuric Nitrate 7200 A	Phenylmercuric Nitrate 7200 B
					Replicate 1							
RI[a] values	84.5	84.1	29.6	31.0	8.2	7.5	94.2	93.7	51.4	49.9	7.3	7.4
					Replicate 2							
	84.0	85.0	30.6	31.1	8.7	8.5	94.3	94.1	51.7	51.0	7.0	8.5
					Replicate 3							
	86.5	84.3	30.0	31.5	8.6	8.3	94.1	94.0	51.2	50.9	7.0	8.1
Average *RI*	85.0	84.6	30.34	31.2	8.5	8.1	94.2	93.9	51.5	50.6	7.1	8.0
$t_{(4)}$	n.s.[b]	0.493	n.s.	1.353	n.s.	0.676	n.s.	1.304	n.s.	1.368	n.s.	1.544

Micrograms added to each system / Group	Chloramphenicol 240 A	Chloramphenicol 240 B	Chloramphenicol 480 A	Chloramphenicol 480 B	Chloramphenicol 960 A	Chloramphenicol 960 B	Benzalkonium Chloride 10,000 A	Benzalkonium Chloride 10,000 B	Benzalkonium Chloride 20,000 A	Benzalkonium Chloride 20,000 B	Benzalkonium Chloride 30,000 A	Benzalkonium Chloride 30,000 B
					Replicate 1							
RI values	78.9	81.2	43.2	45.5	16.1	15.0	72.0	73.1	42.7	44.5	13.6	13.6
					Replicate 2							
	80.9	81.0	44.9	45.0	15.7	15.1	71.2	72.9	43.1	44.6	14.3	13.7
					Replicate 3							
	80.2	80.8	43.9	44.8	16.2	15.3	73.1	73.3	43.2	42.9	14.1	13.5
Average *RI*	80.0	81.0	44.0	45.1	16.0	15.2	72.2	73.1	43.0	44.0	14.0	13.6
$t_{(4)}$	n.s.	0.967	n.s.	1.186	*[c]	2.853	n.s.	0.923	n.s.	1.010	n.s.	1.631

[a] *RI* = reproductive index. [b] n.s. = nonsignificant. [c] * = significant at the 5% level.

Test for Drug Inactivation during Coacervation Process—Into 26 15-ml. graduated cylinders, 2 ml. of A–G Stock Solution and 3 ml. of sterile filtered normal saline were added. Then the cylinders were divided into two groups: A and B. Each cylinder in Group A received 0.8 ml. of 0.1 *N* HNO_3 solution and then the antimicrobial agents were added to the cylinder according to the protocol in Table II. The volume of all cylinders was brought to 15 ml. by the addition of sterile distilled water. After mixing, a clear solution was obtained. Coacervation was prevented in the acidified cylinders (Group A) by addition of sodium chloride [the concentration of sodium chloride in each system was 0.18% (w/v)]. One milliliter of solution from each cylinder was transferred to each of three replicate tubes containing rapidly growing cultures of *E. coli* B/r, which had been inoculated 90 min. previous to the addition of the drugs. Total organism counts were made 4.5 hr. after the antimicrobials were introduced to each culture.

Table III—Components Used to Study Partitioning of Antimicrobials in Gelatin–Acacia Complex Coacervates

Tube	A–G Stock Solution, ml.	0.1 *N* HNO_3 or HCl, ml.	Antibacterial Solution, ml.	Sterile Distilled Water, ml.	Abbreviation of System[a]	Coacervate, ml.
A	4	1.5	0	24.5	C_0	2
B	4	1.5	n[b]	*q.s.* 30	C_+	2
C	4	0	0	26	N_0	0
D	4	0	n[b]	*q.s.* 30	N_+	0

[a] C_0 = coacervate system, no drug; C_+ = coacervate system with drug; N_0 = noncoacervate system, no drug; and N_+ = noncoacervate system with drug. [b] n refers to milliliters antimicrobial shown in Tables IV–XII. (Only one concentration of drug was used in each experiment.)

Drug Partitioning in Complex Coacervate Systems—In each experiment, four 45-ml. centrifuge tubes were designated as A, B, C, and D. Tubes A and B were used to prepare coacervate systems; noncoacervate systems were prepared in C and D. The amounts of different components pipeted into each tube are shown in Tables III–XII. From previously conducted tests, the specific sequence of addition of components was determined to be immaterial. Each of the four tubes was glass stoppered; the stopper was secured to the neck of the tube by means of rubberbands. The system was placed in a reciprocating water bath shaker for 6 hr. as previously described. During this period the partitioning of the drugs was completed. Then the tubes were removed and stored undisturbed at 37° to allow the phases to separate.

Growth Conditions—The techniques of preparing, storing, and subculturing the organisms, the methods of obtaining viable counts and total counts, and the procedures to assess the coincidence of these counts were described in detail elsewhere (10).

Chemical Measurement of Partition Coefficients—*Phenylmercuric Nitrate*—The assay procedure used for this compound was the same as used by Gage (21) in determining trace amounts of phenylmercuric salts in biological materials. The method involves the

Table IV—Partition Coefficient of Phenylmercuric Nitrate (PMN) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *S. aureus* and by Chemical Method

PMN Solution Added to Each System, ml.	PMN (mcg.) Present in 1 ml. Noncoacervate System: Each System		Reproductive Index of Noncoacervate System (RI_n): Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
				6 mcg./ml.				
0.6	3.6	0.12	98.5	99.2	99.7	0.03	46	40.86
0.8	4.8	0.16	98.1	99.0	99.54	0.04	46	41.5
1.0	6.0	0.2	97.3	98.1	99.47	0.05	46	41.2
1.2	7.2	0.24	97.0	97.8	99.30	0.062	44.1	40.11
1.4	8.4	0.28	96.1	97.2	99.07	0.098	36.59	39.23
1.6	9.6	0.32	95.0	96.76	98.92	0.10	34.00	40.30
1.8	10.8	0.36	96.0	96.5	99.75	0.12	31.00	36.71
3.5	21	0.7	93.8	94.4	97.7	0.23	30.8	32.39
7.0	42	1.4	92.0	83.1	95.72	0.48	29.7	30.47
				60 mcg./ml.				
1.4	84	2.8	73.0	70.2	87.5	1.02	27.2	28.1
2.1	126	4.2	50.2	55.15	80.2	1.64	24.5	24.90
3.75	225	7.5	40.4	41.0	63.2	3.12	22.1	22.63
7.5	450	1.5	23.0	24.1	28.1	7.63	15.5	14.91
15.0	900	3.0	2.0	4.0	7.2	16.9	12.6	12.26
17.5	1050	3.5	0	1.8	4.9	21.8	10.5	10.0
0	0	0	100	100	100	0	—	—

Table V—Partition Coefficient of Phenylmercuric Nitrate (PMN) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *E. coli* B/r and by Chemical Method

PMN (600 mcg./ml.) Stock Solution Added to Each System, ml.	PMN (mcg.) Present in 1 ml. Noncoacervate System: Each System		Reproductive Index of Noncoacervate System (RI_n): Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
1.25	750	25	82.1	84.51	88.5	13.84	13.1	13.39
1.5	900	30	77.0	76.1	86.2	16.95	12.55	12.67
2	1200	40	69.1	68.2	79.3	25.4	9.61	9.7
2.5	1500	50	64.3	65.1	75.	32.3	9.22	9.10
3.0	1800	60	57.2	56.4	69.91	39.65	8.70	8.89
3.5	2100	70	48.6	49.7	64.7	47.72	8.0	8.30
4	2400	80	45.0	47.1	60.7	55.5	7.6	8.0
8	4800	160	19.8	20.5	33.7	114.8	6.9	6.51
12	7200	240	8.6	9.2	19.2	175.1	6.59	5.92
15	9300	310	5.0	6.2	12.3	220.3	5.37	5.11
16	9600	320	2.0	3.05	9.02	252.6	5.01	4.62
16.6	9900	330	0	3.01	8.3	260.6	5.00	4.89
17.5	10,500	350	0	3.0	6.7	279.11	5.00	4.87
0	0	0	100	100	100	0	—	—

oxidation of phenylmercuric nitrate with acidic permanganate and the subsequent titration of the mercury with dithizone solutions. The steps were described previously (11).

Furazolium Chloride—One milliliter of the equilibrium liquid from coacervate systems containing furazolium chloride was transferred to a 15-ml. centrifuge tube and made alkaline by the addition of 2 ml. of 0.1 *N* sodium hydroxide solution. The pH was 11.7. Seven milliliters of a hexanol–heptane mixture (4:1) was added to the tube, and the drug was extracted into the organic phase by shaking the tube for 1 min. The phases were separated by centrifugation at 1200×*g* for 10 min. Three milliliters of the upper layer (solvent mixture) was removed, and the absorbance was determined at 420 nm. One milliliter of equilibrium liquid from the coacervate system containing no drug was treated in the same way and served as a zero blank. A standard curve was constructed by plotting the absorbance at 420 nm. against accurately known amounts of drug present in standard aliquots. A least-squares regression line was fitted to the data, using the regression of *X* (mcg. furazolium chloride) on *Y* (absorbance). The amount of drug in the unknown sample was determined by calculation from the standard curve $X = 0.21\,Y$.

Chloramphenicol—The chemical assay of chloramphenicol was performed using a method described in the FDA monographs (22). One milliliter of the equilibrium liquid was placed directly in a 1-cm. quartz cell and the absorbance was determined in a DB-G spectrophotometer at 278 nm. Aliquots of the equilibrium liquid containing no drug were used as the zero blank. An aqueous solution containing 20 mcg./ml. chloramphenicol was used as the standard solution. Standards were freshly prepared for each experiment.

Benzalkonium Chloride—The method described by Scott (23) was adopted for this assay.

1. Preparation of solutions. One-half gram of orange II was dissolved at room temperature in distilled water to make 1 l. This solution was protected from light. Delory–King's carbonate–bicarbonate buffer was prepared by dissolving 170 mg. of exsiccated sodium carbonate and 1.746 g. of sodium bicarbonate in sufficient distilled water to make 100 ml. of a buffer solution with a pH of 9.2 at 23° (24). A reference solution containing 170 mcg./ml. of benzalkonium chloride in distilled water was prepared from a concentrate.

2. Calibration of reference solution. Five aliquots of 1, 2, 3, 4, and 5 ml. each of benzalkonium chloride reference solution, containing 170, 340, 510, 680, and 850 mcg., respectively, of the antimicrobial were transferred to each of five 125-ml. separators. To each separator was added 1 ml. of 95% ethanol, 5 ml. of Delory–King's buffer, 3 ml. of orange II solution, and 12 ml. of chloroform. Water was added to bring the aqueous volume to approximately 35 ml. Each separator was shaken vigorously for 5 min. Then the lower chloroform layer was collected in a 50-ml. volumetric flask. Three

Table VI—Partition Coefficient of Furazolium Chloride (FCl) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *E. coli* B/r and by Chemical Method

FCl (600 mcg./ml.) Stock Solution Added to Each System, ml.	FCl (mcg.) Present in— Each System	1 ml. Noncoacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
1	60	2	88.1	90.0	81.2	1.76	3.00	2.11
1.5	90	3	76.2	75.5	73.7	2.61	3.22	2.53
2	120	4	64.3	65.2	66.9	3.39	3.71	2.99
2.5	150	5	54.2	55.1	61.3	4.16	4.01	3.67
3.0	180	6	48.7	49.0	55.2	4.9	4.36	4.00
4.0	240	8	36.3	39.8	54.7	5.05	9.76	8.93
5.0	300	10	28.7	30.1	52.5	5.4	13.77	13.12
6.0	360	12	25.1	26.0	50.2	5.8	17.00	16.93
7.0	420	14	20.0	20.3	49.0	6.0	21.00	20.12
8.0	480	16	17.0	17.1	44.7	6.7	21.82	22.1
9.0	540	18	11.4	12.0	39.6	7.87	20.30	22.00
10	600	20	8.6	9.05	32.8	9.34	18.10	18.61
11	660	22	7.0	7.80	28.3	10.6	17.12	17.2
12	720	24	5.4	6.0	23.0	12.33	15.20	16.0
13	780	26	4.0	5.02	19.21	13.83	14.21	13.81
14	840	28	3.0	3.5	15.2	15.85	12.50	12.11
15	900	30	1.5	2.71	11.25	18.34	10.50	11.01
16	960	32	0	2.2	8.8	20.48	9.1	9.00
17	1020	34	0	2.05	6.72	22.7	8.50	8.3
18	1080	36	0	2.00	5.41	24.5	8.01	8.00

Table VII—Partition Coefficient of Furazolium Chloride (FCl) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *S. aureus* and by Chemical Method

FCl (300 mcg./ml.) Stock Solution Added to Each System, ml.	FCl (mcg.) Present in— Each System	1 ml. Noncoacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
1	300	10	88.9	90.3	91	5.26	13.8	14.09
2	600	20	75.1	73.2	84	9.31	18.12	17.62
3	900	30	55.3	57.1	71.2	18.3	10.6	12.31
4	1200	40	44.8	45.0	54.1	27.79	7.59	8.01
6	1800	60	32.6	34.8	43.7	44.73	6.12	6.98
8	2400	80	18.9	20.0	30.03	63.22	4.98	5.10
10	3000	100	13.7	14.6	21.3	82.42	4.20	4.00
12	3600	120	10.0	10.63	15.02	100.45	3.92	3.73
13	3900	130	4.0	4.9	14.71	110.73	3.61	3.25
14	4200	140	1.5	4.1	5.0	—	—	3.11
0	0	0	100	100	100	0	—	—

Table VIII—Partition Coefficient of Furazolium Chloride (FCl) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *P. aeruginosa* and by Chemical Method

FCl (10,000 mcg./ml.) Stock Solution Added to Each System, ml.	FCl (mcg.) Present in— Each System	1 ml. Noncoacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
0.3	3000	100	96.1	95.5	92.1	82.6	4.16	4.00
0.6	6000	200	93.2	94.8	89.8	175.95	3.05	3.12
1.2	12,000	300	79.0	78.7	76.2	354.8	2.91	2.78
1.5	15,000	500	69.8	70.8	71.1	450.1	2.63	2.75
3	30,000	1000	44.0	45.2	50.05	914.6	2.4	2.25
6	60,000	2000	23.9	25.0	25.0	1840.5	2.31	2.13
9	90,000	3000	9.1	9.6	12.8	2764.1	2.28	2.10
12	120,000	4000	5.0	5.10	6.3	3690.0	2.28	2.03
15	150,000	5000	1.5	2.4	3.7	—	—	1.99
18	180,000	6000	0	2.2	3.2	—	—	2.00
21	210,000	7000	0	2.02	3.2	—	—	2.07
0	0	0	100	100	100	0	—	—

Table IX—Partition Coefficient of Chloramphenicol (CP) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *E. coli* B/r and by Chemical Method

CP Solution Added to Each System, ml.	CP (mcg.) Present in— Each System	1 ml. Non-coacervate System	Reproductive Index of— Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
			60 mcg./ml.					
0	0	0	100	100	100	0	—	—
2	120	4	91.2	89.9	75.1	4.27	0.04	0.07
2.5	150	5	76.9	78.2	70.2	5.34	0.04	0.069
3.0	180	6	77.3	75.1	64.0	6.40	0.07	0.08
3.5	210	7	67.1	66.0	59.7	7.43	0.1	0.05
4	240	8	63.1	60.1	53.1	8.41	0.21	0.09
5	300	10	52.4	53.0	48.0	10.4	0.3	0.13
6	360	12	44.0	41.3	40.0	12.5	0.45	0.25
7	420	14	38.6	40.0	35.7	14.47	0.5	0.36
8	480	16	28.9	30.2	31.2	16.50	0.57	0.41
9	540	18	28.6	27.1	27.2	18.4	0.62	0.53
10	600	20	23.6	26.2	24.0	20.1	0.91	0.72
11	660	22	22.5	23.9	21.5	21.7	1.2	0.93
12	720	24	20.0	19.1	18.75	23.07	1.61	1.26
13	780	26	14.3	15.1	18.00	24.22	2.1	1.59
14	840	28	13.1	14.3	16.1	25.77	2.3	1.87
15	900	30	10.0	10.6	14.1	26.9	2.71	2.34
16	960	32	9.5	10.01	13.65	28.3	3.1	3.00
17	1020	34	9.14	9.08	12.2	29.5	3.31	3.42
18	1080	36	7.81	8.1	11.9	30.0	4.0	3.95
19	1140	38	6.6	6.6	11.52	30.3	4.82	4.72
20	1200	40	6.0	6.07	11.48	30.6	5.61	5.27
21	1260	42	5.	4.92	11.41	30.7	6.52	5.98
22	1320	44	3.77	4.00	11.07	31.1	7.2	7.00
24	1440	48	2	3.42	9.72	32.7	8.0	8.22
			120 mcg./ml.					
12.5	1500	50	2	2.9	9.6	33	8.7	8.80
13	1560	52	2	2.0	9.25	33.5	9.25	9.11
13.5	1620	54	0	1.5	9.11	33.75	10.0	10.40
14.0	1680	56	0	1.5	8.91	34.13	10.61	10.67
14.5	1740	58	0	1.49	8.45	34.83	10.98	11.02
15.0	1800	60	0	1.5	8.31	35.52	11.34	11.43
15.5	1860	62	0	1.52	7.7	36.24	11.66	11.71
16.0	1920	64	0	1.51	7.18	37.18	11.82	11.80
16.5	1980	66	0	1.5	6.72	38.00	12.05	12.30
17	2040	68	0	1.52	6.21	38.9	12.22	12.45
17.5	2100	70	0	1.51	6.01	39.5	12.58	12.61
20	2400	80	0	1.51	5.4	41.2	15.13	14.99
25	3000	100	0	1.5	4.37	49.1	16.51	16.42

successive chloroform extractions were made from each separator using additional 12-ml. aliquots. The volumetric flasks were brought to volume with chloroform, and the absorbance was determined at 485 nm. with chloroform as the blank. The absorbance of the reference solutions was plotted against the amount of drug in each aliquot. A least-squares regression line was drawn, which served as a standard curve for the determination of the amount of benzalkonium chloride in aliquots withdrawn from coacervate systems.

3. Determination of benzalkonium chloride in coacervate systems. Twenty milliliters of equilibrium liquid was transferred to a 125-ml. separator and treated exactly as already described. The absorbance was determined at 485 nm. Chloroform extractions from an aliquot withdrawn from a coacervate system containing no drug were used as a blank.

RESULTS AND DISCUSSION

Selection of Drugs—Three criteria were observed in the selection of drugs for inclusion in these studies.

Aqueous Solubility—The concentration of the drug in the coacervate system was increased under conditions of the experiment until the partition coefficient became relatively independent of the amount of drug in the system (Figs. 1–4). According to experimental constraints imposed by the model system, the maximum aqueous solution volume that could be added to each system was 24.5 ml. or 6.375 times the volume of the A-G Stock Solution (Table III). Because of this limitation, the concentration of the drug stock solutions and, therefore, the aqueous solubility of the drug itself must be such that the maximum amount of drug required for a microbiological end-point could be achieved within the allowable volumes that might be added to the coacervate systems. The limitation is of practical consideration only for fairly insoluble drugs. Of course, the addition of cosolvents and surfactants or any other changes in the physical conditions might have resulted in a more concentrated drug

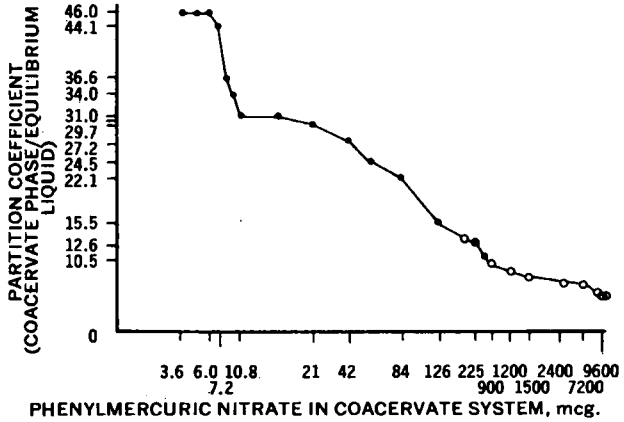

Figure 1—*Partitioning of phenylmercuric nitrate in gelatin–acacia complex coacervate system at 37°. Key:* ●, *determined by* S. aureus; *and* O, *determined by* E. coli *B/r.*

Table X—Partition Coefficient of Chloramphenicol (CP) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *S. aureus* and by Chemical Method

CP Solution Added to Each System, ml.	—CP (mcg.) Present in— Each System	1 ml. Noncoacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
			150 mcg./ml.					
0	0	0	100	100	100	—	—	—
2	300	10	92.1	95.1	86.1	10.3	0.56	0.61
3	450	15	82.9	85.2	79.6	15.3	0.70	0.81
4	600	20	72.6	74.8	75	20.0	1.0	1.10
5	750	25	70.1	69.9	72.3	23.7	1.8	1.72
6	900	30	63.8	64.7	65.2	27.8	2.59	2.43
7	1050	35	62.9	63.02	65.0	29.66	3.7	3.25
8	1200	40	50.8	64.5	30.5	30.5	5.7	5.67
9	1350	45	47.9	48.3	44.02	31.4	7.5	7.39
10	1500	50	45.0	45.2	62.0	33.0	8.7	8.78
15	2250	75	37.3	39.1	55.7	40.2	14.0	15.10
			375 mcg./ml.					
8	3000	100	29.6	28.2	52.2	44.1	20.1	20.71
10	3750	125	13.0	13.9	46.0	53.57	21.0	22.01
12	4500	150	9.2	9.6	39.6	63.38	21.5	22.11
14	5250	175	6.1	7.0	34.2	73.73	21.6	22.00
16	6000	200	4	6.45	28.8	84	21.7	22.00
18	6750	225	2	3.65	25.0	94.01	21.9	22.01
20	7500	250	1.2	2.31	21.7	104.45	21.9	22.07
22	8250	275	0	2.21	6.7	188.3	21.9	22.04
24	9000	300	0	2.00	4.9	205.4	21.9	22.00

Table XI—Partition Coefficient of Benzalkonium Chloride (BZCl) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *S. aureus* and by Chemical Method

BZCl (1080 mcg./ml.) Stock Solution Added to Each System, ml.	—BZCl (mcg.) Present in— Each System	1 ml. Noncoacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
2	2160	72	74.1	75.8	94.71	12.50	72.36	—
3	3240	108	60.2	61.0	91.23	19.12	70.72	—
4	4320	144	47.8	49.2	88.62	27.21	65.30	70.23
5	5400	180	40.3	41.0	84.21	35.3	62.52	69.12
6	6480	216	35.0	34.3	80.0	43.5	60.42	62.31
7	7560	252	33.3	33.1	77.23	51.20	59.83	60.03
8	8640	288	23.0	23.2	73.3	59.9	58.12	57.13
9	9720	324	20.0	20.0	71.0	68.4	57.01	56.81
10	10,800	360	19.1	19.98	68.3	77.6	55.61	54.92
11	11,880	396	15.4	15.5	65.2	87.00	54.27	54.00
12	12,960	432	12.1	12.0	58.51	101.1	50.09	52.36
13	14,040	468	10.1	10.2	54.43	125.3	42.02	44.00
14	15,120	504	8.0	8.01	46.44	154.3	34.99	36.20
15	16,200	540	5.1	6.3	38.62	192.6	28.06	30.10
16	17,280	576	4.0	6.0	33.81	218.7	25.5	27.09
17	18,360	612	2.0	4.8	26.50	270	20.0	21.36
18	19,440	648	2.0	3.91	22.53	301.8	18.2	18.00
19	20,520	684	1.03	3.72	18.26	342.0	16.0	16.80
20	21,600	720	1.0	3.5	15.25	377.6	14.6	15.10
21	22,680	756	0	3.4	12.40	420.0	13.0	13.13
22	23,760	792	0	3.4	10.08	455.1	12.1	11.96
23	24,840	828	0	3.4	8.61	494.8	11.2	11.12
24	25,920	864	0	3.4	6.5	549.1	9.6	9.00
0	0	0	100	100	00	0	—	—

stock solution, but these factors will, in turn, affect the partitioning characteristics of the drug entities as well as the coacervate system itself.

Stability—The pH of coacervation was near 3.67, and all coacervate systems were maintained at 37° for 24 hr. to ensure the attainment of partitioning equilibrium and the complete separation and coalescence of the coacervate phase droplets. These conditions may in themselves cause physical or chemical decomposition of certain drugs such as penicillin. Therefore, the drugs selected for examination in this study were chosen on the basis of chemical and physical stability as well as maintenance of full biological activity under the experimental conditions.

Antimicrobial Spectrum—Since a major objective of this investigation was to examine the partitioning characteristics of drugs in coacervate systems from their lowest measurable concentrations to relatively large amounts where the partitioning behavior in coacer-

Table XII—Partition Coefficient of Benzalkonium Chloride (BZCl) in Gelatin–Acacia Complex Coacervate Systems Determined by Partial Inhibition in the Growth of *P. aeruginosa* and by Chemical Method

BZCl (3000 mcg./ml.) Stock Solution Added to Each System, ml.	BZCl (mcg.) Present in— Each System	1 ml. Non-coacervate System	Reproductive Index of Noncoacervate System (RI_n) Colony Count	Total Count	Coacervate System (RI_c)	*a* Value Calculated from Graph, mcg.	Partition Coefficient Calculated from Microbiological Method	Partition Coefficient Calculated from Chemical Assay
3	9000	300	79.3	81.2	91.1	62.0	58.62	57.5
4	12,000	400	71.4	70.9	90.05	86.2	55.13	55.00
5	15,000	500	46.8	48.4	86.21	118.0	49.51	50.11
6	18,000	600	45.0	46.1	68.8	256.1	21.14	23.12
8	24,000	800	29.0	28.3	51.4	460.0	12.25	14.51
10	30,000	1000	20.0	20.09	35.5	716.5	6.93	7.81
12	36,000	1200	17.1	17.08	25.75	947.4	5.05	5.00
14	42,000	1400	10.8	11.00	18.72	1135.1	4.51	4.43
16	48,000	1600	10.0	10.6	14.92	1318.7	4.2	4.06
18	54,000	1800	9.0	9.03	11.0	1491.7	4.1	4.09
20	60,000	2000	5.0	5.52	9.11	1657.4	4.1	4.01
22	66,000	2200	3.0	4.76	7.2	1823.1	4.1	4.05
24	72,000	2400	0	4.0	5.6	1988.8	4.1	4.1
0	0	0	100	100	100	0	—	—

vates became independent of amount, drugs were selected that offered a wide spectrum of microbiological activity against common organisms such as *E. coli*, *S. aureus*, and *P. aeruginosa*.

On the basis of these criteria, phenylmercuric nitrate, furazolium chloride, chloramphenicol, and benzalkonium chloride were included in this study.

Effect of Drug Concentration on Coacervate Volume—The volume of the coacervate phase was used as a sensitive and reliable indicator of the effects of different factors on the coacervate process itself. Factors such as heat, acids, and sterilization were described previously (10). To design a reproducible model system for the drug partitioning studies reported here, the effect of various drug concentrations was examined to determine the extent to which the coacervation process itself was altered by the presence of the antimicrobial agent. When different amounts of phenylmercuric nitrate, furazolium chloride, chloramphenicol, and benzalkonium chloride were added to coacervating systems, no differences were observed in the volume of the coacervate phase (Table I). The ratio of the volume of the coacervate phase to the volume of the A–G Stock Solution used was consistently 1:2. These observations suggest that the presence of drug in the coacervate system and the partitioning of antimicrobials under the experimental conditions had no measurable effect on the volume of the coacervate layer. Water structure has been postulated as being, in part, a function of the coacervation process itself. Since the volume of the coacervate layer is highly dependent upon the extent and type of water structuring, it is concluded that the coacervation process in the presence of drugs is similar if not identical to the process in the absence of drugs since no measurable change in phase volume occurred. It was shown under a variety of conditions employed in the formation of coacervates that the sequence of addition of the different components, as well as the length of the shaking period to produce a fine dispersion of coacervate droplets during the drug partitioning experiments, had no effect on the volume of the coalesced coacervate phase.

The addition of phenylmercuric nitrate at concentrations above 320 mcg./ml. caused the temporary formation of a coacervate layer when the drug solution was added to the A–G Stock Solution in the absence of acid. The coacervate phase disappeared when the system was mixed. On the other hand, the addition of tetracycline hydrochloride in concentrations above 2 mcg./ml. to the A–G Stock Solution formed permanent coacervate phases which did not disappear on shaking. This indicates that tetracycline hydrochloride sufficiently reduced the pH of the system (to 3.4) to initiate formation of coacervate droplets. This factor alone may explain, at least in part, the decreased absorption of tetracycline from oral dosage forms when ingested in the presence of considerable dietary or milk proteins. The stomach contents acidify the system sufficiently to suppress coacervation; but when the drug–protein complex is passed into the less acidic portions of the small intestine, the pH rises sufficiently to form coacervation droplets at a pH that is slightly below the iso-

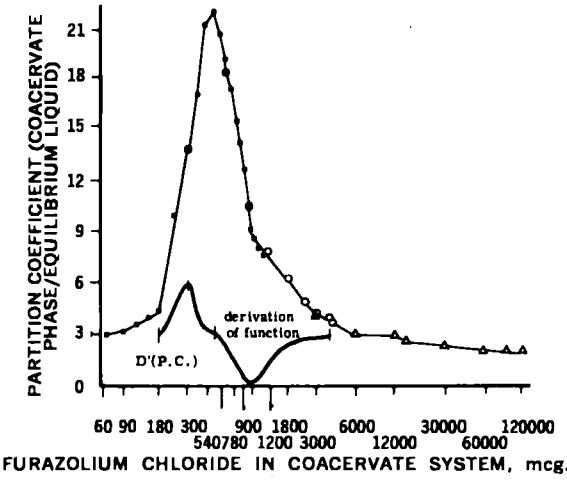

Figure 2—*Partitioning of furazolium chloride in gelatin–acacia complex coacervate system. Key:* ●, *determined by* E. coli *B/r;* O, *determined by* S. aureus; △, *determined by* P. aeruginosa; *and* ——, *derivative of partitioning curve.*

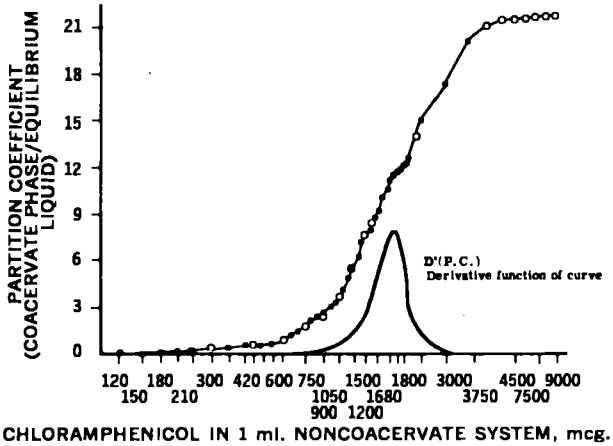

Figure 3—*Partitioning of chloramphenicol in gelatin–acacia complex coacervate system. Key:* ●, *determined by* E. coli *B/r;* O, *determined by* S. aureus; *and* ——, *derivative of partitioning curve.*

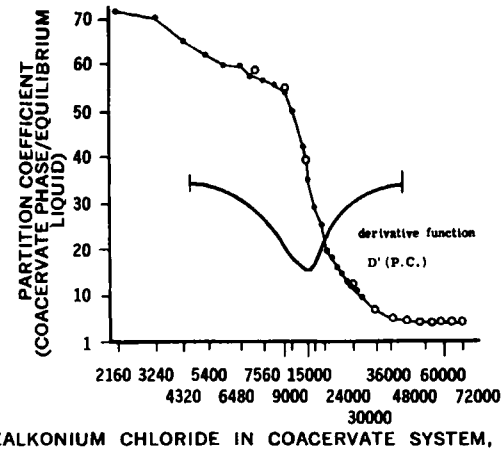

Figure 4—*Partitioning of benzalkonium chloride in gelatin–acacia complex coacervate system. Key:* ●, *determined by* S. aureus; O, *determined by* P. aeruginosa; *and* ——, *derivative of partitioning curve.*

electric point of the dietary proteins. This may provide an additional mechanism to explain the decreased availability of such drugs when coadministered with dietary proteins.

Utilization of Gelatin by Microorganisms—The concentration of gelatin in the equilibrium liquids of coacervate systems is considerably less than in the noncoacervate system, since up to 99% of the colloidal solute of the coacervate system remains in the coacervate phase itself. For this reason, it was important to ascertain whether the growth of the test organisms was directly affected by the difference in the concentrations of gelatin that might be transferred to the incubation tubes along with the antimicrobial agent. The background of all plates inoculated with test organisms turned clear upon the addition of mercuric chloride solution. The zones containing colonies were turbid with a yellow color. Had the organisms utilized gelatin to any extent as a primary nutrient, the background in the areas of colony growth would have appeared clearer than the rest of the plate. From the results of these tests, it was concluded that gelatin was not an important nutrient for any of the organisms under study and that their rate of growth in the nutrient medium used in the bioassay procedure would not be affected by any variation in the concentration of gelatin transferred with the aliquot of antimicrobial agent.

Inactivation of Antimicrobials due to Coacervation Process—To determine whether the reagents used in the preparation of the

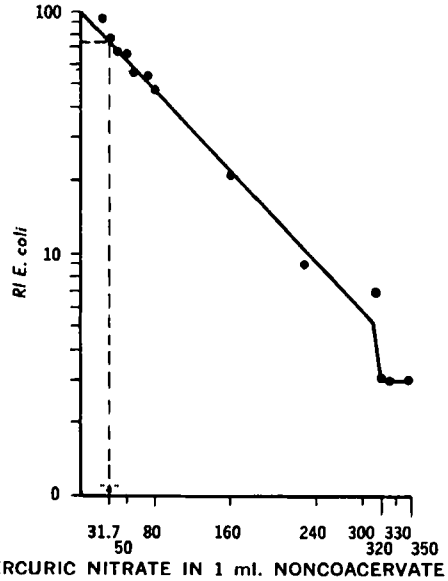

Figure 5—*Effect of phenylmercuric nitrate on the reproductive index* (RI) *of* E. coli *B/r.*

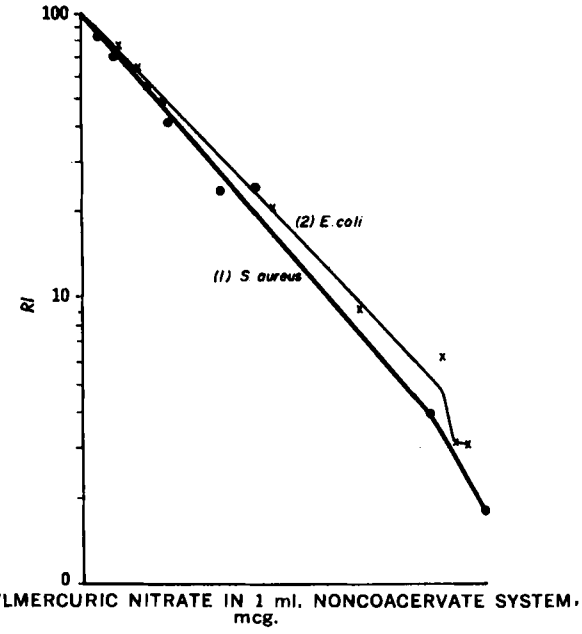

Figure 6—*Effect of phenylmercuric nitrate on the reproductive index* (RI) *of microorganisms. (Information concerning each line and the x-axis is found in Table XIII.)*

coacervate systems or the coacervation process itself had any effect on the potency of the antibacterial agents, several concentrations of each drug were incorporated into both coacervate and noncoacervate systems. Formation of the coacervate phase was suppressed by the addition of 0.18% sodium chloride. Aliquots were removed from each system and tested for antibacterial activity. The total counts of *E. coli* B/r were converted into reproductive index (*RI*) values, and the results are shown in Table II. Among all antimicrobial concentrations examined, the *t* test showed that the differences between the average *RI* values obtained for the noncoacervate and the suppressed coacervate were not significantly different ($p > 0.05$) except for the highest level of chloramphenicol. When 960 mcg. of chloramphenicol was placed into noncoacervate and suppressed coacervate systems, there was a significant ($p < 0.05$) decrease in antibacterial activity in the salt-treated suppressed coacervate system. This observation is attributed to experimental error, since coincidence of viable counts and total counts for much higher concentrations of chloramphenicol using *E. coli* were obtained, and no similar deviations were observed at these increased drug concentrations.

Coincidence of Total and Viable Counts—Agreement between the total (Coulter) counts and the viable (colony) counts of organisms per milliliter in drug-free and drug-treated cultures is evident for all values shown in Tables III–XII. Correlation of the *RI* values obtained by each method indicates that the drug concentration range selected with each organism was sufficient to inhibit partially the growth of the organisms. Furthermore, agreement between viable

Table XIII—Information Concerning Figs. 6–9

Line	Slope (−)	Organism	Antimicrobial[a]	Each Division of *x*-axis, mcg.
1	0.064	*S. aureus*	PMN	0.25
2	0.040	*E. coli*	PMN	2.5
3	0.0518	*E. coli*	FCl	2.5
4	0.0033	*P. aeruginosa*	FCl	50.0
5	0.022	*S. aureus*	FCl	1.0
6	0.04	*E. coli*	CP	0.4
7	0.0064	*S. aureus*	CP	2.5
8	0.0219	*S. aureus*	BZCl	6.0
9	0.00062	*P. aeruginosa*	BZCl	20.0

[a] PMN = phenylmercuric nitrate, FCl = furazolium chloride, CP = chloramphenicol, and BZCl = benzalkonium chloride.

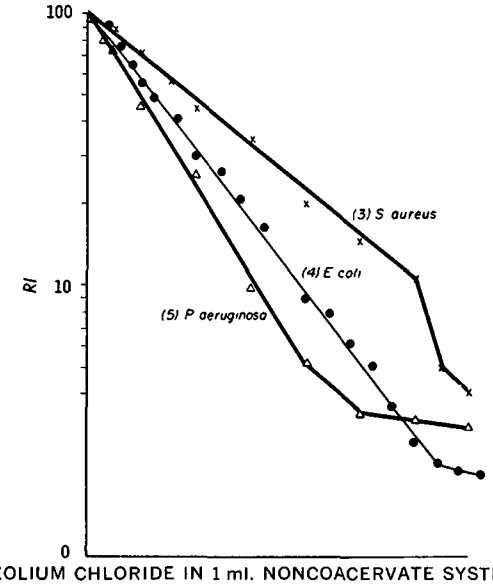

Figure 7—*Effect of furazolium chloride on the reproductive index* (RI) *of microorganisms. (Information concerning each line and the* x-*axis is found in Table XIII.)*

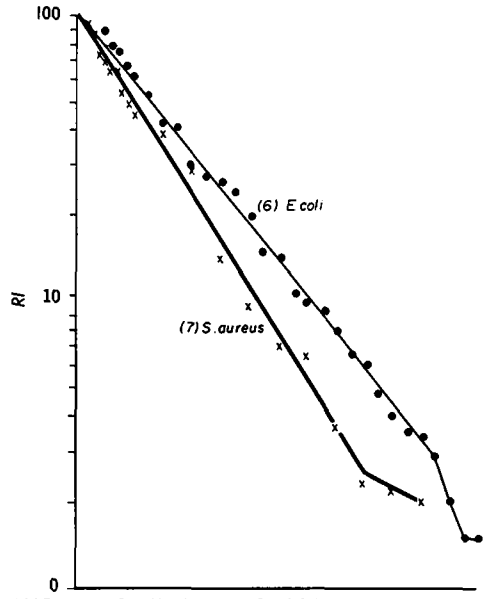

Figure 8—*Effect of chloramphenicol on the reproductive index* (RI) *of microorganisms. (Information concerning each line and the* x-*axis is found in Table XIII.)*

cell counts and particle numbers determined by the Coulter counter technique indicated that only live cells were being counted by the more rapid electronic method. Neither cellular debris nor particulate contamination from the medium was interfering with a true count of the organisms exposed to the effects of the antimicrobial agent. Likewise, the addition of formaldehyde to the cell suspension before electronic counting suppressed further growth but did not disrupt the cells themselves. When systematic deviations occurred between *RI* values obtained from the two types of counting techniques, it was assumed that the amount of drug in the treated systems exceeded the minimum bactericidal concentration for the test organism; only dead organisms were counted by the Coulter counter technique and their number was equal to the number of organisms in the test culture (90 min. after inoculation but immediately before the drug was added).

To study the partitioning of the same drug at higher concentrations in coacervate systems, different microbiological test organisms were selected because of their relative resistance to the concentrations of drug being tested. In this way, a full range of drug concentrations, which exceeded the limit of biological response displayed by any one strain of organism to a particular drug, could be examined in coacervate systems using a variety of organisms (Figs. 1–4). As might be expected, the partition coefficient as determined by the microbiological methods was not dependent upon the particular strain of test organism, but rather there was excellent agreement between the partition coefficients calculated at drug concentrations where more than one organism was used to determine the *RI* values. It is obvious that by selecting organisms with varying sensitivities to the antibacterial or by varying the conditions under which the determinations are made, drug partitioning may be studied over the entire concentration range. In particular, the selection of organisms that were very highly sensitive to the drug permitted the extension of partitioning measurements to ranges far lower than could be detected by conventional chemical techniques.

Drug Partitioning: Calculation of the Partition Coefficient—*Microbiological Method*—In most instances, the experimental conditions were adjusted to produce a constant coacervate volume (V_1) of 2 ml. (Table III) and the total volume of each coacervate system (V) was 30 ml. From these values the volume of the equilibrium liquid (V_2) was calculated to be 28 ml. The partition coefficient of the four antimicrobials was calculated for a variety of total amounts of drug in the system (T) according to the equation developed in a previous paper (11):

$$PC = \frac{T}{aV_1} - \frac{V_2}{V_1} \qquad \text{(Eq. 1)}$$

where PC is the partition coefficient defined as the concentration of drug in the coacervate phase with respect to the concentration of the drug in the equilibrium liquid, and a is the concentration of drug in the equilibrium liquid (micrograms per milliliter). The value of a was determined graphically from growth-inhibition curves for each organism over a biosensitive drug range. The estimation of a may be summarized as follows:

1. Calculate *RI* values based on total organism numbers in flasks in both coacervate and noncoacervate drug systems. Where different sized inocula are used, reproductive coefficients (*RC*) are determined first and *RI* values are computed from the *RC* values. For convenience, designate the reproductive index from noncoacervate systems as RI_n and from coacervate systems as RI_c.
2. Plot log RI_n *versus* the concentration term T/V, where T and V have the same meanings as in Eq. 1. This relationship will yield a straight line which may be used as a "standard" curve for all subsequent determinations with the same drug–organism system (Fig. 5).

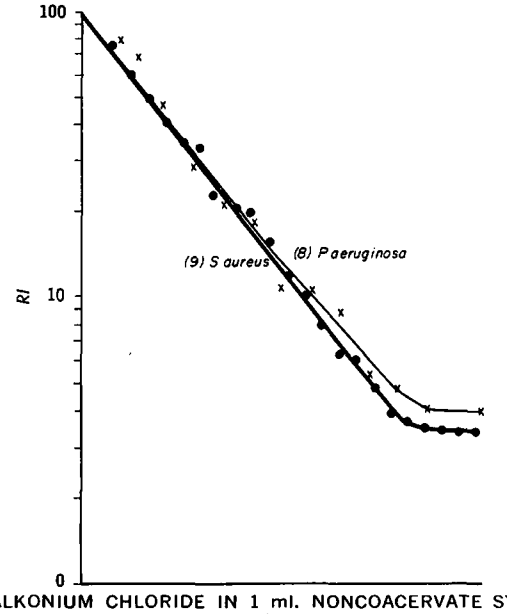

Figure 9—*Effect of benzalkonium chloride on the reproductive index* (RI) *of microorganisms. (Information concerning each line and the* x-*axis is found in Table XIII.)*

3. A drug concentration term may be obtained from the abscissa for each log RI_e value obtained from the coacervate systems. This value (micrograms per milliliter) is termed a and is used directly in Eq. 1 for the calculation of the partition coefficient. Partition coefficients for the antimicrobial agents calculated over a wide range of concentrations are contained in Tables IV–XII and Figs. 6–9.

Chemical Method—The partition coefficient of drug compounds may be calculated directly, using Eq. 1, in a manner that is very similar to that used with the microbiological technique. The concentration term a was determined from an appropriate chemical analysis of an aliquot of equilibrium liquid.

1. Phenylmercuric nitrate. Since phenylmercuric nitrate contains 63.2% (w/w) mercury, the organomercuric assay technique of Gage (21) utilizes a standard titrimetric solution of mercuric chloride which represents 1.1867 mcg. of phenylmercuric nitrate/ml. of titrant consumed in the analysis of 20 ml. of the equilibrium liquid. If n ml. of titrant is used in the assay, then the a value (concentration of drug in the equilibrium liquid) may be calculated directly from the equation:

$$a = \frac{1.1867n}{20} \qquad \text{(Eq. 2)}$$

and the partition coefficient of the drug in the coacervate system is determined once again from Eq. 1. Partition coefficients calculated by this method are given in Tables IV and V.

2. Furazolium chloride. By using the analytical techniques described for furazolium chloride, the concentration of drug in micrograms per milliliter of equilibrium liquid was calculated directly and designated as a. Partition coefficients, calculated from Eq. 1, are presented for furazolium chloride in Tables VI–VIII.

3. Chloramphenicol. The absorptivity (1%, 1 cm.) of the standard solution of chloramphenicol was calculated from the following equation:

$$\text{absorptivity} = \frac{\text{absorbance at 278 nm.} \times 100}{\text{mcg./ml. chloramphenicol standard solution}} \qquad \text{(Eq. 3)}$$

Since absorptivity is concentration independent, the absorbance at 278 nm. of appropriately treated equilibrium liquid samples divided by the absorptivity of chloramphenicol determined under identical conditions yields the concentration of the antimicrobial directly in terms of micrograms per milliliter. From this value the partition coefficient of the drug could be calculated, and Tables IX and X contain values at several drug concentrations.

4. Benzalkonium chloride. When the absorbance values of benzalkonium chloride were plotted against the concentration of the drug in accurately calibrated solutions (micrograms per milliliter), a straight-line standard curve was fitted from which the amount of benzalkonium chloride in the 20 ml. of equilibrium liquid could be calculated. The a value was determined directly, and Eq. 1 was used to calculate the partition coefficient in the usual way. These values are reported in Tables XI and XII.

Reliability of Microbiological Partition Coefficient—One main objective of this investigation was to develop a method whereby the partitioning of drugs in complex coacervate systems could be studied using microbiological end-points over a wide concentration range. The faithful correlation between partition coefficients obtained from both chemical and microbiological methods has established the validity of the newly developed method. However, at low concentrations of drug, deviations arise between the partition coefficient values obtained by the two methods. These are attributed to a general lack of sensitivity of the chemical methods at extremely low drug concentrations. The main advantage of the microbiological method over the chemical analysis (apart from its extended lower limit of sensitivity) is that any subtle physical or chemical alteration in the drug, resulting from the experimental processes, might affect the biological activity of such compounds. Yet such changes are not detected by ordinary chemical methods and generally emerge only when one tests for the biological potency of the compound along with chemical analysis. The sensitivity of microorganisms to biologically active substances provides especially good end-points for the measurement of such compounds. Any changes in the metabolic processes of susceptible organisms as a result of drug action are grossly reflected in the growth rate and reproductive index.

Partition coefficients calculated from the microbiological method described here were plotted *versus* T, the total amount of drug in the coacervate systems (Figs. 1–4). The mechanism of the partitioning of some organic compounds was studied by Takruri (12), who postulated that the effect was based on the differences in water structure between the phases of the experimental model. According to his view, compounds such as benzalkonium chloride and furazolium chloride are "structure makers" and the enhanced structure of water in the coacervate phase at least partially accounts for the unusually high partitioning of these compounds. Diamond (25) showed that tetraalkyl ammonium chlorides exhibited positive deviations from the Debye-Huckel limiting law. According to Diamond, the partitioning of benzalkonium chloride in coacervate systems could be interpreted as a salting-out effect. As seen from Fig. 4, phenylmercuric nitrate shows the same characteristics as furazolium chloride or benzalkonium chloride. The uptake of this salt by the coacervate phase can be interpreted on the basis of an interaction of a hydrophobic ion with water and macromolecules. Chloramphenicol shows a situation opposite to that of the others. But the curves in Figs. 1, 2, and 4 show the same trend; *i.e.*, the partition coefficient is related directly to concentration at low levels of drug and then falls off sharply from a maximum value which varied with each drug.

We postulate that the constant partition coefficient truly represents a saturation of all binding sites on the macromolecular components in the coacervate phase, and the peak found with furazolium chloride (Fig. 2) may represent an alteration in the mechanism of the binding to protein molecules or a complex state of aggregation of the drug in the equilibrium liquid phase. In this way the coacervate model system may become an important adjunct to the study of processes affecting the protein binding of drug molecules to macromolecular components; inflections and peaks in the partition coefficient function with respect to total drug amounts in the system represent the result of ongoing binding and aggregational processes in both the coacervate and equilibrium phases. Further studies are in progress to elucidate more clearly the mechanisms involved in the binding of drug entities with the proteins in the complex coacervate model. Such studies may contribute to the understanding of the component processes involved in the partitioning of small molecules in biological models.

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Steady-State and Nonsteady-State Transport through Membranes Using Rotating-Disk Electrode Polarography: Description and Properties of a Rapid Response New Technique

YIE WEN CHIEN*, CARTER L. OLSON, and THEODORE D. SOKOLOSKI▲

Abstract □ A new rapid response technique was developed to measure both steady-state and nonsteady-state flux (mass transport rate, moles per second) through membranes. The system utilizes a rotating-disk electrode, which is covered with a porous membrane and connected to a polarographic module. The rotating-disk configuration provides a uniform flux density over the entire surface of the membrane. Since flux is directly measured as current, there is no need to construct a concentration *versus* time plot to obtain steady-state permeability and there is no need to rely on lag time to measure effective diffusivity. The use of lag times for this purpose has recently been shown to introduce significant errors in estimating diffusivity. In this technique, the membrane is placed on the surface of the electrode. In the receiving compartment, response to flux is so rapid that nonsteady-state diffusion may be characterized. Moreover, it is possible to determine steady-state flux in the same experimental trial in short time periods. The technique and its use are described and theoretically explained through basic electrochemical and diffusion principles. The reproducibility achieved in transport measurements is seen to be quite good.

Keyphrases □ Transport through membranes—methodology, equations for measuring steady-state and nonsteady-state flux using rotating-disk polarography □ Membranes, characterization of transport—methodology, equations for measuring steady-state and nonsteady-state flux using rotating-disk polarography □ Polarography, rotating disk—used to measure steady-state and nonsteady-state transport through membranes, theory, equations □ Rotating-disk polarography—used to measure steady-state and nonsteady-state transport through membranes, theory, equations

Transport processes occurring across a membrane barrier separating a solution phase from another solution or solid phase continue to be of great interest to pharmaceutical scientists. Among these interests are: (*a*) a better understanding of the mechanisms of transport in order to develop new membrane systems having controlled drug-release properties, and (*b*) the characterization, through the use of models, of the probable mechanisms and behavior of complex biomembranes in terms of physicochemical principles.

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It is apparent from the literature that the design of a suitable diffusion measurement system is complex. Variables such as membrane integrity, area, and thickness; compartment volumes and concentrations; reproducibility of sampling; stirring; and temperature must be controlled. Many diffusional cell prototypes may be found (1–6, 9–16) and, considering that most were designed for similar purposes, their diversity is remarkable. Flynn and Smith (7) classified these cell systems and analyzed their advantages and disadvantages.

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Steady-State and Nonsteady-State Transport through Membranes Using Rotating-Disk Electrode Polarography: Description and Properties of a Rapid Response New Technique

YIE WEN CHIEN*, CARTER L. OLSON, and THEODORE D. SOKOLOSKI▲

Abstract □ A new rapid response technique was developed to measure both steady-state and nonsteady-state flux (mass transport rate, moles per second) through membranes. The system utilizes a rotating-disk electrode, which is covered with a porous membrane and connected to a polarographic module. The rotating-disk configuration provides a uniform flux density over the entire surface of the membrane. Since flux is directly measured as current, there is no need to construct a concentration *versus* time plot to obtain steady-state permeability and there is no need to rely on lag time to measure effective diffusivity. The use of lag times for this purpose has recently been shown to introduce significant errors in estimating diffusivity. In this technique, the membrane is placed on the surface of the electrode. In the receiving compartment, response to flux is so rapid that nonsteady-state diffusion may be characterized. Moreover, it is possible to determine steady-state flux in the same experimental trial in short time periods. The technique and its use are described and theoretically explained through basic electrochemical and diffusion principles. The reproducibility achieved in transport measurements is seen to be quite good.

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One prime concern of making permeability measurements is the ability to determine flux (mass transport

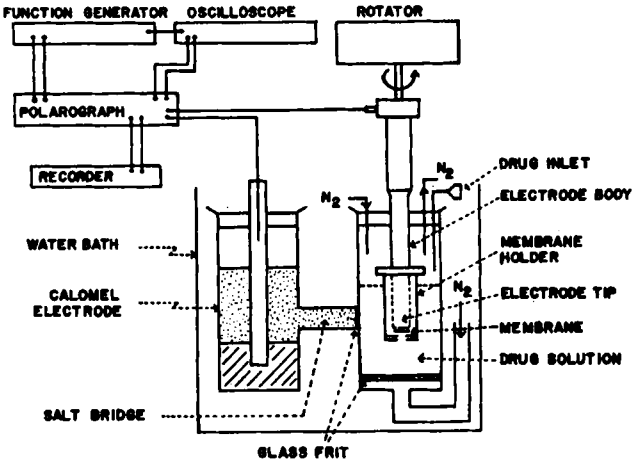

Figure 1—*Schematic representation of the electrochemical system used to measure drug transport.*

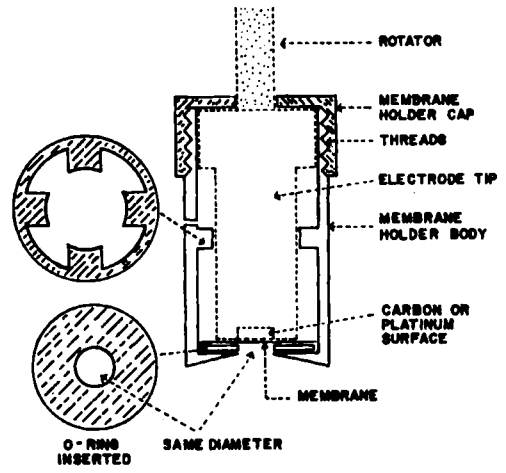

Figure 2—*Schematic representation of the membrane holder assembly showing its relation to the electrode tip. The electrode tip can be removed from the rotator. The cap is placed over the electrode tip with a membrane in place and the body is then attached.*

rate, moles per second) reliably in as short a time as possible. With this in mind, a relatively simple and easily assembled transport cell was constructed. A membrane is positioned smoothly on the immediate surface of a rotating-disk electrode. The flux or transport of material through the membrane is proportional to the current measured polarographically at a constant potential. The response is very rapid, permitting direct measurement of both the initial nonsteady-state and the steady-state membrane transport. The sensitivity of a diffusional cell is proportional to the available diffusional area divided by the solution volume of the receiving compartment (8). The receiving compartment in the present system has a negligible volume because the detector is positioned directly on the membrane surface. Therefore, the detector signal is directly proportional to the flux across the membrane rather than to the receiving compartment concentration.

The high speed rotating-disk configuration has the advantage of uniform, controlled flux to the outside membrane interface. At high rotation speed, the solution diffusion layer thickness may become negligible with respect to membrane thickness. In any case, the thickness of the convective diffusion layer on the surface of the membrane may be controlled and calculated. The concentration at the electrode surface can be set to zero by setting the voltage on the diffusion current plateau. The high sensitivity, rapid response, and nonsampling method utilized permits the observation of transient phenomena which may be significant to the mechanism of membrane transport but which may be overlooked using slower conventional methodology.

The lag time technique of Daynes (17) and Barrer (18) has been popularly applied in conventional mass transport systems to calculate the diffusivity of drug species. Steady-state transport should be calculated from data taken after two lag times; otherwise, significant errors may result in determining the extrapolated lag time and in calculating diffusivity.

Siegel and Coughlin (19) recently made a theoretical analysis of such calculations and they concluded that the relative errors in lag time calculations can be as much as five times larger than the errors obtained from calculations based on the slope of steady-state permeability. Their analysis suggests that the error in diffusivity will always be several times larger than the error in permeability when such techniques are employed. In the method introduced here, diffusivity can be estimated either directly from steady-state permeability or from kinetic analysis of nonsteady-state transport without the use of lag time estimates.

Although extensive experimental and theoretical studies have been made on the permeation of gases through various materials, diffusion in polymer membranes has received relatively limited attention, particularly with regard to the short-time dynamic nature of drug permeation across barriers. In general, two different phenomenological mechanisms are met in the flow of penetrant species through thin membranes. These are molecular transport involving partitioning of the penetrant into the membrane structure, followed by molecular diffusion across it, and passage of penetrant molecules through membrane pore channels filled with solvent. Transport through a nonporous barrier depends on the relative solubility of the penetrant in the barrier as compared to its solubility in the donor and receiving compartments.

The new technique described in this paper provides a direct measurement of flux both at steady-state and nonsteady-state diffusion conditions.

EXPERIMENTAL

Apparatus—The system used in these studies is shown in Fig. 1. A polarographic H-cell is immersed in a constant-temperature bath[1] at 30 ± 0.1°. The left side of the cell contains a saturated calomel reference electrode (S.C.E.) connected to the working half of the cell (on the right) *via* a salt bridge. The working cell contains 10–20 ml. of test solution. The electrode is immersed in this solution and either rotated or held stationary. The working and reference electrodes are connected to a polarograph[2]. Current is measured either with a strip-chart recorder[3] or an oscilloscope[4]. The recorder is used for time measurements greater than 0.5 sec., and the oscilloscope is used for shorter times. For short-time

[1] Sargent Thermonitor, model ST.
[2] Heath polarographic module EVA-19-2.
[3] Varian model G-14.
[4] Hewlett Packard model 141A.

measurements, the potential from the polarograph is controlled by a function generator[6]. The test solution is deaerated with nitrogen gas, and a nitrogen atmosphere is maintained above the solution during measurements. Drug is introduced into the working side of the electrode by injecting a small volume (0.2–1.0 ml.) of a concentrated predeaerated drug solution *via* the drug inlet from a calibrated microsyringe.

A membrane sheet is positioned on the electrode surface using a cell holder made of Teflon (Fig. 2). A small hole is drilled on the upper edge of the holder to balance pressures across the membrane and to minimize hydrodynamic transport through the covering membrane. The entire assembly is rotated at a constant speed with a rotating electrode assembly[6]. Speeds ranging from 0.6 to 6000 r.p.m. are possible with this assembly. Two types of electrode tips were used: a platinum tip[7], which was coated with mercury according to the method of Ramaley *et al.* (20), and a carbon electrode[8]. The carbon electrode permits the use of a wide potential range, has a low residual or background current, provides good reproducibility, and requires little pretreatment. Both electrodes have a smooth surface. The membrane is placed directly on the electrode surface as shown in Fig. 2.

Materials—The supporting electrolyte used in the studies was 0.2 *M* acetate buffer, pH 4.0. The pH selected assures that the electroactive species, *p*-nitrophenol[9], is totally undissociated (pKa 7.12) and that the buffer is of sufficient concentration to obviate pH changes at the electrode surface due to the consumption of protons. *p*-Nitrophenol was found to be 99% pure by potentiometric titration.

Two membranes were used in the study: (*a*) Millipore membranes[10] of cellulose acetate–cellulose nitrate mixtures and (*b*) dialysis membranes[11] with a pore radius of 24 Å and a dry thickness of 25 μ.

Method—All of the membranes were compatible with the solutions used and were presoaked in the buffer system overnight in a refrigerator before use.

The general procedure used for the measurement of steady-state and nonsteady-state transport of *p*-nitrophenol through the various membranes was as follows. Seventeen milliliters of supporting electrolyte was placed in the working compartment of the polarographic H-cell and deaerated for 5 min. with nitrogen gas. A solution of *p*-nitrophenol was separately prepared in the same supporting electrolyte and deaerated for 3 min. The transport cell containing the membrane was assembled (Fig. 2) and placed on the electrode tip. The electrode assembly was placed in the solution and rotated at a fixed speed until equilibration occurred. The current-potential curves for *p*-nitrophenol were determined, and the results are listed in Table I. A constant potential of −1.0 v. *versus* S.C.E. was applied, and the steady-state residual current was measured. One milliliter of the concentrated *p*-nitrophenol solution was rapidly injected into the cell compartment. The current increased with time until it reached a steady-state value. After steady state was reached, an additional 0.4 ml. of solution was introduced, giving rise to a new steady-state current. The incremental procedure was continued until a suitable calibration curve was obtained.

RESULTS AND DISCUSSION

The current–time relation predicted for a plane electrode in a quiet (unstirred) solution is given by the equation:

$$i = \frac{nFAD^{1/2}C}{\sqrt{\pi t}} \qquad \text{(Eq. 1)}$$

where n is the number of electrons transferred (4 for *p*-nitrophenol at pH 4), F is the Faraday constant (96,500 coulombs per equivalent), A is the electrode surface area in square centimeters, D is the diffusion coefficient (9.18×10^{-6} cm.^{2} sec.$^{-1}$ for *p*-nitrophenol), C is the bulk concentration of electroactive species in millimoles per liter, t is the time after start of electrolysis in seconds, and i is the faradaic current in microamperes. A plot of i *versus* $(t)^{-1/2}$ should be a straight line with slope $nFAD^{1/2}C(\pi)^{-1/2}$. When a membrane covers the electrode surface, the effective area of the electrode is reduced to an amount equal to the porosity of the membrane. Figure 3 shows the results for the reduction of *p*-nitrophenol with and without a Millipore membrane on the electrode surface. The experimental slope without a membrane was 2.89×10^3 μa. sec.$^{1/2}$ M^{-1} compared to a theoretical value of 2.87×10^3 (A = 0.232 cm.^{2}), which is excellent agreement. The experimental slope with the Millipore membrane was 2.23×10^3 μa. sec.$^{1/2}$ M^{-1} compared to a theoretical value calculated to be 2.13×10^3, based on the manufacturer's specifications of 74% porosity, which again is in good agreement.

If a disk electrode is placed into solution and rotated at a constant angular velocity, the mass transport of electroactive material to the electrode surface by means of convective diffusion is much greater than obtained by linear diffusion to a stationary electrode of equal area (22–25). A relationship describing the thickness of the convective diffusion layer, δ_D, at a rotating-disk electrode was derived by Levich (26–27):

$$\delta_D = 1.62D^{1/3}\nu^{1/6}\omega^{-1/2} \qquad \text{(Eq. 2)}$$

where D is again the diffusion coefficient, ν is the kinematic viscosity, and ω is the angular rotation in radians per second. The rotating-disk configuration is the only convective diffusion geometry where flux, *i.e.*, δ_D, is a constant over the entire surface. The

Table I—Decomposition and Half-Wave Potential for *p*-Nitrophenol in Acetate Buffer[a]

Electrode	Decomposition Potential, v.	Half-Wave Potential, v.
Mercury-coated platinum disk[b]	−0.48	−0.63
Carbon disk[b]	−0.63	−0.81
Dropping mercury[c]	−0.52	−0.68

[a] The *p*-nitrophenol concentration is 4×10^{-5} *M*. The buffer is 0.2 *M* and pH 4.0. [b] The electrode was rotated at 3000 r.p.m. [c] From *Reference 21.*

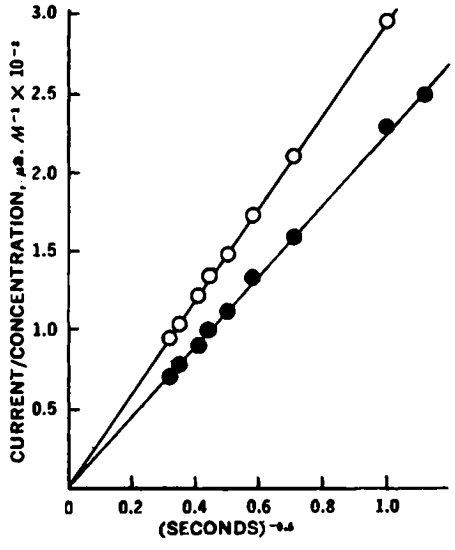

Figure 3—*Relation between current divided by concentration of* p-*nitrophenol and the reciprocal of the square root of time. The carbon disk electrode is not rotating. Key:* ○, *without a covering membrane, concentration of* p-*nitrophenol* = 2×10^{-3} M, *slope* = 2.89×10^3 μa. sec.$^{1/2}$ M^{-1} (*theoretical slope* 2.87×10^3); *and* ●, *with a covering Millipore membrane, type VC (0.1-μ pore, porosity 74%), concentration of* p-*nitrophenol* = 5×10^{-3} M, *slope* = 2.23×10^3 μa. sec.$^{1/2}$ M^{-1} (*theoretical slope* 2.13×10^3).

[6] Wavetec model 112.
[7] Beckman Drive Unit 188501W. Electrode Body 188551W.
[8] Beckman No. 39086.
[9] Beckman No. 39084.
[9] Eastman.
[10] Millipore Filter Co. [Various pore sizes are available (10 nm.–8 μ) but all types have the same approximate thickness (150 μ) and porosity (80%).]
[11] Union Carbide.

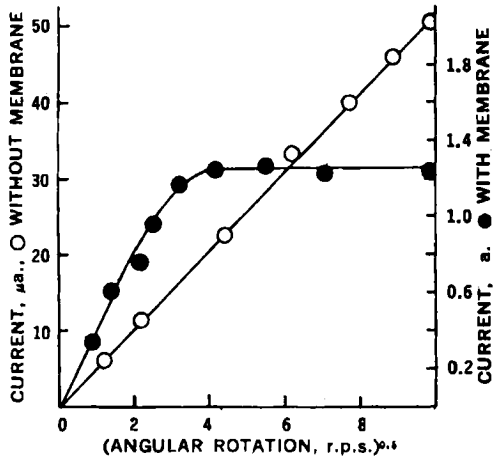

Figure 4—*Effect of the square root of angular rotation (r.p.s.) on the steady-state current of* p-*nitrophenol* (1×10^{-4} M). *Key:* O, *left ordinate, without covering membrane; and* ●, *right ordinate, with a covering dialysis membrane.*

diffusion-limited current at a rotating-disk electrode is calculated as:

$$i = \frac{nFADC}{\delta_D} \qquad \text{(Eq. 3)}$$

or:

$$i = 0.62\, nFAD^{2/3}\, \omega^{1/2}\, \nu^{-1/6}\, C \qquad \text{(Eq. 4)}$$

where the terms are as previously described. The behavior of the rotating-disk electrodes used in these studies was in agreement with theory.

Table II—Determination of the Porosity of Millipore Membranes from Steady-State and Nonsteady-State Transport of *p*-Nitrophenol at pH 4, 30°

Millipore Type	$\pi^2 D/\delta^2$, sec.$^{-1a}$	$P_m \times 10^{-4}$, μa. M^{-1b}	Porosity	
			Measured	Reported[c]
SC	0.333	3.89	0.78	0.74
SM	0.222	3.26	0.80	0.84
SS	0.119	2.52	0.85	0.83
RA	0.0952	2.18	0.82	0.82
GS	0.0054	0.50	0.79	0.75
VC	0.0042	0.44	0.79	0.74
VM	0.0040	0.42	0.77	0.72

[a] Slope of plots of $\ln (i_{ss} - i_t)$ *versus* t. [b] Slope of plots of i *versus* concentration. [c] From Millipore Catalog MF-84.

The current obtained at a membrane-covered rotating-disk electrode is controlled by both the permeability of the membrane and the convective diffusion layer at the outer surface of the membrane. Figure 4 shows a typical plot of current *versus* electrode rotation speed for both a bare and a membrane-covered electrode. When the rotational velocity of the electrode becomes sufficiently large, the transport of material to the electrode surface is controlled primarily by diffusion across the membrane. The measured current, therefore, reaches a plateau, becomes independent of increased rotational velocity, and is directly proportional to the diffusion-controlled transport or flux of material across the membrane.

A typical result obtained in measuring transport through a membrane covering a rapidly rotating disk electrode (3000 r.p.m.) is presented in Fig. 5. *p*-Nitrophenol is rapidly injected into the cell at time zero after allowing the system to come to equilibrium. After a short lag time, the current increases with time, eventually reaching a steady-state flux as defined by Eq. 3, where δ_D becomes the sum of the thickness of the stagnant layer and the effective membrane path. At the steady-state condition, a subsequent addition of *p*-nitrophenol from a concentrated deaerated solution leads to a new

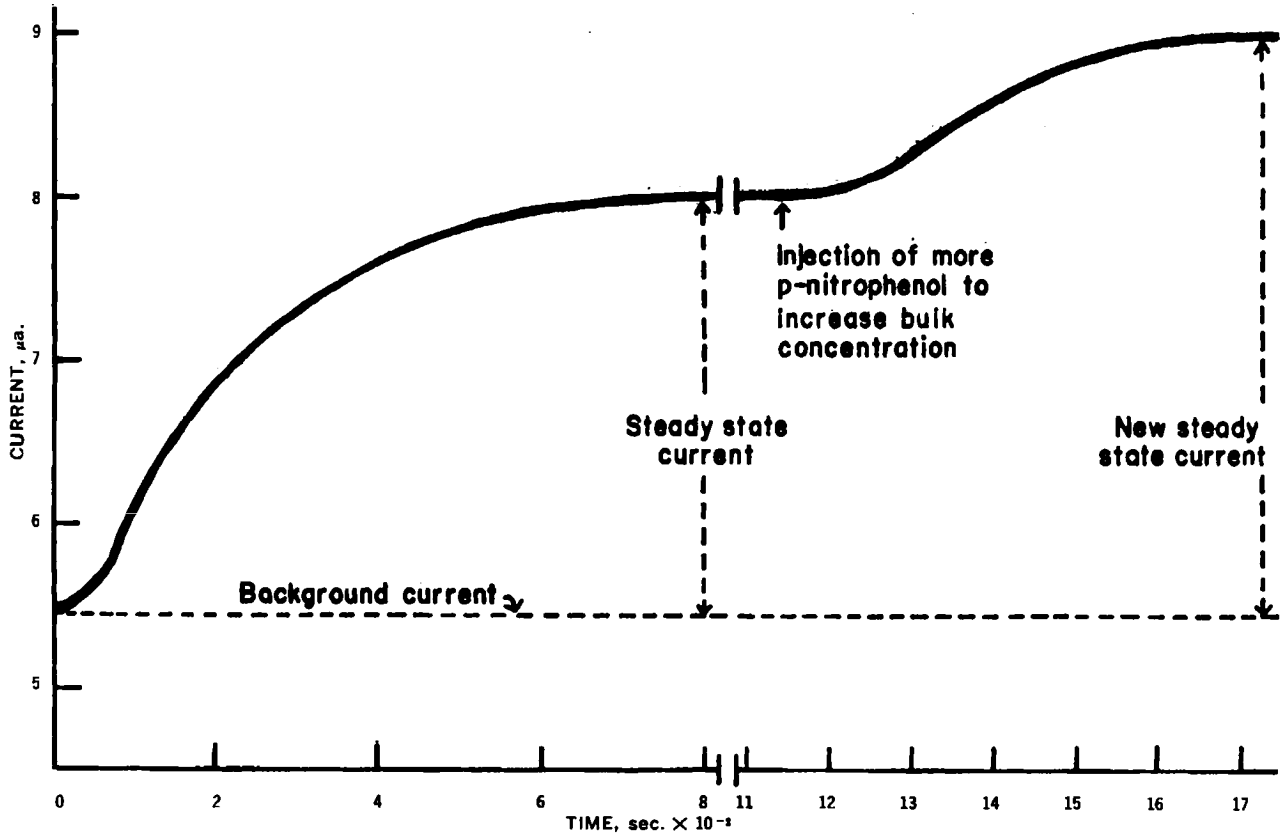

Figure 5—*Relation between current and time in the transport of* p-*nitrophenol through a Millipore membrane, type GS, where successive amounts of compound are added.*

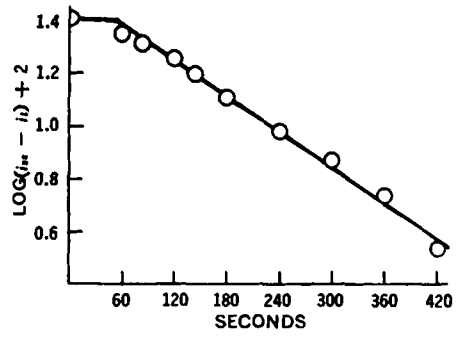

Figure 6—*Relation between the log (steady-state current − current at time t) and the time for p-nitrophenol diffusing through a Millipore membrane, type GS.*

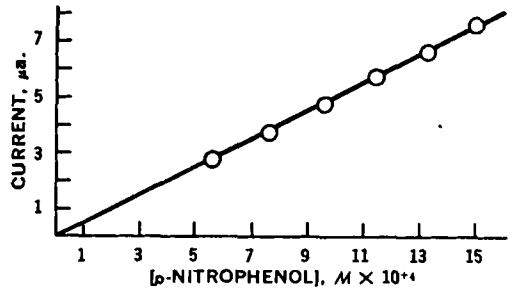

Figure 7—*Relation between steady-state current and concentration of p-nitrophenol at a rotating carbon-disk electrode (3000 r.p.m.) after successive additions of compound. The electrode is covered with a Millipore membrane, type GS.*

steady-state flux. This procedure may be repeated several times to give additional steady-state currents.

Membrane permeability, P_m, calculated from the steady-state current:

$$P_m = \frac{i_{ss}}{nFC} \qquad \text{(Eq. 5)}$$

is determined by two parameters, porosity and effective membrane thickness, which includes tortuosity effects and the stagnant solution layer on the surface of the membrane. To evaluate porosity and effective membrane thickness, additional experimental data are needed. In the present technique the additional data are derived from the time-dependent (nonsteady-state) flux as presented in Fig. 5. Several investigators have derived expressions describing the flux through a membrane as a function of time following a step or rapid change in concentration on one side of the membrane (28–30). In particular, Bowers and Wilson (31) derived an expression relating current to time for a membrane-covered electrode following a step jump in concentration of electroactive species in the stirred solution outside the membrane:

$$i_t = \frac{nFADC}{\delta}\left[1 + 2\sum_{m=1}^{\infty}(-1)^m \exp(-m^2\pi^2)t/\delta^2\right] \qquad \text{(Eq. 6)}$$

where i_t is the current at time t, δ is the effective diffusional thickness, and the other terms are as previously described. Although the electrode type and solution hydrodynamics are different in the Bowers–Wilson experiment, the boundary conditions for transport are the same and the equation is thus valid for a membrane-covered disk electrode when rotational velocity is rapid and constant. Typical experimental values indicate that the summation terms of Eq. 6 converge very rapidly. For example, if it is assumed that $\pi D/\delta^2 = 1$ sec.$^{-1}$, at $t = 1$ sec. an error of 6% would occur in neglecting terms where $m > 1$, and at $t = 2$ sec. the error is less than 0.1%. Neglecting summation terms of $m > 1$ and substituting Eq. 3 into Eq. 6 give:

$$i_t = i_{ss}[1 - 2\exp(-\pi^2 Dt/\delta^2)] \qquad \text{(Eq. 7)}$$

Thus, the value for the effective diffusional path, δ, can be obtained from the slope of a plot of ln $(i_{ss} - i_t)$ *versus* t where the slope is $\pi^2 D/\delta^2$. A typical semilogarithmic plot is shown in Fig. 6. By using the kinetically determined value of δ, it is possible to calculate the porosity, ϵ, of the membrane through the use of the experimentally determined permeability, P_m:

$$P_m = \frac{i_{ss}}{nFC} = \frac{DA}{\delta}\epsilon \qquad \text{(Eq. 8)}$$

Figure 7 shows a typical experimental result demonstrating the applicability of Eq. 8. The slopes of plots of current *versus* concentration give P_m. Thus, by knowing n, F, D, and A (the area of the bare electrode, 0.232 cm.^{2}), the porosity of the membrane, ϵ, may be calculated.

The experimental results obtained for a series of Millipore membranes are given in Table II. The calculated values for porosity are in excellent agreement with the reported values for the Millipore membranes. It is thought that the methodology described is well suited for studying both the steady-state and transient (nonsteady-state) characteristics of membrane transport. From Eqs. 3 and 4, it can be seen that the steady-state current is directly proportional to bulk solution concentration. If a dialysis membrane is placed on the surface of an electrode, macromolecules and molecules bound to macromolecules are excluded from reaching the electrode surface. The use of the methodology as a rapid means to study protein binding is the subject of another paper (32).

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Use of Membrane-Covered Rotating-Disk Electrode to Study Binding of Drugs to Macromolecules: Competitive Binding of 2-(4′-Hydroxybenzeneazo)benzoic Acid and α-(4-Chlorophenoxy)-α-methylpropionic Acid to Serum Albumins

YIE WEN CHIEN*, THEODORE D. SOKOLOSKI▲, CARTER L. OLSON, DONALD T. WITIAK, and RALPH NAZARETH

Abstract □ A new technique is introduced to study the binding of drugs to macromolecules. The method utilizes rotating-disk polarography in which a semipermeable membrane covers the surface of the electrode. The membrane permits passage of an electroactive drug but restricts permeability of the macromolecule and of bound drug. The current generated at the electrode at steady state is directly proportional to the concentration of free drug in bulk solution, from which a calculation of the amount of drug bound may be made. The methodology used provides a complete characterization of binding within about 1 hr. and is, therefore, faster than conventional techniques presently used. Another advantage of this technique is that relatively small amounts of the compounds being studied are required. The method is compared with equilibrium dialysis and ultrafiltration techniques by studying the binding of 2-(4′-hydroxybenzeneazo)benzoic acid (I) to serum albumin and is found to give precisely the same results. α-(4-Chlorophenoxy)-α-methylpropionic acid (II), in previous spectrophotometric studies, showed an anomalous effect on the binding of I to rat serum albumin. At low concentrations, there was an indication that II may increase the binding of I to rat serum albumin. This phenomenon was reinvestigated using the new electrochemical method; it was found that II competitively inhibits the binding of I at all concentration levels of the inhibitor. The conclusion is reached that II affects the nature of the I binding site rather than the number of molecules bound.

Keyphrases □ 2-(4′-Hydroxybenzeneazo)benzoic acid binding to serum albumins—presence, absence of α-(4-chlorophenoxy)-α-methylpropionic acid, rotating-disk polarography method, compared to conventional techniques □ α-(4-Chlorophenoxy)-α-methylpropionic acid—competitive binding with 2-(4′-hydroxybenzeneazo)benzoic acid to serum albumin, rotating-disk polarography method □ Polarography, rotating disk—used in binding studies, compared to equilibrium dialysis and ultrafiltration techniques □ Rotating-disk polarography—used in binding studies, compared to equilibrium dialysis and ultrafiltration techniques □ Serum protein binding, competitive—2-(4′-hydroxybenzeneazo)benzoic acid and α-(4-chlorophenoxy)-α-methylpropionic acid, rotating-disk polarography method □ Binding of drugs to macromolecules—studied using rotating-disk polarography, compared to equilibrium dialysis and ultrafiltration techniques

Classical methods employed to detect and determine the binding characteristics of small molecules to serum albumin have been discussed by several authors (1–3). Techniques such as equilibrium dialysis, ultracentrifugation, light absorption and rotation, and ultrafiltration have been used. The methods conventionally used require long periods of time to collect analyzable data and/or relatively large amounts of reactants, some of which can be costly. Nonequilibrium dialysis techniques (4–7) are generally faster and use less materials than conventional methods but can require cumbersome data analysis. The method of Meyer and Guttman (5), for example, takes about 6 hr. to collect data and requires the use of computer curve-fitting techniques to estimate the number of molecules bound to albumin.

To overcome some of these disadvantages, we applied a technology, developed in these laboratories (8), that was previously used to study mass transport. The technique employed to study mass transport across membranes utilized rotating-disk polarography where a membrane sheet is placed on the surface of a carbon or mercury-coated platinum tip electrode. If the membrane used is semipermeable, allowing only small electrochemically active molecules to penetrate, a system is obtained that is analogous to the thermodynamically well-defined equilibrium dialysis technique. There is,

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however, one notable difference. The steady-state current obtained at the electrode at a constant applied potential is directly proportional to the concentration of electrolyzable material in the bulk solution. Since only small molecules can penetrate the membrane, it is possible to measure in a very short time (from 18 to 30 sec.) the concentration of unbound drug in a solution containing a macromolecule with which it interacts. By successively increasing the total amount of small drug molecule in the bulk solution, enough data can be obtained in 1 hr. (up to 16 points in the present study) to characterize completely the binding of the drug. The membrane also functions to protect the surface of the electrode from contamination by protein (9).

This paper describes the binding of 2-(4′-hydroxybenzeneazo)benzoic acid (I) to various serum albumins in the presence and absence of a competitive inhibitor α-(4-chlorophenoxy)-α-methylpropionic acid (II). In addition to showing that the new methodology gives the same binding results as those obtained by conventional equilibrium dialysis and ultrafiltration techniques, it provides further insight into the effect that II has on the interaction of I to rat serum albumin.

In an earlier work carried out in this laboratory (10), the competitive binding of I and II to several different serum albumins was studied using equilibrium dialysis and extrinsic rotatory dispersion. A unique behavior was observed for rat serum albumin; using spectrophotometry, low concentrations of II appeared to enhance the binding of I to the albumin. This observation and concomitant extrinsic rotatory dispersion studies suggested that II was causing a small molecular perturbation in rat serum albumin. The results could be interpreted in a number of ways. Either II liberates additional binding sites for interaction with I, or II affects the nature of the interaction of I without increasing the number of I molecules bound. This study was undertaken to determine which of these possible mechanisms was taking place.

EXPERIMENTAL

Materials—Bovine serum albumin[1] (Fraction V, fatty acid poor) and rat serum albumin[2] (Fraction V) were used as obtained without further purification. Two brands[3] of 2-(4′-hydroxybenzeneazo)-benzoic acid (I) were used; both showed similar electrochemical and binding properties. α-(4-Chlorophenoxy)-α-methylpropionic acid (II) was prepared according to a method previously described (11). All solutions used were buffered at pH 7.4 using 0.1 *M* sodium phosphate.

Dialysis tubing[4] with an average pore radius of 24 Å and a dry thickness of 0.0041 cm. (0.0016 in.) was used. Disks of 0.9 cm. (0.37 in.) diameter were cut and washed several times with boiling double-distilled water and stored in a preserving solution containing 0.3% benzoic acid and 10% glycerin. One day before use, the disk was washed with distilled water and soaked overnight in refrigerated phosphate buffer. The disk was positioned on the surface of the electrode, as described in an earlier study (8), immediately prior to collection of data.

Binding by Electrochemical Method—The apparatus and techniques used in these studies were the same as those described previously (8). A small modification in procedure was made to cope with the tendency of protein and concentrated drug solutions to foam.

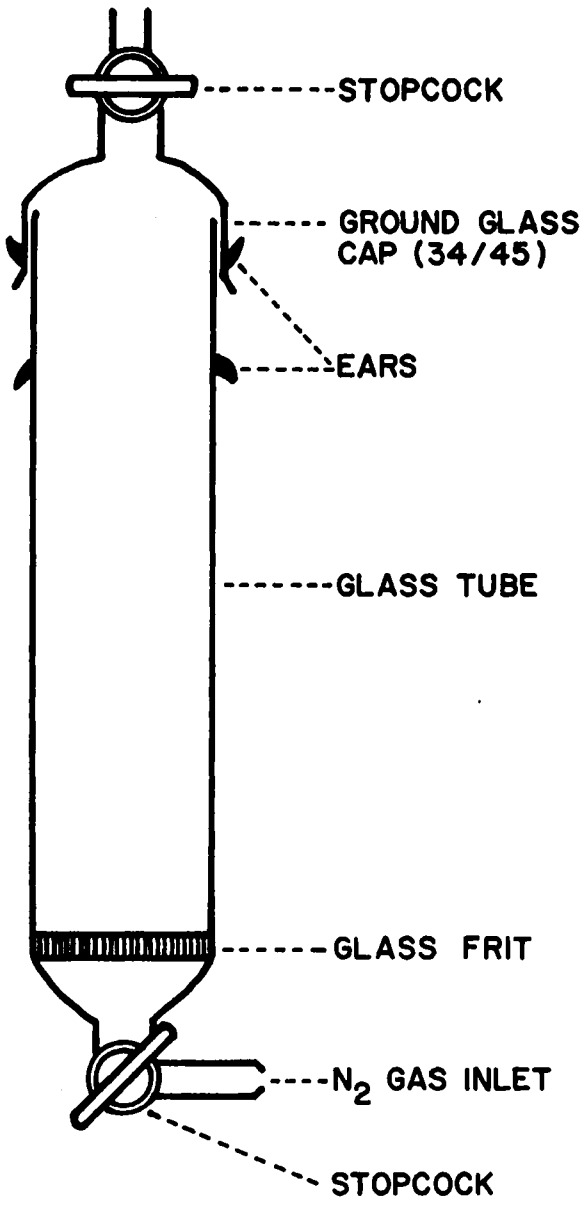

Figure 1—*Deaeration column for concentrated drug solutions or protein-containing solutions.*

Stock solutions of I, II, and albumin were made separately in pH 7.4 phosphate buffer. Deaeration was done in a specially fabricated apparatus (Fig. 1) to avoid subsequent foaming in the electrolysis cell. Adequate deaeration was accomplished in 5 min. Fifteen milliliters of deaerated protein solution was delivered with the use of a syringe to the right-hand side of the polarographic cell; a nitrogen atmosphere was maintained above the solution. The cell holder, containing the dialysis membrane, was assembled with the carbon electrode tip in place as previously described (8). The membrane electrode assembly was mounted on the electrode body and rotated at 1200 r.p.m. This rotation speed was used since it did not produce significant foaming.

A potential of −1.0 v. was applied to the working electrode, and the current was measured[5]. With no I added, the current represents background. Using a calibrated syringe, 1.0 ml. of a concentrated I solution was injected rapidly into the working compartment. After a short induction period, the current increased with time, eventually reaching a steady-state value. Depending on the concentration of I,

[1] Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Pentex, Kankakee, Ill.
[3] Eastman, Rochester, N. Y., and Nutritional Biochemical Corp.
[4] Type 1 7/8 ss, Union Carbide Corp., Chicago, Ill.

[5] The current was recorded on a calibrated Varian model HG-14 recorder.

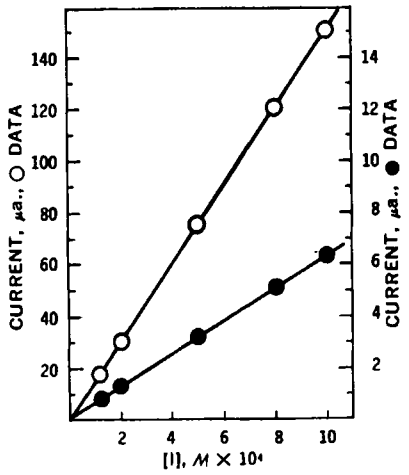

Figure 2—*Relation between steady-state diffusion current and concentration of I in bulk solution. Key: O, bare electrode, slope = 150 × 10³ μa. M⁻¹; and ●, dialysis membrane-covered electrode, slope = 6.3 × 10³ μa. M⁻¹.*

the time needed to reach steady state was 18–30 sec. After the steady-state current was attained, another 0.40 ml. of concentrated solution was injected, thereby increasing the bulk concentration of I and giving a new steady-state current. Additional 0.40-ml. portions of the concentrated solution may be added in a similar manner, giving rise to new steady-state currents. In studying the competitive binding of I and II, 1.0 ml. of a concentrated I solution was added to the protein solution and the steady-state current was recorded. Compound II was then added in consecutively increasing amounts to the I–protein solution, and the change in steady-state current after each addition was recorded. The concentration of all reactants was corrected for the small volume change following the addition of the consecutively added concentrated solutions. The amount of I removed from bulk solution due to reduction at the electrode is negligible. All studies were made at a constant temperature of 30°.

Binding by Equilibrium Dialysis and Ultrafiltration—The procedure used to determine drug binding by equilibrium dialysis studies was identical to the one reported earlier (10). Ultrafiltration measurements were made using a 10-ml. ultrafiltration cell[6] and a membrane[7]. Ten milliliters of drug–protein solution was placed in the cell, and 1 ml. of filtrate was collected. The filtrate was analyzed spectrophotometrically for free I. The absorbance for I at pH 7.4 is 17,682 l. M^{-1}.

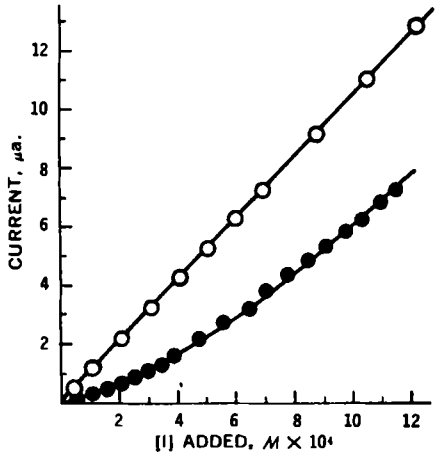

Figure 3—*Effect of bovine serum albumin on the steady-state current of I at various initial bulk concentrations. Key: O, no serum albumin added; and ●, in the presence of 1 × 10⁻⁴ M serum albumin.*

[6] Amicon model 12.
[7] PM10 Diaflo, Amicon Corp.

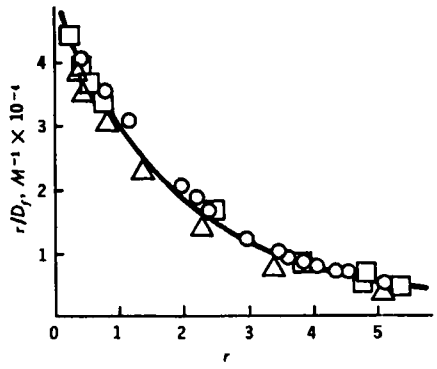

Figure 4—*Scatchard treatment of the binding of I to bovine serum albumin; r is the number of I molecules bound per mole of albumin, and D_f is the concentration of I free in solution. Key: O, electrochemical method; □, ultrafiltration technique; and △, equilibrium dialysis method.*

RESULTS AND DISCUSSION

Use of Rotating-Disk Polarography to Study Protein Binding—At pH 7.4 and 30°, the azo group of I is electrochemically reduced at the carbon electrode, producing two reduction waves with half-wave potentials of −0.40 and −0.60 v. *versus* the standard calomel electrode, respectively. One electron is consumed at each wave. Compound II is electrochemically inactive in this potential range and does not interfere with the reduction of I. The maximum difference in viscosity between I, albumin, and I–albumin mixtures is about 4%. Thus, any viscosity effect on the electrochemical properties of I is negligible.

At an applied constant potential of −1.0 v. (the plateau or diffusion-controlled potential), the steady-state current at a bare electrode due to the reduction of I is a linear function of the concentration of I (Fig. 2). The slope of this linear relation is 1.5 × 10⁶ μa. M^{-1}. A linear relation between current and concentration also was observed for the membrane-covered electrode (Fig. 2). However, owing to the porosity of the membrane which decreases the effective area of the electrode and increases the diffusional path length, the slope of the line is reduced. The ratio of the slope without membrane to slope with membrane in Fig. 2 is 0.042. Even with this reduction in sensitivity, reliable data are obtained.

When serum albumin is present, the current generated at steady state is reduced due to the binding of I to the macromolecule (Fig. 3). Since the steady-state current is proportional to the concentration of free drug, the difference in current in the presence and absence of protein at a particular total concentration of I added is proportional to the drug bound. By using data as presented in Fig. 3, it is possible to calculate the moles of I bound per mole of albumin using a molecular weight of 67,000 for the albumin. Treating the data thus collected by the Scatchard method (12) shows that more

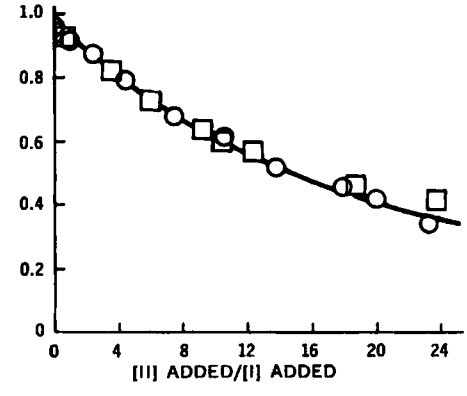

Figure 5—*Relation between the moles of I bound per mole of rat serum albumin r, with varying ratios of II:I added to bulk solution. The rat serum albumin and I concentrations are held constant at 9.4 × 10⁻⁵ and 1 × 10⁻⁴ M, respectively. The concentration of added II varies from 0 to 2.38 × 10⁻³ M.*

than one binding site is involved in the binding of I to bovine serum albumin (Fig. 4). The binding results obtained using the electrochemical method compare very well with those obtained using equilibrium dialysis and ultrafiltration (Fig. 4). However, results utilizing the rotating-disk technique can be obtained in a much shorter time and with considerably smaller amounts of materials.

Competive Binding between II and I—The observation made by Witiak *et al.* (10), indicating an unusual binding of I to rat serum albumin in the presence of low concentrations of II, was reinvestigated using both this electrochemical method and ultrafiltration. The spectrophotometric results in the Witiak *et al.* study could be interpreted in one of two ways: (*a*) II at low concentrations actually increases the number of molecules bound to rat serum albumin, or (*b*) II does not change the number of sites available for interaction but, rather, changes the nature of the binding. Use of the electrochemical method permitted direct measurement of the amount of I free in solution. The early studies (10) were based on indirect spectrophotometric measurement. Ultrafiltration also provided direct measurement of free I.

In the electrochemical studies, the concentrations of I and rat serum albumin were twice those used by Witiak *et al.* (10), but the ratio of I to rat serum albumin was the same (1.07). Hence, the binding character also should be the same. Increasing the II concentration from 0 to 23.8×10^{-4} *M* produced the results shown in Fig. 5. The concentration of II used falls into the range where anomalous effects previously were observed spectrophotometrically. As shown in Fig. 5, identical results were obtained using the ultrafiltration technique. It is clear from this figure that II replaces I from its binding sites even at low concentrations. Therefore, it is apparent that II increases the ϵ^{477} for bound I at lower concentrations (10), and this increase is not a reflection of increased binding of I to rat serum albumin.

The reasons for this increase in ϵ^{477} may, as previously suggested, be due to a small molecular perturbation in rat serum albumin, which changes the I–rat serum albumin extinction coefficient, or to an interaction of bound I with closely bound II.

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Model Transport Studies Utilizing Lecithin Spherules III: Transport of Taurocholic Acid-[cholic-^{3}H(G)] in Buffered D-Glucose Solutions

ZAKA-UD-DIN T. CHOWHAN* and WILLIAM I. HIGUCHI▲

Abstract □ The transport of taurocholic acid-[cholic-^{3}H(G)] in liposome dispersions prepared from lecithin–dicetyl phosphate in buffered glucose solutions at different pH's was studied. The transport experiments were carried out using previously developed techniques in which the release of the solute is studied from dispersions contained in the dialysis bags. The experimental results of the direct-release and the uptake-release cases were analyzed by the monosize, multiconcentric layer Model 1 which was developed, evaluated, and used recently for D-glucose transport studies. Uniformly good fits of the experimental data with the model were observed, and the permeability coefficients and the aqueous–lipid partition coefficients for taurocholate transport in liposome dispersions were calculated. The pH dependence of the permeability coefficient indicated that the unionized form of taurocholic acid was being preferentially transported at low pH and that the taurocholate ion was the main species involved at high pH.

Keyphrases □ Permeability coefficients, taurocholic acid, radiolabeled—liposome dispersions □ D-Glucose solution—transport of taurocholic acid-[cholic-^{3}H(G)], lecithin spherule dispersions, permeability coefficients, pH effect □ Taurocholic acid, radiolabeled—transport in D-glucose solution, lecithin spherule dispersions, permeability coefficients, pH effect □ Lecithin spherules—model transport studies, taurocholic acid-[cholic-^{3}H(G)] in D-glucose solution, permeability coefficients □ Transport studies, model using lecithin spherules—taurocholic acid-[cholic-^{3}H(G)] in D-glucose solution, permeability coefficients, pH effect

In previous investigations (1, 2), quantitative methods were developed and used in the determination of permeability coefficients for D-glucose and 3-*O*-methyl-D-glucose in complex aqueous liposome dispersions. The release of the solutes from dispersions contained in a dialysis bag was studied as a function of time using

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ZAKA-UD-DIN T. CHOWHAN* and WILLIAM I. HIGUCHI▲

Abstract □ The transport of taurocholic acid-[cholic-^{3}H(G)] in liposome dispersions prepared from lecithin–dicetyl phosphate in buffered glucose solutions at different pH's was studied. The transport experiments were carried out using previously developed techniques in which the release of the solute is studied from dispersions contained in the dialysis bags. The experimental results of the direct-release and the uptake-release cases were analyzed by the monosize, multiconcentric layer Model 1 which was developed, evaluated, and used recently for D-glucose transport studies. Uniformly good fits of the experimental data with the model were observed, and the permeability coefficients and the aqueous–lipid partition coefficients for taurocholate transport in liposome dispersions were calculated. The pH dependence of the permeability coefficient indicated that the unionized form of taurocholic acid was being preferentially transported at low pH and that the taurocholate ion was the main species involved at high pH.

Keyphrases □ Permeability coefficients, taurocholic acid, radiolabeled—liposome dispersions □ D-Glucose solution—transport of taurocholic acid-[cholic-^{3}H(G)], lecithin spherule dispersions, permeability coefficients, pH effect □ Taurocholic acid, radiolabeled—transport in D-glucose solution, lecithin spherule dispersions, permeability coefficients, pH effect □ Lecithin spherules—model transport studies, taurocholic acid-[cholic-^{3}H(G)] in D-glucose solution, permeability coefficients □ Transport studies, model using lecithin spherules—taurocholic acid-[cholic-^{3}H(G)] in D-glucose solution, permeability coefficients, pH effect

In previous investigations (1, 2), quantitative methods were developed and used in the determination of permeability coefficients for D-glucose and 3-*O*-methyl-D-glucose in complex aqueous liposome dispersions. The release of the solutes from dispersions contained in a dialysis bag was studied as a function of time using

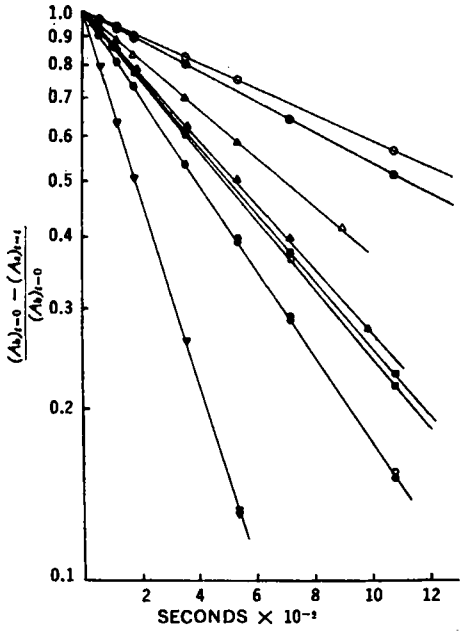

Figure 1—*First-order plots for determining the bag constants giving the transport of taurocholic acid and* D*-glucose through the dialysis bag membrane in buffered isotonic* (D*-glucose) solution. Key to transport of taurocholic acid:* ○, *0.01* M *acetate buffer at pH 3.6;* ●, *0.01* M *acetate buffer at pH 4;* △, *0.01* M *acetate buffer at pH 5;* ▲, *0.01* M *phosphate buffer at pH 7;* ■, *0.008* M *phosphate buffer at pH 8;* □, *0.01* M *phosphate buffer at pH 8;* ⬢, *0.01* M *phosphate buffer in 0.9% sodium chloride at pH 5; and* ○, *0.01* M *phosphate buffer in 0.9% sodium chloride at pH 7. Key to transport of* D*-glucose:* ▼, *0.01* M *acetate buffer at pH 5; and* ▽, *0.01* M *phosphate buffer at pH 7.*

direct-release and uptake-release experimental procedures. These data were analyzed using the monosize, multiconcentric layer models which assume that the spherules consist of multiconcentric bilayers of equal thickness separating aqueous compartments.

The purpose of the present investigation was to test the applicability of the previously developed techniques in the study of the transport behavior of an acidic compound, taurocholic acid. These studies were to be carried out under different pH conditions, and the influence of pH in altering the transport characteristics

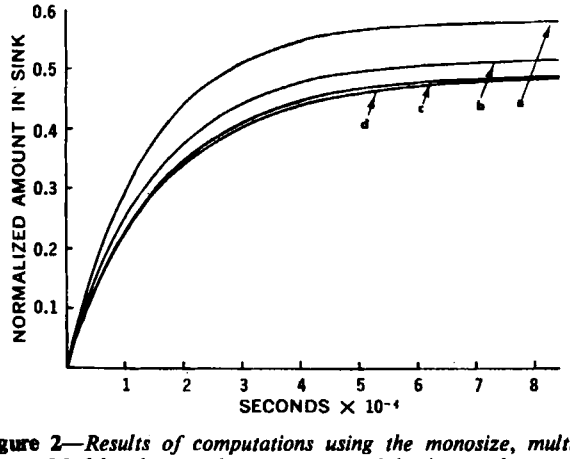

Figure 2—*Results of computations using the monosize, multiconcentric Model 1 showing the convergence of the direct-release curves. For curve a,* n = *5; for curve b,* n = *10; for curve c,* n = *20; and for curve d,* n = *30. Other parameter values are given in Table II under pH 7* (p = 2×10^{-10}).

Table I—Summary of the Parameter Values Determined at Different pH's for the Transport of Taurocholic Acid in Liposome Dispersions

pH	p	P	k_1	k_2
8 (0.01 *M* phosphate buffer)	1×10^{-9}	2.42×10^{-11}	34	140
8 (0.008 *M* phosphate buffer)	5×10^{-10}	1.21×10^{-11}	56	170
7 (0.01 *M* phosphate buffer)	5×10^{-10}	1.23×10^{-11}	50	260
5 (0.01 *M* acetate buffer)	1.5×10^{-9}	3.83×10^{-11}	82	290
4 (0.01 *M* acetate buffer)	4×10^{-8}	99.15×10^{-11}	95	345
3.6 (0.01 *M* acetate buffer)	8×10^{-8}	194.69×10^{-11}	210	600

of this solute was to be determined. It was hoped that the results of this study would help in the understanding of mechanisms that might be important in the movement of such molecules across biological membranes.

EXPERIMENTAL

General Considerations—The aqueous solution containing 5.1% anhydrous D-glucose was used earlier (1, 2) in the preparation of liposome dispersions. The same solution was used in the sink during a release run. Two reasons existed for the choice of this solution:

1. During the determination of the particle-size distribution by means of the Coulter counter, it was found important to make dilutions in 0.9% sodium chloride solution, which is isotonic, with the D-glucose solution used in the preparation of the dispersion.
2. It was not possible to prepare dispersions in 0.9% sodium chloride solution free from aggregates. Therefore, it was decided to conduct the present studies in buffered D-glucose solution at different pH's.

Materials—The procedures used in the purification of egg yolk lecithin were reported previously (1). Dicetyl phosphate[1] was used without further purification. Anhydrous D-glucose, potassium

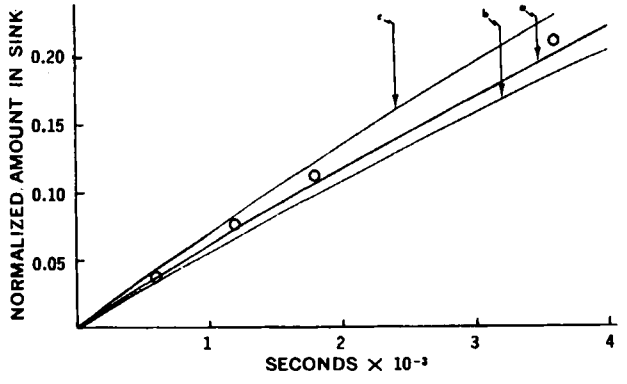

Figure 3—*Determination of* k_2 *from the initial data of the zero-hour uptake-release experiment for taurocholic acid from lecithin–dicetyl phosphate dispersions in buffered glucose solution at pH 7. Key:* ○, *experimental data. Curves represent calculation results. For curve a:* $k_1 = 48$, $k_2 = 260$, $p = 2 \times 10^{-10}$; $k_1 = 25$, $k_2 = 260$, $p = 2 \times 10^{-10}$; *and* $k_1 = 50$, $k_2 = 260$, $p = 5 \times 10^{-10}$. *For curve b:* $k_1 = 48$, $k_2 = 300$, $p = 2 \times 10^{-10}$. *For curve c:* $k_1 = 48$, $k_2 = 200$, $p = 2 \times 10^{-10}$.

[1] Sigma Chemical Co., St. Louis, Mo.

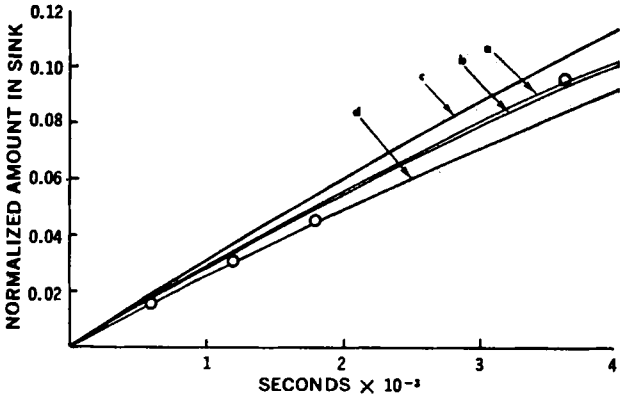

Figure 4—*Determination of* k_1 *from the initial data of direct-release experiment for taurocholic acid from lecithin–dicetyl phosphate dispersions in buffered glucose solution at pH 7. Key: O, experimental data. Curves represent computation results. For curve a:* $k_1 = 48$, $k_2 = 260$, $p = 2 \times 10^{-10}$. *For curve b:* $k_1 = 50$, $k_2 = 260$, $p = 2 \times 10^{-10}$; *and* $k_1 = 50$, $k_2 = 260$, $p = 5 \times 10^{-10}$. *For curve c:* $k_1 = 40$, $k_2 = 260$, $p = 2 \times 10^{-10}$. *For curve d:* $k_1 = 60$, $k_2 = 260$, $p = 2 \times 10^{-10}$.

phosphate monobasic and dibasic, sodium acetate, acetic acid, and chloroform were analytical reagent grade[2] and were used without further purification. Sodium chloride was also reagent grade[3] and used as such. The dialysis bags[4] were used without pretreatment. The water was double distilled in a glass apparatus. Taurocholic acid-[cholic-^{3}H(G)][5] was obtained in the form of methanol–ethanol (1:3) solution with a specific activity of 2–3 c./m*M*.

Apparatus and Procedures—The apparatus used for the taurocholic acid transport studies was exactly the same as reported previously (1). It consisted of a water-jacketed beaker and a dialysis bag holder–stirring assembly. Five milliliters of the dispersion was added to the dialysis bag, and at zero time the dialysis bag holder–stirring assembly was lowered into 100 ml. of sink solution maintained at 25°. The dispersion and the sink solution were stirred at 150 r.p.m. by two different stirring arrangements. At the end of each sampling time, the dialysis bag holder–stirring assembly was transferred to another water-jacketed beaker containing 100 ml. of fresh sink solution.

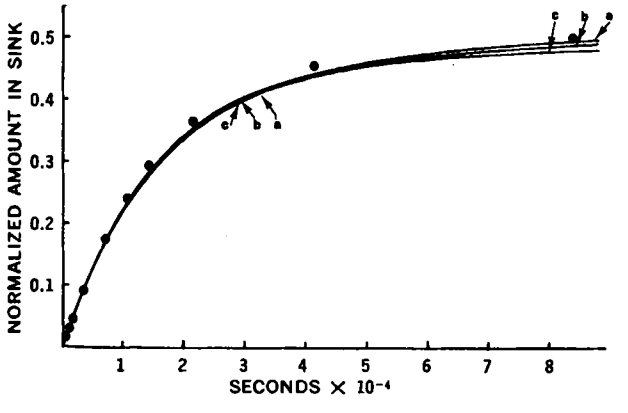

Figure 5—*Comparison of the direct-release experimental data obtained from lecithin–dicetyl phosphate dispersions for taurocholic acid released in 0.01* M *phosphate-buffered glucose solution at pH 7 with the calculations using the monosize, multiconcentric Model 1. Key: ●, experimental data. For curve a:* $k_1 = 50$, $k_2 = 260$, $p = 5 \times 10^{-10}$. *For curve b:* $k_1 = 48$, $k_2 = 260$, $p = 2 \times 10^{-10}$. *For curve c:* $k_1 = 50$, $k_2 = 260$, $p = 2 \times 10^{-10}$. *Other parameter values are taken from Table II.*

[2] J. T. Baker Chemical Co., Phillipsburg, N. J.
[3] Fisher Scientific Co., Fair Lawn, N. J.
[4] Union Carbide Corp., Chicago, Ill.
[5] New England Nuclear, Boston, Mass.

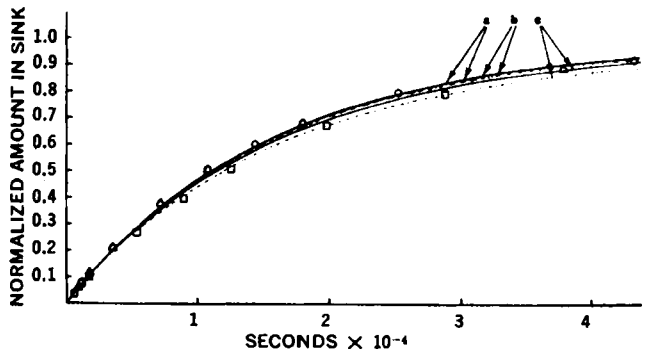

Figure 6—*Comparisons of the uptake-release experimental data obtained from lecithin–dicetyl phosphate dispersions for taurocholic acid release in 0.01* M *phosphate-buffered glucose solution at pH 7 with the calculations using the monosize, multiconcentric Model 1. Key (symbols represent experimental data): O, zero-hour solute uptake prior to release; Δ, 2-hr. solute uptake prior to release; and □, 10-hr. solute uptake prior to release. Curves a, b, and c represent calculations of 0-, 2-, and 10-hr. solute uptake, respectively. For solid curves:* $k_1 = 48$, $k_2 = 260$, $p = 2 \times 10^{-10}$; *and* $k_1 = 50$, $k_2 = 260$, $p = 2 \times 10^{-10}$. *For broken curves:* $k_1 = 50$, $k_2 = 260$, $p = 5 \times 10^{-10}$. *Other parameter values are taken from Table II.*

For the purpose of determining the bag constant (1, 2), the transport of the solute through the cellophane membrane was studied by the same procedure as used for the dispersion except that a buffered glucose solution containing the radioactive solute was used instead of the liposome dispersion.

The dispersions for the direct-release experiments were prepared (1) by mixing the chloroform solution of lecithin–dicetyl phosphate and the buffered glucose solution containing the radioactive solute. The solvent was then slowly removed using a flash evaporator with vacuum from a water aspirator. After the removal of chloroform, the dispersions were allowed to equilibrate for 2 hr. before starting the release run. The dispersions for the uptake-release experiments were prepared by the same procedure except that the radioactive solute was added to the dispersion after preparation.

The procedures for the determination of radioactive counts and for the Coulter counter particle-size distribution were the same as reported earlier (1).

THEORETICAL

Theoretical analyses carried out in the earlier reports (1, 2) indicated that the transport behavior of liposome dispersions could be quantitatively characterized by the physical models, assuming multiconcentric bilayers of lecithin separating aqueous compartments. The permeability coefficient per segment was related to the

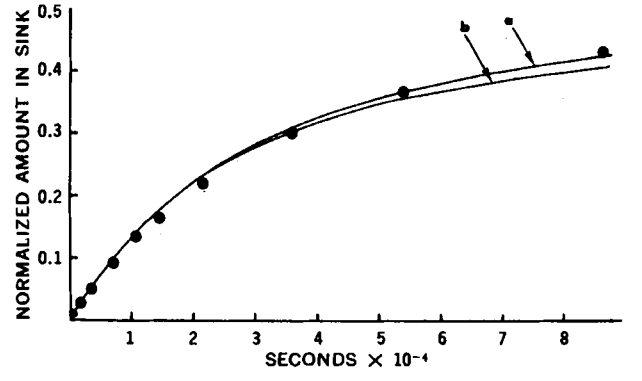

Figure 7—*Comparisons of the direct-release experimental data obtained from lecithin–dicetyl phosphate dispersions for taurocholic acid release in 0.01* M *acetate-buffered glucose solution at pH 5 with the calculations using the monosize, multiconcentric Model 1. Key: ●, experimental data. For curve a:* $k_1 = 82$, $k_2 = 290$, $p = 2.5 \times 10^{-9}$. *For curve b:* $k_1 = 82$, $k_2 = 290$, $p = 1.5 \times 10^{-10}$. *Other parameter values are taken from Table II.*

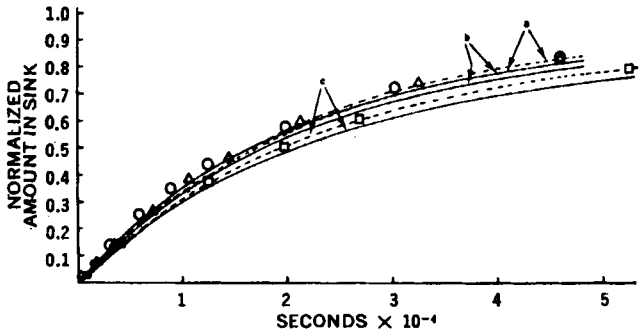

Figure 8—*Comparisons of the uptake-release experimental data obtained from lecithin–dicetyl phosphate dispersions for taurocholic acid release in 0.01* M *acetate-buffered glucose solution at pH 5 with the calculations using the monosize, multiconcentric Model 1. Key (symbols represent experimental data): ○, zero-hour solute uptake prior to release; △, 2-hr. solute uptake prior to release; and □, 10-hr. solute uptake prior to release. Curves a, b, and c represent calculations of 0-, 2-, and 10-hr. solute uptake, respectively. For solid curves:* $k_1 = 82$, $k_2 = 290$, $p = 2.5 \times 10^{-6}$. *For broken curves:* $k_1 = 82$, $k_2 = 290$, $p = 1.5 \times 10^{-6}$. *Other parameter values are taken from Table II.*

effective bulk permeability coefficient by the following equation:

$$p = \frac{P \cdot n}{r} \qquad \text{(Eq. 1)}$$

where p is the permeability coefficient per segment; n and r are the number of concentric layers and radius of the spherule, respectively; and P is the effective bulk permeability coefficient.

It was shown that the theoretical release curves converge at constant P as n is increased. On this basis it was proposed that the best P be determined at sufficiently large n.

Since the partition coefficients for D-glucose (1) and 3-*O*-methyl-D-glucose (2) were relatively small, the outermost lipid could be treated both as a part of the external phase (Model 1) and a part of the spherule (Model 2). Therefore, in the determination of permeability coefficients for 3-*O*-methyl-D-glucose, Model 2 was chosen for the data analysis.

Taurocholic acid transport studies indicated that relatively large amounts of solute bind to the lipid. Model 2, which was used for the transport studies of 3-*O*-methyl-D-glucose, failed to give reasonable agreements between the experiment and theory for this solute. Therefore, in the present studies the theoretical analyses were carried out using the monosize, multiconcentric layer Model 1. The equations describing Model 1 are given in the *Appendix* and were solved by means of a digital computer[6] as reported earlier (1).

RESULTS AND DISCUSSION

The results of the bag control experiments carried out in buffered glucose solutions at different pH's are presented in Fig. 1 in the form of semilogarithmic plots. The slopes of these first-order plots, from which the bag constants were determined (1), represent the bag constant divided by the volume inside the bag. At pH 5 and 7, when the glucose solution was replaced by 0.9% sodium chloride, the transport of taurocholic acid essentially followed the same rate. This indicated that the differences in the rate of taurocholic acid transport through the bag membrane at different pH's were mainly due to the differences in the ionic strength of the buffered glucose rather than to pH. Furthermore, the rate of D-glucose transport through the dialysis membrane remained unchanged in the same buffered glucose solution (pH 5 and 7) which gave significantly different slopes for taurocholate (Fig. 1).

The results of the zero-hour uptake-release experiments generally indicated a much greater binding of taurocholic acid with the outermost lipid layer compared with glucose and methyl glucose. Consequently, relatively large k_2 values were found (Table I). To study

[6] IBM 360.

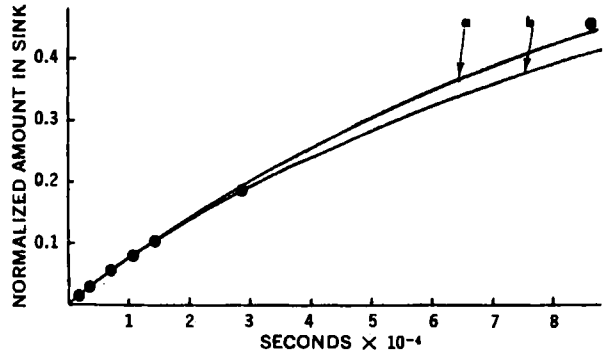

Figure 9—*Comparisons between the direct-release experimental data obtained from lecithin–dicetyl phosphate dispersions for taurocholic acid release in 0.01* M *acetate-buffered glucose solution at pH 4 and the calculations using the monosize, multiconcentric Model 1. Key: ●, experimental data. For curve a:* $k_1 = 120$, $k_2 = 345$, $p = 4 \times 10^{-6}$. *For curve b:* $k_1 = 120$, $k_2 = 260$, $p = 4 \times 10^{-6}$. *Other parameter values are taken from Table II.*

the convergence of the release curves when binding of the solute to the outermost lipid layer is much greater than binding in the inner lipid layers, it is important to keep the amount of solute bound to the last lipid layer constant. The convergence of the theoretical curves with respect to n of this system is typically illustrated by the results of computations given in Fig. 2 for the direct-release case. On the basis of these results, $n = 30$ was considered sufficiently large and was used throughout the present investigation.

The two partition coefficients k_1 and k_2 were determined from the initial rates of the direct-release and zero-hour uptake-release experiments. For a given n, the initial slope of the zero-hour uptake-release experiment is mainly a function of k_2. The determination of k_2 from the zero-hour uptake-release initial data for taurocholic acid in 0.01 M phosphate-buffered glucose solution at pH 7 is illustrated in Fig. 3. The calculations shown in Fig. 3 indicate that the initial slope of the zero-hour uptake-release experiment is insensitive to change in k_1 and p. The initial slope of the direct-release experiment is mainly a function of both k_1 and k_2. Once k_2 was determined, best k_1 values were determined by fitting the initial slope with the direct-release initial experimental points. The initial data of the direct-release experiment carried out in 0.01 M phosphate-buffered glucose solution at pH 7 are presented in Fig. 4. In this figure the theoretical curves show that the initial slope for this situation is insensitive to changes in p and is sensitive to k_1. The results of the direct-release and the uptake-release experiments

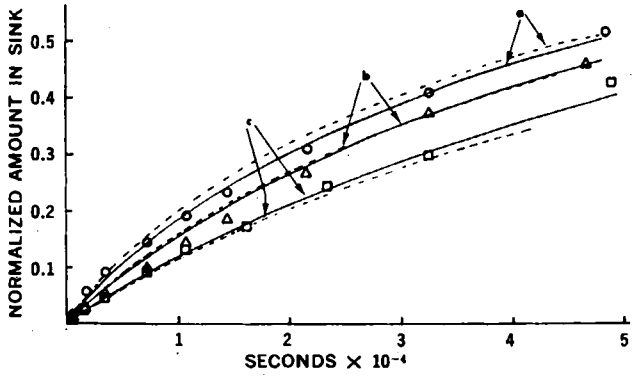

Figure 10—*Comparisons between the uptake-release experimental data obtained from lecithin–dicetyl phosphate dispersions for taurocholic acid release in 0.01* M *acetate-buffered glucose solution at pH 4 and the calculations using the monosize, multiconcentric Model 1. Key (symbols represent experimental data): ○, zero-hour solute uptake prior to release; △, 2-hr. solute uptake prior to release; and □, 10-hr. solute uptake prior to release. Curves a, b, and c represent calculations of 0-, 2-, and 10-hr. solute uptake, respectively. For solid curves:* $k_1 = 95$, $k_2 = 345$, $p = 4 \times 10^{-6}$. *For broken curves:* $k_1 = 120$, $k_2 = 260$, $p = 4 \times 10^{-6}$. *Other parameter values are taken from Table II.*

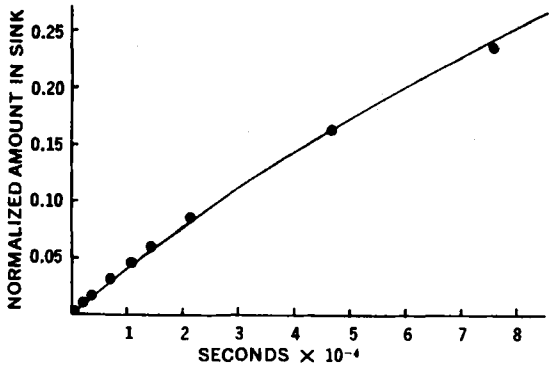

Figure 11—*Comparison between the direct-release experimental data obtained from lecithin–dicetyl phosphate dispersions for taurocholic acid release in 0.01* M *acetate-buffered glucose solution at pH 3.6 and the calculations using the monosize, multiconcentric Model 1. Key:* ●, *experimental data. Curve represents theory based on* $k_1 = 210$, $k_2 = 600$, $p = 8 \times 10^{-4}$, *and the parameter values given in Table II.*

carried out in buffered glucose solutions at different pH's for the transport of taurocholic acid are compared with the corresponding calculations in Figs. 5–12. The parameter values are given in Table II. As these data show, the uncertainty in the determination of the permeability coefficients becomes large when the permeability coefficients become very small. In spite of this limitation, the present results clearly show that the permeability coefficients are dependent on the pH of the media in which the taurocholic acid transport studies are performed.

In Fig. 13 the results of the direct-release experiments for the transport of D-glucose in buffered glucose solution (0.01 *M* acetate and phosphate) at different pH's are presented. The curve in this figure represents the results of computations carried out earlier (1) for the glucose transport studies in unbuffered glucose solution. The comparisons between the release data of D-glucose transport in buffered glucose solution at different pH's and the theoretical curve reproduced from the calculations for glucose transport in unbuffered glucose solution indicate no significant changes in the permeability coefficient of liposome membranes due to buffers or to changes in pH of the aqueous solution. This is a strong indication that the physical characteristics of the liposome membranes are not changed because of the changes in pH of the media, which is used for the preparation of dispersion and for the study of transport.

Since physical characteristics of the liposome dispersions do not seem to be significantly altered by changes in pH of the aqueous

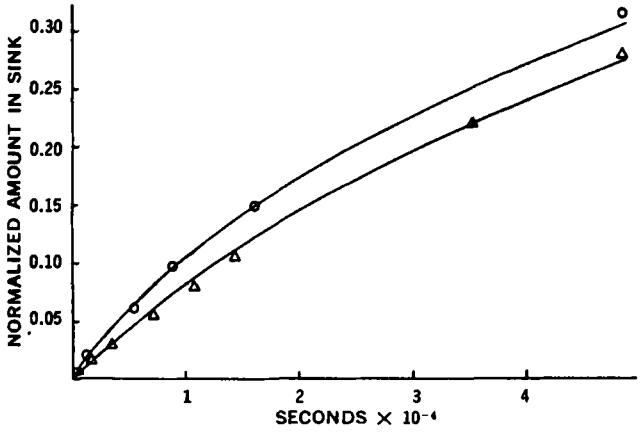

Figure 12—*Comparisons between the uptake-release experimental data obtained from lecithin–dicetyl phosphate dispersions for taurocholic acid release in 0.01* M *acetate-buffered glucose solution at pH 3.6 and the calculations using the monosize, multiconcentric Model 1. Key (symbols represent experimental data):* O, *zero-hour solute uptake prior to release; and* Δ, *2-hr. solute uptake prior to release. Corresponding curves represent calculations based on* $k_1 = 210$, $k_2 = 600$, $p = 8 \times 10^{-4}$, *and the parameter values given in Table I.*

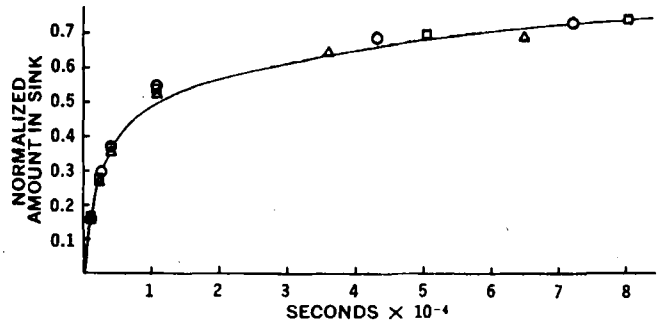

Figure 13—*Results showing little influence of pH for the release (direct release) of glucose from lecithin-dicetyl phosphate dispersion systems in buffered glucose solutions. Key:* O, *0.01* M *phosphate buffer at pH 8;* Δ, *0.01* M *phosphate buffer at pH 7; and* □, *0.01* M *acetate buffer.*

solution, it appears that the effect of pH is most likely related to the differences in the species of taurocholic acid in solution. The relationship between the effective bulk permeability coefficient and pH is given in Fig. 14. This relationship could be explained on the basis of the unionized form of taurocholic acid being preferentially transported. This would be in agreement with the pH-partition hypothesis of Shore *et al.* (3). The small, but finite, flux at high pH might be explained on the basis of the transport of the ionized species being the controlling factor under these conditions. This might have important implications in the *in vivo* absorption of taurocholate from bile.

APPENDIX

Monosize, multiconcentric layer models assume that all spherules are of the same size and consist of $i = 1$ to $i = n$ number of segments. In Model 1 the innermost aqueous phase is defined as segment 1. Segment 2 to segment n consists of an aqueous phase and a lipid phase which are of equal thickness. The outermost lipid phase is considered as a part of Compartment II, which is the external phase of the dispersion. In Model 2 the outermost lipid layer is considered as a part of the spherule so that each segment consists of an aqueous phase and a lipid phase. The basic assumptions, the equations, and the procedure used for solving these equations were reported earlier (1, 2). A summary of the equations used in Model 1 is presented here.

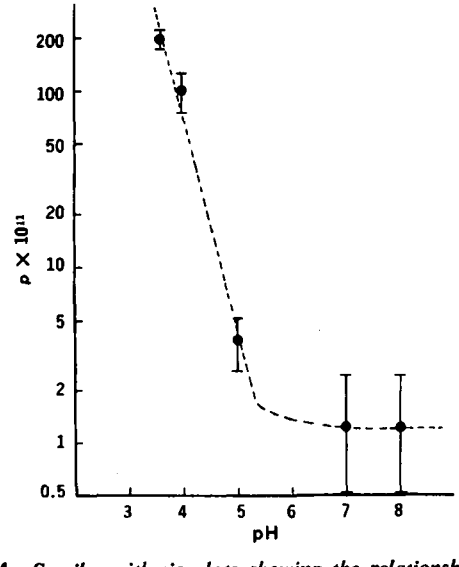

Figure 14—*Semilogarithmic plots showing the relationship between the effective bulk permeability coefficient for taurocholic acid and pH of the buffered glucose solution used in the preparation and in the release studies.*

Table II—Input Data Used in the Calculations Shown in Figs. 5–12 for the Taurocholic Acid Transport at Different pH's

pH	Volume of Spherules, ml.	Mean Surface Radius, μ	Number of Concentric Layers, n	Bag Constant $p_b \cdot S_b \times 10^4$, cm.^{3} sec.$^{-1}$	Permeability Coefficient per Segment, cm. sec.$^{-1}$
8 (0.01 *M* phosphate buffer)	0.922	0.725	30	7.023	1×10^{-9}
8 (0.008 *M* phosphate buffer)	0.922	0.725	30	6.790	5×10^{-10}
7 (0.01 *M* phosphate buffer)	0.930	0.738	30	6.483	5×10^{-10}
5 (0.01 *M* acetate buffer)	1.037	0.766	30	4.986	1.5×10^{-9}
4 (0.01 *M* acetate buffer)	0.973	0.744	30	3.272	4×10^{-8}
3.6 (0.01 *M* acetate buffer)	0.966	0.730	30	2.863	8×10^{-8}

The mass balance considerations for the $i = 2$ to the $i = n$ segment lead to Eq. A1:

$$(V_1 \cdot v_l + V_2 \cdot v_a)C_{2,t} = (V_1 \cdot v_l \cdot k_1 + V_2 \cdot v_a)C_{2,a}$$

$$\vdots$$

$$(V_{n-1} \cdot v_l + V_n \cdot v_a)C_{n,t} = (V_{n-1} \cdot v_l \cdot k_1 + V_n \cdot v_a)C_{n,a}$$

$$(V_n \cdot v_l + V_{II})C_{II,t} = (V_n \cdot v_l \cdot k_2 + V_{II})C_{II,a} \quad \text{(Eq. A1)}$$

where:

$V_1 \ldots V_n$ = volume of segment 1 to segment n
V_{II} = volume of Compartment II (external phase of the dispersion)
v_l = volume fraction of lipid phase
v_a = volume fraction of aqueous phase
$C_2 \ldots C_n$ = average solute concentration of segment 2 to segment n
$C_{II,t}$ = average solute concentration of Compartment II
$C_{2,a} \ldots C_{n,a}$ = aqueous solute concentration of segment 2 to segment n
$C_{II,a}$ = aqueous solute concentration in Compartment II
k_1 = effective lipid–aqueous partition coefficient that applies to the lipid and aqueous phases in the $i = 2$ to $i = n$ segments
k_2 = effective lipid–aqueous partition coefficient that applies to the outermost lipid phase and Compartment II

The rates at which solute leaves segments $i = 1$ to $i = n$ are given by Eq. A2:

$$-V_1 \cdot v_a \cdot \frac{dC_{1,a}}{dt} = S_1 \cdot p(C_{1,a} - C_{2,a})$$

$$(V_1 \cdot v_l \cdot k_1 + V_2 \cdot v_a)\frac{dC_{2,a}}{dt} = S_1 \cdot p(C_{1,a} - C_{2,a}) - S_2 \cdot p(C_{2,a} - C_{3,a})$$

$$\vdots$$

$$(V_{n-1} \cdot v_l \cdot k_1 + V_n \cdot v_a)\frac{dC_{n,a}}{dt} = S_{n-1} \cdot p(C_{n-1,a} - C_{n,a}) - S_n \cdot p(C_{n,a} - C_{II,a}) \quad \text{(Eq. A2)}$$

where:

$S_1 \ldots S_n$ = surface area of segment 1 to segment n
p = permeability coefficient per segment

The rate of appearance of solute in Compartment II is given by Eq. A3:

$$(V_n \cdot v_l \cdot k_2 + V_{II})\frac{dC_{II,a}}{dt} = S_n \cdot p(C_{n,a} - C_{II,a}) - S_b \cdot p_b \cdot C_{II,a} \quad \text{(Eq. A3)}$$

where:

S_b = surface area of dialysis membrane
p_b = effective permeability coefficient of bag

The rate of appearance of solute in the sink is given by Eq. A4:

$$\frac{dAs}{dt} = p_b \cdot S_b \cdot C_{II,a} \quad \text{(Eq. A4)}$$

where As = the amount of solute in the sink normalized with respect to the initial amount in the bag.

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Determination of a Benzothiazepin-2-one in Blood by GLC–Electron-Capture Detection

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Abstract □ A sensitive and specific blood level method was developed for the determination of 8-chloro-1-(3-dimethylaminopropyl)-3,5-dihydro-4,1-benzothiazepin-2(1*H*)-one (I) by GLC-electron-capture detection. It involves the selective extraction of the compound into ether from the blood or plasma made alkaline with sodium hydroxide, followed by reextraction into 0.1 *N* HCl. After washing the acid with ether, the aqueous solution is alkalinized and the compound is reextracted into ether. The ether is evaporated, and the residue is dissolved in a 1% solution of diethylamine in *n*-hexane which contains 5-chloro-2-(2-diethylaminoethylamino)-2′-fluorobenzophenone as a reference standard; this solution is analyzed by GLC. The overall recovery of I-hydrochloride added to blood or plasma is of the order of 85%, and the sensitivity limit is about 0.015 mcg. I/ml. blood or plasma using a 4-ml. sample per assay. The method was applied to the determination of plasma levels of the drug in man following single 100- and 200-mg. oral doses of the drug administered 7 days apart.

Keyphrases □ 8-Chloro-1-(3-dimethylaminopropyl)-3,5-dihydro-4,1-benzothiazepin-2(1*H*)-one—analysis in blood, GLC-electron-capture detection □ Benzothiazepin-2-ones—analysis of 8-chloro-1-(3-dimethylaminopropyl)-3,5-dihydro-4,1-benzothiazepin-2(1*H*)-one in blood, GLC-electron-capture detection □ GLC-electron-capture detection—analysis, 8-chloro-1-(3-dimethylaminopropyl)-3,5-dihydro-4,1-benzothiazepin-2(1*H*)-one in blood

The tranquilizing properties of the phenothiazines (1) has led to the synthesis of a variety of heterocyclic compounds containing the nitrogen and sulfur moieties in the heterocyclic ring. Many of these compounds have antidepressant activity (2). Thiazesim[1] (3) is a member of the benzothiazepine class of compounds (4) that has shown antidepressant activity in animals (5) and man (6).

8-Chloro-1-(3-dimethylaminopropyl)-3,5-dihydro-4,1-benzothiazepin-2(1*H*)-one hydrochloride (I-hydrochloride) (Table I), another member of this class of compounds, was synthesized by Wenner and Uskokovic (7) and is under clinical investigation as a psychostimulant (8, 9).

A sensitive and specific blood level method was developed for the quantitation of I by GLC–electron-capture detection with a linear range of detection of 10–250 ng. (Fig. 1). The method was applied to the determination of plasma levels of the intact drug (I) in man following administration of single 100- and 200-mg. oral doses.

EXPERIMENTAL

Reagents—All reagents were of analytical grade purity (>98%) and were used without further purification. All aqueous solutions were made up in double-distilled water. The following were used: 6 *N* NaOH, aqueous; 2 *N* NaOH, aqueous; 0.1 *N* HCl, aqueous; 1% diethylamine in spectrograde *n*-hexane[2], 1:99 (v/v), prepared as described under *Standard Solutions;* ether[3], analytical grade, anhydrous; sodium sulfate, anhydrous; and ethanol, absolute.

GLC Conditions—A gas chromatograph[4] with a cylindrical foil electron-capture detector (foil strength 225 mc. of titanium tritide) was used.

Column—A 1.22-m., 0.63-cm. (4-ft., 0.25-in.) o.d. and 0.45-cm. (0.18-in.) i.d., borosilicate glass column containing 5% silicone grease[5] on 60–80-mesh silanized Chromosorb W was conditioned for 72 hr. at 260° prior to use.

Carrier Gas—Nitrogen (oil pumped and dry) was used at a column head pressure of 100 psig. and a flow rate of 100–120 ml./min.

Temperature Settings—The injection port temperature was 250°, the detector temperature was 200°, and the column oven temperature was 230°.

Amplifier Range—The range used was 1.0 × attenuation 8.0 for a full-scale deflection for 100 ng. of I.

Detector—It was operated at plateau voltage (−) 70 v. d.c.

Recorder—A 1.0-mv. dual-pen recorder[6] was operated full scale at a chart speed of 101.6 cm. (40 in.)/hr.

Standard Solutions—Stock solutions of I and 5-chloro-2-(2-diethylaminoethylamino)-2′-fluorobenzophenone (III)[7] (the reference standard) have to be prepared, from which the working solutions containing varying amounts of I to a constant amount of III are prepared for making the GLC calibration curve.

Preparation of Stock Solution of I—Weigh out 11.20 mg. of I-hydrochloride, equivalent to 10.00 mg. of the free base, into a 15-ml. centrifuge tube. Dissolve in 1 ml. of water, add 1 ml. of 2 *N* sodium hydroxide to liberate the free base, and extract three times successively with 6 ml. of ether by shaking for 10 min. on a reciprocating shaker. Centrifuge and transfer the first ether extract quantitatively into a tared 10-ml. volumetric flask, evaporate to dryness, add the second and third ether extracts successively to the residue in the tared flask, and evaporate again to dryness. Vacuum dry the sample for 20-min. intervals until constant weight is achieved. Recovery of the free base should be better than 95%. Dissolve the residue in 1 ml. of ethanol and dilute with sufficient *n*-hexane to yield a stock solution containing 1 mg. (free base)/ml.

Preparation of Stock Solution of III (Reference Standard for GLC)—Weigh out 11.10 mg. of III-hydrochloride (Table I), equivalent to 10.00 mg. of free base (oily residue), and make a stock solution containing 1 mg./ml. exactly as described for I.

Preparation of External Standard Solutions of I (Free Base) Containing the Reference Standard (III) for GLC Calibration—Transfer 1 ml. of each of the above solutions into separate 10-ml. volumetric flasks and make up to volume with *n*-hexane. These solutions contain 100 mcg./ml. of I and III, respectively. Make standard solutions of I containing a constant amount of III in 10-ml. volumetric flasks, making to volume with a solution of 1% diethylamine in *n*-hexane such that each solution contains 2.0 mcg./ml. of the reference standard (III) and 2.0, 4.0, 6.0, 8.0, or 10 mcg./ml. of I. Add approximately 300 mg. of sodium sulfate (anhydrous) as a desiccant to each solution to maintain the anhydrous state of these solutions, which are used to make the analytical calibration curve.

A new calibration curve is prepared on each day of analysis from duplicate 10-μl. injections of each standard solution. The peaks due to I and III have retention times (R_t) of 4.2 and 7.5 min., respectively (Fig. 2), and the peak area (cm.²) of each component

[1] 5-[2-(Dimethylamino)ethyl]-2,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one hydrochloride.

[2] Fisher.

[3] Mallinckrodt; may be used for up to 7 days after the can is opened.

[4] Aerograph model 205-1-B.

[5] Dow Corning DC-11.

[6] Westronics.

[7] Synthesized by R. I. Fryer and J. Earley, Department of Chemical Research, Hoffmann-La Roche Inc., Nutley, N. J., 1968.

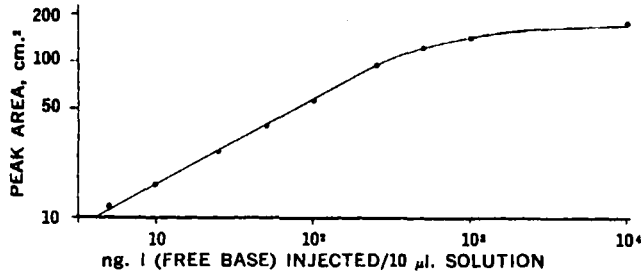


Figure 1—*Linear dynamic range of electron-capture detector response to I.*

is determined by the product of peak height (centimeters) times the width (centimeters) at half-height using the slope–baseline technique. A standard curve is prepared by plotting the peak area ratio of I:III *versus* nanograms of I (Fig. 3).

Preparation of Internal Standards for Determination of Percent Recovery from Blood—Weigh out 11.20 mg. of I-hydrochloride, which is equivalent to 10.00 mg. of free base, and dissolve in 10 ml. of absolute ethanol to give a stock solution containing 1 mcg./ml. Then dilute this solution to yield an internal standard solution containing 1 mcg./100 µl. of ethanol, suitable aliquots of which are added to blood or plasma as internal standards for determining the percent recovery.

Preparation of Stock Solution of 1% Diethylamine in n-Hexane Containing III for GLC Analysis of Biological Samples—Into a 100-ml. volumetric flask, transfer 1 ml. of diethylamine and 0.2 ml. of the 1.0-mg./ml. stock solution of III and make up to volume in spectrograde *n*-hexane. Add about 1 g. of anhydrous sodium sulfate and shake by inversion. This solution contains 200 ng. III/100 µl. and is used for dissolving the residue of the samples prior to GLC analysis.

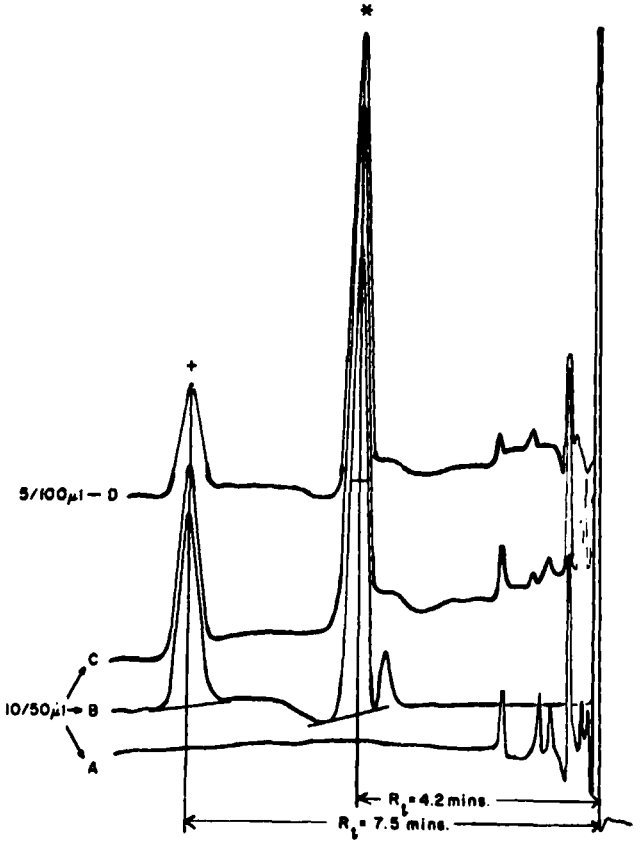


Figure 2—*Gas chromatograms of I extracted from blood. Key: *, I, analytical peak; +, III, reference standard; A, control plasma extract; B, authentic standards of I and III in 1% diethylamine–n-hexane; C, I, recovered from control plasma; and D, plasma extract from a patient dosed with I.*

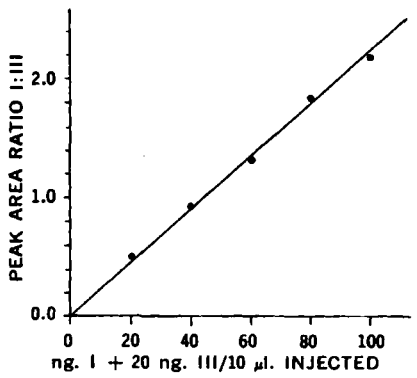


Figure 3—*Calibration curve of electron-capture detector response to I using the benzophenone (III) as a reference standard.*

Experimental Procedure for Extraction from Blood or Plasma—Into a 50-ml. centrifuge tube, add 4 ml. of blood or plasma, 4 ml. of 2 *N* NaOH, and 15 ml. of ether for the first extraction. Along with the samples, run a 4-ml. specimen of control blood or plasma (taken preferably from the patient prior to medication) and four separate 4-ml. specimens of control blood or plasma to which 200, 400, 600, and 800 ng. of I-hydrochloride (20-, 40-, 60-, and 80-µl. aliquots of the internal standard solution evaporated to dryness under nitrogen) are added as internal standards. Seal the stoppers with a drop of distilled water. Shake for 15 min. on a reciprocating shaker, centrifuge for 10 min. at 2000 r.p.m., and transfer the ether into another 50-ml. tube. Repeat the extraction with another 10-ml. portion of ether and combine the ether extracts. Back-extract the ether with 5 ml. of 0.1 *N* HCl, centrifuge, aspirate off the ether, and wash the hydrochloric acid twice with 10-ml. portions of ether; then centrifuge and remove the ether by aspiration as before. Alkalinize the hydrochloric acid to a bromthymol blue end-point with 0.1 ml. of 6 *N* NaOH. Extract with 2 × 10-ml. portions of ether by shaking for 10 min. Then centrifuge and combine the extracts sequentially in a 15-ml. centrifuge tube and evaporate to dryness. Transfer the sample residues to a vacuum desiccator and vacuum dry for 15–20 min. to remove all traces of moisture. Add 50 µl. of a 1% solution of diethylamine in *n*-hexane containing the reference standard (III) (reference standard stock solution) to each tube, and dissolve the residue by mixing for 60 sec. on a mixer[8] at high speed. Prepare two samples at a time for GLC to prevent solvent losses in the tubes. Inject a 10-µl. aliquot for GLC analysis. The peaks due to I and III in the unknowns are identified by their respective retention times of 4.2 and 7.5 min. compared to the retention times of the peaks of the authentic external standards (Fig. 2).

Calculations—The peak areas of I and III are determined as described. The peak area ratios of I:III are calculated, and the

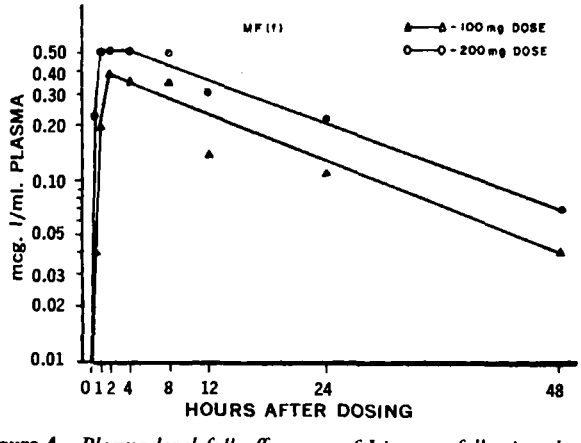
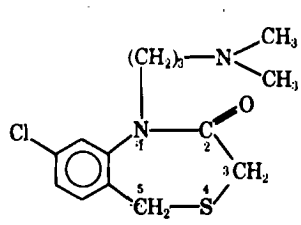


Figure 4—*Plasma level fall-off curves of I in man following the oral administration of 100- and 200-mg. doses given 7 days apart.*

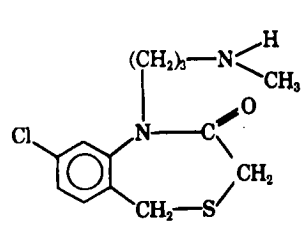
[8] Vortex Super Mixer (Lab-Line).

Table I—Chemical Names, Structural Formulas, and Physical Properties of the Compounds Mentioned

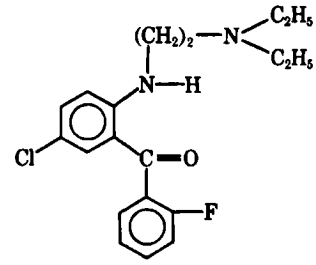
Compound	Chemical Name	Molecular Weight	Melting Point	Reference to Synthesis
I-hydrochloride	8-Chloro-1-(3-dimethylaminopropyl)-3,5-dihydro-4,1-benzothiazepin-2(1*H*)-one hydrochloride	335.31	201–202°	7
II-hydrochloride	8-Chloro-1-(3-methylaminopropyl)-3,5-dihydro-4,1-benzothiazepin-2(1*H*)-one hydrochloride	321.28	230–232°	7
III-hydrochloride	5-Chloro-2-(2-diethylaminoethylamino)-2′-fluorobenzophenone hydrochloride (reference standard for GLC analysis)	385.30	134–143°	



I



II



III

nanogram amounts of I in the unknowns are interpolated from the standard curve of the recovered internal standards or by direct comparison to any given internal standard using the formula:

$$\frac{\text{[peak area ratio] unknown}}{\text{[peak area ratio]internal standard}} \times \frac{\text{mcg. internal standard}}{\text{ml. of sample assayed}} = \text{mcg. I/ml. blood or plasma} \quad \text{(Eq. 1)}$$

The percent recovery of internal standards of I-hydrochloride added to blood or plasma is determined as follows:

$$\frac{\text{ng. I (recovered)}}{\text{ng. I-hydrochloride (added)}} \times \text{aliquot factor} \times 1.122 \times 100 = \%\ \text{recovery of I} \quad \text{(Eq. 2)}$$

where aliquot factor = aliquot of sample injected for GLC (10/50 μl.), and 1.122 = conversion factor for molar weight. The ratio of:

$$\frac{\text{I-hydrochloride}}{\text{I free base}} = \frac{335.31}{298.81} \quad \text{(Eq. 3)}$$

The percent recovery of internal standards should be determined routinely as a check on analytical precision and reproducibility.

DISCUSSION

During the investigation of the intrinsic analytical properties of I, it was noted that the compound had no useful absorbance in either the visible or UV region and, consequently, had no useful intrinsic fluorescence in basic, neutral, or acidic media. However, the free base of the compound was amenable to GLC analysis employing the sensitivity and selectivity of the electron-capture detector response to the halogen, sulfur, and carbonyl groups in the molecule for its detection. The limit of detection of the pure compound was 10 ng. when chromatographed on a 1.23-m. (4-ft.) column of 5% silicone grease[5] at 230°, giving a potential sensitivity limit of 0.015 mcg./ml. of blood or plasma. However, greater sensitivity may be obtained by using the phenyl silicone liquid phases such as OV-1 and OV-17 and the ^{63}Ni (15 mc.) electron-capture detector, especially in the pulsed d.c. operational mode.

The selective extraction of I into ether from the blood or plasma made alkaline with sodium hydroxide was based on the basic nature of the compound (pKa 8.7). Since the compound readily forms acid salts, the free base in ether was back-extracted into 0.1 *N* HCl, which was then subjected to suitable cleanup. The hydrochloric acid solution containing I is made strongly alkaline, extracted into ether, and concentrated by evaporation.

It was noted that I in the final ether residue was not quantitatively dissolved in *n*-hexane, apparently due to strong adsorption on the glass. However, it was determined by GLC that I was solubilized and quantitatively desorbed off the glass with a solution of 1% diethylamine in *n*-hexane. Since diethylamine is very hygroscopic anhydrous sodium sulfate was used to remove traces of moisture in the sample residues which would otherwise severely quench the response of the ^{3}H-electron-capture detector, giving nonlinear and irreproducible results. The reference standard (III) serves as an index of the performance of the electron-capture detector during the assay and monitors the stability of the GLC system.

The electron-capture detector response characteristics of III, its sensitivity limits, and its linear range of detection were compatible with those of I. Its retention time of 7.5 min. ensures complete resolution from I and from extracted impurities, making it a suitable reference standard for GLC analysis.

Application of Method to Biological Specimens—Plasma levels were determined in a female patient (M.F.; age 59 and 68.4 kg. in weight) following single oral doses of 100 and 200 mg. administered 7 days apart. Blood specimens were collected prior to medication (control) and at 0.5, 1, 2, 4, 8, 12, 24, and 48 hr. after dosing; the plasma was separated immediately. Semilogarithmic plots of the plasma levels are shown in Fig. 4 and indicate a slow and prolonged absorption of the drug, with a maximum level being reached 1–2 hr. after administration. These levels were maintained for up to 8 hr. following both the 100- and 200-mg. doses, after which time a progressive decline in the plasma levels was seen up to 48 hr. The plasma level half-life determined by the method of least squares was approximately 15 hr. for both the 100- and 200-mg. doses.

The sensitivity limit of the method was established at 0.015 mcg. I/ml. plasma using a 4-ml. sample per assay in additional studies where blood levels were determined in man following the oral administration of 20 mg. of I-hydrochloride/day in 5-mg. doses (q.i.d.).

Specificity of Assay—Following GLC analysis, the remainder of the 1% diethylamine in *n*-hexane solutions were pooled and analyzed by one-dimensional TLC in acetone–ammonium hydroxide (100:2). The chromatoplates showed the presence of a single spot corresponding to that of authentic I (R_f 0.90). The *N*-monodesmethyl analog (II, R_f 0.17) (Table I), a metabolite seen in dog blood, is absent in human blood extracts after single oral doses. It may, however, appear after chronic administration of the drug. On GLC analysis, Compound II had a retention time of 5.4 min. and was resolved from I whose retention time was 4.2 min. The GLC assay in blood is apparently specific for the intact drug.

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Determination of Coating Thickness of Microcapsules and Influence upon Diffusion

LUU SI-NANG▲, PATRICK F. CARLIER, PIERRE DELORT, JEAN GAZZOLA, and DIDIER LAFONT

Abstract □ Experiments in this study appear to show that the diffusion rate of the encapsulated drug is a function of microcapsule size. The object of this paper is to report the influence of the coating upon diffusion and the determination of the thickness of the coating. An equation was established, which was verified by microscopic measurement of microspheres previously sliced with a microtome.

Keyphrases □ Microcapsules—determination of coating thickness, effect on diffusion rate □ Coating thickness, microcapsules—determination, effect on diffusion rate □ Diffusion rate, encapsulated drugs—influence of nature and thickness of coating

The microencapsulation process is comparatively new (1–7). It consists of coating crystals or microdroplets of liquid with a polymer film. It has been applied successfully to a number of products, from gasoline to tetracycline. Its pharmaceutical use enables one to mix substances incompatible with each other, to mask an unpalatable taste, or to induce a prolonged action. Of course, the nature and thickness of the coating determine the diffusion rate.

THEORETICAL

Influence of Coating upon Diffusion—Studying the dissolution in water of cylinders of benzoic acid and lead chloride, Noyes and Whitney (8) gave the following dissolution equation:

$$\frac{dc}{dt} = K(C_\infty - c) \qquad \text{(Eq. 1)}$$

where K is a constant, C_∞ is the solubility of the substance, and c is the concentration at the expiration of the time t.

According to Brunner (9):

$$K = \frac{DS}{V\delta} \qquad \text{(Eq. 2)}$$

where K is the same constant as in the Noyes–Whitney equation, D is the solute molecule diffusion coefficient, V is the volume of solution, δ is the effective diffusion layer thickness, and S is the surface of the solid–solution interface.

The equation:

$$\frac{dc}{dt} = \frac{DS}{V\delta}(C_\infty - c) \qquad \text{(Eq. 3)}$$

allowed the computation of δ.

As reported by Wurster and Taylor (10): "the idealized film layer is not well defined, but it allows the correlation of experimental data with the physical properties of both the solute and solvent."

Dissolution of Microencapsulated Solute—In this experiment, the material to be encapsulated was viscous and sticky; however, the dry microcapsules did not aggregate. Thus one may disregard the possibility of a spontaneous diffusion of this material through the coating wall, and the phenomenon may be considered as follows: when the microcapsules were suspended in a liquid, the penetration of the solvent into the microspheres occurred first, followed by the dissolution of the encapsulated solute and the diffusion of the solution. The phenomena taking place during this exchange were dependent on osmotic pressure and diffusion.

In the event of the encapsulated substance being only slightly soluble in the liquid, one may disregard the effect of osmotic pressure and consider only diffusion. Thus, Eq. 3 may be expressed as:

$$\left[\frac{dc}{dt}\right]_{\text{coating}} = \frac{DS'\epsilon}{Vh}(C_\infty - c) \qquad \text{(Eq. 4)}$$

where S' is the external surface of a microcapsule, ϵ is a coefficient expressing the porosity and tortuosity of the coating, and h is the thickness of the coating. Other symbols are the same as already defined.

If it is assumed that microcapsules with the same radius have the same coating thickness, and that this thickness as well as that of Brunner's (9) layer are small and negligible with respect to the radius of microcapsules, one may write:

$$S = S' = n4\pi\bar{r}^2 \qquad \text{(Eq. 5)}$$

where $\bar{r}$ is the mean radius of the microcapsules and n is the number of microcapsules. The volume V_1 of the microcapsules is expressed as:

$$V_1 = \frac{4}{3}n\pi\bar{r}^3 = \frac{m}{d} \qquad \text{(Eq. 6)}$$

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Determination of Coating Thickness of Microcapsules and Influence upon Diffusion

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Abstract □ Experiments in this study appear to show that the diffusion rate of the encapsulated drug is a function of microcapsule size. The object of this paper is to report the influence of the coating upon diffusion and the determination of the thickness of the coating. An equation was established, which was verified by microscopic measurement of microspheres previously sliced with a microtome.

Keyphrases □ Microcapsules—determination of coating thickness, effect on diffusion rate □ Coating thickness, microcapsules—determination, effect on diffusion rate □ Diffusion rate, encapsulated drugs—influence of nature and thickness of coating

The microencapsulation process is comparatively new (1–7). It consists of coating crystals or microdroplets of liquid with a polymer film. It has been applied successfully to a number of products, from gasoline to tetracycline. Its pharmaceutical use enables one to mix substances incompatible with each other, to mask an unpalatable taste, or to induce a prolonged action. Of course, the nature and thickness of the coating determine the diffusion rate.

THEORETICAL

Influence of Coating upon Diffusion—Studying the dissolution in water of cylinders of benzoic acid and lead chloride, Noyes and Whitney (8) gave the following dissolution equation:

$$\frac{dc}{dt} = K(C_\infty - c) \qquad \text{(Eq. 1)}$$

where K is a constant, C_∞ is the solubility of the substance, and c is the concentration at the expiration of the time t.

According to Brunner (9):

$$K = \frac{DS}{V\delta} \qquad \text{(Eq. 2)}$$

where K is the same constant as in the Noyes–Whitney equation, D is the solute molecule diffusion coefficient, V is the volume of solution, δ is the effective diffusion layer thickness, and S is the surface of the solid–solution interface.

The equation:

$$\frac{dc}{dt} = \frac{DS}{V\delta}(C_\infty - c) \qquad \text{(Eq. 3)}$$

allowed the computation of δ.

As reported by Wurster and Taylor (10): "the idealized film layer is not well defined, but it allows the correlation of experimental data with the physical properties of both the solute and solvent."

Dissolution of Microencapsulated Solute—In this experiment, the material to be encapsulated was viscous and sticky; however, the dry microcapsules did not aggregate. Thus one may disregard the possibility of a spontaneous diffusion of this material through the coating wall, and the phenomenon may be considered as follows: when the microcapsules were suspended in a liquid, the penetration of the solvent into the microspheres occurred first, followed by the dissolution of the encapsulated solute and the diffusion of the solution. The phenomena taking place during this exchange were dependent on osmotic pressure and diffusion.

In the event of the encapsulated substance being only slightly soluble in the liquid, one may disregard the effect of osmotic pressure and consider only diffusion. Thus, Eq. 3 may be expressed as:

$$\left[\frac{dc}{dt}\right]_{\text{coating}} = \frac{DS'\epsilon}{Vh}(C_\infty - c) \qquad \text{(Eq. 4)}$$

where S' is the external surface of a microcapsule, ϵ is a coefficient expressing the porosity and tortuosity of the coating, and h is the thickness of the coating. Other symbols are the same as already defined.

If it is assumed that microcapsules with the same radius have the same coating thickness, and that this thickness as well as that of Brunner's (9) layer are small and negligible with respect to the radius of microcapsules, one may write:

$$S = S' = n4\pi\bar{r}^2 \qquad \text{(Eq. 5)}$$

where $\bar{r}$ is the mean radius of the microcapsules and n is the number of microcapsules. The volume V_1 of the microcapsules is expressed as:

$$V_1 = \frac{4}{3}n\pi\bar{r}^3 = \frac{m}{d} \qquad \text{(Eq. 6)}$$

where m is the mass of n microcapsules, and d is the density of the microcapsules.

From Eq. 6, one may compute n and, reporting its value in Eq. 5, one has:

$$S = S' = \frac{3m}{\bar{r}d} \qquad \text{(Eq. 7)}$$

$$\left[\frac{dc}{dt}\right]_{\text{coating}} = \frac{3Dm\epsilon}{Vh\bar{r}d}(C_\infty - c) \qquad \text{(Eq. 8)}$$

On the other hand, the diffusion that takes place at the Brunner's (9) layer may also be expressed as:

$$\left[\frac{dc}{dt}\right]_{\text{layer}} = \frac{3Dm}{V\delta\bar{r}d}(C_\infty - c) \qquad \text{(Eq. 9)}$$

And the results of both phenomena are:

$$\left[\frac{dc}{dt}\right]_{\text{layer}} + \left[\frac{dc}{dt}\right]_{\text{coating}} = \left[\frac{dc}{dt}\right]_{\text{total}} \qquad \text{(Eq. 10}a\text{)}$$

$$\left[\frac{dc}{dt}\right]_{\text{total}} = \frac{3Dm}{V\bar{r}d}\left[\frac{1}{\delta} + \frac{\epsilon}{h}\right](C_\infty - c) \qquad \text{(Eq. 10}b\text{)}$$

Thus, diffusion is expressed as the sum of a constant and a variable, the latter being a function of the stirring rate.

One may write the empirical relationship:

$$\delta = \frac{a}{(N)^b} \qquad \text{(Eq. 11)}$$

where N is the agitation rate, and a and b are constants. Substituting this into Eq. 10 gives:

$$\left[\frac{dc}{dt}\right]_{\text{total}} = \frac{3Dm}{V\bar{r}d}\left[\frac{(N)^b}{a} + \frac{\epsilon}{h}\right](C_\infty - c) \qquad \text{(Eq. 12)}$$

$$\frac{dc}{C_\infty - c} = \frac{3Dm}{V\bar{r}d}\left[\frac{(N)^b}{a} + \frac{\epsilon}{h}\right]dt \qquad \text{(Eq. 13)}$$

Integrating this equation gives:

$$\ln(C_\infty - c) = -\frac{3Dm}{V\bar{r}d}\left[\frac{(N)^b}{a} + \frac{\epsilon}{h}\right]t + \text{constant} \qquad \text{(Eq. 14)}$$

If it is assumed that for $t = 0, c = 0$, then constant $= \ln C_\infty$:

$$\ln\left[1 - \frac{C}{C_\infty}\right] = -\frac{3Dm}{V\bar{r}d}\left[\frac{(N)^b}{a} + \frac{\epsilon}{h}\right]t \qquad \text{(Eq. 15)}$$

When C is very inferior to C_∞ (generally $C \leqslant 0.1\ C_\infty$), (C/C_∞) is small with respect to 1 and Eq. 15 reduces to:

$$C = \frac{3Dm}{V\bar{r}d}C_\infty\left[\frac{(N)^b}{a} + \frac{\epsilon}{h}\right]t \qquad \text{(Eq. 16)}$$

The curve $C = f(t)$ where N is a constant is interesting because it expresses the diffusion of the solute; the curve $C = f(N)$ where t is a constant is interesting when extrapolated at $N = 0$.

Thus, beyond the area A where the solution is heterogeneous, the equation of this curve is:

$$C = Co + kN^b \qquad \text{(Eq. 17)}$$

where:

$$Co = \frac{3Dm\epsilon C_\infty}{V\bar{r}dh}t$$

and:

$$k = \frac{3DmC_\infty}{V\bar{r}da}t$$

Co is the coating contribution to diffusion; it is representative of ϵ/h and, therefore, of the nature and thickness of the coating. It affords a global view of the coating's rate of diffusion. Thus, it is possible to compare two batches of microcapsules of the same drug by equalizing all of the other parameters.

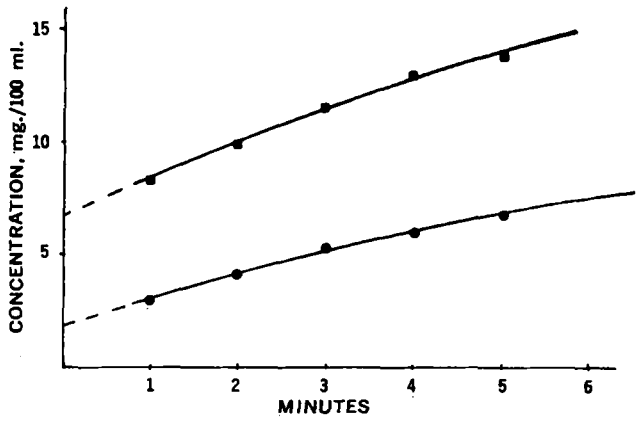

Figure 1—*Liberation rate of microcapsules with* r *different. Key:* ■, r = *140 μ; and* ●, r = *300 μ.*

Determination of Coating Thickness—It follows from Eq. 15 that, for obtaining a prolonged action with microcapsules, it will be advantageous to increase $\bar{r}$ and decrease ϵ/h. Other parameters are determined either by solute and coating (D, d) or by posology (m, V). The formulator is free to work on $\bar{r}$ and h. The mean radius is more easily measured than the coating thickness. The following two methods were used.

Measuring Density and Active Drug Amount—Let us consider a batch of n microcapsules weighing m g. with a mean radius $\bar{r}$. Solute density is d_1, and coating density is d_2.

The solute mass is:

$$m_1 = n\frac{4}{3}\pi(\bar{r} - h)^3 d_1 \qquad \text{(Eq. 18)}$$

The coating mass is:

$$m_2 = n\left[\frac{4}{3}\pi\bar{r}^3 - \frac{4}{3}\pi(\bar{r} - h)^3\right]d_2 \qquad \text{(Eq. 19)}$$

Calling P the relation:

$$P = \frac{\text{solute mass}}{\text{total mass of microcapsules}} \qquad \text{(Eq. 20)}$$

one has:

$$P = \frac{n\frac{4}{3}\pi(\bar{r} - h)^3 d_1}{n\frac{4}{3}\pi(\bar{r} - h)^3 d_1 + n\left[\frac{4}{3}\pi\bar{r}^3 - \frac{4}{3}\pi(\bar{r} - h)^3\right]d_2} \qquad \text{(Eq. 21}a\text{)}$$

$$P = \frac{(\bar{r} - h)^3}{(\bar{r} - h)^3 d_1 + [\bar{r}^3 - (\bar{r} - h)^3]d_2} \qquad \text{(Eq. 21}b\text{)}$$

$$P = \frac{\left[1 - \frac{h}{\bar{r}}\right]^3 d_1}{\left[1 - \frac{h}{\bar{r}}\right]^3 d_1 + \left[1 - \left(1 - \frac{h}{\bar{r}}\right)^3\right]d_2} \qquad \text{(Eq. 21}c\text{)}$$

Assuming that $(h/\bar{r}) \ll 1$, the previous equations reduce to:

$$P = \frac{\left[1 - \frac{3h}{\bar{r}}\right]d_1}{\left[1 - \frac{3h}{\bar{r}}\right]d_1 + \frac{3h}{\bar{r}}d_2} \qquad \text{(Eq. 22}a\text{)}$$

$$1 - P = \frac{\frac{3h}{\bar{r}}d_2}{\left[1 - \frac{3h}{\bar{r}}\right]d_1 + \frac{3h}{\bar{r}}d_2} \qquad \text{(Eq. 22}b\text{)}$$

$$\frac{P}{1 - P} = \frac{\left[1 - \frac{3h}{\bar{r}}\right]d_1}{\frac{3h}{\bar{r}}d_2} \qquad \text{(Eq. 22}c\text{)}$$

Table I—Computation of Coating Thickness from Density and Active Drug Amount Determinations

Mean Radius, μ	Percent of Core Material	Percent of Coating Material	Thickness h, μ
375	0.93	0.07	5.86
300	0.90	0.10	0.80
200	0.89	0.11	5.00
140	0.805	0.195	6.40
87.5	0.755	0.245	5.10

Table II—Computing of Coating Thickness: Number of Observations of Thickness h for the Radius $\bar{r}$

Mean Thickness, μ	Mean Radius, μ 140	200	300	375
3.80	0	1	1	4
4.75	5	6	1	4
5.70	13	10	6	13
6.65	11	8	12	7
7.60	7	10	9	9
8.55	2	4	4	3
9.50	4	5	2	5
Total number	42	44	35	45

$$\frac{3h}{\bar{r}}d_2P = (1 - P)\left[1 - \frac{3h}{\bar{r}}\right]d_1 \qquad \text{(Eq. 22}d\text{)}$$

$$\frac{3h}{\bar{r}}[d_2P + (1 - P)d_1] = (1 - P)d_1 \qquad \text{(Eq. 22}e\text{)}$$

$$h = \frac{\bar{r}}{3}\frac{(1 - P)d_1}{Pd_2 + (1 - P)d_1} \qquad \text{(Eq. 22}f\text{)}$$

d_1 and P are easily measured, and d_2 is computed after measuring of total density and P, from which h is computed.

Direct Measure after Microtome Slicing—Wall thickness is measured with a microscope fitted with a micrometer after slicing with a microtome (slice thickness about 10 μ).

EXPERIMENTAL[1]

This work was carried out using microcapsules containing the liquid organic base, eprazinone, a mucolytic drug, encapsulated by a mixture of gelatin and gum arabic. Eprazinone is 3-[4-(2-methoxy-2-phenylethyl)-1-piperazinyl]-2-methyl-1-phenyl-1-propanone.

The process is carried out in an open top vessel at 40°, and the pH is maintained constant at 6.5 with acetic acid. The core material is dispersed as minute droplets in the solution of the mixture of gelatin and gum arabic; the capsule walls are hardened with glutaraldehyde as a crosslinking agent.

The batch studied was sieve separated into groups with average radii of 375, 300, 200, 140, and 87 μ.

Apparatus—The following were used: a constant-temperature bath kept at 23 ± 0.5°, a mechanical stirrer, a spectrophotometer[2], a chronometer, a pycnometer, a microscope, and a microtome.

Measurement of Diffusion—A volume of 300 ml. of distilled water is stirred until a temperature of 23° is reached. With continuous stirring, 1.5 g. microcapsules is added with the starting of the chronometer. Samplings are made by way of a pipet fitted at its lower end with a small plastic tube filled with glass wool. Each sampling is 3 ml., *i.e.*, 1% of the total volume. After a correct dilution of the sampling, the amount of diffused drug is determined by spectrophotometry at the absorption maximum of 250 nm.

Determination of Thickness—*Calculation*—Densities are determined by way of a pycnometer. Wall density is found by calculation after determination of the total volume of microcapsules and the volume of microencapsulated eprazinone.

[1] The microcapsules were obtained from I.B.F., Paris, France.
[2] Beckman DBG.

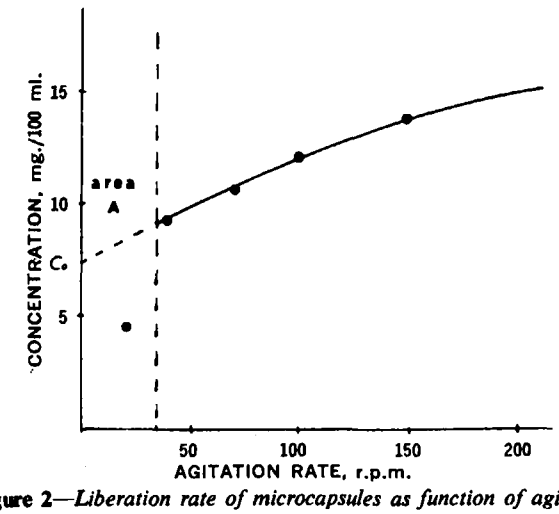

Figure 2—*Liberation rate of microcapsules as function of agitation rate.*

Direct Measure after Microtome Slicing—Microcapsules are imbedded in a gelatin mass according to Baker (11). Paraffin inclusion is unsatisfactory because of the solvent effect of alcohol and toluene and because of the low melting point of the paraffin, which also causes the coating material to melt.

Gelatin is mixed with distilled water in a 1:5 ratio. This mixture is kept 2 hr. at 37° in an incubator. Microcapsules are subsequently added. A separate gelatin bath is used for each size of microcapsules. Cubes of gelatin obtained after hardening are kept at 4°.

The gelatin cubes are subsequently sliced with a congelation microtome. Slice thickness should be about 10 μ. Each slice is placed on an object slide and mounted with levulose syrup, which is water miscible and does not require slice dehydration.

Slides are studied without staining on a microscope fitted with a micrometer and an immersion objective. One can perform a direct measure of coating thickness which appears as a darker zone with respect to the preparation as a whole.

RESULTS AND DISCUSSION

Curves $C = f(t)$ for N = Constant (Fig. 1)—There is good agreement between the experimental curves and the theoretical predictions. The origin of the ordinate is not zero; as a matter of fact, the method for preparing microcapsules requires the pH to be kept constant by adding acetic acid. Thus, some acetate is formed, part of which mixes with the coating. This soluble fraction diffuses at once.

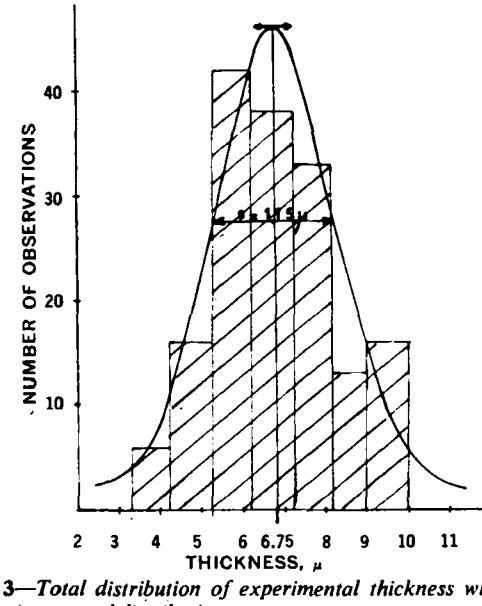

Figure 3—*Total distribution of experimental thickness with an approximating normal distribution.*

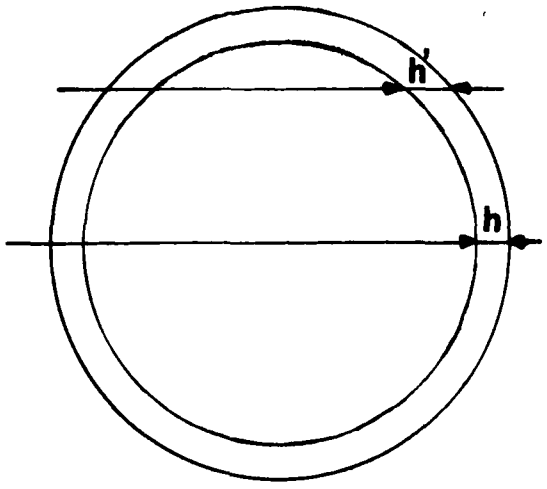

Figure 4—*Section of a microcapsule showing the different values of* h.

Curve $C = f(N)$ for t = Constant (Fig. 2)—Good agreement was found between the experimental curves and the theoretical predictions. The $C0$ value is higher than the ordinate at the origin of the curve $C = f(t)$ for N = constant; acetate dissolution is, therefore, to be disregarded.

Computing Thickness h—*From Densities and Amount of Active Drug*—One finds $d_1 = 1.05$ and $d_2 = 1.60$. The results are given in Table I. The mean value obtained by this method is 5.85 μ.

From Direct Measure—Table II gives the number of observations of thickness h and the total number of measured samples n and $\bar{h}$. The mean value obtained by this method is 6.75 μ.

The coating thickness distribution curve is given with a good degree of precision by microscopic measures (Fig. 3). This curve includes values from 10 to 4 μ. Upper values can be explained by the difficulty in cutting a microcapsule through the center with a microtome (Fig. 4); thus, the resulting coating thickness is larger for one slice than for the other ($h' > h$).

Lower values are not easily explained if thickness is taken as constant. On the other hand, microscopic examination showed that microcapsules are not spherical but rather present a knob at each pole. These knobs appeared only in the smallest microcapsules with a radius between 87 and 140 μ.

For the purpose of simplifying calculation, the microcapsules were assumed to be spherical. The results obtained indicate that this assumption can be made.

In spite of extensive radius differences (×4), the mean coating thickness remains constant, whichever method is used. Differences found with both methods for the mean coating thickness are not significantly different.

CONCLUSIONS

The experiments with microencapsulation seem to demonstrate that, when gelatin and gum arabic are used, the obtained coating is a constant thickness whatever the microcapsule size. Thus, the diffusion rate of the microcapsules will be in direct ratio to the coating material (ϵ) and in inverse ratio to the mean radius ($\bar{r}$).

The proposed equation for determining the coating thickness allows a quick estimation of this thickness, whereas direct microscopic measurement is lengthy and tedious. Knowing the coating thickness would allow the microcapsule manufacturer to determine the best conditions of encapsulation with respect to the objective, and this knowledge would permit the user to verify the constancy of quality of the product.

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Drug Purity Profiles

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Abstract □ Physical constants and purity determinations are given for 115 drug substances. Phase solubility analysis, differential scanning calorimetry, and chromatographic data are reported. High-low TLC, which has emerged as a useful, if imprecise, tool for purity approximation, is described. The concept of purity profile is discussed and examples are given; general observations on the utility of methods are made based on cumulative experience.

Keyphrases □ Drug purity profiles—phase solubility analysis, differential scanning calorimetry, and chromatographic data for 115 drugs □ Purity-indicating methods—phase solubility analysis, differential scanning calorimetry, and chromatographic data for 115 drugs □ TLC, high-low—analysis, purity profiles for 115 drugs □ Differential scanning calorimetry—analysis, purity profiles for 115 drugs □ Phase solubility analysis—purity profiles for 115 drugs

As a service to pharmaceutical analysts, useful physical constants and purity profile data are recorded here for samples of 115 drug substances, which were examined as candidates for adoption or continued official recognition as reference standards by either the USP or the NF. General observations on the purity-indicating methods are offered based on extensive experiences with this large number of drug substances. Such broad experience is rare in purity-indicating methodology, and these observations may be of value to analysts in preparing purity profiles.

These are not the only data available to the USP or the NF in connection with evaluation of candidates for recognition as standards. Moreover, these data do not represent the full scope of this laboratory's contribution to the USP and NF programs; notably, no results of monograph tests and assays are reported here. The samples cannot be presumed to have been found acceptable[1] when evaluated here or in the collaborating industrial and governmental laboratories. Only data generated by this laboratory are reported.

METHODS AND MATERIALS

Phase Solubility Analysis (PSA)—Collection and interpretation of PSA data were described fully elsewhere (1). The usual procedure, described in NF XIII (2), consists of charging eight ampuls which are then flame sealed and rotated end-over-end at 25° for 2 weeks. Initial solubility data are obtained occasionally by the method of Reilly and Rae (3), but most often by the approximate method detailed here. Supernates are examined for evidence of decomposition, when deemed necessary, by TLC. Nitrogen-sparged or degassed solvents are used for easily oxidized compounds.

Results from a PSA experiment may be evaluated graphically or by the least-squares best-fit treatment which affords a confidence range (1). Arbitrary confidence classes are assigned (Table I) based partly on that calculation and partly on experienced inspection of the graph. Extrapolated solubility values in pure solvents or in azeotropes are accurate within 0.3 mg./g. for Classes A–C; mixed solvents, however, introduce yet another variable. With respect to the testing strategy, these classes have the following meanings: A or B, almost conclusive or strong evidence of the (im)purity of a sample; C, good evidence, but requires several supporting data; and D, possibly useful as supporting evidence in conjunction with strong alternative evidence. Where an adequate number of tubes[2] is used, a poor confidence rating may reflect either minor solute decomposition in that solvent or inadequate equilibration or drying time. Similarly, solid solutions of the solute with its impurities may be revealed so that an alternative approach is needed. Points near the intersection of the graphical 45° line and the saturation line, *i.e.*, those with sample charges less than 20% above the solubility, occasionally have been found to reflect higher apparent solubility which can lead to purity values in excess of 100%; this can arise from two interrelated phenomena: failure to reach equilibrium and the known higher solubility of fine particles.

Some PSA data listed in Table I were reported earlier (1) from this laboratory but are repeated here to support the evaluation of high-low TLC.

Approximate Solubility—This method is not as rapid as some other approximate methods, but it is much more conservative of the sample, which is often a consideration with high purity drugs. It does not require assay of the solution and is designed specifically to identify solvents for PSA rather than to establish sample solubility in a given solvent. The resultant value is usually ±1 mg./g. of the actual value determined by solubility analysis.

Accurately weigh about 30 mg. of sample into a screw-capped test tube and add three 1.0-ml. increments of the candidate solvent by pipet, mixing on a vortex-causing mixer after each addition. Solubility levels of 30, 15, and 10 mg./ml., respectively, are revealed.

If the sample dissolves immediately in 1 ml. or does not dissolve appreciably in 3 ml., this solvent is ruled out for solubility analysis. If the sample is almost dissolved at any of the three increments, place the stoppered tube in a 25° bath overnight and then observe the extent of solution. At this point, solubility values of about 10, 15, or 30 mg./ml. or values lying between 10 and 15 or between 15 and 30 mg./ml. are identified. Prepare one or two additional tubes, each containing 1 ml. of solvent. The addition of 12.5 mg. of sample will allow definition of solubility in the 10–15-mg./ml. range after standing at 25° overnight; similarly, a 22.5-mg. sample, followed by yet another intermediate sample, allows determination of approximate solubility in the 15–30-mg./ml. range. A total of three tubes is usually sufficient, and analyst experience normally allows even more simplification. Correction for solvent density is not made in selecting sample and solvent weights for charging the PSA tubes.

[1] About 15% of these were not suitable for reference standard usage.

[2] Adequate with respect to the plateau values used in least-squares calculation. The number (n) used for the entries in Table I is coupled with the assigned confidence class. The data are, for practical purposes, univariant and calculated accordingly.

Thermoanalytical Purity[3]—Estimations of purity from melting behavior are made by the modified, integrated Van't Hoff relationship presented by Gray (4) for use with thermograms recorded by differential scanning calorimetry (DSC) instruments. Temperature correction using the slope of the indium endotherm is always applied. Usually the sealed pans offered by the manufacturers are used, but a nitrogen environment is needed for some compounds. Work in this laboratory[4] has revealed no significant or predictable difference in estimates of impurities where thermograms from the two commercial systems were handled in similar fashion. The published baseline corrections are not necessary for these purposes and are not included in the entries in Table I. This same observation was made independently by other workers[5].

In Table I, T_0 is the extrapolated thermodynamic melting point relative to indium. Separate calibration is needed to express these values in terms of the USP–NF melting range because the methods are substantially different. There is no significance to the fact that a given T_0, converted to Celsius, falls within or outside monograph limits.

The ΔH_f values for ultrapure compounds are reproducible (5) with an internal precision of about 2% by quantitative differential thermal analysis (DTA). Few of the compounds here are ultrapure, and it is known that the ΔH_f values obtained depend on the sample purity; therefore, these data are not to be construed as highly accurate thermodynamic constants even though they may be the best values available. For this same reason, values with defined internal precision were not collected. All these values are corrected for premelt and are based on ΔH_f indium = 6.78 cal./g.

Qualitative TLC—Commercial, precoated glass plates or aluminum sheets are used exclusively. Silica gel[6] is the most common adsorbent, but neutral alumina and microcrystalline cellulose (MN-254) are also used. Four systems are developed for each drug substance and, because most are ionizable, the usual practice is to identify both acidic and basic developing solvents. Systems giving R_f 0.3–0.7 for the main spot are preferred. Three visualization techniques are identified. Nonreactive solvents are used where possible[7]. Artifact identification is made by: (*a*) spotting fresh and day-old solutions, and (*b*) two-dimensional TLC, involving wetting of the main spot of the first chromatogram with spotting solvent, drying, turning, and then developing the second chromatogram. Total spots listed in Table I reflect all four or three out of four systems, corrected for artifacts revealed by two-dimensional TLC. This allows reasonable estimation of the number and type of impurities.

General High-Low TLC Procedure—*Sample Solution*—Prepare a sample solution in a suitable solvent at a concentration of about 10 mg./ml. Nonreactive solvents are necessary, preferably degassed or nitrogen sparged. Dilute portions of this solution to concentrations of 1 and 0.5 mg./ml. These directions are general, and occasional solubility problems require their modification. Usually it is possible to apply 250 mcg. or more of drug sample and not exceed 25 μl. sample volume.

Reference Solution—A 10-mg./ml. solution of the specified reference standard in the same solvent is used to compare the identity of subsequent batches of the same drug substance.

Preparation—Divide a suitable TLC plate, 20 cm. square, coated with a 0.25-mm. layer of chromatographic silica gel mixture, into three parts: 2, 2, and 16 cm. in width. Spot 2 μl. of the reference standard solution (if available) in the first division on a line 1.5 cm. from the lower edge of the plate, spot 20 μl. (or largest volume to be spotted) of solvent in the second, and spot volumes equivalent to 250 (200, 100), 10, 5, 3, 2, 1, and 0.5 mcg. of the sample in the last, taking care to obtain comparable starting spots. Allow the plate to develop in a suitable chamber, usually lined with filter paper, which has been allowed to equilibrate at least 1 hr. with the specified developing solvent.

When the solvent reaches a height of 15 cm. from the origin, remove the plate from the chamber and air dry. Locate and compare spots and relative intensities under long- and shortwave UV light. Spray with 0.5% I_2 in chloroform and again rate the relative intensities of spots. Allow the iodine vapors to dissipate. Spray with 40% H_2SO_4 in methanol and view under a UV handlamp. Heat the plate and again view under UV light. For each visualization, estimate the relative abundance of spots as follows: compare each impurity spot in the 250-mcg. sample spotting to the main spots from the 0.5–10-mcg. spottings and assign a value to the relative intensities. For example, 0.4% impurity in the 250-mcg. sample has an intensity equivalent to the 1-mcg. main spot: (1 mcg./250 mcg.) × 100 = 0.4%. A densitometer may be used for these comparisons. Compare the reference standard mobility to that of the 10-mcg. sample spotting as an identity test. In most cases, acid–base indicators, ninhydrin, or Dragendorff sprays are used in this scheme. The 200- and 100-mcg. spottings are useful where there is evidence of overloading at the 250-mcg. level. Smaller spottings often are not visualized by one or more of the methods, so sensitivity estimates must be revised.

High Pressure Liquid Chromatography—An instrument[8] fitted with a gradient elution accessory is used with 1.0-m. × 2.1-mm. stainless steel columns. Commercial, coated packings with chemically bounded phases[9] are used usually with hydroalcoholic eluants in a reversed phase, gradient elution mode. Temperatures between 30 and 50° are employed at pressures up to 80 atm. to obtain flow rates between 0.5 and 1.5 ml./min. Only the low pressure mercury line, 254-nm., detector was used for the drugs reported herein. Drugs were dissolved wherever possible in one of the eluting solvents, 10–30 mg./ml., just prior to injection of 10–100-mcg. samples.

GLC—A temperature-programming instrument[10] is used with flame-ionization detectors. Only the relatively inert methyl and methylphenyl silicones (OV-1, OV-17, and OV-61)[11] on silanized diatomite are used after curing and conditioning, as instructed in the General Tests section of NF XIII (2).

DISCUSSION

The objective of this laboratory's examination of these samples is to allow an independent recommendation to the compendia as to the suitability of a sample for adoption or continued recognition as a standard. To accomplish this goal, it is not deemed necessary to discern precisely and reliably between, say, 0.3 and 0.4% impurity; rather, it is important to recognize samples containing, for example, 1% or greater impurities, to detect an increase in impurities of a few tenths-percent in a sample during storage, or to identify relative differences in impurity contents of subsequent batches of the same drug. Thus, those obvious experimental enlargements leading to more precise values are not pursued once information adequate to the objective is in hand. The data presented here, on the whole, are to be considered as having moderate precision.

Purity estimates should not be based on a single type of data. No method is universally applicable or reliable. Purity values obtained are in different units (mass or mole purity, visualization response, flame-ionization response, 254-nm. absorptivity, *etc.*), and these measurement units can be interconverted only where the identity, properties, and proportions of all impurities are known. Such thorough definition is unusual and seldom valuable and is not the case for the samples listed here.

The individual purity-indicating data converge to establish a profile of purity. It is this profile of purity that is meaningful and that permits decisions about the scope of analytical utility of a given sample.

There is no practical value to reconciling differences between purity-indicating data of moderate precision where both or all methods show the material to be satisfactory or unsatisfactory for the intended uses. Testing strategy and evaluation must begin with a statement of the intended uses of a standard. Different weights are placed on purity profile data according to intended standard usage, *i.e.*, as a chromatographic assay standard as opposed to an IR identity test or a limit test.

Alternative approaches to purity determination are known. Certified contents of the main or minor component by a defined

[3] Two commercial instrumental systems are used in this laboratory, the Perkin-Elmer DSC-1B and the DuPont 990 thermal analysis system.
[4] Drug Standards Laboratory, unpublished comparative study.
[5] D. L. Sondack, T. E. Cole, and J. K. Frishmann, personal communication.
[6] Merck F_{254}.
[7] Acetone reacts with many drugs and is rarely used as a spotting solvent, although it is a common solubility test for identity.
[8] DuPont 820.
[9] Permaphase-Zipax: octadecylsilane (ODS), a saturated hydrocarbon polymer (HCP), and an ethereal polymer (ETH).
[10] Hybrid of H&P 5750B and F&M 810 modules.
[11] OV-61 has since been discarded due to thermal instability.

Table I—Purity Profile Data

Reference Standards USP[a]	Reference Standards NF[a]	Drug Substance[b]	Solubility Analysis: Solvent	Solubility, mg./g.	Impurity, mass %	Confidence Class-n^c	Calorimetry: T_0[d], °K.	ΔH_f[e], cal./mole	Impurities (Premelt), mole %	TLC: Total Spots[f]	TLC: High-Low, Impurity	Impurities Determined by Other Methods[g]
x		Acetanilid	—	—	—	—	385	4900	<0.02	1+ trace	<0.2	—
x		Acetazolamide										
		(*a*)	Acetone	11.1	1.0	B-4	—	—	—	2	0.4	—
		(*b*)	Acetone	10.8	−0.1	D-5	—	—	—	1	—	—
	x	Acetyldigitoxin	Ethanol	15.4	7.65	B-5	—	—	—	3–4	2	Three spots, paper chromatogram; α and β (5%) isomer mixture
	x	Acetyl sulfisoxazole	Chloroform	15.5	0.02	B-6	dec.	—	—	1	—	—
x		Amitriptyline hydrochloride	Acetone	17.9	0.04	C-6	dec.	—	—	1	—	—
x		Amobarbital	—	—	—	—	433	6380	0.08(3.5)	1	—	<0.1%, GLC (C)
	x	Antipyrine	—	—	—	—	382	6570	0.38(9)	1	—	<0.1%, GLC (A)
x		Aspirin	10% ethanol–benzene	14.4	0.18	C-6	—	—	—	1	—	—
	x	Benzocaine	Absolute ethanol	21.8	1.04	C-6	362	5250	0.06(3)	3	0.1–0.2	<0.1%, GLC (A)
	x	Betamethasone										
		(*a*)	Ethanol	18.7	3.7	C-5	dec.	—	—	3	≥2.0	1.4%, HPLC, five peaks (ETH–water, methanol)
		(*b*)	*p*-Dioxane	10.0	3.5	D-5	—	—	—	—	—	—
		(*c*)	Ethanol	19.4	1.3	C-6	—	—	—	3	1.2	0.7%, HPLC, three peaks
	x	Biperiden										
		(*a*)	Ethanol	15.4	0.51	B-8	383	8350	0.07(1)	1	—	Single polymorph
		(*b*)	Ethanol	16.1	6.0	C-5	—	—	>2.5	2	—	Two polymorphs with 2° difference in melting point, DSC (conversion to higher melting with grinding)
	x	Biperiden hydrochloride	Methanol	16.7	1.0	C-5	—	—	—	1	—	—
	x	Bisacodyl	15% chloroform–ethanol	16.3	−0.5	D-6	407	9850	0.15(3)	1	—	—
	x	Brompheniramine maleate										
		(*a*)	Methyl ethyl ketone	28.6	3.0	A-6	406	12,200	0.54(7)	2	3.8	Possible decomposition during DSC
		(*b*)	*n*-Propanol	23.7	3.3	B-5	—	—	—	—	—	—
x	x	Caffeine	—	—	—	—	508	5010	0.05(4)	1	—	No other peaks, GLC (B) and (C)
x		Chloramphenicol	35% ethanol	17.2	0.58	A-6	423	8930	0.19(4)	—	—	—
x		Chlorpheniramine maleate	*n*-Propanol	28.9	0.4	D-5	406	11,400	0.45(5)	2	<1.0	—
x		Chlorpromazine hydrochloride	—	—	—	—	472	6730	0.18(4.5)	4	0.2	—
x		Chlorpropamide	40% chloroform–benzene	9.0	−1.0	C-5	—	—	—	2	0.4	Polymorphism, DSC
x		Cyclizine hydrochloride	Methanol	24.5	0.2	C-5	dec.	—	—	2	0.4	—
	x	Cyproheptadine hydrochloride	40% ethanol	22.4	0.18	A-5	—	—	—	1	—	—
x		Dapsone	10% water–*n*-propanol	22.7	4.28	C-7	451	4710	0.20(4.2)	1	<0.7	—
x		Dexamethasone	Acetone	21.1	0.6	B-6	dec.	—	—	2–3	0.65	—
	x	Dexchlorpheniramine maleate	Ethyl acetate	26.4	1.58	A-15	—	—	—	—	—	14:1 isomer mixture, PSA
	x	Dextromethorphan hydrobromide	Water	20.4	−0.18	A-7	dec.	—	—	1+ trace	—	<0.1%, GLC (B)
	x	Dienestrol	60% ethanol–water	33.6	4.6	C-7	dec.	—	—	5–7	1.4–3.1	—
x		Diethylstilbestrol	—	—	—	—	443	8040	0.36(11)	1+	<0.2	0.1%, GLC (A)
x		Digitoxin										
		(*a*)	Ethanol	14.6	0.46	A-8	—	—	—	2	0.2	—

Table I—(*Continued*)

Reference Standards USP[a]	Reference Standards NF[a]	Drug Substance[b]	Solubility Analysis: Solvent	Solubility, mg./g.	Impurity, mass %	Confidence Class-*n*[c]	Calorimetry: T_0[d], °K.	ΔH_f[e], cal./mole	Impurities (Premelt), mole %	TLC: Total Spots[f]	TLC: High-Low, Impurity	Impurities Determined by Other Methods[g]
		(*b*)	Ethanol	14.6	1.0	C-7	—	—	—	2	<0.3	—
x		Digoxin	95% aqueous methanol	6.13	2.2	A-8	—	—	—	5	2.0	—
	x	Dimethisterone	60% ethanol	11.8	1.11	D-4	—	—	—	2–3	0.4	—
x		Diphenhydramine hydrochloride	—	—	—	—	443	8160	0.42(7)	1	—	0.1%, GLC (B)
x		Diphenylhydantoin	Ethanol	19.4	0.38	D-6	—	—	—	1	—	0.6%, GLC (A)
	x	Dromostanolone propionate	5% water–methanol	27.8	1.2	B-4	403 dec.	5040	0.56(10)	2–3	1.1	—
	x	Dydrogesterone	Isopropanol	19.5	0.01	A-6	445	6800	0.14(4)	1+	—	—
	x	Ephedrine sulfate	Ethanol	15.0	1.34	A-6	—	—	—	1	—	—
	x	Estradiol	—	—	—	—	453	6880	0.14(3)	2	0.1–0.2	Polymorphism, DSC; <0.1%, HPLC (ODS/water–isopropanol)
	x	Estradiol benzoate (*a*)	—	—	—	—	471	8780	0.26(5)	3	0.7/0.6 +0.1	0.12% (estradiol), GLC (B); 0.5%, HPLC, (ODPN 5% tetrahydrofuran in isooctane)
		(*b*)	—	—	—	—	470	8570	0.71(7.5)	—	—	—
	x	Estradiol cypionate	—	—	—	—	427	7030	0.10(4)	3	0.8	≤0.9% (estradiol), HPLC [ODS/methanol–water (2:1)]
	x	Estradiol dipropionate (*a*)	Ethanol	18.2	0.83	A-6	375	7170	0.55(10)	3	1.0	Four species, one impurity, 15% a/a, HPLC, (HCP, 55% methanol); 2.1%, GLC (B)
		(*b*)	—	—	—	—	376	6150	0.17(3.5)	3	2.3	91.5% drug, 7% main impurity, HPLC, (HCP, 55% methanol); 2.8%, GLC (B)
x		Estradiol valerate	80% ethanol–water	16.8	1.00	A-6	422	7010	0.27(6.5)	2	0.5	—
x	x	Estrone	Acetone	14.6	0.8	C-6	—	—	—	2–3	—	No other peaks, GLC (B)
x		Ethacrynic acid	—	—	—	—	396 dec.	(7860) (7130)	0.17(5) 0.38(7)	2	0.5	—
x		Ethinyl estradiol (*a*)	—	—	—	—	459	6610	0.20(7)	2	0.4	No other UV 254 peaks (ETH-Zipax, methanol, pH 4.7 buffer); 1.0%, GLC (B)
		(*b*)	—	—	—	—	454	5350	1.50(13.5)	5	—	10 UV 254 peaks
x		Ethionamide	Acetone	23.7	−0.14	C-5	—	—	—	1+ trace	—	—
	x	Ethisterone	Chloroform	6.4	−0.3	B-6	dec.	—	—	1	—	One peak, HPLC (ETH/water, methanol)
x		Ethoxzolamide	—	—	—	—	464	9370	0.30(10)	1	—	—
	x	Fluocinolone acetonide	60% ethanol–water	10.9	0.76	B-6	—	—	—	1	—	—
x		Fluoxymesterone	Ethanol	14.3	0.3	B-5	dec.	—	—	1	—	—
	x	Fluphenazine hydrochloride	Ethanol	29.3	0.59	D-5	—	—	—	2–3 trace	0.6	—
	x	Flurandrenolide	60% ethanol–water	21.9	−0.3	D-6	—	—	—	1+ trace	—	—
x		Furosemide	Ethanol	17.1	−0.3	D-6	—	—	—	2–3	<0.2	—

(*continued*)

Table I—(*Continued*)

Reference Standards USP[a]	Reference Standards NF[a]	Drug Substance[b]	Solubility Analysis: Solvent	Solubility, mg./g.	Impurity, mass %	Confidence Class-n^c	Calorimetry: T_0^d, °K.	ΔH_f^e, cal./mole	Impurities (Premelt), mole %	TLC: Total Spots[f]	TLC: High-Low, Impurity	Impurities Determined by Other Methods[g]
	x	Glutethimide	40% ethanol	13.3	0.8	B-5	358	4760	0.25(7)	1+ trace	—	—
x		Hexachlorophene	—	—	—	—	437	8040	0.08(4)	1	—	No other peaks, GLC of trimethylsilyl derivative (B)
x		Histamine dihydrochloride	4% water in *n*-propanol	15.5	−0.5	C-6	dec.	—	—	1	—	—
x		Hydrocortisone	Ethanol	27.8	1.4	D-5	—	—	—	1	—	None, quantitative TLC; 32% polymorph, PSA
x		Hydrocortisone acetate	*p*-Dioxane	9.7	0.6	C-6	dec.	—	—	1	—	0.1%, quantitative TLC
	x	Indomethacin	Ethanol	24.8	0.64	C-5	433	9000	0.12(4)	1+ trace	<0.2	—
	x	Isopropamide iodide	Water	21.6	1.28	C-6	dec.	—	—	3	1.0–1.5	—
	x	Lanatoside C	Absolute ethanol	19.8	23.0	C-6	dec.	—	—	2–3+	—	Glycoside mixture
	x	Menadione	—	—	—	—	376	5130	0.15(5)	—	—	—
x		Meprobamate	—	—	—	—	376	8950	0.40(8)	1	—	1 peak, GLC (A)
x		Mestranol	Ethanol	21.1	0.40	B-6	427	8450	0.09(1.5)	—	—	<0.2%, GLC (B)
	x	Metabutethamine hydrochloride	Isopropanol	26.4	−0.56	A-5	dec.	—	—	1+ trace	—	0.1%, GLC (A, B)
x		Methamphetamine hydrochloride	Isopropanol	32.1	1.11	B-5	447	6370	0.45(12)	1+ trace	—	0.4%, GLC (A)
	x	Methantheline bromide	*n*-Propanol	28.8	1.25	C-6	dec.	—	—	—	—	—
	x	Methdilazine hydrochloride	Acetone	17.6	1.16	D-6	dec.	—	—	3–4	<0.6	—
x		Methimazole	Ethyl acetate	28.6	−0.5	A-6	—	—	—	1	—	—
	x	Methohexital	Ethanol–water (1:1)	13.3	0.39	B-5	365	6740	0.15(4)	1	—	—
	x	Methyclothiazide	Methanol	19.6	−0.05	A-6	—	—	1	1	—	—
	x	Nandrolone phenpropionate	—	—	—	—	367	5700	0:63(7)	1	—	0.9%, quantitative TLC
x		Neostigmine bromide	*n*-Propanol	24.8	−0.04	B-5	dec.	—	—	1	—	—
x		Neostigmine methylsulfate	*n*-Propanol	30.0	0.00	D-4	423	7140	0.04(2)	1	—	—
x		Niacinamide	Ethyl acetate	10.9	−0.9	C-7	402	5460	0.14(4)	1	—	—
	x	Nialamide	40% ethanol	24.9	−0.2	C-5	427	10,920	0.15(1.5)	1	—	—
x		Nitrofurantoin	20% dimethylformamide–acetone	21.6	−0.36	B-6	—	—	—	1	—	One peak, HPLC (SAX/0.1 *N*, pH 9.1 borate buffer)
	x	Norethandrolone	50% ethanol	13.3	4.77	B-6	412	5100	0.13(3)	up to 6	≤3.0	0.2%, GLC (B)
x		Norethindrone	Acetone	19.5	1.38	A-7	481	8740	0.20(5)	1	—	—
	x	Norethindrone acetate	60% ethanol	13.8	1.33	C-5	438	6500	0.49(14.7)	3–5	<1.0	About 9% polymorph, PSA
x		Norethynodrel										
		(*a*)	30% acetone–hexane	21.2	1.68	C-6	459 dec.	6550	1.57(0)	5	<1.5	Up to 3.6% added norethindrone not revealed by DSC; mestranol revealed by DSC
		(*b*)	30% acetone–hexane	20.8	2.95	D-5	455 dec.	7020	1.80(10.5)	3–4	—	—
		(*b*)	Isopropanol	24.6	2.62	D-5	—	—	—	3–4	—	—
		(*c*)	30% acetone–hexane	20.9	−0.01	B-11	—	—	—	1	—	—
	x	Oxandrolone	Acetone	17.3	0.66	A-6	dec.	—	—	2	<1.0	—
x		Phenacetin	Ethyl acetate	23.1	0.40	C-4	410	7480	0.08(8)	2	<0.2	—
	x	Phenazopyridine hydrochloride	Pyridine	10.4	−0.10	B-5	—	—	—	3	0.6	—

Table I—(*Continued*)

Reference Standards USP[a]	Reference Standards NF[a]	Drug Substance[b]	Solubility Analysis: Solvent	Solubility, mg./g.	Impurity, mass %	Confidence Class-n^c	Calorimetry: T_0[d], °K.	ΔH_f[e], cal./mole	Impurities (Premelt), mole %	TLC: Total Spots[f]	TLC: High-Low, Impurity	Impurities Determined by Other Methods[g]
x		Phenformin hydrochloride	—	—	—	—	451	7902	0.06(2)	1	—	—
	x	Phenprocoumon	90% ethanol	31.8	0.59	A-6	456	8530	0.37(7)	1	—	—
x		Phenylbutazone	—	—	—	—	377	7090	0.25(8)	2	<0.2	—
x		Pralidoxime hydrochloride	Ethanol	33.6	0.46	D-5	—	—	—	2	0.1	—
x		Prednisolone acetate										
		(*a*)	*p*-Dioxane	13.5	1.40	D-5	—	—	—	1	—	0.3%, quantitative TLC
		(*b*)	*p*-Dioxane	13.5	4.94	B-5	dec.	—	—	2	1.7	0.5–1.5%, quantitative TLC
x		Prednisone	Ethanol	8.5	0.10	B-5	dec.	—	—	1	—	—
x		Procaine hydrochloride	—	—	—	—	429	8610	0.04(1.5)	1	—	—
	x	Progesterone	—	—	—	—	403	6140	0.06(1)	1–2	<0.1	0.6%, HPLC (ODS/10–40% isopropanol in water)
	x	Promazine hydrochloride	Isopropanol	20.6	0.13	C-12	455	7710	0.20(3)	2	0.2	—
x		Propoxyphene hydrochloride	*p*-Dioxane	25.6	1.52	B-7	—	—	—	1	—	—
x		Pyrazinamide	—	—	—	—	463	6790	0.10(3.2)	1	—	—
x		Pyridoxine hydrochloride										
		(*a*)	Ethanol	13.2	0.80	A-6	dec.	—	—	1	—	—
		(*b*)	Ethanol	13.4	1.6	C-7	—	—	—	3	<0.2	—
	x	Pyrilamine maleate	Ethyl acetate	11.2	−0.03	B-10	—	—	—	2	≤0.6	—
x		Resorcinol	1% ethanol–chloroform	8.2	0.60	C-6	382	4750	0.04(3)	1–2	—	—
	x	Saccharin	Isopropanol	21.4	−0.37	C-4	499	6550	0.32(5)	1+ trace	—	—
	x	Salicylamide	40% ethanol	25.5	−0.07	C-5	414	6390	0.02(1)	1+ trace	—	None, GLC (B) 0.5%, GLC (A)
x		Scopolamine hydrobromide	Absolute ethanol	20.0	−0.3	C-6	dec.	—	—	1	—	0.4%, GLC (A)
x		Spironolactone	60% ethanol–water	16.9	0.4	A-8	dec.	—	—	2	0.1	None, HPLC (ODS, 55°, 10% *n*-propanol–water)
x		Sulfapyridine	—	—	—	—	466	8730	0.23(4)	2	0.2	—
x		Sulfisoxazole	Ethyl acetate	15.4	−0.14	C-5	dec.	—	—	2	<0.4	—
	x	Testosterone	—	—	—	—	429	6250	0.33(1.5)	3–4	<0.3	None, HPLC (ETH/water, isopropanol); 1%, HPLC (ODS/10% *n*-propanol in water)
x		Testosterone cypionate	70% methanol	1.80	0.10	A-7	373	5640	0.13(5)	1	—	—
x		Testosterone enanthate										
		(*a*)	—	—	—	—	312	4960	1.47(15)	6	1.5	1.7%, HPLC, six peaks, (ODS/30% isopropanol in water)
		(*b*)	—	—	—	—	312	5920	1.08(8.5)	6	0.7	—
x		Tetracaine hydrochloride	40% absolute ethanol–isopropanol	11.6	−0.3	B-6	dec.	—	—	2	0.2	—
x		Thiabendazole	Methanol	11.3	0.82	B-6	dec.	—	—	1	—	—
x		Thiopental	10% ethyl acetate	20.7	0.50	D-6	431	7010	0.17(5)	1	—	—
x		Tolbutamide	Benzene	15.6	0.50	A-5	400	6300	0.20(5)	1+ trace	—	—
x		Tolnaftate	—	—	—	—	384	7350	0.72(11)	2+ trace	<1.0	—
	x	Tranylcypromine sulfate	70% ethanol	25.6	1.0	C-5	—	—	—	1	—	—

(*continued*)

Table I—(*Continued*)

Reference Standards			Solubility Analysis			Calorimetry			Impurities (Premelt), mole %	TLC		Impurities Determined by Other Methods[g]
USP[a]	NF[a]	Drug Substance[b]	Solvent	Solubility, mg./g.	Impurity, mass %	Confidence Class-n[c]	T_0[d], °K.	ΔH_f, cal./mole		Total Spots/ Impurity	High-Low, Impurity	
x		Triamcinolone acetonide	Acetone	20.0	0.29	B-7	—	—	—	23	1.7	Triamcinolone not revealed by PSA
	x	Triamcinolone diacetate	70% ethanol	13.2	−0.78	B-5	—	—	—	1+ trace	—	—
x		Trihexyphenidyl hydrochloride	20% ethanol	14.6	0.62	C-5	dec.	—	—	2	<0.4	—
x		Trimethaphan camsylate	Isopropanol	24.5	0.1	C-6	dec.	—	—	1+ trace	—	—
	x	Trioxsalen	Chloroform–methanol (2:1)	25.6	0.8	D-4	508	6790	0.51(8)	3–4	<0.6	—
x		Tripelennamine hydrochloride	—	—	—	—	459	8490	0.37(7)	2	<0.5	—
x		Tropicamide	20% ethanol	23.4	0.8	D-6	368	8990	0.10(2.5)	1	—	No other peaks, GLC (B) and (C)
x		Tubocurarine chloride	Ethanol	30.0	0.29	B-6	dec.	—	—	3	~0.5	*d*-Isomer
x		Vanillin	—	—	—	—	353	(5100)	0.06(2)	1	—	Sublimes, ΔH varies
	x	Vinbarbital	—	—	—	—	439	7270	1.52(12.5)	1	—	0.9%, GLC (A)

[a] Samples cannot be presumed to have been found acceptable by the responsible compendial body. [b] *a*, *b*, and *c* represent different batches. [c] Confidence classes are defined as: A or B, almost conclusive or strong evidence of the (im)purity of a sample; C, good evidence but requires several supporting data; and D, possibly useful as supporting evidence in conjunction with strong alternative evidence. n = number of tubes used. [d] T_0 = extrapolated thermodynamic melting point related to indium. [e] Values are based on H_f indium = 6.78 cal./g. [f] Total number of impurities as determined by qualitative TLC. [g] For GLC systems: A, 3% OV-17 on 100–120-mesh Gas Chrom Q; B, 3% OV-1 on 100–120-mesh Gas Chrom Q; and C, 3% OV-61 on 80–100-mesh Gas Chrom Q. HPLC = high pressure liquid chromatography. ETH = an ethereal polymer; DSC = differential scanning calorimetry. PSA = phase solubility analysis. ODS = octadecylsilane. OPDN = β,β'-oxydipropionitrile. HCP = a hydrocarbon polymer.

method are necessary for such applications as primary titrimetric, calorimetric, or trace standards[12]. Exhaustive definition of the identity and quantity of every impurity in a given lot of drug standard has been achieved elsewhere for a few compounds, but this approach has been judged unsatisfactory and uneconomical[12]. Practical evaluations of a few ultrapure reagent samples[14] and the strategies involved were discussed recently (6).

Profile Interpretations[15].—Estradiol dipropionate was evaluated by PSA, DSC, TLC, and high pressure liquid chromatography. The material was proposed for use partly as a reference standard in a UV assay. Solubility analysis showed 0.8% mass impurity, and scanning calorimetry revealed 0.5 mole % eutectic impurity. High-low TLC and GLC gave a 1.0–1.2% impurity level. At this point, the material could be used as a standard for IR or thin-layer identification purposes. However, liquid partition chromatography revealed six components and that one of the impurities was intensely UV absorbing, about 15% of the area under the curve at 254 nm. Another sample showed 7% in the 254-nm. data but more mass impurities. Collection of the main impurity showed that it interfered about 6 and 3%, respectively, at the monograph wavelength of 268 nm. Therefore, the material was unacceptable as a UV assay standard. *This is a striking illustration of the necessity of interpreting purity profile data in light of the intended uses of a standard.*

Betamethasone was evaluated by liquid partition chromatography; about 1.4% UV absorbing impurity was evident. Quantitative TLC showed only 0.5% and high-low TLC indicated 1.0% impurity. Thermal decomposition precluded an estimate of eutectic impurity. Solubility analysis in a polar alcoholic solvent revealed that the main component was only 96.3% pure. Because polymorphism could account for a discordant solubility analysis, especially for a steroid, a second solubility analysis, this time in an ethereal solvent, was performed. The result, 96.5%, was confirmative, excluding polymorphism as an explanation for the data. The presence of process-related, saturated steroids was suspected, because these would not absorb UV light and, therefore, would not be revealed by either quantitative TLC (recovered *versus* original) or the 254-nm. detector; less detectability is anticipated with most visualization reagents so that the high-low value would register less than the mass present. A subsequent batch assayed 98.7% by solubility analysis, high-low TLC revealed 1.2% impurity, and liquid chromatography showed only 0.7% impurity. The UV absorbance at the monograph wavelength was about 1% greater than that of the former batch, which partially confirmed the interpretation.

Acetanilid was marked for use as a melting-point standard. The material was chromatographically homogeneous. Calorimetric purity analysis indicated that less than 0.02% eutectic impurities could be present, the most desirable feature for a melting-point standard. On the other hand, estradiol cypionate was shown to contain about 0.8% impurity, mostly estradiol, by chromatography, whereas eutectic impurities registered only 0.1%. Such discordance of calorimetric purity with other methods is particularly severe in steroids where solid solutions are commonplace.

Norethynodrel was evaluated by solubility analysis, calorimetry, and TLC. A three- or four-component mixture was indicated by TLC, and a 1.8% eutectic impurity was in evidence. Solubility analyses in two solvent types both demonstrated a high level of impurity, about 2.7%. A subsequent batch showed unusual correlation of PSA, DSC, and high-low TLC values at the 1.6% level which, for a compound of this type, may be currently the best achievable purity.

Dexchlorpheniramine maleate was found by solubility analysis to contain a less soluble component (13.8%) and soluble impurities (1.6%). The 13.8% component was interpreted as the racemic compound due to the presence of a 6.9% *l*-isomer. The solubility of the racemic compound (4.5 mg./g.), allowing for mass action due to maleate, was consistent with the value predicted from the phase diagram, and chromatographic data also were consistent with this interpretation.

[12] The National Bureau of Standards is the major source of such standards.

[13] C. A. Johnson, British Pharmacopoeia, personal communication.

[14] Ultrapure reagents are needed mostly in trace and inorganic analyses.

[15] Only the specialized purity-indicating data are listed here; monograph tests were performed on each, such as moisture content, spectral characteristics, and optical rotation.

Most of the profiles in Table I are straightforward, and the above examples were chosen to illustrate either the interpretations of discordant data or the variable weight assigned to data with respect to the intended uses of a standard.

COMMENTS ON METHODS

PSA—Procedures and interpretation of results were discussed in detail elsewhere (1, 2, 7, 8). This laboratory has made available systems for 120 drugs [including those of an earlier summary (1)]. Approximate solubilities may not be close enough to the PSA-determined value, particularly where substantial impurities exist, so that additional tubes may be required at a later time to fill in the phase diagram. This open-ended feature of PSA is valuable. Where greater precision or definition is required, more tubes may be added. More importantly, additional solvents or temperatures may be chosen. Indeed, identical results of solubility analyses in two chemically different solvents may be taken as conclusive purity evidence.

PSA is the most generally applicable absolute purity determination. Scrupulous attention must be given to technique and choice of systems. A disadvantage of the method is the lengthy elapsed time[16] before interpretation is possible. Solid solutions (9) and disproportionation of salts (10) are encountered occasionally, but recent modifications show promise of extending the scope of solubility analysis to these difficult situations, Successful analysis (10) of atropine sulfate has been reported using a picric acid system, whereas probable disproportionation has prevented analysis in this laboratory by the usual procedure. Apparent polymorphism in the solvent is a more common problem, particularly with steroids, and this may explain the data for hydrocortisone, norethindrone acetate, and prednisolone acetate. A change of systems would be indicated where other purity profile information does not allow interpretation.

High-Low TLC—The need for a semiquantitative TLC test, one that could be used to validate precise but unselective assays such as titrations, has existed for some time. Obvious theoretical arguments against the accuracy of such a test can be overwhelming; nevertheless, it is one purpose of this report to prove the practical value of one such procedure by comparison of the results of (im)-purity estimates with values obtained by more definitive methods. High-low TLC has been instituted in this laboratory out of a suggestion from Johnson[13]. The need for such a test arose from the reference standard evaluation program, in which samples containing minor impurities must be evaluated without benefit of comparison to other standards. This situation is critical in instances where neither solubility nor calorimetric analysis has yielded interpretable purity data.

Typically, varying sample sizes are spotted on a single plate, the chromatogram is developed, and unselective visualization methods are used. Impurity spots in the heaviest spotting are compared in intensity and size to the main spot in each of the smaller sample applications, and the estimated impurities are summed for an overall estimate. The limiting assumption here is that the drug samples are contaminated with chemically related materials. Even with related materials, responses vary so that the usual practice is to use three unselective visualization methods. This method is a realistic approach only where a few percent of impurities are anticipated.

High-low TLC is most useful exactly where quantitative (by extraction) TLC is not reliable, such as distinguishing between 0.5 and 1.0% impurity and ranking subsequent batches of the same drug. TLC data, in general, have not been useful for materials having less than 0.2% impurities.

Correlation of high-low results in Table I with PSA and DSC data shows that this method is reasonably reliable, usually within 0.5% of the total impurity, with PSA–TLC being better correlated than DSC–TLC. High-low TLC and qualitative TLC are valuable tools in constructing purity profiles, and the use of these procedures in conjunction with DSC is recommended strongly for validation.

Other Chromatography—Qualitative and quantitative TLC evaluation of drug purity and stability is commonplace and requires no discussion. It is important to note that recovery and precision considerations easily obscure purity information, particularly where an established standard is not available for direct comparison. In quantitative thin-layer work, the bias is usually in the direction of underestimating the recovered species, and assays are of little value when a standard of known purity is not available.

GLC purity estimates play only a minor role in initial establishment of, as opposed to comparative assay with, a reference standard. GLC purity alone is valid only for distillates, because only the volatile portion of the sample is eluted for measurement. Similarly, only the least polar phases are of value in general purity work. Multiple products are not unusual with derivatization, and any reaction or extraction can constitute a purification step. Thermal-injection artifacts are all too common but are easily distinguished from impurities by comparison of chromatograms resulting from stepwise variation of injection port temperature from that of the column to 150° hotter ("hot-port" experiment).

High-pressure liquid chromatography has substantial potential for purity-indicating data, but applications of this technique are too recent to allow many generalities. At present, it appears that this recent instrumental development, along with excellent new packing materials, has made available a major purity test. Separate evaluation of this tool is planned.

DSC—As a first proposition, this method cannot be regarded as ever yielding absolute estimates of (im)purity. However, where prudently applied, this method can be of great value in drug purity evaluation. Combined with strong supporting evidence such as TLC, the method is well suited to purity and stability evaluations. The method is capable of considerable precision and reproducibility. Discussions of the experimental details and scope are available (5, 11–14). Reliance on this method requires at least the exact knowledge of the history of a sample and the types of likely impurities. Precalibration against other purity-indicating information and use of defined standards are preferred. Most drug substances are not amenable to calorimetry because of thermal decomposition during melting or, in certain situations, significant vapor pressure. Experience with more than 150 compounds, randomly received, indicates that about 30% of official drug substances yield calculable thermograms. The problem of low ΔH_f compounds was recognized earlier (13). In these cases, and for solid solutions as well, melting-point phase diagrams, as discussed by Marti *et al.* (14), may be of value, but samples with proven purity are needed to construct the diagrams. All these remarks apply only to quantitative DSC and not to the major value of thermal analysis for such phenomena as polymorphism.

Several distinctive problems should be discussed. Only eutectic impurities are revealed; solid solutions lead to impurity estimates that are too low. The thermogram does not reveal solid solutions (12). For example, the presence of 3.6% added norethindrone in norethynodrel was not measured by DSC, although this is measurable by UV spectrophotometry; in contrast, the presence of added mestranol was revealed by DSC.

Correction for the fraction of sample melted prior to instrumental response is a feature of the usual DSC method (11–13). This empirical correction (and resulting slope and impurity value) depends on an arbitrary choice of those areas to be used in the temperature *versus* reciprocal fraction-melted plot. For example, varying the choice of areas in the phenacetin analysis resulted in premelt factors ranging from 1 to 15% and in impurity values ranging from 0.02 to 0.40 mole %.

The T_0 and ΔH_f values are affected somewhat by the random selection of areas, but minor variation in these values has negligible effect on the calculated impurity. The premelt correction is varied until the theoretical linear relationship is visually obtained in the plot. However, many drugs yield S curves in the plot, which cannot be even approximately linearized. Premelt correction then is entirely arbitrary, allowing for great variability in the final impurity calculation. Such was the case with diphenhydramine hydrochloride, ethacrynic acid, hexachlorophene, menadione, methamphetamine hydrochloride, norethindrone acetate, norethynodrel, phenacetin, and phenprocoumon. For example, impurity values between 0.2 and 0.4 mole % could have been reported for phenprocoumon because of visual uncertainty in determining the best line for the data. The values reported[17] in the table for these drugs represent results obtained using the premelt correction yielding the least-squares best fit with the lowest standard error. A three-point estimate is

[16] A vibrational method is used elsewhere but has not been found to be reliable here except in instances of large sample charges.

[17] Calculated by means of a programmable desktop calculator.

also used (15). In cases where S character is strong, we recommend that impurity values be calculated using several premelt correction values as an indication of the confidence. Computer programs that calculate a single value for impurity based on a single best fit should be used with caution, as the degree of S character is not specifically determined even though the premelt correction is well controlled. Studies on the impact of this problem are in progress. The magnitude of the premelt correction in Table I correlates roughly with the level of impurity, as should be expected: 0.0–0.2 mole % impurities averaged 3.3% premelt correction, 0.2–0.5 mole % averaged 6.8% premelt correction, and >0.75 mole % averaged 11% premelt correction. A sample that generates a premelt correction not consistent with this trend should be examined more carefully by other methods before such purity data are interpreted.

SUMMARY

Purity constants and analytical systems are reported for samples of 115 official drug substances analyzed by various combinations of PSA, DSC, high-low TLC, GLC, and high pressure liquid chromatography. General experiences are discussed for these purity-indicating techniques. No one method is universally reliable or applicable, and purity decisions should be based on the largest variety of data available.

The individual purity-indicating data on a given sample converge to establish a profile of purity. It is this purity profile that is meaningful and that allows decisions about the scope of usefulness of the sample as a reference standard. Examples of interpretations are given for a few of the purity profiles.

PSA continues to be the most valuable and generally applicable purity technique. DSC has value in the construction of purity profiles, but it can never stand alone and is applicable to only a minority of drugs. GLC is of minor value in establishing purity profiles. On the other hand, high pressure liquid chromatography has demonstrated a potential for major value in purity work.

Despite the inherent inaccuracies in high-low TLC, a direct correlation exists in the majority of cases between the values obtained by PSA and this method. It is possible, where a rapid method is needed, to determine approximate purity within a day using high-low TLC, especially where a marked correlation exists between purity estimates determined by the methods discussed here.

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Freezing Unit Dose Injections prior to Freeze Drying

ARTURO RIGOLI

Abstract □ A new method, used on an industrial scale, for freezing vials to be lyophilized was studied, by which a dried mass in the shape of an internal hollow paraboloid with very thin layers is obtained. The apparatus is also described.

Keyphrases □ Freezing of solutions, emulsions, and suspensions—improvde rotating freezer for liquids in ampuls or small containers □ Injections, unit dose—improved rotating freezer for use prior to freeze drying □ Lyophilization, injectables—improved method for freezing vial contents

Up to the present day, the most used industrial techniques for freezing vials containing pharmaceuticals to be lyophilized have been the: (A) single-cylindric tablet (bottom freezing) (Fig. 1*a*), (B) cylindric tablet composed of two or more separated strata (1, 2) (Fig. 1*b*), (C) shell form (agar slant) tablet (Fig. 1*c*), and (D) double shell (3) (double agar slant) tablet (Fig. 1*d*).

Technique A, being the cheapest and simplest, is the one most used. In this method the vials are placed vertically on suitable trays, which are then transferred to refrigerated shelves or, sometimes, immersed in freezing baths. The freezing is monodirectional and bidirectional in the two cases, respectively, and proceeds from the outer to the inner part of the liquid cylinder. An external layer of frozen liquid is set up which renders rather difficult the heat exchange; as a consequence, a long treatment at low temperature is required to freeze the liquid completely.

Technique C needs specific equipment. The liquid is distributed on part of the vial wall to obtain a larger evaporation surface. By consequence, the mass freezes in thinner layers than in both the A and B techniques while the time required for both freezing the solution and evaporating the solvent is minimized, with an obvious reduction of production costs.

Techniques B and D, much more laborious and expensive than the others, are carried out to avoid the mixing of interreacting substances (4). It is known that the best lyophilization conditions are assured by the quick cooling of large surfaces of material in thin layers (5–7).

During the last 10 years, different kinds of rotating freezers have been patented (8) to reach these conditions. Such equipment allows only the freezing of bottles similar to those containing plasma (9, 10). The liquid mass is spread over most of the internal surface of the bottle, producing a shell form either by a slow or fast rotation of the flasks. The cooling of the bottles is provided either by immersion in a freezing liquid or by spraying the flasks with a cold fluid. This kind of apparatus works with few units at a time, and an external contamination could occur due to the use of the refrigerant fluid. Another type of rotating freezer (11), suggested for flasks, works as a normal centrifugal under vacuum machine, having an inclined rotation axis. The prefreezer does not require a freezing liquid because the cooling is obtained by rapidly rotating the product under vacuum (adiabatic cooling). With this type of freezer, the evaporation may cause the precipitation of the solids even before freezing, with obvious disadvantages in the drying phase and the final product. In fact, with this freezing procedure, fractioned crystallization of some components might occur in relation to their physical properties, and the developing crystals accumulate at the boundary layers of the mass to be frozen due to the centrifugal force.

Up to now, no similar industrial procedure has been applied to liquids to be frozen in ampuls or small containers. We improved and patented (12) a rotating freezer by which frozen solutions, emulsions, and suspensions are obtained in the shape of an internal hollow rotation paraboloid (Fig. 1*e*). With such a system, larger surfaces and thinner layers than those accomplished by the static freezers are obtained and the freezing time is reduced.

EQUIPMENT

The new rotating freezer consists of (Fig. 2):

1. A turning frame (No. 25), with trays containing the ampuls to be frozen, placed in a covered (No. 18) box (No. 17). The box bottom and walls are cooled by a refrigerant group (Nos. 29 and 30), which is able to lower the air temperature of the box to −50° within 25 min.
2. A ventilation system (No. 32) generating a blast of cold air at −45° which passes over the ampuls from the higher to the lower part of the vial at 90 m./sec. and 15 mm. water pressure.
3. A box (No. 17) that can be inclined at a suitable angle, dependent both on the diameter of the ampul and the volume of the liquid to be frozen. In this way, the liquid surface sets itself like an agar slant. By rotating the turning frame at a slow speed (20–30 r.p.m.), the liquid freezes in very thin layers on the inner surface of the ampul, forming a rotating hollow paraboloid (Figs. 3 and 4).

EXPERIMENTAL

The described equipment can be used for freezing mixtures of biological products having different eutectic points. This report concerns the freezing of an aqueous solution containing 15% solids, showing an eutectic point at −12°.

The freezing of 1.2 ml. of solution in vials of 13–13.5-mm. diameter resulted in a frozen mass in which thickness was about 0.2–2 mm. on the walls and 3–3.5 mm. at the bottom (Table I, Column *b*). Under the same conditions using a static freezer, the

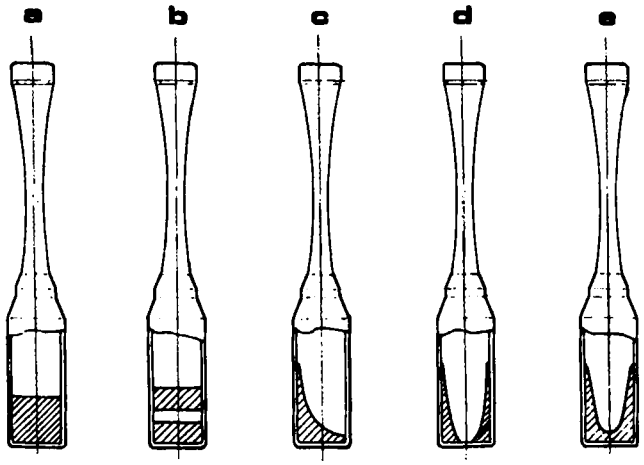

Figure 1—*Freeze-dried vials. Key: a, single-cylindric tablet (bottom freezing); b, cylindric tablet composed of two or more separated stratums; c, shell form (agar slant) tablet; d, double shell (double agar slant) tablet; and e, rotating paraboloid tablet.*

frozen mass thickness was 10–11 mm. (Table I, Column *B*). Moreover, the freezing time of 4–6 hr. (13) (Fig. 5) with static freezers was reduced to 20–25 min. with the study apparatus (Fig. 6) due both to the increased exchange surface (Table I, Column S_2) and the forced circulation of cold air which increases the total heat transfer coefficient. The drying time (Fig. 7) of the frozen vials was also reduced in comparison with that of statically frozen vials (Fig. 8). In fact, the temperature of the heating liquid being 40°, the frozen samples reached 0° within 5 hr., while 13 hr. was required for frozen samples with a cylindric shape.

As shown in Fig. 7, the maximum drying was reached within 1–3.5 hr. while for statically frozen vials the same drying was obtained within 1–10 hr. (Fig. 8). For paraboloid tablets, the maximum difference between the vapor pressure in the drying room and that in the condenser occurs within 3–4 hr. and the pressure in the drying room reaches a minimum value of 10 μ after 9 hr. For the

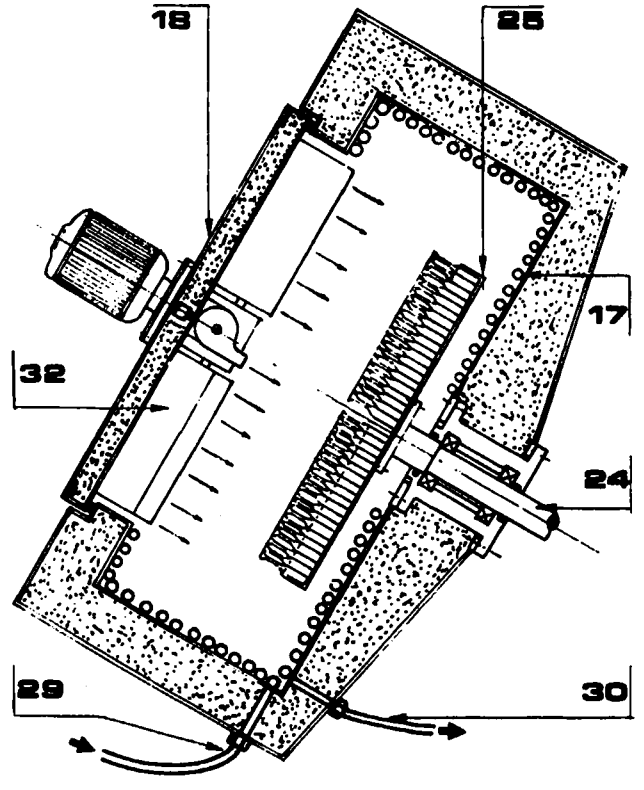

Figure 2—*Cross section of the rotating freezer. (See text for explanation of numbers.)*

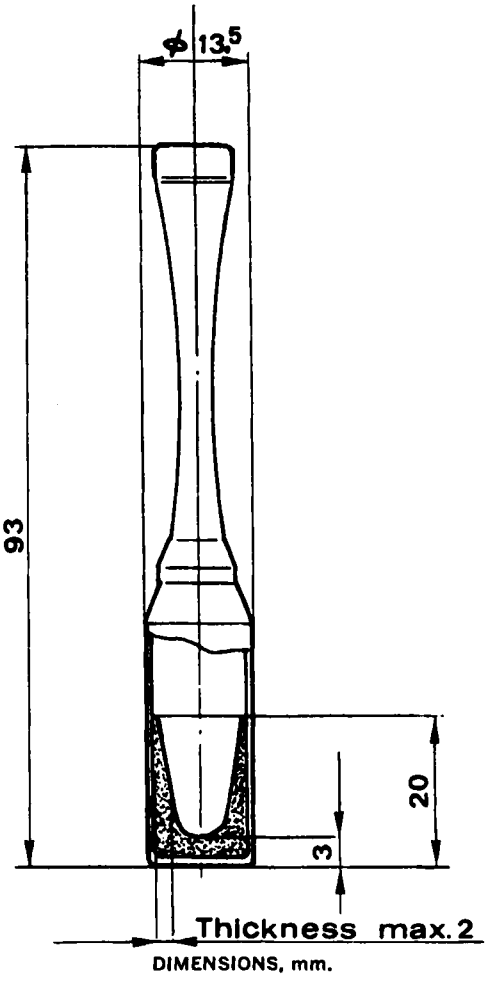

Figure 3—*Frozen vial in the shape of an internal hollow rotation paraboloid. Bottom and sides are 3 and 2 mm. thick, respectively. On the sides the frozen liquid is 20 mm. high.*

cylindric tablets, the maximum pressure difference is reached within 3–7 hr., and the pressure in the drying room reaches the minimum value of 10 μ after 19 hr. These differences are due to the major development of the evaporation surface (Table I, Column *S*), which in the first case is four times (Table I, Column *Rs*) as much as in the second case, and to the reduced resistance offered by the thin layer to the evaporation. In conclusion, the freeze drying of the above-mentioned solution, which would require 22 hr. if performed on the product frozen in a static freezer (Fig. 8), requires only 10–11 hr. in the case of the product frozen in the dynamic freezer (Fig. 7). Therefore, a saving of the required energy and an improved productivity can be obtained.

METHODS

The vials, filled by the usual filling machines, are placed in suitably drilled trays. These trays are placed on the rotating frame of the freezer, previously chilled, and the box is inclined at the suitable angle to distribute the liquid in a shell form, like an agar slant, on the inner side of the vial wall. Both the rotation of the frame and a cold air blast, flowing from the higher to the lower part of the outer surface of the vials, are started up. After 20–25 min., the liquid is completely frozen in contact with the internal wall of the vial. The stratum, having an average thickness of a few millimeters, is formed by several superimposed thin layers showing a spiral form; at the bottom of the vial, the frozen layer is about 3–3.5 mm. thick (Table I, Column *b*), depending on the diameter of the vial and the liquid volume (Table I, Column *VL*). The trays containing the frozen vials are placed into a conventional static freezer until the loading of the drying equipment is completed and are then lyophilized as usual.

Table I[a]—Cubic Paraboloid Measured and Calculated Values[b]

ϕ, mm.	VL, cm.³	V, cm.³	H, mm.	B, cm.	b, cm.	S, cm.²	Rs	S_1, cm.²	S_2, cm.²
12	1.0	1.090	20	0.96	0.28	5.104	4.52	4.77	8.69
13	1.2	1.308	20	0.98	0.32	5.405	4.07	5.33	9.50
14	1.5	1.653	20	1.06	0.42	5.542	3.60	6.20	10.34

[a] For the calculations and solutions, see text. [b] ϕ = vial diameter, VL = volume of 15% aqueous solution still at liquid state, V = VL volume at frozen state, H = height of fluid on wall of frozen paraboloid-shaped vial, B = height and thickness of statically frozen solution, b = thickness of bottom side of solution when frozen in paraboloid shape, S = evaporating surface of frozen paraboloid-shaped solution, Rs = ratio between evaporating paraboloid surface S and one of the same solution statically frozen, S_1 = surface of thermal transfer of statically frozen vial, and S_2 = surface of thermal transfer of same frozen cubic paraboloid-shaped vial as in S_1.

RESULTS

Compared with the usual static procedures, the rotational freezing procedure described here shows the following advantages:

In the Freezing Phase—1. Thinner layers: 0.2–2 mm. on the walls and about 3 mm. at the bottom (Table I, Column *b*) instead of 10–11 mm.

2. Minimized freezing periods (Fig. 6): about 25 min. instead of 4–6 hr. (Fig. 5) (13). This occurs because the heat exchange surface is almost doubled (Table I, Column S_2) and the heat transfer from a paraboloid-shaped mass is much faster than for cylindric tablets; the heat exchange decreases with the increasing frozen layer thickness.

3. Reduced chemical and physical changes: *e.g.*, oxidation reactions on the surface of the tablet, concentration of salts and possible protein (enzymes) denaturation, particle accumulation from suspensions, and emulsion cracking due to the reduced freezing time.

4. Absence of undercooled areas which might be formed in the cylindric tablets due to the rotation of the solution during the freezing time.

5. Absence of fractional crystallization of products contained in the solution to be frozen.

6. Constant thickness of the layers due to the rotation during the freezing. As a consequence, a shorter time and a regular progression in the drying process can be obtained (14).

7. Formation of tiny and compact crystals from which a compact and amorphous freeze-dried product is obtained.

8. Possibility to reduce the amount of support to be added to the solution.

9. Reduced refrigeration energy consumption to overcome the eutectic zone and to get complete freezing.

In the Drying Phase—1. High development of the evaporation surface (Table I, Column *S*), which is about four times as large (Table I, Column *Rs*) as the surface of the solution frozen in single-cylindric tablets in similar vials.

2. Rapid and deep heat penetration into the layer.

3. High drying rate (Fig. 7) due to the minimal resistance of the dried layer to the evaporation of the solvent.

4. Increased drying efficiency as a result of Points 2 and 3.

5. Absence of both visible and invisible fusion areas.

6. Compact and homogeneous aspect of the final product.

7. Higher reconstitution rate of the solution.

8. Reduced thermal energy required for ice elimination.

A comparison of the working conditions of the rotating freezer (Column A) and those of the conventional system (Column B) is shown in Table II. In Case A, a conventional freeze-drying apparatus was used, equipped with a static freezer with 24 trays, a freeze-drier with 24 trays, and a rotating freezer with two trays. In Case B, the same apparatus as in A was used except for the ab-

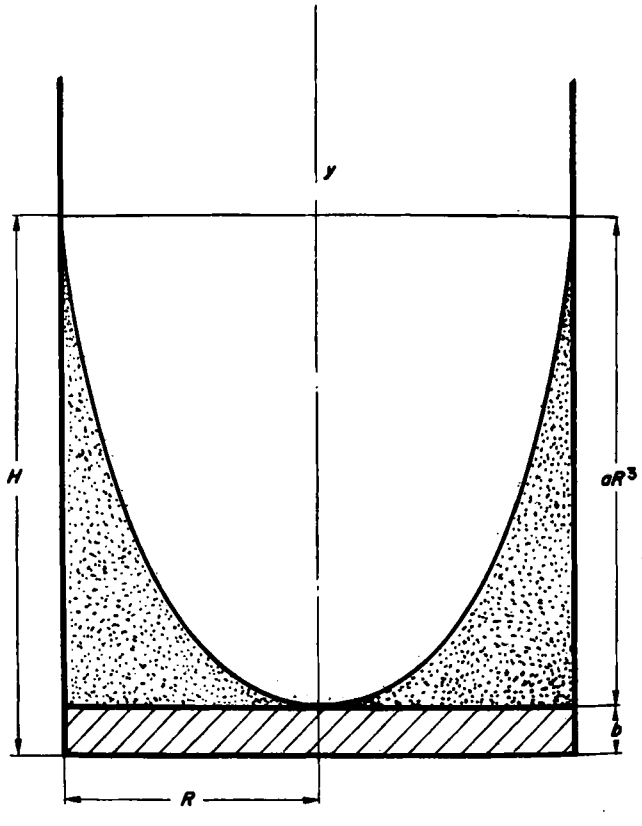

Figure 4—*Geometry of the rotating paraboloid tablet. Key:* H = *total height*, aR^2 = *paraboloid height*, b = *bottom thickness*, R = *paraboloid radius, and* y = *paraboloid axis.*

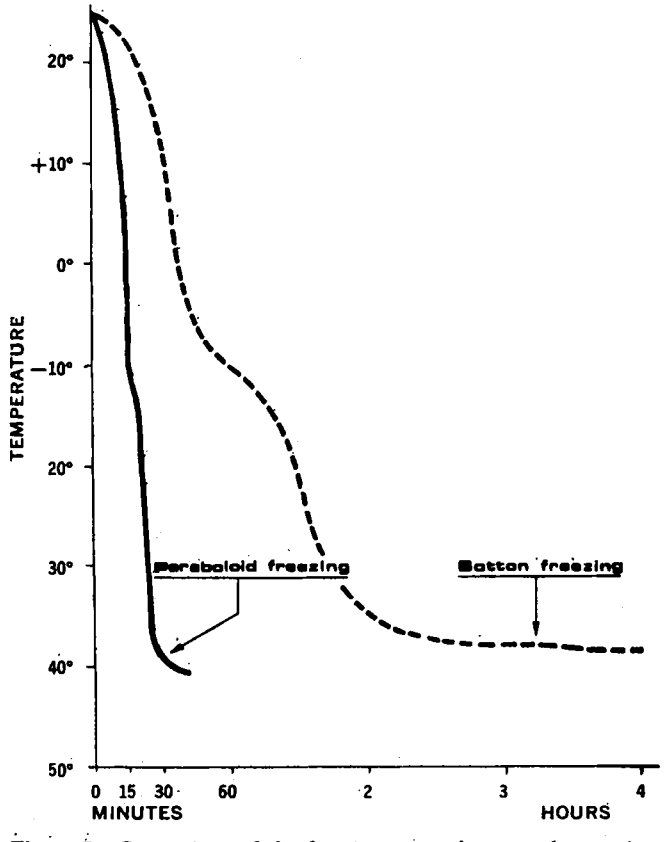

Figure 5—*Comparison of the freezing curves between the rotating paraboloid-shaped vial* (——) *and the cylindric (or bottom freezing) vial* (- - -).

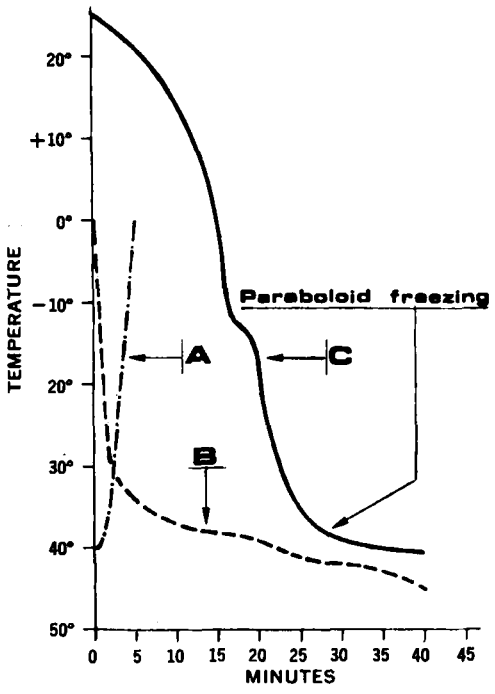

Figure 6—*Graph of the frozen curve of the rotating paraboloid-shaped vial (C). The temperature control was set at −45°. Key: A, temperature of open devia during loading process; B, temperature of closed devia during freezing process; and C, product temperature during freezing.*

sence of the rotating freezer. The minimal times shown in the table are relative to compact cycles without "dead time," while the maximum times represent the real operative time. From the data reported in Table II, it is evident that with A it is possible to reduce the time of operation to 30–50%.

CALCULATIONS

The values of ϕ, VL, and H were measured and the therms were calculated from the cubic paraboloid using the following formulas:

Calculation of V—The volume of the solution when it is at the frozen state is given by:

$$V = \frac{cVL}{0.9} + dVL \qquad \text{(Eq. 1)}$$

where:

VL = volume of solution at liquid state
V = volume of VL at frozen state
c = real percent of water in solution
d = percent of substance dissolved in solution
0.9 = ice density

Calculation of a—This constant of the cubic paraboloid has the following equation:

$$y = ar^2 \qquad \text{(Eq. 2)}$$

Table II—Comparison of Working Hours between Paraboloid (A) and Bottom Freezing (B) Methods

A		B		Remarks
0.5 hr.	6 hr.	6 hr.	15 hr.	Freezing time, 24 trays[a]
10 hr.	12 hr.	20 hr.	24 hr.	Drying time, 24 trays
10.5 hr.	18 hr.	26 hr.	39 hr.	Total hours

[a] This time course is calculated from the moment when the last tray full of dosed vials is placed into the statical freezer to the moment when the first tray with frozen vials is carried from the freezer to the drying room.

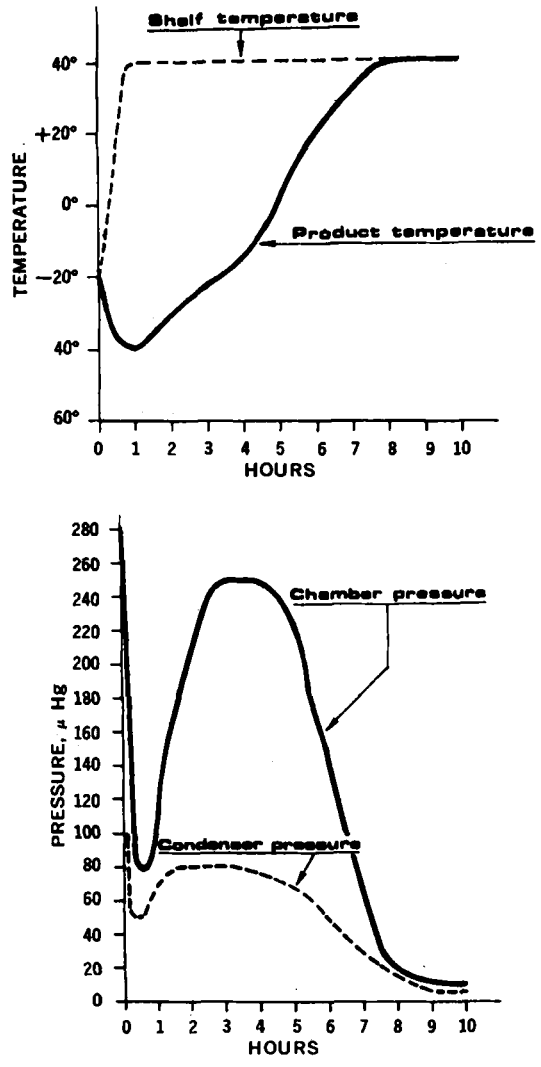

Figure 7—*Temperature–time and pressure–time curves characteristic of the drying cycle for the rotating paraboloid-shaped vials.*

after a series of control measurements. This equation reproduces satisfactorily the shape of the surface of the product frozen in an inclined vial and on a turning support.

$$V = \pi R^2 H - \frac{3}{5}\pi R^5 a \qquad \text{(Eq. 3)}$$

where:

$\pi R^2 H$ = total volume at total height H (Fig. 4)
$\frac{3}{5}\pi R^5 a$ = volume of cubic paraboloid (empty part of Fig. 4)

Equation 3 is used to find out a required to calculate b and S. Thus it becomes:

$$a = \frac{\pi R^2 H - V}{\frac{3}{5}\pi R^5} \qquad \text{(Eq. 3a)}$$

Calculation of b—The bottom thickness (Fig. 4) is given by:

$$b = H - aR^2 \qquad \text{(Eq. 4)}$$

where:

H = total height
aR^2 = paraboloid height

Calculation of S—The inside paraboloid surface is given by:

$$S = 3\pi a \left\{ \frac{R^2}{2} \cdot \left(\frac{1}{9a^2} + R^4 \right)^{1/2} + \frac{1}{18a^2} \ln \left[R^2 + \left(\frac{1}{9a^2} + R^4 \right)^{1/2} \right] - \frac{1}{18a^2} \ln \frac{1}{3a} \right\} \quad \text{(Eq. 5)}$$

Calculation of *Rs*—The ratio between paraboloid (*S*) and the upper surface of a cylindric tablet is given by:

$$Rs = \frac{S}{\pi R^2} \quad \text{(Eq. 6)}$$

Calculation of S_1—The surface of thermal exchange for a statically frozen vial is given by:

$$S_1 = Sf + Scs \quad \text{(Eq. 7)}$$

where:

Sf = base surface
Scs = cylindric lateral surface corresponding to height of statically frozen solution

Calculation of S_2—The surface of the thermal exchange of the frozen paraboloid-shaped vial is given by:

$$S_2 = Sf + Scp \quad \text{(Eq. 8)}$$

where:

Sf = base surface
Scp = cylindric lateral surface corresponding to height of paraboloidally frozen solution

CONCLUSIONS

With the above-described apparatus and technique, solutions, suspensions, and emulsions can be frozen in vials or small containers. An improvement of the freeze-dried product and a reduced cost are obtained due to the large surface and the thickness of the layer.

The increased freezing and drying rates make it possible to carry out two working cycles a day with the same lyostat. The described method is carried out with a rotating freezer, which represents only a further addition to the normally used apparatus of a freeze-drying department. Therefore, it is not necessary to make any substitution or to change the existing plants, except for the collecting vial trays with a drilled bottom. The reported calculations (Table I) are useful to obtain the necessary thickness at the vial bottom with a given height of the frozen paraboloid-shaped liquid. This height depends

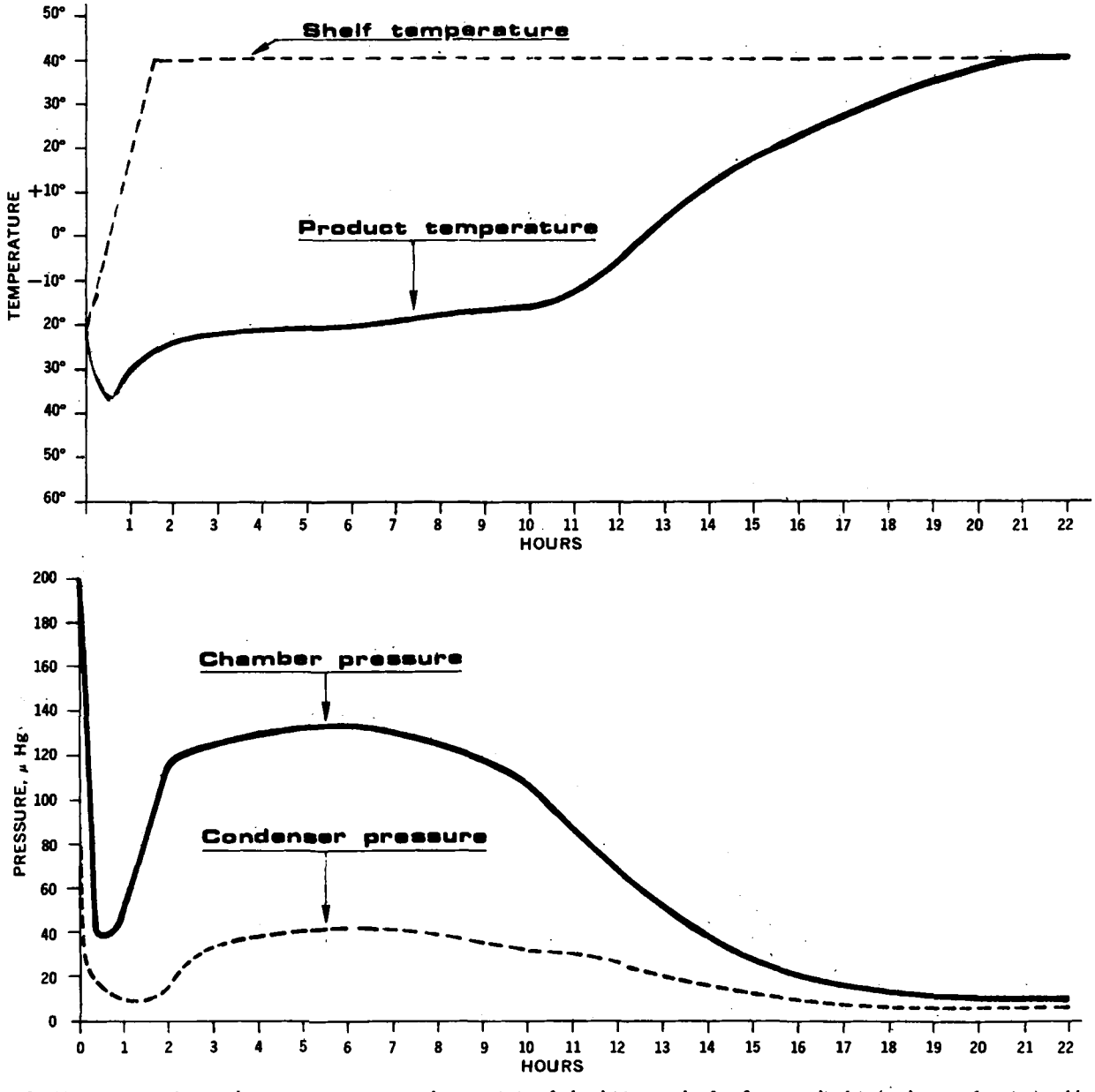

Figure 8—*Temperature–time and pressure–time curves characteristic of the drying cycle for frozen cylindric (or bottom freezing) tablets.*

on the freezer inclination and the volume of the liquid. With a different inclination of the freezer corresponds a different heat exchange surface which, therefore, can be changed according to the working conditions.

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Evaluation of Magnetic Basket Dissolution Apparatus I: Differences in Tablet Formulations

T. E. NEEDHAM▲, R. E. SHEPHERD, and L. A. LUZZI

Abstract □ The use of the magnetic basket dissolution apparatus as a means of following the dissolution of tablets was studied. Several different formulations of pentobarbituric acid tablets were manufactured and used to illustrate the ability of the magnetic basket dissolution apparatus to differentiate between the dissolution of tablets with differences in particle size of active ingredients, in formulation, and in hardness. The magnetic basket dissolution apparatus was compared with the USP XVIII and Levy beaker methods for following dissolution. Log probability plots were used as a means of providing a quick and accurate dimension for analyzing dissolution data.

Keyphrases □ Dissolution equipment—comparison of magnetic basket apparatus with compendial and Levy beaker methods for studying differences in tablet formulations □ Magnetic basket dissolution apparatus—differentiation between tablet formulations, compared with compendial and Levy beaker methods □ Tablet dissolution—magnetic basket apparatus used to study formulation differences, compared with compendial and Levy beaker methods □ Log probability plots—used to analyze dissolution data, magnetic basket apparatus compared with compendial and Levy beaker methods

In vitro dissolution profiles are of importance when they can be correlated to *in vivo* studies or when the results can be used to evaluate and examine relative differences in drug dissolution and, hence, availability for absorption between dosage forms or differences from batch to batch of the same formulation. Implied by these qualities is reproducibility of dissolution profiles for the dosage form and drug in question. An *in vitro* technique incorporating these capabilities is, of course, the ideal for which one strives.

In this quest, Levy and Hayes (1, 2) showed that the round-bottom beaker method in combination with slow agitation rates simulates, in physical appearance, the deposition of the dosage form in the stomach and mimics *in vivo* disintegration. In an earlier report (3), it was shown that the magnetic basket yielded reproducible dissolution results for both capsules and tablets. During the initial investigation of the magnetic basket, the concern was to ascertain the degree of reproducibility possible; in addition, it was found that certain characteristics of capsule disintegration and dissolution could be determined from the results. One type of tablet was examined, and it was determined that dissolution from this tablet could be reproducibly followed using the magnetic basket. No attempt at comparisons with other methods was made at that time.

This report delineates the reproducibility for dissolution of tablets, and it shows that the magnetic basket can be used to detect formulation differences in tablets. In this study, comparisons of the dissolution rate using the magnetic basket, the USP XVIII basket, and the Levy round-bottom beaker are discussed.

EXPERIMENTAL

Material—Pentobarbituric acid powder and pentobarbituric acid micronized were supplied by a commercial source[1]. The dissolution medium consisted of a buffer mixture (4) of hydrochloric acid and potassium chloride at pH 2. Fast flow lactose[2], microcrystalline cellulose[3], starch[4], and stearic acid[5] were purchased.

[1] Abbott Laboratories.
[2] Foremost Dairy, San Francisco, Calif.
[3] Avesil, FMS Corp., Newark, Del.
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Table I—Tablet Formulations

	Formula I	Formula II	Formula III	Formula IV
Pentobarbituric acid	—	—	25.0	25.0
Micronized pentobarbituric acid	25.0	25.0	—	—
Lactose	236.0	243.0	236.0	243.0
Microcrystalline cellulose	43.5	43.5	43.5	43.5
Starch	35.0	35.0	35.0	35.0
Stearic acid	10.5	3.5	10.5	3.5

Equipment—The three dissolution systems used included the USP XVIII dissolution apparatus (5), the round-bottom beaker (1, 2, 6), and the magnetic basket (3).

Tablets were compressed using a 16-station rotary tablet press[6] equipped with an induced die feeder. Standard concave punches, 0.95 cm. (0.37 in.), were used. A hardness tester[7] was employed.

Tablet Formulation—Tablets were compressed from the four formulations listed in Table I. All tablets were weighed and measured for thickness. Fifty percent of those tablets meeting specifications were examined for hardness; at least 75 tablets were examined for each hardness variation of all formulations.

Each of the four formulations was compressed at at least two hardnesses as determined by the hardness tester. For example, the range of hardness used for certain tablets was 8.6–9.0 with an average of 8.8; for softer tablets, a range was 3.1–4.0 with an average of 3.7.

Analysis of Tablets—The weight of each tablet was determined prior to dissolution, and the amount dissolved at any time t, reported as a percent of total acid, was determined at 240 nm. using appropriate blanks. Samples were taken at 2-min. intervals for the first 20 min. and then at 4-min. intervals until dissolution was complete. Reproducibility of results was determined by comparison of the variation produced at time intervals for similar tablets. For example, the Formula III tablets illustrated in Fig. 2 showed a standard error of the mean of 3.44, 5.07, 7.33, and 6.36 for the first four time intervals, respectively, with a reduction to 3.91 or less for the remaining samples. Each dissolution profile is the average of at least five tablets.

RESULTS AND DISCUSSION

Previous work (3) indicated that the magnetic basket could reproducibly follow the dissolution of both capsules and tablets. The additional versatility of this method can be seen if different formulations of the same drug are considered. In Fig. 1 the dissolution of tablets composed of Formulas III and IV are compared. By use of the magnetic basket method, differences between formulations that

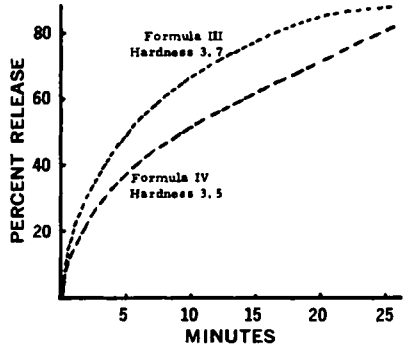

Figure 1—*Comparison of dissolution rates of active ingredients from tablets with different formulations and the same hardness, using the magnetic basket dissolution apparatus.*

[6] Model 216-RP Cherry-Burrell.
[7] Erweka Electronic.

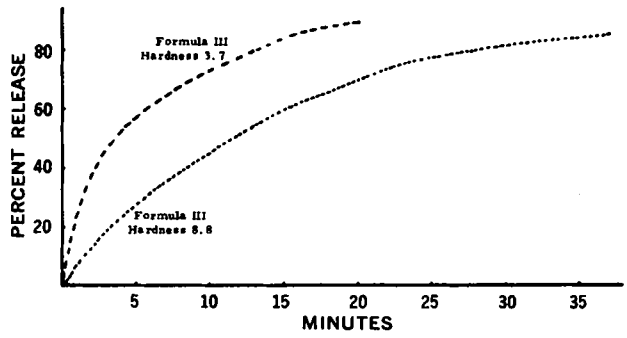

Figure 2—*Comparison of dissolution rate of active ingredients from tablets of the same formulation and different hardness, using the magnetic basket dissolution apparatus.*

are of the same average hardness and weight but differ in stearic acid content may be detected.

Figure 2 illustrates the difference found in dissolution between tablets using Formula III with different average hardnesses of 8.8 and 3.7. A distinct difference in the dissolution profiles of the two different hardnesses is seen, with the softer tablet as expected showing a much faster dissolution rate. The parallel dissolution behavior between the two tablets can be clearly seen using a log probability plot of time *versus* percent dissolved (7). Although the log probability relationship is most frequently used to describe the size distribution of particulate matter, it can be used in this case as a means of illustrating dissolution behavior. In the former case, both straight and curved lines may result. A straight line indicates that the sample examined is composed of a normal distribution, *i.e.*, all particles belong to the same population. A curved line indicates that the sample contains particles from more than one population. If the case of tablet dissolution is considered and the straight line is indicative of dissolution from a relatively unchanging set of parameters, then the correlation of dissolution behavior can be seen.

Figure 3 shows that two straight lines represent the dissolution profile of each tablet. It would seem that the first straight line is indicative of the initial dissolution–disintegration of the tablet when the tablet matrix is still present. That is, the surface area is relatively unchanged since the diminishing surface area of the tablet is offset by the increase in surface area due to disintegration. The second straight line should represent the dissolution phase when the tablet matrix is no longer present and dissolution is taking place from an infinite number of particles residing predominantly at the beaker bottom. Visual observation correlates with the appearance of the second linear segment in the log probability plots. Furthermore, in Wagner's (7) derivation of the rationale for use of log probability

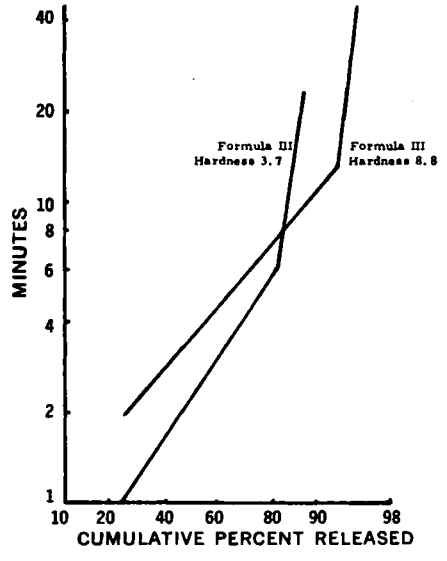

Figure 3—*Cumulative percent released* versus *time as an illustration of the treatment of data for tablets of the same formulation but of different hardness.*

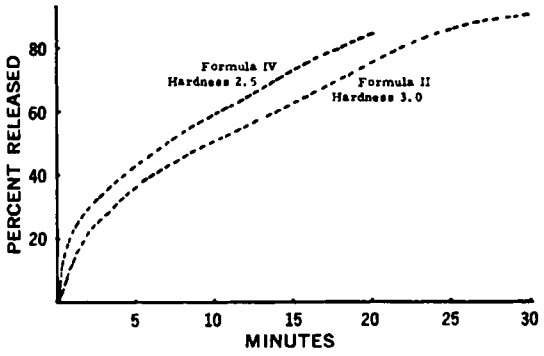

Figure 4—*Comparison of dissolution rates of active ingredient from tablets as a function of particle size of active ingredient, using the magnetic basket dissolution apparatus.*

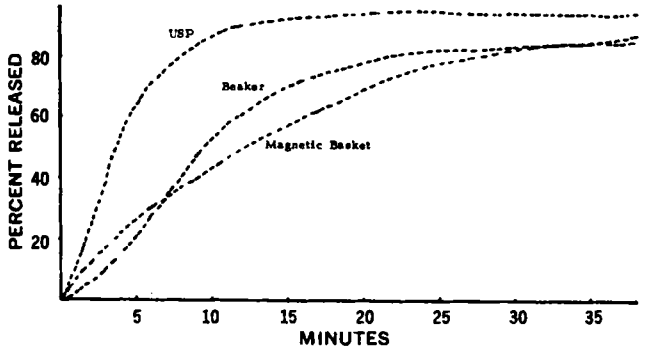

Figure 5—*Comparison of dissolution of active ingredient from tablets of the same formulation using the USP, beaker, and magnetic basket dissolution apparatus. All tablets were from Formula III with a hardness of 8.8.*

plots, his first assumption is that under sink conditions and changing surface area, one may assume that the surface area available for dissolution decreases exponentially with time. This would agree with the second segment of the straight line taking place after the tablet matrix has disintegrated.

One would expect deviation from the straight line concept in two places. The first deviation would be at the apex where the two straight lines intersect, since there is a transitional period when the dissolution–disintegration process and its concomitant "large" particles (small surface area) are changing to a pure dissolution process involving many smaller particles with a large surface area. The second deviation would be at the end of the second straight line when the material is approaching 100% dissolution. Here one is on the plateau of the regular dissolution curve, and the amount of drug dissolving per unit time is somewhat less than that rate witnessed during the initial portions of the second straight line. Therefore, the cumulative percent dissolved would remain virtually unchanged as time increased, giving rise to almost a vertical line. This deviation is small in this case and, although not shown in Fig. 3, may be attributed to a discontinuance of exponential exposure of the particle surface due to an increased shielding of the drug particle by insoluble tablet ingredients. The difference in hardness between the two tablets is readily seen by comparison of the intercepts of the two straight lines. For the tablets with an average hardness of 3.7, this intercept occurs at 6 min. The harder tablets, with an average hardness of 8.8, show this intercept at 13 min., which is what would be expected of a higher compression tablet.

To illustrate the ability of the magnetic basket to differentiate between tablets with different particle size of the active ingredient, Formulations II and IV were used. Formulation II contained micronized pentobarbituric acid, with a mean surface diameter of 4.42 μ; in Formulation IV, the pentobarbituric acid had a mean surface diameter of 9.96 μ. Figure 4 shows the dissolution profiles for these two formulations using tablets of comparable hardness. While the magnetic basket shows a difference between the dissolution profile as a function of particle size, the micronization of pentobarbituric acid does not give faster dissolution. This behavior was also shown to be true when comparing Formulations I and III.

Figure 5 shows a comparison of the official USP XVIII dissolution test, the Levy beaker method, and the magnetic basket method using tablets from Formulation III with an average hardness of 8.8. All three methods show similar dissolution profiles, with the USP method reaching its peak dissolution somewhat faster than the other two methods. A factor that would contribute to faster dissolution rates using the USP XVIII apparatus is the obvious screening, through centrifugal force, imposed on the dosage form placed in the spinning basket. This spinning and resulting rapid dissolution also tends to mask or level relatively small differences in tablet dissolution rates. The Levy beaker and the magnetic basket methods showed similarity in all dissolution comparisons of the various formulations.

While in a previous preliminary study there were indications that reproducibility and detection of formulation differences in both capsules and tablets were possible with the magnetic basket, it may be concluded from this study that the magnetic basket method is versatile in its ability to differentiate between the common parameters of hardness, particle size, and formulation differences found in these tablet studies. Furthermore, it will apparently provide a means of differentiation for both capsules and tablets, presenting the results in a directly comparable manner. Its use in combination with log probability plots provides a quick and accurate means of analyzing dissolution data.

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Adsorption–Dissolution Relationship in Sulfamethazine–Benzoic Acid System

R. N. NASIPURI and SALEH A. H. KHALIL▲

Abstract □ Both the equilibrium solubility and dissolution rate of sulfamethazine were remarkably inhibited in the presence of benzoic acid. The effect was dependent on the concentrations of both the benzoic acid and sulfamethazine in the system. The inhibition of sulfamethazine dissolution was synchronous with the adsorption of benzoic acid on the sulfonamide particles. As low as 5 mg.% polyvinylpyrrolidone prevented the adsorption of benzoic acid on sulfamethazine and, consequently, the suppressive effect of benzoic acid on sulfamethazine dissolution was no longer shown.

Keyphrases □ Sulfamethazine–benzoic acid—adsorption–dissolution relationship, effect of polyvinylpyrrolidone □ Benzoic acid–sulfamethazine—adsorption–dissolution relationship, effect of polyvinylpyrrolidone □ Polyvinylpyrrolidone—effect on adsorption–dissolution relationship in sulfamethazine–benzoic acid system □ Dissolution—sulfamethazine in benzoic acid, effect of polyvinylpyrrolidone

During a study on the adsorption of benzoic acid on sulfamethazine particles, it was observed that in certain systems the equilibrium solubility of the sulfonamide was significantly decreased, sometimes reaching only about 6% of its solubility in water. The present article examines the effect of benzoic acid on the equilibrium solubility and dissolution rate of sulfamethazine and compares the dissolution results with the data of benzoic acid adsorption on the sulfonamide particles. The effect of polyvinylpyrrolidone on both adsorption and dissolution in the system is discussed.

EXPERIMENTAL

Materials—Sulfamethazine, BP grade[1], was used; the powder had a mean volume-surface diameter of 32.6 μ. Benzoic acid was of BP quality[2], and polyvinylpyrrolidone was a sample having an average molecular weight of 40,000[3]. Methylcellulose 20 and sodium carboxymethylcellulose of 50 and 100 viscosity grades[3] were used.

Methods—*Adsorption Isotherm*—The procedure described by Bean and Dempsey (1) was followed at 24 ± 0.2°.

Determination of Dissolution Rate—This was determined at 24 ± 0.2° in an oscillating water bath at a speed of 18 ± 2 strokes/min. After the specified time, the contents of the flask were filtered through a sintered-glass funnel[4]. The concentrations of both benzoic acid and sulfamethazine were determined in the filtrate spectrophotometrically by applying the equations of Tinker and McBay (2) for two-component systems. Measurements were made using a spectrophotometer[5] at 230 and 300 nm. (in 0.05 *N* HCl). The validity of the equations used were tested by assaying known mixtures of sulfamethazine and benzoic acid. The percentage errors were ±1.9% for sulfamethazine and ±2.3% for benzoic acid.

Elution Procedure—The suspension left after the adsorption experiment was filtered through a sintered-glass funnel[4], and the precipitate was digested in water (double the volume used in the adsorption experiment). The procedure described under *Determination of Dissolution Rate* was then followed.

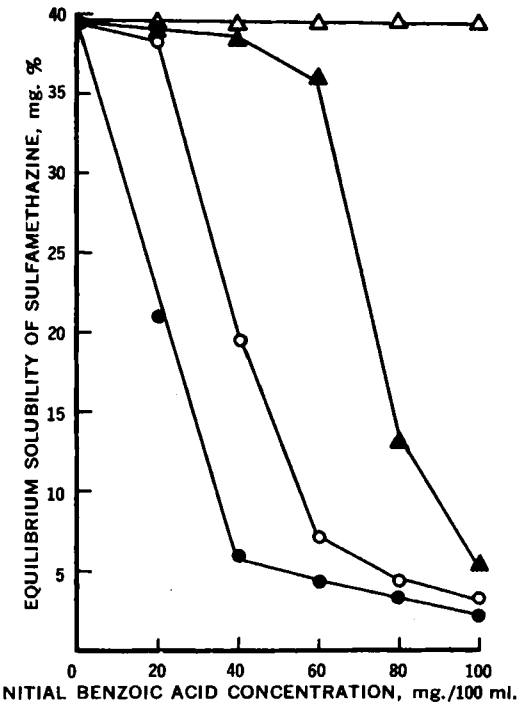

Figure 1—*Effect of benzoic acid on the equilibrium solubility of sulfamethazine. The concentrations of sulfamethazine in the system (g./100 ml.) were: (●) 0.06; (○) 0.1; (▲) 0.2; and (△) 0.3, 0.4, 4, and 10.*

RESULTS AND DISCUSSION

Figures 1 and 2 show, at different initial benzoic acid concentrations, the effect of benzoic acid on the equilibrium solubility of sulfamethazine and its adsorption on the sulfonamide particles. Within the concentration range of benzoic acid used, the equilibrium solubility of sulfamethazine showed a pronounced decrease; the effect was dependent on the concentrations of both benzoic acid and sulfamethazine in the system (Fig. 1). At and above 0.3 g./100 ml., sulfamethazine suffered no significant change in its equilibrium solubility; below 0.3 g./100 ml., a remarkable decrease occurred, reaching about 2.5 mg.% compared with 39.6 mg.% in water.

The decrease in sulfamethazine solubility in the presence of benzoic acid can be attributed to the adsorption of the acid on the sulfonamide particles. The data of benzoic acid adsorption are plotted in Fig. 2. The relationship between the amount of benzoic acid adsorbed per millimole sulfamethazine and the equilibrium

[1] Sulfadimidine, Imperial Chemical Industries, England.
[2] British Drug Houses, England.
[3] Plasdone k 29-32, GAF Corp., New York, N. Y.
[4] Jena 39 G3.
[5] Unicam SP 500.

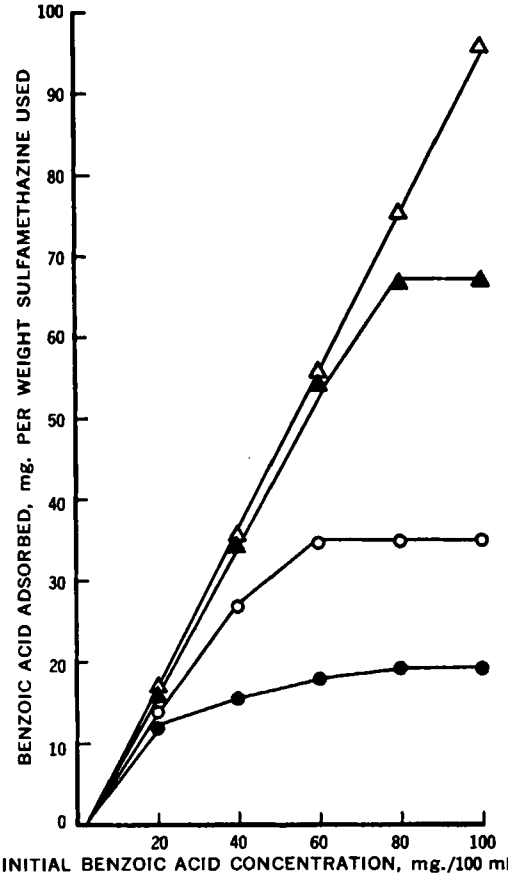

Figure 2—*Adsorption of benzoic acid on sulfamethazine. The concentrations of sulfamethazine in the system (g./100 ml.) were:* (●) *0.06;* (○) *0.1;* (▲) *0.2; and* (△) *0.3, 0.4, 4, and 10.*

solubility of the sulfonamide is shown in Table I. At a constant initial concentration of benzoic acid, the amounts of benzoic acid adsorbed per millimole sulfamethazine were 0.75, 0.77, and 0.76 mmole for systems containing 2.16, 3.60, and 7.20 mmoles sulfamethazine, respectively. These data may suggest that the sulfonamide particles were "saturated" by benzoic acid, as shown by the plateau regions in Fig. 2. As a result, the equilibrium solubility of sulfamethazine showed a pronounced decrease (Table I). At higher sulfamethazine concentrations, the amount of benzoic acid adsorbed showed a gradual decrease and the sulfonamide solubility exhibited an insignificant change (Table I).

The relationship between adsorption of benzoic acid and its effect on sulfamethazine dissolution, both during an adsorption run and an elution experiment, is shown in Fig. 3. After 30 min. of equilibration, the amount of benzoic acid adsorbed per 0.2 g. sulfamethazine was 66.6 mg. In this system the equilibrium solubility of

Table I—Data of Benzoic Acid Adsorption on Sulfamethazine and Its Effect on the Equilibrium Solubility of the Sulfonamide[a]

Sulfamethazine Concentration, mmoles/l.	Benzoic Acid Adsorbed, mmole/mmole Sulfamethazine	Equilibrium Solubility of Sulfamethazine, mmoles/l.[b]
2.16	0.75	0.076
3.60	0.77	0.083
7.20	0.76	0.173
10.80	0.72	1.400
14.40	0.54	1.442
36.00	0.22	1.417
72.00	0.11	1.457
144.00	0.054	1.424
360.00	0.022	1.421

[a] Initial concentration of benzoic acid = 8.18 moles/l. [b] Equilibrium solubility of sulfamethazine in water (pH 5.6) at 24° = 1.424 mmoles/l.

Table II—Effect of Polyvinylpyrrolidone on the Adsorption–Dissolution Relationship in the Sulfamethazine–Benzoic Acid System[a]

Initial Benzoic Acid Concentration, mg. %	Concentration of Polyvinylpyrrolidone, mg. %			
	Benzoic Acid —Adsorbed, mg.—		Sulfamethazine —Solubility, mg. %—	
	0	5	0	5
0	—	—	39.2	40.1
20	15.2	0.0	38.8	39.9
40	34.6	0.0	38.2	40.4
60	54.6	0.1	36.1	40.2
80	65.8	0.1	13.0	39.8
100	66.1	0.2	4.8	39.9

[a] Sulfamethazine concentration = 0.2 g.%.

sulfamethazine dropped from 39.2 to 4.8 mg.%. In the elution experiment, partial desorption of benzoic acid occurred since only about 10% of the amount originally adsorbed was eluted (Fig. 3B). Nevertheless, this seemed to have produced enough unoccupied sites on the sulfonamide particles as the result of partial desorption of benzoic acid, which could be claimed to be responsible for the increase in sulfamethazine dissolution during the elution experiment (Fig. 3A).

The role of polyvinylpyrrolidone in suppressing the adsorption of benzoic acid by sulfamethazine particles is shown in Table II. The incorporation of 5 mg.% of the polymer in the systems studied resulted in almost complete inhibition of benzoic acid adsorption. This was also reflected in the solubility of sulfamethazine, where no significant change was observed (Table II). It is suggested that polyvinylpyrrolidone, being a protective colloid, would be concentrated at the surface of sulfamethazine particles, thus "insulating" or "shielding" the surface. The work of Simonelli *et al.* (3) showed that polyvinylpyrrolidone inhibited the crystal growth of sulfathiazole due to the transport of the polymer molecules from the bulk to the crystal surfaces. Moriguchi and Kaneniwa (4) showed also that polyvinylpyrrolidone was preferentially adsorbed on talc, thus competing for the adsorption of cyanocobalamin on talc. The inhibition of sulfamethazine dissolution by benzoic acid reported in the present

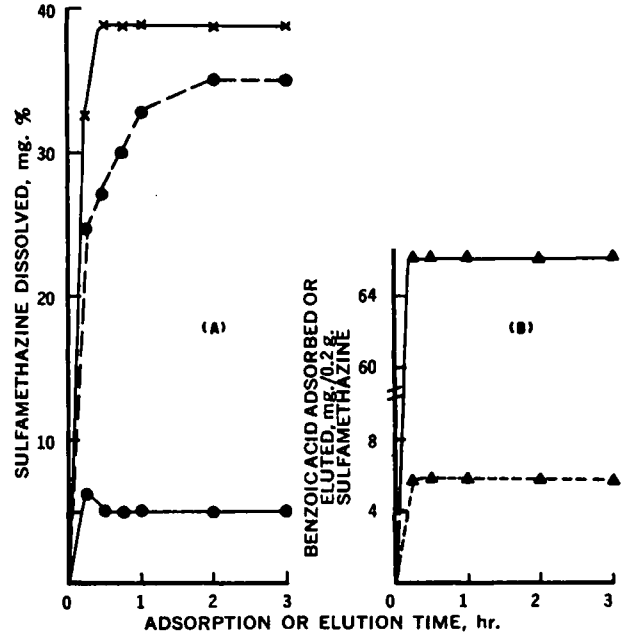

Figure 3—*(A) Effect of benzoic acid on the dissolution rate of sulfamethazine* (●—●) *during an adsorption experiment and* (●- - -●) *during an elution experiment;* (X—X) *is sulfamethazine dissolution in water. (B) Adsorption of benzoic acid on sulfamethazine and its elution. Key:* ▲—▲, *adsorption data; and* ▲- - -▲, *elution data. Sulfamethazine concentration = 0.2 g./100 ml.; initial benzoic acid concentration = 100 mg./100 ml.*

work is analogous to the work of Piccolo and Tawashi (5), who found that at low concentrations of some dyes, inhibition occurred to single crystal dissolution of a number of drugs.

SUMMARY AND CONCLUSIONS

In the presence of benzoic acid, both the dissolution rate and equilibrium solubility of sulfamethazine were suppressed. The phenomenon was attributed to the adsorption of benzoic acid on the sulfonamide particles. The inhibition of sulfamethazine dissolution was only shown when the sulfonamide particles were saturated by benzoic acid. Due to the protective effect of polyvinylpyrrolidone, as low as 5 mg.% of the polymer prevented the adsorption of benzoic acid and, consequently, its suppressive effect on sulfamethazine dissolution.

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K. M. BAKER, A. FRIGERIO▲, P. L. MORSELLI, and G. PIFFERI*

Abstract □ Carbamazepine-10,11-epoxide, a metabolite of carbamazepine found in human urine, undergoes a rearrangement and degradation to 9-acridinecarboxaldehyde during GLC.

Keyphrases □ Carbamazepine-10,11-epoxide—identification of GLC degradation product as 9-acridinecarboxaldehyde □ 9-Acridinecarboxaldehyde—identified as carbamazepine-10,11-epoxide GLC rearrangement product, synthesis, physical-chemical properties

In previous papers (1, 2), it was reported that carbamazepine-10,11-epoxide was found in human urine as a metabolite of carbamazepine. The epoxide was isolated and identified; however, at the same time it was mentioned that subjection of this compound to GLC resulted in a degradation to another material. This paper reports the isolation, identification, and alternative preparation of the degradation product.

EXPERIMENTAL[1]

Carbamazepine-10,11-epoxide—The synthesis described previously (1) was altered to give better, consistent yields for pharmaceutical requirements.

A solution of carbamazepine (50 g., 0.21 mole) and *m*-chloroperbenzoic acid (47.4 g., 0.27 mole) in 2 l. of ethylene chloride was heated under reflux with irradiation by a sun lamp (200 w.) for 3 hr. After cooling and washing with dilute aqueous sodium sulfite solution and with 5% aqueous sodium bicarbonate, the organic phase was dried and evaporated. The residue was taken up in boiling benzene and cooled, and the precipitate collected. The crude product was recrystallized from ethanol to give carbamazepine-10,11-epoxide as pale-yellow needles (16 g., 30%), m.p. 205–207° dec. [lit. (1) m.p. 190–195°]; IR (mineral oil mull): 3500–3100 (NH_2), 1675 (C=O, urea), 865 (C—O, epoxide), 752 (CH out-of-plane bending) $cm.^{-1}$; NMR (acetone–dimethyl sulfoxide, 2:1): 4.38 (s, 2H, —HCO— epoxide), 5.34 (broad s, 2H, $CONH_2$), and 7.7–7.1 (m, 8H, ArH).

9-Methylacridine—Diphenylamine, acetic anhydride, and anhydrous zinc chloride were heated together at 190° for 5 hr. according to Porai-Koshits and Khaskhazov (3). Separation and crystallization from hexane gave pure 9-methylacridine, m.p. 116–118° [lit. (4) m.p. 117–118°]; IR (mineral oil mull): 1150 (CH in-plane bending) and 755 (CH out-of-plane bending) $cm.^{-1}$.

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RESULTS AND DISCUSSION

A clean, single-component gas chromatogram resulted from an ethanol solution of carbamazepine-10,11-epoxide. The mass spectrum (Fig. 1) obtained by hookup to the GLC showed a molecular ion at *m/e* 207 (100%) and a fragmentation to give *m/e* 179 (90%) corresponding to a loss of 28 atomic mass units (CO). This transition was confirmed by the presence of a metastable ion. Also present were ions at *m/e* 152 (15%) and 151 (17%) corresponding

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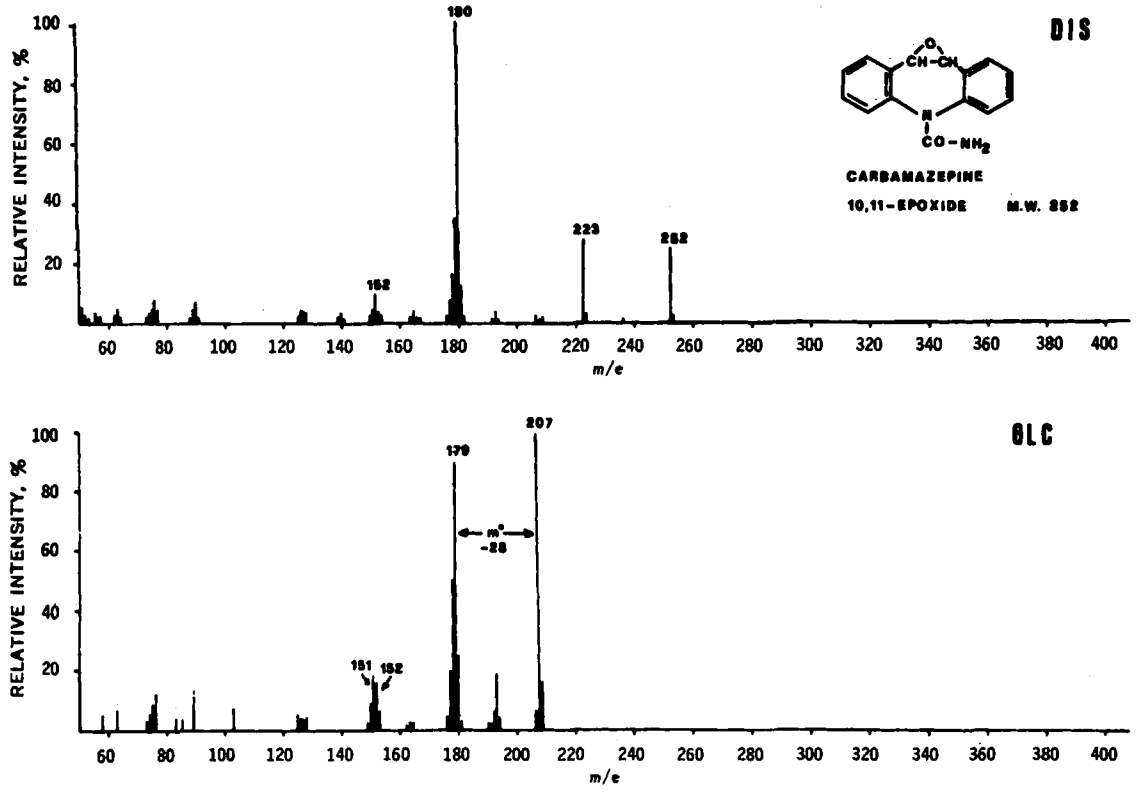


Figure 1—*Mass spectra of carbamazepine-10,11-epoxide. Key: upper panel, direct in the system (DIS); and lower panel, GLC.*

to dibenzocyclobutane (loss of HCN from *m/e* 179, the acridinium ion).

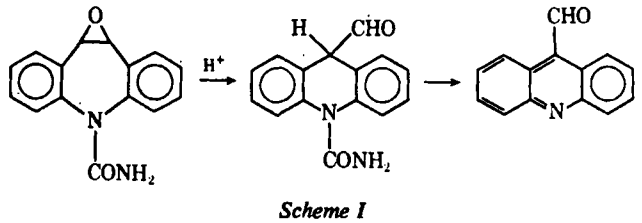
9-Acridinecarboxaldehyde was proposed for this material. For confirmation, the compound was isolated from the GLC column *via* a splitter. Yellow needles, m.p. 138–139°, were obtained [lit. (6) m.p. 147°].

The authentic material prepared was yellow needles, m.p. 145–146° (see *Experimental*). Comparison with the rearranged material proved that they were identical. Both showed the same retention time on GLC, the same UV maximum (264 nm. in methanol), and identical mass spectra.

A mechanism rationalizing the rearrangement is shown in Scheme I. The process is probably catalyzed by the slightly acidic column at high temperature, and then the intermediate imide decomposes to give the much more stable, completely aromatic system, 9-acridinecarboxaldehyde.

It is possible that the rearrangement could take place in a biological system and, therefore, 9-acridinecarboxaldehyde would be found as a metabolite of carbamazepine. Steps are in progress at the moment to determine if this is indeed so.

The result indicates the care that must be taken in identifying drug metabolites. In many cases the quantities available for identification may be very small and GLC–mass spectrometry must be used, but alternative methods should be employed in conjunction wherever possible to check that no rearrangements or degradations take place during one of the physical measurements.



Scheme I

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Chemical and Biological Studies on 1,2-Dihydro-*s*-triazines XVIII: Synthesis of 1-(5,6- and 5,7-Dichloro-2-naphthyl) Derivatives and Related Compounds as Candidate Antimalarial and Antitumor Agents

ANDRE ROSOWSKY▲, KATHERINE K. N. CHEN, RICHARD St. AMAND, and EDWARD J. MODEST

Abstract □ 4,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazines with 5,6- and 5,7-dichloro-2-naphthyl groups at position 1 were synthesized and evaluated for biological activity. The 5,6-dichloro compound caused moderate inhibition of *Streptococcus faecium* and of human epidermal carcinoma (KB cells) *in vitro*, as well as a moderate increase in mean survival of mice bearing P1534 leukemia in the ascitic form. The 5,7-dichloro analog was active against *Plasmodium gallinaceum* in chicks.

Keyphrases □ 4,6-Diamino-1,2-dihydro-*s*-triazines, dichloronaphthyl substituted—synthesis, screened as potential antimalarial and antitumor agents □ 1,2-Dihydro-*s*-triazines, 5,6- and 5,7-dichloro-2-naphthyl derivatives—synthesis, screened as potential antimalarial and antitumor agents □ Antimalarial agents, potential—synthesis and screening of 4,6-diamino-1,2-dihydro-*s*-triazines with dichloronaphthyl substitution □ Antitumor agents, potential—synthesis and screening of 4,6-diamino-1,2-dihydro-*s*-triazines with dichloronaphthyl substitution

The availability in this laboratory of several heteronuclear dichloro derivatives of 2-naphthylamine (1) in connection with another investigation (2) afforded an unusual opportunity for the synthesis and biological evaluation of some hitherto inaccessible 4,6-diamino-1,2-dihydro-*s*-triazine antifolates with a halogenated 2-naphthyl moiety at position 1. These compounds, as well as the previously reported 2-naphthyl (3, 4) and 6-chloro-2-naphthyl (5) derivatives, were viewed with interest because of the high level of antifolate activity displayed in various test systems by other aryldihydrotriazines, including especially the phenyl and 1-naphthyl types (6).

The present paper describes the synthesis of 4,6-diamino-1-(5,6-dichloro-2-naphthyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride (*Ia*·HCl) and its 5,7-dichloro-2-naphthyl analog (*Ib*·HCl). Also described are the corresponding naphthylbiguanide salts (*IIa*·HCl and *IIb*·HCl) and guanidinoquinazoline salts (*IIIa*·HCl and *IIIb*·HCl). Biguanides are known intermediates in the three-component synthesis of dihydrotriazines (7), and guanidinoquinazolines were reported recently to be occasional by-products in this reaction (4, 5).

EXPERIMENTAL[1]

4,6-Diamino-1-(5,6-dichloro-2-naphthyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine Hydrochloride (*Ia*·HCl) (Standard Three-Component Synthesis)—A mixture of 10.7 g. (0.043 mole) of 5,6-dichloro-2-naphthylamine hydrochloride (1), 4.51 g. (0.055 mole) of cyanoguanidine, 15 ml. of acetone, and 20 ml. of absolute ethanol was stirred under reflux for 3 hr., allowed to stand at room temperature for 18 hr., and filtered. The solid was washed with acetone (3 × 10 ml.) and dried, yielding 9.57 g. (60%). Two recrystallizations from water with the aid of decolorizing carbon gave analytically pure colorless needles, m.p. 233–235°.

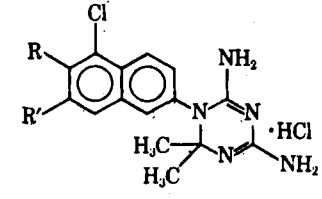
Anal.—Calc. for $C_{15}H_{15}Cl_2N_5 \cdot HCl$: C, 48.34; H, 4.33; Cl, 28.54; N, 18.79. Found: C, 48.46; H, 4.11; Cl, 28.49; N, 18.79.

4,6-Diamino-1-(5,7-dichloro-2-naphthyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine Hydrochloride (*Ib*·HCl) (Two-Component Synthesis)—A mixture of 11.3 g. (0.03 mole) of biguanide *IIb*·HCl, 0.25 ml. of 12 *N* HCl, 50 ml. of acetone, and 50 ml. of absolute ethanol was stirred under reflux for 17 hr., cooled, and filtered. The tan solid was washed with acetone and dried, yielding 9.3 g. (73%). The biguanide test with copper ammonium sulfate (7) was negative (no precipitate). Two recrystallizations from 95% ethanol (charcoal) gave analytically pure *Ib*·HCl as off-white needles, m.p. 221.5–224°.

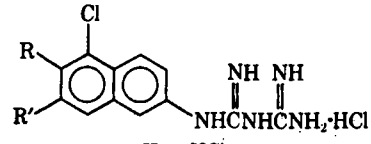
Anal.—Calc. for $C_{15}H_{15}Cl_2N_5 \cdot HCl$: C, 48.34; H, 4.33; Cl, 28.54; N, 18.79. Found: C, 48.43; H, 4.44; Cl, 28.45; N, 18.67.

N^1-(5,6-Dichloro-2-naphthyl)biguanide Hydrochloride (*IIa*·HCl)—A mixture of 10.7 g. (0.0431 mole) of 5,6-dichloro-2-naphthylamine hydrochloride (1), 4.10 g. (0.05 mole) of cyanoguanidine, and 70 ml. of *n*-propanol was stirred under reflux for 3 hr., cooled, and filtered. The tan solid was washed with *n*-propanol (2 × 25 ml.) and dried, yielding 8.18 g. (57%). Three recrystallizations from 95% ethanol (charcoal) afforded colorless needles, m.p. 215.5–216°, with a positive biguanide test (pink solid) (7).

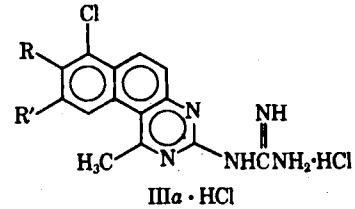
Anal.—Calc. for $C_{12}H_{11}Cl_2N_5 \cdot HCl$: C, 43.33; H, 3.64; Cl, 31.98; N, 21.05. Found: C, 43.23; H, 3.59; Cl, 31.93; N, 20.99.



Ia · HCl
Ib · HCl



IIa · HCl
IIb · HCl



IIIa · HCl
IIIb · HCl

a series: R = Cl, R′ = H
b series: R = H, R′ = Cl

[1] Melting points were taken in Pyrex capillary tubes, using a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, Mass.), and are uncorrected. IR spectra were measured in KCl disks with a Perkin-Elmer model 137B recording spectrophotometer. NMR spectra were determined with a Varian A-60 instrument, with trifluoroacetic acid as the solvent and tetramethylsilane as the internal reference.

N^1-(5,7-Dichloro-2-naphthyl)biguanide Hydrochloride (IIb·HCl)—Use of the foregoing procedure with 5,7-dichloro-2-naphthylamine hydrochloride gave a 54% yield of IIb·HCl as an off-white solid. Two recrystallizations from 95% ethanol furnished the analytical sample, m.p. 255–257°.

Anal.—Calc. for $C_{12}H_{11}Cl_2N_5 \cdot HCl$: C, 43.33; H, 3.64; Cl, 31.98; N, 21.05. Found: C, 43.08; H, 3.41; Cl, 32.17; N, 21.04.

7,8-Dichloro-3-guanidino-1-methylbenzo[*f*]quinazoline Hydrochloride (IIIa·HCl)—A solution of 8.88 g. (0.0418 mole) of 5,6-dichloro-2-naphthylamine (1) and 0.5 g. of iodine in 33 ml. of acetone was stirred under reflux for 60 hr., cooled, and evaporated to dryness under reduced pressure. The resulting dark oil was taken up in 50 ml. of 50% ethanol to which were added 6.25 ml. of 12 *N* HCl and 4.75 g. (0.05 mole) of cyanoguanidine. After being stirred under reflux for 1.5 hr., the mixture was refrigerated until crystallization occurred. The solid was filtered, washed with acetone (5 × 5 ml.), and dried, yielding 7.53 g. (51%). Two recrystallizations from 95% ethanol (charcoal) gave colorless crystals, m.p. 312–313° dec. On treatment with copper ammonium sulfate reagent (7), this material gave a light-green precipitate characteristic of guanidinoquinazolines (3).

Anal.—Calc. for $C_{14}H_{11}Cl_2N_5 \cdot HCl$: C, 47.14; H, 3.39; Cl, 29.82; N, 19.64. Found: C, 47.05; H, 3.35; Cl, 29.98; N, 19.61.

7,9-Dichloro-3-guanidino-1-methylbenzo[*f*]quinazoline Hydrochloride (IIIb·HCl) (Modified Three-Component Synthesis)—A mixture of 5.00 g. (0.0235 mole) of 5,7-dichloro-2-naphthylamine (1), 1.97 g. (0.0235 mole) of cyanoguanidine, 0.2 ml. (0.0024 mole) of 12 *N* HCl, 85 ml. of acetone, and 15 ml. of 95% ethanol was stirred at room temperature for 16 hr. and then under reflux for 4 hr. Another 1.75 ml. (0.0211 mole) of 12 *N* HCl was added, refluxing was continued for 70 min., and the precipitated solid was filtered, washed with acetone, and dried, yielding (two crops collected) 4.8 g. (57%); a light-green solid resulted upon treatment with biguanide reagent (3). Two recrystallizations from absolute ethanol (charcoal) gave analytically pure colorless needles, m.p. 337–341° dec.

Anal.—Calc. for $C_{14}H_{11}Cl_2N_5 \cdot HCl$: C, 47.14; H, 3.39; Cl, 29.82; N, 19.63. Found: C, 47.19; H, 3.29; Cl, 29.69; N, 19.56.

RESULTS AND DISCUSSION

5,6-Dichloro-2-naphthylamine hydrochloride was found to give only dihydrotriazine Ia·HCl under the conditions of the standard three-component synthesis (7), with no evidence of the formation of guanidinoquinazoline IIIa·HCl, a comparison sample of which was synthesized *via* the "acetone anil" route described earlier (3). Although IIIa·HCl might have been expected as a by-product on the basis of work with 2-naphthylamine (3), careful NMR spectral analysis of the crude solid filtered from the reaction mixture failed to reveal any of this compound. On the other hand, the standard three-component synthesis with 5,7-dichloro-2-naphthylamine hydrochloride did produce a small amount of guanidinoquinazoline IIIb·HCl, which made it preferable to obtain dihydrotriazine Ib·HCl *via* the two-component synthesis (8) starting from biguanide IIb·HCl. An authentic specimen of IIIb·HCl was synthesized conveniently *via* the recently described (4) modified three-component synthesis, wherein only a limited amount of acid is used at the outset of the reaction. 6-Chloro-2-naphthylamine has been reported (5) to give some guanidinoquinazoline by-product in the standard three-component synthesis, albeit in a much smaller amount than with 2-naphthylamine. Thus, the findings reported here reinforce the previous conclusion that electron-attracting chloro substitution on the aromatic ring diminishes the tendency for guanidinoquinazoline by-product formation (5). However, it appears that 5,7-dichloro substitution is somewhat less effective than 5,6-dichloro substitution in suppressing this side reaction.

Moderate activity was shown against *Streptococcus faecium* (ATCC 8043) (9) by dihydrotriazine Ia·HCl, which gave an ID_{50} value of 0.08 mcg./ml. at a folate level of 0.001 mcg./ml. This may be compared with the ID_{50} value of 0.035 mcg./ml. reported previously by Foley (10) for 4,6-diamino-1-(3,4-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride, which is closely analogous to Ia·HCl in structure.

Activity was also demonstrated for Ia·HCl against human epidermoid carcinoma (KB cells) in culture (11), with an ID_{50} value of 0.5 mcg./ml. observed. However, both the biguanide IIa·HCl (ID_{50} = 0.2 mcg./ml.) and the guanidinoquinazoline IIIa·HCl (ID_{50} = 0.3 mcg./ml.) were at least as active as the dihydrotriazine. Since IIIa·HCl led to only slight inhibition of *S. faecium* (ID_{50} > 10 mcg./ml.), the cytotoxic action of this compound may perhaps be ascribed to some cause other than folate antagonism such as metal chelation. The ability of guanidinoquinazolines to inhibit KB cell growth was noted previously (3, 5), especially with reference to the possibility of error in the bioassay of dihydrotriazines not completely free from guanidinoquinazoline contamination.

Compound Ia·HCl was tested for experimental antitumor activity *in vivo* as described earlier (12), using two transplantable murine leukemias in ascitic form: L-1210 leukemia in BDF/1 hybrid mice and P1534 leukemia in DBA/2 inbred mice. Against L-1210 leukemia, this compound did not significantly prolong the life of the animals at nontoxic doses (up to 40 mg./kg./day × 4). Against P1534 at a dose of 10 mg./kg./day × 4, however, there was a 27% increase in survival relative to the untreated controls. This may be compared to the 30–40% increase reported previously for the 3,4-dichlorophenyl analog against the P1534 tumor (13).

Also assayed for antitumor activity were biguanides IIa·HCl and IIb·HCl and guanidinoquinazoline IIIb·HCl. Although none of these compounds showed any activity against L-1210, biguanide IIa·HCl produced a 32% increase in survival in the P1534 system at a dose of 10 mg./kg./day × 4. Compounds IIb·HCl and IIIb·HCl were inactive.

The compounds were also tested for antimalarial activity against *Plasmodium berghei* in the mouse and P. *gallinaceum* in the chick (14), as well as in the *in vitro* sporozoite suppression assay employing mosquitoes infected with *P. gallinaceum* or *P. cynomolgi* (15). Compound Ib·HCl was active against *P. gallinaceum* in chicks at 80 mg./kg., prolonging the mean survival time from 4.0 to 8.6 days (a 115% increase); at 320 mg./kg., survival was lengthened to 13.6 days (a 240% increase). Compound Ia·HCl caused 100% sporozoite suppression, with partial to complete occurrence of abnormal oocysts, in the blood of infected mosquitoes at an administered concentration of 0.001%. None of the compounds showed significant activity against *P. berghei* in mice.

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▲ To whom inquiries should be directed.

Vehicle and Route of Administration as Parameters Affecting Operant Behavioral Effects of Δ⁹-Tetrahydrocannabinol

LOWELL A. BORGEN and W. MARVIN DAVIS▲

Abstract □ Four vehicles for Δ⁹-tetrahydrocannabinol were compared after intraperitoneal and subcutaneous administrations, using the disruption of food-reinforced, operant behavior of rats as the test system for cannabinoid activity. Aqueous suspensions based on polyvinylpyrrolidone, polysorbate 80, and a polysorbate 65–sorbitan monolaurate combination all were effective vehicles for intraperitoneal or subcutaneous absorption of the cannabinoid. An olive oil solution was poorly effective. The polyvinylpyrrolidone dispersion appeared to have the most rapid onset of action, while the polysorbate 65–sorbitan monolaurate combination had the longest duration of action.

Keyphrases □ Marijuana—effect of vehicle and route of administration of Δ⁹-tetrahydrocannabinol on operant behavior, rats □ Δ⁹-Tetrahydrocannabinol—effect of vehicle and route of administration on operant behavior, rats □ Cannabinoid activity—studied by operant behavioral effects in rats, different vehicles and routes of administration

The major psychoactive constituent in marijuana (*Cannabis sativa*) is generally accepted to be (−)-Δ⁹-*trans*-tetrahydrocannabinol (I). The elucidation of the structure and synthesis of I has prompted extensive investigation of its pharmacological and behavioral effects in both animals and man (1, 2). However, the extreme aqueous insolubility of I has made parenteral administration difficult. A number of different aqueous suspensions and oil solutions have been tried by various researchers, but no single preparation has found widespread acceptance. At times, conflicting results between studies have been attributed to differences in the drug vehicle or mode of administration.

The present study compared four such vehicles administered *via* two routes of injection to evaluate the drug effect in a test system previously shown to be sensitive to Δ⁹-tetrahydrocannabinol (3–5). The measure chosen was the depression of operant responding of rats for food reinforcement. In this experiment, both the onset and duration of action of I were examined as a function of eight combinations of vehicle and route of injection.

EXPERIMENTAL

Twenty-four male Wistar rats (225–300 g.) were trained to bar-press for food reward on a fixed-ratio-50 (FR50) schedule of reinforcement; the 50th lever-press resulted in the delivery of a 45-mg. Noyes food pellet. The animals were given 60-min. experimental sessions on 5 days each week, with supplementary postsession feeding sufficient to maintain them at 80% of their free-feeding weights. The operant chambers were controlled by solid-state programming equipment. Cumulative recorders and digital counters were used for data collection. Before initial drug sessions, each rat received 4–5 weeks of training until performance stabilized so that there was less than 10% variation in responses per session over three consecutive sessions.

The four preparations of Δ⁹-tetrahydrocannabinol compared in this experiment were: Vehicle A, olive oil solution; Vehicle B, a 1% polysorbate 80[1] dispersion in saline; Vehicle C, a suspension with 1% polysorbate 65[2] and 1% sorbitan monolaurate[3] in 0.9% saline, as described by Moreton and Davis (6); and Vehicle D, a 10% polyvinylpyrrolidone suspension in 0.9% NaCl, prepared according to the procedure of Fenimore and Loy (7). Each vehicle was tested both intraperitoneally and subcutaneously. The dose of I used was 10 mg./kg. given at an injection volume of 1.0 ml./kg. body weight. The injections of I were given at weekly intervals on Wednesday, vehicle alone was given on Tuesday and Thursday, and saline was injected on Monday and Friday. To examine the relative duration of action of I as well as the time of onset, injections were given either immediately prior to the operant session or 1, 2, or 3 hr. before. At least six determinations were made for each vehicle–route–time combination. The drug effect was assessed by comparing the bar-press performance on drug days to performance on the preceding vehicle control session.

RESULTS AND DISCUSSION

Operant responding following the intraperitoneal or subcutaneous injection of the four vehicles alone did not differ from that following saline administration or no injection. In Table I the effects of Δ⁹-tetrahydrocannabinol upon responding are summarized as a function of the route–vehicle–time combinations. The total number of responses during the 1-hr. test session is expressed as a percentage of the responses per hour in the prior vehicle control session. Gen-

[1] Tween 80.
[2] Tween 65.
[3] Arlacel 20.

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[2] Tween 65.
[3] Arlacel 20.

Table I—Effects of Δ^9-Tetrahydrocannabinol on Fixed-Ratio-50 Performance in Rats: Vehicle and Route of Administration as Parameters Affecting Onset and Duration of Behavioral Disruption

Route of Injection	Drug Vehicle	Percent of Predrug Control Responses per Hour[a] 1st hr. Postinjection	2nd hr. Postinjection	3rd hr. Postinjection	4th hr. Postinjection
Intraperitoneal	A, olive oil	102	48	54	105
	B, polysorbate 80	<1	<1	<1	72
	C, polysorbate 65 plus sorbitan monolaurate	4	<1	8	43
	D, polyvinylpyrrolidone	<1	<1	9	90
Subcutaneous	A, olive oil	100	88	83	101
	B, polysorbate 80	54	32	44	59
	C, polysorbate 65 plus sorbitan monolaurate	65	64	68	55
	D, polyvinylpyrrolidone	15	<1	20	83

[a] Each value is the mean of six observations. Δ^9-Tetrahydrocannabinol dose = 10 mg./kg.

erally, all three aqueous suspensions of I showed a prompt onset of action within 1–5 min. following intraperitoneal administration, whereas a significant effect of I in the oil solution did not occur within the 1st hr. after injection. During the 2nd and 3rd hr. after administration of I in aqueous suspension, suppression of operant responding continued, whereas I in oil reduced response output only one-half. When I was administered subcutaneously in these vehicles, a slower onset was observed, as expected. Compound I in Vehicle D appeared to take effect by the subcutaneous route more rapidly than in either Vehicle B or C. Again the olive oil solution was poorly absorbed. Peak behavioral suppression occurred during the 2nd hr. following subcutaneous injection in Vehicles B and D.

Concerning the duration of behavioral depression, data for the 4th hr. after intraperitoneal administration indicate that I in Vehicle C gave the longest activity. In either Vehicle B or C, I given *via* the subcutaneous route still produced significant operant depression 4 hr. postinjection; in Vehicle D, it was only minimally effective. With I in oil, no effect was observed in the 4th hr. after either intraperitoneal or subcutaneous administration.

In a comparison of vehicles for Δ^9-tetrahydrocannabinol, Sophia *et al.* (8) measured the effects of I in four vehicles on the latency to convulsion in mice after electroconvulsive shock. Only the intraperitoneal route was studied. They found that a polyvinylpyrrolidone suspension in saline and a 10% propylene glycol–1% polysorbate 80 suspension in saline were most effective. Polysorbate 80–saline and bovine serum albumin in saline were found to be poorly absorbed preparations. In another recent study (9), emulsions of polysorbate 80 or polyvinylpyrrolidone plus sesame oil in saline were also found to be suitable vehicles for parenteral administration.

By utilizing operant behavioral techniques, the present experiment indicated that Δ^9-tetrahydrocannabinol is absorbed effectively from aqueous suspensions but poorly absorbed from oil solutions following intraperitoneal administration. Absorption from a subcutaneous site is somewhat delayed but is significant. Vehicle D gave the most rapid absorption of the four preparations studied by both routes of administration. To obtain a prolonged duration of action, Vehicle C appeared to be most effective.

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Interaction of Cholesterol with Dibenzanthracenes at Air–Water Interface

ALBERT A. BELMONTE and JAMES SWARBRICK▲

Abstract □ A film balance approach using continuous compression was used to study the interaction of the carcinogen 1,2:5,6-dibenzanthracene and the noncarcinogen 1,2:3,4-dibenzanthracene with insoluble monolayers of cholesterol. Surface pressure and surface potential of mixed dibenzanthracene–cholesterol films spread on water and saline were studied. Results showed an association of 1,2:5,6-dibenzanthracene and no association of 1,2:3,4-dibenzanthracene with cholesterol. Surface potential data support the concept of carcinogen–cholesterol association. The data show a useful difference between a carcinogenic and a noncarcinogenic isomer at the air–water interface.

Keyphrases □ Cholesterol–dibenzanthracene—interaction at air–water interface, film balance approach, comparison of carcinogenic and noncarcinogenic dibenzanthracene isomers □ Dibenzanthracene–cholesterol—interaction at air–water interface, film balance approach, comparison of carcinogenic and noncarcinogenic dibenzanthracene isomers □ Carcinogens, 1,2:5,6-dibenzanthracene—interaction with cholesterol at air–water interface, compared to noncarcinogenic isomer, 1,2:3,4-dibenzanthracene □ Films—mixed dibenzanthracene–cholesterol, surface pressure and surface potential, air–water interface

Carcinogens, materials known to cause cancer, pose an interesting area of cancer research. While many investigations have been conducted on carcinogen interactions with nucleic acids, only a few studies have been directed toward carcinogen–cell membrane interactions. Wallach (1) proposed that oncogenic agents may act to introduce an inappropriate protein into cell membranes. Numerous membrane functions may be affected as a result of such an alteration. Weissmann *et al.* (2) reported the effects of tumor-promoting agents on biological and artificial membrane systems. Their results suggested membrane disruption as a prime tumor-initiating step. Selkirk *et al.* (3) compared the phospholipid profiles from plasma membranes of solid hepatoma cells and liver cells in an attempt to understand cancer–membrane phenomena; differences in composition and degree of fatty acid saturation were found. All of these studies support the premise that cell membranes are affected by neoplasia. Since cell membranes can control passage of materials into the cell, membrane interactions, particularly penetration of cell membrane by carcinogens, may be extremely important in understanding the cancer-initiating process.

Many materials have been shown to be carcinogenic *in vivo*, yet the ability to correlate carcinogenicity to *in vitro* behavior is often difficult. In an attempt to differentiate between closely related polycyclic aromatic hydrocarbons, it was decided to utilize a film balance technique. This approach is based on the well-recognized fact that insoluble monolayers can be employed to simulate biological interfaces (4). Snart (5), in 1967, made a preliminary study of some aromatic hydrocarbons in monolayers, showing some association of hydrocarbons with cholesterol and lecithin. Weiner *et al.* (6) showed an interaction occurring with 3-methylcholanthrene and lecithin–cholesterol mixed films. The present investigation was conducted to study some surface interactions of dibenzanthracene with cholesterol.

The polycyclic aromatic hydrocarbon dibenzanthracene was chosen for study for several reasons. These include the facts that 1,2:5,6-dibenzanthracene was the first synthetic chemical compound to induce cancer and that considerable information has been compiled on its properties (7). In addition, 1,2:5,6-dibenzanthracene has a structural isomer that is noncarcinogenic or weakly carcinogenic, 1,2:3,4-dibenzanthracene (8, 9). Cholesterol was chosen because of its function in cell membrane structure.

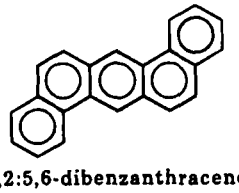
The intent of this report is to relate the monolayer properties of mixed films of cholesterol with dibenzanthracene. The properties will be characterized by: (*a*) the effect of concentration of dibenzanthracene on surface pressure and surface potential parameters, and (*b*) the effect of each dibenzanthracene isomer with respect to their different behavior *in vivo*.

EXPERIMENTAL

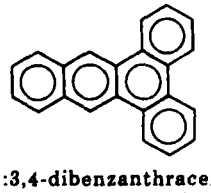
Materials—The mixed films were prepared by dissolving the cholesterol (S.C.W.)[1] in chloroform (nanograde)[2] and the dibenzanthracene[1] in benzene (nanograde)[2]. The appropriate molar ratios of dibenzanthracene–cholesterol were then prepared with the aid of a calibrated syringe[3]. This premixed solution was used as the film-forming material. Double-distilled water from an all-glass still was used as the subphase. The specific conductance of the water was not greater than 1.5 μmhos/cm. When appropriate, normal saline was prepared by adding analytical reagent grade sodium chloride[2].

Film Balance—The film balance was a modified Wilhelmy type which was described previously (10). Surface potential was measured using the ionizing air-gap technique. The apparatus also was described previously (11).

Procedure—Surface pressure was measured continuously using a thin mica plate, of known perimeter, attached to a recording electrobalance. Upon compression, the change in mass of the mica



1,2:5,6-dibenzanthracene (carcinogen)



1,2:3,4-dibenzanthracene (non- or weak carcinogen)

[1] Nutritional Biochemical Corp., Cleveland, Ohio. Purity was checked by TLC using silica gel G and H plates and petroleum ether–ether–acetic acid (90:30:1) and chloroform–methanol–water (65:25:4) as solvents. In all cases, only one spot was found when the plates were developed with bromthymol blue and ammonia gas.

[2] Mallinckrodt, St. Louis, Mo.

[3] Digi-Pet, Manostat Corp., New York, N. Y.

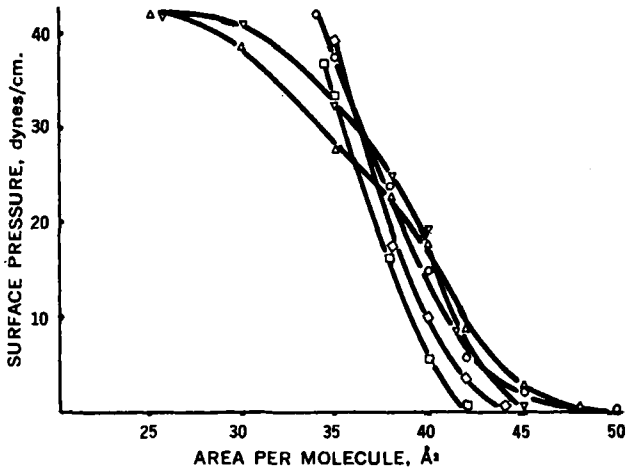


Figure 1—*Surface pressure–area relationship for 1,2:3,4-dibenzanthracene on water. Key: ○, cholesterol; ◇, 1:10 1,2:3,4-dibenzanthracene–cholesterol; □, 1:1 1,2:3,4-dibenzanthracene–cholesterol; ▽, 3:1 1,2:3,4-dibenzanthracene–cholesterol; and △, 5:1 1,2:3,4-dibenzanthracene–cholesterol.*

plate yields a direct measurement of surface pressure. Simultaneous recording of surface potential afforded constant monitoring of monolayers at all stages of compression.

Since preliminary results showed that dibenzanthracene itself does not form surface films, the effect when allowed to react with cholesterol may be evaluated by surface pressure–area per molecule relationships (π–A) directly. Surface potential (ΔV) was also monitored since this can be helpful in interpreting subphase film interactions (4).

Dibenzanthracene–cholesterol films were prepared from solutions containing varying mole fractions. The mixed film solution was pipeted onto the aqueous surface using a micropipet[4] and allowed to spread undisturbed for 10 min. After this equilibration, compression was initiated at a rate of 2.54 cm./min., and data taken from simultaneous recordings of surface pressure and surface potential were used to produce the characteristic π–A isotherms. All runs were performed in triplicate at 25°.

RESULTS

Surface Pressure–Area per Molecule—Figure 1 represents the relationship between surface pressure and area per molecule, expressed in terms of cholesterol, for mixed films of 1,2:3,4-dibenzanthracene–cholesterol. Also shown is the isotherm for pure cholesterol. Similar curves were obtained when saline was substituted as the subphase. The extrapolated area per molecule for cholesterol, which depends on the method of compression, is comparable to that found by Kwong *et al.* (12), who also used continuous compression to generate the surface pressure–area isotherm.

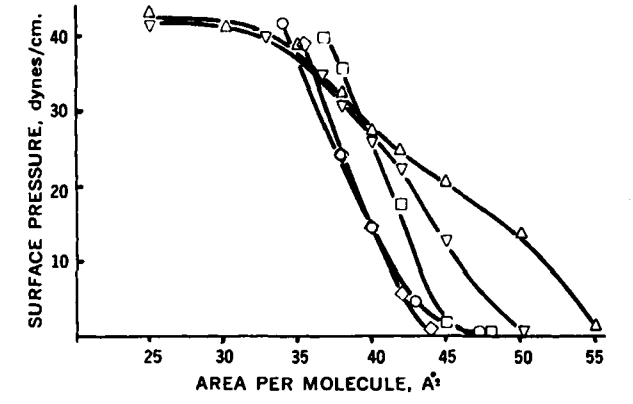


Figure 2—*Surface pressure–area relationship for 1,2:5,6-dibenzanthracene on water. Key: ○, cholesterol; ◇, 1:10 1,2:5,6-dibenzanthracene–cholesterol; □, 1:1 1,2:5,6-dibenzanthracene–cholesterol; ▽, 3:1 1,2:5,6-dibenzanthracene–cholesterol; and △, 5:1 1,2:5,6-dibenzanthracene–cholesterol.*

[4] Bio-Rad Laboratories, New York, N. Y.

Table I—Limiting Surface Potential on Water and Saline[a]

System	Ratio	ΔV, mv. Water	ΔV, mv. Saline
Cholesterol[b]		401 (2.6)[c]	406 (6.1)[c]
1,2:3,4-DBA–chol	1:10	399 (3.1)	411 (7.2)
1,2:3,4-DBA–chol	1:1	403 (8.0)	424 (9.5)
1,2:3,4-DBA–chol	3:1	396 (3.6)	410 (6.6)
1,2:3,4-DBA–chol	5:1	397 (4.7)	427 (4.0)
1,2:5,6-DBA–chol	1:10	378 (13.7)	433 (20.1)
1,2:5,6-DBA–chol	1:1	383 (2.6)	412 (5.5)
1,2:5,6-DBA–chol	3:1	439 (13.6)[d]	464 (1.5)[d]
1,2:5,6-DBA–chol	5:1	436 (18.6)[d]	482 (5.0)[d]

[a] At collapse. [b] DBA = dibenzanthracene; chol = cholesterol. [c] Standard deviation. [d] Significantly different at $p < 0.01$.

Figure 2 illustrates the isotherm resulting from the interaction of the potent carcinogen 1,2:5,6-dibenzanthracene and cholesterol on water. Identical isotherms were observed when experiments were conducted on saline. This figure may be contrasted to Fig. 1, or the noncarcinogenic isomer with cholesterol. The expansion of the carcinogen film at low surface pressures is significant in view of the noncarcinogen film results.

Surface Potential—Surface potential data are shown in Table I. Surface potential was taken at collapse or when the potential failed to change and reached a limiting value. In either case, this generally corresponded to a surface potential of the film at cross-sectional areas per molecule of 35–40 Å². A one-way analysis of variance performed on the data for each subphase showed statistical significance at $p < 0.01$. The individual means within each subphase were then tested using the sequential Q test (13). The 3:1 and 5:1 carcinogenic 1,2:5,6-dibenzanthracene–cholesterol films showed significance with $p < 0.01$ and regardless of subphase.

DISCUSSION

The steric rearrangement of a benzene ring in dibenzanthracene changes this compound from a potent carcinogen to a very weak carcinogen or a noncarcinogen. Although Snart (5) reported some surface interactions of 1,2:5,6-dibenzanthracene, the study did not include 1,2:3,4-dibenzanthracene. The ability to contrast these two compounds *in vitro* may have added importance, since Heidelberger and Moldenhauer (14) showed the noncarcinogen to bind more strongly to protein than the carcinogenic isomer. This binding behavior of dibenzanthracene is generally recognized to be contrary to the usual behavior wherein carcinogens bind more strongly than noncarcinogens.

In the surface interactions with cholesterol, no correlations could be drawn between concentration of noncarcinogenic dibenzanthracene and the isotherms produced. No trend was evident, and the slight differences noted are relatively insignificant when compared to changes evident in the carcinogen films. Mixed films of the strong carcinogenic isomer showed a very different effect with cholesterol. No difference was observed between the cholesterol isotherm and the 1:10 carcinogen–cholesterol film. However, with the films containing higher carcinogen–cholesterol ratios (1:1, 3:1, and 5:1), a significant difference in behavior was noted. There was a dramatic expansion of the film at low surface pressures, attributable to the presence of carcinogen at the interface. There was also a rank correlation between the concentration of 1,2:5,6-dibenzanthracene and the expansion of the film, and this was related to the extrapolated area per molecule.

The extrapolated area per molecule, by tradition, is obtained by continuing a portion of the π–A isotherm to zero film pressure. The assumption was made that, from steric factors and for maximum interaction to occur, the cross-sectional area per molecule of dibenzanthracene was 40–50 Å². By evaluating the expansion effect of the carcinogen–cholesterol films from extrapolated areas per molecule, it was possible to estimate the final molecular association

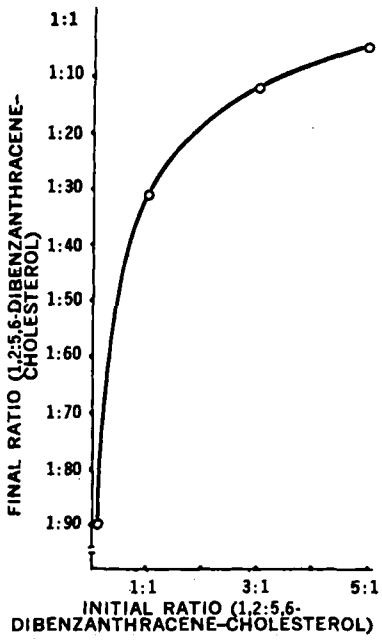


Figure 3—*Degree of association of 1,2:5,6-dibenzanthracene to cholesterol estimated from extrapolated area per molecule.*

present in the film. The molar ratio of carcinogen to cholesterol initially prepared *versus* the association estimated from expansion of the resulting films is plotted in Fig. 3. The data show that after spreading a solution containing a 5:1 carcinogen–cholesterol initial ratio, a 1:4 molecular association results at the interface. Although this association appears to be self-limiting as carcinogen concentration is increased in the film, the limiting value and its significance are still unknown.

Surface potential data reinforced the concept of carcinogen association at the interface. The 3:1 and 5:1 carcinogenic films were statistically different from the noncarcinogenic films regardless of subphase. These results were not unexpected in view of their π–A isotherms, since the 3:1 and 5:1 carcinogen–cholesterol films showed the greatest expanding effect.

These results demonstrate a very useful difference existing between a carcinogenic and a noncarcinogenic or weakly carcinogenic isomer at the air–water interface. This method conceivably may be useful in discriminating between other carcinogenic and noncarcinogenic isomers. Furthermore, cell membrane integrity or disruption at the molecular level may be a cancer-initiating step prior to interaction with other critical targets within the cell.

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Constituents of *Mammea americana* L. XII: Biological Data for Xanthones and Benzophenones

R. A. FINNEGAN▲, K. E. MERKEL, and J. K. PATEL

Abstract □ Sarcoma 180 inhibition data are presented for a number of related xanthones and benzophenones; a few showed significant activity. One of these was also tested *in vivo* against Ehrlich ascites tumor. Four hydroxyxanthones isolated from a polar extract of *Mammea americana* L. (Guttiferae) seeds did not account for the activity of the extract. The results of two antibacterial assays for a few of these compounds are also presented.

Keyphrases □ *Mammea americana* L.—xanthone constituents and related benzophenones, antitumor and antibacterial activities □ Xanthones—from *Mammea americana* L., antitumor and antibacterial activities □ Benzophenones—antitumor and antibacterial activities

A previous paper (1) described the isolation and structure determination of a variety of coumarin derivatives from mamey oil (the dewaxed petroleum ether extract of *Mammea americana* L. seeds) and reported the significant antitumor activity (against Sarcoma 180) of the extract as well as that of a number of the constituent coumarins. During these studies the more polar extract, obtained by extraction of the seed residue with

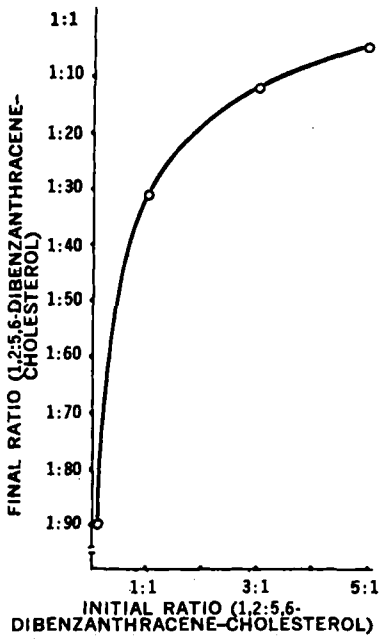

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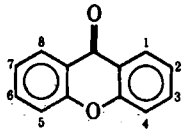
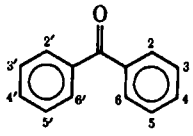
Table I—Growth Inhibitory Activity of Various Simple Xanthones against Sarcoma 180 Tumor Cells

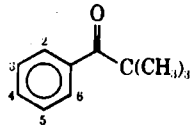


Number	Xanthone	Reference	ID_{50}, mcg./ml.
I	Xanthone[a]	—	11
II	1-Hydroxy	6	21
III	2-Hydroxy	2	25
IV	3-Hydroxy	6	35
V	3-Hydroxy-5,6,7,8-tetrahydro	6	19
VI	4-Hydroxy	3,7	16
VII	1,3-Dihydroxy	7	2.5
VIII	1,3-Dimethoxy	7	120
IX	1,5-Dihydroxy	3,7	6.6
X	1,6-Dihydroxy	3,7	1.8
XI	1,7-Dihydroxy	3,7	8.6
XII	3,4-Dihydroxy	7	23
XIII	3,5-Dihydroxy	3,7	7.0
XIV	3,6-Dihydroxy	6	26
XV	1,3,6-Trihydroxy	6	14
XVI	1,3,8-Trihydroxy	6	10
XVII	1,3,6,8-Tetrahydroxy	6	35
XVIII	1,3,6,7-Tetrahydroxy-2-C-β-D-glucosylxanthone, mangiferin	10	440

[a] Commercially available.

Table II—Growth Inhibitory Activity of Various Benzophenone and Pivalophenone Derivatives against Sarcoma 180 Tumor Cells



Number	Compound	Reference	ID_{50}, mcg./ml.
XIX	Benzophenone[a]	—	33
XX	2-Hydroxy[a]	—	33
XXI	4-Hydroxy[a]	—	29
XXII	2,2',3,4-Tetrahydroxy	7	1.0
XXIII	2,3,3',4'-Tetrahydroxy	3,7	2.6
XXIV	2,2',3,4'-Tetrahydroxy	3,7	18
XXV	2,2',4,4'-Tetrahydroxy[a]	—	17
XXVI	2-Hydroxy-5-*tert*-butyl	11	6.6
XXVII	2-Hydroxy-4,6-di-*tert*-butyl	11	0.066
			
XXVIII	2-Hydroxy-3-*tert*-butyl	12	24
XXIX	4-Hydroxy-3-*tert*-butyl	12	22
XXX	4-Hydroxy-3,5-diisopropyl[b]	—	18

[a] Commercially available. [b] R. A. Finnegan and D. Knutson, unpublished preparation.

benzene (2) and ethanol, also was examined (3, 4). That investigation was prompted by the observation that the ethanol extract provided an ID_{50} value of 5.4 mcg./ml. when assayed against S-180 tumor cells grown in stationary cell culture. Two sets of isomeric xanthones, 2- and 4-hydroxyxanthones (III and VI) and 1,5- and 1,7-dihydroxyxanthones (IX and XI), were subsequently isolated from this polar extract. Their structures were determined by comparison with synthetic materials (2–4). At the same time, a number of other xanthones were prepared (5–7) for comparison purposes and these were also included in the S-180 screen.

EXPERIMENTAL

The Compounds—A few of the test compounds (I, XIX–XXI, and XXV) were commercially available. Some (IX, XXIII, XXIV, XXVI–XXX, and XXXII) were prepared in this laboratory for the first time. The remaining compounds were known and were, in general, prepared by using previously reported procedures. A somewhat improved procedure was used to prepare XXXI. Compound XVIII was used as isolated from *Hiptage madablota* Geartn. Synthetic samples of the compounds isolated from *M. americana* L. (III, VI, IX, and XI) were used.

References to the preparation or isolation of all the compounds are given in the tables. In each case except Compound XXX, experimental details are given as well as relevant literature citations. Compound XXX was prepared by the photo-Fries rearrangement of 2,6-diisopropylphenyl pivalate and details will be provided upon request.

The Tests—A description of the S-180 assay (Tables I and II) was given previously (1). Values for ID_{50} (dose for 50% inhibition of growth) of less than 5 mcg./ml. for a pure compound and less than 20 mcg./ml. for a crude extract are considered significant[1].

The antibacterial results (Table III) were determined[2] using procedures described in the literature (8, 9).

RESULTS AND DISCUSSION

The results shown in Table I indicate that the substances isolated from the extract (III, VI, IX, and XI) are not sufficiently active to account for the activity of the crude extract. However, the synthetic 1,3- and 1,6-dihydroxyxanthones (VII and X) showed noteworthy activity. Further hydroxylation in the equivalent positions of the second ring (Compounds XV–XVII) decreased this activity. Compound XVIII was available in the laboratory (10) and was included for comparison.

A method used for the preparation of certain of the xanthones involves the cyclodehydration of an appropriately substituted benzophenone. Several of these precursors (XXII–XXV and XXXII) were on hand and they were also assayed along with benzophenone (XIX) itself and two of its monohydroxy derivatives (XX and XXI). In addition, a pair of benzophenones (XXVI and XXVII) and a group of pivalophenones (XXVIII–XXX) were available from other studies going on in the laboratory (11, 12) and these were also included. The results are listed in Table II. While two of the tetrahydroxybenzophenones, XXII and XXIII, showed excellent activity, the alkylated hydroxybenzophenone, XXVII, showed activity at least on an order of magnitude greater still. This is reminiscent of the activity exhibited by the alkylated hydroxyketones in the coumarin series (1) and gives a direction to further studies involving alkylated hydroxybenzophenones as well as alkylated hydroxyxanthones.

The acute LD_{50} of Compound XXVII in mice was found to be greater than 500 mg./kg., and the 5-day dose LD_{50} was greater than 100 mg./kg.[3]. Against Ehrlich ascites tumor in mice, the ID_{50} (5-day dosing) of XXVII was 100 mg./kg.[3].

Finally, several of the compounds were examined for antibacterial activity against *Escherichia coli* and *Streptococcus faecalis* with the results shown in Table III. A few of the compounds (I, XXI, and

[1] The authors thank Professor N. Back, Department of Biochemical Pharmacology, State University of New York at Buffalo, for obtaining these data.

[2] Under the direction of Professor A. Bloch, Roswell Park Memorial Institute, Buffalo, N. Y.

[3] These data were obtained through the courtesy of Prof. Peter Hebborn of the Department of Biochemical Pharmacology, State University of New York at Buffalo.

Table III—Antibacterial Activity of Some Xanthones and Benzophenones against *Escherichia coli* (K12) and *Streptococcus faecalis* (8043)

Number	Compound	—ID_{50}, moles/l.— *E. coli*	*S. faecalis*
I[a]		$>10^{-3}$	1×10^{-5} [b]
XV[c]		$>10^{-3}$	9×10^{-4}
XVII[c]		$>10^{-3}$	8×10^{-5}
XIX		$>10^{-3}$	1×10^{-3}
XX[c]		—[d]	—[d]
XXI[e]		3×10^{-4}	3×10^{-5} [f]
XXV		$>10^{-3}$	$>10^{-3}$
XXVII[e]		1×10^{-3}	9×10^{-5}
XXXI[c]	1,8-Dihydroxyxanthone (6)	$\gg 10^{-3}$	$\gg 10^{-3}$
XXXII	2,2′,3,3′-Tetrahydroxybenzophenone (5, 6)	6×10^{-5} [b]	1×10^{-5} [b]

[a] Suspension. [b] One hundred percent inhibition at 10^{-3} *M*. [c] Compound precipitated when test solution was added to medium. [d] No effect at 10^{-5} *M*; at 10^{-3} *M*, a 36% growth enhancement was observed. [e] No effect at 10^{-5} *M*; at 10^{-3} *M*, an 81% growth enhancement was observed. [f] One hundred percent inhibition at 10^{-4} *M*.

XXXII) showed borderline activity (ID_{50} values of about 10^{-5} *M*) against one or the other of these organisms.

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Keyphrases □ *p*-Sulfamoylcarbanilic acid, polyhalophenyl esters—synthesized and screened as potential antifungal agents □ Antifungal agents, potential—synthesis and screening of polyhalophenyl esters of *p*-sulfamoylcarbanilic acid

Recently, it was reported that polyhalophenyl esters of *p*-substituted carbamic acids as well as polyhalophenyl esters of pyridyl- and quinolyl-4-carbamic acids (1, 2) showed significant antifungal activities.

In the present work, a series of polyhalophenyl esters of *p*-sulfamoylcarbanilic acid was prepared by interaction of *p*-sulfamoylbenzoyl azide and the appropriate phenol in boiling toluene or xylene (Scheme I).

$$p\text{-}NH_2SO_2C_6H_4CON_3 + ArOH \xrightarrow[\text{or xylene}]{\text{boiling toluene}} p\text{-}NH_2SO_2C_6H_4NHCOOAr + N_2$$

Scheme I

The physical data of all new compounds are reported in Table I. The antifungal activity of all compounds was determined[1] *in vitro* against *Candida albicans* 1959-2, *Penicillium notatum* 154-3, and *Aspergillus niger* A-23. Concentrations of 5, 10, and 25 mcg./ml. of each compound were used.

Compounds II–IV were dissolved in acetone, Compounds I and V were dissolved in 60% ethanol, and Compounds VI–XI were dissolved in 96% ethanol, all at concentrations of 5 mg./10 ml. These solutions were diluted with hot culture medium to the desired concen-

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[1] Using BBL Sabouraud dextrose agar medium. The microorganisms were obtained from the Department of Parasitology, Public Health Institute, Iran.

Table I—Physical Constants of Substituted Polyhalophenyl Esters of *p*-Sulfamoylcarbanilic Acid

Compound Number	R	Yield, %	Melting Point	Formula	Analysis, %	Calc.	Found
I	Ethyl	70	235°[a]	$C_9H_{12}N_2O_4S$	C	44.26	44.10
					H	4.91	5.11
II	Phenyl	25	160°[b]	$C_{13}H_{12}N_2O_4S$	C	53.42	53.10
					H	4.10	3.90
III	*p*-Tolyl	40	215°[b]	$C_{14}H_{14}N_2O_4S$	C	55.08	55.15
					H	4.59	4.62
IV	*p*-Nitrophenyl	20	235–236°[b]	$C_{13}H_{11}N_3O_6S$	C	48.29	48.26
					H	3.40	3.48
V	*p*-Chlorophenyl	50	225°[a]	$C_{13}H_{11}Cl_2N_2O_4S$	C	47.77	47.80
					H	3.36	3.32
VI	2,4-Dichlorophenyl	70	240°[a]	$C_{13}H_{10}Cl_2N_2O_4S$	C	43.21	43.10
					H	2.77	2.80
VII	2,4,5-Trichlorophenyl	40	212°[a]	$C_{13}H_9Cl_3N_2O_4S$	C	39.44	39.15
					H	2.27	2.30
VIII	2,4,6-Trichlorophenyl	30	215°[a]	$C_{13}H_9Cl_3N_2O_4S$	C	39.44	39.42
					H	2.27	2.24
IX	2,4,6-Tribromophenyl	60	230°[a]	$C_{13}H_9Br_3N_2O_4S$	C	29.48	29.51
					H	1.70	1.65
X	2,4,6-Triiodophenyl	70	220°[a]	$C_{13}H_9I_3N_2O_4S$	C	23.28	23.32
					H	1.34	1.32
XI	Pentachlorophenyl	70	195°[a]	$C_{13}H_7Cl_5N_2O_4S$	C	33.58	33.58
					H	1.93	1.89

[a] Recrystallized from ethanol plus water. [b] Recrystallized from acetone.

Table II—Antifungal Activity of Some Polyhalophenyl Esters of *p*-Sulfamoylcarbanilic Acid[a]

Compound	*Penicillium notatum* 5 mcg./ml.	*Penicillium notatum* 10 mcg./ml.	*Penicillium notatum* 25 mcg./ml.	*Candida albicans* 5 mcg./ml.	*Candida albicans* 10 mcg./ml.	*Candida albicans* 25 mcg./ml.	*Aspergillus niger* 5 mcg./ml.	*Aspergillus niger* 10 mcg./ml.	*Aspergillus niger* 25 mcg./ml.
IX	—	—	+	—	—	+	—	+	2+
X	—	+	2+	+	2+	2+	+	2+	2+
XI	+	2+	2+	2+	2+	2+	—	2+	2+

[a] — equals no inhibition, 2+ equals complete inhibition.

trations and autoclaved at 120° for 2 hr. Five replicates of each concentration were prepared. The antifungal activities of pentachloro-, tribromo-, and triiodophenyl esters are reported in Table II. The antifungal activities of all other compounds were insignificant. The pentachlorophenyl ester was the most active of this series.

EXPERIMENTAL[1]

p-Sulfamoylbenzoylhydrazide was prepared according to Shimizu *et al.* (3).

Preparation of *p*-Sulfamoylbenzoyl Azide—To an ice-cold solution of 2.15 g. (10 mmoles) of *p*-sulfamoylbenzoylhydrazide in 50% acetic acid (25 ml.), a 5% aqueous solution of 0.69 g. (10 mmoles) of sodium nitrite was added with stirring. The resulting precipitate was filtered, washed with cold water, and dried to give 1.92 g. (85%) of *p*-sulfamoylbenzoyl azide, m.p. 100° dec.

Preparation of *p*-Sulfamoylcarbanilic Acid Ethyl Ester—A solution of *p*-sulfamoylbenzoyl azide, 2.26 g. (10 mmoles), in 15 ml. of ethanol was refluxed for 4 hr. The reaction mixture was diluted with water, the resulting precipitate was filtered, and the solid was recrystallized from aqueous ethanol to give 1.70 g. (70%) of the title compound, m.p. 235°; NMR (CF_3CO_2H): τ 9.2 (t, 3H,CH_3), 6.1 (q, 2H, CH_2), 2.7 (q, 4H, ArH), and 2.3 (s, 2H, NH_2).

Preparation of *p*-Sulfamoylcarbanilic Acid Pentachlorophenyl Ester—A solution of 0.452 g. (2 mmoles) of *p*-sulfamoylbenzoyl azide and 0.355 g. (2 mmoles) of pentachlorophenol in 20 ml. of dry toluene was gently refluxed for 4 hr. After evaporation of the solvent under reduced pressure, the residue was recrystallized from 96% ethanol to give 0.650 g. (70%) of the title compound, m.p. 195°; IR (KBr) ν_{max}: 3400, 3320 (C—H and N—H), 1700 (C=O), 1595, 1540 (carbamate)(C—H), 1415, 1390, 1210, 1060 (C—H), 1300, and 1152 (SO_2NH_2) cm.$^{-1}$.

All other carbanilic acid derivatives were prepared similarly (Table I).

[1] Melting points were taken on a Kofler hot-stage microscope and are uncorrected. The IR spectra were determined with a Leitz model III spectrograph. NMR spectra were obtained on a Varian A 60A instrument.

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Topical Mosquito Repellents V: Benzyl Ethers

F. GUALTIERI*, H. JOHNSON, H. TONG, H. MAIBACH†, D. SKIDMORE†, and W. SKINNER▲

Abstract □ A series of benzylic ethers with hydroxyl, epoxy, and unsaturated moieties was synthesized and evaluated on human skin for repellency against female *Aedes aegypti* mosquitoes. Compounds exhibiting the longest durations of repellency were those with boiling points of approximately 125°/0.5 mm. The case of two monobenzyl ethers of 3,4-dihydroxycyclopentene-1 having the same boiling points but different repellent activity is of interest. None of the compounds examined was as effective as *N,N*-diethyl-*m*-toluamide in terms of the duration of topical repellency.

Keyphrases □ Insect repellents, topical—synthesis of benzyl ethers, repellency–volatility relationships □ Benzyl ethers—synthesized and screened as potential insect repellents, repellency–volatility relationships □ Repellency–volatility relationships—benzyl ethers as topical insect repellents

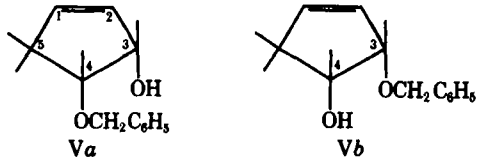
Previous studies (1, 2) suggested that benzyl ethers and polyoxy ethers of the benzyl type were potentially of value in the search for longer lasting topical mosquito repellents. The present compounds were synthesized and evaluated topically on human skin to define the repellency characteristics of such compounds and to determine the effect of other structural moieties such as alicyclic and unsaturated groups.

RESULTS AND DISCUSSION

The compounds (Table I) were obtained by the usual Williamson synthesis using the sodium salt of the alcohol and benzyl chloride; in those cases where a double bond was present, salt formation was *via* sodium hydride in dimethylformamide. With the diols, a double series of compounds, the monobenzyl and dibenzyl ethers, could be obtained using a 1:1 ratio of diol and sodium hydride. The separation of the reaction products by vacuum distillation was not complete, and liquid chromatography was usually required for obtaining suitable analytical samples. The monobenzyl ether fraction of 3,4-dihydroxycyclopentene-1 was further analyzed by VPC, yielding two peaks (FFAP column, 205°, flow rate of 120 ml./min.) with retention times of 10 min. (Compound V*b*, the larger fraction) and 11 min. (Compound V*a*, the smaller fraction).

The indicated structures for the monobenzyl ethers of 3,4-dihydroxycyclopentene-1 were assigned on the basis of NMR as 3-hydroxy-4-benzyloxycyclopentene-1 (V*a*) and 3-benzyloxy-4-hydroxycyclopentene-1 (V*b*). The NMR spectra of these two compounds (Table II) were nearly identical; the major differences were in the τ values of the protons at the 3- and 4-positions.

The 3-proton of Compound V*a* should (5) and does have a lower τ value than the 3-proton of Compound V*b*, since the former is on a carbon with a hydroxyl group and the latter is on a carbon with an ether substituent. Similarly, the 4-proton of Compound V*b* has a lower τ value than that of Compound V*a* for the same reason. Furthermore, the 4-proton of V*a* is a quartet, because of the influence of the 5-methylene protons and the 3-proton. The 4-proton of V*a* also has a higher τ value than its own 3-proton, because the former is an *H*—C—OR proton as compared to an *H*—C—OH proton and because the 3-proton is allylic. In the case of V*b*, its 4-proton should have a lower τ value than its own 3-proton (*H*—C—OH *versus* *H*—C—OR); but since the 3-proton is allylic, it is shifted downfield. The two protons consequently have about the same τ values; 5.6–5.8. The structure of V*b* is further confirmed by its acetyl derivative, whose 4-proton appears at τ 4.65–4.95 as a quartet, having shifted downfield as expected and being split by its neighboring protons; the 3-proton is practically unchanged.



The IR spectrum of the acetate shows the disappearance of the hydroxyl (3 μ) and the appearance of the carbonyl band (5.8 μ). Finally, V*b* is the larger fraction in the reaction product as compared to V*a*, because of the influence of the double bond on the acidity of the 3-hydroxyl group in 3,4-dihydroxycyclopentene-1, thus favoring the formation of the ether derivative in the 3-position.

Similarly, the monobenzyl ether of 3,5-dihydroxycyclopentene-1 was analyzed by VPC. Again, two main peaks were obtained (FFAP column, 210°, flow rate of 120 ml./min., retention times of 17 and 19 min.), collected, and analyzed. Their NMR absorption peaks were identical, suggesting that they are probably *cis*- and *trans*-isomers. Their 4-CH_2 bands (τ 7.8–8.2) confirm the nonallylic nature of these protons in comparison to the 5-CH_2 allylic protons (τ 7.5–7.7) of Compounds V*a* and V*b*.

Compound VI was prepared *via* epoxidation of a mixture of V*a* and V*b*; similarly, the other epoxy compounds were prepared from the corresponding unsaturated ethers. No attempt was made to determine their steric structures.

As in previous studies (1, 2), the present series of compounds exhibited a rather restricted volatility–repellency relationship. Compounds with boiling points higher or lower than those of Compound IV, V*a*, V*b*, and VII did not yield appreciable repellency duration (Table I and Fig. 1). Nonalicyclic benzyl ethers (Compounds XIII–XVIII) of a wide range of boiling points are completely ineffective. Together with previous data (2), the present results suggest that a volatility region corresponding to a boiling point of approximately 115–135°/0.5 mm. is optimal for alicyclic benzyl ethers in terms of topical mosquito repellency. The striking difference in the protection times of Compounds V*a* and V*b*, which are isomeric with the same boiling point, suggests a case of specific structure–activity relationship not commonly reported in insect repellent studies. Similarly, it is of interest that Compounds XIII–XVIII uniformly exhibited poor repellency in spite of the variation in volatility (Table I). The presence of an OH group, an epoxy ring, or a second benzylic moiety does not appear to significantly enhance repellent potency. In terms of duration of protection, Compound V*a* approaches but is inferior to the performance of *N,N*-diethyl-*m*-toluamide (2).

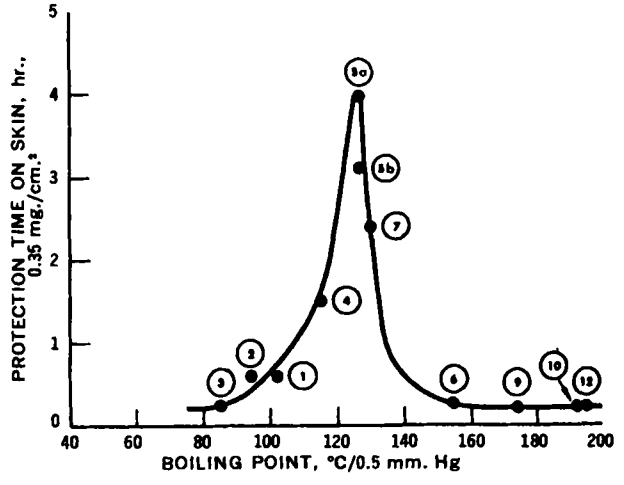
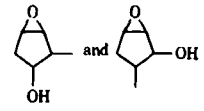
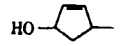
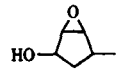
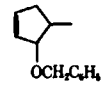
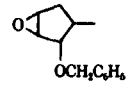
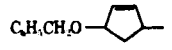
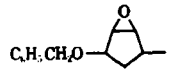
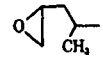
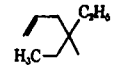
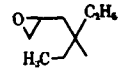
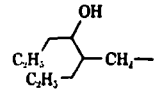
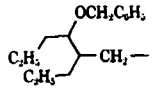


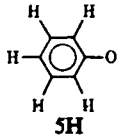
Figure 1—*Alicyclic benzyl ethers.*

Table I—Benzyl Ethers $ROCH_2C_6H_5$

Compound	R	Yield, %	Formula	Analysis, % Calc.	Found	Boiling Point (mm.)	Duration of Repellency, hr. (Number of Determinations)
I		—[a]	—	—	—	127° (2.6)	0.6 (2)
II		—[b]	—	—	—	88° (0.35)	0.6 (5)
III		49	$C_{13}H_{16}O$	C 82.94 H 8.57	83.83 8.75	89° (0.6)	0.25 (2)
IV							
V*a*		37	$C_{12}H_{14}O_2$	C 75.69 H 7.42	76.62 7.46	114° (0.25)	4.0 (4)
V*b*		—	$C_{12}H_{14}O_2$	C 75.69 H 7.42	75.62 7.46	114° (0.25)	2.9 (4)
VI		77	$C_{12}H_{14}O_3$	C 69.89 H 6.84	69.76 6.83	141° (0.25)	0.25 (2)
VII		23	$C_{12}H_{14}O_2$	C 75.69 H 7.42	75.76 7.42	125° (0.4)	2.4 (4)
VIII		71	$C_{12}H_{14}O_3$	C 69.89 H 6.84	69.76 6.85	147° (0.35)	—
IX		45	$C_{19}H_{20}O_2$	C 81.40 H 7.19	81.18 7.39	169° (0.4)	0.25 (2)
X		67	$C_{19}H_{20}O_3$	C 77.00 H 6.80	76.98 6.74	186° (0.35)	0.25 (2)
XI		18	$C_{19}H_{20}O_2$	C 81.40 H 7.19	81.28 7.38	167° (0.4)	—
XII		41	$C_{19}H_{20}O_3$	C 77.06	77.03	187° (0.3)	0.25 (2)
XIII		52	$C_{12}H_{16}O$	C 81.77 H 9.15	81.91 9.26	73° (1.75)	0.25 (2)
XIV		56	$C_{12}H_{16}O_2$	C 74.97 H 10.2	75.09 10.1	99° (1.25)	0.25 (1)
XV		59	$C_{16}H_{22}O$	C 82.52 H 10.2	82.83 10.1	88° (0.25)	0.25 (2)
XVI		86	$C_{16}H_{22}O_2$	C 76.88 H 9.46	76.82 9.67	119° (0.35)	0.25 (4)
XVII		50	$C_{16}H_{24}O_2$	C 76.23 H 10.2	76.17 10.4	111° (0.15)	0.25 (3)
XVIII		50	$C_{22}H_{30}O_2$	C 80.34 H 9.26	80.38 9.45	173° (0.2)	0.25 (3)

[a] See *Reference 3*. [b] See *Reference 4*.

Table II—NMR τ Values of Derivatives of 3,4-Cyclopentenediol

	Aromatic  5H	Vinyl 2H	Benzyl —$OCH_2C_6H_5$ 2H	3-Position 1H	4-Position 1H	5-Methylene 2H	Hydroxyl[a] —OH 1H	Acetate —$OCCH_3$ 3H
4-Benzyl ether	2.70	4.2	5.4	5.5	5.8–6.1 (quartet)	7.5–7.7	7.4–7.6	—
5-Benzyl ether	2.70	4.0–4.3	5.4	5.6–5.8	5.6–5.8	7.5–7.7	7.2–7.4	—
4-Acetate of 3-benzyl ether	2.75	4.1–4.3	5.5	5.6	4.6–4.95 (quartet)	7.4–7.6	—	8.05

[a] Confirmed by D_2O exchange and integration.

EXPERIMENTAL

The starting materials of the best mosquito repellents in this series, 3,4-dihydroxycyclopentene-1 (b.p. 55–65°/0.5 mm. Hg) and 3,5-dihydroxycyclopentene-1 (b.p. 90–100°/0.6 mm. Hg), were obtained commercially[1] as a mixture and were separated by spinning band distillation. The 3,4-dihydroxy fraction (n_D^{25} 1.4971) was further purified by VPC (FFAP column, 205°, flow rate of 120 ml./min.) and had a retention time of 5 min.; the 3,5-dihydroxy fraction (n_D^{25} 1.5012), under the same GC conditions, had a retention time of 11 min. The 3,5-dihydroxy fraction was oxidized to the dione as previously described (5), and the melting point agreed with that reported. A comparison of the NMR spectra of the derivatives V*a*, V*b*, and VII indicated that the assigned structures as 3,4- and 3,5-dihydroxy compounds were correct.

The alcohol (0.1 mole) was added slowly to a stirred suspension of sodium hydride (0.1 mole) in 50 ml. of anhydrous dimethylformamide. After 3 hr. at room temperature, benzyl chloride (0.1 mole) was added dropwise with stirring and cooling. After 24 hr. at room temperature, the mixture was partitioned between hexane and water. The hexane layer was washed and dried (sodium sulfate), yielding primarily the dibenzyl ether, while extraction of the aqueous phase with ethyl acetate gave primarily the monobenzyl ether following fractionation of the oils resulting from the evaporation of the solvents. In some cases (Compounds V, VII, IX, and XI), chromatography on silica gel (eluant 20:80, ethyl acetate–cyclohexane) was necessary to obtain pure analytical samples. Boiling points are uncorrected.

The compounds were tested on the forearm of human subjects as described previously (1, 2) at a concentration of 0.35 mg./cm.[2].

[1] Research Organic.

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Potential Psychoactive Indole Derivatives II: Synthesis of 5-Alkoxyindolines *via* Reduction of 5-Alkoxy-2-indolinones

A. MAHAMADI*, P. PARIMOO†, W. G. HANEY, and B. F. GRABOWSKI‡

Abstract □ As part of a continuing program to define further the role of the indole nitrogen in influencing activity of psychoactive compounds, a series of 5-alkoxy-3-alkylamino-1,3-dimethyl-2-indolinones and indolines were prepared for central activity studies. The intermediate substituted 2-indolinones were prepared by C-5 alkylation of 5-hydroxy-1,3-dimethyl-2-indolinone followed by C-3 alkylation with appropriately substituted chloroalkylamines. The substituted 2-indolinones were then reduced to the indoline derivatives with lithium borohydride. IR and NMR spectroscopy were utilized for the structural characterization of the synthesized compounds.

Keyphrases □ Indolinones, 5-alkoxy-3-alkylamino-1,3-dimethyl substituted—synthesized as potential psychoactive agents, structure-activity relationships of indole derivatives □ Indolines, 5-alkoxy substituted—synthesized as potential psychoactive agents, structure-activity relationships of indole derivatives □ Psychoactive agents—synthesis of 5-alkoxyindolines by reduction of 5-alkoxy-2-indolinones

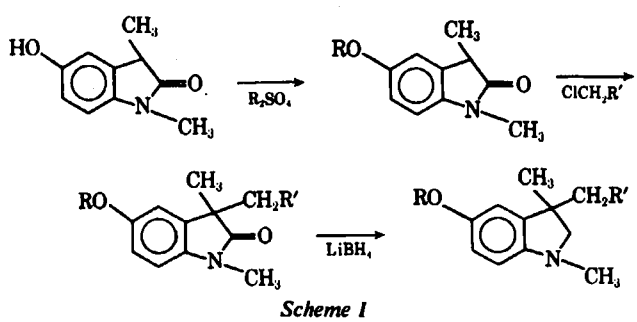
Appropriately substituted indoles have been of interest to the pharmaceutical chemist, especially because of the widespread occurrence of the indole nucleus among both naturally occurring and synthetic psychoactive compounds (1). In particular, those indole derivatives with 3-alkylamino and 4-, 5-, or 6-alkoxy substitutions have been shown to possess central activity, namely antidepressant, depressant, and hallucinogenic (2).

As a result of the large number of indole derivatives that are psychoactive, we became interested in the role of the nitrogen of the indole nucleus in determining both the potency and the spectrum of the psychoactivity of these compounds.

The nitrogen of indoline and of 2-indolinone differs from the nitrogen of indole with respect to electronic character. Hence, the relative psychopharmacological properties of substituted 2-indolinones and indolines should further elucidate the importance of the indole nitrogen in psychoactive compounds.

In a previous work (3), the present authors prepared 3-alkylamino substituted 2-indolinones and indolines for central activity characterization. Since alkoxy substitution in the 4-, 5-, or 6-position of the indole nucleus often leads to changes in psychopharmacological activity, it was of interest to prepare 5-alkoxy derivatives of selected 3-alkylamino substituted 2-indolinones and indolines for their central activity characterization. Therefore, a series of 5-alkoxy-3-alkylamino-1,3-dimethyl-2-indolinones and indolines were prepared in the hope that their psychoactivity relative to that of the corresponding indole derivatives would shed further light on the role of the indole nitrogen in determining central activity of the indole derivatives.

The 5-hydroxy-3-methyl-2-indolinone intermediates were obtained by a procedure based on the methods of Julian *et al.* (4) and Stolle (5). Dialkyl sulfate in benzene was utilized for C-5 alkylation, and further C-3 alkylation was carried out with metallic sodium and the appropriately substituted chloroalkylamine in ethanol. The resulting 5-alkoxy-3-alkylamino-1,3-dimethyl-2-indolinones (Table I) were reduced with lithium borohydride in tetrahydrofuran to give the substituted indoline derivatives (Table II). The synthetic pathway is shown in Scheme I.



Scheme I

EXPERIMENTAL[1]

Preparation of 5-Hydroxy-1,3-dimethyl-2-indolinone Intermediates—The 5-hydroxy-3-monoalkyl-2-indolinones, starting materials for the synthesis of the 3-methyl-3-alkylamino-2-indolinones and indolines, were prepared according to the procedure of Julian and Pikl (6). The yields were in the range of 80% of theory, and IR and NMR spectrophotometric data confirmed the structural assignments.

Synthesis of 5-Alkoxy-3-alkylamino-1,3-dimethyl-2-indolinones (Table I)—The synthetic procedure was adapted from that outlined by Horning and Rutenberg (7). Compounds II*a*–VIII*a* were prepared by the procedure described below for 3-(2-aminoethyl)-5-methoxy-1,3-dimethyl-2-indolinone (Compound I*a*). Pertinent data for these compounds are summarized in Table I.

IR and NMR spectral data were utilized in the characterization of these compounds. The IR spectra showed absorbances at 1725 (amide carbonyl), 1475 (methyl), and 1610, 1500, and 740 (phenyl) cm.$^{-1}$, while the NMR spectra showed peaks (in $CDCl_3$) at 3.8–4.2 (*O*-methyl or methylene), 6.8–7.5 (aromatic substituent), 3.5–3.8 (*N*-methylene), and 0.8–1.1 (3-methyl or methylene) p.p.m.

3-(2-Aminoethyl)-5-methoxy-1,3-dimethyl-2-indolinone (Compound I*a*)—Metallic sodium (2.07 g., 0.09 mole) in ethanol (150 ml.) was mixed with 5-methoxy-1,3-dimethyl-2-indolinone (12.0 g., 0.06 mole) in a three-necked flask equipped with reflux condenser, stirrer, drying tube, and dropping funnel. A solution of 2-chloroethylamine hydrochloride (11.6 g., 0.10 mole) and metallic sodium (2.3 g., 0.10 mole) in ethanol (100 ml.) was added dropwise to the ethanolic substituted 2-indolinone solution. The reaction mixture

[1] Reported melting points are uncorrected. A Thomas-Hoover Unimelt apparatus was used for the melting-point determination. IR spectral analyses were conducted on a Perkin-Elmer model 137 G, while NMR spectra were obtained on a Varian model A-60 spectrophotometer.

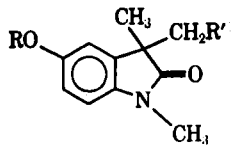


Table I—5-Alkoxy-3-alkylamino-1,3-dimethyl-2-indolinones

Compound	R	R′	Yield, %	Melting Point or Boiling Point (mm. Hg)	Recrystallization Solvent	Formula	Analysis, % Calc.	Found
I*a*	$-CH_3$	$-CH_2NH_2$	41	137–139°[a]	Ether–hexane	—	—	—
II*a*	$-CH_2CH_3$	$-CH_2NH_2$	38	125–126°	Benzene	$C_{12}H_{18}N_2O_2$	C 67.74	67.94
							H 8.06	7.96
							N 11.29	11.27
III*a*	$-CH_3$	$-CH_2N(CH_3)_2$	32	127–128°[b]	Ether	—	—	—
IV*a*	$-CH_2CH_3$	$-CH_2N(CH_3)_2$	39	146–147°	Chloroform–ether	$C_{16}H_{24}N_2O_2$	C 69.57	69.66
							H 8.70	8.58
							N 10.14	10.10
V*a*	$-CH_3$	$-CH_2CH_2N(CH_3)_2$	34	156–160° (1.2)[c]	—	—	—	—
VI*a*	$-CH_2CH_3$	$-CH_2CH_2N(CH_3)_2$	33	121–122°	Ether–hexane	$C_{17}H_{26}N_2O_2$	C 70.34	70.21
							H 8.97	8.91
							N 9.65	9.73
VII*a*	$-CH_3$	$-CH(CH_3)CH_2N(CH_3)_2$	25	151–152°	Chloroform–benzene	$C_{17}H_{26}N_2O_2$	C 70.34	69.92
							H 8.97	8.82
							N 9.65	9.47
VIII*a*	$-CH_2CH_3$	$-CH(CH_3)CH_2N(CH_3)_2$	40	129–130°	Benzene	$C_{17}H_{26}N_2O_2$	C 71.01	71.07
							H 9.27	9.20
							N 9.20	9.13

[a] Lit. (8) b.p. 210–213° (9.0). [b] Lit. (8) b.p. 210–215° (20.0). [c] Lit. (8) b.p. 167–170° (1.0).

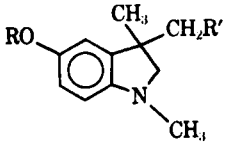


Table II—5-Alkoxy-3-alkylamino-1,3-dimethylindolines

Compound	R	R′	Yield, %	Melting Point or Boiling Point (mm. Hg)	Recrystallization Solvent	Formula	Analysis, % Calc.	Found
I*b*	$-CH_3$	$-CH_2NH_2$	34	120–122° (1.1)	—	$C_{13}H_{20}N_2O$	C 70.88	70.82
							H 9.15	9.12
							N 12.71	12.63
II*b*	$-CH_2CH_3$	$-CH_2NH_2$	28	79–81°	Ether	$C_{14}H_{22}N_2O$	C 71.75	71.45
							H 9.46	9.45
							N 11.95	11.84
III*b*	$-CH_3$	$-CH_2N(CH_3)_2$	37	118–122° (2.0)	—	$C_{15}H_{24}N_2O$	C 72.53	72.53
							H 9.73	9.59
							N 11.28	11.20
IV*b*	$-CH_2CH_3$	$-CH_2N(CH_3)_2$	32	144–145° (1.3)	—	$C_{16}H_{26}N_2O$	C 73.28	73.20
							H 10.08	10.11
							N 10.68	10.56
V*b*	$-CH_3$	$-CH_2CH_2N(CH_3)_2$	38	132–136° (1.4)	—	$C_{16}H_{26}N_2O$	C 73.28	73.44
							H 10.08	9.85
							N 10.68	10.53
VI*b*	$-CH_2CH_3$	$-CH_2CH_2N(CH_3)_2$	26	77–79°	Ether	$C_{17}H_{28}N_2O$	C 73.91	73.56
							H 10.14	9.93
							N 10.14	10.04
VII*b*	$-CH_3$	$-CH(CH_3)CH_2N(CH_3)_2$	33	122–123° (2.4)	—	$C_{17}H_{28}N_2O$	C 73.91	74.10
							H 10.14	10.02
							N 10.14	9.96
VIII*b*	$-CH_2CH_3$	$-CH(CH_3)CH_2N(CH_3)_2$	31	82–83°	Methanol	$C_{18}H_{30}N_2O$	C 74.43	74.62
							H 10.41	10.21
							N 9.64	9.47

was refluxed with stirring for 6 hr. and cooled to room temperature, and then the ethanol was removed under reduced pressure. The resulting brown residue was extracted with ether, and the ether extract was dried over magnesium sulfate. After removal of the ether, the resulting product was recrystallized from ether–hexane to give the compound whose data are summarized in Table I.

Synthesis of 5-Alkoxy-3-alkylamino-1,3-dimethylindolines (Table II)—Reduction of substituted 2-indolinones to indolines was attempted using the procedure outlined by Marion and Kates (8). Since this procedure gave incomplete reduction, the method was altered by utilizing lithium borohydride in tetrahydrofuran. Compounds II*b*–VIII*b* were prepared by the procedure described for 3-(2-aminoethyl)-5-methoxy-1,3-dimethylindoline (Compound I*b*), and their data are summarized in Table II.

The IR spectra of these compounds showed absorbances at 1610, 1500, 740 (phenyl), and 1475 (methyl) $cm.^{-1}$. The amide carbonyl band was absent. The NMR spectra contained peaks at 6.6–7.2

(aromatic), 3.8–4.2 (*O*-methyl or methylene), and 3.5–3.8 (*N*-methylene) p.p.m. with proper multiplicities.

3-(2-Aminoethyl)-5-methoxy-1,3-dimethylindole (Compound Ib)—A solution of 3-(2-aminoethyl)-5-methoxy-1,3-dimethyl-2-indolinone (5.5 g., 0.03 mole) in tetrahydrofuran (30 ml.) was added slowly to a suspension of lithium borohydride (0.04 mole) in tetrahydrofuran (25 ml.). The reaction mixture was refluxed with stirring overnight and then cooled to room temperature. A mixture of methanol–water (10:1) was added slowly to the mixture until effervescence ceased. The tetrahydrofuran was removed under reduced pressure, and the residue was extracted with ether. The ether extract was dried over magnesium sulfate and the ether evaporated. The crude oil was distilled to give the compound whose data are summarized in Table II.

SUMMARY

Sixteen 5-alkoxy-3-alkylamino-1,3-dimethyl-2-indolinones and indolines were prepared. These compounds were characterized with IR and NMR spectroscopy. Pharmacological testing is in progress.

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Comptonia asplenifolia: Low Boiling Components

D. C. CLAGETT▲, W. P. DUBINSKY*, and D. PRZYBYL

Abstract □ Room temperature, low pressure vacuum distillation of 920 g. of the fresh leaves from *Comptonia asplenifolia* Ait. typically yielded 3 mg. of methyl acetate, 1 mg. of ethyl acetate, 2 mg. of diisopropyl acetaldehyde acetal, and 5 mg. of cineol. Methyl acetate, ethyl acetate, and diisopropyl acetaldehyde acetal were not previously reported as components of the essential oil and are important contributors to the odor factors of the plant.

Keyphrases □ *Comptonia asplenifolia* Ait.—isolation and identification of low boiling components □ GLC—identification of low boiling components of *Comptonia asplenifolia*

Comptonia asplenifolia Ait.[1] was first investigated phytochemically by Braun (1) and later by de Nicola and Lynn (2). Using distillation techniques, these workers isolated 0.02–0.5% by weight of an oil from fresh or semidried leaves. Only cineol was identified. Other compounds such as lactones, terpenes, esters, and alcohols were suggested to be present. In a more recent study using GLC, this essential oil was shown to contain at least 32 identifiable terpenoids including cineol (3). This work utilized a cohobation still, which allowed isolation of high boiling components but precluded observation of volatile compounds and those that were heat or water sensitive. It was, therefore, of interest to isolate such low boiling components. Compounds determined would add to the chemotaxonomic information about *C. asplenifolia*[2].

Using a sequence of room temperature, low pressure vacuum distillation, ether extraction, and temperature-programmed GLC, small amounts of methyl acetate, ethyl acetate, diisopropyl acetaldehyde acetal, and cineol were isolated. The previously undetected methyl acetate and ethyl acetate are common, expected odor contributors. Diisopropyl acetaldehyde acetal, which was isolated in amounts comparable to about half that of the previously noted cineol (2, 3), appears to be another important odor contributor. This is the first report of this substance being isolated from natural

[1] This plant, a sweet-scented shrub common to the eastern United States, is alternatively designated *Myrica asplenifolia* Endl. or *Comptonia peregrina* L. Coult. and is colloquially named sweet fern. Identification of the plant material used in this study was made by Dr. Fred Barkley of Northeastern University, and a voucher specimen (Clagett and Dubinsky–1) has been deposited with Dr. Barkley, Curator, Husky Herbarium, Northeastern University, Boston, MA 02115

[2] *Comptonia asplenifolia* has been used for many years as a remedy for high blood pressure and as a sickroom air freshener by residents of Appalachia. Its essential oil is patented for use as a perfume fixative (4). The chemotaxonomic information may be useful for chemosystematic evaluations (3).

(aromatic), 3.8–4.2 (*O*-methyl or methylene), and 3.5–3.8 (*N*-methylene) p.p.m. with proper multiplicities.

3-(2-Aminoethyl)-5-methoxy-1,3-dimethylindole (Compound Ib)—A solution of 3-(2-aminoethyl)-5-methoxy-1,3-dimethyl-2-indolinone (5.5 g., 0.03 mole) in tetrahydrofuran (30 ml.) was added slowly to a suspension of lithium borohydride (0.04 mole) in tetrahydrofuran (25 ml.). The reaction mixture was refluxed with stirring overnight and then cooled to room temperature. A mixture of methanol–water (10:1) was added slowly to the mixture until effervescence ceased. The tetrahydrofuran was removed under reduced pressure, and the residue was extracted with ether. The ether extract was dried over magnesium sulfate and the ether evaporated. The crude oil was distilled to give the compound whose data are summarized in Table II.

SUMMARY

Sixteen 5-alkoxy-3-alkylamino-1,3-dimethyl-2-indolinones and indolines were prepared. These compounds were characterized with IR and NMR spectroscopy. Pharmacological testing is in progress.

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material. Its presence is somewhat surprising when it is considered that its acid-catalyzed hydrolytic lability[3] is about 13 times greater than that of acetal itself (6).

A preliminary phytochemical survey (by referenced methods) of an alcohol extract from the dried leaves remaining after water and volatile oils were removed indicated little or no sterol (7), alkaloid (8), or saponin matter (9). Tannins (10) and flavonoids (11) were suggested[4]. Separations and identifications of these components are being pursued in these laboratories[5].

EXPERIMENTAL[6]

GLC—Temperature-programmed and isothermal column GLC analyses were performed with a chromatograph[7] equipped with a thermal conductance detector block. All temperature-programmed runs were made at a 10°/min. temperature increase between 60° and the indicated temperature limit. All GLC analyses utilized an injector port temperature of 250° and a detector block temperature of 300°. The columns utilized were: Column 1, 25% Dow 710 silicone oil on 60–80-mesh Chromosorb P, acid washed [2.1-m. (7-ft.) × 0.63-cm. (0.25-in.) o.d. copper tubing]; Column 2, 20% Carbowax 20M on 60–80-mesh Chromosorb P, acid washed [3.04-m. (10-ft.) × 0.63-cm. (0.25-in.) o.d. stainless steel tubing]; Column 3, 13% Apiazon L grease on 60–80-mesh Chromosorb P, acid washed [1.8-m. (6-ft.) × 0.63-cm. (0.25-in.) o.d. copper tubing]; and Column 4, 4% SE-30 methyl silicone oil on 60–80-mesh Chromosorb P, acid washed [2.7-m. (9-ft.) × 0.63-cm. (0.25-in.) o.d. copper tubing]. Collections of GLC-purified material for comparison IR measurements were made using dry ice–acetone-cooled U-tubes.

Isolation of Low Boiling Fractions—Fresh *C. asplenifolia* leaves were packed in a 60 × 15-cm. i.d. cylindrical iron vessel. This was connected in turn to a dry ice-cooled 100 × 5-cm. i.d. tubular trap and 25-l./min. vacuum pump. The evacuated iron vessel was maintained at room temperature (approximately 25°) while the pump developed a pressure of 5–10 mm. Hg. Volatiles were frozen out in the trap over a 6-day period. At the end of this time the trap was warmed and the water–oil melt was collected. This mixture was extracted with ether. The ether solution was treated with anhydrous sodium bicarbonate. Ether solvent was removed using a rotary evaporator. In a typical experiment, fresh leaves (920 g.) yielded water (590 g.) and oil (0.12 g., 0.13% of fresh leaf weight). Analysis of the oil by temperature-programmed GLC (Column 1, limit 240°) indicated methyl acetate (0.003 g.), ethyl acetate (0.001 g.), and diisopropyl acetaldehyde acetal (0.002 g.) as the sole components eluted below 125°. At 150°, cineol (0.005 g.) was eluted among other higher boiling components. Positive identification of methyl acetate, ethyl acetate, and diisopropyl acetaldehyde acetal was made by comparing their IR spectra (GLC-collected samples) and programmed retention times (Column 1 and Column 2, limit 240°) with those of authentic samples[8]. Cineol was similarly identified by comparison of its IR spectra and retention times (programmed Column 1, limit 240°, and isothermal Column 3, 150°) with an authentic sample.

Synthesis of Diisopropyl Acetaldehyde Acetal—Diisopropyl acetaldehyde acetal was synthesized by a method analogous to that used for the preparation of the acetal (15). Freshly distilled acetaldehyde (44 g., 1 mole) was added to a mixture of isopropanol (116 g., 1.9 moles) and anhydrous calcium chloride (22 g., 0.2 mole). The vessel was stoppered, shaken, and allowed to stand at room temperature for 24 hr. The solution was decanted onto 0.3-cm. (0.125-in.) activated molecular sieves[9] and dried for an additional 24 hr. The molecular sieves were removed, and anhydrous ether (100 ml.) followed by sodium metal pellets (23 g., 1 mole) was added. After hydrogen evolution ceased, the solution was distilled and the 82–115° boiling fraction was collected. Redistillation yielded a fraction of boiling range 122–124°; yield 42.6 g. (34%); IR (CCl_4): 1390, 1334, 1140, 1085, and 1002 $cm.^{-1}$; NMR (CCl_4): 8.79 d, 8.88 d, 8.91 d (15), 6.16 broad h (2), and 5.29 q (1).

Anal.—Calc. for $C_8H_{16}O_2$: C, 65.71; H, 12.41. Found: C, 65.77; H, 12.20.

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[3] This hydrolytic lability, which results in acetaldehyde formation, probably explains the irritating nature of its concentrated vapors. In this study the ethereal solution obtained after ether extraction, when treated with 2,4-dinitrophenylhydrazine reagent (5), yielded a derivative which was shown to be identical with that of acetaldehyde by melting point (148–150°) and undepressed mixed melting point. Diisopropyl acetaldehyde acetal, when treated similarly, yielded a derivative identical with that of acetaldehyde and that from the natural oil, as shown by melting point and undepressed mixed melting point.

[4] The authors thank Dr. Robert Raffauf for these survey analyses.

[5] Column chromatography of the alcoholic extract, using Dupont nylon 6 on Celite solid phase (12), yielded at least 20 flavonoid substances. Preliminary paper chromatography/color tests indicated the presence of chalcones, isoflavones, flavonols, flavanonols, flavones, and flavanones (12–14).

[6] Fresh *C. asplenifolia* leaves were harvested in the vicinity of Boston, Mass. IR spectrophotometric determinations were made with a Perkin-Elmer model 21 instrument. Samples were dissolved in spectral grade carbon tetrachloride. NMR spectra were obtained using a Varian model T-60 spectrometer. Samples were dissolved in spectral grade carbon tetrachloride, with 1% tetramethylsilane as the internal reference. Melting points were taken using a Thomas-Hoover apparatus, and melting points and boiling points are uncorrected. Carbon–hydrogen analyses were performed by Galbraith Laboratories. Anhydrous calcium chloride, sodium bicarbonate, sodium, isopropanol, methyl acetate, and ethyl acetate were of reagent grade. Technical grade cineol was purchased from Aldrich Chemical Co.

[7] F&M Scientific Corp., model 500.

[8] The synthesis of diisopropyl acetaldehyde acetal was accomplished with complete analytical and spectral data, because all previous syntheses lacked rigorous structure determinations.

[9] Linde Type 5A.

Synthesis and Biological Activity of 1,5-Diphenyl-4-arylazopyrazoles and 5,5-Dimethylcyclohexane-1,2,3-trione Bishydrazones

HARI G. GARG[▲], ANU SINGHAL, and J. M. L. MATHUR

Abstract □ A series of 1,5-diphenyl-4-arylazopyrazoles was synthesized and evaluated for antimalarial activity. The compound possessing the best antimalarial activity of the entire study was 1,5-diphenyl-4-methoxyphenylazopyrazole. Another series of 5,5-dimethylcyclohexane-1,2,3-trione bishydrazones was prepared and tested as anthelmintics. No significant activity was observed.

Keyphrases □ 1,5-Diphenyl-4-arylazopyrazoles—synthesized and screened as potential antimalarial agents □ 5,5-Dimethylcyclohexane-1,2,3-trione bishydrazones—synthesized and screened as potential anthelmintics □ Anthelmintic agents, potential—synthesis and screening of 5,5-dimethylcyclohexane-1,2,3-trione bishydrazones □ Antimalarial agents, potential—synthesis and screening of 1,5-diphenyl-4-arylazopyrazoles

In a previous paper (1) the synthesis and biological data of N^1-substituted arylsulfonylpyrazoles (Ic) were reported. The test results on rodent malaria performed with some of these derivatives (Ic, X = 2-Cl, 4-OCH_3, or 4-NO_2) were not encouraging; the mean survival time of drug-treated, young female, noninbred ICR-HA swiss mice infected with a standard intraperitoneal inoculum of *Plasmodium berghei* (KBG-173) increased by 0.1 day compared to controls. In a search for efficient antimalarials, analogs of 1,5-diphenyl-4-arylazopyrazoles (Ia) were prepared and are reported in this article. The compounds were found to possess marked improvement in potencies over previous candidates.

In view of the anthelmintic activity displayed by Ic, a series of 5,5-dimethylcyclohexane-1,2,3-trione bishydrazones (IV) containing a few of these groupings was synthesized.

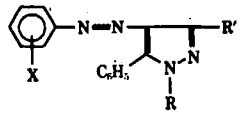


Table I—Chemical and Biological Properties of 1,5-Diphenyl-4-arylazopyrazoles[a]

Number	R	R′	X	Yield, %	Melting Point	Color[b]	Formula	Analysis, % Calc.	Analysis, % Found	Antimalarial Activity[c] D	TD	T/C	Remark
1	C_6H_5	H	H	60	180°	DR	$C_{21}H_{16}N_4$	C 77.77 H 4.93 N 17.28	77.72 4.95 17.24	320	2	3.9	Toxic
2	C_6H_5	H	4-Cl	50	195°	Y	$C_{21}H_{15}ClN_4$	C 70.29 H 4.18 N 15.62	70.24 4.28 15.41	320	2	3.9	Toxic
3	C_6H_5	H	2-OCH_3, 5-OCH_3	60	188°	B	$C_{23}H_{20}N_4O_2$	C 71.87 H 5.20 N 14.58	71.67 5.12 14.34	320	0	1.1	—
4	C_6H_5	H	4-CH_3	55	190°	Y	$C_{22}H_{18}N_4$	C 78.18 H 5.32 N 16.56	78.02 5.22 16.46	500	0	0.1	—
5	C_6H_5	H	4-OCH_3	70	206°	O	$C_{22}H_{18}N_4O$	C 74.57 H 5.08 N 15.81	74.56 5.01 15.61	320	3	4.4	Toxic
6	C_6H_5	H	2-OCH_3	65	193°	LY	$C_{22}H_{18}N_4O$	C 74.57 H 5.08 N 15.81	74.37 4.98 15.91	320	0	2.3	—
7	C_6H_5	H	2-NO_2	50	184°	O	$C_{21}H_{15}N_5O_2$	C 68.29 H 4.06 N 15.21	68.01 4.03 15.11	320	0	0.1	—
8	C_6H_5	H	4-OCH_2CH_3	50	170°	R	$C_{23}H_{20}N_4O$	C 75.00 H 5.43 N 15.21	74.92 5.31 15.11	320	3	3.9	Toxic
9	$C_6H_5CHOHCH_3$	CH_3	2-Br	—	101°[d]	—	—	—	—	320	0	0.3	—
10	$C_6H_5CHOHCH_3$	CH_3	2-NO_2	—	123°[d]	—	—	—	—	640	0	0.5	—
11	$C_6H_5CHOHCH_3$	CH_3	2-CH_3, 3-CH_3	—	78°[d]	—	—	—	—	640	0	0.3	—

[a] J. M. L. Mathur, M.S. thesis, University of Roorkee, Roorkee, India, 1971. Test data supplied by the Medicinal Chemistry Division, Walter Reed Army Institute for Research. [b] B = brown, Bk = brick, Bl = blood, Da = dark, D = dull, L = light, O = orange, R = red, and Y = yellow. [c] D = dose, mg./kg.; TD = toxic deaths attributed to drug toxicity when mice died 2–5 days postinfection of *P. berghei* (KBG-173); and T/C = increase in survival time of treated mice over controls. [d] *Reference 5.*

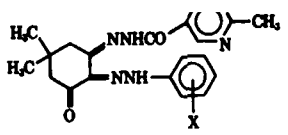


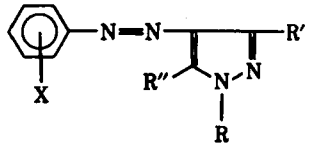
Table II—Chemical and Biological Properties Dimethylcyclohexane-1,2,3-trione Bishydrazones[a]

Number	X	Yield, %	Melting Point	Color[b]	Formula	Analysis, % Calc.	Analysis, % Found	Efficacy at Highest Tested Dose[c] T_g	N	C	O	Dose, mg./kg.
1	H	80	>270°[d]	DR	$C_{20}H_{22}N_6O_2$	C 60.91 H 5.58 N 21.31	60.72 5.34 21.41	—	0	75	0	300[e]
2	4-CH_3	78	>270°/	O	$C_{21}H_{24}N_6O_2$	C 61.76 H 5.88 N 20.58	61.56 5.82 20.68	—	0	0	0	300[e]
3	3-CH_3	75	137°	DaB	$C_{21}H_{24}N_6O_2$	C 61.76 H 5.88 N 20.68	61.66 5.85 20.60	—	—	—	—	—
4	4-OCH_3	78	264–266°/	LB	$C_{21}H_{24}N_6O_4$	C 59.43 H 5.66 N 19.81	59.23 5.43 19.79	—	0	0	0	100
5	2-OCH_3	65	152–154°/	DaB	$C_{21}H_{22}N_6O_4$	C 59.43 H 5.66 N 19.81	59.35 5.52 19.69	—	—	—	—	—
6	4-Cl	80	248–249°[d]	BlR	$C_{20}H_{21}ClN_6O_2$	C 56.00 H 4.60 N 19.60	56.20 4.59 19.42	—	—	—	—	—
7	4-Br	75	255–257°/	DaR	$C_{20}H_{21}BrN_6O_2$	C 50.73 H 4.43 N 17.75	50.53 4.22 17.55	—	0	0	0	100
8	3-NO_2	70	240–241°[d]	R	$C_{20}H_{21}N_7O_4$	C 54.66 H 4.78 N 22.32	54.46 4.52 22.22	0	0	0	0	100
9	2-NO_2	77	250–251°[d]	R	$C_{20}H_{21}N_7O_4$	C 54.66 H 4.78 N 22.32	54.56 4.42 22.30	0	0	0	50	100/400
10	2-Cl, 3-Cl	68	195–196°[d]	BkR	$C_{20}H_{20}Cl_2N_6O_2$	C 51.83 H 4.31 N 18.14	51.62 4.28 18.12	—	—	—	—	—
11	2-Cl, 4-Cl	70	180°	BkR	$C_{20}H_{20}Cl_2N_6O_2$	C 51.83 H 4.31 N 18.14	51.72 4.29 18.04	0	0	0	0	200
12	2-OCH_3, 4-OCH_3	72	215–217°	DaB	$C_{22}H_{26}N_6O_4$	C 58.14 H 5.72 N 18.50	58.04 5.70 18.48	—	—	—	—	—

[a] A. Singhal, M.S. thesis, University of Roorkee, Roorkee, India, 1971. Test data supplied by Dr. W. C. McGuire, Philips Roxane, Inc. [b] See *Footnote b* of Table I. [c] T_g = toxoplasma gondii-RH strain prevention of mortality in mice, N = nematodes, C = cestodes, and O = oxyurids. [d] Charring. [e] Approximate LD_{50}. / Decomposed.

Compounds of general structure I*a* were synthesized by the reaction of the sodium salt of oxymethyleneacetophenone (2) (II) with aryldiazonium salts to give III, which were then condensed with phenylhydrazine.

Bishydrazones (IV) were obtained by the condensation of 2-methyl-4-hydroxy-5-pyrimidylcarbohydrazide (V) with 5,5-dimethylcyclohexane-1,2,3-trione 2-arylhydrazones (VI). Compounds VI were prepared by the coupling of the diazotized anilines with 5,5-dimethylhexane-1,3-dione (3).



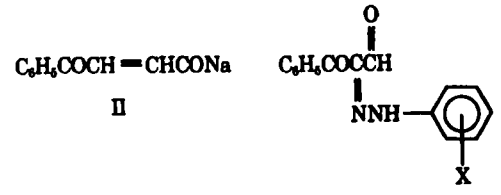
I*a*: R = R″ = C_6H_5, R′ = H

I*b*: R = $C_6H_5CHOHCH_2$, R′ = CH_3, R″ = C_6H_5

I*c*: R = —SO_2—(X), R′ = R″ = CH_3

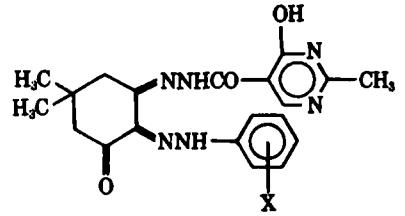
PHARMACOLOGY

Pyrazoles of series I*a* and I*b* appeared weakly active against *P. berghei* 173 in mice, increasing survival time up to 4.4 hr. over controls.



II

III



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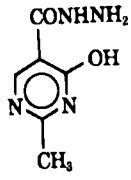

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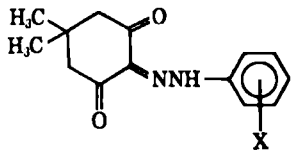

VI

Anthelmintic activity testing of bishydrazone derivatives (IV) revealed that all were inactive except 2-phenyl and 2-(2-nitrophenyl) derivatives.

EXPERIMENTAL[1]

1,5-Diphenyl-4-arylazopyrazoles (Ia)—The appropriate aniline was diazotized as reported previously (4) and treated with sodium salt of oxymethyleneacetophenone (II). The precipitated arylhydrazone (III) (0.01 mole), on treatment with phenylhydrazine (0.01 mole) in ethanol and acetic acid followed by refluxing for several hours and finally cooling to room temperature, afforded pyrazole derivatives (Ia). All of these compounds were recrystallized from acetone (Table I).

5,5-Dimethylcyclohexane-1,2,3-trione, 1-(2-Methyl-n-hydroxy-5-pyrimidylcarbo)-2-(substituted phenyl) Bishydrazones (IV)—A solution of 2-arylhydrazone of 5,5-dimethylcyclohexane-1,2,3-trione (VI) (3) (0.005 mole) in ethanol (15 ml.) and 2-methyl-4-hydroxy-5-pyrimidylcarbohydrazide (V) (0.005 mole) in glacial acetic acid (1 ml.) was refluxed for 1 hr. On cooling, shining crystals of IV separated out and were recrystallized from dimethylformamide (Table II).

[1] All melting points were determined with a Kofler hot-stage-type apparatus and are uncorrected.

REFERENCES

(1) H. G. Garg and N. Kaur, *J. Med. Chem.*, **15**, 554(1972).

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Synthesis and CNS Depressant Activity of Imidazolinone Glyoxylates

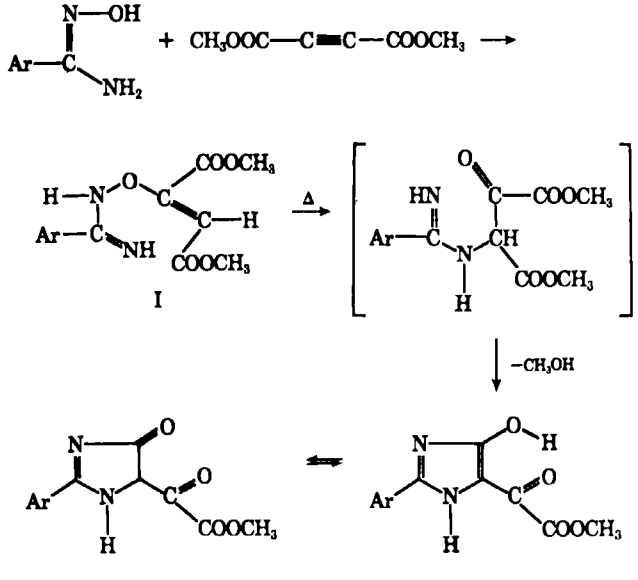
NED D. HEINDEL▲ and MARIA C. CHUN*

Abstract □ Six new imidazolinone glyoxylates, which exist in tautomeric equilibria with hydroxyimidazole glyoxylates, were synthesized by a new type of Claisen rearrangement and evaluated in a neuropharmacological mouse profile. CNS depression was observed in several members of the class.

Keyphrases □ Imidazolinone glyoxylates—synthesized and screened as potential CNS agents □ CNS agents, potential—synthesis and screening of imidazolinone glyoxylates □ Imidazoles—synthesis and CNS depressant activity of six imidazolinone glyoxylates

A recent publication (1) described the synthesis and characterization of a new type of imidazolinone glyoxylate (II) by the Claisen-like rearrangement of amide oxime–dimethyl acetylenedicarboxylate adducts (I). Pyrolysis of these adducts in refluxing diphenyl ether gave 41–68% yields of the tautomeric methyl 2-aryl-4-oxo-2-imidazoline-5-glyoxylates (II) (Scheme I). The impressive history of imidazoles as therapeutic agents (2, 3) and, more relevantly, the recent reports that several imidazoles function as centrally acting depressants (4–6) prompted this report of the evaluation of a new imidazole class in a primary Irwin neuropharmacological mouse profile (7).

The amide oximes utilized as starting materials were prepared by addition of hydroxylamine to aromatic nitriles. These amide oximes were condensed with dimethyl acetylenedicarboxylate to yield 1:1 adducts (I) in which —OH addition to the alkyne had occurred.



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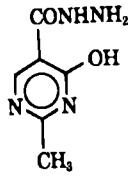
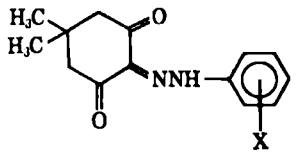


V



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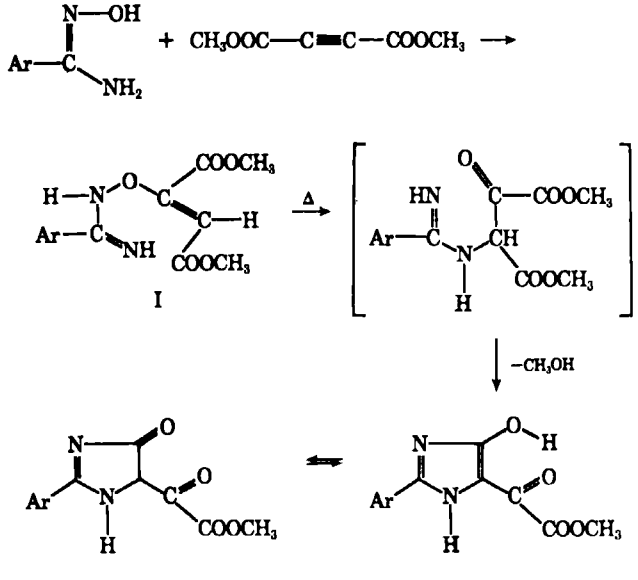

II

Scheme I

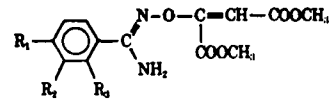

Table I—Amide Oxime–Dimethyl Acetylenedicarboxylate Adducts

Compound	R_1	R_2	R_3	Yield, %	Melting Point	Formula	Analysis, % Calc.	Found
Ia	H	H	H	61	78–79°	$C_{12}H_{14}N_2O_5$	C 56.11	56.04
							H 5.07	5.22
							N 10.07	10.22
Ib	Cl	H	H	64	57–58°	$C_{12}H_{13}ClN_2O_5$	C 49.91	49.63
							H 4.19	4.04
							N 8.96	8.72
Ic	CH_3	H	H	59	69–70°	$C_{14}H_{16}N_2O_5$	C 57.52	57.25
							H 5.52	5.62
							N 9.59	9.55
Id	H	H	CH_3	58[a]	—	—	Compound not isolated	—
								—
								—
Ie	OCH_3	OCH_3	H	64	82–84°	$C_{15}H_{18}N_2O_5$	C 58.81	59.01
							H 5.92	6.02
							N 9.15	9.32
If	CH_3	CH_3	H	62[a]	—	—	Compound not isolated	—
								—
								—

[a] Not obtained in crystalline form; viscous oils used directly for cyclization step.

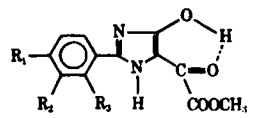

Table II—Imidazolinone Glyoxylates

Compound	R_1	R_2	R_3	Yield, %	Melting Point	Formula	Analysis, % Calc.	Found
IIa	H	H	H	68	240–242°	$C_{12}H_{10}N_2O_4$	C 58.53	58.63
							H 4.09	4.25
							N 11.38	11.20
IIb	Cl	H	H	63	256–257°	$C_{12}H_9ClN_2O_4$	C 51.35	51.49
							H 3.20	3.49
							N 9.98	10.02
IIc	CH_3	H	H	41	256–258°	$C_{13}H_{12}N_2O_4$	C 59.99	60.26
							H 4.64	4.86
							N 10.77	11.01
IId	H	H	CH_3	59	222–224°	$C_{13}H_{12}N_2O_4$	C 59.99	60.12
							H 4.64	4.50
							N 10.77	10.63
IIe	OCH_3	OCH_3	H	52	268–269°	$C_{14}H_{14}N_2O_6$	C 54.89	54.66
							H 4.61	4.63
							N 9.14	9.12
IIf	CH_3	CH_3	H	48	260–264°	$C_{14}H_{14}N_2O_4$	C 61.31	61.27
							H 5.15	5.13
							N 10.22	10.24

Simple ketoximes readily add to such acetylene esters (8). When these adducts (I) are heated to 250°, a thermal rearrangement ensues and the imidazolinones result. Several such Claisen-like transformations were reported recently (2, 8, 9).

BIOLOGICAL ACTIVITY

Compounds II*a–f* were administered as a single intraperitoneal injection in water–methylcellulose solution or suspension to each of four mice. The compounds were nontoxic at doses up to 300 mg./kg., and at that level all but II*f* effected marked depression and reduction of spontaneous motor activity. With the exception of the inactive II*f*, all of the compounds induced some writhing and an abnormal gait in the test animals. With II*a* at 300 mg./kg., there was also a loss of righting reflex. The most active member of the class appeared to be II*b*, since physiological signs (depression, lacrimation, body drop, loss of spontaneous activity, and Staub tail phenomenon) were most rapid in onset (6 min. *versus* a minimum of 15 min. for the other analogs at the 300-mg./kg. level). Furthermore, with II*b*, activity persisted down to 30 mg./kg., while no other test agent was active below 100 mg./kg. Moderate hypothermia (approximately 2.5°) was evident at 300 mg./kg. with II*b*, II*c*, and II*e*. None of the compounds was deemed sufficiently active to merit further study.

EXPERIMENTAL[1]

Preparation of the Amide Oximes—Benzamidoxime (10), *p*-chlorobenzamidoxime (11), *p*-methylbenzamidoxime (10), *o*-methylbenzamidoxime (10), and 3,4-dimethylbenzamidoxime (12) were prepared by the Method A_1 described by Eloy and Lenaers (10) and had physical properties in agreement with those reported. The yet unreported 3,4-dimethoxybenzamidoxime was synthesized by Method A_1 in 73% yield, m.p. 163–164° (from methanol).

Anal.—Calc. for $C_9H_{12}N_2O_3$: C, 55.24; H, 6.17. Found: C, 55.22; H, 6.19.

General Procedure for Preparation of Amide Oxime–Dimethyl Acetylenedicarboxylate Adducts (I*a–f*)—Equimolar quantities (0.1

[1] Melting points were obtained between cover glasses on a Fisher-Johns block and are uncorrected. Elemental analyses were provided by Dr. George I. Robertson, Microanalytical Laboratory, Florham Park, N. J.

mole) of the aromatic amide oxime and dimethyl acetylenedicarboxylate were dissolved in 100 ml. of anhydrous methanol. The mixing of the reactants was often mildly exothermic, but the solution was heated at reflux for 3 hr. to complete the reaction. The methanol was removed *in vacuo*, and the resulting oil was induced to crystallize by chilling and scratching (two exceptions as noted in Table I). These low melting solids were purified by repeated recrystallization from methanol to analytical purity.

General Procedure for Thermal Rearrangement to Imidazolinones (IIa–f)—A mixture of approximately 2.0 g. of the amide oxime–dimethyl acetylenedicarboxylate adduct and 25 ml. of diphenyl ether was heated at reflux for 2 hr. The solution was cooled to room temperature and diluted with *n*-hexane, and the precipitated solid was collected. The crude product was recrystallized from methanol with the aid of decolorizing carbon. Yields and melting points are reported in Table II.

REFERENCES

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Differential Thermal Analysis and X-Ray Diffraction Studies of Griseofulvin–Succinic Acid Solid Dispersions

WIN LOUNG CHIOU▲ and SARFARAZ NIAZI

Abstract □ Differential thermal analysis and X-ray diffraction techniques were employed to study the phase diagrams of griseofulvin–succinic acid systems. The complications of the formation of a thermally decomposed product of succinic acid, succinic anhydride, on the thermograms were discussed. The appearance of typical eutectic peaks and X-ray diffraction peaks of griseofulvin in the resolidified preparations containing 10% or less of griseofulvin indicates that the binary system is a simple eutectic mixture with negligible mutual solid solubilities. This result is partly different from that obtained previously by the microthermal microscope technique, which showed the existence of an extensive solid solution of griseofulvin in succinic acid. The formation of a solid solution was previously concluded to be primarily responsible for the enhanced dissolution of griseofulvin.

Keyphrases □ Griseofulvin–succinic acid solid dispserions—phase diagrams, differential thermal analysis and X-ray diffraction, results compared to microthermal microscope technique □ Succinic acid–griseofulvin solid dispersions—phase diagrams, differential thermal analysis and X-ray diffraction, results compared to microthermal microscope technique □ Differential thermal analysis—phase diagrams of griseofulvin–succinic acid solid dispersions □ X-ray diffraction—phase diagrams of griseofulvin–succinic acid solid dispersions

The classification and pharmaceutical applications of solid dispersion systems were recently reviewed by Chiou and Riegelman (1). A knowledge of the physicochemical properties of a solid dispersion is very important toward an understanding of its applications. Chloramphenicol dispersed in urea was shown to result in faster dissolution and absorption rates (2, 3). The enhancement was thought primarily due to the formation of a solid solution of the drug in the carrier, urea (3). Recently, Chiou (4) used differential thermal analysis and X-ray diffraction methods to reexamine this system, and he concluded that the system is a simple eutectic mixture with negligible mutual solid solubility.

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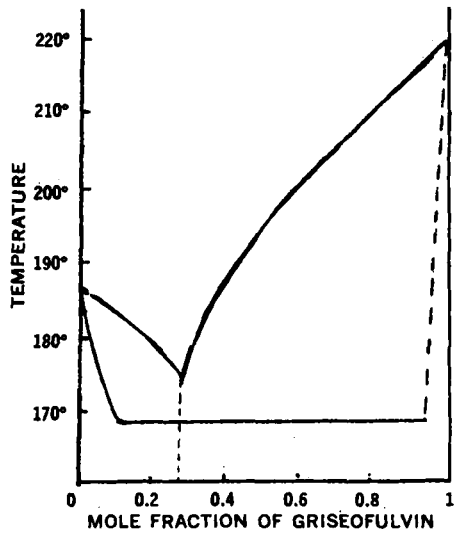

Figure 1—*Phase diagram for griseofulvin–succinic acid mixtures obtained by microthermal techniques. (From* Reference 5.)

lowing the solidification of the melt of the mixture. Griseofulvin dispersed in succinic acid was previously shown by Goldberg *et al.* (5) to produce a much faster dissolution rate and a supersaturation phenomenon. These observations were attributed to the formation of a solid solution of the drug in the carrier. This conclusion was based on their analysis of the phase diagram (reproduced in Fig. 1) determined by the microthermal microscope technique. The purpose of this paper is to report a different finding regarding the griseofulvin–succinic acid phase diagram study obtained through more sophisticated and objective techniques, namely differential thermal analysis and X-ray diffraction.

EXPERIMENTAL

Sample Preparations—Physical mixtures of griseofulvin[1] and succinic acid[2] were obtained by mixing in either a microball mill[3] or mortar. Griseofulvin–succinic acid solid dispersions were prepared by the fusion method (5). The concentration of griseofulvin in each preparation was confirmed spectrophotometrically using a spectrophotometer[4] (5).

Thermal and X-Ray Diffraction Studies—The procedure and instrumentation for differential thermal analysis, X-ray diffraction, and capillary tube melting-point studies were identical to those reported previously (6). Heating rates of 5 and 10°/min. used in this study were found to give almost identical thermograms.

RESULTS AND DISCUSSION

Phase Diagram Determination from Physical Mixtures—It has been generally found that the binary phase diagrams determined from physical mixtures through thermal analyses often correlate well with those determined from fused or evaporated mixtures (7, 8). The phase diagram of the griseofulvin–succinic acid system, determined from their physical mixtures, that has not been previously reported is shown in Fig. 2. The eutectic melting was observed in all samples containing 1–99% (w/w) of griseofulvin. This might be indicative of a lack of an extensive mutual solid solubility between the two components. Similar eutectic peaks were also observed in all physical mixtures of the sulfathiazole–urea binary system (6). However, certain mutual solid solubilities between the two components were found in their solidified fused preparations (6).

[1] Griseofulvin USP (micronized), supplied by McNeil Laboratories, Fort Washington, Pa.
[2] Succinic acid, supplied by Merck & Co., Inc., Rahway, N. J.
[3] Ball mill shaker, Crescent Dental Manufacturing Co., Chicago, Ill.
[4] Beckman DBG.

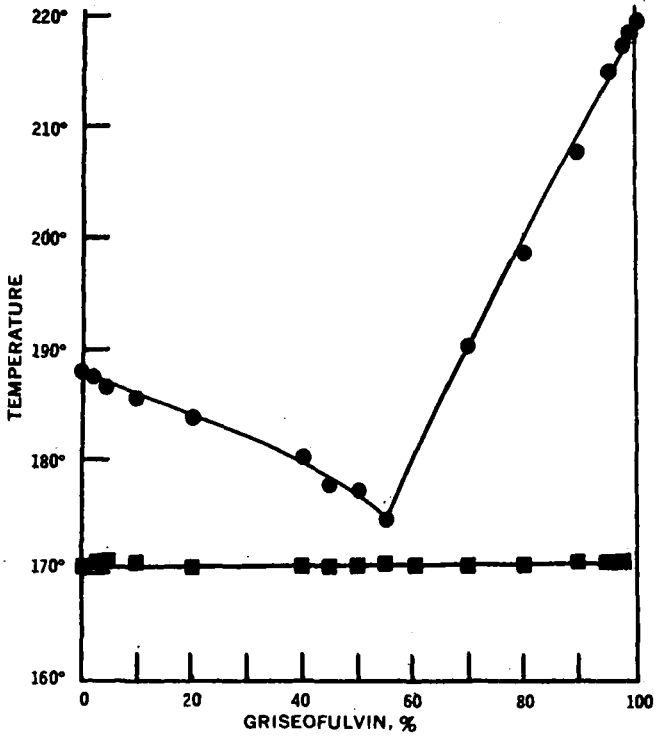

Figure 2—*Phase diagram of griseofulvin–succinic acid binary system determined from physical mixtures. Key:* ■, *thaw point; and* ●, *final melting point.*

The thaw points in this study were determined from the differential thermal analysis thermograms, because differential thermal analysis instruments are more sensitive than the conventional capillary tube method in detecting the beginning of melting of a small fraction of a sample (1, 6). They were all found to be around 170°, which is about 2.5° higher than the previously reported value (5). The final melting points of the samples were determined from both

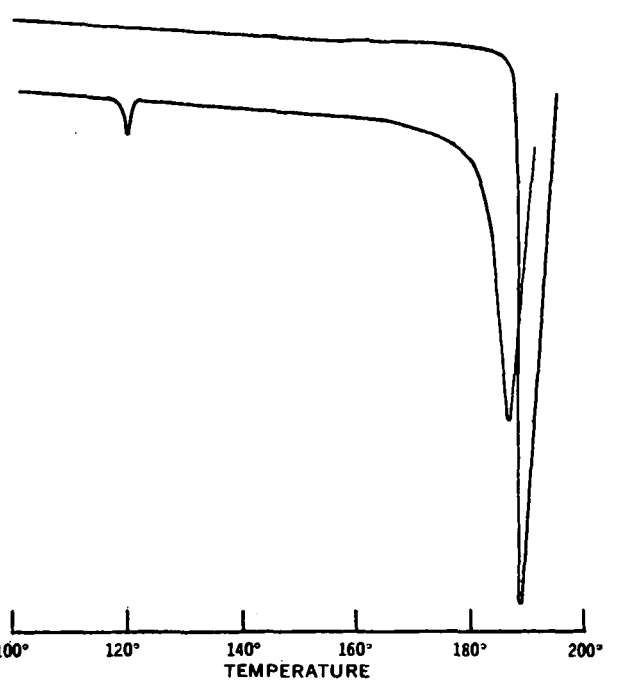

Figure 3—*Thermogram of succinic acid. Key: top, first run; and bottom, rerun of the sample after 5 min.*

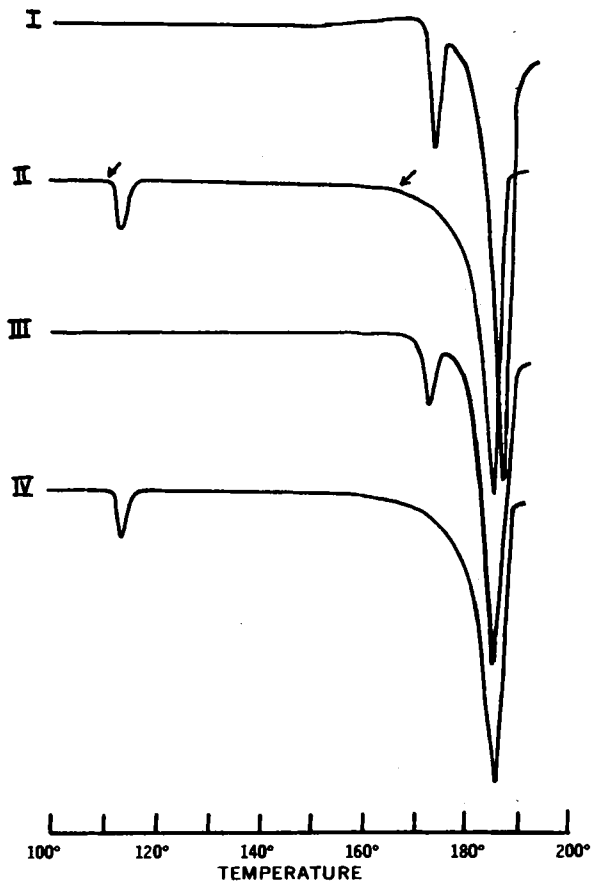

Figure 4—*Thermograms of different samples of 10% griseofulvin. Key: I, physical mixture; II, solid dispersion; III, solid dispersion incubated at 80° for 5 days; and IV, 10% griseofulvin–90% melted succinic acid physical mixture.*

the differential thermal analysis and capillary tube methods. However, for samples that showed ambiguities of the final melting on differential thermal analysis thermograms (6, 8), the values determined from the capillary tube method were used in the construction of the phase diagrams (1, 6).

Thermal Stability of Griseofulvin and Succinic Acid—To determine the phase diagram of a binary system from resolidified fused preparations, it is necessary to study first the thermal stability of both components. If any significant chemical decomposition takes place during the initial melting process, the phase diagram determined might be quite complicated or sometimes misleading because the system now contains three or more components. The thermal stability of griseofulvin was demonstrated by essentially the identical differential thermal analysis thermograms of its original and resolidified pulverized samples. This is in agreement with some previous studies (9, 10). Although succinic acid (m.p. 185–187°, b.p. 235°) has been reported (11) to undergo a partial conversion to succinic anhydride (m.p. 119.6°, b.p. 261°), it appears that no data are available in the literature regarding its thermal stability around its melting point.

The thermogram of the resolidified fused samples obtained from this study showed an extra endothermic peak beginning at 118°. The melting range of its major peak was also widened and considerably shifted to the lower temperature (Fig. 3). Furthermore, the intensity of the extra endothermic peaks and the widening and shifting of the major peaks were all further enhanced when the sample was fused and solidified repeatedly. From a theoretical consideration (1, 13), this observation agrees with a contention that succinic acid will partially decompose after melting and will form a eutectic mixture with its thermally decomposed product. The extent of decomposition also appears to increase with repeated fusion of the acid. It is reasonable to assume that succinic anhydride is also the decomposition product. The thermal decomposition was also confirmed by the sublimed deposition of a thin white layer of film on

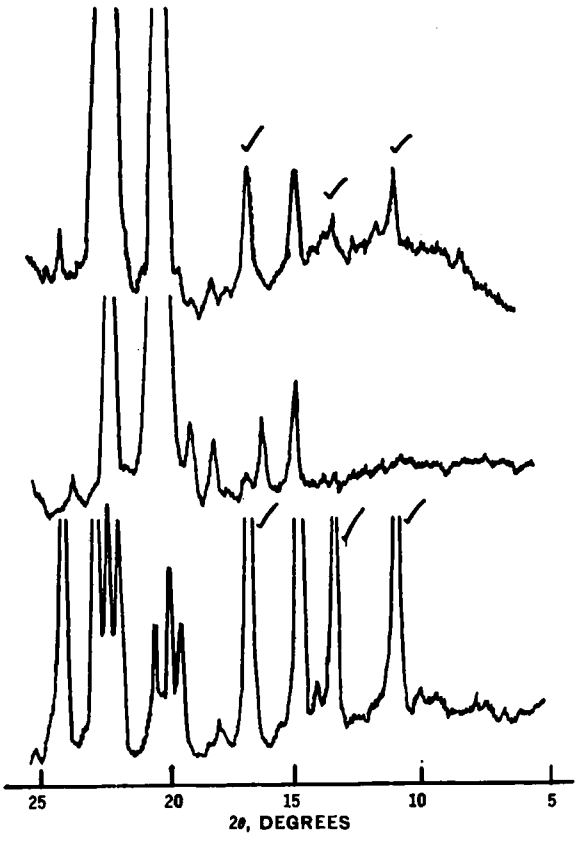

Figure 5—*X-ray diffraction spectra. Key: top, 10% griseofulvin solid dispersion; middle, resolidified succinic acid; and bottom, griseofulvin. Tick marks indicate the characteristic peaks of griseofulvin.*

the cover glass when the resolidified succinic acid powder was heated to about 120° on a hot stage attached to a microscope[5]. Such a sublimation, however, could not be detected when succinic acid was directly heated to 150° without prior fusion and solidification. The sublimation phenomenon of succinic anhydride was reported previously (11). The presence of succinic anhydride in the resolidified succinic acid sample was also confirmed by the preliminary X-ray diffraction study.

Thermal Analyses of Resolidified Fused Mixtures—Although thermal analyses were conducted for many resolidified preparations containing various amounts of griseofulvin and succinic acid, only the 10% (w/w) griseofulvin–90% succinic acid resolidified preparation (or solid dispersion) will be discussed in detail. Since succinic acid might undergo a partial thermal decomposition, its complicating effect on the differential thermal analysis thermogram of the 10% griseofulvin solid dispersion was reasonably anticipated.

Thermograms of both the solid dispersion and the physical mixture are shown in Fig. 4. Two endothermic peaks were present, beginning at 109 and 166°, respectively, in the solid dispersion thermogram (as shown by the arrows). In addition, its final melting peak appeared a few degrees lower than that of the physical mixture. The peak starting at 109° is attributed to the presence of succinic anhydride. This conclusion is also supported by the following evidence: (*a*) a similar sublimation phenomenon was also observed for the solid dispersion at about 120°; and (*b*) a similar thermogram pattern was also obtained from the physical mixture of 10% griseofulvin–90% resolidified succinic acid (Fig. 4) and from the physical mixture of 10% griseofulvin–87.5% succinic acid–2.5% succinic anhydride. It was estimated from the thermograms that about 7% of succinic anhydride was present in the 10% griseofulvin solid dispersion.

During the early stage of this investigation, it was thought that the observed peculiarities of the above solid dispersion thermogram might have been caused by the metastable state of the sample fol-

[5] Kofler.

lowing resolidification of the melt at room temperature. Therefore, the thermogram was again recorded for samples that had been incubated at 80° for 5 days in sealed capillary tubes. Surprisingly, the earlier peak disappeared and the griseofulvin–succinic acid eutectic peak, with an intensity similar to that found in the physical mixture, reappeared (Fig. 4). The disappearance of the earlier peak is thus likely to be due to the sublimation of succinic anhydride to the upper part of the capillary tube during the annealing process. The reappearance of the original eutectic peak also clearly indicates that the majority of griseofulvin is present as griseofulvin crystals and not as solid solution in the solid dispersion.

It must be emphasized that, based on the phase rules and differential thermal analysis principles (1, 4, 6), the thaw point or the beginning of the endothermic peak of the thermogram must be higher than the eutectic temperature if the sample is a solid solution as originally proposed by Goldberg *et al.* (5). The peculiar thermal characteristics of the 10% griseofulvin solid dispersion were also observed in the 1, 2, and 5% griseofulvin solid dispersions. In other words, based on this method of analysis, the solid solubility of griseofulvin in succinic acid at or above the eutectic temperature should be less than 1% or negligible. This is not in agreement with the previous study in which a solid solubility of 25% griseofulvin in succinic acid was reported. As reported (4) earlier for the chloramphenicol–urea system, such discrepancy is probably due to the more subjective visual method used in these previous studies. As beginners in microscopy, the present authors have experienced uncertainty in ascertaining the exact thaw points of the solid dispersion samples using an identical technique to that employed by previous workers (5). The thaw point varied between 110 and 170°. The reported limited solid solubility of succinic acid in griseofulvin was also confirmed by the present study. The resolidified samples containing 95 and 98% of griseofulvin all showed eutectic peaks at about 166°.

X-Ray Diffraction Studies—The X-ray diffraction technique was used recently to study the physical nature of drugs in solid dispersion systems (4, 6, 12). It was employed also in this investigation to support the differential thermal analyses results showing the limited solubility of griseofulvin in succinic acid. Theoretically, the characteristic diffraction peaks of the solid solute should be absent from the diffraction spectrum of a solid solution (1). However, this is not the case for the 10% griseofulvin solid dispersion. Three major peaks of griseofulvin at 10.8, 13.3, and 16.6° (13) were all present in the 10% griseofulvin solid dispersion (Fig. 5). The comparable areas under these peaks between the solid dispersion and physical mixture (10% griseofulvin) also indicate that most of the griseofulvin was present as crystals and not as a solid solution in the preparation. A slight line broadening effect was observed in the freshly prepared solid dispersion which might indicate the presence of ultrafine crystals of griseofulvin. For example, the ratio for the peak height over the peak width at 10.8° was increased about onefold after aging at 80° for 5 days. The effect of peak broadening on the dissolution rate of griseofulvin is currently under investigation in this laboratory.

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Quantitative Determination of Morphine in Poppy Capsules by Differential Spectrophotometry

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Abstract □ A procedure for a rapid quantitative analysis of morphine in poppy capsules is described. It is based on differential spectrophotometry between alkaline and acidic solutions. The method was applied to poppy capsules cultivated in Argentina. The results were statistically compared with those obtained using a TLC method. Both methods showed no significant differences.

Keyphrases □ Morphine, in poppy capsules—determination by differential spectrophotometry □ Poppy capsules, Argentine—analysis of morphine content, differential spectrophotometry □ Opium alkaloids—determination of morphine in Argentine poppy capsules, differential spectrophotometry □ Spectrophotometry, differential—determination of morphine in poppy capsules

Morphine is still important in pharmacy for its direct use and as a source of semisynthetic derivatives. It is obtained from poppy capsules and from opium. Both foreign opium and local poppy capsules are used in Argentina (1), but the latter is obtained from domestic harvests of *Papaver somniferum* var. *nigrum*.

Between 1943 and 1970, poppy was cultivated in different provinces of Argentina in search of favorable growing conditions. The province of Mendoza fulfilled the minimum requirements. It was essential to produce an homogeneous race of alkaloid-rich plants to improve the average morphine content of poppy capsules. Thus, it was necessary to devise a method for determining morphine in a single capsule.

A number of gravimetric (2–4), titrimetric (3, 5), spectrometric (6–17), polarimetric (18), chromatographic (19–28), and electrophoretic (21) methods have been reported for the quantitative determination of morphine. The differential spectrophotometric method seemed the most suitable for a crude extract in which many interfering substances were expected to be present.

Differential spectrophotometry in the analysis of pharmaceutical products was studied by Milos (6) and later by Casinelli and Sinsheimer (8) who used 1 *N* sodium hydroxide solutions, finding it inapplicable to morphine unless it had been previously separated by chromatography. Gupta (12) found 0.1 and 0.01 *N* sodium hydroxide solutions to be more suitable and developed a practical way for determining the alkaloid even in mixtures. Recently, a similar method (29) has been developed and applied to the determination of morphine in tablets, suppositories, and eardrops. Up to now, differential spectrometry has not been applied to the determination of morphine in poppy capsules.

Table I—Absorbance–Concentration Relationships

Morphine Concentration, mg./100 ml.	Absorbance
0	0
0.97	0.059
1.94	0.121
2.91	0.190
3.88	0.254
4.84	0.321
5.81	0.387
6.78	0.450
7.75	0.516
8.72	0.595
9.69	0.655

EXPERIMENTAL

Apparatus—A spectrophotometer[1] with 10-mm. path rectangular cells was used.

Reagents—Analytical grade reagents were used:

0.1 N *Sodium Hydroxide*—Dissolve 4.00 g. of sodium hydroxide in 1.00 l. of water.

30% Ammonium Phosphate—Dissolve 30 g. of ammonium phosphate, monobasic, in water to make 100 ml. total volume.

0.2 N *Hydrochloric Acid*—Add 16.6 ml. of concentrated hydrochloric acid to enough water to make 1.00 l.

2% Sodium Nitrite—Dissolve 2.0 g. of sodium nitrite in 100 ml. of water.

10% Ammonium Hydroxide—Add 350 ml. of concentrated ammonia to enough water to make 1.00 l.

Plant Material—Dried ripe capsules of *Papaver somniferum* L. var. *nigrum* DC., cultivated in the province of Mendoza (Argentina) in 1970, were used. They were chosen at random and obtained from well-grown, healthy plants.

The capsules were opened and the seeds were discarded. The material was powdered, thoroughly mixed, and dried at 60° to constant weight. The same batch was used throughout the study.

Preparation of Alkaloidal Extract—Transfer an accurately weighed aliquot (about 2.50 g.) into a conical flask with a stopper and add a mixture of 2 ml. of 10% ammonium hydroxide and 6 ml. of 96% ethanol. Add 30 ml. of isopropanol–chloroform (1:3) and stir the mixture for 30 min. Filter the solution through paper into a 50-ml. volumetric flask and bring to volume.

Procedure A: Differential Spectrophotometry—*Standard Preparation*—Accurately weigh about 10.0 mg. of anhydrous morphine or its equivalent of a suitable salt. Dissolve it in 0.1 *N* sodium hydroxide to make 100 ml. total volume. Use this solution immediately.

Calibration—Use 0.1 *N* sodium hydroxide to dilute the standard preparation to obtain 11 solutions containing between 0 and 0.100 mg. of anhydrous morphine per milliliter. Transfer two 2.7-ml. portions of each solution to separate cells (*r* and *s*). Add 0.45 ml. of 30% ammonium phosphate to the reference cell (*r*) and 0.45 ml. of 0.1 *N* sodium hydroxide to the sample cell (*s*); mix carefully. Set the wavelength control knob to 320 nm. and adjust to 100% transmittance. Turn the wavelength control knob to 299 nm. and determine the absorbance of the *s versus* the *r* cell. Use the data thus obtained to calculate the absorptivity (*a*) from Eq. 1:

$$a = \frac{\Sigma AC - (\Sigma C \Sigma A/n)}{\Sigma C^2 - [(\Sigma C)^2/n]} \qquad \text{(Eq. 1)}$$

and the A_0 value from Eq. 2:

$$A_0 = \frac{\Sigma A}{n} - a \cdot \frac{\Sigma C}{n} \qquad \text{(Eq. 2)}$$

[1] Beckman DBG.

Table II—Comparison of Assay Methods for Morphine Applied to Poppy Capsules

Sample Number	Morphine Found, g./100 g. Poppy Capsules Procedure A	Procedure B
1	0.516	0.563
2	0.538	0.542
3	0.541	0.528
4	0.513	0.515
5	0.566	0.529
6	0.537	0.501
7	0.510	0.490
8	0.507	0.470
Average	0.5285	0.5173
SD	±3.9%	±5.8%

where A is the absorbance corresponding to the concentration C, n is the number of observations, and A_0 is the theoretical absorbance when $C = 0$.

Procedure—Transfer 10 ml. of the alkaloidal extract to a separator and extract with 0.1 *N* sodium hydroxide (3 × 15 ml.). Collect the alkaline solution in a 50-ml. volumetric flask, and dilute to volume with 0.1 *N* sodium hydroxide. If necessary, centrifuge to clarify the solution. Transfer two 2.7-ml. portions to separate cells (*r* and *s*). Measure the absorbance of the *s versus* the *r* cell as stated under *Calibration*. The amount of morphine in the sample is calculated using Eq. 3:

$$\text{g. morphine/100-g. capsule} = \frac{(A - A_0)}{a} \cdot \frac{25}{q} \qquad \text{(Eq. 3)}$$

where A is the measured absorbance, a is the absorptivity calculated using Eq. 1, A_0 is the theoretical absorbance when $C = 0$ (previously calculated with Eq. 2), and q is the weight in grams of the sample.

Procedure B: TLC—*Standard Preparation*—Accurately weigh about 40.0 mg. of anhydrous morphine or its equivalent of a suitable salt. Dissolve in isopropanol–chloroform (1:3) to make 100 ml. total volume, and keep the solution in a refrigerator. Renew monthly.

Procedure—A modified version of the method of Heusser and Jackwerth (11) was used. The absorbent was silica gel PF_{254}[2]. Chromatography was carried out on 20 × 20-cm. glass plates coated with a 250-μ layer. They were heated at 130° for 30 min. before spotting. The solvent system was chloroform–acetone–methanol–25% ammonia solution (30:40:10:5). Solutions were spotted with a 1-ml. pipet in 2.5-cm. bands. A total of five bands was used per plate as follows: 1, 0.30 ml. standard preparation; 2, 1.20 ml. alkaloidal extract; 3, 0.60 ml. standard preparation; 4, 1.20 ml. alkaloidal extract; and 5, 1.20 ml. standard preparation.

The plates were developed (ascendingly) to 15 cm. After development, they were dried at room temperature and viewed under UV 254-nm. light. The morphine spots were marked and then scraped off with a spatula. Each spot was transferred to a centrifuge tube and extracted by shaking with 3 ml. of 0.2 *N* hydrochloric acid. An extra tube containing 3 ml. of 0.2 *N* hydrochloric acid was added at this point as a reagent blank. To each tube, 2 ml. of 2% sodium nitrite was added. The tubes were allowed to stand at room temperature for 15 min. and then made basic by the addition of 3 ml. of 10% ammonium hydroxide; the silica layer was separated by centrifugation.The absorbance *versus* the reagent blank was immediately determined at 470 nm. The values of a and A_0 were calculated for each plate using Eqs. 1 and 2 (where C refers to the quantity of morphine applied to each standard band). The assay values of morphine were determined using the relationship:

$$\text{g. morphine/100-g. capsule} = \frac{A_2 + A_4 - 2A_0}{12a} \cdot \frac{25}{q} \qquad \text{(Eq. 4)}$$

where A_2 and A_4 are the absorbances corresponding to bands 2 and 4 of the corresponding plate, respectively, and q is the weight in grams of the sample.

[2] Merck.

Table III—Application of the Student *t* test to the Differences between the Data from Table II

Sample Number	Difference[a]
1	−0.047
2	−0.004
3	0.013
4	−0.002
5	0.037
6	0.036
7	0.020
8	0.037
$\bar{d}$	0.01125
s^2	0.000831
$s_{\bar{d}}$	0.01019
t_0'	1.1036
$t_{0.3}$ (from tables)	1.119

[a] Procedure A − Procedure B = d_i.

RESULTS AND DISCUSSION

By using differential spectrophotometry as described, the concentration of a pure morphine solution was shown to be linear with the absorption (Table I). The average of eight determinations of morphine in the batch gave 0.5285 g. of anhydrous morphine/100-g. capsule. The relative standard deviation was 3.9%. The compatibility of these results with the 0.10–0.60% values found in the literature (21, 32, 33) and with the average industrial yield (0.2%) (32, 34) is of interest.

The method of Heusser and Jackwerth (11), in which morphine is previously isolated by TLC, was selected for comparative purposes. The alkaloidal extract was chromatographed, and then morphine was eluted and submitted to the Radulescu reaction (30), a simple case of phenol nitrosation (31). The nitrosomorphine was measured at 470 nm. An average of eight determinations gave 0.5173 g. of anhydrous morphine/100-g. capsule, and the relative standard deviation was 5.8%. Results of both methods are shown in Table II.

A comparison between averages shows a difference of 0.0112 g. of morphine/100-g. capsule. Statistical analysis of the data showed that this difference was not significant.

As the same alkaloidal extract was used for each pair of trials, the Student *t* test could also be applied to the respective differences (Procedure A − Procedure B = d_i) (Table III).

From the table of *t*, it was found that the chance of obtaining a value of *t* greater than or equal to $\pm t_0'$ (1.104) is slightly greater than 30 in 100 ($t_{0.3} = 1.119$). Hence, the null hypothesis ($\bar{d} = 0$) cannot be rejected at the 5% level of significance. The conclusion is also that the two methods of analysis did not produce significantly different results.

CONCLUSION

The present method allows a relatively rapid determination of morphine in poppy capsules without prior chromatography of the crude extract. It is accurate enough to be used in the selection of the alkaloid-rich capsules.

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Inhibition of Pyruvic Acid Oxidation by 2,5-Substituted 1,3,4-Oxadiazoles

H. H. SINGH, S. NAGAR, A. CHAUDHARI, and SURENDRA S. PARMAR▲

Abstract □ Several 2-substituted amino-5-(dichlorophenoxymethyl)-1,3,4-oxadiazoles were synthesized by cyclizing various corresponding 1-(dichloro substituted phenoxyacetyl)-4-substituted thiosemicarbazines. These compounds exhibited the ability to inhibit oxidation of pyruvic acid by rat brain homogenate and showed gross behavioral depression in mice on intraperitoneal administration.

Keyphrases □ Pyruvic acid oxidation—2,5-substituted 1,3,4-oxadiazoles, synthesized and tested as inhibitors, rats, mice □ 1,3,4-Oxadiazoles, 2,5-substituted—synthesis, studied as inhibitors of pyruvic acid oxidation, rats, mice □ Thiosemicarbazines, substituted—synthesis, studied as inhibitors of pyruvic acid oxidation, rats, mice

Several 1-acyl-4-substituted thiosemicarbazines and their cyclized 1,3,4-oxadiazoles have been reported to possess diverse biological properties. The ability of 2,5-disubstituted 1,3,4-oxadiazoles to exhibit analgesic (1, 2), anti-inflammatory (2–4), antipyretic (2), muscle relaxant (5–7), tranquilizing (5, 6), and CNS depressant (8) activities led the present authors to synthesize several 2-substituted amino-5-aryl-1,3,4-oxadiazoles. Furthermore, selective inhibition of nicotinamide adenine dinucleotide-dependent oxidation by CNS depressants such as substituted 4-quinazolones (9–11) prompted the evaluation of the intermediate thiosemicarbazines and cyclized oxadiazoles for their ability to inhibit oxidation of pyruvic acid by rat brain homogenate. Attempts also were made to determine the structure–activity relation of these compounds to their enzyme inhibitory activity. The various oxadiazoles were synthesized according to Scheme I.

EXPERIMENTAL

Ethyl 2,6-Dichlorophenoxyacetate (I)—An equimolar quantity of 2,6-dichlorophenol (8.15 g.), ethyl chloroacetate (6.15 g.), and potassium carbonate (10.35 g.) in dry acetone (50 ml.) was refluxed under anhydrous conditions for 10 hr. The reaction mixture was filtered and the filtrate was poured into 50 ml. of chilled water. The ester was extracted with ether and dried over magnesium sulfate. On removing the excess ether, the remaining liquid was fractionally distilled. The fraction boiling at 180–185° was collected, yielding 9.90 g. (80%).

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EXPERIMENTAL

Ethyl 2,6-Dichlorophenoxyacetate (I)—An equimolar quantity of 2,6-dichlorophenol (8.15 g.), ethyl chloroacetate (6.15 g.), and potassium carbonate (10.35 g.) in dry acetone (50 ml.) was refluxed under anhydrous conditions for 10 hr. The reaction mixture was filtered and the filtrate was poured into 50 ml. of chilled water. The ester was extracted with ether and dried over magnesium sulfate. On removing the excess ether, the remaining liquid was fractionally distilled. The fraction boiling at 180–185° was collected, yielding 9.90 g. (80%).

Table I—1-(Dichlorophenoxyacetyl)-4-substituted Thiosemicarbazines and Their Effect on the Oxidation of Pyruvic Acid

$ArOCH_2CONHNHC(=S)NHR$

Compound Number	R	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Found	Percent Inhibition[b]
			Ar = 2,6-Dichlorophenyl				
III	n-C_4H_9	151°	72	$C_{13}H_{17}Cl_2N_3O_2S$	N 12.00	11.93	13.3 ± 1.0
IV	$CH_2CH{=}CH_2$	176–177°	69	$C_{12}H_{13}Cl_2N_3O_2S$	N 12.59	12.31	62.7 ± 0.4
V	C_6H_{11}	172–174°	70	$C_{15}H_{19}Cl_2N_3O_2S$	N 11.17	11.50	75.0 ± 0.8
VI	C_6H_5	180°	64	$C_{15}H_{13}Cl_2N_3O_2S$	N 11.35	11.57	31.3 ± 0.7
VII	p-BrC_6H_4	197°	70	$C_{15}H_{12}BrCl_2N_3O_2S$	C 40.09	39.91	73.5 ± 1.2
					H 2.67	2.55	
					N 9.33	8.92	
VIII	p-IC_6H_4	175°	85	$C_{15}H_{12}Cl_2IN_3O_2S$	N 8.46	8.08	78.1 ± 1.0
IX	o-$CH_3C_6H_4$	171°	70	$C_{16}H_{15}Cl_2N_3O_2S$	C 50.76	50.56	40.3 ± 1.2
					H 3.85	3.65	
					N 10.93	10.63	
X	p-$CH_3C_6H_4$	196°	65	$C_{16}H_{15}Cl_2N_3O_2S$	N 10.93	10.91	42.1 ± 1.0
XI	m-$OCH_3C_6H_4$	167°	68	$C_{16}H_{15}Cl_2N_3O_3S$	N 10.50	10.84	35.3 ± 1.0
XII	p-$OCH_3C_6H_4$	180–181°	70	$C_{16}H_{15}Cl_2N_3O_3S$	N 10.50	10.86	63.5 ± 1.0
			Ar = 3,4-Dichlorophenyl				
XIII	n-C_4H_9	129°	65	$C_{13}H_{17}Cl_2N_3O_2S$	C 50.32	50.17	15.4 ± 0.8
					H 5.48	5.32	
					N 12.00	12.32	
XIV	$CH_2CH{=}CH_2$	125°	68	$C_{12}H_{13}Cl_2N_3O_2S$	N 12.59	12.64	76.6 ± 0.8
XV	C_6H_{11}	149°	70	$C_{15}H_{19}Cl_2N_3O_2S$	N 11.17	10.77	84.4 ± 0.9
XVI	C_6H_5	165°	72	$C_{15}H_{13}Cl_2N_3O_2S$	N 11.35	11.21	60.9 ± 1.2
XVII	p-BrC_6H_4	180°	78	$C_{15}H_{12}BrCl_2N_3O_2S$	N 9.33	8.96	85.2 ± 1.1
XVIII	p-IC_6H_4	182°	70	$C_{15}H_{12}Cl_2IN_3O_2S$	C 36.29	35.90	84.2 ± 1.0
					H 2.41	2.23	
					N 8.46	8.83	
XIX	o-$CH_3C_6H_4$	123°	70	$C_{16}H_{15}Cl_2N_3O_2S$	N 10.93	11.34	21.3 ± 0.5
XX	p-$CH_3C_6H_4$	160°	66	$C_{16}H_{15}Cl_2N_3O_2S$	N 10.93	11.35	39.7 ± 0.5
XXI	m-$OCH_3C_6H_4$	154°	75	$C_{16}H_{15}Cl_2N_3O_3S$	N 10.50	10.80	67.2 ± 0.8
XXII	p-$OCH_3C_6H_4$	167°	75	$C_{16}H_{15}Cl_2N_3O_3S$	N 10.50	10.82	30.1 ± 1.0

[a] All melting points were taken in open capillary tubes and are corrected. [b] Each experiment was done in duplicate, and the values are the mean of three separate experiments. The oxygen uptake was measured at 5-min. intervals during a 1-hr. incubation period. The percentage inhibition was calculated from the decrease in the oxygen uptake/125 mg. wet brain weight. The final concentrations of pyruvic acid and the thiosemicarbazines were 10 and 1 mM, respectively. The percent inhibition of 37.3 ± 0.8% observed with an anticonvulsant, 2-methyl-3-O-tolyl-4-quinazolone, in a final concentration of 1 mM under similar experimental conditions was determined for the sake of comparison.

Anal.—Calc. for $C_{10}H_{10}Cl_2O_3$: C, 48.19; H, 4.01. Found: C, 48.00; H, 3.80.

2,6-Dichlorophenoxyacetylhydrazine (II*a*)—To a solution of 7.05 g. of methyl 2,6-dichlorophenoxyacetate (0.03 mole) in absolute ethanol (20 ml.) was added 2.25 g. of 99–100% hydrazine hydrate (0.045 mole), and the mixture was refluxed under anhydrous conditions on a water bath for 2 hr. On cooling, a white solid mass which separated out was collected by filtration and recrystallized from ethanol into a white crystalline solid, m.p. 139°, yield 9.4 g. (80%).

Anal.—Calc. for $C_8H_8Cl_2N_2O_2$: N, 11.91. Found: N, 11.67.

3,4-Dichlorophenoxyacetylhydrazine (II*b*)—A mixture of 3,4-dichlorophenol (8.15 g., 0.05 mole), ethyl bromoacetate (7.65 g., 0.05 mole), and potassium carbonate (10.35 g., 0.075 mole) in dry acetone (40 ml.) was refluxed under anhydrous conditions for 10 hr. The reaction mixture was filtered hot, and the excess of solvent was removed by distillation under reduced pressure. To the residue was added 99–100% hydrazine hydrate (3.0 g.) and absolute ethanol (25 ml.). The mixture was refluxed under anhydrous conditions for 3 hr. Ethanol was removed by distillation. On cooling, a white solid mass which separated out was filtered, washed with a little cold ethanol, dried, and recrystallized from ethanol, m.p. 141°, yield 9.9 g. (85%).

Anal.—Calc. for $C_8H_8Cl_2N_2O_2$: N, 11.91. Found: N, 11.80.

Substituted Phenoxyacetylthiosemicarbazines (III–XXII)—An equimolar quantity (0.005 mole) of substituted phenoxyacetylhydrazine and different alkyl or aryl isothiocyanate was refluxed in ethanol (10 ml.) for 2–3 hr. The solvent was removed by distillation. On cooling, the solid products which separated out were filtered and dried. Recrystallization from ethanol gave pure compounds (Table I).

2-Substituted Amino-5-(substituted phenoxymethyl)-1,3,4-oxadiazoles (XXIII–XLII)—To an ethanolic suspension of an appropriate thiosemicarbazine (0.01 mole) was added 5 ml. of 4 N sodium hydroxide with continuous shaking. Iodine in 5% potassium iodide solution was gradually added to the clear solution with stirring until the color of iodine persisted at room temperature. The contents were then refluxed on a water bath, and more iodine solution was added until a permanent tinge of excess iodine remained. The reaction mixture was then poured into ice-cold water (500 ml.), and the precipitated solid mass which separated out was filtered and washed with water and then with warm carbon disulfide. The crude product was finally recrystallized from ethanol. The compounds thus prepared are recorded in Table II.

BIOCHEMICAL STUDIES

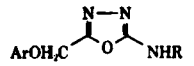
Materials—Commercial chemicals[1] were used in the present study.

Assay of Pyruvic Acid Oxidation by Rat Brain—Male albino rats, kept on an *ad libitum* diet, were used in all experiments. Rat brains isolated from decapitated animals were immediately homogenized in ice-cold 0.25 M sucrose in a Potter–Elvehjem homogenizer. All incubations were carried out at 37°, and the oxygen uptake was measured by the conventional Warburg manometric technique with air as the gas phase. Fresh rat brain homogenate equivalent to 125 mg. wet weight was added to chilled Warburg vessels containing 6.7 mM magnesium sulfate, 20 mM sodium hydrogen phosphate buffer solution (pH 7.4), 1 mM adenosine monophosphate (sodium salt), 33 mM potassium chloride, 10 mM pyruvic acid, and 500 mcg. of cytochrome c in a final volume of 3 ml. The central well contained 0.2 ml. of 20% potassium hydroxide solution. All of the compounds under assay were dissolved in propylene glycol (100%), and an equal volume of the solvent was added in the control vessels.

Effects of Oxadiazoles on Gross Behavior—The studies on the gross behavior in mice were done according to the scheme outlined by Irwin (12). The drugs were taken in 5% gum acacia suspension

[1] Sodium pyruvate, adenosine monophosphate, and cytochrome c were obtained from Sigma Chemical Co., St. Louis, Mo. Other common chemicals were obtained from British Drug House, Bombay, India.

Table II—2-Substituted Amino-5-dichlorophenoxymethyl-1,3,4-oxadiazoles and Their Effect on the Oxidation of Pyruvic Acid



Compound Number	R	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Found	Percent Inhibition[b]
				Ar = 2,6-Dichlorophenyl			
XXIII	n-C_4H_9	145°	65	$C_{12}H_{15}Cl_2N_3O_2$	C 49.36	48.95	55.5 ± 0.9
					H 4.74	4.50	
					N 13.29	12.93	
XXIV	$CH_2CH{=}CH_2$	191–193°	58	$C_{12}H_{11}Cl_2N_3O_2$	C 48.00	48.31	22.5 ± 0.9
					H 3.66	3.22	
					N 14.00	13.90	
XXV	C_6H_{11}	123–125°	54	$C_{15}H_{17}Cl_2N_3O_2$	C 52.63	52.39	3.5 ± 0.8
					H 4.97	5.01	
					N 12.28	11.96	
XXVI	C_6H_5	133–135°	60	$C_{15}H_{11}Cl_2N_3O_2$	C 53.57	53.19	9.1 ± 0.5
					H 3.27	3.52	
					N 12.50	12.16	
XXVII	p-BrC_6H_4	204°	53	$C_{15}H_{10}BrCl_2N_3O_2$	C 43.37	43.10	30.6 ± 1.0
					H 2.40	2.10	
					N 10.12	10.46	
XXVIII	p-IC_6H_4	195°	50	$C_{15}H_{10}Cl_2IN_3O_2$	C 38.96	38.63	42.9 ± 1.1
					H 2.16	2.01	
					N 9.09	8.81	
XXIX	o-$CH_3C_6H_4$	157°	61	$C_{16}H_{13}Cl_2N_3O_2$	C 54.85	54.51	22.6 ± 0.8
					H 3.71	3.80	
					N 12.00	11.68	
XXX	p-$CH_3C_6H_4$	180°	60	$C_{16}H_{13}Cl_2N_3O_2$	C 54.85	54.60	46.2 ± 0.9
					H 3.71	3.30	
					N 12.00	11.70	
XXXI	m-$OCH_3C_6H_4$	135°	56	$C_{16}H_{13}Cl_2N_3O_3$	C 52.45	52.10	—
					H 3.55	3.33	
					N 11.47	11.50	
XXXII	p-$OCH_3C_6H_4$	148–150°	53	$C_{16}H_{13}Cl_2N_3O_3$	C 52.45	52.70	20.6 ± 0.5
					H 3.55	3.79	
					N 11.47	11.22	
				Ar = 3,4-Dichlorophenyl			
XXXIII	n-C_4H_9	190–192°	55	$C_{12}H_{15}Cl_2N_3O_2$	C 49.36	49.60	18.8 ± 1.1
					H 4.74	4.90	
					N 13.29	12.90	
XXXIV	$CH_2CH{=}CH_2$	170°	80	$C_{12}H_{11}Cl_2N_3O_2$	C 48.00	48.36	47.1 ± 1.1
					H 3.66	3.42	
					N 14.00	13.78	
XXXV	C_6H_{11}	133°	60	$C_{15}H_{17}Cl_2N_3O_2$	C 52.63	52.80	73.9 ± 0.9
					H 4.97	5.20	
					N 12.28	12.44	
XXXVI	C_6H_5	155°	63	$C_{15}H_{11}Cl_2N_3O_2$	C 53.57	53.80	8.5 ± 0.9
					H 3.27	3.56	
					N 12.50	12.76	
XXXVII	p-BrC_6H_4	194°	60	$C_{15}H_{10}BrCl_2N_3O_2$	N 10.12	9.97	Nil
XXXVIII	p-IC_6H_4	196–198°	55	$C_{15}H_{10}Cl_2IN_3O_2$	C 38.96	38.84	52.6 ± 0.9
					H 2.16	2.39	
					N 9.09	9.36	
XXXIX	o-$CH_3C_6H_4$	165°	60	$C_{16}H_{13}Cl_2N_3O_2$	C 54.85	54.48	11.1 ± 0.8
					H 3.71	3.36	
					N 12.00	11.80	
XL	p-$CH_3C_6H_4$	190–192°	57	$C_{16}H_{13}Cl_2N_3O_2$	C 54.85	55.10	57.4 ± 0.0
					H 3.71	3.96	
					N 12.00	12.30	
XLI	m-$OCH_3C_6H_4$	171–173°	73	$C_{16}H_{13}Cl_2N_3O_3$	C 52.45	52.04	59.3 ± 0.8
					H 3.55	3.46	
					N 11.47	11.74	
XLII	p-$OCH_3C_6H_4$	154–155°	65	$C_{16}H_{13}Cl_2N_3O_3$	C 52.45	52.72	73.8 ± 1.2
					H 3.55	3.70	
					N 11.47	11.03	

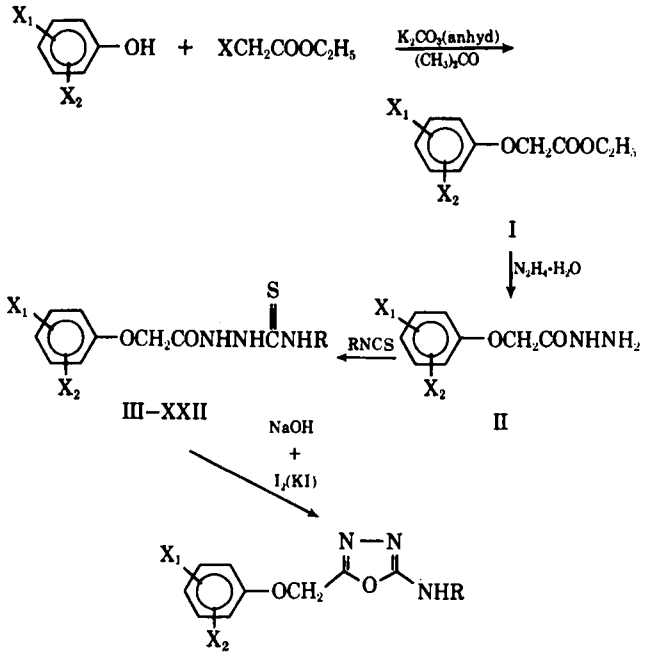
[a] All melting points were taken in open capillary tubes and are corrected. [b] Each experiment was done in duplicate, and the values are the mean of three separate experiments. The oxygen uptake was measured at 5-min. intervals during a 1-hr. incubation period. The percentage inhibition was calculated from the decrease in the oxygen uptake/125 mg. wet brain weight. The final concentrations of pyruvic acid and the oxadiazoles were 10 and 1 mM, respectively.

and administered intraperitoneally. The effect of these compounds on the simple reflexes and gross behavior was observed.

RESULTS AND DISCUSSION

All of the 1-(dichlorophenoxyacetyl)-4-substituted thiosemicarbazines were found to inhibit oxidation of pyruvic acid by rat brain homogenate (Table I). All of the compounds possessing a p-bromophenyl, p-iodophenyl, allyl, or cyclohexyl substituent at position R exhibited greater inhibition. Such an increase in their inhibitory effect was independent of the relative position of the two chlorine atoms present in the phenoxyacetyl moiety of these compounds.

Cyclization of thiosemicarbazines into corresponding oxadiazoles resulted in a significant lowering of their ability to inhibit oxidation of pyruvic acid (Table II). Some oxadiazoles were found to be inactive; a few, however, exhibited greater inhibitory effects than their corresponding thiosemicarbazines. These results failed to provide any direct correlation between the open-chain thiosemicarbazines and their cyclized oxadiazoles. At present, it is difficult



Scheme I

to provide any suitable explanation for the low inhibitory effects of these cyclized oxadiazoles. In general, the steric factors associated with the heterocyclic oxadiazole ring could presumably cause obstruction in the attachment of oxadiazoles on the active site(s) of the enzyme molecule and could, therefore, account for their decreased inhibitory effects. Such a decrease in enzyme inhibitory activities of the oxadiazoles as compared to the thiosemicarbazines could also be due to the loss of C=S, as well as the two acidic hydrogen atoms next to C=O and C=S, respectively, when the thiosemicarbazines are cyclized to their corresponding oxadiazoles. Otherwise, the sulfur and hydrogen atoms in these thiosemicarbazines would form hydrogen bonds with the enzymes to cause greater enzyme inhibition. *In vivo* studies have indicated that almost all compounds produced depression in the gross behavior of experimental mice. Loss of righting reflex was observed with 2-(cyclohexylamino)-5-(3′,4′-dichlorophenoxymethyl)-1,3,4-oxadiazole. In the present study, no correlation could be observed between the enzyme inhibitory activity of substituted thiosemicarbazines and oxadiazoles with behavioral effects.

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Inhibitors of Hydroxyindole-*O*-methyltransferase: Indolealkylpiperazines

BENG T. HO[▲], P. M. GARDNER, and WILLIAM M. McISAAC

Abstract □ Two antipsychotic agents, oxypertine and haloperidol, were both found to be *in vitro* inhibitors of bovine pineal hydroxyindole-*O*-methyltransferase. A series of indolealkylpiperazines structurally related to oxypertine was evaluated for inhibitory activity with the enzyme. The most potent inhibitor of this study, 1-(2-chlorophenyl)-4-[2-(5,6-dimethoxy-3-indolyl)ethyl]piperazine, exhibited a mixed-type inhibition. The possible mode of binding of these inhibitors to the enzyme was discussed.

Keyphrases □ Hydroxyindole-*O*-methyltransferase—indolealkylpiperazines as inhibitors, *in vitro* □ Indolealkylpiperazines—tested *in vitro* as inhibitors of hydroxyindole-*O*-methyltransferase, mode of binding □ Haloperidol—*in vitro* inhibition of hydroxyindole-*O*-methyltransferase, mode of binding □ Oxypertine—*in vitro* inhibition of hydroxyindole-*O*-methyltransferase, mode of binding

Oxypertine, 1-[2-(5,6-dimethoxy-2-methyl-3-indolyl)-ethyl]-4-phenylpiperazine (I), a compound that has been reported to be effective in the treatment of schizophrenia (1–4), was found in our laboratories to be a potent *in vitro* inhibitor of hydroxyindole-*O*-methyltransferase. A widely accepted hypothesis proposes the formation of psychotomimetic compounds from endogenous biogenic amines, such as serotonin (5-hydroxytryptamine), as an etiological factor in schizophrenia. The formation of a compound such as *O*,*N*,*N*-trimethylserotonin (*O*-methylbufotenine) in the body can be prevented by an inhibitor of hydroxyindole-*O*-methyltransferase, and this might explain the mechanism of action of antipsychotic agents such as oxypertine. In the present study, a series of indolealkylpiperazines structurally related to oxypertine was evaluated for their inhibitory activities with hydroxyindole-*O*-methyltransferase.

EXPERIMENTAL[1]

Compounds—The compounds (Table I) were dissolved in glacial acetic acid and then diluted with water to a concentration of 0.2% acetic acid.

Assay—Hydroxyindole-*O*-methyltransferase was isolated from beef pineal gland and purified according to the method of Axelrod and Weissbach (5). Incubation was carried out with *N*-acetylserotonin and *S*-adenosyl-L-methionine-methyl-^{14}C according to the procedure previously described (6).

RESULTS AND DISCUSSION

The inhibitory activity of oxypertine (I) increased with the substitution of H for CH_3 in the 2-position. The 1.5-fold increase in activity of VIII over I could indicate a lack of tolerance of the enzyme for the 2-CH_3 group. The undesirable effect of the methyl group at the 2-position in I could be overcome with a 2-ethyl group; VII was slightly more active than VIII and was twice as active as I. The extended carbon chain might have resulted in an involvement of the terminal CH_3 of CH_2CH_3 group to a hydrophobic region of the enzyme.

The inhibitory activity of I did not increase to a large extent with the substitution of a fluorine atom on the *para*-position of the phenyl ring to give II. The introduction of a hydrophobic group or atom such as CH_3 ($\pi = +0.56$) or Cl ($\pi = +0.71$) (7), however, increased the inhibition of the enzyme. Thus, compounds with *o*-CH_3 (IV) and *m*-CH_3 (VI) groups were both better inhibitors (1.5-fold) than I. Similarly, the *p*-Cl of III doubled the inhibitory activity. No increase in inhibition was observed with substitution of an *o*-OCH_3 group in V; this could be attributed to the hydrophilic nature of the OCH_3 group ($\pi = -0.02$), which differs from the other two hydrophobic CH_3 and Cl groups.

In the 2-H series, no increase in activity of VIII was observed by the *p*-CH_3 group of IX; the substitution of an *o*-Cl group in X, however, increased inhibition by more than fivefold.

Extension of the side chain of VIII by inserting CH_2 between the piperazinyl and phenyl groups resulted in about a 1.5-fold loss of activity, indicating the decrease in the tolerance of the enzyme for the $CH_2C_6H_5$ group of XI.

There was a requirement of at least two methylene (CH_2) groups to bridge the indole nucleus with the piperazinyl moiety of VIII, since shortening of the chain to one CH_2, as in XII, reduced the activity 22-fold.

The binding of the two piperazinyl nitrogen atoms remained to be evaluated. In compounds of the present study, the two nitrogens, both being tertiary amines and one of the two also an anilino nitrogen, are in fact weakly basic. No correlation can be found between the inhibitory activities and their pKa values (Table II).

Haloperidol, another antipsychotic drug, was also found to inhibit hydroxyindole-*O*-methyltransferase *in vitro* (Table I); its activity was about one-sixth of the oxypertine (I).

Kinetic studies were carried out with the four indolealkylpiperazines (I, VIII–X) having low I_{50} values. Lineweaver-Burk

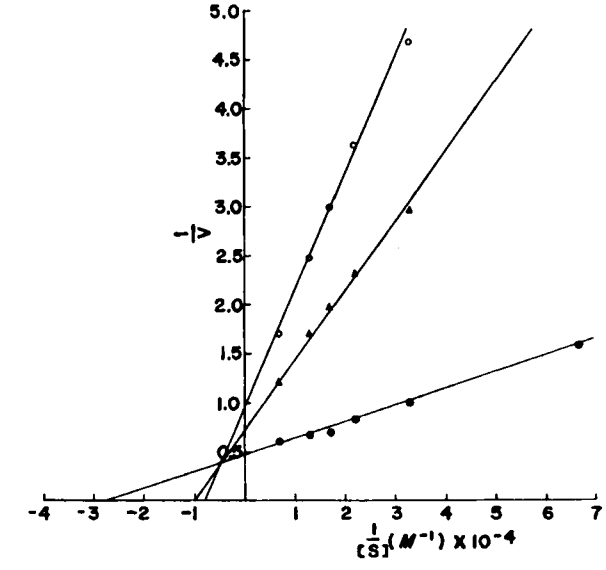
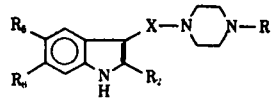


Figure 1—*Lineweaver-Burk plot of inhibition of bovine pineal hydroxyindole-O-methyltransferase by X. Key:* ●, *no inhibitor (0.2% acetic acid);* △, 5×10^{-6} M; *and* ○, 9×10^{-6} M.

[1] All indolealkylpiperazines (Table I) were supplied by Sterling-Winthrop Research Institute, Rensselaer, N. Y., and haloperidol (Haldol) was supplied by McNeil Laboratories, Inc., Fort Washington, Pa.

Table I—Inhibition of Hydroxyindole-*O*-methyltransferase by Indolealkylpiperazines



Compound	R_5	R_6	R_2	X	R	I_{50}[a], m*M*
I[b]	OCH_3	OCH_3	CH_3	CH_2CH_2	C_6H_5	0.078
II[b]	OCH_3	OCH_3	CH_3	CH_2CH_2	C_6H_4F-*p*	0.069
III	OCH_3	OCH_3	CH_3	CH_2CH_2	C_6H_4Cl-*p*	0.038
IV[b]	OCH_3	OCH_3	CH_3	CH_2CH_2	$C_6H_4CH_3$-*o*	0.045
V[c]	OCH_3	OCH_3	CH_3	CH_2CH_2	$C_6H_4OCH_3$-*o*	0.084
VI[b]	OCH_3	OCH_3	CH_3	CH_2CH_2	$C_6H_4CH_3$-*m*	0.047
VII[b]	OCH_3	OCH_3	C_2H_5	CH_2CH_2	C_6H_5	0.039
VIII	OCH_3	OCH_3	H	CH_2CH_2	C_6H_5	0.049
IX	OCH_3	OCH_3	H	CH_2CH_2	$C_6H_4CH_3$-*p*	0.047
X	OCH_3	OCH_3	H	CH_2CH_2	C_6H_4Cl-*o*	0.0089
XI	OCH_3	OCH_3	H	CH_2CH_2	$CH_2C_6H_5$	0.072
XII	OCH_3	OCH_3	H	CH_2	C_6H_5	1.09
XIII	H	H	H	CH_2CH_2	C_6H_5	>0.3
Haloperidol						0.46

[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Hydrochloride salt. [c] Tartrate.

Table II—K_i and pKa values for Indolealkylpiperazines

Compound	K_i, μM[a] Competitive	K_i, μM[a] Noncompetitive	pKa[b]
I	13.2	61.4	6.6
VIII	8.1	53.6	6.8
IX	8.7	53.2	6.9
X	1.5	8.5	7.0
XII	—[c]	—[c]	7.1

[a] Substrate: *N*-acetylserotonin; $K_m = 3.75 \times 10^{-5}$ *M*. [b] A sample of 0.01 mmole of each compound was dissolved in 15 ml. ethanol and made up to 25 ml. with water. To the solution was added 1 ml. of 0.025 *N* HCl, and it was back-titrated with 0.0227 *N* NaOH. The pKa values were calculated by the method of Albert and Serjeant (9). [c] Not determined.

plots, using the method of least squares, showed that all four compounds exhibited a mixed-type inhibition with the bovine pineal hydroxyindole-*O*-methyltransferase. The plot of the best inhibitor, X, is illustrated in Fig. 1. By using the method of Krupka (8), the K_i values were then calculated (Table II). The inhibition of *O*-methylation of *N*-acetylserotonin by these compounds was more competitive than noncompetitive in nature. The K_i values of all compounds, especially the competitive component, are the same order of magnitude as the I_{50} values of the corresponding compounds listed in Table I.

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New Compounds: Synthesis of Alkyl Esters of D,L-Dopa

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Abstract □ In the search for possible prodrugs of levodopa, seven alkyl esters of D,L-dopa were prepared using thionyl chloride as the catalyst. The hydrochloride salts were quite stable to hydrolysis in acidic aqueous solutions.

Keyphrases □ D,L-Dopa—synthesis of alkyl esters □ Levodopa—synthesis of alkyl esters of D,L-dopa as a possible prodrug

Extensive clinical trials undertaken during the last decade have shown that levodopa (L-3,4-dihydroxyphenylalanine) is frequently an effective drug in the treatment of Parkinsonism (1–5). It is generally accepted that orally administered levodopa is absorbed into the blood, passes the blood–brain barrier, and is converted to dopamine in the brain. The resulting increase in brain dopamine and related catecholamines is believed responsible for the effectiveness of the drug in treating Parkinsonism. Although levodopa is now considered the drug of choice, there are still problems associated with its use. A significant number of patients do not respond to treatment, while many who do respond require very large doses and often suffer undesired side effects. A recent report indicated (6) the therapeutic response to levodopa depends on the efficiency of absorption and the attainment of adequate blood levels.

Other workers have also studied the biopharmaceutic and pharmacokinetic aspects of this drug. Peaston and Bianchine (7) measured the total radioactivity in the blood of 22 patients after oral doses of ^{14}C-labeled levodopa and found the ^{14}C to be rapidly and almost completely absorbed. Subsequently, three other laboratories reported studies in which levodopa in the blood was measured following oral administration of the drug (8–10). These studies showed that only about 20–30% of the orally administered dose reached the general circulation as the intact drug and suggested that the remainder is biotransformed in the GI tract and/or the liver during its "first passage" to the general circulation. These results are not surprising when the number of degradative reactions to which levodopa is subject is considered (11–14). One possible method for improving the absorption of levodopa and/or making it less labile could be the administration of a prodrug which can be transformed to the active drug following absorption. In this laboratory the use of alkyl esters as possible prodrugs for levodopa is currently under investigation. The preparation of seven esters of the less expensive D,L-dopa is reported in this article.

Table I—Alkyl Ester Hydrochlorides of D,L-Dopa

Ester	Melting Point	Yield, %	Analysis[a], % Calc.	Analysis[a], % Found	Spectral Data: IR[b] Max. cm.$^{-1}$	Spectral Data: UV[c] Max., nm.	Spectral Data: UV[c] ϵ	Spectral Data: Half Bandwidth, nm.
Methyl	179–180°	73.96	C 48.49 H 5.70 N 5.65	48.10 6.08 5.35	3370; 2940 1724 1205–1290 775– 826	280	2635	22.0
n-Propyl	179°	88.20	C 52.27 H 6.58 N 5.08	52.39 6.68 5.01	3370; 2970 1735 1205–1300 780– 825	280	2865	23.0
Isopropyl	198–199°	63.41	C 52.27 H 6.58 N 5.08	51.99 6.62 5.05	3370; 2950 1724 1200–1300 780– 830	280	2845	23.0
n-Butyl	180–181°	81.66	C 53.89 H 6.96 N 4.83	54.07 7.02 4.82	3370; 2950 1730 1200–1300 780– 825	280	2900	22.5
Isobutyl	217–218°	94.76	C 53.89 H 6.95 N 4.83	54.01 7.01 4.84	3350; 2950 1730 1200–1300 780– 825	280	2845	22.5
n-Pentyl	185–186°	91.74	C 55.35 H 7.30 N 4.61	54.98 7.56 4.58	3360; 2940 1730 1200–1300 780– 825	280	2840	23.0
Isoamyl	197–198°	82.92	C 55.35 H 7.30 N 4.61	55.19 7.34 4.63	3370; 2950 1730 1200–1300 780– 880	280	2795	23.0

[a] Analyses were performed by Atlantic Microlab, Inc. [b] I in KBr pellets. [c] UV absorption spectra of 2×10^{-4} *M* solution in 0.1 *N* HCl.

EXPERIMENTAL[1]

Materials—The D,L-dopa used was 3,4-dihydroxyphenylalanine[2], which decomposed between 270 and 272° and was employed without further purification. All other reagents were of the highest quality commercially available and were used without further purification.

Preparation of the Alkyl Ester Hydrochlorides of Dopa—A modification of the procedure presented by Patel and Price (15), in which thionyl chloride acts as the catalyst, was used to prepare seven alkyl esters of D,L-dopa. One hundred milliliters of the appropriate alcohol was placed in a 200-ml. three-necked flask, which was equipped with a reflux condenser and a tube for nitrogen bubbling. The alcohol was cooled to from −5 to −10° and sparged with nitrogen for 10 min. The thionyl chloride (15 ml.) was added slowly over 15 min., and the reaction mixture was stirred for an additional 15 min. After stirring, 4 g. of D,L-dopa was added, the temperature was adjusted to 40°, and the mixture was refluxed for 12 hr. The hydrochloride salts of the isobutyl, pentyl, and isoamyl esters precipitated directly from the reaction solution. The other four ester hydrochlorides were precipitated by adding just enough petroleum ether to make the solution turbid and then placing them in the refrigerator for a few hours. All of the products were collected by filtration and were recrystallized from an absolute alcohol–ether mixture. The crystals were dried in a vacuum desiccator at room temperature and stored in the desiccator until used. For each ester hydrochloride of D,L-dopa, the melting point, IR and UV spectra, and microanalysis data are presented in Table I. The Arnow (16) test for catechol was positive for each ester.

Paper Chromatography—A paper chromatographic method for the qualitative analysis of D,L-dopa in solutions containing one or more of the alkyl esters of dopa was developed. A solvent system consisting of *n*-butanol–water–acetic acid (16:7:1) was found to effect a separation of the amino acid from the various esters when sample solutions were spotted on Whatman No. 1 paper and developed in an ascending manner. The location of the compounds on the developed chromatograms was determined by exposing the paper to ammonia vapors and observing the brown-yellow color develop as they were oxidized. For duplicate runs on solutions of D,L-dopa with each of the seven esters, the R_f value for dopa was between 0.17 and 0.19, while the esters ranged between 0.30 for the methyl ester and 0.85 for the *n*-butyl ester. The possible hydrolysis at 37° of 1 m*M* solutions of each of the seven esters buffered[3] at pH 1, 3, and 5 was evaluated using the paper chromatographic method. No hydrolysis was detected for up to 12 hr.

[1] All IR spectra were obtained with a Perkin-Elmer 237 B grating spectrophotometer. The UV spectra and analyses were obtained with Perkin-Elmer 202 and Beckman DU spectrophotometers, respectively. The melting points were determined on a Thomas-Hoover capillary melting-point apparatus.

[2] White Label, Nutritional Biochemical Corp.

[3] Using 0.2 *M* HCl and acetate buffers.

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Ureide Ring Scission of Phenobarbital by Sodium Borohydride

Keyphrases ☐ Phenobarbital—ureide ring scission by sodium borohydride ☐ Sodium borohydride—used in ureide ring scission of phenobarbital ☐ Ureide ring scission—reaction of phenobarbital with sodium borohydride

Sir:

Phenobarbital was found to react with sodium borohydride in aqueous and organic media, with ultimate formation of 2-phenyl-1-butanol. This finding was unexpected in view of the report by Smissman *et al.* (1) that phenobarbital was not reduced by sodium borohydride in absolute alcohol, tetrahydrofuran, or diglyme.

Solutions (0.1–1 mg./ml.) of phenobarbital in aqueous alkali, methanol, or methanol–ether mixtures were treated with excess sodium borohydride, added as the solid, and the mixtures were monitored at intervals by TLC. When using 0.25-mm. silica gel GF thin-layer plates with chloroform–methanol (4:1) for development and UV light and iodine vapor for detection, all reaction mixtures showed qualitatively identical patterns with time. At first, shrinkage of the phenobarbital spot at about R_f 0.8 was evident with the appearance of two more polar spots, at R_f's about 0.5 and 0.7. After no more phenobarbital was detected on the plates, a new spot became evident at about R_f 0.9, increasing in size and intensity with concomitant disappearance of the lower mobility spots until, finally, only this spot was visible. The reaction proceeded most rapidly in 0.1 *N* alkali and least in methanol–ether mixtures. Heating a solution of phenobarbital with excess sodium borohydride in 0.1 *N* sodium hydroxide for 20 min. was sufficient to effect complete conversion to R_f 0.9 material.

A quantity of the ultimate reaction product was prepared by dissolving 1.06 g. of phenobarbital in 40 ml. of 0.05 *N* sodium hydroxide, adding 4 g. of sodium borohydride in portions, and allowing the mixture to stand for 22 hr. at room temperature. TLC of the reaction mixture showed a single spot at R_f 0.9. Extraction of the mixture with two 50-ml. portions of hexane, washing the extracts with 10 ml. of water, and evaporation under a stream of nitrogen afforded 0.505 g. of faintly yellow liquid (73.6% yield based on phenobarbital). The product was characterized by NMR[1] spectroscopy in deuterochloroform. The signals obtained and their interpretation are summarized in Table I.

Elemental analysis showed no nitrogen, and the carbon–hydrogen values confirmed the structure of 2-phenyl-1-butanol.

[1] Using a Varian A-60 instrument.

Table I—Summary of NMR Spectrum of Phenobarbital Reduction Product

δ, p.p.m.	Multiplicity	Protons	Assignment
0.80	Triplet	3	$-CH_2-CH_3$
1.65	Multiplet	2	$CH_3-CH_2-C(-H)-$
2.05	Singlet	1	—OH (exchanges in D_2O)
2.6	Multiplet	1	$C_6H_5-CH(-)-CH_2$
3.7	Doublet	2	$-CH(-)-CH_2OH$
7.25	"Singlet"	5	$C_6H_5-CH(-)-$

Anal.—Calc. for $C_{10}H_{14}O$: C, 79.95; H, 9.39. Found: C, 80.30; H, 9.50.

Although the mechanism or reaction route was not investigated, we considered that the first step of the reaction might involve hydrolysis of phenobarbital. This hypothesis appears untenable, however, because no spot corresponding to the primary phenobarbital hydrolysis product, phenylethylacetylurea, was seen in chromatograms of the reaction mixtures. Moreover, phenobarbital was demonstrated to be stable when added to reaction mixtures in which the borohydride had been allowed to decompose to borate.

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1,5-Diphenyl-3-dimethylaminopyrrolidine: A Long-Acting Histamine Antagonist

Keyphrases ☐ 1,5-Diphenyl-3-dimethylaminopyrrolidine—evaluation as potent, long-acting histamine antagonist ☐ Antihistamines, potential—1,5-diphenyl-3-dimethylaminopyrrolidine

Sir:

Antagonism of histamine-induced smooth muscle contraction by antihistamines is assumed to occur as a result of competitive occupation of tissue receptors by

Ureide Ring Scission of Phenobarbital by Sodium Borohydride

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3.7	Doublet	2	$-CH(-)-CH_2OH$
7.25	"Singlet"	5	$C_6H_5-CH(-)-$

Anal.—Calc. for $C_{10}H_{14}O$: C, 79.95; H, 9.39. Found: C, 80.30; H, 9.50.

Although the mechanism or reaction route was not investigated, we considered that the first step of the reaction might involve hydrolysis of phenobarbital. This hypothesis appears untenable, however, because no spot corresponding to the primary phenobarbital hydrolysis product, phenylethylacetylurea, was seen in chromatograms of the reaction mixtures. Moreover, phenobarbital was demonstrated to be stable when added to reaction mixtures in which the borohydride had been allowed to decompose to borate.

(1) E. E. Smissman, A. J. Matuszak, and C. N. Corder, *J. Pharm. Sci.*, **53**, 1541(1964).

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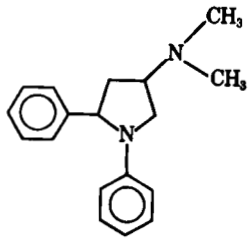
1,5-Diphenyl-3-dimethylaminopyrrolidine: A Long-Acting Histamine Antagonist

Keyphrases ☐ 1,5-Diphenyl-3-dimethylaminopyrrolidine—evaluation as potent, long-acting histamine antagonist ☐ Antihistamines, potential—1,5-diphenyl-3-dimethylaminopyrrolidine

Sir:

Antagonism of histamine-induced smooth muscle contraction by antihistamines is assumed to occur as a result of competitive occupation of tissue receptors by



I

the antagonist drugs (1, 2). We now report pharmacological data demonstrating that 1,5-diphenyl-3-dimethylaminopyrrolidine (I) is a potent histamine antagonist which exhibits a remarkably long duration of action. Compound I is a cyclic analog of the ethylenediamine antihistamine phenbenzamine. It was synthesized as a single racemic diastereoisomer in which the 3-dimethylamino and 5-phenyl substituents have been tentatively assigned the *trans*-configuration. The synthesis of I and related compounds will be the subject of a future paper.

When I was studied for antihistaminic activity *in vitro* using the isolated guinea pig ileum (3), it was found that concentrations as low as 2×10^{-9} *M* inhibited the contractile response to histamine. However, the antagonistic effect of I could not be readily reversed by washing the tissue. Therefore, experiments were undertaken to compare the duration of action of I with that of the potent ethylenediamine antihistamine tripelennamine. The antagonists (as the hydrochloride salts) were kept in contact with the ileal tissue for 15 min. prior to the addition of agonists. The tissue was then washed at 5-min. intervals with fresh Tyrode's solution, and after each wash either histamine (4×10^{-6} *M*) or acetylcholine (4×10^{-7} *M*) was added. The concentration of histamine employed in these experiments produced a near maximal contraction prior to the introduction of antagonists. Figure 1 shows the recovery of the response to histamine with time after a single exposure of the tissues to the antagonists

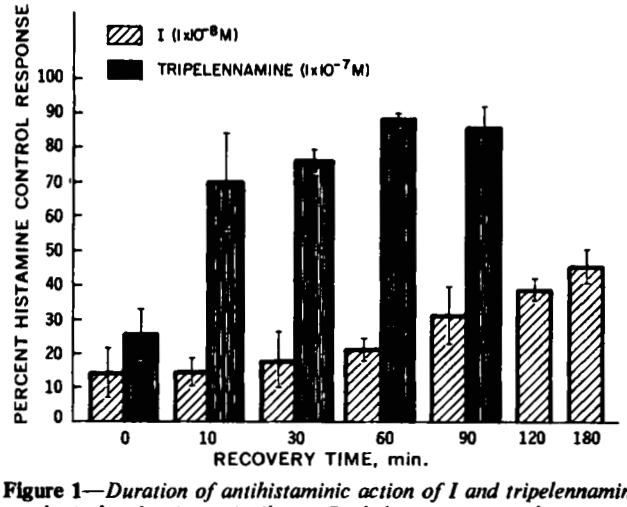


Figure 1—*Duration of antihistaminic action of I and tripelennamine on the isolated guinea pig ileum. Each bar represents the mean of three experiments with I or two experiments with tripelennamine. Vertical lines represent standard error (I) and range (tripelennamine). The 100% control response is defined as the mean response to 4 × 10^{-6} M histamine in these experiments.*

Table I—Percent Decrease in Hind-Limb Perfusion Pressure Produced by Close Intraarterial Injections of Histamine and Methacholine before and after Close Intraarterial Infusion of 5 mg. of I

	Histamine			
	Control			
Experiment	Dose 1 (1–2 mcg.)	Dose 2 (2–4 mcg.)	After Infusion of I[a] Dose 1	Dose 2
1	35	44	4	8
2	14	27	0	3
	Methacholine			
	Control			
Experiment	Dose 1 (0.025–0.1 mcg.)	Dose 2 (0.1–0.4 mcg.)	After Infusion of I[a] Dose 1	Dose 2
1	37	43	35	40
2	15	23	13	18

[a] Agonists were administered in 0.9% saline solution 20–30 min. after infusion of I.

followed by repeated washing. The response to histamine did not exceed 45% of control 3 hr. after exposure to I (1×10^{-8} *M* final bath concentration), whereas recovery after treatment with tripelennamine (1×10^{-7} *M* final bath concentration) reached 70% of control within 10 min. The response to acetylcholine was not inhibited by I or by tripelennamine, and the response to acetylcholine did not fall below control levels during the experiments.

In other studies with the guinea pig ileum, a concentration of 4×10^{-6} *M* of I produced an 85% inhibition of the contractile response to acetylcholine (4×10^{-7} *M*). The response to acetylcholine returned to near control levels within 30 min. after exposure of the tissue to this concentration of I. These experiments demonstrate that I is a much more effective inhibitor of histamine than of acetylcholine on the guinea pig ileum.

Experiments were also conducted on the perfused innervated dog's hind limb, employing the preparation described by Zimmerman (4) to examine the relative effectiveness of I in blocking the direct vasodilator responses to close intraarterial injections of histamine and methacholine. The results, expressing the vasodilator responses as percent decreases in perfusion pressure, obtained in two dogs are shown in Table I. The direct vasodilator effect of histamine was greatly reduced, while the perfusion pressure response resulting from the direct vasodilator action of methacholine was only slightly inhibited.

The results of the experiments reported in this communication demonstrate that I is a potent and selective histamine antagonist which inhibits both the contractile response in guinea pig ileum *in vitro* and the vasodilator response in the dog's hind limb *in vivo*. The very long duration of action of I appears to be unique among selective histamine antagonists and may be an important finding with respect to this new antihistamine. We also prepared the other diastereoisomer of I and found it to be a reversible histamine antagonist, having a pA_2 value of 7.97.

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(3) Staff of the Department of Pharmacology of the University of Edinburgh, "Pharmacological Experiments on Isolated Preparations," 2nd ed., E. and S. Livingstone, London, England, 1970, p. 58.
(4) B. G. Zimmerman, *Amer. J. Physiol.*, **221**, 1171(1971).

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Prediction of Dissolution Rates of Drugs

Keyphrases □ Dissolution rates, slightly water-soluble powders—prediction from simple mathematics □ Powders, slightly water soluble—dissolution rates predicted from simple mathematics □ Powders, volume surface diameter—calculation of surface area

Sir:

Hussain (1) recently showed that, by making certain assumptions and using simple mathematics, it was possible to predict the dissolution rates of slightly water-soluble powders using the formula:

$$Q = \frac{A \cdot S \cdot D \cdot t}{h} \qquad \text{(Eq. 1)}$$

where:

Q = amount of drug (in grams) dissolved in time, t
A = surface area occupied by the total weight of the sample
S = solubility of the drug (in grams per milliliter) in the dissolution medium
D = diffusion coefficient of the drug (in square centimeters per second)
h = thickness of diffusional layer (in centimeters)

By assuming that the diffusion coefficient, D, and the thickness of the diffusional layer, h, are constant at 9×10^{-6} cm.²/sec. and 50×10^{-4} cm., respectively, knowledge of only the surface area exposed, A, and the solubility, S, of the drug enables the dissolution rate Q/t to be calculated.

Despite the remarkable correlation between the experimental and calculated results for the dissolution rates of hydrocortisone, benzoic acid, levodopa, and griseofulvin, there appears to be a considerable argument against the use of such predicted results as a routine measure.

Hussain's Eq. 3 is in error by a density factor (ρ):

$$\text{number of particles in } W \text{ grams of powder} = \frac{W}{4/3\pi r^3 \rho} \qquad \text{(Eq. 2)}$$

assuming spherical particles:

$$V = 4/3\pi r^3 \qquad \text{(Eq. 3)}$$

Since the density for benzoic acid is 1.32 (2), the area would be overestimated without this correction by 32%, with a corresponding overestimation in the calculated dissolution rate.

Hussain also used the average particle size on an arithmetic basis, using the two extremes of the mesh fraction in his calculation of the surface area. While this may not lead to gross errors for the comparatively narrow size ranges considered by Hussain, for larger particle-size distributions a better mean size would be the volume–surface mean diameter, d_{vs} (3):

$$d_{vs} = \frac{\Sigma n d^3}{\Sigma n d^2} \qquad \text{(Eq. 4)}$$

and the surface area computed from:

$$A = \frac{W \times 6}{d_{vs}\rho} \qquad \text{(Eq. 5)}$$

Since the volume–surface mean diameter will also be larger than the arithmetic mean diameter, this will add further to the overestimation of the dissolution rate.

To explain Hussain's apparent correlation requires a compensating decrease in the value of the area used in Eq. 1. This may be due to assuming that the particles are spherical, while deviations from either spherical or cubical shape will lead to an increase in the constant value of 6 given in Eq. 5.

Due to the complexity of these compensating mechanisms, it would be rash to adopt Hussain's method for the prediction of the dissolution rate of sparingly soluble drugs without further evidence. Hussain's article dealt with particles in powder form only. However, for solid dose formulations, where it is known that the total area available may not come into contact with the dissolution medium due to failures of disintegration (4), or where the ingredients may invalidate the diffusion assumptions made by Hussain, we must be prepared to continue to determine experimentally the dissolution rate of the drug.

(1) A. Hussain, *J. Pharm. Sci.*, **61**, 811(1972).
(2) "Handbook of Chemistry and Physics," 49th ed., The Chemical Rubber Co., Cleveland, Ohio, 1968–1969, p. C-180.
(3) G. Herdan, "Small Particle Statistics," Butterworths, London, England, 1960, p. 34.
(4) J. A. Hersey and R. B. Barzilay, *J. Pharm. Pharmacol.*, **21**, 65 (1969).

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Cannabis sativa L. (Marijuana) II: Standardized and Reliable Microscopic Method for Detection and Identification of Marijuana

Keyphrases ☐ Marijuana—microscopic identification, differential staining with fast blue B ☐ Cannabinoid plant tissues—microscopic identification, differential staining with fast blue B ☐ Fast blue B chromogenic reagent—used to identify marijuana ☐ Chromogenic reagents, fast blue B—used to identify marijuana

Sir:

This preliminary communication describes a highly reliable and easily performed procedure for the microscopic identification of marijuana[1].

It is considered desirable for an investigator to utilize one or more chemical methods as well as the microscopic examination of suspect material to confirm the presence or absence of marijuana (1). Although several chemical test procedures have been reported for this purpose (2), detailed methods dealing specifically with the microscopic detection of marijuana have been lacking in the scientific literature.

The positive microscopic identification of marijuana in a particular sample is based on observing the presence of certain morphological structures such as the glandular hairs, cystolith hairs, and nonglandular hairs (3) which are characteristic and diagnostic for *Cannabis sativa*. However, these diagnostic criteria may be complicated by the fact that marijuana is frequently admixed prior to use with a variety of nonmarijuana plant substances including tobacco (*Nicotiana tabacum*), lavender (*Lavendula officinalis*), catnip (*Nepeta cataria*), and oregano (*Origanum vulgare*). Indeed, in many instances we have examined plant material presumed to be marijuana but which instead proved to be composed entirely or in part of catnip, oregano, or lavender. The latter three plant species, as do many other members of the Labiatae (mint family), exhibit glandular hairs which, particularly if they are crushed and fragmented, may be confused with the glandular hairs of marijuana (4). Furthermore, Nakamura (1) recently described more than 80 different plant species containing cystolith hairs similar to those found in marijuana.

The present study was undertaken to develop a microscopic method that would serve to distinguish marijuana unequivocally from various other plant species having similar morphological features. This method, outlined in stepwise fashion, is presented below:

1. Thoroughly mix a small amount of suspect material (1 mg. or less) with 2 drops of clearing solution [chloral hydrate (75 g.), propylene glycol (10 ml.), and distilled water (sufficient quantity to make 100 ml.)] on a clean microscope slide.

2. Carefully heat the slide from below, using a microburner, until the mixture boils for a total of 3 sec. Cool momentarily, add 1 additional drop of clearing solution, and heat to boiling as before. Allow the slide to cool for 1 min. before proceeding. The chloral hydrate clearing solution serves to dissolve starch and plant pigments and thus allows the diagnostic elements to be more readily observed.

3. Treat the wet mount with 2 drops of *freshly prepared* chromogenic reagent [fast blue B salt[2] (0.3 g.) and clearing solution (sufficient quantity to make 100 ml.)], mix well, cover the preparation with a glass (not plastic) coverslip, and examine the prepared mount microscopically (100–400× magnification). It has been shown that fast blue B salt couples with cannabinoid substances to yield characteristic red to purple-colored compounds (5). Accordingly, upon microscopic examination, cannabinoid-rich plant tissues will be observed to acquire a red-purple color. This phenomenon is particularly intense in the case of the marijuana glandular hairs. Furthermore, when one is dealing with a marijuana-containing sample, the entire wet mount rapidly acquires the characteristic red-purple color which is easily observed with the naked eye.

4. Finally, place 1 drop of glacial acetic acid along one edge of the coverslip, draw the acid beneath the coverslip by touching the opposite edge of the coverslip with a piece of filter paper, and observe microscopically. The presence of cystolith hairs is evidenced by the liberation of gas (carbon dioxide) bubbles from the bases of these calcium carbonate-containing hairs.

The most significant aspect of this procedure concerns the fast blue B chromogenic reagent. The use of this reagent allows the marijuana glandular hairs as well as other cannabinoid-containing elements to be differentially stained. Thus, for example, lactiferous tissue and fragments of resin both acquire the characteristic red-purple color. To assess the possibility of false-positive fast blue B reactions, we studied a large number of different noncannabis plant species, including many having glandular hairs, using the described procedure. In no case did we observe staining of the glandular hairs, although in certain instances we noted that some plant tissues or the entire wet mounts acquired diffuse pink to red-brown colors following treatment with fast blue B reagent. These colors, probably the result of the reaction of fast blue B with plant phenolic substances, were distinct and could usually be differentiated from the characteristic red-purple colors observed with the marijuana samples. Thus, under the experimental conditions described, it appears that fast blue B selectively stains cannabinoid-containing plant tissues.

On the basis of the consistently reliable results obtained from evaluating more than 300 different plant species, we have found this easily performed method to be highly reliable for confirming the presence or absence of marijuana in suspect material. The method should find wide application in the area of forensic science. Further studies utilizing this method together with TLC will be reported subsequently.

[1] The term marijuana as used herein refers to the ground stems, leaves, and flowering tops of *C. sativa*.

[2] *o*-Dianisidine diazotate, K & K Laboratories, Plainview, NY 11803

(1) G. R. Nakamura, *J. Amer. Offic. Anal. Chem.*, **52**, 5(1969).
(2) R. Mechoulam, *Science*, **168**, 1159(1970).
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We dedicate this paper to Professor Maynard W. Quimby, University of Mississippi, on the occasion of his appointment to the position of Senior Scientist and Emeritus Professor of Pharmacognosy.

▲ To whom inquiries should be directed.

BOOKS

REVIEWS

Biochemical Applications of Mass Spectrometry. Edited by G. R. WALLER. Wiley, 605 Third Ave., New York, NY 10016, 1972. xiv + 872 pp. 21 × 28 cm. Price $49.95.

The editor and authors of this encyclopedic work have succeeded admirably in assembling into one volume a large amount of theoretical and practical material.

The book is divided into five parts. Part I is an introduction and historical survey. Part II covers the different types of spectrometers and contains an exhaustive section on data acquisition and processing by most of the world's principal mass spectrometric laboratories.

About 80% of the book is devoted to the interpretation of mass spectra. These parts, consisting of 28 chapters, provide both the novice and experienced investigator with a wealth of information on mass spectrometric applications to the entire range of biochemicals and compounds commonly classified as natural products. In addition, there are excellent chapters on the "Origin of Mass Spectra," how to interpret metastable ions, and field and chemical ionization mass spectrometry. Interdisciplinary areas such as those relating to drug metabolism and clinical applications are also covered.

This well-edited volume is about 60% tables and illustrations. The mass spectra are uniformly and clearly presented. Each chapter is adequately, but not excessively, referenced. A good working index is present so that subjects not discussed under a separate chapter, *e.g.*, mass fragmentometry and GC–MS systems, can be quickly found. If anything, the book suffers somewhat from covering the literature *only* through 1969, since it was that long in preparation. Attempts have been made to update, in proof, some of the newer areas like chemical ionization mass spectrometry by the citation of more recent references.

In summary, the book should be useful to investigators working in any of the areas allied with natural products and as a reference work for others using mass spectrometry as an analytical tool. Insofar as it is not out of date, it represents a how-to manual on the subject of data aquisition. As a final point, one would be remiss in not mentioning the efforts of the editor and various contributors in presenting the history and background of the entire field.

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Methods and Techniques in Clinical Chemistry. By P. L WOLF, D. WILLIAMS, T. TSUDAKA, and L. ACOSTA. Wiley, 605 Third Ave., New York, NY 10016, 1972. 417 pp. 14.5 × 23 cm. Price $11.50.

This publication compiles all of the currently significant chemical tests being utilized by the Stanford Hospital Clinical Laboratory. The tests are given in alphabetical order; each test monograph includes a discussion of the principle involved, reagents and equipment, procedure, normal values, references, and clinical interpretation. Throughout the book, the authors have emphasized the practical aspects of clinical chemistry.

Staff Review ■

NOTICES

Proceedings of the International Symposium on Gas Chromatography–Mass Spectrometry. Organized by the Istituto di Richerche Farmacologiche "Mario Negri" of Milan. Edited by ALBERTO FRIGERIO. Tamburini Editore s.p.a. Milano, Italy, 1972. 505 pp. 17 × 24 cm.

Biological Oxidation of Nitrogen in Organic Molecules, Proceedings of the Symposium held at Chelsea College, London, December 1971. Edited by J. W. BRIDGES, J. W. GORROD, and D. V. PARKE. Halsted Press, Wiley, 605 Third Ave., New York, NY 10016, 1972. 269 pp. 17 × 25 cm. Price $21.00.

International Directory of Investigators in Psychopharmacology. U. S. Department of Health, Education, and Welfare, National Institute of Mental Health, 5600 Fishers Lane, Rockville, MD 20852, 1972. 439 pp. 20 × 26 cm.

Die Starke in der Medizin. By JOHANNES SEIDEMANN. Paul Parey, Berlin 61, Lindenstrasse 44–47, Germany, 1972. 92 pp. 16 × 24.5 cm. Price DM 38. (*German*)

Organic-Chemical Drugs and Their Synonyms, Volumes I and II. By MARTIN NEGWER. Akademie-Verlag, DDR 108 Berlin, Leipziger Str. 3–4, Germany, 1971. 1216 pp. 16 × 24 cm. (*German*)

Terpenoids and Steroids, Volume 2, A Specialist Periodical Report. Senior Reporter, K. H. OVERTON. Chemical Society, Blackhorse Road, Letchworth, Herts, SG6 1HN, England, 1972. 450 pp. 13 × 21.5 cm. Price £9.00.

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(4) H. W. Youngken, Sr., "Textbook of Pharmacognosy," Blakiston, Philadelphia, Pa., 1950, pp. 706–749.
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We dedicate this paper to Professor Maynard W. Quimby, University of Mississippi, on the occasion of his appointment to the position of Senior Scientist and Emeritus Professor of Pharmacognosy.

▲ To whom inquiries should be directed.

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The editor and authors of this encyclopedic work have succeeded admirably in assembling into one volume a large amount of theoretical and practical material.

The book is divided into five parts. Part I is an introduction and historical survey. Part II covers the different types of spectrometers and contains an exhaustive section on data acquisition and processing by most of the world's principal mass spectrometric laboratories.

About 80% of the book is devoted to the interpretation of mass spectra. These parts, consisting of 28 chapters, provide both the novice and experienced investigator with a wealth of information on mass spectrometric applications to the entire range of biochemicals and compounds commonly classified as natural products. In addition, there are excellent chapters on the "Origin of Mass Spectra," how to interpret metastable ions, and field and chemical ionization mass spectrometry. Interdisciplinary areas such as those relating to drug metabolism and clinical applications are also covered.

This well-edited volume is about 60% tables and illustrations. The mass spectra are uniformly and clearly presented. Each chapter is adequately, but not excessively, referenced. A good working index is present so that subjects not discussed under a separate chapter, *e.g.*, mass fragmentometry and GC–MS systems, can be quickly found. If anything, the book suffers somewhat from covering the literature *only* through 1969, since it was that long in preparation. Attempts have been made to update, in proof, some of the newer areas like chemical ionization mass spectrometry by the citation of more recent references.

In summary, the book should be useful to investigators working in any of the areas allied with natural products and as a reference work for others using mass spectrometry as an analytical tool. Insofar as it is not out of date, it represents a how-to manual on the subject of data aquisition. As a final point, one would be remiss in not mentioning the efforts of the editor and various contributors in presenting the history and background of the entire field.

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REVIEW ARTICLE

Chemistry and Pharmacology of Heparin

JULIAN EHRLICH and SALVATORE S. STIVALA▲

Keyphrases ☐ Heparin—review of chemistry, pharmacology, therapy, metabolism ☐ Anticoagulants, heparin—review of chemistry and pharmacology ☐ Hypoprothrombinemia—review of heparin pharmacology ☐ Pharmacology of heparin—review

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Heparin is a mucopolysaccharide composed of partially sulfated units of α-D-glucuronic acid and 2-amino-2-deoxy-α-D-glucose joined by 1 → 4 bonds. It is mainly noted for its anticoagulant activity in the treatment of myocardial infarction, but it is also an antilipemic agent. Heparin is thought to originate in the mast cells of connective tissue and is found in beef, hog, and dog livers; beef muscle, adipose tissue, and spleen; hog mucin and alimentary canal; skin of rats; fish plasma; sea clams; and scales of carp.

Since its discovery in 1916 by McLean, many studies have been made on its chemical structure and pharmacology, but progress has been much slower in the elucidation of the macromolecular properties of the polysaccharide, particularly the relationship of physical parameters to its biological action.

The early history, chemistry, and clinical applications of heparin were described by Jorpes (1), Whistler and Smart (2), Foster and Huggard (3), and Stacey and Barber (4). Some general accounts of the mucopolysaccharides and heparin were later written by Blonde (5), Brimacombe and Webber (6), Whistler and Rowell (7), Engelberg (8), Meyer (9), and Jeanloz (10).

Relative to physicochemical aspects of heparin, it exhibits typical polyelectrolyte behavior in aqueous

solution due to its high negative charge. Accordingly, physicochemical measurements are sensitive to ionic strength and pH. Heparin is polydispersed and molecular weights from 6000 to 20,000 have been reported. Physicochemical properties in relation to blood anticoagulation are discussed later in this review.

The purpose of this review is to furnish an up-to-date, comprehensive coverage of the many aspects of this extremely interesting and important drug. This review, although comprehensive, is not necessarily exhaustive. The literature search was carried up to May 1, 1972, and the main body of the review consists of information from the literature of the last decade.

SOURCES AND ISOLATION OF HEPARIN

Most of the heparin for pharmaceutical use is extracted from bovine lung tissue. However, the literature contains many examples of heparin or heparin-like substances isolated from other sources.

Marine life has yielded heparin-like materials with high blood anticoagulant activity. Thomas (11, 12) obtained such a substance from the common surf clam, *Spisula solidissima*, which might be chemically similar to heparin. Frommhagen *et al.* (13) extracted mactin-A and B from two species of clams, *Mactra spissula* and *Artica islandica*, which had a higher activity than heparin and a lower toxicity. Heparin was also found in the blood (14), liver (15), and muscles (15) of fish.

The skin of many animals has been claimed to be a source of heparin. Bollet *et al.* (16) located it in the human dermis and subcutaneous tissue. Horner (17), in 1971, isolated heparin with a molecular weight of over 1 million from rat skin. Injured animal skin has yielded desulfated heparin (18).

Heparin has been found in normal human serum in small amounts (19). Nilsson (20) described a method of extraction of an anticoagulant, probably heparin, from normal horse blood, which exhibited strong metachromasia. The data of Smith and Von Korff (21) indicate a heparin-precipitable complex of a fibrinogen-like protein closely combined with heparin which was found in human plasma. The origin of heparin in the blood may be the basophil cells, which are almost functionally identical with mast cells and might release small amounts of heparin into the plasma (22). On the other hand, Ringertz (23) found that heparin can be liberated from the mast cells by a number of agents that cause the disruption of these cells, but no heparin was detected in the blood.

The kidney of the rat has yielded a heparin-like substance (24). Allalouf *et al.* (25) isolated this material and discovered that it acted similarly to heparin in anticoagulant activity (8 units/mg.), protamine antagonism, and the recalcification test. The major component of the mucopolysaccharides extracted from this source was heparin (26).

Healthy human liver was shown by Schmitz-Moorman (27) to contain uronic acid mucoids consisting of approximately equal amounts of hyaluronic acid, chondroitin, chondroitin sulfate, and heparin. In cases of amyloidosis, the mucopolysaccharides of the amyloid liver were found to contain small amounts of heparin (28, 29), which was 60 times as much acidic mucopolysaccharide content as in healthy liver (30). The capsule surrounding the ox liver was shown by Serafini-Fracassini *et al.* (31) to contain heparin in single chains or as a protein–polysaccharide complex rather than as a multichain proteoglycan. The ox liver capsule also yielded a heparin–protein complex (32).

Other sources of heparin include the aorta (33–35), the loin and ham muscles of the pig (36, 37), the sweat (38), the thyroid (16), and the heart (39). Heparin-related glycosaminoglycans also were isolated from the acid-soluble fraction of Chinese hamster fibroblasts (40), from HeLa L mouse embryo, and from rat embryo cells grown in culture medium in the presence of radioactive inorganic sulfate (41).

The method of Charles and Scott (42), with modifications, is used for most extractions of heparin. In their technique the animal tissues are treated with alkali to remove proteins. Homan and Lens (43) did this removal by shaking with phenol. Jeanloz (44) reported other methods of heparin extraction involving enzymic degradation. Barlow *et al.* (45) described a technique of isolating heparin from sheep, dog, beef, and hog tissues by selective fractionation with quaternary ammonium compounds. They claimed that the heparins were biologically, chemically, and physically similar.

CHEMICAL STRUCTURE OF HEPARIN

Many reviews have sections on the nomenclature and classification of the mucopolysaccharides (46, 47) and the chemistry of heparin (3, 4, 6, 48–53).

The elucidation of the structure of heparin has been concerned with the type of glycosidic linkage, the nature of the uronic acid component, the sulfur content and sulfamino group, the carboxyl and other groups, and the amount of branching.

Cifonelli and Dorfman (54) showed that the major linkage in heparin is uronosyl 1 → 6 hexosamine, while that in heparitin sulfate is mostly 1 → 4. Durant *et al.* (55) and Danishefsky and Steiner (56), on the other hand, found a 1 → 4 linkage also for heparin. This was confirmed by Wolfrom *et al.* (57), who also found that the configuration of the 2-amino-2-deoxy-D-glucuronic acid linkage is α-D. The possibility of some β-configuration might also exist (51, 58). Lloyd and Evans (59) reported a method of linkage analysis of heparin and related mucopolysaccharides by forming *N*-(2,4-dinitrophenyl) derivatives of de-*N*-acylated mucopolysaccharides, rather than affecting the labile sulfamino groups, by breaking acid-resistant 2-amino-2-deoxyglycosidic bonds to achieve a graded depolymerization.

The sulfur content of heparin has been shown to be associated with its anticoagulant activity (60). A method of determination of sulfur as sulfate is precipitation with barium (60, 61). Another technique, proposed by Braselmann (62), permits the determination of 0–12 mcg. of sulfate, with an error of ±0.2 mcg., by heating the heparin sample with hydriodic acid, hypophosphorous acid, and hydrochloric acid to reduce sulfate to hydrogen sulfide. A rapid and sensitive method using GLC to analyze sulfate in mucopolysaccharides, including heparin, in micrograms was devised by Srini-

vasan *et al.* (63). This method was based on the ability of mineral acids to form stable salts with the mucopolysaccharides, which, after reaction with sodium hydroxide, liberate amines that are detected by GLC. Even more recently, Wessler (64) measured the sulfate content in less than a microgram of glycosaminoglycans with a method based on the fact that the electrophoretic mobility in 0.1 *M* HCl is proportional to the sulfate content. Hiyama *et al.* (65) indicated that the anticoagulant activity of heparin is, in general, dependent on the degree of sulfation and the number and position of the *O*-sulfate groups as well as the size of the skeletal polysaccharide molecule.

Durant *et al.* (55) claimed that approximately one-eighth of the hexuronic acid units have no *O*-sulfate groups. The evidence of the report by Wolfrom and Wang (66) establishes that the C-6 hydroxyl group of the 2-amino-2-deoxy-D-glucose unit of heparin is sulfated. In a later work, Wolfrom *et al.* (67) definitively placed the two sulfate groups (per tetrasaccharide unit) on C-6 of the 2-amino-2-deoxy-D-glucose residues and demonstrated that D-glucuronic acid residues are not sulfated. Danishefsky *et al.* (68) investigated the position of the sulfate groups by methylation procedures, and they concluded that about one-third of the glucuronic acid moieties in heparin are sulfated on C-2 whereas the rest are not sulfated. Additionally, most of the glucosamine is sulfated on C-6, but nonsulfated glucosamine units and 3,6-di-*O*-sulfoglucosamine units are also present in small proportions. Helbert and Marini (69), on the basis of elemental analysis and titration studies, found that not all of the sulfate forms an integral part of the heparin molecule but it may be closely associated with it.

The extent of carboxylation may be important in the anticoagulant activity of heparin, as reported by Stivala and Liberti (70) based on copper binding studies. It is possible to convert the carboxyl groups of heparin and other mucopolysaccharides into substitution acids of amino acids (71). These were obtained with glycine methyl ester, phenylalanine methyl ester, and alanine amide.

Heparin is usually considered to be an extended linear molecule. Some authors, however, such as Freeman (72) and Bettleheim (51), still believe in the possibility of a branched structure.

The question of whether heparin contains acetyl groups is still unsettled. Rhadhakrishnamurthy *et al.* (73) found varying amounts of acetyl groups in heparin samples from various sources. The presence of these groups might be due to the presence of heparitin sulfate, as shown by NMR studies (74).

It was concluded by chemical means that D-glucuronic acid is the chief uronic acid component of heparin (75, 76). Wolfrom *et al.* (77) isolated L-iduronic from heparin by a periodate oxidation study. Perlin *et al.* (78–80) showed with NMR spectra that heparin contains a substantial proportion (perhaps one-third or more) of L-idopyranosyluronic residues, in addition to the D-glucosyluronic acid and 2-amino-2-deoxy-D-glucose units. Lindahl and Axelsson (81), through a nitrous oxide degradation of heparin and separation by gel permeation and paper electrophoresis, found that almost all of the uronic acid present in certain fractions was iduronic acid. Rhadhakrishnamurthy *et al.* (82) investigated the nature of the uronic acid by the use of GLC and found that the ratio of iduronic acid to glucuronic acid was 1:2.6.

Through degradation studies with *Flavobacterium heparinum* (83), it was concluded that the heparin molecule is composed largely of a repeating sequence of (1 → 4)-linked 4-*O*-(α-L-idopyranosyluronic acid 2-sulfate)-2-(deoxy-2-sulfamino-α-D-glucopyranosyl-6-sulfate) biose residues. Another proposed structure is that of the tetrasaccharide unit of Helting and Lindahl (58) with both uronic and iduronic residues (Structure I).

A reaction thought by Sampson and Meyer (84) to be important in structural studies is that of heparin or heparitin sulfate with alkali to form 3,6-anhydroglucosamine groups, in which the 6-position is sulfated and the 3-position is unsubstituted.

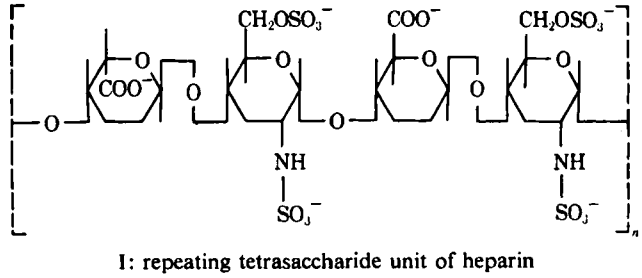
It is evident from the numerous studies conducted on the structure determination of heparin that its true configuration remains to be established unequivocally. The defiance of heparin to be clearly identified may be due to the fact that the heparin derived from different tissues within a given species or from the same tissues from different species may have subtle differences in its structure. Accordingly, it is not surprising that differences in chemical structures have been observed, since heparin is a protein–polysaccharide complex before extraction. The extraction procedure can often leave some undiscernible impurities, which undoubtedly compounds the difficulty in structure determination.

ELECTROPHORESIS AND CHROMATOGRAPHY OF HEPARIN

The techniques of electrophoresis and chromatography of heparin and other acid mucopolysaccharides for separation, identification, fractionation, characterization, and binding studies have been investigated by many workers.

Separation Techniques—One of the oldest separation methods used was electrophoresis. In 1951, Favre-Gilly (85), using the moving boundary method, discovered that the electrophoretic mobility of heparin is 17 × 10^{-5} cm.^{2} v.$^{-1}$ sec.$^{-1}$. Also using the same technique and employing data available on the heparin–streptomycin complex, Barlow and Coen (86) separated heparin free from streptomycin.

Paper and gel electrophoresis have now gained much popularity in separation methods. Bifani and Marcialis (87) discovered that heparin had a higher migration velocity than serum proteins on paper. Conti *et al.* (88) detected heparin in the presence of chondroitin sulfuric



I: repeating tetrasaccharide unit of heparin

acid, hyaluronic acid, or nucleic acid by staining the paper with Auramino O, Malachite green, or Brilliant green. Starch gel electrophoresis has been carried out for the separation of heparitin sulfate from chondroitin sulfate A and B and hyaluronic acid (89). Another sensitive method is zone electrophoresis on cellulose acetate, with alcian blue as a stain (90).

Horner (91) separated heparin and chondroitin sulfate by their differences in charge density on agarose gel. He also noted that heparin had two components on this gel; the slower moving component had the higher anticoagulant activity, whereas the faster moving component had the higher sulfate–carboxyl ratio (92). Jaques *et al.* (93, 94) found that most heparins showed two components by microelectrophoresis on agarose, except for those from the sheep and rat. The use of a microzone cellulose acetate cell[1] enabled Herd (95) to separate heparin; hyaluronic acid; chondroitin sulfates A, B, and C; and keratosulfate. Heparin and heparan sulfate can be separated from other glycosaminoglycans with barium acetate electrophoresis, where the electrophoretic mobility depends primarily on the polysaccharide backbone (96). The relative mobilities of heparin, chondroitin sulfate A (or C), and hyaluronic acid in electrophoresis by polyacrylamide gels were 1.11:1.00:0.67 (97), which are the same as in free solution electrophoresis (98). Another type of electrophoretic separation was performed by Mashburn and Hoffman (99), who used a continuous-flow electrophoretic flow separator for the separation of heparin, chondroitin sulfate, hyaluronic acid, and protein.

Liquid and ion-exchange chromatography are rather old techniques but recently gained new impetus for separations. In 1962, Schmidt (100) obtained a clear separation of the acid mucopolysaccharides on a DEAE-Sephadex anion exchanger. Using the same type of column, Braselmann and Ramm (101) did a microseparation of hyaluronic acid, chondroitin-4-sulfate, dermatan sulfate, chondroitin-6-sulfate, and heparin. In a column chromatographic study, Greiling *et al.* (102) and Egardo *et al.* (103) separated heparin from hyaluronic acid and chondroitin sulfate, utilizing ECTEOLA-cellulose with gradient elution. This type of column was also used for the quantitative estimation of acid mucopolysaccharides in vascular tissue (104). A cellulose microcolumn technique to separate mucopolysaccharides was described by Svejcar and Robertson (105). Microcolumns of Sephadex G-25 (fine) were used to carry out the separation of heparin from D-galactose by Stouffer *et al.* (106), with a hydrogen flame-ionization detector. Another interesting application of this method was the differential precipitation of individual polyanions with quaternary ammonium salts in solutions of specific ionic strength and the subsequent formation of red ion association complexes with the bound quaternary moieties; thus the individual mucopolysaccharides could be identified (107).

Two other major techniques are paper chromatography and TLC. Spolter and Marx (108) separated heparin and chondroitin sulfate by paper chromatography with a solvent of buffer and isopropanol. Bayer (109) used a solvent system containing *n*-propanol. An ascending paper chromatography method with acetate buffer allowed Good (110) to separate heparitin sulfate, heparin, keratosulfate, and chondroitin sulfate B. Aliev and Alekperov (111) used ascending paper chromatography for stepwise control of heparin production. They (112) also reported that commercial heparin gave two spots which might be due to chondroitin sulfate as an impurity. Chondroitin sulfate B (dermatan sulfate) has been identified as a contaminant of bovine heparin during fractionation due to solubility differences of the two substances (see *Physicochemical Studies*). Other methods included microseparation on paper based on the formation of insoluble complexes with cetylpyridinium chloride (113) and the use of salt-gradient ion-exchange paper (114).

In the last few years, TLC has gained support in the separation of mucopolysaccharides. Wustemann *et al.* (115) successfully separated heparin from heparitin sulfate, chondroitin-6-sulfate, and chondroitin-4-sulfate on silica gel, with nitrous indole as a spray specific for detection of the *N*-sulfated aminopolysaccharides. Bischel *et al.* (116) used Sephadex and cellulose as layers, with toluidine blue staining. Other investigators (117) obtained two spots for heparin on cellulose, using ninhydrin as a detection agent.

Fractionation—Chromatography and gel permeation are potent methods for fractionating according to molecular weights and for estimating heterogeneity. In 1958, Payza (118) fractionated heparin on Amberlite IR-45 resin by elution with different molarities of sodium chloride. An ECTEOLA-anion exchanger was later used by Whiteley (119). Laurent (120), after precipitating heparin with cetylpyridinium chloride, used the same system to obtain four fractions. Lasker and Stivala (121) likewise obtained the same number of fractions by ECTEOLA elution, but got 12 fractions by differential solubility in alcohol–water mixtures. (Nine fractions have been obtained by fractional precipitation, using ethanol and dioxane as precipitants; see *Physicochemical Studies*.) The efficiency of DEAE-cellulose as an adsorbent in fractionation was proven by Cleland *et al.* (122), and recently DiFerrante and Popenloe (123) fractionated heparin on Dowex-1.

Further work on fractionation was carried out by Hall *et al.* (124), who submitted pork heparin to gel filtration on a column of Sephadex and found that the heparin was polydispersed with respect to molecular size. Constantopoulos *et al.* (125) and Mathews and Decker (97) determined the degree of heterogeneity of a mixture of acid mucopolysaccharides of different molecular sizes by means of gel filtration.

Characterization—Since exclusion by the gel particles depends on molecular shape, the study of the behavior of heparin on a Sephadex column with changes in the ionic strength of the eluant gave indication that the heparin molecules change in shape and size as well as in solvation, since they are retarded on the column with solvents of higher ionic strength (126).

The work of Hall *et al.* (124) suggests that the anticoagulant activity of a sample could be relatively homogeneous even though it is polydisperse in size. Ferrari and Boffi (127), on the other hand, claimed that

[1] Beckman model R-100.

it was impossible to correlate anticoagulant activity with chromatographic behavior since all samples gave lengthened spots, similar in shape and with the same R_f.

Hilborn and Anastassiadis (128) recently demonstrated how acrylamide gel electrophoresis could be used to: (*a*) estimate the molecular weight of many acidic mucopolysaccharides (including heparin) and (*b*) measure the variations that may exist in their molecular weights and/or the degree of sulfation.

Binding Studies—Boundary electrophoretic studies were conducted by Barlow (129) on the binding of heparin with histamine, using paper electrophoresis. The finding of two different electrophoretic patterns as a function of anticoagulant activity indicates that there is a difference among samples as to their ability to bind histamine; this result could be due to a difference in anticoagulant activity.

Another study of binding was conducted by Accary *et al.* (130) on polysaccharide–sulfate–protein complexes, using paper electrophoresis.

PHYSICOCHEMICAL STUDIES

Deactivation of Heparin and Structure Determination—It has been suggested that anticoagulant activity of heparin is related to sulfation (6, 131–134), to carboxylation (6, 131–134), and to various physical parameters such as molecular weight, shape, and size (6, 131–135). These have been studied by mild hydrolysis of heparin, which results in desulfation (without chain cleavage) accompanied by a decrease in biological activity (deactivation).

Wolfrom and McNeely (136), in 1945, discovered that very mild acidity causes the heparin molecule to lose its blood anticoagulant activity. The rate of loss of activity is proportional to the rate of appearance of a free amino group in the molecule, and substantially complete inactivation occurs when somewhat more than one-half of the nitrogen is released as free amino nitrogen. Jensen *et al.* (135) reported that reaction of heparin with warm acetic acid resulted in a strong decrease in anticoagulant activity without any decrease in molecular weight, sulfate ester, or glucosamine content. There was also an increase in the sedimentation constant and the degree of polydispersity but a decrease in the frictional ratio. They suggested that the amino nitrogen that was first freed during recrystallization of heparin after acid treatment played an important role in anticoagulant activity. The findings of Gauthier *et al.* (137) showed that if the pH was adjusted with acid to 1 or 2 at 25°, there was no change in the biological activity of the heparin within the first 24 hr. Substantiating this finding is the evidence presented by Stock and Warner (138), who observed deterioration of heparin only after 60 hr. at pH 4.4 and 23°. Swarte and Huizinga (139) showed that heparin solutions can be sterilized by steam at 100° for 1 hr. at pH 7.0 with no changes in anticoagulant activity. On the other hand, it is possible to degrade heparin in a strong alkali medium with a rapid loss of antilipemic activity (140).

Foster *et al.* (141) maintained that the anticoagulant activity does not necessarily follow the content of sulfamino groups, since they observed that some heparin preparations with high *N*-sulfate content exhibited low anticoagulant power. In most cases, however, the anticoagulant properties of the sulfated polysaccharides containing no sulfamino groups amounted to only a fraction of that of heparin itself.

Ricketts (142, 143) concluded that the molecular weight of the polysaccharide was more important than the sulfate content, since little activity was found in low molecular weight species of polysaccharides (degree of polymerization = 9) even when the sulfate content was high (2–2.8 sulfate groups per glucose residue). Sulfated dextrans showed a higher anticoagulant activity for the polymer possessing the higher molecular weight. Patat and Elias (144) likewise found that there is a definite dependence of the biological activity of heparin on molecular weight. Laurent (120) claimed a correlation between biological activity and molecular weight in heparin fractions from a single source. Barlow *et al.* (145) and Braswell (146) did not find this relationship; however, their studies were based on unfractionated heparin from various sources. Lasker and Stivala (121) reported that the biological activities of low molecular weight fractions (performed by differential solubility) were uniformly low. Liberti and Stivala (147) discovered that the anticoagulant activity of heparin with molecular weights of 4500–15,500 (fractionation done by precipitation with ethanol and dioxane) increased with molecular weight up to 10,000 but then showed no appreciable change in activity on further increases of molecular weight.

Lasker and Stivala (121) demonstrated that the samples obtained by deactivation of unfractionated heparin with dilute hydrochloric acid showed no detectable changes in molecular weight, intrinsic viscosity, IR spectra, or optical activity. They also found that partially inactivated heparin behaves like active heparin in that there is a drop of activity in an environment of 0.5–1.0 *M* NaCl (148).

The treatment of α-heparin (bovine) and ω-heparin with 40% acetic acid at 37° for almost 1 day, by Yosizawa *et al.* (149), almost completely deprived these heparins of anticoagulant properties, with a loss of 7–8% of *N*-sulfate groups. These results indicate that the anticoagulant activity of α-heparin is more labile and its lipolytic activity less labile than those of ω-heparin. Braswell (146) also degraded heparin with acid, but only until one-half of the activity was removed. There was little change in molecular weight, intrinsic viscosity, or elemental analysis. He postulated that these results are due to the formation of internal esters because acidity is destroyed without losing sulfur.

Opposed to these latter results are the findings of Helbert and Marini (150) who used self-hydrolysis and titration techniques. They found that *N*-sulfate groups were removed twice as fast as *O*-sulfate groups, but they did not perform elemental analyses or anticoagulant assays throughout the course of degradation; therefore, it is difficult to compare their work with that of others.

Stivala *et al.* (133), following the method of Helbert and Marini (150), did a graded hydrolysis on unfractionated heparin and measured the sedimentation co-

efficient, intrinsic viscosity, molecular weight, and axial ratio as functions of hydrolysis time. The data suggest that: (*a*) the decrease in molecular weight is due to desulfation rather than depolymerization, (*b*) hydrolysis results in both *N*- and *O*-desulfation, but the former is greater, and (*c*) the degree of sulfation and size and/or shape are responsible for the biological activity of heparin.

Yuan (151), in a study with Ehrlich (152) on the self-hydrolysis of a heparin fraction, found that the decrease in the anticoagulant activity of the partially hydrolyzed heparin corresponds to the amount of sulfate groups removed. There is also a decrease in the intrinsic viscosity in water, molecular weight, and the axial ratio. Yadlowsky (152A) and Stivala *et al.* (152B) studied the kinetics of the acid hydrolysis of heparin from its Cu (II) complex. They showed that hydrolysis during 5 hr. at various temperatures obeys first-order kinetics, and they obtained specific rate constants of 3.3–26.6 $\times$ 10^{-5} sec.$^{-1}$ in the range of 50–80°. Arrhenius plots of the data yielded 14.7 kcal. as the energy of activation. Yuan (151) also obtained similar results on the self-hydrolysis of heparin. These investigators found that the anticoagulant activities of heparin and the Cu (II) complex were not appreciably different and that the deactivation parallels Cu (II) release from the complex, which in turn parallels desulfation.

Optical rotatory dispersion, NMR, and gel permeation also were utilized to follow deactivation. Stone (153) demonstrated that the induced Cotton effects of the dye diminished in a graded hydrolysis. Changes in conformation were also followed through optical rotatory dispersion by Hirano and Onodera (154). They concluded that *N*-desulfation does not change the original conformation, but complete desulfation causes the original helical conformation of the heparin–dye complex to disappear. Findings by Sumyk and Yocum (155) on elution volumes were consistent in the conformation difference between native and desulfated heparin. Using NMR, Jaques *et al.* (156) found that *N*-desulfation causes a downfield displacement of the signal at 4.75 p.p.m.

The degradation of heparin by UV light and fast electrons was studied by Balazs *et al.* (157) and Sundblad and Balazs (158). Irradiation with UV light gave a decrease in anticoagulant activity and cationic dye binding power of the polyanion. Irradiation with electrons causes depolymerization. The use of γ-irradiation on dilute solutions of heparin by Jooyandeh *et al.* (159) produced depolymerization but no desulfation.

Interaction of Heparin with Dyes, and Optical Rotatory Dispersion—When dyes such as azure A, methylene blue, basic fuchsin, Brilliant green, and other metachromatic dyes are reacted with heparin, there is an absorption at a wavelength that is shorter than with the dye itself. Stone and Bradley (160), in 1967, showed that spectrophotometric titration of mucopolysaccharides with dyes was possible and, because of its high charge density, heparin would best be titrated with proflavine. A review was presented by Stone (161) on the use of metachromasy to induce Cotton effects in glycosaminoglycans.

According to Yen *et al.* (162), two dye molecules react with each tetrasaccharide unit, and apparently the reaction occurs preferentially between two carboxylic or sulfamino groups. Young *et al.* (163), in agreement with the latter investigators, believed that there is an electrostatic interaction between the dye and polyanion sites. Thermodynamic studies on heparin–azure A complexes indicated that the complexation produces a loss of energetic symmetry in the chromaphoric system, resulting in a shift to a lower wavelength. Various other methods, such as electron paramagnetic resonance (164) and pulse radiolysis (165–167), were used by Balazs *et al.* to examine the electrostatic interactions between methylene blue and heparin. Through this latter method, Phillips (168) unequivocally showed that cationic dyes are bound at anion sites of polyanions in such a way that an electronic exchange occurs, which is directed to the metachromasy.

The effect of temperature on polyanion metachromasy was studied by Kelly *et al.* (169). The metachromatic ratios, A_{575}/A_{628}, were found to be inverse linear effects of temperature. Stained sections gave the same results (170).

Dyes have been employed in the identification of heparin. In tissue, the staining with ruthenium red was found by Pihl (171) to be stoichiometric and the bulk of the stain was taken up by heparin in a molar relationship of one dye cationic complex per sulfate group of heparin. Sasai (172), after staining tissues containing acid mucopolysaccharides with alcian blue, discovered that the binding of the dye to mucopolysaccharide decreased as the salt concentration was raised.

The study of the optical rotatory dispersion of heparin has been possible after binding with dyes. Stone (173), in 1969, presented an excellent review on conformations of hexose polysaccharides in solution, dealing with optical rotatory dispersion. In contrast to the views of the previous investigators mentioned, Stone (153) believed in a theoretical card-stack aggregation of dye molecules, with a helical conformation for the heparin–dye complex, since an induced rotation was observed showing anomalous dispersion in the absorption band of the bound dye. Furthermore, the Cotton effect was dependent on helical conformation, since no induced rotation was observed when the dyes were bound to polyglutamic acid in its random coil conformation. This helical structure agrees with that proposed by Velluz (174) for heparin itself. The phenomenon of induced rotation was investigated by Stone and Moss (175), as a function of pH, in the range of ionization of the carboxyl group of heparin, with the result that metachromasy decreased with a decrease in anionic site density. Further investigations by Stone (176) on dye binding indicated that the binding of methylene blue to the negative sites of heparin gives rise to a negative doublet (circular dichroism) in the metachromatic absorption band, indicating a net left-hand chirality for the neighboring dye molecules.

The effect of urea in the destruction of hydrogen bonds that serve to maintain the conformation of the mucopolysaccharides was found (154, 177) to increase slightly the optical rotation of those mucopolysaccharides possessing helical structure. Also, the induced

Cotton effect was lost in urea and a positive plain curve was obtained. A later study by Hirano (178) showed, by optical rotation measured in urea and guanidine hydrochloride, that the tertiary structure of polysaccharide chains is almost destroyed while the tertiary structure remains in water.

Other optical rotatory measurements by Stone (153, 179) on the interaction of histamine and heparin with dye-stacking techniques indicate that there is a decrease in the metachromasy of dye–heparin complexes on the addition of histamine; also, conformational changes in heparin may accompany the release of histamine, which causes anaphylactic shock concomitant with degranulation of the mast cell *in vivo*.

A method of using the optical dispersion effects of mucopolysaccharides was presented by Stone *et al.* (180) for distinguishing between San Filippo and Hurler syndromes, since the UV optical rotatory dispersion and circular dichroism of dermatan sulfate are greatly different from those of heparin sulfate because dermatan sulfate is present only in Hurler's syndromes.

Binding and Complexing of Heparin—Since heparin is a highly charged polysaccharide, binding and complexing with both large and small ions and molecules become possible. It is thought by many to exist in nature bound to proteins as a protein–polysaccharide complex in the mast cells and released when necessary. Also, its anticoagulant and clearing action may be due to binding or complexation.

The binding of heparin to various proteins has been studied by many investigators. Recent reviews were written by Louisot (181) and Meyer (182). Cifonelli *et al.* (183) discovered that concanavalin-A, a globulin protein of Jack-bean meal, combines with heparin to form a precipitate. More recently, DiFerrante and Hrgovcic (184) claimed no interaction between the two, but thought that the presence of other proteins could be responsible for the interaction. However, Doyle and Kan (185) maintained that there is a precipitin-like complex with heparin, but that the interaction is pH, salt, and sugar sensitive.

The binding of heparin to collagen was found to occur in definite proportions by Delaunay and Bazin (186), and an ionic linkage was suspected because of the pH dependence. They (187) also found that other substances, which were unable to form stable combinations with collagen A, had the power to affect the normal combination of collagen–mucopolysaccharide linkage, depending on their acidity. Curves obtained by Serafini-Fracassini (188) of the amount of polyion fixed *versus* pH of heparin and chondroitin sulfate were similar to titration curves of collagen. Németh-Csóka (189) found that the activation energy of collagen at pH 7.35 was increased by heparin. Mathews and Decker (190) showed that heparin increased the rate of fibril formation from collagen solutions. They believed that this is due to electrostatic binding of heparin to collagen.

Kudryashov *et al.* (191, 192) found that complexes of heparin with the blood proteins such as fibrinogen, thrombin, and plasmin had antithrombotic and fibrinolytic activity. The existence of a complex between fibrinogen and heparin was confirmed by Bernfeld (193) with the aid of moving boundary electrophoresis. The mixing of radiolabeled heparin and normal serum was shown by Becker *et al.* (194) to give complexes of heparin in two major electrophoretic regions in starch zone electrophoresis. Lages (195) and Lages and Stivala (195A) quantitatively studied the binding of heparin to fibrinogen in the presence and absence of Cu ions, using equilibrium dialysis and sedimentation velocity. They found that the sedimentation coefficient of the fibrinogen–heparin complex is affected by the Cu ions.

The binding of heparin to other proteins also includes insulin, vasopressin, oxytocin, and corticotropin (196). A heparin–trypsin complex had fibrinolytic activity and showed prophylactic effects against development of thrombosis (197). Aborg and Uvnäs (198) described a protamine–heparin complex which has the ability to bind histamine and monoamines electrostatically. In addition, whale heparin can bind protamine sulfate (199).

The nature of the linkage of heparin to protein has been the subject of much investigation. The findings of Lindahl and coworkers (200–205) indicate that galactose and xylose are both involved in the linkage of heparin to protein. A glycosidic linkage between xylose and the hydroxyl group of serine appears to constitute the carbohydrate–protein linkage. The proposed sequence, given by Helting and Lindahl (58), is shown in Scheme I. Stern *et al.* (206) discovered that dermatan sulfate is similarly bound to proteins. Opposed to this view is that of Scott (207, 208), André *et al.* (209), and Aborg and Uvnäs (198), who believed in an electrostatic linkage.

The binding of neutral pH sulfated polysaccharide to serum low density lipoproteins was observed by Burstein *et al.* (210). Srinivasan *et al.* (211) showed that serum pre-β- and β-lipoproteins formed insoluble complexes with acid mucopolysaccharides in the presence of Ca^{+2}. A mechanism of formation of a complex through Ca^{+2}, acting as a bridge between *N*-sulfate groups of heparin and phosphate groups of the phospholipids of the lipoproteins, was suggested. Similarly, Olivecrona *et al.* (212) presented evidence for an ionic binding of milk lipoprotein lipase to heparin.

The binding of histamine to heparin will be considered in conjunction with the mast cells because heparin may be the histamine binding agent in these cells. The granules of mast cells are known to contain both heparin and histamine (213), and strong evidence has been presented that mast cells produce the heparin. A complex between heparin and histamine was discovered by Werle and Amann (214), which was suggested to be present in the mast cells. Lysis of granules of the mast cells by Hill (215) caused destruction of the protein component and released heparin. The mechanism of binding suggested by Kobayashi (216) was the same type as an ion exchanger. Lagunoff (217) presented evidence that the mast cell granule is a heparin–protein matrix to which histamine and perhaps other nitrogenous bases are attached electrostatically. The findings of Lloyd *et al.* (218, 219) indicate the natural occurrence of mast granule heparin linked to protein *via* alkali-labile covalent bonds. The pH dependence of the binding mechanism of the granules led Aborg and colleagues (220, 221) to suggest that the

histamine is bound ionically, which is easily broken on exposure of the granules to other cations. A study of the heparin–protein complex from ox liver capsule by Serafini-Fracassini *et al.* (222) led to the observation that the mast granules are composed of a random network of elongated heparin–protein macromolecules surrounded by the other components.

The exact correlation between heparin content and mast cell count is not a simple one, according to Jaques and Debnath (223). Also, Drucker-Colin (224) suggested that large amounts of heparin present in the rat intestine are not associated with mast cells. In a study of mast cells in mastocytosis by Zimmer *et al.* (225), it was discovered that heparin, unlike histamine, is only rarely released into the circulation in clinically significant amounts. Experiments by Slorach (226) revealed that both histamine and heparin are released when rat mast cells are exposed to the histamine releasing agent, Compound 48/80. As in the case of mast cells, histamine was shown by Aborg and Uvnäs (227) to be bound to carboxyl groups in a heparin–protein complex in rat thrombocyte granules. The binding of histamine to heparin was used by Barlow (228) to follow desulfation of heparin.

It is possible for heparin to bind with many other organic compounds. Woodside *et al.* (229) interacted heparin with glycogen to form insoluble complexes. Organic dyes such as azure A, toluidine blue, Bismarck brown, and brilliant cresyl blue combine with heparin (230). Acridine orange forms a reversible complex with heparin (231), and alcian blue is fixed to the available binding sites of heparin (232). Heparin combines with many enzymes such as trypsin, chymotrypsin, pepsin, and lysozyme (230). The reaction with lysozyme, according to Bychkov and Kharlamova (233), involves primarily electrovalent forces. Bergman *et al.* (234) mentioned that enzyme–heparin complexes can cause artificial enzyme polymorphism, which can be used both as a diagnostic tool in clinical medicine and in the study of genetic polymorphism. Heparin combines with histones and displaces deoxyribonucleic acid from a bond in deoxyribonucleoprotein (235). It forms complexes with polymyxin B (236), streptomycin (145, 236, 237), monomycin (237), kanamycin (237), and neomycin (237). Popova (238) demonstrated that the binding of chlorpromazine to heparin replaced the storage receptor in the molecule of the acid mucopolysaccharide and released the biogenic amines. In a study of the binding of histamine, carbaminoylcholine, and 5-hydroxytryptamine to heparin, Mordelet-Dambrine *et al.* (239) observed that only histamine was fixed at pH 4, probably because it is most strongly ionized. Evidence was given by Kudryashov and Lyapina (240, 241) that a heparin–adrenalin complex exists which has a possible role in blood coagulation. The complex dissolved nonstabilized clots of fibrin, exhibited antipolymerase activity, and blocked the fibrin-stabilizing activity of factor XIII.

Heparin was shown by Ascoli and Botré (242) to have a high binding capacity for Na^+ and Ca^{+2} ions. Both Jaques (230) and Salminen and Luomanmaki (243) agreed that potassium ion is bound to heparin in preference to the sodium ion. The order of increasing cation activity for heparin was given by Dunstone (244) as Na^+, K^+, Mg^{+2}, Ca^{+2}, Sr^{+2}, and Ba^{+2}. Ohkuma and Furuhata (245) felt that Na^+ ions, in addition to toluidine and albumin, form a complex with heparin, with the suggestion that heparin possesses a cation-exchange behavior. In an investigation of heparin's polyanion affinity to $^{22}Na^+$ and $^{137}Cs^+$, Dorabialska and Plonka (246) discovered that the affinity was greater to the one with the lower total concentration.

The binding of $Co(NH_3)_6^{+3}$ to connective tissue mucopolysaccharides and heparin was investigated by Mathews (247), who concluded that ion-pair formation with $Co(NH_3)_6^{+3}$ is influenced by local binding factors, electrostatic interaction of neighboring charged groups, and competition with other cations for binding sites. Anticoagulant activity was generally associated with affinity for $Co(NH_3)_6^{+3}$. This was also experienced by Moore *et al.* (248) in the interaction of connective tissue and other polyanions with cationic counterions. The binding of Cu^{+2} to heparin forms a basis for studying the anticoagulant activity of heparin (134). It is pH dependent because of competition for sites and is ionic strength dependent because of a change in conformation. In desulfation, reduction in binding sites results in decreased binding of Cu^{+2}. In the work (134) on the Cu^{+2} binding from polarography, it was also shown that the ability of heparin to bind copper is related to blood anticoagulant activity.

The order of calcium binding to hyaluronic acid, chondroitin sulfate, heparin, and heparitin sulfate was examined by Lászlo (249) with the result that chondroitin sulfate had the highest and hyaluronic acid the lowest binding capacity. It was found that most of the Ca^{+2} was bound to the carboxyl groups. The effect of Ca^{+2} ions on the scattering of light by heparin was investigated by Loucas *et al.* (250) who found that the interaction constant was influenced by the ionic strength of the solution.

A study by Quintarelli *et al.* (251) showed that binding between phosphotungstic acid and desulfated heparin only took place between the metal and the desulfated hexosamine amino groups through electrostatic forces. On acetylation of the *N*-desulfated products, no phosphotungstic binding occurred.

Effect of Ionic Strength and pH—An attempt was made by Barlow *et al.* (145) to study the effect of ionic strength on the sedimentation constant and molecular weight, but no previous fractionation was performed on these samples of heparins of different sources. Their results indicated that there is a probable increase of the sedimentation constant with ionic strength until a maximum is reached and then it remains constant. However, they did not use an extrapolated value for the sedimentation constant to allow for concentration dependence. They also found that the molecular weights obtained from light scattering did not change with ionic strength.

Liberti and Stivala (147) found that there is a definite decrease of intrinsic viscosity with an increase of ionic strength. This same effect was experienced by Braswell (146). Yuan (151) also obtained similar results with unfractionated heparin from one source, but the influence of pH was not studied under controlled conditions.

Ehrlich (152) used controlled conditions of pH and ionic strength on a particular fraction and had results for the sedimentation constant that were similar to Barlow's *et al.* (145). Also the axial ratio decreased, the intrinsic viscosity decreased, the molecular weight did not change, and the partial specific volume increased between the ionic strength of 0.5 and 1.0 *M*. This behavior can be explained in that the addition of by-ions results in a lower field strength, with a resulting change in shape offering less frictional resistance to transport.

Size and Shape of Heparin—Stivala *et al.* (252) studied molecular weights, radii of gyration, and persistence lengths of a fractionated sample of heparin by means of low angle X-ray scattering. The molecular weight of the heparin fraction was found to be 12,900. This value is in excellent agreement with the values of 12,600 and 12,500 obtained for the same fraction from sedimentation equilibrium (121) and intrinsic viscosity (147) measurements, respectively. A persistence length of 21.1 Å was calculated. Based on low angle X-ray scattering, the heparin fraction in water (1%) at room temperature may be described as a Gaussian coil molecule.

Since heparin is a polyelectrolyte, plots of specific viscosity *versus* concentration in water are nonlinear. Linearity may be achieved by use of various empirical relationships. Some researchers (121, 133, 147) measured the intrinsic viscosity of numerous heparin fractions of varying molecular weights in water and as a function of pH and ionic strength. Based on various solution theories of linear polymers, heparin exhibits substantial flexibility, approximating a Gaussian coil. From partial specific volume, sedimentation coefficient, molecular weight, and intrinsic viscosity measurements, where charges were swamped (by means of pH and/or ionic strength), Ehrlich (152) and Ehrlich and Stivala (252A) reported that heparin is best approximated as a random coil (Gaussian).

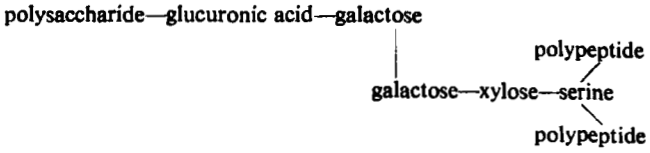
Yuan and Stivala (252B) studied the intrinsic viscosity as a function of dielectric constant and found that the shielded intrinsic viscosity (which is that of a highly shielded polyion) increases linearly with the increasing dielectric constant of the solvent. Furthermore, based on an interaction parameter of a semiempirical relationship containing the shielded intrinsic viscosity, Yuan and Stivala (252B) showed that the interaction parameter is independent of the dielectric constant of the solvent of the various heparin solutions. The significance of this interaction parameter is that it contains a term associated with the equivalent freely jointed segment length when the backbone of a polyion is represented by a Gaussian coil for which the interaction parameter is independent of the dielectric constant.

ASSAY AND ESTIMATION OF HEPARIN

The various methods of heparin determination can be essentially divided into two groups, those involving the reaction of heparin with substances such as protamine or toluidine blue and those measuring directly the specific anticoagulant activity with respect to total blood or plasma.

Metachromatic and Protamine Methods—A recent review of metachromasy of dyes in solution was presented by Padday (253). Awe and Stüdeman (254) found that quantitative measurement of the course of protamine titration and metachromatic dye change from blue to red-violet conformed with USP XV standards, but heparinoids gave erroneous results. They presented two methods for the spectrometric evaluation of the toluidine blue complex of heparin, both involving the metachromatic shift of the complex. The use of azure A and toluidine blue O (tolonium chloride) by Serafin (255), to test activity, gave much larger amounts of heparin in normal blood than previously reported. The ratio between metachromasy and uronic acid content was measured by Bianchini and Osima (256), who found that neither the anticoagulant activity nor the clearing activity is linear with the uronic acid content. According to Jaques *et al.* (257), there is no correlation between activity and chemical analysis except for the finding that increasing metachromatic activity accompanied an increase in sulfur content. A sensitive, accurate, quantitative, colorimetric assay[2] with azure A, with absorption at 505 nm., was presented by Jaques and Wollin (258). A new method for fractionation and determination of heparin by differential colorimetry of metachromatic substances formed by toluidine blue was devised by Satake (259). A method utilizing very small amounts (0.5–5 mcg.) of acidic polysaccharides, employing the shift in the visible absorption spectrum of carbocyanine dye when bound to a polyanion, was given by Edstrom (260). Two colorimetric methods were described by Plekhan *et al.* (261). They involved the: (*a*) reaction of heparin and azure A, and (*b*) reaction with carbazole and concentrated sulfuric acid. A procedure for studying stoichiometry of complexes like heparin and toluidine blue was presented by Singh (262). Qualitative limits of the sensitivity of toluidine blue and azure A as compared with alcian blue and mucicarmine were established by Rahman *et al.* (263). The carbazole method is used widely for determining heparin concentrations. Galambas (264, 265) pointed out that the ratio between maximum absorptivity in sulfuric acid and the carbazole color at 525 nm. is higher for heparin than for the other mucopolysaccharides, probably because of the lack of *N*-acetyl groups in the former. Tsuji *et al.* (266) presented a novel spectrophotometric method for the microdetermination of 2-deoxy-2-sulfamidohexose and 2-acetamido-2-deoxyhexose residues in heparin, using an ion complex which absorbs at 653 nm.

For the determination of tissue heparin, Freeman *et al.* (267) mentioned a method of releasing the heparin from the protein complex that facilitates proteolytic digestion, since proteins interfere with metachromasy of applied dyes (268). The possibility of using acridine



Scheme I—Linkage of heparin to protein

[2] Using a Beckman spectrophotometer.

orange fluorescence for the determination of heparin was explored by Zelenin and Stepanova (269), who found that it had a red fluorescence to pH 7.0 and was resistant to sodium chloride solutions. Zugibe (270) gave many histochemical methods, one of which involves formation of the insoluble polyanion–quaternary salt complex with the mucopolysaccharide, followed by treatment with ferric thiocyanate. Other methods precipitate the acid polysaccharide with dyes such as toluidine blue and alcian blue and then differentially remove the polysaccharides by adding varying amounts of sodium chloride. The critical concentrations of electrolytes for heparin were measured by Sasai (172) by precipitation of alcian blue on cellulose acetate membrane, and he found 0.9 *M* for magnesium chloride and 1.1 *M* for sodium chloride. Scott (271) reviewed the critical electrolyte effects in interactions between acid glycosaminoglycans with organic cations and polycations.

Titration of heparin with protamine sulfate, followed by measurement of the coagulation after a period of time, is another possible method (272). Vincke and Benfey (273) found that 1 mole of protamine sulfate is equivalent to 629 g. of heparin. But Nagasawa and Kimura (274) claimed that there is no relationship between anticoagulant activity and protamine binding capacity. A possible reason for this, given by Lowary *et al.* (275), is that the number of heparin units neutralized by protamine sulfate varies with the source and specific activity of the sodium heparin. Recently, Godal and Gjeugedal (276) found that a transient coagulant effect is observed when heparin is incubated with protamine, possibly due to an excess of protamine. Another method was proposed by Barkagan and Barkagan (277) based on comparison of anticoagulation rates with and without inhibition of heparin by protamine sulfate.

Methods of Determining Heparin Activity in Blood—Reviews on heparin assays were written by Quick (278), Warren and Wysocki (279), Jaques (230, 280), Biggs and MacFarlane (281), and, recently, Jorpes (282). These methods measure directly the specific anticoagulant activity with respect to total blood or plasma.

Blombäck *et al.* (283) determined the heparin level in blood by titrating with thrombin and cofactor. Quick (278) mentioned a thrombin titration method which involves the inactivation of thrombin by heparin and its cofactor albumin X. Another titration method, described by Herbain (284), is a photometric estimation of the specification rate of plasma after the addition of thrombin. Godal (285) argued that the prolongation of the thrombin time is very sensitive to a variation of pH, temperature, and ionic strength; furthermore, calcium ions counteract heparin, especially at alkaline pH. According to Cameron (286), however, the thrombin clotting time is an efficient method of controlling heparin therapy.

The recalcification time was suggested by Belko and Warren (287) to be both useful and timesaving for appraising the heparin effect. The prothrombin time (Quick) method (288) measures the clotting time of recalcified plasma with added thromboplastin. Messerschmidt and Peter (289, 290) used this method with a photometer to measure the beginning of clotting.

Heparin will prolong whole blood clotting time (Howell) to three times the normal level (291). According to Jorpes (282), the main advantage of this technique is that it ensures the presence of the heparin cofactor and all the labile factors entering into the coagulation system without the introduction of additional salts or thromboplastin. Jaques *et al.* (257) found that the results given by whole blood were better than the USP XIV assay which is done in plasma, since the conditions of the former are closer to those used in the clinical control of heparin anticoagulation. The BP assay likewise suffers from the same disadvantages as the USP assay.

Other tests mentioned by Jaques (230) for the anticoagulant activity of heparin include: (*a*) whole blood collected in silicone-clotting tubes, (*b*) plasma time with chicken plasma that does not require recalcification, and (*c*) two-stage tests that are modifications of the previous tests given, with final measurement made on the coagulation time of a separate fibrinogen solution on the addition of an aliquot from the single-stage test.

Other Techniques—Some techniques are not as well known as the ones already discussed. Rosencrantz *et al.* (292) described a method that permits automatic quantitative recording of the formation of clots at 250 nm. An assay of heparin, employing diatomaceous silicate, modified plasma, and a precision coagulation timer[3], was given by Seip *et al.* (293). Attempts to use concanavalin A(I) as a specific reagent for the identification of heparin in biological fluids was found unsuccessful by DiFerrante and Hrgovcic (184). A quantitative method of estimation of acid polysaccharides in the blood of rabbits after the injection of papain, by complexation with cetyltrimethylammonium bromide, was given (294). The determination of heparin activity by an IR spectroscopic method was described by Zhbankov *et al.* (295), where potassium cyanide is introduced as an internal standard and the absorption ratios of 1240–1150 $cm.^{-1}$ and 1240–2065 $cm.^{-1}$ are measured, the latter frequency being due to the CN group. Bohn *et al.* (296) presented an automatic procedure for the determination of mucopolysaccharides (including heparin) based on a turbidity reaction with cetylpyridinium chloride. The thrombo test was described by Owren (297) as differing from earlier methods by measuring the coagulation activity of the absorbable factors in both the intrinsic and extrinsic coagulation systems, thus being insensitive to depression of factor IX during anticoagulant therapy.

For the assay of whale heparin, Matsuno and Hashimoto (298) modified the BP assay by using ox and human sulfated blood and ox thrombokinase. The USP method was more specific than a chemical method based on the shift of the isoelectric point for the estimation of polyanionic compounds like polyestradiol phosphate and polymethylene salicylate (299).

Much controversy exists as to the effectiveness of the various methods. A discrepancy of up to 40% was observed by Walton *et al.* (300) between potencies ex-

[3] Fibrometer.

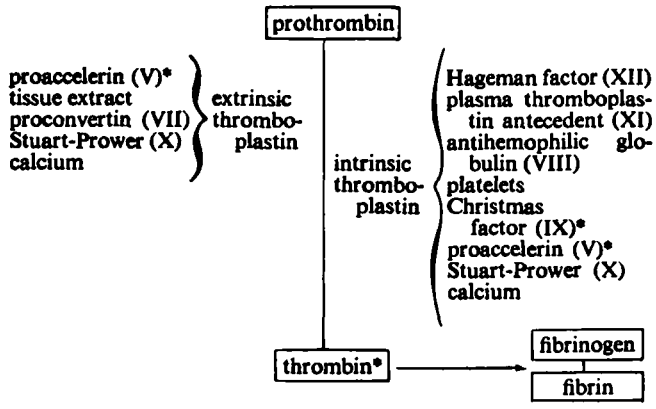
amined by the BP and USP assay procedures. These findings led to a British standard of mucosal heparin of mucosal origin. The results of Bangham and Woodward (301), however, indicate differences between the estimated relative potencies of lung and mucosal heparin, but they were not so consistent as to necessitate a separate international standard for each. The use of animal plasma or human plasma can also alter results (302). The correspondence between the *in vitro* and *in vivo* anticoagulant activities of beef and whale heparin was studied by Hashimoto and Matsuno (303), with the result that the BP unit of whale heparin and its *in vivo* potency in dogs was 1.5-fold more potent than those of beef heparin, whereas USP units of whale and beef heparin were the same. This led to the suggestion that the BP assay method may be better for clinical evaluation than the USP method when the whale lung or intestine is used in the place of the ox lung as the source of heparin.

Andreenko *et al.* (304) compared the method of titration with thrombin in the presence of heparin-cofactor (Blombäck), a colorimetric method based on precipitation of heparin with azure A (Warren–Bozioni method), with the titration with protamine sulfate (Warren–Vysockii). They found that the most suitable for laboratory practice was the first. In a report by Rezansoff and Jaques (305) comparing USP colorimetric, Howell, spectrophotometric, and microelectrophoresis techniques, no assay method consistently described the activity of heparin in another test system, *in vitro* or *in vivo*. This report conforms with that of Magnusson and Nilsson (306) who received different values of anticoagulant activity when using human, bovine, dog plasma, and whole ox blood in one-stage prothrombin, recalcification, and thrombin systems. Kavanagh (307) and Kuo (308) recently discovered no significant correlations between nitrogen, sulfur, acetyl, glucosamine, and other parameters with biological activities determined by Howell assay, USP assay, *in vivo* clotting time, partial thromboplastin time, and *in vivo* lipolytic activity values. Estes (309) claimed that, on comparison of four tests, the whole blood activated partial thromboplastin time provided the greatest precision, information, and economy, followed by (in order) the activated plasma partial thromboplastin time, the whole blood clotting time, and the partial thromboplastin time of plasma. The indication is, nevertheless, that more uniform systems of anticoagulant activity assay must be devised.

PHARMACOLOGY

Pharmacologically, heparin is noted for its anticoagulant action in the treatment of myocardial infarction and in the induction of the clearing reaction as an antilipemic agent in the blood. In addition, many other actions of heparin (antimetastatic, antiviral, and antienzymic) have been reported. Some general surveys of the pharmacology of heparin were written by Jorpes (1), Engelberg (8), Jaques (230, 280), and Gastpar (50).

Effects on Circulatory System—The most important action of heparin is the inhibition of blood coagulation. Surveys on blood anticoagulation were presented by



*Scheme II—Scheme of blood coagulation (*Here it is thought that heparin affects coagulation)*

Wessler and Gaston (310), Rodman (311), Buri and Schneeburger (312), Divald and Jouillié (313), Yen *et al.* (314), and Haas-Weston (315).

Anticoagulants may be divided into two groups: (*a*) the direct type like heparin and the heparinoids, which act rapidly by their presence in the blood, and (*b*) the indirect type (called antivitamin K), the derivatives of coumarin and indandione, which act after some delay. The first class of compounds interferes with the formation of thrombin and thromboplastin, whereas the second class interferes with the formation of prothrombin. The antagonists of the first class are protamine sulfate types, and the antagonists of the second are vitamin K types.

To date, no one has clearly elucidated either the mechanism of blood coagulation or anticoagulation. A recent review on blood coagulation was written in 1971 by Mammen (316). Many theories for blood coagulation have been proposed. A generally accepted scheme of coagulation is presented in Scheme II, according to Fremont (317).

The system offered by Wessler and Gaston (310) is similar to that of Fremont; however, they carry the clotting mechanism to the gelation stage (Scheme III).

Anticoagulant Effects of Heparin—Antithrombic Action—Prothrombin is unaffected in heparinized blood and, therefore, heparin must act on the first part of clotting (318). It is thought to have "antithrombic" activity in the presence of a certain plasma protein named "heparin complement" by Ziff and Chargaff (319) or cofactor (Howell) (317), which is close or identical to antithrombin III (320, 321). The protein forms a complex with heparin which is capable of binding rapidly with thrombin and thus inactivating it. Serotonin and tryptamine can interfere with this reaction (322). Henstell and Kligerman (323) felt that, more likely, three factors are involved: a plasma antithrombin (antithrombin III), a plasma antithrombin inhibitor, and a platelet factor with heparin acting as a catalyst. Porter *et al.* (324) indicated that the anti-

$$\text{fibrin monomer} \xrightarrow{*} \text{fibrin polymer} \quad (1)$$

$$\text{fibrin polymer} \xrightarrow{\text{calcium}} \text{fibrin gel} \quad (2)$$

Scheme III—(Here it is thought that heparin affects coagulation)*

thrombic action of heparin itself is weak in comparison with that of the heparin–cofactor complex.

Inhibition of Thromboplastin—In addition to its antithrombic action, heparin is capable of inhibiting several blood factors for the formation of thromboplastin, which is necessary for the conversion of prothrombin to thrombin, through depression of factors V, IX, XI, and XII (317). The data of Pitlick *et al.* (325) indicate that heparin and hirudin interfere with factor IX*a*, which is the heparin- and hirudin-sensitive factor in plasma when coagulation is initiated *via* factor XI or XII, and that subsequent clotting factors remain in inactive forms. Heparin also inhibits the activation of factor VIII by factor IX*a* (315). Josso (326) pointed out that an important discovery in recent years is that the enzyme activities that operate to transfer prothrombin into thrombin are attached to particulate lipoprotein complexes with which the inactive plasma profactors are transformed into active factors. The interaction between heparin and thromboplastin, observed by Chargaff *et al.* (327), results in a complex formed by heparin and the protein moiety of the lipoprotein. According to Yen *et al.* (314), the scheme of complex formation may be as shown in Scheme IV, where A is the polyanion and C is the polycation. In reaction (4) there is a two-electron transfer. These reactions could also hold for complexation with dyes and heparin antagonists.

Fibrinolysis—In addition to being an anticoagulant, heparin usually enhances fibrinolysis. It was found by Buluk and Januszko (328) to reduce the inhibition of antifibrinolysin. Lackner and Mersk (329) reported markedly accelerated fibrinolytic activity after intravenous injection of heparin. Increased blood heparin in leukemia was thought by Efendiev *et al.* (330) to be responsible for the greater fibrinolytic activity. Heparin and fibrinogen were shown by Godal (331) to react in stoichiometric proportions. Evidence was presented (332) that heparin in solutions of low ionic strengths breaks the bonds holding the fibrin monomers together in clots. It is also able to inhibit the polymerization of fibrinogen. The concentration, according to Silver and Hall (333), in the blood must be greater than 2 mg./kg. to potentiate fibrinolysis.

Olesen (334) suggested that the fibrinolysis is caused by plasminogen activation. However, Holemans *et al.* (335) found that the presence of a cofactor is necessary. Heparin is known also to accelerate catalytically the neutralization of thrombin by antithrombin III and thus prevent reclotting following fibrinolysis (336). The preparation of heparin must be free of protease inhibitors to enhance fibrinolysis (337). Eleparon, a mucopolysaccharide polysulfuric acid ester like heparin, increases fibrinolytic activity (338). Boros (339) discovered that "heparin rebound," the reappearance of heparin in the circulation in open-heart surgery despite its previous complete neutralization, was caused by enhanced fibrinolysis.

$$A:{}^{-}Na^{+} + C^{+}Cl^{-} \rightarrow A:{}^{-}C^{+} + NaCl \quad (3)$$

$$A:{}^{-}C^{+} + A:{}^{-}Na^{+} \rightarrow CA_{2}{}^{-}\,Na^{+} \quad (4)$$

Scheme IV

Effects on Platelets—Platelets contain a factor with some antiheparin activity (320, 340). This was described by Shanberge and Furuta (341) as platelet factor 4. Heparin was shown by Sawyer *et al.* (342) to prevent conversion of degenerated platelets in solution from forming a gel.

According to Jaques (280), heparin inhibits platelet adhesion to intercellular cement. Aznar (343) and Silver (344) found that small doses of heparin had no effect on platelet adhesiveness, but with larger doses the antiaggregating effects increase in proportion to the dose. Moreover, Gröttum (345) reported that the negative charge of the platelets was reduced by the positive charge of heparin and lowered the electrophoretic mobility, but increasing the concentration of heparin increased their electrophoretic mobility but did not aggregate them. A method was developed by Hohage and Hiemeyer (346, 347) for quantification of the aggregation curves of platelet-rich citrate–heparin plasma.

The adhesiveness of platelets to tannic acid-treated red cells was shown by Zbinden and Tomlin (348) to increase when heparin was added. If adenosine diphosphate is added to unstirred, plasma-rich platelets, no aggregation occurs and platelet-bound heparin-neutralizing activity develops immediately (349). The addition of adenosine diphosphate to platelets leads to shape and volume changes, to the development of stickiness, and to heparin-neutralizing activity (platelet factor IV) (350). O'Brien (351) reported that alcian blue also binds heparin and may influence heparin-neutralizing sites and probably the charge on the platelet; thus it can influence platelet aggregation. Eika (352, 353) found that washed human platelets were aggregated by heparin in the presence of divalent cations, but heparin inhibited the thrombin-induced platelet aggregation and release of adenine nucleotides. In the presence of calcium chloride and also in barium sulfate-adsorbed serum, heparin was shown by Murase *et al.* (354) to produce platelet clumping activity, but this was not the case in citrated plasma, edetate plasma, or serum.

Another action of heparin is to prevent platelet disintegration (355, 356) and release of phospholipids (355). Platelet instability, however, is not affected by heparin (357).

The ability of a heparin-like sulfonic polysaccharide to counteract platelet adhesiveness in rabbits and intravascular red cell aggregation in cats was discovered recently by Bicher (358).

Other Actions of Heparin—According to Jaques (280), heparin decreases blood sugar, the fall in lymphocytes and eosinophils, and hemorrhage from stress and hypertension. Hougie (359) reported that heparin gives prompt relief from acute myocardial infarction. Heparin facilitates the flow of blood by vasodilation (280, 360) and so could be important in Buerger's disease (361). The viscosity of blood was reported by Alberto *et al.* (362) and Meda *et al.* (363) to be lowered by heparin because of its anionic character. According to Hoak *et al.* (364), heparin prolongs the time to produce thrombi. *In vitro*, heparin was found to cause hemolysis (365), possibly because there is a decrease in the mechanical resistance of the red blood cells due to nucleoprotein mobilized from leucocytes under the influence of hepa-

rin, but *in vivo* it inhibits hemolysis and alters the erythrocyte sedimentation rate (315). Heparin prevents decrease of lymphocytes and eosinophils caused by ACTH or cortisone (366, 367) and activates alkaline phosphatase and esterase in the blood (367). Another biological effect is its enhancement of vascular permeability (368).

Pathological conditions exist, termed acquired and hereditary hyperheparinemia, where unusually high concentrations of heparin in the blood produce prolonged clotting or thrombin times. These can be corrected by treatment with protamine sulfate, toluidine blue, or polybrene (288).

Clearing Action of Heparin on Blood Lipids—In addition to the anticoagulant action of heparin, another major effect is the clearing of lipemic plasma. Early reviews were written by Levy (369) and Engelberg (370). Heparin stimulates the release of lipoprotein lipase (the clearing factor), an enzyme that catalyzes the hydrolysis of triglycerides associated with chylomicrons (lipoprotein molecules rich in neutral fat), and thus promotes the clearing of lipemic plasma (361, 367). The possible connection between the pathogenesis of atherosclerosis and heart disease and the clearing action of heparin is of great interest.

Antonni *et al.* (371) found that the clarifying action is more evident in atherosclerotic subjects than in normal ones. Also, myocardial infarction is usually accompanied by an increase in plasma lipoproteins and free fatty acids, which are damaging to the myocardial cells, in addition to an accumulation of tissue triglycerides and increased synthesis of fibrinogen (372).

Some properties of the clearing factor were examined by Korn (373), with the conclusion that it is primarily a tissue enzyme, a heparin-activated lipoprotein lipase that catalyzes the hydrolysis of chylomicrons but not simple triglycerides. The possibility of a separate monoglyceridase activity was also suggested by Vogel *et al.* (374). According to Engelberg (375), heparin is a component of the enzyme and its dissociation from the apoenzyme can be readily brought about. The clearing factor showed activation by low concentrations of heparin and inhibition by higher concentrations and protamine. This, with density centrifugation evidence, led Payza *et al.* (376) to suggest that heparin is not a necessary requirement or a cofactor for the action of the clearing factor.

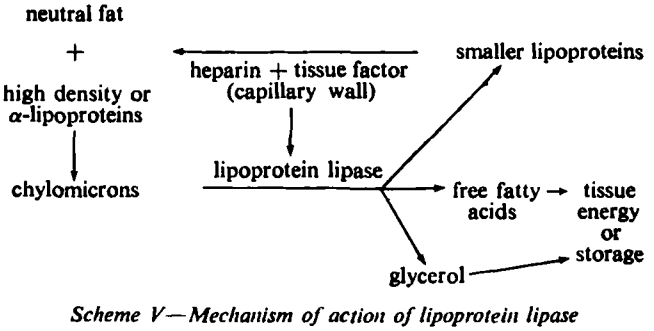
In addition to being found in the plasma, lipoprotein lipase can also be located in the thoracic duct lymph of dogs (377) and near the vascular wall after heparin injection (378). Heparin released lipase from rat epididymal fat pads (379), but Ho *et al.* (380) claimed that heparin only increases the lipolytic activity of the stroma cells of the pads. Ballard *et al.* (381) claimed that heparin increases the release of venous plasma lipolytic activity from perfused canine subcutaneous adipose tissue. From their findings, Gero *et al.* (382) made the observation that heparinoids may act on the tissue level by interfering with the lipid deposition and by locally increasing the lipolytic activity of the vessel wall. Payza *et al.* (383) found that injection of heparin into rats induced an increase in lipase activity of the plasma and a decrease in lipase activity of the tissue. Davis and Heimberg (384) thought that the uptake of chylomicrons from the blood probably is accelerated by a direct action of heparin on the liver.

A mechanism for the action of the postheparin lipidemia clearing factor was given by Engelberg (370) (Scheme V). He believed that the lipoprotein lipase is the major way for the removal of triglycerides from the blood. Rutstein *et al.* (385) argued that heparin leads to increased lipid deposition and that this can be correlated with increased serum levels of nonesterified fatty acids but not with the decreased concentration of triglycerides, cholesterol, and phospholipids. The high nonesterified fatty acid levels are associated with arrhythmias and death in patients with myocardial infarction. Davis and Davis (386) disagreed, their reason being that even though emboli contain lipids, fibrinogen is more important in their formation than lipids. The interpretation of lipase stimulation given by Patten and Hollenberg (387) was that exogenous heparin increases the binding of the enzyme to chylomicrons and then has no effect on either the stability of the extracted enzyme or enzyme activity after the formation of enzyme–chylomicron complexes has occurred. The clearing activity was found by Bianchini and Osima (388) to be independent of the number of sulfate radicals and the presence of uronic acid groups. Also, it does not parallel the anticoagulant activity (389).

Evidence was given by Pav and Wenkeva (390) of the appearance in the blood of another serum esterase in addition to the clearing factor after the injection of heparin. The quantitative inhibition of triton lipidemia by heparin was determined by Haikonen *et al.* (391). Finkelstein *et al.* (392) isolated an extracellular lipase from the culture medium of *Pseudomonas aeruginosa*.

A naturally occurring acid mucopolysaccharide was isolated by Grossman *et al.* (393); it had good lipoprotein lipase-activating effects and cholesterol-reducing ability, with little anticoagulant activity. Sulfovernan, a new heparinoid from a lichen moss, was shown by Stefanovich *et al.* (394) to have anticoagulant activity, but it increased the triglyceride level of the lipids of aortic tissue. The latter effect is not thought to be associated with heparin.

Effects of Heparin on Tumor Growth and Metastasis—Much experimental investigation has been attempted to solve the question of whether heparin actually alters tumor growth and metastasis, but to date this problem remains unsolved. Reviews were written by Balazs and Jacobsen (395), Shats (396), and Regelson (397). The results of Lippman (398) on the Ehrlich ascites tumor in mice show that heparin is a mitotic inhibitor in this



Scheme V—Mechanism of action of lipoprotein lipase

tumor. No effect on the growth of a transplantable lymphosarcoma in mice, however, was found by Kreisler (399). Csaba *et al.* (400) agreed with these results and even thought that some tumors require heparinoid substances for their growth. The findings of Montes de Oca and Dietrich (401) in the incorporation and fate of heparin in L, Le, HeLa, and mouse embryo tissue culture indicate that there is no inhibition by heparin of the four cell lines.

Fisher and Fisher (402) believed that their observed decrease of tumor metastases in rats cannot be explained by an inhibition of tumor cell lodgment and microclot formation but by rheological alterations in the blood as a result of the administration of heparin and previous tumor plasmin. The treatment of tumor-bearing mice by Kudryashov *et al.* (403) did not produce a consistent reduction in tumor growth but actually promoted growth in several animals. If, however, the vegetative nervous system was blocked with chlorpromazine during anticoagulant and fibrinolytic treatment, there was a significant regression of tumor growth. Németh (404), using a combination of heparin and degranol[4], found a reduction in the size of tumors rich in connective tissue.

The effects of heparin and fibrinolysins on pulmonary metastasis were studied by Cliffton and Agostino (405, 406), with the finding that the incidence of metastasis was decreased. Suemasu and Ishikawa (407) discovered a similar result on pulmonary metastasis which they attributed to indicate that tumor cells were flushed away by the anticoagulant through the lung by blocking the formation of fibrin clots around the cells in capillaries that enmesh them. Likewise, Rutstein *et al.* (385) found that heparin has a disseminating influence on pulmonary metastases of experimental tumors. On the other hand, Hagmar and Norrby (408) reported that the average volume of pulmonary metastases became less but their number rose. This was also experienced by Boeryd and Rudenstam (409).

Miscellaneous Effects—The antiviral action of heparin on *Herpes simplex* was mentioned by Jaques (230). The observation of Vaheri (410) was that heparin has a potent reversible inhibitory action on the early interaction of *H. simplex* virus and cells but that it does not have an irreversible effect on the virus. Horváth and Hadházy (411) found that heparin inhibits only the absorption of free virus and does not influence the penetration and cell-to-cell transfer of viruses carried by infected cells. Treatment of chick embryo cells by Mentkevich and Zhdanova (412) inhibited H and B_1 strains of Newcastle disease virus from producing interferon. The stimulation of cell locomotion and pseudopodium formation of the amoeba by heparin was seen by Bell and Jeon (413). Bell (414) suggested that heparin has a depolarizing effect on the cell surface. The antibacterial action of heparin in the presence of blood from regions of injury was mentioned by Regelson (397).

Heparin inhibits the action of many enzymes such as β-glucuronidase (415, 416), renin (417), hyaluronidase (418), alkaline phosphatase (418), chondroitin sulfate glycanhydrolase (418), elastase (418), and collagenase (419). The inhibition of hyaluronidase in the gingiva by heparin was cited by Nord (420) as an important defense mechanism in periodontal disease. Heparin and rifampicin were acclaimed by Neuhoff *et al.* (421) as being the most effective known inhibitors of DNA-dependent RNA polymerase from *Escherichia coli*. It also reduces the action of ribonuclease (367, 421), fumerase (367), serum protease (367), and lecithinase (422). Heparin was found by Wu (423) to inhibit polynucleotide kinase, which catalyzes the transfer of orthophosphate from ATP to the 5^1-hydroxyl terminus of polynucleotides. Freeman (424) suggested that the ability of heparin to inhibit a rather large number of enzyme systems may be due to its acidic character which allows it to combine rather tightly with almost any basic protein. There are some enzymes, such as staphylococcal coagulase, upon which heparin has no action, at least under experimental conditions (425). On other enzymes such as pepsinogen (426) and diamine oxidase (427), it has a stimulating action.

Heparin has some activity in secretion and excretion. Gastric secretion was inhibited by heparin and heparinoids in some dogs (428). It has a diuretic effect and this lowers edema (8, 230). It also inhibits the antidiuretic effect of pitressin (230). The excretion of potassium is decreased and that of sodium is increased (230, 367) after heparin administration.

Its interaction with calcium has been of interest. According to the results of Tidball (429, 430), physiologic histamine release is partially inhibited by heparin, the assumption being that the histamine release may result from the binding of calcium by heparin. Cheymol and Gay (431) found that heparin causes an elevation of alkaline reserve in rabbits. According to Reber and Studer (432), the binding of calcium ions by edetate, sodium citrate, ion exchangers, *etc.*, seems to enhance the intestinal absorption of heparin in rats, as measured by clearing factor activation. However, the work of Koh (433) with dimethyl sulfoxide, diethylsulfone, *etc.*, showed that these also enhance heparin absorption but here they act by a process other than calcium sequestration since they are not chelating agents. Additionally, through chelate-like compounds with calcium ions, heparin may deplete capillary walls and decrease wound strength (281). Heparin inhibits calcification *in vitro* and increases calcification *in vivo*. The explanation for this phenomenon given by Harris *et al.* (434) was that there is binding of calcium by heparin *in vitro* and that heparin acts as a template for calcium deposition *in vivo*. Leonard (435) found that it can cause sequestration of calcium. In addition, it induces a stimulatory effect on bone resorption (367).

Heparin caused developmental anomalies in amphibian and sea urchin eggs. This was explained by DeCarli and Brachet (436) as being due to damage to the transcription of DNA. Skalka *et al.* (437) reported the incorporation of labeled thymidine into the DNA of rat thymocyte and spleen cell nuclei. In the presence of large amounts of heparin, this leads to the disintegration of the nuclei.

The action on various tissues and organs has been given much consideration. Garin and Bassa (438) found that heparin eliminates quickly the action of total spleen

[4] Zitofen.

extract which blocks up medullary inhibition. Sasaki (439) showed that there is a marked decrease of lymphocytes in lymph nodes and the white pulp of the spleen after heparin is administered. The decrease in respiration of animal tissue caused by heparin was described by Schuster (440). Clinical investigation by Fegen *et al.* (441) indicated that heparin aids in prolonged ischemia time in an occluded renal artery after kidney injury. Heparin formed a gel when added to homogenates and nuclear fractions of rat liver, thymus, spleen, and kidney (442). Lazzarini-Robertson (443) found that heparin has a strong inhibitory effect on the uptake of labeled serum-bound lipids by human and animal endothelial-type cells. This is probably due to the very strong negative charge, acting directly at the cell membrane level. Xanthomatosis and burns of the skin are improved by heparin (8), and it stimulates growth of connective tissue (367). The cytotoxic effect of a rabbit antiserum on guinea pig myocardium was eliminated by heparin (444).

Heparin has an inhibitory effect on hormones such as aldosterone (445) and testosterone (446). In euthyroid and hypothyroid patients receiving heparin alone, there were no changes in thyroxine-binding globulin and thyroxine-binding prealbumin; according to Schatz *et al.* (447), it is possible that the heparin competes with thyroxine for binding sites.

In various types of shock, there is an outpouring of heparin from the mast cells (367). However, in a study of anaphylactic manifestations in the rat, Salmon and Lecompte (448) found no inhibition to ovalbumin sensitivity. It was found by Vannas (449) to have a beneficial effect on iritis in the rabbit. In addition to bone loss, another deleterious effect of heparin is increased mortality after its administration in the case of fat embolism (450). Also, the telogen phase in the hair may be precipitated prematurely, producing telogen effluvium (451).

HEPARIN THERAPY AND PHARMACODYNAMICS

Heparin has been reported (452) to be an "ideal" anticoagulant in ease of administration. It is especially desirable because of its safety, rapid onset of activity, and reversibility.

Reviews have been presented on anticoagulant and heparin therapy (8, 311–314, 453–455), discussing indications, dosage, adverse effects, and contraindications.

Indications and Clinical Application—Heparin has its main use in acute thrombotic disease, vascular and cardiac surgery, and acute myocardial infarction (92). Its efficiency in reducing mortality in thromboembolism was questioned by Enger *et al.* (456), but it may decrease chest pain and thromboembolic complications (457).

Its use in the treatment of pulmonary embolism was reported by Zilliacus (458), who claimed that it has certain advantages as compared to vein ligation in legs since it acts on undiagnosed thrombi. Moser (459) found heparin to be the drug of choice in this complication because of its (*a*) immediate onset of action, (*b*) potent inhibition of the coagulation system, (*c*) enhancement of fibrinolytic dissociation of fresh thrombi, (*d*) inhibition of platelet breakdown (and, therefore, serotonin release), and (*e*) prompt reversibility of anticoagulant action.

In the treatment of cerebral thrombosis (460), anticoagulant therapy postpones the arrival of an impending stroke, prevents transient ischemic attacks, and sometimes stops the advance of a progressive thrombotic stroke.

Other uses for heparin were mentioned by Engelberg (8), such as lowering of edema by its diuretic effect, neutralization of snake venom, lowering of hypertension, aid in burns and allergies, and prevention of atherosclerotic disease. Some other possible uses of heparin were said by Jorpes (454) to be in Dupuytren's contracture and fat embolism. It is the anticoagulant of choice for patients under general anesthesia (452).

Method of Administration and Dosage—Heparin is ineffective when given orally. According to Estes (309), the minimum dose required to anticoagulate blood to a clinically desirable level is 35 units/kg. The various routes of administration for acute myocardial infarction and sustaining therapy described by Lomax (461) are: (*a*) continuous intravenous infusion—an initial dose of 5000 I.U. and then 20,000–30,000 I.U. in 1 l. of 5% glucose or 0.9% sodium chloride solution given slowly over the next 24 hr., (*b*) intermittent intravenous infusion—5000 I.U. every 3 hr., (*c*) intramuscular—an initial intravenous dose of 5000–10,000 I.U. and then 20,000–40,000 I.U. injected every 8–10 hr., and (*d*) deep subcutaneous injection—20,000–40,000 I.U. injected subcutaneously, which can prolong coagulation up to 16 hr.

For pulmonary embolism, Moser (459) suggested that 7500 units of heparin be given subcutaneously every 6 hr. or 10,000 units every 8 hr. This dosage is maintained until the patient's cardiopulmonary status has stabilized and all traces of venous thrombosis have departed. Miller *et al.* (462) claimed, however, that streptokinase is more effective after 72 hr. than heparin.

The dose in cerebral thrombosis (460) is 50 mg. every 4 hr. in progressive stroke or with transient ischemic attacks. The therapy is maintained for 1–3 weeks, and then oral coumarin therapy is continued for 1 year or more.

Pharmacodynamics of Heparin—Since heparin is used in emergency prophylactic treatment, its pharmacodynamic properties must be understood. It was found that intravenous injection of heparin follows a logarithmic linear regression curve (463). Studies on the relative volume of distribution and half-life (464) revealed that all log plasma heparin concentration–time curves fitted an equation for first-order kinetics, even at the highest doses. The relative volume of distribution varied with the dose but not the half-life. These data agree with the hypothesis that heparin may leave the plasma by uptake into the reticuloendothelial system and could indicate the reason for the problems found in devising practical and reliable dose schedules. In later investigations, Estes (309, 465) found that the half-life of heparin's anticoagulant activity is approximately 1–1.5 hr., and the equation expressing the relationship between dose ratio and dose interval is:

$$R^* = \frac{D^*}{D} = \frac{1}{1 - e^{-k_{elr}}} \qquad \text{(Eq. 1)}$$

where R^* is the dose ratio, D^* is the initial dose, D is the maintenance dose, k_{el} is the rate constant for elimination, and τ is the dose interval. It is found then that maintenance of anticoagulation to a degree meeting common acceptance is met by administering heparin at intervals approximating the half-life (465).

Contraindications—Heparin should not be given to patients with a history of bleeding diathesis, bleeding ulcer, severe hypertension or cerebral hemorrhage (466), jaundice, purpura, hemophilia, acute bacterial endocarditis, and accessible ulcerated lesions (especially of the GI tract) (378).

Adverse Effects—Rodman (311) stated that prolonged use of large doses of heparin (over 200 mg. daily for 6 months or longer) may produce osteoporosis. He also mentioned as side reactions: (*a*) dysesthesis pedia, which is an intense itching of the soles of the feet, and (*b*) the possibility of inhibition of the adrenal cortical synthesis of aldosterone.

Various other adverse effects, described by Lomax (461), include transient alopecia, spontaneous hemorrhage, hypersensitivity, anaphylactoid reactions, thrombocytopenia, fever, and acute adrenal hemorrhage.

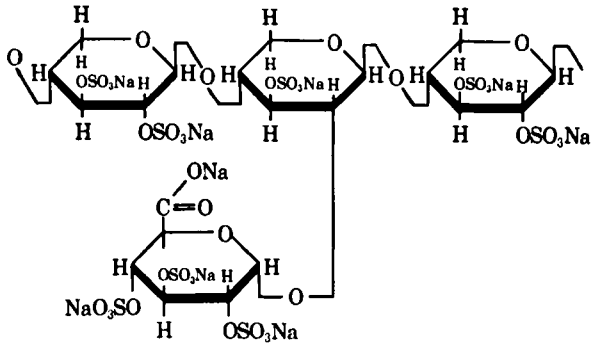
ANTAGONISTS OF HEPARIN

The substances that antagonize the anticoagulant action of heparin are important in the control of heparin therapy and in assay techniques. A review by Agolini (467) discussed the inactivating power of various chemicals on heparin and dextran sulfate. A list of antiheparin agents, given by Stamm (468), includes protamine, clupein, polylysine, lysozyme, polybrene, toluidine blue, fuchsin, and tryptophan.

Garagnini and Fuccini (469) suggested that the inhibiting action of lysozyme on heparin is due to bond formation between the two substances. Protamine sulfate, a low molecular weight protein with a strong basic charge, is antagonistic to the clearing action of heparin (470). The early stages of blood coagulation *in vitro* are inhibited by protamine and polybrene (471) by interfering with the activation of factor IX (plasma thromboplastin component, Christmas factor). Factor IX is activated in the presence of calcium by activated factor XI, a reaction accelerated by thrombin. Protamine prevents this activation by inhibiting the activated XI. Heparin does this by being antithrombic, whereas polybrene acts directly against the inactive IX. This inhibition is probably due to a competitive complex formation since the inhibitory action of each can be overcome by increasing the concentration of its antagonist.

The ratio of protamine sulfate antagonistic to heparin is given as 1:1 by weight (461). According to Lowary *et al.* (472), this is not always true but is also dependent on the tissue source of the heparin in both *in vitro* and *in vivo* studies. Yen *et al.* (473) gave the activity of the antiheparins as β-lipoprotein < thrombin clotting system < Christmas factor < platelet protein < protamine sulfate.

In comparison to toluidine blue, which is very toxic, polybrene administered intravenously promptly and completely neutralizes the anticoagulant action of heparin, with minimal hypotension and no disturbance in pulse rate (474).



II: chemical structure of a sulfonic polysaccharide

Several other less known inorganic and organic substances also have antagonistic properties to heparin. Hexavalent complexes of cobalt and chromium were quite active and complexes of acriflavine, stilbamidine, and viomycin were slightly active (475). Visconti *et al.* (476) discovered that carbon disulfide showed significant antiheparin activity in blood. Substances such as phosphate and sulfate ions, adenosine triphosphate, adenosine diphosphate, and myosin were able to remove the antihemostatic effect of heparin (477).

HEPARINOIDS AND HEPARIN-LIKE SUBSTANCES

This section discusses those substances that have a similar biological action as heparin. The quest for a substance with a high anticoagulant activity and low toxicity still continues.

Sulfur esters of chitin, starch, and cellulose are effective as anticoagulants *in vitro* but are toxic on injection into animals. Dextran sulfate is harmless, but the maximum activity is 15 units/mg. (478). Wood and Mora (479) found that glucose sulfates (degree of substitution 2.6–3.0) had approximately the same activity as clinical preparations of heparin. Amylose, amylopectin, and guaran, after being sulfated, gave low *in vitro* anticoagulant activity, suggesting the importance of *N*-sulfation (480). A sulfated alginic acid[6] has been used as a heparin substitute in therapy (361).

Other heparin-like substances include a sulfonic polysaccharide (Structure II) made by Aznar *et al.* (481). It has marked fibrinolytic activity and is able to inhibit platelet aggregation but has little anticoagulant effect. Wolfrom and Wang (482) prepared an *N*-sulfated heparinoid from amylose which has negligible activity. Chitosan was modified by sulfation and oxidation by Whistler and Kosik (483), converting some of the sugar units to uronic acid. The highest anticoagulant activity attained (60 I.U./mg.) was after all amino groups were sulfated, *O*-sulfate content was increased, and uronic acid groups were introduced. Still another semisynthetic anticoagulant, a Pentosan-polysulfo-ester (SP 54), was introduced by Walb *et al.* (484). In contrast to heparin, *in vitro* this compound has less influence on coagulation and 10 times as great anticomplementary action. Re-

[6] Paritol.

cently, a synthetic sulfated polygalacturonic acid methyl ester[6] was introduced by Jarrett and Jaques (485) which, when administered in conjunction with edetate, has potential as an oral anticoagulant based on the assumption that the chelating agent would form chelate bonds with ions which would otherwise obstruct the absorption of the heparinoid.

Bhattacharya and Singh (299) conducted a comparative study of polyanionic compounds, such as polyestradiol phosphate and polymethylene salicylate, but discovered that they possess only a fraction of the activity of heparin.

Unmodified heparin-like substances from natural sources have shown some promise as anticoagulants. Heparin-like anticoagulants were extracted from clams (11–13, 486). These have acetyl residues but have a high activity (130 I.U./mg. from mantle tissue), low toxicity, and a higher molecular weight than heparin. The polysaccharide, carrageenin, is not an antithrombin and does not inhibit the viscous metamorphosis of thrombocytes as does heparin, but it does decrease fibrin polymerization (487). Matsuoka *et al.* (488) found that charonin sulfuric acid, a polysaccharide from the mucus of *Charonia lampis*, remarkably prolonged whole blood clotting time after intravenous injection in the rabbit but had a toxicity 3.6–7.2 times as strong as heparin. A list of other natural and synthetic anticoagulants was given by Bernfeld (356).

Whale heparin (ω-heparin) was first isolated by Yosizawa (489) from the lungs and intestines of the finback whale (*Balaenoptora physalus*). It contains *N*-acetyl glucosamine, to which the other groups of heparin are attached (490), and has approximately the same molecular weight as heparin (491). The structure was confirmed with NMR by Inoue and Inoue (492). Masao (493) reported that ω-heparin from lung tissue exhibited higher activity than heparin, and it was also found to be longer lasting (494). The heparin isolated from the small intestine of whales showed a much higher anticoagulant activity than bovine heparin (495). Whale intestine heparin was used therapeutically by Abe *et al.* (496) during and after an operation for occlusion of the bilateral iliac arteries. There was prolongation of blood clotting and recalcification time, but the amount of bleeding during and after the operation became insignificant. Nagasawa (497) found, in addition to whale heparin from the small intestines and lungs, that the whale (mucous) heparin also had high activity, 580 units/mg. (BP).

METABOLISM AND ENZYMATIC DEGRADATION

Reviews on the metabolism of heparin were presented in 1966 by Whistler and Rowell (7), in 1968 by Dodgson and Lloyd (498), and in 1969 by Silbert (499).

Studies with Labeled Heparin—With the preparation of a radioactive heparin, the possibility of quantitative metabolic studies could be attempted. A method was described by Lalich (500) for the synthesis of radioactive heparin using a process of *N*-desulfation and *N*-resulfation. The results of Eiber *et al.* (501) indicate that heparin-^{35}S or ordinary heparin given orally would pass into the blood only to a very small extent and would have no effect on blood coagulation. However, when the labeled heparin was injected intravenously, the radioactivity in the blood increased in proportion to increases in clotting time and clearing activity.

Labeling of heparin with tritium and ^{35}S gives a stable product under a variety of conditions which retains the original physicochemical and biological properties (502). Heparin labeled with ^{3}H was found by Barlow and Cardinal (503) to have an activity of 124 USP units.

Dietrich and Montes de Oca (504) isolated radioactive mucopolysaccharides from HeLa, K., mouse, and rat embryo cells grown in the presence of a culture medium with radioactive inorganic sulfate. A ^{51}Cr-labeled heparin was made by refluxing heparin with $^{51}CrCl_3$ by Varga *et al.* (505). This could be important in investigations of the function of heparin *in vivo* and *in vitro* as well as its metabolic fate.

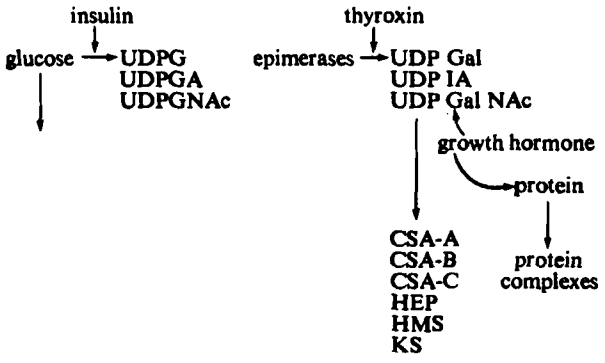
Biosynthesis—Studies by Silbert (506–509) indicate that an *N*-acetylated glycosaminoglycan is transformed into heparin at the polymer stage. Also, the polymerization appears to take place in close proximation to the sulfation in the mast cell. The resulting sulfated polysaccharide remains bound to the same particulate preparation that catalyzes the heparin formation.

In contrast, the results of Rice *et al.* (510) on the mastocytoma showed that there is transfer of sulfate to small molecular weight precursors. Also, the observations of Marx and Eisenman (511) establish that the major portion of the "microsomal" fraction from the homogenates of Furth mast cell tumor was capable of controlling the transfer of radioactive sulfate to *N*-desulfated heparin. Balasubramanian *et al.* (512) concluded that apparently free amino groups are required for the enzymatic *N*-sulfation of glycosaminoglycans.

Most of heparin exists in nature in firm covalent combination with protein and does not appear in appreciable amounts as an extracellular component of connective tissue (498). Helting (513), in 1971, investigated the natural trisaccharide, 3-*O*-β-D-galactosyl-4-*O*-β-D-galactosyl-D-xylose, of the heparin–protein linkage region and found that partial acid hydrolysis of ^{14}C-galactose-labeled endogenous acceptor yielded several fragments with the characteristics of neutral oligosaccharides previously isolated from the heparin–protein linkage regions and, in addition, a compound with the chromatographic qualities of *N*-acetyl lactosamine. This confirmed the biosynthesis scheme of acid mucopolysaccharide production offered by Dorfman (514). Scheme VI (514) shows the biosynthetic pathways of the acid mucopolysaccharides of connective tissue. A more recent scheme (Scheme VII) was presented by Silbert (499).

Studies on Catabolism—Danishefsky and Eiber (515), in 1959, concluded from excretion studies with heparin-^{35}S that the sulfate linkages of heparin were cleaved as a result of metabolic degradation; with small doses, all of the $^{35}SO_4$ in the urine was in the form of inorganic sulfate while larger doses of heparin-$^{35}SO_4$ led to the output of some urinary heparin-$^{35}SO_4$. The findings of Lloyd *et al.* (516) complemented those of Danishefsky and Eiber (515) and also showed the presence in the

[6] G31150.



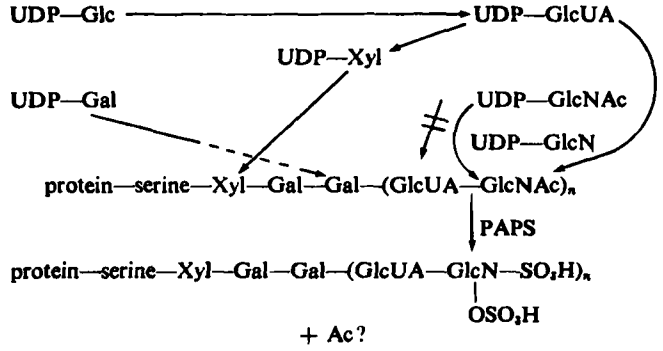
UDPG	=	uridine diphosphoglucose
UDPGA	=	uridine diphosphoglucuronic acid
UDP Gal NAc	=	uridine diphospho-*N*-acetylglucosamine
UDP Gal	=	uridine diphosphogalactose
UDP IA	=	uridine diphosphoiduronic acid
CAS(A,B,C)	=	chondroitin sulfuric acid (A, B, and C)
HEP	=	heparin
HA	=	hyaluronic acid
HMS	=	heparitin sulfate
KS	=	keratosulfate

Scheme VI—Biosynthetic pathways of acid mucopolysaccharides of connective tissue (514)

urine of a material with an electrophoretic mobility similar to heparin but a reduced molecular weight. Thus, it appears that greater catabolism is reserved for higher molecular weight materials.

Lemaire *et al.* (517) found the formation of partially desulfated catabolite in the urine and strongly labeled sulfonucleotides in the liver. The heparin excreted in the urine was termed uroheparin by McAllister and Dennis (518) and was shown to have about half of the anticoagulant activity of ingested heparin; the number of moles of sulfate per repeating unit was 3.94 for uroheparin in contrast to 5.6 for heparin.

Degradation by Enzymes—The change in heparin after absorption in the body is thought to be due mainly to bacterial enzymes. Heparinase, isolated by Walker (519) from *Flavobacterium heparinum*, a Gram-negative soil bacterium, reduces heparin and heparitin sulfate to oligosaccharides, with glucosamine or *N*-acetylglucosamine as the reducing end-group. Linker and Hovingh (520) fractionated a crude heparinase on a Sephadex column and obtained two active fractions. The first had the properties of an eliminase, which degraded heparin and heparitin sulfate nonhydrolytically to di- and oligosaccharides containing α,β-unsaturated glucuronic acid. The second had the properties of a glucuronidase, which hydrolyzed the unsaturated uronides to monosaccharides. Dietrich (521) identified the products of such a bacterial degradation as glucosamine 2,6-disulfate, saturated disaccharides made up of uronic acid and glucosamine and containing two and three sulfate residues, and tetra- and hexasaccharides with the same disaccharide units. From this, he concluded that positions 2 and 6 are the positions for the *N*-sulfate and *O*-sulfate, respectively, in the disulfated glucosamine molecule. He (522, 523) also showed that five enzymes from *F. heparinum*, acting in concert, were able to degrade heparin to its monosaccharides. Karapally and Dietrich (524) isolated a uronic acid isomerase from *F. heparinum* which converts glucuronic acid and galacturonic acid to fructuronic



Scheme VII—Biosynthetic pathway of mucopolysaccharides (499)

acid and tagaturonic acid. Hovingh and Linker (525) found that a heparinase and a heparitinase could be obtained from *F. heparinum*, and the specificity appears to be related to the presence or absence of *N*-acetyl groups. Whale heparin was degraded by Yosizawa (490) by crude heparinase to three major monosaccharides: α-keto acid, *N*-sulfated glucosamine, and *N,O*-desulfated glucosamine. There were also minor products: *N*-acetylglucosamine containing unsaturated oligosaccharides.

A group of enzymes from *F. heparinum*, the sulfamidases capable of liberating inorganic $^{35}SO_4^{-2}$ ions, was discovered by Lloyd *et al.* (526). The sulfamidases extracted from bacterial and mammalian sources differed in that the mammalian enzyme liberated inorganic $^{35}SO_4^{-2}$ ions only from ^{35}S-heparin and the bacterial enzyme preferentially degraded authentic 2-deoxy-2[^{35}S]-sulfamino-D-glucose but to a lesser degree [^{35}S]-labeled polymer (527). The actions of sulfamidase and sulfoesterase in the desulfation of the catabolites of heparin were studied by Dietrich (528, 529). Recent work on heparin degradation (530, 531) established the presence in rat tissues of an entirely normal mammalian system which has the ability to degrade the sulfamate groupings of exogenous [^{35}S]-sulfoamino-heparin and thus exhibits the characteristics of a "heparin sulfamidase."

Testicular hyaluronidase was shown by Ogren and Lindahl (532) to digest and degrade heparin from mouse mastocytoma. The results of this investigation suggest that the molecular weight of the newly synthesized heparin is much higher than that of the polysaccharide stored in the mast cell granules; the heparin released from the mast cell *in vivo* is believed to originate from these granules, thus having partially degraded rather than intact polysaccharide chains.

HEPARITIN SULFATE

Heparitin sulfate, also referred to as heparan sulfate or heparin monosulfate, is closely related to heparin. A general review was written by Spiro (533) in 1965. Heparitin sulfate is obtained as a by-product in the preparation of heparin from lung and liver tissue (534). It has a lower sulfate content than heparin (535) and, unlike heparin, the glucosamine residues are partly *N*-acetylated and partly *N*-sulfated. The most important linkage in this mucopolysaccharide is 1 → 4 and there may be branching (536). The major uronic acid of hepa-

ritin sulfate seems to be glucuronic acid, but the presence of L-iduronic acid was also demonstrated as in the case of heparin (537). In contrast to heparin, heparitin isolated from the human aorta has a higher molecular weight (24,000–29,000) and negligible antithrombin activity (534), and it is bound to protein by way of serine (538). On treatment of heparan sulfate with alkali or proteolytic enzymes, it behaves as a multichain proteoglycan such as chondroitin sulfate or keratan sulfate (539).

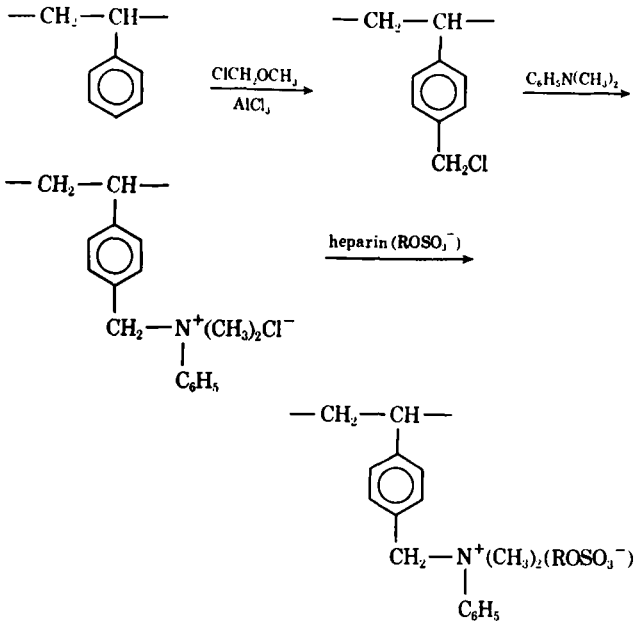
Heparitin sulfate has been isolated in small amounts from human brains (540) and other mammalian brains (541–543). Some evidence (544) indicates that mucopolysaccharides also may be present in the extracellular space in the brain. This could be important in understanding the blood–brain phenomenon, since the distribution of various ions and other high molecular weight substances in the CNS could be influenced by the localization of charged mucopolysaccharides.

Studies report the isolation of small amounts of heparitin sulfate in the dermis of mammalian skin (545) and chicken skin (546). The skin of hogs (547) and that of neonatal rats (548) yielded heparitin sulfate constituents. It was discovered that, in contrast to heparin, the heparitin sulfate content in rat skin increased with age rather than decreased (549).

Other sources of heparitin sulfate include the pig aorta (550), bovine periodontal membrane (551), human umbilical cord (552), and beef lung tissue (553, 554). Normal urine was found to contain heparan sulfate with a molecular weight of 8000–30,000 and calculated Stokes radius of about 60 Å (555). It was separated from dermatan sulfate and chondroitin sulfate in the urine by a cetylpyridinium method (566), electrophoresis (557), and column chromatography (558, 559). Heparitin sulfate was recently isolated from cotton wick granulation tissue of rats (560) and mouse fibroblast line 3T6 (561). It was found also in young mast cells in addition to heparin (562).

Abnormal excretion in the urine of mucopolysaccharides can be related either to disorders of a hereditary character or to a series of conditions where there is a metabolic defect involving the connective tissue. These pathological conditions have been termed the mucopolysaccharidoses, and several reviews have been written concerning them (33, 563–570). Heparitin sulfate was detected in abnormal amounts in the urine of patients with San Filippo's (566), Morquio's (571), Hunter's (565, 567, 571, 572), Hurler's (563, 567, 568, 573–577), and Ulrich-Scheie's (567) syndromes. Some methods of detection of the mucopolysaccharides in Hunter's syndrome were paper chromatography (578) and ion-exchange chromatography (579). Glycosaminoglycans from the liver and spleen of patients with Hurler's syndrome had much lower molecular weights than normal, consisting of single chains of a molecular weight of about 5000 together with multiples of up to four such chains attached to peptide moieties (571).

The mechanism involved in the pathogenesis of mucopolysaccharidosis was indicated by Hers and Van Hoof (580) as: (*a*) the mucopolysaccharides are synthesized in excess by the fibroblasts, released in the circulating fluids, and then excreted in the urine or taken up by the tissues through endocytosis; or (*b*) the mucopolysaccharides are typical inborn lysomal diseases, similar to type II glycogenosis.



Scheme VIII—Reaction for heparinization of quaternary groups on a polymer

BONDING OF HEPARIN TO SURFACES

With the advent of prosthetic circulatory devices, there has been a search for materials that: (*a*) cause no change in the blood, (*b*) stand up for long periods of time, (*c*) are compatible with tissue, (*d*) can be sterilized, and (*e*) are available both as rigid and flexible materials. Of these prerequisites, the first is probably the most urgent, so that clotting is not initiated at the surface and hemolysis of the red cells does not occur.

Heparin appears to be satisfactory for the blood compatibility requirement, but it must be bonded in some way to other materials. In 1963, studies were conducted with colloidal graphite surfaces which had been rinsed with a surfactant capable of bonding heparin (581). The resistance of this new surface was far greater to clot formation and was much better than plastic or silicone, but small amounts of heparin left the surface during a 10-hr. period. Some of the significant features of these heparinized surfaces are that there are no *in vivo* thrombi and the ζ-potential is less than in a benzalkonium surface without heparin (582). Recent reviews were presented by Gott and Furuse (583), Salzman (584), and Yen *et al.* (585).

Polystyrene, polyethylene, butadiene–vinylpyridine, polyvinyl chloride, silicone rubber, and many other materials can be rendered nonthrombogenic by a physical surface bonding of heparin on quaternary groups attached to the polymer (581–591). The procedure involves chloromethylation, quaternization, and heparinization (592) and the typical reaction is shown in Scheme VIII (593). Tests conducted by Fourt *et al.* (594) of some of these heparin-bearing surfaces on nylon and on heparin base with collodion in dilute films present evidence that heparin on the walls may be more effective than heparin in solution.

Milligan *et al.* (595) believed that the antithrombogenic property is not due to heparin, since most of it is eluted and a colloidal graphite coating alone has similar properties to animal tissues. On the other hand, Usdin and Fourt (596) found only partial dilution of heparin from surfaces exposed to whole blood, plasma, or serum. This seems to be dependent on the particular protein fraction used and the nature of the cationic surface (597).

Heparin, chemically bonded to rigid and flexible materials including regenerated cellulose and other hydroxyl-bearing substrates by amination through graft polymerization with ethylene imine, yielded permanently coated surfaces even under high fluid shear (598). Another aminated surface on glass, made from a polymer of 2-hydroxy-3-methacryloyloxypropyltrimethylammonium chloride, showed platelet adhesion but exhibited no attraction of proteins and platelets to the film when exposed to heparin (599). Britton *et al.* (600) demonstrated that heparinized aminoethylated cellulose tubing at low levels of aminoethylation had excellent mechanical strength as well as antithrombogenic properties.

Rubber surfaces that had good blood compatibility were made by chemically binding heparin onto the surface of a diamine-cured polyurethan rubber, but there was a significant thrombosis rate (601). A nonthrombogenic surface on silicone rubber was made by combining graphite, silicone rubber, benzalkonium chloride, and heparin (602). In addition, Hepacone (a combination of heparin and silicone rubber) and Hepacone-6 (a combination of colloidal graphite, heparin, and silicone) were described by Hufnagel *et al.* (603) for intravascular applications.

Other elastomeric complexes were synthesized by Yen and Rembaum (604) from polyether diisocyanates, which had desirable mechanical properties; it was shown that an ionic bond had been formed between the heparin and the quaternized polyether polyurethan. In addition, a new family of linear elastomeric ionenes or cross-linked homo or block polymers containing nitrogen were synthesized, which were capable of bonding with heparin (605, 606).

The use of heparinized polyester fibers in vascular prostheses is finding increased interest. Najjar and Gott (607) suggested that a heparinized, small diameter, Dacron graft would find excellent applications in lower extremity vascular surgery and possibly in coronary artery bypass. Two methods were developed by Hersh *et al.* (608) for heparinizing polyester fiber. One method involves the fixation by heat of a water-insoluble complex of heparin onto the polyester; the second uses an aminosilane coupling agent to bond the heparin ionically to the protonated amine. Conflicting reports as to which is most effective have been issued. According to Hersh and Weetall (609), the thermal method is better since ionically bound heparin eluted from the Dacron quicker. However, in a later report, Hersh *et al.* (610) inferred that the Dacron grafts that had ionically bound heparin yielded the best results on both tissue and blood compatibility.

Membranes for hemodialysis and encapsulation have been constructed from heparin combined with nylon and cellulose. Luttinger and Cooper (611) devised two methods for binding heparin to nylon membranes. In the first, free amino groups are readily quaternized with methyl iodide and the surface is placed in contact with a dilute aqueous heparin solution; in the second, there is surface attachment of quaternary ammonium salt to the membrane surface, followed by heparinization. A heparin-complexed membrane for coating on microcapsules, which might be useful for treating certain enzyme-deficient diseases, was made by combining a benzalkonium–heparin complex with an ether–colloidin solution (612). Promising nonthrombogenic properties were demonstrated by heparinized DEAE CA-cellulose acetate membranes with an indefinite clotting time (613). Measurement of heparin permeability through cellulose membranes gave variable results, possibly because of the polydispersity of commercial heparin (614).

Heparin also may be easily embedded on the surface of biologically derived collagen membranes, which may permit kidney dialysis (615). The effects of heparin on this membrane showed little influence on the recalcified clotting time until levels of 1–10 mg. of heparin/g. of collagen were reached and then there was an increase (616). For extracorporeal connections and membrane assemblies, as well as for potential intravascular implantation, Merrill *et al.* (617) made a hydrogel by heating a homogeneous water solution of polyvinyl alcohol, heparin, glyceraldehyde, and an acid catalyst.

Some other interesting examples of heparin adsorbed physically and chemically on surfaces may find use in cardiovascular surgery. Flexible and rigid epoxy and urethan polymers containing heparin, chemically and uniformly incorporated throughout and/or ionically complexed to quaternized amine groups, were implanted into the vena cava of dogs with no adverse effects on the blood (618). The innominate artery was reconstructed by Murray *et al.* (619) with the use of a temporary heparin-coated shunt bypass. Merenstein (620) flushed umbilical artery catheters with heparinized saline to maintain potency. The absorption of fibrinogen on mica surfaces was inhibited by heparin (621).

An effect that occurs in polymer films after heparin uptake is that only small amounts of protein or oxide and no detectable amounts of fibrinogen or platelets are absorbed at the polymer solid interfaces (622). Another interesting effect is that materials treated chemically so as to introduce negatively charged groups, such as sulfonate, carboxylate, or heparinate, tend to be antithrombogenic, but the surfaces must have a uniform negative charge (623).

The limitation of heparinized surfaces given by Salzman *et al.* (624) is that deposition of platelets occurs on these surfaces which antagonize the action of heparin. Thrombocytopenia, platelet thrombosis, and embolism can complicate heparinized surfaces or extracorporeal surfaces with large surface areas unless methods are developed to reduce heparin platelet interaction. It is also possible for hemolysis to be induced by heparinized polymers (625). According to Friedman and Leonard (626), platelet adhesion on heparinized and nonheparinized surfaces is similar.

According to Salzman (584), some of the problems that remain to be solved are:

1. What is the nature of events that occur at a blood-solid interface?

2. What conditions must be satisfied for a material to be nonthrombogenic?

Since thrombosis is probably most important among the complications with the development of artificial organs, these problems remain foremost in the minds of workers in this field.

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pH–Partition Behavior of Amino Acid-Like β-Lactam Antibiotics

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Abstract □ The pH–partition behavior of the ampholyte antibiotics ampicillin, cephalexin, and cephaloglycin, using *n*-butanol or *n*-octanol as the lipid phase and aqueous phosphate buffers, was determined at 37° over a pH range of 4.9–7.9. By comparing the pH–partition behavior of these drugs with computer-generated plots of the concentration of their various ionic species as a function of pH, the anion was found to be the major partitioning species. Minimum partitioning occurred in the isoelectric region, suggesting that the zwitterion did not partition to a significant extent. The pH–partition behavior of L-phenylalanine, as a model ampholyte, was studied over a pH range of 2.0–8.9; although the L-phenylalanine cation partitioned into the lipid phase, the anion partitioned to a significantly greater extent, while the minimum partitioning in the isoelectric region indicated that the zwitterion was a poor partitioning species. Studies of surface activity as a function of antibiotic concentration in phosphate buffers showed that there is a relationship between the partitioning behavior of these antibiotics and either the reduction in surface tension or the resulting surface excess at the lipid–water interface. To account for the difference in the pH–partition behavior of the compounds employed in this study and the previously reported data for the tetracyclines, two general types of ampholytes capable of forming zwitterions should be considered, depending on whether or not significant amounts of uncharged species are present in the isoelectric region. Maximum partitioning into the lipid phase should occur if uncharged species is present in significant concentrations (*e.g.*, tetracycline); the compounds in this study were of the other type and thus showed reduced partitioning in the isoelectric pH region.

Keyphrases □ pH–partition behavior—amino acid-like β-lactam antibiotics (ampicillin, cephalexin, cephaloglycin), relationships among surface tension, drug concentration, and pH □ Antibiotics, amino acid-like β-lactam—pH–partition behavior, relationships among surface tension, drug concentration, and pH □ Ampholyte antibiotics (ampicillin, cephalexin, cephaloglycin)—pH–partition behavior

The drug absorption mechanism by which organic ions are absorbed from the GI tract is difficult to explain solely on the basis of the pH–partition hypothesis (1, 2), which states that a drug is absorbed in its unionized form. However, drugs like tetracycline (3), ampicillin (4), cephaloglycin (5), and cephalexin (6) are always ionized and yet are sufficiently absorbed from the GI tract to produce therapeutic effects. Fiese and Perrin (7), noting that dextromethorphan is absorbed from the stomach even though it is completely ionized, found that absorption of this drug was related to its surface activity. Higuchi suggested the formation of lipophilic ion-pairs as one possible mechanism for the absorption of organic ions (8).

Colaizzi and Klink (9) studied the pH–partition behavior of tetracyclines in *n*-octanol–aqueous buffer systems and found that there was maximum partitioning into the lipid phase at the pH corresponding to maximum zwitterion concentration. To account for these results, they suggested that a relatively more lipophilic intramolecular ion-pair was formed, resulting from partial charge cancellation which produced more partitioning into the lipid phase. The tetracyclines, as noted in their paper (9), have a relatively complex dissociation pattern. A simpler model such as ampicillin, cephalexin, or cephaloglycin, each of which exhibits a diprotic dissociation pattern with the single amino and carboxylic acid group, might better illustrate the pH–partition behavior of ampholyte drugs.

Thus, the purpose of this study was to investigate the pH–partitioning behavior of several amino acid-like β-lactam antibiotics. Due to the potential relationship between surface activity of these drugs and their pH–partition behavior, the relationships between surface tension and drug concentration and pH were also studied.

EXPERIMENTAL

Materials—L-Phenylalanine[1] and samples of ampicillin, cephaloglycin, and cephalexin were used as supplied from their manufacturers[2]. Phosphate buffers of pH 4.9, 5.7, 6.4 ,7.2, and 7.9, which were employed in the partition studies with the antibiotics, had a total molar buffer concentration of 0.035 and an ionic strength of 0.10; phosphate buffers of pH 2.0, 4.7, 5.2, 6.6, and 8.9, with a total molar buffer concentration of 0.030 and an ionic strength of 0.14, were utilized for similar studies with L-phenylalanine. Sodium chloride[3] was added to the buffers whenever necessary to adjust the ionic strength. The buffer of pH 2.0 was prepared with phosphoric acid[3] and sodium phosphate monobasic[3]. The buffers for pH 4.7, 4.9, 5.2, 5.7, 6.4, 6.6, 7.2, and 7.9 were prepared with anhydrous dibasic sodium phosphate[3] and sodium phosphate monobasic, while the pH 8.9 buffer was prepared with anhydrous dibasic sodium phosphate and tribasic sodium phosphate[3]. All buffer pH's were measured at 25° using a suitably standardized pH meter[4].

The water for these experiments was prepared by passing distilled water through two glass percolator columns containing a mixture of anionic and cationic resins[5]. The conductivity, as determined with a conductivity meter[6], was maintained below 0.1 p.p.m. (as sodium chloride).

Procedure for Determination of Apparent Partition Coefficients of L-Phenylalanine and of Penicillin and Cephalosporin Antibiotics—Exactly 8.84×10^{-5} mole of L-phenylalanine, 5.72×10^{-4} mole of

[1] Eastman Kodak Co., Rochester, N. Y.

[2] The authors are grateful to Eli Lilly and Co. for supplying the cephalexin monohydrate and cephaloglycin dihydrate. They are also grateful to Wyeth Laboratories for supplying the anhydrous ampicillin.

[3] Analytical reagent grade, Mallinckrodt Chemical Works, St. Louis, Mo.

[4] Model DR, E. H. Sargent and Co., Chicago, Ill.

[5] Rexyn AG 501, Fisher Scientific Co., Pittsburgh, Pa.

[6] Barnstead Still and Sterilizer Co., Boston, Mass.

Table I—Apparent Partition Coefficients (Organic Phase–Aqueous Buffer) for Ampicillin, Cephalexin, and Cephaloglycin at 37°

Compound	Organic Phase	Partition Coefficient at pH 4.9	5.7	6.4	7.2	7.9
Ampicillin	Butanol	0.14	0.16	0.19	0.38	0.48
Ampicillin	Octanol	0.18	0.20	0.21	0.23	0.31
Cephalexin	Butanol	0.08	0.12	0.13	0.30	0.87
Cephalexin	Octanol	0.04	0.13	0.11	0.14	0.26
Cephaloglycin	Butanol	0.06	0.11	0.24	0.41	0.93
Cephaloglycin	Octanol	0.04	0.08	0.13	0.26	0.24

Table II—Apparent Partition Coefficients (Organic Phase–Aqueous Buffer) for L-Phenylalanine at 37°

Compound	Organic Phase	Partition Coefficient at pH 2.0	4.7	5.2	6.6	8.9
L-Phenylalanine	Butanol	0.18	0.16	0.14	0.14	0.26

ampicillin, or 3.82×10^{-6} mole[7] of the particular cephalosporin was dissolved in sufficient buffer solution to give a total volume of 100 ml. Exactly 15 ml. of *n*-octanol[8] or *n*-butanol[8] was then placed into each of three 50-ml. glass-stoppered conical flasks, and exactly 15 ml. of the previously prepared drug-buffer solution was added to each of the three flasks; 15 ml. of the drug-buffer solution was placed into a fourth flask as a control. The flasks were stoppered and placed into a metabolic shaking incubator[9], regulated at 37 ± 0.1°, and set to maximum shaking speed. Every 15 min. each flask was individually handshaken to ensure rapid distribution between the two solvent phases and then immediately returned to the metabolic shaker.

When equilibrium was achieved[10], a 10-ml. sample of the aqueous phase was withdrawn from each flask. Its absorbance was determined spectrophotometrically[11] at 260 nm. (257 nm. for L-phenylalanine) using a water blank, and its concentration was determined from the appropriate Beer's law plot. By using the concentration of the drug in the fourth flask and the concentration of drug in the aqueous buffer phase, the concentration of drug in the lipid phase was calculated. The concentration of drug in the lipid phase and that remaining in the aqueous phase were utilized to calculate the *n*-octanol–aqueous buffer apparent partition coefficients (or the *n*-butanol–aqueous buffer apparent partition coefficients). The results of the partitioning studies are summarized in Tables I and II. UV spectral measurements revealed no evidence of degradation of the compounds studied during the partitioning experiments.

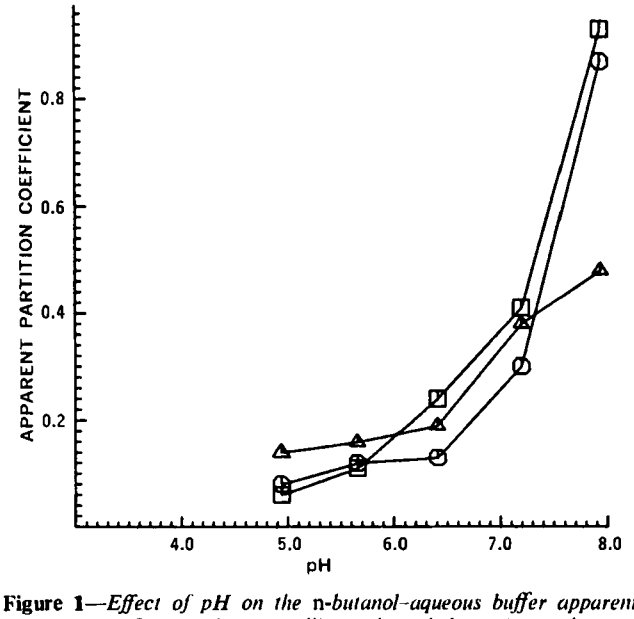

Figure 1—*Effect of pH on the* n-*butanol–aqueous buffer apparent partition coefficients for penicillin and cephalosporin analogs at 37°. Key:* □, *cephaloglycin;* ○, *cephalexin; and* △, *ampicillin.*

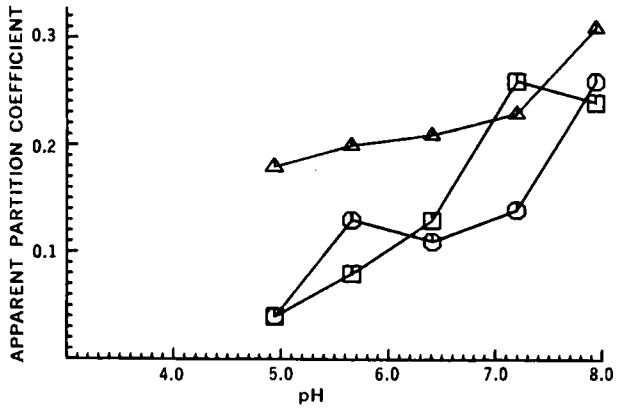

Figure 2—*Effect of pH on the* n-*octanol–aqueous buffer apparent partition coefficients for penicillin and cephalosporin analogs at 37°. Key:* □, *cephaloglycin;* ○, *cephalexin; and* △, *ampicillin.*

Procedure for Determination of Reduction of Surface Tension of Buffered Solutions of Ampicillin, Cephalexin, and Cephaloglycin—Buffered solutions of the drugs were prepared using the phosphate buffers previously prepared for the partition studies in concentrations ranging from 0.25 to 7 mg./ml., depending on the drug[12]. The surface tension of the resulting solutions was determined at 25° using a previously calibrated tensiometer[13] equipped with a platinum-iridium ring with a mean circumference of 5.985 cm.

RESULTS AND DISCUSSION

The apparent partition coefficients for ampicillin, cephaloglycin, cephalexin, and L-phenylalanine as a function of pH can be found in Figs. 1–3. To determine the influence of the different ionic species (cation, zwitterion, and anion) on the apparent partition coefficient, the percent of total concentration of each ionic species was calculated as a function of pH. These calculations were performed by a computer program utilizing Eqs. 1–3 for a diprotic acid, where K_1 and K_2 are the acidity constants (10):

$$\% \text{ cation} = (H_3O^+)^2\, 100/[(H_3O^+)^2 + K_1(H_3O^+) + K_1K_2] \quad \text{(Eq. 1)}$$

$$\% \text{ zwitterion} = K_1(H_3O^+)100/[(H_3O^+)^2 + K_1(H_3O^+) + K_1K_2] \quad \text{(Eq. 2)}$$

$$\% \text{ anion} = K_1K_2\, 100/[(H_3O^+)^2 + K_1(H_3O^+) + K_1K_2] \quad \text{(Eq. 3)}$$

The calculated concentrations were then used to prepare Calcomp[14] plots for each compound (Figs. 4–7).

The apparent partition coefficients for ampicillin, cephalexin, and cephaloglycin with *n*-butanol as the lipid phase, plotted as a function of the pH of the aqueous phase, are found in Fig. 1. When the percent of total concentration of each of the drug's ionic species is compared with Fig. 1 from pH 4.9 to 8.0, the apparent partition coefficients are found to increase as the concentration of anion increases.

For ampicillin the percent of total concentration as zwitterion decreases from 99% at pH 4.9 to 12% at pH 7.9, while the percent of total concentration as anion increases from 0 to about 90% over the same pH range. After noting the variation in the apparent partition

[7] The amount of drug was chosen to be the minimum amount that could be accurately assayed.
[8] Certified reagent grade, Fisher Scientific Co., Pittsburgh, Pa.
[9] Dubnoff Precision Scientific Co., Chicago, Ill.
[10] To achieve equilibrium between the phases, the flasks were shaken for 1 hr. for the antibiotics and for 3 hr. for L-phenylalanine.
[11] Model DB-G, Beckman Instruments, Inc., Fullerton, Calif.
[12] Four different concentrations of drug were used, none of which exceeded 50% of the solubility.
[13] Fisher Surface Tensiomat, Fisher Scientific Co., Pittsburgh, Pa.
[14] IBM 360/50 interfaced digital plotter.

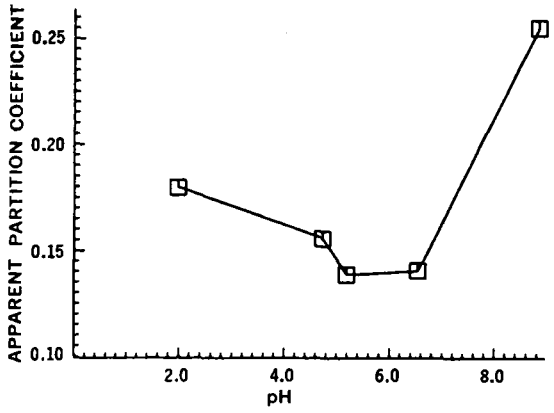

Figure 3—*Effect of pH on the* n-*butanol–aqueous apparent partition coefficients for* L-*phenylalanine* (□) *at 37°.*

coefficients over the same pH range, the anion appears to be the partitioning species. Similar results occurred for cephaloglycin and cephalexin, but, in addition, the cation species (not present in significant concentration over the pH range employed in the partitioning studies for ampicillin) appears to be inhibiting the partitioning into the lipid phase for these drugs.

For cephalexin the percent of total concentration as cation is approximately 60% at pH 4.9 and 0% at around pH 7.5. If it is assumed that the cephalexin anion should partition better than ampicillin anion[15], then the smaller apparent partition coefficients for cephalexin in the pH range from 4.9 to 7.2 may be attributed to the cation forming an ion-pair with the anion and thus reducing the effective concentration of anion that may partition. At pH 4.9 and 5.7, the apparent partition coefficients for cephaloglycin are smaller than for ampicillin; at all other higher pH values, the apparent partition coefficients of cephaloglycin are greater than for ampicillin. The smaller apparent partition coefficients may also be due to the significant concentration of cation reducing the amount of anion available for partitioning.

The apparent partition coefficients for the antibiotics as a function of pH, using *n*-octanol as the lipid phase, are shown in Fig. 2.

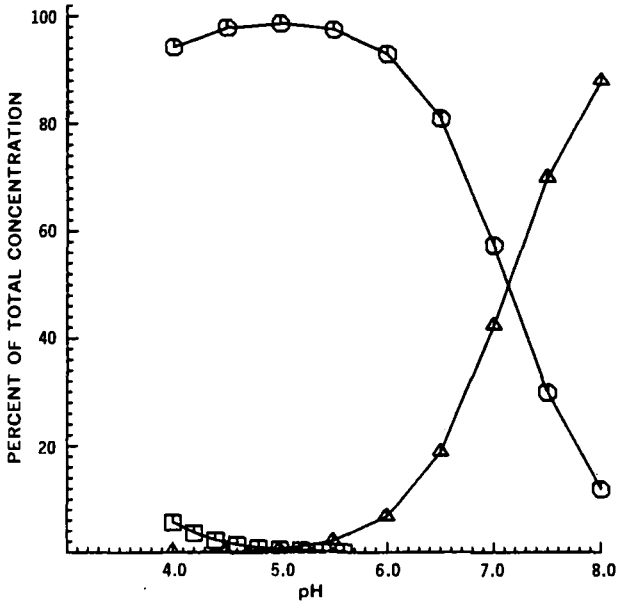

Figure 4—*Percent of total concentration as a particular ionic species of ampicillin in aqueous solution as a function of pH at 25°. Key:* □, *ampicillin cation;* ○, *ampicillin zwitterion; and* △, *ampicillin anion.*

[15] This would be a reasonable assumption since at pH 7.9, where essentially only anion is present for both drugs, the apparent partition coefficient for cephalexin is almost double that of ampicillin.

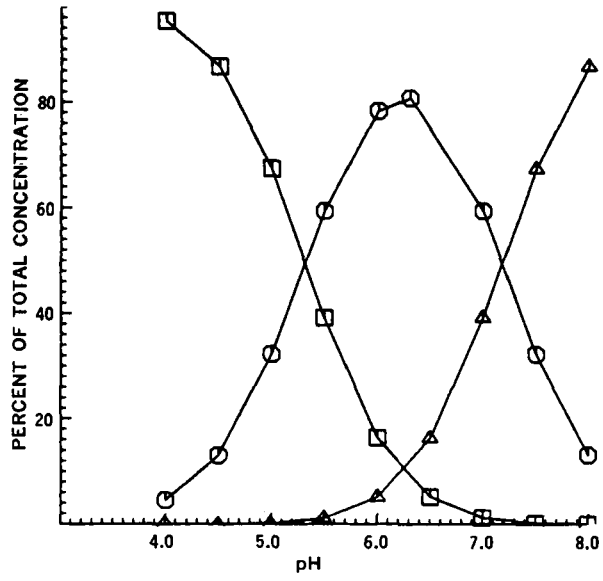

Figure 5—*Percent of total concentration as a particular ionic species of cephalexin in aqueous solution as a function of pH at 25°. Key:* □, *cephalexin cation;* ○, *cephalexin zwitterion; and* △, *cephalexin anion.*

In this system, which is more lipophilic than the *n*-butanol–aqueous buffer system, ampicillin partitions best but the apparent partition coefficients are substantially less than when *n*-butanol is used as the lipid phase. However, the anion appears to be the partitioning species since the apparent partition coefficients appear to be related to the anion concentration. In this system the effect of cation on the partitioning of anion for cephalexin and cephaloglycin is not clear, but it may account for the larger apparent partition coefficients with cephaloglycin than with cephalexin at pH 6.4 and 7.2, since cephalexin has a larger apparent partition coefficient at pH 7.9.

The differences between the apparent partition coefficients in the *n*-butanol–buffer system as compared with those in the *n*-octanol–buffer system are probably due to the large amount of water present in the buffer-saturated *n*-butanol phase, which would tend to increase the amount of ions in the *n*-butanol phase by increasing the dielectric constant of the *n*-butanol phase.

Since the acid instability of the β-lactam antibiotics would cast doubt on the determination of the apparent partition coefficients at a pH where only cation was in high concentration, a model com-

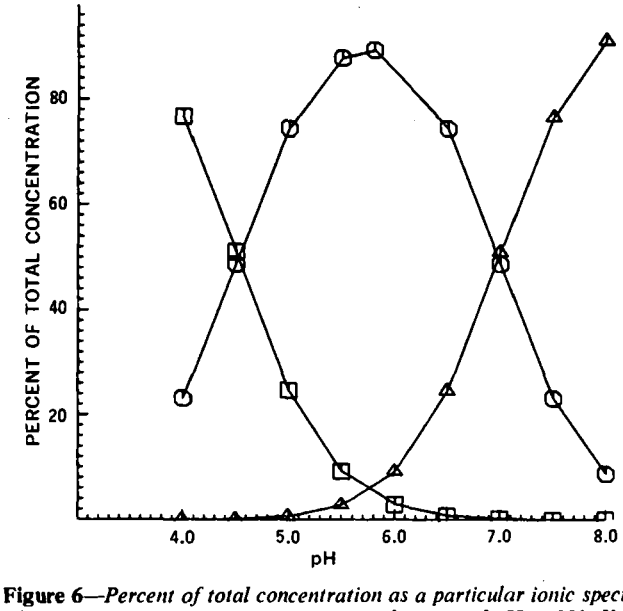

Figure 6—*Percent of total concentration as a particular ionic species of cephaloglycin in aqueous solution as a function of pH at 25°. Key:* □, *cephaloglycin cation;* ○, *cephaloglycin zwitterion, and* △, *cephaloglycin anion.*

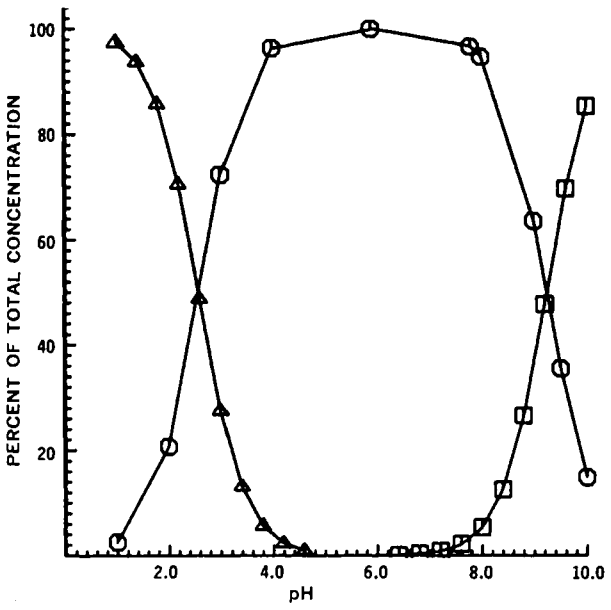

Figure 7—*Percent of total concentration as a particular ionic species of* L-*phenylalanine in aqueous solution as a function of pH at 25°. Key:* △, *phenylalanine cation;* ○, *phenylalanine zwitterion; and* □, *phenylalanine anion.*

pound without such a limitation was desired. Through the use of L-phenylalanine, a relative comparison of the effects of cation, zwitterion, and anion on the apparent partition coefficients in Fig. 3 with the concentration of the ionic species in Fig. 7 shows that the minimum values for the apparent partition coefficients occurred at the maximum zwitterion concentration. Comparing the apparent partition coefficient at pH 2.0, where the cation species is 80% of the total concentration, to pH 8.9, where the anion concentration is about 50% of the total concentration, suggests that the anion partitions better than the cation even though the cation concentration is 1.6 times greater than the anion.

The partitioning results indicate that the zwitterion is not the dominant partitioning ionic species. This would seem to be contrary to the fact that these compounds exhibit minimal water solubility at the isoelectric point (11). However, this decrease in water solubility is a reflection of the high crystal lattice energy of the zwitterion species as compared to its heat of solution in water and not of an increase in lipid solubility (12). The oil–water partition coefficient would be expected to decrease to a minimum at the isoelectric point since the lipid layer, with its low dielectric constant, would have less tendency to solvate the dipolar ion (zwitterion) than water.

Colaizzi and Klink (9) reported maximum partitioning into the *n*-octanol phase in the pH range where the zwitterion concentration of tetracycline and its derivatives would be at its maximum. These results, also seemingly contradictory to the findings reported here, are probably a result of the apparent fact that for tetracycline a significant concentration of the uncharged molecular species exists in equilibrium with the zwitterion present (Fig. 7 of *Reference 9*). This uncharged species would be expected to be highly lipid soluble and thus partition into the *n*-octanol phase. Leeson *et al.* (13) reported the K_{a1} for tetracycline hydrochloride and tetracycline methiodide to be 4.68×10^{-4} and 2.76×10^{-4}, respectively. As stated in their paper (13), the quaternization of the dimethylamino group of tetracycline hydrochloride to form tetracycline methiodide destroys the dimethylamino acidic center of tetracycline hydrochloride but should not affect other acidic centers since the dimethylammonium group should be electronically similar to the quaternary ammonium group. Therefore, the K_{a1} of tetracycline methiodide should represent the sum of the microionization constants ($k_1 + k_2$) associated with the equilibrium of the cation species with the zwitterion species of tetracycline hydrochloride. The difference in acidity between K_{a1} of tetracycline hydrochloride and tetracycline methiodide (1.92×10^{-4}) should approximate the contribution of the proton associated with the dimethylamino group in tetracycline hydrochloride, *i.e.*, the microionization constant k_3 associated with the equilibrium of the cation species with the uncharged species of tetracycline hydrochloride. Since at any pH, each of the isoelectric forms of tetracycline hydrochloride is in equilibrium with the cation species, they must also be in equilibrium with each other.

Table III—Surface Excess as a Function of pH for Ampicillin, Cephalexin, and Cephaloglycin

Compound	pH	Surface Excess, moles/cm.^{2}, $\times 10^{-11}$
Ampicillin	4.94	30.5
Ampicillin	6.49	16.6
Ampicillin	7.20	9.4
Ampicillin	7.94	8.4
Cephalexin	4.94	5.27
Cephalexin	6.49	5.70
Cephalexin	7.20	5.79
Cephalexin	7.94	6.49
Cephaloglycin	4.94	6.43
Cephaloglycin	6.49	9.11
Cephaloglycin	7.20	9.52
Cephaloglycin	7.94	9.67

The ratio between ($k_1 + k_2$) and k_3, which is independent of pH, should represent the tautomeric equilibrium constant (K_z) between the zwitterion species and uncharged species of tetracycline hydrochloride (14).

The value of K_z calculated[16] for tetracycline is 1.44. From this value for K_z, it is a simple matter to calculate[17] that 41% of the isoelectric species will be in the uncharged form. Thus the enhanced partitioning into the lipid phase when the isoelectric forms are in maximum concentration may be due to the uncharged tetracycline molecules. Colaizzi and Klink (9) also reported that the tetracycline methiodide did not partition into the *n*-octanol phase. This would be expected on the basis of the explanation just put forth, since the drug cannot form an uncharged species (although it does form a zwitterion).

In contrast to tetracycline hydrochloride, the pH–partition experiments of this study tend to indicate that very little uncharged species is present in the cases of ampicillin, cephalexin, cephaloglycin, and L-phenylalanine. This was borne out by Hou and Poole (12), who reported that ampicillin, like other amino acids in aqueous solutions at 25° (*e.g.*, L-phenylalanine), exists essentially as zwitterions within the isoelectric region. Similar behavior would be expected for cephalexin and cephaloglycin due to the similarity in structure with ampicillin. This lack of significant amounts of uncharged lipophilic species would also explain the significantly lower oral absorption observed with ampicillin as compared to its acyloxymethyl esters (15), which can exist as an uncharged molecule.

It can be rationalized that the surface activity of the various dissociative species of the drugs employed in this study may be influencing the apparent partition coefficient by changing the surface excess or reducing the interfacial tension at the interface between the lipid and the aqueous buffer phases. Because the concentrations of the various dissociative species change with pH, the surface activity may not remain constant throughout the pH range employed in the partition studies. To evaluate these affects on the pH–partition studies, the Gibbs adsorption equation (4), which defines surface excess as a function of concentration of drug and surface tension, was used (16):

$$s = (-c/RT)(dv/dc)_T \qquad \text{(Eq. 4)}$$

where s is the surface excess in moles/cm.^{2}, R is the universal gas law constant in ergs mole^{-1} deg.$^{-1}$, T is the absolute temperature, c is the concentration of drug in the bulk solution in moles/l., and v is the surface tension in dynes/cm.

The slopes of the regression lines of surface tension as a function of drug concentration at pH 4.9, 6.4, 7.2, and 7.9 were substituted into Eq. 4, along with appropriate values for R and T, to obtain the surface excess for each drug at a concentration of 1 mg./ml. The

[16] $K_z = (k_1 + k_2)/k_3 = 2.76 \times 10^{-4}/1.92 \times 10^{-4} = 1.44$.

[17] K_z = zwitterion species/uncharged species = 1.44. Let zwitterion species = x. Then the percent of total isoelectric concentration as uncharged species would be $(100 - x)$. $K_z = x/(100 - x) = 1.44$, where $x = 59\%$ of the isoelectric concentration as zwitterionic species. Then uncharged species = $(100 - 59) = 41\%$ of isoelectric concentration.

values for surface excess at each pH are summarized in Table III for each drug. The regression lines from which the slopes were calculated had linear correlation coefficients ranging from −0.879 to −0.994.

The surface excess increased for cephalexin and cephaloglycin as the buffer became more alkaline, while for ampicillin the opposite effect was observed. The molar surface tension reduction for most amino acids is at its minimum in the isoelectric region and increases when the pH is increased or decreased from the isoelectric point (17). The observed values for surface excess may account for some of the larger increases in the apparent partition coefficients for cephalexin and cephaloglycin as pH increases from 4.9 to 7.9, as compared to the similar changes observed for ampicillin. The apparent partition coefficients of cephalexin and cephaloglycin in *n*-butanol–aqueous buffer are, respectively, approximately 11 and 16 times greater at pH 7.9 as compared to pH 4.9. For ampicillin in the same partitioning system, only a threefold increase was observed. It may be that an increase in surface excess and a reduction in surface tension occurring in concert with the anion concentration approaching its maximum, as observed with cephalexin and cephaloglycin, enhance the partitioning of the anion into the lipid phase. However, with ampicillin, the largest surface excess (maximum reduction in surface tension) occurs when the zwitterion concentration is at its maximum and the anion concentration is at its minimum. This could explain the observation that there is much less of a difference in the partitioning coefficients of ampicillin over the pH range studied, as compared to the difference observed with cephalexin and cephaloglycin over the same pH range.

In summary, the partitioning results for ampicillin, cephalexin, and cephaloglycin indicate that the anion is the partitioning species. The cation appears to partition to some extent in the case of L-phenylalanine, but it tends to inhibit the partitioning of the anion for the cephalosporins. The zwitterion does not appear to partition to any extent for any of the compounds studied. The surface activity of the drugs appears to modify the partitioning results by increasing the surface excess of the drug and/or by reducing surface tension at the aqueous buffer–lipid interface.

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Pharmacokinetics of Trichloroethanol and Metabolites and Interconversions among Variously Referenced Pharmacokinetic Parameters

EDWARD R. GARRETT[▲] and HOWARD J. LAMBERT

Abstract □ The pharmacokinetics of intravenously administered trichloroethanol, chloral hydrate, sodium trichloroacetate, and trichloroethanol glucuronide were studied in the dog with developed GC analyses of blood, urine, and feces, and analyzed with aid of analog and digital computation. Trichloroethanol pharmacokinetics were evaluated at multiple-dosage levels and showed distribution from a central compartment assignable to extracellular body water into readily available and less available tissues assignable to total body water and lipoidal and other tissues. The major route of removal of trichloroethanol in the dog was by conjugation to the glucuronide, and the first-order rate constants of this metabolic route were dose dependent but did not appear to be a consequence of enzymic saturation or change in apparent volumes of distribution with dose. A possible explanation is that the hepatic lipid–water ratio may vary with the dose of trichloroethanol. The formed trichloroethanol glucuronide, after release from the liver, was rapidly distributed in the extracellular body water of the dog and rapidly eliminated in the urine by glomerular filtration and tubular secretion. Biliary elimination of the glucuronide accounted for about 5% of an intravenous dose of trichloroethanol. Saturation of the bile secretory mechanism was demonstrated at higher trichloroethanol doses, *e.g.*, 100 mg./kg., but the absence of trichloroethanol and its glucuronide in the feces may be explained by hydrolysis of the biliary glucuronide in the GI tract with subsequent reabsorption of the formed trichloroethanol, *i.e.*, an enterohepatic shunt. No assayable trichloroacetic acid was observed in the blood or urine of dogs dosed with trichloroethanol. A long apparent disposition half-life of 75 hr. was observed after intravenous administration of sodium trichloroacetate; this can be explained by the extensive tissue binding of trichloroacetate which gave high apparent volumes of distribution. Intravenously administered chloral hydrate was rapidly and quantitatively converted to trichloroethanol in the dog, with an apparent half-life of 3 min. Protein binding of trichloroethanol and its glucuronide (about 35%) was determined. Although the glucuronide did not partition into the red blood cells, the trichloroethanol did so instantaneously, with a partition coefficient of 2.1 in favor of the red blood cells. Since apparent volumes of distribution and microscopic pharmacokinetic rate constants have quantitative values that vary widely, depending on whether they are referenced to total blood, total plasma, or unbound drug in plasma concentrations, a detailed analysis of the conversions among these variously referenced constants was made and applied to the data of these studies. Estimates of the microscopic rate constants and apparent volumes of distribution referenced to unbound drug in plasma have the most valid physiological significances. These are complex functions of the hematocrit, the degree of protein binding, the true volume of plasma or blood in the animal, and the red blood cell/plasma partition coefficient when the drug is assayed per milliliter of whole blood or per milliliter of plasma. The awareness of these facts will necessitate extensive recalculation of many pharmacokinetic constants now given in the literature.

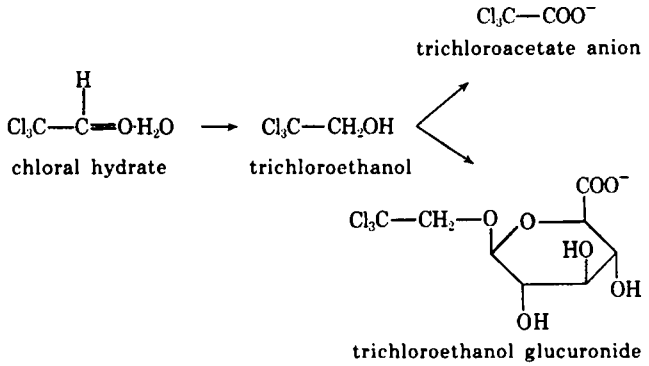
Keyphrases □ Trichloroethanol—pharmacokinetics and metabolites after intravenous administration, dogs □ Chloral hydrate—pharmacokinetics and metabolites after intravenous administration, dogs □ Pharmacokinetics—trichloroethanol, chloral hydrate, and metabolites after intravenous administration, dogs □ GLC—analysis, trichloroethanol and metabolites

The hypnotic effect of 2,2,2-trichloroethanol was first demonstrated in 1882 (1). It was previously reported that the product isolated from the urine of patients dosed with chloral hydrate was a conjugation product of trichloroethanol and glucuronic acid (2). The conjugated glucuronic acid was measured in rabbit urine, and it was concluded that chloral hydrate and trichloroethanol are both converted to the conjugated product in the same proportions (3).

Butler (4) and Marshall and Owens (5) noted that the CNS depression which follows the ingestion of chloral hydrate is due principally, if not entirely, to the trichloroethanol formed by reduction *in vivo*. The same investigators (4–6) demonstrated that the principal metabolic products of trichloroethanol are trichloroacetic acid and trichloroethanol glucuronide (Scheme I). Those metabolites did not appear to have hypnotic activity.

The major route of trichloroethanol metabolism in dogs was glucuronidation, and more than 60% of the dose was found in the urine as the glucuronide (5). This was more than twice that found in the urine of human subjects (5). Some of the glucuronide formed from an intravenous dose of trichloroethanol was secreted and concentrated in the bile of dogs (6). A small fraction of the dose was excreted unconjugated. Comparison of the species difference in trichloroacetate anion formation was stated to be difficult due to its probable metabolic destruction by the dog (5).

This paper presents the results of studies on the pharmacokinetics of trichloroethanol and its metabolites in the dog at various doses administered intravenously. These pharmacokinetic studies were based on analyses expressed in terms of total concentrations of drugs or metabolites in blood that give apparent volumes of distribution and microscopic rate constants referenced to such concentrations. These are different values than would be obtained if the analyses were expressed in terms of total concentrations or unbound concentrations in plasma. The latter values would be most consistent with the physiological referents and the physicochemical realities of drug disposition in the



Scheme I

body. The literature is replete with the presentation of such parameters variously referenced. Since the calculation of one set of reference parameters from another is necessary to compare those of the same and different drugs within and among species, rigorous expressions of such mathematical conversions are developed in a special section and applied to the pharmacokinetic data of this paper. The protein binding and red blood cell partitions necessary for these applications are determined.

The complete model for the pharmacokinetics of trichloroethanol and its metabolites that will be outlined here did not spring into being intuitively as a full-fledged entity. It had to be developed by sequential processes of systems analyses that included graphical analyses, analog simulation, and digital computation in accordance with a consistent model. A section of this paper delineates the systematic technological patterns and sequences of such logical analyses used in model construction and quantification.

The pharmacokinetic studies of trichloroethanol at various intravenously administered dose levels clearly show that the drug distributes by first-order processes into both a shallow and a deep compartment and that the only significant route of removal of trichloroethanol in the dog is by conjugation to the glucuronide. The apparent first-order rate constants of trichloroethanol are dose dependent. This does not appear to be due to changes in the apparent volumes of distribution of the major compartments, nor is it a consequence of enzyme saturation. The latter is denied by the facts that first-order processes persist at all dose levels and that simultaneous challenging *in vivo* with the potent glucuronidation inhibitor sulfobromophthalein indicated a large capacity for glucuronidation. However, an excessively large dose administered did decrease the rate of glucuronidation.

It is shown that the rate of appearance of trichloroethanol glucuronide in the body did not reflect the rate of loss of trichloroethanol. The glucuronide's appearance was delayed, and the maximum blood level was lower than would be predicted from the instantaneous loss of the alcohol. This implies an intermediate storage depot which can be assigned to the liver. The results of balance studies across the liver of a surgically modified dog are consistent with this hypothesis. It is shown that the phenomenon of trichloroethanol dose-dependent pharmacokinetics can be rationalized by postulating that the hepatic lipid–water ratio varies with the dose of trichloroethanol and that this effect on microscopic redistribution of trichloroethanol in the liver from an aqueous metabolic biophase modifies the metabolism rate.

It is explained that the renal clearance of glucuronide that decreases with the higher glucuronide concentration in blood, which results from higher doses of trichloroethanol, can be rationalized by postulating the saturation of the tubular secretory pathway for glucuronide.

The pharmacokinetic study on intravenously administered trichloroethanol glucuronide clearly shows that it is primarily eliminated by filtration and tubular secretion. Although about 5% of the dose is biliary excreted, the lack of significant glucuronide in the feces implicates extensive GI solvolysis and reabsorption of the resultant trichloroethanol. It is demonstrated that saturation of the bile secretory mechanism for glucuronide occurs at higher trichloroethanol doses as well as increased bile flow rates.

The pharmacokinetic studies on intravenously administered trichloroacetic acid demonstrate long dose-independent apparent half-lives of elimination, which can be rationalized by the high apparent volumes of distribution assignable to extensive tissue binding.

The pharmacokinetic studies of intravenously administered chloral hydrate demonstrate that it is rapidly and quantitatively converted to trichloroethanol in the dog, with an apparent half-life of 3 min.

EXPERIMENTAL

Materials—The following were used: glacial acetic acid[1], chloral hydrate USP[1], 5% trichloroacetic acid solution[1], chlorobutanol USP[1], anhydrous ether[2], anhydrous sodium acetate (analytical reagent)[3], mepesulfate (sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside)[4], and sulfosalicylic acid[5]. The β-glucuronidase was bovine liver B grade and had 50,000 units/vial or 360 Fishman units/mg.[6]. Sulfobromophthalein injection was 50 mg./ml.[7]. The heparin solution had 10,000 USP units/ml.[8]. The sodium pentobarbital[9] was in a sterile solution of 50 mg./ml. The trichloroethanol[6,10] was redistilled at 153° before use. The trichloroethanol glucuronide was the sodium salt of 95% purity on assay (7) and was isolated from the urine of dogs given repeated parenteral doses of trichloroethanol. The isolation procedure was described by Seto and Schultze (8). The acidified glucuronide melts at 142° and its triacetyl methyl ester melts at 158° (8).

Analytical Methods—Sensitive and specific GC assays for trichloroethanol, its chlorohydrate precursor, and its metabolites, trichloroethanol glucuronide and trichloroacetic acid, were described previously (7). These methods were modified as described below.

A gas chromatograph[11] equipped with an electron-capture detector and a recorder[12] was used. A pulse interval of 15 μsec. gave linear calibration curves for all physiologically encountered concentrations of trichloroacetic acid, trichloroethanol, and its glucuronide.

The columns used for all studies were either 1.23- or 2.46-m. (4- or 8-ft.) stainless steel tubes, 0.63-cm. (0.25-in.) diameter, containing 20% Carbowax 20M on 60–80-mesh Chromosorb W.

Conditions—The temperature conditions used for assay of trichloroethanol and trichloroacetic acid were: injection port, 160°; column, 135°; and electron-capture detector, 190°. The carrier gas (helium) flow rate was 60 ml./min. (at 50 psig.), while the purge gas (90% argon–10% methane) was fixed at 140 ml./min. (at 30 psig.).

Blood and Urine Assays—Trichloroethanol, chloral hydrate, and trichloroacetic acid were extracted from 1.00 ml. of whole blood or urine admixed with 1.00 ml. of distilled water and 1.00 ml. of a 10% solution of sulfosalicylic acid containing 0.72 mg. of chlorobutanol/100 ml. The extraction was effected with 2.0 ml. ether after protein precipitation with the sulfosalicylic acid. Chlorobutanol was used as an internal standard. The two-phase mixture, in an injection vial of 6- or 10-ml. capacity sealed with a rubber stopper and an aluminum crimped cap, was agitated for 1 min. on a mixer[13] and centrifuged[14]

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[1] Fisher Scientific Co., Fair Lawn, N. J.
[2] J. T. Baker Chemical Co., Phillipsburg, N. J.
[3] Mallinckrodt Chemical Works, New York, N. Y.
[4] Hoffmann-La Roche Inc., Nutley, N. J.
[5] Merck & Co., Rahway, N. J.
[6] Calbiochem, Los Angeles, Calif.
[7] Hynson, Westcott and Dunning, Baltimore, Md.
[8] Organon Inc., West Orange, N. J.
[9] Nembutal Sodium, Abbott Laboratories, North Chicago, Ill.
[10] Aldrich Chemical Co., Inc., Milwaukee, Wis.
[11] F & M model 700, Hewlett-Packard Co., Waltham, Mass.
[12] Minneapolis-Honeywell Apparatus Controls Division, Minneapolis, Minn.
[13] Vortex Jr. Mixer, Scientific Industries, Inc., Queens Village, N. Y.
[14] 1/2 HP International centrifuge (model SBR), International Equipment Co., Boston, Mass.

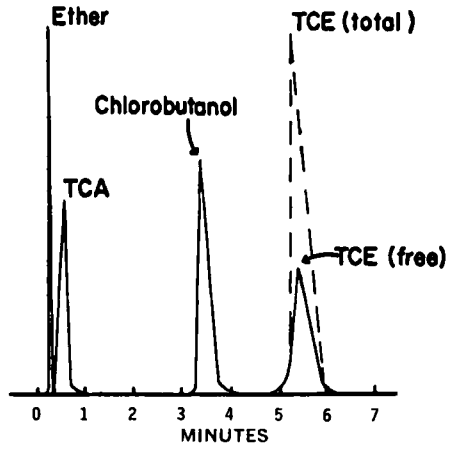


Figure 1—*Typical chromatogram obtained after injection of a 5-μl. aliquot of a 2-ml. ether extract of an acidified 1-ml. blood sample which contained trichloroethanol (TCE), trichloroacetic acid (TCA), and trichloroethanol glucuronide (TCE-G). The solid curve, TCE (free), is a measure of the free trichloroethanol present in the sample, while the broken curve, TCE (total), is a measure of the total trichloroethanol obtained after glucuronidase hydrolysis of the trichloroethanol glucuronide present in the sample. The chlorobutanol was added to the blood sample as an internal standard and permitted calculation of peak height ratios.*

for 2–5 min. until the ether–water emulsion separated and two distinct phases reappeared. The peak height ratios of the peak assigned to the substrate to the peak assigned to the chlorobutanol were used as assay parameters after the injection of a 5-μl. aliquot of the ether solution (Fig. 1).

Trichloroethanol glucuronide in 1.0 ml. of blood or of appropriately diluted urine (1:10, 1:100, or 1:1000) was hydrolyzed with 360 Fishman units (1 mg.) of β-glucuronidase (1.0 ml. of 0.1 *M* pH 4.5 acetate buffer with 1.0 mg. of β-glucuronidase) by incubating at 37°, pH 4.5, for 48 hr., and the trichloroethanol formed was assayed as already described. The difference in the trichloroethanol–chlorobutanol peak height ratios obtained before and after enzymatic hydrolysis was the trichloroethanol glucuronide–chlorobutanol peak height ratio.

The optimum time for complete hydrolysis of trichloroethanol glucuronide by β-glucuronidase was determined by adding a small amount of the purified sodium salt of the glucuronide to urine. One-milliliter aliquots of this urine were treated and incubated as already described for 30, 60, 94, 125, and 180 min. and for 5.0, 8.7, 19.8, 24.5, 27, 31, 50, and 147 hr. Each sample removed at the specified time was immediately refrigerated. The refrigerated samples were chromatographically analyzed for trichloroethanol on the day following the taking of the last sample.

Calibration curves for the substrate were established by adding appropriately graded amounts to the pertinent biological fluid and assaying by the described methods. These methods differed from those previously described (7) in that chlorobutanol was included in the aqueous solution of sulfosalicylic acid added rather than in the ether used for extraction. Also, in the current assay, trichloroacetic acid was directly extracted from acidified blood or urine and subsequently gas chromatographically analyzed in the ether solvent. The previous method cited (7) used an alkaline decarboxylation which yielded assayable chloroform.

The reliability of the assays for trichloroethanol was determined by conducting these analyses on blood (from four different dogs) to which 2.94 mcg./ml. of trichloroethanol had been added. Six samples of similarly spiked, freshly drawn heparinized blood were placed at 5° and analyzed at daily intervals against calibration curves prepared daily.

Protein Binding—Equilibrium dialysis experiments were run with trichloroethanol and its glucuronide to determine protein binding to dog plasma. Two-milliliter samples of dog plasma were separated from whole dog blood by centrifugation and placed inside sealed dialysis sacs[15]. Dialyses were run at 5° for 48 hr. against 10.0-ml. solutions (5, 50, and 500 mcg. trichloroethanol/ml.) in pH 7.4 phosphate buffer. A control dialysis was run at each trichloroethanol concentration with a sac containing only 2.0 ml. of buffer. Aliquots were removed from the solutions outside and inside the dialysis sacs, appropriate dilutions were made, samples were assayed, and the fractional binding was calculated.

Trichloroethanol glucuronide binding to plasma protein was estimated by dialysis of pooled blood plasma containing the glucuronide against pH 7.4 buffer.

Determination of Red Blood Cell/Plasma Distribution of Drug—Since trichloroethanol has high solubility in nonaqueous solvents (9), it was possible that a distribution between red blood cells and plasma would occur when the drug was introduced into a volume of whole blood. Aliquots (0.1 ml.) of trichloroethanol solutions were mixed with 10.0 ml. of heparinized dog blood to give final concentrations of 0.294 and 2.94 mcg./ml., and 1.0 ml. of each blood sample was removed for assay. The remaining 9.0-ml. portions were centrifuged until complete separation of red blood cells occurred and a clear, colorless plasma layer remained. A 1.0-ml. plasma sample was then removed from each tube and assayed. The hematocrit was determined with a hematocrit tube.

Three further determinations were carried out on pooled blood samples obtained from the pharmacokinetic experiments on two other dogs which had been administered 50 mg./kg. of trichloroethanol intravenously. One dog had received 50 mg./kg. of sulfobromophthalein in combination.

Similar studies on red blood cell/plasma distribution were conducted with trichloroethanol glucuronide.

Pharmacokinetics of Trichloroethanol—Female mongrel dogs, 10–20 kg., were fasted for 24 hr. with water *ad libitum*. At 8:30 a.m. on Day 0 (the day of the experiment), the animal was weighed and anesthetized with an intravenous or intramuscular injection of pentobarbital, 30 mg./kg.

One of three pairs of veins was used for cannulation and blood sampling: the external jugular veins of the neck, the cephalic veins of the forelegs, or the lateral saphenous veins of the hind legs. The cannula was frequently flushed with physiological saline solution but was filled with whole blood before 5-ml. samples were taken. These samples were put in 10.0-ml. injectable vials containing 0.1 ml. of 1% mepesulfate solution to prevent blood clot formation.

At least 1 hr. after the pentobarbital was administered, control urine and blood samples were taken. When the animals showed signs of movement and diminished depth of anesthesia, a volume of 5.0% trichloroethanol solution equivalent to the desired dose was injected within 40 sec. The cannula was flushed with 10–20 ml. of physiological saline within an additional 20 sec.

A typical sampling schedule after the time of the saline flush of the trichloroethanol dose was: 2, 4, 6, 8, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min. and then at 30-min. intervals to 720 min. Further samples were taken during the 24–120-hr. interval in several experiments.

Urine samples were collected at least hourly *via* an indwelling urethral catheter in 100-ml. graduated cylinders for 12 hr. on Day 0. When possible, feces were collected after 12 hr. Total urine and feces were collected at t = 24 hr. and at 24-hr. intervals thereafter until no further traces of trichloroethanol or its metabolites were found upon assay.

A minimal period of 14 days was allowed between any two trichloroethanol pharmacokinetic experiments on the same animal. Samples were analyzed for concentrations of trichloroacetic acid, trichloroethanol, and its glucuronide.

Each of four female dogs, A, B, C, and D, received various doses of trichloroethanol ranging from 10 to 150 mg./kg. All studies were grouped within 4 months to avoid possible age, weight, or seasonal effects on the animals.

Pharmacokinetics of Trichloroethanol Glucuronide—Two experiments were performed with intravenous administration of a solution of sodium trichloroethanol glucuronide (100 and 450 mg.) in an anesthetized dog. Blood and urine samples were taken at the intervals previously described for experiments with trichloroethanol and assayed for both trichloroethanol and its glucuronide.

Pharmacokinetics of Trichloroacetic Acid—Twenty milliliters of a 5% trichloroacetic acid solution (1.0 g.) was brought to pH 7.0 with sodium hydroxide and intravenously administered to an anesthetized dog. Blood samples were removed at regular intervals for the first 12 hr. of the experiment, at t = 24 hr., and at 24-hr. intervals thereafter until t = 480 hr.; these samples were assayed for tri-

[15] 0.94-cm. (0.37-in.) flat width, Visking Co., Chicago, Ill.

chloroacetic acid. A second experiment was performed with a 100-mg. dose of trichloroacetic acid.

Pharmacokinetics of Chloral Hydrate—A dose of 500 mg. chloral hydrate (dissolved in physiological saline) was administered through an indwelling jugular vein catheter to a lightly anesthetized dog. Blood samples were taken at intervals and urine samples were taken hourly for a total of 2 hr. and assayed for trichloroethanol, its glucuronide, and for chloral hydrate and trichloroacetic acid.

Studies on Biliary Excretion of Trichloroethanol and Its Glucuronide—Two dogs were anesthetized and laparotomized, and the common bile duct was exposed and cannulated. The renal circulation was not disturbed. The incision was closed with wound clips and covered with gauze pads wet with warm physiological saline solution. Trichloroethanol was injected 1 hr. after surgery was completed.

To obtain maximal data from these experiments, two doses of trichloroethanol were administered in each experiment. In the first experiment, 150 mg. of trichloroethanol (11.6 mg./kg.) was given at $t = 0$ and 600 mg. (46 mg./kg.) at $t = 245$ min. In the second experiment, 500 mg. of trichloroethanol was given at $t = 0$ and 1000 mg. at $t = 370$ min.

Blood samples were collected from the jugular vein and urine samples were collected from a urethral cannula. Bile was collected hourly and assayed for trichloroethanol and its glucuronide in the same manner as urine.

At the end of each experiment, the animal was sacrificed. Gallbladder bile was removed for assay from the second animal but not from the first.

Possible *In Vivo* Inhibition of Trichloroethanol Glucuronidation by Sulfobromophthalein—Dog E, a 10-kg. female, was anesthetized and 600 mg. of trichloroethanol was administered intravenously at $t = 0$. At $t = 250$ min., a second dose of 600 mg. trichloroethanol was given along with 100 mg. of sulfobromophthalein (10 mg./kg.). In addition, a constant intravenous drip of approximately 0.6 mg. sulfobromophthalein/min. was also maintained until $t = 490$ min. The dose of sulfobromophthalein used was twice that recommended (10) for studies on hepatic function.

In a second experiment with the same dog, 600 mg. of trichloroethanol was given at $t = 0$ and samples were taken for 300 min. At $t = 320$ min., a second 600-mg. dose of trichloroethanol was given, which was followed immediately by a dose of 500 mg. sulfobromophthalein. In addition, a constant intravenous infusion of 3 mg. sulfobromophthalein/min. was maintained until $t = 500$ min.

In both experiments, blood and urine samples were taken at regular intervals and assayed for trichloroethanol and its glucuronide.

Hepatic Storage of Trichloroethanol and/or Its Glucuronide—A major problem in the *in vivo* evaluation of hepatic storage of a drug and/or its metabolites by sampling blood flow across the liver is due to the dilution of hepatic venous return by vena cava blood from the lower extremities and kidneys. Whereas blood entering the liver may be easily sampled by catheterization of the hepatic portal vein, hepatic output occurs *via* very short hepatic veins, which immediately merge with the inferior vena cava at the level of the diaphragm in the dog. The vena caval dilution must be circumvented to sample hepatic output *via* the hepatic veins. One approach that was tried was to shunt the vena caval flow into the hepatic portal vein by surgery so that the total blood flow from the lower trunk entered the liver through the hepatic portal vein and all blood exited the liver as hepatic venous output. This surgical approach was attempted on two dogs; one died during surgery and the other expired shortly thereafter.

An alternative solution to the problem was provided by the work of Harris and Riegelman (11). A collateral circulation between a slowly occluded inferior vena cava and the azygous vein was promoted. Slow occlusion was achieved by insertion of a small segment of Tygon tubing in the vena cava 2 weeks prior to the hepatic storage or clearance experiments. An irritation set up by the tubing caused a slow clot and fibrin formation to occlude the vessel. The slow occlusion allowed establishment of the aforementioned collateral circulation. Rapid occlusion of vena caval flow causes death by cardiac arrest. Insertion of the Tygon tubing was performed with sterile surgical technique. The X-rays were taken after cannulation of the inferior vena cava *via* the lateral saphenous vein of the hind leg and clearly showed the occlusion. The radiopaque medium was a 10% solution of meglumine diatrizoate[16] rapidly injected simultaneously with X-irradiation.

One week after X-ray, the animal was anesthetized with pentobarbital and laparotomized and the hepatic portal vein was cannulated *via* a splenic vein. The remaining splenic veins were tied off as was the hepatic artery. Omission of the latter step allows a 25% error to occur due to dilution of hepatic venous flow with hepatic arterial flow. The hepatic venous return was sampled with a cannula inserted in the external jugular vein through the right atrium and into the inferior vena cava (which now only contains hepatic venous blood). After injection of a 50-mg./kg. dose of trichloroethanol *via* the hepatic portal vein, blood samples were simultaneously removed from both cannulas at regular intervals and assayed for trichloroethanol and its glucuronide.

CALCULATIONS, ANALYSIS, AND CURVE FITTING

Calculation of Red Blood Cell/Plasma Distribution Coefficient—The derivation of an expression for the calculation of a red blood cell/plasma distribution coefficient is as follows. The amount of drug, A, in whole blood, A_B, is:

$$A_B = A_P{}^u + A_P{}^b + A_{RBC} \quad \text{(Eq. 1)}$$

where $A_P{}^u$ and $A_P{}^b$ are the amounts of drug in plasma, unbound and bound to plasma proteins, respectively. The amount of drug in red blood cells is represented by A_{RBC}.

When the amount of drug in whole blood is converted to concentration, Eq. 2 becomes:

$$A_B/V_{B_{true}} = A_P{}^u/V_{B_{true}} + A_P{}^b/V_{B_{true}} + A_{RBC}/V_{B_{true}} \quad \text{(Eq. 2)}$$

where $V_{B_{true}}$ is the true volume of whole blood in the animal body.

When $V_{P_{true}}$ and V_{RBC} are the respective true volumes of plasma and red blood cells in the animal body:

$$\frac{A_B}{V_{B_{true}}} = \frac{A_P{}^u/V_{P_{true}}}{V_{B_{true}}/V_{P_{true}}} + \frac{A_P{}^b/V_{P_{true}}}{V_{B_{true}}/V_{P_{true}}} + \frac{A_{RBC}/V_{RBC}}{V_{B_{true}}/V_{RBC}} \quad \text{(Eq. 3)}$$

or:

$$[A_B] = \frac{[A_P{}^u]}{V_{B_{true}}/V_{P_{true}}} + \frac{[A_P{}^b]}{V_{B_{true}}/V_{P_{true}}} + \frac{[A_{RBC}]}{V_{B_{true}}/V_{RBC}} \quad \text{(Eq. 4)}$$

Since the hematocrit (H) may be defined as:

$$H = V_{RBC}/V_{B_{true}} \quad \text{(Eq. 5)}$$

therefore:

$$1 - H = V_{P_{true}}/V_{B_{true}} \quad \text{(Eq. 6)}$$

Substitution of Eqs. 5 and 6 into Eq. 4 yields:

$$[A_B] = [A_P{}^u](1 - H) + [A_P{}^b](1 - H) + [A_{RBC}]H \quad \text{(Eq. 7)}$$

The red blood cell/plasma distribution coefficient is then defined as:

$$D = [A_{RBC}]/[A_P{}^u] \quad \text{(Eq. 8)}$$

Upon rearrangement of Eq. 7:

$$D = \{[A_B]/[A_P{}^u](1 - H) - [A_P{}^b]/[A_P{}^u] - 1\}\,(1 - H)/H \quad \text{(Eq. 9)}$$

If the fraction, f, of drug bound to plasma proteins is known:

$$[A_P{}^b] = [A_P]f \quad \text{(Eq. 10)}$$

and:

$$[A_P{}^u] = [A_P]\,(1 - f) \quad \text{(Eq. 11)}$$

where $[A_P]$ is the total plasma concentration of drug, and Eq. 9 becomes:

$$D = \{[A_B]/[A_P]\,(1 - f)\,(1 - H) - f/(1 - f) - 1\} \times (1 - H)/H \quad \text{(Eq. 12)}$$

[16] Renografin.

$$n[A_P{}^u] + [P] \overset{K}{\rightleftarrows} [A_nP]$$

Scheme II

and the concentration of unbound drug in the plasma can be obtained by a rearrangement of this equation and is:

$$[A_P{}^u] = (1 - f)[A_P] = [A_B] \Big/ \left(DH + \frac{1 - H}{1 - f}\right) \quad \text{(Eq. 13)}$$

The fraction of drug bound to a plasma protein may not be invariant. It may be a function of the unbound concentration of drug (12, 13), *i.e.*:

$$f = 1/(1 + [A_P{}^u]/n[P] + 1/nK[P]) \quad \text{(Eq. 14)}$$

where K is the association constant for the binding of a molecule of drug to one of the equivalent n binding sites of the protein molecule in accordance with Scheme II, where $[P]$ is the concentration of protein.

At low drug concentrations or when the association constant K is small, the second term in the denominator of Eq. 14 becomes negligible and:

$$f \sim 1/(1 + 1/nK[P]) = [A_P{}^b]/[A_P] \quad \text{(Eq. 15)}$$

may be taken as constant for a given protein concentration and is independent of drug concentration.

Distribution of Drug among Blood Components and Fractions of Drug in Blood in Each—The amount of drug in the blood that is in the red blood cells may be calculated on the basis of Eqs. 5, 8, and 11:

$$A_{RBC} = V_{RBC}[A_{RBC}] = HV_{B_{true}}[A_{RBC}] = HDV_{B_{true}}[A_P{}^u] = HDV_{B_{true}}(1 - f)[A_P] \quad \text{(Eq. 16)}$$

The amount of drug in the blood that is bound to plasma protein and in the plasma may be calculated on the basis of Eqs. 6 and 10:

$$A_P{}^b = V_{P_{true}}[A_P{}^b] = (1 - H)V_{B_{true}}[A_P{}^b] = (1 - H)fV_{B_{true}}[A_P] \quad \text{(Eq. 17)}$$

The amount of drug in the blood that is unbound to plasma protein and in the plasma may be calculated on the basis of Eqs. 6 and 11:

$$A_P{}^u = V_{P_{true}}[A_P{}^u] = (1 - H)V_{B_{true}}[A_P{}^u] = (1 - H)(1 - f)V_{B_{true}}[A_P] \quad \text{(Eq. 18)}$$

When Eqs. 1 and 16–18 are considered:

$$A_B = V_{B_{true}}[A_P]\{HD(1 - f) + (1 - H)\} \quad \text{(Eq. 19)}$$

Division of Eqs. 16–18, respectively, by Eq. 19 gives the fraction of drug in the blood that is in the red blood cells as:

$$\gamma_{RBC} = A_{RBC}/A_B = HD(1 - f)/\{HD(1 - f) + (1 - H)\} \quad \text{(Eq. 20)}$$

and the fraction of drug in the blood that is bound to plasma protein and in the plasma as:

$$\gamma_P{}^b = A_P{}^b/A_B = (1 - H)f/\{HD(1 - f) + (1 - H)\} \quad \text{(Eq. 21)}$$

and the fraction of drug in the blood that is unbound to plasma protein and in the plasma as:

$$\gamma_P{}^u = A_P{}^u/A_B = (1 - H)(1 - f)/\{HD(1 - f) + (1 - H)\} \quad \text{(Eq. 22)}$$

Also, the total fraction of drug in the blood that is in the plasma is:

$$\gamma_P = \gamma_P{}^u + \gamma_P{}^b = (1 - H)/\{HD(1 - f) + (1 - H)\} \quad \text{(Eq. 23)}$$

Relations and Conversions among Variously Calculated Apparent Volumes of Distribution of the Central Compartment—Apparent volumes of distribution may be considered as the operational parameters that relate the measured concentrations of drug in the obtainable fluids of the body to the amounts in the individual compartments of the pharmacokinetic multicompartmental model that describe quantitatively the disposition of the drug in the body (12–16).

However, the numerical estimates of these apparent volumes of distribution for these several compartments can vary widely for the same multicompartmental model, dependent on whether the time course of free or total drug concentration is used and on whether blood or plasma is analyzed. Thus, the relations among these several estimates must be delineated to permit the calculation of one from the other. Only then will there be a common basis to compare the pharmacokinetic parameters of the same and different drugs within and among species.

If identification of apparent volumes of distribution with true volumes of body fluids or tissues is to be attempted, the criteria for the choice of the method of calculation of apparent volumes of distribution from pharmacokinetic data must be consistent with the physiological and physicochemical realities of drug disposition in the body.

The concentrations of a drug, A, are measured in aliquots of the fluid of the central compartment and are generally obtained as concentrations in blood, $[A_B]$, or in plasma, $[A_P]$. The apparent volumes of distribution referenced to blood, V_B, or to plasma, V_P, can be estimated from the extrapolation to zero time of the plot of drug concentration in blood or plasma against time on the premise of instantaneous mixing of the intravenously administered drug in the fluids of the central compartment (12, 13).

Thus, the apparent volume of distribution of the central compartment referenced to the total drug concentration in the blood is:

$$V_B = A_0/[A_B]_0 \quad \text{(Eq. 24)}$$

and the apparent volume of distribution of the central compartment referenced to the total drug concentration in the plasma is:

$$V_P = \{A_0 - (A_{RBC})_0\}/[A_P]_0 \quad \text{(Eq. 25)}$$

where A_0 and $(A_{RBC})_0$ are the intravenously administered dose and amount in the red blood cells, respectively; $[A_B]_0$ and $[A_P]_0$ are the total drug concentrations in blood and plasma, respectively, at zero time.

Significance of Variously Calculated Apparent Volumes of Distribution of the Central Compartment—These are fictitious volumes, since they may include the volumes of associated fluids where the drug concentration is in instantaneous equilibration with that of blood or plasma, *i.e.*:

$$V_B = V_{B_{true}} + V_{B_z} \quad \text{(Eq. 26)}$$

where $V_{B_{true}}$ is the true blood volume, and V_{B_z} is the apparent volume of this instantaneously equilibrated fluid or tissue on the presumption that its drug concentration is *exactly* the same as that of the blood. This apparent volume, V_{B_z}, may be termed the *associated* (with the central compartment) apparent volume of distribution *referenced* to the total drug concentration in the blood. Similarly:

$$V_P = V_{P_{true}} + V_{P_z} \quad \text{(Eq. 27)}$$

where $V_{P_{true}}$ is the true plasma volume, and V_{P_z} is the volume of this instantaneously equilibrated fluid or tissue on the presumption that its drug concentration is *exactly* the same as that of the plasma. This apparent volume, V_{P_z}, may be termed the *associated* (with the central compartment) apparent volume of distribution *referenced* to the total drug concentration in the plasma.

If none of the drug is sequestered by, or partitioned into, the red blood cells, all of the drug is assayed in the plasma and a simple relation exists between the respective apparent volumes of distribution. When Eqs. 6, 24, and 25 are considered:

$$V_P = V_{P_s} = A_0/[A_P]_0 = A_0 \Big/ \left(\frac{A_0}{V_{P_{true}}}\right) = A_0 \Big/ \frac{A_0}{V_{B_{true}}(1 - H)} = A_0 \Big/ \frac{[A_B]_0}{1 - H} = (1 - H)V_B \quad \text{(Eq. 28)}$$

and it follows from Eqs. 26–28 that:

$$V_{P_{s_z}} = V_{P_z} = V_{B_z}(1 - H) \quad \text{(Eq. 29)}$$

and the associated apparent volume of distribution referenced to the total drug concentration in plasma is less than the associated apparent volume of distribution referenced to the total drug concentration in blood.

These relations of Eqs. 26–29 are based on the presumption that the concentration in V_{P_x} is the same as that in plasma, $V_{P_{true}}$. The apparent volume V_{P_x} cannot be true plasma; it must be a fluid or tissue readily accessible to drug which is freely diffusible from the plasma water and would be unbound to plasma proteins. If the drug is completely protein bound and not partitioned into the red blood cells, the associated apparent volumes of distribution referenced to total drug concentration in blood and plasma, V_{B_x} and V_{P_x}, are zero and the apparent volumes of distribution, referenced to blood and plasma, V_B and V_P, are the same as the true volumes of blood and plasma, $V_{B_{true}}$ and $V_{P_{true}}$, respectively.

Equations for Calculation of Apparent Volumes of Distribution of the Central Compartment Referenced to Unbound Drug in Plasma from Apparent Volumes of Distribution Referenced to Total Drug Concentration in Blood and Plasma—The apparent volumes of distribution that have the highest probability of physiological significance are those calculated on the premise that only unbound drug in the true plasma volume (*i.e.*, diffusible drug in the plasma water) equilibrates with the other volumes of distribution of the various compartments of the multicompartmental model. The apparent volume of distribution in the central compartment referenced to unbound drug in the plasma water can be formulated as the amount of drug administrated intravenously less the amounts of drug partitioned into red blood cells and bound to plasma proteins at zero time divided by the zero time concentration of unbound drug in the plasma:

$$V_P{}^u = \{A_0 - (A_{RBC})_0 - (A_P{}^b)_0\}/[A_P{}^u]_0 \quad \text{(Eq. 30)}$$

In terms of the respective true volumes of red blood cells and plasma:

$$V_P{}^u = (A_0 - V_{RBC}[A_{RBC}]_0 - V_{P_{true}}[A_P{}^b]_0)/[A_P{}^u]_0 \quad \text{(Eq. 31)}$$

Substitution of values for V_{RBC}, $V_{P_{true}}$, $[A_{RBC}]_0$, $[A_P{}^b]_0$, and $[A_P{}^u]_0$ obtained from Eqs. 5, 6, 8, 10, and 11, respectively, into Eq. 31, with subsequent rearrangement, gives:

$$V_P{}^u = A_0/[A_P]_0\,(1 - f) - HDV_{B_{true}} - (1 - H)V_{B_{true}}f/(1 - f) \quad \text{(Eq. 32)}$$

in terms of the measured total concentration of the drug in plasma at zero time.

The substitution of Eq. 13 into Eq. 32 permits the apparent volume of distribution of the central compartment referenced to unbound drug concentration in the plasma to be expressed in terms of the measured total concentration of the drug in blood at zero time:

$$V_P{}^u = A_0\left(DH + \frac{1 - H}{1 - f}\right)\Big/[A_B]_0 - HDV_{B_{true}} - (1 - H)V_{B_{true}}f/(1 - f) \quad \text{(Eq. 33)}$$

On consideration of Eqs. 6 and 24, this apparent volume of distribution can be expressed in terms of the apparent volume of distribution referenced to the total concentration of drug in blood:

$$V_P{}^u = V_B\left(DH + \frac{1 - H}{1 - f}\right) - HV_{B_{true}}D - (1 - H)V_{B_{true}}f/(1 - f)$$
$$= V_B\left(DH + \frac{1 - H}{1 - f}\right) - \frac{H}{1 - H}V_{P_{true}}D - V_{P_{true}}f/(1 - f) = V_{P_{true}} + V_{P_x}{}^u \quad \text{(Eq. 34)}$$

On consideration of Eqs. 6 and 26, the associated apparent volume of distribution referenced to the unbound drug concentration in plasma can be defined in terms of the associated apparent volume of distribution referenced to the total drug concentration in blood:

$$V_{P_x}{}^u = V_{B_x}\left(DH + \frac{1 - H}{1 - f}\right) \quad \text{(Eq. 35)}$$

When the apparent volume of distribution of the central compartment is referenced to total concentration of drug in the plasma, it is expressed as the quotient of the amount of drug in plasma less the amount in the red blood cells and the total plasma concentration at zero time.

Thus, when Eqs. 25 and 31 are considered, a process similar to the development of Eq. 34 permits the expression of $V_P{}^u$ in terms of the apparent volume of distribution referenced to the total concentration of drug in plasma:

$$V_P{}^u = (V_P - V_{P_{true}}f)/(1 - f) = V_{P_{true}} + V_{P_x}{}^u \quad \text{(Eq. 36)}$$

On consideration of Eq. 27:

$$V_{P_x}{}^u = V_{P_x}/(1 - f) \quad \text{(Eq. 37)}$$

It follows from Eqs. 35 and 37 that:

$$V_{P_x} = V_{B_x}[DH(1 - f) + 1 - H] \quad \text{(Eq. 38)}$$

Pseudoapparent Volumes of Distribution of the Central Compartment and Calculation of Variously Referenced Apparent Volumes—Pseudoapparent volumes of distribution of the central compartment referenced to the total drug concentration in the plasma or to the unbound drug concentration in the plasma have been calculated as:

$$V_{P_s} = A_0/[A_P]_0 \quad \text{(Eq. 39)}$$

and:

$$V_{P_s}{}^u = A_0/[A_P]_0(1 - f) = V_{P_s}/(1 - f) \quad \text{(Eq. 40)}$$

without considering the fact that the numerator of the quotient in Eq. 25 should be lessened by the amount of drug in the red blood cells, $(A_{RBC})_0$.

Thus, consideration of Eqs. 11, 39, and 40 for the pertinent values in Eq. 32 permits the apparent volume of distribution of the central compartment referenced to unbound drug concentration in the plasma to be expressed in terms of these unrealistically calculated pseudoapparent volumes of distribution referenced to total drug concentration (or unbound drug concentration) in plasma:

$$V_P{}^u = V_{P_s}/(1 - f) - HV_{P_{true}}D/(1 - H) - V_{P_{true}}f/(1 - f) = V_{P_{true}} + V_{P_x}{}^u \quad \text{(Eq. 41)}$$

Other pertinent conversions can be obtained. On consideration of Eq. 27:

$$V_{P_x}{}^u = V_{P_{sx}}/(1 - f) - HDV_{P_{true}}/(1 - H) \quad \text{(Eq. 42)}$$

It also follows, on consideration of Eqs. 37 and 42, that:

$$V_{P_x} = V_{P_{sx}} - HDV_{P_{true}}(1 - f)/(1 - H) \quad \text{(Eq. 43)}$$

and:

$$V_P = V_{P_s} - HDV_{P_{true}}(1 - f)/(1 - H) \quad \text{(Eq. 44)}$$

It can be shown from Eqs. 35 and 42 that:

$$V_{B_x} = \frac{V_{P_{sx}} - HDV_{B_{true}}(1 - f)}{DH(1 - f) + 1 - H} \quad \text{(Eq. 45)}$$

so that, on cognizance of Eqs. 6, 23, 26, and 27:

$$V_B = V_{P_s}/[DH(1 - f) + 1 - H] = \gamma_P V_{P_s}/(1 - H) \quad \text{(Eq. 46)}$$

Relations among Variously Referenced Apparent Volumes of Distribution in the Absence of Protein Binding and/or Red Blood Cell Partition—If there is no partitioning into red blood cells, $D = 0$, $V_{P_s} = V_P$, $\gamma_P = 1$, $\gamma_{RBC} = 0$, $\gamma_P{}^b = f$, $\gamma_P{}^u = 1 - f$, and Eqs. 28, 29, 36, and 37 hold.

If there is no protein binding, $f = 0$, $V_P{}^u = V_P$, $V_{P_x}{}^u = V_{P_x}$, $V_{P_s}{}^u = V_{P_s}$, $\gamma_P = \gamma_P{}^u$, $\gamma_P{}^b = 0$ and, from Eq. 34:

$$V_P = V_B(DH + 1 - H) - HV_{B_{true}}D = V_B(DH + 1 - H) - HV_{P_{true}}D/(1 - H) \quad \text{(Eq. 47)}$$

and from Eqs. 35 and 38:

$$V_{P_x} = V_{B_x}(DH + 1 - H) \quad \text{(Eq. 48)}$$

and from Eqs. 41 and 44:

$$V_P = V_{P_s} - HDV_{P_{true}}/(1 - H) \quad \text{(Eq. 49)}$$

and from Eqs. 42 and 43:

$$V_{P_z} = V_{P_{sz}} - HDV_{P_{true}}/(1 - H) \quad \text{(Eq. 50)}$$

and from Eq. 45:

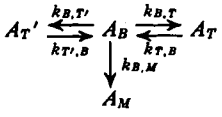
$$V_{B_z} = \frac{V_{P_{sz}} - HDV_{B_{true}}}{DH + 1 - H} \quad \text{(Eq. 51)}$$

and from Eq. 46:

$$V_B = V_{P_s}/(DH + 1 - H) = \gamma_P V_{P_s}/(1 - H) \quad \text{(Eq. 52)}$$

If there is no partitioning of drug into the red blood cells and no binding to plasma proteins, $D = 0, f = 0$, $V_P{}^u = V_P = V_{P_s}$, $V_{P_z}{}^u = V_{P_z} = V_{P_{sz}}$, $\gamma_P = \gamma_P{}^u = 1$, and Eqs. 28 and 29 hold.

Conversions among Microscopic Pharmacokinetic Rate Constants Based on Various Expressions of Drug Concentrations in Blood Components—The differential equation for the loss of drug from the central compartment of the linear three-compartment body model, Scheme III, may be expressed in terms of the amount of drug



Scheme III

in the blood and associated fluids, A_B, and in terms of the amounts of drug in the tissue compartments, A_T and $A_{T'}$:

$$-dA_B/dt = (k_{B,T} + k_{B,T'} + k_{B,M})A_B - k_{T,B}A_T - k_{T',B}A_{T'} \quad \text{(Eq. 53)}$$

It may be expressed in terms of the amounts of drug in the tissues, A_T and $A_{T'}$, and unbound drug, $A_P{}^u$, in the plasma and associated fluids, equilibrated with drug bound to protein and equilibrated with drug partitioned into red blood cells:

$$-\frac{dA_B}{dt} = (k_{P_u,T} + k_{P_u,T'} + k_{P_u,M})A_P{}^u - k_{T,P_u}T - k_{T',P_u}T' \quad \text{(Eq. 54)}$$

When the value for $A_P{}^u$ in Eq. 22 is substituted into Eq. 54:

$$-\frac{dA_B}{dt} = (\gamma_P{}^u k_{P_u,T} + \gamma_P{}^u k_{P_u,T'} + \gamma_P{}^u k_{P_u,M})A_B - k_{T,P_u}T - k_{T',P_u}T' \quad \text{(Eq. 55)}$$

Comparison of Eqs. 53 and 55 clearly shows that $k_{B,T} = \gamma_P{}^u k_{P_u,T}$, $k_{B,T'} = \gamma_P{}^u k_{P_u,T'}$, $k_{B,M} = \gamma_P{}^u k_{P_u,M}$, $k_{T,P_u} = k_{T,B}$, and $k_{T',P_u} = k_{T',B}$.

Estimations of Apparent Volumes of Distribution of Observed Tissue Compartments Referenced to Unbound Drug Concentration in Plasma from Variously Calculated Apparent Volumes of Distribution and Microscopic Pharmacokinetic Rate Constants—The apparent volume of distribution of a tissue compartment in a pharmacokinetic model such as Scheme III should be referenced to the unbound drug concentration in the plasma of the central compartment on the realistic premise that potential equilibration of drug between the central compartment and this tissue is only effected by unbound drug in the plasma water.

On the assumption of the possibility of an eventual equilibration or partition between unbound drug concentration in plasma water and drug concentration in a tissue (*e.g.*, as an ultimate consequence of the steady state resulting from a continuous zero-order infusion):

$$[A_T]_{eq}K = [A_P{}^u]_{eq} = A_P{}^u/V_P{}^u = A_T/KV_{T_P}{}^u \quad \text{(Eq. 56)}$$

where $V_P{}^u$ and $V_{T_P}{}^u$ are the apparent volumes of distribution referenced to the unbound drug concentration in plasma on the presumption that the partition coefficient, K, for the equilibration is unity (12, 13). If the tissue is truly homogeneous and an actual or operational K exists that is not equal to unity, V_T becomes the apparent volume of distribution of the tissue referenced to total drug concentration in that tissue.

The kinetic conditions for this potential steady-state equilibration are:

$$dA_T/dt = dA_P{}^u/dt = 0; k_{P_u,T}A_P{}^u = k_{T,P_u}A_T \quad \text{(Eq. 57)}$$

so that, from Eqs. 56 and 57 on the usually taken premise that $K = 1$, the apparent volume of distribution of the tissue compartment referenced to the unbound drug concentration in the plasma may be defined in terms of the apparent volume of distribution of the central compartment referenced to the unbound drug concentration in plasma and the microscopic pharmacokinetic constants for rate dependence on the amount of unbound drug in the plasma and its associated fluids (12, 13):

$$V_{T_P}{}^u = (k_{P_u,T}/k_{T,P_u})V_P{}^u \quad \text{(Eq. 58)}$$

This relation is valid even when a steady state does not exist between the tissue and plasma compartments.

Thus, as a consequence of the identity between Eqs. 53 and 55, the apparent volume of distribution of the tissue, T, can be related to the microscopic rate constants determined on the postulation of rate dependence on the total amount of drug in the blood as:

$$V_{T_P}{}^u = (k_{B,T}/\gamma_P{}^u k_{T,B})V_P{}^u \quad \text{(Eq. 59)}$$

Similarly:

$$V_{T'_P}{}^u = (k_{B,T'}/\gamma_P{}^u k_{T',B})V_P{}^u \quad \text{(Eq. 60)}$$

where $\gamma_P{}^u$ is defined in Eq. 22.

The apparent volumes of distribution of the tissues, T and T', referenced to the unbound drug concentration in plasma can be calculated from the variously calculated apparent volumes of distribution such as V_B, V_P, and V_{P_s} by appropriate substitutions of the values of $V_P{}^u$ obtained from Eqs. 34, 36, and 41, respectively, into Eqs. 59 and 60.

It can be shown similarly that the apparent volumes of distribution of the tissues, T and T', can be related to the microscopic rate constants determined on the postulation of rate dependence on the total amount of drug in the plasma:

$$V_{T_P}{}^u = (\gamma_P{}^u k_{P,T}/\gamma_P k_{T,P})V_P{}^u \quad \text{(Eq. 61)}$$

and:

$$V_{T'_P}{}^u = (\gamma_P{}^u k_{P,T'}/\gamma_P k_{T',P})V_P{}^u \quad \text{(Eq. 62)}$$

where $\gamma_P{}^u$ and γ_P are defined in Eqs. 22 and 23; and since $\gamma_P{}^u/\gamma_P = 1 - f$, then $k_{P,T} = (1 - f)k_{P_u,T}$, $k_{P,T'} = (1 - f)k_{P_u,T'}$, $k_{P,M} = (1 - f)k_{P_u,M}$, $k_{T,P} = k_{T,P_u}$, and $k_{T',P} = k_{T',P_u}$.

Pharmacokinetic Modeling—At the conclusion of all pharmacokinetic experiments, blood and urine samples were assayed for trichloroacetic acid, trichloroethanol, and its glucuronide. A defined procedure was followed to prepare a model that would be consistent with the observed data and to derive the parameters that quantifiably described the system.

The study of the pharmacokinetics of 30 mg./kg. of trichloroethanol in Dog D (20 kg.) will be used to illustrate the analysis and fitting procedures.

Graphical Analysis of Trichloroethanol Data—A semilogarithmic plot of trichloroethanol concentration in whole blood, given as the trichloroethanol–chlorobutanol peak height ratio per milliliter, is shown in Fig. 2. A peak height ratio per milliliter of 1.0 is equivalent to 8.4 mcg. trichloroethanol/ml.

The concentration of trichloroethanol in blood, $[TCE_B]$, as a function of time can be described by a sum of exponentials (17):

$$[TCE_B] = [A_B] = \sum_{i=1}^{n} [A_B]_i e^{-k_i t} = [A_B]_1 e^{-k_1 t} + [A_B]_2 e^{-k_2 t} + [A_B]_3 e^{-k_3 t} \quad \text{(Eq. 63)}$$

The methods by which the logarithmic plot of such data against time may be analyzed to yield its component $[A_B]_i$ (intercept) and k_i (slope) values were described in detail by Riggs (12).

The sum of the $[A_B]_i$ values, $\Sigma[A_B]_i$, is the time zero concentration, $[A_B]_0$, in the central compartment on intravenous administra-

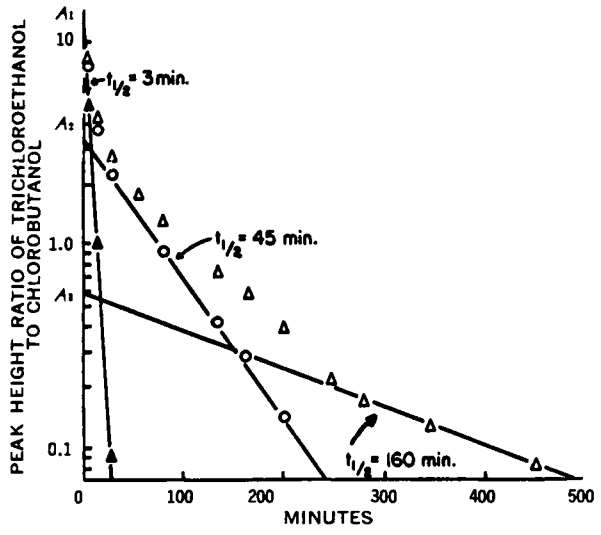


Figure 2—*Feathering of the semilogarithmic plot against time of the peak height ratios (△) of trichloroethanol to the internal standard chlorobutanol derived from the GC assay of 5 μl. of a 2-ml. ether extract of 1 ml. of acidified dog blood after the intravenous administration of 30 mg./kg. of trichloroethanol to the 20-kg. Dog D. A peak height ratio of 1.00 is equivalent to 8.4 mg. of trichloroethanol/ml. of whole blood. The three distinct phases obtained by feathering (the solid triangles, circles, and terminal open triangles) are labeled respectively with their apparent half-lives.*

tion and may be obtained from the calibration curve of peak height ratios *versus* concentrations (7). The trichloroethanol concentration in whole blood can be converted to the concentration of unbound trichloroethanol in plasma (in milligrams per milliliter) by Eq. 13. The apparent volume of distribution, V_B, referenced to the total concentration of trichloroethanol in the blood can be estimated from the dose (in milligrams) by Eq. 24.

The $\Sigma[A_B]_i$ or $[A_B]_0$ in terms of peak height ratio was estimated from the data of Fig. 2 extrapolated to zero time; it was 17.1 and corresponded to an initial whole blood concentration of 0.142 mg. of trichloroethanol/ml. or 0.0237 as percent of dose per milliliter of blood. The separate loading factors for the three exponentials that described the data of Fig. 2 were 13.0, 3.5, and 0.6 for $[A_B]_1$, $[A_B]_2$, and $[A_B]_3$, respectively. Insertion of the $[A_B]_0$ value into Eq. 13 and use of a red blood cell/plasma distribution coefficient D value of 2.1 with a hematocrit, H, of 0.45 (18) and a fraction, $f = 0.35$, of drug bound to plasma proteins (see *Results*) gave an initial concentration of unbound trichloroethanol in plasma, $[A_P{}^u]$, of 0.080 mg./ml.

The fractional amounts of drug in the blood that are in the red blood cells, in the plasma, unbound to protein and in the plasma, and bound to protein and in the plasma are: $\gamma_{RBC} = 0.527$ (Eq. 20), $\gamma_P = 0.475$ (Eq. 23), $\gamma_{P^u} = 0.310$ (Eq. 22), and $\gamma_{P^b} = 0.165$ (Eq. 21), respectively. When each of these fractions is multiplied by the initial milligrams of trichloroethanol per milliliter of blood, 0.142 mg./ml., the amount of drug in each blood component that is initially in 1 ml. of blood is: $(A_{RBC})_0/V_B = 0.075$ mg. for red blood cells, $(A_P)_0/V_{B_{true}} = 0.067$, $(A_P{}^u)_0/V_{B_{true}} = 0.044$, and $(A_P{}^b)_0/V_{B_{true}} = 0.023$. Thus, the respective concentrations are: $[A_{RBC}]_0 = (A_{RBC})_0/HV_{B_{true}} = 167$ mg./ml., $[A_P]_0 = (A_P)/(1 - H)V_{B_{true}} = 122$ mg./ml., $[A_P{}^u] = (A_P{}^u)_0/(1 - H)V_{B_{true}} = 0.080$ mg./ml., and $[A_P{}^b]_0/(1 - H)V_{B_{true}} = 0.043$ mg./ml.

On the premise that $V_{B_{true}} = 2.0$ l. and $V_{P_{true}} = 1.10$ l. for Dog C (20 kg.), where 100 and 55 ml./kg. are given in the literature (18) for dog blood volumes and plasma volumes, respectively, the various apparent volumes of the central compartment can be calculated. The pseudoapparent volume of distribution referenced to total drug concentration in plasma is $V_{P_s} = 4.88$ l. (Eqs. 39 and 46), the apparent volume of distribution referenced to total drug concentration in plasma is $V_P = 3.77$ l. (Eq. 44), the apparent volume of distribution referenced to the concentration of drug not bound to protein in the plasma is $V_P{}^u = 5.10$ l. (Eqs. 34 and 36), and the apparent volume of distribution referenced to drug concentration in blood is $V_B = 4.22$ l. (Eqs. 24 and 46).

The semilogarithmic plot of trichloroethanol concentration in blood *versus* time was resolved by the appropriate "feathering" techniques into three distinct exponential segments. The first exponential, which describes a rapid decline of trichloroethanol concentration in blood to 30% of the calculated initial value within 15 min. after drug administration, may be largely assigned to the diffusion of trichloroethanol from the initial volume of distribution ascribed to the central compartment into a larger volume of a readily available equilibrating body fluid. The low molecular weight of trichloroethanol and its extensive solubility in nonaqueous solvents (9) suggest that such rapid and extensive distribution might be expected.

The sum of the antilogarithms of the extrapolated zero-time intercepts (Fig. 2) assignable to the second and third exponentials of the polyexponential fit of the data (Eq. 63) was $[A_B]_2$ and $[A_B]_3 = 4.1$ in terms of peak height ratio per milliliter. This serves as a preliminary estimate of the assay value of trichloroethanol in blood that would have been observed after the ready equilibration with tissues if no significant drug elimination had occurred in the initial time interval. Insertion of the corresponding whole blood concentration (0.0346 mg./ml.) into Eq. 13 yields a concentration of free trichloroethanol in the plasma that is not bound to proteins of 0.0193 mg./ml. The estimated sum of the apparent volumes of distribution of the central compartment and that of the readily perfused tissues referenced to the total concentration of drug in the blood can be estimated from:

$$V_{D_B} = V_B + V_{T_B} = \text{dose}/([A_B]_2 + [A_B]_3) = A_0/(\Sigma[A_B]_i - [A_B]_1) \quad \text{(Eq. 64)}$$

and was 17.3 l. for a 600-mg. dose where the divisor of the last two terms is the concentration of trichloroethanol in the equilibrated fluids after the ready equilibration on the assumption of insignificant drug excretion or metabolism during the time interval of the equilibration. Subtraction of the initial apparent volume of distribution of the central compartment referenced to total concentration in the blood, $V_B = 4.2$ l., from this value provides an estimated volume of 13.1 l. for the tissue fluids, V_{T_B}, referenced similarly.

It can be argued also (Eq. 30) that if the curves of unbound drug concentration $[A_P{}^u]$ in the plasma against time are analyzed similarly:

$$V_{D_P{}^u} = V_P{}^u + V_{T_P{}^u} = \frac{\text{dose} - (\text{amounts in red blood cells and bound to plasma protein})}{[A_P{}^u]_2 + [A_P{}^u]_3} \quad \text{(Eq. 65)}$$

The estimated sum of the apparent volumes of distribution of the central compartment and of the readily perfused tissues referenced to unbound drug concentration in the plasma, $V_{D_P{}^u}$, can be calculated from the sum of the apparent volumes of distribution, V_{D_B}, referenced to total drug concentration in the blood by an equation derived on the same premises as, and similar to, Eq. 34:

$$V_{D_P{}^u} = V_{D_B}\left(DH + \frac{1 - H}{1 - f}\right) - HDV_{B_{true}} - (1 - H)V_{B_{true}}f/(1 - f) = 28.7 \text{ l.} \quad \text{(Eq. 66)}$$

Subtraction of the initial apparent volume of distribution of the central compartment referenced to unbound drug concentration in the plasma, $V_P{}^u = 5.1$ l., provides an estimated volume, $V_{T_P{}^u}$, of 23.6 l. for the tissue fluids referenced similarly.

More refined estimates of the volumes of distribution were obtained when the data were analyzed with the analog and digital computers, since the value of $[A_B]_0 = \Sigma[A_B]_i$ may be best obtained by computer extrapolation to zero time.

The loss of trichloroethanol from blood during the time period of the second exponential was postulated to be predominantly due to metabolic conversion of trichloroethanol to its glucuronide by hepatic microsomal uridine 5′-diphosphate-glucuronyltransferase. This was verified by the fact that the maximal rates of appearance of the glucuronide in blood and urine occurred in this interval. The rate constant of the second exponential of Eq. 63, k_2, is not equal to $k_{B,M}$, the metabolic rate constant in Scheme III. On the assumption of a predominantly two-compartment body model with rapid

equilibration (13), where k_3 is small or almost zero, k_2 is coincident with k_e (19, 20), the overall disposition rate constant for trichloroethanol where this latter constant can be expressed either in terms of the microscopic rate constants referenced to the total amount of drug in the central compartment of blood and associated fluids or, on consideration of Eq. 22 and the coincidence of Eqs. 53 and 55, in terms of the microscopic rate constants referenced to the amount of unbound drug in the central compartment of plasma and associated fluids:

$$k_2 = k_e = \frac{k_{B,M}}{1 + k_{B,T}/k_{T,B}} = \frac{k_{P_u,M}}{1/\gamma_P{}^u + k_{P_u,T}/k_{T,P_u}} = \frac{k_{P_u,M}}{1 + HD/(1 - H) + f/(1 - f) + k_{P_u,T}/k_{T,P_u}} \quad \text{(Eq. 67)}$$

and where $k_{B,T}$ or $k_{P_u,T}$ and $k_{T,B}$ or k_{T,P_u} are the respective rate constants for diffusion into and out of the rapidly equilibrating fluid compartment responsible for the first exponential of Eq. 63 that characterizes the initial loss of drug from the central compartment referenced to amounts of drug in blood and associated fluids or plasma and associated fluids, respectively.

This latter equation can also be derived from consideration of a general equation when the rate of loss of drug from a complex of rapidly equilibrating compartments is defined as being proportional to the amount of drug in only one of those compartments, *e.g.*:

$$-d(A_B + A_T)/dt = d(A_{RBC} + A_P{}^b + A_P{}^u + A_T)/dt = k_{P_u,M}A_P{}^u \quad \text{(Eq. 68)}$$

When the amount of drug in any one of the components or compartments of this rapidly equilibrating complex can be defined as being in equilibrium with the amount of drug in the rate-determining compartment, *i.e.*, $A_i = K_iA_P{}^u$, where K_i is the respective equilibrium constant for the ith compartment, Eq. 68 can be expressed as:

$$-d(K_{RBC/P_u}A_P{}^u + K_{P_b/P_u}A_P{}^u + A_P{}^u + K_{T/P_u}A_P{}^u)/dt = -dA_P{}^u/dt(1 + K_{RBC/P_u} + K^b_{P_b/P_u} + K_{T/P_u}) = k_{P_u,M}A_P{}^u \quad \text{(Eq. 69)}$$

On consideration of Eqs. 20 and 22:

$$K_{RBC/P_u} = \gamma_{RBC}/\gamma_P{}^u = HD/(1 - H) \quad \text{(Eq. 70)}$$

On consideration of Eqs. 21 and 22:

$$K_{P_b/P_u} = \gamma_P{}^b/\gamma_P{}^u = f/(1 - f) \quad \text{(Eq. 71)}$$

On consideration of Eq. 57:

$$K_{T/P_u} = k_{P_u,T}/k_{T,P_u} \quad \text{(Eq. 72)}$$

Substitution of Eqs. 70–72 into Eq. 69 with subsequent rearrangement reaffirms that Eq. 67 properly represents the overall disposition rate constant for the overall loss of drug from the complex of rapidly equilibrating compartments in terms of microscopic rates and equilibrium constants.

If a third compartment can be discriminated for trichloroethanol distribution in the body and the rate of return from this compartment is extremely slow, the observed k_e may be estimated (19) as:

$$k_e = \frac{k_{B,M} + k_{B,T'}}{1 + (k_{B,T}/k_{T,B})} = \frac{k_{P_u,M} + k_{P_u,T'}}{1/\gamma_P{}^u + k_{P_u,T}/k_{T,P_u}} \quad \text{(Eq. 73)}$$

where $k_{B,T'}$ and $k_{P_u,T'}$ are the rate constants for transfer of drug into this "deep" compartment from the central compartment referenced to the total drug and drug not bound to plasma, respectively.

When such a deep compartment, T', exists and when the return of drug to the central compartment becomes significant, Eq. 73 is inadequate for describing the rate-determining rate constant for the disposition of the drug in the body, and the disposition rate begins to mirror the effects of drug return from the deep compartment (19). The presence of the third exponential demonstrated this deep compartment return, and its k_3 value gave a preliminary estimate of $k_{T',B} = k_{T',P_u}$, the rate constant for transfer of drug from the deep compartment into the central compartment.

To describe mathematically the distribution and metabolism of trichloroethanol, it was necessary to obtain the values of the microscopic rate constants that describe the system of Eq. 63 and the apparent volumes of distribution of the equilibrating compartments.

Initial estimates of $k_{B,T}$, $k_{T,B}$, and $k_{B,T'} + k_{B,M}$ were obtained from the parameters of the first two exponentials that characterize the time course of trichloroethanol in the blood as demonstrated in Fig. 2 ($[A_B]_1$, $[A_B]_2$, k_1, and k_2) by their insertion into the equations of Skinner *et al.* (21) as simplified by Riggs (12). The rate constant estimates, in units of reciprocal minutes (min.$^{-1}$), were: $k_{B,T}$ = 0.128, $k_{T,B} = k_{T,P_u}$ = 0.060, and $k_{B,M} + k_{B,T'}$ = 0.058 so that $k_{P_u,T}$ = 0.413 and $k_{P_u,M} + k_{P_u,T'}$ = 0.187. The k_e estimated from these values by use of Eq. 67 was 0.0185 min.$^{-1}$, which was in good agreement with the value of 0.015 min.$^{-1}$ obtained from 2.303 times the slope of the log A_B plot against time during the period characterized by the second exponential in Fig. 2.

The rate constant for trichloroethanol return from the deep tissue was obtained from 2.303 times the slope for the period characterized by the third exponential in Fig. 2 and was $k_{T',B} \sim k_3$ = 0.004 min.$^{-1}$. The fitting of the data with the analog and/or digital computer was then attempted with utilization of these preliminary estimates of the microscopic pharmacokinetic constants.

Analysis of Trichloroethanol Data by Analog Computation[17]—The bases and details of analog computer programming have been described in several excellent texts and brochures (22–24). Applications to fitting the data to complex pharmacokinetic models have also been given (17, 25–29).

The peak height ratio at any time, PHR_t, of trichloroethanol to chlorobutanol per milliliter of whole blood was plotted against time. The ordinate was scaled from 0 to 100%, where 100% of the dose in the blood was taken as the peak height ratio extrapolated to zero time, *i.e.*, $[A_B]_0 = \Sigma[A_0]_i$, and equated to 10 v., so that the amount of trichloroethanol in the blood at any time could be expressed as the percent of the initial dose that appeared in the blood:

$$\%\ TCE_t = \frac{PHR_t}{[A_B]_0} \times 100 \quad \text{(Eq. 74)}$$

on the assumption of instantaneous homogeneity in the central compartment of the intravenously administered drug. The amount of drug lost in the interval t = 0–30 min. (the time period of the first exponential) was fitted by adjusting the ratio $k_{B,T}/k_{T,B}$ while the rate of its initial disappearance was controlled by the magnitude of these constants (26). A preliminary fit of the remaining data was attempted by holding $k_{T',B}$ at 0.004 min.$^{-1}$ and adjusting the magnitudes of $k_{B,T'}$ and $k_{B,M}$ while their sum remained at 0.058 min.$^{-1}$. Minor adjustments in the rate constant values, to account for the interaction of the two curve segments, were then made to achieve a "best" fit of the data.

The time axis potentiometer was set to generate a time function to drive the X–Y recorder such that 2.54 cm. (1 in.) = 1 v. = 40 sec. of machine time. The data were plotted on a 38.1-cm. (15-in.) abscissa with a scale of 2.54 cm. (1 in.) = 40 min. real time. This choice of time scales permitted the values of rate constants to be read directly from the adjusted rate constant potentiometers. A different choice of settings would have necessitated conversions of potentiometer readings to their corresponding rate constant values.

The analog computer fit of the trichloroethanol blood data for Dog D, dosed at 30 mg./kg. and a total dose of 600 mg., is given in Fig. 3.

Analysis of Trichloroethanol Glucuronide Data by Analog Computation—The total amount of free trichloroethanol excreted in the urine was less than 1% of the administered dose. No assayable trichloroacetic acid was found in blood or urine for the intravenous administration of a 30-mg./kg. dose in Dog D. These facts substantiate the basic pharmacokinetic model for trichloroethanol as given in Scheme III and demonstrate that its only significant pathway for removal from the body in the dog is *via* the route of metabolism to its glucuronide. The analog computer model of Scheme III generated amounts of glucuronide with time in terms of a percent of the total dose of trichloroethanol intravenously administered.

[17] The analog computer used was a model TR-10, Electronic Associates, Long Branch, N. J. A recording X–Y plotter, Moseley model 202, manufactured by Hewlett-Packard/Moseley Division, Pasadena, Calif., was used.

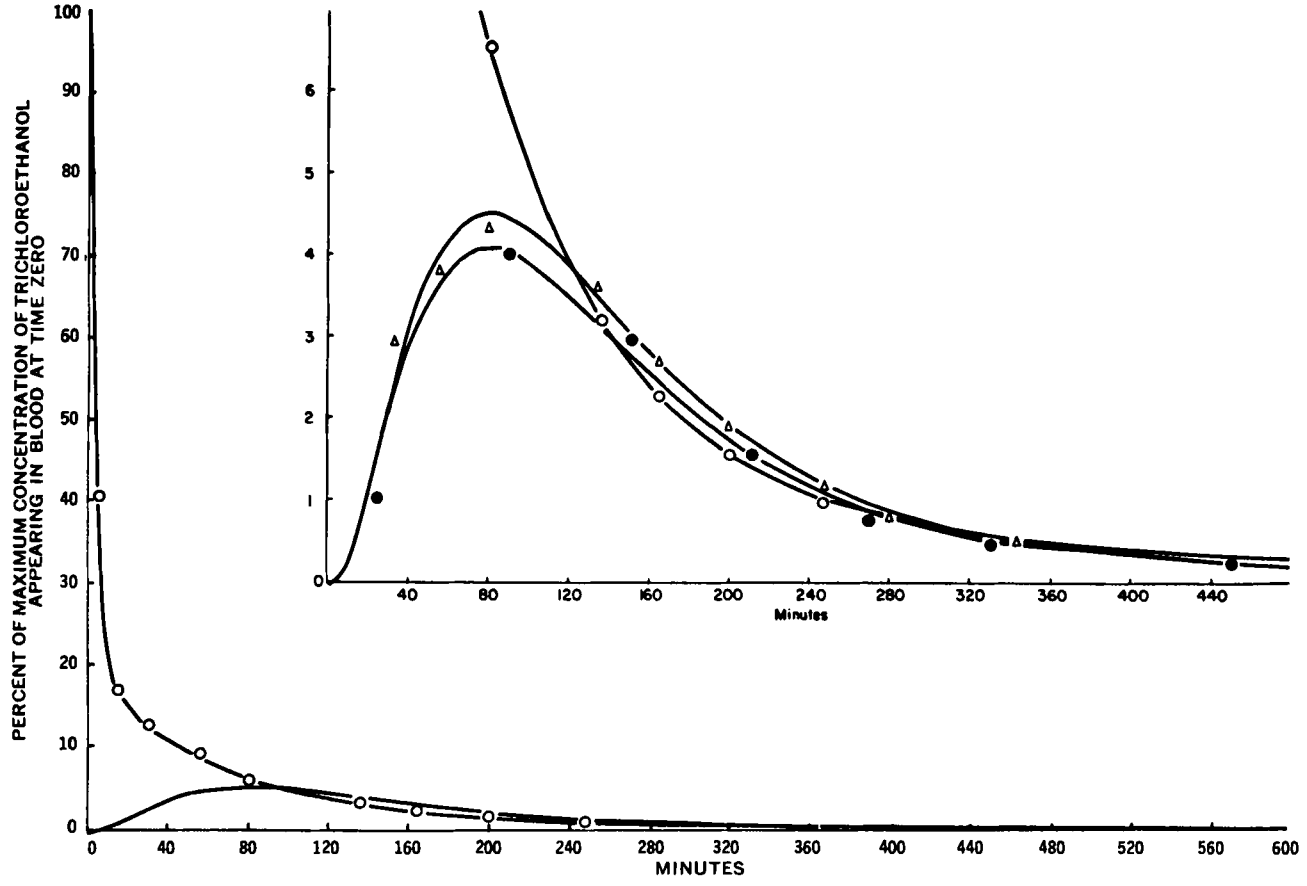


Figure 3—*Analog computer fit of blood and urine data for a dose of 30 mg. trichloroethanol (TCE) per kilogram in the 20-kg. Dog D. The full-scale portion shows the computer fit of the data for trichloroethanol as the percent of the maximum concentration that appears in the blood at zero time* (○), *and the solid line is the predicted trichloroethanol glucuronide in the blood with time. The inset curves are expanded scale plots of the data for trichloroethanol* (○) *and its glucuronide* (△) *with time. The dark circles* (●) *are the urinary excretion rates of trichloroethanol glucuronide (percent dose per minute), which are plotted on a 10-fold smaller scale (0–1%/min.) than that represented on the inset ordinate. The trichloroethanol data were fitted by the program of Scheme V to the specified model by the pertinent microscopic rate constants in Tables I and V, and the computer-generated trichloroethanol glucuronide levels were fitted to the glucuronide data* (△), *which had been multiplied by a factor that represented the ratio of the apparent volumes of distribution of the glucuronide to the trichloroethanol in the central compartment.*

The assayed peak height ratios of trichloroethanol glucuronide to chlorobutanol per milliliter of whole blood were estimated as a percent of the total dose of trichloroethanol administered per volume of whole blood by means of an estimate of the volume of whole blood in the dog on the basis of the preliminary assumption that the glucuronide was only distributed in the central compartment, which was blood. The equation used for this preliminary estimate was:

$$\text{estimated \% of total dose of trichloroethanol as glucuronide in whole blood} = (\text{peak height ratio of glucuronide/chlorobutanol per ml. whole blood}) \times \frac{(8.4 \times 10^{-3}\ \text{mg./peak height ratio})\ (20\ \text{kg.})\ (94\ \text{ml./kg.}) \times 100}{600\ \text{mg.}} \quad \text{(Eq. 75)}$$

where 8.4×10^{-3} mg./peak height ratio is the conversion factor to transform peak height ratios to milligrams of trichloroethanol. The 94-ml./kg. factor has been reported (18) as the whole blood volume of the dog, and 600 mg. is the dose of trichloroethanol administered to Dog D (20 kg.).

The maximum estimated glucuronide in the blood was only 4.2% of the dose. Thus the scale used in the plots of the trichloroethanol was 10-fold, and the estimated percent values as the glucuronide were plotted on the same paper in the expanded range of 0–10% (Fig. 3). This was a reasonable procedure, since the recorder used had a switch that permitted an instantaneous 10-fold amplification of the glucuronide amount in blood generated by the analog computer from an appropriate fitting of the trichloroethanol data. It must be realized that this percent of the dose of trichloroethanol as glucuronide in blood (Eq. 75) was only an estimate of the real situation since the true equilibrated distribution volume, V_{D_B}, of the glucuronide referenced to the total concentration of glucuronide in the blood was not known and only was estimated as being equivalent to the volume of whole blood in the dog. The relationship between the estimated and true values as glucuronide is:

$$\frac{\text{true \% of trichloroethanol dose as glucuronide}}{\text{estimated \% of trichloroethanol dose as glucuronide}} = \frac{[\text{TCE-G}_B] \times V_{D_B}^{\text{true}}}{[\text{TCE-G}_B] \times V_{D_B}^{\text{est}}} = \frac{V_{D_B}^{\text{true}}}{V_{D_B}^{\text{est}}} = \alpha \quad \text{(Eq. 76)}$$

where $[\text{TCE-G}_B]$ is the experimentally observed concentration of glucuronide in whole blood.

The factor α can be estimated from the setting of a variable potentiometer used to multiply the trichloroethanol glucuronide amount in the dog (in terms of percent of trichloroethanol dose as glucuronide) generated from the analog computer fit of the trichloroethanol data in the blood, so that the time course of actual values of glucuronide plotted was fitted when plotted as the estimated values obtained from Eq. 75. This determined α factor would permit a true estimate of the apparent volume of distribution of glucuronide referenced to its total concentration in the blood from a rearrangement of Eq. 75:

$$V_{D_B}^{\text{true}} = \alpha V_{D_B}^{\text{est}} \quad \text{(Eq. 77)}$$

where $V_{D_B}^{\text{est}}$ was taken as 94 ml./kg. (Eq. 76).

The rates of urinary excretion of trichloroethanol glucuronide, $d(\text{TCE-G}_U)/dt$, were determined from the experimental analyses of

total glucuronide in the urine excreted per unit of time in accordance with:

$$\text{urinary excretion rate of glucuronide (\% of dose/min.)} = \frac{100 \times \text{glucuronide excreted in mg. trichloroethanol}}{\text{(dose in mg.) (time interval of excretion in min.)}} \quad \text{(Eq. 78)}$$

These values were plotted on the same graph used for the plotting of estimated glucuronide in the blood as percent of administered trichloroethanol, except that the ordinate was magnified 10-fold to give a range of 0–1% of total trichloroethanol dose excreted per minute (Fig. 3). Since (13):

$$d(\text{TCE-G}_U)/dt = k_e' [\text{TCE-G}_B] \quad \text{(Eq. 79)}$$

the proper multiplication of the amounts of glucuronide in blood as a function of time by a constant k_e' should fit the plotted rates of urinary excretion as a function of time. A selector switch on the recorder permitted instantaneous 10-fold amplification of the generated rate data to conform with the plotted scale. Good fits of both the blood and urine data of glucuronide were necessary before a set of parameters could be said to describe the fate of glucuronide formed from trichloroethanol.

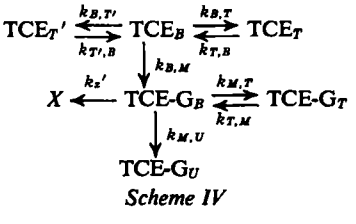
The simplest model for fitting the glucuronide data in blood with the rate constants generated from the fit of trichloroethanol data in blood assumed that the glucuronide appearance in blood reflected trichloroethanol loss as in Scheme III, *i.e.*:

$$d(\text{TCE-G}_B)/dt = k_{B,M}(\text{TCE}_B) \quad \text{(Eq. 80)}$$

If and only if the apparent volumes of distribution of the central compartment referenced to concentrations in blood for both drug, $(V_B)_{TCE}$, and metabolite, $(V_B)_{TCE\text{-}G}$, were the same would this equation be applicable to concentrations. In general:

$$d[\text{TCE-G}_B]/dt = \{(V_B)_{TCE}/(V_B)_{TCE}\} k_{B,M}[\text{TCE}_B] \quad \text{(Eq. 81)}$$

An appropriate expanded model might be as shown in Scheme IV,



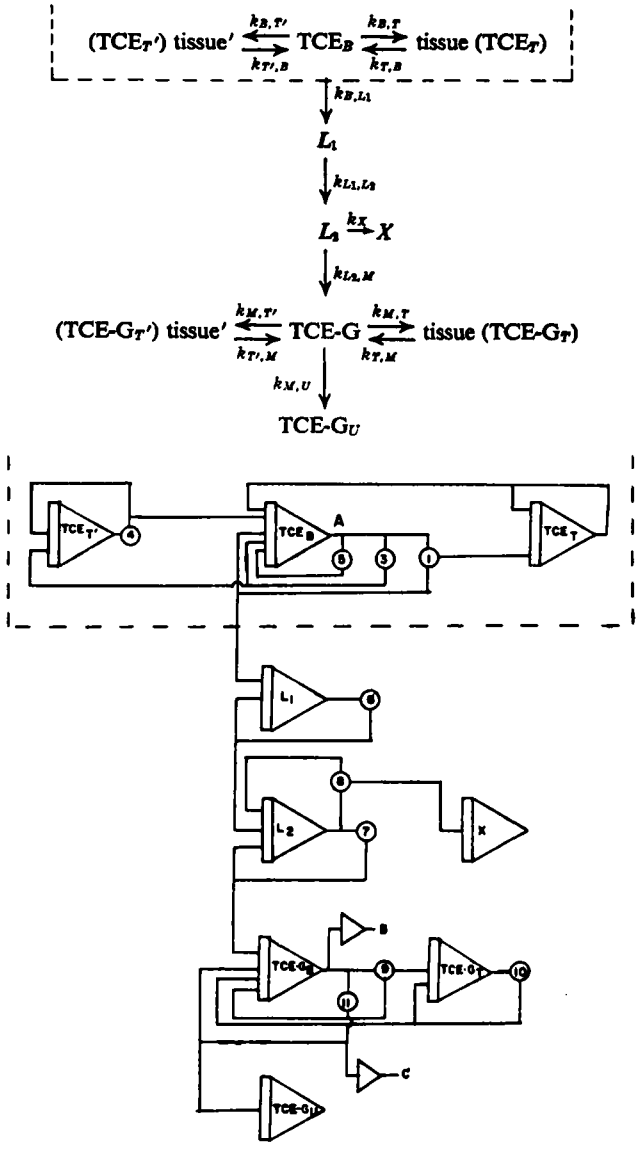
Scheme IV

where the k_e' of Eq. 79 would be defined (13) by the microscopic constants in a manner analogous to Eq. 73 where the glucuronide is rapidly equilibrated with a tissue, TCE-G$_T$, as:

$$k_e' = \frac{k_{M,U} + k_z'}{1 + k_{M,T}/k_{T,M}} \quad \text{(Eq. 82)}$$

where k_z is inserted as a microscopic rate constant for a possible elimination route that occurred simultaneously with the urinary elimination of glucuronide from the body. The urinary excretion rates of glucuronide were always proportional to its concentration in the blood in accordance with Eq. 79. The fact that an intravenous dose of glucuronide was, for all practical purposes, completely excreted into the urine permitted the decision that $k_z' = 0$. The initial estimates of $k_{M,T}$, $k_{T,M}$, and $k_{M,U}$ were obtained from a separate analysis of the pharmacokinetics of the intravenously administered dose of glucuronide.

However, the model of Scheme IV could not be fitted to the glucuronide data plotted as values derived from Eq. 75 against time (Fig. 3). No combination of α values and distribution and elimination rate constants could fit the observed data for glucuronide in blood and urine. The major inconsistencies observed were that the analog computer curves generated from the fits to the trichloroethanol data gave rapid early blood levels, whereas the experimental glucuronide data demonstrated an induction period, a slower rate of glucuronide appearance in the blood, and a broader maximum. It was, therefore, necessary to postulate an additional compartment, L_1, in the model, between trichloroethanol in the blood, TCE$_B$, and the glucuronide in the blood, TCE-G$_B$ (Scheme V), which could serve to accumulate the glucuronide and release it slowly into blood. Although the presence of this compartment improved the fit of calculated to observed data, the lag time required to fit the rate of appearance of glucuronide in blood was still not accommodated. To fit the lag in appearance of glucuronide blood levels while continuing to assume concentration-dependent or first-order processes, it was necessary to insert another compartment, L_2, in the model (Scheme V).



Scheme V—The multicompartmental model and corresponding analog computer program required for the analysis and fitting of pharmacokinetic data. The portions enclosed in dashed lines were used to evaluate trichloroethanol data as analyzed in blood, TCE$_B$, alone while the entire model and program were used to fit trichloroethanol glucuronide data as analyzed in blood, TCE-G$_B$. The potentiometers in the program and their corresponding rate constants in the model were: potentiometer 1, $k_{B,T}$; 2, $k_{T,B}$; 3, $k_{B,T'}$; 4, $k_{T',B}$; 5, k_{B,L_1}; 6, k_{L_1,L_2}; 7, $k_{L_2,M}$; 8, k_x; 9, $k_{M,T}$; 10, $k_{T,M}$; and 11, $k_{M,U}$. Analog computer plots of calculated data were obtained from points A for trichloroethanol in blood, TCE$_B$; B for trichloroethanol glucuronide in blood, TCE-G$_B$; and C for the urinary excretion rate of trichloroethanol glucuronide, d(TCE-G$_U$)/dt.

The presence of compartments L_1 and L_2 (in series between TCE$_B$ and TCE-G$_B$), coupled with an additional compartment (X) to account for the fraction of the dose unaccounted for in urine, allowed for accurate fitting of the glucuronide data in blood and urine in accordance with Scheme V. The analog computer program for this model is also given in the scheme. The portions of the model and program enclosed in dashed lines were used in the preliminary

Table I—Values of Pharmacokinetic Parameters[a] in Dogs Obtained from Computer Fitting of Blood and Urine Data after Intravenous Administration of Trichloroethanol

Dose, mg./kg.	$[A_B]_0$[b]	V_B[c]	V_{P^u}[d]	$V_{T_{P^u}}$[e]	$V_{T'_{P^u}}$[f]	$k_{B,T}$	$k_{T,B}$	$k_{B,T'}$	$k_{T',B}$	k_{B,L_1}	k_{L_1,L_2}	$k_{L_1,M}$	k_e
							Dog A (16 kg.)						
10	0.034	2.94	3.27	14.6	18	0.155 (0.023)	0.112 (0.009)	0.022 (0.003)	0.013 (0.0004)	0.075 (0.008)	0.184	0.018	0.002
50				14.2	22	0.172 (0.049)	0.127 (0.069)	0.039 (0.012)	0.019 (0.001)	0.061 (0.014)	0.160	0.021	0.002
							Dog B (18.6 kg.)						
10	0.030	3.33	3.68	21.6	6	0.069	0.038	0.005	0.009	0.061	0.140	0.016	0.002
25				21.6	6	0.069 (0.012)	0.038 (0.008)	0.005 (0.009)	0.009 (0.001)	0.061 (0.007)	0.140	0.016	0.002
100				19.8	5	0.065 (0.019)	0.039 (0.008)	0.005 (0.006)	0.010 (0.003)	0.039 (0.006)	0.013	0.023	0.001
							Dog C (10 kg.)						
10	0.044	2.27	2.83	13.9	31	0.122 (0.093)	0.080 (0.021)	0.009 (0.004)	0.003 (0.001)	0.070 (0.030)	0.208	0.023	0.002
50				15.7	36	0.186	0.108	0.007	0.002	0.084	0.140	0.019	0.001
100				11.6	36	0.165	0.130	0.007	0.002	0.047	0.025	0.014	0.001
176				16.1	27	0.186	0.106	0.005	0.002	0.033	0.015	0.013	0.001
							Dog D (18 kg.)						
17	0.031	3.22	3.54	22.2	35	0.124 (0.002)	0.064 (0.009)	0.013 (0.002)	0.004 (0.0007)	0.059 (0.001)	0.030 (0.010)	0.022 (0.004)	0.008 (0.003)
50				20.0	19	0.147 (0.020)	0.084 (0.012)	0.009 (0.001)	0.005 (0.0006)	0.045 (0.001)	0.030	0.022	0.008
100				19.6	19	0.161 (0.040)	0.094 (0.011)	0.007 (0.001)	0.004 (0.0003)	0.038 (0.005)	0.010	0.019	0.006
150				19.6	21	0.161 (0.040)	0.094 (0.011)	0.004 (0.0004)	0.002 (0.0007)	0.034 (0.0008)	0.010	0.019	0.006
25[g]				19.2	21	0.158 (0.007)	0.039 (0.006)	0.010 (0.009)	0.005 (0.0009)	0.068 (0.001)	0.032	0.022	0.008
30[g]				28.7	28	0.151 (0.026)	0.060 (0.012)	0.008 (0.003)	0.003 (0.007)	0.050 (0.008)	0.040	0.019	0.006
							Dog E (10 kg.)						
60	0.042	2.38	3.03	10.4	20	0.119	0.112	0.013	0.007	0.063	—	—	—
60[h]				10.4	20	0.119	0.112	0.013	0.007	0.063	—	—	—
60				12.5	19	0.202	0.158	0.021	0.012	0.075	—	—	—
60[i]				11.0	15	0.129	0.115	0.026	0.018	0.035	—	—	—

[a] The microscopic rate constants are defined in Scheme V and are given in minutes^{-1}. The values of $k_{M,T}$, $k_{T,M}$, $k_{M,T'}$, $k_{T',M}$, and $k_{M,U}$ were 0.133, 0.190, 0.008, 0.017, and 0.069 min.$^{-1}$, respectively, and were obtained from the separate studies on trichloroethanol glucuronide pharmacokinetics. The numbers in parentheses are standard deviations of the stated parameters obtained by digital computer fitting with the SAAM program. [b] The computer-fitted zero time concentration of trichloroethanol in blood, $[A_B]_0$, in terms of percent of dose per milliliter of whole blood. The $[A_B]_0$ values were reasonably coincident for all doses in a given dog (Fig. 8). [c] The apparent volume of distribution of the central compartment referenced to total concentration in blood is $V_B = 100\%/[A_B]_0$ in liters. [d] The apparent volume of distribution referenced to the concentration of trichloroethanol in the plasma unbound to protein is $V_{P^u} = V_B\,(1.792) - 1.24\,V_{B_{true}}$ after Eq. 34, where $H = 0.45$, $D = 2.1$, $f = 0.35$, and $V_{B_{true}} = 0.1 \times$ weight of dog in kilograms (16). [e] The apparent volume of distribution referenced to the concentration of unbound trichloroethanol in the plasma of the rapidly equilibrating compartment, T, in liters, where $V_{T_{P^u}} = V_{P^u} - (k_{B,T}/\gamma_{P^u} k_{T,B})$. [f] The apparent volume of distribution referenced to the concentration of unbound trichloroethanol in the plasma of the slowly equilibrating compartment, T', in liters, where $V_{T'_{P^u}} = V_{P^u} - (k_{B,T'}/\gamma_{P^u} k_{T',B})$. [g] The dog weights in these studies were 20 kg. [h] Trichloroethanol, 60 mg./kg. or 600 mg., was administered initially. At 250 min. later, 100 mg. of sulfobromophthalein (10 mg./kg.) was administered concurrently with an additional dose of 600 mg. of trichloroethanol (60 mg./kg.) and an intravenous drip of 0.6 mg. sulfobromophthalein/min. was started and continued until 490 min. [i] Trichloroethanol, 60 mg./kg. or 600 mg., was administered initially. At 320 min. later, 500 mg. of sulfobromophthalein (50 mg./kg.) was administered concurrently with an additional dose of 600 mg. of trichloroethanol (60 mg./kg.) and an intravenous drip of 3 mg. sulfobromophthalein/min. was infused until 500 min.

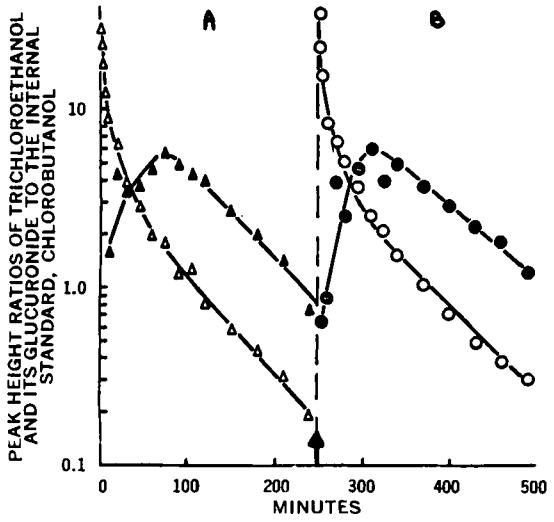


Figure 4—*Semilogarithmic plots of the peak height ratios of trichloroethanol (△,○) and trichloroethanol glucuronide (▲,●) as representative of blood levels with time in the absence and presence of sulfobromophthalein for the 10-kg. Dog E. Segment A is a consequence of dosing 60 mg./kg. of trichloroethanol at zero minute. Segment B is a consequence of dosing 60 mg./kg. of trichloroethanol and 10 mg./kg. of sulfobromophthalein at 250 min. A peak height ratio of 1.0 is equivalent to 8.4 mcg. trichloroethanol or 18.3 mcg. trichloroethanol glucuronide/ml. of whole blood.*

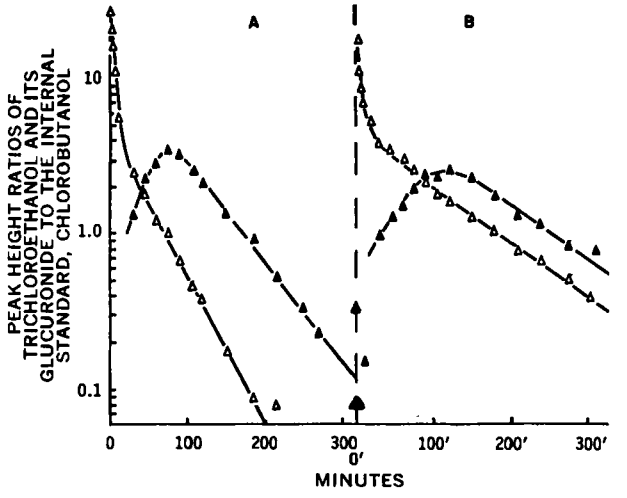


Figure 5—*Semilogarithmic plots of the peak height ratios of trichloroethanol (△) and trichloroethanol glucuronide (▲) as representative of their respective blood levels with time in the absence and presence of sulfobromophthalein for the 10-kg. Dog E. Segment A is a consequence of dosing 60 mg./kg. of trichloroethanol at zero minute. Segment B is a consequence of dosing 60 mg./kg. of trichloroethanol and 50 mg./kg. of sulfobromophthalein at 320 or 0' min. A peak height ratio of 1.0 is equivalent to 8.4 mcg. trichloroethanol or 18.3 mcg. trichloroethanol glucuronide/ml. whole blood.*

fit of the trichloroethanol data in the blood, where k_{B,L_1} is the rate constant given previously as $k_{B,M}$ (Schemes III and IV).

The placement in the multicompartmental model of k_X, a rate constant for elimination of the missing drug fraction, was arbitrary since the route of elimination was unknown. The urinary recovery, for all practical purposes, of all of an intravenous dose of the glucuronide indicated that the TCE-G_B compartment would not be a logical place to remove a fraction of the dose with a rate constant, k_x. Since postulation of this elimination route from the trichloroethanol in the blood, TCE_B, would alter those parameters previously obtained for the loss of trichloroethanol, it was decided that the missing fraction of drug exited from compartment L_2. The fact that an amount of the trichloroethanol dose is secreted into bile as the glucuronide made this choice consistent with physiological reality.

The ratio, $k_X/k_{L_2,M}$, could be estimated (13) from:

$$\frac{k_X}{k_{L_2,M}} = \frac{100 - (\%\ \text{TCE-G}_U)_\infty}{(\%\ \text{TCE-G}_U)_\infty} \qquad \text{(Eq. 83)}$$

where $(\%\ \text{TCE-G}_U)_\infty$ is the total percent of the trichloroethanol dose excreted in the urine.

Analysis of Data by Digital Computation—The major disadvantage of the analog computer in data analysis is that the goodness of fit must be evaluated visually. Statistical determination of the agreement between calculated and observed points would demand a nonlinear regression analysis for every fit. Fortunately, programs are available for the digital computer that include such statistical analysis along with methods to evaluate pharmacokinetic parameters. The SAAM (Simulation, Analysis, and Modeling) program of Berman and Weiss (30) is such a program and was used on a digital computer[18] to fit the pharmacokinetic data.

SAAM is a general-purpose computer program designed to fit physical or mathematical models to data by adjusting the parameter values of the model until a "best" fit is obtained. Any set of mathematical equations (differential, integral, or algebraic) or functions may serve as a model provided an analytical or numerical procedure exists for its solution.

Since the model for trichloroethanol pharmacokinetics (Scheme III) assumed first-order transfers among all compartments, the equations describing the system were linear differential equations with constant coefficients. The equations are solved by the SAAM program with the fourth-order approximative technique of Runge-Kutta (31).

Data to be analyzed were punched out on IBM cards. The data for trichloroethanol in blood were given in terms of percent of total dose, where 100% was the extrapolated zero-time blood concentration (Eq. 74).

Estimates of the rate constants needed to fit the data (lambdas in SAAM program terminology) were those obtained with the preliminary analog computer data fit (Scheme V). These rate constants required iterative adjustment to obtain a least-squares fit of the data. A variable proportionality factor, kappa, was also included within the program to adjust the data in the case of a poor estimate for the initial volume of distribution. If the initial rate constant (lambda) estimates did not provide a good statistical fit of the data, the SAAM program increased or decreased the data by a constant factor, kappa (hence decreasing or increasing the volume of distribution), while simultaneously adjusting the lambda values until the best statistical fit was achieved.

To specify that a parameter be subject to iterative adjustment, upper and lower limits were assigned to each kappa and lambda estimate in the model. Lower limits for lambdas were zero (0.0) while upper limits were twice the value of the initial estimate. Upper and lower limits for kappas were 0.5 and 2.0, twice and one-half the initially estimated volume of distribution, respectively.

A routine printout was produced for every problem deck submitted to SAAM and included: a listing of the problem deck with each card printed as reformatted by SAAM, a computer language reorganization of the information in the program deck, parameter values and a table of the initial solution values (zero iteration), and results for each iteration associated with the fitting of the data and final results including parameter values and the corresponding solution table for the best fit with estimated standard errors and correlation coefficients. An optional arithmetic plot of calculated and observed data could be requested and was used in all trichloroethanol pharmacokinetic analyses.

The final parameter values obtained with the SAAM program were $k_{B,T} = 0.1506$, $k_{T,B} = 0.0602$, $k_{B,T'} = 0.0082$, $k_{T',B} = 0.0030$, and $k_{B,L_1} = 0.0505$ for the 30-mg./kg. dose given Dog D. A kappa value of 1.30 was required to fit the data. This kappa served to raise the calculated initial trichloroethanol concentration from 0.142 mg. trichloroethanol/ml. of blood (or 0.0237 as percent of total dose per milliliter of blood) to 0.185 mg./ml. blood (or 0.031 as percent of total dose per milliliter blood) and hence changed the calculated apparent volume of distribution, V_B, referenced to total concentration in the blood (Eq. 24) from 4.24 to 3.26 l. Thus, the

[18] IBM model 360/50.

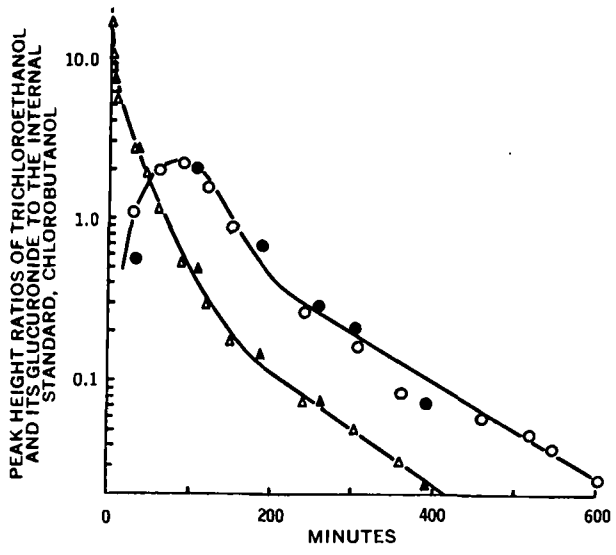


Figure 6—*Semilogarithmic plots of the peak height ratios to the internal standard chlorobutanol of trichloroethanol and trichloroethanol glucuronide blood concentrations with time for a 50-mg./kg. trichloroethanol dose in the absence and presence of pentobarbital anesthesia. The open circles and triangles represent the trichloroethanol and trichloroethanol glucronide blood concentrations for the first dose of trichloroethanol given to the dog in the absence of pentobarbital anesthesia. The solid circles and triangles are the trichloroethanol and trichloroethanol glucuronide data for an identical dose of trichloroethanol given 7 days later to the dog anesthetized with 30 mg./kg. pentobarbital. A peak height ratio of 1.0 is equivalent to 8.4 mcg. trichloroethanol or 18.3 mcg. trichloroethanol glucuronide/ml. whole blood.*

apparent volume of distribution referenced to the concentration of unbound trichloroethanol in the plasma, V_P^u, is 3.37 l. (Eq. 34).

It also follows that the apparent volumes of distribution of the tissue compartments referenced to the unbound drug concentration in plasma are $V_{T_P}^u$ = 26.1 l. from Eq. 59 and $V_{T'_P}^u$ = 29.7 l. from Eq. 60. These are in decided contrast to the apparent volumes of distribution of the tissue compartments if they were referenced to the total drug concentration in blood, since V_{T_B} = 8.1 l. and $V_{T'_B}$ = 8.8 l.

RESULTS AND DISCUSSION

Sensitivity and Reliability of Analytical Methods—A typical chromatogram for a 5-μl. aliquot of the ether extract of a solution of trichloroethanol and its glucuronide, chloral hydrate, and trichloroacetic acid is given in Fig. 1. The dashed line over the trichloroethanol chromatographic peak was the peak observed after 48 hr. of hydrolysis with β-glucuronidase when the glucuronide was present in the sample. It includes free and conjugated trichloroethanol.

The trichloroethanol produced by the β-glucuronidase- (1 mg.) catalyzed hydrolysis of its glucuronide in urine was monitored gas chromatographically as a function of time. A plot of the logarithm of the difference between the peak height ratio (trichloroethanol to chlorobutanol) at infinite time and the peak height ratio at a time, t, against that time was linear and demonstrated a first-order hydrolysis of the glucuronide. The half-life of the solvolysis of glucuronide under the conditions specified in this paper was obtained from the slope of this plot and was 8 hr., so that 98% hydrolysis would be effected after 48 hr. of incubation at 37°.

Concentrations as low as 0.5 mcg. of trichloroethanol, its glucuronide, or chloral hydrate (3.3, 1.54, or 3.0 μmoles, respectively) in 3.0 ml. of water, urine, or blood were assayable. The sensitivity for trichloroacetic acid was 5 mcg. (31 μmoles) in 3.0 ml. Linear calibration curves were obtained for all compounds over a 20-fold concentration range (7). Assay values (trichloroethanol–chlorobutanol peak height ratios) from the trichloroethanol "spiked" blood of four different dogs showed a maximum deviation of less than 3%. Deviation of 2–4.8% resulted between refrigerated blood samples stored up to 168 hr. and calibration standards.

***In Vivo* Inhibition of Trichloroethanol Glucuronidation by Sulfobromophthalein**—The intravenous administration of 10 mg./kg. of sulfobromophthalein concurrently with a 60-mg./kg. dose of trichloroethanol and subsequent infusion of 0.6 mg./min. sulfobromophthalein did not result in any alteration in the pharmacokinetic profile of trichloroethanol and its glucuronide in Dog E when the data were compared with those obtained for an equal dose of trichloroethanol with no sulfobromophthalein administered 4 hr. previously (Fig. 4 and Table I). When the experiment was repeated and the dose of sulfobromophthalein was increased in Dog E to 50 mg./kg., with a subsequent infusion of 3 mg./min. of sulfobromophthalein, a significant decrease in the rate of loss of trichloroethanol from the blood was observed (Fig. 5 and Table I).

Possible Interaction of Pentobarbital Anesthesia with Trichloroethanol Pharmacokinetics—The use of pentobarbital anesthesia in the pharmacokinetic studies of trichloroethanol and its metabolites in the dog was necessitated by the animal's hyperexcitability and difficulty in handling without initial sedation prior to drug administration. However, studies were made in the same animal (Dog E) with the same trichloroethanol doses (50 mg./kg.) with and without pentobarbital anesthesia. The plotted data (Fig. 6) demonstrated no apparent difference in the time course of trichloroethanol or its glucuronide in blood, a valid indication that pentobarbital anesthesia had no significant effect on the pharmacokinetics of trichloroethanol and its glucuronide.

Hepatic Storage of Trichloroethanol and/or Its Glucuronide—The portal and hepatic veins were sampled with time and assayed for trichloroethanol and its glucuronide in the dog that was surgically modified (11) so that the vena cava was occluded and the vena caval flow did not dilute the hepatic output. The trichloroethanol extracted by the liver per milliliter of blood, $[TCE]_L$/ml., was determined from the subtraction of the trichloroethanol concentration in the hepatic vein blood from the concentration in the portal vein blood. The trichloroethanol glucuronide per milliliter of blood, $[TCE\text{-}G]_L$/ml., released by the liver was determined from the subtraction of the glucuronide concentration (as trichloroethanol) in the portal vein blood from the concentration in the hepatic vein blood.

The difference in these two values, $[TCE]_L$/ml. − $[TCE\text{-}G]_L$/ml., represents the storage of trichloroethanol and/or its glucuronide in the liver in terms of concentration per milliliter of blood. When this difference is multiplied by an estimate of the hepatic blood flow, 600 ml./min. for a 15-kg. dog (32), the net amount of trichloroethanol and/or its glucuronide extracted per minute from the hepatic blood is estimated. A plot of such data against time (Fig. 7) indicates that significant accumulation of trichloroethanol and/or its glucuronide in the liver is terminated within an hour of drug administration; *i.e.*, the liver may be considered saturated with respect to the drug and/or its metabolites. When blood hepatic and portal vein samples were analyzed at 90 min., the process was reversed. The amount of glucuronide released by the liver exceeded the trichloroethanol extracted or transformed by the liver so that it can be concluded that the accumulated liver storage of trichloroethanol and/or its glucuronide was being depleted.

The insert of Fig. 7 plots the area under the estimated curve of Fig. 7 against time and thus represents the cumulative amount of trichloroethanol and/or its glucuronide retained by the liver with time. Since the initial rates of drug and/or metabolite retention were estimated from a presumed linear relation with time, the maximum amount of drug and/or metabolite retention in the insert of Fig. 7 is undoubtedly underestimated.

Trichloroethanol Distribution in Dogs—The fractions of trichloroethanol and its glucuronide bound to protein were based on the equilibrium dialysis experiments with dog plasma and calculated from:

$$\text{fraction bound} = \frac{\text{concentration of drug inside dialysis bag} - \text{concentration outside}}{\text{concentration of drug inside dialysis bag}} \quad \text{(Eq. 84)}$$

The values obtained (Table II) were in agreement with those reported by Marshall and Owens (5) for trichloroethanol binding by reconstituted lyophilized human plasma. Control experiments (dialysis in the absence of plasma) gave no evidence that trichloroethanol or its glucuronide was bound by the dialysis sac. Approxi-

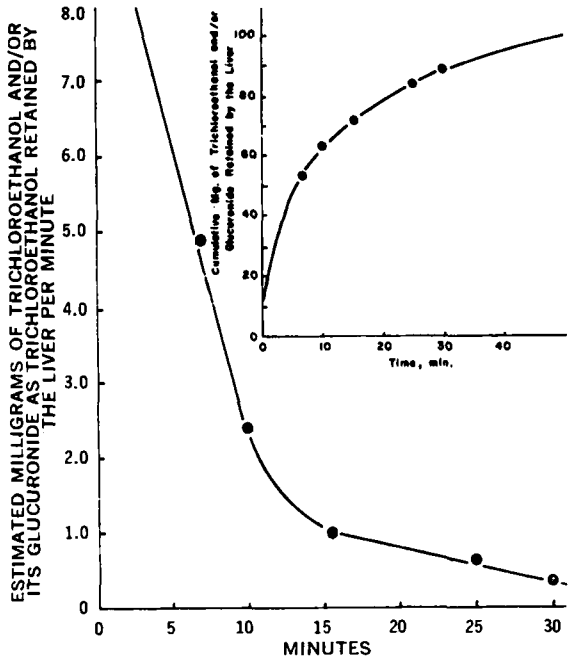


Figure 7—*Estimated amount of trichloroethanol as itself or as its glucuronide that is stored in the liver per minute after injection of a 50-mg./kg. dose of trichloroethanol in the hepatic portal vein in a dog that was surgically modified so that the vena cava was occluded and the vena caval flow did not dilute the hepatic output. The differences in the simultaneously assayed concentrations in milligrams per milliliter of trichloroethanol plus its glucuronide of the hepatic and portal vein bloods were multiplied by an estimated hepatic blood flow of 600 ml./min. to estimate the milligrams of trichloroethanol and/or its glucuronide per minute accumulated in the liver. At 90 min., the glucuronide released by the liver exceeded the trichloroethanol extracted or transformed by the liver. The inset is the cumulative curve for the milligrams of drug and/or its metabolite retained by the liver with time.*

metely 35% of the trichloroethanol found in plasma was bound to protein, and this percent was independent of the plasma concentration of trichloroethanol (Table II). Fifty-three percent of the total trichloroethanol in the entire blood was instantaneously distributed into red blood cells; 16% was bound to plasma proteins and 31% was free (Eqs. 20–22).

The partition coefficient for the ratio of drug concentration in the red blood cells to the drug concentration in plasma unbound to protein, D (Eq. 8), was calculated from Eq. 12 and was taken as 2.1 (Table III). The determined hematocrit, H, was 0.45.

Table II—Data for Estimation of Binding of Trichloroethanol and Its Glucuronide to Plasma Proteins

Compound	Micrograms per Milliliter[a]	Equilibrated Concentration inside Sac[b]	Equilibrated Concentration outside Sac[a]	Percent Bound[d]
Trichloroethanol	5	0.89	0.56	37.4[e]
	50	9.5	5.8	38.7[e]
	500	106.7	70.6	33.8[d]
Trichloroethanol glucuronide	9	0.67	0.4	36.2
	45	2.9	2.2	25.3

[a] Concentrations in 10.0 ml. of pH 7.4 phosphate buffer dialyzed against 2 ml. of dog plasma inside the dialysis sac. [b] Given in terms of trichloroethanol–chlorobutanol peak height ratio, $(TCE/CB)_t$, per milliliter of plasma. [c] Given in terms of trichloroethanol–chlorobutanol peak height ratio, $(TCE/CB)_0$, per milliliter of pH 7.4 phosphate buffer. [d] Percent bound = $100\ [(TCE/CB)_t - (TCE/CB)_c]/(TCE/CB)_t$. [e] The percent errors for total trichloroethanol in the plasma–buffer dialyses *versus* the buffer–buffer controls were 6, 6.7, and 9.4%.

Table III—Red Blood Cell–Plasma Partition Coefficients (D) for Trichloroethanol and Its Glucuronide

Compound	Micrograms per Milliliter	Concentration[a] Whole Blood	Concentration[a] Plasma	D[b]
Trichloroethanol	0.294	0.03	0.02	2.1
	2.94	0.32	0.28	2.0
	47.7	4.68	3.30	2.9[c]
	26.7	3.18	2.17	3.1[c]
	34.0	4.03	3.29	2.3
Trichloroethanol glucuronide	29.1	1.87	3.57	—[d]

[a] Given in terms of trichloroethanol–chlorobutanol peak height ratios per milliliter. [b] Calculated from Eq. 12 for the ratio of concentration of drug in the red blood cells to the concentration of unbound drug in plasma as defined in Eq. 8. [c] Blood samples from a dog that had received 50 mg./kg. of trichloroethanol and 50 mg./kg. of sulfobromophthalein. [d] A value of −0.07 was obtained for the trichloroethanol glucuronide.

The initial apparent volumes of distribution of trichloroethanol referenced to the concentration in whole blood, V_B, and to the unbound concentration in plasma, $V_P{}^u$, were reasonably consistent among the animals studied at all doses (Tables I and IV). The apparent volumes of distribution of the central compartment referenced to the concentration of unbound drug in plasma were approximately equivalent to the extracellular body water of the dog, *i.e.*, 200–350 ml./kg. (18). Thus, it may be assumed that the diffusion of trichloroethanol into the extracellular body water occurred during its first cycles through the circulatory system and the kinetics of its distribution could not be readily observed.

Table IV shows that the apparent volume of distribution of the central compartment which was equated to extracellular water was significantly larger for small dogs (10 kg.) compared to larger dogs (16–20 kg.) on a milliliter per kilogram basis.

The compartment, T (Scheme V), that readily equilibrated with the central compartment had an apparent volume of distribution referenced to the concentration of unbound drug in plasma, $V_{T_P}{}^u$, of 4–6 times the apparent volume of the central compartment similarly referenced (Table IV). Since the total body water is only about 2.5 times that of the extracellular body water (500–750 ml./kg.) in the dog (18), it is implied that trichloroethanol in the central compartment readily equilibrates with lipoidal tissues as well as total body water. The presence of a deep compartment, T', of relatively large capacity in most instances (Table IV) implicates a

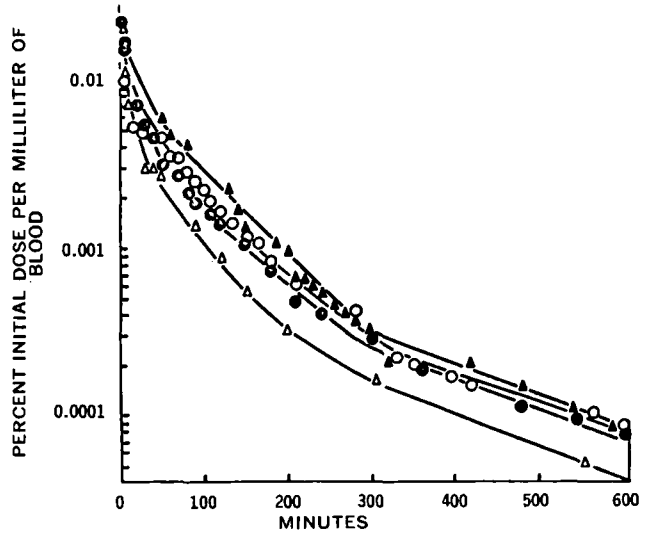


Figure 8—*Semilogarithmic plot of trichloroethanol blood concentrations (as percent initial dose per milligrams whole blood) with time for doses of 17 (△), 50 (●), 100 (○), and 150 (▲) mg. trichloroethanol/kg. in Dog D. The data points were calculated by dividing the trichloroethanol concentration (milligrams per milliliter) by the dose (milligrams) and multiplying by 100.*

Table IV—Range of Apparent Volumes of Distributions for Trichloroethanol (ml./kg.) for All Doses in Dogs

	Dog				
	A	B	C	D	E
Weight, kg.:	16	18.6	10	18	10
V_B	184	180	227	180	240
V_{P^u}	204	198	283	197	303
$V_{T_{P^u}}$	900 ± 10	1130 ± 50	1430 ± 200	1200 ± 100	1100 ± 100
$V_{T'_{P^u}}$	1250 ± 120	310 ± 20	3300 ± 400	1300 ± 300	1800 ± 200

slowly equilibrating, nonvascularized depot in the body which is also most probably lipoidal.

Effect of Dose on Trichloroethanol Pharmacokinetic Parameters in Dogs—Typical semilogarithmic plots of the trichloroethanol per milliliter of whole blood *versus* time for different intravenous doses in a female mongrel dog, Dog D, are given in Fig. 8. The data are plotted as the percent of dose per milliliter of whole blood:

$$\% \text{ dose/ml. whole blood} = \frac{\text{mg. trichloroethanol/ml. whole blood}}{\text{dose in mg.}} \times 100 \quad \text{(Eq. 85)}$$

which normalizes the data for different doses of trichloroethanol to a common scale. If the parameters for drug disposition were independent of dose, the data of Fig. 8 should be superimposable for all doses. This was not so and was typical of the studies in all dogs. Thus, it must be concluded that the pharmacokinetics of trichloroethanol are dose dependent.

Typical semilogarithmic plots for the appearance and subsequent loss of trichloroethanol glucuronide in blood as a function of time after intravenous administration of trichloroethanol are given in Fig. 9 for Dog C. The data are presented as the peak height ratios of trichloroethanol to chlorobutanol per milliliter of whole blood, where a peak height ratio of 1.0 is equal to 19.5 mcg. of the sodium salt of trichloroethanol glucuronide per milliliter of whole blood.

Similar data for Dog D, but given as the percent of the initial dose per milliliter of whole blood *versus* time, are depicted in Fig. 10. The effect of an increasing trichloroethanol dose on the pharmacokinetics of the glucuronide is to delay and broaden the maximum in glucuronide blood level (Figs. 9 and 10). The height of the maximum in terms of percent of the dose is elevated with the increased dose of trichloroethanol (Fig. 10).

The pharmacokinetic parameters of rate constants and volumes of distribution, which describe the distribution and metabolism of trichloroethanol and the appearance, distribution, and urinary elimination of its glucuronide after intravenous administration of different doses of trichloroethanol, are found in Table I for Dogs A–D. Typical analog computer curves generated from the rate constants of Table I are given for widely varied trichloroethanol doses (10 and 176 mg./kg.) in Figs. 11 and 12 for the data of Dog C.

The rate constant, k_{B,L_1} (Scheme V), for the loss of trichloroethanol to the glucuronide decreased as a function of dose in Dogs A–D (Table I and Fig. 13). The dogs formed two groups of similar k_{B,L_1} values, the rate constants for loss of trichloroethanol from the central compartment at an infinitely small trichloroethanol dosage. Curvilinear extrapolation of the data from the two lean dogs, A and C (10 and 16 kg., respectively) gave an estimated k^0_{B,L_1} of 0.086 min.$^{-1}$, while a value of 0.069 min.$^{-1}$ was obtained for the two obese dogs, B and D (18 and 18.6 kg., respectively) (Fig. 13).

The decrease of the rate constant, k_{B,L_1}, for the loss of trichloroethanol to glucuronide with dose is not unique. Similar dose dependency of apparent first-order disappearance of drug from a central compartment was reported for dicumarol (33), ethyl biscoumacetate (34), probenecid (35), and diphenylhydantoin and phenylbutazone (36). As yet, no satisfactory mechanism has been advanced.

A possible explanation is an *in vivo* saturation by a substrate of a metabolic pathway, as described by Levy (37) for salicylate. Probenecid (38) and diphenylhydantoin (39), which also demonstrate dose-dependent pharmacokinetics, also have been reported to be metabolized by glucuronidation. The coumarin derivatives and phenylbutazone have structures that suggest metabolism by the same route. Chloral hydrate (5 m*M*) has been reported to give 50% inhibition of microsomal *O*-aminophenol conjugation (40).

Sulfobromophthalein was shown to be a potent inhibitor of glucuronidation in *in vitro* experiments with rabbit liver homogenate (40). When 10 mg./kg. of this substance was administered simultaneously with 60 mg./kg. of trichloroethanol, no significant change in k_{B,L_1} was observed from the case when sulfobromophthalein was not administered (Dog E, Table I and Fig. 4), even though the sulfobromophthalein dose used was twice that recommended for studies on hepatic function (10). However, when the sulfobromophthalein dose administered simultaneously with 60 mg./kg. of trichloroethanol was increased to 50 mg./kg., a significant 46% decrease in

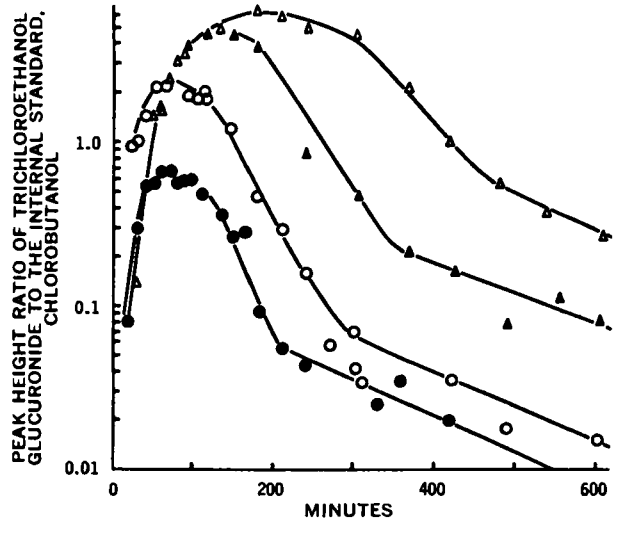


Figure 9—*Semilogarithmic plot of the gas chromatographically obtained peak height ratios for trichloroethanol as trichloroethanol glucuronide to the chlorobutanol internal standard with time for doses of 10 (●), 50 (○), 100 (▲), and 176 (△) mg. trichloroethanol/kg. in Dog C. A peak height ratio, TCE-G/CB, with respect to chlorobutanol (CB) of 1.0 is equivalent to 18.3 mcg. of trichloroethanol glucuronide (or 8.4 mcg. of trichloroethanol)/ml. of whole blood.*

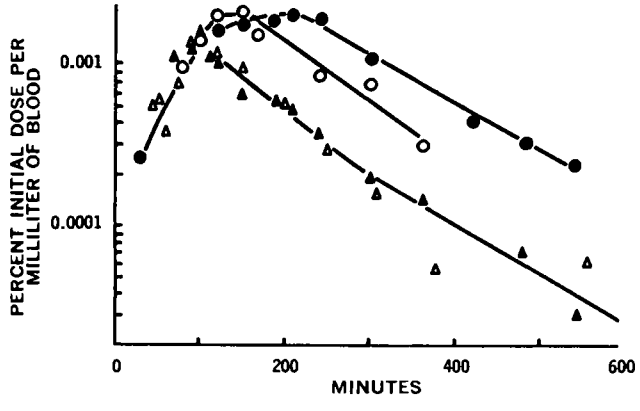


Figure 10—*Semilogarithmic plot of trichloroethanol glucuronide blood levels (as percent initial trichloroethanol dose per milliliter whole blood) with time for doses of 17 (△), 50 (▲), 100 (○), and 150 (●) mg./kg. of trichloroethanol in Dog D. The data points were calculated by converting the glucuronide concentrations to trichloroethanol concentrations (1 mg. glucuronide/ml. = 0.47 mg. trichloroethanol/ml.), dividing by the dose (milligrams), and multiplying by 100.*

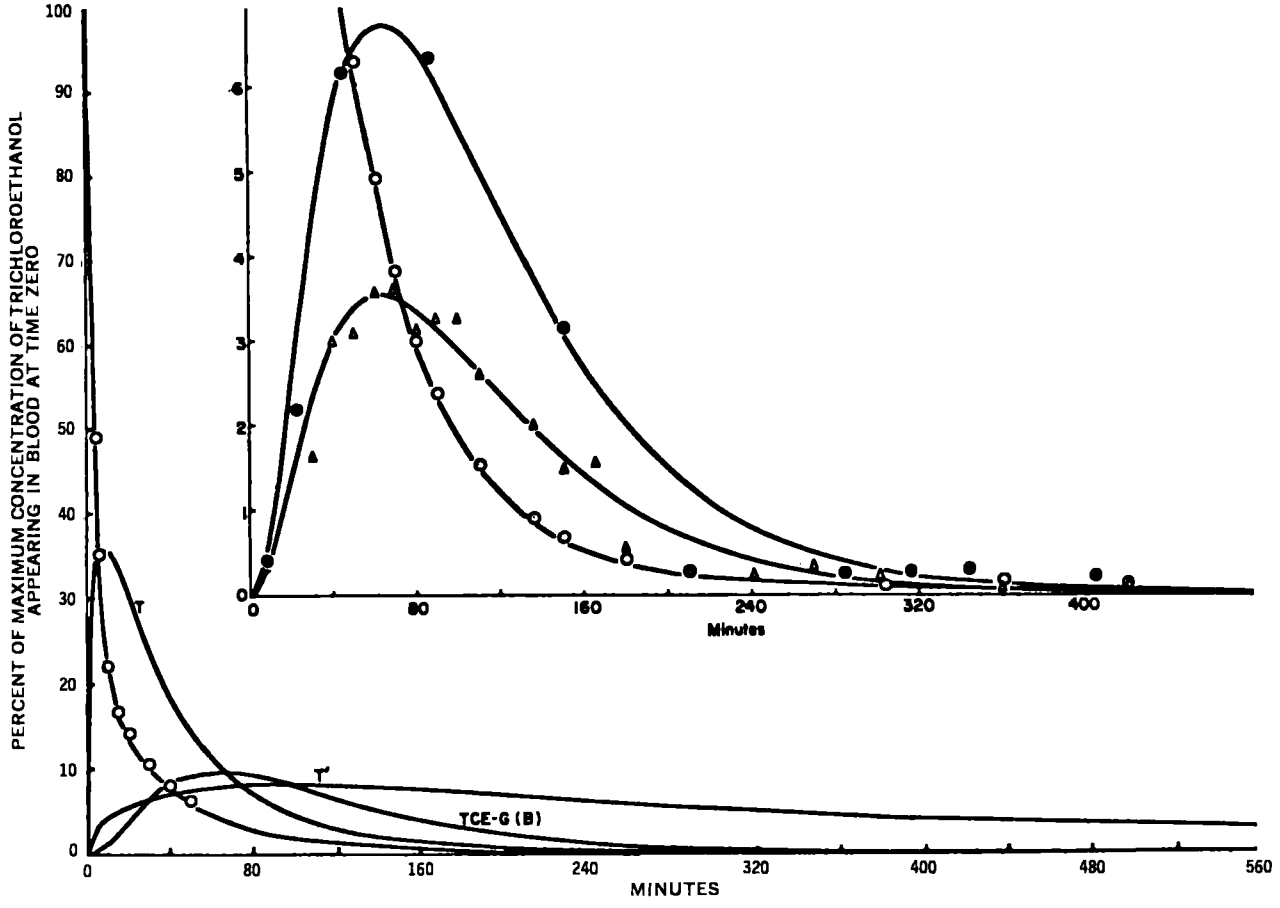
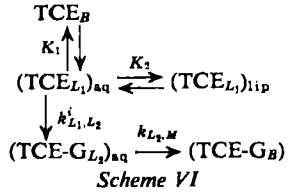


Figure 11—*Analog computer fits of the blood and urine data for a dose of 10 mg. trichloroethanol/kg. in the 10-kg. Dog C. The full-scale portion shows the fit of the data with time for trichloroethanol as the percent of the maximum concentration that appears in the blood at zero time* (○), *as well as the computer curves generated for trichloroethanol in the distribution compartments* T *and* T′ (*Scheme V*). *The remaining curve is for the calculated amount of trichloroethanol glucuronide* (*TCE-G*) *in blood with time. The pertinent microscopic rate constants in Tables I and V were used. The inset curves are expanded scale fittings of the data for trichloroethanol* (○) *and its glucuronide* (△) *in the blood. The latter data were estimated by multiplying the glucuronide blood concentrations by a factor that represented the ratio of the apparent volume of distribution of the glucuronide to that of the trichloroethanol in the central compartment. The solid circles represent the urinary excretion rates of trichloroethanol glucuronide* (*percent dose per minute*) *and are plotted on a 10-fold smaller scale, 0–1.0%/min., than that represented on the inset ordinate.*

$k_{B\cdot L_1}$ was observed (Dog E, Table I and Fig. 5), which indicated a large capacity for glucuronidation. Since sulfobromophthalein is metabolized by conjugation with glutathione and subsequently eliminated by biliary secretion (41), the observed metabolic inhibition of trichloroethanol metabolism was probably not due to a competition for sites on uridine 5′-diphosphate-glucuronyltransferase.

However, these implications of enzyme saturation or substrate inhibition of enzymic action to explain the metabolic rate decrease with increasing dosage are contradicted by the fact that the loss of trichloroethanol from the blood central compartment can be described by first-order kinetic parameters during its entire time course in the blood for any given dosage (Figs. 2, 3, 11, 12, 14, and 15 and Scheme V). It would be anticipated that the rate of trichloroethanol loss would vary from zero-order dependence at high blood levels to first-order dependence on drug concentrations in the central compartment at low blood levels if enzyme saturation or substrate inhibition occurred (37).

Since the reason for metabolic dependence on dose is probably not at the level of the enzymic conjugation, alternative mechanisms may be considered. They could include decreased availability of the uridine 5′-diphosphate-glucuronic acid cofactor with trichloroethanol dose or decreased uptake of trichloroethanol in the biophase of the hepatic cell with dose. This latter could be rationalized by assuming that the lipid–water ratio of trichloroethanol is altered within cells or among liver tissue components by the initial trichloroethanol dose. The fact that significant storage in the liver of trichloroethanol and/or its glucuronide is indicated (Fig. 7) tends to favor this latter explanation.



Scheme VI

The presence of trichloroethanol may stimulate its permeability into a constant volume of liver fat or may cause an increase in the volume of liver lipids. The phenomenon of fatty liver was reported for polychlorinated hydrocarbons (42) and ethanol (43) and might occur for a drug such as trichloroethanol, which possesses the molecular structure of both substances. Such a premise would necessitate the presumption that fat availability is an immediate and persistent consequence of the initial trichloroethanol dose.

An alternative and more probable, although indistinguishable, effect would be the persistent increase in availability of lipid by the action of increased trichloroethanol concentration, which could conceivably lower the surface tension between lipid and aqueous phases or modify the intervening membranic barriers. On the simple assumption that either of these mechanisms is operative, the intermediate steps between the loss of trichloroethanol and the appearance of its glucuronide in the blood in the pharmacokinetic model of Scheme V may be modified to include an instantaneous distribution of trichloroethanol between an aqueous phase $(TCE_{L_1})_{aq}$ and a lipid phase $(TCE_{L_1})_{lip}$ in the liver with an equilibrium constant K_2 (Scheme VI).

If the rate of glucuronide formation in the liver is a function of the trichloroethanol amount in an aqueous biophase:

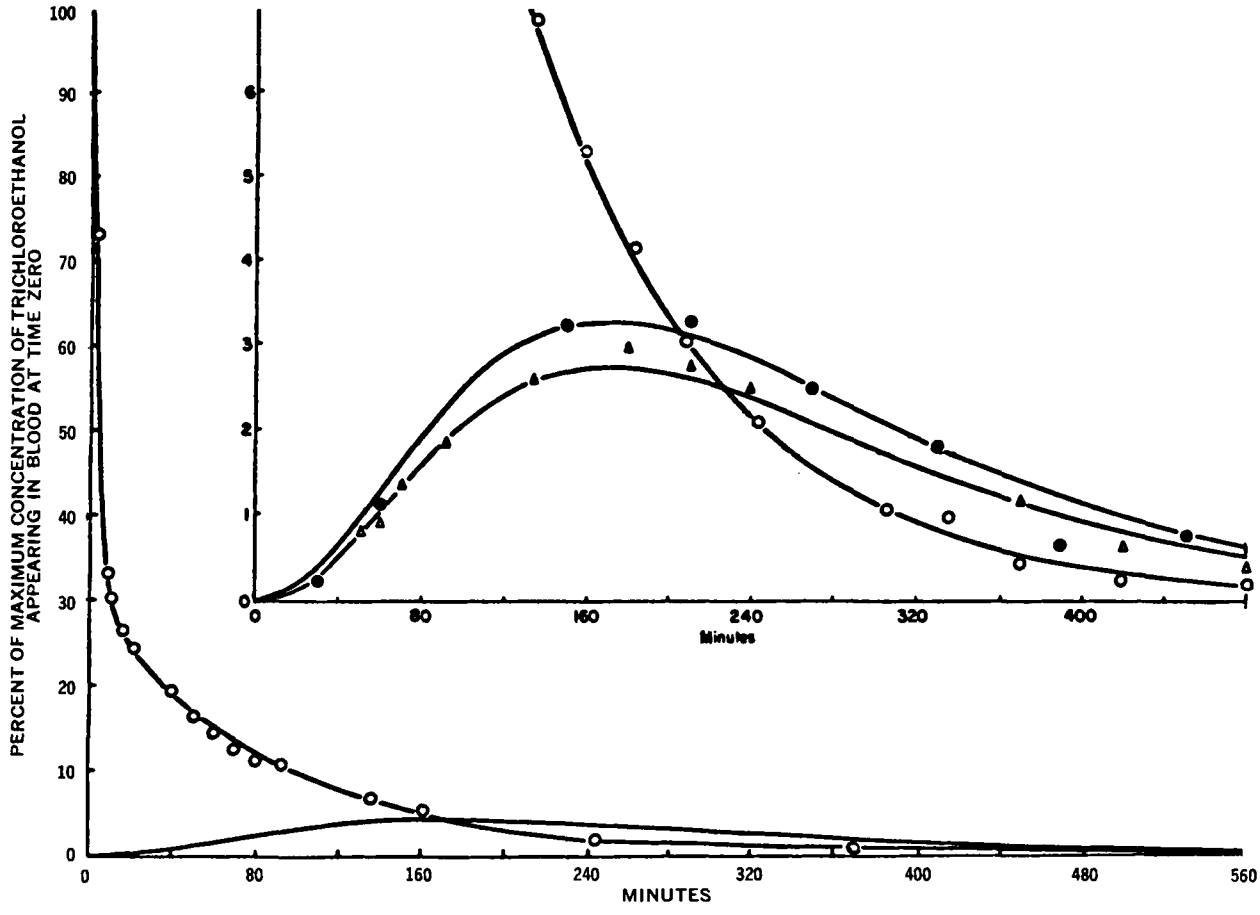


Figure 12—*Analog computer fits of the blood and urine data for a dose of 176 mg. trichloroethanol/kg. in the 10-kg. Dog C. The full-scale portion shows the fit of the data with time for trichloroethanol as the percent of the maximum concentration that appears in the blood at zero time* (○), *as well as the calculated amount of trichloroethanol glucuronide (TCE-G) in blood with time. The pertinent microscopic rate constants in Tables I and V were used. The inset curves are expanded scale fittings of the data for trichloroethanol* (○) *and its glucuronide* (△) *in blood. The latter data were estimated by multiplying the glucuronide blood concentrations by a factor that represented the ratio of the apparent volume of distribution of the glucuronide to that of trichloroethanol in the central compartment. The solid circles represent the urinary excretion rates of trichloroethanol glucuronide (percent dose per minute) and are plotted on a 10-fold smaller scale, 0–1.0%/min., than that represented on the inset ordinate.*

$$d(\text{TCE-G}_{L_1})/dt = -d[(\text{TCE}_{L_1})_{aq} + (\text{TCE}_{L_1})_{lip}]/dt = -d[K_1(\text{TCE}_B) + K_1K_2(\text{TCE}_B)]/dt = k^i_{L_1,L_2}(\text{TCE}_{L_1})_{aq} = k^i_{L_1,L_2}K_1(\text{TCE}_B) \quad \text{(Eq. 86)}$$

It thus follows that:

$$-d(\text{TCE}_B)/dt = \frac{k^i_{L_1,L_2}K_1}{K_1 + K_1K_2}(\text{TCE}_B) = \frac{k^i_{L_1,L_2}}{1 + K_2}(\text{TCE}_B) = k_{B,L_1}(\text{TCE}_B) \quad \text{(Eq. 87)}$$

and the apparent first-order rate constant, k_{B,L_1}, is not an intrinsic constant but a function of the equilibrium, K_2, between the aqueous and fatty phases or its equivalent, the relative volumes of lipid and aqueous phases in the hepatic cell or liver. If either of these factors is a persistent function of the initial trichloroethanol dose so that K_2 increases with dose, the apparent rate constant, k_{B,L_1}, will decrease with dose.

If K_2 is linearly related to dose, A_0, then:

$$K_2 = K_2^0 + mA_0 \quad \text{(Eq. 88)}$$

where K_2^0 is the intrinsic lipid–aqueous distribution in a liver unchallenged by trichloroethanol.

Substitution of Eq. 88 into Eq. 87 yields:

$$k_{B,L_1} = \frac{k^i_{L_1,L_2}}{1 + K_2^0 + mA_0} \quad \text{(Eq. 89)}$$

and, on taking reciprocals:

$$1/k_{B,L1} = \frac{1 + K_2^0}{k^i_{L_1,L_2}} + \frac{mA_0}{k^i_{L_1,L_2}} \quad \text{(Eq. 90)}$$

A plot of the reciprocal of the apparent first-order rate constant for the loss of trichloroethanol from the blood against trichloro-

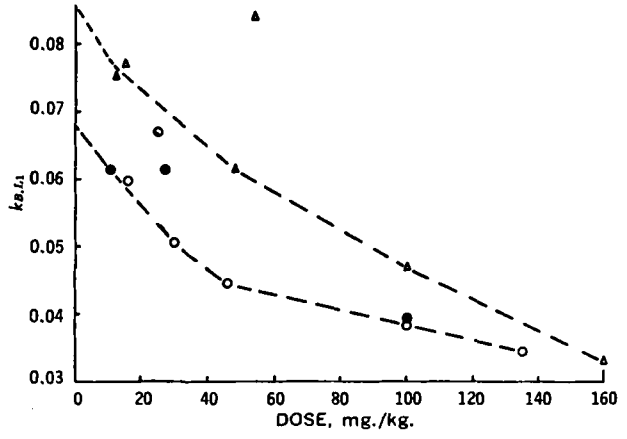


Figure 13—*Plot of the change in the calculated rate constant,* $k_{B,L1}$ *(Scheme V), for the metabolic loss of trichloroethanol with increasing doses of trichloroethanol in Dogs A* (▲), *B* (●), *C* (△), *and D* (○).

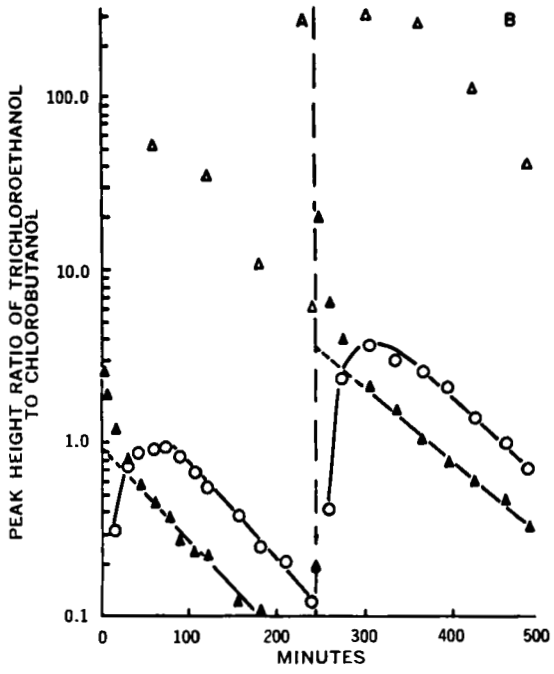


Figure 14—*Semilogarithmic plots of the peak height ratios of trichloroethanol (▲) and trichloroethanol glucuronide (○) from blood and the glucuronide from bile (△) with time for different doses of trichloroethanol. These peak height ratios are referred to the internal standard, chlorobutanol. Segment A is a consequence of dosing 11 mg./kg. of trichloroethanol at zero minute. Segment B is a consequence of dosing 46 mg./kg. of trichloroethanol at 240 min. A peak height ratio of 1.0 is equivalent to 8.4 mcg. of trichloroethanol or 18.3 mcg. of glucuronide/ml. of blood.*

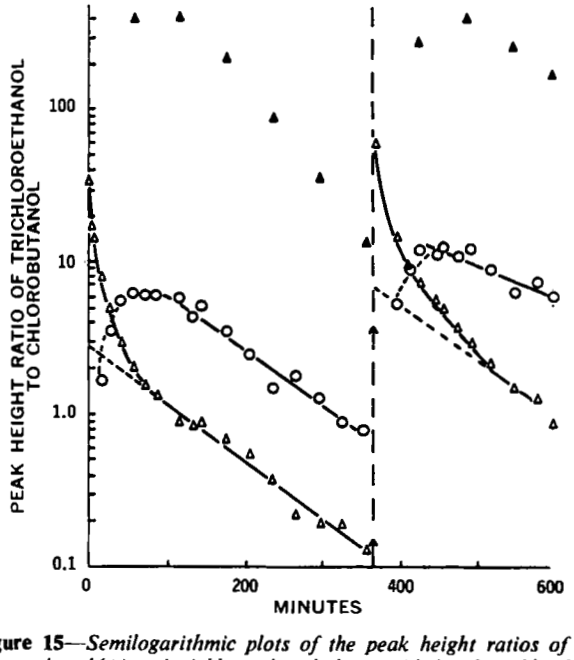


Figure 15—*Semilogarithmic plots of the peak height ratios of trichloroethanol (△) and trichloroethanol glucuronide (○) from blood and the glucuronide from bile (▲) with time for different doses of trichloroethanol. These peak height ratios are referred to the internal standard, chlorobutanol. Segment A is a consequence of dosing 50 mg./kg. of trichloroethanol at zero minute. Segment B is a consequence of dosing 100 mg./kg. of trichloroethanol at 360 min. A peak height ratio of 1.0 is equivalent to 8.4 mcg. of trichloroethanol or 18.3 mcg. of glucuronide/ml. of whole blood.*

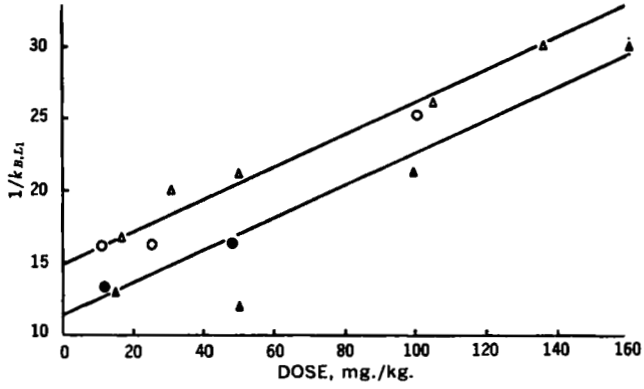


Figure 16—*Plot of the reciprocal of the metabolic rate constant, k_{B,L_1} (Scheme V), for the metabolic loss of trichloroethanol against dose for Dogs A (●), B (○), C (▲), and D (△).*

ethanol dose in accordance with Eq. 90 is given in Fig. 16 and is not inconsistent with the postulated relationship.

Trichloroethanol Glucuronide Distribution and Elimination in the Dog—After immediate equilibration in an initial apparent volume of distribution referenced to the concentration of unbound drug in plasma of 169 ml./kg. (Table V), which is about 3 times the true plasma volume of the dog, the intravenously administered glucuronide rapidly diffused into a compartment (TCE-G_T in Scheme V) of an equivalent apparent volume (Table V). The sum of the two volumes (330 ml./kg.) approximated the 200–380 ml./kg. reported for total extracellular water in dogs (16). The glucuronide did not distribute from plasma into red blood cells. The fraction, f, of glucuronide in plasma bound to protein was taken as 0.35 (Table I).

The urinary elimination of trichloroethanol glucuronide was rapid, with an apparent half-life of 15 min. for the greater portion of drug in the body (Fig. 17). No trichloroethanol or trichloroacetic acid was observed in any assayed blood or urine sample.

The semilogarithmic plot of the decrease in blood concentration of trichloroethanol glucuronide with time (Fig. 17) was triphasic and consistent with the three-component polyexponential expression of Eq. 63. Appropriate feathering, as demonstrated in Fig. 17, permitted preliminary estimates of the parameters of the equation. The cited apparent half-lives in Fig. 17 are inversely related to the k_i constants of Eq. 63. When the logarithm of the amount of unexcreted drug, log $(U_\infty - U)$, obtained from cumulative assays of glucuronide in the urine, was plotted against time (13), similar

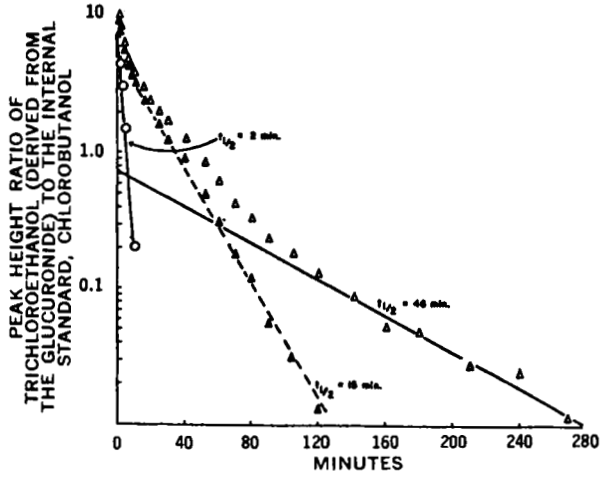


Figure 17—*Semilogarithmic plot of trichloroethanol glucuronide concentrations in blood with time in terms of gas chromatographically obtained peak height ratios of derived trichloroethanol to the internal standard, chlorobutanol, after a dose of 450 mg. (38 mg./kg.) of trichloroethanol glucuronide to Dog C. The original data are represented with open triangles. The three distinct phases obtained by feathering (i.e., the circles, solid triangles, and terminal open triangles) are labeled respectively with their apparent half-lives.*

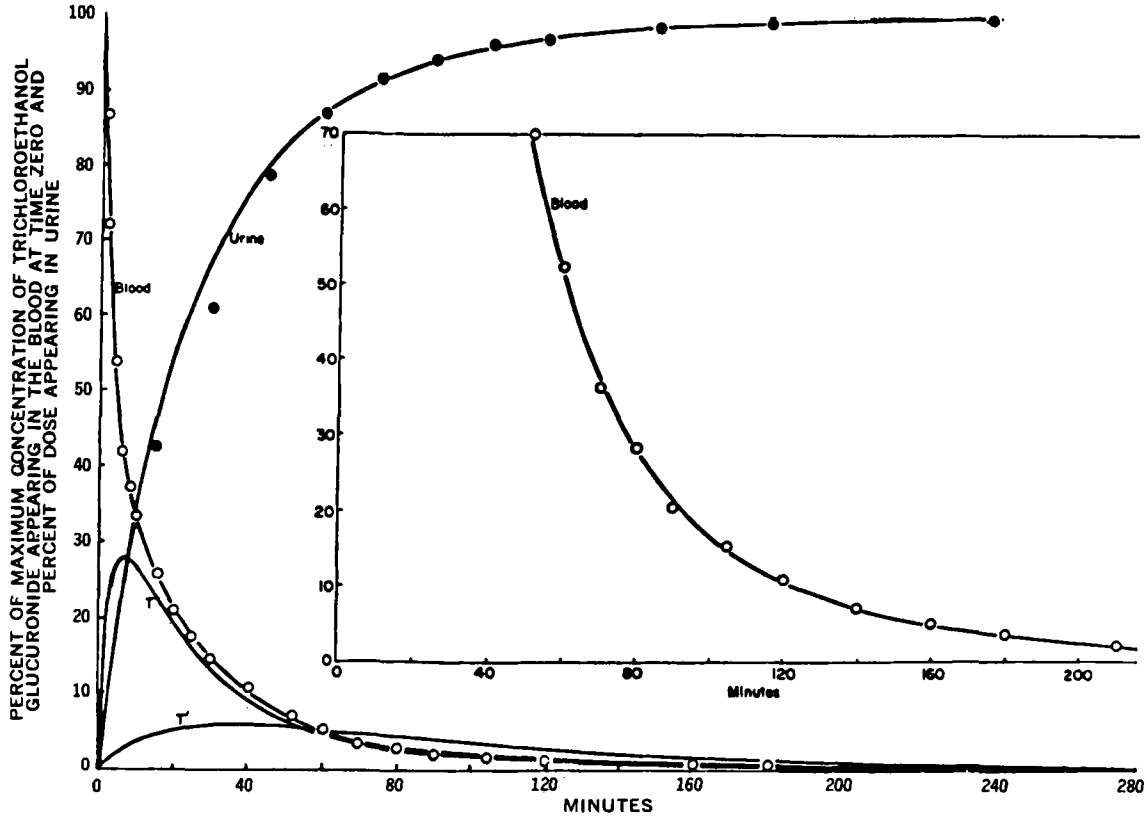


Figure 18—*Analog computer fits of the blood and urine data for trichloroethanol glucuronide in blood and urine after a 39-mg./kg. dose of trichloroethanol glucuronide to the 10-kg. Dog C. The full-scale portion shows the computer fit with time of the data for the glucuronide as the percent of the maximum concentration that appears in the blood at zero time* (○) *and as the percent of the total dose that appears ultimately in the urine* (●). *The curves are also generated for the trichloroethanol glucuronide in the distribution compartments* T *and* T′ *(Scheme V). The inset is an expanded scale fitting of the data for trichloroethanol glucuronide in blood. The microscopic rate constants of Table V were used.*

apparent half-lives for the second and third phases were observed by feathering and were 15 and 45 min., respectively. The first phase of Fig. 17 ($t_{1/2}$ = 2 min.) was not observed in the urinary data. An analog computer fit of trichloroethanol glucuronide data in blood and urine with time in accordance with a model similar to that of Scheme III is given in Fig. 18 (see also Scheme V).

A plot of percent of glucuronide dose excreted in urine per minute against percent of glucuronide dose per milliliter of plasma provided a good linear slope to estimate a clearance of 130 ml./min. This was consistent with the product of $k_{M,U}$ and V_P of 140 ml./min. (13). The $V_P = V_P{}^u(1 - f) + V_{P\text{true}}f$ can be estimated from the $V_P{}^u$ of Table V, where f = 0.30 in accordance with Eq. 36. Since the rena clearance values for inulin (glomerular filtration) for a 12-kg. dog can be calculated to be 51 ±12 ml./min. (44), it is probable that the intravenously administered glucuronide was eliminated by filtration

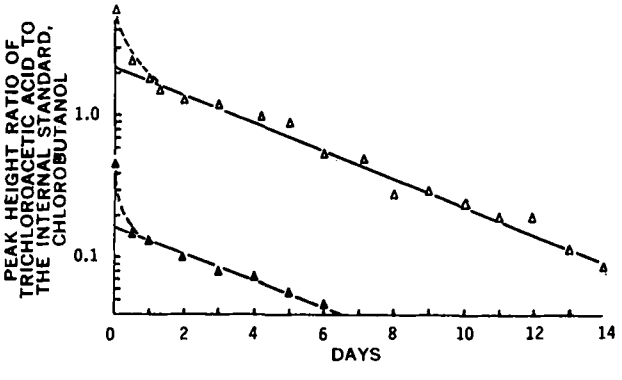


Figure 19—*Semilogarithmic plots of the blood trichloroacetate concentrations with time in terms of the gas chromatographically determined peak height ratios of trichloroacetic acid to the internal standard, chlorobutanol, after doses of 100 mg.* (▲) *and 1.0 g.* (△) *of sodium trichloroacetate to two different dogs. A peak height ratio with respect to chlorobutanol of 1.0 is equivalent to 70 mcg. trichloroacetic acid/ml. of whole blood.*

Table V—Trichloroethanol Glucuronide Pharmacokinetic Parameters[a] of Dog C (12 kg.) Administered 450 mg. of Glucuronide (37.5 mg./kg.)

$[A_B]_0$[b]	0.034
V_B[c]	2.94 l.
$V_P{}^u$[d]	2.03 l.
V_{TP^u}[e]	2.0 l.
$V_{T'P^u}$[f]	1.4 l.
$k_{M,T}$	0.133 (0.013) min.$^{-1}$
$k_{T,M}$	0.190 (0.019) min.$^{-1}$
$k_{M,T'}$	0.008 (0.0006) min.$^{-1}$
$k_{T',M}$	0.017 (0.0007) min.$^{-1}$
$k_{M,U}$	0.069 (0.0006) min.$^{-1}$

[a] The microscopic rate constants are defined in Scheme V for trichloroethanol glucuronide (TCE-G) in the various compartments and are given in minutes^{-1} and are referenced to total amounts in blood. The compartments and transfers for trichloroethanol in Scheme V are to be ignored. The numbers in parentheses are standard deviations of the stated parameters obtained by digital computer fitting with the SAAM program. [b] The computer-fitted zero time concentration of trichloroethanol glucuronide in blood, A_0, in terms of percent of dose per milliliter of whole blood. [c] The apparent volume of distribution of the central compartment referenced to total concentration in blood is V_B = (100%/$[A_B]_0$) in liters. [d] The apparent volume of distribution of the central compartment referenced to the concentration of glucuronide unbound to protein is $V_P{}^u = V_B(2.31) - 0.24V_{B\text{true}}$ after Eq. 34, where H = 0.45, f = 0.30 (Table II), D = 0 (Table III), and $V_{B\text{true}}$ = 0.1 × weight of dog in kg. (16). [e] The apparent volume of distribution referenced to the concentration of unbound glucuronide in the plasma of the rapidly equilibrating compartment, T, in liters, where $V_{TP^u} = V_P{}^u(k_{M,T}/\gamma_P{}^u k_{T,M})$, where $\gamma_P{}^u = 1 - f$ as calculated from Eq. 22. [f] The apparent volume of distribution referenced to the concentration of unbound glucuronide in the plasma of the slowly equilibrating compartment, T', in liters, where $V_{T'P^u} = V_P{}^u(k_{M,T'}/\gamma_P{}^u k_{T',M})$.

Table VI—Renal Clearance of Trichloroethanol Glucuronide at Various Dose Levels of Trichloroethanol

Dog (Weight, kg.)	Trichloroethanol Dose, mg./kg.	Observed Glucuronide Clearance, ml./min.[a]
A	10	139
(16)	50	143
B	10	256
(18.6)	25	256
	100	128[b]
C	10	130
(10)	50	130
	100	60[b]
	176	60[b]
D	17	203
(18)	50	203
	100	101[b]
	150	101[b]

[a] Observed clearance equals percent dose per milliliter plasma/percent dose per minute in urine, as obtained from the slope of plots of glucuronide as percent of trichloroethanol dose excreted into urine per minute plotted against the glucuronide as percent of trichloroethanol dose per milliliter of plasma. [b] At the lower concentrations of glucuronide in plasma, *i.e.*, close to the origin in the plots specified in *Footnote a*, the data follow the straight line drawn through the data for the lower doses of 10–50 mg./kg. of trichloroethanol. The data at the higher concentrations of glucuronide in the plasma fall below this line and become linear at a new and lower slope from which these clearances are estimated.

and tubular secretion at the studied glucuronide dosage of 38 mg./kg.

The glucuronide was distributed into another small compartment of apparent volume, $V_{T'_P}$[u] (Table V). The slow rate of drug return to this compartment was necessary to fit the blood and urine data from 1.5 to 12 hr. after glucuronide dosage (Figs. 17 and 18).

Analyses of the data for glucuronide formed on trichloroethanol dosing in all dogs demanded two compartments between TCE_B and $TCE\text{-}G_B$ (Scheme V) to fit the lag time in glucuronide appearance in the blood and the broad maximum that occurred in the glucuronide blood level–time curves (Figs. 3, 6, 9–12, 14). The rate constant $k_{L_1,M}$ (Table I) from the studies on trichloroethanol administration was remarkably similar to $k_{T',M}$ (Table V) on glucuronide administration. It may be conjectured that the compartment T' for intravenously administered glucuronide represented the hepatic uptake or binding of a small fraction of circulating glucuronide and $k_{T',M}$ was the rate constant for release of this sequestered glucuronide from liver back to blood.

Table VII—Effect of Trichloroethanol Dose on Bile Flow Rate and Biliary Secretion of Trichloroethanol Glucuronide

Dose, mg./kg.	Minutes	Bile Flow, ml./hr.	Percent Dose Excreted in Interval	Total Percent of Dose Excreted as Glucuronide
11.5[a]	60	1.5	0.35	
	120	1.8	0.27	
	180	1.8	0.08	
	240	1.7	0.05	
				0.75
46.0[a]	60	2.5	0.76	
	120	2.1	0.56	
	180	1.8	0.21	
	240	1.7	0.07	
				1.60
50[b]	60	3.8	1.94	
	120	3.8	2.00	
	180	3.0	0.85	
	240	2.4	0.28	
	300	1.7	0.08	
	360	4.3	0.07	
				5.22
100[b]	60	6.7	1.20	
	120	6.2	1.57	
	180	2.0	0.34	
	240	1.7	0.17	
	300	1.0	0.09	
				3.37

[a] Weight of dog was 13.0 kg. [b] Weight of dog was 9.6 kg.

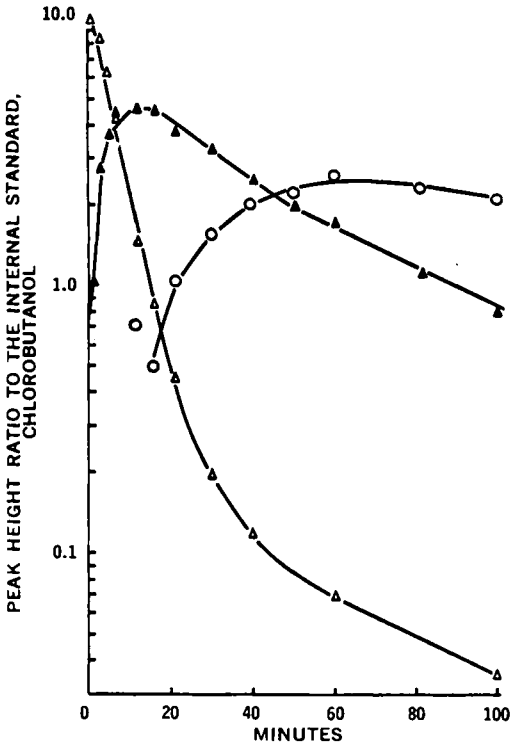


Figure 20—*Semilogarithmic plot of blood concentrations of chloral hydrate (△), trichloroethanol (▲), and trichloroethanol glucuronide (○) in terms of peak height ratio against time for a dose of 500 mg. of chloral hydrate (42 mg./kg.) in Dog C. A peak height ratio to the internal standard, chlorobutanol, of 1.0 is equivalent to 4.3 mcg. chloral hydrate, 8.4 mcg. trichloroethanol, or 18.3 mcg. trichloroethanol glucuronide/ml. whole blood.*

The renal clearances of trichloroethanol glucuronide declined with the higher glucuronide concentration in blood that resulted from the higher doses of trichloroethanol (Table VI). Such clearances were obtained from the slopes of plots of glucuronide as percent of the trichloroethanol dose excreted into the urine per minute against the glucuronide as percent of this dose per milliliter of plasma. These clearances are theoretically the product of the microscopic rate constant for renal excretion of glucuronide, $k_{M,U}$, referenced to total amounts of glucuronide in the plasma and the apparent volume of distribution of the central compartment, V_P, which is also referenced to the drug concentration in the plasma (13) and may be obtained from V_P[u] in Table V by a rearrangement of Eq. 36. The decrease of these clearances with higher blood levels of glucuronide can be assigned to either a lowering of the apparent volume of distribution of the central compartment for glucuronide or a decrease in the $k_{M,U}$ factor for renal excretion of the glucuronide at the higher blood levels. Since the latter is consistent with the claim that a tubular secretory pathway is saturable for carboxylates and other anions (45), it is the preferred explanation.

These decreased glucuronide clearances and the previously discussed lowered rate constant $k_{B,L}$ for the loss of trichloroethanol and glucuronide with increased trichloroethanol dose readily explain the observed retarded time of appearance of maxima in glucuronide blood levels at the higher doses of trichloroethanol (Figs. 9 and 10).

Renal and Fecal Elimination of Trichloroethanol as Its Glucuronide—The excretion of free, unmetabolized trichloroethanol in feces and urine accounted for less than 3% of the administered dose. Essentially, the drug was excreted in the urine as the glucuronide. The average amount of trichloroethanol found in fecal samples collected between 0 and 12 hr. after trichloroethanol administration for 12 experiments was 0.3% of the dose and ranged from 0 to 1.5%. No glucuronide was found in the feces in these experiments. A

mean of 1.5% of the total dose was found in the urine as unconjugated trichloroethanol in 27 experiments. The negligible amounts of fecal elimination of trichloroethanol and its metabolites and urine elimination of trichloroethanol permitted these routes to be ignored in the pharmacokinetic modeling. Any significant amount of trichloroethanol or its glucuronide excreted in the bile must be significantly reabsorbed, with the glucuronide being readily hydrolyzed; *i.e.*, an enterohepatic shunt could be postulated. The highly lipid-soluble trichloroethanol is undoubtedly extensively reabsorbed from the renal tubules and subsequently glucuronidated.

A variable fraction (10–20%) of doses of trichloroethanol could not be accounted for in cumulative urine and fecal assays and most probably was eliminated *via* other routes (*e.g.*, saliva, sweat, and breath), which is to be expected for any low molecular weight alcohol. It is also possible that a small fraction of trichloroacetate was produced and was unavailable for assay because of its tight binding to tissues.

Biliary Secretion of Trichloroethanol and Its Glucuronide—When samples of the bile were assayed from two different dogs administered trichloroethanol, significant biliary concentrations of the glucuronide were obtained. Semilogarithmic plots of the concentration of trichlorethanol and its glucuronide in blood and the glucuronide in bile against time are given in Figs. 14 and 15 for the two experiments.

Maximum biliary concentrations of the glucuronide were 60–85 times the concentrations in whole blood (30–42.5 times the plasma concentration) in the first dog (Fig. 14) for doses of 11.5 and 46.0 mg./kg. However, trichloroethanol concentrations in bile were much less and did not exceed 2.5 times the trichloroethanol blood concentrations so that the total trichloroethanol excreted by this route was not of practical significance.

When the second dog was dosed with 50 mg./kg. of trichloroethanol, the maximum biliary glucuronide concentrations were again greater than 60 times the blood concentration for a 50-mg./kg. dose of trichloroethanol. However, an increase of the trichloroethanol dose to 100 mg./kg. did not increase the biliary concentration of the glucuronide (Fig. 15), which was only 30-fold that of its blood concentration. This apparent inhibition of biliary trichloroethanol glucuronide secretion was concomitant with a slowed loss of trichloroethanol and its glucuronide from the blood (Fig. 15), phenomena which seem to be common to the higher trichloroethanol doses. (See k_{B,L_1} values at various doses in Table I.)

The inhibition by chloral hydrate of the biliary secretion in rats of indocyanine green (a nonmetabolized dye used to evaluate cardiac function) and bilirubin glucuronide was previously reported (40). Doses estimated at 50–100 mg. chloral hydrate/kg. were inhibitory while 10-fold smaller doses had no effect.

Guarino and Schanker (38) reported saturation of biliary secretion of probenecid and its glucuronide at a dose of 0.18 m*M*/kg. The dose of trichloroethanol required for the same effect in these experiments was 0.67 m*M*/kg.

A compilation of the bile flow rates and percentages of the initial dose excreted as the trichloroethanol glucuronide in bile within the specified time intervals is given in Table VII. The data show that bile flow rate increases as a function of increasing trichloroethanol dose. This chloretic effect was previously reported for chloral hydrate (46) and for probenecid (38).

About 5% of the dose was biliary excreted as the glucuronide in the total interval studied. This was consistent with the data of Owens and Marshall (6), from which it was calculated that about 8% of an administered dose was excreted in the bile within 3–4 hr. after intravenous administration of trichloroethanol.

Pharmacokinetics of Trichloroacetic Acid—The apparent disposition half-life for both 0.1- and 1.0-g. doses of trichloroacetic acid as the sodium salt in the dog was 75 hr., which was similar to that in man (47) (Fig. 19). No trichloroacetic acid was found in a pooled 10-day fecal sample. The apparent volume of distribution of 100 and 1000 mg. of sodium trichloroacetate in the central compartment referenced to the concentration in whole blood may be calculated (Eq. 24) from the time zero estimate of blood concentration, $[A_B]_0$ (Fig. 19), as 3.6 l. This value is consistent with the 2.0–3.5 l. (in a 10-kg. dog) or 3.0–5.3 l. (in a 15-kg. dog) of extracellular body water (18), even when a 0.40-l. volume for red blood cells is subtracted. However, since the protein binding of trichloroacetate is in the range of 70–90% (5), the apparent volume of distribution of trichloroacetic acid referenced to unbound drug in the plasma will greatly exceed even the total body water (7.5 l. for a 15-kg. dog). This large value may be the result of the binding of trichloroacetate anions in the readily available tissues and may be the explanation for the comparatively slow elimination of such a highly ionized drug.

The apparent volumes of distribution of 100 and 1000 mg. of sodium trichloroacetate in the total compartments of distribution, *i.e.*, the central compartment plus the more slowly equilibrating tissues, were calculated from the estimates of blood concentrations (Fig. 19) after the linear extrapolation of the terminal points of the semilogarithmic plots of Fig. 13 to zero time and were 7.9 and 6.8 l., respectively, referenced to drug concentration in the blood.

Computer analyses of the data of Fig. 19 in accordance with a model similar to that given in Scheme III (except that $A_{T'} = 0$ and $k_{B,U}$ are substituted for k_B) provided estimates for the microscopic rate constants referenced to blood concentrations for trichloroacetic acid pharmacokinetics, where $k_{B,T} = 0.079$, $k_{T,B} = 0.073$, and $k_{B,U} = 0.22$ hr.$^{-1}$. The overall elimination rate constant, k_e, calculated from Eq. 67 (where $k_{B,U}$ is substituted for the designated $k_{B,M}$) was 0.0105 hr.$^{-1}$ and was consistent with the value obtained from the terminal slopes of the plots of Fig. 19. It was also markedly similar to the value given for man, $k_e = 0.0094$ hr.$^{-1}$ (47).

Pharmacokinetics of Chloral Hydrate—Conversion of chloral hydrate to trichloroethanol in the dog was rapid and quantitative. Blood levels of trichloroethanol (5.5 ± 0.2 mg./ml.) and its glucuronide (36.2 ± 0.2 mg./ml.) 100 min. after intravenous administration of 500 mg. chloral hydrate (42 mg./kg.) were identical (Fig. 20) to those obtained after an equimolar dose of trichloroethanol was given to the same Dog C. The majority (97.5%) of the initial amount of chloral hydrate in blood was lost rapidly, with a half-life of 3 min. ($k_{CH,TCE} = 0.23$ min.$^{-1}$). The semilogarithmic plot was linear for more than five half-lives. The remainder (2.5%) showed a slower decline ($t_{1/2} = 35$ min.).

The appearance of trichloroethanol in the blood was rapid, with a half-life (3 min.) that corresponded to that for loss of chloral hydrate. Maximum trichloroethanol blood levels were observed 12 min. after chloral hydrate administration (Fig. 20). This is consistent with the claim that conversion to trichloroethanol of the chloral hydrate precursor occurs in all body tissues (48). Only a small fraction of the dose shows a deviation from the linear semilogarithmic plot, which can be assigned to a slower return from tissues of equilibration.

The apparent volume of distribution of the 500 mg. of chloral hydrate in the central compartment referenced to concentration in whole blood may be calculated (Eq. 24) from the time zero estimate of blood concentration $[A_B]_0$ (Fig. 20) as 11.5 l., which is very much greater than the values for trichloroethanol (Table I) and its glucuronide (Table V). This undoubtedly implies that chloral hydrate undergoes a more rapid diffusion into, or a more rapid binding with, tissues other than blood during first cycles in the body. No measurable concentrations of trichloroacetic acid were found in blood samples.

The percent of the dose of chloral hydrate excreted in urine as the glucuronide after 2 hr. (46%) was identical to that observed with an equimolar dose of trichloroethanol. In the same period of time, 1.56% of the total dose as unchanged chloral hydrate and 0.27% as trichloroacetic acid were also found in urine.

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GLC Assay for 5-Fluorouracil in Biological Fluids

JORDAN L. COHEN▲ and PATRICK B. BRENNAN

Abstract □ A rapid, sensitive GLC method of analysis for 5-fluorouracil was developed to follow the disposition of the drug in patients on 5-fluorouracil therapy. The free drug is removed from aqueous biological samples in a single extraction step, derivatized by silylation, and chromatographed using flame-ionization detection. An internal standard is utilized to quantitate the results. The procedure is rapid, sensitive (0.2 mcg./ml. plasma), specific for the intact 5-fluorouracil molecule, and suitable to support pharmacokinetic studies of 5-fluorouracil in animals and humans.

Keyphrases □ 5-Fluorouracil—GLC analysis in biological fluids □ GLC—analysis, 5-fluorouracil in biological fluids

Detailed pharmacokinetic studies that would provide information useful to the clinician would be desirable to optimize cancer chemotherapy with 5-fluorouracil. The drug is still given empirically by many different dosage schedules, with some recent evidence suggesting that the oral route of administration differs in activity and toxicity from the intravenous route (1). Disposition studies performed previously were limited by the absence of a rapid, sensitive method of analysis capable of determining free 5-fluorouracil in large numbers of biological samples. Studies using radioactively labeled drugs showed wide variability due to extensive metab-

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Table I—Recovery of 5-Fluorouracil Added to Water, Human Plasma, and Human Whole Blood

5-Fluorouracil Added, mcg./ml.	Water		Plasma		Blood	
	Peak Height Ratio[a]	Percent Recovery[b]	Peak Height Ratio[a]	Percent Recovery[b]	Peak Height Ratio[a]	Percent Recovery[c]
4.83	1.42	78.9 ± 2.50	1.45	80.6 ± 3.24	1.47	81.7
1.94	0.60	82.2 ± 3.10	0.58	79.4 ± 3.47	0.56	76.7
0.96	0.28	80.0 ± 2.74	0.27	77.1 ± 3.05	0.28	80.0
0.48	0.14	82.3 ± 3.85	0.14	82.3 ± 3.75	0.13	76.5

[a] Anthracene concentration was 3.2 ng./μl. injected. [b] Average of three determinations ± standard deviation. [c] Represents a single determination. Human blood was extracted according to the described procedures to determine binding to red blood cells.

olism (2) and illustrated the nonspecificity of this method of analysis for compounds of this type.

Clarkson *et al.* (3) performed some comparative disposition studies on a limited number of patients; they utilized a spectrophotometric assay procedure when the blood levels were greater than 100 mcg./ml. and a microbiological assay for lower blood levels. In most instances the spectrophotometric procedure was not sensitive enough for clinical studies, while the microbiological procedure lacked accuracy and was not readily adaptable to large numbers of samples. Windheuser *et al.* (4) developed a GC method which requires extended (22-hr.) dialysis to remove the drug from blood samples and which would be difficult to perform on a routine basis.

The present work describes an analytical method based upon a single extraction of the drug into a nonaqueous solution, with subsequent formation of a trimethylsilyl derivative for GLC detection. The concentrations of 5-fluorouracil were quantitated using an internal standard. The method is applicable to biological samples including blood and urine and can be performed on a routine basis for clinical studies of patients on 5-fluorouracil therapy.

EXPERIMENTAL

Reagents—All chemicals and solvents used were of analytical reagent grade, and water was glass double distilled.

Procedure for Biological Samples—Blood samples were collected in citrated Vacutainers[1], mixed gently, and centrifuged to allow separation of the plasma. Then the plasma was frozen until the sample was to be analyzed. Urine samples were either collected and directly frozen or lyophilized and stored in a similar fashion.

A plasma or urine sample was adjusted to pH 6 by the addition of a known volume of a pH 4.7, 0.5 *M* acetate buffer (*e.g.*, 125 μl. of acetate buffer was required per milliliter of human plasma). Then 1.0 ml. of this sample was added to a flask already containing 0.4 g. of anhydrous sodium sulfate and 15.0 ml. of a solution of 16% *n*-propanol in ether. This mixture was shaken vigorously for 5 min. and then allowed to stand for 15 min. or until the ether layer cleared. Depending upon the concentration of 5-fluorouracil, a suitable volume (1.0–10.0 ml.) of the ether solution was pipeted into either a: (*a*) 15.0-ml. centrifuge tube if more than 5.0 ml. was used, or (*b*) 5.0-ml. vial[2] if a smaller volume of ether was used. Then 25 μl. of an anthracene solution in pyridine containing 16 mcg./ml. was added as an internal standard, and the solvent was removed under a gentle stream of nitrogen with the reaction vial immersed in a 55° water bath.

Derivatization—The evaporated sample was dissolved in 100 μl. of a freshly prepared solution of 50% *N,O*-bis(trimethylsilyl)-trifluoroacetamide (I), containing 1% trimethylchlorosilane[3] as a catalyst in silation grade pyridine[3]. This reaction mixture was allowed to stand for 5 min. at room temperature, and then 1–10 μl. was injected into the gas chromatograph.

Gas Chromatography—The assay was performed on a gas chromatograph[4] equipped with a flame-ionization detector. A glass coiled column, 1.83 m. (6 ft.) × 0.63 cm. (0.25 in.) o.d., was packed with 3% OV-1 on 100–120-mesh Gas Chrom Q[5], conditioned at 250° for 72 hr. and injected periodically with trimethylsilyl donors[6]. Nitrogen as a carrier gas was maintained at 40 ml./min., while the flow rates for air and hydrogen were 250 and 30 ml./min., respectively. The injector and detector temperatures were both maintained at 200° throughout the procedure.

The sample was injected at a column-oven temperature of 110° and the 5-fluorouracil eluted as a sharp peak at ~5.5 min. The temperature was then increased to 160° (heating rate 50°/min.) and the anthracene (internal standard) peak appeared after 12 min. (from time of injection). After 2–3 days of use, the column was reconditioned at 250° overnight and a mixture of trimethylsilyl donors[6] was injected. The detector was cleaned every 3–4 days with repeated injections of trichlorotrifluoroethane[7] and was removed periodically and further cleaned by sonication in acetone.

RESULTS AND DISCUSSION

5-Fluorouracil is representative of the analytical challenges now faced by workers in biopharmaceutics and clinical pharmacology. Its water solubility, even as the free acid, and nonfluorescent properties make specific, sensitive, routine methods of analysis difficult to develop. The described extraction procedure gives the highest constant percentage extracted (80%) in a single extraction of many explored and was the only method that appeared quantitatively useful. Any increase in the polarity of the "nonaqueous phase" destroys the immiscibility with aqueous samples. Saturation with sodium sulfate also appeared to optimize extraction of 5-fluorouracil by salting it out of the aqueous medium. Since no improvement in extractibility was seen at more acidic pH's, pH 6 was selected, on the basis of the reported pKa of 8.1 (5), to minimize the extraction of other weakly acidic compounds which could be present in blood samples and possibly silate and interfere with the chromatographic assay procedure. This is a critical consideration in this case since the partition coefficient of 5-fluorouracil does not allow a "cleanup" extraction. Plasma blanks were always carried through the assay procedure to verify the absence of any interfering material.

The extraction procedure gave identical results for aqueous, plasma, and blood samples to which 5-fluorouracil had been added[8]. These results (Table I) indicate no interference with the assay in the presence of plasma proteins or red blood cells which would affect the percentage extracted. The percent recovery is based upon the addition of 5-fluorouracil to ether (Table II). In view of this and since the extraction was not complete, a standard curve was prepared by using aqueous samples to which various concentrations of 5-fluorouracil had been added and carrying them through

[1] Becton, Dickinson and Co., Rutherford, N. J.
[2] Reacti-vial, Pierce Chemical Co., Rockford, Ill.
[3] Pierce Chemical Co., Rockford, Ill.
[4] Varian model 1440 (Varian Aerograph, Walnut Creek, Calif.) equipped with a Perkin-Elmer Hitachi recorder, model 156.
[5] Applied Science Laboratories, State College, Pa.
[6] Silyl 8, Pierce Chemical Co., Rockford, Ill.
[7] Freon 113, Pierce Chemical Co., Rockford, Ill.
[8] Obtained from either International Chemical and Nuclear, Irvine, Calif., as a solid, or Hoffmann-La Roche, Nutley, N. J., as the dosage form.

Table II—GLC Data for 5-Fluorouracil Added to Ether

5-Fluorouracil Added, mcg./ml.	Peak Height Ratio[a,b]
4.83	1.80
1.94	0.73
0.96	0.35
0.48	0.17

[a] Anthracene concentration was 3.2 ng./μl. injected. [b] Average of three determinations.

the entire assay procedure. Figure 1 shows a typical chromatographic separation from a human plasma sample. The sharpness of the peaks enabled a quantitation to be made using peak height ratios rather than the more cumbersome peak area ratios. A linear standard curve (Figs. 2 and 3) corresponding to original concentrations of 5-fluorouracil of from 0.2 to 80 mcg./ml. was obtained and used to determine directly the drug levels of samples in this concentration range after accounting for any dilution changes. Higher concentrations of 5-fluorouracil required different concentrations of internal standard and the curve was linear up to 300 mcg./ml.

Working from observations of Windheuser *et al.* (4) that 5-fluorouracil could be silylated quantitatively and chromatographed led to development of the final GLC assay procedure. Compound I had the advantage of much more volatile fluorinated by-products after silylation, which appear under the solvent peak and do not interfere with the quantitation of 5-fluorouracil as did *N,O*-bis-(trimethylsilyl)acetamide under our assay conditions. To be certain of the specificity of the method for the free drug molecule, the analysis was performed on a GLC interfaced with a mass spectrometer[9]. The peak corresponding to 5-fluorouracil at 5.5 min. was shown by fragmentation and the appearance of a parent peak at

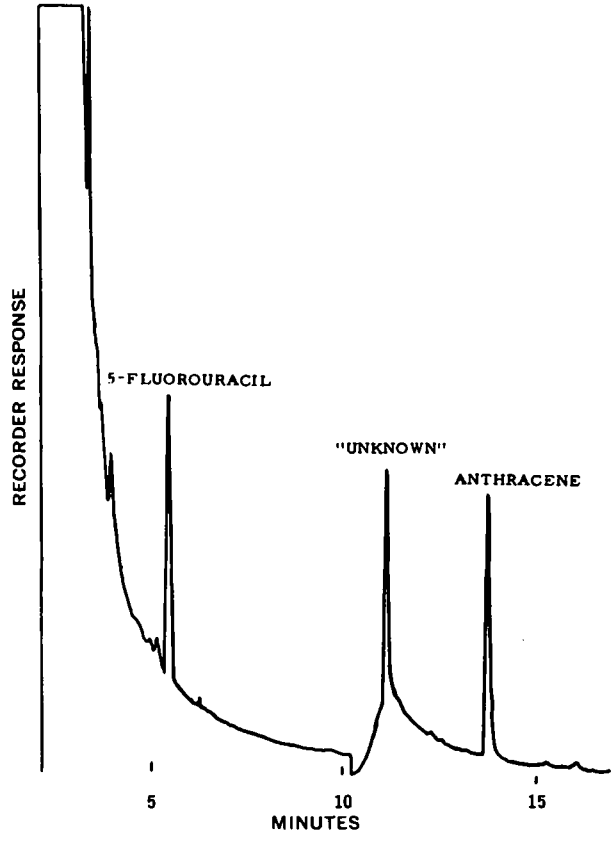

Figure 1—*Gas chromatogram of a plasma sample of 5-fluorouracil with the addition of anthracene. (5-Fluorouracil concentration = 4.83 mcg./ml.; anthracene concentration = 3.2 ng./μl. injected.)*

[9] Varian model CH-7, Varian Mat Divisions, Palo Alto, Calif.

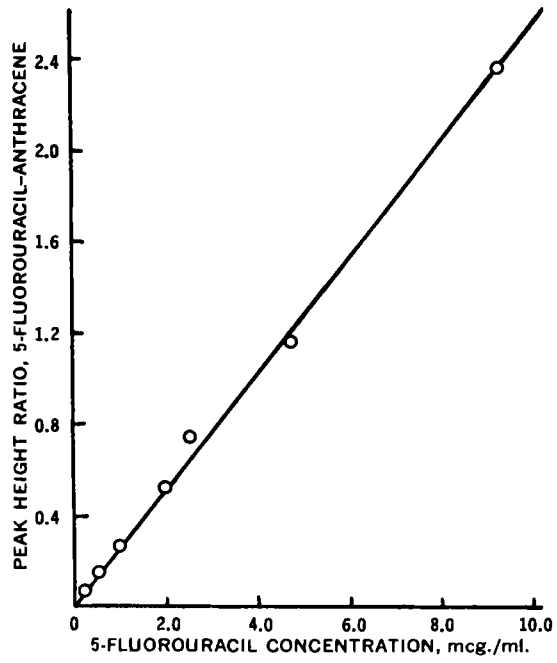

Figure 2—*Calibration curve for 5-fluorouracil extracted from aqueous solution at low 5-fluorouracil concentrations. (Anthracene concentration = 3.2 ng./μl. injected.)*

274 *m/e* to be 5-fluorouracil with the addition of two trimethylsilyl groups.

No suitable internal standard could be found for the column operating temperature at which the assay is performed. Anthracene was selected for its inertness, purity, and good flame-ionization response; however, the column had to be programmed to obtain reproducible results for peak height ratio calculations. This actually turns out to be an advantage, since the higher temperatures elute impurities present in the biological samples before the anthracene peak comes out (the broad peak labeled "unknown" in Fig. 1). Otherwise, to avoid interferences, these impurities would have to be eluted before the next sample could be injected.

The presence of the fluorine atom suggested that electron-capture detection may improve sensitivity and eliminate extraneous plasma peaks and other interferences. Much work has been done correlat-

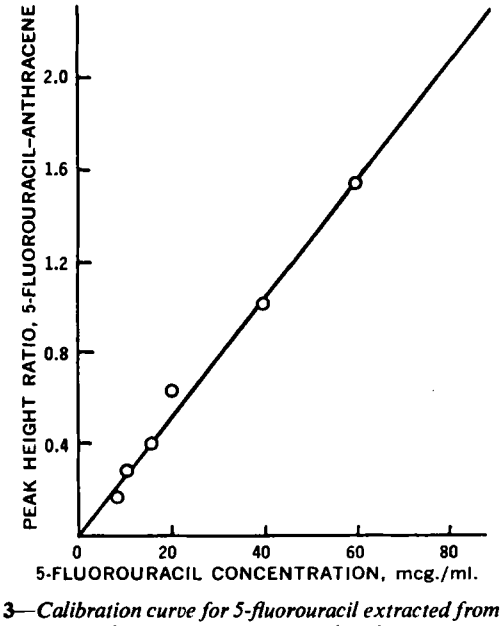

Figure 3—*Calibration curve for 5-fluorouracil extracted from aqueous solution over a wide concentration range. (Anthracene concentration = 3.2 ng./μl. injected.)*

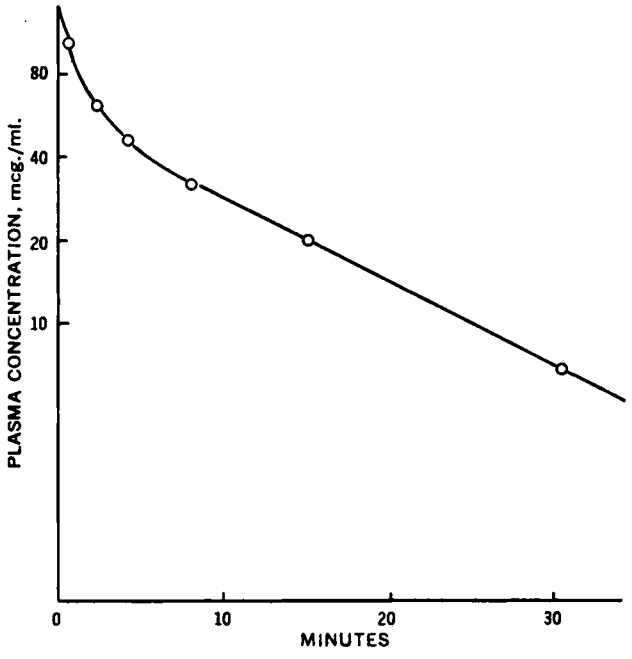

Figure 4—*Plasma concentration of 5-fluorouracil in a patient (E.J.) given a single intravenous dose of 15 mg./kg.*

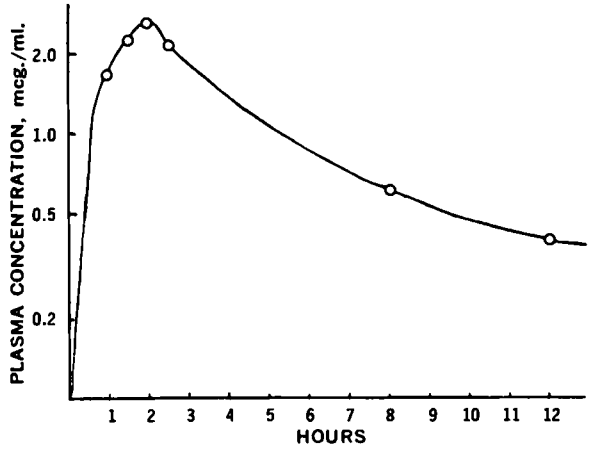

Figure 5—*Plasma concentration of 5-fluorouracil in a patient (I.B.) given a single oral dose (in solution) of 15 mg./kg.*

ing the structures of derivatives with electron-capture sensitivity (6–10), the presence of a single chlorine atom, for example, on chlorpromazine allowed a sensitive electron-capture analysis to be performed (11). Since the analytical conditions were essentially worked out, a ^{63}Ni detector was substituted for the flame-ionization detector; however, sensitivity could not be improved by more than a factor of two using this approach. A modification of the present method is currently being pursued in these laboratories in an effort to increase sensitivity, which may enable the quantitation of 5-fluorouracil in trace concentrations in body tissues and fluids other than plasma and urine.

Since the reason for development of an assay procedure of this type is to support biopharmaceutical and clinical pharmacology studies designed to improve drug therapy, data from two different patients receiving therapeutic doses of 5-fluorouracil have been included. Figure 4 illustrates the plasma time course of the drug in a patient (E.J.) receiving 15 mg./kg. by intravenous push, and Fig. 5 illustrates the same data for a different patient (I.B.) receiving a comparable dose in solution orally. No interfering peaks were encountered either from possible metabolites or other medication taken by these patients. The sensitivity and applicability of the method to these patients indicate that the method is suitable for these types of studies, and several clinical studies are currently being performed.

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Kinetics of Dehydration and Isomerization of Prostaglandins E_1 and E_2

D. C. MONKHOUSE[A], L. VAN CAMPEN, and A. J. AGUIAR

Abstract □ The degradation of prostaglandins E_1 and E_2 was studied at 60° at a pH range of 1–10. A consecutive first-order reaction appeared to be operative above pH 4 for the dehydration and rearrangement reactions. Below pH 3 the dehydration appeared to be first order with respect to hydrogen-ion concentration. The rate of dehydration of E_2 was two to three times faster than that of E_1 under the same conditions over a pH range of 4–10. The rearrangement of E_1 and E_2 proceeded at approximately the same rate at pH 10. The rate of E_2 rearrangement progressively exceeded that of E_1 with a decrease in pH such that at pH 6, E_2 rearranged at a rate 15 times that of E_1. These data suggested that the *cis*-Δ^5 double bond participated actively in the rearrangement reaction of E_2, and was responsible for the greater reactivity of this molecule compared to E_1.

Keyphrases □ Prostaglandins E_1 and E_2—dehydration and isomerization kinetics, pH effect □ Dehydration and rearrangement kinetics—prostaglandins E_1 and E_2 □ Kinetics, dehydration and rearrangement—prostaglandins E_1 and E_2 □ UV spectrophotometry—monitoring, dehydration and isomerization of prostaglandins E_1 and E_2 □ pH effect—dehydration and isomerization kinetics of prostaglandins E_1 and E_2

Prostaglandins affect a wide range of physiological processes, from the contraction of the uterus to secretion from the stomach wall. This diversity of action accounts for the great current interest in their use as pharmacological agents.

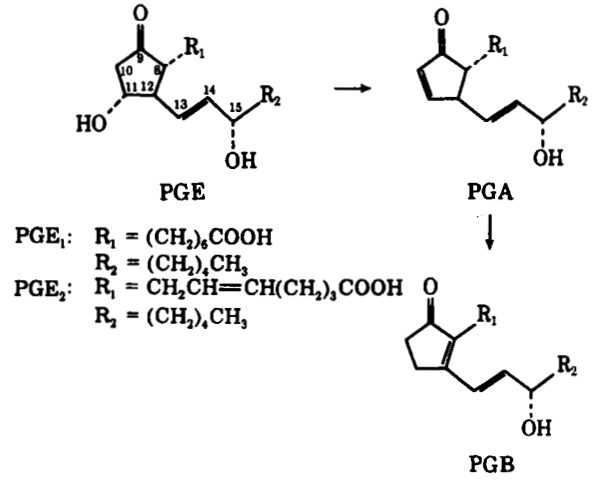
The application of prostaglandins in a number of areas has been severely hampered by their apparent instability in solution. Karim *et al.* (1) measured the biological activity of saline solutions of prostaglandins stored at various pH values at room temperature. The PGE compounds showed 25–40% loss of biological activity after 60 days at pH 5–7. Andersen (2) reported 5–20% conversion to PGA_1, measured spectrophotometrically, in methanolic solutions of PGE_1 after 1 month at 5–10°. Brummer (3) found that, for E_2 in ethanolic solution stored for 4 weeks at 4°, there was no detectable loss of total $E_2 + A_2$; after 6 months, 33% of the E_2 had been converted to PGA_2 and bioassay of the solution showed only 50% potency. As a result of this study, it was recommended that PGE solutions prepared for clinical use be stored as concentrated alcoholic solutions in ampuls. These can be stored for long periods at −20° and diluted with sterile isotonic saline as required within 24 hr. of use.

The β-hydroxyketone moiety of the PGE compounds[1] is extremely reactive. Loss of protons at C-8 or C-10 occurs very readily in both acid and alkaline media. The loss of a proton at C-10 may result in a negative charge, expulsion of the hydroxyl group at C-11, and formation of a Δ^{10} double bond (PGA compound). Under base treatment the PGA compounds undergo internal rearrangement of the double bond ($\Delta^{8,12}$) to form the relatively stable PGB compounds containing a dienone chromophore (Scheme I).

Treatment of PGE_1 (1 mg./ml. in methanol) with 1 ml. of 1 *N* HCl resulted in 80% conversion to a mixture of PGA_1 and methyl PGA_1 after standing for 16 hr. in the dark at room temperature. Systematic studies (4) of the stability of methanolic solutions of PGE_1 in various media for 6 hr. at room temperature revealed that PGA_1 formation was only evident at low pH values (<3) or if the buffer exceeded pH 7.4. Thus, in buffers of carbonate–borate–potassium hydroxide (pH 10.0) or sodium acetate–potassium hydroxide (pH 11–11.5), approximately 50–60% conversion of PGE_1 to PGA_1 was obtained. At the higher pH value, 20% PGE_1 passed into the PGB form after 6 hr. at room temperature. In the presence of oxalic acid (pH 2.5), saturated ammonium chloride in biphthalate buffer (pH 4), potassium acetate–acetic acid (pH 5), or sodium acetate (pH 7.4), PGE_1 was found to be stable (<10% conversion to PGA_1) for 6 hr. at room temperature; longer exposure, however, did result in significant PGA formation.

Oesterling (5) measured the rate of dehydration of E_1 and E_2 and the rate of isomerization of A_1 and A_2 in dilute sodium hydroxide solutions. These processes exhibited a first-order dependency on hydroxide-ion concentration, with activation energies between 13 and 15 kcal./mole. Using TLC and GLC, he found no compounds other than the expected degradation products during the reactions.

Since stability studies appearing in the literature were of a preliminary nature, providing limited kinetic information, the purpose of this study was to obtain



Scheme I

[1] PGE_1 is 11α,15(S)-dihydroxy-9-oxo-13-*trans*-prostenoic acid; PGE_2 is 11α,15(S)-dihydroxy-9-oxo-5-*cis*,13-*trans*-prostadienoic acid.

additional data dealing with the relative rates of the dehydration and rearrangement reactions of both PGE_1 and PGE_2.

EXPERIMENTAL

UV Spectroscopy—An automatic recording spectrophotometer[3] afforded UV measurement at the two wavelengths of choice at definite intervals over extended periods of time. The instrument was equipped with an automatic reference compensator, a wavelength programmer, and an auxiliary dwell unit. A thermistor probe was attached to the sample compartment so that the cell temperature could be automatically recorded prior to the absorbance reading. The temperature was controlled by a constant-temperature circulator[3].

Stock solutions of prostaglandins were accurately prepared at a concentration of 1.0 mg./ml. in 90% aqueous ethanol and stored at 0°. Measurements were made on triplicate stock solutions. A 75-μl. aliquot of the stock solution was measured in a gastight syringe[4] equipped with a Teflon-tipped plunger and Chaney adaption for reproducible delivery. Four 5.0-ml. quantities of aqueous solvent, measured in volumetric flasks, were pre-equilibrated to the temperature at which the study was to be conducted. One aliquot of PGE stock solution was added to each of three solvent flasks at zero time. The fourth solution served as the reference blank. The final concentration of prostaglandin in the solutions was 4.20×10^{-5} *M*. Because absorbance readings were conducted at 218 nm., it was necessary to take certain precautions; for example, special cells[5] were used which have a high percent transmission below 200 nm. In addition, end absorption due to buffer species was overcome by dilution. The buffers used were sodium dihydrogen phosphate–citric acid (pH 2.2–8.0), glycine–sodium hydroxide (pH 8.6–10.6), glycine–hydrochloric acid (pH 2.2–3.6), and hydrochloric acid–potassium chloride (pH 1.1–2.2). In all but the latter system, the buffer solutions were diluted to approximately 0.002 *M*, while the concentration of the hydrochloric acid–potassium chloride buffer was 0.05 *M* in potassium chloride.

Beer's law plots were made for the aqueous PGE_1 solutions, and the values of the absorptivity of the PGA and PGB species were calculated from aqueous acid and base treatments, respectively (λ_{max}PGA = 218 nm., ϵ_{218} = 9500; λ_{max}PGB = 278 nm., ϵ_{278} = 20,500). The measurement of PGE could be made at any time interval during the degradation by calculating the concentrations of PGA and PGB from the absorbances at 218 and 278 nm., respectively. Overlap of the 278-nm. peaks at 218 nm., however, necessitated a correction factor f for the calculation of $A_{PGA(218)}$. This factor maintained a constant value equal to the ratio of the absorbance of PGB at 218 nm. to its absorbance at 278 nm. Therefore:

$$A_{PGA(218)} = A_{obs(218)} - f \cdot A_{PGB(278)} \qquad \text{(Eq. 1)}$$

where $f = 0.135$. Then:

$$X_E + X_A + X_B = 1 \qquad \text{(Eq. 2)}$$

and:

$$X_E = 1 - \frac{A_{PGA(218)}}{\epsilon_{218} \cdot E_i} - \frac{A_{PGB(278)}}{\epsilon_{278} \cdot E_i} \qquad \text{(Eq. 3)}$$

where E_i = initial molar concentration of E, and X represents mole fraction.

The rate constant k_1 in the degradation sequence (Scheme II)

$$PGE \xrightarrow{k_1} PGA \xrightarrow{k_2} PGB$$

Scheme II

was accurately measured by plotting log X_E *versus* time, where $k_1 = -2.303 \cdot$(slope), or by plotting $\log(X_{A\infty} - X_{A_i})$ *versus* time (only in the case where $k_2 \simeq 0$). The rate constant k_2 was estimated by plotting $\log(X_{B\infty} - X_{B_i})$ *versus* time in the region toward the end of the reaction.

[3] Gilford model 2400-S.
[4] Braun Thermomix II.
[5] Hamilton.
[6] Suprasil.

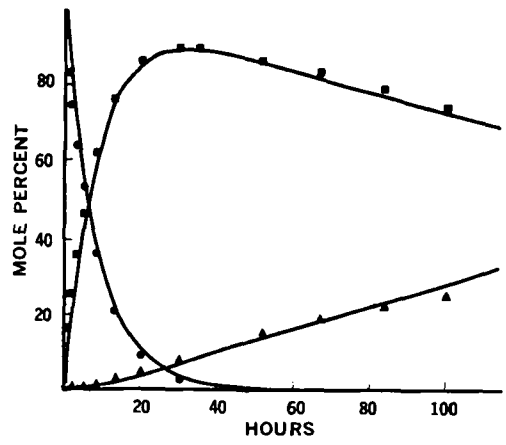


Figure 1—*Reaction profile for $PGE_1 \rightarrow PGA_1 \rightarrow PGB_1$ at pH 8.0 and 60°. Key: ●, PGE_1; ■, PGA_1; and ▲, PGB_1.*

Since the direct measure of B contained the least error, these data points and other required parameters were entered into a computer[6]. By using a consecutive first-order program, the graphically calculated k_1 and k_2 values were used to compute the molar ratios of E, A, and B as a function of time, where:

$$E = E_i e^{-k_1 t} \qquad \text{(Eq. 4)}$$

$$A = E_i k_1 (e^{-k_1 t} - e^{-k_2 t})/(k_2 - k_1) \qquad \text{(Eq. 5)}$$

and:

$$B = E_i[1 - (k_1 e^{-k_2 t} - k_2 e^{-k_1 t})/(k_1 - k_2)] \qquad \text{(Eq. 6)}$$

The average absolute deviation from the observed values was used as a criterion of accuracy for the computed values. The estimated rate constants were varied by an iteration procedure so as to minimize this deviation. The "best fit" values thus obtained were considered to be closer to the true values than were the experimentally observed data calculated from Eqs. 1–3.

TLC—TLC provided a means of checking the initial purity of prostaglandin samples as well as monitoring samples subjected to induced degradation. It also allowed the direct study of PGE's that are transparent to UV light. The chromatographic procedures utilized were extracted from a study of analytical and preparative chromatographic methods for monounsaturated prostaglandins by Andersen (2).

Samples of prostaglandin were prepared for TLC by dissolving PGE in 90% aqueous ethanol at a concentration of 1 mg./ml. Thin-layer plates[7], 15.2 × 20.3 cm. (6 × 8 in.), coated with silica gel GF, were developed in the usual ascending manner following a partial pass in absolute methanol [to approximately 2.54 cm. (1 in.) above the origin] to pull the prostaglandins away from strong base which, if present, interfered with spot mobility. The solvent system used to resolve the parent prostaglandins apart from their degradation products was ethyl acetate–88% formic acid (400:5), and the detection methods were a 5% ethanolic solution of phosphomolybdic acid and 254 nm. UV light.

RESULTS AND DISCUSSION

In the present study the degradation behavior of the two E prostaglandins was investigated under identical buffer conditions covering a wide pH range. A temperature of 60° was chosen to accelerate the decomposition rates so that their measurement would be kinetically convenient. It was assumed that the increased rates were mediated only by thermal phenomena rather than by free radical processes initiated by light or the presence of oxygen.

Since the reaction pathway is of the first-order consecutive type above pH 4, typical concentration *versus* time curves were observed. In Figs. 1 and 2, the change in mole percents of the relevant species with time is shown for the E_1 and E_2 reaction profiles at 60° in pH

[6] Digital PDP-10.
[7] Analtech, Inc.

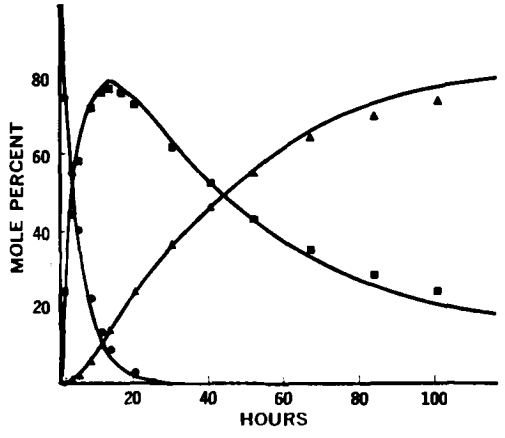


Figure 2—*Reaction profile for $PGE_1 \rightarrow PGA_1 \rightarrow PGB_1$ at pH 8.0 and 60°. Key: ●, PGE_1; ■, PGA_1; and ▲, PGB_1.*

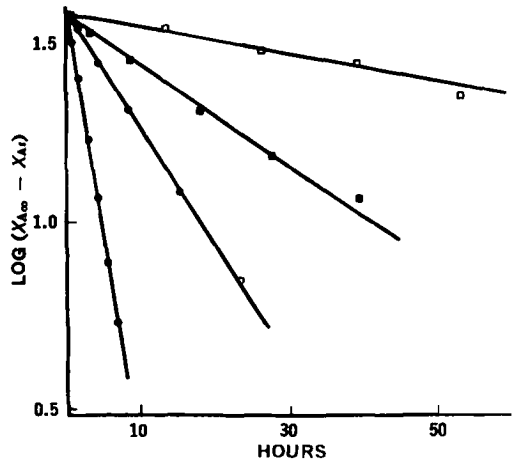


Figure 3—*Dehydration of PGE in acid solutions at 60°. Key: ●, pH 1.22; ○, pH 1.81; ■, pH 2.20; and □, pH 3.01.*

8.0 buffer, respectively. These profiles suggest that the concentration of the original β-hydroxyketone moiety (E) decreased by a first-order rate and that E_2 decreased faster than E_1. The intermediate α,β-unsaturated ketone product (A) rose to a maximum after about 13 hr. for E_2 and after 32 hr. for E_1. In addition, the height of the maximum for A_2 was less than that of A_1 (79% *versus* 89%). After this maximum was reached, the A products decreased in an approximately linear fashion. Following an induction period (3 hr. for B_2 and 10 hr. for B_1), the concentration of the rearranged thermodynamically more stable product (B) rose rapidly and tailed off upon approaching completion of the consecutive reaction. Although mechanistic theory dictates these reactions to be reversible, experimental and resulting thermodynamic considerations showed the reaction to favor overwhelmingly formation of the PGB compounds under all testing conditions, resulting in negligible reverse reaction rate constants.

The initial content of the intact E compound only approached the theoretical concentration of 100%. This was possibly due to a small amount of A compound initially present in the stock solutions. These stock solutions, although stored at zero degrees, showed minor degradation after 1 month. When necessary to obtain an approximate "zero time" at which 100% E was present, the plots were extrapolated to 0% A and 0% B on an appropriately adjusted time axis.

Below pH 4 the concentration of E approached a final value asymptotically as the reaction proceeded toward completion. At the pH levels investigated between 1 and 4, the reaction of A → B became insignificant, while the reaction E → A proceeded by a pseudo-first-order rate law since the solvent (water) was present in large excess. At very high acid concentrations, degradation continued beyond the formation of A, as shown in Scheme III, where X was an unidentified product(s) that formed from either E_1 or E_2 and exhibited a molar absorptivity of approximately 20,000 at a λ_{max} of 325–330 nm. ($R_f \approx 0.8$). Under the conditions of the experiments reported here, $k_1 \gg k_2$, for which reason the A → X reaction was neglected in the kinetic treatment of the E → A reaction. When the dehydration step was treated as a first-order reaction, the values of k_1 could be calculated from the slope of the graph represented by Fig. 3. The reaction was also first order with respect to prostaglandin since little variation in the rate constants was observed upon varying initial reactant concentration.

Above pH 4, however, the rearrangement reaction became significant[8]. In the general consecutive reaction (Scheme II), the E component represented the only component that could be described by a simple first-order equation (Eq. 4). Both the A and B components were biexponential functions which had a time course that was dependent upon both k_1 and k_2 (Eqs. 5 and 6). Data representing A or B could not be analyzed for either k_1 or k_2 by the usual first-order plotting techniques. In general, over the pH range studied the rate of rearrangement was exceeded by the dehydration reaction only by an average factor of 10. While the loss of E was dependent on k_1, the formation of A and B was dependent upon both k_1 and k_2. Thus the overall rate process was not governed by a single constant. For this reason, data collected for A and B as a function of time were first treated graphically to obtain approximate values of k_1 and k_2. These values and other required parameters were then treated by numerical integration on a digital computer to fit calculated values to the observed values of the appearance of B. These values of B were considered to be the most accurate data because of the high sensitivity of the absorbance readings (large absorptivity). The values of A at 218 nm. were frequently subject to interference from end absorption of buffer species present which required a high slit width compensation, contributing greater error to the measurement of A. When the computer-optimized values were plotted as a function of pH, characteristic log rate–pH profiles developed (Figs. 4 and 5).

$$E \xrightarrow{k_1} A \xrightarrow{k_2} X$$

Scheme III

The log k_1–pH profiles for the dehydration reactions of E to A are shown in Fig. 4. The pH of maximum stability in aqueous solutions of E_1 and E_2 was about 3–4. Specific hydrogen-ion catalysis (slope = −1) was evident below pH 3.0 for both E_1 and E_2. The pH-independent region of pH 3–4 probably resulted from solvent catalysis. This pH–rate profile represents the comparative rates observed at identical buffer concentrations less than 0.002 *M* (except at pH < 2.5 where buffer concentration reached 0.05 *M*). At higher buffer concentrations, a study of ionic strength effects

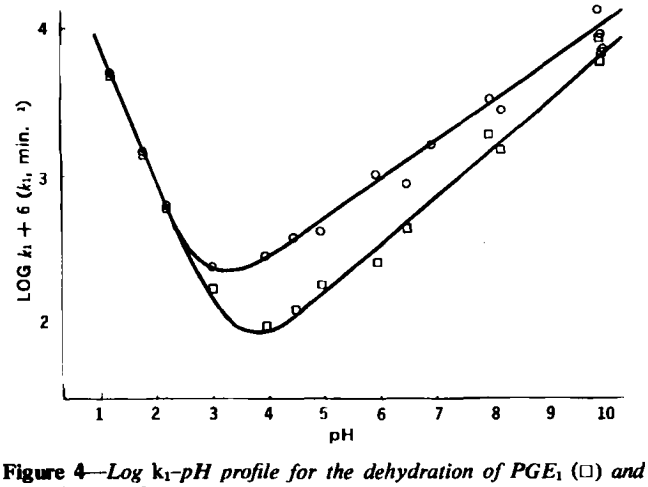


Figure 4—*Log k_1–pH profile for the dehydration of PGE_1 (□) and PGE_2 (○) at 60°.*

[8] Some ester formation could have occurred at the free carboxyl group due to the presence of a small quantity of ethanol in the sample from the stock solution. For present purposes, this esterification was considered to exert an insignificant effect on the rates of dehydration and rearrangement reactions. The occurrence of ester formation was observed on thin-layer chromatograms where the R_f of the ester slightly exceeded that of its corresponding acid.

Table I—Thermodynamic Activation Parameters[a] for Prostaglandins E_1 and E_2

Reaction Type	pH	E_1		E_2	
		E_a, kcal./mole	$\Delta S^{\ddagger}_{298}$, e.u.	E_a, kcal./mole	$\Delta S^{\ddagger}_{298}$, e.u.
Dehydration	1.2	18	−22.4	18	−22.4
Dehydration	8	24	−8.4	24	−7.0
Isomerization	8	20	−25.8	20	−22.3

[a] These are "apparent" values calculated from reaction in dilute aqueous buffer.

was undertaken. Only a minimal effect was noted, however, presumably due to overriding buffer catalysis. No experiments have yet been conducted to extrapolate k values to zero buffer concentration. Therefore, although specific hydroxyl-ion catalysis contributed most significantly to dehydration in alkaline conditions, general acid–base (solvent) catalysis at moderate pH's decreased the slope of the alkaline branch of the catenary to less than +1 (slope ~0.3). The difference in values of the rate constants for the dehydration of E_1 and E_2 under these solvent conditions was evident at alkaline pH's. In general, E_2 dehydrated twice as fast as E_1. Sufficient data were not available at this time to determine if the slopes of the lines were actually parallel. Although the absolute values of k_1 were significantly different, this difference appeared to be relatively independent of pH. At pH 9.0, as in acid media, the rates of reaction were proportional to the initial concentration of reactant, again suggesting first-order dependence on E concentration.

The log k_2–pH profiles for the rearrangement reactions of E_1 and E_2 are represented by the linear graphs shown in Fig. 5. The slopes of these lines were again $<+1$ (0.5 for E_1 and 0.35 for E_2), suggesting that more than simple specific hydroxyl-ion catalysis was involved. Below pH 7.0, the rate of the rearrangement reaction for E_1 became negligible, whereas the rearrangement of E_2 remained significant down to pH 5. In the case of k_2 the relationships of rate and pH between the two E prostaglandins in dilute aqueous buffer were dissimilar. The rate of E_2 rearrangement was significantly faster than E_1 at intermediate pH's, while at high pH their rates became comparable.

From this discussion, no definite conclusions could be reached with respect to the specific mechanisms involved in the reaction. Since k_1 was greater than k_2 only by an order of about 10 over a broad range of pH, the isomerization step could not be considered significantly rate determining. The values of the thermodynamic activation parameters derived from the Arrhenius plot (Fig. 6), listed in Table I, allow some general insight into the mechanistic differences between E_1 and E_2. The differences in their thermodynamic character are attributable to conformational properties that affect molecular orientation during both dehydration and isomerization. Nevertheless, it is difficult to propose a full explanation for the shapes of the reaction profiles at this time.

For the acid-catalyzed dehydration reaction, the slopes of the log rate–pH profiles for E_1 and E_2 were identical (Fig. 4). Furthermore, the rate constants (k_1) under the same conditions were also identical, which implied a similar mechanism of dehydration for both compounds under the given acid conditions. Any intramolecular effect of the carboxyl group on this reaction was presumed negligible because of its lack of physical proximity to the reaction center. Although E_2 is slightly more acidic than E_1 [pKa(E_2) = 5.32, pKa(E_1) = 5.70 as measured in 85% aqueous ethanol], this difference in acidity was considered inconsequential with respect to its influence on reaction rates. Intermolecular participation of the acid moiety also appeared insignificant since studies yielded an invariant rate constant over a moderate range of substrate concentration. The alkaline side of the catenary for the k_1 profile was slightly different for E_1 and E_2. The slopes of their reaction profiles approached the same magnitude, suggesting a constant ratio of k_1 (E_1) to k_1 (E_2), the value of which was approximately 2.5. The apparent energy of activation for E_1 was similar to that of E_2 in the given solvent environment, while their apparent entropies of activation were slightly different. This difference in entropy can be explained by the orientation requirements of solvent molecules around the elimination reaction centers of the two compounds. It is expected that their mechanisms of base-catalyzed dehydration are similar to one another.

In contrast, the slopes in the log rate–pH profile for the rearrangement reactions were markedly different for E_1 and E_2 (Fig. 5). This reaction has two primary driving forces: (*a*) the tetrasubstituted PGB double bond is more stable than that of PGA (6), and (*b*) it contains a system of extended conjugation. The only structural difference between the two molecules is the *cis*-Δ^5 double bond of E_2, which occurs as a saturated bond in E_1. Rotation about the 7–8 carbon–carbon bond could place this double bond of E_2 in a position such that it could interact with the ketone moiety at position 9. Such an interaction, however, would influence the relative reaction rates in the opposite direction to that observed, *i.e.*, inhibit rather than accelerate the E_2 rearrangement with respect to E_1. The apparent minimum energy differences between the reactants and their transition states were identical for both molecules. The apparent

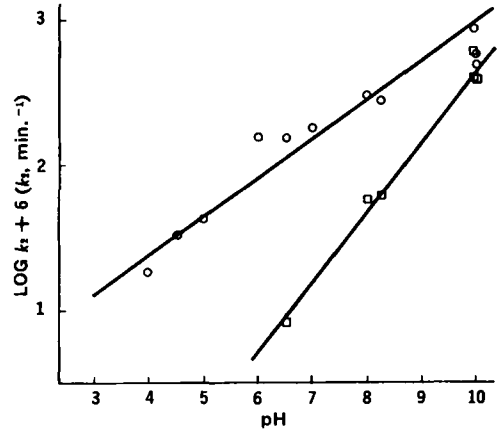


Figure 5—*Log* k_2*–pH profile for the rearrangement of* PGA_1 (□) *and* PGA_2 (○) *at 60°.*

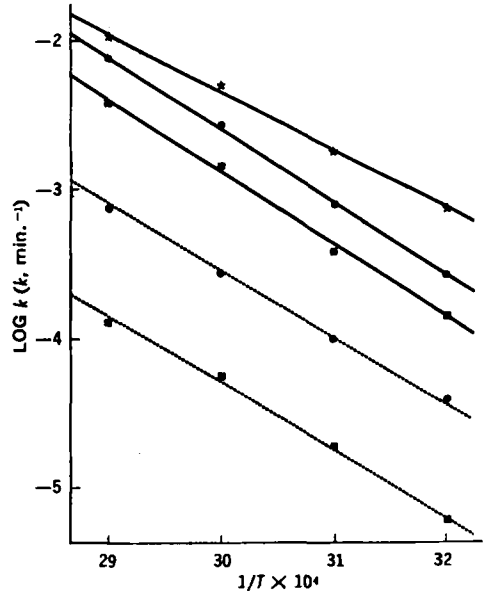


Figure 6—*Arrhenius plot of log* k *against 1/*T *showing temperature dependence of rates of dehydration and rearrangement of* PGE_1 *and* PGE_2. *Key:* ■, PGE_1; ●, PGE_2 *at pH 8.0;* ★, PGE_1 *and* PGE_2 *at pH 1.2;* ——, k_1; *and* - - -, k_2.

entropy of activation values for E_1 and E_2 were different, however, which suggested an explanation for the difference in reaction rates. The large negative values of $\Delta S^{\ddagger}$ reflect a significant requirement in molecular orientation, presumably of the side chains. The *cis*-Δ^5 double bond imposes certain restraints on the free rotation (lowering its degree of freedom) of the C-8 side chain vis-á-vis the C-12 side chain, causing increased nonbonded interactions when both chains lie side by side. These factors become especially important when a carbanion is introduced into the cyclopentenone ring at C-12, forcing the associated side chain and ring to lie flat in one plane. Some of these interactions are relieved if the unsaturation in the C-8 side chain is removed. It then has a more "floppy" nature, which allows it to lie away from the now rigid C-12 side chain. Thus, E_2 contains a lower absolute entropy in its reactant state than E_1, which must accordingly suffer a greater entropy loss during conversion to its carbanion transition state. These interpretations hold only qualitative value since absolute thermodynamic parameters must be determined only at zero buffer concentrations. Nevertheless, the data presented here retain comparative significance and indicate that the difference in behavior between E_1 and E_2 is clearly the result of complicated conformational effects.

In summary, there is a difference in the rates of dehydration and rearrangement between E_1 and E_2. No well-defined mechanism illustrating the reason for this difference can be presented at this time; however, such a mechanism should involve participation of the *cis*-Δ^5 double bond which is present only in E_2.

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Metabolism of the Antihypertensive Agent 1,4-Dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic Acid Diethyl Ester

S. S. WALKENSTEIN▲, A. P. INTOCCIA, T. L. FLANAGAN, B. HWANG, D. FLINT, J. WEINSTOCK, A. J. VILLANI, D. BLACKBURN, and H. GREEN

Abstract □ The absorption, distribution, metabolism, and excretion of 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester, an antihypertensive drug, was studied in rat, dog, and squirrel monkey. The drug, labeled with ^{14}C, was found to undergo aromatization, hydrolysis of its ester groups, and oxidation of a ring methyl and an ethyl group. No dihydropyridyl metabolites or the hypothetical aromatized parent compound were found. The metabolites identified were: 2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid, its monoethyl and mono-2-hydroxyethyl esters, and 2-hydroxymethyl-6-methyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid lactone. The form in which the drug was administered was found to have a profound effect on its absorption. The crystalline powder administered to dogs in gelatin capsules was poorly absorbed; solubilization with polyethylene glycol 200 resulted in a fivefold increase in absorption.

Keyphrases □ 1,4-Dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester, radiolabeled—absorption, distribution, metabolism, and excretion in rats, dogs, and monkeys □ Antihypertensive agents—metabolism of 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester, in rats, dogs, and monkeys □ Absorption—1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester, in rats, dogs, and monkeys

1,4-Dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester[1] (I) is an effective agent for lowering blood pressure by direct vascular dilatation in normotensive, neurogenic, and renal hypertensive dogs. Hypotensive effects were also observed in rats, rabbits, and guinea pigs. The drug also lowered blood pressure in human subjects at relatively low oral doses (10 mg.).

As part of the preformulation and preclinical investigation of this compound, its absorption, distribution, metabolism, and excretion in rats, dogs, and monkeys were studied. These studies were facilitated by the use of the ^{14}C-labeled drug.

EXPERIMENTAL

Synthesis of Labeled Drug—Compound I-4-^{14}C was prepared as shown in Scheme I. ^{14}C-Labeled drug was shown to be free of radiochemical impurity and had a specific activity of 4 μc./mg.

[1] SK&F 24260.

entropy of activation values for E_1 and E_2 were different, however, which suggested an explanation for the difference in reaction rates. The large negative values of $\Delta S^{\ddagger}$ reflect a significant requirement in molecular orientation, presumably of the side chains. The *cis*-Δ^5 double bond imposes certain restraints on the free rotation (lowering its degree of freedom) of the C-8 side chain vis-á-vis the C-12 side chain, causing increased nonbonded interactions when both chains lie side by side. These factors become especially important when a carbanion is introduced into the cyclopentenone ring at C-12, forcing the associated side chain and ring to lie flat in one plane. Some of these interactions are relieved if the unsaturation in the C-8 side chain is removed. It then has a more "floppy" nature, which allows it to lie away from the now rigid C-12 side chain. Thus, E_2 contains a lower absolute entropy in its reactant state than E_1, which must accordingly suffer a greater entropy loss during conversion to its carbanion transition state. These interpretations hold only qualitative value since absolute thermodynamic parameters must be determined only at zero buffer concentrations. Nevertheless, the data presented here retain comparative significance and indicate that the difference in behavior between E_1 and E_2 is clearly the result of complicated conformational effects.

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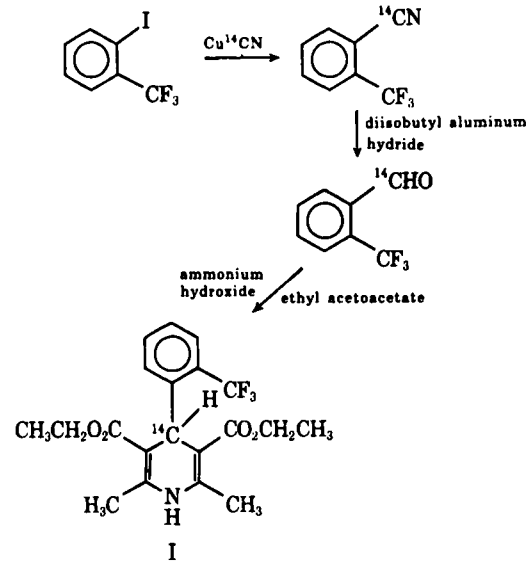
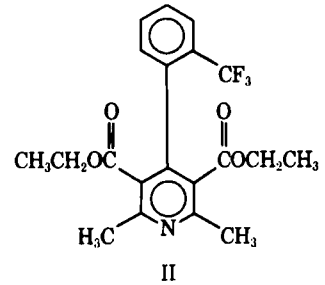


I

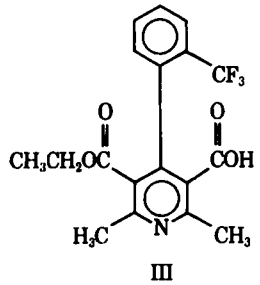
Scheme I—Synthesis of 2,6-dimethyl-3,5-dicarboethoxy-4-(2-trifluoromethylphenyl)-1,4-dihydropyridine-4-^{14}C

GC Assay of I and Metabolites—*Compound I*—Five milliliters of urine containing at least 80 ng. of drug/ml. was adjusted to neutral pH and shaken with 28 ml. *n*-hexane for 20 min. Following centrifugation for 5 min., 25 ml. of the hexane layer was transferred in portions of 10, 10, and 5 ml. to a fresh tube containing 1 ml. methanol. Each portion was blown down, and the sides of the tube were rinsed with methanol before adding a fresh portion. After the final (5 ml.) hexane addition and methanol wash, 0.4 ml. internal standard solution (dichlorodiphenyltrichloroethane, 1.6 mcg./ml. in methanol) was added and the solution was evaporated to dryness under nitrogen in a water bath at 40°. The tubes were kept in a desiccator overnight before derivatizing with an excess of heptafluorobutyric anhydride by heating for 2 hr. at 70°. The samples, after evaporation to dryness at room temperature under a stream of nitrogen, were taken up in 0.5 ml. methanol. A 3-μl. aliquot was taken for injection.

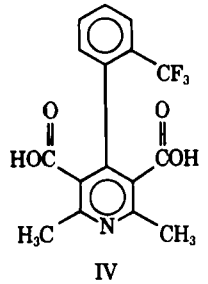
The column was 1.22 m. (4 ft.), glass [0.48-cm. (0.19-in.) o.d.], 3% OV-1, on 100–120-mesh Chrom W at 250°. The carrier gas was argon–methane (95:5); purge 70 ml./min. at 35 ml./min.; purge at 70 ml./min. The electron-capture (Ni) detector temperature was 270°, and the injection block and manifold temperature was 270°. The retention times of I and dichlorodiphenyltrichloroethane were 2.3 and 4.1 min., respectively. The standard curve (peak height ratios) was linear from 80 to 400 ng./ml. urine.



II



III



IV

Table I—R_f Values on Thin-Layer Chromatograms of Compound I and Metabolites

Metabolite[a]	Solvent A	Solvent B	Solvent C
I	0.95	0.24	0.86
II	0.95	0.46	0.90
III	0.75	0.06	0.21
IV	0.46	0.00	0.05
V	0.74	0.00	0.24
VI	0.74	0.00	0.44

[a] See Scheme II.

Table II—Urinary Metabolites of ^{14}C-Labeled I

Metabolite[a]	Dog, 0–24 hr., %	Rat, 0–7 hr., %	Squirrel Monkey, 0–24 hr., %
III	48[b]	68	15.5
IV	7.5	1.5	32
V	9.5	7	4
VI	6	3	3

[a] See Scheme II for structure. [b] Percent of urinary radioactivity.

Compound II[2]—Five milliliters of urine containing at least 1 mcg./ml. of II was adjusted to pH 10 by addition of 2 *N* sodium hydroxide and shaken with 28 ml. ether for 15 min. After centrifuging for 5 min., 25 ml. of the organic phase was transferred to a 40-ml. tapered tube, 0.1 ml. of internal standard (dichlorodiphenyltrichloroethane, 250 mcg./ml. in methanol) was added, and the sample was evaporated to dryness with a stream of nitrogen. The samples were then taken up in 0.1 ml. methanol, and a 2.6-μl. aliquot was taken for injection.

The column was 1.22 m. (4 ft.) stainless steel [0.32-cm. (0.125-in.) o.d.], 3% OV-1 on 100–120-mesh Chrom W at 195°. The carrier gas was helium at 40 ml./min. The flame-ionization detector temperature was 270°, and the injection block temperature was 270°. The retention times of II and dichlorodiphenyltrichloroethane were 3.0 and 6.6 min., respectively. The standard curve was linear from 1 to 5 mcg. II/ml. urine.

Compounds III[3] and *IV*[4]—Five milliliters of urine containing at least 1 mcg. of either III or IV was brought to pH 1 with 6 *N* hydrochloric acid and shaken with 28 ml. of ether for 15 min. After centrifuging for 5 min., 25 ml. of the organic layer were transferred to a 40-ml. tapered tube and evaporated to dryness under a stream of nitrogen. To the dried residue were added 0.1 ml. methanol and 0.3 ml. diazomethane and the contents of the tube were thoroughly mixed. After 1 hr., 0.2 ml. 7 *N* sodium hydroxide and 6 ml. of *n*-hexane were added. The tube was shaken for 20 min. and centrifuged for 5 min. As much of the hexane layer as could be removed with a disposable Pasteur pipet was transferred to a 12-ml. centrifuge tube and, after adding 0.1 ml. of internal standard solution (methyl oleate, 250 mcg./ml. in methanol), evaporated to dryness. The residue was taken up in 0.1 ml. methanol, and a 2.4-μl. aliquot was taken for injection.

Except for the column temperature (180° for these two compounds), the column and conditions were the same as for II. The retention times of III, IV, and methyl oleate were 3.1, 4.1, and 6.1 min., respectively. The standard curves for both compounds were linear from 1 to 5 mcg./ml. urine.

Radioassay—Standard techniques were used to assay 0.1-ml. aliquots of blood and urine and 75–100-mg. aliquots of dried feces.

TLC—Three solvent systems were used to separate I and metabolites on 250-μ plates[5]: A, chloroform–methanol–acetic acid (50:50:5); B, toluene–methanol (95:5); and C, chloroform–acetic acid (95:5). The R_f values found are listed in Table I.

Animal Studies—The animal studies were conducted on Charles River Sprague–Dawley strain rats, squirrel monkeys, and beagle

[2] SK&F 36077.
[3] SK&F 37700.
[4] SK&F 37866.
[5] Merck F_{254}.

Table III—Effect of Dosage Form on Urinary Output of ^{14}C following Oral Administration of ^{14}C-Labeled I

Dosage Form	Dog Number	Percent of Dose Recovered in Urine, 0–96 hr.	Percent of Dose Recovered in Feces, 0–96 hr.
Crystalline drug in gelatin capsule	1	6.9	91.3
	2	7.7	91.9
1.5% solution in polyethylene glycol 200–water (1:1)	3	39	48.9
	4	32	48.9

Table IV—Effect of Dosage Form on Blood Levels of ^{14}C in Dogs after Oral Administration of ^{14}C-Labeled I

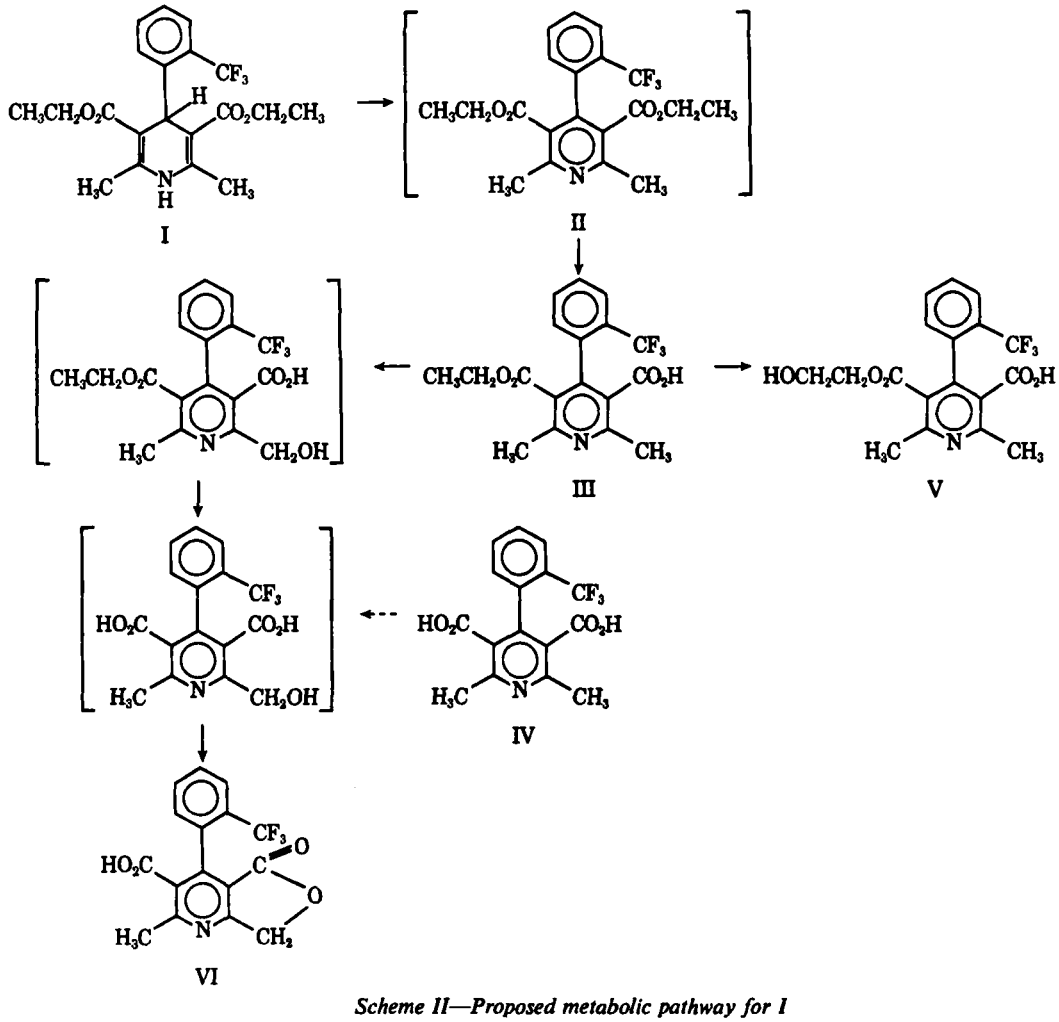
Dosage Form	Dog Number	^{14}C Blood Level (Expressed as ng./ml. of I) Hours 1	2	3	4	6	24
Crystalline drug in gelatin capsule	1	140	150	70	40	30	10
	2	90	140	80	50	40	20
1.5% solution in polyethylene glycol 200–water (1:1)	3	450	920	730	470	260	70
	4	510	910	670	510	360	50

dogs. After an overnight fast, oral doses of suspensions or solutions were administered to rats and squirrel monkeys by gavage. Beagles were given oral doses in gelatin capsules. Polyethylene glycol 200–water (1:1) solutions of drug were used for intravenous dosing. The drug concentration in various dosage media was 1 mg./ml. For collection of urine and feces, animals were kept in metabolism cages.

Enzyme induction studies were conducted in rats kept on an insecticide-free diet for at least 5 days. Following this period and while being maintained on this diet, rats were dosed with a finely ground suspension of drug in 0.5% gum tragacanth, one group receiving a single dose and another group receiving daily doses for 11 days. Amobarbital sleep time and ascorbic acid excretion studies were performed on the same group of rats kept 11 days on the insecticide-free diet and dosed daily with drug (5, 15, or 50 mg./kg.) by the oral route, or with a single intraabdominal dose of phenothiazine (15 mg./kg. in sesame oil) at the end of that period. Ascorbic acid was assayed by a modification of the method of Schaffert and Kingsley (1).

Phenylbutazone plasma half-life studies were conducted in animals kept free of drugs for at least 6 weeks. Phenylbutazone (10 mg./kg.) was injected into a leg vein, and venous blood was sampled from the opposite limb at 0.5, 1, 2, 3, and 5 hr. postdrug. Test drug and phenobarbital groups were dosed for a week and the phenylbutazone treatment was repeated. Plasma assays were performed using the method of Burns *et al.* (2).

For *in vitro* studies, livers were removed from rats kept on the insecticide-free diet. These were divided into groups dosed with saline, I, phenobarbital (chronic dosing), or phenothiazine (single dose). The control (saline) group provided sufficient microsomal enzyme to assay the effects of inhibition or stimulation when I, phenothiazine, or phenobarbital was added directly to the enzyme preparation.



Scheme II—Proposed metabolic pathway for I

Table V—^{14}C Blood Levels and Excretion following Oral Administration of Polyethylene Glycol 200 Solutions[a] of I to Squirrel Monkeys (Expressed as ng./ml. of I)

Animal Number	Blood Level, ng./ml. Hours 1	2	3	4	5	6	24	Percent of Dose Urine, 0–48 hr.	Feces, 0–48 hr.
1	310	510	410	330	340	330	90	8.1	70.6
2	190	320	390	430	410	530	320	11.8	45.5

[a] Polyethylene glycol 200–water (1:1).

Table VI—Effect of I on Drug-Metabolizing Enzyme Systems

Test	Species		Results		
Amobarbital sleep time	Rat		No significant reduction in sleep time as compared with phenobarbital Vehicle: 84 ± 35 min. Phenobarbital: 10 ± 2.1 min. I: 75 ± 30.3 min.		
Ascorbic acid excretion	Rat		Treatment: urinary ascorbic acid in mcg./g. body weight/24 hr. None: 30 I (5 mg./kg.): 42 I (15 mg./kg.): 46 Phenobarbital: 115 (50 mg./kg.)		
Effect of phenylbutazone plasma half-life	Dog		Dosing for 7 days with phenobarbital significantly speeded reduction of phenylbutazone half-life (from 2.5 to 1 hr.); I was without effect		
Liver microsomes *in vitro*	Rat		Phenothiazine, 15 mg./kg.	Compound I, 5 mg./kg.	Compound I, 15 mg./kg.
O-Dealkylation (*p*-nitroanisole)		Single dose	33%[a]	8%	−11%
Phenyl ring hydroxylation (acetanilide)		Single dose	120%	60%	60%
			Sodium phenobarbital, 50 mg./kg.	Compound I, 5 mg./kg.	Compound I, 15 mg./kg.
O-Dealkylation (*p*-nitroanisole)		Chronic dosing	67%	60%	13%
N-Demethylation (aminopyrine)		Chronic dosing	4%	23%	4%
Phenyl ring hydroxylation (acetanilide)		Chronic dosing	100%	78%	27%
Nitro-reduction (*p*-nitrobenzoate)		Chronic dosing	75%	0	0
Aliphatic side-chain oxidation (hexobarbital)		Chronic dosing	300%	225%	22%

[a] Percent increase over saline controls.

The microsomal enzyme was prepared as follows. Freshly removed livers were rinsed in ice-cold 1.15% potassium chloride before homogenization in chilled pH 7.4 0.2 *M* phosphate buffer (2 ml./g. tissue). No more than three to five strokes in an ice-cooled glass homogenizer tube were required. After centrifugation at 9000×*g* for 30 min. at 2–3° and removal of a white layer from the top of the tube, the supernate was decanted for use. This preparation maintained its activity even after being kept 5 months in the frozen state.

Incubations were carried out in 30-ml. beakers in a Dubnoff shaker–incubator at 37°, 70–75 oscillations/min. Oxygen was used as the gas phase in all but the nitro-reductase experiment, in which nitrogen was used. Gas flow was kept at 1 l./min. Each beaker contained 1 ml. of the enzyme preparation, 25 μM glucose-6-phosphate, 1–2 μM nicotinamide adenine dinucleotide phosphate, 34.5 μM magnesium sulfate, substrate (quantities shown below), and 0.2 *M* pH 7.4 sodium phosphate buffer to a final volume of 5 ml. In the *N*-dealkylation reaction assay with aminopyrine, 45 μM semicarbazide was included in the reaction mixture. The enzyme preparation was added last, following a 10-min. incubation period. The reaction was then followed for varying periods: aminopyrine, 5 min.; acetanilide, 5 min.; *p*-nitrobenzoic acid, 10 min.; *p*-nitroanisole, 3 min.; and hexobarbital, 5 min.

Analytical methods used in the *in vitro* studies were as follows. *N*-Demethylation of aminopyrine was determined by trapping formaldehyde derived from the methyl group in semicarbazone and by the Cochin and Axelrod (3) modification of the Nash method. Ring hydroxylation of acetanilide was determined by the method of Brodie and Axelrod (4). Reduction of *p*-nitrobenzoic acid to *p*-aminobenzoic acid was determined by measurement of the latter using the Bratton–Marshall reaction (5). *O*-Demethylation of *p*-nitroanisole was followed by spectrophotometry of the product, *p*-nitrophenol, at 396 nm. (6). Disappearance of hexobarbital was determined by the method of Brodie *et al.* (7).

RESULTS AND DISCUSSION

Biotransformation[6]—The ester linkages of I are highly resistant to hydrolysis, withstanding boiling in the presence of acid or base or incubation in the presence of various enzyme preparations. However, upon oxidation of the dihydropyridyl ring, hydrolysis takes place readily. This was borne out by the failure to find in

[6] Details to be published separately.

Table VII—Distribution of ^{14}C in Blood of Human, Dog, and Rat following Equilibration with ^{14}C-Labeled I *In Vitro*

Species	Percent of Added Drug Found		
	Plasma Proteins	Cells	Plasma Water
Human	83 ± 5	16 ± 5	0.9 ± 0.7
Dog	86 ± 7	13 ± 8	0.8 ± 0.3
Rat	91 ± 4	8 ± 4	1.4 ± 0.3

urine any aromatized parent drug (II), the expected intermediate (Scheme II). Nevertheless, both expected deesterified metabolites were found (III and IV). Two remaining metabolites, both unanticipated, were the results of β-oxidation of an ethyl group (V) and oxidation of a 2-methyl group which apparently underwent spontaneous lactonization with an adjacent carboxyl (VI), respectively.

Quantitative differences in the metabolites were observed in the urine of three species, with III predominating in the rat and dog and IV in the squirrel monkey. Both metabolites, however, account for half or more of the urinary ^{14}C (Table II).

The relative amounts of the dose excreted in urine and feces were highly dependent upon the dosage form. When the drug was given to dogs as the crystalline drug (in gelatin capsules), only about 7% was recovered in urine. However, when dissolved in a mixture of polyethylene glycol 200–water (1:1), a four- to fivefold increase in urinary output was observed, indicating enhanced absorption of the solubilized drug (Table III).

Blood levels were correspondingly higher in the animals dosed with the solution (Table IV). The mean blood level over the 24-hr. period following the dose of the polyethylene glycol 200 solution of drug was almost seven times that attained in the dogs dosed with crystalline drug. Peak levels in the dog dosed with either form appeared at about 2 hr. Similar results were seen in one squirrel monkey following doses of polyethylene glycol solutions of drug (Table V), but another monkey showed a delayed peak at 6 hr.

Irrespective of dosage form, the major portion of ^{14}C excreted in feces of the dog and squirrel monkey appeared from TLC to be the parent drug, which accounted for roughly half of the radioactivity excreted by this route.

Enzyme Induction Studies—Because I is intended for a condition requiring chronic treatment, it was of interest to determine its effect during chronic use on metabolizing enzymes, since either inhibition or stimulation of the enzymes could conceivably alter the physiological response to a given dose. Accordingly, a number of widely used tests were run and the results are summarized in Table VI.

These tests indicated that a weak to moderate induction of some metabolic pathways (ascorbic acid excretion, phenyl ring hydroxylation, *O*-dealkylation, *N*-demethylation, and aliphatic side-chain oxidation) occurred following oral administration of I. The reverse effect, inhibition of the same microsomal enzyme systems, was also tested *in vitro* by incubation of I with the liver microsomal preparations and the various test compounds referred to in Table VI. Compound I was found to be without significant effect on the metabolic pathways investigated.

Protein Binding Studies—Because of the possible influence of binding to plasma proteins on the sojourn and distribution of the drug following absorption, it was of interest to determine the extent to which binding could occur. The results (Table VII) indicate that very little (about 1%) drug remains "free" in the plasma of human, dog, or rat blood; over 80% was bound to protein and the remainder to cellular components.

These findings may not, however, be of much significance since, at times of peak ^{14}C concentrations in blood following an oral dose, only about one-quarter of the low levels present could be accounted for as parent drug (Table VIII) in the rat and squirrel monkey and

Table VIII—^{14}C "Fingerprint" of Animal Blood following Oral Dose of ^{14}C-I[a]

Species	^{14}C Peak Time, hr.	^{14}C Concentration, mcg./ml.	Percent of Dose on TLC at R_f of Metabolites			
			Parent	II	III	IV–V[b]
Rat	7	1.81	30	59	1	5
Dog	2	0.91	2	7	10	45
Squirrel monkey	3	0.40	24	19	26	6

[a] 5 mg./kg. in polyethylene glycol 200–water (1:1). [b] Expressed as equivalent amounts of I.

only 2% in dog. Only the more significant metabolites are shown; at least six or eight additional metabolites appearing on the radioscans were not identified.

CONCLUSION

Compound I, as a consequence of its poor aqueous solubility, was found to require prior solubilization to enhance absorption. It appears to be rapidly metabolized in the species investigated (rat, dog, and squirrel monkey), which may account for its relatively short duration of action[7]. None of the metabolites identified nor the postulated intermediates was found in animal studies[8] to approach the hypotensive effect of the parent drug in potency. Because of the observed weak inductive effect on some of the drug-metabolizing enzyme systems, subjects treated concomitantly with other drugs should be observed for the possibility of drug interactions.

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▲ To whom inquiries should be directed.

[7] B. Walker, preliminary clinical observations.

[8] J. Petta and R. McLean, unpublished observations.

Effect of 3-Phenylpropan-1-ol, 2-Phenylethanol, and Benzyl Alcohol on *Pseudomonas aeruginosa*

R. M. E. RICHARDS[▲] and R. J. McBRIDE

Abstract □ 3-Phenylpropan-1-ol, 2-phenylethanol, and benzyl alcohol were investigated for their inhibitory action against *Pseudomonas aeruginosa*. 3-Phenylpropan-1-ol was the most effective and benzyl alcohol was the least effective as shown by: (*a*) growth rate studies using subinhibitory concentrations of the alcohols, (*b*) determination of minimum inhibitory concentrations, and (*c*) determination of sterilization times. The three compounds enhance the bactericidal action of benzalkonium chloride against *P. aeruginosa* in the same ranking order. It is suggested that 3-phenylpropan-1-ol may be a suitable preservative for oral suspensions and mixtures.

Keyphrases □ 3-Phenylpropan-1-ol effect on *Pseudomonas aeruginosa*—compared to 2-phenylethanol and benzyl alcohol with/without benzalkonium chloride □ 2-Phenylethanol effect on *Pseudomonas aeruginosa*—compared to 3-phenylpropan-1-ol and benzyl alcohol with/without benzalkonium chloride □ Benzyl alcohol effect on *Pseudomonas aeruginosa*—compared to 3-phenylpropan-1-ol and 2-phenylethanol with/without benzalkonium chloride □ Bactericidal activity—comparison of 3-phenylpropan-1-ol, 2-phenylethanol, and benzyl alcohol with/without benzalkonium chloride □ *Pseudomonas aeruginosa* inhibition—effect of 3-phenylpropan-1-ol, 2-phenylethanol, and benzyl alcohol with/without benzalkonium chloride

As the result of a survey of the contamination of pharmaceutical preparations, Hooper (1) stated that: "... aqueous medicines containing no preservative were very favourable breeding grounds for water-borne Gram-negative bacteria including Pseudomonas/Alcaligenes/Achromobacter groups. Mixtures such as magnesium trisilicate, aluminum hydroxide, ammonia and ipecacuanha, kaolin, magnesium carbonate, magnesium sulphate and magnesium hydroxide were often found to contain enormous numbers of organisms, counts of 10^6 per ml were often commonplace." Thus there is an urgent need for a preservative suitable for inclusion in such preparations.

The aim of this present investigation was to compare 3-phenylpropan-1-ol[1], 2-phenylethanol, and benzyl alcohol, both as single compounds and in combination with benzalkonium chloride, for their effects on *Pseudomonas aeruginosa*.

The initial member of this series of phenyl-substituted aliphatic alcohols is benzyl alcohol, which has been used for its antibacterial and analgesic properties in injections over the past 20 years. Gershenfeld (2) stated that, in a concentration of 1% or more, benzyl alcohol is bacteriostatic to a number of organisms. Its sharp burning taste, however, curtails its usefulness in oral products (3).

2-Phenylethanol is active against Gram-negative organisms (4) and is compatible with many ophthalmic solutions (5). Therefore, it is recommended in USP XVIII (6) and NF XIII (7) for preserving ophthalmic solutions. 2-Phenylethanol also has been shown to enhance the activities of a number of preservatives commonly used in ophthalmic solutions (8–13).

3-Phenylpropan-1-ol, the homolog of 2-phenylethanol having the synonym hydrocinnamyl alcohol, is a liquid with a cinnamon-like odor and is slightly soluble in water. The antibacterial properties of this compound have not been reported previously, but it was thought that it may prove to be a suitable preservative for mixtures and suspensions.

EXPERIMENTAL

Materials—Benzyl alcohol[2], 2-phenylethanol[2], and 3-phenylpropan-1-ol[2] were of laboratory reagent grade, and benzalkonium chloride BP[3] was used. The liquid growth medium was oxoid[4] nutrient broth No. 2 and the incubation temperature was 37°. *P. aeruginosa* NCTC 6750[5] was the test organism and was maintained as stab cultures in oxoid[4] nutrient agar at a temperature of 4°. The inactivating medium was that described by Riegelman *et al.* (14).

Methods—The evaluations used either log phase cultures for growth rate studies or overnight cultures for determinations of

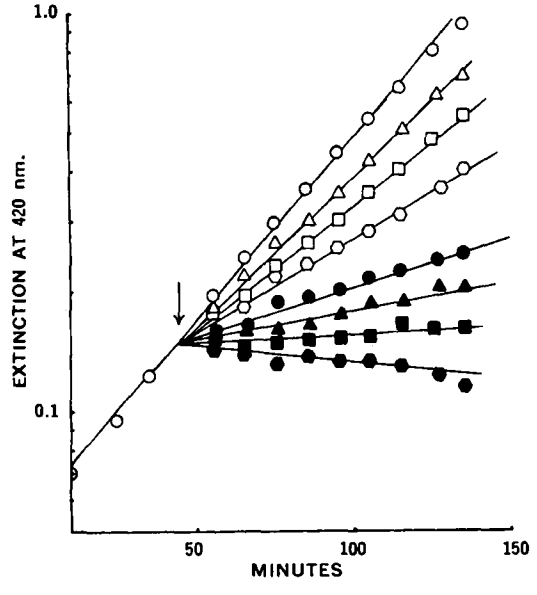

Figure 1—*Effect of various concentrations of phenylethanol on the growth of* P. aeruginosa *NCTC 6750. Sufficient phenylethanol was added at the time indicated by the arrow to give the final concentrations as follows:* ○, *no phenylethanol added;* △, *0.05% phenylethanol;* □, *0.1% phenylethanol;* ○, *0.15% phenylethanol;* ●, *0.2% phenylethanol;* ▲, *0.25% phenylethanol;* ■, *0.3% phenylethanol; and* ●, *0.35% phenylethanol.*

[1] Patent applied for.

[2] British Drug Houses, Poole, Dorset, England.
[3] MaCarthy Ltd., Glasgow, Scotland.
[4] Oxo Ltd., London, England.
[5] National Collection of Type Cultures, Colindale, London, England.

Table I—Minimum Inhibitory Concentrations for 3-Phenylpropan-1-ol, 2-Phenylethanol, and Benzyl Alcohol against *P. aeruginosa* NCTC 6750 in Nutrient Broth at 25°

Alcohol	Minimum Inhibitory Concentration, %—Log Phase Cells	Minimum Inhibitory Concentration, %—1.9×10^7 Overnight Cells/ml.
Benzyl alcohol	0.41	0.42
2-Phenylethanol	0.30	0.34
3-Phenylpropan-1-ol	0.27	0.26

growth inhibition and cell death. Cell numbers were estimated by colony counts as previously described (13).

Effect on Growth Rate—Log phase cultures were prepared by inoculating 100 ml. prewarmed nutrient broth with 1 ml. of an overnight culture, prepared from a stored stab culture of *P. aeruginosa*, and incubating in a shaking incubator[6] at 100 throws a minute. (Each stab culture was used once only.) Growth was followed by measuring the absorbance of samples in a spectrophotometer[7] at 420 nm. When the absorbance reading was 0.3–0.35, 1-ml. samples were transferred to each of the required number of 100-ml. quantities of prewarmed broth in 250-ml. conical flasks. Chemicals were subsequently added to these log phase cultures when the absorbances were approximately 0.1.

Effect of Alcohol Concentration—Varying concentrations of one of the alcohols were added to a number of similar cultures and simultaneously an equivalent volume of water was added to the control culture. (All liquids, as well as the pipets used in making their addition, were prewarmed to 37°.) The growth in each culture was followed by making absorbance measurements over the next 1.5–2.5 hr. The log of the absorbance so obtained for each concentration of alcohol was graphed against the time over which the determinations were made to give a result similar to that in Fig 1. By using the method of least squares, the equation for each line was calculated to obtain the slope which is the growth rate. The growth rate for each concentration, when expressed as a percentage of the growth rate of the control culture, was then plotted against the concentration of alcohol to produce a graph such as Fig. 2. The point where the baseline is crossed indicates the concentration of alcohol that will inhibit log phase cultures of *P. aeruginosa*, and these values are given in Table I.

Comparative Activity of Alcohols—To three log phase cultures, sufficient benzyl alcohol, 2-phenylethanol, and 3-phenylpropan-1-ol were added to give final concentrations of 0.178, 0.2, and 0.223% (w/v), respectively. An equivalent volume of water was added to the control culture. The concentrations used gave equimolar concentrations of the three alcohols. The growth in all flasks was followed as already described; the results are expressed in Fig. 3.

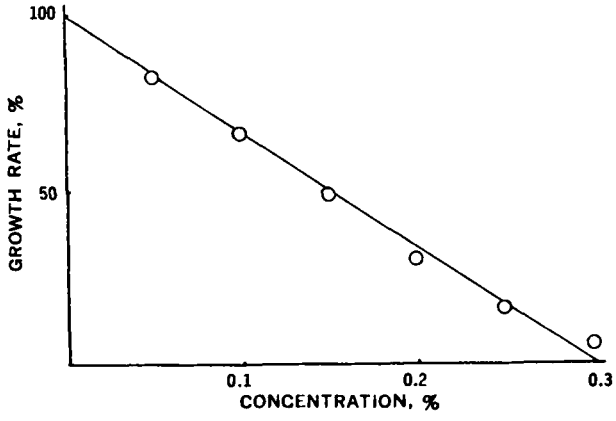

Figure 2—*Correlation between subinhibitory concentrations of phenylethanol and growth rate of* P. aeruginosa *NCTC 6750 in subinhibitory concentrations of phenylethanol expressed as a percentage of the growth rate in the absence of phenylethanol.*

[6] Mickle Engineering Co, Gomshall, Surrey, England.
[7] Unican SP600, Pye Unicam Ltd., Cambridge, England.

Table II—Sterilization Times at 25° for 5.6×10^6 Cells/ml. *P. aeruginosa* NCTC 6750 in Aqueous Solutions of Benzyl Alcohol, 2-Phenylethanol, and 3-Phenylpropan-1-ol Alone and in Combination with Benzalkonium Chloride, 0.01% (w/v)

Antibacterial Concentrations, %	Sterilization Time, min.—Single Substance	Sterilization Time, min.—Combination
Benzalkonium chloride, 0.01	50–60	—
Benzyl alcohol, 0.4	>24 hr.	25–30
2-Phenylethanol, 0.4	>24 hr.	20–25
2-Phenylpropan-1-ol, 0.4	75–90	<5

Alcohol–Benzalkonium Combinations—To eight log phase cultures the following were added: (*a*) water; (*b*) benzalkonium chloride, 0.003%; (*c*) benzyl alcohol, 0.175%; (*d*) 2-phenylethanol, 0.175%; (*e*) 3-phenylpropan-1-ol, 0.175%; (*f*) benzyl alcohol, 0.175%, and benzalkonium chloride, 0.003%; (*g*) 2-phenylethanol, 0.175%, and benzalkonium chloride, 0.003%; and (*h*) 3-phenylpropan-1-ol, 0.175%, and benzalkonium chloride, 0.003%. The growth of each culture was followed as before and the results form Fig. 4.

Effect on Growth Inhibition and Cell Death—*Minimum Inhibitory Concentration*—A series of concentrations of benzyl alcohol, 2-phenylethanol, and 3-phenylpropan-1-ol were prepared in 10-ml. volumes of nutrient broth. Each tube was inoculated with 0.1 ml. overnight culture of *P. aeruginosa* to give a final viable concentration of approximately 1.9×10^7 cells/ml. The tubes were incubated for 5 days and observed for growth daily. The lowest concentration of each alcohol showing no growth is given in Table I.

Sterilization Times—Tubes containing 9.9-ml. volumes of the aqueous solutions under test were equilibrated in a water bath at 25°, after which 0.1 ml. of overnight culture of *P. aeruginosa* was added to give a final inoculum of approximately 5×10^6 cells/ml. At intervals of 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, and 120 min. and 24 hr. after the addition of the inoculum, 0.5-ml. samples were added

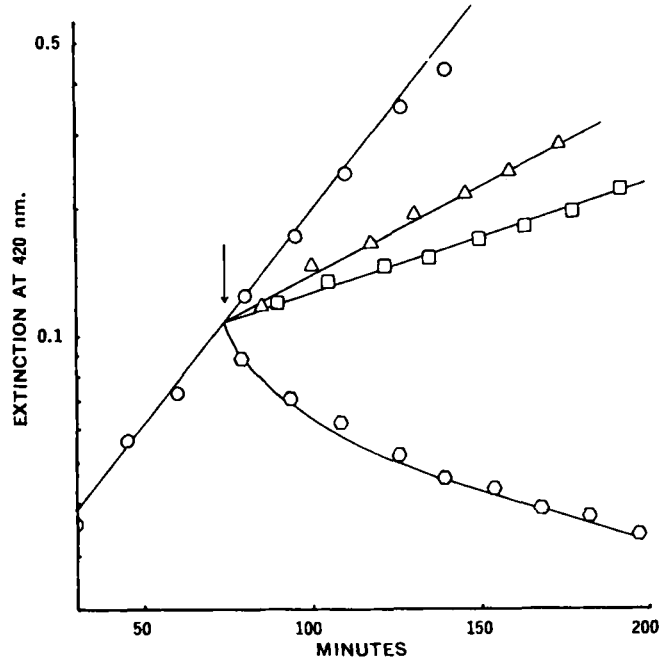

Figure 3—*Comparison of the effect of equimolar concentrations of benzyl alcohol, phenylethanol, and 3-phenylpropan-1-ol on the growth of* P. aeruginosa *NCTC 6750. Sufficient alcohol was added at the time indicated by the arrow to give a final concentration of* 1.6×10^{-3} M. *Key:* ○, *no alcohol;* △, *benzyl alcohol;* □, *phenylethanol; and* ○, *3-phenylpropan-1-ol.*

to 9.5 ml. of inactivating recovery medium and incubated for 3 days. Positive controls were prepared by adding 0.1 ml. of a 10^6 dilution in nutrient broth of an overnight culture of *P. aeruginosa* to tubes of recovery medium separately containing 0.5 ml. of each of the preservative solutions under test; all showed growth. The results are tabulated in Table II.

RESULTS AND DISCUSSION

After the addition of subinhibitory concentrations of either of the three alcohols, the log phase cultures continued to grow exponentially but at a rate dependent on the alcohol concentration. Typical results for phenylethanol are given in Fig. 1. Lang and Rye (15), using a different experimental technique, investigated the growth inhibitory properties of benzyl alcohol and phenylethanol against *Escherichia coli*. These workers found that growth occurring in the presence of benzyl alcohol was exponential, but that the growth rate in the presence of phenylethanol occurred at a gradually decreasing rate throughout the period of incubation. It was not apparent why the response of the *E. coli* to the action of the alcohols was different.

The concentration of each alcohol causing inhibition of growth of log phase cultures was then determined by plotting the growth rate for each concentration of alcohol, as a percentage of the growth rate of the control containing no alcohol, against the alcohol concentration. Figure 2 shows the results for phenylethanol, and the same linear response was obtained with the other two alcohols; these findings demonstrate the effectiveness of these antibacterials under the conditions of the test. Unpublished results[8] with benzalkonium chloride, however, showed a different response pattern to that of the alcohols. When the percentage of the control growth rate was plotted against the concentration of benzalkonium chloride, a straight line was obtained at low concentrations but this became asymptotic at higher concentrations. This lack of linearity shows that the antibacterial action of benzalkonium chloride is inadequate under the conditions of the growth rate experiments using *P. aeruginosa* as the test organism. This technique might prove useful in determining the suitability of a given antibacterial for use as a preservative against a given organism.

The concentrations of the alcohols causing inhibition of log phase cultures of *P. aeruginosa* are given in Table I, together with the minimum inhibitory concentrations for overnight cultures. The closeness of the two sets of values indicates that the alcohols have a uniform activity against *P. aeruginosa* cells in different phases of the growth cycle.

Figure 3 shows that at a concentration of 1.6×10^{-3} *M*, 3-phenylpropan-1-ol is more active against log phase *P. aeruginosa* than 2-phenylethanol, which is more active than benzyl alcohol.

2-Phenylethanol has been shown to enhance the antibacterial effect of benzalkonium chloride on log phase cultures of *P. aeruginosa* (10) and to give shorter sterilization times in ophthalmic preparations (11). Figure 4 shows that all of the alcohols increase the effect of benzalkonium chloride. Again 3-phenylpropan-1-ol is the most effective and benzyl alcohol is the least effective. This result is confirmed by the sterilization times for aqueous solutions of the preservatives alone and in combination with 0.01% benzalkonium chloride against large inocula of *P. aeruginosa* (Table II). 3-Phenylpropan-1-ol sterilized in less than 90 min., but phenylethanol and benzyl alcohol both gave positive samples after 24 hr. No growth was detected in any of the samples from the 3-phenylpropan-1-ol–benzalkonium chloride combination.

SUMMARY AND CONCLUSION

3-Phenylpropan-1-ol is more effective than 2-phenylethanol which, in turn, is more effective than benzyl alcohol in inhibiting the growth of *P. aeruginosa* and in enhancing the activity of benzalkonium chloride against *P. aeruginosa*. The compound 3-phenylpropan-1-ol with its cinnamon-like odor is quite pleasant at a concentration of 0.4% in water and may be suitable, either alone or in combination, for use in the preservation of oral preparations.

[8] R. M. E. Richards and R. J. McBride.

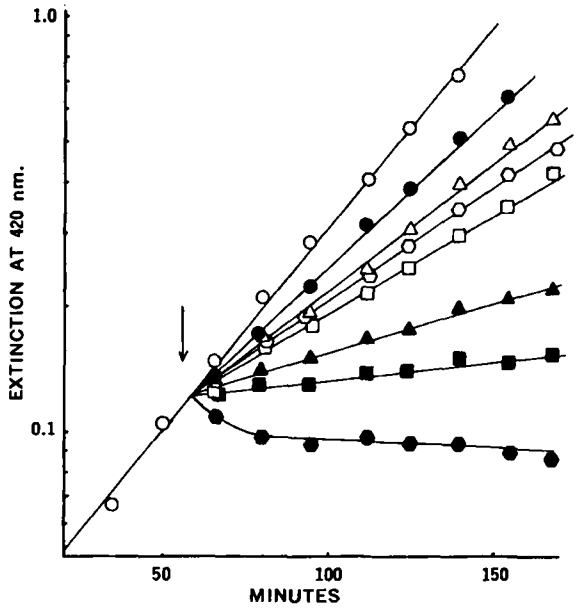

Figure 4—*Effect of 0.175% benzyl alcohol, 0.175% phenylethanol, and 0.175% 3-phenylpropan-1-ol, alone and in combination with 0.003% benzalkonium chloride, on the growth of* P. aeruginosa *NCTC 6750. The substances were added at the time indicated by the arrow. Key:* ○, *water;* ●, *benzalkonium chloride;* △, *benzyl alcohol;* ▲, *benzyl alcohol and benzalkonium chloride;* □, *phenylethanol;* ■, *phenylethanol and benzalkonium chloride;* ○, *3-phenylpropan-1-ol; and* ●, *3-phenylpropan-1-ol and benzalkonium chloride.*

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Ergoline Alkaloidal Constituents of Hawaiian Baby Wood Rose, *Argyreia nervosa* (Burm. f.) Bojer

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Abstract □ Chemical analysis showed that the seeds of *Argyreia nervosa* contain the highest percentage of indole alkaloid constituents (0.5–0.9%) of the genera of the Convolvulaceae thus far studied. A total of 19 indole alkaloids were identified by thin-layer and paper chromatographic procedures. Of these, lysergene, festuclavine, setoclavine, isosetoclavine, agroclavine, elymoclavine, ergine, and isoergine were isolated by column chromatographic procedures and characterized by TLC and IR analyses. Penniclavine, chanoclavine-I, chanoclavine-II, ergometrine, ergometrinine, lysergic acid α-hydroxyethylamide, isolysergol, racemic chanoclavine-II, molliclavine, lysergol, and isolysergic acid α-hydroxyethylamide were identified by TLC only. Of these, lysergene, setoclavine, isosetoclavine, chanoclavine-II, racemic chanoclavine-II, isolysergol, and molliclavine were identified for the first time in species of the Convolvulaceae. Ergine (0.136%) and isoergine (0.188%) were found in the highest concentration. In addition, 11 unidentified indole alkaloids were detected, these being found in very low concentration. Although the pericarp showed the same alkaloid pattern as the seeds, the concentration was much lower (0.0015%). No alkaloids could be detected in the vegetative tissues of nonflowering specimens.

Keyphrases □ Alkaloidal constituents, *Argyreia nervosa* seeds—isolation, identification of 19 indole alkaloids □ *Argyreia nervosa* (Burm. f.) Bojer seeds—isolation, identification of 19 indole alkaloids □ Morning-glory seeds—isolation, identification of 19 indole alkaloids □ Ergoline alkaloids—isolation, identification from *Argyreia nervosa* (morning-glory)

Argyreia nervosa (Burm. f.) Bojer is a member of the Convolvulaceae (morning-glory family) and is characterized by heart-shaped leaves with dense, white, silky hairs beneath. The plant is believed to have its origin in India. Historically, its roots have been used by the Hindus as an alternative, tonic, antirheumatic and in the treatment of diseases of the nervous system (1). The leaves have been used as a local stimulant, rubefacient, and vesicant. Because the under surface of the leaf is covered by a thick layer of silky hairs, it has been used as a natural impermeable temporary covering and poultice for minor skin abrasions by natives in India. However, Dymock (2) reported the leaves to be ineffective for this purpose.

This species is commonly and widely cultivated as a garden plant in several tropical countries because of its showy flowers. The mature capsules are handsome and persistent and are widely used in ornamental dried-flower arrangements. To date, no significant pharmacological or phytochemical studies on *A. nervosa* have appeared in the literature.

Since Aztec times in the uplands of southern Oxaca in Mexico, the seeds of *Rivea corymbosa* (known as "Ololiuqui") and *Ipomoea violacea* (known as "badoh negro") have been used for divinatory and hallucinatory purposes during religious ceremonies. The native use of these seeds for ceremonial purposes was extensively reported by Schultes (3–5) and Wasson (6). Following these publications, there occurred a rash of use of morning-glory seeds for hallucinatory purposes in the United States.

Among the morning-glories that have become quite popular is *A. nervosa* (Hawaiian Baby Wood Rose); it is readily available from the southern parts of California and Florida as well as from Hawaii. The availability of this plant in the United States has led to its use by juveniles who seek hallucinatory experiences through ingestion of the seeds. Contributing to its popularity among juveniles is the potency of seeds of *A. nervosa* compared to seeds of ipomoea species. To achieve a psychotomimetic response, relatively few seeds of *A. nervosa* are needed as opposed to a few hundred of *I. violacea*. Hylin and Watson (7) reported that each gram of seeds contains approximately 3 mg. of total alkaloids, of which 0.36 mg. is represented by one of the psychoactive constituents, ergine. The results from the present study indicate that the seeds contain even higher concentrations of ergine and total indole alkaloid constituents.

Prior to 1960, ergoline-type alkaloids were known to occur only in certain lower fungi, particularly the genus claviceps. Hofmann and Tscherter (8) were the first to find and isolate these ergoline alkaloids from higher plants. Later, several investigators (9–37) showed a wider distribution of the ergoline-type alkaloids in the Convolvulaceae (morning-glory family). These currently include the genera rivea, ipomoea, argyreia, cuscuta, and stictocardia.

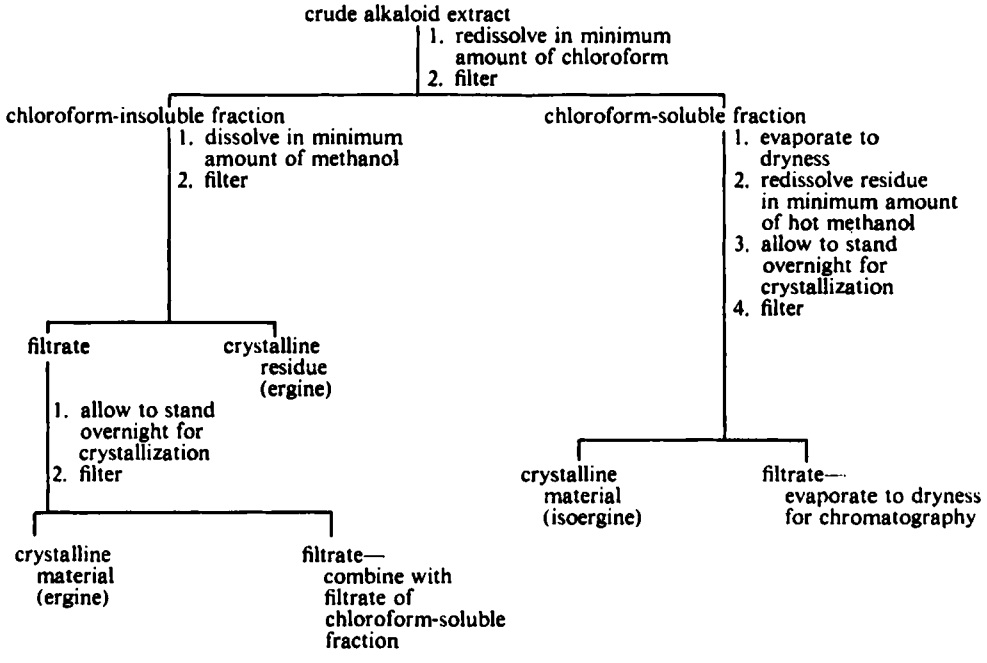
Noteworthy among the genera is argyreia because it possesses very high concentrations of a large number the ergoline alkaloids and until now it has not been subjected to extensive chemical studies. Only two compounds—*viz.*, ergine (lysergic acid amide) and isoergine (isolysergic acid amide), have been identified in *A. nervosa*, by Hylin and Watson (7) using TLC. Recently these two compounds were confirmed by McJunkins *et al.* (35) and isolated by Miller (37).

EXPERIMENTAL

Extraction of Crude Alkaloids and Isolation of Ergine and Isoergine—The total alkaloid was extracted by using a modification of the method of Genest (27). Due to the high concentrations of ergine and isoergine, they could be easily separated first without involving the chromatographic procedure. The diagrammatic isolation procedure is shown in Scheme I.

TLC—The following solvent systems were used for TLC: 1, silica gel plates, methanol–chloroform (20:80); 2, silica gel plates, diethylamine–chloroform (10:90); 3, silica gel plates, methanol–chloroform–concentrated ammonia (20:80:0.2); 4, silica gel plates, ethyl acetate–ethanol–dimethylformamide (86:7:7); 5, alumina plates, ethanol–chloroform (4:96); and 6, alumina plates, chloroform–benzene–glacial acetic acid (45:45:10).

For two-dimensional TLC, the method of Agurell (38) was used. A sample was spotted in the left-hand corner of the plates, 1.5 cm. from each side. The chromatograms were developed first in Solvent



Scheme I—Procedure for isolation of ergine and isoergine

System 1 and, after air drying for 5 min., were run in the second direction in Solvent System 2.

The fluorescent alkaloids were detected using both short- and longwave UV light. Ehrlich's spray reagent was used to locate all indole alkaloids. Dragendorff's reagent was used for general alkaloid detection.

Paper Chromatography—The method reported by Pöhm (39) was used for paper chromatography. The R_A value was calculated by using the R_f value of agroclavine as R_A 100. The solvent system is abbreviated as FBP.

Fractionation of Crude Alkaloids Using Column Chromatography—In these experiments, a minimum of 1.5 g. of crude alkaloid mixture (after ergine and isoergine were isolated) was redissolved in 50 ml. of a chloroform–benzene (1:1) mixture (containing 2% of methanol) and then transferred to a 100 × 2.5-cm. glass column. The column was previously packed with 90 cm. of a thick slurry of an alumina[1] and chloroform–benzene (1:1) mixture. Elution was started with 200 ml. of 50% chloroform in benzene and then developed by increasing the polarity (increasing the percentage of chloroform) in the solvent system. The procedure used was to increase the chloroform percentage by 5% for every 200 ml. of eluent collected until the solvent system contained no benzene. Methanol was then used to increase the polarity of chloroform by increasing its concentration at a rate of 1% for every 200 ml. of solvent collected until the solvent system reached 16% methanol in chloroform. At this point, pure methanol was used to elute the column. Ten-milliliter fractions were collected at a rate of 1 ml./min. until 580 fractions were obtained. All fractions were analyzed using TLC (Solvent System 1) as previously described.

Quantitative Analysis of Indole Alkaloid—The assay for total indole alkaloid was a modification of the procedure of Michelon and Kelleher (40). Isoergine was used as a reference compound. For individual alkaloids, an aliquot of crude alkaloid mixture was separated on a two-dimensional TLC plate as previously described. The individual alkaloids were marked under UV light. Each spot was scraped off the plate and then assayed using the same procedure as for the total indole alkaloid.

RESULTS

Fractionation and Separation of Crude Alkaloids by Column Chromatography—The fractions collected after separation by column chromatography were pooled according to similarity of alkaloid patterns as determined by TLC monitoring of the eluent. A total of 580 fractions was collected, and 30 alkaloids were isolated by this procedure. Nineteen of them were identified by IR or various chromatographic systems.

Thin-Layer and Paper Chromatographic Results (R_f and R_A Values) of Isolated Ergoline-Type Alkaloids—The R_f and R_A values of ergoline-type alkaloids isolated from the seeds of *A. nervosa* in various chromatographic systems are shown in Table I. A diagrammatic map of the two-dimensional TLC system (using Solvent System 1 first and Solvent System 2 second) is given in Fig. 1. Thirty compounds were found by this two-dimensional TLC system.

The crude alkaloidal extract from the pericarp showed the same alkaloidal pattern as the seeds.

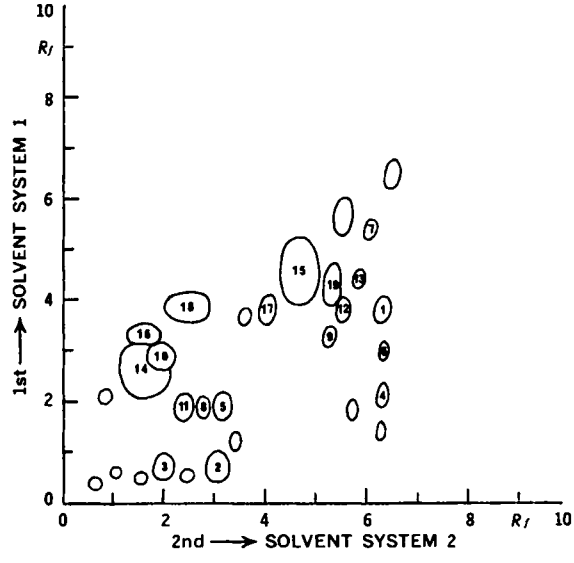


Figure 1—*Two-dimensional TLC of crude alkaloid mixture from the seeds of* A. nervosa *(Burm. f.) Bojer. Key: 1, agroclavine; 2, chanoclavine-I; 3, chanoclavine-II; 4, racemic chanoclavine-II; 5, elymoclavine; 6, festuclavine; 7, lysergene; 8, lysergol; 9, isolysergol; 10, molliclavine; 11, penniclavine; 12, setoclavine; 13, isosetoclavine; 14, ergine; 15, isoergine; 16, ergometrine; 17, ergometrinine; 18, lysergic acid α-hydroxyethylamide; 19, isolysergic acid α-hydroxyethylamide; and* O, *unidentified.*

[1] Alcoa.

Table I—R_f and R_A Values of Ergoline-Type Alkaloids Isolated from Seeds of *A. nervosa* (Burm. f.) Bojer in Various Solvent Systems

Alkaloids	FBP (R_A)	Solvent System[a] 1	2	3	4	5	6
				($R_f \times 100$)			
Agroclavine	100	38	63	69	21	95	34
Reference compound	100	38	62	68	21	95	34
Chanoclavine-I	6	7	30	20	3	33	16
Reference compound	6	7	30	20	3	33	16
Chanoclavine-II	6	8	19	18	3	41	16
Reference compound	—	—	—	—	—	—	—
Racemic chanoclavine-II	54	20	63	50	7	95	20
Reference compound	—	—	—	—	—	—	—
Elymoclavine	30	19	30	45	9	34	9
Reference compound	30	19	30	45	9	35	9
Festuclavine	82	32	63	63	18	91	25
Reference compound	82	32	63	63	18	91	25
Lysergene	118	54	60	77	46	91	32
Reference compound	119	54	60	77	46	91	32
Lysergol	32	19	27	—	—	—	—
Reference compound	32	19	27	—	—	—	—
Isolysergol	65	34	53	65	24	92	14
Reference compound	65	34	53	65	24	93	14
Molliclavine	5	29	19	45	30	18	3
Reference compound	—	—	—	—	—	—	—
Penniclavine	22	20	23	48	12	24	15
Reference compound	22	20	23	48	12	24	15
Setoclavine	84	39	55	67	38	92	16
Reference compound	84	39	55	68	38	92	16
Isosetoclavine	95	44	58	69	55	75	15
Reference compound	95	44	58	69	55	75	15
Ergine	12	27	15	48	15	22	18
Reference compound	12	27	15	48	15	22	18
Isoergine	48	47	45	68	35	55	5
Reference compound	48	47	45	68	35	55	5
Ergometrine	15	33	16	50	14	20	12
Reference compound	15	33	16	50	14	22	12
Ergometrinine	52	39	40	74	30	52	5
Reference compound	52	39	40	75	30	52	5
Lysergic acid α-hydroxyethylamide	19	38	24	56	17	17	12
Reference compound	19	38	24	56	17	17	12
Isolysergic acid α-hydroxyethylamide	54	41	53	—	—	—	—
Reference compound	—	—	—	—	—	—	—

[a] R_A values were calculated by using the R_f value of agroclavine as R_A 100. R_f values were calculated on the basis of a solvent front of 15 cm. and multiplied by 100. —: compounds were not available, the values were not determined.

The FBP solvent system is effective for separating most clavine-type alkaloids except chanoclavine-I and chanoclavine-II; however, it is ineffective for separating mixtures of both clavine- and lysergic acid-type alkaloids. None of the six TLC systems could separate the crude alkaloidal mixture from the seeds of *A. nervosa*. To obtain good separation, the two-dimensional TLC system was used for this mixture. Chanoclavine-I and chanoclavine-II showed very poor resolution in most systems but were separated well in Solvent System 2. Pennicalvine, lysergol, and elymoclavine were not separated by Solvent System 1 but were well separated by Solvent System 2. Agroclavine, festucalvine and racemic chanoclavine-II were well separated by most systems except 2 and 5.

Because of the limited amount of materials isolated, the R_f values of lysergol and isolysergic acid α-hydroxyethylamide were not obtained in Solvent Systems 3–6.

The R_f values represent the average of three chromatograms except for isolysergic acid α-hydroxyethylamide and lysergol; only one chromatogram was run for these compounds.

Quantitative Determination of Total and Individual Alkaloids—Various batches of seeds from different sources showed a 0.5–0.9% range of total indole alkaloid constituents. The results of one typical detailed assay (Table II) indicate the total percent of alkaloid to be 0.60, with isoergine (0.189%) and ergine (0.136%) being found in the highest concentrations in the seeds. These are followed, in order of decreasing indole alkaloid content, by ergometrine, lysergic acid α-hydroxyethylamide, isolysergic acid α-hydroxyethylamide, elymoclavine, ergometrine, chanoclavine-I, and agroclavine.

Table II—Major Alkaloid Content in the Seeds of *A. nervosa* (Burm. f.) Bojer

Alkaloids	Values Expressed as Percent of Total Alkaloid	Values Expressed as Percent of Dry Seed Weight
Agroclavine	1.09	0.006
Chanoclavine-I	2.65	0.016
Elymoclavine	3.62	0.022
Ergine	22.68	0.136
Isoergine	31.36	0.188
Ergometrine	8.20	0.049
Ergometrinine	1.81	0.011
Lysergic acid α-hydroxyethylamide	5.79	0.035
Isolysergic acid α-hydroxyethylamide	3.98	0.024
Tailing, minor, and unidentified alkaloids	18.82	0.113
Total	100.00	0.600

The tailing and minor alkaloids were scraped from the TLC plate and combined in the assay to give a total percentage of 0.113%.

The crude alkaloidal extract of the pericarp showed 0.0015% of total indole alkaloid constituents.

DISCUSSION

In comparison to other members of the Convolvulaceae investigated so far, *A. nervosa* apparently contains one of the largest concentrations of ergoline derivatives. Various batches of seeds have assayed from 0.5 to 0.9% of total indole alkaloid constituents. The various components of the total alkaloidal mixture, as isolated and characterized by chromatographic and IR spectral data, include lysergic acid amide (ergine), isolysergic acid amide (isoergine), agroclavine, elymoclavine, lysergene, festuclavine, setoclavine, isosetoclavine, and racemic chanoclavine-II (no nonindole alkaloids were detected). Those characterized by chromatographic data alone include penniclavine, isolysergol, molliclavine, lysergol, chanoclavine-I, chanoclavine-II, ergometrine, ergometrinine, and the isomeric pair of isolysergic acid and lysergic acid α-hydroxyethylamides. Limited quantities of the isolated clavine- and lysergic acid-type alkaloids have precluded further chemical studies on these constituents.

Nineteen identified ergoline alkaloids are present in the seeds of *A. nervosa*. Of these, lysergene, setoclavine, isosetoclavine, chanoclavine-II, racemic chanoclavine-II, isolysergol, and molliclavine have been identified for the first time in species of the morning-glory family.

The various unknown minor indole alkaloidal constituents are considered to represent *bona fide* minor clavine- or lysergic acid-type alkaloids or decomposition products. The inability in many cases to obtain reproducible R_f values for these tends to indicate the latter, although the former should not be entirely discounted. The possibility that certain of the alkaloids isolated or described are decomposition products has been minimized by careful experimental procedures (avoidance of light, rapid TLC separation, rapid analysis, *etc.*) and reproducibility of results.

The results of the tentative identification of several ergoline alkaloids of 13 other argyreia species and two closely related species, *Stictocardia tiliifolia* and *R. corymbosa*, will be given in a separate paper dealing with the chemotaxonomic implications of these indole alkaloids.

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Clindamycin Dose–Bioavailability Relationships

CARL M. METZLER▲, RAY DeHAAN, DIANA SCHELLENBERG, and WILLIAM D. VANDENBOSCH

Abstract □ Analyses of dose-related changes in pharmacokinetic variables indicating bioavailability and of dosage equivalence were performed on data from single-dose studies on three preparations of the antibiotic clindamycin [7(*S*)-chloro-7-deoxylincomycin]: clindamycin hydrochloride hard-filled capsules, clindamycin palmitate flavored granules, and clindamycin phosphate sterile solution. The variables studied were serum clindamycin bioactivity and parameters calculated from a single-compartment pharmacokinetic model. In general, the changes with dose were linear but not proportional and the pharmacokinetic model fit the data well. There were, however, some suggestions of nonlinear kinetics, particularly in the clindamycin hydrochloride study. Within the dose ranges (150–450 mg. clindamycin hydrochloride, 150–600 mg. clindamycin palmitate, and 300–600 mg. clindamycin phosphate), the average response to a dose could be predicted well. An equivalent dose analysis for the three clindamycin preparations offered some insight into their absorption and disposition characteristics and the relative bioavailability of clindamycin from them. A single equivalent dose could not be computed; there were different equivalent doses for peak serum concentration, area under the serum concentration–time curve, and other variables.

Keyphrases □ Clindamycin formulations—absorption, disposition, and relative bioavailability of hydrochloride capsules, palmitate flavored granules, and phosphate sterile solution □ Dose–bioavailability relationships—clindamycin hydrochloride capsules, clindamycin palmitate flavored granules, clindamycin phosphate sterile solution □ Bioavailability—absorption, disposition of clindamycin capsules, flavored granules, and sterile solution □ Pharmacokinetics—absorption, disposition, and relative bioavailability of three clindamycin formulations

Two problems frequently confront researchers testing new drugs: prediction of response to a particular dosage and equivalence of formulations. In the case of antibiotics, it is often assumed that the desired response, eradication of pathogens, is closely related to the concentration of drug in the serum and, therefore, that

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Clindamycin Dose–Bioavailability Relationships

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Abstract □ Analyses of dose-related changes in pharmacokinetic variables indicating bioavailability and of dosage equivalence were performed on data from single-dose studies on three preparations of the antibiotic clindamycin [7(*S*)-chloro-7-deoxylincomycin]: clindamycin hydrochloride hard-filled capsules, clindamycin palmitate flavored granules, and clindamycin phosphate sterile solution. The variables studied were serum clindamycin bioactivity and parameters calculated from a single-compartment pharmacokinetic model. In general, the changes with dose were linear but not proportional and the pharmacokinetic model fit the data well. There were, however, some suggestions of nonlinear kinetics, particularly in the clindamycin hydrochloride study. Within the dose ranges (150–450 mg. clindamycin hydrochloride, 150–600 mg. clindamycin palmitate, and 300–600 mg. clindamycin phosphate), the average response to a dose could be predicted well. An equivalent dose analysis for the three clindamycin preparations offered some insight into their absorption and disposition characteristics and the relative bioavailability of clindamycin from them. A single equivalent dose could not be computed; there were different equivalent doses for peak serum concentration, area under the serum concentration–time curve, and other variables.

Keyphrases □ Clindamycin formulations—absorption, disposition, and relative bioavailability of hydrochloride capsules, palmitate flavored granules, and phosphate sterile solution □ Dose–bioavailability relationships—clindamycin hydrochloride capsules, clindamycin palmitate flavored granules, clindamycin phosphate sterile solution □ Bioavailability—absorption, disposition of clindamycin capsules, flavored granules, and sterile solution □ Pharmacokinetics—absorption, disposition, and relative bioavailability of three clindamycin formulations

Two problems frequently confront researchers testing new drugs: prediction of response to a particular dosage and equivalence of formulations. In the case of antibiotics, it is often assumed that the desired response, eradication of pathogens, is closely related to the concentration of drug in the serum and, therefore, that

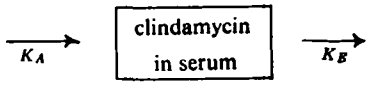
Table I—Subject Characteristics

Study Number	Compound and Dosage	Number of Men	Age (Years), Mean (Range)	Weight (kg.), Mean (Range)	Height (cm.), Mean (Range)
1	Clindamycin hydrochloride				
	150 mg.	10	36.4 (26.0–44.0)	80.0 (67.7–86.8)	182.1 (173.8–190.4)
	300 mg.	8	31.1 (22.0–46.0)	81.6 (70.9–95.0)	183.1 (173.8–198.2)
	450 mg.	8	35.4 (25.0–42.0)	82.5 (71.8–94.1)	181.3 (173.1–192.3)
2	Clindamycin palmitate				
	150 mg.	12	27.8 (23.0–35.0)	75.5 (61.4–91.4)	185.6 (176.9–193.6)
	225 mg. / 300 mg.	12	29.1 (22.0–42.0)	75.9 (65.9–93.6)	177.9 (143.6–191.0)
	600 mg.	6	30.0 (25.0–42.0)	70.6 (62.7–78.6)	176.7 (171.8–182.1)
3	Clindamycin phosphate				
	300 mg. / 450 mg. / 600 mg.	12	36.3 (25.0–48.0)	82.8 (69.1–105.0)	179.3 (172.4–193.6)

equal bioavailability implies therapeutic equivalence. Which measure(s) of drug absorption and distribution best reflects bioavailability remains to be established. This report considers several variables as indicators of bioavailability of three preparations of the antibiotic clindamycin[1] [7(*S*)-chloro-7-deoxylincomycin] undergoing clinical trials: (*a*) clindamycin hydrochloride hard-filled capsules; (*b*) a pediatric oral preparation of the 2-palmitate ester, clindamycin palmitate flavored granules; and (*c*) a parenteral preparation of the 2-phosphate ester, clindamycin phosphate sterile solution. The goal of administering any of the three is to attain therapeutically adequate concentrations of clindamycin bioactivity.

Wagner *et al.* (1) first described the clinical pharmacology of clindamycin hydrochloride, and their observations have been confirmed and extended by others (2–6). Clindamycin palmitate flavored granules, first prepared by Sinkula *et al.* (7), were shown in pharmacokinetic studies in adults (8) and children (9, 10) to be a satisfactory oral delivery agent for clindamycin. Results in clinical efficacy studies[2] in more than 1500 children with infectious diseases are confirmatory. The ester, which is inactive against bacteria *in vitro*, is readily hydrolyzed in the gut to microbiologically active clindamycin before absorption occurs. Preliminary studies did not reveal quantifiable amounts of the intact ester in human serum after a 300- or 600-mg. dose[3].

When clindamycin hydrochloride proved too irritating to use parenterally (11), the 2-phosphate ester was developed (12). Tolerance findings indicate that it is suitable for clinical use. However, to improve local tolerance the concentration of drug was halved from the 300 mg./ml. used in the first clinical pharmacology study reported here to 150 mg./ml., used in all subsequent studies. Microbiologically inactive 2-phosphate ester is absorbed intact after intramuscular injection. The kinetics of hydrolysis of the ester to active clindamycin will be dealt with in future publications.



Scheme I

In the three studies described here, healthy men were given single doses of 150, 300, and 450 mg.[4] clindamycin hydrochloride orally; 150, 225, 300, and 600 mg. clindamycin palmitate orally; or 300, 450, and 600 mg. clindamycin phosphate intramuscularly. Serum clindamycin levels were determined by bioassay, and the observations were subjected to pharmacokinetic analyses. In evaluating antibiotics, serum concentrations at various times after dosing, area under the serum concentration–time curve, peak serum level, and time of peak have all been considered clinically important by some workers. This paper considers these pharmacokinetic variables and others in assessing dose–bioavailability relations. The two aspects focused on are: (*a*) relationship between dose level and values of the pharmacokinetic variables for each compound, and (*b*) comparison of bioavailability of active clindamycin from the three compounds.

Urinary excretion of drug, sometimes used as an indicator of drug absorption, could not be estimated precisely for clindamycin in the studies reported here. Urine assays were run, but the bioactivity measured was due to a mixture of clindamycin and bioactive metabolites, one of which (*N*-demethylclindamycin) is more active and another (clindamycin sulfoxide) much less active than clindamycin itself. To report only the total amount of bioactivity excreted might perpetuate the misleading concept that the amount of bioactivity excreted represents the amount of drug excreted. Definitive studies of excretion of metabolites are not yet completed.

THEORETICAL

A single-compartment model (Scheme I) was used in studying serum clindamycin concentrations after all three clindamycin preparations, where K_A is a first-order rate constant for appearance of clindamycin in the blood (absorption) and K_B is a first-order rate constant for elimination from the blood. Both are expressed in liters

[1] Cleocin, The Upjohn Co.
[2] Case reports on file with The Upjohn Co.
[3] F. Sun and T. Brodasky, The Upjohn Co., personal communication.
[4] All dosages and concentrations expressed in terms of clindamycin base equivalents.

Table II—Dose-Bioavailability Relations after Clindamycin Hydrochloride[a]

Variable	Slope	Intercept	r^2	t-Statistic	Equivalent Dose, mg.	150 mg. Observed Mean[b]	150 mg. Predicted Mean[c]	300 mg. Observed Mean	300 mg. Predicted Mean	450 mg. Observed Mean	450 mg. Predicted Mean
Serum level, mcg./ml.:											
0.25 hr.	0.006	−0.60	0.21	2.54	195	0.48 ±0.69[d]	0.28	0.66 ±0.89	1.16	2.28 ±2.35	2.03
0.50 hr.	0.010	0.39	0.48	4.74	193	2.16 ±1.05	1.95	3.00 ±1.32	3.51	5.33 ±1.74	5.07
0.75 hr.	0.010	0.97	0.59	5.91	187	2.67 ±0.80	2.49	3.57 ±1.06	4.01	5.75 ±1.32	5.53
1.00 hr.	0.009	1.06	0.59	5.89	161	2.69 ±0.77	2.47	3.33 ±0.95	3.88	5.57 ±1.15	5.29
1.50 hr.	0.008	1.18	0.54	5.32	134	2.60 ±0.87	2.40	3.14 ±0.93	3.62	5.09 ±1.00	4.85
2.00 hr.	0.007	1.01	0.52	5.19	133	2.29 ±0.82	2.13	2.82 ±0.83	3.24	4.56 ±0.98	4.35
3.00 hr.	0.006	0.76	0.45	4.50	135	1.82 ±0.76	1.65	2.14 ±0.62	2.55	3.66 ±1.01	3.45
6.00 hr.	0.003	0.25	0.37	3.78	198	0.86 ±0.50	0.77	1.06 ±0.32	1.29	1.93 ±0.81	1.81
Model parameters:											
K_A	0.015	1.61	0.25	2.87	453	3.73 ±1.59	3.86	6.41 ±3.55	6.11	8.20 ±4.65	8.35
$A(^1/_2)$	N.R.	N.R.	0.15	<2	N.R.	0.22 ±0.09	N.R.	0.16 ±0.11	N.R.	0.13 ±0.09	N.R.
K_E	N.R.	N.R.	0.20	<2	N.R.	0.28 ±0.10	N.R.	0.22 ±0.05	N.R.	0.20 ±0.06	N.R.
$E(^1/_2)$	0.003	2.31	0.16	2.17	123	2.82 ±1.13	2.80	3.23 ±0.67	3.28	3.78 ±0.98	3.76
Estimated peak	0.009	1.17	0.61	6.13	174	2.84 ±0.65	2.56	3.44 ±0.87	3.94	5.58 ±1.26	5.33
Time of peak	N.R.	N.R.	0.12	<2	N.R.	1.06 ±0.37	N.R.	0.91 ±0.38	N.R.	0.76 ±0.31	N.R.
Area	0.063	3.64	0.43	4.29	139	14.88 ±7.80	13.18	18.49 ±6.14	22.73	34.40 ±12.55	32.28
Time lag	N.R.	N.R.	0.02	<2	N.R.	0.21 ±0.15	N.R.	0.22 ±0.05	N.R.	0.17 ±0.09	N.R.
Volume of distribution	0.106	33.54	0.43	4.29	153	43.90 ±7.40	49.40	78.90 ±15.90	65.27	74.30 ±14.20	81.10

[a] See text for explanation of terms. [b] Observed mean. [c] Mean predicted from model. [d] ± standard deviation.

per hour. The concentration of clindamycin in the serum as a function of time after dosing is described by the equation:

$$\text{concentration} = \frac{\text{dose } K_A}{V(K_E - K_A)}[\exp(-K_A t^*) - \exp(-K_E t^*)] \quad \text{(Eq. 1)}$$

where $t^* = t - t_0$; t is time measured in hours from dosing and t_0 is a time lag before measurable amounts of clindamycin can be observed in the serum. Dose is in milligrams, and V is the apparent volume of distribution in liters. In addition to being a volume factor, V also adjusts for the fraction of the dose absorbed; in this paper (dose/V) corresponds to the (FD/V) parameter of Wagner and Nelson (13). Parameters that can be calculated from this model include the absorption half-life $A(^1/_2) = (\ln 2)/K_A$ (in hours), the elimination half-life $E(^1/_2) = (\ln 2)/K_E$ (in hours), the peak serum concentration (in micrograms per milliliter), the time of peak (in hours), and the total area under the serum concentration–time curve (in micrograms per milliliter × hours). The use of this model in both single-dose and multidose studies of clindamycin hydrochloride was discussed elsewhere (6).

In this model, serum concentrations are related to dose in a linear manner. If the model holds over all doses, then serum concentrations at a given time should increase in the same proportion as do doses. Thus, if the dose is doubled, serum concentrations at a given time should double, as should the parameters directly dependent upon concentration (peak and area under the curve). However, rate constants, half-lives, time lag, time of peak, and volume should not change. Deviation from this behavior may provide information about the drug and different formulations; it may also indicate that the model does not give a complete description of the drug kinetics.

One way to compare bioavailability of the two formulations is to calculate the dose of one formulation ("equivalent dose") that will produce the same average value of a chosen variable as does a specified dose of the standard formulation. In this paper, 150 mg. clindamycin hydrochloride is used as the standard and, where feasible, the equivalent doses of palmitate and phosphate esters that will produce the same average values for serum concentrations and parameter estimates from the pharmacokinetic model are estimated. The composite means of pharmacokinetic variables found in six studies with 150 mg. clindamycin hydrochloride (6) were used as the reference values. Values obtained with clindamycin hydrochloride in Study 1 described here were also compared with the composite means, using the same method of inverse estimation.

Since for the chosen variables the dose–bioavailability relationship is assumed to be linear over the doses used for each formulation, the equation:

$$\text{value of pharmacokinetic variable} = \text{intercept} + (\text{slope} \times \text{dose}) \quad \text{(Eq. 2)}$$

was used. Thus, for a pharmacokinetic variable, the dose of the ester that is equivalent to 150 mg. clindamycin hydrochloride is given by the equation:

$$\text{equivalent dose} = \frac{P - I}{S} \quad \text{(Eq. 3)}$$

where P is the average value of the pharmacokinetic variable observed in subjects receiving 150 mg. clindamycin hydrochloride in the six studies, and I is the intercept and S is the slope of the linear dose–bioavailability relation computed for all the observed dose

Table III—Dose-Bioavailability Relations after Clindamycin Palmitate[a]

Variable	Slope	Intercept	r^2	t-Statistic	Equivalent Dose, mg.	150 mg. Observed Mean[b]	150 mg. Predicted Mean[c]	225 mg. Observed Mean	225 mg. Predicted Mean	300 mg. Observed Mean	300 mg. Predicted Mean	600 mg. Observed Mean	600 mg. Predicted Mean
Serum level, mcg./ml.:													
0.33 hr.	N.R.	N.R.	0.00	<2	N.R.	1.14 ±0.95[d]	N.R.	0.80 ±0.65	N.R.	1.22 ±0.97	N.R.	1.15 ±0.74	N.R.
0.67 hr.	0.003	1.40	0.14	2.54	370	1.77 ±0.84	1.85	1.94 ±0.84	2.07	2.58 ±1.29	2.30	3.04 ±1.52	3.18
1.00 hr.	0.004	1.13	0.32	4.35	345	1.70 ±0.60	1.80	2.11 ±0.26	2.13	2.63 ±1.00	2.47	3.70 ±1.77	3.80
1.50 hr.	0.005	0.98	0.40	5.20	306	1.70 ±0.55	1.71	2.04 ±0.64	2.07	2.48 ±0.86	2.43	3.86 ±1.66	3.89
2.00 hr.	0.005	0.91	0.43	5.47	206	1.66 ±0.57	1.64	1.93 ±0.57	2.01	2.46 ±0.84	2.38	3.83 ±1.59	3.85
3.00 hr.	0.004	0.83	0.47	5.90	185	1.43 ±0.52	1.46	1.71 ±0.53	1.77	2.20 ±0.61	2.08	3.28 ±1.18	3.34
6.00 hr.	0.002	0.37	0.43	5.48	232	0.76 ±0.31	0.73	0.87 ±0.34	0.90	1.08 ±0.38	1.08	1.80 ±0.70	1.79
12.00 hr.	—	—	—	—	—	0.00	—	0.00	—	0.26	—	0.62 ±0.23	—
Model parameters:													
K_A	N.R.	N.R.	0.07	<2	N.R.	14.25 ±14.38[d]	N.R.	7.17 ±9.39	N.R.	9.30 ±14.54	N.R.	2.00 ±1.07	N.R.
$A(^1/_2)$	N.R.	N.R.	0.04	<2	N.R.	0.24 ±0.41	N.R.	0.24 ±0.16	N.R.	0.31 ±0.50	N.R.	0.46 ±0.27	N.R.
K_B	N.R.	N.R.	0.00	<2	N.R.	0.23 ±0.09	N.R.	0.21 ±0.06	N.R.	0.24 ±0.08	N.R.	0.23 ±0.05	N.R.
$E(^1/_2)$	N.R.	N.R.	0.01	<2	N.R.	3.41 ±1.01	N.R.	3.52 ±1.10	N.R.	3.18 ±1.00	N.R.	3.18 ±0.66	N.R.
Estimated peak	0.005	1.35	0.36	4.71	278	2.02 ±0.64	2.05	2.32 ±0.67	2.40	2.87 ±0.99	2.74	4.08 ±1.63	4.14
Time of peak	N.R.	N.R.	0.09	<2	N.R.	0.96 ±0.71	N.R.	1.18 ±0.54	N.R.	1.09 ±0.74	N.R.	1.62 ±0.49	N.R.
Area	0.031	6.93	0.38	4.90	177	11.92 ±5.06	11.51	13.81 ±4.86	13.80	15.46 ±4.23	16.09	25.67 ±10.83	25.26
Time lag	N.R.	N.R.	0.01	<2	N.R.	0.26 ±0.15	N.R.	0.24 ±0.15	N.R.	0.27 ±0.78	N.R.	0.22 ±0.12	N.R.
Volume of distribution	0.113	55.39	0.18	2.95	−50	59.84 ±25.58	72.39	101.71 ±30.73	80.89	82.18 ±36.53	89.39	121.26 ±46.32	123.39

[a] See text for explanation of terms. [b] Observed mean. [c] Mean predicted from model. [d] ± standard deviation.

levels of that ester. This is called inverse estimation in the statistical literature (14).

EXPERIMENTAL

Design of Studies—Study 1 was described in detail (as Study 7) by DeHaan *et al.* (6). Results after the first 150-mg. dose were included in the six single-dose studies referred to previously.

The other two studies were carried out with prisoners as subjects[5]. In Study 2 the 12 men who took 150 mg. clindamycin palmitate also took 150 mg. clindamycin hydrochloride in a two-way crossover with a week between doses; data from the clindamycin hydrochloride part of the study were reported (as Study 3) previously (6). Another panel of 12 received the 225- and 300-mg. doses in a two-way crossover with a week between doses. In the 600-mg. phase of the palmitate study, six men were given clindamycin palmitate and another six received placebos (vehicle for clindamycin palmitate) as a tolerance control group.

Study 3 was a three-way crossover study in which each of 12 men received intramuscular injections of 300, 450, and 600 mg. clindamycin phosphate with a week between doses.

The volunteers were required to be within 20% of ideal body weight for height and build; to have no known GI, hepatic, or renal disease; and to have screening clinical laboratory values, physical examinations, and vital signs within normal limits. Table I summarizes subject characteristics.

Clindamycin hydrochloride hydrate (Lot 15,759-15) was given as 150-mg. hard-filled capsules, and clindamycin palmitate hydrochloride flavored granules (Lot 16,110-1) were given as a suspension reconstituted with distilled water to a concentration of 15 mg./ml. In both oral studies the subjects fasted (except for water *ad libitum*) overnight before the dose and for 3 hr. after the dose, and they drank 180 ml. (6 fl. oz.) water after the dose. Study 3 utilized clindamycin phosphate sterile solution containing 300 mg. clindamycin base equivalents/ml. (Lot 15,953); this lot did not contain the disodium edetate that was added to subsequent lots. Injections of 1.0, 1.5, or 2.0 ml. drug were made deep into the gluteal muscles, using a 3.8-cm. (1.5-in.) 22-gauge needle.

Microbiological Assay[6]—Serum specimens were obtained for microbiological assay at the times indicated in Tables II–IV, and the samples were frozen promptly and stored at −20°. To estimate clindamycin bioactivity (clindamycin plus bioactive metabolites), a cylinder-plate method was used. Sterile Pen Assay Seed Agar[7] was inoculated with the test organism, *Sarcina lutea* ATCC 9341. Primary standards were prepared in commercial pooled human serum[8], and secondary standards were prepared in the subject's own pretreatment serum specimen. Specimens were assayed undiluted and calculated *versus* their respective secondary standards, all specimens from an individual subject being assayed on the same day. Reported values of less than 0.9 mcg./ml. were extrapolated from the standard curve, and zero indicates less than 0.2 mcg./ml. Before assaying for free clindamycin activity in serum following clindamycin phosphate administration, phosphate buffer was added to the specimen immediately after it was thawed to halt hydrolysis of any clindamycin phosphate present. Wagner *et al.* (1) found that nearly all of the bioactivity in serum after clindamycin hydrochloride is due to clindamycin *per se*.

Tolerance Monitoring—*See Appendix.*

[5] In Jackson Experimental Clinic, State Prison of Southern Michigan, Jackson, Mich.

[6] The Microbiology Section of the Clinical Research Laboratory, The Upjohn Co., performed all bioassays.

[7] Difco.

[8] Hyland.

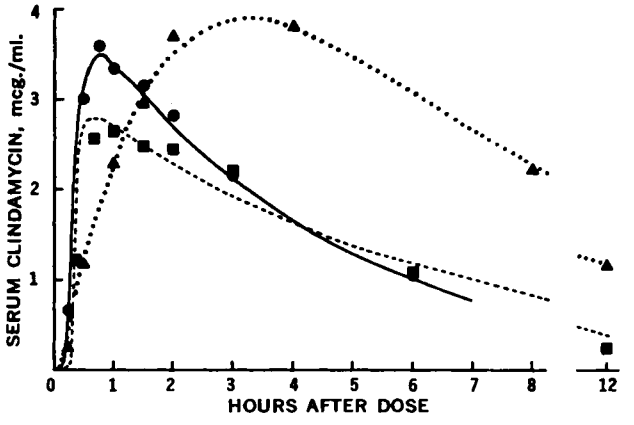


Figure 1—*Mean serum clindamycin (micrograms per milliliter) after a 300-mg. dose of three clindamycin preparations. Key: ●——●, clindamycin hydrochloride (eight men); ■- - -■, clindamycin palmitate (12 men); and ▲...▲, clindamycin phosphate intramuscularly (12 men).*

RESULTS AND DISCUSSION

Serum Levels—Mean serum clindamycin concentrations observed at the various sampling times are listed in Tables II–IV. Figure 1 illustrates the differences and similarities in the serum concentration–time curves following a 300-mg. dose of the three preparations.

Differential assay results in several clindamycin phosphate studies, including Study 3 described here, indicate that a substantial amount of unhydrolyzed ester appears in the serum shortly after intramuscular injection. In this study the ester reached a mean peak value between 1 and 2 mcg./ml. at 30 or 60 min. and virtually disappeared within 4 hr. Presumably the flattened, delayed peak in clindamycin bioactivity (compared with the oral dose curves) reflects hydrolysis of the ester to clindamycin in the serum, as well as various absorption and excretion factors.

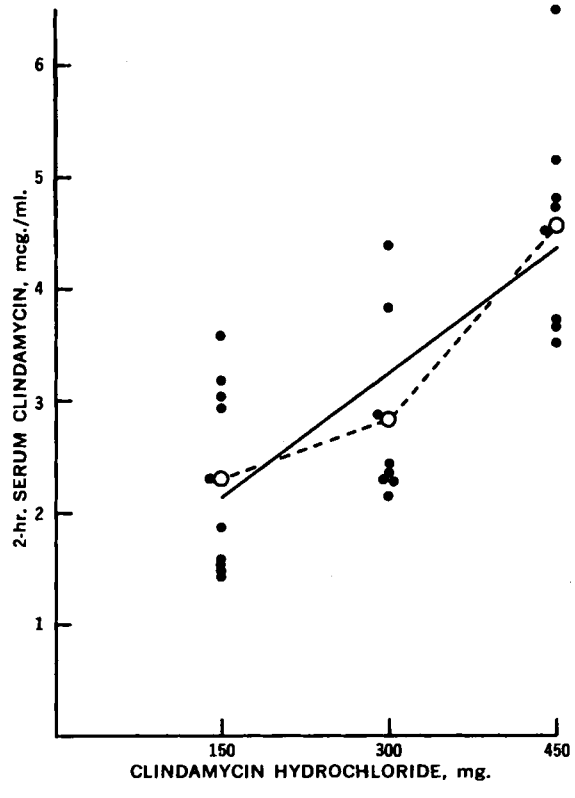


Figure 2—*Dose–bioavailability relation: 2-hr. serum clindamycin concentration after clindamycin hydrochloride. Key: ——, predicted means from linear model; O- - -O, observed means; and ●, individual values.*

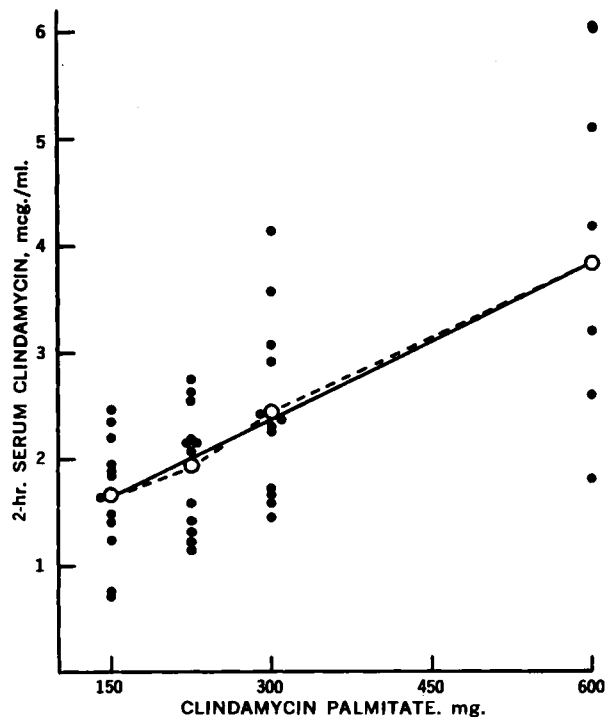


Figure 3—*Dose–bioavailability relation: 2-hr. serum clindamycin concentration after clindamycin palmitate. Key: ——, predicted means from linear model; O- - -O, observed means; and ●, individual values.*

Definitions and units for the estimated model parameters listed in Tables II–IV are given in the *Theoretical* section. The estimates were obtained by use of the nonlinear estimation program NONLIN (15).

Dose–Bioavailability Considerations—For all three formulations and all pharmacokinetic variables considered, where there was a change due to dose, the relation between dose and response was linear. Since at least three dose levels of the formulations were studied, a quadratic relation could be fitted to the data and, in fact, was calculated for all formulations and all variables; however, the resulting fit to the data was not significantly better than the linear fit. This linearity may hold only over the range of doses studied here. Since the intercept was in most cases nonzero, linearity would not hold for small doses and probably would not hold for very large doses.

For each variable (observed value or estimated parameter), a linear regression was computed with the dose as the independent variable and the value of the pharmacokinetic variable as the dependent variable. The strength of the relation was evaluated by a statistical test of the estimated slope; the t-statistic tests whether the slope is significantly different from zero. If the t-statistic was less than 2, that response was judged to be independent of dose [indicated by N.R. (no regression) in Tables II–IV].

Even though the mean values of the pharmacokinetic variables at each dose level fall on a line, there is so much variability about the means at each dose level that only a fraction of the total variability is due to changes in dose. The proportion of the variability of a pharmacokinetic variable that could be explained by change in dose is represented by r^2 (the square of r, the correlation coefficient). To express this proportion as a percent, multiply r^2 by 100. For example, as illustrated in Fig. 3, although the mean 2-hr. serum clindamycin levels at the four dose levels of clindamycin palmitate lie very close to a straight line, the individual values vary considerably about the mean. From Table III in the r^2 column for the 2-hr. serum concentration, it is apparent that only 43% of the total variation of the 2-hr. serum concentration is due to increasing dose levels.

Much of the overall variability in blood level studies is due to between-subject variability (16). Where the same panel of subjects

Table IV—Dose-Bioavailability Relations after Clindamycin Phosphate[a]

Variable	Slope	Intercept	r^2	R^2	t-Statistic	Equivalent Dose, mg.	300 mg. Observed Mean[b]	300 mg. Predicted Mean[c]	450 mg. Observed Mean	450 mg. Predicted Mean	600 mg. Observed Mean	600 mg. Predicted Mean
Serum level, mcg./ml.:												
0.25 hr.	—	—	—	—	—	—	0.23 ±0.49[d]	—	0.31 ±0.39	—	0.53 ±0.71	—
0.50 hr.	N.R.	N.R.	0.08	0.11	<2	N.R.	1.19 ±0.98	N.R.	1.40 ±0.60	N.R.	1.76 ±0.88	N.R.
1.00 hr.	0.002	1.61	0.11	0.20	2.03	450	2.29 ±0.98	2.28	2.59 ±0.54	2.61	2.96 ±0.87	2.95
1.50 hr.	0.004	1.69	0.24	0.40	3.31	140	2.96 ±1.14	2.96	3.59 ±0.50	3.60	4.23 ±1.08	4.23
2.00 hr.	0.005	2.23	0.26	0.45	3.49	−63	3.68 ±1.16	3.75	4.64 ±0.80	4.51	5.20 ±1.22	5.27
4.00 hr.	0.007	1.56	0.43	0.84	5.09	−27	3.79 ±1.03	3.91	5.32 ±1.07	5.09	6.15 ±1.30	6.26
8.00 hr.	0.006	0.29	0.41	0.84	4.88	43	2.22 ±0.65	2.35	3.63 ±1.12	3.38	4.28 ±1.23	4.40
12.00 hr.	0.004	−0.13	0.33	0.76	4.10	90	1.17 ±0.46	1.23	2.02 ±0.90	1.91	2.53 ±1.00	2.59
24.00 hr.	—	—	—	—	—	—	0.09 ±0.21	—	0.27 ±0.47	—	0.50 ±0.36	—
Model parameters:												
K_E	N.R.	N.R.	0.07	0.14	<2	N.R.	0.17 ±0.06[d]	N.R.	0.15 ±0.04	N.R.	0.14 ±0.04	N.R.
$E(^1/_2)$	N.R.	N.R.	0.06	0.10	<2	N.R.	4.50 ±1.57	N.R.	4.77 ±1.21	N.R.	5.32 ±1.29	N.R.
Estimated peak	0.007	1.96	0.43	0.80	5.04	111	4.06 ±1.09	4.18	5.53 ±0.98	5.29	6.29 ±1.17	6.41
Time of peak	0.002	2.06	0.13	0.28	2.29	−620	2.60 ±0.31	2.65	3.03 ±0.80	2.94	3.19 ±0.69	3.24
Area	0.101	5.22	0.40	0.85	4.75	71	37.68 ±13.07	38.92	58.73 ±17.42	55.76	71.38 ±21.20	72.61
Time lag	N.R.	N.R.	0.04	0.07	<2	N.R.	0.31 ±0.21	N.R.	0.34 ±0.25	N.R.	0.22 ±0.11	N.R.
Volume of distribution	N.R.	N.R.	0.09	0.25	<2	N.R.	60.07 ±21.52	N.R.	62.63 ±17.73	N.R.	75.20 ±21.20	N.R.

[a] See text for explanation of terms. [b] Observed mean. [c] Mean predicted from model. [d] ± standard deviation.

is used for all doses of a formulation, it is possible to remove this component. In Study 3 the same 12 subjects received the three dose levels in a crossover design. In Table IV, R^2 is the square of the correlation coefficient for the linear dose-bioavailability relations after the between-subject component of variance is removed. Consider, as an example, the 2-hr. serum concentrations. Of the total variability, only 26% is due to the different dose levels; but after removing the between-subject variability, 45% of the remaining variability is due to the different dose levels. Thus the small values of r^2 in Tables II–IV reflect between-subject variability as much as deviations from the linear dose–bioavailability relations.

Tables II–IV list the t-statistic, r^2, and mean observed value at each dose level. For those pharmacokinetic variables for which the regression was significant (t-statistic >2), the slope and intercept of the regression line and the mean of the pharmacokinetic variable predicted from the linear regression are also reported. By comparing the observed and predicted means at each dose level, it is possible to get some idea of how closely the means fit the computed linear relations. Space limitations prohibit illustrating all these relations, but the 2-hr. serum concentrations for the three preparations are shown in Figs. 2–4. The use of the 2-hr. serum concentrations to illustrate the methods used does not imply that these values have any special therapeutic importance.

Equivalence Considerations—As described in the *Theoretical* section, for appropriate variables the dose of clindamycin ester equivalent to a 150-mg. dose of clindamycin hydrochloride was determined, using the composite means from six studies in adults (6) as the standard of comparison. This comparison was also carried out with the data from Study 1 reported here to see how closely this study fit the overall results from six studies of 150 mg. clindamycin hydrochloride.

Figure 5 illustrates graphically how the equivalent dose was computed. The mean 2-hr. serum concentration is plotted against clindamycin palmitate dose. The arrowed line enters the graph at the composite mean of the 2-hr. serum concentrations observed in the six clindamycin hydrochloride studies (1.94 mcg./ml.). The clindamycin palmitate dose level (206 mg.) at which this arrowed line intercepts the regression line is the "equivalent dose"; this is the estimated dose of clindamycin palmitate that yields the same average value for the pharmacokinetic variable as does 150-mg. clindamycin hydrochloride. The equivalent dose could be computed only for those variables for which the regression with dose was significant (t-statistic >2); other variables are designated N.R. in the tables.

Clindamycin Hydrochloride (Table II)—The relation of the 2-hr. serum concentration to dose (Fig. 2) is representative of all the serum concentrations in that at all sampling times the mean serum level after the 300-mg. dose was less than predicted from the line fitted through all the data. Even though the means did not lie exactly on a straight line, as noted previously, the quadratic component of the regression line was not significant. This is because the variation of the individual observations about the means was greater than the deviations of the means from a straight line. In Study 1 the different dose levels were administered to different panels of subjects at different times. The deviation of the mean responses in the 300-mg. dose group cannot be explained.

At all serum sampling times, there was a highly significant relation between dose and serum level, as evidenced by the large values of the t-statistic. The slopes of the regression lines were quite similar. Because of between-subject variation in serum concentrations at each dose level, no more than 59% ($r^2 = 0.59$) of the variance at any sampling time could be ascribed to changes in dose. Serum concentrations increased only about half as much as dose; *i.e.*, when the dose increased by 100%, the mean serum levels increased by about 50%.

Three of the model parameters that should not be dose dependent, K_A, $E(^1/_2)$, and volume of distribution, did show a significant regression with dose (t-statistics 2.86, 2.17, and 4.29, respectively). As noted previously (6), because of the rapid absorption of clindamycin hydrochloride, it is difficult to obtain enough prepeak serum concentration values to make good estimates of K_A. In all of our clindamycin hydrochloride studies, estimates of K_A have

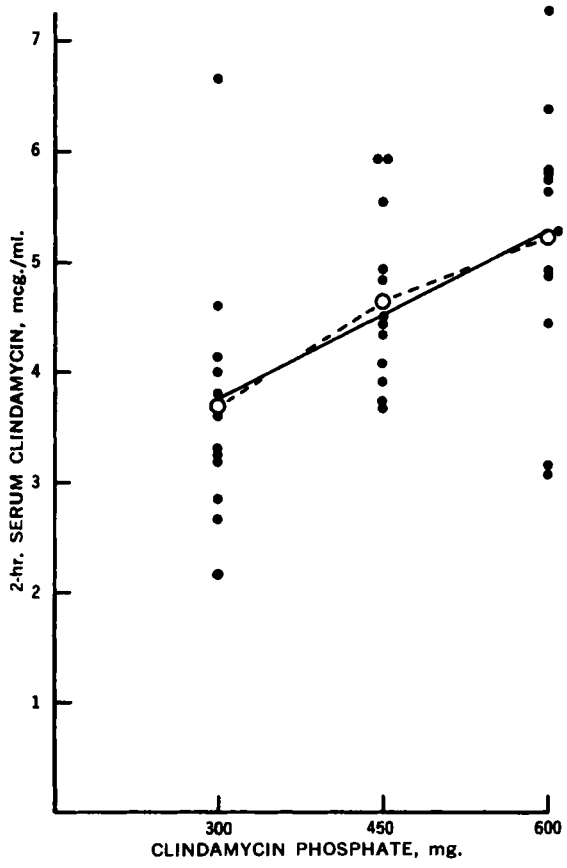


Figure 4—*Dose-bioavailability relation: 2-hr. serum clindamycin concentration after clindamycin phosphate. Key: ——, predicted means from linear model; O- - -O, observed means; and ●, individual values.*

had large standard deviations; K_E is much easier to estimate and has had much smaller standard deviations. While $E(^1/_2)$ tended to increase with dose level for the first dose of clindamycin hydrochloride, after a postequilibrium dose on a *q6h* regimen, $E(^1/_2)$ values did not differ significantly, having mean values of 3.58, 3.12, and 3.32 hr. with 150-, 300-, and 450-mg. regimens, respectively (6). The changes in K_A, $E(^1/_2)$, and volume with changes in the first dose are unexplained but could reflect inappropriateness of the model. Compartmental models of drug kinetics are based on assumptions of linear kinetics. There are several possible causes of nonlinear kinetics; DiSanto and Wagner (17) showed that if one of these, tissue binding, is significant, then the use of a compartmental model will lead to the erroneous conclusion that some of the kinetic parameters change with dose.

The two model parameters that should increase with dose, estimated peak and area under the serum concentration–time curve, did so but, like the serum levels, they did not increase in the same proportion as did dose.

The computed equivalent doses in Table II compared Study 1 with the composite results in six studies of clindamycin hydrochloride. All of these equivalent doses should be close to 150 mg., of course, and any deviation indicates that in some respect this study is different from the average results. Absorption was slower in this study, as reflected in higher equivalent doses at early sampling times and lower ones at later sampling times. That the average total absorption in this study was similar to the overall experience is indicated by the equivalent doses to attain the area under the curve and estimated peak (139 and 174 mg., respectively). The equivalent dose was 153 mg. for volume of distribution.

Clindamycin Palmitate (Table III)—The relation between dose and 2-hr. serum level (Fig. 3) is representative of all the clindamycin palmitate variables. Mean values for the four doses lie very close to the regression line computed from all the data. Despite this good fit, variability within each treatment group was great enough that difference in dose accounted for less than 50% of the variation.

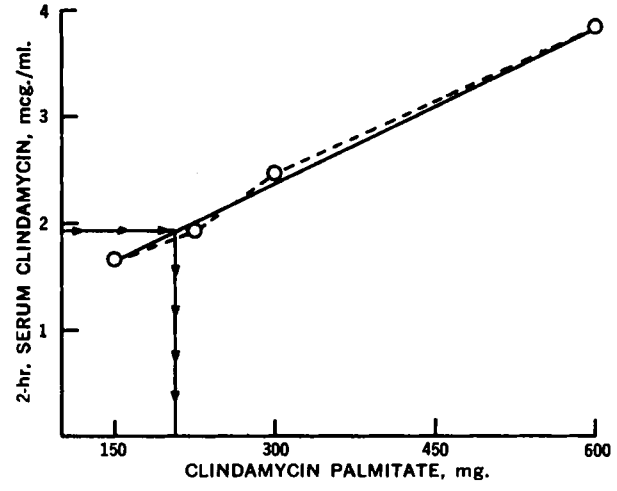


Figure 5—*Estimated dose of clindamycin palmitate required to produce 2-hr. serum clindamycin bioactivity equivalent to that produced by 150 mg. clindamycin hydrochloride in six studies. Key: ——, linear dose–response (predicted means); and O- - -O, observed mean responses. Equivalent dose = 206 mg. clindamycin palmitate.*

Most of the model parameters showed the expected linear relation with dose; rate constants, half-lives, time of peak, and time lag were all independent of dose, while peak and area were dose dependent. Volume, however, did increase significantly with dose (t-statistic, 2.95). Until more information about the distribution and metabolism of clindamycin and about the correctness of the model is available, any explanation for this remains speculative. As noted previously, the volume term in the model includes F, the fraction of dose absorbed. In the 225/300-mg. crossover group, the volume estimate was greater after the lower dose than after the higher dose.

The equivalent doses calculated for clindamycin palmitate illustrate that it is absorbed more slowly than is clindamycin hydrochloride. Equivalent doses are more than double 150 mg. at early serum sampling times but only about one-third larger than 150 mg. at later times. The lower peak levels are reflected in an equivalent dose of 278 mg. to achieve the same peak. However, the equivalent dose (177 mg.) computed for area under the serum curve suggests that total absorption of clindamycin is about the same after the two preparations.

Estimating equivalent doses was a primary purpose in designing Study 2, and the information proved helpful in predicting appropriate dosages for clinical trials with this compound.

Clindamycin Phosphate (Table IV)—Although area under the serum concentration curve almost doubled with a doubling of clindamycin phosphate dose, serum concentrations increased only by about 50% when the dose was doubled. Again the linear dose–bioavailability model, although it fits the serum concentration–time curves very well, dose not completely describe the kinetics of the drug. Although time of peak and time lag had statistically significant dose–bioavailability relations, the estimated equivalent doses are meaningless. Since estimation of absorption of the intramuscular dose is confounded by estimation of rate of hydrolysis of the ester to clindamycin, the absorption rate constant K_A and half-life $A(^1/_2)$ were not included in this analysis.

The delayed peak and flatter, higher serum clindamycin curve after intramuscular clindamycin phosphate injection are reflected in the calculated equivalent doses. As expected from inspection of the curves, calculation of equivalent doses for serum levels at individual sampling times did not yield useful information. However, the equivalent dose for area under the serum concentration–time curve suggests that nearly twice as much clindamycin is absorbed into the circulation from clindamycin phosphate given intramuscularly as from clindamycin hydrochloride given orally.

CONCLUSIONS

For clindamycin hydrochloride, clindamycin palmitate, and clindamycin phosphate, the dose–bioavailability relations for serum

clindamycin bioactivity are linear but not proportional. Means of the pharmacokinetic variables for the different dose levels lie on (or nearly on) a straight line, but the response does not double when the dose is doubled.

Within the dose ranges in the three studies described, the *average* effect of a dose can be predicted well. An individual's serum concentration cannot be predicted as precisely as the average; in no case in these studies did the regression of serum level with dose account for more than 60% of the total variability. In those situations where the between-subject variation could be removed, the regression of serum levels with dose accounts for as much as 84% of the remaining variability.

Analysis of the dose–bioavailability relations is one check on the validity of the one-compartment model as a model of clindamycin absorption and elimination. In general, those model parameters that should be dose related had significant regressions and those that should not change with dose had nonsignificant regressions. The exceptions, especially in the study of clindamycin hydrochloride, suggest that perhaps there is some nonlinearity in the kinetics of clindamycin.

Attempts to compute equivalent doses of the three preparations showed that it is not meaningful to talk about a single equivalent dose, even for compounds producing serum concentration curves as similar as those after clindamycin hydrochloride and clindamycin palmitate. The equivalent doses will be different for peak, area, *etc.* An equivalent dose analysis does provide some understanding of the similarities and differences of formulations.

APPENDIX

Tolerance Monitoring—There were no serious side effects or indications of toxicity in any of the studies. Tolerance of multiple dosing with clindamycin hydrochloride has been described elsewhere (6), but tolerance findings in Studies 2 and 3 have not. The following clinical laboratory determinations[9] were performed during screening and before and after dosing in both studies, except where indicated otherwise: complete blood count, complete urinalysis, serum alkaline phosphatase, serum glutamic oxalacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), serum bilirubin, serum creatinine (Study 2), blood urea nitrogen, and serum creatine phosphokinase (CPK) (Study 3). In Study 2, two subjects developed mildly abnormal SGOT values (normal = <45 K units) after dosing with clindamycin palmitate. One, whose SGPT also became mildly abnormal, was found to have a history of heavy alcohol consumption and slightly elevated SGPT values (although his screening and pretreatment values were normal). The other subject, who had an extremely high CPK but normal SGPT, admitted indulging in strenuous activity; these findings indicated muscle as the source of SGOT elevation in this case. In Study 3, SGOT typically rose after intramuscular injection, along with CPK; SGPT, however, scarcely changed, suggesting muscle trauma as one source of the SGOT increase.

Vital signs (blood pressure, pulse, temperature, and respiration) were recorded before and after each dose. Physical examinations, performed during screening, were repeated after dosing, and in Study 3 audiometric examinations were conducted before and after dosing. Subjects were questioned about systemic side effects. In Study 2, one subject developed signs of a urinary tract infection. Symptoms admitted on repeated questioning were loose or watery stools in three, headache in six, nausea in two, vomiting in one, gas in two, rectal itching in one, and bad taste (1 hr. after dosing) in two. All were transient and most were mild, but one subject had moderate vomiting and loose stools after the 225-mg. dose and then nausea and watery stools after the 300-mg. dose a week later. In the placebo-controlled 600-mg. trial, one subject developed transient dizziness and nausea after drug and one had nausea after placebo. In Study 3, changes in stools (softer, more frequent, or loose stools or diarrhea) were reported in six, rectal itching in four, abdominal pain in one, heartburn and gas in one, and itching of back and buttocks in one. Audiometric evaluations showed no drug effect.

In Study 3 repeated subjective and objective evaluations of tolerance at the intramuscular injection sites indicated that the drug caused more discomfort than saline placebo. However, this was less severe and shorter lived than that reported after intramuscular clindamycin hydrochloride (11).

[9] All determinations were performed by the Clinical Research Laboratory.

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UV Spectrophotometric Method for Determination of Phenacetin in Biological Specimens

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Abstract □ A UV spectrophotometric procedure for determining phenacetin in biological specimens is described. The drug is extracted from biological material by ether and oxidized to a quinone product by cobaltic oxide. The primary metabolite of phenacetin, *N*-acetyl-*p*-aminophenol, is assayed by providing a salting-out step in the extraction process. Better than 90% of phenacetin added to urine and serum specimens *in vitro* at concentrations of 5–50 mcg./ml. was recovered. From homogenized tissue, 83% of added phenacetin was recovered.

Keyphrases □ Phenacetin—UV analysis in biological fluids □ UV spectrophotometry—analysis, phenacetin in biological fluids

Abuse of the analgesic drug phenacetin[1] may lead to certain nephropathies in humans (1, 2). This danger, coupled with the drug's widespread use, necessitates specific and rapid detection of the compound in biological specimens. Phenacetin is a neutral drug that absorbs UV radiation noncharacteristically in the 230–260-nm. portion of the light spectrum. Normal constituents and numerous other pharmacological agents extracted from biological specimens can provide significant interference and make direct UV spectrophotometric analysis of the unchanged drug unrewarding.

Brodie and Axelrod (3) described the metabolic fate of phenacetin in man using an elaborate time-consuming colorimetric procedure. They determined the levels of phenacetin and several metabolites, including *N*-acetyl-*p*-aminophenol and *p*-aminophenol, in biological specimens. Other methods were reported by Welch and Conney (4), Büch *et al.* (5), Cummings *et al.* (6), and Routh *et al.* (7). Each of these techniques is nonspecific, time consuming, or lacking in sensitivity. Prescott (8) reported a sensitive and specific method for the estimation of phenacetin and *N*-acetyl-*p*-aminophenol in plasma and urine by a GLC technique using trimethylsilyl derivatives. The disadvantages of this method are that it has a significant time requisite and absolute dryness is required to achieve an effective silanizing reaction (9, 10).

This report describes a quantitative UV spectrophotometric technique for determining phenacetin in serum, blood, urine, or tissues. Phenacetin is oxidized to a products(s) that has a dissimilar, yet sensitive, response to UV radiation. The phenacetin product is subsequently extracted into sodium hydroxide solution, which results in a highly specific bathochromic shift of the UV spectrum and an increase of sensitivity.

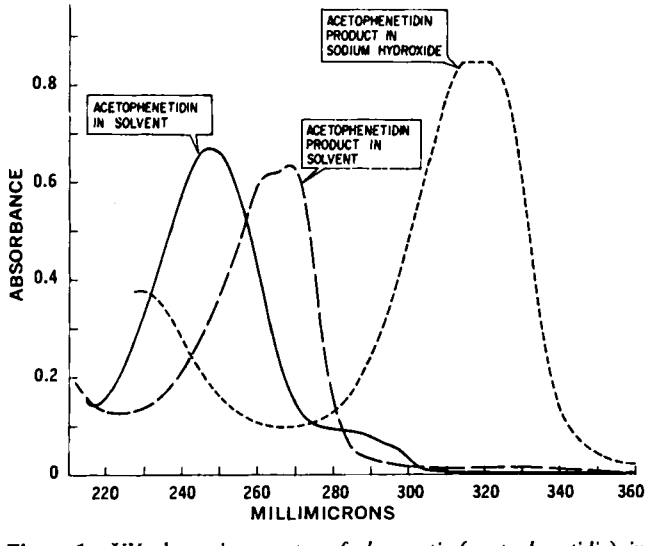

Figure 1—*UV absorption spectra of phenacetin (acetophenetidin) in isopropanol* (——), *the phenacetin product in hexane–*tert*-butanol* (— —), *and the phenacetin product in sodium hydroxide solution* (- - -), *each at a concentration of 8 mcg./ml. corresponding to phenacetin.*

EXPERIMENTAL[2]

Five milliliters of serum or 10 ml. of urine is adjusted to pH 7.0 ± 1.0 and extracted with 50–100 ml. of ether (diethyl). For tissue analysis, 5–10 g. of homogenized tissue is made alkaline (pH 12.0) by addition of several milliliters of 1.0 *N* NaOH and extracted with 100–200 ml. of ether. After extraction the aqueous phase is discarded and the ether layer is washed twice with 20–50 ml. of 1.0 *N* NaOH and once with 20–30 ml. of 1.0 *N* HCl. The organic solvent is filtered through fast-flowing filter paper. The volume recovered is noted and the loss of ether is included in the final calculations.

A major metabolite of phenacetin, *N*-acetyl-*p*-aminophenol, which is itself a commercially available analgesic[3], may be determined by a modification of this procedure. Ten milliliters of urine is adjusted to pH 1.0 ± 0.5 and saturated by the addition of 5 g. of sodium chloride. The original procedure is followed verbatim, except that the two alkali washes of the ether are not performed.

The washed ether is evaporated to dryness under vacuum in a 250-ml. round-bottom flask. Ten milliliters of 2.0 *N* HCl, 20 ml. of a 4:1 mixture of *n*-hexane (spectroquality)–*tert*-butanol, and 200 mg. of cobalt (III) oxide are added to the flask, and the contents are refluxed slowly for 10 min. with constant magnetic stirring. The optimum reflux conditions are achieved by applying 35 v. a.c. to a 270-w. heating mantle containing the flask with attached water-cooled condenser, the mantle being positioned upon a magnetic stirrer. After the flask contents are cooled, the solvent layer is recovered and scanned on the spectrophotometer. A 5-ml. aliquot of the solvent layer is then vigorously extracted with an equal volume of 4.0 *N* NaOH, and the aqueous layer is centrifuged for 5 min. at 3000 r.p.m. and scanned over the 220–360-nm. region; analyses at a single wavelength are achieved by measuring the absorbance at 268 and 320 nm., respectively.

[1] Acetophenetidin.

[2] UV absorption data were obtained with a Beckman DK-2A ratio recording spectrophotometer. A Beckman IR-9 spectrophotometer utilizing KBr disks was used for IR absorption measurements.

[3] Acetaminophen.

Table I—Standard Curve Data of Phenacetin Reaction Product

Phenacetin in Sample, mcg./ml.	Absorbance of Phenacetin Product in Solvent[a]	Absorbance Concentration	Absorbance of Phenacetin Product in Sodium Hydroxide[b]	Absorbance Concentration
10.0	0.798	0.080	1.057	0.106
8.0	0.649	0.081	0.852	0.106
6.0	0.492	0.082	0.623	0.104
4.0	0.321	0.080	0.397	0.099
2.0	0.162	0.081	0.194	0.097

[a] Determined at 268 nm. [b] Determined at 320 nm.

RESULTS

Phenacetin, upon oxidation with cobaltic oxide (Co_2O_3) in dilute hydrochloric acid, was converted to a derivative whose spectrum exhibited maximum absorbance in hexane–*tert*-butanol at 268 nm. and in sodium hydroxide at 320 nm. (Fig. 1). At the levels investigated, a linear relationship exists between the absorbance of the reaction product and the concentrations of drug present (Table I).

Ten milliliters of serum, 10 ml. of urine, and 10 g. of homogenized tissue, to each of which phenacetin was added to provide concentrations of 50, 25, 10, and 5 mcg./ml. (g.), were analyzed; 50 ml. of hexane–*tert*-butanol rather than 20 ml. was used with the 50-mcg./ml. specimens. The recovery of phenacetin by the method of this report is summarized in Table II. Recoveries greater than 90% were obtained from serum and urine, while approximately 83–85% of the drug was recovered from the homogenized liver preparations.

The distribution of phenacetin among the various tissues of the rat, 2 hr. after the animals received 200 mg./kg. body weight of the drug orally, is shown in Table III. Phenacetin, because of low aqueous solubility, was administered to the rats in a suspension of 0.5% methylcellulose in isotonic saline solution. Concentrations of the drug were found in decreasing levels in the following tissues: lungs, brain, kidneys, blood, muscle, and liver. With the exception of the liver, the findings are in agreement with the levels observed by Brodie and Axelrod (3) in dog tissues.

Several drugs, particularly neutral or solvent-soluble agents, were investigated for possible interference (Table IV). The nontherapeutic compound *p*-aminophenol gave an oxidation product with an absorption maximum at 240 nm. in solvent. Extraction of the *p*-aminophenol product into 4.0 *N* NaOH produced a spectrum very similar to that of the phenacetin product. The primary metabolite of phenacetin, *N*-acetyl-*p*-aminophenol, was oxidized by cobaltic oxide to a product with UV spectra (both in hexane–*tert*-butanol and sodium hydroxide solutions) identical to that of the phenacetin derivative. However, *p*-aminophenol and *N*-acetyl-*p*-aminophenol did not interfere with the determination of phenacetin in biological specimens, because both compounds are removed from the final ether extract by the sodium hydroxide wash solution. Compounds other than *p*-aminophenol or *N*-acetyl-*p*-aminophenol provided little or no interference in the determination of phenacetin.

Table II—Recovery of Phenacetin after *In Vitro* Addition to Biological Specimens

Phenacetin Added, mcg./ml.	Recovery[a], Mean[b] ± *SD*		
	Serum	Urine	Liver
50	48.27 ± 1.59	47.31 ± 1.15	43.37 ± 1.08
25	24.26 ± 0.52	22.95 ± 0.78	20.10 ± 0.65
10	9.75 ± 0.24	9.24 ± 0.23	8.30 ± 0.36
5	5.21 ± 0.07	4.71 ± 0.25	—[c]
Average recovery	98.8%	93.2%	83.4%

[a] Determined from readings in 4.0 *N* sodium hydroxide. [b] Average of at least seven determinations. [c] Not determined.

Table III—Distribution of Phenacetin[a] in the Rat

Tissue	Concentration in Tissue: Range of Values, mcg./ml. or g. (Wet Weight)	Concentration in Tissue: Distribution Ratio (Concentration in Tissue/Concentration in Blood), Mean ± *SD*
Blood	20.6–26.4	1.00
Brain	30.8–38.9	1.47 ± 0.12
Kidney	29.4–42.3	1.35 ± 0.13
Lung	31.9–52.9	1.40 ± 0.12
Liver	17.6–20.5	0.70 ± 0.14
Muscle	16.7–24.7	0.92 ± 0.11

[a] Suspension of phenacetin in 0.5% methylcellulose was administered in intraperitoneal doses of 200 mg./kg. body weight, and the animals were sacrificed after 2 hr. Average of determinations for eight rats.

DISCUSSION

Previous methods for the determination of phenacetin in biological specimens are primarily for determining the pharmacological agent in urine and plasma. The method described by Brodie and Axelrod (3) was the only previous report that presented tissue concentrations. Oxidation of phenacetin with cobaltic oxide to a product extractable into aqueous alkali provides a quantitative UV spectrophotometric method for determining the unmetabolized drug in biological specimens. Certain tissues contain large amounts of solvent-extractable material that interfere with the oxidation process. For example, brain tissue is frequently difficult to assay because of its high phospholipid content. The proposed method is applicable to most biological specimens with the exception of fat.

Since *ortho*- and *para*-dihydroxybenzenes, diamines, aminophenols, and related disubstituted benzenes may generally be oxidized to quinones, dependent upon the electronegativity of the substituents and the oxidizing conditions, the method presented in this paper may appear to be quite nonspecific. However, the alkali and acid washes of the ether eliminate all but the most solvent-soluble compounds. Thus, *p*-aminophenol and *N*-acetyl-*p*-aminophenol, very closely related compounds, do not interfere with the analysis of phenacetin. In the absence of solvent washing, considerable specificity is achieved by observing the wavelength of maximum absorbance for different disubstituted benzenes after their oxidation to quinones. Phenacetin and *N*-acetyl-*p*-aminophenol both yield a product absorbing maximally at 268 nm., whereas *p*-aminophenol and *p*-phenetidine apparently yield *p*-benzoquinone which absorbs maximally at 241 nm.

N-Acetyl-*p*-aminophenol, the principal metabolite of phenacetin and itself a commercially available analgesic, is present in high concentrations in the urine. This metabolite is highly water soluble, but it can be determined by the cobalt oxidation technique provided the biological specimen is adjusted to an acidic pH and is saturated with sodium chloride prior to extraction with ether. In addition, the

Table IV—Drugs Found Not to Interfere[a] with the Cobalt Oxide Oxidation Method for Determining Phenacetin[b]

Acetaminophen	Isoniazid
Acetanilide	Meperidine
p-Aminophenol	Meprobamate
Bisacodyl	Methadone
Caffeine	Methenamine mandelate
Carisoprodal	Methyprylon
Chloramphenicol	Morphine
Chlormezanone	Phenacemide
Chloroquine	Phenaglycodol
Dicumarol	Phenylbutazone
Diethylpropion	Phenylephrine
Ethchlorvynol	Propoxyphene
Ethinamate	Salicylamide
Glutethimide	Salicylic acid
Hydroxyphenamate	Trimethadione

[a] Absorbance of the oxidation products in sodium hydroxide was less than 0.01 for 10 mcg./ml. of each drug. [b] Absorbance measured at 320 nm.

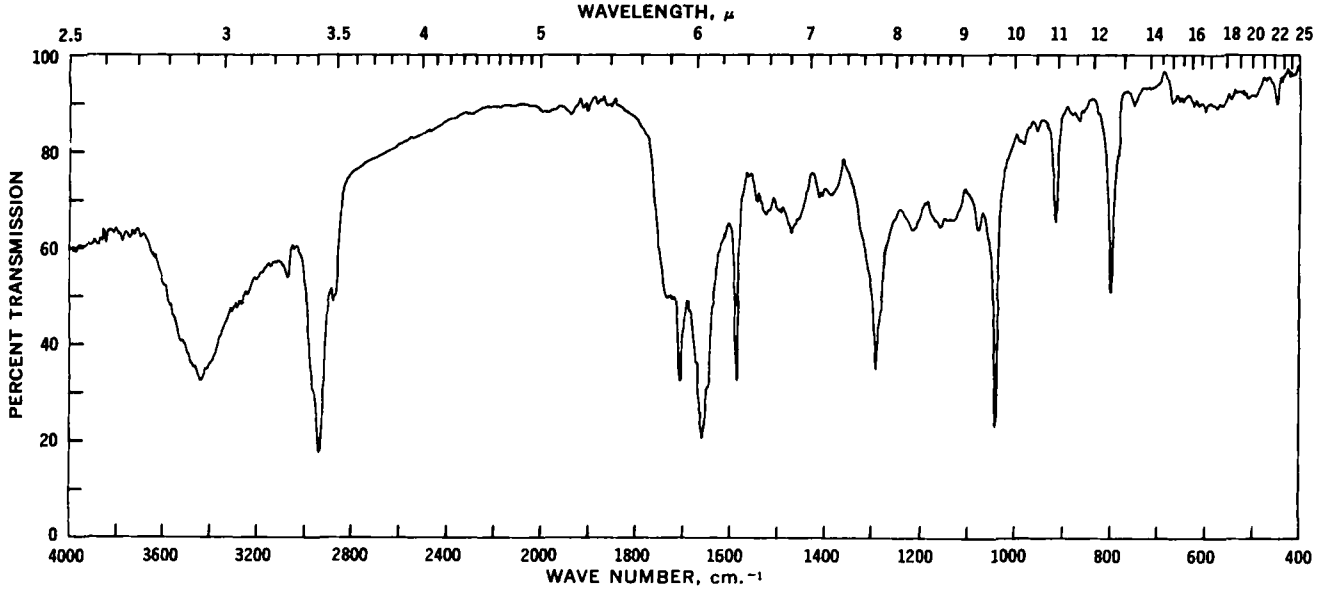

Figure 2—*IR absorption spectrum of the cobaltic oxide oxidation product from phenacetin.*

alkali wash steps must be eliminated to prevent the removal of metabolites from the ether solvent.

IR data indicate that the phenacetin oxidation product(s) consists of a carbonyl group, as evidenced by the strong absorbance (Fig. 2) in the 6-μ region. Aliphatic C—H stretching was conspicuous at 3.4 μ. The phenacetin product(s) is suggested to be a mixture of quinone- or quinonimine-type compounds with aliphatic substituents. The exact chemical structure has not been elucidated for the product(s) but this information is not essential for routine application and effectiveness of the procedure.

SUMMARY

Biological specimens can be analyzed spectrophotometrically for phenacetin after oxidation of the drug by cobaltic oxide. The procedure has an excellent amount of specificity, with no other group of pharmacological agents affording interference. The particular extraction technique required to recover the neutral drug phenacetin from biological environments makes specimens with high lipid concentrations difficult to analyze.

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Drug Permeation through Membranes I: Effect of Various Substances on Amobarbital Permeation through Polydimethylsiloxane

EDWARD G. LOVERING[▲] and DONALD B. BLACK

Abstract □ The effect of some excipients, nutrients, surfactants, and adsorbents on the permeation of amobarbital through polydimethylsiloxane was measured. At a pH of 5, the permeability coefficient, P, of amobarbital is $(7.70 \pm 0.34) \times 10^{-8}$ cm.^{2} sec.$^{-1}$. The rate of drug transfer across the membrane depends on pH because only the unionized species is eligible for transfer. If P is calculated from the actual concentration of unionized drug, then transfer is not a function of pH. Excipients typical of those used in Canadian formulations of amobarbital or its sodium salt have no significant effect on the coefficient of permeability. Bovine albumin has no effect, but the coefficient is depressed by skim milk, perhaps due to binding of the drug. The drug is strongly adsorbed by charcoal. The permeation coefficient is reduced by anionic, cationic, and nonionic surfactants in a way that is consistent with the concept of drug partition between the aqueous and surfactant phases present when surfactant micelles form in water. After dissolution, the permeability coefficients of all Canadian amobarbital preparations tested were found to be identical.

Keyphrases □ Drug permeation through membranes—effect of excipients, nutrients, surfactants, and adsorbents on amobarbital permeation through polydimethylsiloxane □ Amobarbital, permeation through polydimethylsiloxane membranes—effect of excipients, nutrients, surfactants, and adsorbents, permeability coefficients of 14 formulations □ Permeation, amobarbital through polydimethylsiloxane membranes—effect of excipients, nutrients, surfactants, and adsorbents □ Surfactant effect—amobarbital permeation through polydimethylsiloxane membranes □ Excipient effect—amobarbital permeation through polydimethylsiloxane membranes

While always a matter of concern (1), bioavailability and drug absorption have become topics of enlarged interest because of the economically motivated need to compare the effectiveness of different formulations of the same drug. Drug absorption can be envisaged as a two-step process: disintegration and dissolution in the GI tract followed by permeation through the gut wall into the blood. Both of these processes can be modeled *in vitro*. Extensive *in vitro* dissolution studies were carried out and led to USP (2) and NF (3) dissolution specifications for certain drugs.

Drug permeation measurements, while not used as a means to control and evaluate formulations, have been applied to the study of interactions between drugs and other materials liable to affect absorption from the GI tract. The theoretical aspects of drug absorption were discussed by Suzuki *et al.* (4) and Wagner (5), while *in vitro* experimental work with animal membranes was reported by a number of workers (6–8). Synthetic membranes are of value in studying the interactions of drugs that cross the gut wall primarily by a partition–diffusion mechanism, where the rate of transfer across the membrane depends mainly on the membrane–aqueous phase partition coefficient and the solubility of drug in the membrane. If permeation rates of a series of similar drugs through a synthetic membrane are arranged in rank order, one would expect to find the same order of permeation rate *in vivo*, provided no significant interaction between drug and membrane takes place. This behavior is exhibited by the barbiturates (9).

In comparison to animal membranes, synthetic membranes are uniform, reproducible, easy to handle, stable, and cheap. For convenient, reproducible rates of permeation, the membranes should be elastomeric and well above their glass temperature. The material used should be noncrystalline at the temperature of the experiment to avoid variations in permeability due to changes in the degree of crystallinity. The latter depends upon the temperature and rate of crystallization, molecular weight, molecular weight distribution, degree of branching, and other factors (10). Variations in membrane permeability may also arise from the presence of filler, plasticizer, and changes in the degree of crosslinking (11). While a number of synthetic polymers have been tried for drug interaction studies (9), most workers choose polydimethylsiloxane, probably because it is one of the most permeable, nondialysis types of elastomers known (11). The effect of high surface area silica filler, present in commercially available polydimethylsiloxane membranes, was examined by Most (12) and Flynn and Roseman (13). Filler adds to the difficulty of membrane characterization and may lead to variation in the coefficient of permeability between different specimens of polydimethylsiloxane. Drugs and drug interactions studied by this technique include barbiturates, phenylalkylamines (9), aminophenones (14, 15), chlorpromazine (16), and various organic molecules (17).

This paper reports the effect of typical excipients, endogenous substances, adsorbents, emulsifiers, and foods on the permeation of amobarbital through polydimethylsiloxane and rat intestinal membranes. The work is part of an extensive program of biopharmaceutical studies involving the economically most important drugs sold in Canada.

EXPERIMENTAL

Permeation Apparatus—Steady-state permeation coefficients were measured using a cell of the type described by Garrett and Chemburker (18), modified by placing a magnetic stirring bar inside the cell. The cell was placed in a beaker containing a buffered solution of the drug, and both the cell and beaker, stirred magnetically, were thermostated at 37 ± 1.0°. The cell membranes, made of polydimethylsiloxane, were 0.014 cm. thick and contained about 30 parts of silica filler (13). During an experiment the cell contained borate buffer solution at pH 10 and the beaker contained about 160 ml. of sodium amobarbital solution at a concentration in the region of 3.0×10^{-3} *M*. Under these conditions, steady-state diffusion was reached about 1 hr. after beginning the experiment. The permeation coefficient did not change when the same membrane was used in successive experiments.

Table I—Permeability Coefficients Calculated from Measurements Made over a Range of pH Values

pH	Concentration of Unionized Drug, C_0, $M \times 10^3$	Apparent Permeation Coefficient[a], P_a, (cm.^{2} sec.$^{-1}$) $\times 10^6$	Actual Permeation Coefficient[b], P, (cm.^{2} sec.$^{-1}$) $\times 10^6$
6.03	2.428	8.22	8.35
6.98	2.906	6.59	7.46
7.05	2.867	6.24	7.21
7.51	2.204	5.11	7.36
		5.29	7.62
		5.17	7.45
7.52	1.927	5.24	7.61
		5.34	7.75
		5.26	7.64
7.61	2.002	4.92	7.69
7.85	1.725	3.62	7.16
8.03	1.301	2.87	7.12
8.38	0.825	1.66	7.17
		Mean	7.51 ± 0.33

[a] Calculated from the total drug concentration, C. [b] Calculated from C_0, the concentration of unionized drug.

Analysis—The desorbing borate solution was pumped continuously through the cells of a UV spectrometer, and readings at 240.5 nm. were taken manually at appropriate time intervals. The time correction due to the external loop was less than 10 sec. and was neglected. Calibration of the spectrometer was with USP grade sodium amobarbital. Beer's law was obeyed. The extinction coefficient was 9830 in borate buffer containing 3.092 g. l.$^{-1}$ boric acid, 3.782 g. l.$^{-1}$ potassium chloride, and sufficient sodium hydroxide to yield a final pH of 10.00 (about 1.75 g. l.$^{-1}$).

Intestinal Preparation—Intestinal segments were obtained from fasted, male, Sprague–Dawley rats, weighing 240–280 g., after killing by decapitation or carbon dioxide. Ten-centimeter segments of the proximal jejunum under a tension of 20 g. were mounted, noneverted, in a system of glass and polyethylene tubing, which permitted continuous flow of fresh 2.0 $\times$ 10^{-3} M sodium amobarbital in Krebs–Ringer bicarbonate buffer (19) at a pH of 7.4 through the segment to discard (20). Drug transfer across the intestine was into 50 ml. of serosal solution of the buffer without the drug. All solutions, at 37 ± 1°, were continuously oxygenated by bubbling an oxygen–carbon dioxide (95:5) mixture through them. Two-milliliter aliquots were taken at 10-min. intervals for analysis and replaced by fresh buffer solution.

Drug Extraction and Analysis—Blank experiments made in the absence of drug showed that some substance which absorbs strongly in the UV is extracted from the intestinal segments under the experimental conditions, making a direct UV analysis impossible. For analysis, the serosal aliquot pH was lowered to 3.2 by adding 1 ml. of 0.05 N hydrochloric acid. The drug was extracted into 10 ml. chloroform by shaking overnight at room temperature. Eight milliliters of the chloroform layer was shaken for 30 min. with 2 ml. 0.45 N sodium hydroxide. About 76% of the drug was recovered by this procedure. Absorption in the aqueous layer was read at 255 nm., and the drug concentration was read from a calibration curve.

Materials—The following were used: sodium amobarbital USP[1]; lactose USP[2]; calcium sulfate dihydrate[2]; acacia USP[4]; starch USP[4]; calcium stearate, technical[5]; talc, fine powdered, acid purified[6]; activated charcoal[7]; bovine albumin, fraction V[6]; skim milk, household; caffeine USP[8]; sodium lauryl sulfate USP[4]; cetrimonium chloride[8]; polysorbate 80[9]; sodium cholate[10]; and gelatin BP[6].

[1] May and Baker.
[2] Merck.
[3] Analar, British Drug House.
[4] Fisher.
[5] E. H. Sargent.
[6] British Drug House.
[7] Darco G-60, Anachemia.
[8] Eastman.
[9] Tween 80, Fisher.
[10] Sigma Chemical.

Table II—Effect of Excipients on the Permeability Coefficient of Amobarbital

Excipient	Concentration[a], g. l.$^{-1}$		Permeability Coefficient, P, (cm.^{2} sec.$^{-1}$) $\times 10^6$
Lactose	3.4		7.23
	10.5		7.45
	41.3		7.83
		Mean	7.50 ± 0.31
Calcium sulfate dihydrate	1.8		7.34
	1.8		7.10
	10.6		7.73
	39.8		7.71
		Mean	7.47 ± 0.31
Acacia	5.0		7.23
Gelatin	5.0		7.05
	10.2		7.36
	42.8		7.88
		Mean	7.42 ± 0.42
Corn starch	4.0		7.12
	10.8		7.32
	41.2		7.25
		Mean	7.23 ± 0.10
Calcium stearate	2.4		7.77
	2.4		7.54
		Mean	7.65 ± 0.16
Talc	3.0		7.02
	5.2		6.81
	10.6		6.72
	20.0		6.91
		Mean	6.87 ± 0.13

[a] In drug solution at a pH of 5.

RESULTS AND DISCUSSION

Permeability Coefficient—The effect of various substances on the permeation of amobarbital through a polydimethylsiloxane membrane is expressed in terms of a permeability coefficient, P:

$$P = kD \qquad \text{(Eq. 1)}$$

where k is a constant for a given set of experimental conditions and D is the diffusion coefficient. Permeation refers to the transfer of drug from solution on one side of the membrane to solution on the other side, whereas diffusion is movement of drug within the membrane. If the concentration of drug in the membrane at the absorbing side is c and it is zero at the desorbing side and if steady-state conditions prevail, then the quantity of drug, q, transferred across the membrane at time t is:

$$q = \frac{Dc}{l}\left(t - \frac{l^2}{6D}\right) \qquad \text{(Eq. 2)}$$

where l is the thickness of the membrane (21). The concentration of drug in the membrane at the surface in contact with the solution is proportional to C_0, the concentration of unionized drug in solution and to the conditions of the experiment. Thus:

$$c = kC_0 \qquad \text{(Eq. 3)}$$

and Eq. 2 can be written as:

$$q = \frac{DkC_0}{l}t - \frac{kC_0l}{6} \qquad \text{(Eq. 4)}$$

The experimental results were plotted as q *versus* t. Upon reaching the steady state, the slope, dq/dt, is given by DkC_0/l. Substitution into Eq. 1 yields:

$$P = \frac{(dq/dt)l}{C_0A} \qquad \text{(Eq. 5)}$$

where A is the area of membrane in contact with the drug solution and dq/dt is the rate of drug transfer.

Table III—Effect of Charcoal on the Permeability Coefficient

Charcoal Concentration[a], g. l.$^{-1}$	Apparent Permeability Coefficient[b], P_a, (cm.^{2} sec.$^{-1}$) × 10^6	Permeability Coefficient[c], P, (cm.^{2} sec.$^{-1}$) × 10^6
1.0	5.56	—
—	6.34	—
1.8	4.09	6.95
4.5	1.57	8.14
6.8	0.68	7.58
16.7	0.00	—
	Mean	7.55 ± 0.59

[a] The solution pH was 5. [b] Calculated from the original concentration of amobarbital before mixing with charcoal. [c] Calculated from the concentration of amobarbital measured spectroscopically in the supernatant liquid after mixing with charcoal.

Dependence of Permeability on pH—The permeability coefficient of amobarbital going from an acetate buffer at pH 5.00 ± 0.02 was found to be 7.70 × 10^{-6} cm.^{2} sec.$^{-1}$, with a standard deviation of ±0.34 cm.^{2} sec.$^{-1}$ based on 10 determinations. The rate of drug transfer across the membrane is pH dependent because only the nonionized form is soluble in the membrane and hence eligible for transfer. The concentration of nonionized drug in solution, C_0, is given by:

$$C_0 = C[1 + \exp[2.303(\text{pH} - \text{pKa})]]^{-1} \quad \text{(Eq. 6)}$$

where C is the total concentration of drug substance, pH is the pH of the drug solution, and K_a is the ionization constant of the drug. Taking pKa = 7.865 for amobarbital (22), only 0.1% of the drug is ionized at a pH of 5. Permeability coefficients obtained from measurements at pH values in the range where C_0 changes rapidly with pH are given in Table I. The apparent permeability coefficient, P_a, is calculated from Eq. 5 using C, the total concentration of amobarbital present in the system, and reflects the effect of pH on drug transfer. The coefficient P, calculated from the actual concentration of unionized drug, is obtained from Eq. 5. The permeability coefficient P is (7.51 ± 0.33) × 10^{-6} cm.^{2} sec.$^{-1}$ and does not vary significantly over the pH range examined. This constancy indicates the only effect of pH on permeability is the effect on concentration of unionized drug.

Effect of Excipients and Charcoal—The effect of a number of substances, commonly present as excipients in Canadian formulations of amobarbital, on the permeability coefficient was measured. The results (Table II) show these substances to have no significant effect on the permeability coefficient.

The consequence of adding activated charcoal to a drug solution is shown in Table III. This material presumably adsorbs drug, thereby reducing the solvent concentration and hence the coefficient of permeability calculated on the basis of the original drug concentration (23). If the actual concentration of drug is used to calculate the coefficient, it should be, within experimental error, the same as that calculated for drug with no additive, namely (7.70 ± 0.34) × 10^{-6} cm.^{2} sec.$^{-1}$. This is the case with P = (7.55 ± 0.59) × 10^{-6} cm.^{2} sec.$^{-1}$ in the presence of charcoal. There is a good correlation (r^2 = 0.92) between the weight and, hence, surface area of the activated charcoal and the apparent permeability coefficient. The result suggests that administration of activated charcoal might be of value in the early stages of amobarbital poisoning.

Table IV—Effect of Some Nutrients on the Permeability Coefficient

Additive	pH	Concentration, g. l.$^{-1}$	Permeability Coefficient[a], P, (cm.^{2} sec.$^{-1}$) × 10^6
Bovine albumin	5.00	2.0	7.69
	5.00	19.6	7.22
	7.51[b]	4.5	7.02
	7.51[b]	10.7	7.32
	7.51[b]	15.7	6.20
	7.44[b]	31.0	6.80
Skim milk	5.00	38.7	6.34
	5.00	85.6	5.50
	7.30[b]	43.0	7.07
	7.15[b]	80[c]	6.58
	7.01[b]	127	5.45

[a] Calculated from C_0, the concentration of unionized drug. [b] Measured pH after dissolving drug and additive in phosphate buffer at pH 7.40. [c] This is the approximate concentration of reconstituted skim milk.

Table V—Effect of Sodium Lauryl Sulfate on Amobarbital Permeation[a]

Weight of Surfactant, W_m, g./l.	Apparent Permeability Coefficient, P_a, (cm.^{2} sec.$^{-1}$) × 10^6	Total Drug, Q, (moles) × 10^3	$\frac{Q}{C_w W_w}$, (1 g.$^{-1}$) × 10^3	W_m/W_w, × 10^3
24.39	1.36	2.433	5.81	25.1
18.52	1.67	2.821	4.72	18.9
6.17	3.21	2.612	2.42	6.22
0.81	6.65	2.559	1.16	0.81
0.81	6.73	2.559	1.16	0.81

[a] Drug–surfactant solution at pH 5.

Effect of Nutrients—The effect of bovine albumin and skim milk on the permeation of amobarbital is reported in Table IV. There appears to be little, if any, interaction between amobarbital and bovine albumin, at least under the conditions of these experiments. There is a clear interaction with skim milk, but if the interaction occurs to the same extent *in vivo*, it is not of such magnitude as to have much effect on the absorption of the drug by the body. An attempt was made to study the effect of caffeine on the permeation coefficient, but the membrane was found to be permeable to caffeine. The latter absorbs at 240.5 nm., interfering with the amobarbital analysis. The permeation coefficient of caffeine is (2.8 ± 0.5) × 10^{-6} cm.^{2} sec.$^{-1}$.

Surfactant Effects—Drug in an aqueous system containing surfactant micelles is expected to partition between the aqueous phase and the micellar, oil, phase. The total quantity of drug in the system, Q, is given by:

$$Q = C_m V_m + C_w V_w \quad \text{(Eq. 7)}$$

where C_m and C_w are the concentrations in the micellar and water phases, respectively, and V_m and V_w are the corresponding volumes. Substituting weight for volume in Eq. 7 and rearranging yield:

$$\frac{Q}{C_w W_w} = \frac{K_p}{\rho_m} \cdot \frac{W_m}{W_w} + \frac{1}{\rho_w} \quad \text{(Eq. 8)}$$

where ρ_m and ρ_w are the densities of the micellar and water phases, respectively, and K_p is the partition coefficient of drug between the micellar and aqueous phases: $K_p = C_m/C_w$. Equation 8 can be plotted as a straight line with slope K_p/ρ_m and intercept $1/\rho_w$. In Eq. 8, Q and W_m are known directly from the concentrations of drug

Table VI—Effect of Cetrimonium Chloride on Amobarbital Permeation[a]

Weight of Surfactant, W_m, g./l.	Apparent Permeability Coefficient, P_a, (cm.^{2} sec.$^{-1}$) × 10^6	Total Drug, Q, (moles) × 10^3	$\frac{Q}{C_w W_w}$, (1 g.$^{-1}$) × 10^3	W_m/W_w, × 10^3
19.09	1.06	2.865	7.51	19.52
14.35	1.33	2.907	5.89	14.60
9.09	1.84	2.670	4.25	9.20
4.02	4.02	2.893	2.56	4.05
1.24	5.58	2.608	1.40	1.24
0.45	7.12	2.012	1.08	0.45

[a] Drug–surfactant solution at pH 5.

Table VII—Effect of Polysorbate 80 on Amobarbital Permeation[a]

Weight of Surfactant, W_m, g./l.	Apparent Permeability Coefficient, P_a, (cm.^{2} sec.$^{-1}$) × 10^6	Total Drug, Q, (moles) × 10^3	$\frac{Q}{C_w W_w}$, (1 g.$^{-1}$) × 10^3	W_m/W_w, × 10^3
80.6	1.62	2.907	5.20	88.0
70.2	1.68	3.089	5.29	75.7
60.0	1.85	3.554	4.46	64.0
42.3	2.46	2.900	3.28	44.3
37.5	2.38	2.617	3.38	39.1
28.1	2.73	2.900	2.91	29.0
14.5	4.09	2.338	1.91	14.7
3.06	6.24	2.898	1.24	3.07

[a] Drug–surfactant solution at pH 5.0.

and surfactant in the solution and W_w is obtained by taking the density of the surfactant to be unity and the change in volume on mixing with water to be zero. This approximation introduces an error of less than 1% for the surfactants and concentrations used in this study. The concentration of drug in the aqueous phase, C_w, was calculated from the known permeation coefficient (7.70×10^{-6} cm.^{2} sec.$^{-1}$) and the measured apparent permeation coefficient, P_a. This assumes that the membrane was unaffected by surfactant and that the only effect of the latter was to remove drug from aqueous solution by incorporation into micelles. For sodium cholate studied in the region of pH 7.5, the aqueous phase concentration of nonionized drug was calculated from P_a, which was used to calculate the total aqueous concentration, C_w, from Eq. 6. Results for the four surfactants studied are presented in Tables V–VIII. The data for each surfactant were analyzed according to Eq. 8. The slope, intercept, and K_p obtained in each case are given in Table IX, along with the coefficient of correlation r.

The experimental results are adequately described by Eq. 8 and are consistent with the concept that partitioning of amobarbital between the aqueous and micellar phases takes place. Equation 8 is valid only above the CMC, which is about 2.5, 0.4, and 0.1 g. l.$^{-1}$ for sodium lauryl sulfate (24), cetrimonium chloride (24), and polysorbate 80 (25), respectively. Below the CMC, the surfactant has little effect on the permeation rate. This presumably means that there is little if any complexing at a pH of 5 between amobarbital and the surfactants studied. The results also indicate that there is little interaction between the surfactant and the membrane. Generally the type of interaction that could occur would be inbibing of the low molecular weight surfactant by the membrane. The swelling of synthetic elastomers by organic molecules is well known and may lead to substantial increases in the rate at which permeant molecules traverse the membrane (10, 21). The extent of imbibation depends upon the solubility of the low molecular weight species in the membrane, solubility usually being greatest for materials of similar chemical structure. The structures of the surfactants examined here and that of polydimethylsiloxane are quite different and little swelling would be anticipated, as is borne out by the results. The structure of animal membranes is closer to that of typical surfactants. Perhaps it is the dissolution of the surfactant in these membranes that leads to enhanced *in vivo* absorption of certain drugs as, for example, absorption of secobarbital by goldfish (25, 26).

Table VIII—Effect of Sodium Cholate on Amobarbital Permeation[a]

Weight of Surfactant, W_m, g./l.	Apparent Permeability Coefficient, P_a, (cm.^{2} sec.$^{-1}$) × 10^6	Total Drug, Q, (moles) × 10^3	$\frac{Q}{C_w W_w}$, (1 g.$^{-1}$) × 10^3	W_m/W_w, × 10^3
74.8	2.41	3.222	3.109	81.1
39.8	3.77	2.779	1.957	41.6
21.1	5.27	2.733	1.392	21.6
11.8	6.15	2.479	1.185	12.0
4.7	6.75	3.040	1.077	4.7

[a] Drug–surfactant solution at pH ~ 7.5.

Table IX—Surfactant Slopes, Intercepts, and Partition Coefficients Calculated from Eq. 8

Surfactant	$K\rho_m^{-1}$, 1 g.$^{-1}$	ρ_w^{-1}, (1 g.$^{-1}$) × 10^3	r^2	Partition Coefficient, K
Sodium lauryl sulfate	0.191	1.07	0.99	191
Cetrimonium chloride	0.334	1.05	0.99	334
Polysorbate 80	0.049	1.27	0.97	49
Sodium cholate	0.029	0.76	0.99	29

Table X—Effect of Sodium Lauryl Sulfate on Transfer of Amobarbital across Rat Intestine

Concentration of Sodium Lauryl Sulfate, g. l.$^{-1}$	$\bar{R}$[a]
0.00	1.17 ± 0.09
0.01	1.15 ± 0.22
0.30	1.29 ± 0.22
10	0.49 ± 0.07
20	0.36 ± 0.06
40	0.34 ± 0.04
80	0.19 ± 0.05

[a] $\bar{R}$ is the ratio of the average drug transfer rate without surfactant to the average transfer rate in the presence of surfactant.

Results obtained for the transfer of amobarbital across the rat intestine are given in Table X. The quantity, R, is the ratio of transfer rate in the presence, and the absence, of surfactant in the same segment of gut, following the crossover procedure described by Reuning and Levy (6). Results from two or more segments were averaged to obtain $\bar{R}$. At surfactant concentrations greater than the CMC, the transfer rate clearly decreases as the surfactant increases. Results below the CMC are insufficiently precise to permit one to say whether there is a surfactant effect or not.

The partition coefficients, K_p, in Table IX indicate that amobarbital is most soluble in sodium lauryl sulfate and cetrimonium chloride. Both of these compounds have alkyl chains which might be expected to be highly compatible with the nonpolar side chains of amobarbital. There is also a good possibility of reaction between cetrimonium ion and barbiturate ion leading to a decrease in concentration of nonionized drug available for transfer. Polysorbate 80 contains repeating polar groups, while cholic acid has a dense ring structure of perhaps limited compatibility with amobarbital.

Amobarbital Formulations—The permeability of amobarbital in 14 Canadian formulations was measured. Each dosage form was stirred in phosphate buffer at 37° for about 2 hr., and the pH and concentration of the solution were measured before measuring the permeation rate. For all formulations the average coefficient of permeability was (6.87 ± 0.26) × 10^{-6} cm.^{2} sec.$^{-1}$ with a range of 6.50 to 7.29 × 10^{-6} cm.^{2} sec.$^{-1}$. Thus, from the point of view of *in vitro* permeability, all formulations are equivalent.

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Keyphrases □ Dibenzoxazepines, carboxamide and other side chains—tissue distribution, affinity for melanin, dogs □ Carboxamide-substituted dibenzoxazepines—tissue distribution in dogs, affinity for melanin □ Tissue distribution—dibenzoxazepines, melanin affinity, dogs □ Melanin tissue distribution—dibenzoxazepines, dogs □ Ocular tissue distribution—dibenzoxazepines, dogs

It is generally accepted that substituted phenothiazines can adversely affect the eye and skin when large amounts are ingested chronically (1–3). Some compounds, like chlorpromazine, produce primarily opacities of the lens and cornea, whereas others, like thioridazine, can produce a loss of vision by their effect on the retina. Because of these past findings, studies were conducted with some substituted dibenzoxazepines that have exhibited CNS activity in animals (4, 5). The results of these studies show that the presence of a carboxamide side chain alters the localization of the compound and/or its metabolites in the tissues of dogs, including the melanin-containing portion of the eye, as well as the binding of the parent molecules to melanin granules of beef eyeball *in vitro*.

METHODS AND MATERIALS

Purity and Specific Activity of Compounds—The radioactive compounds studied had the following chemical names, radiochemical purities, and specific activities, respectively: I, 4-[3-(7-chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepin-5-yl)propyl]-1-piperazine-$^{14}C_2$-ethanol hydrochloride, 96%, 21.6 μc./mg.; II, 4-[3-(7-(trifluoromethyl)-5,11-dihydrodibenz[*b,e*][1,4]oxazepin-5-yl)propyl]-1-piperazine-$^{14}C_2$-ethanol hydrochloride, 99%, 24.9 μc./mg.; III, 5-[(2-dimethylamino)ethyl-1,2-$^{14}C_2$]-5,11-dihydrodibenz-[*b,e*][1,4]oxazepine maleate, 99%, 6.9 μc./mg.; and IV, 7-chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepine-5-^{14}C-carboxamide, 99%, 5.0 μc./mg.

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catheter was then inserted about 1.27 cm. (0.5 in.) into the common bile duct. The catheter was tied in place, and the midline incision was closed with wound clips.

Analysis of Blood—A sample (0.2 ml.) of heparinized blood was digested in 0.5 ml. of 1 *N* NaOH by heating overnight at 80°. The digest was then bleached with 30% hydrogen peroxide, neutralized with 0.2 ml. of 2-ethylhexanoic acid, and counted with 15 ml. of scintillator (6).

Collection and Analysis of Urine, Bile, and Feces—Purebred beagles (9–11 kg.) and monkeys, *Macaca mulatta* (3.2–4.1 kg.), were fasted overnight prior to drug administration and were housed in metabolism cages[1] designed to allow the separate collection of urine and feces. All animals were dosed between 8 and 10 a.m. Samples of feces were homogenized with 2–3 volumes of methanol. About 50–100 mg. of homogenate was transferred to a weighed scintillation vial, and 1 ml. of a solubilizer[2] was added. The sample was shaken for at least 1 hr., bleached with 30% hydrogen peroxide, and counted in 15 ml. of scintillator (6). Samples of urine and bile were counted directly in 15 ml. of scintillator (6).

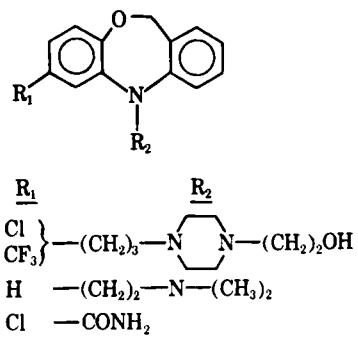
Analysis of Tissue—Samples of brain tissue were homogenized in water, using an all-glass Potter–Elvehjem homogenizer; about 200 mg. of homogenate was transferred to a weighed scintillation vial and digested in 2 ml. of solubilizer. Certain portions of the eye (lens, cornea, aqueous and vitreous humors, and combined retina, choroid, and sclera) were digested directly in 2 ml. of solubilizer. Similarly, representative portions of omental fat, skin (without hair), and the adrenal gland were digested directly in the solubilizer. Samples of tissue from the heart, kidneys, liver, lungs, thigh muscles, and testes were analyzed by first grinding the tissue in a meat grinder and then solubilizing about 100 mg. of the well-mixed sample in 2 ml. of solubilizer. All samples of tissue were counted in 15 ml. of toluene scintillator solution, which contained 5 g. 2,5-diphenyloxazole and 0.3 g. 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene/l. of toluene. Since these analytical methods are normally reproducible to within 5%, replicate determinations were not routinely performed.

Binding to Melanin Granules *In Vitro*—Melanin granules were prepared from the eyes of oxen, according to the method of Potts (7). Since IV is only slightly soluble in water (I, II, and III are readily water soluble), *all* compounds were first dissolved in 100% dimethylformamide. Then a stock solution of each drug at a concentration of 3.5×10^{-3} *M* in 10% dimethylformamide–90% 0.0165 *M* sodium phosphate buffer, pH 6.0, was prepared. The final incubation mixtures, for drug concentrations of 0.75×10^{-4} and 1.5×10^{-4} *M*, contained 2.15 and 4.30 ml. of the stock drug solution and 6.85 and 4.70 ml. of buffer, respectively, and 1 ml. of a suspension of melanin granules (7.1 mg. dry weight/ml.). Thus, the final concentration of dimethylformamide was 2.15% at 0.75×10^{-4} *M* and 4.30% at 1.5×10^{-4} *M*. The final pH was about 6.9. Samples were incubated, with shaking, at 25° for 30 min. and then centrifuged at 35,000×*g* for 15 min. Aliquots of the resulting supernate and of a control sample prepared with water, instead of with melanin granules, were counted in Bray's scintillator. Control experiments with I, II, and III, carried out in an entirely aqueous medium, indicated that the presence of dimethylformamide at the concentrations employed did not appreciably alter the binding of these compounds to melanin granules.

Counting of Samples—Radioactivity in each sample was measured by a liquid scintillation spectrometer[3]. Counting efficiency was determined with automatic external standardization and the use of previously prepared quench curves.

RESULTS

Dogs with externalized bile ducts were dosed intravenously, each with one of the four dibenzoxazepines (5 mg./kg.). The single animals treated with each compound were sacrificed 7 hr. after they had been dosed, and selected tissues were examined for their content of radioactivity (Table I). After the administration of I, II, and III, radioactivity, considered relative to that present in the blood, was highly localized in tissues. The highest concentrations of radioactivity were located in the pigmented portion of the eye,



Compound	R_1	R_2
I	Cl	$-(CH_2)_3-N\langle\rangle N-(CH_2)_2OH$
II	CF_3	
III	H	$-(CH_2)_2-N-(CH_3)_2$
IV	Cl	$-CONH_2$

structures of substituted dibenzoxazepines

consisting of the combined retina, choroid, and sclera, and in the liver and lungs. Most of the other tissues contained levels of radioactivity that were several times those present in the blood but not as high as those just mentioned. By contrast, levels of radioactivity in most of the tissues of the dog that had been dosed with IV were actually lower than those present in the blood, except for the liver and kidneys, the organs of excretion. For IV, there was no localization of radioactivity in the combined retina, choroid, and sclera.

The distribution of radioactivity was examined in selected tissues of intact animals that had been dosed orally with each of the four dibenzoxazepines. The animals were sacrificed 7 or 14 days after being dosed, at which time the residual levels of radioactivity in tissues were determined (Table II). For I, both dogs and monkeys were studied. As it had been after the intravenous administration of I, radioactivity was still localized in certain tissues relative to that present in the blood, most notably in the combined retina, choroid, and sclera of both dog and monkey. For II, radioactivity was localized in the pigmented portion of the eye and in most of the other tissues as it was after the administration of I. The apparent half-life for the elimination of radioactivity from these tissues was about 7 days for most of the tissues and substantially longer for the combined retina, choroid, and sclera. After the administration of III, radioactivity also persisted in the pigmented portion of the eye and in several of the other tissues. The distribution of residual radioactivity in tissues of dogs following the administration of IV, however, was quite different from that observed for the other three dibenzoxazepines. Here, with the marginal exception of the cornea, none of the tissues contained levels of radioactivity higher than that present in the blood and, in most cases, only traces of radioactivity were present.

The distribution of radioactivity after the oral administration of IV was also studied in tissues of intact dogs that were sacrificed 3.5 hr. after being dosed (Table III). Once again, the distribution

Table I—Distribution of Radioactivity in Tissues of Dogs with Externalized Bile Ducts 7 hr. after Intravenous Dosing

Tissue	I[a,b]	II[a,b]	III[a,b]	IV[a,b,c]
	mcg. Drug Equivalents/g. Tissue			
Brain	2.90	3.66	1.86	0.52
Lungs	12.30	24.56	7.43	1.15
Omental fat	0.64	1.57	3.17	1.27
Skin	1.03	1.36	1.94	1.04
Kidneys	3.44	4.83	3.41	1.76
Liver	11.43	14.87	7.67	4.77
Testes	5.60	8.04	5.51	0.90
Heart	2.38	2.95	1.81	0.87
Adrenals	3.19	5.18	4.47	1.17
Skeletal muscle	1.04	1.92	0.96	0.84
Cornea	0.55	0.70	2.35	0.39
Lens	0.13	0.10	1.15	0.35
Aqueous humor	2.34[d]	2.36[d]	0.05	0.15
Vitreous humor			4.31	0.08
Combined retina, choroid, and sclera	23.14	15.01	19.03	0.50
Blood, mcg./ml.	0.45	0.65	0.65	0.98

[a] Five mg./kg. as a 5-min. infusion into the radial vein. [b] Male. [c] Drug administered in 9.6 ml. of 50% aqueous dimethyl sulfoxide. [d] Aqueous and vitreous humors were combined before analysis.

[1] Porter-Mathews Co.
[2] NCS Solubilizer, Amersham-Searle.
[3] Packard Tri-Carb, model 3380.

Table II—Residual Radioactivity in Tissues of Dogs and Monkeys after Oral Administration of Some Substituted Dibenzoxazepines

Tissue	I (14 Days[a]) Dog[b]	I (14 Days[a]) Monkey[c]	II (7 Days), Dog[b]	II (14 Days), Dog[b]	III (7 Days), Dog[b]	IV (7 Days), Dog[d]
	mcg. ± *SE* Drug Equivalents/g. Tissue					
Brain	0.30 ± 0.07	0.067 ± 0.011	0.21 ± 0.00	0.14 ± 0.01	0.016 ± 0.008	0.007 ± 0.007
Lungs	0.34 ± 0.05	0.13 ± 0.03	0.38 ± 0.02	0.19 ± 0.03	0.128 ± 0.040	0.078 ± 0.045
Omental fat	0.12 ± 0.01	0.061 ± 0.013	0.35 ± 0.14	0.15 ± 0.03	0.274 ± 0.253	0.000
Skin	0.16 ± 0.02	0.14 ± 0.03	0.36 ± 0.04	0.18 ± 0.01	0.118 ± 0.020	0.032 ± 0.018
Kidneys	0.33 ± 0.04	0.78 ± 0.67	0.46 ± 0.04	0.22 ± 0.02	0.153 ± 0.011	0.016 ± 0.009
Liver	1.41 ± 0.24	0.69 ± 0.18	2.17 ± 0.03	1.03 ± 0.11	0.510 ± 0.092	0.24 ± 0.04
Skeletal muscle	0.22 ± 0.03	0.11 ± 0.01	0.23 ± 0.02	0.14 ± 0.01	0.049 ± 0.025	0.000
Cornea	0.12 ± 0.01	0.063 ± 0.01	0.15 ± 0.02	0.094 ± 0.009	0.000	0.000
Lens	0.049 ± 0.004	0.032 ± 0.001	0.056 ± 0.005	0.040 ± 0.002	0.008 ± 0.001	0.021 ± 0.009
Aqueous humor and vitreous humor	0.075 ± 0.006[e]	0.033 ± 0.016[e]	0.10 ± 0.01[e]	0.13 ± 0.04[e]	0.000	0.000
Combined retina, choroid, and sclera	0.96 ± 0.28[f]	3.96 ± 1.40	1.04 ± 0.24[f]	0.80 ± 0.05[f]	0.308 ± 0.054[f]	0.070 ± 0.041
Blood, mcg./ml.	0.12 ± 0.02	0.067 ± 0.010	0.15 ± 0.01	0.066 ± 0.013	0.043 ± 0.005	0.15 ± 0.01

[a] The time when the animals were sacrificed is cited in parentheses. [b] Dogs were given 5 mg./kg. by gavage. Two dogs of each sex were employed, except for III for which one male and two females were used. [c] Monkeys (one of each sex) were given 2.5 mg./kg. by nasogastric intubation. [d] Dogs (two of each sex) were given 10 mg./kg. by capsule. [e] Aqueous and vitreous humors were combined before analysis. [f] Concentrations in the combined retina, choroid, and sclera were divided by the concentrations in blood. These ratios were statistically different ($p < 0.025$) by Student's t test from concentrations found in dogs dosed with IV.

of radioactivity found in the tissues of these dogs was very different from that seen for the other substituted dibenzoxazepines. Here, only the liver and kidneys contained levels of radioactivity that were higher than that present in the blood. Radioactivity was rather uniformly distributed in most of the other well-perfused tissues, with the exception of the brain in which low levels were found. Thus, the results obtained with intact dogs dosed orally were similar to those found with the dog having the externalized bile duct that had been dosed intravenously. This finding suggests that the administration of IV intravenously in 50% aqueous dimethyl sulfoxide did not influence the distribution of IV in tissues of dogs.

To determine whether the mode of excretion of the four dibenzoxazepines had any bearing on the distribution of the parent compound or its metabolites in tissues, the excretion of radioactivity in the urine and feces or bile was examined following the administration of each compound (Table IV). The data show that dogs with externalized bile ducts that had been dosed intravenously excreted radioactivity in the urine and bile in a manner very similar to that found in the urine and feces of the dogs dosed orally. Dogs dosed with the nonpiperazine-containing compounds, III and IV, excreted much more radioactivity in the urine than did dogs dosed with I and II, compounds substituted with the piperazine-containing side chains.

To provide additional evidence for the affinity of the four substituted dibenzoxazepines for the melanin-containing portion of the eye, each radioactive parent dibenzoxazepine was incubated *in vitro* with melanin granules from beef eyeball (Table V). Whereas I, II, and III were significantly bound to the malanin granules, IV was bound only to an extent that is within the error of the method.

Table III—Distribution of IV and/or Its Metabolites in Tissues of Intact Dogs

Tissue	Male[a], mcg./g.	Female[a], mcg./g.	Mean, mcg./g.
Brain	0.12	0.046	0.083
Lungs	0.73	0.62	0.68
Omental fat	0.42	0.17	0.30
Skin	0.92	0.53	0.73
Kidneys	4.12	1.87	3.00
Liver	6.47	4.18	5.33
Testes	0.52	—	0.52[b]
Heart	0.56	0.33	0.45
Adrenals	0.59	0.38	0.49
Skeletal muscle	0.51	0.29	0.40
Cornea	0.19	0.13	0.16
Lens	0.070	0.036	0.053
Aqueous humor	0.15	0.088	0.12
Vitreous humor	0.062	0.083	0.073
Combined retina, choroid, and sclera	0.46	0.36	0.41
Blood, mcg./ml.	1.21	0.72	0.97

[a] Dogs were given 10 mg./kg. of IV in a gelatin capsule. The dogs were sacrificed 3.5 hr. after dosing. [b] Single value only.

DISCUSSION

A number of studies showed that chlorpromazine (8–10) and other phenothiazines (11) are localized in various tissues, particularly in the brain, lungs, and liver. The substituted dibenzoxazepines, I, II, and III, and the dibenzoxepin, doxepin (12), are all localized in tissues of dogs to various degrees. In addition, all of these compounds bind to the pigmented portion of the eye. Bolt and Forrest (13) reported on the interaction of chlorpromazine and 7-hydroxychlorpromazine with melanin. The persistence of radioactivity in the fetal and maternal eyes, but not in other tissues, of mice given ^{35}S-chlorpromazine was also reported (14). It is not known if a common mechanism underlies the formation of cataracts and the development of retinopathy. However, chlorpromazine-induced cataracts have been studied experimentally in both albino and pigmented guinea pigs (15). On the basis of those studies, it was concluded that the development of cataracts had no apparent relationship to the presence of melanin. Thus, the binding of compounds to melanin apparently should not be considered as an indicator of potential cataractogenesis.

The excretion of the dibenzoxazepines reported in the present studies appears to follow the pattern established for the phenothiazines (16). Thus, those compounds substituted with piperazine-containing side chains (I and II) were excreted primarily in the feces or bile, whereas the excretion of a compound substituted with the aliphatic amine side chain (III) was shifted toward the urinary route. Compound IV, which bears the carboxamide side chain, was appreciably excreted in the urine after intravenous administration. After oral dosing, IV is incompletely absorbed; nevertheless, 23% of the dose was excreted in the urine, a considerably larger fraction than that excreted in the urine of the dogs dosed with I and II (5–9% of the dose) (17).

An earlier report described the metabolism of I and II in dogs (17). No significant differences could be found in the excretion, distribution, or biotransformation of these two compounds, which differ by the fact that I contains a chloro substituent on the tricyclic ring system whereas II bears a trifluoromethyl group. The binding of these two compounds to melanin granules *in vitro* was comparable. Likewise, III, but not IV, bound to melanin granules *in vitro*. Interestingly, I, II, III, and IV all appear to undergo hydroxylation on the tricyclic ring system, since mass spectral studies of metabolites of I and II showed the presence of the hydroxyl group (17), and other experiments in this laboratory indicated that glucuronide conjugates of all four compounds were excreted in the bile of dogs. For the phenothiazines, chlorpromazine (9) and fluphenazine (18), 7-hydroxylation has been identified as a prominent metabolic pathway. Additional findings in this laboratory showed

Table IV—Excretion of Radioactivity by Dogs after Oral or Intravenous Administration of Some Substituted Dibenzoxazepines

Compound[a]	Dose, mg./kg.	Route[b]	Time of Sacrifice	Percent of Dose ± *SE*		
				Urine	Feces or (Bile)	Total
I (1)	5	i.v.[c]	7 hr.	5.82	(68.01)	73.83
I (4)	5	p.o.	14 days	9.30 ± 1.07	83.28 ± 4.80	92.58 ± 4.50
II (1)	5	i.v.[c]	7 hr.	4.25	(59.58)	63.83
II (4)	5	p.o.	14 days	5.34 ± 0.50	80.88 ± 2.86	86.22 ± 2.42
III (1)	5	i.v.[c]	7 hr.	35.40	(22.76)	58.16
III (2)	5	p.o.	7 days	55.04 ± 9.47	33.96 ± 7.52	89.00 ± 1.95
IV (1)	5	i.v.[c]	7 hr.	31.55	(46.60)	78.15
IV (4)	10	p.o.	7 days	23.25 ± 0.71	62.12 ± 4.15	85.37 ± 3.74

[a] The number of dogs is indicated in parentheses. [b] All drugs were administered orally in aqueous solution by gavage, except IV which was given in capsules. Intravenous dosing for all drugs except IV was as an aqueous solution infused over a 5- or 10-min. interval. IV was administered in 9.6 ml. of 50% aqueous dimethyl sulfoxide. [c] These dogs had their bile ducts externalized.

Table V—Binding of Some Substituted Dibenzoxazepines to Beef Eyeball Melanin Granules *In Vitro*

Compound	Percent Bound[a]	
	0.75×10^{-4} *M*	1.5×10^{-4} *M*
I	52.6	40.1
II	44.1	35.8
III	23.5	19.5
IV	1.3	1.1

[a] The numbers cited are the average of two or three replicate determinations with the same granule preparation. The percent bound is based on the total radioactivity present in the incubation mixture.

that little, if any, unchanged I, II, III, or IV was excreted in the urine and bile of dogs dosed intravenously. Thus, it seems likely that I, II, and III can bind to melanin *in vitro* and that metabolites of these same compounds can bind to the melanin-containing portion of the eye *in vivo*.

In animals, IV has antidepressive, rather than neuroleptic, properties (5) that are similar to some of those exhibited by doxepin and amitriptyline. The latter two compounds bind to melanin, although not to the extent found for chlorpromazine (12), an electron donor that forms charge-transfer complexes with melanin (9, 19, 20). It is suggested that the carboxamide side chain in some way alters the affinity of IV and its metabolites for tissues, including those that contain melanin.

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Microcapsules XIV: Effects of Membrane Materials and Viscosity of Aqueous Phase on Permeability of Polyamide Microcapsules toward Electrolytes

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Abstract □ The effects of membrane materials and the viscosity of the aqueous phase on the permeability of several kinds of polyamide microcapsules toward electrolytes were studied. The permeation rate of electrolytes was higher in the microcapsule membranes consisting of polyamide molecules with a polymethylene chain than in those having an aromatic ring. The viscosity of the microencapsulated aqueous phase decreased the permeation rate of electrolytes to a great extent, indicating that the diffusion of electrolytes in the interior of microcapsules is rate determining in the permeation process.

Keyphrases □ Polyamide microcapsules—permeation rate of electrolytes □ Permeability, polyamide microcapsules—effect of membrane materials and viscosity of aqueous phase □ Microcapsules, polyamide—permeation rate of electrolytes, effect of membrane materials and viscosity of aqueous phase □ Electrolyte permeation through polyamide microcapsules—effect of membrane materials and viscosity of aqueous phase

Permeability is one of the most important properties of microcapsules when they are used in the medical and pharmaceutical fields. However, only a few papers have been published on their permeability. Chang and Poznansky (1) measured the permeability of large nylon microcapsules (200-μ mean diameter), suspended in saline solution, toward low molecular weight solutes labeled with radioisotope. The measurements were made at very early stages of the permeation, using a rapid mixing and sampling technique; the half-time for equilibration of the solutes was less than 60 sec. Takamura *et al.* (2) studied the permeation rate of various electrolytes through the membranes of polyphthalamide microcapsules (2-μ mean diameter) in quasisteady state by means of conductometry, and they reported that the permeability constant for the electrolytes is of the order of 10^{-8} cm./sec. The surprisingly low permeation rate was ascribed to the formation of a stable diffusion layer of the electrolytes in the interior of microcapsules.

Although the works cited have thrown much light on the permeability of microcapsules, many uncertainties remain with regard to the fundamentals of the permeation process. Consequently, extensive work is needed to elucidate the detailed character of the process. This paper deals with the effects of membrane materials and the viscosity of the aqueous phase on the permeability of polyamide microcapsules toward electrolytes.

EXPERIMENTAL

Materials—The diamines used in this work were *p*-phenylenediamine, piperazine, 2,5-dimethylpiperazine, and 1,6-hexamethylenediamine. Sebacoyl dichloride and *p*-phthaloyl dichloride were employed as acid dichlorides. All of these chemicals were of reagent grade. Sodium chloride and sodium sulfate (analytical reagent grade) were used for permeability experiments without further purification. Polyvinylpyrrolidone (mol. wt. 360,000) was a commercial product.

Preparation of Microcapsules—Polyamide microcapsules containing water were prepared by the same method as that described earlier (2) by making use of the interfacial polycondensation reaction between diamine in the aqueous phase and acid dichloride in the organic phase. The polyamide microcapsules prepared from the diamines with the acid dichlorides were as follows: polyphenylene phthalamide, polyphthaloyl piperazine, polyphthaloyl 2,5-dimethylpiperazine, polyhexamethylene phthalamide, and polyhexamethylene sebacamide microcapsules. All of these microcapsules were dialyzed in the same manner as already reported (2) and were dispersed in deionized water containing 1% (v/v) polysorbate 20 as the dispersing agent.

Polyphthaloyl piperazine microcapsules containing aqueous solution of polyvinylpyrrolidone were prepared, dialyzed, and dispersed in 1% polysorbate 20 solution in the same way as already mentioned. In some cases, polyphthaloyl piperazine microcapsules containing aqueous polymer solution were dispersed in polymer solution containing 1% polysorbate 20.

Estimation of Permeability—As in the previous work (2), the permeability of microcapsules was estimated from the change in electrical conductance of the mixture of microcapsule dispersion and electrolyte solution in 1% polysorbate 20 solution.

To 200 ml. of 0.05 *N* NaCl or Na_2SO_4 solution in a conductance cell immersed in a constant-temperature bath, 100 ml. of the microcapsule dispersion previously kept at the same temperature was quickly added with stirring. The conductance reading was started shortly after mixing and was taken at suitable time intervals.

The electrical conductance measurements[1] were carried out at 10 ± 0.1°. The stirring was continued throughout the measurements.

The permeability constant for the electrolyte, P, was calculated by the equation:

$$P = -\frac{2.303 C_f V_m}{C_i A t} \log \frac{C_t - C_f}{C_i - C_f} \qquad \text{(Eq. 1)}$$

which was derived in the earlier work (2) based on Fick's first law of diffusion. In this equation, C_i, C_t, and C_f are the initial, intermediary (at time t), and final concentrations of electrolyte, respectively, in the dispersion medium determined by conductance measurements; V_m is the total volume of microcapsules; and A is the total surface area of microcapsules.

The validity of Eq. 1 can be checked by plotting $\log (C_t - C_f)/(C_i - C_f)$ against time. If the plot gives a straight line, the equation strictly holds and the permeability constant can be evaluated from the slope of the line.

The permeability constant thus obtained was converted to the apparent diffusion coefficient in the microcapsule membrane, D, by the following relation given in the previous paper (2):

$$D = P\Delta x \qquad \text{(Eq. 2)}$$

where Δx denotes the membrane thickness.

Estimation of Membrane Thickness—The thickness of microcapsule membranes was calculated by the equation:

$$\Delta x = \frac{wr}{dA} \qquad \text{(Eq. 3)}$$

where w is the weight of microcapsule membranes in unit volume of dispersion, assuming the complete reaction of diamine and acid dichloride; r is the experimentally determined percent reacted of

[1] Model CM-1DB conductance meter, Toa Electric Co., Tokyo, Japan.

Table I—Apparent Diffusion Coefficients for Electrolytes in Various Microcapsule Membranes

Microcapsule	Apparent Diffusion Coefficients, $\times 10^{14}$ cm.²/sec. Sodium Chloride	Sodium Sulfate
Polyphenylene phthalamide	2.6	2.6
Polyphthaloyl piperazine	2.4	2.5
Polyphthaloyl dimethylpiperazine	3.1	3.2
Polyhexamethylene phthalamide	3.0	3.8
Polyhexamethylene sebacamide	4.4	4.9

diamine; d is the density of microcapsule membrane; and A is the total surface area of microcapsules in unit volume of dispersion.

The percent reacted of diamine was determined in the following way. Polyamide microcapsules were broken down by centrifugation, and a clear aqueous phase containing unreacted diamine was obtained. A 4-ml. portion of the aqueous phase was mixed with 3 ml. of 5% (v/v) *p*-dimethylaminobenzaldehyde solution and 1 ml. of 15% (v/v) sulfuric acid solution in methanol, and the mixture was allowed to react at 60° for 10 min. The amount of diamine remaining unreacted was determined by measuring spectrophotometrically the absorption of colored complex formed between the diamine and *p*-dimethylaminobenzaldehyde (3). The wavelengths of maximum absorption were 425, 415, and 500 nm. for the piperazine, 1,6-hexamethylenediamine, and *p*-phenylenediamine complexes, respectively. The amount of 2,5-dimethylpiperazine could not be determined by this method, because this diamine did not react with *p*-dimethylaminobenzaldehyde. Instead, 2,3-dinitrofluorobenzene was used as the coloring agent. That is, a 2-ml. portion of the aqueous phase was mixed with an equal volume of 1.2% (v/v) 2,3-dinitrofluorobenzene solution in methanol and 1 ml. of aqueous 0.1 *M* $NaHCO_3$ solution, and the absorption at 396 nm. was measured spectrophotometrically (4). In all cases, the percent reacted of diamine was calculated from its concentrations in the aqueous phase before and after microencapsulation.

The microcapsule membranes obtained by breaking down polyamide microcapsules through centrifugation were collected, washed, and dried under reduced pressure. The specific gravity of the dried sample was determined with a pycnometer. The density of the membranes was obtained by multiplying the specific gravity by the density of water.

The total surface area of microcapsules was estimated in a way similar to that adopted in earlier works (5–7).

Measurement of Viscosity—The viscosities of aqueous polyvinylpyrrolidone solutions containing sodium chloride or sodium sulfate of the concentration at distribution equilibrium were measured with a viscometer[1] at 10 ± 0.1°.

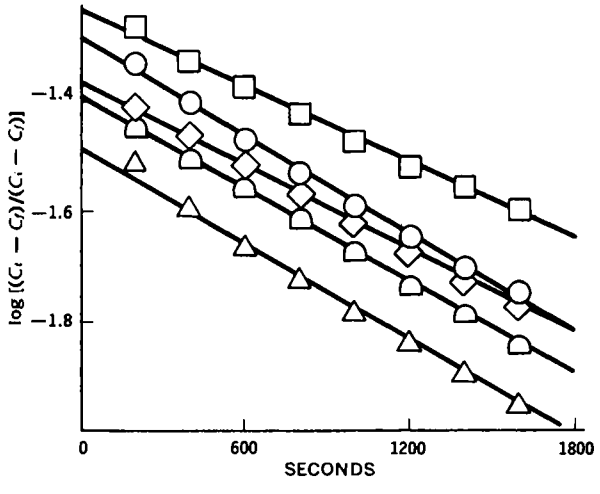

Figure 1—*Plot of log* $(C_t - C_f)/(C_i - C_f)$ *against time for the entry of sodium chloride into polyamide microcapsules. Key: microcapsules:* □, *polyphenylene phthalamide;* ◇, *polyphthaloyl piperazine;* ⬭, *polyphthaloyl dimethylpiperazine;* △, *polyhexamethylene phthalamide; and* ○, *polyhexamethylene sebacamide.*

Table II—Characteristics of Polyamide Microcapsules

Microcapsule	Membrane Density, g./cm.³	Membrane Thickness, *A*	Surface Area, m.²/cm.³
Polyphenylene phthalamide	1.38	123	1.79
Polyphthaloyl piperazine	1.37	133	2.12
Polyphthaloyl dimethylpiperazine	1.35	166	2.03
Polyhexamethylene phthalamide	1.28	167	2.21
Polyhexamethylene sebacamide	1.14	224	2.15

RESULTS AND DISCUSSION

Effect of Membrane Materials—The distribution equilibrium of electrolytes was established within 2 hr. between the inside and outside of all polyamide microcapsules studied in this work.

Figure 1 shows the plot of log $(C_t - C_f)/(C_i - C_f)$ against time for the entry of sodium chloride into polyamide microcapsules, indicating the validity of Eq. 1 between 3 and 30 min. after mixing the electrolyte solution and microcapsule suspension. The electrolyte entry into microcapsules proceeds in a quasisteady state since the permeation process is supposed to have been about 90% completed at the time when the conductance measurement is started.

The permeability constants for sodium chloride and sodium sulfate calculated by Eq. 1 were always of the order of 10^{-8} cm./sec. and were dependent on the membrane constituents. Table I lists the apparent diffusion coefficients in the microcapsule membranes converted from the permeability constants by means of Eq. 2. Inspection of the table reveals that the diffusion rate of the electrolytes is higher in the membranes consisting of polyamide molecules with a polymethylene chain than in those having an aromatic ring.

Table II gives some characteristics of the polyamide microcapsules. The data are useful in discussing the effect of the membrane constituent on the permeation rate of the electrolytes through the polyamide microcapsule membranes.

As stated previously (2), polysorbate 20 molecules are undoubtedly adsorbed on the polymer molecules constituting microcapsules,

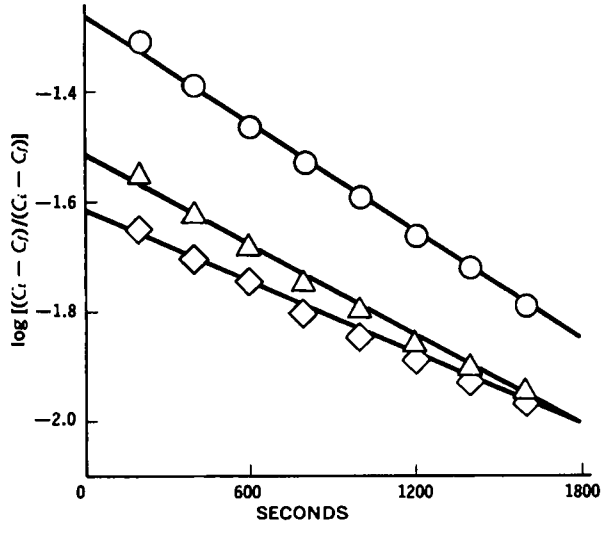

Figure 2—*Plot of log* $(C_t - C_f)/(C_i - C_f)$ *against time for the entry of sodium chloride into polyphthaloyl piperazine microcapsules in the presence of polyvinylpyrrolidone. Key (polyvinylpyrrolidone concentration, %):* ○, *0.05;* △, *0.10; and* ◇, *0.15.*

[1] Ostwald.

Table III—Effect of Viscosity of Microencapsulated and Intercapsular Aqueous Phases on the Permeability Constants of Electrolytes for the Entry into Polyphthaloyl Piperazine Microcapsules

Viscosity, cps.	—Permeability Constant, $\times 10^5$ cm./sec.— Sodium Chloride	Sodium Sulfate
1.31	1.78	1.90
1.43	1.72	1.76
1.54	1.62	1.63
1.68	1.50	1.50

Table IV—Effect of Viscosity of Microencapsulated Aqueous Phase on the Permeability Constants of Electrolytes for the Entry into Polyphthaloyl Piperazine Microcapsules

Viscosity, cps.	—Permeability Constants, $\times 10^5$ cm./sec.— Sodium Chloride	Sodium Sulfate
1.31	1.78	1.90
1.43	1.69	1.74
1.54	1.64	1.57
1.68	1.54	1.59

thereby making the membranes hydrophilic. Consequently, sodium chloride and sodium sulfate are quite likely to diffuse through the interspace filled with water of polymer chains in a random arrangement of the membranes. It is then expected that the dimension of interspace of polymer chains greatly affects the diffusion rate of the electrolytes. Since polyamide molecules having polymethylene groups are more flexible than those with aromatic rings, the dimension of interspace of polymer chains would be larger in polyhexamethylene sebacamide and polyhexamethylene phthalamide microcapsules than in polyphenylene phthalamide, polyphthaloyl piperazine, and polyphthaloyl dimethylpiperazine microcapsules.

This situation is reflected in the densities of the microcapsule membranes shown in Table II and in the diffusion coefficients for sodium chloride and sodium sulfate given in Table I. It may be inferred, therefore, that the diffusion rate of electrolytes is lower in the microcapsule membranes of high density than in those of low density.

The difference in the diffusion rate between sodium chloride and sodium sulfate would be caused either by the different hydration of chloride and sulfate ions or by the different electrostatic interaction of the two anions with polyoxyethylene chains of dispersing agent molecules adsorbed on the polyamide molecules of the microcapsule membranes. However, the former possibility is ruled out because the permeability constants for sodium sulfate are, in general, greater than those for sodium chloride, while sulfate ions are more strongly hydrated and consequently should diffuse more slowly than chloride ions in the interspace of polymer chains. This leads one to the second possibility.

As described earlier (2), anions of higher valency are likely to interact more strongly than those of lower valency with weakly cationic polyoxyethylene chains of dispersing agent molecules adsorbed on the polymer molecules constituting microcapsules. As a consequence, there would be a richer accumulation of sulfate ions than chloride ions in the microcapsule membranes. This may result in the higher permeation rate for sulfate ions than for chloride ions.

Effects of Viscosity of Aqueous Phase—Earlier (2), it was proposed that the diffusion of electrolyte in the interior of microcapsules is rate determining in the permeation process, because the formation of a stable diffusion layer of electrolyte is highly probable inside the microcapsules. If this is really the case, the viscosity of the microencapsulated aqueous phase would affect the permeation of electrolyte through the microcapsule membranes to a great extent.

Figure 2 shows the effect of viscosity of microencapsulated and intercapsular aqueous phases on the rate of entry of sodium chloride into polyphthaloyl piperazine microcapsules. The viscosity was raised by dissolving polyvinylpyrrolidone in the aqueous phases. An increase in the viscosity clearly gives rise to a decrease in the rate of entry of sodium chloride. It is quite unlikely that polyvinylpyrrolidone molecules are adsorbed on the membrane by competing with dispersing agent molecules and thus offer resistance to the entry of sodium chloride since the former molecules are far less surface active than the latter molecules.

Table III lists the permeability constants for sodium chloride and sodium sulfate when both the microencapsulated and intercapsular aqueous phases contain polyvinylpyrrolidone. Table IV gives the permeability constants for sodium chloride and sodium sulfate in the case where the polymer is present only in the microencapsulated aqueous phase. Comparison of the two tables indicates that the permeation rate of electrolytes strongly depends on the viscosity of microencapsulated aqueous phase. This would support the view that the diffusion of electrolyte in the microencapsulated aqueous phase is rate determining in the permeation process. The tables also suggest that the increase in the viscosity of the microencapsulated aqueous phase tends to diminish the difference in the diffusion rate in the microcapsule membranes between sodium chloride and sodium sulfate.

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Relationship between CNS Depressant and Enzyme Inhibitory Properties of Substituted Quinazolone 1,3,4-Oxadiazoles

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Abstract □ Substituted quinazolone 1,3,4-oxadiazoles were synthesized and evaluated for their MAO and acetylcholinesterase inhibitory activity. These compounds were also screened for their anticonvulsant activity, reserpine reversal response, and analgesic activity. Anticonvulsant activity of these compounds against pentylenetetrazol-induced seizures was related to some extent to their MAO and acetylcholinesterase inhibitory properties.

Keyphrases □ Quinazolone 1,3,4-oxadiazoles—synthesis, anticonvulsant and analgesic activity, relationship to MAO and acetylcholinesterase inhibition □ 1,3,4-Oxadiazoles, quinazolone—synthesis, anticonvulsant and analgesic activity, relationship to MAO and acetylcholinesterase inhibition □ Anticonvulsant activity—synthesis and evaluation of quinazolone 1,3,4-oxadiazoles □ MAO inhibitors—synthesis and evaluation of quinazolone 1,3,4-oxadiazoles □ Acetylcholinesterase inhibitors—synthesis and evaluation of quinazolone 1,3,4-oxadiazoles

Anticonvulsant (1) and hypnotic (2) properties exhibited by quinazolones and the specific inhibition of MAO [E.C. 1.4.3.4 monoamine O_2 oxido reductase-(deaminating)] by 1-isonicotinoyl-2-isopropylhydrazine (3) prompted the synthesis of substituted quinazolone hydrazides as MAO inhibitors (4, 5). Furthermore, the ability of substituted 1,3,4-oxadiazoles to exhibit analgesic (6, 7), anti-inflammatory, antipyretic (8, 9), muscle relaxant (9), and CNS depressant activities (10, 11) led us to synthesize substituted quinazolone 1,3,4-oxadiazoles. In the present study, attempts were made to correlate pharmacological properties exhibited by these compounds with their ability to inhibit MAO and acetylcholinesterase.

CHEMISTRY

The various substituted quinazolone 1,3,4-oxadiazoles were synthesized by the route outlined in Scheme I. Acetanthranils (II) were synthesized by refluxing 1 mole of the appropriate anthranilic acid (I) with 2 moles of acetic anhydride. Quinazolone esters (IV) were synthesized by heating equimolar quantities of appropriate acetanthranils (II) and ethyl glycine ester (III). Quinazolone hydrazides (V) were synthesized by refluxing a mixture of quinazolone esters (IV) and hydrazine hydrate. Substituted quinazolone thiosemicarbazides (VI) were prepared by refluxing the appropriate hydrazide (V) with a suitable arylisothiocyanate in ethanol. These quinazolone thiosemicarbazides were cyclized into substituted quinazolone 1,3,4-oxadiazoles (VII).

EXPERIMENTAL

Melting points were taken in open capillary tubes and are uncorrected.

Anthranilic Acids (I)—The method of Atkinson and Milton (12) was used for the synthesis of 5-chloro- and 3,5-dichloroanthranilic acids. 5-Bromoanthranilic acid was prepared by the method of Wheeler and Oats (13), while 5-iodoanthranilic acid was synthesized following the method of Klemme and Hunter (14).

Acetanthranil (II)—Appropriate anthranilic acids (1 mole) were refluxed with acetic anhydride (2 moles) for 1 hr. After excess acetic anhydride was distilled off, the acetanthranil that separated out as a solid mass was dried quickly and used without further purification.

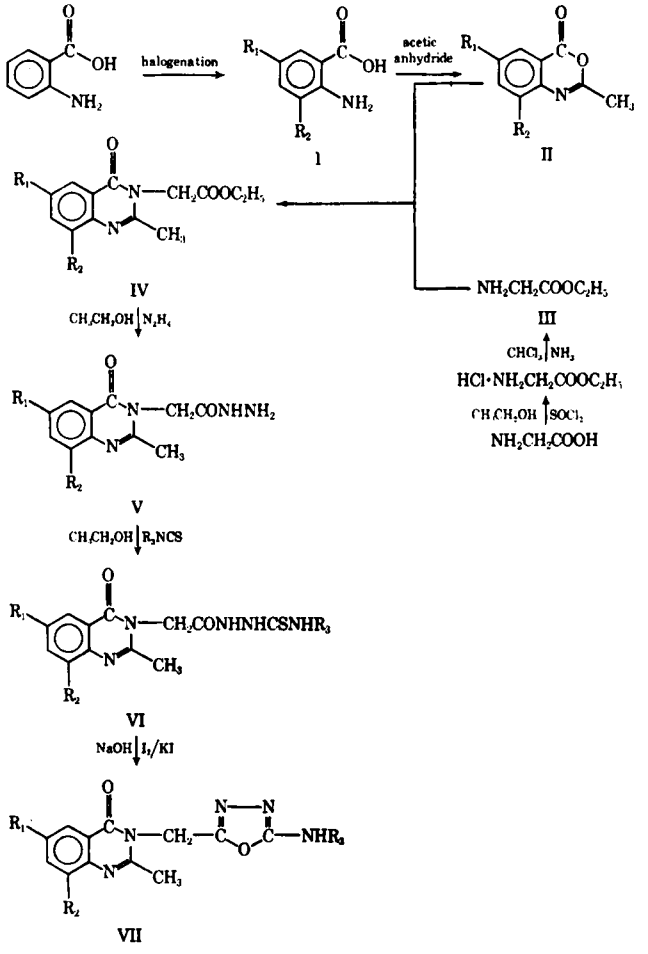
Ethyl Glycine Ester (III)—This ester was prepared by following the method of Kuprozewski and Sokolowaska (15).

Quinazolone Esters (IV)—Equimolar quantities (1 mole) of the appropriatea cetanthranil and ethyl glycine ester were heated directly to yield the desired esters, which were recrystallized from ethanol (Table I).

Quinazolone Hydrazides (V)—A mixture of the appropriate quinazolone ester (0.1 mole) and hydrazine hydrate (98%) (0.12 mole) was refluxed in absolute ethanol for 6–8 hr. On removing the excess solvent, the quinazolone hydrazides that separated out were filtered and recrystallized from ethanol (Table I).

Quinazolone Thiosemicarbazides (VI)—Equimolar quantities of hydrazides and arylisothiocyanate (0.01 mole) were refluxed in ethanol for 3–4 hr. The solution was cooled to obtain corresponding thiosemicarbazides, which were recrystallized from suitable solvents (Table II).

Quinazolone 1,3,4-Oxadiazoles (VII)—The quinazolone thiosemicarbazides were cyclized into quinazolone 1,3,4-oxadiazoles by following the method of Silberg and Cosma (16). To a cold ethanolic suspension of an appropriate thiosemicarbazide (0.01 mole) was added 5 ml. of 4 *N* NaOH solution, followed by 5% solution of iodine in potassium iodide which was gradually added to the clear solution with stirring until the color of iodine persisted at room



Scheme I

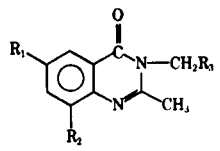


Table I—Substituted Quinazolone Esters and Hydrazides

Compound Number	R_1	R_2	R_3	Melting Point	Yield, %	Molecular Formula (Molecular Weight)	Analysis, % Nitrogen Calc.	Analysis, % Nitrogen Found
1	H	H	$COOC_2H_5$	130–131°	60	$C_{13}H_{14}N_2O_3$	11.38	11.21
2	H	H	$CONHNH_2$	261°	55	$C_{11}H_{12}N_4O_2$	24.13	23.80
3	Cl	H	$COOC_2H_5$	80°	60	$C_{13}H_{13}ClN_2O_3$	9.97	9.53
4	Cl	H	$CONHNH_2$	260°	50	$C_{11}H_{11}N_4O_2$	21.01	20.61
5	Br	H	$COOC_2H_5$	81°	70	$C_{13}H_{13}BrN_2O_3$	8.61	8.24
6	Br	H	$CONHNH_2$	248°	60	$C_{11}H_{11}BrN_4O_2$	18.00	17.60
7	I	H	$COOC_2H_5$	108°	70	$C_{13}H_{13}IN_2O_3$	7.52	7.31
8	I	H	$CONHNH_2$	265°	50	$C_{11}H_{11}IN_4O_2$	18.43	18.21
9	Cl	Cl	$COOC_2H_5$	160°	60	$C_{13}H_{12}Cl_2N_2O_3$	8.88	8.56
10	Cl	Cl	$CONHNH_2$	275°	55	$C_{11}H_{10}Cl_2N_4O_2$	18.60	18.32
11	Br	Br	$COOC_2H_5$	175°	70	$C_{13}H_{12}Br_2N_2O_3$	6.93	6.72
12	Br	Br	$CONHNH_2$	285°	50	$C_{11}H_{10}Br_2N_4O_2$	16.92	16.75

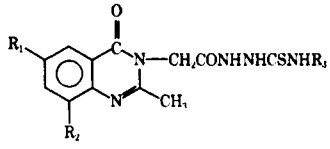


Table II—Substituted Quinazolone Thiosemicarbazides

Compound Number	R_1	R_2	R_3	Crystallization Solvent	Yield, %	Melting Point	Molecular Formula		Analysis, % Calc.	Analysis, % Found
13	H	H	*m*-$CH_3C_6H_4$	Ethanol	60	217–218°	$C_{19}H_{19}N_5O_2S$	C	59.84	59.97
								H	4.98	5.09
								N	18.37	17.89
14	H	H	*p*-$CH_3C_6H_4$	Ethanol	65	226–228°	$C_{19}H_{19}N_5O_2S$	C	59.84	59.87
								H	4.98	5.12
								N	18.37	17.76
15	H	H	*p*-$CH_3OC_6H_4$	Ethanol	70	219–220°	$C_{19}H_{19}N_5O_3S$	C	57.42	57.37
								H	4.78	5.14
								N	17.63	17.94
16	Br	H	*m*-$CH_3C_6H_4$	Ethanol	55	234–235°	$C_{19}H_{18}BrN_5O_2S$	C	49.56	49.88
								H	3.91	4.12
								N	15.22	14.95
17	Br	H	*p*-$CH_3OC_6H_4$	Ethanol	60	235–237°	$C_{19}H_{18}BrN_5O_3S$	C	47.90	48.47
								H	3.78	4.12
								N	14.61	14.42
18	Cl	Cl	*m*-$CH_3C_6H_4$	Acetic acid	70	290–292° dec.	$C_{19}H_{17}Cl_2N_5O_2S$	C	50.66	50.35
								H	3.77	3.87
								N	15.56	15.39
19	Cl	Cl	*p*-$CH_3C_6H_4$	Ethanol	65	260–265° dec.	$C_{19}H_{17}Cl_2N_5O_2S$	C	50.66	50.42
								H	4.77	4.33
								N	15.56	15.32
20	Cl	Cl	*p*-$CH_3OC_6H_4$	Acetic acid–ether	70	284–286°	$C_{19}H_{17}Cl_2N_5O_3S$	C	48.92	48.65
								H	3.64	4.12
								N	15.02	14.95

temperature. The contents were then refluxed on a steam bath, and more iodine solution was added until a slight excess of iodine remained. The reaction mixture was cooled and poured into 500 ml. of ice-cold water. The solid masses that separated out were filtered, washed with water and then with carbon disulfide, and recrystallized from suitable solvents (Table III).

BIOCHEMICAL STUDIES

***In Vitro* Determination of MAO Activity**—MAO activity of rat liver homogenate was determined spectrophotofluorometrically using kynuramine as the substrate (17). The enzyme activity was determined by measuring the 4-hydroxyquinoline formed during oxidative deamination of kynuramine. The reaction mixture, in a final concentration of 3 ml., consisted of 83 m*M* phosphate buffer (pH 7.4) and 1 ml. of the liver homogenate (10% w/v). Water, kynuramine, and the test compounds were added to adjust the final volume to 3.0 ml. The reaction was conducted in test tubes, and incubations were carried out at 37° for 30 min. After incubation, 2 ml. of 10% trichloroacetic acid (w/v) was added to each test tube and the precipitated proteins were removed by centrifugation. Suitable aliquots of the supernate, taken in 1 *N* NaOH solution, were assayed for 4-hydroxyquinoline fluorometrically in a spectrophotofluorometer[1], using activating light of 310 nm. and measuring fluorescence at the maximum of 380 nm. An increase in absorbance provided a direct measurement of 4-hydroxyquinoline as an index of kynuramine utilization and, consequently, of MAO activity. A decrease in kynuramine utilization in experiments containing the test compounds was used to calculate the degree of MAO inhibition.

***In Vitro* Determination of Acetylcholinesterase Activity**—Acetylcholinesterase activity was determined colorimetrically, using acetylthiocholine iodide (0.015 *M*) as the substrate, according to the method of McOskar and Daniel (18). The reaction mixture in final concentration consisted of 43 m*M* tromethamine buffer (pH 7.4), 350 m*M* sodium chloride, and 0.3 ml. brain homogenate (10% w/v).

[1] Aminco-Bowman.

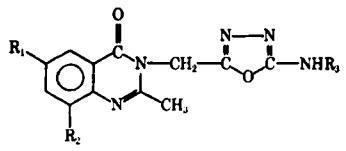


Table III—Substituted Quinazolone 1,3,4-Oxadiazoles

Compound Number	R_1	R_2	R_3	Yield, %	Melting Point	Molecular Formula	Analysis, % Calc.	Found
21	H	H	*m*-$CH_3C_6H_4$	50	180–182°	$C_{19}H_{17}N_5O_2$	C 65.70	65.61
							H 4.89	5.44
							N 20.17	19.80
22	H	H	*p*-$CH_3C_6H_4$	45	210–211°	$C_{19}H_{17}N_5O_2$	C 65.70	65.55
							H 4.89	4.84
							N 20.97	19.69
23	H	H	*p*-$CH_3OC_6H_4$	50	207–209°	$C_{19}H_{17}N_5O_3$	C 62.55	62.75
							H 4.68	4.89
							N 19.29	18.80
24	Br	H	*m*-$CH_3C_6H_4$	45	123–129°	$C_{19}H_{16}BrN_5O_2$	C 53.51	53.64
							H 3.75	4.08
							N 16.43	15.97
25	Br	H	*p*-$CH_3OC_6H_4$	55	222–224°	$C_{19}H_{16}BrN_5O_3$	C 51.58	51.25
							H 3.62	3.84
							N 15.84	15.35
26	Cl	Cl	*m*-$CH_3C_6H_4$	45	203°	$C_{19}H_{15}Cl_2N_5O_2$	C 54.80	54.56
							H 3.60	4.10
							N 16.83	16.35
27	Cl	Cl	*p*-$CH_3C_6H_4$	50	249–250°	$C_{19}H_{15}Cl_2N_5O_2$	C 54.80	55.00
							H 3.60	3.98
							N 16.83	16.31
28	Cl	Cl	*p*-$CH_3OC_6H_4$	40	213–215°	$C_{19}H_{15}Cl_2N_5O_3$	C 52.77	52.71
							H 3.47	3.64
							N 16.20	15.76

Water and acetylthiocholine were added to adjust the final volume to 2.0 ml. The reaction mixture, with or without inhibitor, was incubated at constant temperature (37°) for 10 min. prior to the addition of acetylthiocholine. The incubation was continued for 10 min. more subsequent to the addition of the substrate. The reaction was stopped by the addition of 0.5 ml. of 25% (w/v) trichloroacetic acid, and the resultant solution was centrifuged for 5 min. at 500× *g*. An aliquot of the clear supernate was withdrawn, and the enzymatically formed thiocholine content was determined colorimetrically. Each assay was done in triplicate, and tissue and substrate blanks were substracted to give the actual value for the hydrolysis of acetylthiocholine. Results are expressed as changes in absorbance/100 mg. fresh tissue/10 min.

PHARMACOLOGICAL STUDIES

Toxicity—The approximate 50% lethal dose (LD_{50}) was determined in albino mice by the method of Smith (19). The dose of each compound was taken as one-fifth of its observed LD_{50} in an attempt to evaluate it in its effective therapeutic range. The test compounds were administered to each group of 10 mice orally in 5% gum acacia.

Reserpine Reversal Test for *In Vivo* Determination of MAO Inhibitory Activity—Reserpine was administered intraperitoneally in a dose of 5 mg./kg. after 3 hr. In the control group of mice, without pretreatment of drugs, reserpine always produced sedation, ptosis, and miosis. In animals treated with phenelzine and pargyline, the well-known MAO inhibitors, administration of reserpine caused excitation, piloerection, mydriasis, and increased motor activity. Failure to produce the reserpine reversal response by the test compounds indicated that the compound was inactive in producing *in vivo* MAO inhibition.

Anticonvulsant Activity—The anticonvulsant property was determined in albino mice of either sex, weighing between 20 and 30 g., divided into groups of 10; the group weights were kept equal as far as possible. One group of 10 animals was used as the control. The test compounds were suspended in 5% aqueous gum acacia and were fed orally 3 hr. prior to subcutaneous injection of pentylenetetrazol[2], 80 mg./kg., under the loose skin of the back. This dose of pentylenetetrazol caused convulsions in almost all of the normal control group of animals. The animals were observed for 60 min. for the occurrence of seizures; an episode of clonic spasm persisting for at least 5 sec. was considered a threshold convulsion. Animals not exhibiting even a threshold convulsion during 60 min. were considered protected. The number of animals protected in each group were recorded. Transient intermittent jerks or tremulousness were not taken into account. Phenelzine and pargyline were used for comparison.

Analgesic Activity—*Mouse Tail-Pinch Test*—Morphine-like analgesic activity was tested by the mouse tail-pinch method (20). An artery clip, with branches enclosed in a thin rubber tube, was applied to the root of the tail of a mouse for 30 sec. The animals made continuous attempts to remove the noxious stimulus by biting the clips. Mice were administered test compounds by the intragastric route 3–4 hr. prior to the application of the artery clips. The insensitivity to the noxious stimulus was taken as the positive response, and the results were expressed as percentage of mice showing analgesic activity. Morphine hydrochloride was used as a reference standard for comparing the effective analgesic property of these compounds.

Writhing Test—Aspirin-like analgesic activity of the test compounds was investigated by their ability to protect a painful writhing syndrome. This syndrome is characterized by abdominal torsion, drawing up of hind limbs to the abdominal wall, marked contraction of the abdominal area, and periodic arching of the back to rub the abdominal wall on the glazed surface on which the mouse was kept. It was consistently produced by intraperitoneal injection of 2 mcg. aconitine (100% effective dose) per mouse weighing 20–25 g. The test compounds were given 3 hr. before the induction of the writhing test. The typical response appeared within 5 min. after injection of aconitine and persisted up to 15–20 min. The mice were observed for 30 min., and results are expressed as percent of mice showing protection. Aspirin was taken as a standard reference drug for comparison.

RESULTS AND DISCUSSION

The test compounds exhibited low *in vitro* MAO inhibitory activity (Table IV) as compared to the 78% inhibition produced by phenelzine and pargyline when used at final concentrations of 1×10^{-5} and 1×10^{-6} *M*, respectively.

[2] Metrazol.

Table IV—Biochemical and Pharmacological Studies of Substituted Quinazolones, Substituted Quinazolone Hydrazides, Thiosemicarbazides, and 1,3,4-Oxadiazoles

Compound Number[a]	MAO Inhibition, %		Antiacetylcholinesterase Activity,	LD_{50},	Dose,	Anticonvulsant Activity Protection,	Reserpine Reversal Response	Analgesic Activity	
	6×10^{-4} M	3×10^{-4} M	3×10^{-4} M	mg./kg.	mg./kg.	%		Tail-Pinch	Aconitine Writhing Response Test, %
1	Nil	—	40.53	800	160	10	(—)	Nil	20
2	Nil	—	Nil	800	160	10	(—)	Nil	Nil
3	16.52	—	10.53	800	160	20	(—)	Nil	Nil
4	22.64	—	Nil	800	160	20	(—)	Nil	20
5	Nil	—	26.32	1600	320	10	(—)	Nil	Nil
6	Nil	—	15.79	800	160	20	(—)	Nil	20
7	25.80	—	44.73	400	80	20	(—)	Nil	20
8	19.42	—	23.68	400	80	30	Slight stimulation	Nil	20
9	34.88	—	39.47	1600	320	30	Slight activity	Nil	20
10	38.70	—	21.05	1600	320	50	(—)	Nil	Nil
11	25.80	—	26.32	1600	320	30	(—)	Nil	Nil
12	47.81	—	21.05	400	80	40	Slight stimulation	Nil	80
13	—	77.26	68.42	400	80	50	(—)	Nil	40
14	—	72.29	44.73	1600	320	50	(—)	Nil	Nil
15	—	78.45	47.37	800	160	60	Slight stimulation	Nil	40
16	—	82.22	63.15	1600	320	50	(—)	Nil	20
17	—	80.09	42.10	400	80	50	Slight activity	Nil	Nil
18	—	69.55	57.89	1600	320	70	Slight stimulation	Nil	Nil
19	—	69.38	42.10	400	80	50	(—)	Nil	40
20	—	73.28	42.10	1600	320	50	Slight stimulation	Nil	20
21	—	41.91	21.05	800	160	30	(—)	Nil	20
22	—	56.89	57.89	1600	320	60	(—)	Nil	20
23	—	56.28	21.05	1600	320	20	Slight stimulation	Nil	Nil
24	—	21.12	52.63	400	80	40	(—)	Nil	Nil
25	—	45.10	44.73	800	160	50	Slight activity	Nil	Nil
26	—	23.21	57.89	400	80	50	(—)	Nil	Nil
27	—	Nil	67.89	1600	320	30	(—)	Nil	40
28	—	36.20	52.63	1600	320	70	Slight stimulation	Nil	20

[a] Compound numbers are as recorded in Tables I, II, and III.

As is evident from Table IV, acetylcholinesterase inhibitory properties of substituted quinazolone hydrazides were lower than those of their corresponding esters. Attachment of a halogen substituent at position 6 or 8 of the quinazolone nucleus in no way reflected any basis for their structure–activity relationship with respect to their ability to inhibit MAO and acetylcholinesterase. However, the anticonvulsant activity exhibited by quinazolone hydrazides, as reflected by the protection observed against pentylenetetrazol-induced convulsions, was found to be more than that observed with the corresponding quinazolone esters. The anticonvulsant activity of phenelzine and pargyline, used for comparison, was found to be 50% at a dose of 30 mg./kg. under similar experimental conditions. Most of the quinazolone esters and quinazolone hydrazides were devoid of reserpine reversal response. These results thus indicated their weak *in vivo* MAO inhibitory activity. Furthermore, a number of compounds in the group of quinazolone thiosemicarbazides and 1,3,4-oxadiazoles exhibited slight stimulation on administration of reserpine which, however, was found to have no relationship with their MAO inhibitory potency. Compounds 16, 19, and 22 showed over 50% inhibition but were devoid of reserpine reversal response, while slight stimulation on administration of reserpine was observed with compounds possessing a low MAO inhibitory property (Compounds 7, 8, 12, and 28).

The results in Table IV show that a large number of compounds provided protection against aconitine-induced writhing response where 2-methyl-6,8-dibromo-3-(acetylhydrazino)-4-quinazolone (Compound 12) exhibited appreciable analgesic activity of 80%. None of these quinazolone esters, hydrazides, thiosemicarbazides, and 1,3,4-oxadiazoles gave a positive response when tested for their morphine-like analgesic activity by the tail-pinch method; the standard reference drug, aspirin, showed 80% protection when administered at a dose of 40 mg./kg. Such analgesic activity warrants further detailed investigation for the possible usefulness of this compound as a nonsteroidal, anti-inflammatory agent. In addition, the high LD_{50} values observed with these compounds reflected their low toxicity.

Conversion of the quinazolone hydrazides into the corresponding quinazolone thiosemicarbazides resulted in increased MAO and acetylcholinesterase inhibitory potency. Significant MAO inhibition, ranging from 69.38 to 82.22%, was observed with these substituted quinazolone thiosemicarbazides even at half of their concentration of 3×10^{-4} M as compared to 6×10^{-4} M used for substituted quinazolone esters and hydrazides (Table IV). (Compare Compounds 1 and 2 with 13, 14, and 15; 5 and 6 with 16 and 17; and 9 and 10 with 18, 19, and 20.) Greater inactivation of MAO and acetylcholinesterase was similarly observed with substituted quinazolone 1,3,4-oxadiazole as compared to the corresponding quinazolone esters and hydrazides which, however, were less potent than the quinazolone thiosemicarbazides. Although these results indicated the greater sensitivity of the quinazolone thiosemicarbazides to inhibit MAO and acetylcholinesterase than the corresponding substituted quinazolone hydrazides and quinazolone 1,3,4-oxadiazoles, no clearcut relationship of their enzyme inhibitory effects as a function of their chemical structure could be observed. These results also failed to provide similarity of substituted quinazolone esters, hydrazides thiosemicarbazides, and 1,3,4-oxadiazoles in their inhibitory effects on MAO, a dehydrogenase in which flavins seem to be implicated, and acetylcholinesterase, a hydrolase without any cofactor requirements.

The anticonvulsant activity exhibited by these compounds, by affording protection against pentylenetetrazol-induced seizures, was found to be related with their MAO and acetylcholinesterase inhibitory properties. These studies reflected a greater anticonvulsant property, ranging from 50 to 70% of substituted quinazolone thiosemicarbazides which were also found to cause greater inhibition of MAO and acetylcholinesterase. In the present study, enzyme inhibitory effects and anticonvulsant activity of these compounds were in the order of: thiosemicarbazides > oxadiazoles > hydrazides > esters.

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▲ To whom inquiries should be directed.

Solubility and Dissolution of Triamcinolone Acetonide

L. H. BLOCK▲ and R. N. PATEL

Abstract □ Available data on the solubility of triamcinolone acetonide in aqueous media are neither complete nor in agreement. Solubility of the steroid in distilled water and in potassium chloride solutions of varying ionic strength was determined at 28, 37, and 50°. Dissolution rates of the steroid in the aqueous media were determined at 28° *via* a rotating-disk method. Triamcinolone acetonide solubility in distilled water ranged from 21 mcg. ml.$^{-1}$ at 28° to 33.6 mcg. ml.$^{-1}$ at 50°. The differential heat of solution was 4207 cal. mole^{-1}. The intrinsic dissolution rate constant at the maximum agitation intensity employed was 4.51×10^{-3} hr.$^{-1}$ cm.$^{-2}$. Solubilities and dissolution rates were markedly lower in potassium chloride solutions. "Salting-out" coefficients were calculated, for the effect of potassium chloride on triamcinolone acetonide solubility, using the empirical Setschenow equation.

Keyphrases □ Triamcinolone acetonide—solubility and dissolution in water and potassium chloride solutions □ Solubility, triamcinolone acetonide—water and potassium chloride solutions, Setschenow salting-out coefficients □ Dissolution rates, triamcinolone acetonide—water and potassium chloride solutions

Lange and Amudson (1), using a gravimetric procedure, determined the aqueous solubility of triamcinolone acetonide to be 26.4 mg./100 ml. at 25°. Malkinson and Kirschenbaum (2), in their study of the percutaneous absorption of triamcinolone acetonide, noted that the steroid had an aqueous solubility of 1 mg./100 ml., although neither the temperature of the system nor the method used in determining solubility was specified. Florey (3) recently reported solubilities for the compound in water as well as isotonic saline (pH 7) at 23 and 37° as 0.004 ± 0.002% (40 mcg./ml.). The solubility data available thus far are neither adequate nor in agreement. No data have been reported on the effect of ionic strength on the steroid's solubility, nor are dissolution rate data available. Since the solubility and dissolution of the steroid can ultimately affect its release from matrixes in which it is suspended, these physicochemical properties of triamcinolone acetonide were evaluated.

MATERIALS AND METHODS

Materials—Hydrochloric acid[1], potassium chloride[1], recrystallized blue tetrazolium[1], tetramethylammonium hydroxide (10% in water)[2], and triamcinolone acetonide[3] were used as received.

One potential source of error—the polymorphic form of the steroid—was eliminated following differential thermal analyses of the three lots of steroid received. No marked differences in the thermograms obtained[4] were noted. It could thus be concluded that the steroid employed was of a single polymorphic form.

Reagents—Blue tetrazolium solution (0.2% w/v) was prepared by accurately weighing 100 mg. of blue tetrazolium, suspending the material in 35 ml. of alcohol, and agitating the suspension at 45° until dissolution was complete. Following the attainment of ambient thermal equilibrium, the solution volume was adjusted to 50 ml. with alcohol. The solution was freshly prepared before use. Tetramethylammonium hydroxide solution (1% v/v) was prepared from the commercial 10% solution by accurately transferring 10 ml. of the concentrate to a volumetric flask and adjusting the volume to 100 ml. with alcohol. Hydrochloric acid (1 *N*) was prepared by diluting 12.5 ml. of concentrated acid to 100 ml. with distilled water.

[1] Fisher Scientific Co., Fair Lawn, N. J.
[2] Eastman Organic Chemicals, Rochester, N. Y.
[3] The Squibb Institute for Medical Research, E. R. Squibb and Sons, New Brunswick, N. J.
[4] Fisher differential thermalyzer, model 260, employed through the courtesy of Dr. Omar Steward, Department of Chemistry, Duquesne University.

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▲ To whom inquiries should be directed.

Solubility and Dissolution of Triamcinolone Acetonide

L. H. BLOCK▲ and R. N. PATEL

Abstract □ Available data on the solubility of triamcinolone acetonide in aqueous media are neither complete nor in agreement. Solubility of the steroid in distilled water and in potassium chloride solutions of varying ionic strength was determined at 28, 37, and 50°. Dissolution rates of the steroid in the aqueous media were determined at 28° *via* a rotating-disk method. Triamcinolone acetonide solubility in distilled water ranged from 21 mcg. ml.$^{-1}$ at 28° to 33.6 mcg. ml.$^{-1}$ at 50°. The differential heat of solution was 4207 cal. mole^{-1}. The intrinsic dissolution rate constant at the maximum agitation intensity employed was 4.51×10^{-3} hr.$^{-1}$ cm.$^{-2}$. Solubilities and dissolution rates were markedly lower in potassium chloride solutions. "Salting-out" coefficients were calculated, for the effect of potassium chloride on triamcinolone acetonide solubility, using the empirical Setschenow equation.

Keyphrases □ Triamcinolone acetonide—solubility and dissolution in water and potassium chloride solutions □ Solubility, triamcinolone acetonide—water and potassium chloride solutions, Setschenow salting-out coefficients □ Dissolution rates, triamcinolone acetonide—water and potassium chloride solutions

Lange and Amudson (1), using a gravimetric procedure, determined the aqueous solubility of triamcinolone acetonide to be 26.4 mg./100 ml. at 25°. Malkinson and Kirschenbaum (2), in their study of the percutaneous absorption of triamcinolone acetonide, noted that the steroid had an aqueous solubility of 1 mg./100 ml., although neither the temperature of the system nor the method used in determining solubility was specified. Florey (3) recently reported solubilities for the compound in water as well as isotonic saline (pH 7) at 23 and 37° as 0.004 ± 0.002% (40 mcg./ml.). The solubility data available thus far are neither adequate nor in agreement. No data have been reported on the effect of ionic strength on the steroid's solubility, nor are dissolution rate data available. Since the solubility and dissolution of the steroid can ultimately affect its release from matrixes in which it is suspended, these physicochemical properties of triamcinolone acetonide were evaluated.

MATERIALS AND METHODS

Materials—Hydrochloric acid[1], potassium chloride[1], recrystallized blue tetrazolium[1], tetramethylammonium hydroxide (10% in water)[2], and triamcinolone acetonide[3] were used as received.

One potential source of error—the polymorphic form of the steroid—was eliminated following differential thermal analyses of the three lots of steroid received. No marked differences in the thermograms obtained[4] were noted. It could thus be concluded that the steroid employed was of a single polymorphic form.

Reagents—Blue tetrazolium solution (0.2% w/v) was prepared by accurately weighing 100 mg. of blue tetrazolium, suspending the material in 35 ml. of alcohol, and agitating the suspension at 45° until dissolution was complete. Following the attainment of ambient thermal equilibrium, the solution volume was adjusted to 50 ml. with alcohol. The solution was freshly prepared before use. Tetramethylammonium hydroxide solution (1% v/v) was prepared from the commercial 10% solution by accurately transferring 10 ml. of the concentrate to a volumetric flask and adjusting the volume to 100 ml. with alcohol. Hydrochloric acid (1 *N*) was prepared by diluting 12.5 ml. of concentrated acid to 100 ml. with distilled water.

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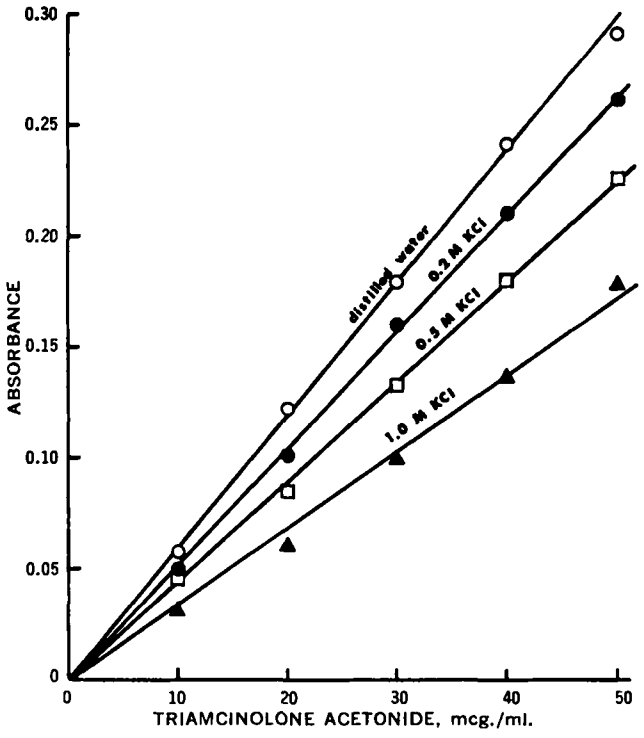

Figure 1—*Beer's plots for triamcinolone acetonide–blue tetrazolium reaction in distilled water and potassium chloride solutions.*

Assay of Aqueous Solutions of Triamcinolone Acetonide—*Standard Solution*—A standard solution of triamcinolone acetonide (1000 mcg./ml.) was prepared by dissolving 100 mg. of steroid, accurately weighed, in enough alcohol to yield a final solution volume of 100 ml.

Assay Procedure—The analytical method employed was that of Umeda *et al.* (4); 1.0 ml. of the steroid-containing solution, 1.0 ml. of alcohol, 2.0 ml. of blue tetrazolium solution, and 1.0 ml. of tetramethylammonium hydroxide solution were transferred to a screw-capped test tube. The mixture was kept at 30–35° in a water bath for 25–30 min., and 1.0 ml. of 1 *N* hydrochloric acid was then added. A blank solution was prepared using 1.0 ml. of the dissolution medium instead of the steroid-containing solution. The absorbance of the steroid-containing solution, relative to the blank, was measured at the λ_{max}, 525 nm., for each dissolution medium employed (distilled water and 0.2, 0.5, and 1.0 *M* potassium chloride)[5]. Beer's plots are shown in Fig. 1. The slopes of the curves were calculated by the method of least squares with the aid of a programmable calculator[6]. The reciprocals of the absorptivities thus calculated were used in the calculation of solution steroid concentrations.

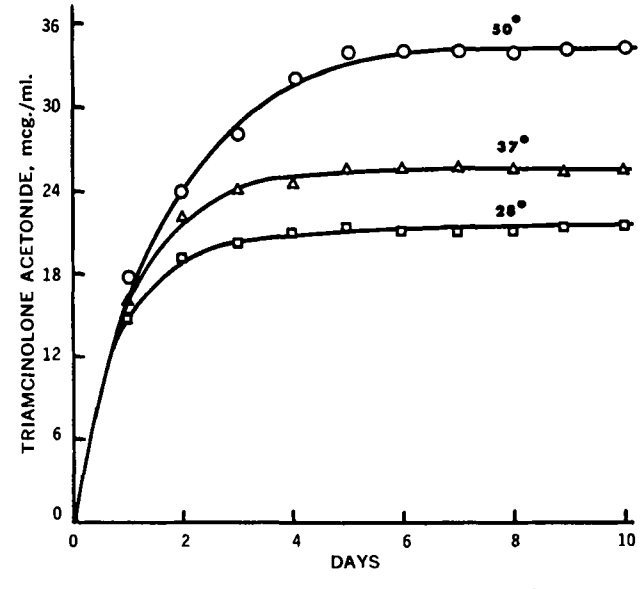

Figure 2—*Solubility of triamcinolone acetonide in distilled water as a function of time.*

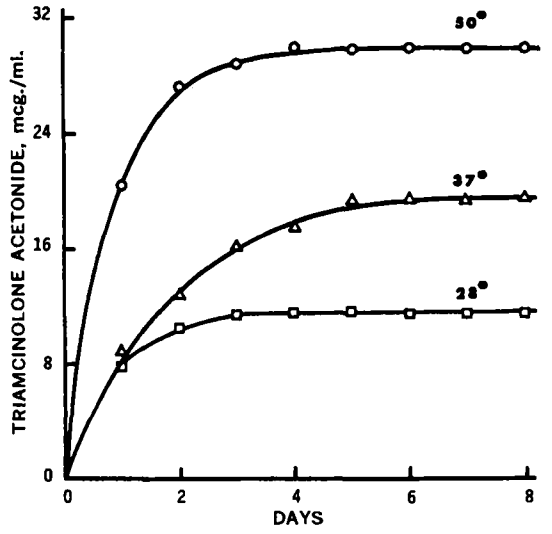

Figure 3—*Solubility of triamcinolone acetonide in 0.2 M potassium chloride solution as a function of time.*

Solubility Determinations—Triamcinolone acetonide solubility in distilled water and in 0.2, 0.5, and 1.0 *M* potassium chloride solutions was determined as a function of temperature in the following manner. An excess of steroid (50 mg.) was added to amber, screw-capped, 180-ml. (6-oz.) bottles containing 100 ml. of the appropriate dissolution medium. A Saran[7] liner was inserted and the bottle cap was tightened in place. The bottles were manually shaken and then suspended in constant-temperature baths[8] maintained[9] at 28 ± 0.5, 37 ± 0.5, and 50 ± 0.5°, respectively. The bottles were shaken periodically. Samples were withdrawn from the con-

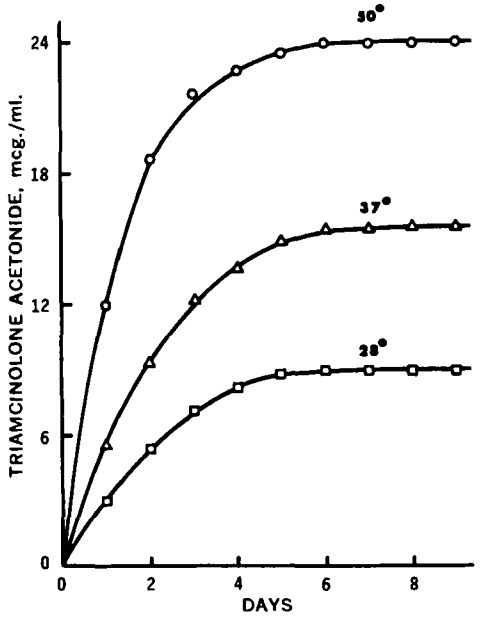

Figure 4—*Solubility of triamcinolone acetonide in 0.5 M potassium chloride solution as a function of time.*

[5] Hitachi Perkin-Elmer spectrophotometer, model 139 UV-VIS, Perkin-Elmer Corp., Norwalk, Conn.
[6] Wang 700B/701, Wang Laboratories, Tewksbury, Mass.
[7] Dow Chemical Co., Midland, Mich.
[8] Unitized constant-temperature water baths, Fisher Scientific Co., Chicago, Ill.
[9] Porta-Temp, Precision Scientific Co., Chicago, Ill.

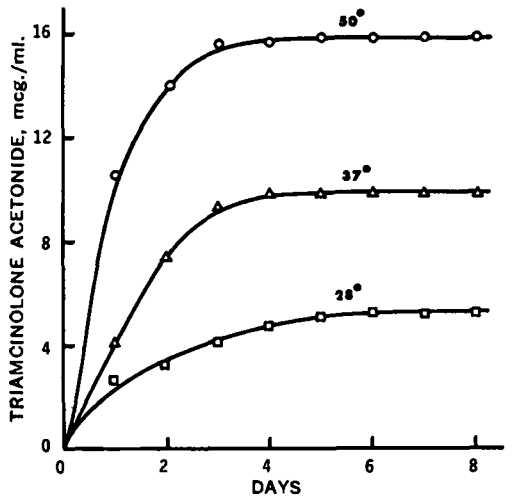

Figure 5—*Solubility of triamcinolone acetonide in 1.0 M potassium chloride solution as a function of time.*

tainers every 24 hr. using 2-ml. tuberculin syringes[10] fitted with Whitacre spinal needles[11]. The samples were filtered through 0.22-μ filters[12] and assayed for triamcinolone acetonide.

Dissolution Rate Studies—The dissolution apparatus employed consisted of a 2000-ml., three-necked, round-bottom flask immersed in a constant-temperature bath and an acrylic turbine-bladed stirrer/pellet holder attached to an electronically controlled precision stirring motor[13].

Flat-faced, 1.3-cm. (0.5-in.) diameter pellets of pure triamcinolone acetonide were prepared by suitable compression with a pellet press[14]. The pellets were then mounted in the acrylic pellet holder, with the aid of beeswax, in such a way that only one flat face of a pellet was exposed. The pellet holder, with the pellet in place, was attached to a metal shaft connected to an electronically controlled precision stirring motor.

After adding 1500 ml. of the dissolution medium to the three-necked flask immersed in the constant-temperature bath, the temperature of the dissolution medium was allowed to equilibrate with

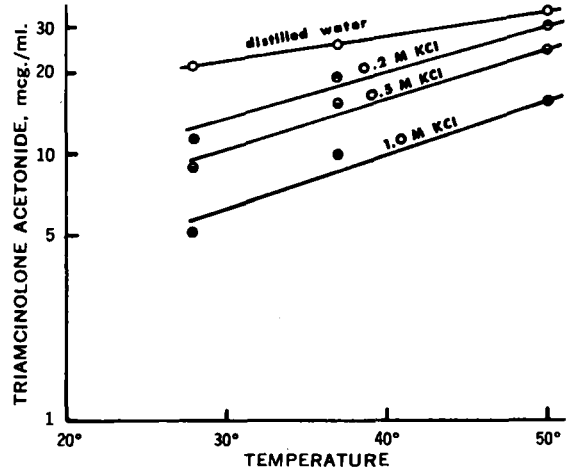

Figure 6—*Solubility of triamcinolone acetonide in dissolution media as a function of temperature.*

[10] B-D Yale 2019, 2yyP, Becton, Dickinson and Co., Rutherford, N. J.
[11] B-D 1331, W46Z, LNR, Becton, Dickinson and Co., Rutherford, N. J.
[12] MF-Millipore filters, GSWP 0 25 00, Millipore Corp., Bedford, Mass.
[13] Laboratory stirrer and motor controller, model GT-21, G. K. Heller Corp., Floral Park, N. Y.
[14] Research and Industrial Instruments Co., London, England.

Table I—Solubility of Triamcinolone Acetonide in Dissolution Media (mcg. ml.$^{-1}$)[a]

Temperature	Distilled Water	Potassium Chloride Solution 0.2 *M*	0.5 *M*	1.0 *M*
28°	21.0	11.4	8.9	5.1
37°	25.5	19.0	15.4	9.9
50°	33.6	29.5	24.1	15.8

[a] Results represent the averages of two determinations.

Table II—Differential Heats of Solution of Triamcinolone Acetonide in Dissolution Media, Calculated from Arrhenius Plots of the Solubility Data

Dissolution Medium	ΔH_{soln}, cal. mole^{-1}
Distilled water	4207
Potassium chloride solution:	
0.2 *M*	8340
0.5 *M*	8722
1.0 *M*	9855

that of the bath (28 ± 0.5°). The pellet holder, with pellet in place, was then immersed in the dissolution medium to a depth of 5 cm. The stirrer was switched on and maintained at constant speed. Aliquots of 2.0 ml. were removed from the flask at appropriate time intervals, using tuberculin syringes fitted with Whitacre spinal needles, and then filtered through 0.22-μ Millipore filters.

RESULTS AND DISCUSSION

Solubility—The approach to equilibrium, as a function of time, is shown in Figs. 2–5. Concentration plateaus were reached within 4–5 days. The solubility of triamcinolone acetonide as a function of temperature in distilled water and in potassium chloride solutions is shown in Fig. 6. The saturation equilibria are summarized in Table I. The differential heats of solution, ΔH_{soln}, of the steroid in the dissolution media were calculated by the method of least squares in accordance with the Arrhenius relationship:

$$\log X = -\frac{\Delta H_{soln}}{2.303R} \cdot \frac{1}{T} + b \qquad \text{(Eq. 1)}$$

where X is the mole fraction of steroid in solution, R is the molar gas constant, T is the absolute temperature, and b is a constant. The values are tabulated in Table II.

The effect of electrolyte on the solubility of the steroid is particularly evident (Fig. 7); it is apparent that a "salting-out" phenomenon

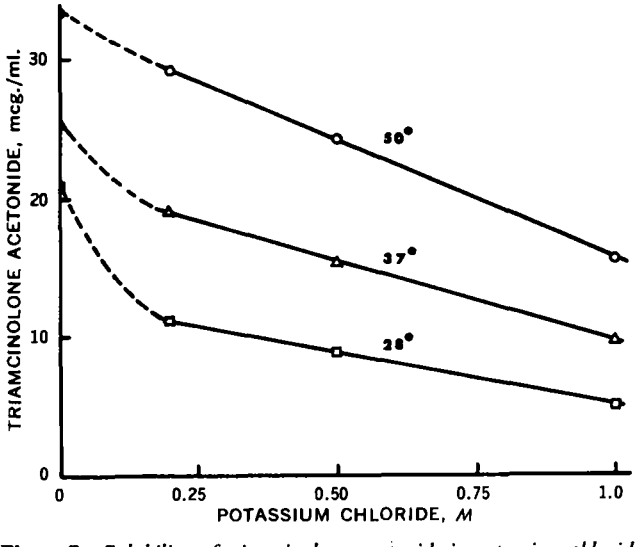

Figure 7—*Solubility of triamcinolone acetonide in potassium chloride solutions.*

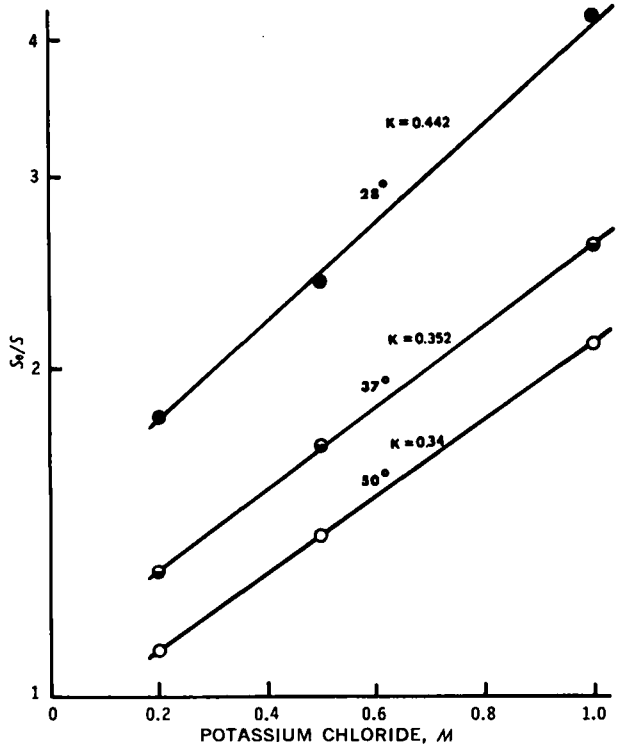

Figure 8—*Setschenow plots for triamcinolone acetonide in potassium chloride solutions.*

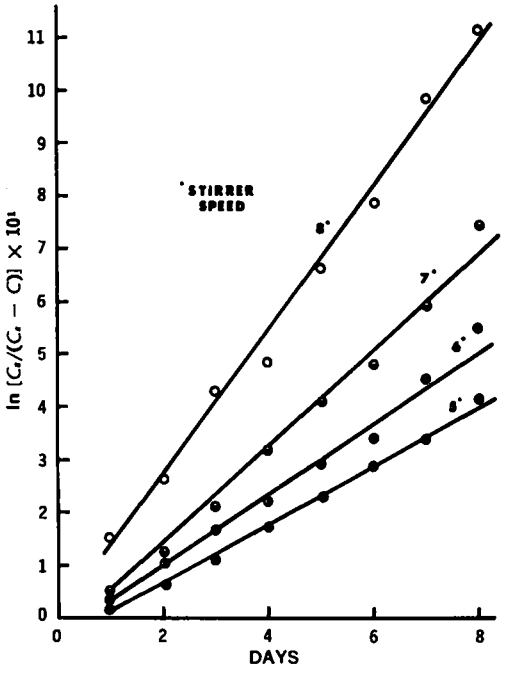

Figure 10—*Triamcinolone acetonide dissolution in distilled water, plotted in accordance with the integrated form of the modified Noyes–Whitney equation.*

is encountered. Setschenow (5, 6) proposed an empirical relationship for the effect of an electrolyte on the solubility of a nonelectrolyte:

$$\log (S_0/S) = kC \qquad \text{(Eq. 2)}$$

where S_0 is the solubility of the nonelectrolyte in pure solvent, S is the solubility of the nonelectrolyte in an electrolyte solution of a given molar concentration C, and k is the salting-out coefficient and is a function of the composition of the system and the temperature. The ratio S_0/S is equal to f, the activity coefficient of the nonelectrolyte in the electrolyte solution. Setschenow plots of the solubility data are shown in Fig. 8 along with the respective slopes or k values for each system. The k values range from about 0.3–0.4 and are higher than those reported for other types of nonelectrolytes such as diacetone alcohol (0.03–0.14) (7), mesityl oxide (0.02–0.18) (7), ethyl acetate (0.02–0.17) (7), and certain barbiturates (0.04–0.21) (8).

The salting-out effect is most apparent in the transition from distilled water to 0.2 M potassium chloride solution. At higher temperatures, however, the effect of added electrolyte is less marked.

Dissolution Rate—Dissolution of triamcinolone acetonide in distilled water at 28° under sink conditions is shown in Fig. 9 as a function of time for various agitation intensities. The data are in accordance with the integrated form of the modified Noyes–Whitney equation (9):

$$\ln \frac{C_s}{C_s - C} = k'At = \frac{DA}{Vh} \cdot t \qquad \text{(Eq. 3)}$$

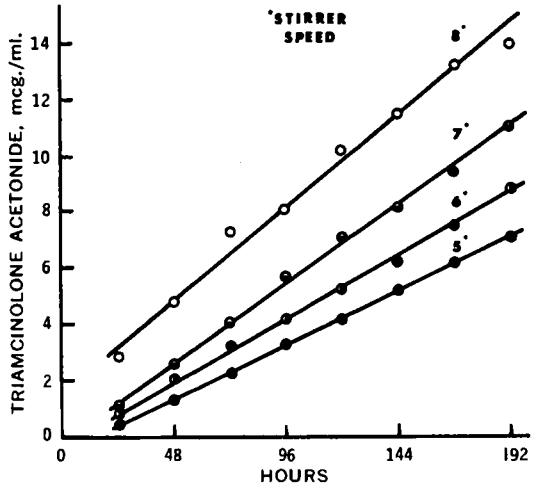

Figure 9—*Triamcinolone acetonide dissolution in distilled water as a function of agitation intensity (stirrer speed 5 = 147 r.p.m., 6 = 248 r.p.m., 7 = 308 r.p.m., and 8 = 398 r.p.m.).*

Table III—Dissolution Rates and Dissolution Rate Constants for Triamcinolone Acetonide in Distilled Water at 28°

Stirrer Speed, r.p.m.	dC/dt, (mcg. $ml.^{-1}$ $hr.^{-1}$) × 10^2	k'^a, ($hr.^{-1}$ $cm.^{-2}$) × 10^3
147	4.01	1.86
248	4.70	2.33
308	5.82	3.15
398	6.63	4.51

[a] Surface area of pellet exposed to dissolution medium was ~1.271 $cm.^2$.

Table IV—Dissolution Rates and Dissolution Rate Constants for Triamcinolone Acetonide in Potassium Chloride Solutions at 28°[a]

Potassium Chloride Solution	dC/dt, (mcg. $ml.^{-1}$ $hr.^{-1}$) × 10^2	k'^b, ($hr.^{-1}$ $cm.^{-2}$) × 10^3
0.2 M	3.61	3.91
0.5 M	1.98	2.37
1.0 M	1.85[c]	3.60[c]

[a] Stirrer speed = 308 r.p.m. [b] Surface area of pellet exposed to dissolution medium was ~1.271 $cm.^2$. [c] For data within the period of 96–192 hr. only.

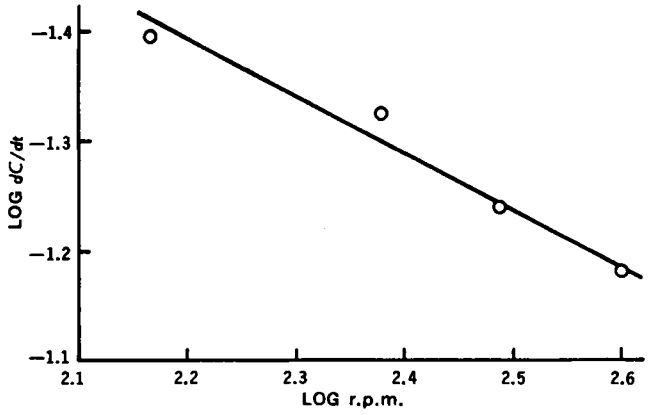

Figure 11—*Log-log plot of triamcinolone acetonide dissolution rate in distilled water* versus *stirrer speed (r.p.m.).*

where C_s and C are the concentrations of a saturated solution of the solute and of a solution of the solute at time t, respectively; k' is the intrinsic dissolution rate constant; A is the surface area of the solid exposed to the dissolution medium; D is the diffusion coefficient of the solute in the dissolution medium; V is the volume of the solution; and h is the diffusion layer thickness. The integrated Noyes–Whitney plot of the dissolution data is shown in Fig. 10. The dissolution rates and dissolution rate constants for triamcinolone acetonide in distilled water at 28° are given in Table III.

The influence of agitation intensity in the unbaffled system employed in this study is quite evident. Cooper and Kingery (10) showed that dissolution from a rotating-disk face is proportional to the square root of angular velocity, ω, *i.e.:*

$$dC/dt = K\omega^{1/2} \qquad \text{(Eq. 4)}$$

where K is a proportionality constant. If the angular velocity is given in revolutions per minute and if Eq. 4 is transformed logarithmically, then:

$$\log (dC/dt) = \log K + {}^1/_2 \log \text{r.p.m.} \qquad \text{(Eq. 5)}$$

A double logarithmic plot of dC/dt as a function of stirrer speed should yield a straight line with a slope of 0.5. Linear regression analysis of the data depicted in Fig. 11 indicates a slope of 0.515, with a correlation coefficient of 0.981.

The evaluation of steroid solubility in potassium chloride solutions as a function of time under sink conditions was only determined at speed 7 (~308 r.p.m.), since vortex formation tended to occur in this system at higher speeds. A plot of the data in accordance with the integrated Noyes–Whitney equation is shown in Fig. 12. The data, however, are not at all indicative of adherence to Eq. 3, since a zero ln $[C_s/(C_s - C)]$ intercept at $t = 0$ is not obtained. Rather, a lag in dissolution is apparent, particularly for dissolution in 1.0 M potassium chloride solution. Dissolution rates and dissolution rate constants for the steroid at speed 7, as a function of potassium chloride concentration, are given in Table IV.

The data for solubility and dissolution appear to be reasonable in view of the most reliable data available to date (3) and in view of the results of linear regression analysis of Beer's plots which yielded correlation coefficients of 0.9985 to 0.9998 for the systems employed. Additional work is underway involving the synthesis of ^{14}C-labeled triamcinolone acetonide. Isotopic methods will be employed to measure its solubility in comparison with the data reported here.

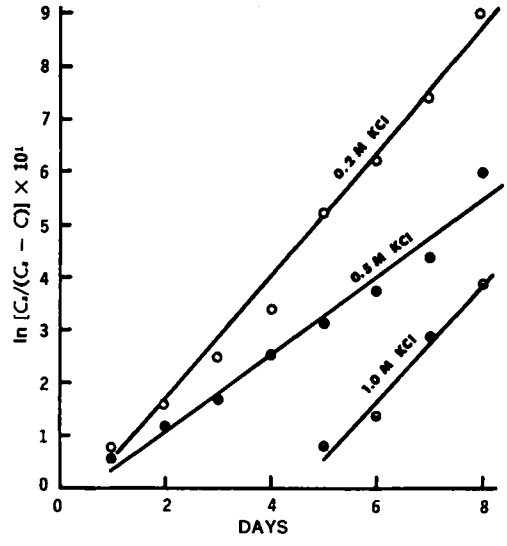

Figure 12—*Triamcinolone acetonide dissolution in potassium chloride solutions at speed 7 (308 r.p.m.) and 28°, plotted in accordance with the integrated form of the modified Noyes–Whitney equation.*

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▲ To whom inquiries should be directed.

Preformulation Studies I: Molindone Hydrochloride

J. DUDZINSKI, L. LACHMAN, E. SHAMI, and J. TINGSTAD▲

Abstract □ Preformulation studies on molindone hydrochloride, a dihydroindolone tranquilizer, are described. A TLC assay is outlined, degradation products are identified, and results of stability studies are presented. The dissociation constant is estimated using pH determinations.

Keyphrases □ Molindone hydrochloride—preformulation assay development, identification of degradation products, stability, and estimated dissociation constant □ Preformulation assay development, identification of degradation products, stability, and estimated dissociation constant—molindone hydrochloride □ Drug development, preformulation studies—assay development, identification of degradation products, stability, and estimated dissociation constant

This series of articles will present examples of preformulation studies that attain a proper balance between exhaustive quantitative experiments and those that are only semiquantitative. Studies of this type should consider the overall time-economic factors present in new drug development. This paper is concerned with assay development, identification of degradation products, stability studies, and an estimation of the dissociation constant for a dihydroindolone tranquilizer, molindone hydrochloride [3-ethyl-6,7-dihydro-2-methyl-5-(morpholinomethyl)indol-4(5*H*)-one hydrochloride].

EXPERIMENTAL

Selection of Assay—Molindone hydrochloride has UV absorption peaks at 255 and 299 nm., which can be used to determine quantitatively the pure drug substance. However, preliminary studies indicated that obvious chemical degradation (as evidenced by color and precipitate formation) was not accompanied by a concomitant decrease in UV absorption. This showed that degradation products absorb significant amounts of light at these wavelengths and necessitate a separation procedure prior to the determination of UV absorbance. TLC was selected as the separation procedure; the general technique described by Spencer and Beggs (1) was used, with some modifications.

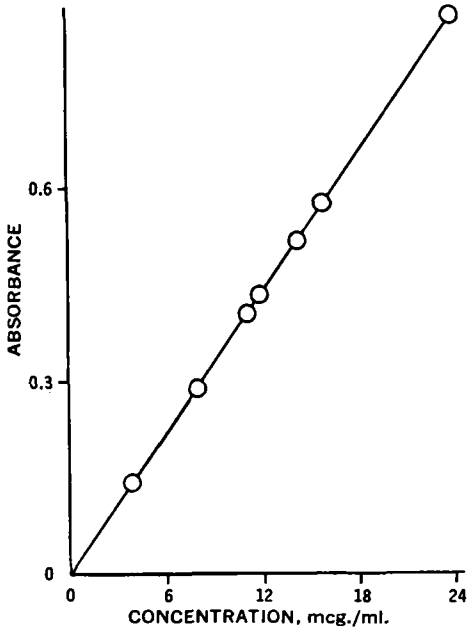


Figure 1—*Standard Beer's law plot for molindone hydrochloride at 255 nm.*

Development of TLC System—Precoated silica gel plates[1] were employed; the developing solvent system was butanol–methanol–1 *N* HCl (85:10:5). Drug solutions (either freshly prepared or degraded by heat[2] or light) were spotted with previously calibrated disposable pipets. The system was then developed for 10 cm. in a sealed glass jar lined with saturated filter paper. Inspection under shortwave UV light provided initial qualitative analysis.

Quantitative TLC Separation and UV Analysis—From 20 to 40 μl. of the solution to be assayed (representing 100–600 mcg. of drug) was applied to the plate and developed as already outlined. The plate was then dried and examined under shortwave UV light; the area containing the drug was removed from the plate with a razor blade and quantitatively transferred to a syringe fitted with a Millipore filter holder containing a 0.22-μ filter. The silica gel was washed in the syringe with four 5-ml. portions of 0.1 *N* HCl; the washings were then filtered directly into a 25-ml. volumetric flask. The solution was brought to volume with 0.1 *N* HCl (also passed through the filter) and read at 255 nm. against 0.1 *N* HCl. A 0.1 *N* HCl blank was subjected to the same treatment.

Preparation of Standard Curve—Because complete recovery of drug from silica gel is seldom accomplished, it was necessary to construct a standard curve. Solutions of molindone hydrochloride were freshly prepared in distilled water at concentrations of 0.5, 0.7, 0.9, and 1.0% and subjected to the described quantitative TLC separation and UV analysis. Each determination was done in quadruplicate.

Determination of Adsorption Isotherm—Results from the standard curve data confirmed that extraction of the drug from silica gel was not complete. Consequently, an adsorption isotherm for this system was constructed to determine whether the amount of drug lost was constant. The average amount of silica gel scraped off the plate (119.5 mg.) was added to standard solutions of molindone hydrochloride (100, 200, 300, 400, and 600 mcg./25 ml.). The flasks were then shaken for 5 min. and filtered through a 0.22-μ filter. The absorbance of each solution was determined at 255 nm. against a silica gel-treated blank. No effort was made to control temperature carefully; this procedure would correspond to the conditions present during the assay.

Identification of Degradation Products—The additional spots from the TLC treatment of degraded solutions were sprayed with test solutions (see *Results and Discussion*) to indicate the chemical nature of the degradation products. In addition, the brown precipitate found in degraded samples was collected and decolorized by dissolving in acetone–methanol (50:50) and treating with activated charcoal. After evaporating the filtered solution to dryness, the residue was recrystallized from methanol. Identification was made by comparison with a known compound and by elemental (C, H, N) analysis.

Stability Studies—The stability of molindone hydrochloride (in solution and in dosage forms) was investigated in the usual manner. Constant-temperature baths were used for studies on the drug substance, while ovens were used for dosage forms.

Determination of Dissociation Constant—The dissociation constant of molindone was estimated by the procedure described by Stern *et al.* (2). Molindone hydrochloride (0.001 or 0.0006 mole) was added to 450 ml. of recently boiled, distilled water. Two milliliters of 0.1 *N* NaOH was added, along with sufficient sodium chloride

[1] Brinkmann HF 254, 250 μ, with fluorescent indicator.
[2] All samples in this report, other than light-degraded ones, were protected from light.

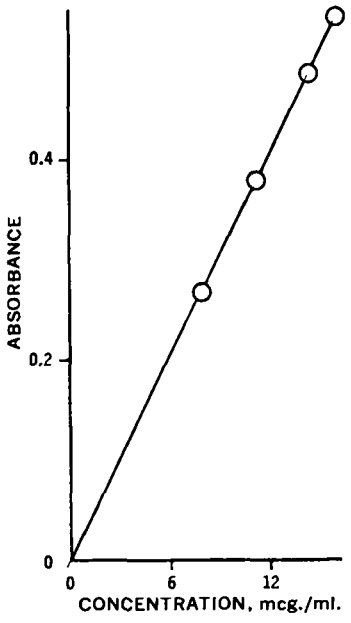


Figure 2—*Standard curve for TLC assay of molindone hydrochloride.*

to bring the ionic strength to 0.2; then the solution was made up to 500 ml. with recently boiled, distilled water. The pH of the solution at 25° was immediately measured using an expanded scale pH meter[3].

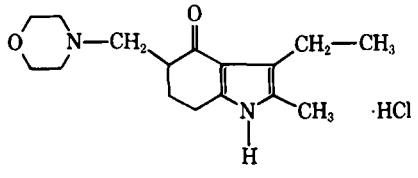
RESULTS AND DISCUSSION

TLC of Freshly Prepared Solutions—Two spots with R_f values of 0 and 0.5 are visible under shortwave UV light. The R_f 0.5 spot stains blue with iodoplatinate reagent (I, specific for heterocyclic nitrogen), stains blue with modified Ehrlich's reagent (II, specific for the indole ring), and exhibits a green fluorescence under longwave UV light after spraying with Prochazka reagent (III, specific for the indole ring); all of these results indicate the presence of molindone. The R_f 0 spot is very faint and does not respond to any of the spray treatments.

TLC of Heat-Degraded Samples—Molindone samples, whether heat degraded in the solid state or in aqueous solution, produce four spots visible under shortwave UV light. The R_f 0 spot does not stain with any of the three reagents. The R_f 0.24 spot (faint) yields a blue color with I and a faint blue color with II, and it produces a faint green fluorescence under longwave UV light in the presence of III. The R_f 0.5 spot produces a blue color with I and a strong blue color with II, and it reacts positively to III. The R_f 0.79 spot does not react with I but yields a faint blue reaction to II and III.

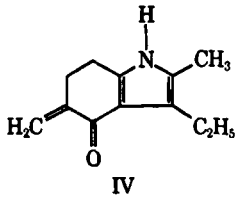
TLC of Light-Degraded Samples—Light-degraded samples of molindone, whether solid or aqueous solutions, show three spots with R_f values of 0, 0.24, and 0.5. These spots react in the same way as the corresponding spots of heat-degraded molindone; however, no R_f 0.79 spot is present in this case.

Quantitative Assay Results—A standard Beer's law plot appears in Fig. 1 (absorptivity with 1-cm. cell = 36.00 ml./mg.). The standard curve for the TLC assay is shown in Fig. 2 (slope = 34.00 ml./mg.). Based upon the slopes of the least-squares lines in Figs. 1 and 2, the average recovery in the standard curve data was 94.5%. To check the validity of the standard curve, nine determinations with known concentrations of drug were performed, reading the calculated concentrations off the standard curve. The average figure



molindone hydrochloride

[3] Corning.



IV

was 98.7% with a standard deviation of 0.23%, indicating that the assay procedure was valid.

Determination of Adsorption Isotherm—The adsorption isotherm for the silica gel–molindone hydrochloride system is shown in Fig. 3. The slope of the line is 33.50 ml./mg., which is in excellent agreement with the 34.00 value obtained for the standard curve. This indicates that a constant percentage of the amount of drug present is recovered from the silica gel; hence, it is valid to use the standard curve in assaying samples (initial and stability) of molindone hydrochloride. It should be mentioned at this point that no drug was adsorbed by the filters used in the assay procedure.

Identification of Degradation Products—Chemically, molindone hydrochloride is 3-ethyl-6,7-dihydro-2-methyl-5-(morpholinomethyl)indol-4(5*H*)-one hydrochloride. From the TLC studies, the R_f 0.5 spot was identified as molindone hydrochloride, staining with Reagent I (specific for heterocyclic nitrogen, *i.e.*, the morpholine substituent) and Reagents II and III (specific for the indole ring). The staining behavior of the R_f 0.79 spot, present in the heat-degraded samples but absent in the light-degraded material, indicated that the indole structure was present (positive II and III) but the morpholine substituent was not (negative I). This suggests an amine elimination reaction similar to that reported by Koshy and Mitchner (3), yielding morpholine and the following compound: 3-ethyl-6,7-dihydro-2-methyl-5-methyleneindol-4(5*H*)-one (IV), used in the synthesis of molindone.

Confirmation that the R_f 0.79 spot is, in fact, Compound IV was accomplished by comparing the recrystallized precipitate from heat-degraded samples with a known sample. The melting points coincided (known 217.5–219° *versus* unknown 218–219°), as did their UV and IR spectra. TLC treatment of the two yielded R_f values of 0.80 and 0.79, and the staining properties were identical with each other and with the aforementioned 0.79 spots. Finally, the elemental analysis of the precipitate yielded the following results.

Anal.—Calc.: C, 76.15; H, 7.99; N, 7.40. Found: C, 76.05; H, 8.12; N, 7.59.

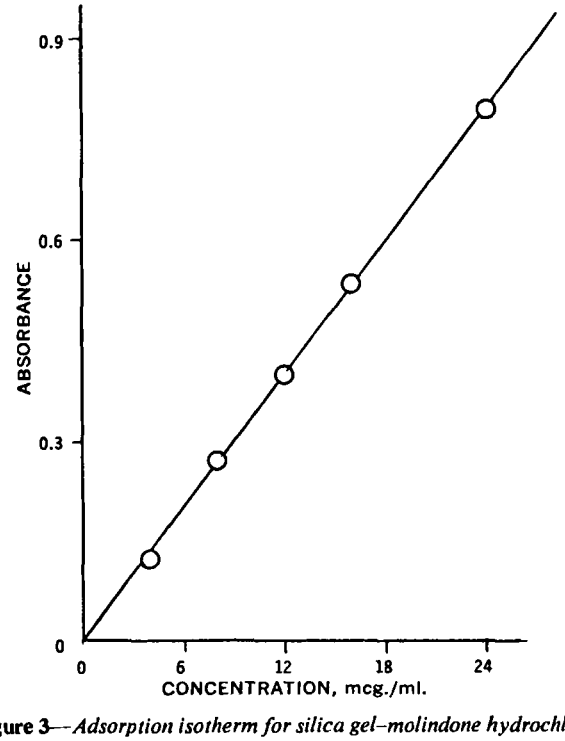


Figure 3—*Adsorption isotherm for silica gel–molindone hydrochloride at room temperature.*

Table I—Pseudo-First-Order Rate Constants and Half-Lives for Molindone Hydrochloride Degradation at Various Temperatures and pH's

pH	Temperature	k, day^{-1}	Half-Life, days
1.24	50°	0.0051	136
1.46	50°	0.0056	124
1.86	50°	0.0044	157
2.24	50°	0.0045	154
1.23	60°	0.0161	43
1.47	60°	0.0140	50
1.86	60°	0.0128	54
2.22	60°	0.0140	50

Trace amounts of this degradation product could be detected on the plates (R_f 0.79) by spraying with 0.5% potassium ferricyanide in a 10% aqueous solution of ferric chloride. While this reagent is not specific (it will react with most substances that are easily oxidized), it is very sensitive and stains the R_f 0.79 spot blue when the amount of the degradation product is too small to be detected by any of the aforementioned methods including UV light.

The R_f 0.24 spot probably represents oxidation products of molindone (4); the morpholine substituent probably hydrolyzes open and is not visible on the developed TLC plates. Because the work on the 0.79 spot indicated the nature of the primary degradation reaction, no definitive experiments were performed on the 0.24 spot.

Stability Studies—While time did not permit a complete investigation of molindone's stability to heat, light, oxygen, and pH, sufficient preformulation studies were conducted to point the way to successful formulation conditions. Fortunately, color development could be used as a semiquantitative end-point to determine the effect of light, oxygen, and pH on the drug in solution. Results from these studies indicated that molindone was susceptible to light degradation and oxidation; these conclusions were confirmed by the TLC studies. Brief semiquantitative experiments indicated that the usual protection from light (amber containers) and oxygen (inclusion of an antioxidant) was sufficient to prevent color formation; subsequent studies concentrated on the effect of pH, buffer species and concentration, and heat.

Because of various formulation restrictions, including solubility, taste (oral liquids), and tissue irritation (injectables), it was desirable to formulate solutions of the drug between pH 3 and 5. When using color formation as a rough indicator, it was found that a pH range of 2.8–3.5 was most desirable from a stability standpoint. With this preliminary information, the following quantitative experiments were performed.

To show the effect of high concentrations of hydrogen ion on stability, four different aqueous solutions (0.1%) of molindone hydrochloride (pH adjusted to 1.2–2.2 with hydrochloric acid and ionic strength held constant with sodium chloride) were stored at 50 and 60° and assayed periodically. The results (Table I) indicated that: (*a*) the reaction (first order) was not particularly sensitive to hydrogen ion, and (*b*) the drug was relatively stable, having a half-life of about 140 days at 50° in this pH range. From these and subsequent experiments, the heat of activation was estimated to be in the "normal" range of about 20 kcal./mole.

To show the effect of buffer species and concentration and to determine whether the pH 3 or 5 area was more desirable, three solutions (A, pH 5.0, 0.167 *M* citrate buffer; B, pH 5.0, 0.333 *M* citrate buffer; and C, pH 3.5, 0.333 *M* citrate buffer, all at constant ionic strength) were stored at 70° and assayed periodically. The results (Fig. 4) confirmed the first-order nature of the reaction and indicated that: (*a*) the drug was about four times more stable at pH 3.5 than at 5.0, and (*b*) the reaction was catalyzed by citrate. No additional spots appeared on the TLC plates, eliminating the possibility of a molindone–citrate reaction as might be suspected from the work of Higuchi and Miki (5). Similar results were obtained with bitartrate and phosphate buffers (still keeping ionic strength constant). Because of these results and because unbuffered solutions of molindone hydrochloride remain at a relatively constant pH with respect to time, no further attempts at buffering solutions of the drug were made.

As a result of these experiments, formulation of solution dosage forms (oral and parenteral) was relatively routine; the stability of the resulting formulations was satisfactory. Preformulation work also included testing the stability of the drug with various tableting excipients. As expected from the pH–stabilty studies in solution, basic materials (*e.g.*, dicalcium phosphate) proved to be unacceptable while acidic components (*e.g.*, calcium sulfate) were acceptable.

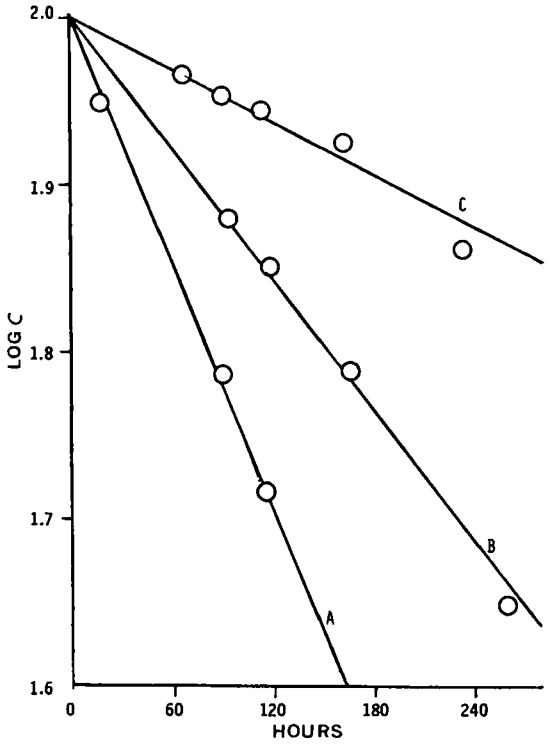


Figure 4—*First-order plot of molindone hydrochloride degradation at 70°. Key: A, pH 5.0, 0.167 M citrate buffer; B, pH 5.0, 0.333 M citrate buffer; and C, pH 3.5, 0.333 M citrate buffer.*

Determination of Dissociation Constant—The pKa of molindone was estimated to be 6.89 (using 0.001 mole of drug) and 6.76 (using 0.0006 mole of drug). As Stern *et al.* (2) pointed out, the values of the dissociation constant obtained with this method are "semi-classical," since the hydrogen ion is expressed in activity units and the other figures in concentration units. The usefulness of this method for estimating dissociation constants is shown by the fact that subsequent determination of pKa by potentiometric titration in water–dioxane mixtures (then extrapolating to zero dioxane concentration), according to the method of Van Uitert and Haas (6), yielded a value of 6.94.

CONCLUSION

The information presented provides a good example of preformulation studies that lead to meaningful semiquantitative and accurate quantitative results useful in product formulation.

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Colorimetric Determination of Aromatic Compounds by Hydroxylation

KENNETH S. ALBERT and KENNETH A. CONNORS▲

Abstract □ Aromatic hydroxylation has been developed into a widely applicable analytical method for aromatic compounds. A modified Hamilton hydroxylating system, consisting of hydrogen peroxide, ferric ion, catechol, and glucose, converts aromatic compounds to phenols, which are measured colorimetrically after oxidative coupling with 4-aminoantipyrine. The method is applicable to 10^{-4}–10^{-3} *M* aqueous solutions of many substituted benzene derivatives, linear absorbance–concentration plots being obtained with apparent molar absorptivities in the range of 1–5 × 10^3. The method has been applied to numerous aromatic drugs. Many common tablet excipients do not interfere at normal formulation levels. As one of the few methods available for the detection of the phenyl group, this technique has the advantages and limitations of a general functional group method.

Keyphrases □ Aromatic compounds—hydroxylation, colorimetric determination after oxidative coupling with 4-aminoantipyrine □ Hydroxylation of aromatic compounds—modified Hamilton system (hydrogen peroxide–ferric ion–catechol–glucose), colorimetric determination after coupling with 4-aminoantipyrine □ Phenyl group determination—modified Hamilton hydroxylation, colorimetry after oxidative coupling with 4-aminoantipyrine □ Colorimetry—determination of aromatic compounds, hydroxylation followed by oxidative coupling with 4-aminoantipyrine

A method (Scheme I) for the quantitative determination of aromatic compounds was recently described (1). An aromatic compound is converted to a phenol by the action of the Hamilton hydroxylating system (2–4), consisting of hydrogen peroxide and catalytic amounts of ferric ion and catechol; this system was modified by the incorporation of a cyclodextrin. After hydroxylation, the product phenol is oxidatively coupled with 4-aminoantipyrine for spectrophotometric measurement (5–7). Many benzene derivatives were determined in this manner (1).

Significant improvements have now been made in this aromatic hydroxylation method. The present paper describes the basis for these improvements and the application of the new method to numerous compounds of pharmaceutical interest.

EXPERIMENTAL

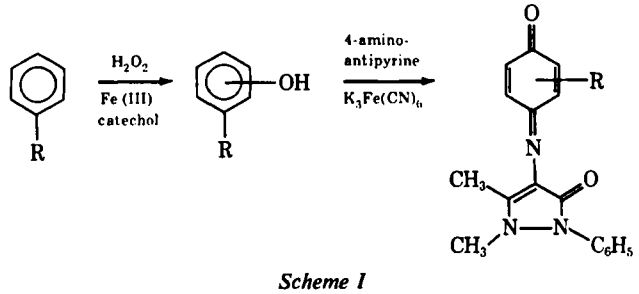
Materials—The following chemicals were used as received: ferric perchlorate hexahydrate[1], potassium ferricyanide[2], ethyl acetate[3], acetone[3], chloroform[3], methanol[3], benzene[3], toluene[3], isopropyl alcohol[3], benzoic acid[3], phenol[3], glucose[3], aniline[3], 30% hydrogen peroxide[3], lactose[3], stearic acid[3], benzaldehyde[3], nitrobenzene[3], hydroquinone[3], sorbitol[4], acetonitrile[4], dioxane[4], talc[5], starch[6], 4-aminoantipyrine[7], *N,N*-dimethylformamide[8], Schardinger α- and β-dextrins[9], L-phenylalanine[10], L-tryptophan[11], quinine sulfate[4], *m*-cresol[8], *p*-cresol[8], *m*-chlorophenol[8], *m*-hydroxyacetophenone[8], chloramphenicol[12], benzocaine[13], phenacetin[12], *d,l*-mandelic acid[12], antipyrine[12], *d,l*-ephedrine hydrochloride[8], benzenesulfonamide[7], sulfanilamide[8], atropine sulfate, and chlorpheniramine maleate. Anisole[7] was found to be gas chromatographically pure (10% SE-30, 165°).

Catechol[13] was recrystallized twice from toluene, m.p. 103–104° [lit. (8) m.p. 104°]; hydrocinnamic acid[8] was recrystallized from petroleum ether, m.p. 47–48° [lit. (9) m.p. 47.5–48°]; benzamide[8] was recrystallized from water, m.p. 125.5–127° [lit. (10) m.p. 125–126°]. The three methoxybenzoic acids were recrystallized from water: *o*-methoxybenzoic acid, m.p. 98.5–100° [lit. (10) m.p. 99–101°]; *m*-methoxybenzoic acid, m.p. 104.5–105° [lit. (10) m.p. 104–105°]; and *p*-methoxybenzoic acid, m.p. 183–184° [lit. (11) m.p. 184°].

N-Methylbenzylamine[8] was distilled at reduced pressure, b.p. 71°/2 mm. Hg [lit. (12) b.p. 78°/14 mm. Hg]; methyl benzoate[8] was distilled, b.p. 199.6° [lit. (13) b.p. 200°]; ethylbenzene[8] was distilled, b.p. 135° [lit. (14) b.p. 135°]; benzyl alcohol[8] was distilled at reduced pressure, b.p. 70°/15 mm. Hg [lit. (15) b.p. 104–105°/20 mm. Hg]; *o*-cresol[8] was distilled, b.p. 191–192° [lit. (16) b.p. 192°]; guaiacol[8] was distilled at reduced pressure, m.p. 28° [lit. (17) m.p. 27.4°]; *p*-methoxyphenol[8] was distilled under reduced pressure, m.p. 53° [lit. (18) m.p. 53°]; and salicylaldehyde[8] was distilled at reduced pressure, b.p. 65–66°/2 mm. Hg [lit. (19) b.p. 197°/760 mm. Hg].

Salicylic acid[8] was recrystallized from methanol–water, m.p. 159–160° [lit. (20) m.p. 158°]; *m*-hydroxybenzoic acid[8] was recrystallized from water, m.p. 200° [lit. (21) m.p. 202°]; *p*-hydroxybenzoic acid[8] was recrystallized from xylene–ethanol, m.p. 213–214° [lit. (22) m.p. 212–213°]; *o*-aminophenol was recrystallized from toluene–methanol, m.p. 174.5–176° [lit. (23) m.p. 171°]; *m*-aminophenol was recrystallized from toluene–methanol, m.p. 122–123° [lit. (24) m.p. 123°]; *o*-nitrophenol[8] was recrystallized from ethanol, m.p. 44.5–45° [lit. (25) m.p. 45°]; *m*-nitrophenol[8] was recrystallized from aqueous hydrochloric acid, m.p. 95–96° [lit. (26) m.p. 95–96°]; *p*-nitrophenol was recrystallized from toluene, m.p. 113–114° [lit. (27) m.p. 114°]; and *m*-hydroxybenzaldehyde[8] was recrystallized twice from water, m.p. 101–103° [lit. (28) m.p. 101–102°].

Acetanilid[5] was recrystallized from water, m.p. 113–114° [lit. (29) m.p. 114°]; acetylsalicylic acid[5] was recrystallized from acetone, m.p. 135–136° [lit. (30) m.p. 135°]; 2-naphthalenesulfonic acid[13] was recrystallized from dioxane–benzene, m.p. 121–123° [lit. (31) m.p. 120–122°]; phenobarbital[14] was recrystallized from water–methanol, m.p. 174–176° [lit. (32) m.p. 174.5–175.5°]; barbital[14] was recrystallized from water, m.p. 188–189.5° [lit. (33) m.p. 190°]; and *d,l*-



Scheme I

[1] G. Frederick Smith Co.
[2] J. T. Baker Chemical Co.
[3] Mallinckrodt.
[4] Fisher Scientific Co.
[5] Merck.
[6] Beaver Dam Wholesale Co.
[7] Aldrich Chemical Co.
[8] Eastman.
[9] Pierce Chemical Co.
[10] Schwartz/Mann.
[11] Nutritional Biochemical Corp.
[12] Ruger Chemical Co.
[13] Matheson, Coleman and Bell.
[14] Z. D. Gilman, Inc.

tropic acid was recrystallized from benzene, m.p. 116–117° [lit. (34) m.p. 116–117°].

Standard buffers were prepared according to Bates (35). Water was purified by passing once-distilled water through an ion-exchange column[15]. Acetate buffer (pH 4.0), in which hydroxylation reactions were run, was prepared to be 5×10^{-3} *M* in total buffer concentration and 0.1 *M* in ionic strength; ionic strength was adjusted with potassium chloride. Borate buffer, used to quench the hydroxylation reaction and as the color development medium, was prepared to be 0.4 *M* in total boric acid and 0.2 *M* in sodium hydroxide. Hydrogen peroxide (3%) was prepared by dilution of the 30% solution and was standardized by titration with potassium permanganate. Aqueous catechol solutions (3×10^{-3} *M*) were stored in the dark. Ferric perchlorate stock solution (1.5×10^{-2} *M*) was stabilized by adding 1 drop of 60% perchloric acid/50 ml. of solution. Ferric perchlorate reagent solution (1.5×10^{-3} *M*) was prepared by diluting the stock solution.

Apparatus—Spectrophotometric measurements were made in spectrophotometers[16] equipped with thermostatted cell compartments. The pH measurements[17] were made at 25.0°. Reaction temperatures were maintained to ±0.1° with constant-temperature water baths. Thermometers were calibrated against thermometers carrying either an NBS or an ASTM certificate.

Procedures—*Analytical Method*—To a 25-ml. volumetric flask are added 1.0 ml. of 3×10^{-3} *M* aqueous catechol, 1.0 ml. of 1.5×10^{-3} *M* ferric perchlorate, 5.0 ml. of 1.8×10^{-2} *M* glucose, and enough of the aromatic sample compound such that its concentration in the diluted solution is in the range of 10^{-4}–10^{-3} *M*. The solution is diluted to the mark with pH 4.0 acetate buffer, and the pH of the final solution should be in the range of 3.5–4.0.

The flask is placed in a 75° water bath, and reaction is initiated by adding 0.1 ml. of 3% hydrogen peroxide solution. After 10 min., a 5.0-ml. aliquot is pipeted into 5.0 ml. of borate buffer. The color is developed by adding 0.1 ml. of 3% (w/v) aqueous 4-aminoantipyrine followed by 0.1 ml. of 10% (w/v) aqueous potassium ferricyanide. The absorbance is measured immediately at the absorption maximum against a reagent blank carried through the same procedure. A standard curve is prepared by subjecting known concentrations, bracketing the unknown concentration, of the same aromatic compound to the procedure.

Chloroform Extraction Method—To achieve greater sensitivity or to circumvent fading of the color with some compounds (discussed later), substances giving a neutral extractable coupling product can be determined as follows. After hydroxylation is complete, 15.0 ml. of the reaction mixture is transferred to a 60-ml. separator containing 10 ml. of borate buffer. To this is added 0.1 ml. of 3% 4-aminoantipyrine, about 4 ml. of chloroform, and then 0.1 ml. of 10% potassium ferricyanide; the mixture is shaken for about 1 min. The chloroform layer is filtered through glass wool into a 5-ml. volumetric flask. Then the separator is rinsed with an additional 0.75 ml. of chloroform, which is added to the first chloroform extract, and this solution is diluted to volume with chloroform. Its absorbance is measured at the absorption maximum against a reagent blank carried through the same procedure.

Optimization Studies—During the development of the proposed method, many system variables were investigated including time, temperature, pH, concentrations and ratios of concentrations of reactants, other colorimetric finishes, other "stabilizers" (see *Discussion*), and interferences. These studies were carried out in much the same manner as described under *Analytical Method*, with appropriate variation in the feature being studied.

RESULTS

Unmodified Hamilton System—When catechol is subjected to the 4-aminoantipyrine colorimetric method, no significant interference is observed at wavelengths used for monohydric phenols. This lack of interference makes the 4-aminoantipyrine method the finish of choice. It is, therefore, possible to carry out the color development directly in the hydroxylation solution.

Table I—Effect of Reagent Age on Yield in Hydroxylation of Anisole[a]

Age of Hamilton System before Addition of Anisole, min.	A_{500}[b]
0	0.461
5	0.429
10	0.317
15	0.230
30	0.205
60	0.146
120	0.068
120[c]	0.461
180	0.012
180[d]	0.036

[a] 5.36×10^{-4} *M* anisole, 5.90×10^{-5} *M* Fe(III), 1.17×10^{-4} *M* catechol, 1.06×10^{-3} *M* H_2O_2, pH 3.9, 25°, 1-hr. reaction time. [b] 4-Aminoantipyrine finish. [c] Additional catechol (1.17×10^{-4} *M*) and H_2O_2 (1.06×10^{-3} *M*) added at this time. [d] Additional H_2O_2 (1.06×10^{-3} *M*) added at this time.

Dependence of Yield on Peroxide–Substrate Ratio—Typical initial reactant concentrations employed by Hamilton *et al.* (3, 4) were catechol, 1.5×10^{-4} *M*; Fe(III), 4×10^{-5} *M*; hydrogen peroxide, 2×10^{-3} *M*; and substrate, 10^{-2} *M*. The reaction was followed by monitoring the loss of peroxide. As an analytical reagent, however, the hydrogen peroxide in the Hamilton system must be in excess of the aromatic substrate (sample). With these more practical conditions, the yield of phenolic product, as reflected by the absorbance from the 4-aminoantipyrine-coupled product, was markedly dependent upon time of reaction and initial ratio of peroxide to substrate. Moreover, when phenol was substituted for an aromatic substrate in such a system, hydroxylation occurred as shown by the absorbance decrease.

Figure 1 shows the time course for the hydroxylation of anisole. At initial ratios of peroxide–anisole much greater than 2, production and then loss (by further hydroxylation) of monohydroxyanisoles are evident. When this ratio is about 2 or less, stable absorbance values are obtained after 1–2 hr.; however, the yield is sharply dependent upon this ratio, being depressed as the ratio is decreased. In a practical analytical situation, this ratio is not known and cannot be controlled. Under such conditions (namely $[H_2O_2]_0$ is constant and $[\text{anisole}]_0$ is variable), the percent yield of phenolic product depends upon the sample concentration, and a Beer's law plot with curvature is observed. The direction of curvature depends upon the $[H_2O_2]_0$–$[\text{substrate}]_0$ ratio, being negative if this ratio is 2 or less and positive for values greater than 2.

Dependence of Yield on pH, Catechol, and Iron—Initial experiments revealed measurable but small differences in yield between studies carried out under nitrogen and in the presence of air. Most subsequent work was done with an air environment.

Studies of yield (in these, as in other experiments unless otherwise noted, it is to be understood that yields are based upon absorbance values of the 4-aminoantipyrine-coupled products) established optimal conditions for several system variables when $[H_2O_2]_0$–$[\text{anisole}]_0$ was held at about 2 so as to give stable yields. The yield was found to be essentially independent of pH in the range of 3.5–4.2. The optimum concentration of catechol is about 1.2×10^{-4} *M*, and the optimum ratio of Fe(III) to catechol is 0.5, although the yield is not very sensitive to this ratio[18]. These optimum conditions were incorporated into the recommended analytical method.

Aging of Hamilton System—Catechol, which is itself an aromatic, should be hydroxylated, possibly altering its catalytic effectiveness as well as depleting peroxide. This anticipated aging phenomenon is demonstrated in Table I, which shows that the time of addition of substrate to a pre-prepared Hamilton system affects the yield of hydroxylated product. When additional catechol plus peroxide is added, the yield returns to its expected value, but addition of peroxide alone does not restore the yield. These observations were used in designing the analytical method, the reaction being initiated by adding peroxide, that is, by completing the Hamilton hydroxylating system.

[15] Continental Deionized Water Service.
[16] Cary models 14 and 16.
[17] Radiometer pH meter model 25 with Corning combination electrode 476051, or Sargent model DR pH meter with Sargent combination electrode S-30072-15.

[18] Data substantiating these and other findings were reported elsewhere (36).

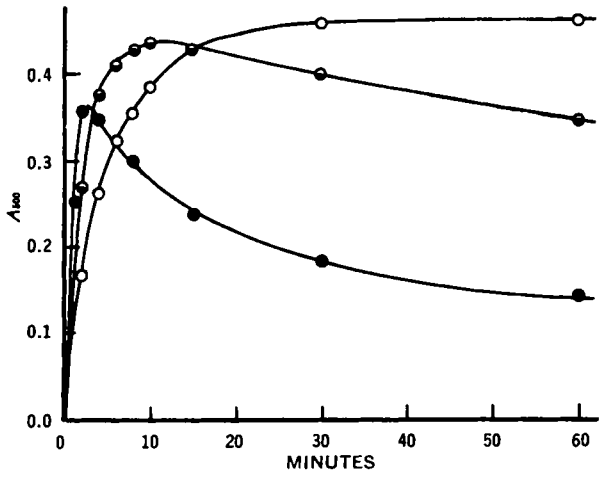


Figure 1—*Change of absorbance with time (4-aminoantipyrine finish) for hydroxylation of anisole at 25° (nitrogen atmosphere). Initial conditions: 5.26 × 10⁻⁴ M anisole, 4.26 × 10⁻⁵ M Fe(III), 1.17 × 10⁻⁴ M catechol, pH 3.9. Key: ○, $[H_2O_2]_0$–$[anisole]_0$ = 2.3; ◒, 4.7; and ●, 23.6.*

Cyclodextrin-Modified Hydroxylating System—Figure 1 shows that under analytical conditions, where the hydrogen peroxide in the Hamilton system is in large excess of the aromatic sample compound, hydroxylation continues beyond the introduction of the first hydroxy group. This phenomenon reduces the attractiveness of the Hamilton system as an analytical reagent.

The hypothesis was conceived that a cyclodextrin (ring compounds of 1,4-linked D-glucose monomers) might have a differential rate effect on the sequential hydroxylation steps as a consequence of its capability of forming inclusion complexes with aromatic compounds (37, 38). Figure 2 shows the time course for the hydroxylation of benzene in the presence and absence of cyclodextrins at an initial peroxide–substrate ratio of 6.75. Evidently the cyclodextrins have a profound stabilizing effect. About 10^{-3} *M* cyclodextrin gives an optimum effect; too high a concentration results in depressed yields. Under the conditions given in Fig. 2, a linear Beer's law plot was obtained for benzene[19] over a concentration range corresponding to initial peroxide–benzene ratios of 3.15–31.5. Incorporation of a cyclodextrin, therefore, provides a satisfactory analytical method.

This method was successfully applied to 15 mono- and disubstituted benzenes, using 1–2-hr. reaction times at 25° (1). Its extension to some drugs and related compounds is shown in Table II. Chlorpheniramine and benzalkonium chloride gave standard curves with marked negative curvature. Benzocaine could not be determined because the product precipitated from solution. The phenobarbital standard plot showed positive curvature. No significant interference (p = 0.05) was observed by tablet excipients in the determination of acetanilid[20]; the mixture contained 35 mg. acetanilid, 100 mg. lactose, 10 mg. starch, 10 mg. talc, and 2 mg. stearic acid.

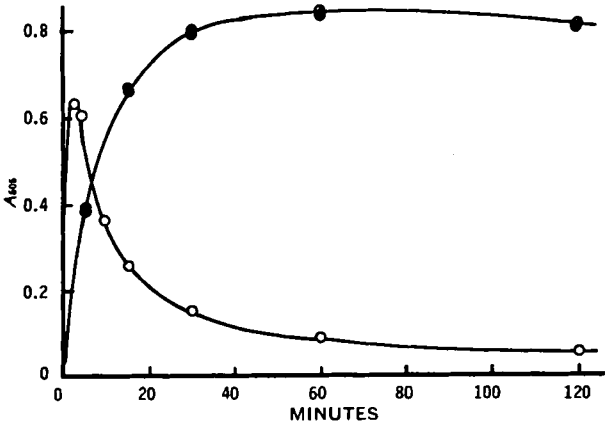


Figure 2—*Change of absorbance with time (4-aminoantipyrine finish) for hydroxylation of benzene at 25°. Initial conditions: 5.38 × 10⁻⁴ M benzene, 1.17 × 10⁻⁴ M catechol, 5.94 × 10⁻⁵ M Fe(III), 3.63 × 10⁻³ M H_2O_2, pH 3.7. Key: ○, no cyclodextrin; ◑, 1.13 × 10⁻³ M β-cyclodextrin; and ●, 1.26 × 10⁻³ M α-cyclodextrin.*

[19] For this substrate, an apparent molar absorptivity of 4.5 × 10^3 is obtained, corresponding to a 37% yield of phenol.

Table II—Aromatic Compounds Determined by the Cyclodextrin-Modified Hydroxylation Method[a]

Compound	Reaction Time, hr.	Analytical Wavelength, nm.[b]	10^{-3} ϵ_{app}[c]
Acetanilid	2	510	3.9
Phenacetin	1.3	490	2.7
Aspirin	2	490	1.7
Benzenesulfonamide	2	505	1.1
Sulfanilamide	2	435	1.5
Chloramphenicol	2	390	0.7
Mandelic acid	2	500	2.1
L-Phenylalanine	1	495	2.6
Tropic acid	2	500	1.4
Atropine	2	500	2.6
Ephedrine	1	505	2.7
Phenobarbital	2	515	1.1
Antipyrine	2	500	0.9
2-Naphthalenesulfonic acid	2	510	0.6
L-Tryptophan	2	485	0.5
Quinine	2	490	0.5
Physostigmine	2	495	0.2

[a] 1.17 × 10^{-4} *M* catechol, 5.94 × 10^{-5} *M* Fe(III), 3.4 × 10^{-3} *M* H_2O_2, 1.0–1.3 × 10^{-3} *M* β-cyclodextrin, pH 3.6–4.0, 25°. [b] Color developed by the 4-aminoantipyrine method except with chloramphenicol and sulfanilamide, which were measured through the nitrophenolate absorption. [c] Apparent molar absorptivity based upon total sample concentration, regardless of fate, in the final colorimetric solution. For more examples, see *Reference 1.*

Glucose-Modified Hydroxylating System—As a reasonable first step in determining the mechanism of stabilization by cyclodextrins, a time course for hydroxylation was carried out with the incorporation of α-D-glucose instead of cyclodextrin. Figure 3 shows the effect of glucose on the hydroxylation of acetanilid. Evidently, glucose has a stabilizing effect similar to that of cyclodextrin, although higher concentrations of glucose are required. Figure 4 is a Beer's law plot for the hydroxylation of acetanilid, showing that glucose provides a very satisfactory analytical result.

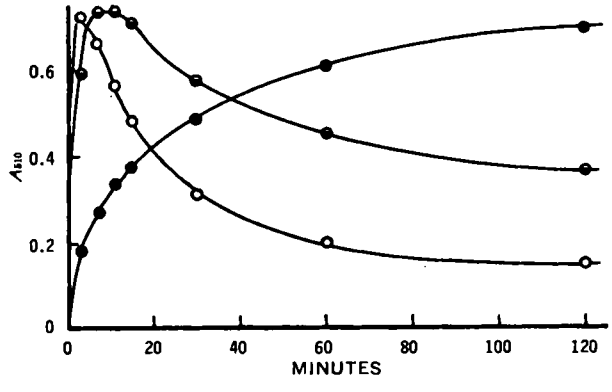


Figure 3—*Time course for hydroxylation of acetanilid (4-aminoantipyrine finish) at 25° in the presence and absence of glucose. Initial conditions: 4.70 × 10⁻⁴ M acetanilid, 1.17 × 10⁻⁴ M catechol, 5.98 × 10⁻⁵ M Fe(III), 4.13 × 10⁻³ M H_2O_2. Key: ○, no glucose; ◒, 1.26 × 10⁻³ M glucose; and ●, 1.26 × 10⁻² M glucose.*

[20] The mixture, contained in a 50-ml. volumetric flask, was shaken with 30 ml. of water for 15 min. After diluting to volume, a 2.0-ml. aliquot of the filtrate was taken for analysis.

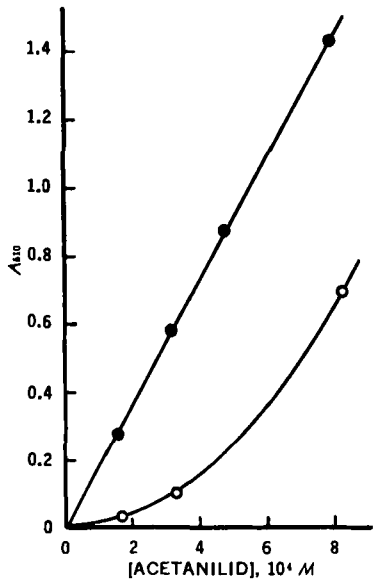


Figure 4—*Concentration–response curves for hydroxylation of acetanilid at 25° in the presence and absence of glucose. Conditions: 1.17 × 10⁻⁴ M catechol, 5.98 × 10⁻⁵ M Fe(III), 4.13 × 10⁻³ M H_2O_2; 2-hr. reaction time. Key: ○, no glucose; and ●, 7 × 10⁻³ M glucose (4-aminoantipyrine finish).*

The effect of temperature on time of hydroxylation is shown in Fig. 5. The hydroxylation reaction reaches a stable yield value after about 10 min. above 70°. At these higher temperatures, yields are slightly lower than at 25°. Moreover, the glucose concentration giving maximum yield, which was established by varying the glucose concentration and measuring the resultant absorbances, is less at higher temperatures and is mildly dependent upon the substrate[18]. At 75° the glucose concentration should be in the range of 2–5 × 10^{-3} *M* for maximal yield and for yields independent of substrate concentration. With such conditions, phenobarbital gave a linear working curve. It was found that yield is independent of catechol concentration in the range of 1–5 × 10^{-4} *M*.

Table III gives analytical results on aromatic compounds subjected to the glucose-modified hydroxylation method as described in the *Experimental* section. Linear working curves were observed for most compounds at concentrations up to 1 × 10^{-3} *M*. Chlorpheniramine showed linear behavior up to 6 × 10^{-4} *M*. Antipyrine gave a standard curve with negative curvature. Many of the Beer's law plots appear to intersect the abscissa close to the origin. This is probably a consequence of the very high peroxide–substrate ratio at low substrate concentration; it is not a serious analytical drawback.

A typical tablet formulation consisting of 65 mg. sodium phenobarbital, 26 mg. lactose, 20 mg. starch, 20 mg. talc, and 0.3 mg. stearic acid gave an average recovery of 98.4 ± 0.6% (four determinations) when compared with a standard phenobarbital solution[21]. When the lactose was increased to 200 mg. in the same formulation, a 10% decrease in response resulted. However, when the phenobarbital was extracted from the tablet formulation prior to hydroxylation, an average recovery of 98.8 ± 1.2% (four determinations) was achieved[22].

Other compounds were found to function effectively as stabilizers, although for analytical purposes they offer no advantages over glucose. The optimum stabilizer concentration depends upon the identity of the stabilizers. These approximate values were observed: lactose, 3 × 10^{-3} *M*; sorbitol, 4 × 10^{-3} *M*; acetonitrile, 0.15 *M*; isopropyl alcohol, 0.01 *M*; dioxane, 10^{-3} *M*; and acetone, 0.1 *M*.

Table III—Aromatic Compounds Determined by the Glucose-Modified Hydroxylation Procedure[a]

Compound	Analytical Wavelength, nm.[b]	$10^{-3}\ \epsilon_{app}$[c]
Benzene	505	2.9
Anisole	505	4.4
Acetanilid	510	3.4
Phenacetin	490	3.1
Benzoic acid	495	1.7
o-Methoxybenzoic acid	493	3.7
m-Methoxybenzoic acid	493	2.8
p-Methoxybenzoic acid	485	5.5
Mandelic acid	500	1.3
Atropine	500	2.1
Ephedrine	505	2.2
Phenobarbital	515	1.3
Chloramphenicol	390	0.8
Chlorpheniramine	500	2.7

[a] 1.17 × 10^{-4} *M* catechol, 5.94 × 10^{-5} *M* Fe(III), 3–4 × 10^{-3} *M* H_2O_2, 3.7 × 10^{-3} *M* glucose, pH 3.6–4.0, 10-min. reaction time, 75°. [b] Color developed by the 4-aminoantipyrine method except with chloramphenicol, which was measured through the nitrophenolate absorption. [c] Apparent molar absorptivity based upon total sample concentration, regardless of fate, in the final colorimetric solution.

Studies on Colorimetric Finish—The stability of the 4-aminoantipyrine-coupled dye has been the subject of several studies (7, 39, 40). Color instability has been associated with the presence of electron-withdrawing groups on the parent phenol (7). In the present work, a series of monosubstituted phenols was subjected to the 4-aminoantipyrine color development and the stability of the color was observed. It has been verified that electron-withdrawing groups enhance instability. Although the rate observations were semiquantitative, it can be stated that significant fading will be observed if the Hammett substituent constant σ is greater than about +0.3. An apparent exception is the amino group; thus, *o*-aminophenol gave an unstable color. It is possible that this group was oxidized to (for example) the *N*-nitroso function.

In the analytical hydroxylation studies with compounds carrying electron-withdrawing groups, the absorbance after 4-aminoantipyrine coupling was measured as a function of time and extrapolated to zero time. With nitro compounds (and with sulfanilamide, which may be oxidized to a nitro compound under hydroxylation conditions), the yellow color produced by the nitrophenolate absorption upon addition of the borate buffer provided a suitable finish.

Extraction of the 4-aminoantipyrine dye into chloroform has been proposed to stabilize and to concentrate the color (7, 41). This procedure was also successful when applied to the hydroxylation method, as described in the *Experimental* section.

DISCUSSION

Stabilization Effect—A simplified view of the Hamilton hydroxylating system (Scheme II) incorporates the idea of sequential hydroxylation as revealed by the present data. Only the monohydroxy compounds (and not even all of these) are expected to couple with 4-aminoantipyrine. Since Hamilton and coworkers (2–4) observed that anisole and nitrobenzene are hydroxylated at comparable rates, the reagent is fairly nonselective, and it may be anticipated that $k_1 \approx k_2$. The kinetic behavior is not as simple as Scheme II implies, however, because of the aging effect described earlier. The Hamilton reagent decomposes (by hydroxylation of catechol), and this phenomenon not only alters the catalytic effectiveness of the catechol but also consumes peroxide.

The hypothesis leading to the use of a cyclodextrin as a potential stabilizer invokes a stabilization resulting from inclusion compound formation, the hope being that a phenol will bind more firmly within the cyclodextrin cavity than will the parent aromatic; thus the ratio

$$\text{aromatic compound} \xrightarrow{k_1[H_2O_2]} \text{monohydroxy derivatives} \xrightarrow{k_2[H_2O_2]} \text{dihydroxy derivatives}$$

Scheme II

[21] The mixture, contained in a 50-ml. volumetric flask, was shaken with 30 ml. of water for 15 min., the pH was adjusted to 3.9 with 5% HCl, and the solution was diluted to volume with water. A 4.0-ml. aliquot was taken for analysis.

[22] The mixture, contained in a 60-ml. separator, was shaken with 10 ml. of water for 15 min., acidified with hydrochloric acid, and extracted with one 20-ml. and three 15-ml. portions of chloroform. The chloroform extracts were filtered through glass wool and evaporated to dryness. The residue was dissolved in acetate buffer and quantitatively transferred to a 100-ml. volumetric flask, and the solution was diluted to volume with buffer. An 8.0-ml. aliquot was taken for analysis.

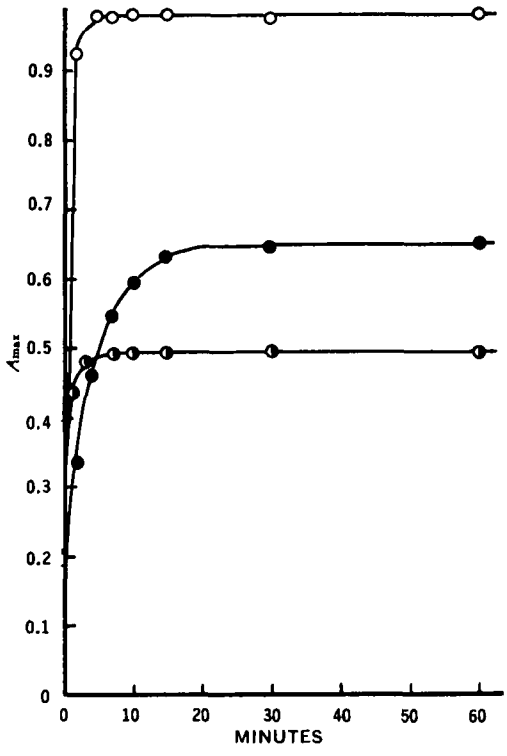


Figure 5—*Effect of temperature on time course for hydroxylation of phenobarbital and acetanilid with a glucose-modified Hamilton system. Conditions: 1.17 × 10⁻⁴ M catechol, 5.94 × 10⁻⁵ M Fe(III), 3.90 × 10⁻³ M H_2O_2, 7.24 × 10⁻³ M glucose. Key:* ○, *5.94 × 10⁻⁴ M acetanilid, 74°;* ◑, *8.64 × 10⁻⁴ M phenobarbital, 73°; and* ●, *8.64 × 10⁻⁴ M phenobarbital, 55° (4-aminoantipyrine finish).*

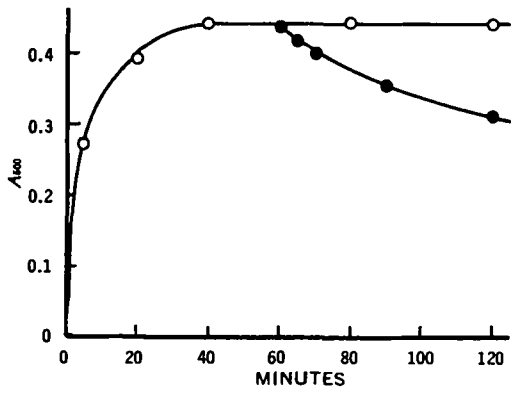


Figure 6—*Effect of addition of Hamilton reagent at the stable state (d[P]/dt = 0) on the time course for hydroxylation in a cyclodextrin-modified system. Conditions: 1.16 × 10⁻⁴ M catechol, 5.94 × 10⁻⁵ M Fe(III), 3.55 × 10⁻³ M H_2O_2, 6.37 × 10⁻⁴ M anisole, 1.08 × 10⁻³ M cyclodextrin, 25°, 4-aminoantipyrine finish. Key:* ○, *normal time course; and* ●, *Hamilton reagent (iron, catechol, and peroxide) added at 60 min.*

k_2/k_1 would be effectively decreased. Although inclusion complexes are certainly formed in many of these solutions, their role in stabilizing the monohydroxy product appears to be a minor one, as evidenced by the similar effects produced by glucose and cyclodextrin. The stabilization effect must, therefore, have a more general basis than the one originally hypothesized for cyclodextrin. The nature of the time course curves suggests this basis. Addition of a stabilizer substance like glucose or cyclodextrin causes behavior that can be duplicated (at least qualitatively) by reducing the peroxide concentration. The kinetic scheme should therefore be modified to provide an additional pathway for depletion of peroxide. In Scheme III, "ox" denotes the actual hydroxylating species, postulated by Hamilton to result from a 1:1:1 complex of hydrogen peroxide, iron, and catechol; S is the aromatic substrate; P is monohydroxylated product; I is the stabilizer or "inhibitor" substance; and P'' is the product of unspecified nature arising from the action of "ox" on I. This kinetic scheme achieves generality with the stipulation that I is any substance other than S or P that can consume peroxide. In the recommended analytical procedure, I is (mainly) glucose, but other solution components can function in the same way; thus, the acetate buffer, catechol, product P', and components of the sample other than S can all serve as I and account for the consumption of peroxide. Stabilization results from the depletion of peroxide.

An expression for this effect is easily obtained by applying the steady-state approximation to the intermediate "ox":

$$\frac{d[P]}{dt} = [ak_S - yk_P]\left[\frac{r_t k'[S]_0}{k_{-1}/[S]_0 + ak_S + yk_P + zk_I}\right] \quad \text{(Eq. 1)}$$

where $k' = k_1[\text{Fe(III)}][\text{catechol}]$, $r_0 = [H_2O_2]_0/[S]_0$, $r_t = [H_2O_2]/[S]_0$, $a = [S]/[S]_0$, $y = [P]/[S]_0$, $x = [I]_0/[S]_0$, and $z = [I]/[S]_0$. The condition of a stable state with respect to P means that $d[P]/dt = 0$; this can be met if $r_t = 0$ or if $ak_S = yk_P$. That the former condition holds is indicated by Fig. 6, which shows that the addition of fresh Hamilton reagent at the stable state results in loss of monohydroxylated product.

Equations 2 and 3 are the material balances on substrate and peroxide, where it is assumed that [ox] ≪ $[H_2O_2]$ and that P' is dihydroxylated (hence the stoichiometric factor 2):

$$[S]_0 = [S] + [P] + [P'] \quad \text{(Eq. 2)}$$

$$[H_2O_2]_0 = [H_2O_2] + [P] + 2[P'] + n([I]_0 - [I]) \quad \text{(Eq. 3)}$$

For convenience, n is taken to be unity and $[I]_0$ is adjusted accordingly; that is, the inhibitor concentration is specified on an equivalent rather than a molar basis. Then with the earlier definitions:

$$r_0 = r_t + 2(1 - a) + x - y - z \quad \text{(Eq. 4)}$$

Applying the stable-state condition $r_t = 0$ to Eq. 4 gives Eq. 5 for the yield y of monohydroxylated product:

$$y = 2(1 - a) + x - r_0 - z \quad \text{(Eq. 5)}$$

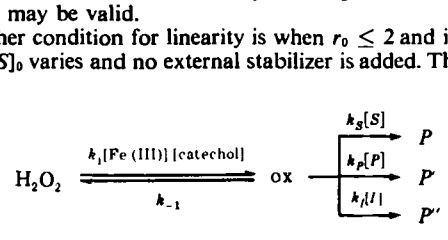
If ϵ_{av} is the weighted average molar absorptivity of the 4-aminoantipyrine-coupled monohydroxy products, then $A = \epsilon_{av}b[P] = \epsilon_{av}by[S]_0$, or:

$$A = \epsilon_{av}b[2(1 - a) + x - r_0 - z][S]_0 \quad \text{(Eq. 6)}$$

Lacking quantitative data on all system components, it is not possible to specify the behavior of a and z; the quantities x and r_0, however, are initial conditions, and it appears that the magnitude and sign of $(x - r_0)$ may control the shape of the concentration–response plot of A against $[S]_0$. When $x \approx r_0$, $d(x - r_0)/d[S]_0 = 0$ and the yield is independent of substrate concentration (with respect to the influences of x and r_0); a linear Beer's law plot is expected. When $x < r_0$, $d(x - r_0)/d[S]_0$ is positive, leading to a positive curvature in the Beer's law plot. When $x > r_0$, negative curvature is predicted.

Some of these predicted phenomena appear to match the results described earlier. There are two conditions under which a linear Beer's law plot is observed. One of these, the typical analytical situation, is when both r_0 and x are large and variable. The suggestion above is that $x \approx r_0$ is the essential condition for a linear response. If the number of oxidizable carbon atoms in the added stabilizer molecule is taken to define the number of equivalents per mole of I, then the optimal range of x values observed is about 20–400 for cyclodextrin, glucose, sorbitol, and lactose, whereas r_0 was 4–40 in these same studies. This overlap of ranges indicates that the approach may be valid.

The other condition for linearity is when $r_0 \leq 2$ and is held constant as $[S]_0$ varies and no external stabilizer is added. Then the role



Scheme III

of I is played by normal system components, including P', so x may remain small and essentially constant. In comparison, when $r_0 \leq 2$ and $[H_2O_2]_0$ is held constant as $[S]_0$ varies, x tends to become larger than r_0 as $[S]_0$ is increased (in the absence of added stabilizer) because of the contribution of P'; negative curvature is seen in the Beer's law plot. When r_0 is large and no stabilizer is added ($r_0 > x$), positive curvature is seen (Fig. 4, open circles).

Analytical Method—The proposed analytical method is applicable to the determination of many aromatic compounds in the range of 10^{-4}–10^{-3} M in aqueous solution. For it to be applicable, the aromatic sample compound must be hydroxylated and the resulting phenol must couple with 4-aminoantipyrine. These are not highly restrictive limitations, although the latter may be important for some samples. For example, pyridine gives no color when subjected to the procedure. Separate experiments showed that *o*- and *p*-hydroxypyridines do not yield a color upon treatment with 4-aminoantipyrine, whereas *m*-hydroxypyridine does give a color. Apparently, pyridine does not produce *m*-hydroxypyridine in significant yield upon treatment with the hydroxylating agent, although the other isomers may be produced. It may usually be expected that a *para*-substituted phenol will not couple with 4-aminoantipyrine unless the substituent is eliminated in the coupling reaction. The sensitivity of the method, which is quantitatively expressed in the apparent molar absorptivities, is therefore a function of the total yield of monohydroxy products, of the positional isomer distribution, and of the molar absorptivities of the 4-aminoantipyrine-coupled dyes of the various isomers.

The analytical data presented here and earlier (1) permit a comparison between the responses of a series of sample compounds subjected to the cyclodextrin-modified reagent at 25° and the glucose-modified reagent at 75°. Although some individual differences appear to be significant (possibly because of the inclusion complexing capability of cyclodextrin), the average molar absorptivity for 13 compounds subjected to the cyclodextrin system was $(2.8 \pm 1.1) \times 10^3$, and it was $(2.7 \pm 1.3) \times 10^3$ for the same compounds with the glucose system. On the average, then, the two analytical systems give the same response, so the glucose-modified reagent, with a 10-min. reaction time at 75°, is preferred for practical reasons.

The most serious limitation to this method of analysis is its susceptibility to interference. The manifestation of this interference is a decreased response (lower absorbance values than anticipated). Its basis is a competition for the available peroxide. In effect, aliphatic components of the sample act as added stabilizers (I in Scheme III) and, since the analytical reagent already contains an optimum stabilizer concentration, they depress the yield of hydroxylated aromatic. This is probably why equimolar concentrations of several organic solvents gave varying degrees of interference, with dioxane being the worst participant (1). If the concentration (that is, its normality with respect to peroxide concentration) is low enough, it will not seriously interfere, but higher concentrations cause a decreased response. This effect was described earlier for a tablet formulation, where it was shown that a simple prior separation eliminated the interference.

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Trichloroacetic Acid-Induced Binding of Phenylthiourea to Erythrocytes

ALAN B. COMBS▲ and SHRI N. GIRI

Abstract □ Trichloroacetic acid precipitation causes the binding of phenylthiourea to the erythrocytes of a number of vertebrates. In rats, mice, and dogs, Scatchard plot presentations of the binding data indicate that there may be two sets of receptors involved in the binding. The total binding capabilities of the sets of receptors are of the same order of magnitude, but one set is of much greater affinity than the other. The induced binding of phenylthiourea to erythrocytes is probably related to acidity because a neutral precipitating agent did not cause binding.

Keyphrases □ Phenylthiourea binding to erythrocytes—induced by trichloroacetic acid □ Erythrocytes—trichloroacetic acid-induced binding of phenylthiourea □ Trichloroacetic acid—induced binding of phenylthiourea to erythrocytes □ Binding of phenylthiourea to erythrocytes—effect of trichloroacetic acid

The edema-producing thioureas, phenylthiourea and α-naphthylthiourea, exhibit a number of pharmacologically interesting properties. Rats are among the animals most susceptible to the acute toxic effects (1), which include pulmonary edema, pleural effusion (2, 3), and disturbances in carbohydrate metabolism (4–6).

In this laboratory, thiourea and phenylthiourea, after intraperitoneal injection in rats, have been shown to have apparent volumes of plasma distribution greater than the body size, an indication either of storage of the drug or of binding to some physiological component (7). Williams and Kay (8) showed that thiourea appears to be taken up by rat erythrocytes (red blood cells). Giri *et al.* (9, 10) reported that thiourea is not released from rat red blood cells by trichloroacetic acid precipitation, a method frequently used to facilitate drug recovery from biological samples (7, 11). Heating of the rat red blood cell samples, however, did cause the release of thiourea. The trichloroacetic acid-associated binding of thiourea to rat red blood cells was of sufficient magnitude to be a possible explanation for the large apparent volumes of distribution.

The present paper reports the extension of the binding study to phenylthiourea. Because of the large apparent volumes of distribution shown for phenylthiourea, it was of interest to determine if a binding phenomenon similar to that reported for thiourea occurred with phenylthiourea. In addition, since rats are uniquely susceptible to the toxic effects of the thioureas, the possibility that this susceptibility might be related to species differences in the binding of phenylthiourea to red blood cells was explored by measuring the binding in a number of species. The quantitative nature of the binding was determined using binding isotherms and Scatchard plots of the data (11). Finally, preliminary equilibrium dialysis studies with phenylthiourea and rat red blood cells gave evidence of only slight binding in comparison to the binding observed after trichloroacetic acid precipitation of the red blood cells. This indication that the binding might be trichloroacetic acid induced was investigated.

EXPERIMENTAL

Chemicals—The chemicals included Folin-Ciocalteu phenol reagent[1], a carbonate–tartrate solution consisting of 10% sodium carbonate and 0.1% potassium sodium tartrate (0.4 H_2O), practical grade phenylthiourea[2] recrystallized twice from hot 95% ethanol, freshly prepared heparin[3] containing no preservatives, 50% trichloroacetic acid, 1.32 and 5.3 *N* sodium hydroxide, freshly prepared 0.3 *N* barium hydroxide, and 0.3 *N* zinc sulfate. All aqueous reagents were prepared in deionized water.

Sources of Blood—Blood from male Sprague–Dawley rats, weighing over 150 g., was collected by decapitation and pooled. Freshly prepared heparin was used in all cases to prevent clotting. Ten-milliliter samples of blood from each of five mongrel dogs were obtained by venipuncture and pooled. Blood from approximately 150 male Swiss–Webster mice was collected by cardiac puncture after ether anesthesia.

Characterization of Binding—Samples of pooled blood were centrifuged at 4000 r.p.m. for 10 min. and the plasma was removed by aspiration and discarded. The erythrocytes (red blood cells) were rinsed with saline and centrifuged, and the rinse solution was discarded. Then a known volume of saline was added to the packed red blood cells and the suspension was decanted into a graduated cylinder. The volume of packed red blood cells was determined and enough additional saline was added to the cylinder so that 4 ml. of the final dilution contained 1 ml. of packed red blood cells.

Triplicate samples of the red blood cell suspension were incubated with varying amounts of phenylthiourea dissolved in saline. Table I shows the typical incubation scheme used; the items are listed in the order of addition to 25-ml. screw-capped tubes. After the final addition and mixing, the samples were incubated over ice with shaking for 30 min. An additional run at 37° was performed with the mouse red blood cells. After incubation, 2.5 ml. of cold 50% trichloroacetic acid was added to each sample. The samples were then mixed and stored over ice for 30 min. before removing the supernates.

Analysis of Phenylthiourea—A colorimetric method for phenylthiourea, based upon its reaction with the Folin-Ciocalteu phenol reagent, was used for analysis (12). The trichloroacetic acid supernates were neutralized with one-tenth of their volume of 5.3 *N* sodium hydroxide. Each supernate and its appropriate blank were diluted 5–50-fold with deionized water, depending upon the amount of phenylthiourea originally present. A 3.0-ml. aliquot of each diluted supernate was placed in a 25 × 150-mm. test tube. To this tube were added 1.0 ml. of 1.32 *N* sodium hydroxide and 1.0 ml. of the carbonate–tartrate solution. Then 1.0 ml. of the phenol reagent (diluted 1:1 with deionized water) was added rapidly while the contents of the test tube were mixed on an electric mixer. Absorbance was measured at 750 nm.[4] after 1.5 hr.

Calculations—The phenylthiourea recoveries from each triplicate sample were averaged. The amounts not recovered were assumed to be bound. The limited solubility of phenylthiourea prevented addition of concentrations greater than 2 mg./ml. to the incubation

[1] Fisher Scientific Co., Fair Lawn, N. J.
[2] J. T. Baker Chemical Co.
[3] Sigma.
[4] Gilford photometer with Beckman monochromator.

Table I—Typical Incubation System for Analysis of Trichloroacetic Acid Associated Binding of Phenylthiourea[a]

Saline, ml.	Phenylthiourea Solution[b], ml.	Milliliters of Red Blood Cell Dilution	Milliliters of Packed Red Blood Cells
6.0	0.0[c]	4.0	1.0
5.0	1.0	4.0	1.0
3.0	3.0	4.0	1.0
0.0	6.0	4.0	1.0
2.0	6.0	2.0	0.5
3.0	6.0	1.0	0.25
3.5	6.0	0.5	0.125
4.0	6.0[d]	0.0	0.0
10.0	0.0[e]	0.0	0.0

[a] The first three columns show the order of addition to 25-ml. screw-capped tubes, making 10.0-ml. final volume. Each sample was in triplicate. [b] The phenylthiourea was dissolved in isotonic saline; the concentrations used were either 1.90 or 1.00 mg./ml. [c] Blank used to estimate color reactions from sources other than phenylthiourea. [d,e] Internal standard and its blank, respectively.

systems. *Relative* increases in phenylthiourea were achieved by decreasing the amount of red blood cells incubated (Table I). The amounts of phenylthiourea incubated with the decreased volumes of packed red blood cells were converted to the value:

$$\frac{\text{micromoles of phenylthiourea incubated per milliliter of solution}}{\text{milliliter of packed red blood cells}}$$

Then the binding isotherm functions: micromoles of phenylthiourea bound per milliliter of packed red blood cells *versus:*

$$\frac{\text{micromoles of unbound phenylthiourea per milliliter of solution}}{\text{milliliter of unpacked red blood cells}}$$

were calculated.

Equilibrium Dialysis—One-centimeter diameter dialysis tubing was cut into 20-cm. strips, and the strips were boiled for 0.5 hr. in deionized water. They were rinsed, flushed with deionized water, and stored under refrigeration until needed. Duplicate 10-ml. total volume dialysis systems, containing the equivalent of 1 ml. of packed rat red blood cells inside the tubing and varying amounts of phenylthiourea in saline outside the bag, were incubated with shaking for 18 hr. at 3–5° in a cold room. After incubation, the solutions outside the dialysis bags were analyzed. The amounts of phenylthiourea not recovered were assumed to be bound.

Red Blood Cell Sedimentation—Rat red blood cells were used in an incubation similar to that shown in Table I. After incubation, the red blood cell suspensions were centrifuged for 10 min. at 2000 r.p.m. in a refrigerated centrifuge at 0°. A 1-ml. aliquot of supernate from each sample was analyzed for phenylthiourea. The cells were then resuspended and precipitated with trichloroacetic acid. The trichloroacetic acid supernates were neutralized and analyzed for phenylthiourea.

Nonacidic Precipitating Agent—An incubation similar to that shown in Table I was performed with dog red blood cells. Triplicate samples of cells were precipitated by a neutral Somogyi (13) method. Then 1.0 ml. of each cell suspension was added to 10.0-ml. samples of freshly prepared 0.3 *N* barium hydroxide and mixed; 10.0 ml. of 0.3 *N* zinc sulfate was added to each with mixing. The resulting precipitate was centrifuged and the supernate was analyzed for phenylthiourea. The remainders of each original red blood cell suspension were precipitated with trichloroacetic acid, and the supernates were analyzed.

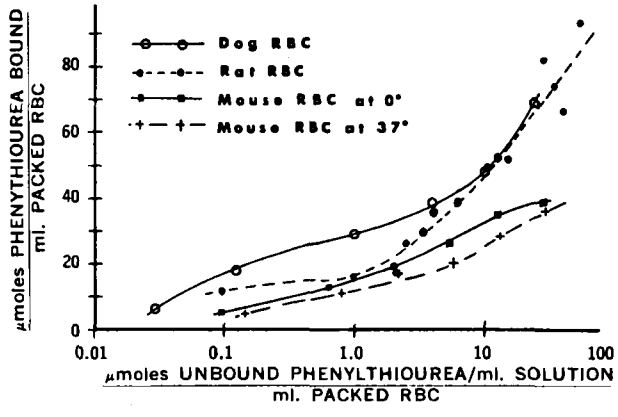

Figure 1—*Isotherm presentation of the binding data. Pooled red blood cells (RBC) were incubated for 30 min. and then precipitated with trichloroacetic acid as described in the text. Each point represents the average of triplicate samples.*

Table II—Dialysis Data[a]

Theoretical Concentration of Phenylthiourea, μmoles/ml.[b]	Measured Concentration of Phenylthiourea, μmoles/ml.	Phenylthiourea Bound by Red Blood Cells, %	Phenylthiourea Bound if Precipitated by Trichloroacetic Acid[c], Approximate %
3.93	3.03	23.0	60
1.97	1.55	21.5	85
1.31	1.20	8.0	90
0.66	0.57	13.0	100

[a] Duplicate 10.0-ml. total volume dialysis systems containing the equivalent of 1 ml. of packed rat red blood cells inside the dialysis bag and varying amounts of phenylthiourea outside were incubated with shaking for 18 hr. at 3–5° in a cold room. [b] The theoretical equilibrium concentration of the added phenylthiourea if no binding occurred. [c] These quantities were extrapolated from the rat red blood cell binding isotherm (Fig. 1).

RESULTS

Characterization of Binding—Phenylthiourea was bound to the red blood cells of rats, dogs, and mice after trichloroacetic acid precipitation. The results in binding isotherm form are presented in Fig. 1. Internal standard recoveries varied from 94 to 101%. A small amount of human and White Leghorn rooster blood was available. These samples also bound phenylthiourea after trichloroacetic acid precipitation.

Equilibrium Dialysis—The results of the dialysis experiment are shown in Table II. The recoveries were erratic but were always much greater than the amount that would be expected after trichloroacetic acid precipitation was extrapolated from the rat red blood cell binding isotherm (Fig. 1).

Red Blood Cell Sedimentation—The results shown in Table III indicate that little phenylthiourea was bound by centrifuged, unprecipitated red blood cells and that much more was bound after trichloroacetic acid precipitation.

Nonacidic Precipitation—The results presented in Table IV show that much more phenylthiourea was bound after trichloroacetic acid precipitation than after the neutral Somogyi precipitation.

Table III—Red Blood Cell Sedimentation Data[a]

Theoretical Concentration of Phenylthiourea, μmoles/ml.[b]	Measured Concentration of Phenylthiourea in Supernate before Trichloroacetic Acid, μmoles/ml.	Measured Concentration of Phenylthiourea in Supernate after Trichloroacetic Acid, μmoles/ml.	Percent Bound before Trichloroacetic Acid	Percent Bound after Trichloroacetic Acid
1.25	1.19	0.000	4.8	100.0
3.74	3.52	0.475	5.8	87.3
7.48	7.33	3.17	2.0	57.6

[a] Triplicate 10.0-ml. total volume incubation systems containing 1 ml. of packed rat red blood cells, saline, and varying amounts of phenylthiourea were incubated for 30 min. over ice. After incubation the suspensions were centrifuged and 1.0 ml. of each supernate was analyzed. Then the red blood cells were resuspended and precipitated with trichloroacetic acid, and the supernate was again analyzed. [b] The theoretical equilibrium concentration of the added phenylthiourea if no binding occurred.

Table IV—Nonacidic Precipitation Data[a]

Theoretical Concentration of Phenylthiourea, μmoles/ml.[b]	Measured Concentration of Phenylthiourea in Supernate after Neutral Somogyi Precipitation, μmoles/ml.	Measured Concentration of Phenylthiourea in Supernate after Trichloroacetic Acid, μmoles/ml.	Percent Bound after Neutral Somogyi Precipitation	Percent Bound after Trichloroacetic Acid
3.95	3.54	0.93	10.4	76.4
7.90	7.46	4.10	5.6	48.1
15.80	14.91	11.22	5.6	29.0

[a] Triplicate 10.0-ml. total volume incubation systems containing 1 ml. of packed dog red blood cells, saline, and varying amounts of phenylthiourea were incubated for 30 min. over ice. After incubation, a 1.0-ml. aliquot from each cell suspension was precipitated with 10.0 ml. of 0.3 *N* barium hydroxide followed by 10.0 ml. of 0.3 *N* zinc sulfate. The resulting supernate was analyzed for phenylthiourea. The remainder of each original cell suspension was precipitated with trichloroacetic acid, and the supernates were analyzed for phenylthiourea. [b] The theoretical equilibrium concentration of the added phenylthiourea if no binding occurred.

Table V—Binding Constants and Receptor Capacities[a]

Source of Red Blood Cells	Binding Constants: High Affinity Receptors	Binding Constants: Low Affinity Receptors	Receptor Capacities, μmoles/ml. of Packed Red Blood Cells: High Affinity Receptors	Receptor Capacities, μmoles/ml. of Packed Red Blood Cells: Low Affinity Receptors	Receptor Capacities, μmoles/ml. of Packed Red Blood Cells: Total Capacity
Rat	37.6	0.069	11	87	98
Dog	9.60	0.044	29	79	108
Mouse (red blood cells incubated at 0°)	6.82	0.126	13	34	47
Mouse (red blood cells incubated at 37°)	6.30	0.105	10	38	48

[a] Data were derived from Scatchard plots of the binding data (Fig. 1). Two sets of receptors were assumed, and the binding constants and capabilities were determined by trial-and-error substitution to give the best visual fit to the data.

DISCUSSION

Characterization of Binding—The binding data do not fall into the simple sigmoid curves that would be expected from such log-linear plots (Fig. 1) if a single set of similar receptors were involved (14). For this reason, Scatchard plots (11) were constructed of the data in terms of:

$$\frac{\text{micromoles of phenylthiourea bound per milliliter of packed red blood cells}}{\left[\dfrac{\text{micromoles of bound phenylthiourea per milliliter of solution}}{\text{milliliter of packed red blood cells}}\right]}$$

versus micromoles of phenylthiourea bound per milliliter of packed red blood cells. The regression curves giving the best visual fit to the data, assuming two classes of receptors, were determined by trial-and-error substitution. The resulting binding constants and receptor capacities are shown in Table V. The binding of phenylthiourea in all of the animals tested suggests the ubiquity of the phenomenon, but the data are not sufficient to allow any conclusions about the differential species toxicity shown by phenylthiourea.

The comparison of mouse red blood cells incubated at two different temperatures is shown in Fig. 1 and Table V. The total binding capacities are not appreciably changed by the increase in temperature from 0 to 37°, but the binding constant for each set of receptors is decreased. These findings are consistent with the observation of Giri *et al.* (9) and Giri and Combs (10) that as incubation temperature increased, the binding of thiourea to rat red blood cells decreased. The explanation might be that temperature-dependent structural changes in the receptors decrease their affinity for phenylthiourea.

Mechanism of Binding—Preliminary equilibrium dialysis studies gave rise to the suspicion that the trichloroacetic acid-associated binding might, in fact, be induced by trichloroacetic acid. This was confirmed by the dialysis results (Table II) and red blood cell sedimentation results (Table III). Only a small amount of phenylthiourea was bound after dialysis with intact red blood cells. The same amount of red blood cells would have bound much more phenylthiourea if they had been precipitated with trichloroacetic acid. As shown in Table III, the recovery of phenylthiourea from the supernates of centrifuged red blood cell suspensions was nearly quantitative, while recovery after trichloroacetic acid precipitation of the identical suspensions was much less. All of the evidence indicates that the binding is induced by trichloroacetic acid precipitation rather than being resistant to trichloroacetic acid precipitation.

Acidic protein precipitating agents other than trichloroacetic acid (perchloric acid and tungstic acid) have been shown in this laboratory to be associated with the binding of thiourea to rat red blood cells. After it was established that the binding of phenylthiourea to red blood cells was induced by trichloroacetic acid, it was decided to test the effect of nonacidic precipitation upon the binding. The data in Table IV indicate that much less phenylthiourea is bound after neutral Somogyi precipitation than after trichloroacetic acid precipitation. It seems likely, therefore, that induction of binding is associated with low pH. Because dialysis, red blood cell sedimentation, and neutral Somogyi precipitation each gave some evidence of binding of a lower magnitude than the induced binding, it may be that a low pH causes activation of the receptors responsible for the slight binding shown in these studies. Characterization of the noninduced binding and its possible relationship to the large apparent volumes of distribution found with phenylthiourea in rats is currently under study.

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Kinetics of Synthesis of d_4-Succinic Anhydride from Succinic Anhydride and d_1-Acetic Acid

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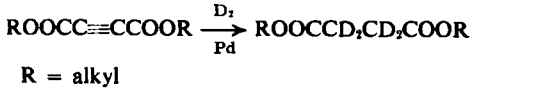
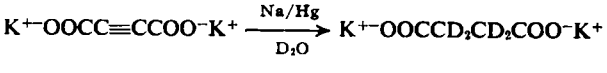
Abstract □ The synthesis and the kinetics of the synthesis of d_4-succinic anhydride from succinic anhydride and d_1-acetic acid, catalyzed by sodium acetate, are presented. The results show that the conversion is both base and solvent catalyzed, suggesting that the deuteration may occur through the enolization of succinic anhydride. The synthesis is faster and simpler than previous methods and makes use of relatively accessible and inexpensive materials.

Keyphrases □ d_4-Succinic anhydride—synthesis and kinetics from succinic anhydride and d_1-acetic acid □ Deuteration of succinic anhydride—synthesis of d_4-succinic anhydride □ d_1-Acetic acid—deuteration of succinic anhydride, kinetics

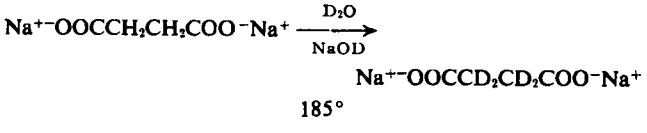
The use of isotopically labeled pharmaceutical compounds as analytical tools and the selective deuteration at various points in drug molecules to investigate drug activity kinetic isotope and stability effects have been achieving wider coverage in recent years. The need to find a fast inexpensive synthesis of d_4-succinic anhydride and deuterated succinic acid is exemplified by deuterated succinic acid's use as an important carbon source for the growth of algae, fungi, and bacteria in deuterium oxide (1–3). Growth of these organisms in deuterium oxide is severely hindered if carbon source substrates are not present. The use of these culture techniques employing deuterated and nondeuterated substrates has led to the biosynthesis of fully deuterated penicillin (4, 5), deuterated griseofulvin (6–8), deuterated clavine alkaloids (9, 10), and, more recently, L-deuterophenylalanine (11). On the basis of these needs and in the course of preparation of a number of d_4-succinyl derivatives of drug molecules for a later study, it was indeed advisable to find a high yielding, fast synthesis of d_4-succinic anhydride[1]. Previous methods of preparation of d_4-succinic acid and subsequently d_4-succinic anhydride involved the catalytic reduction of acetylenedicarboxylic acid derivatives (12–19) (Schemes I and II), the reaction of disodium succinate with alkali deuterium oxide under vigorous conditions (20–23) (Scheme III), or an enzymatic synthesis (24). All of these procedures have problems either in the preparation of starting materials (Schemes I and II and the enzymatic synthesis) or in the very vigorous conditions required for complete reaction (Scheme III). Attempts at Scheme III in these laboratories under reflux conditions proved extremely slow and costly, both in time and glassware.



Scheme I



Scheme II



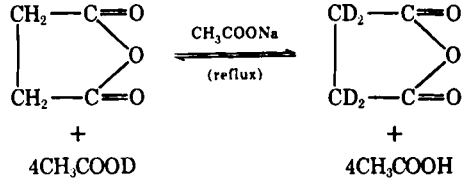
Scheme III

Stella and Higuchi[2] attempted the preparation of the *N*-succinyl derivative of phthalimide by the reaction of potassium phthalimide with succinic anhydride in nonaqueous aprotic solvents. The reaction products suggested that, under such conditions, succinic anhydride may undergo an enolization, although this was not fully investigated at the time. The partial acidic nature of the α-hydrogens of carboxylic acids (20–23, 25–30), carboxylic acid amides (23), carboxylic acid esters (31, 32) in the Stobbe reaction, and carboxylic acid anhydrides (32) in the Perkin condensation reaction has been well established. This acidic nature of the α-protons and the possible enolization of succinic anhydride prompted the present line of investigation.

In this study the synthesis and kinetics of synthesis of d_4-succinic anhydride from succinic anhydride and d_1-acetic acid in the presence of sodium acetate were evaluated (Scheme IV). This system fulfilled the requirements of a nonaqueous solution, a base, and a deuterium source. Haddadin (34) showed that succinic anhydride, in the presence of acetic acid at room temperature, results in the retention of the anhydride character by the succinate, with only one part in a hundred of the succinic anhydride being converted to succinic acid and acetic anhydride.

EXPERIMENTAL

Materials—Succinic anhydride was prepared by refluxing succinic acid in acetic anhydride, allowing the succinic anhydride to crystallize, and subsequently washing and recrystallizing from chloroform. d_1-Acetic acid was prepared by stirring equimolar quantities of 99.6% deuterium oxide and acetic anhydride at 60°



Scheme IV

[1] d_1-Succinic acid or d_0-succinic acid can be prepared from d_1-succinic anhydride by hydrolysis from water or deuterium oxide, respectively.

[2] V. J. Stella and T. Higuchi, University of Kansas, Lawrence, KS 66044, unpublished work.

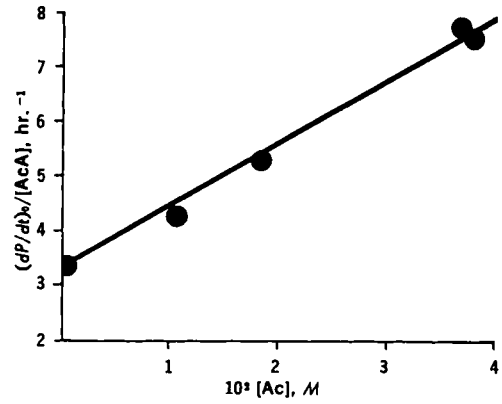


Figure 1—*Plot against acetate concentration of the initial rate of formation of acetic acid carboxyl protons divided by acetic anhydride concentration for the reaction of 0.516* M *acetic anhydride in* d_1-*acetic acid in the presence of varying amounts of acetate ions at 99.8 ± 0.2°.*

until the reaction was complete. Reagent grade anhydrous sodium acetate, dried in a hot air oven for 24 hr., and reagent grade benzene were also used.

Kinetics—Reaction solutions were prepared by adding an appropriately weighed amount of each ingredient of the reaction mixture to a 10-ml. volumetric flask and making up to volume with d_1-acetic acid. The solutions were transferred to 2-ml. hard-glass ampuls, flushed with nitrogen, sealed, and placed in a mineral oil bath at 99.8 ± 0.2°. Ampuls were removed at appropriate times, the reaction was quenched by cooling in ice water, and their contents were analyzed by NMR[3].

The reactions involving the rearrangement of CH_3COOD to CDH_2COOH were followed by integrating the increase of acetic acid carboxyl proton signal relative to a reference material of known concentration. The reference signal in all reactions was benzene, in a concentration of 200 mg./10 ml. The reactions involving the deuteration of succinic anhydride were followed by comparing the loss of α-proton signal of succinic anhydride relative to the internal reference signal. The results and subsequent rate constants will be expressed as succinic anhydride giving d_4-succinic anhydride, even though the overall reaction scheme would be succinic anhydride giving d_1-succinic anhydride which, in turn, gives d_2-succinic anhydride, *etc.*

Synthesis of d_4-Succinic Anhydride—Eight grams (0.0800 mole) of succinic anhydride and 350 mg. (0.0043 mole) of sodium acetate were refluxed in 40 ml. of d_1-acetic acid. After 2 hr. the solution was flash evaporated, replaced with another 40 ml. of fresh d_1-acetic acid, and again refluxed. This procedure was repeated for a total of six times. After the last reflux and on cooling, 3 g. of d_4-succinic anhydride, m.p. 118–120°, crystallized out of solution. NMR suggested the product to be better than 97% deuterated. The filtrate was evaporated down to 10 ml., and 5 ml. of acetic anhydride was added. The suspension was heated to effect solution and allowed to cool, yielding another 3 g. of product, m.p. 119–120°. The solution was further concentrated to give 1 g. of product, m.p. 110–116°. The deuterated product, on recrystallization from anhydrous chloroform, gave m.p. 119–121°. The percentage of deuteration was assessed by dissolving 104 mg. of d_4-succinic anhydride in a 2% sodium carbonate–deuterium oxide solution by warming. The procedure was repeated for 100 mg. of succinic anhydride in the same volume of solution. The NMR spectra of the two solutions were then compared, and the proton peak from HDO was used as the internal standard to estimate the percentage of deuteration. The results showed the d_4-succinic anhydride to be better than 97% deuterated.

RESULTS AND DISCUSSION

In an initial attempt to synthesize d_4-succinic anhydride *via* Scheme IV, the amount of acetic acid carboxyl proton formed did not correspond stoichiometrically with the amount of protons lost from the isolated, partially deuterated succinic anhydride. It was postulated that the excess protons had come from acetic anhydride by incorporation of deuterium into the methyl groups of acetic anhydride and, subsequently, acetic acid; *i.e.*, acetic anhydride present in the solution was competing with the succinic anhydride for the deuterium. The acetic anhydride resulted from: (*a*) the reaction of succinic anhydride with acetic acid to form succinic acid and acetic anhydride (34), (*b*) excess acetic anhydride from the production of d_1-acetic acid, or (*c*) the fact that, under reflux conditions, acetic anhydride (and the corresponding amount of water) is formed in low concentration from acetic acid (35–37).

To check if this side reaction could be controlled, a kinetic investigation was conducted to observe the incorporation of deuterium into the methyl groups of acetic anhydride and, subsequently, acetic acid from d_1-acetic acid. The rate of production of carboxyl proton, dP/dt, at 99.8° was followed as a function of sodium acetate concentration, [Ac], and acetic anhydride concentration, [AcA]. The curves obtained from the reaction, complete to equilibrium, followed neither strict first- nor second-order kinetics. The results, therefore, are presented in terms of initial rates. The results suggest that the rate of production of carboxyl protons follows Eq. 1:

$$\left(\frac{dP}{dt}\right)_0 = k_1[\text{AcA}] + k_2[\text{AcA}][\text{Ac}] \qquad \text{(Eq. 1)}$$

where $(dP/dt)_0$ is the initial rate of production of carboxyl protons in molar concentration per hour, k_1 is the solvent-catalyzed rate constant, and k_2 is the acetate-catalyzed rate constant.

The rate constants k_1 and k_2 are pseudo-first-order constants in that they assume the d_1-acetic acid concentration to remain relatively constant in the initial time period. Equation 1 predicts that a plot of $(dP/dt)_0/[\text{AcA}]$ *versus* [Ac] would give a straight line of slope k_2 and intercept k_1 (Fig. 1). This gave values for k_2 of 1.15 M^{-1} hr.$^{-1}$ and k_1 of 3.35×10^{-2} hr.$^{-1}$. Eq. 1 also predicts that a plot of $(dP/dt)_0$ *versus* [AcA] for a given acetate concentration would give a straight line of slope equal to $k_1 + k_2$[Ac] and intercept of zero (Fig. 2). The least-squares slope of this line is 7.88×10^{-2} hr.$^{-1}$. The slope of the line calculated using the data obtained from Fig. 1 and the corresponding acetate concentration would have predicted a slope of 7.60×10^{-2} hr.$^{-1}$. It does appear, therefore, that under the conditions of the experiment and the limited accuracy of the NMR

Table I—Comparison of k_1 and k_2 for Succinic Anhydride *versus* Those for Acetic Anhydride

Anhydride	k_1, hr.$^{-1}$	k_2, M^{-1} hr.$^{-1}$
Acetic anhydride	3.35×10^{-2}	1.15
Acetic anhydride[a]	2.23×10^{-2}	7.70×10^{-1}
Succinic anhydride	9.75×10^{-2}	2.37

[a] Corrected for the fact that there are six exchangeable protons in acetic anhydride relative to four in succinic anhydride.

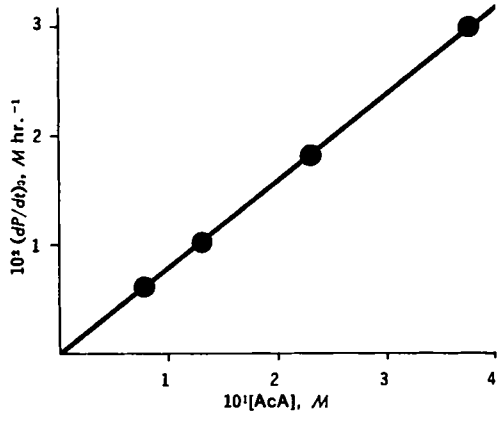


Figure 2—*Plot against acetic anhydride concentration of the initial rate of formation of acetic acid carboxyl protons for the reaction of acetic anhydride in* d_1-*acetic acid at 99.8 ± 0.2° in the presence of* 3.69×10^{-2} M *acetate ions.*

[3] Varian A60.

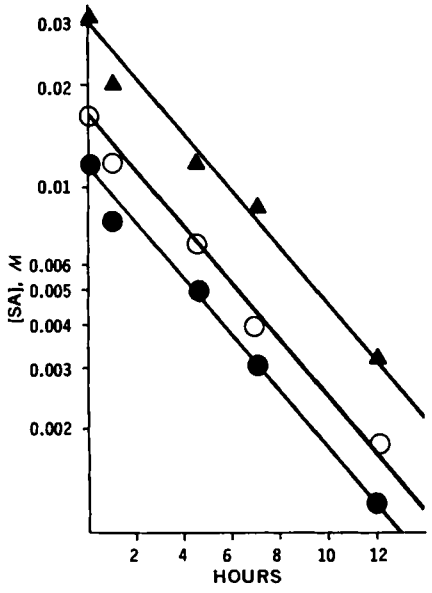


Figure 3—*Plot against time of logarithms of residual concentration of succinic anhydride during its deuteration at 99.8 ± 0.2° in* d_1*-acetic acid containing 3.64 × 10⁻² M sodium acetate as a catalyst. Initial succinic anhydride concentrations were 3.16 × 10⁻² M (▲), 1.60 × 10⁻² M (○), and 1.12 × 10⁻² M (●).*

technique, Eq. 1 does adequately describe the production of the carboxyl protons.

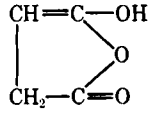
The formation of d_4-succinic anhydride was followed by observing directly the loss of succinic anhydride protons. Because of the conditions used, *i.e.*, an excess of d_1-acetic acid, the loss of succinic anhydride protons followed pseudo-first-order kinetics. The pseudo-first-order rate constants for the disappearance of the α-protons of succinic anhydride were independent of succinic anhydride concentration, [SA] (Fig. 3). The dependency on acetate concentration is seen in Figs. 4 and 5. The results appeared to follow the same kinetics as for acetic anhydride and were described by Eq. 2:

$$-\frac{dP'}{dt} = k_1[SA] + k_2[SA][Ac] \qquad \text{(Eq. 2)}$$

where $-dP'/dt$ is the rate of loss of succinic anhydride protons in molar concentration per hour.

From Figs. 4 and 5, k_2 was found to equal 2.37 M^{-1} hr.$^{-1}$ and k_1 to equal 9.75 × 10⁻² hr.$^{-1}$. The rate of carboxyl proton formation again could not be totally accounted for on the basis of what had been formed from the succinic anhydride. However, in light of the results presented earlier in this discussion, the small amount of extra protons could be accounted for in terms of the analogous reaction of acetate and solvent reacting with the small amounts of acetic anhydride present in solution.

As described by Eqs. 1 and 2, the deuterium incorporation in acetic anhydride (and, subsequently, acetic acid) and succinic anhydride is both base and solvent catalyzed. The incorporation of deuterium *via* enolization into other carbonyl compounds such as ketones and aldehydes is both acid and base catalyzed, suggesting that a possible rate-determining transition step in the present study involves the enolization of succinic anhydride to Compound I and a similar structure for acetic anhydride. The rate dependency on acetate ions leads to the possibility of acetate catalyzing an enolization *via* a transition state of the type shown in Scheme V, while the solvent catalyzes the reaction *via* an acid-catalyzed enolization. These results do not preclude a concerted acid- and base-catalyzed pathway. On regeneration of the nonenol form of succinic anhydride, the molecule picks up a deuterium instead of a proton. This process continues until equilibrium is established.



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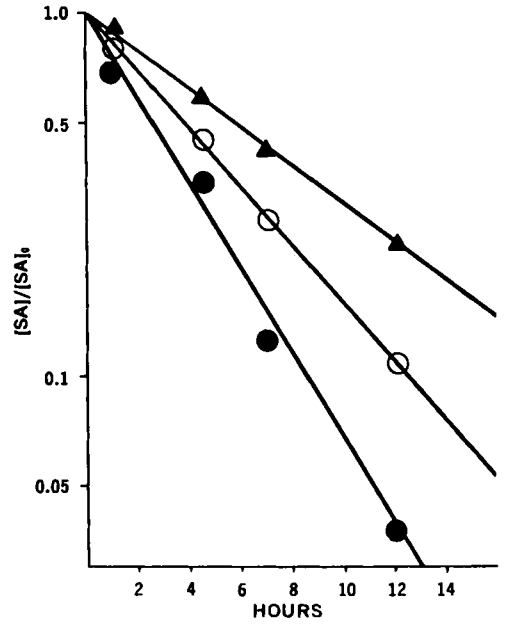


Figure 4—*Plot against time of logarithms of residual/initial concentration of succinic anhydride during its deuteration at 99.8 ± 0.2° in* d_1*-acetic acid containing 1.28 × 10⁻² M (▲), 3.64 × 10⁻² M (○), and 7.31 × 10⁻² M (●) sodium acetate.*

No attempts were made at this stage to determine the deuterium–hydrogen isotope effects for any steps in these reactions or the catalytic effects of other acids or bases. Work along these lines will be continued. Because anhydrides are known to undergo the Perkin condensation reaction under basic conditions by the reaction of the anhydride-α-anion at carbonyl centers, it seemed advisable to check whether products like 2-acetylsuccinic anhydride might form. Succinic anhydride was refluxed for 18 hr. in acetic acid, with sodium acetate present in approximately the same concentration as in the kinetic study. No new peaks or anomalies were seen by NMR, which would have suggested the presence of 2-acetylsuccinic anhydride or similar products.

Table I shows the values of k_1 and k_2 for succinic anhydride relative to those of acetic anhydride. These results show that succinic anhydride is more susceptible to exchange (and enolization) than acetic anhydride, a fact probably attributable to its ability to undergo delocalization of its electrons more favorably because of its planar nature. The values compared in Table I also are not a true indication of the differences between succinic anhydride and acetic anhydride exchange rates and actually minimize the difference. When the increase in carboxyl proton signal was being measured for the reaction involving the proton release from acetic anhydride, the reaction being measured was d_1-acetic acid reacting with acetic anhydride to give acetic acid and d_1-acetic anhydride.

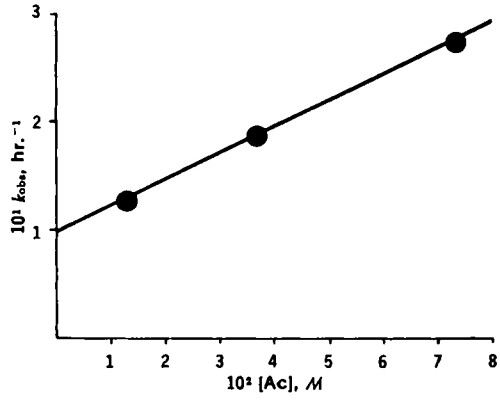
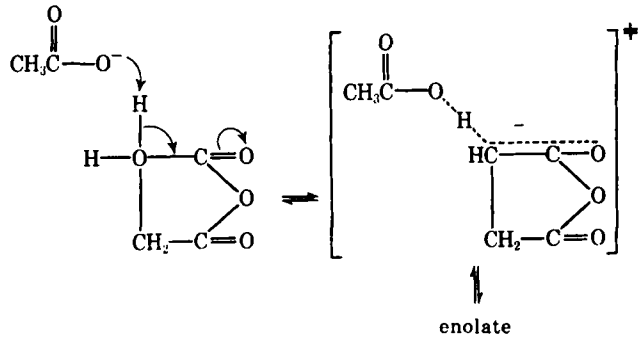


Figure 5—*Plot against acetate concentration of the pseudo-first-order rate constant for the deuteration of succinic anhydride to* d_4*-succinic anhydride at 99.8 ± 0.2° in* d_1*-acetic acid.*



Scheme V

However, d_1-acetic anhydride reacts with d_1-acetic acid or acetic acid to give d_1'-acetic acid (CDH_2COOH) or d_2-acetic acid (CDH_2COOD) and acetic anhydride. Equilibrium is established when the concentration of RCOOH approaches that of RCOOD. At equilibrium the acetic anhydride is present mainly as d_1-acetic anhydride, which still has five exchangeable protons. Under the same reaction conditions, succinic anhydride would have been converted to d_1-succinic anhydride and, subsequently, d_2-succinic anhydride, d_3-succinic anhydride, and d_4-succinic anhydride. If the reaction is not stopped at this stage and acetic acid and d_1'-acetic acid are allowed to build up, d_4-succinic anhydride is slowly converted back to d_3-succinic anhydride, *etc.*

The results of the kinetic study show that succinic anhydride can be successfully deuterated in d_1-acetic acid as long as attempts are made to ensure that the smallest possible amount of acetic anhydride is present. A synthetic scale reaction was successfully attempted and has been described. By carefully preparing d_1-acetic acid from deuterium oxide and acetic anhydride with deuterium oxide in very slight excess and reacting this with succinic anhydride in the presence of sodium acetate, the synthesis resulted in a product that was better than 97% deuterated and a yield of 86%. The reaction uses simple chemicals and makes use of the partial acidic nature of the α-protons of succinic anhydride and the probable tendency of the molecule to enolize.

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Synthesis and Antimicrobial Properties of 17β-Isopentyloxy-4-aza-5α-androstane and the 4-Methyl Derivative

NORMAN J. DOORENBOS and WILLIAM E. SOLOMONS▲

Abstract □ Synthesis of 17β-isopentyloxy-4-aza-5α-androstane was accomplished through a set of reactions involving oxidative opening of an A-ring α,β-unsaturated ketone, ring closure, and two reductions. The Williamson synthesis of ethers was employed to introduce the 17β-isopentyloxy group; ketal formation of the 3-oxo group protected the α,β-unsaturated ketone during this reaction. A serial dilution assay showed the title compound and the 4-methyl derivative to have antimicrobial activity against Gram-positive bacteria, yeasts, and molds.

Keyphrases □ 17β-Isopentyloxy-4-aza-5α-androstane and 4-methyl derivative—synthesis, antimicrobial activity □ 4-Aza-steroids—synthesis, antimicrobial activity of 17β-isopentyloxy-4-aza-5α-androstane and 4-methyl derivative □ Antimicrobial activity—17β-isopentyloxy-4-aza-5α-androstane and 4-methyl derivative □ Structure–activity relationships—4-aza-steroids (17β-isopentyloxy-4-aza-5α-androstane) and antimicrobial activity

Doorenbos and his coworkers (1–4) synthesized 4-aza-steroids which have a wide variety of pharmacological properties such as coronary dilatory, hypocholesterolemic, hypercholesterolemic, hypotensive, and antimicrobial. In certain of these compounds (*e.g.*, 4-methyl-4-aza-5α-cholestane), antimicrobial activity was quite high, *i.e.*, approximately 1 mcg./ml. bactericidal (1) against Gram-positive bacteria, yeasts, and molds.

Previous studies indicated that 4-aza-5α-androstanes possessing a free 17β-hydroxyl (2) and 4-aza-5α-pregnanes possessing a free 20-hydroxy (3, 4) are devoid of antimicrobial activity except at very high concentrations. This showed a necessity for the lack of a hydrophilic group in this region of the molecule. Further explanation for the lack of activity in these molecules may be microbial degradation enhanced by hydroxyl substitution. It is commonly known that testosterone (17β-hydroxyl) is inactive orally but that deactivation is blocked by a 17α-methyl group. This hypothesis was applied to 4-aza-5α-androstan-17β-ol by the synthesis and testing of 17α-methyl-4-aza-5α-androstan-17β-ol, but this compound was also inactive in antimicrobial testing (1).

Alkylation of the 20-hydroxy group in the pregnane series (5) produced a highly active agent, 4-aza-22α-oxa-5α-cholestane. To evaluate structure–activity relationships along these lines, the synthesis and antimicrobial properties of 17β-isopentyloxy-4-aza-5α-androstane and the 4-methyl derivative are reported here.

DISCUSSION

Synthesis—Testosterone was utilized as starting material for the synthesis of 17β-isopentyloxy-4-aza-5α-androstane (VIII) and the 4-methyl derivative IX (Scheme I). The 3-ethylenedioxy derivative II was prepared by the usual method (6, 7). The reaction was very sensitive to traces of water and acid, but the addition of anhydrous potassium carbonate and a few drops of pyridine immediately before workup improved the yield. The ketal II was formed to prevent alkylation of the α,β-unsaturated ketone in the conversion of II to III.

The ether linkage was formed *via* a base-catalyzed condensation of the alcohol II with isopentyl *p*-toluenesulfonate. Yields from this reaction were about 30% until large excesses of newly purchased and carefully protected (anhydrous storing) potassium *tert*-butoxide and addition of dimethyl sulfoxide boosted yields to a respectable range.

Hydrolysis of the ketal-ether III in an acetic acid–water medium, with a small amount of *p*-toluenesulfonic acid as a strong acid catalyst, produced the isopentyl ether of testosterone, IV. It was necessary to conduct the hydrolysis under a nitrogen atmosphere since air oxidation caused formation of dark-colored oils which were not characterized.

Ozonolysis of IV yielded 17β-isopentyloxy-3,5-*seco*-4-norandrostan-5-on-3-oic acid (V). Ethers are known to react with ozone, although at a slower rate than olefins and unsaturated ketones, to produce carboxylic acids, esters, and aldehydes. TLC analyses of reactions run with varied amounts of ozone showed that 0.4 mole excess of ozone per mole of steroid gave the desired product V containing a little starting material. This material was suitable for the subsequent ring closure.

The keto-acid-ether V, when heated under nitrogen in concentrated ammonium hydroxide in a pressure vessel, gave 17β-isopentyloxy-4-aza-5-androsten-3-one (VI). This compound resisted attempts at recrystallization and persisted in forming gels. Elemental analyses were not obtained but the IR, NMR, and UV spectra were consistent with the assigned structure. In addition, the next step gave a crystalline product whose structure was confirmed by all spectral data and accurate elemental analyses.

The enamine-lactam-ether VI was converted to the lactam-ether VII by low pressure catalytic hydrogenation, giving the 5α-product only (8).

The final products, 17β-isopentyloxy-4-aza-5α-androstane (VIII) and 17β-isopentyloxy-4-methyl-4-aza-5α-androstane (IX), were produced from VII by lithium aluminum hydride reduction and the Eschweiler–Clarke *N*-methylation, respectively.

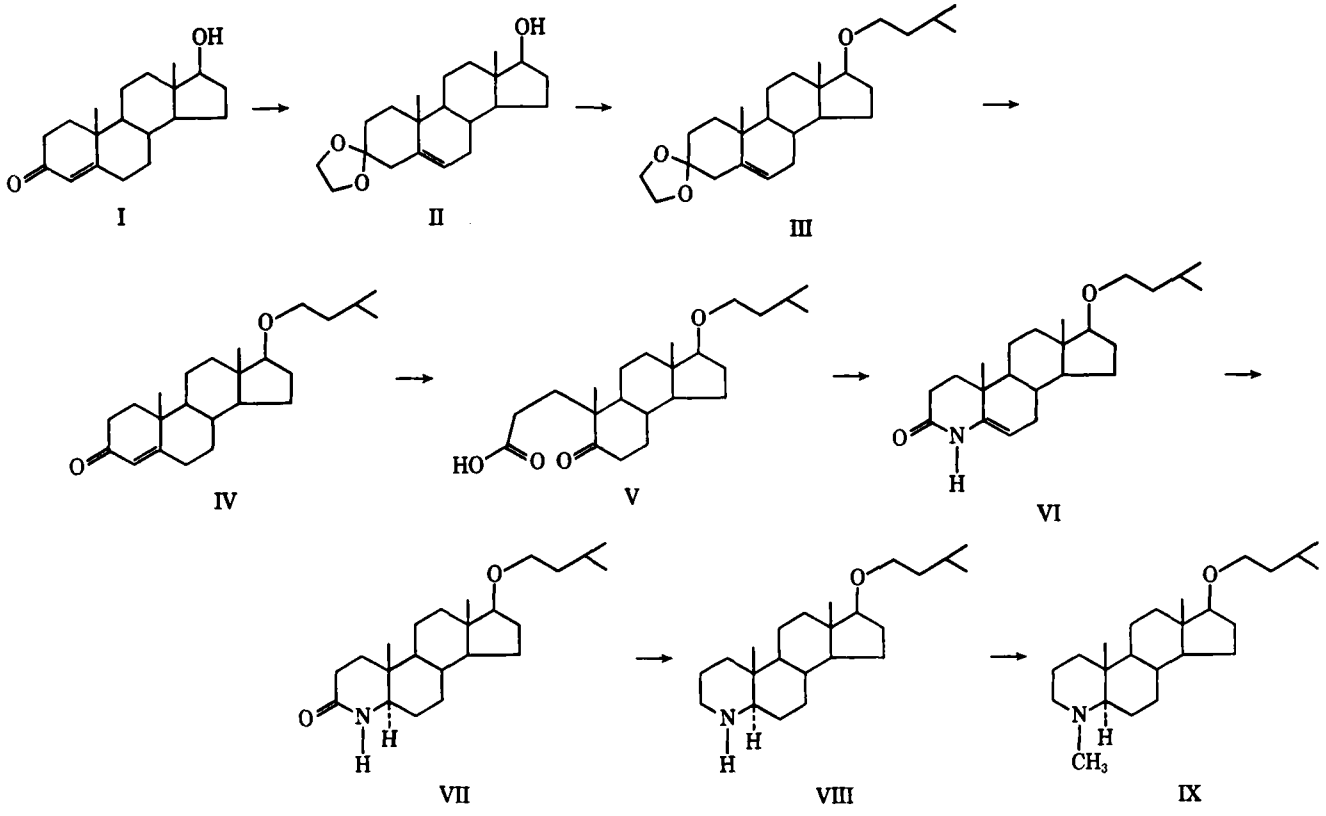
Biological[1]—A twofold serial dilution assay was used to determine the approximate antimicrobial potency of 17β-isopentyloxy-4-

Table I—Inhibitory Concentrations (Expressed in Micrograms per Milliliter)

Microorganisms	Steroids		
	VIII	IX	4-Aza-steroid[a]
Saccharomyces cerevisiae	12.5	12.5	12.5
Candida albicans	12.5	50.0	50.0
Aspergillus niger	3.12	3.12	50.0
Staphylococcus aureus	12.5	50.0	12.5
Bacillus cereus	12.5	12.5	25.0
Escherichia coli	—[b]	—	—

[a] 4-Methyl-4-aza-5α-cholestane (ND-502). [b] No inhibition.

[1] The cell cultures used were obtained from the Department of Biology, University of Mississippi. For *Candida albicans*, *Staphylococcus aureus*, and *Escherichia coli*, the strains are identified by the numbers ATCC-10231, K-257, and ATCC-4157, respectively. *Saccharomyces cerevisiae*, *Aspergillus*, *niger*, and *Bacillus cereus* cultures have been part of University stock cultures for many years and are typed periodically by students using microscopic and biochemical methods.



Scheme I

aza-5α-androstane (VIII) and the *N*-methyl derivative IX. For comparison, 4-methyl-4-aza-5α-cholestane[2], a 4-aza-steroid of known activity, was tested by the same procedure.

The first tube of each series contained 100 mcg. of sample; succeeding tubes contained 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mcg. The level of activity of the compounds was determined by choosing the last tube in which there was no noticeable growth of the organism. No statistical analyses were employed. To ensure that the cells were viable under test conditions, tubes containing no steroid were used as blanks. The assay was duplicated.

The biological data are presented in Table I. The growth medium for the bacteria was tryptic soy broth; for the fungi, it was mycophil broth.

EXPERIMENTAL[3]

3-Ethylenedioxy-5-androsten-17β-ol (II)—A solution of 25 g. (0.086 mole) of testosterone, 55 ml. (61.35 g., 1.011 mole) of ethylene glycol, 1.2 g. (0.007 mole) of *p*-toluenesulfonic acid, and 1.5 l. of benzene was refluxed 5 days in a 3-l. boiling flask fitted with a Dean-Stark trap, condenser, and drying tube. Water was removed from the trap each day. After adding 10–15 g. of anhydrous potassium carbonate and stirring until room temperature was reached, the solution was divided in half. Each half was washed with three 500-ml. portions of 4% sodium carbonate and dried over anhydrous potassium carbonate. A few drops of pyridine were added and the solvent was evaporated to dryness *in vacuo*. The slightly yellow solid residue was recrystallized from acetone and a few drops of pyridine to yield 22.87 g. (79%) of product, m.p. 180–182° [lit. (9) m.p. 182–183°]; IR: 3450 (OH), 1130, 1095, 1055, and 1025 (C—O stretching) cm.$^{-1}$ and absence of carbonyl absorption.

3-Ethylenedioxy-17β-isopentyloxy-5-androstene (III)—To a solution of 13.98 g. (0.042 mole) of II and 500 ml. of dry tetrahydrofuran, in a carefully dried 1-l. three-necked boiling flask fitted with dropping funnel, condenser, and drying tubes, was added 8.0 g. (0.071 mole) of potassium *tert*-butoxide with refluxing under nitrogen over 5 hr. To this solution was added dropwise 10.2 g. (0.042 mole) of isopentyl *p*-toluenesulfonate and 8.5 ml. of dimethyl sulfoxide in 100 ml. of tetrahydrofuran. Refluxing was continued for an additional 19 hr. Addition of potassium *tert*-butoxide and isopentyl *p*-toluenesulfonate was repeated as follows: (*a*) 8.0 g. (0.071 mole) potassium *tert*-butoxide with refluxing for 5 hr. and 6.0 g. (0.028 mole) isopentyl *p*-toluenesulfonate in 100 ml. tetrahydrofuran with refluxing 19 hr., and (*b*) 8.0 g. (0.071 mole) of potassium *tert*-butoxide with refluxing for 17 hr. After cooling and removing the solvent *in vacuo*, the residue was digested in refluxing benzene; the benzene mixture was washed with six portions of 500 ml. of water, dried over anhydrous potassium carbonate, and evaporated to dryness. Recrystallization of the solid residue from methanol and a few drops of pyridine gave shiny leaflets, 12.00 g. (70%), m.p. 130–131°; IR: 1135, 1105, 1065, and 1025 (C—O stretching) cm.$^{-1}$ and absence of hydroxyl absorption.

Anal.—Calc. for $C_{26}H_{42}O_3$: C, 77.56; H, 10.51. Found: C, 77.31; H, 10.37.

17β-Isopentyloxy-4-androsten-3-one (IV)—A solution of 8.31 g. (0.021 mole) of III, 0.13 g. (0.68 mmole) of *p*-toluenesulfonic acid, 2 ml. (0.111 mole) of water, and 200 ml. of acetic acid in a 250-ml. boiling flask was stirred under nitrogen for 1 hr. at ambient temperature. After mixing the reaction solution with 500 ml. of ether, it was washed with three 1-l. portions of water, three 340-ml. portions of cold 4% sodium carbonate solution, and 1 l. of water. Then it was dried over anhydrous potassium carbonate, filtered, and evaporated *in vacuo* to give 7.01 g. of a viscous oil which solidified.

Purification Method A—An analytical sample was obtained by passing 1.63 g. of this substance through 25 g. of grade III neutral alumina in a 2-cm. column with *n*-hexane. Evaporation of the solvent and several recrystallizations from methanol gave 157 mg. of

[2] ND-502.

[3] All melting points were determined on a Thomas-Hoover capillary melting-point apparatus, and the elemental analyses were obtained from Galbraith Laboratories, Knoxville, Tenn. The maximum wavelength of absorption in the UV spectra was determined on a Perkin-Elmer model 202, and the absorbance was determined on a Beckman model DU spectrophotometer. The IR spectra were recorded on a Perkin-Elmer model 137B and, unless indicated otherwise, all spectra were determined in KBr. Specific rotations were determined on a Perkin-Elmer model 141 automatic polarimeter as 2% solutions in chloroform.

Appreciation is extended to Mrs. R. L. Settine of the Chemistry Department, University of Mississippi, for the NMR spectra, which were recorded with a Varian model A-60-A spectrometer. The NMR spectra sample solutions were 40 mg. of sample/0.5 ml. of chloroform-*d*, with tetramethylsilane as the internal reference.

white solid, m.p. 65–66°; IR 1680, 1620 (3-carbonyl and 4-double bond), 1135, 1114, and 1095 (C—O stretching) cm.$^{-1}$.

Anal.—Calc. for $C_{24}H_{38}O_2$: C, 80.39; H, 10.68. Found: C, 80.22; H, 10.77

Purification Method B—Sublimation of 7.0 g. of IV through a distance of 2.6 cm. at 180° (wax bath temperature) and 0.250 mm. gave 6.61 g. (90%) of a white solid, m.p. 63–64° and IR identical to that obtained by Method A. Method B was routinely used for purification of this compound.

17β-Isopentyloxy-3,5-*seco*-4-norandrosten-5-on-3-oic Acid (V)—To a solution of 6.61 g. (0.018 mole) of IV, 50 ml. of ethyl acetate, and 150 ml. of glacial acetic acid was added 0.025 mole of ozone at ice bath temperature. After addition of 10 ml. of 30% hydrogen peroxide and 20 ml. of water and stirring for 16 hr., the solution was mixed with 600 ml. of ether. This solution was washed with four 500-ml. portions of water and extracted with two 300-ml. portions of 8% sodium hydroxide, and the basic solutions were made acidic to litmus by addition of 47% (v/v) phosphoric acid with cooling. After extracting the oily precipitate of each fraction with three 250-ml. portions of ether, washing with four 500-ml. portions of water, drying over anhydrous magnesium sulfate, and filtering, the filtrate was evaporated *in vacuo* to give a viscous oil which was not further purified. The IR spectrum (liquid film) gave major bands at 3500–3000 (carboxyl OH), 1725, 1700 (5-ketone and carboxyl carbonyl), 1130, 1085, and 1040 (C—O stretching) cm.$^{-1}$.

17β-Isopentyloxy-4-aza-5-androsten-3-one (VI)—A solution of the viscous oil V dissolved in 200 ml. of concentrated ammonium hydroxide and cooled in a dry ice–acetone bath was placed in an autoclave, evacuated at the water pump, sealed, and heated to 170° for 22 hr. After cooling, a creamy-white solid was collected, washed with water, and dried *in vacuo* at 70° for 6–8 hr., giving 3.36 g. of VI (51% based on the quantity of IV used), m.p. 226–227° dec.; IR: 3190, 3050 (NH), 1690, 1675 (C=C and lactam carbonyl), 1135, 1100, and 1090 (C—O stretching) cm.$^{-1}$; $UV_{max}^{95\%\,EtOH}$: 235 nm. (ε 13,600); NMR (CCl_4): δ 7.77 (broad, NH), 4.75 (broad, vinyl), 3.30 (t, CH and CH_2 adjacent to the ether linkage), 1.04 (s, C-19 methyl), 0.88 (d, isopropyl group in the side chain), and 0.72 (s, C-18 methyl).

17β-Isopentyloxy-4-aza-5α-androstan-3-one (VII)—A mixture of 3.36 g. (9.3 mmoles) of VI, 0.5 g. of platinum oxide, and 100 ml. of glacial acetic acid was hydrogenated at 46 p.s.i. and 60° for 36 hr. The UV spectrum indicated the presence of a small amount of non-reduced compound, so hydrogenation was continued at 74° and 46 p.s.i. for 15 hr. (after adding an additional 0.2 g. of platinum oxide). After removing the catalyst, evaporating the solvent *in vacuo*, and recrystallizing the residue from acetonitrile, 2.30 g. (68%) of white needles was obtained, m.p. 241–242°; IR: 3125, 3025 (NH), 1675, 1620 (lactam carbonyl), 1135, 1115, 1110, and 1100 (C—O stretching) cm.$^{-1}$; NMR (CCl_4): δ 7.96 (broad, NH), 3.36 (t, CH and CH_2 adjacent to the ether linkage), 0.90 (d, isopropyl group in the side chain), 0.90 (s, C-19 methyl), and 0.72 (s, C-18 methyl).

Anal.—Calc. for $C_{23}H_{39}NO_2$: C, 76.40; H, 10.87; N, 3.87. Found: C, 76.49; H, 11.01; N, 3.78.

17β-Isopentyloxy-4-aza-5α-androstane (VIII)—A slurry of 5.0 g. (0.132 mole) of lithium aluminum hydride in 200 ml. of ether was added dropwise with magnetic stirring to a solution of 2.30 g. (6.4 mmoles) of VII and 600 ml. of dry ether in a 1-l. three-necked flask fitted with a dropping funnel and condenser with drying tube. Refluxing was continued for 41 hr. After decomposing excess lithium aluminum hydride with methanol and then water, adding 350 ml. of ether and 200 ml. of water, separating the salts and water from the ether phase, and washing with 500 ml. of water, 500 ml. of 9% sodium hydroxide, and two 500-ml. portions of water, the solvent was evaporated *in vacuo* to yield 2.26 g. of a yellow oily solid. Crystallization from acetonitrile gave 1.34 g. (62%) of prisms, m.p. 68–70°; $[\alpha]_D^{23}$ + 25.1° ($CHCl_3$); IR: 3220 (NH), 1140, 1120, 1100, and 1095 (C—O stretching) cm.$^{-1}$ and absence of carbonyl absorption; NMR ($CDCl_3$): δ 3.60 (t, CH and CH_2 adjacent to the ether linkage), 0.92 (s, C-19 methyl), 0.86 (d, isopropyl group in the side chain), 0.76 (s, C-18 methyl), and a proton under the methylene absorption which exchanged with D_2O.

Anal.—Calc. for $C_{23}H_{41}NO$: C, 79.48; H, 11.89; N, 4.03. Found: C, 79.26; H, 11.94; N, 4.23.

17β-Isopentyloxy-4-methyl-4-aza-5α-androstane (IX)—A solution of 0.51 g. (1.47 mmoles) of VIII, 5 ml. (0.098 mole) of 90% formic acid, and 5.0 ml. (0.063 mole) of formalin was heated for 6.5 hr. at 90° and 12 hr. at 55°. After cooling, concentrated ammonium hydroxide was added dropwise until the solution turned red litmus blue. The mixture was cooled in an ice bath and filtered, and the precipitate was washed with water and dried at 70° *in vacuo* to give 0.51 g. of white solid. Recrystallization from acetonitrile yielded 0.47 g. (87%) of colorless needles, m.p. 94–95°; $[\alpha]_D^{23}$ + 6.3° ($CHCl_3$); IR: 2925, 2825, 2750, 2700 [Bohlmann bands (10)], 1140, 1120, 1100, and 1070 (C—O stretching) cm.$^{-1}$ and absence of NH stretching; NMR ($CDCl_3$): δ 3.48 (t, CH and CH_2 adjacent to the ether linkage), 2.22 (s, *N*-methyl), 0.98 (s, C-19 methyl), 0.92 (d, isopropyl group of the side chain), and 0.77 (s, C-18 methyl).

Anal.—Calc. for $C_{24}H_{43}NO$: C, 79.72; H, 11.99; N, 3.87. Found: C, 79.54; H, 11.93; N, 3.69.

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Prazepam Metabolism in the Rat

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Abstract □ Groups of rats were treated by gavage with ^{14}C-labeled prazepam (10 mg./kg.), and the fate of the isotope was determined by measuring the radioactivity in the blood plasma, urine, feces, and selected tissues. The half-life of the drug and its metabolites in the plasma was found to be 2.5 hr. Twenty-four hours after drug administration, 17.3% of the drug radioactivity was excreted in the urine, 33.6% was excreted in the feces, and 35.1% was present in the GI tract. Fractionation of the 24-hr. urine collection showed its radioactivity to consist of 14.7% unconjugated compounds, 14.7% glucuronides, 52.0% sulfates, and 18.6% unclassified polar compounds. Unchanged prazepam was identified in the unconjugated fraction along with oxazepam and desalkylprazepam. The glucuronides identified were conjugates of 4'-hydroxydesalkylprazepam and 4'-hydroxyoxazepam. The sulfate fraction included 4'-hydroxydesalkylprazepam sulfate and 4'-hydroxyoxazepam sulfate.

Keyphrases □ Prazepam, radiolabeled—absorption, excretion, and tissue distribution in rats, identification of metabolites □ Metabolism, radiolabeled prazepam—absorption, excretion, and tissue distribution in rats, identification of metabolites □ Absorption—radiolabeled prazepam, rats □ Excretion—radiolabeled prazepam, rats

Consistent with the pharmacological activity of prazepam in animals (1, 2), the tranquilizer is effective in treating a variety of anxiety states in man (3–10). Earlier reports dealt with the metabolism of prazepam in man (11–13) and the dog (14, 15) and with the biotransformation of prazepam by the 9000×*g* fractions prepared from human, canine, and rodent livers (13, 16). The present report describes prazepam metabolism in the rat; it covers drug absorption, excretion, and tissue distribution and the identification of drug metabolites excreted in the urine.

EXPERIMENTAL

Reference Compounds—Ring-labeled prazepam [7-chloro-1-(cyclopropylmethyl) - 1,3-dihydro - 5 - phenyl - 2*H* - 1,4 - benzodiazepin-2-one-5-^{14}C] was synthesized with a specific activity of 1.31 mc./g.; its chemical purity and radiochemical purity were >99% (17). Also available were authentic samples of desalkylprazepam, 3-hydroxyprazepam, oxazepam, 4'-hydroxydesalkylprazepam, 4'-hydroxyoxazepam, 2-amino-5-chlorobenzophenone, and 4'-hydroxy-2-amino-5-chlorobenzophenone.

Radioactivity Counting—Quantitative assays for ^{14}C were performed using a liquid scintillation spectrometer[1]. The external standardization method was employed for quench corrections.

Animals and Dosing—Male Wistar rats[2], 280 g. mean weight, were treated by gavage with a solution of ^{14}C-prazepam in polyethylene glycol 400. The dose was 10 mg./kg. body weight. The animals were housed in individual glass metabolic cages without food or water during the experiments. Four rats were used to determine the half-life of plasma radioactivity, and six animals were employed for the tissue distribution, excretion, and biotransformation studies.

Plasma Half-Life—Blood samples were withdrawn by heart puncture at the following intervals after dosing: 0.5, 1, 2, 4, 6, and 24 hr. The heparin-treated samples were centrifuged in a desktop clinical centrifuge for 20 min., and the separated plasma preparations were assayed for ^{14}C by scintillation spectrometry.

Distribution of Drug Radioactivity in Tissues—Twenty-four hours after drug treatment, the rats were killed and selected tissues were excised. The fresh tissues were pooled by type, homogenized in a blender[3] with 75% aqueous dioxane, and filtered. The residues were extracted twice in the same manner. Aliquots (1.0 ml.) of the appropriately combined extracts were counted by scintillation spectrometry.

Fecal and Urinary Excretion—The fecal collections were washed from the cages with water and 75% dioxane. The mixtures were kept at 40° overnight and filtered. The residues were reextracted three times with 75% dioxane. Then 1.0-ml. aliquots of the combined filtrates (650 ml.) were counted by scintillation spectrometry. After the urine collections were combined with the vessel rinsings and diluted to 80 ml., aliquots (0.5 ml.) were counted for radioactivity.

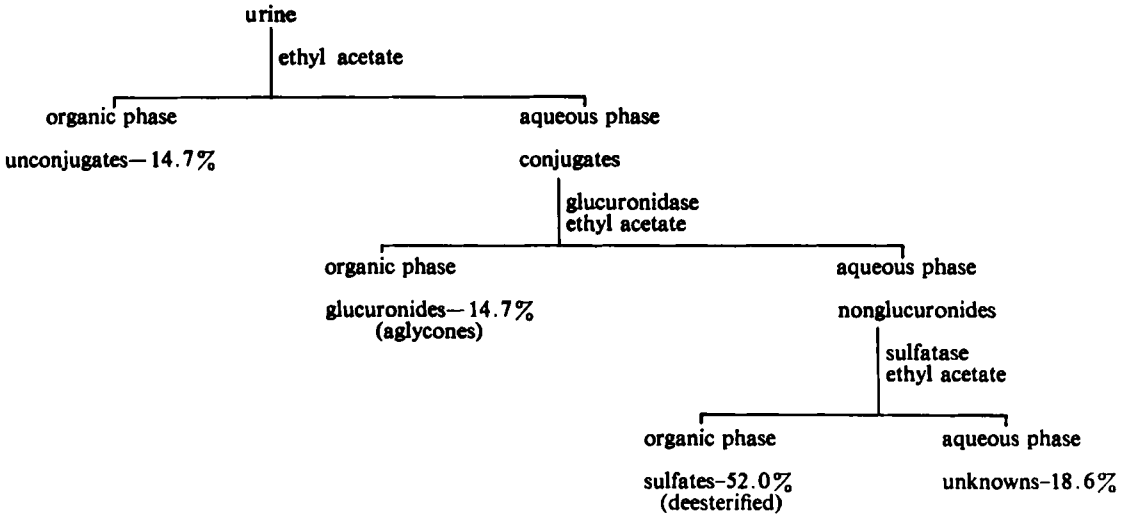
Fractionation of Urinary Drug Metabolites—The fractionation scheme is outlined in Scheme I. It involved: (*a*) extraction of unconjugated urinary metabolites, (*b*) enzymatic hydrolysis of the glucuronide metabolites followed by extraction of the aglycones, and (*c*) enzymatic hydrolysis of the sulfate metabolites followed by extraction of the liberated compounds. The distribution of radioactivity among these fractions was determined by scintillation spectrometry. To obtain the unconjugated compounds, the pH of the entire urine collection (80 ml.) was adjusted to 7.0 and extracted with five 50-ml. portions of ethyl acetate. The combined ethyl acetate extracts were then evaporated to approximately 1 ml. for TLC.

After extracting the unconjugated metabolites, the urine was brought to pH 5.0–5.5 and incubated for 48 hr. at 37° with 240,000 units of β-glucuronidase. Then the pH was adjusted to 7.0 and the solution was extracted five times with 40-ml. portions of ethyl acetate to collect the aglycones. The combined organic phase was evaporated to about 2 ml. for TLC. Before liberating the free phenols from their sulfate conjugates, the ethyl acetate dissolved in the extracted urine was removed by aeration. Then the pH of the pool was adjusted to 6.8 with 1 *N* NaOH and treated with 240,000 units of β-glucuronidase-aryl sulfatase. After incubating for 48 hr. at 37°, the solution was extracted five times with 100-ml. portions of ethyl acetate. The combined extracts were evaporated to approximately 4 ml. for TLC.

Chromatography—Five solvent systems were used for TLC on glass plates coated with silica gel G. The solvents were prepared by volume as follows: 206, benzene–ethyl acetate (5:1); 301, heptane–chloroform–ethanol (1:3:1); 306, chloroform–ethanol–acetone (8:1:1); 307, chloroform–acetone (9:1); and 309, chloroform–acetic acid–methanol (18:1:1). All chromatograms were scanned with a radiochromatogram scanner[4] to locate radioactive bands, and the areas under the peaks were quantified with a planimeter. The cochromatographic technique for the direct comparison of known and unknown compounds was described previously (15).

Hydrolysis of Drug Metabolites and Testing of Split Products for Primary Amine and Phenol Groups—The metabolite under investigation was scraped from TLC plates into a 15-ml. centrifuge tube and extracted with 5 ml. of ethyl acetate by shaking the tube for 30 min. Following evaporation to dryness, the eluted material was hydrolyzed in 1 ml. of 6 *N* HCl for 1 hr. at 100° (18). The hydrolysate was kept ice cold during partial neutralization by the addition of 44 mg. of sodium hydroxide (48 mg. required for neutralization). The acidic solution was extracted with an equal volume of ethyl acetate to collect the 2-amino-5-chlorobenzophenones. All of the ^{14}C was recovered in the ethyl acetate. The extract was concentrated to 100 μl. for chromatography. After TLC, the Bratton–Marshall

[1] Packard Tri-Carb, model 3324.
[2] Marland Farms.
[3] Waring.
[4] Packard model 7201.



Scheme I—Plan for fractionating and identifying prazepam metabolites in rat urine. The unconjugated metabolite fraction, aglycone fraction, and desulfated fraction were submitted to TLC in three solvents. The percentages refer to urinary radioactivity.

test for primary aromatic amines (19) and the ferric ferrocyanide test for phenols (20) were run directly on the chromatograms.

RESULTS

Plasma Half-Life, Tissue Distribution, and Excretion of Prazepam Radioactivity—The mean values for plasma ^{14}C following ^{14}C-prazepam administration are presented in Fig. 1 in terms of prazepam equivalents per milliliter. The data show that the tranquilizer was absorbed rapidly after oral administration to the rat and that the peak plasma level of radioactivity was attained within 30 min. Figure 1, plotted by the method of least squares, indicates that the half-life of plasma ^{14}C was 2.5 hr.

Twenty-four hours after drug treatment, the tissues contained 44.3% of the isotope (Table I). There was no unusual accumulation of radioactivity in any of the tissues. The fecal excretion (33.6% of the dose) was almost twice the urinary excretion (17.3%), and the total recovery of drug radioactivity was 95.2%.

Fractionation of Labeled Urinary Compounds—Scheme I shows that the sulfates comprised more than half of the urinary drug metabolites. The other three fractions (unconjugates, glucuronides, and unknown polar metabolites) contained the balance in comparable quantities.

Chromatography—Table II lists the R_f values of all of the reference compounds in five solvents, and it is evident that their use permitted the separation of each listed compound from any other. Solvents 301, 306, and 309 were especially valuable for the identification of oxazepam, 4′-hydroxyoxazepam, and 4′-hydroxydesalkylprazepam. Solvents 206 and 307 gave excellent resolutions of 2-amino-5-chlorobenzophenonone, 2-cyclopropylmethylamino-5-chlorobenzophenone, and 4′-hydroxy-2-amino-5-chlorobenzophenone.

Identification of Labeled Unconjugated Compounds—As indicated in Scheme I, 14.7% of the excreted radioactivity was in the form of unconjugated compounds. TLC in Solvent 306 revealed the presence of five radioactive areas. Unchanged prazepam, identified by TLC and its hydrolytic conversion to 2-cyclopropylmethylamino-5-chlorobenzophenone, accounted for 5% of the ^{14}C in the unconjugated fraction (Table III). With the same techniques the only metabolites identified were oxazepam and desalkylprazepam, which accounted for 55 and 4%, respectively, of the radioactivity of the unconjugated fraction. Thus, prazepam, oxazepam, and desalkylprazepam accounted for 0.7, 8.1, and 0.6%, respectively, of the total urinary radioactivity. Unknown metabolites represented 36% of the radioactivity of the unconjugated fraction.

Identification of Labeled Glucuronides—This fraction contained 14.7% of the urinary radioactivity (Scheme I). Chromatography of 100-μl. aliquots in Solvent 306 revealed the presence of five radioactive peaks. The major peak, comprising 61% of the radioactivity of this fraction, was scraped from several plates and eluted with ethyl acetate. Rechromatography in Solvent 309 gave rise to three peaks. Two of these peaks were identified tentatively as oxazepam and 4′-hydroxydesalkylprazepam based upon their chromatographic behavior when they were cochromatographed with authentic reference compounds in Solvents 301, 306, and 309 (Table II). When the aglycone that migrated as oxazepam was hydrolyzed as already described, it yielded a labeled split product, which traveled with authentic 2-methylamino-5-chlorobenzophenone in cochromatographic tests conducted in several solvent systems. Additionally, the labeled hydrolysis product gave a positive Bratton–Marshall test and a negative phenol test. Hydrolysis of the aglycone that behaved chromatographically as 4′-hydroxydesalkylprazepam gave a product that migrated with synthetic 4′-hydroxy-2-amino-5-chlorobenzophenone in all solvent systems. Furthermore, this labeled hydrolysis product responded positively to Bratton–Marshall and phenol tests.

To collect the third aglycone, the R_f 0.45 zone was scraped from a series of plates developed in Solvent 306 and the radioactive material was extracted from the gel with ethyl acetate. Upon TLC in Solvents 301, 306, and 309, the extracted compound gave the

Table I—Tissue Distribution and Excretion of ^{14}C after Oral Administration of Ring-Labeled Prazepam to Rats (10 mg./kg.)

Specimen	Percent of ^{14}C Dose Found after 24 hr.
Brain	0.03
Cadaver	6.98
GI tract	35.10
Heart	0.01
Kidney	0.27
Liver	1.24
Lung	0.03
Spleen	0.65
Feces	33.6
Urine	17.3
Total recovery	95.2

Table II—R_f Values of Reference Compounds

Compound	R_f in Solvent 206	301	306	307	309
Prazepam	0.45	—	0.88	0.60	—
Desalkylprazepam	0.11	—	0.72	0.30	—
Oxazepam	0.04	0.70	0.55	0.15	0.65
4′-Hydroxyoxazepam	—	0.55	0.43	—	0.25
4′-Hydroxydesalkylprazepam	—	0.68	0.50	—	0.20
2-Amino-5-chlorobenzophenone	0.77	—	0.90	0.85	—
2-Cyclopropylmethylamino-5-chlorobenzophenone	0.95	—	0.95	0.97	—
4′-Hydroxy-2-amino-5-chlorobenzophenone	0.44	—	0.80	0.55	—

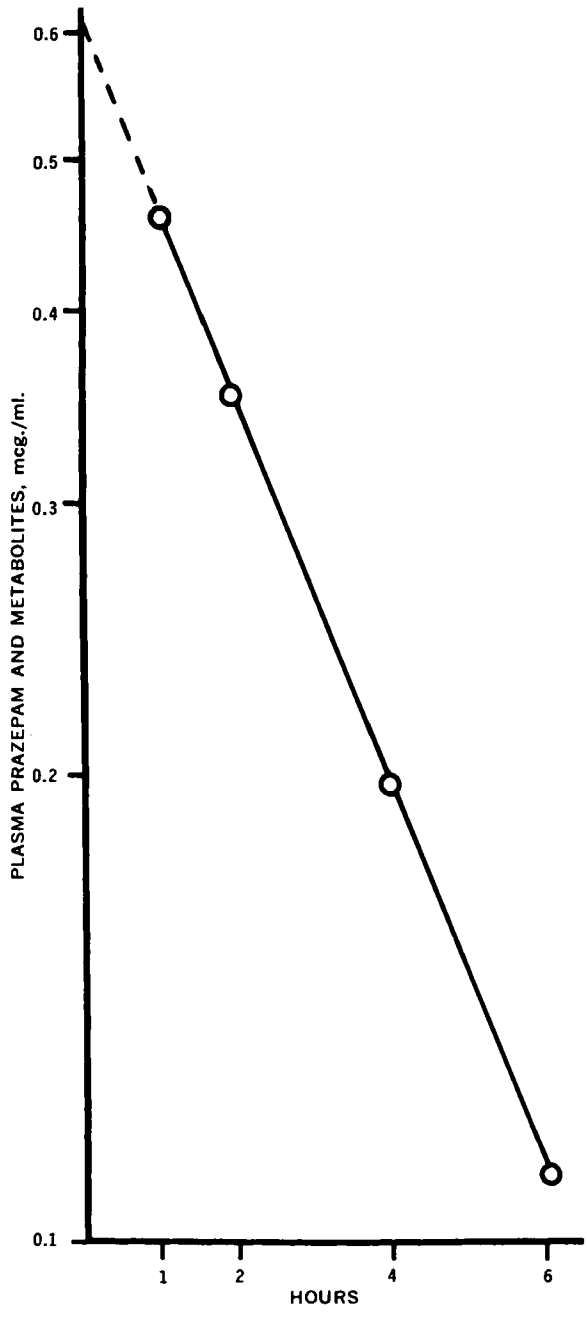


Figure 1—*Concentrations of prazepam and metabolites in the blood plasma of rats.*

same R_f values as 4'-hydroxyoxazepam, and cochromatography did not resolve the urinary and synthetic preparations. Hydrolysis of the urinary aglycone yielded a phenolic primary aromatic amine, which was identified as 4'-hydroxy-2-amino-5-chlorobenzophenone by the test procedures already described.

As shown in Table III, the radiolabeled urinary glucuronide fraction consisted of 39% oxazepam glucuronide, 17% 4'-hydroxyoxazepam glucuronide, 22% 4'-hydroxydesalkylprazepam glucuronide, and 22% unknown glucuronides.

Identification of Labeled Sulfates—It can be seen from Scheme I that the sulfate fraction accounted for 52% of the urinary radioactivity. Chromatography of 100-μl. aliquots in Solvent 306 showed five radioactive peaks. The major peak was scraped from several plates and eluted with ethyl acetate. Cochromatography of this unknown with 4'-hydroxydesalkylprazepam in Solvents 301, 306, and 309 revealed identical chromatographic behavior between the unknown and reference compound. Hydrolysis of the main sulfate metabolite followed by cochromatography with 4'-hydroxy-2-amino-5-chlorobenzophenone in three solvent systems also showed

Table III—Qualitative and Quantitative Assay of Urine Collected from a Group of Six Rats 24 hr. after ^{14}C-Prazepam Administration

Identification	Percent of Fraction[a]	Percent of Total Urinary Radioactivity	
Unconjugates			
Prazepam	5	0.7	
Desalkylprazepam	4	0.6	
Oxazepam	55	8.1	
Unknowns	36	5.3	
			14.7
Glucuronides			
Oxazepam	39	5.8	
4'-Hydroxyoxazepam	17	2.5	
4'-Hydroxydesalkylprazepam	22	3.2	
Unknowns	22	3.2	
			14.7
Sulfates			
4'-Hydroxyoxazepam	7	3.6	
4'-Hydroxydesalkylprazepam	65	33.8	
Unknowns	28	14.6	
			52.0
Unclassified polar compounds			
Unknowns	100	18.6	
			18.6
			100.0

[a] Based upon data obtained with Solvent 306 and resolution of oxazepam from 4'-hydroxydesalkylprazepam with Solvent 309.

agreement between the hydrolyzed metabolite and authentic 4'-hydroxy-2-amino-5-chlorobenzophenone. This metabolite gave positive phenol and Bratton–Marshall tests.

A peak with an R_f value 0.45 was scraped from several plates developed in Solvent 306 and was eluted with ethyl acetate. This metabolite was cochromatographed with 4'-hydroxyoxazepam in Solvents 301, 306, and 309. Agreement was observed between the unknown metabolite and authentic 4'-hydroxyoxazepam in all cases. Hydrolysis of this metabolite followed by cochromatography with 4'-hydroxy-2-amino-5-chlorobenzophenone in three solvent systems showed agreement between the hydrolyzed metabolite and the reference compound. Spraying with the phenol reagent or the Bratton–Marshall reagent gave positive tests in both instances.

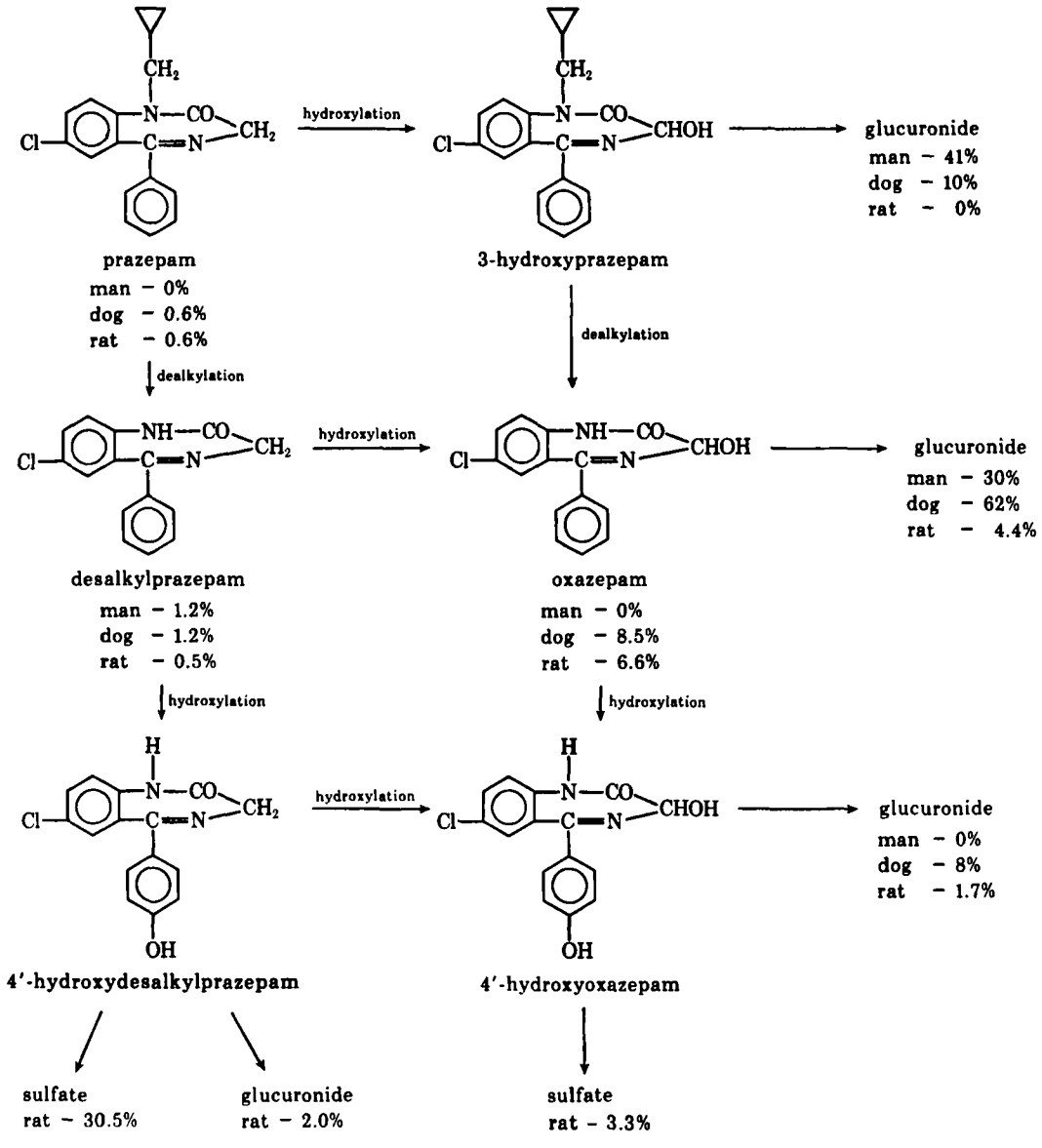
Table III summarizes these findings. 4'-Hydroxydesalkylprazepam sulfate accounted for 65% of the radioactivity of this major urinary fraction, whereas 4'-hydroxyoxazepam sulfate represented 7% of this fraction. Expressed in terms of the total urinary radioactivity, 4'-hydroxydesalkylprazepam sulfate accounted for 33.8% and 4'-hydroxyoxazepam sulfate represented 3.6%. Unknown metabolites were present to the extent of 14.6% of the urinary ^{14}C.

DISCUSSION

Orally administered prazepam was absorbed rapidly by the rat and reached plasma levels that were far greater than those found in the dog treated with the same dose (14). While the half-time of prazepam metabolites in blood plasma was not determined accurately for the dog, the mean values for man (13) and rat were found to be very different, 67 and 2.5 hr., respectively. The urinary excretion of prazepam by the rat in 24 hr. (17%) was more similar to man (14%) than to the dog (3%).

Scheme II illustrates pathways of prazepam metabolism and summarizes the urinary excretion of the end-products by rats, dogs, and humans. Clearly, the rat showed the most complex pattern of prazepam biotransformation. Nine end-products in rat urine accounted for only 50% of the isotope present. By contrast, three metabolites represented 71% of the drug radioactivity in human urine (11) and six end-products represented 90% of the drug ^{14}C in dog urine (15).

In humans and dogs, prazepam undergoes aliphatic hydroxylation and dealkylation as phase 1 reactions. There is no evidence that the rat employs more than one initial attack, namely, dealkylation. All of the metabolites identified in rat urine can be derived from desalkylprazepam (Scheme II). This interpretation agrees



Scheme II—Prazepam biotransformation in man, dog, and rat (For clarity, the sequences involving 4'-oxidation of prazepam and 3-hydroxyprazepam were omitted)

with the results of prazepam metabolism *in vitro*. Incubating prazepam with rat liver microsomal enzymes produced desalkylprazepam but no 3-hydroxyprazepam even after pretreating the animals with prazepam (13) and phenobarbital (16).

Prazepam-treated rats and dogs excreted free oxazepam as well as oxazepam glucuronide. Sisenwine *et al.* (21) made the same observation after administering a high oxazepam dose (40 mg./kg.) to rats. One possibility is that the conjugating system became saturated. However, the absence of unconjugated 4'-hydroxydesalkylprazepam and 4'-hydroxyoxazepam suggests either that oxazepam is a poorer substrate for glucuronyl transferase than these phenolic metabolites or that oxazepam glucuronide is more susceptible to β-glucuronidase than are the other glucuronides.

Prazepam biotransformation in the rat is further distinguished by aromatic hydroxylation and by conjugation with sulfate, reactions that have not yet been observed in the other species. While it is possible that prazepam is hydroxylated directly like diazepam (22), this conversion has no present experimental support because 4'-hydroxyprazepam was detected neither *in vivo* nor *in vitro* (13, 16). Diazepam also undergoes no aromatic hydroxylation by rat liver microsomes (23), although 4'-hydroxydiazepam was found in rat urine (22). It appears that the ability of rat liver microsomes to oxidize phenyl groups is inhibited by the benzodiazepine nucleus. Marcucci *et al.* (24) showed that diazepam inhibits the oxidative dealkylation of 3-hydroxydiazepam to oxazepam *in vitro*. A review of the metabolism of benzodiazepines *in vitro* (25) illustrates the emphasis placed upon the enzymology of the liver and leads one to consider that the aromatic hydroxylations observed *in vivo* but not *in vitro* may involve extrahepatic enzymes.

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Structure Side-Effect Sorting of Drugs I: Extrapyramidal Syndrome

ERIC J. LIEN▲ and GEDY A. GUDAUSKAS*

Abstract □ A computer program using PL/I language was developed to sort the side effects of 540 clinically useful drugs. The common structural features of the drugs that produce the extrapyramidal syndrome are: (*a*) the drug contains at least one tertiary amino group separated by three carbon atoms from a coplanar hydrophobic region, and (*b*) the coplanar hydrophobic region can be a phenothiazine ring, its isosteres, or two benzene rings held close together (*e.g.*, trimethobenzamide and haloperidol). Other tranquilizers and anticonvulsants such as diazepam and diphenylhydantoin are shown to have entirely different structural features.

Keyphrases □ Side effects, computer sorting of 540 drugs—extrapyramidal syndrome related to structure □ Computer sorting of side effects of 540 drugs—extrapyramidal syndrome related to structure □ Structure-activity relationships—features related to extrapyramidal side effects, result of computer sorting of 540 drugs □ Extrapyramidal syndrome—related to structural features, result of computer sorting of side effects of 540 drugs □ Drug sorting by side effects—structure-activity relationships

In recent years, numerous publications on chemical structure-pharmacological activity have appeared in various journals (1–13). However, due to several reasons, little systematic work has been done in sorting and correlating undesirable side effects of various drugs with their chemical structures. One reason is that side effects are usually more variable and more difficult to measure than the major pharmacological effect. In addition, when several drugs are concurrently administered to a patient, it is not easy to pinpoint the agent causing the most undesirable side effect or to detect the presence of drug interactions.

Many potentially useful drugs are not used primarily because of the severity of their side effects, not because of the lack of efficacy. On the other hand, there are many instances of an annoying side effect promoted later to be an appreciated therapeutic effect, such as the development of oral antidiabetic drugs from the observation of hypoglycemia in patients treated with certain sulfonamides. Therefore, it was considered worthwhile to employ a computerized program to sort the side effects of all clinically useful drugs and to correlate these effects with the chemical structure. It is believed that any effect of a drug, desired or undesired, direct or indirect, is determined by its physicochemical properties which are, in turn, governed by its structure.

This report discusses the structural features of the drugs known to cause extrapyramidal syndrome and why other tranquilizers (*e.g.*, diazepam) do not cause this side effect. Extrapyramidal syndrome consists of parkinsonian-like symptoms (*e.g.*, tremors, rigidity, and salivation), akathisia (a psychosis marked by an inability to sit still or to remain seated), and akinesia (loss of the power of voluntary motion).

METHOD

A data bank consisting of 540 clinically useful drugs (14–17) was established, and each side effect is represented by a unique three-digit hexadecimal code. The name of the side effect associated with each code is stored separately in another file, the symptom file. The code is also the key for each record, allowing direct retrieval of the name.

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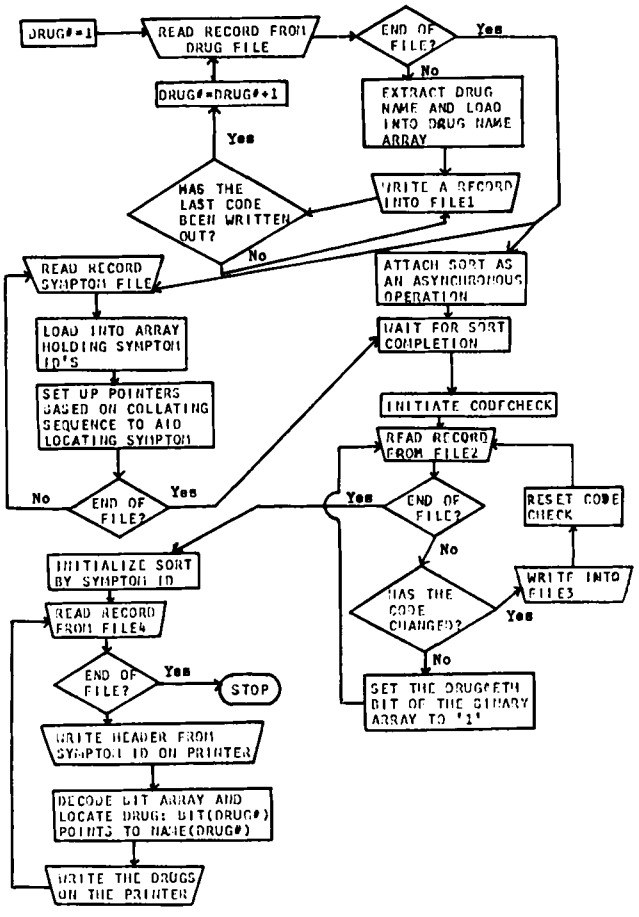


DRUG FILE—a sequential input file, with each record organized as follows: NAME, CODE 1, CODE 2, CODE 3, . . ., CODE n, where NAME is the name of the drug, and CODE n represents a three-digit hexadecimal code for each side effect associated with the drug.

SYMPTOM FILE—a sequential input file, with each record organized as follows: CODE n, SYMPTOM-ID, WORD, where CODE n is the three-digit hexadecimal code for the symptom, SYMPTOM-ID is the name of the symptom, and WORD is a binary string of length 20 categorizing the symptom by anatomical region.

FILE 1, FILE 2, FILE 3, FILE 4—sequential input and output files used by the sort routine.

FILE 1 organization: CODE n, DRUG #; CODE n is the three-digit hexadecimal number code, and DRUG # is the relative position of the drug in the drug bank.

FILE 2—sorted output of FILE 1.

FILE 3—a sequential input file which is organized: SYMPTOM -ID, BINARY (504); BINARY (DRUG #) = '1' if the drug #eth drug has the side effect, otherwise it is = to '0'.

FILE 4—sorted output of FILE 3 alphabetically.

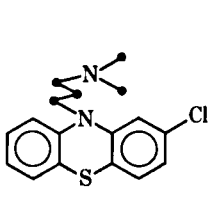
Scheme I—Flow chart of sorting program

and lists the drugs that have been reported to cause it. The program consists of three steps (Scheme I):

1. A procedure that creates records in a new file comprised of the code followed by the sequence number of the drug. For example, if the side effect code is 123 and it is associated with drug A, the 50th drug, the record will be "12350." This is done for the entire data bank, the output being stored on a temporary data set.

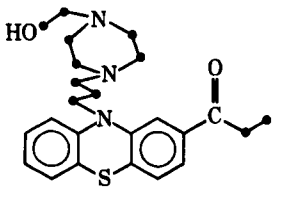
2. A link with the IBM Sorting Program, through the facilities of PL/I, that initiates sorting of the temporary data set based on the first three characters of each record. Output of 12301, 12338, 12345, and 12350 would then indicate that drugs 01, 38, 45, and 50 have the side effect corresponding to code 123.

3. A procedure that examines the output of the sort routine, retrieving the name of the side effect from the symptom bank based on the code and retrieving the names of the drugs based on their sequence number. The printout would have the following format: "THE FOLLOWING DRUGS HAVE THE SIDE EFFECT OF XYZ: A, B, C, D, E."



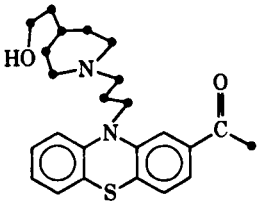
I

chlorpromazine



II

carphenazine



III

piperacetazine

RESULTS AND DISCUSSION

By sorting the data bank of 540 drugs, the following striking common features were found for the drugs that produce the extrapyramidal syndrome:

1. A phenothiazine derivative with a tertiary amino group which is separated from the ring by three carbon atoms [chlorpromazine (I), promazine, trifluoperazine, fluphenazine, mesoridazine, thioridazine, thiopropazate, acetophenazine, carphenazine (II), piperacetazine (III), *etc.*]. (In the structures illustrated here, the hydrogens on the carbon atoms are not shown.)

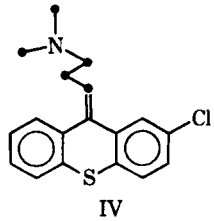
2. Isosteres of phenothiazine, such as chlorprothixene (IV), doxepin (V), and thiothixine, cause the same type of side effect (16–19).

3. Two compounds, trimethobenzamide (VI) and haloperidol (VII), are structurally quite different from phenothiazines and yet produce the extrapyramidal syndrome. By making Corey–Pauling–Kaltun (CPK) molecular models, it was noticed that these molecules could assume many different conformations, ranging from a more or less linear to a phenothiazine-like shape. When these molecules assumed the phenothiazine-like conformation, the tertiary amino group occupied the apex of a triangle, with two benzene rings occupying the baseline.

Since the pKa value of a tertiary amino group is approximately 10–11, it would be protonated under a physiological condition. If one assumes that the phenothiazine-like conformation is necessary for production of the extrapyramidal syndrome, then it appears that the protonated tertiary amino group would bind to a negatively charged site on the receptor while the coplanar ring system would occupy a flat nonpolar region by hydrophobic interactions.

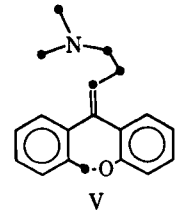
The distance between the tertiary amino group and the ring system of three carbon atoms appears to be very critical. It is known that phenothiazines with a two-carbon side chain show antiparkinsonian and antihistamine effects (20).

Since the drugs were not administered on an equimolar basis, no attempt was made to correlate the frequency of occurrence or the



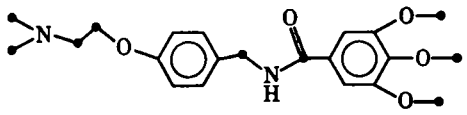
IV

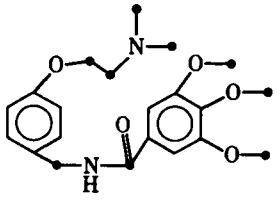
chlorprothixene



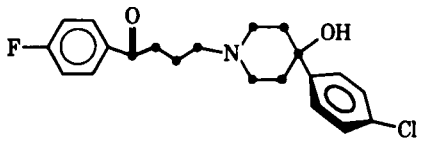
V

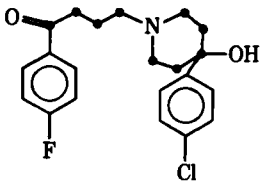
doxepin



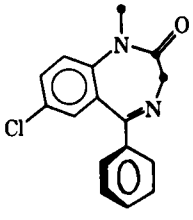


VI
trimethobenzamide

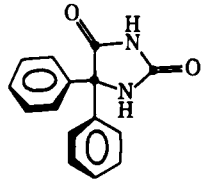




VII
haloperidol



VIII
diazepam



IX
diphenylhydantoin

degree of severity of the extrapyramidal syndrome with the structure. However, it is generally known that extrapyramidal effects are most commonly associated with the piperazine derivatives (21).

In clinical applications, it is known that tranquilizers like diazepam do not give extrapyramidal effects. Comparison of the CPK model of diazepam with those of phenothiazines revealed significant differences. An X-ray crystallographic study (22) showed that the phenyl group in diazepam (VIII) made an angle of 124° with the chlorophenyl ring. In the same study it was found that diphenylhydantoin (IX), a potent anticonvulsant, exhibited very similar structural features with diazepam, which is a tranquilizer and a muscle relaxant and is also used in treating emergency cases of status epilepticus (23). The two phenyl groups of diphenylhydantoin lie at angles of 114 and 113° to the hydantoin ring and at an angle of 90° to each other. These are, of course, quite different from the phenothiazines where the two benzene rings are essentially coplanar. Furthermore, neither of these two anticonvulsant drugs possesses a tertiary amino group.

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Effects of Antihistamine–Epinephrine Combinations on Adjuvant Arthritis in Rats

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Abstract □ Three antihistaminic agents—diphenhydramine, doxylamine, and methapyrilene—alone and in combination with epinephrine, were examined for their effectiveness against adjuvant arthritis in Wistar–Lewis inbred rats. Simultaneous administration of diphenhydramine (75 mg./kg. p.o.) and epinephrine (0.4 mg./kg. s.c.) reduced adjuvant-injected and contralateral noninjected hind-paw volumes during a 21-day dosing period as compared to saline-treated controls; however, considerable toxicity was evident at these doses. Protective activity was minimal or absent when lower dosage combinations, which produced no overt evidence of toxicity, were employed. Doxylamine and methapyrilene combinations with epinephrine were effective only against the primary response to adjuvant administration, *i.e.*, early swelling of the injected paw. The individual agents were largely ineffective against adjuvant disease in rats. Sustained protective activity could be achieved only with dosage combinations that elicited toxic manifestations; thus, the potential utility of antihistamine–epinephrine combinations appears limited.

Keyphrases □ Antihistamine–epinephrine combinations—effect on adjuvant arthritis, rats □ Diphenhydramine with epinephrine—effect on adjuvant arthritis, rats □ Doxylamine with epinephrine—effect on adjuvant arthritis, rats □ Methapyrilene with epinephrine—effect on adjuvant arthritis, rats □ Arthritis, adjuvant—effect of antihistamine–epinephrine combinations, rats

Adjuvant arthritis in rats is a widely investigated animal model of chronic inflammation. This experimental disorder, induced by the injection of Freund's complete adjuvant or of mycobacteria suspended in oil, is considered to be an example of delayed hypersensitivity reactions against antigen(s) associated with the peptide-glycolipids of the wax D fraction of mycobacteria (1). The disease cannot be transferred by serum from a sensitized animal, but transmission *via* lymphoid cells has been achieved (2). Certain manifestations of adjuvant-induced arthritis resemble those of clinical disorders such as rheumatoid arthritis, ankylosing spondylitis, and Reiter's disease (3–6). Immunosuppressants and several other classes of drugs (7–9) have been employed to modify this inflammatory process.

Histamine has been implicated as one mediator of the early stages of allergic and inflammatory reactions (10). Experimental evidence exists that histamine antagonism can alter the development of adjuvant arthritis. Pelczarska (11) reported that hypostamine, a histidine decarboxylase inhibitor, reduced the inflammation and edema associated with the developing disease. Perrine and Takesue (12) and Walz *et al.* (9) demonstrated a similar effect with chlorpheniramine. In a series of publications, Henson and coworkers (13–15) examined the inflammatory suppressive effect of a combination of epinephrine and propiomazine, a phenothiazine derivative with antihistaminic and other "protective" properties (16). Henson and his associates postulated that epinephrine might enhance the anti-inflammatory activity of propiomazine by increasing lymphoid cell susceptibility to the actions of the latter drug. An inhibitory effect was demonstrated on the development of adjuvant arthritis (13), the Arthus reaction (14), and experimental allergic encephalomyelitis (15), and the suggestion was made that similar drug combinations might offer an alternative approach to treatment with toxic immunosuppressants.

The purpose of this study was to examine the effects of three antihistaminic agents—diphenhydramine, doxylamine, and methapyrilene—alone and in combination with epinephrine, on adjuvant arthritis in rats.

EXPERIMENTAL

Male Wistar–Lewis inbred rats[1], weighing initially between 100 and 130 g., were used throughout this study. Food and water were supplied *ad libitum*. The animals were assigned randomly to treatment groups. Adjuvant arthritis was produced by a single subcutaneous injection (20-gauge needle) of 0.3 mg. of *Mycobacterium butyricum*[2], suspended in 0.06 ml. of light mineral oil NF, into a foot pad of the left hind paw. Normal control animals were injected with an identical volume of mineral oil vehicle.

Hind-paw volumes were determined plethysmographically by a modified method of Van Arman *et al.* (17). A mercury well was connected to a pressure transducer[3], and output from the transducer was led to a polygraph[4] equipped with a low-level d.c. preamplifier[5]. Throughout the study the instrument was calibrated repeatedly by the addition and withdrawal of a known volume of mercury. For measurement of hind-paw volumes, the hind limbs were immersed up to the hairline in the mercury pool. Determinations of paw volumes were made at regularly spaced intervals throughout each experiment; body weights were recorded daily.

The following drugs were studied: diphenhydramine hydrochloride[6], doxylamine succinate[7], methapyrilene hydrochloride[8], and epinephrine bitartrate. Phenylbutazone[9] was included as a positive control. Doses of all compounds are expressed as the base. Antihistaminic agents were dissolved in purified water; phenylbutazone was suspended with 2.5% (w/v) tragacanth with the aid of a blender[10]. Saline (0.9% NaCl), prepared with 0.15% sodium metabisulfite as a catecholamine antioxidant, was used as the solvent for epinephrine bitartrate.

Volumes administered were 10 ml./kg. orally and 2 ml./kg. subcutaneously. In each animal the oral dosing immediately preceded subcutaneous injection. Drugs were administered once daily for 21 consecutive days, beginning 1 day before adjuvant injection, with the exception of phenylbutazone in the methapyrilene–epinephrine study, in which the dosage schedule was reduced to 15 days. Normal control (mineral oil injected) and adjuvant control animals received 10 ml./kg. oral doses of water and 2 ml./kg. subcutaneous injections of saline–metabisulfite solution daily for the specified duration of each drug treatment schedule.

Six series of experiments were performed, four with diphenhydramine and epinephrine in various combinations and two with

[1] Obtained from Charles River Laboratories.
[2] Difco Laboratories, Detroit, Mich.
[3] Statham Model P23BC, 0–5 cm. Hg.
[4] Grass model 7B.
[5] Model 7P1B.
[6] Donated by Parke-Davis & Co., Detroit, Mich.
[7] Donated by Merrell-National Laboratories, Cincinnati, Ohio.
[8] Donated by Eli Lilly & Co., Indianapolis, Ind.
[9] Donated by Ciba Pharmaceutical Co., Summit, N. J.
[10] Waring.

Table I—Effect of Diphenhydramine and Epinephrine on Paw Edema and Body Weight Gain in Adjuvant Arthritic Rats

Treatment	Dose, mg./kg.	Hind-Paw Volume, ml.[a] Injected (Left) Paw Day 4	Injected (Left) Paw Day 11	Injected (Left) Paw Day 18	Injected (Left) Paw Day 26	Noninjected (Right) Paw Day 11	Noninjected (Right) Paw Day 18	Noninjected (Right) Paw Day 26	Body Weight Increase, g., Day 18	Number Surviving at Day 26 / Number Tested
Diphenhydramine	75	1.48	1.93	2.78	3.46	1.43	2.16	2.65	31.5	11/14
Diphenhydramine + epinephrine	75 0.4	1.31[d]	1.64[d]	2.24[b]	3.15	1.24[d]	1.65[b]	2.20[b]	38.0	7/14
Diphenhydramine	37.5	1.63	2.07	2.87	3.65	1.56	2.29	3.00	28.4	8/15
Diphenhydramine + epinephrine	37.5 0.4	1.38[d]	1.90	2.70	3.57	1.34[c]	2.01	2.38	51.2	9/14
Epinephrine	0.4	1.54	2.00	2.79	3.75	1.47	2.20	3.07	38.9	12/14
Phenylbutazone	50	1.50	1.76[b]	2.05[d]	2.94[d]	1.41	1.67[d]	2.33[b]	55.0	12/12
Normal control		1.21[d]	1.45[d]	1.45[d]	1.53[d]	1.38[c]	1.41[d]	1.49[d]	83.9	14/14
Adjuvant control		1.57[e] (1.4–1.9)	2.00 (1.7–2.4)	2.91 (1.9–4.2)	3.66 (2.3–4.6)	1.55 (1.3–2.0)	2.15 (1.4–2.7)	2.83 (1.9–3.5)	42.0 (13–77)	14/14

[a] All values represent means of the number of rats surviving to Day 26. [b] Differs from adjuvant control at $p < 0.05$. [c] Differs from adjuvant control at $p < 0.01$. [d] Differs from adjuvant control at $p < 0.001$. [e] Mean (range in parentheses).

Table II—Effect of Diphenhydramine and Epinephrine on Paw Edema and Body Weight Gain in Adjuvant Arthritic Rats

Treatment	Dose, mg./kg.	Hind-Paw Volume, ml.[a] Injected (Left) Paw Day 4	Injected (Left) Paw Day 11	Injected (Left) Paw Day 18	Noninjected (Right) Paw Day 11	Noninjected (Right) Paw Day 18	Body Weight Increase, g., Day 18	Number Surviving at Day 18 / Number Tested
Diphenhydramine + epinephrine	75 0.4	1.66[d]	1.89[c]	2.93[b]	1.32[d]	2.15[b]	49.3	11/11
Diphenhydramine + epinephrine	75 0.2	1.64[d]	1.95[b]	3.33	1.37[d]	2.50	54.1	11/11
Diphenhydramine + epinephrine	75 0.1	1.68[d]	1.97	3.20	1.37[d]	2.32	55.4	13/13
Diphenhydramine + epinephrine	75 0.05	1.79	2.03	3.47	1.35[d]	2.64	42.2	12/12
Adjuvant control		1.93[e] (1.6–2.3)	2.17 (1.8–2.6)	3.63 (2.8–4.7)	1.63 (1.4–2.1)	2.89 (1.5–4.0)	42.5 (24–72)	11/11

[a] All values represent means of the number of rats surviving to Day 18. [b] Differs from adjuvant control at $p < 0.05$. [c] Differs from adjuvant control at $p < 0.01$. [d] Differs from adjuvant control at $p < 0.001$. [e] Mean (range in parentheses).

either doxylamine–epinephrine or methapyrilene–epinephrine. The selection of doses was based upon preliminary dose-range studies, wherein the drugs were administered from 1 to 4 days. In these studies, the minimum symptomatic doses were approximated for each of the three antihistamines, alone and in combination with epinephrine (0.4 mg./kg.). Henson and Brunson (13) stated that repeated administration of epinephrine, at a dose of 0.5 mg./kg. s.c., produced about a 50% incidence of mortality. In the present experiments, epinephrine was employed at doses ranging from 0.05 to 0.4 mg./kg.

All paw volumes and body weight data represent mean values derived from the number of rats surviving at the termination of the experimental period. Statistical significances of differences between drug-treated and adjuvant control animals were determined by an analysis of variance.

RESULTS

Development and Course of Adjuvant Disease—Subcutaneous injection of adjuvant produced swelling of the injected hind paw within 24 hr. This initial inflammatory reaction (primary response) usually reached a peak at Day 4 and became stabilized at Days 7–11. Thereafter, a rapid and marked further increase in swelling developed. Progression of the disease was followed to Day 26, at which time hind-paw volumes had increased 110–280% above normal paw volumes prior to adjuvant injection. Swelling of the contralateral noninjected limbs and nodules on the ears and tail appeared in approximately 90% of the animals between Days 7 and 11 and increased progressively in severity to Day 26. Paw volumes of the noninjected hind limbs ranged from 60 to 235% above normal. Enlargement of both noninjected and injected paws, which occurred from Day 7 and thereafter, was designated as the secondary response and constituted the disseminated disease. In rats with adjuvant disease, the gain in body weight was significantly diminished in comparison to normal control animals.

Diphenhydramine in Adjuvant Disease—Table I contains a summary of results obtained with diphenhydramine administered alone and in conjunction with epinephrine. The hind-paw and body weight gain values represent data pooled from two replicate experiments.

A combination of diphenhydramine (75 mg./kg. p.o.) and epinephrine (0.4 mg./kg. s.c.) significantly reduced noninjected and injected paw volumes during the 21-day period of dosing as compared to saline-treated adjuvant control animals. On Day 26, injected paw volumes of drug-treated animals were not significantly different from controls. A lower dose of diphenhydramine (37.5 mg./kg.), in combination with epinephrine, was active only against the primary response. The apparent effectiveness of these treatments was tempered by the observed toxicity.

Additional studies were performed to determine whether protective activity could be obtained at essentially nontoxic doses of the drug combination. Epinephrine, at doses ranging from 0.05 to 0.4 mg./kg., was administered in conjunction with diphenhydramine at doses of 75 mg./kg. (Table II) and 18.8 mg./kg. (Table III). Mortality did not occur in either of these 18-day studies, in contrast to the previous 26-day experiments (Table I).

The duration of protective activity of epinephrine with diphenhydramine, 75 mg./kg. (Table II), was related to the dose of epinephrine. The regimen of diphenhydramine, 75 mg./kg., with epinephrine, 0.4 mg./kg., was effective throughout the 18-day experimental period. Doses of 0.2 and 0.1 mg./kg. of epinephrine in conjunction with 75 mg./kg. of diphenhydramine reduced paw volumes only to Day 11; the lowest dose of epinephrine (0.05 mg./kg.) in combination with 75 mg./kg. of diphenhydramine was largely ineffective. With the exception of a statistically significant decrease

Table III—Effect of Diphenhydramine and Epinephrine on Paw Edema and Body Weight Gain in Adjuvant Arthritic Rats

Treatment	Dose, mg./kg.	Hind-Paw Volume, ml.[a] Injected (Left) Paw Day 4	Injected (Left) Paw Day 11	Injected (Left) Paw Day 18	Noninjected (Right) Paw Day 11	Noninjected (Right) Paw Day 18	Body Weight Increase, g., Day 18	Number Surviving at Day 18 / Number Tested
Diphenhydramine + epinephrine	18.8 0.4	1.64[b]	2.28	3.52	1.56	2.42	54.7	11/11
Diphenhydramine + epinephrine	18.8 0.2	1.66	2.29	3.45	1.52	2.44	49.1	11/11
Diphenhydramine + epinephrine	18.8 0.1	1.73	2.31	3.98	1.51	2.47	45.8	11/11
Diphenhydramine + epinephrine	18.8 0.05	1.89	2.52	4.00	1.60	2.64	35.0	11/11
Diphenhydramine	18.8	1.85	2.66	4.02	1.51	2.59	33.6	11/11
Adjuvant control		1.92[c] (1.5–2.6)	2.44 (1.6–4.1)	3.58 (2.1–4.4)	1.53 (1.2–1.9)	2.46 (1.5–3.2)	42.4 (14–87)	11/11

[a] All values represent means of the number of rats surviving to Day 18. [b] Differs from adjuvant control at $p < 0.05$. [c] Mean (range in parentheses).

Table IV—Effect of Doxylamine and Epinephrine on Paw Edema and Body Weight Gain in Adjuvant Arthritic Rats

Treatment	Dose, mg./kg.	Hind-Paw Volume, ml.[a] Injected (Left) Paw Day 4	Injected (Left) Paw Day 11	Injected (Left) Paw Day 18	Injected (Left) Paw Day 26	Noninjected (Right) Paw Day 11	Noninjected (Right) Paw Day 18	Noninjected (Right) Paw Day 26	Body Weight Increase, g., Day 18	Number Surviving at Day 26 / Number Tested
Doxylamine	100	1.64	2.11	3.21	3.59[b]	1.37	2.21	2.70	39.1[b]	7/8
Doxylamine + epinephrine	100 0.4	1.30	1.90	3.10	4.20	1.20	1.50	2.20	36.0	1/8
Doxylamine	50	1.83	2.19	3.38	3.90	1.49	2.31	2.60	33.5	8/8
Doxylamine + epinephrine	50 0.4	1.53[c]	2.20	3.30	4.15	1.45	2.33	2.75	47.3[b]	6/6
Epinephrine	0.4	1.47[b]	1.93	2.90	3.80	1.37	2.30	2.97	48.3	3/8
Phenylbutazone	50	1.56[d]	1.85	2.50[d]	3.41[d]	1.34	1.79[b]	2.27[b]	43.5[b]	8/8
Normal control		1.38[d]	1.54[d]	1.61[d]	1.73[d]	1.45	1.48[d]	1.57[d]	98.9[d]	7/8
Adjuvant control		1.85[e] (1.6–2.2)	2.00 (1.7–2.3)	3.51 (2.8–4.1)	4.11 (3.4–4.6)	1.44 (1.2–1.8)	2.37 (1.3–3.0)	3.16 (1.5–4.4)	29.7 (21–39)	7/8

[a] All values represent means of the number of rats surviving to Day 26. [b] Differs from adjuvant control at $p < 0.05$. [c] Differs from adjuvant control at $p < 0.01$. [d] Differs from adjuvant control at $p < 0.001$. [e] Mean (range in parentheses).

in injected paw volumes at Day 4 in rats treated with the high dose of epinephrine (0.4 mg./kg.) in conjunction with 18.8 mg./kg. of diphenhydramine, combinations of epinephrine with this dose of diphenhydramine did not evidence protective activity against adjuvant arthritis (Table III).

Doxylamine and Methapyrilene in Adjuvant Disease—Evaluation of doxylamine and methapyrilene, alone and in combination with epinephrine, revealed that these treatments had little effect on the progression of adjuvant disease. For example, doxylamine (50 mg./kg.) in combination with epinephrine (0.4 mg./kg.) reduced the primary edema only at Day 4 (Table IV). Similarly, methapyrilene (15 and 30 mg./kg.) in combination with epinephrine, and methapyrilene alone (30 mg./kg.) reduced inflammation of the injected hind paw at Day 4 (Table V). No other significant differences from adjuvant controls were noted with respect to primary or secondary reactions. Animals treated with doxylamine (100 mg./kg.) and doxylamine (50 mg./kg.) in combination with epinephrine evidence significantly greater body weight gain than the adjuvant controls; a similar pattern was observed with methapyrilene (30 mg./kg.) and methapyrilene (15 mg./kg.) in combination with epinephrine. The combination of doxylamine (100 mg./kg.) and epinephrine (0.4 mg./kg.) was extremely toxic; 87% of the animals in this group died prior to termination of the experiment.

In all experiments, phenylbutazone (50 mg./kg.) significantly reduced both injected and noninjected hind-paw volumes as compared to adjuvant control animals. When phenylbutazone was given for 21 days, activity persisted during the latter stages of the 26-day experimental period (Tables I and IV); when the dosage schedule was reduced to 15 days, activity was noted only against the primary response (Table V). Extended treatment with 50 mg./kg. of phenylbutazone was not associated with grossly apparent toxicity. Epinephrine (0.4 mg./kg.) alone was essentially inactive against adjuvant disease; however, this dose produced overt toxicity.

DISCUSSION

The combination of epinephrine and diphenhydramine afforded the greatest degree of protection against adjuvant arthritis in this series of experiments. However, this protective activity was frequently associated with evidence of drug-induced toxic effects. Animals treated with high dose combinations (diphenhydramine, 37.5 and 75 mg./kg., with epinephrine, 0.4 mg./kg.) lost their well-groomed appearance, became lethargic, and exhibited respiratory difficulties. Alopecia of the neck and back (epinephrine injection site) was apparent after 8–12 days of dosing and was not reversible upon cessation of treatment. When mortality occurred, it was preceded by dyspnea, cyanosis, loss of righting reflex, and clonic convulsions. Autopsy revealed macroscopic cardiac lesions and pulmonary hemorrhage, a profile that suggests that lethality might be due primarily to epinephrine rather than to the antihistamine.

Attempts to dissociate drug toxicity from protective activity by reducing the dose levels of both diphenhydramine and epinephrine proved largely unsuccessful. Although lower dosage combinations of diphenhydramine and epinephrine did not produce overt toxicity (based on gross appearance, behavior, body weight gain, and survival incidence), they failed to provide sustained inhibition of adjuvant disease.

Henson and Brunson (13) proposed that the enhanced activity of propiomazine with epinephrine resulted from an epinephrine-induced increase in lymphoid cell susceptibility to anti-inflammatory actions of propiomazine. The possibility exists that antihistamine–epinephrine combinations may act through a similar

Table V—Effect of Methapyrilene and Epinephrine on Paw Edema and Body Weight Gain in Adjuvant Arthritic Rats

Treatment	Dose, mg./kg.	Hind-Paw Volume, ml.[a] Injected (Left) Paw Day 4	Injected (Left) Paw Day 11	Injected (Left) Paw Day 18	Noninjected (Right) Paw Day 11	Noninjected (Right) Paw Day 18	Body Weight Increase, g., Day 18	Number Surviving at Day 18 / Number Tested
Methapyrilene	30	2.09[b]	2.60	4.05	1.67	2.93	5.9[c]	9/9
Methapyrilene + epinephrine	30 0.4	1.97[d]	1.97	3.73	1.40	2.78	−0.5	9/10
Methapyrilene	15	2.66	2.56	3.94	1.73	2.94	−3.0	9/9
Methapyrilene + epinephrine	15 0.4	2.04[b]	2.18	4.15	1.47	2.95	8.6[d]	8/10
Phenylbutazone	50	2.08[b]	2.38	3.44	1.64	2.51	13.7[b]	9/9
Normal control		1.64[d]	1.68[d]	1.87[d]	1.58	1.78[d]	71.9[d]	9/9
Adjuvant control		2.42[e] (2.0–2.9)	2.18 (1.7–2.6)	3.69 (2.9–4.3)	1.54 (1.3–2.1)	2.89 (1.4–3.4)	−12.0 (−35–+5)	9/9

[a] All values represent means of the number of rats surviving to Day 18. [b] Differs from adjuvant control at $p < 0.05$. [c] Differs from adjuvant control at $p < 0.01$. [d] Differs from adjuvant control at $p < 0.001$. [e] Mean (range in parentheses).

mechanism, but alternative explanations are also plausible. Their effectiveness may be due to competitive inhibition of histamine by the antihistaminic agent, together with physiological antagonism by epinephrine of certain critical effects of histamine, *i.e.*, microvascular dilation and increased capillary permeability. In support of this concept, all antihistamine–epinephrine dosage combinations examined in this study significantly reduced the primary or early response to adjuvant, a phase during which histamine mediation of the inflammatory reaction is reputedly prominent (18). Of the three antihistaminic drugs evaluated, only diphenhydramine in combination with epinephrine produced extended protection; the reason for this longer duration of activity is not apparent. This protective effect against adjuvant disease could be achieved only at dosage levels that elicited overt toxic manifestations. Thus, the potential usefulness of such a drug combination appears limited.

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Interaction of Dextroamphetamine Sulfate with Dextrates in Solution

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Abstract □ The interaction between dextroamphetamine sulfate and dextrates (a partially hydrolyzed starch composed of 92% dextrose combined with 8% higher saccharides) was studied in buffered solutions at three different temperatures and dextroamphetamine sulfate concentrations. On heating, solutions containing dextroamphetamine sulfate and dextrates became progressively darker than solutions containing dextroamphetamine sulfate or dextrates alone. Also, a new absorption maximum appeared at 298 nm. in the solutions containing dextroamphetamine sulfate and dextrates. The rate of browning in these solutions increased with increasing temperature and pH and decreased with increasing dextroamphetamine sulfate concentration. From the relationship shown in the plots of absorbance against time, the browning reaction was assumed to follow an apparent zero-order rate law and the rate constants, k, were calculated for all solutions containing dextroamphetamine sulfate and dextrates at pH 6 and 8 at 50, 60, and 70°. Arrhenius-type plots were also obtained and were used to approximate the browning rate at 25°. By using TLC, IR spectroscopy, and elemental analysis, the brown material isolated from the discolored solutions was identified as amphetamine-hydroxymethylfurfural. A Schiff base-type reaction was postulated involving the primary amine and the carbonyl groups of the sugar.

Keyphrases □ Dextroamphetamine sulfate and dextrates in solution—browning reaction, effect of temperature and pH, isolation of amphetamine-hydroxymethylfurfural □ Dextrates and dextroamphetamine sulfate in solution—browning reaction, effect of temperature and pH, isolation of amphetamine-hydroxymethylfurfural □ Amphetamine-hydroxymethylfurfural—isolated as brown material in solutions of dextroamphetamine sulfate and dextrates

Koshy *et al.* (1) studied the browning of spray-dried lactose and USP conventionally processed lactose in aqueous solutions buffered to various pH's. Spray-dried lactose was found to have a UV absorption maximum, whereas the USP conventionally processed and analytical reagent grade lactose did not. A relationship between the intensity of this maximum and the 5-hydroxymethylfurfural content was shown. This absorbance was related to browning. The pH profiles for lactose, dextrose, and galactose were similar except that galactose solutions discolored over a wider pH range, 5 and above, whereas the other two sugars discolored at pH 7 and above. The relative intensity of the browning of the three sugars was lactose > galactose > dextrose. These studies were conducted in the absence of added amines. The same authors also studied the browning of lactose spray-dried powder at different temperatures and relative humidities. Their results indicate that a combination of heat and moisture accelerates browning more than either heat or humidity. Castello and Mattocks (2) reported that the presence of amines accelerated the browning reaction in tablets containing lactose, particularly when the tablets were stored at elevated temperature and high humidity.

Brownley and Lachman (3) showed that the browning of lactose was related to the amount of free 5-hydroxymethylfurfural in the lactose. This relationship between free 5-hydroxymethylfurfural and browning was also found to exist for dextrose (4). However, the relative browning of dextrose and lactose formulations indicated that lactose was more susceptible to browning than dextrose.

The browning of lactose and dextrose in the presence of added amines was shown by Duvall *et al.* (5) to be predominantly a primary amine–carbonyl reaction. A relative visual comparison of the formulations under all accelerated conditions indicated that amine browning was worse with dextrose than with lactose. This type of browning, which has been referred to as Maillard-type browning (6), requires a relatively low order of energy for its initiation and exhibits autocatalytic qualities once it has begun.

Dextrates[1] were recently developed for use as an excipient in direct compression tablet production. Dextrates are produced by the controlled hydrolysis of starch and are composed of 92% dextrose combined with 8% higher saccharides. Henderson and Bruno (7) studied the physical properties and stability of dextrates with respect to tableting by direct compression. With regard to color stability, tablets prepared with dextrates and stored at different temperatures and humidities showed no substantial improvement over those prepared with spray-dried lactose.

To study and explain the browning reaction reported (7) to occur in solid–solid mixtures containing an amine and dextrates, it was necessary to investigate the effect of temperature and pH on the rate of the browning reaction in solutions containing dextrates and an amine such as dextroamphetamine sulfate. A later study will report the interaction between an amine and dextrates in solid–solid mixtures.

EXPERIMENTAL[2]

Reagents—The following were used: dextroamphetamine sulfate USP, m.p. > 300°; dextrates, m.p. 150–152°; silica gel GF_{254}[3] for TLC; citric acid monohydrate[4]; analytical reagent grade dibasic anhydrous sodium phosphate[5]; and iodine USP.

Procedure—*Transmittance Measurements*—The reaction of dextrates with dextroamphetamine sulfate was studied in buffered aqueous solution at pH 6 and 8 using citric acid–disodium hydrogen phosphate buffers. These pH values were chosen since the browning of lactose, dextrose, and galactose solutions was reported

[1] Celutab, Penick and Ford Ltd., Cedar Rapids, Iowa.

[2] All transmittance measurements were made using the Beckman DK-2 and DU spectrophotometers. IR spectra were obtained using a Beckman IR-10 spectrophotometer equipped with sodium chloride optics and 0.1-mm. cells. Constant temperature was maintained with a Sargent heater and circulator equipped with a microset thermoregulator. All pH measurements were performed using a Beckman Zeromatic II pH meter.

[3] Hexagon Laboratories, New York, N. Y.

[4] Fisher certified, Fisher Scientific Co., Fair Lawn, N. J.

[5] Mallinckrodt Chemical Works, St. Louis, Mo.

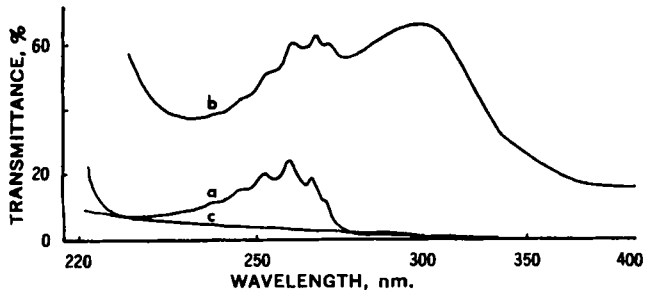


Figure 1—*Absorption spectra of solutions containing:* (a) *1% dextroamphetamine sulfate,* (b) *1% dextroamphetamine sulfate and 10% dextrates, and* (c) *10% dextrates in citric acid–phosphate buffer, pH 8. All solutions were heated for 2 days at 60°.*

(1) to be minimal at pH 5 and accelerated at pH 8 in the absence of added amines. Duvall *et al.* (5) studied the reaction of dextrose and galactose with amphetamine in pH 8 buffer and reported that browning was apparent in 1 day with both monosaccharides. Three solutions were prepared: (*a*) a 1% dextroamphetamine sulfate solution, (*b*) a solution containing 1% dextroamphetamine sulfate and 10% dextrates, and (*c*) a 10% dextrate solution. The solutions were heated at 60 ± 0.1° in a constant-temperature oil bath for 2 days. At the end of this time, 2-ml. aliquots were withdrawn and diluted to 100 ml. with water. The absorption spectra of these solutions were determined from 700 to 220 nm. using a spectrophotometer[6]. The blank consisted of a 2-ml. aliquot of the buffer solution that had been diluted to 100 ml. with water. The absorption spectra obtained at pH 8 are shown in Fig. 1. Similar spectra were obtained at pH 6.

Identification of Reaction Products—Identification of the reaction products was carried out using TLC techniques and IR spectrometry. TLC plates were prepared with an applicator[7], using silica gel GF_{254} (0.25 mm. thick) as an adsorbent. Solutions containing: (*a*) 1% dextroamphetamine sulfate, (*b*) 1% dextroamphetamine sulfate plus 10% dextrates, and (*c*) 10% dextrates, buffered to pH 8 and heated for 2 days at 60° were spotted on a prepared plate. Also spotted on the plate were: (*d*) a chloroform solution of the precipitated brown material which had appeared in Solution (*b*), and (*e*) an aqueous solution containing 1.0% dextroamphetamine sulfate and 0.05% 5-hydroxymethylfurfural. The lower phase of a mixture of ethyl acetate–pyridine–water (2:1:2) was used for development, and iodine vapor and UV light were used for visualization. A composite chromatogram is shown in Fig. 2.

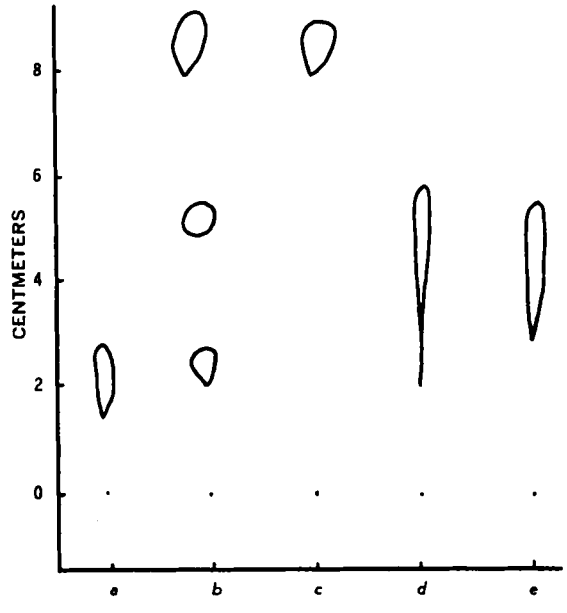


Figure 2—*Composite thin-layer chromatogram of dextroamphetamine sulfate–dextrates solutions (buffered to pH 8 and heated for 2 days at 60°). Key:* a, *1% dextroamphetamine sulfate solution;* b, *1% dextroamphetamine sulfate and 10% dextrate solution;* c, *10% dextrate solution;* d, *chloroform solution of precipitate from solution* b; *and* e, *1% dextroamphetamine sulfate and 0.05% 5-hydroxymethylfurfural solution. The spots were detected by iodine vapor and UV light.*

[6] Beckman DK-2.
[7] Beckman model S.

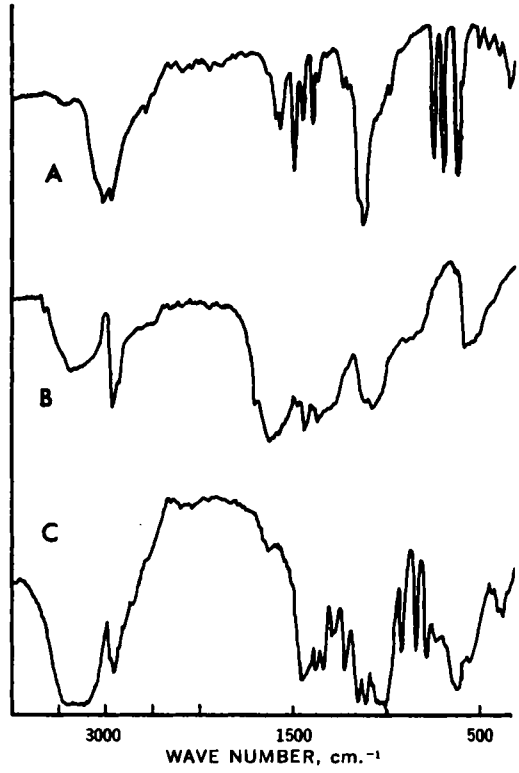


Figure 3—*IR spectra of:* (*A*) *dextroamphetamine sulfate,* (*B*) *the brown precipitate isolated from a solution containing 1% dextroamphetamine sulfate and 10% dextrates, and* (*C*) *dextrates. All samples were obtained after heating for 2 days at 60°.*

IR spectra of dextroamphetamine sulfate and dextrates were obtained, using KBr pellets, after heating each powder for 2 days at 60° in a constant-temperature oven. The IR spectrum of the brown material obtained from Solution (*b*) was determined in chloroform (Fig. 3).

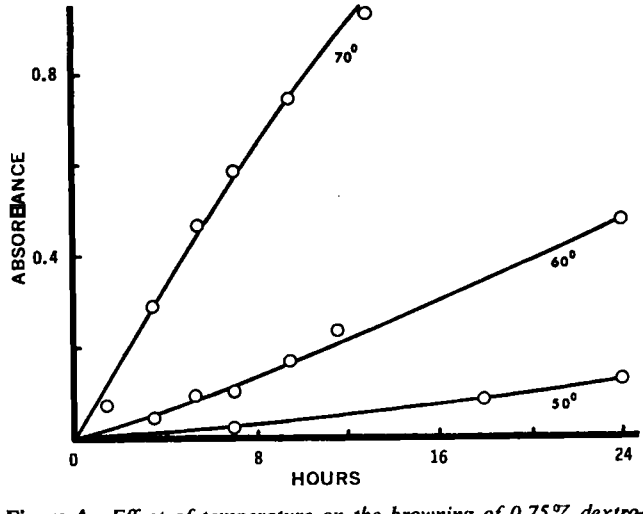


Figure 4—*Effect of temperature on the browning of 0.75% dextroamphetamine sulfate + 10% dextrate solutions in citrate–phosphate buffer, pH 8.*

Table I—Apparent Zero-Order Rate Constants for the Browning of Dextroamphetamine Sulfate–Dextrate Solutions at pH 6

Temperature	0.75% Dextroamphetamine Sulfate + 10% Dextrates—k, day^{-1}	0.75% Dextroamphetamine Sulfate + 10% Dextrates—log k	1% Dextroamphetamine Sulfate + 10% Dextrates—k, day^{-1}	1% Dextroamphetamine Sulfate + 10% Dextrates—log k	1.25% Dextroamphetamine Sulfate + 10% Dextrates—k, day^{-1}	1.25% Dextroamphetamine Sulfate + 10% Dextrates—log k
50°	0.115	−0.9393	0.070	−1.1549	0.065	−1.1871
60°	0.286	−0.5436	0.200	−0.6990	0.100	−1.0000
70°	1.063	0.0265	0.800	−0.0969	0.450	−0.3468

Table II—Apparent Zero-Order Rate Constants for the Browning of Dextroamphetamine Sulfate–Dextrate Solutions at pH 8

Temperature	0.75% Dextroamphetamine Sulfate + 10% Dextrates—k, day^{-1}	0.75% Dextroamphetamine Sulfate + 10% Dextrates—log k	1% Dextroamphetamine Sulfate + 10% Dextrates—k, day^{-1}	1% Dextroamphetamine Sulfate + 10% Dextrates—log k	1.25% Dextroamphetamine Sulfate + 10% Dextrates—k, day^{-1}	1.25% Dextroamphetamine Sulfate + 10% Dextrates—log k
50°	0.326	−0.4868	0.220	−0.6576	0.140	−0.8539
60°	0.834	−0.0788	0.652	−0.1858	0.400	−0.3979
70°	1.810	0.2577	1.313	0.1183	0.813	−0.0899

Effect of Temperature, pH, and Drug Concentration on Browning Reaction in Aqueous Solution—Solutions containing 10% dextrates and 0.75, 1.0, and 1.25% dextroamphetamine sulfate, in pH 6 and 8 citric acid–disodium hydrogen phosphate buffers, were placed in a constant-temperature oil bath at 50, 60, and 70 ± 0.1°. Buffer solutions containing 10% dextrates were also heated in the constant-temperature baths for use as blanks. At periodic time intervals, 2-ml. aliquots of these solutions were withdrawn and diluted to 100 ml. with water. The absorbance of each solution was determined at 298 nm. in a spectrophotometer[8] equipped with 1-cm. silica cells, using the diluted dextrate solutions as blanks. Plots of absorbance against time were prepared for all solutions at pH 6 and 8 and at 50, 60, and 70 ± 0.1°. Because of the similarity in the plots, the effect of temperature on the browning reaction is shown in Fig. 4 for a solution containing 0.75% dextroamphetamine sulfate and 10% dextrates at pH 8 and the effect of pH on the browning reaction is shown in Fig. 5 for a solution containing 1% dextroamphetamine sulfate and 10% dextrates at 70°. From the relationships shown, the browning reaction was assumed to follow an apparent zero-order rate law and the rate constants, k, were calculated for all solutions containing dextroamphetamine sulfate and dextrates at pH 6 and 8 at 50, 60, and 70°. The calculated values are shown in Tables I and II. Arrhenius-type plots indicating the temperature dependence for the browning of dextroamphetamine sulfate–dextrate solutions in citrate–phosphate buffer, pH 8, are shown in Fig. 6.

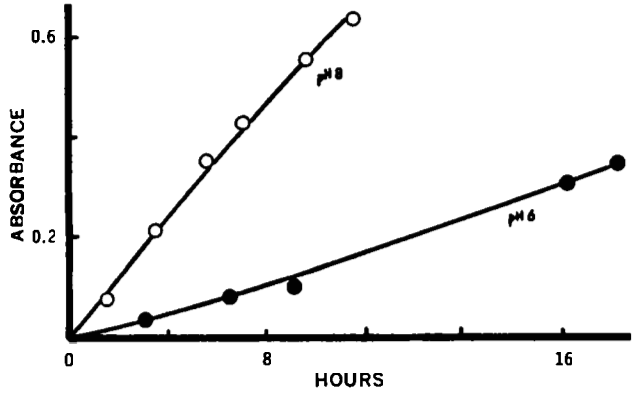


Figure 5—*Effect of pH on the browning of 1% dextroamphetamine sulfate + 10% dextrate solutions at 70°.*

[8] Beckman DU.

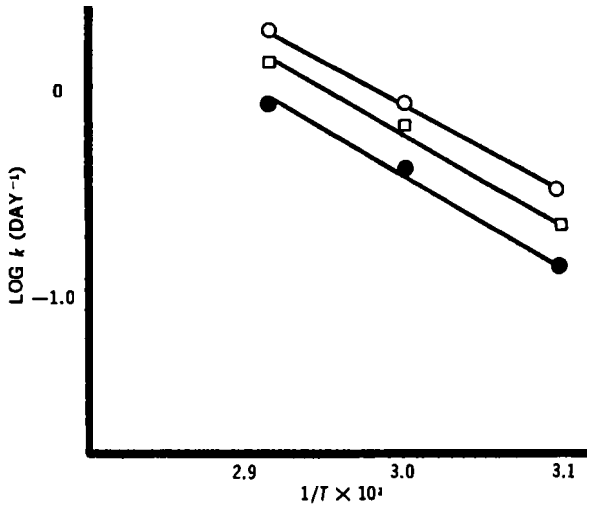


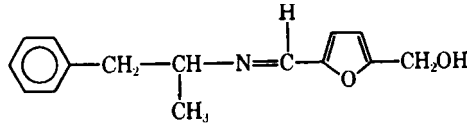
Figure 6—*Arrhenius-type plots showing the temperature dependence for the browning of dextroamphetamine sulfate–dextrate solutions in citrate–phosphate buffer, pH 8. Key: ○, 0.75% dextroamphetamine sulfate + 10% dextrates; □, 1% dextroamphetamine sulfate + 10% dextrates; and ●, 1.25% dextroamphetamine sulfate + 10% dextrates.*

RESULTS AND DISCUSSION

The absorption spectra shown in Fig. 1 indicate a marked change in the absorbance curve of Solution (*b*) when compared with the spectra obtained on Solutions (*a*) and (*c*) at pH 8. The results obtained at pH 6 were the same as those obtained at pH 8, except for a decrease in the rate of browning at pH 6. On heating at 60°, Solution (*b*) became progressively darker than Solutions (*a*) and (*c*), and a brown-black precipitate appeared in Solution (*b*) after 2 days at 60°. The intensity of absorbance at 257 nm. increased considerably in curve (*b*), and a new peak appeared at 298 nm. On dilution of Solution (*b*) with water, there was a marked decrease in the absorbance at 298 nm. This phenomenon, associated with the maximum at 298 nm., is consistent with a report (8) concerning the absorption characteristics of a solution following the reaction of a primary amine with a carbonyl compound. Pigman (9) showed that reaction occurs between primary amines and aldehyde groups of open chain forms of the carbohydrates to form a Schiff base, as well as with ring forms of the carbohydrates to form glycosylamines. In either reaction, the resulting solution was colored.

Solution (*a*) did not discolor on heating at 60° and it showed a very slight increase in the intensity of the absorbance at 257 nm., the characteristic absorption maximum of dextroamphetamine sulfate, when compared with an unheated dextroamphetamine sulfate solution.

After Solution (*c*) was heated, an absorption maximum appeared at about 283 nm., which was not present in the spectrum of the unheated solution. The undiluted solution was light brown in color; however, when a 2-ml. aliquot was diluted to 100 ml. with water, the solution became colorless and the absorption peak at 283 nm. disappeared. The appearance of an absorption maximum at 283 nm. and discoloration of the dextrate solution were attributed to the decomposition of dextrose, the major constituent (92%) in dextrates. Wolfram *et al.* (10) reported that the decomposition involved the conversion of dextrose to 5-hydroxymethylfurfural, with the subsequent breakdown of this material to formic and levulinic acids and a colored material. Scallet and Gardner (11) and Joslyn (12) at-



amphetamine-hydroxymethylfurfural

tributed the discoloration of dextrose solutions to the polymerization of 5-hydroxymethylfurfural.

The discoloration of the dextrate solution was negligible compared to the browning reaction that occurred in Solution (*b*) which contained both dextrates and dextroamphetamine sulfate. Duvall *et al.* (4) reported that amines increased the extent of the browning reaction in tablets prepared from dextrose. The solubility characteristics of the brown material obtained from Solution (*b*), which had been heated for 2 days at 60°, differed from those of the brown material obtained from Solution (*c*). The residue from Solution (*b*) was soluble in chloroform, ethanol, and acetone and could be extracted from aqueous solution by these organic solvents. However, the residue from Solution (*c*) was very water soluble and could not be extracted with organic solvents.

Identification of the reaction product(s) was carried out using TLC. A composite chromatogram is shown in Fig. 2. It indicated the presence of one reaction produced at R_f 0.513, which was present in Solutions (*b*), (*d*), and (*e*).

As shown in Fig. 3, the appearance of a strong absorption band at 1650 $cm.^{-1}$ (C=N) in the IR spectrum of the brown material isolated from Solution (*b*) offered further proof of the occurrence of a reaction between the amine and the carbonyl group of the sugar (dextrates). Since decomposition of dextrose involves its conversion to 5-hydroxymethylfurfural, the reaction product was postulated as amphetamine-hydroxymethylfurfural.

The brown residue obtained from Solution (*b*) was subjected to elemental analysis.

Anal.—Calc. for $C_{15}H_{17}NO_2$ (amphetamine-hydroxymethylfurfural): C, 74.07; H, 6.99; N, 5.76. Found: C, 73.01; H, 6.69; N, 5.98.

Elemental analysis of the precipitate was compatible with the postulated structure of the reaction product, amphetamine-hydroxymethylfurfural.

Results of the study on the effect of temperature, pH, and drug concentration on the rate of the browning reaction in solutions containing dextroamphetamine sulfate and dextrates are shown in Figs. 4 and 5 and Tables I and II. Figures 4 and 5 show that the rate of browning was faster at higher temperatures and at pH 8. In fact, the browning reaction was so slow at 50° and pH 6 that no discoloration occurred even after heating for 1 day. Data in Tables I and II also show that the rate of browning increased substantially with decreasing concentrations of dextroamphetamine sulfate. Temperature and pH were major factors contributing to the browning reaction. Browning was faster and more intense at 70° and pH 8 than it was at lower temperatures and pH 6. The calculated rate constants at 50, 60, and 70° and at pH 6 and 8 are shown in Tables I and II for the three dextroamphetamine sulfate–dextrate solutions.

Typical Arrhenius-type plots obtained at pH 8 are shown in Fig. 6. A straight-line relationship between log k and $1/T$ was obtained, with a negative slope at each dextroamphetamine sulfate concentration studied. The time required for browning to occur at 25° can be obtained from such plots by determining the rate constant, k, at 25°. For example, the time required for browning to occur at 25° and pH 8 in the solutions containing 0.75, 1, and 1.25% dextroamphetamine sulfate and 10% dextrates was calculated as 33.4, 40.94, and 43.02 days, respectively. Except for slower browning rates, similar plots would be obtained at pH 6.

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Potentiometric Determination of Overlapping Dissociation Constants

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Abstract □ The dissociation constants of polyprotic acids can be determined potentiometrically by defining a variable, P, as the average number of protons dissociated. The proton balance equation for the titration of these acids with base led to a general expression which could be solved graphically or through the use of linear regression or nonlinear regression analysis. Experimental data for a number of acids and for a series of simulated titration curves were analyzed using the three methods. The graphical solution was found to be applicable in all instances. As the ratio of K_1 to K_2 for diprotic acids increased from 50 to 1000, the value of K_2 estimated by the use of linear regression became progressively worse and finally could not be determined at all. In all instances, the value of K_1 was determined to within a relative error of 0.5%. In all instances in which nonlinear regression analysis was used, the values for K_1 were determined to within 0.3% and those for K_2 were determined to within 0.02%. This technique requires an initial estimate of each constant. These initial estimates are then refined in an iterative procedure until satisfactory convergence is obtained. The digital computer program used in this work is unique in that initial estimates are obtained within the computer program itself. The program also determines the total number of dissociable protons. This latter property can be extremely useful for compounds with an unknown number of acidic sites.

Keyphrases □ Polyprotic acids—potentiometric determination of overlapping dissociation constants, computer estimation of initial constants □ Dissociation constants, overlapping, of polyprotic acids—potentiometric determination, computer estimation of initial constants □ Potentiometry—determination of overlapping dissociation constants for polyprotic acids, computer estimation of initial constants □ Computer estimation of initial dissociation constants—used in potentiometric determination of overlapping dissociation constants for polyprotic acids

Determination of the thermodynamic dissociation constants for polyprotic acids is quite complicated, particularly if the ratio of the successive dissociation constants is less than 1000. One major difficulty is the estimation of individual ion activity coefficients; because of this, most of the data appearing in the literature have been obtained by extrapolating to infinite dilution the dissociation constants obtained in media of known ionic strength. This procedure, however, can lead to erroneous results for the second and successive dissociation constants (1).

Speakman (2) developed an equation which permitted a simple graphical solution for the thermodynamic dissociation constants of diprotic acids and which obviated the need for extrapolation to infinite dilution. It was suggested (2) that the equation would be useful for triprotic and higher order acids if conditions could be chosen such that only two successive dissociation constants would be operative at one time. In many instances, this is not possible. For example, the ratios of K_1/K_2 and K_2/K_3 for citric acid were both shown to be approximately 44 at 25° (3). Other investigators (3–5) developed a general procedure based upon electromotive force measurements in cells without liquid junctions. This method yields results with a high degree of accuracy and precision, but it does require the preparation of a relatively large number of precisely made buffer solutions.

Litchinsky *et al.* (1) extended the method of Speakman to cover any polyprotic acid, regardless of whether successive dissociation constants overlap. This method, although it obviates the need for preparing a large number of precisely made buffer solutions, requires the use of a digital computer. It is apparent, however, that these methods (1, 3–5) are the methods of choice for determining the thermodynamic dissociation constants of polyprotic acids.

However, in many instances, thermodynamic dissociation constants must be corrected for both ionic strength and temperature before they can be utilized. Due to the uncertainties in these corrections, many investigators prefer to obtain either an apparent (K') or a concentration-based (K_c) dissociation constant for an acid under the exact conditions of their study. Numerous methods are available for the potentiometric determination of dissociation constants for monoprotic acids and for polyprotic acids whose dissociation constants do not overlap (6, 7). Perhaps the most widely used method for determining practical dissociation constants for polyprotic acids is that of Parke and Davis (8). This method, however, was subjected to a critical evaluation by Garrett (9), who found that it had little advantage over the half-neutralization method for acids whose pKa values ranged from 4 to 10. The purpose of this article is to report the authors' experiences with a general method for the determination of either K' or K_c values for polyprotic acids with overlapping dissociation constants.

EXPERIMENTAL

Reagents—The following were used: succinic acid[1], citric acid[2], glycine hydrochloride[3] (m.p. 182°), potassium chloride (reagent grade), and carbonate-free potassium hydroxide (0.0997 N).

Equipment—The acids were titrated using a 1-ml. microburet[4] calibrated to 0.0002 ml. The pH measurements were made with a digital readout pH meter[5] standardized against 0.05 M potassium hydrogen phthalate (pH 4.01) and 0.1 M borate (pH 9.00).

Method—Dilute solutions of the acids to be titrated (about 1.00×10^{-3} M) were made in distilled, deionized water which had been degassed by boiling for 30 min. Sixty milliliters of the solutions, adjusted to an ionic strength of 0.1 with potassium chloride, was placed in a thermostated vessel (30 ± 0.05°), and nitrogen was bubbled through the solution. Small increments of base were added, and the pH was noted after each addition.

[1] Certified 99.9%, Fisher Scientific Co.
[2] Reagent grade, J. T. Baker Chemical Co.
[3] Nutritional Biochemical Co.
[4] Manostat Digi-Pet.
[5] Orion model 801.

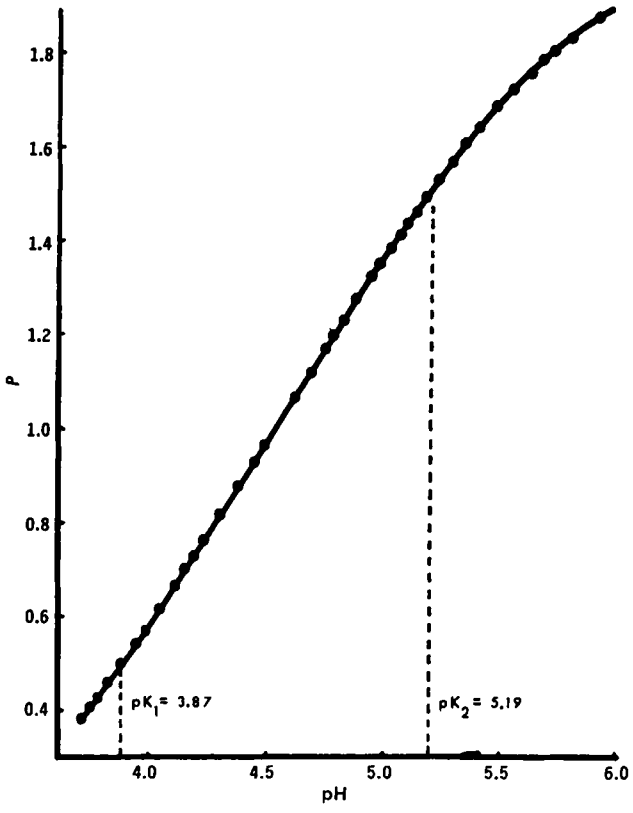

Figure 1—*Plot of* P versus *pH for succinic acid.*

THEORETICAL

The dissociation constants for a polyprotic acid, H_nA, in which n represents the total number of dissociable protons, can be determined potentiometrically by defining a variable P as the average number of protons dissociated or:

$$P = \frac{\sum_{j=1}^{n} j[H_{n-j}A^{-j}]}{C_a} \qquad \text{(Eq. 1)}$$

in which j is the number of protons dissociated and C_a is the stoichiometric concentration of acid.

The proton balance equation for the acid when titrated with base is:

$$B + [H_3O^+] = [OH^-] + \sum_{j=1}^{n} j[H_{n-j}A^{-j}] \qquad \text{(Eq. 2)}$$

or:

$$Z = \sum_{j=1}^{n} j[H_{n-j}A^{-j}] = B + [H_3O^+] - [OH^-] \qquad \text{(Eq. 3)}$$

in which B is the total concentration of base added. Inserting Eq. 3 into Eq. 1 gives:

$$P = Z/C_a \qquad \text{(Eq. 4)}$$

which enables the experimental value for P to be determined at each data point. The concentration of all species derived from the acid, H_nA, as given by Eq. 1, can be defined solely in terms of hydronium-ion concentration and dissociation constants, using the method described by Niebergall (10), to give:

$$P = \frac{\sum_{j=1}^{n} j\left([H_3O^+]^{n-j} \prod_{m=1}^{j} K_m\right)}{[H_3O^+]^n + \sum_{j=1}^{n}\left([H_3O^+]^{n-j} \prod_{m=1}^{j} K_m\right)} \qquad \text{(Eq. 5)}$$

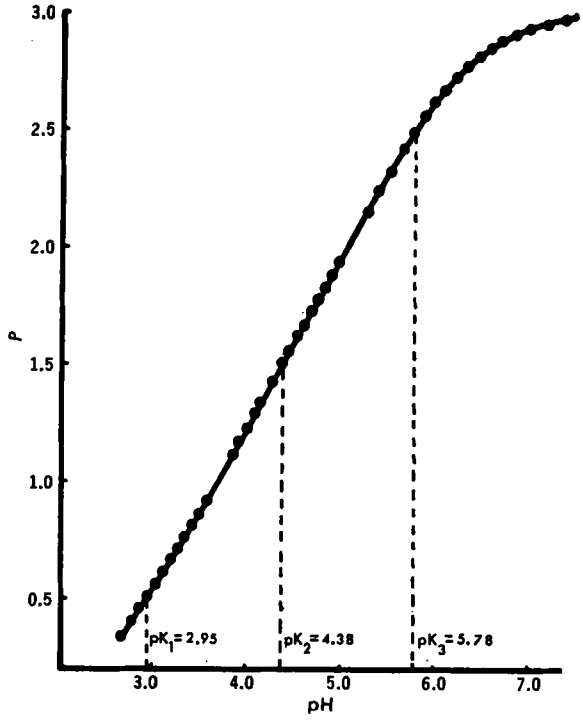

Figure 2—*Plot of* P versus *pH for citric acid.*

in which K_m represents the acid dissociation constants.

In the case of a diprotic acid, Eq. 5 becomes:

$$P = \frac{K_1[H_3O^+] + 2K_1K_2}{[H_3O^+]^2 + K_1[H_3O^+] + K_1K_2} \qquad \text{(Eq. 6)}$$

which can be rearranged to:

$$\frac{[H_3O^+]^2P}{(2 - P)} = K_1K_2 + \frac{K_1[H_3O^+](1 - P)}{(2 - P)} \qquad \text{(Eq. 7)}$$

which can be analyzed using the usual methods of linear regression analysis. Equation 7 is equivalent to the linear equation used by Speakman (2), with the ordinate and abscissa values being the same for both. One advantage of using the variable P rather than the variables given by Speakman is that rather good approximate values for the dissociation constants can be obtained from a plot of P *versus* pH, in a manner similar to the Bjerrum (11) half $\bar{n}$ method for obtaining association constants. The value of pK_j is equal to the pH at which $P = j - 0.5$.

Equation 5 can be analyzed directly using the techniques of nonlinear regression analysis. The general method and digital computer

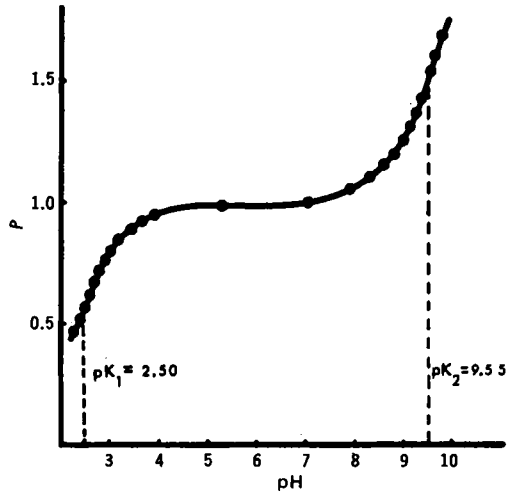

Figure 3—*Plot of* P versus *pH for glycine hydrochloride.*

Table I—pK Values Obtained at 30° and Ionic Strength 0.1

Constant	*P* Plots	Eq. 7	Eq. 5	Literature[a]
		Citric Acid		
pK_1	2.95	—	3.023	2.993
pK_2	4.38	—	4.395	4.386
pK_3	5.78	—	5.750	5.791
		Succinic Acid		
pK_1	3.87	3.952	3.956	3.953
pK_2	5.19	5.116	5.117	5.151
		Glycine Hydrochloride		
pK_1	2.50	2.131	2.512	2.432
pK_2	9.55	5.192[b]	9.558	9.532

[a] Corrected to ionic strength 0.1. L. G. Sillen and A. E. Martell, "Stability Constants," Metcalfe and Cooper, Ltd., London, England, 1964. [b] Deemed not significantly different from zero at the 95% confidence level using the *t* test.

program utilized to analyze the data presented in this report were given in detail by Niebergall *et al.* (12). Briefly, the method consists of taking the partial derivatives of Eq. 5 with respect to each dissociation constant and evaluating each derivative at each data point, using the experimental variables *P* and $[H_3O^+]$ and initial estimates for each constant. Summing these calculated values in the manner described (12) yields a set of normal equations which can be solved to give correction vectors for each constant. The initial estimates are corrected and the process is repeated until two sets of constants agree to within a specified degree.

RESULTS AND DISCUSSION

Plots of *P versus* pH are shown in Figs. 1, 2, and 3 for succinic acid, citric acid, and glycine hydrochloride, respectively. A plot of Eq. 7 using the data for succinic acid is shown in Fig. 4. The dissociation constants obtained using the *P* plots, Eq. 7, and nonlinear regression analysis of Eq. 5 are given in Table I. In general, there was good agreement between the methods, and the experimentally determined constants were in good agreement with literature values. The value for K_2 of glycine hydrochloride obtained using Eq. 7, however, was not significantly different from zero at the 95% confidence level when analyzed using the *t* test, and it was markedly different from the literature value.

To determine whether the problem was with the data or with the analysis of the data and to give a further test for the applicability of Eqs. 5 and 7, the authors decided to simulate titration curves for a number of hypothetical diprotic acids in which K_1 was equal to 3.00×10^{-3} and K_2 was varied to give K_1/K_2 ratios ranging from 50 to 1000. This was done by combining Eqs. 3, 4, and 6 to give:

$$B + [H_3O^+] - (K_w/[H_3O^+]) = \frac{(K_1[H_3O^+] + 2K_1K_2)C_a}{[H_3O^+]^3 + K_1[H_3O^+] + 2K_1K_2} \quad \text{(Eq. 8)}$$

in which K_w is the ion product of water. The Newton–Raphson method of successive approximations and the digital computer program described by Niebergall (13) were used to solve for $[H_3O^+]$ after each hypothetical addition of a small quantity of base. The

Table II—Effect of the Ratio of K_1/K_2 upon the Suitability of Eq. 7 for Determining K_1 and K_2

Ratio of K_1/K_2	Percentage Difference between Theoretical and Calculated K_1	Percentage Difference between Theoretical and Calculated K_2
50	0.37	3.17
100	0.30	6.07
180	0.20	8.80
250	0.37	14.25
300	0.23	15.68
500	0.27	27.95
1000	0.40	61.73[a]

[a] The value for K_2 was found to be not significantly different from zero at the 95% confidence level using the *t* test.

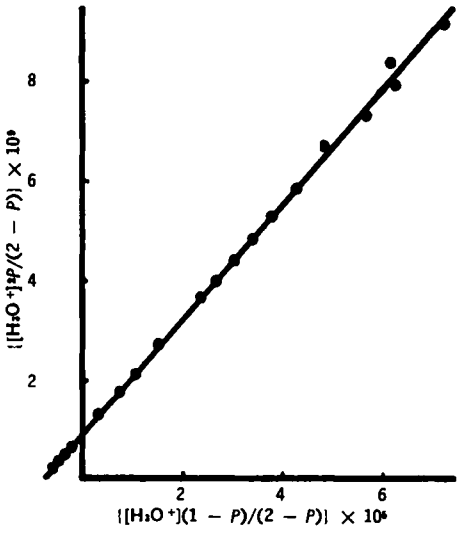

Figure 4—*Plot of Eq. 7, using data obtained for succinic acid.*

actual concentration of base after each increment was obtained from:

$$B = \frac{(\text{milliliters base})(\text{normality})}{\text{Vol} + (\text{milliliters base})} \quad \text{(Eq. 9)}$$

in which Vol represents the initial volume of the solution before any base had been added. The term C_a was corrected for volume changes during titration (although these were less than 2%) by:

$$C_a = \frac{(\text{initial concentration of acid})(\text{Vol})}{\text{Vol} + (\text{milliliters base})} \quad \text{(Eq. 10)}$$

The utilization of Eqs. 8–10 would give unambiguous data for the titration curves, since no assumptions were made (except for the use of concentrations in the place of activities) and all possible equilibria in solution were considered. Thus, any discrepancy between the constants used to build the simulated titration curves and those obtained by analyzing the data using Eq. 5 or 7 would have to be due to the method of analysis. A small amount of scatter, representing an accuracy of ±0.005 pH unit, was built into the data through the use of a random numbers table. This scatter should result in the dissociation constants being determined to within 0.5% of their true values. Data were generated for 60 ml. of a 0.01 *M* solution of acid being titrated with 0.1-ml. increments of 0.205 *N* base at 30°. The data were analyzed using Eq. 7 (Table II). The values for K_1 were determined and agreed well with the theoretical values. The values for K_2, however, became progressively worse as the ratio of K_1/K_2 increased, which was rather surprising. When the ratio reached 1000, the value for K_2 was determined to be not significantly different from zero at the 95% confidence level, using the *t* test. Thus, the failure to determine K_2 for glycine hydrochloride, in which the ratio of K_1/K_2 is approximately 10^7, using the linear plotting technique was due to the technique itself and not to any lack of reliability within the data.

The fact that the estimates of K_2 became progressively worse as the ratio of K_1/K_2 increased appears to be due to the fact that the

Table III—Effect of the Ratio of K_1/K_2 upon the Suitability of Eq. 6 for Determining K_1 and K_2

Ratio of K_1/K_2	Percentage Difference between Theoretical and Calculated K_1	Percentage Difference between Theoretical and Calculated K_2
50	0.20	0.02
100	0.27	0.03
180	0.21	0.05
250	0.20	0.04
300	0.19	0.03
500	0.27	0.08
1000	0.24	0.07

intercepts, as defined by Eq. 7, became progressively smaller as compared to the ordinate values as this ratio increased. Since excellent linearity was observed in all instances when the data were plotted according to Eq. 7, the experimenter has little basis for deciding whether the values of K_2 obtained in this manner are valid. He can be sure of this only when the ratio of K_1/K_2 approaches 50 or less, and he can be certain that the results are not valid only when K_1/K_2 approaches 1000 and the t test shows K_2 to be not significantly different from zero. Thus it would appear, based on this study, that the use of linear equations equivalent to Eq. 7 should be avoided if reliable estimates of the value of K_2 for diprotic acids are to be obtained.

The same data were analyzed using Eq. 6 and the techniques of nonlinear regression analysis (Table III). In all instances, the agreement between the actual and calculated values for the dissociation constants was excellent. Although the emphasis in this report is directed toward the determination of overlapping dissociation constants for di- and triprotic acids, Eq. 5 is perfectly general and can be used for monoprotic acids as well[6], and n is not limited to a maximum of three.

Since plots of P *versus* pH also gave reasonable results for all of the systems studied, including the simulated titration data, it would appear that either these P plots or nonlinear regression analysis should be used for obtaining overlapping dissociation constants for polyprotic acids.

DIGITAL COMPUTER PROGRAM[7]

The utilization of nonlinear regression analysis requires an initial estimate for each dissociation constant which, in this instance, would ordinarily be obtained from plots of P *versus* pH. However, this program is written in such a manner that if care is taken to obtain a number of data points around the addition of 0.5, 1.5, and 2.5 moles of base/mole of acid, good initial estimates will be obtained automatically from the input data as the program is run. The only necessary input data are the stoichiometric concentration and volume of acid, the ion product of water, the normality of the titrant, and the experimental values of pH and volume of base added. In the process of obtaining its own initial estimates from the raw input data, the program also determines the total number of dissociable protons for the acid. Thus, it would be extremely valuable for obtaining dissociation constants for an acid with an unknown number of dissociable protons.

[6] The dissociation constants for a large number of monoprotic acids were evaluated using the Benet and Goyan (6) linear equation:

$$Z = C_a - \frac{Z[H_3O^+]}{K}$$

in which the symbols have the meanings as given in this paper, and the following version of Eq. 5:

$$P = \frac{[H_3O^+]}{[H_3O^+] + K}$$

In all instances, the sum of squares of residuals (SS) between the experimental and calculated values of P were reduced by at least 95% when the nonlinear regression analysis was used. For example, the SS for sulfisomidine decreased from 3.21×10^{-9} to 0.17×10^{-9}, and the SS for sulfacetamide decreased from 46.0×10^{-9} to 0.04×10^{-9}.

[7] A copy of the program, along with its documentation, can be obtained by writing the authors.

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UV Spectrophotometric Analysis of Aminobenzoic Acid Tablets

W. LARRY PAUL

Abstract □ An accurate UV spectrophotometric procedure was developed for the assay of aminobenzoic acid tablets. The crushed tablet was treated with 95% ethanol, and this alcoholic solution was diluted with distilled water. The resulting solution was assayed by absorption spectroscopy. The reproducibility and short-time requirement of the assay suggest that such a UV spectrophotometric method should be considered as the official assay method for *p*-aminobenzoic acid samples.

Keyphrases □ Aminobenzoic acid tablets—UV spectrophotometric analysis □ UV spectrophotometry—analysis, aminobenzoic acid tablets

An accurate analysis procedure for any pharmaceutical preparation is obviously a prerequisite before the preparation can be marketed. From the standpoint of industrial application, it is desirable that the analytical procedure be rapid and easily automated.

It was decided to investigate the development of such an analytical procedure for aminobenzoic acid tablets for several reasons. First, the USP XVIII (1) assay procedure for *p*-aminobenzoic acid is time consuming because it is based on the titration of a *p*-aminobenzoic acid–hydrochloric acid–ice solution with 0.1 *M* sodium nitrite until a blue color is produced immediately when a glass rod dipped in the solution is touched to starch iodide paper. This procedure is subject to variations between individuals in terms of deciding when the endpoint is reached.

Aminobenzoic acid tablets are sold over-the-counter as a B-complex factor in a number of health food stores. Based on the growing popularity of the health food diet, it was felt that the development of a faster assay procedure and the analysis of several aminobenzoic acid tablets selected at random would yield valuable information concerning the potency of the health food aminobenzoic acid formulation.

In addition to the official USP XVIII titration method for *p*-aminobenzoic acid analysis, numerous other titration procedures have been reported (2–8). Other reported analytical procedures include reflectance spectrometry (9), the Bratton–Marshall method (10), diazotization followed by coupling to acid (11), photocolorimetry (12), microbiological methods (13), and coprecipitation by zinc ferrocyanide of procaine in a procaine–*p*-aminobenzoic acid mixture (14). Analytically useful fluorescence and phosphorescence signals have also been obtained for *p*-aminobenzoic acid (15).

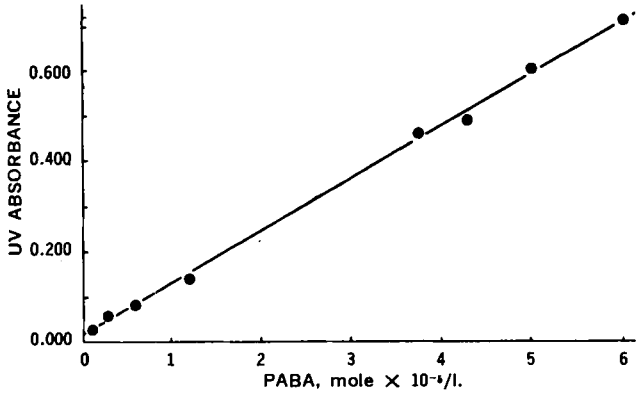

Figure 1—*Plot of UV absorbance* versus p-*aminobenzoic acid* (*PABA*) *concentration.*

EXPERIMENTAL

Materials—*p*-Aminobenzoic acid[1] was used without further purification in preparing the standard solutions for the analytical curve. Two bottles of 100-mg. aminobenzoic acid tablets[2] were purchased from a local health food store. For the UV measurements, a grating spectrophotometer[3] was used.

Analytical Curve—Five samples of *p*-aminobenzoic acid with weights of 90.05, 95.00, 100.14, 105.05, and 110.03 mg. were weighed, and each sample was treated in the following manner. The sample was placed in a 50-ml. volumetric flask, and 50 ml. of 95% ethanol was added. The solution was shaken and placed in an ultrasonic cleaner to ensure complete dissolution of the *p*-aminobenzoic acid.

Using a Lambda pipet, 0.1 ml. of the alcoholic stock solution was added to 50 ml. of distilled water in a volumetric flask. Four such solutions were prepared, and the absorbance at 268 nm. of each solution at ambient temperature was then recorded. The absorbance was plotted against the milligrams *p*-aminobenzoic acid in the original alcoholic stock solution.

***p*-Aminobenzoic Acid Isolation from Tablets**—Each tablet was wrapped inside glassine weighing paper and then crushed. The powder was transferred to a 50-ml. volumetric flask, and 50 ml. of 95% ethanol was added. The solution was shaken, placed in the ultrasonic cleaner, and then filtered. Five 0.1-ml. aliquots were taken and each was added to 50 ml. of distilled water. The absorbance at 268 nm. was measured for each solution.

RESULTS AND DISCUSSION

To determine the limit of detection and to check Beer's law for the UV analysis, a series of *p*-aminobenzoic acid solutions, ranging from 1.2×10^{-6} to 6.04×10^{-5} *M*, was prepared. Figure 1 is a plot of absorbance *versus* *p*-aminobenzoic acid concentration. The UV plot is linear over the range considered. The limit of detection for UV analysis is approximately 1.2×10^{-6} *M*. This is equivalent to a tablet containing 4.14 mg. of *p*-aminobenzoic acid.

Table I summarizes the data for the absorbance values of the analysis of 90–110 mg. *p*-aminobenzoic acid. This is in the range of the commercial tablets tested.

[1] Fisher Lot No. 280288.
[2] Distributed by Radiance Products Co., Alhambra, Calif. (Lot Nos. 1337809 and 1347610).
[3] Beckman BD-GT.

Table I—UV Absorbance Data for Various Concentrations of *p*-Aminobenzoic Acid

Solution	*p*-Aminobenzoic Acid Initially Weighed, mg.	*p*-Aminobenzoic Acid Concentration in the Analyzed Solution, *M*	UV Absorbance (268 nm.)	$X \pm \sigma$
A1	90.05	2.61×10^{-5}	0.326	0.328 ± 0.002
A2			0.371[a]	
A3			0.328	
A4			0.329	
B1	95.00	2.75×10^{-5}	0.359	0.350 ± 0.006
B2			0.348	
B3			0.350	
B4			0.345	
C1	100.14	2.91×10^{-5}	0.378	0.366 ± 0.011
C2			0.360	
C3			0.359	
C4			0.410[a]	
D1	105.05	3.05×10^{-5}	0.373	0.377 ± 0.005
D2			0.376	
D3			0.382	
D4			0.430[a]	
E1	110.3	3.19×10^{-5}	0.400	0.400 ± 0.006
E2			0.399	
E3			0.393	
E4			0.407	

[a] Value excluded from data.

Table II—UV Absorbance Readings and Milligrams *p*-Aminobenzoic Acid for the Two Lots of Aminobenzoic Acid Tablets Tested

Lot	Tablet	Average Absorbance ± σ[a]	Average Milligrams *p*-Aminobenzoic Acid ± σ[a]	Average Milligrams *p*-Aminobenzoic Acid/Tablet/Lot
1337809	1	0.394 ± 0.011	108.67 ± 3.20	103.40 ± 4.90
	2	0.379 ± 0.014	104.26 ± 4.02	
	3	0.383 ± 0.011	105.38 ± 3.22	
	4[b]	0.365 ± 0.012[b]	100.14 ± 3.46[b]	
	5	0.357 ± 0.007	97.93 ± 1.98	
1347610	1	0.372 ± 0.009	102.15 ± 2.71	104.22 ± 4.47
	2	0.372 ± 0.009	102.21 ± 2.60	
	3	0.388 ± 0.015	106.84 ± 4.31	
	4	0.395 ± 0.007	108.84 ± 2.29	
	5[b]	0.365 ± 0.016[b]	100.29 ± 4.59[b]	

[a] Average of five determinations. [b] Average of four determinations.

Five tablets from each of the two lots were analyzed, and five absorbance determinations were made on each tablet. These data are summarized in Table II. The tablets from Lot No. 1337809 had an average value of 103.40 ± 4.90 mg. *p*-aminobenzoic acid per tablet, while those from Lot No. 1347610 had an average value of 104.22 ± 4.47 mg. *p*-aminobenzoic acid per tablet.

Statistical analysis of the data in Fig. 1 gave the following linear regression equation:

$$Y = 0.114874X + 0.0136 \qquad \text{(Eq. 1)}$$

The linear correlation coefficient is 0.999. The statistical analyses were performed on a calculator[4] using standard programs. Statistical analysis of the regression data in Table I (UV absorbance *versus* milligrams *p*-aminobenzoic acid initially weighed) gave the following linear regression equation:

$$Y = 0.003408X + 0.023469 \qquad \text{(Eq. 2)}$$

with a linear correlation coefficient of 0.971.

Based on the fact that this is a completely randomized experiment with equal sample sizes, the statistical tests based on a nested classification of the hypotheses that the *p*-aminobenzoic acid content in the two lots and among the individual tablets is equal is shown in Table III. The ANOVA values were obtained from the five *p*-aminobenzoic acid milligram values for each of the 10 tablets.

[4] Monroe Epic 3000.

Table III—ANOVA for the Assayed Weight of *p*-Aminobenzoic Acid

Source	*df*	*SS*	*MS*	*F*, calc.
Lots	1	0.0000245	0.0000245	0.02
Tablets/lot	8	0.0083615	0.0010452	4.64
Determinations/ tablet	40	0.0090040	0.0002251	—
Total	49	114.24	—	—

To test the hypothesis of equal *p*-aminobenzoic acid content between the lots, $F = 0.02$. From the *F*-table (16), $F^{1}_{8(0.05)} = 5.32$. Therefore, the hypothesis should be accepted. To test the hypothesis of equal *p*-aminobenzoic acid content among the tablets, $F = 4.64$. From the *F*-table (16), $F^{8}_{40(0.05)} = 2.18$. Therefore, the hypothesis should be rejected.

The statistical analysis of the data shows that there is no significant variation between the two lots tested but that the variation among the tablets is significant.

These results show that the UV method of analysis is applicable to aminobenzoic acid tablet analysis. This UV method is fast and accurate, and it offers a substantial improvement over the USP XVIII titration procedure.

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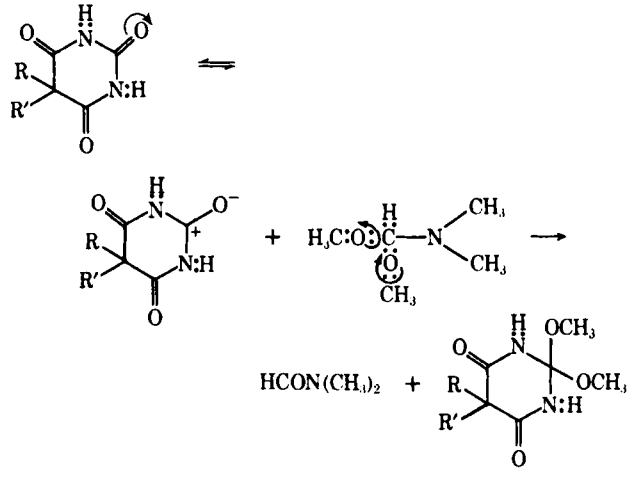
Dimethylformamide Dimethylacetal as a Derivatizing Agent for GLC of Barbiturates and Related Compounds

VINCENT S. VENTURELLA▲, VITO M. GUALARIO, and R. E. LANG

Abstract □ Dimethylformamide dimethylacetal reacted quantitatively and reproducibly with glutethimide, phenobarbital, hexobarbital, secobarbital, amobarbital, aprobarbital, and pentobarbital to form the corresponding acetals. The acetal derivative could be chromatographed easily on a 3% OV-17 column using either isothermal or programmed conditions (for multiple separations), permitting the separation and determination of the compounds studied with good precision and speed. NMR evidence and mass fragmentography was used to confirm the structure of the derivative. The glutethimide derivative was susceptible to solvent-induced reversibility.

Keyphrases □ Dimethylformamide dimethylacetal—used as derivatizing agent for GLC of barbiturates, glutethimide □ Barbiturates—GLC analysis, dimethylformamide dimethylacetal as derivatizing agent □ Glutethimide—GLC analysis, dimethylformamide dimethylacetal as derivatizing agent □ GLC—analysis, barbiturates and glutethimide, dimethylformamide dimethylacetal as derivatizing agent

The vast use of the 5,5-disubstituted barbituric acid derivatives, glutethimide, and methyprylon as sedatives as well as the interest they have commanded in forensic science has led to numerous reports on the use of GLC as an analytical method for their determination (1–5). The most recent literature (1–3) relied on the formation of their *N*-methyl derivatives prior to GLC (2) or on *in situ* formation in the chromatograph injector port (1–3). The derivative was formed by the action of trimethylanilinium hydroxide, a reagent requiring a time-consuming synthesis[1] (3). While the formation of the methylated compounds removed the disadvantages of adsorption, tailing, and column contamination previously experienced with GLC of the parent barbiturates, most of the columns and conditions used did not remove the failure of baseline separation when chromatographing mixtures.



Scheme I

The inadequacies prompted this laboratory to investigate other derivative routes, which led to a study of the chromatographic behavior of the product of barbiturates with dimethylformamide dimethylacetal (I)[2].

Depending on the reacting compounds and conditions employed, dimethylformamide–dialkylacetals react to form either acetals with imides (6) or formamidine derivatives with amides (7). Since I decomposes during the reaction to form both CH_3^+ and OCH_3^-, either *N*-methylation or acetal formation with barbiturates was seemingly possible. The path taken would depend only on the relative ease of proton abstraction from the subject compound, as opposed to carbonyl polarization.

[1] After completion of this work, Pierce Chemical Co. began marketing the reagent as MethElute.
[2] Aldrich Chemical Corp., Milwaukee, Wis.

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Dimethylformamide Dimethylacetal as a Derivatizing Agent for GLC of Barbiturates and Related Compounds

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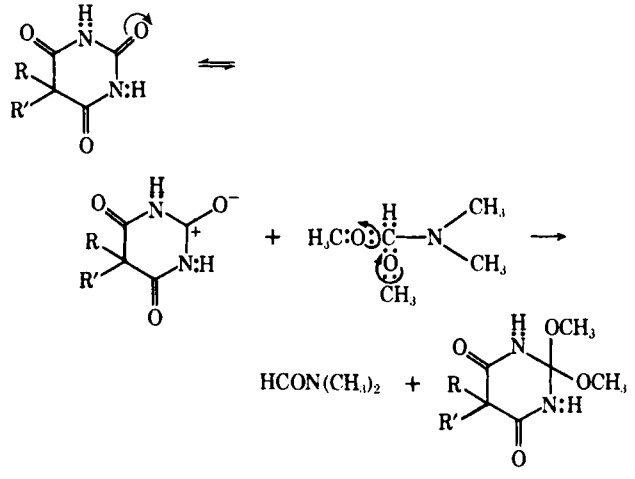
Abstract □ Dimethylformamide dimethylacetal reacted quantitatively and reproducibly with glutethimide, phenobarbital, hexobarbital, secobarbital, amobarbital, aprobarbital, and pentobarbital to form the corresponding acetals. The acetal derivative could be chromatographed easily on a 3% OV-17 column using either isothermal or programmed conditions (for multiple separations), permitting the separation and determination of the compounds studied with good precision and speed. NMR evidence and mass fragmentography was used to confirm the structure of the derivative. The glutethimide derivative was susceptible to solvent-induced reversibility.

Keyphrases □ Dimethylformamide dimethylacetal—used as derivatizing agent for GLC of barbiturates, glutethimide □ Barbiturates—GLC analysis, dimethylformamide dimethylacetal as derivatizing agent □ Glutethimide—GLC analysis, dimethylformamide dimethylacetal as derivatizing agent □ GLC—analysis, barbiturates and glutethimide, dimethylformamide dimethylacetal as derivatizing agent

The vast use of the 5,5-disubstituted barbituric acid derivatives, glutethimide, and methyprylon as sedatives as well as the interest they have commanded in forensic science has led to numerous reports on the use of GLC as an analytical method for their determination (1–5). The most recent literature (1–3) relied on the formation of their *N*-methyl derivatives prior to GLC (2) or on *in situ* formation in the chromatograph injector port (1–3). The derivative was formed by the action of trimethylanilinium hydroxide, a reagent requiring a time-consuming synthesis[1] (3). While the formation of the methylated compounds removed the disadvantages of adsorption, tailing, and column contamination previously experienced with GLC of the parent barbiturates, most of the columns and conditions used did not remove the failure of baseline separation when chromatographing mixtures.



Scheme I

The inadequacies prompted this laboratory to investigate other derivative routes, which led to a study of the chromatographic behavior of the product of barbiturates with dimethylformamide dimethylacetal (I)[2].

Depending on the reacting compounds and conditions employed, dimethylformamide–dialkylacetals react to form either acetals with imides (6) or formamidine derivatives with amides (7). Since I decomposes during the reaction to form both CH_3^+ and OCH_3^-, either *N*-methylation or acetal formation with barbiturates was seemingly possible. The path taken would depend only on the relative ease of proton abstraction from the subject compound, as opposed to carbonyl polarization.

[1] After completion of this work, Pierce Chemical Co. began marketing the reagent as MethElute.
[2] Aldrich Chemical Corp., Milwaukee, Wis.

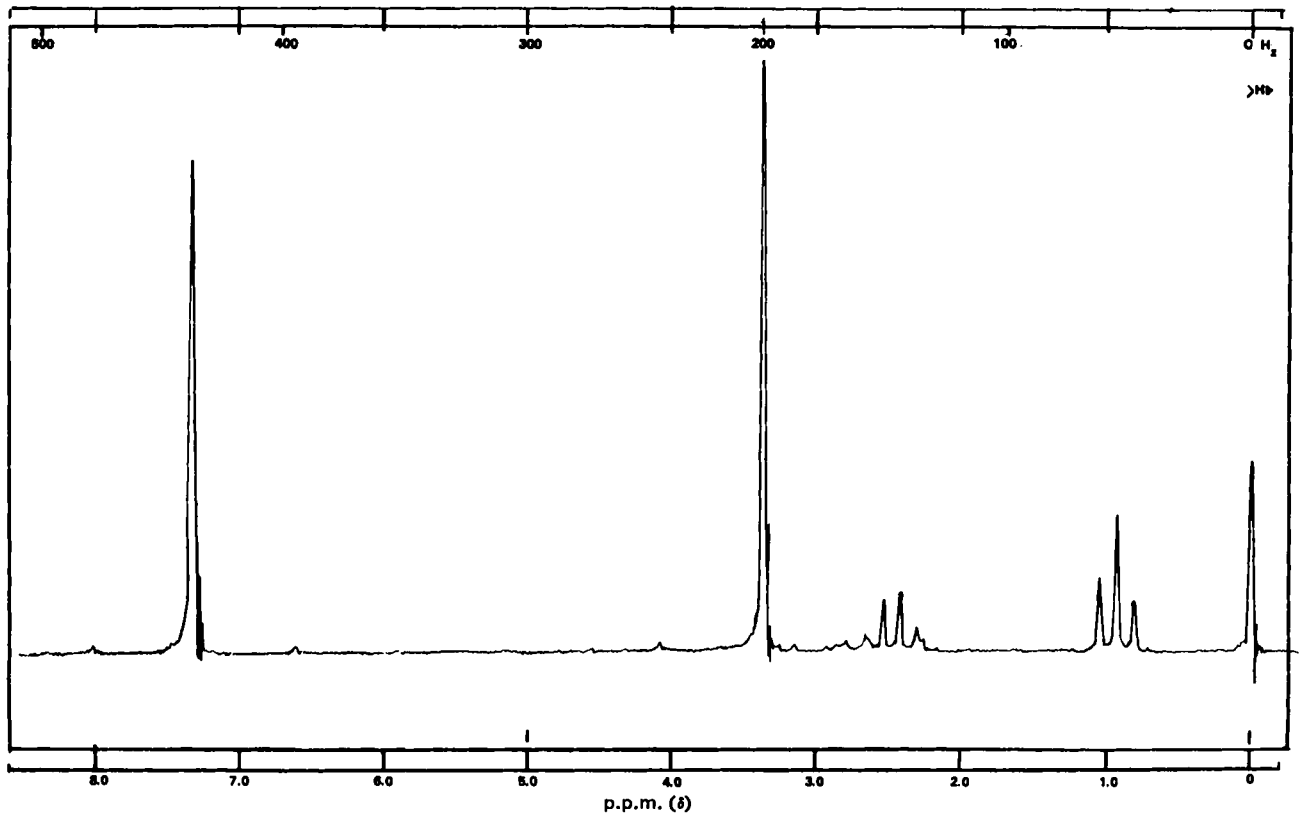


Figure 1—*NMR spectrum of phenobarbital dimethylformamide dimethylacetal adduct at 60 MHz. (concentration 45 mg./0.5 ml. $CDCl_3$; spectrum amplitude 2.0; 40 r.p.s. spin; 250 sec./500 Hz. sweep rate; filter 1; R_f level 0.08).*

Table I—Retention Time and Response Ratio Data

Compound[a]	Relative Retention Time [Dibutyl Phthalate]	Relative Retention Time [*n*-Eicosane]	Response Ratio (Triplicate Average) Dibutyl Phthalate	Response Ratio (Triplicate Average) *n*-Eicosane	Concentration, mg./ml.
Aprobarbital	0.413	0.565	0.7938	0.5442	7.96
Amobarbital	0.481	0.661	0.8529	0.5819	8.15
Pentobarbital	0.519	0.714	0.8516	0.5712	8.05
Secobarbital	0.572	0.786	0.8334	0.5806	6.99
Hexobarbital	0.865	1.19	0.7962	0.5499	4.81
Phenobarbital	0.923	1.27	0.9197	0.6270	8.84
Methyprylon	0.542	—	0.8599[b] (0.8702)[c]	—	4.52[b]
Glutethimide	—	1.29[d,e]	—	0.2749	12.08
Dibutyl phthalate	1.00 (10.4 min.)	1.38	—	—	—
n-Eicosane	0.754 (7.49 min.)	1.00[e]	—	—	—

[a] As the acetal (Compounds 1–6). [b] Direct solution in dimethylformamide. [c] Solution in 0.5 ml. AcOH in dimethylformamide (5 ml. total volume). [d] As the acetal; no solvent; *n*-eicosane standard. [e] At 240° isothermal, retention times of *n*-eicosane = approximately 1.9 min. and of glutethimide acetal = 3.1 min.

EXPERIMENTAL[3]

Instrument Parameters—The gas chromatograph for the analytical studies was equipped with dual U-shaped Pyrex columns, 1.8 m. × 0.6 cm. (6 ft. × 0.25 in.) × 4 mm. i.d. ("on-column" inserted), packed with 3% OV-17 on Supelcoport (100–120 mesh)[4]. Sensitivity was 10^{-10} amp./mv., with helium flow at 55 ml./min., hydrogen flow at 40 ml./min., and air at 1.6 ft.³/hr. The injector temperature was 285°, the detector temperature was 300°, and the column was programmed from 160 to 220° at 7°/min.

GC–mass spectrometry conditions were: glass coil column, 0.6 cm. × 1.8 m. (0.25 in. × 6 ft.) × 2.8 mm. i.d.; injector temperature, 210°; separator temperature, 210°; column, 160–200° at 8°/min.; flow, 25 ml./min.; high vacuum, 8 × 10^{-6} torr; transfer line, 200°, manifold, 120°; beam current, 0.30 ma.; ion energy, 9.2 v.; electron energy, 70.3 v.; collector, 34.2 v., emission, 0.70 ma.; extractor, 10.2 v.; lens, 100.9 v.; and high voltage, 1.55.

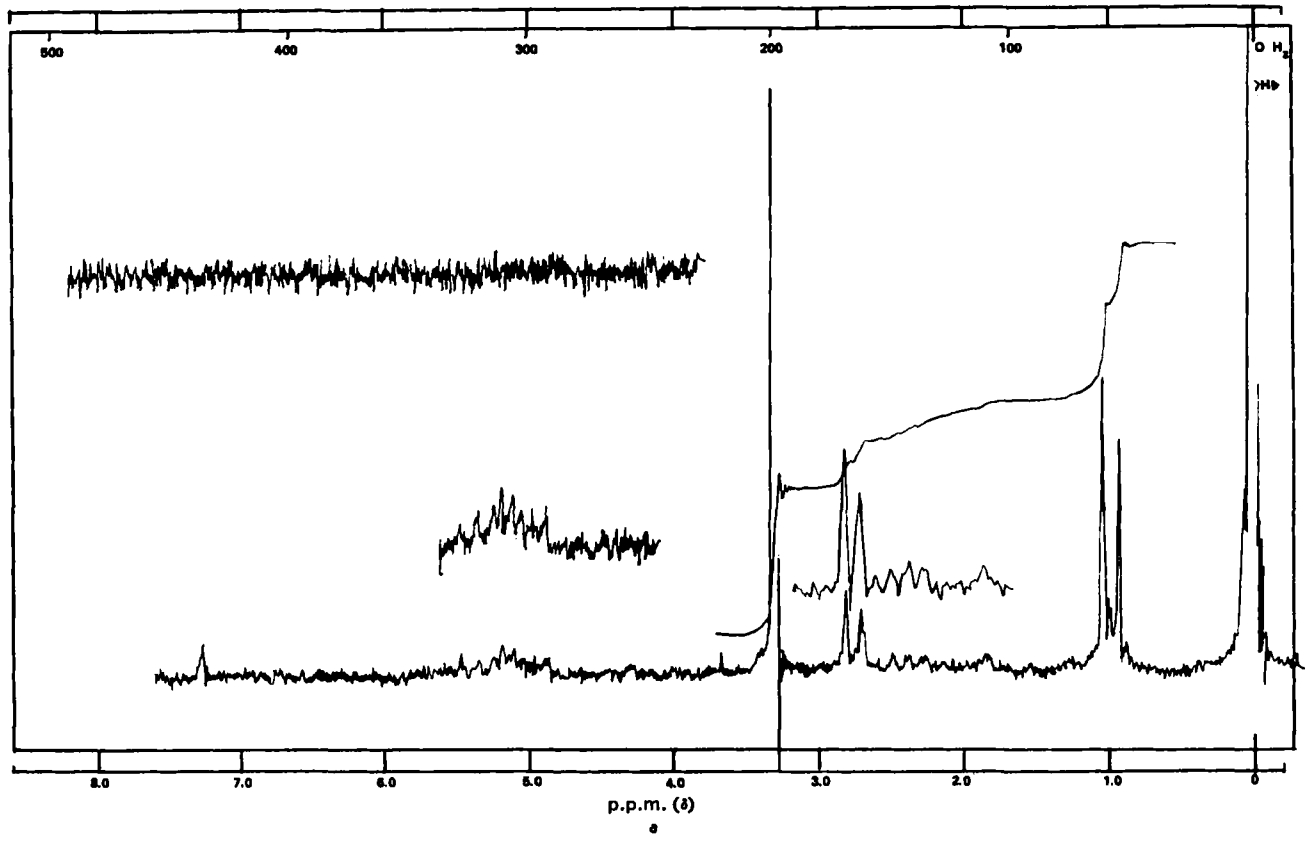
Standard Procedure—Accurately weigh 5–40 mg. of the subject compound[5] into a 5-ml. volumetric flask, add exactly 1.0 ml.[6] of I, swirl gently until the compound dissolves, and warm on a steam bath for 10 min. Cool, dilute with internal standard solution, and

[3] GLC analyses were performed using a Bendix model 2500 chromatograph equipped with a flame-ionization detector, Honeywell Electronik 194 recorder, and a Disc integrator. All area measurements were taken from the integrator with the aid of a model 610 Disc printer. Mass fragmentography was performed on a Varian 1400 chromatograph linked to a Finnigan Corp. 3000/PDP 8-E system. NMR analyses were performed on a Varian T-60 spectrometer.

[4] Supelco Corp.

[5] Reference standards were obtained from the respective manufacturers except phenobarbital (Merck) and pentobarbital (Ganes Chemical Co.).

[6] For small amounts of compound (5–10 mg.), 0.5 ml. of reactant will suffice.



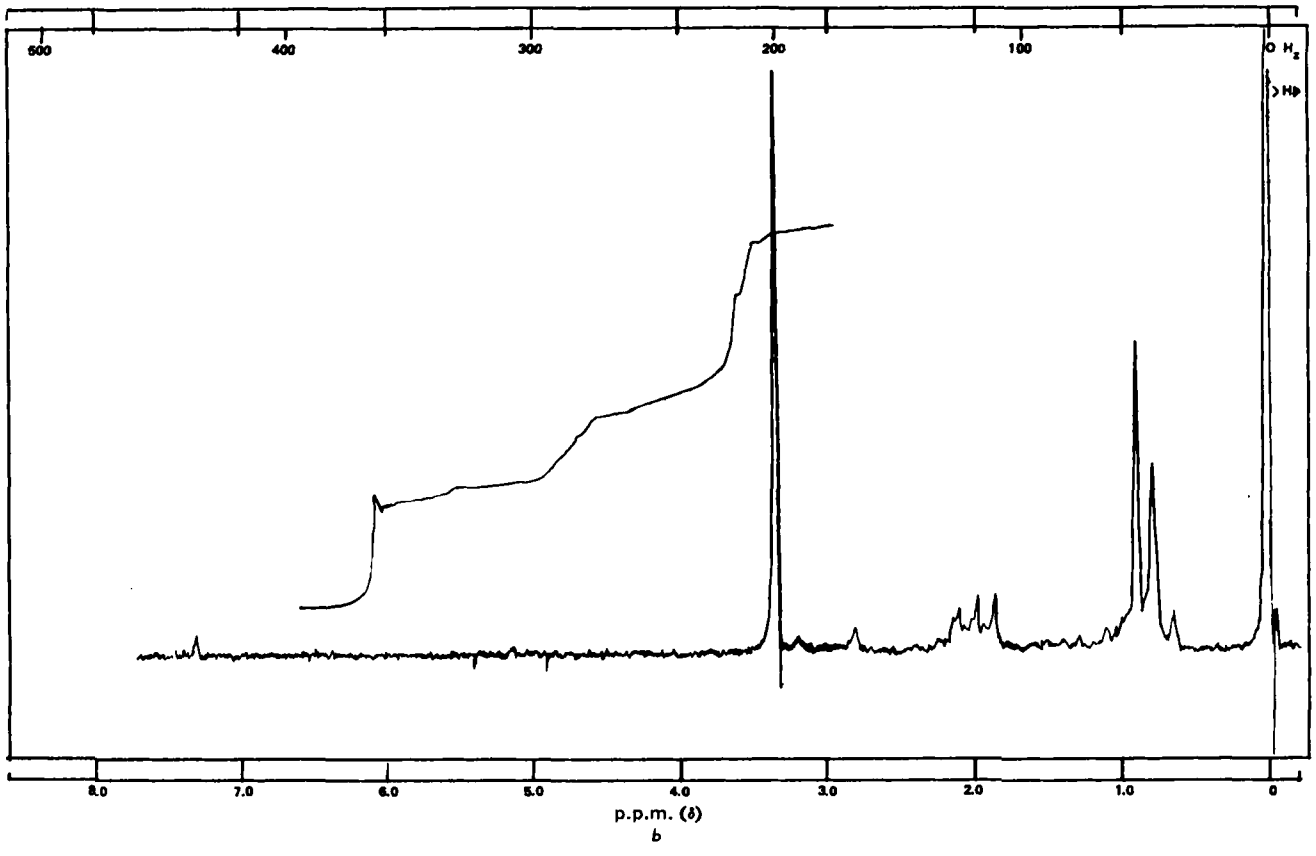


Figure 2—(a) *NMR spectrum of aprobarbital dimethylformamide dimethylacetal adduct at 60 MHz. (concentration 23 mg./0.4 ml. $CDCl_3$; amplitude 20; 52 r.p.s.; 250 sec./500 Hz. sweep rate; filter 2; R_f level 0.11; upper trace amplitude 40; insert sweep offset 280 Hz., amplitude 50).* (b) *NMR spectrum of amobarbital dimethylformamide dimethylacetal adduct at 60 MHz. (concentration 44 mg./0.5 ml. $CDCl_3$; amplitude 12.5; 58 r.p.s.; 250 sec./500 Hz. sweep rate; filter 2; R_f level 0.065).*

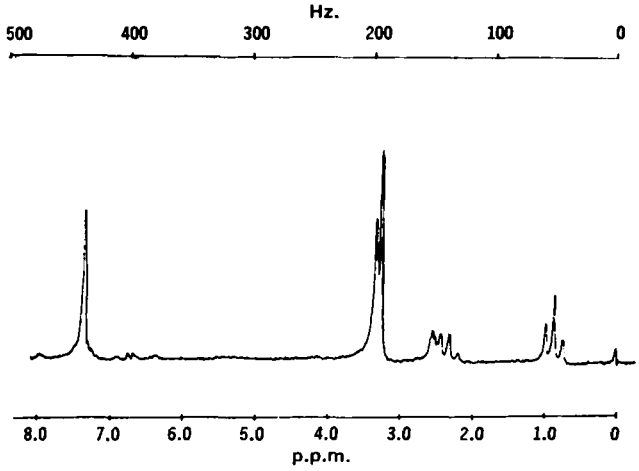


Figure 3—*NMR spectrum of metharbital dimethylformamide dimethylacetal adduct at 60 MHz. (concentration 46 mg./0.6 ml. dimethyl sulfoxide-d_6; amplitude 12.5; 48 r.p.s.; 250 sec./500 Hz. sweep rate; filter 2; R_f level 0.09; parts per million on σ scale). Note dimethyl sulfoxide-d_6 signal at 152 Hz. overlaps —CH_2 quartet of —C_2H_5 group at 142 Hz.*

Methyprylon Procedure—Since methyprylon reacts with the reagent only with difficulty, it is simply dissolved in dimethylformamide or chloroform and chromatographed directly in the concentrations specified for the other sedatives.

Mixtures of Sedatives—Samples containing mixtures in the 5–40-mg. range can be prepared in identical fashion. If the combined total in the mixture is in excess of about 100 mg., use an additional 0.5 ml. of reactant for each additional 50 mg.

RESULTS AND DISCUSSION

Dimethylformamide reacted quantitatively with the sedatives studied (except methyprylon) in such a manner that simple —N—H to N—CH_3 transformation was not considered a possibility. Since the compounds studied are chemically related to the imides studied by Meerwein *et al.* (6), the mechanism shown in Scheme I was favored. This mechanism takes advantage of both the CH_3^+ and the OCH_3^- formed by the acetal. A derivative of this nature is somewhat unusual but would depend only upon ease of polarization of the carbonyl on C-2. Since this position is known to polarize easily (8) and is less hindered, it was considered the only logical position of reaction. While the actual derivatives formed showed GLC retention times similar to the 1,3-dimethyl derivatives noted previously (1–3), simple 1,3-dimethyl formation was excluded because of ease of formation and because it was noted that the barbiturate must be present as the keto tautomer for smooth reaction to occur. Thus,

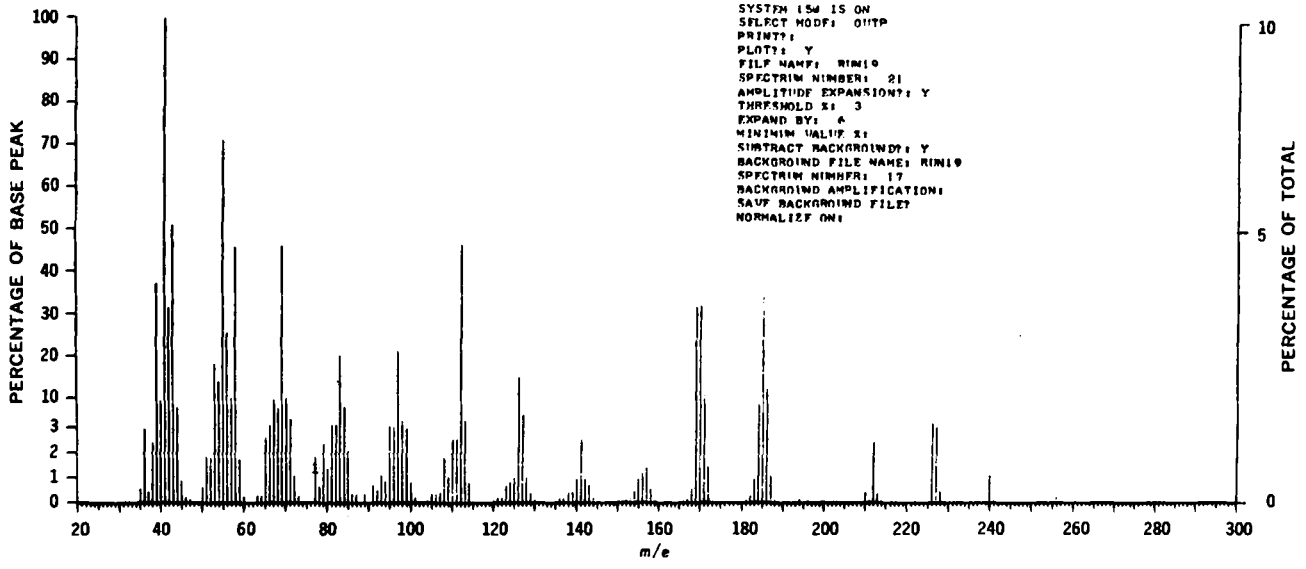


Figure 4—*Mass fragmentogram of amobarbital dimethylformamide dimethylacetal adduct from 1-μl. injection of 8.25 mg. of amobarbital, 1 ml. of reagent/5 ml. volume in dimethylformamide.*

then dilute to the mark with dimethylformamide[7]. For the internal standard, weigh accurately about 4 g. of dibutyl phthalate[8] into a 100-ml. volumetric flask, mix, and dilute to volume with reagent grade chloroform. Use 1.0 ml. of internal standard for approximately 20 mg. of barbiturate.

The sample solution with standard was injected (3 μl.) into the chromatograph using the prescribed operating conditions. In place of dibutyl phthalate, 1 ml. of a chloroform solution of *n*-eicosane/20 mg. of compound (concentration 12 mg./ml.) may be used.

Record the peaks and areas using a recorder equipped with an integrator (Disc) or digital integrator and calculate the response ratio and concentration of compounds of unknown purity using the appropriate standard equations.

Glutethimide Procedure—The standard procedure is changed by the addition of about 12 mg. (accurately weighed) of *n*-eicosane to the I-glutethimide mixture after heating. After the *n*-eicosane dissolves, 1 μl. is injected into the chromatograph, using the prescribed conditions, without further solvent dilution.

[7] Baker Instra Analyzed.
[8] Fisher reagent grade.

it was noted that the sodium salts of secobarbital and amobarbital reacted in a nonpredictable fashion, resulting in multiple chromatographic peaks. However, both reacted smoothly as the free acids alone or in a mixture.

Proof of acetal formation rather than methylation was obtained by the use of NMR and mass fragmentography. NMR analysis in $CDCl_3$ of the isolated adduct from the reaction of phenobarbital (Fig. 1), aprobarbital (Fig. 2*a*), and amobarbital (Fig. 2*b*) as well as metharbital in dimethyl sulfoxide-d_6 (Fig. 3) clearly indicated derivative formation of the methoxy type. Figure 1 shows singlet resonance at 203 Hz. of relative area 6, 2*a* shows a singlet at 199 Hz. (area 6) and N—H at 308 Hz., and 2*b* shows a singlet at 201 Hz. (area 6). In support of these findings, the isolated product of the reaction of metharbital gave an NMR spectrum which showed two nonequivalent —CH_3 patterns, the resonance at 199 Hz. being attributed to the N—CH_3 and that at 195 Hz. resulting from >$C(OCH_3)_2$. The former matches that shown in the spectrum of unreacted metharbital, while the peak at 195 Hz. (6 area units compared with the phenyl area of 5) could only indicate acetal formation at a carbonyl because of the existence of CH_3 on position 1 (area 3) and the absence of any other possible nonequivalent CH_3 resonance in the vicinity of 195 Hz.

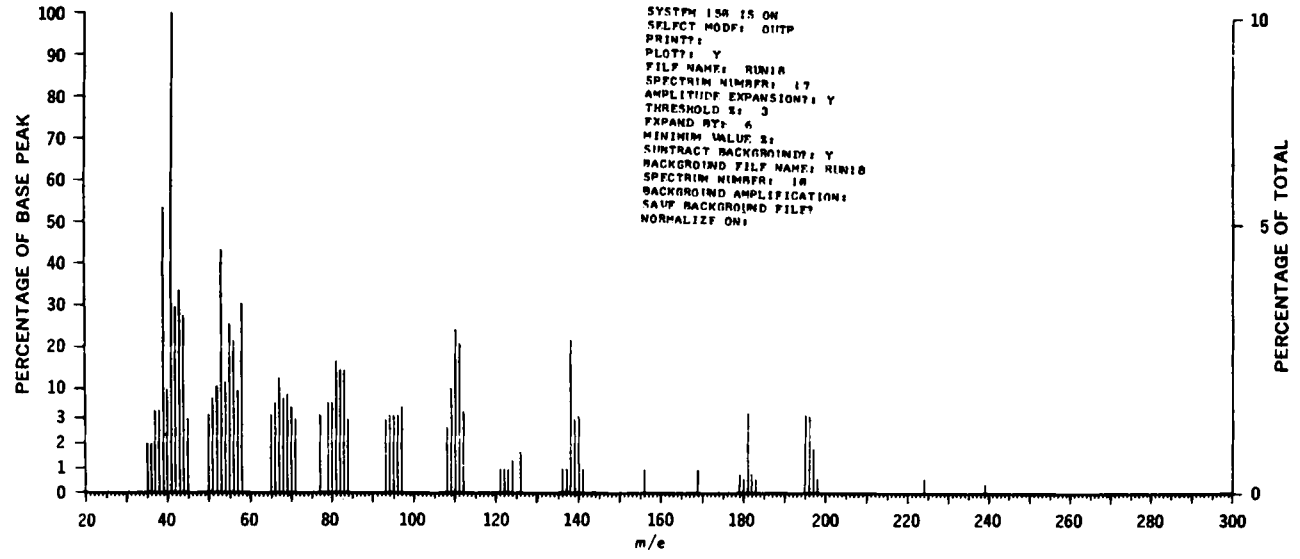


Figure 5—*Mass fragmentogram of aprobarbital dimethylformamide dimethylacetal adduct from 1-μl. injection of 8.77 mg. aprobarbital, 1 ml. of reagent/5 ml. volume in dimethylformamide.*

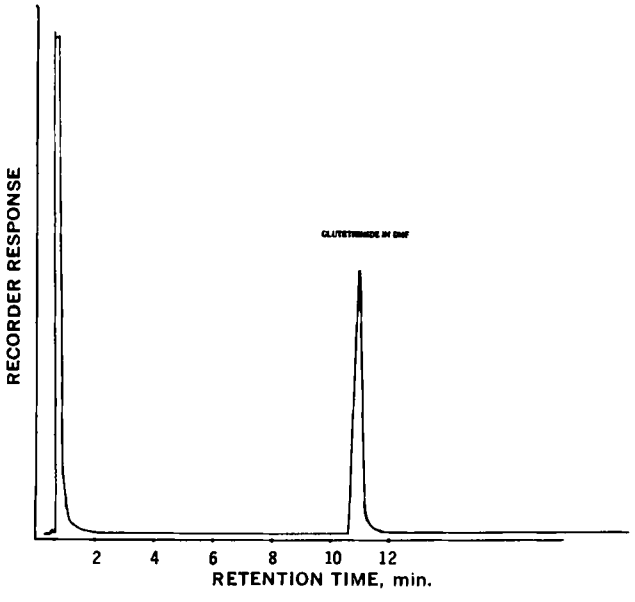
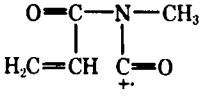


Figure 6—*Gas–liquid chromatogram of glutethimide (12.12 mg./ml.) in dimethylformamide; programmed conditions. Retention time = 11.05 min.*

Mass fragmentography of the reaction mixtures of amobarbital and aprobarbital, using the conditions previously given, was carried out as further proof of derivative formation. The resulting data were compared directly with those published by Knight (2). The patterns for amobarbital acetal (A) (Fig. 4) and aprobarbital acetal (B) (Fig. 5) are dissimilar to those given for the corresponding 1,3-dimethyl barbiturates (2). A partial analysis of the fragmentations typically showed what might be expected for acetals (9, 10). Distinguishing features in the pattern of A are *m/e* 257 ($M^+ - 15$, $-CH_3$), *m/e* 226 ($M^+ - 46$; $-CH_3$, $-OCH_3$), and *m/e* 195 ($M^+ - 77$; $-2-OCH_3$, $-CH_3$, $-H$). A distinguishing feature in the pattern of B was the absence of a significant *m/e* 91 peak, which showed 35% of base peak abundance during the fragmentation of 1,3-dimethylaprobarbital (2). This may be attributed to the loss of CH_3 from:



which is possible to a significant extent only in the dimethyl compound having an unsubstituted carbonyl at position 2. Other mass fragments suggestive of acetal presence in B were *m/e* 224 ($M^+ - OCH_3$, $-H$) and *m/e* 181 [$M^+ - OCH_3$, $-H$, $-CH(CH_3)_2$], along with $M^+ - 73$, $M^+ - 87$, and $M^+ - 101$, typical of the fragmentation of acetals (10).

The proposed mechanism explains, in part, the reason why methyprylon formed the acetal derivative with extreme difficulty and why the glutethimide derivative was susceptible to solvent-induced reversibility. In the case of methyprylon, both the 2- and the 4-carbonyl position are sufficiently hindered by the 3,3-diethyl substituent. In addition, the 4-position does not have imide character and, therefore, should not derivatize in this manner. With glutethimide, the imide carbonyl at C-6 is open to acetal formation but would not be as stable as the $-NHC(OCH_3)_2NH$ formed with the barbiturates. The reversibility of the glutethimide reaction can be noted by a comparison of Figs. 6 and 7.

Linearity of detector response was established for the six barbiturates examined (concentration range 2–30 mg./ml.). Secobar-

Table II—Recovery Data: Known Standards

Compound	Replicate Analyses	Concentration, mg./ml.	Percent Recovery[a]	σ	Coefficient of Variation, %
Hexobarbital	6	10.02	100.4	±0.4412	0.439
Phenobarbital	7	10.14	98.54	±0.4702	0.477
Amobarbital	6	9.93	98.93	±0.7712	0.780
Aprobarbital	6	9.74	98.50	±0.5899	0.599
Pentobarbital	6	9.43	99.13	±0.7033	0.709
Secobarbital	6	9.97	99.48	±0.5270	0.530
Methyprylon[b]	6	4.52	99.40	±0.5254	0.529

[a] Values are based upon replicates on a single preparation. [b] Direct injection of solution made with 0.5 ml. AcOH in dimethylformamide (5 ml. total volume).

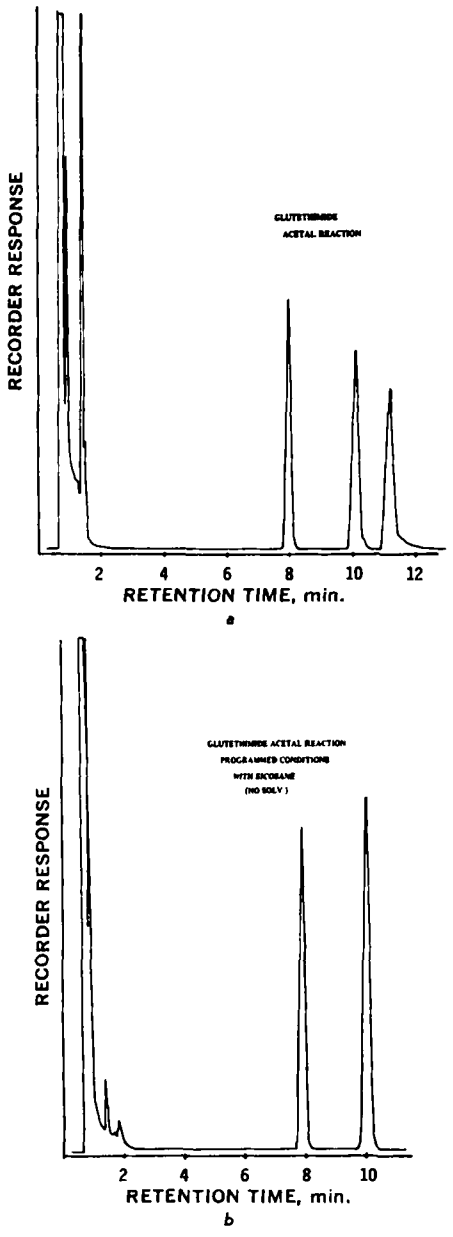


Figure 7—(a) *Gas-liquid chromatogram of glutethimide dimethylformamide dimethylacetal product with dibutyl phthalate internal standard (solvent mixture; $CHCl_3$; dimethylformamide acetal). Peak 1 = glutethimide acetal (8.03 min.); 2 = dibutyl phthalate (10.15 min.); and 3 = free glutethimide (11.2 min.). Chromatogram taken 60 min. after reaction.* (b) *Gas-liquid chromatogram of glutethimide dimethylformamide dimethylacetal product with* n-*eicosane (peak 1, 7.84 min.) without added solvent under programmed conditions. Chromatogram taken 60 min. after reaction.*

bital and amobarbital linearity showed coincident slopes. The retention time and response ratio data are presented in Table I. The relative retention times are, in increasing order, aprobarbital through phenobarbital, as expected by virtue of the effect of the substituents at the 5-position on the overall polarity and adsorbability of the molecule. No generalizations can be made for methyprylon and glutethimide with regard to elution characteristics since they do not follow the general barbiturate analyses.

An inspection of Table I as well as Figs. 8 and 9 clearly shows that, under programmed conditions, most combinations of barbiturates and methyprylon or glutethimide could be determined with minor procedural modifications. It is possible that methyprylon would interfere with baseline separation of pentobarbital and/or secobarbital (dibutyl phthalate standard) or glutethimide with phenobarbital (*n*-eicosane standard), although the probable need for such a separation would be remote.

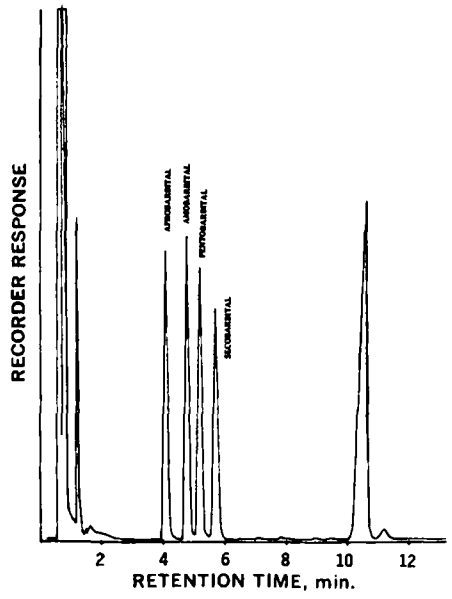


Figure 8—*Chromatogram showing the separation of the four noted barbiturates in a single mixture. Last major peak = dibutyl phthalate (minor peak is impurity in dibutyl phthalate).*

To evaluate the precision of the method and the quantitative recovery that can be anticipated, a series of standard barbiturates were analyzed (Table II). The recoveries obtained ranged from 98.54 to 100.4% based upon response to dibutyl phthalate internal standard. The standard deviation range was from ±0.4412 to ±0.7712.

Since one facet of the proposed method is the ease of determination of mixtures, two standard mixtures of bulk drugs were tested. Table III shows the results of determinations of: (*a*) pentobarbital plus hexobarbital, and (*b*) phenobarbital plus hexobarbital (Fig. 10) under programmed conditions and also at 215° isothermal. In addition, a commercial sample of sodium amobarbital with secobarbital sodium capsules[9] and a sodium pentobarbital capsule were

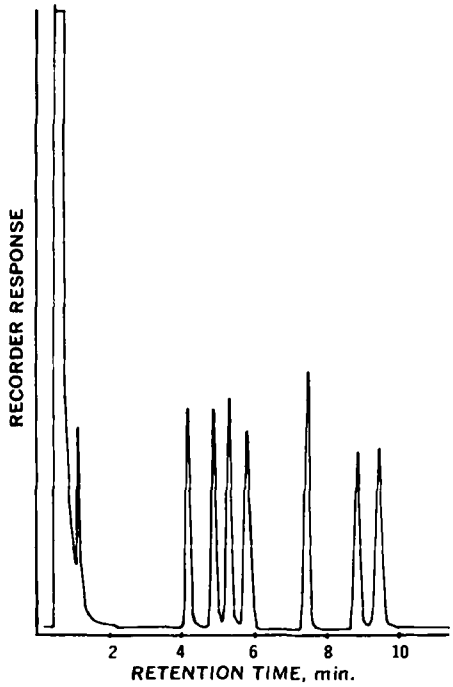


Figure 9—*Chromatogram showing the separation of aprobarbital, amobarbital, pentobarbital, secobarbital,* n-*eicosane, hexobarbital, and phenobarbital in their order of elution. The barbitals are as their acetals.*

[9] Tuinal.

Table III—Recovery Data: Barbiturate Mixtures

Mixture	Amount Used, mg.	Amount Found, mg.[a]	Percent Recovery[b]	Percent Theoretical
Phenobarbital–hexobarbital[c]	24.18 22.00	24.34 22.04	52.47 47.71	52.37 47.63
Phenobarbital–hexobarbital[d]	24.48 20.44	24.53 20.40	54.61 45.42	54.50 45.50
Phenobarbital–hexobarbital	19.93 21.85	20.05 22.05	47.98 52.77	47.70 52.30

[a] Average of duplicate determinations. [b] Relative to the mixture. [c] Programmed conditions. [d] Isothermal conditions (215° column temperature. Retention times: hexobarbital, 5.0 min.; phenobarbital, 5.82 min.; and dibutyl phthalate, 7.01 min.).

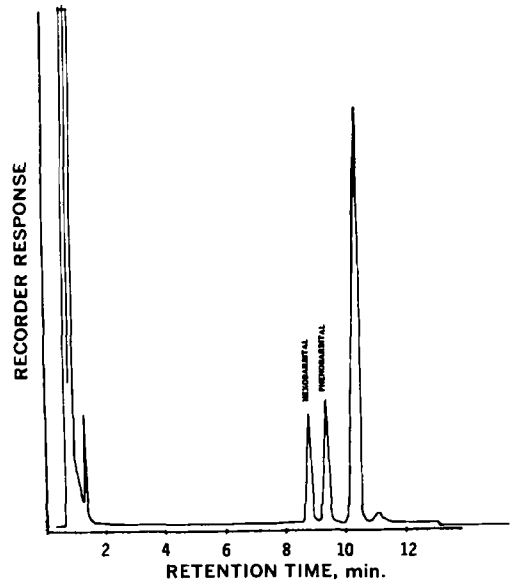


Figure 10—*Chromatogram of a mixture (Table III) of hexobarbital and phenobarbital with dibutyl phthalate internal standard, using the standard programmed conditions.*

assayed. The former assayed at 95.0% of the labeled amount (200 mg./capsule) when determined on two separate samplings. In the latter case, a single capsule assayed at 92.3% of the labeled amount (assuming 100 mg./capsule). The USP XVIII extraction procedure (11) for the respective dosage form was used, followed by evaporation of an aliquot of the chloroform extract to dryness (25°, stream of nitrogen) prior to acetal reaction.

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Fluorometric Determination of Epinephrine in Low Dosage Injections and in Lidocaine Hydrochloride–Epinephrine Combinations

THERON JAMES

Abstract □ A procedure is reported for determining epinephrine in low dosage injections and in combination with lidocaine hydrochloride. Epinephrine is eluted from a buffer column as its ion-pair with di(2-ethylhexyl)phosphoric acid in ether, extracted into hydrochloric acid, and determined fluorometrically. The method determines unchanged epinephrine by isolating it from its sulfonic acid and other decomposition products; it is a measure of the stability of epinephrine in solution and can be modified to include an assay for lidocaine hydrochloride. When synthetic mixtures of epinephrine and epinephrine–lidocaine hydrochloride were analyzed by the proposed procedures, recoveries for epinephrine ranged from 98.5 to 101.3%, and recoveries for lidocaine hydrochloride ranged from 98.6 to 101.7%. Assay results on 23 different commercial samples of varying potencies and combinations are also reported.

Keyphrases □ Epinephrine—separation, analysis in low dosage injections and combinations with lidocaine hydrochloride □ Lidocaine hydrochloride–epinephrine mixtures—separation, analysis of epinephrine □ Partition chromatography—extraction of epinephrine as ion-pair with di(2-ethylhexyl)phosphoric acid □ Di(2-ethylhexyl)phosphoric acid—used in ion-pair partition chromatography of epinephrine □ Spectrophotofluorometry—analysis, epinephrine in low dosage injections and combinations with lidocaine hydrochloride

Recent evidence suggests that low dosage epinephrine solutions (0.01–0.1 mg./ml.) are not very stable in the presence of bisulfite, which reacts with epinephrine to produce epinephrine sulfonic acid, a physiologically inactive derivative. Conditions for this ester reaction were discussed previously (1, 2). Nevertheless, bisulfite is commonly added to this type of preparation because it is necessary to prevent adrenochrome formation.

Compounding an already complex situation, assay procedures for epinephrine are limited in scope and specificity and involve very tedious and complex manipulations. The USP XVIII (3) procedures for epinephrine injection and solution are based on its optical rotation and, as such, require at least 30 mg. of epinephrine for the assay. Obviously, this method is not applicable to low dosage epinephrine solutions.

Colorimetric methods (4, 5) can be extended to low levels of epinephrine but will not selectively determine epinephrine in the presence of epinephrine sulfonic acid and other decomposition products.

Hellberg's fluorometric procedure (6) is applicable to low levels of epinephrine and detects only the unchanged epinephrine. This method is a modification of the trihydroxyindole reaction first observed by Loew (7), and many other variations have been tried (8–17). The large number of published modifications attests to the fact that the assay is subject to many variables and interferences. Time, temperature, pH, type of oxidizing agent, presence of reducing agents, and composition of the final alkali–ascorbate mixture all affect the stability and sensitivity of the fluorescent lutine produced. Additionally, the bisulfite generally found in commercial preparations hampers the development of fluorescence (6). The USP XVIII procedure for epinephrine in lidocaine hydrochloride injections (18), still another variation of Hellberg's procedure, compensates for the presence of bisulfite by adding it to the standard.

Boon and Mace (19) reported a GC determination of epinephrine which involved ion-pair extraction and the formation of a derivative with *N,O*-bis(trimethylsilyl)acetamide. The method also included a concentration step for low dosage forms. Reproducibility was estimated to be only ±10%.

The trend now appears to be toward automated or semi-automated methods (20–22). Automated methods offer several advantages, chief of which are the reduction of human error and the normalization of errors inherent in manual procedures. However, in spite of the recent advances in automation, a reliable manual method would still be a more efficient and expedient way to analyze an occasional sample.

Levine and Doyle (23) extracted phenylephrine quantitatively by using partition chromatography with an ether solution of di(2-ethylhexyl)phosphoric acid. The same general procedure was used by Welsh and Sammul (24) to determine isoproterenol in deteriorated inhalations and injections. In their work the di(2-ethylhexyl)-phosphoric acid–ether mobile phase extracted the isoproterenol–di(2-ethylhexyl)phosphoric acid ion-pair, while the isoproterenol sulfonic acid remained in the stationary phase.

Welsh and Sammul (24) also reported standard recovery data for several substances related to isoproterenol, including epinephrine. By using their extraction procedure, a method has been developed to assay unchanged epinephrine in low dosage forms. A portion of sample is buffered to pH 5.6 and incorporated into a diatomaceous earth[1] column. Epinephrine is eluted with an ether solution of di(2-ethylhexyl)phosphoric acid, extracted into dilute hydrochloric acid, and determined fluorometrically.

This partition system, with some modification, is also effective in the analysis of epinephrine and lidocaine hydrochloride in combination. An ether wash of the sample column, prior to the elution of epinephrine, removes most of the lidocaine hydrochloride, which is subsequently trapped on a hydrochloric acid column. Chloroform removes the last traces of lidocaine hydro-

[1] Celite 545, Johns-Manville Corp., New York, N. Y.

Table I—Recovery of Epinephrine and Lidocaine Hydrochloride from Synthetic Mixtures

Sample	Epinephrine Recovery, %	Lidocaine Hydrochloride Recovery, %
I (0.01 mg./ml. epinephrine)	98.5	—
	100.2	—
	100.2	—
	100.5	—
	99.5	—
	Average = 99.8	—
	SD = 0.80%	—
II (0.01 mg./ml. epinephrine, 1% lidocaine hydrochloride)	99.9	99.4
	98.9	99.4
	100.9	101.1
	98.8	101.7
	98.9	100.2
	99.1	99.2
	Average = 99.4	Average = 100.2
	SD = 0.83%	*SD* = 1.03%
III (0.01 mg./ml. epinephrine, 2% lidocaine hydrochloride)	100.0	99.7
	101.3	99.9
	100.0	99.7
	100.0	98.6
	Average = 100.4	Average = 99.5
	SD = 0.64%	*SD* = 0.59%

chloride from the sample column, and additional chloroform elutes lidocaine hydrochloride quantitatively from the acid column.

EXPERIMENTAL

Apparatus—Fluorescence measurements were made using a recording spectrophotofluorometer[2] with 1-cm. cells. The following instrument parameters were employed: xenon lamp; meter multiplier, 0.01; sensitivity, 20–25; 1P21 photomultiplier; slit arrangement No. 4; excitation wavelength, about 281 nm.; and emission wavelength, about 334 nm.

UV spectra were obtained using a recording spectrophotometer[3].

Buffer (pH 5.6)—Mix 1.5 volumes of 1 *M* dibasic potassium phosphate with 8.5 volumes of 1 *M* monobasic potassium phosphate. Adjust to pH 5.60 ± 0.05.

Standard Solutions—The following were used: (*a*) epinephrine bitartrate, 2.16 mcg./ml. (about 1.2 mcg./ml. epinephrine base) in *ether-saturated* 0.1 *N* HCl; and (*b*) lidocaine hydrochloride monohydrate, 0.33 mg./ml. in 0.1 *N* HCl.

Sample Preparation—If necessary, dilute an accurately measured volume of sample to an epinephrine concentration of 10 mcg./ml. using pH 5.6 buffer solution.

Procedure—Use water-saturated solvents throughout.

A: Samples without Lidocaine Hydrochloride—Transfer a mixture of 1 g. of acid-washed diatomaceous earth with 1 ml. of pH 5.6 buffer to a glass chromatographic column containing a pledget of glass wool, and tamp to uniform mass. Into a beaker, pipet 3.0 ml. of sample preparation, add 3.0 ml. of pH 5.6 buffer, and swirl to mix. Add 7 g. of diatomaceous earth, mix thoroughly, and add to column in portions, tamping moderately after each addition.

Wash prepared column with 100 ml. of ether and discard wash. Place a separator containing 10 ml. of 0.1 *N* HCl as a receiver under the column. Elute epinephrine with 50 ml. of a 1.5 in 50 solution of di(2-ethylhexyl)phosphoric acid in ether, followed by 25 ml. of ether.

After all ether has passed through the column, shake separator vigorously for 2 min. Allow layers to separate and then collect the acid layer in a 25-ml. volumetric flask. Repeat extraction with another 10 ml. of 0.1 *N* HCl, again collecting the acid in the volumetric flask. Finally, dilute to volume with 0.1 *N* HCl and mix.

[2] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Silver Spring, Md.

[3] Cary model 15 spectrophotometer, Applied Physics Corp., Monrovia, Calif.

Table II—Assay of Commercial Epinephrine Samples

Sample	Epinephrine Declared, mg./ml.	Epinephrine Found, mg./ml.	Percent of Declared
1	0.01	0.0062	62
2	0.01	0.0052	52
3	0.01	0.0076	76
4	0.02	0.0140	70
5	0.02	0.0182	91
6	0.1	0.078	78
7	0.1	0.100	100
8	0.1	0.107	107
9	0.1	0.108	108
10	0.1	0.088	88
11	0.1	0.111	111
12	0.1	0.110	110
13	0.1	0.113	113
14	10	8.45	85

Adjust the spectrophotofluorometer to about 70% relative fluorescence intensity at 334 nm., reading percent fluorescence at maximum. Use ether-saturated 0.1 *N* HCl as a blank.

B: Samples Containing Lidocaine Hydrochloride—Prepare the sample column as under Procedure A. Mount a second column, prepared with 3 g. of diatomaceous earth and 2 ml. of 1 *N* HCl, directly below the sample column. Pass 150 ml. of ether through both columns and discard wash. Change receivers to a 400-ml. beaker and elute through both columns with 50 ml. of chloroform. Rinse the tip of sample column with chloroform and separate columns.

Continue eluting lidocaine hydrochloride from second column with chloroform, collecting about 200 ml. Evaporate eluate to dryness, dissolve residue in 0.1 *N* HCl, and dilute to a lidocaine hydrochloride concentration of about 0.3 mg./ml. Determine the absorbance of this solution at 262 nm.

Wash sample column with 25 ml. ether and elute epinephrine as under Procedure A.

RESULTS AND DISCUSSION

The method isolates unchanged epinephrine from its sulfonic acid and from lidocaine hydrochloride by utilizing ion-pair formation and partition chromatography. The ether wash removes parabens from the buffer column, along with 90–95% of the lidocaine hydrochloride, if present. Lidocaine hydrochloride is trapped on the 1 *N* hydrochloric acid column while parabens pass through and are discarded. The lidocaine hydrochloride remaining on the buffer column is eluted with chloroform, and the total lidocaine hydrochloride is then quantitatively eluted from the acid column with additional chloroform.

The procedure for epinephrine, while not as sensitive as the USP XVIII (18) procedure, does afford more simplicity and stability. However, two precautions should be observed:

1. The pH of the sample column should not be below 5.2; otherwise, incomplete extraction might occur.
2. The final standard solution should be prepared in ether-saturated 0.1 *N* HCl. Ether-saturated hydrochloric acid apparently enhances the fluorescence of epinephrine by about 8%.

Three synthetic solutions, each containing 0.01 mg./ml. epinephrine and from 0 to 2% lidocaine hydrochloride, were prepared

Table III—Assay of Commercial Epinephrine–Lidocaine Hydrochloride Samples

Sample	Epinephrine, mg./ml. Declared	Epinephrine, mg./ml. Found	Lidocaine Hydrochloride, mg./ml. Declared	Lidocaine Hydrochloride, mg./ml. Found
1	0.01	0.0021	10	9.40
2	0.01	0.0051	10	9.59
3	0.01	0.0083	10	10.0
4	0.01	0.0061	20	19.0
5	0.01	0.0073	20	19.0
6	0.01	0.0083	20	20.4
7	0.01	0.0081	20	20.6
8	0.01	0.0081	20	20.2
9	0.01	0.0102	20	19.3

with the common excipients methyl paraben and sodium chloride in a buffer solution. Replicate analyses of these solutions are presented in Table I.

Several commercial samples were assayed by the proposed procedure (Tables II and III). Judging from the results, there appears to be a significant problem of epinephrine deterioration. Most samples over 6 months in age showed some decomposition. Samples containing 0.02 mg./ml. or less of epinephrine had higher and more severe incidences of decomposition. Additionally, samples that assayed less than 75% of the declared value often had wide variations from vial to vial; individual vial analyses of one sample ranged from 48 to 71%. This would indicate that the rate of decomposition is not constant. Several factors, including storage conditions, pH, substrate composition, and bisulfite concentration, may affect the rate of decomposition, but a discussion of these factors is not within the scope of this report. The cogent element of the method presented here is that it measures the stability of epinephrine in commercial preparations, whatever the factors affecting that stability may be.

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PHARMACEUTICAL TECHNOLOGY

Design and Evaluation of a Rotating Filter–Stationary Basket *In Vitro* Dissolution Test Apparatus I: Fixed Fluid Volume System

ASHOK C. SHAH▲, CRAIG B. PEOT, and JOHN F. OCHS

Abstract □ The apparatus described in this report provides a reliable and convenient means for determining *in vitro* dissolution characteristics of tablets, capsules, powders, suspensions, and most other solid drug dosage forms. Basic components of the apparatus are a perforated stationary sample basket, a rotating filter–stirrer assembly, and a closed jacketed dissolution fluid container. Among major advantages of the apparatus are: (*a*) precision-controlled variable intensity of mild laminar liquid agitation; (*b*) continuous or intermittent filtration of representative dissolution fluid samples through a nonclogging, microporous, *in situ* filter for automated or manual dissolution rate determinations; (*c*) convenient means for introducing solid samples in a stationary basket and positioning at a set level in the fluid medium; (*d*) minimal mechanical impacts, abrasion, and wear of the solid sample, with the retainment of its microenvironment during the dissolution process; and (*e*) simultaneous determinations of disintegration–dissolution rates of tablets and capsules. Studies performed using this apparatus are described to demonstrate its reproducibility, reliability, and application versatility as a research, development, and quality control test apparatus. Dissolution rates of five different tablet lots of an antidiabetic drug evaluated by this apparatus correlated with their *in vivo* activity. A multiple-test system for the simultaneous automated determination of six dissolution rates is described.

Keyphrases □ Dissolution equipment—design and evaluation of a rotating filter–stationary basket apparatus, compared to compendial methods □ Rotating filter–stationary basket dissolution apparatus—design and evaluation, compared to compendial methods

It is now generally recognized that the dissolution rate of a drug from its solid dosage form can become the rate-limiting process in the physiological availability and *in vivo* absorption of the drug. In recent years, therefore, considerable interest has been focused on the development of a reliable *in vitro* dissolution test

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It is now generally recognized that the dissolution rate of a drug from its solid dosage form can become the rate-limiting process in the physiological availability and *in vivo* absorption of the drug. In recent years, therefore, considerable interest has been focused on the development of a reliable *in vitro* dissolution test

method, which can positively characterize the *in vivo* dissolution rate-controlled absorption of drugs administered in solid dosage forms. One basic requirement to achieve this goal, however, seems to be the availability of a reliable and flexible dissolution test apparatus, which is not only suitable for characterizing *in vivo* dissolution behavior of essentially all types of solid drug dosage forms but is also convenient to use for research, development, and quality control. Such an apparatus should meet most of the following criteria:

1. The design, dimensions, and positioning of each individual component of the apparatus must be exactly specified. Prototype units can be economically fabricated from commercially available equipment.

2. The apparatus should be relatively simple in design, convenient to operate, flexible for use under a variety of test conditions, and give reproducible results upon repeated tests.

3. Dissolution rates evaluated by the apparatus under appropriate physiological test conditions should correlate with the *in vivo* dissolution rate-controlled drug absorption process.

4. The apparatus should permit controlled variable intensity of mild, uniform, nonturbulent liquid agitation.

5. It must provide a convenient means for introducing a test sample (tablet, capsule, *etc.*) into the dissolution medium and holding it at a set position such that the sample is completely immersed in the fluid medium. During the dissolution process, the test sample must be subjected to minimal mechanical impacts, abrasion, and wear in order to retain its microenvironment.

6. The dissolution fluid container must be closed to prevent solvent evaporation, thermostated to regulate fluid temperature, and preferably transparent to permit visual observation of the disintegration characteristics of the test sample and fluid flow conditions during dissolution. It should be possible to maintain solvent sink conditions by employing either a relatively large volume of dissolution fluid or other convenient means such as continuous flow dilution of the bulk fluid with fresh solvent.

7. Withdrawal of representative fluid samples of the bulk medium for analysis, either by manual or automated methods, must be possible without interrupting solution agitation. As an automated system it should be possible to conduct continuous filtration of the fluid samples efficiently without encountering any operational or analytical problems.

8. The apparatus must be applicable for the evaluation of disintegrating, nondisintegrating, dense, or "floating" tablets and capsules; finely powdered drugs; and all other types of solid drug forms.

Although numerous types of dissolution test apparatus are reported in the literature (1–14), many, including those recognized by the official compendia (15, 16), do not seem to meet all of the stated criteria desired. In addition, the nature and extent of solvent agitation, treatment of the test sample during the dissolution study, and liquid sampling techniques employed in some of these systems can yield unreliable dissolution rate results. For example, in NF XIII Method II the fluid undergoes violent, turbulent agitation, while in both the official test methods (USP–NF Method I and NF Method II) the sample container itself serves as a liquid stirring device. Under these conditions, there can be excessive abrasion and wear of the sample due to its mechanical impacts with the container surface, thereby adversely influencing its microenvironment.

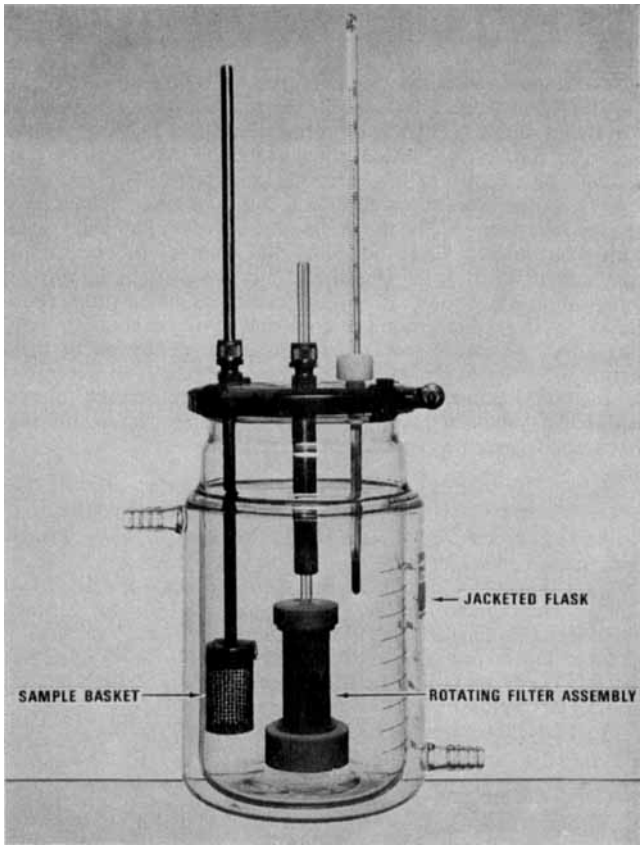

Figure 1—*Photograph of the rotating filter–stationary basket apparatus.*

Another major problem present in most automated and continuous flow dissolution test apparatus is the difficulty in conducting continuous, efficient filtration of the dissolution fluid sample. Continuous monodirectional filtration through a conventional, static, coarse-filter element (stainless steel wire cloth screen, sintered glass, membrane, *etc.*) frequently clogs the filter with undissolved solid particles, which results in progressive diminution of the fluid flow rate (9, 17, 18). Liquid filtration through a relatively coarse-filter screen permits fine particles to escape and accumulate in the circulation tubing as well as in the spectrophotometer flow cell. These filtration problems not only create serious operational difficulties due to the diminishing fluid flow rates but may yield erroneous dissolution rate results due to: (*a*) continual passage of dissolution fluid through a filter covered with drug particles and (*b*) interference in the spectrophotometric analysis of the filtered fluid by solid particles collected in the spectrophotometer flow cell.

The design and evaluation of a reliable, rotating filter–stationary basket dissolution test apparatus[1],

[1] Patent is pending on the design and application of this apparatus.

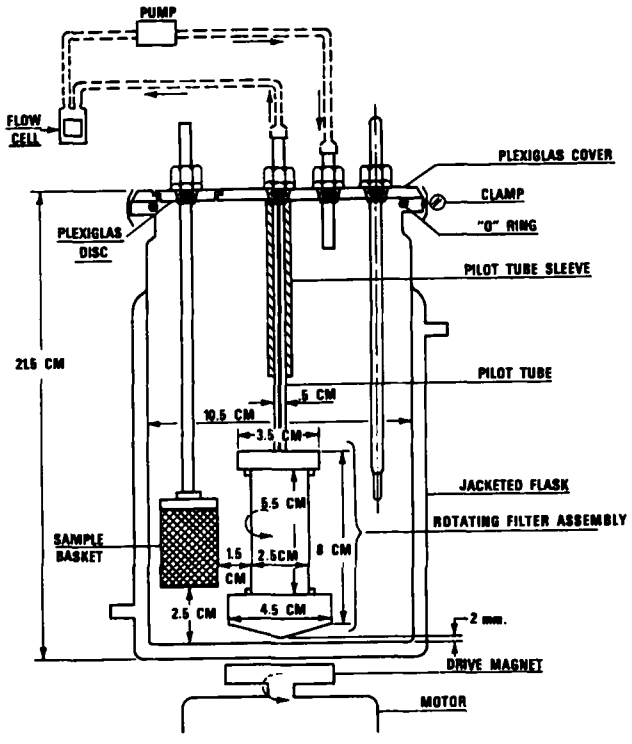

Figure 2—*Schematic diagram of the rotating filter–stationary basket apparatus. Broken lines represent setup for the automated spectrophotometric analysis.*

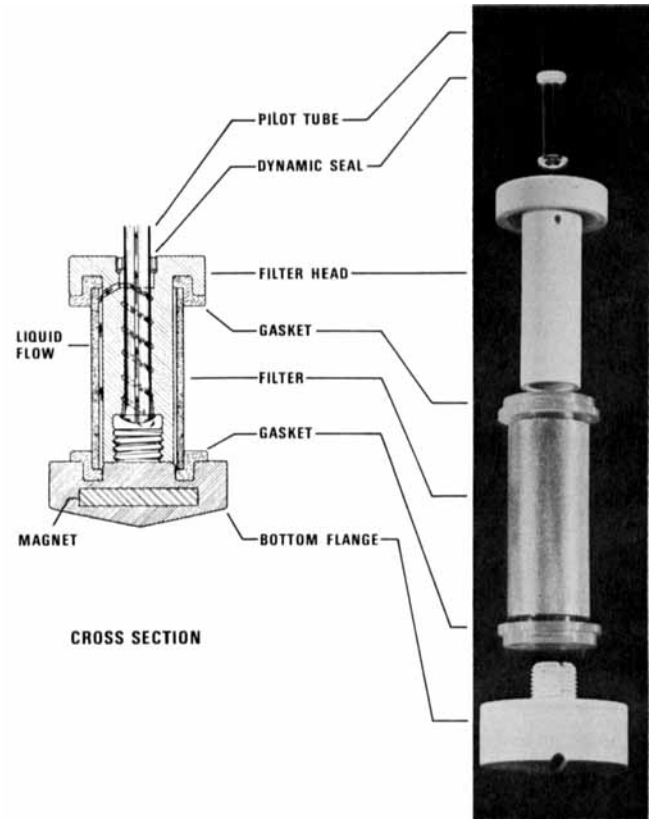

Figure 3—*Rotating filter assembly.*

which eliminates these problems and meets essentially all of the above-mentioned criteria, are reported here. Essential features of the apparatus are a stationary sample basket, a large volume fluid container, and a rotating filter assembly. The rotating filter system, employed previously in the cultivation of mammalian cells (19), functions as a liquid agitation device as well as an efficient fluid sampling system.

APPARATUS DESCRIPTION

Single-Test Apparatus—The rotating filter–stationary basket single-test apparatus was constructed by incorporating necessary modifications into a commercially available microbial cell culture cultivation flask[1]. Basic features of the apparatus (Figs. 1 and 2) are a jacketed dissolution fluid container flask, a stationary sample basket, a rotating filter assembly, and an external variable speed magnetic stirrer[2]. Broken lines in Fig. 2 represent the optional setup for automated dissolution rate determinations, in which filtered fluid samples are cycled through a spectrophotometer flow cell by means of 2-mm. i.d. flexible polyethylene tubing and a peristaltic pump[3]. The description of individual parts of the apparatus follows.

Fluid Container Flask and Cover—The jacketed glass flask, suitable for holding up to 1.5 l. of dissolution fluid, has a removable Plexiglas cover secured firmly to the neck of the flask by means of an O-ring and a flexible metal belt. The cover serves as a support for the sample basket and the rotating filter assembly. A rabbeted edge circular opening in the cover, 3 cm. in diameter, provides an entrance pathway for the sample basket. There are three other ports in the cover: one in the center for a glass capillary pilot tube, another for a thermometer, and the third one for the return of the dissolution fluid from the spectrophotometer flow cell.

Sample Basket—The design features of the stationary sample basket are similar to the basket assembly employed in the official USP–NF Method I apparatus (15), with the exception of using 12-mesh instead of 40-mesh wire cloth screen to facilitate fluid movement through the basket and prevent plugging of the basket screen with solid particles (4, 20, 21). A rabbeted edge circular Plexiglas disk is attached to the basket holding rod with a compression fitting, so that when the basket is introduced into the flask through the cover opening, this disk rests in the corresponding rabbeted edge opening and holds the basket at a preset level in stationary position. The basket level can be varied by moving the position of the disk along the holding rod. In all experiments, the basket was held 2.5 cm. from the flask bottom (Fig. 2).

Rotating Filter Assembly—The rotating filter assembly provides variable intensity of mild laminar liquid agitation and it also functions as an *in situ* nonclogging filter to permit intermittent or continuous filtration of the dissolution fluid samples efficiently during the dissolution process. The assembly is suspended in the center of the flask on the flared end of a glass capillary pilot tube. Since the pilot tube is secured firmly to the cover with a compression fitting,

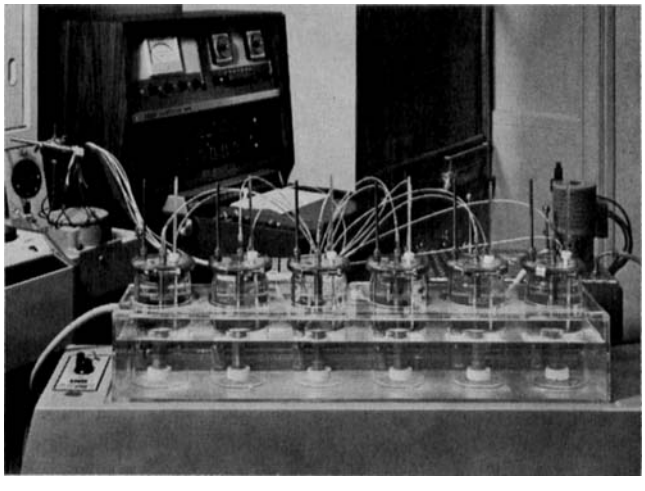

Figure 4—*Photograph of the multiple-test rotating filter–stationary basket apparatus.*

[1] Flask model BSC 1000 CA and magnetic stirrer model MS-1, The Virtis Co., Gardiner, NY 12525

[2] Model 1210, Harvard Apparatus Co., Millis, MA 02054

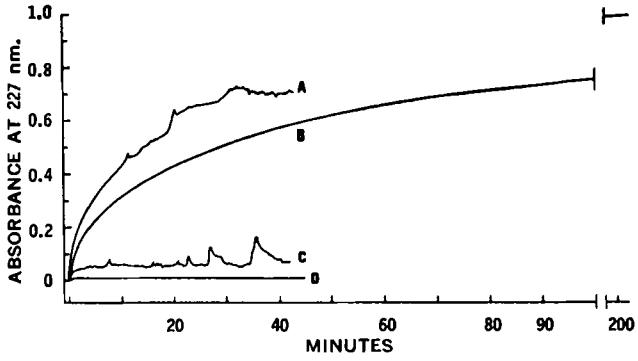

Figure 5—*Comparison of the rotating filter with the conventional wire mesh screen filter used in the automated spectrophotometric dissolution rate analysis of an active tablet sample (A and B) and placebo tablet sample (C and D). Key: A and C, screen filter; and B and D, rotating filter.*

it remains in a fixed position while the assembly can freely rotate on the flared end of the tube. The assembly rotates by means of a controlled, variable speed, external magnetic stirrer coupling with a magnetic bar embedded in the bottom part of the assembly. The level of the assembly in the flask can be varied simply by raising or lowering the pilot tube. A stainless steel pilot tube-sleeve provides support to the pilot tube and prevents subtle vibration of the assembly.

The design features of the assembly (Fig. 3) consist of a filter head, bottom flange, cylindrical filter, two flexible gaskets, and a dynamic seal. The filter head and bottom flange are fabricated from 20% glass-filled Teflon with a magnet embedded in the bottom flange. Cylindrical filters of glass fiber, Teflon, ceramic, or sintered stainless steel are available in the 0.2–3-μ porosity range. In the present studies, either a 0.5- or 1-μ porosity stainless steel filter was employed. In assembling these parts, first the filter head is suspended on the flared end of the pilot tube, then the cylindrical filter with one flexible gasket on each end is slipped over the filter head, and finally the bottom flange is screwed into the filter head threads. The spring-action dynamic seal slid over the pilot tube positions into the filter head and prevents passage of liquid through the space between the pilot tube and the filter head. Arrows in the cross-sectional diagram of Fig. 3 show the liquid filtration system and flow through the assembly. Dissolution fluid upon filtration through the cylindrical filter flows through a hole in the filter head, channels through a helical path around the pilot tube, and then enters into the pilot tube capillary. Fluid samples can be withdrawn continuously or intermittently from the upper end of the pilot tube.

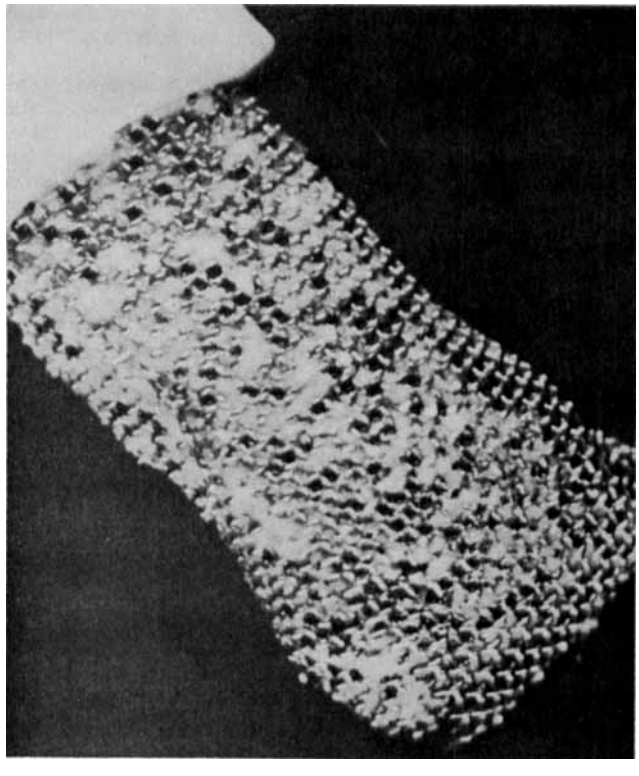

Figure 6—*Photograph of the clogged screen filter.*

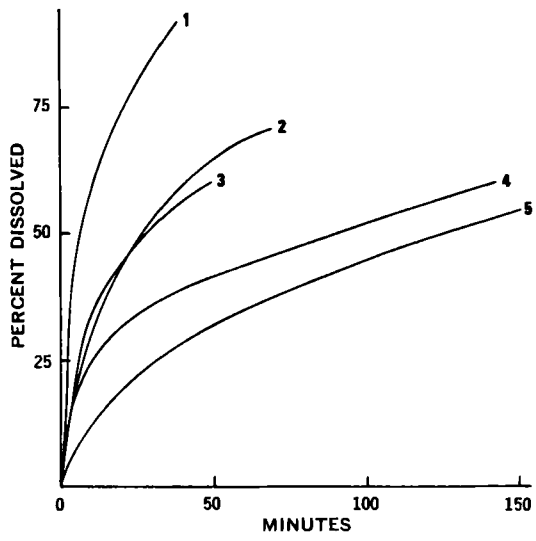

Figure 7—*Dissolution profiles of five different tablet formulations of an antidiabetic drug measured by the automated rotating filter–stationary basket apparatus.*

Multiple-Test Apparatus—An automated multiple-test rotating filter–stationary basket apparatus (Fig. 4) is capable of monitoring up to six dissolution tests simultaneously. The basic design of this apparatus is similar to the single-test unit, with the exception of using a Plexiglas water bath to thermostat all six units and a six-stage controlled variable speed magnetic stirrer operated by a single motor[4]. The upper panel of the stirrer housing is illuminated by a fluorescent light placed underneath the housing, which aids in visual observation of the test environment. Dissolution fluid samples from each flask are continuously cycled by a multichannel pump[5] through one of six flow cells located in the cell compartment of the spectrophotometer[5]. Dissolution rates are monitored by the spectral absorbance recording of the cycling fluid samples at programmed time intervals.

EXPERIMENTAL

Procedure—In a typical experiment, the sample basket is removed from the flask and a measured volume of dissolution fluid is transferred into the flask through the rabbeted edge opening in the cover. The fluid is allowed to equilibrate at 37° and is stirred by rotation of the filter assembly at set revolutions per minute. Filtered fluid samples are continuously withdrawn through the capillary pilot tube at a 40-ml./min. flow rate and are circulated through a spectrophotometer. When all the test conditions (*i.e.*, fluid temperature, stirring rate, cycling through the flow cell, and calibration of the spectrophotometer) are properly adjusted, the dissolution experiment is started by introducing the sample basket into the flask through the rabbeted edge opening in the cover.

For powdered drugs, a weighed amount of the sample is first dispersed into a small volume (5 ml.) of the dissolution medium and then immediately transferred into the bulk fluid medium. In the case of metastable drug–polyvinylpyrrolidone coprecipitates, the weighed powder sample was added directly into the fluid medium and was dispersed by a momentary rapid liquid agitation. In all powder dissolution studies, a 0.5-μ porosity filter was used,

[4] Jar test machine model 7303, Coffman Industries, Kansas City, KS 66101

[5] Model DBG or Kintrac VII spectrophotometer, Beckman Instruments, Fullerton, CA 92634

Table I—Tablet Dissolution Rates ($T_{50\%}$)[a] Determined by the USP-NF Method I and Rotating Filter-Stationary Basket Method

Apparatus	$T_{50\%}$, min. Experiment Number 1	2	3	4	5	6	Mean	*SD*	Coefficient of Variation, %
USP-NF Method I	23.6	44.7	44.9	53.6	15.6	57.0	39.9	16.6	41.7
Rotating filter-stationary basket method	34.0	34.0	31.3	34.0	33.0	33.0	33.4	1.09	3.28

[a] Time required for 50% of the drug to dissolve.

whereas in the tablet dissolution studies a 1-μ porosity filter was employed.

Upon completion of the dissolution run, the apparatus can be conveniently cleaned by discarding dissolution fluid through the opening in the cover and adding water through the same opening to clean the flask and the filter assembly. If necessary, however, the cover can be removed from the flask by loosening the metal belt and each component can be separately cleaned. The filter can be cleaned either by circulating water through it or by placing it in an ultrasonic cleaning bath.

Results—*Comparison of Static* versus *Dynamic Liquid Filter Systems*—The influence of filtration efficiency in the fluid sampling procedure upon the accuracy of dissolution rate results is demonstrated by comparing the dynamic rotating filter system described in this report with a conventional static filter consisting of a stainless steel 100-mesh wire cloth screen filter (3). Dissolution profiles of an active and a placebo tablet sample (Fig. 5) were determined by the automated spectrophotometric analysis of the fluid samples filtered continuously through the static screen filter (curves A and C) and the dynamic rotating filter system (curves B and D). Except for the difference in the filtration system, these studies were performed under identical experimental conditions.

Continuous filtration of the test fluid through the static screen filter resulted in clogging of the filter screen by accumulation of solid particles on the filter surface. Consequently, there was progressive diminution of the fluid flow rate from 40 ml./min. initially to only 2 ml./min. after about 45 min. A photograph of the clogged screen filter is illustrated in Fig. 6. A noticeable amount of fine particles escaped through the screen filter and accumulated in the flexible tubing and spectrophotometer flow cell. These apparent filtering problems encountered with the static filter system may account for the erratic spectral response evident in curves A and C and a false absorbance reading recorded with the placebo tablet sample (curve C). Similar types of filter clogging problems were also observed with other static filter systems such as sintered-glass and Millipore filters.

The dynamic rotating filter system permitted continuous, efficient filtration of the test fluid for prolonged time periods (up to 200 min.) without any noticeable sign of filter clogging, change in the fluid flow rate, or escape of solid particles through the filter pores. The efficiency of the rotating filter system is reflected in the smooth spectral recording of the dissolution profiles (curves B and D, Fig. 5) and, as expected, the almost zero absorbance reading recorded upon dissolution of the placebo tablet (curve D).

Reproducibility of USP-NF Method I and Rotating Filter-Stationary Basket Apparatus—The dissolution rate results (Table I) were obtained upon six repeated runs for a tablet sample employing an automated USP-NF Method I apparatus (21), at 300-r.p.m. stirring, and the automated rotating filter-stationary basket apparatus, at 600-r.p.m. stirring. These stirring speeds were selected because of the comparable tablet dissolution rates ($T_{50\%}$) obtained under these conditions in both apparatus. Other test conditions employed were one tablet sample per test, 900 ml. of simulated intestinal fluid without the enzyme, and 37°. It is apparent from the data listed in Table I that, for the tablet samples examined, the results obtained with the rotating filter-stationary basket apparatus are considerably more reproducible than with the USP-NF Method I apparatus.

In Vitro–In Vivo *Correlation*—Dissolution profiles for five different tablet lots of an antidiabetic drug (Fig. 7) were determined using the rotating filter-stationary basket apparatus. The test conditions employed for these studies were: one 2.5-mg. tablet sample, 1 l. of simulated intestinal fluid without the enzyme, 37°, and 400-r.p.m. stirring speed. Solvent sink conditions were maintained during the dissolution because the maximum drug concentration in solution reached upon complete dissolution was about 12% of the saturation concentration. The disintegration time for all of these tablet samples was less than 45 sec.

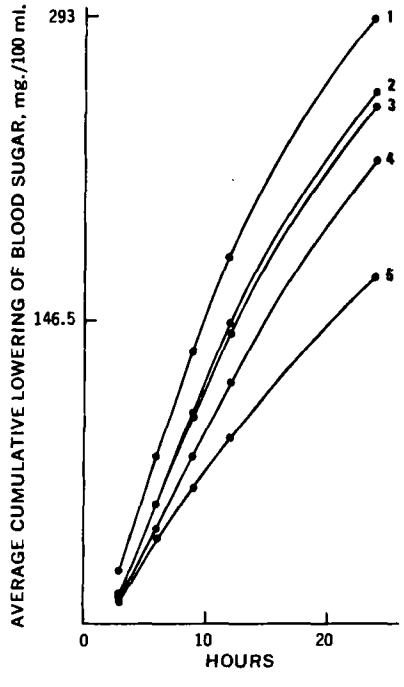

Figure 8—*Average cumulative blood sugar-lowering response in dogs after administration of the antidiabetic tablets.*

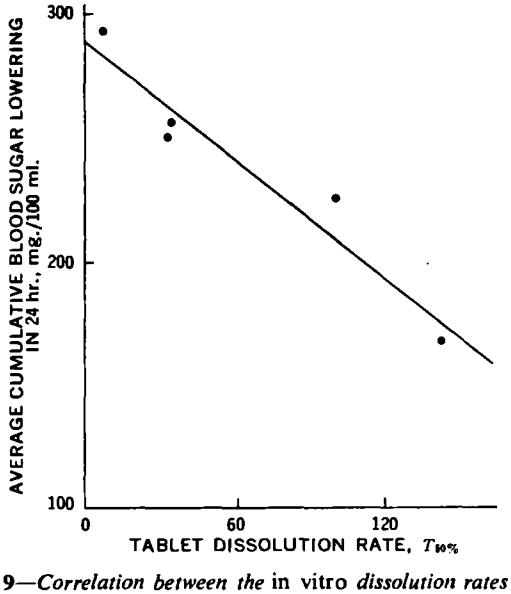

Figure 9—*Correlation between the* in vitro *dissolution rates* ($T_{50\%}$) *and total* in vivo *response obtained with the antidiabetic tablets.*

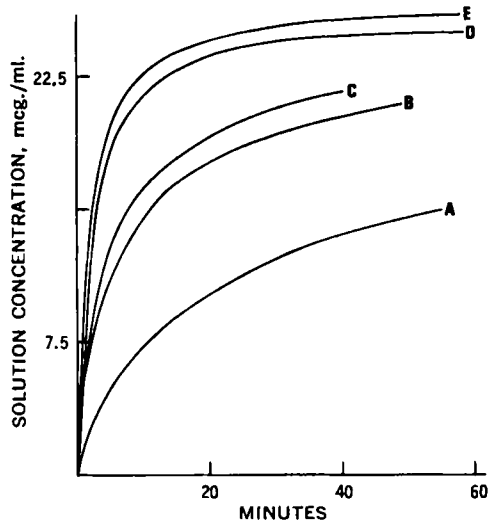

Figure 10—*Dissolution profiles of the powdered drug samples with varying particle size determined by the automated rotating filter–stationary basket apparatus. Key (surface area, m.²/g., of the powder samples): A, 0.87; B, 1.38; C, 1.54; D, 2.10; and E, 2.54.*

In a crossover study, these tablet samples were administered orally to mongrel female dogs, and their blood sugar concentrations were analyzed at various time intervals up to 24 hr. The average cumulative blood sugar-lowering response produced at various time intervals after administration is shown in Fig. 8. Comparison of these results with the dissolution profiles shown in Fig. 7 suggests a rank order *in vitro–in vivo* correlation. This correlation is further evident from a linear relationship (Fig. 9) obtained upon plotting the dissolution rate $T_{50\%}$ against the total 24-hr. blood sugar-lowering response (correlation coefficient = −0.9287).

Quality Control Dissolution Test for Powdered Bulk Drug Lots—During formulation development studies, it became apparent that the variation in the particle size among various milled bulk drug lots of a sparingly soluble drug seemed to have a noticeable effect upon the dissolution characteristics of tablets manufactured from these lots. Dissolution profiles of several bulk drug lots were, therefore, determined utilizing the rotating filter–stationary basket apparatus under the test conditions of 50 mg. of powdered drug suspended in 1 l. of 0.05 *M* phosphate buffer, pH 7.5, containing 0.05% polysorbate 80 as a wetting agent, 400 r.p.m., and 37°. From the dissolution profiles (Fig. 10), it is evident that the finer bulk drug with greater surface area showed faster dissolution than the coarse particle-size drug. Establishment of routine dissolution rate specifications for the bulk drug lots based on these results has considerably improved the lot-to-lot uniformity of the tablet dissolution rate.

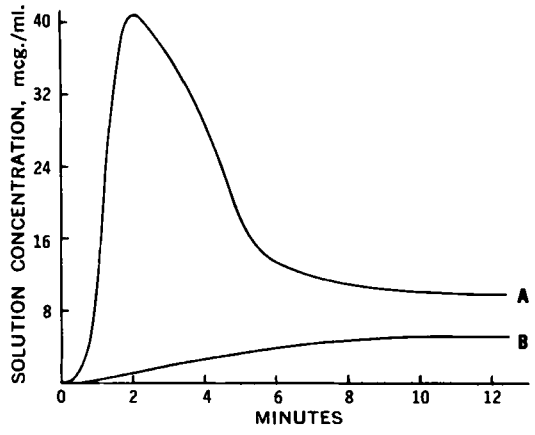

Figure 11—*Dissolution behavior of a metastable drug–polyvinylpyrrolidone coprecipitate (curve A) and the stable crystalline drug powder (curve B) evaluated by the automated rotating filter–stationary basket apparatus.*

Table II—Comparison of the Official Dissolution Test Apparatus with the Rotating Filter–Stationary Basket Apparatus[a]

Desired Features	Rotating Filter–Stationary Basket Apparatus	USP–NF Method I Apparatus	NF Method II Apparatus
Enclosed container	A	A	NA
Variable intensity of liquid agitation	A	A	NA
Definable (laminar) liquid flow	A	A	NA
Visibility of the test sample and fluid environments	A	?	?
Representative fluid sampling under mild agitation conditions	A	?	?
Automation with a continuous, efficient, clog-free fluid filtration system	A	NA	NA
Minimal mechanical impacts, abrasion, and wear of the test sample during dissolution	A	NA	NA

[a] A = available, NA = not available.

Dissolution Behavior of a Metastable Drug Form—The rotating filter–stationary basket apparatus was utilized as a preformulation research tool for the evaluation of dissolution and crystallization behavior of a drug in its metastable drug–polyvinylpyrrolidone (1:9) coprecipitate form. The coprecipitates were prepared by vacuum evaporation of a methanol solution of drug and polyvinylpyrrolidone. The test procedure employed was essentially similar to the previously described procedure for the powdered bulk drug dissolution studies. Rapid dissolution of the coprecipitates with the attainment of peak solution concentration, followed by the concentration decline due to precipitation of the drug, occurs within a few minutes (curve A, Fig. 11). Such a rapid transient dissolution behavior of the metastable drug form can be conveniently studied by the automated rotating filter–stationary basket apparatus. Curve B in Fig. 11 was obtained with the stable crystalline form of the drug.

In addition to these studies, the apparatus was utilized for dissolution rate determinations of capsules, granules, and timed-release dosage forms and also in evaluating solubility–solubilization of various drugs.

DISCUSSION

The rotating filter–stationary basket dissolution test apparatus was employed extensively by the authors over the past 18 months for testing various drugs and their dosage forms. The typical studies described here demonstrate its application as a preformulation research, formulation development, and quality control dissolution test apparatus. Its application as a quality control dissolution test system for both the active drug and the final dosage form offers a two-pronged approach to ensure drug quality from the standpoint of biological availability, a type of approach considered valuable by the NF Board (22).

Dissolution rates can be determined by either a manual or an automated test method. In the manual test method, filtered aliquots of the dissolution fluid are withdrawn at periodic time intervals and then assayed individually. This method is laborious and inconvenient for routine quality control testing of dissolution rates and unsuitable for evaluating very rapid dissolution processes; in addition, there is a human error involved in the accurate sampling and analysis.

Since the automated method overcomes these problems and provides a convenient means for monitoring dissolution rates, there have been increasing applications of the automated method in the pharmaceutical field. Aside from an automated titration tech-

nique (23), in most automated test methods a portion of the dissolution fluid is filtered continuously through a stainless steel wire cloth screen, sintered glass, membrane, or similar other static system and then cycled through a spectrophotometer flow cell or other analytical system for the continuous analysis of the circulating dissolution fluid (3, 5, 9, 11, 13, 20). However, use of such static filter elements for the continuous filtration of the dissolution fluid often presents filtration problems, such as clogging of the filter with solid particles and escape of solid particles through the filter element, which may yield erroneous dissolution rate results. The occurrence of these filtration problems has been acknowledged by other workers (9, 17, 18) and is demonstrated in the present study.

The rotating filter system employed in the present apparatus provides a dynamic *in situ* microporous filter element which, because of the boundary effect (centrifugal force), prevents the filter from becoming clogged with solid particles and permits continuous, efficient filtration of the dissolution fluid. Another advantage of the rotating filter system is its relatively large filter area extending over the greater portion of the dissolution fluid, which permits representative sampling of the bulk dissolution medium. From the dimensions of the rotating filter assembly, it may appear that there might be a considerable amount of liquid held inside the assembly, but, in fact, there is very little void space in the assembly so that the hold-up volume inside the assembly is less than 1 ml.

Dissolution rate determinations under solvent sink conditions can be performed by: (*a*) employing a relatively large fixed volume of dissolution fluid, (*b*) partitioning the dissolved drug into an organic solvent phase, (*c*) adsorbing the dissolved drug on a solid adsorbent, and (*d*) continuous flow replacement or dilution of the fluid with fresh solvent. In the adsorption and solvent partition methods, certain additional factors such as partition coefficient of the drug in the organic phase, rate of partitioning, and rate of adsorption are involved, which further complicate the dissolution rate determination. In a continuous flow system, replacement of dissolution fluid with fresh solvent medium necessitates continuous filtration of the fluid. Most of the existing continuous flow systems are, therefore, faced with the same types of filter clogging and other filtering problems as are the automated methods. Dissolution rate determination in a fixed fluid volume offers perhaps the simplest test method, provided it is possible to maintain a relatively dilute solution concentration by utilizing a large volume of the dissolution fluid. The present apparatus provides a relatively large size (1.5-l.) fluid container suitable for dissolution rate determinations under fixed fluid volume conditions. This apparatus can be employed as a continuous flow system by replacing a portion of the fluid with the fresh solvent medium, as described by Pernarowski *et al.* (11). However, achievement of rapid dilution of a large volume of the bulk fluid requires relatively fast fluid flow rates and consumption of appreciable amounts of fluid in a single dissolution test. For instance, to dilute 1-l. volume of the bulk fluid at a rate of 20%/min., one must replace 200 ml. of the bulk fluid per minute with the fresh solvent (*i.e.*, 200 ml./min. fluid flow rate); if the experiment is continued for 30 min., a total of 7 l. of dissolution fluid will be consumed. While these test conditions can be maintained, they obviously are not very convenient and practical for routine dissolution rate studies. A modification of the present apparatus capable of holding small volumes of the fluid, which is suitable as a continuous flow system, will be reported in a subsequent publication.

The importance of relatively mild agitation conditions for the determination of dissolution rates to reflect *in vivo* conditions was emphasized by Levy (24). Furthermore, the liquid agitation conditions must be reproducible upon repeated tests, and the hydrodynamics of the system must be quantitated to describe the dissolution process in terms of theoretical dissolution rate relationships. The agitation system employed in the present apparatus meets these criteria. The smooth cylindrical surface of the rotating filter assembly without impeller blades and its agitation action over a long vertical axis provide uniform, mild, laminar, nonturbulent, and reproducible liquid stirring even at relatively high revolutions per minute.

Retainment of the microenvironment of the test sample and accurate positioning of the sample are necessary requirements for reliable dissolution rate studies. These have been accomplished in the present apparatus by the use of a stationary sample basket held in position at a precise level. The sample can be conveniently introduced into the fluid after all other test conditions have been adjusted. Visual observation of the test sample through the transparent flask and stationary basket permits simultaneous disintegration time–dissolution rate evaluation. A 12-mesh wire cloth screen basket was employed primarily to achieve unhindered liquid flow through the basket, to enable visual observation of the test sample, and to prevent plugging of the basket screen with the solid particles, as experienced by other workers with the official test method using a 40-mesh screen basket (4, 20, 21).

Recognition of the two dissolution test apparatus in the official compendia (15, 16) suggests wider use of these apparatus in the pharmaceutical field. Hence, a comparison of these two apparatus with the rotating filter–stationary basket apparatus is presented in Table II to illustrate the availability of some of the major features desired in a standard dissolution test apparatus.

SUMMARY

Requirements of a standard dissolution test apparatus suitable for all types of solid drug forms and applicable as a research, development, and quality control tool are discussed. The design of a rotating filter–stationary basket apparatus, which meets essentially all these criteria, is presented. Results are described to demonstrate its reproducibility and versatility of application in solubility and dissolution rate studies. *In vitro* dissolution rates of five different tablet samples of an antidiabetic drug determined by this apparatus are shown to correlate with the *in vivo* drug activity.

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▲ To whom inquiries should be directed.

Phytochemical Investigation of *Convolvulus arvensis* (Convolvulaceae)

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Abstract □ A phytochemical investigation of the aerial parts of *Convolvulus arvensis* resulted in the isolation and identification of several *n*-alkanes and *n*-alkanols, α-amyrin, campesterol, stigmasterol, and β-sitosterol.

Keyphrases □ *Convolvulus arvensis* L.—phytochemical investigation of aerial parts, isolation and identification of *n*-alkanes, *n*-alkanols, α-amyrin, campesterol, stigmasterol, and β-sitosterol □ Medicinal plants—phytochemical investigation of *Convolvulus arvensis* □ European bindweed—phytochemical investigation

Convolvulus arvensis (Convolvulaceae), commonly referred to as European bindweed, is a vine that grows wild in Europe and the United States. Phytochemical studies on this plant have been limited to the detection of saponins (1), flavonoids and caffeic acid (2), alkaloids (3), and lipids (4) and to the identification of δ-aminolevulinic acid (5). Aqueous extracts of the plant have been reported to have antihemorrhagic properties (6) and phytotoxic effects by inhibiting seedling growth and germination of wheat, flax, alfalfa, and oats (7). Resins obtained from the plant have been reported to have a cathartic action in rats (8), while total alkaloid extracts, when administered to cats and rabbits, gave a hypotensive effect with vasodilation and an increase in the coronary circulatory rate (9).

EXPERIMENTAL

Plant Material—The plant material[1] used in this investigation was collected as a weed growing in Chicago, Ill., during August 1971. The aerial parts were harvested, air dried, and milled to a coarse powder.

Preparation of Crude Fractions—The coarsely milled plant material (2.3 kg.) was extracted in a soxhlet apparatus for 36 hr. with petroleum ether (b.p. 30–60°) which, after evaporation *in vacuo*, yielded 22.8 g. of petroleum ether-soluble extract. After air drying the defatted plant material, it was percolated with methanol. The methanol extract yielded 215 g. of residue after evaporation *in vacuo*.

The petroleum ether fraction (22.8 g.) was chromatographed over a column containing 1.5 kg. of Woelm neutral alumina (activity III). Elution with petroleum ether (15 l.) followed by benzene (10 l.) gave 11.1 g. of an oily residue (A) and a residue of 7.2 g. (B), respectively. The material remaining on the column was eluted with chloroform and methanol to yield a residue of 2.8 g. (C).

Identification of Alkanes in Fraction A—A mixture of alkanes (1.2 g.) was isolated by crystallization of Fraction A with hot acetone. The mixture melted at 53–54°. An IR spectrum (KBr) was typical for alkanes, with no evidence of hydroxyl or carbonyl absorption. The isolate was subjected to GC analysis using an instrument[2] fitted with a 0.05-cm. (0.02-in.) o.d. × 15.2-m. (50-ft.) SE-30 support-coated open tubular (SCOT) column. Helium (15 ml./min.) was the carrier gas. The column was maintained at 220°. Under these conditions, the sample separated into nine distinct peaks which, by comparison with reference samples, were identified (10) as *n*-pentacosane ($C_{25}H_{52}$) (4%), *n*-hexacosane ($C_{26}H_{54}$) (4%), *n*-heptacosane ($C_{27}H_{56}$) (15.1%), *n*-octacosane ($C_{28}H_{58}$) (9.7%), *n*-nonacosane ($C_{29}H_{60}$) (14.5%), *n*-triacontane ($C_{30}H_{62}$) (13.7%), and *n*-hentriacontane ($C_{31}H_{64}$) (38.6%). The remaining two peaks (<1%) were not identified.

Identification of Alkanols in Fraction D—While attempting to dissolve Fraction B in benzene, some amorphous material separated and was removed by filtration. This sample weighed 1.5 g. and was designated as Fraction D. The filtrate was dried and designated as Fraction E. A mixture of alkanols (0.161 g.) was isolated from Fraction D by crystallization from hot acetone. The mixture melted at 76–77°. An IR spectrum (KBr) showed absorption peaks at 3300 (OH), 730, and 720 [$(CH_2)_x$] cm.$^{-1}$. Analysis of this isolate was effected by gas chromatography, using an instrument[3] fitted with a 0.63-cm. (0.25-in.) o.d. × 1.8-m. (6-ft.) glass column packed with 3% OV-1. Helium (120 ml./min.) was the carrier gas. The column was maintained at 220°. Under these conditions, the isolate separated into seven peaks which were identified by reading from a linear curve constructed by plotting reference sample homologs against the log of their respective retention times. The peaks were identified as *n*-octadecanol (C_{18}) (trace), *n*-nonadecanol (C_{19}) (trace), *n*-eicosanol (C_{20}) (trace), *n*-heneicosanol (C_{21}) (22.8%), *n*-tetracosanol (C_{24}) (37.5%), and *n*-hexacosanol (C_{26}) (35.3%). A peak (3%) between the C_{20} and C_{21} alcohols was unidentified.

Isolation and Identification of α-Amyrin—TLC of Fraction E on silica gel G plates, developed with a solvent system composed of benzene–acetone (6:1) and sprayed with sulfuric acid followed by heating, showed four spots. The sample was chromatographed over a column packed with 600 g. of silica gel PF_{254}. The adsorbent was first activated by heating at 105° overnight and then deactivated with 10% (w/v) distilled water. It was packed with the above-mentioned solvent system. The column was eluted with the same solvent system and 20-ml. fractions were collected. Fractions 1–10 from the column gave a yellow oil (3.1 g.), fractions 12–20 (1.45 g.) yielded a crude triterpene, and fractions 23–27 (1.08 g.) afforded a mixture of sterols. The crude triterpene was crystallized from ethanol to afford 50 mg. of colorless needles, m.p. 178–180° [lit. (11) m.p. 183°); $[\alpha]_D^{25}$ + 92° (concentration, 0.5 in benzene) [lit. (11) $[\alpha]_D^{25}$ + 90° in benzene]. An acetate was prepared in the usual manner and it was found to have m.p. 219–221° [lit. (11) m.p. 220–227°]. The identity of the compound as α-amyrin was further established by an IR spectrum (KBr), which was superimposable with that of an authentic sample of α-amyrin, and a mixed melting point with an authentic sample of α-amyrin, which was undepressed. A mass spectrum of the isolate gave a molecular ion at *m/e* 426 and exhibited a fragmentation pattern that was in agreement with that published for α-amyrin (12).

Identification of Campesterol, Stigmasterol, and β-Sitosterol—The mixture of sterols isolated from Fraction E was crystallized from ethanol to yield a product exhibiting m.p. 134–135°. TLC of the isolate showed a single spot identical with β-sitosterol, but GC

[1] Voucher specimens were prepared and identified as *Convolvulus arvensis* L. (I-2344) and are deposited in the Herbarium of the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center.

[2] Perkin-Elmer model 800.

[3] Varian Associates.

analysis showed it to be a mixture of three sterols. The mixture was analyzed using an instrument[4] fitted with a 0.63-cm. (0.25-in.) o.d. × 1.8-m. (6-ft.) glass column packed with Gas Chrom Q, 100–120 mesh, and coated with 5% OV-101. Helium was used (80 ml./min.) as the carrier gas, and the column was maintained at 250°. Cholestane was used as the internal standard. The sterols were identified as campesterol (15.6%), stigmasterol (6.2%), and β-sitosterol (78.2%) by comparison of the relative retention times of the eluted sterols with reference samples of authentic sterols.

[4] Perkin-Elmer model 881.

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Synthesis of Potential Antineoplastic Agents XXII: Compounds Related to 1-Nitro-3-[(*p*-phenylbenzylidene)amino]guanidine

FRANK D. POPP

Abstract □ The nitroguanylhydrazone of 4-biphenylcarboxaldehyde exhibits antineoplastic activity in the Walker 256 and KB test systems. Other nitroguanylhydrazones and other derivatives of 4-biphenylcarboxaldehyde were prepared and found to be devoid of antineoplastic activity.

Keyphrases □ 1-Nitro-3-[(*p*-phenylbenzylidene)amino]guanidine, related compounds—synthesized and screened as potential antineoplastic agents □ Antineoplastic agents, potential—synthesis of compounds related to 1-nitro-3-[(*p*-phenylbenzylidene)amino]-guanidine, screened against Walker 256 and KB test systems □ 4-Biphenylcarboxaldehyde derivatives—synthesized and screened as potential antineoplastic agents □ Nitroguanylhydrazones—synthesized and screened as potential antineoplastic agents

In connection with other work in progress in this laboratory, the nitroguanylhydrazone of 4-biphenylcarboxaldehyde (I) was prepared. In routine screening, this compound was found to possess antineoplastic activity against Walker carcinosarcoma 256 (Table I) and had confirmed activity against KB cell culture. To explore this lead further, a series of nitroguanylhydrazones and some derivatives of 4-biphenylcarboxaldehyde were prepared for screening. Nitroguanylhydrazones were previously used to identify aldehydes and

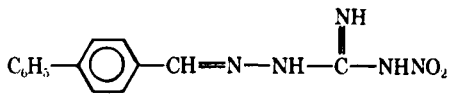

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Table I—Summary of Screening of 1-Nitro-3-[(*p*-phenylbenzylidene)amino]guanidine against Walker Carcinosarcoma 256 (Subcutaneous) in Fischer 344 Rats[a]

Vehicle	Day of First Injection	Number of Injections	Dose, mg./kg.	Animal Weight —Percent— Difference T−C[b]	T/C[c]
Hydroxypropylcellulose[d]	1	9	400	−6	150
	1	9	200	−2	138
	1	9	100	−2	116
	3	4	400	−1	166
	3	4	200[e]	1	177
	3	4	100	−2	116
Saline with polysorbate 80	1	9	400	−7	144
	1	9	200	−2	133
	1	9	100	−5	144
	1	9	50	1	150
	3	4	400	−8	144
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[a] Supplied by Drug Research and Development, Chemotherapy, National Cancer Institute. Intraperitoneal administration was made daily, with evaluation on Day 30. In all cases, there was 6/6 survivors. [b] Average weight change of test group minus average weight change of control animals in grams. [c] Ratio of survival time of treated to control animals expressed as percent. [d] Klucel. [e] Two cures.

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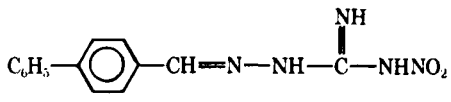

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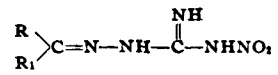
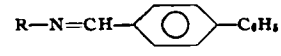
Table II—Nitroguanylhydrazones



Aldehyde or Ketone Used (RR_1CO)	Melting Point	Yield, %	Formula	Analysis, % N Calc.	Analysis, % N Found
4-Biphenylcarboxaldehyde	225–227°	90	$C_{14}H_{12}N_6O_2$	24.72	24.40
Acetylferrocene	205–207°	80	$C_{13}H_{15}FeN_6O_2$	21.28	20.95
Acetylisatin	224–226°	30	$C_{11}H_{10}N_6O_4$[a]	28.96	28.93
1-Benzylindole-3-carboxaldehyde	208–209°	91	$C_{17}H_{16}N_6O_2$	24.99	24.76
4-Bromobenzaldehyde	220–221°	92	$C_8H_8BrN_5O_2$	24.48	24.41
Cyclopentanone	139–141°	65	$C_6H_{11}N_5O_2$	37.82	37.74
Ferrocenecarboxaldehyde	195–196°	76	$C_{12}H_{13}FeN_6O_2$	22.23	22.05
4-Fluorobenzaldehyde	206–207°	83	$C_8H_8FN_5O_2$	31.10	30.94
Isatin	268–270°	98	$C_9H_8N_6O_3$[b]	33.86	33.87
Pentafluorobenzaldehyde	233–234°	94	$C_8H_4F_5N_5O_2$	23.57	23.41
Pyridine-3-carboxaldehyde	230–231°	67	$C_7H_8N_6O_2$	40.37	40.50
Quinoline-2-carboxaldehyde	228–229°	61	$C_{11}H_{10}N_6O_2$	32.55	32.40
3,4,5-Trimethoxybenzaldehyde	208–209°	76	$C_{11}H_{15}N_5O_5$	23.56	23.46

[a] Calc.: C, 45.52; H, 3.47. Found: C, 45.36; H, 3.61. [b] Calc.: C, 43.55; H, 3.25. Found: C, 43.68; H, 3.39.

Table III—4-Biphenylcarboxaldehyde Derivatives



RNH_2 Used	Melting Point	Yield, %	Formula	Analysis, % N Calc.	Analysis, % N Found
Diaminomaleonitrile	231–232°	83	$C_{17}H_{12}N_4$[a]	20.58	20.36
1,1-Dimethylhydrazine	86–87°	63	$C_{15}H_{16}N_2$	12.49	12.41
4-Phenylsemicarbazide	207–208°	95	$C_{20}H_{17}N_3O$	13.32	13.40
4-Phenyl-3-thiosemicarbazide	197–198°	99	$C_{20}H_{17}N_3S$	12.68	12.66

[a] Calc.: C, 74.98; H, 4.48. Found: C, 74.81; H, 4.35.

ketones (1) and showed antitubercular activity (2). Previously unreported compounds in these two series are shown in Tables II and III.

Screening results indicate a complete lack of antineoplastic activity (T/C of 125% for tumor survival systems and T/C 42% for tumor weight-inhibition systems) for all except the title compound in Tables II and III. Furthermore, of a large number of known nitroguanylhydrazones that have been screened, only three showed even marginal activity. KB cell culture activity was observed, however, in some of these compounds.

EXPERIMENTAL[1]

Nitroguanylhydrazones—To a warm solution of 1.19 g. (0.01 mole) of 1-amino-3-nitroguanidine in 10 ml. of ethanol, 10 ml. of water, and 20 ml. of acetic acid was added 0.01 mole of the carbonyl compound in 25 ml. of ethanol. The mixture was heated on the steam bath for 30 min., cooled, and filtered to give, after recrystallization from ethanol, the compounds listed in Table II.

4-Biphenylcarboxaldehyde Derivatives—To 1.82 g. (0.01 mole) of 4-biphenylcarboxaldehyde in 20 ml. of ethanol was added 0.01 mole of the appropriate carbonyl reagent in ethanol. The mixture was heated on the steam bath for 30 min., cooled, and filtered to give, after recrystallization from ethanol, the compounds listed in Table III.

[1] Analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. Melting points were determined in capillary tubes and are corrected.

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Transport Cell for Examining Solute Transfer across Biological Membranes

M. MAYERSOHN▲ and K. SURYASAPUTRA

Abstract □ A transport cell was designed for examining *in vitro* solute transfer across biological membranes. When using the intestine as a model membrane, a primary advantage of the method is that there is no need to evert the intestine. This eliminates the influence of eversion on the structural and functional integrity of the intestine and thus its possible influence on solute transfer. Preliminary studies using salicylamide were performed to quantitate various parameters of the apparatus. Clearance values for the mucosal-to-serosal transfer of salicylamide across the everted rat intestine were calculated per unit of membrane surface area. The clearance per area values obtained agree reasonably well with those reported in the literature using different methods. The reproducibility of the transport data agrees favorably with other methods. The utility of the present method is explored.

Keyphrases □ Cell, transport—for examining solute transfer across biological membranes, clearance values, salicylamide □ Biological membranes—transport cell for examining solute transfer □ Solute transfer, *in vitro*—transport cell for biological membranes, clearance values, salicylamide

A number of preparations are available for studying *in vitro* drug transport across biological membranes. Methods utilizing intestinal membranes were recently reviewed (1). Probably the most widely used *in vitro* preparation in drug transport studies is the Crane and Wilson (2) modification of the everted intestinal sac technique (3), with various minor alterations on this basic approach. Although this preparation has a number of advantages over other *in vitro* techniques, it may be criticized on the basis that relatively little is known about the influence of the eversion process on the structural and functional properties of the membrane and the resulting influence on drug transport, and appropriate experiments are not easily designed to answer these questions. A method is reported here for examining drug transfer which obviates the need for membrane eversion. The technique should provide a useful approach for examining directional drug transfer as well as the influence of eversion on drug transport.

EXPERIMENTAL

Transport Cell and Preparation of Intestine—The apparatus may be accurately tooled from Plexiglas (Figs. 1 and 2). The entire apparatus is self-contained and may be placed into a water bath to maintain a constant temperature. The edges of the inner face of one-half of the cell, where contact is made when the unit is closed, are lined with flexible plastic adhesive[1] for a watertight seal. One end of each of the four screws is embedded in one-half of the cell. These screws pass through holes in the opposite half of the cell and are tightened with wing-nuts once the membrane is placed into position.

Male, Sprague–Dawley rats[2], weighing approximately 250 g., were starved 18–22 hr. prior to the experiment with water allowed *ad libitum*. The animals were anesthetized with ether, and a midline abdominal incision was made. The intestine was severed at the ileocecal and pyloric junctions, and the lumen was washed with normal saline and placed into a dish of normal saline at room temperature.

In the studies reported here, the intestine was everted by attaching the proximal part *via* a ligature to a glass rod and gently sleeving the intestine over itself. The first 15 cm. of the proximal intestine was discarded. The intestine was then cut lengthwise with scissors along the point of attachment of adhering tissue. An approximate 6-cm. length of flat intestine was placed over the "window" of one-half of the cell containing embedded pins whose points were sharpened. To prevent the intestine from falling through the window, a solid piece of Plexiglas was placed into the window to provide a solid surface for positioning the membrane. The edges of the membrane were then forced over the pins to secure it in place. During this process the intestine was handled only at the edges with the use of forceps. The other half of the cell was then placed in position, the intestine was sandwiched, and wing-nuts attached to the screws were tightened. The plastic adhesive along the inner cell surface prevented the membrane from being damaged during this process. The Plexiglas insert was then removed, and the cell was placed into a water bath to maintain the drug and buffer solutions at 37 ± 1°. Twenty milliliters of drug in the buffer solution was placed into one compartment of the cell (bathing the mucosal surface), and 20 ml. of buffer solution devoid of drug was placed into

Table I—Comparison of the Mucosal-to-Serosal Salicylamide Clearance Value Obtained in This Study to Those Reported in the Literature

Reference	This Study	*Reference 6*	*Reference 8*	*Reference 9*
Method	Transport cell	Everted gut	Everted gut	Everted gut
Mucosal solution, buffer and pH	Bicarbonate, pH 7.4	Phosphate, pH 6.0	Phosphate, pH 6.6	Bicarbonate, pH 7.4
Solution volume, ml.:				
Mucosal	20	80	140	100
Serosal	20	2	15	2
Mucosal surface area[a], cm.[2]	1.79	13	13	13
Mucosal salicylamide concentration, mg./ml.	4.0	2.0	1.0	1.0
Mucosal-to-serosal salicylamide clearance, ml./hr.	0.287	3.87	5.24	3.42
Clearance per surface area, ml./hr./cm.[2]	0.160	0.298	0.403	0.263

[a] The surface area represents the gross dimensions and not necessarily the effective area available for drug transfer. The area reported for this study represents a measurable area corresponding to the window dimensions. The other values are calculated based upon a membrane segment of 10-cm. length with a circumference of 1.3 cm., assuming a simple cylinder.

[1] Silicone Seal, Canadian General Electric Co., Toronto, Ontario, Canada.

[2] Canadian Breeding Laboratories, Montreal, Quebec, Canada.

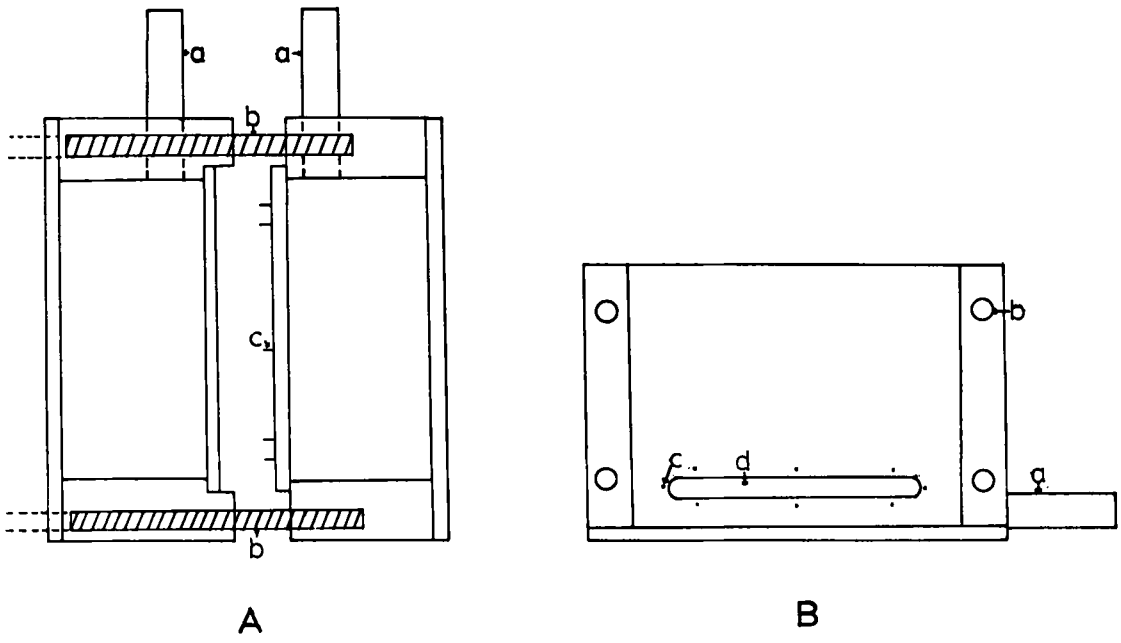

Figure 1—*Diagrammatic sketch of the transport cell. (A) Top view. Key: a, tube outlet to vacuum for removing entire volume of fluid in compartment; b, connecting screws for closing unit; and c, pins for securing intestinal membrane. Inside compartment dimensions are: height, 5.2 cm.; length, 6.0 cm.; width, 2.9 cm. (B) Front view of inside surface of one-half of cell. Key: a, tube outlet to vacuum; b, receiving holes for connecting screws (0.4-cm. diameter); c, receiving holes for pins; and d, membrane window (length 4.85 cm.; width, 0.37 cm.). The bottom edge of the window is approximately 0.5 cm. above the inside bottom surface of the compartment.*

the other compartment (bathing the serosal surface). Solutions in both compartments were gassed with 95% O_2–5% CO_2 *via* thin polyethylene tubing, and underwater magnetic stir plates provided the stirring of small magnetic stirrers in the transport cell. The compartment into which drug was diffusing was easily sampled by pipet or, alternatively, by total removal of the volume in that compartment by vacuum *via* tubing connected to the vacuum outlet tubes.

This procedure was repeated using a second transport cell of dimensions identical to the first for another 6 cm. of intestine which was just distal to the first segment.

Transport Experiments—Salicylamide[3] was the test compound used for examining mucosal-to-serosal transfer to quantitate various parameters of the transport cell. The mucosal drug solution contained 4 mg./ml. salicylamide in a modified Kreb's sodium bicarbonate buffer, pH 7.4 (4). The serosal solution was buffer devoid of drug. Two-milliliter samples of the serosal solution were taken every 10 min. during the 2-hr. experiment, and each sample was immediately replaced by 2 ml. of fresh buffer. Corrections were made for the quantity of salicylamide removed by this sampling technique. Because of the large concentration of drug in the mucosal solution, sink conditions were maintained and a constant rate of transport was obtained. The drug was assayed spectrophotometrically[4] by the method of Trinder (5) as modified by Feldman and Gibaldi (6). All chemicals were of reagent grade.

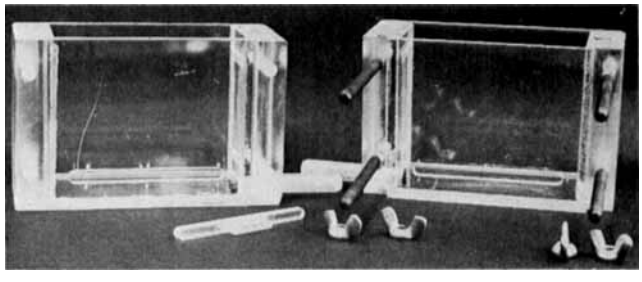

Figure 2—*Photograph of the transport cell separated into both units.*

[3] Reagent grade, Lot No. 2278020, British Drug House, Toronto, Ontario, Canada.

[4] Zeiss PMQ-II spectrophotometer, Oberkochen, Germany.

RESULTS AND DISCUSSION

Figure 3 is a representative plot of the cumulative amount of salicylamide transferred as a function of time from the mucosal to the serosal solution. The data points of the straightline portion of the curve (*i.e.*, transfer after steady state has been reached) were fitted by least-squares regression. An alternative approach to plotting transfer data, which was used previously (7), is illustrated in Fig. 3; one graphs the rate of transfer per unit of time as a function of the midpoint of that time interval. This graphical method

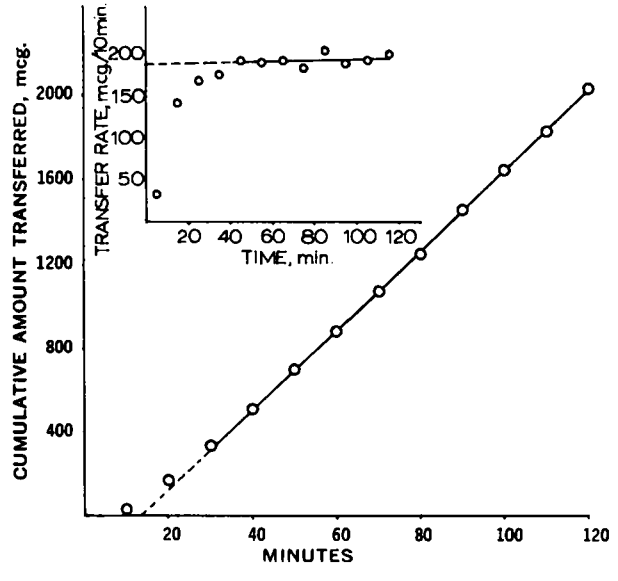

Figure 3—*Representative plot of the cumulative amount of salicylamide transferred across the everted rat intestine from the mucosal to the serosal solution as a function of time. Data were taken from one experiment. The inset graph illustrates an alternative method of plotting transport data. The rate of transfer per 10 min. as a function of the midpoint of the time interval was plotted for the same data as in the cumulative plot.*

provides a valuable assessment of variation in transfer rate in a given experiment. As may be seen, there was relatively little fluctuation in the rates of transfer over the time period of the experiment. Since there were no significant differences ($p > 0.05$) in transfer rates between segments obtained from the same animal, as determined by a paired Student's t test, rates of transfer from all segments were lumped together and averages were determined from all the data. The steady-state transfer rates were determined from a least-squares fit of cumulative amount transferred plots. The steady-state transfer rates were transformed into a clearance value (milliliters per hour) by dividing the rate of transfer by the initial mucosal drug concentration. In seven individual experiments, the arithmetic mean ± standard deviation is 0.287 ± 0.034 ml./hr. The range of values in this series of experiments is 0.223–0.315 ml./hr., with a percent coefficient of variation of approximately 12. Based upon the type of variation normally encountered in transport studies utilizing biological membranes, these values appear quite reproducible. For example, the percent coefficient of variation for salicylamide transfer across the everted rat intestine may be calculated from several literature reports to be approximately 8 (6) or 10 (8).

A comparison of the mucosal-to-serosal salicylamide clearance values obtained in this study to other literature values was attempted (Table I). Several other pertinent parameters of those systems cited are included in the table. The values to be compared are those in the last line of the table: clearance per unit surface area. The surface area of the membrane in the transport cell used in this study is a constant and is calculated from the dimensions of the window. This area is based only on the gross measurable dimensions of the window and, as with the other preparations reported in Table I, is not necessarily the effective membrane surface area available for transfer. A number of assumptions were made to estimate the surface area of the other preparations listed in Table I. The length of all segments listed in those reports (6, 8, 9) is 10 cm. From our own experiments, the circumference of the rat intestine (*i.e.*, the width of the flat membrane once the intestine is cut lengthwise) was estimated to be approximately 1.3 cm. The luminal diameter varies from animal to animal and depends upon the portion of the intestine being used. Other workers showed the intestinal circumference of the rat to be approximately 1.7–1.8 cm. (10). Nonetheless, the clearance values per square centimeter listed in Table I are in relatively good agreement. A number of reasons might explain the fact that the smallest clearance per area value is found in the present study. As mentioned, there is likely to be considerable variation in the diameter of the intestinal lumen from animal to animal, which alters the calculated value of the membrane surface area of the other preparations reported here. In addition, the serosal fluid in the everted gut method has a tendency to distend the intestine laterally. This distension might result in an increased luminal diameter or a more complete exposure of the villi to the mucosal drug solution. Either effect would increase the area available for transfer. Finally, in the other reports cited, there is some type of lengthwise distension of the gut segment during the experiment other than the eversion process itself.

From the preliminary data presented here, it appears that the method reported provides reproducible transport data in reasonably good agreement with previous reports using different methods. The apparatus is easily used and has a number of advantages to recommend it over other *in vitro* techniques. In addition to the fact that there is no need to evert the intestine (one is able to examine the influence of eversion on directional drug transport), relatively small volumes of solution may be used in these studies. This latter point might prove useful when small quantities or expensive materials are to be examined. The primary disadvantage of the transport cell is the small surface area of the membrane available for drug diffusion. This creates a problem when one is performing classical transport studies where amounts or concentrations of drug are determined by analytical methods. Although this transport cell was originally designed for a different approach to studying drug transport, the membrane area available for diffusion will limit the analytical method to be used. If sensitive analyses are available, there should be little difficulty in obtaining valid data. When using experimental animals that have intestines of widths different from the rat, the apparatus may be easily modified by altering the height of the window. The utility of the apparatus described here for *in vitro* drug transport studies will be the subject of future reports.

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Interaction of 3-Quinuclidinol and Its Derivatives with Acetylcholinesterase

R. PYTTEL and J. B. ROBINSON▲

Abstract □ The preparation of R-(−)-3-quinuclidinol and its acetate ester is reported. These compounds, together with their racemates, were tested as inhibitors and substrates of the enzyme acetylcholinesterase. The results suggest that the enzyme active site is stereospecific toward R-(−)-3-quinuclidinol hydrochloride when acting as an inhibitor. Both (±)- and R-(−)-3-acetoxyquinuclidine hydrochloride are substrates of the enzyme, but the S-enantiomer probably has some affinity for the enzyme also.

Keyphrases □ 3-Quinuclidinol and derivatives—tested as inhibitors and substrates of acetylcholinesterase □ Acetylcholinesterase—3-quinuclidinol and derivatives tested as substrates and inhibitors □ R-(−)-3-Acetoxyquinuclidine hydrochloride—tested as substrate of acetylcholinesterase

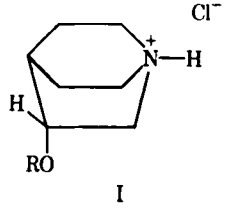
The activity of the enantiomers of 3-acetoxyquinuclidine methiodide, when tested as muscarinic agonists or as substrates for the enzyme acetylcholinesterase (1), has been shown to reside predominantly in the enantiomer of the R-configuration (2). However, the tertiary amine derivative, (±)-3-acetoxyquinuclidine hydrochloride, has been shown to be a more potent muscarinic agonist than its quaternary analog (3, 4) and to act as a substrate for the enzyme acetylcholinesterase (5).

The present work reports the preparation of R-(−)-3-quinuclidinol hydrochloride (I: R = H) and R-(−)-3-acetoxyquinuclidine hydrochloride (I: R = CH_3CO), together with the corresponding racemates, and the testing of these compounds as inhibitors and substrates, respectively, of the enzyme acetylcholinesterase.

EXPERIMENTAL[1]

(±)-3-Quinuclidinol [m.p. 220–222°, lit. (6) m.p. 221–223°] hydrochloride [m.p. >300° dec., lit. (6) m.p. >300°] was resolved according to literature methods (1, 6) using camphor-10-sulfonic acid. Four recrystallizations gave a salt, $[\alpha]_D^{22}$ −0.73° (c, 6.8, water), from which R-(−)-3-quinuclidinol was isolated, m.p. 219–220° (benzene), $[\alpha]_D^{22}$ −36° (c, 3.1, 1 *N* HCl) [lit. (1) $[\alpha]_D^{22}$ −37.1° (c, 3.0, 1 *N* HCl)]. The hydrochloride had m.p. >300° dec. (ethanol), $[\alpha]_D^{22}$ −27.6° (c, 1.1, water).

On acetylation (1), R-(−)-3-quinuclidinol gave R-(+)-3-acetoxyquinuclidine, b.p.$_{11}$ 101–102°; $[\alpha]_D^{22}$ +24.1° (c, 2.5, water) [lit. (1) $[\alpha]_D^{22}$ +28.5 (c, 3.0, ethanol)]. The IR spectrum (liquid film) was identical to that of an authentic sample. The hydrochloride salt had m.p. 200–201°, $[\alpha]_D^{22}$ −4.50° (c, 1.8, CH_3OH).



I

(±)-3-Acetoxyquinuclidine had a boiling point of 103–104°/10 mm. [lit. (7) b.p. 113–115°/11 mm.] and an IR spectrum (liquid film) identical to that of an authentic sample.

Bovine erythrocyte acetylcholinesterase[2] was used, and the compounds were tested as substrates or inhibitors as previously described (1, 8). All solutions of quinuclidine derivatives were freshly prepared immediately before use, and the solutions were adjusted to pH 7.4 with the appropriate titrant before being made up to the required volume. Values of $K_{m\ app}$, V_{max}, and K_i were calculated from conventional double-reciprocal plots (Table I).

Table I—Enzymatic Hydrolysis and Inhibition of Ox Erythrocyte Acetylcholinesterase

Substance	$K_{m\ app} \times 10^4$ *M*	$V_{max} \times 10^7$ *M*/min.	$K_i \times 10^3$ *M*
Acetylcholine	2.7	9.5	—
(±)-3-Quinuclidinol hydrochloride	—	—	3.4
R-(−)-3-Quinuclidinol hydrochloride	—	—	1.74
(±)-3-Acetoxyquinuclidine hydrochloride	11.0	1.9	—
R-(−)-3-Acetoxyquinuclidine hydrochloride	4.2	1.8	—

DISCUSSION

Both (±)- and R-(−)-3-quinuclidinol hydrochloride are weak competitive inhibitors of the enzyme-catalyzed hydrolysis of acetylcholine, the interaction being highly stereoselective in favor of the enantiomer having the R-configuration. Theoretical calculations (9) suggest that the S-(+)-enantiomer has some very weak inhibitory activity, but the precision of such calculations under the present conditions is extremely limited.

Tests of the substrate specificity of 3-acetoxyquinuclidine hydrochloride show a similar selectivity, the activity being resident primarily in the R-(−)-enantiomer. The derived Lineweaver–Burk plots for the racemate and R-(−)-enantiomer do suggest, however, some slight enzyme inhibitory effect of the S-(+)-enantiomer and this is reflected in the computed values of V_{max} and $K_{m\ app}$ (Table I). Thus, S-(+)-3-acetoxyquinuclidine hydrochloride would appear to have some affinity for the enzyme active site. This is in contrast to the previously reported results (1) on the enantiomers of 3-acetoxyquinuclidine methiodide, in which the value of $K_{m\ app}$ was, within experimental error, equal for the (±)- and R-(−)-enantiomers (8.12 and 7.95 × 10^{-4} *M*, respectively) and V_{max} for the R-(−)-enantiomer was approximately double that of the racemate (1.50 and 0.835 × 10^{-6} *M*/min., respectively), indicating a lack of affinity of S-(+)-3-acetoxyquinuclidine methiodide for the enzyme active site.

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[2] Sigma.

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Biochemical Interactions of Dimethyl Sulfoxide I: Respiratory Inhibition

ROBERT L. BEAMER▲, JAMES E. WYNN, and RAFAEL E. LEDESMA*

Abstract □ The effect of dimethyl sulfoxide upon rabbit liver homogenates was determined with the Warburg respirometer. Lineweaver–Burk treatment of the data indicated that the respiration was competitively inhibited by dimethyl sulfoxide.

Keyphrases □ Dimethyl sulfoxide—effect on rabbit liver homogenates, Warburg respirometer □ Respiratory inhibition—dimethyl sulfoxide, effect on rabbit liver homogenates, Warburg respirometer □ Warburg respirometer—effect of dimethyl sulfoxide on rabbit liver homogenates

During recent years there has been widespread interest in the clinical effects and applications of dimethyl sulfoxide. Toxicological studies have demonstrated a low acute toxicity, as indicated by higher LD_{50} values from intravenous, oral, and subcutaneous administration (1, 2).

Long-term administration of dimethyl sulfoxide produced changes in tissue morphology, such as reduced relucency of the lens cortex in dogs (3) and changes in hepatic cell morphology in rats (4). Teratogenic effects of dimethyl sulfoxide have been observed in various experimental animals (*e.g.*, chicks and golden hamsters) (5–7). However, few investigations have been reported concerning the effects of dimethyl sulfoxide on biochemical systems (8). This study was designed to provide preliminary data concerning the effects of dimethyl sulfoxide at the enzyme level.

EXPERIMENTAL

Materials—*Dimethyl Sulfoxide*—Experimental drug grade dimethyl sulfoxide[1] was redistilled under reduced pressure (100° at 2 mm.).

Serum Substrate—The substrate was prepared by diluting lyophilized bovine serum[2] to a specific gravity of 1.0210 with glass-redistilled water.

Apparatus—*The Warburg Instrument*—A Warburg respirometer[3], equipped with one-arm, 15-ml. reaction vessels with center wells, was used. The center wells contained 0.2 ml. of 15% KOH and a Whatman No. 1 filter paper wick. The amplitude of flask travel was 4 cm. at a rate of 60 c.p.m.; the water bath temperature was 37°. Warburg manometers were filled with Kreb's manometric fluid (9, 10), density 1.0334.

C–H–N Analyzer—A carbon–hydrogen–nitrogen analyzer[4] was utilized to determine the nitrogen content of animal tissue.

Preparation of Animal Tissues—Four albino rabbits, weighing approximately 1.4 kg. (3 lb.) each and in apparent good health, were quarantined for 2 weeks; they were fed rabbit pellets[5] and given water *ad libitum*. Each day they were examined for symptoms of disease.

At the end of the quarantine period, the animals were sacrificed by placing them in a closed container with dry ice. The livers immediately were removed, rinsed in cold Ringer's solution, weighed, inspected for gross abnormalities, and placed in cold (0–4°) 0.06 *M* sodium phosphate buffer at pH 7.4 (1 ml. buffer/g. wet tissue). The wet liver appeared normal and weighed 54.80–56.51 g. Small segments of each liver were dried at 100°, and their dry-to-wet ratios and nitrogen contents were determined. The mean value of the dry-to-wet weight ratio was 0.7735 with a standard deviation of 0.256; the mean nitrogen content was 10.62% with a standard deviation of 0.21.

The livers were homogenized individually in a cold Virtis homogenizer, pooled, and further homogenized in a test tube homogenizer[6]. The homogenates were placed in test tubes, sealed with sulfide-free rubber stoppers, and rinsed with glass double-distilled water. The homogenates were stored in a freezer.

Inhibition Studies—Inhibition studies were performed using the Warburg technique (11). The homogenates were thawed for approximately 60 min. at 6°. A constant liver homogenate volume containing 3.84×10^{-2} g. of nitrogen was used throughout. The flasks and their contents were incubated for 10 min. at 37° prior to initiating the reaction. The lyophilized serum substrate concentration for apparent maximal liver respiration (apparent V_{max}) (12) was found to be 1.7×10^{-2} g./ml.

Studies were then performed to determine the minimal concentration of dimethyl sulfoxide that would produce significant respiratory inhibition. The results were analyzed statistically and the kinetics of inhibition were evaluated by Lineweaver–Burk plots con-

[1] Crown Zellerbach Corp.
[2] Nutritional Biochemicals Corp.
[3] Model 18U, Precision Scientific.
[4] Hewlett-Packard model 185.
[5] Purina.
[6] Model 8335, Ace Glass Co.

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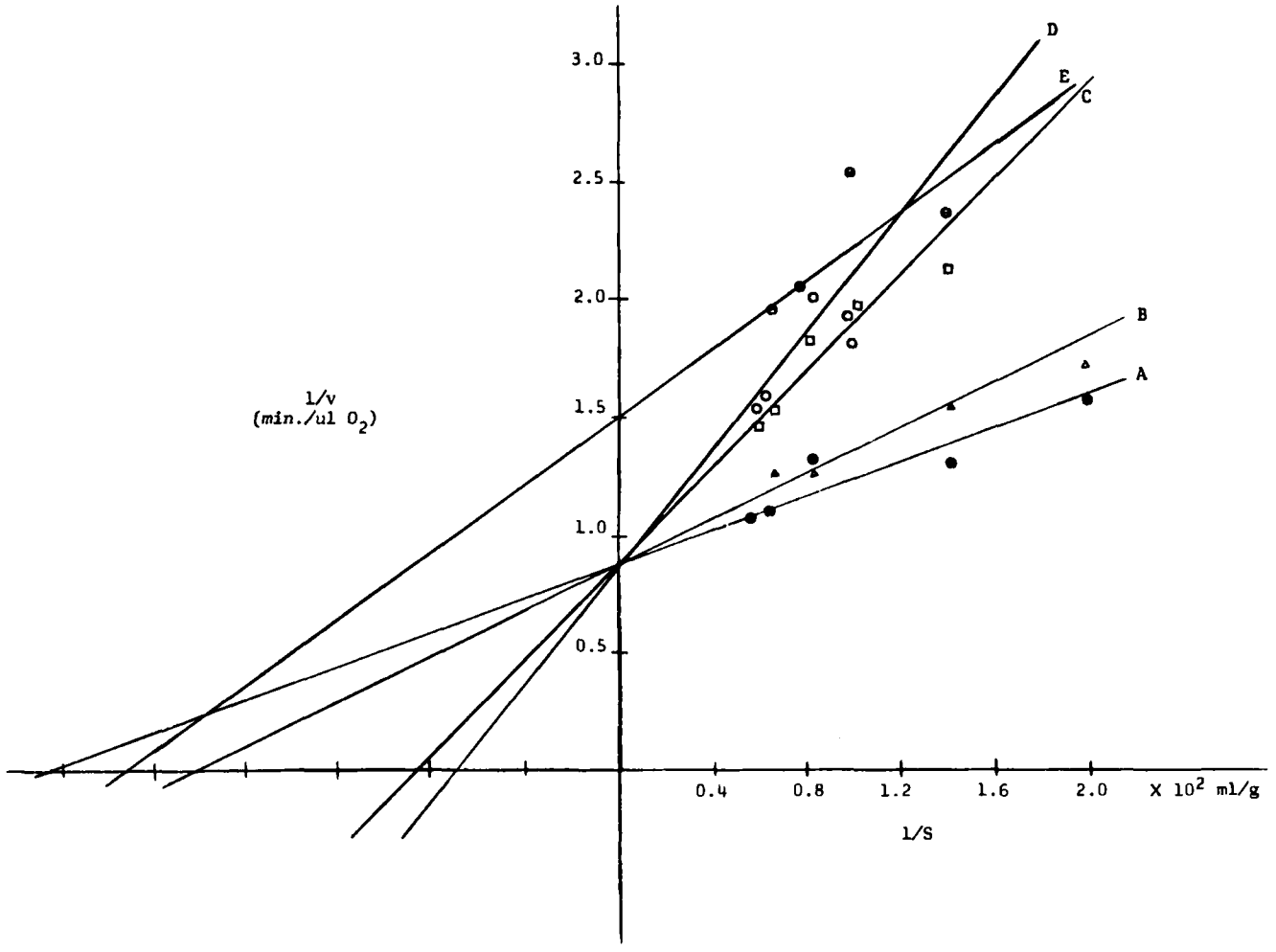

Figure 1—*Standard Lineweaver-Burk plots of the inhibited* versus *noninhibited liver homogenate. Key: A, ●, control; B, △, 10^{-3} M dimethyl sulfoxide; C, □, 10^{-2} M dimethyl sulfoxide; D, ○, 10^{-1} M dimethyl sulfoxide; and E, ⊖, 10^{-3} M sodium cyanide.*

structed by the method of least squares (13). Values for the apparent Michaelis constant of the uninhibited reaction (apparent K_m)[7] and for the inhibited reactions (apparent K_m')[7] were obtained by extrapolation. Calculations of the apparent inhibitor constant (apparent K_i)[7] were made by utilizing the equation (14):

$$K_i = \frac{K_m\,(I)}{K_m' - K_m} \qquad \text{(Eq. 1)}$$

Comparative experiments were carried out using 1×10^{-3} *M* sodium cyanide as the inhibitor.

RESULTS

The maximal rate of the control reaction was obtained with a substrate concentration of 1.7×10^{-2} g./ml. of lyophilized serum. The rates of reaction were linear up to 40 min., at which time the rates decreased. This decrease in rate is typical of Warburg studies with animal tissues and can be attributed to either complete utilization of the substrate or product inhibition. This deviation from linearity was observed in all inhibited and control studies at approximately 40 min. Therefore, the kinetic studies are based on 40-min. oxygen uptake values.

A concentration of 10^{-3} *M* dimethyl sulfoxide was observed to be the minimal concentration producing a significant inhibition of the reaction. A concentration of 10^{-4} *M* dimethyl sulfoxide produced an insignificant decrease in the reaction rate.

Lineweaver-Burk plots for the data in Table I (dimethyl sulfoxide- and sodium cyanide-inhibited reactions) are presented in Fig. 1. The plots of the dimethyl sulfoxide-inhibited reaction are of the competitive type, and the plot of the sodium cyanide reaction is of the noncompetitive type. Sodium cyanide proved to be a stronger inhibitor than dimethyl sulfoxide. The V_{max} (0.925 μl. O_2/min.) of the plots remained constant, while both the Michaelis constant (K_m) and the inhibitor constant (K_i) increased with increased inhibitor concentration.

DISCUSSION

The choice of lyophilized bovine serum as the substrate for the studies was made because of the presence of a large number of possible reactions for liver respiration. A single substrate approach would have been too limited for a preliminary investigation.

In considering the inhibition of respiration in a liver homogenate, one must remember that he or she is dealing with a multienzyme system. At this time the points of inhibition or the mechanism by which inhibition is accomplished cannot be identified.

The overall resultant inhibition of the system was competitive and could result from any one or a combination of mechanisms, *i.e.*, competitive, partially competitive, and substrate trapping.

Although V_{max} was constant in the dimethyl sulfoxide-inhibited reactions, K_m' and K_i increased with increasing inhibitor concentration. Variations in K_m' are expected for competitively inhibited reactions, and these values should deviate from one another by a factor of $1 + [(I)/K_i]$ in purified enzyme systems (15).

In complex multiple-enzyme systems, this constant deviation in K_m' usually is not observed. The inability to determine an adequate value for the inhibitor constant in multienzyme systems is a primary

[7] Since the investigations were carried out using a whole homogenate, the Michaelis constants (K_m and K_m'), the inhibitor constants (K_i and K_i'), and the maximal velocity (V_{max}) should be considered apparent.

Table I—Kinetics of Dimethyl Sulfoxide Inhibition of the Respiration of Rabbit Liver Homogenates

Inhibitor Concentration, *M*	Substrate Concentration, g./ml.	Oxygen Consumption, μl. O_2/40 min.	Percent Inhibition[a]	Standard Deviation	Apparent V_{max}, μl. O_2/min.	Apparent K_m, g./ml.	Apparent K_i, moles
0.00 Dimethyl sulfoxide	0.5×10^{-2}	24.88	—	5.17	1.162	4.25×10^{-3}	—
	0.7×10^{-2}	30.33	—	4.60			
	1.5×10^{-2}	35.28	—	4.91			
	1.7×10^{-2}	36.18	—	6.01			
10^{-3} Dimethyl sulfoxide	0.5×10^{-2}	23.07	7.28	0.95	1.162	5.81×10^{-3}	2.72×10^{-3}
	0.7×10^{-2}	25.89	14.64	1.40			
	1.0×10^{-2}	25.17	—	0.88			
	1.2×10^{-2}	30.99	—	2.66			
	1.5×10^{-2}	30.85	12.56	1.73			
10^{-2} Dimethyl sulfoxide	0.5×10^{-2}	18.73	24.72	2.86	1.162	1.01×10^{-2}	7.39×10^{-2}
	0.7×10^{-2}	18.70	38.35	3.32			
	1.0×10^{-2}	22.42	—	1.21			
	1.2×10^{-2}	21.73	—	3.36			
	1.5×10^{-2}	24.99	29.17	1.88			
	1.7×10^{-2}	27.21	24.80	2.13			
10^{-1} Dimethyl sulfoxide	0.5×10^{-2}	17.75	28.66	3.50	1.162	1.39×10^{-2}	4.39×10^{-1}
	0.7×10^{-2}	18.83	37.92	3.00			
	1.0×10^{-2}	21.65	—	1.56			
	1.2×10^{-2}	19.83	—	4.22			
	1.5×10^{-2}	24.97	29.23	4.64			
	1.7×10^{-2}	32.89	9.10	4.25			
10^{-3} Sodium cyanide	0.5×10^{-2}	15.92	36.02	2.97	0.675	4.94×10^{-3}	—
	0.7×10^{-2}	16.81	44.58	1.50			
	1.0×10^{-2}	15.63	—	3.36			
	1.2×10^{-2}	19.13	—	2.41			
	1.5×10^{-2}	20.26	42.58	1.70			

[a] Percent inhibition is given for those substrate concentrations in the inhibited reactions for which there were corresponding substrate concentrations in the controls. Additional points were obtained in the inhibited reactions to provide more points for the Lineweaver–Burk plots.

reason for the lack of a constant deviation in K_m' (15). The fact that K_i is observed to increase with increasing inhibitor concentration is evidence that more than one enzyme is being inhibited and that the inhibitor has a greater affinity for certain enzymes than for others (15).

The inhibition by sodium cyanide was used as a control to test the reliability of this system to detect competitive *versus* noncompetitive-type inhibitions. That sodium cyanide is a noncompetitor of respiration is well known, and the fact that it is a stronger inhibitor of respiration than dimethyl sulfoxide is not surprising.

This study was preliminary, and future investigations are planned to identify both the points of inhibition (*i.e.*, the enzymes being inhibited) and the mechanism of inhibition.

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Effect of Vehicle Viscosity and an Anticholinergic Agent on Bioavailability of a Poorly Absorbed Drug (Phenolsulfonphthalein) in Man

JOHN J. ASHLEY* and GERHARD LEVY▲

Abstract □ The purpose of this study was to determine if an increase in the viscosity of the solution vehicle, or premedication with an anticholinergic agent, can increase the bioavailability of a poorly absorbed drug in man. The absorption of phenolsulfonphthalein by healthy adults was decreased significantly during the 1st hr. when the drug was administered in very viscous sodium alginate solution or when the subjects had been premedicated with propantheline. Sodium alginate had no effect on the total amount of phenolsulfonphthalein absorbed; pretreatment with the anticholinergic agent increased the total amount of phenolsulfonphthalein recovered in the urine from an average of 16 to 24% of the dose. This effect is attributed to a decrease of the GI transit rate.

Keyphrases □ Drug absorption—effect of vehicle viscosity and anticholinergic agent on bioavailability of poorly absorbed drugs (phenolsulfonphthalein), man □ Phenolsulfonphthalein absorption—effect of vehicle viscosity and anticholinergic agent, man □ Anticholinergic agents—effect on bioavailability of poorly absorbed drugs (phenolsulfonphthalein) in man □ Viscosity of solution vehicle—effect on bioavailability of poorly absorbed drugs (phenolsulfonphthalein) in man □ Bioavailability of poorly absorbed drugs (phenolsulfonphthalein)—effect of vehicle viscosity and anticholinergic agent, man

Some orally administered drugs, particularly those that are almost completely ionized in the pH range of GI fluids and have a relatively high molecular weight, are not completely absorbed. It is of interest to determine if the bioavailability of such drugs may be increased by pharmaceutical or pharmacological means. Previous studies with riboflavin, a vitamin absorbed by a specialized transport process, showed that administration in a very viscous sodium alginate solution vehicle or premedication with the anticholinergic drug propantheline increases the total amount of riboflavin absorbed upon oral administration of a large and incompletely absorbed dose (1, 2). This increased absorption was presumably due to prolonged retention of the vitamin at intestinal absorption sites as a result of decreased intestinal transit rate. Extrapolation of these results to drugs may be limited by the rather unique absorption characteristics of riboflavin (3, 4). The previous studies were extended by determining the effect of vehicle viscosity and premedication with propantheline on the bioavailability of a poorly absorbed drug, phenolsulfonphthalein (phenol red).

EXPERIMENTAL

Eight healthy male volunteers received 20 mg. phenolsulfonphthalein USP[1], dissolved in 50 ml. of aqueous vehicle, in the morning on an empty stomach. For the control experiments, the drug was dissolved in about 2 ml. water with the aid of 20 mg. sodium bicarbonate, and this was added to 48 ml. of a vehicle containing 1% citric acid, 0.1% sodium saccharin, 0.01% terpeneless lemon oil, and distilled water.

To determine the effect of viscosity on phenolsulfonphthalein absorption, 2% sodium alginate[2] was incorporated in the solution. The rheologic characteristics of the alginate solution were similar to those described previously (1).

To determine the effect of an anticholinergic agent, the subjects received 30 mg. propantheline bromide[3] the night before and again 1 hr. before the experiment. Subject T received only one-half this dose because of his low body weight. Phenolsulfonphthalein was administered in the same vehicle as was used in the control experiments.

The three types of experiments were carried out in random order on each subject at least 1 week apart. No food was permitted until 4 hr. after phenolsulfonphthalein administration, at which time the subjects ate lunch. Urine was collected hourly for the first 12 hr. and then at longer intervals for a total of 24 hr. The subjects drank sufficient water to maintain an average urine flow of 50–100 ml./hr. Phenolsulfonphthalein in the urine was determined colorimetrically as described by McLeod *et al.* (5), except that the pH and capacity

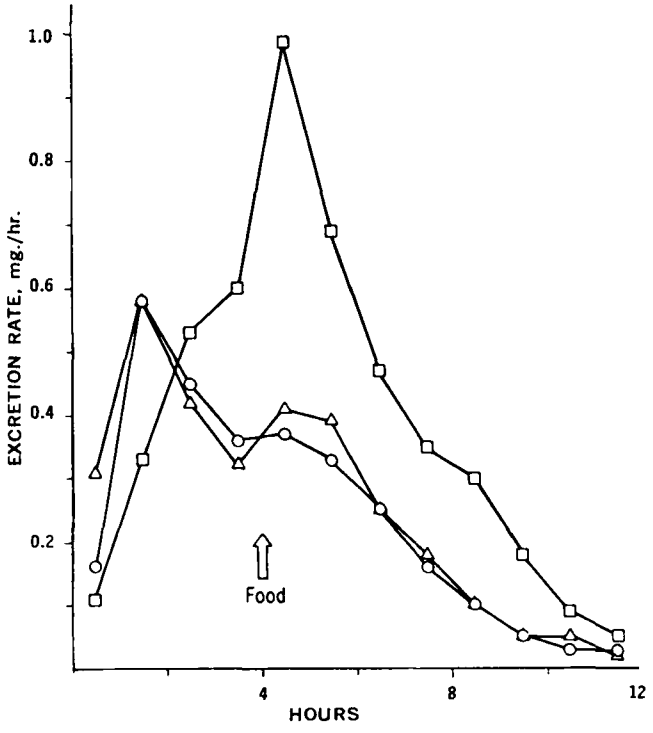

Figure 1—*Mean urinary excretion rates of phenolsulfonphthalein after oral administration of 20 mg. in solution to eight normal adult subjects. Key: △, control; ○, in sodium alginate solution; and □, after pretreatment with propantheline. Some of the individual data were obtained by interpolation.*

[1] Lot No. 851p, Allied Chemicals Corp.

[2] Sodium alginate XRD-1000, Marine Colloids, Inc., New York, N.Y.

[3] Pro-Banthine tablets, 15 mg., Searle.

Table I—Effect of Sodium Alginate and Propantheline on Phenolsulfonphthalein Absorption in Man

Subject	Age, years	Weight, kg.	Amount of Phenolsulfonphthalein Excreted, mg. 1 hr. Control	1 hr. Sodium Alginate	1 hr. Propantheline	24 hr. Control	24 hr. Sodium Alginate	24 hr. Propantheline
A	36	84	0.21	0.20	0.21	2.9	3.9	3.9
G	23	66	0.26	0.09	0.05	3.7	3.2	5.7
K	27	68	0.27	0.20	0.14	3.3	2.7	5.9
L	37	64	0.42	0.33	0.11	4.1	4.1	4.8
O	28	75	0.42	0.18	0.03	4.3	2.5	3.3
S	27	83	0.24	0.08	0.09	3.3	3.2	5.5
T	29	55	0.20	0.01	—[a]	1.7	1.6	4.1
Y	26	68	0.42	0.19	—[a]	2.7	2.9	5.4
Mean			0.30	0.16	0.10	3.2	3.0	4.8
Relative *SD*			32	61	62	26	26	20
Statistical significance[b] *versus* control				$p < 0.005$	$p < 0.01$		N.S.	$p < 0.01$

[a] These subjects were unable to void until after the 1st hr. [b] Paired *t* test.

of the alkaline buffer were increased to avoid underestimation of phenolsulfonphthalein concentrations in particularly acidic urine samples. The modified buffer solution consisted of 38.0 g. $Na_3PO_4 \cdot 12H_2O$, 26.8 g. $Na_2HPO_4 \cdot 7H_2O$, and enough distilled water to make 1 l. (pH approximately 11.5). The samples were centrifuged at $1000 \times g$ for 10 min., and part of the supernatant phase was removed for determination of the absorbance at 560 nm., with water in the reference cell. Blank determinations were made in the same manner, except that the alkaline buffer was replaced by 0.1 *N* acetic acid. The blank values averaged 0.38 mg. apparent phenolsulfonphthalein/24 hr. (0.09 mg. *SD*).

Binding of phenolsulfonphthalein to sodium alginate was determined by equilibrium dialysis at 37° using 5 ml. of the sodium alginate solution (without phenolsulfonphthalein) employed in the absorption study and 5 ml. 0.9% NaCl in 0.01 *N* HCl in the cellophane bag and using 30 ml. of a solution of 0.0015% phenolsulfonphthalein and 0.9% NaCl in 0.01 *N* HCl as the outer phase. The external phase was assayed at 24 hr. and both phases were assayed at 48 hr. The concentrations of phenolsulfonphthalein in the external phase were identical at 24 and 48 hr., indicating that equilibrium had been reached in less than 24 hr.

RESULTS

Administration of phenolsulfonphthalein in sodium alginate solution or premedication with propantheline decreased significantly the amount of phenolsulfonphthalein excreted 1 hr. after administration of the dye (Table I). Equilibrium dialysis showed that there was no measurable binding of phenolsulfonphthalein by sodium alginate under the experimental conditions. Phenolsulfonphthalein excretion continued for less than 24 hr.; the total amount excreted in 24 hr. was not significantly different from the control when phenolsulfonphthalein was administered in sodium alginate solution. On the other hand, premedication with propantheline increased the total recovery of phenolsulfonphthalein from 16 to 24% of the dose, a statistically significant difference (Table I). The time course of phenolsulfonphthalein excretion in the control, sodium alginate, and propantheline experiments is shown in Fig. 1. The averaged data in the figure are a good reflection of the individual results and are not distorted by the averaging process. A secondary excretion maximum was noted in all three experiments. It occurred immediately after lunch, *i.e.*, 4 hr. after phenolsulfonphthalein administration.

DISCUSSION

Phenolsulfonphthalein, a weak acid with a molecular weight of 354, is almost completely ionized at pH above 1. About 85% of a 6-mg. dose is recovered in the urine within 6 hr. after intravenous administration to normal subjects (5). About 3% of the dose is eliminated in the bile (5). The dye is excreted partly by renal tubular secretion, with a transport maximum of 35.8 mg./min./1.73 $m.^2$ (6). Intestinal perfusion studies in rats have shown that phenolsulfonphthalein is equally well absorbed in the proximal and distal regions of the small intestine (7). In the 0.5–20-mg./l. concentration range, phenolsulfonphthalein absorption from the intestinal tract of the rat occurs mainly by passive diffusion (8). At lower concentrations, there is an appreciable contribution by a specialized transport process which can be inhibited by *p*-aminohippuric acid (9). Oral administration of 6, 30, 60, and 120 mg. phenolsulfonphthalein to normal human subjects resulted in the recovery of 13.2, 8.0, 6.5, and 5.3% of the dose in the urine, respectively (5). A double reciprocal plot of maximum excretion rates or amounts recovered in the urine in 24 hr. *versus* the oral dose shows a good linear relationship suggestive of a specialized transport process, but similar results could be obtained by other mechanisms (such as partial precipitation of phenolsulfonphthalein due to interaction with a component of the GI fluids or nonlinear binding to mucosal proteins as the first step in absorption). Two colectomized patients absorbed phenolsulfonphthalein as well as normal subjects, suggesting that absorption of orally administered phenolsulfonphthalein occurs mainly in the small intestine (5).

The decreased excretion of phenolsulfonphthalein in the 1st hr. after administration in sodium alginate solution and after premedication with propantheline may be attributed to slower gastric emptying. The sodium alginate solution was slightly thixotropic and had a viscosity of more than 6000 cps. at a shear rate of 5.14 $sec.^{-1}$ and a temperature of 37°. It formed a stiff gel when acidified with 0.01 *N* hydrochloric acid. This should result in appreciable slowing of phenolsulfonphthalein transfer from the stomach to the intestines. Binding or complexation of phenolsulfonphthalein with sodium alginate could also reduce the rate of absorption of the dye, but this unlikely possibility (both are weak acids) was ruled out by equilibrium dialysis studies. Propantheline is a potent anticholinergic agent and inhibits gastric emptying by pharmacological means. Intravenous injection of propantheline has been reported (in abstract) to retard significantly gastric emptying and to decrease the rate, but not the extent, of acetaminophen absorption from *tablets* by human subjects (10).

Administration of phenolsulfonphthalein in sodium alginate solution did not increase the total amount of phenolsulfonphthalein absorbed relative to the aqueous control (Table I). On the other hand, pretreatment with propantheline increased phenolsulfonphthalein recovery by 50%, presumably due to longer retention of the dye at intestinal absorption sites because of slower transit of intestinal contents. The secondary excretion rate peak after lunch is most probably not due to enterohepatic cycling since very little phenolsulfonphthalein is excreted in the bile. It may reflect transfer of residual phenolsulfonphthalein from the stomach to the intestines or enhanced absorption due to discharge of bile into the intestinal lumen.

The effect of propantheline on phenolsulfonphthalein absorption is very similar to that on riboflavin absorption (2). Pretreatment with the anticholinergic agent delayed the absorption of both substances but increased appreciably the total amount absorbed. The variability of the 1-hr. excretion data was increased and that of the total amounts excreted was decreased with both substances by propantheline.

The results of this study show that the initial rate of absorption of a poorly absorbed substance such as phenolsulfonphthalein *from solution* can be decreased by pharmaceutical and pharmacological

means and that an anticholinergic drug can increase appreciably the bioavailability of such a substance.

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Effect of Various Steroids and ACTH on Plasma Levels of Zoxazolamine and Dicumarol

P. KOUROUNAKIS▲, S. SZABO*, J. WERRINGLOER†, and H. SELYE

Abstract □ Comparative experiments were performed on rats given pregnenolone-16α-carbonitrile, estradiol, progesterone, triamcinolone, hydroxydione, or ACTH (corticotropin) to study correlations between the *in vivo* conditioning effects upon resistance to zoxazolamine and dicumarol and the plasma concentrations of these drugs. The conditioners were classified as catatoxic when a decrease in toxicity was associated with increased blood clearance of the drug, and they were classified as syntoxic when *in vivo* protection was accomplished without a simultaneous fall in the plasma drug level. With these criteria, pregnenolone-16α-carbonitrile was markedly protective and catatoxic whereas triamcinolone and ACTH were moderately protective and syntoxic against zoxazolamine. The remaining steroids elicited very mild changes in zoxazolamine paralysis and blood clearance. Of all the conditioners tested, only estradiol protected against dicumarol intoxication and accelerated plasma clearance of this drug. Hence, only estradiol exhibited a manifest catatoxic effect against this anticoagulant.

Keyphrases □ Steroids (pregnenolone-16α-carbonitrile, estradiol, progesterone, triamcinolone, and hydroxydione)—effect on plasma levels of zoxazolamine and dicumarol □ ACTH—effect on plasma levels of zoxazolamine and dicumarol □ Zoxazolamine, plasma levels—effect of various steroids and ACTH, resistance–plasma concentration correlations □ Dicumarol, plasma levels—effect of various steroids and ACTH, resistance–plasma concentration correlations

It is well known that certain pharmacological agents, including steroidal hormones, can reduce the biological half-life of others by the induction of drug-metabolizing enzymes (1). During the past few decades, numerous observations have confirmed that, in addition to their classic hormonal functions as regulators of reproduction and general metabolism, steroids also play a decisive role in determining the resistance of the body against the most varied types of injury (2). These adaptive steroids can be classified according to their mechanism of action into two main groups: (*a*) "syntoxic" steroids, which improve host-tissue tolerance by permitting coexistence with the substrate (*e.g.*, by suppressing nonspecific inflammatory or allergic reactions against it); and (*b*) "catatoxic" steroids, which enhance the detoxication of endogenous and exogenous toxicants *via* induction, activation, decreased degradation of drug-metabolizing enzymes, and/or accelerated substrate elimination from the body. To obtain the best catatoxic effect, the steroids are usually administered 2–3 days before the toxicant. However, recent findings (3) indicate that even posttreatment facilitates protection against certain intoxications.

Systematic studies (2, 4) in these laboratories revealed that, among more than 1200 steroids tested, pregnenolone-16α-carbonitrile and its close derivatives exert the greatest prophylactic effect *in vivo*. Biochemical investigations (5) on rats showed that pregnenolone-16α-carbonitrile stimulates catatoxic mechanisms. On the other hand, steroids such as estradiol protect *in vivo* only against a limited number of drugs, and it is not known whether their actions are syntoxic or catatoxic (6, 7).

In view of these observations, an attempt was made to investigate the correlation between the *in vivo* effects of steroids and the plasma concentrations of zoxazolamine and dicumarol after treatment with pregnenolone-16α-carbonitrile, estradiol, triamcinolone, progesterone, hydroxydione, or ACTH. The steroids were selected on the basis that the syntoxic effects are virtually limited to glucocorticoids, whereas the catatoxic properties appear to be independent of any other known steroid

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Table I—Effect of Various Steroids and ACTH upon Plasma Level of Zoxazolamine

Conditioner	Plasma Concentration of Zoxazolamine, mcg./ml.			Paralysis Time, min.	
	Conditioned[a] Animals	Control I[b]	Control II[a]	Conditioned Animals	Control II
Pregnenolone-16α-carbonitrile	26.5 ± 0.7 (18)[c]	41.4 ± 1.3 ***[d] (16)	28.1 ± 2.0 NS (5)	19 ± 1 ***	115 ± 17
Estradiol	25.3 ± 0.7 (15)	30.5 ± 1.1 *** (14)	24.7 ± 1.8 NS (7)	166 ± 10 NS	203 ± 29
Progesterone	22.0 ± 1 (16)	27.5 ± 0.7 *** (15)	25.1 ± 1.6 NS (4)	123 ± 15 NS	100 ± 3
Triamcinolone	35.0 ± 0.9 (20)	31.1 ± 1.4 * (16)	26.2 ± 1.1 *** (9)	89 ± 8 ***	130 ± 6
Hydroxydione	28.7 ± 0.7 (17)	33.0 ± 1.6 * (18)	26.4 ± 1.2 NS (6)	188 ± 9 *	252 ± 24
ACTH	32.6 ± 0.9 (17)	34.8 ± 0.8 NS (19)	27.0 ● 0.8 *** (10)	111 ± 8 ***	160 ± 10

[a] Killed when the righting reflex was regained. [b] Killed when the conditioned group regained the righting reflex. [c] Figures in parentheses indicate number of animals. [d] The plasma levels of zoxazolamine in Controls I and II are compared with the plasma concentrations of the drug in the conditioned rats: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, and NS = not significant.

hormone action. ACTH protects against several toxic compounds (8); hence, it was used in this experimental series to stimulate endogenous glucocorticoid secretion.

EXPERIMENTAL

Two series of experiments were performed on female ARS/Sprague–Dawley rats[1], averaging 100 g. (range 90–110 g.) and maintained *ad libitum* on laboratory food[2] and tap water.

In the zoxazolamine series, the animals were divided into three groups, of which the first and second received 1 ml. of water twice daily *per os*. The rats of the remaining group were given pregnenolone-16α-carbonitrile[3] (3β-hydroxy-20-oxo-5-pregnene-16α-carbonitrile), estradiol[4], progesterone[4], hydroxydione hemisuccinate[5], triamcinolone[6] (10 mg. in 1 ml. water twice daily *per os*), or ACTH[7] (5 I.U. in 0.2 ml. water three times daily subcutaneously) throughout the experiment. Zoxazolamine[8] (10 mg./100 g. body weight in 1 ml. water) was administered intraperitoneally to all groups on the 4th day, 1 hr. after the last pretreatment. Blood samples were taken from control (Group I) and conditioned (Group III) animals when the latter regained the righting reflex. Samples were removed from Group II when their righting reflex reappeared.

In the dicumarol series, the animals were divided into four groups, of which the first two received 1 ml. of water twice daily *per os*. The remaining two groups were given the conditioners as described. Only triamcinolone was administered at the individual dose of 1 mg. because of its toxicity at a higher dose level during 4 days of pretreatment plus 5 days of treatment. Dicumarol[9] (13 mg./100 g. body weight in 1 ml. water *per os*) was given once daily to all groups beginning on the 4th day, always 1 hr. after the first daily dose of the conditioners. Blood samples were taken from Groups I and III on the 7th day, 5 hr. after dicumarol treatment. Mortality was recorded in the remaining two groups on the 9th day.

Zoxazolamine was extracted from plasma into ethylene dichloride and from the organic solvent into hydrochloric acid. The plasma concentration of the drug was measured spectrophotometrically at 278 nm., where it exhibits a pronounced absorption maximum (9). Dicumarol was extracted from acidified plasma into heptane and from heptane into a sodium hydroxide solution. The drug concentration was determined spectrophotometrically at 315 nm. (10). All measurements were performed with a spectrophotometer[10]. Drug-free plasma from conditioned and nonconditioned animals was used for preparing standards and blanks.

[1] Madison, Wis.
[2] Purina Laboratory Chow, Ralston Purina Co. of Canada.
[3] The Upjohn Co.
[4] Roussel Corp.
[5] Pfizer Laboratories.
[6] E. R. Squibb & Sons.
[7] N. V. Organon.
[8] K & K Laboratories.
[9] Abbott Laboratories.
[10] Unicam SP 8000.

RESULTS AND DISCUSSION

These experiments were performed to establish whether the protection offered by a steroid or ACTH is due to a catatoxic or syntoxic effect, *i.e.*, to the enhanced elimination of, or increased tolerance to, zoxazolamine and dicumarol.

The plasma levels of zoxazolamine were compared in control and conditioned animals when the latter regained the righting reflex as well as when paralysis disappeared in both groups.

The following changes in plasma drug concentrations were regarded as characteristic of protection through catatoxic or syntoxic mechanisms:

1. The zoxazolamine concentration in the blood of conditioned animals was lower than that of the controls at the time when the former regained the righting reflex; it corresponded to the level in control Group II when paralysis disappeared in these rats.

This characteristic catatoxic pattern was clearly exhibited by pregnenolone-16α-carbonitrile. The duration of zoxazolamine-induced paralysis was shortened from 115 to 20 min. and the plasma drug concentration decreased from 41 to 26 mcg./ml. as compared with the controls when the righting reflex reappeared in the former. A similar concentration (28 mcg./ml.) was noted in the controls that were sacrificed when they regained the righting reflex.

2. If a steroid acts syntoxically, the plasma levels of zoxazolamine should be higher in the pretreated rats than in control Group II when paralysis disappears in both groups. On the other hand, the blood levels of the drug should be the same in both the conditioned and control Group I when blood samples are taken from the conditioned rats after they regain the righting reflex.

Triamcinolone and ACTH reduced zoxazolamine paralysis from 130 to 89 and from 160 to 111 min., respectively. This reduction took place although the plasma levels of the drug in the conditioned rats were significantly higher than in the controls when both groups regained the righting reflex (Table I).

When paralysis disappeared, the plasma concentrations of zoxazolamine in the estradiol- or hydroxydione-treated rats were slightly lower than in the controls killed at the same time. However, the plasma drug levels in the conditioned groups were not significantly different from those in the controls sacrificed when they regained the righting reflex. Since paralysis was slightly reduced, these two steroids seem to have a mild catatoxic effect.

The action of progesterone on the plasma concentration of zoxazolamine was comparable to that of estradiol and hydroxydione. Although paralysis was prolonged by this steroid, the apparent difference was not significant. This discrepancy may be attributed to the anesthetic properties of progesterone, administered 1 hr. before zoxazolamine.

The plasma level of dicumarol was influenced by the steroids in three different ways (Table II). The *in vivo* protection offered by estradiol was associated with a significant decrease in the plasma drug concentration. Triamcinolone and progesterone, on the other hand, markedly elevated the plasma level of dicumarol as compared with the concentration of the drug in nonconditioned rats. The increased plasma drug concentration after pretreatment (*e.g.*, with triamcinolone or progesterone) might have been due to inhibition

Table II—Effect of Various Steroids and ACTH upon Plasma Level of Dicumarol

Conditioner	Plasma Concentration of Dicumarol[a], mcg./ml.		Mortality[b]	
	Conditioned Animals	Control	Conditioned Animals	Control
Pregnenolone-16α-carbonitrile	19.4 ± 1.5 NS (17)[c]	18.6 ± 2.2 (17)	9/10 NS	7/9[e]
Estradiol	13.7 ± 1.5 ***[d] (18)	26.6 ± 2 (18)	1/10 ***	7/9[e]
Progesterone	43.3 ± 2.9 *** (16)	31.1 ± 2.3 (11)	9/10 NS	9/10
Triamcinolone	55.8 ± 4.1 ** (6)	19.3 ± 2.6 (7)	10/10 NS	7/9[e]
Hydroxydione	26.3 ± 3.3 NS (15)	20.6 ± 3.3 (11)	12/15 NS	11/15
ACTH	29.9 ± 2.8 NS (14)	25.1 ± 2.8 (17)	6/9 NS	8/10

[a] Determined on the 7th day, 5 hr. after dicumarol treatment. [b] Recorded on the 9th day. [c] Figures in parentheses indicate number of animals. [d] ** = $p < 0.01$, *** = $p < 0.005$, and NS = not significant. [e] *Cf. Reference 5.*

of the hepatic microsomal enzyme system responsible for the metabolism of these steroids (1, 11, 12). The mortality rates were comparable in the control and pretreated groups. Pregnenolone-16α-carbonitrile, hydroxydione, and ACTH, which also did not offer *in vivo* protection, did not markedly influence the plasma concentration of dicumarol. Hence, only estradiol demonstrated a significant catatoxic effect against dicumarol intoxication.

The results of these investigations indicate that steroids can offer protection against drugs through syntoxic or catatoxic mechanisms. The catatoxic effect is not dependent upon any particular hormonal activity whereas the syntoxic action is limited to triamcinolone, a glucocorticoid, and to ACTH, a stimulator of endogenous glucocorticoid secretion. Most of the catatoxic actions of steroids, particularly those of pregnenolone-16α-carbonitrile, are mediated through enhanced hepatic microsomal enzyme activity. Since zoxazolamine is a substrate of this system, there is little doubt that the blood levels are lowered and paralysis time reduced through increased hydroxylation of this drug (13). However, pregnenolone-16α-carbonitrile does not act against dicumarol, which is known to be detoxified through microsomal hydroxylation (14).

The catatoxic effect of estradiol against dicumarol is uncommon; it could be attributed to the fact that this steroid induces drug-metabolizing enzymes in female but not in male rats (15–18). The influence of triamcinolone and ACTH on zoxazolamine paralysis is even more difficult to explain. It may be due to reduced receptor sensitivity, altered drug distribution in the body, decreased substrate excretion, or interactions at receptor sites. Future work will have to clarify whether these factors play decisive roles in diminishing zoxazolamine paralysis.

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Flowmeter for Pharmaceutical Powders

Keyphrases ☐ Flowmeter for pharmaceutical powders—determination of flow rates ☐ Powders, pharmaceutical—flowmeter, determination of flow rates

Sir:

Monodisperse powders have been reported by many authors to have a maximum flow rate (Q) at a particular diameter (x) (1–3). We have found that beyond the maximum the flow rates often follow a parabolic decline in the sense that:

$$\log Q = a \cdot \log x + B \qquad \text{(Eq. 1)}$$

where a and B are product-dependent constants (Figs. 1 and 2).

The flow rates here were determined in a flowmeter[1]. Both the glass beads and granulations tested were of normal flow patterns in other respects. For example, the flow rates increased with increasing length of efflux tube (Fig. 3), as reported by Jones and Pilpel (4) and Bingham and Wikoff (5).

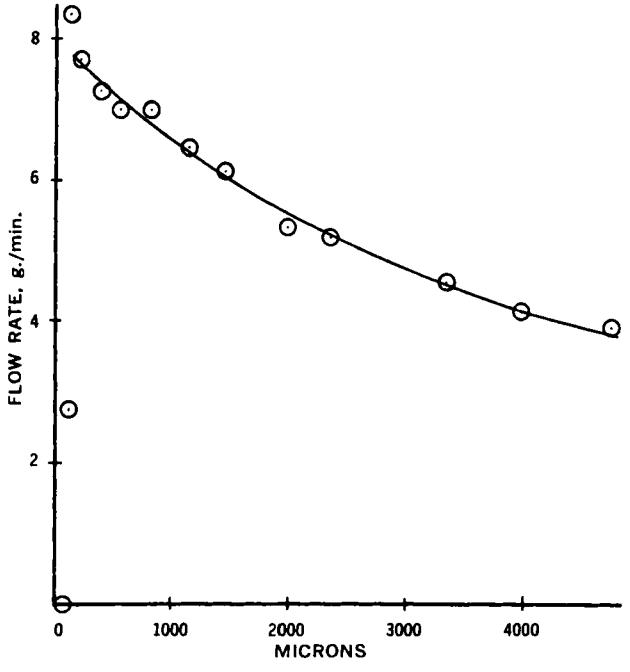

Figure 1—*Flow pattern of a granulation showing maximum flow rate at 210 μ.*

[1] The Lewis-Howe flowmeter developed at Lewis-Howe Co. in 1954. The Erweka flowmeter (marketed at a later date) is based on somewhat the same design.

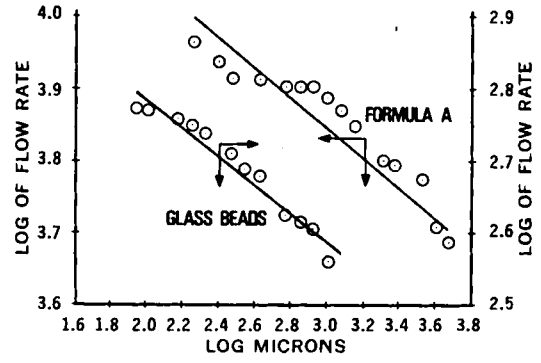

Figure 2—*Flow rates of glass beads and of a granulation (Formula A) plotted according to Eq. 1.*

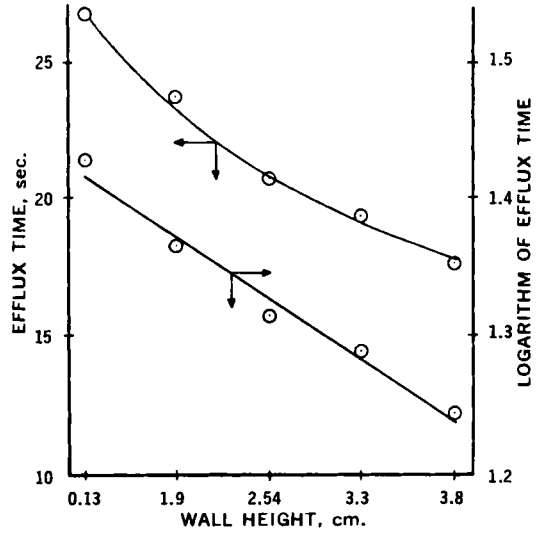

Figure 3—*Flow rate as a function of height of efflux tube of 500 g. of spray-dried lactose. Semilogarithmic plotting (right ordinate) shows adherance to the Bingham–Wikoff (5) equation.*

Equation 1 allows a good estimation of optimum diameter in four determinations (two below x_{max} and two above), assuming (for calculation purposes) that the curve is linear on the low side of x_{max}.

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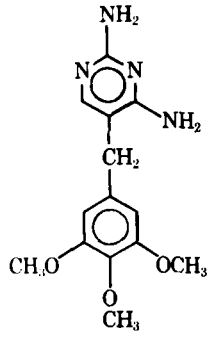
Identification of Trimethoprim 3-Oxide as a New Urinary Metabolite of Trimethoprim in Man

Keyphrases ☐ Trimethoprim 3-oxide—isolation, identification as urinary metabolite of trimethoprim, man ☐ Urinary metabolite of trimethoprim—isolation, identification of trimethoprim 3-oxide, man ☐ TLC—isolation, identification of trimethoprim 3-oxide as new urinary metabolite, man

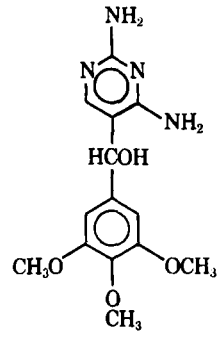
Sir:

Trimethoprim, 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine (I), is a broad spectrum antibacterial (1) and is used in combination[1] with sulfonamides (2). The metabolism of trimethoprim in the rat, dog, and man was investigated by Schwartz *et al.* (3) using the ^{14}C-labeled drug. The ^{14}C-metabolites were separated on silica gel plates, located by fluorescence quench spectrofluorometry or by autoradiography, scraped from the plate, and eluted, and the radioactivity was measured. The isolation, identification, and quantitation of the same four metabolites were described in all three species. In our recent work on the development of a fluorescence thin-layer assay for trimethoprim and metabolites (4), we reinvestigated the thin-layer system of Schwartz *et al.* (3) and found that one of the TLC spots assigned to Metabolite II is a mixture of two compounds. A new metabolite, which is cochromatographed with II, has been isolated and identified as trimethoprim 3-oxide (III)[2].

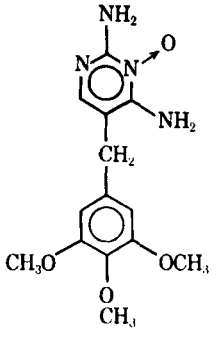
For preparative work, cumulative 24-hr. urines from four adult male volunteers who had received 160 mg. of trimethoprim[3] were combined and adjusted to pH 1.5 with 1 *N* H_2SO_4. The acidified urine was washed with chloroform, and sodium hydrogen phosphate (170 g./l.) was added. The solution was adjusted to pH 8 with 1 *N* NaOH and extracted with isopropanol–dichloromethane (1:9). The extract was evaporated under vacuum to dryness. The residue was triturated with methanol, and the methanol-soluble portion was chromatographed successively on two columns (SilicAR CC-7) by elution with methanol–chloroform and ethanol–benzene mixtures, respectively. Final purification was achieved by preparative TLC on silica gel[4]. Upon further TLC in additional solvent systems, the isolated material appeared as a single spot with the same R_f as synthetic III. A few hours after development and exposure to light, a characteristic yellow-green fluorescence under long wavelength (360 nm.) UV light developed for both the isolated and synthetic III.



I



II



III

Isolated III crystallized from water to afford colorless needles, m.p. 217–219°[2]. The IR, UV, and mass spectra of isolated and synthetic III were identical. The accurate mass of the molecular ion of isolated III was determined by peak matching at 10,000 resolution, 10% valley definition, and found to be 306.1326 (calc. for $C_{14}H_{18}N_4O_4$: 306.1328).

The amount of III excreted in human urine during 24 hr. was measured by assaying samples of urine individually from seven healthy male volunteers who had received 160 mg. of I[3]. An average of 2.1% of the administered dose was present as III. Urine levels were determined by a direct measurement of the fluorescence which developed on silica gel plates[4] for the 3-oxide spot by scanning with a spectrodensitometer[5]. Other pertinent details of the method were reported recently (4) for trimethoprim and four other metabolites and also apply directly to the assay of the 3-oxide (III).

Some revision in the reported metabolic pattern of trimethoprim in man is required. Schwartz *et al.* (3) found that II accounted for about 2.5% of the dose[6]. The present study indicates that the reported levels of Metabolite II included a substantial amount of the 3-oxide. When separated from the 3-oxide, II is barely detectable by our TLC method and is present in human urine in lower concentrations than previously determined. A more detailed report on the metabolic distribution of trimethoprim in man was presented recently (7).

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[1] Trimethoprim–sulfamethoxazole (1:5 combination); Septra, Burroughs Wellcome Co. and Bactrim, Hoffmann–La Roche.

[2] We thank Dr. L. A. Nielsen of Burroughs Wellcome Co. for a reference sample of trimethoprim 3-oxide. The sample was prepared according to the procedure of Rey-Bellet and Reiner (5) and had m.p. 216–218° [lit. (5) m.p. 225–227°] with identical spectral properties (UV, NMR, and mass spectra) to those reported (5).

[3] Two tablets of Septra (see *Footnote 1*).

[4] Plates were developed in ethanol–benzene (3:7) twice to 15 cm. followed by one development in chloroform–*n*-propanol–25% ammonium hydroxide (80:20:1). In this system, III has R_f 0.26 and is well separated from the other metabolites.

[5] Schoeffel.

[6] The data of Schwartz *et al.* (3) expressed in "percent urinary concentrations" were converted to a "percent of dose" figure by using their observation that 59% of the dose is present in the urine in 24 hr. as unchanged drug and metabolites (6).

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Participation of Micelle at Crystal–Solution Interface in Rate-Determining Step for Cholesterol Gallstone Dissolution in Unsaturated Bile Media

Keyphrases ☐ Cholesterol gallstone dissolution rate in unsaturated bile media—effect of micelle at crystal–solution interface ☐ Gallstone dissolution rate in unsaturated bile media—effect of micelle at crystal–solution interface ☐ Micellar effect—cholesterol gallstone dissolution rate in unsaturated bile media ☐ Bile salt–lecithin micelle—effect on transport of cholesterol in interface, effect on gallstone dissolution rate

Sir:

Recent investigations (1–3) in this laboratory have established that the *in vitro* dissolution of human cholesterol gallstones in bile salt–lecithin media is largely controlled by crystal–solution interfacial kinetics rather than by bulk solution diffusion. Apparent interfacial barriers for dissolution were determined (2, 3) for a variety of cholesterol stones; these were typically 15–20 times greater than bulk diffusion barriers in the *in vitro* experiments (2, 3). Similar dissolution rate experiments with compressed pellets of cholesterol monohydrate crystals have yielded comparable results—*viz.*, interfacial barriers 20 times greater than bulk diffusion in sodium taurocholate–lecithin and sodium cholate–lecithin solutions (2, 3). These results have encouraged the mechanistic exploration of the rate-determining step(s) at the cholesterol monohydrate crystal–solution interface.

This communication reports data showing that the bile salt–lecithin micelle is critically involved in the "activated complex" for the transport of cholesterol in the interfacial region. Table I gives the results of a dissolution rate study showing the relative constancy of the effective interfacial transport coefficient, P, when the bile salt–lecithin concentration is changed while the bile salt–lecithin ratio is kept constant. As can be seen, P remained essentially constant when the solubility, C_s, varied by about a factor of 10. Since it was already demonstrated (2, 3) that the process is interface-kinetics controlled, these data can only be interpreted by some mechanism in which the bile salt–lecithin–cholesterol micelle is involved in the rate-determining step.

Table I—Dissolution Rate[a] of Cholesterol Monohydrate Pellets Showing the Direct Dependence of the Rate, J/A, upon the Solubility, C_s, and the Relative Independence of the Rate upon the Interface Transport Coefficient, P

Sodium Cholate + Lecithin, % (in 0.1 M Phosphate, pH 7.4)	$(J/A)^b 10^4$, mg./cm.²/sec.	C_s[c], mg./cm.³	$D^d \times 10^{6e}$, cm.²/sec.	$P^f \times 10^5$, cm./sec.
1 + 0.5	0.057	0.30	1.46	1.90
2 + 1.0	0.16	1.05	1.49	1.52
2 + 1.0 + 0.07 M NaCl	0.14	1.00	1.49	1.40
ionic strength (correct to 5% cholate)				
5 + 2.5	0.56	3.00	1.30	1.87

[a] Experimental methods for determining J/A, C_s, and D are given in *Reference 3*. [b] $J/A = PC_s$ = dissolution rate (mg./cm.²/sec.). [c] C_s = total solubility of cholesterol monohydrate. [d] D = effective diffusivity of cholesterol in micellar solution. [e] Diffusion coefficient data are presented mainly to show that the size of the micelle is not changing greatly over the concentration range studied. [f] P = interface transport coefficient (cm./sec.).

This finding is exciting for a number of reasons. The recent studies of Surpuriya and Higuchi (4, 5) showed that the oil–water transfer of a variety of sterols in several bile salt–lecithin systems is also interfacial-barrier controlled and that the bile salt–lecithin–cholesterol micelle is involved in the rate-determining step. Thus the basic mechanisms may be very similar for the two situations, and other parallel characteristics are anticipated. For example, Surpuriya[1] showed that the presence of 0.01 M calcium chloride increases P by a factor of 5 in his oil–water system. It would indeed be interesting to see similar effects in the gallstone and/or cholesterol monohydrate dissolution.

There is a significant clinical aspect with regard to the present findings. Recently, Danzinger *et al.* (6) showed that oral administration of chenodeoxycholic acid to patients suffering from gallstone disease can lead to *in vivo* dissolution of gallstones. In four of the seven patients studied, these investigators found that gallstones either disappeared completely or diminished significantly in size during the 14–22 months of chenodeoxycholic acid treatment. In the remaining three patients, no changes in gallstone size could be determined. These clinical studies support the idea that increasing the *in vivo* dissolution rate by a factor of 10 or more should yield material patient benefits in that practical medical treatment times may be approached.

The present findings and the parallel work on the oil–water transport of sterols could be of value in seeking out agents, additives, and other biopharmaceutical factors important to the safe and efficacious treatment of gallstone sufferers.

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Participation of Micelle at Crystal–Solution Interface in Rate-Determining Step for Cholesterol Gallstone Dissolution in Unsaturated Bile Media

Keyphrases ☐ Cholesterol gallstone dissolution rate in unsaturated bile media—effect of micelle at crystal–solution interface ☐ Gallstone dissolution rate in unsaturated bile media—effect of micelle at crystal–solution interface ☐ Micellar effect—cholesterol gallstone dissolution rate in unsaturated bile media ☐ Bile salt–lecithin micelle—effect on transport of cholesterol in interface, effect on gallstone dissolution rate

Sir:

Recent investigations (1–3) in this laboratory have established that the *in vitro* dissolution of human cholesterol gallstones in bile salt–lecithin media is largely controlled by crystal–solution interfacial kinetics rather than by bulk solution diffusion. Apparent interfacial barriers for dissolution were determined (2, 3) for a variety of cholesterol stones; these were typically 15–20 times greater than bulk diffusion barriers in the *in vitro* experiments (2, 3). Similar dissolution rate experiments with compressed pellets of cholesterol monohydrate crystals have yielded comparable results—*viz.*, interfacial barriers 20 times greater than bulk diffusion in sodium taurocholate–lecithin and sodium cholate–lecithin solutions (2, 3). These results have encouraged the mechanistic exploration of the rate-determining step(s) at the cholesterol monohydrate crystal–solution interface.

This communication reports data showing that the bile salt–lecithin micelle is critically involved in the "activated complex" for the transport of cholesterol in the interfacial region. Table I gives the results of a dissolution rate study showing the relative constancy of the effective interfacial transport coefficient, P, when the bile salt–lecithin concentration is changed while the bile salt–lecithin ratio is kept constant. As can be seen, P remained essentially constant when the solubility, C_s, varied by about a factor of 10. Since it was already demonstrated (2, 3) that the process is interface-kinetics controlled, these data can only be interpreted by some mechanism in which the bile salt–lecithin–cholesterol micelle is involved in the rate-determining step.

Table I—Dissolution Rate[a] of Cholesterol Monohydrate Pellets Showing the Direct Dependence of the Rate, J/A, upon the Solubility, C_s, and the Relative Independence of the Rate upon the Interface Transport Coefficient, P

Sodium Cholate + Lecithin, % (in 0.1 M Phosphate, pH 7.4)	$(J/A)^b 10^4$, mg./cm.²/sec.	C_s[c], mg./cm.³	$D^d \times 10^{6e}$, cm.²/sec.	$P^f \times 10^5$, cm./sec.
1 + 0.5	0.057	0.30	1.46	1.90
2 + 1.0	0.16	1.05	1.49	1.52
2 + 1.0 + 0.07 M NaCl	0.14	1.00	1.49	1.40
ionic strength (correct to 5% cholate)				
5 + 2.5	0.56	3.00	1.30	1.87

[a] Experimental methods for determining J/A, C_s, and D are given in *Reference 3*. [b] $J/A = PC_s$ = dissolution rate (mg./cm.²/sec.). [c] C_s = total solubility of cholesterol monohydrate. [d] D = effective diffusivity of cholesterol in micellar solution. [e] Diffusion coefficient data are presented mainly to show that the size of the micelle is not changing greatly over the concentration range studied. [f] P = interface transport coefficient (cm./sec.).

This finding is exciting for a number of reasons. The recent studies of Surpuriya and Higuchi (4, 5) showed that the oil–water transfer of a variety of sterols in several bile salt–lecithin systems is also interfacial-barrier controlled and that the bile salt–lecithin–cholesterol micelle is involved in the rate-determining step. Thus the basic mechanisms may be very similar for the two situations, and other parallel characteristics are anticipated. For example, Surpuriya[1] showed that the presence of 0.01 M calcium chloride increases P by a factor of 5 in his oil–water system. It would indeed be interesting to see similar effects in the gallstone and/or cholesterol monohydrate dissolution.

There is a significant clinical aspect with regard to the present findings. Recently, Danzinger *et al.* (6) showed that oral administration of chenodeoxycholic acid to patients suffering from gallstone disease can lead to *in vivo* dissolution of gallstones. In four of the seven patients studied, these investigators found that gallstones either disappeared completely or diminished significantly in size during the 14–22 months of chenodeoxycholic acid treatment. In the remaining three patients, no changes in gallstone size could be determined. These clinical studies support the idea that increasing the *in vivo* dissolution rate by a factor of 10 or more should yield material patient benefits in that practical medical treatment times may be approached.

The present findings and the parallel work on the oil–water transport of sterols could be of value in seeking out agents, additives, and other biopharmaceutical factors important to the safe and efficacious treatment of gallstone sufferers.

(1) W. I. Higuchi, F. Sjuib, D. Mufson, A. P. Simonelli, and A. F. Hofmann, *J. Pharm. Sci.*, in press.
(2) W. I. Higuchi, S. Prakongpan, V. Surpuriya, and F. Young, *Science*, **178**, 633(1972).

[1] Data to be published.

(3) W. I. Higuchi, S. Prakongpan, and F. Young, *J. Pharm. Sci.*, in press.
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Kinetic Assay of Single Nitroglycerin Tablets

Keyphrases ☐ Nitroglycerin (single) tablets—kinetic assay, compared to compendial method ☐ Tablets, single nitroglycerin—kinetic assay, compared to compendial method ☐ Kinetic analysis—single nitroglycerin tablets, compared to compendial method

Sir:

Current interest in the stability of nitroglycerin tablets (1, 2) demonstrates the need for a simple, rapid, and precise single-tablet assay procedure for nitroglycerin. The present USP method (3) requires separation of the drug from potentially interfering substances by column chromatography followed by acid hydrolysis, which yields the nitrate ion, and subsequent spectrophotometric determination of nitrated phenoldisulfonic acid. Its complexity of manipulation does not permit this method to be rapid and efficient for the determination of nitroglycerin in single tablets. The GC (4) and polarographic (5) methods are accurate and specific but require extensive instrumentation. Simpler methods, such as that developed by Bell (6), are nonspecific; they can be subject to interference either by nitrate or nitrite ions.

The recent accelerated research activities in nitroglycerin formulations prompted us to report our preliminary data on a kinetic assay of nitroglycerin in tablets. This assay method is simple, rapid, and precise and appears to be superior to existing methods for the single-tablet assay of nitroglycerin.

Nitroglycerin has no significant UV absorption above 240 nm.; but in alkaline solutions, it degrades *via* a chromophoric intermediate which exhibits an absorption peak around 336 nm. For example, in 99% methanol and at a sodium hydroxide concentration of 0.03 *M*, the appearance and disappearance of peak intensity at 336 nm. (Fig. 1) can be ascribed to $A \rightarrow B \rightarrow C$ kinetics. The kinetic order, rate of degradation, and maximum peak absorbance are dependent on nitroglycerin and sodium hydroxide concentrations and solvent composition. A detailed description of the kinetics and mechanism of nitroglycerin degradation in alcoholic sodium hydroxide solutions will be presented elsewhere[1].

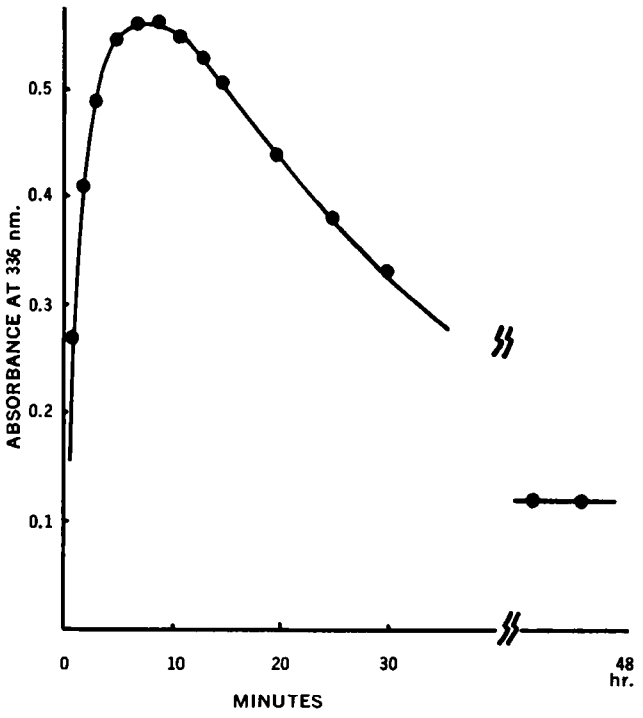

Figure 1—*Degradation of nitroglycerin as followed by absorbance change at 336 nm.; [nitroglycerin] ≃ 0.4 mg./3 ml. of reaction mixture; [sodium hydroxide] = 0.03* M; *solvent = 99% methanol; 25°.*

At a constant sodium hydroxide concentration and solvent composition, the observed maximum absorbance at 336 nm. was found to be a linear function of nitroglycerin concentration. A convenient system was 33% water in methanol and 0.033 *M* NaOH in the reaction mixture. The kinetic assay could be initiated either by placing 1 ml. of aqueous nitroglycerin solution or dissolving the sublingual tablet with 1 ml. of water directly in a 1-cm. cell, followed by addition of 0.05 *M* NaOH in methanol. The absorbance at 336 nm. was then followed until a maximum value was reached.

The validity of the present assay technique was checked against the official USP method. Different quantities of nitroglycerin tablets[2] and powders[3] were dissolved in water, and the solutions were assayed simultaneously by the USP and the kinetic methods. In the USP assay, 1-ml. aliquots of nitroglycerin solutions were mixed with the indicated amount of column material[4] and applied to the column. The same volume of nitroglycerin solution was used in the kinetic assay. Figure 2 shows a plot of absorbance maximum at 336 nm. as a function of the concentrations of nitroglycerin in the prepared solutions as determined by the USP method. The excellent linearity observed (correlation coefficient = 0.994 for 14 points) strongly testifies to the validity of the kinetic assay.

The slight positive intercept observed in Fig. 2 was apparently contributed by the tablet diluent (lactose)

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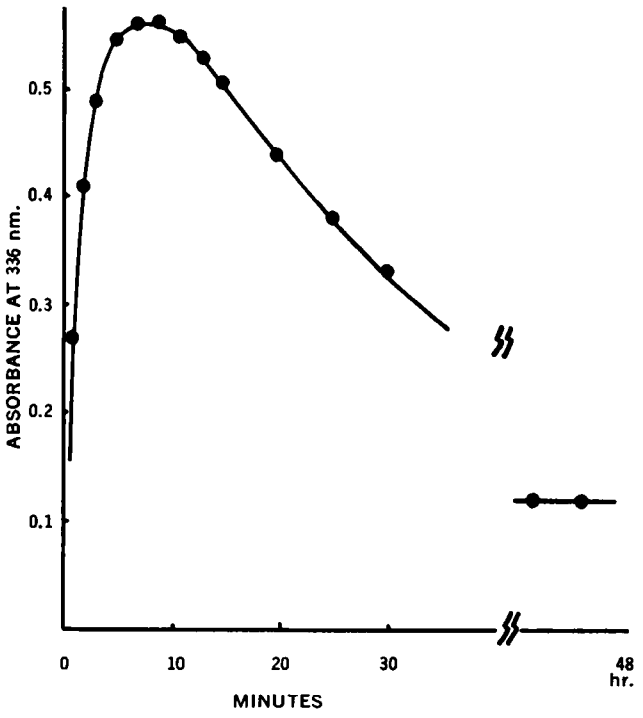

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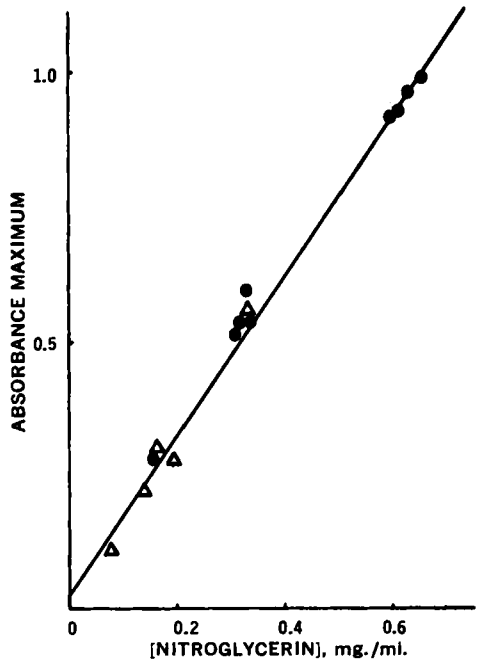

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BOOKS

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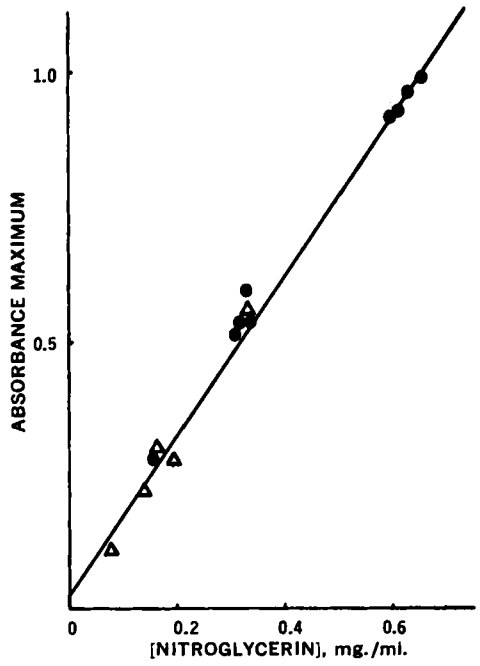

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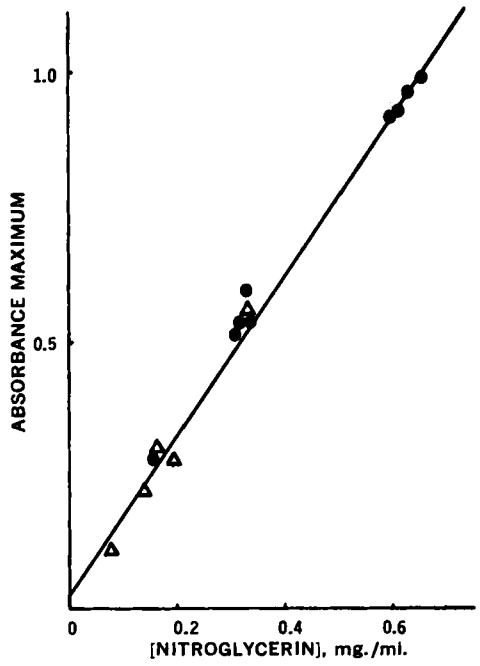

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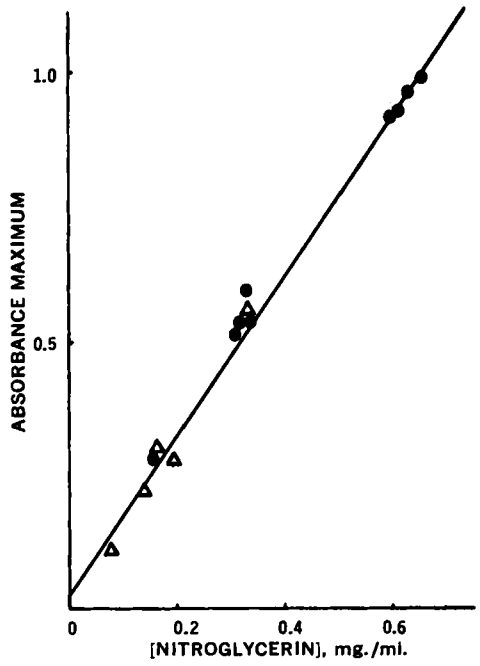

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REVIEWS

Neuropharmacology and Behavior. By V. G. LONGO. W. H. Freeman, 660 Market St., San Francisco, CA 94104, 1972. 184 pp. 15 × 23.5 cm. Price $6.95.

This relatively brief book does not attempt to cover all aspects of psychotropic drugs. The author, by drawing on his own research in this field, has written a very readable and interesting book covering the discovery, development, and use of psychotropic drugs. It is divided into four chapters—Antipsychotic Drugs, Antidepressant Drugs, Tranquilizing Drugs, and Hallucinogenic Drugs. For the reader interested in a historical perspective or a good overview, this book is recommended.

Staff Review ■

International Drug Monitoring: The Role of National Centres. World Health Organization Report Series. World Health Organization, Geneva, Switzerland. (Available from American Public Health Association, 1015 18th St., N.W., Washington, DC 20036), 1972. 47 pp. 16 × 24 cm. Price $1.00.

The development of systems for detecting adverse drug reactions has recently received increased attention. This report offers guidelines on the methodology and organization of national detection centers and the role of such national centers in an international reporting system. Since resources vary from country to country, a flexible approach is stressed. Recommendations concerning the principal activities of national drug monitoring centers are given as well as suggestions relative to the means for carrying out these activities.

Staff Review ■

Automation and Management in the Clinical Laboratory. Edited by GEORGE E. WESTLAKE and JAMES L. BENNINGTON. University Park Press, Chamber of Commerce Building, Baltimore, MD 21202, 1972. 274 pp. 13.5 × 20.5 cm. Price $12.50.

Hospitals have been the subject recently of allegations of inefficiency and wastefulness. This book represents the updated and revised proceedings of a conference, held in May 1971, on effective management of clinical laboratories. This meeting attempted to apply industrial engineering techniques to the medical laboratory. Individuals from laboratory management and systems design discuss how the principles of industrial management, accounting, and engineering may be used to develop greater economy, reliability, accuracy, and speed of delivery in the clinical laboratory. Computerized laboratory systems are examined in terms of objectives,

cost justifications, and interpretation of manufacturers' claims. The 15 articles are divided into three sections: Systems Techniques, Cost Control, and Evaluation of Equipment.

Staff Review ■

Predictive Toxicometrics—Basic Methods for Estimating Poisonous Amounts of Foods, Drugs, and Other Agents. By ELDON M. BOYD. Williams & Wilkins, Baltimore, MD 21202, 1972. vi + 408 pp. 14 × 22 cm. Price $32.00.

Even though there is increasing interest in the broad field of toxicology, one is often hard put to find a suitable text which can be used in undergraduate and graduate programs. A few years ago, Dr. Ted A. Loomis published a modern text on toxicology under the title *Essentials of Toxicology* (Lea & Febiger, 1968). The book has served a very useful purpose for those teachers presenting an introductory course in toxicology. For those courses in which methodology was emphasized, a book edited by Dr. G. E. Paget, *Methods of Toxicology* (F. A. Davis Co., 1970), became popular. More recently, a revised text by Drs. Linton H. Thienes and Thomas J. Haley, under the title *Clinical Toxicology* (5th Edition, Lea & Febiger, 1972), became available. To these three texts now can be added *Predictive Toxicometrics*, authored by a recognized toxicologist, Dr. Eldon M. Boyd, Professor of Pharmacology, Queen's University, Canada. The book is primarily directed toward methods of testing and evaluating the toxicity of drugs and other agents using many examples taken from the author's own laboratory. One of the unique features of this book is that many little details are presented that other books have not included.

In thumbing through the text, one is immediately confronted with words which often are not used by toxicologists in this country. These include "toxicometrics," "uniposal toxicometrics," and "multiposal predictive toxicometrics." This should not really cause the reader to shy away from the book, since the terms become well defined on reading the text. On the first page of the book, Boyd defines "toxicometrics" by drawing attention to two Greek words, *toxicon*, or poisons, and *metrikos*, or measures. One versed in etymology would thus have no difficulty with this work. "Predictive toxicometrics" can then be considered as determining "the effect of a range of doses (toxic and nontoxic) of a substance in representative groups of plants, animals, or man and, from the results, predict the effect of such doses on the human or animal or plant population."

In the introductory chapter, Boyd reviews very briefly the training required to undertake toxicometric studies. He also presents a list of general references, texts, and journals which fit into the field of toxicometrics. Several paragraphs are also directed at the history of predictive toxicometrics, while several pages deal with food and drug legislation, including both Canadian and U. S. laws. Boyd clearly identifies at the end of the first chapter the scope and direction of the book:

> *I shall confine my discussion of predictive toxicometrics to what I consider to be the essential or minimal amount of information which should be obtained by the predictive toxicologist. There is practically no limit to the total amount of data he could collect. I shall also confine my review to areas in which I have had practical experience.*

The remaining portions of the book are divided into three segments, with appropriate chapters under each segment. The first segment is called "Factorial Toxicometrics," with further subdivisions entitled "Host Factors," "Factors Associated with Drug Administration," and "Factors Associated with the Environment." A second segment is entitled "Uniposal Toxicometrics," which can be defined as the toxicity of a substance as a single dose (or a series of doses repeated at short intervals usually from several minutes to an hour). The last segment is called "Multiposal Predictive Toxicometrics" or the toxicity of a substance administered daily for varying periods of time.

Coming back to the first segment, "Factorial Toxicometrics," the author includes seven chapters under "Host Factors." These range from a discussion on species and strains of animals to idiosyncratic toxicity. In this same segment, but under the classification of "Factors Associated with Drug Administration," he includes chapters on physical and chemical properties of drugs, route of drug administration, simultaneous administration of other agents, and tolerance to repeated drug administration. The segment ends with a review on the effect the environment can play on altering toxicity of a substance. Environment here includes the presence of the same animals (as a group rather than single), other species (including man), and the physical environment. For example, the attention the animal care personnel direct to animals may have a dramatic effect upon the biological response. Both the uniposal and multiposal segments of the book are filled with very valuable information for the experimental toxicologist. Chapter 14, under uniposal considerations, presents an interesting account of the effect various solvents can play in altering toxic response. Unfortunately, in many toxicity studies, the investigators have not given sufficient attention to the solvent. Boyd suggests in this chapter that pilot studies employing one to five animals be used in order to save time before a full study is to be undertaken. Chapter 15 deals with clinical parameters which should be observed in toxicity studies. He stresses the importance of cage-side observation by a person who is knowledgeable in the normal behavior of the animals he or she is studying. Further emphasis is given to daily clinical measurements such as change in body weight, food intake, water intake, colonic temperature, urinary volume, and urinary analysis. Even though many toxicologists employ routinely hematology studies, Boyd feels that the information derived may be minimal in relation to the time required to perform the tests.

In Chapter 16, the author reviews uniposal autopsy parameters and describes these under four headings: (1) gross pathology, (2) histopathology, (3) organ weights, and (4) organ water levels. He pays close attention to many details which unfortunately are not generally followed by many toxicologists for one or more reasons. For instance, he shows by examples changes in water content of body organs of rats stored for 24 hr. under several different temperatures. Even though many toxicologists have not placed much emphasis on organ weights in toxicology studies, Boyd emphasizes that this measurement is extremely useful since it can be a very sensitive indicator of toxicity. Under the segment on "Multiposal Predictive Toxicometrics," specifically Chapter 19, Boyd presents the disadvantages of administering a substance in the diet if the agent, such as a drug, is to be taken orally on a daily basis. He prefers to administer the drug to animals by gastric lavage.

Other chapters appear in the last segment which deal with duration of daily dosing, analysis of clinical signs, analysis of autopsy signs, and multiposal toxicity syndromes.

One of the interesting aspects of this very valuable collection of toxicology information is that the author does not refer to such terms as "acute toxicity," and prefers the terms "uniposal" and "multiposal" to "chronic toxicity." He does not discuss his reasons for this in the book, but in no way does this detract from the book once the terms are understood.

If I were to find a fault with the book, it would be that the author has perhaps included too many details and data which may, at times, hinder a student of toxicology from following the theme the author wishes to emphasize. This fault is minor, however. This book should be of great value in particular to teachers presenting graduate courses in toxicology and to those toxicologists in the field who can always use a little help in refreshing their knowledge.

Reviewed by John Autian
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REVIEW ARTICLE

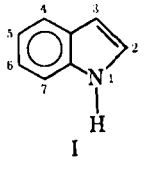
Influence of Ergot Alkaloids on Pituitary Prolactin and Prolactin-Dependent Processes

H. G. FLOSS▲, J. M. CASSADY, and J. E. ROBBERS

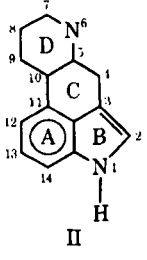
Keyphrases ☐ Ergot alkaloids—review of occurrence, structure, and general pharmacology, effect on pituitary prolactin, mammary tumors, and nidation inhibition ☐ Prolactin—review of secretion control and physiological activity, effect of ergot alkaloids on lactation and prolactin release, relationship to mammary tumors ☐ Pituitary hormone release—effect of ergot alkaloids on pituitary prolactin and prolactin-dependent processes, review ☐ Lactation and prolactin release—effect of ergot alkaloids, review ☐ Mammary tumors—relationship to prolactin, effect of ergot alkaloids, review ☐ Nidation—inhibition by ergot alkaloids, review

The ergot alkaloids occur in various species of claviceps including *Claviceps purpurea* (Fries) Tulasne (1, 2), other closely related fungi (3), and certain species of the Convolvulaceae, mainly in the genera ipomea, rivea, and argyreia (4).

The ergot alkaloids are all 1,4-substituted derivatives of indole (I), with the majority possessing the tetracyclic ring structure which has been designated ergoline (II).



I



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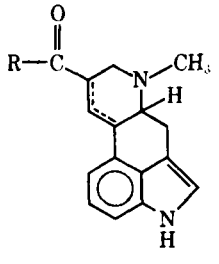
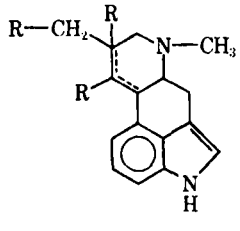


III

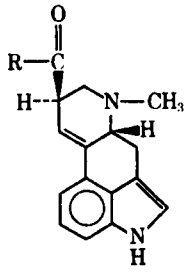


IV

The naturally occurring ergot alkaloids can be conveniently divided into two groups according to their chemical structure (1): the lysergic acid derivatives (III) and the clavines (IV). The clavines are substituted 6,8-dimethylergolines and include a few members, namely the chanoclavines, with a 6,7-*seco* D-ring.

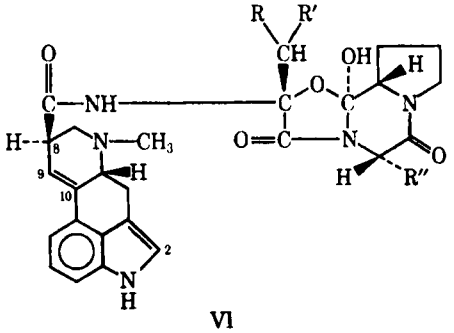
The lysergic acid derivatives can be further divided into the simple amide derivatives (V*a*–V*e*) and the cyclic peptides (VI*a*–VI*o*). All are derivatives of (+)-lysergic acid with the absolute configuration 5*R*,8*S* as shown. Stereoisomers at C-8 also exist in nature and are members of the (+)-isolysergic acid group.

The various clavines (VII*a*–VII*p*) are divided into three groups; $\Delta^{8,9}$, $\Delta^{9,10}$, and those containing a saturated D-ring. The tricyclic clavines, a relatively small group, consist of the isomeric chanoclavines and the interesting lactones, rugulovasine A and B (5).



V

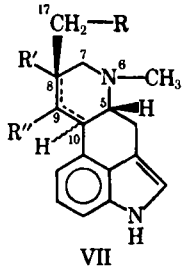
	R
V*a:* (+)-lysergic acid	—OH
V*b:* ergine	—NH_2
V*c:* (+)-lysergic acid α-hydroxyethylamide	—NH—CH(OH)—CH_3
V*d:* ergonovine (ergobasine, ergometrine)	—NH—CH(CH_2OH)—CH_3
V*e:* lysergic acid L-valine methyl ester	—NHCH($COOCH_3$)—$CH(CH_3)_2$



VI

	R	R′	R″
VI*a:* ergotamine	H	H	—CH_2—C_6H_5
VI*b:* ergotaminine*	H	H	—CH_2—C_6H_5
VI*c:* ergosine	H	H	—CH_2—$CH(CH_3)_2$
VI*d:* ergosinine*	H	H	—CH_2—$CH(CH_3)_2$
VI*e:* ergocristine	CH_3	CH_3	—CH_2—C_6H_5
VI*f:* ergocristinine*	CH_3	CH_3	—CH_2—C_6H_5
VI*g:* α-ergokryptine	CH_3	CH_3	—CH_2—$CH(CH_3)_2$
VI*h:* α-ergokryptinine*	CH_3	CH_3	—CH_2—$CH(CH_3)_2$
VI*i:* β-ergokryptine	CH_3	CH_3	—$CH(CH_3)CH_2CH_3$
VI*j:* β-ergokryptinine*	CH_3	CH_3	—$CH(CH_3)CH_2CH_3$
VI*k:* ergocornine	CH_3	CH_3	—$CH(CH_3)_2$
VI*l:* ergocorninine*	CH_3	CH_3	—$CH(CH_3)_2$
VI*m:* ergostine	H	CH_3	—CH_2—C_6H_5
VI*n:* ergostinine*	H	CH_3	—CH_2—C_6H_5
VI*o:* ergovaline	H	H	—$CH(CH_3)_2$

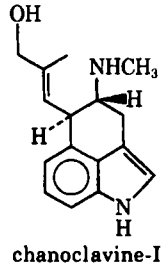
* epimeric at C-8; isolysergic acid series.



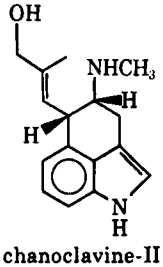
VII

	R	R′	R″
Δ^{8,9}-ergolenes			
VII*a:* agroclavine [10(*R*)]	H	—	H
VII*b:* elymoclavine [10(*R*)]	OH	—	H
VII*c:* molliclavine	OH	—	OH
Δ^{9,10}-ergolenes			
VII*d:* lysergene (Δ^{8,17} and Δ^{9,10})	—	—	H
VII*e:* lysergine	H	H	H
VII*f:* isolysergine*	H	H	H
VII*g:* lysergol	OH	H	H
VII*h:* isolysergol*	OH	H	H
VII*i:* setoclavine	H	OH	H
VII*j:* isosetoclavine*	H	OH	H
VII*k:* penniclavine	OH	OH	H
VII*l:* isopenniclavine*	OH	OH	H
ergolines (D-ring saturated)			
VII*m:* festuclavine [10(*R*)]	H	H	H
VII*n:* pyroclavine* [10(*R*)]	H	H	H
VII*o:* costaclavine [10(*S*)]	H	H	H
VII*p:* fumigaclavine A	H	H	CH_3COO—

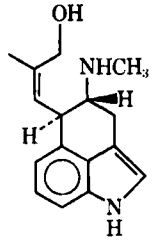
* epimeric at C-8, C-17 is α.



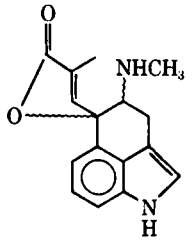
chanoclavine-I



chanoclavine-II (and enantiomer)



isochanoclavine-I



rugulovasine A and B

naturally occurring tricyclic clavines

GENERAL PHARMACOLOGY

The pharmacology of ergot and its alkaloids finds its beginnings in the myriad historical accounts from the Middle Ages of ergot toxicity in man and animals. This toxicity, called ergotism, was due to the ingestion of the grain from rye or products from the grain that had been infected with the ergot fungus, *Claviceps purpurea*. From the accounts of ergotism reviewed by Barger (6) and Bové (7), it is apparent that during the Middle Ages there were two types of ergotism in Europe. The form of ergotism referred to as St. Anthony's fire or ignis sacer was common west of the Rhine, particularly in France, where vasoconstriction of the peripheral blood vessels resulted first in intense burning pain in the extremities, with gangrene eventually setting in. East of the Rhine the poisoning usually resulted in CNS effects, with the most prominent symptom being convulsions. The basis for the occurrence of these two general types of toxic manifestations was attributed by Barger (6) to differ-

ences in diet, principally a deficiency in vitamin A being a factor in convulsive ergotism. However, the difference in toxic manifestations still remains one of the mysteries of history which has never been adequately explained.

What prompts the initial use of a toxic material as a drug is difficult to determine; however, in 1582, Adam Lonicer mentions in his Kreuterbuch the use of ergot to induce childbirth. For centuries it was used by midwives and physicians for this purpose until, in 1808, ergot moved from folkloric medicine to official status when an account of its use to quicken childbirth appeared in the *Medical Repository of New York*. Since this introduction into established medicine, the pharmacological effects of ergot and its alkaloids have been extensively documented (8, 9). The effects which have given ergot importance in medicine are related to its ability to stimulate both vascular and nonvascular smooth muscle. The effect apparently is direct, although the mechanism of action is not known. All natural alkaloids of the lysergic acid type have qualitatively the same effects on smooth muscle, but they exhibit differences in potency.

Stimulation of the vascular smooth muscle causes constriction of the blood vessels in the vascular bed, and the resulting constriction of intracranial arteries is useful in the treatment of migraine. The intensity of pain in migraine headache has been related to the amplitude of pulsations of the cranial arteries, chiefly the branches of the external carotid. Relief of headache by ergotamine is attributed to a reduction in amplitude of cranial artery pulsations because of vasoconstriction. The sensitivity of the smooth muscle of the uterus to stimulation by ergot increases along with the stage of gestation, so the resulting contractions at the end of the third trimester may induce labor. The principal use of the ergot alkaloids, however, involves their administration postpartum to prevent hemorrhage. For this purpose, ergonovine and its semisynthetic derivative, methylergonovine, are the drugs of choice. They are more potent, may be administered orally, have a more rapid onset of action, and are less toxic than ergotamine.

Mention should also be made of the pharmacological effects that have minor importance in current therapeutics. One of these effects is α-adrenergic blockade, which was reviewed by Nickerson (9) and Nickerson and Hollenberg (10). The ergot alkaloids were the first adrenergic blocking agents discovered, and only those alkaloids with a peptide side chain have this activity. The 9,10-dihydro derivatives of ergotamine and ergotoxine, which is a mixture of ergocornine, ergocristine, and ergokryptine[1], are the most potent of these blocking agents; consequently, they have been used to control hypertension and in the treatment of peripheral vascular diseases. Unfortunately, at doses where these compounds are effective, the side effects which include nausea and vomiting and, occasionally, headache, dizziness, blurring of vision, rashes, anorexia, nasal stuffiness, and abdominal cramps are usually too great to allow for their general use in therapeutics.

[1] Many authors in the English language, including *Chemical Abstracts*, have used the spelling "ergocryptine," apparently in analogy to ergocornine and ergocristine. However, the ending -kryptine is derived from the Greek "kryptos" = hidden; therefore, ergokryptine is the correct spelling.

An additional neurohormonal effect exhibited by the alkaloids of ergot is serotonin antagonism. Takeo (11) reported that the clavine-type alkaloids including agroclavine, elymoclavine, and their dihydro derivatives had moderate antiserotonin activity *in vitro* and *in vivo*. In later work, Yui and Takeo (12) found an increase in antiserotonin activity by 1-methylation of the clavine-type alkaloids, and Fanchamps *et al.* (13) showed that 1-methylation of several lysergic acid derivatives resulted in an increased antiserotonin activity over their original compounds. Consequently, it is not surprising that 1-methyl-methylergonovine, methysergide, is the most potent serotonin antagonist of the ergot compounds. Methysergide is effective in preventing all types of migraine headache (common, classic, and cluster), but it is of no value in treating an acute attack of migraine. It has been suggested (14) that serotonin, together with other humoral agents such as noradrenalin, exerts a tonic vasoconstriction effect on cranial arteries. Consequently, the prophylactic effect of methysergide in migraine is probably due to the potentiation of serotonin vasoconstriction rather than serotonin antagonism. Serotonin antagonism has been implicated in a rather simplistic explanation of the psychotomimetic activity of lysergic acid diethylamide, a semisynthetic derivative of lysergic acid (15). While it is now apparent that the mechanism behind psychotomimetic activity cannot be explained in such a simple fashion, it is noteworthy that there is a structural similarity between serotonin and psychotomimetic agents such as lysergic acid diethylamide, psilocybin, and nitrogen-substituted tryptamines.

A new phase of ergot pharmacology was initiated in 1954 when Shelesnyak (16) reported that the systemic injection of ergotoxine into rats inhibited deciduoma formation. Since that time, Carlsen *et al.* (17) demonstrated that a single subcutaneous injection of ergocornine methanesulfonate terminated early pregnancy in mice, and Zeilmaker and Carlsen (18) found that ergocornine administered to lactating rats temporarily inhibited milk production, the effect being prevented by treatment with luteotropic hormone (prolactin). Lactation inhibition by ergot was first reported in 1676, when Dodart noted that agalactia occurred among nursing women suffering from gangrenous ergotism (6). By far the most exciting aspects of this new phase of ergot pharmacology are the reports by several groups that ergocornine and ergokryptine induced regression of carcinogen-induced mammary adenocarcinomas in rats (*e.g.*, 19–21) and inhibited the growth of spontaneous mammary tumors which appear in the postreproductive phase of life of female rats (22). All these effects of the ergot alkaloids can be related to an inhibition of prolactin secretion from the anterior pituitary.

PROLACTIN AND ITS CONTROL

Prolactin is a peptide hormone secreted by the adenohypophysis. It is generally assumed that a prolactin is present in the pituitary of all vertebrate species, and Bern and Nicoll (23) reviewed the comparative endocrinology of prolactin. The prolactins and the pituitary growth hormones comprise groups of protein hormones with similar structural features, homologous amino

acid sequences, and, in some species, overlapping biological properties, even though there is considerable species specificity within each group.

Prolactin has been isolated and purified from the pituitary glands of a number of species of mammals including the sheep, ox, pig, and rat. Li *et al.* (24) reported the complete amino acid sequence of ovine prolactin, and Seavey and Lewis (25) determined that bovine prolactin differed from ovine prolactin by only two amino acids in the peptide map. On the other hand, the identification and isolation of prolactin as a protein distinct from growth hormone in primates have been difficult to achieve. This difficulty, coupled with the fact that highly purified human growth hormone possesses substantial prolactin activity (26, 27), raised some doubt as to the existence of prolactin as a separate entity in man.

Because of the difficulties encountered in the isolation and purification of prolactin, studies on the physiological role of prolactin, which require quantitation of the hormone, have had to rely on bioassays. The most practical of these assays in terms of efficiency and sensitivity is the pigeon crop-sac assay. Columbiforme birds, such as pigeons and doves, secrete a special fluid into their crop which they use to feed their young. This secretion, called pigeon's milk, is under the control of prolactin, since injections of this hormone stimulate growth of the special cells involved in this process. The essential features of this assay, which has undergone various modifications since Lyons (28) first described it in 1937, are that a standard amount of prolactin is injected on one side of the crop gland while the material to be assayed is injected on the other side. In some cases, different birds are used for the control than for the unknown. A ratio of the degree of proliferation of crop cells of the control to the unknown is determined from a standard curve (29).

The recent development of radioimmunoassays has made it possible to measure blood levels of prolactin for the first time. These assays generally involve a double antibody method. Prolactin obtained from the animal species to be assayed is injected into another species of animal to prepare antiserum against the prolactin. Antiserum to the immunoglobulin G of the animal which manufactures the prolactin antiserum is prepared by using a different species of animal. Highly purified prolactin from the animal species to be assayed is radioactively tagged by treating it with radioactive iodine, *e.g.*, ^{131}I. Blood serum from the animal to be assayed for prolactin levels is incubated with prolactin antiserum and radioiodinated prolactin. The nonlabeled hormone from the test animal competes with the ^{131}I-labeled hormone for the antibody so that the ratio of bound to free labeled hormone decreases. When enough time has elapsed for maximum binding, immunoglobulin G antiserum is added to precipitate the prolactin–immunoglobulin G complex, which is then assayed for radioactivity. Various adsorbents such as charcoal and cellulose can also be used for this separation in place of the second antibody. Recent publications that described radioimmunoassays for prolactin are those of Niswender *et al.* (30), Johke (31), Neill and Reichert (32), Davis *et al.* (33), Hwang *et al.* (34), Raud and Odell (35), and McNeilly (36).

Recently, Frantz *et al.* (37) reviewed their previous works in which they determined unequivocally the presence of human prolactin separate from human growth hormone in plasma. These workers used an *in vitro* mammary gland culture system from a midpregnant mouse breast tissue and found high bioassayable prolactin activity in human plasma samples that had low immunoassayable growth hormone. They then demonstrated that antihuman growth hormone antiserum was incapable of neutralizing prolactin activity, whereas it completely neutralized the prolactin activity of growth hormone itself.

The isolation and purification of human prolactin in sufficient quantities for chemical and biological characterization are well on the way to being achieved. Guyda and Friesen (38) separated monkey prolactin from monkey growth hormone using affinity chromatography. Sepharose-coupled antibodies to human placental lactogen were able to remove more than 99% of monkey growth hormone present in incubation media or tissue homogenates of monkey pituitaries. The potency of prolactin in these fractions, however, remained unaltered or even increased after the immunoadsorption of monkey growth hormone.

Chrambach *et al.* (39), using polyacrylamide gel electrophoresis, identified human prolactin and determined several of its physical characteristics. They found that biologically active serum human prolactin is a single, homogeneous protein, which is distinct from the human growth hormone species hGH-β. Human prolactin is more highly charged than growth hormone but has the same molecular size. Lewis *et al.* (40) isolated human prolactin from pooled human pituitary glands (nearly 500 were used), using a combination of Sephadex chromatography and DEAE–cellulose chromatography. Their product had a potency of 22 I.U./mg. in the pigeon crop-sac assay for prolactin but only 0.4 USP unit/mg. in the tibial line assay for growth hormone. The product was less electronegative than growth hormone at pH 9.5 and had a molecular weight of about 22,000. Most recently, Hwang *et al.* (41), after gel filtration and ion-exchange chromatography of a discard fraction obtained in human growth hormone purification, obtained a preparation with 30.5 I.U./mg. of prolactin activity in an *in vitro* mouse mammary tissue culture assay. Human growth hormone contamination was less than 0.5% by radioimmunoassay. The authors claimed that the method of purification can easily be scaled up for larger batches of material so that prolactin of sufficient quantity and purity can be obtained for the determination of the primary structure of the molecule.

Since it is not within the scope of this article to provide references to the specific experimental literature documenting the current concept of prolactin action in the body, the reader is referred to the extensive review papers of Meites and Nicoll (42) and Meites *et al.* (43). It is the purpose to limit this discussion to the salient features of prolactin activity, with emphasis on the control of prolactin secretion.

Regulation of prolactin secretion from the adenohypophysis, as with the other anterior pituitary hormones,

is mediated in various ways through the hypothalamus. Prolactin secretion is unique in that it appears to involve hypothalamic inhibition, hypothalamic stimulation, and prolactin feedback control. There is evidence that the hypothalamus continually inhibits prolactin secretion by the adenohypophysis. This is exactly opposite to most pituitary hormones whose release is stimulated by hypothalamic secretions. It is presumed that the hypothalamus is the source of a continuously secreted substance that inhibits prolactin synthesis as well as release. Meites and coworkers (42, 43) called this hormone prolactin-inhibiting factor, PIF. Everett (44) demonstrated that pituitary transplants in hypophysectomized rats could secrete prolactin for months, as evidenced by maintenance of luteal function, and transplantation studies in certain teleosts and amphibians showed that their anterior pituitary can secrete prolactin autonomously while the secretion of other hormones is decreased (23).

Bilateral lesions placed in the median eminence of intact female rats, which destroyed the connection between the hypothalamus and the anterior pituitary, have resulted in a rapid increase in serum prolactin levels in rats (45, 46). This effect is similar to that seen after pituitary stalk section (47*a*). In this regard, Turkington *et al.* (47*b*) demonstrated elevated serum prolactin levels in patients subjected to surgical section of the pituitary stalk for metastatic breast cancer or for diabetic retinopathy. In tissue culture of human fetal pituitaries, Pasteels (48) showed that the secretion of prolactin increased with the length of the culture period; however, if crude hypothalamic extracts were added, there was a significant decrease in the amount secreted. At the present time the chemical structure of prolactin-inhibiting factor is not known. It may be a small polypeptide by analogy with the structures of the various hypothalamic-releasing factors. Experimental determination of prolactin-inhibiting factor activity must rely on bioassays, and Nicoll *et al.* (49) reviewed the several *in vitro* and *in vivo* assays employed.

To complicate the picture of prolactin secretion control, Nicoll *et al.* (49) discussed the evidence indicating that the hypothalamus may also secrete a prolactin-releasing factor (PRF). Early studies indicated that the avian hypothalamus exerts a stimulatory effect, rather than an inhibitory effect as in mammals, on prolactin release. Kragt and Meites (50) showed that an extract of pigeon hypothalamus stimulates prolactin secretion by the pigeon pituitary *in vitro*.

It is now apparent that prolactin-releasing factor is present in mammals. Nicoll *et al.* (49) presented evidence that there is both prolactin-inhibiting activity and prolactin-stimulating activity in rat hypothalamic extract when incubated with rat pituitary tissue. Tashjian *et al.* (51) obtained direct evidence that thyrotropin-releasing hormone stimulated the production of prolactin and inhibited growth hormone production in rat pituitary cell cultures. In this regard, Bowers *et al.* (52*a*), using a radioimmunoassay, demonstrated that synthetic pyroglutamyl-histidyl-prolinamide (thyrotropin-releasing hormone) stimulated the secretion of prolactin as well as thyrotropin in normal men and women. Tyson *et al.* (52*b*) found that synthetic thyrotropin-releasing hormone administered to parturient, lactating women and to women with normal menstrual cycles provoked a maximal rise in prolactin levels 15 min. after injection, with a return to preinjection levels within 60 min. Valverde-R. *et al.* (52*c*) presented evidence that there is a prolactin-releasing hormone distinct from thyrotropin-releasing hormone. By using Sephadex G-10 chromatography and testing in male rats previously sensitized with estrogen and progesterone, they were able to separate from a porcine hypothalamic extract a fraction with prolactin-releasing activity that was different from thyrotropin-releasing hormone.

The final aspect of prolactin secretion to be discussed is the matter of feedback control. MacLeod *et al.* (53) transplanted prolactin-secreting pituitary tumors into intact rats and found a decrease in pituitary prolactin content. Chen *et al.* (54) in the same type of experiment also found an increase in hypothalamic prolactin-inhibiting factor activity. Also, implantation of prolactin into the median eminence of rats was demonstrated by Clemens and Meites (55) to cause a decrease in pituitary and serum prolactin levels as well as an increase in prolactin-inhibiting factor activity. These investigations lend support to the postulation that prolactin inhibits its own secretion by the pituitary gland through influencing the hypothalamus to secrete more prolactin-inhibiting factor. Hökfelt and Fuxe (56) recently established that the tubero-infundibular dopamine neurons are involved in the regulation of prolactin secretion. These authors postulated that the tubero-infundibular dopamine neurons stimulate the release and/or synthesis of prolactin-inhibiting factor at the level of the median eminence and that they are a link in a feedback system by which serum prolactin levels influence prolactin-inhibiting factor secretion. Supporting data for this hypothesis come from the work of Lu and Meites (57), who found that L-dopa and various MAO inhibitors increased catecholamic activity in the hypothalamus as well as hypothalamic prolactin-inhibiting factor activity.

The physiological effect of prolactin on female mammals is complex, and many differences in effect are found between species (43). The principal functions of prolactin, however, are in the initiation and maintenance of lactation and in promoting the growth of the mammary glands. In rats, prolactin has an additional importance in inducing luteolysis of the previous corpora lutea during each estrus cycle and helping maintain corpora lutea together with luteinizing hormone during pseudopregnancy and early pregnancy.

In studies on the pregnant female rat, Voogt *et al.* (58*a*) showed that serum prolactin (except for the first 3 days), ACTH, and blood corticosterone levels are low during gestation, although Butcher *et al.* (58*b*) showed a semicircadian rhythm in plasma levels of prolactin during early gestation in the rat. The absence of lactation during pregnancy is believed to be due to an insufficiency of adrenal cortical hormones or prolactin or both. At the time of parturition there is an increase in blood prolactin, ACTH, and corticosterone and these are thought to initiate lactation. To develop this idea further, it is important to note that estrogen is a potent stimulator of prolactin release in mammals. Apparently estrogen can directly stimulate the anterior pituitary to secrete prolactin without intervention of the hypothala-

mus (59). Therefore, the low serum prolactin in the rat during pregnancy is probably associated with low estrogen secretion during gestation (60) and perhaps with the ability of progesterone to prevent the stimulating action of estrogen on prolactin secretion. Also significant is that an increase in pituitary prolactin content at the time of parturition was observed in humans by Frantz and Kleinberg (61). This effect may be due to a decrease in progesterone levels and an increase in estrogen level.

A definite stimulus for prolactin secretion during postpartum lactation is suckling. Ratner and Meites (62*a*) showed that stimulation of the numerous sensory nerves in the nipples and surrounding skin evokes a reflex release of prolactin from the pituitary by depressing prolactin-inhibiting factor activity. Johke (62*b*) reported that in lactating cows plasma prolactin levels rapidly increased and reached a peak 4–20 min. after the start of milking; in lactating goats, milking caused a 40–100-fold increase in prolactin release. Hwang *et al.* (34) demonstrated that serum prolactin increased by 10–20-fold 30 min. after the start of breast feeding in postpartum humans.

Prolactin is clearly one of the major hormones involved in mammary growth along with somatotropic hormone (growth hormone), and practically the entire endocrine system has some regulatory influence on the mammary gland. The ovarian hormones, interestingly, have little effect on mammary growth in the absence of the pituitary gland (43). This observation suggests that the ovarian hormones act in part *via* the pituitary, and it has been postulated that the ovarian hormones sensitize the mammary gland to the effects of prolactin and somatotropin (42).

INFLUENCE OF ERGOT ALKALOIDS ON LACTATION AND PROLACTIN RELEASE

As mentioned earlier, the knowledge that ergot alkaloids can inhibit lactation dates back several hundred years to Dodart, who noted that agalactia could be observed among nursing women suffering from gangrenous ergotism (6). This knowledge was reinforced repeatedly in the following centuries and its potential clinical usefulness was discussed (*cf.*, 63, 64). Animal work similarly indicated suppression of lactation by ingestion of crude ergot sclerotia or by some of the constituent alkaloids (65–71). Such studies have involved the effect in rats, mice, guinea pigs, dogs, sows, and cows. One of the first systematic studies was that of Sommer and Buchanan (69) in albino rats. They showed that 0.5–1.0 mg./kg. of several ergot alkaloids given twice daily during the second half of pregnancy significantly reduced the rate of weight gain of the litter after birth. Ergotamine was most effective in this respect, followed by ergotoxine, whereas dihydroergotoxine and methylergonovine showed little activity. Later studies showed the same effect in mice who were kept on a diet containing the clavine alkaloid, agroclavine (71). At the lowest dose tested, about 15 mg./kg. daily until 3–8 days before parturition, all animals completely failed to raise their litter. In addition, an almost complete inhibition of the hypertrophy of mammary tissue during pregnancy and lactation was observed (71), as was also noted by Sommer (72). A recent study in cows showed a slightly more differentiated picture. Administration of the prolactin inhibitor, 2-bromo-α-ergokryptine[2], to lactating cows, while drastically reducing the serum prolactin levels, had only little effect on the milk yield (73). However, treatment immediately before parturition effectively inhibited the onset of lactation (74).

An understanding of the mechanism of lactation inhibition by ergot alkaloids came about only gradually and it can, even today, not be said to be complete. While there were some indications in the literature of effects of ergotoxine on the pituitary gland (75, 76), early workers apparently did not suspect this to be the site through which the ergot effect was mediated. A "lack of normal maternal instincts" (67, 68), inadequate development of the mammary glands (65), and reduced food intake by the mothers (77) were suggested as the causes for impaired lactation following administration of ergot alkaloids. Grosvenor (78) found that daily administration of ergotamine during lactation interfered with the milk-ejection reflex in rats and later showed this to be due to blocking of the release of oxytocin in response to suckling (79). Zeilmaker and Carlsen (18) apparently were the first to establish a relationship between the inhibition of prolactin release from the pituitary and the inhibition of lactation by ergot alkaloids. They found that in rats the lactation inhibition caused by a single injection of 1 mg. ergocornine given after parturition could be overcome by simultaneously administering prolactin.

The picture of these relationships has since been considerably refined, particularly by a recent study by Shaar and Clemens (80) which correlates the effect of a number of ergot alkaloids on serum prolactin levels, as measured by radioimmunoassay, with parameters like litter weight gain, body weight gain of mothers, and mammary tissue weight. The authors concluded that the inhibition of lactation is at least partly due to the suppression of prolactin release. The reduction in the body weight of the mothers is almost entirely accounted for by the reduction in mammary weight. Whether other factors, like the inhibition of milk ejection, contribute to the effect on lactation is not entirely clear. It seems likely that for some alkaloids such other factors contribute significantly to their overall effect on lactation, whereas with other alkaloids the effect on prolactin levels may be the main mode of action. Several pieces of circumstantial evidence support this view. For example, in the work of Shaar and Clemens (80) the ability of several alkaloids to decrease the weight gain of litter did not strictly parallel their effect on serum prolactin levels. A comparison of the ability of various alkaloids to inhibit lactation (69) with their activity as inhibitors of nidation (81) (see below) also points in that direction. For example, ergotamine is a good lactation inhibitor but has little activity in inhibiting nidation. Ergotoxine, on the other hand, is much less active in lactation inhibition but is very effective as a nidation inhibitor. This was studied systematically by Flückiger and Wagner (82) in a comparison of ergokryptine and 2-bromo-α-ergokryptine as lactation and nidation inhibitors. They found that the dose–response curves for lactation and nidation inhibition by ergokryptine converged toward one dose which com-

[2] CB 154, Sandoz.

pletely blocked both effects, suggesting a common primary effect. The same is true for ergocornine but not for 2-bromo-α-ergokryptine. Therefore, the latter must act in a somewhat different way than the other two. Whether the difference lies in a specific antifertility effect of the bromo derivative, as the authors suggested, or whether these compounds have different effects on oxytocin release, as suggested by Nicoll *et al.* (83), remains to be established. Another possibility is that differences in the pharmacokinetic properties could well account for the differences in the quantitative effectiveness of various ergot alkaloids in lactation and nidation inhibition. Finally, it should be mentioned that Edwardson carried out experiments in rats with cannulated mammary ducts and found that the alkaloid agroclavine did not inhibit the rise in intramammary pressure produced by oxytocin (84). Therefore, inhibition of the milk-ejection response can probably be excluded as a major factor in the mode of action of this alkaloid.

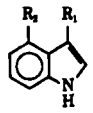
Most of the efforts in recent years have been directed toward understanding the mode in which ergot alkaloids influence prolactin levels. The work of Zeilmaker and Carlsen (18) with hypophysectomized rats bearing an autotransplanted pituitary under the kidney capsule showed that ergocornine still counteracts the prolactin effects, and thus presumably inhibits prolactin release, when the hypothalamic–pituitary connection is interrupted. As discussed later, their conclusion that ergot alkaloids inhibit prolactin release by direct action on the pituitary has been repeatedly challenged by other workers who felt that the action on the pituitary is mediated through the hypothalamus. More recent work seems to indicate that both sites may be involved. Wuttke *et al.* (85) found that pituitaries incubated *in vitro* with hypothalamic extract from ergocornine-treated rats released less prolactin and luteinizing hormone (LH) than ones incubated with hypothalamic extract from untreated animals. Ergocornine treatment, therefore, seems to increase the hypothalamic concentration of prolactin-inhibiting factor and decrease that of luteinizing hormone releasing hormone (LH-RH), suggesting an action *via* the hypothalamus. On the other hand, there is also overwhelming evidence for a direct action of ergot alkaloids on the pituitary.

Additional experiments with hypophysectomized, pituitary-grafted animals confirmed Zeilmaker and Carlsen's (18) earlier findings, both by following the influence of ergocornine on prolactin-dependent phenomena, *e.g.*, the luteolysis of nonfunctional corpora lutea (86), and by directly measuring serum prolactin levels using radioimmunoassay techniques (59, 80). Further *in vivo* evidence for a direct action of ergot alkaloids on the pituitary was obtained in rats in which the vascular continuity between the hypothalamus and the pituitary had been eliminated by median eminence lesions (87). The increase in serum prolactin levels resulting from the removal of the control of pituitary prolactin release by prolactin-inhibiting factor could be prevented by ergocornine but not by ovariectomy. *In vitro* studies with explanted rat (59, 83, 88) and human (89) hypophyses in culture fully confirmed these findings. Ergocornine (59, 88, 89), 2-bromo-α-ergokryptine (89), and ergotamine (83) all significantly inhibited prolactin release at concentrations in the range of 1–20 mcg./ml. No effect of ergotamine on the secretion of growth hormone was detected (83). Prolactin secretion can be restored by washing out the ergot alkaloid, indicating that the effect is not due to cellular destruction (89). According to Pasteels *et al.* (89), preliminary electron microscopic evidence suggests a direct action on prolactin cells to inhibit the excretion of secretory granules. Ergot alkaloids also inhibit prolactin release and prolactin formation in cultures of pituitary tumor cells without affecting the levels of growth hormone (90). Finally, a recent study indicates that ergot alkaloids also inhibit pituitary prolactin release in certain species of fish (91) in which prolactin is required to maintain plasma sodium levels when the animal is in fresh water. In this case the eta cells of the pituitary, which most likely are the source of prolactin, seemed to be inactivated as a result of administration of 2-bromo-α-ergokryptine.

Only a limited amount of information is available in the literature on relationships of the structure of ergot alkaloids and their prolactin-inhibitory or lactation-inhibitory activity. Particularly, quantitative information on structure–activity relationships is almost completely missing. Compounds that have been reported to have activity as lactation inhibitors include ergotamine (69, 80), ergotoxine (69), ergocornine (18, 80), ergokryptine (80, 82), dihydroergotoxine (weak) (69), dihydroergocornine (80), methylergonovine (weak) (69), ergonovine (weak) (80), 2-bromo-α-ergokryptine (82), agroclavine (71), and D-6-methyl-8-ergolinylacetamide (92). Activity toward inhibiting prolactin release *in vivo* or *in vitro* has been reported for ergocornine (59, 80, 85, 87–89), 2-bromo-α-ergokryptine (73, 74, 89), ergotamine (80, 83), lysergic acid diethylamide (93), ergokryptine (80), dihydroergocornine (80), and ergonovine (80). Also active are agroclavine and elymoclavine (94–96).

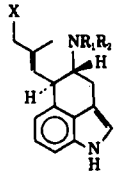
In a collaborative study (97) aimed at defining the minimal structural requirements for prolactin inhibition, the activity of a variety of simple ergolines and some of their analogs and derivatives was determined. The assay involved injecting 10 mcg. of the compound into reserpinized male rats (average body weight ~200 g.), which were killed 2 hr. later and assayed for their serum prolactin level using the rat prolactin radioimmunoassay kit (NIAMD). In each group of tests, ergocornine was included as a standard and the observed values were normalized according to the inhibition by ergocornine. The data, grouped according to structural types, are summarized in Table I. The figures in parentheses give the prolactin-inhibitory activity relative to ergocornine = 100. The actual inhibitions observed with ergocornine under these conditions ranged from 58 to 75%. Compounds having relative activities of less than 50 were considered marginally active or inactive. It can be seen that simple 3,4-substituted indoles, even if they have essentially all the carbon atoms of the ergoline system, as well as tricyclic clavines are completely inactive. Apparently, the complete tetracyclic ergoline ring system is required for activity. Quaternization of the nitrogen atom 6 completely abolishes activity, possibly because of reduced permeability. The double bond in the 8,9-position is not absolutely essential for activity, but shifting it to the 9,10-position reduces activity. A methyl

Table I—Ergolines and Related Compounds as Inhibitors of Prolactin Release



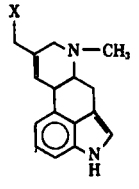
I: Substituted indoles

Compound	Activity
1. 1-(3-Indolyl)-2-amino-5-methyl-4-hexene acetate R_1 = —CH_2—CH(—$NH_3^+CH_3COO^-$)—CH_2—CH=$C(CH_3)_2$ R_2 = H	(−)
2. 4-(γ,γ-Dimethylallyl)-skatylformamidomalonic acid diethyl ester R_1 = —CH_2—C(—$CO_2CH_2CH_3$)—NHCHO—$CO_2CH_2CH_3$ R_2 = —CH_2—CH=$C(CH_3)_2$	(−)
3. 4-(γ,γ-Dimethylallyl)gramine R_1 = —CH_2—$N(CH_3)_2$ R_2 = —CH_2—CH=$C(CH_3)_2$	(−)
4. 4-(γ,γ-Dimethylallyl)tryptophan R_1 = —CH_2—CH(—NH_3)—COOH R_2 = —CH_2—CH=$C(CH_3)_2$	(−)



II: Clavines, tricyclic

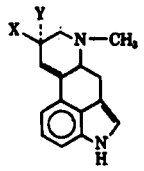
Compound	Activity
1. Chanoclavine-I R_1 = H, R_2 = CH_3, X = OH	(−)
2. *N*-Methylchanoclavine-I R_1 = R_2 = CH_3, X = OH	(−)
3. *N*-Methyldesoxychanoclavine-I R_1 = R_2 = CH_3, X = H	(−)



III: Clavines, tetracyclic, $\Delta^{8,9}$

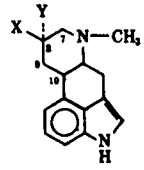
Compound	Activity
1. Agroclavine X = H	(+)
2. Agroclavine methiodide	(−)
3. Elymoclavine X = OH	(+)
4. Dihydroelymoclavine	(+) (82)
5. Elymoclavine-*O*-acetate	(+) (80)
6. Elymoclavine-17-carbamate	(+) (65)
7. Elymoclavine-17-benzoate	(+) (65)
8. Elymoclavine-β-D-fructoside	(+) (60)
9. Elymoclavine-*N*-oxide	(−)
10. Elymoclavine methiodide	(−)

Table I—(*Continued*)



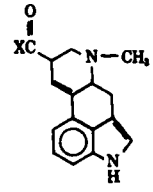
IV: Clavines, tetracyclic, $\Delta^{9,10}$

Compound	Activity
1. Lysergine X = CH_3, Y = H	(−) (24)
2. Isolysergine X = H, Y = CH_3	(−)
3. Isolysergol X = H, Y = CH_2OH	(+) (52)
4. Lysergene X, Y = CH_3	(−)
5. 6-Methyl-8-acetoxymethylene-9-ergolene X, Y = CHOCOCH$_3$	(+) (48)



V: Clavines, tetracyclic

Compound	Activity
1. 6-Methyl-8-aminoergoline	(+) (52)
2. Dihydrolysergic acid lactam X, Y = CH_2, 7-keto	(−)
3. Dihydrolysergic acid dihydrolactam X = CH_3, Y = H, 7-keto	(−)
4. Dihydroelymoclavine X = CH_2OH, Y = H	(+) (82)



VI: Lysergic acid derivatives

Compound	Activity
1. Ergocornine	(+) (100) control
2. Lysergic acid α-hydroxyethylamide X = —NHCH(CH_3)OH	(+) (74)
3. Dihydrolysergic acid azide·HCl X = N_3	(−)

group seems to be the minimal size substituent in the 8-position for activity, and a somewhat larger substituent seems to be advantageous particularly with a $\Delta^{9,10}$ double bond. A keto function in the 7-position completely eliminates activity. This defines some of the structural elements needed for activity, and further studies are underway to refine this picture.

Since the systematic study of lactation and prolactin inhibition by ergot alkaloids is only relatively recent, few reports of clinical trials or applications are found in the literature. Various ergot alkaloids have been repeatedly and successfully used in the treatment and prevention of puerperal mastitis in humans (*e.g.*, 98, 99) or of milk-fever (agalactia toxemia) in sows (100).

Their usefulness in these disorders has usually been ascribed to their sympatholytic and spasmolytic activity, but the possibility that their lactation-inhibitory effect may be a contributing factor certainly deserves consideration. A recent study reports experiences with 2-bromo-α-ergokryptine in the treatment of nonpuerperal galactorrhoea in three women. In all three cases, the galactorrhoea was terminated (101*a*). Even more recently, the same group (101*b*) extended this study to 14 women with galactorrhoea with good results. In a double-blind trial, they found 2-bromo-α-ergokryptine to be as effective as diethylstilbestrol in terminating lactation in women and they reported that they now use the compound commonly for that purpose (in 60 successful treatments so far).

RELATIONSHIP BETWEEN PROLACTIN AND MAMMARY TUMORS

It has been established that mammary cancer in humans is hormone responsive and susceptible at least temporarily to treatment by removal of the ovaries, testes, adrenals, or pituitary or by administration of steroid hormones (102*a*,*b*,*c*). Pearson *et al.* (103) and Pearson and Ray (104) found that a pituitary factor is probably involved in the estrogenic stimulation of mammary tumor growth in humans, since after hypophysectomy-induced remission the administration of estrogen to the patient did not reactivate tumor growth, whereas it did following an oophorectomy-induced remission.

Turkington *et al.* (47*b*) studied the effect of pituitary stalk section on human serum prolactin levels in 11 patients with metastatic carcinoma of the breast. Seven patients showed substantial elevations in the serum prolactin activity while four showed no increase. Eight of these patients evidenced objective remissions for periods ranging from 7 months to 12 years. Five of these eight patients had elevated prolactin levels during this period. Among the three patients who showed no objective remission, two had elevated prolactin levels. The authors concluded that prolactin does not promote the growth of mammary carcinoma during remission but perhaps is involved in "reactivation" of the disease.

A number of studies have been carried out with model tumor systems in mice and rats. These studies have provided a reasonably clear picture of the factors involved in the induction and maintenance of mammary tumor growth in these animals (105–108).

A series of publications (109–114) has appeared which establish that hypophyseal isografts produce a stimulatory effect on the mammary glands of mice, leading to mammary tumors in mice carrying the mammary tumor virus. Mühlboch and Boot (115) extended this research and found that multiple subcutaneous hypophyseal isografts in most mammary tumor virus-free mice also induced tumor formation. These grafts were found to produce prolactin continuously, and development of the mammary tumors was ascribed at least in part to the action of prolactin along with progesterone and/or estrogen. Boot and coworkers, in a series of publications, reported the effects of site of implantation, age and sex of donor (116), size of the growth (117), and steroid hormones on the growth and thereby indirectly the amount of prolactin on the production of mammary tumors (118–121). As a result of these studies, Boot (106) concluded that prolactin is the hormone most directly involved in mammary tumor formation in mice. Yanai and Nagasawa (122) also found that prolactin continuously secreted by isografted pituitaries promoted the maintenance and growth of mammary hyperplastic nodules in adreno-ovariectomized mice. In a later study (123), these same workers reported that mammary tumors were produced in about 51% of the adreno-ovariectomized mice by placement of pituitary isografts, and they concluded that prolactin is the primary hormonal factor in spontaneous mammary tumorigenesis in mice.

Prolactin along with estrogen is also involved in the development and growth of mammary tumors in rats. Spontaneous mammary tumors occur in old female rats and are stimulated by administration of estrogen (124) and inhibited by ovariectomy (125). It has been established that estrogen can promote the synthesis and release of prolactin by action on both the hypothalamus and pituitary (42). The spontaneous mammary tumors, which occur generally as a single benign adenoma, differ considerably from the multiple adenocarcinomas induced by carcinogens. Early induction and increased incidence of the spontaneous mammary tumors occurred on grafting multiple pituitaries (126) or on placing lesions in the median eminence of the hypothalamus (46, 127). Both of these treatments have been shown to result in increased serum prolactin levels.

A somewhat different pattern has been established in the induction and maintenance of carcinogen-induced mammary adenocarcinomas in rats. Dao (107, 108) established that pregnancy and lactation inhibit induction of mammary cancer by the carcinogen 3-methylcholanthrene. Stimulation of an increase in prolactin levels before administration of 7,12-dimethylbenz[*a*]anthracene results in an inhibition of the development of 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors in the rat. Placement of lesions in the median eminence in rats prior to a single dose of 5 mg. of 7,12-dimethylbenz[*a*]anthracene significantly inhibited induction by the carcinogen (128, 129). Complete inhibition was effected if rats with bilateral lesions were also castrated. These results were subsequently confirmed by Klaiber *et al.* (130). Prolactin-secreting pituitary homografts (131), reserpine (132), or mestranol–norethynodrel[3] treatment (133) also inhibited tumor induction by 7,12-dimethylbenz[*a*]anthracene. This method of inhibition apparently involves the induction of mammary hyperplasia by prolactin prior to carcinogen administration. It appears that the functionally active mammary gland is protected from carcinogens.

Following the development of 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors, an increase in prolactin secretion results in a definite enhancement of tumor growth, in contrast to the situation prior to induction. At this stage, a decrease in prolactin secretion results in inhibition of mammary tumor growth. Thus, after tumor development placement of lesions in the

[3] Enovid.

median eminence (128, 129), grafting of multiple pituitaries (131) or treatment with reserpine (132) or mestranol–norethynodrel[3] (133) stimulated the growth of 7,12-dimethylbenz[*a*]anthracene-induced mammary cancers. Kim and Furth (134) and Kim *et al.* (135) also found that prolactin was important in promoting and maintaining the growth of mammary tumors in rats.

Pearson *et al.* (105) used rats bearing 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors to show that the development of such tumors was dependent on the presence of prolactin in the serum. Their data suggested that prolactin may be the only hormone of significance in maintaining tumor growth. Further evidence for the role of prolactin in the development of such tumors was obtained in experiments in which antibodies to rat prolactin were injected into rats bearing 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinomas, leading to a decreased growth of the tumors and an increased incidence of tumor regression (136).

It is apparent on the basis of the preceding discussion that prolactin and estrogen are the primary hormones controlling the maintenance and growth of mammary tumors in the rat, mouse, and probably the human. The exact mechanisms by which these two hormones effect carcinogenesis is not completely elucidated (107, 108); however, several reports dealing with this subject have appeared in the literature (138–142*b*).

EFFECTS OF ERGOT ALKALOIDS ON MAMMARY TUMORS

Yanai and Nagasawa (143, 144) studied the effects of ergocornine and 2-bromo-α-ergokryptine on the formation of hyperplastic alveolar nodules of the mammary gland in female C3H/He multiparous mice. It has been established (145) that hyperplastic alveolar nodules represent the preneoplastic state in spontaneous mammary tumor formation in mice. Ergocornine and 2-bromo-α-ergokryptine were found to have a significant effect, involving inhibition of development and growth of hyperplastic alveolar nodules. This effect was ascribed to the measured ability of these substances to inhibit pituitary prolactin secretion. These same workers (123, 146*a*) investigated the effect of subcutaneously implanted pellets of ergocornine methanesulfonate and 2-bromo-α-ergokryptine on the appearance of spontaneous mammary tumors in C3H/He strain mice. Mammary tumor development was enhanced by a subsequent (4 weeks after pellet implantation) pituitary isograft (122). The appearance of mammary tumors was significantly diminished in mice treated with the ergot alkaloids, with treated mice showing a mammary tumor incidence of 10 and 16% (2-bromo-α-ergokryptine) and 20 and 23.5% (ergocornine) compared to a 74% incidence in the controls. The mammary tumor appearance in a few of the treated animals was attributed to small, nonpalpable tumors already in existence at the start of the experiment and to loss of the implanted pellet during the experiment. Recently, Singh *et al.* (146*b*) reported the effect of ergocornine on transplanted Dz-mammary tumors in BALB/C mice. They found that the growth of tumors was decreased by ergocornine at doses ranging from 20 to 100 mg./day for 5 weeks. There was no significant regression of the tumors at these doses.

Several groups have studied the effects of ergot alkaloids on 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors in rats. Nasr and Pearson (147) studied the effect of ergocornine on 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinomas and found that a dose of 2 mg. daily produced a 60% reduction in tumor size within 18 days. Henson *et al.* (148) studied the effect of 2-bromo-α-ergokryptine on this same tumor and observed a 67% reduction of tumor growth as a result of injecting 2-bromo-α-ergokryptine at a daily dose of 3 mg./kg. for 3 weeks followed by a 6-mg./kg. dose for an additional 3 weeks. Concurrently, Nagasawa and Meites (19) found that ergocornine inhibited the growth of 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors in rats when injected daily for 15 days at a dose level of 0.6 mg./day. In a subsequent paper, Cassell *et al.* (20) reported a study involving a longer treatment period (28 days) using both ergocornine and 2-bromo-α-ergokryptine. At the doses used, both ergocornine and 2-bromo-α-ergokryptine suppressed tumor growth; however, ergocornine produced complete disappearance of some tumors and was more effective than 2-bromo-α-ergokryptine in inhibiting growth. Termination of treatment with ergocornine resulted in a marked increase in the size and numbers of mammary tumors. Quadri and Meites (22) also reported that these same drugs were equally effective in inducing regression of spontaneous mammary tumors in old female rats. These spontaneous tumors appear in the postreproductive phase of life and, unlike the carcinogen-induced mammary adenocarcinomas, they are usually benign fibroadenomas and occur as a single tumor per rat[4].

Stähelin *et al.* (149) tested the effect of 2-bromo-α-ergokryptine on the 7,12-dimethylbenz[*a*]anthracene-induced rat tumors by treatment at the time of tumor induction. The previous studies reported treatment after the tumors had reached a certain size. In this study, injections of 6 mg./kg. of 2-bromo-α-ergokryptine were given daily (except Sunday), beginning on the day of the second 7,12-dimethylbenz[*a*]anthracene application. The experiment was extended for 243 days, with the result that mean tumor size in the 2-bromo-α-ergokryptine-treated group was less than 50% of that of the 7,12-dimethylbenz[*a*]anthracene control up to at least 119 days. Tumor inhibition was minimal at a dose of 1 mg./kg./day for 80 days. However, at the higher dose, 2-bromo-α-ergokryptine clearly delayed tumor appearance and increased survival time in addition to reducing tumor growth. When treatment was terminated after 80 or 100 days, tumor growth was initiated as would be expected on the basis of the hypothesis that 2-bromo-α-ergokryptine depresses mammary tumor growth by reversibly depressing prolactin secretion. These workers also reported that 2-bromo-α-ergokryptine had no effect on sarcoma 137 (a hormone-nondependent transplantable tumor in the mouse), had no cytotoxic effect in cell cultures, and did not inhibit multiplication of P-815 mastocytoma cells *in vitro*.

In a recent paper, Clemens and Shaar (21) reported a similar study involving the effects of ergocornine on

[4] Both papers (20, 22) frequently refer to "ergokryptine," one of them even in the title. However, it is apparent that the compound actually used was 2-bromo-α-ergokryptine.

7,12-dimethylbenz[*a*]anthracene-induced mammary tumors as a function of tumor size. In this study, rats were treated with ergocornine before and after mammary tumor appearance. Treatment of rats with ergocornine methanesulfonate and ergocornine hydrogen maleate in corn oil at a dose of 0.4 mg. daily significantly inhibited the induction of mammary tumors in rats treated with 7,12-dimethylbenz[*a*]anthracene. Ergocornine administration after tumor appearance caused a consistent and rapid regression of tumors of a size up to about 1.8 $cm.^3$ to the point that they disappeared to palpation. This incidence of complete tumor regression was not obtained in other studies and is significant. The authors suggested that the more rapid and complete regression (62% of the established tumors) in this study was perhaps due to the mode of administration of the compound (suspension in corn oil *versus* saline plus alcohol or aqueous dimethyl sulfoxide). Tumors approximately 14.1 $cm.^3$ and over did not respond to the treatment, suggesting that as the tumors grow larger they lose their hormone dependence—a very important factor to consider in planning the optimum therapeutic usage of these compounds.

Quadri *et al.* (150) also studied the effect of ergocornine, ergonovine, and 2-bromo-α-ergokryptine on rat prolactin and growth hormone-secreting pituitary (MtW15) tumors. Daily injections of ergocornine (0.1 and 0.2 mg.) or ergonovine (0.2 mg.) for 3 weeks induced significant regression or inhibition of tumor growth. Ergokryptine (0.3 mg.) had no significant effect on this tumor. At the end of the 3 weeks, tumors in the control animals showed a 33.6% gain in average diameter, while those in rats injected with 0.1 and 0.2 mg. ergocornine showed a 14.8 and 30.6% loss in average diameter, respectively. Ergonovine was less effective at the same dose but did reduce tumor growth.

To date, only one clinical trial has been reported (151). In this study, 2-bromo-α-ergokryptine was given orally in dosages of 6 mg./day for 2 days, 9 mg./day for the next 2 days, 12 mg./day for the next 2 days, and 15 mg./day for an additional 36 days. Nineteen patients with advanced breast cancer were treated and no objective remission was observed.

NIDATION INHIBITION BY ERGOT ALKALOIDS

In the course of studies on the mechanism of egg implantation, Shelesnyak (152–154) examined the effect of various drugs on deciduoma formation in the uterus of pseudopregnant rats. The formation of deciduoma, an artificial maternal placenta, is a convenient model system for the exploration of the processes of ova implantation and decidua initiation. It has two essential requirements: a proper hormonal sensitization of the uterus, *i.e.*, a specific estrogen–progesterone ratio, and a "nonspecific" stimulation (traumatization) of the endometrium, which may be brought about by mechanical, electrical, or chemical means. Experimentally, pseudopregnancy is induced in adult rats by electrical stimulation of the cervix, followed on the 4th day of the diestrual phase by chemical (histamine) or mechanical (clamping, scratching) traumatization of one or both horns of the uterus. The weight of deciduomata is determined 96 hr. after uterine stimulation (*cf.*, 81). Using this system, Shelesnyak (152) observed that while a variety of compounds inhibited deciduoma formation when instilled into the uterus, only one compound, ergotoxine, was effective when administered parenterally (16). A single subcutaneous dose of 0.25 mg. ergotoxine at the time of uterine traumatization effectively inhibited the development of deciduomata. This observation was the starting point for extensive investigations by Shelesnyak's group and by others into the effects of ergot alkaloids on reproductive physiology.

It was found that the inhibition of deciduoma formation by ergotoxine could be reversed with progesterone (16). However, it was concluded that ergotoxine does not act as an antagonist or competitor of progesterone, since the minimum effective dose of the latter was independent of the ergotoxine concentration and since progesterone did not protect against the toxic effects of the alkaloid (158). Rather, the inhibition of decidual growth is the result of a disturbance in the hormonal balance (estrogen–progesterone) caused by the alkaloid (154). The same paper reported the finding that single injections of ergotoxine into pregnant rats, in a dose of 0.5 mg./animal or higher during the first 6 days postcoitus, terminated pregnancy in all animals with reappearance of the estrus cycle. Injections of 3 mg./animal on Days 7 and 8 postcoitus terminated pregnancy in six out of seven and one out of seven cases, respectively, and were ineffective on later days. In all cases where pregnancy had not been terminated, the animals delivered normal litters. A dose of 0.25 mg. injected on Day 5 was effective in 17 out of 20 cases, whereas 0.1 mg. was ineffective. The alkaloid was also found to terminate pseudopregnancy with reestablishment of the normal cycle (154). The basic finding that ergot derivatives can terminate early pregnancy was subsequently confirmed by a number of laboratories using both rats (82, 84, 92, 155, 156) and mice (17, 157) as experimental animals.

The effectiveness of ergotoxine and ergocornine as fertility-control agents was tested on a larger scale in rats. In these trials, as a result of 1303 coital contacts only 23 pregnancies occurred. The fertility index was decreased to between 1.5 and 4% from a normal 96.5% in the same colony (158). As in the inhibition of decidual growth, the termination of early pregnancy by ergotoxine was reversed by progesterone, although a higher dose of the hormone was required and the effect was not complete (159). The treatment with ergot alkaloids had little or no effect on the subsequent fertility of the animals (157, 158). At this point it had become fairly clear that ergotoxine terminated early pregnancy by preventing nidation as a result of disturbing the hormonal balance rather than by any direct effect. However, the nature of the primary action of the alkaloid was completely unknown and Shelesnyak directed his attention to this question. He (160) showed that adrenalectomy did not interfere with the effect, indicating that the the action of ergotoxine was not mediated through the adrenal gland. Ergotoxine was found to have no estrogenic or gonadotropic activity (161), and a single injection of the alkaloid had no effect on the gonadotropin and prolactin content of the pituitaries of pregnant or

pseudopregnant rats (162). A limitation in the interpretation of the latter experiments is that the production of these hormones may have been influenced but, because of altered excretion into the system, this did not manifest itself in the hormone content of the gland (162). Injection of prolactin, on the other hand, counteracted the effect of ergotoxine and sustained pregnancy, indicating that the alkaloid did not interfere with the ability of the ovary to produce progesterone (163). These experiments also indicated that ergotoxine did not act by direct competition with prolactin (163).

After having thus ruled out many possible modes of action, Shelesnyak (81) speculated that ergocornine might affect the metabolism of progesterone. A preliminary study in women, in which the change in urinary levels of various steroids in response to ergocornine was measured, resulted in the observation that ingestion of a single dose of 2 mg. ergocornine in the immediate postovulatory phase markedly decreased urinary levels of pregnanediol and estrogens and increased those of ketosteroids and hydroxycorticosteroids (164). This finding, which was confirmed by Sterba (165), led to the more specific suggestion that the alkaloid might act by blocking 3β-hydroxysteroid dehydrogenase (164). However, later experimentation did not bear out this proposal. After administration of ergocornine to rats, the enzyme was still demonstrable histochemically (166, 167) and by assay in homogenates (168). In the ovaries of pseudopregnant rats, a drop of progesterone content following ergocornine injection was only observed after a latency period of 12 hr. and at the same time the 20α-hydroxypregn-4-en-3-one content increased. This change in the ratio of the two steroids is similar to that observed during normal estrus; it can account for the termination of the pregnancy but is clearly not the result of an inhibition of β-hydroxysteroid dehydrogenase (169).

Finally, an extended reinvestigation measuring the plasma and urinary levels of a number of steroids in normal and ergocornine-treated women failed to reproduce the dramatic decreases in urinary pregnanediol levels observed earlier and provided no evidence for a specific blockade of the Δ^5-3β-hydroxysteroid dehydrogenase system (170). The results of these studies (168–170) were, however, in accord with the emerging view that the action of the alkaloid was in some way mediated by the pituitary.

While Shelesnyak's early results seemed to rule out the pituitary as being involved in the effect of ergot alkaloids on ova implantation (162), a study by Zeilmaker and Carlsen (18) specifically pointed to this possibility. Their work with hypophysectomized rats carrying transplanted pituitaries suggested that ergocornine administration leads to a temporary inhibition of prolactin release by the pituitary, which brings about irreversible changes in the corpus luteum. As a result the progesterone level decreases, preventing the development of decidual tissues and leading to the onset of estrus. Their paper also quotes experimental evidence to the effect that the change in the pituitary function is not the indirect result of an influence of the alkaloid on the hypothalamus. This picture of the sequence of events has, for the most part, stood the test of time. Lobel *et al.* (167) observed that the corpora lutea showed necrosis and dissolution of cells 24 hr. after ergocornine administration, and Varavudhi *et al.* (171) confirmed the results with hypophysectomized rats carrying autotransplanted pituitaries. Further experiments indicated that ergocornine did not interrupt gestation in the presence of an extra source of luteotropic activity, *e.g.*, in the presence of fetal placenta (172) or the pseudopregnancy induced by ectopic trophoblast tissue (173). Finally, it was found that treatment of rats with ergocornine induced 20α-hydroxysteroid dehydrogenase, giving rise in the corpora lutea of pregnancy to a 100-fold increase in enzyme activity, and that this action was prevented by exogenous prolactin (174). The enzyme 20α-hydroxysteroid dehydrogenase appears in the late diestrus in newly formed normal corpora lutea but is absent in the corpora lutea of pregnancy or pseudopregnancy. Ergocornine acts to increase 20α-hydroxysteroid dehydrogenase activity when given during the first days of pregnancy when the corpora lutea are mainly under the control of the pituitary but not at later stages when the chorionic secretion of luteotropic activity plays a significant role (175). This indicates, as does other evidence (176–178), that the alkaloids of ergot inhibit release of luteotropic activity from the pituitary but not from the placenta and that the interruption of early pregnancy is most likely mediated through the pituitary.

These experiments indicate in which manner prolactin influences progesterone levels and how the drug-induced prolactin deprivation irreversibly leads to a change in the luteal cell which results in decreased progesterone secretion. While these subsequent results thus largely confirm the later steps in the sequence of events as postulated by Zeilmaker and Carlsen (18), their suggestion that the first step is a direct action of ergocornine on the pituitary to inhibit prolactin release has been repeatedly challenged. Experiments in which ergocornine was placed directly on the uterus, the ovaries, or the pituitary showed that the drug was inactive under these conditions (179), leading to the consideration that the effect of the alkaloid on the pituitary might be mediated by the hypothalamus.

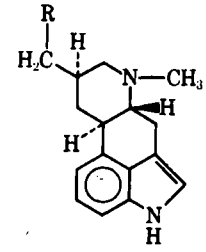
Later publications also discussed the possibility that the alkaloid acts by way of the CNS (169, 171, 180). Seda *et al.* (181*a*), in a recent study in rats using the nidation-inhibiting synthetic ergot derivative 6-methyl-8-cyanomethylergoline, noted an increase in gonadotropic [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] secretion in addition to inhibition of prolactin release. This is supported by observations that lactation-inhibiting ergot alkaloids restore the estrus cycle or normalize it in the case of disturbances both in animals (181*b*) and in humans (101*b*). Seda *et al.* (181*a*) felt that this effect on these three hormones could hardly be explained by a direct action of the alkaloid on the pituitary but would more logically seem to be the result of a stimulation of the hypothalamus to release LH- and FSH-releasing and prolactin-inhibiting factors. Since the nidation inhibition by ergot alkaloids is overcome by prolactin alone (163, 178, 182), the effect on the release of this hormone seems to be at least the main mode of action. Therefore, the question comes down to the problem, which has been already discussed,

whether ergot alkaloids inhibit prolactin release directly at the level of the hypophysis or *via* the hypothalamus, or both.

Whether nidation inhibition by ergot alkaloids is solely due to their effect on pituitary prolactin release is not entirely clear. There seems to be a reasonable empirical correlation between lactation inhibition and nidation inhibition, in the sense that all ergot alkaloids which are active as nidation inhibitors also seem to inhibit lactation or prolactin release. However, a detailed study comparing dose–response curves of two compounds, ergokryptine and 2-bromo-α-ergokryptine, for nidation and lactation inhibition in rats raised doubt as to whether both responses depend entirely on the same primary effect (82). While the ED_{50} of 2-bromo-α-ergokryptine for nidation inhibition (0.75 mg./kg. s.c.) is smaller than that of the parent compound (1.15 mg./kg.), the ED_{50} for lactation inhibition of the halogenated derivative (3.5 mg./kg.) is larger than that of the parent compound (0.7 mg./kg.). As would be expected for a common mechanism, in the case of ergocornine and ergokryptine the dose–response curves for nidation and lactation inhibition converge toward one dose which completely inhibits both responses. However, this is not the case with 2-bromo-α-ergokryptine and, therefore, at least for this alkaloid the assumption of a common primary effect does not seem to be valid (82). As mentioned earlier, this conclusion was criticized by Nicoll *et al.* (83), who pointed out that a difference between these two drugs in their ability to depress oxytocin release could account for a difference in their potency as lactation inhibitors; therefore, the result would not necessarily disprove that nidation inhibition is due entirely to the inhibition of prolactin release. Furthermore, as mentioned earlier, differences in the pharmacokinetic properties could account for this result.

Only a limited amount of information is found in the literature on the relationship between the structure of ergot alkaloids and their activity as nidation inhibitors. Most of this information relates to the peptide-type ergot alkaloids. In early studies, Shelesnyak (81) found little activity for ergotamine (VI*a*) and its 9,10-dihydro derivative, for ergonovine (V*d*) and methylergonovine (lysergic acid butanolamide), for tochergamine, a synthetic analog belonging to the ergotamine group, and for ergocristine (VI*e*) and its 9,10-dihydro derivative. The active components of the ergotoxine complex were found to be ergocornine (VI*k*) and ergokryptine (VI*g*), of which the latter was more active. Their 9,10-dihydro derivatives were less active than the parent compounds. Dose–response curves established in later work indicated an ED_{50} of 335 mcg./rat (subcutaneous, average body weight 167 ± 7.4 g.) for ergocornine (183, 184), compared to 175 mcg. for ergokryptine, and values of 505 and 945 mcg. for ergosine (VI*c*) and ergovaline (VI*o*), respectively, two new alkaloids found to be active (184). Another newly tested alkaloid, ergostine (VI*m*), had only weak activity (184). As already mentioned, substitution of ergokryptine in the 2-position with bromine leads to a compound with somewhat lower ED_{50} for nidation inhibition (82). The main advantage of this substitution is a marked decrease in toxicity.

More recent work has shown that the peptide portion of the alkaloids is not required for activity. The groups of Semonsky *et al.* (92) and of Rezabek *et al.* (156) prepared D-6-methyl-8-cyanomethylergoline (VIII*a*) and a series of amides of D-6-methylergoline-8-acetic acid (VIII*b*) and found these to be very effective nidation as well as lactation inhibitors. Compound VIII*a* inhibits nidation when given orally to rats in a single dose of 10 mg./kg. or in five daily doses of 1 mg./kg. during the first 7 days of pregnancy (92), as does apparently its $\Delta^{9,10}$-unsaturated analog (five times 5 mg./kg.) (185). This compares to an LD_{50} of 110 mg./kg. p.o. for VIII*a*. The figures are particularly impressive for the amide of 6-methylergoline-8-acetic acid (VIII*c*), which interrupted pregnancy in all animals when given in a single oral dose of 5 mg./kg. at any time between the 1st and 7th day of pregnancy or as a single oral dose of 0.4 mg./kg. on Day 6 or 7. This compound has an acute LD_{50} of 93 mg./kg. given intravenously and about 1 g./kg. upon oral administration (92). Very recently the same group reported additional structure–activity relationships (181*b*). Lysergic acid α-hydroxyethylamide (V*c*) was tested in mice and found to terminate pregnancy in all cases in a daily dose of 250 mcg./animal given orally (157). Finally, it was found that not only lysergic acid derivatives possess this activity but also clavine-type alkaloids. Agroclavine (VII*a*), a known lactation inhibitor (71), also inhibited nidation effectively in mice (250 mcg. daily orally during Days 3 and 4 or 5 and 6 of pregnancy) (157) and rats (1.5–2.5 mg. daily orally on Days 1–5 of pregnancy) (84, 182). As in the case of ergotoxine, the nidation inhibition by simpler ergolines was overcome by simultaneously administering progesterone (84) or prolactin (92, 178, 182). Interestingly, while the ergotoxine-type alkaloids are active both orally and when injected systemically (158), agroclavine is completely inactive when injected intraperitoneally (84) or subcutaneously (157). The question has been examined whether the ergot alkaloids themselves are the active entities or whether they require some kind of metabolic activation. In this study (186), ergocornine was perfused through a liver *in vitro* before administering it to rats or was directly introduced into the liver *in vivo* through the portal system. The results did not indicate any activation but strongly suggested metabolic inactivation of the alkaloid in the liver.



VIII*a*: R = CN
VIII*b*: R = $CONR_1R_2$
VIII*c*: R = $CONH_2$

Some consideration has been given to the question whether ergot alkaloids might have a teratogenic effect. Several studies on nidation inhibition in rats or mice reported that in the cases where pregnancies had not been terminated as a result of administering ergot alkaloids, normal litters were born (17, 154, 156). It was also shown that ergocornine in doses which are

effective in inhibiting decidualization and nidation does not have a direct toxic effect on the blastocyst of pregnant rats (187). However, at least one study reported very pronounced effects of ergocornine on fetal morphology (176). A dose of 1 mg. of ergocornine given to rats on the 8th day of pregnancy, while no longer effective in terminating pregnancy, caused a very significant increase in the number of fetal malformations. No such effect was seen when the same dose was given on the 12th day, and the detrimental effect on the 8th day could be averted by simultaneously administering progesterone. The authors concluded that the teratogenic effect of ergocornine was a result of the progesterone deficiency it induced.

It is evident from the above discussion that ergot alkaloids have been quite instrumental in the studies on the mechanism of nidation. Likewise, they have been and continue to be useful tools in the study of the complex hormonal relationships during the estrus cycle. It has already been mentioned that the termination of pregnancy or pseudopregnancy by ergotoxine or ergocornine induced the appearance of estrus and usually resulted in a new ovulation within 3–4 days of treatment with the alkaloid (154, 188, 189). While ergot alkaloids can thus stimulate ovulation, Kraicer and Strauss (190) reported that ergocornine can also act as an ovulation blocker under certain circumstances. A single dose of 1 mg./rat inhibits ovulation when given during the time period of 50–10 hr. before normal ovulation, with a maximum of effectiveness when given during proestrus at about 14 hr. before ovulation. The mechanism of this ovulation inhibition is not really understood, and in a more recent study by another group, the investigators did not observe an ovulation block by ergocornine (191). The discrepancy may, however, be due simply to differences in methodology. In other studies, ergot alkaloids were instrumental in securing evidence that prolactin, in addition to its luteotropic activity during early pregnancy, also has a luteolytic function during the estrus cycle in rats (192–194). Blocking the secretion of prolactin during the estrus cycle with ergocornine (193) or 2-bromo-α-ergokryptine (194) led to the accumulation of corpora lutea which failed to undergo normal involution. From this it was suggested that the normal surge of blood prolactin on the day of proestrus serves to induce luteolysis of the previous crop of corpora lutea formed during each cycle.

The crucial question in evaluating the potential of ergot alkaloids as fertility control agents is, of course, whether the effective nidation inhibition observed in rats and mice also holds true in other animals and in man. Unfortunately, there is only a very limited amount of information on this point in the literature. As discussed earlier, there is no doubt that the inhibition of pituitary prolactin release is a general phenomenon. However, the question is whether in other animals and in man prolactin plays a similar essential role in the process of ova implantation as it does in rats. Two studies have indicated that ergocornine is not effective in inhibiting implantation in rabbits (195) and guinea pigs (196). However, a more recent investigation in sows reported that doses of 125–250 mg. of ergocornine can terminate pseudopregnancy in swine with regression of the corpora lutea and reappearance of estrus (197). The limited studies in women (164, 165, 170, 198, 199) have so far not produced entirely conclusive results. All the studies agree that ergocornine has no effect on the basal body temperature curve. One study (170), in contrast to earlier results from the same group (164), found no significant changes in serum progesterone levels or urinary pregnanediol excretion, whereas other groups observed at least some decrease in urinary pregnanediol levels following ergocornine (165, 198, 199). In only one out of five women treated during early pregnancy did spontaneous abortion occur after oral administration of 10 mg. of ergocornine (198). It is apparent that this question cannot yet be considered settled.

FUTURE DEVELOPMENTS

It is probably too early to judge whether and to what extent the effect of ergot alkaloids on pituitary hormone release will turn out to be clinically useful. Ergot alkaloids, by virtue of this activity, have been and continue to be very useful tools in the study of prolactin-dependent physiological functions. Beyond this, Rezabek *et al.* (200), in a recent discussion of their work, suggested the following clinical applications in which ergot alkaloids might be utilized:

1. For decreasing milk production in puerperal mastitis or in painful milk retention and breast swelling.
2. For decreasing the prolactin secretion in the treatment of breast tumors, mastopathy, premenstrual mastodynia, and galactorrhoea.
3. For shortening or interrupting the luteal phase of the menstrual cycle.
4. For promoting follicle-stimulating hormone and luteinizing hormone secretion and for induction of ovulation.
5. In exceptional cases for the suppression of the initial phase of pregnancy.

One might add to the list the possibility of preventive treatment of breast cancer in high risk women, for example in the form of slow-release implants.

In addition, the same authors consider a number of potential applications in zootechnical practice:

1. For the induction and synchronization of estrus.
2. For suppression of lactation and for provocation of estrus and ovulation to obtain a higher frequency of litters.
3. For suppression of undesired pregnancies, *e.g.*, in bitches.
4. For suppression of clucking in the fowl and for stimulation of egg laying.

Obviously, the exploration of the practical and clinical usefulness of the pituitary effects of ergot alkaloids has only just begun and most of these potential applications still remain to be investigated in detail. At the moment the most promising compounds for clinical applications seem to be 2-bromo-α-ergokryptine, D-6-methyl-8-cyanomethylergoline-I, and D-6-methyl-ergolin-I-yl-8-acetamide (Compounds 6605 VUFB and 6683 VUFB of Semonsky). However, there is certainly room for further developments in this area.

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RESEARCH ARTICLES

Pharmacology of Mono- and Disubstituted Chlorpromazine Metabolites

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Abstract □ Chlorpromazine and 10 of its mono- and disubstituted metabolites were investigated for their effects on the CNS of male rats and mice. All of the compounds decreased motor activity and respiration, and all but two of the compounds decreased heart rate in rats. The most potent metabolite in depressing spontaneous motor activity of mice was 3,7-dihydroxychlorpromazine, with an ED_{50} of 5.1 mg./kg. i.p. in comparison to chlorpromazine which had an ED_{50} of 2.0 mg./kg. i.p. However, the 3,7-dihydroxy derivative was approximately twice as toxic as chlorpromazine in mice. 7,8-Dihydroxychlorpromazine did not alter forced motor activity, but it did induce a dose-related depression of spontaneous motor activity; 7-hydroxy-8-methoxy derivative also produced a marked decrease in spontaneous motor activity with minimal effects on forced motor activity. None of the compounds demonstrated anticonvulsant activity, and only 7-hydroxy-chlorpromazine elicited effects suggesting possible antidepressant activity. Barbiturate sleeping time was potentiated by all of the compounds, mainly due to their CNS depressant properties.

Keyphrases □ Chlorpromazine and 10 mono- and disubstituted metabolites—effects on CNS of rats and mice □ CNS depression—effects of chlorpromazine and 10 mono- and disubstituted metabolites, rats, mice □ Motor activity, spontaneous and forced—effects of chlorpromazine and disubstituted metabolites compared, rats, mice

Fishman and Goldenberg (1) identified several metabolites of promazine and chlorpromazine in human urine including 3-hydroxyphenothiazine and 7-hydroxychlorpromazine. These and other monohydroxylated and methoxylated derivatives have been investigated and found to be pharmacologically active (2–7); it also

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Fishman and Goldenberg (1) identified several metabolites of promazine and chlorpromazine in human urine including 3-hydroxyphenothiazine and 7-hydroxychlorpromazine. These and other monohydroxylated and methoxylated derivatives have been investigated and found to be pharmacologically active (2–7); it also

Table I—Experimental Compounds

Compound Number	Compound Name
I	8-Hydroxy-7-methoxychlorpromazine
II	7-Hydroxy-8-methoxychlorpromazine
III	7-Hydroxy-8-methoxy-nor$_1$-chlorpromazine
IV	7-Hydroxy-8-methoxy-nor$_2$-chlorpromazine hydrochloride
V	3,7-Dihydroxychlorpromazine
VI	7,8-Dihydroxychlorpromazine hydrochloride
VII	7-Hydroxychlorpromazine
VIII	7-Hydroxy-nor$_1$-chlorpromazine
IX	7-Hydroxy-nor$_2$-chlorpromazine
X	3-(2-Chloro-7-hydroxy-10-phenothiazinyl)-propionic acid

has been shown that some metabolites contribute to the total effect of the parent drug. The purpose of this present investigation was to evaluate several mono- and disubstituted metabolites of chlorpromazine for their possible effects on the CNS.

EXPERIMENTAL

Nine mono- and disubstituted metabolites of chlorpromazine and 3-(2-chloro-7-hydroxy-10-phenothiazinyl)propionic acid (Table I) were used.

Male Swiss-Webster mice[1], weighing 20–30 g., and Wistar descendent rats[2], 200–300 g., were used after being acclimated to laboratory conditions for 4–5 days. Each compound or solvent was usually tested in groups of 10 mice or six rats, which were permitted food and water *ad libitum*. The free bases were dissolved in sufficient 0.1 *N* HCl and then adjusted to volume with normal saline, except for Compounds IV and IX for which propylene glycol USP was used and Compound X for which 0.5 *N* NaOH was used. Compound VI, synthesized as the hydrochloride salt, was dissolved in normal saline; however, since this solution changed color almost immediately, 0.1% ascorbic acid was added to prevent oxidation. The solutions were freshly prepared and the injection volume was kept constant at 10 ml./kg. for mice or 1.0 ml./kg. for rats. The injection time was over a period of 15 sec. The results that differed from control values at $p < 0.05$ level (Student's t test) were considered to be statistically significant.

METHODS

The acute 72-hr. intraperitoneal lethal effects were determined in mice using three dose levels, and the LD_{50} was estimated using probit analysis as described by Goldstein (8). The intravenous LD_{50} of Compound VI was also determined with three dose levels.

Gross Behavior in Rats—All 10 compounds were evaluated by a gross-observation rating scale as described by Watzman *et al.* (7). The time course of the drug effect was ascertained by checking items on the scale at 15 min. prior to and 30, 60, 120, 180, and 240 min. following drug administration with special emphasis on behavioral and autonomic effects.

Spontaneous Motor Activity—The effects of the compounds on the spontaneous motor activity of mice were measured in three photocell cages[3]. Two animals treated with identical doses of the same compound were placed in each of two photocell cages 0.5–1 hr. after drug administration, and counts were recorded every 15 min. over a 1-hr. period. Each dose was tested in a factorial design in each of the three activity cages to negate the differences in sensitivity among units. Control animals were tested simultaneously in one activity cage at the same time intervals after administration of an equal volume of saline or the particular solvent used, and the ED_{50} (defined as the dose that decreased the level of performance to 50% of the control scores) was calculated for each compound.

Forced and Spontaneous Motor Activity—The effects of the compounds on forced motor activity of mice were studied using the rotarod as described by Watzman and Barry (9). The wooden rod rotated at 4 r.p.m. during the first 30 sec., at 6 r.p.m. during the next 30 sec., and at progressively increasing speeds thereafter at 30-sec. intervals until the animals fell off. Six animals were tested simultaneously and were given two trials spaced 4–6 hr. apart on each of 2 consecutive days for a total of four trials. The last trial was preceded by a 0.5–1-hr. interval before the administration of saline, solvent, or one of the selected doses of chlorpromazine or the metabolites. The drug or placebo effect for each animal was computed as the ratio of performance time on the fourth trial divided by performance time on the third trial. Ten minutes following the initiation of the fourth rotarod test, the six animals were put into three photocell activity cages as described for spontaneous motor activity and the counts were recorded for 15 min. The percentage of control activity (spontaneous and forced motor activity) at each dose level of the compounds used was plotted in an attempt to make an early evaluation of the possible type of activity of each compound.

Table II—Acute LD_{50}[a], ED_{50}, and Safety Index of the Compounds in Mice

Compounds	LD_{50}, mg./kg. i.p.	ED_{50}, mg./kg. i.p.	Safety Index, $\frac{LD_{50}}{ED_{50}}$
Chlorpromazine	119.7	2.0	59.9
I	114.6	44.5	2.6
II	130.3	27.2	4.8
III	77.8	35.4	2.2
IV	67.5	31.7	2.1
V	52.8	5.1	10.4
VI	24.8	6.6	3.8
VII	119.4	7.5	15.9
VIII	58.1	16.1	3.6
IX	48.2	13.7	3.5
X	133.7	75.3	1.8

[a] The 72-hr. LD_{50}.

Anticonvulsant Activity—The effects of the compounds on convulsions and death produced by pentylenetetrazol (100 mg./kg. s.c.) and strychnine sulfate (2 mg./kg. i.p.) were studied in mice, as described by Watzman *et al.* (7), and on maximal electroshock seizure, as described by Manian *et al.* (6). The doses of chlorpromazine and of the metabolites were identical to those used in the spontaneous activity studies.

Antireserpine Activity—Male mice, weighing 20–25 g., were used. Each experiment was carried out on five groups (five animals in each group) (10). The doses of the metabolites were identical to those used in the spontaneous motor activity studies. The animals were permitted food and water *ad libitum*, and the experiment was conducted at a temperature of 18–20° (64.5–68°F.). One hour was allowed for the animals to stabilize in the room, and then the rectal temperature was determined by inserting the thermometer[4] 2 cm. into the rectum. Five groups were used simultaneously: Groups I, II, and III received different doses of chlorpromazine or a metabolite; Group IV received desipramine (desmethylimipramine) (20 mg./kg. i.p.); and Group V received saline (10 ml./kg. i.p.). One hour following treatment, the rectal temperature was again determined and the animals were examined for development of ptosis, diarrhea, and change in motor activity. Reserpine (1 mg./kg. i.p.) was then administered, and 4 hr. later the rectal temperature was obtained and alterations in ptosis, diarrhea, and motor activity were noted using the saline group as the control. Hypothermia was calculated in terms of the decrease in rectal temperature from the individual control value of each mouse. The control value was the temperature recorded after chlorpromazine or a metabolite was administered, since these compounds were found to decrease body temperature.

Barbiturate Sleeping Time—One hundred and twelve male mice, 20–24 g., were used. Half of these (56 animals) were divided into 14 groups of four animals each. Each group received normal saline (10 ml./kg.), a dose of chlorpromazine, or one of five test compounds intraperitoneally. Thirty to sixty minutes after administration of the drug, either sodium hexobarbital (100 mg./kg. i.p.) or sodium bar-

[1] Carworth Laboratories.
[2] Hilltop Laboratories.
[3] Woodard Research Corp.
[4] Tele-thermometer, Yellow Spring Instrument Co.

bital (300 mg./kg. i.p.) was injected. The onset of sleeping time (loss of righting reflex) and the length of sleeping time (recovery from immobility) were determined. Sleeping time is defined as the interval between the loss and return of the righting reflex. The criterion for the loss and return of the righting reflex is defined as the inability and ability, respectively, of the animal to right itself within 5 sec. in three successive trials when placed on its back. The test was terminated if the mouse continued to sleep 4 hr. after the loss of the righting reflex.

On the following day the study was repeated with the remaining five test compounds using the remaining 56 mice. The 2-day experiment was replicated so that a total of eight mice received one dose of each compound. Each metabolite was tested for effects on sodium hexobarbital sleeping time at three dose levels and on sodium barbital sleeping time at two dose levels in mice. Since sodium barbital is not metabolized by the liver microsomal enzymes, these data can distinguish between effects on the CNS *per se* and inhibition or enhancement of liver microsomal enzyme activity.

Since mice receiving the highest doses of chlorpromazine and of some of its derivatives did not recover their righting reflex in less than 4 hr., the problem was approached by using the HD_{10} of barbital (11). Male mice, in groups of 10, received various doses of sodium barbital intraperitoneally in order to estimate the HD_{10} of barbital. HD_{10} is defined as the dose of sodium barbital estimated to cause 20% of the mice to lose their righting reflex for at least 5 min. during the 1st hr. following the injection of sodium barbital. The intraperitoneal HD_{10} of sodium barbital was 147.9 mg./kg., determined according to the method of Litchfield and Wilcoxon (12). Male mice, 20–24 g., in groups of eight received the highest dose of either chlorpromazine or one of the 10 metabolites 30–60 min. prior to the administration of the HD_{10} of sodium barbital.

RESULTS

Gross Behavior—Chlorpromazine and its metabolites decreased motor activity and rate of respiration within 30 min. Heart rate was decreased by all compounds except III (7-hydroxy-8-methoxy-nor$_1$-chlorpromazine) and VIII (7-hydroxy-nor$_1$-chlorpromazine). The pupil was slightly dilated by all compounds except IX. Body temperature was decreased by all compounds except V, and ptosis occurred with all of the phenothiazines except III and V.

The intraperitoneal 72-hr. LD_{50}, ED_{50} (effects on spontaneous motor activity), and the safety index (LD_{50}/ED_{50}) in mice are summarized in Table II. Compound VI was the most toxic compound tested on a milligram per kilogram basis; however, Compound X had the lowest safety index and, therefore, the lowest safety range. As can be seen in Table II, chlorpromazine was more potent on a milligram per kilogram basis in depressing spontaneous motor activity than the metabolites and had a greater safety index than any of the compounds investigated. The 7-hydroxy (Compound VII) and 3,7-dihydroxy (Compound V) derivatives had the highest safety indexes of the metabolites investigated, having indexes of 15.9 and 10.4, respectively.

Table III—Effects of the Experimental Compounds on Spontaneous Motor Activity in Mice[a]

Compound	Dose, mg./kg. i.p.	Percent of Control	Dose, mg./kg. i.p.	Percent of Control	Dose, mg./kg. i.p.	Percent of Control
Chlorpromazine	1	87.0	2	53.8	4	5.2[b]
I	20	122.5	40	68.2	60	11.5[b]
II	13	101.6	26	50.0[b]	39	15.9[b]
III	16	97.2	32	59.4	48	23.4[b]
IV	12.5	85.1	25	61.7	50	30.3[b]
V	5	49.6	10	32.5[b]	20	11.8[b]
VI	3	90.2	5	66.7	10	28.5[b]
VII	6.25	63.8	9	34.1	12.5	9.0[b]
VIII	12	78.9	18	32.0[b]	24	22.8[b]
IX	5	110.4	10	47.1	30	17.3[b]
X	40	94.6	60	87.3	80	37.8[b]

[a] The 0.5-hr. counting time. [b] Significantly different from saline-treated control groups ($p < 0.05$).

Table IV—ED_{50} for Spontaneous Motor Activity Data for 0.25-hr. Test Shown in Figs. 1 and 2 and 10 min. following the Test of Forced Motor Activity

Compound	ED_{50}, mg./kg. i.p.	95% Confidence Limit
Chlorpromazine	1.9	1.3–2.4
I	47.2	28.3–78.9
II	22.2	15.4–31.9
III	28.8	19.7–42.3
IV	53.2	30.0–94.4
V	8.5	4.8–15.1
VI	11.6	5.2–25.7
VII	8.2	4.9–13.9
VIII	27.6	19.7–38.6
IX	24.6	12.0–50.4
X	197.2	95.3–408.3

Spontaneous Motor Activity—As seen in Table III, almost all doses of the 10 compounds exhibited a dose-dependent depressant effect on spontaneous motor activity as measured in the photocell activity cage.

Forced and Spontaneous Motor Activity—Figures 1 and 2 show the comparative effects of chlorpromazine and the 10 experimental compounds using data in which effects on spontaneous activity were investigated 10 min. following initiation of the rotarod test. Chlorpromazine clearly decreased motor coordination in doses of 1, 2, and 4 mg./kg. (Fig. 1), whereas much higher doses of the experimental compounds were needed to produce detrimental effects on forced and spontaneous motor activities. Most of the compounds had a greater detrimental effect on spontaneous than on forced motor activity. In particular, Compounds II, III, and VI decreased spontaneous motor activity with minimal effects on forced motor activity. However, Compounds V, VIII, and IX produced similar magnitudes of effect on both forced and spontaneous motor activities, suggesting maximal effects on cerebral cortical activity. Compound X did not significantly alter either forced or spontaneous motor activity under these experimental conditions, even though it did produce some decrease in spontaneous motor activity in the initial studies (Table III). This difference in activity could possibly be due to stimulating effects induced during the rotarod portion of this phase of the study, although the ED_{50}'s for depression of spontaneous motor activity in this phase of the study (Table IV) were similar for most of the compounds to those obtained earlier (Table II).

Anticonvulsant Activity—None of the compounds investigated protected the animals from convulsions produced by pentylenetetrazol (100 mg./kg. s.c.), strychnine sulfate (2 mg./kg. i.p.), or maximal electroshock.

Antireserpine Activity—Since chlorpromazine and its derivatives decreased body temperature, the rectal temperature was measured after the administration of the test compounds and again 4 hr. following the administration of reserpine. Compound VII was the only experimental compound that significantly counteracted the decrease in body temperature due to reserpine (Table V), and then the difference was significant only at the lowest dose used, 6.25 mg./kg. i.p. Two compounds, IV and V, significantly potentiated the hypothermic effect of reserpine (Table VI). None of the 11 compounds tested showed any effect on the reserpine-induced ptosis, and only des-

Table V—Antagonism of Hypothermic Effects of Reserpine (1 mg./kg. i.p.) by Compound VII

Drugs	Dose, mg./kg. i.p.[a]	N[b]	Δ Temperature[c], $\bar{X} \pm SE$	p
Saline	10 ml./kg.	5	3.62 ± 0.34°	—
Desmethylimipramine	20	5	1.32 ± 0.26°	<0.001
VII	6.25	5	2.10 ± 0.54°	<0.025
VII	9.00	5	2.68 ± 0.53°	<0.10
VII	12.50	5	2.74 ± 0.49°	<0.10

[a] Administered 1 hr. prior to reserpine. [b] Groups of five mice each. [c] Decrease in rectal temperature 4 hr. after the administration of reserpine.

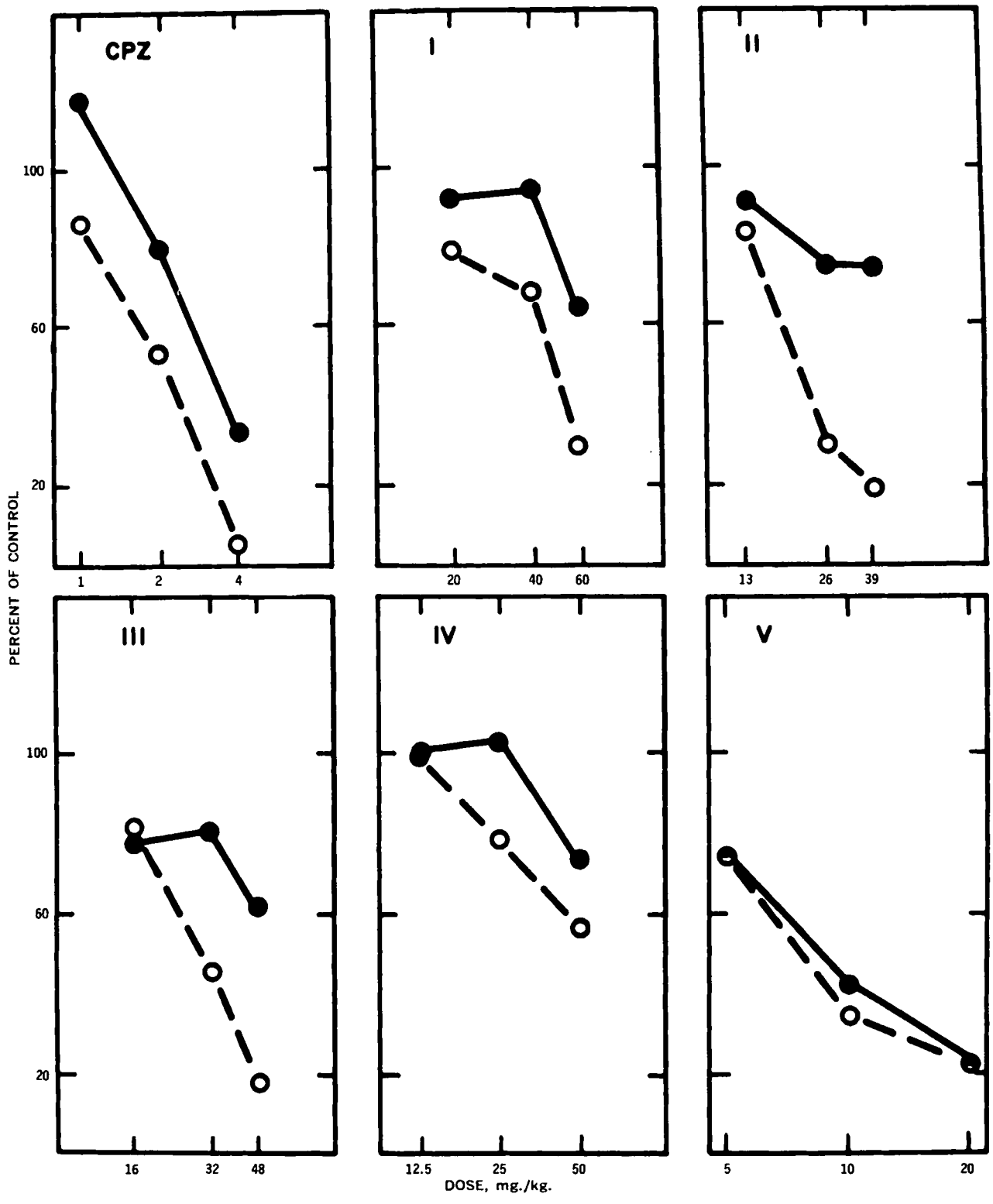

Figure 1—*Effects of chlorpromazine and Compounds I–V on spontaneous* (○) *and forced* (●) *motor activities in mice.*

ipramine was capable of blocking this effect. All animals showed a decrease in motor activity, and diarrhea developed in all mice except those receiving desipramine.

Barbiturate Sleeping Time—Only chlorpromazine and Compounds VII and VIII significantly increased hexobarbital sleeping time in the lowest dose used (Fig. 4). The low doses of Compounds VII and VIII increased hexobarbital sleeping time from 38.6 ± 1.5 to 52.0 ± 7.3 min. (34.7%) and from 38.6 ± 1.5 to 57.9 ± 5.1 min. (50.0%), respectively. In the medium dose range, all compounds except III, V, VI, and X increased sleeping time whereas the high doses of all compounds produced a significant increase in the sleeping time; however, chlorpromazine was by far the most potent of the compounds tested (Figs. 3 and 4).

In medium doses, chlorpromazine and Compounds III, VI, VII, VIII, and IX produced a significant increase in the duration of barbital sleeping time. The mean sleeping time of sodium barbital in the saline-treated animals ranged from 65.0 ± 4.8 to 88.3 ± 13.2 min. Table VII shows that when the HD_{20} dose of sodium barbital was given to mice that were pretreated 30–60 min. prior to the administration of sodium barbital with the high dose of either chlor-

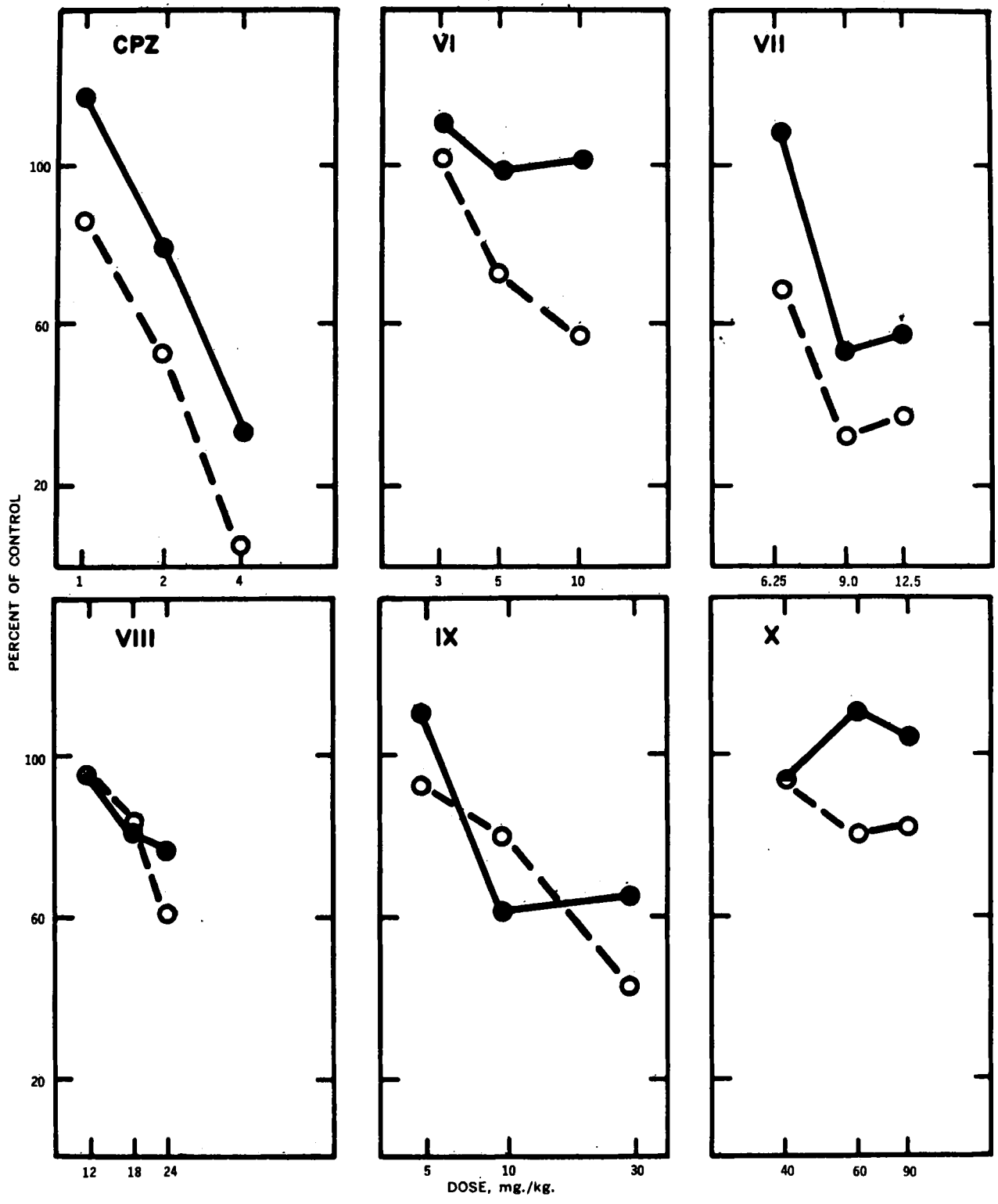

Figure 2—*Effects of chlorpromazine and Compounds VI–X on spontaneous* (○) *and forced* (●) *motor activities in mice.*

promazine or one of its 10 metabolites, all but Compound X potentiated the sedative action of sodium barbital; however, Compounds VII and VIII produced only a slight increase in the number of animals losing their righting reflex.

DISCUSSION

Chlorpromazine and 10 mono- and disubstituted metabolites were studied in a battery of tests designed to compare their effects on the CNS. The tests used were: gross behavior, spontaneous motor activity, forced motor activity, chemoshock antagonism, maximal electroshock seizures, antireserpine activity, and barbiturate sleeping time.

Chlorpromazine was by far the most potent compound in inhibiting spontaneous motor activity (ED_{50} 2.0 mg./kg. i.p.). Since the LD_{50} of chlorpromazine was higher than most of the metabolites and the safety index was 3.8–33.2 times greater than the metabolites investigated, it would appear that the metabolites not only are less

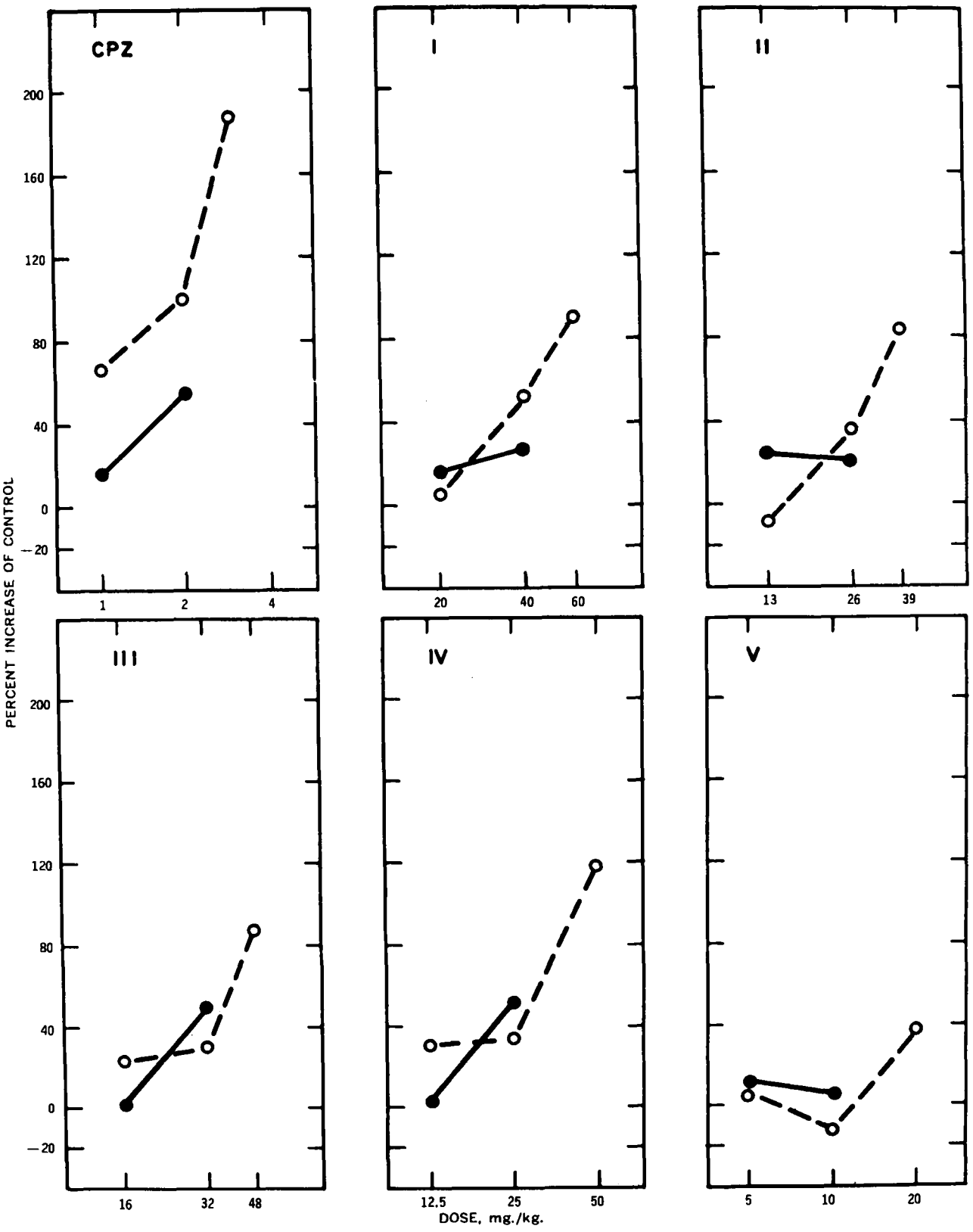

Figure 3—*Effects of chlorpromazine and Compounds I–V on hexobarbital* (○) *and barbital* (●) *sleeping time in mice.*

effective in depressing CNS activity but also are more toxic. Although Compound VI was extremely toxic, Compound II had an LD_{50} 5 times greater than that of Compound VI. Therefore, the replacement of the 8-hydroxy by an 8-methoxy group markedly decreased the toxicity of the compound; however, it still took a dose of Compound II approximately 5 times greater to equal the depressant effects produced by Compound VI. Compound V produced approximately equivalent dose–response effects on spontaneous and forced motor activities, suggesting maximal effects on cerebral cortical activity, whereas Compounds II, IV, and VI produced marked effects on spontaneous motor activity with minimal or no effects on forced motor activity. Compound VII had the highest

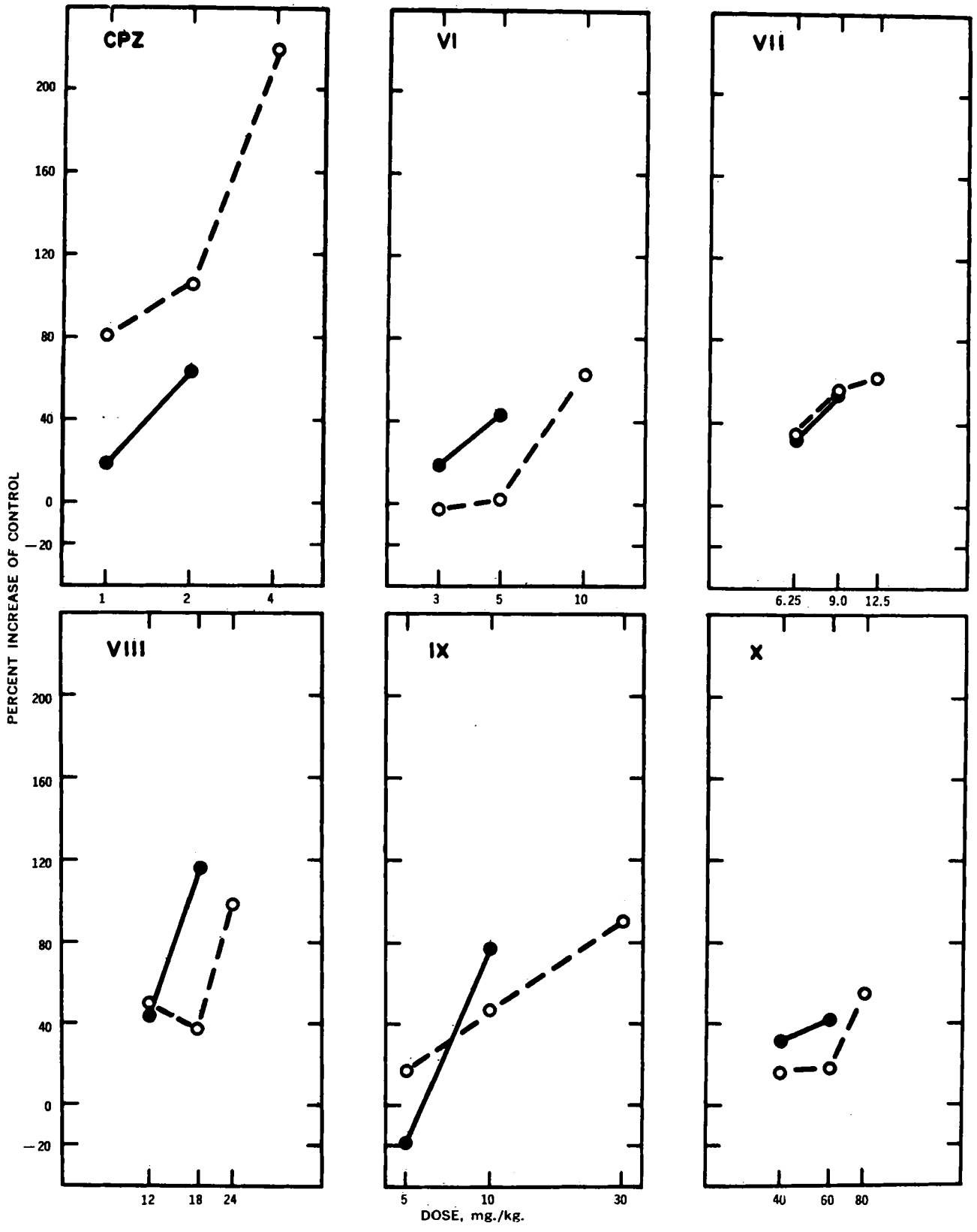

Figure 4—*Effects of chlorpromazine and Compounds VI–X on hexobarbital* (○) *and barbital* (●) *sleeping time in mice.*

safety index of the metabolites studied; however, it was slightly less than one-third as effective as chlorpromazine in inhibiting spontaneous motor activity, whereas the LD_{50}'s of the two compounds were approximately equal. The data obtained in this portion of the study suggest that the metabolites are less efficient in depressing CNS activity and more toxic than chlorpromazine.

None of the phenothiazine compounds investigated protected the animals from convulsions produced by pentylenetetrazol, strychnine sulfate, or maximal electroshock seizures.

Only Compound VII showed significant antireserpine activity in the low dose tested, and Compounds IV and V showed a significant potentiation of the effect of reserpine in decreasing rectal tempera-

Table VI—Potentiation of Hypothermic Effects of Reserpine (1 mg./kg. i.p.) by Compounds IV and V

Drugs	Dose, mg./kg. i.p.[a]	N[b]	Δ Temperature[c], $\bar{X} \pm SE$	p
Saline	10 ml./kg.	5	4.02 ± 0.26°	—
Desmethylimipramine	20	5	1.62 ± 0.17°	<0.001
V	20	5	5.48 ± 0.39°	<0.01
IV	50	5	5.58 ± 0.25	<0.01

[a] Administered 1 hr. prior to reserpine. [b] Groups of five mice each. [c] Decrease in rectal temperature 4 hr. after the administration of reserpine.

ture. No conclusion can be drawn from these data as to the effects of the dihydroxy and methoxy substitution to the chlorpromazine molecule as related to potential antidepressant activity. Halliwell *et al.* (13) tested imipramine, desipramine, chlorpromazine, and compounds structurally related to chlorpromazine. They found that while imipramine and its derivative markedly antagonized the reserpine-induced mydriasis and ptosis, chlorpromazine did not demonstrate these effects; thus their results were similar to the present findings.

The compounds affected sleeping time induced by both sodium hexobarbital, which is oxidatively metabolized by the hepatic microsomal enzymes (14), and sodium barbital, which is excreted unmetabolized (15). There were quantitative differences in the effects of each compound on the degree of potentiation of hexobarbital and barbital sleeping time. For example, if the effects of the medium dose of the compounds were compared, chlorpromazine and Compounds I and II had a greater effect on hexobarbital sleeping time; Compounds III, IV, VI, VIII, IX, and X had a greater effect on barbital sleeping time; Compound VII produced equivalent effects on both barbiturates; and Compound V produced a slight decrease in hexobarbital sleeping time without significantly affecting barbital activity. The enhancement of sleeping time induced by barbital, which is not metabolized, suggests that in those instances where the compounds potentiated hexobarbital, the activity was due to a direct depressant effect on the CNS and not merely to inhibition of hexobarbital metabolism. Compound V, the compound that had the lowest ED_{50} of the metabolites investigated (using effects on spontaneous motor activity as the assay procedure), produced minimal effects on barbital sleeping time in both doses used, increasing hexobarbital sleeping time only 6.5% with the low dose (5 mg./kg. i.p.) and 38.6% with the high dose (20 mg./kg. i.p.).

Compound X, in which the 10-[3-(dimethylamino)propyl] group of chlorpromazine is replaced by propionic acid, was the least active compound tested.

The inhibitory effects of Compound VII on spontaneous and forced motor activity, its relatively high safety index, and its antireserpine activity suggest that the metabolite may have potential utility as a therapeutic agent.

Table VII—Percentage of Mice Losing the Righting Reflex after Intraperitoneal Administration of Sodium Barbital (147.9 mg./kg.) at 30–60 min. after Intraperitoneal Injection of Chlorpromazine or the Experimental Compound

Compound	Dose, mg./kg.	Percent Losing Righting Reflex
Chlorpromazine	4	88
I	60	88
II	39	100
III	48	75
IV	50	75
V	20	75
VI	10	75
VII	12.5	38
VIII	24	38
IX	30	88
X	80	13
Saline	10 ml./kg.	25

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Muscle Relaxant Action of β-Hydroxylamine Cinnamonyl Hydroxamic Acid in Isolated Organs

HORST KEHL

Abstract □ β-Hydroxylamine cinnamonyl hydroxamic acid produced muscle relaxation in the guinea pig trachea, the rabbit jejunum, and the rabbit atria. This relaxing action was not blocked by α- or β-blocking agents, atropine and diphenhydramine; however, it could be antagonized with epinephrine, acetylcholine, and histamine. The degree of muscle relaxation was dose dependent and expressed itself in decreased force exerted and decreased frequency of spontaneous contractions. The β-hydroxylamine cinnamonyl hydroxamic acid-induced muscle relaxation could not be explained in terms of any known neurohumoral mediator inhibition and appears to be direct and attributable to the unique molecular structure of the hydroxamic acid itself.

Keyphrases □ β-Hydroxylamine cinnamonyl hydroxamic acid—muscle relaxant activity *in situ* □ Muscle relaxant activity, *in situ*—hydroxylamine cinnamonyl hydroxamic acid □ Hydroxamic acid, hydroxylamine cinnamonyl—muscle relaxant activity *in situ*

β-Hydroxylamine cinnamonyl hydroxamic acid (I) has been studied as an example of the generic group of hydroxamic acids. Earlier it was reported (1) that this compound showed vasomotor properties and seems to be active as a muscle-relaxing agent. The cardiovascular properties established the hypotensive action of this hydroxamic acid.

During these studies it also was observed that I was able to antagonize physostigmine-induced muscle fasciculations of skeletal muscles. Previously, it had been concluded that the hypotensive and cardiovascular actions of I were a result of interference of energy-generating mechanisms and that this property was inherent in the molecular structure of this hydroxamic acid (1).

To examine the muscle-relaxant properties of this compound in more detail and to lend support for its direct action, it was studied in three isolated organ systems. The three organ systems are representative of the adrenergic, cholinergic, and histaminergic systems.

To examine the effect on the adrenergic system, the isolated rabbit atria were selected; this tissue also exemplifies a muscle system with a strong autogenous contractile drive. The rabbit jejunum served as the model of the cholinergic system, a muscle contractile system with a moderate autogenous drive. The guinea pig trachea served as the representative histaminergic system and as a muscle system with a weak autogenous contractile drive.

EXPERIMENTAL

Adrenergic System—Twenty-three mongrel rabbits of either sex, weighing 2–3 kg., were used. Each animal was stunned with a blow on the back of the neck and the heart was excised immediately. The organ was quickly washed with warm oxygenated Locke solution to remove any residual blood. While the heart remained submerged in Locke solution, the atria were separated from the ventricles. The spontaneously beating atria were suspended in warm (37.5°) oxygenated Locke solution in a bath of 50 ml. Contractions were measured with a force-displacement transducer[1] and recorded with a polygraph[2]. The instrument was calibrated so that a 2-cm. deflection was equal to 1 g. tension force. The organ was permitted to attain equilibrium in the organ bath for 20–40 min., with frequent washings, before any experimental or control readings were taken.

After equilibrium, the number of beats per minute (frequency) was obtained in the following manner: the number of beats was counted for every alternate 6 sec. in a 2-min. period and averaged as the mean number of beats per minute. This was repeated at least three times for each experimental or control event taken. The respective means so obtained were then averaged as the mean of the means. The Student *t* test with self-paired analysis was applied for statistical evaluation.

Cholinergic System—Twenty-one mongrel rabbits of either sex were sacrificed in the same manner as already described. A midline incision was made, and approximately 5 cm. of jejunum was excised 20–25 cm. distal to the pylorus. The gut lumen was gently washed with warm (37.5°) Tyrode solution to remove all contents. Approximately 2.5 cm. of jejunum was suspended in warm Tyrode solution saturated with 95% oxygen and 5% carbon dioxide in an organ bath of the dimensions already described. Contractions were measured by the same force transducer and polygraph. The calibration was that a 2-cm. deflection equaled 1 g. force. The frequency of contractions was counted manually and evaluated in the same manner as described for the atria. The jejunum was equilibrated for 30 min. in the organ bath, with frequent washings, before any experimental or control readings were taken.

Histaminergic System—Twenty-four Hartley short hair guinea pigs of either sex were sacrificed in the similar manner as the rabbits. The ventral side of the neck was opened and about 3 cm. of trachea was removed. The trachea was washed and cleaned. Spiral tracheal strips of 0.5-cm. width and 2.0-cm. length (2) were prepared and mounted in the organ bath containing warm Tyrode solution saturated with 95% oxygen and 5% carbon dioxide. The organ bath used was the same type as already described. The trachea was permitted a 15–30-min. equilibrium period, with frequent washings, before any experimental or control readings were taken. Gram-force was recorded as described for the rabbit jejunum. No frequency measurements were taken.

All drugs used were diluted in either Locke or Tyrode solution and given in the least volume possible to produce minimal osmotic shock to the tissues. The hydroxamic acid was dissolved in either Tyrode or Locke solution without altering the pH of these solutions. The concentration of the β-hydroxylamine cinnamonyl hydroxamic acid was 50 mg./ml. for the atria, 10 mg./ml. for the jejunum, and 2.5 mg./ml. for the trachea, which, in turn, made the bath concentrations of 1 mg./ml. for the atria, 0.2 mg./ml. for the jejunum, and 0.05 mg./ml. for the trachea possible.

Calcium determinations of the organ bath fluid were carried out by the method of Sherrick and de la Huerga (3).

RESULTS

Effects of β-Hydroxylamine Cinnamonyl Hydroxamic Acid on Spontaneously Beating Isolated Rabbit Atria—Alterations of the contractile rate of the spontaneously beating rabbit atria were observed only after relatively high doses of I (0.8–1.0 mg./ml. bath). Changes in the contractile force become evident at 0.4 mg./ml. bath; significant changes were observed at concentrations of 1 mg./ml. bath (Figs. 1 and 2). Incremental increases in concentrations of I from 0.8 to 2.0 mg./ml. bath produced a log dose linear reduction for both frequency and gram-force exerted by the atria (Figs. 1 and 2).

[1] FT03C, Grass.
[2] Grass model 15.

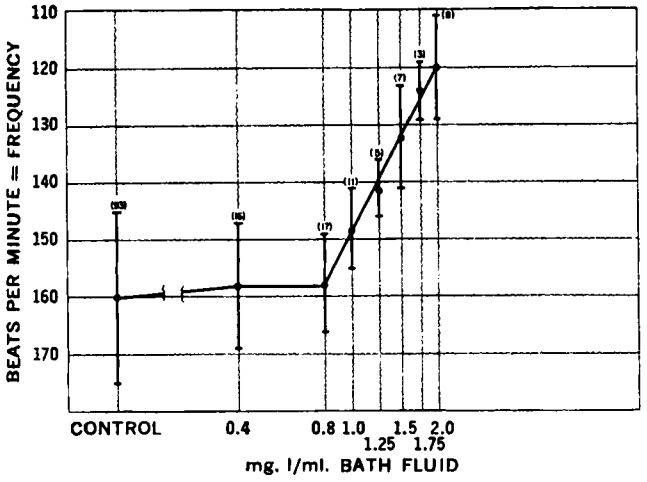

Figure 1—*Log dose-response plot of beats per minute (frequency) of the isolated rabbit atria. A log linear response was observed with incremental increases of concentrations of I when a least-squares fit was applied for the range of 0.8–2.0 mg./ml. bath. Frequencies were recorded as the mean of the means with the standard deviations indicated. The number of means used for each concentration is indicated by the number in parentheses.*

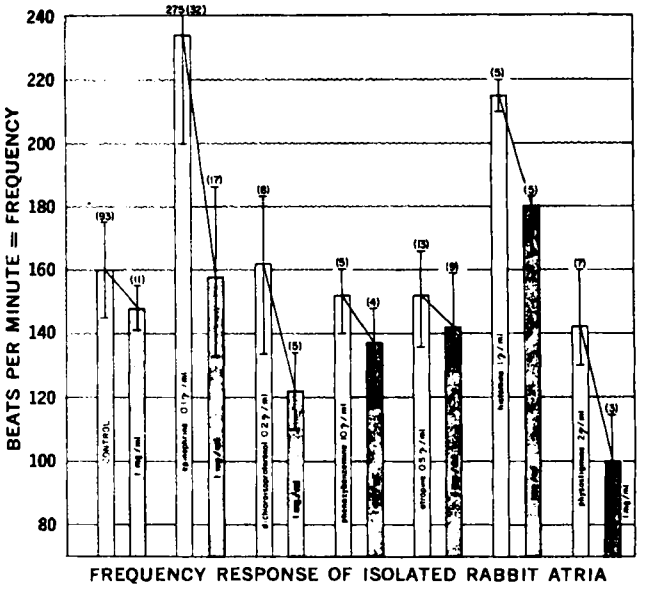

Figure 3—*Histogram of beats per minute (frequency) of the isolated rabbit atria treated with I in the presence of various agonists and blocking agents. The shaded bars represent the addition of 1.0 mg. I/ml. bath. Frequencies were recorded as the mean of the means with the standard error indicated. The number of means evaluated is indicated by the number in parentheses. The concentrations for the agonists and blocking agents were expressed as per milliliter of bath. In the histogram the effect of the agonist after the block was omitted since no significant change of frequency was recorded.*

Higher concentrations of I produced a state of contracture of the atria associated with an increase in tension and a further reduction of frequency with no alteration of amplitude.

To determine the efficacy of I on the force and rate of the atrial contractions, the agonists epinephrine and histamine were selected. Under control conditions a standard dose of I (1 mg./ml. bath) produced an average reduction of the frequency of the contractions by 12 beats/min., while a simultaneous reduction of an average of 0.48 gram-force occurred. After stimulating the atria with 0.1 mcg. epinephrine/ml. bath, the addition of the standard dose produced an average reduction of 76 beats/min. while at the same time a 0.79-g. average reduction of force occurred. Stimulating the atria with 1 mcg. histamine/ml. bath followed with the standard dose of I led to an average reduction of 35 beats/min. and an average reduction of 1.40 gram-force (Figs. 3 and 4).

To ascertain if the actions of I were direct and not through release or inhibition of mediators, the effects of several blocking agents on the response of I were studied. In all cases the efficacy of the block was tested by challenging the tissue with the appropriate agonist. After the addition of 0.2 mcg. dichloroisoproterenol/ml. bath, no changes in frequency and gram-force from the control values were observed. The addition of the standard dose of I in the presence of the challenger epinephrine and the β-blocking agent in the tissue bath now produced an average reduction of frequency of 40 beats/min. and an average reduction of gram-force of 0.53 g. The addition of 10 mcg./ml. phenoxybenzamine to the organ bath resulted in a moderate reduction in frequency of the atria with no change in gram-force. After verifying the adequacy of the block with norepinephrine, the standard dose of I produced, in the presence of the block and the agonist, an average reduction of 15 beats/min. and a 0.22-

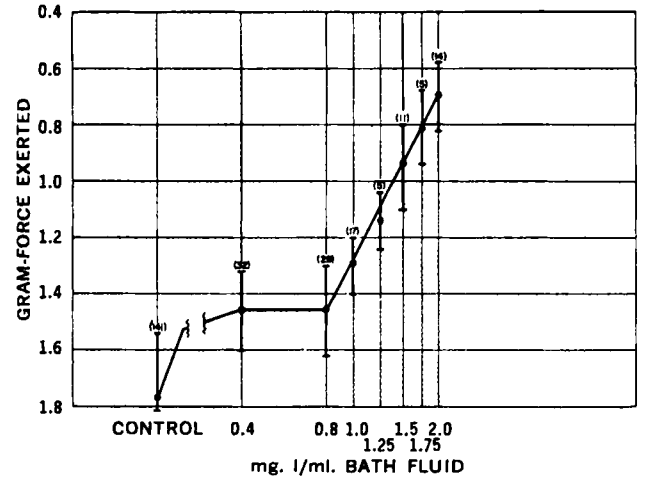

Figure 2—*Log dose-response plot of the gram-force exerted by the isolated rabbit atria after incremental concentration increases of I. A log linear response was obtained when a least-squares fit was applied for the concentration range of 0.8–2.0 mg./ml. bath. Gram-force was recorded as the mean of the means with the standard deviations indicated. The number of means used for each incremental increase is indicated by the number in parentheses.*

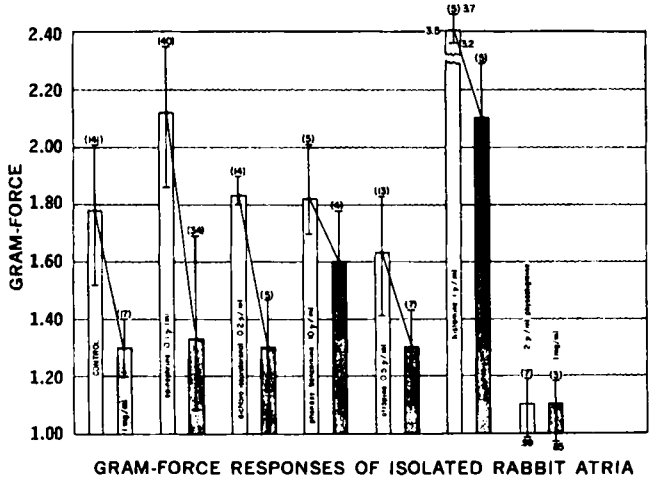

Figure 4—*Histogram of the reduction of gram-force exerted by the isolated rabbit atria. The shaded bars represent I (1.0 mg./ml. bath). The gram-force was tabulated as the mean of the means with the standard deviation indicated for each bar. The number in parentheses represents the means evaluated for this bar. The responses of the atrium are shown after agonists and in the presence of blocking agents. The effect of agonists after blocking agents was omitted since no significant change in response was calculated.*

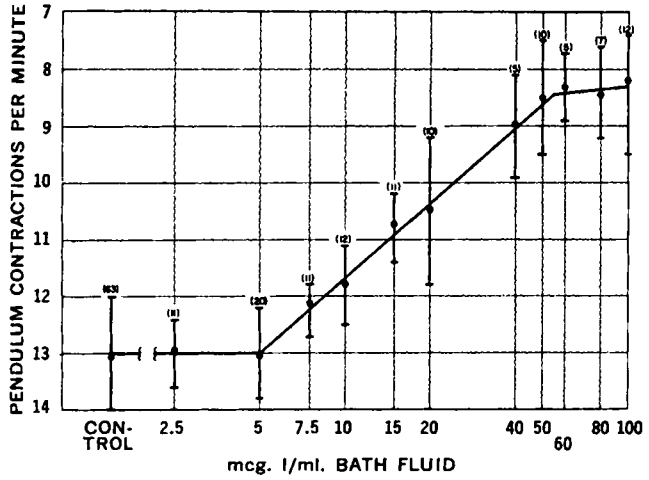

Figure 5—*Log-dose response plot of pendulum contractions (frequency) of the isolated rabbit jejunum. A log linear response was observed with incremental increases of concentrations of I when a least-squares fit was applied for the concentration range of 5.0–50.0 mcg./ml. bath. Before 5 mcg./ml. bath of the compound, a level response was noted; at concentrations greater than 50.0 mcg./ml., a ceiling effect was observed. Frequencies were recorded as the mean of the means with the standard deviations indicated. The number of means used for this concentration is indicated by the number in parentheses.*

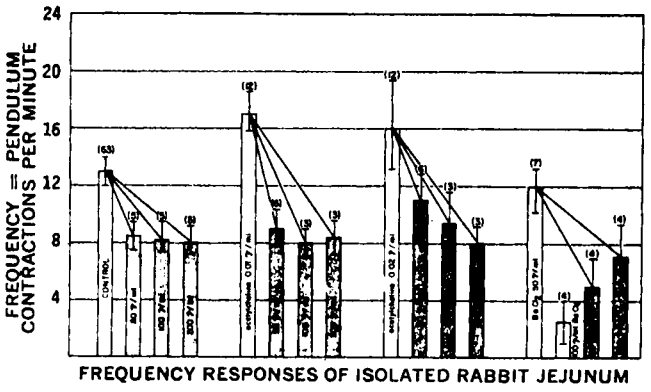

Figure 7—*Histogram of the pendulum contractions per minute (frequency) of the isolated rabbit jejunum treated with I in the presence of two agonists and two different concentrations for one agonist. The shaded bars represent I at 50, 100, and 200 mcg./ml. as indicated. All concentrations are per milliliter of bath. The frequencies were recorded as the mean of the means for each bar with the standard deviations shown. The number of means used for each bar is indicated by the number in parentheses.*

gram-force reduction. The addition of 0.5 mcg./ml. of atropine to the bath produced a moderate reduction in both the frequency and gram-force of the atria in comparison to the control values. After ascertaining the adequacy of the atropine block with 0.01 mcg. acetylcholine/ml. bath, the standard dose of I produced an average reduction of 10 beats/min. in frequency and a 0.33-gram-force reduction from the atropine values. Physostigmine (2 mcg./ml. bath) produced a marked reduction of the frequency and gram-force exerted by the atria. The addition of the standard dose of I to the bath produced a further reduction of the frequency of 42 beats/min., while no change was observed in the gram-force (Figs. 3 and 4).

Effects of β-Hydroxylamine Cinnamonyl Hydroxamic Acid on Isolated Rabbit Jejunum—Minimum concentrations of I to inhibit spontaneously occurring rhythmic pendulum contraction and the gram-force exerted by the jejunum were 7.5 mcg./ml. Incremental increases of I from 5 to 50 mcg./ml. yielded a linear log dose reduction in frequency of the spontaneous contractions. At concentrations above 50 mcg./ml., a ceiling effect was observed (Fig. 5). Incremental increases of I in the range of 5–20 mcg./ml. produced a linear log dose reduction in the gram-force exerted by the jejunum. At concentrations above 20 mcg./ml., a ceiling response was produced (Fig. 6). A concentration of 500 mcg./ml. of I produced a total paralysis of the jejunum which, upon washing, was restored to the normal control rhythm and force.

The actions of I were examined on the spontaneously contracting rabbit jejunum, on the acetylcholine-stimulated jejunum, and in the presence of barium chloride as a nonspecific stimulator for the jejunum. Control pendulum contractions of 13/min. were reduced to an average of 8.5 contractions/min. with 50 mcg. I/ml. bath, to 8.2 contractions/min. with 100 mcg. I/ml. bath, and to 8.0 contractions/min. with 200 mcg. I/ml. The average control gram-force was reduced to a 0.91 gram-force with 50 mcg./ml. and to 0.85 gram-force at 100 mcg. I/ml. and to the same average with 200 mcg. I/ml. (Figs. 7 and 8). Stimulating the jejunum with 0.01 mcg. acetylcholine/ml.

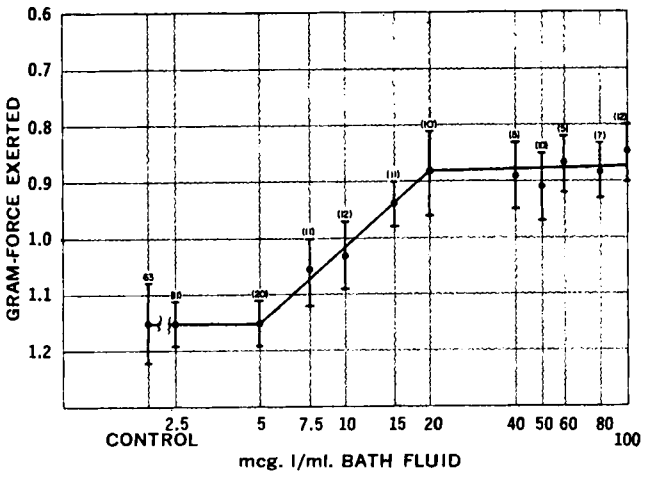

Figure 6—*Log-dose response plot of the reduction of the gram-force exerted by the isolated rabbit jejunum after incremental increases of I concentrations in the bath. A log linear response was obtained when a least-squares fit was applied for the concentration range of 5.0–20 mcg. I/ml. bath. No response was seen before 5 mcg./ml., while a ceiling effect was noted after 20 mcg./ml. The gram-force was recorded as the mean of the means with the standard deviations shown. The number of means used for this concentration is indicated by the number in parentheses.*

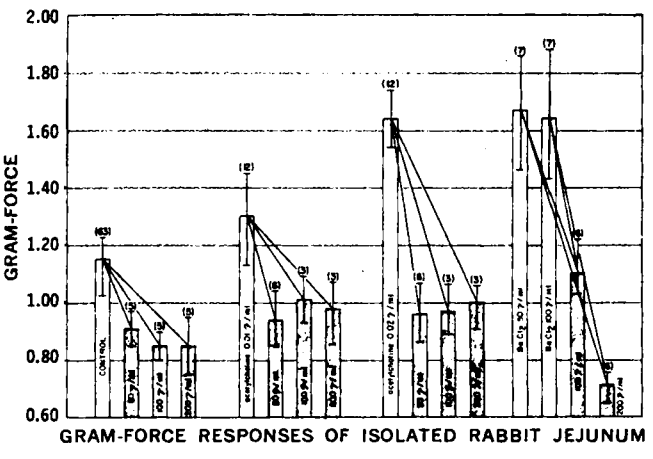

Figure 8—*Histogram of the reduction of gram-force exerted by the isolated rabbit jejunum treated with I at three different concentrations. The shaded bars represent the compound at concentrations of 50, 100, and 200 mcg./ml. of bath. The gram-force was tabulated as the mean of the means with the standard deviations shown. The number in parentheses represents the number of means evaluated for each bar. The concentrations of the two agonists are per milliliter of bath. The responses induced by the compound after barium chloride were tabulated together since no significant difference of the response to the two concentrations of barium chloride was noted.*

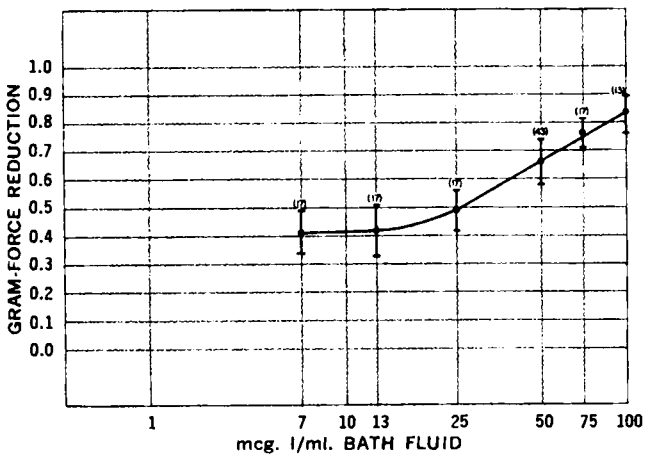

Figure 9—*Log-dose response plot of the reduction of gram-force exerted by the isolated guinea pig trachea after incremental increases of I in the bath. A log linear response was obtained with a least-squares fit applied for the concentration range of 25–100 mcg./ml. bath. No significant response was seen before 25 mcg./ml. bath. The gram-force was recorded as the mean of the means with the standard deviation shown for each incremental increase of I. The number in parentheses represents the number of means evaluated for this concentration.*

bath led to an increase in both frequency of rhythmic contraction and gram-force exerted over the control values: 17 contractions/min. and 1.30 gram-force, respectively (Figs. 7 and 8). A dose of 50 mcg. I/ml. bath in the presence of the same amount of acetylcholine led to a reduction of the frequency of the pendulum contractions to an average of 9.0 contractions/min., while a reduction of the gram-force to an average of 0.94 g. occurred. Increasing the concentration of I to 100 and 200 mcg./ml. did not produce changes significantly different from the responses produced by the addition of 50 mcg./ml. (Figs. 7 and 8).

The addition of 0.02 mcg. acetylcholine/ml. bath led to an average response of 16 contractions/min. and 1.64 gram-force. When repeating the concentrations of 50, 100, and 200 mcg. I/ml. bath, the following values for the frequency of contractions were observed: 11.0, 9.4, and 8.0 contractions/min., respectively, and gram-force response was 0.96, 0.97, and 1.00 g., respectively. The isolated rabbit jejunum was exposed to barium chloride (50 and 100 mcg./ml. bath) and a marked increase in gram-force (1.67 and 1.64, respectively) was observed. The frequency of pendulum contractions for 50 mcg. $BaCl_2$/ml. bath was not significantly different from the control value; 100 mcg. $BaCl_2$/ml. bath led to a pronounced reduction of the pendulum frequency: 2.5 contractions/min. A concentration of 100 mcg. I/ml. bath in the presence of either 50 or 100 mcg. $BaCl_2$/ml. led to a reduction of gram-force to 1.10 g. Using 200 mcg. I/ml. bath in the presence of either 50 or 100 mcg. $BaCl_2$/ml. produced a reduction to 0.72 gram-force; 100 mcg. I/ml. bath produced 5.0 pendulum contractions/min. in the presence of 50 mcg. $BaCl_2$/ml., while 200 mcg. I/ml. produced 7.2 contractions/min. in the presence of 50 mcg. $BaCl_2$/ml. No pendulum contractions were measured after 100 mcg. $BaCl_2$/ml. since the jejunum went into spasmodic contracture even after a small amount of I (25 mcg. I/ml.) (Figs. 7 and 8). The reductions of the pendulum contractions and gram-force after the addition of I to the bath could be counteracted by the addition of acetylcholine or the removal of I from the bath by washing. Normal control values for both the contractions per minute and the gram-force could be restored.

Effects of β-Hydroxylamine Cinnamonyl Hydroxamic Acid on Isolated Guinea Pig Trachea—The minimum amount of I required to produce a degree of relaxation in the rather quiescent guinea pig trachea was 25 mcg. I/ml. bath. Increasing the concentration of I from 25 to 100 mcg./ml. bath produced a linear log dose reduction of the gram-force exerted by the guinea pig trachea (Fig. 9). No pendulum contractions could be measured in this organ system. The average control value for the gram-force of the trachea was 1.6 g. This could be reduced to an average of 0.94 g. or a reduction of 0.66 g. from the control values after the addition of 50 mcg. I/ml. bath. The addition of 8 mcg. histamine hydrochloride/ml. bath produced a sustained contraction of the trachea (1.82 gram-force), which could be antagonized with a dose of 50 mcg. I/ml. bath. Introducing 50 mcg. I/ml. to the bath fluid before the addition of histamine (8 mcg./ml.) diminished the histamine response by an average of 0.76 gram-force. In this case, I behaved much as an antihistamine would by inhibiting the histamine response (Fig. 10).

A similar observation was made when acetycholine was substituted for histamine as the agonist. The addition of 1 mcg. acetylcholine/ml. bath increased the force to 1.78 g., and this could be antagonized with 50 mcg. I/ml. bath to reduce the force to 1.12 g. Placing 50 mcg. I/ml. into the bath before the addition of acetylcholine diminished the acetylcholine response by an average of 0.68 gram-force. Relaxation of the trachea could be achieved with I when the organ had been pretreated with dichloroisoproterenol followed by histamine stimulation and β-block challenge with isoproterenol. The gram-force produced during the histamine stimulation was 1.82 g., while isoproterenol (0.08 mcg./ml. bath) produced a reduction of 1.40 g. which did not differ from the control value. When this preparation was followed with 50 mcg. I/ml., a reduction to 0.88 g. could be observed. Stimulating the trachea with histamine (8 mcg./ml.) produced a force increase of 1.82 g. Dichloroisoproterenol (10 mcg./ml.) in the same bath led to a moderate reduction of force of 1.66 g. Adding 50 mcg. I to this preparation produced a reduction of force to 1.00 g. Diphenhydramine (10 mcg./ml.) inhibited the histamine response to an average value of 1.44 gram-force. Adding 50 mcg. I/ml. to this preparation produced a reduction of 0.40 gram-force (Fig. 10).

Calcium Analysis of Organ Bath in the Presence of β-Hydroxylamine Cinnamonyl Hydroxamic Acid—To exclude the possibility that I may interfere with the availability of ionic calcium in the bath and, therefore, inhibit contractions, a series of calcium anal-

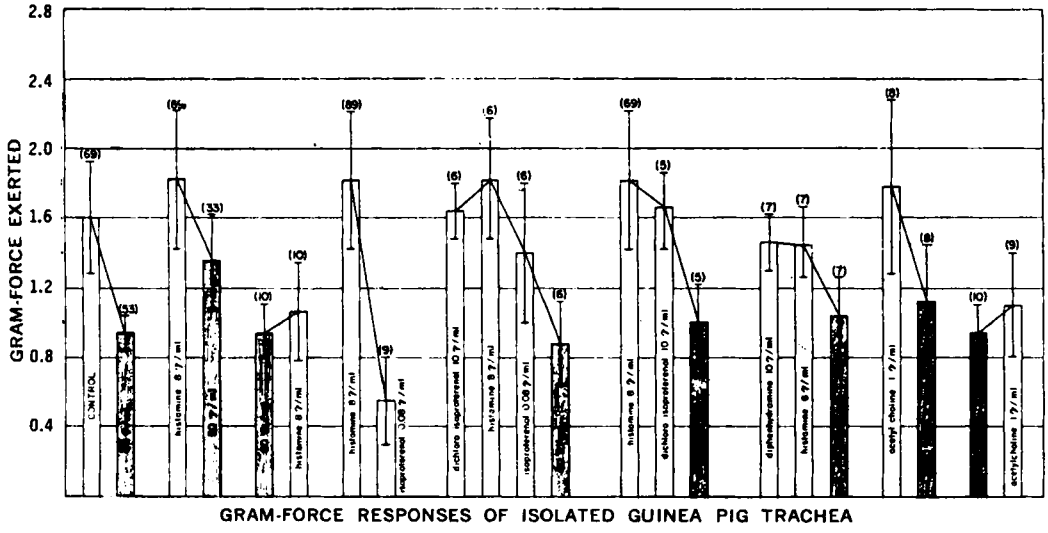

Figure 10—*Histogram of the reduction of gram-force exerted by the isolated guinea pig trachea after treatment with I (50 mcg./ml. bath; shaded bars) in the presence of agonist and blocking agents. The gram-force was tabulated as the mean of the means with the standard deviation shown for each tabulation. The number in parentheses represents the number of means evaluated for each condition studied. The concentrations for the agonists and blocking agents are expressed as per milliliter of bath.*

yses was carried out on organ bath preparations of the atria. Table I reveals no difference in values for the control concentrations of calcium and the calcium concentrations in the bath when I was present. By using ethylenediaminetetraacetate disodium salt as a chelating agent, ionic calcium was removed from the bath in amounts equal to 25, 50, 75, or 100% of the total. This produced morphologically distinct different relaxation in the atria than I produced. While I produced a contracture of the atria at higher concentrations, ethylenediaminetetraacetate produced a total relaxation with paralysis at 100% removal of calcium ion.

Table I—Ionic Calcium Analysis (3)[a]

	Control, mg. Ca^{+2}/ 50 ml. Bath	1 mg. I/ml. Bath, mg. Ca^{+2}/ 50 ml. Bath
	3.9	4.1
	4.2	4.1
	4.1	4.2
	4.5	4.4
	4.4	4.4
	4.3	4.2
	4.1	4.3
	3.7	3.5
	3.9	4.4
	4.3	4.3
	4.7	4.1
	4.3	4.3
Average[b]	4.2 mg. Ca	4.2 mg. Ca

[a] The calculated theoretical calcium concentration in the Locke solution used for the rabbit atria based on the addition of anhydrous $CaCl_2$ for the preparation of the solution should be 4.3 mg./50 ml. bath. Both the control and experimental values represent the mean of three samples in a series of 12 analyses from 12 different experiments with the atria. [b] The average values represent 36 control and 36 experimental analyses.

DISCUSSION

These isolated organ experiments clearly show that β-hydroxylamine cinnamonyl hydroxamic acid (I) produced inhibition of autogenous contractile mechanisms. This inhibition was, however, not limited to the autogenous drive but was also active in the presence of an agonist that magnified the autogenous contractile drive. It became evident that this inhibition of the contractile drive was not limited to one particular mediator system. The degree of inhibition was related to the concentrations of I in the bath as well as to the strength of the autogenous contractile drive. In the atria, an organ system with a very strong autogenous contractile mechanism, the minimum concentration of I to show any degree of inhibition was 400 mcg./ml. bath. This is thought to be a high concentration when compared with the amount of I needed for inhibition of the jejunum and guinea pig trachea, organs with a much weaker autogenous contractile mechanism.

The inhibition of the contractile mechanism by I was, in all cases, reversible by washing the organ preparation and, therefore, by removing I from the bath as well as from the hypothetical receptor site. The inhibitory effect of I could also be neutralized in the case of the atria with epinephrine or histamine, both potent agonists (4) for the atria that enhance its contractile force and frequency (Figs. 3 and 4).

With the rabbit jejunum, the minimum amount of I needed to effect a reduction of both the force and the frequency of the spontaneously occurring pendulum contractions was 7.5 mcg. I/ml. Inhibition of these parameters by I could be counteracted by acetylcholine or barium chloride. Normal control values could be restored by washing the jejunum and removing I from the bath fluid. The apparent smaller amount of I needed to induce some degree of inhibition in the jejunum in comparison to the atria is to be related to the much stronger spontaneous autogenous drive of the atria in contrast to the weaker drive in the jejunum. For the compound to be pharmacodynamically active, the internal contractile drive mechanism must be overcome; since the atria exhibit a much greater force and frequency of autogenous drive, the amounts of I to inhibit this drive would logically be larger than in the jejunum. In the jejunum, I produced a ceiling effect over a wider concentration range in contrast to the atria. While the atria underwent contracture after the dose of 2.00 mg. I/ml. bath had been reached, the jejunum had a latitude of 50–500 mcg. I/ml. bath before paralytic contracture occurred. In the case of the gram-force, a dose range of 20–500 mcg./ml. bath was needed for the jejunum.

Pretreatment of the jejunum preparation with acetylcholine (0.01 or 0.02 mcg./ml.) produced an increase in contractile frequency and force. When acetylcholine was followed by the introduction of I into the same bath fluid, a notable reduction of force and contractile frequency could be recorded. This indicates that I was able to interfere with either hormonally induced or spontaneously occurring contractions. The addition of barium chloride (50 mcg./ml.) to the bath produced a marked increase in the force of contraction while no significant change in frequency occurred. The addition of I to the bath in the presence of barium chloride did lead to a reduction of both frequency and force. The ability of I to antagonize the direct stimulation of the jejunum by barium chloride would indicate that I was interfering with the energy-generating machinery of the cell.

The reduction in the force of contraction of the guinea pig trachea produced by I was counteracted by the addition of either histamine (8 mcg./ml.) or acetylcholine (1 mcg./ml.). Conversely, I was able to reduce the histamine or acetylcholine-induced increases in force, behaving, in the case of the histamine stimulation, not unlike isoproterenol. However, when I was placed into the bath prior to the addition of histamine, the response produced by histamine or acetylcholine was suppressed. Moreover, I was able to produce a reduction of force in the presence of dichloroisoproterenol and diphenhydramine, indicating that the actions of I are not through any of the known mediator systems operating within tracheal tissue.

The persistent actions of I in the presence of a β-block (dichloroisoproterenol) and a histamine block (diphenhydramine) are not limited to the guinea pig trachea, since similar observations were made with the rabbit atria. As with the trachea, I was able to exert its depressing action on the force and rate of contractions of the atria in the presence of a β-block, in the presence of an α-block (phenoxybenzamine), and in the presence of atropine. These observations are in agreement with the earlier observed and postulated mode of action of I (1).

The maintenance of active contractile force in any tissue is dependent on the availability of the enzymes involved in an intact ATP system (5–7). It is suggested that I interferes with this enzyme system and, therefore, reduces the energy available to the cells for contraction. These enzymes are mostly bound to the cell membrane (8) and it is postulated that it is here that I exerts its pharmacodynamic actions, possibly by interfering with conformational changes in the receptor enzyme protein system needed to propagate the energy impulse.

Compound I did not reduce the calcium concentration in the bath and, therefore, did not change the availability of calcium to the tissues. Neither was the calcium concentration increased, excluding calcium extraction for the organ preparation. However, alteration of the calcium pump at the sarcoplasmic level so necessary for the generation of the contractile forces was not excluded.

Fishbein and Carbone (9) stated that hydroxyurea was converted to acetohydroxamic acid and that the hydroxamic acid diminished oxidative phosphorylation with a reduced ATP content in the mitochondria. In other work, it was reported (10) that hydroxyurea prevents incorporation of labeled phosphor into DNA. Since Fishbein and Carbone (9) reported that the hydroxyurea was converted to acetohydroxamic acid and Coutts (11) reported that hydroxamic acids were preferentially reacting with the coenzyme pyridoxal phosphate, it is possible that the inhibition shown by I was interference with phosphate transfer necessary for energy generation of a contraction. Coutts also reported that hydroxylamine cleaves the acetyl coenzyme A and that this leads to a cellular as well as a mitochondrial inhibition of ATP. Since I possesses a hydroxylamine group in the β-position, it is probable that not only the hydroxamic acid moiety is responsible for the pharmacodynamic action, but the hydroxylamine group may aid in the production of the muscle relaxation characteristic of I. The observations that I exerted its inhibitory action in the presence of various agonists, in different tissues and in the presence of blocking agents, would indicate that the relaxation was a result of inhibition of a common energy system.

It was reported (12) earlier that decanohydroxamic acid possesses papaverine-like, antiacetylcholine-like, and antihistamine-like ac-

tions. It may be that the generic group (hydroxamic acids), due to their peculiar molecular structure such as the R—C(=O)—N(—H)—OH moiety, are a group of oxidative phosphorylation uncoupling agents and compete for the energy available within the cellular contractile mechanism.

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NMR Study on 1,2,4,5-Tetrasubstituted 3,3,6,6-Tetradeuterated Cyclohexanes: Conformational Contributions and 1,3-Diaxial and Vicinal Deshielding Effects of Amino and Alkylated Amino Groups and Their Protonated Forms

BARTLETT D. WHELTON*, BETTY R. LOWRY, JOHN B. CARR†, and ALAIN C. HUITRIC▲

Abstract □ The partially deuterated compounds *trans*-2-*o*-tolyl-*cis*-4-hydroxy-*trans*-5-aminocyclohexanol-3,3,6,6-d_4 and *trans*-2-*o*-tolyl-*cis*-4-amino-*trans*-5-hydroxycyclohexanol-3,3,6,6-d_4, the corresponding *N*-methyl and *N*,*N*-dimethyl derivatives, the trimethylammonium iodide salts, the C-1 benzoyloxy esters of all these compounds, and a few C-4 acetoxy derivatives were investigated by NMR for conformational changes brought about by alkylation and protonation of axially oriented amino groups and for 1,3-diaxial and vicinal deshielding effects of amino and protonated amino groups. In these compounds, hydrogens H-1 and H-2 and hydrogens H-4 and H-5 make up two separate *AX* systems where, in a given chair conformation, the hydrogens of one pair have *trans*-diaxial orientations and those of the other pair have *trans*-diequatorial orientations. Changes in coupling constants J_{12} and J_{45} give a sensitive measure of conformational changes. The most pronounced conformational changes occurred with protonation of the dimethylamino derivatives in formic acid and in chloroform, where the equilibrium highly favors the inverted chair conformations with an axially oriented *o*-tolyl group. The 1,3-diaxial deshielding of the primary amino group was found to be somewhat comparable to that of the hydroxyl group, with little change in the effect upon protonation. The effects of *N*-methylation are discussed. Geminal and vicinal deshielding by the amino group and the role of *N*-methylation and protonation on these effects are given. Further data supporting the long-range deshielding by the carbon–carbon double bond and the epoxy group are also presented.

Keyphrases □ Cyclohexanes, 1,2,4,5-tetrasubstituted 3,3,6,6-tetradeuterated—NMR study, conformation contributions and deshielding effects of amino groups and protonated forms □ Coupling constants, 1,2,4,5-tetrasubstituted 3,3,6,6-tetradeuterated cyclohexanes—conformation changes produced by alkylation and protonation of axial amino groups □ Deshielding effects, 1,3-diaxial and vicinal—1,2,4,5-tetrasubstituted 3,3,6,6-tetradeuterated cyclohexanes, conformation changes produced by alkylation and protonation of axial amino groups □ NMR spectroscopy—tetrasubstituted cyclohexanes, conformation changes produced by alkylation and protonation of axial amino groups, vicinal deshielding effects of amino groups, coupling constants

A number of partially deuterated six-membered ring compounds have been prepared in this laboratory in recent years for NMR conformational analysis studies (1–4) and for study of long-range 1,3-diaxial deshielding of ring hydrogens by the hydroxyl group (5). On the basis of this previous work, the substituted 3,3,6,6-tetradeuterated cyclohexene (III) was easily available as an ideal intermediate for the synthesis of the tetradeuterated compounds of series VI–IX, of known stereochemistry, through the epoxides IV and V. The stereochemistry for IV and V was established for the nondeuterated epoxides (6). The deuterated compounds of series VI through IX were of special interest for the study of possible conformational changes as a function of solvent resulting from successive alkylation of the amino group and/or from protonation of the primary, secondary, and tertiary amines. The tetradeuterated compounds also provide useful systems for the study of the relative 1,3-diaxial deshielding properties of primary, secondary, and tertiary amino groups and the effect of protonation on this property. The series further proved useful in demonstrating the deshielding effect of protonation of the amino groups on the chemical shifts of vicinal hydrogens.

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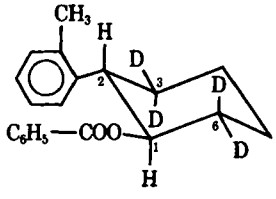
A very important feature of the tetradeuterated compounds, VI–IX, is that the four hydrogens on the cyclohexane ring form two separate *AX* systems, whose coupling constants give a very sensitive NMR probe for the study of conformational changes. Since each ring hydrogen is adjacent to only one other hydrogen, with the exception of the hydrogen on the amino-bearing carbon, the NMR signal of each gives a simple doublet which allows accurate measurements of coupling constants and chemical shifts from first-order treatment of the spectra. The components of each doublet are broadened slightly by weak coupling with the deuterium atoms on adjacent carbons, but this does not interfere with the measurement of coupling constants between hydrogens of individual *AX* pairs. Occasionally, there was coupling with the hydroxyl hydrogen, especially in dimethyl sulfoxide-d_6, but this could be circumvented by addition of a small quantity of D_2O.

RESULTS AND DISCUSSION

Conformational Study—Coupling constants between axial hydrogens or adjacent carbon atoms (J_{aa}) are normally around 11 Hz., and those between equatorial hydrogens (J_{ee}) are usually in the neighborhood of 3 Hz. (1). In the 1,2,4,5-tetrasubstituted 3,3,6,6-tetradeuterated cyclohexanes under consideration, there are two separate *AX* systems of *trans*-hydrogens. In structures having the chair conformation, the hydrogens of one vicinal pair have axial–



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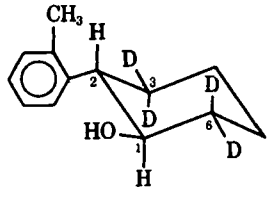

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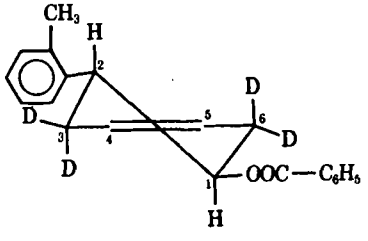

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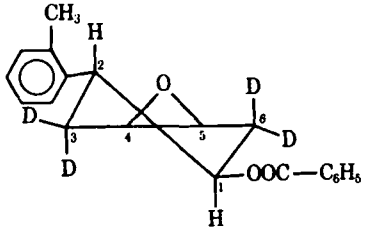

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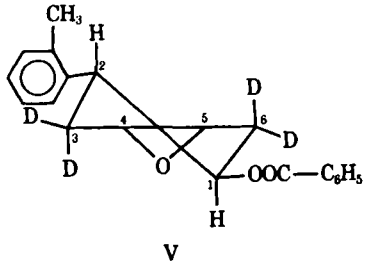

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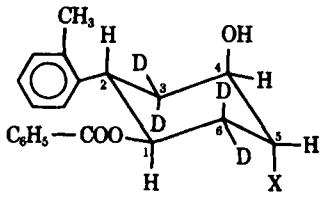

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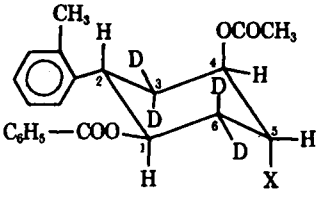

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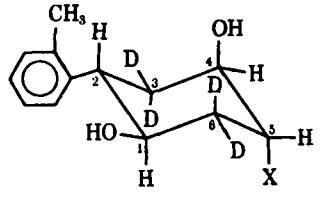
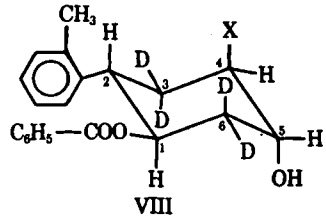


VII



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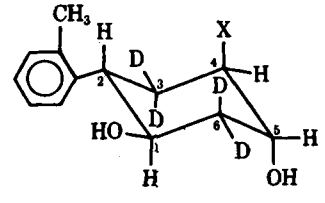
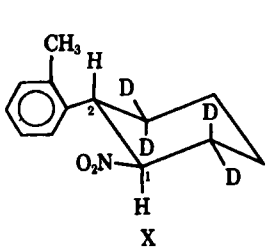


IX

VI-IX*a*: X = NH_2
VI-IX*b*: X = NH—CH_3
VI-IX*c*: X = $N(CH_3)_2$
VI-IX*d*: X = $\overset{+}{N}(CH_3)_3Cl^-$
VI-IX*e*: X = deuterium
VI-IX*f*: X = OH
VI-IX*g*: X = N=C(CD_3)(CD_3)



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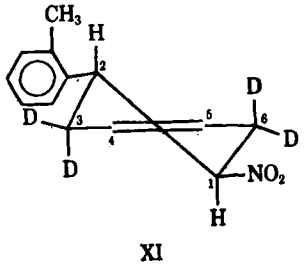
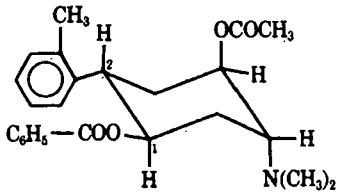


XI



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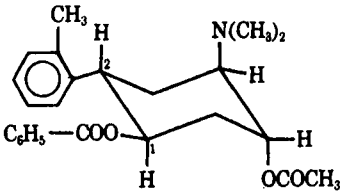

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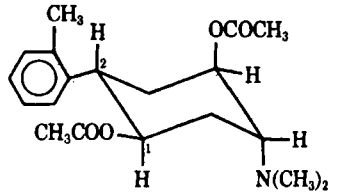

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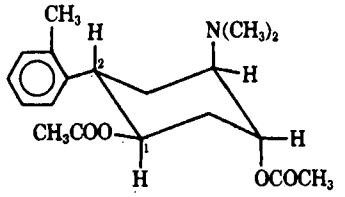

XV

methiodides of Compounds XII–XV are included

axial orientations while those of the other pair are equatorial–equatorial. Since the assignment of signals was possible in all cases, the coupling constants J_{12} and J_{45} provide a unique NMR probe for determining the preference between the two chair conformers in systems where the equilibrium favors a large predominance of one of the two possible chair conformations. In such a system, either J_{12} or J_{45} will be the normal J_{aa} value, while the other one will be the normal J_{ee} value. In cases where J_{12} and J_{45} are essentially equal, the system does not give a direct differentiation between an equilibrium involving nearly equal populations of the two chair conformers with the exclusion of other conformers and a situation where the equilibrium involves partial or total contribution from boat and other flexible conformations, because in the flexible conformations the average dihedral angle between H-1 and H-2 will be the same as between H-4 and H-5. It is important to realize that unequal J_{12} and J_{45} values indicate a predominance of one chair conformation over the other; but in cases where neither coupling constant approaches the normal J_{aa} value, the system does not provide a direct differentiation between an equilibrium involving only the two chair conformers in unequal populations and an equilibrium involving one or both chair conformers and flexible forms.

The normal J_{aa} value is seen from Table I to be 11.0 Hz. in the reference benzoate I and about 10.0 Hz. in the corresponding hydroxy Compound II in the various solvents investigated. The data also show conformational changes in the 1,2,4,5-tetrasubstituted compounds which vary from partial to total chair inversion. The roles of solvent, alkylation, protonation, quaternization, and intramolecular hydrogen bonding on conformational changes are also demonstrated by the J_{12} and J_{45} values in Table I.

Effects of Intramolecular Hydrogen Bonding—The J_{12} and J_{45} values indicate that in hydrogen-bonding solvents, which act as hydrogen acceptors, the equilibria for the free base forms of the primary, secondary, and tertiary amines of classes VI through IX favor a high predominance of the normal chair conformation having the *o*-tolyl group on C-2 and the benzoate or hydroxyl group on C-1 in equatorial orientations, with the amino and hydroxyl or acetoxy group on C-4 and C-5 in axial orientations. In deuterated chloroform, the J_{12} and J_{45} values of 7.3 and 5.9 for the dimethylamine VI*c* indicate a reduction in the population of the normal chair conformation. Threefold dilution did not change the J_{12} and J_{45} values. The acetoxy derivative VI*c*Ac shows a high predominance of the normal chair form. The conformational change in VI*c* in chloroform is attributed to the stabilization of the inverted chair or some flexible conformation through intramolecular hydrogen bonding between the C-4 hydroxyl and C-5 amino groups. Because of intermolecular hydrogen bonding between the compounds and solvent molecules in acetone, pyridine, and dimethyl sulfoxide-d_6, intramolecular hydrogen bonding will not have the same degree of stabilization of the inverted conformation in these solvents as in chloroform. For reasons discussed later, the inverted chair is favored over flexible forms. The presence of intramolecular hydrogen bonding in chloroform was demonstrated in the nondeuterated Compounds VI*c* and VIII*c* by IR spectroscopy. In each case, the spectra showed a nonbonded OH stretching band at 3610 cm.$^{-1}$ and a broader bonded OH band at 3440 cm.$^{-1}$. This latter band persisted when the solution was diluted to 0.01 *M*.

The conformation free energy values ($-\Delta G$) for amino, methylamino, and dimethylamino groups have been studied by several investigators by different methods (7–10). The general trend, as expected, is that of an increase in $-\Delta G$: (*a*) with increased alkylation, (*b*) in going from nonhydrogen-bonding to hydrogen-bonding solvents, and (*c*) with protonation. Conformational equilibrium studies have also been reported for 1,2-aminocyclohexanol systems (11, 12) and, to no surprise, the combined $-\Delta G$ value for the *trans*-1,2-aminohydroxy groups, in *trans*-2-amino-*trans*-5-isopropylcyclohexanol in tetrachloroethylene, was found to be larger than the sum of the individual values (12). This effect is attributed to hydrogen bonding in the chair conformation with the two functional groups in equatorial orientations. Tichý *et al.* (11) also found a slight contribution from an intramolecularly hydrogen-bonded conformer of *cis*-4-*tert*-butyl-*trans*-2-dimethylaminocyclohexanol, and they favored an inverted chair conformation, with the *tert*-butyl group axial, in preference to contributions from flexible forms for this conformer.

Effects of Methylation—For the free bases of the primary, *N*-methyl, and *N,N*-dimethylamines of classes VI through IX, the equilibrium in every case favors a high predominance of the normal chair conformation in all hydrogen-bonding solvents that act as hydrogen acceptors. In these solvents, the combined $-\Delta G$ values of the adjacent *trans*-hydroxyl and amino groups are not sufficient to overcome the combined $-\Delta G$ values of the *trans*-*o*-tolyl and the C-1 substituent, and no conformational change can be observed upon successive methylation. In deuterochloroform, the combined $-\Delta G$ values of the *trans*-hydroxyl and amino groups are larger, presumably because of a greater contribution from intramolecular hydrogen bonding, and the differences between the combined $-\Delta G$ values for the substituents on C-1 and C-2 *versus* those on C-4 and C-5 fall within the range where conformational changes can be observed in VI*c* and VII*c*. Comparison of J_{12} and J_{45} values between VI*a*, VI*b*, and VI*c* in chloroform shows that there is a considerable reduction of the normal chair in going from the *N*-methyl to the *N,N*-dimethyl derivative but that there is very little difference in conformation between the primary and secondary amines.

Effects of Protonation—Protonation brings about changes in conformation which vary with degree of alkylation and which are highly solvent dependent in the limited solvents investigated. The most pronounced changes are found with the trifluoroacetate salts in chloroform and the formate salts in formic acid–D_2O solutions. With the latter, the changes seem more pronounced in solutions containing 5% D_2O than in those with 25% D_2O. Very little difference was seen between formate and hydrochloride salts in formic acid–D_2O solutions, and the data for the hydrochloride salts in those solutions are not included in Table I.

Of special interest is the high predominance of the inverted chair conformation with the *o*-tolyl group in axial orientation for the salts of the *N,N*-dimethyl derivatives VI*c*, VII*c*, and VIII*c* in formic acid and for the trifluoroacetate of VI*c* in chloroform-*d*. In dimethyl sulfoxide the equilibrium favors a high predominance of the normal chair conformation, with the *o*-tolyl group in equatorial orientation for the hydrochloride salts of VI*c* and VIII*c* and the trifluoroacetate of VI*c*. A comparison of the data on the salts of the primary and secondary amines shows that in dimethyl sulfoxide-

Table I—NMR Data for Tetradeuterated Six-Membered Ring Compounds

Compound	Solvent	Coupling Constants, Hz. J_{12}	J_{45}	Chemical Shifts in δ Units, p.p.m. N—CH_3	H-1	H-2	H-4	H-5	Deshielding Shifts $(\nu - \nu^\circ)^a$, Hz. at 60 MHz. $\nu_1 - \nu_1^\circ$	$\nu_2 - \nu_2^\circ$
I	Carbon tetrachloride	11.0	—	—	5.21	3.09	—	—	—	—
	Acetone-d_6	11.0	—	—	5.29	3.23	—	—	—	—
	Dimethyl sulfoxide-d_6	10.9	—	—	5.25	3.17	—	—	—	—
	Pyridine	11.0	—	—	5.53	3.28	—	—	—	—
	Formic aicd–D_2O (95:5)	11.0	—	—	5.35	3.28	—	—	—	—
	Chloroform-*d*	10.8	—	—	5.25	3.13	—	—	—	—
II	Acetone-d_6	10.0	—	—	3.71	2.73	—	—	—	—
	Dimethyl sulfoxide-d_6	10.1	—	—	3.60	2.62	—	—	—	—
	Pyridine	10.0	—	—	3.89	2.90	—	—	—	—
	Formic acid–D_2O (75:25)	10.2	—	—	4.01	2.86	—	—	—	—
	Chloroform-*d*	9.8	—	—	3.66	2.67	—	—	—	—
III	Carbon tetrachloride	9.2	10.0	—	5.38	3.40	5.72[b]	5.61[b]	8	19
	Acetone-d_6	10.3	10.2	—	5.50	3.55	5.83[b]	5.73[b]	12	19
	Dimethyl sulfoxide-d_6	10.2	10.2	—	5.45	3.49	5.83[b]	5.72[b]	12	19
	Pyridine	10.0	10.1	—	5.70	3.61	5.81[b]	5.71[b]	10	20
IV	Carbon tetrachloride	11.2	—	—	5.12	3.40	3.09	3.09	−5	19
	Acetone-d_6	11.0	—	—	5.30	3.49	3.23	3.23	0	16
	Dimethyl sulfoxide-d_6	11.6	—	—	5.27	3.42	3.27	3.27	1	15
	Pyridine	11.5	—	—	5.46	3.64	3.19	3.19	−5	22
V	Carbon tetrachloride	11.2	3.5	—	5.33	3.15	3.20[b]	3.06[b]	7	4
VI*a*	Dimethyl sulfoxide-d_6	11.0	3.2	—	5.76	3.70	3.75	3.25	31	32
	Pyridine	10.6	3.3	—	6.27	4.19	4.18	3.71	44	55
	Chloroform-*d*	9.3	4.3	—	5.63	3.66	3.85	3.30	22	32
VI*a*-HCl	Dimethyl sulfoxide-d_6	10.2	3.2	—	5.75	3.73	4.19	3.55	30	34
VI*a*-HCl	Pyridine[c]	9.8	3.8	—	6.17	4.13	5.00	4.41	38	51
VI*a*-FA[d]	Formic acid–D_2O (95:5)	5.6	7.2	—	5.71	3.83	4.66	4.11	—	—
VI*a*-TFA[e]	Chloroform-*d*	4.8	8.0	—	5.57	3.72	4.57	—	—	—
VI*b*	Acetone-d_6	11.2	3.0	2.44[f]	5.71	3.80	3.97	2.96	25	35
	Dimethyl sulfoxide-d_6	11.1	3.0	2.44	5.65	3.71	3.86	2.86	24	33
	Pyridine	10.5	3.0	2.49[f]	6.12	4.17	4.22	3.23	35	53
	Chloroform-*d*	9.9	3.7	2.51	5.57	3.65	3.95	2.91	19	32
VI*b*-HCl	Dimethyl sulfoxide-d_6	10.2	3.2	2.72	5.62	3.70	4.24	3.39	22	32
VI*b*-HCl	Pyridine[c]	9.8	3.8	3.23	6.07	4.13	4.94	4.08	32	52
VI*b*-FA	Formic acid–D_2O (95:5)	5.8	7.2	3.13	5.72	3.83	4.67	3.91	—	—
VI*b*-TFA	Chloroform-*d*	4.1	8.8	2.91	5.62	3.70	4.58	—	—	—
VI*c*	Acetone-d_6	10.9	3.1	2.39	5.62	3.83	4.26	—	19	36
	Dimethyl sulfoxide-d_6	11.0	2.8	2.37	5.53	3.72	4.12	—	17	34
	Pyridine	10.8	3.2	2.42	6.00	4.20	4.50	—	28	55
	Chloroform-*d*	7.3	5.9	2.35	5.53	3.62	4.15	—	—	—
VI*c*-HCl	Dimethyl sulfoxide-d_6	9.6	3.6	2.94	5.46	3.68	4.33	3.51	13	31
VI*c*-TFA	Dimethyl sulfoxide-d_6	8.0	5.0	3.00	5.41	3.67	4.31	—	—	—
VI*c*-FA	Formic acid–D_2O (95:5)	3.8	9.2	3.20[g] 3.15	5.74	3.79	4.69	3.97	—	—
VI*c*-TFA	Chloroform-*d*	2.7	10.2	2.88[g] 2.98	5.69	3.67	4.63	—	—	—
VI*c*Ac	Acetone-d_6	11.5	3.2	2.42	5.58	3.63	5.26	—	20	30
	Chloroform-*d*	11.0	3.2	2.43	5.56	3.55	5.28		19	35
VI*c*Ac-FA	Formic acid–D_2O (95:5)	6.0	7.4	3.21	5.69	3.79	5.68	4.11	—	—
VI*c*Ac-TFA	Chloroform-*d*	4.5	8.0	2.96[g] 3.03	5.64	3.65	5.63	4.01	—	—
VI*d*	Dimethyl sulfoxide-d_6	6.7	6.4	3.28	5.61	3.95	4.49	—	—	—
	Pyridine	6.5	7.1	3.79	6.12	—	5.25	4.69	—	—
	Formic acid–D_2O (95:5)	5.8	7.0	3.39	5.79	3.75	4.82	4.08	—	—
VI*d*Ac	Pyridine	~8	~5	3.90	6.07	—	6.21	4.99	—	—
	Pyridine–D_2O (~50:50)	7.0	6.0	3.60	5.96	—	5.95	4.55	—	—
	D_2O	—[h]	—[h]	3.61	5.94	—	5.94	4.55	—	—
	Formic acid–D_2O (~50:50)	7.0	6.0	3.34	5.78	3.64	5.75	4.28	—	—
VII*a*	Dimethyl sulfoxide-d_6	10.0	2.6	—	4.02	3.13	3.54	3.04	25	30
	Pyridine	10.0	3.2	—	4.61	3.82	4.04	3.58	43	55
	D_2O	10.0	3.2	—	4.15	3.24	3.78	3.19	—	—
VII*a*-HCl	Dimethyl sulfoxide-d_6	9.3	3.7	—	4.08	3.19	3.98	3.33	29	34
VII*a*-HCl	Pyridine[c]	8.2	4.7	—	4.85	3.89	4.91	4.33	—	—
VII*a*-FA	Formic acid–D_2O (75:25)	7.6	6.0	—	4.42	3.52	4.41	3.92	—	—
VII*a*-HCl	D_2O	9.0	4.3	—	4.21	3.36	4.15	3.67	—	—
VII*b*	Acetone-d_6	10.0	2.9	2.36[f]	4.13	3.32	3.84	2.81	25	36
	Dimethyl sulfoxide-d_6	10.4	2.8	2.32	3.96	3.14	3.70	2.68	21	31
	Pyridine	10.2	3.0	2.47	4.62	3.88	4.22	3.19	44	59
	D_2O	10.4	2.9	2.35[f]	4.09	3.21	3.92	2.86	—	—
VII*b*-HCl	Dimethyl sulfoxide-d_6	~8.3	~3.9	2.61	4.07	3.21	4.10	3.25	—	—
VII*b*-HCl	Pyridine[c]	8.4	5.0	3.18	4.71	3.80	4.79	3.96	—	—
VII*b*-FA	Formic acid–D_2O (75:25)	6.9	6.0	3.00	4.39	3.52	4.43	3.70	—	—

(continued)

Table I—(*Continued*)

Compound	Solvent	Coupling Constants, Hz. J_{12}	J_{45}	Chemical Shifts in δ Units, p.p.m. N—CH_3	H-1	H-2	H-4	H-5	Deshielding Shifts ($\nu - \nu^\circ$)[a], Hz. at 60 MHz. $\nu_1 - \nu_1^\circ$	$\nu_2 - \nu_2^\circ$
VII*b*-HCl	D_2O	~8.7	~4.9	2.86	4.21	3.38	4.26	3.51	—	—
VII*c*	Acetone-d_6	9.2	3.8	2.27	4.11	3.35	4.09	—	24	37
	Dimethyl sulfoxide-d_6	9.8	3.2	2.22	3.91	3.15	3.94	—	18	32
	Pyridine	9.4	3.2	2.25	4.50	3.81	4.35	—	36	54
	Chloroform-*d*	8.2	4.5	2.29	4.12	3.27	4.05	2.41	—	—
VII*c*-HCl	Dimethyl sulfoxide-d_6	6.7	6.7	2.80	4.04	3.23	4.16	3.38	—	—
VII*c*-FA	Formic acid-D_2O (75:25)	3.8	9.4	3.10[e] 3.00	4.44	3.53	4.49	3.79	—	—
VII*c*-HCl	D_2O	5.0	8.6	2.99	4.27	3.43	4.39	3.63	—	—
VII*d*	Dimethyl sulfoxide-d_6	5.8	7.2	3.19	4.11	—	4.32	3.69	—	—
	Formic acid-D_2O (75:25)	5.9	7.6	3.30	4.58	—	4.64	3.89	—	—
	D_2O	~6.5	~7.3	3.33	4.56	—	4.65	3.82	—	—
VIII*a*	Pyridine	10.6	3.2	—	6.29	4.07	3.51	4.30	45	48
VIII*a*-HCl	Dimethyl sulfoxide-d_6	10.2	3.5	—	5.64	3.75	3.41	4.24	23	35
VIII*a*-HCl	Pyridine	9.2	3.8	—	6.26	4.28	4.24	5.19	44	60
VIII*a*-FA	Formic acid-D_2O (95:5)	5.8	7.0	—	5.71	3.75	4.02	4.64	—	—
VIII*b*	Pyridine	11.0	3.0	2.52	6.35	4.03	3.09	4.31	49	45
VIII*b*-HCl	Dimethyl sulfoxide-d_6	10.0	3.0	2.69	5.62	3.77	3.33	4.38	22	36
VIII*b*-HCl	Pyridine	9.3	3.7	3.10	6.23	4.33	3.93	5.18	42	63
VIII*b*-HCl	Formic acid-D_2O (95:5)	6.2	7.2	3.13	5.74	3.78	—	4.70	—	—
VIII*c*	Acetone-d_6	10.7	3.1	2.33	5.75	3.70	—	4.42	27	28
	Dimethyl sulfoxide-d_6	11.0	3.0	2.27	5.64	3.56	—	4.25	23	24
	Pyridine	10.7	3.3	2.30	6.24	3.89	—	4.66	42	37
VIII*c*-HCl	Dimethyl sulfoxide-d_6	9.0	3.9	2.93	5.59	3.88	3.47	4.51	20	43
VIII*c*-FA	Formic acid-D_2O (95:5)	3.8	9.5	3.16	5.69	3.84	3.80	4.69	—	—
VIII*d*	Dimethyl sulfoxide-d_6	6.2	8.0	3.33	5.42	3.59	—	4.61	—	—
	Pyridine	5.8	8.4	3.88	5.88	—	4.66	5.24	—	—
	Formic acid-D_2O (95:5)	4.6	9.2	3.42	5.63	3.82	4.01	4.87	—	—
IX*a*-FA	Formic acid-D_2O (75:25)	8.1	4.8	—	4.45	3.36	3.78	4.47	—	—
IX*b*	Pyridine	10.2	2.8	2.45	4.79	3.64	2.98	4.38	54	44
IX*b*-HCl	Dimethyl sulfoxide-d_6	~8.9	~3.6	2.60	4.05	3.15	3.11	4.21	—	—
IX*b*-HCl	Formic acid-D_2O (75:25)	8.2	4.8	2.99	4.46	3.36	3.58	4.56	—	—
IX*c*	Pyridine	9.9	3.4	2.23	4.67	3.57	—	4.53	46	40
IX*c*-HCl	Dimethyl sulfoxide-d_6	6.0	6.5	2.82	4.01	3.29	3.33	4.29	—	—
IX*c*-FA	Formic acid-D_2O (75:25)	4.6	8.5	3.03	4.42	3.47	3.57	4.53	—	—
	D_2O	6.0	7.4	2.96	4.28	3.32	3.44	4.47	—	
VII*e*	Acetone-d_6	10.6	3.0	—	3.81	3.34	4.02	1.83	6	37
	Dimethyl sulfoxide-d_6	10.4	2.8	—	3.64	3.17	3.87	1.71	3	33
	Pyridine	10.4	3.0	—	4.15	3.85	4.30	2.10	15	57
	Formic acid-D_2O (75:25)	10.4	3.1	—	4.06	3.35	4.23	1.97	3	30
	Chloroform-*d*	10.2	2.9	—	3.79	3.27	4.07	1.88	8	36
VII*f*	Acetone-d_6	10.4	3.1	—	4.22	3.32	4.00	3.82	30	36
	Dimethyl sulfoxide-d_6	10.2	3.2	—	3.99	3.11	3.79[b]	3.61[b]	24	29
	Pyridine	10.2	3.2	—	4.93	3.98	4.65[b]	4.44[b]	62	65
	Formic acid-D_2O (75:25)	10.4	3.4	—	4.43	3.36	4.28	4.09	25	30
VI*f*	Acetone-d_6	11.4	3.4	—	5.82	3.83	4.18[b]	4.02[b]	31	36
	Dimethyl sulfoxide-d_6	11.0	3.2	—	5.74	3.74	4.03[b]	3.88[b]	29	34
	Pyridine	11.2	3.5	—	6.45	4.30	4.68[b]	4.51[b]	55	61
	Formic acid-D_2O (95:5)	10.7	3.7	—	5.79	3.83	4.49[b]	4.35[b]	26	33
VI*g*	Acetone-d_6	11.2	3.4	—	5.88	3.90	3.92	3.74	35	41
VII*g*	Acetone-d_6	10.0	3.6	—	4.26	3.38	3.76	3.60	33	39
VIII*g*	Acetone-d_6	10.8	3.4	—	5.89	3.91	3.69	3.88	35	41
X	Carbon tetrachloride	11.0	—	—	4.71	3.40	—	—	—	—
XI	Carbon tetrachloride	11.0	—	—	4.93	3.69	5.71	5.71	13	18

[a] The symbols ν_1° and ν_2° refer to the chemical shifts of H-1 and H-2 in the appropriate reference Compounds I, II, and X, and ν_1 and ν_2 are the chemical shifts of H-1 and H-2 for the compound under consideration. [b] Differentiation between H-4 and H-5 is not certain. [c] From 2 to 5 drops of D_2O was added to solubilize the salts in pyridine. [d] FA signifies the formate salt, obtained by dissolving the amine in formic acid. [e] TFA signifies the trifluoroacetate salt. This was obtained by the addition of a few drops of trifluoroacetic acid to a solution of the amine in the given solvent. [f] Differentiation between the NCH_3 and $ArCH_3$ signals is not certain because the two signals are very close. [g] Two methyl signals. [h] The coupling constants cannot be determined. All signals are very broad and poorly resolved, suggesting micellar aggregation of the quaternary salt in D_2O. Addition of a few drops of formic acid causes sharpening of all signals.

d_6, and in pyridine containing a few drops of D_2O, the equilibrium favors the normal chair conformation. In chloroform and in formic acid–D_2O solutions, the equilibrium is shifted predominantly toward the inverted chair conformation. It is also noteworthy that the formate and trifluoroacetate salts of VI*c*, in formic acid and chloroform-*d*, respectively, exhibit a higher population of the inverted chair conformation than the corresponding salts of the acetoxy derivative VI*c*Ac.

Figure 1 shows the spectra and predominant conformers of the hydrochloride salt of VIII*c* in dimethyl sulfoxide-d_6 and formic acid–D_2O solutions. The inversion of the values of J_{12} and J_{45} in the two solvent systems is obvious. On the basis of the extreme shifts in equilibrium positions between the two chair conformers for the salts of VI*c* and VIII*c* with a change of solvent, it seems reasonable to conclude that the observed intermediate J_{12} and J_{45} values for the primary, secondary, and tertiary amines and their salts in certain

solvent systems represent equilibria of different proportions of the two conformers, but the possibility of contributions from twist and boat forms is not ruled out.

There is no doubt that protonation of the amino groups, especially the dimethylamino group, increases the steric requirements of the groups by forcing the nitrogen into the sp^3 hybrid form and preventing strain release by nitrogen inversion. Undoubtedly this factor contributes in part to the conformational differences seen between VI*a*, VI*b*, VI*c*, and their respective trifluoroacetate salts in, for example, chloroform-*d*. But since one is dealing with thermodynamic equilibria, it is the overall free energy differences between the various conformers in the given medium that affect the relative populations at equilibrium and it would be an oversimplification to attribute the observed differences strictly to steric factors of the protonated amino groups. The significant differences seen between solvent systems indicate that in the salts, as was the case in the free amines, intermolecular hydrogen bonding with the solvent molecules *versus* intramolecular hydrogen bonding between the hydroxyl and the amino function at the C-4 and C-5 positions in the inverted chair contributes significantly to the relative free energy states of the systems. A comparison of the data for the quaternary ammonium salts with those for the protonated dimethylamines also lends support to this contention.

In the case of the free amines, the intramolecular hydrogen bonding will be mostly between the hydroxyl hydrogen and the nitrogen; in the salts, it is the oxygen that is the hydrogen acceptor. The strength of these hydrogen bonds is not necessarily comparable. The differences in energy barriers to rotation of the amino group upon alkylation and/or protonation will also affect the strength of hydrogen bonds. The apparent importance of intramolecular hydrogen bonding in formic acid is noteworthy. There is a possibility of a bridging of the two equatorially oriented functional groups by the formate anion or a formic acid molecule. If this plays any role in the conformational state of the system, the resulting free energy differences would involve differences between two intermolecular hydrogen-bonding states rather than competition strictly between intermolecular and intramolecular hydrogen bonding. The same phenomenon could be operative with the trifluoroacetates in chloroform and dimethyl sulfoxide-d_6, but in dimethyl sulfoxide-d_6 the intermolecular association with solvent molecules is expected to play a much more important role than in chloroform.

Another factor which must not be overlooked in considering factors that affect the relative thermodynamic stabilities of conformers of the salts is the stronger basicity of an equatorial than an axial amino group, as shown in *cis*- and *trans*-4-*tert*-butylcyclohexylamines (7, 13).

Effects of Quaternization—With the quaternary ammonium salts, none of the systems shows a high predominance of chair conformers with the *o*-tolyl group equatorial and the trimethylammonium group axial. This is understandable because the trimethylammonium group probably has steric requirements that are as demanding as those of the tertiary butyl group. What is of greater interest is the much lower population of the inverted chair with the quaternary salts VI*d* and VII*d* in formic acid in comparison to the salts of the corresponding dimethylamines VI*c* and VII*c* in the same solvent. This again suggests the importance of intramolecular hydrogen bonding in the secondary amines, but the possibility of a greater contribution from twist or other flexible conformers with the quaternary salts is not ruled out. The contributions to the overall equilibria from flexible forms cannot be assessed from the present data, but it is noteworthy that almost all systems of the quaternary salts show unequal populations of the two chair conformers, showing that the systems cannot consist of flexible forms only. The highest population of the inverted chair form is seen with VIII*d* in formic acid–D_2O solutions.

A deformation involving a flattening of the chair ring was invoked by Tichý *et al.* (11) for strain release in *trans*-4-*tert*-butyl-*cis*-2-dimethylaminocyclohexanol and *cis*-5-*tert*-butyl-*cis*-2-dimethylaminocyclohexanol, where the dimethylamino group is axial in each case. A flattening of this type about the C(2)—C(3) and C(3)—C(4) bonds, with little change in the geometry of the rest of the molecule, was found by Shefter and Smissman (14) in 3(a)-dimethylamino-2(a)-acetoxy-*trans*-decalin methiodide in the solid state by single crystal X-ray crystallography. The results with the systems in solution in the present study give no evidence of significant deformation of this type. The effect of such a deformation would be to increase the dihedral angle between H-4 and H-5, bringing this angle closer to

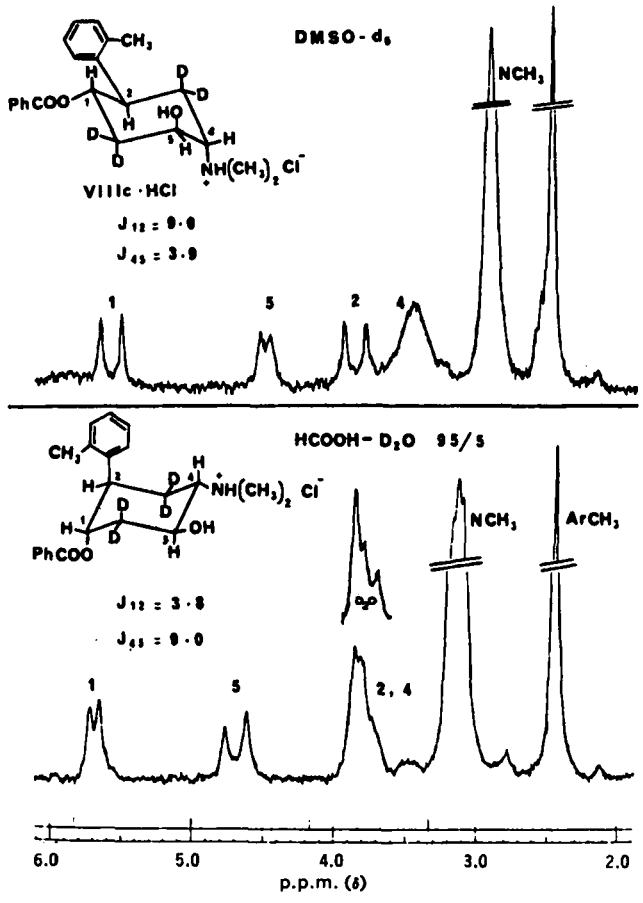

Figure 1—*The 60-MHz. NMR spectra and predominant conformations of* trans-2-o-*tolyl*-cis-4-N,N-*dimethylamino*-trans-5-*hydroxycyclohexyl-3,3,6,6-*d_4 *benzoate hydrochloride in dimethyl sulfoxide-*d_6 *and in formic acid–*D_2O *(95:5) solution.*

90° from its normal value of 60°, thus causing a decrease in the J_{45} value with little if any change in the value of J_{12}. No significant change of this type is seen with increased steric requirements of the amino group either by methylation to the dimethylamino or by protonation. In fact, the sum of J_{12} and J_{45} remains quite constant throughout the series. Assessment of contribution from this type of deformation in the systems is more direct where J_{12} has the normal J_{aa} value, and it would be hazardous to eliminate such contributions in the quaternary salts on the basis of the consistencies of the sums of the J_{12} and J_{45} values.

Half-Chair Conformations of Cyclohexene and Epoxides—The data in Table I also show a very high predominance of the half-chair conformations, with the aromatic and benzoate substituents in equatorial orientations in the cyclohexenes III and XI and the epoxides IV and V.

Equatorial–Equatorial Coupling Constants—The data in Table I also show that for those systems having the chair conformation with H-4 and H-5 in equatorial orientations the J_{ee} values are not very sensitive to changes in substituents at C-4 and C-5, as seen by comparison of the various aminohydroxy compounds, the dihydroxy Compounds VI*f* and VII*f*, and the deuterated hydroxy Compound VII*e*.

Deshielding Effects—*1,3-Diaxial Deshielding*—The spatial 1,3-diaxial deshielding effect of a hydroxyl group on ring hydrogens is a well-documented phenomenon in NMR spectroscopy of six-membered ring compounds (5, 15–20). It is also known that the effect varies with solvents. There is enhanced deshielding in pyridine over chloroform and many other solvents (5, 19). Acetylation to the acetoxy group reduces the deshielding effect (15, 16, 18, 20). The tetradeuterated compounds of series VI–IX are well suited for the investigation of similar deshielding effects by primary, secondary, and tertiary amino groups and for a comparison of the effects in protonated and nonprotonated forms. This has been done by comparison of the chemical shifts of H-1 in compounds of series VI and VII and

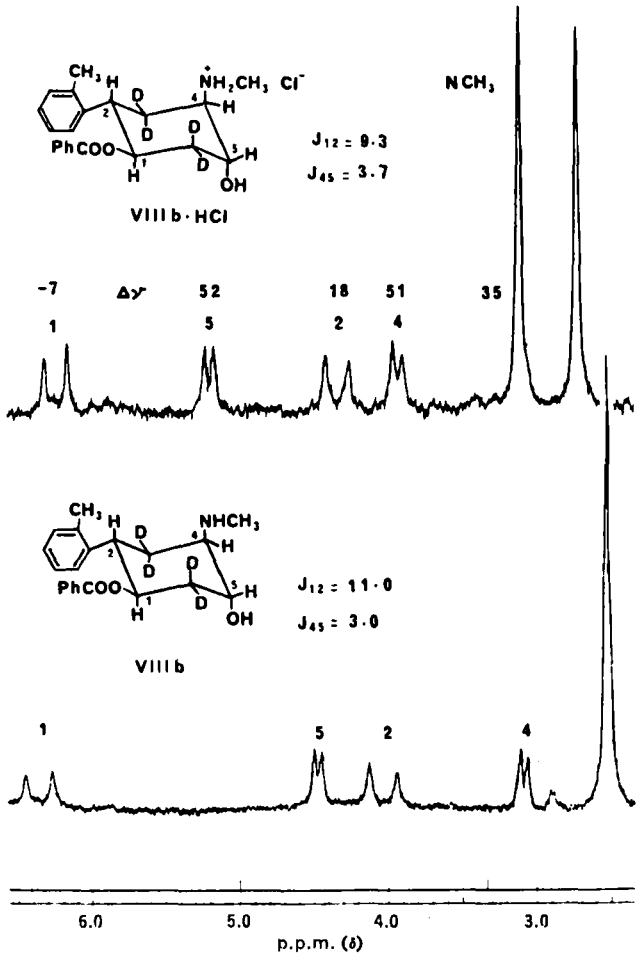

Figure 2—*The 60-MHz. NMR spectra of* trans-*2*-o-*tolyl*-cis-*4*-N-*methylamino*-trans-*5-hydroxycyclohexyl-3,3,6,6*-d_4 *benzoate and the hydrochloride salt in pyridine. The shifts upon protonation are designated in hertz.*

of H-2 in compounds of series VIII and IX with those of H-1 and H-2 in the appropriate reference Compound I or II in the same solvent. Obviously, the same spectra also provide information on the deshielding effects of the hydroxyl group in the same compounds. The comparison could not be done in acetone with the primary amines because of rapid imine formation, but there was no evidence of any imine formation between acetone and the secondary amines. The observed shifts are given in hertz at 60 MHz. in Table I.

The 1,3-diaxial deshielding by the amino and hydroxyl substituents has meaning only in those systems known to have a high predominant population of the chair conformer with the amino and hydroxyl groups in axial orientations. Therefore, the deshielding shifts have been listed in Table I only for those systems where the J_{12} values are 9 Hz. or above. In systems where the inverted chair predominates, the downfield shifts of H-1 and H-2, compared to the reference Compounds I and II, are mostly the result of a change of the orientation of these hydrogens from axial to equatorial.

The pertinent data for the 1,3-diaxial deshielding by amino groups are more complete for the compounds of series VI and VII, those compounds in which H-1 is deshielded by the amino groups and H-2 is deshielded by the hydroxyl group. It is important to note the consistency of the deshielding ($\nu_2 - \nu_2°$) by the hydroxyl group in a given solvent for those systems having a high predominance of the normal chair conformation (tolyl group equatorial). These observed $\nu_2 - \nu_2°$ deshielding values are generally in excellent agreement with the corresponding values in the reference Compound VII*e*, having a deuterium at C-5, as well as for those of the 4,5-dihydroxy Compounds VI*f* and VII*f* and the previously published values (5) of 38 Hz. in acetone and 58 Hz. in pyridine for *trans*-2-*o*-tolyl-*cis*-4-hydroxycyclohexanol-3,3,6,6-d_4. This information reinforces the conclusion arrived at from observed coupling constants that there is no evidence for a significant deformation involving a flattening of the chair ring about the C(5)—C(4) bond in these compounds in solution.

1,3-Diaxial deshielding of H-1 by the NH_2 group at C-5 in Compounds VI*a* and VII*a* is comparable to the deshielding of H-2 by the C-4 hydroxyl group in dimethyl sulfoxide-d_6. In pyridine, however, a slightly lower deshielding effect by the NH_2 compared to the OH group is observed. Alkylation to methylamino and dimethylamino derivatives reduces the deshielding in the two solvents. In acetone the deshielding of H-1 by the secondary and tertiary amino groups is somewhat comparable to the results in dimethyl sulfoxide-d_6. There are undoubtedly several factors involved in the reduction of the 1,3-diaxial deshielding effect upon methylation. Changes in the time-average orientations of the amino group will affect any deshielding resulting from the anisotropies of the unshared electrons and the N—H and N—CH_3 bonds, and there are undoubtedly differences in the anisotropic properties of the N—H and N—CH_3 bonds. Successive methylation should also affect solvation of the amino group, and differences in solvation can affect the time-average orientations of the anisotropic solvent molecules associated through hydrogen bonding with the NH hydrogens. The role played by solvation in the decreased deshielding in going from primary to secondary to tertiary amines in the free bases is difficult to assess from the available data. If orientation of the solvent molecules through hydrogen bonding with N—H hydrogens played a dominant role in 1,3-diaxial deshielding by the amino group, a larger difference should be expected in pyridine than in the other solvents between primary, secondary, and tertiary amines. It is interesting that there is very little difference in the 1,3-diaxial deshielding effects of the protonated *versus* nonprotonated amino groups in VI*a*, VI*b*, VI*c*, and VII*a* in dimethyl sulfoxide-d_6 and in VI*a* and VI*b* in pyridine containing slight amounts of D_2O. Surprisingly, a larger difference is not found in pyridine for VI*a* and VI*b* upon protonation, where hydrogen bonding of the protonated form with pyridine is expected to play a larger role. Such a difference is seen in pyridine for the protonated *versus* nonprotonated forms in Compounds VIII*a* and VIII*b*, where the deshielding is on H-2. Comparisons of 1,3-diaxial deshielding effects of protonated and free bases are limited to those compounds that have a high predominance of the normal chair conformation under both conditions. The conformational changes brought about by protonation restricts the available data for such comparisons. The overall results suggest important contributions from the anisotropic properties of the ring C—N and the N—CH_3 bonds to the observed long-range 1,3-diaxial deshielding effects caused by free amino groups and aminium cations. If this is the case, the C(5)—N bond contributes to deshielding of H-1 while the N—CH_3 bond contributes to shielding of H-1.

Table I shows that in the imines VI*g*, VII*g*, and VIII*g* in acetone, the 1,3-diaxial deshielding by the N=$C(CD_3)_2$ group is of about the same magnitude as the deshielding caused by an NH_2 or an OH group. When the primary amines are added to acetone-d_6 at an NMR concentration of about 100 mg. in 0.5 ml. of solvent, the equilibrium is reached within a few minutes, and at equilibrium the NMR spectra show no evidence of any amine. Dropwise addition of D_2O shifts the position of the equilibrium, and the NMR spectra now show the presence of the free amine by the appearance of second H-1 and H-5 signals. After the addition of 10 drops of D_2O to the NMR sample of VI*g* in acetone-d_6, the ratio of the imine to amine was about 3 to 1. The chemical shifts of H-1 and H-5 of the primary amine were δ 5.68 and 3.38, respectively. The difference in 1,3-diaxial deshielding of H-1 by the N=$C(CD_3)_2$ and NH_2 groups in this medium is only 4 Hz. at 60 MHz.

Geminal Deshielding—A comparison of the chemical shift of H-5 in VII*e* with those of H-5 in VII*a* and VII*b* gives an indication of the geminal deshielding effects of the amino group in dimethyl sulfoxide and pyridine and of the methylamino group in acetone, dimethyl sulfoxide, and pyridine. Accurate chemical shifts could not be obtained for H-5 of VII*c* in these solvents because the signal is overlapped by the signals of the aromatic and amino methyl groups. The deshielding is more pronounced with the primary amino group and decreases successively with the methyl and dimethylamino groups. At 60 MHz. there is a geminal deshielding of 80 Hz. in dimethyl sulfoxide-d_6 and 89 Hz. in pyridine by the NH_2 group in going from VII*e* to VII*a;* a deshielding of 59 Hz. in acetone, 58 Hz. in dimethyl sulfoxide-d_6, and 65 Hz. in pyridine by the $NHCH_3$ group in going from VII*e* to VII*b;* and a deshielding of 32 Hz. in $CDCl_3$ in going from VII*e* to VII*c*. A comparison of the chemical

shifts of *N*-methyl hydrogens in the monomethyl and dimethyl compounds shows that the signal of the monomethyl group is further downfield by from 3 to 14 Hz., with the largest differences occurring in pyridine.

Protonation of the primary amino group of VI*a* and VII*a* causes a downfield shift of H-5 (geminal deshielding) of 17 or 18 Hz. in dimethyl sulfoxide-d_6 and of 42–45 Hz. in pyridine. With the secondary amines VI*b* and VII*b*, the downfield shift of H-5 upon protonation is of the order of 32–34 Hz. in dimethyl sulfoxide-d_6, while in pyridine it is from 46 to 52 Hz. Thus, there is a significant difference in the geminal deshielding of H-5 between the primary and secondary amines upon protonation in series VI and VII in dimethyl sulfoxide-d_6 but not in pyridine. However, the protonation geminal deshielding effect is considerably larger in pyridine than in dimethyl sulfoxide-d_6. The downfield shift of the N—CH_3 group upon protonation in series VI and VII in dimethyl sulfoxide-d_6 is about twice as large for the dimethylamino as for the corresponding monomethyl compounds.

Vicinal Shielding and Deshielding—A comparison of the chemical shift of H-4 in VII*e* with those of H-4 in Compounds VII*a*, VII*b*, and VII*c* gives information on the vicinal shielding effects of the NH_2, $NHCH_3$, and $N(CH_3)_2$ groups where the dihedral angle between H-4 and the C(5)—N bonds is essentially 60°. The data show an upfield shift of 19 and 16 Hz. (at 60 MHz.) for H-4 in dimethyl sulfoxide and pyridine, respectively, in the primary amine VII*a* compared to the reference Compound VII*e*. The methylamino group causes a shielding of H-4 in VII*b* of 11, 10, and 5 Hz. in acetone, dimethyl sulfoxide-d_6, and pyridine, respectively. The dimethylamino group causes a deshielding of H-4 by 4, 4, and 3 Hz. in acetone, dimethyl sulfoxide-d_6, and pyridine, respectively, and practically no change in chloroform-*d*.

Protonation of the amino group brings about a significant downfield shift of vicinal H-4 in compounds of series VI and VII in comparison to the chemical shifts of H-4 in the free amines. There is little difference in this effect between the primary and secondary amines of series VI and VII in a given solvent, but there is a significant difference between dimethyl sulfoxide-d_6 and pyridine for a given compound. The shifts in VI*a* and VI*b* in dimethyl sulfoxide-d_6 are 26 and 23 Hz., respectively, but they are 49 and 42 Hz. in pyridine. For VII*a* and VII*b* the shifts are 26 and 24 Hz., respectively, in dimethyl sulfoxide-d_6 and 52 and 35 Hz. in pyridine. For the dimethyl Compounds VI*c* and VII*c*, the shift of H-4 in dimethyl sulfoxide-d_6 upon protonation is 13 Hz. in each case, but only VI*c*-HCl of the two salts is shown to have a high predominance of the normal chair form.

The shifts upon protonation are demonstrated for VIII*b* in pyridine in Fig. 2.

Although there is a significant vicinal deshielding upon protonation in these compounds, the net vicinal deshielding effects of the aminium cation, as seen by comparison of the chemical shift of H-4 in VII*e* with those of H-4 in the protonated amines, is not as large as the shift observed in going from the free amine to the protonated form with the primary and secondary amines. This results from the fact that the free NH_2 and $NHCH_3$ groups cause a vicinal shielding of H-4 in VII*a* and VII*b*.

Demarco *et al.* (19) compiled data on deshielding effects in pyridine relative to chloroform for geminal and vicinal deshielding by the hydroxyl group, and they pointed to the dihedral angle dependency for the solvent difference of the vicinal deshielding by the hydroxyl group. The data on the chemical shifts of H-4 and H-5 of Compound VII*e* are of special interest, because they provide information on these effects in a system where the hydroxyl function and the deshielded hydrogens are remote from other functional groups. The observed downfield shift differences of 0.23 and 0.22 p.p.m. for H-4 and H-5, respectively, in pyridine relative to $CDCl_3$ are in good agreement with differences listed by Demarco *et al.* (19). The differences in these two solvents are even greater for the chemical shifts of H-1 and H-2 in VII*e*, but in this *AX* system the presence of the aromatic group may affect the chemical shift of H-1 differently in the two solvents. With respect to H-2, in addition to the solvent difference on the vicinal deshielding by the OH group is added the difference of these solvents on the 1,3-diaxial deshielding by the C-4 hydroxyl group. There is also a possibility that the geminal deshielding of H-2 by the aromatic group could be different in the two solvents. The observed difference of 0.58 p.p.m. in the chemical shift of H-2 in the two solvents is the result of a summation of these effects. The differences in the other three solvents are also of interest.

Deshielding by the Double Bond—The long-range deshielding effect of the double bond is seen by comparison of the chemical shifts of the hydrogens on the nitro- and aromatic-bearing carbons, respectively, between Compounds X and XI. Deshielding values of 0.22 p.p.m. for H-1 and 0.29 p.p.m. for H-2 in XI, measured in carbon tetrachloride, are in agreement with reported values in an analogous system in chloroform-*d* (20). Similar results are seen by comparison of the chemical shifts of H-1 and H-2 between I and III in carbon tetrachloride, acetone-d_6, dimethyl sulfoxide-d_6, and pyridine. The deshielding of H-1 varies from 0.17 to 0.21 p.p.m. in the four solvents, and the deshielding of H-2 varies between 0.31 and 0.33 p.p.m. The larger deshielding of H-2 could be associated with a difference in the average orientation of the *o*-tolyl group in the saturated *versus* the unsaturated compounds, causing a slight difference in the deshielding of H-2 by the magnetic anisotropy of the tolyl group. It is also possible that the magnetic anisotropy of the benzoate functional group has different effects on H-2 and/or H-1 in the unsaturated and saturated compounds.

Deshielding by the Epoxy Group—The anisotropic effects of the epoxy group in substituted epoxycyclohexanes were reported previously (20). In the half-chair conformation of *trans*- and *cis*-4,5-epoxy-*trans*-2-(*p*-chlorophenyl)nitrocyclohexane, the epoxy group was found to cause a deshielding of the axial hydrogen *cis* to the epoxy group, and two carbons removed from it, by about 0.2–0.26 p.p.m. when measured in chloroform. Little effect was found on the chemical shift of the *trans*-axial hydrogen two carbons removed from the epoxy group. For epoxides IV and V, again the J_{12} values indicate a large predominance of the expected half-chair conformation. A comparison of the chemical shifts of H-1 and H-2 in epoxide IV with the chemical shifts of H-1 and H-2 in the reference ester I shows a deshielding of H-2 ranging from 0.25 to 0.36 p.p.m. in the four solvents, with little change in the chemical shift of H-1. This substantiates the previous results (20). Epoxide V was investigated only in carbon tetrachloride. It shows a deshielding of H-1 by 0.12 p.p.m. and of H-2 by 0.06 p.p.m.

EXPERIMENTAL[1]

Compounds III, IV, V, and XI—These compounds were prepared by the methods previously described for the corresponding hydrogen-containing compounds (6, 21), except that *m*-chloroperbenzoic acid was used instead of perbenzoic acid for the epoxidation. The selective incorporation of deuterium on carbons 3 and 6 was accomplished by using butadiene-1,1,4,4-d_4 (1, 22) instead of butadiene in the Diels–Alder condensation step of the synthetic scheme (6).

Compound I—This compound was obtained by catalytic hydrogenation of III using 5% palladium-on-carbon in ethyl acetate, m.p. 71–73°. Mass spectral analysis for the molecular ion gave *m/e* 298.1880 (calc. for $C_{20}H_{18}D_4O_2$: 298.1866).

Compound II—This compound was obtained by basic hydrolysis of I. The corresponding hydrogen-containing compound was reported previously (22).

Compound VII*e*—This compound was obtained by lithium aluminum deuteride reduction of IV. The corresponding hydrogen-containing compound was reported previously (6).

Compound VI*f*—This compound was obtained by acid-catalyzed hydrolysis of a mixture of IV and V by warming for 5 min. in a solution of 7% sulfuric acid in a mixture of 60% dioxane in water. The corresponding hydrogen-containing compound was also prepared and recrystallized from a mixture of dioxane, benzene, and hexane, m.p. 173.5–174°.

Anal.—Calc. for $C_{20}H_{22}O_4$: C, 73.60; H, 6.79. Found: C, 73.43; H, 6.80.

Compound VII*f*—This compound was obtained from basic hydrolysis of VI*f*. The corresponding nondeuterated analog was also prepared and recrystallized from water. The compound under-

[1] The NMR spectra were recorded on a Varian A-60 spectrometer at an operating temperature of about 37° for all compounds except XII, XIII, and XV, which were obtained on a Varian T-60 instrument, also at 37°. Tetramethylsilane was used as the internal reference in all solvents, except in D_2O where sodium 2,2-dimethyl-2-silapentane-5-sulfonate was used. The spectra were obtained on freshly prepared solutions.

Table II—Physical Constants and Analytical Data for *trans*-2-*o*-Tolyl-*cis*-4-hydroxy-*trans*-5-methylaminocyclohexanol, *trans*-2-*o*-Tolyl-*cis*-4-methylamino-*trans*-5-hydroxycyclohexanol, and the Corresponding Dimethylamino and 1-Benzoate Derivatives

Compound	Melting Point			Analysis[a], %	
	Amine	Hydrochloride	Methiodide	Calc.	Found
VI*b*	162.5–164°	276–279°	—	C —	—
				H —	—
				N —	—
VII*b*	148–149°	263–264.5°	—	C 61.85	62.01
				H 8.16	7.79
				N 5.16	5.20
VIII*b*	—	240–241.5°	—	C —	—
				H —	—
				N —	—
IX*b*	—	211–213°	—	C 61.85	61.94
				H 8.16	8.17
				N 5.16	4.99
VI*c*	157–158°	214–217°[c]	202.5–204°	C 74.75	74.79
				H 7.70	7.45
				N 3.96[b]	3.85[b]
VII*c*	163–163.5°	260–263.5°	205–207°	C 63.04	63.14
				H 8.46	8.20
				N 4.90	4.85
VIII*c*	142–143.5°	240.5–245.5°	234–234.5°	C 74.75	74.56
				H 7.70	7.91
				N 3.96[b]	4.01[b]
IX*c*	51–74°[d]	248–250°	89–162°[e]	C 63.04	63.22
				H 8.46	8.37
				N 4.90	5.15

[a] The analyses are on the hydrochloride salts unless stated otherwise. [b] Analysis on the free amine. [c] Appears to undergo polymorphic changes, melts at about 210°, resolidifies, and remelts between 214–217°. [d] Crystallizes as a hydrate from an acetone–water mixture. [e] Crystallizes as a 1:1 dioxane complex from dioxane–methanol solution. The ratio of dioxane was determined by NMR.

goes polymorphic changes upon heating: melts at 150–153°, resolidifies at 156–160°, and remelts at 177.5°.

Anal.—Calc. for $C_{13}H_{18}O_2$: C, 70.24; H, 8.16. Found: C, 69.97; H, 8.15.

Compound X—This compound was reported previously (1).

***trans*-2-*o*-Tolyl-*cis*-4-hydroxy-*trans*-5-aminocyclohexyl-3,3,6,6-d_4 Benzoate (VI*a*)**—This compound and the corresponding *N*-methyl (VI*b*) and *N*,*N*-dimethyl (VI*c*) derivatives were obtained from the epoxide IV; likewise, the *trans*-2-*o*-tolyl-*cis*-4-amino-*trans*-5-hydroxycyclohexyl-3,3,6,6-d_4 benzoate (VIII*a*) and the corresponding *N*-methyl (VIII*b*) and *N*,*N*-dimethyl (VIII*c*) derivatives were obtained from epoxide V by heating the appropriate epoxide with either ammonia, methylamine, or dimethylamine in peroxide-free tetrahydrofuran at temperatures ranging from about 100 to 120° in a stainless steel pressure bomb for 1–4 days (usually for 2 days). The method is analogous to that described by Mousseron *et al.* (23) for opening of epoxides with amines. The yields were generally quite good, mostly above 80% and often above 90%. The degree of aminolysis of the benzoate group was usually low. Treatment of the benzoates with potassium hydroxide in methanol gave the corresponding dihydroxyamino compounds, VII*a*, VII*b*, VII*c*, IX*a*, IX*b*, and IX*c*. The quaternary trimethylammonium chlorides were obtained from the corresponding dimethylamino compounds by heating with a large excess of methyl chloride in anhydrous, peroxide-free tetrahydrofuran at 90–95° for 48 hr. in a stainless steel pressure bomb.

Analytical data and physical constants for the nondeuterated *N*-methyl and *N*,*N*-dimethylamino derivatives of Compounds VI–IX are given in Table II. The melting points of the methiodide salts obtained from the *N*,*N*-dimethylamino compounds are also included. The nondeuterated primary amines were not prepared. Melting points of the deuterated primary amines and their hydrochloride salts were as follows: VI*a*, 122.5–123.5°; VI*a*-HCl, 254–256.5°; VII*a*, 139.5–141°; VII*a*-HCl, 259–263°; VIII*a*, 167–167.5°; VIII*a*-HCl, 228.5–231°; and IX*a*, 215.5–217.5°. Mass spectral analyses were obtained for the diols VII*a* and IX*a*, which, in turn, were obtained by basic hydrolysis of the benzoates VI*a* and VIII*a*. Mass spectral analysis for the molecular ion gave *m/e* 225.1654 for VII*a* and 225.1648 for IX*a* (calc. for $C_{13}H_{15}D_4NO_2$: 225.1660). The mass spectral analyses were carried out on the hydrochloride salts. The loss of hydrochloric acid is a normal fragmentation of salts of amines.

Nondeuterated Compounds VI*c* and VIII*c* were converted to the corresponding acetates XII and XIII by treating with acetic anhydride in anhydrous pyridine, and similar treatment of the nondeuterated diols VII*c* and IX*c* produced the corresponding diacetates XIV and XV, respectively.

***trans*-2-*o*-Tolyl-*cis*-4-acetoxy-*trans*-5-dimethylaminocyclohexyl Benzoate (XII)**—This compound had a melting point of 140.5–141.5°; NMR (pyridine): δ 5.90 (td, 1, poorly resolved, w $\sim$ 25 Hz., H-1), 5.48 (m, 1, w $1/2$ = 7 Hz., H-4), 3.81 (td, 1, J = 11.5, 4.8 Hz., H-2), 2.48 (s, 3, $ArCH_3$), 2.43 (s, 6, $N—CH_3$), and 2.17 (s, 3, Ac).

Anal.—Calc. for $C_{24}H_{29}NO_4$: C, 72.88; H, 7.39; N, 3.54. Found: C, 73.05; H, 7.50; N, 3.65.

The hydrochloride had a melting point of 197–202.5°, and the methiodide had a melting point of 215–217.5°.

***trans*-2-*o*-Tolyl-*cis*-4-dimethylamino-*trans*-5-acetoxycyclohexyl Benzoate (XIII)**—This compound had a melting point of 85.5–87°; NMR (pyridine): δ 5.92 (td, 1, partially overlapped by H-5, H-1), 5.67 (m, 1, overlaps H-1, H-5), 3.83 (dt, 1, J = 11.0, 4.8 Hz., H-2), 2.50 (s, 3, $Ar—CH_3$), 2.30 (s, 6, $N—CH_3$), and 2.13 (s, 3, Ac).

The hydrochloride salt melts at about 208°, resolidifies, and remelts at 214–217°.

Anal.—Calc. for $C_{24}H_{30}ClNO_4$: C, 66.73; H, 7.00; N, 3.24. Found: C, 66.46; H, 6.90; N, 3.16.

The methiodide had a melting point of 208–211°.

***trans*-2-*o*-Tolyl-*cis*-4-acetoxy-*trans*-5-dimethylaminocyclohexyl Acetate (XIV)**—This compound had a melting point of 88.5–91°; NMR (pyridine): δ 5.62 (dt, 1, J = 11.0, 4 Hz., H-1), 5.40 (m, 1, H-4), 3.58 (dt, 1, J = 11.2, 5 Hz., H-2), 2.37 (s, 3, $ArCH_3$), 2.34 (s, 6, $N—CH_3$), 2.13 (s, 3, Ac), and 1.72 (s, 3, Ac).

Anal.—Calc. for $C_{19}H_{27}NO_4$: C, 68.44; H, 8.16; N, 4.20. Found: C, 68.13; H, 7.97; N, 4.31.

The hydrochloride had a melting point of 220.5–224.5°. The methiodide melts around 237°, resolidifies, and remelts at 246–248°.

***trans*-2-*o*-Tolyl-*cis*-4-dimethylamino-*trans*-5-acetoxycyclohexyl Acetate (XV)**—This compound had a melting point of 90–91°; NMR (pyridine): δ 5.59 (td, 1, overlapped by H-5, H-1), 5.56 (m, 1, overlaps H-1, H-5), 3.60 (dt, 1, J = 10.5, 4.8 Hz., H-2), 2.42 (s, 3, $Ar—CH_3$), 2.26 (s, 6, $N—CH_3$), 2.08 (s, 3, Ac), and 1.68 (s, 3, Ac).

Anal.—Calc. for $C_{19}H_{27}NO_4$: C, 68.44; H, 8.16; N, 4.20. Found: C, 68.10; H, 8.10; N, 4.24.

The hydrochloride had a melting point of 185–188.5°, and the methiodide had a melting point of 210.5–211.5°.

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Excretion of *trans*-Δ⁹-Tetrahydrocannabinol and Its Metabolites in Intact and Bile Duct-Cannulated Rats

ROBERT F. TURK*, WILLIAM L. DEWEY†▲, and LOUIS S. HARRIS†

Abstract □ Tritiated Δ⁹-tetrahydrocannabinol (I) was administered both orally and intravenously to groups of bile duct-cannulated rats and those with their bile duct intact. From 55.8 to 66.9% of the total radioactivity was excreted during the 96-hr. period following administration. The excretion of radioactivity was minimal in each group beyond 48 hr. after drug administration. The major route of excretion following the intravenous administration in bile duct-cannulated rats was by way of bile (59.4%; feces, 2.7%), whereas more radioactivity was excreted in feces (41.5%) than bile (21.5%) when the drug was given orally. The radioactivity excreted in the feces of the orally medicated rats was mainly extractable in petroleum ether. This extract was found to contain I by TLC and GLC analysis. After intravenous administration, the radioactivity in the feces was not extractable in petroleum ether but appeared in ether, methanol, and water extracts. TLC confirmed that the radioactivity in these solvents was associated with metabolites of I. Bile contained mainly metabolites of I, as did the urine. Less than 10% of the radioactivity was excreted in the urine of each group of rats.

Keyphrases □ *trans*-Δ⁹-Tetrahydrocannabinol, radiolabeled, and metabolites—excretion kinetics after oral and intravenous administration, intact and bile duct-cannulated rats □ Excretion kinetics—radiolabeled *trans*-Δ⁹-tetrahydrocannabinol and metabolites after oral and intravenous administration, intact and bile duct-cannulated rats □ Cannulation *versus* noncannulation of bile duct—excretion kinetics of radiolabeled tetrahydrocannabinol after oral and intravenous administration, rats

The earliest reports describing the distribution of Δ⁹-tetrahydrocannabinol (I) were made by Miras (1) and Joachimoglu *et al.* (2). These investigators described the distribution of ^{14}C-Δ⁹-tetrahydrocannabinol, isolated from marijuana grown in a $^{14}CO_2$ atmosphere, in the rat. The findings of King and Forney (3) using a fluorometric method and those of Turk (4) using TLC were consistent with the earlier reports. Human studies by Miras and Coutselinis (5) indicated that only 5.2% of smoked radiolabeled hashish was secreted by the bile and 16.9% was excreted through the urine.

Due to the recent synthesis of highly purified tritium- and carbon-labeled I and their availability to the research community, more progress has been made concerning the metabolism and excretion of I. Lemberger *et al.* (6, 7) showed that radiolabeled I is mainly excreted by man in the feces and to a less extent in the urine, mainly as polar metabolites. Agurell *et al.* (8, 9) studied the metabolism and excretion of radiolabeled I in the rat and the rabbit. More recently, Klausner and Dingell (10) also reported the excretion of I in the rat. Their data agree with the excretion data reported by

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Due to the recent synthesis of highly purified tritium- and carbon-labeled I and their availability to the research community, more progress has been made concerning the metabolism and excretion of I. Lemberger *et al.* (6, 7) showed that radiolabeled I is mainly excreted by man in the feces and to a less extent in the urine, mainly as polar metabolites. Agurell *et al.* (8, 9) studied the metabolism and excretion of radiolabeled I in the rat and the rabbit. More recently, Klausner and Dingell (10) also reported the excretion of I in the rat. Their data agree with the excretion data reported by

Agurell *et al.* (8, 9); however, there was a difference in the rate of excretion between the two studies. The work of Klausner and Dingell (10) as well as that of Agurell *et al.* (8) showed that the radioactive material was mainly excreted in the feces and in a polar form. Liver perfusion experiments suggest that the main route of excretion is by way of the bile (10). This was also suggested in the early work of Joachimoglu *et al.* (2). To our knowledge, no one has reported the quantitative excretion of I and its metabolites in the urine, bile, and feces after oral and intravenous administration of radiolabeled I. Nor has the quantitative excretion of I and its metabolites been reported following cannulation of the rat bile duct *versus* noncannulation of the bile duct to determine the major route of excretion after oral and intravenous administration of the drug.

We have examined the excretion kinetics of intravenously and orally administered tritium-labeled I and its metabolites in the feces, urine, and bile of bile duct-cannulated rats and in urine and feces of noncannulated rats. The results of these experiments comprise the body of this report.

EXPERIMENTAL

Tritium-labeled I (25.4 μc./mg.), labeled in positions 2, 4, 8, and 10, with radiopurity of 100% and GLC purity of 97%, was obtained from the National Institute of Mental Health. Male Sprague–Dawley rats, weighing between 230 and 260 g., were used in these experiments.

The tritium-labeled I was prepared for intravenous and oral administration by suspending it in bovine albumin (fatty acid poor) (11).

Excretion of Tritium-Labeled I Administered Intravenously—Tritium-labeled I (4 mg./kg., 5 μc./rat) was administered intravenously to each rat in a total albumin suspension volume of 0.2 ml. Two groups, consisting of four rats each, were used in this experiment. One group was bile duct cannulated and the other was not. All rats were housed individually in metabolism cages with water and food available *ad libitum*.

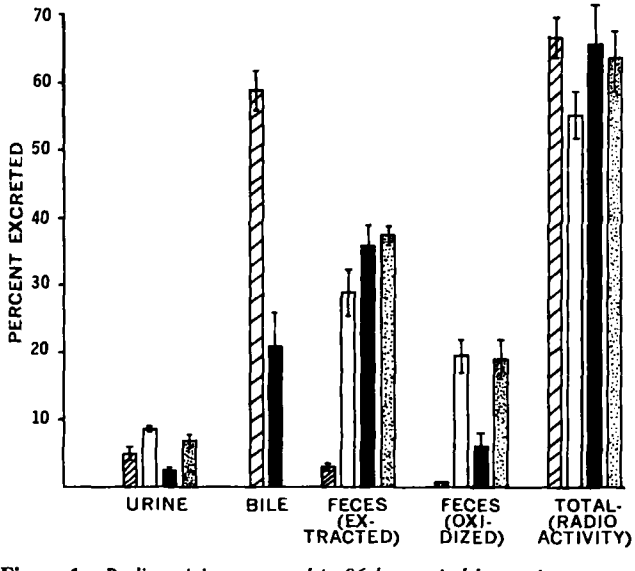

Figure 1—*Radioactivity excreted in 96-hr. period by various routes of excretion following intravenous injection of I (4 mg./kg., 4.7 μc./rat) or oral administration (100 mg./kg., 10 μc./rat); the bars represent the standard error of the mean. Key:* ■, *intravenous, bile duct cannulated* (n = 4); □, *intravenous, bile duct not cannulated* (n = 4); ■, *oral, bile duct cannulated* (n = 5); *and* ▣, *oral, bile duct not cannulated* (n = 5).

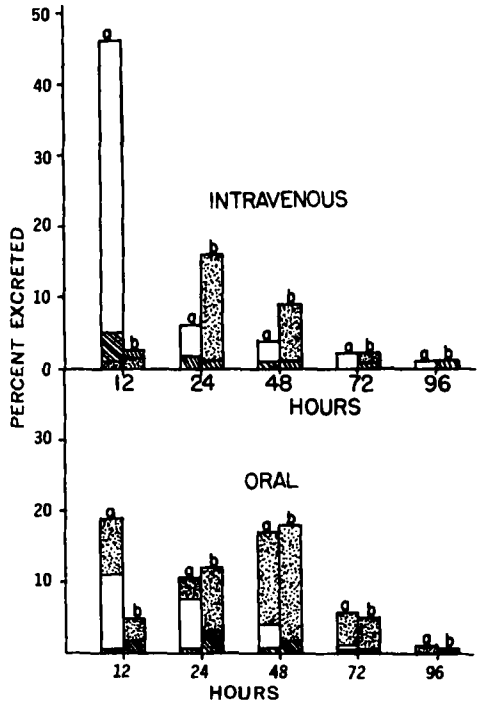

Figure 2—*Radioactivity excreted during various time periods following intravenous administration (4 mg./kg., 4.7 μc./rat) or oral administration (100 mg./kg., 10 μc./rat). These columns are not cumulative; for example, during the first 12 hr., 1% was excreted in urine, 10.5% in bile, and 19% in feces following oral administration in bile duct-cannulated rats. Key: (a) bile duct cannulated; (b), bile duct not cannulated;* □, *bile;* ■, *urine; and* ▣, *feces. As in Fig. 1, each group medicated intravenously contained four rats and each group medicated orally contained five rats.*

The bile duct cannulation was performed 4 days prior to the injection. Bile and urine were collected separately at the following times: 3, 6, 12, 48, 72, and 96 hr. after administration of drug. Feces were collected at 12, 24, 48, 72, and 96 hr. after administration of drug.

Excretion of Tritium-Labeled I Administered Orally—Two groups of rats (one bile duct cannulated, the other not), consisting of five rats each, were used in this experiment. The only variation from the intravenous study was that these rats were administered 100 mg./kg. of Compound I (10 μc.) orally in a total albumin suspension volume of 2 ml.

Determination and Solvent Extraction of Radioactivity in Excreta—After collection of the excreta, its volume (bile and urine) or dry weight (feces) was recorded. The feces, after drying, were ground in a mortar to assure a uniform sampling. After the dried feces samples were ground, each sample was extracted three times for 15 min., first with petroleum ether (30–60°), second with ethyl acetate, third with methanol, and finally with water. Each solvent extract was pooled, evaporated to dryness, and made up to 10 ml.; then a 0.2-ml. aliquot of each was counted in a liquid scintillation counter using a scintillation fluid containing 1 l. of a purified

Table I—Comparison of the Radioactivity Excreted in Bile following the Oral or Intravenous Route of Administration

Collection Period, hr.	Route	Percent of Total Radioactivity Excreted, Mean ± *SE*
0–3	Intravenous	26.03 ± 1.47
	Oral	2.42 ± 0.63
3–6	Intravenous	10.82 ± 0.59
	Oral	1.98 ± 1.13
6–12	Intravenous	9.29 ± 0.71
	Oral	2.85 ± 1.16

Table II—Fecal Excretion of ^3H in Rats Treated with ^3H-Δ^9-Tetrahydrocannabinol

Route of Administration	Bile Cannulation	Percent of ^3H Excreted in Feces Extracted in Solvents: Petroleum Ether	Ethyl Acetate	Methanol	Water	Oxidized, Not Extracted
Intravenous	Yes	Negative	Negative	Negative	Negative	Negative
Intravenous	No	3	12	27	16	42
Oral	Yes	47	29	8	0	16
Oral	No	10	16	24	16	34

nonionic detergent[1], 8 g. of 2,5-diphenyloxazole, 200 mg. of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, and 2 l. of toluene. After the last water extraction, the remaining fecal sample was dried and oxidized in a combustion flask and counted in the liquid scintillation counter to determine the remaining radioactivity. The amount of radioactivity in the oxidized sample and that found in each extraction solvent were combined to determine the total radioactivity excreted in feces for each time period.

Aliquots of bile (0.2 ml.) and urine (0.4 ml.) were counted directly in the liquid scintillation counter, and the total radioactivity excreted by each route was calculated for each collection time. All samples were corrected for quenching by internal standardization. The remaining bile and urine were each extracted three times with petroleum ether and ether as described for the fecal samples.

Identification of Radioactive Compounds Excreted—The various solvent extracts of the bile, feces, and urine were chromatographed on TLC System 1 reported by Turk *et al.* (4, 12, 13) and on the TLC system (System 2) reported by Lemberger *et al.* (6). This was done to identify the components present in each solvent extract. System 1 allows the separation of I from its metabolites, and System 2 separates the metabolites 8,11-dihydroxy-Δ^9-tetrahydrocannabinol (II) and 11-hydroxy-Δ^9-tetrahydrocannabinol (III). The presence of I was further identified by GLC (4).

RESULTS

Intravenous Route of Administration—*Bile Duct-Cannulated Rats*—The excretion of tritium in the bile duct-cannulated rats is shown in Figs. 1 and 2. In the bile duct-cannulated rats, 66.9% of the radioactivity was excreted during the first 96 hr. Of this amount, 4.8% was excreted in the urine, 59.4% in the bile, and 2.7% in the feces. The major portion of the radioactivity that was excreted in the bile was excreted in the first 3 hr.; after 12 hr., excretion by the biliary route dramatically decreased (Table I).

Excretion of radioactivity in the urine was essentially complete within 48 hr. The excretion of radioactivity in the feces was detectable only during the first 24 hr.

Noncannulated Rats—In the noncannulated group of rats, 55.8% of the radioactivity was excreted during the first 96 hr. (Fig. 1). Of this radioactivity, 7.4% was excreted in the urine and 48.4% was excreted in the feces.

Excretion of radioactivity in the urine was greatest in the first 12 hr. and was negligible for the remainder of the 96-hr. period. Of the radioactivity excreted in the feces, the greatest excretion occurred between the 12- and 48-hr. interval and appeared to be complete by 72 hr. (Fig. 2).

Oral Route of Administration—*Bile Duct-Cannulated Rats*—The bile duct-cannulated group of rats excreted 65.5% of the radioactivity in 96 hr. Of this radioactivity, only 2.7% was excreted in the urine, 41.5% was excreted in the feces, and 21.5% was excreted in the bile (Fig. 1).

Noncannulated Rats—The noncannulated rats excreted 63.6% of the total radioactivity in the 96-hr. period. Of this amount, 6.9% was excreted in urine and 56.7% was excreted in the feces (Fig. 1).

Identification of Radioactive Compounds Excreted—After visualization with diazo blue B salt, both TLC Systems 1 and 2 indicated the presence of I in the petroleum ether extract of feces and no evidence for the presence of metabolites. The presence of I was further substantiated by GC and by zone scraping of the TLC plate. Counting of the zone scrapings confirmed that the major radioactivity was in the zone of the Compound I spot. The ethyl acetate, methanol, and water extracts of the feces were plated. Some of the components identified in these fractions had the same R_f values as II and III. There also existed, in these fractions, radioactivity of a more polar nature than II and III. The quantification of radioactivity in the various solvent extracts of bile, feces, and urine is given in Tables II–IV.

DISCUSSION

The results presented in this paper show that 55–67% of the radioactivity is excreted within 96 hr. after either an oral or intravenous injection of tritium-labeled I. These data do not agree with those reported by Agurell *et al.* (8), who found that the excretion of I was very slow in the rat and that less than 50% of the dose was excreted during the 1st week. However, the present data agree with those of Klausner and Dingell (10), who reported that the excretion of radioactivity was essentially complete within 72 hr. after an injection of I. The present data also show that the major route of excretion of I is by way of the bile, mainly as polar metabolites (II and III).

Analytical data indicate that when the bile duct was not cannulated, the radioactivity excreted in the feces following oral dosing represented mainly metabolites. However, when the bile duct was cannulated, these metabolites were found in the bile but not in the feces. Determination of radioactivity following intravenous administration showed that there was negligible excretion of radioactivity in the feces of bile duct-cannulated animals. However, ^3H excreted in the feces in the noncannulated rats was associated with metabolites II and III. On this basis, one might theorize that the long duration of action of I might be due to enterohepatic circulation of either one or more of its metabolites.

Table III—Biliary Excretion of Radioactivity in Rats Treated with ^3H-Δ^9-Tetrahydrocannabinol

Route of Administration	Excreted in Bile, d.p.m. × 10^6	Extracted in Petroleum Ether, d.p.m.	Extracted in Ether, d.p.m.
Intravenous	5.49	79,766 (0.64%)[a]	792,770 (7.17%)
Oral	4.82	71,414 (1.48%)	393,554 (8.17%)

[a] Percent of total excreted in bile that was extractable in the solvent.

[1] Triton X-100.

Table IV—Urinary Excretion of Radioactivity in Rats Treated with ^3H-Δ^9-Tetrahydrocannabinol

Route of Administration	Bile Cannulation	Excreted in Urine, d.p.m. × 10^6	Extracted in Petroleum Ether, d.p.m.	Extracted in Ether, d.p.m.
Intravenous	Yes	0.56	498 (0.09%)[a]	26,112 (4.69%)
Intravenous	No	2.73	406 (0.01%)	31,232 (1.15%)
Oral	Yes	1.12	692 (0.06%)	5022 (0.45%)
Oral	No	2.18	1246 (0.06%)	99,426 (4.55%)

[a] Percent of total excreted in urine that was extractable in the solvent.

A considerable quantity of the radioactivity (47% in bile duct-cannulated and 10% in intact rats) excreted in the feces of the rats given tritium-labeled I orally was identified as the parent compound. This material probably represents Compound I, which was not absorbed from the GI tract.

Radioactivity in the urine was in the form of metabolites of I. These data agree with previous reports (6, 9) of the appearance of polar metabolites in the urine. Zone scraping of the TLC plates indicates the presence of metabolites other than 11-hydroxy-Δ^9-tetrahydrocannabinol and 8,11-dihydroxy-Δ^9-tetrahydrocannabinol. Identity of this large polar fraction has not been achieved. This may be accounted for, in part, by the recently described (14) diacetyl derivative found in bile. The identity of the material remaining in the feces after exhaustive extraction is also a mystery. Unfortunately, sufficient samples of material were not available for hydrolysis studies. These are planned.

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H. KIMIZUKA* and L. G. ABOOD▲

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Keyphrases □ Adsorption, interfacial, ^{3}H-labeled psychotomimetic agent—studied using liquid scintillation counting □ Interfacial adsorption and oil–water partitioning of radiolabeled compounds—studied using liquid scintillation counting □ 2-Pyrrolidylmethyl *N*-methyl cyclopentylphenylglycolate, radiolabeled—interfacial phospholipid adsorption and oil–water partitioning studied using liquid scintillation counting □ Anticholinergic psychotomimetic agents, radiolabeled—interfacial phospholipid adsorption and oil–water partitioning studied using liquid scintillation counting □ Liquid scintillation counting—used to measure interfacial adsorption and oil–water partitioning of radiolabeled psychotomimetic agent □ Partitioning (oil–water) of ^{3}H-2-pyrrolidylmethyl *N*-methyl cyclopentylphenylglycolate—studied using liquid scintillation counting

For a number of years, interest in this laboratory has focused on the mechanism of action of a group of anticholinergic psychotomimetic agents, particularly with regard to their ability to modify the physicochemical properties of excitatory membranes (1). A useful approach has been to examine the effect of the agents on the interfacial properties of surface films of phospholipids and membranous proteins using conventional interfacial techniques, including the measurement of surface adsorption with radioactive compounds (2).

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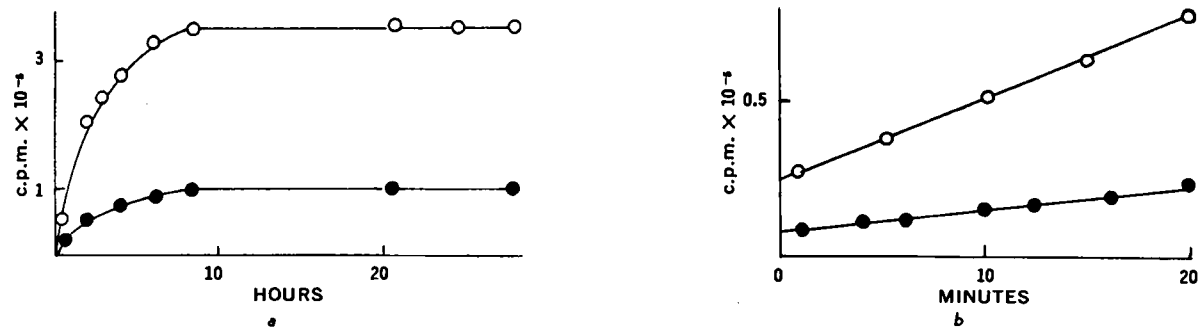

Figure 1—(a) *Time course of partitioning of I from the aqueous to the scintillator phase. Concentration of I was 0.16 mM in the presence* (○) *and in the absence* (●) *of II.* (b) *Time course of partitioning of I from the aqueous to the scintillator phase during initial period. Concentration of I was 0.16 mM in the presence* (○) *and in the absence* (●) *of II.*

interacts with a surface film (comprised of a lipid or other surfactant) by means of a geiger-mueller tube (3). One difficulty, however, has been the insensitivity of the geiger-mueller tube to soft β-radiation, particularly in the case of ^{3}H-labeled compounds.

To overcome this limitation, a new technique was developed for the measurement of interfacial interaction as well as oil–water partitioning by use of the liquid scintillation method. By means of this technique, the interaction of ^{3}H-labeled drugs with lipoidal substances can be determined with greater accuracy and simplicity. The present study is concerned with the kinetic aspects of the interfacial interaction and oil–water partitioning of the ^{3}H-labeled psychotomimetic agent, ^{3}H-2-pyrrolidylmethyl *N*-methyl cyclopentylphenylglycolate (I) employing a phospholipid, didodecyl phosphate (II).

EXPERIMENTAL

The scintillator solution was prepared by dissolving 4 g. of 2,5-diphenyloxazole and 0.25 g. of *p*-bis[2-(5-phenyloxazolyl)]benzene in 1:1 toluene (*S*). This solution (*S*) was diluted twice by volume with either toluene (*S* + *T*), or toluene solution of 1 m*M* didodecyl phosphate (*S* + *D*). After 10 ml. of an aqueous solution of ^{3}H-I was introduced in a vial, 10 ml. of the scintillator solution was carefully layered on the top solution and the radiation was counted with a liquid scintillation counter. The samples were counted immediately (within 1 min.) after preparation. By extrapolation to zero time, the time course of adsorption during the first few minutes could be determined.

The specific activity of I was determined in a mixture of 1 ml. aqueous ^{3}H-I solution of known concentration, 10 ml. ethanol, 5 ml. (*S* + *T*), and 5 ml. (*S* + *D*). The specific activity of I in this mixture was found to be (3.76 ± 0.19) × 10^{11} c.p.m./mole. By means of this standardization procedure, the specific activities (mean ± *SD*) of I were determined in the scintillator solutions (*S* + *T*) and (*S* + *D*) and were found to be (5.09 ± 0.25) × 10^{12} c.p.m./mole in (*S* + *T*) and (3.10 ± 0.16) × 10^{11} c.p.m./mole in (*S* + *D*), respectively. The aqueous I solution was adjusted to pH 2 with hydrochloric acid unless otherwise stated.

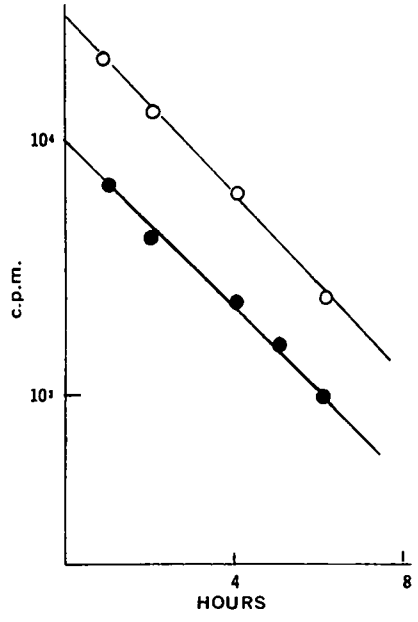

Figure 2—*Time course of partitioning, log* (I_∞ − I) versus t *in the presence* (○) *and in the absence* (●) *of II.*

RESULTS AND DISCUSSION

The increase in counting due to the partitioning of I into the scintillator phase was measured with time, and an example is shown in Figs. 1*a* and 1*b*. These figures indicate that the partition coefficient was greater in the presence of didodecyl phosphate than in its absence, although the time constants for partitioning were approximately the same under both conditions.

The semilogarithmic plots for the data are given in Fig. 2, which are expressed by the relation:

$$\ln (I_\infty - I) = \ln I_\infty - kt \qquad \text{(Eq. 1)}$$

where *I* denotes the radioactive counts at time *t*, I_∞ is the equilibrium value, and *k* is the rate constant. From Figs. 1 and 2, it is apparent that the radioactivity arises from the scintillator phase. Since the counting is proportional to the amount of ^{3}H-I in the scintillator phase, Eq. 1 may be rewritten as:

$$\ln (m_{II_\infty} - m_{II}) = \ln m_{II_\infty} - kt \qquad \text{(Eq. 2)}$$

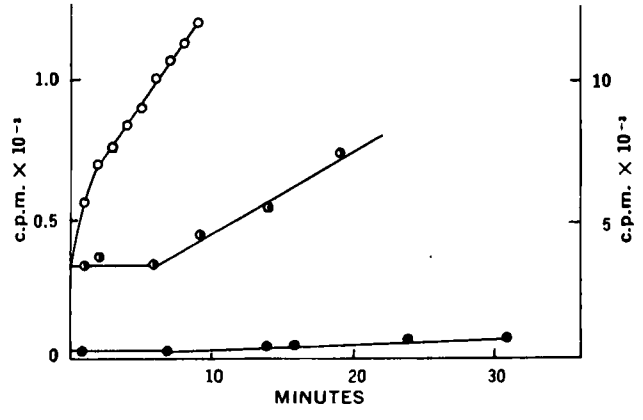

Figure 3—*Time course of partitioning of I during initial period, with II present in scintillator phase. Initial concentrations of I in the aqueous phase were: 1.6 × 10^{-5} M* (○), *1.8 × 10^{-6} M* (◑), *and 1.8 × 10^{-7} M* (●).

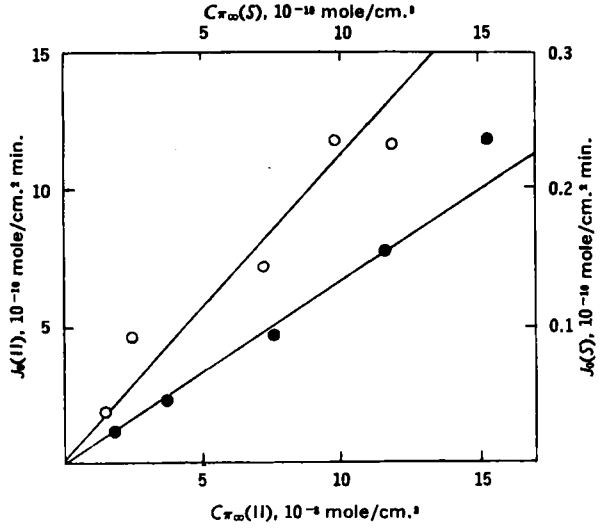

Figure 4—*Initial rate of permeation* versus *equilibrium concentration of I in the scintillator phase in the presence* (○) *and in the absence* (●) *of II.*

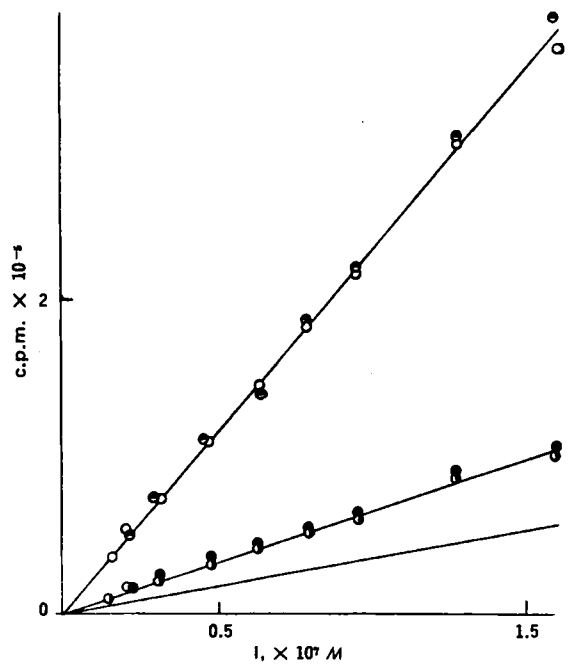

Figure 5—*Partitioning of I between scintillator and aqueous phases in the presence or the absence of II. Key:* ○ *and* ◔, *activity in presence of II in 5 ml. scintillator phase and 5 ml. scintillator + 10 ml. aqueous phase, respectively; and* ● *and* ◑, *same as above but in absence of II. Lower curve refers to the calibration by the standardization procedure described in text.*

where m_{II} denotes the amount of I present in the scintillator phase at a time t, and m_{II_∞} is the equilibrium value.

When t is small, the counting is linear with time after the first 3–7 min. (Fig. 3). At low values of t, m_{II} is also very small as compared with m_{II_∞}, so Eq. 2 may be reduced to:

$$m_{II} = m_{II_\infty} kt \qquad \text{(Eq. 3a)}$$

or:

$$C_{II} = C_{II_\infty} kt \qquad \text{(Eq. 3b)}$$

where C_{II} and C_{II_∞} are the concentrations at a time t and infinity; respectively. By differentiating Eq. 3*a* with respect to t and dividing by the interfacial area A:

$$j_0 = \frac{1}{A}\frac{dm_{II}}{dt} = \frac{m_{II_\infty}}{A} k_0 = \frac{V_{II}}{A} C_{II_\infty} k_0 \qquad \text{(Eq. 4)}$$

where j_0 and k_0 denote the rate of partitioning per unit area and the rate constant, respectively, at the small values of t; and V_{II} is the volume of scintillator phase. Values for j_0 and C_{II_∞} were determined for various concentrations of I, and the results (Fig. 4) verified the relation given by Eq. 4.

According to a previous study (4), the permeation of an ion through a membrane is expressed as:

$$j = P[C_I e^{-(ZFV+\eta)/2RT} - C_{II} e^{(ZFV+\eta)/2RT}] \qquad \text{(Eq. 5)}$$

where j denotes the steady ion flux; P is the membrane permeability; C_I and C_{II} are the concentrations of diffusible ion in phases I and II, respectively, which are separated by a membrane; Z is the charge of the ion; F is the Faraday constant; V is the transmembrane potential; η is the free energy of partition between phases I and II; R is the gas constant; and T is absolute temperature. For nonionic species, $Z = 0$ and Eq. 5 reduces to:

$$j = P[C_I e^{-(\eta/2RT)} - C_{II} e^{(\eta/2RT)}] \qquad \text{(Eq. 6)}$$

Table I—Rate Constant in Permeation

	With II	Without II	Ratio
$\tau = k^{-1}$	150 min.	120 min.	1.25
Partition constant	$K_2 = 2.89$	$K_1 = 1.12 \times 10^{-2}$	258
P	9.33×10^{-5}/cm./sec.	2.25×10^{-5}/cm./sec.	3.66
k_1	1.59×10^{-4}/cm./sec.	2.70×10^{-6}/cm./sec.	50.9
k_2	5.50×10^{-5}/cm./sec.	2.41×10^{-4}/cm./sec.	0.23

Equation 6 may also be applied to the permeation process through an oil–water interface, since the interface can be regarded as the boundary separating the aqueous (I) from the scintillator (II) phase.

Since the permeation of I from the aqueous to scintillator phase was observed in the present study, it follows that:

$$V_I C_I + V_{II} C_{II} = V_I C_0 \qquad \text{(Eq. 7)}$$

where V_I and V_{II} denote the volumes of the aqueous and scintillator phases, respectively; and C_0 is the initial concentration in the aqueous phase. When the equilibrium partition is attained, one has from Eq. 6:

$$C_{II_\infty}/C_{I_\infty} = e^{-\eta/RT} = K \qquad \text{(Eq. 8)}$$

where K denotes the partition constant, and the subscript ∞ refers to the equilibrium value. Combining Eqs. 6–8, one finds:

$$j = P(e^{-\eta/2RT} + e^{\eta/2RT})(C_{II_\infty} - C_{II}) \qquad \text{(Eq. 9)}$$

provided $V_I = V_{II} = 10$ ml. (see *Appendix*).

Since the total flux, J, is given by j multiplied by the area of the interface, A, one has from Eqs. 4 and 9:

$$J = Aj = \frac{dm_{II}}{dt} = k(m_{II_\infty} - m_{II}) \qquad \text{(Eq. 10)}$$

where:

$$k = AP(e^{\eta/2RT} + e^{-\eta/2RT})/V_{II} \qquad \text{(Eq. 11a)}$$

$$m_{II} = C_{II} V_{II} \qquad \text{(Eq. 11b)}$$

$$m_{II_\infty} = C_{II_\infty} V_{II} \qquad \text{(Eq. 11c)}$$

Integrating Eq. 10 with respect to time, Eq. 2 is obtained, which may be reduced to Eq. 4 when t is small. The values of the time constant, τ, or the reciprocal of the rate constant in the presence or absence of II in the scintillator phase are given in Table I.

After partition equilibrium was attained, 5 ml. of scintillator solution was transferred to another vial, and the radioactivity was measured in both vials (Fig. 5). It was found that the I in the scin-

tillator phase was homogeneously distributed, while the radiation from the aqueous part was within experimental error and, hence, was disregarded. The lower curve refers to the calibration by the standardization procedure described in the *Experimental* section. The standardization procedure, by permitting the determination of the concentrations of I in the aqueous and scintillator phases, makes it possible to calculate the partition constant.

Equation 6 may also be written as:

$$j = k_1 C_I - k_2 C_{II} \qquad \text{(Eq. 12)}$$

where:

$$k_1 = Pe^{-\eta/2RT} \qquad \text{(Eq. 13}a\text{)}$$

$$k_2 = Pe^{\eta/2RT} \qquad \text{(Eq. 13}b\text{)}$$

The values of P, k_1, and k_2 may be evaluated according to Eqs. 1 and 11, provided the partition constant, the volume of scintillator phase, and the area of interface are known. By assuming the interface to be spherical and measuring the meniscus height of the interface and the inner diameter of the vial, the interfacial area was found to be 5.2 cm.^{2}. The values of P, k_1, and k_2 are given in Table I, together with the time constant and the partition constant. Also given are the ratios of these values both in the presence and absence of II.

According to Eq. 4, the time constant for initial sorption, τ_0, can also be estimated from Fig. 4. The estimated values of time constant τ_0 were near to those of τ estimated from the whole curves of the time course, as indicated in Table I. The turbulence of the system involved in the initial stage of sorption would be responsible for the slight deviation of τ_0 from τ.

As seen in Table I, the partition constant K_2 is much greater than K_1, a finding suggesting the formation of a II salt of I. The formation constant, K_s, of the salt in the scintillator phase can be expressed by:

$$K_s = \frac{C_{II_\infty}^{salt}}{C_D \cdot C_{II_\infty}} \qquad \text{(Eq. 14)}$$

where $C_{II_\infty}^{salt}$ and C_{II_∞} denote the concentrations of salt and free I, respectively; and C_D is the concentration of free II.

Since the volumes of aqueous and scintillator phases are identical, the following relations hold:

$$C_{I_\infty} = C_I^0 - (C_{II_\infty} + C_{II_\infty}^{salt}) \qquad \text{(Eq. 15)}$$

$$C_{II_\infty}^{salt} = (C_{II_\infty}^{salt} + C_{II_\infty}) - C_{II_\infty} \qquad \text{(Eq. 16)}$$

and:

$$C_D = C_D^0 - C_{II_\infty}^{salt} \qquad \text{(Eq. 17)}$$

where C_I^0 and C_D^0 are the initial concentrations of I in the aqueous phase and those of II in the scintillator phase, respectively. In Eq. 15, $(C_{II_\infty} + C_{II_\infty}^{salt})$ indicates the total concentration of I in the scintillator phase that may be estimated from the radiation intensity. The concentration of free I in the scintillator phase, C_{II_∞}, may be determined from the relation:

$$K_1 = C_{II_\infty}/C_{I_\infty} \qquad \text{(Eq. 18)}$$

where K_1 is the partition constant for free I. The value of K_1 in the absence of II was found to be $(1.12 \pm 0.02) \times 10^{-2}$. Since the presence of II does not alter K_1, $C_{II_\infty}^{salt}$ can be determined according to Eqs. 16 and 18 and C_D can be determined according to Eq. 17. The values of K_s calculated according to Eq. 14 by using the data at various concentrations of I were found to be a constant within experimental error, $K_s = (5.7 \pm 0.4) \times 10^5\ M^{-1}$ (Table II). This verifies Eq. 4, which suggests the formation of a 1:1 salt between the cation of I and anion of II.

Since K_2, the partition constant in the presence of II, is given by:

$$K_2 = \frac{C_{II_\infty} + C_{II_\infty}^{salt}}{C_{I_\infty}} \qquad \text{(Eq. 19)}$$

combining this equation with Eqs. 14–19 (see *Appendix*) gives:

$$K_2 = K_1 + \frac{K_s K_1 C_D^0}{1 + K_s K_1 C_{I_\infty}} \qquad \text{(Eq. 20)}$$

When $K_s K_1 C_{I_\infty}$ is much less than unity, K_2 may be reduced to:

$$K_2 \simeq K_s K_1 C_D^0 \qquad \text{(Eq. 21)}$$

provided K_1 is much less than K_2. Since the observed value of K_2 (2.89 ± 0.25) is much greater than K_1 and $K_s K_1 C_{I_\infty}^0$ is less than unity, the approximate value of K_2 may be evaluated according to Eq. 21. A value of 3.02 is obtained, which is close to the observed value, 2.89.

The partition of I between the $(S + T)$ and the aqueous phase at pH 7.4 was also measured and the partition coefficient was found to be 9.6 ± 1.1 (Table III). The pH dependence of I may be attributed to the dissociation of I ion in aqueous phase, *i.e.*:

$$K_a = \frac{[H^+]_W [PMCG]_W}{[PMCG^+]_W} \qquad \text{(Eq. 22)}$$

where the subscript W refers to the aqueous phase, and PMCG = I.

Since the PMCG exists in the form of $PMCG^+$ and PMCG, one has from Eq. 22:

$$\frac{[PMCG]_{TW}}{[PMCG]_W} = \frac{[PMCG^+]_W + [PMCG]_W}{[PMCG]_W} = \frac{[H]_W}{K_a} + 1 \qquad \text{(Eq. 23)}$$

where $[PMCG]_{TW}$ is the total concentration of I in the aqueous phase. On the other hand, the partition coefficient of the neutral I molecule between the $(S + T)$ and the aqueous phase is defined as:

$$K_{0W} = \frac{[PMCG]_0}{[PMCG]_W} \qquad \text{(Eq. 24)}$$

Since the concentrations of I in the $(S + T)$ phase, $[PMCG]_0$, and in the aqueous phase are equivalent to C_{II_∞} and C_{I_∞} of Eq. 18, respectively:

$$K_1 = \frac{[PMCG]_0}{[PMCG]_{TW}} \qquad \text{(Eq. 25)}$$

and, hence:

$$K_{0W} = K_1\left(\frac{[H]_W}{K_a} + 1\right) \qquad \text{(Eq. 26)}$$

Table II—Constants in Partition Phenomenon[a]

Concentration					10^5
$10^{-5}\ M$					M^{-1}
C_I^0	C_{I_∞}	$C_{II_\infty}^{salt}$	C_D	C_{II_∞}	K_s
16	4.7	11.25	38.75	0.052	5.6
12.8	3.1	9.64	40.36	0.035	6.8
9.6	2.6	6.97	43.03	0.029	5.6
8.0	1.5	5.48	43.52	0.017	7.4
6.4	1.9	4.46	45.54	0.021	4.7
4.8	1.54	3.24	46.76	0.017	4.1
3.2	0.78	2.41	47.59	0.009	5.7
2.4	0.59	1.80	48.20	0.007	5.4
		Mean $K_s = (5.7 \pm 0.4) \times 10^5\ M^{-1}$			

[a] $K_1 = (1.12 \pm 0.02) \times 10^{-2}$
$K_2 = 2.89 \pm 0.24$
$K_s = (5.7 \pm 0.4) \times 10^5\ M^{-1}$
$K_2 \doteqdot K_s K_1 C_D^0 = 3.02$
Formation constant, K_s, of salt.
$(C_D^0 = 50 \times 10^{-5}\ M)$

Table III—Partition Coefficient of I

	pH	K_1
1.8 *M* I	7.4	8.0
1.8 *M* I (hyaluronic acid)[a]	7.4	8.0
9.6 I	7.4	8.0
Desorption experiment 1	7.4	13.0
Desorption experiment 2	7.4	11.0
Mean		9.6 1.1

[a] The hyaluronic acid (0.01%) was present in the aqueous phase.

Table IV—Partition Coefficients and pKa of I Ion

pH	2	7.4
K_1	1.12×10^{-2}	9.6
K_a	10^{-5}	
pKa (calc.)	5	
pKa (obs.)	5.3 ± 0.2	
K_{ow}	10	

The stability constant of I ion in aqueous solution may be estimated from the measurements of the partition coefficients of I at various pH's since K_{ow} is a constant. The value of pKa calculated from K_1's at pH 2 and 7.4 was found to be 5, in agreement with 5.3 ± 0.2 which was determined by pH titration. K_{ow} which could be determined from Eq. 26, provided K_1, K_a, and pH were known, was found to be 10 (Table IV).

In investigating the effect of II on the permeation rate of I at pH 7.4, it was found that the rate of permeation was not influenced by the presence of II in the scintillator phase (Fig. 6). At pH 7.4, virtually all of the I in the aqueous phase would be in the neutral form, so that no ionic interaction with II would occur.

Finally, the transfer of I from the scintillator to the aqueous phase was examined under various conditions (Fig. 7). At pH 7.4, the transfer of I was negligible, indicating the high partition of I in the scintillator phase at this pH. On the other hand, the presence of a strong polyanion, such as the hyaluronic acid, in the aqueous phase at pH 2 enhanced the transfer rate of I from the scintillator to the aqueous phase with or without II in the scintillator phase.

In living cells, the membrane as well as the cell interior possesses components exhibiting a high affinity to diffusible substances such as inorganic ions, metabolites, and drugs. Interfacial techniques have proven to be effective tools for examining such phenomena and, thereby, have contributed to the development of an effective experimental model of a biological membrane. The adaptation of liquid scintillation to the measurement of oil–water partitioning and interfacial adsorption should extend the usefulness of the radiotracer technique for measuring surface adsorption.

APPENDIX

Derivation of Eq. 9—Combining Eq. 6 with Eq. 7, one derives:

$$j = P[C_{I}e^{-\eta/2RT} - C_{II}e^{\eta/2RT}]$$

$$= P[(C_0 - C_{II})e^{-\eta/2RT} - C_{II}e^{\eta/2RT}]$$

$$= P[C_0e^{-\eta 2RT} - C_{II}(e^{\eta/2RT} + e^{-\eta/2RT})]$$

$$= P(e^{\eta/2RT} + e^{-\eta/2RT})\left[\frac{C_0e^{-\eta/2RT}}{e^{\eta/2RT} + e^{-\eta/2RT}} - C_{II}\right] \quad \text{(Eq. A1)}$$

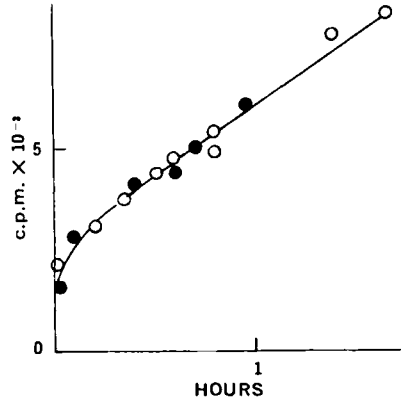

Figure 6—*Effect of II on the permeation rate of I, pH 7.4. Concentration of I was 1.8 × 10⁻⁶ M in the presence* (○) *and in the absence* (●) *of II.*

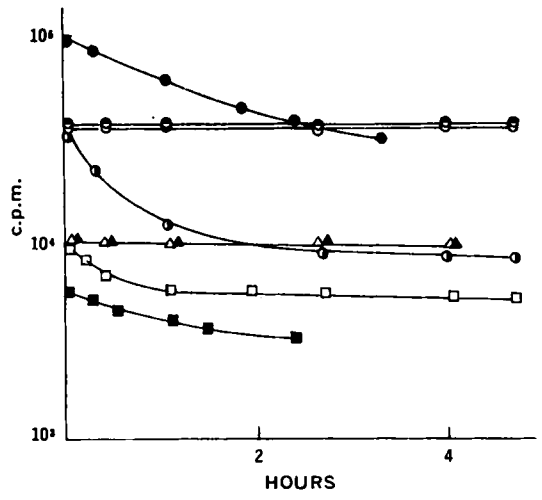

Figure 7—*Effects of adding various substances to scintillator and aqueous phases on the partition rate of I from scintillator phase to aqueous phase. Key:* ●, *in the presence of II alone, pH 2;* ○ *and* ◓, *in the presence of II at pH 7.4 with and without 0.01% hyaluronic acid (III), respectively;* ◑ *in the presence of II and 0.01% III, pH 2;* △ *and* ▲, *absence of II at pH 7.4 with and without 0.01% III, respectively; and* □ *and* ■, *absence of II at pH 2 with and without 0.01% III, respectively. III dissolved in aqueous phase and II dissolved in scintillator phase.*

From Eq. 8:

$$\frac{C_{I_\infty}}{C_{II_\infty}} + 1 = e^{\eta/RT} + 1 \quad \text{(Eq. A2)}$$

Since $V_I = V_{II}$, one has:

$$C_I + C_{II} = C_{I_\infty} + C_{II_\infty} = C_0 \quad \text{(Eq. A3)}$$

and combining Eq. A2 with Eq. A3:

$$C_{II_\infty} = \frac{C_0}{e^{\eta/RT} + 1} = \frac{C_0e^{-\eta/2RT}}{e^{\eta/2RT} + e^{-\eta/2RT}} \quad \text{(Eq. A4)}$$

Comparing Eq. A1 with Eq. A4, one obtains Eq. 9.

Derivation of Eq. 20—From Eq. 14:

$$C_{II\ salt} = K_aC_{II_\infty}C_D \quad \text{(Eq. A5)}$$

Combining Eqs. 14 and 17 and substituting in Eq. A5, one obtains:

$$C_D^0 - C_D = K_aC_{II_\infty}C_D \quad \text{(Eq. A6}a\text{)}$$

$$C_D = \frac{C_D^0}{K_aC_{II_\infty} + 1} \quad \text{(Eq. A6}b\text{)}$$

$$C_{II\ salt} = \frac{K_aC_{II_\infty}C_D^0}{K_aC_{II_\infty} + 1} \quad \text{(Eq. A6}c\text{)}$$

From Eq. 18, $C_{II_\infty} = K_1C_{I_\infty}$. Substituting Eqs. A5 and A6*c* in Eq. 19, one obtains:

$$C_{I_\infty}K_2 = K_1C_{I_\infty} + \frac{K_aK_1C_{I_\infty}C_D^0}{K_aK_1C_{I_\infty} + 1} \quad \text{(Eq. A7)}$$

Finally:

$$K_2 = K_1 + \frac{K_aK_1C_D^0}{1 + K_aK_1C_{I_\infty}} \quad \text{(Eq. A8)}$$

which is Eq. 20.

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ANWAR A. HAKIM

Abstract □ Epinephrine solutions were exposed to ultrasonic frequencies or to oxidation by passing oxygen through the solution until a pink color was formed. The effects of these epinephrine solutions were investigated on the physiological functions of heart papillary muscles and their contractile element, *i.e.*, actomyosin. When added to the bathing medium containing papillary muscle from beating rat hearts, the modified epinephrine produced a gradual increase in the maximum response of the muscle to electrical stimulation. If the incubating medium contained papillary muscle from quiescent dog heart, the muscle did not respond to electrical stimulation alone or in the presence of epinephrine. However, when added to the bathing medium, the sonified or the oxidized epinephrine caused initiation and potentiation of isometric contractions in response to electrical stimulation. The effects of the altered epinephrines on papillary muscles from both types of hearts were concentration and time dependent. When added with either the sonified or the oxidized epinephrine, a "serum protein" isolated from human serum intensified and stabilized the effects of both forms of epinephrine on the papillary muscle from rat or dog quiescent heart. When added to a standard actomyosin solution in imidazole–potassium chloride–ethyleneglycol tetraacetate salt buffer at pH 7.0, adenosine triphosphate caused precipitation of the contractile protein, which was assessed by the increase in absorbance and was measured spectrophotometrically at 660 nm. When the incubation mixture contained increments of the sonified or the oxidized epinephrine, the precipitating effect of adenosine triphosphate was not changed. But, if added with either modified epinephrine, the serum protein caused two- or fivefold increases in the precipitation of the contractile elements from solutions incubated in the presence of the oxidized or sonified epinephrine, respectively.

Keyphrases □ Epinephrine, ultrasonicated or oxidized—potentiation of isometric contraction in isolated heart papillary muscle □ Isometric contraction in isolated heart papillary muscle—potentiation by ultrasonicated or oxidized epinephrine □ Heart papillary muscle—potentiation of isometric contraction by ultrasonicated or oxidized epinephrine □ Ultrasonicated epinephrine—potentiation of isometric contraction in isolated heart papillary muscle

Epinephrine inotropy in cardiac muscle is characterized by two distinct alterations in the contractile process. In isolated tissue preparations (1) as well as *in situ* (2, 3), administration of epinephrine brings about an increase in systolic force or shortening, with an appreciable reduction in the duration of contraction (4).

In recent years, most studies on cardiac muscle have been performed on papillary muscle from beating cat or rat heart. When bathed or perfused with physiological buffer solution, isolated myocardial preparations spontaneously undergo contractile failure. Clark *et al.* (5) suggested that the addition of serum to the buffer solution stabilized these failing preparations. Addition of various plasma fractions or dialysates also delayed the onset of cardiac decay. This led Gabel *et al.* (6) to hypothesize that fatigue in isolated muscle preparations might also be influenced by the presence or absence of blood fractions. Other investigators (7, 8) showed that cardiac muscle maintained in plain buffer solutions lost the poststimulation potential, but it was restored by serum; they confirmed the presence of cardioactive principles in blood plasma.

The aim of the present studies is to demonstrate the initiation and potentiation of isometric contractions in electrically stimulated dog heart papillary muscle with epinephrine modified by exposure to gaseous cavitation produced by ultrasonic frequencies or by limited oxidation of the amine.

EXPERIMENTAL

Materials—Epinephrine solutions were transformed to a light-pink pigment, metaphrine, by two methods:

1. Solutions (10^{-6} *M*) of epinephrine in physiological saline were exposed to ultrasonic frequencies. A sonic oscillator[1] was employed at 20 w. at the machine maximum output of 10 kc., 0.78 amp. and 60 cycles. The solutions were sonified for 120 min. at 0–4° until the formation of metaphrines, which was assessed by the appearance of a light-pink color (9).

[1] Raytheon, model DF 101.

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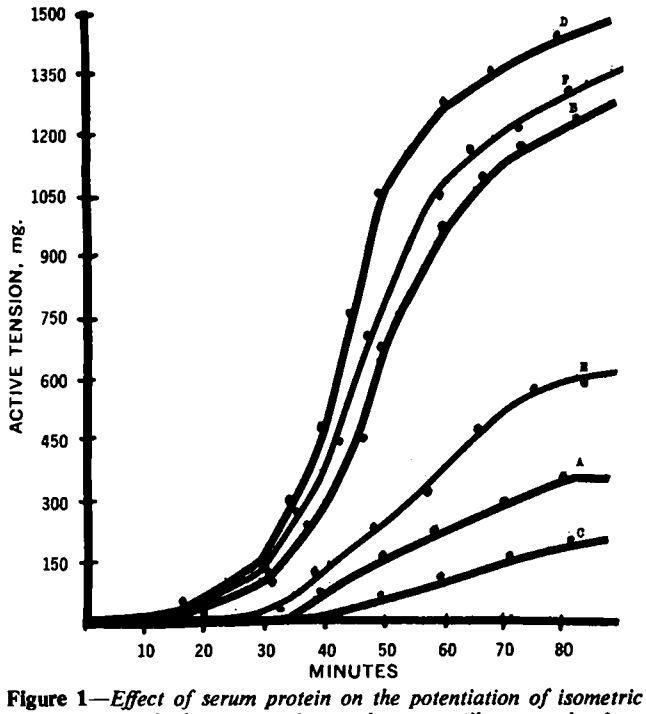

Figure 1—*Effect of serum protein on the potentiation of isometric contractions with ultrasonicated epinephrine: papillary muscles from quiescent dog heart incubated in the presence of 10^{-6} M epinephrine (A) and 10^{-6} M ultrasonicated epinephrine (B); tension developed by the papillary muscle incubating with 100 mcg. protein serum alone (C) and in the presence of 10^{-6} M ultrasonicated epinephrine (D); and tension generated in the muscle incubating with 10^{-6} M oxygenated epinephrine alone (E) and in the presence of 100 mcg. of the serum protein (F). The concentration of the ultrasonicated or oxidized epinephrine is taken as the concentration of epinephrine prior to treatment.*

2. A thin stream of oxygen was passed through solutions (10^{-6} *M*) of epinephrine in physiological saline to the appearance of a light-pink color, *i.e.*, 30 min. at 0–4°.

Dog hearts[2] were removed immediately after the animals expired and placed in cold preoxygenated bicarbonate–dextrose solution. Series of papillary muscles were removed, and the remaining tissue was frozen and saved for extraction of the contraction elements.

The papillary muscles were excised from the right ventricle and suspended from a force transducer[3] into a 50-ml. chamber containing 154.0 m*M* NaCl, 5.4 m*M* KCl, 7.4 m*M* $NaHCO_3$, 5.6 m*M* dextrose, and 5.6 m*M* $CaCl_2$ [*i.e.*, modified Krebs–Henseleit solution as described by Lefer (10)]. The papillary muscles were prepared with a preload of 1 g. and stimulated continuously at a frequency of 1/sec. for a duration of 3 msec. at 32 v. above threshold voltage. Under these experimental conditions, electrical stimulation alone did not generate isometric contractions in the papillary muscle from dog quiescent hearts. To the incubating medium containing the papillary muscle, aliquots of 0.05–0.20 ml. of 10^{-6} *M* ultrasonication modified epinephrine solution was added, and electrical stimulation was continued until maximum isometric contractions were generated at a steady state. The bathing medium was then drained, and the muscles were rinsed several times (5–10 times) with fresh buffer. Electrical stimulation was reinstated and continued to maximum steady state. Aliquots of the ultrasonication modified or the oxidized epinephrine, each alone or with the "serum protein," were then added into the incubating medium, and the isometric contractions were continuously recorded.

The human serum protein was prepared from freshly drawn blood as described by Hakim (11, 12).

Preparation of Human Heart Actomyosin—The hearts were obtained[4] from gunshot victims. The muscle was cleaned from adhering fat, minced, and then homogenized for 1 min. with three parts of Weber–Edsall solution (0.6 *M* KCl, 0.04 *M* NaCl, 0.04 *M* $NaHCO_3$, and 0.01 *M* Na_2CO_3). The homogenate was stirred continuously for 20 hr. at 4°, and the insoluble material was removed by centrifugation at $1000 \times g$ for 60 min. The pH of the supernate was adjusted to 7.0, and the actomyosin was precipitated with nine volumes of cold distilled water as described by Fanburg *et al.* (13).

Superprecipitation and clearing of actomyosin was followed by measuring the absorbance of an actomyosin suspension at 660 nm. in a spectrophotometer[5] after the addition of adenosine triphosphate. When added to an incubating mixture containing 0.1 *M* KCl, 0.5 m*M* imidazole, 0.1 m*M* ethylene glycol tetraacetate salt buffer at pH 7.0 and 0.50 mg. actomyosin (determined as protein), 0.2 m*M* adenosine triphosphate caused precipitation of the contractile protein, which was assessed by the increase in absorbance. When the incubating mixture contained aliquots of normal human serum or the isolated human serum protein, adenosine triphosphate caused an increase in the absorbance of the solution. The incubation mixtures were run in triplicate. The average deviation between the triplicate reactions was approximately 5.2%.

Determination of Adenosine Triphosphate, Phosphocreatine, and Lactic Acid—Immediately after the last contraction at the indicated time, the muscle was removed and quickly frozen in a dry ice–acetone mixture. An extract was then made in 0.25 *M* perchloric acid at 0°. All muscles were analyzed for adenosine triphosphate (14), phosphocreatine (15), and lactic acid (16).

RESULTS

Chemical Characterization of Modified Solutions of Epinephrine—When 1.0 ml. of either the ultrasonicated or the oxidized epinephrine was diluted with 4 ml. of pH 4.0 phthalate buffer and 1.0 ml. of 0.1 *N* iodine, followed by mixing for 5 min. and the addition of 2.0 ml. of 0.1 *N* sodium thiosulfate, a deep-red color was produced.

When 1.0 ml. of either modified epinephrine solution was diluted with 5 ml. of glacial acetic acid, followed by addition of 0.1 ml. of crystal violet test solution (0.5% solution) and titration with 0.1 *N* perchloric acid, 25.00 mg. of epinephrine, ultrasonicated epinephrine, or oxidized epinephrine consumed 1.36, 1.00, or 0.30 ml. of the acid, respectively.

When stored in the refrigerator for 3 months, epinephrine or its ultrasonicated form (10^{-6} *M* solutions in normal saline in 0.1 *N* HCl) remained clear with their initial specific optical rotation of −51 to −53°. Solutions of the oxidized form became brown in color with insoluble dark-brown sedimented particles.

These observations suggest that the ultrasonicated epinephrine is a metaphrine, while the oxidized epinephrine is mostly adrenochrome.

Physiological Effects on Rat Heart Papillary Muscle—When added to the bathing medium of rat heart papillary muscle obtained from beating heart, epinephrine at 10^{-6} *M* concentration produced a 15 ± 5.5% increase in the developed active tension and increased the rate of isometric contractions. Higher concentrations of epinephrine did not increase further the contractile tension but increased the rate of contraction to a magnitude that caused progressive decrease in the developed active tension where the muscle became refractive to electrical stimulation. The magnitude of the increase in the rate was concentration dependent.

If added to the bathing medium of papillary muscles obtained from beating hearts, ultrasonicated epinephrine solution at 10^{-6} *M* intensified the active tension without influencing the rate of the isometric contractions. The magnitude of the increase in tension was concentration and time dependent. Under the same experimental conditions, oxidized epinephrine at 10^{-6} *M* intensified the active tension without influencing the rate of the isometric contractions. The magnitude of the effect was also concentration and time dependent, equivalent to one-tenth the effect obtained with the ultrasonicated epinephrine.

Physiological Effects on Papillary Muscles from Dog Quiescent Hearts—Papillary muscles from quiescent dog hearts did not respond to electrical stimulation. Epinephrine or norepinephrine caused the muscle to respond to electrical stimulation after a latent period of 120–150 min. The magnitude of the response was time

[2] Obtained through Dr. L. Malasanos and Dr. S. Marotta of the Department of Physiology.
[3] TF .03.
[4] Cook County Morgue.
[5] Beckman model DU 2.

and concentration dependent. As shown by curves A in Figs. 1–3, an optimal concentration between 2 and 4 × 10^{-6} *M* produced a maximum tension of 575 ± 25 mg./mm.^{2}.

The data depicted by curves B of Figs. 1–3 suggest that, within 15 min., ultrasonicated epinephrine initiated and potentiated active tension in electrically stimulated dog heart papillary muscle. The magnitude of the developed active tension was concentration and time dependent. Electrical stimulation of papillary muscles incubating in the presence of 10^{-7} *M* ultrasonicated epinephrine for longer periods than 110 min. caused a progressive decrease in the developed active tension. These observations suggested that longer periods of electrical stimulation lead to the fatigue and decay of the papillary muscle.

Curves C of Figs. 1 and 2 indicate that 0.10 mg. of the serum protein prevented or delayed the fatigue or decay of the electrically stimulated papillary muscles.

Curve E of Fig. 1 suggests that, within 28–30 min., oxidized epinephrine initiated and potentiated the development of active tension in electrically stimulated dog heart papillary muscle. The magnitude of the effect was time and concentration dependent but equivalent to approximately one-third of the effect obtained with the ultrasonicated epinephrine. Curves F of Figs. 1 and 2 indicate that 0.10 mg. of the serum protein prevented or delayed the fatigue or decay of the electrically stimulated papillary muscle.

The results of experiments summarized in Fig. 1 demonstrate a lag period for the potentiation of isometric contractions in papillary muscle from quiescent dog heart with epinephrine, epinephrine derivatives, or the serum protein. Ultrasonicated epinephrine potentiated isometric contractions within 15 min. When combined with the ultrasonicated epinephrine, the serum protein enhanced and intensified the potentiation of the developed tension.

The data in Fig. 2 suggest that the maximum active tension potentiated with the ultrasonicated epinephrine decreased after 110 min. of continuous electrical stimulation. The presence of the serum protein prevented the effect of longer periods of electrical stimulation on the active tension of the dog heart papillary muscle. Therefore, the experiments suggest that the serum protein delays the fatigue and decay of the muscle and stabilizes its contractility and its response to electrical stimulation.

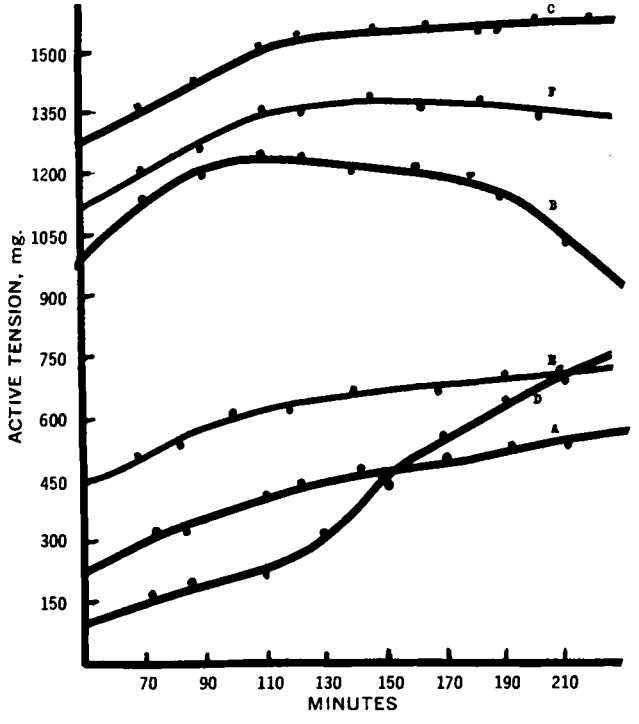

Figure 2—*Effect of serum protein on the decay of active contraction in papillary muscle from quiescent dog heart: the papillary muscles incubated with 10^{-6} M epinephrine (A) and with 10^{-8} M ultrasonicated epinephrine (B); developed tension with 100 mcg. of the serum protein alone (D) and in the presence of 10^{-8} M ultrasonicated epinephrine (C); and generated tension by muscles incubating with 10^{-8} M oxygenated epinephrine alone (E) and in the presence of 100 mcg. of the serum protein (F). The concentration of the modified forms of epinephrine is taken as the concentration of epinephrine prior to treatment.*

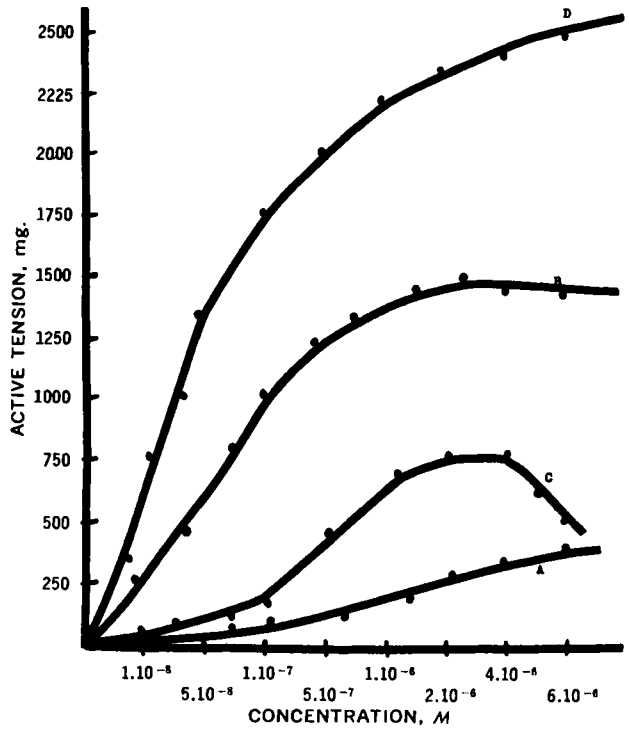

Figure 3—*Effect of concentration of the ultrasonicated epinephrine on the potentiation of active tension: papillary muscles from quiescent dog heart incubated with increasing concentration of epinephrine (A) or of ultrasonicated epinephrine (B); and tension developed by papillary muscles incubating with increasing concentrations of the serum protein (C) or with 100 mcg. of the serum protein and increasing concentrations of ultrasonicated epinephrine (D).*

The data in Fig. 3 suggest that the maximum tension developed by the papillary muscle in response to electrical stimulation depended on the concentration of epinephrine or its ultrasonicated or oxidized forms. An optimum concentration for epinephrine was found to be between 1 and 4 × 10^{-6} *M*. For the ultrasonicated or oxidized derivatives of epinephrine, as well as for the serum protein, the developed active tension was concentration dependent. There appears to be a certain relationship between concentration and effect in the action of the ultrasonicated epinephrine and the response to electrical stimulation. The combined effect of the serum protein and increasing concentrations of the ultrasonicated epinephrine was also dependent on the concentration of the derivative. Since the optical rotation of the ultrasonicated form was similar to that of epinephrine, the concentration of the ultrasonicated form was considered to be equivalent to the concentration of epinephrine prior to ultrasonication.

If added to the incubating medium containing papillary muscles from dog quiescent heart, the ultrasonicated epinephrine initiated isometric contraction in response to electrical stimulation. Tracings A, C, and E of Fig. 4 represent the typical response of papillary muscles from dog quiescent heart to electrical stimulation in the presence of ultrasonicated epinephrine. Tracings B and D show the effect of the epinephrine derivative on the developed active tension of the papillary muscle. In these two cases, the muscles were potentiated first with the ultrasonicated form to a maximum active tension; then the muscles were washed thoroughly with 5 l. of the oxygenated bicarbonate-dextrose solution and placed in fresh medium. The tracings suggest that the ultrasonicated epinephrine initiated and potentiated the development of active tension in the papillary muscle from dog quiescent heart. The magnitude of the potentiation and increase in the active tension induced by the ultrasonicated form is time dependent.

The tracings shown in Fig. 5 represent isometric contractions of papillary muscles from dog quiescent heart that was first potentiated

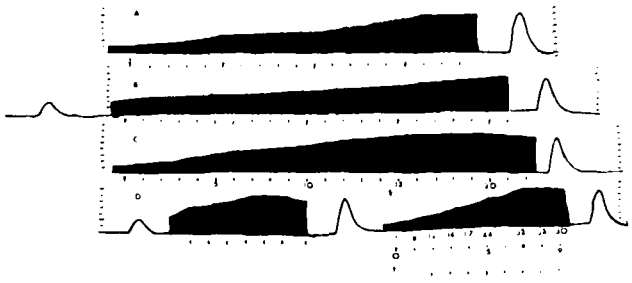

Figure 4—*Potentiation of active contraction of papillary muscle from quiescent dog heart. The arrow indicates addition of 0.05 ml. 10^{-6} M ultrasonicated epinephrine solution to electrically stimulated, nonresponding (patterns A, C, and E) and responding (B and D) papillary muscles of dog heart. The vertical scale is the tension of 36 mg./unit, and time (in minutes) is the horizontal scale. The top scale of tracing E indicates the tension potentiated with 0.10 ml. of ultrasonicated epinephrine solution in centigrams.*

to respond to electrical stimulation and then rinsed as described under *Experimental*. When the reinstated maximum active tension reached a steady state, aliquots of 50, 70, and 100 mcg. of the serum protein were added to the incubating medium. The tracings demonstrate the generation of a new maximum of active tension and suggest that the rate to attain this new maximum steady state depended on the concentration of the serum protein.

The tracings shown in Fig. 6 represent the isometric contractions of the papillary muscles from dog quiescent heart that was first activated to respond to electrical stimulation and then rinsed. When the reinstated maximum active tension reached a steady state, aliquots of 0.20 and 0.40 ml. of 10^{-6} *M* ultrasonicated epinephrine were added to the incubating medium. These tracings demonstrate that the rate of muscular decay or fatigue is faster at the higher concentration levels of the ultrasonicated epinephrine. If combined with the ultrasonified epinephrine, the serum protein delayed and reduced the magnitude of the decay caused by electrical stimulation (tracing C). The tracings indicate that the serum protein changed the mechanics of the contraction and produced an increase in the rate to peak tension and in the contraction–relaxation time. These observations suggest the role of the serum protein in the coupling excitation–contraction cycle of the contractile elements of the cardiac muscle.

The data in Fig. 7 summarize the ultrasonicated epinephrine concentration response alone and in the presence of three concentrations of the serum protein. Again, the concentration of the ultrasonicated form was taken as equivalent to the initial concentration of epinephrine prior to ultrasonication. The results suggest a linear relationship for the ultrasonified epinephrine between 10^{-9} and

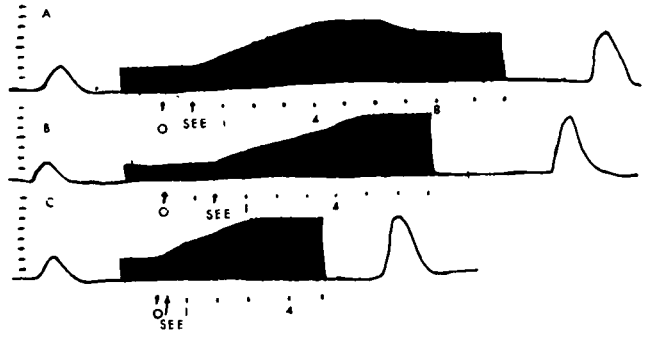

Figure 5—*Effect of concentration of the serum protein on the developed active tension. Papillary muscle from quiescent dog heart was first potentiated to respond to electrical stimulation with the ultrasonicated modified epinephrine and then rinsed as described in the text. At the reinstated maximum active tension at the steady state, aliquots of 50, 70, and 100 mcg. (tracings A, B, and C, respectively) of the serum protein were added to the incubating medium. The vertical scale is the tension of 36 mg./unit, and time (in minutes) is the horizontal scale.*

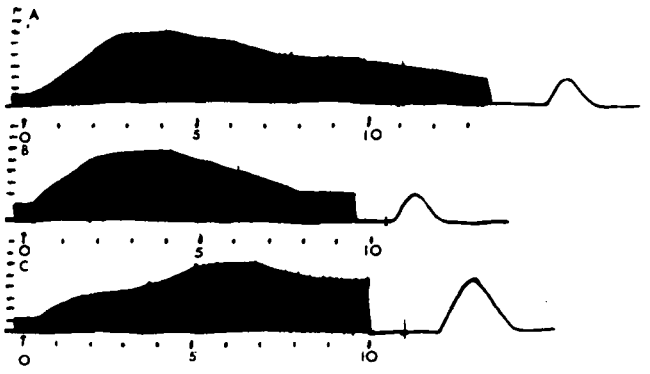

Figure 6—*Potentiation of active contraction with 0.20 and 0.40 ml. of 10^{-6} M ultrasonicated epinephrine alone (tracings A and B, respectively) and 0.40 ml. of 10^{-6} M ultrasonicated epinephrine with 100 mcg. of the serum protein together (tracing C).*

5×10^{-7} *M* concentration and the generated active tension in the rat heart papillary muscle. These experiments indicate that the serum protein enhanced the effect of the ultrasonicated epinephrine in the papillary muscle model. The magnitude of the enhancing effect is dependent on the employed serum protein concentration.

Effect of Epinephrine, Ultrasonicated Epinephrine, Oxidized Epinephrine, and Serum Protein on Superprecipitation of Human Heart Actomyosin—When added to a standard incubating mixture containing 0.50 mg. of actomyosin from human heart, adenosine triphosphate caused precipitation of the contractile protein, described as the superprecipitation of the contractile protein, and was assessed by the increase in absorbance of the solution (curve A, Fig. 8).

The human heart actomyosin preparation responded well to

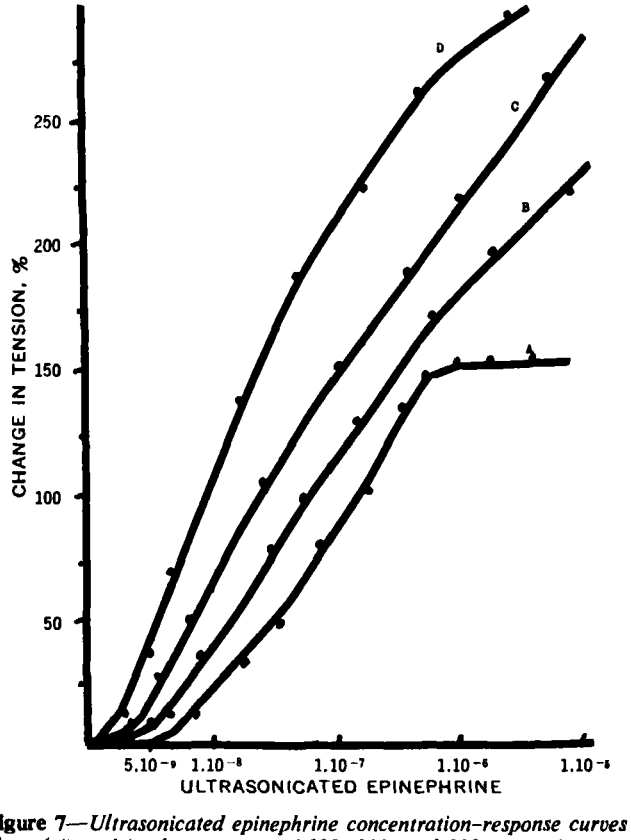

Figure 7—*Ultrasonicated epinephrine concentration–response curves alone (A) and in the presence of 100, 200, and 300 mcg. of serum protein (B, C, and D, respectively) in rat heart papillary muscles. Each point is the average of four muscles with an average deviation of ±5.3. Isometric tension force of the rat heart papillary muscle before the ultrasonicated epinephrine = control, 3.8 ± 0.8 g./mm.².*

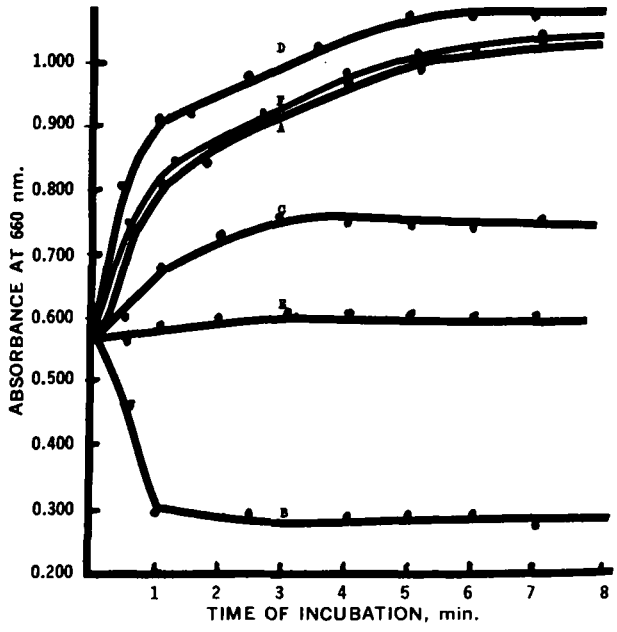

Figure 8—*Effect of ultrasonicated and oxidized epinephrine on actomyosin superprecipitation. The incubation mixture contained: 5.0 mM $MgCl_2$, 5.0 mM adenosine triphosphate, 20 mM tromethamine buffer of pH 7.5, 0.05 mM KCl, and 0.50 mg. actomyosin/ml. (A); all of A with 0.1 mM ethylene glycol tetraacetic acid (B); all of A with ultrasonicated epinephrine, 10^{-6} M (C), 10^{-3} M (D); all of A with epinephrine, 10^{-3} M (E); or all of A with oxidized epinephrine, 10^{-3} M (F).*

ethylene glycol tetraacetic acid, a chelating agent (Fig. 8). Removal of calcium from the medium with ethylene glycol tetraacetic acid resulted in the reversal of superprecipitation and clearing of the actomyosin suspension, which was followed by the decrease in absorbance (curve B). Addition of small amounts of the ultrasonicated epinephrine, 10^{-6} or 10^{-3} *M*, caused reversal of the clearing effect of the ethylene glycol tetraacetic acid. When added to the ethylene glycol tetraacetic acid, epinephrine at 10^{-3} *M* inhibited both the precipitation effect of adenosine triphosphate and the clearing effect of the chelating agent.

The data depicted by curves C and D suggest a concentration-dependent response for the ultrasonicated epinephrine. The action differed from that of epinephrine in its intensifying and enhancing effects on the superprecipitation of the actomyosin.

Figure 9 shows the effect of the ultrasonicated epinephrine alone (curve B), with the serum protein (curve C), and with the heat-denatured serum protein (curve D). In contrast to the effect of the serum protein, the heat-denatured serum protein did not influence the effect of the ultrasonicated epinephrine.

Effect of Ultrasonicated and Oxidized Epinephrine on Concentration of Adenosine Triphosphate, Phosphocreatine, and Lactic Acid of Dog Heart Papillary Muscle—Extracts of nonresponding and responding papillary muscles were prepared at time intervals during the potentiation period and analyzed for adenosine triphosphate, phosphocreatine, and lactic acid. The data (Table I) revealed no change in the concentration of adenosine triphosphate, phosphocreatine, or lactic acid in muscle which did not respond to electrical stimulation. When the ultrasonicated or oxidized epinephrine was added to the incubation medium, the initiation and potentiation of isometric contractions in response to electrical stimulation were accompanied by a progressive increase in adenosine triphosphate, phosphocreatine, and lactic acid (micromoles per gram of fresh weight) of the muscle. These experiments suggest that the amount of chemical energy released from the high energy phosphate compounds was utilized during the generation of active tension. The data also indicate that the ultrasonicated epinephrine stimulated the conversion of chemical energy in the endogenous adenosine triphosphate and phosphocreatine to the contractile, *i.e.*, mechanical, energy. When combined with the ultrasonicated epinephrine, the serum protein maintained the adenosine triphosphate and phosphocreatine at levels of 280 ± 10.5 and 350 ± 15 μmoles/gram weight, respectively[6]. These experiments suggest that the major functions of the serum protein are in the adenosine triphosphate regeneration systems and in maintaining the high energy phosphate compounds at a concentration capable of supporting development of active tension in muscular contraction.

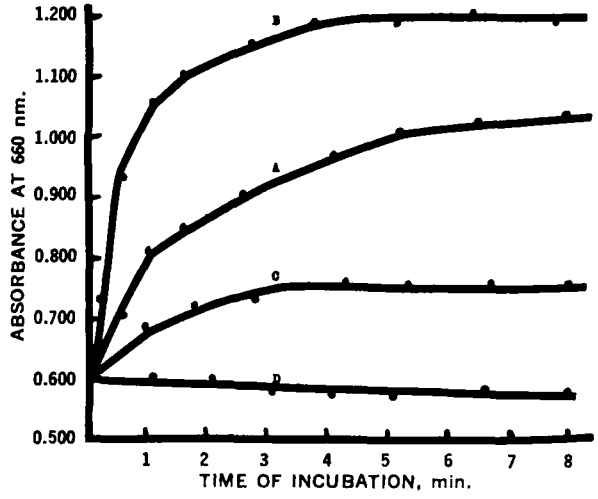

Figure 9—*Effect of ultrasonicated epinephrine and of the serum protein on actomyosin superprecipitation. The incubation mixture contained: 5.0 mM $MgCl_2$, 5.0 mM adenosine triphosphate, 20.0 mM tromethamine buffer of pH 7.5, 0.05 mM KCl, and 0.5 mg. of actomyosin/ml. (A); all of A with 10^{-6} M ultrasonicated epinephrine with 100 mcg. of the serum protein (B); all of A with 10^{-6} M ultrasonicated epinephrine, 0.1 mM ethylene glycol tetraacetic acid with 100 mcg. of the serum protein (C); all of A with 100 mcg. of the 80° denatured serum protein, 10^{-6} M ultrasonicated epinephrine with ethylene glycol tetraacetic acid (D).*

DISCUSSION

The effect of epinephrine on the cardiac muscle is well known (1–4). Epinephrine slightly increased the active tension, *i.e.*, developed tension of the muscle, and the rate of cardiac muscle, *i.e.*, the number of heart beats. The lag period required for the potentiation and initiation of active contraction in the presence of epinephrine or norepinephrine supports the suggestion that the effect of these amines on the developed tension of the papillary muscle is the result of the action of a modified form of the amine. Possible modification is the oxidation of epinephrine by the stream of oxygen gas employed to supply oxygen to the muscle and to stir the incubation medium surrounding the papillary muscle.

The superprecipitation of natural actomyosin was accelerated by a minute amount of calcium-removing substance, such as chelating agents, or the relaxing factor (17, 18). In view of the importance of this phenomenon in the physiological events of muscle contraction (19), the effect of epinephrine and of its sonified and oxidized forms, alone and in presence of the serum protein, was studied on human cardiac actomyosin. Paddle and Hauggard (20) found that epinephrine failed to initiate contraction in the arrested heart; their measurements of lactate production, glucose 6-phosphate content, and phosphorylase "a" activity showed that epinephrine also had no effect on glycolysis. Norepinephrine also had no effect on the papillary muscles from dog or human quiescent hearts[6]. The tracings of the initiation and potentiation of isometric contractions, with the enhancing effects on the precipitation of the purified actomyosin, suggest that ultrasonicated epinephrine, alone or in presence of the serum protein, stimulates the *in vivo* contractile elements to initiate the development of isometric contractions.

Current concepts of excitation-contraction coupling suggest that procedures that increase the sarcotubular calcium pool augment myocardial contractility by increasing the amount of calcium released at the actomyosin junction following membrane depolarization resulting from electrical stimulation (21, 22) and, conversely,

[6] Unpublished data.

Table I—Concentration of Adenosine Triphosphate, Phosphocreatine, and Lactic Acid in Papillary Muscle from Quiescent Dog Heart

Muscle	Minutes	Adenosine Triphosphate, mcg./Gram Weight	Phosphocreatine, mcg./Gram Weight	Lactic Acid, mcg./Gram Weight
Nonresponding	0	4.87 (5)[a] 2.50 to 7.24	14.67 (4) 10.55 to 18.41	1.63 (5) 0.95 to 2.31
	15	4.95 (6) 4.05 to 5.85	14.50 (4) 12.25 to 16.75	1.62 (4) 1.05 to 2.15
	30	5.15 (5) 4.25 to 6.05	14.25 (5) 12.15 to 16.35	1.75 (5) 0.95 to 2.55
	45	4.92 (4) 4.25 to 5.55	14.60 (5) 13.10 to 16.20	1.68 (5) 1.15 to 2.21
	60	4.85 (5) 4.05 to 5.65	14.75 (4) 12.25 to 17.25	1.65 (4) 1.15 to 2.15
	120	5.05 (5) 4.60 to 5.50	15.15 (5) 12.75 to 17.55	1.76 (4) 1.15 to 2.37
Responding (ultrasonicated epinephrine)	0	5.65 (6) 4.25 to 7.05	15.75 (5) 14.25 to 17.25	7.68 (5) 4.60 to 10.76
	15	77.25 (5) 70.25 to 84.15	82.15 (4) 68.50 to 95.80	16.75 (5) 11.50 to 22.00
	30	110.75 (5) 92.15 to 119.65	136.20 (4) 102.50 to 169.65	24.50 (6) 20.75 to 23.50
	45	177.10 (4) 150.5 to 204.3	205.25 (5) 165.4 to 255.6	90.25 (5) 65.25 to 115.25
	60	231.25 (5) 215.2 to 247.40	264.50 (4) 215.25 to 323.75	190.75 (5) 110.25 to 270.25
Responding (oxygenated epinephrine)	0	5.60 (4) 4.15 to 7.05	15.15 (4) 13.80 to 16.50	5.60 (5) 4.12 to 7.08
	15	55.35 (4) 40.75 to 69.95	64.35 (4) 53.25 to 75.45	13.20 (4) 12.15 to 14.25
	30	85.75 (4) 73.15 to 98.35	105.45 (4) 98.75 to 112.15	19.75 (4) 18.25 to 21.25
	45	126.35 (4) 124.50 to 128.20	167.25 (4) 165.45 to 169.05	57.65 (4) 56.25 to 59.05
	60	185.35 (4) 184.25 to 186.45	205.45 (4) 202.75 to 208.15	108.25 (4) 106.95 to 109.55

[a] Number of experiments is indicated in parentheses.

methods that decrease the calcium pool diminish contractility (23).

When a muscle is stimulated electrically, chemical reactions occur that result in activation of the contractile elements and constitute the active state of the muscle. Alone, electrical stimulation of papillary muscle from dog or human heart did not potentiate or initiate isometric contractions and did not cause changes in the concentration of adenosine triphosphate, phosphocreatine, or lactic acid. Therefore, in these muscles, electrical stimulation did not activate the contractile elements. In the presence of ultrasonicated or oxidized epinephrine in the incubating medium, electrical stimulation of the papillary muscle from quiescent hearts did potentiate isometric contractions, with a progressive increase in the concentration of adenosine triphosphate, phosphocreatine, and lactic acid. The magnitude of the developed active tension of the isometric contractions and the magnitude of the increase in adenosine triphosphate, phosphocreatine, and lactic acid concentrations were time dependent. These observations suggest that the presence of the modified forms of epinephrine caused the activation of certain metabolic processes, which involve the high energy phosphate compounds adenosine triphosphate and phosphocreatine, with the conversion of chemical energy to the contractile element actomyosin. When combined with ultrasonicated or oxidized epinephrine, the serum protein stabilized the initiation and potentiation of the developed tension. These observations are in agreement with the mechanism of action of the serum protein reported earlier (11, 12) and with the effect of serum on the fatigue and decay of normal muscles (5–8).

Preliminary chemical studies suggest similarities in the structure of the ultrasonicated and oxidized forms of epinephrine prepared as described in the *Experimental* section. The physiological effects on the papillary muscles from dog quiescent hearts also indicate similarities between these two modified forms of epinephrine. Difference in the stability of solutions containing the modified forms of epinephrine indicate certain differences in the identity of oxidized and ultrasonicated epinephrine.

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Adsorption of Nonionic Surfactants on Sulfathiazole and Naphthalene and Flocculation–Deflocculation Behavior of These Suspensions

A. OTSUKA▲, H. SUNADA, and Y. YONEZAWA

Abstract □ The adsorption of polyoxyethylated nonyl-phenols and octyl-phenols, having different polyoxyethylene chain lengths, on sulfathiazole and naphthalene was studied. Adsorption isotherms for these surfactants on sulfathiazole are S-shaped, and the adsorption amounts at saturation were greater than those for the closest packing at the air–solution interface. These results suggest that some multilayer adsorption or aggregate formation on the surface of solid occurred above a certain concentration. In the case of naphthalene, all isotherms are of the Langmuir type and the areas per molecule calculated from the saturation adsorption were found to be considerably larger than the values for the air–solution interface. It is likely that the hydrocarbon chains adhere to the surface of naphthalene, lying flat, while the polyoxyethylene chains are directed toward the solution. Flocculation–deflocculation measurements for both systems were carried out by the sedimentation volume method, and the results support the adsorption mechanisms described.

Keyphrases □ Polyoxyethylated octyl- and nonyl-phenols—adsorption on sulfathiazole and naphthalene, flocculation–deflocculation behavior of suspensions □ Surfactants, nonionic—adsorption on sulfathiazole and naphthalene, flocculation–deflocculation behavior of suspensions □ Adsorption of polyoxyethylated octyl- and nonyl-phenols on sulfathiazole and naphthalene—adsorption isotherms, flocculation–deflocculation behavior □ Sulfathiazole—adsorption behavior of polyoxyethylated octyl- and nonyl-phenols □ Naphthalene—adsorption behavior of polyoxyethylated octyl- and nonyl-phenols □ Flocculation–deflocculation behavior—suspensions of polyoxyethylated octyl- or nonyl-phenols and sulfathiazole or naphthalene

Many surfactants are capable of affecting the state of dispersion of powder particles in suspension. To examine the mechanism of action of surfactants in flocculation–deflocculation phenomena, it is desirable to investigate the adsorption of surfactants on powders. Several workers have studied the adsorption behavior and/or the dispersing action of nonionic surfactants. The adsorption of polyoxyethylated alkyl-phenols on sand and of ethylene oxide–propylene oxide condensates on quartz has been reported (1, 2). Kuno and Abe (3) reported the adsorption of polyoxyethylated nonyl-phenols on calcium carbonate and carbon black from both aqueous and cyclohexane solutions. The adsorption of nonionic surfactants by textile fibers was studied by Weatherburn and Bayley (4), Nemoto and Miwa (5), and Schott (6).

Corkill *et al.* (7) studied the adsorption of polyoxyethylene glycol monoalkyl ethers on graphon at different temperatures and obtained the adsorption isotherms. They also measured the heats of adsorption calorimetrically. Other investigators (8, 9) commented on the influence of the adsorption of polyoxyethylene glycol monoalkyl ethers on the stability of silver iodide and polystyrene latex sols. In most of these studies, the adsorbents examined were inorganic compounds or polymers. Only a few workers have performed this kind of study using pharmaceutical or organic powders. The adsorption of polyoxyethylated nonyl-phenol on sulfamines (10) and of several nonionic surfactants on griseofulvin (11) has been reported.

The purpose of this work was to investigate the adsorption of nonionic surfactants on some organic powders and to find a certain relation between the flocculation–deflocculation behavior of these suspensions and the adsorption results. The surfactants used were polyoxyethylated nonyl-phenols and octyl-phenols. Sulfathiazole (polar compound) and naphthalene (nonpolar) were selected as adsorbents.

EXPERIMENTAL

Materials—Series of polyoxyethylated nonyl-phenols and octyl-phenols were prepared by repeated distillation of commercial products[1]. The average polyoxyethylene chain lengths were determined from their hydroxyl values. Table I gives the CMC and

[1] Nippon Nyukazai Co. Ltd., Tokyo, Japan.

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A. OTSUKA▲, H. SUNADA, and Y. YONEZAWA

Abstract □ The adsorption of polyoxyethylated nonyl-phenols and octyl-phenols, having different polyoxyethylene chain lengths, on sulfathiazole and naphthalene was studied. Adsorption isotherms for these surfactants on sulfathiazole are S-shaped, and the adsorption amounts at saturation were greater than those for the closest packing at the air–solution interface. These results suggest that some multilayer adsorption or aggregate formation on the surface of solid occurred above a certain concentration. In the case of naphthalene, all isotherms are of the Langmuir type and the areas per molecule calculated from the saturation adsorption were found to be considerably larger than the values for the air–solution interface. It is likely that the hydrocarbon chains adhere to the surface of naphthalene, lying flat, while the polyoxyethylene chains are directed toward the solution. Flocculation–deflocculation measurements for both systems were carried out by the sedimentation volume method, and the results support the adsorption mechanisms described.

Keyphrases □ Polyoxyethylated octyl- and nonyl-phenols—adsorption on sulfathiazole and naphthalene, flocculation–deflocculation behavior of suspensions □ Surfactants, nonionic—adsorption on sulfathiazole and naphthalene, flocculation–deflocculation behavior of suspensions □ Adsorption of polyoxyethylated octyl- and nonyl-phenols on sulfathiazole and naphthalene—adsorption isotherms, flocculation–deflocculation behavior □ Sulfathiazole—adsorption behavior of polyoxyethylated octyl- and nonyl-phenols □ Naphthalene—adsorption behavior of polyoxyethylated octyl- and nonyl-phenols □ Flocculation–deflocculation behavior—suspensions of polyoxyethylated octyl- or nonyl-phenols and sulfathiazole or naphthalene

Many surfactants are capable of affecting the state of dispersion of powder particles in suspension. To examine the mechanism of action of surfactants in flocculation–deflocculation phenomena, it is desirable to investigate the adsorption of surfactants on powders. Several workers have studied the adsorption behavior and/or the dispersing action of nonionic surfactants. The adsorption of polyoxyethylated alkyl-phenols on sand and of ethylene oxide–propylene oxide condensates on quartz has been reported (1, 2). Kuno and Abe (3) reported the adsorption of polyoxyethylated nonyl-phenols on calcium carbonate and carbon black from both aqueous and cyclohexane solutions. The adsorption of nonionic surfactants by textile fibers was studied by Weatherburn and Bayley (4), Nemoto and Miwa (5), and Schott (6).

Corkill *et al.* (7) studied the adsorption of polyoxyethylene glycol monoalkyl ethers on graphon at different temperatures and obtained the adsorption isotherms. They also measured the heats of adsorption calorimetrically. Other investigators (8, 9) commented on the influence of the adsorption of polyoxyethylene glycol monoalkyl ethers on the stability of silver iodide and polystyrene latex sols. In most of these studies, the adsorbents examined were inorganic compounds or polymers. Only a few workers have performed this kind of study using pharmaceutical or organic powders. The adsorption of polyoxyethylated nonyl-phenol on sulfamines (10) and of several nonionic surfactants on griseofulvin (11) has been reported.

The purpose of this work was to investigate the adsorption of nonionic surfactants on some organic powders and to find a certain relation between the flocculation–deflocculation behavior of these suspensions and the adsorption results. The surfactants used were polyoxyethylated nonyl-phenols and octyl-phenols. Sulfathiazole (polar compound) and naphthalene (nonpolar) were selected as adsorbents.

EXPERIMENTAL

Materials—Series of polyoxyethylated nonyl-phenols and octyl-phenols were prepared by repeated distillation of commercial products[1]. The average polyoxyethylene chain lengths were determined from their hydroxyl values. Table I gives the CMC and

[1] Nippon Nyukazai Co. Ltd., Tokyo, Japan.

Table I—CMC and Close-Packed Areas at the Air–Solution Interface for the Surfactants Used

Surfactant	CMC × 10⁴ moles/l.	Area per Molecule at Air–Solution Interface, Å²
Polyoxyethylated nonyl-phenol-5.8[a]	0.25	39
Polyoxyethylated nonyl-phenol-6.6	0.4	44
Polyoxyethylated nonyl-phenol-8.5	0.4	53
Polyoxyethylated nonyl-phenol-9.8	0.6	61
Polyoxyethylated nonyl-phenol-11.7	0.8	62
Polyoxyethylated octyl-phenol-7.7	2.7	59
Polyoxyethylated octyl-phenol-9.7	3.4	65

[a] Numbers indicate the average polyoxyethylene chain length.

average cross-sectional areas per molecule at the air–solution interface obtained from the data of surface tension using a tensiometer[2]. These values are in fair agreement with those in the literature (3, 12, 13).

Sulfathiazole[3] (JP VII) was finely ground and dried before use. Naphthalene was recrystallized from ethanol and then ground. The specific area for sulfathiazole was determined by the Brunauer–Emmett–Teller method with nitrogen adsorption and by gas-phase[4] and liquid-phase permeametry (14) to give 0.50 m.²/g. for sulfathiazole-A and 0.46 m.²/g. for sulfathiazole-B. Neither the nitrogen adsorption method nor gas-phase permeametry was applicable to naphthalene because the reproducibility of the data was poor. The value obtained by liquid-phase permeametry was 0.34 m.²/g.

Procedure—To 2.5 g. sulfathiazole or 5.0 g. naphthalene in a stoppered flask was added 50 ml. of surfactant solution. The flasks were shaken in water bath at 25° for 24 hr., after which the suspensions were centrifuged quickly. The equilibrium concentration for the supernate was determined by the cobalt blue method (15). The amount of surfactant adsorbed was calculated from the difference in the concentrations before and after adsorption.

The flocculation-deflocculation characteristics of suspensions were evaluated by the sedimentation volume method. A 2.5-g. sample of the test powder and 50 ml. of surfactant solution were placed in a graduated test tube, which was shaken at 25° for 24 hr. and subsequently allowed to stand for an hour; the sedimentation volume was then measured. There was no appreciable change in the general tendency of the sedimentation volume even after standing overnight.

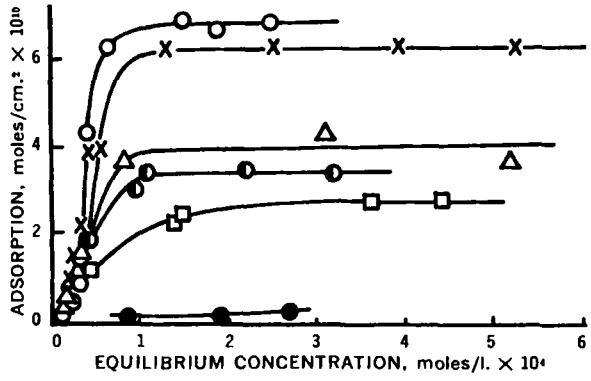

Figure 1—*Adsorption isotherms for polyoxyethylated nonyl-phenols and polyoxyethylene glycol 400 on sulfathiazole. Key (average polyoxyethylene chain lengths of polyoxyethylated nonyl-phenol): ○, 5.8; ×, 6.6; △, 8.5; ◐, 9.8; □, 11.7; and ●, polyoxyethylene glycol 400.*

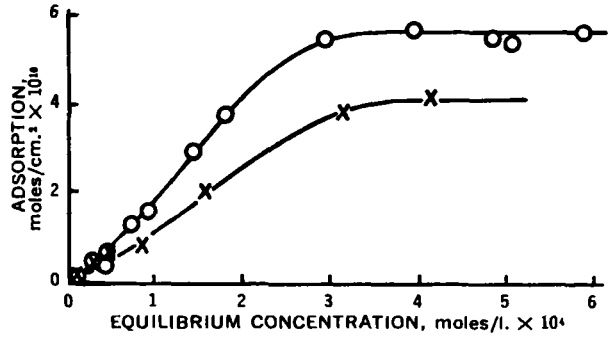

Figure 2—*Adsorption isotherms for polyoxyethylated octyl-phenols on sulfathiazole. Key (average polyoxyethylene chain length of polyoxyethylated octyl-phenol): ○, 7.7; and ×, 9.7.*

RESULTS AND DISCUSSION

Figures 1 and 2 show the adsorption isotherms of polyoxyethylated nonyl-phenols and octyl-phenols on sulfathiazole, respectively. All isotherms are nearly S-shaped; *i.e.*, the amount of adsorption is found to be less in the low concentration region and rises rapidly as the concentration increases, and then a plateau is reached at a concentration slightly above the CMC in solution.

Nakagaki *et al.* (10) reported the adsorption of polyoxyethylated nonyl-phenol on sulfamines, but the adsorption behavior at low concentrations of the surfactant remains unanalyzed. Kuno and Abe (3) demonstrated that the adsorption isotherms of polyoxyethylated nonyl-phenols, having five to six ethylene oxide units, on calcium carbonate from aqueous solution are S-shaped and that the areas per molecule calculated from the saturation adsorption are smaller than the values obtained at the air–solution interface. They considered that the adsorbed surfactant is not in the state of individual molecules but is in the state of micelles. However, as Mathai and Ottewill (9) pointed out, it may be misleading to assume that the aggregate on the solid–solution interface has the same form as the micelles in bulk solution. Somasundaran *et al.* (16, 17) found that the adsorption of ionic surfactants on quartz and alumina increased markedly at a certain critical concentration less than that required for the micelle formation in the bulk solution. These workers concluded that this abrupt change in adsorption is due to the association of adsorbed surfactant ions to form two-dimensional aggregates or "hemimicelles" at the solid–solution interface.

The areas per molecule obtained from the saturation adsorption of polyoxyethylated nonyl-phenols and octyl-phenols on sulfathiazole are listed in Table II. From these values and the shape of the

Table II—Areas per Molecule at the Solid–Solution Interface Calculated from the Saturation Adsorption

Surfactant	Area per Molecule, Å², Solid Sulfathiazole	Naphthalene
Polyoxyethylated nonyl-phenol-5.8[a]	24	110
Polyoxyethylated nonyl-phenol-6.6	26	110
Polyoxyethylated nonyl-phenol-8.5	42	120
Polyoxyethylated nonyl-phenol-9.8	48	120
Polyoxyethylated nonyl-phenol-11.7	60	130
Polyoxyethylated octyl-phenol-7.7	29	180
Polyoxyethylated octyl-phenol-9.7	40	280

[a] Numbers indicate the average polyoxyethylene chain length.

[2] DuNouy.
[3] Sankyo Co. Ltd., Tokyo, Japan.
[4] The instrument was prepared by Shimazu Seisakusho Ltd., Kyoto, Japan.

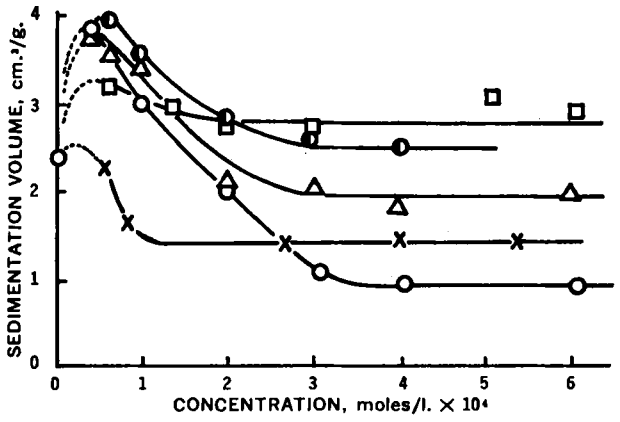

Figure 3—*Sedimentation volume of sulfathiazole in the presence of polyoxyethylated nonyl-phenols. Key (average polyoxyethylene chain length of polyoxyethylated nonyl-phenol): O, 5.8; ×, 6.6; △, 8.5; ◐, 9.8; and □, 11.7.*

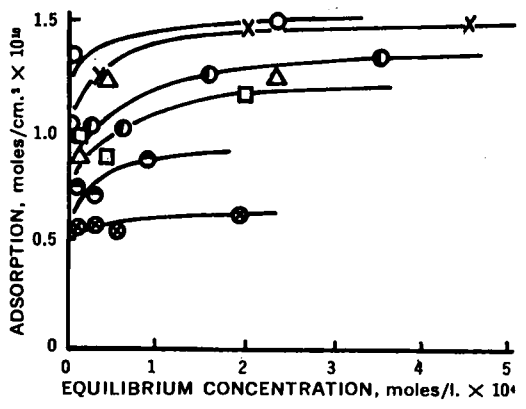

Figure 5—*Adsorption isotherms for polyoxyethylated nonyl-phenols (A) and octyl-phenols (B) on naphthalene. Key (A—average polyoxyethylene chain length of polyoxyethylated nonyl-phenol): O, 5.8; ×, 6.6; △, 8.5; ◐, 9.8; and □, 11.7. Key (B—average polyoxyethylene chain length of polyoxyethylated octyl-phenol): ◔, 7.7; and ⊗, 9.7.*

adsorption isotherms, it is apparent that some multilayer adsorption or aggregate formation occurs on the surface above a certain concentration.

Flocculation–deflocculation measurements should be useful in the study of the behavior of surfactants at the solid–solution interface. Figures 3 and 4 show the sedimentation volume for sulfathiazole in the presence of polyoxyethylated nonyl-phenols and octyl-phenols, respectively. It is well known that, in coarse suspensions, completely deflocculated particles give a small sedimentation volume as they settle and flocculated ones tend to form a loose structure of large volume. In all cases in the present experiments, the sedimentation volume was large in the very low concentration region and fell gradually as concentration increased. Furthermore, maximum ease of transfer from the aqueous to the benzene phase was found at the concentration where a maximum in the sedimentation volume was observed. It seems likely, therefore, that initially surfactant molecules are adsorbed nearly flat with polyoxyethylene chains attached to the surface of sulfathiazole, which renders the surface more or less hydrophobic. With an increase in concentration, the surface aggregation of the surfactant molecules will be initiated before a close-packed monolayer of vertically orientated molecules is completed. Since most of the polyoxyethylene chains in such aggregates are supposed to be exposed to the aqueous phase, the surface of sulfathiazole will become hydrophilic.

The contribution of polyoxyethylene chain length to the adsorption of the surfactants on sulfathiazole is remarkable. Table II indicates that the degree of close-packing of molecules at the saturation adsorption becomes greater as the polyoxyethylene chain length decreases. This is in accordance with the works of Corkill *et al.* (7) and Elworthy and Guthrie (11). In the case of polyoxyethylated nonyl-phenol-11.7[b], the area per molecule at the solid–solution interface is rather close to the value at the air–solution interface, so that it is possible to assume a close-packed monolayer. But it would be more reasonable to assume that some aggregate formation also occurs at the surface of the solid, although the tendency is less than in the cases of the surfactants containing shorter polyoxyethylene chains.

The results of the sedimentation volume measurements in the high concentration region simply reflect the adsorption results; *i.e.*, the sedimentation volume decreases with a decrease in polyoxyethylene chain length. On the other hand, at low concentrations, there seems to be no simple correlation between the sedimentation volume and the polyoxyethylene chain length. This may be due to the following two effects which counteract each other: (*a*) the smaller the hydrophilic group, the higher the magnitude of surface aggregate formation, and (*b*) the larger the hydrophilic group, the greater the steric stabilization against flocculation.

Although there was no essential difference between polyoxyethylated nonyl-phenols and octyl-phenols in the adsorption behavior onto sulfathiazole, the adsorption rise for polyoxyethylated octyl-phenols with an increase in concentration was slower than that for polyoxyethylated nonyl-phenols. This would be consistent with the fact that the values of the CMC for polyoxyethylated octyl-phenols are much larger than those of nonyl-phenols. Reflecting the adsorption results, the deflocculating action of polyoxyethylated octyl-phenols on sulfathiazole occurs at higher concentrations compared with polyoxyethylated nonyl-phenols.

The adsorption behavior and the deflocculating action of the surfactants on naphthalene seem to be simple, unlike sulfathiazole.

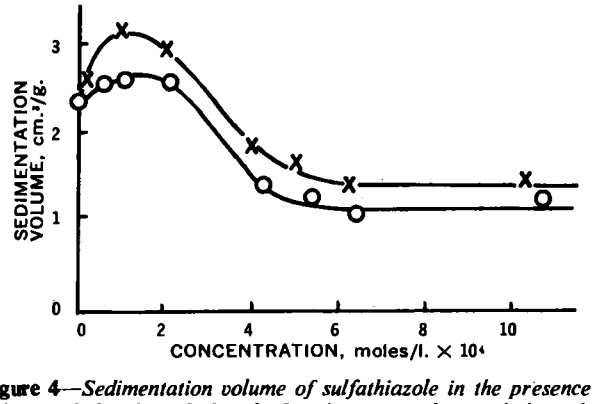

Figure 4—*Sedimentation volume of sulfathiazole in the presence of polyoxyethylated octyl-phenols. Key (average polyoxyethylene chain length of polyoxyethylated octyl-phenol): O, 7.7; and ×, 9.7.*

[b] Number indicates the average polyoxyethylene chain length.

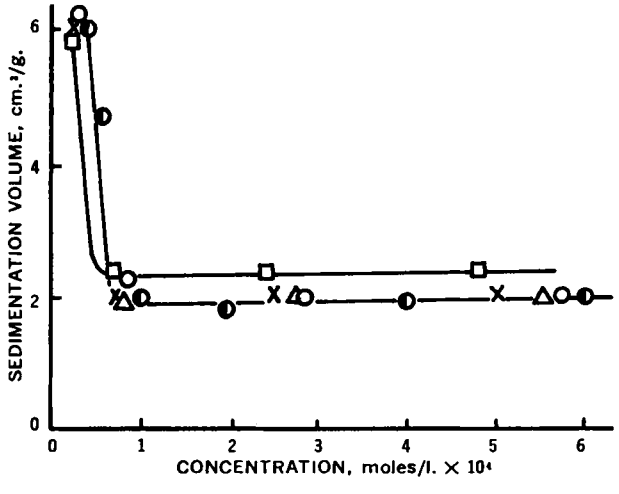

Figure 6—*Sedimentation volume of naphthalene in the presence of polyoxyethylated nonyl-phenols. Key (average polyoxyethylene chain length of polyoxyethylated nonyl-phenol): O, 5.8; ×, 6.6; △, 8.5; ◐, 9.8; and □, 11.7.*

As shown in Fig. 5, all isotherms are of the Langmuir type. Each curve starts from the origin and the amount adsorbed reaches a saturation value at or close to the CMC in solution. The areas per molecule calculated from the saturation adsorption were considerably larger than the values for the air–solution interface and were also larger than the dimensions of the hydrocarbon part of the molecule given by a molecular model. With an increasing polyoxyethylene chain length, the value of saturation adsorption decreases, but this trend is not so evident as compared with sulfathiazole.

Figure 6 presents the sedimentation volume of naphthalene in the presence of polyoxyethylated nonyl-phenols. At very low concentrations, naphthalene was only slightly wet by water and some portions of it floated, so measurement of the sedimentation volume was difficult. The sedimentation volume rapidly decreased with an increase in concentration and a highly deflocculated state was reached. The deflocculating action of the surfactants was not so influenced by the polyoxyethylene chain length.

It is likely, therefore, that the hydrocarbon chains of the surfactant molecules adhere to the surface of naphthalene and are lying flat, while the polyoxyethylene chains are directed toward solution.

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GLC Determination of Nitroglycerin and Isosorbide Dinitrate in Human Plasma

M. T. ROSSEEL and M. G. BOGAERT*▲

Abstract □ A procedure is described for the identification and quantitative determination of nitroglycerin and isosorbide dinitrate in plasma. The nitrates are extracted from plasma with ethyl acetate and measured by GLC with electron-capture detection. Quantitation is done using an internal standard. The lower limit of sensitivity for both substances is around 0.5 ng./ml. Results obtained with this method in humans are given.

Keyphrases □ Nitroglycerin—identification, GLC–electron-capture analysis, human plasma □ Isosorbide dinitrate—identification, GLC–electron-capture analysis, human plasma □ GLC–electron-capture detection—identification, analysis, nitroglycerin and isosorbide dinitrate in human plasma

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The present authors previously described a GC method for the separation and identification of different organic nitrates in the nanogram range, using electron-capture detection (9). Only incomplete data about electron-capture detection for assay of organic nitrates in biological material have been published (10).

This report describes the use of GLC for the identification and quantitation of nitroglycerin and isosorbide dinitrate in plasma after administration of therapeutic doses in man.

EXPERIMENTAL

Reagents—Benzene[1], trimethylchlorosilane[2], and activated charcoal[3] filters were used. Ethyl acetate[1] was shaken three times with 10% ferrous sulfate and two times with water distilled in glass, stored over calcium chloride (95% minimum)[2], and distilled immediately before use. Anhydrous sodium sulfate[2] was washed with ethyl acetate and dried at 100° for several hours. Nitroglycerin was available as a 1% solution in ethanol[2], and isosorbide

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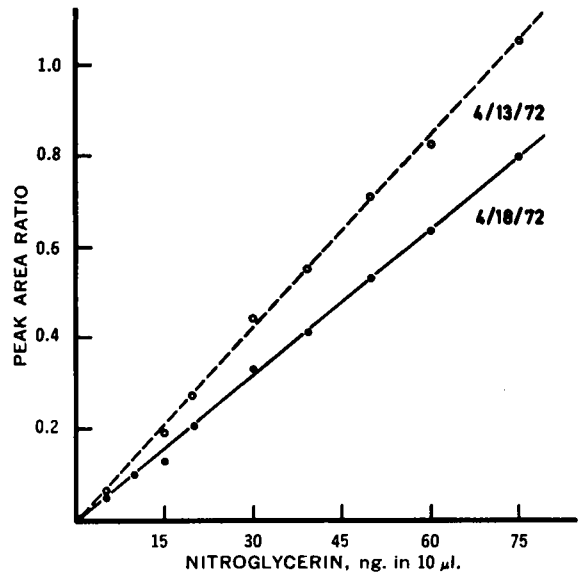


Figure 1—*Standard curves using plasma samples of 5 ml. to which are added 15 ng. of isosorbide dinitrate and varying amounts of nitroglycerin. From the extract in 10 μl. of benzene, 1.6 μl. is injected in the gas chromatograph with electron-capture detection. The two curves shown were obtained on 2 different days.*

dinitrate was available as a powder[4]; isoidide dinitrate was synthesized as described previously (11).

Glassware—All glassware was silanized by soaking for 1 hr. in 5% trimethylchlorosilane in benzene; rinsed with benzene, methanol, and water distilled in glass; and dried.

Apparatus—A gas chromatograph[5] with a tritium electron-capture detector (150 mc.) was used. Glass columns, 1.83-m. × 2-mm. i.d., were packed with 3.5% of QF-1[6] on 60–80-mesh Gas Chrom Q[7]. The injection port temperature was 160°, the column temperature was 120° for nitroglycerin determinations and 117° for isosorbide dinitrate determinations, and the detector block temperature was 180°. The nitrogen carrier gas flow was 30 ml./min. Pulse voltage was 25 v., and the electrometer range was 1×10^{-9}.

Extraction Procedure—Five milliliters of plasma and 5 ml. of ethyl acetate were shaken for 5 min. in glass-stoppered tubes; after centrifugation for 10 min. at 4000 r.p.m., the organic phase was transferred over an activated charcoal filter to a conical tube by means of a Pasteur pipet, avoiding the lipoprotein interface. The filter was rinsed before use with ethyl acetate and covered with sodium sulfate; afterward it was rinsed with 0.5 ml. of ethyl acetate. The organic phase was then evaporated under nitrogen to near dryness at room temperature. The plasma was extracted two more times. The yellow evaporation residue from the three extractions was dissolved in 0.5 ml. of ethyl acetate, filtered again through a filter, reevaporated to dryness, and immediately dissolved in 10 μl. benzene to prevent evaporation of nitroglycerin. The benzene solution (1.6 μl.) was injected into the gas chromatograph. The injection was done as soon as possible after the extraction procedure; in between the stoppered samples were stored at −18° to minimize evaporation of the benzene.

Quantitation—Quantitation of the amount of nitroglycerin or isosorbide dinitrate present in plasma was done by using an internal standard; this internal standard was isosorbide dinitrate when determining nitroglycerin and isoidide dinitrate when assaying isosorbide dinitrate. A known amount of the internal standard was added to the plasma samples before the extraction procedure. The peak areas of the nitrates on the chromatogram were calculated by multiplying peak height times width at half height; from the ratio of the peak areas of the product under study and the internal standard, the amount of product present in the samples can be calculated. A calibration factor was obtained by analyzing samples to which known amounts of both internal standard and test product were added. The presence of a wide front influences an early peak (*e.g.*, nitroglycerin) more than a late peak (*e.g.*, isosorbide dinitrate). Since the extraction solvent determines front width, the calibration samples are run through the whole extraction procedure used for the unknown samples (12).

[4] Cedona, The Netherlands.
[5] Packard Series 7400.
[6] Applied Sciences Laboratories.
[7] Supelco, Inc.

Table I—Efficacy of Extraction and Accuracy for the GLC Determination of Nitroglycerin and Isosorbide Dinitrate Added to Blank Plasma[a]

	Quantity[b], ng.	Mean Assay, ng.	*RSD*[c], %	*RE*[d], %
Extraction				
Nitroglycerin	25	24.3	±6.1	−2.8
Isosorbide dinitrate	15	14.5	±3.2	−2.9
Accuracy				
Nitroglycerin	10	10.2	±13.3	+2.2
Isosorbide dinitrate	5	4.9	±10.1	−2.0

[a] All determinations were done five times. [b] Added to 5 ml. of plasma. [c] Relative standard deviation. [d] Relative error.

RESULTS AND DISCUSSION

Retention Times—The relative retention time for nitroglycerin as compared to isosorbide dinitrate is 0.44; for isosorbide dinitrate as compared to isoidide dinitrate, it is 1.69. As described previously, nitrated metabolites of isosorbide dinitrate and of nitroglycerin have retention times different from those of the parent compounds (9); this is also true for the mononitrate of isoidide.

Extraction—From previous work (13) with TLC coupled with densitometry, it was known that microgram concentrations of nitroglycerin and isosorbide dinitrate can be extracted from plasma quantitatively. For concentrations in the nanogram range, the efficacy of extraction is given in Table I.

Activated charcoal filters are very helpful in working with biological material (14) but do not influence the impurities introduced by the use of the extraction solvent. Different brands of ethyl acetate were tried, as well as different purification methods, and the best results were obtained with the procedure described under

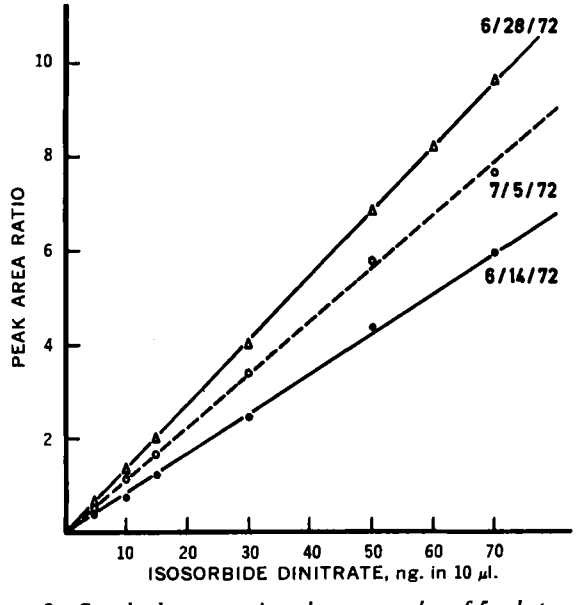


Figure 2—*Standard curves using plasma samples of 5 ml. to which are added 5 ng. of isoidide dinitrate and varying amounts of isosorbide dinitrate. From the extract in 10 μl. of benzene, 1.6 μl. is injected in the gas chromatograph with electron-capture detection. The three curves shown were obtained on 3 different days.*

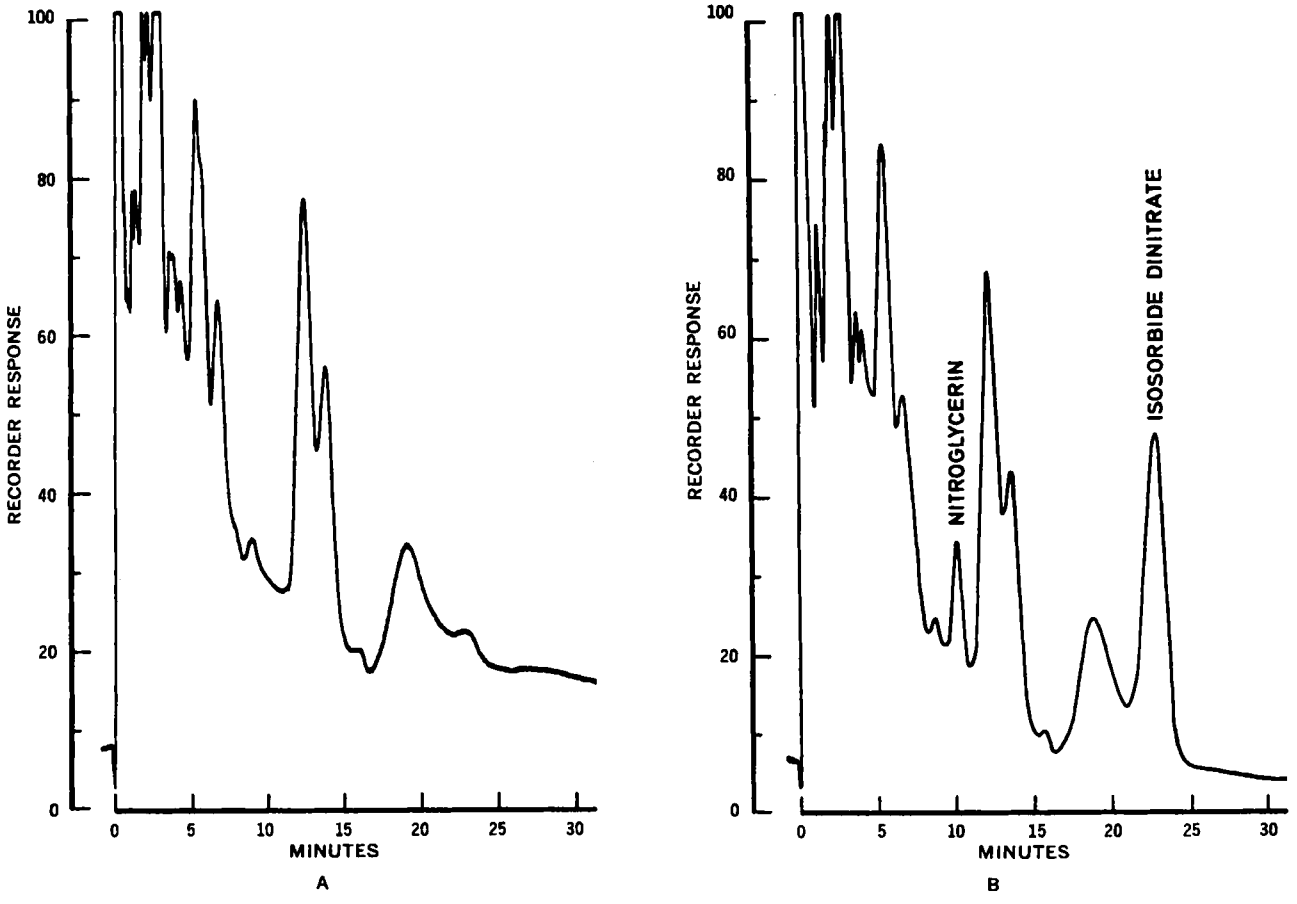


Figure 3—*Gas chromatograms of extracted human plasma. Key: A, extract from 5 ml. blank plasma; and B, isosorbide dinitrate (15 ng.) added to 5 ml. plasma of the same subject after sublingual administration of nitroglycerin, 800 mcg. In both cases the extraction was done with ethyl acetate, and 1.6 μl. of the extract in 10 μl. of benzene was injected.*

Experimental. Although the wide front and baseline irregularities still exist, none of these irregularities interferes with the peaks of the three nitrates. Some large peaks due to ethyl acetate impurities appear much later than the nitrates, so the chromatograms have to run for 90 min. before one can inject another sample.

Sensitivity—If 5 ml. of plasma is used for the extraction, the lower limit of detection is approximately 0.5 ng./ml. for nitroglycerin and somewhat lower for isosorbide dinitrate. As always with electron-capture detection, sensitivity varies from day to day; part of the variation is due to changes in front width and in baseline.

Quantitation—A prerequisite for the use of an internal standard for purposes of quantitation is a linear relationship between the ratio of the peak areas of internal standard and compound and the quantity of the compound. This is the case (Fig. 1) for control plasma samples of 5 ml. to which both nitroglycerin (5–75 ng.) and isosorbide dinitrate (15 ng.) were added. After extraction, 1.6 μl. of the 10-μl. benzene extract was injected into the gas chromatograph; the calibration line is linear and passes through the origin.

Table II—Plasma Levels (Nanograms per Milliliter) of Isosorbide Dinitrate at Different Times after Sublingual Administration of 1.25 mg. of This Substance[a] in Five Human Volunteers

Subject	Minutes[b] 4	6	15	30	50	80
M.B.	17.5	34.5	11.1	4.5	3.2	2.2
J.W.	3.1	14.4	5.2	3.8	2.7	1.0
M.D.	8.5	21.7	7.8	3.7	2.7	1.1
R.M.	15.8	11.5	2.6	1.2	1.9	0
M.V.	3.5	10.5	7.3	0.5	0.6	0.2

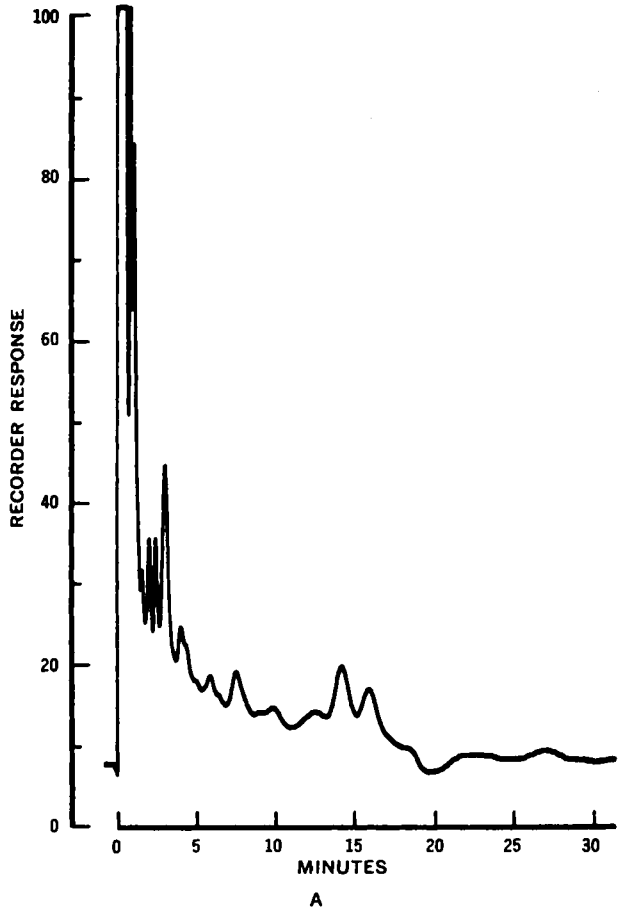
[a] A quarter of a commercially available tablet (Cedocard, 5 mg.) was put underneath the tongue. [b] Administration at $t = 0$ min.

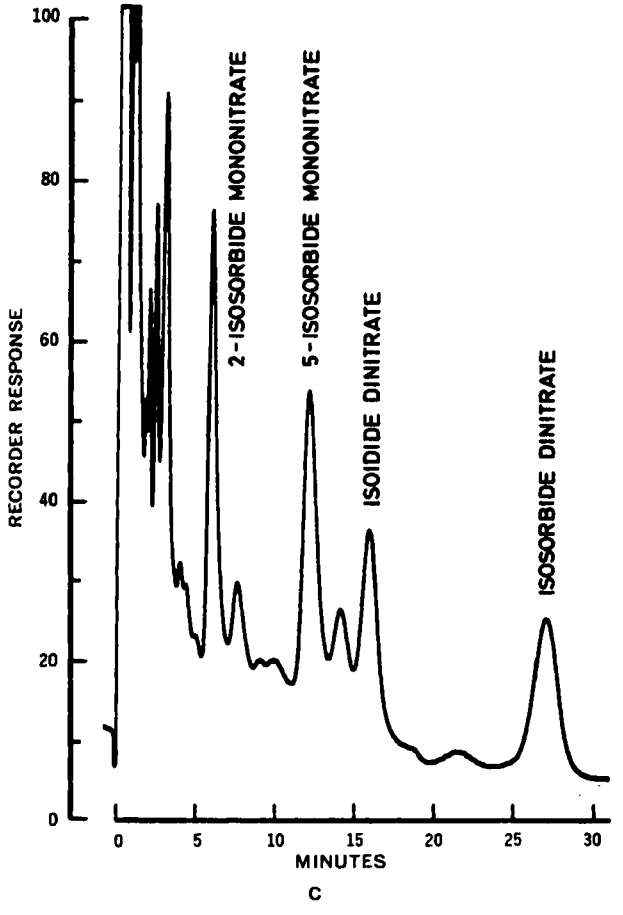
The slope of the curve is, however, different from day to day. Obviously, the length of the procedure precludes the preparation of a new calibration curve every day. Therefore, with each set of samples to be assayed for nitroglycerin, two calibration samples are run through the assay procedure: to each 5-ml. plasma sample, 15 ng. of isosorbide dinitrate and 25 or 50 ng. of nitroglycerin are added before the extraction procedure. From these two values, the slope of the calibration curve can be inferred.

For determination of isosorbide dinitrate, isoidide dinitrate is added as internal standard. Here, too, the relationship between ratio of peak areas and ratio of concentrations is linear, as ascertained by experiments in which 5 ng. of isoidide dinitrate and 5–70 ng. of isosorbide dinitrate were added to 5-ml. plasma samples (Fig. 2).

The accuracy of the method was assessed as follows. To each 5-ml. plasma sample, 10 ng. of nitroglycerin and 15 ng. of isosorbide dinitrate were added. After extraction and injection into the gas chromatograph, the nitroglycerin content was calculated from the respective peak areas, using a calibration factor obtained on the same day from a calibration sample containing 25 ng. of nitroglycerin and 15 ng. of isosorbide dinitrate for 5 ml. of plasma. Likewise, to plasma samples of 5 ml., 5 ng. of isosorbide dinitrate and 5 ng. of isoidide dinitrate were added. The isosorbide dinitrate content was calculated using a calibration sample containing 15 ng. of isosorbide dinitrate and 5 ng. of isoidide dinitrate for 5 ml. of plasma. The results of these accuracy tests are given in Table I.

As an illustration of the method, two chromatograms are shown in Fig. 3 from an experiment in which 800 mcg. of nitroglycerin was taken sublingually by a human volunteer. Figure 4 shows chromatograms from an experiment in which a volunteer was given 5 mg. isosorbide dinitrate orally; this figure also demonstrates the good separation of isosorbide dinitrate from its mononitrated metabolites.





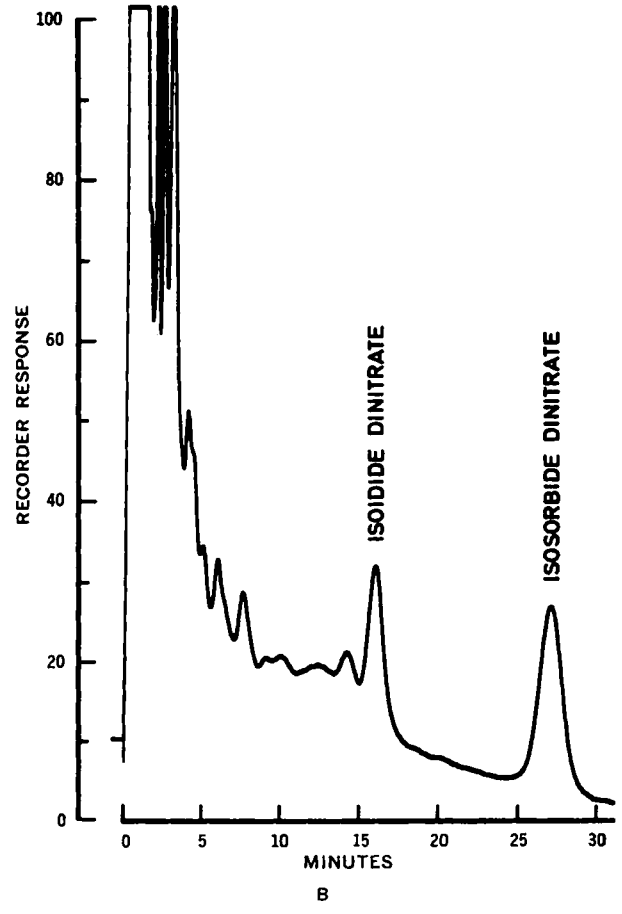


Figure 4—*Gas chromatograms of extracted human plasma. Key: A, extract from 5 ml. blank plasma; B, isosorbide dinitrate (15 ng.) and isoidide dinitrate (5 ng.) added to 5 ml. of blank plasma of the same subject; and C, isoidide dinitrate (5 ng.) added to 5 ml. of plasma of the same subject after oral administration of 5 mg. of isosorbide dinitrate. Plasma was extracted with ethyl acetate; and from the extract in 10 µl. of benzene, 1.6 µl. was injected into the chromatograph. Note the presence of mononitrated metabolites in the plasma after oral administration of isosorbide dinitrate.*

The method described here allows specific determination of small amounts of organic nitrates in plasma. Plasma levels of nitroglycerin, after sublingual administration of the substance in man using the method described here, have been published elsewhere (15). The results obtained with sublingual isosorbide dinitrate are given in Table II.

Use of the method described here for the study of orally given nitrates will hopefully lead to a better understanding of the problem of the long-term prophylaxis of angina pectoris by these products.

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Quantification *in vivo* of the action of drugs that can interfere in the cerebral energy-yielding sequences or that can modify cerebral reactions at metabolic branch points is a problem involving theoretical and methodological considerations. Since several experimental conditions such as anesthesia, hypothermia, or hypercapnia can produce large changes in the cerebral oxygen metabolic rate without disrupting the energy balance (1–3), changes in oxygen consumption cannot be used to define the action of a drug on the cerebral energy state. On the other hand, not all of the factors regulating the cerebral blood flow have been clarified, so flow changes cannot readily be used to evaluate drug action on energy states.

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Furthermore, recent results have demonstrated that the phosphocreatine and lactate concentrations and the

lactate–pyruvate ratio are influenced by the intracellular pH, even if hypoxia is absent (4), so the lactate–pyruvate ratio must be used with caution. Therefore, the changes in the energy charge of the adenylate system were estimated.

The adenosine monophosphate level may be important in determining whether glycolysis or gluconeogenesis will predominate (5, 6), and the balance between the adenine nucleotide concentrations may be a major regulating factor at every point where a metabolite is partitioned between energy-yielding and energy-demanding or energy-storing processes (7–9). This is stated in terms of the energy charge of the adenylate pool (10, 11), a parameter that is intended to quantify the energy state of the cell. The control of adenosine triphosphate-regenerating sequences and, especially, regulation of interaction between adenosine triphosphate regeneration and processes that utilize adenosine triphosphate must clearly respond to the energy balance of the cell rather than to the concentration of a single metabolite (12). Then any drug-induced increase or decrease in the cerebral energy charge potential will be the result of the power to increase or decrease the rates of reactions that control adenosine triphosphate regeneration and to decrease or increase the rates of reactions controlling adenosine triphosphate use; both of these changes counteract the modifications in the energy charge of the brain.

This report concerns the drug-induced changes in the cerebral energy charge of the dog with normal tension, in hypovolemic hypotension, during control conditions, during hypoxia, and during recovery after hypoxia. Bemegride, nicergoline, and dipyridamole were chosen because of their metabolic action (13–18).

METHODS

Animals and Anesthesia—The experiments were carried out on 48 male beagle dogs at 240–360 days of age and weighing from 10.4 to 13.2 kg. Before the experiments the dogs were maintained under the same environmental conditions (22 ± 1°, relative humidity = 60 ± 5%) and were fed only on standard diet as powder (before 60 days of age) or as pellets (after 60 days of age), with water *ad libitum*.

The surgical procedure was performed on animals preanesthetized with urethan (0.4 g./kg. i.p.). Electrical activity of the brain (as portrayed by electroencephalography) was used to determine the degree of anesthesia (19), which was induced and maintained only during the surgical procedure by chloralose (30–40 mg./kg. i.v.). Because anesthetics affect labile phosphates and the extra- and intracellular brain lactate and pyruvate concentrations (2, 3, 20–23), electroencephalographic (EEG) pattern restoration indicated the removal of the anesthetic from the brain before the start of the experiment on cerebral energy metabolism. The dogs were artificially ventilated with room air or a nitrogen–oxygen mixture with an intratracheal Warne tube. During the operative procedure and the experiment, the animals were paralyzed by intravenous injection of gallamine triethiodide (2–3 mg./kg.) to permit unresisted artificial respiration and maintain a constant $PaCO_2$.

Operative Procedure—This procedure consisted mainly of isolation of both common carotid arteries, with ligature of all their branches except the internal carotid arteries and the superior thyroid ones. The zygomatic, maxillary, auricular, and supraorbital vessels were all occluded by ligature or compression. Each of the isolated superior thyroid arteries was cannulated with a polystan tube and connected by a two-way cannula to a perfusion apparatus (to infuse the intracarotid drug or saline solution) and to a glass syringe (to draw arterial blood samples).

The head was fixed to a head holder with the confluence of the cerebral venous sinuses approximately 10 cm. higher than the heart. Monopolar electrodes were inserted in the left and right frontal, parietal, and occipital areas. Heated thermocouples were placed on the brain surfaces to evaluate the changes in cortical blood flow. The flow probe was placed obliquely in the parietal cortex, with the tip adjacent to the cortical gray mantle. The small hole in the skull for the probe was carefully sealed to prevent untoward ambient heat exchange (24). After a longitudinal incision, a 2–2.5-cm. diameter hole was made in the frontoparietal area contralateral to the thermistor flow probe. A plastic funnel was fitted into the hole and the skin was sutured tightly around the funnel (25, 26); subsequently, the plastic funnel was sealed with a rubber stopper and thermally insulated.

Both femoral arteries were cannulated: one connected to a physiological pressure transducer and the other to a heparinized Wolfe bottle to withdraw arterial blood from the systemic circulation. The Wolfe bottle was connected to a femoral vein to infuse the drained blood into the systemic circulation. Connection was made by a large silicone-coated pararubber cannula joined to a peristaltic pump.

Cerebral blood flow, EEG, systemic pressure, and arterial P_{O_2} and P_{CO_2} were measured (using anaerobic samples from the superior thyroid artery). The rectal temperature was monitored with a telethermistor, and intermittent heating was applied to keep the dog's temperature as close to 37° as possible.

These parameters were recorded using a 12-channel recorder.

Design of Experiment—Previous research on the diphosphopyridine nucleotide concentration in the intact brain, as well as on the adenosine triphosphate or phosphocreatine concentration in the tissues, has shown changes for inspired oxygen concentrations below 7% (27–29). On the other hand, some studies have indicated the absence of changes in labile tissue phosphates with 4–5% oxygen in the inspired gas mixture (30). When the arterial P_{O_2} was reduced below 35 mm. Hg, the phosphocreatine content in the brain was reduced, but there were not even small changes in the adenylate system until the P_{O_2} was reduced below 25 mm. Hg. In any case, PaO_2 could be reduced to below 20 mm. Hg without causing major changes in the adenosine phosphates as long as the mean arterial blood pressure was held close to normal levels (4). Also, the cerebral circulation (31, 32) and the brain concentration of labile phosphate (33–36) are not significantly changed until the arterial hypotension results in a cerebral perfusion pressure of about 40 mm. Hg. Nevertheless, P_{O_2} below 25 mm. Hg causes changes in the adenosine phosphates to be exaggerated by even small decreases in the blood pressure (4). Therefore, the hypoxia experiments were carried out on beagle dogs in hypovolemic hypotension.

After the operative procedure, the EEG was followed until the anesthetic had been removed, and then a period of 30 min. was allowed before the hypotension was induced. During this period the cerebral vascular reactivity was tested by administration of an 8% carbon dioxide–air mixture through the respirator; the experiment was continued only if cerebral blood flow was increased, which indicated a qualitatively appropriate response to carbon dioxide in the cerebral vessels (24). Subsequently, the dogs were slowly bled from the arterial femoral cannula into a heparinized Wolfe bottle. A mean blood pressure of 60 mm. Hg was attained within 8–20 min., and then cautious arterial withdrawals or venous infusions by peristaltic pump were made so that the mean blood pressure was held as constant as possible for 25 min. Normally, the blood pressure could be held at the specified level to within ±4 mm. Hg; if unduly large variations occurred or if a substantial part of the blood had to be reinfused to keep the blood pressure constant, the dog was discarded (36). The induced arterial hypotension changes $PaCO_2$ and the hemoglobin concentration. The controls had a mean blood pressure of 112 ± 8 mm. Hg, and the working mean blood pressure was 60 ± 4 mm. Hg; in this range the carbon dioxide tension decreased from 41 ± 4 to 36.5 ± 3 mm. Hg, while the hemoglobin concentration decreased from 18.4 ± 0.45 to 14.6 ± 0.94 g./100 ml.

The experiments were carried out as follows.

1. Evaluation of the drug action in curarized dogs with the systemic blood pressure within the normal range of 100–140 mm. Hg and with controlled ventilation with room air. The drug was perfused for 6 min. into the carotid artery at the rate of 0.5 ml./min., at the molar concentration indicated below.

2. Evaluation of the drug action in curarized dogs during and after arterial hypoxemia. The three periods in each experiment were

Table I—Curarized Beagle Dog: Cerebral Cortex Levels of Lactate, Pyruvate, Adenosine Triphosphate (ATP), Adenosine Diphosphate (ADP), and Adenosine Monophosphate (AMP), Expressed in Micromoles per Gram of Wet Tissue, after 6 min. of Intracarotid Perfusion (0.5 ml./min.) with Saline Solution, Nicergoline, Dipyridamole, and Bemegride

—Intracarotid Perfusion— Substance	Molar Concentration	Number of Dogs	Lactate, μmoles/g.	Pyruvate, μmoles/g.	Lactate / Pyruvate	ATP, μmoles/g.	ADP, μmoles/g.	AMP, μmoles/g.	ATP / ADP	ATP / AMP	Energy Charge Potential[a]
Saline solution	—	3	1.46 ±0.08	0.092 ±0.001	16.37 ±0.83	2.19 ±0.04	0.46 ±0.04	0.07 ±0.01	4.80 ±0.41	32.06 ±6.61	0.89 ±0.03
Nicergoline	1×10^{-4}	3	1.63 ±0.05	0.091 ±0.001	18.03 ±1.64	2.27 ±0.16	0.41 ±0.06	0.11 ±0.02	5.82 ±1.21	20.40 ±2.19	0.88 ±0.02
Dipyridamole	2.5×10^{-4}	3	1.51 ±0.19	0.094 ±0.011	17.07 ±4.24	2.22 ±0.08	0.41 ±0.03	0.11 ±0.04	5.42 ±0.40	21.20 ±1.19	0.89 ±0.04
Bemegride	6.4×10^{-4}	3	4.95[b] ±0.55	0.125[b] ±0.002	39.65[b] ±1.21	1.22[b] ±0.04	0.51 ±0.07	0.87[b] ±0.08	2.53[b] ±0.43	1.43[b] ±0.16	0.57[b] ±0.03

[a] Calculated as [(ATP) + 0.5 (ADP)]/[(ATP) + (ADP) + (AMP)]. [b] Significant difference ($p < 0.05$) from the value after saline solution perfusion, calculated by t test.

usually: (*a*) a steady-state period of hypovolemic hypotension of 10 min. during room air ventilation; (*b*) 12 min. of acute hypoxia induced with controlled $PaCO_2$ by altering the inspired oxygen concentration with a 6% oxygen–nitrogen mixture in the respirator (in some cases, saline perfusion or drugs were given during the last 6 min. of hypoxia); and (*c*) 3 min. of recovery of respiration with room air, and perfusion through one of the thyroid superior arteries with saline solution or drugs.

3. Evaluation of drug action after hypoxia and hypoglycemia in curarized dogs in hypovolemic hypotension. Fasting dogs were prepared in the same manner as already described. During the steady-state period, 800–1200 units of regular insulin was injected intravenously; 40–60 min. later, the dog was given 6% oxygen–nitrogen gas mixture.

The following drugs were perfused through the superior thyroid arteries at 0.5 ml./min. for 3 or 6 min.: (*a*) 4-methyl-4-ethyl-2,6-dioxopiperidine (bemegride), 6.4×10^{-4} *M*; (*b*) 1,6-dimethyl-8β-(5-bromonicotinoylhydroxymethyl)-10α-methoxyergoline tartrate (nicergoline or nimergoline), 1×10^{-4} *M*; (*c*) 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine (dipyridamole), 2.5×10^{-4} *M*; (*d*) sodium malonate, 5×10^{-2} *M*; and (*e*) cocaine hydrochloride, 5×10^{-3} *M*. These concentrations were chosen in accordance with the clinical doses employed in man: 20–80 mg. of bemegride; from 2–4 to 10–15 mg. of nicergoline, and from 12.5–25 to 50–100 mg. of dipyridamole.

Analytical Techniques—At the set time, the motor area of the cortex was frozen *in situ* by pouring liquid nitrogen into the plastic funnel fitted into the cranial vault; the ventilation with room air or oxygen–nitrogen mixture was continued for at least 2 min. after the beginning of the regional brain freezing. A portion of the frozen brain was chiseled out using a rotating cold hollow tube (15-mm. i.d.) during continuous irrigation with liquid nitrogen. The frozen cerebral material was then immersed in liquid nitrogen for 10–15 min. It was then extracted with perchloric acid. The initial extraction was performed at from −10 to −15°, using frozen 3 *M* perchloric acid (4, 21), while the subsequent steps were carried out at 0–5° until a neutral perchlorate-free extract was obtained (4, 37, 38). The steps (4) were: extraction with 3 *M* perchloric acid at subzero temperatures, homogenization for 15 sec., additional extraction with 3% perchloric acid, centrifugation, neutralization of the combined extracts with 5 *N* KOH to pH 5.5, and separation of the potassium perchlorate by centrifugation. The neutralized, potassium perchlorate-free extract was then used for immediate analyses. Lactate, pyruvate, and adenosine mono-, di-, and triphosphates were assayed (37–39), a blank sample being run for each individual adenosine monophosphate analysis because the enzyme used contained adenosine monophosphate itself as a contaminant (21).

Estimation of Energy State of the Brain—The energy charge potential (10) was estimated assuming that the balance among concentrations of adenine nucleotides responds to the energy state of the cell rather than to the concentration of a single nucleotide (12).

The adenosine triphosphate regeneration or the adenosine triphosphate utilization in energy metabolism can be described by Scheme I:

$$2ADP + 2P_i \leftrightharpoons 2ATP + 2H_2O$$

Scheme I

while the reaction catalyzed by adenylate kinase is indicated as shown in Scheme II:

$$ATP + AMP \rightleftarrows 2ADP$$

Scheme II

where ATP = adenosine triphosphate, ADP = adenosine diphosphate, AMP = adenosine monophosphate, and P_i = inorganic phosphate. The adenylate energy storage system may be described (Scheme III) by the sum of the processes indicated in Schemes I and II:

$$AMP + 2P_i \rightleftarrows ATP + 2H_2O$$

Scheme III

The number of anhydride-bound phosphates per adenosine moiety varies from 2 to 0; division by 2 gives a parameter varying between 1 and 0, which has been named *energy charge* of the adenylate system.

The energy charge potential is defined by the ratio: half of anhydride-bound phosphates/concentration of the components of the adenylate system. The number of anhydride-bound phosphates is: 2(ATP) + 1(ADP) + 0(AMP); division by 2 gives: (ATP) + 0.5(ADP). Therefore, the energy charge is defined in terms of actual concentrations as:

$$\text{energy charge potential} = \frac{[(ATP) + 0.5(ADP)]}{[(ATP) + (ADP) + (AMP)]} \quad \text{(Eq. 1)}$$

2 —(division by 2)→ 1 → full charge: only ATP present

⇧ energy charge potential

0 —(division by 2)→ 0 → complete discharge: only AMP present

When only adenosine triphosphate and adenosine monophosphate are evaluated, Eq. 1 can be expressed as:

$$\text{energy charge potential} = \frac{(ATP)}{[(ATP) + (AMP)]} \quad \text{(Eq. 2)}$$

Equations 1 and 2 are compatible with Scheme III, but they are not identical because the concentrations of all three substances (adenosine mono-, di-, and triphosphates) contribute to the energy dynamic, but only two of these (adenosine di- and triphosphates) are involved in hydrolysis. Equation 1 was used in the present study.

Table II—Curarized Beagle Dog in Hypovolemic Hypotension: Cerebral Cortex Levels of Lactate, Pyruvate, Adenosine Triphosphate (ATP), Adenosine Diphosphate (ADP), and Adenosine Monophosphate (AMP), Expressed as Micromoles per Gram of Wet Tissue; Arterial PaO_2 and $PaCO_2$; and Relative Cortical Blood Flow in Various Experimental Conditions (the Rate of the Intracarotid Perfusion was 0.5 ml./min.)

Experimental Conditions	Intracarotid Perfusion—Substance	Molar Concentration	Number of Dogs	PaO_2, mm. Hg	$PaCO_2$, mm. Hg	Relative Cortical Blood Flow[a]	Lactate, μmoles/g.	Pyruvate, μmoles/g.	Lactate/Pyruvate	ATP, μmoles/g.	ADP, μmoles/g.	AMP, μmoles/g.	ATP/ADP	ATP/AMP	Energy Charge Potential[b]
Control condition[c]	Saline solution	—	5	96.4 ±2.3	36.5 ±1.3	100	2.28 ±0.10	0.122 ±0.009	18.85 ±0.67	2.28 ±0.06	0.45 ±0.03	0.09 ±0.01	5.20 ±0.42	27.64 ±4.54	0.89 ±0.01
Hypoxia[d] 2 min.	Saline solution	—	3	45.0 ±4.4	37.8 ±1.2	144[e] ±10	2.87 ±0.15	0.140 ±0.014	20.64 ±0.98	2.46 ±0.09	0.41 ±0.05	0.09 ±0.02	6.18 ±0.69	30.22 ±7.87	0.90 ±0.05
6 min.	Saline solution	—	3	27.3[e] ±1.3	36.0 ±1.8	180[e] ±13	7.60[e] ±0.56	0.209[e] ±0.017	36.20[e] ±1.90	2.22 ±0.44	0.44 ±0.04	0.12 ±0.01	5.10 ±0.47	18.35 ±1.72	0.87 ±0.01
12 min.	Saline solution	—	3	19.3[e] ±0.7	40.2 ±0.9	222[e] ±16	23.03[e] ±1.72	0.267[e] ±0.025	88.60[e] ±13.29	1.68[e] ±0.16	0.52 ±0.05	0.64[e] ±0.17	3.23[e] ±0.30	3.23[e] ±1.15	0.68[e] ±0.06
After 3-min. period of recovery of room air ventilation	Saline solution	—	3	98.3 ±2.8	37.8 ±1.0	143 ±13	11.87 ±1.23	0.166 ±0.025	78.50 ±22.99	1.98 ±0.09	0.53 ±0.05	0.37 ±0.05	3.84 ±0.49	5.68 ±1.09	0.78 ±0.02
	Nicergoline	1×10^{-4}	3	96.0 ±3.2	35.8 ±1.0	162 ±20	12.80 ±1.36	0.176 ±0.025	75.00 ±13.92	2.16 ±0.10	0.45 ±0.06	0.13[f] ±0.02	4.88 ±0.44	16.91[f] ±1.15	0.87[f] ±0.02
	Dipyridamole	2.5×10^{-4}	3	97.7 ±2.2	36.3 ±1.3	151 ±14	8.93 ±0.81	0.128 ±0.017	73.73 ±14.30	2.12 ±0.08	0.46 ±0.05	0.17[f] ±0.02	4.69 ±0.31	12.72[f] ±1.83	0.85[f] ±0.01
	Bemegride	6.4×10^{-4}	3	98.0 ±2.3	36.2 ±1.3	181[f] ±16	30.53[b] ±0.82	0.298[f] ±0.025	103.17[f] ±7.31	1.36[f] ±0.07	0.54 ±0.04	0.97[f] ±0.10	2.54[f] ±0.18	1.45[f] ±0.23	0.57[f] ±0.03

[a] Expressed as percent of the control value equal 100. [b] Calculated as [(ATP) + 0.5 (ADP)]/[(ATP) + (ADP) + (AMP)]. [c] Room air controlled ventilation and 6 min. of saline solution perfusion. [d] Induced by controlled ventilation with 6% oxygen–nitrogen mixture. [e] Statistical difference ($p < 0.05$) *versus* the value of the control condition. [f] Statistical difference ($p < 0.05$) *versus* the value after 3-min. period of recovery of room air ventilation and intracarotid perfusion with saline solution.

Obviously the generalization proposed by the quantification of the drug action on the energy-yielding sequences by the cerebral energy charge of the adenylate pool is necessarily oversimplified because little satisfactory information is available as to: (*a*) the extent of the importance of barrier compartmentation as compared with kinetic compartmentation, the selective permeabilities and transport properties being modulated by both the energy charge and the levels of specific metabolites on the sides of the barrier; (*b*) the interactions between feedback modifiers and energy charge in regulation of biosynthetic sequences and in production of storage compounds, or in regulation of sequences that regenerate adenosine triphosphate and supply primary biosynthetic intermediates; and (*c*) the sequences involved in the control of the phosphopyridine nucleotide system.

Statistical Analysis—The *t* test was applied to the differences in all instances.

RESULTS AND DISCUSSION

Drug Action during Room Air Ventilation—Table I shows that a curarized normal beagle dog receiving room air responds to intracarotid perfusion (0.5 ml./min. for 6 min.) with nicergoline (1×10^{-4} *M*) and dipyridamole (2.5×10^{-4} *M*), with no change in the levels of lactate, pyruvate, and labile phosphates in the cerebral cortex or in the cerebral energy charge; perfusion with bemegride (6.4×10^{-4} *M*) increases the lactate, pyruvate, and lactate–pyruvate ratio in the cerebral cortex. Previous observations on curarized monkeys (14) have shown that arterial and venous cerebral blood lactate rapidly increase by about twofold after bemegride injection. In the present study, the animals were curarized so any effect of bemegride on muscular contraction and muscle metabolism could be ignored. Furthermore, intracarotid perfusion with bemegride (Table I) induced a decrease in the adenosine triphosphate level and an increase in the adenosine monophosphate concentration in the cerebral cortex, the adenosine diphosphate level being quite unchanged. The energy charge potential was significantly reduced. These data are in agreement with the observation (39, 40) that agents inducing seizures are able to lower the levels of adenosine triphosphate and phosphocreatine in the brain, even if the seizure activity may be associated with unchanged levels of cerebral high energy compounds (47).

Drug Action during Hypoxia and Hypotension—*Influence of Arterial Hypoxemia*—Table II shows that hypoxia induced by a 6% oxygen–nitrogen mixture caused PaO_2 in hypotensive beagle dogs to fall rapidly, while the $PaCO_2$ varied little because of the controlled ventilation. The cerebral cortical blood flow increased significantly at PaO_2 below about 50 mm. Hg. Furthermore, there were changes in the lactate, pyruvate, and lactate–pyruvate ratio in the brain at PaO_2 below about 30–40 mm. Hg, but there were no significant variations in the cerebral energy charge potential until PaO_2 fell below 20 mm. Hg (Table II). This finding indicates that the brain lactate and pyruvate can change at PaO_2 levels which are compatible with unchanged energy charge, which confirms that the cerebral lactate content or production can increase at oxygen tensions compatible with unchanged levels of labile phosphates (27, 30, 36, 41–43).

Different anatomical regions of the brain vary in sensitivity to energy deprivation (44) and the time course of energy deprivation (45, 46). In fact, gross irreversible damage from total asphyxia is limited almost exclusively to the brain stem (45), while in partial asphyxia damage to the cortex is the major finding (46). In the present research, during oxygen–nitrogen mixture respiration, the cerebral energy charge potential on the cortex of the frontal, parietal, and occipital areas was simultaneously evaluated in three dogs. At $PaO_2 = 19.3 \pm 0.7$ mm. Hg, the mean values were 0.66 ± 0.07, 0.69 ± 0.05, and 0.70 ± 0.04, respectively, which are not significantly different.

After 12 min. of hypoxia, PaO_2 rose rapidly to the control value following normal ventilation and perfusion for 3 min. through the superior thyroid artery with saline solution (Table II). The increased cortical blood flow fell when the gas was replaced by room air but only by about 45% within the 3 min. of observation. Also, the lactate, pyruvate, and adenosine triphosphate levels and the energy charge potential only partially recovered within the 3 min. (Table II).

Effect of Pharmacological Treatment with Drugs—After hypoxia, room air ventilation and perfusion through the superior thyroid

Table III—Curarized Beagle Dog in Hypovolemic Hypotension: Cerebral Cortex Level of Adenosine Triphosphate (ATP), Adenosine Diphosphate (ADP), and Adenosine Monophosphate (AMP), Expressed as Micromoles per Gram of Wet Tissue, in Various Experimental Conditions (Rate of Intracarotid Perfusion was 0.5 ml./min.)

Experimental Conditions	Intracarotid Perfusion—Substance	Molar Concentration	Number of Dogs	ATP, μmoles/g.	ADP, μmoles/g.	AMP, μmoles/g.	ATP/ADP	ATP/AMP	Energy Charge Potential[a]
After 12-min. period of hypoxia[b]	Saline solution	—	3	1.68 ±0.16	0.52 ±0.05	0.64 ±0.17	3.23 ±0.30	3.23 ±1.15	0.68 ±0.06
	Nicergoline	1×10^{-4}	3	1.63 ±0.12	0.57 ±0.06	0.59 ±0.12	2.90 ±0.15	3.07 ±0.72	0.68 ±0.04
After 3-min. period of recovery of room air ventilation	Saline solution	—	3	1.98[c] ±0.09	0.53 ±0.05	0.37[c] ±0.05	3.84 ±0.49	5.86[c] ±1.09	0.78[c] ±0.02
	Nicergoline	1×10^{-4}	3	2.16[c] ±0.10	0.45 ±0.06	0.13[c,d] ±0.02	4.88 ±0.44	16.91[c,d] ±1.15	0.87[c,d] ±0.02
	Nicergoline + malonate	1×10^{-4} + 5×10^{-2}	3	1.71[d] ±0.15	0.49 ±0.01	0.64[d] ±0.17	3.49 ±0.39	3.14[d] ±0.92	0.68[d] ±0.06
	Nicergoline + cocaine	1×10^{-4} + 5×10^{-3}	3	1.60[d] ±0.10	0.54 ±0.04	0.74[d] ±0.11	3.02 ±0.38	2.30[d] ±0.47	0.65[d] ±0.04
After 3-min. period of recovery of room air ventilation in hypoglycemia[e]	Nicergoline	1×10^{-4}	3	1.63[d] ±0.17	0.54 ±0.03	0.67[d] ±0.19	3.02 ±0.25	2.43[d] ±1.06	0.67[d] ±0.06

[a] Calculated as [(ATP) + 0.5 (ADP)]/[(ATP) + (ADP) + (AMP)]. [b] Induced by controlled ventilation with 6% oxygen–nitrogen mixture. [c] Statistical difference ($p < 0.05$) *versus* the value after 12-min. period of hypoxia. [d] Statistical difference ($p < 0.05$) *versus* the value after 3-min. period of recovery of room air ventilation and intracarotid perfusion with saline solution. [e] Induced by intravenous injection of 800–1200 units of regular insulin.

artery with bemegride (6.4×10^{-4} *M* at 0.5 ml./min.) for 3 min. (Table II) inhibited the cerebral metabolic restoration and decreased the adenosine triphosphate level and the energy charge potential, while the lactate and pyruvate concentrations and the adenosine monophosphate level increased later. Bemegride also inhibited the normal reduction in the cerebral blood flow occurring during the recovery with room air respiration. In fact, the residual enhancement in cortical blood flow after 3 min. of saline solution perfusion was about 45%; after bemegride perfusion, it was about 80%. During bemegride perfusion, no significant differences in PaO_2 or $PaCO_2$ were present relative to saline perfusion.

After hypoxia, recovery with room air ventilation, and intracarotid perfusion (0.5 ml./min.) with nicergoline (1×10^{-4} *M*) or dipyridamole (2.5×10^{-4} *M*) for 3 min. (Table II), the energy charge potential was significantly different from that induced by saline perfusion. The drugs decreased significantly the adenosine monophosphate concentration. Conversely, no action on the lactate and pyruvate levels or on the lactate–pyruvate ratio of the cortex was observed. During drug perfusion, no PaO_2 and $PaCO_2$ values significantly different from the saline case were found.

The mechanism was elucidated by perfusing nicergoline (1×10^{-4} *M*) at 0.5 ml./min. during hypoxia; the drug induced no significant change in the cerebral energy charge (Table III). Furthermore, during the recovery with room air ventilation after hypoxia, nicergoline (1×10^{-4} *M*) was tested during simultaneous perfusion with sodium malonate (5×10^{-2} *M*) or cocaine (5×10^{-3} *M*) at 0.5 ml./min. for 3 min. Both agents blocked the effect of nicergoline action on the cerebral energy charge (Table III). On the other hand, hypoglycemia inhibited the action of nicergoline on the energy charge potential of the brain during recovery with room air ventilation after hypoxia (Table III).

Mode and Mechanism of Drug Action—It was possible to distinguish drugs that reduce the energy charge (*e.g.*, bemegride) from ones that increase it (*e.g.*, nicergoline and dipyridamole).

Bemegride affects the energy metabolism of the brain in the following four respects:

1. After injection of bemegride in animals immobilized with curare, appearance of spike activity in the EEG is correlated with a marked increase in the cerebral blood flow and cerebral metabolic rate for oxygen (13).

2. Cerebral glucose consumption and cerebral blood lactate levels increase significantly, showing a condition compatible with increases in aerobic and anaerobic glycolyses during seizure activity induced by bemegride (14).

3. Electric shock and drugs inducing seizures are known to decrease the levels of adenosine triphosphate and phosphocreatine in brain (39, 40).

4. The eventual reduction in cerebral levels of high energy phosphates during seizures reflects the discrepancy between the high energy consumption and the normal resynthesis of labile phosphates, as occurs during hypoxia localized in the brain itself (39, 40, 48).

In the present study, intracarotid perfusion with bemegride in beagle dog induced: (*a*) an increase in the lactate and pyruvate levels of the cortex and a decrease in the energy charge, the adenosine triphosphate being decreased and the adenosine monophosphate being increased in the brain tissues; and (*b*) inhibition of the normal restoration of the cerebral energy charge potential after hypoxia. All these results support the hypothesis that bemegride may exert a hypoxic effect on the brain because of a discrepancy between the energy-demanding processes and the oxygen that the increased cerebral blood flow can supply. In any case, bemegride reduced the stored energy in spite of the increase in some pharmacodynamic (EEG and cerebral blood flow) or metabolic parameters (cerebral glucose consumption and cerebral metabolic rate for oxygen).

There are three notable points about the action of drugs that raise the depressed energy charge potential of the brain: (*a*) nicergoline (as well as dipyridamole) is inactive during the control condition, the cerebral energy charge being normal; (*b*) nicergoline is inactive during hypoxemia; and (*c*) malonate, cocaine, and hypoglycemia inhibit the action of nicergoline on cerebral labile phosphates during the recovery of room air ventilation after hypoxia. The mechanism of action of ergoline derivatives is only partially elucidated by these observations. As regards malonate inhibition, it should be noted that: (*a*) the well-known inhibitory effect on succinate oxidation makes malonate a potent inhibitor of the citric acid respiratory cycle (49), (*b*) potassium-stimulated brain respiration is highly malonate sensitive (50, 51), and (*c*) the stimulation of metabolism by a changed K^+/Ca^{+2} balance is inherently associated with effects at the brain cell membrane (52). Malonate inhibition of nicergoline action was observed in the present study, and it supports the hypothesis that the drug increases the K^+/Ca^{+2} balance and the associated activity in the citric acid cycle, because ionic movements at cell membranes occur by the initiation of reactions involving energy change in the adenosine phosphate system. The hypothesis concerning nicergoline interference in the K^+/Ca^{+2} balance is supported also by the nicergoline antagonism against the Ca^{+2}

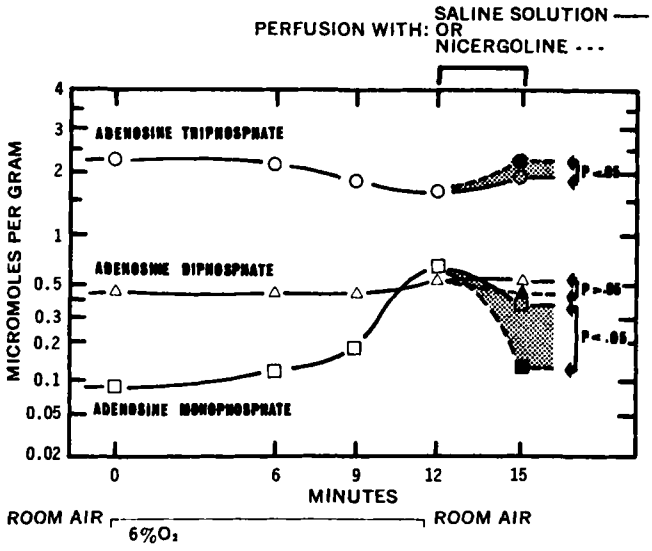

Figure 1—*Variation in the cerebral cortex levels of adenosine mono-, di-, and triphosphates induced in beagle dogs by hypoxia and recovery with room air ventilation and intracarotid perfusion (0.5 ml./min. for 3 min.) with saline solution or nicergoline (5 × 10⁻⁴ M). The abscissa shows the time of observation. The numbers by the lines for the concentrations represent the probability levels of the difference between the saline solution perfusion and the nicergoline perfusion. Each point represents the average value for three dogs.*

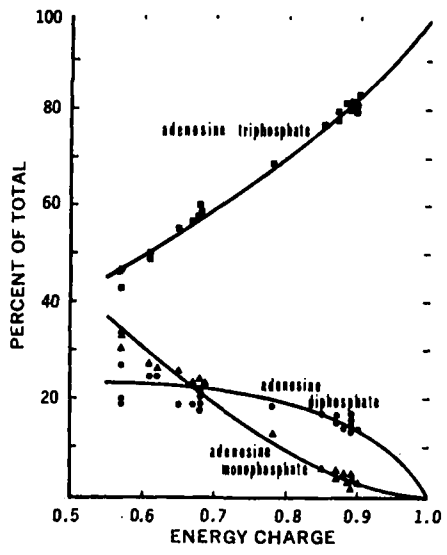

Figure 2—*Cortical motor region of the brain of beagle dog in various experimental conditions: relative concentrations of the adenine nucleotides as a function of energy charge.*

accumulation by epinephrine (53) and by cocaine inhibition of the nicergoline effect on brain energy-coupling processes. In fact, cocaine acts in a manner similar to Ca^{+2}, its presence resulting in an effective diminution of a K^+/Ca^{+2} ratio at the brain cell surface (52).

The estimation of adenine nucleotides in the cortical motor region of the brain shows that hypoxia in the hypovolemic, hypotensive dog leaves the adenosine diphosphate level little changed, while the decrease in adenosine triphosphate is related to an increase in adenosine monophosphate (Fig. 1), which confirms the previous suggestion that the level of adenosine monophosphate may be considered a measure of the stress upon the brain (39). After 3 min. of recovery with room air ventilation, the adenosine monophosphate concentration varied inversely with the adenosine triphosphate concentration, the adenosine diphosphate being little changed. Intracarotid nicergoline perfusion (1×10^{-4} *M*, 0.5 ml./min. for 3 min.) enhanced significantly the decrease in adenosine monophosphate and the increase in adenosine triphosphate, while adenosine diphosphate remained unchanged (Fig. 1). This confirms that the ordered changes among the adenine nucleotides are a consequence of the constant sum of adenosine mono-, di-, and triphosphates and the slight changes in adenosine diphosphate, which together force an inverse relationship between adenosine monophosphate and adenosine triphosphate. The relative proportions of the individual adenine nucleotides are functions of the cerebral energy charge (Fig. 2).

CONCLUSIONS

It is difficult to quantify the action of drugs upon cerebral energy-yielding sequences because there is doubt as to the parameter to measure and the conditions inducing change in the energy-coupling processes.

The oxygen consumption and the cerebral blood flow cannot be used, because the cerebral metabolic rate for oxygen and the cerebral blood flow can be modified without change or with opposite change in the cerebral energy balance. On the other hand, the factors regulating the cerebral blood flow have not yet been clarified (1–3, 47, 54). In the present *in vivo* study, the lactate and pyruvate levels of the brain and the cerebral energy charge *via* the adenosine mono-, di-, and triphosphates were evaluated. There was some difference between these two groups of parameters since the lactate and pyruvate of the brain can change at PaO_2 values which are compatible with unchanged levels of the stored energy. Therefore, it seems advisable to interpret the lactate–pyruvate changes with caution, since no other parameter of the cerebral energy state can be used.

The changes in the energy state of the brain induced by drugs are best evaluated from the cerebral energy charge potential in the adenine nucleotide pool, but two points should be stressed:

1. A change in the energy charge of the motor area does not imply that an equal change has occurred in the whole cerebral tissue or in any other important local region of the brain.
2. A change in energy charge evaluated from the adenine nucleotide pool does not imply that an equal change has occurred in other substrates and, particularly, in the creatine phosphate system. Nevertheless, several enzymes that are involved in energy-yielding sequences, or that catalyze reactions at metabolic branch points, are affected by adenine nucleotides. The pattern of the responses suggests that they participate in the regulatory interactions that must underlie orderly metabolic integration. The creatine phosphate can be affected by hypoxemia before changes in the adenosine phosphate pool have occurred; in fact, at PaO_2 of about 35 mm. Hg, the cerebral phosphocreatine level is significantly reduced, but there are no changes in the adenosine mono-, di-, or triphosphate concentration (4). In any case, it must also be stressed that isolated adenosine triphosphate values give relatively restricted information (54).

The cerebral energy state is remarkably resistant to various experimental conditions. Thus, PaO_2 could be reduced to below 20 mm. Hg without causing major changes in adenosine phosphates as long as the mean arterial blood pressure is held close to normal levels, while more pronounced changes are induced in hypotensive animals (4). In the present research, the action of the drugs on the cerebral energy-coupling processes *in situ* was evaluated by inducing a hypoxic condition in the hypovolemic, hypotensive dog.

Bemegride lowered the cerebral energy charge, while nicergoline and dipyridamole increased the depressed energy charge. The drugs showed significant effects on the relative proportions of the individual components of the adenylate system, which are related to the energy-yielding, energy-demanding, and energy-storing processes, the cerebral blood flow being unchanged or changing in the opposite direction. Nicergoline resembles dipyridamole in being inactive in the normal brain. Its action on the cerebral energy, as depressed by hypoxemia and hypotension, is antagonized by malonate, cocaine, and hypoglycemia.

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Quantitative Determination of Pentazocine in Plasma and of Pentazocine and Metabolites in Urine

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Abstract □ GLC methods for the determination of plasma pentazocine and for the determination of urinary pentazocine and its known metabolites are described. The determination of plasma pentazocine involves the extraction of pentazocine and a suitable internal standard, the derivatization of these compounds with heptafluorobutyric acid anhydride, and the chromatography of the derivatives using tritium-foil, electron-capture detection. The determination of urinary pentazocine and two of its metabolites involves the extraction of these compounds and an internal standard, derivatization with a silylating reagent, and the chromatography of the derivatives using flame-ionization detection. The determination of a third urinary metabolite involves adsorption chromatography followed by the same derivatization and chromatographic analysis. The sensitivity and reliability of the techniques are discussed and compared to other methods in the literature.

Keyphrases □ Pentazocine in plasma, pentazocine and metabolites in urine—GLC analysis □ GLC—analysis, pentazocine in plasma, pentazocine and metabolites in urine

Pentazocine is a strong analgesic and weak narcotic antagonist possessing little dependence potential (1). Both parenteral and oral formulations are available and have found widespread clinical use. Although clinical studies of the efficacy of the compound and comparisons of it with other analgesics abound, only limited attempts have been made to study the behavior of the drug in man. Blood levels and the urinary excretion of free pentazocine in man after various routes of administration have been reported (2–5), as have some studies of its total human excretion and metabolism (6, 7) and of its metabolism in animals (8–13). With low and moderate doses of pentazocine, plasma levels in man rarely exceeded 0.2 mcg./ml. and were usually less than 0.1 mcg./ml.

Pentazocine is extensively metabolized in animals and in man. The dimethylallyl side chain is oxidized to two isomeric alcohols and the phenolic hydroxyl is conjugated. Both alcohols may be conjugated and one, the *trans*-isomer, is known to be further oxidized by man to a carboxylic acid. This acid is probably conjugated at the phenolic hydroxyl but to a lesser extent than are the other compounds. The urinary excretion of free pentazocine varies from less than 5 to more than 20% of the dose, depending upon the subject and the route of administration. The remainder of the 60–65% of the dose excreted in 24 hr. can be found in the form of the other known metabolites.

To compare various doses and routes of administration and to develop a pharmacokinetic model for the absorption, distribution, and excretion of pentazocine, adequate methods for the measurement of urinary metabolites, as well as of parent compound, and a sensitive method for the determination of plasma pentazocine were needed. This paper reports the development of methods that we have used in the analysis of a large number of plasma and urine samples from human subjects receiving pentazocine. Those data will be reported subsequently.

EXPERIMENTAL

Reagents—Technical grade ethylene dichloride was purified by fractional distillation in an all-glass apparatus. Nanograde benzene[1] and spectroquality hexane[2] were used without further treatment. Heptafluorobutyric acid anhydride[3] was distilled, 0.2-ml. amounts were sealed under nitrogen individually in small ampuls, and the ampuls were stored at −20°. *N,O*-Bis(trimethylsilyl)acetamide[4], *N,O*-bis(trimethylsilyl)trifluoroacetamide[4], β-glucuronidase[5], and the packings for GLC[6] were used as purchased. Amberlite XAD-2 resin[7] was cleaned by repeatedly and serially suspending it in water, methanol, acetone, methanol, and, finally, water. All other chemicals used were reagent grade.

Pentazocine [1,2,3,4,5,6-hexahydro-*cis*-6,11-dimethyl-3-(3-methyl-2-butenyl)-2,6-methano-3-benzazocin-8-ol], its "*trans*-alcohol" [1,2,-3,4,5,6-hexahydro-8-hydroxy-α,6(eq),11(ax)-trimethyl-2,6-methano-3-benzazocin-3-*trans*-2-buten-1-ol] and its "*trans*-acid" [1,2,3,4,5,6-hexahydro-8-hydroxy-α,6(eq),11(ax)-trimethyl-2,6-methano-3-benzazocin-3-crotonic acid] metabolites, and the internal standard (1,-2,3,4,5,6-hexahydro-*cis*-6,11-dimethyl-3-cyclopentyl-2,6-methano-3-benzazocin-8-ol) were prepared[8]. Solutions of these benzazocines were kept in the dark and refrigerated, and they are stable for at least 3 months under such conditions. Biological samples were stored in the frozen state (−20°), and they are stable for over 1 year.

Treatment of Plasma Samples—To analyze plasma for unconjugated pentazocine, the following procedure was used. From 0.5- to 2-ml. plasma samples were placed in 150 × 16-mm. test tubes, and exactly 0.50 ml. of a 4×10^{-5}% solution of the internal standard in 0.001 *N* HCl, 0.2 ml. of a buffer consisting of a solution saturated with both sodium bicarbonate and sodium carbonate, and 5 ml. of nanograde benzene were added to each tube. The contents of the tubes were stirred in a vortex mixer and centrifuged, and the benzene layers were transferred to 175 × 20-mm. tubes. The aqueous phases were extracted with a second 5 ml. of benzene as before. The combined benzene phases were extracted as before with 2 ml. of 0.1 *N* HCl. The organic phases were removed and discarded and, after the addition of 0.2 ml. of the carbonate buffer, the aqueous phases were extracted as before with 5 ml. of benzene. The benzene extracts were transferred to 150 × 16-mm. tubes, and the aqueous phases were extracted again with 3 ml. of benzene.

The combined benzene extracts were centrifuged to remove the last traces of the aqueous phases, decanted into 100 × 16-mm. tubes, and dried in a heating block at 65° under a gentle stream of filtered air. The residues were taken up in 0.4 ml. of a 1.25% solution of heptafluorobutyric acid anhydride in ethyl acetate, and clean carborundum chips were added to each tube. The solutions were taken to dryness at 65° in a vacuum oven, the residues were taken up in approximately 0.25 ml. of ethyl acetate, and these solutions were dried in the vacuum oven again. At least 15 min. was allowed for each drying step. The residues were taken up in 0.1 ml. of ethyl acetate for chromatography; once dissolved, they

[1] Mallinckrodt Chemical Works.
[2] Matheson, Coleman and Bell.
[3] Pierce Chemical Co.
[4] Regisil, Regis Chemical Co.
[5] Glusulase, Endo Research Laboratories.
[6] Applied Science Laboratories.
[7] Rohm and Haas Co.
[8] By Dr. N. F. Albertson, Sterling-Winthrop Research Institute.

had to be analyzed on the same day. However, the dry heptafluorobutyryl esters could be kept in a desiccator for several days.

Treatment of Urine Samples—To analyze urine for unconjugated pentazocine and unconjugated *cis*-alcohol and *trans*-alcohol metabolites, the following procedure was used. Five-milliliter urine samples were placed in 50-ml. extraction tubes, and exactly 0.100 ml. of a 0.005% solution of the internal standard in 0.1 *N* HCl, 20 ml. of ethylene dichloride, and 0.5 g. of an equal mixture of sodium bicarbonate and sodium carbonate were added to each tube. The tubes were closed with polyethylene stoppers and shaken, horizontally, for 5 min. at 360 oscillations/min. All buffer had to be in solution at this point. The tubes were centrifuged and the aqueous phases were removed and discarded. The organic phases were washed in the same manner with 5 ml. of water and the washes were discarded. The organic phases were then extracted in the same manner with 5 ml. of 0.1 *N* HCl, and the acid phases were transferred to clean 50-ml. extraction tubes. The organic phases were discarded and, after adding 0.5 g. of the buffer used above to the aqueous phases, they were extracted with 15 ml. of ethyl acetate.

After centrifugation, approximately 10 ml. of the ethyl acetate phases was transferred to 15-ml. conical centrifuge tubes and dried in a heating block at 70–75° under a gentle stream of air. Fifty microliters of 10% *N,O*-bis(trimethylsilyl)acetamide in dimethylformamide was added to each tube, and the tubes were closed with disposable polyethylene stoppers. The desired trimethylsilyl derivatives formed almost immediately and had to be analyzed on the same day.

To analyze urine for total (unconjugated plus conjugated) pentazocine and *cis*-alcohol, *trans*-alcohol, and *trans*-acid metabolites, the following procedure was used. Five-milliliter urine samples were placed in 50-ml. extraction tubes and 0.100 ml. of the 0.005% solution of internal standard, 15 ml. of 0.1 *N* acetate buffer (pH 5.5), 0.125 ml. of β-glucuronidase, and approximately 0.05 ml. of chloroform were added to each tube. The tubes were stoppered and incubated over 3 nights at 37°. Then the buffer salts were added as before and the tubes were stoppered and shaken by hand until all of the salts had dissolved. Twenty milliliters of ethylene dichloride was added to each tube and the tubes were mixed by inverting them by hand 100 times. The tubes were centrifuged, the aqueous phases were transferred to 100-ml. extraction tubes, the organic phases were washed in the same manner with 5 ml. of water, and the aqueous phases were combined.

At this point, the organic phases were carried through the rest of the procedure used earlier to obtain derivatives of pentazocine and its hydroxylated metabolites for GLC. The combined aqueous phases were adjusted to pH 4 by adding 3.0 ml. of glacial acetic acid gently down the side of each tube. The tubes were then agitated sufficiently to expel most of the carbon dioxide formed. The solutions were then passed through 1 × 10-cm. columns of Amberlite XAD-2 at a flow rate of 1 ml./min. The contents of each tube were rinsed into the columns with 20 ml. of water, and the columns were washed with one more 20-ml. portion of water. The columns were drained after the last rinse; all of the aqueous solutions were discarded. The columns were eluted with one 10-ml. and two 20-ml. portions of methanol. Flow was stopped halfway through the second elution, and the column bed was stirred to expel gas bubbles. The combined methanol eluates were dried on a steam bath, and the residues were transferred quantitatively with two 2.5-ml. washes of the upper phase of an equilibrated mixture of water–methanol–chloroform–hexane (1:3:4:4 v/v) to 20-ml., round-bottom centrifuge tubes. These solutions were extracted twice with 10-ml. portions of the lower phase of the same solvent mixture, the lower phases being discarded each time. To each tube was added 0.250 ml. of a 0.04% solution of the internal standard in 0.1 *N* HCl, and the contents were dried as in the other procedures. The residues were reacted for 15 min. at 60° with 100 μl. of *N,O*-bis(trimethylsilyl)trifluoroacetamide[4] before being chromatographed. The solutions had to be analyzed on the same day.

GLC—For the analysis of the heptafluorobutyryl derivatives of pentazocine, a gas chromatograph[9] equipped with a tritium-foil, electron-capture detector was used. Separation was performed isothermally on a 1.5-m. (5-ft.), 2-mm. i.d. glass column packed with 10% OV-1 on 100–120-mesh Gas Chrom Q. Injection volumes were 3–5 μl. The following conditions were used for all analyses: injection temperature, 250°; column oven temperature, 215°; detector temperature, 215°; and nitrogen carrier gas flow, 50 ml./min.

Table I—Relative Retention Times of Derivatives of Pentazocine and Its Metabolites[a,b]

Compound	Derivative: Heptafluorobutyryl	Derivative: Trimethylsilyl
Pentazocine	0.75	0.75
cis-Alcohol metabolite[c]	—	1.47
trans-Alcohol metabolite[c]	—	1.72
trans-Acid metabolite[c]	—	2.23

[a] Values were determined on 1.8-m. (6-ft.) columns packed with 10% OV-1 on Gas Chrom Q. [b] Retention times are relative to the appropriate derivative of the internal standard (1,2,3,4,5,6-hexahydro-*cis*-6,11-dimethyl-3-cyclopentyl-2,6-methano-3-benzazocin-8-ol). [c] These metabolites were characterized previously (8).

For the analysis of trimethylsilyl derivatives of pentazocine and its metabolites, a gas chromatograph[10] equipped with flame-ionization detectors was used. Separation was performed isothermally on 1.8-m. (6-ft.), 2-mm. i.d. glass columns packed with 10% OV-1 on 100–120-mesh Gas Chrom Q. The injection volume was 2 μl. The following conditions were used for all analyses: injector and detector temperatures, 270°; nitrogen carrier gas flow, 55 ml./min.; hydrogen flow, 33 ml./min.; and air flow, 300 ml./min. The column oven temperature was varied between 245 and 265°, depending upon the particular analysis performed and the particular column used. Columns had to be replaced monthly under conditions of daily use.

Calculations—Standard curves were constructed by plotting the ratios of the peak heights of known amounts of authentic standards to the peak heights of the internal standard against the amounts of the standards added to human plasma or human urine and extracted by the methods outlined earlier in this paper. Since no authentic *cis*-alcohol metabolite was available, the standard curve established for the *trans*-alcohol metabolite was used, and all peak heights of the *cis*-alcohol were reduced by multiplying them by the ratio of the $^1/_3$ height width of the *cis*-alcohol to the $^1/_3$ height width of the *trans*-alcohol, on the assumption that the areas subtended by equal amounts of these two isomers would be equal.

RESULTS

Choice of Internal Standard—An analog of pentazocine was chosen because it could be expected to: (*a*) form the same derivatives as pentazocine and its metabolites, (*b*) behave very similarly to pentazocine and the hydroxylated metabolites in the extraction procedures involved, and (*c*) have a retention time near to that of the other compounds. Table I lists the relative retention times of pentazocine and its metabolites relative to the internal standard in the GLC systems used. The retention time of the internal standard conveniently falls just after that of pentazocine. Table II lists the absolute recovery of these various compounds from plasma and from urine after using the extraction procedures and indicates that the internal standard does behave very similarly to the compounds of interest.

Determination of Plasma Pentazocine—A chromatogram of the heptafluorobutyryl ester of the internal standard and of pentazocine extracted from human plasma shows the compounds to be well separated with symmetrical peaks, although a small contaminant often appears as a shoulder just prior to the peak representing the internal standard. Sample drying is a particularly important step in the procedure. If the temperature is too great, the derivatives are lost; if too slow, substances derived from the heptafluorobutyric anhydride are not removed and gross contamination occurs. Generally, further heating *in vacuo* of the latter type of samples will satisfactorily clean them.

The response of the electron-capture detector is linear with these benzazocine derivatives to about 150 ng. in the sample (about 5 ng. on the column). The 95% confidence interval of the slope of

[9] Packard series 7300.

[10] Varian Aerograph series 2100.

Table II—Absolute Recovery Obtained, by the Extraction Procedures Used, of Pentazocine, Its Hydroxylated Metabolite, and the Internal Standard from Plasma and Urine

Extracted Fluid	Compound Recovered, %: Pentazocine	*trans*-Alcohol	Internal Standard[a]
Urine[b]	66.4	64.2	61.5
Hydrolyzed urine[c]	68.8	60.0	56.5
Plasma[b]	95	—	97

[a] 1,2,3,4,5,6-Hexahydro-*cis*-6,11-dimethyl-3-cyclopentyl-2,6-methano-3-benzazocin-8-ol. [b] Values represent the mean recovery at 10 concentrations between 0.1 and 4.0 mcg./ml. of original sample. [c] Values represent the mean recovery at five concentrations between 0.1 and 4.0 mcg./ml. of original sample.

the line of the standard curve generally falls within ±10–15% of the value of the slope. If an unknown exceeds the range of the standard curve, it may be diluted and reinjected. A series of about 100 control plasma samples from 11 human volunteers was analyzed, giving an average blank value equivalent to 2.7 ± 2.5 (*SD*) ng. pentazocine/ml. While recovery of extracted standards relative to direct standards approaches 100%, extracted standards from plasma obtained before drug administration from subjects involved in experiments have been employed routinely. Duplicate series of 13 replicate plasma samples with 25 and 100 ng. pentazocine added/ml. were analyzed on consecutive days. At 100 ng./ml., the recoveries were 95 and 92 ng./ml. with percent standard deviations of 13 and 11, respectively. At 25 ng./ml., recoveries were 25 and 18 ng./ml. with percent standard deviations of 14 and 44, respectively.

Determination of Urinary Pentazocine and Its Metabolites—A chromatogram of the trimethylsilyl derivatives of the internal standard and of pentazocine and its metabolites run under the conditions described in the *Experimental* section shows the compounds to be well separated and their peaks to be symmetrical. The following basic drugs were checked and found not to interfere with peaks due to the internal standard, pentazocine, or its metabolites: amphetamine, atropine, caffeine, cocaine, codeine, meperidine, methadone, methamphetamine, morphine, nalorphine, nicotine, and normeperidine. Quinine interferes with the *trans*-alcohol metabolite. Acidic and neutral drugs would not appear in the final extract. The response of the gas chromatograph to all of the pertinent compounds was linear over a wide range of concentrations, extending above and below the concentrations that were expected to be measured in urine (Fig. 1). Each point on the graph is the result of the measurement of a single injection. Since no crystalline *cis*-alcohol metabolite was available, an amount sufficient to obtain the results shown in Fig. 1 was isolated from a human urine sample by extraction and TLC; the approximate amount present was determined by reference to known amounts of the *trans*-alcohol metabolite.

Whenever a column became unsuitable for use, the first compound to be affected was the *trans*-acid metabolite. Pronounced tailing of this compound was noted, and the linearity of response disappeared at lower concentrations. The primary reason for the deterioration of the quality of the chromatograms seemed to be the accumulation of salts and charred organic material in the glass-wool plug at the head of the columns. The source of the contamination probably was the prepared samples of the *trans*-acid metabolite, which remain very impure preparations. Often, chromatograms could be improved or restored to acceptable appearance simply by changing the glass-wool plug.

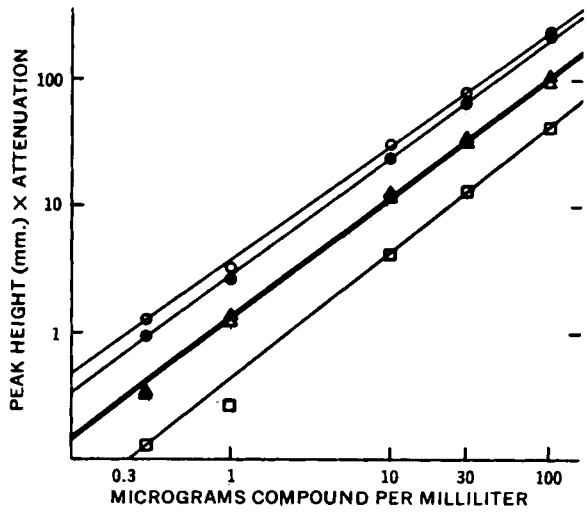

Figure 1—*Response of the flame-ionization detector to various amounts of trimethylsilyl derivatives of pentazocine and related compounds. A single measurement of pentazocine* (○), *the internal standard* (●), *the* cis*-alcohol metabolite* (△), *the* trans*-alcohol metabolite* (▲), *and the* trans*-acid metabolite* (□) *was made at each concentration. (A Varian model 2100 gas chromatograph was used.)*

Preliminary experiments using urine from human subjects given pentazocine were carried out to establish satisfactory conditions for the complete enzymatic hydrolysis of conjugates of pentazocine and its metabolites. Incubation periods less than 2 days or a β-glucuronidase concentration one-tenth that recommended resulted in less than maximum release of pentazocine or its metabolites from their conjugated forms. No degradation of unconjugated pentazocine or of its unconjugated metabolites occurs under the conditions used for enzymatic hydrolysis.

Typical standard curves for extracted standards of pentazocine and the *trans*-alcohol and *trans*-acid metabolites show low blank values and good linearity. The 95% confidence intervals of the slopes of the lines of the standard curves for pentazocine and the *trans*-alcohol and *trans*-acid metabolites generally fall within ±5–10% of the value of the slope. More variation in the *trans*-acid metabolite curve might be expected, since the internal standard could not be carried through the entire operation with this compound.

The values presented in Table III were obtained in separate analyses of nine sets of samples containing 10 premedication samples of human urine each. They indicate the magnitudes and variabilities of the experimental blanks that would be expected to be included in the values obtained for pentazocine and its metabolites if a sample of human urine were analyzed as described earlier. Much of the variability in the overall means, as expressed by their percent standard deviations, is due to day-to-day variation in the slope–*y* intercept of the standard curves. As indicated by the mean percent standard deviations (Table III) calculated from the standard deviations of each set of samples, the variability within samples analyzed on a given day is less. Therefore, the reliability of a deter-

Table III—Analysis for Apparent Pentazocine and Metabolites in Urine Taken from Human Subjects Prior to Medication

Compound	Microgram Equivalents[b] per Milliliter: Urine: Mean	Urine: % *SD*[c]	Urine: % $\overline{SD}$[d]	Hydrolyzed Urine: Mean	Hydrolyzed Urine: % *SD*[c]	Hydrolyzed Urine: % $\overline{SD}$[d]
Pentazocine	0.048	133	56	0.026	234	101
cis-Alcohol metabolite	0.058	236	64	0.018	728	124
trans-Alcohol metabolite	0.012	842	156	−0.033	215	90
trans-Acid metabolite	—	—	—	0.66	100	84

[a] A total of 90 samples of human urine was analyzed, by the methods presented in the text, in nine sets of 10 samples each. [b] Microgram equivalents = micrograms compound (mol. wt. pentazocine per mol. wt. compound). [c] The percent standard deviation (% *SD*) was calculated by comparing each sample to the overall mean. [d] The average percent standard deviation (% $\overline{SD}$) is the mean of the nine relative standard deviations, each calculated by comparing the samples in a set to the mean for that set.

Table IV—Accuracy and Precision of the Measurement of Pentazocine and Its Known Metabolites in Human Urine[a]

Treatment of Urine	Compound Added	Microgram Equivalents of Compound Added per Milliliter[b]	Microgram Equivalents of Compound Recovered per Milliliter[b] Day 1	% SD[c]	Day 2	% SD[c]	Pooled SE[d]
None	Pentazocine	1.00	0.91	5	1.04	6	0.03
	trans-Alcohol metabolite	1.00	0.92	9	0.96	7	0.02
Hydrolysis	Pentazocine	1.00	0.97	4	1.00	3	0.01
	trans-Alcohol metabolite	1.00	1.12	5	0.94	5	0.03
	trans-Acid metabolite	3.00	3.31	12	2.76	10	0.13

[a] Seventeen replicate samples, prepared by adding known amounts of the compounds to premedication human urine, were analyzed on each of 2 consecutive days, against extracted standards, by the methods described in the text. [b] Microgram equivalents = micrograms compound (mol. wt. pentazocine per mol. wt. compound). [c] The percent standard deviations were calculated by comparing each value obtained on a given day to the mean value for that day. [d] The pooled standard error includes the error due to the "day of analysis" effect, which was significant in every case ($p < 0.05$ to $p < 0.001$).

mination of urinary pentazocine and its metabolites would be increased if a sample of urine known to be free of those compounds would be analyzed at the same time and used as an experimental blank.

An estimate of the accuracy and precision of the measurement of pentazocine and its metabolites in urine and in hydrolyzed urine was obtained by adding known amounts of the compounds to human urine and subjecting replicate samples to analysis either directly or after hydrolysis. Replicate sets were analyzed in this manner on 2 consecutive days (Table IV).

DISCUSSION

The advantages of the internal standard technique in multistep procedures are obvious. The fact that the internal standard used is so close in its physicochemical properties to that of pentazocine and its hydroxylated metabolites is insurance against variations in temperature and salt concentrations changing significantly the relative amounts of these compounds recovered in the final step of the assay. Also, this internal standard can be expected to behave similarly to pentazocine in derivatization procedures. However, any such compound with the proper retention time and polarity should be satisfactory. For example, Beckett *et al.* (4) used α-3-hydroxy-6-dimethylamino-4,4-diphenylheptane (α-methadol) as an internal standard in the GC assay of pentazocine in blood and urine, and Ahmad and Medzihradsky (15) used cyclazocine as an internal standard.

It was not possible to find an internal standard for use in the analysis of the *trans*-acid metabolite of pentazocine more suitable than the one used for pentazocine analysis. Since this internal standard cannot be added at the beginning of the procedure for the analysis of the *trans*-acid metabolite, the power of the internal standard technique is correspondingly diminished.

The technique used here for the determination of pentazocine in plasma is more sensitive and/or specific than other methods developed to date. The fluorometric technique developed by Berkowitz *et al.* (2) is less specific, since a metabolite of pentazocine interferes (2), and has a practical limit of determination of about 30–50 ng./ml. of plasma (2)[11]. The procedures used by Borg and Mikaelsson (14) and by Ahmad and Medzihradsky (15) are more specific but not more sensitive than the fluorometric one. The procedure of Beckett *et al.* (4), although specific, is not likely to be as sensitive as one involving the electron-capture detection of a highly quenching derivative. They reported a recovery of pentazocine of 98 ± 5% from plasma containing 0.2 mcg. of drug/ml., but no information concerning the lower limit of detection was provided. In addition, the Beckett *et al.* procedure requires large volumes of blood, up to 15 ml., for the determination of small amounts of pentazocine. In the present study, the use of benzene as the extracting solvent and of a carbonate buffer system conforms to the general experience that benzene results in a clean extract and, at the pH of 10 or so obtained, a quantitative extraction. Borg and Mikaelsson (14) discussed the pKa's and extraction of pentazocine. Benzene is not a suitable extraction solvent for the hydroxylated, metabolites of pentazocine, because only about 10% can be extracted by use of the same procedure used for pentazocine, nor for the acid metabolite, because none is extracted. The plasma technique, as described, is useful only for pentazocine.

The fluorometric method, with some modification, has been applied to the analysis of urinary pentazocine (3). With a lower limit of detection of about 0.2 mcg. pentazocine/ml., it should possess adequate sensitivity. Hydrolyzed urine has also been used for pentazocine and pentazocine conjugates. The direct GC method as used for the analysis of blood was used by Beckett *et al.* (4) in the analysis of urinary pentazocine. An average recovery of pentazocine of 93 ± 3% from urine containing 1 mcg. of drug/ml. was reported, but no indication of the lower limit of detection was mentioned, nor was any attempt made to apply this method to other metabolites. This method also should be adequate for determining urinary pentazocine. The technique described herein for the analysis of urine is not more sensitive for pentazocine than are the other techniques already available. The use of benzene as the extracting solvent in the other GLC procedures probably results in a cleaner final extract. The advantage of the procenures reported here is that they allow the determinationof pentazocine, its major metabolites, and most of their conjugates by a unified approach. Based on the values in Tables III and IV, the practical lower limit for the quantitative determination of pentazocine and of the hydroxylated metabolites is about 0.1–0.2 mcg./ml. and that for the acid metabolite is about 1–2 mcg./ml.

[11] K. A. Pittman and C. Davison, unpublished data.

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Keyphrases □ Carbamates—mass spectral characterization of nine drugs □ Meprobamate—mass spectral characterization, fragmentation pathways □ Mebutamate—mass spectral characterization, fragmentation pathways □ Carisoprodol—mass spectral characterization, fragmentation pathways □ Emylcamate—mass spectral characterization, fragmentation pathways □ Bethanechol chloride—mass spectral characterization, fragmentation pathways □ Styramate—mass spectral characterization, fragmentation pathways □ Hydroxyphenamate—mass spectral characterization, fragmentation pathways □ Mephenesin carbamate—mass spectral characterization, fragmentation pathways □ Methocarbamol—mass spectral characterization, fragmentation pathways □ Mass spectroscopy—characterization, nine medicinal carbamates

There are several reports in the literature on the mass spectral behavior of carbamates (1–5), but all of these studies are concerned with *N*-substituted and *N*,*N*-disubstituted carbamates, including many of the carbamate pesticides. Unsubstituted carbamates are employed in medicine as minor tranquilizers and muscle relaxants and for other purposes. Their mass spectra have not been examined in detail.

The study now reported is a continuation of previous investigations (6, 7) on the characterization of medicinal compounds by means of their mass spectra. For this study, nine medicinal carbamates were available. The mass spectrum of each was recorded, and fragmentation pathways were proposed either as a result of deuterium labeling or accurate mass measurements. At the outset, each spectrum was inspected to see whether the chosen carbamates lost the carbamate grouping early in the fragmentation sequence and, if so, whether it was consistently lost as an identifiable molecule or radical such as HNCO or $NH_2COO^{\cdot}$. It soon became clear that the carbamate grouping was expelled at various stages during fragmentation and that it was lost in a variety of ways. However, one diagnostic peak was observed in five of the carbamates. An ion of mass 62 was formed from meprobamate, mebutamate, carisoprodol, mephenesin carbamate, and methocarbamol. An accurate mass measurement in each instance revealed that this ion was $NH_2COOH_2^+$. The elimination of NH_2COOH (or simultaneous expulsion of NH_3 and CO_2) at some stage during the fragmentation sequence was also common to the same five compounds and to emylcamate. The formation of the NH_2COOH molecule is considered to be the result of a hydrogen-transfer mechanism such as that illustrated in the fragmentation of emylcamate.

RESULTS[1] AND DISCUSSION

In the nine carbamates investigated (I–IX), the molecular ion generally either was present in very low abundance or was absent from each spectrum.

The spectrum of meprobamate (I) (Fig. 1) displayed six fragment ions at *m/e* 144 [146], 114 [115], 101 [102], 96 [96], 84 [84], and 83 [83], which could be useful for characterization purposes. Tetradeuterated meprobamate (I, NH_2, groups replaced with ND_2) gave a spectrum with corresponding deuterated fragment ions. This information, together with the appearance of appropriate metastable ions, permits a rationalization of fragmentation pathways as illustrated in Scheme I. (In all schemes, the mass of a deuterated ion is enclosed in a square bracket, and an asterisk indicates the presence of a supporting metastable ion.)

The chemical structures of mebutamate (II) and carisoprodol (III) are closely related to that of meprobamate. As expected, some of the fragment ions that appear in the spectra of II and III arise in ways similar to those just described for meprobamate. Thus, the spectrum of mebutamate (Fig. 1) possesses abundant ions of *m/e* 158 [160], 128 [129], 110 [110], 115 [116], 97 [97], and 69 [69], which arise in the same manner as the ions *a* and *c–g*, respectively, illustrated in Scheme I.

Other abundant ions in the spectrum of mebutamate were located at *m/e* 97, 72, 71, 62, and 55. A rational explanation of their formation is given in Scheme II. In addition, accurate mass measurements were made of all ions and, in this way, the elemental composition of each ion was confirmed as shown. The peak at *m/e* 71 was a doublet,

[1] Mass spectra were recorded by Dr. A. M. Hogg and his associates using an AEI MS-9 mass spectrometer at an ionizing potential of 70 ev. Samples were introduced using the direct probe method. The source temperature was between 75 and 155°, depending on the compound being examined. Accurate mass measurements were carried out by the peak matching method.

All of the compounds examined were gifts from pharmaceutical firms. All had literature melting points and gave IR spectra consistent with their structures. Each deuterated compound was prepared by boiling, under reflux for 4 hr., a solution of the compound in dioxane containing deuterium oxide and then repeated recrystallization from the same solvent until the O—H and N—H stretching bands in the IR spectra of the unlabeled carbamate were replaced by O—D and N—D stretching bands at longer wavelengths.

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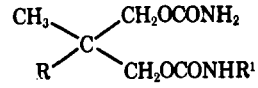
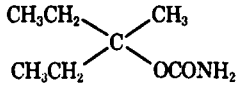
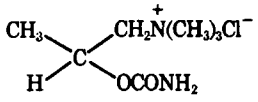


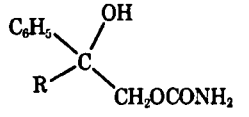
I: meprobamate, R = $CH_3(CH_2)_2$, R_1 = H
II: mebutamate, R = $CH_3CHCH_2CH_3$, R_1 = H
III: carisoprodol, R = $CH_3(CH_2)_2$, R_1 = $CH(CH_3)_2$



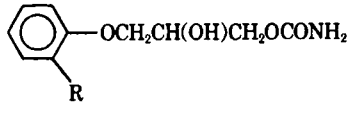
IV: emylcamate



V: bethanechol chloride



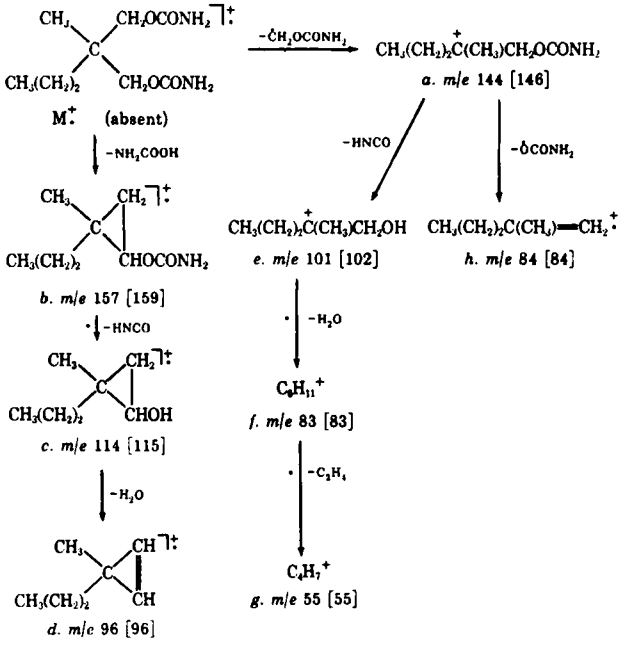
VI: styramate, R = H
VII: hydroxyphenamate, R = CH_2CH_3



VIII: mephenesin carbamate, R = CH_3
IX: methocarbamol, R = OCH_3

C_4H_7O (60%) and C_5H_{11} (40%). Mechanisms for the formation of the latter and for fragment *i* (Scheme II) would be speculative and are not suggested.

The mass spectrum of carisoprodol (III) (Fig. 1) is more complex than the spectra of Compounds I and II. The spectra of III and trideuterated carisoprodol contained fragment ions and appropriate metastable ions which enable the identification of fragments *a*, *c*, *d*, and *f–h* (Scheme I) but, with the exception of the ion *m/e* 55, all of these ions are of low abundance in the spectrum of carisoprodol



Scheme I—Fragmentation of meprobamate

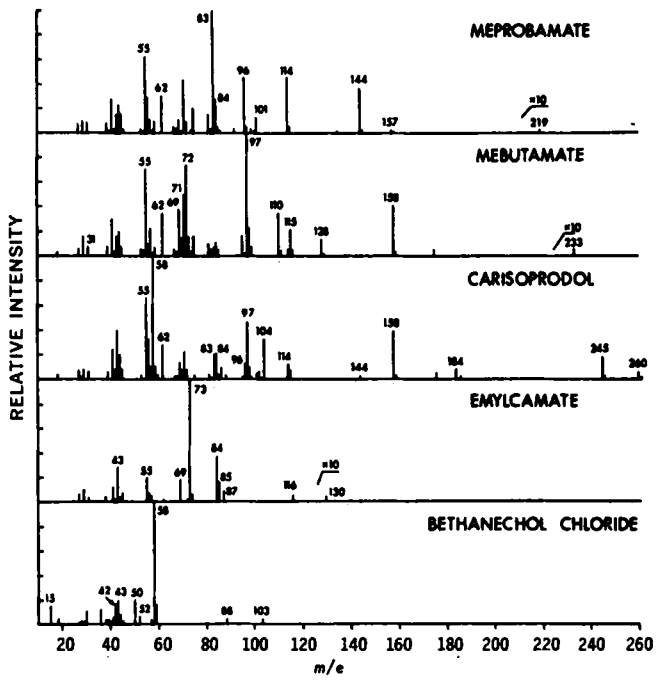
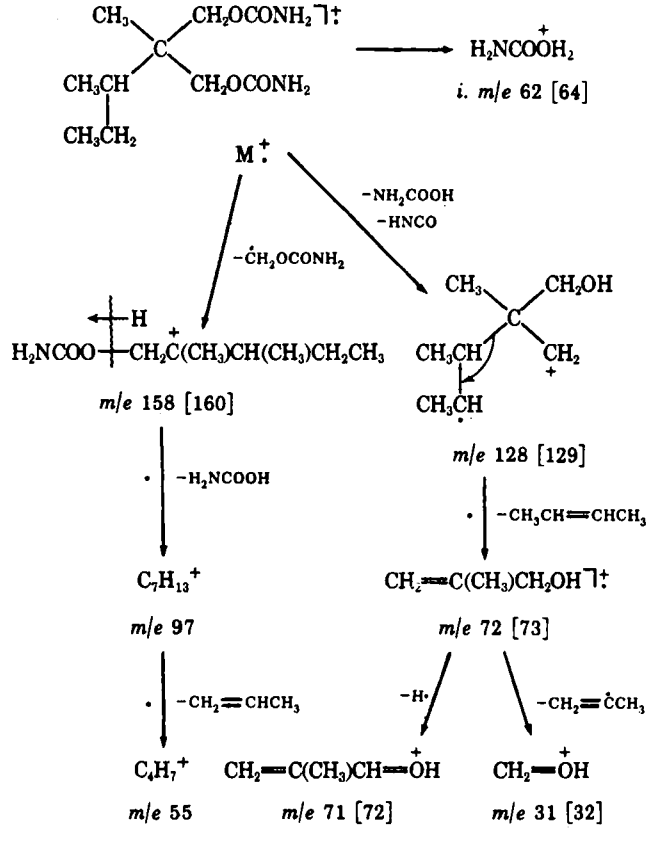


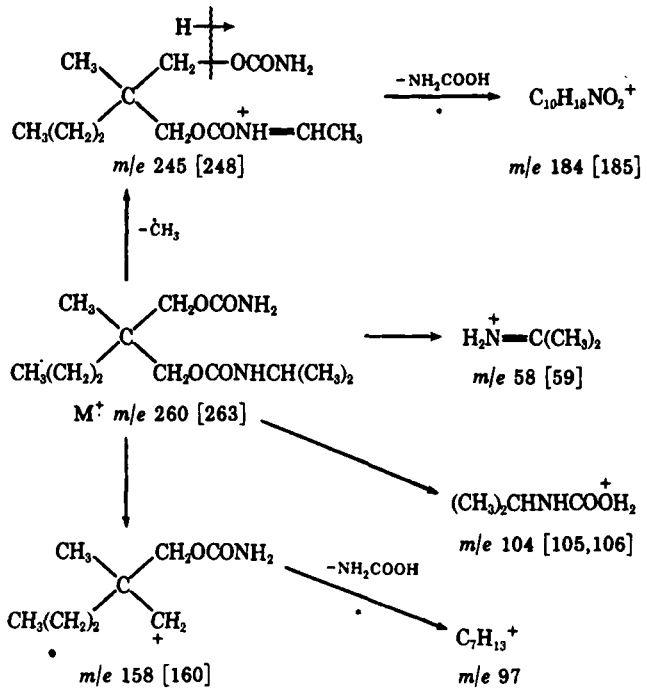
Figure 1—*Mass spectra.*

and of little value for characterization purposes. Fragment ions that are of more value in identification are the molecular ion *m/e* 260; the fragment ions *m/e* 245, 184, and 158, which appear as isolated peaks in the spectrum; and the abundant ions *m/e* 104, 97, and 58 (base peak). Mechanisms for their formation are suggested in Scheme III. Accurate mass measurements of the ions *m/e* 104 and 58 confirmed their elemental compositions.

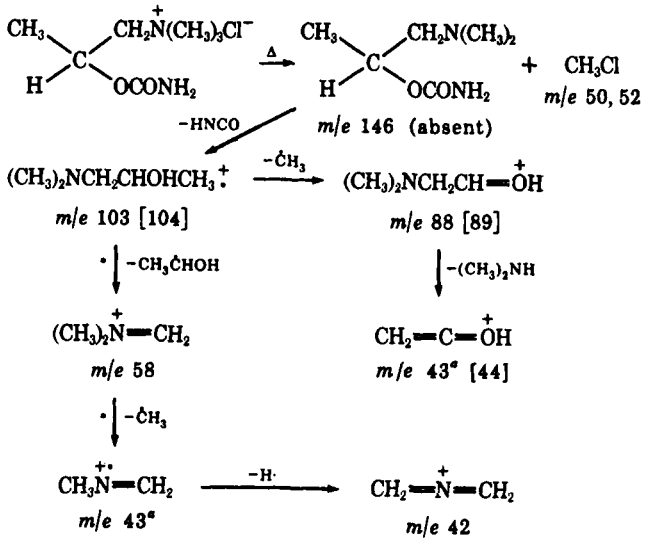
The simplest compound examined was emylcamate (IV), the mass spectrum (Fig. 1) of which lacked a molecular ion; an M−15



Scheme II—Some fragmentations of mebutamate



Scheme III—Some fragmentations of carisoprodol

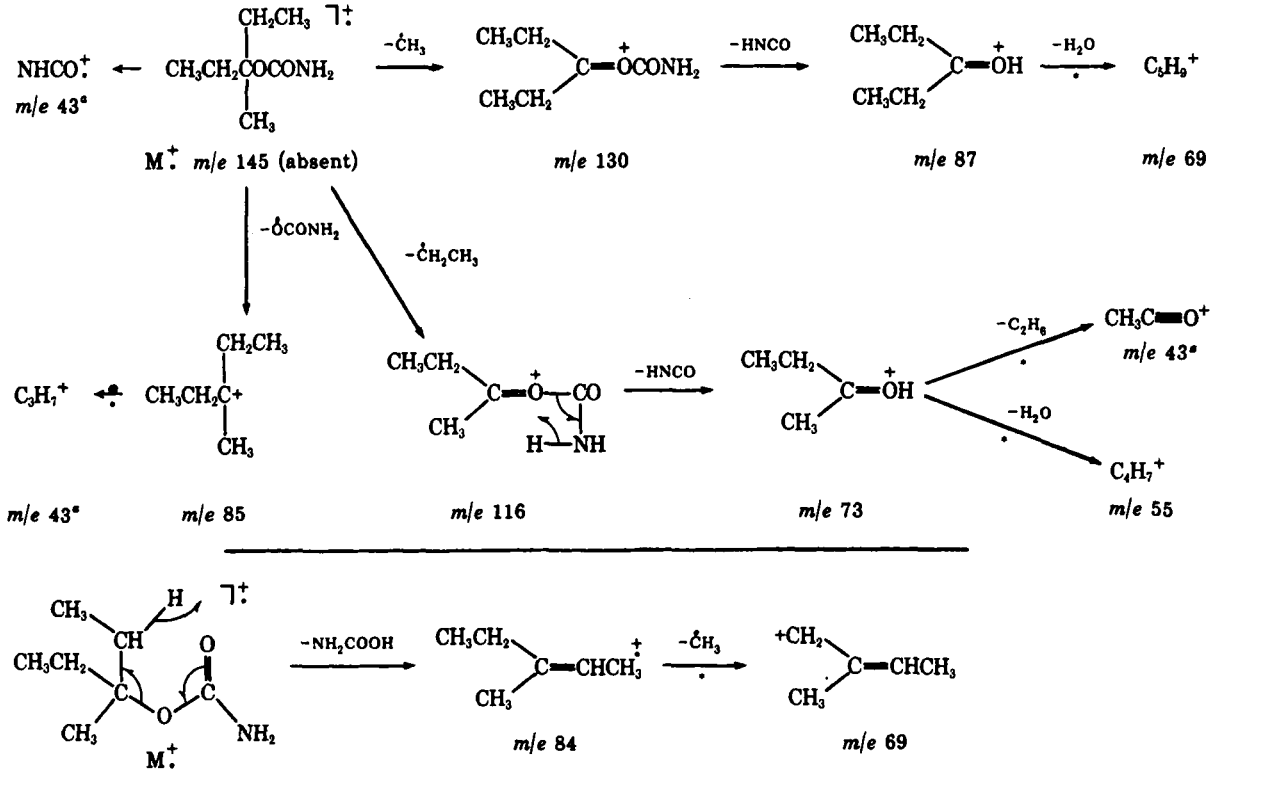


Scheme V—Fragmentation of bethanechol chloride (ᵃdoublet)

ion, due to the loss of a methyl radical from the molecular ion, was less than 1% abundant. A comparison of the spectrum of emylcamate with those of Compounds I–III and with that of *tert*-butyl alcohol (8) permitted the elucidation of the unexceptional fragmentation pathways depicted in Scheme IV. Accurate mass measurements of the ions *m/e* 87, 85, 84, 73, 69, 55, and 43 confirmed elemental compositions in agreement with Scheme IV. The peak, *m/e* 43, was a triplet: C_3H_7 (45%), C_2H_3O (34%), and HNCO (21%).

The quaternary salt, bethanechol chloride (V), also behaved predictably in the mass spectrometer (Fig. 1) by decomposing initially to methyl chloride (*m/e* 50 and 52) and the corresponding tertiary base (9). The molecular ion of the latter was not detected; the ions of greatest mass in the spectrum were located at *m/e* 103 and 88; and although both were of low intensity, their presence is of value in identifying this compound. Additional ions that could assist in the identification of this spectrum are the ion *m/e* 50 (CH_2-^{35}Cl), the base peak (*m/e* 58), and fragments derived from it. Accurate mass measurements of the ions *m/e* 88, 43, and 42 showed them to be $C_4H_{10}NO$, C_2H_3O (90%) and C_3H_8N (10%), and C_2H_4N, respectively. Proposed mechanisms for the formation of fragments in the spectrum of bethanechol chloride are presented in Scheme V.

The formation of the ion C_2H_3O by the expulsion of dimethylamine from the fragment of *m/e* 88 is not supported by a metastable but parallels a similar fragmentation observed (10) in the spectrum of glycerol—*viz.*, $CH_2OHCH{=}O^+H \rightarrow CH_2{=}C{=}O^+H$.



Scheme IV—Fragmentation of emylcamate (ᵃtriplet)

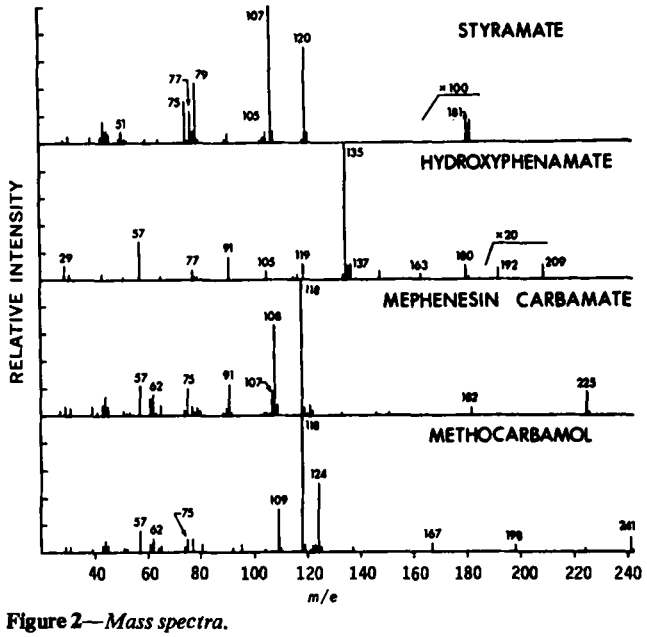
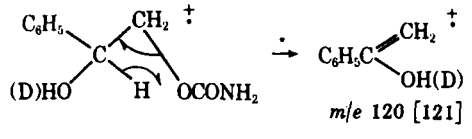


Figure 2—*Mass spectra.*

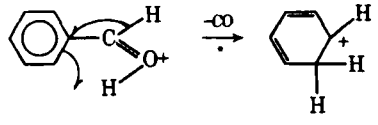
Despite the presence of a stabilizing phenyl substituent, the spectra of styramate (VI) (Fig. 2) and trideuterostyramate did not show molecular ions. The styramate molecule fragmented in the manner typical of a benzyl alcohol derivative (11) by expelling $\cdot CH_2OCONH_2$ and yielding successively ions of *m/e* 107 [108] (base peak, $C_6H_5CH{=}O^+H$), 105 ($C_6H_5C{\equiv}O^+$), 77 ($C_6H_5^+$), and 51 ($C_4H_3^+$). Appropriate metastable ions were present. Other ions of interest were located at *m/e* 120 [121], 79 [80], and 75 [78]. The former was the second most abundant ion in the spectrum. The presence of an appropriate metastable ion (*m/e* 79.6) supported the contention that this *m/e* 120 fragment was derived by expulsion of NH_2COOH from the molecular ion. The location of this fragment at *m/e* 121 in the spectrum of deuterated styramate indicates that a hydrogen atom other than that of the alcohol group is involved in the formation of the NH_2COOH molecule. One appropriate explanation would be the involvement of the H atom of the α-carbon (Scheme VI).

The ions of *m/e* 79 and 75 are readily explained as shown in Schemes VII and VIII.

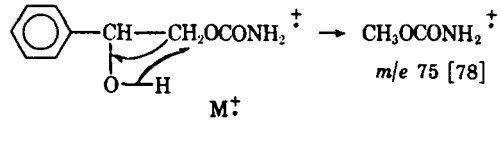
As was observed with styramate, the major fragmentation observed in the spectrum of hydroxyphenamate (VII) (Fig. 2) was the loss of a $\cdot CH_2OCONH_2$ radical and the formation of the base peak at *m/e* 135 [136]. The presence of the ethyl substituent on the α-carbon, however, influenced subsequent fragmentation (Scheme IX), which differed from that observed in the spectrum of styramate.



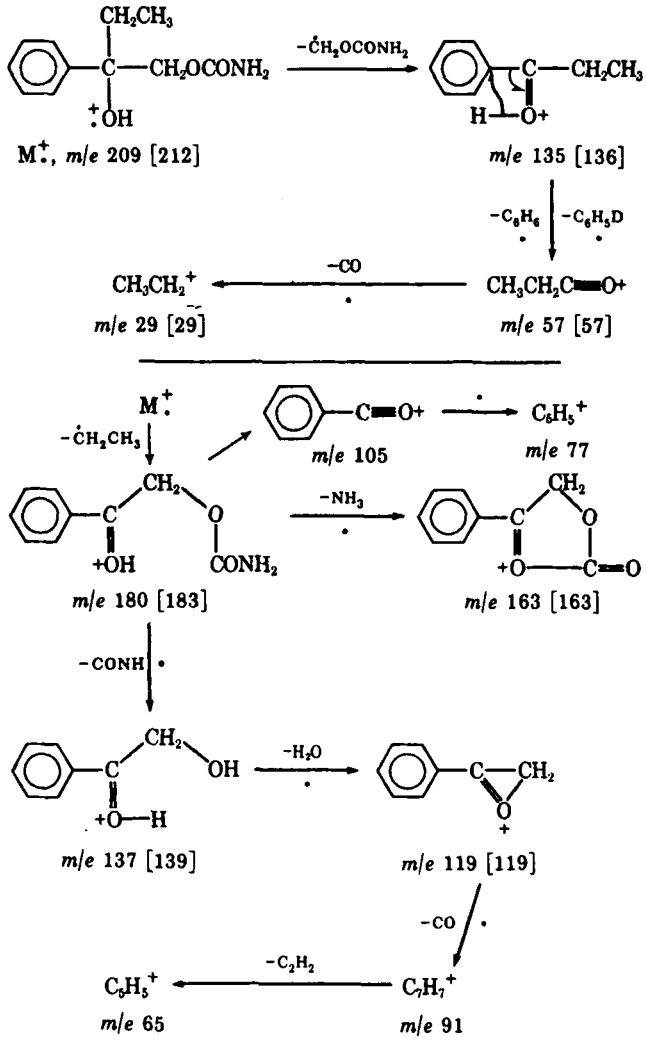
Scheme VI



Scheme VII



Scheme VIII



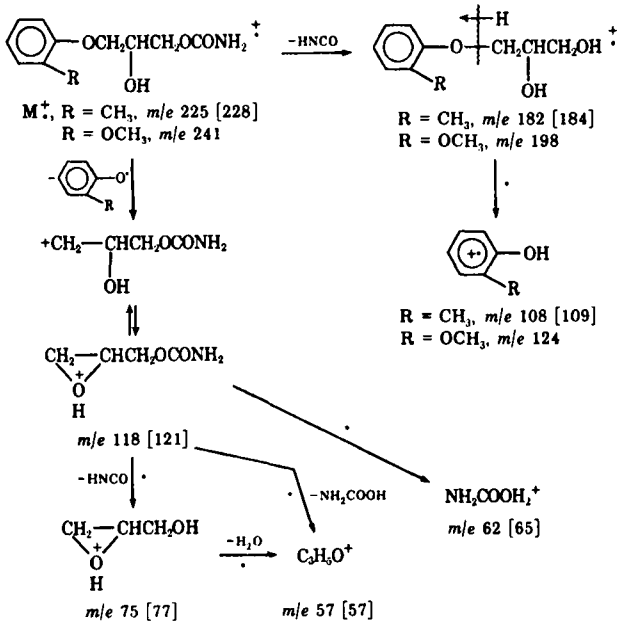
Scheme IX—Fragmentation of hydroxyphenamate

The *m/e* 135 and 57 ions are the most characteristic peaks in the spectrum.

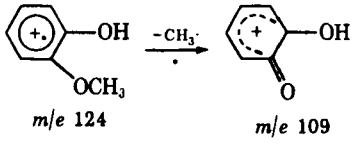
Relatively minor fragmentations which occur with hydroxyphenamate (Scheme IX) are also of some interest and arise as a result of an initial expulsion of an ethyl radical from the molecular ion. An ion of *m/e* 119 is detected in the spectrum of hydroxyphenamate which is absent from the spectrum of styramate. The ion of *m/e* 163 in the spectrum of hydroxyphenamate is of very low abundance (3%) but it is interesting because that same ion is also located at *m/e* 163 in the deuterated compound. Its formation from *m/e* 180 [183], supported by an appropriate metastable ion, must involve the expulsion of the three hydrogen atoms of the NH_2 and OH groups. The proposed mechanism of this unexpected expulsion of an ammonia molecule is illustrated in Scheme IX. An accurate measurement of the ion *m/e* 163 confirmed its elemental analysis as $C_9H_7O_2$.

Mephenesin carbamate and methocarbamol are closely related structures and fragmentation pathways are common to both molecules (spectra given in Fig. 2). Most major ions of value in identification are shown in Scheme X (fragments present in the spectrum of trideuterated mephenesin carbamate are enclosed in parentheses). Additional characteristic ions are located at *m/e* 107 [108] and 91 in the spectrum of mephenesin carbamate; both ions are absent from the spectrum of methocarbamol. The former ion is identified as the hydroxytropylium ion, formed by the expulsion of a hydrogen atom from the *m/e* 108 ion (12). The tropylium ion, *m/e* 91, is presumably formed by expulsion of the ether side chain from either the molecular ion or the fragment *m/e* 182.

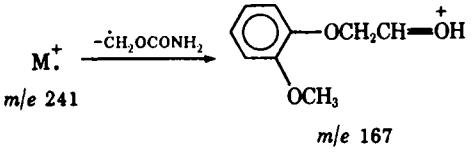
A metastable ion, *m/e* 95.8, supports the postulate that the fragment *m/e* 109 present in the spectrum of methocarbamol (but absent from the spectrum of mephenesin carbamate) is formed as a result



Scheme X—Fragmentations of mephenesin carbamate ($R = CH_3$) and methocarbamol ($R = OCH_3$)



Scheme XI



Scheme XII

of the expulsion of a methyl radical from the ion *m/e* 124 (Scheme XI).

The spectrum of methocarbamol (Fig. 2) also contains a weak, isolated fragment of *m/e* 167. An accurate mass measurement revealed an elemental composition of $C_9H_{11}O_3$, the formation of which is readily explained (Scheme XII).

The present study has shown that medicinal carbamates are readily characterized by means of their mass spectra.

REFERENCES

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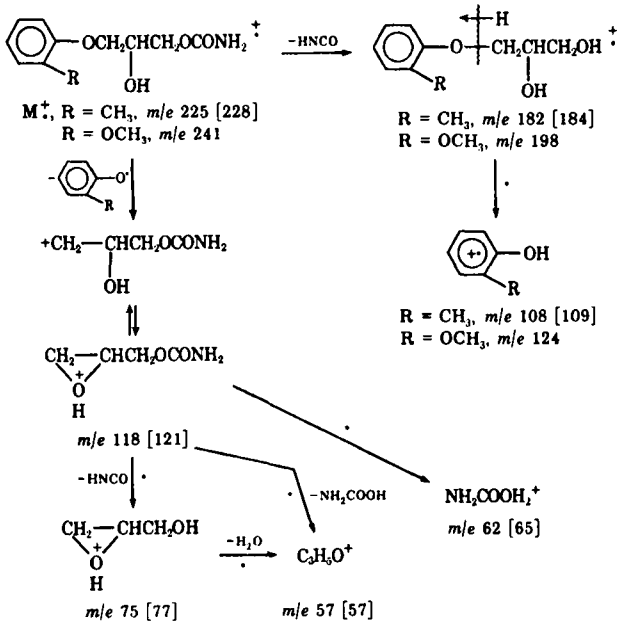
S. CASADIO▲, A. DONETTI, and G. COPPI

Abstract □ The preparation and the antifungal activity *in vitro* of nine *N*-trisubstituted methylimidazoles related to clotrimazole and of two congeneric compounds, having the 2-pyrrylmethylidenimino and the 2-pyrimidinylamino groups in place of the imidazole nucleus, are presented. Significant activity against six different organisms was found for three compounds. The *in vivo* activity of one selected compound was determined against *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*. Structure–activity relationships of the tested compounds are also discussed.

Keyphrases □ *N*-Trisubstituted methylimidazoles—synthesis, screened for antifungal activity □ Antifungal agents, potential—*N*-trisubstituted methylimidazoles synthesized and screened □ structure–activity relationships—*N*-trisubstituted methylimidazoles, antifungal activity

Clotrimazole [1-(*o*-chloro-α,α-diphenylbenzyl)imidazole] (1) is a new type of antifungal, which has been reported (2) to possess broad spectrum activity including that of amphotericin B and griseofulvin. The consistent results obtained in patients with severe systemic mycoses and the advantages that this drug



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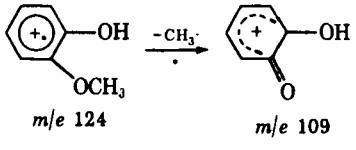

Scheme XI

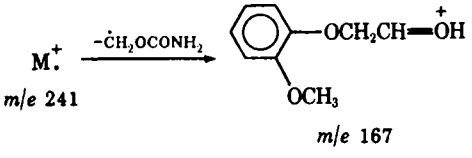

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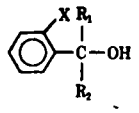
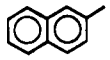
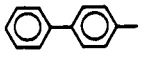
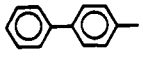
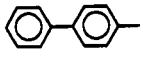
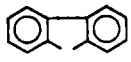
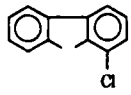
Table I—Trisubstituted Carbinols (I)



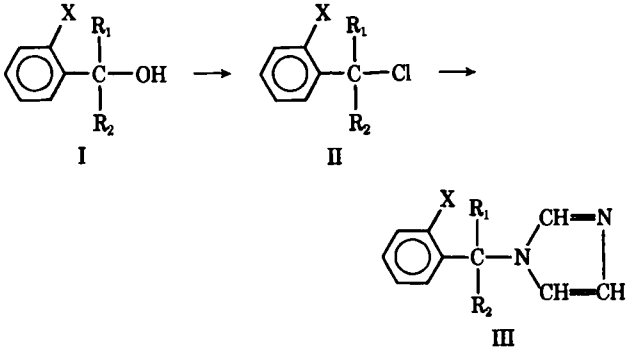
Number	R_1	R_2	X	Method	Crystallization Solvent[a]	Melting Point	Yield, %[b]	Molecular Formula	Analysis, % Calc.	Found
1	H		Cl	A	C–P	87–88°	65	$C_{17}H_{13}ClO$	C 75.98 H 4.87 Cl 13.19	75.65 4.62 13.52
2			Cl	B	P	152–154°	55	$C_{23}H_{17}ClO$	C 80.11 H 4.97 Cl 10.28	79.73 4.82 10.12
3			Cl	A[c]	—	—[d]	41	$C_{23}H_{17}ClO$	C 80.11 H 4.97 Cl 10.28	80.22 4.58 10.45
4			Cl	B	P	147–148°	82	$C_{25}H_{19}ClO$	C 80.96 H 5.16 Cl 9.56	80.77 5.25 9.66
5			Cl	A[c]	C–P	143–144°	62	$C_{31}H_{23}ClO$	C 83.30 H 5.19 Cl 7.93	82.51 5.16 7.97
6			Cl	B	E–H_2O	125–126°	38	$C_{18}H_{14}ClNO$	C 73.09 H 4.77 Cl 11.98 N 4.73	73.15 4.90 11.71 4.76
7			H	A	Et–P	114–115°[e]	62	$C_{16}H_{13}NOS$	C 71.88 H 4.90 N 5.24 S 11.99	71.71 4.85 5.36 11.78
8			Cl	A	E	137–138°[f]	50	$C_{19}H_{13}ClO$	C 77.95 H 4.47 Cl 12.11	77.72 4.68 12.38
9			H	B	C	131–132°	86	$C_{19}H_{13}ClO$	C 77.95 H 4.47 Cl 12.11	78.15 4.35 12.31

[a] B = benzene, C = cyclohexane, E = ethanol, Et = ether, and P = petroleum ether. [b] Crystallized product. [c] Chromatographed on a silica gel column using benzene–carbon tetrachloride (2:8) as the eluent. [d] Glassy solid. [e] R. P. Kurkjy and E. V. Brown, *J. Amer. Chem. Soc.*, 74, 6260(1952), reported m.p. 116–117°. [f] N. Campbell and A. Marks, *J. Chem. Soc.*, 1951, 2941, reported m.p. 140–141°.

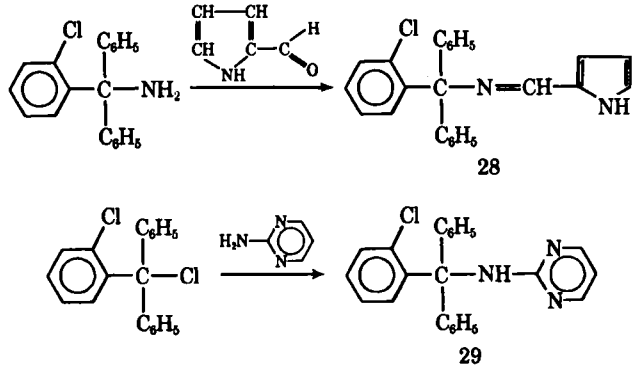
appears to possess over the classical antifungal agents (3) encouraged the synthesis and the examination of some congeners.

The synthesis of the title compounds (Scheme I) was achieved starting from the corresponding carbinols (I, Table I), obtained by reaction of the required lithium or Grignard reagent with the appropriate carbonyl compound. Treatment of the carbinols with thionyl chloride afforded the corresponding chlorides (II, Table II) which were, in turn, converted into *N*-substituted imidazoles (III, Table III) by reaction with imidazole.

Compounds 28 and 29 (Table IV), in which the imidazole moiety of clotrimazole was replaced by the 2-pyrrylmethylidenimino and 2-pyrimidinylamino groups, were prepared by condensing 2-pyrrolaldehyde and 2-aminopyrimidine with bisphenyl-*o*-chlorophenylcarbinylamine and bisphenyl-*o*-chlorophenylcarbinyl chloride, respectively (Scheme II).

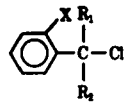


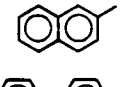
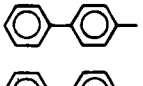
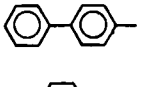
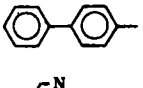
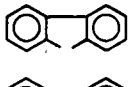
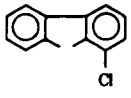
Scheme I



Scheme II

Table II—Trisubstituted Methyl Chlorides (II)



Number	R_1	R_2	X	Method	Crystallization Solvent[a]	Melting Point	Yield, %	Molecular Formula	Analysis, % Calc.	Found
10	H		Cl	A	P	117–118°	95	$C_{17}H_{13}Cl_3$	Cl 24.69	24.35
11			Cl	B	—	—[b]		$C_{23}H_{16}Cl_2$	Cl 19.52	19.73
12			Cl	A	—	—[c]	90	$C_{23}H_{16}Cl_2$	Cl 19.52	19.03
13			Cl	A	Et	168–169°	84	$C_{25}H_{18}Cl_2$	Cl 18.21	18.53
14			Cl	A	P	160–161°	88	$C_{31}H_{22}Cl_2$	Cl 15.23	15.51
15			Cl	B	P	137–138°	94	$C_{15}H_{12}Cl_2N$	Cl 22.56 N 4.46	22.31 4.35
16			H	A	—	—[d]	93	$C_{16}H_{12}ClNS$	Cl 12.40 N 4.90	12.52 4.81
17			Cl	A	P	142–143°	90	$C_{19}H_{12}Cl_2$	Cl 11.22	10.95
18			H	A	P	109–110°	89	$C_{19}H_{12}Cl_2$	Cl 22.78	22.51

[a] See *Footnote a* of Table I. [b] Glassy product. [c] Waxy product. [d] Thick oil.

The antifungal activity of the compounds was studied *in vitro* against six different organisms (Table V). After this preliminary screening, the *in vivo* activity of one selected compound was determined against *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* in mice infections (Table VI).

EXPERIMENTAL[1]

The intermediate trisubstituted carbinols (I) were synthesized by the action of the required Grignard or lithium reagent on the appropriate carbonyl compound. The characteristics of these carbinols are listed in Table I, and their preparation is illustrated by the following methods.

Bis(4-biphenylyl)-*o*-chlorophenylcarbinol (Compound 5, Table I)—*Method A*—A solution of 13.36 g. (0.04 mole) of 4,4′-diphenylbenzophenone (4) in 150 ml. of dry benzene was added dropwise to a Grignard reagent prepared from 23 g. of *o*-chlorobromobenzene (0.12 mole) and 2.52 g. of magnesium turnings (0.104 mole) in 75 ml. of dry ether. The mixture was stirred for 1 hr. at room temperature and then refluxed for an additional 1 hr. The reaction mixture was decomposed with 10% sulfuric acid, and the organic layer was washed with water, saturated sodium carbonate solution, and water. Removal of the solvent left a thick oil, which was steam distilled until no appreciable amount of water-insoluble material was present in the distillate (2 hr.). The undistilled carbinol was extracted with ether, and the extract was dried over anhydrous magnesium sulfate. After evaporation of the solvent, the residue was chromatographed on silica gel, using benzene–carbon tetrachloride (2:8) as the eluent, and then crystallized from cyclohexane–petroleum ether to give 11.07 g. of Compound 5 as colorless crystals.

The following carbinols (I) were prepared in an analogous manner: Compound 1 (from *o*-chlorobenzaldehyde and 1-naphthylmagnesium bromide), Compound 3 [from 2-benzoylnaphthalene (5) and *o*-chlorophenylmagnesium bromide], Compound 7 [from benzophenone and 2-thiazolylmagnesium bromide (6)], and Compound 8 (from fluorenone and *o*-chlorophenylmagnesium bromide).

9-Phenyl-1-chlorofluoren-9-ol (Compound 9, Table I)—*Method B*—A solution of 7.34% phenyllithium in 280 ml. of ether was added to a stirred solution of 35 g. 1-chlorofluorenone (0.163 mole) (7) in 600 ml. of dry ether. After 19 hr. of stirring at room temperature, the mixture was decomposed with water and extracted with ether. The ethereal layer was washed with water until neutral, dried over anhydrous magnesium sulfate, and evaporated to dryness to give 45.6 g. of a light-brown waxy product which, on crystallization, furnished 41.04 g. of Compound 9 as colorless crystals.

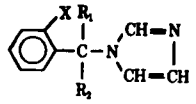
Compound 2 [from *o*-chlorobenzophenone (8) and 1-naphthyllithium], Compound 4 [from 4-(2-chlorobenzoyl)-biphenyl (9) and phenyllithium], and Compound 6 (from *o*-chlorobenzophenone and 2-pyridyllithium) were prepared by a similar procedure.

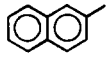
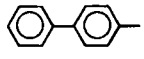
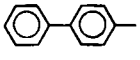
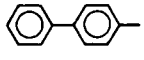
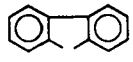
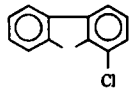
Trisubstituted Methyl Chlorides (II, Table II)—*Method A*—A solution of 0.1 mole of the appropriate carbinol in 150 ml. of thionyl chloride was refluxed for 4 hr. The excess thionyl chloride was evaporated under reduced pressure, and the residue was used as crude product or crystallized.

Method B—A solution of 0.04 mole of thionyl chloride in 10 ml. of dry benzene was added dropwise to a stirred solution of 0.02 mole of the carbinol and 0.03 mole of pyridine in 80 ml. of benzene. The mixture was refluxed for 5 hr., cooled, and then poured into excess

[1] All melting points were determined on a Büchi capillary melting-point apparatus and are uncorrected. The IR spectra were determined on a Perkin-Elmer 337 spectrophotometer; the NMR spectra were taken using a Varian A 60-A instrument and tetramethylsilane as the internal standard. IR and NMR spectra agreed in each case with the structural assignments.

Table III—*N*-Trisubstituted Methylimidazoles (III)



Number	R_1	R_2	X	Crystallization Solvent[a]	Melting Point	Yield, %[b]	Time, hr.	Molecular Formula	Analysis, % Calc.	Found
19	H		Cl	P	145–146°	38	5	$C_{20}H_{15}ClN_2$	C 75.35 H 4.74 Cl 11.12 N 8.79	75.12 4.52 11.31 8.51
20			Cl	—[c]	120–121°	51	30	$C_{26}H_{19}ClN_2$	C 79.08 H 4.85 Cl 8.98 N 7.09	78.88 4.91 8.53 6.61
21			Cl	Et	197–199°	48	42	$C_{26}H_{19}ClN_2$	C 79.08 H 4.85 Cl 8.98 N 7.09	78.91 4.68 8.58 6.95
22			Cl	Et	169–170°	78	44	$C_{28}H_{21}ClN_2$	C 79.89 H 5.03 Cl 8.42 N 6.65	79.57 4.82 8.30 6.43
23			Cl	Et	206–207°	78	40	$C_{34}H_{25}ClN_2$	C 82.16 H 5.07 Cl 7.13 N 5.63	82.33 4.95 6.92 5.55
24			Cl	Et	150–151°	68	40	$C_{21}H_{16}ClN_3$	C 72.93 H 4.66 Cl 10.25 N 12.15	72.75 4.49 10.12 12.31
25			H	Et	212–214°	86	20	$C_{19}H_{15}N_3S$	C 71.90 H 4.76 N 13.24 S 10.10	72.10 4.90 13.50 9.75
26			Cl	P	160–161°	84	20	$C_{22}H_{15}ClN_2$	C 77.08 H 4.41 Cl 10.34 N 8.17	76.95 4.33 10.12 8.31
27			H	P	135–136°	62	40	$C_{22}H_{15}ClN_2$	C 77.08 H 4.41 Cl 10.34 N 8.17	76.85 4.36 9.98 8.25

[a] See *Footnote a* of Table I. [b] Crystallized product. [c] Purified by column chromatography on silica gel using benzene–ether (1 : 1) as the eluent.

water. The organic layer was washed with water, dried over anhydrous magnesium sulfate, and evaporated to dryness. The products were isolated as shown in Table II.

***N*-Trisubstituted Methylimidazoles (III, Table III)**—A solution of 0.1 mole of the appropriate chloride and 0.3 mole of imidazole was refluxed in 120 ml. of dry benzene for the time indicated in Table III. After cooling to room temperature, the mixture was poured into water and extracted with benzene. The organic layer was washed with water, dried over anhydrous magnesium sulfate, and evaporated to dryness; the residue was purified as shown in Table III.

Bisphenyl-*o*-chlorophenylcarbinylamine—A solution of 31.32 g. (0.1 mole) of bisphenyl-*o*-chlorophenylcarbinyl chloride (1) in 900 ml. of ether was added under stirring to 900 ml. of 32% ammonia solution. After 22 hr. of stirring at room temperature, the organic layer was separated, extracted with 10% hydrochloric acid, and washed with water. The acidic solution was combined with the aqueous washings and filtered with charcoal, and the filtrate was basified with 10% sodium hydroxide. The basic material was extracted with ether, and the extract was washed with water, dried over anhydrous magnesium sulfate, and evaporated to dryness to give 24 g. of a white solid (81.7% yield, m.p. 95–97°).

***N*-Bisphenyl-*o*-chlorophenyl-2-pyrrylmethylideniminomethane (Compound 28, Table IV)**—A solution of 8.81 g. (0.03 mole) of bisphenyl-*o*-chlorophenylcarbinylamine and 7.13 g. (0.075 mole) of 2-pyrrolaldehyde (10) in 50 ml. of dry benzene was refluxed in the presence of catalytic amounts of *p*-toluenesulfonic acid, while the water azeotrope was removed with a takeoff adapter. After all of the water had been removed (23 hr.), the solution was allowed to cool to room temperature; the product, which crystallized on standing, was collected by filtration.

Bisphenyl-*o*-chlorophenyl-2-pyrimidinylaminomethane (Compound 29, Table IV)—A solution of 6.26 g. (0.02 mole) of bisphenyl-*o*-chlorophenylmethyl chloride and 7.61 g. (0.08 mole) of 2-aminopyrimidine in dry benzene was refluxed for 43 hr. The solution was then cooled, diluted with benzene, washed with water, and dried over anhydrous magnesium sulfate. Removal of the solvent left a yellow, thick oil which was purified by crystallization.

Microbiological Experiments (11)—In Vitro—The test organisms used in this experiment were *C. albicans* (DM), *Crypt. neoformans* (ATCC 5006), *Nocardia asteroides* (C.B.S.), *Trichophyton mentagrophytes* (I.D.A.), *A. niger* (I.S.M.), and *Penicillium chrysogenum* (I.D.A.). The organisms were incubated for 48 hr. at 37° (*C. albicans*, *Crypt. neoformans*, and *N. asteroides*) and for 96 hr. at 25° (*T. mentagrophytes*, *A. niger*, and *P. chrysogenum*) in Sabouraud broth (Difco). The cultures were then centrifuged and washed twice with sterile 0.9% saline and were finally resuspended in sterile saline. They were then diluted with sterile saline to give 90% light trans-

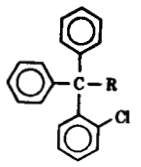
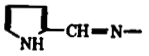
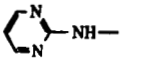


Table IV—Chlorotrityl Derivatives Bearing 2-Pyrrylmethylidenimino and 2-Pyrimidinylamino Groups (IV)

Number	R	Crystallization Solvent[a]	Melting Point	Yield, %[b]	Molecular Formula	Analysis, % Calc.	Found
28		B	152–153°	57	$C_{24}H_{19}ClN_2$	C 77.72 H 5.16 Cl 9.56 N 7.55	77.95 5.32 9.72 7.83
29		E	159–160°	61	$C_{23}H_{18}ClN_3$	C 74.29 H 4.88 Cl 9.53 N 11.30	74.15 4.95 9.62 11.15

[a] See *Footnote a* of Table I. [b] Crystallized product.

Table V—*In Vitro* Antifungal Activity of *N*-Trisubstituted Methylimidazoles[a]

Test Organism	Compound 19	20	21	22	23	24	25	26	27	28	29	Clotrimazole	Nystatin
C. albicans	>100	100	>100	>100	>100	50	>100	50	3.12	>100	>100	3.12	0.40
Crypt. neoformans	>100	>100	>100	>100	>100	0.80	>100	0.10	6.25	>100	>100	0.40	0.40
N. asteroides	>100	0.40	6.25	6.25	>100	3.12	100	0.10	3.12	50	12.5	1.56	50
T. mentagrophytes	0.20	0.40	>100	>100	>100	1.56	0.80	0.10	0.40	>100	>100	0.10	0.20
A. niger	>100	>100	>100	>100	>100	6.25	>100	0.80	3.12	>100	>100	1.56	3.12
P. chrysogenum	3.12	>100	>100	>100	>100	0.40	>100	0.10	1.56	>100	>100	0.80	1.56

[a] Minimum inhibitory concentrations in micrograms per milliliter.

Table VI—*In Vivo* Antifungal Activity

Compound Tested	*C. albicans* Number Animals Dead/Number Treated	Efficacy, %	*A. fumigatus* Number Animals Dead/Number Treated	Efficacy, %	*Crypt. neoformans* Number Animals Dead/Number Treated	Efficacy, %
27	26/48	45.84	23/29	20.69	27/27	0.00
Clotrimazole	23/49	53.07	20/30	33.34	27/27	0.00
Nystatin	30/65	53.85	15/29	48.28	23/65	64.62

mission at 625-nm. wavelength on the colorimeter[2].

In all cases, exactly 0.05 ml. of this standardized culture was used for the inoculum. Compounds to be tested were suspended with 0.1% of polysorbate 80[3] in sterile distilled water and serially diluted in Sabouraud broth. The inoculated tubes were incubated as already described and were finally observed for growth. Nystatin and clotrimazole were used as reference standards.

In Vivo—The test organisms in this study were *C. albicans* (DM), *Crypt. neoformans* (ATCC 5006), and *A. fumigatus* (I.I.). The organisms were incubated for 24 hr. at 37° (*C. albicans* and *Crypt. neoformans*) in Gremy broth and for 96 hr. at 24° (*A. fumigatus*) in Sabouraud broth. The cultures were then centrifuged and washed twice with sterile 0.9% saline and were finally resuspended in sterile saline to give an appropriate light transmission at 625 nm. (*C. albicans*, $T = 15\%$; *Crypt. neoformans*, $T = 20\%$; and *A. fumigatus*, $T = 2\%$). The inoculum used for each mouse was 0.15 ml. of these suspensions intravenously. All infected and untreated animals were dead within 9 days. Compound 27 and clotrimazole were administered orally and nystatin was administered subcutaneously suspended in 5% acacia gum (10 ml./kg.) at the dose of 100 mg./kg. The administrations were performed on the 1st day 2 hr. before and 3 and 6 hr. after infections and on the 2nd, 3rd, and 4th days at 9 a.m. and 4 p.m. All mice were observed for a period of 9 days.

[2] Spectronic 20, Bausch & Lomb, Rochester, N. Y.
[3] Tween 80.

RESULTS AND DISCUSSION

Compounds 19–29 were subjected to preliminary antifungal screening. The *in vitro* activity against six different organisms is reported in Table V. On the basis of this preliminary screening, it would appear that the ring size of the substituents plays some part in antifungal activity of compounds of the clotrimazole type. In fact, replacement of phenyl groups in the clotrimazole molecule by α- or β-naphthyl groups (Compounds 19, 20, and 21) or substitution in the *para* position by phenyl radicals (Compounds 22 and 23) led to compounds practically devoid of antifungal activity. No inhibition was also observed for the thiazole Compound 25 and when the imidazole was replaced by different groups (Compounds 28 and 29). The activity was retained when one of the two phenyl groups of clotrimazole was replaced by a 2-pyridyl radical (Compound 24) and in Compounds 26 and 27, where two phenyl groups are *ortho* connected to form a fluorene system. Actually, Compounds 24 and 26 inhibited *in vitro* the growth of the considered organisms, with the exception of *C. albicans*. In this respect, the activity of Compound 26 was even better than that of clotrimazole. On the other hand, Compound 27 was less active than Compound 26, but it showed inhibition against *C. albicans*. The activity of the latter compound was only slightly inferior to that of the reference standards.

During the completion of this study, the authors became acquainted with two patents (12, 13) reporting the preparation and antifungal activity of compounds having formulas corresponding to Compounds 24 and 26, so only Compound 27 was chosen for the study of the *in vivo* activity (Table VI). Compound 27 confirmed its

activity *in vivo* against *C. albicans* and *A. fumigatus*. Like clotrimazole, it was inactive against *Crypt. neoformans*.

The data obtained in this study indicate that Compound 27 is endowed with good inhibitory activity *in vitro* and *in vivo*, which is only slightly inferior to that of clotrimazole.

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(13) W. Draber, K. H. Büchel, and M. Plempel (to Farbenfabriken Bayer A.-G.), Ger. pat. 1,811,654 (1970); through *Chem. Abstr.*, **73**, 45510q(1970).

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Toxicological and Pharmacological Actions of Methacrylate Monomers I: Effects on Isolated, Perfused Rabbit Heart

G. N. MIR, W. H. LAWRENCE, and J. AUTIAN▲

Abstract □ A series of 12 methacrylate esters and methacrylic acid was investigated for activity upon the isolated, perfused rabbit heart. Each compound was dissolved in the perfusing fluid and tested at concentrations of 1:1000, 1:10,000, and 1:100,000 (v/v). All compounds reduced heart rate and force of contraction at one or more of these concentrations, and most, but not all, of the compounds reduced coronary flow rate.

Keyphrases □ Methacrylate monomers—toxicological and pharmacological actions, isolated, perfused rabbit heart □ Medical devices—toxicity and pharmacological actions of methacrylate esters from dental and surgical uses □ Methyl acrylic acid esters—toxicity and pharmacological actions, related to use in dentistry and surgery, cardiovascular effects, isolated, perfused rabbit heart □ Toxicity—methacrylate monomers, effects on isolated, perfused rabbit heart

Methacrylate esters are esters of methyl acrylic acid, $CH_2{=}CH(CH_3)COOH$, and the lower members of the series have a penetrating, disagreeable odor which may be recognized in tissues of treated animals (1). These esters may be polymerized by heat, oxygen, and oxygen-yielding compounds. The methacrylate polymers are widely used in dentistry and surgery as well as in many industrial applications.

Toxicity studies by Deichmann (1) and by Spealman *et al.* (2) indicated that lower members of the methacrylate esters were acutely lethal to laboratory animals due to respiratory depression by all routes of administration employed. Lawrence *et al.* (3) subjected 11 of the present 13 compounds to structure–toxicogenic analyses and found eight of these 11 compounds to be consistent with the hypothesis of a common mode of lethal activity.

Various investigators have reported a mild to marked irritant effect from application of some methacrylate monomers to skin, rabbit eye, wall of the stomach, respiratory membranes, and peripheral nerve tissue (1, 2, 4, 5). Strain (6) reported contact allergic reactions in sensitive patients to the residual monomer from incompletely polymerized methyl methacrylate dentures. Chronic toxicity feeding studies were conducted on rats and dogs by Borzelleca *et al.* (7) over a 2-year period. They did not find an increased mortality in the methacrylate-treated animals and, in fact, noted only a few instances of abnormal findings.

Acrylic polymers, primarily self-curing methyl methacrylate, have found some use as "bone cements" in orthopedics, especially for intramedullary placement of prosthetic devices. Hypotensive responses in patients shortly after clinical use of the cement have been reported by several workers (8–10). Cardiac arrests, some of which were fatal, have been reported in a few patients undergoing such treatment (9, 10) in which methyl methacrylate monomer was suggested as the primary or contributory cause of the cardiac arrest (10).

The present study was undertaken to investigate the pharmacological and toxicological effects of methacrylate monomers upon cardiac function, independent of their effect upon respiration and peripheral vasodilator activity.

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Table I—Response of Isolated Rabbit Heart to Methacrylate Perfusion (Mean ± *SE*)

	Cardiac Rate per Minute			Force of Contraction, g.			Coronary Flow, ml./min.		
	Control	Response[a]	Change	Control	Response[a]	Change	Control	Response[a]	Change
Methacrylic acid									
1:100,000	182.8 ± 3.9	170.2 ± 4.2[b]	−6.9%	2.36 ± 0.05	1.90 ± 0.12[b]	−19.4%	10.4 ± 1.0	10.0 ± 0.3	−3.8%
1:10,000	180.0 ± 3.5	90.4 ± 16.8[b]	−49.0%	2.28 ± 0.37	1.00 ± 0.30[b]	−56.1%	10.8 ± 0.9	10.3 ± 0.5	−5.0%
1:1000	181.6 ± 2.7	0.0[b]	−100.0%	2.34 ± 0.15	0.00[b]	−100.0%	9.8 ± 1.4	0.0[b]	−100.0%
Methyl methacrylate									
1:100,000	184.0 ± 2.8	163.6 ± 1.6[b]	−10.9%	2.44 ± 0.15	1.26 ± 0.15[b]	−48.3%	10.1 ± 0.5	7.0 ± 0.6[b]	−30.5%
1:10,000	181.6 ± 2.6	79.6 ± 10.4[b]	−56.2%	2.36 ± 0.11	0.62 ± 0.21[b]	−73.7%	10.3 ± 0.8	5.0 ± 0.7[b]	−51.4%
1:1000	183.2 ± 2.1	0.0[b]	−100.0%	2.34 ± 0.08	0.00[b]	−100.0%	10.1 ± 1.3	0.0[b]	−100.0%
Ethyl methacrylate									
1:100,000	184.6 ± 3.5	173.8 ± 7.5[c]	−5.8%	2.42 ± 0.04	1.94 ± 0.08[b]	−19.8%	10.4 ± 0.6	7.6 ± 0.5[b]	−26.1%
1:10,000	184.4 ± 5.3	151.6 ± 11.7[b]	−17.8%	2.38 ± 0.04	0.66 ± 0.11[b]	−72.2%	9.7 ± 0.4	4.1 ± 0.4[b]	−57.9%
1:1000	184.0 ± 2.8	0.0[b]	−100.0%	2.48 ± 0.13	0.0[b]	−100.0%	11.0 ± 1.1	0.0[b]	−100.0%
n-Propyl methacrylate									
1:100,000	184.0 ± 3.7	177.2 ± 3.6	−3.7%	2.36 ± 0.08	2.02 ± 0.04[b]	−14.4%	9.48 ± 1.0	9.40 ± 0.5	−0.84%
1:10,000	185.2 ± 3.0	164.8 ± 4.3[b]	−11.0%	2.38 ± 0.08	1.32 ± 0.10[b]	−44.5%	10.7 ± 0.8	10.3 ± 0.4	−3.5%
1:1000	186.4 ± 4.3	145.2 ± 6.9[b]	−22.1%	2.38 ± 0.04	0.74 ± 0.08[b]	−68.9%	11.7 ± 1.7	10.0 ± 1.0[b]	−14.1%
n-Butyl methacrylate									
1:100,000	184.0 ± 3.1	169.2 ± 2.2[b]	−8.0%	2.38 ± 0.17	2.00 ± 0.15[b]	−15.9%	10.3 ± 0.4	10.2 ± 0.4	−0.38%
1:10,000	184.8 ± 3.0	159.2 ± 1.0[b]	−13.8%	2.44 ± 0.15	1.14 ± 0.25[b]	−53.3%	10.3 ± 0.2	6.1 ± 0.2[b]	−41.2%
1:1000	183.2 ± 2.2	132.4 ± 4.5[b]	−27.7%	2.44 ± 0.05	0.76 ± 0.11[b]	−68.8%	10.3 ± 0.5	4.9 ± 0.1[b]	−51.7%
Isobutyl methacrylate									
1:100,000	183.2 ± 2.6	166.4 ± 2.6[b]	−9.1%	2.44 ± 0.11	2.16 ± 0.08[b]	−11.4%	10.1 ± 0.2	10.0 ± 0.1	−1.1%
1:10,000	183.6 ± 2.1	151.6 ± 2.6[b]	−17.4%	2.50 ± 0.15	1.86 ± 0.05[b]	−25.6%	10.8 ± 0.4	10.5 ± 0.3	−2.0%
1:1000	184.8 ± 1.7	128.4 ± 1.6[b]	−30.5%	2.38 ± 0.08	0.44 ± 0.05[b]	−81.5%	10.1 ± 0.1	9.1 ± 0.1[c]	−9.5%
1,3-Butylene dimethacrylate									
1:100,000	184.0 ± 3.1	181.2 ± 5.2	−1.5%	2.32 ± 0.13	2.08 ± 0.22[c]	−10.3%	10.2 ± 0.4	9.7 ± 0.4	−5.8%
1:10,000	184.0 ± 3.7	166.0 ± 6.7[c]	−9.7%	2.30 ± 0.07	1.74 ± 0.11[b]	−25.6%	10.2 ± 0.2	9.5 ± 0.4	−6.4%
1:1000	183.8 ± 3.0	131.2 ± 5.9[b]	−28.6%	2.40 ± 0.07	0.68 ± 0.04[b]	−71.6%	11.3 ± 0.1	10.2 ± 0.5[c]	−9.5%
2-Ethylhexyl methacrylate									
1:100,000	184.8 ± 1.7	180.0 ± 1.4	−2.5%	2.52 ± 0.33	2.36 ± 0.11[c]	−6.7%	10.0 ± 0.3	10.1 ± 0.4	+0.6%
1:10,000	184.0 ± 2.8	172.4 ± 3.8[c]	−6.3%	2.40 ± 0.07	1.96 ± 0.05[b]	−18.3%	9.9 ± 0.2	11.0 ± 0.8	+11.0%
1:1000	182.8 ± 1.7	153.2 ± 1.7[b]	−16.1%	2.40 ± 0.07	1.54 ± 0.05[b]	−35.8%	10.0 ± 0.2	12.9 ± 1.4[b]	+28.1%
Isodecyl methacrylate									
1:100,000	184.0 ± 2.4	179.6 ± 1.6	−2.4%	2.40 ± 0.12	2.34 ± 0.13	−2.5%	10.4 ± 0.4	11.6 ± 0.8[c]	+12.3%
1:10,00	184.0 ± 3.7	173.8 ± 3.0[c]	−5.5%	2.44 ± 0.05	2.16 ± 0.13[b]	−11.4%	10.3 ± 0.4	11.8 ± 0.7[c]	+14.7%
1:1000	182.6 ± 1.9	162.6 ± 1.9[b]	−10.9%	2.40 ± 0.07	1.78 ± 0.08[b]	−25.8%	10.2 ± 0.2	12.2 ± 1.7[c]	+18.9%
Lauryl methacrylate									
1:100,000	183.6 ± 3.5	183.0 ± 4.1	−0.21%	2.38 ± 0.08	2.36 ± 0.08	−0.84%	10.2 ± 0.1	10.4 ± 0.1	+2.1%
1:10,000	184.4 ± 3.5	179.6 ± 1.6	−2.6%	2.34 ± 0.19	2.18 ± 0.10[c]	−6.8%	10.1 ± 0.1	10.4 ± 0.1	+2.7%
1:1000	184.8 ± 2.2	170.0 ± 2.4[c]	−8.0%	2.40 ± 0.07	1.94 ± 0.11[b]	−19.1%	10.0 ± 0.2	11.1 ± 0.7[b]	+11.6%
Substituted Methacrylates:									
Hydroxyethyl methacrylate									
1:100,000	183.6 ± 3.2	163.2 ± 2.6[b]	−11.1%	2.42 ± 0.04	2.28 ± 0.22	−5.7%	9.7 ± 0.4	9.8 ± 0.4	+1.0%
1:10,000	183.6 ± 2.9	144.8 ± 3.9[b]	−21.1%	2.38 ± 0.08	1.56 ± 0.05[b]	−34.4%	9.7 ± 0.3	10.8 ± 1.3	+11.0%
1:1000	181.6 ± 1.6	106.4 ± 3.5[b]	−41.4%	2.36 ± 0.05	0.42 ± 0.04[b]	−82.2%	9.5 ± 0.8	10.8 ± 0.3[b]	+13.3%
tert-Butylaminoethyl methacrylate									
1:100,000	184.0 ± 3.1	172.8 ± 3.3[c]	−6.0%	2.42 ± 0.08	2.32 ± 0.14	−4.1%	10.0 ± 0.2	10.0 ± 0.1	0.0
1:10,000	181.8 ± 2.0	163.8 ± 3.0[b]	−9.9%	2.42 ± 0.04	1.96 ± 0.05[b]	−19.0%	10.2 ± 0.6	10.1 ± 0.4	−0.39%
1:1000	182.2 ± 2.2	143.6 ± 3.5[b]	−21.8%	2.36 ± 0.20	1.64 ± 0.15[b]	−30.5%	11.0 ± 1.1	10.3 ± 0.9	−5.6%
Dimethylaminoethyl methacrylate									
1:100,000	184.2 ± 3.1	149.2 ± 12.9[b]	−19.0%	2.26 ± 0.20	1.02 ± 0.08[b]	−54.8%	9.8 ± 1.1	9.6 ± 1.1	−2.4%
1:10,000	181.6 ± 1.6	0.0[b]	−100.0%	2.38 ± 0.16	0.0[b]	−100.0%	9.4 ± 1.1	0.0[b]	−100.0%
1:1000	183.2 ± 4.8	0.0[b]	−100.0%	2.34 ± 0.15	0.0[b]	−100.0%	10.2 ± 0.6	0.0[b]	−100.0%

[a] Response represents the stabilized level of altered activity within the first 3 min. following methacrylate perfusion and return to normal Locke's solution. [b] $p < 0.01$ when compared to control. [c] $p < 0.05$ when compared to control.

Table II—Reversibility of Heart Response to Methacrylates

Group	Effect
I: Methacrylic acid, Methyl methacrylate, Ethyl methacrylate, Dimethylaminoethyl methacrylate	Produced an irreversible effect on the isolated heart at all three concentrations
II: *n*-Propyl methacrylate, *n*-Butyl methacrylate, Isobutyl methacrylate, Hydroxyethyl methacrylate, *tert*-Butylaminoethyl methacrylate	Produced an irreversible effect on the isolated heart at the 1:1000 concentration but not at lower concentrations
III: 1,3-Butylene dimethacrylate, 2-Ethylhexyl methacrylate, Isodecyl methacrylate, Lauryl methacrylate	Produced a reversible effect on the isolated heart at all three concentrations

MATERIALS AND METHODS

Materials—The monomers included in this study are all liquids at room temperature, and they constitute the following: methacrylic acid[1]; methyl[1], ethyl[1], *n*-butyl[1], isobutyl[1], isodecyl[1], lauryl[1] (dodecyl), hydroxyethyl[1], *tert*-butylaminoethyl[1], dimethylaminoethyl[1], *n*-propyl[2], and 2-ethylhexyl[2] methacrylates; and 1,3-butylene dimethacrylate[1].

Methods—Isolated rabbit hearts were perfused using an Anderson coronary perfusion apparatus. A twin reservoir system was employed, one containing normal Locke's solution and the other the test substance dissolved in Locke's solution[3], in which a uniform hydrostatic pressure was maintained to provide a constant perfusion pressure and to permit rapid changes from one solution to the other. Heart rate and force of contraction were recorded with a polygraph[4] by placing a small hook in the apex of the heart and passing a ligature over a pulley to a force-displacement transducer[5] which was calibrated to produce 1 cm. deflection/g. of force applied. The outflow from the heart was collected in a small graduated cylinder at specified time intervals to determine the coronary flow rate.

Hearts from small rabbits (1.5–2.0 kg.) were used throughout this study. Each heart was perfused for a 20-min. equilibration period prior to initiating the experiment, with the tests being conducted during the following 90 min. Preliminary studies using only normal Locke's solution did not reveal any functional abnormality in the isolated, perfused heart for approximately 2 hr. or more.

All compounds were tested at three fixed concentrations, 1:1000, 1:10,000, and 1:100,000 (v/v), in Locke's solution. When cardiac activity had stabilized, the test solution was introduced as the perfusate for 1 min. and immediately thereafter normal Locke's solution was perfused to permit recovery of the heart. The effect of a test solution was deemed "irreversible" if cardiac activity had not made a significant return toward control levels within 30–35 min. of perfusion with normal Locke's solution. If at any time the heart failed to return to control levels of activity, it was replaced with a fresh one before proceeding with the next test. Each methacrylate concentration was tested five times. Effects of the test solution were determined by quantitating the three parameters (rate and force of contraction and coronary flow rate) of the heart at its stabilized activity immediately prior to perfusion of test solution and comparing these to the values obtained from the heart at its plateau activity near the end of, or immediately following, perfusion with the test solution.

[1] Rohm & Haas, Philadelphia, Pa.
[2] K & K Labs., Inc., Plainview, N. Y.
[3] The Locke solution contained 9.0 g. NaCl, 0.42 g. KCl, 0.24 g. $CaCl_2$, 0.15 g. $NaHCO_3$, and 1.0 g. glucose/l. It was oxygenated with 95% oxygen and 5% carbon dioxide, and the perfusate temperature was maintained at 37 ± 0.3°.
[4] Grass model 7.
[5] Grass FT 03C.

RESULTS

When dissolved in Locke's solution and perfused through isolated rabbit hearts, methacrylic acid and the 12 methacrylate esters produced significant effects upon the heart at one or more of the three concentrations employed. A summary of the mean values and standard errors is presented in Table I for cardiac rate, force of contraction, and coronary flow. The data also show which changes are significant by Student's *t* test and the magnitude of the change as percent change from control values. The methacrylate perfusion produced cardiac standstill in five of the test solutions employed. In these cases, the heart stopped in systole and coronary flow ceased or was reduced to negligible amounts (responses were recorded as zero), with a −100% change from control.

These methacrylates may be rather equally divided into three categories (Table II) as to those which, under the conditions of these experiments: (*a*) produced irreversible effects upon the isolated heart at all three concentrations tested, (*b*) produced irreversible effects on the isolated heart at highest concentrations but reversible effects at lower concentrations, and (*c*) produced reversible effects upon the isolated heart at all three concentrations tested.

DISCUSSION

Although the acute lethality of methacrylate monomers has been reported to result from respiratory depression (1, 2), more information was needed concerning the pharmacodynamic (or toxicological) activity of these compounds upon a relatively simple system: the isolated, perfused rabbit heart. Examination of the data in Table I reveals that most of the methacrylate-containing perfusing solutions produced a significant ($p \leq 0.05$) reduction in cardiac rate and force of contraction, with the effect upon contraction generally being of a greater magnitude. This was true whether or not the effect was reversible. In approximately one-half of the test groups, there was also a significant ($p \leq 0.05$) change in coronary flow rate; this change was usually a reduction but, in several instances, it was an increase.

From the toxicological aspect, it would be desirable to rate or rank such compounds according to their toxicological liability. However, there appears to be no single criterion upon which to base such a comparison since the magnitude of change in these three characteristics varies from one methacrylate to another. For example, the 1:10,000 concentration of *n*-butyl methacrylate reduced heart rate by 13.8%, force of contraction by 53.3%, and coronary flow by 41.2%, while the same concentration of isobutyl methacrylate reduced rate by 17.4%, force by 25.6%, and flow by 2.0%; this concentration of ethyl methacrylate reduced rate by 17.8%, force by 73.3%, and flow by 57.9%. Thus, while the reduction of rate was comparable for all three monomers (13.8, 17.4, and 17.8%), the reduction in force of contraction and coronary flow varied tremendously (force = 53.3, 25.6, and 73.7%, respectively; flow = 41.2, 2.0, and 57.9%, respectively). For any particular methacrylate, the response obtained demonstrated a concentration-dependent relationship for each experimentally measured variable.

Table III presents these 13 methacrylates ranked in order of their relative effects upon the parameters indicated. The compounds are listed in decreasing order of acute toxicity to mice (intraperitoneally), with the acute LD_{50} values for each. The effect of the compound upon each function of the isolated heart was expressed as the average mean response to the three standard concentrations, and this value is given in parentheses following each ranking number. These were ranked from 1 to 13, with 1 producing the greatest reduction and 13 the least reduction. Examination of these data or the more detailed data in Table I suggests a relatively greater reduction in force of contraction and cardiac rate than upon coronary flow.

The data presented, their reproducibility, and discussions to this point have been based upon volume quantities or concentrations of methacrylates. To obtain an estimate of the activity of each methacrylate upon these three parameters by molar concentrations of the compounds, the mean percent change from control was plotted against the molar concentration in the perfusate and a "best-fit" curve was drawn through these three points. The molar concentrations *versus* percent change for heart rate, force of contraction, and coronary flow rate are shown in Figs. 1, 2, and 3, respectively. It is readily apparent that these three-point curves are estimates;

Table III—Relative Activity of Methacrylates of Equal Volume to Volume Dilutions[a]

Compound (Monomer)	Acute LD_{50}, Intraperitoneal, Mice, ml./kg.	Relative Effect upon Isolated Rabbit Heart: Rate	Contraction	Coronary Flow
Methacrylic acid	0.048	3 (−52.0%)	4 (−58.5%)	4 (−36.3%)
Dimethylaminoethyl methacrylate	0.104	1 (−73.0%)	1 (−84.9%)	1 (−67.5%)
tert-Butylaminoethyl methacrylate	0.190	9 (−12.6%)	11 (−17.9%)	9 (−2.0%)
Hydroxyethyl methacrylate	0.497	5 (−24.5%)	7 (−40.8%)	+11 (+8.4%)[b]
n-Propyl methacrylate	1.121	10 (−12.3%)	6 (−42.6%)	7 (−6.1%)
Methyl methacrylate	1.198	2 (−55.7%)	2 (−74.0%)	3 (−60.6%)
Isobutyl methacrylate	1.340	6 (−19.0%)	8 (−39.5%)	8 (−4.2%)
Ethyl methacrylate	1.369	4 (−41.2%)	3 (−64.0%)	2 (−61.5%)
n-Butyl methacrylate	1.663	7 (−16.5%)	5 (−46.0%)	5 (−31.1%)
2-Ethylhexyl methacrylate	2.614	11 (−8.3%)	10 (−20.3%)	+12 (+13.2%)[b]
1,3-Butylene dimethacrylate	3.598	8 (−13.3%)	9 (−35.8%)	6 (−7.2%)
Isodecyl methacrylate	3.688	12 (−6.3%)	12 (−13.2%)	+13 (+15.3%)[b]
Lauryl methacrylate	24.897	13 (−3.6%)	13 (−8.9%)	+10 (+5.5%)[b]

[a] Relative activity determined by using the mean responses obtained at the three standard concentrations tested (1×10^{-3}, 10^{-4}, and 10^{-5} v/v). [b] + indicates an increase over controls, with higher numbers indicating a greater increase.

Table IV—Relative Effect of Perfusing Methacrylates upon Three Functions of the Isolated Heart at Equimolar Concentration

Compound (Monomer)[a]	Acute LD_{50}, Intraperitoneal, Mice, moles/10^6 g.	Relative Effect upon Isolated Heart[b]: Rate	Contraction	Coronary Flow
Methacrylic acid	0.564	3 (−63.5%)	5 (−64.9%)	7 (−7.5%)
Dimethylaminoethyl methacrylate	0.618	1[c]	1[c]	1[c]
tert-Butylaminoethyl methacrylate	0.937	10 (−16.5%)	11 (−28.0%)	9 (−1.9%)
Hydroxyethyl methacrylate	4.060	5 (−28.5%)	7 (−48.0%)	+11 (+11.5%)
n-Propyl methacrylate	7.821	9 (−17.4%)	6 (−57.5%)	6 (−7.9%)
Isobutyl methacrylate	8.398	6 (−23.5%)	9 (−43.9%)	8 (−5.0%)
n-Butyl methacrylate	10.481	8 (−20.0%)	4 (−65.5%)	4 (−48.9%)
Ethyl methacrylate	10.896	4 (−36.9%)	2 (−85.5%)	2 (−70.5%)
Methyl methacrylate	11.217	2 (−75.0%)	2 (−85.5%)	3 (−64.5%)
2-Ethylhexyl methacrylate	11.571	11 (−13.0%)	10 (−30.4%)	+13 (+20.4%)
Isodecyl methacrylate	14.251	12 (−11.0%)	12 (−21.9%)	+12 (+18.0%)
1,3-Butylene dimethacrylate	16.063	7 (−21.0%)	8 (−47.0%)	5 (−8.0%)
Lauryl methacrylate	84.531	13 (−8.0%)	13 (−17.3%)	+10 (+8.9%)

[a] Compounds listed in decreasing order of lethal potency on a mole basis. [b] Ranking is based upon relative effects produced by the compound at a concentration of 2×10^{-3} mole/l. in the perfusing solution as estimated from Figs. 1–3. A rank of 1 represents the greatest depressant effect, while that of 13 is the least depressant. In the case of coronary flow rate, a plus value indicates an increase in flow rate, with the higher number representing the greater increase. Percentage change from control is given in parentheses. [c] This compound produced cardiac standstill at a concentration of 0.59×10^{-3} *M*, while a concentration of 0.059×10^{-3} mole/l. reduced rate by 19%, contraction by 55%, and coronary flow by 2%.

arguments for other "lines of best fit" could be made for some, particularly those in which cardiac standstill has occurred, since the −100% point could readily be shifted to the left by data intermediate between those presented. Such a shift would, of course, show an increased potency for the compound upon the parameters in question. Although only approximate, the curves show the patterns of activity and the relative magnitudes of potency of the various compounds on a molar basis.

Examination of these figures reveals considerable similarity between the effects that a compound produces upon heart rate and force of contraction; this appears to be true on a qualitative basis for all compounds in this series. When comparing these to changes in coronary flow rate, a few of the compounds (*e.g.*, methyl and ethyl methacrylates) still produce similar curves; however, many of these compounds produce response curves of a grossly different shape or magnitude.

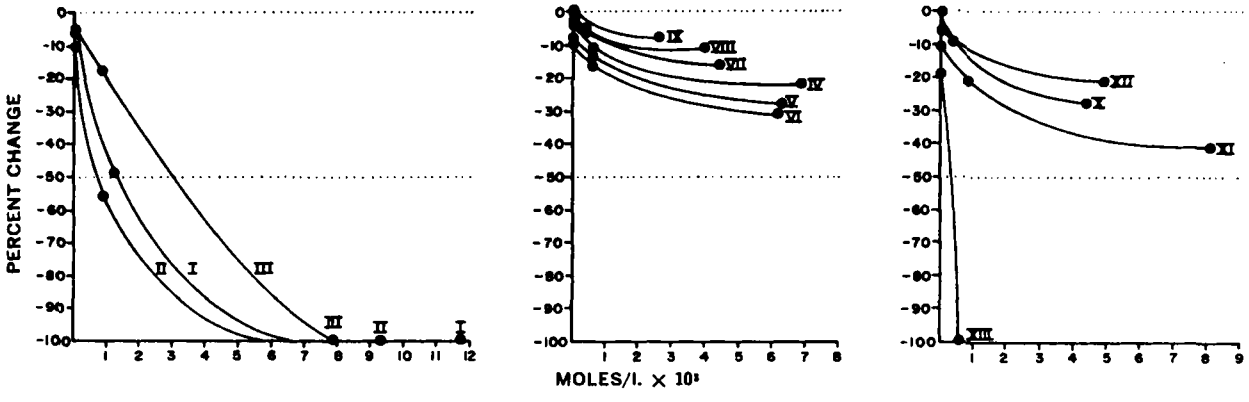

Figure 1—*Effects of methacrylates upon rate of contraction of isolated rabbit heart. Key: I = methacrylic acid, II = methyl methacrylate, III = ethyl methacrylate, IV = n-propyl methacrylate, V = n-butyl methacrylate, VI = isobutyl methacrylate, VII = 2-ethylhexyl methacrylate, VIII = isodecyl methacrylate, IX = lauryl methacrylate, X = 1,3-butylene dimethacrylate, XI = hydroxyethyl methacrylate, XII = tert-butylaminoethyl methacrylate, and XIII = dimethylaminoethyl methacrylate.*

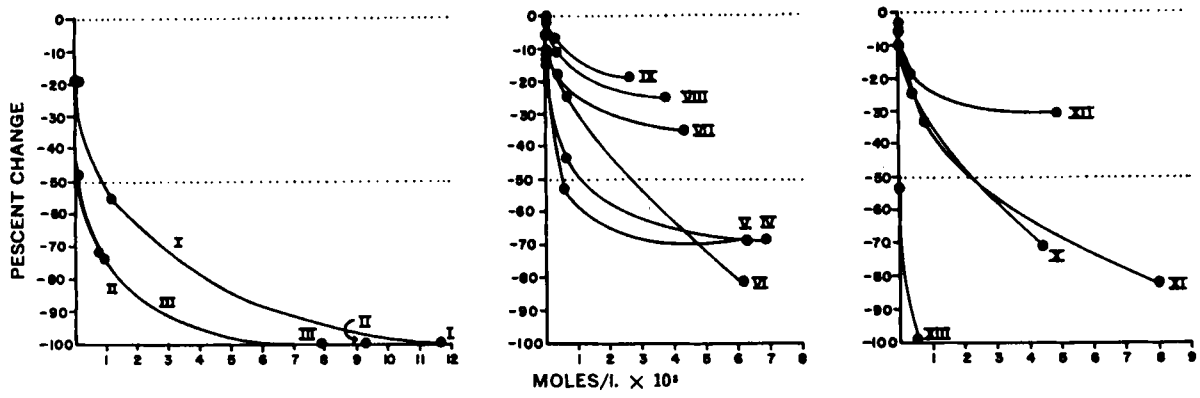

Figure 2—*Effects of methacrylates upon force of contraction of isolated rabbit heart. (For Key, see Fig. 1.)*

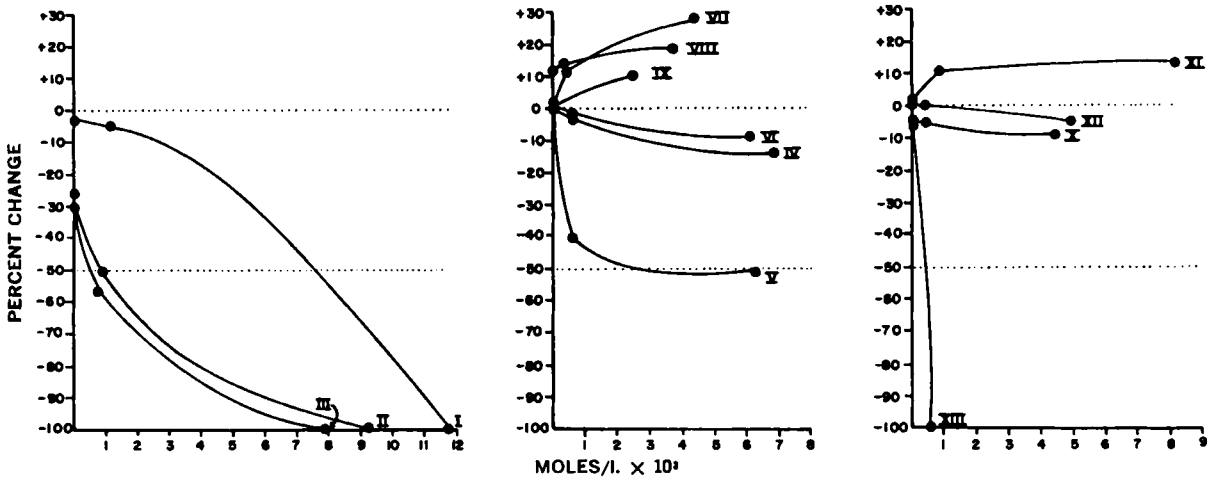

Figure 3—*Effects of methacrylates upon coronary flow rate of isolated rabbit heart. (For Key, see Fig. 1.)*

To facilitate equimolar comparisons, a perfusate concentration of 2×10^{-3} mole/l. was selected and the approximate responses were obtained from the curves shown in Figs. 1–3 and ranked for each cardiac parameter. These data are presented in Table IV, in which the monomers are ranked in order of decreasing acute intraperitoneal toxicity to mice, with LD_{50} values expressed as moles/10^6 g. of mouse.

A comparison of Table IV with Table III indicates that most of the compounds exhibited similar ranking according to their effect upon the isolated heart, whether they were considered on the basis of average response to the three standard volume to volume concentrations (Table III) or response produced by a 2×10^{-3} *M* solution (Table IV). The relative ranking of these compounds by these two methods of comparison differed by more than the adjacent rank in only one case (effect of methacrylic acid upon coronary flow rate). There were a number of instances, however, where the rank moved up or down to the adjacent number.

SUMMARY

The action of a number of methacrylates was examined using the isolated, perfused rabbit heart. Dimethylaminoethyl methacrylate exhibited the most potent effect upon the isolated heart, producing cardiac standstill at a dilution of 1:10,000 (v/v) (0.59×10^{-3} mole/l.), while lauryl methacrylate showed the least depressant effect upon the isolated heart at the concentrations tested. All of the methacrylates tested, at all concentrations employed, produced a relative increase in coronary flow (coronary flow per gram of contraction) or did not alter it when compared to pretreatment control values. The only exception observed was when the test solution produced cardiac standstill. Although cardiac failure may not be the primary cause of death from methacrylate esters in animals, these compounds do show some marked effects upon the isolated heart, and some clinicians have implicated one of them (methyl methacrylate) in certain clinical cases of cardiac arrests observed during the use of "acrylic bone cement." Dose-response data are presented based upon both molar concentrations and volume dilutions of these compounds.

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Kinetics of β-Lactamase Inactivation of Penicillins I: Effect of Side-Chain Structure, Ionic Strength, pH, and Temperature

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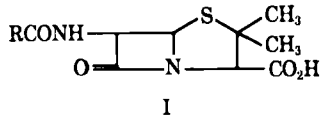
Abstract □ The *Staphylococcus aureus* Tex-2 β-lactamase-catalyzed hydrolysis of several penicillins was shown to follow Michaelis–Menten kinetics. Penicillins with a lipophilic side chain had the highest affinity for the enzyme. Introduction of a polar group (either positively or negatively charged) into the side chain and/or a bulky diester group on the penicillin nucleus drastically lowered the binding. Of the penicillins studied, the α-aminopenicillins were the most susceptible. The effect of ionic strength on the staphylococcal β-lactamase hydrolysis of penicillin G and ampicillin was small, but the effect of pH was conspicuous. At 25°, a V-shaped pH–K_m profile with a pH optimum at 6.8 was obtained for penicillin G, while a sigmoid pH–K_m profile with bell-shaped pH–V_{max} profile at 6.4 was obtained for the amphoteric ampicillin. The heats of activation of penicillin β-lactamase-catalyzed hydrolysis of penicillins was shown to vary with the enzyme species and with the substrate. The amphoteric penicillin had a much lower heat of activation value than that of the monobasic penicillin. For the *S. aureus* Tex-2 catalyzed hydrolysis of penicillin G and ampicillin, the heat of activation values were 7.18 and 5.17 kcal./mole at pH's of 6.8 and 7.4, respectively, while a value of 5.10 kcal./mole was obtained for the *Bacillus cereus* B569 catalyzed hydrolysis of ampicillin at pH 6.2. The temperature optimum for the staphylococcal β-lactamase inactivation of ampicillin at pH 7.4 may be 35–37°.

Keyphrases □ Penicillins—kinetics of β-lactamase hydrolysis, effect of side chain, ionic strength, pH, temperature □ β-Lactamase hydrolysis of penicillins—kinetics, effect of side chain, ionic strength, pH, temperature □ Penicillinase inactivation of penicillins—kinetics, effect of ionic strength, pH, temperature, side chain □ α-Aminopenicillins—kinetics of β-lactamase hydrolysis, effect of side chain, ionic strength, pH, temperature

Considerable research has been conducted on the kinetics of the interactions between the β-lactam antibiotics (the penicillins and cephalosporins) and the β-lactamases produced by various bacteria. Abraham and Newton (1), Banfield (2), Citri and coworkers (3–7), and Hamilton-Miller (8) investigated the kinetics of the bacillary β-lactamases; Knox and Smith (9), Hamilton-Miller (8), Crompton *et al.* (10), Novick (11), Richmond (12), Gourevitch *et al.* (13), Smith *et al.* (14), and Depue *et al.* (15) studied the kinetics of the staphylococcal β-lactamases. The hydrolysis of penicillins by *Klebsiella aerogenes* and *Bacillus licheniformis* enzymes has also been reported (8). Comprehensive reviews on β-lactamases and β-lactamase kinetics were recently published by Citri and Pollock (16), Citri (17), Abraham (18), and Rauenbusch (19).

Of all the penicillins, ampicillin is the most unstable toward the β-lactamases. Penicillin G, phenoxymethyl penicillin (penicillin V), phenethicillin, and propicillin



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are all hydrolyzed at a similar rate and all are more stable than ampicillin (1, 9). Penicillin N, cephalosporin C (1), and 6-aminopenicillanic acid (11) are only slightly inactivated. Methicillin, oxacillin, and cloxacillin (3–7, 11) are resistant.

The ability of a penicillin (I) to resist hydrolysis depends, to a large degree, on the nature of the side chain R (13, 20). Susceptible penicillins have low Michaelis constants (K_m) and very high maximum rates (V_{max}). Moderately resistant penicillins have K_m's 30–80 times larger than those of the susceptible penicillins and have somewhat lower V_{max}'s; the resistant penicillins have very low V_{max}'s but K_m's similar to those of the moderately resistant penicillins (13).

A previous article (21) reported the applicability of a pH-stat alkalimetric titration method in the investigation of β-lactamase activity, substrate specificity, and penicillin stability. The present paper reports the effects of environmental factors, such as ionic strength, temperature, and pH, on the interactions between the β-lactamases and the penicillins. The main interest was the kinetic behavior of the staphylococcal and bacillary β-lactamase (penicillinase) catalyzed hydrolysis of penicillins with different side-chain groups and nucleus structures. It is hoped that such studies will add to the understanding of the mechanism of the penicillinase inactivation of penicillins.

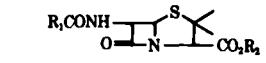
EXPERIMENTAL

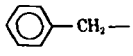
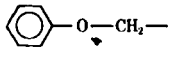
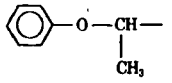
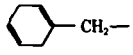
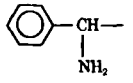
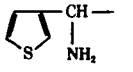
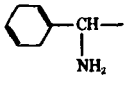
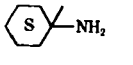
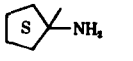
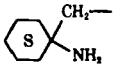
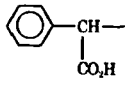
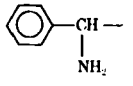
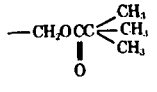
Materials—The following penicillins were used, all of which are either new or established products of this laboratory[1]: the potassium salts of penicillin G, phenoxymethyl penicillin, phenethicillin, ampicillin, and cyclacillin[2]; the sodium salt of 6-[2-(1,4-cyclohexadien-1-yl)acetamido]penicillanic acid[3] (I); and zwitterions of 6-(1-aminocyclopentanecarboxamido)penicillanic acid[4] (II) and 6-[2-(1-aminocyclohexyl)acetamido]penicillanic acid[5] (III). Also used were the disodium salt of carbenicillin[6], zwitterions of epicillin[7] (IV) and 6-[2-amino-(3-thienyl)acetamido]penicillanic acid[8] (V), and the hydrochloride salt of pivampicillin[9].

β-Lactamases—*Staphylococcus aureus* Tex-2 β-lactamase was obtained from the supernatant fluid of a fresh broth culture of this organism[10]. *Bacillus cereus* B569 β-lactamase was obtained commercially[11] in crystalline form and was dissolved in a dilute (0.1%) gelatin A USP solution to the desired strength.

[1] Wyeth Laboratories.
[2] Wy-4508.
[3] Wy-12955.
[4] Wy-7953.
[5] Wy-14903.
[6] Beecham Laboratories.
[7] 6-[2-Amino-(1,4-cyclohexadien-1-yl)acetamido]penicillanic acid, SQ 11,302, Squibb Institute.
[8] BE-P875, Bristol Laboratories.
[9] Leo Laboratories.
[10] Prepared by the Antibiotics Section, Research Division, Wyeth Laboratories.
[11] Riker Co.

Table I—*S. aureus* Tex-2 β-Lactamase-Catalyzed Hydrolysis of Penicillins at 25° and pH 7.0[a]



Number	Penicillin	Side Chain R_1	Side Chain R_2	K_m, μM	V_{max}, μM/min.	Ratio to V_{max} of Penicillin G
1	Penicillin G		H	50	130	1.00
2	Phenoxymethyl penicillin		H	150	165	1.27
3	Phenethicillin		H	100	113	0.87
4	I		H	150	85	0.65
5	Ampicillin		H	550	303	2.33
6	V		H	500	292	2.24
7	IV		H	650	320	2.46
8	Cyclacillin		H	750	78	0.60
9	II		H	350	117	0.90
10	III		H	900	320	2.46
11	Carbenicillin		H	4200	160	1.23
12	Pivampicillin (ampicillin diester)			3000	30–35	0.23–0.27

[a] Substrate concentrations ranging from 2 to 12 × 10^{-3} M and total ionic strength of 0.026 were used for each penicillin except carbenicillin. The K_m and V_{max} were calculated from Lineweaver–Burk plots.

Kinetics—The standardization of the enzyme preparations, the kinetic procedures, and calculations of initial rates were identical to those reported previously (21). The enzyme kinetic parameters were obtained in the following manner.

According to conventional enzyme kinetics (22), the enzyme E and substrate S interact to form an enzyme–substrate complex ES and then decompose to enzyme E and a product P (Scheme I). The

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E$$

Scheme I

rate of formation of product is given by:

$$v = \frac{d(p)}{dt} = \frac{K_3(E)(S)}{(S) + K_m} = \frac{V_{max}(S)}{(S) + K_m} \qquad \text{(Eq. 1)}$$

By taking the reciprocal of Eq. 1 and multiplying through by (S), the following final equation is obtained:

$$\frac{(S)}{v} = \frac{(S)}{V_{max}} + \frac{K_m}{V_{max}} \qquad \text{(Eq. 2)}$$

where (S) is the initial substrate concentration and v is the initial rate. From Lineweaver–Burk plots (23), by plotting the term $(S)/v$ *versus* (S), the V_{max} can be obtained from the slope of the plot and the K_m either by dividing the ordinate intercept by the slope or by extrapolating the slope to the abscissa intercept, which is the same as the negative K_m. A calculator[12] was utilized for the initial rate computations, and the kinetic parameters were obtained graphically.

RESULTS AND DISCUSSION

The *S. aureus* Tex-2 β-lactamase-catalyzed hydrolysis was shown to follow Michaelis–Menten kinetics. By using 2.5 units of the enzyme, where the unit of activity of the β-lactamase is defined as the amount of enzyme that will hydrolyze 1.0 μmole of potassium penicillin G in 1 min. at 25° and pH 7.0 (buffer free), and 2.0–12.0 × 10^{-3} M each of penicillin G, phenoxymethyl penicillin, phenethicillin, ampicillin, cyclacillin, Compounds I, II, III, IV, and V, and pivampicillin, essentially pseudo-zero-order kinetics were maintained in each case over most of the reaction, particularly with higher initial substrate concentrations. With carbenicillin, higher (fivefold) substrate concentrations had to be used due to its rather high K_m value. In these studies, except those on carbenicillin and pivampicillin, a constant ionic strength was also maintained for each sample. In almost all cases, rather reproducible initial rates were obtained.

[12] Olivetti Underwood Programma 101.

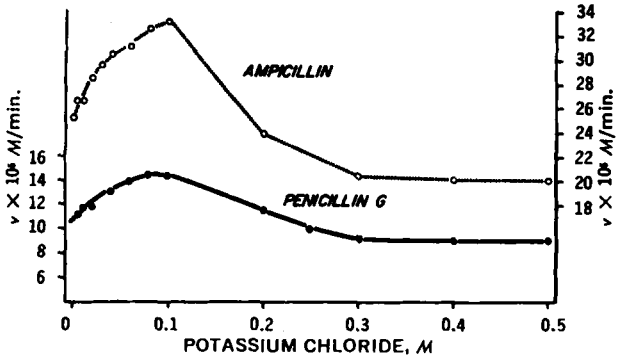


Figure 1—*Effect of ionic strength on the* S. aureus *Tex-2 β-lactamase- (2.5 units) catalyzed hydrolysis of ampicillin and penicillin G at 25°. The pH = 6.8 for penicillin G and 6.4 for ampicillin.* (S) = 2×10^{-3} M *for either penicillin.*

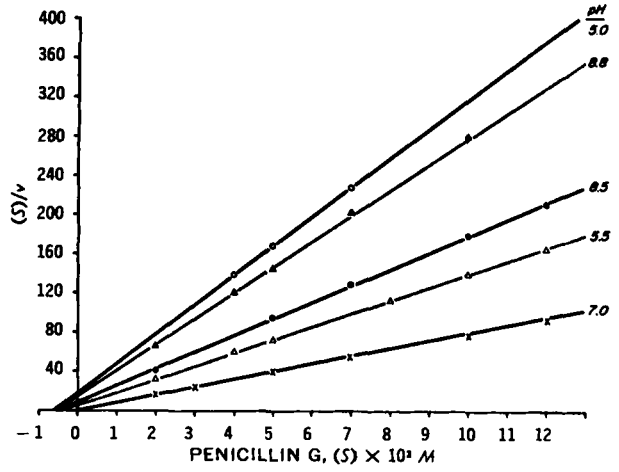


Figure 2—*Effect of substrate concentration* (S) *at various pH's on kinetics of* S. aureus *Tex-2 β-lactamase- (2.5 units) catalyzed hydrolysis of penicillin G at constant temperature (25°).* (S)/v *was plotted* versus (S) *at various indicated pH's to give maximal rates* (V_{max}) *and Michaelis constants* (K_m).

Effect of Penicillin Side Chain on Enzyme Kinetic Parameters—Table I shows the calculated K_m and V_{max} of 12 penicillins studied at pH 7.0 and 25°. Among the penicillins studied, penicillin G, phenoxymethyl penicillin, phenethicillin, and Compound I had the lowest K_m values (50–150 μM); *i.e.*, they have the highest affinity for the active sites on the enzyme. The α-aminopenicillins (ampicillin and Compounds IV and V) and the amino alicyclic penicillins (cyclacillin and Compounds II and III) had a much lower affinity, as reflected in the higher K_m values (350–900 μM). Carbenicillin and pivampicillin had the lowest affinity among the penicillins studied, their K_m values (3000–4000 μM) being about 60–80 times higher than that of penicillin G.

Thus, the penicillins with the highest affinity for β-lactamase are those having aromatic benzyl (penicillin G), phenoxyalkyl, and 1,4-dihydrophenylmethyl (Compound I) side chains. Introduction of a polar group (either positively or negatively charged) into the side-chain group significantly decreases the affinity. A similar finding was previously reported by Depue *et al.* (15), who showed that penicillins with a positively charged nitrogen atom (as in an amino or a nitro group in the side chain) have substantially lower affinity.

Despite the rather low affinities of the α-aminopenicillins for the enzyme, their maximum rates of hydrolysis were about two times as fast as that of penicillin G or phenoxymethyl penicillin. The kinetic parameters of carbenicillin were even more unusual. Its extremely low affinity seemed to have little effect on its susceptibility toward the enzyme. Cyclacillin, on the other hand, has relatively low affinity for the enzyme and also demonstrates a rather low V_{max} value. However, this property was altered as a result of the addition of a methylene group between the amino alicyclic group and the peptide linkage of cyclacillin as in Compound III. The different kinetic behavior of carbenicillin and Compound III from that of penicillin G and cyclacillin is, no doubt, related to the steric effect of their side chains. In general, the maximum rates of the penicillins obtained in the present studies were in accordance with their relative stability pattern reported previously (21).

The drastically increased stability of pivampicillin could be related to its greatly decreased affinity for the enzyme. Conceivably, the stretched bulky diester group of pivampicillin is playing an important role.

Variation in the penicillin side-chain group (R) could be expected to produce a drastic change in the overall lipophilic and hydrophilic properties of the molecule and in the enzyme kinetic parameters. Variation in the side chain, however, had a much greater effect on the K_m than on the V_{max}. Penicillin G, phenoxymethyl penicillin, and carbenicillin are good examples of this difference; their V_{max} values were essentially identical, but their K_m's differed by a factor of 50–80. Ampicillin and Compounds IV and V, which are all α-aminopenicillins and have similar biological properties[13] (24–26), were equally susceptible toward the β-lactamases. They were inactivated about 2–3 times faster (greater V_{max}) than penicillin G despite their much lower affinity (higher K_m) for the active sites of the enzyme. Thus, hydrolysis may depend on "favorable conformational change" or "favorable orientation" of the active sites in the enzyme which, in turn, may depend on the presence of a charge amino group in the substrate. On the other hand, the significantly increased insusceptibility of cyclacillin and pivampicillin to the β-lactamase is probably due to catalytically "unfavorable orientation" of the active centers in the enzyme as a result of some steric effect of the substrate molecule. The faster and higher serum levels of pivampicillin attained compared to its parent compound (27–29) may be partially due to its higher resistance toward β-lactamase inactivation *in vivo*.

Effect of Ionic Strength—At 25° and the pH optimum (21), *S. aureus* Tex-2 β-lactamase (2.5 units) was allowed to react with penicillin G and ampicillin (2.0×10^{-3} M), the total ionic strength being varied with the range of 0.005–0.5 by adding potassium chloride. The initial rates for both penicillin G and ampicillin were shown to increase directly in proportion to the salt concentration until a maximum rate was reached, then to slow down slightly, and finally to level off with a further increase in salt concentration (Fig. 1). In each case the maximum rate was reached at an ionic strength of 0.1 and began to level off at 0.3. The rate increased about 30% as the apparent potassium chloride concentration progressed from 0 to 0.1 M and decreased about 20% as it continued on to 0.3 M. Accordingly, the neutral salt concentration seemed to have no significant effect on the rate of β-lactamase-catalyzed hydrolysis, at least within the salt range used.

Effect of pH on Kinetic Parameters—As mentioned previously, the hydrophilic and lipophilic groups on the penicillin side chain could effectively influence the kinetic parameters of the β-lactamase-catalyzed hydrolysis of penicillins at a neutral pH. It was of interest to observe the change in kinetic behavior at different pH's, particularly between two distinctly different types of penicillin such as monobasic penicillin G and amphoteric ampicillin. Accordingly, *S. aureus* Tex-2 β-lactamase (2.5 units) was allowed to interact with various initial substrate concentrations of either penicillin G or ampicillin, holding the temperature at 25°, maintaining a constant total ionic strength[14] (0.026 M), and varying the pH from 5 to 9.

Virtually pseudo-zero-order kinetics were obtained for penicillin G at all pH's, *i.e.*, the enzyme apparently was saturated with the substrate. Ampicillin, when applied within the usual range of initial substrate concentrations ($2–12 \times 10^{-3}$ M), appeared quite sufficient to saturate the enzyme at neutral and higher pH's, although at lower pH's (<6.0) a relatively higher initial substrate concentration had to be used. The enzyme kinetic parameters K_m and V_{max} were obtained from Eq. 2 by plotting $(S)/v$ against

[13] All three are acid stable, readily absorbed after oral administration, and have a broad spectrum of antimicrobial activities.

[14] The ionic strengths contributed by the substrates were calculated using pK 2.64 as the dissociation constant for the carboxyl group of penicillins and 7.25 for the amino group of ampicillin (30).

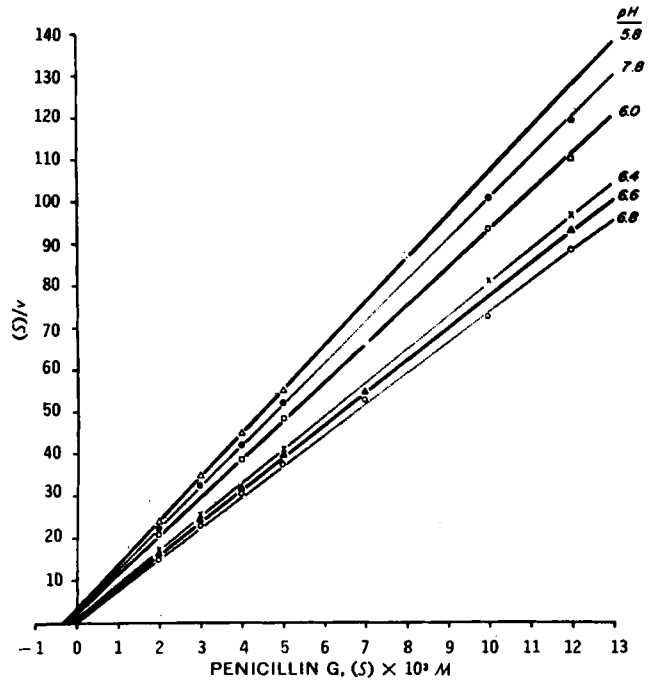


Figure 3—*See Fig. 2 for explanation.*

(*S*). Figures 2 and 3 are the resulting plots for penicillin G; similar plots were obtained for ampicillin but are not shown.

Penicillin G had the highest affinity for the active sites of the *S. aureus* Tex-2 β-lactamase or a lowest K_m value at pH 6.8 than at other pH's. The K_m values were increased about 15-fold at pH's of 5 and 8.5 over that at pH 6.8. By plotting the K_m against the pH (Fig. 4), a V-shaped pH–K_m profile was obtained. This type of curve may be typical for the monobasic penicillin G type of drugs. By plotting the V_{max} *versus* pH (right-hand side of Fig. 4), a nearly bell-shaped pH–V_{max} profile was obtained which is also in accordance with the pH optimum value of *S. aureus* Tex-2 reported previously (21). The greatest affinity occurred at the point of highest rate. At pH's other than optimum, the hydrolysis rate decreases significantly and simultaneously diminishes its affinity for the enzyme.

The effect of pH on the affinity characteristics of ampicillin were quite different from those of penicillin G. As shown in Fig. 5, a sigmoid pH–K_m profile was obtained for this amphoteric penicillin, although its pH–V_{max} curve was bell-shaped like those of all the other penicillins studied (21). Since it depends on the state of ionization of the substrate (and possibly on the free enzyme and enzyme–substrate complex), the pH has a significant effect on the K_m. Thus, at a pH of about 5, ampicillin exists virtually in zwitterionic form and has the highest K_m (>2000 μ*M*) or the lowest affinity for the

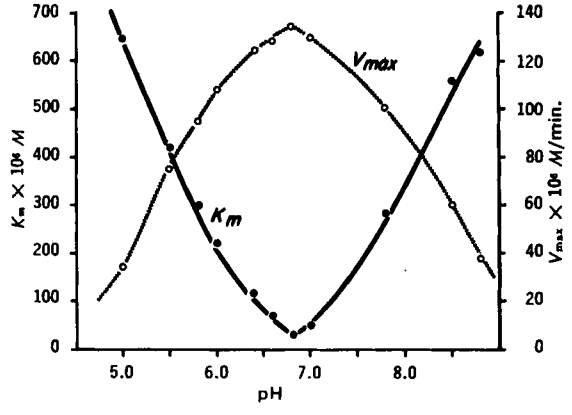


Figure 4—*Effect of pH on maximal rate* (V_{max}) *and Michaelis constant* (K_m) *for the* S. aureus *Tex-2 β-lactamase- (2.5 units) catalyzed hydrolysis of penicillin G at 25°.*

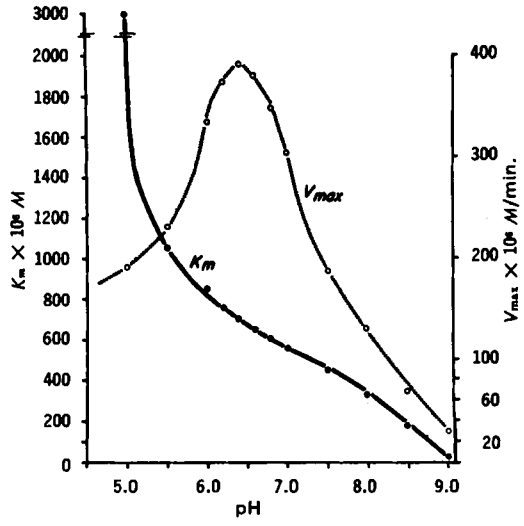


Figure 5—*Effect of pH on maximal rate* (V_{max}) *and Michaelis constant* (K_m) *for the* S. aureus *Tex-2 β-lactamase- (2.5 units) catalyzed hydrolysis of ampicillin at 25°.*

active sites of the enzyme, but at pH 6–7 it exists in both zwitterionic and anionic species and its affinity is increased about fivefold; at a pH above 8.5, essentially all of the ampicillin molecules are in anionic form and the K_m (<200 μ*M*) drops about 10–20-fold compared with that at a pH of 5. Accordingly, further evidence is provided that only the positively charged amino group on the side chain of ampicillin is needed to account for the decreased affinity for the enzyme.

The maximum *S. aureus* Tex-2 β-lactamase activity occurred at a slightly lower pH with ampicillin than with penicillin G (6.4 instead of 6.8). The difference probably involves the state of ionization of the enzyme–substrate complex. The maximum rate (V_{max}) was shown to be nearly halved at pH 5 and decreased about 10-fold at pH 9 compared with the value at the pH optimum (6.4). The decreased V_{max} at pH's other than optimum observed in the present studies may have been due to a conformational change or to the state of ionization of the enzyme–substrate complex rather than to an acid–base inactivation of the enzyme protein.

Effect of Temperature on Rate—Experiments were conducted on the *S. aureus* Tex-2 β-lactamase-catalyzed hydrolysis of penicillin

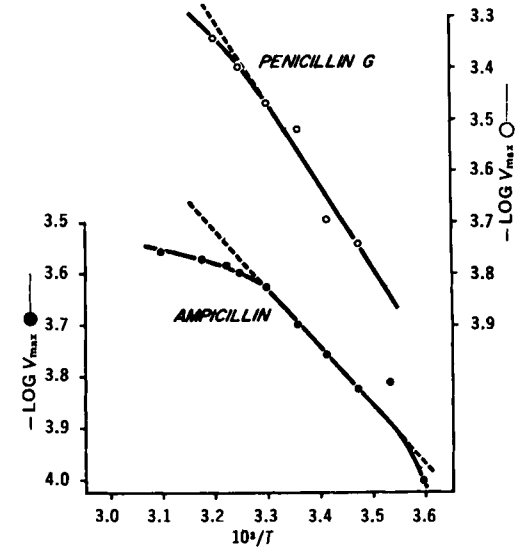


Figure 6—*Effect of temperature on the maximal rate* (V_{max}) *of the* S. aureus *Tex-2 β-lactamase-catalyzed hydrolysis of two penicillins. The logarithm of the maximal rate (log* V_{max}*) is plotted against the reciprocal of the absolute temperature (10³/T°). Ampicillin was hydrolyzed at pH 7.4 with 2.5 units of the enzyme; penicillin G was hydrolyzed at 6.8 with 5.0 units of the enzyme.*

Table II—Effect of Temperature on *S. aureus* Tex-2 Kinetic Parameters[a]

Temperature	Penicillin G		Ampicillin	
	$K_m \times 10^6$ *M*	$V_{max} \times 10^6$ *M*/min.	$K_m \times 10^6$ *M*	$V_{max} \times 10^6$ *M*/min.
5°	—	—	800	100
10°	—	—	750	155
15°	200	180	650	160
20°	80	202	600	175
25°	35	268	460	200
30°	25	340	350	235
35°	30	398	320	245
37°	—	—	300	258
40°	50	455	—	—
42°	—	—	340	267
50°	—	—	500	278

[a] Substrate concentration ranging from 40 to 240 μmoles ($2 \times 12 \times 10^{-3}$ *M*) and a total ionic strength of 0.026 were maintained closely for each penicillin. The *S. aureus* Tex-2 catalyzed hydrolysis of penicillin G and ampicillin was conducted at pH 6.8 and 7.4, respectively. The strength of the enzyme was 5.0 units for penicillin G and 2.5 units for ampicillin.

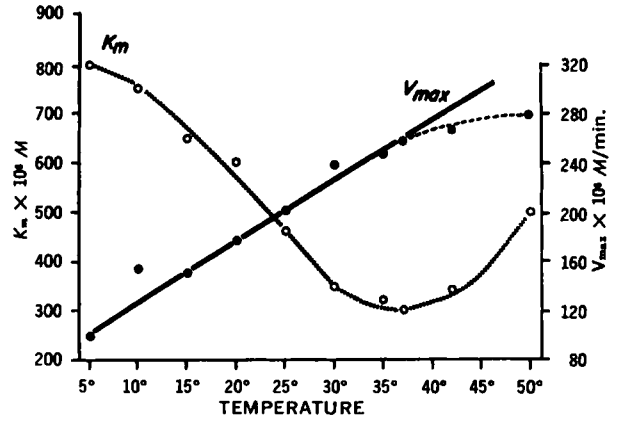


Figure 7—*Effect of temperature on the* S. aureus *Tex-2 β-lactamase-(2.5 units) catalyzed hydrolysis of ampicillin at pH 7.4. The Michaelis constant* (K_m) *is shown on the left-hand scale; the maximum rate* (V_{max}) *is shown on the right-hand scale.*

G at 15–40° and pH 6.8 and of ampicillin at 5–50° and pH 7.4. A constant enzyme concentration was maintained (5.0 units for penicillin G and 2.5 units for ampicillin) as well as a nearly constant ionic strength (0.026 *M*), but the substrate concentration was varied ($2\text{–}12 \times 10^{-3}$ *M*). The V_{max} values for different temperatures were obtained from Lineweaver–Burk plots (Eq. 2). Figure 6 was constructed by plotting log V_{max} *versus* $1/T$ for both penicillin G and ampicillin. The heats of activation for the staphylococcal β-lactamase hydrolysis of these two antibiotics, as calculated from the Arrhenius equation, were 7.18 and 5.17 kcal./mole, respectively.

The *B. cereus* B569 β-lactamase- (5.0 units) catalyzed hydrolysis of ampicillin was also investigated at 4–45° and pH 6.2. From the plot of the logarithms of the initial rates *versus* the reciprocals of the absolute temperatures (not shown), the heat of activation for this hydrolysis was 5.10 kcal./mole.

The curves plotted in Fig. 6 show a slight downward concavity, which indicates that either the breakdown of the enzyme–substrate complex or the affinity of the substrate for the enzyme, or both, may depend on the heat of activation over the indicated range of temperatures. The room temperature range of 15–30° was chosen for calculating the heat of activation, simply because within this range the rates of hydrolysis fall essentially on a straight line. As the temperature was increased toward the so-called "optimum" for the enzyme, the heat of activation dropped to values somewhat lower than those in the room temperature region. For penicillin G at 30–40°, it dropped from 7.18 to about 5.0–6.0 kcal./mole. At 15° and below, on the other hand, it increased from 7.18 to about 9.5 kcal./mole. Similar variations were noted for the interactions of ampicillin with *S. aureus* Tex-2 and *B. cereus* B569 β-lactamases.

Prior to the present study, Smith and Hamilton-Miller (31) had already presented data showing that the heat of activation of the penicillin hydrolysis is higher when catalyzed by β-lactamases from Gram-positive than from Gram-negative bacteria (7–9 *versus* about 3–5 kcal./mole). No one had previously demonstrated, however, that the heat of activation also varies from enzyme to enzyme (species) in accordance with the temperature and the type of substrate used. The variance of heat of activation with temperature will be discussed further in the following section.

Effect of Temperature on Kinetic Parameters—With the pH for the *S. aureus* Tex-2 β-lactamase-catalyzed hydrolysis of ampicillin held at 7.4 and of penicillin G held at 6.8, the temperature was varied and the resulting kinetic parameter values, were obtained (Table II). The affinity of penicillin G and ampicillin for the enzyme (as shown by the decrease in K_m) increased with the increase in temperature, the highest affinity falling in the region of 30–35° for penicillin G and 35–37° for ampicillin and thereafter decreasing with any further increase in temperature. Similarly, the V_{max} increased proportionately with an increase in temperature up to the body temperature region for both penicillins, and then the rate increased only slightly with a further increase in temperature. The effect of temperature, like pH and the penicillin side chain, was shown to be definitely greater on the K_m than on the V_{max}. Figure 7 clearly depicts the effect of temperature on the kinetic parameters of the *S. aureus* Tex-2 β-lactamase-catalyzed hydrolysis of ampicillin at pH 7.4. In this system, both the rate and affinity are at a maximum at an apparent temperature optimum of the body temperature (35–37°).

The heat of activation varying with temperature in β-lactamase kinetics is not unexpected, since β-lactamases, like other biologically active enzymes, are proteinaceous macromolecules. The active sites on the enzyme and their three-dimensional structures are extremely sensitive to environmental factors, particularly pH and temperature. The observed high and low heat of activation values at relatively lower and higher temperatures in comparison with that at room temperature in the present staphylococcal β-lactamase studies was believed due to the physical structure changes of the enzyme. It is conceivable that at the apparent optimal conditions (pH optimum and temperature optimum) of an enzyme, its active form molecules are overwhelming; accordingly, a faster reaction rate, higher affinity for a substrate, and lower heat of activation for the reaction are characteristic.

SUMMARY

The *S. aureus* Tex-2 β-lactamase-catalyzed hydrolysis of several penicillins was shown to follow Michaelis–Menten kinetics. Variations in side-chain structure as well as pH and temperature had a much greater effect on the K_m than on the V_{max}. Either a positively or negatively charged group on the penicillin side chain caused a drastic decrease in binding to the active sites of the enzyme. The α-aminopenicillins were the most readily hydrolyzed of the penicillins studied. Some side-chain groups may introduce a steric effect which renders the penicillin molecule less accessible to the enzyme, or the same reduced accessibility may result from esterification of the penicillin nucleus carboxyl group. The effect of a neutral salt on the enzyme kinetics was insignificant, but the effect of pH was conspicuous. At 25°, a V-shaped pH–K_m profile with a pH optimum at 6.8 was obtained for penicillin G, while a sigmoid-shaped pH–K_m profile with a maximum rate at pH 6.4 was obtained from ampicillin. In the temperature range of 15–30°, the heat of activation values for the *S. aureus* Tex-2 β-lactamase-catalyzed inactivation of penicillin G and ampicillin were 7.18 and 5.17 kcal./mole at pH's of 6.8 and 7.4, respectively, while that for the *B. cereus* B569 β-lactamase-catalyzed hydrolysis of ampicillin was 5.10 kcal./mole at pH 6.2. The heat of activation varies from enzyme to enzyme species in accordance with temperature and the type of substrate used. The temperature optimum for the *S. aureus* Tex-2 β-lactamase may be 35–37°.

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Fenfluramine Blockade of CNS Stimulant Effects of Amphetamines

HARVEY J. BERGER▲, CLINTON C. BROWN, and JOHN C. KRANTZ, Jr.

Abstract □ Fenfluramine, 15 mg./kg. s.c., produced a slight but significant decrease in spontaneous motor activity in male Swiss mice as measured in a circular photocell activity cage. Treatment with fenfluramine 15 min. prior to either dextroamphetamine or methamphetamine, 2.5 mg./kg. i.p., significantly diminished the hyperactivity caused by these compounds. In a second experiment in mice, fenfluramine prolonged pentobarbital sleeping time, whereas dextroamphetamine and methamphetamine shortened barbiturate narcosis. Pretreatment with fenfluramine reversed the reduction of pentobarbital sleeping time induced by amphetamine. In a final experiment, it was found that fenfluramine did not produce amphetamine stereotypy in male Wistar rats. Fenfluramine administered prior to dextroamphetamine or methamphetamine, however, prevented the appearance of typical compulsive gnawing behavior in 80% of the animals. These results, using three different indexes of CNS excitation, demonstrate that fenfluramine reliably antagonizes amphetamine-induced stimulation in mice and rats. The observed reduction of the CNS activity of amphetamines by fenfluramine points to a possible clinical application of this agent in the prophylactic management of amphetamine abuse. Preliminary clinical trials using various psychological tests tend to confirm that fenfluramine decreases the effects of dextroamphetamine in man.

Keyphrases □ Amphetamine-induced CNS stimulation—blockade effect of fenfluramine, rats, mice □ Fenfluramine blockade—amphetamine-induced CNS stimulation, rats, mice □ CNS stimulation, amphetamine induced—blockade effect of fenfluramine, rats, mice

The anorexigenic agent fenfluramine, *N*-ethyl-α-methyl-*m*-(trifluoromethyl)phenethylamine hydrochloride, differs from its structural analog amphetamine (1–3) in that it does not produce CNS stimulation yet may produce a sedative action (4). Furthermore, fenfluramine reduction of amphetamine toxicity in grouped mice has been demonstrated (5).

Recently, Jonsson *et al.* (6) showed the blockade of intravenous amphetamine euphoria in man by oral pretreatment with α-methyl-*p*-tyrosine, an inhibitor of

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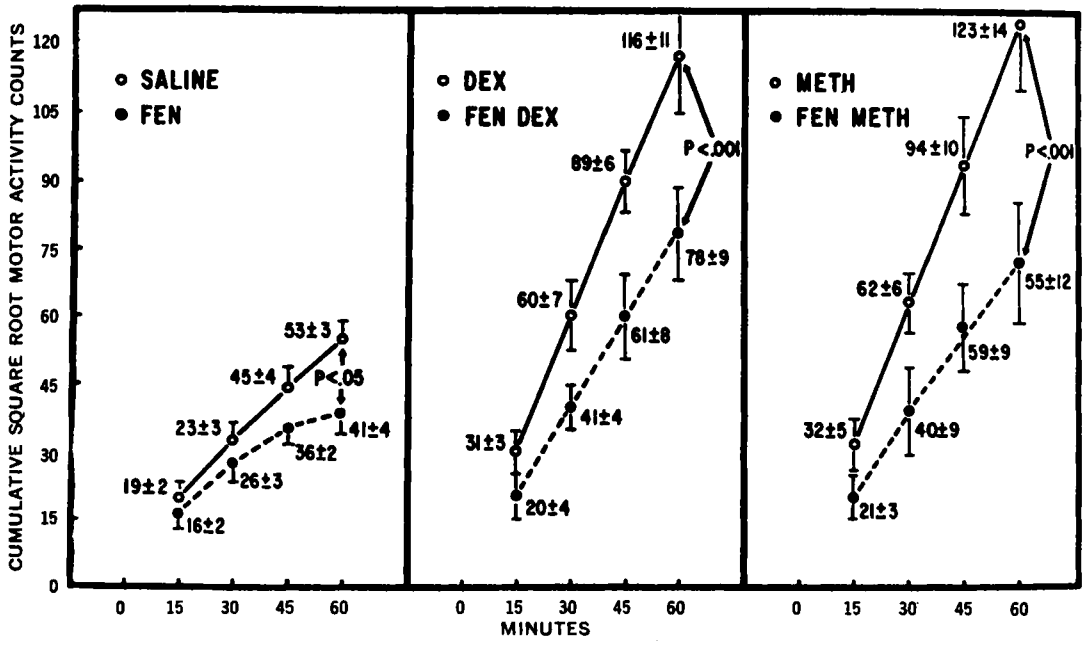

Figure 1—*Effect of fenfluramine (FEN) on dextroamphetamine- (DEX) and methamphetamine- (METH) induced increase in spontaneous motor activity in mice. Spontaneous motor activity counts are presented in a cumulative fashion and expressed in square roots as a function of time. Each value represents the mean ± standard deviation for 20 mice. Fenfluramine was administered at 15 mg./kg. s.c., with dextroamphetamine and methamphetamine at 2.5 mg./kg. i.p. Fenfluramine was injected 15 min. prior to dextroamphetamine or methamphetamine.*

tyrosine hydroxylase. This finding suggests the use of α-methyl-*p*-tyrosine as a drug-blocking agent in amphetamine abuse.

The results of four separate experiments in the present study show that fenfluramine pretreatment antagonizes the CNS stimulant properties of dextroamphetamine sulfate and methamphetamine in mice, rats, and man. The effects of fenfluramine on three amphetamine-induced responses were studied: (*a*) increased spontaneous motor activity in mice, (*b*) decreased pentobarbital sleeping time in mice, and (*c*) typical stereotyped behavior in rats.

EXPERIMENTAL

Male Swiss white CD-1 mice[1], 32–38 g., and male Wistar rats[1], 250–300 g., were used. Mice were caged in groups of eight 4 weeks prior to testing. Rats were isolated in individual cages in a room separate from mice. Animals were maintained at 23–26° on laboratory food[2] and water *ad libitum*. Housing and experimentation were performed in the same room with regulated 12-hr. light and dark cycles.

Fenfluramine was administered at 15 mg./kg. s.c.; dextroamphetamine and methamphetamine were administered at 2.5 mg./kg. i.p. Controls received 0.2 ml. of the vehicle, physiologic saline. The routes of administration were chosen to ensure that fenfluramine would not merely interfere with the uptake and absorption of the amphetamines from the peritoneal cavity.

The experimental design called for the random selection of 20 animals for each of six treatment groups as outlined in Figs. 1–3. In all three experiments, fenfluramine was injected 15 min. prior to either dextroamphetamine or methamphetamine.

Spontaneous Motor Activity—Three circular photocell activity cages with six beams[3], 33 cm. in diameter, were utilized simultaneously. The circuits were wired so that activation of any photocell was independent of the other beams. Spontaneous motor activity counts were registered on inaudible digital counters during four 15-min. trial periods, totaling 1 hr. Individual mice were placed in the activity cages immediately following the second drug injection, approximately 3 hr. after the start of the light cycle.

Pentobarbital Sleeping Time—The time from the loss to the regaining of the righting reflex was recorded as the "sleeping time." Whenever one animal was checked for the return of the righting reflex, animals still asleep were also checked to standardize external stimulation. Equal numbers of mice in the compared drug treatments were injected simultaneously.

Amphetamine Stereotypy—One hour following the first drug administration, a trained observer, unfamiliar with the agents injected, recorded upon observation for 5 min. the presence or absence of typical amphetamine-induced gnawing behavior in naive rats (7). Continuous sniffing and small head movements with periodic exploratory activity were chosen as indicative of standard stereotypy.

RESULTS

The effect of fenfluramine on amphetamine-induced increase in spontaneous motor activity in mice is shown in Fig. 1. Results are expressed as mean cumulative square root counts with standard deviations. The data were transformed because the frequency distribution of raw scores is usually skewed in the positive direction, with higher scores being farther from the mean than the lower scores. Thus, skewness of the distribution is minimized by taking square root scores (8, 9).

Fenfluramine-treated mice produced a mean of 40.9 ± 8.6 motor activity counts in 60 min. as compared to 52.7 ± 9.8 for saline controls. This significant difference ($p < 0.05$) reflects the sedative action of fenfluramine in contrast to the stimulant effect of its structural analog, dextroamphetamine. Such a finding is in agreement with that of Ziance *et al.* (4), indicating that fenfluramine showed no signs of CNS stimulation. However, both dextroamphetamine and methamphetamine significantly increased ($p < 0.001$) spontaneous motor activity to 116.4 ± 16.2 and 123.3 ± 8.9 counts, respectively, in the hour. Fenfluramine treatment prior to either dextroamphetamine and methamphetamine significantly lowered ($p < 0.001$) the amphetamine-induced increase in random motor activity to 77.9 ± 12.7 counts. While either dextroamphetamine or methamphetamine caused a 130% increase above saline control, fenfluramine limited this increment to only 46%, indicating that fenfluramine is able to block partially the hyperactivity produced by amphetamines.

[1] Charles River Breeders.
[2] Purina.
[3] Actophotometers, Metro Industries.

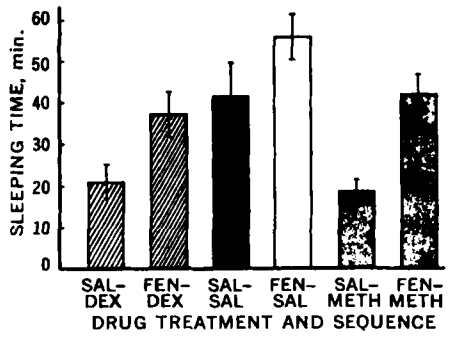

Figure 2—*Effect of fenfluramine (FEN) on dextroamphetamine- (DEX) and methamphetamine- (METH) induced antagonism of pentobarbital sleeping time in mice. Pentobarbital sleeping times are expressed as time in minutes from loss to regaining of the righting reflex. Each value represents the mean ± standard deviation for at least 20 mice. Fenfluramine was administered at 15 mg./kg. s.c., with dextroamphetamine and methamphetamine at 2.5 mg./kg. i.p. Fenfluramine was injected 15 min. prior to dextroamphetamine or methamphetamine. All mice received pentobarbital (40 mg./kg. i.p.) as the third injection. The dashed bars represent animals that received dextroamphetamine, while the shaded bars represent animals that received methamphetamine. The solid and open bars are both controls. SAL = saline.*

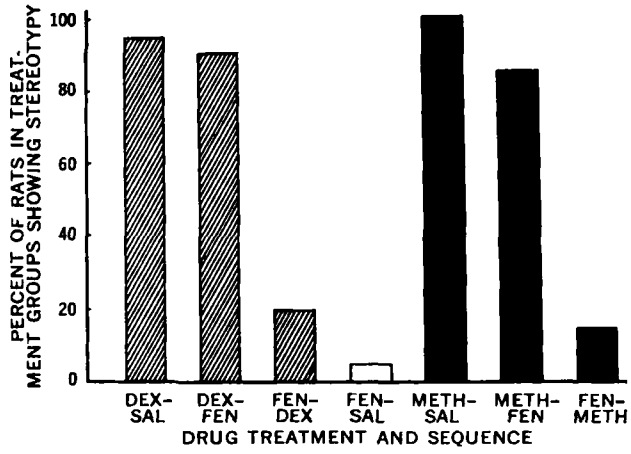

Figure 3—*Effect of fenfluramine (FEN) on amphetamine stereotypy in rats. The presence of compulsive gnawing behavior in rats following drug treatments is expressed as percent of the total number of animals in each group. Drug treatments consisted of 20 rats. Fenfluramine was administered at 15 mg./kg. s.c., with dextroamphetamine (DEX) and methamphetamine (METH) at 2.5 mg./kg. i.p. Fenfluramine was injected 15 min. prior to dextroamphetamine or methamphetamine. Observations were made 1 hr. following the first drug administration. SAL = saline.*

In mice treated with saline or fenfluramine, a large proportion of the counts was recorded within the initial 15-min. period, suggesting that much of the spontaneous activity was exploratory in nature. However, mice receiving dextroamphetamine or methamphetamine showed a more uniform distribution of activity counts over the four 15-min. periods. Thus, the spontaneous motor activity measured by the photocell cages reflects increased stimulation and not merely increased general exploration.

As shown in Fig. 2, control mice receiving pentobarbital alone slept for 42 ± 8.1 min. Pretreatment with fenfluramine produced a significant increase ($p < 0.01$) in pentobarbital sleeping time to 56 ± 6.1 min. Conversely, dextroamphetamine and methamphetamine each decreased pentobarbital sleeping time to 21 ± 3.5 and 19 ± 2.7 min., respectively. When fenfluramine was administered prior to dextroamphetamine or methamphetamine, the sleeping time induced by pentobarbital was essentially the same as that evoked by the barbiturate alone (37 ± 5.2 or 43 ± 3.9 min., respectively). Because of the significant increase in pentobarbital sleeping time following fenfluramine (from 42 to 56 min.), it would appear that the depressant properties of fenfluramine were principally responsible for this antagonism.

The experiments on amphetamine stereotypy (Fig. 3) yielded further evidence of blockade by fenfluramine of the CNS activity of amphetamines. Fenfluramine was observed to induce gnawing behavior in only one of 20 rats, while dextroamphetamine and methamphetamine produced typical stereotypy in almost all animals observed (19/20 and 20/20, respectively). When administered prior to amphetamines, fenfluramine prevented the appearance of the stereotyped behavior in 80% of the rats. However, when fenfluramine was given after dextroamphetamine or methamphetamine, no antagonism was evident, and the animals exhibited standard signs of amphetamine stereotypy. This would suggest that the blockade produced by fenfluramine might be due to interference with receptors specific to amphetamine activity.

DISCUSSION

The precise mechanism by which fenfluramine produces the observed blockade of dextroamphetamine and methamphetamine needs further elucidation. It has been reported (5) that fenfluramine does not antagonize all of the effects of amphetamines. For example, fenfluramine neither depletes brain norepinephrine nor does it alter elevated levels of free fatty acids evoked by amphetamines. Whether the antagonism by fenfluramine of dextroamphetamine and methamphetamine is due to changes in the metabolism of amphetamine leading to the formation of a physiologically active metabolite or to interaction with the adrenergic receptor mechanism is not yet known.

In the motor activity study, individual mice were used in the photocell cages, instead of the more common groups of three to five, because of the effects of group size on amphetamine toxicity and activity (10, 11). All experiments were carried out at the same time of day, because circadian rhythm and the time of testing relative to the length of the light and dark cycles have been shown to affect amphetamine responses (12).

Amphetamines administered orally (10–30 mg.) in man evoke stimulation of the sensory cortex, which is manifested in increased alertness, motor and speech activity, and varying degrees of euphoria (13). In addition, characteristic stereotyped behavior shown by repetitious ponderous movements is common. Each of the three experimental parameters in mice and rats was chosen to parallel a clinical expression of amphetamine use.

The observed acute blockade of the psychoactivity of amphetamines by fenfluramine might suggest an application in man in the prophylactic blockade of the euphoric effects of high doses of amphetamines. To determine whether fenfluramine is actually valuable as a drug-blocking agent in amphetamine abuse, long-term administration of fenfluramine is required; such studies are now planned in both experimental animals and man.

A double-blind study on a fenfluramine–amphetamine combination in man involving a battery of psychomotor performance tasks was recently reported (14). Preliminary results indicate that: (*a*) fenfluramine alone was insignificantly different from placebo for each of the tasks, (*b*) fenfluramine and dextroamphetamine were significantly different from each other in four of eight tests, and (*c*) the fenfluramine–amphetamine combination produced effects similar to, but *less marked* than, those produced by dextroamphetamine alone.

Fenfluramine had a sedative and dysphoric effect but was not able to suppress completely the amphetamine-induced changes in psychomotor task performance. The possible clinical implications of this work in the blockade of amphetamine abuse are being considered.

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Dehydration of Crystalline Theophylline Monohydrate and Ampicillin Trihydrate

ELI SHEFTER▲, HO-LEUNG FUNG, and OLIVIA MOK

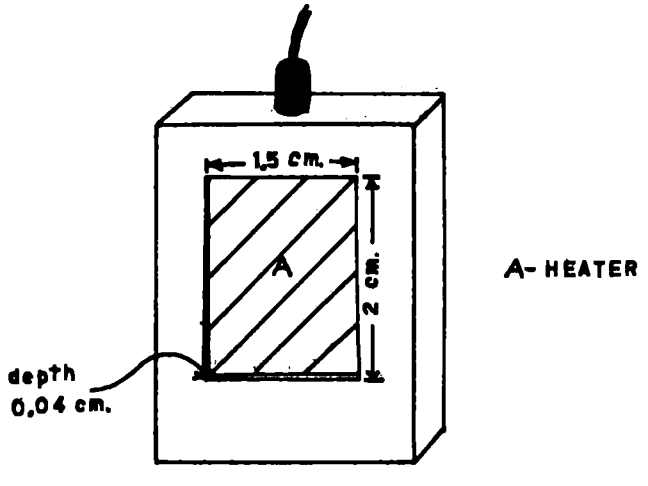
Abstract □ The dehydration kinetics of theophylline monohydrate and ampicillin trihydrate were measured by an X-ray powder diffraction technique in an open system. In the case of theophylline, the hydrate transforms directly to a crystalline anhydrous form with apparent zero-order kinetics. The loss of water from ampicillin trihydrate results in an amorphous state. Commercial micronized ampicillin trihydrate, which contains a small amount of excipients, exhibits a different kinetic order and a faster rate of transformation.

Keyphrases □ Theophylline monohydrate—dehydration kinetics measured using X-ray powder diffraction technique □ Ampicillin trihydrate—dehydration kinetics measured using X-ray powder diffraction technique □ Solid-state phase transformations—dehydration of theophylline monohydrate and ampicillin trihydrate studied using X-ray powder diffraction technique □ X-ray powder diffraction technique—used to measure dehydration kinetics for theophylline monohydrate and ampicillin trihydrate □ Phase stability, theophylline monohydrate and ampicillin trihydrate—dehydration kinetics studied using X-ray powder diffraction technique

The solid-state phase transformation of the active ingredient in a dosage form could dramatically alter the pharmaceutical properties of the preparation. The solid phase of the administered drug can influence such important properties as bioavailability (1). Where highly energetic (metastable) forms are incorporated into the formulation, it is exceedingly important that, in addition to chemical stability, the phase integrity of the pharmaceutical be monitored.

Many organic medicinal agents are known to crystallize with solvent molecules as an integral part of their structure. The most widely found group of solvates are the hydrates. The phase stability of these multicomponent solids is governed by temperature, pressure, and the concentration of solvent in the system. However, even though thermodynamics might indicate that a particular solvate is metastable under normal storage conditions, the conversion rate to a desolvated phase could be relatively slow in pharmaceutical terms. The rates at which these desolvation processes take place are exceedingly important to the formulation.

The desolvation process of two crystalline hydrates (theophylline monohydrate and ampicillin trihydrate) was studied using an X-ray powder diffraction method. This investigation is part of a continuing study to delineate the various physical parameters controlling the kinetics of solid-state phase transformations. The principal point of this study is to demonstrate the scope of



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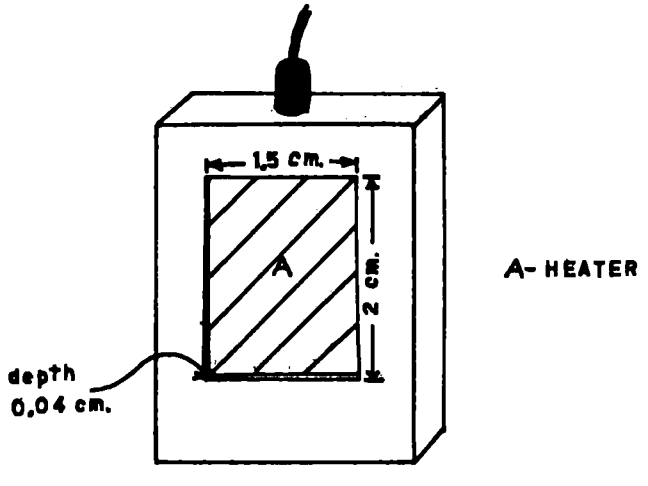

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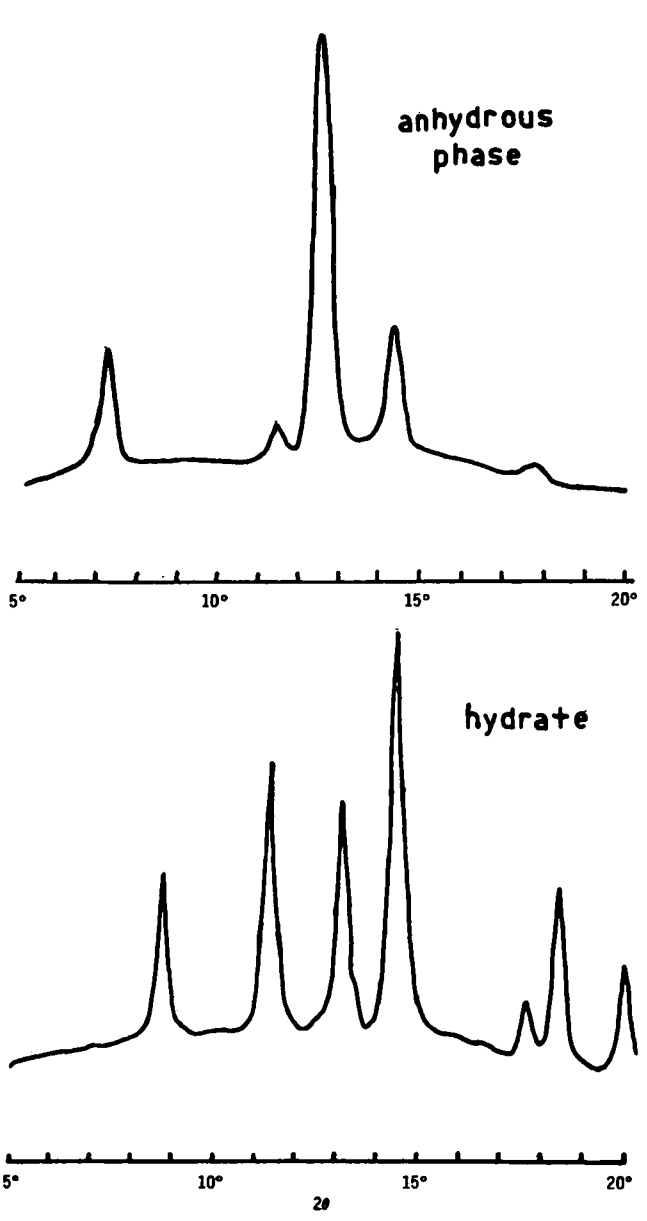

Figure 2—*Portions of the X-ray powder patterns for theophylline monohydrate and an anhydrous form.*

the X-ray powder technique for routine control of phase stability.

EXPERIMENTAL

Materials—Theophylline USP was recrystallized from water. After filtering, the crystals were ground to a fine powder in a mortar and stored in a tightly stoppered container.

Ampicillin trihydrate and the two anhydrous polymorphs were used[1]. The trihydrate sample consisted of very small needles and had a measured potency of 842 mcg./mg. (biological assay based on anhydrous ampicillin). A commercial sample of ampicillin trihydrate[2] (capsule form) was also used. Microscopic examination revealed that the ampicillin material was in a micronized state in the capsules. Weight analysis of the capsules indicated that a small amount of excipient was present (approximately 7% by weight).

No attempt was made to measure the particle-size distribution in each batch of material, but the same batch was used for each set of kinetic studies.

[1] Batch Nos. 71F1064 (trihydrate), 67F3041 (form I), and 68F2199 (form II), supplied by Bristol Laboratories, Syracuse, N. Y.

[2] Penbritin, Batch No. A60410L, 500-mg. capsules, supplied by Ayerst Laboratories, Rouses Point, N. Y.

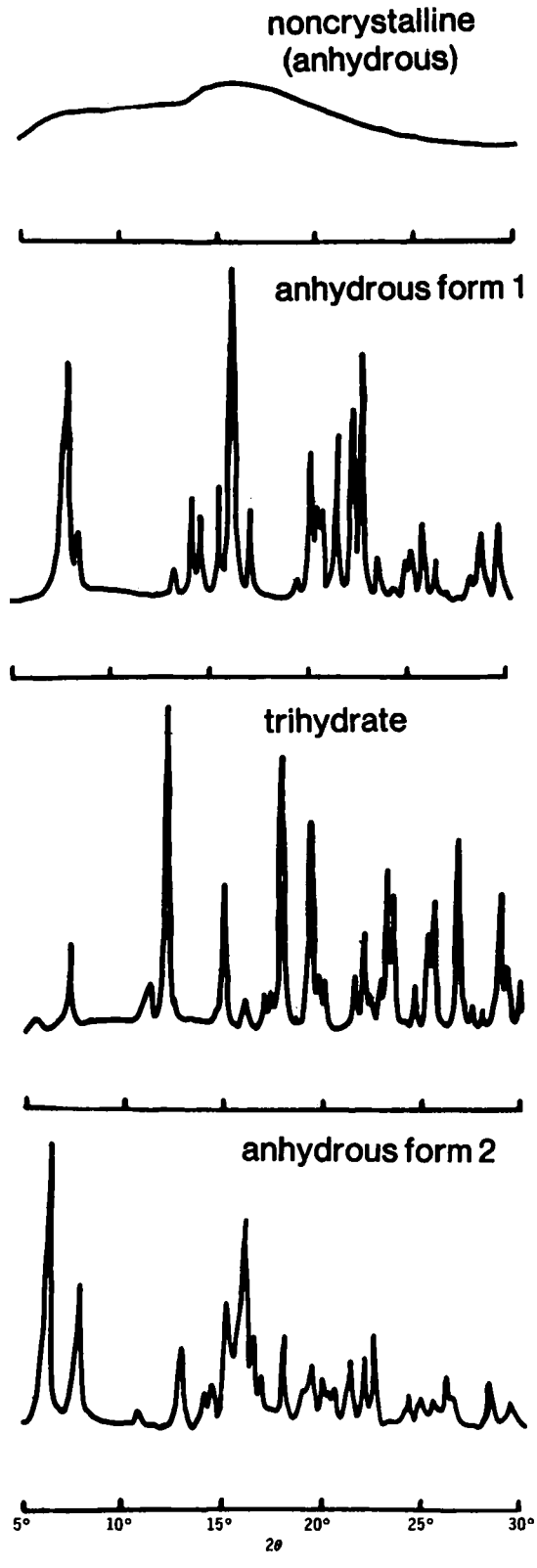

Figure 3—*Diffractograms for various forms of ampicillin.*

X-ray diffraction patterns run on all starting materials were used as controls. In the case of the hydrates, no evidence of any anhydrous form was observed.

Apparatus—All X-ray powder patterns were collected on a diffractometer[3]. Nickel-filtered CuKα radiation was used.

[3] Toshiba ADG-301 Diffpet. The detector used was a Geiger–Mueller type.

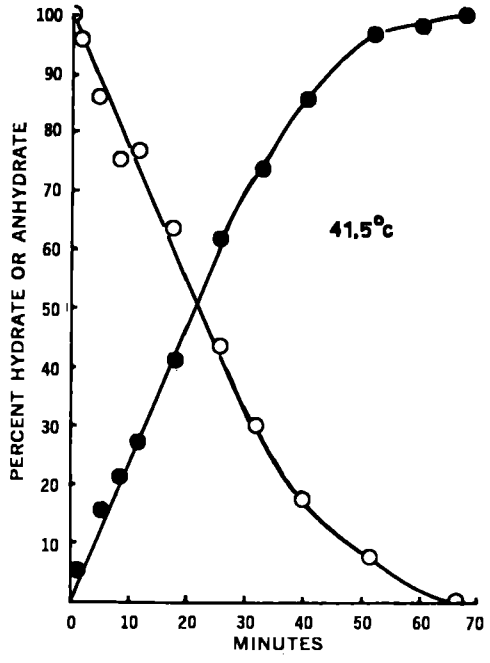

Figure 4—*Fraction (in percent) of monohydrate* (○) *and anhydrous* (●) *forms in powder as a function of time.*

A sample holder, made of Bakelite, was designed to maintain the powder bed at a relatively constant temperature (Fig. 1). The temperature on the underside of the powder was maintained to ±0.5° of the set temperature. By placement of a thermocouple on the powder surface, the temperatures on the two sides were monitored. Since the atmospheric temperature in the diffraction chamber was not controlled in these experiments (room conditions 25 ± 3°), the surface material exhibited a lower temperature than those particles in contact with the heater. The temperature differential remained fairly constant throughout each experiment. The difference was greater at higher temperatures: approximately 10° when the heater was maintained at 95° and less than 2° when set at 38°.

X-Ray Powder Patterns—The X-ray powder diffraction patterns for theophylline monohydrate and the crystalline anhydrous form into which it directly converts are shown in Fig. 2. Ampicillin trihydrate was found to transform to an amorphous anhydrous state. (Diffraction patterns for these forms are shown in Fig. 3.) During the experiment with ampicillin, it was of interest to see if the two crystalline anhydrous forms entered into a phase transformation in the temperature range studied; their X-ray diffractograms are also illustrated in Fig. 3.

Kinetic Study—The sample to be studied was firmly packed into the holder with a spatula. After placing the holder in the diffraction chamber, a zero-time diffractogram of the material was recorded and then the heater and timer were initiated. The diffraction pattern for theophylline (between 10.5 and 14.5° in 2θ) was recorded at various time intervals. For the theophylline studies, the disappearance of a hydrate diffraction maximum at 11.3° (2θ) and the appearance of an anhydrate crystal peak at 12.6° (2θ) were simultaneously followed as a function of time. In the case of ampicillin, only the disappearance of a trihydrate peak could be followed (maximum at 12.2° in 2θ was selected) because the material transformed to an amorphous powder. The fractional amount of each species relative to the zero time (100% hydrate) was derived from the ratio of peak intensities over background in a manner similar to that described by Alexander and Klug (2). The linear absorption coefficients and

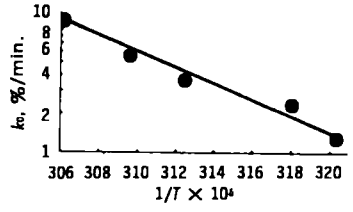

Figure 5—*Arrhenius plot of zero-order dehydration of theophylline monohydrate.*

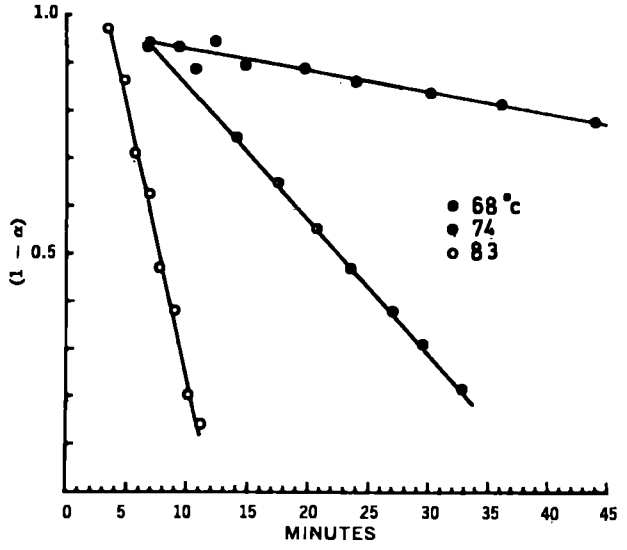

Figure 6—*Fraction of crystalline ampicillin trihydrate remaining in heated sample as a function of time.*

density of the dehydrated material were assumed to be approximately equal to the hydrated samples.

The time necessary for the sample holder to reach a prescribed temperature was approximately 5 min. at 95° and much less at lower temperatures. Thus, the initial points on the dehydration plots could not be relied on. In general, the kinetic plots indicated a lag time that reasonably reflected the lag time required to achieve temperature equilibrium. The temperature ranges (lower surface) used in these studies were 68–95° for ampicillin and 38–54° for theophylline.

RESULTS AND DISCUSSION

A typical experimental dehydration plot for theophylline is shown in Fig. 4. The observed rates of appearance of the anhydrous form

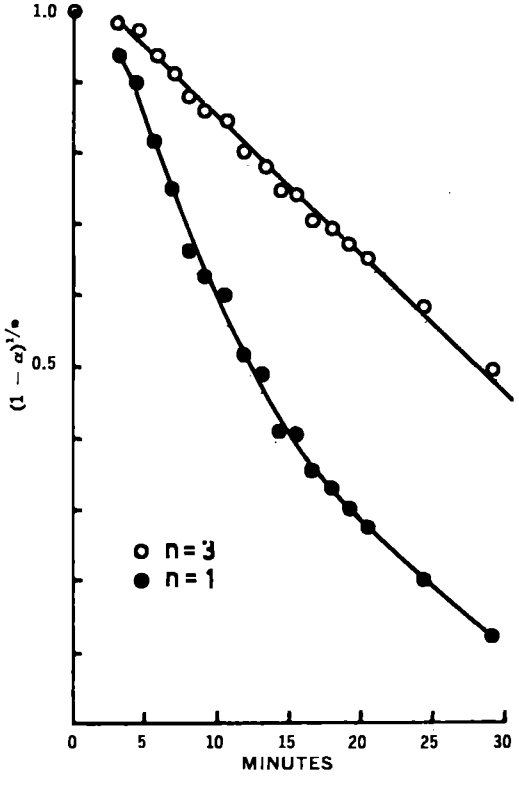

Figure 7—*Fraction of ampicillin trihydrate present in the capsule formulation (1 − α) at various times at 74.5° (plotted in two ways).*

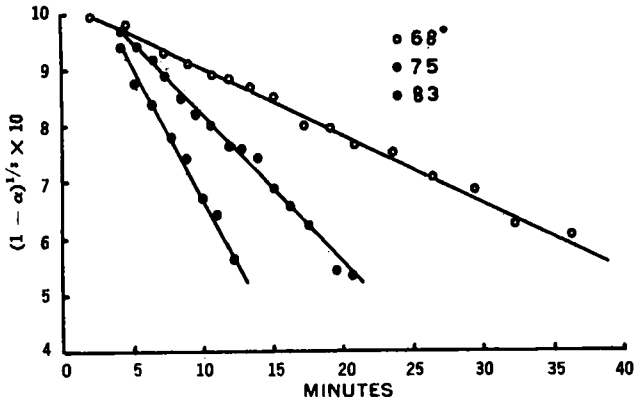

Figure 8—*Kinetic data for ampicillin trihydrate capsule formulation plotted by diminishing sphere model.*

and the disappearance of the hydrate were essentially the same. The 1:1 relationship need not occur because physical intermediates can form in some systems (3). The initial transformation rate appeared to be zero order for at least 1 half-life at all of the temperatures studied. Many solid-state dehydrations typically show a decrease in rate as the anhydrous material accumulates on the surface (4). Since the theophylline hydrate used in these studies was composed of fine needles, the retardation of the dehydration process did not appear to take place as rapidly as it probably would for spheres and flat platelets. The activation energy for the dehydration process can be estimated from an Arrhenius plot (Fig. 5). A value of approximately 140 kJ./mole was obtained.

In a few experiments carried out with nitrogen (dried over sulfuric acid) constantly flushed through the reaction chamber vessel, the zero-order rate constant increased slightly. This observation is expected inasmuch as any decrease in water vapor pressure should promote the reaction: theophylline hydrate ⇄ theophylline (anhydrous) + water (vapor).

The needle-shaped crystals of ampicillin trihydrate generally exhibited zero-order decomposition (Fig. 6). In the case of the capsule formulation, this was not found (Fig. 7). A good fit of the experimental data was obtained using the "diminishing sphere" model (4) for this material. The equation for this model is:

$$(1 - \alpha)^{1/3} = -k_{d_s} \cdot t + A \qquad \text{(Eq. 1)}$$

where α is the fraction transformed, k_{d_s} is the transformation rate constant, t is the time, and A is a constant associated with the lag time involved in the attainment of thermal equilibrium. The data plotted in this manner are shown in Fig. 8.

Microscopic examination of the ampicillin trihydrate samples showed that the commercial preparation contained micronized particles which were fairly equiaxial (spherical).

Both ampicillin trihydrate samples are converted to an amorphous anhydrous light-yellow powder. A bioassay run on an ampicillin trihydrate sample (capsule formulation held at 65° until it turned amorphous) suggested that a significant amount of decomposition (approximately half) had taken place[4]. It was of interest to see if, in the temperature range of this study, any physical or chemical degradation takes place in the two anhydrous forms. No significant changes in the diffractograms (distribution of peaks and their intensities) were found on maintaining these two forms at 90° for 24 hr. A detailed analysis of the decomposition mechanism which takes place in the trihydrate is contemplated.

At room temperature the trihydrate is the most stable form in water (5). It was, therefore, of interest to look at the relative stabilities of the two anhydrous forms in the presence of water. Slurries of the two forms were prepared and their X-ray diffraction patterns

[4] Assay carried out by A. P. Granatek of Bristol Labs., Syracuse, N. Y.

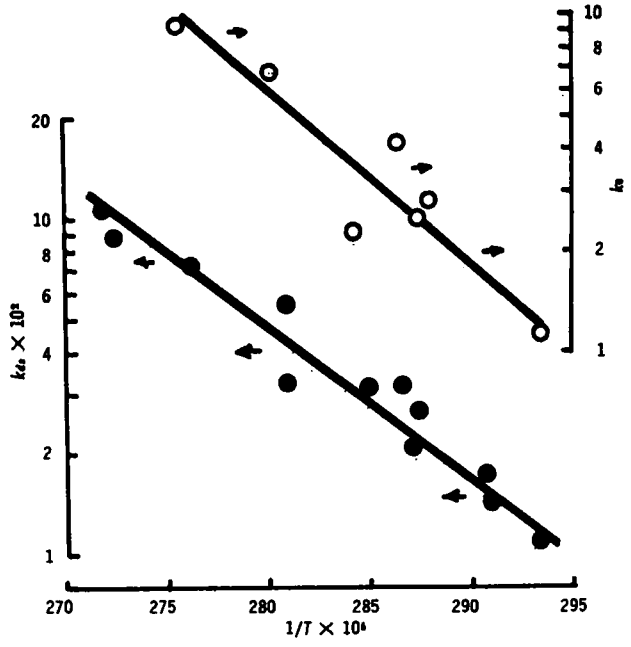

Figure 9—*Arrhenius plots for ampicillin trihydrate dehydration. Key: ●, zero-order k_0 for small needle crystals; and ○, k_{d_s} (diminishing sphere model) of capsule formulation.*

were measured. Form II was found to convert to the trihydrate very rapidly (converted while making slurry). Form I, on the other hand, showed no significant change in its diffraction pattern for 2 days (time of study), which is in agreement with the Poole and Bahal (5) findings.

The activation energies for the two trihydrate samples are essentially the same (estimated to be approximately 95 kJ./mole from the Arrhenius plots shown in Fig. 9). The gradients in these plots are parallel within the limits of experimental error. On a zero-order basis, the commercial preparation exhibits a significantly faster transformation rate; the zero-order rate constant at any temperature studied for the capsule material is nearly twice the value found for the other sample. The observed differences could result from the marked differences in particle size and/or the presence of the small amount of excipients in the capsule preparation.

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▲ To whom inquiries should be directed.

Dissolution Profile of Log-Normal Powders: Exact Expression

DANA BROOKE

Abstract □ A rigorously correct derivation is given for the time course of dissolution of powders that follow the log-normal probability distribution. The exact expression, based on reasonable assumptions, requires no integration but can be evaluated by use of a calculator and readily available mathematics tables. The approximations of earlier workers for the dissolution patterns of log-normal powders are tested against the exact equation.

Keyphrases □ Dissolution profiles—log-normally distributed powders, exact derivation □ Powders, dissolution—exact solution of log-normal distribution profile □ Particle dissolution—log-normally distributed powders, exact derivation

Recently, Carstensen and Musa (1) presented approximate dissolution patterns for powders obeying the log-normal distribution law. They, like earlier workers (2, 3), refrained from an exact treatment of this subject because the expressions involved tended to become "analytically unmanageable." Instead, Carstensen and Musa (1) used a computer to gain approximate numerical solutions of their complicated probability expressions.

In this paper, a rigorously correct expression is derived for the dissolution profiles of log-normally distributed powders. The exact expression for weight fraction undissolved with time can be evaluated by use of a calculator and a handbook of mathematics tables including the standardized normal probability distribution. The calculations are tedious but can be accomplished without the use of a computer.

THEORETICAL

Consider a powder containing spherical particles of diameters a_0 which are distributed, on a numbers basis, such that ln a_0 is normal with population mean μ and population standard deviation σ[1]. The probability frequency f of log diameters is given by:

$$f = \frac{1}{\sigma\sqrt{2\pi}} e^{-(\ln a_0 - \mu)^2/2\sigma^2} \qquad \text{(Eq. 1)}$$

When particles are placed in a medium, the diameters diminish as dissolution proceeds. If it is assumed that the particles dissolve isotropically under sink conditions and that the solubility C_s is independent of particle size, then the diameter a_τ of a particle at some function τ of time is described (1) by:

$$a_\tau = a_0 - \frac{2kC_s}{p}t = a_0 - \tau \qquad \text{(Eq. 2)}$$

where a_0 is the initial (time = 0) diameter, k is a dissolution rate constant, t is time, and p is particle density. Here the function τ of time is $2kC_st/p$, which is essentially the notation of Carstensen and Musa (1). Obviously, the diameter is linear with time. The function τ has dimensions of length.

If the total number of particles in the powder is N, then the number dN of particles of initial diameter a_0 is given by:

$$dN = Nfd \ln a_0 \qquad \text{(Eq. 3)}$$

Initially, the contribution dw of particles of diameter a_0 to the total weight of the powder is the weight of one particle times the number in that cut or $\pi pa_0^3 dN/6$. As dissolution continues, the diameters of particles decrease; but as long as $a_\tau > 0$, the number of particles in that cut remains the same. Thus, the contribution to the total weight after some time such that $0 < \tau < a_0$ is:

$$dw = \frac{\pi p}{6}a_\tau^3 dN = \frac{\pi p}{6}(a_0 - \tau)^3 Nfd \ln a_0 \qquad \text{(Eq. 4)}$$

The weight of undissolved solid at τ is given by an integration of Eq. 4 for all remaining particles. The integration is in terms of initial diameters, since the numbers of particles were described on that basis. The lower limit of integration is rationalized by the fact that at any value of τ the smallest remaining particles ($a_0 - \tau \to 0$) were initially of size $a_0 = \tau$. Therefore, the weight w_τ is:

$$w_\tau = \int_{\ln a_0 = \ln \tau}^{\ln a_0 = \infty} \frac{\pi p}{6}(a_0 - \tau)^3 Nfd \ln a_0 \qquad \text{(Eq. 5)}$$

For purposes of integration, τ is a constant.

By expanding the cubic term and letting $r = \pi pN/6$, Eq. 5 becomes:

$$w_\tau = r\int_{\ln \tau}^{\infty} a_0^3 fd \ln a_0 - 3r\tau\int_{\ln \tau}^{\infty} a_0^2 fd \ln a_0 + 3r\tau^2\int_{\ln \tau}^{\infty} a_0 fd \ln a_0 - r\tau^3\int_{\ln \tau}^{\infty} fd \ln a_0 \qquad \text{(Eq. 6)}$$

When considering any of the four integrals in Eq. 6, let there be a number β ($\beta = 0, 1, 2, 3$) such that:

$$I_\beta = \int_{\ln \tau}^{\infty} \frac{a_0^\beta}{\sigma\sqrt{2\pi}} e^{-(\ln a_0 - \mu)^2/2\sigma^2} d \ln a_0 \qquad \text{(Eq. 7)}$$

It is convenient to express a_0^β as $e^{\beta \ln a_0}$ and then to manipulate the resulting integral in much the same manner as is done when developing the moment generating function for the normal distribution (4). First, the powers of e in the integral are collected so that:

$$\beta \ln a_0 - \frac{(\ln a_0 - \mu)^2}{2\sigma^2} = -\frac{[\ln^2 a_0 - 2(\mu + \beta\sigma^2)\ln a_0] + \mu^2}{2\sigma^2} \qquad \text{(Eq. 8)}$$

By completing the square of the bracketed part of Eq. 8, that is, by adding and subtracting $(\mu + \beta\sigma^2)^2$, and collecting terms, the exponent of e becomes:

$$\beta\left(\mu + \frac{\beta}{2}\sigma^2\right) - \frac{[\ln a_0 - (\mu + \beta\sigma^2)]^2}{2\sigma^2}$$

and I_β becomes:

$$I_\beta = e^{\beta(\mu + \beta\sigma^2/2)} \int_{\ln \tau}^{\infty} \frac{e^{[\ln a_0 - (\mu + \beta\sigma^2)]^2/2\sigma^2}}{\sigma\sqrt{2\pi}} d \ln a_0 \qquad \text{(Eq. 9)}$$

The new integral in Eq. 9 is that of a random variable (ln a_0) which is normally distributed with mean ($\mu + \beta\sigma^2$) and standard

[1] The symbols μ and σ in this paper have the sense of population parameters. Since the normal variable, ln a_0, is in (natural) logarithmic terms, so must be μ and σ. This is different from *Reference 1* where the standard deviation is in common log terms. Since the distribution is log-normal, then the population mean μ is equivalent to the natural log of the geometric mean diameter.

Table I—Dissolution Patterns for Log-Normal Powders with a Geometric Mean of 40 μm. (μ = 3.68888) as Calculated from Eq. 13 for Exact and for Truncated (at $\mu + 3\sigma$) Distributions

	Calculated Weight Fraction Remaining			Percent Relative Error[a]
	τ	Exact	Truncated	
σ = 0.03178	0	1.0000	1.0000	—
	5	0.6707	0.6707	—
	10	0.4231	0.4231	—
	15	0.2455	0.2455	—
	20	0.1263	0.1263	—
	25	0.0538	0.0538	—
	30	0.0164	0.0165	—
	36.36(τ_c)[b]	0.0010	0.0010	—
σ = 0.10000	0	1.0000	1.0000	—
	10	0.4337	0.4333	0.1
	20	0.1381	0.1376	0.4
	29.63(τ_c)	0.0255	0.0254	0.4
	30	0.0234	0.0233	0.4
	35	0.0063	0.0059	6.4
σ = 0.30000	0	1.0000	1.0000	—
	5	0.7320	0.7294	0.4
	10	0.5215	0.5173	0.8
	16.26(τ_c)	0.3273	0.3221	1.3
	25	0.1575	0.1521	3.4
	40	0.0372	0.0330	11.3
σ = 0.50000	0	1.0000	1.0000	—
	8.93(τ_c)	0.6929	0.6793	1.0
	10	0.6625	0.6476	2.3
	20	0.4326	0.4097	5.3
	40	0.1831	0.1567	14.4
	80	0.0361	0.0195	46.0
σ = 1.08304	0	1.0000	1.0000	—
	1.55(τ_c)	0.9938	0.9877	0.6
	40	0.8632	0.7380	14.5
	80	0.7594	0.5543	28.9
	120	0.6769	0.4201	38.0
	200	0.5529	0.2438	58.4
	280	0.4634	0.1404	69.8
	350	0.4034	0.0842	79.2

[a] Percent relative error = (exact − truncated)/exact × 100. [b] τ_c refers to the value of τ equivalent to a diameter given by $\ln a_0 = \mu - 3\sigma$.

deviation σ. If $x = [\ln a_0 - (\mu + \beta\sigma^2)]/\sigma$ and $dx = d \ln a_0/\sigma$, then Eq. 9 can be changed to:

$$I_\beta = e^{\beta(\mu+\beta\sigma^2/2)} \int_{x=[\ln\tau-(\mu+\beta\sigma^2)]/\sigma}^{\infty} \frac{e^{-x^2/2}}{\sqrt{2\pi}}\,dx \quad \text{(Eq. 10)}$$

which can be evaluated by use of tables of the standard normal distribution. If:

$$F(x) = \int_{-\infty}^{x} \frac{e^{-x^2/2}}{\sqrt{2\pi}}\,dx \quad \text{(Eq. 11)}$$

then it follows that I_β is to be evaluated by:

$$I_\beta = e^{\beta(\mu+\beta\sigma^2/2)}\left(1 - F\left[\frac{\ln\tau - (\mu+\beta\sigma^2)}{\sigma}\right]\right) \quad \text{(Eq. 12)}$$

where $F\{[\ln\tau - (\mu + \beta\sigma^2)]/\sigma\}$ is a tabled value.

The exact and rigorously correct expression for the weight undissolved, Eq. 6, becomes:

$$w_\tau = re^{3(\mu+3\sigma^2/2)}\left(1 - F\left[\frac{\ln\tau - (\mu+3\sigma^2)}{\sigma}\right]\right) - 3r\tau e^{2(\mu+\sigma^2)}\left(1 - F\left[\frac{\ln\tau - (\mu+2\sigma^2)}{\sigma}\right]\right) + 3r\tau^2 e^{(\mu+\sigma^2/2)}\left(1 - F\left[\frac{\ln\tau - (\mu+\sigma^2)}{\sigma}\right]\right) - r\tau^3\left(1 - F\left[\frac{\ln\tau - \mu}{\sigma}\right]\right) \quad \text{(Eq. 13)}$$

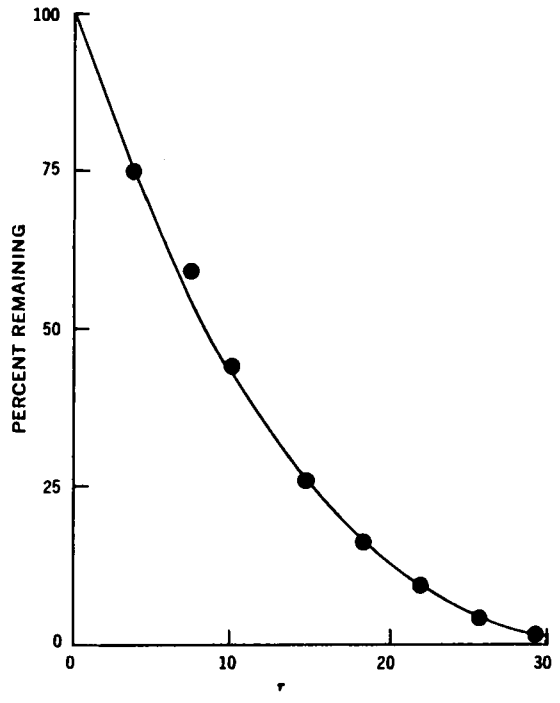

Figure 1—*Dissolution pattern for a log-normal powder with a geometric mean diameter of 40 μm. The population parameters were μ = 3.68888 and σ = 0.03178. The solid line was calculated from Eq. 13. The circles are the Carstensen and Musa (1) estimates for this distribution as reported in their Table II.*

It is clear that an expression of weight fraction undissolved, w_τ/w_0, is a function only of τ, μ, and σ.

RESULTS AND DISCUSSION

The dissolution profiles for five log-normally distributed "powders" were treated for this paper using an electronic programmable calculator[2]. In each case the powder was assigned a geometric mean diameter of 40 μm. (μ = 3.68888). Standard deviations were chosen so that the distributions ranged from rather narrow to very broad. A dissolution pattern, as calculated from Eq. 13, for a powder with σ = 0.03178 is shown in Fig. 1. Such a powder would have 99.74% of its particles in the range 36.36–44.00 μm. This is perhaps a narrower distribution than could be expected for most pharmaceutical powders.

The distribution treated in Fig. 1 is the same one treated by Carstensen and Musa (1) by computer techniques and reported in their Table II. The circles in Fig. 1 are the values of Carstensen and Musa. The excellence of their approximations is obvious.

Carstensen and Musa assumed a powder containing no particles outside of a range within 3 standard deviations from the mean. In practical situations it is probable that many (or even most) actual powders follow truncated distributions. If this be the case, then it is possible that using an ideal (as opposed to truncated) log-normal distribution for actual powders could lead to errors in the calculated dissolution profile. Conversely, when dealing with a powder that is "ideally" log-normal, arbitrary truncation for the convenience of computer fitting could lead to errors. Whether or not significant errors occur because of these considerations depends upon the parameters (μ and σ) of the distribution. More specifically, the larger the value of the standard deviation σ, the more chance that application of the exact equation (Eq. 13) to a truncated distribution will lead to errors. The converse is also true.

Let it be assumed that there is a powder that follows a truncated log-normal numbers distribution, with the smallest particle given by $\ln a_0 = \ln \tau_c = \mu - c\sigma$ and the largest particle given by $\mu + c\sigma$, where c is a constant. Use of the ideal log-normal distribution

[2] Monroe 1665.

Table II—Dissolution Patterns as Calculated Exactly by Eq. 13, as Calculated by the Carstensen and Musa (1) Approximation with a Knowledge of Weight Fraction at τ^*, and as Calculated by the Carstensen and Musa (1) Approximation by Assuming the Weight Fraction to be Zero at τ_c

		Weight Fraction Remaining		
		Calculated by		
			Carstensen and Musa (1) Approximation	
	τ	Exact Equation	Using Known Weight Fraction at τ^*	Assume Weight Fraction 0 at τ_c
σ = 0.03178	0	1.0000	1.0000	1.0000
μ = 3.68888	5	0.6707	0.6728	0.6416
	10	0.4231	0.4261	0.3810
	15	0.2455	0.2485	0.2027
	20	0.1263	0.1288	0.0911
	25	0.0538	0.0554	0.0350
	30	0.0164	0.0171	0.0054
	36.36 τ_c,τ^*	0.0010	0.0010	0
σ = 0.10000	0	1.0000	1.0000	1.0000
μ = 3.68888	10	0.4337	0.4424	0.2909
	20	0.1381	0.1438	0.0344
	29.63 τ_c,τ^*	0.0255	0.0256	0
σ = 0.30000	0	1.0000	1.0000	1.0000
μ = 3.68888	5	0.7320	0.7398	0.3321
	10	0.5215	0.5295	0.0571
	16.26 τ_c,τ^*	0.3273	0.3274	0
	25	0.1575	0.1424	—
	40	0.0372	0.0130	—
σ = 1.08304	0	1.0000	1.0000	—
μ = 3.68888	40	0.8632	0.8962	—
	80	0.7594	0.8000	—
	120	0.6769	0.7108	—
	200 τ^*	0.5529	0.5529	—
	280	0.4634	0.4203	—
	350	0.4034	0.3232	—

for this case has the effect of erroneously adding a few larger particles and a few smaller particles than actually exist. If $c = 3$, the number of particles added to each end represents only 0.13% of the total number of particles in the powder. However, it is not the small *number* of particles wrongly added that should cause worry—it is the *weight* that they represent.

If there is a log-normal distribution on a *numbers basis* with mean μ and standard deviation σ, then, by the Hatch and Choate equations (5), the distribution of particles on a *weight basis* is log-normal with mean $(\mu + 3\sigma^2)$ and standard deviation σ. Let z be the standard normal variable for the weight distribution. Thus:

$$z = \frac{\ln a_0 - (\mu + 3\sigma^2)}{\sigma} \qquad \text{(Eq. 14)}$$

If the lower limit of particle sizes is $\ln a_0 = \ln \tau_c = \mu - c\sigma$ for the numbers distribution, then for the weight distribution the limiting z is:

$$z = \frac{\mu - c\sigma - (\mu + 3\sigma^2)}{\sigma} = -(c + 3\sigma) \qquad \text{(Eq. 15)}$$

Thus the weight wrongly added to the small end by using the ideal log-normal where the truncated distribution should have been used can be calculated. If $c = 3$, the error corresponds to no more than about 0.13% of the actual weight. Even if $c = 2$, the error is still less than about 2.27% of the actual weight. Thus it can be assumed that truncating (or not truncating) at the small end of the distribution will rarely lead to significant errors in calculated profiles.

At the large end, if the largest particle is given by $\ln a_0 = \mu + c\sigma$, then:

$$z = \frac{\mu + c\sigma - (\mu + 3\sigma^2)}{\sigma} = c - 3\sigma \qquad \text{(Eq. 16)}$$

If σ were vanishingly small, the errors involved would be relatively small for $c = 2, 3, \ldots$. But if $c = 3$ and (say) $\sigma = 1$, then the standard

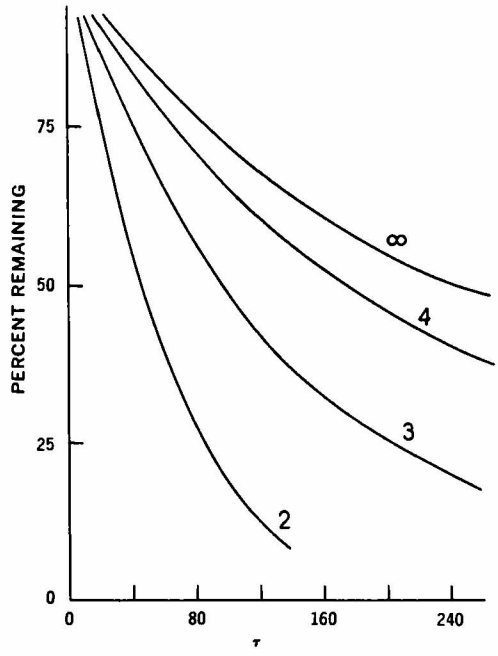

Figure 2—*Plot showing the effect of truncating the log-normal distribution at* $\mu + c\sigma$ *on the calculated dissolution patterns where* c = *2, 3, 4, or* ∞. *The distribution parameters are* μ = *3.68888 and* σ = *1.08304.*

normal variable z would be zero. The effect would be the erroneous addition of a weight of particles *equal to* the actual weight of the powder. The resulting errors in the calculated profile would be great.

The exact equation (Eq. 13) that was developed for an ideally log-normally distributed powder can be altered to take into account a truncation at the upper end of the distribution at $\mu + c\sigma$. This simply involves changing the integral terms I_β so that:

$$I_\beta = e^{\beta(\mu+\beta\sigma^2/2)}\left[F(c - \beta\sigma) - F\left(\frac{\ln \tau - (\mu + \beta\sigma^2)}{\sigma}\right)\right] \qquad \text{(Eq. 17)}$$

It is now clear that the dissolution profile calculated exactly and shown in Fig. 1 excellently reflects the results of the Carstensen and Musa (1) approximation, because the standard deviation was vanishingly small.

The effect of arbitrarily truncating at $\mu + 3\sigma$ when dealing with a number of exact distributions that vary only as to standard deviation is shown in Table I. The effect of truncating an ideal log-normal distribution at various upper limits is shown in Fig. 2.

The standard deviations of the particle distributions treated by Carstensen and Musa (1) are all small, and any truncating effects would be negligible. Actually, the dissolution of powders of the distributions treated by those authors was essentially complete before the smallest particles in those distributions began to disappear. Their smallest particles were assigned by $\ln \tau_c = \mu - 3\sigma$, where τ_c was the diameter of the smallest particle considered. The calculations in Table I show that for σ = 0.03178, 99.9% of the weight had disappeared before τ_c was reached. For σ = 0.1, 97.45% of the weight had dissolved before τ_c was reached. If such were always the case, *i.e.*, if σ were vanishingly small when compared with the mean, Eq. 13 could be changed to:

$$w_{\tau\,(\sigma\to 0)} = re^{3(\mu+3\sigma^2/2)} - r\tau e^{2(\mu+\sigma^2)} + r\tau^2 e^{(\mu+\sigma^2/2)} - r\tau^3 \qquad \text{(Eq. 18)}$$

because the $(1 - F)$ terms are essentially unity through most of the dissolution profile. However, it seems likely that powders like that of Table I, with μ = 3.68888 and σ = 0.3000, will often be encountered in pharmacy so that calculations by Eq. 13 will be required for precision.

Carstensen and Musa (1) developed equations to approximate the dissolution patterns. Their Eq. 8 was offered to approximate the dissolution time course before $\tau = \tau_c$. That equation in terms

of the notation used in this report was:

$$w_0^{1/3} - w_\tau^{1/3} = \frac{50\tau}{(\sigma/2.303)^{1.05}\,M} = \alpha\tau \qquad \text{(Eq. 19)}$$

where M is the geometric mean diameter of the distribution and the sense of α is obvious. This equation, rearranged to give:

$$w_\tau/w_0 = [1 - (\alpha/w_0^{1/3})\tau]^3 \qquad \text{(Eq. 20)}$$

was tested for its fidelity. This was done by calculating α and then finding a value for $w_0^{1/3}$ by plugging in a point τ^*, w_τ^*/w_0 from the exactly calculated profile. Then the entire dissolution pattern was calculated from Eq. 20 and compared with the exactly calculated values. These comparisons are shown in Table II for several different powder distributions. The point τ^*, w_τ^*/w_0 is indicated in Table II. For standard deviations that are small or of moderate size, the approximation of Carstensen and Musa as rearranged in Eq. 20 gives excellent values when compared with the precise dissolution curve even for values $\tau > \tau_c$. For large σ, the approximation is not quite as good. In those distributions where dissolution is essentially complete before $\tau = \tau_c$, Eq. 20 can be employed without a knowledge of the exact profile. The initial condition can be set by $(w_\tau/w_0) = 0$ at $\tau = \tau_c$. This works well for the distribution where only 0.1% of the initial weight remains at τ. It does not work well for the distribution where 2.56% of the weight remains at τ_c (Table II).

The growing importance of dissolution rate technology in pharmacy, coupled with the fact that many powders follow the log-normal distribution law, should lend importance to the exact equation derived here. In practical terms, however, the use of the exact equation for the dissolution of log-normal powders requires a knowledge of population parameters, solubility, and dissolution rate constant, none of which may be readily available. The population parameters found may depend on the method of measurement. The solubility of the smallest particles should be greater than that of the largest particles. Few powders are composed of spherical particles that dissolve isotropically. And, finally, it would undoubtedly be difficult to design a dissolution rate experiment in which the agitation and, therefore, the diffusion barrier were the same for the entire surface of each particle and for every particle.

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Chronic Isoproterenol Treatment of Mice: Effects on Catecholamines and Rectal Temperature

GERDA I. KLINGMAN▲, GENIE McKAY, ALLEN WARD*, and LUANA MORSE†

Abstract □ The chronic administration of isoproterenol (5 mg./kg. twice a day) to male and female mice resulted in increased wet weights and tissue weight–body weight ratios of the submaxillary and parotid glands. For the heart the ratio was elevated in male but not in female mice. The first dose of isoproterenol produced a decrease in the rectal temperature. Continuation of the treatment led to hyperthermia, which became maximum after treatment for longer than 10 days (20 doses). In these animals the predose rectal temperatures were lower than the pretreatment control values and the temperatures of control animals. A smaller, single dose (2.5 mg./kg.) did not alter the rectal temperature. The norepinephrine concentrations of the parotid and submaxillary glands were reduced in male and female mice, but the total norepinephrine content of these glands was decreased only in male mice. The cardiac norepinephrine levels were not affected. Chronically treated animals were less active than controls for about 90 min. after dosing and showed rarification of fur and hair loss. Isoproterenol treatment of dams before, during, and after pregnancy did not alter the body weight, gross appearance, and wet weight of organs and tissues of pups examined on the 3rd postnatal day.

Keyphrases □ Isoproterenol—effect on catecholamines and rectal temperature after chronic administration, mice □ Catecholamine concentration—effect of chronic isoproterenol administration, mice

A number of investigators have reported physiological and pathological changes in various species following acute and chronic isoproterenol treatment (1–14). Depending on the species and duration of treatment, the reported effects have included hypertrophy and hyperplasia of the submaxillary and parotid glands, infarct-like lesions and fatty degeneration of the heart, growth stunting, premature opening of the eyes, sparse hair growth, prostration, lethargy, and changes in rectal temperature, norepinephrine levels, and wet weights of several peripheral tissues.

To gain a better understanding of the mode of action of isoproterenol, we investigated several effects of chronic treatment in mice, a species in which this drug has not been studied in detail. The factors studied included the wet weights and norepinephrine levels of several tissues and organs and the effects of acute and chronic administration on the rectal temperature.

METHODS

Animals—In one study, 16 adult male Swiss Webster mice were injected subcutaneously twice a day, except Sundays, with 5 mg./kg. isoproterenol hydrochloride and another 16 mice were injected with 0.85% sodium chloride solution for 15, 23, and 31 days. The animals were sacrificed by exsanguination under sodium pentobarbital anesthesia. Whole brain (minus the cerebellum) or brain-

of the notation used in this report was:

$$w_0^{1/3} - w_\tau^{1/3} = \frac{50\tau}{(\sigma/2.303)^{1.05}\,M} = \alpha\tau \qquad \text{(Eq. 19)}$$

where M is the geometric mean diameter of the distribution and the sense of α is obvious. This equation, rearranged to give:

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To gain a better understanding of the mode of action of isoproterenol, we investigated several effects of chronic treatment in mice, a species in which this drug has not been studied in detail. The factors studied included the wet weights and norepinephrine levels of several tissues and organs and the effects of acute and chronic administration on the rectal temperature.

METHODS

Animals—In one study, 16 adult male Swiss Webster mice were injected subcutaneously twice a day, except Sundays, with 5 mg./kg. isoproterenol hydrochloride and another 16 mice were injected with 0.85% sodium chloride solution for 15, 23, and 31 days. The animals were sacrificed by exsanguination under sodium pentobarbital anesthesia. Whole brain (minus the cerebellum) or brain-

Table I—Ratios of the Tissue Weight to the Body Weight (mg./g.) of the Heart, Parotid Glands, and Submaxillary Glands in Male and Female Mice, Treated Chronically with Isoproterenol Hydrochloride (ISO)

Treatment and Body Weight, g. ± *SE*	Sex	Number of Mice	Ratio, Tissue Weight/Body Weight, mg./g. ± *SE*		
			Heart	Parotid Glands	Submaxillary Glands
Control, 31.1 ± 0.9	Male	9	3.9 ± 0.1	3.7 ± 0.2	5.2 ± 0.2
ISO, 29.4 ± 0.5	Male	9	5.4 ± 0.1[a]	12.0 ± 0.4[a]	11.0 ± 0.5[a]
Control, 32.6 ± 2.5	Female	7	4.6 ± 0.2	3.3 ± 0.5	5.1 ± 0.5
ISO, 43.6 ± 1.0	Female	8	4.8 ± 0.1	14.5 ± 1.6[a]	8.9 ± 0.5[a]

[a] $p < 0.001$ treated to control of the same sex, *t* test in nonpaired experiments (29).

stem[1], heart, submaxillary glands, parotid glands, spleen, kidneys, small intestine, and adrenal glands were dissected in the cold and weighed on a Mettler balance or Cahn electrobalance. The tissues were either immediately analyzed for catecholamines or frozen for subsequent measurement, always within 3 days after dissection.

In the second study, 25 female Swiss Webster mice were injected subcutaneously twice a day with 0.85% sodium chloride solution (eight controls) or with 5 mg./kg. isoproterenol hydrochloride for 65 days (10 mice) and 107 days (two mice) before, during, and after pregnancy. Five isoproterenol-treated mice died during the treatment. The mice were sacrificed, and the same tissues were dissected and prepared as already described.

Neonatal mice were sacrificed 3 days after birth, and the peripheral organs and tissues, including sympathetic ganglia, were examined grossly and weighed on a Mettler balance or Cahn electrobalance.

Tissue Weights and Ratios—For the statistical analysis of the effect of chronic isoproterenol treatment on the tissue weights, the ratios of the tissue weights to the body weights (milligrams per gram) were used. There were no differences in the body weights within and between the groups of treated and control male mice. The body weights of the female control mice were lower than the body weights of the isoproterenol-treated female mice before treatment and remained so throughout the experiment.

Catecholamine Analysis—Norepinephrine and epinephrine were extracted with 1-butanol as previously described (15) and measured in a spectrophotofluorometer[2] by a modification (16, 17) of the ferricyanide oxidation method of von Euler and Floding (18).

Rectal Temperature—The rectal temperatures of female mice were measured with a telethermometer[3] every 30 min. for up to 150 min. (room temperature 22–24°) before and after the administration of the first and third doses of isoproterenol or 0.85% sodium chloride solution. During the chronic treatment, the rectal temperatures were determined periodically. Rectal temperatures were measured in another group of female mice following a single lower dose (2.5 mg./kg.) of isoproterenol hydrochloride.

Other Observations—During the entire experimental period the gross appearance of chronically treated male and female mice was observed. These observations included the appearance of the fur and the occurrence and severity of lethargy and salivation after dosing. The results of the histopathological study of tissues from isoproterenol-treated and control mice will be reported elsewhere.

Within the groups of female and male mice, no variation in the intensity of the effects on the tissue weights, the ratios, and the tissue catecholamines was noted that could be attributed to the different durations of the chronic treatment. For this reason, these data were pooled (65 and 107 days for female mice and 15, 23, and 31 days for male mice).

RESULTS

Tissue Weights—The wet weights of the hearts, submaxillary glands, and parotid glands were larger in the treated mice of both sexes than in the control mice. In treated male mice the ratio of the heart weights to the body weights was significantly increased, while this ratio was not altered in the female mice. The ratio was larger in female control mice when compared to male control mice ($p < 0.01$); the opposite was noted in the isoproterenol-treated male and female mice ($p < 0.001$). The ratios of the parotid and submaxillary glands from isoproterenol-treated mice of both sexes were significantly increased over control values (Table I). The ratios of parotid glands from male and female treated mice were not statistically different, but those of the submaxillary glands were larger in male than in female treated mice ($p < 0.01$). In control male and female mice the gland to body weight ratios were similar. Spleen, intestine, kidneys, adrenal glands, superior cervical ganglion, brain, and brainstem showed no alteration in the tissue weight to body weight ratios as a result of chronic isoproterenol treatment. Gross appearance and the wet weights of tissues of neonatal animals from isoproterenol-treated female mice were not different from those of neonatal controls.

Rectal Temperature—A decrease in the rectal temperature of female mice occurred 30, 60, 90, 120, and 150 min. after the administration of a single dose of 5 mg./kg. isoproterenol when the experimental temperatures were compared to the predrug control values and control mice (Table II). After three doses the hypothermic response was no longer demonstrable, and continuation of the isoproterenol treatment led to hyperthermia. The rectal temperature increased significantly within 30 min. after dosing, remained elevated for approximately 1 hr., and then began to return to the control value. The maximum increase in the rectal temperature was not reached at 7 (Table II) or 10 days but was observed after 29 days of treatment. The final temperature measurements were taken 24 hr. before sacrifice after 65 and 107 days of treatment. The mean increase amounted to 2.2° with a range from 1.8 to 3.2°. In half of the mice, peak temperatures were reached at 30 min.; in the other half they were reached at 60 min. after the administration of the dose. During the chronic treatment the rectal temperatures recorded before the administration of a daily dose of isoproterenol were lower than the temperatures of these mice before the chronic treatment was started, during the earlier periods of the treatment, and in control mice; these differences were statistically significant. Treatment of female mice with a single dose of 2.5 mg./kg. isoproterenol did not affect the rectal temperature during the observation period (Table II).

Catecholamines—The norepinephrine concentration of the parotid glands was significantly decreased in treated mice of both sexes when compared to their respective controls. The norepinephrine content of these glands was also decreased in chronically treated male mice but not in the treated female mice. In female but not in male mice, isoproterenol treatment reduced the epinephrine concentration of parotid glands by 28%. A fourfold increase in the total epinephrine content (nanograms per glands) occurred in the parotid glands of both male and female treated mice.

Chronic isoproterenol treatment reduced the norepinephrine concentration to 37% and the content to 80% of control values in the submaxillary glands of male mice. Female treated mice also showed a decreased norepinephrine concentration (to 69% of control value), but the norepinephrine content was increased to 163%. The norepinephrine concentration and content of the submaxillary glands from male control mice were 2.5-fold greater than those from female control mice. Lower norepinephrine concentration and content of parotid glands were also noted in female control than in male control mice (Table III). Similar differences were obtained in a subsequent study which was undertaken to verify these findings. The epinephrine concentration and content of the submaxillary glands from male mice were greater regardless of the treatment when compared with the respective glands from

[1] Brainstem is designated as the medulla, the pons, the midbrain, and the diencephalon.
[2] Aminco-Bowman.
[3] Yellow Springs Instrument Co., probe No. 402.

Table II—Rectal Temperatures of Female Mice Treated Subcutaneously with 5 mg./kg. Isoproterenol Hydrochloride (ISO)

Treatment	Number of Mice	Rectal Temperature ± *SE* before and Every 30 min. after Treatment					
		0	30	60	90	120	150
Controls	13	38.1 ± 0.1°	38.1 ± 0.1°	37.9 ± 0.1°	37.8 ± 0.1°	37.7 ± 0.1°[a]	37.7 ± 0.1°[a]
First dose ISO	14	38.1 ± 0.1°	37.2 ± 0.2°[4d]	37.4 ± 0.2°[1c]	37.2 ± 0.2°[3d]	37.2 ± 0.1°[3d]	37.3 ± 0.1°[1d]
First dose ISO, 2.5 mg./kg.	11	38.1 ± 0.2°	37.9 ± 0.2°	37.7 ± 0.3°	37.5 ± 0.3°	37.4 ± 0.3°	37.3 ± 0.3°[a]
Third dose ISO	14	38.1 ± 0.2°	37.8 ± 0.2°	37.7 ± 0.2°	37.6 ± 0.1°	37.5 ± 0.2°[a]	37.3 ± 0.2°[b]
Controls	11	37.6 ± 0.2°	37.4 ± 0.2°	37.2 ± 0.2°	37.2 ± 0.2°	—	—
7 days ISO	14	37.7 ± 0.1°	39.1 ± 0.2°[4d]	39.0 ± 0.2°[4d]	38.6 ± 0.2°[4d]	—	—
65–107 days ISO	12	36.8 ± 0.1°[3]	39.0 ± 0.3°[4d]	38.6 ± 0.2°[4d]	37.9 ± 0.2°[1d]	—	—

[1] $p < 0.05$, [2] $p < 0.02$, [3] $p < 0.01$, [4] $p < 0.001$ } *t* test in nonpaired experiments (29), treated to control.

[a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$, [d] $p < 0.001$ } *t* test in nonpaired experiments (29), zero-time temperature (predrug) to experimental temperature (postdrug).

Table III—Norepinephrine and Epinephrine Concentration and Content of Tissues of Male and Female Mice Treated Chronically with Isoproterenol Hydrochloride (ISO)

Tissue	Number of Mice	Treatment	Sex	Norepinephrine		Epinephrine	
				ng./g. ± *SE*	ng./Tissue ± *SE*	ng./g. ± *SE*	ng./Tissue ± *SE*
Submaxillary glands	9	Control	Male	4320 ± 330[4d]	686 ± 36[1d]	192 ± 28[b]	31 ± 5[4a]
	9	ISO		1610 ± 165[4c]	527 ± 63[1]	226 ± 29[c]	75 ± 9[4b]
	7	Control	Female	1690 ± 95[3d]	270 ± 19[4d]	105 ± 17[b]	17 ± 3[a]
	8	ISO		1170 ± 99[3c]	442 ± 29[4]	99 ± 23[c]	38 ± 10[b]
Parotid glands	9	Control	Male	1270 ± 120[4c]	141 ± 7[4c]	120 ± 12	14 ± 1[4]
	9	ISO		238 ± 21[4]	82 ± 5[4]	142 ± 25	50 ± 8[4]
	7	Control	Female	827 ± 73[4c]	86 ± 15[c]	120 ± 10[3]	12 ± 1[4]
	8	ISO		173 ± 44[4]	91 ± 17	87 ± 5[3]	54 ± 8[4]
Heart	9	Control	Male	545 ± 59	65 ± 6	65 ± 12[3]	8 ± 2
	9	ISO		467 ± 37	74 ± 6[c]	25 ± 5[3]	4 ± 1
	7	Control	Female	492 ± 37	76 ± 12[3]	72 ± 17	10 ± 2
	8	ISO		556 ± 49	115 ± 10[2c]	48 ± 16	10 ± 3

[1] $p < 0.05$, [2] $p < 0.02$, [3] $p < 0.01$, [4] $p < 0.001$ } *t* test in nonpaired experiments (29), control to treated of the same sex.

[a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$, [d] $p < 0.001$ } *t* test in nonpaired experiments (29), control male to control female or treated male to treated female.

female mice. Chronic isoproterenol treatment doubled the total epinephrine content (nanograms per glands) of the submaxillary glands of both sexes.

The cardiac norepinephrine concentration was not significantly different in control and isoproterenol-treated mice of the same sex nor in control animals of opposite sexes. However, treated female mice showed a significantly higher norepinephrine content of the heart than the female controls and the treated males (Table III). In male mice, chronic isoproterenol treatment reduced the cardiac epinephrine concentration.

The brainstem norepinephrine and epinephrine of male mice were not altered by chronic isoproterenol treatment. Chronic treatment of female mice, in which the catecholamines of the whole brain were measured, resulted in an increase in norepinephrine concentration (127% of control value; $p < 0.05$). No change in the brainstem epinephrine concentration was noted. The adrenal norepinephrine concentration of chronically treated female mice showed a mean increase to 164%; this was statistically nonsignificant. The adrenal epinephrine concentration was not altered. The adrenal catecholamines of control and treated male mice were not measured. Concentrations of norepinephrine and epinephrine in spleen, intestines, and kidneys were similar to those of control animals.

Other Observations—After the first few doses, the mice of both sexes were less active than the control animals for approximately 60–90 min. following administration of isoproterenol. There was little or no salivation. Continuation of the treatment accentuated the depression and salivation became marked. Rarification of fur and hair loss, resulting in bald areas, were also noted. The number of pups per litter produced by the chronically treated dams was similar to the litter sizes of controls. The body weights, the gross appearance, and the wet weights of the organs and tissues of pups from isoproterenol-treated dams were not different from those of control animals when examined on the 3rd postnatal day.

DISCUSSION

The histopathological and biochemical effects of isoproterenol on the peripheral tissues of mice have not been explored to the same extent as in the rat. The weight increases of the submaxillary and parotid glands of mice were similar to those reported in rats (3, 5, 13, 14). According to Brown-Grant (11), large doses (30 and 40 mg./100 g.) in mice produced hypertrophy but no hyperplasia of submaxillary and parotid glands. Baserga (12) found a 10-fold increase in DNA synthesis and stimulation in cell proliferation in submaxillary glands of mice after treatment. It is not known whether isoproterenol treatment produces histopathological lesions in the mouse heart comparable to those observed in the rat. In the current study, an increase in the wet weight of the heart was demonstrated in male mice only. This increase, expressed as the ratio of the tissue weight to the body weight, amounted to 138% of the control value ($p < 0.001$). In rats treated chronically with 10–15 mg./kg. isoproterenol twice a day, the ratio increased in male and female animals (145.2 and 147.5%, respectively, of control values) (13).

A similar pattern of hypothermic and hyperthermic responses following single and repeated doses of isoproterenol, as seen in the current study with mice, has also been noted in rats (13, 19). In both species, chronic treatment for longer than 10 days was required before a maximum hyperthermic response (increase of 2–3°) was reached. In the group of male mice, no temperature measurements were made. However, isoproterenol treatment affected the rectal temperature similarly in male and female rats (13).

A smaller, single dose, 2.5 mg./kg., did not produce hypothermia (Table II). From the data obtained in the current study with mice and in another study with rats (19), one may conclude that the effect of a single dose of isoproterenol on the rectal temperature is dose related. As shown by Brittain and Handley (20), the intracerebral injection of isoproterenol resulted in an increase in esophageal temperature with small doses, 5–20 mcg./mouse, and a

decrease with a larger dose, 200 mcg./mouse. These authors suggested that the adrenergic α-receptor is involved in the hypothermic response and that the adrenergic β-receptor is involved in the hyperthermic response. Feldberg and Myers (21) postulated that hypothermia is the result of centrally released norepinephrine. Evidence for and against this proposed mechanism is available. For instance, reduction of central norepinephrine antagonized the 6-hydroxydopamine-induced hypothermia in the cold (22) but not at room temperature (23).

In the rat the cardiac norepinephrine concentration and content were significantly reduced after acute and short-term isoproterenol treatment (14, 24, 25). Mueller and Axelrod (25) presented evidence indicating that the reduction of the cardiac norepinephrine may be due to a storage defect. The nerve endings of the heart responded with a normal uptake but showed a decreased capacity to retain labeled norepinephrine. These authors linked the storage defect to an imbalance of the cardiac electrolytes, particularly sodium. An increase in sympathetic activity is believed to contribute to the decreased cardiac norepinephrine level.

After chronic isoproterenol treatment of rats only the norepinephrine concentration—not the total content of the heart—was significantly decreased (13). The fact that the cardiac norepinephrine concentrations in mice were not affected by chronic isoproterenol treatment, even though there was an increase in the heart weight of male mice, might indicate differences in species, sex, and/or long-term treatment (>1 month) to the effects of the drug that require further study. The statistically significant increase in the cardiac norepinephrine content in female treated mice (Table III) complicated the interpretation of the data even more in regard to the state of endogenous norepinephrine after chronic and short-term isoproterenol treatment.

It is not known whether a similar mechanism, as postulated by Mueller and Axelrod (25), may be responsible for the norepinephrine reduction found in the submaxillary and parotid glands after isoproterenol treatment. With certain dose regimens in rats, the extent of the reduction of the norepinephrine concentrations in these glands was similar to that in the heart, but the effect on the norepinephrine content of the glands by acute and short-term isoproterenol treatment was statistically nonsignificant (14). However, in chronically treated male mice the norepinephrine content of the submaxillary and parotid glands was reduced ($p < 0.05$ and 0.001, respectively), while in treated female mice the norepinephrine content of the submaxillary glands was elevated and no change was noted in parotid glands.

The lethargy observed with mice in the present studies and with rats (13) following isoproterenol treatment may have been described previously. Bertaccini *et al.* (2) reported prostration in rats after the administration of 400 mg./kg. of isoproterenol for 12 days. Fukuda (26) used the same term to describe the effect produced in rats by 5 mg./kg. administered once a day for 14 days. In Fukuda's study the prostration apparently occurred with the first dose and did not become more marked with subsequent dosing. In the present studies the lethargy following the first dose of isoproterenol was not as severe as that seen after repeated doses, particularly with long-term chronic treatment, and was not as pronounced in mice as in rats. However, the mice received only 5 mg./kg. while the rats received 15 mg./kg. (13). The rarification of the fur was less marked in the chronically treated mice than in the rats (13, 19). This also might have been due to the smaller dose administered chronically to the mice.

Five of the 17 female mice died in the course of the chronic treatment. Compared to the acute intraperitoneal LD_{50} in female Swiss Webster mice (250 mg./kg.; 95% *CL* 221–283; mice were observed for 1 week; death occurred within 18 hr.[4]), the dose of 5 mg./kg. used in these studies is very low. No deaths occurred in the group of 16 male mice treated with isoproterenol. According to Barka (27), male rats were more sensitive than female animals to the lethal effect of a three-dose course of intraperitoneally injected isoproterenol.

According to Geber (9), single doses of isoproterenol (0.003–34.8 mg./kg.) administered subcutaneously to pregnant hamsters on the 8th day of gestation produced a variety of anomalies and the percentages of dead, resorbed, and runt fetuses were increased over those in control litters. Vogin *et al.* (28) exposed pregnant rats and rabbits to isoproterenol aerosol to provide daily dosage levels of 150–450 mcg./kg. There was no evidence of teratologic effects due to the drug or the methodology. The present study in mice supports the observations reported by Vogin *et al.*, since no gross abnormalities were found in neonatal mice and their organs and tissues. However, histopathological lesions cannot be ruled out. The tissues of the neonatal animals were not examined histologically.

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Quantitative Determinations of Phenylephrine and Phenylpropanolamine Hydrochlorides in Combination

BHAMY B. SHENOY and V. DAS GUPTA▲

Abstract □ The quantitative determinations of phenylephrine and phenylpropanolamine hydrochlorides in combination are reported. Phenylephrine hydrochloride is assayed using the Koshy–Mitchner method without interference from phenylpropanolamine hydrochloride. Phenylpropanolamine hydrochloride is assayed using an acid-dye technique with negligible interference from phenylephrine hydrochloride. Beer's law is followed, and the best pH range for the extraction of the propanolamine–dye complex was determined to be 5.8–6.4. Buffer concentration does not appear to have any effect on the sensitivity of the assay method. In both assay methods, there is no interference from the antioxidant, sodium metabisulfite, and the preservatives, methylparaben and propylparaben.

Keyphrases □ Phenylephrine and phenylpropanolamine hydrochlorides in combination—analysis using the Koshy–Mitchner method and acid-dye technique □ Phenylpropanolamine and phenylephrine hydrochlorides in combination—analysis using Koshy–Mitchner method and acid-dye technique □ Drug mixtures —analysis of phenylephrine ahd phenylpropanolamine hydrochlorides in combination

The biggest challenge to a pharmaceutical analyst is how to assay one active ingredient in the presence of another. One such problem arises when two decongestants, phenylephrine hydrochloride (I) and phenylpropanolamine hydrochloride (II), are mixed together. The quantitative determinations of I and II in separate formulations have been reported by many workers (1–5). Ponder (6) reported a column chromatography technique for the separation of I from codeine, dextromethorphan, and II. Recently, Brown and Portmann (7) reported a modified version of the periodate technique of Chafetz (3) for the analysis of these two decongestants in combination in a mixture with aspirin. According to these authors, the method requires: "...careful adjustment of pH, [for] selective separation of two oxidative compounds, benzaldehyde and *m*-hydroxybenzaldehyde..."

The purpose of this paper is to report simple and accurate methods for the analysis of I and II in combination without prior separation. Compound I can be assayed by the Koshy–Mitchner method (2), and Compound II can be assayed by an ion-pair extraction technique using an acid dye, bromthymol blue.

EXPERIMENTAL

Chemicals and Reagents—All of the chemicals and reagents used were USP, NF, or ACS grade. Phenylephrine hydrochloride[1] and phenylpropanolamine hydrochloride[2] were used without further purification.

Preparation of Buffer Solutions—Phosphate buffers (0.05 *M*) of various pH values were prepared according to USP XVII (8). The phosphate buffers of other concentrations (0.025, 0.075, 0.1, and 0.125 *M*) were prepared similarly.

Preparation of Bromthymol Blue Solutions—Solutions of bromthymol blue (1×10^{-4} *M*) in phosphate buffers of various pH values and concentrations were prepared using a procedure reported earlier (9). The pH of each solution was determined with a pH meter[3].

Preparation of Standard Solutions of I and II—Stock solutions of I and II were prepared by dissolving 100 mg. of the powder in enough distilled water to make 250 ml. These stock solutions were used to prepare solutions of lower concentrations as needed.

Preparation of Assay Solutions of I and II in Combination—The following three assay solutions were prepared in distilled water using a simple solution method: 1, 0.125% of I and 1% of II; 2, 0.25% of I and 1% of II; and 3, 0.5% of I and 1% of II.

Determination of Effect of Buffer Concentration on Extraction of Amine–Bromthymol Blue Complexes—A 5.0-ml. quantity of an appropriate amine salt solution (I, II, chlorpheniramine maleate, or methapyrilene hydrochloride) containing 15 mcg./ml. was mixed with 5.0 ml. of dye solution (pH 6.4) of appropriate buffer concentration, ranging from 0.025 to 0.125 *M* in a 250-ml. separator. A 10.0-ml. quantity of chloroform was added and the mixture was shaken for 60 sec. The phases were allowed to separate, and the absorbance of the clear chloroform layer was determined at 420 nm. A blank was prepared by substituting 5.0 ml. of distilled water for the amine salt solution in the procedure. The results are presented in Fig. 1.

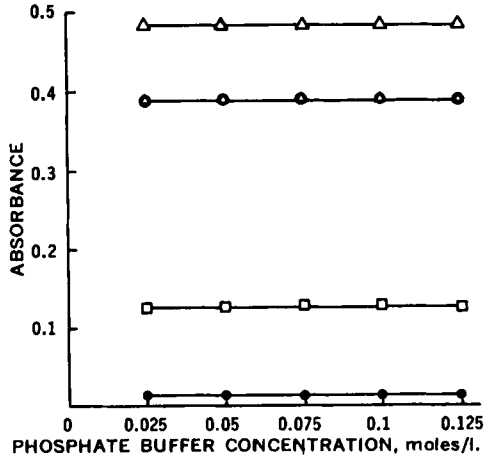

Figure 1—*Effect of buffer concentration on extraction of amine–bromthymol blue complexes. Key:* △, *ephedrine–dye complex;* ⊙, *chlorpheniramine–dye complex;* □, *phenylpropanolamine–dye complex; and* ●, *phenylephrine–dye complex.*

[1] Winthrop Laboratories.

[2] S. B. Penick.

[3] Zeromatic.

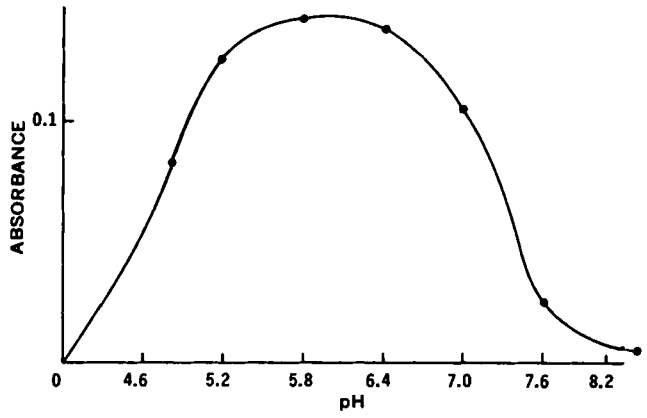

Figure 2—*Effect of pH on extraction of phenylpropanolamine–dye complex with chloroform.*

Determination of Effect of pH on Extraction of II–Bromthymol Blue Complex—The effect of pH on the extraction of a II–dye complex with chloroform was determined using the procedure reported under *Determination of Effect of Buffer Concentration on Extraction of Amine–Bromthymol Blue Complexes*, except that dye solutions of various pH values in 0.05 *M* phosphate buffer solutions were used. The results are presented in Fig. 2.

Preparation of Calibration Curve for II—A 5.0-ml. quantity of an appropriate solution containing various concentrations of II was mixed with 5.0 ml. of a dye solution (pH 6.4) in phosphate buffer (0.05 *M*). Then 10.0 ml. of chloroform was added, and the mixture was treated in the manner described under *Determination of Effect of Buffer Concentration on Extraction of Amine–Bromthymol Blue Complexes*. The results are presented in Fig. 3.

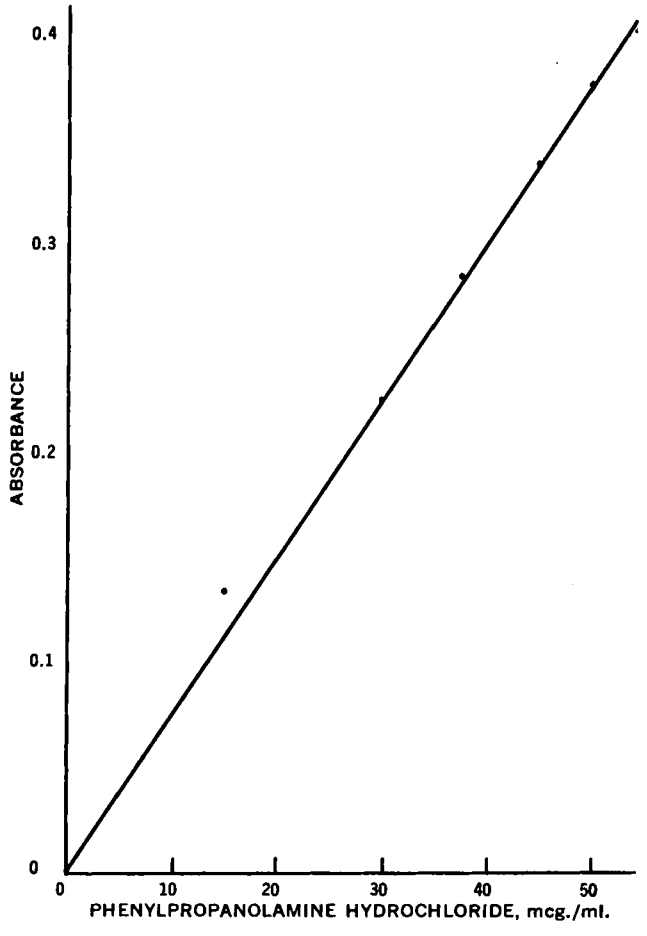

Figure 3—*Standard curve for phenylpropanolamine hydrochloride.*

Table I—Correction Factors for II

Solution Number	Factor to be Substracted from Absorbance Value
1	0.003
2	0.006
3	0.012

Table II—Assay Results on I and II

Solution Number	Results in Percent of Claim I	Results in Percent of Claim II
1	99.47	97.77
	100.80	97.77
	99.47	99.24
	Average 99.91	Average 98.26
	Avg. dev. ±0.59	Avg. dev. ±0.65
2	98.15	99.29
	99.47	97.77
	98.15	97.77
	Average 98.59	Average 98.26
	Avg. dev. ±0.59	Avg. dev. ±0.65
3	100.80	100.70
	100.80	97.77
	99.47	100.70
	Average 100.36	Average 99.72
	Avg. dev. ±0.59	Avg. dev. ±0.61

Determination of Interference from Sodium Bisulfite, Methylparaben, and Propylparaben—To determine the interference from the antioxidant, sodium bisulfite, and the preservatives, methylparaben and propylparaben, a solution containing 0.2% of sodium bisulfite, 0.02% of methylparaben, and 0.01% of propylparaben was prepared in distilled water, using a simple solution method. These are the usual concentrations added to nasal drops. The solution was assayed in the same manner as described under *Assay Procedure for II in Combination with I*. This solution was also assayed for I using the Koshy–Mitchner method[4] (2). There was no interference in the assay procedures from the preservatives and the antioxidant.

Determination of Interference from I—A solution containing 15.0 mcg./ml. of I was assayed in the same manner as described under *Assay Procedures for I*. There was a minor interference in the assay procedure. The correction factors are presented in Table I.

Determination of Interference from II—A solution containing eight times more of II than I was assayed using the Koshy–Mitchner method[4] (2) for the determination of I. There was no interference in the assay procedure.

Assay Procedure for I—Compound I was assayed using the Koshy–Mitchner method[4] (2). The results are presented in Table II.

Assay Procedure for II in Combination with I (Assay Solutions 1, 2, and 3)—A 5.0-ml. quantity of the appropriately diluted assay solution containing 40.0 mcg./ml. of II was mixed with 5.0 ml. of the dye solution (pH 6.4, buffer concentration 0.05 *M*). Then 10.0 ml. of chloroform was added, and the mixture was treated in the manner described under *Determination of Effect of Buffer Concentration on Extraction of Amine–Bromthymol Blue Complexes*.

Calculation of Assay Results on II—Provided that the results on I are within limits as determined above by the Koshy–Mitchner procedure (2), the correction factors reported in Table I are suggested because of a minor interference from I.

After making these corrections, the results were determined with the help of the calibration curve (Fig. 3) (Table II).

DISCUSSION AND CONCLUSIONS

The assay results (Table II) indicate that II can be assayed using an acid-dye technique. Beer's law is followed within a narrow range of 30–50 mcg./ml. of II (Fig. 3). The interference from I was almost

[4] Using borate buffer.

nil because I is not as strong a base as II since only the latter contains a primary amino group. There was no interference from the antioxidant, sodium bisulfite (0.2%), and the preservatives, methylparaben (0.02%) and propylparaben (0.01%). The best pH range for the extraction of the phenylpropanolamine–dye complex with chloroform appears to be from 5.8 to 6.4 (Fig. 2). A pH value of 6.4 was preferred for these studies due to low blank values. The effect of buffer concentration on the extraction of the complex appears to be negligible (Fig. 1). To confirm this finding, three more amines (chlorpheniramine, ephedrine, and phenylephrine) were tested and the results were identical (Fig. 1). Compound I can be easily assayed (Table II) using the Koshy–Mitchner method (2) with borate buffer without any interference from II, the antioxidant, and the preservatives.

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Quantitative GLC Determination of Resorcinol Monoacetate in Dermatological Products

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Abstract □ A GLC procedure employing an internal standard of orcinol is described for the analysis of resorcinol monoacetate in dermatological preparations. The analysis of a cream or lotion is performed by the addition of an internal standard, acetylation, extraction with benzene, evaporation of benzene, addition of chloroform, and then chromatography on a 5% cyano ethyl silicone column.

Keyphrases □ Resorcinol monoacetate creams and lotions—GLC analysis □ Dermatological creams and lotions, resorcinol monoacetate—GLC analysis □ Cream, resorcinol monoacetate—GLC analysis □ Lotion, resorcinol monoacetate—GLC analysis □ GLC—analysis, resorcinol monoacetate in creams and lotions

Due to its mild action, resorcinol monoacetate has been incorporated in dermatological products primarily for the treatment of eczema, psoriasis, and seborrheic dermatitis. In addition to the base, the dermatological preparations frequently contain sulfur, hydrocortisone, and hexachlorophene.

Due to the complex matrix present in dermatological creams and lotions, the quantitative determination of resorcinol monoacetate requires extensive cleanup procedures.

Methods of analysis for resorcinol monoacetate reported in the literature have included UV absorption (1, 2) and photometry after reaction with picric acid (3) or *p*-dimethylaminobenzaldehyde (4). Paper chromatography and TLC techniques have also been used extensively (5, 6), and methods describing the use of GLC for phenolic compounds have been reported (7, 8). None of these methods has been used for the quantitative determination of resorcinol monoacetate in a pharmaceutical matrix.

Table I—Statistical Data from GC Analysis of Resorcinol Monoacetate (RMA) in Cream Base Placebo

RMA Added, mg.	RMA Found, mg.	Bias, mg.	*SD*, mg.	*df*	*CV*, %
13.50	13.45	−0.05	0.193	5	1.43
15.00	14.94	−0.06	0.187	6	1.25
16.50	16.53	+0.03	0.249	5	3.51

The method described here utilizes an internal standard technique and a simple cleanup procedure involving acetylation and extraction. It allows the separation and determination of resorcinol monoacetate by GLC without interference from the excipients commonly present in dermatological creams and lotions. This method can be adapted to the quality control of resorcinol monoacetate in creams and lotions.

EXPERIMENTAL[1]

Chromatographic Conditions—A 1.22-m. (4-ft.), 2-mm. i.d., stainless steel column packed with 5% cyano ethyl silicone on diatomite aggregate (high performance)[2], 80–100 mesh, was used for the assay. The column temperature was 170°, and the detector and injection port temperatures were 220°. The helium carrier gas flow rate was 15 ml./min. A flame-ionization detector was used with a hydrogen flow rate of 30 ml./min. and an air flow rate of 450 ml./min.

Reagents and Solutions—The following were used: resorcinol monoacetate NF; orcinol, 95–99% pure[3]; acetic anhydride, reagent grade; and pyridine, reagent grade.

[1] A Hewlett-Packard 7620A research chromatograph with 7127A strip chart recorder and a 7670A automatic sampler was used.

[2] Five percent XE-60 on Chromosorb G (HP), Supelco, Inc., Bellefonte, Pa.

[3] K & K Laboratories, Plainview, NY 11803

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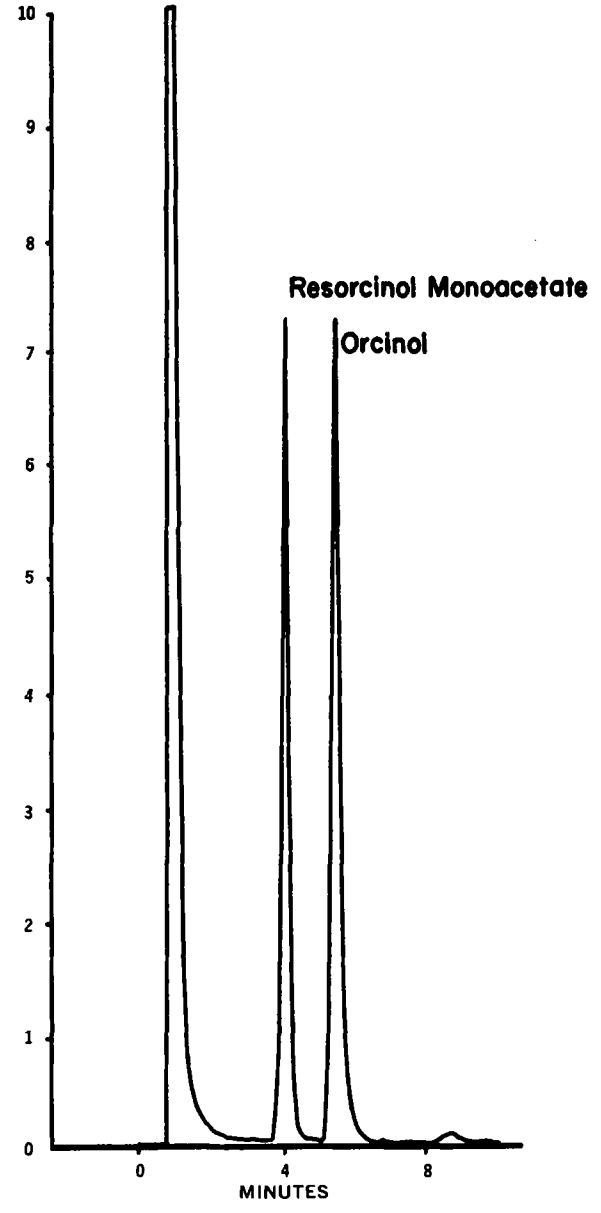

Figure 1—*A typical chromatogram of standard solution of resorcinol monoacetate and orcinol as internal standard, acetylated and extracted.*

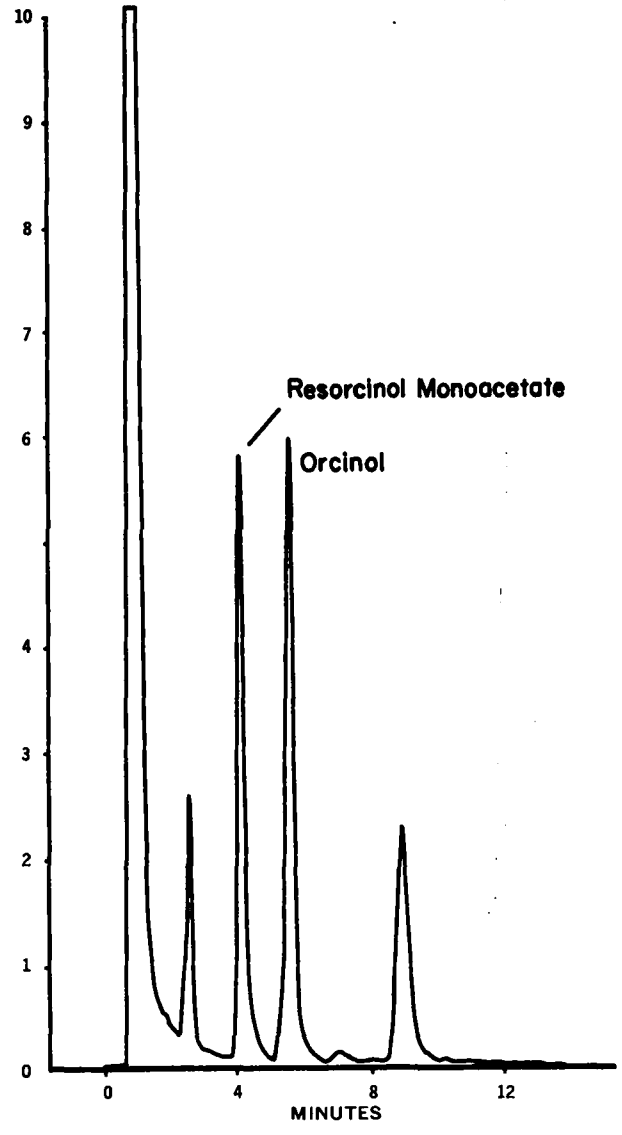

Figure 2—*Chromatogram of sample solution containing orcinol as internal standard, acetylated and extracted.*

For the standard resorcinol monoacetate solution, prepare a standard solution containing, accurately weighed, about 300 mg. of resorcinol monoacetate in 100 ml. of pyridine. For the internal standard orcinol solution, prepare an internal standard solution containing about 300 mg. of orcinol in 100 ml. of pyridine.

For the working standard solution preparation, pipet 5.0 ml. of standard solution and 5.0 ml. of internal standard solution into a 125-ml. glass-stoppered boiling flask. Pipet 10 ml. of pyridine and 4 ml. of acetic anhydride into the flask. Connect an air condensor to the flask and heat on a steam bath for 30 min.

Sample Preparation—Transfer an accurately weighed sample containing about 15 mg. of resorcinol monoacetate into a 125-ml. boiling flask. Pipet 5.0 ml. of orcinol internal standard solution, 15 ml. of pyridine, and 4 ml. of acetic anhydride into the flask. Heat on a steam bath for 30 min. using an air condenser.

Procedure—Cool the working standard and sample preparations. Add 50 ml. of benzene to each, mix well, and transfer to respective separators. Wash each mixture three times with 50 ml. of distilled water, discarding the lower (aqueous) layer each time. Gravity filter the upper (benzene) layer (through anhydrous sodium sulfate in a folded glass fiber filter) into a 125-ml. glass-stoppered boiling flask. Place the flasks in a rotary vacuum evaporator in a hot water bath and evaporate the solvent until only an oily residue remains. Add 5 ml. of chloroform to each flask and swirl to dissolve the sample. Inject 1 or 2 μl. of the sample and standard solutions into a gas chromatograph.

Calculation—Resorcinol diacetate elutes before orcinol diacetate under the conditions of the analysis. Determine the peak height ratio for each standard and sample solution:

$$\frac{A}{W} \times \frac{\text{(RDA/ODA) for sample}}{\text{(RDA/ODA) for standard}} \times 100 = \text{percent resorcinol monoacetate} \quad \text{(Eq. 1)}$$

where:

RDA = resorcinol diacetate peak height
ODA = orcinol diacetate peak height
A = milligrams of resorcinol monoacetate in working standard solution
W = sample weight in milligrams

RESULTS AND DISCUSSION

To determine the accuracy and precision of the proposed method, a statistical study was done. Samples were prepared containing three different levels of known amounts of resorcinol monoacetate. The

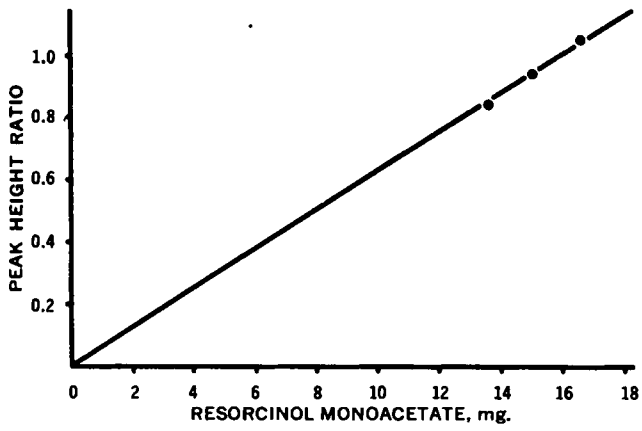

Figure 3—*Standard curve of resorcinol monoacetate concentration* versus *peak height ratio.*

statistical data are shown in Table I. The sample cream base formulated was composed of sorbitan monolaurate, glycerin, polyoxyethylene (20), glyceryl monostearate, hexachlorophene, hydrocortisone acetate, propylene glycol, cetyl alcohol, and colloidal sulfur. The statistical evaluation of the results indicates that the method had no bias at any of the three levels of resorcinol monoacetate. The average coefficient of variation was 1.40%. For 16 replicate samples, the average recovery was 99.76%.

A typical chromatograph of the standard mixture is shown in Fig. 1, and a chromatograph obtained from the extract of the dermatological preparation is shown in Fig. 2. The correlation coefficient for concentration of resorcinol monoacetate *versus* peak height ratio (resorcinol diacetate/orcinol diacetate) was found to be 1.025. A calibration curve for milligrams of resorcinol monoacetate *versus* peak height ratio is shown in Fig. 3.

The statistical results indicate that the method is accurate and reproducible for quality control of resorcinol monoacetate in dermatological preparations.

Table II—Determination of Resorcinol Monoacetate in Commercial Preparations

Sample	Number of Samples	Label Claim, %	Found, % ($\bar{x}$)
Cream I	20	3.0	3.03
Lotion I	12	3.0	3.09
Cream II	16	3.0	3.04
Lotion II	9	3.0	2.88

This method has been used in these quality control laboratories for over a year. The results obtained during this period are shown in Table II.

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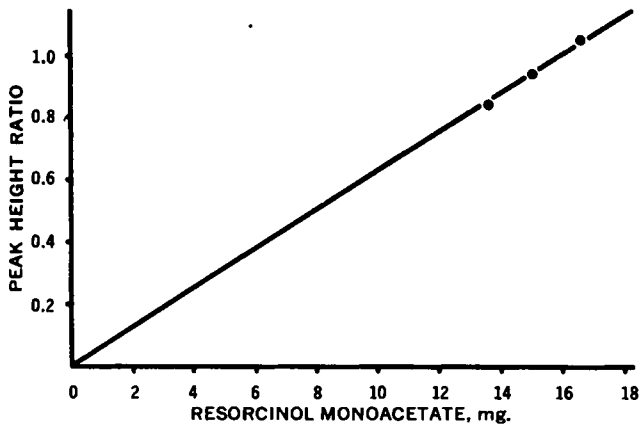
B. F. GRABOWSKI*▲, B. J. SOFTLY†, B. L. CHANG*, and W. G. HANEY, Jr.*

Abstract □ A GLC assay was developed for the determination of homatropine methylbromide in both tablets and elixirs. The method allows for assaying the drug in the presence of other tropine derivatives and the usual constituents of tablets and elixirs. An aqueous suspension of tablets or a sample of elixir was adjusted to pH 2.2 with hydrochloric acid buffer USP and extracted with ether. This preliminary extraction of the buffered sample removes interfering substances and excludes any hydrolyzed material in the sample from analysis. The homatropine methylbromide was then hydrolyzed by adding 10% sodium hydroxide solution to pH 10–11 and boiling the solution for 20 min. After acidification with hydrochloric acid buffer, the resulting mandelic acid was extracted with ether. The trimethylsilyl derivative of the mandelic acid resulting from hydrolysis was then chromatographed, with the trimethylsilyl derivative of 2-naphthol as the chromatographic standard. A blank elixir preparation with added homatropine methylbromide (0.12 mg./ml.) assayed with an accuracy of 99.12% of the calculated value. This procedure was applied to various commercial preparations containing homatropine methylbromide, with reproducible results ranging from 97.59 to 100.86% of the labeled amount of homatropine methylbromide.

Keyphrases □ Homatropine methylbromide tablets and elixir—GLC analysis □ GLC—analysis, homatropine methylbromide tablets and elixir

Many esters of the amino alcohol tropine exhibit strong anticholinergic activity (1) and, as a result, are found in several pharmaceutical preparations. Analysis of both naturally occurring and semisynthetic derivatives, particularly those contained in pharmaceutical preparations, has been of interest to the pharmaceutical chemist for many years (2). Several of the official procedures (3) for the analysis of these compounds, how-



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ever, lack both sensitivity and specificity (4, 5). Because of these deficiencies, many procedures for the analysis of tropine derivatives have been developed (6, 7) and one of these (8) has been adopted as the official analysis procedure for atropine sulfate in various dosage forms (9).

Analytical methods utilizing spectrophotometry (10), TLC (11), and GLC (12) appear to provide useful procedures for the analysis of these compounds. Of these methods, the latter would be the most useful for the analysis of tropine derivatives since it has the potential advantages of speed, sensitivity, and specificity.

Quaternary ammonium salts of tropine esters would, however, present difficulties in GLC analysis. Analysis of quaternary ammonium salts *via* GLC is limited by the fact that chromatographic characteristics of permanent cations are unsuitable for precise quantification (13). Additionally, because of the polarity of these salts, their solubility in organic solvents is low and, therefore, both their preliminary extraction from pharmaceutical dosage forms and their purification are difficult. These problems would be applicable to the GLC analysis of homatropine methylbromide, which is contained in a number of pharmaceutical preparations.

Since homatropine methylbromide is a semisynthetic product, it would be desirable to assay for the drug in the presence of closely related natural products, *e.g.*, atropine and scopolamine, and the usual constituents of the pharmaceutical dosage forms in which it is contained. Furthermore, the assay should be specific for the intact drug molecule and be independent of the hydrolysis products of homatropine methylbromide, *i.e.*, tropine methylbromide and mandelic acid.

With these considerations in mind, a GLC procedure for the assay of homatropine methylbromide was developed which is based upon the amount of mandelic acid produced upon its hydrolysis. This procedure was applied, with satisfactory results, to several commercial tablet and elixir preparations containing homatropine methylbromide.

EXPERIMENTAL

Materials—*N,O*-Bis(trimethylsilyl)acetamide[1] was used as both the silylating solvent and the silylating agent. Hydrochloric acid buffer (pH 2.2) was prepared according to the procedure outlined in USP XVIII (14). Homatropine methylbromide[2], *d,l*-mandelic acid[3], and 2-naphthol[4] were used (the latter two melted at reported temperatures after recrystallization). Standard solutions used in analyses were prepared by diluting 60.0 mg. homatropine methylbromide to 100 ml. with distilled water and by diluting 20.7 mg. *d,l*-mandelic acid and 60.0 mg. 2-naphthol, respectively, to 100.0 ml. with anhydrous ether[5]. Both tablets[6] and elixirs[7] containing homatropine methylbromide were used for analysis.

Apparatus—A gas chromatograph[8] with isothermal control, flame-ionization detector, and an electronic digital integrator[9] was used. The columns were 1.52 m. long, spiral-shaped, borosilicate glass tubing (3.2-mm. i.d.) packed with 15% phenylmethyl silicone

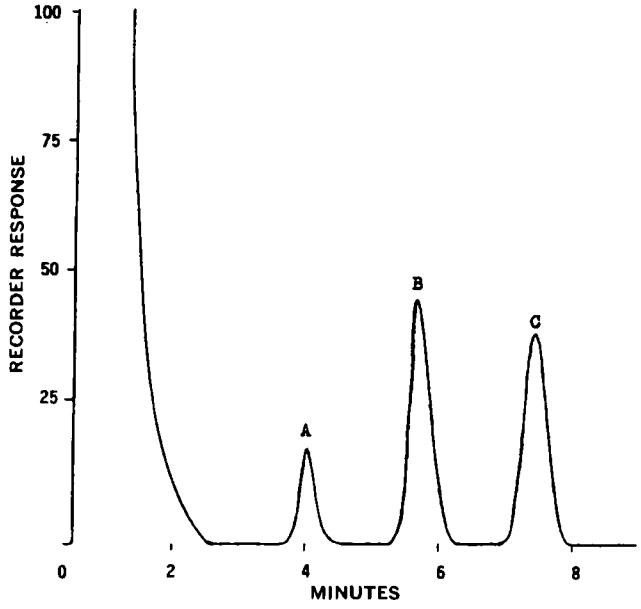

Figure 1—*Chromatogram trace of the silyl derivatives of: A, mandelic acid, 0.207 mg./ml.; B, tropic acid, 0.60 mg./ml.; and C, 2-naphthol, 0.6 mg./ml. Conditions were as listed in the text.*

on 60–80-mesh silanized, acid-washed, flux-calcined diatomite[10] which, prior to use, was conditioned at 275° for 24 hr. The injector port (fitted with a glass injection sleeve), column, and detector temperatures were 195, 160, and 210°, respectively. The gas flow rates were: hydrogen, 40 ml./min.; compressed air, 180 ml./min.; and nitrogen as the carrier gas, 50 ml./min.

Procedure—*Chromatographic Standards*—Mix together 1.0 ml. each of the *d,l*-mandelic acid and 2-naphthol standard solutions in a cone-shaped vial[11] fitted with a Teflon septum. Evaporate the solution to dryness with a stream of dry nitrogen, and add 200-μl. of the silylating agent to the residue contained in the vial. Cover and allow to stand at room temperature for 20 min., with occasional shaking. Inject an appropriate volume of the solution (1–3 μl.), on column, into the chromatographic system. Obtain the peak areas A_m for the trimethylsilyl derivative of *d,l*-mandelic acid and A_n for the 2-naphthol derivative. Calculate R_s for the chromatogram as follows:

$$R_s = A_m/A_n \qquad \text{(Eq. 1)}$$

Tablets—Weigh and finely powder not less than 20 tablets containing homatropine methylbromide. Weigh accurately a portion of the powder, equivalent to about 0.60 mg. of homatropine methylbromide, and transfer with the aid of 10 ml. of the hydrochloric acid buffer to a separator. Add 10 ml. of hydrochloric acid buffer, and extract with three 20-ml. portions of anhydrous ether, discarding the ether extracts. Adjust the solution to pH 10–11 with 10% sodium hydroxide solution, and boil the solution on a hot plate for 20 min. Cool to room temperature, adjust the solution to pH 2.2 with hydrochloric acid buffer, and transfer to a separator. Extract the solution with three 20-ml. portions of anhydrous ether, receiving the extracts in a flask. Dry the combined ether extracts over anhydrous sodium sulfate and filter. Transfer the combined ether extracts to a cone-shaped vial, and evaporate to dryness with a stream of dry nitrogen. Add 200 μl. of the silylating agent to the residue contained in the vial, and proceed as directed under *Chromatographic Standards* beginning with: "Cover and allow to stand. . . ." Calculate R_u for the chromatogram as follows:

$$R_u = A_m/A_n \qquad \text{(Eq. 2)}$$

where A_m is the peak area for the trimethylsilyl derivative of mandelic acid and A_n is the peak area for the derivative of 2-naphthol.

[1] Silylation grade, Pierce Chemical Co., Rockford, Ill.
[2] Reference standard, NF XIII.
[3] Baker grade, J. T. Baker Chemical Co., Phillipsburg, N. J.
[4] Certified, Fisher Scientific Co., Fair Lawn, N. J.
[5] Ether anhydrous, Fisher Scientific Co., Fair Lawn, N. J.
[6] Commercial products.
[7] Dia-Quel elixir and placebo elixir, supplied by Marion Laboratories, Kansas City, Mo.
[8] Varian model 575.
[9] Varian model 475.
[10] QF-1 on Gas Chrom Q, Applied Science Labs., Walnut Creek, Calif.
[11] Reacti-Vials, Pierce Chemical Co., Rockford, Ill.

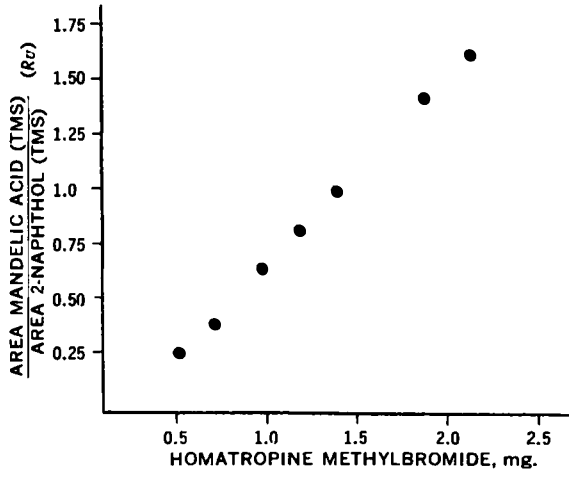

Figure 2—*Standard curve.* R_U versus *concentration.*

Calculate the quantity, in milligrams, of homatropine methylbromide in the sample as follows:

$$W_u = W_s \frac{R_s}{R_u} 2.43 \qquad \text{(Eq. 3)}$$

where W_u is the weight, in milligrams, of homatropine methylbromide in the sample and W_s is the weight, in milligrams, of *d,l*-mandelic acid in the standard solution.

Elixir—Transfer a volume of elixir equivalent to 0.60 mg. of homatropine methylbromide to a separator. Add 20 ml. of the hydrochloric acid buffer, and extract with three 20-ml. portions of anhydrous ether, discarding the ether extracts. Proceed as directed under *Tablets*, beginning with: "Adjust the solution. . . ."

RESULTS AND DISCUSSION

Studies of the hydrolysis of homatropine methylbromide indicated both the conditions and minimum time necessary to assure its complete hydrolysis. The present studies of the hydrolysis of homatropine methylbromide in 10% sodium hydroxide solution at various saponification times showed that complete hydrolysis is achieved after 8 min. Solutions of homatropine methylbromide in the hydrochloric acid buffer (pH 2.2) were also studied. After 30 min., no mandelic acid was detected in the buffer solution, which suggests that hydrolysis of the salt during the preliminary extraction process is negligible. These results support the work of Patel and Lemberger (15) on the stability of tropine esters in alkaline and acidic media.

Ether extraction of the acidic solution prior to hydrolysis removes many potentially interfering substances. Mandelic acid, which would be liberated as a result of any prior hydrolysis of homatropine methylbromide in the dosage form, does not interfere with the results of the assay since it is removed by ether extraction. This assay, therefore, can also be used to study the hydrolytic stability of the drug. In addition, other possible components of the dosage forms analyzed, such as benzoic acid and *p*-hydroxybenzoate esters, are also removed by this ether extraction.

Other possible contaminants, such as the methylbromide derivatives of atropine and scopolamine, are quantified in the chromatographic scheme. For example, solutions containing both atropinium methylbromide and homatropine methylbromide were analyzed by this procedure. The results indicate that the retention time of the trimethylsilyl derivative of tropic acid relative to that of the trimethylsilyl derivative of *d,l*-mandelic acid in this system is 1.54, and the resolution factor, calculated according to the method of Gadzinowicz (16), is 4.60.

Silylation rates of *d,l*-mandelic acid and 2-naphthol at room temperature were also studied. The results indicate that the maximum quantity of the trimethylsilyl derivative is obtained after 9.5 min. These derivatives were also found to be stable at room temperature for a least 24 hr. when protected from light and moisture.

Studies of liquid stationary phases on the column packing material indicated that a relatively high charge (15%) of the liquid stationary phase was necessary to obtain proper separation of the trimethylsilyl derivatives of tropic and *d,l*-mandelic acids. While this charge leads to less than optimal chromatographic parameters (asymmetry: 1.20; tailing: 1.20) (17), the resulting baseline separation (Fig. 1) allows for accurate peak area determinations.

Table I—Assay of Commercial Homatropine Methylbromide Preparations

Sample	Other Known Components	Homatropine[a] Methylbromide	*N*	Standard Deviation
Elixir A	Opium tincture, pectin, preservatives	98.64	10	2.94
Elixir B	Phenobarbital, preservatives	97.59	8	2.86
Tablet A	Phenobarbital	99.46	9	1.80
Tablet B	—	100.86	8	2.10

[a] Expressed as percent of labeled amount.

A plot of R_u and concentration of homatropine methylbromide, in milligrams, for the analysis of placebo elixir containing added amounts of homatropine methylbromide was constructed and was found to be linear (Fig. 2). This calibration curve should be constructed and used to determine the amount of homatropine methylbromide analyzed by this procedure.

Samples of placebo elixir containing added amounts of homatropine methylbromide were analyzed, with reproducible results, on successive days by the procedure outlined here. The mean calculated value of the results was 99.12% of the added homatropine methylbromide, with a coefficient of variation of 2.6%. Several commercial tablet and elixir preparations were also assayed by this procedure, and the results are reported in Table I. In all cases, the trimethylsilyl derivative of tropic acid was absent, and there were no interfering peaks in the chromatogram.

In conclusion, a GLC determination of the mandelic acid liberated by the saponification of homatropine methylbromide was developed and is proposed as an assay for the drug contained in both tablets and elixirs. The assay is specific for the determination of the mandelic acid esters of quaternary ammonium salts, and the usual components of tablets and elixirs do not interfere. This procedure was applied, with satisfactory results, to several tablet and elixir preparations containing homatropine methylbromide

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Abstract □ The application of high-pressure liquid chromatography to the separation and analysis of trisulfapyrimidines in pharmaceutical dosage forms is demonstrated. The preparation of samples of both tablet and suspension dosage forms is simple and rapid. The chromatographic conditions chosen optimize the separation of sulfadiazine, sulfamerazine, and sulfamethazine and allow quantitative analysis of these trisulfapyrimidines in a reasonable time.

Keyphrases □ Trisulfapyrimidine formulations—analysis, high-pressure liquid chromatography □ Sulfa drugs, sulfadiazine-sulfamerazine-sulfamethazine—separation, analysis, high-pressure liquid chromatography □ High-pressure liquid chromatography—analysis, trisulfapyrimidine formulations

The separation and quantitative analysis of sulfadiazine, sulfamerazine, and sulfamethazine in pharmaceutical dosage forms present a difficult problem to the pharmaceutical analyst. Current methods and proposed modifications of them are slow and tedious. The USP method (1) uses a paper chromatographic separation prior to colorimetric determination, by means of the Bratton–Marshall reaction, of the eluted individual sulfonamides. Modifications proposed by Kunze and coworkers (2, 3) require extreme care for satisfactory results. Banes and Riggleman (4) recently proposed a hybrid assay for trisulfapyrimidine preparations in which total sulfonamides are measured colorimetrically by the Bratton–Marshall procedure. Sulfadiazine is then measured colorimetrically by means of its specific reaction with thiobarbituric acid; sulfamethazine is separated from its homologs by column partition chromatography and is then determined by UV spectrophotometry. The third sulfonamide is obtained by difference. A qualitative chromatogram is used to confirm that only the three sulfonamides are present.

The determination of trisulfapyrimidines in dosage forms by high-pressure liquid chromatography was first reported by Poet and Pu (5). A recent paper by Kram (6) reported the conditions for the separation of a number of sulfapyrimidines. The determination of trisulfapyrimidines by high-pressure liquid chromatography overcomes or circumvents many shortcomings of the previously reported methods. The preparation of samples is simple and rapid, and separation and analysis times are reasonably short.

EXPERIMENTAL[1]

Mobile Phase and Reagents—The mobile phase, 0.2 *M* disodium phosphate solution adjusted to pH 6.0 with 85% phosphoric acid, was prepared fresh daily. Sodium hydroxide and sulfuric acid solutions (1 *N*) were required.

Internal Standard Solution—The stock internal standard solution, 1200 mcg./ml., was prepared by first dissolving 120 mg. of sulfadimethoxine in 5 ml. of 1 *N* sodium hydroxide solution and then diluting to 100 ml. with distilled water.

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Standard Curve Solutions—These solutions were prepared by suitable dilution of the stock standard solution with distilled water. They contained each of the trisulfapyrimidines at a concentration between 108 and 132 mcg./ml. and the internal standard at a concentration of 120 mcg./ml.

Preparation of Tablet Sample—For a single-tablet analysis, the weight of a single tablet was determined. For a batch analysis, the weight of a pool of several tablets was determined and the average tablet weight was calculated. The sample was ground to a fine powder. An accurately weighed portion of the powdered sample, equivalent to 36 mg. of total trisulfapyrimidines, was transferred to a 100-ml. volumetric flask containing 10 ml. of 1 *N* sodium hydroxide solution. The stoppered flask was shaken on a mechanical reciprocal shaker for 15 min. A total of 9 ml. of 1 *N* sulfuric acid was added while the flask contents were swirled. Some distilled water was added, followed by 10 ml. of internal standard solution and enough additional distilled water to make 100 ml. of solution. A portion of the well-shaken extract, transferred to a glass-stoppered test tube, was centrifuged for 10 min. at 2000 r.p.m., and the supernate was used for analysis.

Preparation of Suspension Sample—A weight of well-shaken trisulfapyrimidine suspension, equivalent to 36 mg. of total trisulfapyrimidines, was transferred to a 100-ml. volumetric flask, and 10 ml. of 1 *N* sodium hydroxide solution was added. Preparation of the sample was continued as described earlier for tablet samples.

Conditions for Chromatographic Separation—The important features of the liquid chromatograph used were described in detail elsewhere (7, 8). The degassed mobile phase was passed through the cation-exchange column under a pressure of 1000 psig., to obtain a flow rate of 0.7–0.8 ml./min. at room temperature, until a stable

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Table I—Effect of the Mobile Phase Molarity on Retention Time[a]

Compound	0.01 *M*	0.1 *M*	0.2 *M*
Sulfadiazine	64	67	70
Sulfamerazine	97	103	112
Sulfadimethoxine	140	155	182
Sulfamethazine	240	270	308

[a] Expressed as the number of seconds elapsed between injection and attainment of the chromatographic peak maximum.

baseline was obtained at a photometer attenuation of 8×10^{-3}. Replicate 4-μl. injections of standard and sample solutions were made, using a 10-μl. syringe[2]. The chart recorder provided a record of the elution of the sulfonamides from the column as peaks on a chromatogram, while the electronically calculated peak areas were printed out on paper tape.

RESULTS AND DISCUSSION

The purpose of this study was to determine the operating conditions for chromatography that would optimize the resolution of the trisulfapyrimidines in a reasonable time with a suitable level of precision.

The use of an internal standard was intended to minimize the effects of minor procedural variations. Sulfadimethoxine was chosen as the internal standard because it was eluted between two existing peaks in the chromatographic system employed and, in solution, demonstrated properties similar to those of the other trisulfapyrimidines.

The operating conditions studied included pH and molarity of the mobile phase, as well as pressure and flow rate. The effect of pH of the mobile phase on the separation of the trisulfapyrimidines is illustrated in Fig. 1. Although no separation of sulfamerazine and sulfadimethoxine was obtained at pH 5.7, adequate separation was obtained at pH 6.0. The compounds were well separated at pH 6.3; however, the higher the pH, the longer was the time required for elution of the more strongly retained sulfonamides. Therefore, pH 6.0 was chosen for the separation to minimize the time required. The effect of the molarity of the mobile phase on the time that a sulfonamide is retained on the column is shown in Table I. These data were obtained in a preliminary screening study using a faster mobile phase flow rate than was finally chosen. Despite an unexpected moderate increase in the retention time for the two more strongly retained sulfonamides, a 0.2 *M* disodium phosphate buffer solution, pH 6.0, was selected for the mobile phase to ensure the buffering stability of the system. Because buffer solutions that had been stored or used over extended periods of time adversely affected the chromatogram baseline, a fresh supply of buffer solution was prepared daily. The chosen pressure of 1000 psig., which produced a flow rate of 0.7–0.8 ml./min., resulted in separation of the sample components within 15–20 min.

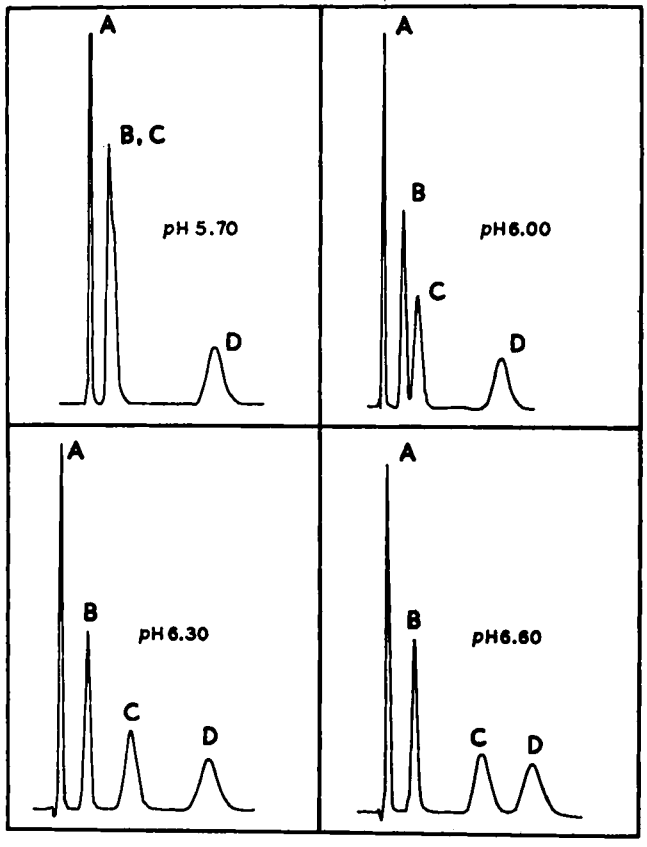

Figure 1—*Effect of pH on the separation of sulfadiazine (A), sulfamerazine (B), sulfadimethoxine (C), and sulfamethazine (D).*

[2] Hamilton 701N.

Table II—Response Factors for Standard Solutions[a]

Sulfonamide	Average Response Factor	Standard Deviation	Variance	Coefficient of Variation, %
Sulfadiazine	1.2827	±0.0332	0.0011	2.6
Sulfamerazine	1.2356	±0.0246	0.0006	2.0
Sulfamethazine	0.9162	±0.0221	0.0005	2.4

[a] These data represent four injections of each of five standard solutions.

Table III—Concentrations of Trisulfapyrimidines in Sulfonamide Tablets and Suspensions

Sample	Sulfadiazine mg.	Sulfadiazine %	Sulfamerazine mg.	Sulfamerazine %	Sulfamethazine mg.	Sulfamethazine %
Tablet A[a]	161.7	96.8	164.9	98.7	163.0	97.6
Tablet B	167.2	100.1	167.8	100.5	172.4	103.2
Tablet C	165.6	99.2	162.0	97.0	164.7	98.6
Tablet D	161.3	96.6	166.9	99.9	169.7	101.6
Suspension A[b]	165.9	99.3	158.7	95.0	158.9	95.2
Suspension B	180.4	108.0	169.1	101.3	171.8	102.9

[a] Theory = 167.0 mg. of each sulfonamide per tablet. [b] Theory = 167.0 mg. of each sulfonamide/5 ml. of suspension formulation. In these calculations, the specific gravity for the particular suspension, previously determined, was employed.

The area under the curve for each peak on the chromatograms was calculated electronically. The response factor, the ratio of each sulfonamide peak area to the area of the internal standard, was calculated for each chromatogram. Because the response factors were linearly related to sulfonamide concentration, average response factors for a 120-mcg./ml. concentration of each sulfonamide were computed from the ratios (Table II). Response factors for each sulfonamide were determined for a limited number of standard solutions on the same day that samples were assayed. Thus, the effect of changes in response factors due to possible changes in the operating conditions or in the column could be minimized.

Analytical data obtained for four representative lots of tablet formulations and two suspension formulations are presented in Table III. The calculated coefficients of variation for replicate sample injections ranged from 0.9 to 4.0%. The data in Table III demonstrate the utility of high-pressure liquid chromatography for the analysis of these trisulfapyrimidines in pharmaceutical dosage forms.

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PHARMACEUTICAL TECHNOLOGY

Evaluation of Inhalation Aerosols Using a Simulated Lung Apparatus

ARNOLD W. KARIG*, GARNET E. PECK▲, and GLEN J. SPERANDIO

Abstract □ A model lung chamber was designed for the evaluation of oral inhalation aerosols. The lung chamber was a compartmentalized unit based on certain parameters of the human respiratory tract. A vacuum system was used to regulate the flow rate through the chamber. Based on studies of air flow rate and evaluations with medicinal aerosol units, a vacuum of 30.4 cm. (12 in.) of mercury was chosen as the most suitable pressure for analysis of the aerosol samples within the chamber. Sampling of the chamber was by gravity, deposition of the nebula in sample collection vials attached to the base of each compartment, or full rinsing of each compartment. Samples were analyzed spectrophotometrically. The particle-size distributions of aerosolized talc samples from each chamber compartment were determined to evaluate the separation characteristics of the model lung chamber. Solutions of varying strengths of isoproterenol hydrochloride and phenylephrine hydrochloride aerosolized using several common aerosol devices demonstrated the model lung chamber to be a suitable device for evaluating medicinal and pharmaceutical aerosol units.

Keyphrases □ Inhalation aerosols—evaluated using simulated lung chamber □ Aerosols, inhalation—evaluated using simulated lung chamber □ Simulated lung chamber—design used to evaluate inhalation aerosols □ Lung chamber, simulated—design used to evaluate inhalation aerosols

Until recently, aerosol inhalation therapy has received comparatively little attention when compared with the more conventional dosage routes for drugs. However, with the development of pressurized aerosol technology and portable aerosol-generating equipment and an increasing appreciation of inhalation as a route for the administration of medicinals, considerable interest has developed in this area.

Inhalation therapy may be used to administer drugs for local and/or systemic response. By effecting depth of penetration and retention of inhaled medicinals, it is possible to obtain a purely local action without systemic effects or a combination of local and systemic action (1–3). Several parameters influence the penetration and deposition of inhaled materials in the lung.

Extensive research (4–12) has been conducted on the relationship of particle size to distribution and retention in the lungs. The conflict of theories and experimental results from these investigators has been attributed to such variable factors as species of animal used, nonuniform breathing rates, methods of measurement of particle-size distribution, methods of administering the agents used, and effects of lung moisture content on the size of inhaled particles (3, 6, 7, 13, 14). All authors agree, however, that depth of penetration increases with decreasing particle size while whole lung retention increases with increasing particle size (1, 6, 15, 16). The optimum particle-size range for inhalation of medicinals into the lungs is currently accepted as 0.5–5.0 μ (17, 18).

While the importance of particle size in inhalation therapy has been well documented, the other parameters effecting deposition and retention of inhaled matter have received less attention. In addition, pharmaceuticals are available in pressurized form for administration as either liquid droplets or fine solid particles. However, no evidence in the literature documents the superiority of solution systems of inhalation aerosols over suspension systems or vice versa.

Since *in vivo* evaluation of inhaled materials can lead to a disparity of results due to variable factors, there is a need for a good *in vitro* method of evaluating inhaled materials. This study describes the development of a collection chamber for *in vitro* analysis of materials from pressurized pharmaceutical aerosols or other aerosol-generating equipment.

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Table I—Model Lung Chamber Flow Rates

Vacuum Setting, cm. (in.) of Mercury	Air Flow Rate through Lung Chamber, ft./sec.
7.6 (3)	2.5
15.2 (6)	3.0
22.8 (9)	3.5
30.4 (12)	3.5
38.1 (15)	4.0
50.8 (20)	5.0

EXPERIMENTAL

Construction of Model Lung Chamber—In the design of the lung chamber, it was desired to create a device that would relate to the pathway followed by an inhaled aerosol in the respiratory tract of man. The superstructure of the lung chamber was fabricated from an acrylic plastic tube 15.2 cm. (6.0 in.) in diameter [13.9-cm. (5.5-in.) i.d.] and 92.1 cm. (36.25 in.) in length.

The tube was partitioned into six compartments by cementing in place plastic plates at specified intervals. Each compartment was designed to represent a given segment of the human respiratory tract. The basic parameters used in design of the lung chamber were based on the measurements of Findeisen (4) and Landahl (8). Compartment 1 represented the trachea, Compartment 2 the primary bronchi, Compartment 3 the secondary bronchi, Compartment 4 the tertiary bronchi, Compartment 5 the quatenary bronchi, and Compartment 6 the remainder of the bronchial tree. The pathlength from the entrance into a compartment to the exit from that compartment was four times the length of the corresponding lung segment represented by the compartment. For example, the pathlength from the entrance orifice of Compartment 1 to the opening into Compartment 2 represented approximately four times the length of man's trachea.

The diameters of the openings into each lung compartment were five times the diameter of the corresponding component of the human lung. The sole exception was the tracheal opening (opening into Compartment 1) which was approximately actual size for more effective screening of large aerosol particles normally too large to penetrate into the lungs. Openings were located in a staggered up and down and off-center arrangement. The pathway from one orifice to the next was at varying angles. This arrangement was chosen to give a rough approximation of the tortuous path followed by inhaled aerosol particles in the lungs.

Entrance to the tracheal compartment of the lung chamber was *via* a segment constructed to represent the approximate actual size of the human mouth. The mouth segment was constructed by cementing a 7.6-cm. (3-in.) diameter [6.3-cm. (2.5-in.) i.d.] tube, 5.9 cm. (2.36 in.) in length, to the front portion of the chamber. The opening into the mouth compartment was designed to fit the mouthpiece of commercial aerosol units. The opposite end of the lung chamber was sealed with a plastic plate through which a center vacuum spout was affixed.

Threaded sample collection ports were centrally located at the base of Compartments 1–5. These threaded ports were fitted with 20-ml. glass vials for collection of aerosol samples from each compartment for spectrophotometric or particle-size analysis. For cleaning purposes the lung unit could be disassembled into four parts or sections. Flat rubber gaskets were used as seals between the lung sections. The sections were held together by two large plastic plates fitted on each end of the lung and pulled together by three threaded rods having wing nuts on each end.

Sample Collection Procedures—Two sample collection procedures were employed in the study of liquid aerosols. In the first method, a 3.0-ml. portion of distilled water was placed in each of the five collection vials. Samples of the nebula were collected by gravity deposition of the mist during aerosol delivery. Following the sampling sequence, the collection vials were removed and an additional 7.0-ml. portion of distilled water was added to each vial. After thorough mixing, the samples were analyzed spectrophotometrically.

The second method of sample collection employed rinsing of each chamber compartment and analysis of the resultant solution. The aerosol was delivered to the chamber, with dry collection vials attached to each collection port. Following aerosol delivery, the chamber was inverted, the collection vials were removed, and 20.0-ml. portions of distilled water were added to each compartment using a hypodermic syringe. In Compartments 1 and 2, the 20.0 ml. of distilled water was added directly into the compartment proper. In Compartments 3–5, the 20.0 ml. of distilled water was first directed around the orifice of the compartment endplate by means of a plastic tubing extension on the syringe to remove the impacted sample. The chamber was slowly rotated to wash thoroughly all sample from the sides. The collection vials were then attached, and the sample solutions were drained into the vials. Spectrophotometric analysis of the samples was then conducted.

Table II—Average (Three Trials) Deposition Patterns for Two Commercial Medicinal Aerosol Units at Several Vacuum Settings

Compartment	Mercury 7.6 cm. (3 in.)	15.2 cm. (6 in.)	22.8 cm. (9 in.)	30.4 cm. (12 in.)	38.1 cm. (15 in.)	50.8 cm. (20 in.)
Isoproterenol HCl[a], % Deposition per Compartment of Aerosol Depositing in Chamber Compartments 1–5						
1	55.9	58.1	46.7	45.0	42.8	35.5
2	17.5	12.9	11.7	10.7	12.5	12.4
3	6.4	5.5	6.4	6.1	7.2	9.0
4	5.3	6.0	10.9	11.8	12.6	17.0
5	14.9	17.5	24.3	26.4	24.9	26.1
Isoproternol Sulfate[b], % Deposition per Compartment of Aerosol Depositing in Chamber Compartments 1–5						
1	39.4	38.6	34.3	33.6	33.0	30.7
2	23.0	22.1	21.5	22.9	23.4	16.8
3	12.9	10.9	13.9	10.9	11.3	7.7
4	10.8	12.2	13.9	12.9	14.5	18.8
5	13.9	16.2	16.4	19.7	17.8	26.0

[a] Isuprel Mistometer, Winthrop Laboratories, New York, NY 10016. [b] Medihaler-Iso, Riker Laboratories, Northridge, CA 91324

For sample collection of powder aerosols, dry sample vials were attached to each collection port of the lung chamber. Following delivery of the powder aerosol, the powder collected in each compartment was shaken into the collection vials or pushed into the vials using a spatula. The samples were analyzed using a Coulter counter[1] to determine the solid particle distribution of the powder sample from each compartment of the lung chamber.

Aerosol Devices—Two metered commercial medicinal aerosol units and one commercial pharmaceutical aerosol unit were employed in evaluating the operating characteristics of the lung chamber. One of the commercial medicinal aerosol units was a fine-particle suspension of isoproterenol sulfate in an inert propellant of fluorochlorohydrocarbons. Each metered dose delivered 75 mcg. of isoproterenol sulfate. The second metered aerosol employed was a solution of isoproterenol hydrochloride, alcohol, and fluorochlorohydrocarbon propellants. Each measured dose of the unit delivered approximately 125 mcg. of isoproterenol hydrochloride. For delivery of aerosol to the chamber, the units were in the inverted operating position with the mouthpiece inserted into the mouth of the chamber by a clamp arrangement. The requisite number of actuations was then made. One commercial pharmaceutical aerosol unit, a pressurized perfumed talc spray, was used in the study. Delivery of powder to the lung chamber was by means of short bursts directed into the mouth of the lung.

Several nonpressurized aerosol devices were employed, including an air-displacement aerosol unit, a hand nebulizer for solutions and a powder nebulizer adapted for hand operation. The air-displacement aerosol unit consisted of two pieces, a compressor and a nebulizer. The compressor was connected to the nebulizer by means of a plastic air hose. The diaphragm-type compressor was designed to deliver oilless air to the nebulizer at approximately 12 p.s.i. pressure. The nebulizer unit was baffled and vented so that a reverse air flow produced an increased delivery of finer particles. For use with the lung chamber, the air-displacement aerosol unit was adapted for continuous flow, the rate of delivery being approximately 5 ml. of solution/min. Solutions of isoproterenol hydrochloride or phenylephrine hydrochloride were delivered on a timed basis. The nebulizer unit was positioned with its tip inserted into

[1] Model B, Coulter Electronics, Hialeah, Fla.

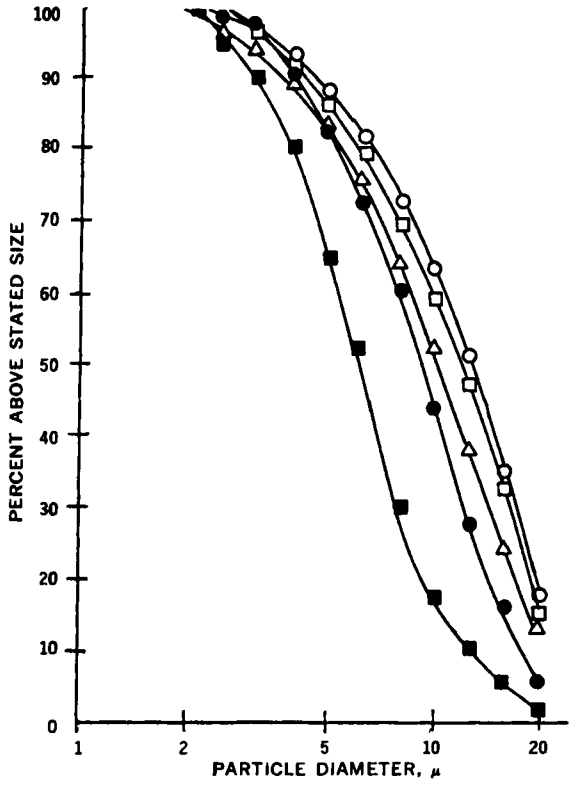

Figure 1—*Particle-size distributions for nebulized talc USP samples collected from lung chamber compartments. Key: ○, Compartment 1; □, Compartment 2; △, Compartment 3; ●, Compartment 4; and ■, Compartment 5.*

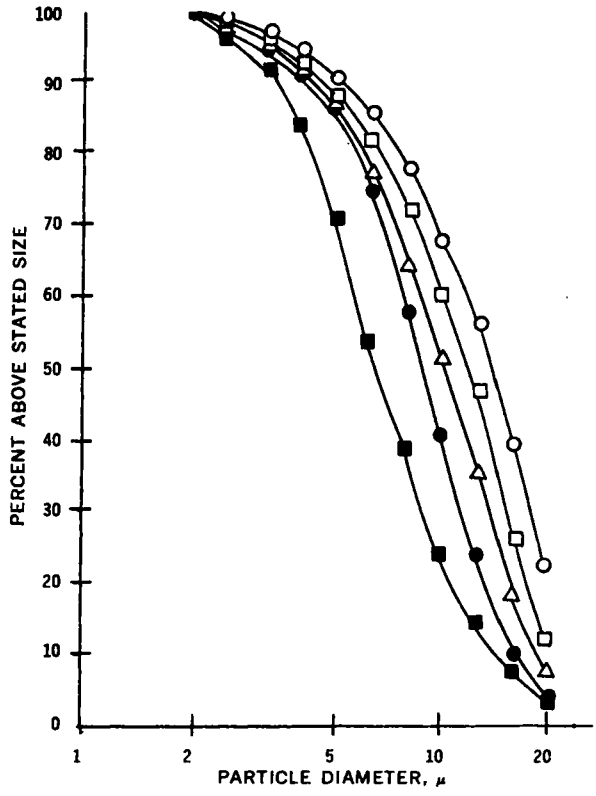

Figure 2—*Particle-size distributions for commercial talc spray samples collected from lung chamber compartments. Key: ○, Compartment 1; □, Compartment 2; △, Compartment 3; ●, Compartment 4; and ■, Compartment 5.*

the chamber mouth using the same arrangement employed for the pressurized medicinal aerosols.

Both the solution nebulizer and the powder nebulizer were operated by means of a rubber hand bulb. Solutions of isoproterenol hydrochloride were aerosolized by a given number of compressions of the hand bulb per trial. The powder blower was used to nebulize talc USP for delivery to the lung chamber.

Analytical Instrumentation—Solutions of phenylephrine hydrochloride and isoproterenol hydrochloride were analyzed using a spectrophotometer[2]. A slit width of 0.3 and a sensitivity setting of 0.4 were used for all solutions analyzed. Absorbances were determined in 1-cm. silica cells using distilled water as the blank. Absorbance determinations were made at 279 nm. for isoproterenol hydrochloride and 273 nm. for phenylephrine hydrochloride. A standard plot of absorbance *versus* concentration was prepared for each drug. Samples from the lung chamber were analyzed within 2 hr. after collection to minimize possible formation of degradation products and their possible influence on the results.

Particle-size determinations of powder aerosol samples were made using the Coulter counter with a converter[3]. Each sample was analyzed using both the 70- and 140-μ aperture tubes. A 0.85% solution of sodium chloride was used as the dispersion medium. Samples were dispersed for counting by use of a suitable mechanical stirrer.

RESULTS

Establishment of Operating Vacuum—Measurements of the air flow rate through the chamber at several vacuum settings, as well as evaluations of the two commercial medicinal aerosol units at the same vacuum settings, were conducted to establish the operating vacuum of the lung chamber. Measurements of the flow rate were made at several different vacuum settings using a meter[4] (Table I). As expected, there was a gradual increase in flow rate through the chamber as the vacuum setting was increased. The measured flow rate at 22.86 and 30.4 cm. (9 and 12 in.) of mercury was identical.

Evaluations of aerosol samples delivered by the commercial medicinal aerosol units were undertaken at vacuum settings of 7.6, 15.2, 22.8, 30.4, 38.1, and 50.8 cm. (3, 6, 9, 12, 15, and 20 in.) of mercury. Three trials were conducted for each vacuum setting, with sample collection being by full chamber rinsing of each compartment. A total of 2250 mcg. of isoproterenol sulfate or isoproterenol hydrochloride was delivered to the chamber per trial. The average deposition patterns are presented in Table II for two commercial inhalation aerosol products.

The data in Table II reveal that at either end of the vacuum range there are marked changes in deposition pattern. The pattern of percent deposition per compartment at a setting of 7.6 cm. (3 in.) of mercury vacuum is markedly different from the pattern at 50.8 cm. (20 in.) of mercury. On the other hand, at vacuum settings in the middle of the range studied, deposition patterns do not reflect wide deviation. Deposition patterns at 22.8, 30.4, and 38.1 cm. (9, 12, and 15 in.) of mercury vacuum are similar. These results correlate well with the measurements of the flow rate through the chamber. Based on these findings, a vacuum setting of 30.4 cm. (12 in.) of mercury was chosen for operation of the lung chamber.

A check of the total sample delivered from the medicinal aerosol units revealed absorbance values twice as large as expected. The interfering substance in the isoproterenol hydrochloride solution was the ascorbic acid present in the formulation. Sorbitan trioleate[5] was determined to be the ingredient interfering with the analysis of the isoproterenol sulfate suspension aerosol. Solutions of varying concentrations of both the ascorbic acid and the sorbitan trioleate were analyzed spectrophotometrically at 279 nm. A linear absorbance *versus* concentration plot was obtained for each drug. All available evidence indicated approximately the same interference by each compound.

[2] Beckman DU-2, Beckman Instrument Co., Fullerton, Calif.
[3] Model M.
[4] Wallac-Thermex GGA2, Gelman Instrument Co., Chelsea, Mich.

[5] Span 85, ICI Atlas Chemical Industries, Wilmington, DE 19899

Table III—Average (Six Trials) Absorbance and Deposition Values for 30-min. Air-Displacement Aerosol Unit Delivery of Solutions of Isoproterenol Hydrochloride and Phenylephrine Hydrochloride

5.0% Isoproterenol Hydrochloride Solution

Compartment Number	A[a]	Drug, mcg.[b]	Percent Deposition per Vial of Total Aerosol Sample Collected
1	0.600	535	37.3
2	0.334	298	20.8
3	0.191	170	11.9
4	0.319	285	19.8
5	0.164	146	10.2
Total	1.608	1434	

5.0% Phenylephrine Hydrochloride Solution

Compartment Number	A	Drug, mcg.[c]	Percent Deposition per Vial of Total Aerosol Sample Collected
1	0.552	627	36.1
2	0.338	384	22.1
3	0.183	208	12.0
4	0.306	348	20.0
5	0.149	169	9.8
Total	1.528	1736	

1.25% Isoproterenol Hydrochloride Solution

Compartment Number	A	Drug, mcg.[b]	Percent Deposition per Vial of Total Aerosol Sample Collected	$\frac{A\ 1.25\%\ \text{Solution}}{A\ 5.0\%\ \text{Solution}} \times 100, \%$
1	0.156	139	39.0	26.0
2	0.092	82	23.0	27.5
3	0.046	41	11.5	24.1
4	0.069	62	17.3	21.6
5	0.037	33	9.2	22.9
Total	0.400	357		0.400/1.608 = 24.9

2.5% Phenylephrine Hydrochloride Solution

Compartment Number	A	Drug, mcg.[c]	Percent Deposition per Vial of Total Aerosol Sample Collected	$\frac{A\ 2.5\%\ \text{Solution}}{A\ 5.0\%\ \text{Solution}} \times 100, \%$
1	0.260	295	36.7	47.1
2	0.158	180	22.3	44.1
3	0.089	101	12.5	48.6
4	0.127	144	17.9	41.5
5	0.075	85	10.6	50.3
Total	0.709	805		0.709/1.528 × 100 = 46.4

1.0% Isoproterenol Hydrochloride Solution

Compartment Number	A	Drug, mcg.[b]	Percent Deposition per Vial of Total Aerosol Sample Collected	$\frac{A\ 1.0\%\ \text{Solution}}{A\ 5.0\%\ \text{Solution}} \times 100, \%$
1	0.113	100	36.6	18.8
2	0.070	62	22.6	20.9
3	0.037	33	12.0	19.4
4	0.059	53	19.1	18.5
5	0.030	29	9.7	18.3
Total	0.309	275		0.309/1.608 × 100 = 19.2

1.25% Phenylephrine Hydrochloride Solution

Compartment Number	A	Drug, mcg.[c]	Percent Deposition per Vial of Total Aerosol Sample Collected	$\frac{A\ 1.25\%\ \text{Solution}}{A\ 5.0\%\ \text{Solution}} \times 100, \%$
1	0.121	137	39.2	21.9
2	0.065	74	21.0	19.2
3	0.031	35	10.0	16.9
4	0.063	72	20.4	20.6
5	0.029	33	9.4	19.5
Total	0.309	351		0.309/1.528 × 100 = 20.2

[a] A = absorbance. [b] Drug = isoproterenol hydrochloride. [c] Drug = phenylephrine hydrochloride.

Calibration with Solid Particle Aerosols—To calibrate the particle separation of an aerosol sample by the model lung chamber, two trials employing talc were performed. In one experiment, talc USP was nebulized using a powder blower. The second experiment involved the delivery of talc to the chamber from a pressurized commercial perfumed talc spray. A setting of 30.4 cm. (12 in.) of mercury vacuum was used. Samples were analyzed using the Coulter counter, and analysis was conducted with both the 70- and 140-μ aperture tubes (Figs. 1 and 2).

Examination of the data reveals a similar type of selective particle deposition occurring in the lung chamber as occurs in the respiratory tract of man. The larger particles in a disperse system of particle sizes are filtered out by the trachea and upper regions of the respiratory tract, with the finer particles penetrating to greater depths before depositing. Thus, as the aerosol sample proceeds into the respiratory tract, the particle-size distribution changes from high percentages of larger particles to high percentages of fine particles. The same type of deposition pattern is shown by the results of the talc analysis presented in Figs. 1 and 2. Compartment 1 shows the highest percentage of the larger particles of the talc sample, while Compartment 5 shows the highest percentage of the smaller particles from the talc sample. As the sample proceeds from Compartment 1 through to Compartment 5, the percentage of large particles in the distribution gradually decreases while the percentage of fine particles in the distribution gradually increases.

Evaluations with an Air-Displacement Aerosol Generator—When using the air-displacement aerosol generator, experiments were conducted using both high volume and low volume aerosol delivery. In the high volume aerosol delivery experiments, solutions of 5.0, 1.25, and 1.0% isoproterenol hydrochloride and 5.0, 2.5, and 1.25% phenylephrine hydrochloride were delivered to the chamber for 30 min./trial. Six trials were conducted for each solution strength listed. Sample collection was by gravity deposition of the nebula in collection vials containing 3 ml. of distilled water. Table III lists the average absorbance values and patterns. Reference to the table shows that the deposition pattern is the same for all solutions tested. This demonstrates that the deposition at a particular vacuum flow setting is a function of this particular aerosol-generating device and not the drug or strength of drug in solution.

The deposition values of the 1.25 and 1.0% isoproterenol hydrochloride solution trials are approximately 25 and 20%, respectively, of the 5.0% isoproterenol hydrochloride solution values, as expected. This same type of relationship is also demonstrated by the results from the three different strength phenylephrine hydrochloride solutions (Table III). Although the relationship is not as close as for the isoproterenol hydrochloride solutions, the deposi-

Table IV—Average (Six Trials) Absorbance and Deposition Values for Hand-Operated Nebulizer Aerosols

Compartment Number	2% Isoproterenol Hydrochloride Solution: A[a]	Drug, mcg.[b]	Percent Deposition per Compartment of Aerosol Depositing in Compartments 1–5
1	0.247	442	32.1
2	0.160	286	20.8
3	0.113	202	14.7
4	0.108	193	14.1
5	0.141	252	18.3
Total	0.769	1375	

Compartment Number	1% Isoproterenol Hydrochloride Solution: A	Drug, mcg.	Percent Deposition per Compartment of Aerosol Depositing in Compartments 1–5	$\frac{A\ 1\%\ \text{Solution}}{A\ 2\%\ \text{Solution}} \times 100$, %
1	0.150	268	31.8	60.7
2	0.097	173	20.6	60.6
3	0.072	129	15.2	63.7
4	0.072	129	15.2	66.7
5	0.081	145	17.2	57.4
Total	0.472	844		0.472/0.769 × 100 = 61.4

[a] A = absorbance. [b] Drug = isoproterenol hydrochloride.

tion patterns are similar. The low volume aerosol delivery experiments did not show significantly different patterns.

Evaluations with a Hand-Operated Nebulizer—The aerosol produced by a hand-operated nebulizer (for solutions) was evaluated in the lung chamber. Solutions of 1.0 and 2.0% isoproterenol hydrochloride were nebulized using 50 actuations/trial. Six trials were conducted for each solution at a vacuum setting of 30.4 cm. (12 in.) of mercury. Sample collection was accomplished by rinsing each compartment with 20.0-ml. portions of distilled water.

The deposition patterns of the 1.0 and 2.0% solutions appear to be nearly identical (Table IV). The absorbance values from the 1.0% isoproterenol hydrochloride samples are about 61% of the 2.0% solution values. One primary disadvantage of the hand nebulizer is a lack of accurate dosage control, the amount of pressure applied to each squeeze of the rubber hand bulb varying the amount of aerosol produced (Table IV).

SUMMARY

The design of a model lung chamber was shown to be a suitable method for evaluating medicinal or pharmaceutical aerosols, free from the many variables of *in vivo* analysis. Construction of the unit was based on literature values for the size of the human respiratory tract. An operating vacuum of 30.4 cm. (12 in.) of mercury was established based on measurements of air flow and evaluations with commercial medicinal aerosol units. The solid particle segregation of solid particle aerosols by the lung chamber was used to evaluate the aerosols produced by different aerosol-generating devices. By using the lung chamber unit, it should be possible to compare liquid solution aerosols to solid suspension aerosols of a drug to determine if there is any difference in their deposition in the respiratory tract.

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Semiautomated Determination of Mandelic Acid as Benzaldehyde in Content Uniformity Testing of Methenamine Mandelate Tablets

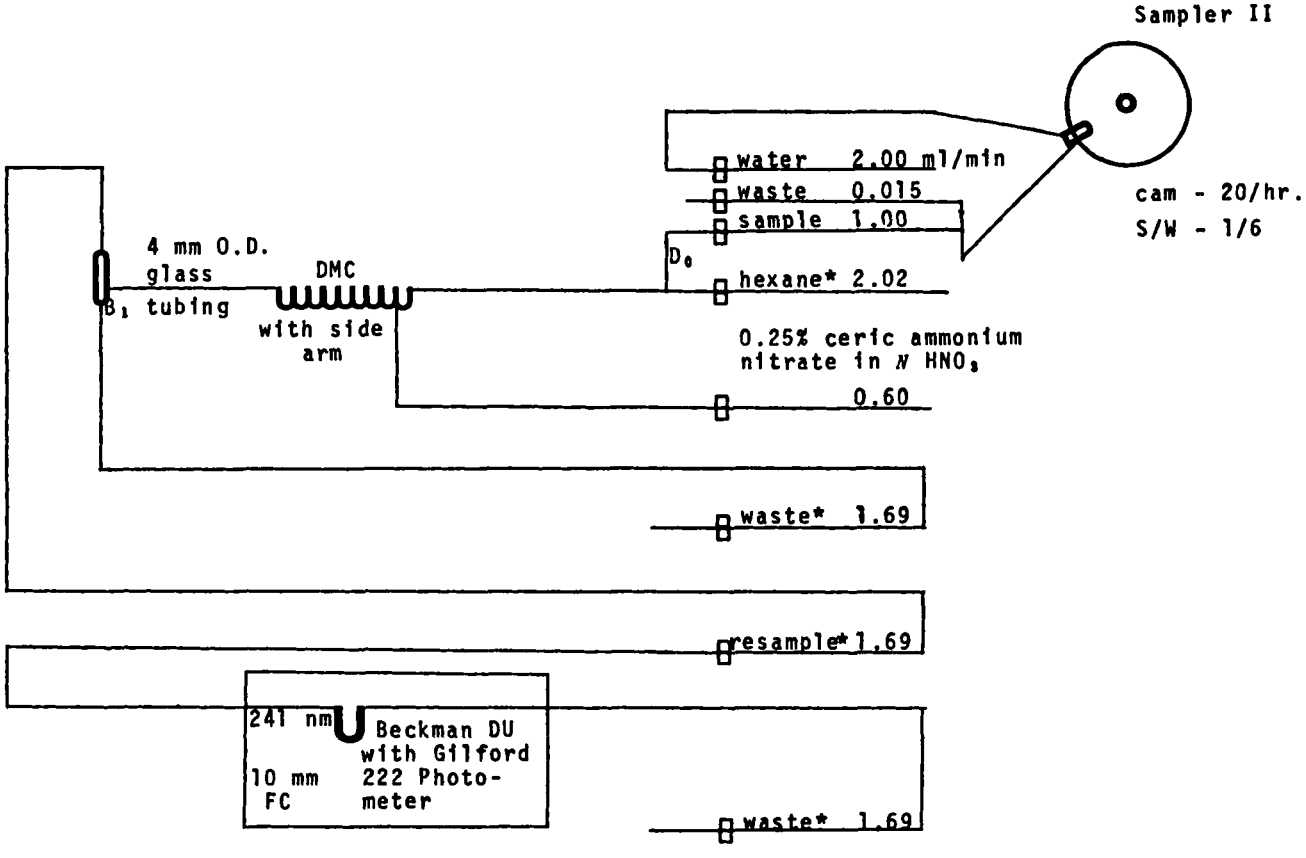
ROBERT E. DALY▲ and LESTER CHAFETZ

Abstract □ A semiautomated method for the determination of the mandelic acid component of methenamine mandelate tablets is described. A programmed cam rate of 20 samples/hr., excellent recoveries, and relative standard deviations of about 1.5% were obtained. Benzaldehyde, formed by ceric-ion oxidation of the arylglycolic acid, is determined in the system described.

Keyphrases □ Methenamine mandelate tablets—semiautomated spectrophotometric determination of content uniformity, mandelic acid as benzaldehyde □ Tablet content uniformity, methenamine mandelate—semiautomated spectrophotometric determination □ Content uniformity, methenamine mandelate tablets—semiautomated spectrophotometric determination □ Arylglycolate ester drugs, content uniformity—semiautomated spectrophotometric determination □ UV spectrophotometry—determination of content uniformity for methenamine mandelate tablets

Dow *et al.* (1) provided an autoanalytic method for methenamine mandelate tablets based on colorimetry of formaldehyde released from the methenamine moiety of the drug. An alternative method based on the mandelic acid component, which is potentially useful for control of a number of different drugs, is presented here.

Chafetz (2) described a method for spectrophotometric determination of mandelic acid and other arylglycolic acids as benzaldehyde or aryl ketones formed by oxidation with ceric ion. This method is used in the NF XIII (3) for assay of oxyphencyclimine hydrochloride tablets and for content uniformity determina-



Scheme I—Flow diagram: methenamine mandelate content uniformity manifold

tion of homatropine methylbromide tablets (4). Cerimetric titration methods using the same chemistry have been described for methenamine mandelate formulations (5) and for homatropine hydrobromide (6). Automation of the spectrophotometric method is described here, wherein 20 units of methenamine mandelate tablets can be determined in an hour.

EXPERIMENTAL[1]

Materials—Methenamine mandelate USP, 0.25% ceric ammonium nitrate in 1 *N* nitric acid, and hexane were used.

Assay—*Standard Preparation*—Transfer about 100 mg. of methenamine mandelate USP [previously assayed by direct cerimetric titration (2)], accurately weighed, to a 100-ml. volumetric flask and dilute to volume with distilled water. Further dilute 10.0 ml. to 100.0 ml. with water to obtain a concentration of about 100 mcg./ml.

Assay Preparation—Transfer one tablet to the jar of the blender, add exactly 100.0 ml. of distilled water, and blend for about 15 sec. With a disposable pipet, withdraw a portion of the sample and completely rinse the cap and the sides of the jar. Blend the sample for an additional 45 sec. Dilute the solutions to a final concentration of about 100 mcg. methenamine mandelate/ml.

Procedure—Assemble the apparatus as shown in Scheme I. Allow the recorder and spectrophotometer to stabilize for at least 30 min. and charge the reagent lines. Insert the 20/hr. sample cam with a 1:6 sample–wash ratio, place 2-ml. sample cups in the sampler, and arrange so that duplicate assays are obtained for each tablet and each 10-assay preparation is bracketed with a standard preparation. Set the recorder on the 50-mv. scale, adjust the wavelength of the spectrophotometer to 241 nm., and adjust the absorbance meter to an absorbance of 1.200 and the ratio switch on the power supply to 0.25.

Calculations—For a 1-g. tablet, milligrams per tablet = $A_u/A_s \times 10C$; for a 0.5-g. tablet, milligrams per tablet = $A_u/A_s \times 5C$; and for a 0.25-g. tablet, milligrams per tablet = $A_u/A_s \times 2.5C$; where A_u and A_s are the absorbance of the sample and standard, respectively, and C is the weight, in milligrams, of standard taken.

RESULTS AND DISCUSSION

A 0.25% solution of ceric ammonium nitrate in 1 *N* nitric acid was sufficient for reproducible oxidation of methenamine mandelate in the concentration range employed. Adherence to Beer's law was demonstrated over a concentration range of from 50 to at least 150 mcg./ml.

Methenamine mandelate tablets[2] USP (declaring 1, 0.5, and 0.25 g./tablet) were assayed. Recoveries of added methenamine mandelate in six replicate assays of each formulation by the automated method were 99.8 ± 1.27% for the formulation declaring 1 g./tablet, 99.4 ± 1.38% for the formulation declaring 0.5 g./tablet, and 99.2 ± 1.87% for the formulation declaring 0.25 g./tablet.

SUMMARY AND CONCLUSIONS

A rapid, sensitive, and repetitive semiautomated method has been described for determination of the content uniformity of methenamine mandelate tablets. Excellent recovery data were obtained for methenamine mandelate added at the 0.25-, 0.5-, and 1-g./tablet levels. On the basis of previous work on spectrophotometric determination of arylglycolate ester drugs (2), it is expected that the method could easily be extended to the content uniformity determination of the tablet preparations of clidinium bromide, glycopyrrolate, homatropine methylbromide, mepenzolate bromide, oxyphencyclimine hydrochloride, and penthienate bromide in NF as well as to unofficial drugs with similar structure.

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[1] A Waring blender equipped with a liter glass jar, a Sargent SRLG recorder, a Beckman DU spectrophotometer modified with a Gilford 222 photometer and equipped with an elevated cell chamber cover and automated apparatus (Technicon AutoAnalyzer, Technicon, Inc., Tarrytown, N. Y.), which included a sampler (Sampler II) and a proportionating pump (Proportionating Pump I) were employed.

[2] Mandelamine tablets, Warner-Chilcott Laboratories, Division of the Warner-Lambert Co.

Variables Involved in an Automated Tablet-Coating System

ALLEN HEYD

Abstract □ Operational characteristics of an automatic airless spray tablet-coating system were evaluated. The major variables considered were volume of fluid delivery and spray pattern characteristics. The study showed that: (*a*) a linear relationship existed between the pressure employed and the volume of fluid delivered, (*b*) viscosity had a small effect on the volume of fluid delivered, (*c*) spray tip orifice size was directly related to fluid delivery volume, and (*d*) spray patterns can be effectively used in formulating and selecting the proper conditions for spray application of a tablet-coating liquid with an automatic coating system.

Keyphrases □ Tablet-coating system, automatic, airless—evaluation of volume of fluid delivery and spray pattern characteristics, relationships □ Coating of tablets, automatic, airless—evaluation of volume of fluid delivery and spray pattern characteristics, relationships

The art of tablet coating has become, in recent years, a scientific process in which an exact and reproducible amount of coating material can be delivered in a uniform manner onto a tablet mass. The evolution of this scientific process began with the design of an air suspension technique by Wurster (1), which was later refined for special automatic techniques. The impetus for developing better tablet-coating techniques was continued by Lachman and Cooper (2) and Mody *et al.* (3), who developed automatic film coating. More recently, Heyd and Kanig (4) described an improved automated tablet-coating system.

Once these automated systems were developed, the need for a better coating procedure became evident. The conventional method of completely wetting down the tablets and then allowing them to dry with the aid of forced hot air could no longer be used. Instead, some variation of short intermittent wetting and drying cycles was introduced to apply the tablet coat more uniformly. In addition, a complete reformulation of the coating solution was sometimes necessary when converting from the conventional tablet-coating technique to an automated process. Accordingly, the spray apparatus selected in an automated system must be evaluated with respect to the variables involved in the use of diverse coating solutions as well as for the mechanical aspects of the process.

The purpose of this study was to evaluate the influence of the automated coating system *per se* and the nature of the coating liquid on the volume of coating fluid delivery and its spray pattern.

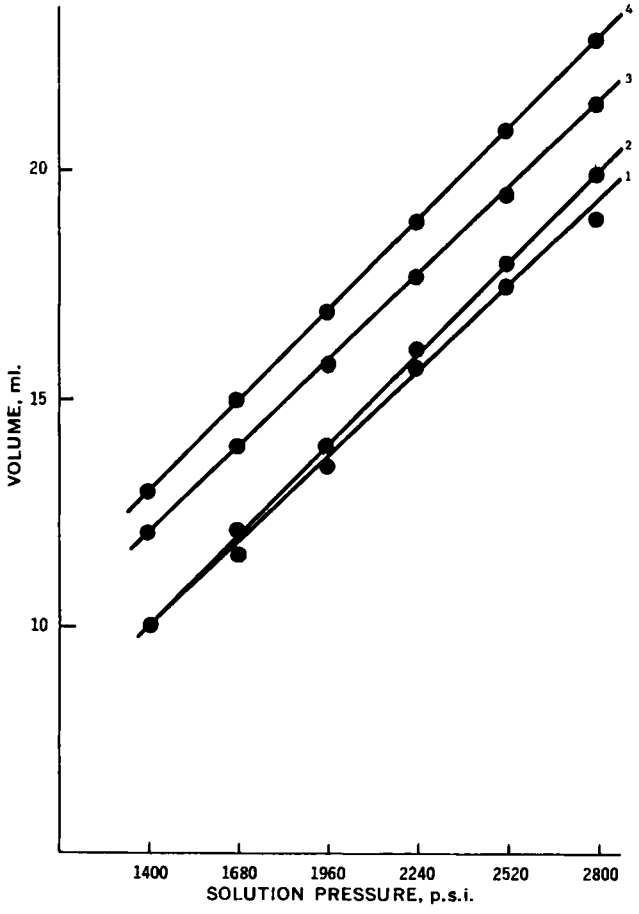

Figure 1—*Pressure-volume curves, 3-sec. delivery. Key: 1, calcium carbonate, 5%; 2, syrup USP; 3, water; and 4, alcohol USP.*

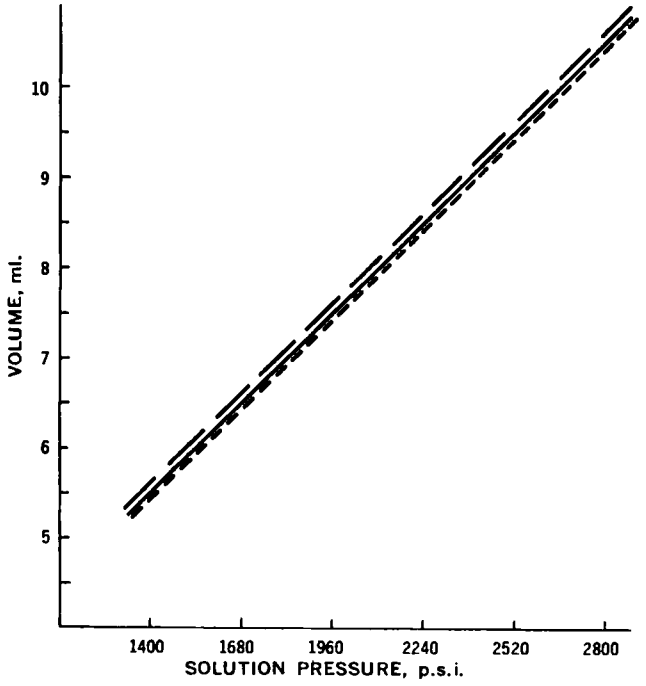

Figure 2—*Pressure-volume curves, calcium carbonate suspensions. Key: — —, 5%; ——, 15%; and ---, 35%.*

Table I—Delivery Volume as a Function of Pressure

Liquid	Rate, ml./p.s.i. (10^3)	Total Volume Delivered, ml. 1400 p.s.i.	2100 p.s.i.	2800 p.s.i.
Calcium carbonate, 5%	6.7	10	14.7	19
Syrup USP	7.1	10	15	20
Water	6.8	12	16.8	21.5
Alcohol USP	7.1	13	18	23

EXPERIMENTAL

Materials and Equipment—The liquids used in this study were: distilled water; syrup USP; shellac, 2.7 kg. (6 lb.) cut; 5, 15, and 35% (w/v) aqueous suspensions of calcium carbonate; and a 2% (w/v) solution of methylcellulose[1], 500 cps.

The equipment utilized to evaluate the factors involved in an automated tablet-coating system was a self-programming automated tablet-coating system with two automatic spray guns[2] as previously described (4).

Fluid Delivery Studies—Fluid delivery experiments were conducted to ascertain the influence of pressure, spray tip orifice size, and type of liquid on the volume of spray delivered.

The volume of fluid delivery in milliliters was determined from 1400 to 2800 p.s.i. nozzle pressure in 140 p.s.i. pressure increments. The tip orifice sizes varied from 0.023 cm. (0.009 in.) to 0.053 cm. (0.021 in.) in diameter. All experiments were conducted in an air conditioned room at approximately 25° and 30–40% relative humidity.

Three-second fluid spray delivery cycles were used, and five replicate samples of each liquid under study were collected, at each pressure and nozzle tip size, in a 100-ml. graduated cylinder; the resulting volumes were recorded.

Spray Pattern Characteristics—Spray patterns were determined for the liquids studied, each containing 1% (w/v) FD&C Red No. 2 dye for contrast. The spray from the activated gun of the automated tablet-coating system was directed at a specially designed target which consisted of a 61-cm. square pane of glass with five concentric circles painted 10 cm. apart. The tip of the spray gun was positioned 30 cm. from the glass surface and at the center point of the target. The spray cycle was set for a 0.25-sec. solution delivery. The patterns were subsequently observed at 1400-, 1960-, and 2800-p.s.i. nozzle pressure (the lower, middle, and upper pressure range, respectively) for all tip sizes ranging from 0.023 cm. (0.009 in.) to 0.053 cm. (0.021 in.) in orifice diameter. After each spray, the pattern dimensions were taken and pattern uniformity was noted.

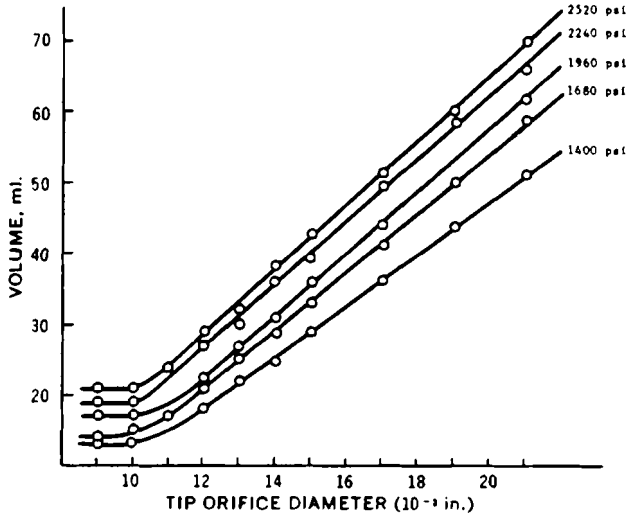

Figure 3—*Change in delivery with respect to change in tip orifice size using water.*

[1] Methocel MC, Dow Chemical Co., Midland, Mich.
[2] Graco Hydro-Spray Unit, Gray Co., Minneapolis, Minn.

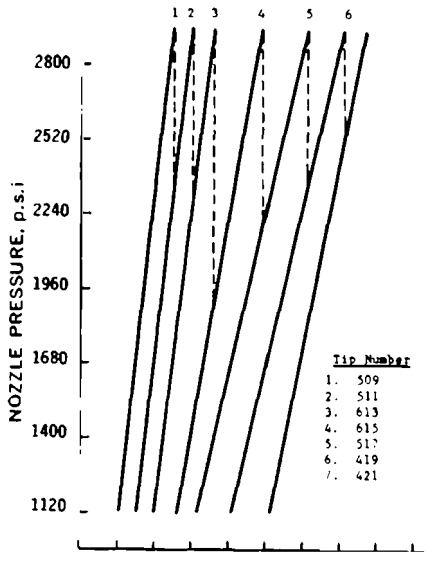

Figure 4—*Fluid delivery curves, 3-sec. cycle.*

RESULTS AND DISCUSSION

Fluid Delivery—The pressure–volume curves in Fig. 1, constructed from fluid delivery data obtained with a 0.023-cm. (0.009-in.) spray tip and a 3-sec. delivery, show that the change in fluid volume delivered as a function of pressure was constant. The curves are representative of some of the liquids studied, and each shows excellent linearity. The rate of change of delivery (Table I) for the liquids represented in Fig. 1 was essentially the same.

A 3–4-ml. difference in total volume delivered at each pressure increment was found between the four liquids studied. These small volume differences do not appear to be significant, but the data show a trend toward a decrease in volume delivered with an increase in fluid viscosity and solids content when comparing syrup and calcium carbonate with water and alcohol. A significant difference would be expected at a much lower or at atmospheric pressure. The data, therefore, indicate that fluid volume delivery is essentially independent of the physical properties of the fluid at very high pressures.

Fluid delivery curves for the three calcium carbonate suspensions, which varied from a "watery" to an "ointment-like" consistency (Fig. 2)[3], are in essence identical and confirm that the elevated pressures used in the study obliterated the effects of the physical properties of the fluids delivered.

In Fig. 3, the volume of water delivered in 3 sec. *versus* tip orifice diameter shows a good linear relationship within the range of 0.030 cm. (0.012 in.)–0.053 cm. (0.021 in.). This finding is unusual because one would expect an orifice–volume relationship based on

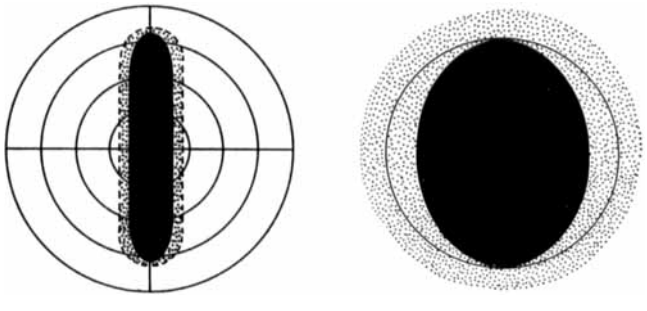

Figure 5—*Spray patterns with water. Left: 0.023-cm. (0.009-in.) tip size and 30 × 9-cm. pattern size. Right: 0.053-cm. (0.021-in.) tip size and 40 × 30-cm. pattern size.*

[3] Data points for Fig. 2 were omitted for the purpose of clarity.

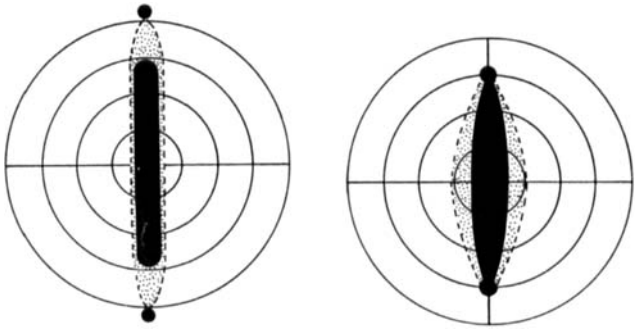

Figure 6—*Spray patterns with syrup USP. Left: 0.023-cm. (0.009-in.) tip size and 29 × 8-cm. pattern size. Right: 0.053-cm. (0.021-in.) tip size and 32 × 12-cm. pattern size.*

the square of the orifice diameter. All fluids studied showed similar results.

Since a pressure–volume relationship was found for the liquids studied, it can be postulated that pressure–volume graphs for specified spray time cycles and tip orifice sizes can be prepared for all coating liquids used in tablet-coating systems utilizing an automatic spray system. From these graphs, delivery volume at various pressures and tip orifice sizes can be estimated. Figure 4 is a theoretical example of such a graph constructed for water as the vehicle. For a specific delivery volume, a different pressure level at a constant tip size or a different tip orifice size at a constant pressure can be chosen from these constructed graphs. The vertical broken lines on the curves show the points where the tip orifice size and pressure parameters overlap so that either combination may be used.

Spray Pattern Characteristics—In general, the solutions studied formed rather individual spray patterns (Figs. 5–8). All patterns consisted of an unbroken, uniform film, usually with sharp edges and surrounded by a "corona" effect. Each solution possessed an individual spray pattern which changed in fan length and width with an increase in tip orifice size. Water produced an elongated oval when using a 0.023-cm. (0.009-in.) tip orifice size and more of a circular oval when using a 0.053-cm. (0.021-in.) tip orifice size (Fig. 5). The 85% syrup solution (Fig. 6) formed a peculiar long, slender, round-edged pattern with two dots equidistant from the ends of the long axis which were joined to the main body of the pattern by a corona. This pattern slowly changed to a dumbell shape as the tip size increased to 0.053 cm. (0.021 in.). Methylcellulose and shellac solutions as examples of polymeric substances, showed distinct rectangular forms with small coronas, which increased in width from the 0.023-cm. (0.009-in.) to the 0.053-cm. (0.021-in.) tip orifice size. The methylcellulose showed a decrease in pattern length and the shellac showed an increase in length with increased tip orifice size (Figs. 7 and 8, respectively).

Spray pattern studies serve to illustrate the type, the uniformity, and the area of surface coverage that can be achieved on a mass of moving tablets with coating solutions. They are especially useful

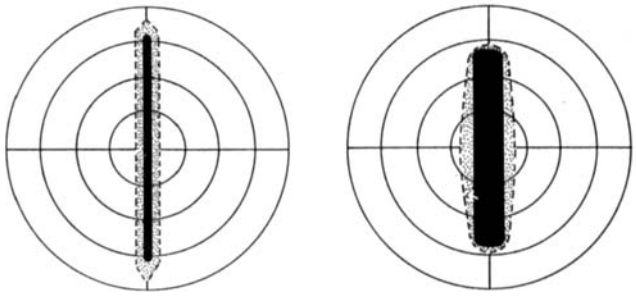

Figure 7—*Spray patterns with methylcellulose, 500 cps. Left: 0.023-cm. (0.009-in.) tip size and 34 × 3-cm. pattern size. Right: 0.053-cm. (0.021-in.) tip size and 27.5 × 7.5-cm. pattern size.*

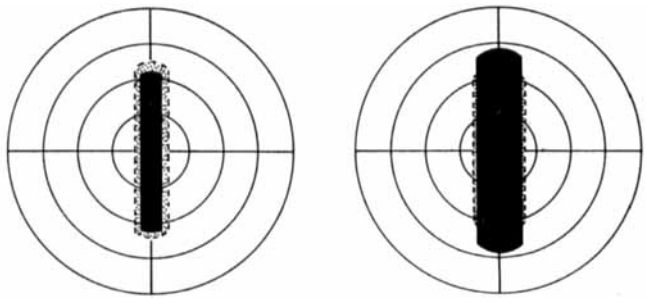

Figure 8—*Spray patterns with shellac, 2.7 kg. (6 lb.) cut. Left: 0.023-cm. (0.009-in.) tip size and 24 × 5.5-cm. pattern size. Right: 0.053-cm. (0.021-in.) tip size and 29 × 12-cm. pattern size.*

in the development of new tablet-coating formulations and in choosing the proper tip orifice size, spray pressure, and spraying distance from the moving tablet mass. Spray patterns relate directly to the moving surface of the tablet mass, because each pattern is illustrative of the pattern that will appear on the surface of the mass at any given moment in time.

SUMMARY

The operational characteristics of an automatic airless spray system were evaluated and the variables affecting the delivery of various liquid systems were studied. The major variables studied produced data that permitted the development of several relationships:

1. A linear relationship between the pressure employed and the volume delivered existed for all solutions studied, regardless of the nature of the solvent.
2. When compared to water, increases in viscosity or in insoluble solids content caused a very small decrease in volume of solution delivered. The volume delivered for any suspension was not materially changed with an increase in solids concentration at a given pressure.
3. Spray tip orifice size was directly related to delivery volume at a constant pressure. Normally, as previously mentioned, the orifice–volume relationship would be expected to follow a curve based on the square of the orifice diameter.
4. Spray patterns obtained under the experimental conditions were potentially useful in estimating area, shape, uniformity, and dimensions produced by a coating solution striking a mass of tablets. Estimates of this nature are valuable in selecting the proper conditions of spraying and other features such as orifice size, spray pressure, and spraying distance.
5. Pressure–volume plots were developed as prototypes of related plots which should be developed for other coating solutions. These data are useful as guides in properly coordinating spray tip, pressure, volume, and type of solution to produce optimum effects.

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Synthesis of *N*-Pentafluorobenzoyl-S-(−)-prolyl 1-Imidazolide, A New Electron-Capture Sensitive Reagent for Determination of Enantiomeric Composition

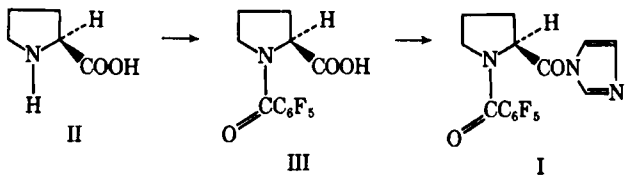
SHAIKH B. MATIN, MALCOLM ROWLAND, and NEAL CASTAGNOLI, Jr.▲

Abstract □ The synthesis of *N*-pentafluorobenzoyl-S-(−)-prolyl 1-imidazolide, a new electron-capture sensitive derivatizing reagent, is described. Its potential usefulness in the detection and quantitative estimation of the enantiomeric composition of amines at nanogram levels is discussed.

Keyphrases □ *N*-Pentafluorobenzoyl-S-(−)-prolyl 1-imidazolide—synthesized as electron-capture sensitive reagent for the GLC determination of enantiomeric composition □ Enantiomers, amines—determination of nanogram levels using *N*-pentafluorobenzoyl-S-(−)-prolyl 1-imidazolide as an electron-capture sensitive derivatizing reagent □ Electron-capture detection, nanogram levels of enantiomeric amines—use of *N*-pentafluorobenzoyl-S-(−)-prolyl 1-imidazolide as a derivatizing reagent

It is well established that derivatization of enantiomers with a chiral reagent yields diastereomers which often can be easily resolved and quantitatively estimated by gas–liquid partition chromatography (1–6). Utilizing this principle, Halpern and Westley (7) introduced the use of *N*-trifluoroacetyl-S-(−)-prolyl chloride to analyze the enantiomeric composition of amino acids. Gordis (8), Gunne (9), and later Wells (10) extended the use of this reagent to the determination of the enantiomeric composition of microgram quantities of amphetamine.

A second type of derivatizing reagent for primary and secondary amines utilizes moieties that are electron-capture sensitive. Such a process may enhance the gas–liquid partition chromatographic detectability of these amines several thousandfold (11). The pentafluorobenzamides of primary and secondary amines afford excellent electron-capture sensitivity (11), in contrast to the corresponding trifluoroacetamides which give almost no electron-capture response (12). For example, the *N*-pentafluorobenzoyl derivative of amphetamine can be detected at 1/6000th the level of the corresponding trifluoroacetamide. In an attempt to combine the electron-capture sensitive properties of the pentafluorobenzoyl moiety with the enantiomeric resolving property of the prolyl moiety, *N*-pentafluorobenzoyl-S-(−)-prolyl 1-imidazolide (I) was synthesized by the following route. S-(−)-Proline (II) was converted to its *N*-pentafluorobenzoyl derivative (III) under Schötten–Baumann conditions. The resulting amido acid was treated with *N,N'*-carbonyldiimidazole to yield the desired 1-imidazolide (I), a high melting, crystalline solid which proved to be stable when stored under anhydrous conditions (Scheme I).



II III I

Scheme I

EXPERIMENTAL

***N*-Pentafluorobenzoyl-S-(−)-proline (III)**—To a stirred suspension of S-(−)-proline (1.0 g., 8.7 mmoles) in 10 ml. water, maintained at 0° by an ice bath, was added dropwise a 10-ml. solution of ice-cold 0.2 *N* NaOH. When dissolution of the amino acid was complete, pentafluorobenzoyl chloride (2.0 g., 8.7 mmoles) was added slowly followed by the periodic addition of 0.2 *N* NaOH over a 2–3-hr. duration to maintain the pH of the reaction mixture at 8.0. After 30 min. of additional stirring in the cold, the reaction mixture was acidified with 0.2 *N* HCl and the resulting mixture was extracted with ether. After drying (sodium sulfate), the ether was removed *in vacuo* to yield 2.1 g. (6.8 mmoles, 73%) of a hygroscopic, white solid, m.p. 80–81°; $[\alpha]_D$ −91° (c 0.22%, C_2H_5OH).

Anal.—Calc. for $C_{12}H_8F_5NO_3$: C, 46.60; H, 2.58; N, 4.53. Found: C, 46.39; H, 2.52; N, 4.68.

***N*-Pentafluorobenzoyl-S-(−)-prolyl 1-Imidazolide (I)**—A solution of *N,N'*-carbonyldiimidazole (1.47 g., 9.07 mmoles) in 20 ml. anhydrous tetrahydrofuran was added over a 15-min. period with stirring to an ice-cold solution of Compound III (2.94 g., 9.51 mmoles) in 50 ml. anhydrous tetrahydrofuran. Stirring was continued for an additional 30 min., after which the white crystalline solid that had separated during the reaction was collected to yield 2.2. g. (6.13 mmoles, 64.4%) of the analytically pure imidazolide, m.p. 175–178°; $[\alpha]_D$ −34° (c 0.10%, $CH_3CO_2C_2H_5$).

Anal.—Calc. for $C_{15}H_{10}F_5NO_2$: C, 50.14; H, 2.80; N, 11.69. Found: C, 50.20; H, 2.90; N, 11.63.

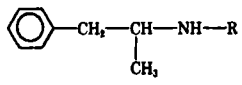
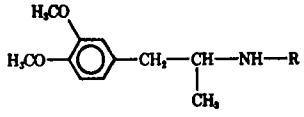
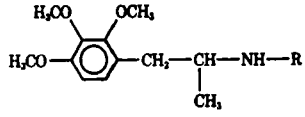
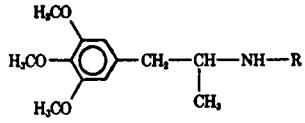
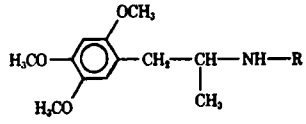
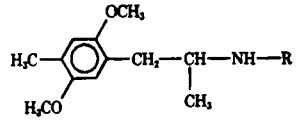
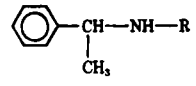
(−)-*N*¹-Pentafluorobenzoyl-*N*²-S-β-phenylisopropyl-S-prolylamide (IV*b*)—A solution of S-(+)-β-phenylisopropylamine (187 mg., 1.39 mmoles) in 10 ml. dry benzene was added to a suspension of Compound I (500 mg., 1.39 mmoles) in 2 ml. dry tetrahydrofuran. One hour later, the reaction mixture was shaken with 2 ml. water. The benzene layer was separated, dried (sodium sulfate), and concentrated *in vacuo*. The resulting solid was crystallized from 50% aqueous methanol to yield 500 mg. (1.17 mmoles, 84.7%) of amide IV*b*, m.p. 70–74°; $[\alpha]_D$ −84° (c 0.2%, C_2H_5OH).

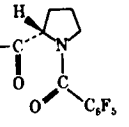
Anal.—Calc. for $C_{21}H_{19}F_5N_2O_2$: C, 59.11; H, 4.49; N, 6.57. Found: C, 59.29; H, 4.62; N, 6.39.

Apparatus—The GLC work was performed on a chromatograph[1] equipped with a ^{63}Ni detector. A 2.12-m. (6-ft.) × 0.31-cm. (0.125-in.) diameter glass column, packed with 3% OV-17 coated onto Chromo-

[1] Varian 1200.

Table I—Gas–Liquid Partition Chromatography[a] Retention Times of *N*-Pentafluorobenzoyl-S-prolyl Derivatives of Various Amines

Compound		Retention Time, min. Derived from			
Number	Structure[b]	Levorotatory Amine *a*	Dextrorotatory Amine *b*	Column Temperature	Resolution Factor[c]
IV		11.25	12.00	250°	0.6
V		12.48	14.40	250°	1.1
VI		16.50	17.75	270°	1.0
VII		13.00	15.00	270°	1.2
VIII		11.8	13.2	270°	1.2
IX		11.4	12.7	270°	1.1
X		10.5	9.0	245°	1.2

[a] Nitrogen flow, 25 ml./min.; injection temperature, 270°; and detector temperature, 300°. [b] R =  [c] See *Reference 14*. Note that when the resolution factor = 1, the peak resolution is approximately 98% complete.

sorb W AWDMCS HP, 100–120 mesh, was used. The column was conditioned for 48 hr. at 280°, the first 24 hr. without nitrogen flow.

Procedure for GLC Analyses—The amine (0.01–0.02 mmole) in anhydrous benzene (1 ml.) was treated with an equimolar amount of Compound I dissolved in 1 ml. anhydrous benzene for 30 min. at room temperature. The reaction mixture then was shaken with 1 ml. water for 30 min. and centrifuged at 2000 r.p.m. for 1 min. The benzene solution (1–5 μl.) was then injected onto the column.

Measurement of Electron-Capture Response—Amide IV*b*, or the diastereomeric mixture obtained with racemic amphetamine and Compound I, was injected onto the column in an amount giving a response that did not exceed 30% of the standard potential (11). The area under the peak was calculated as the product of peak height and width at half the peak height. By knowing the current for full-scale deflection at the sensitivity setting of the amplifier and the chart speed used, the area measurements were converted to coulombs. Response was then expressed as coulombs per mole injected.

RESULTS AND DISCUSSION

It was previously shown (11, 13) that the enhanced electron-capture sensitivity of the pentafluorobenzoylamides of primary and secondary amines over the corresponding trifluoroacetyl derivatives is a consequence of the greater resonance stabilization of the captured electron by the pentafluorobenzoyl moiety. Consequently, it was anticipated that pentafluorobenzoylprolyl derivatives would display electron-capture sensitivity. Quantitative estimations of the amide IV*b*, derived from S-(+)-amphetamine and Compound I, established the electron-capture sensitivity to be 2.4×10^3 coulombs/mole, comparable to the reported sensitivity of related derivatized secondary amines (11). The diastereomeric mixture consisting of amides IV*a* and IV*b*, obtained with racemic β-phenylisopropylamine and Compound I, gave two well-resolved peaks (Table I) of equal intensity on gas–liquid partition chromatographic analysis. When utilizing this derivatizing agent, as little as 1 ng. of these diastereomeric amides can be simultaneously detected. Thus, a sensitive method to estimate enantiomeric composition as well as the potential for quantitative estimation of these amines from biological fluids is established.

To investigate further the potential value of this reagent, the amines listed in Table I that were available either fully or partially resolved were converted to their corresponding *N*-pentafluorobenzoylprolyl derivatives and analyzed by gas–liquid partition chromatography. In the case of the six β-phenylisopropylamines (IV–IX, R = H), it was observed that the retention times of the levorotatory compounds were consistently shorter than the retention times of the corresponding dextrorotatory compounds. The absolute configurations of amphetamine (IV) (15), 1-(3,4-dimethoxyphenyl)-2-aminopropane (V) (16), 1-(3,4,5-trimethoxyphenyl)-2-

aminopropane (VII)[2], and 1-(2,5-dimethoxy-4-methyl)-2-aminopropane (IX)[2] were all established as R(−) and S(+). Consequently, it is tempting to assume a correlation between retention time and absolute configuration. Consistent with such an assumption is the observation that the amide of R-(+)-α-methylbenzylamine (X*b*) has a shorter retention time than the amide of S-(−)-α-methylbenzylamine (X*a*). However, until more information on the molecular nature of the interactions of such diastereomers with column materials is available, this suggestion must remain speculative. Similar differences in retention times with configuration of a series of camphorsulfonamides (17) of α-methoxy-α-methylpentafluorophenylacetamides (18) and of *N*-trifluoroacetylprolylamides (19) have been reported.

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Degradation of Bronchodilator Agents in Oxymix System

GEORGE H. KRONBERG▲ and BERTIL H. TAKMAN

Abstract □ Known amounts of isoproterenol, phenylephrine, and epinephrine were added to the aqueous oxymix system and thereby exposed to potential redox destruction. Thirty minutes later, recoveries of isoproterenol and phenylephrine were 89–100% and 89–108%, respectively, while the recovery of epinephrine was 53–79%.

Keyphrases □ Bronchodilator agents—degradation in oxymix system, clinical implications of concurrent use □ Oxymix–isoproterenol, phenylephrine, or epinephrine—degradation of bronchodilators, clinical implications of concurrent use □ Fluorometry—determination of stability of isoproterenol, phenylephrine, and epinephrine in aqueous oxymix system

Oxymix[1] is a mixture of ascorbic acid, cupric sulfate, sodium percarbonate, a buffer system, and excipients and is essentially a redox system. It has mucolytic properties and has been clinically administered as an aerosol in the treatment of several pathologic pulmonary conditions (1). Because bronchodilating agents are often used for the same indications, knowledge of the compatibility of representative bronchodilators and oxymix was considered necessary, particularly since concurrent administration might be preferred. Since no investigations of the stability of catecholamines or catecholamine-like compounds under such conditions had been reported previously, the stability of epinephrine, isoproterenol, and phenylephrine in the oxymix system was determined using fluorometric assay methods.

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EXPERIMENTAL

Apparatus—The fluorometric measurements were performed with two types of fluorometers[2]. The ion-exchange columns were of the same design and dimensions as described by Kelly and Auerbach (2) but contained 50 × 5-mm. resin beds.

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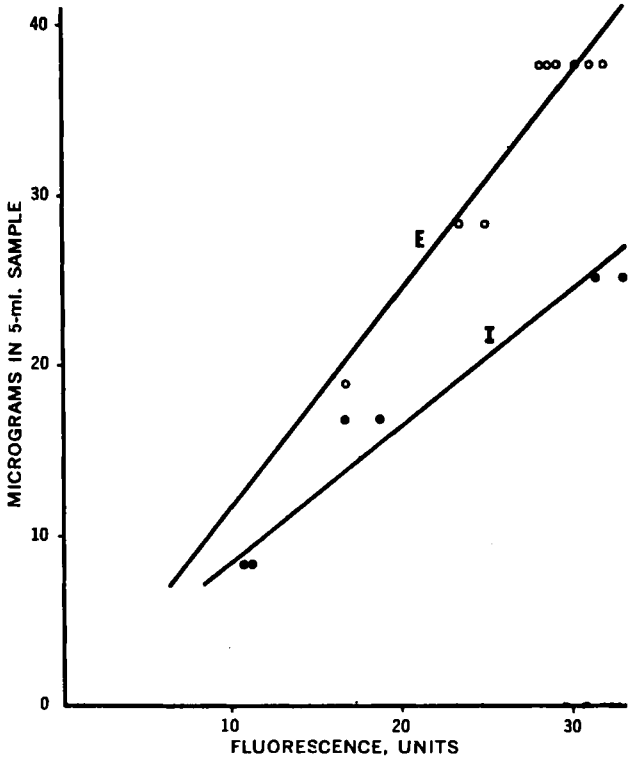

Figure 1—*Standard curves for isoproterenol (I) and epinephrine (E): fluorescence (units)* versus *amount in 5-ml. sample (micrograms of base form).*

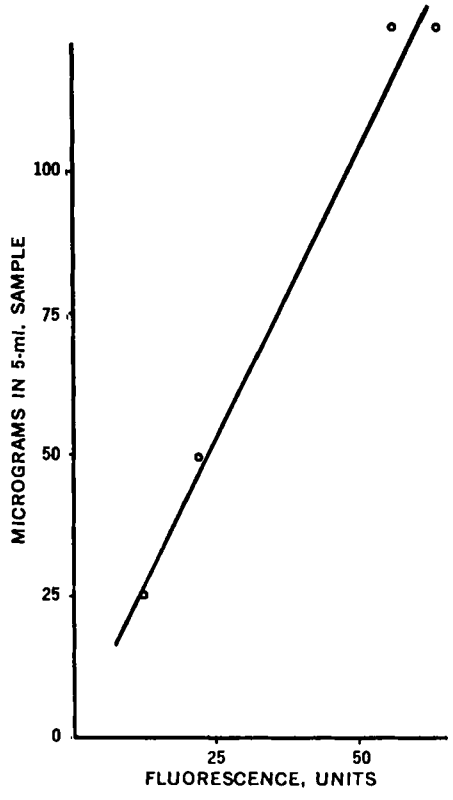

Figure 2—*Standard curve for phenylephrine: fluorescence (units)* versus *amount in 5-ml. sample (micrograms of base form).*

Reagents and Chemicals—The following were used: Amberlite CG 120 (type 2, 200 mesh, Na^+ form); bromthymol blue, 0.04% in ethanol; potassium carbonate, 2 *N* solution; phosphate buffer, 0.2 *M*, pH 6.5; zinc sulfate, 0.25% solution; potassium ferricyanide, 0.25% solution; sodium hydroxide, 5 *N* solution; ascorbic acid, 2% solution (prepared fresh daily); sodium hydroxide–ascorbic acid solution (9:1 v/v, freshly prepared within a few minutes of use); hydrochloric acid, 0.1 and 2 *N* solutions; oxymix (granulate) with a quantitative composition (percent) of: ascorbic acid 33.33, sodium percarbonate 19.67, cupric sulfate 0.07, polyvinylpyrrolidone 7.67, erythrosin 0.003, tartaric acid 16.00, sodium bicarbonate 21.51, mannitol 1.27, menthol 0.08, and saccharin 0.40; epinephrine bitartrate USP[2]; isoproterenol hydrochloride USP[2]; phenylephrine hydrochloride USP[2]; and sodium edetate, 0.1% solution (I), prepared by treating reagent grade ethylenediaminetetraacetic acid with 0.02 *N* NaOH to form a solution of pH 7.

Stock Solutions—Stock solutions of epinephrine, isoproterenol, and phenylephrine were made to contain the equivalent of 1 mg. base/ml. in 0.1 *N* HCl and were kept in amber bottles at 4° for subsequent dilutions. They were found to be quite stable for the duration of these experiments.

Ion-Exchange Columns—The ion exchangers were prepared by stirring about 20 g. of the resin with 100 ml. of 2 *N* NaOH solution (containing 1% ethylenediaminetetraacetic acid) for 15 min. Excess alkali was decanted and the resin was washed with distilled water. This procedure was repeated using 100 ml. 3 *N* HCl. The resin was allowed to settle and the fines were removed by decantation. The prepared resin was then slurried into columns (5 mm. i.d.) to form beds 50 mm. long. Further washing of the beds with distilled water compacted the resin to give flow rates varying from 10 to 20 ml./hr. Small plugs of glass wool were placed on top of the resin beds. At this point, the columns were ready for use.

Solution Systems—

1. *Reagent Blank*—Five milliliters of distilled water containing 0.5 ml. of I was used.

2. *Oxymix Blank (Aged 30 min.)*—To a 400-ml. tall-form beaker containing 75 ml. of distilled water, 12 g. of oxymix granulate was added as rapidly as allowed by the marked foaming that occurred during mixing. Cooling of the mixture suppressed the foaming. As soon as the first portion of oxymix was added, timing was begun. The mixture was stirred for 15 min., then transferred to a 100-ml. volumetric flask, diluted to the mark with distilled water, and centrifuged. A 1-ml. aliquot of this solution was transferred to a 10-ml. volumetric flask. At 30 min., 1 ml. of I was added and the mixture was diluted to the mark with distilled water.

3. *Pure Drug (Standard Solution)*—The requisite amount of drug (either from acid stock solution or from the solid powder form of its salt) was diluted to 100 ml. with distilled water in a volumetric flask. A 2.5-ml. aliquot of this solution was then transferred to a 25-ml. volumetric flask, followed by 2.5 ml. of I, and diluted to the mark with distilled water. If the drug was taken from its acid stock solution, the pH was adjusted to 6–7 with sodium hydroxide before the final dilution was completed.

4. *Drug in 30-min.-old Oxymix*—Oxymix granulate (12 g.) was dissolved in 50 ml. of distilled water and allowed to stir for 30 min. before the drug (salt form) was added. After transfer of the drug quantitatively by rinsing, the mixture was diluted to 100 ml. Aliquots of this solution were mixed with equal parts of I and the mixture was diluted fivefold. No pH adjustment was necessary.

5. *Oxymix Granulate Added to Drug Solution*—A quantity of drug (as the salt) corresponding to the concentration desired was accurately weighed and dissolved in 50 ml. of distilled water contained in a 400-ml. tall-form beaker. To this solution, 12 g. of oxymix granulate was added and the mixture was allowed to stir for 15 min. from the time that the first portion of oxymix was added. The mixture was then transferred and diluted in a 100-ml. volumetric flask at 30 min. Final dilutions were prepared as described for Solution System 4.

6. *Drug Added to Fresh Oxymix Solution*—In this solution system, 12 g. of oxymix granulate was dissolved in 50 ml. of distilled water and the salts of the drugs were then added quantitatively. Aging for 30 min. was again timed from the beginning of the addition of oxymix. In this case, the drug was actually introduced into the active oxymix system within 1–3 min.; loss in activity of the redox system could be considered almost negligible under these conditions. After the mixture had stirred for 15 min., it was transferred to

[2] Winthrop.

Table I—Stability of Bronchodilators in the Presence of Oxymix

Drug	Solution System	Amount Added, mg.	Number of Samples	Recovery, %
Isoproterenol	4	3.33	1	100
	5	3.33	2	89, 97
	6	3.33	2	89
Phenylephrine	4	5, 25	2	108, 93
	5	5–25	7	89–100
Epinephrine	4	7.5	5	73–79, 98[a]
	5	7.5	2	53, 65
	6	7.5	2	65, 71

[a] For unknown reasons, a discordant value of 98% was obtained on one sample.

a 100-ml. volumetric flask and diluted to the mark with distilled water. Thirty minutes later, final dilutions were prepared as described for Solution System 4.

Ion-Exchange Chromatography—The reagent blank (5 ml.) was pipeted onto an ion-exchange column, allowed to pass through, eluted with 15 ml. of 2 *N* HCl into a 50- or 100-ml. volumetric flask, and diluted to the mark with distilled water.

For the oxymix blank and for the other four solution systems, the above procedure was employed, except that the 5-ml. aliquot was allowed to seat properly on the column, washed with 3 × 10-ml. portions of distilled water, eluted with 15 ml. acid, and diluted to proper volumes. The volume of acid used in eluting the columns was sufficient to regenerate automatically the resin beds (to the H^+ form). Washing with water then removed excess acid so that the ion exchangers were ready for subsequent experiments. No loss of drug occurred on water washing the columns after the samples had been applied to the resin beds (prior to elution with acid).

Fluorometric Assay Procedures (3–5)—After epinephrine and isoproterenol had been collected from the ion-exchange columns, 2-ml. aliquots were transferred to 16 × 150-mm. test tubes, followed by addition of 1 ml. of distilled water to each sample. One drop of bromthymol blue indicator was added, and the solution was adjusted with vigorous stirring or shaking to approximately pH 6 by the dropwise addition of 2 *N* potassium carbonate. This was followed by addition of 0.5 ml. of 0.2 *M* phosphate buffer. Oxidation was then performed by addition, with thorough mixing, of 0.1 ml. of 0.25% zinc sulfate and 0.1 ml. of 0.25% potassium ferricyanide. After exactly 3 min., 1 ml. of the freshly prepared sodium hydroxide–ascorbic acid reagent was added and mixed thoroughly.

The fluorometer was blanked on the dummy cell. The solutions were transferred to 12 × 75-mm. cells and were read, at a range selector setting of ×10, 10–20 min. after the last reagent was added.

For the determination of phenylephrine, the acid eluate was first adjusted to pH 1 by addition of 5 *N* NaOH solution and was then diluted to a final volume of 25 ml. with 0.1 *N* HCl. The natural fluorescence of the drug was then measured[4] by merely introducing a few milliliters of the solution into the sample cell and reading its "percent transmission" on the meter, using 0.1 *N* HCl as a blank. (See also *Reference 6.*)

The reagent blanks (Solution Systems 1 and 2) gave fluorescence readings in the following ranges: in the epinephrine experiments, 0.8–0.9 fluorescence unit; and in the isoproterenol experiments, 1.1–1.2. In the phenylephrine experiments, blank readings of 1.3–1.8 were obtained.

RESULTS AND DISCUSSION

An arbitrary time of 30 min. was chosen at which to measure residual drug activity, since it was known that the clinical treatment is usually completed well inside this time span and that potential redox destructibility of the oxymix system decays to a minimum value during 15–30 min.[5]. Two methods of addition (drug to oxymix and oxymix to drug) were investigated, since in clinical situations both procedures may be used.

The standard solutions (System 3) gave the data presented in Figs. 1 and 2. The lines were constructed from the experimental data by the method of least squares (7).

The results of the investigation of the stability of the bronchodilators in the presence of oxymix are compiled in Table I. It was evident that a reduction in epinephrine concentration occurred, even in a 30-min. old oxymix solution, and to a somewhat greater extent in the freshly prepared solutions[6]. However, of more importance from a clinical point of view, at least 50% of the initially added amount of epinephrine remained unchanged in all solutions. No significant degradation could be observed in the experiments with isoproterenol and phenylephrine.

Aerosols containing 6–12% of oxymix have been used clinically (1). In the present study, all experiments were performed at or initially even somewhat above the high limit of this range. This is probably adequate because of the extremely high molar ratio of redox components to bronchodilator in all clinical compositions (>100:1). From the fact that a loss of epinephrine was also observed when it was combined with oxymix that had been kept in water solution for 30 min., it is clear that a reaction occurs with some component(s) of the aged system and that isoproterenol and phenylephrine do not undergo this reaction.

The stability of phenylephrine in a pharmaceutical composition containing, *inter alia*, ascorbic acid was reported (10). Even when such a mixture had been kept at 70° for 36 hr., almost no change in the phenylephrine content was observed. The authors also found that, under certain conditions, phenylephrine can be changed in its side chain in such a manner that this structural change could not be observed spectroscopically. However, by the use of an ion-exchange method, these types of formed derivatives could be separated completely, thus allowing quantitative measurement of the intact phenylephrine.

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▲ To whom inquiries should be directed.

[4] Aminco-Bowman fluorometer.

[5] P. Levins and N. Adler, Arthur D. Little, Inc., Cambridge, Mass., personal communication.

[6] In water solutions of epinephrine alone, the degradation is very slow in the acid and neutral pH range (8, 9); during a 30-min. period, no significant change in epinephrine concentration can be observed.

Isolation and *In Vitro* Antifungal Activity of 6,6′-Dihydroxythiobinupharidine

W. P. CULLEN*, R. T. LaLONDE*▲, C. J. WANG†, and C. F. WONG*

Abstract □ The isolation procedure for obtaining a new nuphar alkaloid, 6,6′-dihydroxythiobinupharidine (formerly named 6,6′-dihydroxythionuphlutine-A), and its *in vitro* activity against eight human pathogenic fungi are described.

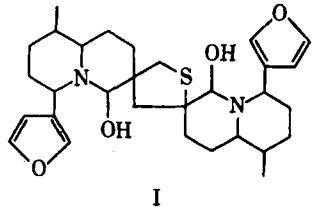
Keyphrases □ 6,6′-Dihydroxythiobinupharidine—isolation from *Nuphar luteum* subsp. *macrophyllum*, tested for antifungal activity □ *Nuphar luteum* subsp. *macrophyllum* (Beal)—isolation of 6,6′-dihydroxythiobinupharidine, tested for antifungal activity □ Antifungal agents, potential—isolation and testing of 6,6′-dihydroxythiobinupharidine from *Nuphar luteum* subsp. *macrophyllum*

The usefulness of various preparations of nuphar rhizomes in treating enteritis, gingivitis, and skin diseases was reported in 1945 (1). Subsequently, other accounts have dealt with the isolation of alkaloid mixtures, specific compounds of unknown structure, and the activity of these materials against specific organisms (2–5). This report describes the isolation of 6,6′-dihydroxythiobinupharidine[1] (I) and the results of preliminary studies dealing with the activity of this substance against eight human pathogenic fungi. The study leading to the structure of 6,6′-dihydroxythiobinupharidine and to that of a companion alkaloid, 6,6′-dihydroxythionuphlutine-B, was reported previously (6).

EXPERIMENTAL[3]

Plant Material[2]—*Nuphar luteum* subsp. *macrophyllum* (Beal) (9) rhizomes were harvested from the flats along the lower Hudson River near Columbianville, N. Y., during August 1968. The rhizomes were sliced into 0.63-cm. (0.25-in.) sections and dried under electric fans at room temperature with frequent turning. The dried rhizome sections were ground to a fine powder in a Wiley mill immediately before extraction.

Isolation of 6,6′-Dihydroxythiobinupharidine—A 500-g. quantity of powdered plant material was soaked with 1 l. of 10% aqueous ammonia for 24 hr. The resulting mixture was extracted with



I

ethylene chloride (3 × 5 l.). Each separate extract was washed sequentially with 1000-, 750-, and 625-ml. portions of 10% sulfuric acid. The combined acid wash was made alkaline with aqueous ammonia in ice. The resulting turbid mixture was extracted with ~3 l. of chloroform. The deep amber-colored extract was dried over sodium sulfate. Removal of the solvent at the rotary evaporator (<40°) yielded <6 g. of dark-brown viscous oil, which was dissolved in 70 ml. of ethanol and treated with 15% ethanolic methylene-bis(salicylic) acid. The brown precipitate which resulted was filtered off and shaken with 60 ml. of 10% aqueous ammonia, and the mixture that formed was extracted with chloroform. The chloroform extract was dried over sodium sulfate. Removal of the solvent on the rotary evaporator yielded 4.2 g. of oil, 4 g. of which was eluted from a column of neutral alumina (activity grade III, 170 g.) with 13 50-ml. portions (fractions A1–A13) of *n*-hexane–methylene chloride (99:1). The column was eluted further with 50-ml. portions (fractions A14–A62) of *n*-hexane–methylene chloride containing gradually increasing amounts of the latter. After the fraction of methylene chloride had reached 50%, the column was eluted with 50-ml. portions of pure methylene chloride (fractions A63–A86) and finally with 50-ml. portions of methylene chloride–10% methanol. Fractions A72–A86 amounted to 402 mg. of a glass-like solid, 6,6′-dihydroxythiobinupharidine; $[\alpha]_D^{25}$ +44.5° (methylene chloride, c 1.2); TLC (0.25 mm. alumina GF_{254} on a 20-cm. plate); R_f (methylene chloride–0.6% methanol) 0.3; R_f (methylene chloride–1% methanol) 0.67.

Anal.—Calc. for $C_{30}H_{42}N_2O_4S$: C, 68.41; H, 8.04; N, 5.33; S, 6.09. Found: C, 68.64; H, 8.10; N, 5.26; S, 5.95.

6,6′-Dihydroxythiobinupharidine was dissolved in methylene chloride and was treated with 2 equivalents of dilute perchloric acid in the sonic oscillator. The solvents were removed at reduced pressure and the crystalline residue was recrystallized from methanol to give the bisiminium diperchlorate, m.p. (powder) 226–228° and (large crystals) 232–234°; $[\alpha]_D^{25}$ +184° [c, 0.5 methanol–acetone (16.5:3.5)].

Anal.—Calc. for $C_{30}H_{42}Cl_2N_2O_{10}S$: C, 52.09; H, 5.84; Cl, 10.25; N, 4.05; S, 4.63. Found: C, 52.19; H, 5.60; Cl, 10.28; N, 4.01; S, 4.84.

Other physical properties of 6,6′-dihydroxythiobinupharidine and its bisiminium diperchlorate were reported previously (6).

Combined fractions A47–A62 (356 mg.) were rechromatographed on 25 g. of neutral alumina (activity grade III), which was eluted sequentially with four 50-ml. portions of benzene (fractions B1–B4), three 50-ml. portions of methylene chloride (fractions B5–B7), and finally with 25 ml. of methanol. A 156-mg. portion of fraction B5 (175 mg.) was rechromatographed on 10 g. of neutral alumina (activity grade IV), which was eluted with 50 ml. of *n*-hexane (fraction C1) and three 25-ml. portions of benzene (fractions C2–C4). Fraction C3 consisted of 85 mg. of a pure, glass-like solid, 6,6′-dihydroxythionuphlutine-B; $[\alpha]_D^{25}$ −69° (c, 10 mg./ml., methylene chloride); TLC (0.25 mm. alumina GF_{254} on a 20-cm. plate); R_f (methylene chloride–1% methanol) 0.75.

Anal.—Calc. for $C_{30}H_{42}N_2O_4S$: C, 68.41; H, 8.04; N, 5.33; S, 6.09. Found: C, 68.38; H, 8.21; N, 5.34; S, 5.79.

Other physical properties of 6,6′-dihydroxythionuphlutine-B were reported previously (6).

Determination of Antifungal Properties—Twenty-five milliliters of a 0.01 *M* aqueous acetic acid solution containing the alkaloid at a concentration of 490 mcg./ml. was sterilized by filtering through a Seitz filter. The sterile alkaloid was added to four portions of sterile, melted (50°) Sabouraud dextrose agar to make the final concentrations of the alkaloid in the Sabouraud dextrose agar 100, 10, 1, and 0.1 mcg./ml. They were distributed into sterile "perfume" bottles and made into slants.

The eight human pathogenic fungi tested in this study were: *Histoplasma capsulatum* Darling (No. 1098), *Blastomyces dermati-*

[1] The alkaloid was originally named 6,6′-dihydroxythionuphlutine-A (6). While elucidating the structure of this alkaloid, it was reduced to thionuphlutine-A (6). Recently, the latter alkaloid was observed to be identical with thiobinupharidine (7), an alkaloid whose structure had remained unknown until the discovery of its identification with thionuphlutine-A. Since the name thiobinupharidine has precedence (8), the name thionuphlutine-A should be deleted from the literature.

[2] Verification of the plant material was made by Mr. S. Smith of the New York State Botanists Office, Albany, N. Y. A voucher specimen has been deposited at the New York State Botanists Office, Albany, N. Y.

[3] Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn., and the analytical laboratories of Stanford Research Institute, Menlo Park, Calif.

Table I—Sensitivities of Histoplasma and Blastomyces to 6,6′-Dihydroxythiobinupharidine as Expressed by Weekly Increments of the Diameter of Colony (in Millimeters) on Sabouraud Dextrose Agar at 25° (Mycelial Phase)

Concentration of Alkaloid, mcg./ml.	Week	*Histoplasma capsulatum* — Isolate No. 108						*Histoplasma capsulatum* — Isolate No. 1106						*Blastomyces dermatitidis* — Isolate No. 1099						*Blastomyces dermatitidis* — Isolate No. 1107					
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
100		0	0	0	T[a]	4[b]	7	0	0	0	0	T	T	0	0	0	T	T	T	0	0	0	2	5	5
80		0	0	2	4	5	8	0	0	0	0	0	0	0	0	T	T	T	T	0	0	2	3	6	7
60		0	3	4	7	13	17	0	0	0	0	0	T	0	0	T	2	2	2	0	0	2	3	7	7
40		0	3	8	12	14	16	0	0	0	T	2	5	0	2	3	3	4	4	0	0	8	11	13	21
20		0	5	11	16	19	24	0	6	12	17	20	23	0	6	9	11	15	18	0	0	7	12	15	25
10		2	9	14	20	23	26	2	4	7	7	13	18	2	7	9	14	19	20	4	10	16	25	28	30
Acetic acid control		4	14	25	29	31	31	0	6	14	19	28	31	8	19	26	28	29	29	6	26	31	32[c]	32	32
Control		7	12	22	26	27	27	2	10	15	27	31	31	8	18	26	28	30	30	8	25	31	32	32	32

[a] Trace of growth. [b] Average diameter of two cultures. [c] Maximal growth; surface area of this bottle is 32 × 32 mm.

tidis Gilchrist and Stokes (No. 1099), *Trichophyton mentagrophytes* (Robin) Blanchard (No. 1100), *T. tonsurans* Malmsten (No. 1101), *Microsporum gypseum* (Bodin) Guiart and Grogorakis (No. 1102), *M. canis* Bodin (No. 1103), *Cryptococcus neoformans* (Sanfelice) Vuillemin (No. 1104), and *Candida albicans* (Robin) Berkhour (No. 1105).

Each bottle was center-inoculated with a 2 × 2-mm. portion of a 2-week-old mycelial culture or a loopful of yeast-like candida or cryptococcus. All cultures were made in duplicate and incubated at 25°. Linear expansion of each colony was measured weekly up to 5 weeks and at the end of 8 weeks.

Subsequent tests were performed with two isolates each of *H. capsulatum* (Nos. 1098 and 1106) and *B. dermatitidis* (Nos. 1099 and 1107) and Sabouraud dextrose agar slants containing 100, 80, 60, 40, 20, or 10 mcg. of the alkaloid per milliliter of Sabouraud dextrose agar.

The toxicity of 6,6′-dihydroxythiobinupharidine in mice was ascertained from results (NSC 141542) obtained by the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.

RESULTS AND DISCUSSION

The effect of the alkaloid 6,6′-dihydroxythiobinupharidine on the growth of histoplasma and blastomyces was measurable. At 100 mcg./ml., the alkaloid inhibited the growth of *H. capsulatum* up to 3 weeks, whereas it completely suppressed the growth of *B. dermatitidis*. At the same concentration the alkaloid suppressed the growth rate of *M. gypseum* and *M. canis* up to 3 weeks; the growth of *T. mentagrophyte* and *T. tonsurans* was suppressed up to 5 weeks. However, the alkaloid at a concentration of 100 mcg./ml. exerted no inhibitory effect against yeast-like candida or cryptococcus.

Table I reveals that isolate No. 1106 of *H. capulatum* was more sensitive to the alkaloid than isolate No. 1098. Likewise, *B. dermatitidis* isolate No. 1099 was more sensitive to the alkaloid than was No. 1107. Clearly, the alkaloid at a concentration of 40 mcg./ml. may inhibit the mycelial growth of some strains of *H. capsulatum* and *B. dermatitidis*.

These results show that 6,6′-dihydroxythiobinupharidine possesses antifungal properties *in vitro*. The effect of this alkaloid in treating animal mycoses will be investigated in the near future. In preliminary tests on mice, no deaths occurred upon intraperitoneal administration of 100 and 200 mg. of alkaloid per kilogram of mouse once daily for 30 days. At a level of 400 mg. of alkaloid per kilogram of mouse, four of six mice died within 30 days.

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Fluorometric Measurement of 5-Fluorocytosine in Biological Fluids

DENIS WADE[A] and GILLIAN SUDLOW

Abstract □ A spectrofluorometric method is described for the assay of 5-fluorocytosine in biological fluids. The method has the advantage of high specificity and has proved to be equally suitable for assaying the drug in human plasma and urine.

Keyphrases □ 5-Fluorocytosine—spectrophotofluorometric determination in biological fluids □ Spectrophotofluorometry—determination of 5-fluorocytosine in biological fluids

The concentration of 5-fluorocytosine in biological fluid can be measured by a microbiological technique involving the inhibition of the growth of *Candida albicans*[1]. Although this technique has high sensitivity (1–1.5 mcg./ml.), there is a problem of interference by unknown constituents in the urine and the specificity of the method may not allow measurement when there is simultaneous administration of other antifungal drugs.

The fluorometric method described here has the advantages of specificity and is equally applicable to plasma and urine. The method is less sensitive than the *C. albicans* assay but encompasses the therapeutic range and the very high plasma levels seen in some patients with renal insufficiency.

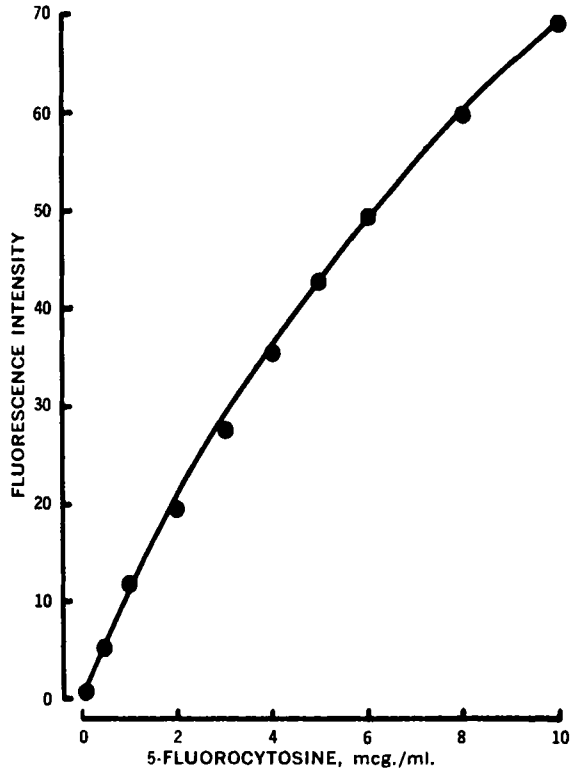

Figure 1—*Relationship between the concentration of 5-fluorocytosine in alkaline aqueous solutions and fluorescence intensity. Solutions were excited at a wavelength of 300 nm., and fluorescence was measured at 365 nm.*

[1] Method report: "Agar diffusion assay of 5-fluorocytosine in human serum," F. Hoffmann–La Roche & Co., Basel, Switzerland.

EXPERIMENTAL

Principle—5-Fluorocytosine is isolated from protein-free biological fluids by TLC. After elution from the chromatoplate, the fluorescence of the eluate is measured in alkaline solution.

Reagents—The following were used: (*a*) 5-fluorocytosine[2]; (*b*) *n*-butanol, analytical reagent grade; (*c*) glacial acetic acid, analytical reagent grade; and (*d*) 0.75 *M* NaOH. All solutions were made up in glass-distilled water.

Procedure—*Serum Estimations*—Two-milliliter serum samples are ultrafiltered through a pressure filtration funnel[3] using an ultrafiltration membrane[4] at 90 p.s.i. of oxygen. Twenty microliters of the first drop of the ultrafiltrates is spotted on thin-layer chromatoplates[5] alongside 5 mcg. of authentic 5-fluorocytosine as a standard. Chromatograms are then developed in one direction using the solvent system of butanol–acetic acid–water (6:1:2). Developed chromatoplates are dried and viewed under UV light at 254 nm. Squares, 2.54 cm. (1 in.), corresponding to the 5-fluorocytosine spots and serum blanks at an R_f of 0.59 are marked and cut out. The silica is scraped from these squares into centrifuge tubes, and the 5-fluorocytosine is eluted by thorough mixing with 2 ml. of distilled water. After centrifugation, the supernate is made alkaline with 2 drops of 0.75 *M* NaOH and the fluorescence is measured, exciting at a wavelength of 300 nm. and measuring emission at 365 nm.

A standard curve of the relationship between 5-fluorocytosine concentration and fluorescence intensity is established using freshly prepared standard solutions of 0.2, 0.5, and 1 mcg./ml. Since the ultrafiltrate is diluted by a factor of 100, these standards correspond to 20, 50, and 100 mcg./ml. of 5-fluorocytosine in serum, respectively.

Urine Estimations—5-Fluorocytosine in urine is measured using the same method. Two-milliliter urine samples are filtered through Whatman No. 1 filter paper, and 2-μl. aliquots of the filtrate are spotted on the chromatoplates. Standard solutions of 0.1, 0.5, 1, and 1.25 mcg./ml. are used to establish the standard curve from which the 5-fluorocytosine concentration in the eluting solution is read. These standards correspond to urine 5-fluorocytosine levels of 100, 500, 1000, and 2000 mcg./ml., respectively, since a dilution factor of 1000 is involved.

Calculation—The concentration of 5-fluorocytosine in the eluting solutions is established from their fluorescence measurements by reference to the standard curves. Since these standard curves are obtained from solutions in water and the recovery of 5-fluorocytosine from serum and urine averages approximately 80%, each value obtained for 5-fluorocytosine concentration is corrected accordingly.

RESULTS

Fluorescence of 5-Fluorocytosine—5-Fluorocytosine fluoresces strongly in alkaline solution. The emission maximum is at 365 nm. when excited at 300 nm. The fluorescence of alkaline solutions of 5-fluorocytosine increases with concentration up to 25 mcg./ml. and decreases at higher concentrations due to quenching. The relationship is sufficiently linear up to 10 mcg./ml. (Fig. 1) for the assay of solutions containing from 0.05 to 10 mcg./ml. of 5-fluorocytosine.

5-Fluorocytosine in Plasma—There is good linear correlation between fluorescence and concentration up to 250 mcg./ml. of 5-fluoro-

[2] Ro 29915 E/265'601, Roche Products Pty Ltd., Sydney, Australia.
[3] Gelman.
[4] Diaflo type UM-20E.
[5] Kieselgel F_{254}, Merck.

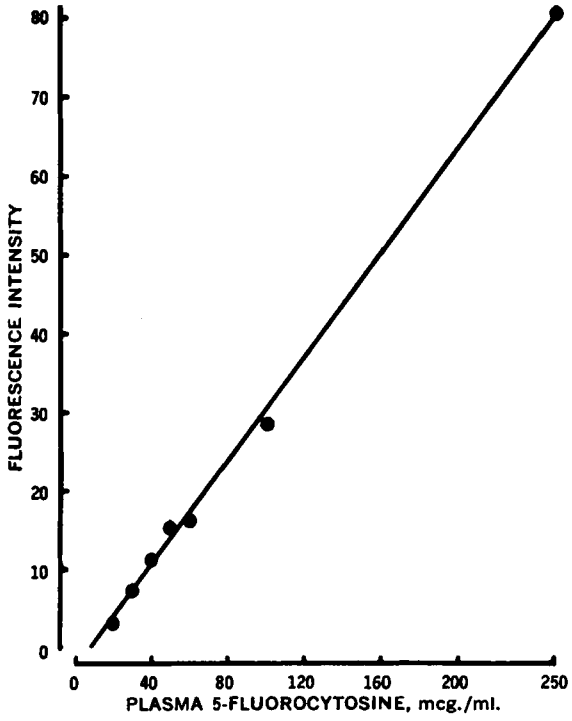

Figure 2—*Relationship between fluorescence intensity of the chromatoplate eluate and the concentration of authentic 5-fluorocytosine added to normal human plasma.*

cytosine in plasma (Fig. 2). Concentrations up to 1000 mcg./ml. can be read if standards are applied to the chromatogram to correct for altered recovery in this range. The upper and lower limits of the linear region lie outside the therapeutic blood levels and those high levels that may develop in patients with renal insufficiency (1).

5-Fluorocytosine in Urine—There is good linear correlation between fluorescence and concentration up to 5000 mcg./ml. of 5-fluorocytosine in urine. Since 5-fluorocytosine in the urine can be up to 100 times more concentrated than in the plasma, the fluorescence of eluates from undiluted urine samples may fall outside the linear region of the standard curve.

Specificity—*Normal Human Plasma and Urine*—The method appears to be highly specific for 5-fluorocytosine in plasma. Control estimations on the plasma from eight drug-free normal individuals gave fluorescence readings corresponding to only 1.6–3.8 mcg./ml. of 5-fluorocytosine in plasma, with a mean and standard deviation of 2.6 ± 0.82 mcg./ml., well below the minimum therapeutically useful plasma level of the drug.

A similar high degree of specificity is seen with estimations in human urine. Control estimations on the urine from eight drug-free normal individuals gave fluorescence readings corresponding to 13–75 mcg./ml. of 5-fluorocytosine in urine, with a mean and standard deviation of 35.7 ± 24.5 mcg./ml. This again represents levels well below minimum values seen with therapeutic use of the drug.

Possible Metabolites of 5-Fluorocytosine—It was shown previously (1) that little, if any, 5-fluorocytosine is metabolized in normal subjects and that 90% of a single dose is excreted as unchanged drug in the urine within 48 hr. If any 5-fluorocytosine (I) was converted to 5-fluorouracil (II), this would not interfere with the assay because the two compounds are widely separated by the chromatography method described (R_f I 0.59; R_f II 0.77).

Other Drugs—In patients receiving no other drugs, 5-fluorocytosine may be assayed directly in the plasma ultrafiltrate without the chromatography step. Ultrafiltered plasma blanks from drug-free individuals showed no significant interfering fluorescence; a standard curve showing a linear relationship between 5-fluorocytosine added to plasma and fluorescence in the ultrafiltrate was established over the range 10–200 mcg./ml. of plasma. However, aspirin and its metabolite, salicylic acid, fluoresce strongly at the wavelengths used in the assay and show significant interference with this direct assay at therapeutic blood levels. Chromatography using the solvent system described separated 5-fluorocytosine (R_f 0.59) from salicylate (R_f 0.88) which thus does not interfere with the assay. Other drugs likely to be given simultaneously with 5-fluorocytosine, including amphotericin B, nystatin, and azathioprine, do not interfere with the assay.

Sensitivity—The effective and safe range of blood levels probably depends in part on the particular organism and the site and extent of the infection. This range is not yet known with certainty, but this assay is clearly sufficiently sensitive to measure below the minimum therapeutically effective blood level. The relative sensitivity limit (RSL) was calculated from the blank values of plasma and urine samples from eight drug-free normal individuals using the formula:

$$\text{RSL} = t_{0.05} \cdot s \sqrt{1 + \frac{1}{N}} \qquad \text{(Eq. 1)}$$

where s = standard deviation of the readings, $t_{0.05}$ = one-sided t-deviate for the probability of error $P = 5\%$ and $N - 1$ degrees of freedom, and N = number of observations.

In human plasma and in human urine, the relative sensitivity limit had a value of 1.65 and 49.1 mcg./ml. of 5-fluorocytosine, respectively.

Reproducibility—The method gives good reproducibility both in plasma and urine. The reproducibility was tested by eight analyses of the same sample of human plasma containing 80 mcg./ml. of 5-fluorocytosine. A value of 68.01 ± 2.28 mcg./ml. (mean and standard deviation) was obtained.

In the case of human urine, the reproducibility was determined from 10 analyses of the same urine sample containing 2000 mcg./ml. of 5-fluorocytosine. A value of 1902 ± 106 mcg./ml. (mean and standard deviation) was obtained.

Recovery—The recovery from human plasma was determined from seven analyses of samples containing known 5-fluorocytosine concentrations in the 20–100-mcg./ml. range. It varied between 78 and 89.5%, with a mean and standard deviation of 81.72 ± 4.07%. The recovery from human urine was determined from 10 analyses of samples containing known 5-fluorocytosine concentrations in the 500–5000-mcg./ml. range. It varied between 70 and 92%, with a mean and standard deviation of 80.2 ± 7.7%. Recovery from plasma did not vary systematically with concentration, but recovery from urine did decrease with increasing concentration.

SUMMARY

A specific spectrofluorometric method is described for the assay of 5-fluorocytosine in plasma and urine. The method has sufficient sensitivity to cover the therapeutic range and higher values occasionally seen with cumulative overdose. The drugs likely to be used in association with 5-fluorocytosine do not interfere with the method, which would recommend it for the monitoring of blood levels in patients on long-term therapy.

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▲ To whom inquiries should be directed.

Open-Chain Analogs of Muscarine Derivatives

JOSEPH G. CANNON*▲, PATRICK J. MULLIGAN*, JOHN PAUL LONG†, and SHARON HEINTZ†

Abstract □ A short series of choline ethers containing a free or esterified OH group was prepared as open-chain analogs of desmethylmuscarine stereoisomers. The acetate ester of one alcohol showed some degree of muscarinic activity. Contrary to the implication of an old literature report, 2-(dimethylaminoethoxy)-ethanol methiodide exhibited a low order of muscarinic activity.

Keyphrases □ Choline ethers—prepared as open-chain analogs of desmethylmuscarine stereoisomers, evaluated for muscarinic activity □ Muscarine derivatives—synthesis and evaluation of open-chain desmethylmuscarine stereoisomer analogs □ Desmethylmuscarine stereoisomer analogs—synthesis of choline ether series, muscarinic activity evaluated

In the course of other work, dimethylaminoethoxyethanol (I) was required. The quaternary salt (II) of this compound closely resembles an open-chain analog of desmethylmuscarine or desmethylepimuscarine (III), which was reported (1) to possess some weak muscarinic properties.

Callsen (2) described the therapeutic effect of II as being similar to that of arecoline, and he suggested possible use as a laxative by subcutaneous injection. The age and questionable validity of this report suggested the evaluation of II for muscarinic effect. The acetate (IV) and carbamate (V) esters were prepared, on the basis that the acetate ester of muscarine is markedly muscarinic (1).

Compound I has been prepared from dimethylamine in 75% yield by treatment with 2-chloroethoxyethanol (3) and in poor yield by treatment with ethylene oxide (4). In the present work, the most convenient method utilized a reaction between dimethylaminoethanol and ethylene oxide in the presence of base. A reaction of dimethylamino-2-propanol with propylene oxide gave a poor yield of VI. The quaternary derivative (VII) was prepared as an open-chain analog of a muscarine-like system.

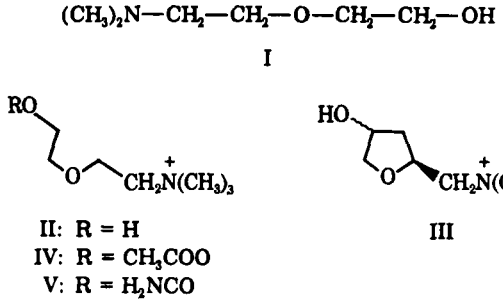
IR and NMR data on all of the compounds prepared were consistent with the structures proposed.

PHARMACOLOGY

The compounds were evaluated for muscarinic effect in a superfused guinea pig ileum preparation (5). Muscarinic activity relative to acetylcholine (Table I) was estimated using the method of Litchfield and Wilcoxon (6). At least three different animals were used for assaying each compound. Muscarinic effects were blocked by atropine (0.2 mcg./ml.) but not by hexamethonium (20 mcg./ml.). The entire series revealed no properties of pharmacological interest; the claim of the older report (2) that II mimics acetylcholine or arecoline with respect to action on smooth muscle tissue was verified, but activity relative to acetylcholine was very low.

Table I—Relative Muscarinic Activity

Compound	Relative Potency (95% *CI*)
Acetylcholine	1.0
II	0.003 (0.0007–0.02)
IV	0.004 (0.0006–0.013)
V	0.0003 (0.00007–0.018)
VII	<0.00001



EXPERIMENTAL[1]

2-(Dimethylaminoethoxy)ethanol (I)—Ethylene oxide (132 g., 3 moles) and sodium methoxide (5 g.) in 1.5 l. of methanol were added slowly to 222.5 g. (2.5 moles) of dimethylaminoethanol in 2 l. of methanol, and the resulting mixture was refluxed for 10 hr. The methanol was removed under reduced pressure, and the liquid residue was distilled at 95° (8 mm.) to afford 120 g. (36%) of product [lit. (4) b.p. 78–79° (3.5 mm. Hg)].

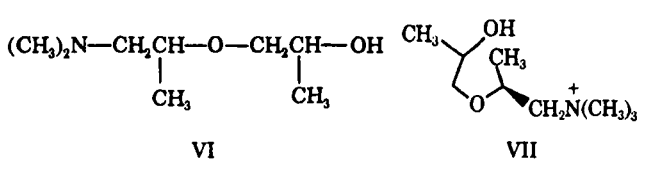
2-(Dimethylaminoethoxy)ethanol Methiodide (II)—Compound I (13.3 g., 0.1 mole) was stirred with a 2 *M* excess of methyl iodide in 100 ml. of ether at room temperature for 24 hr. The solid which separated was recrystallized from *n*-butanol and then from acetone to yield 24 g. (87%) of crystals, m.p. 116–117° [lit. (2) m.p. 116–117°].

2-(Dimethylaminoethoxy)ethyl Acetate Methiodide (IV)—Compound II (2.5 g., 0.0088 mole) was stirred in 25 ml. of acetic anhydride for 10 days at room temperature. The resulting solution was poured into an excess of ether, and the solid which separated was collected and recrystallized from acetone to yield 2.0 g. (72%), m.p. 123–125°.

Anal.—Calc. for $C_9H_{20}INO_2$: C, 34.07; H, 6.30; I, 40.00; N, 4.40. Found: C, 34.16; H, 6.30; I, 39.80; N, 4.50.

2-(Dimethylaminoethoxy)ethyl Carbamate Methiodide (V)—Trifluoroacetic acid (4.56 g., 0.04 mole) was added dropwise with stirring to 5.5 g. (0.02 mole) of II and 3.08 g. (0.04 mole) of sodium cyanate in 100 ml. of methylene chloride. The resulting mixture was stirred at room temperature until solution occurred and then was stirred overnight. The crystals which separated were recrystallized from *n*-butanol–ether to yield 2.5 g. (39%) of needles, m.p. 158–160°.

Anal.—Calc. for $C_8H_{19}IN_2O_3$: C, 30.17; H, 5.90; I, 39.90; N, 8.80. Found: C, 29.96; H, 5.99; I, 40.10; N, 8.70.



[1] Boiling points are uncorrected. Melting points were determined in open glass capillaries using a Thomas-Hoover Uni-Melt apparatus and are corrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

1-Dimethylamino-2-(2-hydroxy-1-propyloxy)propane (VI)—1-Dimethylamino-2-propanol (103 g., 1 mole) and 65 g. (1.1 mole) of propylene oxide were refluxed in 800 ml. of methanol for 18 hr. Methanol was evaporated under reduced pressure and the residue was fractionated. The fraction with a boiling point of 65° (0.2 mm. Hg) was the desired product; the yield was 24 g. (15%).

Anal.—Calc. for $C_8H_{19}NO_2$: C, 59.63; H, 11.79. Found: C, 59.86; H, 11.89.

1-Dimethylamino-2-(2-hydroxy-1-propyloxy)propane Methiodide (VII)—Compound VI (1 g., 0.006 mole) and 3 g. (0.021 mole) of methyl iodide in 25 ml. of ether containing a few drops of methanol were kept at 0° for 2 days. The resulting crystals were recrystallized from acetone–ether and then from acetone to afford 0.93 g. (55%) of material, m.p. 147–148°.

Anal.—Calc. for $C_9H_{22}INO_2$: C, 35.64; H, 7.26; I, 41.91; N, 4.60. Found: C, 35.79; H, 7.21; I, 41.72; N, 4.52.

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Colorimetric Method for Determination of Guanazole in Blood and Urine

MAHMOUD N. MUSA▲, GERALD M. LOWER, Jr., and GEORGE T. BRYAN*

Abstract □ A colorimetric method, based on diazotization and coupling, for the determination of guanazole in aqueous solution, blood, and urine is described. The average relative standard deviation of the method is about 0.9%. Determination in blood involves precipitation of proteins with trichloroacetic acid and diazotization and coupling of guanazole in the filtrate. Determination in urine involves separation of guanazole from the diazotizable, naturally occurring urinary aromatic amines using ion-exchange chromatography.

Keyphrases □ Guanazole (3,5-diamino-1,2,4-triazole)—colorimetric analysis in aqueous solution, blood, and urine □ 3,5-Diamino-1,2,4-triazole (guanazole)—colorimetric analysis in aqueous solution, blood, and urine □ Colorimetry—analysis, guanazole in aqueous solution, blood, and urine

Guanazole (3,5-diamino-1,2,4-triazole)[1] is an experimental drug beneficial in the treatment of adult acute leukemia (1–6). No methods for its determination are published in the literature. This paper describes a method for the determination of guanazole in aqueous solution, blood, and urine. The method is based on diazotization and coupling (7, 8).

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with a recording spectrophotometer[2]. Matched cells with a 1-cm. optical path were used.

Materials—Dowex-50W (H^+) (12% cross-linkage, 200–400 mesh) was prepared as previously described (9). Dowex-1 (Cl^-) (10% cross-linkage, 200–400 mesh) was sedimented to remove the very fine and very coarse particles. Dowex-1 (Cl^-) was converted to Dowex-1 formate by passage of sodium formate and formic acid over the resin followed by appropriate water washes.

Guanazole was obtained in bulk[3].

The following reagent grade chemicals were used: concentrated hydrochloric acid, sodium nitrite, ammonium sulfamate, *N*-(1-naphthyl)ethylenediamine dihydrochloride, dimethyl sulfoxide, and trichloroacetic acid.

Procedure for Aqueous Solution—Two milliliters of 5.0 *N* HCl and 2 ml. of the guanazole aqueous solution were added to 2 ml. of dimethyl sulfoxide. Then 0.2 ml. of 0.5% $NaNO_2$ was added to this mixture. Immediately 0.2 ml. of 5.0% ammonium sulfamate was added and mixed thoroughly, followed by 0.2 ml. of 0.5% *N*-(1-naphthyl)ethylenediamine dihydrochloride[4]. After 10 min. the absorbance of the sample was compared with a blank at 504 nm. in the spectrophotometer. Quantitative comparisons were made with a guanazole standard curve prepared in a similar fashion.

Procedure for Blood—Eight milliliters of a 15% solution of trichloroacetic acid was added to 2 ml. of blood. This was then filtered through a prewashed filter paper, which was then washed with a few milliliters of distilled water. The filtrate was made up to 25 ml. in a volumetric flask. Two milliliters of the filtrate was added to 2 ml. of 5.0 *N* HCl, diazotized and coupled as described for the aqueous solution, and compared with a blank made up with water and the other reagents.

Procedure for Urine—Fifty milliliters of the urine sample was passed through a Dowex-1 formate column (1.0 cm. o.d. × 10 cm.) under a pressure of 1.0–2 p.s.i. The column was washed with 50 ml. of deionized water, the effluent and wash were combined, and the pH was adjusted to 1–2. The acidified sample was passed through a Dowex-50W (H^+) column (1.0 × 10 cm.) by gravity. The eluate was discarded, and the column was then washed with 50 ml. of 0.1 *N* HCl. Guanazole was eluted from the column with 200 ml. of 2.5 *N* HCl. This chromatographic procedure separates guanazole from the diazotizable, naturally occurring urinary aromatic amines. An

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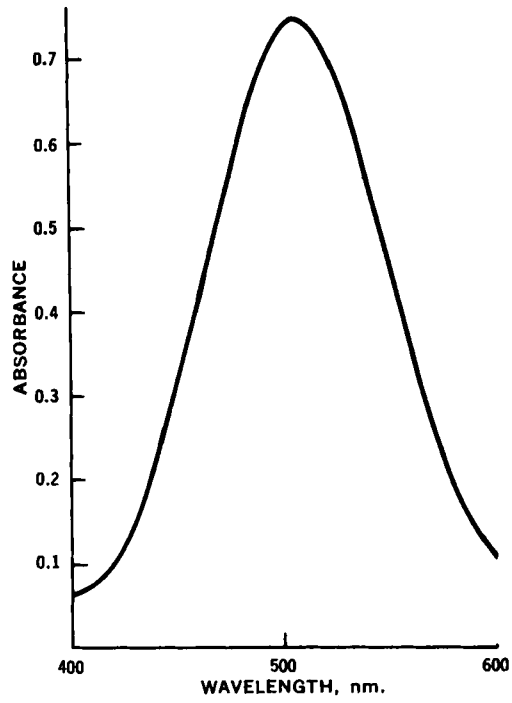

Figure 1—*Visible spectrum of the dye formed. Guanazole was added to water and then diazotized and coupled.*

aliquot of the 2.5 *N* HCl eluate was taken for diazotization and coupling as described for the aqueous solution and was compared with a blank urine sample treated as already described. If the eluate is too dilute to be assayed accurately, it can be concentrated by evaporating some of the water to a known volume.

RESULTS AND DISCUSSION

Figure 1 shows the visible spectrum of the dye formed; the maximum absorbance is at 504 nm. Absorbance is linear with concentration up to an absorbance value of 0.7. The molar absorptivity in the final solution is 17,500 l. M^{-1} cm.$^{-1}$. Table I shows the recovery of guanazole from samples of aqueous solution, human blood, and human urine. The recovery seems to be quantitative. The average relative standard deviation is 0.9%.

The absorbance value of the blank for human blood is very close to zero; the value for human urine in a 1-cm. cell is 0.12 ± 0.11 (standard error). Treatment of urine with ion-exchange chromatography, as described under the *Experimental* section, decreases the value of absorbance of the blank to 0.005 (0.05 in a 10-cm. cell).

Dimethyl sulfoxide is used to prevent the dye from precipitating. It is not needed if absorbance is read immediately after the addition of the coupling reagent or if the concentration of guanazole is low.

Table I—Recovery of Guanazole from Samples of Aqueous Solution, Blood, and Urine

	Guanazole Added, mcg./ml.	Recovery, %
Aqueous Solution		
	1.0	99.5
	3.0	99.2
	7.0	98.4
	9.9	100.5
	15.0	100.7
Average recovery ± *SD*		99.7 ± 1.0
Human Blood		
	12.0	98.4
	36.0	97.5
	84.0	99.2
	120.0	98.7
	180.0	97.0
Average recovery ± *SD*		98.2 ± 0.9
Human Urine		
	4.0	97.2
	10.0	99.2
	25.0	98.4
	40.0	97.5
	60.0	97.8
Average recovery ± *SD*		98.0 ± 0.8

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Biological and Phytochemical Evaluation of Plants XII: Isolation of Acetylakuammidine from *Aspidosperma quebracho-blanco* Leaves

R. L. LYON, H. H. S. FONG▲, and N. R. FARNSWORTH

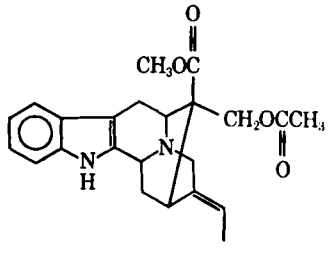
Abstract □ A continuing investigation of the leaves of *Aspidosperma quebracho-blanco* Schlecht. (Apocynaceae) has resulted in the isolation of natural acetylakuammidine, in addition to the previously reported alkaloids aspidospermine and quebrachidine. Methods used for the isolation and characterization of these bases are described.

Keyphrases □ *Aspidosperma quebracho-blanco* Schlecht. (Apocynaceae) leaves—biological and phytochemical evaluation, isolation of acetylakuammidine □ Acetylakuammidine—isolation from *Aspidosperma quebracho-blanco* leaves □ Phytochemical evaluation—*Aspidosperma quebracho-blanco* leaves, isolation of natural acetylakuammidine

In a previous article, the isolation of aspidospermine, quebrachidine, (−)-pyrifolidine, akuammidine, and rhazinilam from the leaves of *Aspidosperma quebracho-blanco* was reported (1). Rhazinilam, a new type of alkaloid from the Apocynaceae, was subsequently studied and its structure was solved by X-ray crystallography (2). An investigation of the chloroform-soluble Fraction C, currently reported, has yielded acetylakuammidine, as well as additional large quantities of aspidospermine and quebrachidine. Until now, acetylakuammidine (I) has only been prepared synthetically (3).

EXPERIMENTAL

Preparation and Chromatography of Alkaloid Fraction—Two hundred grams of the alkaloid Fraction C (1) was dissolved in ether and partitioned four times with two volumes of 10% acetic acid. The acid solution was separated, evaporated *in vacuo* to remove entrained ether, and placed into five cellulose dialysis tubes, each 100 × 2.65 cm. The tubes, each containing 600 ml. of the acidic alkaloid solution, were suspended in a 9 × 115-cm. glass column, with distilled water being perfused at a rate of 150 ml./hr. at 5°. A total of 9 l. of the dialysate was collected, made alkaline with ammonium hydroxide, and extracted with ether. The ether extract was dried over anhydrous magnesium sulfate, and 40.0 g. of aspidospermine was obtained by direct crystallization of the fraction. It corresponded in all respects with a reference sample of aspidospermine previously isolated from this plant. Removal of the ether from the alkaloid mother liquor yielded 75.0 g. of residue, which was chromatographed over a column of 3.2 kg. of silica gel PF_{254} as previously described (1), using a solvent mixture of benzene–ethyl acetate–95% ethanol (2:2:1).



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Isolation of Acetylakuammidine—Using methanol as the solvent, 0.327 g. of an alkaloid was obtained as colorless prisms from fractions 48–56 from the column. Concentration of the mother liquor yielded an additional 0.073 g. of the same alkaloid. Recrystallization of the pooled crystals from methanol afforded an analytical sample which sublimed at 245°, and the sublimate subsequently melted at 278–279° dec. The isolate exhibited $[\alpha]_D^{25}$ + 13° (c 1.0, chloroform), and its UV spectrum in methanol exhibited absorptions at λ_{max} 225 (log ϵ 4.80), 278 (4.06), and 291 (3.96) nm., which were indicative of a tetrahydro-β-carboline base. The IR spectrum (KBr) resembled that of akuammidine except for the presence of the acetate absorptions (1735 and 1260 cm.$^{-1}$). The mass spectrum revealed a molecular ion at *m/e* 394 ($C_{23}H_{26}N_2O_4$), and the fragmentation pattern of ionic species at *m/e* 168, 169, 182, 249, 321, and 335 confirmed the presence of the akuammidine nucleus. The base peak at *m/e* 335 conforms to the M − 56 fragment reported for acetylakuammidine (akuammidine acetate) (3). A mixed melting-point determination with a reference sample of synthetic acetylakuammidine[1] showed no depression, and the IR spectra of the isolate and the reference samples were superimposable, thus confirming the identity of the isolate as acetylakuammidine (I).

Isolation of Aspidospermine and Quebrachidine—Fractions 63–72 and 73–100 from the column yielded 1.59 and 2.50 g., respectively, of aspidospermine from methanol, whereas fractions 131–200 afforded 0.81 g. of quebrachidine from the same solvent. Identification of these bases was made by comparison of their physical data with those of reference samples.

SUMMARY

Acetylakuammidine, quebrachidine, and aspidospermine have been reported isolated from the leaves of *Aspidosperma quebracho-blanco*. This is the first report of the natural occurrence of acetylakuammidine[2] in nature.

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▲ To whom inquiries should be directed.

[1] Supplied by Professor Carl Djerassi, Department of Chemistry, Stanford University, Stanford, Calif.

[2] Reaction of akuammidine with 10% acetic acid at room temperature did not produce synthetic acetylakuammidine, when analyzed at 24 and 48 hr. and at 7 and 14 days.

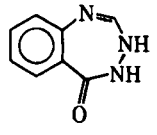
Reaction of Methyl 2-[2′,2′-Bis(carbethoxy)vinylamino]benzoate with Hydrazine

MILTON J. KORNET

Abstract □ Previous workers incorrectly assigned the 1,3,4-benzotriazepine-5-one structure to the product resulting from the reaction of methyl 2-[2′,2′-bis(carbethoxy)vinylamino]benzoate with hydrazine. Spectral data and independent synthesis show that the product is actually 3-amino-4-quinazolone.

Keyphrases □ Methyl 2-[2′,2′-bis(carbethoxy)vinylamino]benzoate—reaction with hydrazine, correction of product structure to 3-amino-4-quinazolone □ 3-Amino-4-quinazolone—product of reaction of methyl 2-[2′,2′-bis(carbethoxy)vinylamino]benzoate with hydrazine, previously assigned 1,3,4-benzotriazepine-5-one structure

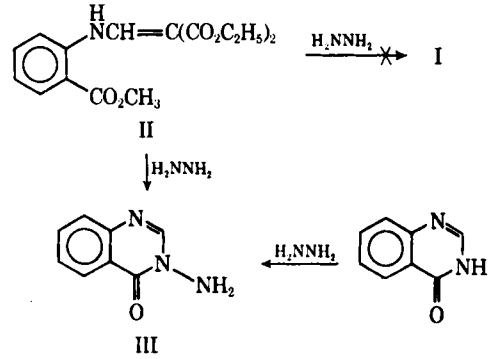
The reaction of methyl 2-[2′,2′-bis(carbethoxy)vinylamino]benzoate with hydrazine was reported by Podesva *et al.* (1) in 1969. These workers obtained a compound, m.p. 214–215°, to which they assigned the 1,3,4-benzotriazepine-5-one structure (I). This reaction



I

appeared to offer a convenient route to additional analogs of I for study as potential psychotherapeutic agents[1].

This transformation has been reproduced, giving a product with a melting point of 214–215°, but the NMR showed the presence of a sharp singlet at 5.87 δ integrating for two protons, a sharp singlet (1H) at 8.35 δ, and a complex multiplet (4H) at 7.31–8.29 δ. The absence in the spectrum of a broad signal due to an amide proton is inconsistent with Structure I. Another possible structure for the product from the reaction of methyl 2-[2′,2′-bis(carbethoxy)vinylamino]benzoate (II) with hydrazine



Scheme I

is the isomeric aminoquinazolone (III, Scheme I). The NMR data are completely consistent with this structure.

An authentic sample of III was prepared from 4-quinazolone (2) and hydrazine according to the method of Leonard and Ruyle (3)[2]. Comparison of the IR and NMR spectra of this compound with the product obtained from II showed them to be identical. In addition, a mixed melting point was undepressed.

By analogy, the compound described as 7-chloro-3,4,5-benzotriazepine-5-one by Podesva *et al.* (1) must also have the corresponding quinazolone structure.

EXPERIMENTAL[3]

Methyl 2-[2′,2′-Bis(carbethoxy)vinylamino]benzoate (II)—This compound was prepared according to the procedure of Podesva *et al.* (1) from 7.55 g. (0.05 mole) of methyl anthranilate and 10.8 g. (0.05 mole) of ethyl ethoxymethylenemalonate. The first crop amounted to 9.62 g. (60%), m.p. 51–55°. Recrystallization from hexane gave clusters of fine white needles, m.p. 56.5–57° [lit. (1) 78% yield, m.p. 71–72°]. Further recrystallizations from hexane did not change the melting point. IR (KBr): 5.9 (broad ester C=O) and 3.11 (NH) μ; NMR ($CDCl_3$): δ 12.62 (broadened d, 1, J = 13.5 Hz., NH), 8.56 (d, 1, J = 13.5 Hz., N—CH), 6.89–8.17 (m, 4, ArH), 4.40 (q, 2, OCH_2), 4.28 (q, 2, OCH_2), 3.98 (s, 3, OCH_3), 1.38 (t, 3, C—CH_3), and 1.33 (t, 3, C—CH_3).

Anal.—Calc. for $C_{16}H_{19}NO_6$: C, 59.81; H, 5.96; N, 4.36. Found: C, 60.09; H, 6.04; N, 4.47.

3-Amino-4-quinazolone (III)—*Method A*—Treatment of 2.50 g. (7.79 mmoles) of II with 1.25 g. (25.0 mmoles) of 100% hydrazine hydrate in 12 ml. of ethanol, according to the procedure of Podesva *et al.* (1), gave a solid which was filtered and washed thoroughly with water. After drying in an evacuated desiccator containing phosphorus pentoxide overnight, 1.03 g. (82%) of colorless glistening needles, m.p. 213–214°, was obtained. Recrystallization from 95% ethanol afforded needles, m.p. 214–215°; IR (KBr): 5.98 (C=O), 6.12 (C=N), and 3.04, 3.17 (NH_2) μ; NMR (dimethyl sulfoxide-d_6): δ 8.35 (s, 1, N=CH—N), 7.31–8.29 (m, 4, ArH), and 5.87 (s, 2, NH_2).

Method B—Colorless needles, m.p. 213.5–215°, were prepared from 4-quinazolone (2) and hydrazine hydrate according to the procedure of Leonard and Ruyle (3). Recrystallization from 95% ethanol gave colorless needles, m.p. 213–214° [lit. (3) m.p. 209–210°]. This compound proved to be identical with the product described in Method A by comparison of their IR and NMR spectra. In addition, a mixed melting point was not depressed.

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[1] The only other compound related to I that has been reported in the literature is the 7-chloro analog. It was prepared similarly by the same authors.

[2] The compound obtained by Leonard and Ruyle was characterized as its benzal derivative, thereby ruling out Structure I for this product.

[3] Melting points were determined with a Fisher-Johns apparatus and are corrected. IR spectra were obtained on a Beckman IR-8 spectrophotometer. NMR spectra were determined on a Varian A-60A spectrometer, using tetramethylsilane as the internal reference.

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GLC Determination of Methaqualone in Plasma

J. F. DOUGLAS▲ and S. SHAHINIAN

Abstract □ A simple GLC method was developed for the determination of methaqualone in plasma. The method is rapid and quantitative over the 0.1–1.0-mcg./ml. range.

Keyphrases □ Methaqualone—GLC determination in plasma □ GLC—determination of methaqualone in plasma

Methaqualone, 2-methyl-3-*o*-tolyl-4(3*H*)-quinazolinone, is a well-known compound which has been available for use as a hypnotic since 1960. Determination of this drug in biological fluids has been investigated using spectrophotometric (1) and GC (2, 3) procedures. The former technique is not suitable for precise measurement because of high blank values, while the latter procedure, which eliminates the blank problem, is tedious and time consuming. In addition, it requires 5.0 ml. of plasma. This report describes a GC method for the determination for methaqualone that is simple, rapid, and reliable.

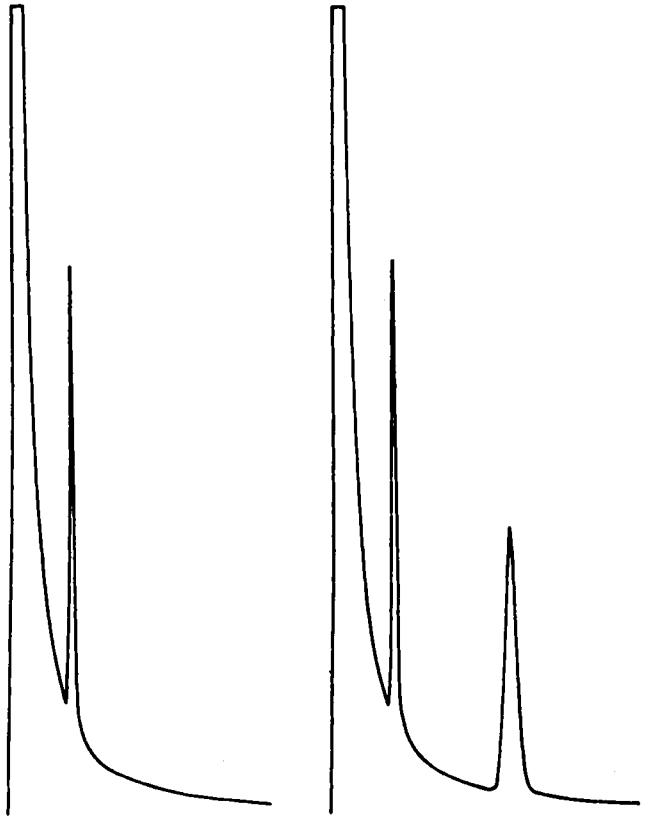

Figure 1—*Gas chromatograms of plasma treated as described. Left: chromatogram from normal plasma. Right: chromatogram from normal plasma with 0.5 mcg./ml. of methaqualone added.*

Table I—Recovery of Methaqualone from Plasma

Methaqualone Added, mcg./ml.	Methaqualone Recovered, mcg./ml.	Percent Recovery[a]
0.20	0.14	70
0.50	0.37	74
1.0	0.77	77
2.0	1.4	70
5.0	3.5	70
10.0	6.9	69
	Average	71.7 ± 1.3[b]

[a] Recoveries are based on a comparison to GC response to pure solutions of methaqualone. [b] Standard error.

Table II—Methaqualone Plasma Concentrations (Micrograms per Milliliter) in Human Subjects[a]

Methaqualone Dose, mg.	Hours						
	0	0.25	0.5	1	2	4	8
400	None detected	0.6	1.7	2.3	1.4	1.1	0.5
800	None detected	1.1	2.3	3.5	4.3	3.5	2.0

[a] Each value given is an average of three subjects.

EXPERIMENTAL

GC—A dual-column gas chromatograph[1] equipped with a hydrogen flame-ionization detector and a 1-mv. recorder[2] was employed. The chromatographic columns used were 0.6-m. (2-ft.) × 0.6-cm. (0.25-in.) glass tubes packed with 3% XE-60 on 100–120-mesh Gas Chrom Q[3]. The instrument settings were: column temperature, 180°; injection port temperature, 240°; and detector block temperature, 230°. Gas flow rates were: hydrogen, 25 ml./min.; and helium (carrier gas), 50 ml./min. Sensitivity settings were: range, 10; and attenuation factor, 2×. The retention times under these conditions were 1.6 min. for butyl stearate and 4.5 min. for methaqualone (Fig. 1).

Reagents—The reagents were redistilled chloroform[4] and butyl stearate[4].

Procedure—Plasma, 1.0 ml., was made alkaline with 0.05 ml. of 1 *N* NaOH, and 0.2 ml. of chloroform containing 0.5 mcg./ml. of

[1] F & M model 402.
[2] Minneapolis-Honeywell.
[3] Applied Science.
[4] Regis Chemical.

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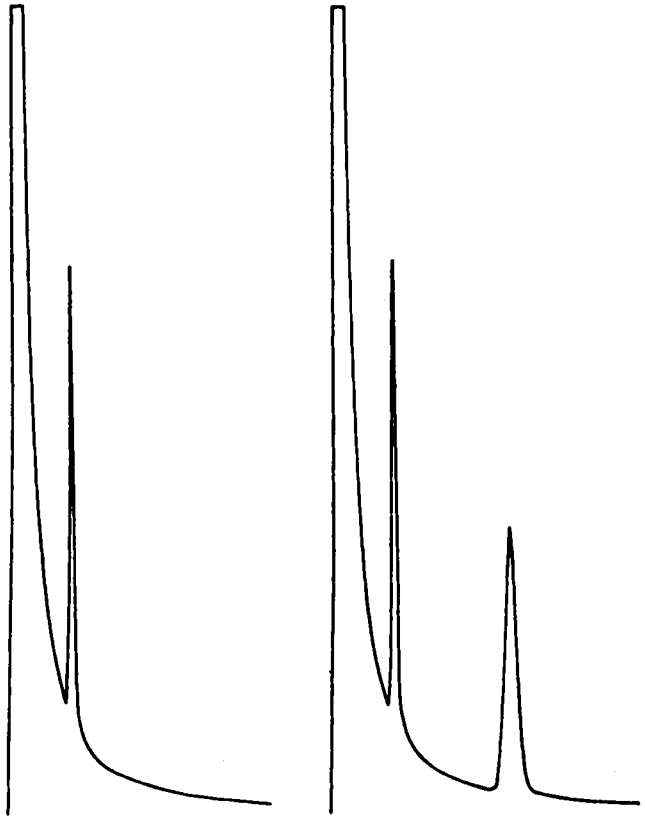

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[a] Each value given is an average of three subjects.

EXPERIMENTAL

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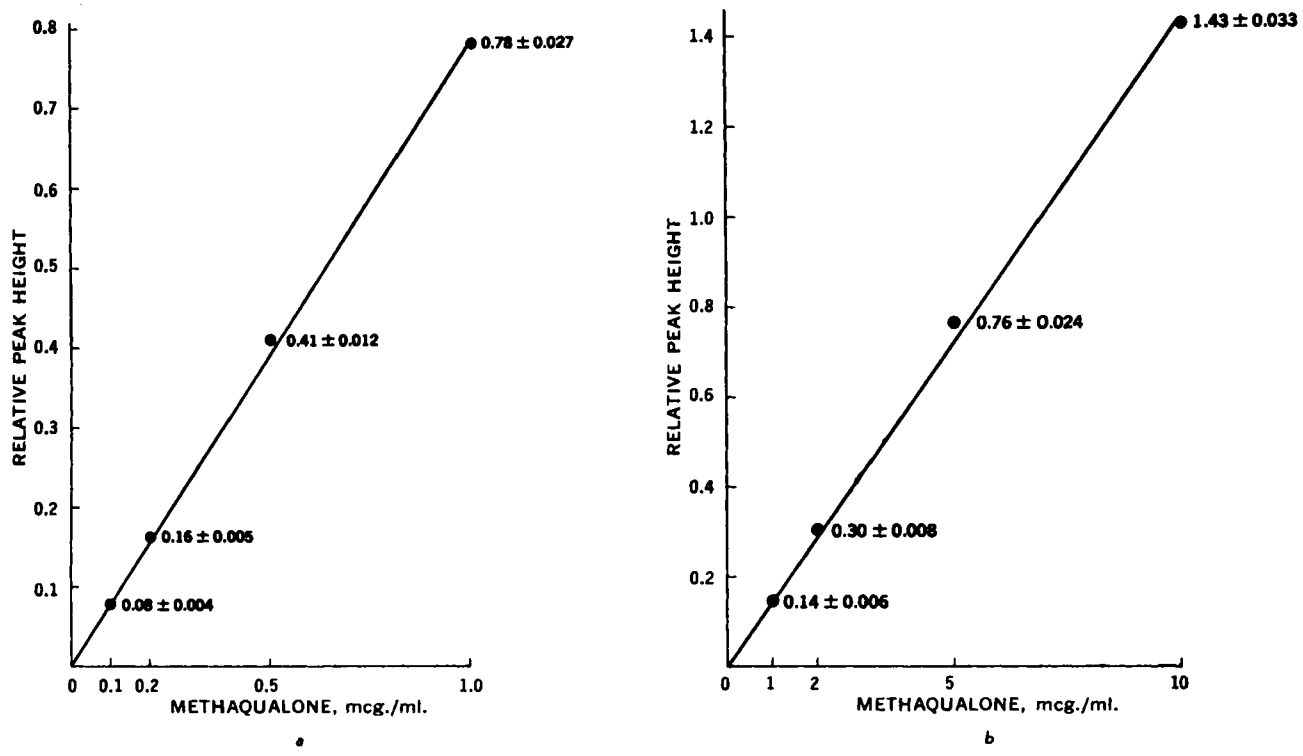

Figure 2—*Relationship between relative peak height and methaqualone concentration in plasma. Key:* a, *0.1–1.0 mcg./ml.; and* b, *1.0–10.0 mcg./ml.*

butyl stearate was added. The mixture was swirled using a Vortex mixer for 20 sec. and then was centrifuged for 10 min., and the aqueous layer was aspirated. One hundred microliters of the chloroform solution was transferred to a clean tube, and the solvent was removed under a stream of nitrogen. The residue was dissolved in distilled chloroform, 25 μl., and 2.6 μl. of this solution was injected into the gas chromatograph. The concentration of methaqualone was determined by the relative peak height method, using butyl stearate as internal standard.

RESULTS AND DISCUSSION

The relationship between relative peak height and methaqualone concentration in the range of 0.1–1.0 mcg./ml. of plasma is shown in Fig. 2*a*. The reproducibility of the procedure, as indicated by the standard error of quadruplicate determinations, is also shown. Concentrations of methaqualone greater than 1.0 mcg./ml. can readily be determined using the same extraction technique while omitting the chloroform concentration step. In this procedure, an aliquot of the chloroform solution was injected directly into the gas chromatograph after aspiration of the aqueous layer. The results obtained with plasma concentrations of 1.0–10.0 mcg./ml. are shown in Fig. 2*b*.

The extraction technique effectively separates methaqualone from normally interfering plasma constituents since determinations with normal plasma give little or no blank (Fig. 1). About 70% of the plasma methaqualone is reproducibly extracted regardless of drug concentration (Table I) over a range of 0.2–10.0 mcg./ml.

The blood-depletion pattern of methaqualone was studied by this procedure following oral administration of this drug to human subjects. The results obtained (Table II) are similar to those reported previously by Berry (2). The drug reached only low levels in the plasma, probably due to its rapid uptake by adipose tissue (4). A 400-mg. dose gave a peak plasma level at about 1 hr., while with a higher dose, 800 mg., the peak plasma level was obtained at 2 hr.

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▲ To whom inquiries should be directed.

Ascorbic Acid: Preferential Lysis and Catalase Induction in Antibiotic-Resistant Strains of *Staphylococcus aureus*

B. D. RAWAL

Abstract □ Experiments revealed that antibiotic-sensitive staphylococci produce more catalase per unit viable cell than the antibiotic-resistant strains. Ascorbic acid induces catalase activity in the antibiotic-resistant strains. In addition to the variations in catalase formation, ascorbic acid preferentially lyses antibiotic-resistant but not sensitive staphylococci in the exponential phase of growth. By using this criterion, a series of 36 hospital strains of antibiotic-resistant and sensitive strains were differentiated ($p < 0.005$) by ascorbic acid after a 90-min. incubation period.

Keyphrases □ Ascorbic acid—preferential lysis and catalase induction in antibiotic-resistant *Staphylococcus aureus* □ *Staphylococcus aureus*, antibiotic resistant—ascorbic acid-induced catalase activity and preferential lysis □ Antibiotic-resistant staphylococci—ascorbic acid-induced catalase activity and preferential lysis

The antimicrobial action of ascorbic acid is attributed to hydrogen peroxide resulting from the decomposition of ascorbic acid (1). It is well known that microorganisms overcome this lethal action of hydrogen peroxide by producing catalase. Indeed, ascorbic acid has been reported (2) to induce catalase in an antibiotic-resistant strain of *Staphylococcus aureus*. These authors also observed that catalase activity in cultures adapting to antibiotics *in vitro* gradually diminished or even stopped. It is, therefore, likely that the established antibiotic-resistant strains of staphylococci may produce less catalase than the antibiotic-sensitive strains. If this is the case, then these two groups of staphylococci may respond differently to ascorbic acid with regard to growth and/or catalase activity; antibiotic-resistant and sensitive staphylococci could then be distinguished on this basis. The present investigation was aimed at determining if catalase activity, response to ascorbic acid, and antibiotic resistance in staphylococci were associated.

EXPERIMENTAL

Test Organisms—*S. aureus* strain oxford and the phage propagating strain PS84 were used as typical representatives of antibiotic-sensitive and resistant staphylococci, respectively. In addition, 34 hospital strains of staphylococci were tested. These strains were maintained by serial passage on nutrient agar (oxoid).

Culture Media—Tryptone soya broth (oxoid) was used for all experiments. Ascorbic acid (2.5 mg./ml.) was dissolved in this broth, and the pH was adjusted to 7.3 with sodium hydroxide. This ascorbic acid broth was sterilized by membrane filtration, while tryptone soya broth was autoclaved at 15 lb. pressure for 15 min.

Antibiotic Sensitivity—All cultures were tested for antibiotic sensitivity using the multodisks (oxoid) on nutrient agar plates. Strains PS84 and oxford were also tested by the tube dilution method.

Growth Curve—Four sterile McCartney bottles (A, B, C, and D) were used in the growth curve experiment. Bottles A and B contained tryptone soya broth (20 ml. each); Bottles C and D contained tryptone soya broth fortified with ascorbic acid (2.5 mg./ml.) neutralized to pH 7.3. All transfers were made in a laminar work station. Bottles A and C were inoculated with *S. aureus* (oxford strain) grown overnight in tryptone soya broth. The absorbance of the final medium in these bottles after inoculation was 0.06 at 560 nm.[1]. Similar inoculations were made in Bottles B and D with *S. aureus* (PS84 strain). A blank set of sterile tryptone soya and ascorbic acid broths was also included. All bottles were incubated at 37° under agitation. Aliquots from each bottle were aseptically removed at 0, 1, 2, 3, and 4 hr. during incubation to determine the number of viable organisms, absorbance, and catalase activity.

Table I—Catalase Activity in Oxford and PS84 Strains of *S. aureus*

	Medium			
	Tryptone Soya Broth (pH 7.3)		Tryptone Soya Broth plus 2.5 mg./ml. Ascorbic Acid (pH 7.3)	
S. aureus	Oxford	PS84	Oxford	PS84
Rate of multiplication (generations/hour)	4.5	1.7	3.7	1.3
Catalase activity[a]:				
Milligrams hydrogen peroxide	8.350	5.601	2.811	7.110
Percent	100	67	100	309
Catalase activity[b]	100	100	67	126
χ^2 test		11.82 ($p < 0.001$)		

[a] Per 10^8 colony-forming units up to 4 hr. of growth. [b] Expressed as percent of catalase activity in tryptone soya broth.

Measurement of Growth—Viable counts were made by plating suitable dilutions of the sample in 0.1-ml. amounts in nutrient agar plates. Colonies formed after 16 hr. of incubation at 37° were counted.

The absorbance of each sample was measured at 560 nm.[1], using tryptone soya broth and ascorbic acid broth blanks as controls.

Estimation of Catalase—Catalase activity was estimated iodometrically (3). An aliquot of 1.0 ml. of each culture was added to 10.0 ml. of 0.1 *N* hydrogen peroxide contained in a conical flask. Five minutes later, the enzyme was inactivated by adding 2.0 ml. of 2 *N* sulfuric acid. This was followed by 5 ml. of freshly prepared 10% aqueous solution of potassium iodide and 2–3 drops of 15% ammonium molybdate. Liberated free iodine was then titrated against 0.1 *N* sodium thiosulfate. Blank readings were obtained by titrating the uninoculated broth; sulfuric acid was added to hydrogen peroxide prior to adding the broth for this determination. Catalase activity in each sample, computed from the amount of sodium thiosulfate required, was then expressed as milligrams hydrogen peroxide decomposed in 5 min. Catalase activity was also expressed per 10^8 colony-forming units. This value was calculated by dividing the observed catalase activity by the actual number of colony-forming units per milliliter determined by the viable count method described.

Growth Response (Absorbance) of Antibiotic-Resistant and Sensitive Staphylococci to Ascorbic Acid—The basic standardization to determine the minimal amounts of ascorbic acid, the type of culture media, the amounts of inoculum, the minimum period of incubation, *etc.*, was made with the oxford and PS84 strains of *S. aureus*. Up to 2–3 hr. of incubation of the ascorbic acid broth did

[1] A Spectronic-20 was used.

Table II—Growth[a] of Oxford and PS84 Strains of *S. aureus* in Tryptone Soya Broth Containing 2.5 mg./ml. Ascorbic Acid, pH 7.3

Time of Incubation, hr.	Absorbance		Colony-Forming Units	
	Oxford	PS84	Oxford	PS84
1	100	84	100	92.0
2	120	70	104	96.5
3	112	77	104	96.5
4	105	94	108	96.5

[a] Expressed as percent of growth observed in corresponding tryptone soya broth.

not cause significant darkening. Furthermore, these strains completed their exponential growth within 3 hr. of incubation under agitation. The test was based on the comparison of the absorbance formed in tryptone soya broth and the ascorbic acid broth. Consequently, at zero hour the inocula were adjusted so as to obtain identical absorbances in both media. The cultures were then incubated at 37° and absorbance was read at 560 nm. at varying time intervals. The absorbance in the ascorbic acid broth was expressed as the percent absorbance of that observed in the corresponding tryptone soya broth culture. Viable counts of the PS84 and oxford strains were made at each observation during the growth.

RESULTS AND DISCUSSION

The response of the antibiotic-resistant and sensitive staphylococci to ascorbic acid was measured by using four different parameters: (*a*) rate of growth, (*b*) catalase activity, (*c*) catalase induction, and (*d*) susceptibility to ascorbic acid.

Under the experimental conditions described, the PS84 strain multiplied at a significantly lower rate than the oxford strain (Table I). Such behavior of antibiotic-resistant strains was previously reported (4). What is unknown, however, is the significant difference in the catalase activity of antibiotic-resistant and sensitive staphylococci. In the present study, 10^8 colony-forming units of the antibiotic-resistant PS84 strain produced 67% of the catalase formed by 10^8 colony-forming units of the antibiotic-sensitive oxford strain in tryptone soya broth. In ascorbic acid broth, however, the catalase activity of the PS84 strain was 309% of that of the oxford strain per 10^8 colony-forming units. These differences ($\chi^2 = 11.82$, $p = 0.001$) indicate that the antibiotic-resistant PS84 strain exhibited an impaired catalase activity as compared with the antibiotic-sensitive oxford strain. Increased catalase production may then be a characteristic of antibiotic-sensitive organisms.

When ascorbic acid was added to the medium, however, the PS84 strain formed catalase in considerably larger amounts. Is it then likely that antibiotic-resistant staphylococci growing in the presence of ascorbic acid become increasingly susceptible to an antibiotic? This, indeed, is the case. Synergistic inhibition of *S. aureus* with ascorbic acid and penicillin was previously reported (5). The author has also observed synergistic action between ascorbic acid and several antibiotics *in vitro* on the PS84 strain[2]. Furthermore, ascorbic acid acting singly and in combination with sulfamethoxazole-trimethoprim inhibits *Pseudomonas aeruginosa* but not *Streptococcus faecalis* (6).

[2] These data will be published elsewhere.

Table III—Differential Response to Ascorbic Acid by Antibiotic-Resistant and Sensitive Strains of Staphylococci after 90 min. of Growth *In Vitro*

	Antibiotic-Resistant Strains	Antibiotic-Sensitive Strains
Number of strains tested	28	8
Mean absorbance[a] in ascorbic acid broth	78	117
Standard deviation	3.7417	8.4853
t test	11.1 ($p < 0.005$)	

[a] Expressed as percent absorbance of the corresponding tryptone soya broth culture.

The variations in the catalase activity were also associated with the susceptibility of the strains to ascorbic acid (Table II).

In the presence of ascorbic acid, the absorbance of the oxford strain was 100% or more of the control throughout the observation period; the absorbance of the PS84 culture was well below 100% of the control throughout. Viable count determinations were not sensitive enough to highlight these differences, although the pattern was suggested. The data show preferential lysis of some antibiotic-resistant cells during exponential growth in the presence of ascorbic acid. It might then be said that exponentially growing antibiotic-resistant cells are sensitive to the lytic effect of ascorbic acid while antibiotic-sensitive staphylococci are resistant to it. Whether or not such a generalization is applicable to hospital strains of staphylococci was then investigated with 36 strains (Table III).

The data in Table III reveal that antibiotic-sensitive hospital strains of staphylococci also produced a significantly higher absorbance, like the oxford strain, than the antibiotic-resistant strains in the presence of ascorbic acid (2.5 mg./ml.).

In conclusion, this investigation reveals that antibiotic-resistant staphylococci produce significantly less amounts of catalase and are preferentially lysed by ascorbic acid during exponential growth. Antibiotic-sensitive staphylococci exhibit the opposite pattern. Further work would bear out the importance of this observation.

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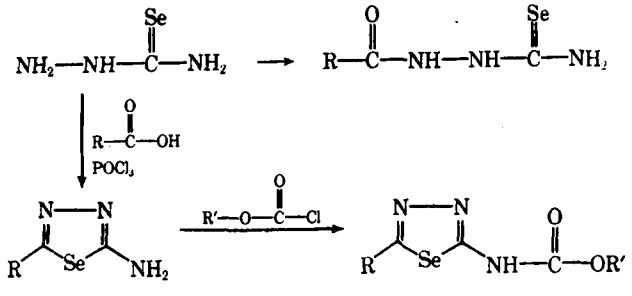
Selenium Heterocycles VIII: Synthesis and Antibacterial Activity of Selenosemicarbazide and 1,3,4-Selenadiazolylcarbamic Acid Esters

A. SHAFIEE, I. LALEZARI▲, S. YAZDANY, and A. POURNOROUZ

Abstract □ The antibacterial activity of selenosemicarbazide and its acyl derivatives was compared with the activity of their sulfur and oxygen analogs. A series of 1,3,4-selenadiazolylcarbamic acid esters was also prepared and tested. Some of these compounds showed significant antibacterial activity.

Keyphrases □ Selenosemicarbazide and acyl derivatives—synthesis, antibacterial activity compared with sulfur and oxygen analogs □ 1,3,4-Selenadiazolylcarbamic acid esters—synthesis, antibacterial activity □ Antibacterial activity—synthesis of selenosemicarbazide derivatives and 1,3,4-selenadiazolylcarbamic acid esters

Recently, we reported (1, 2) that 1,3,4-thiodiazolylcarbamic acid esters have potent antibacterial and antiviral activity. Bhamaria *et al.* (3) reported that 1-acyl 4-alkyl (or aryl) thiosemicarbazides have no significant antibacterial activity against *Escherichia coli* and *Salmonella typhosa* and very limited activity against *Staphylococcus aureus*, but the majority of the compounds tested were active against *Mycobacterium tuberculosis*. Bednarz (4) reported that selenosemicarbazides of aldehydes were active against *M. tuberculosis*, *S. aureus*, *Bacillus subtilis*, and *E. coli*. Therefore, the comparative study of antibacterial activity of a series of selenium compounds and their analogs was of special interest.



Scheme I

EXPERIMENTAL[1]

The desired compounds were prepared according to Scheme I. The physical data of these compounds are summarized in Tables I and II.

All compounds listed in Table III were tested against *B. subtilis* (NCTC 3610), *S. aureus* (ATCC 6538), *Klebsiella pneumoniae* (ATCC 10031), and *Sarcina lutea* (ATCC 9341).

The compounds were dissolved in sterile distilled water and diluted to a 0.5% concentration. Standard paper disks of 6 mm. diameter were immersed in solution and were placed on inoculated assay medium surface[2]. None of the 1-acyl semicarbazides or 1-acyl thiosemicarbazides and their derivatives showed significant activity (Table III).

All compounds listed in Table II were tested against *S. aureus* (ATCC 6538p) and *K. pneumoniae* (ATCC 10031) at different concentrations in liquid medium[3]. Compounds 6, 7, and 12 at the concentration of 0.6 mg./ml. inhibited the growth of these organisms. No significant inhibition was observed with the other compounds up to the concentration of 2.5 mg./ml.

Selenosemicarbazide was prepared according to the literature (5).

1-Formylselenosemicarbazide (Compound 1)—Selenosemicarbazide (2.76 g., 0.02 mole) and 10 ml. of 99–100% formic acid were refluxed for 20 min. The reaction mixture was filtered hot, allowed to crystallize at room temperature, and recrystallized from water.

1-Propionylselenosemicarbazide (Compound 2)—This compound was prepared from propionic acid and selenosemicarbazide similar to 1-formylselenosemicarbazide.

Other 1-acyl selenosemicarbazides were prepared by the method reported previously (6).

1-Benzoylselenosemicarbazide (Compound 3)—Selenosemicarbazide (2.76 g., 0.02 mole) was dissolved in 40 ml. of 4% sodium hydroxide and, while stirring at ice bath temperature, benzoyl chloride (2.8 g., 0.02 mole) was added dropwise. After standing at room temperature for 1 hr., charcoal was added to the reaction mixture, which was then filtered and acidified with 10% hydrochloric acid and the precipitate was recrystallized from ethanol–water.

***N*-(5-Trifluoromethyl-1,3,4-selenadiazol-2-yl)carbamic Acid Ester (Compound 12)**—2-Amino-5-trifluoromethyl-1,3,4-selenadiazole (6)

Table I—1-Acyl Selenosemicarbazides RCONHNHCSeNH₂

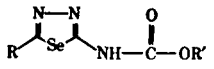
Compound	R	Melting Point	Yield, %	Crystallization Solvent	Formula	Analysis, % Calc.	Found
1	H	172°	50	Water	$C_2H_5N_3OSe$	C 14.45	14.33
						H 3.01	2.96
2	C_2H_5	182°	40	Ethanol	$C_4H_9N_3OSe$	C 24.72	24.89
						H 4.63	4.55
3	C_6H_5	210–214°	76	Ethanol–water	$C_8H_9N_3OSe$	C 39.66	39.39
						H 3.70	3.80
4	*p*-FC_6H_4	198–200°	72	Water	$C_8H_8FN_3OSe$	C 36.92	37.02
						H 3.07	2.99

[1] Melting points were taken on a Kofler hot stage microscope and are uncorrected. Their spectra were recorded using a Leitz spectrograph. NMR spectra were recorded on a Varian A60A instrument.

[2] Antibiotic assay medium, British Pharmacopoeia, 1968.

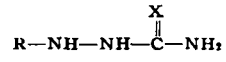
[3] Antibiotic assay medium without agar, British Pharmacopoeia, 1968.

Table II—5-Substituted-1,3,4-selenadiazol-2-yl-carbamic Acid Esters



Compound	R	R′	Melting Point	Yield, %	Crystallization Solvent	Formula	Analysis, % Calc.	Found
5	CH_3	CH_3	170°	63	Water	$C_5H_7N_3O_2Se$	C 27.14	27.21
							H 3.16	3.09
6	CH_3	n-C_4H_9	165°	69	Water	$C_8H_{13}N_3O_2Se$	C 36.50	36.29
							H 4.92	5.01
7	CH_3	iso-C_4H_9	155°	77	Water	$C_8H_{13}N_3O_2Se$	C 36.50	36.66
							H 4.92	4.99
8	C_2H_5	CH_3	147°	82	Water	$C_6H_9N_3O_2Se$	C 30.63	30.77
							H 3.82	3.78
9	C_2H_5	C_2H_5	119°	80	Water	$C_7H_{11}N_3O_2Se$	C 33.73	33.71
							H 4.41	4.49
10	C_6H_5	CH_3	148°	53	Water	$C_{10}H_9N_3O_2Se$	C 42.40	42.43
							H 3.18	3.17
11	C_6H_5	C_2H_5	132°	66	Water	$C_{11}H_{11}N_3O_2Se$	C 44.44	44.26
							H 3.70	3.66
12	CF_3	C_2H_5	204°	77	Water	$C_6H_6F_3N_3O_2Se$	C 24.91	25.06
							H 2.07	2.11

Table III—Average Zone Size, mm.



R	X	*B. subtilis*	*K. pneumoniae*	*S. aureus*	*Sar. lutea*
H	Se	23	48	30	40
HCO	S	—	—	—	—
HCO	Se	20	24	19	45
CH_3CO	S	—	—	—	—
CH_3CO	Se	21	35	29	32
C_6H_5CO	S	—	—	—	—
C_6H_5CO	Se	14	34	23	36
C_6H_5CO	O	—	—	—	—
C_6H_5CO	S	—	—	—	—
C_6H_5CO	Se	19	39	26	41
p-FC_6H_4CO	Se	19	38	27	42

(0.55 g., 0.025 mole) and chloroformic acid ethyl ester (0.28 g., 0.026 mole) in 18 ml. chloroform were refluxed for 2 hr. After removing the solvent, the residue was crystallized from water to give 0.55 g. (77%) of Compound 12.

All other compounds were prepared similarly (Table II).

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Effect of Radiation on Activity of Antibiotics

K. G. GUPTA*, K. K. VYAS*, and N. S. SEKHON†▲

Abstract □ Thirteen antibiotics belonging to different groups were exposed to 5, 10, and 15 megarads of neutron and γ-radiations, and their activity in comparison to nonirradiated antibiotics for *Escherichia coli* and *Staphylococcus aureus* was studied. With γ-irradiation, oxytetracycline at 15 megarads and penicillin G at 5 and 10 megarads showed considerable decrease in activity. With penicillin G, the activity was somewhat restored at the 15-megarad dose. Other antibiotics did not show a significant change in activity by this method. Neutron irradiation did not change the activity.

Keyphrases □ Antibiotics—effect of neutron and γ-radiation on activity □ Neutron radiation—effect on activity of 13 antibiotics of different groups □ γ-Radiation—effect on activity of 13 antibiotics of different groups

UV light is known to inactivate urease, papain, and trypsin (1), although the degree of inactivation may vary depending upon the type of the molecule. Similarly, amino acids have been shown to be modified when exposed to radiation (2). Iodine compounds such as potassium iodide, potassium iodate, and iodoacetic acid have been shown to possess higher toxicity to bacteria when irradiated (3). Irradiated antibiotics have also been found to have changed activity against sensitive and resistant strains (4). γ-Radiation with cobalt 60 does not bring about any chemical or biological changes in oxytetracycline and chlortetracycline in the dry state or in organic solvents, but it inactivates them significantly in aqueous solutions (5).

In the present investigation, various antibiotics were exposed to different doses of neutron and γ-radiations and the activity of such treated antibiotics against Gram-negative and Gram-positive bacteria was studied.

EXPERIMENTAL

Staphylococcus aureus (NCTC 6571)[1] and *Escherichia coli* (06)[2] were maintained on nutrient agar at 4°. Subculturing was done every month. Incubation temperatures for *S. aureus* and *E. coli* were 31 ± 1° and 36 ± 1°, respectively.

Antibiotics—The following antibiotics were used in this study: ampicillin[3], bacitracin[4], chloramphenicol[3], cloxacillin[5], erythromycin[3], kanamycin[5], neomycin sulfate[3], nisin[6], oxytetracycline[3], penicillin G[3], streptomycin sulfate[3], spiramycin[3], and vancomycin[7].

Standard solutions of antibiotics were made in glass double-distilled water.

Irradiation of Antibiotics—A known quantity of each antibiotic (in triplicate) was irradiated in the dry state[8]. Neutron irradiation was done with a neutron flux of 1.28×10^{12} neutrons cm.^{2}/sec.; the duration was 12, 24, and 36 min. for 5, 10, and 15 megarads, respectively. The rate of γ-irradiation of the antibiotics was 0.356 megarad/hr.; the doses given were 5, 10, and 15 megarads.

Antibiotic Testing—*S. aureus* and *E. coli* were grown in nutrient broth. A 24-hr.-old culture containing approximately 10^8 cells/ml. was prepared. One milliliter of this suspension was mixed with every 15 ml. of nutrient agar and plated in 85–90-mm. diameter petri dishes so that the thickness of the medium in each plate was between 3.4 and 3.6 mm. The dishes were then allowed to dry in an incubator at 37° for 0.5 hr.

Disks, 6.25 mm. in diameter, were punched from Whatman No. 1 filter paper. These disks were sterilized by dry heat at 140° for 1 hr.

Table I—Effect of γ-Radiation on the Activity of Antibiotics

Antibiotic	Radiation Dose, megarads	Activity[a] (in Terms of Zone Diameter, mm.) against *E. coli*	against *S. aureus*
Ampicillin	—[b]	19	32
	5	18	30
	10	18	30
	15	18	30
Bacitracin	—	Nil	14
	5	Nil	14
	10	Nil	14
	15	Nil	14
Chloramphenicol	—	18	20
	5	19	21
	10	19	21
	15	19	21
Cloxacillin	—	Nil	29
	5	Nil	28
	10	Nil	28
	15	Nil	29
Erythromycin	—	Nil	13
	5	Nil	13
	10	Nil	12
	15	Nil	12
Kanamycin	—	16	23
	5	16	23
	10	15	23
	15	16	23
Neomycin	—	14	17
	5	13	16
	10	14	16
	15	14	16
Nisin	—	Nil	9
	5	Nil	9
	10	Nil	8
	15	Nil	8
Oxytetracycline	—	23	27
	5	23	26
	10	23	26
	15	Nil	16
Penicillin G	—	12	40
	5	Nil	29
	10	Nil	30
	15	9	35
Streptomycin	—	21	15
	5	21	15
	10	21	14
	15	21	15
Spiramycin	—	Nil	14
	5	Nil	13
	10	Nil	13
	15	Nil	13
Vancomycin	—	Nil	22
	5	Nil	21
	10	Nil	21
	15	Nil	20

[a] Strength of the disk used = 5 mcg. [b] Nonradiated.

[1] Obtained from P.G.I., Chandigarh, India.
[2] Obtained from WHO International Escherichia Centre, Copenhagen, Denmark.
[3] Local market.
[4] Pfizer.
[5] Bristol Laboratories.
[6] Aplin and Barrett, Ltd., Yeovil, Somerset, England.
[7] Lilly Laboratories.
[8] At the Bhaba Atomic Research Centre, Bombay, India.

Table II—Effect of Neutron Radiation on the Activity of Antibiotics

Antibiotic	Radiation Dose, megarads	Activity[a] (in Terms of Zone Diameter, mm.) against *E. coli*	against *S. aureus*
Ampicillin	—[b]	20	30
	5	19	30
	10	19	29
	15	19	30
Bacitracin	—	Nil	12
	5	Nil	12
	10	Nil	12
	15	Nil	12
Chloramphenicol	—	19	20
	5	21	21
	10	22	22
	15	22	21
Cloxacillin	—	Nil	30
	5	Nil	30
	10	Nil	29
	15	Nil	30
Erythromycin	—	Nil	15
	5	Nil	15
	10	Nil	14
	15	Nil	14
Kanamycin	—	17	24
	5	16	24
	10	16	23
	15	16	23
Neomycin	—	13	16
	5	13	15
	10	13	15
	15	12	15
Nisin	—	Nil	10
	5	Nil	9
	10	Nil	9
	15	Nil	9
Oxytetracycline	—	21	25
	5	21	25
	10	21	25
	15	21	25
Penicillin G		Not done	
Streptomycin	—	20	13
	5	19	13
	10	19	13
	15	19	13
Spiramycin	—	Nil	12
	5	Nil	11
	10	Nil	11
	15	Nil	9
Vancomycin	—	Nil	23
	5	Nil	22
	10	Nil	22
	15	Nil	22

[a] Strength of the disk used = 5 mcg. [b] Nonradiated.

in batches of 100 in screw-capped bottles. For preparing disks containing 5 mcg. of antibiotic each, 1 ml. of the solution containing 500 mcg. of the antibiotic was added to each bottle of 100 disks. Because all of this solution was absorbed, it could be assumed that each disk contained approximately 5 mcg. of the antibiotic.

Finally, four disks of each antibiotic (one nonradiated and three radiated at 5, 10, and 15 megarads) were placed on the medium, suitably spaced apart, in one petri dish and incubated overnight at 37°. For each disk the diameters of the circular areas of inhibition were measured.

The above method was adopted from Cruickshank (6). This method is semiquantitative, but it was used because the authors have observed only gross changes in the antibiotic activity. Moreover, by testing radiated and nonradiated samples of each antibiotic on the same petri dish, the difference in zone inhibition diameter due to the differences in inoculum size or thickness of the medium, *etc.*, is overruled.

Results shown (Table I) are the mean values of three replications.

RESULTS AND DISCUSSION

The effect of various antibiotics subjected to different radiation doses upon *E. coli* and *S. aureus* was studied. Results (Table I) indicate that the activity of penicillin G and oxytetracycline was affected considerably. In the case of penicillin, the activity against *E. coli* disappeared when it was exposed to 5- and 10-megarad doses of γ-radiation but some activity reappeared when it was exposed to a dose of 15 megarads. Similarly, in the case of *S. aureus*, the activity of penicillin decreased at 5- and 10-megarad doses as compared to the control but partially reappeared at the 15-megarad dose. In the case of oxytetracycline, the activity decreased against both the organisms at 15 megarads. In all other antibiotics, the increase or decrease in the activity was not significant.

When the antibiotics were irradiated with neutrons, very little effect or no effect upon the activity of antibiotics was obtained against *E. coli* and *S. aureus* except in the case of spiramycin, which showed decreased activity against *S. aureus* when irradiated at a 15-megarad dose (Table II).

In the case of penicillin where there was an increase in the activity at the 15-megarad dose as compared to 5- and 10-megarad doses, no explanation can be made at this time for the strange behavior. The results indicate that, at least for oxytetracycline and penicillin, the activity can be affected by irradiation of antibiotics in the dry state. These results differ from earlier observations of Holland *et al.* (5); these authors reported no effect of γ-radiation on the antibiotics in the dry state up to a 10-megarad dose. Although cloxacillin, ampicillin, and penicillin are related antibiotics, only penicillin seems to be susceptible to γ-radiation. Although polypeptides are known to be more susceptible to radiation, the polypeptide antibiotics, nisin and bacitracin, did not show any change in activity whereas oxytetracycline was affected considerably.

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Relationship of Physicochemical Properties of Isomeric Trifluoromethylphenols with Their Bactericidal Effect on *Escherichia coli*

J. C. DEARDEN▲ and G. R. WEEKS

Abstract □ The bactericidal activities of isomeric trifluoromethylphenols against *Escherichia coli* were found to be in the order: $m > p > o$. These activities are related to the partition coefficients of the compounds between polar organic phases and aqueous buffer and these, in turn, are related to hydrogen bonding and polar characteristics.

Keyphrases □ Trifluoromethylphenols, isomeric—physicochemical properties–bactericidal effect correlation, *Escherichia coli*, partition coefficients, hydrogen bonding, polar characteristics □ Bactericidal activity, isomeric trifluoromethylphenols—relationship to physicochemical properties, *Escherichia coli* □ Partition coefficients, isomeric trifluoromethylphenols—related to bactericidal activity

Many studies have been made of the bactericidal activities of halogen-substituted phenols (*e.g.*, 1, 2), but these concern only ring-halogenated compounds. In an investigation of the bactericidal effectiveness of phenols substituted with halogen in a side chain, the effects of the three isomeric α,α,α-trifluoromethylphenols on *Escherichia coli* were examined, and these effects were related to certain physicochemical properties of the compounds.

EXPERIMENTAL

Materials—The phenols were prepared from the corresponding amines by diazotization, and approximately 40% yields were obtained. Purification was effected by vacuum distillation, followed in the cases of the *o*- and *p*-isomers by repeated vacuum sublimation. In each case, GC of the purified material on 3% E30 methyl silicone gum gave only a single peak.

The organism used was *E. coli* (NCIB8114), grown and counted on oxoid nutrient agar (pH 7.4) consisting of 0.1% Lab-Lemco, 0.2% yeast extract, 0.5% peptone, and 0.5% sodium chloride, gelled with 1.5% agar. The nutrient agar was made up at six-fifths of these concentrations to allow for dilution by the reaction mixture.

The buffer used for the bactericidal evaluations was McIlvaine's standard buffer solution, prepared from citric acid and disodium hydrogen phosphate, at a total concentration of approximately 0.04 *M*. The pH of the buffer was adjusted to 5.0, since *o*- and, particularly, *p*-trifluoromethylphenols were found to decompose to the corresponding hydroxybenzoic acid in aqueous solution above pH 5.5.

Table I—Bactericidal and Physicochemical Properties of the Trifluoromethylphenols

Property	*Meta-*	*Para-*	*Ortho-*
Percent w/v concentration for 0.1% survival of *E. coli* in 10 min. (*C*)	0.041	0.069	0.110
Partition coefficient, oleyl alcohol–buffer, pH 5	525	393	294
Partition coefficient, methyl oleate–buffer, pH 5	393	306	220
Partition coefficient, cyclohexane–buffer, pH 5	1.45	1.18	2.39
pKa	8.27	8.08	7.74
$\Delta\nu_{OH}$, CCl_4 to 10% v/v ether in CCl_4 ($cm.^{-1}$)	249	257	269
$\Delta\nu_{OH}/\nu_{OH}$ (free)	0.0690	0.0712	0.0742
Dipole moment	2.78	3.07	3.29

Methods—Bactericidal evaluation was by the method of viable counts. *E. coli* suspensions were prepared from the washed 24-hr. surface growth. The suspensions were diluted with water to give about 1×10^7 viable cells/ml. (viability of the initial suspensions was 45–75%). The reaction mixture was prepared by adding 5 ml. of buffered phenol solution to 45 ml. of bacterial suspension. Replicate 1-ml. aliquot samples were taken from the mixture at appropriate intervals and immediately diluted appropriately with sterile 0.9% saline to prevent further bactericidal activity. One milliliter of the final dilution was added to 5 ml. of melted nutrient agar in roll-tubes. The tubes were incubated at 37° for 48 hr. and then counted.

The pKa values were determined by automatic titration of 0.01 *N* solutions against 0.1 *N* sodium hydroxide solution on a potentiograph[1] calibrated at pH 3.99 and 9.20. It was established spectroscopically that during the time taken (about 1.5 min.) for a titration, no detectable decomposition of the *o*- or *p*-isomers occurred.

Dipole moments were determined[2] in benzene solution at 25°. Results were calculated by the method of Smith (3).

Partition coefficients were obtained by measuring concentrations spectrophotometrically after thorough mixing of the two phases at 25°, following the method of Clarke *et al.* (4). The aqueous phase was McIlvaine's buffer, pH 5.0, and the nonaqueous phases were oleyl alcohol, methyl oleate, and cyclohexane, respectively.

IR spectra were taken[3] at a concentration of about 0.05 *M* so as to preclude self-association. Variable pathlength cells with KBr windows[4] were used in the determinations. Frequencies are estimated to be accurate to $\pm 2\ cm.^{-1}$.

RESULTS AND DISCUSSION

The bactericidal and physicochemical properties of the trifluoromethylphenols are given in Table I.

The bactericidal effects of the isomers, based on a 99.9% kill in 10 min., are in the order: $m > p > o$. This is similar to the findings of Pinney and Walters (2) who showed that the bactericidal effectiveness of the monofluorophenols against *E. coli* was in the order: $m > p = o$.

The bactericidal effects of the trifluoromethylphenols correlate well with their partition coefficients (*P*) between aqueous buffer and *polar* nonaqueous phases (Fig. 1). No such correlation is observed if cyclohexane is used as the nonaqueous phase, because *o*-trifluoromethylphenol has a partition coefficient three times higher than would be expected from Collander's (5) rule relating, for a set of congeners, partition coefficients determined using various nonaqueous phases. A similar observation was made by Pinney and Walters (2) for the monofluorophenols and by Burton *et al.* (1) for a number of substituted phenols. The latter workers attributed the anomalous partition coefficients of the *o*-isomers to their intramolecular hydrogen bonding, and they suggested that this gave rise to low partition coefficients in hydrogen-bonding solvents because of low solute–solvent interaction.

However, it must be remembered that a partition coefficient is a measure of relative, not absolute, solubility. Water is more polar and is a better hydrogen-bonding agent than is oleyl alcohol. Since the

[1] Metrohm E436.
[2] Using a Dipolmeter, type DM01, and a Bellingham and Stanley Abbe "60" refractometer.
[3] Grubb-Parsons Spectromaster.
[4] Research and Industrial Instruments Co., London, England.

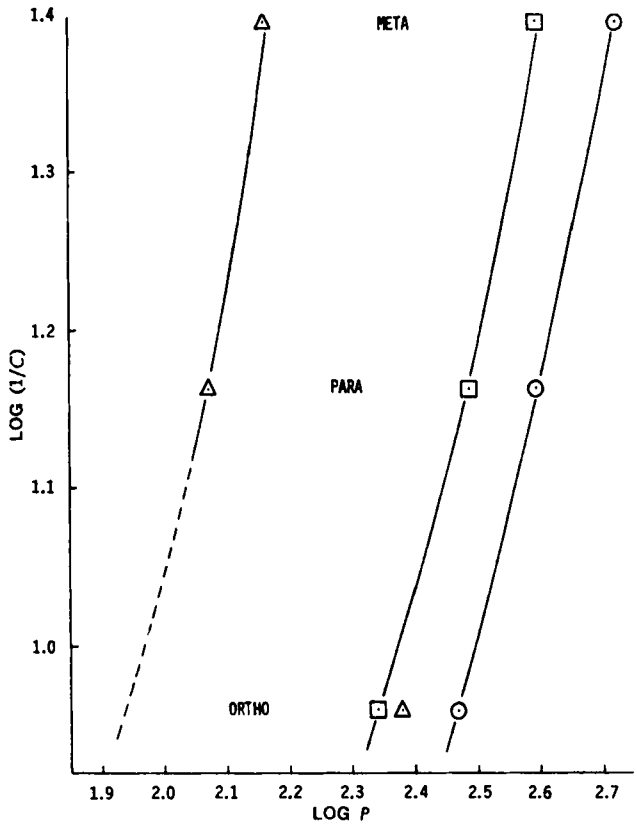

Figure 1—*Correlation between bactericidal effects and partition coefficients of the isomeric α,α,α-trifluoromethylphenols. Partition coefficients are between aqueous buffer, pH 5, and oleyl alcohol* (⊙), *methyl oleate* (⊡), *and cyclohexane* (△). *Partition coefficients in cyclohexane–buffer are shown* × *100.*

effect of intramolecular hydrogen bonding is to reduce polarity and intermolecular hydrogen bonding, an intramolecularly hydrogen-bonded compound should have a higher partition coefficient than its *p*-isomer in oleyl alcohol–buffer.

Nonetheless, the present results and those of Burton *et al.* (1) show that if an *o*-substituted phenol can form an intramolecular hydrogen bond, then it generally has a lower partition coefficient than has its *p*-isomer in oleyl alcohol–buffer. It is suggested that this is largely because the intramolecular hydrogen bond is ruptured in polar solvents, and the compound then distributes itself according to its polarity and/or intermolecular hydrogen-bonding ability, which must be greater than those of the corresponding *p*-isomer. Table I shows that, for the compounds examined here, both the dipole moment[5] and hydrogen-bonding ability (as measured by the shift of the hydroxyl stretching frequency produced by a hydrogen-accepting solvent) are greater for the *o*-isomer than for the *p*-isomer. It thus becomes clear, as emphasized in Fig. 1, that the partition coefficient of an *o*-isomer, capable of intramolecular hydrogen bonding, is anomalous *in inert solvent* and not, as Burton *et al.* (1) suggested, in polar solvent.

A further confirmation of this comes from the isomeric chlorophenols. *o*-Chlorophenol is known to participate in intramolecular hydrogen bonding; yet the solubility of this isomer is greater in water (28.5 g. l.$^{-1}$ at 20°) than is that of the *p*-isomer (27.1 g. l.$^{-1}$ at 20°). It is, therefore, not surprising that Burton *et al.* (1) found the partition coefficient (oleyl alcohol–buffer) and effectiveness against *Pseudomonas aeruginosa* of *o*-chlorophenol to be lower than those of the *p*-isomer.

On the other hand, if an intramolecular hydrogen bond is sufficiently strong to resist rupture in polar solvent, then the *o*-isomer may have the higher biological activity. This is the case for the isomeric nitrophenols. Although partition coefficients are not available, aqueous solubility data (*o*-isomer, 2.1 g. l.$^{-1}$ at 20°; *p*-isomer, 15 g. l.$^{-1}$ at 20°) show that the intramolecular hydrogen bond is not ruptured in water; Fujimoto (6) showed that the *o*-isomer is a more effective bactericide against *Salmonella typhosa*.

If the *o*-isomer cannot form an intramolecular hydrogen bond, then the rank order of partition coefficients should not be affected by choice of nonaqueous phase; Table II shows this to be the case with the isomeric cresols. In all solvent systems, *o*-cresol has the highest partition coefficient; and it is significant that Burton *et al.* (1) reported that this isomer also has the highest bactericidal effectiveness of the three cresols against *P. aeruginosa*.

The pKa values of the isomeric trifluoromethylphenols parallel their partition coefficients, *i.e.*, $m > p > o$. This is probably fortuitous, since the partition coefficients were determined using buffer at pH 5, when all three isomers would be virtually completely undissociated. Furthermore, since the bactericidal activities were also determined at pH 5, one would not expect the observed small difference in pKa to be significant. However, the bactericides act on or in the cell, where the pH is about 7.4. Here the extent of ionization will be significant; and since primarily it is undissociated phenol which acts as bactericide, effectiveness will be a function of pKa. Thus, the effect of pKa in this series of compounds is to reinforce, at the site of action, the effect of partition in the random walk to the site of action.

In conclusion, solvent systems for the determination of *in vitro* parameters of biological activity must be chosen carefully. Rytting *et al.* (7) recently argued, from a thermodynamic standpoint, for the use of hydrocarbon solvents. However, this could lead to erroneous predictions of biological activity, as would have been the case for the present results. From a practical viewpoint, we prefer the use of polar liquids such as oleyl alcohol or octanol.

Table II—Partition Coefficients of the Cresols between Certain Organic Phases and McIlvaine's Buffer, pH 5

Organic Phase	*Meta-*	*Para-*	*Ortho-*
Oleyl alcohol	65.1	66.5	74.4
Methyl oleate	42.2	38.4	57.8
Cyclohexane	0.74	0.77	1.55

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▲ To whom inquiries should be directed.

[5] Strictly speaking, dipole moments should be determined in a hydrogen-bonding solvent in which weak intramolecular hydrogen bonds may be assumed to be ruptured.

Determination of Hydroxy Compounds by 4-Dimethylaminopyridine-Catalyzed Acetylation

KENNETH A. CONNORS[▲] and KENNETH S. ALBERT

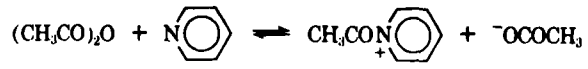
Abstract □ 4-Dimethylaminopyridine is an effective catalyst of analytical acylations by acetic anhydride, having a specific catalytic activity about 10^4 times greater than that of pyridine. The conventional titrimetric determination of hydroxy groups is carried out with 4-dimethylaminopyridine as the catalyst and pyridine as the solvent. Typical reaction times for primary and secondary alcohols are 5–10 min. at 54–70°.

Keyphrases □ *Hydroxy compounds—determination* by 4-dimethylaminopyridine-catalyzed acetylation, compared to pyridine catalysis □ Alcohols—4-dimethylaminopyridine-catalyzed acetylation, titrimetric determination, compared to pyridine catalysis □ 4-Dimethylaminopyridine—as a catalyst of acylations by acetic anhydride, titrimetric determination of hydroxy compounds

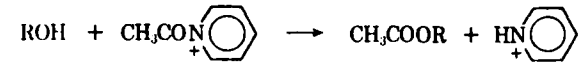
Pyridine-catalyzed acetylation (1) and phthalation (2) are standard methods for the determination of hydroxy compounds and other acylable substances. The mechanism involves nucleophilic catalysis with the intermediate formation of the acylpyridinium ion (3), as shown in Schemes I and II for the reaction of an alcohol with acetic anhydride.

Although pyridine is an effective catalyst in such acylations, typical reaction times are 0.5–1 hr. at reflux temperature. Schenk *et al.* (4) compared several tertiary amines as catalysts in the acetylation of cyclohexanol; they found that triethylenediamine is about 50 times more effective than pyridine. Because of the greater base strength of this saturated amine, it was necessary to modify the classical titrimetric finish (4). Triethylenediamine is also a better catalyst than is pyridine in the acylation of alcohols with the acid chloride of the 2,4-dinitrophenylhydrazone of pyruvic acid (5). Imidazole catalyzes acylations by pyromellitic dianhydride (6). Analytical acylations were thoroughly reviewed by Mehlenbacher (7) and Schenk (8).

Recently, Steglich and Höfle (9) reported that 4-dimethylaminopyridine is superior to pyridine as a catalyst for some synthetic acylations. The present article describes the application of 4-dimethylaminopyridine (I) to the determination of hydroxy compounds by reaction with acetic anhydride.



Scheme I



Scheme II

EXPERIMENTAL

Materials—4-Dimethylaminopyridine[1] was used directly.

Reagents—*Acetic Anhydride Solution*—Ten milliliters of reagent grade acetic anhydride was mixed with 40 ml. of reagent grade pyridine. This reagent should be prepared fresh daily.

Catalyst Solution—Two grams of 4-dimethylaminopyridine was dissolved in enough pyridine to make 100 ml. Each 5-ml. aliquot of this solution contained 100 mg. of I.

Procedure—A 2–3-meq. sample of a hydroxy compound contained in 1.0 ml. of pyridine solution was pipeted into a 50-ml. conical flask. Five milliliters of the catalyst solution, followed by 2.0 ml. of the acetic anhydride solution, was added, and the solution was well mixed. The stoppered flask was maintained at the selected temperature for an appropriate time (see Table I and *Results and Discussion*). Then 25 ml. of water was added to the flask and the contents were brought to room temperature. If the mixture was not homogeneous, 5 ml. of *n*-butyl alcohol was added. Three drops of 1% phenolphthalein solution was added, and the solution was titrated to the pink color with standard 0.5 *N* sodium hydroxide. A blank determination, omitting only the sample, was carried out in exactly the same way.

The milliequivalents of hydroxy compound contained in the sample taken for assay is given by $N(V_b - V_s)$, where V_b and V_s are the volumes (in milliliters) of sodium hydroxide solution of normality N required to titrate the blank and sample, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the time course for acetylation of isopropyl alcohol in a pyridine–acetic anhydride mixture at different concentrations of I. A profound catalysis of this esterification by I clearly occurs. In the development of the analytical procedure, pyridine has been retained as the solvent, although it makes little contribution to the overall catalysis.

An estimate of the relative catalytic efficiencies of I and pyridine (II) was obtained as follows. It is anticipated that the rate for acylation in a I–pyridine–acetic anhydride mixture will be given by Eqs. 1–3:

$$v = (k_{\mathrm{I}}[\mathrm{I}] + k_{\mathrm{II}}[\mathrm{II}])[\mathrm{ROH}][(\mathrm{CH_3CO})_2\mathrm{O}] \quad \text{(Eq. 1)}$$

$$v = (k_{\mathrm{I}}' + k_{\mathrm{II}}')[\mathrm{ROH}][(\mathrm{CH_3CO})_2\mathrm{O}] \quad \text{(Eq. 2)}$$

$$v = k_{\mathrm{obs}}[\mathrm{ROH}][(\mathrm{CH_3CO})_2\mathrm{O}] \quad \text{(Eq. 3)}$$

Table I—Analytical Results of 4-Dimethylaminopyridine-Catalyzed Acetylation of Hydroxy Compounds

Sample Compound	Amount[a] I, mg.	Temperature	Reaction Time, min.	Percent Recovery—Mean[b]	Percent Recovery—*SD*
n-Propyl alcohol	50	54°	20	100.1	0.2
Isopropyl alcohol	50	54°	20	100.1	0.4
Benzyl alcohol	50	54°	20	100.4	0.6
n-Butyl alcohol	100	54°	10	99.0	0.5
Phenol	100	54°	10	101.2	0.3
Cinnamyl alcohol	100	54°	10	98.1	0.4
Isobutyl alcohol	100	70°	5	100.6	0.4
Ethylene glycol[c]	100	70°	5	99.2	0.2
p-Methoxyphenol[d]	100	70°	10	99.4	—

[a] Amount of I in the 8-ml. reaction volume. [b] Mean of three determinations except as noted. [c] Both hydroxy groups acetylated. [d] Two determinations.

[1] Aldrich Chemical Co. A sample of I recrystallized from Skellysolve A gave the same results. It has been found convenient to refer to I as DMAP.

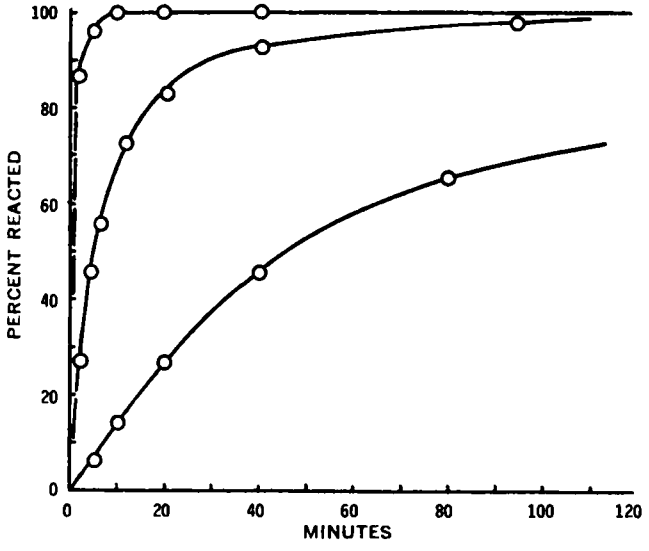
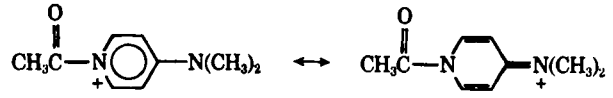


Figure 1—*Time course for acetylation of isopropyl alcohol by acetic anhydride at 54° and varying I concentration. The solvent was acetic anhydride–pyridine (1:4 v/v). Compound I concentrations (slowest to fastest reactions) were: zero, 4.65 × 10⁻³, and 4.48 × 10⁻² M.*

From the titrimetric data, the apparent second-order rate constant, k_{obs}, could be obtained. The plots showed some deviations from strict second-order kinetics, but for the present purpose it was possible to obtain these estimates: for reaction with isopropyl alcohol at 54°, $k_{II} = 3.6 \times 10^{-6}\ M^{-2}$ sec.$^{-1}$ and $k_I = 7 \times 10^{-2}\ M^{-2}$ sec.$^{-1}$. Therefore, I is about 2×10^4 times more effective than is pyridine as a catalyst in this reaction. This rate enhancement may be a consequence of stabilization of the acylpyridinium ion, or rather of the transition state leading to it, as shown in Scheme III.

Table I lists analytical results for the I-catalyzed acetylation of various hydroxy compounds. The accuracy and precision of the method are comparable with those of other acylation procedures. Given a sample compound, the analyst has available for manipulation three experimental variables: I concentration, temperature, and reaction time. Sufficient variation in these quantities is represented in Table I for the analyst to select appropriate conditions to suit his or her requirements. The attempted determination of *tert*-butyl alcohol (100 mg. I, 70°) gave 26% acetylation in a 75-min. reaction time.



Scheme III

A yellow color in the presence of high I concentrations limits the accuracy of the visual end-point detection. In the procedure as described, it appears that 100 mg. of I in the 8-ml. assay volume is about the maximum practical amount. Higher concentrations of I give increasingly erratic results, which are associated with definite consumption of reagent even in the blank solution. Possibly this very powerful catalyst is promoting C-acylation of acetic anhydride in a Perkin-type reaction.

The 4-dimethylaminopyridine-catalyzed method given here appears to be the most reactive of available anhydride acylation methods utilizing nucleophilic catalysis. Extension of 4-dimethylaminopyridine catalysis to other acylating agents, types of samples, and finish methods should be analytically fruitful.

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Anticonvulsant Activity of Substituted Benzoylpyridines

M. P. BREEN*, E. M. BOJANOWSKI*, R. J. CIPOLLE*, W. J. DUNN, III, E. FRANK, and J. E. GEARIEN▲

Abstract □ A series of substituted benzoylpyridines was prepared and evaluated for their ability to prevent convulsions induced by electroshock. Results of regression analysis indicate that electron-releasing substituents enhance anticonvulsant activity in this series. A log P_0 of 2.66 was found, which is near the average value of 2 found for 16 series of hypnotics.

Keyphrases □ Benzoylpyridines, substituted—synthesized as potential anticonvulsants, structure-activity relationships □ Anticonvulsants, potential—synthesis of series of substituted benzoylpyridines, structure-activity correlations □ Structure-activity relationships—series of substituted benzoylpyridines, synthesized as potential anticonvulsants □ Partition coefficients—series of substituted benzoylpyridines, synthesized as potential anticonvulsants

The ability of 4-benzoylpyridine (I) to protect mice from convulsions induced by electroshock prompted the synthesis and study of a series of benzoylpyridines in which the phenyl group was replaced by a variety of alkyl groups (1). While these compounds still possessed anticonvulsant activity, they were less potent than 4-benzoylpyridine (1). In the hope of obtaining more potent compounds, a series of substituted benzoylpyridines was prepared and examined for anticonvulsant activity. The physical constants and analytical data for the compounds from the Grignard reaction are reported in Table I. 4-Nitrobenzoylpyridine was prepared following the procedure of Bryans (2). To gain additional information for structure-activity relationships, the results of the biological evaluation were subjected to regression analysis. For this purpose, the previously reported compound, 4-benzoylpyridine, was included in the analysis (1).

EXPERIMENTAL[1]

Melting points were determined in capillary tubes and are uncorrected. The structures of the compounds were confirmed by their IR and proton magnetic resonance (PMR) spectra. 1-Octanol–water partition coefficients were determined according to published procedures (3) and are the averages of at least three determinations. For some of the compounds, it was necessary that log P be estimated. This was done by taking advantage of the additive-constitutive nature of log P (3). The π values used in the estimations were from the phenoxyacetic acid system (4), with the exception of that for the 4-*tert*-butyl group which was taken from the benzene system (4). The anticonvulsant activities were determined according to Turner (5) and are reported as pC, where C is the concentration (in moles per kilogram) required to protect 50% of the mice from electroshock. Regression analyses were carried out using a nonweighted multiple linear regression program and a computer[2].

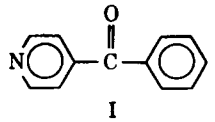
Substituted Benzoylpyridines—To a stirred suspension of 0.10 mole magnesium turnings was added dropwise, with stirring, a solution of 0.10 mole of substituted bromobenzene in 50 ml. of anhydrous ether. It was sometimes necessary to add a crystal of iodine to initiate the reaction. After the addition was completed, the resulting mixture was heated at reflux temperature for 1.5 hr. and then cooled to room temperature. A solution of 5.2 g. (0.05 mole) of 4-cyanopyridine in 100 ml. of anhydrous ether was added dropwise with constant stirring over 1 hr. The reaction mixture was heated at reflux temperature for 24 hr. and then treated with 200 ml. of 10% hydrochloric acid. The aqueous layer was separated, and the ether layer was washed three times with 50-ml. portions of 25% hydrochloric acid.

These washings were added to the aqueous layer, and the combined aqueous phase was made alkaline (pH 8) with 40% sodium hydroxide solution. This alkaline mixture was cooled to room temperature and extracted six times with ether. The ether extracts were combined and dried (sodium sulfate). The amino-ketone was obtained by evaporation of the ether; if solid, it was purified by recrystallization or, if liquid, it was purified by distillation (Table I).

4-Isonicotinylbenzenesulfonamide—To 107.2 g. (0.92 mole) of chlorosulfonic acid, cooled to 10–15°, was added 26.7 g. (0.16 mole) of 4-benzylpyridine in small portions with constant stirring. After the addition was complete, the reaction mixture was allowed to warm to room temperature and was then heated to 70° for 30 min. It was cooled to room temperature and then poured slowly, with stirring, into 600 g. ice in 100 ml. water. The mixture was kept at 0° for 3 hr., during which time a white precipitate formed. This precipitate was filtered, dried, and used without further purification.

The crude product (34.8 g., 0.13 mole) was treated with 75 ml. of ammonium hydroxide solution (28%). After the vigorous exothermic reaction had subsided, the orange mixture was heated over a steam bath for 45 min. At the end of this time, the mixture was cooled to room temperature and 6 *N* hydrochloric acid was added dropwise with constant stirring. At pH ~5, a precipitate formed. The flask was placed in an ice bath, and more hydrochloric acid was added with constant stirring until a pH of 4 was obtained. The mixture was kept at 0° for 2 hr., and the solid precipitate was removed by filtration. Following recrystallization from water–isopropyl alcohol (1:1), 23.2 g. (72%) of a slightly tan solid, m.p. 148–151°, was obtained.

This sulfonamide (4.7 g., 0.019 mole) was treated with potassium permanganate (8.0 g., 0.05 mole) in 200 ml. of water. The reactants were thoroughly mixed and then heated at reflux temperature for 1.5 hr. At this time, an additional 8 g. (0.05 mole) of potassium permanganate was added and heating was continued for 30 min. A final 2-g. (0.012-mole) portion of potassium permanganate was added, and the mixture was heated at reflux temperature for a final 60 min. The mixture was then cooled to room temperature, decolorized with 3–4 drops of alcohol, and filtered. The brown filter cake was digested with 300 ml. of ethanol on a steam bath, cooled to room temperature, and filtered. The alcoholic filtrate was then concentrated under vacuum. A gray crystalline product formed and was recrystallized from ethanol, giving 1.6 g. (32.1%) of white needles, m.p. 185–187°. The initial aqueous filtrate was made neutral with 6 *N* hydrochloric acid and was concentrated to 50 ml. This slightly yellow solution was then placed in an ice bath, and further quantities of 6 *N* hydrochloric acid were added until a precipitate began to appear at pH ~5. The beaker was scratched with a glass rod and placed in the freezer for 60 min. At this time



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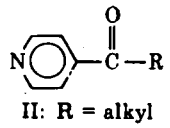
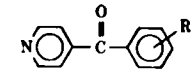


II: R = alkyl

[1] Analyses for carbon, hydrogen, and chlorine were performed by Micro-Tech Laboratories, Skokie, Ill. Analyses for nitrogen were performed with a Coleman automatic nitrogen analyzer.

[2] IBM 370/155.

Table I—4-(Substituted benzoyl) Pyridines



R	Boiling Point or Melting Point	Yield, %	Crystallization Solvent[a]	Formula	Analysis, % Calc.	Found
4-F	81–84°	67	A	$C_{12}H_8FNO$	C 71.64	71.69
					H 3.98	4.09
					N 6.97	7.00
3-CF_3	116–117°[b]	40	A	$C_{13}H_8ClF_3NO$	C 54.26	54.58
					H 3.13	3.41
					N 4.87	4.69
2-OH	76–77°	44	B	$C_{12}H_9NO_2$	C 72.36	72.41
					H 4.52	4.41
					N 7.04	7.02
2-OCH_3·HCl	215–218°[b]	60	A	$C_{13}H_{12}ClNO_2$	C 62.53	62.46
					H 4.81	4.87
					Cl 14.23	14.35
					N 5.61	5.70
4-OCH_3	118–120°[c]	64	A	$C_{13}H_{11}NO_2$	C 73.24	73.36
					H 5.16	5.21
					N 6.57	6.81
4-CH_3	93–95°	51	A	$C_{13}H_{11}NO$	C 79.19	79.05
					H 5.58	5.58
					N 7.11	7.25
2-CH_3	113–115°(0.07)	65		$C_{13}H_{11}NO$	C 79.19	78.92
					H 5.58	5.87
					N 7.11	7.24
2,5-$(CH_3)_2$·HCl	176–178°[b]	54	A	$C_{14}H_{14}ClNO$	C 67.88	67.62
					H 5.66	5.75
					Cl 14.34	14.19
					N 5.66	5.66
4-$(CH_3)_3$-C	62–64°	44	A	$C_{16}H_{17}NO$	C 80.33	80.32
					H 7.11	7.25
					N 5.86	5.97

[a] A = ethanol, and B = 2-propanol. [b] Isolated as the hydrochloride. [c] H. Marxer, *Helv. Chem. Acta*, **52**, 262(1969), reported a melting point of 118–122°.

the mixture was filtered and the solid was air dried for 3 hr. and then recrystallized from ethanol to yield an additional 1.5 g. (total yield 61.7%) of white needles, m.p. 184–187.5°.

Anal.—Calc. for $C_{12}H_{10}N_2O_3S$: C, 54.96; H, 3.82; N, 10.69. Found: C, 54.60; H, 3.51; N, 10.42.

RESULTS AND DISCUSSION

Attempts to correlate the anticonvulsant activity of the substituted benzoylpyridines with various physicochemical parameters are summarized in Eqs. 1–3:

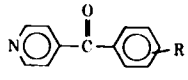
$$pC = 2.84(\pm0.49) + 0.15(\pm0.20)\log P$$
$$n = 13\ s = 0.26\ r = 0.46 \quad \text{(Eq. 1)}$$

$$pC = 2.17(\pm0.79) + 0.80(\pm0.68)\log P - 0.14(\pm0.14)(\log P)^2$$
$$n = 13\ s = 0.22\ r = 0.69 \quad \text{(Eq. 2)}$$

$$pC = 2.37(\pm0.64) - 0.35(\pm0.28)\sigma + 0.77(\pm0.54)\log P - 0.14(\pm0.11)(\log P)^2$$
$$n = 13\ s = 0.18\ r = 0.84\ \log P_0 = 2.66(2.16 \text{ to } 4.22) \quad \text{(Eq. 3)}$$

In these equations, n is the number of compounds used to derive each equation, s is the standard deviation of the regression, and r is the correlation coefficient. The reduction in variance that results from addition of the Hammett σ constant to Eq. 2 is significant at the 95% level of confidence; $F_{1,9} = 7.932$, $F_{1,9,\alpha 0.05} = 5.117$. Although the correlation of Eq. 3 is not exceedingly sharp, the "unexplained" variance is small (0.27) and is probably due mainly to experimental error. The values in parentheses are 95% confidence intervals on the coefficients that they follow. For the derivatives of this study, there is no interrelationship between the independent variables (r^2 for correlation of log P with σ is 0.102). The negative coefficient with the σ term indicates that electron-releasing substituents enhance anticonvulsant activity in the series. The series contains only three compounds with negative σ groups. More derivatives with electron-donating groups, as measured by σ, and lipophilic groups, as measured by π, balanced so as to give a log P near the log P_0 of 2.66 found for this series, should be made. An example is the 4-isopropoxy compound with $\sigma = -0.45$ and log P estimated to be 2.74. Its activity, calculated from Eq. 3, is 3.60. The most interesting parameter determined from this analysis is the log $P_0 = 2.66$. This is very near the average value of 2.00 found for optimum lipophilicity for 16 sets of hypnotics (6). It has also been found that log $P_0 = 2.30$ gives optimum concentration of benzeneboronic acids in

Table II—Anticonvulsant Activity of 4-(Substituted benzoyl) Pyridines



	R	log P	σ[a]	pC Obs.	pC Calc. (Eq. 1)	(ΔpC)
1	4-F	2.13[b]	0.06	3.51	3.34	0.17
2	3-CF_3	3.05[c]	0.47	3.43	3.22	0.21
3	2-OCH_3	1.85[d]	0.12	3.41	3.26	0.15
4	2-CH_3	2.82[e]	0.29	3.41	3.29	0.12
5	H	1.98[d]	0.00	3.39	3.33	0.06
6	4-OCH_3	1.94[d]	−0.27	3.27	3.42	0.15
7	2,5-$(CH_3)_2$	3.33[f]	0.22	3.25	3.25	0.00
8	4-Cl	2.61[d]	0.23	3.24	3.32	0.08
9	4-CH_3	2.51[d]	−0.17	3.19	3.45	0.26
10	4-$(CH_3)_3C$	3.96[g]	−0.20	3.17	3.22	0.05
11	4-NO_2	1.75[d]	0.78	3.07	3.00	0.07
12	2-OH	2.14[d]	1.22	2.70	2.93	0.23
13	4-SO_2NH_2	0.57[d]	0.57	2.56	2.57	0.01
14	4-OH	1.37[d]	−0.37	Inactive	3.29	—

[a] G. B. Barlin and D. D. Perrin, *Quart. Rev.*, **20**, 75(1966). [b] log P = log P $C_6H_5C(=O)C_5H_4N$ + $\pi_{4\text{-}F}$ = 1.98 + 0.15 = 2.13. [c] log P = log P $C_6H_5C(=O)C_5H_4N$ + $\pi_{3\text{-}CF_3}$ = 1.98 + 1.07 = 3.05. [d] Experimental value, this laboratory. [e] log P = log P $C_6H_5C(=O)C_5H_4N$ + $\pi_{2\text{-}CH_3}$ = 1.98 + 0.84 = 2.82. [f] log P = log P $C_6H_5C(=O)C_5H_4N$ + $\pi_{2\text{-}CH_3}$ + $\pi_{5\text{-}CH_3}$ = 1.98 + 0.84 + 0.51 = 3.33. [g] log P = log P $C_6H_5C(=O)C_5H_4N$ + $\pi_{4\text{-}C(CH_3)_3}$ = 1.98 + 1.98 = 3.96.

mouse brain (6), and optimum anesthetic potency to mice (7) of a series of 26 ethers administered in vapor phase was associated with $\log P_0 = 2.35$. Our result is consistent with these observations.

The inactivity of the 4-hydroxy derivative is difficult to explain on the basis of lipophilicity and the electronic nature of the OH group. Its predicted activity according to Eq. 3 is 3.29. Since the compound showed no activity, it must not be accessible to the CNS. This could be due to metabolism but further work would be necessary to substantiate this.

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* Undergraduate research participant.

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Topical Mosquito Repellents VI: Sulfonamides and Quinoline-4-carboxylic Acid Derivatives

F. GUALTIERI*, P. TSAKOTELLIS†, W. SKINNER▲, H. JOHNSON, D. SKIDMORE‡, and H. MAIBACH‡

Abstract □ Certain sulfonamides derived from butanesulfonic acid are repellent to female *Aedes aegypti* mosquitoes. Exploration of the structure–activity relations revealed that the boiling point is the most important factor controlling duration of activity as topical repellents. Quinoline-4-carboxylic acid derivatives were explored for their repellency, and amides were found less active than were esters, primarily due to their low volatility.

Keyphrases □ Mosquito repellents, topical—synthesis, evaluation of butane sulfonamides and quinoline-4-carboxylic acid derivatives □ Repellents, mosquito, topical—synthesis, evaluation of butane sulfonamides and quinoline-4-carboxylic acid derivatives □ Quinoline-4-carboxylic acid derivatives—synthesis, evaluated as topical mosquito repellents □ Sulfonamides, butane—synthesis, evaluated as topical mosquito repellents □ Volatility, quinoline-4-carboxylic acid derivatives—relationship to mosquito repellency

In 1967, Pervomaisky *et al.* (1) reported that certain butane sulfonamides were effective mosquito repellents. The most active of these was reported to be the hexamethyleneimino derivative. This compound and several other analogs were synthesized in the present study for evaluation as topical repellents for *Aedes aegypti* mosquitoes (Table I). The syntheses were straightforward, utilizing *n*-butanesulfonyl chloride in benzene with an excess of the amine. The products were purified by vacuum distillation.

All derivatives were evaluated topically on human subjects as previously described (2). None of the analogs of Compound 1 improved on its activity as a mosquito repellent, although Compounds 3 and 4 had similar activities. As in compounds previously studied as repellents, duration of repellency in a series is a function of the boiling point of the compounds. In this series, maximum duration seems to be achieved with boiling points of 120–130°/0.5 mm. whereas *N,N*-diethyl-*m*-toluamide, which lasts longer, boils at 100°/0.5 mm.

Another interesting lead to mosquito repellents was reported in 1968 (3, 4). These investigators found that certain cinchoninic acid esters were highly repellent. These compounds were evaluated in the present study and several amide analogs were synthesized (Table II). None of the amides exhibited any degree of repellency, probably due to the fact that volatility is reduced too much going from the esters to the amides.

Four of the sulfonamides were evaluated on cloth by the U. S. Department of Agriculture (Table III). The superiority of Compound 1 in these tests over dimethyl phthalate is interesting.

EXPERIMENTAL

n-Butyl Sulfonamides—A solution of *n*-butanesulfonyl chloride (0.03 mole) in anhydrous benzene was slowly added to a solution of the amine (0.06 mole) in anhydrous benzene with cooling and stirring. The solution was then heated to reflux for 1 hr. and, after cooling, the benzene solution was washed with water and dried with sodium sulfate. The oil remaining after evaporation of the solvent was distilled through a short Vigreux column.

Quinoline-4-carboxylic Acid Pyrrolidine Amide—Quinoline-4-carboxylic acid chloride (0.1 mole) (7) was added in small portions to a solution of 0.25 mole of pyrrolidine in anhydrous benzene. After 1 hr. at room temperature, the mixture was heated to reflux for 1 hr. and then cooled, and the benzene solution was washed with water. After drying with sodium sulfate and evaporation of the solvent, the oil was distilled *in vacuo*.

Biological Evaluation: Cloth Tests—Fifty milligrams of the test material is placed in a 2-dram vial to which 0.75 ml. of acetone or other solvent is added. When the chemical is thoroughly dissolved, a 50-cm.^{2} (5 × 10-cm.) piece of muslin bandage is rolled, placed in

mouse brain (6), and optimum anesthetic potency to mice (7) of a series of 26 ethers administered in vapor phase was associated with $\log P_0 = 2.35$. Our result is consistent with these observations.

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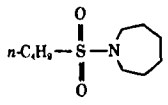
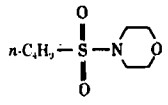
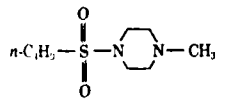
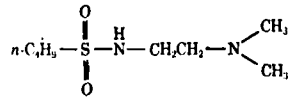
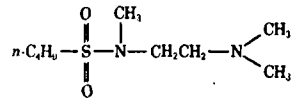
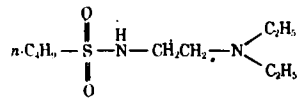
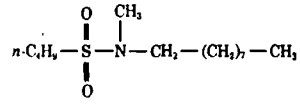
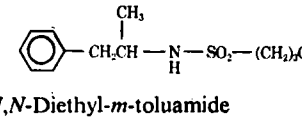
Quinoline-4-carboxylic Acid Pyrrolidine Amide—Quinoline-4-carboxylic acid chloride (0.1 mole) (7) was added in small portions to a solution of 0.25 mole of pyrrolidine in anhydrous benzene. After 1 hr. at room temperature, the mixture was heated to reflux for 1 hr. and then cooled, and the benzene solution was washed with water. After drying with sodium sulfate and evaporation of the solvent, the oil was distilled *in vacuo*.

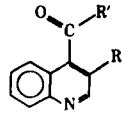
Biological Evaluation: Cloth Tests—Fifty milligrams of the test material is placed in a 2-dram vial to which 0.75 ml. of acetone or other solvent is added. When the chemical is thoroughly dissolved, a 50-cm.^{2} (5 × 10-cm.) piece of muslin bandage is rolled, placed in

Table I—Sulfonamides

Compound Number	Structure	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found	Boiling Point (0.5 mm.)	Repellency, hr. (0.15 mg./cm.²)	Repellency, hr. (0.35 mg./cm.²)
1		—[a]	$C_{10}H_{21}NO_3S$	—[a]	—[a]	130°	1	2
2		67	$C_8H_{17}NO_3S$	C 46.45 H 8.26 N 6.75 S 15.47	46.16 8.19 6.62 15.54	140°	—	1
3		47	$C_9H_{20}N_2O_3S$[b]	C 40.09 H 5.16 N 15.58 S 7.13	40.13 5.02 15.51 7.02	124°	—	2
4		63	$C_8H_{20}N_2O_2S$[b]	C 38.44 H 5.30 N 16.01 S 7.33	38.34 5.26 15.76 7.54	130°	1	—
5		72	$C_9H_{22}N_2O_2S$[b]	C 39.91 H 5.58 N 15.51 S 7.10	40.05 5.52 15.72 7.25	115°	—	1
6		34	$C_{10}H_{24}N_2O_2S$[b]	C 42.58 H 6.09 N 14.61 S 6.69	42.61 6.02 14.49 6.61	147°	—	1
7		63	$C_{14}H_{31}NO_2S$	C 59.23 H 10.81 N 5.28	59.28 11.10 5.32	145°	—	0.25
8		96	$C_{13}H_{31}NO_2S$	C 60.90 H 8.35 N 5.94	61.14 8.29 5.48	170°	—	0.25
9	*N,N*-Diethyl-*m*-toluamide					100°	1–2	3–5

[a] See *Reference 1*. [b] Analyzed as picrate salt.

Table II—Amides and Esters of Quinoline-4-carboxylic Acid



Compound Number	R	R′	Boiling Point (mm.)	Repellency, hr. (0.35 mg./cm.²)
10	H	$N(C_2H_5)_2$	145° (0.2)[a]	0.25
11	H		175° (0.2)[b]	0.25
12	H	OC_2H_5	109° (0.25)[c]	0.25
N,N-Diethyl-*m*-toluamide				2–3
13	CH_3	OC_2H_5	143° (1.0)[d]	6[e]
14	CH_3	O-*n*-C_3H_7	149° (1.0)[d]	5[e]
15	CH_3	O-*n*-C_4H_9	163° (1.0)[d]	6.5[e]
16	C_2H_5	OC_2H_5	155° (1.0)[d]	5.5[e]
N,N-Diethyl-*m*-toluamide			100° (0.5)	10[e]

[a] See *Reference 5*. [b] Synthesized in 87% yield. Calc. for C, H, N: 74.31, 6.24, 12.38. Found: 74.26, 6.47, 12.30. [c] See *Reference 6*. [d] These compounds were obtained from Dr. G. H. Yeoman, Astra-Hewlett, Ltd., Veterinary Research Station, Wheathampstead, Herts, England. [e] Evaluated at 1.75 mg./cm.².

the vial, and mixed with the solution, and the vial is sealed until the chemicals are to be tested. The rate of cloth treatment is 1.0 mg./cm.². After treatment the cloths are kept in the vials in a refrigerator for at least 24 hr.

At the start of a test, vials are removed from the refrigerator and allowed to warm to room temperature. The cloth is then removed from the vial and stapled over a 5 × 9-cm. rectangular opening cut in a 12.7 × 20.3-cm. (5 × 8-in.) file card. The cloth is allowed to dry for 15 min. before testing.

The subject testing the candidate materials covers his arms with a woman's nylon stocking and wears a rubber glove over his hand and wrist to protect against bites. The card and attached cloth patch are then taped over the nylon-covered forearm so that only the treated cloth allows access to the skin by the mosquitoes. The arm is then exposed to a stock cage of *A. aegypti* mosquitoes (approximately 1500) for 1 min. Two or more bites through the treated cloth in 1 min. denotes failure of the chemical. If the chemical does not fail, it is stored at room temperature and retested in 24 hr.

Two standard repellents are tested with each group of candidate material: dimethyl phthalate at the rate of 1.0, 0.5, 0.25, and 0.125 mg./cm.² and diethyltoluamide at 0.25 and 0.125 mg./cm.².

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Table III—Repellent Evaluations on Cloth

Compound	Rate of Application on Cloth, mg./cm.²	Number of Bites Obtained at Indicated Hour 0	24	96	144	192
1	1.0	0	0	0	0	0
2	1.0	30	—	—	—	—
3	1.0	35	—	—	—	—
4	1.0	25	—	—	—	—
Dimethyl phthalate	1.0	0	0	10	—	—
	0.5	0	20	—	—	—
	0.25	2	10	—	—	—
N,N-Diethyl-*m*-toluamide	0.25	0	10	—	—	—
	0.125	0	25	—	—	—

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Comparison of Theoretical Equations for Potential Energy of Electrostatic Repulsion of Colloidal Particles at Constant Surface Charge

N. F. H. HO▲, H. TOGUCHI, and W. I. HIGUCHI

Abstract □ Recently, derived and relatively simple expressions for the energy of electrostatic repulsion between flat plates and spheres at constant surface charge were compared with the more rigorous expressions of Jones and Levine and of Müller. They are quantitative of the electrostatic barrier to the collision of colloidal particles in electrolyte solutions within the limitation of small surface potentials, $\psi_0 < 25$ mv. They are also useful in the general approximation of colloidal stability of most practical systems, despite the limitations on the validity of the expression at higher surface potentials.

Keyphrases □ Colloidal particles, potential energy of electrostatic repulsion, at constant surface charge—comparison and discussion of simple and complex equations □ Surface potential—comparison of equations for the potential energy of electrostatic repulsion of colloidal particles □ Electrostatic barrier—discussion of several equations for determining potential energy of electrostatic repulsion of colloidal particles at constant surface charge

In those dispersed systems in which the primary barrier to flocculation is electrical, the classical theory of the repulsive interaction of overlapping electrical double layers between two particles combined with the attractive interaction due to dispersion forces is used. Moreover, the usual model employed for the repulsive energy requires that the surface potential remains constant during the collision of particles, although the model of constant surface charge, in which case the surface potential increases during the encounter, is applicable in most dispersed systems (1).

Frens *et al.* (2, 3) showed that the collision of silver iodide colloidal particles in aqueous electrolyte solutions was more appropriately explained by the constant surface charge condition. They employed the exact solution of the Poisson–Boltzmann equation in the form of elliptical integrals. Recently, while examining the question of the surface potential or charge remaining constant during the mutual approach of particles, Jones and Levine (4) derived approximate expressions in series form and compared their equations

Table I—Comparison of the Potential Energy of Electrostatic Repulsion Calculated from Various Equations for the Constant Surface Charge Model at Various Interplate Distances

$Z\eta_0$	$\frac{\kappa}{16nkT} V_R$ at $2\kappa d = 1$: Müller (5)	Jones and Levine (4)	Equation 1	$\frac{\kappa}{16nkT} V_R$ at $2\kappa d = 2$: Müller (5)	Jones and Levine (4)	Equation 1
0.5	0.0330	0.0302	0.0364	0.0095	0.0094	0.0098
1.0	0.1108	0.1087	0.1455	0.0347	0.0347	0.0391
2.0	0.3219	0.3213	0.5820	0.1106	0.1099	0.1565
4.0	0.8087	0.8567	2.3280	0.2765	0.2651	0.6261

Table III—Repellent Evaluations on Cloth

Compound	Rate of Application on Cloth, mg./cm.²	Number of Bites Obtained at Indicated Hour 0	24	96	144	192
1	1.0	0	0	0	0	0
2	1.0	30	—	—	—	—
3	1.0	35	—	—	—	—
4	1.0	25	—	—	—	—
Dimethyl phthalate	1.0	0	0	10	—	—
	0.5	0	20	—	—	—
	0.25	2	10	—	—	—
N,N-Diethyl-*m*-toluamide	0.25	0	10	—	—	—
	0.125	0	25	—	—	—

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In those dispersed systems in which the primary barrier to flocculation is electrical, the classical theory of the repulsive interaction of overlapping electrical double layers between two particles combined with the attractive interaction due to dispersion forces is used. Moreover, the usual model employed for the repulsive energy requires that the surface potential remains constant during the collision of particles, although the model of constant surface charge, in which case the surface potential increases during the encounter, is applicable in most dispersed systems (1).

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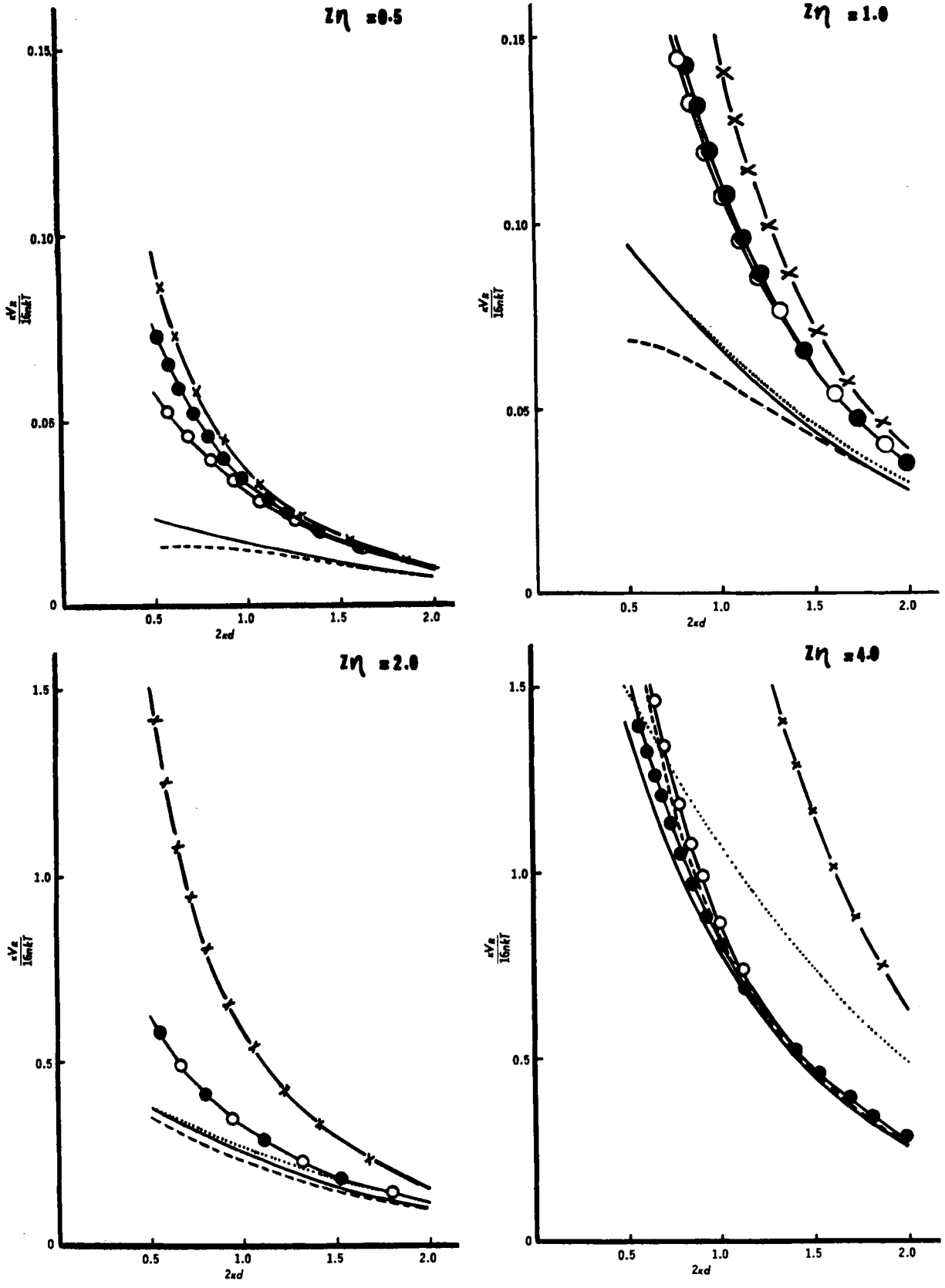

Figure 1—*Potential energy–interplate distance profiles for the constant surface charge and constant surface potential models at various surface conditions of* $Z\eta_0$. *Key:*

	ψ_0 const.	σ_0 const.
Müller (5)		●—●
Jones–Levine (4)	---	○—○
Derjaguin, Landau, Verwey, Overbeek	...	
Eq. 1		×—×

under the conditions of constant plate potential and charge. Likewise, Müller (5) analyzed exact equations expressed in elliptical integral form. In this paper, some rather simple, approximate equations, previously reported (6), for the potential energy of electrostatic repulsion of colloidal particles at constant surface charge are compared with those equations of Jones and Levine and of Müller. Also included is a brief discussion of the equations for the constant potential case.

COMPARISON OF THEORETICAL EQUATIONS

Recently (6), simple expressions for the case of constant surface charge were derived based on the linear approximations of the Poisson–Boltzmann equation, the free energy of the single electrical double layer, and the region of overlapping double layers. For flat plates, the potential energy is:

$$V_R = \frac{2nkTZ^2\eta_0^2}{\kappa}\left[\frac{1 - \tanh \kappa d}{\tanh \kappa d}\right] \qquad \text{(Eq. 1)}$$

σ_0 const. $\qquad (\kappa d \gg 1;\ Z\eta_0 \ll 1)$

and for equal spheres:

$$V_R = \frac{\epsilon a \psi_0^2}{2} \ln\left(\frac{1}{1 - e^{-\kappa H}}\right) \qquad \text{(Eq. 2)}$$

σ_0 const. $\qquad (\kappa a \gg 1;\ \psi_0 \ll 25$ mv.)

where $\eta_0 = e\psi_0/kT$, e is the electronic charge, Z is the valence, ψ_0 is the surface potential, k is the Boltzmann constant, T is the absolute temperature, n is the total number of ions per milliliter, κ is the Debye–Huckel reciprocal length parameter, ϵ is the dielectric constant of the medium, a is the radius, σ_0 is the surface charge, d is the middistance between the plates, and H is the closest distance between the spheres. Equations 1 and 2 are analogous to the classical expressions for the constant surface potential case; *i.e.*, for flat plates:

$$V_R = \frac{2nkTZ^2\eta_0^2}{\kappa}(1 - \tanh \kappa d) \qquad \text{(Eq. 3)}$$

ψ_0 const. $\qquad (\kappa d \gg 1;\ Z\eta_0 \ll 1)$

and for spheres:

$$V_R = \frac{\epsilon a \psi_0^2}{2} \ln(1 + e^{-\kappa H}) \qquad \text{(Eq. 4)}$$

ψ_0 const. $\qquad (\kappa a \gg 1;\ \psi_0 \ll 25$ mv.)

Upon comparing these expressions for flat plates or spheres, one observes that the coefficients of the interparticle distance function are identical. In both the constant surface potential and constant surface charge models, the ψ_0 is the surface potential of a single particle. However, in the later model the increase in the surface potential as two particles approach each other is implicitly accounted for by the distance functions. Furthermore, it is the distance function that characterizes the essential differences of the two models. It is well known that Eqs. 3 and 4 are poorer approximations of the energetics of electrostatic repulsion when $\psi_0 \gtrsim 25$ mv. at room temperature. However, the simplicity of these equations lends itself to rapid qualitative interpretation and semiquantitative estimation of the barrier to the collision of colloidal particles, and herein lies the reason for their wide usage. In this respect, Eqs. 1 and 2 for the constant surface charge model are also useful approximations for most practical systems.

The approach of Jones and Levine (4) is more rigorous. Their expressions are shown here. For flat plates at constant surface potential:

$$V_R = \frac{64nkT}{\kappa} \cdot \tanh^2(Z\eta_0/4) \cdot \exp(-2\kappa d) \cdot [1 - C_{\psi_0} \tanh^2(Z\eta_0/4) \cdot \exp(-2\kappa d)] \qquad \text{(Eq. 5)}$$

ψ_0 const. $(\kappa d > 0.5;\ Z\eta_0 \leq 4)$

and for spheres:

$$V_R = \frac{64\pi nkTa}{\kappa^2} \cdot \tanh^2(Z\eta_0/4) \cdot \exp(-\kappa H) \cdot [1 - \tfrac{1}{2} \cdot (1 + C_{\psi_0}) \cdot \tanh^2(Z\eta_0/4) \cdot \exp(-\kappa H)] \qquad \text{(Eq. 6)}$$

ψ_0 const. $(\kappa a \gg 1;\ Z\eta_0 \leq 4)$

For the flat plates and spheres of the constant surface charge case, the function C_{ψ_0} in Eqs. 5 and 6 is replaced by C_{σ_0} where:

$$C_{\psi_0} = 4\kappa d - 3 + \frac{4\cosh(Z\eta_0/2)}{\sinh^2(Z\eta_0/2)} \qquad \text{(Eq. 7)}$$

$$C_{\sigma_0} = C_{\psi_0} - \frac{8}{\cosh(Z\eta_0/2) \cdot \sinh^2(Z\eta_0/2)} \qquad \text{(Eq. 8)}$$

Figure 1 shows the potential energy–interplate distance profiles at constant surface charge and potential according to Eqs. 1, 3, 5, 7, and 8 at various η_0 values. Müllers' (5) Eqs. 2.8 and 1.8 for the constant ψ_0 case and Eqs. 3.10 and 3.11 for the constant σ_0 case were also used. When comparing the curves according to Eqs. 1 and 3, it is observed that the electrostatic repulsion between particles is always greater for the constant σ_0 condition than that at constant ψ_0. In contrast, the profiles of Jones and Levine (4) and Müller (5) indicate that there are differences in the repulsive energy between the two conditions at small plate potentials; however, when $Z\eta_0 \geq 4$, the two models are essentially indistinguishable in the region of importance to colloidal stability, *i.e.*, $1 \lesssim 2\kappa d \leq 2$. Furthermore, the results of the equations by Jones and Levine are in good agreement with those of Müller at $2\kappa d > 1$. It is readily observed that Eq. 1 is an increasingly poor approximation of the more rigorous expressions at constant σ_0 for $Z\eta_0 > 1$. A comparison of the repulsive energy calculated from the various equations for the constant σ_0 model at various interplate distances is shown in Table I. Although calculations were not made for spherical particles, the discussion of the results would be analogous to those made for flat plates.

CONCLUSION

Relatively simple expressions for the energy of electrostatic repulsion between flat plates (Eq. 1) and spheres (Eq. 2) at constant surface charge have been compared with the more rigorous expressions of Jones and Levine (4) (Eqs. 3 and 4) and of Müller (5). They have utility in the rigorous quantitation of the electrical barrier to the collision of colloidal particles in electrolyte solutions to surface potential conditions of $Z\eta_0 < 1$. As a point of reference, for a 1–1 electrolyte and at room temperature, $\psi_0 = 25$ mv. when $Z\eta_0 = 1$ and $\psi_0 = 50$ mv. when $Z\eta_0 = 2$, *etc.* In the general approximation of colloidal stability, Eqs. 1 and 2 may even be extended to surface conditions of $Z\eta_0 = 2$.

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Interaction of Mechlorethamine and Isophosphamide with Bovine Serum Albumin and Rat Liver Microsomes

LARRY M. ALLEN▲ and PATRICK J. CREAVEN

Abstract □ The interaction of the alkylating metabolites of isophosphamide and mechlorethamine with microsomal protein and bovine serum albumin was studied. The procedure of freezing and thawing of rat liver microsomal fraction greatly increases the inhibition by mechlorethamine of its ethylmorphine demethylase activity. Microsomal metabolites of isophosphamide bind covalently to microsomal protein and bovine serum albumin. Mechlorethamine interacts with thiol and tyrosyl hydroxyl groups of bovine serum albumin. The use of the mechlorethamine–bovine serum albumin interaction as a model system for the study of the reactions of isophosphamide metabolites with protein is proposed.

Keyphrases □ Isophosphamide alkylating metabolites—interaction with microsomal protein and bovine serum albumin, compared to mechlorethamine □ Bovine serum albumin—interaction with isophosphamide alkylating metabolites, compared to mechlorethamine □ Microsomal protein binding—isophosphamide alkylating metabolites □ Mechlorethamine—interaction with microsomal protein and bovine serum albumin, model for isophosphamide interaction

Isophosphamide [3-(2-chloroethyl)-2-(2-chloroethyl)-aminotetrahydro-1,3,2-oxazaphosphorine-2-oxide][1] is an antineoplastic analog of cyclophosphamide currently undergoing clinical trial. It was previously reported (1, 2) that isophosphamide is activated by rat liver microsomes to compounds with alkylating activity. These compounds could react with microsomal protein and so affect the metabolism of isophosphamide itself or of concurrently administered drugs. Indirect evidence for such an interaction is the finding of Sladek (3) that the microsomal metabolism of cyclophosphamide requires fresh microsomes; the process of freezing and thawing even once destroys most of the activity, whereas most other microsomal enzyme activities are relatively resistant to this procedure (4). Similar observations with isophosphamide (2) have been made, and it was postulated that freezing and thawing of microsomes could expose sensitive thiol groups which would be susceptible to alkylation by metabolites of isophosphamide. Therefore, the interaction between the metabolites of isophosphamide and both microsomal protein and bovine serum albumin was examined. As a model of this interaction, the reaction of an alkylating agent of known structure, mechlorethamine, and bovine serum albumin was studied.

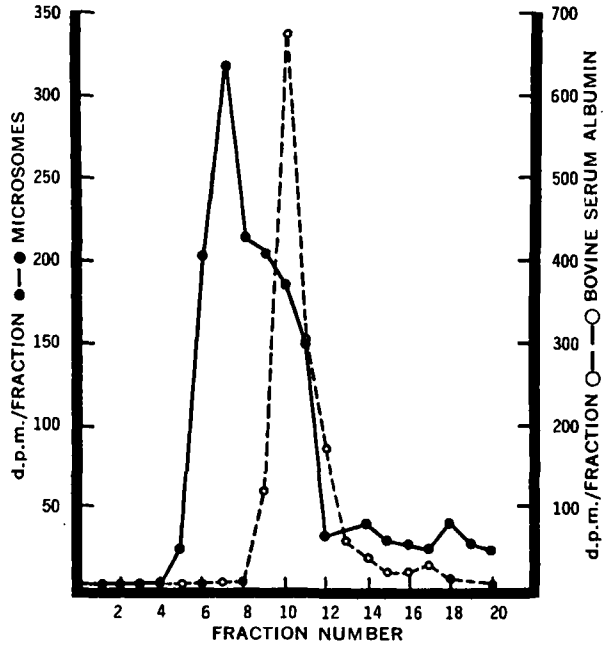

Figure 1—*Gel filtration of ^{14}C-isophosphamide-labeled bovine serum albumin (○) and rat liver microsomes (●). Bovine serum albumin and rat liver microsomes were labeled with ^{14}C-isophosphamide as described in the text and were gel filtered through Biogel P-30 in 0.05 M phosphate buffer, pH 7.4; 1-ml. fractions were collected and counted. ^{14}C-Isophosphamide had an elution volume of 40 ml.*

MATERIALS AND METHODS

Isophosphamide[2], (U-chloroethyl-^{14}C)-isophosphamide[2], crystalline bovine serum albumin[3], mechlorethamine[2], and dithiobisnitrobenzoic acid[3] were used. ^{14}C-Isophosphamide (190 μc./μmole), 3.8 m*M* final concentration, was incubated for 15 min. with a rat liver microsomal suspension (3 ml.) containing microsomes (≡ 250 mg. wet weight liver) (2) in a dialysis bag suspended in 0.5% bovine serum albumin (10 ml.). After incubation the microsomal protein and bovine serum albumin were precipitated with 28% perchloric acid, washed, resuspended, dialyzed against water (10 l.), and subjected to gel filtration on Biogel P-30 to remove noncovalently

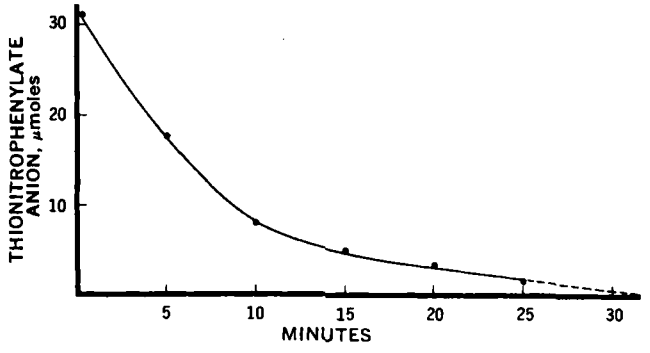

Figure 2—*Inhibition of the thiol group of bovine serum albumin by mechlorethamine. Bovine serum albumin was treated with mechlorethamine and assayed for free sulfhydryl groups with dithiobisnitrobenzoic acid as described in the text, and the decrease in thionitrophenylate anion with the incubation time of mechlorethamine was plotted.*

[1] NSC-109724; Z-4942, Asta Werke.

[2] Drug Research and Development, National Cancer Institute, Bethesda, MD 20014

[3] Sigma Chemical Co.

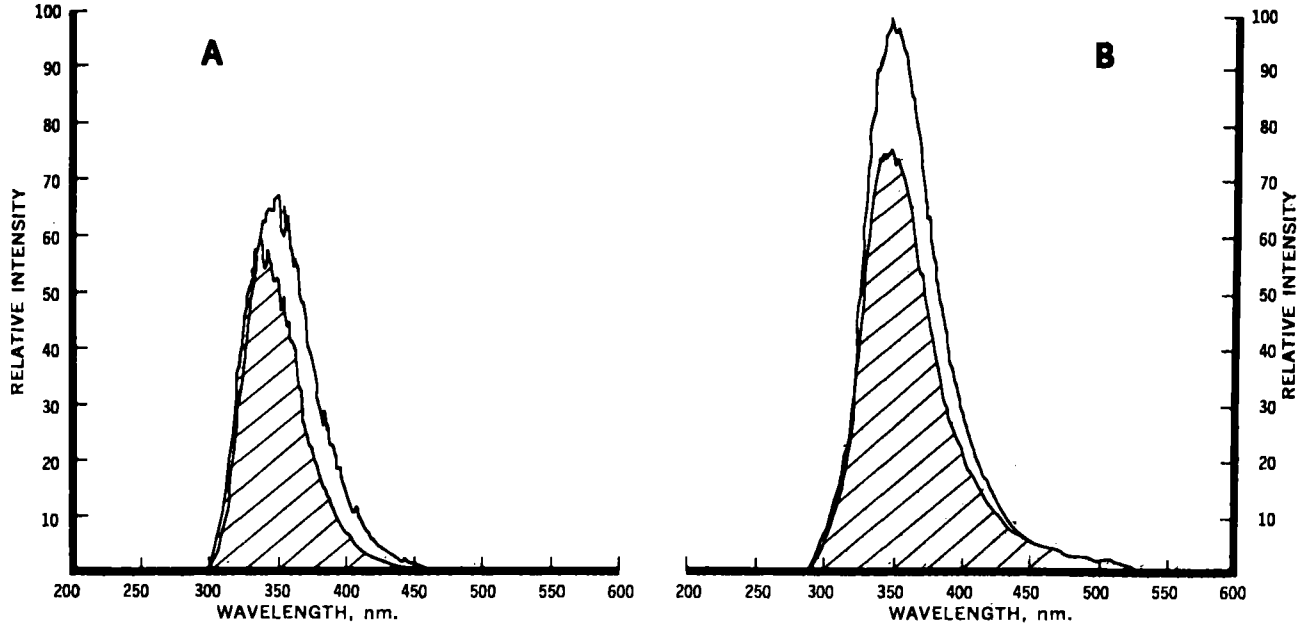

Figure 3—*Fluorescence spectral analysis of bovine serum albumin interaction with mechlorethamine. (A), Fluorescence emission at 287-nm. excitation of bovine serum albumin before (unshaded) and 15 min. after (shaded) addition of mechlorethamine (10.7 μmoles). (B), Fluorescence emission at 305-nm. excitation of bovine serum albumin before (unshaded) and 15 min. after (shaded) addition of mechlorethamine (10.7 μmoles). The slit width was 2.0 mm., sensitivity was 0.001, and photomultiplier was 0.5.*

bound isophosphamide (elution volume 40 ml.). Fractions were counted by liquid scintillation spectroscopy.

The interaction of mechlorethamine with thiol groups and the tyrosine and tryptophan residues of bovine serum albumin was studied by the use of dithiobisnitrobenzoic acid (5) and fluorometry, respectively. Bovine serum albumin (10 mg. in 1.5 ml. of Sorenson's buffer, 0.1 *M*, pH 7.4) was incubated with mechlorethamine (10.7 μmoles) at 22°. After incubation periods of 0–30 min., dithiobisnitrobenzoic acid (2 mg. in 0.5 ml. of water) was added and the concentration of thiophenylate anion was determined at 412 nm. using an absorptivity of $a = 1360.0$ (5). Fluorescence spectral analysis of bovine serum albumin (0.22 mg./ml. in 0.067 *M* phosphate buffer, pH 7.4) was performed on a spectrophotofluorometer[4] at λ_{exc} 287 and λ_{fl} 310 nm. in the presence and absence of mechlorethamine (10.7 μmoles, final concentration).

Ethylmorphine demethylase activity of microsomes was estimated according to Davies *et al.* (6).

RESULTS

The freezing and thawing procedure of a microsomal preparation greatly increases the inhibition of ethylmorphine demethylase activity by mechlorethamine (Table I). Incubation of isophosphamide with rat liver microsomes leads to covalent binding of isophosphamide metabolites to both microsomal protein and bovine serum albumin (Fig. 1).

A model reaction between mechlorethamine and bovine serum albumin leads to the complete loss of available thiol groups as measured by the dithiobisnitrobenzoic reaction (Fig. 2). Fluorometric determinations indicate no reaction with tryptophan residues [measured by fluorescence at λ_{exc} 305 nm. (6)] but there is a transient decrease in fluorescence intensity at λ_{exc} 287 nm. followed by a 12-nm. shift in λ_{fl} (Fig. 3), indicating an interaction with tyrosine residues (7).

DISCUSSION

The reaction between alkylating agents and nucleophilic centers of proteins is well known (8). Isophosphamide is metabolized into alkylating metabolites by liver microsomes (1, 2), the production of which occurs in intimate contact with the active sites of microsomal protein and could affect the activity of these enzymes, especially as it is known that microsomal oxidations are inhibited by sulfhydryl reagents (9).

The fact that freezing microsomes greatly reduces their ability to metabolize cyclophosphamide and isophosphamide suggested indirectly that such a reaction could be taking place and that freezing, by exposing essential sulfhydryl groups, was accentuating this effect. The present findings that mechlorethamine inhibits microsomal demethylase activity and that this inhibition is much greater after freezing and thawing the microsomal preparation are in agreement with this concept. The results also show that a reaction does take place between microsomal metabolites of isophosphamide and microsomal protein.

Microsomal protein is heterogeneous and particulate, and the metabolites of isophosphamide are still undefined; consequently, a model for their interaction, consisting of a known alkylating agent and a protein with defined spectral qualities (7) and containing a single thiol group (9), was studied. Interactions between mechlorethamine and both thiol and phenolic residues of bovine serum albumin were demonstrated. A reaction between bovine serum albumin and isophosphamide metabolites was also demonstrated, and it seems to be a convenient, simple model with which to study the interaction between isophosphamide and proteins.

Table I—Effect of Mechlorethamine Pretreatment on Ethylmorphine Demethylase Activity of Rat Liver Microsomes[a]

Mechlorethamine, m*M* Final Concentration	Ethylmorphine Demethylase Activity, μmoles/g. Liver/hr. Fresh Microsomes	Inhibition, %	Frozen Microsomes	Inhibition, %
0	2.44 ± 0.03	—	2.08 ± 0.00	—
0.5	2.09 ± 0.03	14.3	1.23 ± 0.04	40.9
5.0	0.73 ± 0.03	70.1	0.00 ± 0.00	100

[a] Microsomes prepared as previously described (2) were pretreated with mechlorethamine for 30 min. at 22°. Cofactor mixture (3) and substrate were added and the mixture was incubated for 15 min. at 37° in air with shaking. Each value represents the mean of determinations carried out on three sets of livers, each set being the pooled liver homogenates from two rats.

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Biosynthesis of Deuterated Benzylpenicillins III: Relative Antibiotic Potency of Highly Deuterated Benzylpenicillin

BRUCE C. CARLSTEDT*, HENRY L. CRESPI, MARTIN I. BLAKE▲, and JOSEPH J. KATZ

Abstract □ The relative antibiotic potency of a highly deuterated benzylpenicillin and ^{1}H-benzylpenicillin was compared using the official cup-plate bioassay, with *Sarcina lutea* as the test organism. A relative potency (H/D) of 1.23 was obtained. Although the penicillin analogs act by the same mechanism, deuterium apparently affects the potency in an adverse way.

Keyphrases □ Benzylpenicillin, highly deuterated—potency compared to nondeuterated benzylpenicillin, cup-plate bioassay, *Sarcina lutea* □ Penicillin analogs—effect of high deuteration on potency of benzylpenicillin □ Antibiotics—potency of highly deuterated benzylpenicillin, compared to ^{1}H-benzylpenicillin □ Deuteration—effect on potency of benzylpenicillin

The participation of solvent deuterium oxide in the biosynthesis of benzylpenicillin (1) and the isolation and characterization of a highly deuterated benzylpenicillin have been reported (2). Proton magnetic resonance spectra revealed an average replacement of 89% of the protons (^{1}H) with deuterium (^{2}H) atoms. Complete replacement by ^{2}H is observed in the phenylacetyl group, the C-3 position of the thiazolidine ring, and the C-6 position of the β-lactam ring. Partial substitution is noted in the C-5 position of the β-lactam ring (64%) and in the methyl groups (77%) at the C-2 position of the thiazolidine ring.

In the present study, the relative antibiotic potency of this highly deuterated benzylpenicillin and ^{1}H-benzylpenicillin is compared.

EXPERIMENTAL

Highly Deuterated Benzylpenicillin—Isolation, identification, and characterization of a highly deuterated benzylpenicillin was described earlier (2).

Assay—USP XVII (3) describes a relative potency assay which is useful for determining benzylpenicillin activity as compared to a standard. The cup-plate bioassay, involving *Sarcina lutea* (ATCC 9341) as the test organism (1), was used for this study. Penicillin concentrations are expressed in moles rather than in units per milliliter or milligrams per milliliter. Expression of the concentrations on a weight basis would introduce a factor involving the difference in molecular weights and would influence the slope in a dose–response relationship (4).

RESULTS AND DISCUSSION

Figure 1 shows the regression lines calculated for highly deuterated potassium benzylpenicillin (D) and potassium ^{1}H-benzylpenicillin (H) when the results of the assay were represented as inhibition zone diameters (millimeters) on the *Y*-axis (random variable) and the antibiotic concentration was expressed as log moles × 10^{11} on the *X*-axis. The statistical methods used by Laskar and Mrtek (4) for their comparisons were utilized here, and a summary of the results is presented in Table I. There was an observed difference in the regression coefficients for the two penicillins, and it was necessary to test the significance. The null hypothesis stated that there was no difference between the slopes of the lines. The results of the slope test were that $t = 0.1625$ (76 *df*) (not significant), the null hypothesis was accepted, and the parallelism of the slopes was retained. A further test on the regression lines must establish the fact that, although the lines were parallel, intercepts for the two lines were not identical. This test arises from the fact that the intercepts may describe the same locus, and the null hypothesis then was that both regression lines describe the same locus. Application of an identity test reveals that $t = 12.90$ (77 *df*) ($p < 0.001$) and the null hypothesis can be rejected, indicating that the lines are not identical.

Horizontal displacement may be used to give the relative potency of the ^{1}H- and highly deuterated benzylpenicillins. This calculation revealed a relative potency (H/D) of 1.23 (95% confidence interval, 1.20–1.26) and indicated that, although the analogs presumably work by the same mechanism of action, deuterium in the molecule affected the potency adversely. Laskar and Mrtek (4) found a relative potency of 1.25 (H/D) for deuteriobenzyl-d_7-penicillin; but a comparison, although tempting, between the results of these workers and the present study would be only cursory since two methods of assay (turbidimetric and cup plate, respectively), two organisms (*Staphylococcus aureus* and *S. lutea*, respectively), and two salts (*N*-ethylpiperidine and potassium, respectively) were used.

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Biosynthesis of Deuterated Benzylpenicillins III: Relative Antibiotic Potency of Highly Deuterated Benzylpenicillin

BRUCE C. CARLSTEDT*, HENRY L. CRESPI, MARTIN I. BLAKE▲, and JOSEPH J. KATZ

Abstract □ The relative antibiotic potency of a highly deuterated benzylpenicillin and ^{1}H-benzylpenicillin was compared using the official cup-plate bioassay, with *Sarcina lutea* as the test organism. A relative potency (H/D) of 1.23 was obtained. Although the penicillin analogs act by the same mechanism, deuterium apparently affects the potency in an adverse way.

Keyphrases □ Benzylpenicillin, highly deuterated—potency compared to nondeuterated benzylpenicillin, cup-plate bioassay, *Sarcina lutea* □ Penicillin analogs—effect of high deuteration on potency of benzylpenicillin □ Antibiotics—potency of highly deuterated benzylpenicillin, compared to ^{1}H-benzylpenicillin □ Deuteration—effect on potency of benzylpenicillin

The participation of solvent deuterium oxide in the biosynthesis of benzylpenicillin (1) and the isolation and characterization of a highly deuterated benzylpenicillin have been reported (2). Proton magnetic resonance spectra revealed an average replacement of 89% of the protons (^{1}H) with deuterium (^{2}H) atoms. Complete replacement by ^{2}H is observed in the phenylacetyl group, the C-3 position of the thiazolidine ring, and the C-6 position of the β-lactam ring. Partial substitution is noted in the C-5 position of the β-lactam ring (64%) and in the methyl groups (77%) at the C-2 position of the thiazolidine ring.

In the present study, the relative antibiotic potency of this highly deuterated benzylpenicillin and ^{1}H-benzylpenicillin is compared.

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RESULTS AND DISCUSSION

Figure 1 shows the regression lines calculated for highly deuterated potassium benzylpenicillin (D) and potassium ^{1}H-benzylpenicillin (H) when the results of the assay were represented as inhibition zone diameters (millimeters) on the *Y*-axis (random variable) and the antibiotic concentration was expressed as log moles × 10^{11} on the *X*-axis. The statistical methods used by Laskar and Mrtek (4) for their comparisons were utilized here, and a summary of the results is presented in Table I. There was an observed difference in the regression coefficients for the two penicillins, and it was necessary to test the significance. The null hypothesis stated that there was no difference between the slopes of the lines. The results of the slope test were that $t = 0.1625$ (76 *df*) (not significant), the null hypothesis was accepted, and the parallelism of the slopes was retained. A further test on the regression lines must establish the fact that, although the lines were parallel, intercepts for the two lines were not identical. This test arises from the fact that the intercepts may describe the same locus, and the null hypothesis then was that both regression lines describe the same locus. Application of an identity test reveals that $t = 12.90$ (77 *df*) ($p < 0.001$) and the null hypothesis can be rejected, indicating that the lines are not identical.

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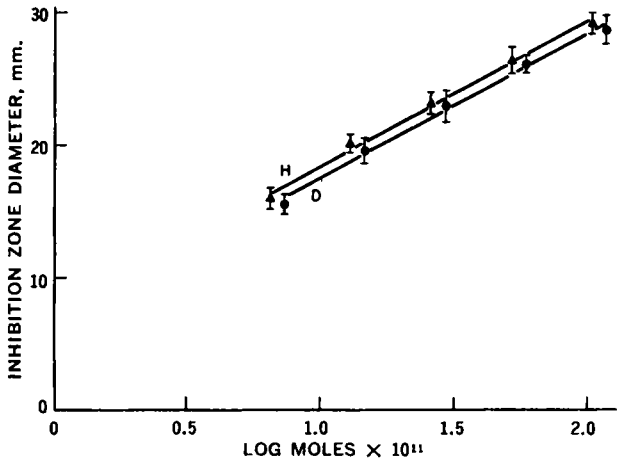

Figure 1—*Regression lines calculated for potassium ^{1}H-benzylpenicillin (H) and highly deuterated potassium benzylpenicillin (D).*

the protio analog, but no difference in activity was found between partially deuterated and ^{1}H-griseofulvins. Laskar and Mrtek (4) synthesized deuteriobenzyl-d_7-penicillin and compared the biological activity of the *N*-ethylpiperidine salts of the protio- and deuterio-analogs; an H/D ratio of 1.25, indicating a decrease in potency of the deuterio-analog, was observed. Kutter and Machleidt (7) prepared α-deuteriochloramphenicol and compared relative potencies against *Escherichia coli*. A decreased activity of the deuterium-substituted antibiotic was reported.

Penicillin acts by inhibiting cell wall formation, but the exact mechanism of this inhibition is not well understood. Although Collins and Richmond (8) showed a similarity between *N*-acetylmuramic acid and penicillin, Wise and Park (9) concluded that, since penicillin did not prevent formation of a polysaccharide backbone in a staphylococcus *in vitro* system, the *N*-acetylmuramic acid component was not involved and that penicillin probably acted at the cross-linking level in mucopeptide synthesis. They showed that the penicillin molecule fits the active site of a hypothetical transpeptidase. The β-lactam ring of the penicillin molecule presumably reacts specifically with the enzyme, and inactivation of the enzyme results. Tipper and Strominger (10) further postulated that penicillin has a configuration like that which is normally at the end of the acetylmuramylpentapeptide fragment. When the penicillin is fixed to the substrate binding site of the transpeptidase, the β-lactam ring opens and a penicilloyl enzyme, which is inactive, is formed. Penicillin has been shown (11) to be irreversibly bound to a "penicillin-binding component" of bacterial cell walls, and this component is presumably the enzyme transpeptidase.

Elison *et al.* (12) reported that the substitution of ^{2}H for ^{1}H in the N—CH_3 of morphine resulted in a weaker binding of the deuterio-analog to the *N*-demethylating enzyme. Hattori *et al.* (13) showed that the introduction of ^{2}H in nonexchangeable ^{1}H positions of protein phycocyanin appeared to decrease nonpolar side-chain interactions. Fisher and Jardetzky (14) and Katz and Crespi (5) observed that the phenyl group was the portion of the benzylpenicillin molecule involved in binding to serum protein albumin. If the phenyl group assists in the binding of benzylpenicillin to the enzyme, a decrease in the binding strength of highly deuterated benzylpenicillin when compared to ^{1}H-benzylpenicillin would be expected. The acylation of the highly deuterated benzylpenicillin to the enzyme would be slowed, resulting in a decreased potency of the highly deuterated benzylpenicillin. This result, taken with the result of Laskar and Mrtek (4), indicates that deuteration of any site of penicillin has only a small effect on the biological potency.

Table I—Calculation Results for Regression Analysis of ^{1}H- and Highly Deuterated Benzylpenicillin

	Protio	Highly Deuterated
N	40	40
Σy^2	1279.519	1141.624
Σx^2	7.251	7.246
b_{yx} (regression coefficient)	10.838	10.875
F_{ratio} (nonzero slope)	70.28 ($p < 0.001$)	107.36 ($p < 0.001$)
$\bar{X}$ (log moles × 10^{11})	1.417	1.473
$\bar{Y}$ (mm.)	22.96	22.59
$\hat{Y}_{byx}$ (regression line)	$10.838X + 7.60$	$10.875X + 6.57$
$SS_{dev.\ from\ regression}$	3.99	4.702

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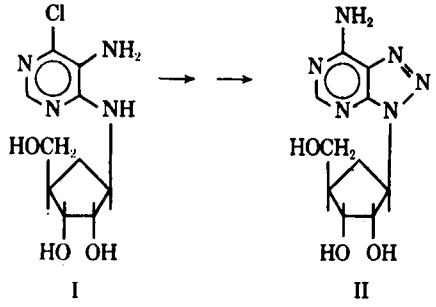
Carbocyclic Analog of Purine Ribonucleosides with Antileukemic Activity

Keyphrases ☐ Carbocyclic analogs of purine nucleosides (8-azaadenosine analog)—synthesis, antileukemic activity ☐ *v*-Triazolo[4,5-*d*]pyrimidines—synthesis of 8-azaadenosine analog of purine nucleoside, antileukemic activity ☐ Antileukemic activity—synthesis of a *v*-triazolo[4,5-*d*]pyrimidine (carbocyclic analog of purine nucleoside) ☐ Purine ribonucleosides—synthesis of carbocyclic analog with antileukemic activity

Sir:

Carbocyclic analogs of several naturally occurring and biologically active purine nucleosides have been synthesized (1–4). In these analogs a methylene group occupies the position of the ring oxygen atom of the ribo- or deoxyribofuranosides. Because of the presence of a stable carbon–nitrogen bond at position 9 of the purine moiety, these analogs are not subject to cleavage by enzymes that remove or transfer the ribofuranosyl moiety of the nucleosides or nucleotides. Biochemical studies (5–7) have shown that the racemic analog (III) of adenosine can serve either as a substrate for or as an inhibitor of some of the enzymes involved in purine nucleotide metabolism, one of the more interesting findings being the inhibition of guanylic acid kinase by the phosphate (7). The antibiotic aristeromycin (8), the optically active form (9, 10) of the adenosine analog, inhibits certain plant pathogens (8, 11). Several racemic carbocyclic analogs of purine nucleosides are cytotoxic to neoplastic cells growing in culture (4). In this communication, antileukemic activity *in vivo* by a carbocyclic analog, the 8-azaadenosine analog (II), is reported.

The 8-azaadenosine analog II, (±)-*trans*-3-(7-amino-3*H*-*v*-triazolo[4,5-*d*]pyrimidin-3-yl)-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol, was synthesized from the diaminopyrimidine I (Scheme I) (1, 2). The pyrimidine was diazotized with sodium nitrite in hydrochloric acid. Since 6-chloro-8-azapurines are known to be highly reactive, the 6-chloro-8-azapurine formed by this reaction was not isolated. The reaction mixture was neutralized and then lyophilized, and the residue was treated with anhydrous ammonia. The yield of II after recrystallization from water was 76%, m.p. 276–278° dec.; UV_{max}: 264 (ε 12,400) and 275 (sh) in 0.1 *N* HCl, 279 (ε 12,400) in phosphate buffer (pH 7), and 279 nm. (ε 12,300) in 0.1 *N* NaOH.



Scheme I

Anal.—Calc. for $C_{10}H_{14}N_6O_3$: C, 45.11; H, 5.30; N, 31.57. Found: C, 44.90; H, 5.46; N, 31.39.

In tests of II against leukemia P-388[1] in mice, the median survival time of treated animals was prolonged by 40–70%. Thus, at doses of 100, 75, 67, and 37 mg./kg./day given intraperitoneally, q.d. 1–9, the observed median increases in lifespan (ILS) were 68, 53, 63, and 42%, respectively. Higher doses produced evidence of toxicity; in two tests at 150 mg./kg./day and in one at 225 mg./kg./day, ILS were 73, 18, and 36%, respectively, but the average differences in weight change between treated and untreated animals were −2.5, −3.4, and −5.1 g., respectively. In comparison, the carbocyclic analog (III) of adenosine was not active against P-388 leukemia in a dose–response test at 50 (toxic), 25 (toxic), 12.5, and 6.25 mg./kg./day performed simultaneously with the dose–response test of II (at 225, 150, 100, and 67 mg./kg./day).

In tests *versus* leukemia L-1210, Compound II displayed, at best, borderline activity. Average ILS were 18–22% after doses of 150, 100, 67, or 33 mg./kg./day given intraperitoneally, q.d. 1–9. Average ILS were 23 and 26% after doses of 200 and 100 mg./kg., respectively, given on Days 1, 5, and 9; but in a second test at 150 and 100 mg./kg., ILS were 11 and 14%, respectively.

Both II and the carbocyclic analog of adenosine (III) are highly cytotoxic to human epidermoid carcinoma cells, No. 2, in culture. The concentration of II producing 50% inhibition of growth (ED_{50}), determined by protein measurements, was about 0.3 mcg./ml. The cytotoxicity of III is essentially the same; values of ED_{50} determined by this method and by the clone-colony method were about 0.7 (4) and 0.2 mcg./ml. (6), respectively. The fact that II is tolerated *in vivo* at higher doses than III suggests that the observed activity *in vivo* may be due to greater selectivity in the action of II.

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Failure of USP Tablet Disintegration Test to Predict Performance in Man

Keyphrases ☐ Aminosalicylic acid enteric-coated tablets—results of compendial disintegration testing compared to plasma levels in man ☐ Plasma levels, aminosalicylic acid and metabolites—after administration of enteric-coated tablets, compared to dissolution data ☐ Tablet disintegration, compendial method—results for enteric-coated aminosalicylic acid tablets compared to plasma levels in man ☐ Excretion, fecal—enteric-coated aminosalicylic acid tablets, man

Sir:

An enteric-coated tablet of aminosalicylic acid[1], purchased on the open market in the United States by the Food and Drug Administration (FDA), was shown to pass all USP specifications in the FDA laboratories. In a four-way crossover study in eight normal adult volunteers, single oral doses of either 1 g. of aminosalicylic acid or the equivalent of 1 g. of the acid were administered as the enteric-coated tablet[1], a compressed tablet of aminosalicylic acid, a suspension of aminosalicylic acid in water, and a solution of the sodium aminosalicylate in water.

Blood samples were taken at 0, 0.33, 0.67, 1, 2, 3, 4, 6, 8, 12, and 24 hr. postadministration of each dose. The plasma samples, derived from the blood, were assayed for both aminosalicylic acid and its metabolite, *N*-acetyl-*p*-aminosalicylic acid, by a new specific analytical method (1). Plasma samples of all eight subjects at each sampling time assayed "zero" for both drug and metabolite following oral administration of the enteric-coated tablet. The assay sensitivity level was 0.5 mcg./ml. The average peak plasma levels of unchanged drug were 43.5, 16.7, and 8.86 mcg./ml. following oral administration of the solution, the suspension, and the compressed tablet, respectively. Drug levels were measurable over 6–8 hr. following the latter three dosage forms. The average peak plasma levels of the metabolite were 11.6, 12.3, and 10.9 mcg./ml. following the solution, the suspension, and the compressed tablet, respectively. Metabolite levels were measurable over 6–8 hr. following these dosage forms. Detailed results of this study will be published.

Since we did not know about the zero plasma levels of unchanged drug and metabolite in every subject following the enteric-coated tablet until all assays were completed, we never thought to ask the subjects to check their stools for intact tablets or large fragments of tablets. After the results were known, however, one of the eight subjects volunteered to take two more of the enteric-coated tablets. One tablet was excreted in his feces essentially intact (but with a small "hole" in one face) about 30 hr. postingestion. The other tablet did break up, but large pieces were excreted in the same feces. Figure 1 is a photograph showing the original intact tablet as it appeared before ingestion and the essentially intact tablet and the large fragments of the other tablet that were isolated from the feces. After photographing, the drug content of the material isolated from the feces was determined. The essentially intact tablet isolated from the feces assayed 489 mg. (98% of labeled dose), and the fragments[2] of the other tablet assayed 240 mg. (48% of labeled dose).

It is obvious that an enteric-coated tablet that gave zero plasma levels of bioactive aminosalicylate and that was excreted in the feces would be clinically ineffective. It would be unethical to perform a clinical study in patients with tuberculosis to prove such a point. The results obtained are attributable to both poor disintegration of the tablets and slow dissolution of drug from the fragments of the tablets once they did disintegrate or the coating ruptured. This was readily

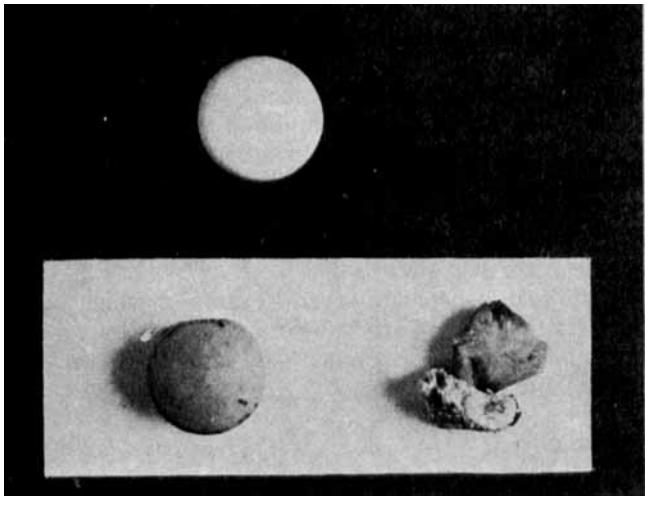

Figure 1—*(Top) Enteric-coated tablet before ingestion. (Bottom) Enteric-coated tablets excreted in the feces.*

[1] 4-Aminosalicylic acid; Parasal enteric-coated 0.5-g. tablet (Lot No. 722165B), containing 0.5 g. aminosalicylic acid USP (Panray Division of Ormont Drug and Chemical). The other dosage forms had the same trademark and were made by the same manufacturer.

[2] Only some fragments were recovered. Since some remained in the feces, the assay is lower than it should be.

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Sir:

An enteric-coated tablet of aminosalicylic acid[1], purchased on the open market in the United States by the Food and Drug Administration (FDA), was shown to pass all USP specifications in the FDA laboratories. In a four-way crossover study in eight normal adult volunteers, single oral doses of either 1 g. of aminosalicylic acid or the equivalent of 1 g. of the acid were administered as the enteric-coated tablet[1], a compressed tablet of aminosalicylic acid, a suspension of aminosalicylic acid in water, and a solution of the sodium aminosalicylate in water.

Blood samples were taken at 0, 0.33, 0.67, 1, 2, 3, 4, 6, 8, 12, and 24 hr. postadministration of each dose. The plasma samples, derived from the blood, were assayed for both aminosalicylic acid and its metabolite, *N*-acetyl-*p*-aminosalicylic acid, by a new specific analytical method (1). Plasma samples of all eight subjects at each sampling time assayed "zero" for both drug and metabolite following oral administration of the enteric-coated tablet. The assay sensitivity level was 0.5 mcg./ml. The average peak plasma levels of unchanged drug were 43.5, 16.7, and 8.86 mcg./ml. following oral administration of the solution, the suspension, and the compressed tablet, respectively. Drug levels were measurable over 6–8 hr. following the latter three dosage forms. The average peak plasma levels of the metabolite were 11.6, 12.3, and 10.9 mcg./ml. following the solution, the suspension, and the compressed tablet, respectively. Metabolite levels were measurable over 6–8 hr. following these dosage forms. Detailed results of this study will be published.

Since we did not know about the zero plasma levels of unchanged drug and metabolite in every subject following the enteric-coated tablet until all assays were completed, we never thought to ask the subjects to check their stools for intact tablets or large fragments of tablets. After the results were known, however, one of the eight subjects volunteered to take two more of the enteric-coated tablets. One tablet was excreted in his feces essentially intact (but with a small "hole" in one face) about 30 hr. postingestion. The other tablet did break up, but large pieces were excreted in the same feces. Figure 1 is a photograph showing the original intact tablet as it appeared before ingestion and the essentially intact tablet and the large fragments of the other tablet that were isolated from the feces. After photographing, the drug content of the material isolated from the feces was determined. The essentially intact tablet isolated from the feces assayed 489 mg. (98% of labeled dose), and the fragments[2] of the other tablet assayed 240 mg. (48% of labeled dose).

It is obvious that an enteric-coated tablet that gave zero plasma levels of bioactive aminosalicylate and that was excreted in the feces would be clinically ineffective. It would be unethical to perform a clinical study in patients with tuberculosis to prove such a point. The results obtained are attributable to both poor disintegration of the tablets and slow dissolution of drug from the fragments of the tablets once they did disintegrate or the coating ruptured. This was readily

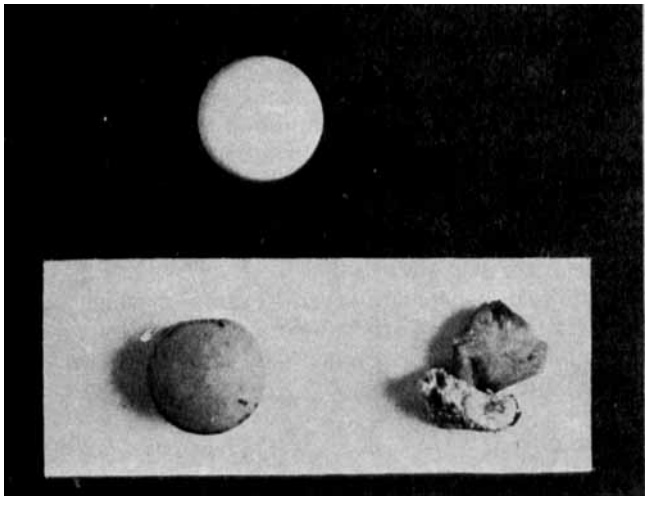

Figure 1—*(Top) Enteric-coated tablet before ingestion. (Bottom) Enteric-coated tablets excreted in the feces.*

[1] 4-Aminosalicylic acid; Parasal enteric-coated 0.5-g. tablet (Lot No. 722165B), containing 0.5 g. aminosalicylic acid USP (Panray Division of Ormont Drug and Chemical). The other dosage forms had the same trademark and were made by the same manufacturer.

[2] Only some fragments were recovered. Since some remained in the feces, the assay is lower than it should be.

shown by subjecting the tablets to *in vitro* test conditions in which the intensity of agitation was very much less than exists during performance of the USP tablet disintegration test. The flask and stirrer method of Poole (1969), as described by Wagner (2), was employed. For the first 0.5 hr., the tablets were subjected to 0.1 *N* HCl with a stirring rate of 50 r.p.m. For the remainder of the test, the tablets were stirred in pH 6.9 buffer, first at a stirring rate of 50 r.p.m. and then at a stirring rate of 200 r.p.m. Three of the enteric-coated tablets tested under these conditions exhibited initial cracks in the coatings after 230, 290, and 300 min. at 50 r.p.m. and then slowly released 22.7, 24.0, and 24.0% of the labeled dose over additional periods of 160, 60, and 40 min., respectively. When the stirring rate was increased to 200 r.p.m., these tablets eventually released essentially the labeled dose of drug. A fourth tablet, tested under the same conditions, showed no release of drug after 420 min. at 50 r.p.m.

Under the same conditions, the sodium aminosalicylate solution, when added to acid such that the final concentration was 0.1 *N* HCl, showed no precipitation of aminosalicylic acid (*i.e.*, infinite rate of dissolution). The suspension gave apparent first-order dissolution in 0.1 *N* HCl at 50 r.p.m., with a rate coefficient of 4.28 $hr.^{-1}$; at the end of 30 min., an average of 89.0% of drug was in solution (average of five tests). The compressed tablet similarly released drug in 0.1 *N* HCl at 50 r.p.m., with a coefficient of 2.58 $hr.^{-1}$; at the end of 30 min., an average of 71.3% of the labeled dose of drug was in solution (average of four tests with individual tablets). The enteric-coated tablets did not disintegrate in the classical sense when stirred at 50 r.p.m. in the pH 6.9 buffer. The coatings merely cracked open, and the drug was released extremely slowly from the ruptured tablets. It was not until the stirring rate was increased to 200 r.p.m. that the tablets really disintegrated and eventually released their labeled content of drug. These results strongly suggest that the intensity of agitation in the USP tablet disintegration test is too intense, at least for enteric-coated aminosalicylic acid tablets, and that the USP test does not predict performance in man. Thus, this is a documented case where a commercial product met compendia standards but was not suitable from a standpoint of effectiveness.

Feldmann (3) stated: "In other words, from a statistical viewpoint, the number of cases in which products have been found to meet compendia standards, but are not suitable from the standpoint of effectiveness or safety, are negligible."

Historically there are several examples which could be used to challenge the above quotation, but in this communication we document an example discovered in our laboratory.

(1) R. G. Stoll, P. D. Holmes, S. B. Roth, and J. G. Wagner, *Res. Commun. Chem. Pathol. Pharmacol.*, 4, 327(1972).

(2) J. G. Wagner, "Biopharmaceutics and Relevant Pharmacokinetics," 1st ed., Drug Intelligence Publications, Hamilton, Ill., 1971, p. 112.

(3) E. G. Feldmann, Statement of the National Formulary of the American Pharmaceutical Association to the Select Committee on Small Business—Subcommittee on Monopoly of the Senate of the United States, 90th Congress, 1st Session, Washington, D. C., June 8, 1967.

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Reliable and Simple Method of Intravenous Injection into the Laboratory Rat

Keyphrases ☐ Injection techniques, intravenous—use of dorsal penis vein, rats ☐ Drug administration—intravenous injection *via* dorsal penis vein, rats ☐ Intravenous injection of drugs into small laboratory animals—dorsal penis vein method

Sir:

In a previous article, Salem *et al.* (1) demonstrated a technique for intravenous injection of drugs into small laboratory animals *via* the dorsal penis vein. The advantages of this procedure compared to the tail vein method (2) lie in its simplicity, rapidity, reproducibility, and ease of injection. Tail vein injections into rats weighing over 100 g. are usually difficult and poorly reproducible, and they generally require a great degree of skill. On the other hand, the dorsal penis vein technique requires very little prior experience, so a relatively untrained laboratory worker can master the procedure in a short time, *i.e.*, one to two trials. In addition, tissue damage at the site of injection will have a minor overall effect on the animal compared to intracardial injection, and surgical manipulations such as those involved with femoral vein injections (2) are not required.

Due to these advantages it is not surprising that this method of drug administration is being employed in biopharmaceutical and pharmacokinetic studies. A review of the literature indicates that little information has been published concerning the physiological fate of drugs administered *via* this route. It is important to know whether this vein is part of the portal or general circulation. Drugs administered orally or intraperitoneally are absorbed *via* the mesentery blood system. These vessels combine to form the portal vein which leads directly to the liver. The drug plasma concentra-

shown by subjecting the tablets to *in vitro* test conditions in which the intensity of agitation was very much less than exists during performance of the USP tablet disintegration test. The flask and stirrer method of Poole (1969), as described by Wagner (2), was employed. For the first 0.5 hr., the tablets were subjected to 0.1 *N* HCl with a stirring rate of 50 r.p.m. For the remainder of the test, the tablets were stirred in pH 6.9 buffer, first at a stirring rate of 50 r.p.m. and then at a stirring rate of 200 r.p.m. Three of the enteric-coated tablets tested under these conditions exhibited initial cracks in the coatings after 230, 290, and 300 min. at 50 r.p.m. and then slowly released 22.7, 24.0, and 24.0% of the labeled dose over additional periods of 160, 60, and 40 min., respectively. When the stirring rate was increased to 200 r.p.m., these tablets eventually released essentially the labeled dose of drug. A fourth tablet, tested under the same conditions, showed no release of drug after 420 min. at 50 r.p.m.

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Due to these advantages it is not surprising that this method of drug administration is being employed in biopharmaceutical and pharmacokinetic studies. A review of the literature indicates that little information has been published concerning the physiological fate of drugs administered *via* this route. It is important to know whether this vein is part of the portal or general circulation. Drugs administered orally or intraperitoneally are absorbed *via* the mesentery blood system. These vessels combine to form the portal vein which leads directly to the liver. The drug plasma concentra-

Table I—Urinary Excretion Data of 4-Aminoantipyrine

Mean[a] Parameters	Route of Injection[b] Intracardial (± *SE*)	Penal Vein (± *SE*)
K_E (hr.)$^{-1}$	0.232 ± 0.031	0.246 ± 0.031
$t_{1/2}$ (hr.)	3.24 ± 0.41	3.03 ± 0.32
R_1[c]	11.14 ± 0.64	10.94 ± 1.66
R_2[d]	88.87 ± 0.64	89.10 ± 1.65
k_e (hr.)$^{-1}$	0.026 ± 0.004	0.032 ± 0.009
k_m (hr.)$^{-1}$	0.201 ± 0.027	0.246 ± 0.030
k_e/k_m	0.126	0.130

[a] Mean of six animals. [b] No statistical difference in parameters due to route of injection as determined by the Student *t* test. [c] R_1 = ratio of cumulative amount excreted at time infinity of unmetabolized to total drug times 100 = k_e/K_E. [d] R_2 = ratio of cumulative amount excreted at time infinity of metabolized to total drug times 100 = k_m/K_E.

tion is relatively high during this first pass through the liver; for substances that are biotransformed at this site, the rate of metabolism can be expected to be higher than when the drug is administered *via* a route leading directly to the heart. This first-pass effect can be associated with an increase in the fraction of drug metabolized. The present study was undertaken to examine the fate of a drug after penis vein injection and to determine the influence of this route of administration on metabolism. In addition, the feasibility of performing urinary excretion studies after penis vein injection was explored.

Male Sprague–Dawley rats[1] were used. The animals (six in each group), weighing 257–268 g., were lightly anesthetized with ether and subsequently injected with a pH 7.4 Sorensons phosphate buffer solution of 4-aminoantipyrine (70 mg./kg.) by either the intracardial or dorsal penis route. Blood and urine samples were collected as a function of time and assayed for both the free drug and the acetylated metabolite according to the method of Brodie and Axelrod (3). Urinary drug recovery after 72 hr. expressed as percent of total dose recovered was 71.9 ± 4.6 and 73.2 ± 2.7% for the intracardial and dorsal penis vein injections, respectively.

The cumulative amount of unmetabolized drug excreted in the urine was calculated as a function of time, and the data were appropriately plotted in accordance with the sigma-minus method (4). The overall elimination rate constant (K_E) and the biological half-life ($t_{1/2}$) were determined from the least-squares slopes of such plots. A representative graph is shown in Fig. 1. As expected, the half-life of the drug was identical for both groups, regardless of the administration technique. In addition, the half-life of the drug calculated from blood level data after penis vein injection was essentially similar (2.9 ± 0.3) to that obtained *via* urinary excretion (3.0 ± 0.3).

Measurement at time infinity, 72 hr., of the total cumulative amount of unchanged and metabolized drug recovered in the urine allows one to calculate the excretion rate constants for unchanged (k_e) and metabolized (k_m) 4-aminoantipyrine. The data shown in Table I summarize the pharmacokinetic parameters determined from the urinary excretion data. It appears

[1] Blue Spruce Farms, Altamont, N. Y.

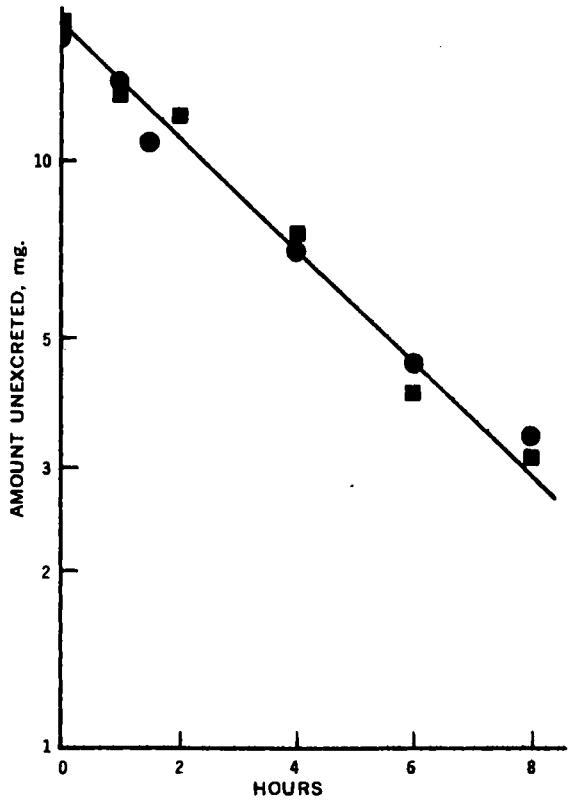

Figure 1—*Mean 4-aminoantipyrine excretion data after a 70-mg./kg. intravenous dose. Key: ●, intracardial; and ■, dorsal penis vein.*

that the degree of 4-aminoantipyrine metabolism is similar for both intracardial as well as penis vein injection. This suggests that drug injected *via* the dorsal penis vein does not directly enter the portal circulation. This was confirmed by following the suggested procedure of Conway[2], where 0.25–0.50 ml. of air was injected into the penis vein and the air bubbles were observed flowing through the inferior vena cava and not the portal vein. Injection of air into the femoral vein produces the same result.

Based on these experiments, it can be concluded that injection of drug into the dorsal penis vein leads to the general circulation and that a first-pass effect on drug metabolism is not to be expected.

(1) H. Salem, M. H. Grossman, and D. J. Bilbey, *J. Pharm. Sci.*, **52**, 794(1963).

(2) "The Rat in Laboratory Investigation," E. J. Farris and J. Q. Griffith, Eds., Hafner, New York, N. Y., 1967, p. 289.

(3) B. B. Brodie and J. Axelrod, *J. Pharmacol. Exp. Ther.*, **98**, 171(1950).

(4) E. Nelson, *J. Pharm. Sci.*, **50**, 181(1961).

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[2] Walter Conway, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, personal communication.

Identification of a Major Impurity in Methamphetamine

Keyphrases ☐ Methamphetamine—*N*-methyl-*N*-(α-methylphenylethyl)formamide isolated and identified as impurity in illicit samples ☐ *N*-Methyl-*N*-(α-methylphenylethyl)formamide—isolated, identified as major impurity in illicit samples of methamphetamine

Sir:

A major impurity in samples of illicit methamphetamine was isolated and identified as *N*-methyl-*N*-(α-methylphenylethyl)formamide (I).

Initial TLC investigations into the impurities present in exhibits of methamphetamine indicated I to be present in all samples. Qualitative analysis by TLC can be accomplished on silica gel plates using either of two solvent systems: A, chloroform–methanol (9:1); and B, chloroform–acetone–triethylamine (5:4:1). Both methamphetamine and I may be visualized by spraying successively with iodoplatinate[1] and *p*-dimethylaminobenzaldehyde[2] reagents. The R_f values of I in Solvent Systems A and B are 0.69 and 0.76, respectively.

Compound I was identified as the impurity by extraction from a TLC plate to yield a yellow oil. An IR spectrum of the oil showed aromatic absorption at 3050 cm.$^{-1}$ and aliphatic absorption at 2990 and 2950 cm.$^{-1}$. A weak band at 2860 cm.$^{-1}$ suggested the presence of a formyl group. The spectrum also indicated the presence of an amide carbonyl at 1675 cm.$^{-1}$ and absorption due to a monosubstituted phenyl group at 700 and 750 cm.$^{-1}$.

NMR spectrum[3] of the oil showed two singlets at 7.93 and 7.70 δ (formyl), a singlet at 6.93–7.50 δ (phenyl), two multiplets at 4.53–4.93 and 3.47–4.03 δ (methine), a multiplet at 2.66–2.83 δ (benzyl and *N*-methyl), and a pair of doublets at 1.13 and 1.27 δ (C-methyl). The duplicate NMR spectrum for the aliphatic protons is expected on the basis of restricted rotation about the C—N bond in formamides (1). A mass spectrum[4] of the oil yielded a parent peak at *m/e* 177 and a cracking pattern consistent with the proposed structure.

Further confirmation of the identity of I was obtained by formylation of methamphetamine by a modification of the method of Sheehan and Yang (2). A mixture of 10 ml. of acetic anhydride and 5 ml. of 88% formic acid was added to 1.5 g. of methamphetamine, yielding an almost quantitative yield of the *N*-formyl derivative. Comparison of the synthetic material with that isolated from exhibits by TLC, IR, and NMR showed the two to be identical.

Quantitative analysis by GLC[5] on 30 randomly selected samples indicated I to be present in amounts ranging from 10 to 39% relative to methamphetamine.

Production of illicit methamphetamine apparently is accomplished by a method involving production of I from phenylacetone and *N*-methylformamide (3) and hydrolysis to methamphetamine. The residue of I would arise due to incomplete hydrolysis.

(1) L. A. LaPlanche and M. T. Rogers, *J. Amer. Chem. Soc.*, **86**, 337(1964).

(2) J. C. Sheehan and D.-J. Yang, *ibid.*, **80**, 1154(1958).

(3) P. Mastagli and G. de Bievre-Gallin, *Compt. Rend.*, **224**, 1290 (1947).

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[1] Dissolve 1 g. platinum chloride in 10 ml. of water, mix with 250 ml. of 4% (w/v) aqueous potassium iodide, and dilute to 500 ml. with water.

[2] Dissolve 125 mg. *p*-dimethylaminobenzaldehyde in 100 ml. of 50% (v/v) sulfuric acid and add 2 drops of 10% (w/v) ferric chloride solution.

[3] Varian T-60 spectrometer.

[4] Hitachi Perkin-Elmer RMU-6 spectrometer.

[5] Bendix 2500, equipped with a 1.8-m. × 0.63-cm. (6-ft. × 0.25-in.) glass column, 5% OV-7 on Gas Chrom Q, at 165°. Phenacetin was used as an internal standard.

REVIEWS

Perspectives in Clinical Pharmacy. Edited by DONALD E. FRANCKE and HARVEY A. K. WHITNEY, JR. Drug Intelligence Publications, Hamilton, IL 62341, 1972. xvi + 535 pp. 18 × 25 cm. Price $15.00.

Perspectives in Clinical Pharmacy, according to the title page, is "a textbook for the clinically-oriented pharmacist wherever he may practice." The book consists of a series of 25 chapters written by 32 authors, each chapter written to stand alone but with the intent that all chapters relate to what the editors refer to in their preface as "the concept of clinical pharmacy." The work has been prepared under joint editorship, the senior editor being a well-recognized hospital pharmacist and pharmacy journalist, the junior editor, a director of pharmacy service in a university teaching hospital. An easily read two-column format is used throughout the book which is illustrated with tables, figures, and drawings. Chapters contain rather complete bibliographies, some of which are supplemented with a secondary list of references. This feature adds greatly to the book's utility to students and practitioners. The index is carefully prepared.

Perspectives is one of the first textbooks to be prepared under the general title of "clinical pharmacy." In this sense, it has a certain historic significance. Since any first effort of this nature made during periods of rapid transition may fall short of the original goals set for it, *Perspectives* must be accepted in a certain provisional light. If in the years ahead, the concept of clinical pharmacy becomes an enduring component of pharmacy practice and education, and if the book in succeeding editions keeps pace with the field as it evolves, *Perspectives* may become, indeed, a valuable asset to the literature of the profession.

Prior to reviewing *Perspectives*, the reviewer raised two questions which he hoped would be answered. "What is clinical pharmacy?" and "Is clinical pharmacy something new or is it only a concept, an emphasis, an attitude, or a hope of things longed for?"

A critical reader of a new book usually wants to know why the book was written, and if a specific need exists which the author(s) is attempting to fulfill. The reviewer found that *Perspectives* failed to supply this information since the preface was less informative in this regard than had been hoped for. Of the seven paragraphs in the preface, the first identifies clinical pharmacy as representing "a revolt by a group young in years and in spirit against the way the profession has developed," when in fact it seems to be stemming from more than just the disenchantment of the young. The second paragraph acknowledges that no one has succeeded in defining clinical pharmacy but when the concept matures it will bring with it the clinical pharmacist who "will perhaps best be described as an applied pharmacologist generalist." The third paragraph states the objectives which dictated the selection of subject matter: *first*, to orient the reader to the health-care system; *second*, to acquaint the reader with those continually evolving concepts and practices which relate to clinical pharmacy; and *third*, to present information "the clinical pharmacist will use in his daily effort to promote the safe and effective use of drugs...." The remaining four paragraphs in the preface deal with acknowledgments.

The reviewer examined the book in light of the three objectives which directed the selection of the subject matter. The first, dealing with an orientation to the health-care system is dealt with in Chapters 1 and 4—principally in Chapter 1. While it includes data relating to health manpower, health-care costs, and morbidity and mortality data, the chapter contains very little that has not appeared regularly in the literature of the past 5 years. The author chooses not to discuss the financing of health care because of the many schemes that have been proposed and "the uncertain direction they will take." Admittedly, inclusion of the financing of health care in a textbook of this nature presents a difficult decision since omission of it, even for purposes of orientation, makes a discussion incomplete. It appears deficient in still another sense, in that it fails to provide guidelines which the reader, presumed to be a pharmacist, can use in adjusting his attitudes and behavior to the new delivery system. The chapter does treat, in a generally readable style, some of the issues that have been at work in the present health-care situation, but which bring to the reader very little new information.

Chapter 4 approaches the health team concept from the point of view of the interrelationships among the pharmacist, physician, and nurse. The discussion, while well written, is largely conceptual and philosophical and again, represents a restatement of many of the ideas that have been discussed in recent years in the pharmacy literature.

One turns to Chapters 2, 3, 5, 6, 7, 8, 10, 11, 12, 14, and 15 for an appraisal of the subject matter which relates to the second objective: "to acquaint him with some of the still evolving concepts and practices of clinical pharmacy...." Here one has several well-written and well-organized chapters, each prepared by an author or authors well qualified for the assignment. For a pharmacist who wants to know "how" and "why," several discussions are worthy of note: Developing a Clinical Pharmacy Program in the Hospital; Recent Advances in Drug Distribution Systems and Their Economic Feasibility; Developing a Parenteral Admixture Service; Building a Clinically-Oriented Drug Information Service; the Clinical Pharmacist in an Interprofessional Group Practice; and Pharmacy Services in Small Hospitals.

That portion of the subject which deals with the pharmacist's role as drug expert (objective three) is found in Chapters 9, 13, 16, 17, 18, 19, 20, 21, 22, 23, and 24. The subject matter ranges from drug interactions and the clinical pediatric specialist to biological half-lives and pKa values with clinical applications; formulation of sterile dosage forms; and intraperitoneal dialysis and interference of drugs with laboratory tests. These sections, too, are well written and show clearly the scientific sophistication and quantification that is now used in the study of pharmaceutical systems. Of particular note is the increasing application of the biological sciences to drug-related phenomena. These observations illustrate the growth that has occurred during the past 25 years in the body of scientific knowledge encompassed by the pharmaceutical sciences.

Three chapters are deserving of special note. Chapter 2 is an excellent chronological recounting of the development of clinical pharmacy according to concept, practice, and education. In this section, the reviewer found answers or partial answers to the two questions raised prior to his review. *Question 1.* "What is clinical pharmacy?" *Answer:* "Evolvement of clinical pharmacy in the seventies appears to progress toward greater involvement in a patient-oriented practice by pharmacists in both hospitals and the community.... Despite the continued effort to *practice*, to *educate* for and to *write* about, there is yet no clear definition of 'clinical pharmacy.' The concept of clinical pharmacy tends to bend—depending on whether one refers to education or to practice—to its applications in the institutional setting or in the community."

Question 2. "Is clinical pharmacy something new or is it only a concept, an emphasis...?" *Answer:* "It is difficult to assess the extent of actual clinical practice by any definition.... However, more often than not, clinical pharmacy may be a new term attached to old roles. Nevertheless, those pharmacists who have taken on new roles... have found a mission in which is deeply rooted a professional service role intrinsic to the delivery of health care and the needs of the patient. ... Clinical pharmacy is not a completely new concept but admittedly the emphasis has changed during recent years."

This chronology of the evolvement of clinical pharmacy is recommended reading for all pharmacists.

Chapter 3 is entitled "The Social Psychological Role of the Pharmacist in Drug Usage." The scope is extremely broad, embodying such topics as the professional self-image, physician–patient relationships, patient behavior in illness, patient's compliance, factors influencing patient's response to drugs, pharmacist–patient relationships, pharmacists' practice patterns, and separation of pharmacies and drugstores. The chapter, well written and well documented, highlights a number of important issues: professionalism, the need for instruction in the social and behavioral sciences in the pharmacy curriculum, and factors that influence drug utiliza-

tion. It has an editorial quality which calls for reform in both pharmacy education and pharmacy practice.

Chapter 25, "Principles of Experimental Design for Clinical Drug Studies," makes a unique contribution to *Perspectives*. It is written by a biostatistician who obviously is thoroughly familiar with his subject and lays out the concepts, requirements, design, and evaluation of clinical trials. For those who participate in clinical drug studies, this chapter is a valuable resource.

Perspectives, as with many first editions has both strengths and weaknesses. The chief weakness, in the reviewer's opinion, lies in the limited achievement of objective 1 for selection of subject matter. One might question the feasibility of attempting to include an "orientation to the health care system" in a textbook on clinical pharmacy because of the vast and complex nature of the subject. It is also obvious in some chapters that the authors are making a deliberate attempt to relate their subject matter to clinical pharmacy—a "bending" to accommodate a new concept. This appears to illustrate a statement under "Conclusions and Definitions," Chapter 2, which speaks of the loss of the medicine-making function (by pharmacists) to the pharmaceutical industry: "...the profession has sought to replace it with a 'new' thing—and today clinical pharmacy is the 'thing.'"

A new textbook that enjoys certain historical significance because of the time of its appearance and its subject matter often causes a critical reader to question the impact that the book will have on others, in this case the profession. A foreword for *Perspectives* was written by Professor Alex Berman who addressed himself to this question: "If the perspectives in clinical pharmacy revealed in this work become widely accepted in American pharmacy, can anyone doubt that this would have a profound impact on the profession and that it would grow enormously in social esteem? But such an acceptance will require a conscious change of direction and goals as well as a planned restructuring of economic and institutional aspects of pharmacy." The reviewer can only agree with Professor Berman's statement.

In summary, *Perspectives in Clinical Pharmacy*, has many features which will direct its use both as a textbook and a reference source. The reader will be left to ponder some of the philosophical matters that the book raises, particularly those that deal with the clinical pharmacy concept. In the final analysis, these matters are of lesser significance than those raised by the subject matter of the book. There are chapters in *Perspectives* that approach state-of-the-art presentations of certain areas of pharmacy practice and these will be the ultimate determinants of its usefulness to students and practitioners.

Reviewed by Donald C. Brodie
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Stereochemistry and Its Application in Biochemistry, The Relation between Substrate Symmetry and Biological Stereospecificity. By WILLIAM L. ALWORTH. Wiley-Interscience, 605 Third Ave., New York, NY 10016, 1972. 311 pp. 14.5 × 22.5 cm. Price $16.95.

Appropriately, the opening chapter of the book starts with a discussion of the valuable contribution made by Ogston who pointed out that an asymmetric enzyme can distinguish between identical groups on a symmetrical enzyme. This point is further elaborated in a separate chapter on biochemical differentiation between chemically like, paired groups. The stereospecificity of fumarase, citrate synthetase, aconitase, and alcohol dehydrogenase reactions is nicely condensed. For the nonchemistry majors, the introductory information on molecular asymmetry and biological stereospecificity, the standard configuration designations such as D, L system, and sequence rule are provided. Simple, selected examples for the determination of absolute configurations of the isomer are well presented. About 15 pages are devoted to the substrate stereospecificity of the well-studied enzyme, α-chymotrypsin. It emphasizes the value of stereoisomers in mapping the active site of the enzyme. The information will be useful to pharmacologists and other biologists who are interested in using D and L isomers as a tool to probe drug-sensitive pharmacological receptors.

The final chapter covers the additional examples of biological stereospecificity of the tricarboxylic acid cycle. The biological stereospecificities in the squalene biosynthetic pathways should be particularly useful to the medicinal chemist. A proper design of anticholesterol agents should come from the proper understanding of biochemistry and stereochemistry of the system. One related topic on *cis* and *trans* isomers of retinine is not discussed in the text.

Throughout the book, the important statements and words are presented in italics. The illustrations are simple, clear, and creative. In the preface, the author states that "all students will profit by considering their study of molecular symmetry and biologic stereospecificity as a vital part of an introduction to the field of biochemistry." The statement is very true. Due to his background in bioorganic chemistry, the author has kept a *proper* balance between biochemistry and stereochemistry.

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REVIEW ARTICLE

Pharmacokinetics and Molecular Modification: Implications in Drug Design and Evaluation

ROBERT E. NOTARI

Keyphrases ☐ Pharmacokinetics and molecular modifications—review of application to drug design, consideration of absorption, drug–receptor interactions, pH–partition effects, prodrugs, interpretation of parameters ☐ Drug design—implications of pharmacokinetics and molecular modification, review of absorption processes and kinetics, drug–receptor interactions, pH–partition effects, prodrugs, interpretation of parameters ☐ Absorption processes and kinetics—review of application to drug design ☐ Drug–receptor interactions—review of application to drug design ☐ Antibiotics (penicillins and tetracyclines)—molecular modifications, optimization of pharmacokinetic parameters, review of implications in drug design ☐ pH–partition effects—optimizing oral absorption by chemical modification, implications in drug design, review ☐ Prodrugs and bioavailability—interpretation of pharmacokinetic parameters, application to drug design, review

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INTRODUCTION

This is a review (and in some cases an analysis) of selected pharmacokinetic studies on drugs with closely related chemical structures. It is not an attempt to survey all of the work that has been reported in the field of biopharmaceutics and pharmacokinetics, nor is it a summary of all studies involving alteration of drug absorption, distribution, and excretion through molecular modification. Several recent publications collectively serve that purpose, and these are cited within the text. This review is an attempt to stress the significance of pharmacokinetics in drug design and to illustrate the main points using examples selected from the literature.

The concept of pharmacokinetics and compartmental analysis is not a new one, although the level of sophistication and interest in this subject has rapidly increased during the last decade. As early as 1920, Widmark and Tanberg (1) and Widmark (2) studied the kinetics of drug elimination and drug plasma levels after multiple doses. Compartmental analysis was first introduced by Teorell (3, 4) in 1937 when he reported the kinetics of the distribution of substances in the

body. Marshall *et al.* (5, 6) made early contributions to what is now called pharmacokinetic analysis with their work on the elimination of sulfanilamide, as did Stewart *et al.* (7).

The first review of pharmacokinetics appeared in 1961 when Nelson (8) surveyed the literature on the kinetics of drug absorption, distribution, metabolism, and excretion. Also in 1961 Wagner (9) published a review of the fundamental principles involved in the absorption aspects of biopharmaceutics. A very fine text was written by Rescigno and Segré in 1961 covering the mathematical aspects of modeling and compartmental analysis. The English edition appeared in 1966 (10). Several texts are available (11, 12) which discuss the fundamentals of drug metabolism, disposition, and evaluation using pharmacokinetic principles, and guidelines have been published for the evaluation of bioavailability of drugs (13). The principles and methods used in biopharmaceutics and pharmacokinetics are the subjects of several texts (14–19) for the beginning and advanced student. Pharmacokinetics have been applied to the evaluation of oral timed-release drugs and used to determine the effects of dissolution rate, particle size, inert tablet ingredients, and urinary pH on the absorption and excretion of drugs. The pharmacokinetic parameters involved in drug–receptor interactions, binding to nonreceptor tissues, passive and active transport, metabolism, and excretion have been the subject of papers too numerous to list here.

Absorption Aspects—Although the use of compartmental analysis of drugs in the body is quite common and well publicized, relatively little use has been made of *a priori* considerations of pharmacokinetic parameters in drug design. Most of what has been reported applies primarily to GI absorption and may be described as attempts to:

1. Maximize the absorption rate by increasing the dissolution rate (as in micronization, salts of acids or bases, buffers, amorphous or metastable polymorphs, *etc.*).
2. Extend duration by decreasing the release rate from the dosage form (timed release, repository injections of slowly soluble salts, macrocrystals, free acid or base instead of salt, *etc.*).
3. Decrease loss to degradation in the stomach (acid-insoluble esters or salts, chemically stable derivatives, enteric coating, *etc.*).
4. Decrease loss due to complexation with foods (chemical modification, *etc.*).

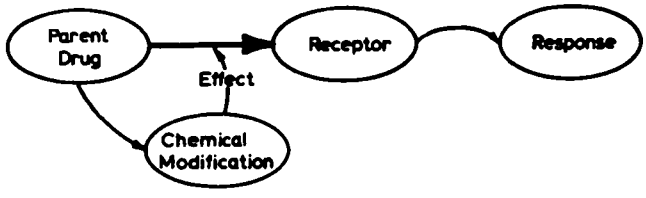
Although modification in the formulation (or of the dosage forms themselves) has received the most attention, many of the above alterations may be considered as chemical changes. While these modifications usually occurred in response to a particular problem with the parent compound, it is imperative that this aspect of drug design move (and no doubt it will) from an era of "corrective" research into one of "predictive" and "preventative" research. Obviously, the evaluation and thus the control of pharmacokinetic parameters must be introduced in the early phases of drug design to optimize all considerations that will become significant when the drug is in clinical use.

Need for Pharmacokinetics in Drug Design—Very few studies have been reported wherein the goal was to define substituent group effects on the total drug–organism interaction, including not only biological responses but also correlations between the observed pharmacological effects and the absorption, distribution, and excretion of the derivatives. Generally, examples illustrating such effects must be found in retrospect by examining classes of drugs where both pharmacokinetic studies and biological responses have been reported and then deducing, where possible, the connection between them. While this is not an easy task, the results do point up three important facts:

1. Pharmacokinetic parameters do influence biological response, and they are indeed critical in drug design.
2. Pharmacokinetic parameters can be modified by rather subtle structural changes.
3. There is a great need for basic studies in this field to arrive at the level of sophistication necessary to make *a priori* judgments practical.

The ultimate goal is to design a drug molecule having a desired pharmacological effect resulting from the proper balance of absorption, distribution, intrinsic activity, metabolism, and excretion without resorting to the costly and time-consuming process of screening large numbers of analogs.

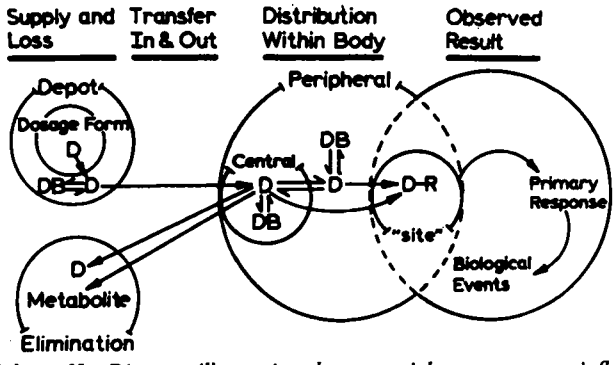
Drug–Receptor Interactions—It is common practice to consider the effects of substituent groups in a series of molecules upon the "drug–receptor" interaction (Scheme I). Typically, some assumptions are made



Scheme I—Diagram of molecular modification effects at the drug–receptor interaction

regarding the interaction between parent compound and receptor. Molecular modifications are made, and the basic assumptions are tested. Generally, abnormalities (unexpected results) are explained by modifying the concept of the receptor and occasionally even by modifying the concept of the drug structure, as in the case where the observed activity is explained by arguing for a particular conformation being the preferred one for that molecule *only* when it is in the vicinity of *that* receptor. In many cases, the conclusions are based upon dose–response curves and the dose is assumed to be responsible for the magnitude of the response. However, it is well recognized (albeit seldom evaluated) that the time course for a drug at the receptor must also be considered. The onset, duration, and intensity of effect may be considered as a function of at least two factors: (*a*) transport processes affecting the time course at the receptor site: delivery and removal from the site, absorption, distribution, excretion, metabolism, binding, *etc.*, and (*b*) interaction between drug and receptor after arrival at the site.

Time Course for Drug–Receptor Interactions—Scheme II illustrates how modification of a parent structure



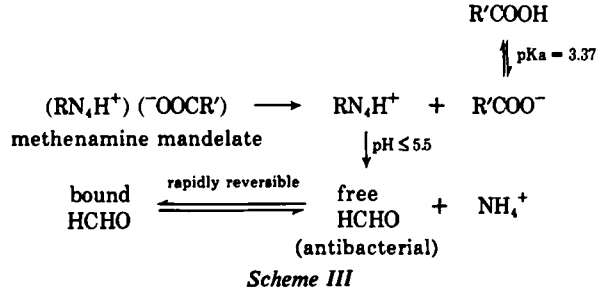
Scheme II—Diagram illustrating the potential rate processes influencing the time course for a drug at the receptor. The drug–receptor interaction is indicated as D–R *and dosage form is placed in depot. See text for further definition of symbols.*

can influence the drug available to the receptor site. The following processes in Scheme II can be altered by changing a substituent group on a drug (*D*):

1. Supply and loss
   (*a*) Release from dosage form (rate and/or amount)
   (*b*) Stability in depot
   (*c*) Binding in depot (*DB*)
   (*d*) Transfer from depot to central compartment (rate and/or amount)
   (*e*) Elimination rate from central compartment
2. Distribution
   (*a*) Binding in central compartment (*DB*)
   (*b*) Binding in peripheral compartment (*DB*)
   (*c*) Rate and volume of distribution
   (*d*) Transfer to receptor site
3. Drug–receptor interaction

An immediate problem challenging those who would consider and "optimize" all of these factors is physically locating the receptor site and defining an ideal time course for the drug–receptor interaction, *e.g.*, "hit and run," sustained effect, *etc.* Although quite arbitrary, it has been stated (14) that an *ideal drug* should: (*a*) reach the site of action, (*b*) arrive rapidly in sufficient quantity, (*c*) remain for a sufficient duration, (*d*) be excluded from other sites, and (*e*) be removed from the site when appropriate.

Consider a relatively simple problem and a well-known example: the design of a urinary tract anti-infective. For the simplest case, the site of infection will be regarded as the urinary tract itself. The example chosen is the prodrug, methenamine. In acid pH, methenamine is converted to formaldehyde, which acts as the antibacterial agent as illustrated in Scheme III. Tablets of methenamine are often enteric coated to prevent conversion to formaldehyde in the stomach



Scheme III

(20). Thus, absorption occurs from the intestine into the bloodstream where the pH is too alkaline for conversion in both cases. Methenamine is cleared intact in the urine where it becomes converted to formaldehyde if the pH is less than 5.5. The rate of conversion can be controlled by management of urinary pH.

This example is somewhat simplistic since the site of action has been limited to the urinary tract. Thus, the physiological location for delivery of the active agent can be defined and monitored. The time course for drug at the site can be determined by assay of urine samples for formaldehyde as a function of time. The goal of product design would thus be to achieve the optimum time course of formaldehyde concentration in the urine.

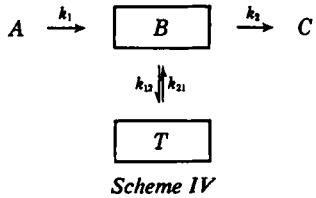
In general, the problem is a more difficult one because the site of action cannot be easily defined or sampled. Pharmacokinetic analysis presents a powerful tool for understanding the entire time course of a drug in the patient by kinetic modeling. The following discussions are intended to illustrate this premise as well as to suggest possible approaches in defining substituent effects.

DEFINING PHARMACOKINETIC RESULTS OF MOLECULAR MODIFICATION

Interpretation of Data—Which parameters have changed? How have they changed? Why? These are the basic questions in the evaluation of substituent effects on pharmacokinetic behavior. However, the more significant question ultimately is: "Does this alteration in pharmacokinetic behavior improve the course of therapy with this drug?" It is difficult to envision a model system that will lend itself to simple assessments of these questions. Indeed, this is the challenge of future structure modification in drug design.

This review is limited to pharmacokinetic parameters associated with compartmental analysis. Several recent texts define the methodology involved in this approach (14–19). Although the terminology employed is fairly standard, the symbols vary somewhat throughout the literature. The most frequently encountered terminology is summarized in Table I, where typical symbols and their references are also listed. The symbols in Column 1, taken from *Reference 14*, are used in this review.

Scheme IV illustrates the model that corresponds to the symbols in Column 1, Table I. The meaning of this scheme is defined in Table I. This model is used to represent both one- and two-compartment systems. A one-compartment system is arbitrarily defined as one that immediately achieves equilibrium ratios of T/B following a rapid intravenous dose. Graphically, this would result in a monoexponential first-order plot for concentration in *B versus* time. When distribution



Scheme IV

Table I—Survey of Symbols Commonly Used in Pharmacokinetic and Biopharmaceutical Literature (Those Taken from *Reference 14* Are Used throughout This Review)

Description of Parameter and Its Units	References 14	15	21	16, 22	19
First-order rate constant for absorption ($time^{-1}$)	k_1	K	k_a	k_a	k_{ab}
First-order rate constant for elimination from central compartment[a] ($time^{-1}$)	k_2	K_2	k_{el}	k_{el}	k_{10}
First-order rate constant for elimination from central compartment by excretion ($time^{-1}$)	k_e	—	k_e	k_e	—
First-order rate constant for elimination from central compartment by metabolism ($time^{-1}$)	k_m	—	k_m	k_m	—
First-order rate constant for transfer from central[a] to peripheral[b] compartment ($time^{-1}$)	k_{12}	K_1	k_{12}	k_{12}	k_{12}
First-order rate constant for transfer from peripheral[b] to central compartment[a] ($time^{-1}$)	k_{21}	K_{-1}	k_{21}	k_{21}	k_{21}
Apparent first-order rate constant for elimination from body ($time^{-1}$)	β	K	β	β	β
Apparent first-order rate constant for elimination from body by excretion ($time^{-1}$)	β_{ex}	k_r	—	—	k_{ex}
Apparent first-order rate constant for elimination from body by metabolism ($time^{-1}$)	β_{met}	k_{nr}	—	—	k_{mX}, k_{mY}, *etc.*
Apparent volume of distribution (volume)	Vd	V	Vd	Vd	V
Apparent volume of central compartment[a] (volume)	V_p	V_1	V_p	V_c	V_c
Concentration of drug in bloodstream or plasma (amount/volume, mg. %, *etc.*)	P	C	Cp	Cp	D_t
Dose of drug in body (amount)	D	$f \times D$	—	X^0	D
Biological half-life for elimination from body (time)	$t_{1/2}$	$t_{1/2}$	$t_{1/2}$	$t_{1/2}$	$t_{1/2}$
Renal clearance (ml./min.)	C	Cl_d	—	—	Cl_r
Site of administration of drug	A	—	D	—	—
Central compartment[a]	B	C_1	P	C	1
Compartment accounting for total elimination[b]	C	—	ME	—	—
Peripheral compartment[c]	T	C_2	T	T	2

[a] The central compartment is often referred to as the blood or plasma although it is recognized that it can in fact be larger in volume (23). [b] See Scheme IV. [c] The peripheral compartment is often referred to as the tissue compartment.

between T and B is slow relative to elimination, Scheme IV represents a two-compartment model. In practice, this is evidenced by a biexponential first-order plot for drug in compartment B. In either case the biological half-life is defined as:

$$t_{1/2} = 0.693/\beta \qquad \text{(Eq. 1)}$$

where β represents the negative value of the slope of the first-order plot for a one-compartment model or the slope of the terminal portion of the plot for a two-compartment model.

Table II—Reported (or Calculated) Values for Selected Pharmacokinetic Parameters[a] for Several Penicillins

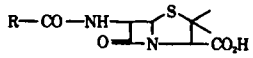
Penicillin	$t_{1/2}$, hr.	Vd, l.	C, ml./min.[b]
Ampicillin	1.0 (41), 0.8 (42), 1.2 (43)	22 (41), 20[c] (42), 43 (43), 25 (49)[d], 30 (50)	283 (41), 210 (49)[e], 312 (50)
Carbenicillin	1.0 (42)	10[c] (42)	86 (42)
Cloxacillin	0.42 (44), 0.6 (42)	10[c] (44), 11 (42), 23 (51)	162 (44), 287 (50)
Dicloxacillin	0.88 (41), 0.71 (44), 0.7 (42)	13 (41), 10[c] (44), 9.4 (42), 16 (50)	88 (41), 162 (44), 130 (50)
Methicillin	0.43 (41)	22 (41)	350 (41)
Nafcillin	0.55 (45), 1.2 (43)	21[c] (45), 57 (43)	160 (45)[e]
Oxacillin	0.70 (41), 0.38 (44), 0.40 (45)	27 (41), 14[c] (44), 13 (42), 15[c] (45), 26 (50)	190 (45)[e], 402 (50)
Penicillin G	0.70 (41), 0.5 (42), 0.84–0.93 (46)[f], 0.6–0.99 (47)[g], 0.54 (24), 0.65[h], 0.78 (48)	26 (41), 22[c] (42), 37–37 (46)[f], 35 (24)	433 (41), 386 (49)[e], 340–480 (46)[f], 377[h]
Phenethicillin	0.77 (48)	Appears to be $0.6 \times Vd$ of penicillin G (48)	—
Phenoxymethyl penicillin	0.53 (24), 0.43[h], 0.52 (48)	51 (24), 54[h]	393[h]

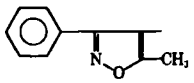
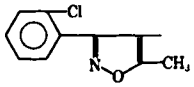
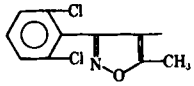
[a] The reference for each value listed is given in parenthesis. [b] Values for renal clearance. See Table I for symbols. [c] Calculated from $Vd = k_a/\beta P_o$ (14, 40) using the 3-hr. constant-infusion data reported in the reference cited. [d] Average value calculated from P_o data using all three $t_{1/2}$ estimates in Column 1 of this table. [e] Units are ml./min./1.73 m.^{2}. [f] Variation due to ambulatory *versus* bed rest. [g] Variation attributed to size of dose. [h] Calculated from data in *Reference 51*.

Optimizing Parameters: Antibiotics—Methods for assessing structural effects on pharmacokinetic parameters are illustrated using literature data for penicillin derivatives. As stated earlier, a more basic concern is that of tailoring pharmacokinetic parameters for optimum therapeutic response. It has been emphasized, for example, that steady-state levels of carbenicillin are twice that of ampicillin. These high blood levels of carbenicillin following intravenous administration have been said to be a primary factor for its efficacy in treatment of relatively resistant infections such as pseudomonas (42). However, the reasons for the observed differences in blood levels must be determined to compare the drugs rationally. It is demonstrated in the following section that the reason for this difference is the larger volume of distribution for ampicillin since the elimination rate constants are similar.

If all other factors were equal, it might be argued that an increased value for Vd is a clinical advantage. Spitzy and Hitzenberger (24) stated that "bacteria germinate more frequently in the tissues than in the blood," while Pratt (25) stated that "bacteria are more common in other tissues than blood." Several other authors stressed the importance of tissue concentrations (26–28), and Fabre *et al.* (29) stated that an antibiotic's effectiveness depends upon its penetration into tissues, particularly inflamed tissues. Thus, if plasma protein binding is equal, an antibiotic derivative with a larger Vd would appear to be reaching the site of action with better efficiency. Although this is by no means unequivocal, the spectrum of research activity in antibiotic derivatives would imply that the following goals for

Table III—Isoxazolyl Penicillins



Penicillin	Structure, R	P_{ss}, mg./l.[a]	C, ml./min.[a]	Vd, l.[b]	β, hr.$^{-1}$[b]	β_{ex}, hr.$^{-1}$[b]	β_{met}, hr.$^{-1}$[b]
Oxacillin		9.7	226	14	1.83	1.02	0.81
Cloxacillin		15	162	10	1.65	1.02	0.63
Dicloxacillin		25	113	10	0.98	0.72	0.26

[a] Taken from *Reference 44.* [b] Calculated from *Reference 44.*

molecular modification are generally pursued: (*a*) increased distribution to tissues; (*b*) increased $t_{1/2}$ to maintain higher postdistribution body levels and to decrease the dosage frequency; (*c*) decreased binding capacity to foods and also to plasma protein; (*d*) increased oral absorption through increased solubility, dissolution rate, GI stability, and partition coefficient; and (*e*) decreased minimum inhibitory concentration (MIC) with resultant decrease in the required dose.

It is interesting (and perhaps amusing) to note that these goals often appear more obvious in promotional literature for antibiotic derivatives than in the scientific literature itself. While this list is not all inclusive, it can serve as the basis for a pharmacokinetic comparison.

Penicillins—Antibiotics probably represent the principal group wherein minor molecular modifications have been made on parent molecules with resultant alterations in pharmacokinetic parameters. Since penicillin marked the beginning of antibiotic chemotherapy, there have been a great number of studies on these derivatives. Schwartz and Buckwalter (30) discussed the relative gastric stability of penicillins as the primary factor in determining bioavailability. Hou and Poole (28) reviewed the relationship of penicillin structure to biological activity. Structures as well as typical MIC values for the penicillins discussed here may be found in their excellent review. A number of other reviews on penicillins and cephalosporins are also available (31–39).

The reported values for three pharmacokinetic parameters ($t_{1/2}$, Vd, and C) are summarized in Table II together with their reference sources. The $t_{1/2}$ values, reported by the various investigators for a given penicillin, appear to be in good agreement. Wagner (15) suggested that the values for the $t_{1/2}$ of a drug would be expected to be distributed normally or log-normally within a total population of subjects. Somewhat surprising, perhaps, is the observation that generally all of the penicillins in Table II have half-lives in the rather narrow range of 0.5–1 hr.

There appears to be a much greater variation in the reported values for the Vd of a given penicillin. Biological or subject-to-subject variation surely accounts for some of these observed differences. This is especially true where absolute values are compared, as they are in Table II where the distribution volumes are given in liters. It is probably more rational to state these on a relative basis such as percent of body weight or milliliters per gram, as illustrated in *References 24* and *47*. Levy (46) illustrated that the Vd of penicillin G is increased during bed rest (47 l.) compared to that of ambulatory patients (37 l.). The rate constant for metabolism of penicillin G, β_{met}, was also significantly decreased ($p < 0.05$) during bed rest. Studies comparing drug derivatives must be designed to control factors such as biological variation and patient activity for a meaningful experimental design.

There is a more basic problem in evaluating the Vd of a drug. If a drug is truly distributed according to a one-compartment open model, the calculated value for Vd may be expected to be independent of the method used to determine it (52) since equilibrium ratios of T/P will be achieved. However, if the drug is distributed according to a multicompartment model, the value for Vd is dependent upon the kinetics. For example, the value obtained by the extrapolation method will not agree with that obtained by the steady-state method (52). The influence of the elimination rate constant, k_2, on the calculated value for Vd was illustrated by Gibaldi and Perrier (53) and Jusko and Gibaldi (54). By using constant intravenous infusion to obtain a steady-state plasma level, the T and B compartments (Scheme IV) can be brought to equilibrium. The steady-state distribution volume is then defined as:

$$Vd_{ss} = \frac{k_{12} + k_{21}}{k_{21}} Vp \qquad \text{(Eq. 2)}$$

which is independent of k_2. This offers one approach to comparing distribution of analogs using a method that should produce more reliable results than one that is influenced by elimination.

The isoxazolyl penicillins represent a group of three closely related penicillins with varied pharmacokinetic behavior (Table III). Their structural differences are limited to the number of chlorine atoms. Early studies attributed higher dicloxacillin serum levels to increased oral absorption. The ratios of serum levels following oral administration in one study were roughly 2:1 for dicloxacillin–cloxacillin (55). In another study, similar ratios of 2.3:1.4:1 for dicloxacillin–cloxacillin–oxacillin were obtained for both the oral and intravenous routes

of administration (50). This precludes the possibility of absorption being the sole factor for different plasma profiles. The percent absorption was also calculated from:

$$\% \text{ absorption} = 100\% \, (\text{area}_{\text{oral}}/\text{area}_{\text{i.v.}}) \qquad \text{(Eq. 3)}$$

using the areas under the curves. Some improvement in absorption with chlorine substituents was detected; 80% of dicloxacillin, 77% of cloxacillin, and 67% of oxacillin were absorbed. However, the differences are not sufficient to explain the dramatic contrast in plasma levels following oral administration. Such curves cannot be compared directly. In spite of the seemingly minor structural differences in the isoxazolyl penicillins, each must be regarded pharmacokinetically as a different drug. Dittert *et al.* (41) demonstrated that penicillins are distributed according to a two-compartment open model as represented by Scheme IV. Therefore, the values for all four constants (k_1, k_{12}, k_{21}, and k_2) may change with the addition of chlorine atoms, and their individual contributions to the time course must therefore be considered.

The data of Rosenblatt *et al.* (44) provide a means for illustrating how pharmacokinetic substituent effects might be assessed. The steady-state plasma levels following 250 mg./hr. intravenous infusions, as reported in their studies, are given in Table III. The ratio of these steady-state levels is quite similar to the previously discussed results; the dicloxacillin–cloxacillin–oxacillin ratio is 2.6:1.7:1. Why are these plasma levels different? There are three parameters that may be examined: Vd, β_{ex}, and β_{met}. The values for Vd have been calculated from the equation (14, 40):

$$Vd = k_0/\beta P_{ss} \qquad \text{(Eq. 4)}$$

and results are given in Table III. Now it can be concluded that the dicloxacillin–cloxacillin steady-state plasma ratio of 1.7 cannot be attributed to differences in distribution since the Vd values are the same. The values for β have been calculated using Eq. 1. It can now be concluded from the values for P_{ss}, Vd, and β that plasma level differences and their reasons are: dicloxacillin > cloxacillin due to decreased elimination; cloxacillin > oxacillin due to decreased Vd; dicloxacillin > oxacillin due to both decreased elimination and Vd.

It is generally agreed that extending the duration of penicillin blood levels represents a clinical advantage, especially since these antibiotics have such short $t_{1/2}$ values (42, 44). Dicloxacillin has the longest $t_{1/2}$ (smallest β) of the isoxazolyl penicillins in Table III. The addition of two chlorine atoms to oxacillin has thus extended its duration. But why has this occurred? It is necessary to determine the mechanism responsible for the change if one is to develop a rational approach to further molecular modification.

The total elimination constant, β, can be divided into renal, β_{ex}, and nonrenal, β_{met}, components using the data for the fraction excreted in the urine (44). The value for β_{met} appears to decrease with the addition of chlorine atoms to oxacillin (Table III). A similar trend can be observed with the values for renal clearance. Both renal and nonrenal excretion decrease with increasing lipophilicity. Nayler (56) suggested that active transport systems for organic anions in the kidney tubules and the liver represent the most important pathways for elimination of penicillins. Since both tubular secretion and inactivation involve passage across lipid membranes, there should be an optimum partition coefficient (56). The isoxazolyl penicillins have the most hydrophobic side chains of all penicillins in clinical use and presumably have partition coefficients in excess of the optimum (56). These observations are somewhat supported by the fact that penicillin G has a renal clearance value near 400 ml./min., which approaches the renal plasma flow rate of 650 ml./min. Addition of chlorine atoms to the already lipophilic oxacillin further depresses the clearance value from 226 to 113, which is roughly the value for glomerular filtration (14).

The decrease in metabolism of cloxacillin as compared to oxacillin might have been overlooked if a comparison of $t_{1/2}$ (or β) were made. The single chlorine atom appears to have no effect on excretion when $t_{1/2}$ is used as the sole criterion. Yet the ratio of renal clearance values is 226:162, indicating that oxacillin is cleared 1.4 times faster than cloxacillin by the kidney. The $t_{1/2}$ values are the same, since this parameter represents the time to remove half of the drug from the entire body, and the ratio of the Vd of oxacillin to cloxacillin is 14:10 or 1.4 times larger so that the difference in renal clearance is offset by the increase in Vd. From a structural point of view, the additional chlorine atom has markedly depressed renal clearance.

Similar analyses can be extended to other penicillins. Differences in steady-state plasma levels of related penicillins are frequently attributed solely to their rates of elimination (42, 44, 49, 56). Table IV summarizes the observed steady-state plasma levels following constant intravenous infusion of 500 mg./hr. The values for the elimination rate constant, β, are also given, together with the predicted plasma level rank order if only elimination is considered. Since infusion rate is constant, the lowest elimination rate would result in the highest plasma level, *etc.* A similar ranking is given using only the Vd as a criterion. Here it is assumed that larger volumes will give more dilute blood samples and thus lower levels. Neither of these approaches is sufficient to account for the observed ranking. A few simple comparisons can be made. For example, the rank order of carbenicillin > dicloxacillin > cloxacillin can be attributed solely to elimination differences. Conversely, cloxacillin levels are higher than oxacillin due to distribution. The correct ranking for all penicillins in Table IV can be obtained by calculating values for P_{ss} using Eq. 4. Results are in good agreement with observed values, illustrating that both Vd and β must be considered to compare blood levels of analogs.

One problem that continues to confuse the issue when comparing various antibiotic derivatives is their difference in protein binding. The binding of drugs to plasma protein was discussed in an excellent review by Meyer and Guttman (57) and will not be repeated here. It should be emphasized, however, that the significance of protein binding (as well as tissue binding) in relation to therapeutic efficacy remains unclear for

Table IV—Observed and Predicted Rank Orders (Highest to Lowest) for Plasma Steady-State Concentration of Various Penicillins during Constant Intravenous Infusion of 500 mg./hr. (See Text for Explanation)

Penicillin	β, hr.$^{-1}$	Rank	*Vd*, l.	Rank	P_{ss}, mg./l. Calc.[a]	P_{ss}, mg./l. Obs.	Rank	Reference
Carbenicillin	0.69	1	10	1	72	73	1	42
Dicloxacillin	0.98	3	10	1	51	51	2	44[b]
Cloxacillin	1.65	6	10	1	30	30	3	44[b]
Ampicillin	0.71	2	25	4	28	29	3	49
Oxacillin	1.78[c]	7	14[c]	2	19	19	4	44,45[b]
Nafcillin	1.26	5	21	3	19	18	4	45
Penicillin G	1.13	4	24[d]	4	18	16	5	49

[a] P_{ss} = (500 mg./hr.) ÷ (β)(*Vd*). [b] Observed P_{ss} × 2 to correct for infusion rate of 250 mg./hr. [c] Average value from *References 44* and *45*. [d] Average value from *References 41* and *42*.

many antibiotics. Warren (27), in his thorough review of that subject, points out that readily reversible binding to serum or tissues can serve as a pool of active antibiotic to prolong therapeutic levels. In contrast, several authors who hold the position that penicillins bound to proteins lose their activity are cited in that review. The ultimate criteria in comparing serum levels of derivatives must always be based on how much is required to treat the disease in question. A derivative producing a plasma level twice as high as the original drug is insignificant if 10 times the original is required. Warren contended that absorption, distribution, and inactivation may be more important than binding in determining penicillin therapy, presumably due to reversibility.

Kunin (58) demonstrated that the MIC of eight penicillins in human serum is the same as that in broth when corrected for the bound fraction. That report stresses a decrease in bioavailability of penicillins by inactivation through protein binding. Methicillin, the least active in broth, not only required the smallest concentration to kill more than 99% of inoculum in serum but it was also more rapid than any other penicillin. A decrease in distribution of penicillins to tissues was also predicted as an expected result of the inability of bound penicillin to diffuse from the plasma. The work of Dittert *et al.* (41) and Doluisio *et al.* (59) does not support the latter hypothesis, since similar values for *Vd* were obtained for four penicillins whose percent binding in plasma varied from 22 to 94 (58). The fraction of the dose in the peripheral compartment was considered similar for all five penicillins studied (41, 59). One report suggests that the antibacterial activity of three long-acting sulfonamides is independent of their protein binding but that this binding determines their duration (60).

While the significance of protein binding of penicillins is still in dispute, there appears to be some agreement that reversibility of bound drug is the key factor. It has been noted and documented by authors with seemingly opposing views that reversible binding can serve to prolong drug action (27, 58) whereas only irreversible binding removes drug from the biophase. It has been suggested that plasma protein binding will not affect the $t_{1/2}$ if a drug is cleared by secretion in kidney tubules, but increased binding may prolong the $t_{1/2}$ of drug excreted solely by glomerular filtration (61). It would appear, from present evidence, that the significance is probably dependent upon the strength of binding. It was previously calculated that protein binding will affect drug distribution only if the binding constant exceeds 10^4 (57).

Tetracyclines—Development of tetracycline analogs may be considered as illustrative of research aimed at achieving the goals listed previously under *Optimizing Parameters*. It became apparent soon after the introduction of chlortetracycline in 1948 that concurrent administration of aluminum hydroxide gel resulted in decreased bacteriological activity for the antibiotic (62). Decreased GI absorption due to complexation with divalent and trivalent cations such as calcium, aluminum, and magnesium precluded coadministration with milk or antacids to reduce the known potential for anorexia, nausea, vomiting, *etc.*, observed during tetracycline therapy. Development of tetracyclines with a decreased propensity for complexation thus became a clinically significant research goal. Some measure of success has been realized with the newer tetracyclines. Demethylchlortetracycline (demeclocycline) blood levels, for example, are extremely sensitive to milk and aluminum hydroxide gel (63). By using those data, it was calculated that, relative to equal doses in the fasting state, only 13% oral absorption takes place when the antibiotic is administered with 240 ml. (8 oz.) of milk and 22% oral absorption takes place with 20 ml. of aluminum hydroxide gel (14). The data of Rosenblatt *et al.* (64) can be treated in a similar fashion, with the results showing that doxycycline absorption with food and milk is reduced to about 50% whereas demethylchlortetracycline is absorbed about 25% when both are compared to the fasting state during an 8-hr. period following ingestion of 100 and 300 mg., respectively. This comparison may reflect initial absorption since elimination was not completed. Ingestion of antacids containing divalent and trivalent cations resulted in negligible absorption for both drugs.

A similar result, although more pronounced, was noted by Schach von Wittenau and Twomey (65) in their discussion of unpublished work. When doxycycline was administered orally to humans together with aluminum hydroxide gel, the observed plasma concentrations (at an unspecified time) were reduced to 10% of normal. In another report, doxycycline plasma levels following administration with milk were not notably different from those in either the fasting state or when administered with food (66). However, comparisons were made only at 1, 3, and 12 hr. on Day

1 and at 0 and 12 hr. on Day 5 of a twice-a-day regimen. Thus, insufficient sampling could mask differences in absorption. Adequate data should include several values before and after the plasma maximum so that areas under the curves could be compared to establish relative bioavailability in each case.

The extension of biological $t_{1/2}$ to increase duration and decrease frequency of administration was reviewed by Fabre *et al.* (67) as follows. Chlortetracycline first appeared in 1948 with $t_{1/2}$ of 5.6 hr. and a dosage interval of 6 hr. Oxytetracycline and tetracycline soon followed with half-lives of 8.2 and 9.2 hr., respectively, and thus slightly longer durations. In 1958, demethylchlortetracycline ($t_{1/2}$ of 11.8 hr.) and later methacycline were introduced as useful on a 12-hr. regimen. Doxycycline ($t_{1/2}$ of 18–22 hr.) marked a new stage in this evolution with the advent of once-a-day therapy.

This discussion was published in 1967 and was most encouraging to those interested in improvement of tetracyclines by molecular modification. However, the $t_{1/2}$ values employed for the discussion do not appear to be unequivocal. Doluisio and Dittert (68) redetermined the $t_{1/2}$ values for several tetracyclines and compared their results with those previously reported (Table V). Comparison of the $t_{1/2}$ values following a single oral dose to those determined during steady-state plasma levels within the same study indicates that the $t_{1/2}$ of each of the four antibiotics increased significantly during the 6-day study. It is also apparent that the $t_{1/2}$ values for the four tetracyclines do not vary as much as indicated in the earlier review (67). The range of the four $t_{1/2}$ values in the steady state, for example, is only 14 ± 3 hr. Therapy with an antibiotic drug involves the administration of multiple doses and not just a single dose. The values obtained during multiple dosing would therefore appear to be more significant in clinical use of the drugs. Doluisio and Dittert demonstrated that $t_{1/2}$ values from single-dose studies do not predict steady-state serum levels of tetracyclines during multiple dosing whereas the steady-state $t_{1/2}$ values adequately describe their data.

Many factors can alter the $t_{1/2}$ value for a given drug and these were reviewed elsewhere (69, 70). The simplest equation that defines the value for β in a manner independent of the kinetic model may be written:

$$\beta = f_c k_2 \qquad \text{(Eq. 5)}$$

where f_c is the fraction of drug in the central compartment under postdistributive conditions (71). The $t_{1/2}$ value for a drug distributed according to a one-compartment model would be expected to be independent of the mode of administration since f_c is constant and equal to $1/(1 + K)$, so that:

$$t_{1/2} = 0.693/\beta = 0.693(K + 1)/k_2 \qquad \text{(Eq. 6)}$$

where:

$$K = k_{12}/k_{21} = T/P \qquad \text{(Eq. 7)}$$

Conversely, a two-compartment drug does not achieve preequilibrium following rapid intravenous injection and its $t_{1/2}$ cannot, therefore, be defined by a simple equation employing the equilibrium constant, K. In this case the ratio of drug in the tissues to that in the central compartment during the β-phase is larger than that predicted by assuming the equilibrium condition described in Eq. 7 (14). Under steady-state conditions imposed during constant-rate intravenous infusion, the tissue–plasma ratio again becomes defined by Eq. 7. Thus the fraction, f_c, can be shown to be larger during steady-state conditions than it is during the β-phase of a single intravenous dose of drug whose distribution is not instantaneous (71). Gibaldi (72) estimated that the T/P ratio for a single dose of oxacillin would be about twice that achieved during infusion equilibrium. Since β is directly proportional to f_c (Eq. 5), the value for β_{ss} would be larger than the value obtained from a single dose of a two-compartment drug. During steady-state plasma levels, the value of f_c becomes constant and so does $t_{1/2_{ss}}$, which is then defined by Eq. 6. Thus, the $t_{1/2}$ that is operative during the steady-state would be expected to be shorter than that observed following a single dose. Therefore, the increased values for $t_{1/2}$ observed during continuous dosing (*Reference 68* also cites two similar previous reports for doxycycline) cannot be explained from the standpoint of approaching steady-state equilibrium.

Table V—Literature Values for Apparent Biological Half-Lives (in Hours) for Tetracyclines[a]

Drug	Single Dose	Repeated Dosing: Days 5–6	Steady State
Tetracycline	6.3[b], 5.6–9.3, 8.2, 8, 7.2, 7.3	10[b], 9.5, 11	10.8[b]
Demethylchlortetracycline (Demeclocycline)	9.0[b], 6.3–13.3, 12.6, 10, 11, 12.7	14.7[b], 14.7, 15	13.6[b]
Methacycline	7.0[b], 14.3, 8.5, 9.6, 7.7	11.0[b], 10.5, 11.5	14.3[b]
Doxycycline	8.3[b], 11.7, 15, 15.1	14.5[b], 22[c]	16.6[b]

[a] Taken from *Reference 68.* [b] Determined in *Reference 68.* [c] This value appears to be incorrect since predicted steady-state serum levels using $t_{1/2}$ = 22 hr. do not agree with experimentally observed values.

It has been suggested that the increased $t_{1/2}$ values might arise from a failure to obtain an accurate assessment of the terminal (or β) slope following a single oral dose (73). Inclusion of part of the distribution phase could tend to make the $t_{1/2}$ appear smaller with a single dose whereas this problem is eliminated during the steady state. This behavior was illustrated using a hypothetical three-compartment model (73). However, it would also apply to a two-compartment case which might be appropriate for assessing tetracyclines (74).

A significant observation is the fact that all four tetracyclines have reasonably similar steady-state $t_{1/2}$ values (Table V). Since the sizes of the doses were varied, it was observed that all four drugs produced comparable serum levels when administered every 12 hr. This is easily understood in terms of what may now be considered classical approaches to dosage regimen development. Schumacher (75) reviewed the methodology employed in calculating a dosage regimen in an applied paper that illustrates the use of the techniques on tetracyclines and penicillins. Five approaches are outlined, one of which may be described as using a fixed dosage interval equal to the $t_{1/2}$ and varying the dosage size to achieve the desired plasma concentrations during the steady state. In the study under consideration (68), the doses of the analogs were varied and the dosage

Table VI—Comparison of Reported Plasma Levels for Tetracyclines

	Dose[a], mg.	P_{max}[a], mcg./ml.	Dosage Regimen[b], mg./Δhr.	P[c,d], mcg./ml. Max.[c,d]	Min.[c,d]	Max.[e,f]	Max.[e,g]
Tetracycline	500	3.25[h]	500/12	3.5	1.9	4.0	1.9
Demethylchlortetracycline (Demeclocycline)	300	1.74[h]	300/12	2.8	1.5	2.7	1.2
Methacycline	300	2.40[h]	300/12	2.2	1.2	2.3	0.7
Doxycycline	150	1.49[i]	100/12 (Day 1) then 50/12	2.1	1.2	2.51	1.5

[a] Taken from *Reference 77*. [b] Administered during a 4-day period. [c] Taken from *Reference 68*. [d] Steady-state levels. [e] Taken from *Reference 76*. [f] Determined at peak. [g] Highest value obtained during first 12 hr. [h] Determined 4 hr. after single dose. May not be true maximum. [i] First determination at 2 hr. is highest.

interval was held constant at 12 hr. It was fortuitous that this interval also approximated the values determined for the steady-state half-lives. Thus, the regimen may be considered as one of a fixed interval equal to the $t_{1/2}$ with variable dose. Statistical analysis showed that levels of tetracycline were higher than doxycycline and methacycline but similar to demethylchlortetracycline after 4 days of therapy. The authors concluded that all four drugs produced comparable levels of antibiotic activity when administered every 12 hr. in their recommended doses.

A similar protocol was employed in a comparison of plasma concentrations which did not employ any pharmacokinetic analyses. The maximum observed plasma values in that study (76) are in excellent agreement with those previously reported (68). However, the maximum observed values obtained during the first 12 hr. appear to be lower than those reported for a single oral dose (77), except for the case of doxycycline which gave equal maximum plasma levels despite a decreased dose (Table VI). Minocycline was reported to yield higher plasma levels than the other tetracyclines (77), but the fact that the fraction of the dose recovered in the urine is smaller than the others attests to the necessity of examining the effect of nonabsorption factors when comparing plasma levels of different chemical entities.

Considerable effort has been applied to examining the distribution of various tetracyclines into body tissues (78–83). The ratio of total tetracycline in muscle to free concentration in serum in dogs has been correlated with chloroform–water partition coefficients (78, 79). Doxycycline had both the highest distribution and partition coefficient of the marketed tetracyclines. The order of values of the tetracyclines studied was demethyldeoxychlortetracycline ≫ doxycycline ≫ tetracycline > demethylchlortetracycline = methacycline ≫ oxytetracycline. Concentrations of tetracycline, demethylchlortetracycline, and chlortetracycline were compared in no less than 47 organs, tissues, or fluids in dogs using radiolabeled compounds (80). Results indicate that all nonfat tissues are penetrated by the antibiotics within 4.5 hr. after intravenous injection, with tissue distribution of tetracycline being generally less than that of chlortetracycline and demethylchlortetracycline. Increased lipophilicity of doxycycline allegedly increased oral absorption (66). Computer analysis showed an absorption $t_{1/2}$ of 28 min., with 80% of the administered dose absorbed within 1 hr.

Doxycycline content in 12 organs sampled operatively in 81 patients was determined with concomitant serum levels, and the tissue to serum ratio was determined for each organ (81). These ratios varied from approximately 2 for kidneys, lungs, and bladder to less than 0.7 for appendix and adipose tissue. When corrected for binding, these ratios became 13.4 for kidneys, 12.2 for lungs, 6.3 for muscle, and 3.9 for lymph node. The concentration in all organs (usually 2–4 mcg./g. and always above 1 mcg./g.) was considered to be amply bacteriostatic. Other reports on distribution of tetracyclines in human organs were indicative of good tissue penetration in general, with ratios for tissue to plasma of 1 (oxytetracycline in gallbladder, methacycline in muscles and lungs) and 0.77 (demeclocycline in uterus) (81). Fat solubility was claimed to play a major role in influencing distribution of tetracyclines into tissues, and serum protein binding appeared to be less of a factor. Tetracyclines permeate both extracellular fluid and intracellular fluid. Calculated values for the apparent volume of distribution in man exceed the volume of total body water (84) and probably reflect protein binding. For example, the following values were reported (results in percent v/w as given in *References 24* and *83*, respectively): chlortetracycline, 92, 148; demethylchlortetracycline, —, 179; oxytetracycline, 90, 189; and tetracycline, 95, 159.

Tetracyclines tend to accumulate during renal insufficiency. Chlortetracycline and doxycycline are notable exceptions; chlortetracycline is excreted primarily in the bile. However, doxycycline urinary clearance decreases during renal insufficiency without a concurrent increase in hepatic excretion despite a relatively constant $t_{1/2}$ in normal and anuric patients. It was suggested that renal insufficiency is accompanied by a compensatory increase in inactivation of doxycycline microbial activity (81). More recent work negated this possibility (65). Studies into the extent of enzymatic degradation (or metabolism) of doxycycline in man and dog were conducted in response to the question of why doxycycline does not appear to accumulate during renal insufficiency. More than 90% of intact drug was recovered from urine and feces in both species. It was concluded that excretion of intact doxycycline in feces probably compensates for decreased renal excretion in uremic patients.

The $t_{1/2}$ appears to be inversely related to protein binding only for those drugs that are primarily cleared by glomerular filtration. In this case the concentration of drug in the filtrate would be expected to be equal to the concentration of free drug in the blood. However, a drug that is excreted by tubular secretion does not appear to be influenced by protein binding, as illus-

trated previously when penicillins were discussed. Penicillins have very high clearance values reflecting tubular secretion; for that reason, their $t_{1/2}$ values are relatively insensitive to protein binding. Conversely, it has been stated that the renal clearance of tetracyclines decreases with increased protein binding (81). Urinary clearance is greatest for oxytetracycline (73% free) and least for doxycycline (18% free), with chlortetracycline and minocycline being considered as exceptions since they are mainly excreted in the bile. Data suggest that tetracyclines are primarily excreted by glomerular filtration rather than tubular secretion, although the lack of precise serum protein binding measurements makes it difficult to calculate individual contributions.

OPTIMIZING ORAL ABSORPTION BY CHEMICAL MODIFICATION

pH–Partition Effects—The pH–partition hypothesis for GI drug absorption was first proposed in 1957 (85, 86). The basic principles are now discussed in many texts and are not reviewed here. A few early reviews summarize the pertinent data (87–89).

Basically this hypothesis predicts that absorption of drugs occurs in the unionized form and that increased lipophilicity increases absorption. Undoubtedly, many compounds behave according to this hypothesis, but many exceptions have been reported within the last decade. Levine (90) reviewed some of the exceptions. For example, phenobarbital and pentobarbital, acids of pKa 7.2 and 8.1, respectively, would be predicted to be absorbed more rapidly from the stomach than the intestines. Instead, 2–3 times as much is absorbed from the intestines during 10 min. than is absorbed from the stomach in 1 hr.

Rate constants for absorption of both ionized and unionized species of sulfaethidole and barbital were calculated using the *in situ* rat stomach and rat small intestinal lumen (91). The ratio of the first-order rate constant for the neutral species to that of the ionized form was roughly 5 for sulfonamide and 3 for barbital. Consequently, at pH 6.1, 42% of the absorption occurs as the ionized form.

Linear Relationships—Quantitative relationships that predict structural effects on GI absorption would be most valuable for *a priori* "tailoring" of physicochemical properties by the medicinal chemist. While this is not yet a reality, considerable progress has been made in the form of linear relationships that are predictive for a given series.

Beckett and coworkers applied their buccal absorption techniques to the evaluation of amines (92), amphetamines (93), alkyl-substituted acids (94), series of amines and acids (95), substituted phenylacetic acids (96), and a series of carboxylic acids (97). Several linear relationships were reported. Studies designed to evaluate structural effects on partition coefficients and absorption determined the buccal absorption of nine aliphatic acids of chain lengths from butyric to dodecanoic as a function of pH. Absorption was shown to be passive and increased by low pH and increasing chain length (94). Rate constants for buccal absorption of 10 carboxylic acids from solutions at pH 4 were determined in a single subject. Rate constants, expressed as clearance values, correlated (correlation coefficient = 0.89) with previously determined *n*-heptane–1 *N* HCl partition coefficient values (97). Substituted benzoic acids were subject to decreased absorption when pKa decreased. A linear relationship was found between log partition coefficient and the buccal absorption of a series of amines and acids under conditions of 1 and 10% ionization. The plot of alkyl chain length and either log partition coefficient or buccal absorption was also linear for amphetamines and 10 fluramines at 1% unionized conditions (95, 96). Stehle (98) had earlier calculated that the log transfer rate through a lipoidal barrier would be expected to increase linearly with log partition coefficient until a maximum constant value was approached.

Ho and Higuchi (99) used the previously reported data (92–94) to test a diffusional model for buccal absorption considering both the pKa and partition coefficient. They determined a factor of 2.3 per methylene group upon the incremental partition constant for the buccal lipoidal membrane–aqueous environment. Later this same model was satisfactorily tested on data for buccal absorption of nonionized species, thus providing further evidence for the applicability of the diffusion model proposed earlier (99) and for the significance of the diffusion layer in buccal transport (100).

Hansch and Dunn (101) recently reviewed the methods of linear free energy calculations (generally bearing Hansch's name) for correlating physicochemical properties with biological response[1]. The partition coefficient of drug derivatives can be calculated by the Hansch treatment of π additivity values for the *n*-octanol–water distribution. Flynn (102) showed that π values for a given substituent on a steroid are position dependent but can be used successfully once the contributions are known for the position in question. The log of the water solubility of 156 liquid organic compounds was shown to be inversely related to the log partition coefficient (103). Linear plots were obtained for log solubility *versus* π values using various alkyl esters of testosterones (104), substituted phenothiazines (105), and *p*-hydroxybenzoate esters (106). The slopes of the lines for these solids varied from approximately 2.4 to 3.1 per methylene group.

In an excellent study and discussion, Flynn and Yalkowsky (107) examined the effect of alkyl chain length for a series of *p*-aminobenzoate esters on physicochemical properties and their resultant ability to penetrate inert barriers. Their model, which is based on solubility-limited diffusion through a membrane diffusion layer composite barrier, predicts a parabolic dependency as the homologous series is ascended, and both the controllable and uncontrollable factors in designing optimum chain length for absorption are discussed. A linear relationship of unit slope was found between log of intrinsic partition coefficient values and extraction constants of ion-pairs for protonated chlorides of *p*-alkylpyridines (108). Methylene increments were concluded to be similar for the free base and the

[1] The parabolic case has now been reviewed by C. Hansch and J. M. Clayton, *J. Pharm. Sci.*, **62**, 1(1973).

ion-pair due to the distance of the alkyl substituent from the polar portion of the molecule.

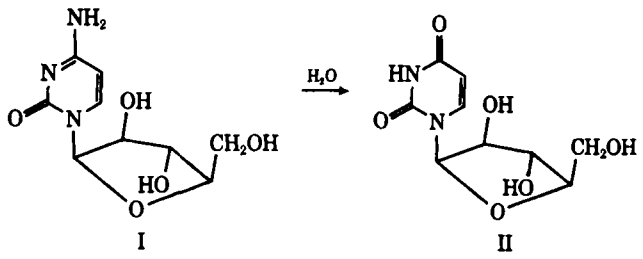
In studies involving more than 50 chemical compounds, Nogami *et al.* (109, 110) examined relationships between chemical structure and absorption rate. Their results indicate that the absorption rate constant can be predicted from an additivity rule and that the values for a given combination of interacting groups are constant and referred to as the "intramolecular interaction constant." Recently, the absorption of pralidoxime methanesulfonate was compared to pralidoxime hydrochloride following oral administration to healthy young men (111). Plasma concentrations were considered equivalent on a molar basis.

Morozowich (112) suggested that an increasing partition coefficient can affect not only absorption characteristics but also distribution in lipids and ultimately the biological $t_{1/2}$. He cited as an example the 3-cyclopentyl ether of 17α-ethinyl estradiol which has been reported to exert an estrogenic effect which lasts for 3 months after oral or intramuscular administration of 5 mg. (113).

PRODRUGS: BIOREVERSIBLE DERIVATIVES

Nucleosides—The first report describing a chemical modification of a nucleoside that dramatically increased oral absorption in humans appeared in 1961 (114). Psicofuranine was not significantly absorbed from the GI tract in humans. Acetate esters of the sugar hydroxyls were prepared, and the tetraacetate was chosen for human trials based upon observed efficacy equivalent to the parent compound compared subcutaneously in mice. Although no detectable levels could be determined after oral administration of psicofuranine in humans, its tetraacetate was well absorbed. Assays with and without preliminary hydrolysis indicated that the tetraacetate was rapidly converted to free psicofuranine in the blood. The outstanding difference in physicochemical properties between the drug and its prodrug appears to be the enhanced lipophilicity in the ester. The water–chloroform partition coefficient was 992 for psicofuranine and 0.041 for the tetraacetate. This increased lipophilicity was deemed responsible for the increased absorption.

It is quite common to associate the term bioavailability with absorbability from an oral dosage form. This is probably a natural result of the overwhelming research activity in that area. However, three additional rate processes in Scheme IV can be involved in limiting bioavailability. These are indicated by the constants k_{12}, k_{21}, and k_2. This may be said of any drug since its degree of success is dependent upon its ability to reach the site of action and the resultant time course at that site. This point was illustrated by Skipper *et al.* (115) using animal tumor systems *in vivo*. In the case of arabinosylcytosine, its bioavailability may be said to be limited by k_2 (in this case, metabolism) which, in turn, limits its effectiveness. After pharmacokinetic studies successfully defined this problem, therapy was improved by appropriate dosage regimens and derivatives. The details of this research are summarized here as an illustrative example of the potential significance of pharmacokinetics in drug design.



Scheme V

1-β-D-Arabinofuranosylcytosine (I, Scheme V), a cytotoxic agent, exerts antitumor and antiviral activity in a variety of animal and human neoplasms. It is highly effective for the treatment of acute myelogenous leukemia in man (116, 117). The greatest response in a single disease was observed in acute granulocytic leukemia where 35 of 144 adequately treated patients (or 24%) achieved complete or partial remissions (117, 118) and, more recently, 21 out of 49 patients (43%) attained complete remission status through rapid drug injection followed by continuous drug infusion to maintain steady-state therapy for 4 hr./day (119).

This somewhat encouraging remission rate is rather astonishing when one considers the brief duration of bioavailability of this drug. When injected intravenously in human subjects, the resulting blood level data exhibit biphasic first-order plots (120) characteristic of a two-compartment open model. The initial loss from the blood following rapid intravenous injection is extremely fast, has a $t_{1/2}$ of approximately 12 min. (120–122), and may be attributed to simultaneous distribution and elimination. At the end of the initial phase (or α-phase), over 80% of the drug remaining in blood and urine is in the form of a single inactive metabolite, 1-β-D-arabinofuranosyluracil (II, Scheme V), indicating both rapid and extensive deamination during the distribution phase (120).

It is well known that cytosine nucleosides undergo hydrolytic deamination to their corresponding uracils and this may be catalyzed enzymatically (123, 124) or chemically (125–131). The elimination phase (or β-phase) has a half-life of approximately 111 min. and results in nearly complete conversion of I to II (120) *via* catalysis by pyrimidine nucleoside deaminase (123). Recovery of the metabolite in the urine was 86–96% of the total 48-hr. recovery in one study (122) and 90% of the total 24-hr. recovery (representing 80% of the total dose) in another (120). Uptake of I by red blood cells is rapid and within 5 min. cells attained 60% of the concentration of the plasma (120). However, the half-life within the cell is only 2–3 min. Thus, I exhibits a relatively short duration of bioavailability and its loss is almost entirely due to deamination to II, which is apparently inactive as an inhibitor of cell growth (132).

It is reasonable to expect that an increase in the biological duration of I might lead to increased remission rates. Evidence for this hypothesis already exists in the work of Baguley and Falkenhaug (133) who measured plasma $t_{1/2}$ of I following 30-min. intravenous infusions in leukemic patients. Patients who experienced complete remissions had significantly longer $t_{1/2}$ values than those who did not respond to therapy, and this

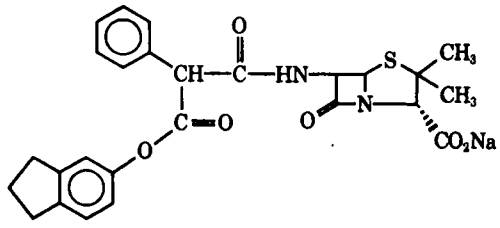
variation was presumably due to variations in deaminase activity. These results suggest that degradation of I may cause failure of the drug to produce hematologic remission in certain patients (133). Further support for this proposal may be taken from the demonstration that the dosage regimen markedly affects its therapeutic index. For example, superior results were obtained using either constant intravenous infusion or injections every 8 hr. compared to single daily injections (134–136). The most effective present schedule appears to be 200 mg./m.²/day given by continuous intravenous infusion (116), which provides steady-state plasma levels of approximately 0.15 mcg./ml. (120).

Another approach to increasing duration is that of slow release of I from a poorly soluble prodrug. A single dose of 1-β-D-arabinofuranosylcytosine-5′-adamantoate was nearly as effective as an optimum schedule of I given every 3 hr. by injection to L-1210 leukemic mice. The duration of cytotoxic plasma levels of I was greatly increased by administration of the prodrug (137). Gray *et al.* (138) showed that several 5′-acylates were equal to or better than the activity of the 5′-adamantoate. These derivatives appear to be more stable to pyrimidine nucleoside deaminase and capable of yielding I upon hydrolysis in the blood.

Since pharmacokinetic studies indicate rapid loss of I to inactive metabolite *via* enzymatic deamination, the design of new antimetabolites which are more stable and potentially more effective has evolved. Cyclocytidine, effective against L-1210 leukemia (139), was synthesized from cytidine (140, 141). It appeared less toxic and more effective than I administered once daily for 5 days in L-1210 leukemic mice (141). If hydrolyzed *in vivo*, it would yield I and act as a prodrug. It is reportedly more resistant to deaminase.

Additional studies have been carried out on nonbioreversible derivatives with the intent of establishing increased stability to deamination. For example, Panzica *et al.* (142) determined that 1-β-D-arabinofuranosylcytosine-3-*N*-oxide is a potential inhibitor of lymphoid leukemia L-1210. Although it is designed to resist deamination, no enzyme stability studies have been reported as yet.

Studies on the effects of substituent groups on the chemical stability of cytosines to hydrolytic deamination and the possibility that chemical reactivity might be related to the enzyme-catalyzed transformations have been initiated (125, 126, 131). These investigations are designed to provide a systematic study of substituent group effects on the chemical hydrolytic deamination of cytosines and to apply these findings to enzymatic studies with the ultimate goal of extending the biological duration of I or one of its analogs. The effect of the sugar on deamination was previously studied by comparing the deamination kinetics of three cytosines (126). A major difference in the case of I is that of intramolecular attack by the 2′-hydroxyl of the sugar on the C-6 position in the pyrimidine ring. However, support for a common deamination pathway is evidenced by the nucleophilic addition of bisulfite HSO_3^- to C-6 of various cytosine nucleosides (129). Since addition at the C-6 position is involved in the chemical catalysis of deamination, appropriate substitution at



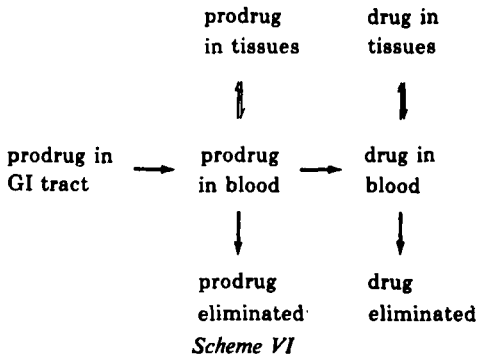
III

that position should hinder catalysis and thereby decrease the deamination rate. To test this hypothesis, three derivatives (1-β-D-arabinofuranosyl-6-methylcytosine, 1-β-D-ribofuranosyl-6-methylcytosine, and 6-methylcytosine) were synthesized and the kinetics of their transformations in aqueous buffer were compared to the unsubstituted compounds (131). Results of the comparisons were only partly as predicted. While 6-methylcytosine was more stable to deamination than cytosine, the substituted nucleosides were found to undergo competing reactions which were not observed for the parent compounds that underwent deamination under identical conditions. The net result is an increased stability for 6-methylcytosine but an increased rate of loss for the 6-methylnucleosides. Relative stability to deaminase has not yet been reported, and biological testing has not been carried out.

Antibiotic Prodrugs—There are many possible reasons for developing prodrugs, and several successful examples of antibiotic prodrugs may be cited to illustrate some advantages. Carbenicillin was initially marketed only as an injectable due to its poor oral bioavailability resulting from poor absorption. This was presumably due to either its high polarity (it is a dicarboxylic acid) or acid instability or both. Recently, studies have indicated that the prodrug carbenicillin indanyl sodium (III) is orally absorbed and active (143–147) and it is being marketed as such.

Hetacillin is a prodrug of ampicillin, which may be considered as a condensation product of acetone and ampicillin. Once absorbed, hetacillin is rapidly converted to ampicillin (148). Both are marketed in oral dosage forms as well as in dry powder form for reconstitution before parenteral use. Solutions of hetacillin have been shown to be more stable (149). Six hours was required for 10% loss of activity at room temperature, whereas ampicillin itself showed 10% loss in 1 hr. under similar conditions. There is an advantage of extended shelflife to the user of reconstituted parenteral hetacillin. The oral absorption of hetacillin may be somewhat greater than that of ampicillin based on urinary recovery data (150, 151), although one study reported decreased urinary recovery with the prodrug (152). There have been a number of comparisons based on the plasma concentration time-profiles, but the meaning of these results is equivocal since assays often failed to differentiate between drug and prodrug. This problem will be discussed later in the section dealing with potential pitfalls in data interpretation.

One reason for prodrug formation is to increase stability in the GI tract and thereby increase bioavailability. Erythromycin is rapidly destroyed at pH values less than 4, and numerous esters have been synthesized



Scheme VI

with the goal of increasing stability and, in turn, oral absorption. Lee *et al.* (153) examined the absorption of a series of erythromycin esters following oral administration in rats. Based on areas under the plasma curves, the acetate and propionate appeared to be better absorbed than the parent drug while the butyrate was absorbed markedly less. However, these curves may not be indicative of absorption differences alone since intact prodrug was present in significant amounts. Scheme VI illustrates the several rate processes that potentially affect the time course of drug and prodrug in plasma. If blood level curves represent total erythromycin (by hydrolysis of ester to erythromycin before assay), one cannot attribute differences to absorption without further data.

Erythromycin base has a pKa of approximately 8.9. It would be expected to be ionized throughout the entire GI tract, and intestinal absorption would be expected to be better than absorption from the stomach. In rats, erythromycin was most rapidly absorbed in the upper small intestines (153). Two prodrugs, designed to bypass the stomach, are in common use. Erythromycin stearate resists dissolution in the stomach but dissociates rapidly in the intestines to yield free erythromycin. Erythromycin estolate, by contrast, is probably absorbed largely as intact ester. Once in the blood the ester hydrolyzes to yield free erythromycin. The $t_{1/2}$ for hydrolysis in human serum was reported to be 93 min. (154).

The advantages of high serum levels of the propionyl ester prodrug have been a matter of controversy. In a four-way crossover study (fasting and nonfasting), blood levels obtained after single and multiple doses of erythromycin estolate and erythromycin stearate were compared (155). The estolate achieved higher blood levels at a faster rate both with and without food. However, the assay used was probably capable of hydrolyzing estolate esters, since the $t_{1/2}$ for *in vitro* hydrolysis has been reported to vary from 0.5 hr. at pH 8 to 5.0 hr. at pH 5 (156). Stephens *et al.* (157) reported 20–35% free base and 65–80% ester after the fifth dose of ester in humans, so that the average free base was higher than that obtained by administration of the salt.

Esters of lincomycin and clindamycin have been prepared in an effort to mask undesirable taste and maintain (or improve) oral absorption. Four lincomycin monoesters were compared for activity by subcutaneous and oral routes in mice (158). Earlier, 15 esters were compared in a similar manner (159). Sinkula *et al.* (160) reported comparative activity in mice for 16 clindamycin esters and their areas under plasma level–time profiles following oral and intramuscular injections in dogs. The 2-hexanoate, 2-laurate, and 2-palmitate appear to be absorbed as well or better than clindamycin itself when all are administered as hydrochloride salts at doses of 25 mg./kg. equivalent to clindamycin base. In contrast to the erythromycin estolate case already discussed, these esters apparently hydrolyze prior to absorption or immediately thereafter so that intact ester does not present a problem in comparing plasma level–time profiles. At least one product has been marketed from these efforts in the form of clindamycin palmitate hydrochloride flavored granules for oral suspension.

A pharmacokinetic study involving clindamycin and its palmitate in 52 children showed that mean serum levels of clindamycin were lower when administered as the palmitate in a single dose (161). It was concluded that rapid predictable absorption occurred using the palmitate, and neither drug accumulation nor increase in metabolism due to induction occurred. The 8–16-mg./kg./day doses were deemed adequate for the age group.

Related Publications—The publications on prodrugs and drug derivatives are too numerous to discuss completely in this review. Examples have been chosen (admittedly arbitrarily) to support the author's various theses. Several reviews consider, at least in part, the effects of molecular modification on pharmacokinetic properties (162–169). In this article, an attempt has been made to avoid repetition of discussions found in the previously published reviews.

In an excellent review of prodrugs and their properties, Sinkula (170) outlined some pharmaceutical reasons for drug modification. He listed the properties which may be altered through derivative formation as follows: (*a*) mask the taste and/or odor of bitter or obnoxious drugs, (*b*) increase or decrease aqueous solubility, (*c*) improve formulatability of drugs difficult to formulate, (*d*) increase stability, (*e*) decrease gastric and intestinal irritation, (*f*) decrease pain on injection, (*g*) improve depot action, (*h*) improve drug absorption, and (*i*) facilitate transport of drug to site of action.

Except for those cases where derivatives revert back to the parent drug before or immediately after absorption, the modification must be considered as potentially capable of affecting the absorption, distribution, and elimination kinetics in addition to the primary mission. Sinkula (170) surveyed a number of marketed drug derivatives and their stated reasons for the modification. Some of these are listed in Table VII.

PITFALLS IN DATA INTERPRETATION

Meaning of Parameters—Although it is imperative that the field of drug design and evaluation involves consideration of pharmacokinetic effects, it is equally imperative that the meaning and limitation of each parameter under comparison be understood. Earlier the values for *Vd* were compared at steady-state concentrations for three isoxazolyl penicillins. The steady state was chosen to avoid the effect of k_2 upon the values for *Vd*.

The calculation of *Vd* for a two-compartment model has been the subject of considerable confusion in the

Table I—Delivery Volume as a Function of Pressure

Liquid	Rate, ml./p.s.i. (10^3)	Total Volume Delivered, ml. 1400 p.s.i.	2100 p.s.i.	2800 p.s.i.
Calcium carbonate, 5%	6.7	10	14.7	19
Syrup USP	7.1	10	15	20
Water	6.8	12	16.8	21.5
Alcohol USP	7.1	13	18	23

EXPERIMENTAL

Materials and Equipment—The liquids used in this study were: distilled water; syrup USP; shellac, 2.7 kg. (6 lb.) cut; 5, 15, and 35% (w/v) aqueous suspensions of calcium carbonate; and a 2% (w/v) solution of methylcellulose[1], 500 cps.

The equipment utilized to evaluate the factors involved in an automated tablet-coating system was a self-programming automated tablet-coating system with two automatic spray guns[2] as previously described (4).

Fluid Delivery Studies—Fluid delivery experiments were conducted to ascertain the influence of pressure, spray tip orifice size, and type of liquid on the volume of spray delivered.

The volume of fluid delivery in milliliters was determined from 1400 to 2800 p.s.i. nozzle pressure in 140 p.s.i. pressure increments. The tip orifice sizes varied from 0.023 cm. (0.009 in.) to 0.053 cm. (0.021 in.) in diameter. All experiments were conducted in an air conditioned room at approximately 25° and 30–40% relative humidity.

Three-second fluid spray delivery cycles were used, and five replicate samples of each liquid under study were collected, at each pressure and nozzle tip size, in a 100-ml. graduated cylinder; the resulting volumes were recorded.

Spray Pattern Characteristics—Spray patterns were determined for the liquids studied, each containing 1% (w/v) FD&C Red No. 2 dye for contrast. The spray from the activated gun of the automated tablet-coating system was directed at a specially designed target which consisted of a 61-cm. square pane of glass with five concentric circles painted 10 cm. apart. The tip of the spray gun was positioned 30 cm. from the glass surface and at the center point of the target. The spray cycle was set for a 0.25-sec. solution delivery. The patterns were subsequently observed at 1400-, 1960-, and 2800-p.s.i. nozzle pressure (the lower, middle, and upper pressure range, respectively) for all tip sizes ranging from 0.023 cm. (0.009 in.) to 0.053 cm. (0.021 in.) in orifice diameter. After each spray, the pattern dimensions were taken and pattern uniformity was noted.

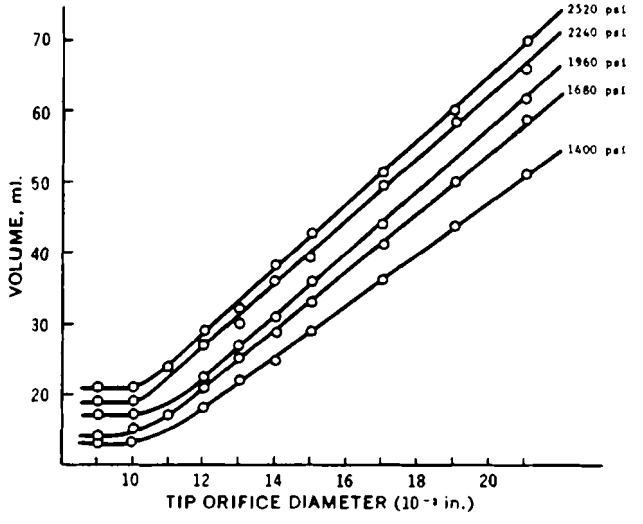


Figure 3—*Change in delivery with respect to change in tip orifice size using water.*

[1] Methocel MC, Dow Chemical Co., Midland, Mich.
[2] Graco Hydro-Spray Unit, Gray Co., Minneapolis, Minn.

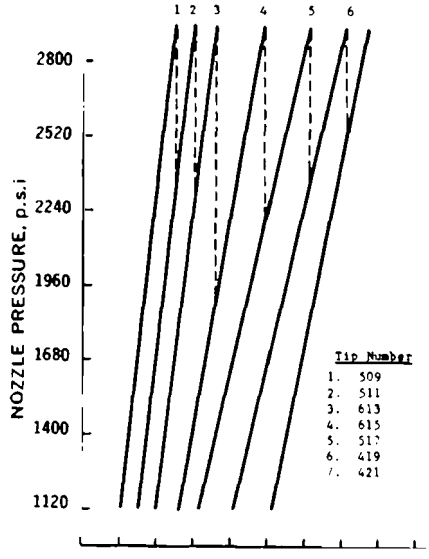


Figure 4—*Fluid delivery curves, 3-sec. cycle.*

RESULTS AND DISCUSSION

Fluid Delivery—The pressure–volume curves in Fig. 1, constructed from fluid delivery data obtained with a 0.023-cm. (0.009-in.) spray tip and a 3-sec. delivery, show that the change in fluid volume delivered as a function of pressure was constant. The curves are representative of some of the liquids studied, and each shows excellent linearity. The rate of change of delivery (Table I) for the liquids represented in Fig. 1 was essentially the same.

A 3–4-ml. difference in total volume delivered at each pressure increment was found between the four liquids studied. These small volume differences do not appear to be significant, but the data show a trend toward a decrease in volume delivered with an increase in fluid viscosity and solids content when comparing syrup and calcium carbonate with water and alcohol. A significant difference would be expected at a much lower or at atmospheric pressure. The data, therefore, indicate that fluid volume delivery is essentially independent of the physical properties of the fluid at very high pressures.

Fluid delivery curves for the three calcium carbonate suspensions, which varied from a "watery" to an "ointment-like" consistency (Fig. 2)[3], are in essence identical and confirm that the elevated pressures used in the study obliterated the effects of the physical properties of the fluids delivered.

In Fig. 3, the volume of water delivered in 3 sec. *versus* tip orifice diameter shows a good linear relationship within the range of 0.030 cm. (0.012 in.)–0.053 cm. (0.021 in.). This finding is unusual because one would expect an orifice–volume relationship based on

Figure 5—*Spray patterns with water. Left: 0.023-cm. (0.009-in.) tip size and 30 × 9-cm. pattern size. Right: 0.053-cm. (0.021-in.) tip size and 40 × 30-cm. pattern size.*

[3] Data points for Fig. 2 were omitted for the purpose of clarity.

of distribution in patients with renal failure as compared to normal patients (53, 176). Also, administration of probenecid, an inhibitor of renal tubular secretion of organic acids, reduces the apparent volume of distribution of penicillin derivatives (43, 176). This observation is not surprising in view of certain facts. Since the *Vd* does not necessarily represent a real volume, observed changes may not reflect any change in the tissues through which the drugs are distributed. The values obtained may depend upon the size of k_2 for a two- (or more) compartment drug. Thus, comparisons of *Vd* for derivatives should be carried out at steady-state plasma levels or be interpreted in terms of the effects of k_2 on the distribution value.

In addition, equilibrium between nonequivalent compartments involves primarily the unionized drug or unbound species which can pass through the biological membrane. This has important consequences in the calculation of *Vd* since many drugs, such as erythromycin, sulfonylureas, salicylates, and coumarin anticoagulants are bound to plasma protein. The apparent volume of distribution should be calculated on the basis of freely diffusing drug, so a correction must be made for the fraction bound. If this correction is not made, two types of error can occur. When the assay method determines only free drug, the bound drug is counted with drug distributed to the tissues and the *Vd* calculated is too large. On the other hand, if the assay is for total drug, the value of *Vd* is too small. If corrections are not made for protein binding, *Vd* values calculated at different doses for a drug whose extent of binding varies with dose also vary (177).

Absorption Rate Constants—The determination of absorption rate constants from blood level data is a common problem when biopharmaceutics and pharmacokinetics are applied to design and evaluation of drugs and their derivatives. Wagner and Nelson (178) developed a method for calculating the absorption rate constant for transfer from an extravascular depot into the body for a drug whose distribution can be described by a one-compartment model. Similarly, Loo and Riegelman (179) reported a method for calculating the absorption rate constant, k_1, for the case where the drug is distributed according to a two-compartment model. When simultaneous loss of drug occurs from the site of administration, the competing rate increases the calculated apparent value for the absorption constant (180). For the case where the competing loss of drug is by a first-order process, the apparent rate constant calculated by either method yields the sum of the competing first-order rate constants (180).

The significance of this potential problem is readily apparent. False impressions of rapid absorption could result from rapidly hydrolyzable analogs. The compound with the fastest apparent absorption rate constant in a series may be the most susceptible to biotransformation. The total amount of drug absorbed from the depot should be determined for an accurate interpretation of the calculated rate constants for absorption. If a drug is well absorbed from the depot, the calculated absorption rate constant may be considered to be a good estimate. If, however, the drug is poorly absorbed, the reason for the incomplete absorption must be determined before one can assign a physical meaning to the calculated value of the apparent absorption rate constant.

This problem is not unique to oral dosage forms. Doluisio *et al.* (181) showed that intramuscular injections of sodium dicloxacillin and sodium ampicillin solutions were only 75–78% absorbed. They suggested that the drug may have undergone chemical or enzymatic decomposition at the injection site. If 22–25% of the drug placed into the muscle is indeed lost to some simultaneous first-order rate process, the apparent absorption rate constants would be approximately one-third larger than the actual value.

Bioavailability of Prodrugs: Hetacillin—Scheme VI illustrates one potential problem in prodrug evaluation. If conversion of prodrug to drug is not instantaneous, then the distribution, elimination, and conversion kinetics for the prodrug must be determined to describe the system adequately.

A second potential problem may be illustrated by considering hetacillin, a prodrug of ampicillin. Early studies indicated increased ampicillin plasma levels from intravenous administration of hetacillin (182). However, this result was later shown to be due to an artifact of the assay method (183). Because of the long incubation period for the microbiological assay, hetacillin was also converted to ampicillin and thus measured as drug. Thus, plasma levels after hetacillin injection appeared to exceed those obtained by rapid intravenous injection of the drug itself. Application of a differential assay technique showed the areas under the ampicillin curves to be identical from either source (148, 183). This represents a good didactic example since an understanding of pharmacokinetic parameters would dictate *a priori* that no increase in bioavailability would be expected beyond that observed by an intravenous injection of the drug itself.

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Transport and Binding of Methotrexate *In Vivo*

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Abstract □ A mathematical model is presented for time-dependent uptake of drugs in tissues exhibiting significant membrane resistance to transport during periods of rapidly changing plasma concentration *in vivo*. The model includes joint effects of blood flow, membrane transport, and binding. It is used to interpret data on the uptake of methotrexate in bone marrow, spleen, and small intestine of the bile-cannulated rat following intravenous doses of 0.05, 0.25, 2.5, and 25 mg./kg. The transport is saturable and influenced by strong intracellular binding. The Michaelis constant ranges from 0.45 mcg./ml. (1.0 μM) to 1.1 mcg./ml. (2.4 μM), in general agreement with *in vitro* studies in a number of mammalian cell lines.

Keyphrases □ Methotrexate—*in vivo* transport and binding, mathematical model for time-dependent uptake in tissues, rats □ Transport parameters, *in vivo* methotrexate—mathematical consideration of blood flow, membrane permeability, and binding □ Tissue distribution, *in vivo* methotrexate, rats—transport parameters, mathematical model for time-dependent uptake □ Drug transport —mathematical model for time-dependent uptake in tissues during rapidly changing plasma concentration *in vivo*

The mechanism of action of methotrexate[1] has been reviewed by Bertino and Johns (1). It is a strong inhibitor of the enzyme dihydrofolate reductase, which catalyzes the formation of tetrahydrofolate. DNA synthesis is inhibited if tetrahydrofolate is not available for the production of a coenzyme required for thymidylate formation. Additional sites of action have been proposed, which complicate the complete description of the biochemical events leading to cell death or recovery following exposure to methotrexate.

Methotrexate can be highly toxic to rapidly proliferating normal cells because it kills cells in S-phase (the DNA synthetic phase). Therapeutic application of methotrexate to a tumor-bearing host exposes both tumor and sensitive normal cells to the drug at concentrations and with time courses determined jointly by the dose schedule, route of administration, and physiological disposition. The therapeutic framework must be optimized so that tumor cells are destroyed more rapidly than they are produced, with constraints imposed by toxicity to the host. Within this framework, rather subtle differences in cell kinetics, drug transport, and tissue perfusion may be exploited despite the rather general biochemical mechanism of action.

Defective membrane transport has been proposed as a mechanism of drug resistance in the murine leukemia L5178Y (2). Drug transport has been associated with sensitivity or resistance of a number of other tumors including the Yoshida sarcoma in rats (3, 4), P-388 leukemia in mice (5), Ehrlich ascites tumor (6), a variety of mouse leukemias (7), human leukemia (8), and mouse L-1210 leukemia (9).

Transport of methotrexate into mammalian tumor cells has been studied extensively *in vitro*. A few studies in addition to those already cited include the mouse L-1210 leukemia (10–13) and Sarcoma-180 (14, 15). Werkheiser (16) studied the uptake of methotrexate by ascitic P-388 leukemia in mice. A number of aspects of methotrexate transport in mammalian cells were discussed by Goldman (17). Werkheiser (18) also developed a theoretical mathematical model which includes the effect of finite permeability on the action of methotrexate *in vivo*. Data on rates of diffusion of methotrexate into sensitive normal tissues *in vivo* have not been published. Past investigations of the distribution of methotrexate *in vivo* (19, 20) indicated that blood flow and protein binding could not explain the rate of uptake in bone marrow, the spleen, and the GI tract. That these are important sites of action is suggested by clinical findings of leukopenia, thrombocytopenia, and intestinal toxicity (21).

The purposes of this paper are: (*a*) to report transport parameters for entry of methotrexate into bone marrow, the small intestine, and the spleen of the rat and (*b*) to present a mathematical model for the interpretation of the observed uptake during time intervals in which the plasma concentration is decreasing rapidly because of drug redistribution and elimination. Data were obtained following pulse intravenous injections at four doses ranging from 0.05 to 25 mg./kg. A mathematical model has been developed to incorporate the joint effects of blood flow, transport across cell membranes, and strong intracellular binding (presumed to be due to dihydrofolate reductase). The mathematical treatment is general and can be adapted to other normal tissues or to tumors in which blood flow, membrane permeability, and binding must be considered together.

EXPERIMENTAL

The concentration of methotrexate in the various tissues of the rat was obtained as follows. The tritiated drug, 3′,5′,9-^{3}H-methotrexate[2], in chromatographically pure form (22) was mixed with nonlabeled drug[3] dissolved in 2% sodium bicarbonate. Each animal received an injection into the femoral vein of 0.001 ml. labeled methotrexate solution/g. body weight; radioactivity (3–9 μc./kg.) was sufficient to give tissue levels with counting errors of 5% or less. Doses ranged from 0.05 to 25 mg./kg.

[1] *N* - [*p* - [(2,4 - Diamino - 6 - pteridinylmethyl)methylamino]benzoyl] glutamic acid. Methotrexate is currently used in the treatment of leukemia and other tumors.

[2] New England Nuclear.
[3] NCS 740, Lederle.

The rats were anesthetized with pentobarbital (60 mg./kg. i.p.) and maintained at a reflex-free level of anesthesia by supplementary doses as necessary. A midline laparotomy was performed, and the bile duct was cannulated with polyethylene 20 tubing. Control bile was collected until a constant flow of 20 μl./min. was achieved. Body temperature was maintained at 36 ± 0.5° throughout the experiment by a heating lamp and monitored constantly with a thermistor-type rectal thermometer[4].

The animals were decapitated at selected times after injection of methotrexate and tissue samples were collected. The entire small intestine minus expressible contents was homogenized as were the spleen and other tissues; the bone marrow from two femurs was removed by cracking open the bones and aspirating the contents into a tared micropipet; the blood was centrifuged to collect plasma. Aliquots of the tissue homogenates, plasma, and the entire sample of bone marrow were combusted and counted (23). The counts were converted to micrograms of drug per unit of tissue after appropriate corrections for quenching, volumes, and weights of tissues.

As reported previously, significant metabolism of methotrexate by intestinal bacteria occurs in rats after 6 hr. (24). With this consideration in mind, the comparison of model predictions with experimental data was limited to short times so that the tritium measured actually represents intact drug.

The diversion of the bile from the intestinal lumen made it possible to analyze the rate of methotrexate uptake into the small intestine from the vascular compartment without the complication of enterohepatic recirculation.

In those studies in which extracellular space of tissues was determined by inulin-^{14}C, the same experimental procedure as already described was used. Inulin-^{14}C[2] in 0.1 *M* phosphate buffer at pH 7.0 was the injection fluid. The inulin-^{14}C was purified just prior to use by a Sephadex chromatography procedure (25).

DEVELOPMENT OF MODEL

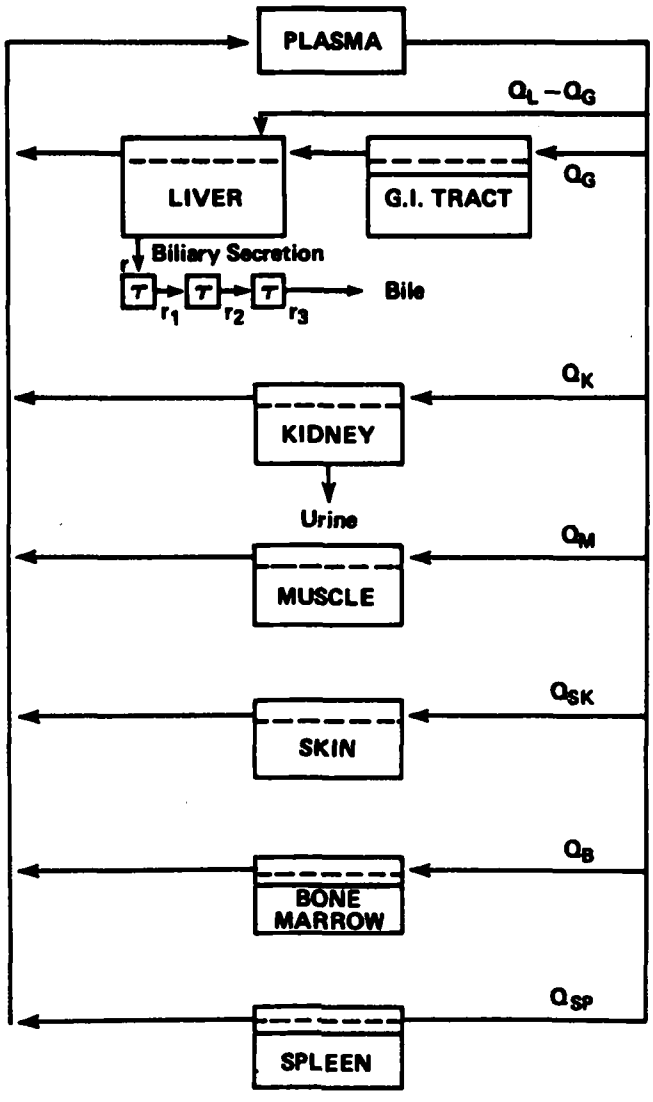
A mathematical model has been published which simulates the distribution and disposition of methotrexate in several mammalian species (19, 26) and in the sting ray (20). Since this model and its key assumptions were described previously, only a summary development is presented here. Careful attention is given to the operational formulation of transport within a region which may be influenced by membrane transport as well as flow.

Scheme I is a flow diagram showing the compartments in the mathematical model. The spleen and marrow compartments are small and contribute little to the systemic distribution of methotrexate, but they are included because they are important sites of action for the drug. This scheme differs from the previously published diagram in a number of ways. The bile is led directly out of the common bile duct. Diversion of the bile minimizes drug metabolism and eliminates the enterohepatic circulation. With this experimental design, reabsorption kinetics, which are not well quantified in the intact rat, are not required in the model. In addition, elimination of bile secretion into the contents of the small intestine provided the opportunity to observe transport directly between blood and gut tissue with a greatly reduced role of luminal drug contents in obscuring pharmacokinetic events in the tissue.

A skin compartment has been included, which contributes more to the distribution of the drug than had been previously realized and which serves to maintain elevated plasma concentrations at long times, since it is poorly perfused.

The bone marrow, spleen, and GI compartments include a detailed representation of drug transport across the cell membrane. Although bone marrow and spleen are small and play no significant role in influencing the systemic drug distribution, analysis of their kinetic behavior is important. Transport within the GI tract, the bone marrow, and the spleen shows time-dependent behavior that is quite different from the apparent flow limitation observed for a number of other tissues. This suggests a more complex process involving a significant role of cell membranes in determining the rate of drug uptake by these tissues.

A mass-balance equation is written for each compartment in the mathematical model. The resulting set of differential equations is solved simultaneously to simulate the concentration of drug in each compartment as a function of time. For example, the balance equation on the kidney is:



Scheme I—Diagram of compartmental model for bile-cannulated rat, where Q represents plasma flow rate to various compartments (milliliters per minute), r represents rate of flow of drug in bile (micrograms per minute), and τ represents the bile transport parameter (minutes). Values of these parameters are given in Table I.

$$V_K \frac{dq_K}{dt} = Q_K \left(q_p - \frac{q_K}{R_K}\right) - k_K \frac{q_K}{R_K} \qquad \text{(Eq. 1)}$$

where:

- Q = plasma flow rate, grams per minute or milliliters per minute
- V = size of compartment, grams or milliliters
- q = total (free plus bound) concentration in the tissue, micrograms per gram or micrograms per milliliter
- k = "clearance" (defined by Eq. 1), milliliters per minute
- R = equilibrium tissue-to-plasma distribution ratio, $(q/q_p)_{eq}$
- t = time, minutes

and the subscript, K, identifies the compartment.

The rate of flow of drug in the urine from the kidney at any time is: $k_K(q_K/R_K)$, and the cumulative amount of drug in the urine is:

$$U = \int_0^t k_K \frac{q_K}{R_K} d\tau \qquad \text{(Eq. 2)}$$

Implicit in Eq. 1 is the assumption of flow limitation in the sense that the plasma leaving the compartment is in equilibrium with the compartment so that a thermodynamic distribution ratio can be used. This idea is inferred from Fig. 1, in which the concentrations

[4] Yellow Springs Instrument Co.

Table I—Model Parameters for 200-g. Rat

Parameter	Compartment											
						Gut		Spleen		Bone Marrow		
	Plasma	Muscle	Kidney	Liver	Skin	ECF[a]	ICF[a]	ECF[a]	ICF[a]	ECF[a]	ICF[a]
Volume, ml.	9.0	100.0	1.9	8.3	35.0[b]	11.0		0.54[c]		4.0[d]	
V						3.7	7.3	0.11	0.43	0.80	3.2
Flow rate, ml./min.	22.7	3.0	5.0	6.5	2[e]	5.3	—	0.25[e]	—	0.64[f]	—
Q											
Linear distribution ratio	—	0.15	3.0	3.0	1.0	—	—	—	—	—	—
R											
Strong binding constant, mcg./ml.	0	0	0.3	0.5	0	0	0.2	0	0.1	0	0.2
a'											
Dissociation constant, mcg./ml.	—	—	0.00001	0.00001	—	—	0.00001	—	0.00001	—	0.00001
ϵ											
"Clearance," ml./min.	—	—	2.0	5.7	—	—	—	—	—	—	—
Bile transit parameter, min.	—	—	—	1.0	—	—	—	—	—	—	—
τ											
Maximum facilitated transport, mcg./(min.)(ml.)	—	—	—	—	—	0.014		0.009		0.012	
k											
Michaelis constant, mcg./ml.	—	—	—	—	—	0.45		1.1		0.9	
K											

[a] ECF = extracellular fluid, and ICF = intracellular fluid. [b] Jansky and Hart (32). [c] *Reference 33.* [d] Mapleson (34), ratioed to 200-g. rat. [e] Mandel and Saperstein (35), for 45% hematocrit. [f] Brookes (36).

in the liver, kidneys, and skeletal muscles of mice are plotted as functions of the plasma concentration. Time does not appear explicitly in this plot. An intermediate point on the curve may have been obtained at a short time following a small dose or after a longer time following a larger dose. This suggests that (on the time scale of the experiments at least) a unique relationship exists between the plasma concentration and the concentration in each of these three tissues. Distribution into muscle is linear, and the volume of distribution is approximately equal to the extracellular fluid volume. At high plasma concentrations, the drug is concentrated in the liver and kidneys about 10:1 and 3:1, respectively. At low plasma concentrations, these tissue concentrations appear to approach constant values presumed to be associated with strong binding of methotrexate to dihydrofolate reductase. *In vivo* binding isotherms, therefore, consist of a linear part and a saturable part with a very low dissociation constant, and the distribution coefficients are functions of plasma concentration.

If the concentrations in the bone marrow, the spleen, and the small intestine are plotted in the manner of Fig. 1, no unique thermodynamic relationship is seen between the drug in the tissue and that in the plasma. This is illustrated for bone marrow in Fig. 2. The dependence of the concentration of drug in the tissue on dose (and thus prior drug exposure) as well as on plasma concentration suggests an analysis which includes membrane resistance to transport. The mathematical analysis presented here is rather general and can be applied or easily adapted to any lumped compartment in which flow, membrane transport, and binding must be considered jointly. It is developed in some detail with explicit citation of model assumptions.

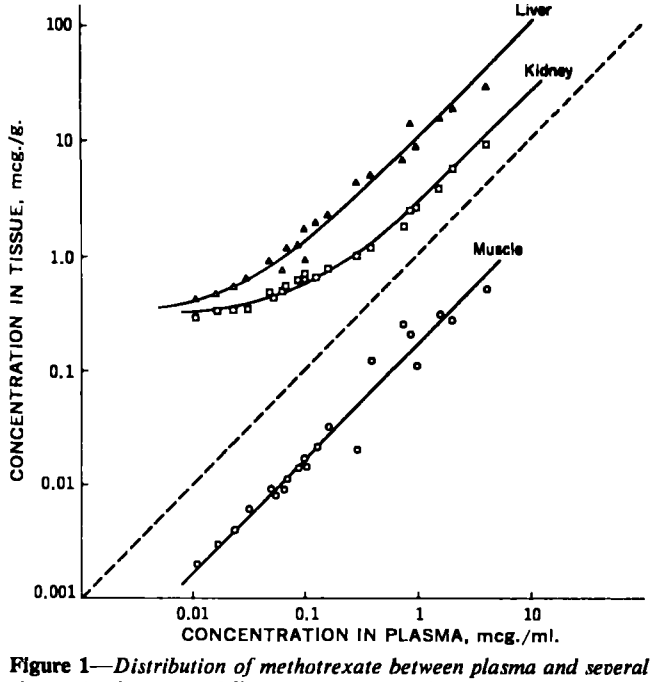


Figure 1—*Distribution of methotrexate between plasma and several tissues in the mouse following intravenous doses of 0.1, 0.3, and 3 mg./kg. at times from 5 to 120 min.*

General Balance Equations—A mass balance on each section of the compartment in Scheme II gives:

$$V_1\frac{dq_1}{dt} = Q(q_p - q_1) - (jA)_{12} \qquad \text{(Eq. 3)}$$

$$V_2\frac{dq_2}{dt} = (jA)_{12} - (jA)_{23} \qquad \text{(Eq. 4)}$$

$$V_3\frac{dq_3}{dt} = (jA)_{23} \qquad \text{(Eq. 5)}$$

where j = net flux of drug, mcg./(min.)(cm.^{2}); A = area for transport, cm.^{2}; and the single subscripts 1, 2, and 3 refer to plasma, interstitial fluid, and intracellular fluid, respectively. The double subscripts 12 and 23 indicate the directions shown in the scheme. The flux, j, and the area, A, are defined separately here; however, values were not obtained for each. Their product is treated as a single concept (rate of transport) as is common practice in both physiology and engineering. If an independent measure of the area becomes available, the actual fluxes can be calculated.

Binding—In each compartment, the total concentration is given as the sum of the free and the bound concentration:

$$q_j = f_jC_j + (1 - f_j)x_j \qquad j = 1, 2, 3 \qquad \text{(Eq. 6)}$$

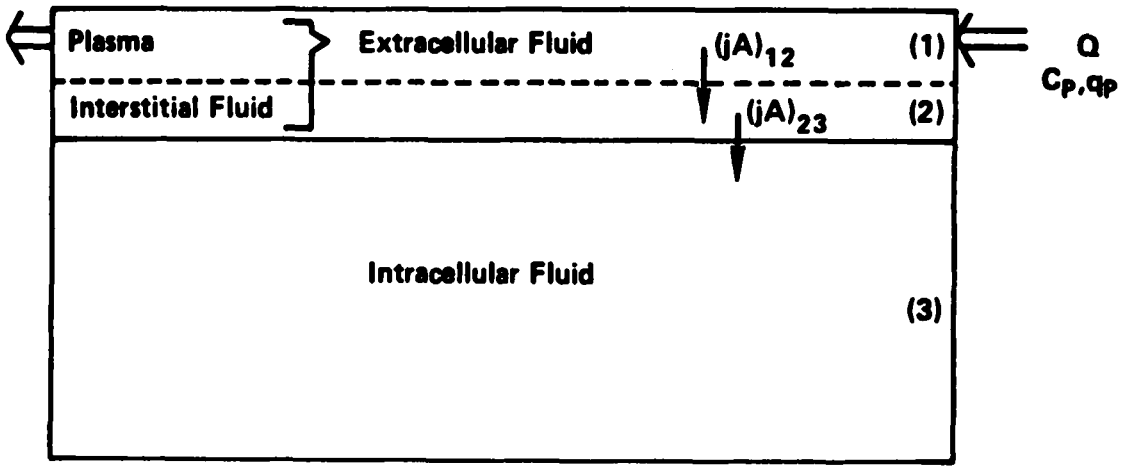
with:

$$x_j = x_j(C_j) \qquad j = 1, 2, 3 \qquad \text{(Eq. 7)}$$

where:

- f_j = fraction of water in the jth compartment
- C_j = free concentration, micrograms per gram or micrograms per milliliter
- x_j = bound concentration based on $(1 - f_j)$, micrograms per gram or micrograms per milliliter

In the intracellular compartment there may be both linear nonspecific binding to proteins and other cellular constituents (B_3C_3) and strong specific binding to dihydrofolate reductase (a_3C_3)/($\epsilon_3 + C_3$), where a_3 is the strong binding capacity and ϵ_3 is the dissociation constant of the drug–enzyme complex. Thus, the total



Scheme II—Diagram of a compartment (e.g., *bone marrow*) *which shows inter- and intratissue drug transport, where* Q *is the plasma flow rate to the compartment* (*milliliters per minute*), C_p *is the plasma concentration of free drug* (*micrograms per milliliter*), Q_p *is the total plasma concentration of drug* (*free + bound*) (*micrograms per milliliter*), *and* (jA) *is the transmembrane rate of transport* (*micrograms per minute*).

bound concentration inside the cells is expressed as $x_3 = B_3C_3 + (a_3C_3)/(\epsilon_3 + C_3)$.

From Eq. 6, the total intracellular concentration, free plus bound, becomes:

$$q_3 = f_3C_3 + (1 - f_3)x_3 \quad \text{(Eq. 8a)}$$

$$q_3 = f_3C_3 + (1 - f_3)B_3C_3 + (1 - f_3)\frac{a_3C_3}{\epsilon_3 + C_3} \quad \text{(Eq. 8b)}$$

or:

$$q_3 = \phi_3C_3 + \frac{a_3'C_3}{\epsilon_3 + C_3} \quad \text{(Eq. 8c)}$$

where $\phi_3 \equiv f_3 + (1 - f_3)B_3$ and $a_3' \equiv (1 - f_3)a_3$.

Linear binding to plasma proteins and cell membranes may occur in the extracellular compartment, giving $x_1 = B_1C_1$ and $x_2 = B_2C_2$ so that:

$$q_1 = [f_1 + (1 - f_1)B_1]C_1 = \phi_1C_1 \quad \text{(Eq. 9)}$$

$$q_2 = \phi_2C_2 \quad \text{(Eq. 10)}$$

The previous equations can be simplified by assuming that the linear protein binding terms in all compartments are small[6]. Then B_1, B_2, $B_3 \approx 0$; f_1, f_2, $f_3 \approx 1$; and ϕ_1, ϕ_2, $\phi_3 \approx 1$. From Eqs. 8–10, the total concentration in each compartment is now:

$$q_1 = C_1 \quad \text{(Eq. 11)}$$

$$q_2 = C_2 \quad \text{(Eq. 12)}$$

$$q_3 = C_3 + \frac{a_3'C_3}{\epsilon_3 + C_3} \quad \text{(Eq. 13)}$$

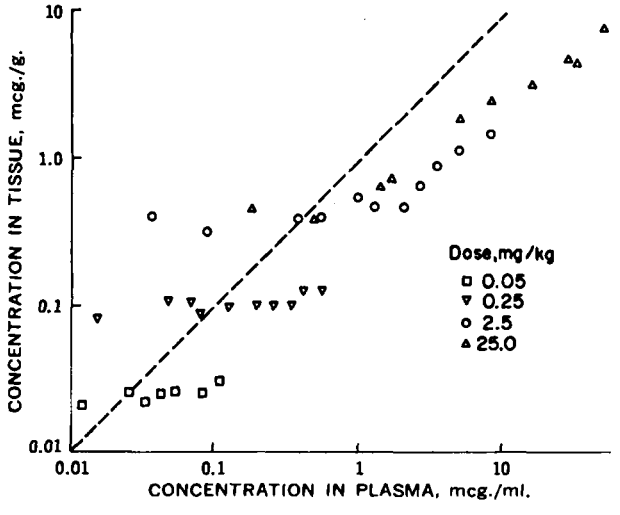


Figure 2—*Distribution of methotrexate between plasma and bone marrow of the rat at several doses.*

[6] The binding of methotrexate to rat plasma is about 15%.

Transport—It is assumed that the rate of transport, $(jA)_{13}$, is very fast, *i.e.*, very rapid equilibration across the capillary membrane so that plasma leaving the compartment and interstitial fluid have the same concentration at all times: $C_1 = C_2 = C_e$ and $C_3 = C_i$, where the subscripts *e* and *i* refer to extracellular and intracellular, respectively. Let:

$$(jA)_{23} = \frac{kVC_e}{K + C_e} - \frac{kVC_i}{K + C_i} + bV(C_e - C_i) \quad \text{(Eq. 14)}$$

Equation 14 describes saturable transport (first two terms on right-hand side) occurring in parallel with passive transport (third term). The transport parameters k (maximum facilitated rate) and b (passive permeability) are arbitrarily based on a unit volume or weight of tissue. The Michaelis constant for transport is K. Equation 14 incorporates the assumption that the facilitated transport is symmetric in the sense that $k_e = k_i = k$ and $K_e = K_i = K$.

If one adds balance Eqs. 3 and 4 and uses Eqs. 11 and 12 with $C_1 = C_2 \equiv C_e$ and $V_e = V_1 + V_2$, one gets:

$$V_e\frac{dC_e}{dt} = Q(C_p - C_e) - (jA)_{23} \quad \text{(Eq. 15)}$$

Inserting Eq. 13 for q_3 into balance Eq. 5 with $C_3 = C_i$ and carrying out the appropriate differentiation give Eq. 16, explicit in free intracellular concentration C_i:

$$V_i\frac{dC_i}{dt} = \frac{(jA)_{23}}{1 + \epsilon_i a_i'/(\epsilon_i + C_i)^2} \quad \text{(Eq. 16)}$$

Equations 14–16 are the working forms of the equations used for computation. They require estimates of the extracellular and intracellular volumes (V_e and V_i), the plasma flow rate to the compartment (Q), the transport parameters (k, K, and b), and the binding constants (ϵ_i and a_i').

RESULTS

Table I lists the model parameters used for simulation of plasma and tissue concentrations in the rat. They include those from a previous study (19) with a few exceptions: (*a*) there was no requirement for gut–lumen parameters in the anesthetized bile-cannulated rat, (*b*) clearance of methotrexate by the kidney was increased by the anesthesia so that the kidney clearance as defined by Eq. 1 was found to be 2.0 ml./min., (*c*) the bile secretion values were revised on the basis of data from the current study, and (*d*) binding constants in the GI tract were replaced by a more complete model of transport and binding in the small intestine.

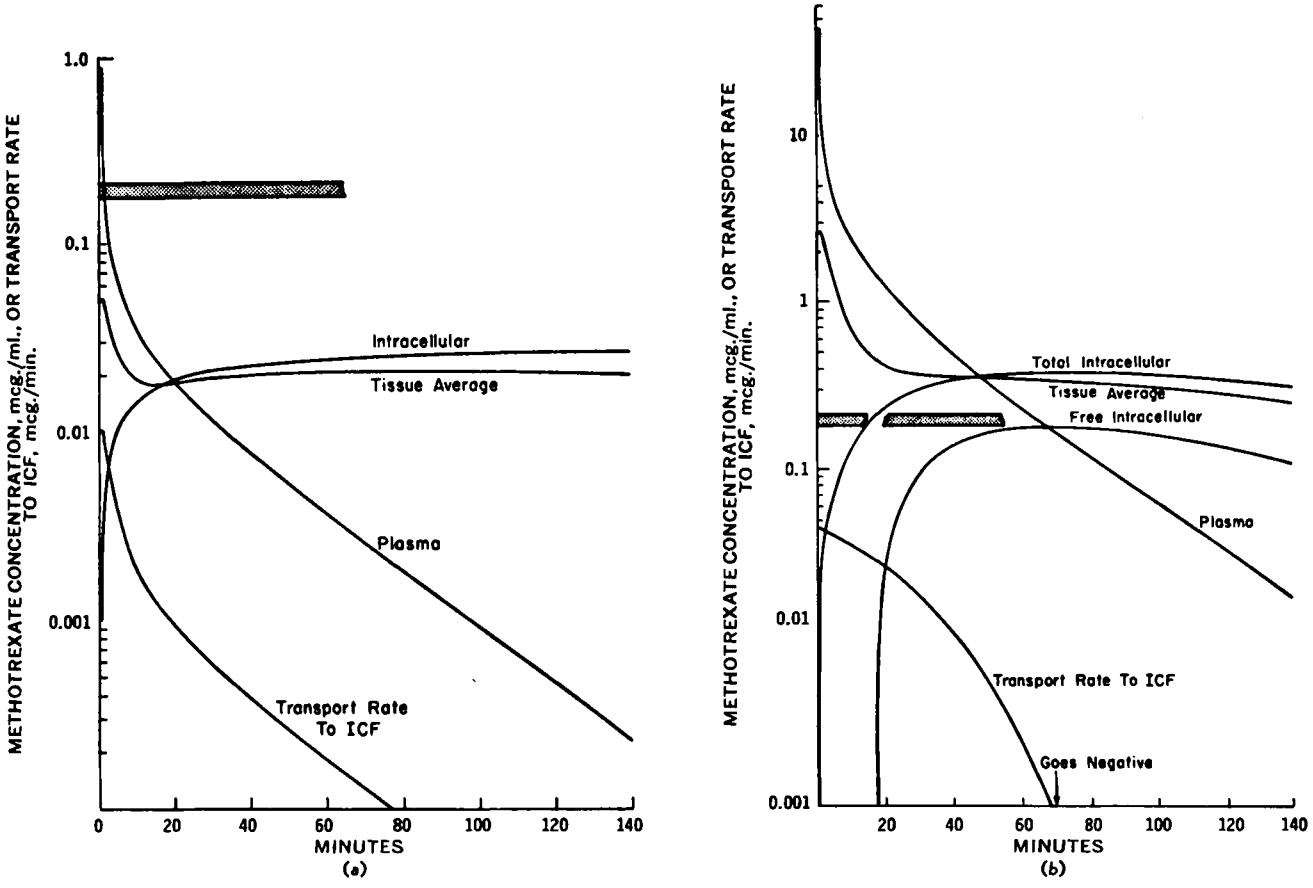


Figure 3—*Simulated plasma concentration and concentrations and transmembrane transport rate of methotrexate in bone marrow of rat. Shaded bar represents strong binding capacity, presumably dihydrofolate reductase, micrograms methotrexate per milliliter cell contents. It is assumed constant for the duration of the experiment. Key:* (a), *0.05 mg./kg. i.v.; and* (b), *2.5 mg./kg. i.v. ICF = intracellular fluid.*

As discussed previously, additional parameters were required to describe saturable transport and binding in the bone marrow, the spleen, and the small intestine and to incorporate a skin compartment. Plasma flow and volume of the skin, the spleen, and the bone marrow were obtained from the literature as documented in the table. Parameters not discussed in the previous work (19) include V_s, V_t, k, K, b, and a_t' in the compartments exhibiting time-dependent uptake. These were obtained from the data to simulate the measured time course of drug concentration in these compartments as discussed below.

Extracellular Volume (V_e)—This was first based on the tissue to plasma concentration ratio for inulin after a pulse injection. A better second estimate was obtained from tissue to plasma methotrexate ratios obtained at short times following the highest dose. Because of transport saturation under these conditions, intracellular drug appears to comprise a very small fraction of measured drug. The methotrexate extracellular "space" of 20% in the bone marrow is slightly greater than the 15% observed for inulin, a finding consistent with observations on muscle.

Intracellular Volume (V_i)—$V_i = V - V_e$.

Membrane Transport Parameters (k, K, b)—There was proportionality between the amount of drug in a tissue and dose (and area under the plasma concentration curve) at the two lowest doses. This suggests a linear transport. For a dose that does not saturate the dihydrofolate reductase in the cells, efflux is negligible. Then, from Eq. 14, with $b = 0$:

$$(jA)_{12} = \frac{kVC_e}{K + C_e} \qquad \text{(Eq. 17)}$$

and for $C_e \ll K$:

$$(jA)_{12} = \frac{kV}{K} C_e \qquad \text{(Eq. 18)}$$

The amount in the cells at any time, t, is:

$$\text{amount} = \int_0^t \frac{kV}{K} C_e d\tau \qquad \text{(Eq. 19)}$$

Since $C_t \approx C_P$, this equation provides a first estimate of the ratio k/K. The data on tissue uptake *versus* time could then be simulated within the context of the model to give a more precise estimate of k/K and of K from the data at higher doses when saturation of the transport process occurred. It was not necessary to use a finite value of the permeability to passive diffusion, b; however, a very small value cannot be ruled out on the basis of the data.

Intracellular Binding Constant (a)—Following doses thought sufficient to saturate the intracellular dihydrofolate reductase, the tissue concentration at longer times remained nearly constant after correction for extracellular drugs. This provided an initial estimate of a'. Inspection of numerous simulations provided a better estimate.

The dissociation constant (ϵ_i) for the dihydrofolate reductase-methotrexate complex was estimated from *in vitro* data of Werkheiser (27) as discussed previously (19).

Following a pulse intravenous injection of methotrexate, the plasma concentration decreases very rapidly for a few minutes, followed by a longer and slower exponential phase which results when the bile is diverted, since no significant intestinal reabsorption occurs. A dynamic and complex set of events occurs in those tissues that have a significant membrane resistance. Figures 3*a* and 3*b* illustrate these events for bone marrow at two different doses as interpreted by the model.

At 0.05 mg./kg. (Fig. 3*a*), the predicted extracellular concentration peaks very rapidly and then closely follows the predicted plasma concentration. Drug enters the intracellular compartment through the cell membranes; however, there is insufficient transport to saturate all the intracellular dihydrofolate reductase. The total marrow concentration is a weighted average of the extracellular and intracellular compartments. Since ϵ is of the order of 10^{-11} M or less (27), the free intracellular concentration remains near zero as long

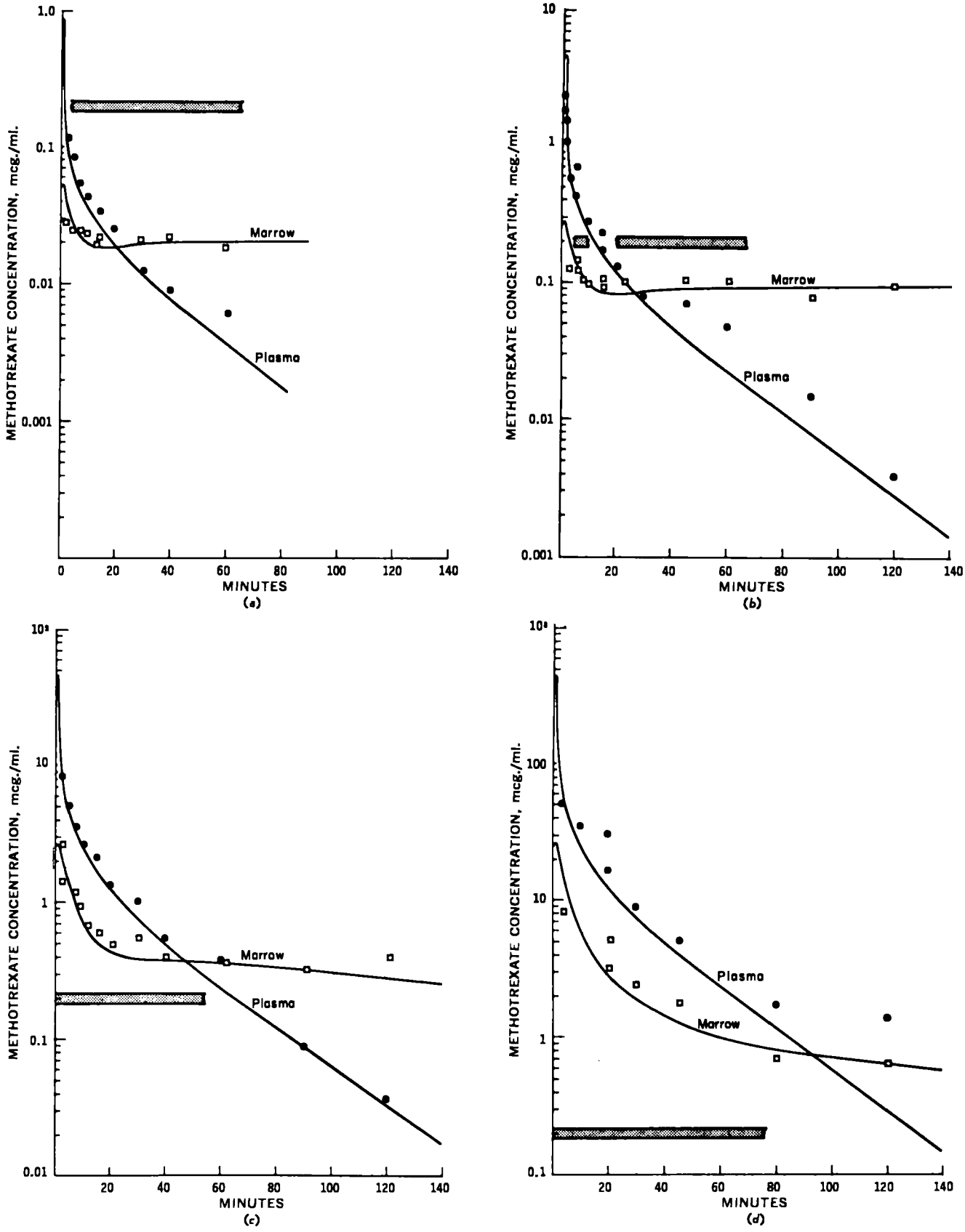


Figure 4—*Plasma and average bone marrow concentrations of methotrexate in rat. Solid lines represent model simulations. Data points were obtained from one rat at each time. Key:* (a), *0.05 mg./kg. i.v.;* (b), *0.25 mg./kg. i.v.;* (c), *2.5 mg./kg. i.v.; and* (d), *25 mg./kg. i.v.*

as free dihydrofolate reductase is available. Therefore, influx of methotrexate can occur even after the plasma concentration is less than the total intracellular concentration. It is sufficiently slow during this time interval, however, that the measured marrow concentration remains about constant.

Following a dose of 2.5 mg./kg. (Fig. 3*b*), the simulated intra-

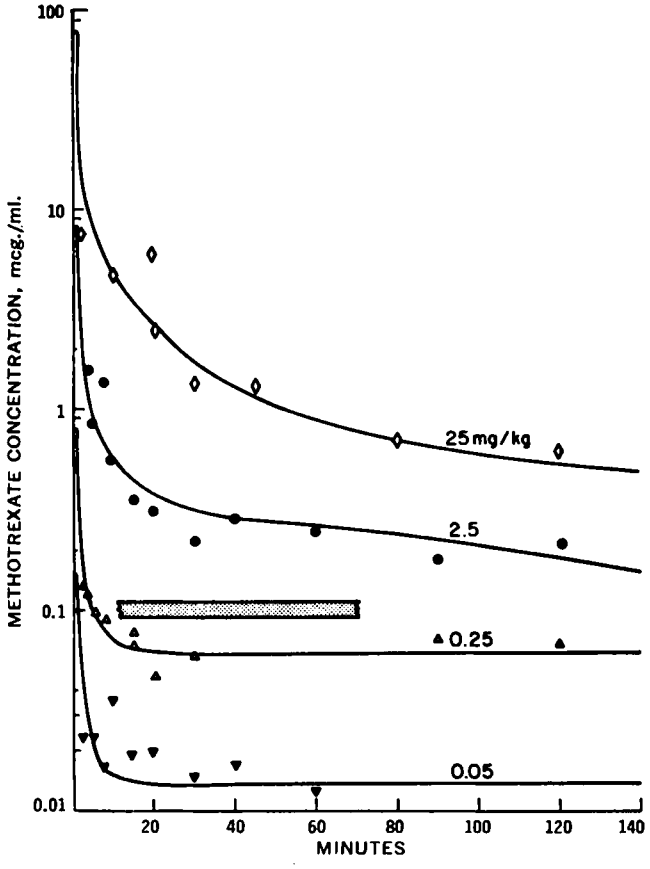


Figure 5—*Average concentrations of methotrexate in spleen of rat. Solid lines represent model simulations. Each data point was obtained from a single rat.*

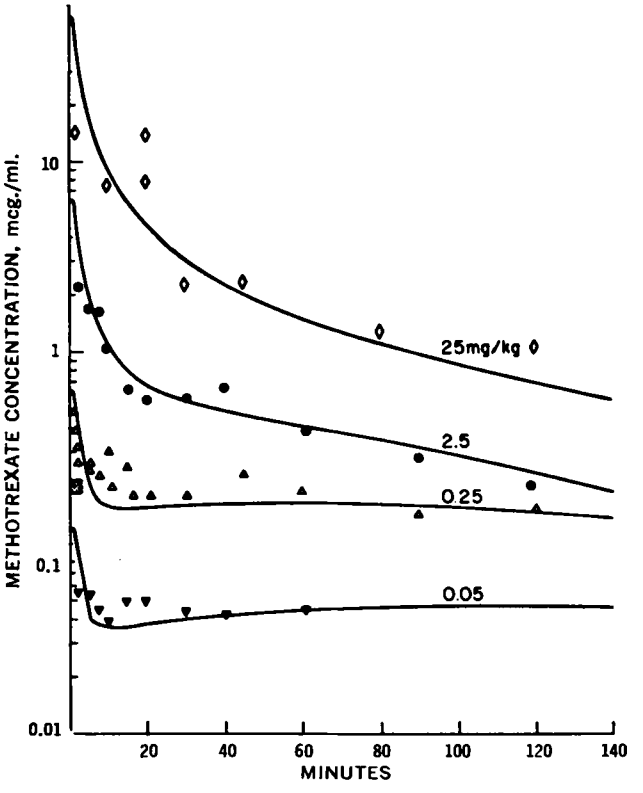


Figure 6—*Average concentrations of methotrexate in small intestine of rat with bile diverted. Solid lines represent model simulations. Each data point was obtained from a single rat.*

cellular events are somewhat different. There is sufficient entry of drug by about 20 min. to saturate the dihydrofolate reductase. At this time, there is an abrupt rise in free intracellular drug concentration. Efflux of drug occurs beyond 75 min. since the *free* intracellular drug concentration exceeds the extracellular concentration which is constantly decreasing with plasma. It is apparent that total tissue concentration is not a valid measure of intracellular drug at all times. Most of the measured drug is located extracellularly at the termination of a short experiment. In fact, washout of drug with plasma from the extracellular space makes the measured tissue concentration decrease rapidly during an early phase, even though there is actually uptake by the cells.

Figures 4*a*–4*d* compare model simulations with experimental data for bone marrow at four doses, ranging from 0.05 to 25 mg./kg. for time periods up to 2 hr. Both the shape of the bone marrow curves and their position with respect to the plasma curves change. There is a systematic variation from a relatively flat shape at the lowest dose to one exhibiting a pronounced decrease with time at the highest dose. The plasma concentration is approximately proportional to dose at all times, consistent with earlier observations (28); however, the measured marrow concentration is not. There is approximate proportionality in the marrow for a few minutes, which reflects the dominant role of extracellular fluid concentration. At longer times, increasing the dose by a factor of 5 from 0.05 to 0.25 mg./kg. increases the marrow concentration from 0.02 to 0.1 mcg./g., also a factor of 5. Since the plasma and extracellular concentrations are much lower than the total marrow concentration, these values represent intracellular drug. Further increases in dose to 2.5 and 25 mg./kg. do not result in proportional increases in marrow concentrations at long times. This behavior strongly suggests a saturable transport process. Symmetric transmembrane-facilitated diffusion with no significant passive component (Eq. 14) gave a satisfactory simulation of the data. The maximum facilitated rate (k), the Michaelis constant (K), and the methotrexate equivalent of intracellular dihydrofolate reductase (a') are given in Table I.

Figures 5 and 6 compare tissue concentrations in the spleen and small intestine with model simulations following the same four doses. The same qualitative picture exists. It may be explained by the general considerations discussed with Figs. 3*a* and 3*b*. Satisfactory transport and binding parameters are listed in Table I. These are similar for all three tissues.

DISCUSSION

A rather complex model was required to explain the observations concerning the pharmacokinetics of methotrexate in several tissues critical to its toxicity. Part of the complexity arose from the desire to simulate *in vivo* processes with changing plasma concentrations that occur after a single dose. This situation contrasts with studies *in vitro*, which have consistently been conducted at constant medium concentrations. The basic model was derived from earlier work which was shown to simulate adequately the concentrations in plasma and a number of tissues. The large number of parameters required are mostly physiological, anatomical, or physicochemical. The additional complexity involved the incorporation of saturable transport between extracellular and intracellular compartments in certain tissues. Other tissues are still satisfactorily simulated by a lumped, single compartment and flow-limited conditions.

A detailed analysis of the sensitivity of the model to each parameter was not undertaken. The model was perturbed by varying a number of parameters individually, and simulations were made to illustrate its behavior.

Figure 7 shows the necessity for saturability of the transport. If Eq. 14 is written:

$$(JA)_{23} = \frac{kV}{K} C_e - \frac{kV}{K} C_t \qquad \text{(Eq. 20)}$$

it describes transport that is linear in free concentration at all doses. The simulation is satisfactory at the lowest dose, since the plasma concentration is much less than the *in vivo* K, and essential linearity exists. At the higher doses, there is a systematic and serious deviation from the data and previous simulations.

For sufficiently high cell permeability, blood flow to a compartment becomes the limiting factor for drug transport. Figure 8

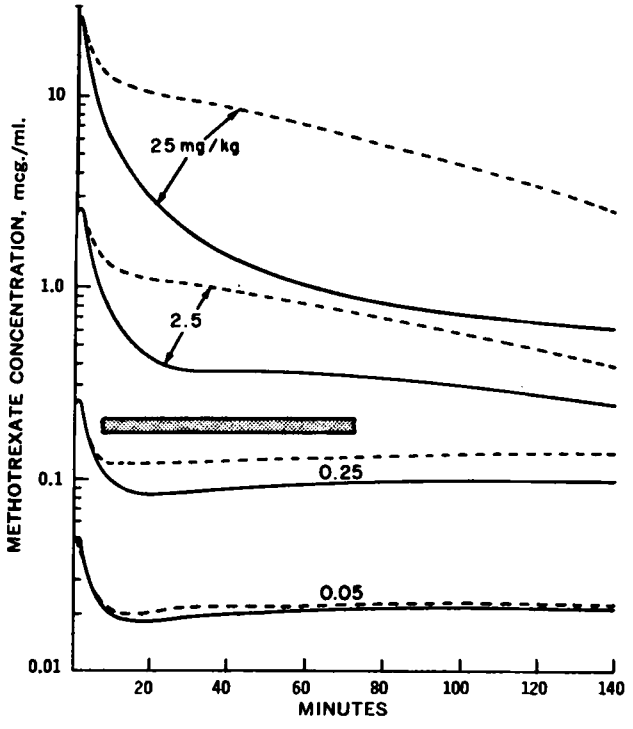


Figure 7—*Comparison of model simulation of linear and saturable transport in bone marrow at several doses. The dashed lines represent the model simulations for linear transport to the intracellular compartment of bone marrow as described by Eq. 20. The solid lines represent model simulations with saturable transport (Eq. 14).*

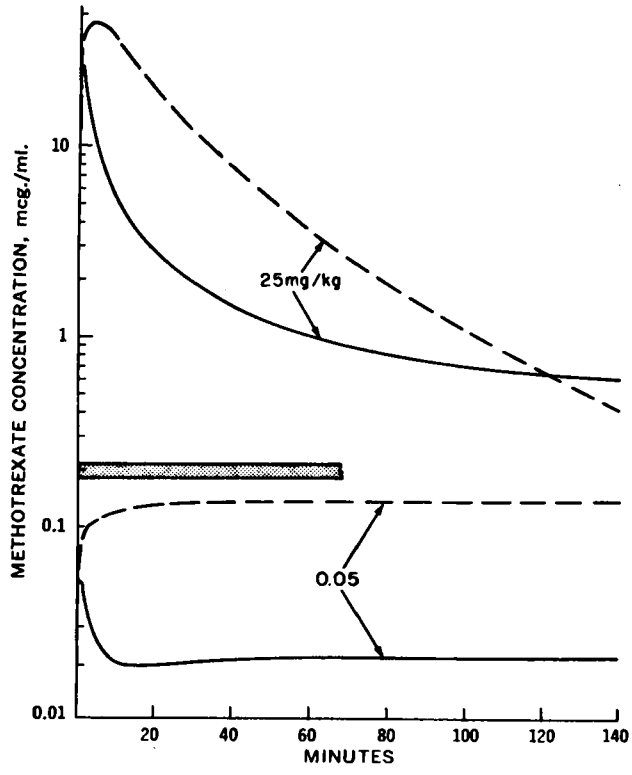


Figure 8—*Effect of very high permeability (flow limitation) on model simulations in bone marrow at two doses. The dashed lines represent bone marrow modeled as an equilibrium, flow-limited compartment. The solid lines represent model simulations with saturable transport.*

illustrates the results for a model simulation of bone marrow for this flow-limited case. There are very large deviations from previous simulations. Membrane resistance is significant if the permeability (k/K) for linear transport is much smaller than the tissue perfusion (29). For the bone marrow:

$$\frac{k}{K} = \frac{0.012 \text{ mcg./(min.)(ml.)}}{0.9 \text{ mcg./ml.}} = 0.013 \text{ min.}^{-1} \quad \text{(Eq. 21)}$$

which is much less than the plasma flow rate per unit volume:

$$\frac{Q}{V} = 0.16 \text{ min.}^{-1} \quad \text{(Eq. 22)}$$

Comparable values for the other tissues are: spleen, $(k/K) = 0.0082$ min.$^{-1}$, $(Q/V) = 3.3$ min.$^{-1}$; and small intestine, $(k/K) = 0.031$ min.$^{-1}$, $(Q/V) = 0.48$ min.$^{-1}$.

The plasma flow rates to the bone marrow, the spleen, and the small intestine are estimates based on the literature already cited. No attempt was made to measure these parameters under the actual conditions of anesthesia used in these experiments. The type and duration of anesthesia may have some effect on blood flow to these tissues; however, the calculated values of the transport parameters k and K are not highly sensitive to blood flow in very well-perfused tissues. Any possible effect of anesthesia on k or K is unknown.

As a final simulation, the strong binding parameter, a', for bone marrow was changed from 0.2 mcg./g. to zero or 2.0 mcg./g. The resulting marrow concentrations are compared with previous simulations in Fig. 9. The high level of binding does not affect the results at the two lowest doses, since the dihydrofolate reductase would not be expected to saturate at 0.2 mcg./g. methotrexate equivalent so that free intracellular concentration remains near zero. The higher value of a' shows a marked effect at the highest dose, because the drug that enters the cell is effectively trapped and is not free to be transported out during the experiment. Setting a' equal to zero has a profound effect at the low doses, because now intracellular drug can be transported out rather than remain bound to the intracellular enzyme.

No valid comparison can be made at present between the strong binding parameter and some independent measure of dihydrofolate reductase content. For reference, strong binding in mouse and rat kidney and liver is in the range of 0.3–0.5 mcg./g. of tissue *in vivo*. Attempts to extract the dihydrofolate reductase and quantitate it *in vitro* would raise questions about both recovery and activity in the intracellular environment. The spleen, the bone marrow, and the small intestine were assayed for methotrexate for up to 2 days following doses of 2.5 and 25 mg./kg. to obtain independent data on strong binding in these tissues. At the lower dose, the amount of strong binding observed experimentally was approximately the same as the strong binding parameter used in the model, although significant scatter of the data was observed. Agreement was poor after the higher dose, and the scatter was much worse. These long time data are not proper validation of the strong binding at short times, because there can be significant synthesis of new enzyme and loss of drug-bound enzyme due to cell turnover on a time scale of 2 days. This problem is even more acute following the administration of a cytotoxic drug, which has been shown to induce cell death and increase enzyme activity (30, 31).

The transport process was modeled as symmetric facilitated diffusion (Eq. 14 with $b = 0$). The data *in vivo* do not permit a rigorous assertion that the efflux parameters k and K are identical to the corresponding influx parameters. Goldman *et al.* (11) reported different values for the influx and efflux processes in the L-1210 cell *in vitro*. Neither do the data prove that b cannot be a small positive number so that passive diffusion may be a factor at very high doses.

The Michaelis constants obtained during this work *in vivo* range from 0.45 mcg./ml. (small intestine) to 1.1 mcg./ml. (spleen). This narrow range implies that the transport mechanism may be the same for all three tissues. In fact, the values are very similar to those observed during *in vitro* experiments with a number of cell lines: 0.20–0.59 mcg./ml. for three lines of L-1210 leukemia (9), 1.8 mcg./ml. for entry of methotrexate into L-1210 cells (10), and 0.9 mcg./ml. for entry of methotrexate into rabbit reticulocytes (17).

The concepts incorporated in this model are necessary to elucidate transport processes occurring *in vivo*. Blood flow, saturable membrane transport, and complex binding can be examined jointly, and the effect of any one can be interpreted within a formal and operational context.

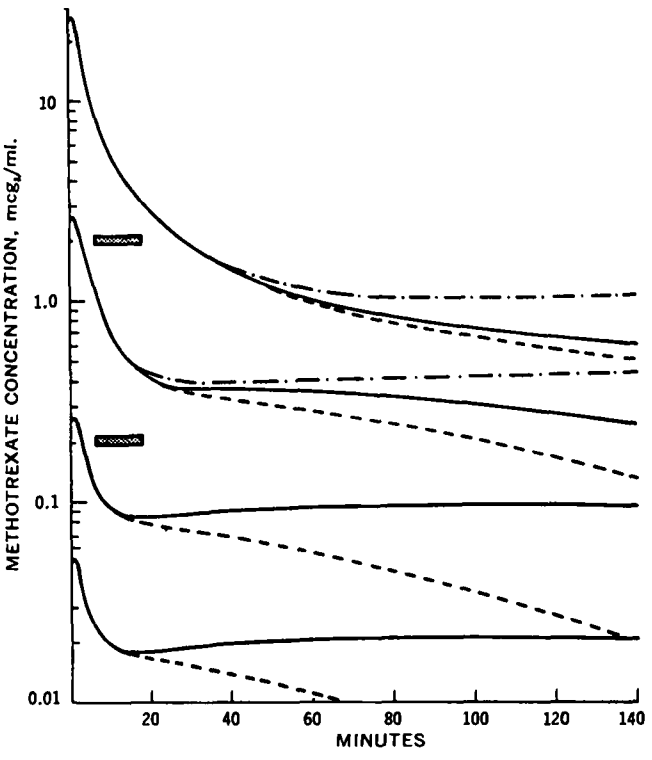


Figure 9—*Effect of change in dihydrofolate reductase content, a' (micrograms methotrexate equivalent per milliliter cell contents), on model simulations in bone marrow of rat. Key: - - -, a' = 0; —·—, a' = 2; and ——, a' = 0.2.*

While the several concepts are necessary, they may not always be sufficient. The model compartments are considered uniform in their description and behavior. No attempt was made to incorporate nonuniformities in flow. Neither was the problem of intratissue variations in the thermodynamics of binding or kinetic characteristics addressed explicitly. All of the tissues in which significant membrane limitation to transport was observed are microscopically heterogeneous. Bone marrow, for example, contains a variety of cell types in various stages of differentiation and maturity. It is probable that this distribution is accompanied by a distribution in numerous biochemical characteristics of the cells so that transmembrane transport rates and details of binding vary among the cells. Measured parameters, thus, are averages. They should not be assumed to be appropriate for every cell or even cell type within a tissue. Nevertheless, they can provide substantial pharmacokinetic insight and estimates of average local environment at appropriate biochemical sites of action.

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Ergosterol Photoisomerization Reaction Scheme

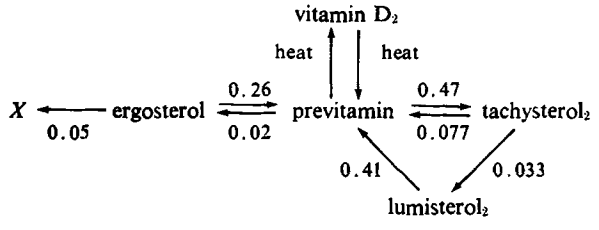
RENÉ MERMET-BOUVIER and ELIANE ABILLON▲

Abstract □ The kinetics of the various isomers formed at low temperature by UV irradiation of ergosterol were studied experimentally at the wavelength λ = 253.7 nm. The characteristics of the photostationary state were used to determine the isomer precursor of the toxisterols as well as the quantum transformation yields of one isomer into another.

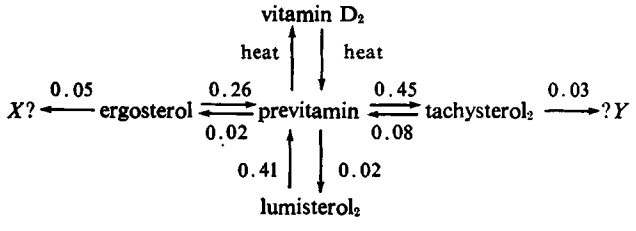
Keyphrases □ Ergosterol photoisomerization—reaction scheme, kinetics, transformation quantum efficiencies between isomers, formation and quantum yields of toxisterols □ Photoisomerization, ergosterol—reaction scheme, kinetics, transformation quantum efficiencies between isomers, formation and quantum yields of toxisterols □ Isomerization of ergosterol by UV irradiation—reaction scheme, kinetics, quantum transformation yields between isomers

Since vitamin D_2 is obtained by submitting ergosterol to UV irradiation, the system of isomers formed under radiation has already been the subject of many works (1–3). The three latest reaction schemes (Schemes I–III), summarizing the experimental results achieved, were successively submitted by Rappoldt in 1958 (4), Sanders and Havinga in 1964 (5), and Sanders in 1967 (6).

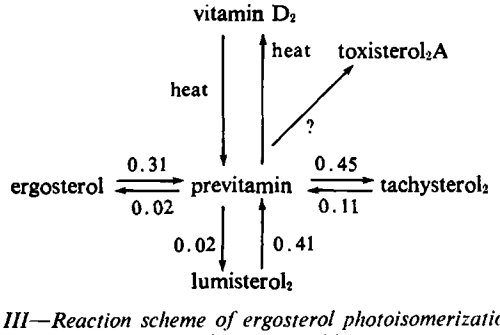
The solely thermal nature of previtamin $D_2 \leftrightarrows$ vitamin D_2 transformations was brought to light by Velluz in



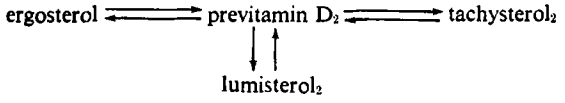
Scheme I—Reaction scheme of ergosterol photoisomerization suggested by Rappoldt (4)



Scheme II—Reaction scheme of ergosterol photoisomerization suggested by Sanders and Havinga (5)



Scheme III—Reaction scheme of ergosterol photoisomerization suggested by Sanders (6)



Scheme IV—Internal structure of the reaction scheme at low temperature suggested by Mermet-Bouvier and coworkers (7–11). In the internal structure, the irreversible transformations do not figure.

1948 (2); all other transformations are photochemical in nature. The values of the corresponding quantum yields are shown in each scheme.

Experimental work recently carried out in this laboratory (7–11), at a sufficiently low temperature for the previtamin $D_2 \rightarrow$ vitamin D_2 transformation to be negligible, led to the reaction scheme shown in Scheme IV. For this scheme, the present authors attempted to determine: (*a*) the value of the transformation quantum efficiencies between the various isomers, and (*b*) the isomers that act in starting the irreversible formation of toxisterols and the corresponding quantum yields.

THEORETICAL (12)

Let 1, 2, 3, and 4 be the classes whose elements are molecules of ergosterol, previtamin D_2, tachysterol, and lumisterol, respectively.

Let f_{ij} be the probability per unit time for a j class element to get into an i class element.

The reaction photoisomerization scheme of ergosterol given in Scheme IV corresponds to a true exotropic graph[1] (13), whose internal structure is shown in Scheme V.

Let external function f_{je} be the exit probability per unit of time of an element of the system, as from class j.

The problem consisted of determining: (*a*) the value of the internal interaction functions f_{ij}, and (*b*) the existence and value of the external functions f_{je}.

Evolution Equations—Let $\{N(t)\}$ be the vector whose components:

$$\begin{vmatrix} N_1(t) \\ N_2(t) \\ N_3(t) \\ N_4(t) \end{vmatrix}$$

are the populations of the various classes at time t.

The evolution equation of the system is:

$$\frac{d\{N(t)\}}{dt} = \boxed{a}\{N(t)\} \qquad \text{(Eq. 1)}$$

in which $\boxed{a}$ is the interaction matrix whose nondiagonal elements are the internal functions $a_{ij} = f_{ij}$ and whose diagonal elements are the sum of the divergent functions related to a class j bearing the minus sign:

$$a_{jj} = -f_{je} - \sum_k f_{kj} \qquad \text{(Eq. 2)}$$

The solutions of the differential system (Eq. 1) appear as a sum of four exponential functions:

$$N_j(t) = \sum_{i=1}^{i=4} A_{ij} e^{\lambda_i t} \qquad \text{(Eq. 3)}$$

where λ_i is the eigenvalues of matrix $\boxed{a}$.

[1] A true exotropic graph is a maximum, strongly connected component to which are added boundary functions that are all divergent.

After a sufficiently long irradiation time, Eq. 3 becomes equivalent to:

$$N_j(t) \simeq A_{1j}e^{\lambda_1 t} \qquad \text{(Eq. 4)}$$

where λ_1 is the eigenvalue of the smallest absolute value.

If the evolution of the four isomers is presented as semilogarithmic coordinates, two states may be distinguished as shown in Fig. 1:

1. The transient state during which at least two exponentials enter into each $N_j(t)$ expression.
2. The photostationary state or the permanent evolution state, for which one exponential only directs the evolution of each isomer, that is represented in semilogarithmic coordinates by parallel straight lines of slope λ_1.

The reaction schemes given in the literature express the experimental results obtained in a transient irradiation state. This report concerns the permanent evolution state characteristics, and the parameters used are: (*a*) the slope λ_1, and (*b*) the relative concentrations of the various isomers:

$$C_j = \frac{N_j(t)}{\sum_j N_j(t)} = \frac{A_{1j}}{\sum_j A_{1j}} \qquad \text{(Eq. 5)}$$

Relationship between Internal Functions f_{jk}, External Functions f_{j*}, Slope λ_1, and Relative Concentrations C_j in Permanent Evolution State—The population N_j of class j obeys the following balance equation:

$$\frac{dN_j}{dt} = -X_jN_j + \sum_{k \neq j} f_{jk}N_k \qquad \text{(Eq. 6)}$$

in which:

$$X_j = f_{j*} + \sum_{k \neq j} f_{kj} \qquad \text{(Eq. 7)}$$

Now, in the permanent evolution state:

$$\frac{dN_j}{dt} = \lambda_1 N_j \qquad \text{(Eq. 8)}$$

By identifying Eqs. 6 and 8 and dividing by N_j:

$$\lambda_1 + X_j = \sum_{k \neq j} f_{jk}\frac{C_k}{C_j} \qquad \text{(Eq. 9)}$$

If N is the total number of elements at time t:

$$N = \sum_j N_j \qquad \text{(Eq. 10)}$$

The number of elements leaving the system is per time unit:

$$-\frac{dN}{dt} = \sum_j f_{j*}N_j \qquad \text{(Eq. 11)}$$

Now, in the permanent state:

$$\frac{dN}{dt} = \sum_j \frac{dN_j}{dt} = \lambda_1 \sum_j N_j = \lambda_1 N \qquad \text{(Eq. 12)}$$

By identifying the relations Eqs. 11 and 12 and dividing by N:

$$\lambda_1 = -\sum_j f_{j*}C_j \qquad \text{(Eq. 13)}$$

The internal functions f_{jk} and external functions f_{j*} will be determined on the basis of Eqs. 9 and 13.

Table I—Photochemical Constants of the Four Isomers

	Ergosterol	Previtamin D_2	Tachysterol$_2$	Lumisterol$_2$
ϵ_j, liters/mole cm.	4800	8210	9915	4760
ρ_j, molecule/photon absorbed	0.31	0.49	0.11	0.41
X_j, sec.$^{-1}$	5.69	15.4	4.17	7.46

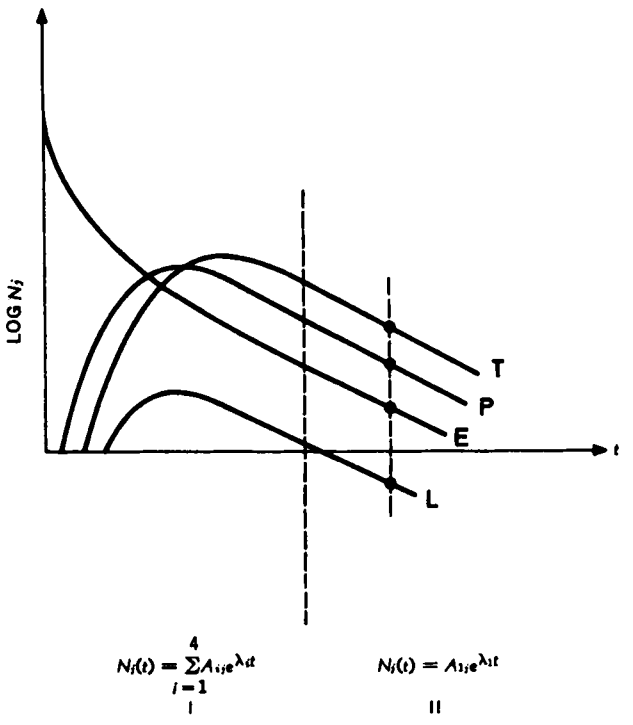


Figure 1—*Evolution states of the system. Key: I, transient state; II, permanent evolution state; E, ergosterol; T, tachysterol; L, lumisterol; and P, previtamin D_2.*

X_j Values Used—Let ϵ_j be the coefficient of molecular extinction related to the isomer j, expressed in liters per mole centimeter; and let ρ_j be the quantum disappearance yield of the isomer j, expressed in molecules per photon absorbed by the isomer j.

The exit probability per time unit of a class j element is given, in reciprocal seconds, by the expression (8):

$$X_j = 3.822 \times 10^{-3}\,\epsilon_j\rho_j \qquad \text{(Eq. 14)}$$

The values given by Havinga *et al.* (14) were taken for ϵ_j and ρ_j. They are summarized in Table I with the corresponding X_j values.

The slope λ_1 and the relative concentrations C_j were the subjects of the following experimental determination.

EXPERIMENTAL

The experimental values of λ_1 and of the relative concentrations C_j in the permanent evolution state at a wavelength of 253.7 nm. were determined by UV spectrophotometry and GC (7), respectively.

Determination of λ_1—The experimental determination of λ_1 was made with very weak ergosterol solutions (2×10^{-5} *M*) by following the evolution of absorbances of the four substances ergosterol, previtamin D_2, tachysterol, and lumisterol at such a wavelength that the substances outside the true graph (toxisterols) only showed insignificant absorption, since experimental determination techniques do not allow this value to be obtained by determining the slope of the evolution graph of each isomer.

Although the concentration used in the irradiated solutions is low, the 253.7-nm. wavelength incident radiation is slightly absorbed. Therefore, the correcting factor F (8), which reflects the flux decrease in the solutions, was introduced:

$$F = \frac{1 - e^{-A(t)}}{A(t)} \qquad \text{(Eq. 15)}$$

where $A(t)$ is the absorbance at time t at the incident radiation wavelength (253.7 nm.).

This factor enables the mean intensity $\bar{I}(t)$ in the irradiated solution to be known, since the incident intensity I_0 can only be used, strictly, in a very shallow system. The values $A(t)$ are determined by the experimental measurements of the change in absorbance as a function of the irradiation time at the *incident radiation wavelength* (λ = 253.7 nm.).

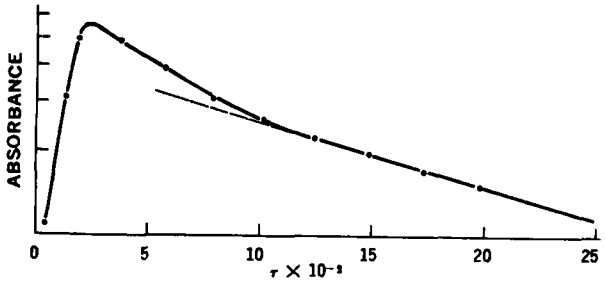


Figure 2—*Changes of absorbance with the variable* $\tau = \int_0^t [I(t)/I_0]dt$. *The slope in the rectilinear part of the curve is the slope* λ_1 *of the permanent evolution state.*

The mean intensity is then calculated, from the experimental values obtained, by the formula:

$$\bar{I}(t) = I_0 \times F \quad \text{or} \quad \bar{I}(t) = I_0 \times \frac{1 - e^{-A(t)}}{A(t)} \qquad \text{(Eq. 16)}$$

The transition from variable t, irradiation time that could be used in a very thin system, to the variable τ, which is necessary to make the system used linear (this system was approximately but not really a very thin system since there was an attenuation of the incident flux in the irradiation medium), is given by the formula:

$$\tau = \int_0^t \frac{\bar{I}(t)}{I_0}\, dt \qquad \text{(Eq. 17)}$$

Figure 2 shows the experimental results obtained with an ergosterol solution as the initial product; τ is on the abscissa, and the changes in absorbance with irradiation time, for a wavelength λ = 290 nm. where the substances outside the true graph have no input, are on the ordinate.

The value of slope λ_1 is calculated with the following formula:

$$\lambda_1 = \frac{\log A_1 - \log A_2}{\tau_1 - \tau_2} \qquad \text{(Eq. 18)}$$

where A_1 and A_2 are the measured absorbances corresponding to τ_1 and τ_2, respectively.

Measurements made with previtamin D_3, lumisterol$_3$, and tachysterol$_3$ as starting products gave, within experimental errors, the same value of slope λ_1. It was thus checked that the elements belonging to a same true graph have, in fact, the same slope λ_1 (12) in the same permanent evolution state, irrespective of the initial conditions.

The experimental value of slope λ_1 equals -0.27 ± 0.01 sec.$^{-1}$.

Determination of Relative Concentrations C_i—The very small amount of substance obtained after UV irradiation of a low concentration ergosterol solution (2×10^{-5} *M*) in ether does not allow the various isomers formed to be determined separately. Stronger solutions were, therefore, used (1.25×10^{-2} *M*). The *F* factor, characterizing the flux decrease, becomes zero, and the transition through the variable τ is no longer possible. But the system then tends to an asymptotic state whose relative compositions are the same as those of the permanent evolution state defined previously. Therefore, the determinations were made as soon as this asymptotic state was reached (7).

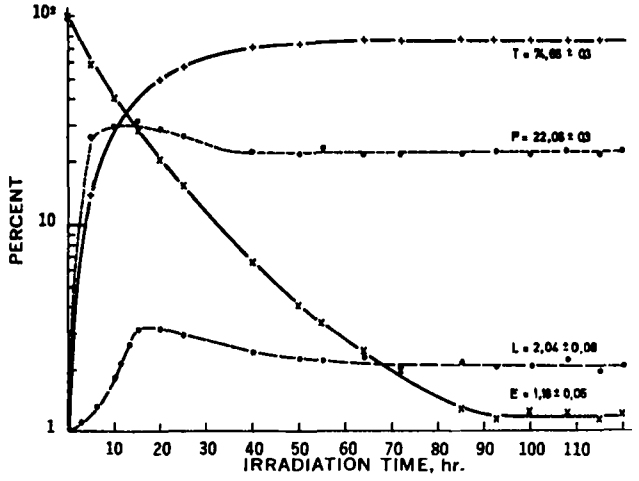
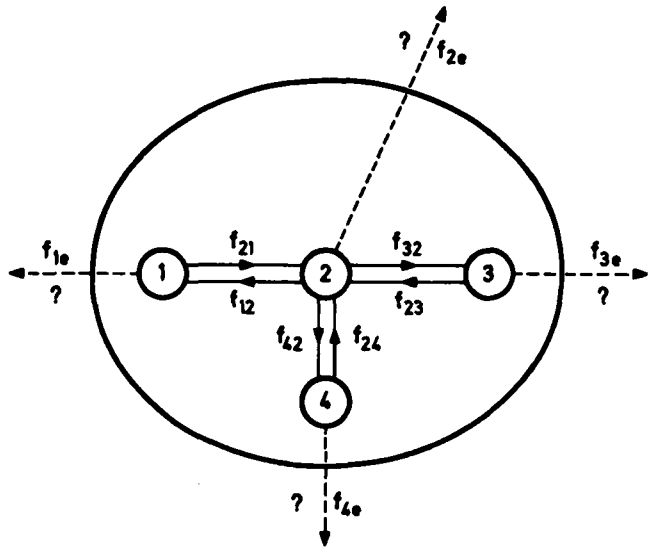


Figure 3—*Evolution with the irradiation time of the relative concentration of the four isomers. Key: E, ergosterol; T, tachysterol; L, lumisterol; and P, previtamin* D_3.

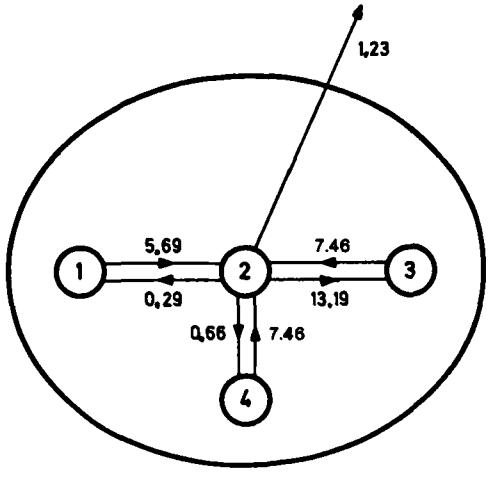


Scheme V—Internal structure of the true exotropic graph of the photoisomeric system of ergosterol. The possible irreversible transformations are represented by external functions f_{je}.

The separation methods used for the qualitative analysis (9–11) showed that for the long irradiation times needed to reach the asymptotic state the previtamin and one of the toxisterols were not separated; only GC, with 3.04-m. (10-ft.) long and 2-mm. × 0.6-cm. (0.25-in.) diameter Pyrex columns filled with 3% XE-60 on Gas Chrom Q, 100–120 mesh, permitted adequate separation of all isomers.

Before being used, the column was placed in an oven heated to 230° for 2 days, with a convection gas (helium) flow of 20 ml./min. The temperature of the oven was then regulated to 150°, and several injections of 10 μl. each of hexamethyldisilazane were made.

When the baseline of the recorder was stabilized, the temperature of the oven was again set at 230°. As soon as the flame-ionization detector gave a stable response, several injections of 2 μl. each of an irradiated ergosterol solution were made. This procedure reduces the absorption effects on the support, which are frequently encountered with new columns, and allows aging of the column.



Scheme VI—Interaction graph of the system corresponding to experimental results

Table II—Quantum Transformation Yields of Previtamin D_2

Previtamin D_2 →	Ergosterol	Tachysterol$_2$	Lumisterol$_2$	Toxisterol$_2$
Sanders (6)	0.02	0.45	0.02	—
This study	0.0092	0.42	0.021	0.039

Finally, it was necessary to make sure that the GC, owing to the high temperature used, did not cause any distortion of the structure of the eluted isomers. After trapping the various eluates in the oven and directly at the exit of the column and analyzing them by UV spectrophotometry, UV spectra of each isomer were obtained identical to those obtained at low temperature.

The internal standardization procedure was used for the quantitative analysis, and the measurement of the areas of the peaks representing the four isomers of the true graph (ergosterol, previtamin D_2, tachysterol, and lumisterol) was made with an electronic digital integrator[2].

The results obtained with a chromatograph[3] are given in Fig. 3. The ergosterol solutions in ether had a concentration of 1.25×10^{-2} *M*. They were irradiated with an incident flux of 1.24×10^{-5} photon/cm.^{2} sec.

The experimental values of λ_1 and of the relative concentrations were: $\lambda_1 = -0.27 \pm 0.01$ sec.$^{-1}$, $C_1 = 1.18 \pm 0.05\%$, $C_2 = 22.06 \pm 0.3\%$, $C_3 = 74.66 \pm 0.3\%$, and $C_4 = 2.04 \pm 0.08\%$.

RESULTS AND DISCUSSION

Values of Internal Divergent Functions Relative to Class 2—Let Eq. 9 be applied in turn to classes 1, 3, and 4:

$$f_{12} = (\lambda_1 + X_1)\frac{C_1}{C_2} = 0.29 \text{ sec.}^{-1} \qquad \text{(Eq. 19)}$$

$$f_{32} = (\lambda_1 + X_3)\frac{C_3}{C_2} = 13.19 \text{ sec.}^{-1} \qquad \text{(Eq. 20)}$$

$$f_{42} = (\lambda_1 + X_4)\frac{C_4}{C_2} = 0.66 \text{ sec.}^{-1} \qquad \text{(Eq. 21)}$$

Value and Unicity of External Function f_{2e}—The value of f_{2e} is given by:

$$f_{2e} = X_2 - (f_{12} + f_{32} + f_{42}) \qquad \text{(Eq. 22)}$$

giving $f_{2e} = 1.229$ sec.$^{-1}$. This external function is unique because, using Eq. 13, the term corresponding to class 2—allowing for the value of f_{2e} previously determined—is:

$$-f_{2e}C_2 = 0.271 \qquad \text{(Eq. 23)}$$

The value of λ_1 being $\lambda_1 = -0.27$, the term $-f_{2e}C_2$ suffices to express it and the three other terms are, therefore, zero. Thus, $f_{1e} = f_{3e} = f_{4e} = 0$.

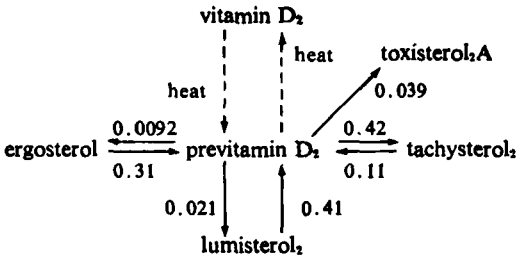
Value of Converging Functions Related to Class 2—Classes 1, 3, and 4 were not given external functions:

$$f_{21} = X_1 = 5.69 \text{ sec.}^{-1} \qquad \text{(Eq. 24)}$$

$$f_{24} = X_4 = 7.46 \text{ sec.}^{-1} \qquad \text{(Eq. 25)}$$

$$f_{23} = X_3 = 4.17 \text{ sec.}^{-1} \qquad \text{(Eq. 26)}$$

[2] Varian Aerograph model 480.
[3] Varian Aerograph model 2100.



Scheme VII—Suggested reaction scheme for the photoisomerization of ergosterol

Proposed Reaction Scheme—All these results are summarized in Scheme VI in which the transfer functions are expressed in reciprocal seconds.

As the quantum yield ρ_{ij} of the transformation of isomer j into isomer i is obtained, as from the transfer function f_{ij} by the relation (8):

$$\rho_{ij} = 2.616 \times 10^3 \frac{f_{ij}}{\epsilon_j} \qquad \text{(Eq. 27)}$$

the reaction scheme shown in Scheme VII is obtained. This scheme is structurally identical to those proposed by Velluz (2) and Sanders (6) (Scheme III). It differs from them by the value of the quantum transformation yields of previtamin D_2.

The values submitted by Sanders (6) and the values from the present study are summarized in Table II.

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Mechanism of Action of Retinyl Compounds on Wound Healing III: Effect of Retinoic Acid Homologs on Granuloma Formation

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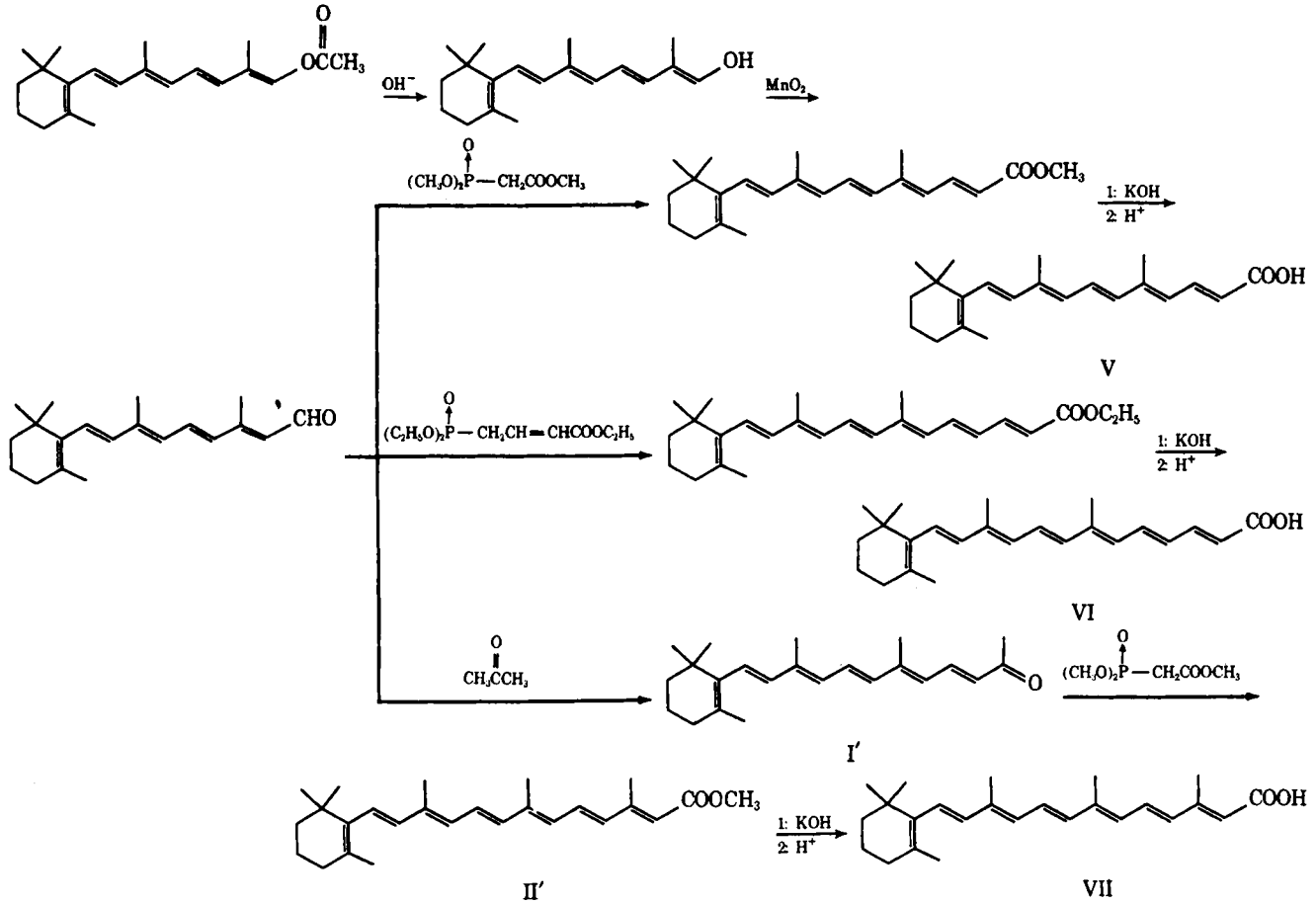
Abstract □ A series of homologs of retinoic acid was synthesized. The activities of these compounds on granuloma formation induced by implanted cotton pellets were studied. The compound (β-C_{22} acid) with two more carbons on the side chain of retinoic acid is the most active. The hexosamine and hydroxyproline contents in the granuloma affected by these compounds are also increased. The mechanisms of action of these compounds on wound healing are discussed.

Keyphrases □ Retinoic acid analogs—synthesis, effect and mechanism on granuloma formation □ Wound healing—synthesis of retinoic acid analogs, effect and mechanism on granuloma formation □ Granuloma formation—synthesis, effect and mechanism of retinoic acid analogs □ Structure–activity relationships—effect of retinoic acid analogs containing 2,6,6-trimethylcyclohex-1-ene ring on granuloma formation

Previous reports showed that a few retinol-related compounds promote skin wound healing in rats (1–4). The structure–activity relationships of a few naturally occurring compounds related to retinol were studied (3). The fact that retinol, retinyl acetate, and retinoic acid are all active in promoting wound healing indicates that the primary alcohol group is not essential. β-Carotene, the natural precursor of retinol, is active. Lycopene has essentially the same structure as β-carotene except that it does not have a closed ring at either end of the molecule. Lycopene is inactive. β-Ionone has essentially the same trimethylcyclohexene ring as retinol or β-carotene, but it has a shorter side chain. β-Ionone is active, but it is less active than retinol. α-Ionone, with only the position of a double bond in the ring different from β-ionone, is not active. It is clear that the 2,6,6-trimethylcyclohex-1-ene ring is essential for activity.

The present study concerned the structure–activity relationship of side-chain length of retinoic acid and tissue regeneration. The growth of granuloma induced by the implanted cotton pellet technique, as



Scheme I

described previously (4), was used. Jackson *et al.* (5) pointed out that the repair of connective tissue is the most basic feature in wound healing, and they used the formation of granuloma induced by polyvinyl sponges to study healing. Sandberg and Zederfeldt (6) found that the rate of gain in tensile strength and hydroxyproline in granuloma was directly related in both rats and rabbits. We have found that only those compounds that are active in increasing tensile strength also increase the size and weight of granuloma induced by either cotton pellet or polyvinyl sponge. Granuloma formation is a useful quantitative method to study wound healing.

EXPERIMENTAL[1]

Materials and Special Chemicals—Materials and chemicals used in this study included the following: crystalline hydroxy-L-proline[2]; retinoic acid[3], all-*trans*, Sigma grade, Type XX; D-glucosamine hydrochloride[3], A grade; sodium hydride[4], 57% oil dispersion; lithium aluminum hydride powder[4], 97.5%; triethyl phosphite[5], 97%; β-ionone[5], n_D^{20} 1.584°; trimethylphosphonoacetate[5]; ethyl 4-bromocrotonate[5], 75%; tetrahydrofuran[6], 99.5%; 1,2-dimethoxyethane[6]; *p*-dimethylaminobenzaldehyde[6], reagent grade; ether[7], anhydrous; phosphoric acid NF[7], 85%; and dental cotton roll[8], size I.

Implantation of Cotton Pellet—The effect of retinoic acid and its homologs (I–VII, Table I) on granuloma formation in rats was evaluated by the cotton pellet method (7). Cotton pellets are disks sliced from dental rolls with a sharp razor blade. Disks weighing 20 ± 0.5 mg. were placed in petri dishes for sterilization in a steam autoclave for 40 min. at 115°. The sterilized cotton disks were handled with sterilized instruments and an aseptic technique.

Growth of granulation tissue in cotton pellets was induced by subcutaneous implantation at two symmetrical dorsolateral sites on Sprague–Dawley male rats, weighing 120 ± 2 g., under ether anesthesia.

The cotton pellet implanted on the right side contained 2 mg. of the test compound, and the cotton pellet implanted on the left side served as the control. The compound under study was dissolved in tetrahydrofuran, and 0.05 ml. of the solution was introduced into the pellet from a 0.25-ml. syringe with a size 20 needle. The tetrahydrofuran was completely evaporated in a vacuum desiccator connected to a life vacuum line under total darkness. Two hours was sufficient to evaporate all of the tetrahydrofuran. On the 7th day after implantation, the granulomas formed were carefully removed and the wet weight was measured right after removal. The granulomas formed were dried at 65° for 3 days, and the dry weights of the granulomas were measured.

Chemical Analysis—All of the dried granulomas resulting from a test compound of one experiment were combined in a digestion tube. One milliliter of 6 *N* HCl was added for each granuloma. The control dried granulomas were digested in a separate tube.

The lower part of the digestion tube is 25 mm. in diameter and 100 mm. in length; the upper part of the tube is 10 mm. in diameter and 100 mm. in length. It can be easily made from a 25 × 200-mm. Pyrex test tube. The digestion tube was sealed *in vacuo*, and the contents were hydrolyzed at 115° in a constant-temperature heating block or in an oil bath for 4 hr. After cooling to room temperature, the seal of the tube was broken and the contents were carefully neutralized with 6 *N* NaOH. The contents were then filtered with the aid of suction. The original digestion tube and the filter were rinsed several times with small portions of distilled water, and the filtrate was brought to volume in a suitable volumetric flask. Aliquots were used for hydroxyproline and hexosamine analysis according to the methods described by Woessner (8) and Cessi and Piliego (9), respectively.

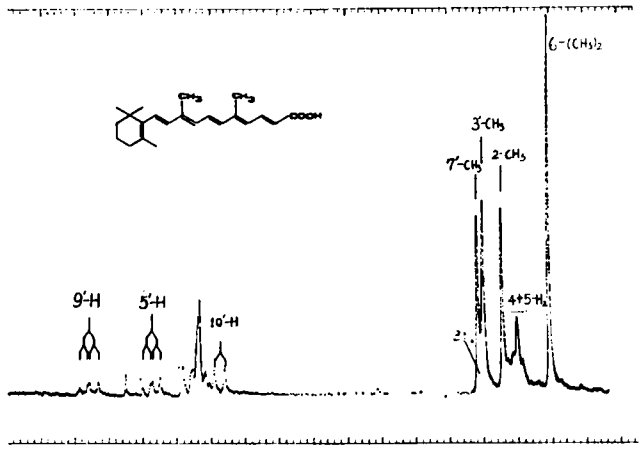


Figure 1—*NMR spectrum of β-C_{12} acid.*

Synthesis—Small quantities of analytically and spectroscopically pure Compounds II and III were used as received[9]. More of these compounds were synthesized by following essentially the published methods (10) with good success. The method for synthesizing Compound I was modified. Compounds V and VI are new. Compounds V, VI, and VII were synthesized from retinol acetate according to Scheme I.

2,6,6-Trimethyl-1-(2'-carboxy-1'-vinyl)cyclohex-1-ene (I) (β-C_{12} Acid)—Sodium hydroxide, 240 g. (6 moles), was dissolved in water to make 600 ml. solution in a 2-l. conical flask with a magnetic stirrer. The weight of the flask and its contents was recorded. The sodium hydroxide was then cooled to −5° in an acetone–dry ice bath. Chlorine gas was bubbled into the sodium hydroxide solution until 126 g. had been introduced. The temperature was kept at 0°. This takes about 1 hr. The solution was then poured into a 4-l. beaker, and 100 g. (0.52 mole) of β-ionone was added into the beaker in a slow stream with stirring. The stirring was continued at room temperature after the addition of β-ionone for 3 hr.

Methanol (120 ml.) was slowly added to the contents in the beaker with stirring. Vigorous reaction was started about 3 min. after the addition of methanol, and chloroform bubbled out from the solution. The mixture was kept just below 80° by adding crushed ice. After the reaction subsided, the mixture was cooled to 2–3°. The mixture was neutralized slowly with concentrated phosphoric acid (approximately 200 ml.). The final pH was adjusted to about 3.5. The β-C_{12} acid solidified and separated out of the solution and was filtered in a Büchner funnel. The acid was dissolved in ether. The ether solution was extracted with 10% sodium hydroxide solution four times, and the ether layer was discarded. The alkaline extract was acidified with 6 *N* HCl slowly at cold temperature to pH 4. The β-C_{12} acid was extracted with ether, and the extract was dried overnight with anhydrous magnesium sulfate. The dried ether was filtered by gravity and washed with anhydrous ether, and the ether of the combined extracts was evaporated. The yield of crude C_{12} acid was 92 g. (92%). The crude acid was recrystallized from 60% ethanol, and the yield was about 74 g. (74%), m.p. 107.5–108.0° (uncorrected) [lit. (10) m.p. 105.5–108°]; UV absorption spectrum (ethanol): λ_{max} = 278 nm., ϵ = 10,000; IR spectrum (KBr): C=O 1690 cm.$^{-1}$, C=C 1620 cm.$^{-1}$; NMR spectrum ($CDCl_3$): gem-dimethyl δ = 1.09 (singlet), $C_{(2)}$—CH_3 δ = 1.80 (singlet), $C_{(2')}$—H δ = 5.90 (doublet), and $C_{(1')}$—H δ = 7.60 (doublet).

Anal.—Calc. for $C_{12}H_{18}O_2$: C, 74.19; H, 9.34. Found: C, 73.88; H, 9.16.

2,6,6-Trimethyl-1-(10'-carboxy-3',7'-dimethyldeca-1',3',5',7',9'-pentaenyl)cyclohex-1-ene (V) (β-C_{22} Acid)—Sodium hydride (3.1 g. 57% in mineral oil, 0.07 mole) was washed with ether and then sus-

[1] The NMR spectra of the synthesized compounds were measured with either a Varian A-60 analytical spectrometer or Jeolco J N M-4H-100 spectrometer. A Cary 15 double-beam UV spectrophotometer and Perkin-Elmer IR spectrometer were used to measure UV and IR spectra. Elemental analysis was performed by the Chemistry Department's Microchemical Analysis Laboratory, University of California, Berkeley, Calif.

[2] Sigma Chemical Co., St. Louis, Mo.

[3] Calbiochem, Los Angeles, Calif.

[4] Alpha Inorganics, Beverly, Mass.

[5] Aldrich Chemical Co., Milwaukee, Wis.

[6] Eastman Kodak Co., Rochester, N. Y.

[7] Mallinckrodt Chemical Works, St. Louis, Mo.

[8] Johnson & Johnson, New Brunswick, N. J.

[9] Supplied by Professor H. O. Huisman, University of Amsterdam, The Netherlands.

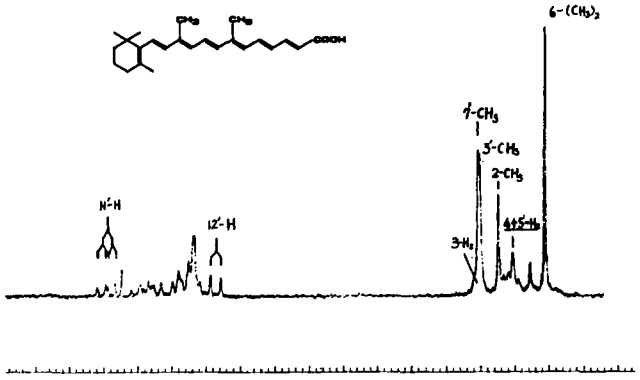


Figure 2—*NMR spectrum of β-C_{24} acid.*

pended in dry tetrahydrofuran at 0°. Trimethylphosphonoacetate (15.7 g., 0.07 mole) was added slowly to this suspension so that the temperature remained below 5°. The reaction mixture was kept at 0° with stirring for 30 min. Retinal (10 g., 0.035 *M*) was added as a solution in tetrahydrofuran over 15 min. The reaction mixture was kept at 0° for 30 min. and then at 35° for an additional 30 min. Saturated sodium chloride solution was cautiously added to the mixture and cooled to 0°. Extraction (petroleum ether, b.p. 30–60°), drying over anhydrous magnesium sulfate, and removal of solvent gave a dark-red oil containing the ethyl ester of β-C_{22} acid. The ester was immediately hydrolyzed in 100 ml. of ethanol and 100 ml. of a 20% potassium hydroxide solution at room temperature overnight. The reaction mixture was diluted with water, and the nonacidic impurities were extracted with petroleum ether. The aqueous layer was extracted with ether after acidifying with hydrochloric acid to pH 4, and the ether extract was dried over anhydrous magnesium sulfate. After removing the solvent, β-C_{22} acid (9 g., 79%) was isolated. Recrystallization from 80% ethanol three times gave pure *trans*-β-C_{22} acid, m.p. 180.5–181.0° (uncorrected); UV absorption spectrum (ethanol): λ_{max} = 378 nm., ϵ = 46,400; IR spectrum (KBr): C=O 1680 cm.$^{-1}$, C=C 1600 cm.$^{-1}$; NMR spectrum ($CDCl_3$) (Fig. 1): gem-dimethyl δ = 1.04 (singlet), $C_{(3)}$—CH_3 δ = 1.72 (singlet), $C_{(3')}$—CH_3 δ = 2.00 (singlet), $C_{(7')}$—CH_3 δ = 2.08 (singlet), $C_{(10')}$—H δ = 5.88 (doublet) (J = 13 Hz., $C_{(5')}$—H δ = 6.88 (quartet), and $C_{(9')}$—H δ = 7.80 (quartet) (J = 13 Hz.).

Anal.—Calc. for $C_{22}H_{30}O_2$: C, 80.87; H, 9.20. Found: C, 80.77; H, 9.25.

2,6,6-Trimethyl-1-(12′-carboxy-3′,7′-dimethyldodeca-1′,3′,5′,7′,9′,11-hexaenyl)cyclohex-1-ene (VI) (β-C_{24} Acid)—β-C_{24} acid was synthesized essentially as β-C_{22} acid except that 17.5 g. (0.07 mole) of triethylphosphonocrotonate was used to replace trimethylphosphonoacetate for 10 g. (0.035 mole) of retinal. The β-C_{24} ester was refluxed in alcohol (50%) containing 10% potassium hydroxide under nitrogen for 3 hr. The yield was 10 g. (81%). After recrystallization from 80% ethanol three times, pure β-C_{24} acid was obtained, m.p. 188.0–188.5° (uncorrected); UV absorption spectrum (cyclohexane): λ_{max} = 410, ϵ = 69,800; IR spectrum (KBr): C=O 1680 cm.$^{-1}$, C=C 1600 cm.$^{-1}$; NMR spectrum ($CDCl_3$) (Fig. 2): gem-

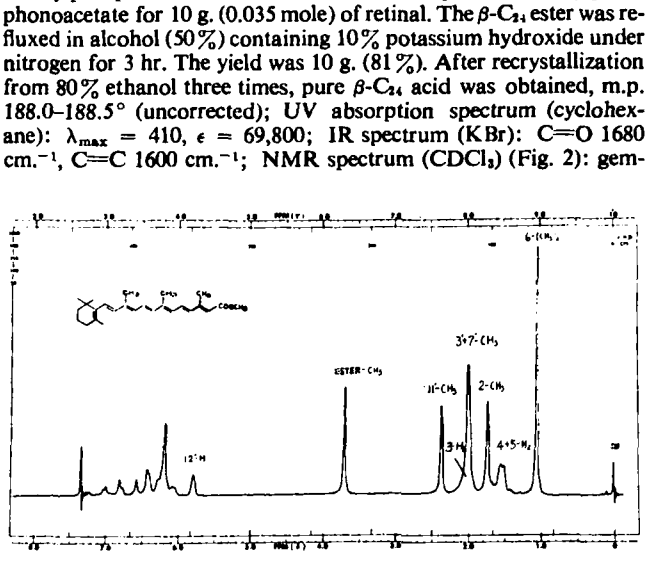
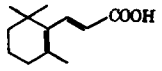
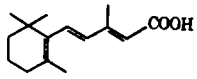
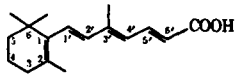
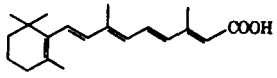
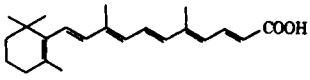
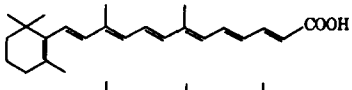
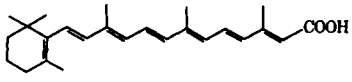


Figure 3—*NMR spectrum of β-C_{25} acid methyl ester.*

Table I—Structural Formulas of Retinoic Acid Homologs Studied

Compound Number	Structure	Trivial Names
I		All-*trans*-β-C_{12} acid
II		All-*trans*-β-C_{16} acid
III		All-*trans*-β-C_{17} acid
IV		All-*trans*-β-C_{20} acid
V		All-*trans*-β-C_{22} acid
VI		All-*trans*-β-C_{24} acid
VII		All-*trans*-β-C_{25} acid

dimethyl δ = 1.04 (singlet), $C_{(3)}$—CH_3 δ = 1.72 (singlet), $C_{(3')}$—CH_3 δ = 2.00 (singlet), $C_{(7')}$—CH_3 δ = 2.02 (singlet), $C_{(12')}$—H δ = 5.87 (doublet) (J = 13 Hz.), and $C_{(11')}$—H δ = 7.49 (quartet) (J = 13 Hz.).

Anal.—Calc. for $C_{24}H_{32}O_2$: C, 81.77; H, 9.15. Found: C, 81.82; H, 9.09.

3,5,7,11-Dodecapentaein-2-one-6,10-dimethyl-12-(2′,6′,6′-trimethyl-1′-cyclohexene-1′-yl) (I′) (β-C_{22} Ketone)—A mixture of 28 g. (0.098 mole) of retinal, 75 ml. of petroleum ether (b.p. 30–60°), 300 ml. of acetone, and 100 ml. of 0.5 *N* NaOH solution was refluxed for 2.5 hr. After cooling, the reaction mixture was poured into 500 ml. of ice water and 35 ml. of 2 *N* H_2SO_4. The organic layer was separated and the aqueous layer was extracted with petroleum ether. The combined organic phases were washed with water and dried over anhydrous magnesium sulfate. Evaporation of solvent gave 15 g. of solid residue (46.3%). It was crystallized from hexane, yielding 11.3 g. (34.8%), m.p. 104–105° [lit. (11) m.p. 104–105°]; UV absorption spectrum (cyclohexane): λ_{max} = 392, ϵ = 52,700; IR spectrum (KBr): C=O 1650 cm.$^{-1}$, C=C 1600 cm.$^{-1}$; NMR spectrum ($CDCl_3$): gem-dimethyl δ = 1.03 (singlet), $C_{(2')}$—CH_3 δ = 1.73 (singlet), $C_{(10)}$—CH_3 δ = 2.00 (singlet), $C_{(6)}$—CH_3 δ = 2.10 (singlet), and $C_{(1)}$—CH_3 δ = 2.30 (singlet).

2,6,6-Trimethyl-1-(12′-carbomethoxy-3′,7′,11′-trimethyldodeca-1′,3′,5′,7′,9′,11′-hexaenyl)cyclohex-1-ene (II′)—Trimethylphosphonoacetate, 5.46 g. (0.03 mole), in 20 ml. tetrahydrofuran was added dropwise to a well-stirred suspension of 0.72 g. (0.03 mole) sodium hydride in 200 ml. tetrahydrofuran. The temperature was kept at −5–0° for 1 hr. and at room temperature for 1 hr. A solution of β-C_{23} ketone, 4.86 g. (0.015 mole), in 20 ml. tetrahydrofuran was then added dropwise. The mixture was refluxed for 5 hr. under nitrogen. It was then poured into 500 ml. of water and extracted with petroleum ether. The organic phase was washed with water. After removal of the solvent, 8 g. of crystalline residue was obtained. It was recrystallized from 95% ethanol to give 4 g. of β-C_{25} acid methyl ester (70.1%), m.p. 108.5–109° (uncorrected); UV absorption spectrum (cyclohexane): λ_{max} = 402, ϵ = 68,200; IR spectrum (KBr): C=O 1700 cm.$^{-1}$, C=C 1600 cm.$^{-1}$; NMR spectrum ($CDCl_3$) (Fig. 3): gem-dimethyl δ = 1.01 (singlet), $C_{(3)}$—CH_3 δ = 1.70 (singlet), $C_{(3')}$ + $C_{(7')}$—CH_3 δ = 2.00, $C_{(11')}$—CH_3 δ = 2.35, ester—CH_3 δ = 3.7 (singlet), and $C_{(12')}$—H δ = 5.8 (singlet).

2,6,6-Trimethyl-1-(12′-carboxy-3′,7′,11′-trimethyldodeca-1′,3′,5′,7′,9′,11-hexaenyl)cyclohex-1-ene (VII) (β-C_{25} Acid)—A mixture of 4 g. (0.011 mole) β-C_{25} acid methyl ester, 5 g. potassium hydroxide,

Table II—Effect of Retinoic Acid Homologs on Cotton Pellet-Induced Granuloma

Compound Number	Number of Animals	Compound Applied	Body Weight Change, Average	Granuloma Wet Weight, mg. Experimental	Granuloma Wet Weight, mg. Control	Granuloma Weight Experimental/Control (p)	Granuloma Dry Weight, mg. Experimental	Granuloma Dry Weight, mg. Control	Experimental/Control (p)
I	6	C_{12}	+48	213.8 ± 7.5	215.3 ± 5.1	1.0	28.3 ± 3.1	26.8 ± 2.9	1.0
II	6	C_{15}	+38	214.5 ± 2.3	215.4 ± 5.0	1.0	27.6 ± 1.0	27.8 ± 0.8	1.0
III	12	C_{17}	+50	220.5 ± 8.2	218.0 ± 6.0	1.0	30.2 ± 2.7	29.9 ± 3.0	1.0
IV	12	C_{20}	+50	332.2 ± 4.9	218.7 ± 2.5	1.5 (<0.001)	59.9 ± 1.7	35.1 ± 1.9	1.5 (<0.001)
V	12	C_{22}	+47	438.0 ± 5.4	225.9 ± 7.4	1.9 (<0.001)	69.8 ± 2.6	36.1 ± 2.5	1.9 (<0.001)
VI	10	C_{24}	+46	302.0 ± 12.7	216.5 ± 6.9	1.4 (<0.001)	47.4 ± 2.1	31.3 ± 2.2	1.5 (<0.001)
VII	7	C_{25}	+61	278.4 ± 26.4	198.7 ± 4.3	1.4 (<0.005)	35.0 ± 2.7	20.8 ± 1.3	1.6 (<0.001)

25 ml. water, and 150 ml. ethanol was stirred under nitrogen in a 50° water bath for 4 hr. After diluting with water, the nonacidic impurities were extracted with ether and the water layer was acidified with sulfuric acid. Extraction with ether and evaporation of ether gave 3.5 g. of orange-red crystalline residue. It was crystallized from isopropyl alcohol to give 1.5 g. (90.0%) of pure β-C_{25} acid, m.p. 197–198°; UV absorption spectrum (cyclohexane): λ_{max} = 405 nm., ϵ = 70,300; IR spectrum (KBr): C=O 1700 cm.$^{-1}$, C=C 1600 cm.$^{-1}$.

Anal.—Calc. for $C_{25}H_{24}O_2$: C, 81.96; H, 9.29. Found: C, 81.95; H, 9.47.

The acid was converted into methyl ester by treatment with diazomethane and its NMR spectrum is identical with that shown in Fig. 3.

RESULTS AND DISCUSSION

The structural formulas for the compounds studied are shown in Table I. The effects of these compounds on granuloma formation induced by implantation of cotton pellets are shown in Table II. Compounds with a side chain shorter than retinoic acid did not stimulate granuloma formation at the amount of 2 mg./pellet, while compounds with a side chain longer than that of retinoic acid stimulated granuloma formation. These findings are in agreement with the previous finding (4) that, besides the 2,6,6-trimethylcyclohex-1-ene ring, the length of the side chain is also important for activity. Compound V is the most active.

Meier *et al.* (7) introduced a simple quantitative method for measuring the degree of inflammation. They used a cotton pellet as a foreign body to induce granuloma formation. Application of cortisone caused a diminution of granuloma size, which can be expressed quantitatively by determining the weight of the granuloma formed. Since then, this method has been extensively used for assaying steroid and nonsteroid anti-inflammatory agents. Retinoic acid and its active homologs, like retinol (4), cause an increased granuloma mass induced by cotton pellets. These active compounds are "inflammatory agents."

Grindlay and Waugh (12), at about the same time, applied essentially the same implantation technique to study tissue regeneration. Jackson *et al.* (5) pointed out that the repair of connective tissue is the most basic feature in wound healing, and they used the formation of granuloma induced by implanted polyvinyl sponges to study wound healing. Sandberg and Zederfeldt (6) found that the rate of gain in tensile strength and the rate of hydroxyproline synthesis in granuloma were directly related in both rats and rabbits. This was confirmed previously in this laboratory (4). Granuloma formation is a technique used for measuring inflammation as well as wound healing.

Inflammation and mucopolysaccharide synthesis are the two known important features in wound healing. Anti-inflammatory

Table III—Effect of β-C_{20} Acid on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control	Experimental
Number of animals	6	
Granuloma dry weight, mg.	36	61.2
Experimental/control	1.7	
Hydroxyproline weight, mcg.	395.6	662.5
Experimental/control	1.67	
Hydroxyproline, mcg./granuloma, mg.	11	10.8
Hexosamine weight, mcg.	330	493
Experimental/control	1.50	
Hexosamine, mcg./granuloma, mg.	9.17	8.1

Table IV—Effect of β-C_{22} Acid on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control	Experimental
Number of animals	8	
Granuloma dry weight, mg.	31.8	70.4
Experimental/control	2.2	
Hydroxyproline weight, mcg.	343.7	637.5
Experimental/control	1.85	
Hydroxyproline, mcg./granuloma, mg.	10.8	9.1
Hexosamine weight, mcg.	306	640
Experimental/control	2.1	
Hexosamine, mcg./granuloma, mg.	9.6	9.1

Table V—Effect of β-C_{24} Acid on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control	Experimental
Number of animals	6	
Granuloma dry weight, mg.	30.2	47.9
Experimental/control	1.6	
Hydroxyproline weight, mcg.	350	600
Experimental/control	1.7	
Hydroxyproline, mcg./granuloma, mg.	11.6	12.5
Hexosamine weight, mcg.	284	460
Experimental/control	1.62	
Hexosamine, mcg./granuloma, mg.	9.4	9.6

Table VI—Effect of β-C_{25} Acid on Hydroxyproline and Hexosamine Contents of Granuloma Induced by Cotton Pellets

	Control	Experimental
Number of animals	7	
Granuloma dry weight, mg.	20.8	35
Experimental/control	1.6	
Hydroxyproline weight, mcg.	287.5	412.5
Experimental/control	1.44	
Hydroxyproline, mcg./granuloma, mg.	13.8	11.8
Hexosamine weight, mcg.	223	333
Experimental/control	1.5	
Hexosamine, mcg./granuloma, mg.	10.7	9.5

agents retard healing by their anti-inflammatory and inhibitory actions on mucopolysaccharide synthesis (2). Retinoic acid reverses the healing retardation action of anti-inflammatory agents (13). Active retinoic acid homologs studied in the present paper promote wound healing by increasing granuloma mass.

The healing-promoting action of these active retinoic acid homologs is further evidenced by the increase of hydroxyproline and hexosamine contents of the granuloma (Tables III–VI). Hydroxyproline is an important constituent of collagen, which is an essential component of connective tissue. Hexosamine is a component of mucopolysaccharide, and the role of mucopolysaccharide in healing was extensively discussed previously (2). As shown in Tables III–VI, both the hydroxyproline and hexosamine contents were increased in the granuloma with either one of the active compounds. This fact indicated that these compounds increased connective tissue regeneration. However, the amount of hydroxyproline or hexosamine per unit of weight of granuloma of the experimental was lower than that of the control. This fact indicated that these active compounds also stimulated inflammation.

It is suggested that active retinoic acid homologs, like retinol, promote healing by inducing inflammation and increase mucopolysaccharide and collagen synthesis mechanisms of action.

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Extraction and Separation of Anthraquinone Glycosides

STEPHEN CHAO YUNG SU and N. M. FERGUSON▲

Abstract □ A method was developed for extracting anthraquinone aglycones and their corresponding glycosides from plant materials and for separating them in pure form. The method consists of rendering the glycosides and aglycones insoluble in chloroform and then removing, stepwise, the interfering extractable substances. The method makes use of the fact that the aglycones are chloroform soluble whereas the glycosides are not. The method was applied with good results to the isolation of the aglycones and glycosides of cascara and senna.

Keyphrases □ Anthraquinone aglycones and corresponding glycosides—extraction, separation from plant material □ Cascara bark—extraction, separation of anthraquinone aglycones and glycosides □ Senna leaves—extraction, separation of anthraquinone aglycones and glycosides

The methods that have been used to date for the isolation and purification of the anthraquinone derivative glycosides from cascara bark and senna leaflets fall into three categories. The first involves an aqueous or hydroalcoholic menstruum (1), and the second uses a pure solvent such as methanol (2, 3), ethanol (4), propyl alcohol, or isopropyl alcohol (1). The final method uses a solvent such as chloroform or ether (5) to remove certain interfering substances, and then extraction proceeds using an alcoholic menstruum. Purification of the various fractions is then accomplished by recrystallization from one of these solvents. In some procedures, the fractions are acetylated in benzene (1, 3, 6) prior to being further purified by fractional crystallization.

In view of the fact that interfering plant constituents in cascara bark and senna leaflets made a clearcut separation of their constituents impossible by these methods, it was decided to try to develop a new procedure which would produce purer fractions of the anthracene derivatives, particularly derivatives of hydroxyanthraquinones, both free and in the form of glycosides.

EXPERIMENTAL

Extraction Procedure—The extraction procedure chosen (Scheme I) is based on the fact that the hydroxyanthraquinone derivatives and their corresponding glycosides are present both free and as magnesium, potassium, and sodium salts combined either through a hydroxy or a carboxylic acid group. In addition, the separation method capitalizes on the differential solubility in chloroform of the glycosides and their aglycones as well as other anthracene derivatives. The glycosides are insoluble in chloroform whereas the free aglycones, as well as the other anthracene derivatives, are chloroform soluble.

The extraction procedure consisted of macerating the ground drug for 24 hr. with 5% acetic acid. The purpose of this step was to liberate the free anthraquinones and their corresponding glycosides from their magnesium, potassium, or sodium salts, in which form

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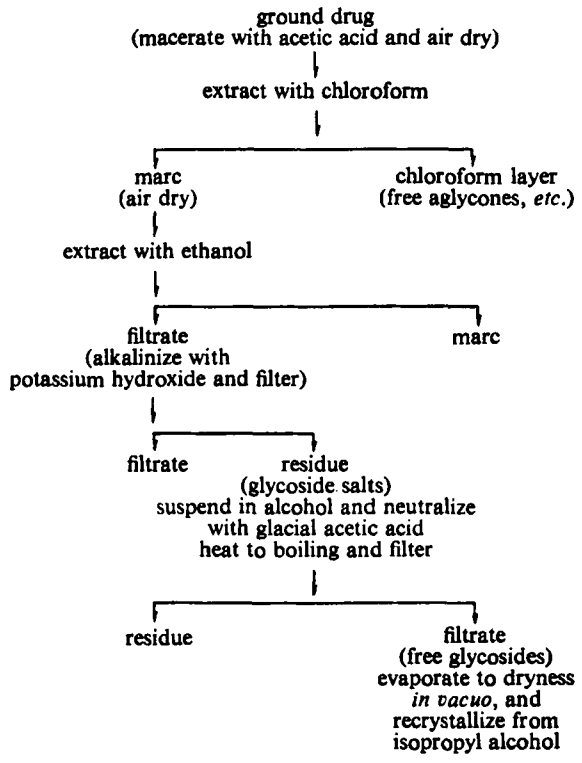

Scheme I—General Extraction Procedure

some of them are present in the plant. The maceration mixture was then dried and extracted with chloroform until free of anthracene derivatives. The drug was again air dried and then extracted with warm 95% ethanol. The alcoholic extract was then treated with 5% alcoholic potassium hydroxide solution until precipitation of the anthraquinone glycosides was complete. Following this step, the residue was filtered and washed with 95% ethanol to remove the last traces of potassium hydroxide and was then sucked dry on a Büchner funnel. The anthraquinone glycoside salt residue was suspended in warm ethanol, and the free glycosides were liberated by adding glacial acetic acid dropwise with stirring until the color

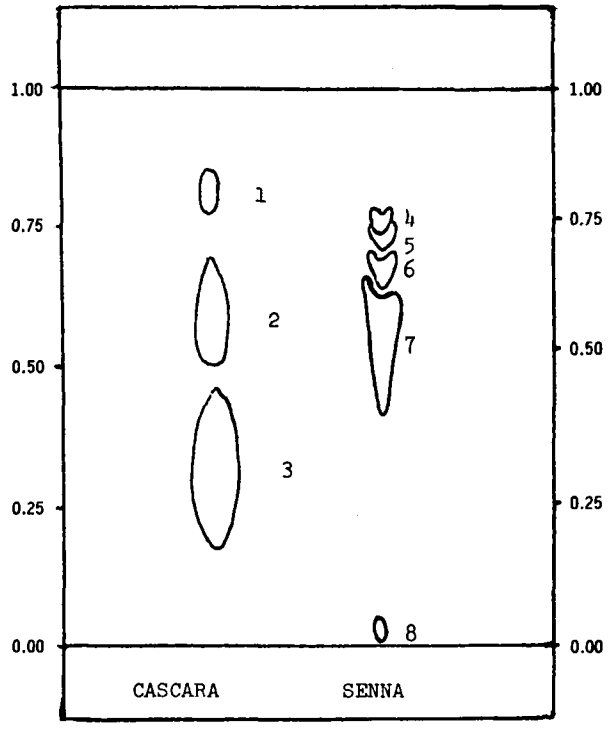

Figure 1—*Chromatogram of glucosides.*

Table I—R_f Values of Cascara Glucosides Calculated from Fig. 1

Spot Number	R_f Value
1	0.80
2	0.57
3	0.26

Table II—R_f Values of Senna Glucosides Calculated from Fig. 1

Spot Number	R_f Value
4	0.75
5	0.71
6	0.65
7	0.48
8	0.05

change was complete. The solution was then filtered hot, and the residue was further extracted with warm ethanol. The combined filtrates were then evaporated to dryness under reduced pressure, and the residue was recrystallized from isopropyl alcohol.

Cascara Sagrada—Powdered cascara sagrada bark (*Rhamnus purshiana*, D. C.)[1], 300 g., was macerated with 850 ml. of 5% acetic acid for 24 hr. in a 2000-ml. stoppered conical flask. The mixture was air dried, after which it was exhaustively extracted with chloroform to remove free anthraquinones as well as many impurities. This required 11 600-ml. portions of chloroform. The absence of a red color imparted to a small portion of the chloroform filtrate on the addition of a few drops of 5% alcoholic potassium hydroxide indicated complete extraction of the hydroxyanthraquinones. The chloroform extracts were then combined and evaporated in air to give 21.6 g. of residue.

The marc obtained was air dried and extracted exhaustively with 600-ml. portions of warm ethanol until successive extracts showed no red or pink coloration when treated with a few drops of a 5% alcoholic potassium hydroxide solution. For complete extraction, 8100 ml. of ethanol was required. The combined filtrates were then evaporated to 3000 ml. *in vacuo* at 40°. To this solution, 5% alcoholic potassium hydroxide was added until no further precipitation occurred. The solution was then filtered, and the residue was washed with cold ethanol to remove the last traces of potassium hydroxide and then dried by suction on the Büchner funnel. The residue thus obtained, consisting of the potassium salts of the glycosides, was suspended in warm ethanol; the free glycosides were liberated by adding glacial acetic acid slowly with stirring. The solution was then filtered hot, and the residue was further extracted with warm ethanol. The combined filtrates were evaporated to dryness *in vacuo* at 40°, and the residue thus obtained was recrystallized from isopropyl alcohol. The yield of glycosides from 300 g. of cascara bark was 5.5 g. or 1.83%.

Senna—Powdered senna[2] (*Cassia angustifolia*, Delile), 300 g., was extracted by the same method as was used for cascara bark. The chloroform extracts, when combined and evaporated in air, gave 22.4 g. of residue. The yield of glycosides from the marc was 5.2 g. or 1.73%.

Chromatographic Separation—*Glucosides of Cascara and Senna*—Because of the difference in the solubility of the hydrophilic anthraquinone glycosides compared to that of the lipophilic aglycones, it is impossible to carry out the separation of both these compounds simultaneously in one system. It became necessary, therefore, to use two different sets of conditions for their separation.

In the chromatographic separation of the glucosides, the method of Betts *et al.* (7) was used. This consisted of using a mixture of water, acetone, and benzene (2:1:4); the mixture was shaken vigorously and then allowed to separate. The lower aqueous phase (*i.e.*,

[1] Cascara sagrada bark USP XVI, 100 mesh. Control No. BKP 1853, S. B. Penick and Co., New York, N. Y.

[2] Senna leaves, *Cassia angustifolia*, Delile, Tinnevelley No. 1, NF X, 40 mesh. Lot No. WEB-414, E-10317, S. B. Penick and Co., New York, N. Y.

Table III—R_f Values of Aglycones Calculated from Fig. 2

Aglycone	Spot	Pure Aglycone R_f Value	Cascara Observed R_f Value	Senna Observed R_f Value
Chrysophanic acid	A	0.98	0.98	0.98
Aloe emodin	B	0.83	0.83	0.83
Frangula emodin	C	0.53	—	0.53
Rhein	D	—	0.035	—

the running solvent) and a beaker containing the upper phase of the mixture were placed on the bottom of a chromatographic jar. After equilibrium was established, the glucoside mixture, dissolved in alcohol, was spotted on Whatman No. 1 paper and placed in the jar. When the solvent front reached a suitable height, the paper was removed, dried, sprayed with 0.5% magnesium acetate in methanol, and heated to about 100° for 3–5 min. The glucosides appeared as yellow to orange spots. The chromatograms are shown in Fig. 1, and the R_f values are listed in Tables I and II.

In the chromatogram for the mixture of the glucosides of cascara, three distinct spots developed on the paper, showing three separate cascara glucosides. The spot marked "1" and having an R_f value of 0.80 corresponds to Frangula emodin glucoside, the spot marked "2" and having an R_f value of 0.57 corresponds to Aloe emodin glucoside, and the spot marked "3" and having an R_f value of 0.26 corresponds to chrysophanic glucoside.

In the chromatogram for the mixture of the glucosides of senna, it is seen that five spots appear for senna corresponding to five separate glucosides.

Aglycones of Cascara and Senna Glycosides—To determine the exact composition of the glycosides of cascara and senna as extracted by the developed procedure, it was thought advisable to hydrolyze each of the glycosidal fractions thus obtained and to identify the aglycones.

Hydrolysis of Glycosides of Cascara and Senna—A solution consisting of 20 mg. of the appropriate isolated glycosidal fraction was dissolved in 25 ml. of a 1:1 methanol–water mixture and refluxed in a boiling water bath for 30 min., using a mixture of hydrochloric acid and ferric chloride as the hydrolytic agent (7, 8). The mixture was then cooled and extracted with ether, using three 25-ml. portions. The combined ether fractions were then washed with three 15-ml. portions of water to remove the hydrochloric acid and ferric chloride. The ether solution was diluted to 50 ml. with ether and dried over anhydrous sodium sulfate.

Chromatographic Separation of Aglycones—A small amount of the filtered ether solution of the aglycones was spotted on Whatman No. 1 paper and chromatographed by the ascending method according to Betts *et al.* (7), using toluene as the running solvent.

The aglycone chromatograms are shown in Fig. 2. The aglycones hydrolyzed from the glycosides extracted and the R_f values for these aglycones are listed in Table III, where they are compared with published values for the pure aglycones. It is seen that the R_f values of the pure aglycones are just reversed in value when converted to their corresponding glucosides. This is due to the fact that, in the polar solvent system used, the addition of the sugar to the aglycone as a glucoside increases the polarity of the molecule. Thus, chrysophanic acid has an R_f value of 0.98, whereas chrysophanic acid glucoside has an R_f value of 0.26. Similarly, Aloe emodin has an R_f value of 0.83 and Aloe emodin glucoside has an R_f value of 0.57. Frangula emodin shows an R_f value of 0.53, whereas the corresponding Frangula emodin glucoside R_f value is 0.80. This general principle is borne out in the literature (8). The literature (8) value given as the R_f for rhein is 0.00–0.03, since there is considerable tailing in this constituent. The results obtained here of 0.035 are certainly within experimental error for this determination.

The paper chromatograms, after being developed through spraying, were dipped in melted paraffin to preserve them. The chromatograms then could be stored for long periods without loss of color and could serve as references for comparison with later work.

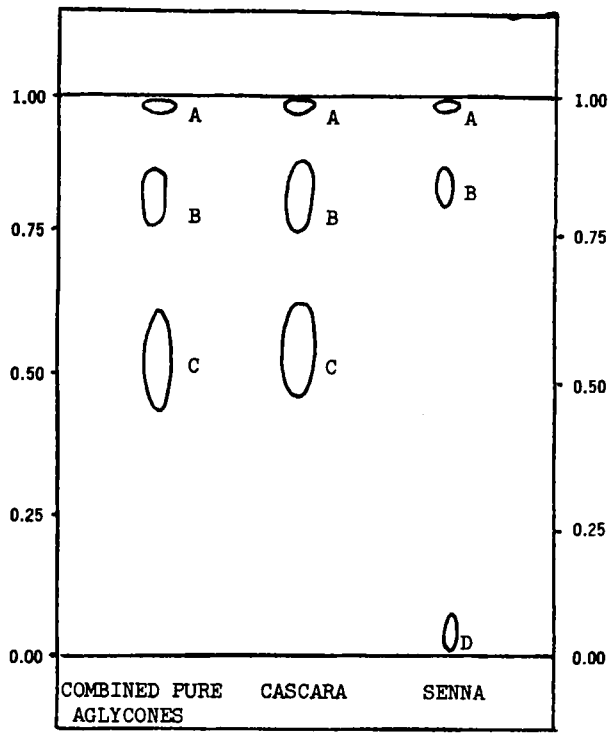

Figure 2—*Chromatogram of aglycones.*

SUMMARY AND CONCLUSIONS

A method was developed for extracting anthraquinone aglycones and their corresponding glycosides from plant materials and for separating them in pure form. This method eliminates the difficulties of filtration and extraction encountered in previous methods by rendering the glycosides insoluble from the start. In this way, many interfering substances are eliminated. Paper chromatograms and corresponding R_f values were tabulated for the aglycones and glycosides of cascara and senna.

A method was also developed for the preparation and preservation of paper chromatograms.

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ortho Effect in Alkaline Hydrolysis of *ortho*-Substituted Benzoylcholine Esters

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Abstract □ Second-order rate constants were determined for the alkaline hydrolysis of *ortho*-substituted benzoylcholine esters in 0.1 *M* aqueous NaCl at pH 9.0. Activation parameters for the reactions were calculated from the slopes of Arrhenius plots, with all plots being linear in the range of 25–45°. One derivative, *o*-methylbenzoylcholine, exhibited an abrupt change in the Arrhenius slope above 50°. Over the same extended temperature range, benzoylcholine and *o*-chlorobenzoylcholine gave no such change. An average isergonic temperature, β = 267°K., was calculated for the series from three independent correlations involving activation ($\Delta H^{\ddagger}$ *versus* $\Delta S^{\ddagger}$), rate (log k_1 *versus* log k_2), and linear free energy (ρ_T *versus* $1/T$) data. The parameters $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ each correlate independently with σ_I, the Taft inductive constant, indicating that the hydrolysis reactions are "well behaved." By comparison, thermodynamic parameters for acetic acid ionizations (*i.e.*, a model process defining σ_I) are not well behaved: ΔH° *versus* ΔS° is nonlinear, but ΔG°, ΔH°_{int}, and ΔS° correlate independently with σ_I. Rho–sigma correlations and an isergonic relationship for the benzoylcholine reactions are interpreted on the basis of the Hammett and Hepler analyses, respectively. Entropy values for this congeneric series are discussed in relation to the positive charge on the quaternary nitrogen and its effect in potentially neutralizing the substituent charge.

Keyphrases □ *Ortho* effect—alkaline hydrolysis of *ortho*-substituted benzoylcholine esters, second-order rate constants, thermodynamic parameters correlated with Taft inductive constant, relation of entropy values to nitrogen charge □ Benzoylcholine esters, *ortho*-substituted—*ortho* effect in alkaline hydrolysis, second-order rate constants, thermodynamic parameters correlated with Taft inductive constant, rho–sigma correlations □ Thermodynamic parameters—alkaline hydrolysis of benzoylcholine esters, *ortho* effect, correlated with Taft inductive constant □ Hydrolysis, alkaline—effect of *ortho*-substitution, benzoylcholine esters, thermodynamic parameters calculated

The substituent effects for a number of aliphatic (1–3) and aromatic (4, 5) reaction series have been successfully correlated using the Taft inductive constant, σ_I, originally defined according to Eq. 1 (3):

$$\sigma_I = 0.45\sigma^* = 0.45\,[\log(k/k_{CH_3})_A - \log(k/k_{CH_3})_B] \quad \text{(Eq. 1)}$$

where σ^*, the Taft polar constant, was obtained as the difference in the logarithms of the relative hydrolysis or esterification constants for acetate esters in alkaline (B) and acidic (A) solutions; k and k_{CH_3} refer to rate constants for substituted and nonsubstituted acetate esters, respectively. This definition (Eq. 1) is an inconvenient one and, more recently, it has been shown that σ_I may be expressed as a linear function of acetic acid ionizations in terms of Eq. 2 (6, 7):

$$\sigma_I = 0.276\log(K/K_{CH_3}) \quad \text{(Eq. 2)}$$

where K and K_{CH_3} are the ionization constants determined in aqueous solution for substituted and nonsubstituted acetic acid, respectively.

The electronic mechanism represented by σ_I is not entirely clear, but some evidence suggests that such a substituent effect is transmitted by a direct field effect instead of by the classical inductive mechanism (*i.e.*, induction through the chain) (8, 9). Based upon the field effect interpretation, correlations with σ_I have been used to explain the nature of the *ortho* effect in the hydrolysis of benzoate esters. For example, the effects of *ortho*-substituents in the alkaline hydrolysis of methyl and ethyl benzoates (4) and benzoylcholine iodides (5) produce rate changes which are highly correlated with σ_I. Resonance (σ_R) and steric (r_s) effects are unimportant for these reactions, and apparently only field effects (σ_I) are involved.

While the proportionality of substituent effects between acetic acid ionizations (*i.e.*, the model process defining σ_I) and *ortho*-substituted benzoate hydrolysis reactions is explicable in terms of free energy or internal potential energy changes (7), a question remains regarding the similarities or differences that exist between the respective solvation mechanisms of these reactions. A consideration of the mechanistic differences alone would suggest a dissimilarity in the solute–solvent interactions for the two processes and, hence, a difference in their observed enthalpy–entropy relationships. In this regard, previous analyses of substituent effects on enthalpy and entropy changes have greatly augmented the usual mechanistic information obtained from rho–sigma correlations; Leffler and Grunwald (10) listed a number of organic reaction series which exhibit linear enthalpy–entropy profiles in accordance with Eq. 3, the isergonic relationship[1]:

$$\delta_R\Delta H^{\ddagger} = \beta_i\delta_R\Delta S^{\ddagger} \quad \text{(Eq. 3)}$$

where δ_R = the Leffler–Grunwald operator for substituent effects (*i.e.*, the change incurred upon a reaction as a result of substituent variation); the superscript, ‡, refers to activation data; and the slope, β_i, is the isergonic temperature. For most isergonic reactions, β_i is positive in sign ($\simeq$100°–1300°K.), and a compensatory effect is imposed upon the expression for the free energy of activation (Eq. 4):

$$\delta_R\Delta G^{\ddagger} = (\beta_i - T)\delta_R\Delta S^{\ddagger} = (1 - T/\beta_i)\delta_R\Delta H^{\ddagger} \quad \text{(Eq. 4)}$$

From Eq. 4 it is seen that β_i may be defined operationally as the temperature at which a particular interaction mechanism vanishes from $\delta_R\Delta G^{\ddagger}$. Thus, when isergonic reactions are conducted near $\beta_i = T$, more useful mechanistic information is potentially available from $\delta_R\Delta S^{\ddagger}$ and $\delta_R\Delta H^{\ddagger}$ than from the simplified free energy term.

[1] The term isergonic was suggested [see R. L. Schowen, *J. Pharm. Sci.*, 56, 931(1967)] as a general term to include both isokinetic and isoequilibrium phenomena. In keeping with this suggestion, this term was adopted in the present report.

Table I—Second-Order Rate Constants for the Alkaline Hydrolysis of *ortho*-Substituted Benzoylcholine Iodides as a Function of Temperature

ortho-Substituent	k_2, M^{-1} sec.$^{-1}$[a] 25°	30°	35°	40°	45°	50°	55°	57°	60°
Hydrogen	1.22	1.65	2.05	2.87	3.66	4.77	6.50	6.61	7.69
Methyl	—	0.620	0.658	0.838	0.959	1.04	1.49	1.88	2.42
Methoxy	—	0.848	1.09	1.32	1.83	—	—	—	—
Iodo	1.66	2.16	2.82	3.64	—	—	—	—	—
Bromo	1.96	2.50	3.61	4.73	—	—	—	—	—
Chloro	2.27	2.88	4.08	5.66	—	8.82	10.4	12.4	14.7
Fluoro	2.96	3.73	5.43	7.42	—	—	—	—	—
Nitro	3.36	4.50	7.08	9.44	—	—	—	—	—

[a] Reported as an average of duplicate runs. With the exception of the methyl derivative, the mean deviation for these averages was less than 1%, excluding occasional erratic runs. The mean deviation for the methyl derivative was usually less than 3%.

In an effort to elucidate more clearly the mechanism of the *ortho* effect in the alkaline hydrolysis of *ortho*-substituted benzoylcholine esters, the activation parameters for this congeneric series were determined and the results were analyzed for the existence of an isergonic relationship. Both the Leffler–Grunwald (10) and Exner (11, 12) approaches for establishing $\Delta H^{\ddagger}$ *versus* $\Delta S^{\ddagger}$ profiles were considered to provide three independent estimates of β_i, the isergonic temperature. It was also of primary interest to compare the results of the present study with those of Hepler's (13) analysis for acetic acid ionizations to determine the analogies that may or may not exist between the two series with respect to isergonic relationships and rho–sigma dependencies.

EXPERIMENTAL

Materials—The synthesis of the ester substrates was reported previously (5). An aqueous solution of each ester was prepared just prior to investigation and stored under refrigeration during a 4-day study. All solutions were 0.1 *M* in reagent grade sodium chloride and, except for the methyl derivative, contained an ester concentration of 5×10^{-4} *M*. *o*-Methylbenzoylcholine was used at a concentration of 2.44×10^{-4} *M*. Purified nitrogen was used to provide a carbon dioxide-free environment in the reaction chamber, with the gas being passed through an aqueous solution before entering the chamber. The normalities of the sodium hydroxide titrant solutions were determined by titration with standard potassium acid phthalate solutions. A titrant normality of 0.0243 *N* was used for the methyl derivative, and one of 0.050 *N* was used for all other esters.

Kinetic Assays—Assays for the hydroxide-ion-catalyzed reactions were carried out at a constant pH of 9.00 using a pH-stat assembly (Radiometer): automatic titrator (TTT-1c), titrigraph recorder (SBR-2), syringe buret unit (SBU-1), and manual temperature compensator (PHA 924). The titration unit for this assembly consisted of a 50-ml. water-jacketed reaction chamber and an electrode head fitted with a general-purpose glass electrode (Type G 202c), reference calomel electrode (K 401), mechanical stirrer, inlet tube for nitrogen, and a titrant delivery tube for slow titrations. Solutions were maintained at desired temperatures (±0.02°) using a bath and circulator[2].

Kinetic assays were conducted in the following way. Prior to daily runs, the electrode assembly was equilibrated at the desired temperature and the pH meter was standardized with pH 9.18 buffer[3]. The 45-ml. reaction solutions were routinely flushed with nitrogen to the neutral point for the given temperature and then allowed to reach the equilibrium temperature. Nitrogen was subsequently directed across the surface of the solution during the kinetic run. Reactions were started by adding sodium hydroxide titrant to a pH of 9.00 with the automatic titrator. Base additions were continued automatically to maintain a constant pH of 9.00 during the ester hydrolysis. Depending upon the particular ester and temperature used, the length of the kinetic runs varied from 4 to 352 min., with the average percent hydrolysis for all esters being 27.9 ± 3.3%.

Since benzoic acid is virtually totally ionized at pH 9.00, the *x*–*y* recorder plot of titrant added during any kinetic run also provides a record of the acid released from the ester: $d[OH^-]/dt = d[BA]/dt = -d[RCOOR]/dt$. Data points were taken from the exponential *x*–*y* recorder tracings in calculating the pseudo-first-order rate constants. Accurate, readable plots were obtained by using appropriate chart speeds at the various temperatures.

RESULTS AND DISCUSSION

Kinetic Constants—Pseudo-first-order rate constants, k_{obs}, were obtained from the slopes of plots of log $[ester]_{remaining}$ *versus* time. Over the hydrolysis range investigated, all plots were linear with correlation coefficients of not less than 0.998. Figure 1, for the hydrolysis of *o*-bromobenzoylcholine at four temperatures, is typical of the data.

The k_{obs} for the hydrolysis of *m*-nitrobenzoylcholine at 37° was previously (5) shown to be a linear function of $[OH^-]$ in the pH region of 7.4–8.6. A zero intercept for the plot further indicated that any contribution from k_0, the water hydrolysis constant, was insignificant. In an identical manner for the present data, k_{obs} at 37° for *o*-bromobenzoylcholine is linear in $[OH^-]$ from pH 7.4–9.0, producing an apparent zero intercept and a correlation coefficient of 0.999. Values of k_2, the second-order rate constant, were therefore calculated for each derivative from the equation: $k_2 = k_{obs}/[OH^-]$. The average values from duplicate runs for k_2 at various temperatures are listed in Table I.

Activation Parameters—Arrhenius plots of the data given in Table I are illustrated by Fig. 2. The activation parameters calculated from these data are given in Table II and are based on a least-squares fit of the equation: $\log k = [-E_a/2.303R][1/T] + A$, where

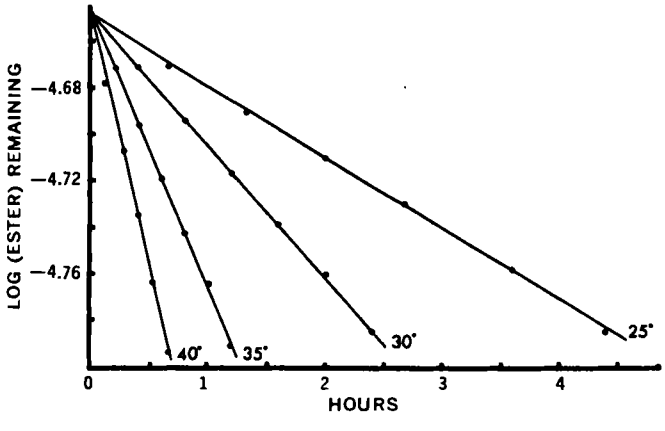

Figure 1—*Pseudo-first-order plots as a function of temperature for the alkaline hydrolysis of o-bromobenzoylcholine iodide in 0.1 M aqueous NaCl at pH 9.0.*

[2] Forma-Temp. Jr.
[3] Fisher Scientific.

Table II—Activation Parameters for the Alkaline Hydrolysis of *ortho*-Substituted Benzoylcholine Iodides[a]

ortho-Substituent	E_a, kcal./mole	$\Delta H^{\ddagger}$[b], kcal./mole	log A, M^{-1} sec.$^{-1}$	$\Delta S^{\ddagger}$[c], cal./mole/deg.
Hydrogen	10.3	9.67	7.63	−25.8
Methyl[d]	4.86	4.25	3.29	−45.7
	(18.3)	(17.7)	(12.4)	(−3.76)
Methoxy	9.60	8.98	6.84	−29.3
Iodo	9.72	9.11	7.35	−27.0
Bromo	11.2	10.6	8.48	−21.8
Chloro	11.4	10.8	8.71	−20.7
Fluoro	11.6	11.0	8.96	−19.6
Nitro	12.8	12.2	9.92	−15.2

[a] The average standard error associated with E_a and $\Delta H^{\ddagger}$ = 0.616 kcal., with log A = 0.438 unit and with $\Delta S^{\ddagger}$ = 2.00 cal. [b] $\Delta H^{\ddagger}$, the enthalpy of activation, was calculated from the relation $\Delta H^{\ddagger} = E_a - RT$, where T = 308°K. (A. A. Frost and R. G. Pearson, "Kinetics and Mechanisms," 2nd ed., Wiley, New York, N. Y., chap. 5). [c] $\Delta S^{\ddagger}$, the entropy of activation, was calculated from the relation $k_2 = (RT/Nh) \exp(\Delta S^{\ddagger}/R) \exp(-\Delta H^{\ddagger}/RT)$, where h = Planck's number and N = Avogadro's number (reference of *Footnote b*). [d] Values in parentheses were obtained using the rate constants at 50, 55, 57, and 60°.

E_a = Arrhenius activation energy, A = frequency factor, T = °K., R = 1.987 cal./deg./mole, and k_2 = second-order rate constant. The correlation coefficients for these fits were not less than 0.994, except for the methyl derivative in the temperature region 25–50° for which the value was 0.958.

A comparison of the activation enthalpies (Table II) with corresponding rate constants (Table I) reveals an unexpected trend between these parameters. Thus, within the congeneric series, rate constants are seen to parallel increases in enthalpy values. For *o*-methylbenzoylcholine, the most slowly hydrolyzed ester, the value of $\Delta H^{\ddagger}$ is 4.25 kcal./mole; for *o*-nitrobenzoylcholine, the most rapidly hydrolyzed ester, the value of $\Delta H^{\ddagger}$ is 12.2 kcal./mole. These results are seen more clearly from the tabulation of substituent effects on the free energy, enthalpy, and entropy of activation given in Table III. With benzoylcholine taken as the standard, the resulting difference in the activation parameters show that, in each case except for the *I* derivative, the $-T(\delta_R \Delta S^{\ddagger})$ term contributes more strongly to the free energy of activation than does the $\delta_R \Delta H^{\ddagger}$ term.

Over an extended temperature range of 30–60°, nonlinearity was observed in the Arrhenius plot for the *o*-methyl derivative. An attempt to fit these data nonlinearly using the equation $\log k_2 = a(1/T) + b(\log T) + c$ produced only statistically nonsignificant results. As seen in Fig. 3, however, the log k_2 values for *o*-methylbenzoylcholine may be represented as two straight-line segments when plotted against $1/T$. Such a result suggests an abrupt change in the mechanism or rate-limiting step for the reaction in the vicinity of 50°. By contrast, the Arrhenius plots for the parent compound

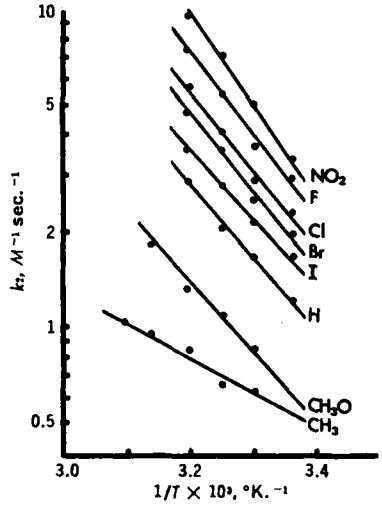

Figure 2—*Arrhenius plots for the alkaline hydrolysis of* ortho-*substituted benzoylcholine iodides in 0.1 M aqueous NaCl at pH 9.0. Kinetic constants are from Table I.*

Table III—Substituent Effects on Activation Parameters for *ortho*-Substituted Benzoylcholine Iodides[a]

ortho-Substituent	$\delta_R \Delta G^{\ddagger} = \delta_R \Delta H^{\ddagger} - T(\delta_R \Delta S^{\ddagger})$[b]		
	$\delta_R \Delta G^{\ddagger}$	$\delta_Z \Delta H^{\ddagger}$	$-T(\delta_R \Delta S^{\ddagger})$
Hydrogen	0	0	0
Methyl	0.70	−5.43	6.13
Methoxy	0.389	−0.691	1.08
Iodo	−0.196	−0.567	0.371
Bromo	−0.351	0.889	−1.24
Chloro	−0.44	1.13	−1.55
Fluoro	−0.60	1.31	−1.91
Nitro	−0.76	2.51	−3.27

[a] Units in kcal./mole; 35°C. [b] δ_R is the Leffler–Grunwald (10) operator for substituent effects (see text).

and the *o*-chloro derivative exhibit a strictly linear trend over the same temperature range.

Isergonic Relationships and Rho–Sigma Dependencies—Hepler (16) recently showed that Eq. 3 may be derived by an exact thermodynamic analysis of the Hammett equation (Eq. 5) when $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are temperature independent (*i.e.*, $\delta_R \Delta C_p^{\ddagger} = 0$):

$$\delta_R \Delta G^{\ddagger} = -2.303 RT \rho \sigma \qquad \text{(Eq. 5)}$$

Other important relationships which resulted from his analysis are given by Eqs. 6–8:

$$\delta_R \Delta H^{\ddagger} = 2.303 R \beta_i \rho_\infty \sigma \qquad \text{(Eq. 6)}$$

$$\delta_R \Delta S^{\ddagger} = 2.303 R \rho_\infty \sigma \qquad \text{(Eq. 7)}$$

$$\rho_T = \rho_\infty (1 - \beta_i/T) \qquad \text{(Eq. 8)}$$

where β_i and ρ_∞ are temperature-independent constants of integration. The enthalpy and entropy of activation are derived as linear functions of the sigma constant; ρ_T, the reaction constant for a linear free energy relationship at any given temperature, is shown to be inversely proportional to the absolute temperature. While these relationships are written in terms of σ, they would also apply to other σ constants such as σ_I.

Experimentally, β_i may be obtained as the slope of Eq. 3 or as the slope-intercept ratio from Eq. 8. Exner (11, 12), however, criticized the use of Eq. 3 on the basis of computational and experimental arguments. As an alternative, he proposed still another method of calculating β_i according to Eq. 9:

$$\log k_1 = \lambda \log k_2 + b \qquad \text{(Eq. 9)}$$

where k_1 and k_2 are rate constants at two temperatures with $T_1 > T_2$. By this method, β_i is obtained indirectly from the slope, λ, of a log k_1 *versus* log k_2 plot with the aid of Eq. 10 (14):

$$\beta_i = T_1 T_2 (T_1\lambda - T_1)/(T_1\lambda - T_2) \qquad \text{(Eq. 10)}$$

Nevertheless, it should be cautioned that β_i values calculated from

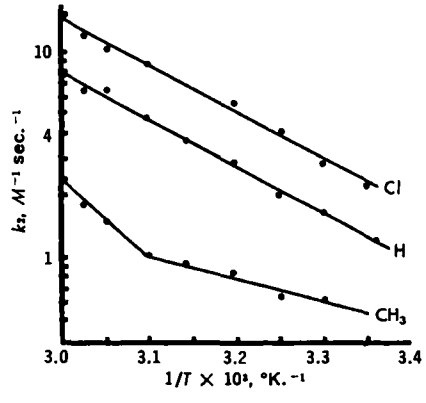

Figure 3—*Arrhenius plots over an extended temperature range for the alkaline hydrolysis of benzoylcholine iodide and* o-*chloro- and* o-*methylbenzoylcholine iodides in 0.1 M aqueous NaCl at pH 9.0. Kinetic constants are from Table I.*

Table IV—Estimates of β_i from a Fit of Experimental Data for *ortho*-Substituted Benzoylcholine Iodides[a]

Regression Equation	n	r	F	β_i (°K.)	Equation Number
$\Delta H^{\ddagger} = 258.1\Delta S^{\ddagger} + 16.18$	8	0.997	1131	258	11
$\log k_1 = 1.204 \log k_2 + 0.1875$	8	0.999	318	281	12
$\rho_T = -2904(1/T) + 0.1106$[b]	4	0.988	84	263	13
				β_i (average) = 267	

[a] n = number of data points used in the least-squares fit; r = correlation coefficient; and F = F-ratio. The β values were obtained as discussed in text. [b] The ρ_T values were obtained as the slopes of log k_2 *versus* σ_I correlation for data at 25, 30, 35, and 40° according to the equation $\log k_2^{\ddagger} = \rho\sigma_I + \log k_2^{CH_3}$, where $k_2^{\ddagger}$ = second-order rate constants given in Table I for substituents at a given temperature, $k_2^{CH_3}$ = calculated second-order rate constant for the methyl derivative, and σ_I = Taft's inductive constant (1–3). The regression analyses for data at the four temperatures produced correlation coefficients of 0.954, 0.959, 0.971, and 0.973, respectively.

Eq. 10 are highly dependent upon λ and may be in considerable error when scatter exists in the log k_1 *versus* log k_2 profiles.

***ortho*-Substituted Benzoylcholine Iodides**—By using the rate constants and activation parameters obtained for the hydrolysis of *ortho*-substituted benzoylcholine iodides, Eqs. 3 and 9 were plotted in Figs. 4 and 5, respectively. The Leffler–Grunwald and Exner methods are observed to produce similar values for the isergonic temperature, *i.e.*, β_i = 258°K. (Fig. 4) and β_i = 281°K. (Fig. 5). The dotted line in Fig. 4 is a transformation of the solid line from the Exner plot. There is an obvious close visual correspondence in the slopes for the two lines, but there is a slight vertical displacement because of the use of an average temperature value in the transformation equation converting rate constants to activation parameters[4]. The third estimate of β_i = 263°K. derived from Eq. 8

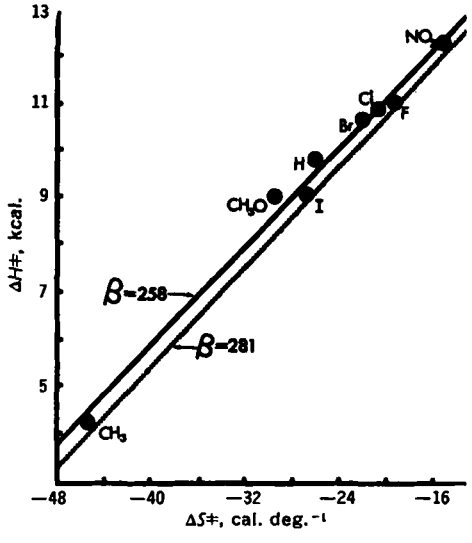

Figure 4—*Isergonic relationship for the alkaline hydrolysis of* ortho-*substituted benzoylcholine iodides in 0.1* M *aqueous NaCl at pH 9.0. Activation parameters are from Table II. Dotted line is a transformation of the solid line from Fig. 5.*

[4] The rate data of Fig. 5 are converted into the activation data of Fig. 4 using the equations (17): $\Delta H^{\ddagger} = [2.303RT_1T_2/(T_1 - T_2)](\log k_1 - \log k_2) - R(T)_{ave}$ and $\Delta S^{\ddagger} = [2.303RT_1/(T_1 - T_2)][\log k_1 - (T_2/T_1)\log k_2] - 2.303R \log[ek(T)_{ave}/h]$. When the solid line in Fig. 5 is drawn onto an E *versus* log A plot, the resulting lines coincide almost exactly since then a transformation involving only discrete T values is involved. The conversion of rate data to Arrhenius parameters is accomplished with the following expressions (11): $E = [2.303RT_1T_2/(T_1 - T_2)](\log k_1 - \log k_2)$ and $\log A = [T_1/(T_1 - T_2)][\log k_1 - (T_2/T_1)\log k_2]$.

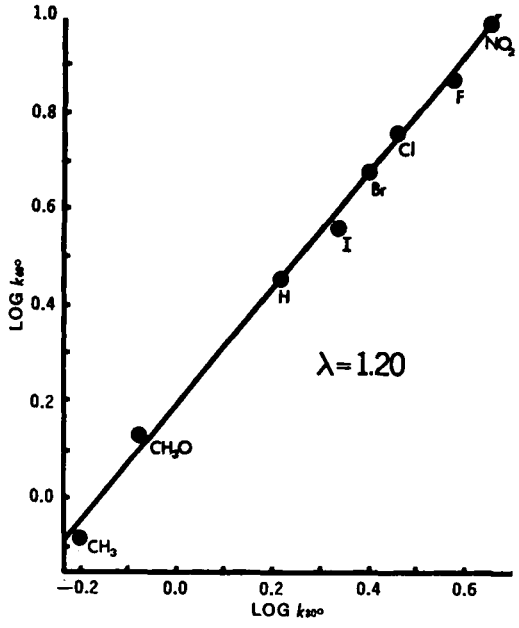

Figure 5—*Relationship between the logarithms of the second-order rate constants at 30 and 40° for the alkaline hydrolysis of* ortho-*substituted benzoylcholine iodides in 0.1* M *aqueous NaCl at pH 9.0. Kinetic constants are from Table I.*

is intermediate to the values of 258 and 281°K., resulting in an average for the three of β_i = 267°K. Correlations (Eqs. 11–13) for the fit of the experimental data to the theoretical equations are given in Table IV.

The relationships of the activation parameters for the benzoylcholine series to σ_I are given by Eqs. 14–16 in Table V. Each parameter is excellently correlated by σ_I. These results, together with those for Eqs. 11 and 13, indicate that the present congeneric series fits the "well-behaved" category of rho–sigma correlations (10).

Table V—Correlations of Activation Parameters for Acetic Acid and Benzoylcholine Series[a,b]

	n	r	s	F	Equation Number
***ortho*-Substituted Benzoylcholine Iodides**[c]					
$\Delta G^{\ddagger} = -2.312\sigma_I + 0.2626$	7	0.971	0.137	84	14
$\Delta H^{\ddagger} = 11.43\sigma_I + 4.641$	7	0.981	0.543	131	15
$\Delta S^{\ddagger} = 44.60\sigma_I - 44.77$	7	0.991	1.440	285	16
α-Substituted Acetic Acids[d]					
$\Delta H^{\circ} = 9.504\Delta S^{\circ} - 0.9597$	14	0.063	0.610	0.05	17
$\Delta H^{\circ} = 0.7186\sigma_I - 0.6207$	14	0.308	0.582	1.25	18
$\Delta G^{\circ} = -5.146\sigma_I + 6.441$	14	0.965	0.366	162	19
$\Delta H^{\circ}_{int} = -4.889\sigma_I + 6.004$	14	0.966	0.342	168	20
$\Delta S^{\circ} = 14.89\sigma_I - 23.66$	14	0.960	1.137	141	21
Enthalpy and Entropy Comparisons between Series[e]					
$\Delta H^{\circ}_{int} = -0.4184\Delta H^{\ddagger} + 8.013$	6	0.985	0.208	130	22
$\Delta S^{\circ} = 0.246\Delta S^{\ddagger} - 12.07$	6	0.987	0.434	152	23

[a] n = number of data points used in the least-squares fit; r = correlation coefficient; s = standard error of estimate; F = F-ratio (F = 106 at the 0.0005 level for 1,4 degrees of freedom, and F = 63.6 at the same level for 1,5 degrees of freedom). [b] The superscripts, ° and ‡, refer to acetic acid and benzoylcholine series, respectively. [c] Fluoro, chloro, bromo, iodo, methyl, methoxy, and nitro (hydrogen consistently produced lower correlations and was not included in these analyses) (5); 35°. [d] Fluoro, chloro, bromo, iodo, methoxy, ethyl, propyl, isopropyl, butyl, isobutyl, cyano, carboxymethyl, and hydrogen (data for nitro were not available for analysis). The experimental data for methoxyacetic acid at 25° were taken from E. J. King, *J. Amer. Chem. Soc.*, 82, 3575(1960). All other values for acetic acids (25°) were taken from *Reference 15*. The H°_{int} values were calculated from the relation: $\Delta H^{\circ}_{obs.} - 280\ \Delta S^{\circ}_{obs.}$. [e] Fluoro, chloro, bromo, iodo, methyl, and methoxy.

α-Substituted Acetic Acids—An analysis of the thermodynamic constants of α-substituted acetic acids is presented in Table V by Eqs. 17–21. Analogous to the benzoylcholine series, $\Delta G°$ and $\Delta S°$ for the acetic acid series both correlate independently with σ_I. The $\Delta H°$ *versus* $\Delta S°$ relationship, however, is highly scattered ($r = 0.063, n = 14$), and $\Delta H°$ is not a linear function of σ_I. Furthermore, $\delta_R\Delta C_p° \neq 0$ for these reactions (15), and it may be shown under such conditions that β_i is no longer a constant but is temperature dependent (16). While the observed enthalpy changes for acetic acid ionizations are not well behaved, $\Delta G°$ values for the series still approximate a linear free energy model for σ_I because of the compensatory effect inherent in Eq. 4.

An explanation of the compensatory effect was offered by Hepler (13, 16) based upon an extrathermodynamic model for substituent and solvent effects. Accordingly, the observed free energy changes may be written as a sum of two interaction energies:

$$\delta_R\Delta G°_{obs} = \delta_R\Delta G°_{int} + \delta_R\Delta G°_{env} \qquad \text{(Eq. 24)}$$

where "int" signifies the internal or intrinsic effect of the substituent on the reaction center, and "env" refers to the environmental effect due to the interaction between solvent and substituent. In a similar manner, the observed enthalpy and entropy may be expressed according to Eqs. 25 and 26, respectively:

$$\delta_R\Delta H°_{obs} = \delta_R\Delta H°_{int} + \delta_R\Delta H°_{env} \qquad \text{(Eq. 25)}$$

$$\delta_R\Delta S°_{obs} = \delta_R\Delta S°_{int} + \delta_R\Delta S°_{env} \qquad \text{(Eq. 26)}$$

From evidence which suggests that substituent effects on $\Delta S°_{int}$ are negligible (*i.e.*, $\delta_R\Delta S°_{int} \simeq 0$) and that the solvation enthalpy and entropy are isergonically related (*i.e.*, $\delta_R\Delta H°_{env} = \beta_e\Delta S°_{env}$), Eq. 27 may be written:

$$\delta_R\Delta H°_{obs} = \delta_R\Delta H°_{int} + \beta_e\delta_R\Delta S°_{obs} \qquad \text{(Eq. 27)}$$

where β_e is now a parameter related to solvent and is not necessarily identical to β_i. Finally, upon combining the appropriate equations, the free energy is expressed in terms of Eq. 28:

$$\delta_R\Delta G°_{obs} = \delta_R\Delta H°_{int} + (\beta_e - T)\delta_R\Delta S°_{obs} \qquad \text{(Eq. 28)}$$

Since it is observed that $\beta_e \simeq T$ for many reactions conducted in aqueous solution, Eq. 28 reduces further to an expression (Eq. 29) that underlies the general success of the Hammett equation:

$$\delta_R\Delta G°_{obs} \simeq \delta_R\Delta H°_{int} \simeq -2.303RT\rho\sigma \qquad \text{(Eq. 29)}$$

The result of Eq. 20 (Table V) shows that a correlation between σ_I and the calculated $\delta_R\Delta H°_{int}$ values for acetic acid ionizations does indeed produce the linear fit predicted by Eq. 29. Thus, experimental measures of the free energy clearly contain contributions from intrinsic substituent effects ($\delta_R\Delta G°_{int}$), but the effects from solvent–substituent interactions are apparently not seen because of compensatory phenomenon (*i.e.*, $\delta_R\Delta G°_{env} \simeq 0$). A set of equations analogous to Eqs. 24–29 may also be written for kinetic reactions in aqueous solution.

Mechanistic Inferences—In drawing mechanistic inferences from the results for the benzoylcholine series, it is important to distinguish between the meaning of the rho–sigma correlations and the linear $\Delta H^{\ddagger} - \Delta S^{\ddagger}$ profiles. From the Hammett propositions (17), it is understood that linear rho–sigma correlations based on $\Delta G^{\ddagger}$ reflect solely internal potential energy changes of substituents. Mechanistic contributions from such analyses would then be limited normally to those for inductive and/or resonance effects exclusive of solvent effects. For the present series, therefore, Eq. 14 reflects the high dependency of $\Delta G^{\ddagger}$ on the inductive effect of the substituents. Furthermore, the negative slope for the relationship indicates that hydrolysis rates increase as the electron-withdrawing power of the substituents increases. An increasingly positive character of the carbonyl carbon facilitates attack by hydroxide ion. It is not necessary to invoke a description that bases the *ortho* effect on resonance or steric interactions.

Information from the $\Delta H^{\ddagger} - \Delta S^{\ddagger}$ profile can be interpreted in light of the Hepler analysis. The value of β_i for the *ortho*-substituted esters approximates that of β_e for solvation processes in general; thus it is likely that the results of Fig. 4 are highly dependent upon solvent–substituent interactions. Such a linear profile need not reflect the existence of only a single-interaction mechanism. Leffler and Grunwald (10) indicated that linear $\Delta H^{\ddagger} - \Delta S^{\ddagger}$ profiles are also obtained for multiple-interaction mechanisms when the β values for the interactions are identical or nearly so. In these cases, the regression lines for the different mechanisms cannot be separated, and they are superimposed upon one another on the isergonic plot. For the present series, it is likely that β_i and β_e are nearly identical since both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ have been shown to correlate independently with σ_I (Eqs. 15 and 16, respectively). The $\Delta H^{\ddagger}$ is interpreted to include both internal potential energy and solvation enthalpies, while $\Delta S^{\ddagger}$ should reflect solely the solvation entropy involved. A similar consistency between β values is not obtained for the acetic acid series. The correlation between $\Delta H°_{int}$ and $\Delta S°$ values produces an isergonic temperature of −465.8°K. when β_e = 280°K. A further comparison between the two series for a limited number of congeners shows a surprisingly close correlation between their respective enthalpy and entropy values as indicated by Eqs. 22 and 23.

While the solvation entropy does not contribute to the observed free energy changes or the ordering of the substituents within the benzoylcholine series, the $\Delta S^{\ddagger}$ values given in Table II can be utilized to gain further insight into the solvation mechanism. An unexpected trend is observed to exist between entropy and the charge on the substituent. Since it has been shown that σ_I is an increasing linear function of substituent charge (18), it would be expected that the most highly charged substituent would be the most strongly solvated, producing the greatest decrease in entropy. This is clearly not the case for the present series; the most highly charged substituent, nitro, exhibits the greatest entropic freedom (*i.e.*, the most positive entropy value); the least highly charged substituent, methyl, displays the greatest entropy requirement (*i.e.*, the most negative entropy value). The apparent inconsistency is explained potentially by considering the positive charge on the choline moiety. Thus, the close proximity of a diffuse positively charged electron cloud, such as that of a quaternary nitrogen (19), could be expected to neutralize partially the substituent charge and lead to an alteration of expected solvent effects. In such a case, the conformation of the benzoylcholine molecule would be a critical factor in altering the substituent–solvent interactions. From conformational analysis studies (19), there is evidence for acetylcholine, suggesting that minimum energy conformations do exist which would bring the quaternary nitrogen and the α-carbon of the acyl group into reasonably close proximity. A similar analysis will be necessary for the benzoylcholine molecule to verify this hypothesis.

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Abstract □ A GLC technique utilizing a flame-ionization detector is described for the analysis of meperidine in blood plasma. The meperidine is extracted with benzene from plasma that has been made basic with sodium carbonate. A linear calibration curve is found in the range 0.1–1.25 mcg./ml., with the precision of the assay estimated to be ±9.9% (*RSD*). The method has been used in the determination of the half-life of meperidine in dog plasma.

Keyphrases □ Meperidine—GLC-flame-ionization analysis in dog plasma, determination of half-life □ Plasma levels, meperidine—determination of half-life in dogs, GLC-flame ionization □ GLC-flame-ionization detection—analysis, meperidine in dog plasma, determination of half-life

Until recently, the colorimetric technique of Burns *et al.* (1), with a sensitivity of 0.3 mcg./ml., was the best method available for analysis of meperidine[1]. This method, which depends upon the formation of a methyl orange–base complex, is applicable for most amines. Thus, its adaptation for analysis of meperidine raises the question of specificity. Beckett *et al.* (2) showed that, even with modifications such as buffer washes, nicotine interferes with the assay. This should be true of many other bases as well. Dal Cortivo *et al.* (3) developed a fluorometric assay for meperidine, but a lower limit again of only 0.3 mcg./ml. was established. Jenkins *et al.* (4) used a GLC method for the estimation of meperidine in a study of its placental transfer in pregnant ewes. A sensitivity of 0.025 mcg./ml. was reported; however, in our hands this sensitivity was not obtainable. Moreover, there is a limitation of only two analyses per hour and a relatively large volume of plasma is needed (3–4 ml.).

Although other papers have been published concerning GLC techniques, either complete details of the methodology were not reported (5) or no attempt was made to adapt the method for the analysis of biological fluids (6–11). Other approaches used for the determination of meperidine in biological fluids include another colorimetric approach utilizing bromthymol blue (12, 13), a UV absorption spectrophotometric technique (14), and two methods using column chromatography, to separate meperidine from contaminating material, coupled with either paper chromatography (15) or colorimetry (16) for quantitation. Most of the therapeutic serum concentrations have been estimated using the method of Burns *et al.* (1) with widely varying values (1, 17–21). Consequently, a method having greater sensitivity and greater specificity was desired. A GLC method was developed which has a sensitivity slightly better than 0.1 mcg./ml. and the required specificity.

[1] Demerol, Sterling-Winthrop Research Institute.

EXPERIMENTAL

Apparatus—A gas chromatograph[2] equipped with a flame-ionization detector was used with gas flows of 45, 60, and 600 ml./min. for nitrogen, hydrogen, and air, respectively. A column oven temperature of 180° was used; injection port and detector temperatures were maintained at 220 and 210°, respectively.

Column—A 2-mm. i.d. × 180-cm. glass U tube column was packed with 3% OV-17 on 100–120-mesh Gas-Chrom Q[3]. Before it was packed, the empty glass column was thoroughly rinsed with methanol and acetone, dried, and conditioned 1 hr. with a 5% solution of dimethyldichlorosilane[3] in toluene to silylate reactive sites. The column was again rinsed with acetone and dried.

Analytical Procedure—Two milliliters of blood plasma[4] was transferred to a 50-ml. glass-stoppered centrifuge tube made basic with 1.5 ml. 10% sodium carbonate and extracted with 15 ml. analytical reagent grade benzene by shaking for 2 min. The tubes were centrifuged for 3 min., and 12.5 ml. of the benzene phase was then transferred to a screw-top test tube. One drop of methanolic 1 *N* HCl (prepared from 12 *N* HCl) was added to the benzene phase. The benzene was evaporated at no more than 40° under a flow of nitrogen. The residue was taken up in 30 μl. of analytical reagent grade dimethylformamide and 20 μl. of hexamethyldisilazane[3], taking care to rinse the sides of the tube well. GLC determinations were made by injecting 4 μl. of the dimethylformamide solution onto the column with a 1-μl. solvent wash. Quantitation was accomplished by the measurement of peak heights.

Standards for a calibration curve were prepared by adding aliquots of an aqueous solution of meperidine hydrochloride to 50-ml. glass-stoppered centrifuge tubes containing 2 ml. of blood plasma[5] to give final plasma concentrations of 0.1–1.25 mcg./ml. The tubes were shaken to ensure mixing and then analyzed as mentioned. To determine percent recoveries, it was necessary to compare the calibration curve data with a detector response curve. This was obtained by taking aliquots of an aqueous solution of meperidine hydrochloride to give standards of 0.2–2.5 mcg. The aliquots were evaporated to dryness and taken up in 30 μl. of dimethylformamide and 20 μl. hexamethyldisilazane, and 4-μl. samples were injected onto the column.

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[3] Applied Science Laboratories, State College, Pa.
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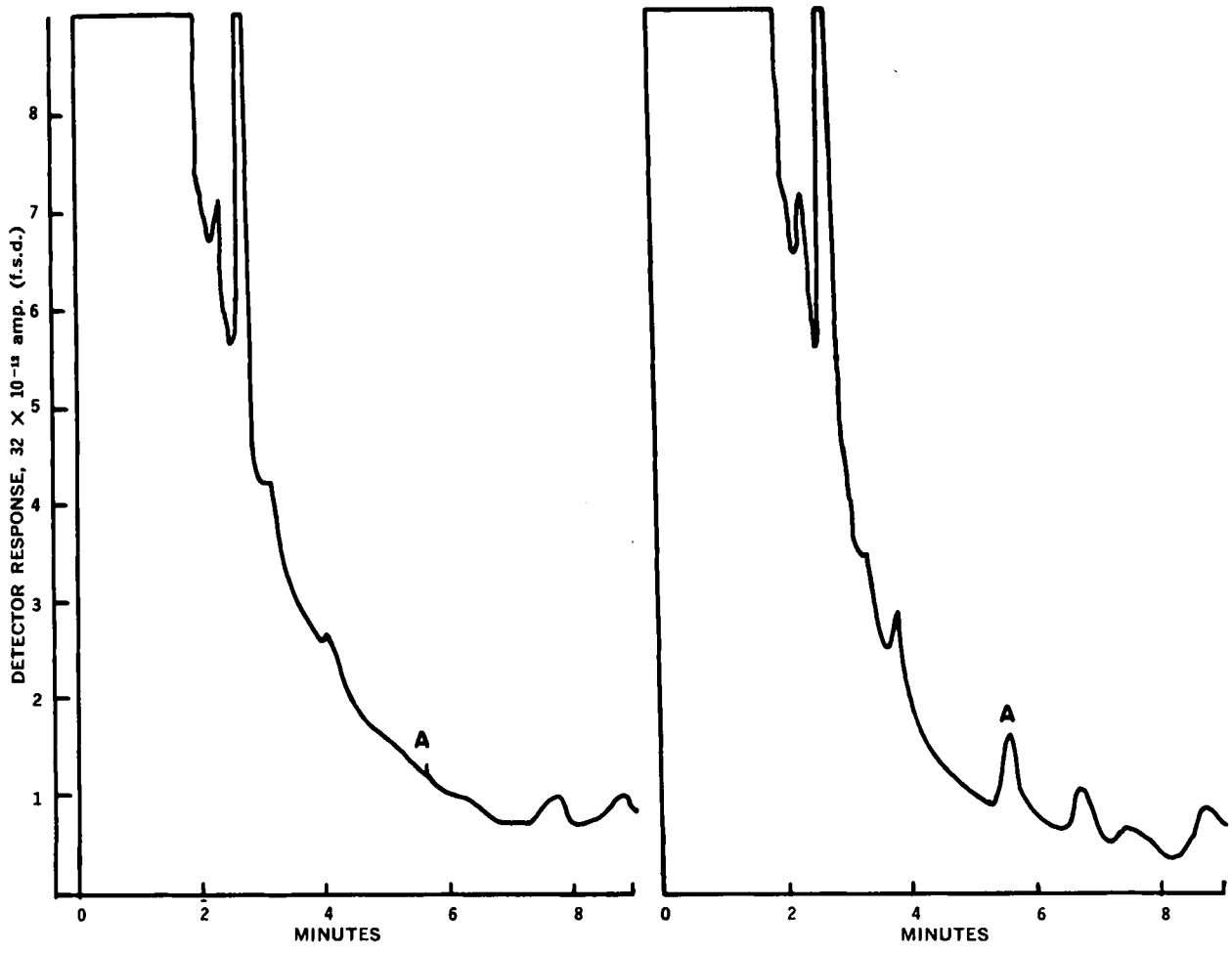

Figure 1—*Gas chromatogram of human plasma. Left: control plasma. Right: plasma containing 0.2 mcg./ml. meperidine (A).*

Precision and accuracy were determined by analyzing replicate plasma samples to which known amounts of meperidine had been added.

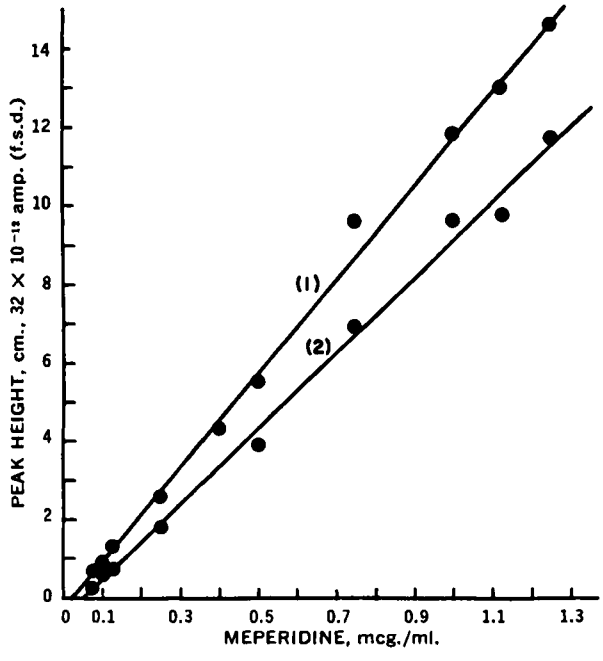

Figure 2—*Detector response curve (1) and calibration curve (2) for meperidine in plasma. Peak height was not corrected for aliquot of extraction solvent used in the analyses.*

To illustrate the utility of the method, four mongrel dogs were medicated intravenously with meperidine; the doses ranged between 15 and 25 mg. drug/kg. body weight. Multiple blood samples were taken through 2 hr. postmedication. The samples were then analyzed for meperidine, and the half-life of distribution and the half-life of elimination from blood were determined.

RESULTS AND DISCUSSION

The retention time for meperidine was found to be 5.8 min., with no interfering peaks in this area (Fig. 1). The detector response curve and the calibration curve were linear over the 0.1–1.25-mcg./ml. range (Fig. 2). No advantage was found by plotting peak areas instead of peak heights. The peak heights for the calibration curve are plotted without correcting for the volume of extraction solvent used; *i.e.*, only 12.5 ml. of benzene out of 15 ml. was used. After taking this into consideration, the percent recoveries from spiked blood plasma averaged 96%.

The precision was determined to be ±9.9% (*RSD*) for the standard curve shown (Fig. 2). For data obtained from running replicate

Table I—Precision in Measurement of Meperidine Added to Human Plasma

Added, mcg./ml.	Measured[a], mcg./ml.	*RSD*[b], %
0.2	0.22 (0.20–0.24)	±8.8
0.5	0.53 (0.52–0.55)	±2.9
1.0	1.03 (0.93–1.06)	±5.8

[a] Mean of five replicate samples (range). [b] Relative standard deviation [$(s/\bar{y})$].

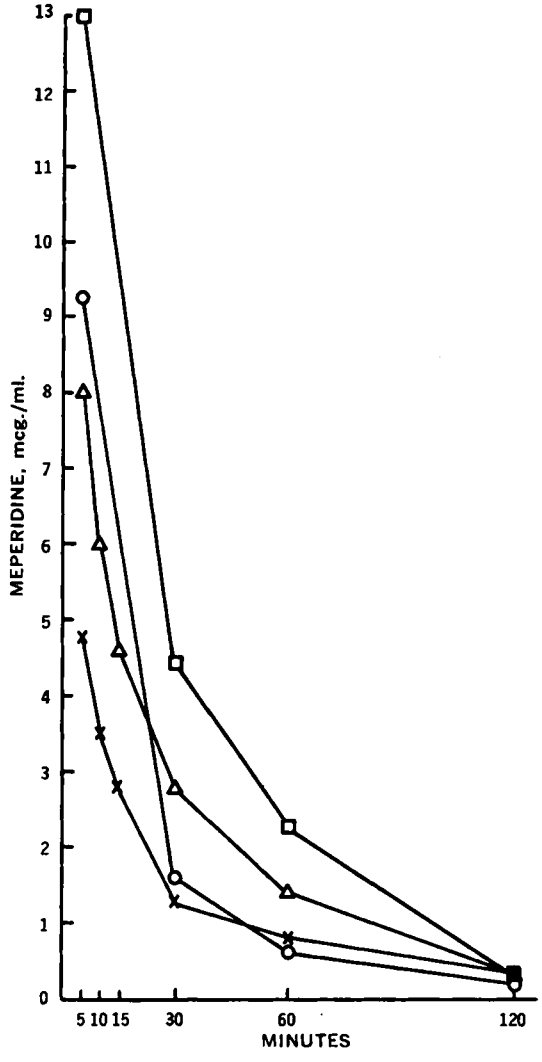

Figure 3—*Meperidine plasma concentrations in the dog after a single intravenous medication. Key: □, 25 mg./kg.; ○, 20 mg./kg.; △, 15 mg./kg.; and ×, 15 mg./kg.*

samples in the range of 0.2–1.0 mcg./ml., the precision varied from ±2.9 to 8.8% (*RSD*) (Table I).

The use of the hexamethyldisilazane along with dimethylformamide for taking up the meperidine for injection proved advantageous in two respects: (*a*) the rinsing of the walls of the tube was facilitated since the solution wet the glass very well, which was not the case with dimethylformamide alone; and (*b*) it inhibited the binding of drug to the glass, which occasionally was noticed when only dimethylformamide was used.

The facts that some tailing was noted in the chromatogram and that the standard curve, determined by the least-squares method, did not pass through zero at the 95% confidence level pointed to an interaction between the compound and the column packing. Attempts were made to overcome this problem by trying 3% OV-1 on Gas Chrom Q as the packing as well as silylating the reactive sites in the packing by hexamethyldisilazane injections; neither variation was successful. This interaction problem was not serious at the concentrations used.

Another problem was seen when on-column amounts of meperidine five times the normal range were used. A fraction of these larger amounts remained on the column and was eluted by the next dimethylformamide injection. Incomplete volatilization at the injection port seems to be a plausible explanation. However, this problem can be avoided by using on-column amounts of meperidine of 0.2 mcg. or less.

That protein binding might be a minor consideration at the lower concentrations was indicated by comparing data for extractions of meperidine from water and from plasma. Whereas the average percent recovery was greater than 90% through the 0.1–1.0-mcg./ml. range, the percent recoveries at the lower concentrations of spiked plasma dipped into the lower 80's.

It is recommended that four spiked plasma standards be run with each set of plasma samples as a routine procedure. By using the standard curve and the peak height of an unknown sample, the concentration of meperidine in a sample can be determined.

Under the conditions of the column, other peaks were noted at approximately 14 and 90 min. These could pose a problem for subsequent analyses; however, by running a sample every 10 min., it was possible to avoid interference by each 14-min. peak and thus nine samples were run before the first very large broad 90-min. peak interfered. It was then necessary to wait 90 min. until all of these latter peaks were eluted.

For this half-life study in dogs, it was shown that the metabolites of meperidine do not interfere. For future clinical studies, it was established that lidocaine, mepivacaine, bupivacaine, and nicotine do not interfere in the assay.

The analyses of the plasma samples from the dogs showed a rapid decline in the concentration of meperidine (Fig. 3). It was reported (1) previously that meperidine was metabolized at a rate of about 70%/hr., which is equivalent to a half-life of about 30 min. In this present study, the arithmetic mean and standard deviation for the half-life of distribution and the half-life of elimination from blood were calculated to be 6.5 ± 0.8 and 31.2 ± 6.4 min., respectively.

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Synthesis and Antimalarial Activity of Benzotrifluoride Derivatives

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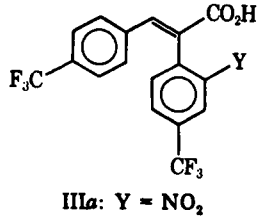
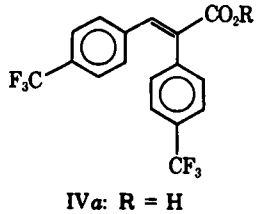
Abstract □ The preparation of the potential antimalarial agents 4,4′-bis(trifluoromethyl)stilbene, 4,4′-bis(trifluoromethyl)-α-phenylcinnamic acid, and several trifluoromethyl-bearing intermediates is described. Use of a modified Willgerodt synthesis provided the best synthetic route to 4-trifluoromethylphenylacetic acid. A modification of the Grignard synthesis of 4-trifluoromethylbenzaldehyde and 1-(4-trifluoromethylphenyl)ethanol, suitable for largescale preparation, is reported.

Keyphrases □ Benzotrifluoride derivatives—synthesized as potential antimalarials □ 4,4′-Bis(trifluoromethyl)stilbene—synthesized as potential antimalarial □ 4,4′-Bis(trifluoromethyl)-α-phenylcinnamic acid—synthesized as potential antimalarial □ Antimalarial agents, potential—synthesis of benzotrifluoride derivatives

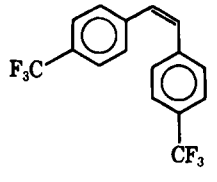
The antimalarial activity of a number of 3- and/or 6-trifluoromethyl-substituted phenanthrenemethanol analogs (1–4) has prompted extensive studies of one of the most promising, α-(2-piperidyl)-3,6-bis(trifluoromethyl)-9-phenanthrenemethanol hydrochloride (1, 3, 5). Practical largescale preparation of this and related compounds requires use of inexpensive starting materials in synthetic routes amenable to scale-up. This article reports the preparation of several benzotrifluoride derivatives useful for the preparation of the related phenanthrenes. The reaction sequences described represent new approaches to the preparation of these compounds. Of particular interest is a new preparation of the Grignard of 4-chlorobenzotrifluoride suitable to the largescale synthesis of 4-trifluoromethylbenzaldehyde (I) and 1-(4-trifluoromethylphenyl)-ethanol (II). The aldehyde (I) is used in the preparation of such important antimalarial intermediates as 4,4′-bis(trifluoromethyl)-2′-nitro-α-phenylcinnamic acid (III) (1), 4,4′-bis(trifluoromethyl)-α-phenylcinnamic acid (IV) (both prepared by the Perkin condensation of I with the appropriate phenylacetic acid), and 4,4′-bis(trifluoromethyl)stilbene (V), prepared using the Wittig reaction of I with 4-trifluoromethylbenzyltriphenylphosphonium chloride (VI). Compound III*b* is converted to a phenanthrene derivative by the Pschorr process (1), while IV*b* and V have been photocyclized to the corresponding phenanthrenes[1] (4) for subsequent conversion to 3,6-bis(trifluoromethyl)-9-phenanthrene carbinolamines (6). In addition to its use in the sequence herein, the ethanol (II) is a starting material for the preparation of 2,6-bis(4-trifluoromethylphenyl)-4-pyridinemethanol derivatives, another important family of antimalarial compounds (7).



III*a*: Y = NO_2
III*b*: Y = NH_2



IV*a*: R = H
IV*b*: R = CH_3



V

SYNTHESES

Two routes were investigated for the preparation of the intermediate 4-trifluoromethylphenylacetic acid (VII) from 4-chlorobenzotrifluoride. In one (Route B), 4-chlorobenzotrifluoride was converted to the magnesium Grignard and then to 4-trifluoromethylbenzaldehyde (I) (8) in 43% yield. This was transformed into 4-trifluoromethylbenzyl alcohol (VIII) (9) in 95% yield and then to 4-trifluoromethylbenzyl chloride (IX) (9, 10) in 98% yield. The chloride (IX) was converted to 4-trifluoromethylbenzonitrile (X) (11) (66% yield). Hydrolysis of X gave the acid (VII) in 72% yield. The overall yield by this route is 19%.

4-Chlorobenzotrifluoride was chosen as initial starting material due to its lower cost and because of a hazard associated with the use of 3- (and possibly 4-) bromobenzotrifluoride (12). Preparation of I was facilitated by use of an entrainment procedure (13) in tetrahydrofuran (14) to prepare the intermediate Grignard reagent[2]. As a result of this modification, the yield of Grignard reagent has improved from 9% (14) to more than 75%. Preparation of the Grignard and its subsequent reaction with appropriate aldehydes have been successfully carried out in these laboratories up to the 50-mole scale.

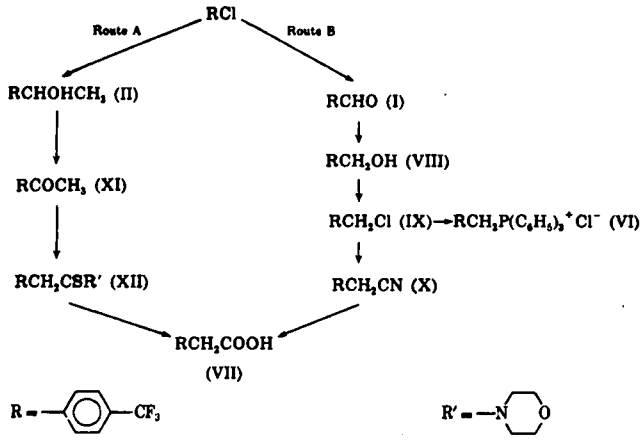
In the conversion of IX to the nitrile (X), a 10% yield of α,β-bis(4-trifluoromethylphenyl)propionitrile (XIII) was formed as a side-product. In an attempt to prepare VIII directly from 4-chlorobenzotrifluoride, the Grignard was reacted with formaldehyde. This produced a 19% yield of the desired VIII and 35% of bis(4-trifluoromethylbenzyl)formal (XIV).

In the second (and preferred) route to 4-trifluoromethylphenylacetic acid (VII) (Route A, Scheme I), the Grignard of 4-chlorobenzotrifluoride was reacted with acetaldehyde to give 1-(4-trifluoromethylphenyl)ethanol (II) (15) (74% yield), which was converted to 4-trifluoromethylacetophenone (XI) (15, 16) by dichromate oxidation in 83% yield. The Willgerodt reaction on XI gave a 72% yield of 4-trifluoromethylphenylthioacetomorpholide (XII). This was hydrolyzed to the acid (VII) in 80% yield. The higher overall yield (35%), the convenience of scale-up to 10 or 20 moles or more, and the lack of complicating side reactions make this Route A the preferred route to VII.

The condensation of 4-trifluoromethylbenzaldehyde (I) with 4-trifluoromethylphenylacetic acid (VII) gave 4,4′-bis(trifluoromethyl)-α-phenylcinnamic acid (IV*a*) in 94% yield. This was converted to its methyl ester (IV*b*) by treatment with methanol over molecular sieves.

[1] Largescale photocyclization of both IV*b* and V was carried out by Dr. R. Srinivasan, IBM Corp. (personal communication).

[2] The preparation of 4-trifluoromethylbenzylmagnesium chloride was developed by Dr. C. E. Maxwell, Pfizer, Inc., and was communicated to the authors by Dr. Barry M. Bloom of that company.



Scheme I

The 4,4′-bis(trifluoromethyl)stilbene (V) was obtained both from 4-trifluoromethylbenzaldehyde (I) and 4-trifluoromethylbenzyltriphenylphosphonium chloride (VI) and by decarboxylation of IV*a*. Both methods produce mixtures of the *cis*- and *trans*-isomers which could be separated by fractional distillation. Both isomers (and their mixture) give the same product, 4,4′-bis(trifluoromethyl)bibenzyl (XV), on hydrogenation.

BIOLOGICAL

The benzotrifluoride derivatives described were tested for antimalarial activity against *Plasmodium berghei*-infected mice by the test of Osdene *et al.* (17). None of these compounds showed antimalarial activity[3].

EXPERIMENTAL[4]

4-(Trifluoromethyl)benzaldehyde (I)—To 121.5 g. (4.998 g.-atoms) of magnesium turnings and 100 ml. of dry tetrahydrofuran was added 14.6 g. (0.134 mole) of ethyl bromide. After the reaction subsided, the mixture was augmented with 1775 ml. of tetrahydrofuran. With stirring, a solution of 257.9 g. (2.367 moles) of ethyl bromide and 457.5 g. (2.534 moles) of 4-chlorobenzotrifluoride[5] was added dropwise at a rate that maintained a gentle reflux; about 3 hr. was required[2]. The reaction mixture was refluxed for 30 min. and then cooled to 30–35°. While temperature was maintained, 365.0 g. (4.994 moles) of dimethylformamide was added dropwise. After being stirred at 25–30° for 2 hr. the reaction mixture was poured onto ice containing 238 ml. of concentrated sulfuric acid. The tetrahydrofuran was distilled off, and the product was recovered from the aqueous residue by hexane extractions. Crude material was distilled *in vacuo*, yielding 188.8 g. (43%); b.p. 68–70°/13 mm. Hg [lit. (8) b.p. 66–67°/13 mm. Hg]. The product was homogeneous to VPC; λ_{max} ($\epsilon \times 10^{-3}$): 236 (11.4), 280 (1.3), and 287 (1.2) nm.

Anal.—Calc. for $C_8H_5F_3O$: C, 55.18; H, 2.90; F, 32.73. Found: C, 54.96; H, 2.82; F, 32.34.

4-(Trifluoromethyl)benzyl Alcohol (VIII)—A solution of 87 g. (0.50 mole) of I in 100 ml. of ether was added dropwise to 7.6 g. (0.20 mole) of lithium aluminum hydride in 200 ml. of ether. The suspension was refluxed for 1 hr., cooled, hydrolyzed by the addition of 100 ml. of water, and then filtered. The ether was removed from the filtrate by spin-evaporation to produce an oily residue of the benzyl alcohol suitable for further transformation, yielding 83.5 g. (95%). Analytically pure material was obtained by distillation *in vacuo*; b.p. 97°/21 mm. Hg [lit. (9) b.p. 86°/8 mm. Hg]. The product was homogeneous to VPC and TLC; λ_{max} ($\epsilon \times 10^{-3}$): 257 (3.2), 262 (3.3), and 268 (2.5) nm.

Anal.—Calc. for $C_8H_7F_3O$: C, 54.55; H, 4.01; F, 32.36. Found: C, 54.49; H, 4.10; F, 32.16.

4-(Trifluoromethyl)benzyl Chloride (IX)—To a solution of 17.6 g. (0.100 mole) of crude VIII and 7.9 g. (0.10 mole) of pyridine in 100 ml. of chloroform was added dropwise 15.7 g. (0.132 mole) of thionyl chloride. The reaction mixture was refluxed overnight, cooled, washed with water (3400 ml.), dried, and then filtered. The filtrate was dried and then spin-evaporated to an oil, yielding 19.0 g. (98%). The product was suitable for further transformation. Analytically pure material was obtained by distillation *in vacuo;* b.p. 67°/10 mm. Hg [lit. (9, 10) b.p. 61°/8 mm. Hg]. This was homogeneous to both VPC and TLC; λ_{max} ($\epsilon \times 10^{-3}$): 264 (5.0) and 271 (4.0) nm.

Anal.—Calc. for $C_8H_6ClF_3$: C, 49.38; H, 3.11; Cl, 18.22; F, 29.29. Found: C, 49.47; H, 3.25; Cl, 17.99; F, 29.42.

4-(Trifluoromethyl)phenylacetonitrile (X)—To a stirred suspension of 11.7 g. (0.239 mole) of sodium cyanide in 55 ml. of dimethylformamide at 50° was added dropwise 359 g. (0.181 mole) of IX. Heating was continued for 1.5 hr. The reaction mixture was cooled and diluted with 500 ml. of water, and two layers resulted. This mixture was extracted into ether. The ether extracts were dried and the ether was removed to yield a crude product, which was purified by distillation to yield 22 g. (66%) of X; b.p. 117–126°/14 mm. Hg [lit. (11) b.p. 110–112°/14 mm. Hg]. The compound crystallized on standing; m.p. 43–45° [lit. (11) m.p. 44–45°]. It was homogeneous to TLC; λ_{max} ($\epsilon \times 10^{-3}$): 256 (3.2), 262 (3.8), and 267 (3.2) nm.

Anal.—Calc. for $C_9H_6F_3N$: C, 58.39; H, 3.26; F, 30.78; N, 7.56. Found: C, 58.13; H, 3.12; F, 30.50; N, 7.49.

1-(4-Trifluoromethylphenyl)ethanol (II)—A solution of 201 g. (4.56 moles) of acetaldehyde in 100 ml. of dry tetrahydrofuran was added during 90 min. to a Grignard reagent prepared from 97.2 g. (4.00 moles) of 4-chlorobenzotrifluoride as described for I. The crude II was isolated in a similar fashion and distilled *in vacuo* to yield 296 g. (74%) of product suitable for further transformation; b.p. 91–103°/10 mm. Hg [lit. (15) b.p. 106–107°/18 mm. Hg].

4-(Trifluoromethyl)acetophenone (XI)—A solution of 426 g. (2.24 moles) of II in 610 ml. of glacial acetic acid was added in six portions (at 10°) to 305 g. (1.04 moles) of sodium dichromate in 460 ml. of glacial acetic acid. The reaction mixture was heated at 80° for 1 hr., cooled, added to 15 l. of cold water, and then extracted into ether (8 × 5 l.). The ether was washed with saturated aqueous sodium carbonate (1500 ml.) and water (2 × 3 l.), dried, and then filtered. The filtrate was spin-evaporated to a residue. The product was distilled to yield 330 g. (83%); b.p. 81–83°/10 mm. Hg [lit. (14, 15) b.p. 79–80°/8 mm. Hg] and further purified by recrystallization from 170 ml. of petroleum ether (b.p. 30–60°); m.p. 30–31°. The product was homogeneous to VPC and TLC; λ_{max} ($\epsilon \times 10^{-3}$): 233 (13.1) and 278 (1.4) nm.

Anal.—Calc. for $C_9H_7F_3O$: C, 57.45; H, 3.75; F, 30.29. Found: C, 57.64; H, 3.76; F, 30.20.

4-(Trifluoromethyl)phenylthioacetomorpholide (XII)—A mixture of 2171 g. (11.50 moles) of XI, 1503 g. (17.25 moles) of morpholine, and 550 g. (17.3 g.-atoms) of sulfur was refluxed for 16 hr., cooled, and then poured into 2300 ml. of ethanol containing 45 ml. of benzene. The crystalline product which precipitated was suitable for further transformation, yielding 2396 g. (72%). Analytically pure material was obtained by recrystallization from ethanol, m.p. 120–120.5°. The compound was homogeneous to TLC (four solvent systems); λ_{max} ($\epsilon \times 10^{-3}$): 282 (14.4) nm.

Anal.—Calc. for $C_{13}H_{14}F_3NOS$: C, 53.97; H, 4.88; N, 4.84. Found: C, 53.88; H, 4.92; N, 4.86.

4-(Trifluoromethyl)phenylacetic Acid (VII)—*Method A*—A solution of 2396 g. (8.030 moles) of XII in 32.4 l. of 10% aqueous potassium hydroxide was refluxed for 16 hr. The mixture was filtered, and the filtrate was added slowly to a cold (5°) solution of 7.8 l. of concentrated hydrochloric acid in 32 l. of water. The resulting crystalline precipitate was collected and recrystallized from petroleum ether (b.p. 30–60°), yielding 1300 g. (80%), m.p. 81–83° [lit. (11) m.p. not recorded]. The product was homogeneous to TLC; λ_{max} ($\epsilon \times 10^{-3}$): 257 (2.8), 202 (3.0), and 268 (2.3) nm.

Anal.—Calc. for $C_9H_7F_3O_2$: C, 52.95; H, 3.46; F, 27.92. Found: C, 53.14; H, 3.45; F, 28.00.

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[3] The test results were provided by the Walter Reed Army Institute of Research through the courtesy of Dr. T. R. Sweeney and Dr. B. T. Poon.

[4] All temperatures reported are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. VPC was carried out using a Hewlett-Packard model 5750B, temperature programmed at 10°/min. from 40 to 250°, using a 2-m. × 3-mm. column packed with 10% UC-W982 on 80–100-mesh Diataport S. TLC samples were developed on Eastman Chromagram Sheet 6060 using at least three solvent systems for each compound. UV spectra were measured in ethanol on a Bausch & Lomb Spectronic 505 spectrophotometer.

[5] Hooker Chemical Co.

Method B—A mixture of 10 ml. concentrated sulfuric acid, 10 ml. water, 15 ml. glacial acetic acid, and 10 g. (0.05 mole) of X was refluxed for 2 hr. and then cooled. Two layers formed which were saturated with ether (4 × 50 ml.). The ether extracts were dried and spin-evaporated to yield an oil which crystallized on standing. Recrystallization yielded 6.9 g. (72%) of VII, m.p. 81–83°, identical to that obtained above.

4,4′-Bis(trifluoromethyl)-α-phenylcinnamic Acid (IV*a*)—To a suspension of sodium methoxide (11.6 g., 0.215 mole) in 1000 ml. of acetic anhydride was added, under a nitrogen atmosphere, 43.9 g. (0.215 mole) of VII. The solution was stirred 20 min., 25 g. (0.14 mole) of I was added, and the mixture was stirred at 100° for 18 hr. While still at 100°, four portions of water were added to hydrolyze the acetic anhydride. During this process a crystalline product appeared which was collected after cooling the mixture to 20°, yielding 48.8 g. (94%), m.p. about 140°. This material contained mostly *cis*-IV*a* with some *trans*-IV*a* and was suitable for further transformation. Pure *cis*-IV*a* was obtained by chromatographing the mixture on silica gel with a 7:3 mixture of petroleum ether (b.p. 30–60°) and ethyl acetate, m.p. 184–185° (lit.[6] m.p. 183–186°). This material was homogeneous to TLC; λ_{max} ($\epsilon \times 10^{-4}$): 275 (1.4) nm.

Anal.—Calc. for $C_{17}H_{10}F_6O_2$: C, 56.68; H, 2.80; F, 31.64. Found: C, 56.62; H, 2.78; F, 31.73.

Methyl 4,4′-Bis(trifluoromethyl)-α-cinnamate (IV*b*)—A flask containing 48.8 g. (0.135 mole) of crude IV*a* in 250 ml. of methanol and 25 ml. of concentrated sulfuric acid was attached to an apparatus (soxhlet) containing 85 g. of No. 3A molecular sieves immersed in 1000 ml. of methanol. After refluxing for 40 hr., the reaction mixture was poured into 1000 ml. of cold water containing 66 g. of K_2CO_3. The mixture was extracted with ether. The ether extracts were washed with water, dried, and spin-evaporated to a residue which was chromatographed on 100 g. basic alumina with ether as the eluate. After removing the ether, the resulting oil crystallized on standing, yielding 47 g. (93%). This was further purified by distillation *in vacuo*, b.p. 127–133°/0.6 mm. Hg. VPC and TLC revealed the presence of two isomers, with *cis*-IV*b* predominating (VPC); λ_{max} ($\epsilon \times 10^{-4}$): 279 (1.65) nm.

Anal.—Calc. for $C_{18}H_{12}F_6O_2$: C, 57.76; H, 3.23; F, 30.46. Found: C, 57.80; H, 3.40; F, 30.21.

Pure *cis*-IV*b* was obtained by fractional distillation *in vacuo*, b.p. 141–143°/1.5 mm. Hg, followed by crystallization from hexane, m.p. 70–71° (lit.[6] m.p. 69–72°). *cis*-IV*b* was homogeneous to VPC and TLC; λ_{max} ($\epsilon \times 10^{-4}$): 277 (2.0) nm.

Anal.—Calc. for $C_{18}H_{12}F_6O_2$: C, 57.76; H, 3.23; F, 30.46. Found: C, 57.99; H, 3.06; F, 30.28.

4-(Trifluoromethyl)benzyltriphenylphosphonium Chloride (VI)—A mixture of 8.3 g. (43 mmoles) of IX and 11.8 g. (44.8 mmoles) of triphenylphosphine in 200 ml. of dry xylene was refluxed overnight and then cooled. The crystalline product which separated was filtered off and dried, yielding 17.3 g. (88%), m.p. 275–281° dec. Analytically pure material was obtained by a single recrystallization from ethanol, m.p. 283–285° dec. It was homogeneous to TLC; λ_{max} ($\epsilon \times 10^{-3}$): 267 (3.7) and 274 (2.9) nm.

Anal.—Calc. for $C_{26}H_{21}ClF_3P$: C, 68.35; H, 4.63; F, 12.48; P, 6.78. Found: C, 68.13; H, 4.74; F, 12.66; P, 6.79.

4,4′-Bis(trifluoromethyl)stilbene (V)—*Method A*—To a suspension of 21.5 g. (47.1 mmoles) of VI under nitrogen was added 36.7 g. of hexane solution containing 61 mmoles of butyllithium. The reaction mixture was stirred for 2 hr., and then 9.9 g. (57 mmoles) of I was added. The suspension was stirred for 16 hr., diluted with petroleum ether (b.p. 30–60°) (1175 ml.), filtered, and then spin-evaporated to an oil. Addition of 500 ml. of petroleum ether (b.p. 30–60°) precipitated the triphenylphosphonium oxide, which was discarded. The filtrate was spin-evaporated *in vacuo*, and the resulting residue was distilled to give 4.8 g. (32%) of *cis*-V, b.p. 80–82°/2 mm. Hg, m.p. 49–52°. *trans*-V, 7.4 g. (50%), was recovered from the residue, m.p. 124–127°. Assignment of configuration was by comparison of physical properties and spectra with those of authentic samples of the unsubstituted stilbene isomers (18–20). Each isomer was homogeneous to VPC (retention time 14.2 and 16.7 min. for *cis*- and *trans*-V, respectively); λ_{max} (*cis*) ($\epsilon \times 10^{-4}$): 225 (2.1) and 280 (1.3) nm.; λ_{max} (*trans*) ($\epsilon \times 10^{-4}$): 226 (1.4), 298 (3.1), 308 (3.2), and 320 (2.0) nm.

Anal.—Calc. for $C_{16}H_{10}F_6$: C, 60.79; H, 3.19; F, 36.04. Found for *cis*-V: C, 60.80; H, 3.27; F, 35.85. Found for *trans*-V: C, 60.78; H, 3.14; F, 36.20.

Method B—A stirred mixture of 20.0 g. (0.056 mole) of IV*a*, 70 ml. of quinoline, and 1 g. of copper powder was refluxed (at 195°) for 17 hr. The reaction mixture was poured into 280 ml. of 10% HCl and extracted into ether. The ether was removed, and the product mixture was chromatographed on alumina using hexane. Distillation under vacuum gave 9.5 g. (54%) of mixed *cis*- and *trans*-V, identified by VPC. Fractional distillation gave 0.7 g. of *cis*-V and 8.5 g. of *trans*-V.

1,2-Bis(4-trifluoromethylphenyl)ethane (XV)—A mixture of *cis*- and *trans*-V (6.4 g., 20 mmoles) in glacial acetic acid was hydrogenated for 30 min. over 400 mg. of palladium oxide. The solution was neutralized with 50% NaOH and extracted with ether. The extracts were dried and spin-evaporated to a crystalline residue. Analytically pure material was obtained by recrystallization from hexane, yielding 5.2 g. (81%), m.p. 78–79°. The product was homogeneous to VPC and TLC; λ_{max} ($\epsilon \times 10^{-2}$): 253 (5.5), 258 (7.0), 263.5 (5.9), and 268.5 (3.9) nm.

Anal.—Calc. for $C_{16}H_{12}F_6$: C, 60.38; H, 3.80; F, 35.82. Found: C, 60.30; H, 3.90; F, 35.61.

α,β-Bis(4-trifluoromethylphenyl)propionitrile (XIII)—This product was obtained as a by-product of the reaction to produce X from IX. The distillation residue was triturated with petroleum ether (b.p. 30–60°) to give a solid, which was then recrystallized twice from absolute ethanol (charcoal), m.p. 78–79°. The material was homogeneous to TLC. The compound was characterized by its molecular weight and IR and NMR spectra; λ_{max} ($\epsilon \times 10^{-3}$): 258 (8.4), 263 (9.6), and 269 (7.9) nm.

Anal.—Calc. for $C_{12}H_{11}F_6N$: C, 59.48; H, 3.63; F, 33.21; N, 4.08. Found: C, 59.49 and 59.76; H, 3.11 and 3.16; F, 33.55; N, 3.99.

4,4′-Bis(trifluoromethyl)benzylformal (XIV)—This material was obtained during an attempt to prepare VIII directly from 4-chlorobenzotrifluoride, using paraformaldehyde on the intermediate magnesium Grignard. A 19% yield of the desired product, VIII, was obtained. Further fractionation produced XIV in 35% yield, b.p. 119–121°/0.25 mm. Hg. It was homogeneous to VPC and TLC. The compound was identified by its IR and NMR spectra; λ_{max} ($\epsilon \times 10^{-3}$): 257 (8.0), 262 (9.0), and 268 (7.3) nm.

Anal.—Calc. for $C_{17}H_{14}F_6O_2$: C, 56.05; H, 3.85; F, 31.29. Found: C, 56.18; H, 4.09; F, 30.99.

[6] This product was also prepared and characterized by Dr. D. W. Henry, Stanford Research Institute (personal communication).

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Fluorometric Estimation of Oxytetracycline in Blood and Plasma

BRYAN SCALES▲ and DAVID A. ASSINDER

Abstract □ A sensitive spectrofluorometric method was developed for the estimation of oxytetracycline in whole blood or plasma. The method involves the use of protein precipitation, solvent extraction, and alkaline degradation procedures. Reproducible recoveries of oxytetracycline are obtained by adding β-mercaptopropionic acid at various stages of the extraction procedure. Whereas the alkaline decomposition product of oxytetracycline fluoresces at 337/410 nm., the reproducibility of the assay is improved by scanning the fluorescence spectrum from 300 to 500 nm. This enables any variation in background fluorescence of different samples to be estimated and accounted for when measuring low levels of oxytetracycline. The method will measure oxytetracycline to better than 0.1 mcg./ml. blood or plasma and is therefore suitable for monitoring the blood levels obtained after a therapeutic dose. The results obtained in man illustrate the variability of blood level profiles of oxytetracycline following its oral administration under closely controlled conditions.

Keyphrases □ Oxytetracycline—spectrophotofluorometric analysis in whole blood or plasma □ Plasma levels, oxytetracycline—after oral administration in man, spectrophotofluorometric analysis □ Spectrophotofluorometry—analysis, oxytetracycline in whole blood or plasma

A growing understanding of the importance of the blood levels of drugs and their relevance to the clinical evaluation makes it imperative that relatively simple and reliable methods are available for estimating this class of organic compounds in biological fluids. The present study started as an attempt to show biological equivalence, or a lack of it, in oxytetracycline hydrochloride[1] formulations and to correlate blood levels with disintegration times or dissolution times.

Initial problems occurred in the development of a satisfactory analytical method for oxytetracycline in serum, plasma, or whole blood. Although bioassay techniques have been recommended and widely used (1), an alternative method was preferred. Estimations by conventional polarography offered a rapid, although insensitive, method of analysis, and this could not be improved sufficiently even by the use of polarography (a.c.)(2). The more recent colorimetric assays for tetracyclines (3) also lack sensitivity. A variety of fluorescence methods have been reported in the literature; Hayes and DuBuy (4) utilized the conversion to the anhydroderivative on heating in acid, followed by a measurement of the fluorescence intensity at alkaline pH. Ibsen *et al.* (5) developed a method based on the increase in fluorescence of the oxytetracycline on chelation with magnesium, and Kahn (6) described its estimation in biological systems utilizing the extractability of fluorescent calcium–barbital complexes.

In this laboratory the reported fluorescence methods were largely unworkable and at best gave irreproducible results, probably due to photodecomposition of the oxytetracycline. During the investigations it was found that the ready decomposition which occurred during the various isolation stages could be most satisfactorily prevented by the inclusion of thiol reagents during the work-up procedures. This led to the development of a reliable, sensitive fluorometric method for the quantitation of oxytetracycline at the therapeutic levels encountered in whole blood or plasma.

EXPERIMENTAL[2]

Reagents—All hydrochloric acid solutions were prepared from concentrated hydrochloric acid[3] for Foodstuffs Analysis grade. This was free from the interfering materials often added to some commercially standardized solutions to prevent bacterial growth.

The following aqueous solutions were prepared in glass-distilled water: 0.1 *N* HCl containing β-mercaptopropionic acid (1.0 mg./ml.), trichloroacetic acid (30% w/v), 1.0 *N* NaOH, 0.02 *N* HCl, and

[1] Oxytetracycline hydrochloride is the active drug substance in the pharmaceutical formulation Imperacin, marketed by I.C.I. Ltd., Pharmaceuticals Division.

[2] All fluorometric measurements were carried out using either the Aminco-Bowman spectrophotofluorometer or the Hitachi MPF 2A or MPF 3 instruments. All glassware was cleaned preferably in Haemosol, rinsed in tap water, soaked overnight in 1 *N* HCl, and, after rinsing in distilled water, dried.

[3] British Drug Houses.

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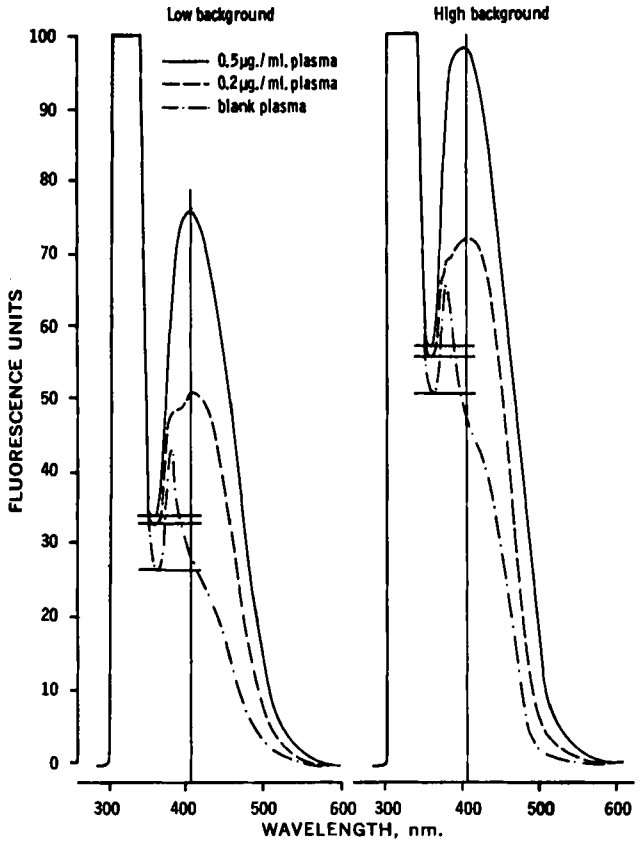

Figure 1—*Fluorescence spectra of low and high background extracts of oxytetracycline obtained from control and spiked whole blood.*

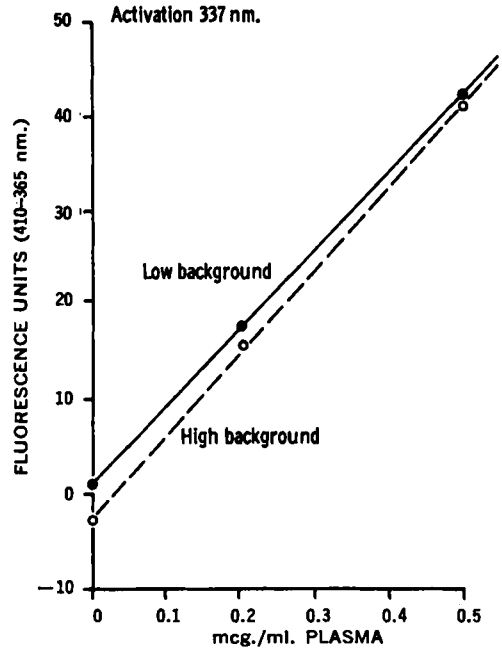

Figure 2—*Calibration curves obtained from the low and high background extracts illustrated in Fig. 1.*

a citrate–phosphate buffer prepared by diluting a mixture of 0.5 *M* citric acid (30.7 ml.) and 1.0 *M* disodium hydrogen phosphate (19.3 ml.) to 100 ml.

The organic solvents required were ether, freshly distilled ethyl acetate, and a solution of β-mercaptopropionic acid (50 mg./ml.) in methanol.

Standard Solutions—Oxytetracycline hydrochloride was dissolved at 1.00 mg./ml. in 0.1 *N* HCl containing β-mercaptopropionic acid (1 mg./ml.). This solution was then diluted 100- and 1000-fold, using the same solvent mixture, to give solutions of 10.0 and 1.00 mcg./ml., respectively.

Spectrofluorometric Determination of Oxytetracycline in Blood or Plasma—Into a 10-ml. glass-stoppered tube were placed 1.0 ml. whole blood or plasma, 1.0 ml. water, 1.0 ml. 0.1 *N* HCl containing β-mercaptopropionic acid (1.0 mg./ml.), and 1.0 ml. trichloroacetic acid (30% w/v). The mixture was thoroughly shaken for 5 min. and centrifuged for 10 min. at 2000 r.p.m. (1500×*g*), and a 3.0-ml. aliquot of the supernate was placed in a 50-ml. tube. The trichloroacetic acid was extracted by shaking with 2 × 10-ml. portions of ether; after the last traces of ether had been aspirated, the pH of the aqueous phase was adjusted to 4.0 by the addition of 0.10 ml. 1.0 *N* NaOH and 0.60 ml. citrate–phosphate buffer. This buffered aqueous phase was extracted with 15.0 ml. ethyl acetate. After centrifugation, a 14.0-ml. aliquot was transferred to a tube containing 0.10 ml. of a methanol solution of β-mercaptopropionic acid (50 mg./ml.) and evaporated to dryness at 60° in a stream of dry nitrogen.

The residue obtained was dissolved in 3.0 ml. 0.02 *N* HCl, and the solution was made alkaline by the addition of 0.5 ml. 1.0 *N* NaOH prior to heating in a boiling water bath for 15 min. to develop the maximum fluorescence. The fluorescence intensity of the cooled solution was determined over the 300–500-nm. range while exciting at the peak activation wavelength of 337 nm. (Fig. 1).

Calibration Curves—Two types of calibration curves were constructed to aid in the development of the method and to check the recovery during routine analysis.

Standard Calibration Curves for Extracted Oxytetracycline—Aliquots of the standard solutions of oxytetracycline (10.0 and 1.00 mcg./ml.) were added to the mixture of 1.0 ml. biological fluid and 1.0 ml. water. The volume was increased to 3.0 ml. where necessary by the addition of 0.1 *N* HCl containing β-mercaptopropionic acid; 1.0 ml. trichloroacetic acid (30% w/v) was then added and the samples were taken through the full extraction and fluorescence development procedure already described. Such a standard curve was prepared with every batch of samples to be analyzed.

Calibration Curves for Oxytetracycline Added to Extracts of Blood or Plasma—Samples of whole blood or plasma that were free from oxytetracycline were taken through the full extraction procedure. After evaporation of the ethyl acetate extracts to dryness, standard solutions of oxytetracycline (0.0–10.0 mcg.) were added to the residue. Then 3.0 ml. 0.02 *N* HCl was added to dissolve the residue, the solution was made alkaline, and the fluorescence was developed as already described.

Preparation of Oxytetracycline Solution for Oral Administration—Solutions of oxytetracycline sufficient for a single oral dose were prepared by dissolving a mixture of oxytetracycline hydrochloride (500 mg.) and ascorbic acid (2.30 g.) in 20.0 ml. distilled water in screw-capped vials. The proportion of these two components was similar to that used in intravenous formulations[4]. The solution was administered within 15 min. of preparation.

Administration and Blood Sampling—Normally healthy male volunteers were fasted for at least 10 hr. prior to, and 3 hr. after, the dose of oxytetracycline (500 mg.), which was taken at about 9:00 a.m. Any formulation remaining in the vials was washed out and swallowed with at least 60 ml. water.

Venous blood (8.0 ml.) was taken and oxalated at the times shown in the figures, usually at 0, 1, 2, 3, 5, 7, and 24 hr. When plasma samples were required, the blood was centrifuged and the plasma separated, usually within 30 min. of being obtained.

One week elapsed between subsequent doses to each volunteer, ensuring that plasma levels had fallen well below the limits of detection. All samples were assayed in duplicate, the second assay being carried out 1–5 days after the first assay.

RESULTS AND DISCUSSION

Development of Method—Once it had been established that the fluorescence intensity of the alkaline oxytetracycline solution was sufficient to permit the detection of oxytetracycline at levels of 0.01 mcg./ml. of final solution, it became a relatively simple task to develop the extraction technique and eliminate or control unreliable

[4] Pfizer.

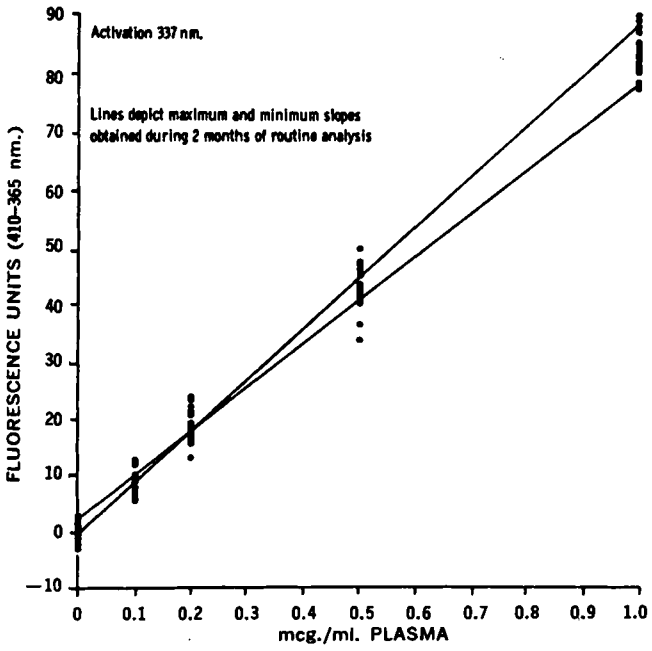

Figure 3—*Calibration curves obtained for oxytetracycline extracted from spiked whole blood and plasma. Points are those accumulated over a 2-month period. The lines show the extremes in slopes obtained during this time.*

stages. This was done most expediently by examining the latest stages first. Thus, it was found that the simple evaporation of solutions of oxytetracycline in ethyl acetate (1 mcg. in 15 ml.) was sufficient to destroy all oxytetracycline. This effect was presumably due in part to photodecomposition since, when the evaporation was carried out in darkness, a 20–45% recovery was usually obtained. The addition of antioxidants such as β-mercaptoethanol and β-mercaptopropionic acid resulted in a 98–100% recovery of oxytetracycline from the solvent. Ascorbic acid could not be used because of the production of fluorescent impurities during treatment of the residue with alkali.

The use of hydrochloric acid free from metallic antibacterials was essential, since their presence led to a large and variable reduction (5–30-fold) in fluorescence of the oxytetracycline.

The trichloroacetic acid precipitation of protein and the removal of excess trichloroacetic acid by ether extraction prior to pH adjustment are advisable, since these procedures helped to reduce the background fluorescence to acceptable levels. The extraction of excess trichloroacetic acid also simplifies the problem of pH adjustment to 4.0 prior to the extraction of oxytetracycline into the ethyl acetate.

The addition of higher concentrations of hydrochloric acid to the whole blood or plasma prior to protein precipitation did not increase the recovery of oxytetracycline; and since the recovery was virtually the same from whole blood, plasma, and water, it is presumed that the standardized conditions were adequate to prevent any absorption and losses due to protein binding.

The assay was also carried out on a number of occasions using 4.0 ml. whole blood. The stages up to that of pH 4.0 adjustment were scaled up proportionately; the volume of ethyl acetate used for the oxytetracycline extraction was doubled, but subsequent stages were unchanged. As expected, the background contamination was slightly increased, but the method could still be used to detect 0.1 mcg. oxytetracycline in the 4.0-ml. sample. This largescale method was not used because it was more cumbersome and time consuming, and the sensitivity of the assay based on a 1-ml. sample was perfectly adequate.

Stability of Oxytetracycline in Whole Blood and Plasma—The stability was checked by spiking samples of the fluids, as in the preparation of calibration curves, and storing at 0–4° or at room temperature for up to 3 weeks prior to assaying. There was no distinction between calibration curves prepared from freshly spiked and from stored samples. The stability of oxytetracycline in whole blood or plasma was also independent of the presence of β-mercaptopropionic acid, although it was invariably added during routine assays since it was present in the standard aqueous solutions used for spiking samples.

Further evidence for the stability of oxytetracycline in the biological samples was obtained when carrying out replicate assays of unknown samples. Identical results were obtained when the replicate assays were carried out over a 2-week period.

Table I—Comparison of Plasma Level Profiles after Oral Administration of 500 mg. Oxytetracycline Hydrochloride in Solution to Four Subjects (see Fig. 4)

Subject	Weight, kg.	Dose Sequence	Maximum Plasma Level, mcg./ml.	Area under Curve (0–7 hr.), hr. × mcg./ml.
M.B.C.	58.0	1	2.95	17.4
		2	2.71	14.4
		3	2.46	14.8
		Mean ± *SD*	2.71 ± 0.24	15.53 ± 1.63
D.A.A.	65.8	1	1.22	5.8
		2	2.46	8.0
		3	2.00	11.6
		Mean ± *SD*	1.89 ± 0.63	8.47 ± 2.93
A.J.W.	72.5	1	2.10	12.4
		2	3.40	15.2
		3	2.06	10.8
		Mean ± *SD*	2.52 ± 0.76	12.80 ± 2.23
D.M.F.	86.6	1	1.58	9.0
		2	1.46	7.1
		3	1.31	7.3
		Mean ± *SD*	1.45 ± 0.14	7.80 ± 1.04
Overall mean			2.14	11.15

Fluorescence Spectrum of Oxytetracycline and Its Quantitative Interpretation—The fluorescence spectra of extracts of whole blood containing 0.00, 0.20, and 0.50 mcg. oxytetracycline/ml. are shown in Fig. 1. For the majority of samples, the background fluorescence due to the Raman spectrum and to tissue impurities is low. In this case, the calibration curves obtained by plotting total fluorescence intensity at 410 nm. *versus* oxytetracycline concentration are quite acceptable, although there is a positive fluorescence blank value equivalent to about 0.25 mcg. oxytetracycline/ml. blood. Occasionally, the background scatter is increased due to poor sample preparation or to aging of the biological sample. In these circumstances, the use of such samples would normally be unacceptable in the analytical procedure. The value of scanning the full fluorescence spectrum rather than reading the fluorescence at a selected wavelength lies in the fact that this enables the background contribution of each sample to be estimated from the scan. On measuring the background fluorescence of blank samples, the fluorescence intensity of the valley at 365 nm. is equivalent to that at 410 nm.

The equivalence of fluorescence intensity of blank samples at the valley and 410 nm. is independent of the instrument and of the bandpass used in the excitation and fluorescence monochromators. For these reasons, a more accurate method of estimating fluorescence intensity due to oxytetracycline in any sample is to measure the peak height at 410 nm. above the valley at 365 nm. An example of one of the worst cases of a high background due to poor sample preparation is shown in Fig. 1, and even in this case the background is only equivalent to −0.025 mcg. oxytetracycline/ml. blood when calculated by this difference method. In general, calibration curves prepared by this method (Fig. 2) have a blank value which falls within the range of 0.00 ± 0.02 mcg. oxytetracycline/ml. biological sample.

Sources of Variability in Analysis—The day-to-day variation in overall extraction efficiency of oxytetracycline (42 ± 2%) and in instrument sensitivity was quite small. The individual points from extracted calibration curves obtained from whole blood and plasma over 2 months[5] are accumulated in Fig. 3. Also illustrated are the

[5] The Hitachi MPF 2A was used.

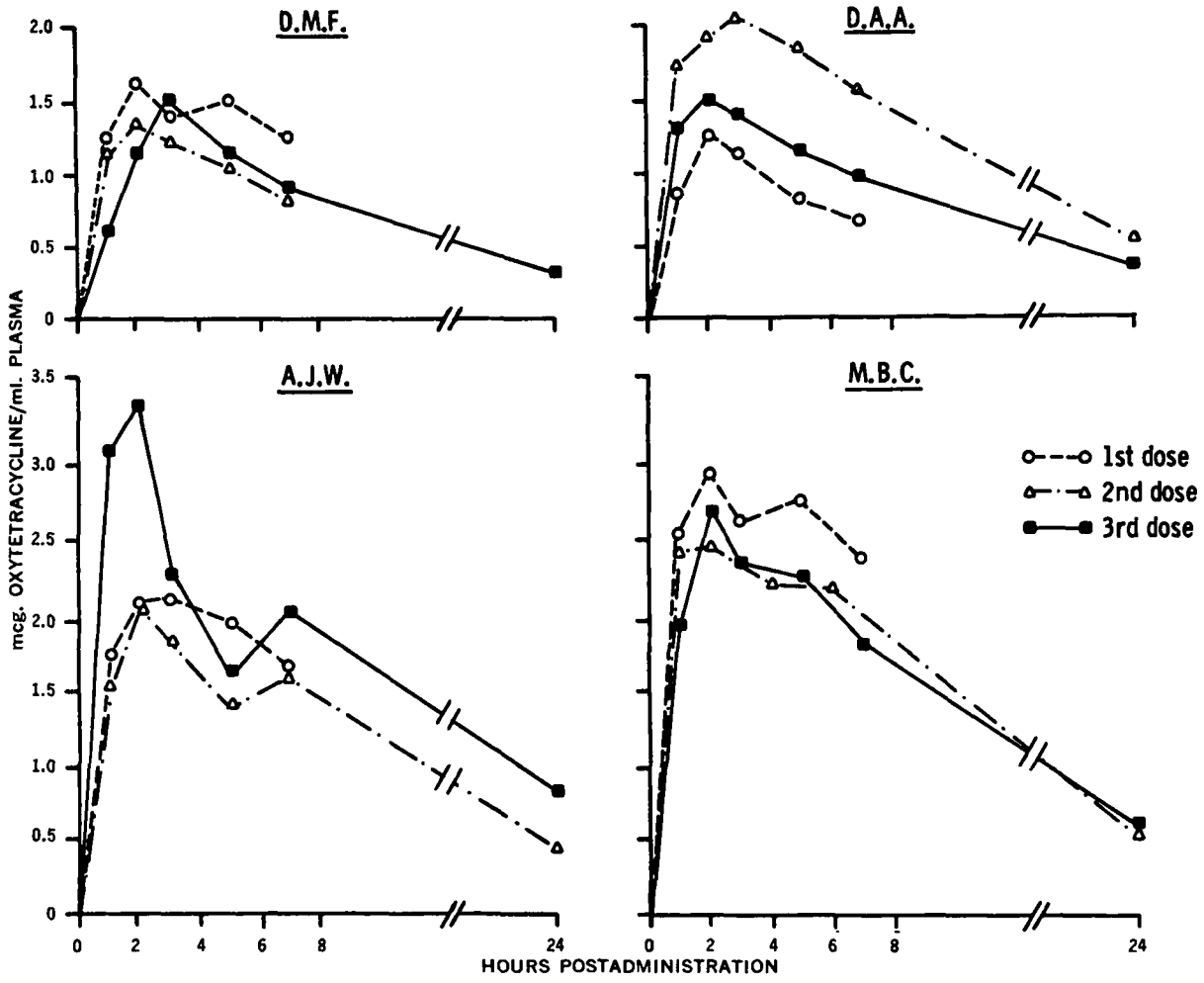

Figure 4—*Plasma level curves of oxytetracycline in four volunteers following the oral administration of a solution of 500 mg. oxytetracycline hydrochloride.*

extremes of slopes encountered during this period of routine assays. It was not necessary to adjust instrument sensitivity during the analytical runs, although this could have been done by use of fluorescence standards. The only instrument checks carried out during routine use were that the activation wavelength was correctly set at the optimum of about 337 nm. and that the activation and fluorescence slits were each set for a bandpass of 8 nm.

The slight fluctuations in slope (Fig. 3) encountered over a 2-month period were usually caused by the necessary periodic renewal of extraction solvents and reagents. For this reason it was preferred to calculate the concentration of oxytetracycline in the biological samples by direct application of the calibration curve from extracted spiked blood samples, rather than to rely solely on calculations based on a 42% extraction efficiency and a nonextracted standard curve.

Table II—Analysis of Variance of Data in Table I

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	*F*-Ratio
Maximum Plasma Level				
Between subjects	3	3.01	1.00	3.80[a]
Within subjects	8	2.11	0.26	—
Area under Curve				
Between subjects	3	121.08	40.36	9.34[b]
Within subjects	8	34.55	4.32	—

[a] Marginally significant ($p < 0.1$). [b] Highly significant ($p < 0.01$).

Plasma Levels after Oral Dosing—The solution was administered to four fasted male volunteers on three separate occasions at weekly intervals. The results of the plasma level analyses are given for each volunteer in Fig. 4. All samples were assayed in duplicate, and agreement was better than ±4% for levels above 1.2 mcg./ml. and better than ±8% for levels in the 0.2–1.2-mcg./ml. range. The drug appears to be fairly rapidly absorbed, with peak plasma levels being achieved between 2 and 3 hr. after dosing, and by 24 hr. the level had fallen to about 23 ± 2% of the peak value in all subjects. The absence of measured plasma levels in the 7–24-hr. period permits only a rough estimate of the plasma half-life, which in these volunteers probably falls in the 8–10-hr. range; this compares favorably with the literature value of 9.2 hr. (7).

The most disconcerting observation in this brief study was that although attempts were made to obtain reproducible plasma level profiles by: (*a*) administering the oxytetracycline in a form free from complications of variable disintegration and dissolution rates, and (*b*) minimizing disturbances in absorption due to variations in the contents of the GI tract, the results showed that this had not been achieved.

Any assessment of the reproducibility of plasma level profiles in one individual should include *inter alia* a comparison of the times to attain the peak level, the actual level achieved at the peak, and the area under the curve (hr. × mcg./ml.) over a chosen period of time. The peak plasma levels that occurred reproducibly at from 2 to 3 hr. after dosing and the area under the curves for the 0–7-hr. period are listed in Table I. Although the number of subjects was small and the within-subject variability was occasionally high, the results of the analysis of variance for these two parameters (Table II) show that there are significant differences between individuals with respect to their plasma level profiles.

The variability encountered in the plasma level profiles of any one individual was not an artifact due to changes in protein binding since, unlike microbiological assay techniques, the present fluorometric method measures total oxytetracycline. Neither was the variation between subjects caused by weight differences in the subjects, since none of the analyses was improved by taking the weight of the subjects into account. It is possible that plasma level profiles could be made more reproducible by stabilizing physiological conditions, *e.g.*, transit times (8). However, such changes could only be of use in bioavailability studies and not in general clinical practice; even then, attempts at such stabilization could be beneficial to one formulation and detrimental to another.

In the present studies the mean peak plasma levels of oxytetracycline (2.14 mcg./ml.) attained after oral administration of a solution of 500 mg. of the hydrochloride are only 40% higher than the serum levels of the reference oxytetracycline (1.55 mcg./ml.) used in the study of Blair *et al.* (9), in which the dose of 250 mg. of the hydrochloride was administered in a capsule. This difference could be unreal, caused by the relatively few subjects used in the present study, but it could equally be the result of different physiological conditions of the GI tract.

The present results illustrate the difficulty in obtaining reproducible plasma level profiles of oxytetracycline, even under fairly carefully controlled conditions. The variability could be much greater after the oral administration of more complicated formulations than that used here. One way around the problem is to study plasma levels achieved after multiple doses, as in normal clinical practice, and the results of such studies will be the basis of further reports.

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▲ To whom inquiries should be directed.

Specificity of Antibodies Produced by Linear Antigenic Polypeptides of a Known Primary Structure: Synthesis and Use of Poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine Methyl Ester

BRIAN J. JOHNSON▲ and CHARLES CHENG

Abstract □ The specificity of antibodies formed in rabbits in response to the antigenic challenge of poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I) was studied with respect to the role of the glutamyl residue. For this purpose the polypeptide poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine methyl ester (II) was prepared. When using this material, it was found by cross-reaction and absorption studies that the antibodies to the antigen I possess a high affinity for the γ-carboxyl groups of the glutamyl residue.

Keyphrases □ Polypeptides, linear—synthesis, immunochemical properties □ Antibodies, polypeptide produced—specificity □ Poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine methyl ester—synthesis, used to study antibody specificity

It has previously been reported that the antibodies produced by rabbits against the polypeptide poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I) (1–3) are most probably dependent upon the conformation of the antigen (4–7). These antibodies also have been shown to possess a specificity for the phenolic hydroxyl groups and the aromatic moiety of the tyrosyl residue (8). However, no such specificity has been shown for the alanyl residue (9). The next phase of this work has been to study the specificity of these antibodies pertaining to the role of the glutamyl residue. This paper reports the characterization of the specificity of antiserum produced by rabbits against the antigen I as studied by cross-reactions and absorption studies. For this purpose the following polymer was synthesized and used: poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)-glycine methyl ester (II).

DISCUSSION[1]

The polypeptide II was prepared from the polymerizing unit, L-tyrosyl-γ-(*tert*-butyl-glycyl)-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (IX), which was synthesized as outlined in Scheme I and as detailed in the *Experimental* section. The polymerization of IX was performed by the procedure that has been shown to produce linear high molecular weight polypeptides (1, 2, 4–8). After removal of the protecting *tert*-butyl groups, the

[1] All amino acids are of the L-variety.

The variability encountered in the plasma level profiles of any one individual was not an artifact due to changes in protein binding since, unlike microbiological assay techniques, the present fluorometric method measures total oxytetracycline. Neither was the variation between subjects caused by weight differences in the subjects, since none of the analyses was improved by taking the weight of the subjects into account. It is possible that plasma level profiles could be made more reproducible by stabilizing physiological conditions, *e.g.*, transit times (8). However, such changes could only be of use in bioavailability studies and not in general clinical practice; even then, attempts at such stabilization could be beneficial to one formulation and detrimental to another.

In the present studies the mean peak plasma levels of oxytetracycline (2.14 mcg./ml.) attained after oral administration of a solution of 500 mg. of the hydrochloride are only 40% higher than the serum levels of the reference oxytetracycline (1.55 mcg./ml.) used in the study of Blair *et al.* (9), in which the dose of 250 mg. of the hydrochloride was administered in a capsule. This difference could be unreal, caused by the relatively few subjects used in the present study, but it could equally be the result of different physiological conditions of the GI tract.

The present results illustrate the difficulty in obtaining reproducible plasma level profiles of oxytetracycline, even under fairly carefully controlled conditions. The variability could be much greater after the oral administration of more complicated formulations than that used here. One way around the problem is to study plasma levels achieved after multiple doses, as in normal clinical practice, and the results of such studies will be the basis of further reports.

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Specificity of Antibodies Produced by Linear Antigenic Polypeptides of a Known Primary Structure: Synthesis and Use of Poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine Methyl Ester

BRIAN J. JOHNSON▲ and CHARLES CHENG

Abstract □ The specificity of antibodies formed in rabbits in response to the antigenic challenge of poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I) was studied with respect to the role of the glutamyl residue. For this purpose the polypeptide poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine methyl ester (II) was prepared. When using this material, it was found by cross-reaction and absorption studies that the antibodies to the antigen I possess a high affinity for the γ-carboxyl groups of the glutamyl residue.

Keyphrases □ Polypeptides, linear—synthesis, immunochemical properties □ Antibodies, polypeptide produced—specificity □ Poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine methyl ester—synthesis, used to study antibody specificity

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DISCUSSION[1]

The polypeptide II was prepared from the polymerizing unit, L-tyrosyl-γ-(*tert*-butyl-glycyl)-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (IX), which was synthesized as outlined in Scheme I and as detailed in the *Experimental* section. The polymerization of IX was performed by the procedure that has been shown to produce linear high molecular weight polypeptides (1, 2, 4–8). After removal of the protecting *tert*-butyl groups, the

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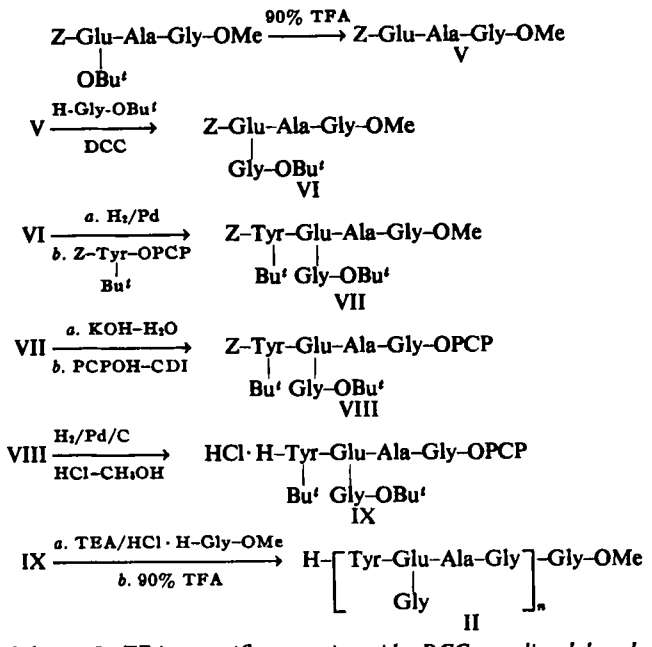

Scheme I—TFA = trifluoroacetic acid, DCC = dicyclohexylcarbodiimide, PCP = pentachlorophenyl, CDI = 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate, and TEA = triethylamine

crude polypeptide II was purified by dialysis and then fractionated by successive diafiltrations through membranes (Diaflo) into four different molecular weight fractions: $>5 \times 10^4$, $2\text{–}5 \times 10^4$, $1\text{–}2 \times 10^4$, and $<1 \times 10^4$.

Eight rabbits were immunized against I using the previously reported protocol (3). It was found that each serum gave a positive precipitin reaction with the homologous polymer I. The serum from each animal was pooled, because it was assumed that each rabbit had responded to the same antigenic determinants in this time interval (10). Incremental amounts of the polypeptide II were added to 1-ml. aliquots of this pooled antiserum and a cross-reaction occurred. The relative precipitin curves for the polypeptides I and II are shown in Fig. 1. To quantitate the amount of antibody not precipitated by polymer II, a separate experiment was performed. A quantity equal to the equivalence point amount of the heterologous polypeptide II was reacted with the pooled serum. After removal of the precipitate, 30 mcg. of the homologous polypeptide I was added to the resulting supernatant liquid. Further precipitation was obtained and quantitated by analysis for nitrogen (Kjeldahl) as shown in Table I.

It has been found that the heterologous polypeptide II cross-reacts with anti-I-serum; however, it precipitates less antibody than the homologous antigen I. The amount of the heterologous polymer II required to attain the equivalence point is slightly larger than that necessary for the homologous material. It would appear from these cross-reactions that the conformation of polymer II is similar to that of the homologous antigen I. The lower precipitating ability of polymer II indicates a decreased affinity of the antibody-combining site for this material. By using the rationale that the determinants of the heterologous polypeptide II are in the same orientation as those of the antigen I, it is suggested that the observed differences in the binding ability of the heterologous polypeptide II is due only to the modification of the glutamyl residue. Thus, it would appear that changes in the distance of the side-chain carboxyl group from the backbone polypeptide cause a large decrease in precipitating ability. It was, therefore, concluded that the antibody-combining sites have a high affinity for the γ-carboxyl groups of the glutamyl residue.

EXPERIMENTAL[1]

N-Benzyloxycarbonyl-L-glutamyl-L-alanylglycine Methyl Ester (V)—To 20 g. (0.0417 mole) of N-benzyloxycarbonyl-γ-*tert*-butyl-L-glutamyl-L-alanylglycine methyl ester (1) was added 90 ml. of 90% trifluoroacetic acid. The solution was stirred at room temperature for 50 min. and then concentrated under reduced pressure to yield a solid. This material was crystallized from ethyl acetate to yield 14.5 g. (82%) of the product, m.p. 178–180°; $[\alpha]_D^{24}$ −7.2° (c 4.2 in dimethylformamide).

Anal.—Calc. for $C_{19}H_{25}N_3O_8$: C, 53.90; H, 5.95; N, 9.93. Found: C, 53.75; H, 5.94; N, 9.70.

N-Benzyloxycarbonyl-γ-(*tert*-butyl-glycyl)-L-glutamyl-L-alanylglycine Methyl Ester (VI)—To a cooled solution of 14.5 g. (0.0343 mole) of N-benzyloxycarboxyl-L-glutamyl-L-alanylglycine methyl ester (V) and 5.0 g. (0.038 mole) of glycine *tert*-butyl ester in 200 ml. of methylene chloride was added 7.8 g. (0.0378 mole) of dicyclohexylcarbodiimide. This mixture was stirred overnight at room temperature and then concentrated under reduced pressure to yield a solid. This material was taken up in ethyl acetate, and the insoluble urea filtered off. The filtrate was washed successively with 1 *N* HCl, water, 10% sodium bicarbonate solution, and water and then dried (sodium sulfate). This solution was concentrated under reduced pressure to yield a solid, which was chromatographed on a column of Silicar CC-7 using chloroform–ethyl acetate (1:1) as the eluent to give 7.2 g. (40%) of the tetrapeptide, m.p. 141°; $[\alpha]_D^{24}$ −8.6° (c 3.2 in dimethylformamide).

Anal.—Calc. for $C_{25}H_{36}N_4O_9$: C, 55.95; H, 6.76; N, 10.44. Found: C, 55.91; H, 6.65; N, 10.37.

N-Benzyloxycarbonyl-*O-tert*-butyl-L-tyrosyl-γ-(*tert*-butyl-glycyl)-L-glutamyl-L-alanylglycine Methyl Ester (VII)—A fine suspension of 7.2 g. (0.0135 mole) of VI and 0.6 g. of 10% palladium-on-charcoal in 100 ml. of methanol was treated with 0.492 g. (0.0135 mole) of dry hydrochloric acid in methanol, and the suspension was hydrogenated for 2 hr. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure to give an oil. To this material, dissolved in 200 ml. of methylene chloride and 1.36 g. (0.00135 mole) of triethylamine, was added 9.2 g. (0.00148 mole) of N-benzyloxycarbonyl-*O-tert*-butyl-L-tyrosine pentachlorophenyl ester (1). This mixture was stirred overnight at room temperature and then concentrated under reduced pressure to give a solid; the solid was suspended in ethyl acetate, washed successively with 1 *N* HCl and water, and dried (sodium sulfate). Concentration of this solution produced a solid, which was chromatographed on a column of Silicar CC-7 using chloroform–ethyl acetate (1:1) as eluent. The major fraction obtained was crystallized from ethyl acetate–hexane to yield 5.8 g. (59%) of the heptapeptide, m.p. 204.5–206°; $[\alpha]_D^{24}$ −15.1° (c 4.3 in dimethylformamide).

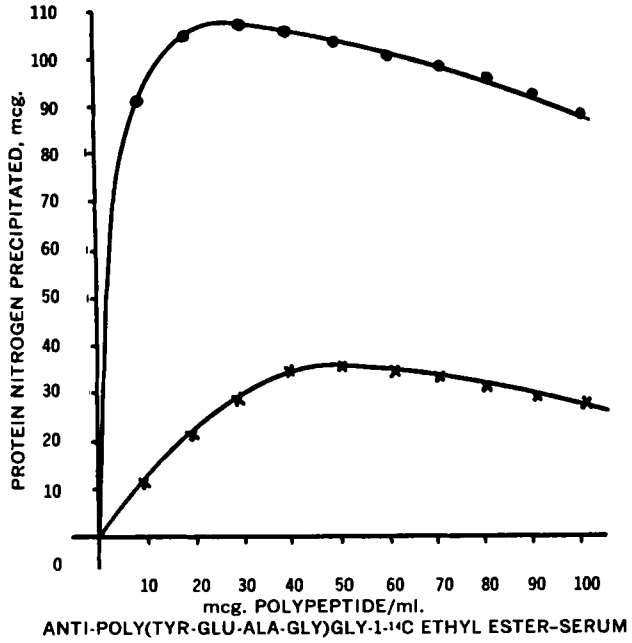

Figure 1—*Precipitin curves. Key:* ●, *Poly(Tyr-Glu-Ala-Gly)$_n$Gly; and* ×, *Poly(Tyr-Glu-Ala-Gly)$_n$Gly.*
Gly

[1] Melting points were taken with a Mel-Temp apparatus and are uncorrected.

Table I—Absorption Data

Polypeptide	Protein Nitrogen (Kjeldahl) Precipitated at Equivalence Point[a], mcg.	Protein Nitrogen (Kjeldahl) Precipitated by I after Absorption[a], mcg.	Protein Nitrogen (Kjeldahl) Precipitated by Polypeptide[a], %
(Tyr–Glu–Ala–Gly)$_n$Gly (I)	106	0	100
(Tyr–Glu–Ala–Gly)$_n$Gly (II) Gly	33	71	32

[a] Per milliliter of anti-poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C ethyl ester-serum.

Anal.—Calc. for $C_{38}H_{53}N_5O_{11}$: C, 60.40; H, 7.06; N, 9.26. Found: C, 60.18; H, 6.99; N, 9.16.

***N*-Benzyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-γ-(*tert*-butyl-glycyl)-L-glutamyl-L-alanylglycine Pentachlorophenyl Ester (VIII)**—To a solution of 7.4 g. (0.0098 mole) of VII in 350 ml. of methanol was added 9.8 ml. of 1 *N* KOH. The solution was stirred for 1 hr. at room temperature and then concentrated under reduced pressure. The residue was flooded with water, acidified with 10% citric acid solution, and extracted into ethyl acetate. This solution was dried (sodium sulfate) and concentrated under reduced pressure to give a solid. To this material dissolved in 150 ml. of methylene chloride were added 2.88 g. (0.0108 mole) of pentachlorophenol and 4.6 g. (0.0108 mole) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide *metho-p*-toluenesulfonate. The mixture was shaken at room temperature for 2 days and then concentrated under reduced pressure to give a solid, which was washed with water and crystallized from methanol to yield 5.2 g. (49%) of the activated ester, m.p. 203.5–205.5°; $[\alpha]_D^{24}$ −15.0° (c 2.66 in dimethylformamide).

Anal.—Calc. for $C_{43}H_{50}Cl_5N_5O_{11}$: C, 52.15; H, 5.09; N, 7.08. Found: C, 51.90; H, 5.00; N, 6.94.

***O*-*tert*-Butyl-L-tyrosyl-γ-(*tert*-butyl)-L-glutamyl-L-alanylglycine Pentachlorophenyl Ester Hydrochloride (IX)**—A suspension of 5.1 g. (0.00515 mole) of the pentapeptide activated ester VIII and 0.7 g. of 10% palladium-on-charcoal in 300 ml. of methanol was treated with 0.188 g. (0.00515 mole) of dry hydrochloric acid in methanol, and the suspension was hydrogenated for 2 hr. The mixture was filtered and the filtrate was concentrated under reduced pressure to give a solid, which was crystallized from methanol–ether to yield 2.3 g. (50%) of the polymerizing unit IX, m.p. 186°; $[\alpha]_D^{24}$ −4° (c 2.50 in dimethylformamide).

Anal.—Calc. for $C_{35}H_{45}Cl_6N_5O_9$: C, 47.10; H, 5.08; N, 7.85. Found: C, 46.85; H, 5.07; N, 8.12.

Poly-(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine Methyl Ester (II)—To a solution of 0.82 g. (0.00815 mole) of triethylamine and 0.585 mg. of glycine methyl ester hydrochloride in 5 ml. of dimethyl sulfoxide was added a solution of 2.1 g. (0.00233 mole) of the polymerizing unit IX in 18 ml. of dimethyl sulfoxide. The mixture was shaken for 1 week and then centrifuged to yield the fully protected polymer. This polymer was washed with three 35-ml. portions of water, three 35-ml. portions of methanol, and three 35-ml. portions of ether and dried to give the fully blocked polymer. This material was treated with 30 ml. of 90% trifluoroacetic acid, stirred for 50 min. at room temperature, and then concentrated under reduced pressure to yield the crude polypeptide II. This material was suspended in 20 ml. of water and dissolved by the addition of 1 *N* NaOH to pH 7.5. The solution was filtered and the filtrate was dialyzed against distilled water overnight, acidified with 6 *N* HCl to pH 2.5, and dialyzed against distilled water for a day. The precipitated polypeptide II was collected by centrifugation and then lyophilized to yield 0.3 g. (27%); the amino acid ratio of an acid hydrolysate was: $Tyr_{0.9}$ $Glu_{1.0}$ $Ala_{1.0}$ $Gly_{2.1}$.

Molecular Weight Determination—The polypeptide II (0.3 g.) was dissolved in 50 ml. of water by the addition of 1 *N* NaOH to pH 7.4. This solution was separated into four different molecular weight ranges by successive diafiltrations through membranes[1]. Each fraction was acidified with 6 *N* HCl, dialyzed, and lyophilized to yield 0.10 g. mol. wt. >50,000, 0.02 g. mol. wt. 20,000–50,000, 0.03 g. mol. wt. 10,000–20,000, and 0.14 g. mol. wt. <10,000.

Immunochemical Procedures—Eight rabbits were treated with I at weekly intervals, using the immunization schedule previously described (3); 25 days after the last injection, all rabbits were bled using the standard heart puncture technique. Serum from each rabbit was tested for the precipitin reaction with the homologous antigen I, and serum from each animal gave a positive precipitin reaction. The serum from each animal was pooled, and this combined serum was used for the following experiments. It was assumed that the antibodies produced by each rabbit after the same time interval was directed against the same antigenic determinants of the antigen I.

Quantitative Precipitin Reactions—To 1-ml. aliquots of the pooled rabbit serum were added incremental amounts of the polypeptide II. Each tube was made up to a total of 2 ml. with buffer (0.1 *M* NaCl–0.05 *M* $NaHCO_3$), incubated for 1 hr. at 37°, and then kept at 4° for 48 hr. The tubes were centrifuged in the cold and the precipitates were washed twice with 1 ml. of buffer (0.05 *M* K_2HPO_4–NaOH), pH 7.0. The total amount of protein that precipitated was estimated by analysis for nitrogen (Kjeldahl). An identical procedure was used for the homologous polymer I, which was run simultaneously with that used for the polypeptide II. The comparative precipitin curves are shown in Fig. 1.

Absorption Studies—The pooled rabbit serum was treated with the equivalence point amount of the heterologous polypeptide II as already described. The precipitate was centrifuged out and the supernatant liquor was poured off into a separate tube. To this was added 30 mcg. of the homologous antigen I. The mixture was incubated at 37° for 1 hr. and then stored at 4° for 48 hr. The precipitate was collected by centrifugation and washed twice with 1 ml. of buffer solution (0.05 *M* K_2HPO_4–NaOH), pH 7.0. The amount of precipitate obtained using this procedure is shown in Table I. A control in which the serum was first absorbed with the homologous antigen I ascertained that the homologous antigen precipitated all of the antibody, since the supernatant liquor gave no further precipitin reaction when 30 mcg. of I was added.

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[1] Diaflo OM 50, UM 20E, and UM 10, Amicon Corp., Lexington, Mass.

Interaction Forces Involved in Wetting of Human Skin

ALLAN ROSENBERG▲, ROBERTA WILLIAMS, and GEORGE COHEN

Abstract □ Wetting of human skin by several liquids was investigated by contact angle measurements. Excised and live skin exhibited similar wetting properties. The skin surface was found to be hydrophobic, with little or no polar interaction occurring between the wetting liquid and the skin surface. The Good–Girafalco–Fowkes–Young equation can be used to predict contact angles of liquids on human skin.

Keyphrases □ Wettability of human skin—hydrophobic interactions, determination of critical surface tension, contact angles predicted □ Skin (human) wettability—hydrophobic interactions, determination of critical surface tension, contact angles predicted □ Surface tension, critical—determination for human skin, used to predict contact angles □ Contact angles of liquids on excised and live human skin—predicted from critical surface tension determinations, used to study wettability

The subject of wetting has been a major concern of surface scientists from both an applied and a fundamental approach. Important industrial applications such as transfer of ink in printing, dirt removal, flotation, and disintegration of tablets all involve wetting phenomena. Fundamental investigations of wetting are concerned with surface energies of solids, surface roughness, forces involved in the wetting process, and thermodynamic considerations. Several reviews of various aspects of wetting are available in the literature (1–5).

Wettability is normally described in terms of contact angles (6) (*i.e.*, the internal angle between the solid surface and a liquid droplet applied to that surface). High contact angles mean poor wetting while low angles signify good wetting. If a liquid spreads on the solid surface, the contact angle is considered zero and complete wetting is said to occur.

Relatively few studies of wetting of human skin have been reported. Adamson *et al.* (7) and Ginn *et al.* (8) reported that water contact angles on clean degreased human skin usually exceed 100°. Additionally, Ginn *et al.* determined the critical surface tension of skin to be 26.8 dynes/cm. using an acetone–water series. More recently, Schott (9) reported contact angles of water on human skin which are somewhat lower compared to those reported previously.

The above work clearly shows that human skin is a hydrophobic surface. The wetting of any surface can be considered as related to a combination of two types of forces operative between the liquid and the surface: polar and nonpolar (10, 11). The relative contribution of each of these forces in human skin has not previously been determined. By measuring contact angles of several liquids of varying polarity and employing the Good–Girafalco–Fowkes–Young equation (GGFY) (10–13), we intend to establish the dominant type of interaction occurring between skin and the wetting liquid, determine the critical surface tension of skin, and predict contact angle values from a knowledge of the critical surface tension of skin and the polar and dispersion components of surface tension of the wetting liquid. We also report that there is no significant difference between wetting properties of excised and live human skin.

Table I—Contact Angles (θ) Obtained on Live and Excised Human Skin[a]

Compound	θ Live, degrees	θ Excised, degrees
Water	108 ± 4.1	107 ± 2.0
Glycerol	86 ± 3.1	91 ± 4.3
Formamide	79 ± 6.9	85 ± 5.4
Diidomethane	55 ± 6.9	54 ± 6.3
Tetrabromoethane	42 ± 4.6	39 ± 4.9
Ethylene glycol	70 ± 4.5	69 ± 3.6
Benzyl alcohol	41 ± 5.1	32 ± 2.6
Tetrachloroethane	0	0
Mineral oil	0	0
Decahydronaphthalene	0	0

[a] Results are quoted ± *SD* of the parameter.

EXPERIMENTAL

Contact angles were measured with a contact angle goniometer[1] equipped with a specially designed finger holder. For live subjects the dorsal areas of the ring and forefingers were used. Female breast skin was employed for excised skin investigations. The excised skin was kept frozen before use.

All measurements were made by the sessile drop method using advancing angles at 25°. For each contact angle determination, the average value of three droplets, reading both sides of the drop, was designated as the contact angle. Generally, 2–10-μl. droplets were employed. Ten subjects were used for live studies, while six pieces of excised skin were employed. Angles obtained in this manner were reproducible to within ±2° for each individual liquid on a specific subject or piece of excised skin.

To obtain contact angles on clean degreased skin, the skin was thoroughly washed with soap and water, rinsed several times with absolute ethanol, rinsed with distilled water, and finally hot air dried. Contact angles of water on skin cleaned in this manner were always greater than 100°.

The purity of the liquids was checked by GC. Surface tensions were measured with a tensiometer[2] using appropriate ring correction factors.

Paraffin[3] [specified as wax, refined, m.p. 56.1–57.1° (133–135°F.)] was used for contact angle determinations. Smooth paraffin surfaces were obtained by melting the wax on glass slides.

RESULTS AND DISCUSSION

***In Vivo–In Vitro* Comparison**—Table I shows a comparison of contact angles of several liquids on clean excised skin with those measured using live skin. Good agreement was obtained, demonstrating that excised skin can be used as a model for studying the wetting properties of living human skin. The use of excised skin can simplify wetting studies since the problem of obtaining and controlling live panels is avoided. Additionally, toxic liquids can be used if excised skin is employed.

[1] Ramé-Hart.
[2] Cenco-Du-Nuöy.
[3] Mobil Oil Corp.

Table II—Dispersion and Polar Components of Total Surface Tension of Acetone–Water Mixtures Calculated from Contact Angles on Paraffin

Weight Percent Acetone	Contact Angle (θ), degrees	cos θ	Total Surface Tension (γ_L), dynes/cm.	Dispersion Component ($\gamma_L D$), dynes/cm.	Polar Component ($\gamma_L P$), dynes/cm.
0.00	108	−0.309	71.3	23.8	47.5
4.77	95	−0.087	56.5	26.1	30.4
9.67	90	0.000	49.6	24.1	25.5
14.58	85	0.087	44.4	22.9	21.5
19.87	77	0.225	41.4	25.2	16.2
36.60	65	0.423	34.2	23.2	11.0
45.97	59	0.515	31.7	22.6	9.1
50.00	53	0.602	30.4	23.3	7.1
60.20	53	0.602	28.9	21.0	7.9

Wetting of Skin—As described by Dann (10, 11), the GGFY equation relates contact angles to various physiochemical parameters as follows:

$$\cos\theta = (-\gamma_L + 2\sqrt{\gamma_S D \gamma_L D} - \pi_S)/(\gamma_L - \pi_L) \quad \text{(Eq. 1)}$$

Here θ is the contact angle, γ_L is the surface tension of the liquid, $\gamma_S D$ is the dispersion force component of the solid, $\gamma_L D$ is the dispersion force component of the liquid, π_S is the reduction in surface energy of the solid resulting from adsorption of vapor from liquid L, and π_L is the reduction in surface energy of the liquid resulting from adsorption of a film of solid material from the solid. As a first approximation, π_S and π_L are assumed to be negligible for low energy solids (10) and are neglected in the calculation.

Dann (10, 11) showed that the determination of critical surface tensions, as obtained by the Zisman method (6), can be misleading as it does not account for the specific type of interaction force involved in the wetting process as used in Eq. 1.

Figure 1 is a Zisman plot (6) for measurements made using an acetone–water series. The critical surface tension for excised skin (γ_C) is determined to be 27.5 dynes/cm., in good agreement with that previously published (8). From contact angle measurements of the acetone–water series on paraffin, the contribution of dispersion and polar forces to the surface tension of each mixture was determined from the following equation:

$$\sqrt{\gamma_L D} = (\cos\theta + 1)\gamma_L/2\sqrt{\gamma_S D} \quad \text{(Eq. 2)}$$

which is a rearranged version of Eq. 1, assuming $\pi_L = \pi_S = 0$

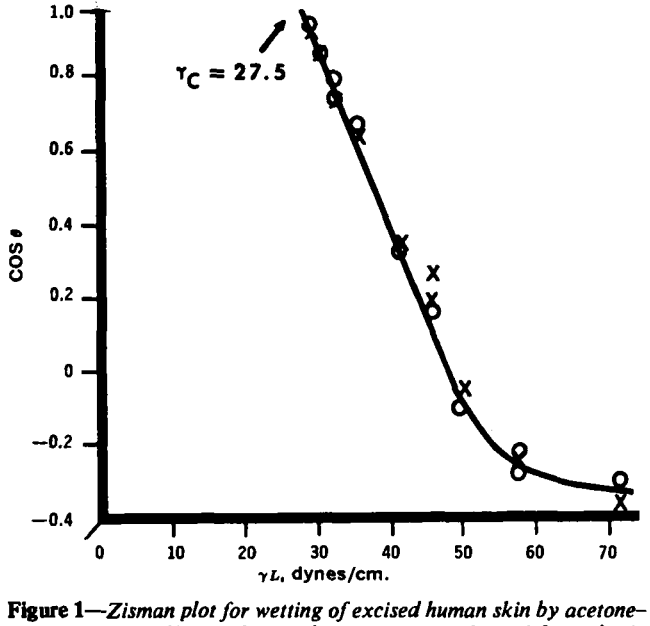

Figure 1—*Zisman plot for wetting of excised human skin by acetone–water mixtures. Extrapolating the curve to cos θ = 1.0 results in γ_C = 27.5 dynes/cm.*

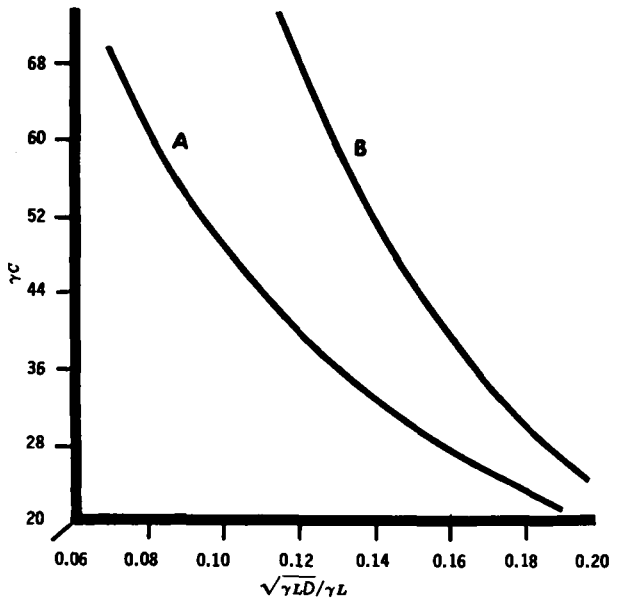

Figure 2—*Critical surface tension conversion plot according to the method of Dann (10). Curve A is obtained using the data in Table II for the acetone–water series. Curve B is constructed assuming a hypothetical series of liquids which have no polar surface energy component, so that $\gamma_L = \gamma_L D$.*

and $\gamma_S D$ for paraffin is 25.5 dynes/cm. (13), and from the relationship (10):

$$\gamma_L = \gamma_L D + \gamma_L P \quad \text{(Eq. 3)}$$

where $\gamma_L P$ is the polar force component of the surface tension. It is assumed that only dispersion forces are operative between paraffin and the liquid series (13). Table II shows the results of these calculations. It can be seen that dispersion components of the series do not vary greatly throughout the entire surface tension range, while the polar component varies considerably. When relating these results to Fig. 1, it is apparent that high contact angles on human excised skin are observed when the water–acetone mixtures have large polar energy contributions to the total surface tension. Thus, it appears that the skin surface has little interaction with polar forces of the liquids under investigation.

Figure 2 is a critical surface tension conversion plot from which, according to the method of Dann (10), $\gamma_S D$ for skin is determined in the following manner.

If γ_C is defined as the point where cos θ equals 1 for a particular liquid series on a particular surface, then at this point Eq. 1 can be given as:

$$1 = -1 + 2\sqrt{\gamma_S D}\,\frac{\sqrt{\gamma_L D}}{\gamma_L} \quad \text{(Eq. 4a)}$$

Table III—Comparison of Observed Contact Angles on Live Skin and Calculated Values Using the GGFY Equation[a]

Compound	γ_L, dynes/cm.	$\gamma_L D$, dynes/cm.	θ Observed, degrees	θ Calculated, degrees
Water	72.0	22.0	108 ± 4	102
Glycerol	62.2	30.4	86 ± 3	85
Formamide	58.2	32.0	79 ± 7	79
Diidomethane	50.6	50.6	55 ± 7	45
Tetrabromo-ethane	49.7	49.7	42 ± 5	43
Ethylene glycol	47.6	27.1	70 ± 5	71
Benzyl alcohol	39.2	34.9	41 ± 5	34
Tetrachloroethane	36.1	36.1	0	0
Mineral oil	31.9	31.9	0	0
Decahydro-naphthalene	30.8	30.8	0	0

[a] Results are quoted ± SD of the parameter.

Table IV—Comparison of Observed and Calculated Contact Angles on Skin for Liquids Having Poorly Determined $\gamma_L D$ Values[a]

Liquid	γ_L, dynes/cm.	$\gamma_L D$[b], dynes/cm.	θ Observed, Excised Skin, degrees	θ Observed, Live Skin, degrees	θ Calculated[c], Skin, degrees
α-Bromonaphthalene	44.5	66.4	43 ± 1.4	44 ± 4.4	34
Acetophenone	39.2	44.5	47 ± 5.1	47 ± 5.6	20
Chlorophenylbenzene	41.7	56.4	37 ± 4.6	43 ± 5.8	28

[a] Results are quoted ± SD of the parameter. [b] From contact angles on paraffin and Eq. 2. [c] From Eq. 1 using $\gamma_S D$ = 37.0 dynes/cm. for skin and assuming $\gamma_L D = \gamma_L$ for all liquids.

or:

$$\frac{\sqrt{\gamma_L D}}{\gamma_L} = \frac{1}{\sqrt{\gamma_S D}} \qquad \text{(Eq. 4b)}$$

Since by definition at γ_C the value of γ_L equals γ_C, the curves in Fig. 2 were obtained by plotting γ_L *versus* $\sqrt{\gamma_L D}/\gamma_L$. Curve A in Fig. 2 was constructed using γ_L and $\gamma_L D$ values obtained for the acetone–water series on paraffin as shown in Table II. Curve B in Fig. 2 represents a hypothetical liquid series of pure hydrocarbons having only dispersion energies so that $\gamma_L = \gamma_L D$. To obtain $\gamma_S D$ for skin, pass a vertical line through curve A at the point where γ_C is 27.5 dynes/cm., the measured value of γ_C for skin using the acetone–water series. Where the line intercepts curve B is the expected value of $\gamma_S D$ for skin, since for the hypothetical liquid series of pure hydrocarbons γ_C is equal to $\gamma_S D$. The $\gamma_S D$ for skin was equal to 37 dynes/cm. Thus, nonpolar liquids ($\gamma_L P$ = 0) having surface tensions less than 37 dynes/cm. should completely wet skin.

Using Eq. 1, with the stated assumptions, values of contact angles on skin were calculated for several organic liquids (Table III). Values of $\gamma_L D$ for each liquid were determined by contact angle measurements on paraffin and use of Eq. 2. Contact angles of each liquid were measured on human skin and, as shown in Table III, the agreement between the measured and calculated angles is satisfactory.

The accuracy of the determination of $\gamma_L D$ values will determine the accuracy of calculated θ values. In some cases, wide variations between calculated and observed θ values for liquids on skin were observed. Table IV shows poor agreement between calculated and observed θ values for α-bromonaphthalene, acetophenone, and chlorophenylbenzene[4] on skin. For these liquids it is seen that $\gamma_L D$ values determined from contact angles on paraffin and Eq. 2 are greater than γ_L values. This is due to the chemical or physical interaction between the liquid and the paraffin surface. Thus, it is clear that reliable $\gamma_L D$ values are essential for the proper use of the GGFY equation.

[4] A mixture of 2-chloro-, 3-chloro-, and 4-chlorophenylbenzene (Aroclor 1232, Applied Science Laboratories, State College, Pa.) was used.

CONCLUSIONS

The results of these studies strongly indicate that little, if any, polar interaction occurs between liquids and clean skin surfaces. The hydrophobic nature of the skin surface is in agreement with the work of Ginn *et al.* (8) and Adamson *et al.* (7). Additionally, the GGFY equation as described by Dann satisfactorily predicts contact angles of liquids on human skin.

A possible explanation of the hydrophobicity of skin is that polar groups present in the α-keratin structure of the stratum corneum are oriented away from the skin–air interface. Scheuplein's (14) hypothesis that detergents may uncoil and extend the α-keratin structure suggests that the coiled, unextended structure could be such that polar groups are unaccessible for polar interactions with liquids in the wetting process.

In summary, it was shown that the GGFY equation satisfactorily describes wetting of skin by several liquids if accurate $\gamma_L D$ values are employed. The observed hydrophobic nature of the skin surface can be attributed to polar groups oriented away from the skin surface.

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Osmotic Properties of Aqueous Solutions Containing Nucleosides

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Abstract □ The thermoelectric osmometer described in earlier publications was used to study the osmotic properties of 2′-deoxyadenosine, 2′-deoxyguanosine, uridine, cytidine, and thymidine in aqueous solutions ranging from 0.01 to 0.2 molal. Changes in resistance of a thermistor, ΔR, represent colligative properties which were plotted against corresponding molal concentrations. All points fell on intersecting straight lines, the points of intersection representing critical concentrations which are analogous to the critical concentrations reported previously for caffeine. This critical concentration is shifted to higher values by the presence of a fixed concentration of another nucleoside. Osmotic coefficients and complexing capacity numbers may be calculated by the methods used for caffeine data.

Keyphrases □ Osmotic properties—aqueous nucleoside solutions, calculation of osmotic coefficients and complexing capacity numbers □ Nucleosides, aqueous solutions—determination of osmotic properties, calculation of osmotic coefficients and complexing capacity numbers □ Complexing capacity numbers—determined for nucleosides in aqueous solutions □ Thermoelectric osmometry—osmotic properties of nucleosides in aqueous solutions

Because of the biological importance of nucleosides, it was decided to extend the thermoelectric osmometry reported for caffeine (1) to include certain nucleosides. Dilute solutions of cytidine, 2′-deoxyadenosine, 2′-deoxyguanosine, thymidine, and uridine in the concentration range between 0.01 and 0.2 molal were studied at 25° separately in pure water and also in dilute solutions of other members of the group.

This paper is concerned primarily with the presentation of experimental data which, like the caffeine data, can be given in terms of the slopes, intercepts, and intersections of straight lines representing the colligative property, ΔR, plotted against molal concentration. All measured points fall on a line within the limit of experimental accuracy. This treatment expedites usual calculations and is essential to show clearly the shift in point of intersection used to calculate complexing capacity numbers similar to those shown for caffeine.

EXPERIMENTAL

Instrumentation—The osmometer used in this work was described previously (1–4). It is essentially an ebulliometer in that condensing vapor elevates the temperature of a sample suspended in air which is saturated with solvent vapor. Solvent is held against the inner wall of the containing cell immersed in a water bath held at constant temperature by means of an air bath and an auxiliary heater. Slow temperature drifts of the order of 0.001° are compensated for by a reference thermistor in a different cell in the water bath.

New circuits developed after this work was completed provide greater convenience and economy (4). However, all of the other essential improvements described were used, but with greater need for manual control of temperature. The older bridge circuit was used throughout without loss of final accuracy. Absolute resistance values are of secondary importance because ΔR is the difference between the resistance of the measuring thermistor when in contact with pure solvent and when in contact with the sample under study. Both solvent and sample are suspended on the same thermistor in the same way.

Table I—Data Falling on Two Intersecting Straight Lines

Variable	Vehicle	Intercept[a], ohms	First Slope, $\Delta R/\Delta M$	Intersection	Second Slope, $\Delta R/\Delta M$
2′-Deoxyadenosine	Water	0.04	927.2	0.0152	597.4
2′-Deoxyadenosine	Thymidine, 0.02 molal	19.16	691.8	0.0360	330.0
2′-Deoxyadenosine	Uridine, 0.02 molal	18.36	767.0	0.0358	378.0
2′-Deoxyadenosine	2′-Deoxyguanosine, 0.01 molal	9.14	672.0	0.0565	342.0
2′-Deoxyguanosine	Water	0.03	925.0	0.0123	565.0
2′-Deoxyguanosine	Cytidine, 0.003 molal	2.72	897.0	0.0155	384.6
2′Deoxyguanosine	2′-Deoxyadenosine, 0.003 molal	2.98	833.8	0.0213	400.0
Uridine	Water	0.02	922.5	0.0568	789.4
Uridine	2′-Deoxyadenosine, 0.015 molal	13.83	799.8	0.1900	651.0
Uridine	2′-Deoxyguanosine, 0.012 molal	10.87	846.4	0.1631	659.5
Cytidine	Water	0.03	923.5	0.0412	823.5
Cytidine	Thymidine, 0.02 molal	18.29	846.4	0.1212	683.5
Cytidine	2′-Deoxyadenosine, 0.015 molal	13.98	805.0	0.1307	657.0
Cytidine	2′-Deoxyguanosine, 0.012 molal	11.08	834.5	0.1285	694.4
Thymidine	Water	0.04	924.4	0.0563	747.1
Thymidine	2′-Deoxyguanosine, 0.012 molal	11.10	811.2	0.1369	620.9
Thymidine	Cytidine, 0.02 molal	18.43	845.6	0.1154	660.0
Thymidine	2′-Deoxyadenosine, 0.015 molal	13.84	783.0	0.1458	604.5

[a] The intercepts are not experimental points.

Solutions—Solutions were made with distilled water on a molal basis. No further purification was carried out on the solid chemicals obtained from commercial sources[1]. However, an assay for percentage of water was made in each case by drying a small sample to constant weight over phosphorus pentoxide in a vacuum drying apparatus at the boiling point of absolute alcohol. The percentage of water found was used to correct the weight of solid used in preparing the various solutions.

As a test of purity, the following molecular weights were calculated by Eq. 4 from the osmotic data given in Table I and compared

[1] Solid chemicals were obtained from Nutritional Biochemicals except for 2′-deoxyadenosine which was obtained from General Biochemicals.

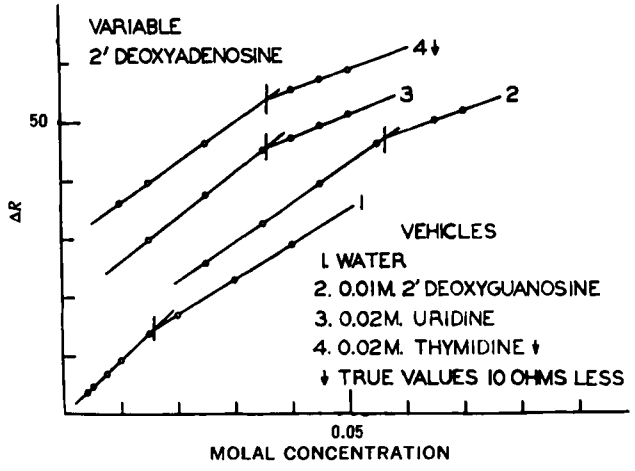

Figure 1—ΔR *in ohms* versus *molal concentration of 2'-deoxyadenosine. Arrow indicates displacement by 10 ohms to avoid crowding.*

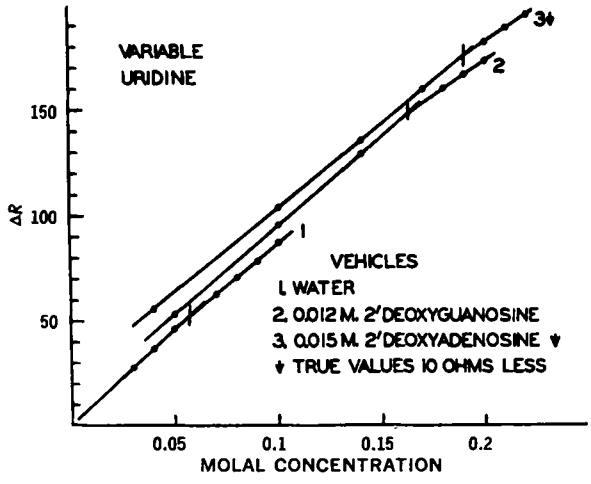

Figure 3—ΔR *in ohms* versus *molal concentration of uridine. Arrow indicates displacement by 10 ohms to avoid crowding.*

with the established formula weights (given in parentheses): 2'-deoxyadenosine (251.2), found: 250.3; 2'-deoxyguanosine (267.2), found: 266.8; cytidine (243.2), found: 243.3; thymidine (242.2), found: 242.0; and uridine (244.2), found: 244.5. Reagent grade (ASC Code 2376) B&A sucrose crystals were used to prepare the solutions used as reference standards.

Standardization—Aqueous solutions of sucrose at 0.01, 0.03, and 0.05 molal were found to have ΔR values of 9.3, 27.5, and 46.2 ohms, respectively. These three values may be treated by the method of least squares to give a slope of 922.3 ohms molal^{-1}. When the experimental values of 93.3 and 187.8 ohms for 0.1 and 0.2 molal, respectively, are corrected by dividing by the corresponding osmotic coefficients given by Robinson and Stokes (5) and included with the above values for the lower concentrations, a slope of 923.7 ohms molal^{-1} is found. This slope was used with all nucleoside data and should not be confused with values given in other papers. Large differences in standardization represent different thermistors from the same shipment. Small differences close to estimated experimental error are caused by slight changes in the cell itself. It is important to restandardize after any change in the instrument.

RESULTS

The experimental data on the nucleosides are given in Figs. 1–5 and are summarized in Table I. The figures present a fair estimate of the accuracy of the measurements and the goodness of fit. Each ΔR value shown is the average of two or more measurements on separate samples of the same solution. The slopes and intercepts shown in Table I were obtained by the method of least squares and are reported to one more significant figure than is probably warranted by the accuracy of the experimental work. This treatment is justified because these values are used in computing molecular weights, osmotic coefficients, and points of intersection. Intercepts are included as a measure of internal consistency.

Table I also provides data needed to calculate osmotic coefficients, ϕ, assuming the validity of the equation:

$$\phi = \Delta R/\Delta R_0 \qquad \text{(Eq. 1)}$$

The following equations can be derived by the application of elementary analytic geometry:

$$\phi = S_1/S_0 \qquad \text{(Eq. 2)}$$

$$\phi = S_1C_1/S_0M + S_2(M - C_1)/S_0M \qquad \text{(Eq. 3)}$$

Equation 2 applies at molal concentrations below the critical concentration (intercept) designated C_1. Equation 3 applies above the critical concentration, where S_2 is the second slope and M is the molal concentration under consideration. In both equations, S_1 is the first slope and S_0 is the slope obtained by standardization with sucrose. In Eq. 1, ΔR_0 is S_0M, and ΔR represents the solution under investigation at the molal concentration, M.

Osmotic coefficients are calculated from Table I by the method already described and are compared with values reported by other workers (6–8). These results and comparisons are given in Table II for selected concentrations and also for the exact concentration

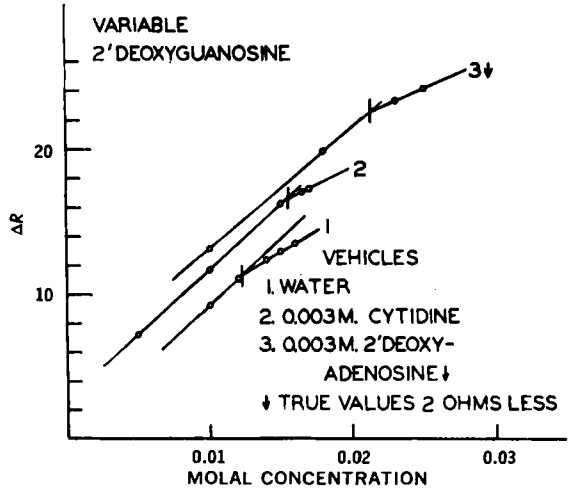

Figure 2—ΔR *in ohms* versus *molal concentration of 2'-deoxyguanosine. Arrow indicates displacement by 2 ohms to avoid crowding.*

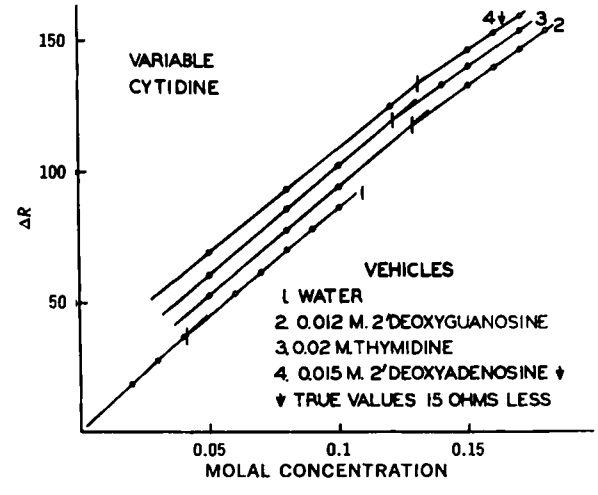

Figure 4—ΔR *in ohms* versus *molal concentration of cytidine. Arrow indicates displacement of 15 ohms to avoid crowding.*

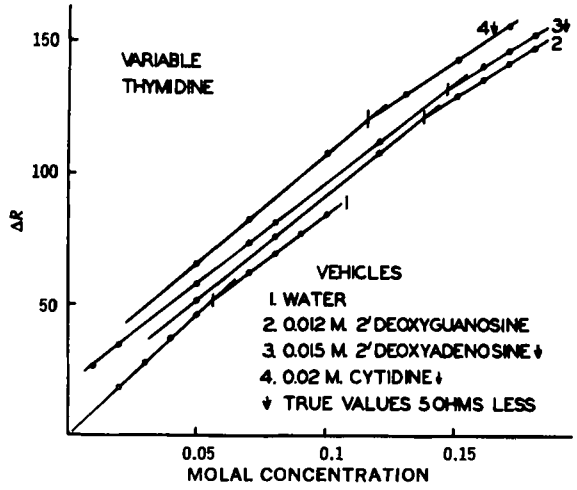

Figure 5—ΔR *in ohms* versus *molal concentration of thymidine. Arrow indicates displacement of 5 ohms to avoid crowding.*

specified by other workers. The earlier work involved curve fitting and was not primarily directed to this region of concentration.

DISCUSSION

Osmolal Concentrations and Molecular Weights—All ΔR values may be converted to osmolal concentration by dividing by the corresponding value for sucrose, which is conveniently expressed as $S_0M = \Delta R_0$, using the symbolism of Eqs. 1 and 3. Below the critical concentration, it may be assumed that dissociation is negligible and that osmolal and molal concentrations are the same. It is, therefore, possible to calculate molecular weights from ΔR values in this region. Assume that any arbitrary value, F, was used for the molecular weight of the solute in calculating a tentative molal concentration, T, from weight, F, of solute in 1 kg. of solvent. On this basis, $\Delta R = S_1T$, $\Delta R_0 = S_0T$, and the osmolal concentration, $\Delta R/\Delta R_0$, the true molal concentration in this region, becomes S_1/S_0 which is F/mol. wt. Therefore, molecular weight is given by the equation:

$$\text{mol. wt.} = F(S_0/S_1) \qquad \text{(Eq. 4)}$$

Equation 4 was used to calculate the molecular weights listed in connection with the preparation of solutions.

Complexing Capacity Numbers—Calculations based on the values given in Table I and the method outlined for caffeine (1) show that, just as for caffeine, the complexing capacity numbers approach integers. However, higher values predominate, especially for the complexing capacity numbers with uridine, cytidine, and thymidine where values of 9, 6, and 6, respectively, are found for 2'-deoxyadenosine and values of 9, 7, and 9, respectively, are found for 2'-deoxyguanosine.

Self-Association—The fact that a critical concentration is observed for all pure substances studied suggests self-association, possibly of the type $nA = A_n$ or $2A = A_2$, $A_2 + A = A_3$, *etc.* By writing appropriate equilibrium expressions, both molal and osmolal concentrations can be generated by assigning reasonable values to K and, for the first type, n also. For each choice, the monomer concentration (A) was varied systematically over a sufficient range to show that calculated osmolal concentrations plotted against calculated molal concentrations did indeed generate curves that approached intersecting straight lines for some values chosen for the parameters. The choice of $n = 4$ and $K = 100$ generates such a curve for equilibria of the first type, and the choice of $K_1 = 1$, $K_2 = 2$, and $K_3 = 4$ serves approximately for the multiple-equilibrium hypothesis. These calculations were greatly facilitated by the use of a computer[2].

It must not be assumed that the authors are offering evidence favoring specific equilibrium constants or models. It is quite possible that combinations occur that are not approximated by either hypothesis considered here. However, it is important to show that the presentation of accurate measurements by giving slopes and intersection is not inconsistent with more conventional treatment involving a consideration of complex equilibria.

[2] IBM 360 computer programmed by Mr. Phillip Johnson of the University of California San Francisco Information Service.

Table II—Comparison between Calculated Osmotic Coefficients (ϕ) and Values Reported in the Literature at 25°

Molal Concentration	ϕ Calc.	ϕ Lit.
	Deoxyadenosine in Water	
0.005	1.015	—
0.010	1.014	—
0.015	1.009	—
0.01725	0.965	0.84 ± 0.07[a]
0.020	0.921	—
0.025	0.867	0.900 ± 0.01[b]
0.030	0.830	—
0.0345	0.806	0.760 ± 0.04[a]
0.040	0.785	—
0.050	0.757	0.800 ± 0.005[b]
	2'-Deoxyguanosine in Water	
0.010	1.006	—
0.012	1.006	—
0.014	0.957	—
0.015	0.934	—
0.016	0.914	—
	Uridine in Water	
0.030	1.001	—
0.040	1.001	—
0.050	1.001	0.969[c]
0.060	0.993	—
0.070	0.973	—
0.080	0.958	—
0.094	0.943	0.952, 0.962[c]
0.095	0.942	0.933[c]
0.096	0.941	0.937[c]
0.100	0.938	0.943[c]
	Cytidine in Water	
0.030	1.002	—
0.040	1.002	—
0.050	0.983	0.967[c]
0.070	0.957	—
0.080	0.949	—
0.0941	0.941	0.941[c]
0.0948	0.940	0.939[c]
0.0955	0.940	0.936[c]
0.1000	0.938	0.935[c]
	Thymidine in Water	
0.040	1.003	—
0.050	1.003	—
0.070	0.965	—
0.090	0.931	—
0.100	0.918	0.905[b]

[a] From *Reference 8.* [b] From *Reference 7.* [c] From *Reference 6.*

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Chemical Constituents of the Gentianaceae V: Tetraoxygenated Xanthones of *Swertia chirata* Buch.-Ham.

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Abstract □ Nine tetraoxygenated xanthones [1,5,8-trihydroxy-3-methoxyxanthone (I), 1-hydroxy-3,5,8-trimethoxyxanthone (II), 1-hydroxy-3,7,8-trimethoxyxanthone (III), 1,8-dihydroxy-3,5-dimethoxyxanthone (IV), 1,8-dihydroxy-3,7-dimethoxyxanthone (V), 1,3,6,7-tetrahydroxyxanthone-C_2-β-D-glucoside (mangiferin, VI), 1,3,8-trihydroxy-5-methoxyxanthone (VII), 1,3,5,8-tetrahydroxyxanthone (VIII), and 1,3,7,8-tetrahydroxyxanthone (IX)] were isolated from the roots and aerial parts of *Swertia chirata* Buch.-Ham. (Gentianaceae) collected from Nepal and India. The identity of the xanthones was established by direct comparison with reference materials in most cases, preparation of derivatives, and spectral evidence (UV, IR, proton magnetic resonance, and mass spectrometry). Among these xanthones, II was not encountered before in nature and VI was found for the first time in the genus swertia. The biogenetic significance of the co-occurrence of several tetraoxygenated xanthones, of varying oxygenation patterns, in absence of the "standard" 1,3,5- and 1,3,7-trioxygenated xanthones in *S. chirata* and in related species is appraised. In addition to the tetraoxygenated xanthones (I–IX), a number of heterosides, triterpenes, and monoterpene alkaloids were isolated from this plant. Preliminary pharmacological screening of the total xanthones of *S. chirata* indicated that the medicinal properties ascribed to the plant extracts were due to these constituents.

Keyphrases □ *Swertia chirata* Buch.-Ham. (Gentianaceae)—isolation, identification of nine tetraoxygenated xanthones □ Xanthones, tetraoxygenated—isolation, identification from *Swertia chirata* Buch.-Ham. (Gentianaceae) □ Medicinal plants—isolation, identification of tetraoxygenated xanthones from *Swertia chirata* Buch.-Ham.

Swertia chirata Buch.-Ham. (Gentianaceae) is widely distributed in India in the temperate Himalayas between 4000 and 10,000 ft., from Kashmir to Bhutan, and in Khasia Hills between 4000 and 5000 ft. (1). It also grows abundantly in Nepal. The plant is well known for its uses in the Indian system of medicine for a variety of purposes (1, 2). The extract of the plant is used as a bitter stomachic, a febrifuge, an anthelmintic, a remedy for scanty urine, in epilepsy, and for certain types of mental disorders. The total annual requirement of this pharmacopeial drug in India is about 400 quintals (3).

Previous phytochemical investigation by Dalal and Shah (4) reported the presence of only one xanthone, 1,8-dihydroxy-3,5-dimethoxyxanthone, in the whole plant. But no attempt was made to determine the active principle of this vegetable drug. Interest in the detailed investigation of this plant was piqued for two reasons: (*a*) xanthone-bearing plants generally elaborate multiple xanthones (5), and (*b*) in *Calophyllum inophyllum* L. (Guttiferae), variations in the types of xanthonic constituents were recorded (6–8) due to ecological variations. In the present study, *S. chirata* plants[1] were collected from Nepal and India to test these possibilities.

EXPERIMENTAL[2]

The general procedure described under *Isolation of Xanthones from Aerial Parts of* S. chirata was followed for the isolation of xanthones from the roots. The other chemical constituents were isolated following a procedure shown in Scheme I.

Isolation of Xanthones from Aerial Parts of *S. chirata*—Dried and milled aerial parts of *S. chirata* (1 kg.) were continuously extracted (soxhlet) for 20 hr. with petroleum ether (60–80°), and the defatted plant material was subsequently extracted (20 hr.) with ethanol. The two extracts were separately processed.

Treatment of Petroleum Ether Extract—The petroleum ether extract was concentrated (about 250 ml.) under reduced pressure, and the concentrate was kept overnight at ordinary temperature when a yellow amorphous solid (Fraction A) separated. The solid was collected by filtration, and the mother liquor was evaporated to dryness (Fraction B).

Separation of Xanthones Present in Fraction A—Fraction A (1.8 g.) was dissolved in ether (500 ml.), and the phenolic and nonphenolic components were separated by extraction with aqueous

[1] The plant material of Indian origin was a gift from Dr. S. P. Wahi, Department of Pharmaceutics, Banaras Hindu University, and that from Nepal was supplied by Mr. R. A. Panjiar, Janakpur Dhum, Nepal. Voucher specimens have been preserved at the Department of Pharmaceutics, Banaras Hindu University, Varanasi-5, India.

[2] Melting points were determined on a Toshniwal melting-point apparatus, in open capillaries, and are uncorrected. UV spectra were recorded in a Cary 14 spectrophotometer in aldehyde-free ethanol (95%). IR spectra were recorded with Perkin-Elmer 237/257 instruments in KBr and mineral oil. Proton magnetic resonance (PMR) spectra were obtained on a Varian A-60 D spectrometer, using deuteriochloroform and dimethyl sulfoxide-d_6 as the solvents. Mass spectra were determined on an AEI MS-9 instrument operated at 70 ev. Combustion analyses were performed by the Central Drug Research Institute, Lucknow, India. Separation by column chromatography was carried out by using silica gel (60–120 mesh, British Drug Houses), and layer chromatographic experiments were conducted with silica gel G (E. Merck). Four solvent systems [benzene–acetic acid (60:1, Solvent 1), chloroform–benzene (5:2, Solvent 2), chloroform–benzene (1:1, Solvent 3), and *n*-butyl alcohol–acetic acid–water (4:1:2, Solvent 4)] were used as the developers, and iodine vapor was used for staining purposes.

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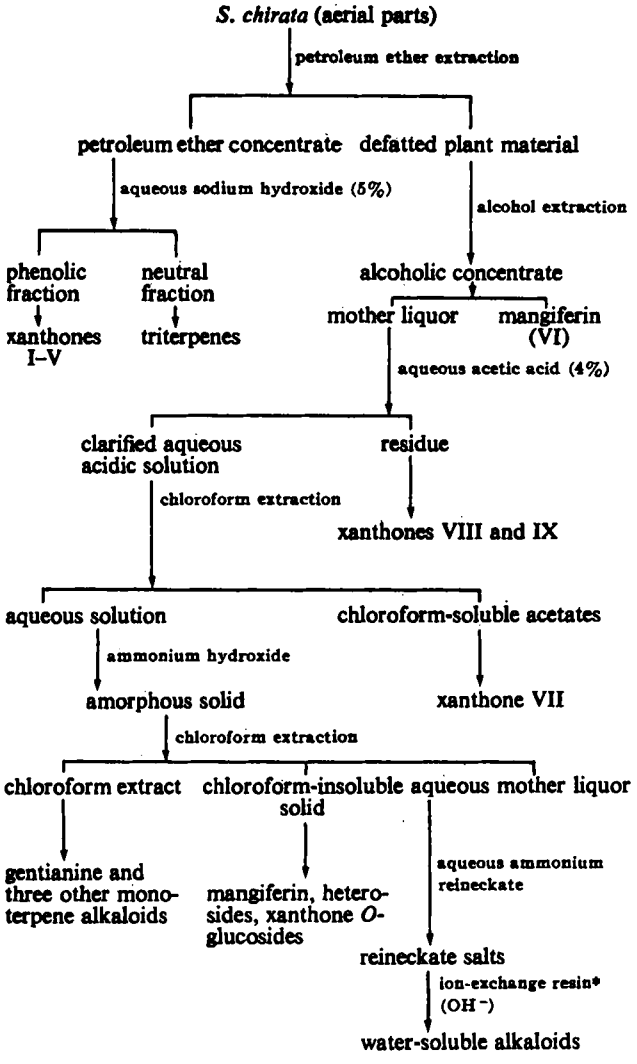


Scheme I—Isolation of chemical constituents of S. chirata (**Amberlite IRA 400*)

sodium hydroxide (5%, four 25-ml. portions). The phenolic constituents from the alkaline aqueous extracts were liberated with hydrochloric acid (2 *N*) and then extracted with chloroform (three 50-ml. portions). The neutral fraction, obtained from the ethereal layer, consisted mainly of triterpenes and was kept aside. The phenolic constituents (118 mg.) showed one major spot and one minor spot on analytical thin-layer chromatoplates. The major component was purified by column chromatography (silica gel, 100 g.), using petroleum ether and benzene as the eluents. The petroleum ether eluates did not give any solid, and the small amount of the gummy material obtained was not processed further.

Xanthone I (1,5,8-Trihydroxy-3-methoxyxanthone)—The benzene eluates, on concentration, gave a yellow solid (52 mg.), which crystallized from ethanol as yellow shining needles, m.p. 271° [lit. (9) m.p. 270–271°]; R_f 0.5 (Solvent 2); ν_{max} (KBr): 3400, 1668, 1628, 1610, 1045, and 995 cm.$^{-1}$.

The 5,8-dimethyl ether, prepared with ethereal diazomethane, crystallized from alcohol as pale-yellow needles, m.p. and mixed m.p. 205°.

Separation of Xanthones Present in Fraction B—Fraction B (0.8 g.) was dissolved in ether (300 ml.), and the phenolic and nonphenolic constituents were separated as described for Fraction A. The phenolic constituents (248 mg.), obtained as a brown gum, were dissolved in chloroform (15 ml.) and chromatographed over silica gel (250 g.). Elution was carried out with petroleum ether (2.5 l.) and benzene (4 l.). The eluates, on evaporation, gave amorphous solids.

The solid (132 mg.), obtained from the petroleum ether eluates, showed several spots on analytical thin-layer chromatoplates. It was dissolved in benzene (10 ml.) and again chromatographed over silica gel (150 g.). The middle petroleum ether–benzene (1:9) eluates showed two major spots on analytical thin-layer plates. These were separated by preparative layer chromatography, using Solvent 3 as the developer.

Xanthone II (1-Hydroxy-3,5,8-trimethoxyxanthone)—The yellow upper zone from the preparative layer chromatography was scraped and eluted with chloroform. Evaporation of the solvent gave a solid (61 mg.), which crystallized from ethanol as pale-yellow needles, m.p. and mixed m.p. with an authentic synthetic sample (12) 205°; ν_{max} (KBr): 3428 (broad), 2912, 2858, 1662, 1615, 1598, and 1060 cm.$^{-1}$.

Xanthone III (1-Hydroxy-3,7,8-trimethoxyxanthone)—The deep-yellow lower layer, from the preparative layer chromatography, gave yellow needles from ethanol, m.p. and mixed m.p. with authentic decussatin (10) 148–149°. The IR spectra were also superimposable.

The solid (124 mg.), obtained from the benzene eluates (Fraction B), showed several spots on analytical thin-layer chromatoplates. It was dissolved in chloroform (10 ml.) and rechromatographed on silica gel (150 g.). Petroleum ether, benzene, and different proportions of their mixtures were used as the eluents. The petroleum ether–benzene (1:1) fraction showed two major spots on TLC, which were separated by preparative layer chromatography using Solvent 3 as the developer.

Xanthone IV (1,8-Dihydroxy-3,5-dimethoxyxanthone)—The yellow upper zone from the layer chromatography afforded a solid (48 mg.), which crystallized from ethanol as yellow needles, m.p. 185–186° [lit. (9) m.p. 185–186°]; R_f 0.68 (Solvent 3); ν_{max} (KBr): 3438, 2925, 2855, 1688, 1632, 1600, 1050, and 978 cm.$^{-1}$.

The 8-methyl ether, prepared with ethereal diazomethane, crystallized from ethanol as pale-yellow needles, m.p. and mixed m.p. with xanthone II 205°.

Xanthone V (1,8-Dihydroxy-3,7-dimethoxyxanthone)—The yellow lower layer from the preparative layer chromatography afforded a solid (21 mg.), which crystallized from ethanol as bright-yellow needles, m.p. 175–176°; R_f 0.36 (Solvent 1); co-TLC with authentic 1,8-dihydroxy-3,7-dimethoxyxanthone (10) showed a single spot having the same R_f value. The 8-methyl ether, prepared with ethereal diazomethane, crystallized from ethanol as yellow needles, m.p. and mixed m.p. with decussatin (10) 148–149°.

Treatment of Alcoholic Extract—The alcoholic extract of the defatted plant material was concentrated under reduced pressure, and the concentrate was allowed to stand at ordinary temperature overnight. The precipitated dull-yellow solid (1.2 g.) was collected by filtration.

Xanthone VI (1,3,6,7-Tetrahydroxyxanthone-C$_2$-β-D-glucoside)—The above solid crystallized from dioxane as yellow needles, m.p. 270–271°. The mixed melting point with authentic mangiferin (10), m.p. 270–271°, remained undepressed. Co-TLC with mangiferin showed a single spot having the same R_f value; their IR spectra were also superimposable.

Mangiferin heptaacetate, prepared with boiling acetic anhydride–pyridine, crystallized from chloroform–hexane (1:4) as colorless needles, m.p. 214–216° [lit. (11) m.p. 228–230°, 141°]. The heptaacetate was also prepared with acetic anhydride and sulfuric acid (1 drop) at ordinary temperature. The derivative crystallized from chloroform–hexane as colorless microcrystals, m.p. 212–214°. The mixed melting point of the heptaacetates, prepared under basic and acidic conditions, remained undepressed. The heptaacetate did not give any color with ferric chloride. The IR, PMR, and mass spectral data of the compound were also consistent with the assigned structure.

The alcoholic mother liquor, after separation of mangiferin, was concentrated to a syrupy liquid. It was poured into aqueous acetic acid (4%, 400 ml.). The mixture was kept at ordinary temperature overnight when an amorphous solid separated. It was filtered and the solid was extracted with chloroform. The chloroform-insoluble solid was processed for polyhydroxylated xanthones (Fraction C). The chloroform-soluble fraction was combined with the chloroform-soluble acetates. The clarified aqueous acidic solution was extracted with chloroform (four 100-ml. portions) to remove the chloroform-soluble acetates. The combined chloroform extracts were washed with water, dried (anhydrous sodium sulfate), and evaporated to give a yellow solid (82 mg.).

Xanthone VII (1,3,8-Trihydroxy-5-methoxyxanthone)—The above solid was triturated with ethanol when a further crop (18 mg.) of mangiferin (VI) was obtained from the alcohol-insoluble frac-

Table I—UV Spectral Data of the Xanthones[a] of *S. chirata*

Xanthone	λ_{max}^{EtOH}, nm. (log ε)
I	220 (4.28), 240 (4.15), 255 (4.20), 277 (4.10), 305–310 sh (3.71), 329 (3.92)
II	220 (4.08), 230–235 sh (4.15), 250 (4.40), 274 (3.92), 332 (3.88)
IV	235 (4.30), 251 (4.42), 275 (4.21), 295–297 (3.80), 332 (3.98)
VII	220 (4.20), 252 (4.32), 276 (4.18), 305–310 sh (3.73), 339 (4.08)
VIII	239 (4.28), 267 (4.52), 332 (4.10), 390 (3.88)

[a] UV data of other xanthones were previously recorded (10).

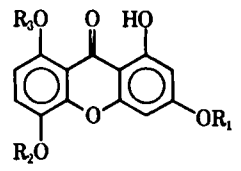
tion. The alcohol-soluble fraction, on concentration, afforded pale-yellow needles, m.p. 263–265° [lit. (12) m.p. 263–264°]; R_f 0.48 (Solvent 2); ν_{max} (mineral oil): 3435, 3082, 1652, 1630, 1610, 1592, 1508, 1262, 1240, 1202, 1180, and 1150 cm.$^{-1}$.

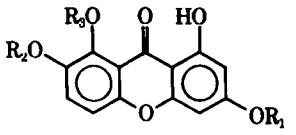
The 3,8-dimethyl ether, prepared with ethereal diazomethane, crystallized from ethanol as light-yellow needles, m.p. and mixed m.p. with xanthone II 205°.

Polyhydroxyxanthones (Xanthone VIII and Xanthone IX)—The chloroform-insoluble solid (Fraction C) (1.34 g.) showed several spots on TLC plates (Solvent 4). Attempts to separate them by preparative layer chromatography failed. A portion of the solid (about 0.2 g.) was methylated with ethereal diazomethane. The ether-soluble fraction, consisting of a mixture of methyl ethers, was subjected to preparative layer chromatography using Solvent 3 as the developer when two major zones were separated. The upper zone afforded 1-hydroxy-3,5,8-trimethoxyxanthone (II), while the lower zone gave 1-hydroxy-3,7,8-trimethoxyxanthone (III). No spot corresponding to 1-hydroxy-3,6,7-trimethoxyxanthone or the methyl ethers of 1,3,5- or 1,3,7-trioxygenated xanthones could be detected in the presence of authentic markers. Complete demethylation of the two methyl ethers with hydrobromic acid (48%) afforded the respective tetrahydroxyxanthones—*viz.*, 1,3,5,8-tetrahydroxyxanthone (VIII) and 1,3,7,8-tetrahydroxyxanthone (IX). The natural mixture of the polyhydroxylated xanthones, present in Fraction C, was subjected to cochromatography with the two tetrahydroxyxanthones (VIII and IX) when their presence in the mixture was detected. The appropriate fractions from the natural mixture were separated by preparative chromatography and examined spectrometrically (Tables I–III), which established their identities.

RESULTS AND DISCUSSION

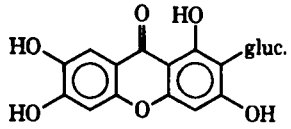
Nine tetraoxygenated xanthones (I–IX) were isolated from the aerial parts of *S. chirata* Buch.-Ham. (Gentianaceae). Isolation and purification of the compounds involved solvent extraction, fractional crystallization, repeated column and preparative layer chromatography, and preparation of derivatives where possible. The identity of the xanthones was established by direct comparison (mixed melting point, co-TLC, and superimposable IR spectra) with authentic reference materials and from spectral (UV, IR, PMR, and mass spectrometry) evidence (Tables I–III). The characterization of the xanthones is described here in the order of their isolation from the plant extract.





I: $R_1 = CH_3$; $R_2 = R_3 = H$
II: $R_1 = R_2 = R_3 = CH_3$
IV: $R_1 = R_2 = CH_3$; $R_3 = H$
VII: $R_1 = R_3 = H$; $R_2 = CH_3$
VIII: $R_1 = R_2 = R_3 = H$

III: $R_1 = R_2 = R_3 = CH_3$
V: $R_1 = R_2 = CH_3$; $R_3 = H$
IX: $R_1 = R_2 = R_3 = H$



VI

Xanthone I—This xanthone, $C_{14}H_{10}O_6$ (M^+, 274), m.p. 271°, formed a dimethyl ether with ethereal diazomethane. The UV absorption spectrum of the xanthone (Table I) indicated its close similarity with 1,3,5,8-tetraoxygenated xanthones (9). The UV spectrum remained unchanged in the presence of sodium acetate. It showed one methoxyl group and four aromatic protons in its PMR spectrum (Table II). The aromatic protons appeared as *meta* and *ortho* split doublets associated with H-2, H-4, H-6, and H-7 protons, respectively. A broad two-proton signal appeared at 13.5 p.p.m. and was assigned to the chelated 1- and 8-OH protons. The mass spectrum of the compound showed, aside from the molecular ion as the base peak, a significant peak due to the fragment ion M − 29 (Table III) associated with the loss of CHO from the C_3—OH group (10). On the basis of these data, together with its insolubility in 5% aqueous sodium carbonate, xanthone I is identified as 1,5,8-trihydroxy-3-methoxyxanthone.

Xanthone II—This xanthone, $C_{16}H_{14}O_6$ (M^+, 302), m.p. 205°, formed a monomethyl ether with dimethyl sulfate and potassium carbonate. It remained unchanged upon treatment with ethereal diazomethane. The UV absorption spectrum of the compound indicated its close similarity with 1,3,5,8-tetraoxygenated xanthones (9). In the PMR spectrum of the xanthone, nine protons due to three methoxyl groups and four aromatic protons appeared as *meta* and *ortho* split doublets due to H-2, H-4, H-6, and H-7 protons, respectively. A one-proton signal appeared at 13.33 p.p.m., which remained unchanged upon treatment with deuterium oxide, and was ascribed to the strongly chelated 1-OH. The mass spectrum of the xanthone showed, aside from the molecular ion peak, significant peaks at *m/e* 287 (M − 15) and 259 (M − 43), associated with the losses of the CH_3 radical and C_2H_3O complex from the molecular ion. On the basis of these observations, xanthone II is identified as 1-hydroxy-3,5,8-trimethoxyxanthone. This conclusion was established by a direct comparison with an authentic synthetic sample.

Xanthone III—This xanthone, $C_{16}H_{14}O_6$ (M^+, 302), m.p. 148–149°, was found to be identical with decussatin in all respects (10).

Xanthone IV—Xanthone IV, $C_{15}H_{12}O_6$ (M^+, 288), m.p. 185–186°, is a dihydroxydimethoxyxanthone since it formed a diacetate and

Table II—PMR Spectral Data[a] of the Xanthones[b] of *S. chirata*

Xanthone	Chelated Hydroxyl	Methoxyl Protons	H-2	H-4	H-5	H-6	H-7	H-8
I[c]	13.5 (broad)	3.90 (3H)	6.32/6.29	6.58/6.54	—	7.38/7.22	6.75/6.60	—
II[d]	13.33 (1-OH)	4.0–3.95 (9H)	6.48/6.43	6.65/6.60	—	7.40/7.24	6.88/6.72	—
IV[d]	11.96, 11.33 (1- and 8-OH)	4.0–3.95 (6H)	6.42/6.37	6.64/6.60	—	7.40/7.24	6.84/6.68	—
VII[c]	13.45 (broad 1-OH)	3.90 (3H)	6.30/6.26	6.58/6.55	—	7.33/7.17	6.74/6.59	—

[a] The signals (in parts per million) were recorded from tetramethylsilane. [b] PMR data of other xanthones were previously recorded (10). [c] Solvent dimethyl sulfoxide-d_6. [d] Solvent deuteriochloroform.

Table III—Mass Spectral Data of the Xanthones[a] of *S. chirata*

Xanthone	Molecular Ion, *m/e* (%)	Significant Peaks, *m/e* (%)	Metastable Peaks, *m/e* Calc.	Found
I	274 (100)	259 (3), 246 (7), 245 (17), 231 (14), 217 (8), 216 (7)	$274 \xrightarrow{m^*} 245$ 219;	219
II	302 (100)	287 (18), 273 (7), 259 (8), 231 (3)	$302 \xrightarrow{m^*} 287$ 272.7;	273
IV	288 (100)	273 (22), 258 (5), 245 (7), 230 (16)	$288 \xrightarrow{m^*} 273$ 258.7;	259
VII	274 (100)	259 (14), 231 (18), 203 (8)	$274 \xrightarrow{m^*} 259$ 244.8;	245
VIII	260 (100)	242 (8), 232 (7), 231 (18), 203 (6)		

[a] Mass spectral data of other xanthones were previously recorded (10).

a monomethyl ether (CH_2N_2). The UV spectrum of the xanthone showed its close similarity with 1,3,5,8-tetraoxygenated xanthones (9). The UV spectrum remained unchanged in the presence of sodium acetate. It showed two methoxyl groups and two *meta* and two *ortho* split doublets in its PMR spectrum. These data and its insolubility in 5% sodium carbonate located one of the methoxyl groups at C_3. The abundance of the M − 15 and M − 43 peaks in the mass spectrum of the xanthone (Table III) is consistent (10) with the location of the other methoxyl group at C_5. The monomethyl ether of the xanthone was identical with xanthone II. On the basis of these observations, xanthone IV is identified as 1,8-dihydroxy-3,5-dimethoxyxanthone.

Xanthone V—This xanthone, $C_{16}H_{12}O_6$ (M^+, 288), m.p. 175–176°, is a dihydroxydimethoxyxanthone. Its UV absorption spectrum was similar to those of 1,3,7,8-tetraoxygenated xanthones (10. 12). The UV spectrum remained unchanged in the presence of sodium acetate. The PMR spectrum of the xanthone showed two methoxyl groups and four aromatic protons associated with H-2, H-4, H-5, and H-6 (10). The mass spectrum of the xanthone indicated that the two methoxyl groups are at the C_3 and C_7 positions (10). This conclusion was confirmed by direct comparison of its monomethyl ether with decussatin (10); they were identical. Xanthone V is, therefore, identified as 1,8-dihydroxy-3,7-dimethoxyxanthone.

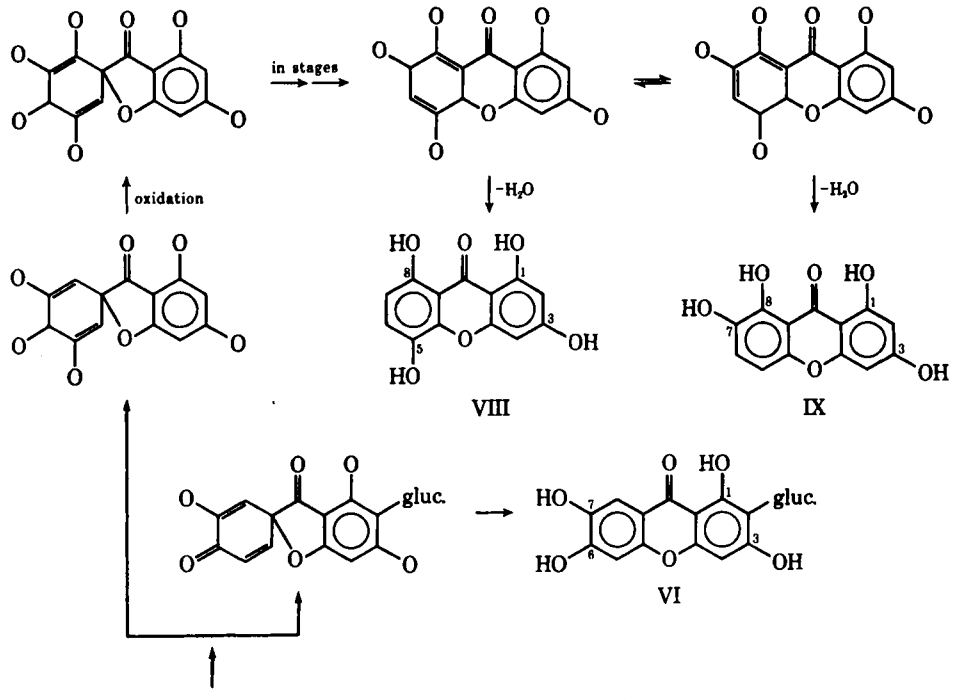
Xanthone VI—Xanthone VI, $C_{19}H_{18}O_{11}$ (M^+, 422), m.p. 270–271°, was identical with mangiferin in all respects (10).

Despite several reports in the literature (11, 12, 14) that mangiferin gave an octaacetate under certain experimental conditions, the present investigation conclusively proved that the acetyl derivative formed, under both basic and acidic conditions, was only the heptaacetate. Furthermore, no trace of isomangiferin, which is commonly known to accompany mangiferin in nature (13), was observed in *S. chirata*.

Xanthone VII—This xanthone, $C_{14}H_{10}O_6$ (M^+, 274), m.p. 263–265°, is a trihydroxymonomethoxyxanthone since it formed a dimethyl ether with ethereal diazomethane and showed one chelated hydroxyl, one methoxyl, and four aromatic protons in its PMR spectrum. The UV spectrum of the xanthone was closely similar to those of 1,3,5,8-tetraoxygenated xanthones. There was a bathochromic shift of the longer wavelength maximum by about 20 nm. in the presence of sodium acetate. The xanthone was also soluble in 5% aqueous sodium carbonate. These properties are consistent with a free C_3-OH group in the molecule. The xanthone exhibited abundant fragment ions at *m/e* 259 (M − 15) and 231 (M − 43) in its mass spectrum (Table III), which located the methoxyl group at C_5. On the basis of these data, xanthone VII is identified as 1,3,8-trihydroxy-5-methoxyxanthone.

Xanthones VIII and IX—The tetrahydroxyxanthones (VIII and IX) could not be separated from the mixture of polyhydroxyxanthones. Methylation with diazomethane and preparative chromatography provided separation of the corresponding trimethyl ethers, which were identified as 1-hydroxy-3.5,8-trimethoxyxanthone and 1-hydroxy-3,7,8-trimethoxyxanthone by direct comparison with reference samples. Complete demethylation of the methyl ethers afforded the tetrahydroxyxanthones as the pure entities. The natural mixture of the polyhydroxyxanthones from *S. chirata* was shown to contain these components by co-TLC and preparative layer chromatography. Comparison of the spectral properties of these compounds finally established their structures as 1,3,5,8-tetrahydroxyxanthone (VIII) and 1,3,7,8-tetrahydroxyxanthone (IX).

This study was the first demonstration of the occurrence of 1-hydroxy-3,5,8-trimethoxyxanthone (II) in nature and of mangiferin



Scheme II—Spiran intermediates in the biogenesis of tetraoxygenated xanthones of S. chirata

(VI) in the genus swertia. Mangiferin was encountered only once before in this family (Gentianaceae) in *Canscora decussata* Schult (10), along with other polyoxygenated xanthones (14). This finding is of considerable systematic significance since mangiferin has a uniquely interesting taxonomic character; both in its distribution and biogenesis, it seems to be more closely related to the flavonoids than to other xanthone derivatives (5).

The roots of *S. chirata* contained the same tetraoxygenated xanthones as in the aerial parts, but the content of mangiferin was less in these parts. No qualitative nor quantitative difference was observed in the xanthonic constituents in *S. chirata* plants collected from Nepal and India. The content of mangiferin declined upon preservation of the plants.

Despite the abundance of the tetraoxygenated xanthones in *S. chirata*, the corresponding "standard" 1,3,5- and 1,3,7-trioxygenated patterns are completely missing from this plant. In the review of Carpenter *et al.* (5), *S. chirata* was wrongly reported to contain 1,3,7-trioxygenated xanthones, since in the original paper by Dalal and Shah (4) the plant was reported to contain only 1,8-dihydroxy-3,5-dimethoxyxanthone. In other members of the genus swertia, no trioxygenated xanthone was encountered (15). This is particularly germane to the biosynthesis of the 1,3,5,8- and 1,3,7,8-tetraoxygenated xanthones of swertia plants. The tetraoxygenated xanthones are envisioned as arising from the standard trioxygenated (1,3,5- and 1,3,7-) xanthones by further oxidation at one of the activated sites (C_6 or C_8). The absence of the trioxygenated xanthones and also of the 1,3,5,6-tetraoxygenated patterns in the members of swertia returns the question to a stand-off. It remains to be seen whether these compounds actually represent different modes of cyclization in different taxa, *e.g.*, the coupling route (18, 19) or the spiran intermediate proposed by Gottlieb (20), or whether there is a single path clouded by branching and/or adventitious oxidation. A probable route, involving spiran intermediates, is proposed for the genesis of the tetraoxygenated xanthones and of mangiferin present in *S. chirata*. The general scheme (Scheme II) could also account for the genesis of mangiferin and the more complex polyoxygenated xanthones co-occurring in *C. decussata* (10, 16).

In addition to the tetraoxygenated xanthones, several heterosides, triterpenes, and monoterpene alkaloids were isolated (Scheme I) from the roots and the aerial parts of *S. chirata*. Structure elucidation of these constituents is currently underway.

The results of detailed pharmacological screening of mangiferin was previously reported (21). Preliminary screening of the total xanthones of *S. chirata* showed definite signs of CNS depression in albino mice and rats. The effect was manifested by initial transient hyperactivity followed by moderate to deep depression, potentiation of hexobarbital sleeping time (by about 145%), and antagonism to 5-methoxy-*N*,*N*-dimethyltryptamine (22). Hydrocholeretic, cardiostimulant, and anticonvulsant effects were also observed. These activities were determined according to the methods reported earlier (21). The percentage protection offered by the total xanthones of *S. chirata* against convulsions produced by the administration of pentylenetetrazol was taken as the anticonvulsant activity and was found to be about 65%. The total heterosides showed only feeble activities with these parameters. These observations seem to indicate that the curative properties ascribed to the *S. chirata* plant extracts, in the Indian system of medicine (1, 2), are due to the contained tetraoxygenated xanthones and their *O*-glucosides.

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Physiological Disposition of a New Diuretic, ^{14}C-Metolazone, in Dogs

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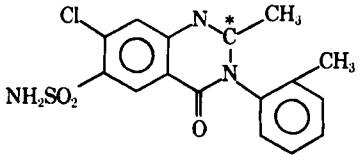
Abstract □ Counting procedures using ^{14}C were used to follow the concentration of metolazone and its metabolites in the blood, plasma, plasma water, bile, urine, and feces of dogs given single oral or intravenous doses of metolazone. Doses were administered orally from 0.1 to 50 mg./kg. and intravenously from 0.1 to 5 mg./kg. Regardless of the route of administration, a log–log relationship existed between the peak blood level and the dose. Within the blood, the drug was distributed among the fractions according to the approximate proportion: 80% erythrocyte, 15% plasma, and 5% plasma water. The appreciable hemoglobin and albumin protein binding of drug may explain the relatively long half-life, 5–6 hr., of the total counts in circulation from an intravenous dose of 5 mg./kg. Hydroxylated and further products of oxidation accounted for 40–60% of the excreted drug. There did not appear to be any accumulation or preferential retention of drug or metabolites. This was coupled with a recovery of 90–100% of the dose in urine and feces. The rate at which the metabolites were excreted *via* the urine was equivalent to that of the intact drug. In intravenous studies, excretion of drug occurred primarily in the urine; about 25% of the dose may be excreted in the bile.

Keyphrases □ Metolazone, radiolabeled—absorption, distribution after oral and intravenous administration, dogs □ Blood level–time relationships—metolazone after oral and intravenous administration, dogs □ Plasma protein binding—metolazone after oral and intravenous administration, dogs □ Absorption, drug—metolazone after oral and intravenous administration, dogs □ Excretion, drug—metolazone and metabolites after oral and intravenous administration, dogs

Metolazone[1] (7-chloro-1,2,3,4-tetrahydro-2-methyl-4-oxo-3-*o*-tolyl-6-quinazolinesulfonamide) is a new diuretic agent (1, 2) structurally (I) related to quinethazone. This quinazolinone derivative has been shown in both the rat and dog to be a potent, long-acting diuretic and natriuretic agent, exhibiting low kaliuretic activity and possessing virtually no toxic side effects (3, 4).

In the present studies, dogs were given single oral or intravenous doses of the ^{14}C-labeled drug over a wide dosage range to elucidate several parameters of drug disposition in this species. Among the parameters studied were blood level–time relationships, the biological half-life in plasma, and the extent of binding to plasma proteins and erythrocytes. Several possible routes of drug metabolism were deduced from identification and quantitation of metolazone and metabolites in urine and feces. The extent of biliary secretion was also examined.



I: metolazone (the asterisk indicates the position of the labeled carbon atom)

[1] Zaroxolyn, Pennwalt Corp.

MATERIALS AND METHODS

Purebred female beagle dogs, weighing 7–10 kg., were housed in individual stainless steel metabolism cages. The dogs were fed daily between 2:00 and 4:00 p.m. and allowed water *ad libitum*. The drug was administered between 8:00 and 9:00 a.m. on the day of the experiment. For intravenous administration, the compound was dissolved in a few drops of acetone and brought to volume with water or physiological saline. For oral administration, exact amounts of the compound were weighed on edible cachets, which were then placed into gelatin capsules.

Metolazone was synthesized with ^{14}C in the 2-position of the ring system (I) and had a specific activity[2] of 0.97 mc./mmole. Radioactivity in the blood, urine, feces, and bile was calculated as metolazone from the specific activity. The extent to which these values represent metolazone or its metabolites is unknown in blood and bile. In some experiments with urine and fecal samples, however, certain metabolites were identified and their concentrations were followed through the time course. The biologically derived samples were counted at 2° using a liquid scintillation system[3]. An external standard was utilized for correction of the quenching effect by relating the counting rate for the isotope in the sample to the increase in the counting rate of the sample when exposed to a γ source (^{133}Ba).

Blood was collected from the jugular vein without stasis in heparinized tubes (Vacutainer). Urine was collected at prescribed

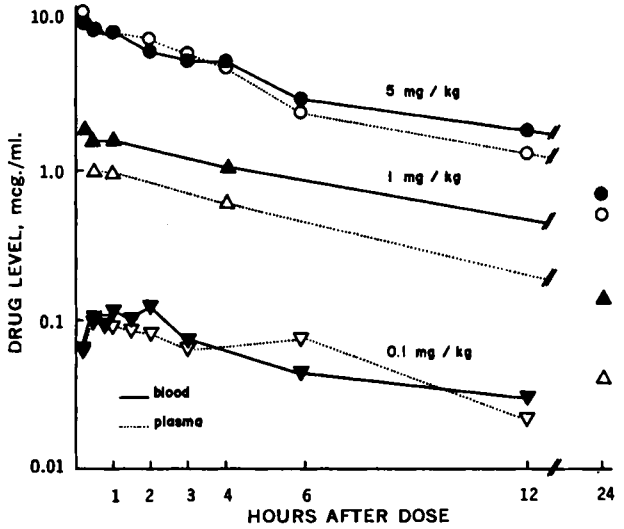


Figure 1—*Relationship of blood and plasma levels of metolazone and/or metabolites as a function of time following intravenous administration to dogs.*

[2] Lot No. 758-961. The authors thank Dr. T. A. Davidson and Dr. B. V. Shetty of the Organic Chemistry Department for the synthesis of the labeled drug, as well as Dr. T. L. Thomas and Mr. L. A. Campanella for their preparation of predicted metabolites.

[3] Mark I, Nuclear Chicago.

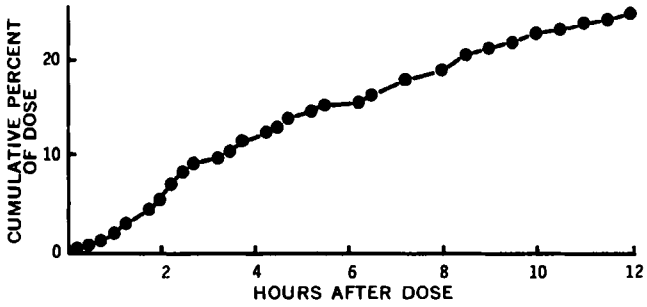


Figure 2—*Cumulative excretion of metolazone and/or metabolites in the bile of a dog given a 2-mg./kg. i.v. dose of the drug. Bile was collected through a polyethylene catheter which had been surgically placed in the common bile duct the preceding day.*

intervals with a metal catheter and, between catheterizations, from a metal pan in the metabolism cages. The volume collected over a given interval included the urine voided into the pan of the metabolism cages as well as that obtained by catheterization. Aliquots of blood and plasma (0.2 ml.) were prepared for liquid scintillation counting according to the method of Mahin and Lofberg (5). One milliliter urine and 1 ml. 95% ethanol were mixed with 15 ml. Bray's solution for liquid scintillation counting (6). All samples were prepared in duplicate, and results are reported as the average values. While substantiating this method, known amounts of labeled metolazone, which had been added to blood, plasma, and urine, were fully recovered (mean $\pm$ SD = 102.5 $\pm$ 3.2, n = 18).

The values for metolazone associated with the erythrocyte fraction were determined from the differences in the concentration of the drug in 1 ml. of whole blood and in the plasma thereof as determined from the hematocrit value. To calculate the total amount of drug in the circulation, total blood volume was assumed to be 10% of body weight. Ultrafiltrates for the measurement of drug in the plasma water were prepared by a modification (7) of the method described by Coolidge (8). For liquid scintillation counting, 1 ml. water was added to the aliquot of the ultrafiltrate (which was usually less than 1 ml.) plus 15 ml. Bray's solution.

Feces were collected from the metabolism cages and frozen until analyzed. The thawed feces were homogenized in acetone and extracted by refluxing (soxhlet) overnight. One-milliliter aliquots of the acetone extracts were counted as described for urine samples. To be certain that all radioactivity was extracted, 30-mg. samples of the dried acetone-extracted fecal material were prepared in the same manner as already described for blood and plasma and were found to contain no counts significantly higher than background. Since most of the urine was collected by catheterization at frequent

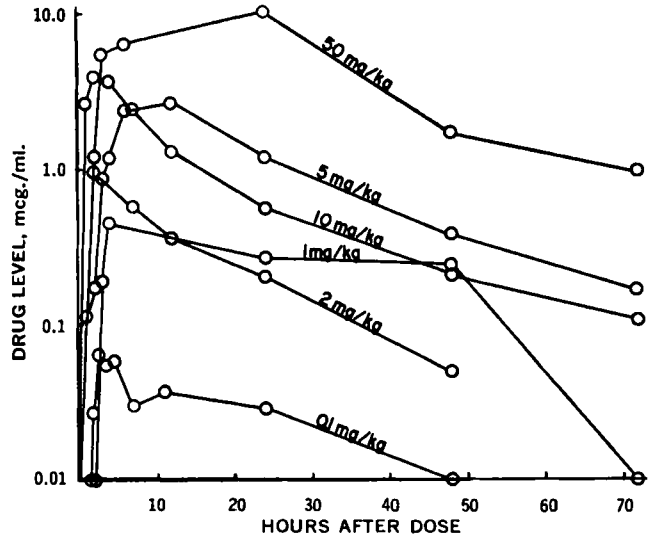


Figure 3—*Levels of metolazone and/or metabolites in whole blood following oral administration of the drug at the doses indicated. Each curve represents the blood levels obtained from a single dog.*

Table I—Half-Lives of ^{14}C-Metolazone in Plasma and Whole Blood of Dogs Receiving Single Intravenous Doses of the Drug[a]

Dose, mg./kg.	Whole Blood, hr.	Plasma, hr.
0.1	5.8	5.8
1.0	6.5	5.3
5.0	6.4	5.3

[a] The rate constant for disappearance was calculated from the disappearance portion of the blood and plasma concentration–time curves by least-squares regression analysis. The half-lives were calculated from the rate constant.

intervals, there was little chance for contamination of the feces by voided urine. In the daytime, fecal material was removed as soon as it was observed in the cage.

To study biliary excretion, two in-dwelling polyethylene catheters were inserted into the common bile duct of a dog under pentobarbital anesthesia. One catheter was directed upward toward the gallbladder; the other was directed in the opposite direction toward the duodenum. The two free ends were united to form a loop outside of the animal which permitted uninterrupted bile flow. The morning following surgery, a 5-mg./kg. dose of ^{14}C-metolazone was given intravenously and the loop was disconnected to allow bile collections from the proximal tube. The distal tube was clamped until the end of the initial 12-hr. collection period. During the 12–24-hr. interval of the experiment, the loop was reconnected to allow biliary flow to the duodenum. Aliquots of 0.1 ml. of the bile were prepared for liquid scintillation counting as described for blood and plasma.

The disappearance rate of drug from whole blood and plasma was determined using least-squares regression analysis (9). A number of predicted metolazone metabolites, which included hydroxylated and further oxidized products (Scheme I), were synthesized[4] to serve as standards for the identification and quantitation of metabolites in urine and feces by a method previously described (10). Briefly, this procedure involves a liquid chromatographic analysis of the chloroform-extractable tagged drug and its metabolites. These materials were extracted into chloroform, concentrated by removal of the solvent under nitrogen, reconstituted with 100 ml. tetrahydrofuran, and injected into a liquid chromatograph[5].

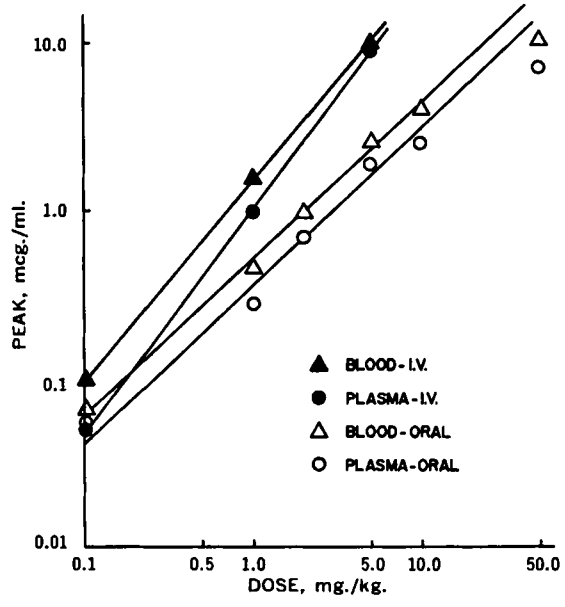


Figure 4—*Relationship between peak blood and plasma levels of metolazone and/or metabolites as a function of dose. Metolazone was administered by both the oral and intravenous routes.*

[4] By the Organic Chemistry Department, Pennwalt Corp.
[5] Waters ALC-100.

Table II—Metolazone and Converted Metolazone in Feces of Dogs Administered 2 mg./kg.[a]

Hours Postdose	Distribution in Feces: Intravenous Percent of Dose			Distribution in Feces: Oral Percent of Dose		
	Total	Metolazone	Converted Metolazone	Total	Metolazone	Converted Metolazone
12	—	—	—	54.8	51.6	3.2
24	0.6	NM[b]	NM	2.4	1.8	0.6
30	—	—	—	3.0	1.8	1.2
48	17.6	3.0	14.6	3.3	1.9	1.3
72	2.4	0.2	2.2	1.7	NM	NM

[a] Metolazone and metabolites were determined in acetone extracts of the fecal samples by separation by two-dimensional TLC, comparison of the R_f's with known standards, and quantitation by liquid scintillation counting. [b] NM = not measured.

Measurements were obtained by either utilizing peak height proportions or collecting individual fractions and obtaining the disintegrations per minute count. This method led to the identification of metabolites that were extractable into chloroform from the aqueous phase at about pH 5. The remaining counts in the aqueous phase were not identified.

RESULTS

Blood Levels and Excretion following Single Intravenous Doses—The concentrations of total radioactivity expressed as metolazone in the whole blood and plasma after intravenous doses of 0.1, 1.0, and 5.0 mg./kg. are shown in Fig. 1. Linear semilogarithmic plots of drug levels *versus* time were obtained over the major portion of the curves prepared from these data. A half-life of 5–6 hr. for metolazone in blood and plasma was obtained (Table I). Metolazone was no longer detectable in the blood at the end of 24 hr. for the low doses and 72 hr. for the highest dose. Close to 70% of the label was recovered in the urine of these animals. Fecal material was not analyzed.

Biliary Excretion of Metolazone and Metabolites—The cumulative percent excretion of radioactivity in the bile after a 5-mg./kg. intravenous dose is depicted in Fig. 2. The bile was collected at 15–30-min. intervals. At the end of the initial 12-hr. collection period, approximately 25% of the labeled dose was recovered through the bile duct fistula. Several peaks in both concentration and the total amount of drug excreted over the collection intervals were observed and were interpreted as intermittent contractions of the gallbladder. Initial attempts to chromatograph biliary extracts and to identify unconverted drug and its metabolites were not successful. To determine indirectly the extent of excretion of metabolites in the bile, the distribution of metabolites was determined in the feces of a dog receiving a 2-mg./kg. i.v. dose. About 20% of the label was excreted in the feces in 72 hr., of which about 80% was metabolites (Table II). For an equivalent oral dose, 65% of the dose was excreted in the feces in 72 hr., of which only about 10% was metabolites. These results would indicate that incomplete absorption occurred from the GI tract and that biliary excretion consists primarily of metabolites.

Absorption and Excretion following Single Oral Doses—The blood levels of metolazone and/or metabolites at various time intervals following oral doses of 0.1, 1.0, 2.0, 5.0, 10.0, and 50.0 mg./kg. are shown in Fig. 3. Excellent correlations exist between the log of the different dose levels studied and both the log of their respective peak blood levels (Fig. 4) and the cumulative 24-hr. urinary drug excretion. A good relationship also exists between peak blood level and cumulative 24-hr. excretion in urine, as shown in Table III. There was a substantial rise in the peak blood level (Figs. 3 and 4) as well as the duration of measurable blood levels at each increment of oral dose up to 50 mg./kg. (Fig. 3). The same relationships held for intravenous doses of 0.1–5 mg./kg. (Figs. 1 and 4).

The cumulative urinary and fecal excretion of the orally administered drug is shown in Table IV. Over the dose range administered, 74–100% of the dose was recovered. With increasing dosage, the percent of the administered dose excreted in the urine decreased, with concomitant increases being found in the feces. This would imply an inverse relationship between bioavailability and dose.

Distribution of Drug in Blood Fractions—The distribution of drug and/or metabolites between erythrocytes and plasma at peak blood levels for oral and intravenous doses is shown in Table V. More

Table III—Relationship between Dose, Peak Blood Levels, and 24-hr. Urinary Excretion of Metolazone

Dose		24 hr. Urinary Excretion		Peak Blood Level Reached within 24 hr.	
mg./kg.	Ratio[a]	mg./kg.	Ratio[a]	mcg./ml.	Ratio[a]
0.1	1	0.055	1.0	0.07	1.0
1.0	10	0.26	4.6	0.47	6.7
2.0	20	0.60	10.9	0.97	13.9
5.0	50	2.06	36.8	2.65	37.8
10.0	100	2.08	37.8	4.03	57.6
50.0	500	8.22	145.4	10.22	146.0

[a] Ratio of value at lowest dose level to the indicated value. All animals received the dose by oral administration.

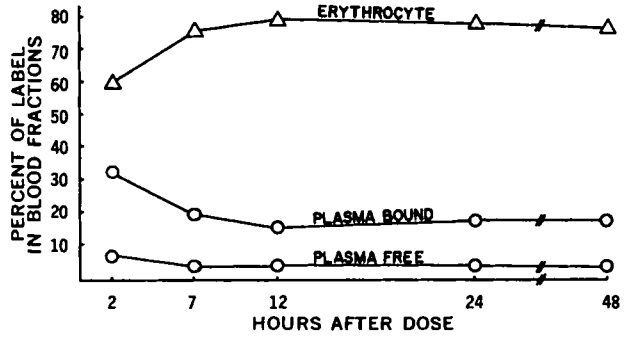


Figure 5—*Distribution of metolazone and/or metabolites as a function of time in the blood fractions of a dog given an oral 2-mg./kg. dose of metolazone. The whole blood concentration is considered to be 100%.*

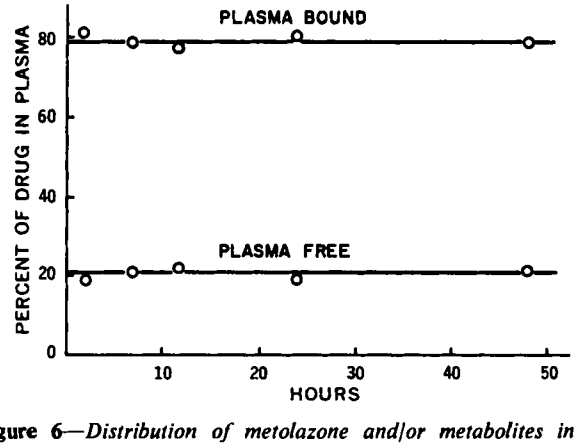


Figure 6—*Distribution of metolazone and/or metabolites in the plasma of a dog given an oral 2-mg./kg. dose of metolazone. The whole plasma concentration is considered to be 100%.*

Table IV—Recovery and Distribution of Metolazone Radioactivity as Found in Urine and Feces of Dogs after Administration of Various Oral Dose Levels

Dose, mg./kg.	Cumulative Percent of Dose in Urine, Hours					Cumulative Percent of Dose in Feces, Hours					Total Recovery, %
	12	24	48	72	96	12	24	48	72	96	
0.1	46.4	55.6	65.2	—	—	—	—	—	—	—	—
1.0	—	26.2	43.8	56.3	58.5	—	—	—	24.0	28.5	87.0
2.0	23.2	30.2	34.0	34.8	—	54.8	57.2	63.5	65.1	—	99.9
5.0	26.1	41.1	50.0	51.0	—	0.2	9.7	41.7	—	—	92.7
10.0	17.8	20.8	22.1	22.7	—	—	74.4	74.7	75.3	—	98.0
50.0	—	16.4	24.9	25.1	25.2	—	37.5	47.8	48.5	48.6	73.8

drug was associated with the erythrocyte fraction, as demonstrated by the fact that the erythrocyte to plasma ratio was greater than 1. The only exception was the 5-mg./kg. i.v. dose, in which the ratio was less than 1. This may be a function of the dose and the route of administration.

The distribution of radioactivity as a function of time between the three blood fractions—erythrocytes, plasma proteins, and plasma water—at the 2-mg./kg. oral dose is shown in Fig. 5. Close to 80% of the drug was bound to erythrocytes and about 15% was found in whole plasma, with only 5% or less actually in solution in the plasma water following equilibration of the labeled material between the three blood fractions. The consistency of the distribution within the plasma with time for this dog is shown in Fig. 6. In the plasma fraction, over a concentration range of 18–699 ng./ml., 80% of the drug was bound to proteins. Only 20% appeared free in the water.

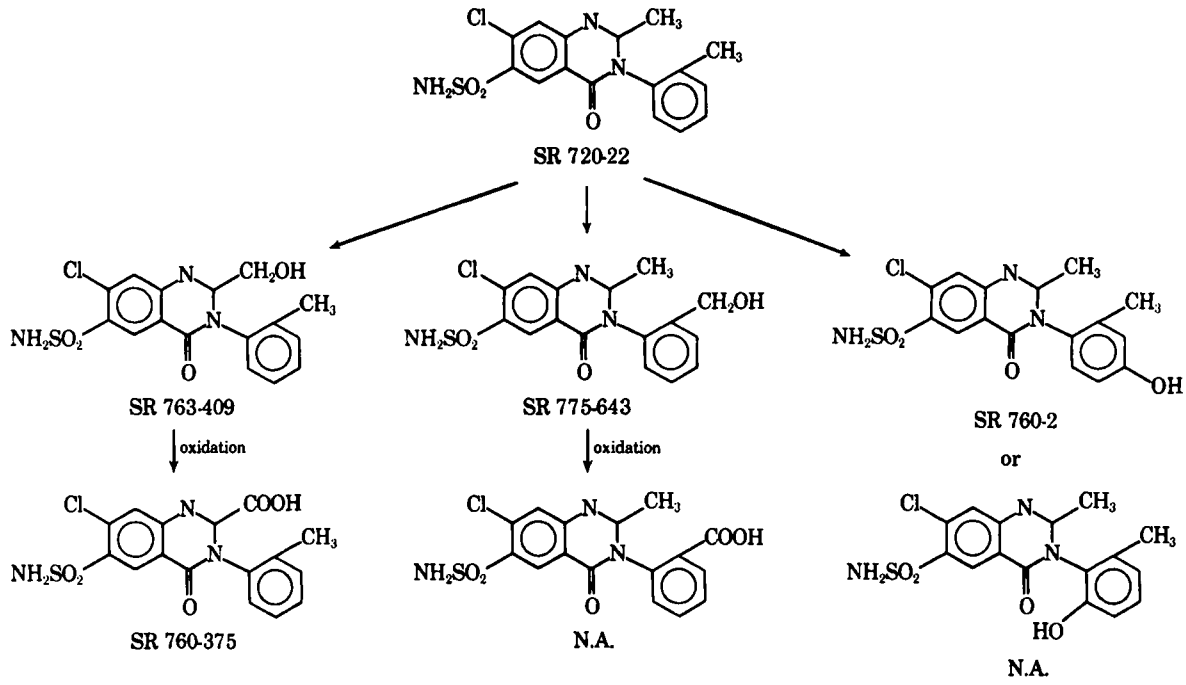
The disappearance half-lives of total ^{14}C in the case of the oral doses were similar to the intravenous values, as calculated from results in Fig. 3. The half-life was about 6 hr. with the exception of the dog that received 1 mg./kg. orally and had a half-life of 29 hr. The reason for this result has not been explained.

The volumes of distribution that could be estimated from the plasma drug levels following the intravenous doses approximated a volume equivalent to the total body water. If the amount of drug dissolved in the plasma water was used, the volume of distribution greatly exceeded the total body water, indicating substantial binding of the drug in the body tissues.

Where data were available from both intravenous and oral dosages, the bioavailability of the oral dose was estimated by comparing the area under the plasma concentration–time curve to that of its respective intravenous dose. At the 0.1-mg./kg. dose, the bioavailability of the oral dose was 80% that of the intravenous dose. When the doses were raised to 1 and 5 mg./kg., the bioavailability decreased to 52 and 38%, respectively.

Metabolite Identification—Some predicted metabolites are shown in Scheme I. The distribution of drug and metabolites found in the total 24-hr. urine and fecal samples for dogs receiving oral and intravenous doses is shown in Table VI. The bulk of the metabolites are hydroxylated derivatives of the native drug. As much as 25–50% of the label remained unextractable and in the aqueous phase. This fraction represents more polar derivatives, although this cannot be stated with certainty since the partition coefficients for the known metabolites in this extraction system are not known.

The rate of urinary excretion of unconverted metolazone and of total metabolites in the urine of dogs receiving 5 mg./kg. of metolazone by either the oral or intravenous route is shown in Fig. 7. The rate is expressed as the amount of metolazone to be excreted in the urine as a function of time following drug administration. The amount to be excreted in the urine is defined as the total amount of drug eliminated by this route minus the amount found in the urine up to the time indicated on the abscissa. When administered intravenously, both metabolites and unconverted metolazone were excreted rapidly at equivalent first-order rates for the first 8 hr. After this time, a second slower first-order rate loss was evident. Following oral administration, the rate of excretion of both unconverted metolazone and metabolites was very slow for the first 6–8 hr. Following this slow phase of elimination, a rapid first-order loss of metolazone and metabolites was observed, which appeared to parallel the first phase of elimination following the intravenous



Scheme I—Structures of some possible metabolites of metolazone. N.A. indicates that a suitable standard was not available.

Table V—Effect of Dose and Route of Administration on Drug Distribution between Erythrocytes and Plasma at the Peak Blood Level

Dose, mg./kg.	Peak Time, hr.	Percent Dose			Ratio Erythrocyte
		Blood	Erythrocyte	Plasma	Plasma
Intravenous Route					
0.1	—	12.3	7.8	4.5	1.7
1.0	—	14.6	9.8	4.8	2.0
5.0	—	18.7	5.8	12.9	0.4
Oral Route					
0.1	2.5	6.4	4.1	2.3	1.8
1.0	4.0	4.4	2.9	1.5	1.9
2.0	2.0	4.9	2.9	2.0	1.4
5.0	6.0	5.5	3.6	1.9	1.9
10.0	2.0	4.0	2.6	1.4	1.8
50.0	24.0	2.2	1.3	0.9	1.4

dose. At lower doses, administered both orally and intravenously, a single exponential rate of excretion in the urine was observed, which was equivalent to the slower phase for the intravenous dose in Fig. 7.

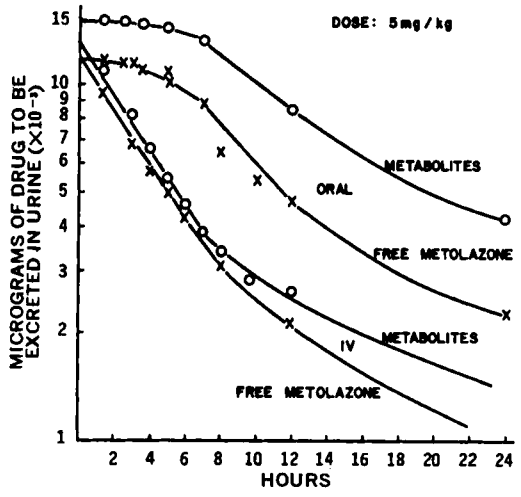


Figure 7—*Urinary excretion of metolazone and metabolites following the oral and intravenous administration of 5 mg./kg. to dogs. The amount of drug to be excreted is defined as the total drug excreted in the urine minus the total amount of drug excreted at any given time period.*

DISCUSSION

Drug distribution and excretion studies were conducted with single intravenous and oral doses of metolazone. Peak blood levels are related exponentially to the amount of drug administered over a wide dosage range, up to and including the 50-mg./kg. dose. Studies conducted in the rat (11) demonstrated a loss of linearity above this dose. This relationship has not been studied in dogs at higher oral doses, but there is a tendency (Fig. 4) that indicates that linearity may have been lost at the 50-mg./kg. dose level. This phenomenon may be explained in part by the low solubility of the drug and decreased absorption at higher doses, as evidenced by the inverse relationship between bioavailability and dose. In addition, Table III shows that the ratios (value at selected dose/value of lowest dose) for drug excreted over 24 hr. and also for peak blood levels reached during each 24-hr. interval are less than the ratios for the doses. The ratios for these two parameters, however, are approximately equal for any given dose level. By implication, therefore, one would expect that urinary drug excretion would follow a pattern related to the peak blood levels.

An important property of metolazone is believed to be the extent to which it is bound by erythrocytes and plasma proteins of many species, including man (12). In the present studies, the red cell/plasma ratio of metolazone plus its metabolites was always greater than unity; with chlorothiazide, this ratio was always less than unity (13). Another important parameter is the extent of protein binding in plasma, since the concentration of the unbound drug in this fraction is believed to be a reasonable estimate of both the free concentration in the extravascular fluids and the pharmacologically active concentration *in vivo* (14). Within the plasma fraction, approximately 80% of the metolazone is bound. Other diuretic drugs are bound to plasma proteins to a variable extent in mammals: chlorothiazide (15, 16), 40–45%; hydrochlorothiazide (16), 66%; trichlormethiazide (16), 80%; mefruside (16), 80–85%; polythiazide (15, 16), 85%; and hygroton (17), 85%. The apparent volume of distribution of metolazone, calculated from the blood level data, was found to be in excess of the total body water, indicating extensive binding by not only blood but by tissue proteins as well.

Because of the limited intestinal absorption and low water solubility of this drug, it is difficult, if not impossible, to obtain high enough blood levels of the drug to saturate the binding sites on plasma proteins and to decrease the ratio of bound to free drug. In the case of the 2-mg./kg. oral dose (Figs. 4 and 5), only about 20% of the total drug found in the plasma of dogs was actually in solution as an unbound entity throughout the 2-day period of the study, regardless of the drug concentration in the whole plasma. A similar inability to saturate the plasma binding capacity was observed in the rat where doses as high as 1.25 g./kg. were administered (11, 12).

Rather than express the association of metolazone with plasma proteins as percent bound, it would be more informative to calculate the dissociation constant of the drug–protein complex. To gain further insight into this interaction, the apparent dissociation constant (k_{dp}) was calculated from the plasma levels of the dog given 2 mg./kg., using certain assumptions. Preliminary *in vitro* experiments from this laboratory, with the use of various canine plasma protein fractions, indicated that under *in vivo* conditions only a small percentage of the total drug in plasma could be bound by proteins other than albumin. When assuming the plasma albumin concentration to be approximately 5×10^{-4} M, the apparent dissociation constants (k_{dp}) were calculated using standard procedures (18, 19) from the plasma levels of the drug in the dog that received the oral 2-mg./kg. dose. Over a range of plasma concentrations of 18–699 ng./ml., measured at five different time intervals, the calculated dissociation constant was $(1.23 \pm 0.6) \times 10^{-4}$ (mean ± *SD*). The uniformity of the dissociation constants over this range

Table VI—Distribution of Metolazone and Its Metabolites[a] in 24-hr. Urine Samples from Dogs

Dose, mg./kg.	Route	Percent Distribution of Counts Appearing in Urine					
		720-22	763-409	760-2	775-643	760-375	Aqueous Solution
0.1	Intravenous	33.25	9.60[b]		8.07	3.20	45.88
1.0	Intravenous	41.37	4.01	1.98	1.25	1.19	50.20
5.0	Intravenous	62.29	0.39	1.04	2.56	4.18	29.54
0.1	Oral	57.73	1.0	0.25	0.87	2.35	37.80
2.0	Oral	61.00	5.07[b]		4.80	2.93	26.20
5.0	Oral	44.92	0.77	0.67	0.52	2.40	50.72

[a] Metabolites were identified by comparison of their known R_f's in two-dimensional TLC and quantitated by liquid scintillation counting. Structures are shown in Scheme I. [b] Sum of metabolites 763-409 and 760-2.

of concentration is attributed to the fact that only one binding site on plasma albumin is involved. The molar ratio of drug to albumin at the highest drug concentration was 3.8×10^{-3}.

The half-life of metolazone in plasma and whole blood of dogs receiving intravenous doses of the drug ranged from 5 to 6 hr. The enterohepatic circulation of the drug and the strong binding affinity of the red cell and plasma proteins no doubt strongly influenced the half-life. At this time it is not known how much unconverted drug and metabolites were present in the total concentration of label found in the bile, blood, or plasma. By implication, the high distribution of converted drug found in the feces of the animal given a 2-mg./kg. i.v. dose suggests that most of the biliary excretion consists of metabolic products. Bioavailability determinations suggest that above the 0.1-mg./kg. oral dose level, a large percentage of unconverted drug in the feces of these dogs was due to a lack of absorption.

The total percent of the administered dose recovered in the excretion was between 90 and 100% in four of the five orally dosed dogs. The one dog showing a lower percent of drug recovery had been given a very high dose of 50 mg./kg. No doubt this was due to some residual drug in the body. The total metabolites are excreted at a rate comparable to that of metolazone. This observation, coupled with the good recoveries with therapeutic doses, indicates that there is no residual drug remaining in the tissues and that no buildup of drug or metabolites should be expected.

It has been reported[6] that quinethazone, which is closely related to metolazone, is not metabolized in experimental animals. The extent to which thiazides and other related diuretics are metabolized in mammals has been reported to be variable. Chlorothiazide, hydrochlorothiazide, flumethiazide, and hydroflumethiazide have been reported to be rapidly excreted without undergoing metabolic transformation (16). By contrast, bendroflumethiazide, trichlormethiazide (16), and polythiazide (16, 20, 21) are partially metabolized by the dog. The metabolism of metolazone in animal species would be classified in the latter group. The metabolites of metolazone, which are products of hydroxylation and oxidation, have been found in both the urine and feces. The origin of the metabolites in the feces are presumably the results of the biliary secretion of the drug.

The extent of metabolism of metolazone was unexpectedly high for such a polar drug. In a 24-hr. period following administration of the drug, 30–60% of the drug appearing in the urine of dogs was metabolites. In the human, by contrast, the amount of metabolites found in the urine in 24 hr. was only 10–15% of the excreted drug (12). In view of the unexpectedly high degree of metabolism in dogs, the metabolites were synthesized and assayed for diuretic activity. The activities of these metabolites administered orally have been considerably less than that of metolazone and, in addition, exhibited no evidence of toxic reactions.

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Urinary Metabolic Profiles for Choosing Test Animals for Chronic Toxicity Studies: Application to Naproxen

GEOFFREY F. THOMPSON[A] and JUDITH M. COLLINS

Abstract □ Gradient elution ion-exchange chromatography was employed to determine urinary metabolic profiles of naproxen in test animals and humans. Retention molarities were found to be very reproducible, and the quantitation and column recovery were good. The profiles add another dimension to the basic information required to pick the most appropriate test animal for the chronic toxicological evaluation of a new drug. Based on the profiles, the mini pig and rat appeared to be the animals of choice for the chronic toxicological evaluation of naproxen.

Keyphrases □ Naproxen urinary metabolic profiles—used to determine appropriate test animal for toxicology evaluation, gradient elution ion-exchange chromatography □ Urinary metabolic profiles—determination of appropriate test animal for toxicology evaluation, naproxen □ Toxicology studies, naproxen—choice of appropriate test animal based on urinary metabolic profiles □ Ion-exchange chromatography, gradient elution—determination of naproxen urinary metabolic profiles, animals, humans

Naproxen [(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid[1], I] is a new nonsteroidal anti-inflammatory agent as determined by the carrageenin-induced rat paw edema assay (1, 2). During the development of this new drug, it became important to generate information to determine which rodent and nonrodent test animals resemble the human most closely in the disposition of the drug. This information, which includes data on the absorption, excretion, distribution, rate of metabolism, and metabolic pattern of the drug, provides the rationale for the choice of the most appropriate test species in the chronic toxicological evaluation of a new drug (3, 4). A study of the absorption, distribution, and excretion of naproxen in various test animals and man was recently reported (5). The present report describes an approach taken for the determination of the urinary metabolic profiles of naproxen, based on gradient elution ion-exchange chromatography.

EXPERIMENTAL[2]

Species, Dosing, and Urine Collection—The test animals and humans employed in this investigation received intravenous injections of tritium-labeled naproxen. A description of the preparation of the dosing solutions, the method of dosing, and the method of urine collection was previously described (5). A description of the species and the dosing data employed in this investigation is summarized in Table I. The urine specimens were preserved with formaldehyde and stored in the frozen state.

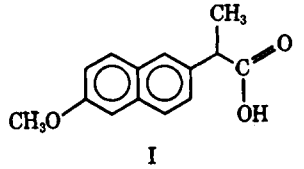
Naproxen—The synthesis of naproxen[3] was previously reported (1). Tritiated naproxen[3] (6), labeled on the ring system, had a specific activity of 92 mc./mmole (5). Naproxen-3-^{14}C[3] had a specific activity of 25.6 mc./mmole.

2-(6′-Hydroxy-2′-naphthyl)propionic Acid[1] (II)—The structure of this compound was verified by its spectral properties and elemental analysis; IR (KBr): 3400, 1705, 1605, 1500, 1450, 1380, 1360, 1280, 1200, and 1145 cm.$^{-1}$; UV (CH_3OH): 225 (log ε 5.10), 256 (3.49), 265 (3.62), 274 (3.65), 322 (3.18), and 334 (3.24) nm.; NMR (d_6-acetone): δ 7.55–7.80 (m, 3), 7.36 (d of d, 1, J = 10 and 2.0 Hz.), 7.03–7.20 (m, 2), 3.83 (q, 1, J = 7 Hz.), and 1.49 (d, 3, J = 7 Hz.); mass spectrum (70 ev.) m/e: base peak 171, molecular ion 216.

Anal.—Calc. for $C_{13}H_{12}O_3$: C, 72.21; H, 5.59. Found: C, 72.26; H, 5.62.

Methyl 2-(6′-Methoxy-2′-naphthyl)propionate—To 4.08 g. (0.018 mole) of naproxen was slowly added 120 ml. (0.108 mole) of 14% (w/v) boron trifluoride–methanol[4]. The mixture was refluxed for 15 min., cooled to room temperature, and taken up in 1.2 l. of petroleum ether (30–60°). The solution was washed with water and dried over anhydrous sodium sulfate. The solution was filtered, and the petroleum ether was removed by rotary evaporation to give 3.97 g. (92%) of crystalline methyl 2-(6′-methoxy-2′-naphthyl)-propionate, m.p. 90–91°.

2-(6′-Methoxy-2-naphthyl)propionuric Acid (III)—The synthesis of this compound was based on Ford's (7) procedure for the synthesis of phenaceturic acid. A solution of sodium methoxide in methanol was prepared by the reaction of 0.51 g. (0.022 g.-atom) of sodium metal in 20 ml. of anhydrous methanol. A 1.31-g. (0.018 mole) portion of glycine was added to the alkoxide solution, and the solution was refluxed for 20 min. to dissolve the glycine. A 3.87-g. (0.016-mole) portion of methyl 2-(6′-methoxy-2′-naphthyl)propionate was then added to the glycine solution, and the mixture was refluxed (91°) for 5 days. The methyl alcohol was removed by distillation through a short Vigreux column, and the residue was taken up in 200 ml. of 5% sodium bicarbonate and washed with two 50-ml. portions of ether. The aqueous solution was adjusted to pH 2 with hydrochloric acid, precipitating 3.41 g. of crystalline material. TLC on silica gel GF, using benzene–methanol–acetic acid (90:16:8), showed this material to be approximately a 50:50 mixture of naproxen and the desired product. Preparative TLC on 0.51 g. of the crude material, using toluene–acetic acid–water (50:50:5) followed by elution with methanol, recrystallization from methanol–water (50:50), and drying over phosphorus pentoxide (0.01 mm.), gave 0.24 g. of the desired product, m.p. 156–157°; IR (KBr): 3350, 1750, and 1625 cm.$^{-1}$; UV (CH_3OH): 232 (log ε 4.84), 253 (3.58), 262 (3.67), 272 (3.67), 324 (3.09), 303 (2.88), 316 (3.14), and 331 (3.24) nm.; NMR (d_6-dimethyl sulfoxide): δ 8.30 (broad t, 1), 7.68–7.86 (m, 3), 7.46 (d of d, 1, J = 8.5 and 1.7 Hz.), 7.28 (d, 1, J = 2.0 Hz.), 7.15 (d of d, 1, J = 9.0 and 2.5 Hz.), 3.86 (s, 3), 3.76 (m, 3), and 1.41



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[1] This name is the correct *Chemical Abstracts* index name, but this compound was identified previously in the chemical and biological literature as *d*-2-(6′-methoxy-2′-naphthyl)propionic acid.

[2] All reagents were of analytical grade. NMR spectra were determined on a Varian HA-100 spectrometer, using tetramethylsilane as an internal standard. Ion-exchange fractions were collected on a GME model 45 volumetric fractionator, and radioactivity was monitored on a Nuclear-Chicago Unilux II scintillation counter. The scintillation fluid contained 260 mg. of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (Arapahoe Chemical Co., Boulder, Colo.), 13 g. of 2,5-diphenyloxazole, and 208 g. of naphthalene in 600 ml. of methanol, 1000 ml. of toluene, and 1000 ml. of dioxane.

[3] Obtained from the Institute of Organic Chemistry, Syntex Research, Palo Alto, Calif.

[4] Applied Science Laboratories, Inc., State College, Pa.

Table I—Species and Dosing Data

Species	Number, Sex	Weight, kg.	Urinary Half-Life, hr.[a]	Urine Specimen Used, hr.	Dose, Cold, mg./kg.	Dose, Hot, μc./kg.
Human	2M 1F	49–86	13	1–3	93[b]	40[c]
Mini pig	2F	63–70	5	0–24	2.5	11.6
Rhesus monkey	2F	5–6	2	0–0.5	10.0	200
Guinea pig	1M	0.82–1.1	10	0–24	5.0	49.0
Rat (Sprague–Dawley)	3M	0.45–0.50	8	0–24	2.88	28.8

[a] Obtained from *Reference 5*. [b] The total cold human dose was 93 mg. [c] The total hot human dose was 40 μc.

Table II—Retention Data for the Biotransformed Products of Naproxen in Test Animals and Man

Compound Number	Rhesus Monkey	Guinea Pig	Rat	Mini Pig	Human Male	Human Female	Authentic III	Authentic II
			Retention Molarity					
1	0	0	0	0	0	0	—	—
2	0.01	0.01	0.01	—	—	—	—	—
3	—	0.09	—	0.09	—	—	—	—
4	0.14	0.16	0.16	0.14	—	—	—	—
5 (Naproxen, I)	0.20	0.20	0.20	0.20	0.19	0.20	0.20	0.20
6	0.22	—	—	0.22	0.23	0.25	—	—
7 (III)	0.25	0.27	0.27	0.27	0.26	—	0.26	—
8 (II)	—	—	—	—	—	0.29	—	0.29
9	0.31	0.31	0.31	0.30	0.30	0.31	—	—
10	—	—	0.36	0.35	0.35	0.37	—	—
11	—	—	0.40	0.40	0.40	0.41	—	—
12	0.46	0.45	0.45	0.45	—	—	—	—
13	—	—	0.49	0.48	0.48	0.50	—	—
14	—	0.57	—	0.58	—	—	—	—
15	0.81	—	0.80	—	—	—	—	—
16	0.85	—	0.84	—	—	—	—	—
			Retention pH					
17	—	—	3.85	—	—	—	—	—
18	4.45	—	4.45	4.45	—	—	—	—
19	—	4.65	4.65	—	—	4.65	—	—
20	—	—	—	—	6.50	—	—	—

(d, 3, J = 7.0 Hz.); mass spectrum (70 ev.) m/e: base peak 185, molecular ion 287.

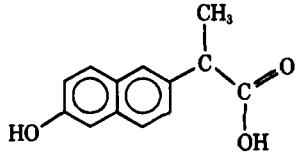
Anal.—Calc. for $C_{16}H_{17}NO_4$: C, 66.89; H, 5.96. Found: C, 66.72; H, 6.00.

Ion-Exchange Resin—All ion-exchange separations described here were carried out with portions of the same batch of diethylaminoethyl Sephadex in the formate form prepared as follows. A 200-g. portion of diethylaminoethyl Sephadex (chloride) A-25[5] in a 12 × 60-cm. chromatography column was treated successively with 1 *M* NaOH until chloride was not detectable in the eluate, with distilled water until the eluate was neutral, with 0.5 *M* formic acid until the pH of the eluate was equivalent to that of 0.5 *M* formic acid, and finally with distilled water until formate ion was not detectable in the eluate. Formate ion was monitored by the method of Feigl (8).

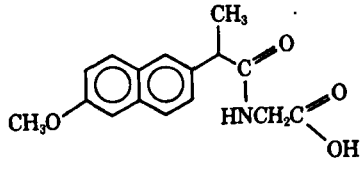
Ion-Exchange Chromatography—The procedure employed was similar to that described by Lethco and Brouwer (9). Composite urine specimens were prepared by combining the specimens (same percent by volume of each) of all animals of the same species. Aliquots (5–40 ml.) of the composite specimens were taken for chromatography so as to contain about 3×10^6 d.p.m. of tritium for the animal specimens and about 7.5×10^5 d.p.m. of tritium for the human specimens. An internal standard of about 3.5×10^5 d.p.m. of ^{14}C-naproxen was introduced into each specimen used for chromatography by mixing the specimens in a centrifuge tube in which a solution of ^{14}C-naproxen had been evaporated. Male and female human urine specimens were chromatographed separately; however, the two female specimens were combined.

Urine composite aliquots were pipeted onto a 35 × 1.8-cm. i.d. column charged with 35 meq. of ion-exchange resin[6]. The biotransformed components related to naproxen were separated by successive elution with distilled water (200 ml.), pH 3.5 ammonium formate buffer (4000 ml.) over a linear concentration gradient from 0 to 1.0 *M*, 1.0 *M* ammonium formate buffer (2000 ml.) over a pH gradient from 3.5 to 5.5, and 1 *M* ammonium formate buffer (1000 ml.) at pH 6.5. Final washing of the resin in a conical flask with methanol gave less than 0.5% of the initially present tritium radioactivity in each case.

The eluate was collected in 10-ml. fractions, and 5-ml. aliquots of every other fraction were evaporated to dryness, diluted with scintillation fluid, and subjected to double isotope counting using an external standard for quench correction. Plots of tritium radioactivity *versus* fraction number gave the unperturbed metabolic profiles shown in Fig. 1. Retention molarities and retention pH's (Table II) were determined from fraction number *versus* formate molarity and fraction number *versus* pH calibration curves, respectively. Every fraction of each biotransformed product (as defined by the profile)



II



III

[6] Obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

Table III—Quantitative Distribution of the Biotransformed Products of Naproxen in Test Animals and Man[a]

Compound Number	Rhesus Monkey	Guinea Pig	Rat	Mini Pig	Human Male	Human Female
1	12.1	0.3	0.2	19.7	0.3	0.2
2	0.6	0.8	0.7	—	—	—
3	—	0.3	—	0.5	—	—
4	0.6	0.5	0.2	0.9	—	—
5 (Naproxen, I)	6.7	39.8	2.8	3.6	11.5	7.3
6	6.4	—	—	1.3	[	[
7 (III)	31.9	4.5	8.2	16.9	28.0[b]	22.7[b]
8 (II)	—	—	—	—	]	]
9	19.9	8.2	1.0	7.7		
10	—	—	0.5	2.3	38.2	40.1
11	—	—	1.1	16.1	7.9	6.6
12	2.2	2.6	2.1	1.2	—	—
13	—	—	0.5	3.0	10.3	13.9
14	—	1.8	—	8.2	—	—
15	0.9	—	7.4	—	—	—
16	1.2	—	4.1	—	—	—
17	—	—	2.2	—	—	—
18	2.9	—	24.4	1.7	—	—
19	—	15.1	32.8	—	—	2.5
20	—	—	—	—	5.6	—

[a] The percentage values are based on the total tritium radioactivity applied to the column. [b] These values represent the total concentration of products eluted after Compound 5 and before Compound 10.

was combined, and an aliquot was counted to determine the quantitative distribution of each product (Table III).

The elution of authentic II and III was followed by monitoring their UV absorption at 225 and 232 nm., respectively.

RESULTS

The data in Table IV demonstrate the good total recovery using the specified column. The mean recovery based on seven separations was 93.8%.

The urinary metabolic profiles presented in Fig. 1 show that at least 20 biotransformed products of naproxen were detected. For purposes of comparison, each biotransformed product, defined by a retention molarity or pH, was given a compound number. These data are presented in Table II.

Compounds 10 and 13 were identified[4] as the glucuronides of naproxen and II, respectively, by incubation with β-ketodase. Naproxen, II, and III (Compounds 5, 8, and 7, respectively) were identified in the various urine specimens by comparison of their retention molarities with those of authentic compounds.

The reproducibility of the retention molarities from run to run is demonstrated by the data in Table II for naproxen (Compound 5), in which identification is unambiguous because of the presence of the naproxen-^{14}C internal standard. For naproxen, a retention molarity of 0.20 was obtained in each of the eight runs (Table II), except for the human male for which the value was 0.19. Additionally, the data in Table V show that except for Compound 9 (retention molarity 0.30 and 0.29) the retention molarities are identical for duplicate runs on the human male specimen.

The retention molarity range for each compound in Table II is 0.02, except for Compound 6 for which the retention molarity varied from 0.22 in the mini pig to 0.25 in the human female (range 0.03).

The assignment of compound numbers for most of the biotransformed products based on retention molarities was straightforward. Some very minor components, however, were not given compound numbers, such as the shoulder on the descending side of Compound 6 and on the ascending side of Compound 12 in the rhesus monkey.

The assignment of compound numbers for the biotransformed products in the human specimens between the retention molarities of 0.22 and 0.31 was difficult due to the poor resolution of the products in this region. However, an attempt was made to assign some compound numbers to the components in this region, but these assignments should be considered with reservation. This is the region where II and III elute.

Although reference compounds were not employed in the retention pH region, the data in Table II indicate that the retention pH's are reproducible. The only exception to this is the retention pH values of 6.5 and 5.1 (Table V) obtained from duplicate runs on the human male urine specimens. Since in this specimen only one component eluted in the retention pH range and since the remainders of both profiles were virtually identical, it has been assumed for convenience that the two retention pH's represent the same component. Following a similar line of reasoning, Compound 20 found in the human male may be equivalent to Compound 19 found in the human female, rat, and guinea pig.

Since the column may not be selective when distilled water is used as the eluting solvent, the radioactive naproxen-related materials eluted in the first 200 ml. in all of the urine specimens may or may not represent the same substance. For convenience, however, the material eluted in this region was assigned as Compound 1.

Inspection of the percent distribution data in Table V shows that the quantitative capabilities of the method are sufficiently reproducible to make quantitative comparisons of the naproxen-related products produced by the different species. The percent distribution data in Table III show that less than 1% of the biotransformed products of naproxen was eluted with water in the human. However, the material eluted in this region represents about 12 and 20% of the naproxen products in the rhesus monkey and mini pig, respectively. Compounds 2, 3, and 4 were not found in the human samples and represent less than 1% of the products found in the test animals. Naproxen (Compound 5) was found in every specimen. It represents about 7–12% of the products in the human specimen and was the major product (about 40%) in the guinea pig. Compounds 6, 7, 8, and 9, which were poorly resolved in the human runs, represent a total of about 23–28% of the products. The glycine conjugate of naproxen (Compound 7) was a major product in both the rhesus monkey (about 32%) and the mini pig (about 17%). Compound 9, unknown, represents about 20% of the products in the rhesus monkey.

Compound 10, the glucuronide conjugate of naproxen, was the major biotransformed product of naproxen in the human (about

Table IV—Column[a] Recovery for the Biotransformed Products of Naproxen in Test Animals and Humans

Species		Recovery, %[b]
Rhesus monkey		92.5
Mini pig		89.9
Rat		91.3
Guinea pig		86.1
Human male		102.8, 99.9
Human female		94.2
	Mean	93.8

[a] Diethylaminoethyl Sephadex (formate). [b] Based on tritium radioactivity.

[4] To be published.

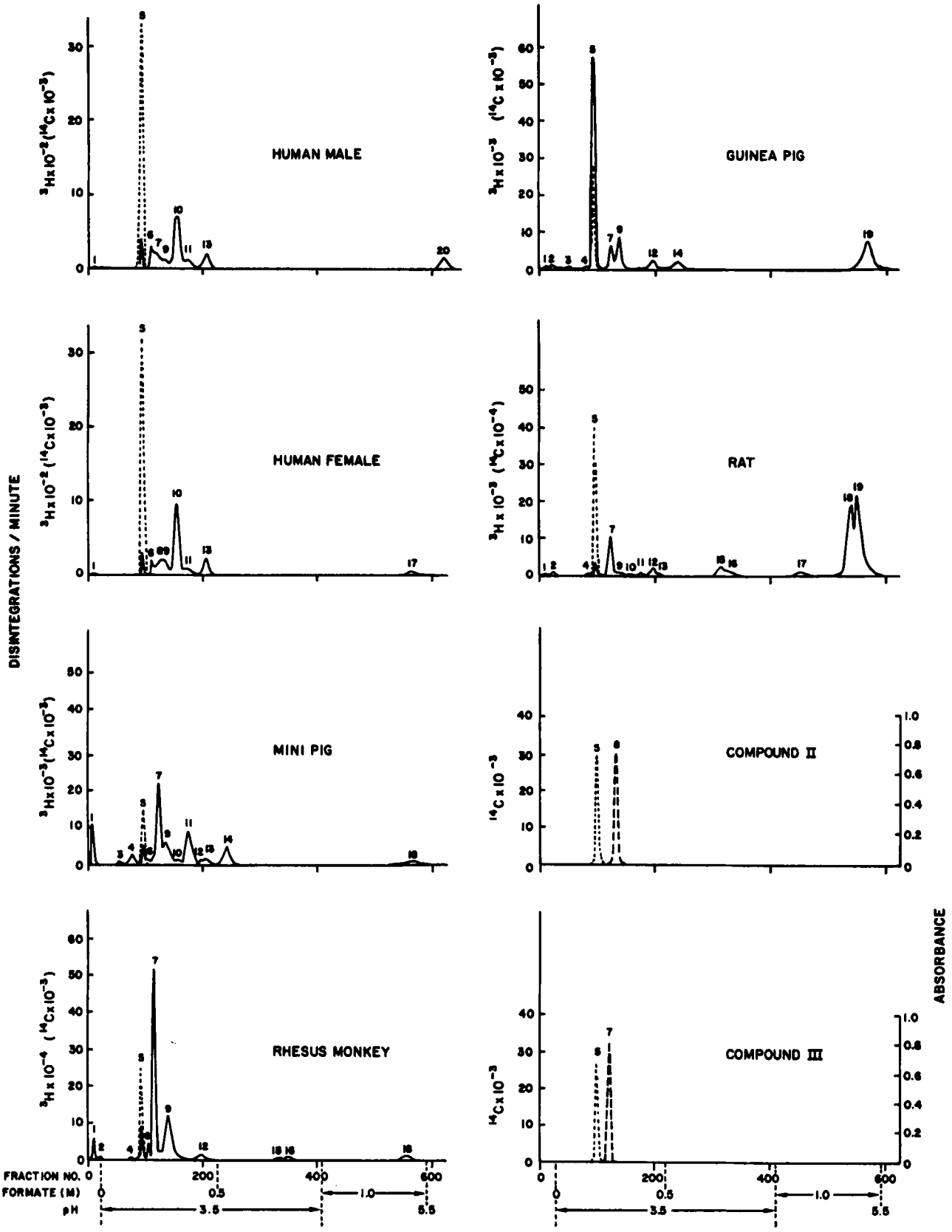


Figure 1—*Metabolic profiles for naproxen in humans and test animals. Key:* ——, *^3H measurements;* - - -, *^{14}C measurements; and* — — *absorbance measurements.*

40%). It was present in very small quantities in the mini pig and rat but not present in the guinea pig or rhesus monkey. Unidentified Compound 11, which represents about 7% of the products in the human was a major product (about 16%) in the mini pig. Unknown Compound 12 was not found in the human and represents less than 3% of the products in each of the test animals. Compound 13, a glucuronide of II, was a major product in the human (10–14%) and was also found in the mini pig (3%) and rat (0.5%). Unknown Com-

Table V—Comparison of Retention and Percent Distribution Data for Duplicate Separations (Human Male) of the Same Specimen[a]

Compound Number	Retention Molarity Run 1	Retention Molarity Run 2	Percent Distribution[b] Run 1	Percent Distribution[b] Run 2
1	0	0	0.3	0.3
2	—	—	—	—
3	—	—	—	—
4	—	—	—	—
5	0.19	0.19	11.5	11.5
6	0.23	0.23	[28.0[c]	[27.5[c]
7	0.26	0.26		
8	—	—		
9	0.30	0.29		
10	0.35	0.35	38.2	32.7
11	0.40	0.40	7.9	8.0
12	—	—	—	—
13	0.48	0.48	10.3	10.4
14	—	—	—	—
15	—	—	—	—
16	—	—	—	—
	Retention pH Run 1	Retention pH Run 2		
17	—	—	—	—
18	—	—	—	—
19	—	—	—	—
20	6.50	5.70	5.6	9.3

[a] Run 1, column eluted with an ammonium formate buffer; run 2, column eluted with a sodium formate buffer. [b] The percentage values are based on the total tritium radioactivity applied to the column. [c] These values represent the total concentration of products eluted after Compound 5 and before Compound 10.

pounds 14, 15, 16, 17, and 18 were not found in the human. Compound 18 was a major product in the rat. Unknown Compound 19 was found to the extent of 2.5% in the human female and was the major product in the rat. About 5% of Compound 20, which may or may not be equivalent to Compound 19, was found in the human male.

DISCUSSION

When employing metabolic profiles as the only criterion, the ideal test animal for chronic toxicity studies is that test animal whose metabolic profile is identical with that of the human. Since this is a very unlikely or even impossible goal for most drugs, the next best criterion is to pick that animal whose profile is most similar to that of the human. When using the metabolic profiles as the only criterion, the mini pig appears to be the best nonrodent test animal (of those studied) for the chronic toxicological evaluation of naproxen. This choice is based on the presence in the mini pig of Compounds 10, 11, and 13. These are the three major biotransformed products of naproxen in the human and are not found in the rhesus monkey. Again, based only on the profiles, the rat appears to be the choice as a rodent test species. This choice is also based on the presence in the rat (albeit smaller quantities) of Compounds 10, 11, and 13 which are not present in the guinea pig. Additionally, the guinea pig contained about 40% free naproxen whereas the rat contained only about 3%, a value more comparable to that found in the human specimens.

Since the biotransformed products of most drug substances are amenable to ion-exchange chromatography, the method appears to have general applicability. The good column recovery allows the development of a complete unperturbed picture of the number and quantity of biotransformed products produced. This method appears to have advantages over other methods that include extraction and derivatization procedures which may perturb the urinary products. The generally good reproducibility of the retention data demonstrates the utility of the profiles for both comparison purposes and for the tentative identification of the drug-related components when authentic compounds are available.

In addition to supplying part of the information for choosing the best test animals, metabolic profiles form a good basis for the planning and development of experiments to give additional metabolic information. The method provides separated samples of the individual biotransformed products which can be subjected to other analytical techniques.

The major limitation of the method is that it is fairly time consuming. It was primarily for this reason that the urine specimens from the same test animals were pooled. Although it can be argued that this results in an average picture for a particular test animal, it does not give a picture of animal-to-animal variation. It appears at this point that one method of circumventing the time requirement would be the use of linear gradient high pressure chromatography monitored by a flowthrough scintillation (and UV) detector such as was described by Hunt (10).

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Dissolution Kinetics of Gallstones: Physical Model Approach

W. I. HIGUCHI[▲], F. SJUIB, D. MUFSON, A. P. SIMONELLI, and A. F. HOFMANN

Abstract □ Recent studies suggested that *in situ* dissolution of gallstones in man might be dissolution rate controlled. In this work, several physical models for the dissolution rate of cholesterol-type stones were developed. One is based upon simple diffusion-controlled dissolution and another upon a leaching process. Two others relate to interfacial coat-type barriers. Calculations were carried out employing models with reasonable input parameters. The results show, for example, that the theoretical dissolution rate based on the simple dissolution model would predict that a 2.5-mm. stone should dissolve in several days into an undersaturated bile solution. Both clinical and *in vitro* experiments show that much longer times, *e.g.*, months, are needed.

Keyphrases □ Gallstones—dissolution kinetics, various physical models proposed and discussed, calculations □ Dissolution rate, gallstones—proposed physical models, calculations □ Cholesterol-type stones—proposed physical models, dissolution kinetics, calculations □ Diffusion—in dissolution kinetics of gallstones, physical models proposed, calculations □ Interfacial barriers—as a factor in dissolution kinetics of gallstones, physical models proposed, calculations

Cholesterol is transported in vertebrate bile dissolved in bile acid–lecithin micelles. Recently, the phase equilibria present in bile acid–lecithin–cholesterol–water systems were defined and shown to relate to human cholesterol cholelithiasis (1, 2). In man, cholesterol cholelithiasis appears to be associated with the formation of bile by the liver which is saturated or supersaturated with cholesterol (3, 4). Recent work by Thistle and Schoenfield (5) indicated that the administration of chenodeoxycholic acid to patients with cholesterol gallstones results in a significant reduction in the ratio of cholesterol to bile acids and lecithin. Nevertheless, despite the achievement of altering bile composition from saturated to unsaturated with respect to cholesterol, gallstone size appears to diminish only slightly or not at all during several months of treatment, indicating that rapid equilibrium between gallstones and bile may not occur. That is, stone dissolution appears to be rate limiting *in vivo*.

This report presents various possible physical models for gallstone dissolution kinetics. This study was expected to be useful in several ways with regard to gallstone dissolution. First, a semiquantitative perspective developed from these models should help identify possible rate-limiting situations and establish preliminary correlations with already published clinical and *in vitro* data. Second, these models, in conjunction with suitable experiments involving "synthetic" gallstones, should be valuable in designing significant clinical or *in vitro* experiments. And third, the models and the related mathematics should provide the vehicle for the appropriate mechanistic analysis of the data, both clinical and *in vitro*.

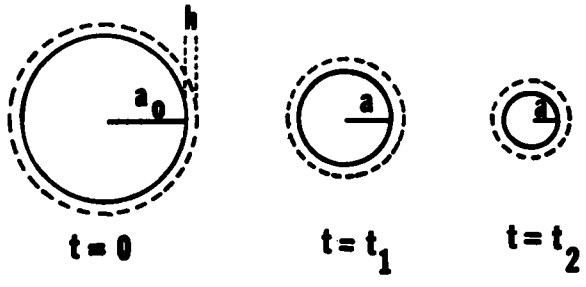

Figure 1—*Simple dissolution case. The radius of the sphere* a *changes with time* t; h *is the liquid diffusion layer thickness.*

Figure 2—*Simple matrix case. Cholesterol embedded in a spherical matrix is dissolved and leached out as a function of time.*

PHYSICAL MODELS

The present discussion is restricted to the problem of the rate of dissolution of "cholesterol" gallstones where the principal constituent of the stones is cholesterol (~70–98%). Thus, the primary processes under consideration are the dissolution and transport of cholesterol from the gallstone and into the solvent. It is assumed that the other constituents (*e.g.*, bile pigments, proteins, and calcium salts) may be important only to the extent that they may provide a barrier to transport of cholesterol.

The fastest dissolving gallstone would be essentially pure cholesterol, where the rate-determining step is the transport of cholesterol from the surface into the solvent. This implies that no significant barriers to transport are present at the solid–solution interface.

This maximum rate situation can be decreased significantly by such factors as interfacial barriers and inert residues which can form a diffusion barrier. Interfacial barriers may arise from intrinsically slow (6) crystal–surface solution equilibria or from adsorbed or deposited substances (7, 8) on the stone surfaces. Gallstones containing appreciable amounts of noncholesterol components may lead to the formation of residue-type barriers (9–12). These barriers arise when the cholesterol is dissolved and leached faster than the slower dissolving or disintegrating noncholesterol substance. The residues then can form a continuous matrix through which the cholesterol must be transported.

Case I: Simple Dissolution Model (No Matrix-Diffusion Barriers or Interfacial Barriers)—This model would be appropriate if either: (*a*) the stone is pure cholesterol or (*b*) the other constituents of the stone slough off sufficiently rapidly from the gallstone surface during cholesterol dissolution.

For simplicity, let the stone have a spherical geometry so that the situation illustrated in Fig. 1 would apply. This model then assumes that during dissolution the solvent adjacent to the surface of the stone is saturated with cholesterol and that the rate of dissolution, J, is governed by the diffusion of cholesterol across the solvent diffusion layer of thickness h. The dissolution rate J is then expressed mathematically by Eq. 1:

$$J = \frac{AD(C_s - C_B)}{h} \qquad \text{(Eq. 1)}$$

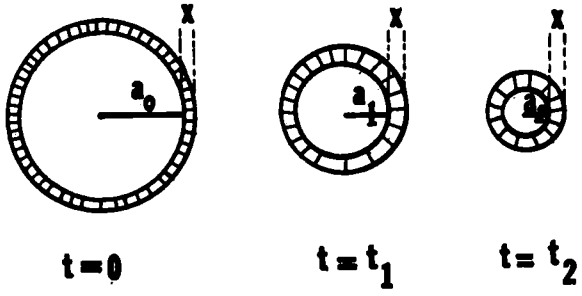

Figure 3—*Interfacial "coating" Model I: gallstone dissolution in the presence of interfacial barrier of thickness* x.

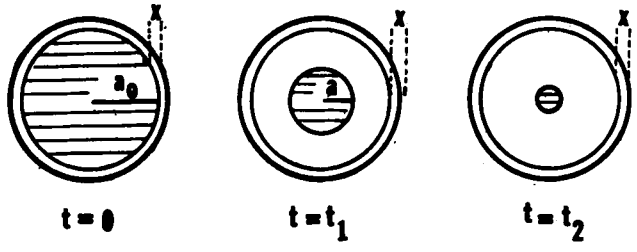

Figure 4—*Interfacial "coating" Model II: gallstone dissolution in the presence of indestructive coating.*

where A = surface area of the sphere, D = diffusion coefficient for cholesterol in the solvent, C_s = solubility of cholesterol, and C_B = concentration of cholesterol in the bulk solvent. J may be also related to the rate of change in mass, W, expressed by Eq. 2:

$$J = -dW/dt = 4\pi a^2 \rho(da/dt) \qquad \text{(Eq. 2)}$$

where ρ = density of cholesterol, and a = radius of the sphere. Therefore, by combining Eqs. 1 and 2 and noting that $A = 4\pi a^2$:

$$\rho \frac{da}{dt} = \frac{D(C_s - C_B)}{h} \qquad \text{(Eq. 3)}$$

In the special case where C_B is constant, Eq. 3 may be integrated to give:

$$a = a_0 - \frac{D(C_s - C_B)}{\rho h} t \qquad \text{(Eq. 4)}$$

where a_0 is the initial ($t = 0$) radius of the gallstone. Equation 4 may also be expressed on a weight basis:

$$W^{1/3} = W_0^{1/3} - \frac{D(C_s - C_B)}{\rho h} \left(\frac{4\pi\rho}{3}\right)^{1/3} t \qquad \text{(Eq. 5)}$$

where W_0 is the initial ($t = 0$) weight of the gallstone, and W is the weight at time t. Equation 5 is a form of the well-known Hixon-Crowell cube-root law (13).

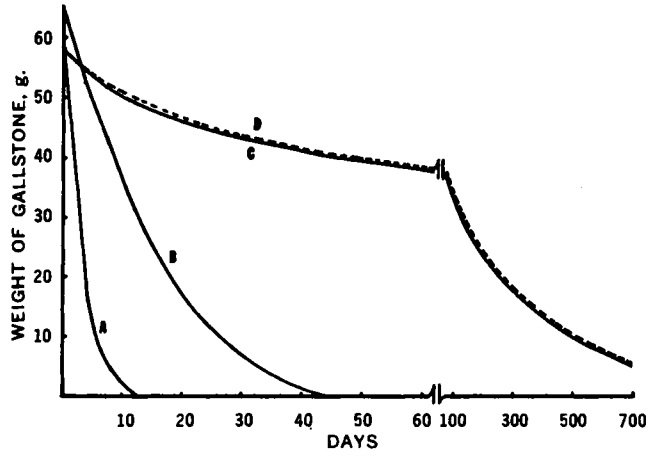

Figure 5—*Calculated dissolution of gallstone of radius* a_0 = *2.5 cm. and solubility* C_s = *26.4 mM/l. with A for Case I and* h = *50 μ, B for Case I and* h = *200 μ, C for Case II and* h = *50 μ, and D for Case II and* h = *200 μ.*

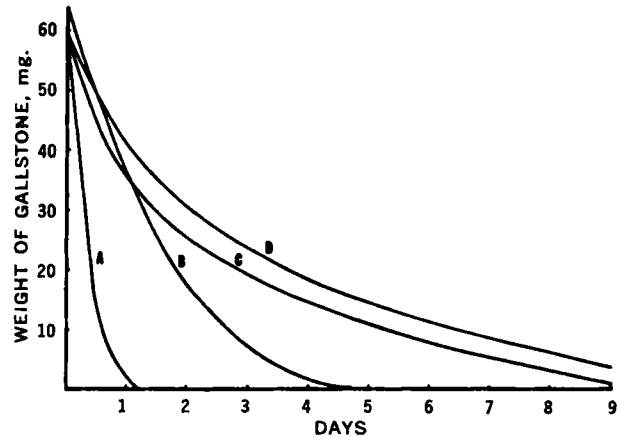

Figure 6—*Calculated dissolution of gallstone of radius* a_0 = *2.5 mm. and solubility* C_s = *26.4 mM/l. See Fig. 5 for meaning of A, B, C, and D.*

The general solution to Eq. 3, *i.e.*, where C_B is not a constant, may easily be obtained (13). If, for example, C_B changes appreciably during the dissolution period, it may be necessary to use the more general equation which is somewhat unwieldy. However, in most of the present discussion, Eqs. 1 and 4 are sufficient.

Case II: Leaching of Cholesterol from Uniform Matrix Model—If an insoluble, nondisintegrating, porous residue remains in place of the original gallstone substance when cholesterol dissolves into the solvent, this model may be appropriate. Figure 2 illustrates this problem for the sphere geometry case.

In this model, the dissolution rate is governed by diffusion of cholesterol from the surface ($r = a$, where r is the distance from the center of the sphere outward) of the cholesterol core through the solvent-filled but leached (no cholesterol) portion ($a \leq r \leq a_0$) of the gallstone and through the solvent diffusion layer of thickness h. It is again assumed that the solvent at $r = a$ is saturated with cholesterol. The rate of cholesterol transport, J, for this case is given by Eq. 6, where it is again assumed that C_B, the cholesterol concentration in the bulk solvent, remains constant during dissolution:

$$J = \frac{4\pi a_0^2 aD}{(\tau/\epsilon)a_0(a_0 - a) + ha}(C_s - C_B)t \qquad \text{(Eq. 6)}$$

where ϵ and τ are the porosity and tortuosity of the leached region ($a \leq r \leq a_0$), respectively. The other symbols have already been defined. Equation 6 thus considers both the effects of the solvent diffusion layer resistance and the matrix-diffusion resistance to cholesterol dissolution.

When Eq. 6 is combined with Eq. 2, one obtains the final expression which relates the size of the cholesterol core to time:

$$-2\left(\frac{h}{a_0} - \frac{\tau}{\epsilon}\right)\left(\frac{a}{a_0}\right)^3 - 3\frac{\tau}{\epsilon}\left(\frac{a}{a_0}\right)^2 + \frac{\tau}{\epsilon} + 2\frac{h}{a_0} = \frac{6D(C_s - C_B)}{a_0^2 S} \qquad \text{(Eq. 7)}$$

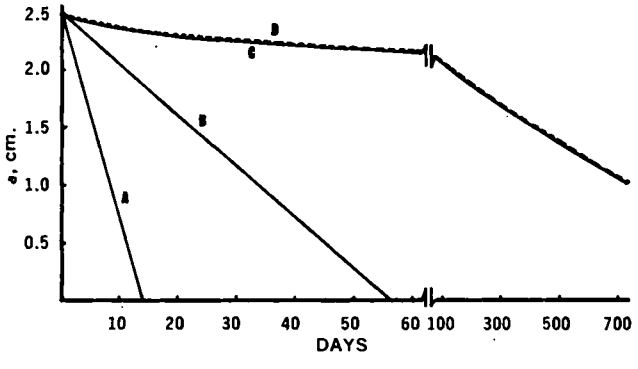

Figure 7—*Calculated dissolution of gallstone with radius* a versus *time* t. *Values of* a_0 = *2.5 cm. and* C_s = *26.4 mM/l. were used. See Fig. 5 for meaning of A, B, C, and D.*

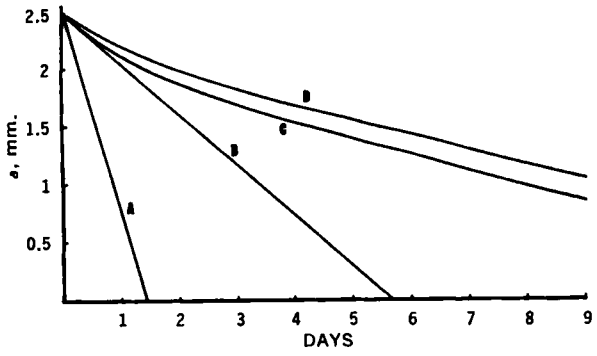

Figure 8—*Calculated dissolution of gallstone with radius* a versus *time* t. *Values of* a_0 = *2.5 mm. and* C_s = *26.4 mM/l. were used. See Fig. 5 for meaning of A, B, C, and D.*

where S = solid concentration of cholesterol in the gallstone matrix. Equation 7 may also be expressed on a weight basis:

$$-2\frac{(4\pi\rho)^{1/3}h}{(3W_0)^{1/3}}-\frac{W}{W_0}-3\frac{\tau}{\epsilon}\left(\frac{W}{W_0}\right)^{2/3}+\frac{\tau}{\epsilon}+\frac{2(4\pi\rho)^{1/3}h}{(3W_0)^{1/3}}=\frac{6(4\pi\rho)^{2/3}D(C_s-C_B)}{(3W_0)^{2/3}S} \quad \text{(Eq. 8)}$$

Case III: Interfacial Barrier or Coat Model I—Figure 3 illustrates a model that may be appropriate when: (*a*) a moderate degree of disintegration and/or dissolution of a residue (matrix) barrier (Case II) takes place, or (*b*) a reversible or collapsible barrier film is present on the stone surface during dissolution. Equation 9 describes the dissolution rate behavior for the sphere situation:

$$W^{1/3}=W_0^{1/3}-\frac{P(C_s-C_B)}{\rho}\left(\frac{4\pi\rho}{3}\right)^{1/3}t \quad \text{(Eq. 9)}$$

It is assumed that the permeability coefficient P of this barrier remains constant. All symbols in Eq. 9 have been defined. The physical meaning of the permeability coefficient P depends upon the particular situation (see *Discussion* section).

Case IV: Interfacial Barrier or Coat Model II—This situation (Fig. 4) might arise if a stable coating (*e.g.*, proteins, calcium phosphates, or calcium carbonate) is present on the stone surface. The outer dimensions do not change with time, while the inner cholesterol dissolves. Equation 10 describes the time change in the weight for this situation:

$$\frac{\epsilon}{\tau}+\frac{2(4\pi\rho)^{1/3}D}{P(3W_0)^{1/3}}-3\frac{\epsilon}{\tau}\left(\frac{W}{W_0}\right)^{2/3}+\left\{2\frac{\epsilon}{\tau}-\frac{2(4\pi\rho)^{1/3}D}{P(3W_0)^{1/3}}\right\}\frac{W}{W_0}=\frac{6(4\pi\rho)^{2/3}(C_s-C_B)\epsilon D}{\tau(3W_0)^{2/3}S} \quad \text{(Eq. 10)}$$

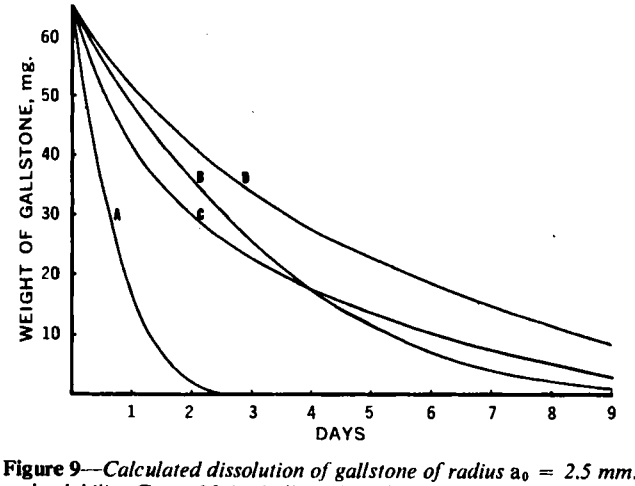

Figure 9—*Calculated dissolution of gallstone of radius* a_0 = *2.5 mm. and solubility* C_s = *26.4 mM/l. with A for Case III and* P = *2* × *10⁻⁴ cm./sec., B for Case III and* P = *5.0* × *10⁻⁵ cm./sec.; C for Case IV and* P = *2* × *10⁻⁴ cm./sec., and D for Case IV and* P = *5.0* × *10⁻⁵ cm./sec.*

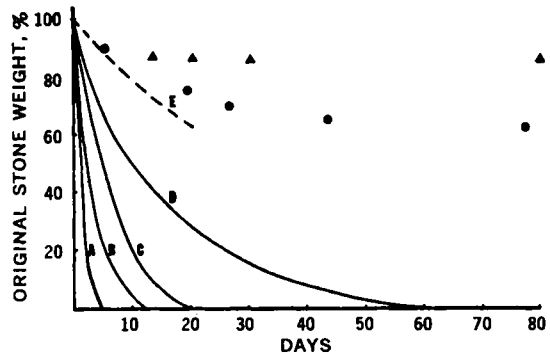

Figure 10—*Comparison of calculated percent gallstone dissolved for cases of* h = *200 μ and Small's (2) data with A for Case I and* C_s = *26.4 mM/l., B for Case II and* C_s = *26.4 mM/l., C for Case I and* C_s = *5.6 mM/l., and D for Case II and* C_s = *5.6 mM/l. Key (Small's data):* ▲, *dissolution in bile salt;* ●, *dissolution in bile salt–lecithin (2:1); and E, bile salt–lecithin corrected to sink condition by changes in* ΔC *with time.*

Here again the physical meaning of the permeability coefficient P depends upon the particular situation.

Other Models—Other models may be developed that relate to physical situations not already presented. Also, very complex combinations are possible. Before much serious consideration is given to very elaborate models, however, those presented in this paper should be evaluated thoroughly.

THEORETICAL CALCULATIONS

While the models and equations in the preceding section may be utilized in a rather rigorous and quantitative manner with experiments on "synthetic" and real gallstones, the present applications of the theoretical relations are concerned primarily with the identification of and "range-finding" in a number of situations. These "order-of-magnitude" calculations should be helpful in suggesting preliminary correlations and expectations and establishing limiting behavior.

Results Involving Case I—Some results of calculations with Eqs. 5 and 6 are shown in Figs. 5–8 (curves A and B). In these calculations, meaningful values for the parameters were selected that correlate to either *in vivo* or *in vitro* situations. The two values for h (50 and 200 μ) should represent reasonable[1] lower and upper limits for moderate and low agitation conditions (14). The two values for a_0, the initial stone radius, are typical of cholesterol stones. The value for the cholesterol solubility (C_s = 26.4 mmoles/l.) was reported to be that for gallbladder bile (15). For the diffusion coefficient D, a value of 1×10^{-6} cm.²/sec. was selected because this is close to that observed for bile salt–cholesterol micelles (16).

These calculations, being based on Case I and sink conditions ($C_B = 0$), represent optimum or maximum dissolution rates. Thus, it is probably significant from a clinical standpoint that it may take about a week to a month for a significant reduction in size of large gallstones ($a_0 \sim 2.5$ cm.) under such optimal conditions. The calculations predict that smaller stones ($a_0 \simeq 0.25$ cm.) may dissolve in a few days under these optimal (Case I and sink) conditions.

Results with Other Models—In curves C and D of Figs. 5–8, results of calculations with Eqs. 7 and 8 (Case II) are presented. As expected, the rates are lower than those for simple dissolution. The effect of agitation (*i.e.*, dependence upon h) is significant for small gallstones following this model but not for large stones.

Figure 9 shows the results of some calculations based on Cases III and IV.

DISCUSSION

The models considered and the equations deduced in this paper should serve as baselines for research on the dissolution kinetics of gallstones *in vitro* and *in vivo*. Experimental results in these laboratories have shown, for example, that gallstones dissolve according

[1] If the medium is much more viscous than water, however, these values should be greater.

to Model I in ethanol–water and acetone–water solvent mixtures. In 5% cholate, the rates appear to be somewhat slower than the predictions of Model I; in bile salt–lecithin solutions, the rates appear to be more than a factor of 10 slower. In Fig. 10, the data of Small (2) are compared to some theoretical calculations. As can be seen, rates based upon Model I appear to be significantly greater than Small's *in vitro* results. These preliminary comparisons of experimental results with the theoretical relationships show that under physiologically important conditions, the simple diffusion-controlled model may or may not be applicable and that careful scrutiny of the various factors is necessary.

From the clinical standpoint, the idea that a 2.5-mm. gallstone might be dissolved in a few days is very appealing. Therefore, the question of why the observed rates might be significantly slower than diffusion controlled is important clinically.

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WILLIAM I. HIGUCHI▲, SOMPOL PRAKONGPAN, and FUDAH YOUNG

Abstract □ Experiments were designed for investigating and comparing the *in vitro* dissolution kinetics of human cholesterol gallstones and cholesterol monohydrate compressed pellets. The dissolution rates were determined in 66% acetone–water, in 63% ethanol–water, in bile acids, and in bile acid–lecithin solutions. It was found that the rates of dissolution of the stones compared well with the dissolution rates obtained with the cholesterol monohydrate pellets in all solvents investigated. The dissolution rates for both stones and pellets in the organic–aqueous solvents were extremely rapid and of the order of magnitude expected for a bulk diffusion-controlled process. In sodium cholate solutions, the dissolution rates were about 2–3 times slower than rates predicted by diffusion theory and the data suggested a modest interfacial resistance to dissolution. The rates obtained in 2% bile acid–1% lecithin solutions were about 17 times slower than diffusion-controlled processes, and these results point to an interfacial barrier to dissolution that may be very important clinically.

Keyphrases □ Gallstones, human—mechanisms of dissolution, dissolution rates in different media, compared to prepared cholesterol pellets, existence of interfacial barriers □ Cholesterol gallstones, human—mechanisms of dissolution, dissolution rates in different media, compared to prepared cholesterol pellets, existence of interfacial barriers □ Dissolution, human cholesterol gallstones—mechanisms, dissolution rates in different media, compared to prepared cholesterol pellets, existence of interfacial barriers □ Diffusion—role in dissolution of human cholesterol gallstones, existence of interfacial barriers □ Interfacial barriers—as a factor in the dissolution of human cholesterol gallstones, mechanisms of dissolution

During recent years much research has been done to evaluate the thermodynamic equilibria existing in the bile acid–lecithin–cholesterol–water systems and their relation to cholelithiasis (1, 2). It has been shown that the degree of cholesterol saturation or supersaturation in gallbladder bile is a critical factor in the formation or dissolution of cholesterol gallstones *in vivo*. While it is now clear that thermodynamic factors can play important roles, relatively little is known about the kinetic factors involved. Questions concerning the *kinetics* of gallstone dissolution have become especially important since the finding of Thistle and Schoenfield (3) and Danzinger *et al.* (4) that oral administration of chenodeoxycholic acid in patients with gallstones can result in the simultaneous "normalization" of bile and the dissolution of stones.

The present article reports results of initial physicochemical investigations on the mechanisms of dissolution of cholesterol gallstones. An attempt was made to determine: (*a*) whether or not the rate of cholesterol gallstone dissolution *in vitro* compares closely to the dissolution rate of "synthetic" gallstones of compressed cholesterol monohydrate pellets, and (*b*) whether or not the dissolution rates of gallstones in various solvent media are governed by diffusion in the bulk or by interfacial factors (5).

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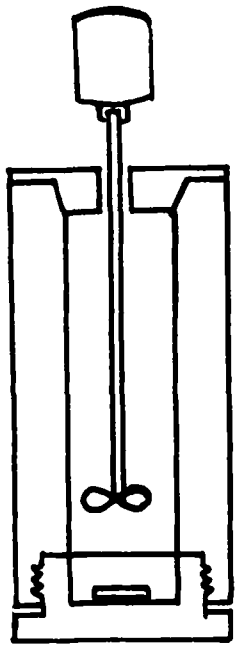

Figure 1—*The diffusion cell.*

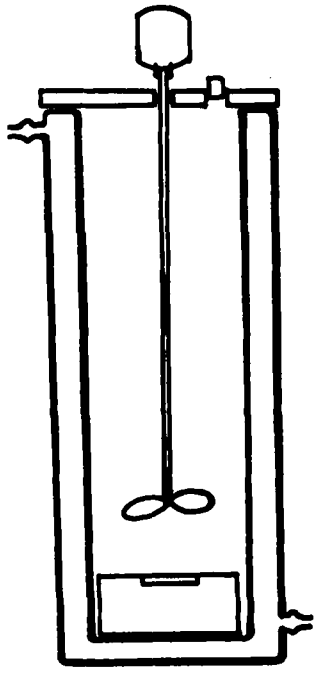

Figure 2—*The dissolution apparatus.*

Table I—Dissolution Rates (mg. cm.$^{-2}$ sec.$^{-1}$ × 10^4) of Human Gallstones[a] and Cholesterol Monohydrate Pellets in Solvent Media at 37°

Gallstones	63% Ethanol	66% Acetone	5% Cholate[b]	5% Glycocholate[b]	2% Cholate[b] and 1% Lecithin
Black					
o[c]	1.10	3.01	1.64	0.51	—
i[d]	2.70	5.28	2.62	0.88	—
Dark brown					
o	2.40	—	1.36	—	—
i	2.57	—	2.90	—	—
Light brown					
o	3.41	5.90	1.20	—	0.24
i	2.58	3.89	2.33	—	0.25
Gray					
o	1.87	3.90	1.94	—	0.17
i	2.33	5.66	2.21	—	0.21
White					
o	2.12	5.28	2.09	—	—
i	2.76	6.66	3.46	—	—
Cholesterol monohydrate pellets	2.60	4.45	1.95	0.34	0.18

[a] Spherical gallstones of diameters ranging from 0.60 to 1.10 cm. were used. [b] Containing 0.1 *M* phosphate buffer. [c] o = dissolution from external surface of gallstone. [d] i = dissolution from cross-sectional cut surface of gallstone.

EXPERIMENTAL

Materials—Radioactive cholesterol monohydrate was prepared by mixing 5 g. of a three-times recrystallized cholesterol[1] with 50 μc. of benzene solution of ^{14}C-cholesterol[2] and drying *in vacuo*. The mixture was dissolved in 400 ml. of 95% ethanol at 60°. It was then seeded with a few crystals at room temperature. After 48 hr., the mixture was filtered and dried *in vacuo* overnight.

Various aqueous compositions of the following materials were used as solvent media. Sodium cholate and glycocholate[3] were used as received. Sodium taurocholate[4] was prepared by the method of Pope (6). Egg lecithin was prepared by the method of Small *et al.* (7), except that commercial egg lecithin of purified grade[5] was used as starting material. Human gallstones were obtained from Hofmann and his group[4].

Solubility Determination—The solubilities of cholesterol monohydrate in various media were determined by introducing an excess amount of radioactive cholesterol monohydrate of about 100 mg. into 20 ml. of a solvent in a 50-ml. volumetric flask. The flask was simultaneously flushed with nitrogen gas and shaken by a wrist-action shaker[6] in a water bath at 37°. An aliquot of 5 ml. was taken and filtered through a 0.22-μ filter[6] in a controlled atmosphere at 37°. Ten milliliters of scintillation fluid (8) was added to 1.0 ml. of the filtrate, and the radioactivity was determined in a scintillation system[7]. Aliquots were taken and assayed for cholesterol daily. Solubility of cholesterol monohydrate in the medium was obtained when the concentration in the filtrate reached a constant level.

Diffusion Coefficient Determination—Measurements were made at 37° in a diaphragm diffusion cell made of Lucite (Fig. 1) and by a method similar to that described by Keller *et al.* (9), except that the two reservoirs were separated by double silver filter membranes[8] of 1.2-μ pore size. The lower reservoir had a capacity of 3.4 ml. and was completely filled with a solution of radioactive cholesterol during operation. The upper reservoir was filled with the same amount of solvent medium. The stirring speed in both reservoirs was maintained at 150 r.p.m. A solution of 0.1 *N* KCl with a known diffusion coefficient[9] of 2.41 × 10^{-5} cm.^{2} sec.$^{-1}$ at 37° was used as standard in the measurements.

Dissolution Rate Determination—*Cholesterol Monohydrate or Benzoic Acid Pellets*—Pellets of radioactive cholesterol monohydrate or benzoic acid were prepared by directly compressing 300 mg. of the material in a die, 1.3-cm. (0.5-in.) i.d., under a force of 3000 lb. using a laboratory press[10]. The surface area of the resulting pellets was 1.267 cm.^{2}. The pellet was held firmly in a die by covering the inside surface with melted paraffin. This die was then mounted at the bottom of a water-jacketed cylinder, with the pellet facing a

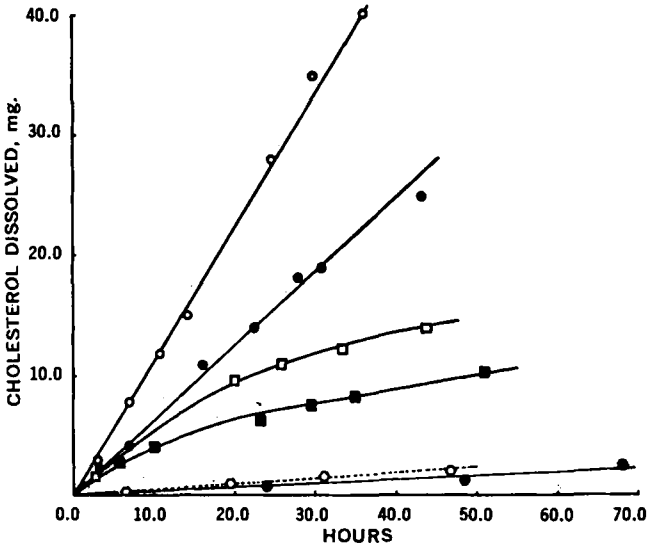

Figure 3—*Dissolution of human cholesterol gallstones at 37° in various solvent media. Key: ○, from external surface; ●, from cross-sectional inner surface of stone in 5% cholate and 0.1 M phosphate, pH 7.4; □, from external surface; ■, from cross-sectional surface of stone in 63% ethanol; ○, from external surface; and ●, from cross-sectional surface in 2% cholate + 1% lecithin + 0.1 M phosphate buffer, pH 7.4.*

[1] Eastman Kodak Co., Rochester, N. Y.
[2] New England Nuclear Corp., Boston, Mass.
[3] Schwarz/Mann, Orangeburg, N. Y.
[4] Supplied by A. F. Hofmann, Gastroenterology Unit, Mayo Clinic and Mayo Foundation, Rochester, Minn.
[5] Burrel Corp., Pittsburgh, Pa.
[6] Millipore Filter Corp., Bedford, Mass.
[7] Beckman Instruments Inc., Fullerton, Calif.
[8] Selas Flotronics, Spring House, Pa.
[9] This value was determined by the method of Saraf *et al.* (10).
[10] Model B, Fred Carver, Inc., Summit, N. J.

Table II—Solubilities (C_s), Diffusion Coefficients (D), Theoretical Dissolution Rates (J_{Th}/A), and Measured Dissolution Rates (J_{obs}/A) of Solutes in Solvent Media at 37°

Solute	Solvent Medium	C_s, mg. cm.$^{-3}$	$D \times 10^6$, cm.^{2} sec.$^{-1}$	$J_{Th}/A \times 10^4$, mg. cm.$^{-2}$ sec.$^{-1}$	$J_{obs}/A \times 10^4$, mg. cm.$^{-2}$ sec.$^{-1}$	J_{Th}/J_{obs}
Cholesterol monohydrate pellet	63% Ethanol	0.26	3.10	1.61	2.60	0.62
	66% Acetone	0.28	—	—	—	—
	2% Cholate[a]	0.53	2.17	2.30	0.67	3.43
	5% Cholate[a]	1.34	1.90	5.09	1.95	2.61
	2% Taurocholate[a]	0.12	2.15	0.52	0.17	3.03
	2% Cholate[a] + 1% lecithin	1.05	1.49	3.13	0.18	17.4
	2% Taurocholate[a] + 1% lecithin	0.51	1.24	1.26	0.075	16.9
Benzoic acid pellet	0.01 N HCl	4.70	14.0	131.0	131.0	1.00

[a] Containing 0.1 M phosphate buffer at pH 7.4.

stirring paddle inserted at the top of the cylinder (Fig. 2). The stirring speed was maintained at 150 r.p.m. during dissolution. Seventy milliliters of solvent preequilibrated at 37° was used. To measure the dissolution rates, the amount of cholesterol or benzoic acid dissolved in the solvent was analyzed against time.

Human Cholesterol Gallstones—Gallstones of various colors and sizes, ranging from 0.6 to 1.1 cm. in diameter, were used. Dissolution rates were measured from both the intact outer surface and the cross-sectioned inner surface by embedding a gallstone in a die with melted paraffin exposing half of the stone to the bulk solvent medium. The inner surface was obtained by cutting a spherical stone into halves cross sectionally. The surface areas for dissolution were estimated according to the specific geometry of the surfaces. The same apparatus and procedures as already described were then used. Samples of 5 ml. were taken and replaced with fresh solvent. Cholesterol in these samples was analyzed by the method of Dam *et al.* (11) or of Sperry and Webb (12) when lecithin was present in the medium.

RESULTS AND DISCUSSION

Figure 3 shows typical raw data obtained for the dissolution of human cholesterol gallstones in several solvent media. As can be seen, the dissolution in some solvents gave nearly linear plots; in these cases, the solvents remained much undersaturated with cholesterol during the dissolution period. In other solvents, apparent bending of the dissolution curves occurred as a result of the more rapid increase in cholesterol saturation with time.

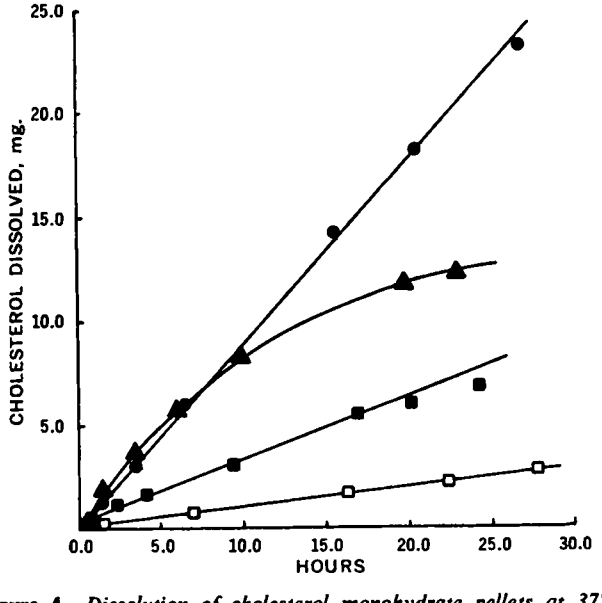

Figure 4—*Dissolution of cholesterol monohydrate pellets at 37°. Key: ●, in 5% cholate and 0.1 M phosphate buffer, pH 7.4; ■, in 2% cholate + 0.1 M phosphate buffer, pH 8.0; □, in 2% cholate + 1% lecithin + 0.1 M phosphate buffer, pH 8.0; and ▲, in 63% ethanol.*

The dissolution rate data of the compressed pellets of cholesterol monohydrate crystals are shown in Fig. 4. The dissolution patterns in Fig. 4 are similar to those in Fig. 3 for the gallstones. The dissolution rates (in mg. cm.$^{-2}$ sec.$^{-1}$) were obtained by taking the initial slopes of the dissolution curves such as those in Figs. 3 and 4 and correcting for the surface area.

Correlation between Gallstone and Cholesterol Monohydrate Dissolution Rates—The dissolution rates of various human gallstones and cholesterol monohydrate pellets in the organic-aqueous solvents and the bile media are shown in Table I. In each solvent studied, the dissolution rates of gallstones were found to compare closely with those for cholesterol monohydrate pellets. The variations in the gallstone dissolution rates, with a few exceptions, are well within a factor of two of the dissolution rates for the cholesterol monohydrate pellets in all solvents.

The dissolution rates determined from the inner cross-sectional surfaces were, in general, greater than those determined from the external surfaces. These differences and the variations found among the stones may be due to deposited film and matrix-forming substances derived from the biliary environment.

In view of the reported cholesterol content in human gallstones running as high as 98% (13), the consistent agreement between the dissolution rates of gallstones and cholesterol monohydrate pellets is not surprising. The data in Table I show that cholesterol monohydrate appears to be a good experimental model for the dissolution of the gallstones in these experiments. The presence of insoluble residues, bile pigments, mucus, and proteins may create physical situations where the rate of dissolution can be affected (5).

Diffusion-Controlled Model—In Table II, the experimental dissolution rate data for the cholesterol monohydrate pellets are compared to the dissolution rates predicted by the Nernst equation (14):

$$J = \frac{ADC_s}{h} \qquad \text{(Eq. 1)}$$

where A is the surface area of the pellet, D is the diffusivity of cholesterol in the solvent, C_s is the solubility of cholesterol monohydrate in the solvent, and h is the effective diffusion layer thickness. The theoretical rate calculations for J/A involved the use of Eq. 1 and the experimental D and C_s values, with an h value of 50 μ determined from a benzoic acid pellet dissolution experiment. This analysis points out several interesting aspects of the dissolution kinetics. First, the dissolution rates in the organic-aqueous solvents are maximal, *i.e.*, essentially diffusion controlled. Second, in the bile acid media, the rates are about 2–3 times slower than diffusion-controlled processes for the hydrodynamic situation prevailing in those experiments. Finally, when the solvent is 2% bile acid–1% lecithin, the rates are about 17 times slower than diffusion-controlled values and a very large interfacial barrier clearly dominates the kinetics.

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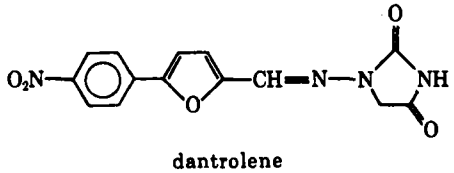
Keyphrases □ Dantrolene—effects on skeletal muscle, CNS, and smooth or cardiac muscle □ Skeletal muscle relaxants, potential—dantrolene, proposed site of action

The synthesis of a series of 1-[(5-arylfurfurylidene)-amino]hydantoins and their identification as skeletal muscle relaxants were reported by Snyder *et al.* (1). One member of this series, dantrolene, 1-{[5-(*p*-nitrophenyl)furfurylidene]amino}hydantoin, subsequently received further evaluation (2, 3). This report details the general pharmacology of dantrolene and the proposed site of action as a skeletal muscle relaxant[1].



dantrolene

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[2] Taconic Farms.
[3] Grass SIU-5.
[4] Grass S88.
[5] Grass FT 10.
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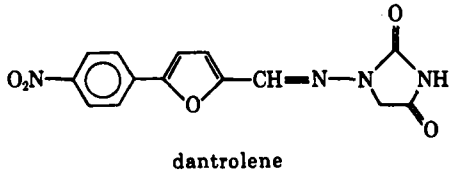

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Cross-Circulation Studies—Mongrel dogs of either sex were anesthetized with sodium pentobarbital (35 mg./kg. i.v.), tracheotomized, and prepared for trunk-to-limb cross-circulation studies similar to these described by Margolin *et al.* (8). Contractions of the tibialis cranialis muscle in the perfused hindlimb were induced and recorded as described for the cat flexor-reflex studies. Drugs were administered: (*a*) to the recipient animal to evaluate the central effects of the compounds, and (*b*) to the donor animal to test peripheral inhibition. Drugs administered to the recipient dog reached only the spinal cord, while drug administered to the donor reached the perfused hindlimb with its motor nerve and contracting muscle.

Indirect versus *Direct Muscle Stimulation*—Adult Sprague-Dawley male rats were anesthetized with allobarbital-urethan[7], 1 ml./kg. i.p., and artificially respired. Carotid blood pressure was monitored, and an external jugular vein was cannulated for drug injections. The Achilles tendon was isolated and detached with a piece of the calcaneous bone at its insertion, and the gastrocnemius muscle was freed from surrounding tissue. The free tendon was connected with a ligature to a force-displacement transducer[8], and muscle contractions were recorded on a dynagraph[9]. Indirect stimulation of the muscle was accomplished through the peripheral stump of the severed sciatic nerve. Square-wave stimuli of 10-msec. duration, 0.33 Hz., and at supramaximal voltage were delivered by the apparatus described in the cat flexor-reflex studies. The muscle was stimulated directly with stainless steel needle electrodes[10] inserted directly into the muscle, with the anode in the belly and the cathode in the Achilles tendon. Stimulus parameters were as already stated, but the voltage was adjusted so that magnitude of the contraction response approximated that produced with indirect stimulation. The nerve and muscle were kept moist with frequent applications of warm (37°) mineral oil saturated with Krebs-Ringer's solution.

Denervated Muscle (*9*)—Under ether anesthesia the right sciatic nerve in rats (adult Sprague-Dawley male) was severed and the animals were allowed to recover. Fifteen days later, gastrocnemius muscle contractions were elicited by direct muscle stimulation as already described.

Decerebrate Cat (*10*)—Cats were anesthetized with ether, and a midline incision was made from the base of the nose to the occiput. After trephination the skull bone was removed to expose the right cerebral hemisphere and, by using the tentorium as a guide, the brain stem was sectioned at this level with a spatula. The right hemisphere and then the left were removed, and the cranial vault was lightly packed with cotton.

Decerebrate rigidity ensued when the animal was positioned with its anterior-posterior axis vertical to the horizon and the head was allowed to fall backward.

Other Neuropharmacological Tests—Hexobarbital, 80 mg./kg. i.p., and paraldehyde, 0.8 ml./kg. i.p., "sleep" times were evaluated in mice by the methods of Cook *et al.* (11) and Iyer (12), respectively. Maximal electroshock seizures, produced by a method similar to that described by Swinyard *et al.* (13), and pentylenetetrazol- or strychnine-induced convulsions (14) were utilized as anticonvulsant tests in mice.

Local anesthetic activity was evaluated by the procedures of Wielding (16) on rabbit cornea and by an intradermal method in guinea pigs (17).

Autonomic Pharmacology—Anesthetized dogs (sodium pentobarbital, 35 mg./kg. i.v.) were tracheotomized and placed on artificial respiration, and a thoracotomy was performed through the fifth intercostal space. Coronary blood flow was measured with an extracorporeal flowmeter probe[11], and myocardial contractile force was evaluated with a strain gauge arch[12] in the usual manner. A femoral artery and vein were cannulated for recording of blood pressure and the injection of drugs, respectively. Doses of acetylcholine chloride (1 mcg./kg.), histamine phosphate (2 mcg./kg.), or epinephrine bitartrate (4 mcg./kg.) were administered several times before and at various times after dantrolene or vehicle (polyethylene glycol 300).

Nonvascular smooth muscle activity was evaluated on the GI tract of dogs by the procedures of Goldenberg and Burns (18).

[7] Dial-Urethane, Ciba Pharmaceutical Co.
[8] Grass FT 03.
[9] Type R. S., Beckman.
[10] Grass subdermal E-2.
[11] Medican.
[12] Walton-Brodie.

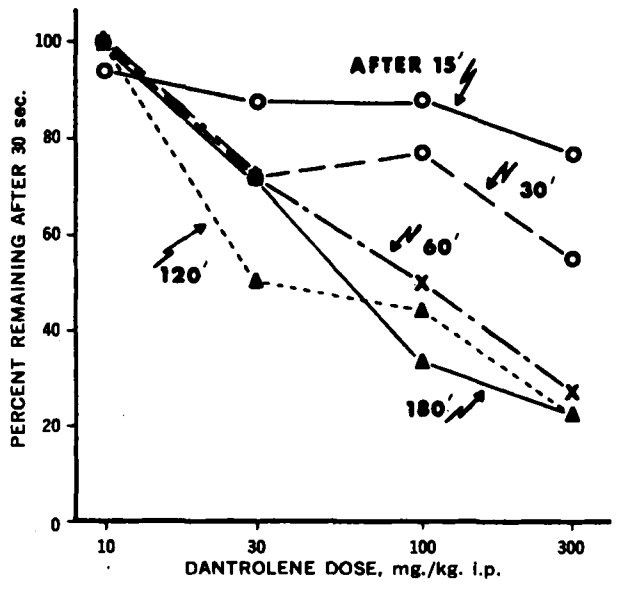

Figure 1—*Dantrolene effects on muscle incoordination in mice using the rotarod (20 r.p.m.), 18 trials per dose-time interval.*

Drugs—The compounds used were: acetylcholine chloride[13], physostigmine salicylate[13], allobarbital with urethan[7], α-chloralose[14], barium chloride[15], paraldehyde[15], dantrolene[16], decamethonium bromide[17], tubocurarine[17], histamine phosphate[17], epinephrine bitartrate[18], sodium hexobarbital[18], mephenesin[19], sodium pentobarbital[20], pentylenetetrazol[21], and strychnine sulfate[22].

RESULTS AND DISCUSSION

Dantrolene, at doses from 10 to 1600 mg./kg. i.p. in mice, induced dose-dependent decreases in spontaneous motor activity, muscle tone (leg and abdomen), and responsiveness to external stimuli. Movements became slow and occurred only when the

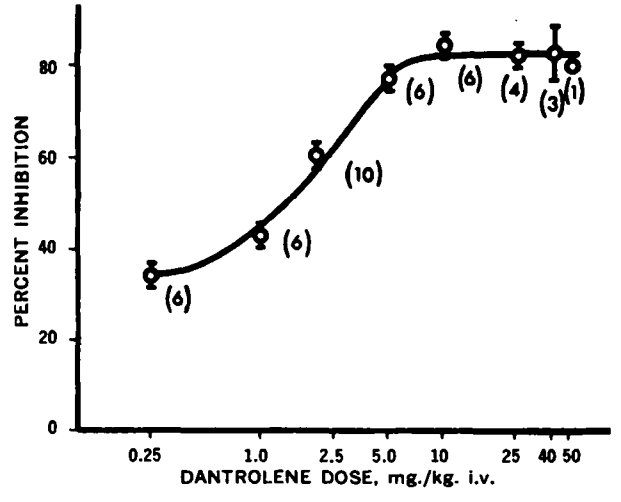

Figure 2—*Dantrolene inhibition of indirectly induced twitch contractions of rat gastrocnemius muscle. Shown are mean values and standard deviations (vertical bars); the number of animals is given in parentheses.*

[13] Merck and Co.
[14] Fisher Scientific Co.
[15] Matheson, Coleman and Bell.
[16] Norwich Pharmacal Co.
[17] Burroughs Wellcome Co.
[18] Winthrop Laboratories.
[19] E. R. Squibb and Sons.
[20] Abbott Laboratories.
[21] Knoll Pharmaceutical Co.
[22] Mallinckrodt Chemical Works.

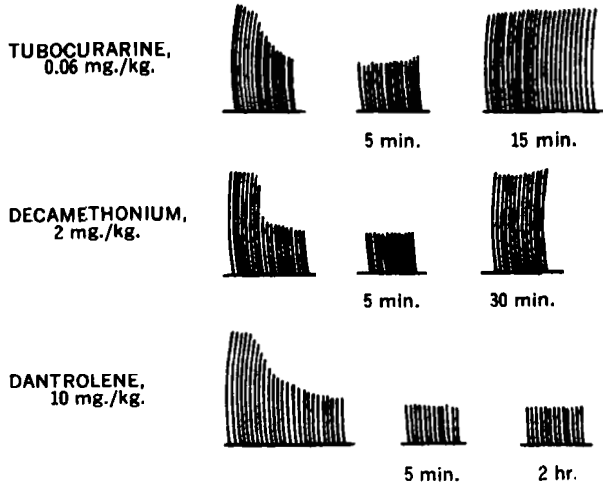

Figure 3—*A comparison of tubocurarine and decamethonium inhibition of the indirect twitch response in the rat with the inhibition caused by dantrolene.*

animals were prodded. Muscle relaxation at doses >200 mg./kg. was present for longer than 8 hr., and at doses >400 mg./kg. it was present for >18 hr. No deaths occurred after 72 hr. The only autonomic effects observed were increased frequency of urination and defecation. The urine was dark yellow following >40 mg./kg. i.p., and there were indications of chromodacryorrhea at >80 mg./kg. i.p. Similar effects in the whole animal were seen with other routes of administration (oral and intravenous) and in other species of animals (rats, cats, and dogs).

Dantrolene effects on motor coordination were evaluated using the rotarod (Fig. 1). The percentage of animals remaining on the rotarod for 30 sec. was plotted for four doses at time intervals up to 180 min. The time to peak drug effect was 60 min.; at that time, when the dose range was expanded, the ED_{50} for muscle incoordination was calculated as 153 mg./kg. i.p. with 19/20 confidence limits of 86 and 271 mg./kg. This ED_{50} for muscle incoordination was considerably above the intraperitoneal dose producing observable muscle relaxation (10–20 mg./kg.) and indicated that these two effects were readily separable.

Dantrolene's production of dose-dependent decreases in spontaneous motor activity, responsiveness to external stimuli, and skeletal muscle relaxation characterized a "sedative-like" profile. However, this profile was atypical in that sedation was secondary to muscle relaxation rather than the reverse, as characterizes sedative and hypnotic drugs. Muscle relaxation was the most pronounced and consistent feature. Unlike the centrally acting muscle relaxants and sedative and hypnotic drugs, dantrolene had no effect on the corneal and pinna reflexes and caused no loss of righting reflex.

Dantrolene (5–100 mg./kg. p.o.) increased the duration of hexobarbital and paraldehyde sleep times at doses >25 mg./kg. Since both methods are of a nonspecific nature and measure only the return of righting reflex, a long acting skeletal muscle relaxant would be expected to prolong such sleep times. Dantrolene (500–1500 mg./kg. p.o.) inhibited maximal electroshock seizures only 40% at 1500 mg./kg., but it had no effect on pentylenetetrazol-

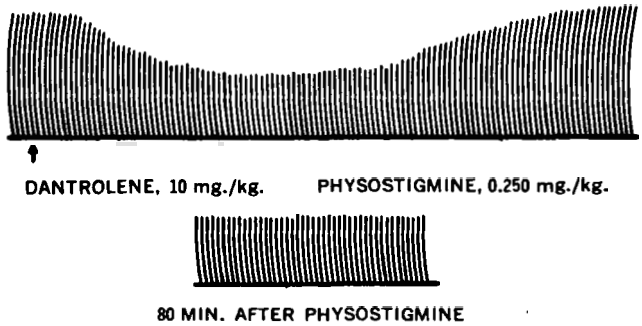

Figure 4—*Physostigmine effects on dantrolene depression of the indirect twitch response in the rat.*

Table I—Dantrolene Effects on Muscle Contractions in the Perfused Hindlimb of the Dog

Drug[a]	*n*	Percent Inhibition
Dantrolene (mg./kg.):		
1.0, monosynaptic peripheral response	3	63
2.5, polysynaptic central response	4	4
2.5, monosynaptic peripheral response	1	84
Mephenesin (mg./kg.):		
30, polysynaptic central response	4	75.2

[a] Dissolved in polyethylene glycol 300, all volumes <1.5 ml./kg.

or strychnine-induced convulsions at doses up to 500 mg./kg. p.o. or i.p. Decreased muscle strength was also observed in the rat analgesic test with 100–500 mg./kg. i.p. Although these doses produced significant muscle relaxation and slowed the reaction time for the tail withdrawal, the response was never blocked. No local anesthetic activity was observed with dantrolene (2–600 mg./ml.) as evaluated on rabbit cornea or by intradermal injections in guinea pigs.

No effect on blood pressure, coronary blood flow, heart rate, or contractile force was elicited by dantrolene (1–10 mg./kg. i.v.) in anesthetized dogs. Moreover, the cardiovascular responses induced by the various autonomic drugs were not affected by dantrolene. Only a slight increase and decrease of short duration in the contractile activity of the duodenum and colon, respectively, were observed after dantrolene (0.5–5 mg./kg. i.v.). There was no effect on the ileum. A slight antagonism of barium chloride-induced spasticity (19) of the ileum and colon was produced by dantrolene.

Dantrolene's muscle relaxant activity led to its being evaluated specifically as a skeletal muscle relaxant. Decerebrate cats that evidenced rigid extension of their legs and catatonic contraction of the trunk musculature were treated with dantrolene intravenously. At a dose of 1.8 mg./kg., the extensor rigidity was markedly reduced for a duration of 35 min., while 4.0 mg./kg. had the same effect for 85 min. Decerebrate rigidity was inhibited totally with 5 mg./kg. in two animals, whereas reduction in the rigidity and tremors, but not complete abolition, was observed in three other cats treated with the same dose. All animals treated with dantrolene (1.8–5 mg./kg.) exhibited at least some reduction in rigidity.

Centrally acting skeletal muscle relaxants are well known (7) for their preferential inhibition of polysynaptic reflex functions. This type of action is readily demonstrated in flexor-reflex preparations in which drug effects on monosynaptic and polysynaptic reflexes can be determined simultaneously. Dantrolene (0.25–10 mg./kg. i.v.) was studied in such preparations, and the results indicated that dantrolene had no preferential effect on polysynaptic reflex responses.

It was further demonstrated that dantrolene produced its effect on skeletal muscle without central actions (inhibition of polysynaptic transmission or otherwise) in the isolated perfused hindlimb of the dog in the cross-circulation preparation. Dantrolene (2.5 mg./kg.) was administered to the recipient dog and found to produce no effect (Table I) on either the sensory- or motor nerve-induced contractions. Mephenesin (30 mg./kg.) administered to the recipient dog for comparison inhibited contractions elicited *via* the polysynaptic pathway by 75%. Following mephenesin, when muscle contractions had returned to control levels (after 1 hr.), dantrolene (1.0 mg./kg.) was administered to the donor dog (periph-

Table II—Effect of Dantrolene, Tubocurarine, and Decamethonium on Denervated Rat Gastrocnemius Muscle

Compound	Dose, mg./kg. or ml./kg.	*n*	Percent Inhibition ± *SE*
Dantrolene	2.5	6	39.1 ± 15.
Polyethylene glycol 300 control	1.0	3	9.7 ± 1.9
Tubocurarine	0.05	6	4.0 ± 1.1
Decamethonium	1.0	6	61.3 ± 2.8
Saline control	1.0	3	0

eral) and the muscle contractions were reduced in the recipient dog by 63 and 84% at 1.0 and 2.5 mg./kg., respectively. This lack of effect in the recipient dog and the marked effect in the donor animals clearly demonstrated that only the peripheral elements of the twitch response were affected by dantrolene.

The peripheral elements of the twitch response were studied in the gastrocnemius muscle stimulated through its motor nerve (Fig. 2). There was a wide effective dose range that caused twitch inhibition, and the dose–response relations were unique for this type of preparation in that the dose–response curve plateaued at less than 100% inhibition of twitch. Increasing the dose of dantrolene to 50 mg./kg. (five times the maximally effective dose) did not increase the level of twitch inhibition.

The inhibition of the indirect twitch response produced by dantrolene was not unlike that produced by neuromuscular blocking agents. This was studied further by comparing dantrolene (0.3–15.0 mg./kg.) with the competitive antagonist tubocurarine (0.01–0.1 mg./kg.) and the depolarizing blocker decamethonium (0.5–15 mg./kg.). The responses at roughly equieffective doses (producing 40–60% level of twitch inhibition) are illustrated in Fig. 3. The time course of the development of the twitch inhibition was similar for all three agents, but the duration of action of the dantrolene was much greater (>2 hr.) than that produced by either tubocurarine (15 min.) or decamethonium (30 min.). Complete abolition of the twitch response was never accomplished with dantrolene at any dose, whereas it was readily accomplished with curare and decamethonium at 0.075 and 15 mg./kg., respectively.

Anticholinesterase agents potentiate the twitch response and antagonize the twitch inhibition induced by competitive neuromuscular blockers such as tubocurarine (20). They do not antagonize the effects of depolarizing blocking agents such as decamethonium (20). The twitch inhibition produced by dantrolene was found to be similar to that of tubocurarine in that physostigmine (an anticholinesterase agent) reversed the twitch depression for a short time (Fig. 4). After the addition of physostigmine, the twitch response steadily increased for 2 min., at which time the twitch height was at the predantrolene level. The physostigmine effects lasted for 80 min., when the twitch response returned to the dantrolene-treated level.

Physostigmine antagonism of dantrolene twitch depression indicated that dantrolene did not produce a depolarizing blockade like decamethonium and implied that dantrolene had anticholinergic properties. However, this anticholinergic character was not substantiated when the effects on denervated muscle were studied. Dantrolene and decamethonium inhibited the twitch response, while curare did not (Table II). (Vehicle control values are included for comparison because of the extreme sensitivity of the preparation to twitch inhibition by a variety of chemical agents.)

The effectiveness of dantrolene in denervated muscle indicated that it did not require neuromuscular transmission to produce its effects and that the reversal of the twitch depression by physostigmine was a physiological type antagonism. Greater quantities of acetylcholine, causing twitch potentiation, masked the dantrolene depression until the effects of the anticholinesterase had disappeared after 80 min. and dantrolene twitch depression was again evident.

The physiological antagonism by physostigmine and the lack of neuromuscular blocking properties pointed to a site of action of dantrolene that was beyond the neuromuscular junction. This post-neuromuscular junction action of dantrolene was tested in experiments with direct and indirect twitch responses in the same preparation. At a dose of 2.5 mg./kg. i.v., dantrolene inhibited the direct twitch response by 56.8 ± 7.6%, while the indirect twitch responses were attenuated by 57.1 ± 3.0%. Although stimulation of the muscle undoubtedly released acetylcholine from some nerve terminals, the responses were >70% direct (75–80% of the direct response remained in the totally curarized muscle). Equal inhibition of both responses confirmed that the blocking action of dantrolene was beyond the neuromuscular junction.

CONCLUSIONS

Dantrolene is a new type of skeletal muscle relaxant. It has a specific action on skeletal muscle that is beyond the neuromuscular junction. It represents a new class of skeletal muscle relaxants and should be a useful tool for studying skeletal muscle contraction mechanisms.

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Hypocholesterolemic Agents II: Inhibition of β-Hydroxy-β-methylglutaryl Coenzyme A Reductase by Arylalkyl Hydrogen Succinates and Glutarates

MARVIN R. BOOTS*▲, SHARON G. BOOTS*, CAROLE M. NOBLE†, and KENNETH E. GUYER†

Abstract □ A series of arylalkyl hydrogen succinates and glutarates was synthesized and assayed for inhibition of yeast β-hydroxy-β-methylglutaryl coenzyme A reductase. The following compounds were found to be particularly active as inhibitors: 1-(4-biphenylyl)-pentyl hydrogen succinate (5S), 1-(4-biphenylyl)-hexyl hydrogen succinate (6S), 1-(4-biphenylyl)-pentyl hydrogen glutarate (5G), 1-(2-fluorenyl)-pentyl hydrogen succinate (13S), and 1-(2-fluorenyl)-pentyl hydrogen glutarate (13G). Further studies with Compound 5S indicated that the inhibition was reversible and noncompetitive with respect to β-hydroxy-β-methylglutaryl coenzyme A and NADPH. Dixon and modified Hill plots were used to calculate a cooperativity number of 3 for 5S.

Keyphrases □ Arylalkyl hydrogen succinates and glutarates—synthesis, inhibition of yeast β-hydroxy-β-methylglutaryl-CoA reductase □ β-Hydroxy-β-methylglutaryl-CoA reductase—inhibition by arylalkyl hydrogen succinates and glutarates □ Inhibition of yeast β-hydroxy-β-methylglutaryl-CoA reductase—synthesis and evaluation of arylalkyl hydrogen succinates and glutarates □ Hypocholesterolemic agents—inhibition of β-hydroxy-β-methylglutaryl-CoA reductase by arylalkyl hydrogen succinates and glutarates

Atherosclerosis and its complications constitute a growing problem in public health. Although the term atherosclerosis is ill defined (1), it is generally agreed to include lipid accumulation within the arterial intima as one of its early causative factors.

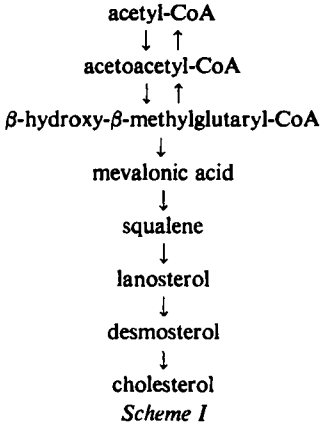
The discovery of cholesterol in atherosclerotic arteries (2) coupled with the observation of experimentally induced atherosclerosis after cholesterol feeding (3) provided some of the first indications of the implication of cholesterol in atherosclerosis and a suggestion of its causative role.

Because of the importance attached to the plasma cholesterol level in atherosclerosis, many approaches (4, 5) have been investigated to control it. These methods may be summarized as: (*a*) decreased ingestion of cholesterol, (*b*) decreased absorption of cholesterol, (*c*) increased conversion of cholesterol to bile acids, and (*d*) decreased synthesis of cholesterol.

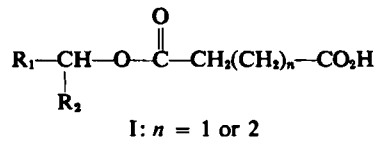
The inhibition of the endogenous cholesterol synthesis could lead to a lowering of the serum level. A brief outline of the biosynthetic pathway is shown in Scheme I.

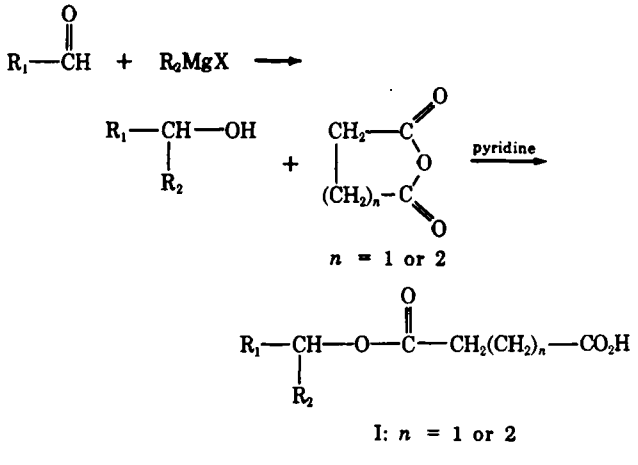
The specific step of the biosynthetic pathway to be inhibited or blocked is an important factor (6). Inhibition of the first portion of the pathway (acetyl-CoA → acetoacetyl-CoA) is not desirable because acetoacetyl-coenzyme A (CoA) is involved in fatty acid biosynthesis. Antimetabolites related to acetoacetyl-CoA would possibly tend to inhibit fatty acid biosynthesis as well as β-hydroxy-β-methylglutaryl-CoA synthesis; thus the earliest feasible step for control would appear to be the conversion of β-hydroxy-β-methylglutaryl-CoA to mevalonic acid. Later possible sites of inhibition would involve the reaction sequence between mevalonic acid and squalene. However, it appears that any site after mevalonic acid requires almost a complete enzyme inhibition to affect significantly overall cholesterol biosynthesis (7). If the pathway is blocked after squalene synthesis, large amounts of the intermediate accumulate since the reactions after β-hydroxy-β-methylglutaryl-CoA synthesis are irreversible. This accumulation has led to serious toxic effects, as in the case of triparanol (6).

Thus, the conversion of β-hydroxy-β-methylglutaryl-CoA to mevalonic acid seems to be the ideal



Scheme I

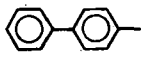
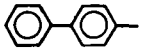
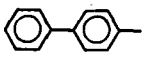
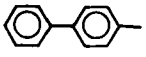
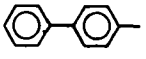
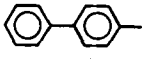
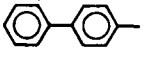
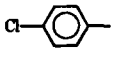
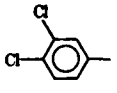
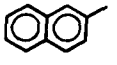
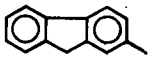
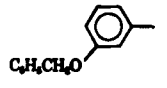
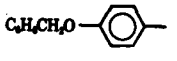




Scheme II

Table I—Properties of Alkylarylcarbinols

$R_1—CHOH$
R_2

Compound Number	R_1	R_2	Yield, %	Melting Point or Boiling Point/mm.	Formula	Analysis, % Calc.	Found
1		H—	—	99–101°[a]	—	—	
2		CH_3—	86	96–98°[b]	—	—	
3		CH_3CH_2—	63	57–59°[c]	—	—	
4		$CH_3(CH_2)_2$—	58	59–61°	$C_{16}H_{18}O$	C 84.9 H 8.0	85.1 8.3
5		$CH_3(CH_2)_3$—	89	78–79°	$C_{17}H_{20}O$	C 85.0 H 8.3	85.1 8.4
6		$CH_3(CH_2)_4$—	69	81–82°	$C_{18}H_{22}O$	C 85.0 H 8.7	85.3 9.1
7		$CH_3(CH_2)_5$—	73	61–63°	$C_{19}H_{24}O$	C 85.1 H 9.0	84.7 9.1
8		$CH_3(CH_2)_6$—	—	100–104°/3[a]	—	—	
9		$CH_3(CH_2)_6$—	67	110°/1[d]	—	—	
10		$CH_3(CH_2)_6$—	94	135°/0.2	$C_{11}H_{14}Cl_2O$	C 56.7 H 6.0	56.5 6.0
11		$CH_3(CH_2)_6$—	69	63–65°[e]	—	—	
12		$CH_3(CH_2)_6$—	58	49–50°	$C_{15}H_{18}O$	C 84.0 H 8.4	83.8 8.3
13		$CH_3(CH_2)_6$—	90	102–102.5°	$C_{18}H_{20}O$	C 85.7 H 7.9	85.6 8.3
14		$CH_3(CH_2)_6$—	73	189°/0.3	$C_{18}H_{22}O_2$	C 80.0 H 8.1	79.8 7.9
15		$CH_3(CH_2)_6$—	92	67–69.5°	$C_{18}H_{22}O_2$	C 80.0 H 8.1	80.1 8.2

[a] Sample obtained from Aldrich Chemical Co. [b] Lit. (20) m.p. 98–99°. [c] Lit. (13) m.p. 58°. [d] Lit. (13) b.p. 164°/27. [e] Lit. (21) m.p. 65–66°.

point for the inhibition of cholesterol biosynthesis. The selection of this step is based on the following reasons:

1. All reactions prior to mevalonic acid synthesis are either reversible directly or reversible through other enzymatic pathways. Therefore, the problem of accumulation of intermediates is eliminated.

2. This conversion is the rate-limiting step in the synthesis of cholesterol.

3. The physiological regulation of hepatic cholesterogenesis appears to be the result of a feedback inhibition of this step (8, 9) by cholesterol or a lipoprotein containing cholesterol by decreasing the amount, rather than simply the activity, of β-hydroxy-β-methylglutaryl-CoA reductase (10).

The enzyme β-hydroxy-β-methylglutaryl-CoA reductase (E.C.1.1.1.34), which catalyzes this step, is readily available from yeast (11, 12). This enzymatic reduction can be measured spectrophotometrically and thereby provides an *in vitro* assay.

Palazzo *et al.* (13) and Bizzi *et al.* (14) reported that arylalkyl hydrogen succinates and glutarates (I) exhibited hypocholesterolemic activity in rats and also markedly inhibited the *in vitro* incorporation of acetate and mevalonate into both cholesterol and fatty acids. These data suggest that multiple sites of inhibition are involved. The similarity of β-hydroxy-β-methylglutaryl-CoA to the inhibitors (I) in terms of a free carboxyl group, an esterified carboxyl group, and the distance separating these two functional groups suggests that one site of inhibition may be the conversion of β-hydroxy-β-methylglutaryl-CoA to mevalonic acid. Therefore, a series of arylalkyl hydrogen succinates and

Table II—Properties of Succinates and Glutarates

$$R_1\text{—}\underset{\displaystyle R_2}{\overset{\displaystyle |}{C}}HO_2CCH_2(CH_2)_nCO_2H$$

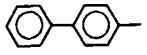
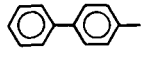
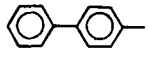
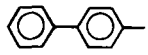
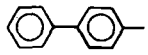
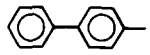
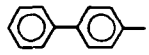
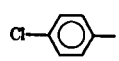
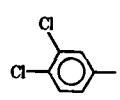
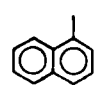
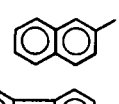
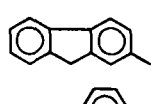
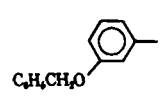
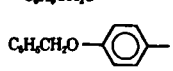
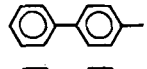
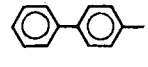
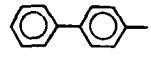
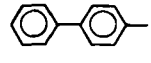
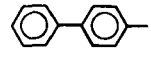
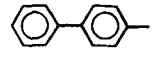
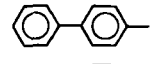
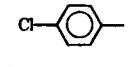
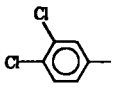
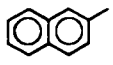
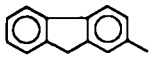
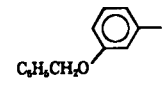
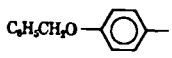
Compound Number	R_1	R_2	Yield, %	Melting Point	Melting Point (Salt)	Formula	—Analysis, %[a]— Calc.	Found
			Succinates, $n = 1$					
1S		H—	98	135–138°	—	$C_{17}H_{16}O_4$	C 71.8 H 5.6	71.7 5.6
2S		CH_3—	60	109–111°	—	$C_{18}H_{18}O_4$	C 72.5 H 6.0	72.7 6.4
3S		CH_3CH_2—	27	64–66°[b]	—	—	—	—
4S		$CH_3(CH_2)_2$—	45	73–74°	—	$C_{20}H_{22}O_4$	C 73.6 H 6.6	73.5 6.9
5S		$CH_3(CH_2)_3$—	68	77–79°[c]	—	—	—	—
6S		$CH_3(CH_2)_4$—	60	59–61°	—	$C_{22}H_{26}O_4$	C 74.5 H 7.4	74.6 7.7
7S		$CH_3(CH_2)_5$—	66	49–52°	—	$C_{23}H_{28}O_4$	C 75.0 H 7.6	75.4 7.9
8S		$CH_3(CH_2)_3$—	67	Oil	127–128°	$C_{22}H_{30}N_2O_4S$	C 64.2 H 7.0 N 6.5	64.0 7.0 6.3
9S		$CH_3(CH_2)_3$—	76	Oil	—	$C_{15}H_{19}ClO_4$	C 60.7 H 6.4	60.6 6.5
10S		$CH_3(CH_2)_3$—	33	Oil	123–123.5°	$C_{32}H_{28}Cl_2N_2O_4S$	C 55.3 H 5.6 N 5.6	55.2 5.6 5.5
11S		$CH_3(CH_2)_3$—	67	Oil	118–118.5°	$C_{27}H_{32}N_2O_4S$	C 67.5 H 6.7 N 5.6	67.4 6.8 5.8
12S		$CH_3(CH_2)_3$—	55	72–72.5°	—	$C_{19}H_{22}O_4$	C 72.7 H 7.0	72.8 7.0
13S		$CH_3(CH_2)_3$—	53	104–106°	—	$C_{22}H_{24}O_4$	C 75.0 H 6.8	75.0 7.1
14S		$CH_3(CH_2)_3$—	37	78–80°	—	$C_{22}H_{26}O_5$	C 71.4 H 7.0	71.4 7.2
15S		$CH_3(CH_2)_3$—	25	75.5–77°	—	$C_{22}H_{26}O_5$	C 71.4 H 7.0	71.3 7.3
			Glutarates, $n = 2$					
1G		H—	80	90–91°	—	$C_{18}H_{18}O_4$	C 72.5 H 6.1	72.3 6.1
2G		CH_3—	40	58–61°	—	$C_{19}H_{20}O_4$	C 73.1 H 6.4	73.1 6.7
3G		CH_3CH_2—	40	52–55°[d]	144–145°	$C_{23}H_{22}N_2O_4S$	C 68.3 H 6.5 N 5.7	68.4 6.5 5.8
4G		$CH_3(CH_2)_2$—	31	Oil	141–142°	$C_{29}H_{34}N_2O_4S$	C 68.7 H 6.7 N 5.5	68.5 6.9 5.6
5G		$CH_3(CH_2)_3$—	53	Oil	122–123°	$C_{30}H_{36}N_2O_4S$	C 69.2 H 6.9 N 5.4	69.4 7.1 5.3
6G		$CH_3(CH_2)_4$—	40	Oil	106–107°	$C_{31}H_{38}N_2O_4S$	C 69.7 H 7.1 N 5.3	70.0 7.1 5.5
7G		$CH_3(CH_2)_5$—	43	Oil	105–107°	$C_{32}H_{40}N_2O_4S$	C 70.1 H 7.3 N 5.1	69.9 7.0 5.0
8G		$CH_3(CH_2)_3$—	52	Oil[e]	—	—	—	—
9G		$CH_3(CH_2)_3$—	48	Oil	—	$C_{16}H_{21}ClO_4$	C 61.4 H 6.7	61.7 6.6

Table II—(*Continued*)

Compound Number	R_1	R_2	Yield, %	Melting Point	Melting Point (Salt)	Formula	Analysis, %[a] Calc.	Found
			Glutarates, $n = 2$					
10G		$CH_3(CH_2)_3$—	29	Oil	118–120°	$C_{24}H_{30}Cl_2N_2O_4S$	C 56.1 H 5.9 N 5.5	56.2 6.1 5.7
11G		$CH_3(CH_2)_3$—	48	Oil	112–114°	$C_{28}H_{34}N_2O_4S$	C 68.0 H 6.9 N 5.7	68.2 7.1 5.7
12G		$CH_3(CH_2)_3$—	53	Oil	116–117°	$C_{28}H_{34}N_2O_4S$	C 68.0 H 6.9 N 5.7	68.2 7.0 5.5
13G		$CH_3(CH_2)_3$—	40	Oil	129–130°	$C_{31}H_{36}N_2O_4S$	C 70.0 H 6.8 N 5.3	69.8 6.8 5.1
14G		$CH_3(CH_2)_3$—	21	Oil	116.5–118.5°	$C_{31}H_{38}N_2O_5S$	C 67.3 H 6.9 N 5.1	67.4 7.0 4.9
15G		$CH_3(CH_2)_3$—	40	Oil	110–112°	$C_{31}H_{38}N_2O_5S$	C 67.3 H 6.9 N 5.1	67.2 7.1 5.0

[a] Analytical data were obtained on the solid monoesters, while the oils were characterized as their solid S-benzylthiuronium salts. Th) formula indicates the compound upon which analytical data were obtained. [b] Lit. (13) m.p. 66°. [c] Lit. (13) m.p. 80°. [d] Lit. (13) 62°. [e] Lit. (13e n_D^{20} 1.4961. Found: n_D^{20} 1.4957.

glutarates (I) were prepared and assayed as inhibitors of the reductase.

The synthetic scheme for the inhibitors is shown in Scheme II. Treatment of the appropriate Grignard reagent with an ethereal solution of a suitable aldehyde gave the desired alcohols (Table I). Acylation of the alcohols with succinic or glutaric anhydride in dry pyridine afforded the desired monoesters (Table II).

The monoesters[1] that failed to crystallize were chromatographed on silicic acid and characterized as their solid S-benzylthiuronium salts.

EXPERIMENTAL[2]

Preparation of Alkylarylcarbinols—The carbinols listed in Table I were prepared by treatment of the appropriate aromatic aldehyde with the required Grignard reagent. The preparation of 1-(4-biphenylyl)pentanol may serve as an example.

1-(4-Biphenylyl)pentanol (Compound 5)—The Grignard reagent was prepared from 4.1 g. (0.17 g.-atom) of magnesium turnings and 20 ml. (26 g., 0.192 mole) of *n*-butyl bromide in 100 ml. of ether. To the prepared Grignard was added dropwise, without heating or cooling, 25 g. (0.137 mole) of 4-biphenylcarboxaldehyde in 200 ml. of ether. The mixture was stirred at 25° for 45 min. after the addition was complete. A solution of saturated ammonium chloride, adjusted to pH 7 with ammonium hydroxide, was added dropwise with cooling until a heavy granular precipitate settled out. The mixture was filtered and the solvent was removed under reduced pressure to afford 29.5 g., 89%, of a white solid, m.p. 77–78°. Recrystallization from ethyl acetate–petroleum ether, b.p. 60–75°, afforded an analytical specimen, m.p. 78–79°. Table I lists the analytical data.

Preparation of Arylalkyl Hydrogen Succinates and Glutarates—The succinates and glutarates listed in Table II were prepared using a modification of the pyridine method described by Palazzo *et al.* (13). The preparation of 1-(4-biphenylyl)-pentyl hydrogen glutarate may serve as an example.

1-(4-Biphenylyl)-pentyl Hydrogen Glutarate (Compound 5G)—A mixture of 3.6 g. (0.015 mole) of the alcohol (Compound 5), 1.71 g. (0.015 mole) of glutaric anhydride, and 20 ml. of pyridine (distilled from and stored over barium oxide) was heated at reflux for 8 hr. The resulting solution was poured on an ice–hydrochloric acid mixture and then extracted with ether. The organic phase was washed twice with water and then twice with a 5% sodium hydroxide solution. The basic aqueous extract was acidified with a 5% hydrochloric acid solution and then was extracted with ether. The organic phase was washed with water, washed with a saturated sodium chloride solution, and then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to give 2.9 g. of a colorless oil. The colorless oil was chromatographed on 50 g. of silicic acid. Elution with 20% ether–petroleum ether, b.p. 60–75°, afforded 2.5 g. of a colorless oil (Compound 5G), 46%. The NMR spectrum ($CDCl_3$) exhibited a 9:1 ratio of aromatic protons to the carboxyl proton, indicating the desired monoester. Analytical data were obtained for the solid monoesters, while the oily monoesters were characterized as their solid S-benzylthiuronium salts (Table II).

Preparation of S-Benzylthiuronium Salts—The method of Donleavy (15) was modified. To a solution of 0.17 g. (0.49 mmole) of 5G in 15 ml. of 95% ethanol was added 5.1 ml. of 0.095 *M* sodium hydroxide solution. To this solution was added 0.5 g. (2.4 mmoles) of 2-benzyl-2-thiopseudourea hydrochloride[3] in 5 ml. of hot 95% ethanol and then 20 ml. of water. The salt precipitated upon cooling to give 0.21 g. of a white solid. Two recrystallizations from ethyl acetate–petroleum ether, b.p. 60–75°, afforded an analytical specimen as white prisms, m.p. 122–123°. Table II lists the analytical data.

Enzyme Preparation—For the comparative inhibitor studies, β-hydroxy-β-methylglutaryl-CoA reductase was prepared from locally obtained baker's yeast[4] by the procedure of Durr and Rudney (11) as previously reported (12). The aqueous enzyme preparation was transferred to capped serum bottles and stored at −10° until used.

[1] The monoester 9S was characterized as an oil.

[2] Melting points, determined with a Thomas-Hoover capillary melting-point apparatus, are uncorrected. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. NMR spectra were obtained using a Varian A-60 NMR spectrophotometer.

[3] Eastman.

[4] Fleischman's.

Table III—Inhibition of β-Hydroxy-β-methylglutaryl-CoA Reductase

Compound Number	Activity I/S[a]	Activity Percent Inhibition	Compound Number	Activity I/S[a]	Activity Percent Inhibition
1S	150	0	1G	150	0
2S	Insoluble	—	2G	Insoluble	—
3S	150	16	3G	150	50
4S	150	44	4G	90	50
5S	66	50	5G	62	50
6S	62	50	6G	75	50
7S	Insoluble	—	7G	50	30
8S	100	0	8G	100	0
9S	100	0	9G	100	0
10S	100	30	10G	100	50
11S	150	50	11G	125	50
12S	115	50	12G	95	50
13S	70	50	13G	65	50
14S	125	50	14G	125	50
15S	115	50	15G	130	50

[a] The inhibition index, I/S, equals the ratio of the nM concentration of the inhibitor to the nM concentration of substrate. The I/S ratios are reported for 50% inhibition unless the solubility properties of the inhibitor precludes this determination. If sufficiently soluble, a maximum ratio of 150 was used for compounds exhibiting a low order of inhibition. The $I/S_{10\%}$ ratios were determined by interpolation from a plot of percent inhibition *versus* log I/S ratio. At least three sets of duplicate determinations between 30 and 80% inhibition were used, and one set within 45–55% inhibition was used for confirmation.

Enzyme Inhibition—Enzyme activity was determined at 30° in a recording spectrophotometer[5] by measuring the decrease in absorbance at 340 nm. for the oxidation of NADPH (12). To each cell were added 1.6 ml. of 0.15 M NaH_2PO_4 (pH 7.0) containing 1 mM ethylenediaminetetraacetate, 0.2 ml. ethylene glycol monoethyl ether containing inhibitor, 0.5 ml. of a solution containing 6.8 mg. cysteine, 50 μl. reduced triphosphopyridine nucleotide (NADPH) solution containing 0.36 μmole NADPH, and 0.1 ml. enzyme solution (about 14 mg. protein/ml. in buffer). In control cells, ethylene glycol monoethyl ether was added without inhibitor. The absorbance was allowed to stabilize after stirring prior to adding 50 μl. of β-hydroxy-β-methylglutaryl-CoA solution containing 44 nmoles of *dl*-β-hydroxy-β-methylglutaryl-CoA (12) and stirring again.

Kinetic Studies—For the kinetic studies, a more highly purified enzyme was prepared from baker's yeast by a modification of the methods of Durr and Rudney (11) and Kirtley and Rudney (16) as reported previously (17). This procedure adds a 30–45% saturated ammonium sulfate precipitation and substitutes 1 mM dithiothreitol for cysteine.

Reversibility of inhibition by dialysis was also investigated. Various combinations of 5S, substrate, coenzyme, and enzyme were dialyzed for 6, 12, or 24 hr. against buffer; activity was compared to enzyme alone subjected to similar dialysis (Table IV).

RESULTS AND DISCUSSION

Thirty monoesters of succinic and glutaric acids were prepared and assayed as inhibitors of yeast β-hydroxy-β-methylglutaryl-CoA reductase (Table III). The compounds are composed of two series, the succinates (S) and the glutarates (G). This study involved the modification of the alcohol moiety of the ester function to determine what structural aspects were essential for activity. The complex alcohol moiety is composed of aromatic (R_1) and alkyl (R_2) groups (Structure I). To differentiate between the effects of the aromatic and alkyl groups, Compounds 1S–7S and 1G–7G were prepared and assayed. The aromatic group (R_1) was maintained as a biphenyl group with the alkyl group (R_2) being varied from hydrogen to hexyl. As the chain length is increased from R_2 = hydrogen to R_2 = *n*-hexyl, activity increases dramatically, with a maximum resulting when R_2 = *n*-butyl or *n*-pentyl (5S, 5G, 6G). In the glutarate series, the ethyl compound, 3G, begins to show

[5] Gilford.

Table IV—Dialysis of Enzyme in the Presence of Substrate, Coenzyme, and Inhibitor 5S[a]

	Hours of Dialysis: Experiment I, 6	Experiment I, 12	Experiment II, 12	Experiment II, 24
Control	100.0%	100.0%	100.0%	100.0%
Inhibitor (1.58 mM)	79.4	83.3	97.2	100.5
Inhibitor (1.58 mM), β-hydroxy-β-methylglutaryl-CoA (15.8 μM)	22.4	42.8	47.6	91.1
Inhibitor (1.58 mM), NADPH (144 μM)	80.9	93.3	104.7	94.2
Inhibitor (1.58 mM), β-hydroxy-β-methylglutaryl-CoA (15.8 μM), NADPH (144 μM)	34.0	51.7	68.8	102.1

[a] The indicated enzyme solutions containing 14.8 mg. enzyme were diluted to 6.0 ml. with phosphate buffer (pH 7.6) containing 1 mM dithiothreitol, mixed, incubated for 5 min. at 30°, and transferred to dialysis tubing. After dialysis at 5° against phosphate buffer (pH 7.6) containing 1 mM dithiothreitol for 6, 12, or 24 hr., enzyme activity was determined as before and calculated as a percentage of control activity (dialyzed without inhibitor, β-hydroxy-β-methylglutaryl-CoA, or NADPH).

reasonable activity ($I/S_{0.5}$ = 150), whereas the ethyl compound in the succinate series, 3S, indicates only minimal activity (I/S = 150, 16% inhibition). However, when R_2 = *n*-butyl in both series, 5S and 5G, the activities become equal. An additional methylene group, Compound 6S, fails to increase activity in the succinate series; in the glutarate series, Compound 6G, a slight decrease in activity results. A further increase of chain length of R_2 is precluded by solubility problems with Compounds 7S and 7G. Thus, it appears that a hydrophobic bonding area on the enzyme has such dimensions that it can accommodate a chain length up to

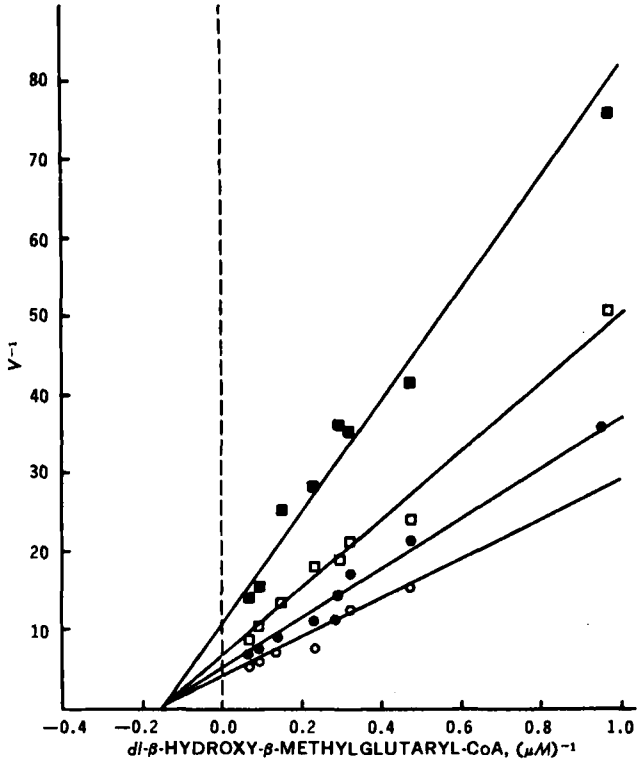


Figure 1—*Double-reciprocal plot of velocity* (ΔA_{340}/*min.*) versus *substrate concentration. All reaction mixtures consist of 7.4 mg. enzyme, 0.1 ml. ethylene glycol monoethyl ether, 2.4 ml. of 0.15* M *phosphate buffer containing 1* m*M dithiothreitol (pH 7.6), 0.36 μmole of NADPH, and varying amounts of substrate in a total volume of 2.5 ml. Key:* ○, *uninhibited;* ●, *0.79* m*M 5S;* □, *1.19* m*M 5S; and* ■, *1.58* m*M 5S.*

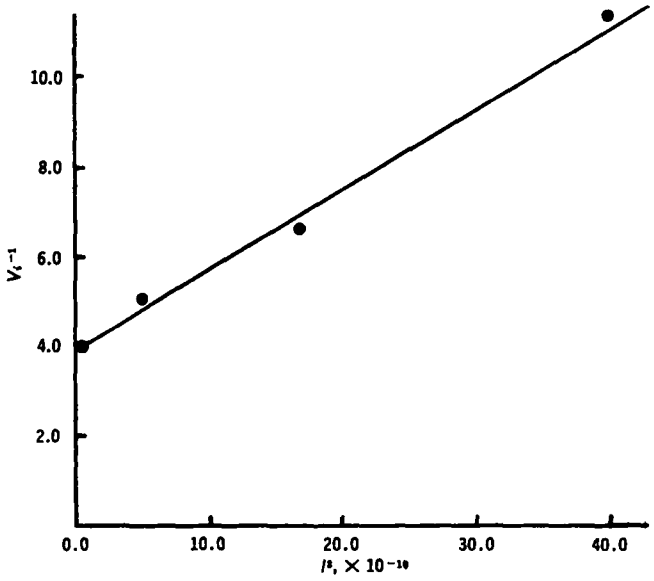


Figure 2—*Dixon plot for inhibition by 5S (data from Fig. 1).*

five carbon atoms.

The effects of the variation of the aromatic group (R_1) was investigated to determine if hydrophobic and electronic effects are important in binding of the inhibitor to the enzyme. The *n*-butyl chain (R_2) was retained in these inhibitors. The activity of Compounds 5S, 5G, 8S–10S, and 8G–10G appears to indicate that hydrophobic bonding is of primary importance in determining the degree of binding. As the hydrophobic bonding characteristics of the group (R_1) is increased (biphenyl > 3,4-dichlorophenyl > 4-chlorophenyl > phenyl), the activity of the monoesters increases. Coplanarity of the biphenyl group also appears to be important, as shown by the similar activity of the planar fluorenyl group, Compounds 13S and 13G, when compared to the biphenyl group, Compounds 5S and 5G.

In an attempt to determine if the position of attachment of the hydrophobic group (R_1) to the carbon atom bearing the ether oxygen of the ester was critical, the isomeric α- and β-naphthyl derivatives (11S, 11G, 12S, and 12G) were examined. No appreciable differences in activity were observed. An attempt also was made to detect electronic effects by evaluating positional isomers (14S, 14G, 15S, and 15G) containing an electron-donating substituent. This variation produced no significant change in activity.

In an effort to obtain more detailed information concerning the inhibition, Compound 5S was selected for additional studies on the type of inhibition, cooperativity, and reversal of inhibition by dialysis. From a double-reciprocal plot of velocity *versus* β-hydroxy-β-methylglutaryl-CoA (Fig. 1), it may be seen that the inhibition is noncompetitive with respect to substrate. A similar double-reciprocal plot of velocity *versus* NADPH (not shown) indicated that the inhibition is noncompetitive with respect to coenzyme as well.

A Dixon plot (18) and a modified Hill plot (19) were used to investigate cooperativity. A cooperativity number of 3 was calculated from the Dixon plot (Fig. 2) as well as a modified Hill plot (not shown). This is subject to a number of alternative explanations:

1. The enzyme molecules must combine with three molecules of inhibitor before any inhibition occurs.
2. The binding of one molecule of inhibitor to an enzyme molecule facilitates further binding of inhibitor to a total of three.
3. The molecules of enzyme which bind fewer than three molecules of inhibitor are quite unstable, either losing all of their bound inhibitor or binding up to their total of three.

Although subunits of this enzyme have not been reported, these data are compatible with an enzyme model consisting of subunits. However, subunits and their interactions are only one explanation of cooperativity.

It may be seen from Table IV that inhibition of the enzyme with inhibitor 5S is reversible by 24 hr. of dialysis. In the absence of substrate, however, inhibition is reversed in 12 hr. This would suggest the formation of a more stable ternary complex of enzyme, inhibitor, and substrate. The presence of NADPH appears to have little effect on the reversal of inhibition.

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Biophasic Availability of Ophthalmic Carbachol I: Mechanisms of Cationic Polymer- and Surfactant-Promoted Miotic Activity

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Abstract □ The influence of the topical application of cationic benzalkonium chloride and diethylaminoethyl dextran (mol. wt. $\simeq 2 \times 10^6$) adjuvants on the time variation of the miotic response intensity elicited by intraconjunctivally and intraaqueously administered carbachol was studied. The inclusion of either adjuvant in a 0.1% carbachol ophthalmic solution topically administered in the eye was observed to augment the miotic response activity attributable to direct transcorneal absorption into the biophase of the treated eye; the effect was nearly comparable to that achieved with similar dosing of a 2.0% solution containing carbachol alone. Simultaneous miotic activity in untreated control eyes of the rabbits, as well as other cholinergic side effects observed with the 2.0% carbachol solutions, did not occur with the 0.1% adjuvant-containing solutions, indicating that the enhanced local effects were not accompanied by a concomitantly promoted undesirable systemic absorption. The topical application of the adjuvants to the cornea following injection of the carbachol into the anterior chamber of the eye also appreciably promoted the miotic activity of the drug when it was administered by this mode. The principal mechanism of the observed adjuvant effects on carbachol biophasic availability is postulated as attributable to a promoted tissue permeability and release of the drug from corneal tissue binding sites; these occurrences result from an inductively implemented diminution of carbachol tissue binding affinities. The inductive effects originate and are propagated from fixed anionic corneal sites which interact with the cationic adjuvants. The impermeability of the cornea prevents the penetration of the adjuvant molecules to the deeper tissue layers where they could more directly influence carbachol tissue interactions.

Keyphrases □ Carbachol ophthalmic solutions—effect of cationic adjuvants on miotic activity □ Adjuvants, cationic—effect on miotic activity of carbachol solutions □ Miosis—effect of cationic adjuvants on carbachol activity □ Cationic adjuvant effect—miosis of carbachol ophthalmic solutions □ Ophthalmic solutions, carbachol—effect of cationic adjuvants on miotic activity □ Benzalkonium chloride—effect on miotic activity of carbachol solutions □ Diethylaminoethyl dextran—effect on miotic activity of carbachol solutions

Studies in this laboratory have been directed toward the elucidation of factors that influence the dynamic pharmacological response behavior of ophthalmic drugs (1–9) and the biophysical mechanisms by which they are operative (3, 6, 9). Since they are salutary to the development of an ultimate predictive capability with respect to the optimal control (7, 8) of drug response dynamics, quantitative treatments of observed phenomena in terms of pharmacokinetic and biophysical models have been developed and implemented whenever practicable. The requisite quantitative data have been derived from the implementation of two unique experimental methodologies, which allow results generally considered to be attainable only by destructive and *in vitro* techniques to be derived *in vivo* under normal physiological drug product use conditions without causing any injury. These methods thereby allow repeated measurements to be performed on the same subjects which can also serve as their own control. These techniques include a pharmacological method of drug absorption analysis (1–8, 10, 11) and a bioelectrometric method (9, 12–18) for the study of drug and other solute interactions with tissues or any other electrolytically conducting materials.

The bioelectrometric technique permits the determination of adsorption isotherms (12, 18) to describe drug–tissue interactions and the performance of kinetic studies of sorption and desorption (12, 18); the results can be quantitatively characterized in terms of thermodynamic and kinetic parameters (9, 12, 14, 18). The interpretation of results provided by the bioelectrometric method, concerning the dependency of the fixed-charge density of tissue surfaces on the composition of applied drug formulations, provides valuable insights into the submolecular mechanisms operative in drug–tissue interactions and transport across biological barriers; such information is generally unattainable by other techniques.

The use of transformed temporal pharmacological data (10) coupled with conventional pharmacokinetic approaches and/or techniques of engineering systems analysis and optimization (7, 8) provides a powerful approach to performing drug absorption analysis (1–5). The use of pharmacological data, as contrasted to the use of direct assay data derived by chemical or radiological assay, in addition to allowing the time course and rates of cumulative amounts of drug absorbed systemically to be determined, also permits the biophasic availability of the drug, *i.e.*, the amounts of drug that gain access to their sites of action, to be studied for any route of administration. The capability to compute the time course of drug absorption to a vicinal biophase adjacent to a site of topical administration, as well as the drug absorption profile for the quantities of drugs that simultaneously become systemically absorbed, renders this approach particularly valuable for ophthalmic drug bioavailability studies (1–6). Similar to the bioelectrometric method for the study of drug–tissue interactions, the use of pharmacological data for the study of drug transport is nondestructive and noninvasive.

The present report is the first of a series concerned with the kinetics and mechanisms of the biophasic availability of ophthalmically administered carbachol. Carbachol is an effective drug for the treatment of glaucoma. The seemingly erratic and sensitive dependency of its pharmacological response behavior on the formulation of the ophthalmic vehicle has contributed to its not being more commonly employed. However, it was hypothesized that the same and similar formula-

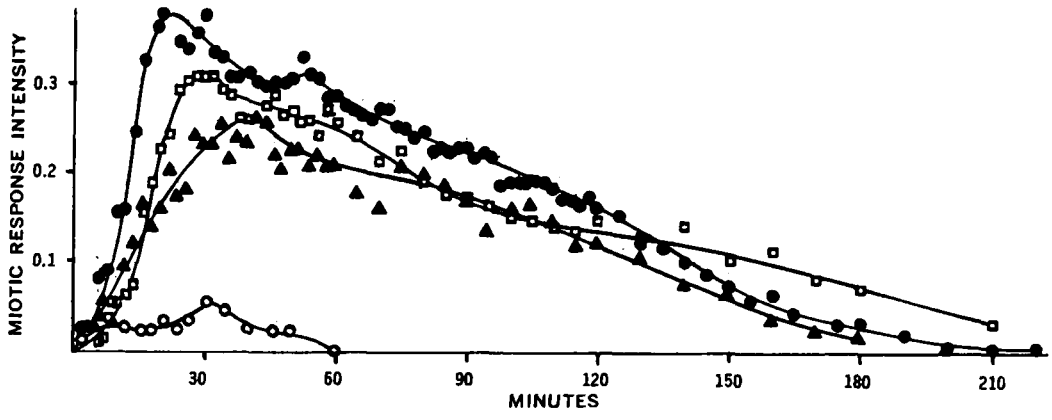

Figure 1—*Time variation of miotic response intensity following topical administration of 0.1 ml. of 0.1% carbachol (○), 2.0% carbachol (●), and 0.1% carbachol solutions containing 0.02% benzalkonium chloride (▲) and 0.06% diethylaminoethyl dextran (□). Each point represents the average of four replications performed on separate rabbits.*

tion factors to those responsible for the apparent erratic response behavior of ophthalmic carbachol, when properly understood, could conceivably provide the means by which to implement an optimal predictive control of the pharmacological response dynamics; this capability would permit a rational approach to the design of carbachol ophthalmic vehicle formulations.

The present report describes the results of relatively simple preliminary experiments concerned with the gross mechanistic nature of the very appreciable enhancement of the miotic activity observed to be induced by the inclusion of benzalkonium chloride (I) and diethylaminoethyl dextran (II) in carbachol ophthalmic vehicles. Subsequent reports will describe the quantitation of such results through the implementation of the bioelectrometric technique and the pharmacological method of drug bioavailability analysis.

EXPERIMENTAL

Materials—The ophthalmic solutions containing carbachol[1], I[2], and II[3], which were employed for topical and/or intraaqueous administration to the eye, were each prepared in a pH 7.4, isotonic phosphate buffer containing 0.1884 g./l. $NaH_2PO_4 \cdot H_2O$, 1.8293 g./l. $Na_2HPO_4 \cdot 7H_2O$, and 7.8910 g./l. NaCl. Distilled water was used and all the inorganic components were of reagent grade. The cationic dextran polymer has a supplier-reported molecular weight of approximately 2×10^6. The molecular weight of I was determined in this laboratory as 370 and 370.2 by UV spectrophotometry and chloride electroanalysis, respectively. It possessed a critical micelle concentration, determined by a liquid junction potential technique (19), of 8×10^{-4} *M*. The carbachol was at least USP grade. The solutions were prepared fresh prior to experimentation.

The apparent equivalent weight of the cationic polymer, II, was determined from Donnan potential measurements to have a value of 704 g. per equivalent of charge. The method involved separating a 5% (w/v) solution of II in pH 7.4 buffer from the buffered vehicle itself across a dialyzing membrane[4]. Dialysis equilibrium was achieved by storing the dialysis cell arrangement in a 37° water bath for 30 hr. with constant agitation until the Donnan potential, measured with a digital pH/voltmeter[5] using saturated calomel electrodes[6], became constant. The concentration of cationic charge in the solution resulting from the polymer and its equivalent weight was computed using previously reported (9, 12) theoretical expressions. By using these results, the 0.06% (w/v) II solution used in the present studies may be computed to be 8.5×10^{-4} *N* in polymer-contributed cation c charge. The normalities of the 0.02 and 0.03% I solutions are 5.4×10^{-4} and 8.1×10^{-4} equivalents of cationic charge per liter, respectively.

Approximately 4-month-old, male, New Zealand white rabbits, weighing 3.3–3.6 kg., were used as experimental animals. Twenty rabbits were screened on the basis of their similitude in pupillary accommodation to varying light intensity, magnitude of peak miotic response to test doses of carbachol administered intravenously, and clarity of pupil definition. Four of the rabbits were selected for use in the study.

Topical Administration to the Eye—The solutions administered intraconjunctivally to the rabbits contained 0.1 or 2.0% carbachol or 0.1% carbachol with 0.03% I or 0.06% (w/v) II. Solutions of 0.03% I and 0.06% II alone were also administered topically following intraaqueous dosing with carbachol.

Topical doses of 0.1 ml. of solution were instilled into the rabbit's eye using a 1.0-ml. syringe; the lower eyelid was retracted from the cornea and the solution was dropped into the conjunctival sac. The eyelids were held closed for 30–45 sec. following instillation to keep the rabbit from blinking, thereby preventing a large initial loss of the solution from the eye.

Intraaqueous Administration of Carbachol—Approximately 1 ml. of a 1%, isotonic, xylocaine solution[7] was dropped into the rabbit's eye to anesthetize the cornea. A 4-mcg./kg. dose of carbachol, contained in volumes of 0.05% (w/v) solutions ranging between 0.020 and 0.029 ml., was carefully injected through the cornea into the anterior chamber of the right eye. A similar injection of the same volume of solution vehicle alone provided a control. The influence of the adjuvants was studied by the topical instillation of 0.1 ml. of the 0.03% I or 0.06% II solution immediately following the intraaqueous injection.

Miotic Response Monitoring—The details of monitoring the time variation of pupillary diameters following dosing of rabbits were reported previously (1, 2). Briefly, however, the rabbits were restrained in a box during the procedure in which the diameter of the pupil was measured with a vernier caliper supported at a constant distance from the eye. A standard initial value of 4.5 mm. was obtained by adjusting the light intensity produced by two microscope lamps[8] using a rheostat. Subsequently, the eye was illuminated only during the time of the measurements (30–45 sec.) to avoid accommodation of the pupil to the light. The minimum pupil diameter during the period of illumination was recorded along with the time at which it was observed. Experience has demonstrated that this procedure, coupled with screening the rabbits prior to their selection for the study, considerably decreases both intrasubject and intersubject variations in the results. The miotic response intensity, I, was computed as: $I = 1 - (d_t/4.5)$, where d_t is the pupillary response at time t following dosing, and the value of 4.5 refers to the millimeter value of the pupillary diameter at time zero. The overall average relative error in the values of I obtained in this study is less than 6%. Since the variation in replicate experiments performed on the same animals was of the same magnitude as for replicates performed on different rabbits, results from different animals could be combined as averages.

[1] Alcon Labs, Inc., Fort Worth, Tex.
[2] K&K Labs., Plainsview, N. Y.
[3] Pharmacia, Uppsala, Sweden.
[4] Brasite Machine Co., New York, N. Y.
[5] Orion model 601.
[6] Beckman.
[7] Astra Pharmaceutical.
[8] Bausch & Lomb.

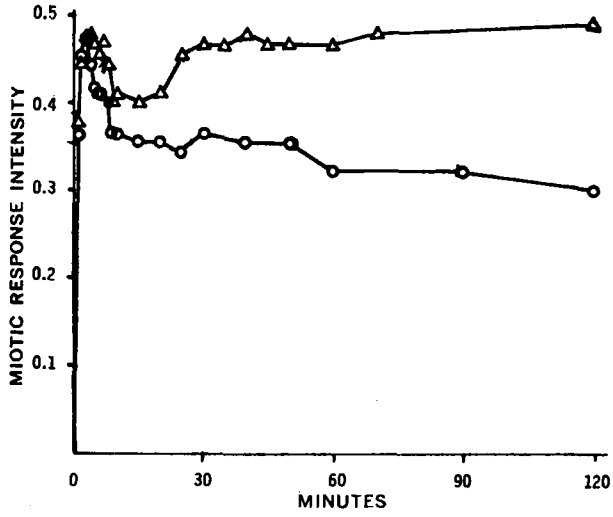

Figure 2—*Time variation of miotic response intensity following intraqueous administration of 4 mcg./kg. of carbachol to two rabbits with (Δ) and without (O) the topical administration of 0.1 ml. of 0.03% benzalkonium chloride immediately afterward.*

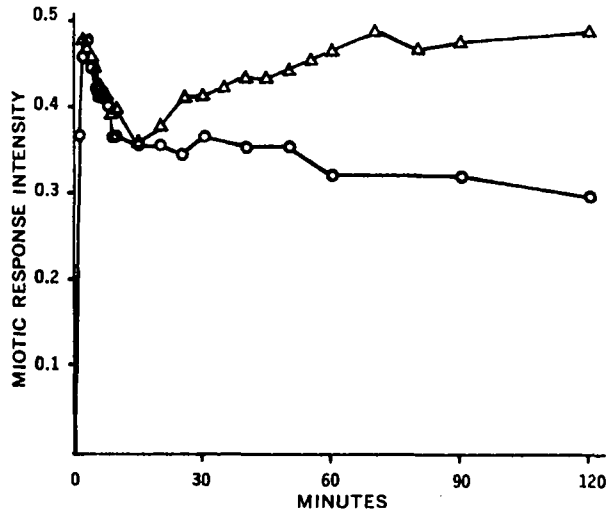

Figure 3—*Time variation of miotic response intensity following intraqueous administration of 4 mcg./kg. of carbachol to two rabbits with (Δ) and without (O) the topical administration of 0.1 ml. of 0.06% diethylaminoethyl dextran immediately afterward.*

RESULTS AND DISCUSSION

Adjuvant-Promoted Miotic Activity of Topically Administered Carbachol—Figure 1 presents the results of monitoring the time variation of miotic response following intraconjunctival administration. The results represent the averages of four replications performed on separate animals. The lowest curve depicts the miotic activity observed following the administration of 0.1% carbachol, which may be compared to the approximately eightfold increase in peak response intensity observed with an equal volume dose of a 2.0% carbachol solution. The middle curves demonstrate the influence of the inclusion of 0.02% I or 0.06% II in the ophthalmic solutions. The response *versus* time profiles clearly demonstrate the appreciable enhancement of miotic activity induced by the relatively small concentrations of these cationic adjuvants. Concentrations of II other than 0.06% were not studied. However, increasing the concentration of I beyond 0.02% up to 0.04%, which was the maximum level studied, causes further, although less pronounced, enhancement to the point where the response to the 0.1% carbachol solution is approximately tantamount to the results of administering the 2.0% carbachol solution alone. The details of all results with I will be presented in a subsequent report in the present series. It may be expected that an increase in the concentration of II would behave similarly to I in augmenting the response.

Except for topical dosing with the 2.0% carbachol solution without adjuvants, which produced a peak response in the left eye of 0.055–0.06 unit and a duration of approximately 100 min., the 0.1% carbachol solutions with and without adjuvants did not elicit any miotic activity in the left eyes of the rabbits used as carbachol-untreated controls. This observation and the observed relative lack of other systemic effects clearly indicate that the adjuvant-induced promotion of the pharmacological activity is attributable to an enhanced transcorneal biophasic availability directly to the sites of action as compared to an increase in the systemic bioavailability as can occur through schleral absorption, aqueous flow, and volume loss through the lacrimal-nasal drainage duct (3, 5, 6). By the latter routes, the drug enters the systemic circulation prior to gaining access to its biophase. Such systemic absorption is obviously undesirable because of the cholinergic side effects that can result from the systemic presence of carbachol. Inclusion of the adjuvants in the topically administered 0.1% carbachol solutions induced a local pharmacological activity nearly equivalent to the 2.0% carbachol, without apparent systemic activity. The significance of this observation with respect to the possibilities for the formulation of both safer and more effective carbachol ophthalmic preparations is apparent.

Adjuvant-Promoted Miotic Activity of Intraaqueous Injected Carbachol—Figure 2 presents the average results of the intraaqueous administration of 4-mcg./kg. doses of carbachol alone (lower curve) and the same dose followed by the topical administration of 0.1 ml. of 0.03% I (upper plot). Intraaqueous dosing results similarly obtained with the topical administration of II are shown in Fig. 3. In each case, a peak miotic response intensity was observed within 2–4 min. following dosing, after which an initially rapid decline in effect was apparent. The effects of the treatment with intraaqueous carbachol alone continued to diminish in a second phase, appearing between approximately 5 and 10 min., during which the decay was considerably slower. However, the miotic response observed in adjuvant-treated eyes again rose to a secondary maximum and plateau for a prolonged period in excess of the 180-min. interval over which the miotic activity was monitored. The pattern and magnitude of the influence of I and II were similar. Although the molar concentrations of I and II solutions are quite different, their normality with respect to cationic charge is nearly the same.

Mechanisms of Adjuvant-Promoted Miotic Activity—The topical application of the cationic adjuvants to the cornea has been demonstrated by the discussed results to augment the miotic activity of both topically and intraaqueously administered carbachol. Nonspecific physical mechanisms operative at the corneal surface—such as an increased viscosity of the vehicle or a Donnan exclusion effect which may be expected to be imparted by the positively charged polymeric II, or a lowering of surface tension attributable to the inclusion of the surface-active I—which could conceivably contribute to promoting the transcorneal absorption of carbachol following topical dosing, cannot reasonably be considered responsible for the enhanced activity observed following intraaqueous administration. Any explication in terms of a principal mechanism common to both routes of administration must obviously account for apparent transmission of the influence of the adjuvants through the thickness of the cornea to effect the disposition of the bolus of carbachol injected directly into the anterior chamber in such a manner as to promote the availability of pharmacologically active drug to its sites of action. The adjuvants themselves are miotically inactive; any appreciable penetration of the adjuvants across the minute lesional area in the periphery of the cornea resulting from the needle puncture may be considered as unlikely. The permeation of the highly charged II, possessing a molecular weight of approximately 2 million, across the corneal barrier to influence directly the interaction of carbachol with deeper tissue structures and thereby effect its disposition is untenable.

As a remaining alternative, it may be hypothesized that it is not necessary for the adjuvant molecules themselves to be physically transported into and through the cornea to effect changes in the nature of the interaction of carbachol with affected tissue structures; it is only required for the cationic adjuvants to interact with anionic corneal tissue surface sites. Such interactions can allosterically operate to induce changes which are inductively propagated (20, 21) by delocalization of mobile electrons to neighboring sites and deeper tissue levels where they alter tissue binding affinities for carbachol. These changes, in turn, result in the release of free, pharmacologi-

cally active drug. Although this postulation concerning the propagation of seemingly ethereal influences over relatively long ranges through tissues may initially appear highly speculative, it is consistent with theoretical considerations appearing in reports by other investigators (20–25) as well as from this laboratory (12, 13, 15, 16, 18). A further discussion and the details of experimental results which lend considerable credence to this hypothesis will be presented in subsequent reports. Briefly, however, it was found through the application of the pharmacological method of drug absorption analysis that larger quantities of carbachol were computed to be ultimately absorbed following topical administration with adjuvant-containing vehicles than were actually contained in the volume of solution with which the rabbits were dosed. This obviously violates the requirement for material balance. This anomaly does not occur when carbachol is administered alone, suggesting that the adjuvants act to displace and/or cause a release of tissue-bound, miotically inactive, carbachol from tissue binding sites. This action of the adjuvants is manifest in a lesser "apparent volume" of distribution; therefore, a higher concentration of free carbachol is available to its sites of action relative to that occurring in the absence of the influence of the adjuvants.

The above phenomenon may be further understood by considering an analogy to drug binding to plasma proteins. The volume of the plasma compartment computed as the ratio of an amount of drug known to be present in the plasma to the concentration of free drug for an extensively protein-bound drug will be determined as quite large. An adjuvant-induced release of the drug from its binding sites will obviously cause an increased free concentration of drug. When this concentration is multiplied by the value for an apparent volume of distribution determined in the absence of the adjuvant effect, values of apparent quantities of drug in excess of those which may be known to be actually present will be obtained. This is, in effect, analogous to what was observed with carbachol. The results of applying the bioelectrometric method to the study of adjuvant and carbachol interactions (to be reported later) also clearly imply further evidence that binding of cationic adjuvants to anionic corneal surface sites can profoundly influence the extent to which gangs of other anionic sites are occupied by cationic sorptates such as carbachol. Briefly, the results reveal a complex behavior in which I was observed to: (*a*) create new anionic tissue binding sites for carbachol through causing a cooperative, all-or-none type, deprotonation and/or release of other cations, *e.g.*, Na^+ and K^+, which can normally occupy these sites, (*b*) compete with carbachol for tissue binding sites, and (*c*) cause a sudden release of carbachol, as well as possibly other cations. This release occurs over nearly undiscernably small concentration ranges; such behavior is characteristic of cooperative mechanisms (21). The contribution of each of these phenomena to the tissue interaction behavior at any time was found to be dependent upon the relative magnitudes of carbachol and I concentrations simultaneously present.

SUMMARY AND CONCLUSIONS

In summary, the mechanism of the adjuvant effects is tentatively postulated on the basis of the present evidence as follows. Carbachol enters the corneal tissue through its surface following topical administration or back-diffuses into the cornea after intraaqueous dosing; the carbachol strongly binds to the tissues to a considerable extent. The bound, miotically inactive, drug could serve as a reservoir to prolong the activity, except that, under normal circumstances in the absence of adjuvants, the strongly bound drug is released too slowly to induce or contribute appreciably to observable levels of pharmacological activity. The interaction of the cationic adjuvants with anionic corneal surface sites results in the competitive displacement of bound carbachol from the surface and a diminution in the affinities of surface and deeper tissue binding sites. There is also a probable enhancement of the permeability of carbachol through the cornea (21). These effects permit the existence of relatively higher concentrations of the free drug to be available to its site(s) of action.

It is particularly notable that the observed adjuvant-induced augmentation of the miotic response activity apparently occurred by a promoted transcorneal absorption route rather than an enhanced systemic availability, which appeared to remain unaffected. The significance of this observation resides in an ability to formulate safer carbachol ophthalmic preparations using smaller concentrations of the drug, with a resulting diminution in systemic side effects; this can be accomplished without impairing the ophthalmic potency of the formulations.

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Esters of Hydantoic Acids as Prodrugs of Hydantoins

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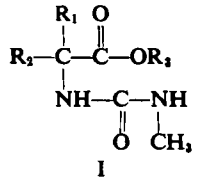
Abstract □ Hydantoins used as drugs possess low water solubility, which has led to erratic absorption patterns and dissolution patterns and possible precipitation in the blood after intravenous infusions of solutions of their sodium salts. The aim of this investigation was to elucidate the chemistry, physical properties, and pharmacological activity of some more water-soluble derivatives of hydantoins to determine the feasibility of their use as prodrugs. Various esters of the hydantoic acids of mephenytoin and 3-methylphenytoin were prepared and found to be quantitatively converted to the respective hydantoin under neutral to basic conditions. The β-*N'*,*N'*-diethylaminoethyl esters, in particular, were soluble in water up to pH 8 (presumably in the protonated form) and were converted to the respective hydantoin at pH 7.4 and 37°, with half-lives of under 20 min. At 25° and pH 6.0, the esters were stable for a few hours. An animal study using mice to test for anticonvulsant activity of the esters after intraperitoneal injection gave encouraging results (ED_{50} = 60 mg./kg., LD_{50} = 600 mg./kg.). The results suggested that β-*N'*,*N'*-diethylaminoethyl esters of hydantoic acids were candidates as water-soluble prodrugs of the respective hydantoin.

Keyphrases □ Hydantoic acid esters—evaluated as potential hydantoin prodrugs □ Hydantoin prodrugs—evaluation of water-soluble hydantoic acid esters □ Prodrugs for hydantoins—evaluation of water-soluble hydantoic acid esters

A number of hydantoins, including phenytoin, mephenytoin, and nitrofurantoin, are widely used as drugs. Their medical and veterinary usages, however, have been hampered by their low water solubility combined with their weakly acidic nature. Phenytoin, for example, has shown erratic absorption patterns and dissolution patterns as well as possible precipitation in the body after intravenous infusions of highly alkaline solutions of its sodium salt (1–4). Gastric upset associated with oral doses of sodium phenytoin similarly may be due to this necessary high alkalinity of aqueous solutions of the salt form.

The aim of this investigation was to determine the feasibility of obtaining transient prodrug (5) forms of these drugs which would confer acceptably high water solubility within the physiological pH range but convert to the desired active structure within the body system. Results obtained based on various esters (I) of the



I

R_1, R_2 = aryl or alkyl substituents
R_3 = alkyl (simple esters) or $-C_2H_4N(C_2H_5)_2$ (amino esters)

respective hydantoic acid of the hydantoin, especially those esters that have an ionizable amino group in the alcohol portion of the ester grouping, are reported at this time. Esters of hydantoic acids are known to cyclize *via* an intramolecular reaction under basic conditions to their respective hydantoin (Scheme I), but no actual kinetic data are available. Kinetic data are available, however, on the cyclization of esters of the related *o*-ureidobenzoic acid under aqueous conditions in the neutral to alkaline pH range (6). Intramolecular cyclizations have been postulated as a means of latentiation by Levine *et al.* (7) in their work on the conversions of ω-haloalkylamines to their quaternary analogs. In the past, many derivatives of drugs formed through an ester linkage have been dependent on enzymatic reactions to release the parent compound (8, 9). Intramolecular reactions are known to be many thousand times faster than their intermolecular equivalent (10), forming a basis for the release of the parent compound at a speed that is not totally dependent on enzymatic mediation.

Table I lists the four esters investigated in the present study along with their compound numbers and formulas. Compounds IV and V cyclize to give mephenytoin, while II and III cyclize to give 3-methylphenytoin. Both mephenytoin and 3-methylphenytoin are anticonvulsant drugs.

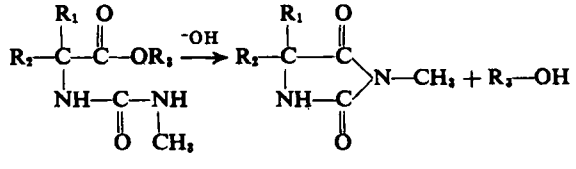
EXPERIMENTAL

Equipment—A recording spectrophotometer[1] with a thermostated cell compartment capable of maintaining temperatures to ±0.1° was used for all spectroscopic measurements. The pH measurements were carried out using a research pH meter[2] standardized at 25° with phosphate buffer (pH 7.40 at 25°) and borate buffer (pH 9.18 at 25°). NMR spectra were measured on an NMR spectrometer[3].

Materials and Reagents—All chemicals used were of analytical or reagent grade. Mephenytoin was supplied commercially[4]. Buffers were made from triply distilled water and analytical grade phosphates and borates, with the ionic strength adjusted to unity with potassium chloride. Buffer concentration was 0.1 *M*.

The four esters were prepared *via* identical pathways. Analyses of the compounds are shown in Table II, and Table III gives some spectral data. An example of the synthetic procedure is listed here, and any differences associated with the synthesis of the other three esters are explained under the particular step.

Synthesis of V—*Step 1*—Ten grams (0.046 mole) of mephenytoin was placed in a plunging autoclave with 200 ml. of 20% NaOH under nitrogen. The mixture was heated at 180–190° for 24 hr., filtered through charcoal, and diluted to 500 ml. The solution was neutralized with glacial acetic acid and concentrated to 150 ml.



Scheme I

[1] Cary 14.
[2] Corning model 12.
[3] Varian T60.
[4] From Sandoz Drug Co.

Table I—Hydantoic Acid Esters Studied with R_i (i = 1, 2, or 3) Referring to the Groups in Scheme I

Chemical Name	Compound Number	R_1	R_2	R_3
Ethyl 5-methyl-2,2-diphenylhydantoate	II	C_6H_5	C_6H_5	C_2H_5
β-N′,N′-Diethylaminoethyl 5-methyl-2,2-diphenylhydantoate	III	C_6H_5	C_6H_5	$C_2H_4N(C_2H_5)_2$
Methyl 2-ethyl-5-methyl-2-phenylhydantoate	IV	C_6H_5	C_2H_5	CH_3
β-N′,N′-Diethylaminoethyl 2-ethyl-5-methyl-2-phenylhydantoate	V	C_6H_5	C_2H_5	$C_2H_4N(C_2H_5)_2$

The crystalline material that precipitated was filtered and washed with 200 ml. of water and 200 ml. of ether. This gave a product of m.p. 288° dec., corresponding to 2,2-ethylphenylglycine (VI) [lit. (11) m.p. 275° dec.]. The yield was 5.5 g.

Step 2—Three grams (0.017 mole) of VI was dissolved in 30 ml. of 1 *N* NaOH. An excess of ethyl chloroformate (5 g., 0.047 mole) was added dropwise to this solution, while the pH of the solution was maintained alkaline by the addition of sodium hydroxide pellets. The solution was allowed to stir until no odor of ethyl chloroformate was detectable. After extraction with 2 × 50-ml. portions of ether (to remove any ether-soluble impurities), the aqueous layer was acidified with hydrochloric acid and again extracted with 4 × 50-ml. portions of ether. After drying over magnesium sulfate, the second ether extraction was evaporated by a rotary evaporator for 1 hr. Fifteen milliliters of benzene was added and warmed to complete solution. *n*-Hexane was added dropwise to cause cloudiness and precipitation of a white solid of m.p. 130–131° and a yield of 1.5 g. This was presumed to be *N*-ethoxycarbonyl-2,2-ethylphenylglycine (VII).

Step 3—Five milliliters of thionyl chloride was refluxed with VII (700 mg., 0.003 mole) for 1 hr. The excess thionyl chloride was removed by a rotary evaporator, leaving a yellow solid. The solid was taken up in benzene and precipitated with *n*-hexane, leaving a white compound of m.p. 99–101° and a yield of 550 mg. This was presumed to be 4,4-ethylphenyloxazolidinedione (2, 5) (VIII).

Step 4—To VIII (500 mg., 0.002 mole), freshly distilled diethylaminoethanol (400 mg., 0.003 mole) was added in a dry flask at 140°. An atmosphere of hydrogen chloride gas was passed over the melt. These conditions were maintained for 20 min. No product was actually isolated in this step.

If the ethyl or methyl esters were being prepared, VIII was refluxed in a solution of the appropriate alcohol which had hydrogen chloride gas passed through it.

Step 5—The syrupy melt of Step 4 was taken up in chloroform and extracted with 0.1 *N* NaOH and water. After drying, the chloroform layer was evaporated to 0.5 ml. and methylisocyanate was added (500 mg., 0.009 mole). The solution was allowed to stand for 20 min. and then evaporated to remove the excess methylisocyanate. The remaining oil was taken up in *n*-hexane and eventually precipitated as a solid of m.p. 83–87°. The solid was taken up in chloroform and extracted with 2 × 50-ml. portions of water. After drying and evaporating, the chloroform layer gave a white solid of m.p. 88–89°, corresponding by elemental analysis and NMR to β-N′,N′-diethylaminoethyl 2-ethyl-5-methyl-2-phenylhydantoate (V).

Table II—Elemental Analyses and Melting Points of the Prepared Esters

Esters	Analysis, % Calc.	Found	Melting Point
II	C 69.25	69.20	220°
	H 6.41	6.69	
	N 8.98	8.84	
IV	C 62.35	62.17	168–169°
	H 7.20	7.42	
	N 11.20	11.37	
III	C 68.95	69.10	144–145°
	H 7.60	7.49	
	N 10.97	10.66	
V	C 64.50	64.77	88–89°
	H 8.66	8.86	
	N 12.52	12.76	

Table III—NMR Data for the Four Esters

Ester	δ Value, Integration, Assignment[a]
II	1.20 δ (triplet), 3, $-OCH_2CH_3$; 2.65 δ (singlet), 3, $-NHCH_3$; 4.25 δ (quartet), 2, $-OCH_2CH_3$; 5.45 δ (broad), 1, $-NHCH_3$; 6.85 δ (broad), 1, $-CONHC(C_6H_5)_2-$; 7.30 δ (multiplet), 10, $-C(C_6H_5)_2$
IV	0.85 δ (triplet), 3, $-C_6H_5C(CH_2CH_3)-$; 2.60 δ (quartet), 2, $-C_6H_5C(CH_2CH_3)-$; 2.65 δ (singlet), 3, $-NHCH_3$; 3.65 δ (singlet), 3, $-OCH_3$; 7.35 δ (multiplet), 5, $-C_6H_5C(C_2H_5)-$
III	0.96 δ (triplet), 6, $-N(CH_2CH_3)_2$; 2.40 δ (quartet), 2.50 δ (singlet), 2.55 δ (triplet) all integrated for 9 protons, $-CH_2N(CH_2CH_3)_2$ and $-NHCH_3$; 4.20 δ (triplet), 2, $-OCH_2CH_3-$; 4.60 δ (broad), 1, $-NHCH_3$; 6.20 δ (broad), 1, $-CO-NH-C(C_6H_5)_2-$; 7.35 δ (multiplet), 10, $-C(C_6H_5)_2-$
V	0.85–1.55 δ (multiple peaks), 9, $-N(CH_2CH_3)_2$ and $-C_6H_5(CH_2CH_3)-$; 2.45–3.00 δ (multiple peaks), 11, $-CH_2N(CH_2CH_3)_2$ and $-NHCH_3$ and $-C_6H_5C(CH_2CH_3)-$; 4.40 δ (triplet), 2, $-OCH_2CH_3$; 5.00 δ (broad), 1, $-NHCH_3$; 6.35 δ (broad), 1, $-CONHC-$; 7.60 δ (multiplet), 5, $-C_6H_5C(C_2H_5)-$

[a] All esters run in $CDCl_3$.

Kinetic Measurements—All kinetic measurements were carried out directly in the thermostated cell compartment of a recording spectrophotometer[1]. At the elevated temperatures, the buffer solution was placed in the cell compartment and allowed to equilibrate before the ester was introduced as a standard dioxane solution. For II and IV, the least water-soluble esters, the final concentration of dioxane was 5%; for III and V, the concentration of dioxane was 2%. The formation of the hydantoin was followed at 240 nm.; the rate constants were determined from plots of log ($A_\infty - A_t$) *versus* time, where A_∞ and A_t are the absorbance readings at infinity and at time t, respectively. Some rate constants were also determined by the Guggenheim (12) method. The closure rates in the presence and absence of horse serum cholinesterase[1], with a concentration of 2 mg./ml. of enzyme in pH 7.4 buffer, were followed directly in the cell compartment of the spectrophotometer.

Product Analysis—All of the esters gave their respective hydantoins on reaction with base. This was checked by spectral comparisons and TLC [solvent system of chloroform using microscopic slide technique (13)]. On heating in water, the four esters gave a near quantitative yield of the parent hydantoin.

Solubility Study—The solubility of the hydantoins and their derivatives in chloroform–carbon tetrachloride (1:3 v/v) at 25° was determined semiquantitatively using a gravimetric technique. The semiquantitative scale was necessary because of the small amounts of materials available. The partitioning of the esters and hydantoins between the chloroform–carbon tetrachloride nonaqueous layer and the pH 6.0 phosphate buffer was also estimated *via* the gravimetric technique by determining the amount of hydantoin or derivative in the nonaqueous layer before and after extraction with the aqueous buffer. The aqueous solubility of V and III in pH 6.0 phosphate buffer was estimated by placing 50-mg. fractions of the esters in 10 ml. of the buffer at room temperature until a saturated solution was obtained. This simple semiquantitative procedure was necessary, because even at 25° and pH 6.0 the esters are converted to their hydantoins with half-lives of under 10 hr.; since the hydantoins (the products of the reaction) are very water insoluble, they would show up as saturated solutions when only a simple observation was being used as the criterion for saturation.

Animal Studies—The animal studies presented here are not meant to represent a full comprehensive study but rather to show that the particular derivatives synthesized and studied in this work are, in fact, pharmacologically active and relatively nontoxic on a short-term scale.

[1] Sigma Chemical Co.

Table IV—Effect of Varying Borate Buffer Concentration on Cyclization[a] of IV at 25°, pH 9.0 (μ = 1.0)

Borate Concentration, M	k_{obs}, min.$^{-1}$
0.017	8.50×10^{-2}
0.029	8.65×10^{-2}
0.050	8.60×10^{-2}
0.100	8.16×10^{-2}
Average	8.58×10^{-2}

[a] k_2 for the cyclization of IV at 25° from the average value of k_{obs} at pH 9.0 is equal to 8.58×10^{3} M^{-1} min.$^{-1}$.

Random bred, albino, female mice, Ha/ICR, age 3–6 weeks, with an average weight of 11 g. (variation 8–15 g.), were obtained[6] and acclimatized for 3 days before use.

Toxicity Studies—Fifty mice were separated into lots of 10. Compound V was dissolved in a 0.1 *M* phosphate buffer of pH 5.9. The concentration of drug was such that when given intraperitoneally, ~0.1 ml. or ~0.2 ml. of the solution gave 100 mg./kg., 200 mg./kg., *etc.*, of drug; *i.e.*, all intraperitoneal injections were of 0.2 ml. or less. The animal weight was used to calculate what volume of solution was necessary to give the desired dose. With the first set of 10 animals, each animal received 100 mg./kg. of V. The second set was given 200 mg./kg. The other three sets were given 400 and 800 mg./kg. and the buffer solution, respectively.

Anticonvulsant Activity Tests—The ED_{50} anticonvulsant activity test is basically an electroshock test[7] where a supramaximal current of 20 ma. is applied across the ears of a mouse for 0.2 sec. to induce convulsion. Forty mice were separated into four sets of 10. Each mouse was submitted to the electroshock and any insensitive mice were discarded. All of the mice required severe resuscitation after the current was disconnected. The first set of mice was then given 50 mg./kg. i.p. of V, the second set 100 mg./kg., and the third set

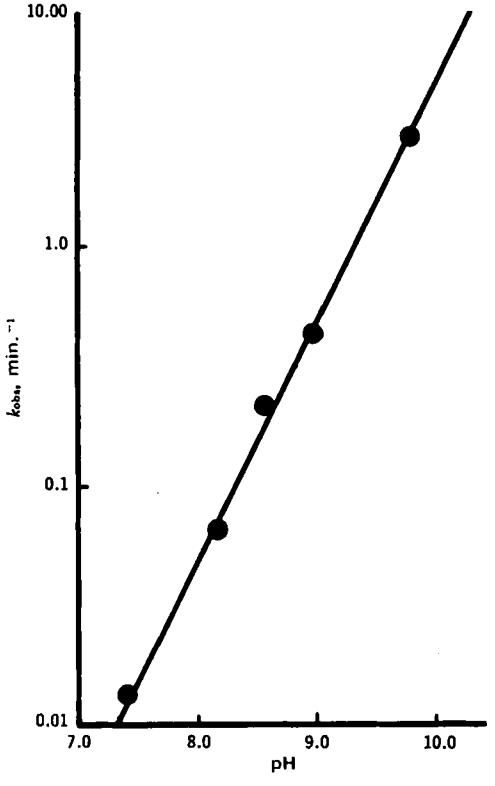


Figure 1—*Log* k_{obs} versus *pH profile for the closure of II at 37°* (μ = *1.0, with potassium chloride*).

[6] From A. R. Schmidt of Wisconsin.
[7] Similar to that used as an undergraduate assignment in the University of Kansas Pharmacology Department.

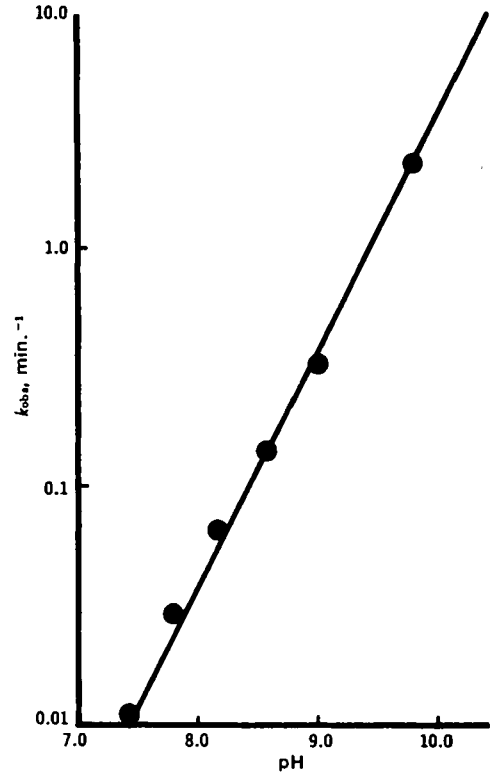
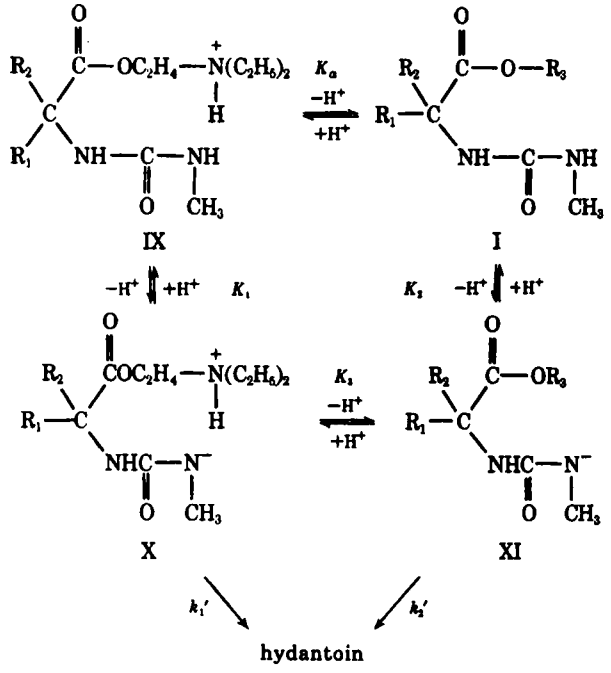


Figure 2—*Log* k_{obs} versus *pH profile for the closure of IV at 37°* (μ = *1.0, with potassium chloride*).

150 mg./kg. The fourth set of mice was given 25 mg./kg. of III. After the injection the mice were returned to their cages for 1 hr. After this 1 hr., each mouse was again submitted to the electroshock test to observe if any protection against electroshock had resulted from the injections.

RESULTS AND DISCUSSION

Chemistry of Cyclization of II, III, IV, and V to Their Respective Hydantoins under Neutral to Basic Aqueous Conditions—In the pH range studied, 7.43–10.80, and at temperatures of 25 and 37°, all



Scheme II

Table V—Effect of Varying Borate Buffer Concentration on Cyclization[a] of II at 25°, pH 9.0 (μ = 1.0)

Borate Concentration, M	k_{obs}, min.$^{-1}$
0.017	1.47×10^{-1}
0.029	1.48×10^{-1}
0.050	1.50×10^{-1}
0.100	1.46×10^{-1}
Average	1.48×10^{-1}

[a] k_2 for the cyclization of II at 25° from the average value of k_{obs} at pH 9.0 is equal to 1.48×10^4 M^{-1} min.$^{-1}$.

four esters cyclized *via* first-order kinetics to their respective hydantoins with the general reaction shown in Scheme II. All reactions were irreversible and complete, as evidenced by spectral comparisons, TLC, and near quantitative recovery of the hydantoins from boiling water solutions of the esters.

Plots of log k_{obs} *versus* pH for the cyclization of the four esters are shown in Figs. 1–4. For the simple esters, II and IV, plots of log k_{obs} *versus* pH (Figs. 1 and 2) gave straight lines whose slopes[b] were approximately one (0.98 and 0.96, respectively), suggesting that the reaction was first-order dependent on hydroxyl ions. For the amino esters, V and III, the plots (Figs. 3 and 4) were predictably nonlinear over part of the pH range studied but did approach linearity at the limits of the plots.

The rates of cyclization of all four esters were found to be buffer concentration independent at constant ionic strength (Tables IV and V), which, along with the log k_{obs} *versus* pH plots, suggest that like the esters of *o*-ureidobenzoic acid (6), the closures to the cyclic hydantoins are specific base catalyzed.

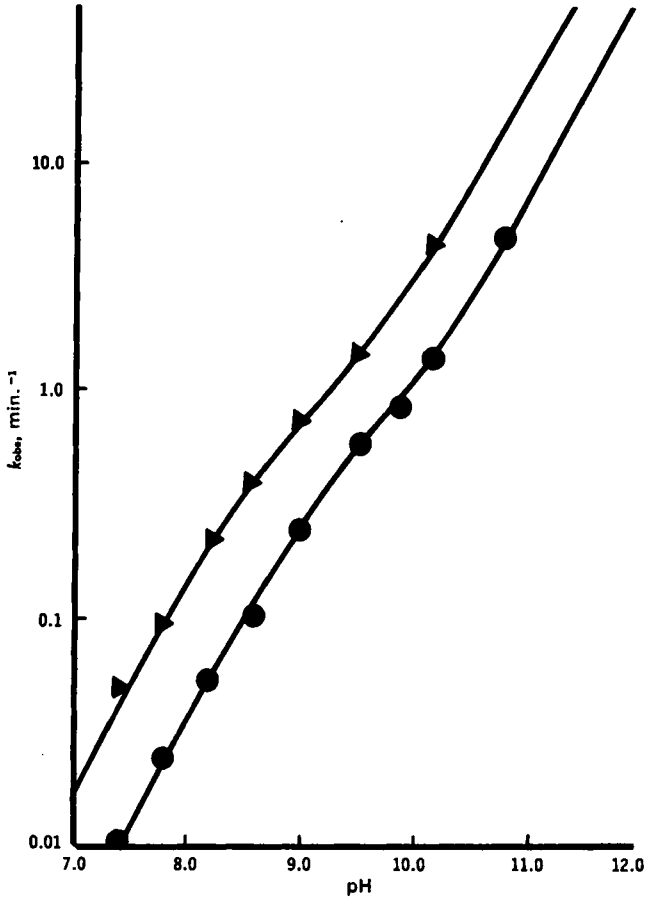


Figure 3—*Log* k_{obs} versus *pH profile for the closure of V at 25°* (●) *and 37°* (▲) (μ = *1.0, with potassium chloride*).

[b] Calculated by standard least-squares techniques, Olivetti Programma.

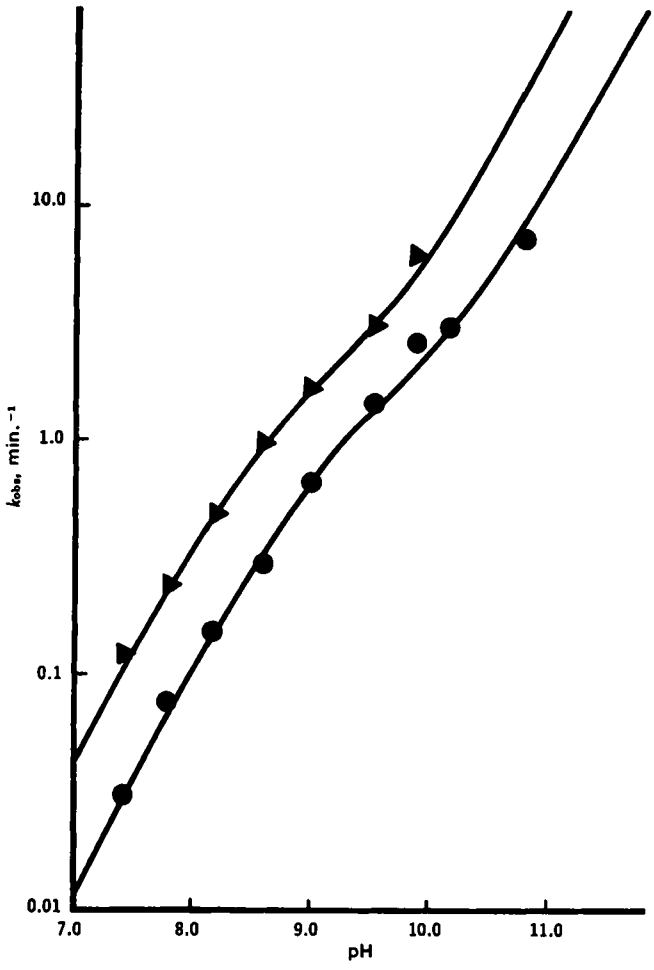


Figure 4—*Log* k_{obs} versus *pH profile for the closure of III at 25°* (●) *and 37°* (▲) (μ = *1.0, with potassium chloride*).

The results presented in Figs. 1–4 and Tables IV and V could be rationalized in terms of the general mechanistic Scheme II, where R_3 = alkyl or $-C_2H_4N(C_2H_5)_2$, and k_1' and k_2' are the spontaneous rate constants for the hydrolytic decomposition of X and XI.

The ionizations described by K_1 and K_2 are due to the microscopic ionization of the ureido group ($-NHCONHCH_3$) to the ureido anion ($-NHCON^-CH_3$). Both K_a and K_3 are due to the dissociation of the protonated amino group which can exist only for the amino esters; *i.e.*, Scheme II is complete for the amino esters but the terms IX, X, K_1, K_3, and K_a are all eliminated from Scheme II for the simple esters. If these terms are eliminated, then the rate of formation of the hydantoin, $d[P]/dt$, from the simple esters may be expressed as:

$$\frac{d[P]}{dt} = k_2'[XI] \quad \text{(Eq. 1a)}$$

$$= k_2'K_2[I]a_{OH^-} \quad \text{(Eq. 1b)}$$

$$= k_2a_{OH^-}[I] \quad \text{(Eq. 1c)}$$

where $k_2 = k_2'K_2$.

The total ester concentration is $[ester]_T = [I] + [XI]$, but since K_2 represents the ionization constant of the ureido group to the ureido anion and this constant is considered to be smaller than 10^{-14} (6), the term [XI] is negligible when compared to [I]. Therefore:

$$\frac{d[P]}{dt} = k_2[ester]_T a_{OH^-} \quad \text{(Eq. 2a)}$$

or:

$$k_{obs} = k_2a_{OH^-} \quad \text{(Eq. 2b)}$$

Since K_2 was not determinable, the results will be expressed in terms of k_2. The a_{OH^-} and a_{H^+} used later refer to the activity of hy-

Table VI—Second-Order Rate Constants and Ionization Constants for the Cyclization of Esters II–V to Their Respective Hydantoins at 25 and 37° in Aqueous Solution (μ = 1.0)

Ester	Temperature	k_1, × 10^{-4} (M^{-1} min.$^{-1}$)	k_2, × 10^{-4} (M^{-1} min.$^{-1}$)	pKa
II	25°	—	1.48	—
	37°	—	2.00	—
IV	25°	—	0.86	—
	37°	—	1.52	—
III	25°	10.5	1.10	9.20
	37°	17.3	1.93	8.65
V	25°	3.82	0.64	9.25
	37°	6.70	1.01	8.75

droxyl ion and hydrogen ion, respectively, which were calculated from pH measurements. Equation 2*b* predicts that a plot of log k_{obs} *versus* pH will be linear with a positive slope of unity, which is consistent with the present findings.

For the amino esters (III and V), the rate equation was more complex:

$$\frac{d[P]}{dt} = k_1'[X] + k_2'[XI] = k_1'[IX]K_1a_{OH^-} + k_2'[I]K_2a_{OH^-} \quad \text{(Eq. 3)}$$

The total ester concentration is:

$$[\text{ester}]_T = [IX] + [I] + [X] + [XI] \quad \text{(Eq. 4)}$$

Since K_1 and K_2 represent the ionization constants of the ureido group (substituted urea) and are considered to be smaller than 10^{-14} (6), the terms [X] and [XI] are negligible compared to [IX] and [I]. The fraction of ester present as [IX], expressed as β, therefore, can be defined as:

$$\beta = \frac{[IX]}{[I] + [IX]} = \frac{a_{H^+}}{a_{H^+} + K_a} \quad \text{(Eq. 5a)}$$

$$\frac{[dP]}{dt} = [\text{ester}]_T \{k_1a_{OH^-}\beta + k_2a_{OH^-}(1 - \beta)\} \quad \text{(Eq. 5b)}$$

$$k_{obs} = k_1a_{OH^-}\beta + k_2a_{OH^-}(1 - \beta) \quad \text{(Eq. 5c)}$$

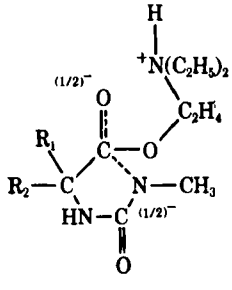
where $k_1 = k_1'K_1$, and $k_2 = k_2'K_2$.

Since K_1 and K_2 could not be determined, the results will be expressed in terms of k_1 and k_2. Equation 5*c* predicts that plots of log k_{obs} *versus* pH for the amino esters will be complex and nonlinear in the pH range where β does not approximately equal zero or one. The plot resulting from such an equation has been adequately described (14) and will not be discussed here.

The rate and equilibrium constants consistent with the experimental results are shown in Table VI. Variation of pKw with temperature was taken into account in the calculation of the rate and equilibrium constants. The solid lines drawn in Figs. 1–4 have been calculated by substituting the rate and equilibrium constants shown in Table VI into Eqs. 2*b* and 5*c*.

The rate constant k_1 has been defined by Scheme II and Eq. 5*c* as the second-order base-catalyzed rate constant for the attack of the ureido group on the protonated amino ester. However, its kinetic equivalent, the spontaneous attack of the neutral ureido group on the free base form of the ester, cannot be fully discounted.

In summary, the cyclization of esters of hydantoic acid in neutral to alkaline solution appears to be a specific base-catalyzed intramolecular closure, with the rate-determining transition state involving the attack of the ureido anion at the ester carbonyl group.



XII

Table VII—Half-Lives for Closure of Esters of Hydantoic Acids to Their Hydantoins

Ester	$t_{1/2}$, min.[a]	$t_{1/2}$, min.[b,c]
II	57.3	4680
IV	73.0	8170
III	6.8	600
V	16.9	1710

[a] pH 7.4 and 37°. [b] pH 6.0 and 25°. [c] Estimated from Figs. 3 and 4 and Tables IV and V.

It appears that the diethylamino moiety helped increase the ester's rate of closure at pH's below the pKa of the amino group. For example, k_{III}/k_{II} at pH 7.40 and 37° is 8.5. This increased rate of closure over the simple esters and the free base form of the amino esters may be due to electrostatic stabilization of the negatively charged reaction center (see XII), although the possibility of intramolecular general acid catalysis cannot be discounted. The transition states for the esters where $-C_2H_4N^+(-H)(C_2H_5)_2$ is replaced by a simple alkyl group or $-C_2H_4N(C_2H_5)_2$ would be the same as XII, except that no electrostatic stabilization of the negative reaction center would be possible.

Hegarty and Bruice (6) recently demonstrated the intramolecular nucleophilicity of the ureido group. They showed a rate acceleration of 1.5 × 10^5-fold for the attack of the ureido group in the intramolecular cyclization of the methyl ester of *o*-ureidobenzoic acid to that of its intermolecular equivalent. The results and conclusion presented here are in perfect accordance with their findings.

Table VII shows the half-lives for the conversion of the four esters to their hydantoin at pH 7.4 and 37°, suggesting that at least under *in vitro* conditions the esters are quickly converted to their hydantoin, while at pH 6.0 and 25° the conversion is quite slow. This means that the esters could be used in a reconstitutable injection if they are water soluble. The solubility of the esters will be discussed later.

Because the derivatives appeared promising, a test was run to observe the effect of an esterase enzyme on the product and rate of cyclization of one of the esters, III. Horse serum cholinesterase[5] at a concentration of 2 mg./ml. in buffer solution did not affect the hydrolytic intramolecular closure of the ester. The $t_{1/2}$ values at pH 7.43 and 23.6° with and without enzyme were 31.1 and 31.9 min., respectively. The products of the two reactions, *i.e.*, with and without enzyme, gave the same final UV spectrum corresponding to 3-methyl-5,5-diphenylhydantoin. Of particular importance was the enzyme's inability to cleave the ester linkage before the intramolecular reaction was able to take place. The product of such a

Table VIII—Results of LD_{50} Study of V[a]

Dose, mg./kg.	Mortality, %	Observations
100	0	All mice acted quite normal with no observable toxic effects over 4 hr.
200	0	Slightly sedated and ataxic over 4 hr. but no critical effects.
400	0	After 1 hr., the mice were very ataxic and it did appear that a few of them may die. Their breathing appeared normal, but there was kicking of the hindlegs. After 4 hr., all the mice had recovered and appeared normal.
800	100	All mice died within 10–15 min. They became very ataxic, with increased breathing rate and kicking of the hindlegs, indicative of convulsions.
Buffer solution (no drug)	0	All mice appeared quite normal.

[a] The mice given III in the ED_{50} experiment at a dose of 25 mg./kg. appeared quite normal over a 3-hr. range with no obvious toxic effects.

Table IX—Effect of V and III on Protection of Mice against Electroshock (an ED_{50} Study)

Number	V, 50 mg./kg.	V, 100 mg./kg.	V, 150 mg./kg.	III, 25 mg./kg.
1	−+[a]	+	+	−
2	−[a]	+	+	−−+
3	−++	+	+	−+
4	−	+	+	+
5	+[a]	+	+	−+
6	−	+	+	−
7	+	+	+	−
8	−	+	+	−+
9	+	−	+	−
10	−+	+	+	−

[a] Key: −, no protection; +, full protection; and −+, borderline; *i.e.*, mice actually extended their hindlegs, but there was a very definite lag time before this actually occurred.

reaction would have been the hydantoic acid, and the hydantoic acid under *in vitro* physiological conditions is closed very slowly to the hydantoin (15).

Solubility Studies—The order of solubility of the esters in chloroform–carbon tetrachloride (1:3 v/v) on a molar basis compared to the hydantoins was found to be amino ester (in the free base form) > parent hydantoin > simple ester. A study of the partitioning between chloroform–carbon tetrachloride (1:3 v/v) and 0.1 *M* phosphate buffer (pH 6.0) showed that very little of the hydantoin and the simple ester passed into the aqueous layer from the organic layer in which they were dissolved, whereas virtually all the amino ester did pass into the aqueous layer presumably in the protonated form. The aqueous solubility of V and III in 0.1 *M* phosphate buffer (pH 6.0) was estimated at 2 and 1% w/v, respectively. An accurate estimate could not be made because of the fast cyclization of the esters to the hydantoins. It appears, therefore, that the diethylamino moiety not only helped increase the esters' rate of closure under *in vitro* physiological conditions but also helped increase the aqueous solubility of the esters below their pKa.

Toxicity and Activity Studies—A problem that may arise when attempting chemical modification of a drug is that the derivative itself may possess increased toxicity or have a pharmacological effect of its own. The β-*N'*,*N'*-diethylaminoethyl moiety is found in a diverse group of compounds: local anesthetics, cholinergic drugs, antiasthmatics, and tranquilizers.

Table VIII shows the results of LD_{50} determinations on mice *via* intraperitoneal injections of V dissolved in 0.1 *M* phosphate buffer (pH 5.9). Table IX shows the results of an anticonvulsant activity ED_{50} determination on mice *via* intraperitoneal injections of V dissolved in the same buffer used for the LD_{50} determination. The results of one run using III at a concentration of 25 mg./kg. is also given.

The results displayed in Tables VIII and IX suggest that the ED_{50} of V against electroshock in mice after intraperitoneal injection is approximately 60 mg./kg. and its LD_{50} is between 400 and 800 mg./kg. For III an ED_{50} and LD_{50} could not be estimated; but at 25 mg./kg., 16% protection was observed. In the ED_{50} experiments with doses that gave only slight or no protection, the animals after convulsion did not require any resuscitation, whereas the mice not given the drug had to be revived for breathing to begin. This suggests that even at low doses the drugs were having an effect. Efforts to compare solutions of V and III with the parent hydantoins were not successful, because the hydantoins were not soluble in the buffer of pH 5.9. Suspensions of the hydantoin given intraperitoneally through a wide bore needle and tested for anticonvulsant activity after 1 hr. offered no protection, *i.e.*, the lack of protection against electroshock was probably due to the method of administration.

SUMMARY

The results of the present study showed that esters of hydantoic acids may be of use as prodrugs for the respective hydantoin. The β-*N'*,*N'*-diethylaminoethyl esters in particular showed superior physical properties to those of the parent hydantoin. Their cyclization rates, half-lives of under 20 min., under physiological conditions were unaffected by an esterase enzyme, and a preliminary animal study showed interesting and encouraging results. The amino esters may be of use as components of a reconstitutable injection, because their rate of cyclization and aqueous solubility at physiological pH would not lead to the precipitation in the bloodstream that was seen with the sodium hydantoin. Similarly, because solutions can be maintained at around neutral pH, the solution would be more compatible in intravenous infusions with other possible components of the infusion.

Normally, prodrugs formed through the formation of an ester linkage require enzyme mediation to regenerate the parent drug. Intramolecular nucleophilic ureido participation at an ester linkage has been shown to give a rate acceleration of 10^5-fold over the intermolecular equivalent. If the product of the intramolecular reaction happens to be the parent drug, then the acylic ester is a chemical modification whose closure rate is independent of the need of enzymatic mediation. In the present study, an ionizable group has been built into the alcohol portion of a hydantoic acid ester, giving it increased aqueous solubility and an increased rate of closure under physiological conditions. These desirable properties make V and III possible candidates as prodrugs of their respective hydantoin.

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Hydrolytic Behavior of *N*-Acyl Phthalimides

V. STELLA* and T. HIGUCHI▲

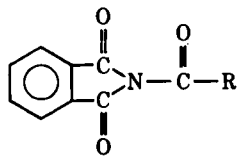
Abstract □ The hydrolysis of *N*-acyl phthalimides was studied to determine the effect of the *N*-acyl moiety on the hydrolytic rate and hydrolytic products, *i.e.*, whether hydrolysis leads to the regeneration of the parent phthalimide or to formation of the corresponding *N*-acyl phthalamic acid. A series of *N*-acyl phthalimides was prepared and their hydrolysis was observed in the neutral to alkaline pH range at 25°. It was found that *N*-acetyl-, *N*-propionyl-, *N*-(ethoxycarbonyl)-, and *N*-(morpholinocarbonyl)phthalimides gave their corresponding *N*-acyl phthalamic acid as their major hydrolytic product while *N*-chloroacetyl- and *N*-bromoacetylphthalimides gave phthalamide and the corresponding carboxylic acid. The change in path depending on the *N*-acyl group was rationalized in terms of the susceptibility of the appropriate carbonyls to attack.

Keyphrases □ *N*-Acyl phthalimides—hydrolysis, effect of *N*-acyl moiety on rate and formation of phthalimide parent or *N*-acyl phthalamic acid □ Phthalimides, *N*-acyl—hydrolysis, effect of *N*-acyl moiety on rate and formation of phthalimide parent or *N*-acyl phthalamic acid □ Hydrolysis of *N*-acyl phthalimides—effect of *N*-acyl moiety on rate and phthalimide or phthalamic acid products □ Imides (phthalimides)—formation by hydrolysis of *N*-acyl phthalimides

Imides form a group of compounds which have found wide use in the pharmaceutical and chemical industries. The major pharmaceuticals having the imide functionality within their structural formulas are glutethimide and thalidomide (sedatives), ethosuximide and phensuximide (anticonvulsants), bemegride (barbiturate antagonist), and a number of antibiotic, antiviral, and carcinostatic agents (1).

The present study was concerned with the hydrolysis of *N*-acyl phthalimides (I) and the possible use of acylimides as prodrugs of imides. The aim of the work was to observe the effect of the *N*-acyl moiety on the hydrolytic rate and hydrolytic products of *N*-acyl phthalimides, *i.e.*, whether hydrolysis leads to the regeneration of the imide or to formation of the corresponding *N*-acyl phthalamic acid. If *N*-acyl imides are to be considered for prodrugs of imides, they must regenerate the imide on hydrolysis.

The solvolysis and aminolysis of *N*-acyl cyclic imides under various conditions were the subjects of a limited number of studies (2–9), most of which were intended as observations of the acylating power of the acylated imides; however, no quantitative kinetic data were presented. There also appears to be some confusion as to the mechanism of reaction because the products vary depending on the nucleophile, solvent, and temperature of reaction.



I

R = alkyl, alkoxy, or amino

EXPERIMENTAL

Equipment—A recording spectrophotometer[1], with a thermostated cell compartment maintained at 25 ± 0.1°, was used for all kinetic and product measurements. NMR spectra were obtained using spectrometers[2] with all spectra run in $CDCl_3$. IR spectra were acquired using an IR spectrometer[3]. All pH measurements were obtained on a research pH meter[4] at 25°, standardized with standard phosphate and borate buffers.

Reagents and Materials—Dioxane and phthalimide were purified according to the method of Perrin *et al.* (10). All other chemicals were of analytical or reagent grade and were used without further purification. A TLC sorbent[5] was used. *N*-Ethoxycarbonylphthalimide (II) was obtained commercially[6], and all other phthalimide derivatives studied were synthesized.

Synthesis of *N*-Acetylphthalimide (III)—Five grams (0.034 mole) of phthalimide was dissolved in 25 g. (0.250 mole) of acetic anhydride. The solution was refluxed for 2 hr. and then fractionally distilled (30-cm. vigreux column). The first distillate was collected at 123° (acetic acid plus acetic anhydride), and the reaction was terminated when the distillate temperature rose to 138°. The residue was then taken up in toluene, hot filtered through charcoal, and, on cooling, diluted with 50 ml. of ether. The resulting white precipitate was recrystallized three times from the toluene–ether mixture to give a solid of m.p. 136–137° [lit. (11) m.p. 135–136°]. NMR showed a symmetrical A_2B_2 pattern at 7.92 and 7.98δ (four protons) and a singlet at 2.69δ (three protons), confirming *N*-acylation. The IR spectrum gave a large peak area between 1800 and 1600 $cm.^{-1}$, and the UV spectrum gave peaks at 270 and 295 nm.

Synthesis of *N*-Propionylphthalimide (IV)—This was prepared in a similar manner to *N*-acetylphthalimide, except that propionic anhydride was used in place of acetic anhydride. A product of m.p. 153–155° was obtained [lit. (12) m.p. 143–144°]. NMR showed a symmetrical A_2B_2 pattern at 7.92 and 7.96δ (four protons), a triplet at 1.29δ (three protons), and a quartet at 3.10δ (two protons), confirming *N*-acylation. The IR spectrum gave a large peak area between 1800 and 1600 $cm.^{-1}$, and the UV spectrum gave peaks at 270 and 295 nm.

Synthesis of *N*-Chloroacetylphthalimide (V)—To a solution of 10 g. (0.059 mole) of chloroacetic acid was added 6.1 g. (0.059 mole) of dicyclohexylcarbodiimide in 120 ml. of tetrahydrofuran. After standing for 4 hr., the urea was filtered off, leaving a solution of the anhydride of approximately 10% (w/v). Two grams of potassium phthalimide was added to 20 ml. of the solution, and the suspension was refluxed for 5 hr. with stirring. The solution was filtered and evaporated to 10 ml., and the precipitate was collected. After three recrystallizations from tetrahydrofuran, a white compound was collected, m.p. 177–179° [lit. (13) m.p. 179–180°]. NMR showed a symmetrical A_2B_2 pattern at 7.92 and 7.98δ (four protons) and a singlet at 4.70δ (two protons), confirming *N*-acylation.

Synthesis of *N*-Bromoacetylphthalimide (VI)—The synthesis was identical to that of V, except that bromoacetic acid was used in the initial step. A product of m.p. 153–155° was obtained.

Anal.—Calc. for $C_9H_{10}BrNO_3$: C, 44.94; H, 2.27; N, 5.24. Found: C, 45.24; H, 2.49; N, 5.37.

NMR showed a symmetrical A_2B_2 pattern at 7.92 and 7.98δ (four protons) and a singlet at 4.60δ (two protons), confirming *N*-acylation.

Synthesis of *N*-(Morpholinocarbonyl)phthalimide (VII)—This compound was synthesized according to the method of Grigat (14), except that the starting material was *N*-(ethoxycarbonyl)phthalimide instead of *N*-(phenoxycarbonyl)phthalimide. The product showed m.p. 204–205° [lit. (14) m.p. 207–208°]. NMR showed a sym-

[1] Cary 14.
[2] Varian T60 or A-60A.
[3] Perkin-Elmer 700.
[4] Corning model 12.
[5] Silicar, TLC-7G1, Mallinckrodt Chemical Works, St. Louis, Mo.
[6] Aldrich Chemical Co., Milwaukee, Wis.

Table I—Delivery Volume as a Function of Pressure

Liquid	Rate, ml./p.s.i. (10^3)	Total Volume Delivered, ml. 1400 p.s.i.	2100 p.s.i.	2800 p.s.i.
Calcium carbonate, 5%	6.7	10	14.7	19
Syrup USP	7.1	10	15	20
Water	6.8	12	16.8	21.5
Alcohol USP	7.1	13	18	23

EXPERIMENTAL

Materials and Equipment—The liquids used in this study were: distilled water; syrup USP; shellac, 2.7 kg. (6 lb.) cut; 5, 15, and 35% (w/v) aqueous suspensions of calcium carbonate; and a 2% (w/v) solution of methylcellulose[1], 500 cps.

The equipment utilized to evaluate the factors involved in an automated tablet-coating system was a self-programming automated tablet-coating system with two automatic spray guns[2] as previously described (4).

Fluid Delivery Studies—Fluid delivery experiments were conducted to ascertain the influence of pressure, spray tip orifice size, and type of liquid on the volume of spray delivered.

The volume of fluid delivery in milliliters was determined from 1400 to 2800 p.s.i. nozzle pressure in 140 p.s.i. pressure increments. The tip orifice sizes varied from 0.023 cm. (0.009 in.) to 0.053 cm. (0.021 in.) in diameter. All experiments were conducted in an air conditioned room at approximately 25° and 30–40% relative humidity.

Three-second fluid spray delivery cycles were used, and five replicate samples of each liquid under study were collected, at each pressure and nozzle tip size, in a 100-ml. graduated cylinder; the resulting volumes were recorded.

Spray Pattern Characteristics—Spray patterns were determined for the liquids studied, each containing 1% (w/v) FD&C Red No. 2 dye for contrast. The spray from the activated gun of the automated tablet-coating system was directed at a specially designed target which consisted of a 61-cm. square pane of glass with five concentric circles painted 10 cm. apart. The tip of the spray gun was positioned 30 cm. from the glass surface and at the center point of the target. The spray cycle was set for a 0.25-sec. solution delivery. The patterns were subsequently observed at 1400-, 1960-, and 2800-p.s.i. nozzle pressure (the lower, middle, and upper pressure range, respectively) for all tip sizes ranging from 0.023 cm. (0.009 in.) to 0.053 cm. (0.021 in.) in orifice diameter. After each spray, the pattern dimensions were taken and pattern uniformity was noted.

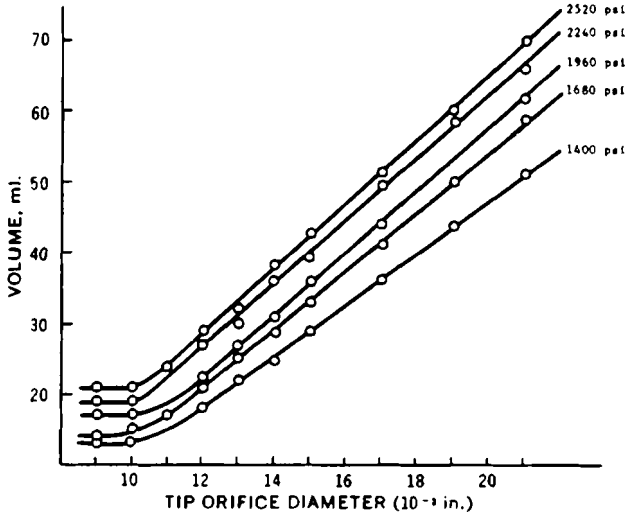


Figure 3—*Change in delivery with respect to change in tip orifice size using water.*

[1] Methocel MC, Dow Chemical Co., Midland, Mich.
[2] Graco Hydro-Spray Unit, Gray Co., Minneapolis, Minn.

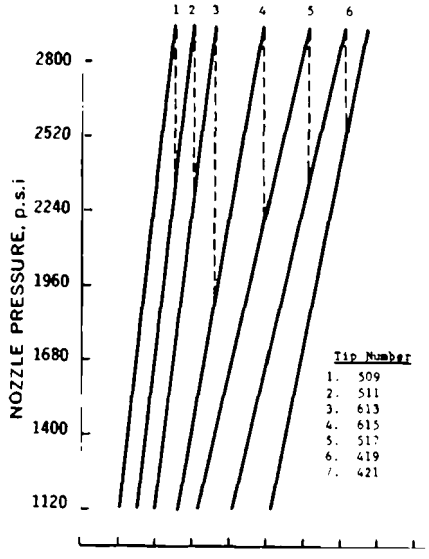


Figure 4—*Fluid delivery curves, 3-sec. cycle.*

RESULTS AND DISCUSSION

Fluid Delivery—The pressure–volume curves in Fig. 1, constructed from fluid delivery data obtained with a 0.023-cm. (0.009-in.) spray tip and a 3-sec. delivery, show that the change in fluid volume delivered as a function of pressure was constant. The curves are representative of some of the liquids studied, and each shows excellent linearity. The rate of change of delivery (Table I) for the liquids represented in Fig. 1 was essentially the same.

A 3–4-ml. difference in total volume delivered at each pressure increment was found between the four liquids studied. These small volume differences do not appear to be significant, but the data show a trend toward a decrease in volume delivered with an increase in fluid viscosity and solids content when comparing syrup and calcium carbonate with water and alcohol. A significant difference would be expected at a much lower or at atmospheric pressure. The data, therefore, indicate that fluid volume delivery is essentially independent of the physical properties of the fluid at very high pressures.

Fluid delivery curves for the three calcium carbonate suspensions, which varied from a "watery" to an "ointment-like" consistency (Fig. 2)[3], are in essence identical and confirm that the elevated pressures used in the study obliterated the effects of the physical properties of the fluids delivered.

In Fig. 3, the volume of water delivered in 3 sec. *versus* tip orifice diameter shows a good linear relationship within the range of 0.030 cm. (0.012 in.)–0.053 cm. (0.021 in.). This finding is unusual because one would expect an orifice–volume relationship based on

Figure 5—*Spray patterns with water. Left: 0.023-cm. (0.009-in.) tip size and 30 × 9-cm. pattern size. Right: 0.053-cm. (0.021-in.) tip size and 40 × 30-cm. pattern size.*

[3] Data points for Fig. 2 were omitted for the purpose of clarity.

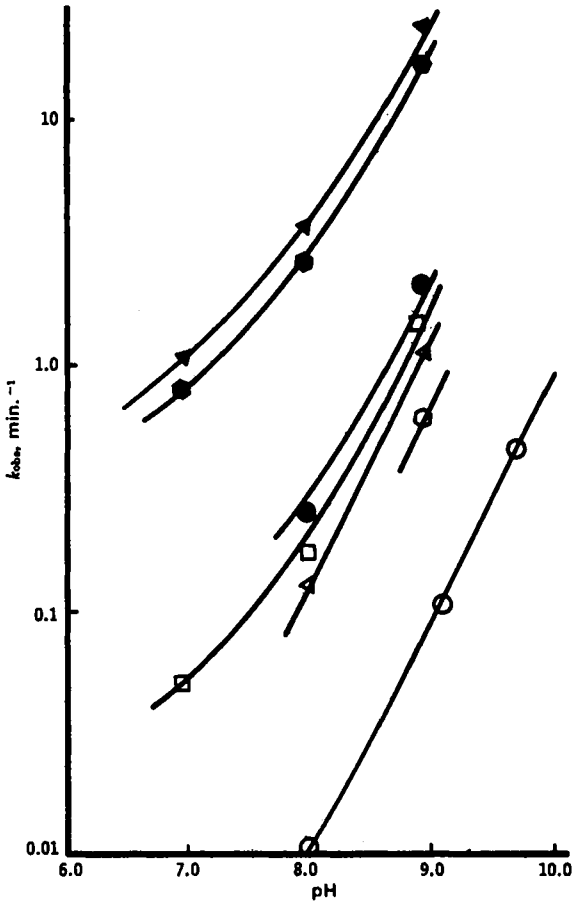


Figure 1—*Plot of log* k_{obs} versus *pH for hydrolysis of* N-*acyl phthalimides. Key:* ○, $R = N(CH_3)_2$[a]; ○, *R = morpholino;* △, $R = C_2H_5$; □, $R = C_2H_5O$; ●, $R = CH_3$; ●, $R = CH_2Br$; *and* ▲, $R = CH_2Cl$. *(For definition of R, see Scheme I.)* ([a]*Unpublished data, T. Higuchi and F. Kametani.*)

(*b*) help disperse any negative charge built up on the nitrogen in the transition state.

Similarly, for the reactions that took place at the *N*-acyl carbonyl, the rates were found to be proportional to the electron-withdrawing power of R, again referring to R in Scheme I.

Effects of R on Reaction Path—If the rates for the attack of base at the phthalimide carbonyls are to be compared to those at the *N*-acyl carbonyl, it is important to consider that there are two phthalimide carbonyls as compared to one *N*-acyl carbonyl. Statistical considerations, therefore, predict that more of the *N*-acyl phthalamic acid would be formed than phthalimide.

Which path (Path I or II) provides the better leaving group could be a matter for further discussion. However, for the task at hand, the route of reaction can be explained on the basis of the susceptibility of the various carbonyl groups to attack by a nucleophile. A close look at the compounds that hydrolyzed *via* Path I revealed a common property. The R group for each of the compounds was electron donating to the *N*-acyl carbonyl; *i.e.*, dimethylamino and morpholino can donate electrons to the *N*-acyl carbonyl by resonance because the amine group possesses a free pair of electrons. Where R is a methyl or an ethyl group, electrons are donated to the *N*-acyl carbonyl by induction. If R is an ethoxy group, a carbamate linkage results. The specific interactions of the R group with the *N*-acyl carbonyl actually change the character of the reaction centers, making the comparison of attack at the phthalimide carbonyls to the *N*-acyl carbonyl difficult. All these R groups deactivate the *N*-acyl carbonyl while, at the same time, as the *N*-acyl group (RCO), they activate the phthalimide carbonyls by being electron withdrawing at those carbonyls.

Phthalimide–acyl cleavage occurs only if the *N*-acyl carbonyl is sufficiently activated by electron-withdrawing R groups, such as $-CH_2Br$ and $-CH_2Cl$, so it can compete with attack at the phthalimide carbonyls. Thus, the product of reaction appears to be determined by susceptibility of the appropriate carbonyl to attack. The slightest activation of the *N*-acyl carbonyl, even by the phenoxymethylene R group (9), has been shown to lead to reaction at the *N*-acyl carbonyl. The idea that the carbonyl activation is the product-determining factor in the hydrolysis of *N*-acyl phthalimides may be best explained in terms of free energy of activation. Electron-withdrawing *N*-acyl groups lower the free energy of activation for Path I. Proportionally, however, there may be greater effect on the free energy of activation for Path II by the changes in R; *i.e.*, for VII, $\Delta G^{\ddagger}_{\text{Path II}} >>> \Delta G^{\ddagger}_{\text{Path I}}$, whereas for III, $\Delta G^{\ddagger}_{\text{Path II}} > \Delta G^{\ddagger}_{\text{Path I}}$. For V and VI, $\Delta G^{\ddagger}_{\text{Path I}} \gg \Delta G^{\ddagger}_{\text{Path II}}$, thus predicting that compounds like *N*-(phenylacetyl)phthalimide with a poor electron-withdrawing R group should degrade *via* Paths I and II, *i.e.*, both pathways being detectable.

In summary, chemical modification of imides through *N*-acylation requires a detailed study of the products of the *in vitro* hydrolysis because it cannot be assumed that the product will be the parent imide. This study has shown that *N*-acylated imides, when hydrolyzed, can regenerate the parent imide if the *N*-acyl carbonyl is sufficiently activated to compete with possible reaction at the imide carbonyls.

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Drug Permeation through Membranes II: Simultaneous Dissolution and Permeation of Drug from Solid Dosage Forms

EDWARD G. LOVERING[▲] and KEN B. McRAE

Abstract □ The simultaneous dissolution and permeation of a drug from solid oral dosage forms were analyzed by a treatment based on Fick's second law of diffusion. The analysis shows that dissolution rate constants can be obtained from the permeation lag time, while the coefficients of permeability are estimated, as usual, from the rate of drug transfer across the membrane under steady-state conditions. Drug–excipient interactions, if any, would be expected to affect the rate of drug transfer across the membrane. Therefore, from a single experiment, the dissolution rate and the extent of drug–excipient interaction can be obtained. The equation was tested by dissolution–permeation measurements on two amobarbital formulations.

Keyphrases □ Drug permeation—membranes, amobarbital, analysis of dissolution and permeation from solid oral dosage forms, application of Fick's second law of diffusion, relevance to drug–excipient interactions, equations □ Drug–excipient interactions—application of Fick's second law of diffusion to determine both dissolution and membrane permeation, amobarbital from a solid oral dosage form, equations □ Dissolution–membrane permeability data—generated by application of Fick's second law of diffusion, relevance to drug–excipient interactions, amobarbital from solid oral dosage form, equations □ Permeation, membrane—used to evaluate drug–excipient interactions and drug dissolution by application of Fick's second law of diffusion, amobarbital from a solid oral dosage form □ Membrane permeation–dissolution data—generated by application of Fick's second law of diffusion, relevance to drug–excipient interactions, amobarbital from a solid oral dosage form, equations

It is now well recognized that the *in vivo* dissolution characteristics of drug formulations may have a pronounced effect on their bioavailability. Because *in vivo* dissolution rates are difficult to measure directly, attempts to assess this aspect of drug quality are commonly made from *in vitro* dissolution rate data. Dissolution specifications *in vitro* have been promulgated for a number of drugs (1, 2), and attempts to correlate these with bioavailability have met with varying degrees of success (3–5).

In addition to *in vivo* dissolution, bioavailability depends upon transfer of the drug across the GI membrane. For typical oil-soluble drugs, the transfer rate depends upon the concentration of free, unionized drug in the GI tract. The concentration may be adversely affected by adsorption, absorption, reprecipitation, partitioning into bile micelles or fat particles, or any other factor that reduces the concentration of free drug (6). Substances having an adverse effect on absorption rates may be endogenous to the GI tract or may be introduced as nutrients or tablet excipients. *In vitro* assessment of the latter can be made by comparing the permeation rate of unformulated drug to that of drug released from formulations dissolved, or dispersed, in an appropriate buffer. Drug–excipient interactions may account for the low bioavailabilities observed in some formulations.

This paper describes a method for the simultaneous determination of the dissolution rate constant of drug in a formulation and the permeability coefficient of the drug in that formulation.

THEORETICAL

The experiment involves dissolution of drug from a solid dosage form, followed by drug transfer across the membrane of a permeation cell mounted in the dissolution vessel. The term permeation refers to transfer of drug from the aqueous solution in which it dissolves, across the membrane, and into the aqueous solution within the permeation cell, while diffusion refers to movement of drug molecules within the cell membrane.

Movement of drug within a homogeneous membrane can be described by Fick's second law of diffusion:

$$\frac{\partial C^*}{\partial t} = D\frac{\partial^2 C^*}{\partial x^2} \qquad \text{(Eq. 1)}$$

where C^* is concentration of drug within the membrane, t is time, D is the diffusion coefficient, and x is the space coordinate normal to the surface of the membrane (7). For a membrane of thickness l, x lies between zero and l. On the absorbing side of the membrane, where $x = 0$, the concentration of drug is time dependent and given by:

$$C^* = C_0^* G(t) \qquad \text{(Eq. 2)}$$

where C_0^* is the drug concentration in the membrane at $x = 0$ when the dosage form has completely dissolved, and $G(t)$ is a particular driving function with range [0, 1]. The drug concentration is zero when time is zero. On the desorbing side, where $x = l$, the drug concentration is maintained at zero by buffering the desorbing solution at an appropriate pH. These considerations lead to the following boundary conditions:

$$C^*(l,t) = 0 \qquad \text{(Eq. 3)}$$

$$C^*(x,0) = 0 \qquad \text{(Eq. 4)}$$

$$C^*(0,t) = C_0^* G(t) \qquad \text{(Eq. 5)}$$

Solution of Eq. 1 for C^* as a function of x and t is given in *Appendix I*. The rate at which drug diffuses across the plane of the membrane at $x = l$ is given by:

$$-D\left.\frac{\partial C^*}{\partial x}\right|_{x=l} \qquad \text{(Eq. 6)}$$

so that, for a membrane of unit area, the quantity of drug crossing the plane during the time interval from 0 to t is:

$$q = -D\int_0^t \left(\frac{\partial C^*}{\partial x}\right)_{x=l} dt \qquad \text{(Eq. 7)}$$

The particular driving function, $G(t) = (1 - e^{-\alpha t})$, was chosen to represent the rate of drug dissolution from a solid dosage form. It probably represents actual dissolution behavior as well as any other function and it is convenient to handle mathematically. The

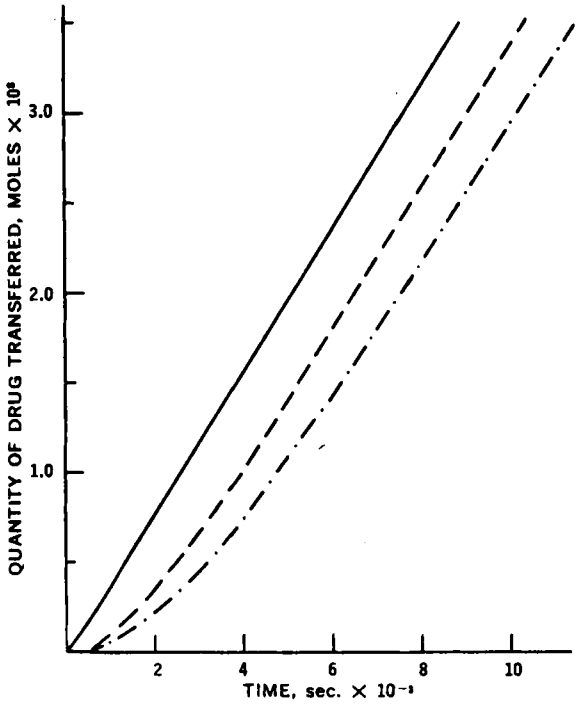

Figure 1—*Plots of theoretical* q versus t *for various half-times of dissolution. Key:* ——, $t_{1/2}$ = *0 min.;* - - -, $t_{1/2}$ = *16.6 min.;* -.-., $t_{1/2}$ = *30 min.;* P = 10^{-6} *cm.*^{2} *sec.*$^{-1}$, C* = 10^{-6} *mole cm.*$^{-3}$, *and* l = 2.54×10^{-3} *cm.*

constant κ is characteristic of a given dosage form and is a measure of the rate at which it dissolves. Solution of Eq. 1 for this particular case is given in *Appendix II*. Differentiation with respect to x at $x = l$ and integration over time lead to:

$$q = C_0^* \left\{ \frac{Dt}{l} - \frac{l}{6} - \frac{D}{\kappa l} + \frac{e^{-\kappa t}}{\sqrt{\kappa/D}\sin\{l\sqrt{\kappa/D}\}} + \frac{2\kappa l}{\pi^2 D}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\frac{e^{-(n\pi/l)^2 Dt}}{(n\pi/l)^2 - \kappa/D}\right\} \quad \text{(Eq. 8)}$$

provided that $(l/\pi)\sqrt{\kappa/D} \neq n = 1, 2, 3 \ldots$.

If the limit of q in Eq. 8 is taken as t becomes large, then:

$$q = \frac{C_0^* AD}{l}t - C_0^* A\left(\frac{l}{6} + \frac{D}{\kappa l}\right) \quad \text{(Eq. 9)}$$

where A is the surface area of the membrane. To apply Eq. 9 to permeation, it is necessary to replace DC_0^* by PC_0, where P is the permeation coefficient and C_0 is the concentration of drug in solution when dissolution is complete. Thus, the partition coefficient,

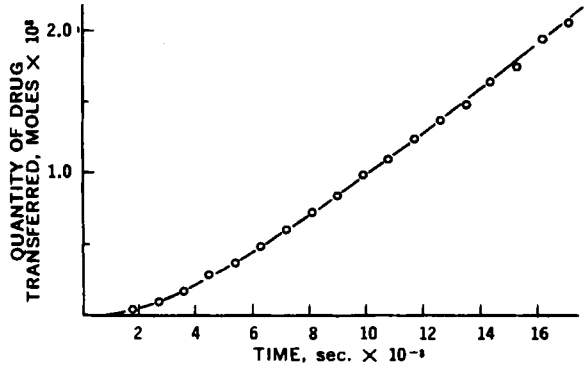

Figure 2—*Experimental points and fitted theoretical curve of* q versus t *for amobarbital Formulation B. The curve was calculated for* C* = 2.90×10^{-7} *mole cm.*$^{-3}$, P = 8.2×10^{-6} *cm.*^{2}*/sec.*$^{-1}$, l = *0.014 cm., and* $\kappa = 3.11 \times 10^{-4}$ *sec.*$^{-1}$. P *and* κ *were calculated from the steady-state portion of the curve.*

Table I—Experimentally Determined Dissolution and Permeability Coefficients of Amobarbital Formulations

Tablet Number	Lag Time, t^*, sec.	Permeability Coefficient, P, cm.^{2} sec.$^{-1}$ × 10^6	Dissolution Constant, κ, sec.$^{-1}$ × 10^4	Dissolution Half-Time, $t_{1/2}$, min.
		Formulation A		
1	1450	8.99	9.2	12
2	1180	8.42	12.7	9
3	990	7.84	17.4	7
4	1080	7.84	15.1	8
5	—	—	—	9[a]
6	—	—	—	9[a]
		Formulation B		
1	4608	7.42	2.40	48
2	4480	7.74	2.46	47
3	5280	8.35	2.04	56
4	3610	8.23	3.11	37
5	—	—	—	20[a]
6	—	—	—	65[a]
7	—	—	—	82[a]

[a] Dissolution half-times determined by direct measurement.

K_p, is C_0^*/C_0. This substitution assumes that the concentration of drug in the membrane at $x = 0$ is proportional to the concentration of drug in solution. The term P includes diffusion of the drug across the aqueous boundary layer in contact with the membrane. Thus:

$$q = \frac{C_0 AP}{l}t - C_0 A\left(\frac{l}{6} + \frac{P}{\kappa l}\right) \quad \text{(Eq. 10)}$$

Equation 10 is applicable to dissolution–permeation experiments of sufficient duration to reach the steady state, where the plot of q *versus* t is linear. Under these conditions:

$$P = \frac{(dq/dt)l}{C_0 A} \quad \text{(Eq. 11)}$$

and:

$$\kappa = \left(t^* - \frac{l^2}{6D}\right)^{-1} \quad \text{(Eq. 12)}$$

where t^*, the lag time, is the value of t when q is zero.

Before attempting to use Eqs. 8–12 to determine κ and P from experimental measurements of q and t, it is important to know whether κ and P vary significantly with changes in the experimental parameters. Figure 1 gives curves of q *versus* t for various κ's between 10^3 and 3.851×10^{-4} sec.$^{-1}$, corresponding to a range in half-times of dissolution from 0 to 30 min. Clearly, the curves and lag times are well separated with the lag time increasing from 2 to 45 min. It is, therefore, feasible to determine κ by this technique. Plots of Eq. 8 for various values of P (not shown) demonstrate that while t^* depends on P, the change in t^* is less than a factor of 2 when P is varied over an order of magnitude. However, highly permeable membranes are preferred since the time required to carry out an experiment increases as P decreases.

The significance of changes in lag time can be visualized by recasting Eq. 12:

$$t^* = \frac{l^2}{6D} + \frac{1}{\kappa} \quad \text{(Eq. 13)}$$

showing t^* to be made up of a diffusion term and a dissolution term. As l becomes smaller, the contribution of the first term to t^* decreases, enhancing the relative importance of κ^{-1} and facilitating its determination. In addition, small values of l reduce the time required to reach the steady state and, hence, the overall time of the experiment. The effect of an increase in l on q is to decrease the drug transfer rate, increase the lag time, and prolong the period required to reach a steady state.

EXPERIMENTAL

Dissolution–permeation experiments were carried out in a 1-l. resin kettle at 37 ± 1°. The kettle contained a wire basket of the

type used in the USP dissolution procedure (1) placed near the rim of the kettle, about halfway down, and rotated at 150 r.p.m. A diffusion cell of the type described by Garrett and Chemburkar (8), equipped with 0.014-cm. membranes of polydimethylsiloxane, was placed opposite the rotating basket.

Dissolution–permeation experiments were carried out on two tablet formulations of amobarbital, A and B. The experiment was started by placing 900 ml. of acetate buffer, pH 5, in the kettle and 15 ml. of borate buffer in the diffusion cell. A tablet was put into the wire basket at time zero; at appropriate time intervals, the concentration of amobarbital in the diffusion cell was measured by UV spectroscopy at 240.5 nm., following the procedure already described (6). Tablet dissolution rates were measured directly by sampling the resin kettle at appropriate time intervals. These samples were diluted with borate buffer, and the amobarbital concentration was read spectrophotometrically.

RESULTS AND DISCUSSION

To measure the dissolution constant κ and the permeability coefficient P by the method described, it is necessary for the transfer rate of drug across the membrane to reach the steady state. The steady state cannot be achieved before the tablet has completely dissolved. The time taken to reach the steady state also increases as the membrane thickness increases.

The dissolution constant κ for two brands of amobarbital, A and B, was determined in the dissolution–permeation apparatus by two methods: (*a*) from the lag time as described in this paper, and (*b*) by measurement of the drug concentration in the dissolving medium.

The results (Table I) show $t_{1/2}$ as determined by the two methods to be in agreement, remembering that large intralot variations in tablet dissolution times are commonly observed. The permeability coefficients are, within experimental error, the same as those reported previously for amobarbital (6). The component of the lag time due to the nature of the drug and the membrane thickness, $l^2/6D$, in Eq. 13 is about 425 sec. for this experiment. The difference between this quantity and t^*, the experimentally observed lag time, is a reflection of the time required for the tablet to dissolve.

Equation 8 was tested by calculating the theoretical curve of q *versus* t for an amobarbital tablet with a dissolution half-time of 37 min. and plotting the experimental points of Tablet 4, Formulation B, on the same graph (Fig. 2). It is evident that the experimental results are adequately described by Eq. 8.

In conclusion, it was possible to determine the dissolution constant and the permeability coefficient of a drug in a solid dosage form by a single experiment. The time required to determine κ by this method is greater than in a single dissolution experiment, but additional information is gained. Comparison of P as determined from tablet dissolution, with P for the pure drug in question, indicates whether or not a portion of the drug is unavailable for membrane transfer. Drug availability could be reduced by interaction with tablet excipients. Further studies are planned to determine if dissolution–permeation experiments are of value in monitoring drug quality and whether the results can be correlated with human bioavailability data.

APPENDIX I

Suppose $C^*(x,t)$ is well defined for $0 < x < l$, $t > 0$, so that for fixed x, $C^*(x,t)$ is sectionally continuous in every finite interval $0 < t < N$ and of exponential order γ for $t > N$, where N and γ are finite numbers. Then by the method of Laplace transformations (9), the partial differential Eq. 1 can be transformed into the ordinary differential equation:

$$sc^*(x,s) - C^*(x,0) = D\frac{d^2c^*}{dx^2} \qquad \text{(Eq. A1)}$$

where the Laplace transformation of $C^*(x,t)$ is denoted by the lower case function $c^*(x,s) \overset{\text{defn}}{\equiv} \int_0^\infty e^{-st}\, C^*(x,t)\, dt$.

The Laplace transforms of boundary conditions (Eqs. 3–5) are, respectively:

$$c^*(l,s) = 0 \qquad \text{(Eq. A2)}$$

$$c^*(x,0) = 0 \qquad \text{(Eq. A3)}$$

$$c^*(0,s) = C_0^* g(s) \qquad \text{(Eq. A4)}$$

The general solution of Eq. A1 after the substitution of Eq. 4 is given by:

$$c^*(x,s) = \kappa_1 \cosh \sqrt{s/D}x + \kappa_2 \sinh \sqrt{s/D}x \qquad \text{(Eq. A5)}$$

Using boundary condition A2, one finds:

$$\kappa_2 = \kappa_1 \cosh\{\sqrt{s/D}l\}/\sinh\{\sqrt{s/D}l\} \qquad \text{(Eq. A6)}$$

and:

$$c^*(x,s) = \kappa_1 \sinh\{\sqrt{s/D}(l-x)\}/\sinh\sqrt{s/D}l \qquad \text{(Eq. A7)}$$

After imposing boundary condition A4, one has:

$$c^*(x,s) = C_0^* g(s) \sinh\{(l-x)\sqrt{s/D}\}/\sinh\sqrt{s/D}l \qquad \text{(Eq. A8)}$$

The solution to the partial differential equation is given by the inverse Laplace transformation of Eq. A8 and can be found by use of the convolution theorem. That is:

$$C^*(x,t) = L^{-1}\left\{C_0^* g(s)\frac{\sinh(l-x)\sqrt{s/D}}{\sinh l\sqrt{s/D}}\right\} = \int_0^t \left(L^{-1}\frac{\sinh(l-x)\sqrt{s/D}}{\sinh l\sqrt{s/D}}\bigg|_{x=u}\right) C_0^* G(t-u)\,du = \frac{2\pi D}{l^2} C_0^* \sum_{n=1}^{\infty}(-1)^{n-1} n \int_0^t e^{-(n\pi/l)^2 Du} G(t-u)du \times \sin n\pi(l-x)/l \qquad \text{(Eq. A9)}$$

The rate of transfer at $x = l$ at time t is given by:

$$-D\frac{\partial C^*}{\partial x}\bigg|_{x=l}$$

so that the quantity of diffusing substance during the interval $[0,t]$ is:

$$q = -D\int_0^t \left(\frac{\partial C^*}{\partial x}\right)_{x=l} d\tau \qquad \text{(Eq. A10)}$$

APPENDIX II

For the particular driving function $G(t) = (1 - e^{-\kappa t})$, it is necessary to evaluate:

$$\int_0^t e^{-(n\pi/l)^2 Du}\{1 - e^{-\kappa(t-u)}\}\,du = \int_0^t e^{-(n\pi/l)^2 Du}\,du - e^{-\kappa t}\int_0^t e^{-\{(n\pi/l)^2 D-\kappa\}u}du = [(1-e^{-\kappa t})/\{(n\pi/l)^2 D - \kappa\}] - [\kappa\{1 - e^{-(n\pi/l)^2 Dt}\}/(n\pi/l)^2 D\,\{(n\pi/l)^2 D - \kappa\}] \qquad \text{(Eq. A11)}$$

provided $(n\pi/l)^2 D \neq \kappa$. Substituting into Eq. A9, one has:

$$C^*(x,t) = \frac{2C_0^*\pi D}{l^2}\left[(1-e^{-\kappa t})\sum_{n=1}^{\infty}\frac{(-1)^{n-1}n}{(n\pi/l)^2 D - \kappa}\times \sin\frac{n\pi(l-x)}{l} + \kappa\sum_{n=1}^{\infty}\frac{(-1)^n n}{\{(n\pi/l)^2 D - \kappa\}(n\pi/l)^2 D}\times \{1 - e^{-(n\pi/l)^2 Dt}\}\sin n\pi(l-x)/l\right] = C_0^*(1-e^{-\kappa t})\times \frac{\sin(l-x)\sqrt{\kappa/D}}{\sin l\sqrt{\kappa/D}} + \frac{2\kappa C_0^*}{\pi D}\sum_{n=1}^{\infty}\frac{(-1)^n}{\{(n\pi/l)^2 - \kappa/D\}n}\times \{1 - e^{-(n\pi/l)^2 Dt}\}\sin\frac{n\pi(l-x)}{l} \qquad \text{(Eq. A12)}$$

where the first summation was executed by series 557 in Jolley (10).

After differentiating Eq. A12 with respect to x and using summation formula 558 in Jolley (10), one obtains:

$$\left(\frac{\partial C^*}{\partial x}\right)_{x=l} = C_0^* \left\{\sqrt{\frac{\kappa}{D}}\frac{e^{-\kappa t}}{\sin l\sqrt{\kappa/D}} - \frac{1}{l} + \frac{2\kappa}{Dl}\sum_{n=1}^{\infty}\frac{(-1)^n e^{-(n\pi/l)^2 Dt}}{(n\pi/l)^2 - \kappa/D}\right\} \quad \text{(Eq. A13)}$$

Consequently, upon integration and simplifying by means of series 337 and 558 in Jolley (10), one obtains Eq. 8. Under experimental conditions, l, D, and κ will be rational numbers while π^2 is irrational so that $l^2\kappa/\pi^2 D \neq 1, 4, 9, 16, \ldots$. However, it may be sufficiently close to an integer to cause "round off" difficulties in computing the infinite sum.

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Previous reports from this laboratory have analyzed the occurrence of locomotor stimulation following low doses of morphine in nontolerant rats (1–4). This effect had been little emphasized and had not been systematically evaluated in earlier works, which did, however, describe repeatedly an enhancement of motility by morphine occurring after an interval of repeated dosing in the study of tolerance and/or physical dependence (5–9). Certain studies have cited gross observations or limited data concerning such an effect in nontolerant rats (9–12). Locomotor excitation provides evidence for behavioral arousal in response to morphine in the nontolerant rat despite the classification of this species among those showing predominantly a response of depression and behavioral inhibition (13, 14).

Interest in this excitatory component of the CNS pharmacology of the opiates led to a consideration of the generality of the motility response seen with low doses of morphine. Specifically, it was of interest whether similar effects might be found not only after the synthetic narcotic analgesics but also after an agent of the narcotic antagonist–analgesic class. Therefore, methadone, meperidine, levorphanol, and penta-

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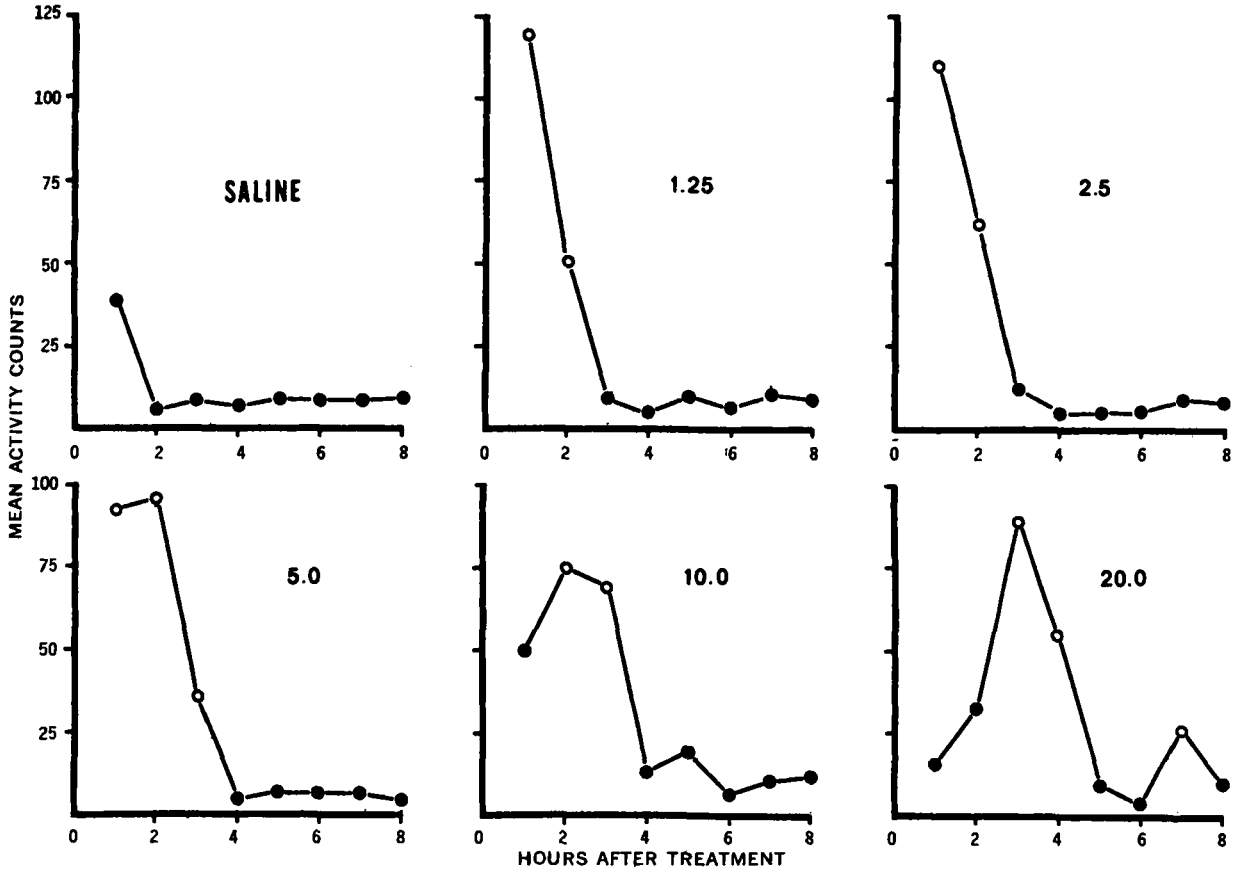

Figure 1—*Locomotor activity of rats following saline or several doses of morphine sulfate as indicated on the individual graphs (1.25–20.0 mg./kg.). Open circles represent hourly totals differing significantly from corresponding values of saline controls at* $p \leq 0.05$. *For each point,* $N = 18$.

zocine were examined to compare their actions to that of morphine. In addition to measuring the locomotor response to such agents alone, their interaction was tested with α-methyl-*p*-tyrosine, an agent that was previously found to be an effective inhibitor of the motor excitatory response to morphine in nontolerant rats (3, 4).

EXPERIMENTAL

Experimental subjects were male Wistar rats[1], which were assigned randomly on receipt to treatment groups and housed three per cage in metal cages (41 × 24.5 × 18 cm.) with food and water available *ad libitum*. They were placed in a room with an altered light cycle of 4:00 p.m. to 4:00 a.m., and 7–9 days was allowed for adaptation to the environmental conditions. Most rats attained a desired weight range of 225–235 g. by the time the locomotor activity study was begun. Each rat received only one dose of only one drug.

The photocell actometer units used (15) consisted of a circular alley, 7.6 cm. wide and 15 cm. high, with an external diameter of 37.5 cm. The inner wall held a container for food and a water bottle. Photocells of this apparatus cannot be deactivated several times in succession. An adjacent photocell must be subsequently deactivated to reset the first one, so that only large horizontal movements may be recorded. The actometers were located in a windowless, sound-shielded room, which also contained a masking white-noise generator to attenuate further any outside sounds. The temperature was generally maintained at 24 ± 1°.

Experiments began 1.5–2 hr. after light onset. On the 1st day, all rats were injected with saline, placed in actometer units individually, and allowed 24 hr. for adaptation to the actometer. On the 2nd day, they were injected with saline or with the test drug. Beginning immediately following injection, motility data were recorded hourly for 8 hr. Statistical analyses tested for differences between the saline control and each treatment group at each hourly observation period by means of one-way analysis of variance and Duncan's multiple range test (16).

The locomotor activity responses to several dosages of morphine and its surrogates were evaluated first. With the exception of methadone, all drugs were tested at the same five levels: 1.25, 2.5, 5.0, 10, and 20 mg./kg. For methadone the highest dosage was reduced to 16 mg./kg. because of toxicity. Each of the six treatment groups was composed of 18 subjects. During every actometer session (a 48-hr. period), three rats were tested from each treatment group.

Subsequent experiments tested for a relationship between central adrenergic function and the excitation produced by morphine and certain surrogates by determining the effect of α-methyltyrosine pretreatment on drug-induced motility. For this purpose, a dose of each analgesic agent was chosen that had produced the greatest activity response at the 1st hr. after treatment. For comparison, the effect of α-methyltyrosine on the activity evoked by 1.0 mg./kg. dextroamphetamine also was tested. These studies utilized four treatment groups: saline–saline, α-methyltyrosine–saline, saline–drug, and α-methyltyrosine–drug. On the 2nd day of the experimental session, saline or α-methyltyrosine pretreatment was administered 3.5–4 hr. prior to the injection of the test drug or saline. For the interaction study with dextroamphetamine, α-methyltyrosine was administered in a dose of 50 mg./kg. However, in experiments with analgesics, 100 mg./kg. was used.

A total recording period of only 4 hr. was utilized for the interaction studies because earlier data showed that the maximum activity produced by the selected dosages occurred within the first 3 hr. postinjection. Analysis of variance was applied to the cumulative motility counts over the first 3 hr. and separately for the 2nd and 3rd hr., but only the former values are reported here.

For each individual experiment, a solution or suspension of the drugs to be used was freshly prepared. Saline (0.9%) was used to

[1] National Laboratory Animal Co., Creve Coeur, Mo.

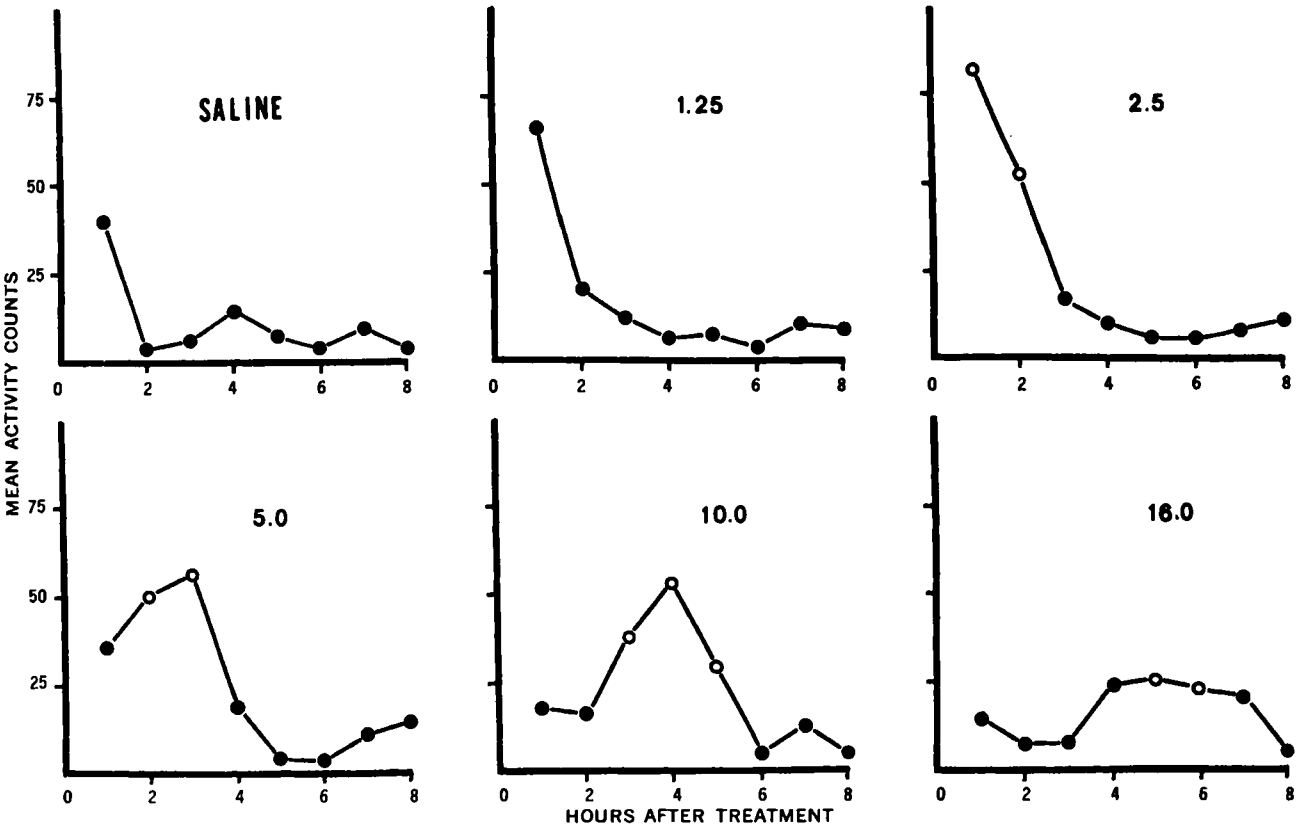

Figure 2—*Locomotor activity of rats following saline or several doses of methadone hydrochloride as indicated on the individual graphs (1.25–16.0 mg./kg.). Open circles represent hourly totals differing significantly from corresponding values of saline controls at* $p \leq 0.05$. *For each point,* $N = 18$.

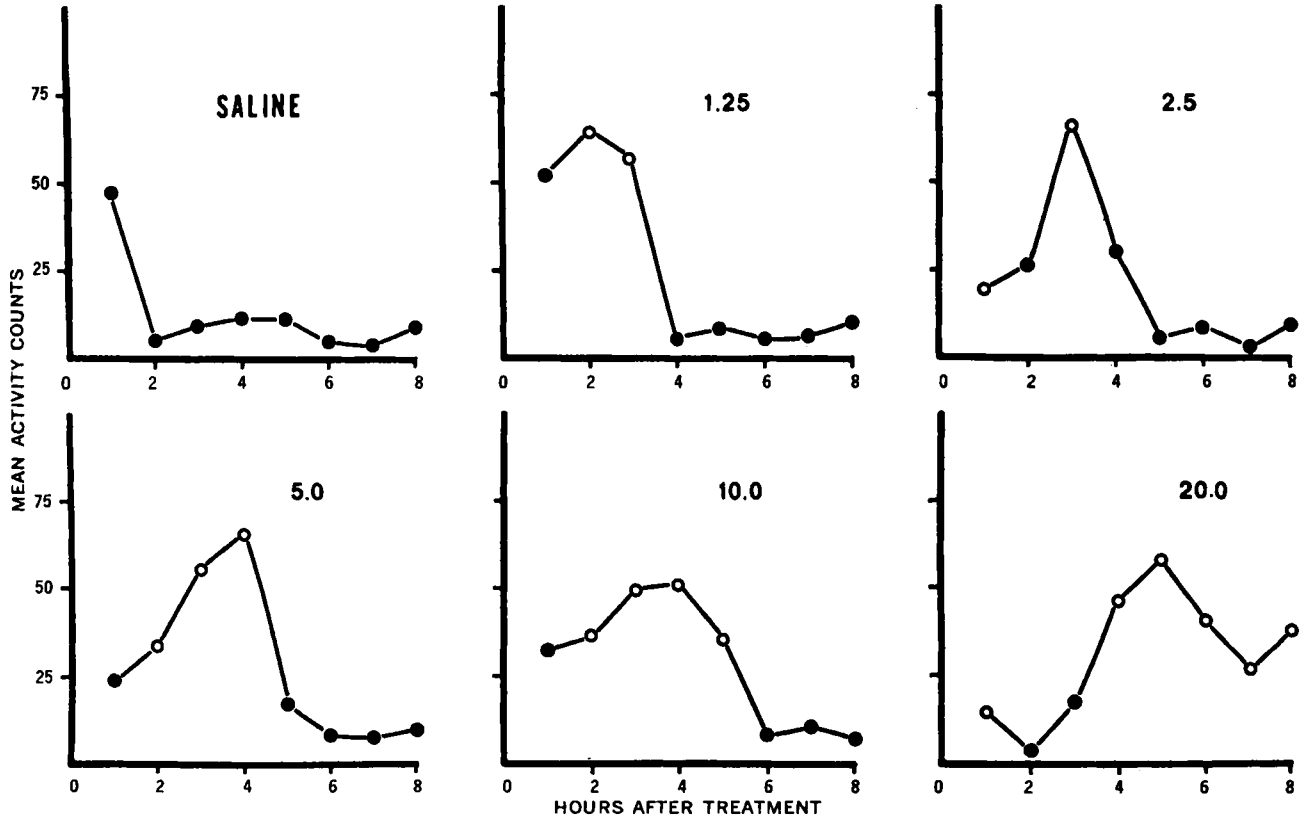

Figure 3—*Locomotor activity of rats following saline or several doses of levorphanol tartrate as indicated on the individual graphs (1.25–20.0 mg./kg.). Open circles represent hourly totals differing significantly from corresponding values of saline control at* $p \leq 0.05$. *For each point,* $N = 18$.

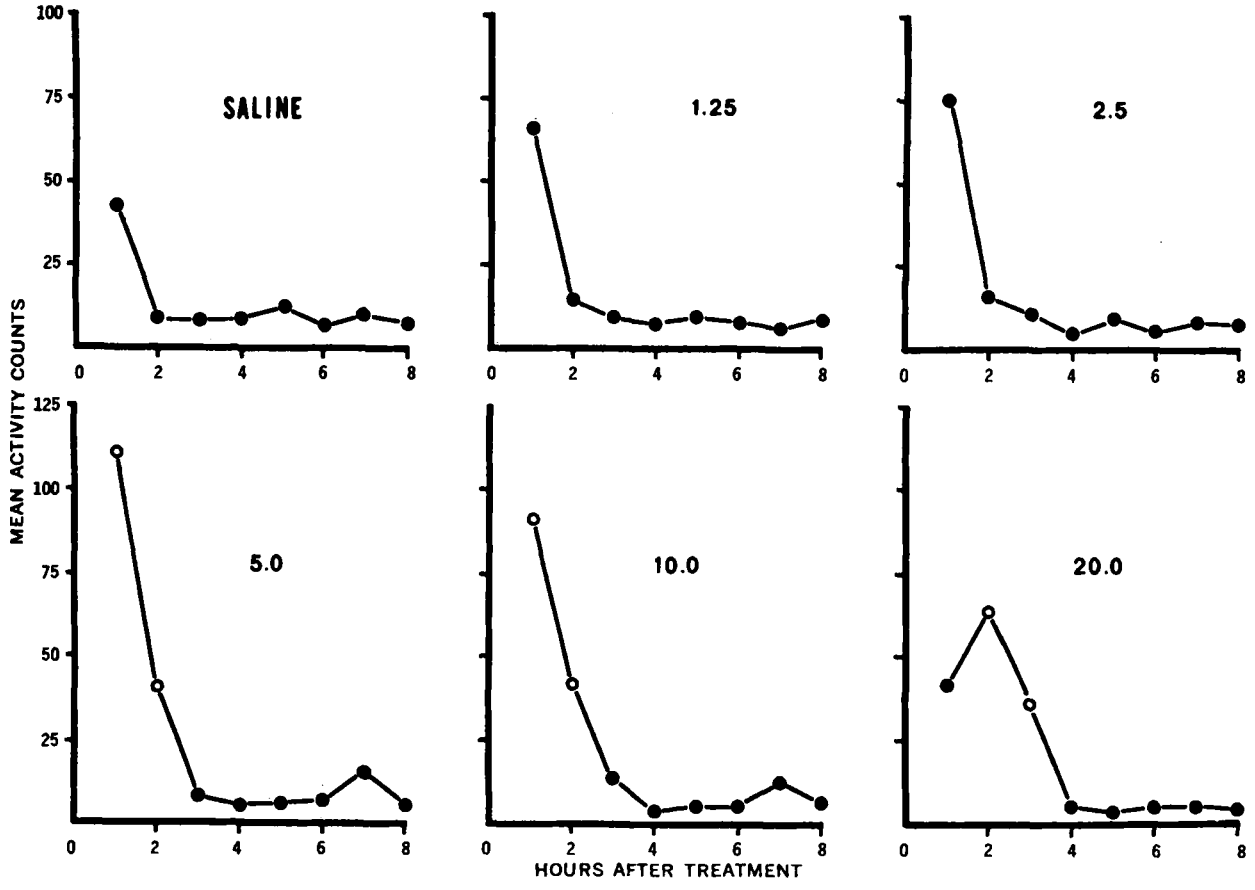

Figure 4—*Locomotor activity of rats following saline or several doses of pentazocine lactate as indicated on the individual graphs (1.25–20.0 mg./kg.). Open circles represent hourly totals differing significantly from corresponding values of saline controls at* $p \leq 0.05$. *For each point,* $N = 18$.

prepare solutions of morphine sulfate, levorphanol tartrate, and dextroamphetamine sulfate and a suspension of D,L-α-methyl-*p*-tyrosine[1]. Solutions of methadone hydrochloride, meperidine hydrochloride, and pentazocine lactate were prepared by dilution of commercial injectable solutions with saline. All drugs were administered in a volume of 1.0 ml./kg. As a control for each drug, an equal volume of 0.9% saline was injected. All injections were by the intraperitoneal route. Dosages given are in terms of the salts used.

RESULTS

Dose–Response Relationships for Analgesics on Locomotor Activity—Graphical presentations of results are given in Figs. 1–5. Saline-treated control rats characteristically displayed a moderate level of activity during the 1st hr., which is attributable to a combined effect of handling and injection procedures and of the behavioral arousal evoked by being removed from and returned to the actometer. Activity fell greatly in the 2nd hr. and remained low throughout the remaining 6 hr. of recording, except for a slight rise often found between the 3rd and 6th hr.

The activity patterns over time for several dosage levels of morphine are shown in Fig. 1. These are in close agreement with previous results from this laboratory (1, 2). The three lower doses produced a high initial excitatory response. For the lower two the peak activity was at the 1st hr., with significant activity continuing only during the 2nd hr. With the 5.0-mg./kg. dose, the peak was observed at the 2nd hr., and the duration was greater since a lower but still significant activity was evident at the 3rd hr. A further delay in peak activity was produced by increasing the dosage to 10 and 20 mg./kg. The former dose had no significant effect at the 1st hr. but caused peak activity at the 2nd hr., which was maintained with only slight reduction through the 3rd hr. The response to 20 mg./kg. suggested a biphasic effect: initial depression followed by excitation. A biphasic pattern has been more clearly demonstrated in other observations for which the 24-hr. adaptation was omitted so that activity at the 1st hr. was elevated. Peak excitation occurred at the 3rd hr., and high activity continued through the 4th hr.

The activity patterns for all of the other analgesics except meperidine showed a general similarity to that of morphine. The only apparent differences were in the level of peak activity achieved, in the duration of either depressant or stimulatory phase, and in the dosage producing a particular response pattern.

Methadone never showed as high a peak excitation as morphine (Fig. 2). The response to a 2.5-mg./kg. dose was like that of morphine at 1.25 or 2.5 mg./kg., while the response to 5.0 mg./kg. resembled morphine at 10 mg./kg. The response to methadone at 10 mg./kg. matched that with 20 mg./kg. morphine, and at 16 mg./kg. it was comparable to activity found in another study (2) for 40 mg./kg. morphine.

Levorphanol in doses of 1.25 and 2.5 mg./kg. (Fig. 3) produced responses closely resembling those after 10 and 20 mg./kg. of morphine, respectively. There was a suggestion of a biphasic response after levorphanol doses of 2.5, 5.0, and 10 mg./kg., while 20 mg./kg. clearly caused initial depression followed by excitation. No dose caused as high a degree of excitation as did morphine. There was a delay in time of peak excitatory response and an increased duration of excitation as the levorphanol dose was increased from 1.25 to 20 mg./kg.

Pentazocine at 1.25 and 2.5 mg./kg. had no significant effects (Fig. 4). However, the time-activity curves after 5.0 and 10 mg./kg. were very similar to those of morphine at 1.25 and 2.5 mg./kg., respectively. The pattern of activity following 20 mg./kg. pentazocine was comparable to that of 10 mg./kg. morphine.

Results with meperidine were not comparable to morphine at any dose tested (Fig. 5). Only 10 mg./kg. gave a small but significant increase in activity at the 2nd hr. The 20-mg./kg. dose showed a significant 1st hr. depressant effect along with a small but significant increase in activity at the 3rd hr.

[1] Regis Chemical Co., Chicago, Ill.

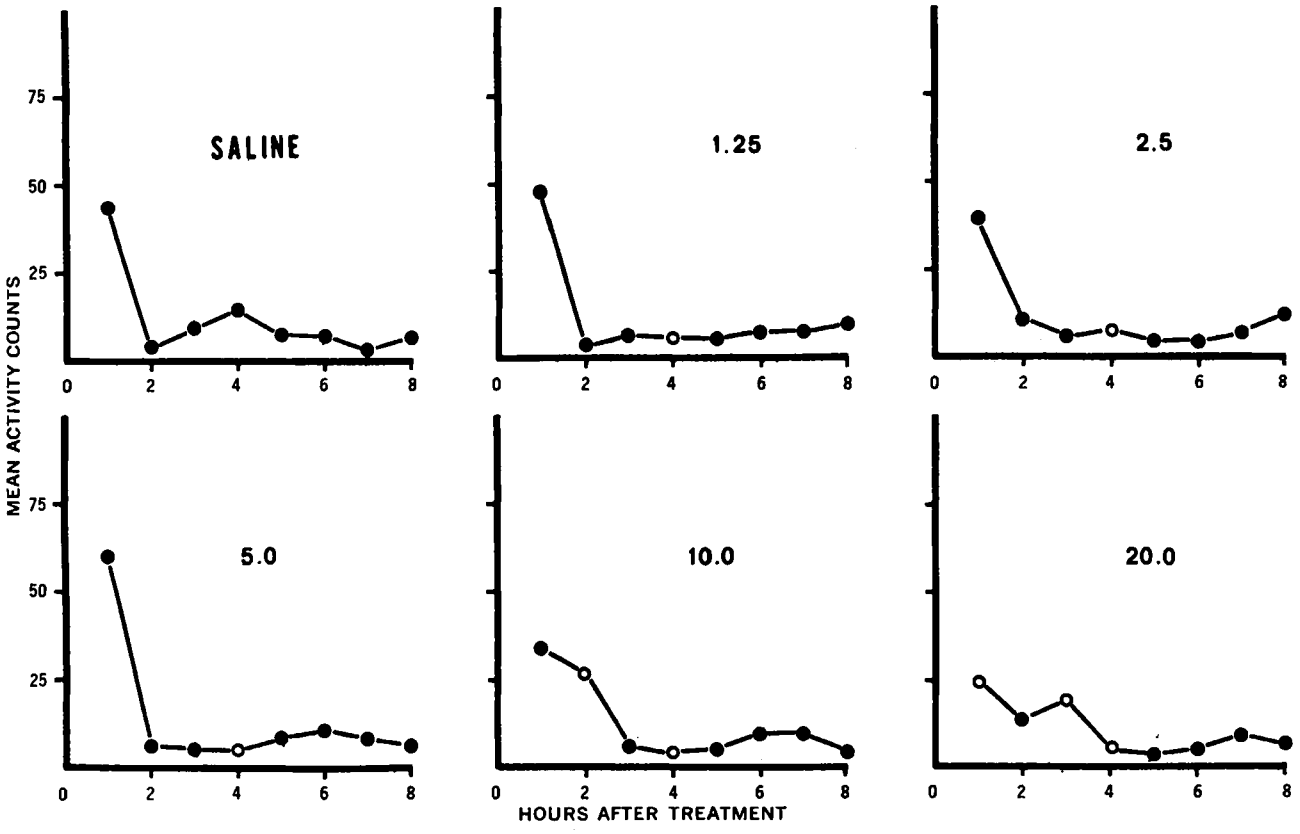

Figure 5—*Locomotor activity of rats following saline or several doses of meperidine hydrochloride as indicated on the individual graphs (1.25–20.0 mg./kg.). Open circles represent hourly totals differing significantly from corresponding values of saline controls at* $p \leq 0.05$. *For each point,* $N = 18$.

Thus, these results indicate that characteristic dose–activity patterns observed with morphine were matched by methadone at about one-half, by levorphanol at about one-tenth, and by pentazocine at 2–4 times the morphine dosage. On the other hand, meperidine failed to show clearly any pattern corresponding to those found with morphine.

Effect of α-Methyltyrosine on Locomotor Activity—The inhibition by α-methyltyrosine of the initial excitatory response to dextroamphetamine and to selected dosages of the analgesics (except for meperidine) is shown in Fig. 6. Statistical comparisons between means for total activity over the first 3 hr. after drug showed that in all cases α-methyltyrosine blocked the drug-induced excitatory response; *i.e.*, the activity of the combination did not differ from the saline controls or α-methyltyrosine–saline groups. Although the 3-hr. means for the α-methyltyrosine control groups were, in most instances, somewhat lower than saline controls, the differences were nonsignificant, and the apparent deficit in activity occurred mainly during the 1st hr.

DISCUSSION

While morphine in low doses (1, 2) has been known to stimulate motility of nontolerant rats, reports of such an effect have been lacking for the synthetic analgesics (methadone, levorphanol, and meperidine), although all have shown such activity in mice (17). One previous report of increased motility in the rat after a narcotic antagonist analgesic appeared recently for pentazocine (18). This work showed that α-methyltyrosine pretreatment blocked the motility response to pentazocine, another result that is confirmed by the present study. In addition, the morphine data of this study, both alone and in combination with α-methyltyrosine, provide confirmation for earlier findings (3, 4) in a different strain of rat.

The activity response to methadone and levorphanol was qualitatively similar to that after morphine, although of lesser magnitude. With both drugs the rats displayed a progressive delay in onset of peak activity with increasing dosage, as has been found characteristic of morphine (2). Pentazocine caused a maximum activation comparable in magnitude to that of morphine, but the shift over time with increasing dosage was less clearly evident in this dosage range. Meperidine in the doses reported here caused no consistent elevation of activity over controls. However, a later supplementary study with a dose of 30 mg./kg. gave a result very similar to that shown here for 20 mg./kg. of levorphanol (Fig. 3), having a quite delayed onset and peak of activity. Even with this observation, the pattern of response to meperidine in rats clearly differed from that of the other analgesics.

It has been postulated that locomotor excitatory effects of morphine are elicited through an action at a receptor site which is separate from and more sensitive than the one that causes locomotor inhibitory effects (2). The similarity in response patterns between morphine and the analgesics tested here (except meperidine) would allow the extension of this concept to these morphine surrogates. Such quantitative differences as were found could be attributed largely to differences in rates of metabolic inactivation. In view of these results in rats, it is interesting to note that meperidine in mice also caused the least increase in motor activity among several narcotic analgesics including morphine, methadone, and levorphanol (17). A difference in relative affinities for excitatory and inhibitory receptor sites could possibly explain the differing response pattern of meperidine compared to the other narcotic analgesics.

While a 50-mg./kg. dose of α-methyltyrosine blocked most of the motility response to dextroamphetamine, a dose of 100 mg./kg. was required to block completely the response to the four analgesics. The latter dose was the same that Holtzman and Jewett (18) found effective against the motility effects of pentazocine. The failure of morphine, methadone, levorphanol, and pentazocine to produce locomotor excitation after α-methyltyrosine suggests the dependence of their stimulatory effects upon a release of brain catecholamines. The same supposition was made earlier for morphine on the basis of a lowering of brain catecholamine levels during its action (9, 14, 17) as well as the interaction of morphine and α-methyltyrosine (4). In the case of pentazocine, this supposition also was put forth recently on the basis of both types of data (18). However, differences were observed in brain amine effects and

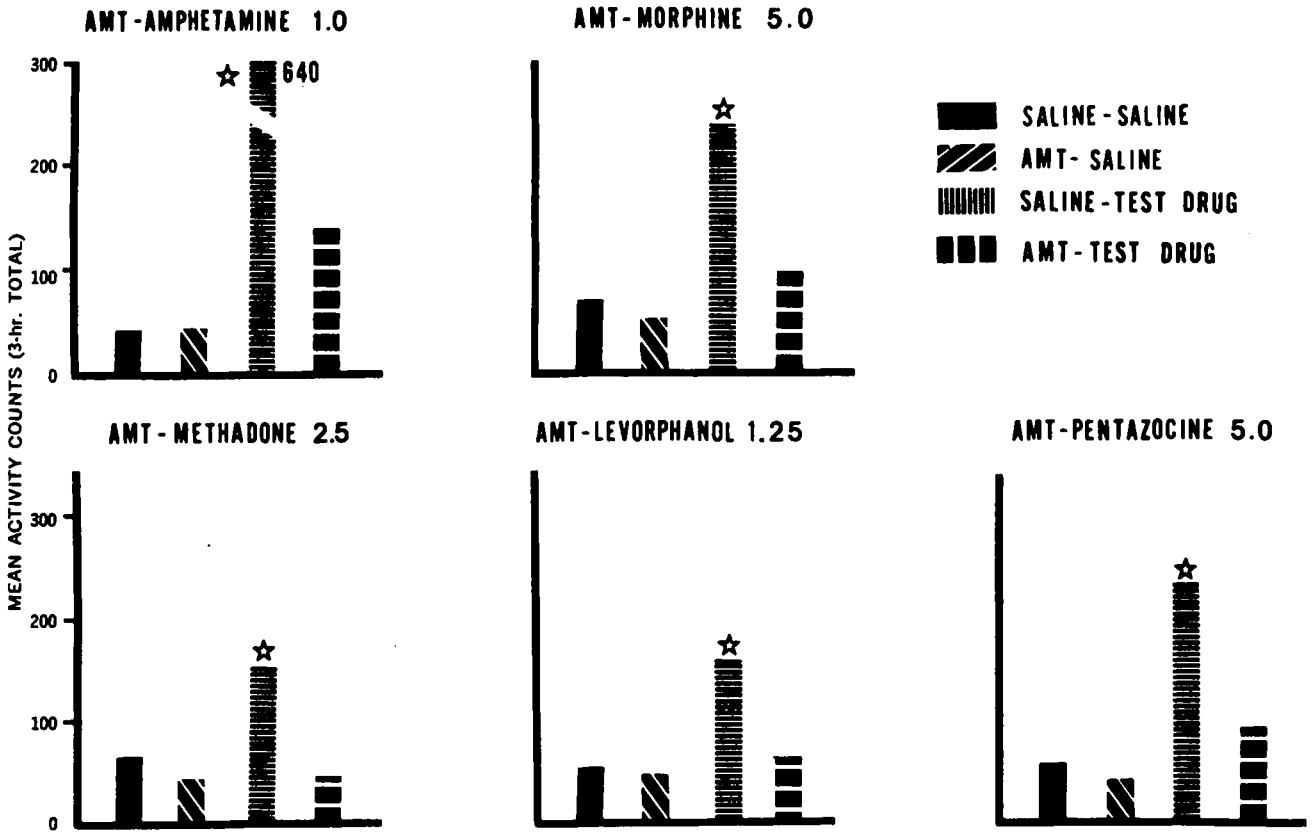

Figure 6—*Effect of α-methyltyrosine (I, AMT) on drug-induced locomotor activity of rats. Saline or I was injected intraperitoneally 3.5–4 hr. before each test drug. Dosages of drugs tested were dextroamphetamine, 1.0 mg./kg.; morphine sulfate, 5.0 mg./kg.; methadone hydrochloride, 2.5 mg./kg.; levorphanol, 1.25 mg./kg.; and pentazocine lactate, 5.0 mg./kg. Values indicated by stars were the only ones differing significantly* ($p \leq 0.05$) *among the respective treatment groups. For each group,* $N = 18$.

interactions with naloxone, which were taken to suggest that some agonist actions of pentazocine are mediated by receptors distinct from those on which morphine acts (18). The present data do not provide a basis for speculation on that point but do indicate some qualitative similarity in the responses to the agonist actions of morphine and pentazocine in terms of a locomotor activity measure.

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Polarography of Cephalosporin C Derivatives I: 3-(5-Methyl-1,3,4-thiadiazol-2-ylthiomethyl)-7-[2-(3-sydnone)-acetamido]-3-cephem-4-carboxylic Acid, Sodium Salt

D. A. HALL

Abstract □ 3-(5-Methyl-1,3,4-thiadiazol-2-ylthiomethyl)-7-[2-(3-sydnone)acetamido]-3-cephem-4-carboxylic acid, sodium salt was found to be polarographically reducible in acidic medium, producing two polarographic waves: the first a two-electron reduction, and the second a six-electron reduction. Both waves are diffusion controlled; however, only the first can be used to follow acid and base hydrolysis and β-lactamase degradation of the compound.

Keyphrases □ 3-(5-Methyl-1,3,4-thiadiazol-2-ylthiomethyl)-7-[2-(3-sydnone)acetamido]-3-cephem-4-carboxylic acid, sodium salt—polarographic reduction □ Cephalosporin C derivatives—polarographic reduction of 3-(5-methyl-1,3,4-thiadiazol-2-ylthiomethyl)-7-[2-(3-sydnone)acetamido]-3-cephem-4-carboxylic acid, sodium salt □ Polarography—reduction of 3-substituted cephalosporin C derivatives [3-(5-methyl-1,3,4-thiadiazol-2-ylthiomethyl)-7-[2-(3-sydnone)acetamido]-3-cephem-4-carboxylic acid, sodium salt]

The initial report of the polarographic reducibility of cephalosporins, specifically, cephalosporin C and two of its derivatives, cephalothin [7-(thiophene-2-acetamido)-cephalosporanic acid] and cephaloridine [7-(2-thienylacetamido)-3-(1-pyridylmethyl)-3-cephem-4-carboxylic acid betaine], was that of Jones *et al.* (1). The polarographic reduction wave of cephaloridine was used for quantitative analysis, while those of cephalothin and cephalosporin C were used for qualitative comparison.

This paper reports the d.c. polarographic reduction of 3-(5-methyl-1,3,4-thiadiazol-2-ylthiomethyl)-7-[2-(3-sydnone)acetamido]-3-cephem-4-carboxylic acid, sodium salt (I) and its analytical utilization for control and stability assays.

EXPERIMENTAL

Apparatus—Polarograms were recorded by an IR compensating automatic recording polarograph[1], employing a water-jacketed three-compartment polarographic cell (the indicating-electrode compartment was between the reference and counter-electrode compartments). The cell was maintained at 25.0 ± 0.2° and contained a saturated calomel reference electrode and a platinum foil auxiliary electrode. The compartments of the cell were separated by medium-porosity sintered-glass disks and 4% agar-saturated potassium chloride salt bridges. The dropping mercury electrode had an $m^{2/3}t^{1/6}$ value of 1.555 at open circuit in air-saturated 0.1 *M* potassium chloride solution, unless noted otherwise.

Coulometric runs were made using a mercury pool cathode in the same polarographic cell. Stirring was accomplished by means of a 600-r.p.m. synchronous rotator[2] and a one-turn screw-type impeller.

Procedure—Test solutions were prepared by weighing approximately 2.5 mg. of the compound of interest into a 25-ml. volumetric flask, adding the buffer, and diluting to the mark with deionized water; the pH of this solution was measured. Without delay, about 12 ml. of test solution was transferred to the polarographic cell, purged with argon[3] for about 10 min., and polarographed. The half-wave potential, $E_{1/2}$, and the diffusion current, i_d, were determined graphically using the maximum of the recorder trace.

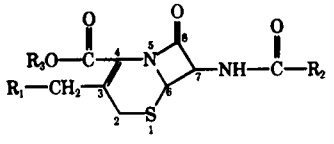
Buffer solutions were prepared from analytical reagent grade chemicals. Phosphate–citric acid–potassium chloride buffers were used at constant ionic strengths (2) of 0.4 and 0.6 *M* over the pH range of 2.3–6.0. A hydrochloric acid–potassium chloride buffer was used for pH 1.6 and was 0.1 *N* in hydrochloric acid. All buffer solutions were polarographically clean within the potential region of interest.

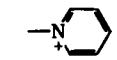
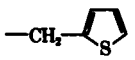
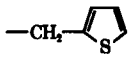
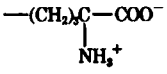
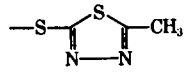
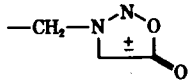
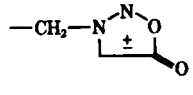
The total number of coulombs passed during coulometry was determined graphically. All coulometric runs were corrected for background current and were considered completed when the current was equivalent to the steady-state coulometric current of the background electrolyte.

RESULTS

Effect of pH—Compound I exhibits two polarographic waves (Fig. 1, solid lines), and both half-wave potentials are pH dependent. The results of the pH study at a concentration of 0.43 m*M* are shown in Table I. Waves I and II at this concentration show a pH dependence of $E_{1/2} = -0.45$–0.09 pH and $E_{1/2} = -0.76$–0.10 pH, respectively. As can be seen from Table I, the pH range in which both reduction waves are present is very narrow. A pH of 2.3 and an ionic strength of 0.6 *M* were selected for analytical purposes and were used throughout the rest of the study, unless noted otherwise.

Effects of Temperature and Mercury Height—Both waves I and II show a temperature dependence of +1.0% per degree. Waves I and II are dependent on the square root of the mercury height, corrected for back-pressure, within a relative standard deviation of ±1.6 and ±4.1%, respectively. These results indicate that both reduction waves are diffusion controlled.



	R_1	R_2	R_3
cephaloridine			zwitterion
cephalothin	$-OCOCH_3$		H^+
cephalosporin C	$-OCOCH_3$		H^+
I			Na^+
III	$-OCOCH_3$		Na^+

[1] Beckman Electroscan 30 Electroanalytical System, Beckman Instruments, Inc., Fullerton, Calif.

[2] E. H. Sargent & Co., Chicago, Ill.

[3] The Matheson Co., Inc., East Rutherford, N. J.

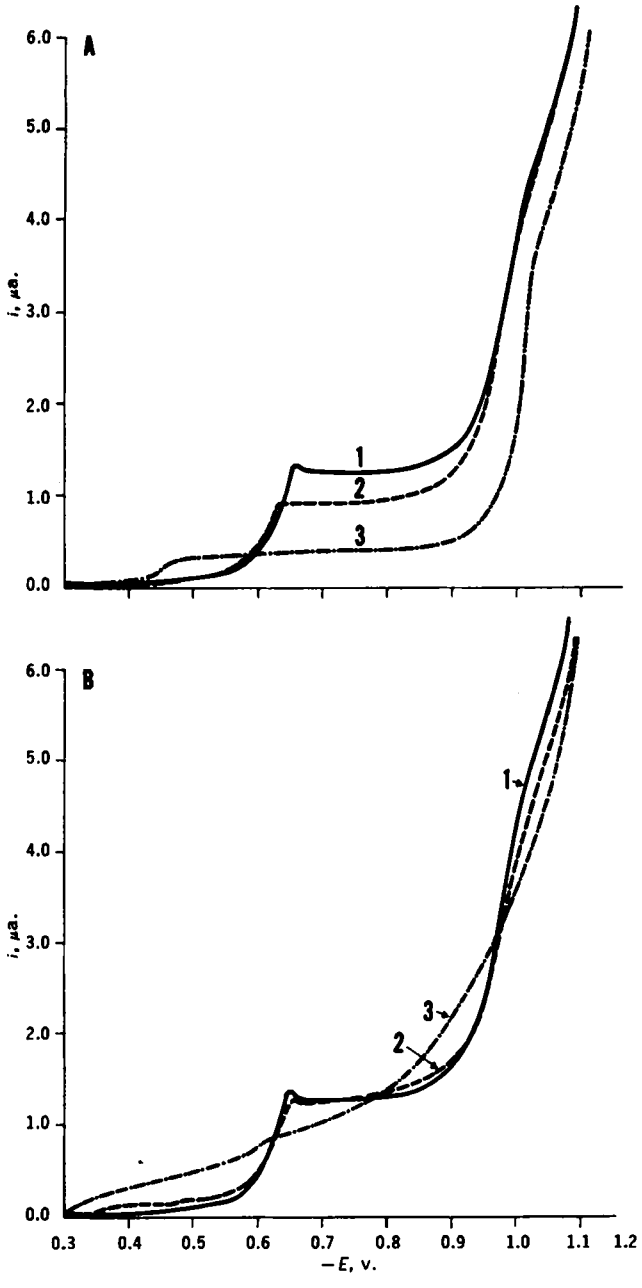


Figure 1—*(A) Polarography of acid hydrolysis sequence. Key: 1, initial; 2, 10 min. at 60°; and 3, 20 min. at 80°. (B) Polarography of base hydrolysis sequence. Key: 1, initial; 2, 0.1 meq. NaOH for 10 min. at room temperature; and 3, 0.1 meq. NaOH for 10 min. at 45°. All solutions were 0.22 m*M *in 1 at pH 2.0. The* $m^{2/3}t^{1/6}$ *of the dropping mercury electrode at open circuit in air-saturated 0.1* M *potassium chloride is 1.720.*

Concentration Dependence—The half-wave potentials of waves I and II are dependent on concentration, becoming more negative with increasing concentration (Table II). Wave I exhibits what appears to be a maximum of the first kind (3) that decreases more rapidly than the diffusion current with decreasing concentration and shows a negative dependence on surfactant[4] concentration; however, the addition of surfactant results in wave II merging with background. An increase in the ionic strength of the buffer from 0.4 to 0.8 *M* had no effect on the maximum. All data reported are in the absence of surfactant.

In the concentration range examined, *i.e.*, 0.07–0.43 m*M*, the diffusion currents of waves I and II are directly proportional to concentration (Table II). The dependences are i_{dI} = 4.41 μa./m*M* + 0.03 μa. and i_{dII} = 12.63 μa./m*M* − 0.15 μa., respectively. The *RSD* of waves I and II for four individually weighed 0.20 m*M*, 0.6 *M* ionic strength, pH 2.3 samples are ±2.6 and ±2.4%, respectively.

[4] Triton X-100.

Table I—Effect of pH on Polarographic Reduction of I

	Wave I			Wave II		
pH[a]	$-E_{1/2}$, v.	i_d, μa.	i/C, μa./m*M*	$-E_{1/2}$, v.	i_d, μa.	i/C, μa./m*M*
1.60	0.58	2.04	4.77	—	—	—
2.32	0.66	1.95	4.56	1.00	5.76	13.46
3.30	0.73	1.90	4.44	1.08	4.58	10.70
4.84[b]	—	—	—	1.26	4.52	10.56
5.98[c]	—	—	—	—	—	—

[a] Buffer systems described in *Procedure*. [b] Very poorly defined wave I. [c] Very poorly defined waves I and II.

Table II—Effect of Concentration on Polarographic Reduction of I at pH 2.3

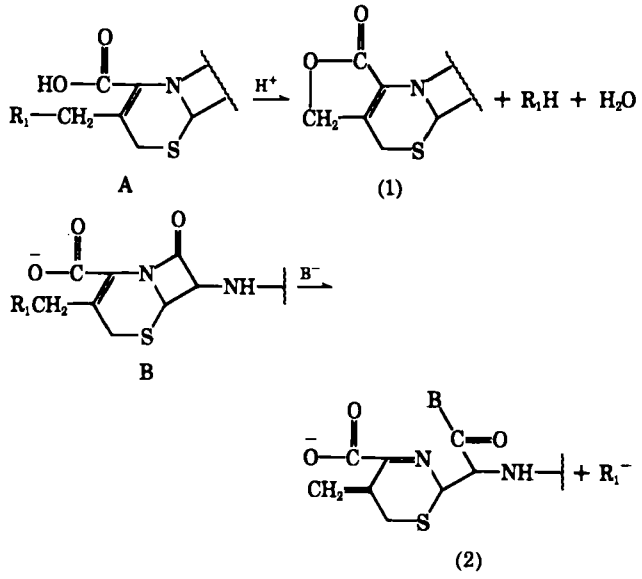
	Wave I			Wave II		
Concentration, m*M*	$-E_{1/2}$, v.	i_d, μa.	i/C, μa./m*M*	$-E_{1/2}$, v.	i_d, μa.	i/C, μa./m*M*
0.07	0.60	0.34	4.86	0.97	0.78	11.15
0.15	0.62	0.70	4.65	0.98	1.81	12.06
0.22	0.63	1.02	4.64	0.98	2.60	11.81
0.26	0.64	1.19	4.58	0.99	3.05	11.72
0.37	0.66	1.65	4.46	1.00	4.58	12.38
0.43	0.66	1.95	4.56	1.00	5.76	13.46

Acid Hydrolysis Stability—A 0.22 m*M*, 0.6 *M* ionic strength, pH 2.0 solution of I was subjected to three heating conditions. Results of two are shown in Fig. 1A. The polarograms show a decrease in wave I, essentially no effect on wave II, and the development of a reduction wave at −0.45 v. The third hydrolysis at 25° for 1 hr. did not detectably degrade I. A 1.95 m*M*, pH 2.3 solution of I was degraded at 80° for 15 min. and examined by an iodometric β-lactam assay and TLC. The iodometric assay, as compared to nondegraded I, indicated a 61% reduction in iodine uptake and a very large increase in the blank iodine uptake. The blank of the degraded material used approximately 1.7 times the iodine absorbed by the nondegraded sample. TLC in acetic acid–dioxane–acetone (1:2:8) on silica gel indicated a total loss of I, some material at the point of application, and two spots, both more mobile than I (iodine chamber development). The lead spot corresponded in R_f to the thiol-thiadiazole side chain of I. The less mobile spot corresponded to the lactone of I (Scheme IA) (1).

Base Hydrolysis Stability—Two 5.5-mg. samples of I were reacted with 0.1 meq. of base as 0.05 *N* NaOH under two different heating conditions. Polarograms of the resulting material at a concentration of 0.22 m*M* with respect to I and at a pH of 2.0 are shown in Fig. 1B. There is a decrease in both waves I and II and development of two ill-defined reduction waves at −0.35 and −0.48 v. A 2.5-mg. sample of I was degraded in 6 ml. of 0.17 *N* NaOH for 80 min. at room temperature. TLC, using the system previously described, revealed two new spots. The spot more mobile than I corresponded to the thiol-thiadiazole side chain of I. The second spot was less mobile than I and could be better described as a smear, most probably made up of material containing degraded β-lactam (see *Discussion*).

β-Lactamase Stability—Treatment of I with β-lactamase from *B. cereus* at pH 7 and 25° resulted in a 54% decrease in the diffusion current of wave I at pH 2.3 over 2 hr. The degradation produced a very small reduction wave at −0.36 v.

Polarography of 5-Methyl-1,3,4-thiadiazole-2-thiol (II)—Since II is indicated as a product of at least two different degradation paths of I and is also a probable intermediate in the synthesis of I (see *Discussion*), it seemed reasonable to try to develop a polarographic method to determine II. The compound is not reducible; therefore, it was decided to look into the possibility of an oxidation. The McIlvaine buffers used to determine I contain chloride ion, which results in an early anodic discharge at the dropping mercury electrode. However, II gives a well-defined anodic wave in pH 5.7, 0.7



Scheme I—Cephalosporin degradative pathways: A, acid hydrolysis; and B, base hydrolysis

M acetate buffer which is proportional to concentration within a relative standard deviation of ±6.0% (Table III). The half-wave potential is quite concentration dependent. The oxidation is assumed to involve the formation of an insoluble mercurous salt. This oxidation can be used as a supplemental analytical method to follow the degradation pathways of I which involve the loss of II and also as a control method for II in nondegraded I.

Coulometry—Coulometric reduction of I in pH 2.3 buffer on the limiting current plateau of wave I resulted in *n* values of 1.85 and 1.94. No anodic waves corresponding in $E_{1/2}$ to wave I were found in the electrolyzed solutions. Wave II was considered to be too close to background discharge for coulometric *n* value determination. However, comparison of diffusion currents for waves I and II indicates a five- or six-electron transfer for wave II.

Polarography of 3-Acetoxymethyl-7-[2-(3-sydnone)acetamido]-3-cephem-4-carboxylic Acid, Sodium Salt (III)—A 0.54 m*M* solution of III, in which an acetoxy group has been substituted for the thiol-thiadiazole group, in pH 2.3 McIlvaine buffer exhibits only one reduction wave at −1.01 v. with an *i/C* value of 14.3 μa./m*M*.

Polarography of 7-(Thiophene-2-acetamido)cephalosporanolactone (IV)—Compound IV, with a thiophene in place of a sydnone and an α,β-unsaturated lactone at the 3–4 position (Scheme IA) (1), showed no reduction waves in pH 2.3 McIlvaine buffer.

DISCUSSION

Cephaloridine, cephalothin, and cephalosporin C, as reported by Jones *et al.* (1), each exhibit one reduction wave. The polarographic $E_{1/2}$'s of the three compounds are pH dependent and concentration dependent, and the reduction wave of cephaloridine is diffusion controlled. Compound I exhibits two reduction waves, both showing the same characteristics with respect to pH and concentration as has been demonstrated. The $E_{1/2}$ concentration dependence of all four compounds is indicative of a polarographically irreversible reduction. Final solutions, completely electrolyzed with respect to the first reduction wave of I, showed no anodic wave with an $E_{1/2}$ corresponding to that of wave I, further proof of an irreversible reduction. Based on preliminary diffusion current data, Jones *et al.* (1) assumed that cephaloridine was reduced *via* a two-electron transfer. Coulometry, on the first reduction of I, confirms a net two-electron transfer for the compound and, based on *i/C* data, suggests a five- to six-electron transfer for the second reduction wave.

Jones *et al.* (1) made no attempt to assign the functionality in cephaloridine that undergoes reduction. Comparison of the reduction patterns of I and cephaloridine shows similar half-wave potentials for the second reduction of I and the reduction of cephaloridine. However, the former is a five- to six-electron transfer, and the latter is assumed to be a two-electron transfer. Sydnones, as reported by Zuman (4), are reduced in a single six-electron step under acidic conditions, which supports a hypothesis that wave II represents the reduction of the sydnone side chain of I, a functionality not present in cephaloridine.

Table III—Effect of Concentration on the $E_{1/2}$ and i_d of II

Concentration, m*M*	$-E_{1/2}$, v.	$-i_d$, μa.
0.30	0.09	0.76
0.60	0.11	1.67
0.91	0.12	2.64
1.21	0.12	3.52
1.51	0.13	4.41

The acid hydrolysis of I, as followed by TLC, results in the loss of II and the formation of the lactone of I. Polarographically, acid hydrolysis results in the loss of the first reduction wave and the retention of the second reduction wave. These two observations point to the first reduction of I as involving the side chain (II) at the 3-position. This conclusion is supported by the fact that III, which has an acetoxy group in the 3-position in place of the thiol-thiadiazole and the same acetamido grouping at the 7-position, exhibits only one reduction wave at −1.01 v., corresponding to the proposed sydnone reduction of I. Compound II is not reducible under similar conditions.

It was recently reported (5) that cephalosporin C, upon macroelectrolysis at a mercury pool electrode at pH 6.6, is converted to the deacetoxy compound, *i.e.*, the acetoxy group at the 3-position is reductively eliminated. It seems reasonable to assume, then, that the first reduction wave of I represents a similar elimination in which the thiol-thiadiazole group is expelled from the molecule. Conclusions from an extension of these arguments to the reduction waves of cephalosporin C and cephalothin, as reported by Jones *et al.* (1), are obvious. The reduction of cephaloridine, which probably also involves the 3-position side chain, may be complicated by its zwitterion character. Moreover, the first reduction wave of I is approximately 300 mv. more positive than the reduction wave of cephaloridine and approximately 600 mv. more positive than the reductions of cephalosporin C and cephalothin at the same pH. This indicates a substituent effect on the reduction caused by the functionality at the 3-position.

Cephalosporins, in general, easily undergo acid (6) and base (7) hydrolysis (Scheme I). Acid hydrolysis results in the loss of the R group at the 3-position and formation of an α,β-unsaturated lactone. Base hydrolysis opens the β-lactam moiety and simultaneously eliminates the R group at the 3-position. TLC has tentatively supported these degradation pathways for I. Enzymatic deactivation of cephalosporins is believed (7) to follow a reaction path similar to that of base hydrolysis, again with loss of the R group. As can be seen from Figs. 1A and 1B, the acid and base hydrolysis of I can be followed by polarography. The enzymatic degradation also results in loss of wave I of I. The key to this specificity is believed to be the loss of the R group at the 3-position. The identities of the new polarographic waves produced *via* the three degradation paths have not been established. The lactone functionality, from the polarography of IV, is not responsible for any of them. However, Structure 2 in Scheme IB could be polarographically reducible.

As mentioned under the polarography of II, II should also be a contaminant in nondegraded I. This conclusion is based on the reported (7) method of synthesis of compounds containing R groups at the 3-position other than —H or —$OCOCH_3$. The reaction is a nucleophilic displacement wherein an excess of free side chain is used to replace the naturally occurring —$OCOCH_3$ function of the fermentation product, cephalosporin C. The oxidation of II, therefore, should not only be useful in following the degradation of I, and presumably other cephalosporins with similar 3-position substituents, but should also be useful in examining newly synthesized cephalosporin for side-chain contaminant.

CONCLUSION

The polarographic reduction of I consists of two waves. The first is believed to be the two-electron reductive elimination of the 3-position substituent. The second is believed to be the six-electron reduction of the sydnone acetamido 7-position substituent. Both waves can be utilized as control methods for the analytical determination of I. Only the first wave, however, can be used to follow the

acid, base, and enzymatic degradations of I which involve the loss of the 3-position substituent.

When taking into consideration the work of Jones *et al.* (1), it is believed that the polarographic method should be generally applicable to all cephalosporins containing a leaving group at the 3-position. Those free leaving groups containing a thiol functionality should also be amenable to polarographic determination *via* oxidative mercurous salt formation.

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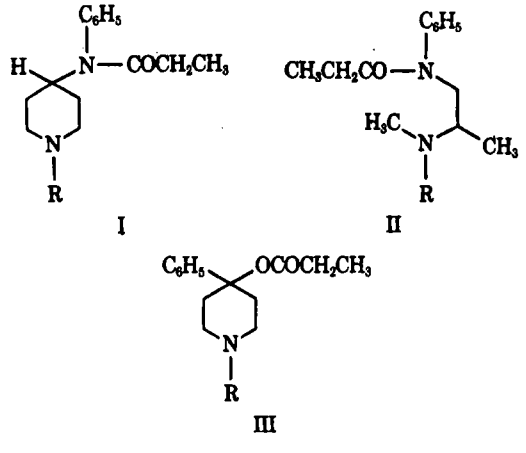
THOMAS N. RILEY▲, DANNY B. HALE*, and MARVIN C. WILSON†

Abstract □ In view of the potency-enhancing effect of methyl substitution of the piperidine ring of the 4-phenylpiperidine analgesics and the alkylene chain of the acyclic basic anilide analgesics, the 1-methyl, 1-benzyl, and 1-phenylethyl derivatives of 2-methyl-, 3-methyl-, and 2,5-dimethyl-4-propananilidopiperidine were prepared. The analgesic activity of these compounds indicates that 3-methylation has the greatest effect in enhancing analgesic potency whereas 2-methyl and 2,5-dimethyl substitution is detrimental to analgesic activity.

Keyphrases □ 4-Anilidopiperidine analgesics—synthesis and activity of ring-methylated 1-substituted 4-propananilidopiperidines □ Analgesics, potential—synthesis of ring-methylated 1-substituted 4-propananilidopiperidines, structure–activity relationships □ Structure–activity relationships—4-anilidopiperidines and analgesic activity, effect of ring methylation

Fentanyl[1] (I, R = $C_6H_5CH_2CH_2$) is a potent narcotic analgesic which possesses a rapid onset and short duration of action (1). Its pharmacological profile is very similar to other morphinomimetic compounds, except that fentanyl is a considerably more potent narcotic analgesic (2). Structurally, fentanyl may be characterized as a 4-anilidopiperidine derivative. This class of synthetic narcotic analgesics exhibits structural features also found in the acyclic basic anilide analgesics (II) and the 4-phenylpiperidine analgesics (III).

In general, studies of structure–activity relationships in the 4-anilidopiperidine class (3–6) indicate that structural requirements for analgesic activity are similar to those established for both the acyclic basic anilides and the 4-phenylpiperidines. One aspect of the structure–activity relationships of the 4-anilidopiperidines that has not as yet been reported is the effect of piperidine ring methylation in this class. It is well established that methylation of the piperidine ring of the 4-phenylpiperidine analgesics (*e.g.*, the prodines) results in a significant increase in analgesic activity. In addition, introduction of a methyl substituent on the alkylene chain of the acyclic basic anilides provides for compounds of high analgesic activity. In this regard, however, it is important to note that the increase in analgesic activity in these analogs is highly dependent on the position of the methyl substituent relative to the basic nitrogen. In the 4-phenylpiperidines, 3-methylation and 2,3-, 2,5-, and 3,5-dimethylation result in high analgesic activities (7). Other methyl substitution patterns in this class generally reduce analgesic activity. In the acyclic basic anilide analgesics, methyl substitution α to the basic nitrogen affords the greatest enhancement of analgesic activity (3).



[1] Fentanyl citrate, Sublimaze, McNeil Laboratories, Inc.

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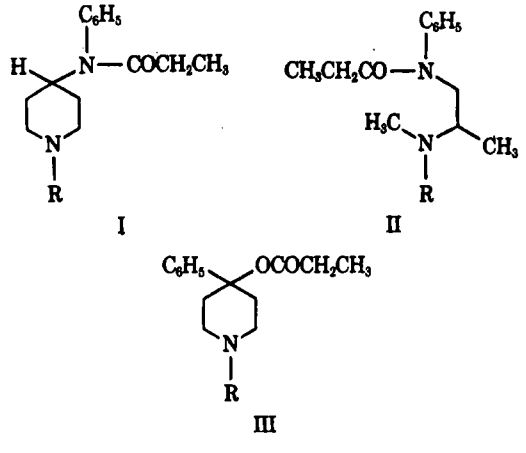

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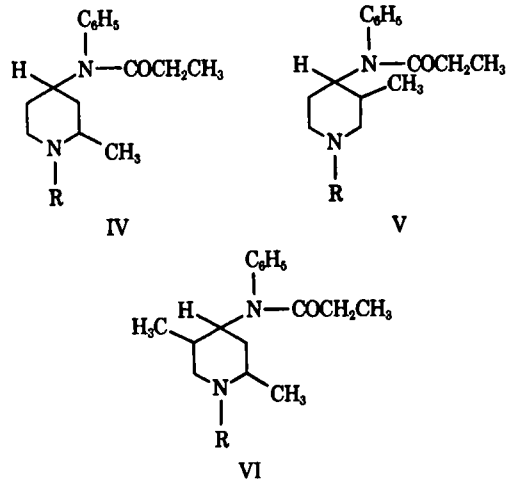


IV V VI

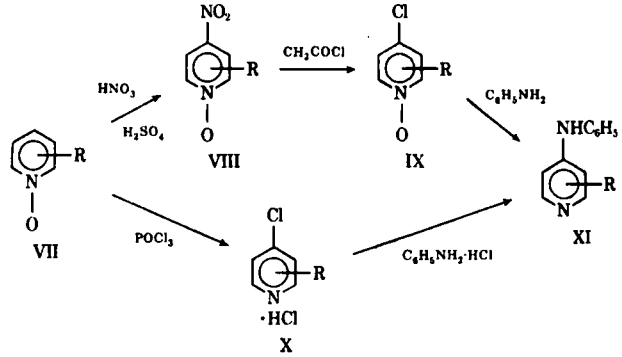
R = (*a*) CH_3, (*b*) $C_6H_5CH_2$, or (*c*) $C_6H_5CH_2CH_2$

These considerations prompted this study of the effect of ring methylation in conjunction with 1-substitution on the analgesic activity of the 4-propananilidopiperidines. The 1-substituted 2-methyl analogs (IV) possess structural features shown to confer high analgesic activity on the acyclic basic anilides. The 1-substituted 3-methyl-4-propananilidopiperidines (V) and 2,5-dimethyl-4-propananilidopiperidines (VI) exhibit structural features important for potent analgesic activity in the 4-phenylpiperidine class.

SYNTHESIS

Literature schemes for the synthesis of 4-anilidopiperidines usually proceed through the 1-substituted 4-piperidones with the formation of the Schiff base with aniline, followed by reduction and subsequent acylation of the 4-anilino moiety (5, 8). In this study, however, the desired 4-anilidopiperidines were most readily accessible *via* the appropriate 4-anilinopyridines (XI, Scheme I). These key intermediates were prepared according to literature procedures (9) *via* nucleophilic displacement of chloride by aniline from the appropriate 4-chloropyridine *N*-oxides (IX) or 4-chloropyridine hydrochlorides (X). Syntheses of X proved to be more facile than the preparation of IX, and treatment of X with aniline hydrochloride gave higher yields of the desired 4-anilinopyridines.

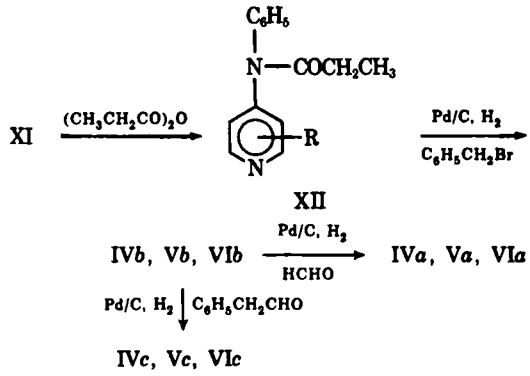
The propananilides (XII, Scheme II) were obtained by heating the 4-anilinopyridines (XI) in propionic anhydride. Hydrogenation of the pyridine ring in XII was achieved using 10% palladium-on-charcoal in glacial acetic acid in a catalyst to a compound ratio of 1:3. The use of platinum oxide as a catalyst in this conversion proved unsatisfactory because of partial pyridine ring reduction and reduction of the aniline ring.



VII VIII IX X XI

R = (*a*) 2-CH_3, (*b*) 3-CH_3, (*c*) 2-CH_3, 5-CH_3

Scheme I



XI XII IV*b*, V*b*, VI*b* IV*a*, V*a*, VI*a* IV*c*, V*c*, VI*c*

Scheme II

The ring-methylated 4-propananilidopiperidines obtained from the reduction of the 4-anilidopyridines proved very difficult to purify. Preparation of the 1-benzyl-4-propananilidopiperidines provided products that could be easily purified by adsorption chromatography. The other two series of 1-substituted 4-propananilidopiperidines were conveniently prepared by catalytic hydrogenolysis of the 1-benzyl derivatives in the presence of either formaldehyde or phenylacetaldehyde.

The synthetic procedures employed for the preparation of the ring-methylated 4-anilidopiperidines of this study undoubtedly gave rise to diastereomeric mixtures of the products. Isomeric mixtures were indicated in certain cases by separation during chromatographic purification. However, in all cases that this was observed the isomers were combined to give homogeneous products with regard to elemental analyses. The results with adsorption chromatography will be of value in future studies of the isomers of these compounds.

ANALGESIC ACTIVITY

The analgesic activity of the ring-methylated 4-anilidopiperidines prepared in this study was determined in rats by the D'Amour–Smith (10) tail-flick method. The compounds were tested as the hydrochloride salts and were dissolved in normal saline immediately prior to intraperitoneal injection. The ED_{50} value for the compound studied is defined as the dose of the drug which, in 50% of the animals tested, increased the reaction time by 50% at 40 min. postinjection. Compounds were deemed inactive if the rats did not exhibit significant analgesia at dose levels of 100 mg./kg.

EXPERIMENTAL[2]

4-Anilino-2-methylpyridine (XI*a*)—A mixture of 8.2 g. (0.05 mole) of 4-chloro-2-methylpyridine hydrochloride (X*a*) [prepared by standard procedures by treatment of 2-methylpyridine *N*-oxide (VII*a*) with phosphorus oxychloride] and 10.3 g. (0.08 mole) of aniline hydrochloride was placed in a sealed vessel and heated for 4 hr. at 130°. The resulting tarry material was purified by steam distillation. After 800 ml. of distillate had been collected, the residue was filtered and the filtrate was treated with charcoal and filtered through diatomaceous earth[3]. The filtrate was extracted with chloroform and the chloroform was dried (sodium sulfate) and evaporated *in vacuo*, leaving 5.2 g. (56%) of XI*a* as white crystals, m.p. 146–147°; NMR ($CDCl_3$): δ 8.40 (d, 1, C-6 H), 7.45–7.85 (m, 5, N—C_6H_5), 7.28 (s, 1, C-3 H), 7.20 (d, 1, C-5 H), 6.78 (broad s, 1, N—*H*), and 2.50 (s, 3, C-2 CH_3).

[2] All melting points were determined on a Thomas-Hoover melting-point apparatus and are uncorrected. The IR spectra for all compounds were as expected. The NMR spectra were taken using a C-60HL Jeolco instrument, using tetramethylsilane as the internal standard. Elemental analyses were performed by Chemlytics, Inc., Tempe, Ariz. Hydrochloride salts of amine oxides were formed by dissolving the amine oxide in dilute aqueous hydrochloric acid and evaporating *in vacuo*.

[3] Celite.

Anal.—Calc. for $C_{15}H_{13}N_3$: C, 78.26; H, 6.52; N, 15.22. Found: C, 78.21; H, 6.67; N, 14.64.

4-(*N*-Propananilido-2-methylpyridine) (XII*a*)—An 18.4-g. (0.10-mole) sample of XI*a* was dissolved in 100 ml. of propionic anhydride and heated at 80° for 12 hr. The excess propionic anhydride was removed *in vacuo*, and the dark residue was purified in 10-g. quantities by passage through a 1000 × 25-mm. chromatographic column packed with 190 g. of silica gel. Elution was carried out with ethyl acetate and 30-ml. fractions were collected. Fractions 11–20 were found to contain the product. This procedure gave 19.5 g. (81%) of XII*a* as a light oil; NMR ($CDCl_3$): δ 8.40 (d, 1, C-6 H), 7.40–7.85 (m, 5, N—C_6H_5), 7.31 (s, 1, C-3 H), 7.25 (d, 1, C-5 H), 2.54 (s, 3, C-2 CH_3), 2.48 (m, 2, $COCH_2CH_3$), and 1.25 (t, 3, $COCH_2CH_3$). The hydrochloride salt was prepared by standard procedures and recrystallized from ethanol–ether, m.p. 152°.

Anal.—Calc. for $C_{15}H_{16}N_2O \cdot HCl$: C, 65.10; H, 6.15; N, 10.13. Found: C, 64.91; H, 6.22; N, 9.64.

1-Benzyl-2-methyl-4-(*N*-propananilido)piperidine (IV*b*)—A 15.6-g. (0.064-mole) sample of XII*a* was dissolved in 150 ml. of glacial acetic acid, and 4.0 g. of 10% palladium-on-charcoal was added to the solution. The mixture was hydrogenated for 24 hr. at 75°. The catalyst was removed by filtration, and the filtrate was carefully basified with sodium hydroxide and then extracted with chloroform. The chloroform extract was dried (sodium sulfate) and concentrated *in vacuo*, giving a viscous oil. This oil was dissolved in acetonitrile, and 13.3 g. (0.078 mole) of benzyl bromide was added. The solution was refluxed for 11 hr. followed by concentration *in vacuo*. The residual oil was taken up in a saturated solution of potassium carbonate and extracted with chloroform. The chloroform extract was dried (sodium sulfate) and concentrated *in vacuo* to yield a yellow oil, which was then passed through a 500 × 25-mm. chromatographic column packed with 90 g. of silica gel. Elution with ethyl acetate gave 10.5 g. (48%) of IV*b* as a clear oil; NMR ($CDCl_3$): δ 7.20–7.75 (m, 10, N—C_6H_5 and N—$CH_2C_6H_5$), 4.50–5.05 (m, 1, C-4 H), 3.15 and 4.33 (*AB* quartet, 2, N—$CH_2C_6H_5$), and 0.80–1.45 (m, 6, $COCH_2CH_3$ and C-2 CH_3). The picrate of IV*b* was recrystallized from ethanol, m.p. 210–212°.

Anal.—Calc. for $C_{22}H_{28}N_2O \cdot C_6H_3N_3O_7$: C, 59.46; H, 5.50; N, 12.38. Found: C, 59.72; H, 5.40; N, 12.36.

1-(2-Phenylethyl)-2-methyl-4-(*N*-propananilido)piperidine (IV*c*)—A 3.0-g. (0.009-mole) sample of IV*b* was mixed with 1.44 g. (0.012 mole) of phenylacetaldehyde and 0.5 g. of 10% palladium-on-charcoal in ethanol and hydrogenated for 4 hr. at room temperature. The reaction mixture was filtered and concentrated *in vacuo*, leaving a dark oil. The oil was purified by passing it through a 500 × 25-mm. chromatographic column packed with 90 g. of silica gel. Elution with ethyl acetate provided 2.24 g. (71%) of IV*c* as an oil; NMR ($CDCl_3$): δ 7.20–7.85 (m, 10, N—C_6H_5 and N—$CH_2CH_2C_6H_5$), 4.33–4.80 (m, 1, C-4 H), and 0.80–1.50 (m, 6, $COCH_2CH_3$ and C-2 CH_3). The oil formed a picrate which was recrystallized from ethanol, m.p. 207–208°.

Anal.—Calc. for $C_{23}H_{30}N_2O \cdot C_6H_3N_3O_7$: C, 60.09; H, 5.74; N, 12.08. Found: C, 59.90; H, 5.75; N, 12.12.

1,2-Dimethyl-4-(*N*-propananilido)piperidine (IV*a*)—A mixture of 2.0 g. (0.006 mole) of IV*b*, 0.90 g. (0.01 mole) of 37% formaldehyde solution, and 0.5 g. of 10% palladium-on-charcoal was hydrogenated for 4 hr. The reaction mixture was filtered, concentrated *in vacuo*, and passed through a 250 × 20-mm. chromatographic column packed with silica gel. Elution with ethyl acetate gave 1.2 g. (79%) of viscous oil; NMR ($CDCl_3$): δ 7.20–7.75 (m, 5, N—C_6H_5), 4.50–5.00 (m, 1, C-4 H), 2.07 (s, 3, N—CH_3), and 0.80–1.50 (m, 6, $COCH_2CH_3$ and C-2 CH_3). The oil formed a picrate which was recrystallized from ethanol, m.p. 194–196.5°.

Anal.—Calc. for $C_{16}H_{24}N_2O \cdot C_6H_3N_3O_7$: C, 53.98; H, 5.56; N, 14.31. Found: C, 54.00; H, 5.68; N, 14.76.

4-Anilino-3-methylpyridine (XI*b*)—A mixture of 7.2 g. (0.05 mole) of 4-chloro-3-methylpyridine *N*-oxide (IX*b*), prepared by standard procedures from the 4-nitro derivative (VIII*b*) (9), and 9.3 g. (0.10 mole) of aniline was heated in a sealed vessel at 130° for 4 hr. The deep-violet tar obtained from this procedure was purified as described in the synthesis of XI*a*, leaving a green crystalline solid which was recrystallized from ethanol–water (1:3) to yield 3.51 g. (38%) of product, m.p. 119–120° [lit. (9) m.p. 122°].

4-(*N*-Propananilido)-3-methylpyridine (XII*b*)—A 20.3-g. (0.11-mole) sample of 4-anilino-3-methylpyridine (XI*b*) dissolved in 100 ml. of propionic anhydride was stirred and heated at 80° for 12 hr. Purification by the procedure described for XII*a* gave 21.1 g. (80%) of a light-yellow oil; NMR ($CDCl_3$): δ 8.63 (d, 1, C-6 H), 8.70 (s, 1, C-2 H), 7.55–7.80 (m, 5, N—C_6H_5), 7.35 (d, 1, C-5 H), 2.45 (m, 2, $COCH_2CH_3$), and 1.20 (t, 3, $COCH_2CH_3$). The hydrochloride salt of XII*b* was formed in the usual way and recrystallized from ethanol–ether, m.p. 136–137°.

Anal.—Calc. for $C_{15}H_{16}N_2O \cdot HCl$: C, 65.09; H, 6.15; N, 10.12. Found: C, 64.94; H, 6.25; N, 9.98.

1-Benzyl-3-methyl-4-(*N*-propananilido)piperidine (V*b*)—A 9.6-g. (0.04-mole) sample of XII*b* was dissolved in 100 ml. of glacial acetic acid, and 4.0 g. of 10% palladium-on-charcoal was added to the solution. The mixture was hydrogenated for 24 hr. at 75°. After filtration, basification, and concentration, the residual oil was refluxed with 11.9 g. (0.07 mole) of benzyl bromide in acetonitrile. The oil obtained by this procedure was purified as described for IV*b*, yielding 7.8 g. (58%) of V*b* as a clear oil; NMR ($CDCl_3$): δ 7.45 (s, 5, $CH_2C_6H_5$), 7.30–7.80 (m, 5, N—C_6H_5), 4.45–4.80 (m, 1, C-4 H), 3.55 (s, 2, N—$CH_2C_6H_5$), and 1.10 (d, 3, C-3 CH_3). The hydrochloride salt of V*b* was prepared and recrystallized from ethanol–ether, m.p. 202.5–204°.

Anal.—Calc. for $C_{22}H_{28}N_2O \cdot HCl \cdot H_2O$: C, 67.59; H, 7.99; N, 7.17. Found: C, 67.83; H, 7.55; N, 7.02.

1-(2-Phenylethyl)-3-methyl-4-(*N*-propananilido)piperidine (V*c*)—A 4.0-g. (0.012-mole) sample of V*b* was mixed with 1.0 g. (0.016 mole) of phenylacetaldehyde and treated according to the procedure described for the synthesis of IV*c*, resulting in 3.32 g. (79%) of V*c* as a clear oil; NMR ($CDCl_3$): δ 7.42 (s, 5, N—$CH_2CH_2C_6H_5$), 7.30–7.77 (m, 5, N—C_6H_5), 4.40–4.80 (m, 1, C-4 H), and 1.10 (d, 3, C-3 CH_3). The hydrochloride salt of V*c* was prepared and recrystallized from ethanol–ether, m.p. 164–166°.

Anal.—Calc. for $C_{23}H_{30}N_2O \cdot HCl$: C, 71.38; H, 8.08; N, 7.24. Found: C, 71.01; H, 8.24; N, 6.95.

1,3-Dimethyl-4-(*N*-propananilido)piperidine (V*a*)—A mixture of 4.0 g. (0.012 mole) of V*b* and 0.13 g. (0.015 mole) of formaldehyde solution (37%) was treated according to the procedure described in the preparation of IV*a* to yield 2.6 g. (82%) of V*a* as a clear oil; NMR ($CDCl_3$): δ 7.10–7.65 (m, 5, N—C_6H_5), 4.24–4.75 (m, 1, C-4 H), 2.23 (s, 3, N—CH_3), and 1.10 (d, 3, C-3 CH_3). The picrate of V*a* was prepared in the usual manner, m.p. 164°.

Anal.—Calc. for $C_{16}H_{24}N_2O \cdot C_6H_3N_3O_7$: C, 53.98; H, 5.56; N, 14.31. Found: C, 54.04; H, 5.56; N, 14.73.

4-Chloro-2,5-dimethylpyridine (X*c*)—A 154.5-g. (0.88-mole) sample of 2,5-dimethylpyridine *N*-oxide hydrochloride was prepared by standard procedures and added to 475 g. (3.10 moles) of phosphorus oxychloride. The reaction was heated to 120° for 1 hr. and then refluxed for 4 hr. The solution was allowed to cool and poured into ice. The solution was basified (potassium carbonate) and extracted with ether, and the ethereal extracts were dried (sodium sulfate) and concentrated *in vacuo*, leaving an impure liquid which was distilled at 40–50° (1 mm.) to give 89.7 g. (72%) of X*c*; NMR ($CDCl_3$): δ 8.60 (s, 1, C-6 H), 7.15 (s, 1, C-3 H), 2.50 (s, 3, CH_3), and 2.40 (s, 3, CH_3). The hydrochloride of X*c* was prepared, m.p. 240–241°.

Anal.—Calc. for $C_7H_8ClN \cdot HCl$: C, 47.21; H, 5.09; N, 7.69. Found: C, 47.29; H, 5.55; N, 7.74.

4-Anilino-2,5-dimethylpyridine (XI*c*)—Following the procedure used to prepare XI*a*, 81.6 g. (0.63 mole) of aniline hydrochloride and 80.1 g. (0.45 mole) of X*c*·HCl reacted to yield XI*c* as a gray solid. Recrystallization from dioxane yielded 61.2 g. (49%) of a white solid, m.p. 145°; NMR ($CDCl_3$): δ 7.05–7.90 (m, 6, C-3 H and N—C_6H_5), 2.50 (s, 3, CH_3), and 2.40 (s, 3, CH_3).

Anal.—Calc. for $C_{13}H_{14}N_2$: C, 78.78; H, 7.07; N, 14.14. Found: C, 78.70; H, 7.15; N, 14.16.

4-(*N*-Propananilido)-2,5-dimethylpyridine (XII*c*)—A 29.7-g. (0.15-mole) sample of XI*c* was heated in 150 ml. of propionic anhydride for 12 hr. at 80°. The reaction was worked up as described for XII*a* to give 31.7 g. (83%) of XII*c* as an oil which solidified on standing at room temperature. Recrystallization of this solid from ether gave white crystals, m.p. 87–89°; NMR ($CDCl_3$): δ 8.15 (s, 1, C-6 H), 7.20–7.55 (m, 5, N—C_6H_5), 7.13 (s, 1, C-3 H), 2.57 (s, 3, C-2 CH_3), 2.26 (s, 3, C-5 CH_3), 2.33 (m, 2, $COCH_2CH_3$), and 1.16 (t, 3, $COCH_2CH_3$).

Anal.—Calc. for $C_{16}H_{18}N_2O$: C, 75.79; H, 7.09; N, 11.02. Found: C, 75.53; H, 6.90; N, 10.77.

1-Benzyl-4-(*N*-propananilido)-2,5-dimethylpiperidine (VI*b*)—A solution of 10.1 g. (0.04 mole) of XII*c* in 120 ml. of glacial acetic acid was hydrogenated and then treated with 8.5 g. (0.05 mole) of

Table I—Analgesic Activities of 4-Propananilidopiperidines

1-Substituent	ED_{50}, mg./kg.[a,b] 2-CH_3 (IV)	3-CH_3 (V)	2-CH_3, 5-CH_3 (VI)
(*a*) CH_3	Inactive	14.34	Inactive
(*b*) $C_6H_5CH_2$	Inactive	36.67	65.14
(*c*) $C_6H_5CH_2CH_2$	0.665	0.004	0.803

[a] The ED_{50} of fentanyl hydrochloride as determined in these studies was 0.04 mg./kg. [b] Significance level: $\alpha = 0.05$.

benzyl bromide, as previously described for the preparation of IV*b*, to yield 7.3 g. (52%) of VI*b* as a clear oil; NMR ($CDCl_3$): δ 7.20–7.75 (m, 10, N—C_6H_5 and N—$CH_2C_6H_5$), 4.30–4.75 (m, 1, C-4 H), 2.95–4.20 (*AB* quartet, 2, N—$CH_2C_6H_5$), and 0.80–1.40 (m, 9, C-2 CH_3, C-5 CH_3 and $COCH_2CH_3$). The oil formed a picrate which was recrystallized from ethanol, m.p. 209–210°.

Anal.—Calc. for $C_{23}H_{30}N_2O \cdot C_6H_3N_3O_7$: C, 60.09; H, 5.74; N, 12.08. Found: C, 59.71; H, 5.68; N, 11.81.

1-(2-Phenylethyl)-2,5-dimethyl-4-(*N*-propananilido)piperidine (VI*c*)—A solution of 1.0 g. (0.008 mole) of phenylacetaldehyde and 2.0 g. (0.006 mole) of VI*b* was hydrogenated over 10% palladium-on-charcoal. The product was purified, as previously described in the preparation of IV*c*, to provide 1.16 g. (53%) of VI*a* as a clear oil; NMR ($CDCl_3$): δ 7.35–7.75 (m, 10, N—C_6H_5 and N—$CH_2CH_2C_6H_5$), 4.30–4.75 (m, 1, C-4 H), and 0.80–1.40 (m, 9, C-2 CH_3, C-5 CH_3 and $COCH_2CH_3$). The oil formed a hydrochloride salt which was recrystallized from ethanol–ether, m.p. 235.5–237°.

Anal.—Calc. for $C_{24}H_{32}N_2O \cdot HCl$: C, 71.88; H, 8.30; N, 6.99. Found: C, 71.70; H, 8.24; N, 6.96.

4-(*N*-Propananilido)-1,2,3-trimethylpiperidine (VI*a*)—Two grams (0.006 mole) of VI*b* and 0.9 g. (0.01 mole) of formaldehyde were hydrogenated. The product was purified, as previously described in the preparation of IV*a*, to yield 1.28 g. (78%) of VI*a* as a clear oil; NMR ($CDCl_3$): δ 7.20–7.75 (m, 5, N—C_6H_5), 4.30–4.75 (m, 1, C-5 H), 2.07 (s, 3, N—CH_3), and 0.80–1.40 (m, 9, C-2 CH_3, C-5 CH_3 and $COCH_2CH_3$). The oil formed a picrate which was recrystallized from ethanol, m.p. 242–244°.

Anal.—Calc. for $C_{17}H_{26}N_2O \cdot C_6H_3N_3O_7$: C, 54.86; H, 5.81; N, 13.91. Found: C, 55.86; H, 5.95; N, 14.13.

RESULTS AND DISCUSSION

Analgesic activities of the ring-methylated 4-propananilidopiperidines prepared in this study are given in Table I. The results of analgesic activities of the 4-propananilidopiperidines in this study are in accord with previous findings of structure–activity relationships for morphinomimetic compounds in that phenethylation of a basic nitrogen in these agents provides compounds of the highest analgesic activity. The results further indicate that 3-methylation of the piperidine ring of the 4-propananilidopiperidines significantly enhances analgesic activity, whereas 2-methylation and 2,5-dimethylation lead to a significant reduction in analgesic activity. It is interesting to correlate the effect of 1-substitution on the analgesic activities of the 4-propananilidopiperidines. In the fentanyl (I) and the 2,5-dimethylated (VI) series, potency is observed to decrease in the order $C_6H_5CH_2CH_2 > C_6H_5CH_3 > CH_3$, whereas in the 3-methylated series (V) the dependence of analgesic activity on the 1-substituent is in the order $C_6H_5CH_2CH_2 > CH_3 > C_6H_5CH_2$. These relationships suggest that introduction of a methyl substituent in the piperidine ring of the 4-propananilidopiperidine analgesics results in a fundamental alteration of the analgesic activity of these compounds which is possibly related to the mode of binding of the molecules at the analgesic receptor.

A meaningful interpretation of the nature of the interaction of the 4-propananilidopiperidines with the analgesic receptor is rather difficult to derive from the analgesic activities given in Table I. In addition to reflecting differences in receptor affinities, these results also reflect differences in distribution and metabolism of the various compounds. These latter factors can result in significant differences in biophase concentrations and, hence, apparent differences in analgesic activities (11).

Introduction of a ring methyl into the 4-propananilidopiperidines results in chiral molecules. In view of the reported stereoselectivity of the narcotic analgesic receptor (12), it is anticipated that separation of the geometric and optical isomers of the compounds in this study will provide even more potent compounds and will afford pertinent information with regard to the nature of the drug–receptor interaction. Studies on stereostructure–activity relationships for the ring-methylated 4-propananilidopiperidines are currently underway in these laboratories.

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Reaction of Helenalin with Hydrogen Chloride

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Abstract □ Reaction of helenalin (I) with hydrogen chloride–chloroform and deactivated neutral alumina yielded three products: mexicanin A (II), 1-epiallohelenalin (IV), and a new oxide (V). These products were formed *via* intermediates (VII, IX, and VIII, respectively) by the initial addition of hydrogen chloride to the exocyclic methylene grouping of the γ-lactone followed by elimination of hydrogen chloride upon treatment with neutral alumina. Exposure of II to hydrogen chloride–chloroform gave the same intermediates (VII, IX, and VIII) which, upon treatment with the neutral alumina, regenerated II, IV, and V, respectively. Thus, II was the common intermediate for IV and V in the reaction of I with hydrogen chloride–chloroform and neutral alumina. The conversion of IV to its intermediate [11,13-dihydro-13-chloro-1-epiallohelenalin (IX)] with hydrogen chloride–chloroform and the complete recovery of the starting material (IV) with neutral alumina were also observed. Treatment of I with concentrated hydrochloric acid afforded 2,3-dihydro-2-chlorohelenalin (X). Compound X was also readily converted to I upon treatment with either neutral alumina or water. Further reaction of I with deactivated neutral alumina in chloroform led to the direct formation of II. The structures of these compounds were assigned on the basis of chemical as well as spectral evidence. The mode of formation of these compounds was also postulated.

Keyphrases □ Helenalin—reaction with hydrogen chloride–chloroform, identification of products and intermediates, mechanism of formation □ Sesquiterpene lactones—reaction of helenalin with hydrogen chloride–chloroform, identification of products and intermediates □ Hydrogen chloride—reaction with helenalin, identification of products and intermediates, mechanism of formation

The conversion of helenalin (I) to a mixture of mexicanin A (II) and neohelenalin (III) by treatment with hydrogen chloride–chloroform was previously reported (1–3). In connection with the study of the structure–activity relationship among the helenalin-related sesquiterpene lactones for cytotoxic or antitumor activity (4–6), this reaction was repeated; the chemical as well as spectroscopic data on the resulting products provided some interesting information on their structures and the mode of their formation.

When helenalin (I) was dissolved in anhydrous chloroform saturated with hydrogen chloride at room temperature and the resulting products were chromatographed on deactivated neutral alumina according to Herz *et al.* (1), the expected mexicanin A (II) was obtained in 25% yield as reported (1); but instead of neohelenalin (III)[1] accompanying II, traces of 1-epiallohelenalin (IV) and a new compound, an oxide (V), isolated in 5% yield, were obtained. However, these compounds (II, IV, and V) were not obtained before the treatment of the reaction products with neutral alumina. This finding will be covered later.

DISCUSSION

Compound V, m.p. 188–190°, had the composition $C_{15}H_{18}O_4$ and showed a molecular ion peak at *m/e* 262 in the mass spectrum. Compound V revealed IR bands at 1760 and 1655 cm.$^{-1}$ and a pair of low-field doublets in the NMR spectrum at 6.29 (1H, J = 3), and 5.61 (1H, J = 3). This is characteristic of a γ-lactone conjugated with an exocyclic methylene grouping, a feature common to helenalin (I) and mexicanin A (II). The NMR spectrum of V showed the lactonic proton at C-8 as a multiplet at 4.60. Two methyl groups at C-10 and C-5 in V were seen as a doublet at 1.18 (3H, J = 6) and a sharp singlet at 1.07 (3H), respectively. The presence of a cyclopentanone ring system in V was first suggested by the appearance of a strong IR band at 1740 cm.$^{-1}$ and substantiated by the absence of the characteristic conjugated cyclopentenone olefinic protons.

The IR spectrum of Compound V taken in mineral oil showed the absence of a hydroxyl group. This was further confirmed by the fact that V could not be acetylated with acetic anhydride in pyridine. This evidence, coupled with the fact that the empirical formula of Compound V ($C_{15}H_{18}O_4$) was identical with those of helenalin and mexicanin A, would indicate that the remaining fourth oxygen must form an oxide ring. Models show that one of the two possible structures, V or VI, can be assigned to this compound[2]. The former is regarded as the most likely because the signal for the proton at H-2 in Structure V appeared as a multiplet at 4.76, and it would be expected to move further downfield if Structure VI is assigned since H-3 in VI is adjacent to the C-4 carbonyl group. The upfield shift of the oxide proton at C-6 (3.79, 1H, d, J = 5.3) in V, compared with that in the NMR spectrum of helenalin (I) in which the H-6 signal appeared at 4.45 (1H, d, J = 4.5), might be due to the anisotropic effect of the α-methylene grouping of the γ-lactone.

The foregoing evidence suggests the assignment of Structure V with complete stereochemistry for the oxide.

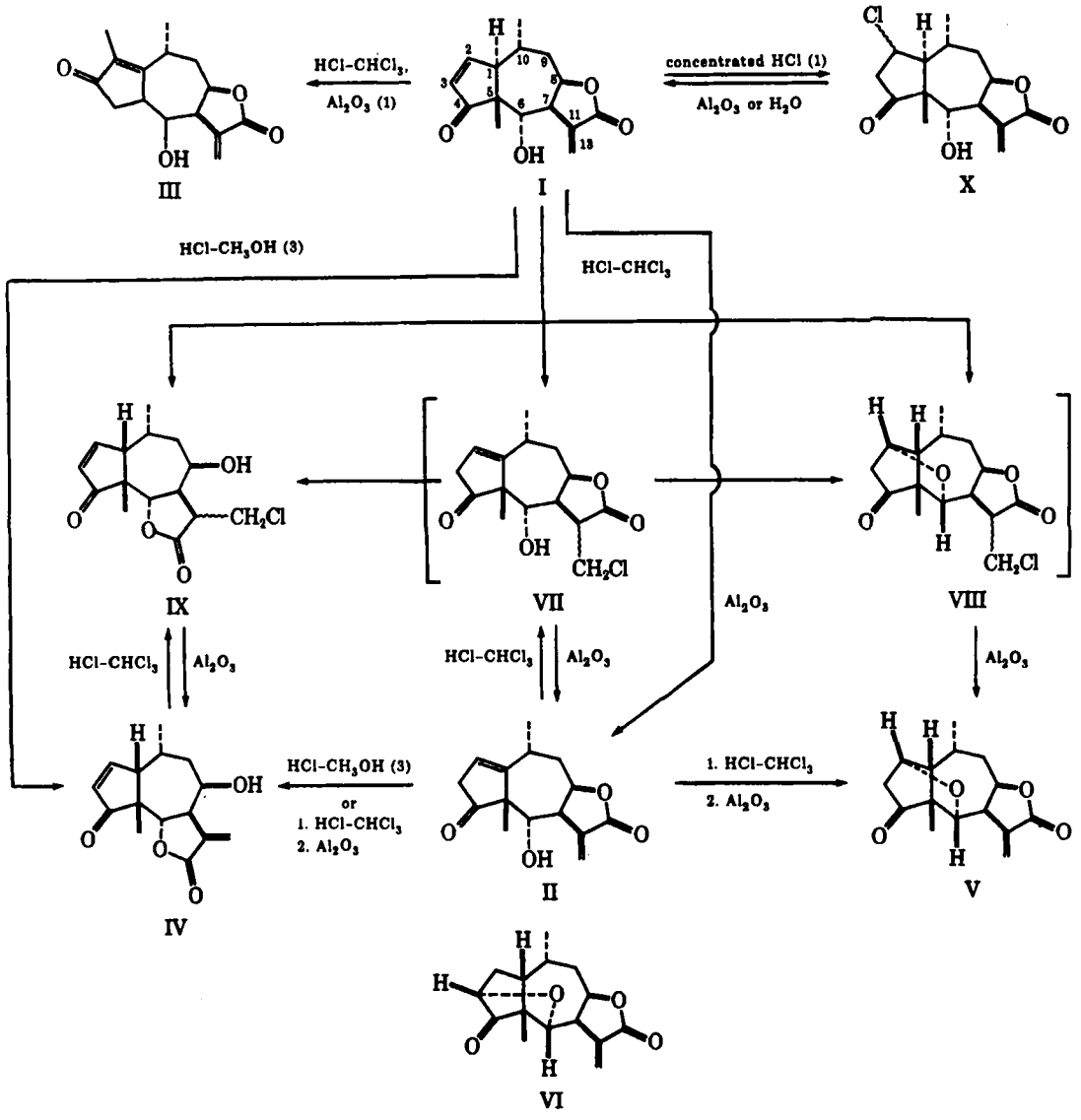
The other product besides II and V in this reaction of I with hydrogen chloride–chloroform and neutral alumina was the 1-epiallohelenalin (IV). This compound was obtained in only about 2% yield and was shown to be identical with the major product obtained by the treatment of I with hydrogen chloride–methanol as reported previously (3).

As already mentioned, during the action of hydrogen chloride–chloroform on helenalin, it was found that the initial reaction products prior to chromatography on neutral alumina were not mexicanin A (II), 1-epiallohelenalin (IV), and the oxide (V). They were mainly the new intermediates that resulted from the addition of hydrogen chloride to the exocyclic methylene grouping of the γ-lactone. These observations were verified by the fact that the intermediates were obtained in almost quantitative yield and showed the characteristic isotopic molecular ions at *m/e* 298 and 300 ($C_{15}H_{19}{}^{35}ClO_4$ and $C_{15}H_{19}{}^{37}ClO_4$) in the ratio of approximately 3:1, indicating the incorporation of 1 mole of hydrogen chloride into the molecule. These intermediates have proven to be elusive as far as separation is concerned[3], but their isolation as a mixture (likely Compounds VII, VIII, and IX) was easily achieved by silica gel chromatography. The NMR spectrum of this mixture further confirmed the absence of the characteristic pair of low-field doublets which corresponds to the α-methylene-γ-lactone grouping. Treatment of mexicanin A (II) with hydrogen chloride–chloroform in a manner similar for helenalin (I) gave reaction products whose NMR spectrum was completely identical with the one obtained from the

[1] Analysis of the crude reaction mixture in high concentration by NMR spectroscopy revealed the absence of any detectable vinyl methyl protons in the high field region (1.60 ~ 2.10). Neohelenalin was reported to show a vinyl methyl group at C-2 as a doublet at 1.68 (J = 2) (1).

[2] Dreiding models indicate that the formation of such an oxide ring between C-6 and C-2 or C-3, as shown in V or VI, requires the inversion of the asymmetric center at C-1 of helenalin. No other oxygen bridge could be constructed when H-1 is in an α disposition. As shown in Structure V, the oxide bridge involved C-6 α, C-2 α bonds.

[3] These mixtures were not very stable and tended to generate mexicanin A gradually upon prolonged sitting at room temperature.



Scheme I

reaction of I with hydrogen chloride–chloroform and yielded products II, IV, and V upon chromatography on neutral alumina.

In conclusion, the results obtained indicate that mexicanin A (II) is the common intermediate for the 1-epiallohelenalin (IV) and the oxide (V) in the reaction of helenalin with hydrogen chloride–chloroform and neutral alumina (Scheme I).

Similar treatment of 1-epiallohelenalin (IV) with hydrogen chloride–chloroform led to the formation of the corresponding hydrogen chloride adduct of the α-methylene-γ-lactone grouping (IX) in quantitative yield. Compound IX, m.p. 234–236°, analyzed for $C_{15}H_{19}ClO_4$, showed a molecular ion peak at *m/e* 298 along with an isotopic ion at *m/e* 300. The NMR spectrum of IX showed the disappearance of the characteristic exocyclic methylene protons and the generation of a new two-proton multiplet at 3.80, attributable to the protons attached to chlorine atom. Compound IX regenerated IV upon treatment with neutral alumina.

Reaction of helenalin (I) with concentrated hydrochloric acid elaborated 2,3-dihydro-2-chlorohelenalin (X) in quantitative yield. The following evidence supported Structure X for this reaction product: the presence of the isotopic molecular ions at *m/e* 298 and 300 in the mass spectrum, the absence of signals corresponding to the olefinic protons at C-2 and C-3 as found in I, and the presence of the well-defined pair of low-field doublets ascribed to the γ-lactone-α-methylene protons in the NMR spectrum. Further treatment of X with neutral alumina or water led to the complete recovery of I. Further reaction of I with deactivated neutral alumina in chloroform led to the direct formation of II in 15% yield.

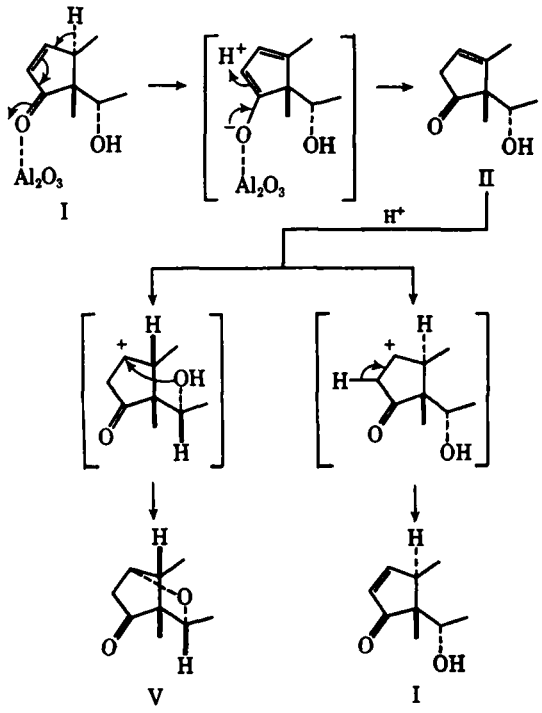
The formation of II and V can be accounted for by the mechanism postulated in Scheme II in which alumina is considered to act as a Lewis acid.

EXPERIMENTAL[4]

Helenalin (I) was obtained from extracts of either *Helenium microcephalum* or *Balduina angustifolia* as previously reported (4, 7).

Treatment of Helenalin (I) with Hydrogen Chloride–Chloroform: Compounds VII, VIII, and IX—This reaction was carried out in the same manner as that reported in the literature (1); *i.e.*, a solution of helenalin (1 g.) in anhydrous chloroform saturated with hydrogen chloride (35 ml.) was allowed to stand at room temperature overnight. The mixture was further refluxed for 30 min., and the product

[4] Melting points were determined on a Thomas-Hoover melting-point apparatus and are corrected. Unless otherwise specified, IR spectra were determined in mineral oil mulls with a Perkin-Elmer 257 grating IR spectrophotometer. NMR spectra were measured in $CDCl_3$ with a Jeolco C 60 HL NMR spectrometer, using tetramethylsilane as an internal standard. All chemical shifts were reported in δ (p.p.m.) values and the coupling constants were reported in hertz values. Signals are characterized in the usual way: s, singlet; d, doublet; t, triplet; q, quartet, and m, multiplet. Mass spectra were determined on an A.E.I. MS-902 instrument at 70 ev. using a direct inlet system. Silica gel for column chromatography refers to Baker A.R. No. 3405, and silica gel for TLC refers to Merck silica gel G developed with chloroform–acetone (3:1) and visualized by spraying with concentrated sulfuric acid and heating. Deactivated neutral alumina for column chromatography refers to neutral alumina AG-7 (100–200 mesh), Bio-Rad, activity grade III. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga.



Scheme II

was washed with 5% sodium hydroxide, washed with water, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to yield a brown syrup. The syrup showed the characteristic isotopic molecular ions at *m/e* 298 and 300 as described in the text. It was chromatographed on silica gel (1.3 × 30 cm.) activated at 120° and impregnated with 10% of water using *n*-hexane–benzene (1:1), benzene, and ethyl acetate as the developing solvents, successively. Thirty-one 25-ml. fractions were collected, and the composition of the fractions was determined by examining thin-layer chromatograms. The first hexane–benzene (1:1) eluate (fractions 1–19) yielded, after evaporation of the solvent, a colorless oil (380 mg.), which appeared as a single fast moving spot. The NMR spectrum of this substance indicated that this was a mixture of the hydrogen chloride adducts, *i.e.*, Compounds VII, VIII, and IX. The subsequent benzene (fractions 20–26) (180 mg.) and ethyl acetate (fractions 27–31) (400 mg.) eluates all contained a mixture of mainly the above-mentioned, fast moving spot and traces of others, and they were not investigated further.

Treatment of Helenalin (I) with Hydrogen Chloride–Chloroform and Deactivated Neutral Alumina—The brown syrup obtained from the repetition of the above experiment with I (1 g.) and hydrogen chloride–chloroform (35 ml.) was chromatographed on deactivated neutral alumina (1.5 × 25 cm.). Elution was effected in 2-ml. fractions with benzene, benzene–chloroform, chloroform, and acetone.

The Oxide (V)—The first benzene eluates (fractions 1–10) afforded, after evaporation of the solvent, a crystalline residue. This residue was recrystallized from dichloromethane–absolute ethanol to give V as colorless needles (50 mg., 5%), m.p. 188–190°. The relevant (IR, NMR, and mass) characteristics have been described in the text.

Anal.—Calc. for $C_{15}H_{18}O_4$: C, 68.68; H, 6.92. Found: C, 68.46; H, 7.01.

Mexicanin A (II)—The subsequent benzene–chloroform (1:1) and chloroform eluates (fractions 11–25) provided a crystalline material, which was recrystallized from acetone–ether to furnish II as colorless prisms (250 mg., 25%), m.p. 140–142° [lit. (1) m.p. 138–140° (acetone–ether)]. The IR spectrum of II was identical with that of mexicanin A as described in the literature (1). The NMR spectrum exhibited signals at 1.21 (3H, s, C-5 CH_3), 1.32 (3H, d, J = 6, C-10 CH_3), 3.59 (1H, d, J = 7.5, H-6), 4.67 (1H, dt, J = 3, 8.3, H-8), 5.89 (1H, q, J = 3.8, 1.5, H-2), 6.02 (1H, d, J = 3, H-13), and 6.35 (1H, d, J = 3, H-13) and was in complete agreement with the indicated structure (II), *i.e.*, mexicanin A.

1-Epiallohelenalin (IV)—The final acetone eluates (fractions 26–39) gave an oily residue, which was seeded with one crystal of 1-epiallohelenalin obtained from the treatment of helenalin with hydrogen chloride–methanol (q.v.). The colorless crystals which formed were collected and recrystallized from dichloromethane–ether to yield 20 mg. of IV, m.p. 179–180°. The identity of IV was established by direct comparison with an authentic sample of 1-epiallohelenalin by mixed melting point, TLC, and superimposable IR and NMR spectra.

Attempted Acetylation of Oxide (V)—A solution of V (35 mg.) in acetic anhydride–dry pyridine (2:1) (0.5 ml.) was kept at room temperature overnight. The product, recovered by working up the reaction mixture in the usual way, was the unchanged starting material (33 mg.).

Treatment of Mexicanin A (II) with Hydrogen Chloride–Chloroform and Deactivated Neutral Alumina: Compounds VII, VIII, and IX and Compounds II, V, and IV—Compound II (300 mg.) was treated with anhydrous chloroform saturated with hydrogen chloride (15 ml.) in an analogous manner as that for helenalin, furnishing a brown oily residue. The residue was identified as the mixture of Compounds VII, VIII, and IX by their superimposable NMR spectra in comparison with the reaction products of helenalin and hydrogen chloride–chloroform already described. The oily residue was chromatographed on deactivated neutral alumina (1.5 × 12 cm.). Elution with benzene (20 ml.) afforded V (30 mg., 10%). Elution with benzene and chloroform–ether (1:1) (25 ml.) yielded II (180 mg., 60%). Elution with chloroform–ether (1:2) (20 ml.) gave IV (18 mg., 6%).

Treatment of 1-Epiallohelenalin (IV) with Hydrogen Chloride–Chloroform: 11,13-Dihydro-15-chloro-1-epiallohelenalin (IX)—Compound IV (100 mg.) was treated with anhydrous chloroform–hydrogen chloride (5 ml.) in an analogous manner as described for helenalin. The oily residue resulting from the removal of solvent crystallized upon trituration with a small amount of anhydrous ether. Recrystallization from dichloromethane–ether gave IX as white crystals in quantitative yield, m.p. 234–236° dec. The IR bands were at 3420 (OH), 1775 (saturated γ-lactone), 1690, and 1590 (cyclopentenone) cm.$^{-1}$. The NMR spectrum exhibited signals at 1.22 (3H, d, J = 6.8, C-10 CH_3), 1.38 (3H, s, C-5 CH_3), 3.80 (2H, m, CH_2Cl), 4.05 (1H, m, H-8), 4.77 (1H, d, J = 8, H-6), 6.33 (1H, dd, J = 6, 2.3, H-3), and 7.82 (1H, dd, J = 6, 2.3, H-2).

Anal.—Calc. for $C_{15}H_{19}ClO_4$: C, 60.29; H, 6.41. Found: C, 60.25; H, 6.51.

1-Epiallohelenalin (IV) from 11,13-Dihydro-13-chloro-1-epiallohelenalin (IX) and Neutral Alumina—Chromatography of IX (20 mg.) on deactivated neutral alumina (1.2 × 7 cm.) and elution with chloroform afforded IV as colorless needles in quantitative yield. Compound IV showed undepressed melting point on admixture of an authentic sample, and their IR and NMR spectra were identical.

Treatment of Helenalin (I) with Concentrated Hydrochloric Acid: 2,3-Dihydro-2-chlorohelenalin (X)—A solution of I (100 mg.) in concentrated hydrochloric acid (1 ml.) was heated at 40° for 5 min. The colorless crystalline product that deposited was filtered and dried to give quantitative yield of X, m.p. 134–135° dec. [lit. (3) m.p. 143–145° dec.]. Compound X showed IR bands at 3480 (OH), 1765 (sh), 1752, 1650 (α-methylene-γ-lactone), and 1735 (cyclopentanone) cm.$^{-1}$. Compound X also displayed NMR (dimethyl sulfoxide-d_6) signals at 0.87 (3H, s, C-5 CH_3), 1.15 (3H, d, J = 6, C-10 CH_3), 5.86 (1H, d, J = 3, H-13), and 6.19 (1H, d, J = 3, H-13).

Helenalin (I) from 2,3-Dihydro-2-chlorohelenalin (X)—Chromatography of X (50 mg.) on deactivated neutral alumina (1.7 × 7 cm.) with elution with acetone (50 ml.) recovered I quantitatively. Compound I was also obtained by extraction of X with water and chloroform. The chloroform extract was dried and evaporated to yield a crystalline residue, which was recrystallized from benzene–ether to give I as colorless needles, m.p. 170–172°. The IR spectrum of this compound was identical with that of helenalin.

Treatment of Helenalin (I) with Deactivated Neutral Alumina: Mexicanin A (II)—A solution of I (70 mg.) in chloroform (5 ml.) was added to deactivated neutral alumina (1 g.). The reaction mixture, after standing at room temperature for 3 days with occasional shaking, was filtered and evaporated to yield an oil[5]. Preparative TLC on silica gel[6] furnished mexicanin A (II) (10.5 mg., 15%), m.p. 140–142°, identical (IR and mixed melting point) with an authentic sample, and unreacted I (43 mg.).

[5] Examination of this oil by TLC revealed the absence of Compounds IV and V.

[6] Merck silica gel GF-254 with chloroform–acetone (3:1).

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DRUG STANDARDS

Quantitative Fluorometric Determination of Pancreatic Lipase in Pharmaceuticals

S. S. WAGLE▲ and D. G. HOUSE

Abstract □ A rapid and reliable assay for pancreatic lipase is described. The method utilizes microquantities of enzyme and a fluorescent substrate. Among the many fluorescent substrates available, 4-methylumbelliferone laurate was chosen as the most suitable for this method. The rate of hydrolysis at different enzyme levels was determined at 25°. Interference due to nonspecific esterases and the effect of some pharmaceutical agents were evaluated. The fluorometric assay procedure was compared to the NF XIII potentiometric method. Pancreatic lipase in concentrations as low as 0.15 NF unit of activity could be detected. Various complex pharmaceutical preparations were compared using the fluorometric and the potentiometric methods. The fluorometric procedure was found to be reproducible and useful in determining content uniformity.

Keyphrases □ Pancreatic lipase—quantitative fluorometric determination in pharmaceuticals □ Fluorometry—quantitative determination of pancreatic lipase in pharmaceuticals □ Enzymes, pancreatic lipase—quantitative fluorometric determination in pharmaceuticals

For many years, pancreatic enzymes have been used as digestive aids. A number of pharmaceutical preparations contain pancreatic enzymes alone or in combination with other therapeutic agents. Many assay procedures have been devised to determine the potencies and activities of the lipolytic enzymes in these preparations. Aldridge (1) first described a manometric method utilizing carbon dioxide liberation from bicarbonate buffer to detect lipases in biological extracts. However, titrimetric measurement of acid liberation from natural fats was found to be more convenient (2, 3). Over the years, this procedure has been greatly modified and refined to improve its reliability and reproducibility (4). The potentiometric titration method, although reliable, is cumbersome and time consuming for routine analysis. This procedure also lacks the versatility needed for testing complex pharmaceutical preparations and ascertaining content uniformity.

Fluorometric procedures have a distinct advantage over other methods in that they are very sensitive. For a number of years, biochemists and clinical chemists detected minute amounts of esterase in microorganisms by utilizing fluorogenic substrates such as 1-naphthyl esters (5). Most fluorometric procedures for hydrolytic enzymes involve hydrolyzing a nonfluorescent substrate to a highly fluorescent product. 1-Naphthyl phosphate, a nonfluorescent substrate, is readily hydrolyzed by the enzymes, acid phosphatase and alkaline phosphatase, producing the free fluorescent product, 1-naphthol, which is then measured quantitatively by the fluorometric method. Similarly, umbelliferone and 4-methylumbelliferone are highly fluorescent compounds while their ester derivatives are nonfluorescent. These derivatives are ideal substrates for many enzymes (6). Recently, a number of investigators (6–8) reported new derivatives of fluorescein and 7-hydroxy-4-methylcoumarin to detect and locate minute quantities of lipase in plants

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Table I—Relative Fluorescence of Fluorogenic Substrates and Their Hydrolysis Products

	Wavelength, nm.		Fluorescence Values: 1×10^{-5} *M* Solutions in pH 9.0, 0.05 *M* Phosphate Buffer		
	Excitation	Fluorescence	Substrate	Hydrolysis Product	Product/Substrate Ratio
Fluorescein dibutyrate	493	570	11	951	86.5
4-Methylumbelliferone laurate	380	450	29	397	13.7
1-Naphthyl acetate	335	450	11	26	2.36

Table II—Effects of Pharmaceutical Agents on Pancreatic Lipase-Catalyzed Hydrolysis of Fluorescein Dibutyrate and 4-Methylumbelliferone Laurate

	Relative Hydrolysis Rate, nmoles/min.				
	Control[a]	Sodium Taurocholate[b]	Dioctyl Sodium Sulfosuccinate[c]	1-Hyoscyamine Sulfate[d]	Trypsin[e]
Fluorescein dibutyrate	19.0	19.15	73.4	1.8	15.9
4-Methylumbelliferone laurate	42.83	43.0	44.1	43.6	13.5

[a] Control experiment contained 0.3 NF unit of lipase standard. [b] Sodium taurocholate, 5 mg./ml. [c] Dioctyl sodium sulfosuccinate, 25 mcg./ml. [d] Hyoscyamine sulfate, 50 ng./ml. [e] Trypsin, 1.2 NF units.

and biological fluids. The suitability of the fluorogenic substrates for quantitative analysis of pancreatic lipase in pharmaceutical preparations has not been investigated.

This paper reports a systematic study of some fluorogenic substrates and a simple fluorometric assay procedure for rapid and quantitative determination of lipase in pharmaceutical preparations.

EXPERIMENTAL

Apparatus—The rate of hydrolysis of the fluorogenic substrate is measured as the rate of increase in fluorescence and is simultaneously recorded by a fluorometer[1] with a recorder attachment. Clear fused Pyrex glass cells (10 × 10 × 65 mm.) were used as reaction vessels, and the instrument supplier's slit arrangement No. 1 was used throughout the work. The excitation and fluorescent wavelengths used for various substrates are listed in Table I.

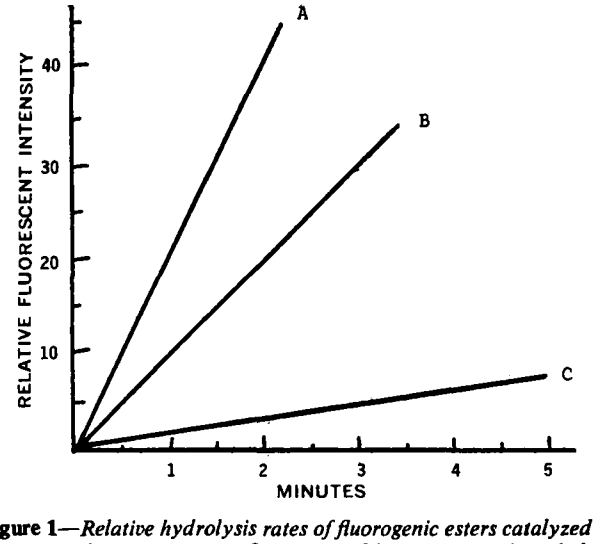

Figure 1—*Relative hydrolysis rates of fluorogenic esters catalyzed by pancreatic lipase. Key: A, fluorescein dibutyrate; B, 4-methylumbelliferone laurate; and C, 1-naphthyl acetate.*

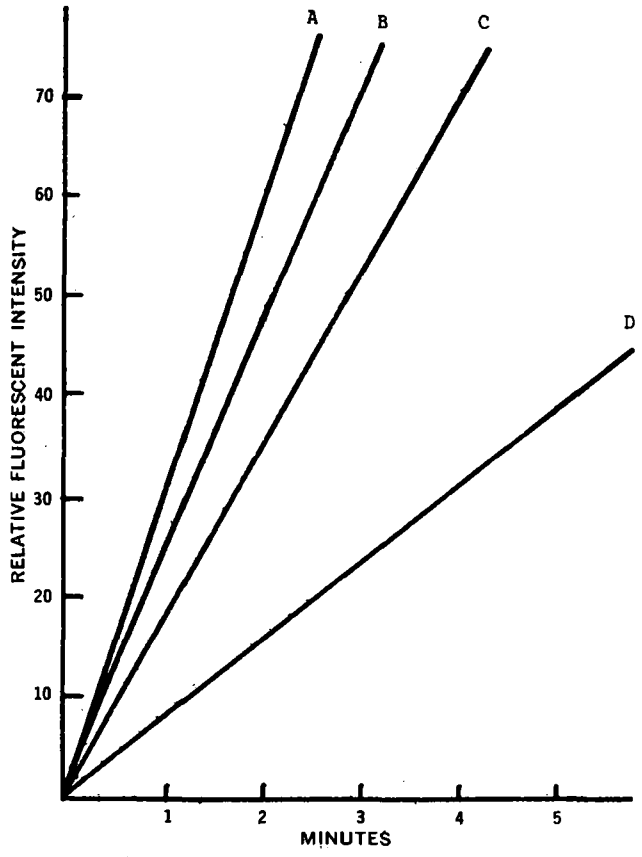

Figure 2—*Lipolysis of 4-methylumbelliferone laurate. Incubation mixture contained 5 × 10^{-5} mole of substrate and varying amounts of pancreatic lipase standard. Key: A, 0.6 NF unit; B, 0.45 NF unit; C, 0.30 NF unit; and D, 0.15 NF unit, in a total volume of 5 ml. of 0.05* M *phosphate buffer, pH 9.0, at 25°.*

Reagents and Chemicals—Stock solutions (1×10^{-2} *M*) of 1-naphthyl acetate[2], fluorescein dibutyrate[2], and 4-methylumbelliferone laurate[3] were prepared in 2-methoxyethanol[4]. These stock solutions are stable for at least 1 year when frozen. A fresh working solution (1×10^{-5} *M*) was made by diluting 1 ml. of the stock solution to 1 l. with 0.05 *M* disodium hydrogen phosphate buffer, pH 9.0.

Samples—At least 20 capsules (or tablets) were emptied into a beaker and mixed well, and a quantity equivalent to one capsule content was accurately weighed into a beaker and mixed thoroughly with 2–3 drops of glycerin. When the contents were thoroughly wet, 25 ml. of phosphate buffer (0.05 *M*) was added. The mixture was stirred for 5–10 min., quantitatively transferred into a centrifuge tube, and spun at 3000×*g* for 10 min. The supernatant liquid was transferred into a suitable flask and diluted with phosphate buffer to obtain a desired concentration of lipase activity.

Standard Solution—One hundred milligrams of the NF pancreatin reference standard (for protease and lipase activity) was accurately weighed and was treated in the same manner as the sample to obtain a desired concentration.

Other Pharmaceutical Agents—Dioctyl sodium sulfosuccinate USP, hyoscyamine sulfate NF, sodium taurocholate NF, and trypsin[5] were either dissolved or suspended in 0.05 *M* phosphate buffer, pH 9.0.

[1] Model 111, G. K. Turner.
[2] Eastman Kodak Co.
[3] Nutritional Biochemicals, Cleveland, Ohio.
[4] Aldrich Chemical Co., Milwaukee, Wis.

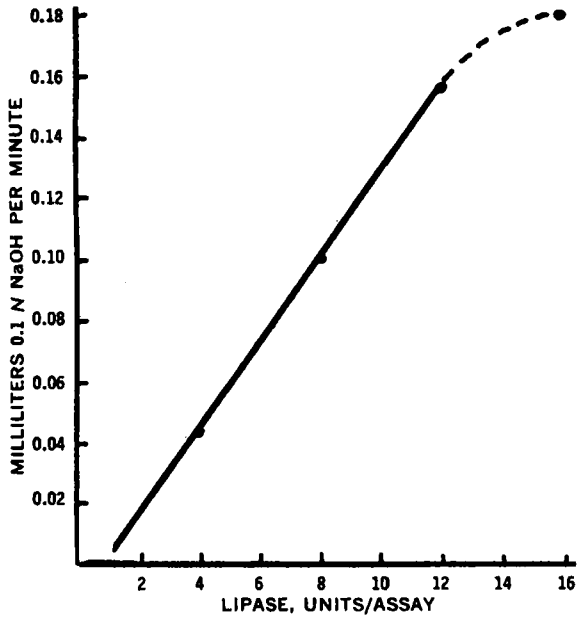

Figure 3—*Representative standard curve for pancreatic lipase by NF method.*

Procedure—Enzymatic reactions were conducted at 25° in the fluorometer cells. The reaction mixture contained 1 ml. of the substrate (1×10^{-5} *M*), with or without inhibiting or potentiating agents, and variable amounts of lipase (between 0.2 and 0.6 NF unit of activity per assay) in a total volume of 5 ml. The cell was placed immediately in a fluorometer, and the increase in fluorescence was recorded for the next 5–7 min. The rate of hydrolysis was calculated from the slope of the curve. For comparison, the potentiometric assay for lipase was performed as described in the second supplement of NF XIII (4).

RESULTS AND DISCUSSION

The effects of temperature and pH on lipolysis of fluorogenic substrates have been extensively studied by several investigators (6, 7, 11). In the present investigation, the hydrolytic reaction was conducted in phosphate buffer at pH 9.0 to reduce the possible deactivation of lipases by proteolytic enzymes which may be present in the formulations. No special precautions were necessary to control the cell temperature since the study indicated that the temperature of the reaction mixture varied less than a degree during the entire procedure.

Three fluorogenic esters were investigated as possible lipase substrates. Several important criteria, such as substrate sensitivity and specificity and enzyme inhibition and potentiation by pharmaceutical agents normally employed in the enzyme preparations, were tested and evaluated. To demonstrate hydrolysis at a very low enzyme concentration, the substrate should possess a relatively high fluorescence after hydrolysis. Table I shows the relative fluorescence of the three prospective fluorogenic substrates and their hydrolysis products. All fluorogenic substrates tested were hydrolyzed by lipase; however, fluorescein dibutyrate and 1-naphthyl acetate produced the highest and the lowest fluorescence, respectively, after hydrolysis and the latter exhibited the lowest hydrolytic rate (Fig. 1).

Table III—Comparative Analysis of Commercial Preparations by NF Method and Fluorometric Method[a]

Product	NF Method	Fluorometric Method[b]	Variation, %
A	16.49	16.8 ± 0.28	1.87
B	14.69	15.0 ± 0.40	2.11
C	20.80	22.2 ± 0.44	6.73

[a] Assay values presented in NF units. [b] Mean of at least six determinations.

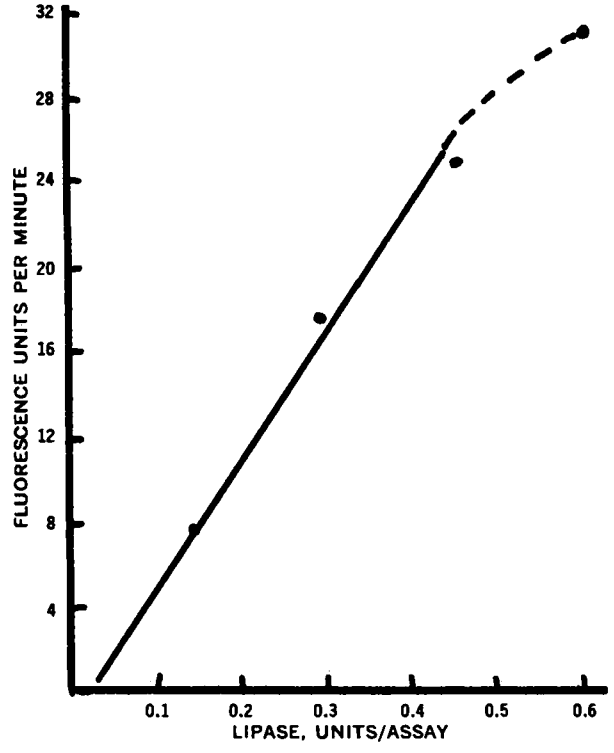

Figure 4—*Representative standard curve for pancreatic lipase by fluorometric method.*

It is imperative that minute quantities of pharmaceutical agents and excipients employed in the formulation have a minimal effect on the lipolysis of the substrate. The effects of some pharmaceutical agents and other interfering enzymes are summarized in Table II.

Trypsin is found in significant amounts in pancreatic extracts and possesses high esterase activity. Many substrates used in fluorometric assays are nonspecific and are hydrolyzed by both esterases and lipases. Therefore, a substrate that is specific for lipase will have a distinct advantage. Experiments revealed that fluorescein dibutyrate was readily hydrolyzed by both trypsin and lipase. 4-Methylumbelliferone laurate, on the other hand, was specifically hydrolyzed by lipase and was least affected by trypsin (Table II).

Some pharmaceutical and medicinal agents exhibit biological activity by virtue of their potentiating or inhibiting actions on enzyme catalysis (9). Bile salts and other surfactants are commonly used to enhance lipolysis of olive oil or other fatty esters (10). The data, however, indicate that sodium taurocholate had no potentiating effect on the rate of hydrolysis of fluorescein dibutyrate or 4-methylumbelliferone laurate. Another potent surfactant, dioctyl sodium sulfosuccinate, at 25-mcg./ml. concentration, enhanced the lipolysis of fluorescein dibutyrate 400%, but no such effect could be demonstrated on the hydrolysis of 4-methylumbelliferone laurate. Belladonna alkaloids, particularly hyoscyamine sulfate, are frequently formulated in combination with digestive enzymes. Hyoscyamine sulfate inhibited the enzymatic hydrolysis of fluorescein dibutyrate but had no apparent effect on the hydrolysis of 4-methylumbelliferone laurate. The data summarized in Table II clearly suggest that 4-methylumbelliferone laurate is a preferable substrate for fluorometric determination of lipase.

Figure 2 shows a typical continuous recorder tracing of hydrolysis of 4-methylumbelliferone laurate at different concentrations of NF lipase standard. The slope of the curve thus represented the rate of hydrolysis. This, when plotted against the concentration of enzyme, provided a standard curve for quantitative estimation of lipase. Using the NF lipase standard, comparison was made of the rates of hydrolysis obtained by the NF method and the fluorometric method. The data show that essentially the same linear rate was obtained using either method (Figs. 3 and 4). The fluorometric method, however, was 10–20 times more sensitive than the potentiometric procedure.

Comparative analyses were conducted on commercial preparations using potentiometric and fluorometric methods. All products

tested contained, in addition to lipases, other hydrolytic enzymes of pancreatic, plant, or microbial origin mixed with lactose and smaller quantities of lubricants. The data were analyzed statistically, and excellent agreement between the two methods was observed (Table III). The reproducibility of the method was evaluated by performing repeated analysis of the same enzyme solution. The precision of the method was determined from the variability obtained upon repeated analysis of lipase standard or samples. A standard deviation of 0.76, with a coefficient of variation of 3.05%, was obtained.

CONCLUSION

The advantages of a fluorometric procedure are many. In contrast to earlier methods, the fluorometric assay is more sensitive and requires smaller amounts of substrate and enzyme. The lipolytic measurements can be made at such a great dilution that interfering enzymes and other excipients are frequently diluted out. 4-Methylumbelliferone laurate was found to be a most suitable substrate for the fluorometric assay, because it was unaffected by a number of pharmaceutical agents and enzymes tested.

The method shows a high degree of precision and accuracy. The procedure is simple and requires little preparation. The substrate is stable under the experimental conditions and can be stored frozen for long periods. The method may be valuable in determining content uniformity of pharmaceutical preparations containing lipase.

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HENRY S. I. TAN

Abstract □ A sensitive colorimetric method was developed for the assay of isoniazid. The method is based on measuring the absorbance at 467 nm. of the orange-colored product produced by the interaction of isoniazid with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole in the presence of sodium tetraborate in absolute methanol. The Beer–Lambert law was obeyed over the concentration range of 0.3–5 mcg./ml, and the color was stable for at least 90 min. The coefficient of variation as determined on nine replicate samples containing 2 mcg. isoniazid/ml. was 0.87%. Applied to the assay of isoniazid tablets and syrups, the method gave results comparable to those obtained by the USP XVIII method. It also afforded improvements in sensitivity, ease, and speed over the official method. It was established that the major metabolites of isoniazid (acetylisoniazid and isonicotinic acid) produced no color with the reagent under the proposed experimental conditions.

Keyphrases □ Isoniazid—colorimetric analysis, compared to compendial method □ Colorimetry—analysis, isoniazid, compared to compendial method

Isoniazid (isonicotinyl hydrazine, I) is a remarkably effective drug which is now considered a primary drug for the chemotherapy of tuberculosis. It is not surprising that the determination of this drug has been, and is still, the subject of much investigation (1, 2). The need for further investigation is evidenced by the fact that many of the assay procedures reported appear not to be very satisfactory. For example, the current official assay method for isoniazid employs an iodometric titration in the presence of sodium bicarbonate (3). This method, originally developed by Canbäck (4), involves a slow reaction; 90 min. is needed for the completion of the reaction before a back-titration of the excess iodine can be performed. In addition, at several stages during the assay period, loss of iodine by volatilization can occur. The USP XVIII method for the assay of isoniazid tablets is time consuming and laborious; *at least* 2.5 hr. is required for the extraction of the drug from the tablet mass, evaporation of the extract, and completion of the reaction (5).

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[1] NBD Chloride, Aldrich Chemical Co., Milwaukee, Wis.

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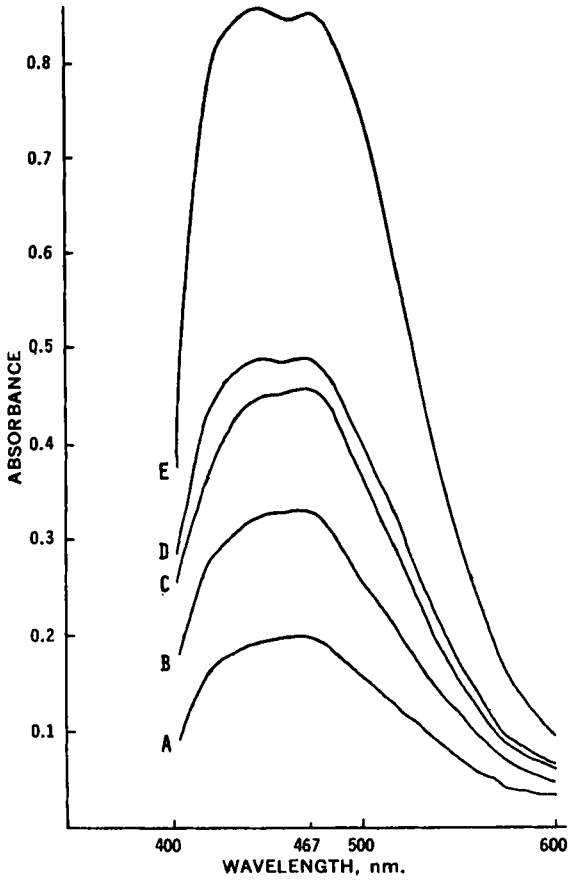


Figure 1—*Absorption spectra of reaction product at various isoniazid concentrations (micrograms per milliliter). Key: A, 2.0; B, 3.3; C, 4.5; D, 5.0; and E, 9.0.*

with isoniazid. The color of the product seemed to be pH dependent.

This work served as the basis for the present study, in which II in alkaline medium was used for the quantitative colorimetric assay of isoniazid.

EXPERIMENTAL[2]

Materials and Reagents—The following were used:

Isoniazid—Highest purity grade isoniazid[3] recrystallized from 80% methanol and dried under reduced pressure for 24 hr., m.p. 172–172.5°.

Methanol—Acetone-free absolute methanol containing 0.008% water[4].

Isonicotinic Acid—Isonicotinic acid[5], m.p. 310–315°.

1-Isonicotinyl-2-acetylhydrazine—Prepared by a slightly modified procedure of Fox and Gibas (9). To a stirred solution of 4 g. isoniazid in 25 ml. warm glacial acetic acid was added dropwise 3 ml. of acetic anhydride. The mixture was heated in a boiling water bath for 30 min., cooled, and evaporated under reduced pressure. Upon treatment of the residue with 35 ml. benzene, a white precipitate (4.5 g.) was formed. Recrystallization from isopropyl alcohol–ethyl acetate afforded fine needles, m.p. 161–162.5° [lit. (9) m.p. 162–163°].

[2] A Beckman Acta V UV-visible double-beam spectrophotometer (Beckman Instruments, Fullerton, Calif.), with 1-cm. Bausch and Lomb cells and a slit width of 0.36 mm., was used for the absorption measurements. A Blue M Magniwhirl model MW 1110A-1 constant-temperature water bath (Blue M Electric Co., Blue Island, Ill.) and a Mettler type H18 analytical balance (Mettler Instrument Corp., Princeton, N. J.), were also utilized in the assay procedure.

[3] Fisher Scientific Co., Pittsburgh, Pa.

[4] Matheson, Coleman and Bell, Norwood, Ohio.

[5] Aldrich Chemical Co., Milwaukee, Wis.

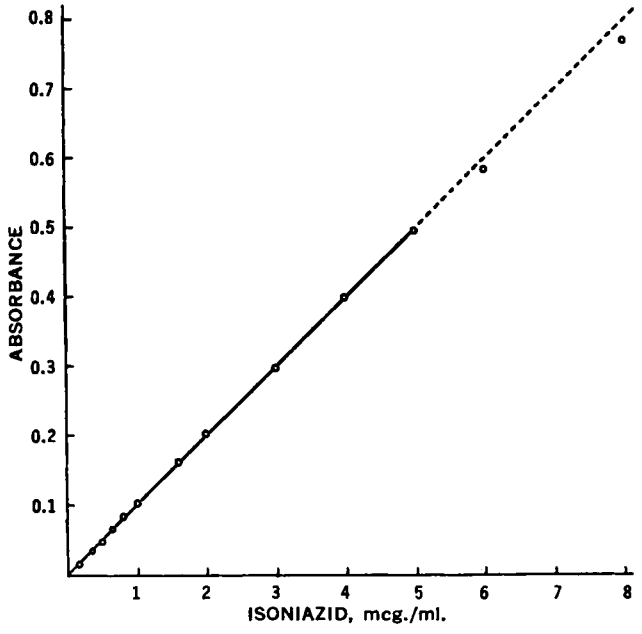


Figure 2—*Relationship between absorbance and concentration of isoniazid.*

Color Reagent—A 0.10% (w/v) solution of II (m.p. 97–99°)[6] in methanol. This reagent is stable for 2 weeks if kept under refrigeration.

Borax Solution—A 2.5% solution of sodium tetraborate decahydrate (ACS grade)[3] in methanol.

Preparation of Standard Curve—A stock solution of isoniazid was prepared by dissolving 50.0 mg. of isoniazid in 100 ml. of methanol. Further dilutions were made to obtain standard solutions, each containing 1.6, 2.0, 3.2, 4.0, 5.0, 6.4, 8.0, 10.0, 12.0, 16.0, 20.0, 30.0f 40.0, 50.0, 60.0, 80.0, and 100.0 mcg. isoniazid/ml. One milliliter or each solution was utilized for color development as described unde, *Assay Procedure for Isoniazid.* The stock and standard solutions must be freshly prepared.

Assay Procedure for Isoniazid—Pipet 1.0 ml. of a methanolic solution containing 3–50 mcg. of isoniazid into a 10-ml. glass-stoppered volumetric flask. To this solution, add successively 1 ml. of color reagent and 2 ml. of borax solution. Dilute the mixture to the mark with methanol. Stopper the flask tightly and place in a constant-temperature water bath at 60 ± 1° for 1 hr. Allow the mixture to cool to room temperature by immersing the flask in a tap-water bath (3–5 min.). Adjust the volume with methanol, if necessary, and measure the absorbance at 467 nm. against a blank prepared as already described but omitting the isoniazid. Obtain the amount of isoniazid present from a calibration curve.

Assay Procedure for Isoniazid Tablets—Weigh and finely powder 20 tablets. Weigh accurately a portion of powder, equivalent to about 100 mg. of isoniazid, and transfer to a 100-ml. volumetric flask. Add methanol to the mark and shake well for about 5 min. Filter the mixture quickly through a dry filter into a dry flask, rejecting the first 10 ml. of filtrate and keeping the funnel covered during the filtration. Pipet 1.0 ml. of the filtrate into a 50-ml. volumetric flask and make up to volume with methanol. Pipet 1.0 ml. of the resulting solution into a 10-ml. glass-stoppered volumetric flask and proceed as described in the *Assay Procedure for Isoniazid*, beginning with: "To this solution, add . . .."

Assay Procedure for Isoniazid Syrups—Transfer an accurately measured volume of syrup, equivalent to about 100 mg. of isoniazid, to a 100-ml. volumetric flask. Add methanol to the mark and shake well for about 1 min. Pipet 1.0 ml. of the resulting solution into a 50-ml. volumetric flask and make up to volume with methanol. Pipet 1.0 ml. of the latter solution into a 10-ml. glass-stoppered volumetric flask and proceed as described in the *Assay Procedure for Isoniazid*, beginning with: "To this solution, add . . .."

Effect of Reagents on Color Formation—*Color Reagent*—A series of 10-ml. glass-stoppered volumetric flasks, each containing 1.0 ml. of a methanolic solution of isoniazid (20 mcg./ml.), was prepared. Varying volumes of 0.05% II in methanol were added to each flask.

Table I—Assay of Solutions of Known Concentrations of Isoniazid at Different Levels

Amount Weighed, mg.	Analyzed at Concentration Level, mcg./ml.	Amount Found, mg.		Percent Recovery		
		Proposed Method[a]	USP XVIII Method	Proposed Method		USP XVIII Method
				Individual Samples	Average	
50.0	0.32	49.5		99.1	100.4	
	0.64	50.8		101.6		
50.0	0.64	50.8		101.6	101.4	
	1.6	50.6		101.2		
100.1	0.5	99.0		98.9	100.4	
	1.0	102.0		101.9		
	4.0	100.6		100.5		
100.0	0.5	100.0		100.0	100.0	
	4.0	100.9		100.9		
	5.0	99.2		99.2		
199.9	1.0	196.0		98.0	98.8	
	2.0	198.0		99.0		
	4.0	198.5		99.3		
200.0	1.0	202.0		101.0	100.9	
	2.0	203.0		101.5		
	4.0	200.0		100.0		
98.9			98.2			99.3
100.2			99.5			99.3
Overall percent recovery					100.3	
Standard deviation					0.88	

[a] Each value is the average of three replicate assays.

After 2 ml. of borax solution was added to each flask and the mixture was diluted to the mark with methanol, the flasks were warmed at 60 ± 1° for 1 hr. The flasks were cooled to room temperature, the volume of the solution was adjusted, if necessary, and the absorbance was measured at 467 nm. against a blank[6].

Borax Solution—A series of 10-ml. glass-stoppered volumetric flasks, each containing 1.0 ml. of a methanolic solution of isoniazid (20 mcg./ml.) and 1 ml. of color reagent, was prepared. Varying volumes of 2.5% borax solution were added to each flask, and the experiment was completed as described for the color reagent. The absorbance of each solution was measured at 467 nm. against a blank[6].

Effect of Isonicotinic Acid and Acetylisoniazid on Assay Results of Isoniazid—Two solutions of isonicotinic acid in methanol containing 20 and 40 mcg./ml., respectively, were prepared. Two solutions of acetylisoniazid in methanol containing 20 and 40 mcg. acetylisoniazid/ml., respectively, were also prepared. One milliliter of each solution was assayed as described under *Assay Procedure for Isoniazid.*

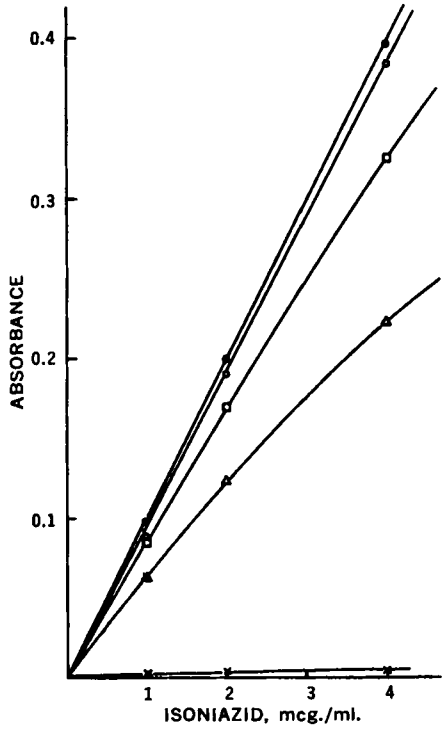


Figure 3—*Effect of heating on color development. Key: ×, 30 min. at room temperature; △, 30 min. at 50°; □, 60 min. at 50°; ○, 30 min. at 60°; and ●, 60 min. at 60°.*

[6] Each blank contained the same amount of II and sodium tetraborate as in the solution studied but contained no isoniazid.

RESULTS AND DISCUSSION

The orange-colored product obtained from the interaction between isoniazid and II in alkaline medium shows an absorption peak at 467 nm. in its absorption spectrum (Fig. 1). At concentrations above 3 mcg. isoniazid/ml., another peak appears around 445 nm. This absorption maximum shifts gradually toward lower wavelengths as the concentration of isoniazid is increased. As might be expected, absorbances measured at this second peak are not proportional to the concentrations of isoniazid. At 467 nm., however, the procedure as described shows adherence to the Beer–Lambert law up to 5 mcg. isoniazid/ml. A typical standard curve is shown in Fig. 2. Under the experimental conditions described in the assay procedure, the molar absorptivity, ϵ, is 13,715 l. mole^{-1} cm.$^{-1}$. Since isoniazid has a molecular weight of 137.15, an absorbance value of 0.100 corresponds to a concentration of 1 mcg./ml.

The interaction between isoniazid and II in the presence of sodium tetraborate in methanol was favored by heating. The time and tem-

Table II—Reproducibility of Color Development of Replicate Isoniazid Samples Containing 2 mcg./ml.

Solution	Absorbance at 467 nm.
1	0.200
2	0.197
3	0.202
4	0.200
5	0.201
6	0.202
7	0.202
8	0.200
9	0.198
Average	0.200
Standard deviation	0.0017
Coefficient of variation	0.87%

Table III—Assay of Isoniazid Tablets and Syrups[a]

Dosage Form	Manufacturer	Claim, mg./Tablet or mg./ml.	Found mg./Tablet or mg./ml.		Percent of Claim	
			Proposed Method	USP XVIII Method	Proposed Method	USP XVIII Method
Tablet	A	100	97.9	99.4	97.9	99.4
Tablet	B	100	98.3	98.9	98.3	98.9
Tablet	C	300	287.4	289.7	95.8	96.6
Syrup	D	10	9.81	9.97	98.1	99.7
Syrup[b]	—	10	9.82	9.79	98.2	97.9

[a] Tablets: average of duplicate assays. Syrups: average of triplicate assays. [b] Isoniazid, 1.00 g.; water (40°), 3 ml.; and sufficient syrup USP to make 100 ml.

perature of heating affected the color formation. At room temperature, hardly any color was formed (Fig. 3). Since heating at 60° required about 60 min. to obtain satisfactory absorbance values, attempts were made to speed up the reaction time by increasing the reaction temperature. Heating at temperatures above the boiling point of methanol presented problems since screw-capped tubes must be used to prevent significant evaporation of the solvent. Furthermore, heating at these high temperatures caused a shift in the absorption maximum to 462.5 nm. At this wavelength, adherence to the Beer–Lambert law could not be observed. Therefore, 60° was chosen as the heating temperature.

The time of heating was determined by following the color development. Absorbance values increased through 70 min. and then decreased. It was found that a heating period of 1 hr. at 60 ± 1° was quite satisfactory. The color produced appeared to be stable for at least 90 min. after the heating. Under these conditions the reference blank showed a pale-yellow coloration.

Gosh and Whitehouse (10) reported the determination of certain amino acids with II in aqueous solutions. When using water or 95% ethanol as solvents for the assay of isoniazid with II, it was found that the color formation was greatly accelerated. However, variations in color intensity occurred with the use of these solvents. The presence of water in the system caused nonreproducibility of the results. In these cases, a deep-yellow color was obtained with the reference blank.

The effect of the concentrations of reagent and sodium tetraborate on color formation was also studied. Higher absorbance values were obtained with the increasing amount of II used. At the same time the reference blank also exhibited a more intense yellow color. This caused a decrease in the sensitivity of the proposed procedure and made the determination of isoniazid at the lower limits of the concentration range obeying the Beer–Lambert law less reliable. The presence of more than 5 mg. sodium tetraborate/ml. in the assay solution caused a decrease in the absorbance value. In view of these results, 1 ml. 0.10% II and 2 ml. 2.5% sodium tetraborate were selected as optimum concentrations for the assay.

The stability of the color reagent solution was evaluated by recording its UV spectrum (λ_{max} 335 nm.) over an extended period. Molar absorptivities remained unchanged for 14 days, indicating that the reagent preparation, when kept refrigerated, could be kept for 2 weeks without significant deterioration.

As little as 0.16 mcg. isoniazid/ml. can be detected by the proposed procedure. The method, however, is reliable only for concentrations above 0.2 mcg./ml. The percent recoveries of known samples analyzed at 0.16- and 0.20-mcg. isoniazid/ml. levels were only 87.5 and 95.0%, respectively, with relative standard deviations from the mean of 38.5 and 3.5%, respectively. Very good recovery data were obtained for the concentration levels of 0.3–5 mcg./ml. The recovery experiments were performed by dissolving an accurately weighed amount of about 50 mg. isoniazid in 100 ml. methanol. Appropriate dilutions were then prepared to afford different concentrations (Table I) which were assayed by the proposed method. The recovery experiments were also conducted on 100 and 200 mg. isoniazid. Data presented in Table I indicate the accuracy of the method; the results were reproducible and quantitative. It was found that slight changes in absorbance reading can result in fairly substantial changes in percent recovery. The overall percent recovery for the six samples was 100.3%, with a standard deviation of ± 0.88%. In comparison, the average percent recovery for two samples analyzed by the USP XVIII method was 99.3%.

The precision of the analytical method was determined by running replication studies on nine 1.00-ml. aliquots of a standard isoniazid solution containing 2.0 mcg./ml. These solutions were assayed by the proposed procedure. The coefficient of variation for the nine replicate samples was 0.87% (Table II).

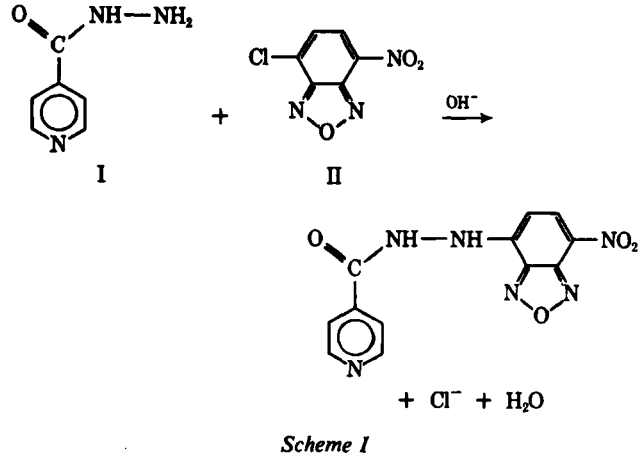
Results obtained by applying the assay procedure to commercially available isoniazid tablets are presented in Table III. Comparison of the experimental data with those obtained by the official method shows a relatively good correlation. The proposed extraction of the drug by shaking the tablet mass with methanol at room temperature for 5 min. appeared to be complete. In contrast, the official method requires heating on a steam bath for 30 min. Generally speaking, the present method offers ease and speed over the official method.

Table III also shows that the proposed colorimetric procedure is applicable for the assay of isoniazid syrups. The results of the proposed method are in agreement with those obtained by the USP XVIII procedure. Since the amount of water in the final assay solution is very low due to the two-step dilution procedure, it does not interfere with the reaction. Commercial Syrup D (Table III) also contained a flavoring agent and a yellow dyestuff. It appeared that these agents do not affect the absorbance measurements.

Several workers (6, 11) have suggested that, analogous to the König reaction, 1-chloro- or 1-fluoro-2,4-dinitrobenzene reacts with the pyridine nitrogen of isoniazid to form a polymethine dyestuff. The structurally related II may be expected to react with isoniazid in a similar fashion. Present studies revealed that neither acetylisoniazid nor isonicotinic acid produced any color with II under the experimental conditions described (Table IV). This would indicate that a free amino function in the molecule is necessary for the reaction with II. These findings are in agreement with those of Poole and Meyer (7), who stated that the reactivity of 1-fluoro-2,4-dinitrobenzene with the pyridine nitrogen of isoniazid is not probable.

Table IV—Effect of Acetylisoniazid and Isonicotinic Acid on Assay Results of Isoniazid

Compound	Concentration Assayed, mcg./ml.	Absorbance at 467 nm.
Acetylisoniazid	2.0	0.000
Acetylisoniazid	4.0	0.000
Isonicotinic acid	2.0	0.001
Isonicotinic acid	4.0	0.000



Scheme I

Therefore, it is concluded that the reaction between isoniazid and II under the experimental conditions of the assay method likely proceeds as depicted in Scheme I.

The present method can be modified to make it applicable to the determination of *free* isoniazid in biological fluids since none of the most important metabolites of isoniazid that have been identified (acetylisoniazid, isonicotinic acid, isonicotinylglycine, pyruvic acid isonicotinylhydrazine, and α-oxoglutaric acid isonicotinylhydrazine) (12) possesses a free amino function in the molecule. Further studies are in progress, and the results will be reported at a later date.

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SERGIO PINZAUTI▲, VITTORIO DAL PIAZ, and ENZO LA PORTA

Abstract □ A simple and accurate potentiometric titration procedure for the analysis of five antithyroid drugs is described. The drugs dissolved in aqueous solution at particular pH values are titrated with 0.01 *M* mercuric acetate. The end-point is determined potentiometrically by the use of three different indicating electrodes (mercury drop, amalgamated gold, and silver). Quantitative recoveries are reported.

Keyphrases □ Thyroid inhibitors—analysis, potentiometric titration with mercuric acetate □ Methimazole—analysis, potentiometric titration with mercuric acetate □ Thiobarbituric acid—analysis, potentiometric titration with mercuric acetate □ Thiouracil—analysis, potentiometric titration with mercuric acetate □ Methylthiouracil—analysis, potentiometric titration with mercuric acetate □ Propylthiouracil—analysis, potentiometric titration with mercuric acetate □ Potentiometric titrimetry—analysis of five thyroid inhibitors using mercuric acetate □ Mercuric acetate—used in potentiometric titration of five thyroid inhibitors

Methimazole (1-methylimidazole-2-thiol), 2-thiobarbituric acid, 2-thiouracil, 6-methyl-2-thiouracil, and 6-propyl-2-thiouracil are commonly employed in treatment of hyperthyroidism. Many types of titrimetric procedures for their determination have been proposed (1–15). The mercurimetric method of Abbot (4) was the basis in the BP monographs (16) for the determination of 6-methyl-2-thiouracil and 6-propyl-2-thiouracil. Accordingly, thiouracil (0.35 g.) is dissolved in an excess of sodium hydroxide and the resulting disodium salt is titrated in a buffered acetate system with mercuric acetate (0.05 *M*); the end-point is determined by the use of diphenylcarbazone as the indicator. Although satisfactory results are attainable by this method, the indicator change is not sharp and the procedure is not suitable for other antithyroid thiols, *e.g.*, methimazole.

In the present study, a simple, rapid, and accurate direct potentiometric titration is reported for the determination of methimazole USP, thiouracil, methylthiouracil BP, propylthiouracil USP and BP, and 2-thiobarbituric acid. The use of three different indicating electrodes is described.

EXPERIMENTAL

Apparatus—Titrations were performed potentiometrically with a pH meter[1] equipped with a mercury drop electrode (17) and mercurous sulfate electrode[2]. An amalgamated gold electrode (a gold flag dipped into metallic mercury for 20 sec.) or a silver electrode (a silver coil) was also employed as indicating electrodes. The mercury drop and amalgamated gold electrodes can be used for many titrations if rinsed with water between titrations.

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Borax (0.025 M)—Dissolve 9.535 g. of $Na_2B_4O_7 \cdot 10H_2O$ (reagent grade) in sufficient water to make 1000 ml.

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Table I—Results of Potentiometric Titration of Antithyroid Drugs with Mercuric Acetate and Three Different Indicating Electrodes

Drug	Weighed Range, mg.	Proposed Method, % Recovery with Mercury Drop Electrode	Proposed Method, % Recovery with Amalgamated Gold Electrode	Proposed Method, % Recovery with Silver Electrode	BP Method, % Recovery
Methimazole	20.20–57.05	99.90 ± 0.22[a]	99.78 ± 0.27[a]	99.69 ± 0.15[a]	—
Thiouracil	21.06–55.34	99.67 ± 0.36	99.58 ± 0.24	99.71 ± 0.38	—
Methylthiouracil	23.20–62.15	99.83 ± 0.38	99.70 ± 0.16	99.59 ± 0.33	99.26 ± 0.25[a]
Propylthiouracil	20.90–56.50	100.06 ± 0.33	100.03 ± 0.25	100.37 ± 0.18	99.20 ± 0.16
2-Thiobarbituric acid	24.84–67.56	100.14 ± 0.29	100.36 ± 0.32	99.93 ± 0.40	—

[a] Standard deviation based on six analyses.

grade) in 200 ml. of water; add 2.3 ml. of glacial acetic acid and sufficient water to make 400 ml.

Mercuric Acetate (0.01 M)—Dissolve 800 mg. of mercuric acetate (reagent grade) in 250 ml. of water, and add 0.3 ml. of glacial acetic acid to prevent hydrolysis. Titrate the solution potentiometrically with 0.01 *M* ethylenediaminetetraacetic acid in pH 4.6 acetate buffer, using a mercury drop–mercurous sulfate electrode system.

Procedure—*Assay of Methimazole*—About 20–60 mg. of methimazole, accurately weighed, was dissolved in 50 ml. of 0.025 *M* borax. The solution, magnetically stirred, was titrated potentiometrically with 0.01 *M* mercuric acetate. The end-point in the titration was determined from the inflection in the curve obtained by plotting milliliters of titrant *versus* millivolt readings. Each milliliter of 0.01 *M* mercuric acetate is equivalent to 2.283 mg. of methimazole.

Assay of Methylthiouracil and Propylthiouracil—About 20–60 mg. of the drug, accurately weighed, was dissolved in 50 ml. of pH 7.2 phosphate buffer, with warming to complete solution. After cooling to room temperature, titration was performed in the same way (as for methimazole). Each milliliter of 0.01 *M* mercuric acetate is equivalent to 2.844 mg. of methylthiouracil or 3.405 mg. of propylthiouracil.

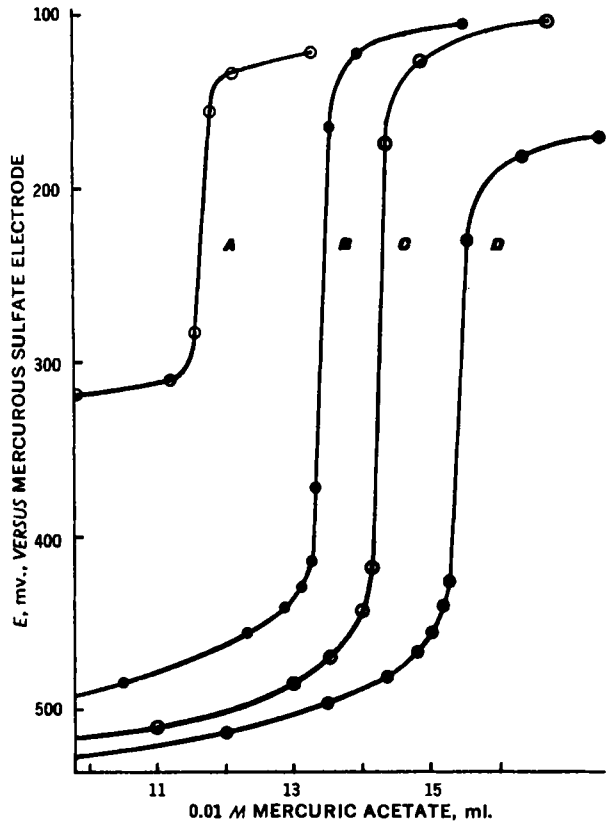

Figure 1—*Typical titration curves for antithyroid drugs. Key: A, 2-thiobarbituric acid, 33.82 mg. (silver electrode); B, methylthiouracil, 38.25 mg. (silver electrode); C, propylthiouracil, 48.50 mg. (amalgamated gold electrode); and D, methimazole, 35.46 mg. (mercury drop electrode).*

Assay of Thiouracil—About 20–60 mg. of the drug, accurately weighed, was dissolved in 50 ml. of pH 4.6 acetate buffer, with warming to complete solution. After cooling to room temperature, titration was performed as already described. Each milliliter of 0.01 *M* mercuric acetate is equivalent to 2.563 mg. of thiouracil.

Assay of 2-Thiobarbituric Acid—About 20–60 mg. of the drug, accurately weighed, was dissolved in 50 ml. of water. The solution was adjusted to pH 1 with nitric acid (20% w/w), and titration was performed as already described. Each milliliter of 0.01 *M* mercuric acetate is equivalent to 2.883 mg. of 2-thiobarbituric acid.

RESULTS AND DISCUSSION

Table I shows the results of mercurimetric determination of five antithyroid agents with potentiometric end-point detection. The recoveries by the proposed method are satisfactory, and only small amounts of drug are required for analysis. A mercury drop electrode, an amalgamated gold electrode, and a silver electrode were used with success as pHg indicators for the potentiometric titration. The silver electrode behaves as reversible with respect to the anion RS^-.

For the analysis of methylthiouracil, propylthiouracil, and methimazole, the largest change in potential at the end-point [about 200 mv./0.1 ml. Hg^{+2} (0.01 *M*)] was observed using the amalgamated gold electrode (Fig. 1). However, the sharp rise produced with the mercury drop and silver electrodes was also sufficiently steep (about 160 mv./0.1 ml. of titrant). In the titration of thiouracil, a lower rise in the potential was noted with each electrode used (about 90 mv./0.1 ml. of titrant). In the assay of 2-thiobarbituric acid, the best results were obtained with the silver electrode (about 160 mv./0.2 ml. of titrant), but the inflections observed with the mercury drop or amalgamated gold electrode (about 100 mv./0.2 ml. of titrant) were also analytically useful.

Antithyroid thiols react with mercuric acetate to form very slightly soluble sulfides. Two moles of the sulfido form (RS^-) of the thiol reacts with 1 mole of the mercuric titrant. The quantitative presence of the sulfido form is pH dependent (20). In preliminary experiments for methimazole in the pH 4–6.8 region, no detectable end-point was apparent. The best results for the assay of methimazole were attained at pH 8.2–9.4, using borax dilute solutions. Methylthiouracil and propylthiouracil gave the most satisfactory titraton curves at pH 7.2–8 (phosphate buffer). The greater sensitivity in end-point detection was noted for 2-thiouracil at pH 4.6 and for 2-thiobarbituric acid in the pH range of 1–2.2.

The potential of the mercury electrode depends on pH by formation of mercuric oxide (18) (the electrode reaction may be written as: $Hg + 2OH^- \leftrightharpoons HgO + H_2O + 2e^-$). In the case of methylthiouracil, propylthiouracil, and methimazole, the solubility products of the precipitate sulfides are evidently lower than HgO ($pSP_{HgO} = 25.5$) (19), permitting sharp breaks in the titration curves under alkaline conditions.

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Determination of Dopa in Pharmaceutical Dosage Forms Based on Oxidation at Tubular Carbon Electrode

WILLIAM D. MASON

Abstract □ A method for the determination of dopa [3-(3,4-dihydroxyphenyl)alanine] in pharmaceutical dosage forms based on electrochemical oxidation at the tubular carbon electrode is presented. A comparison with a colorimetric method shows this new method to be considerably faster and simpler without a significant loss in precision or accuracy. Between 25 and 30 samples may be determined each hour, for which the variance due to the method results in a standard deviation no greater than ±0.9%.

Keyphrases □ Dopa—analysis in dosage forms, electrochemical oxidation at tubular carbon electrode □ Electrochemical analysis—dopa in dosage forms, tubular carbon electrode

The problems associated with the fluorometric or colorimetric determination of levodopa [(−)-3-(3,4-dihydroxyphenyl)-L-alanine] in pharmaceutical dosage forms were recently summarized (1). Although highly sensitive and specific, these methods are inconvenient due to lengthy procedures involving separation and other manipulative steps. A new colorimetric method by Maggi and Cometti (1), designed specifically for the determination of levodopa in dosage forms, eliminates separatory steps but requires a number of manipulations including a precisely controlled reaction for color formation. A possible alternative to the colorimetric approach may be an electrochemical method similar to the one presented for ascorbic acid (2).

The method for ascorbic acid is based on continuous analysis in flowing streams by oxidation of the drug at the tubular carbon electrode (3). This electrochemical method has advantages over other commonly employed methods of being extremely fast and simple without any significant loss in accuracy or precision. Studies (4) of the electrochemical oxidation of catecholamines, similar in structure to levodopa, suggested that the drug may be assayed in this manner. Thus, an investigation of the oxidation of levodopa at the tubular carbon electrode was undertaken with the idea of developing an analytical method. In this paper a method for the determination of dopa in pharmaceutical dosage forms is presented and compared to the colorimetric method of Maggi and Cometti (1). In addition, the utility of the new electrochemical method in continuously monitoring the dissolution of solid dosage forms is demonstrated.

EXPERIMENTAL

Instrumentation—The electrode assembly, flow system, pump, and polarography system were the same as reported earlier (2). The tablet dissolution apparatus was described by Levy and Hayes (5).

Chemicals—All chemicals were of the highest quality commercially available. Standard solutions of dopa were prepared using the powder[1].

Procedures—Current–voltage curves were determined using a tubular carbon electrode (TCE) which had previously been cleaned with ethyl acetate. Volume flow rates between 4 and 10 ml./min. were commonly employed and were controlled to ±1%. The voltage was scanned anodically from 0 v. *versus* the saturated calomel electrode (SCE) at 0.20 v./min. Standard buffers (6) were used to determine the effect of pH on the oxidation half-wave potential of the compounds studied.

Solid dosage forms were assayed by dissolving weighed quantities in sufficient 0.1 *N* HCl to give approximately 10^{-4} *M* dopa solutions. The sample solutions were pumped through a pledget of glass wool to the TCE (with the potential set at 0.90 v. *versus* the SCE) and the limiting currents were recorded. A calibration plot of the limiting current *versus* concentration was determined daily, and intermittent standards were run to check and adjust the calibration.

The accuracy of the TCE method was evaluated by comparison with a colorimetric method (1). Three tablets were weighed and powdered, and weighed aliquots were taken for analysis; the capsules were emptied and weighed, and similar aliquots were taken. The sample aliquots were dissolved in 1 ml. of 1 *N* HCl and diluted to 1 l. with water; then each aliquot was assayed by the colorimetric method three times. These same aliquots were then

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Table I—Current–Voltage Scans of Possible Interfering Substances

Compound	Half-Wave Potential[a] ±0.005 v. *versus* SCE
Levodopa	0.250
3-Amino-L-tyrosine	0.230
Hydroquinone	0.135
Resorcinol	N.W.[b]
Tryptophane	N.W.
Tyrosine	N.W.

[a] Determined at pH 6.0, and subject to slight variance due to electrode history. [b] No wave (N.W.) before 0.60 v. *versus* the SCE.

Table II—Dopa Analysis

Dosage Form	Amount Declared per Sample, mg.	—Mean Amount, mg. ± *SD*[a]— TCE[b] Method	Colorimetric Method[c]
Tablet (250 mg.)	20.1	20.1 ± 0.1	20.1 ± 0.1
	42.7	42.8 ± 0.2	42.6 ± 0.1
Capsule (250 mg.)	20.0	20.1 ± 0.1	20.1 ± 0.1
	38.9	38.7 ± 0.2	39.0 ± 0.2

[a] Calculated on the basis of three assays of a single aliquot. [b] With 0.1 *N* HCl as medium. [c] *Reference 1.*

diluted with 0.1 *N* HCl to about 10^{-4} *M* and assayed by the TCE method.

For the dissolution study the beaker was filled to 1 l. with 0.1 *N* HCl and the propeller was set at 60 r.p.m. The solution was pumped through a Swinnex-25 unit[2] without a filter to the TCE and returned to the beaker. After setting the electrode potential at +0.90 v. *versus* the SCE and establishing a baseline current, a tablet was added and the limiting current was recorded as a function of time. From a previously determined calibration plot, the chart paper was labeled in terms of percent dissolution (taking the plateau current to represent 100%) as well as microamperes.

RESULTS AND DISCUSSION

A typical current–voltage curve for the oxidation of levodopa at the TCE is shown in Fig. 1. The limiting current was linear with respect to concentration over the 10^{-6}–10^{-3} *M* range. With the volume flow rate at 5 ml./min., a typical calibration plot has a slope of 6.9 μa./10^{-4} *M* with a zero intercept. A plot of the logarithm limiting current *versus* logarithm flow rate had a slope of 0.33,

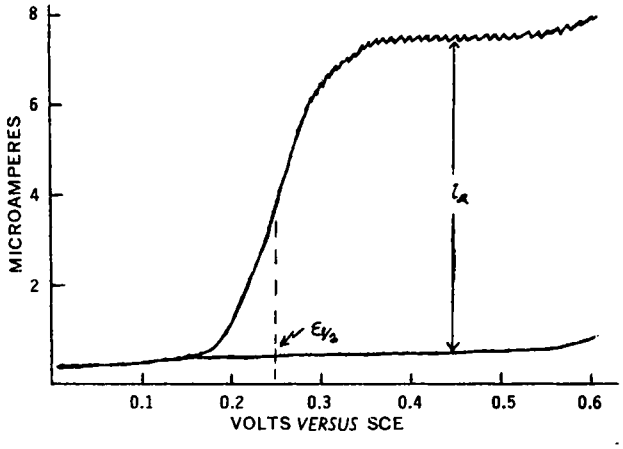

Figure 1—*Levodopa current–voltage curve; run at pH 6. (See text for details.)*

[2] Millipore Corp., Bedford, Mass.

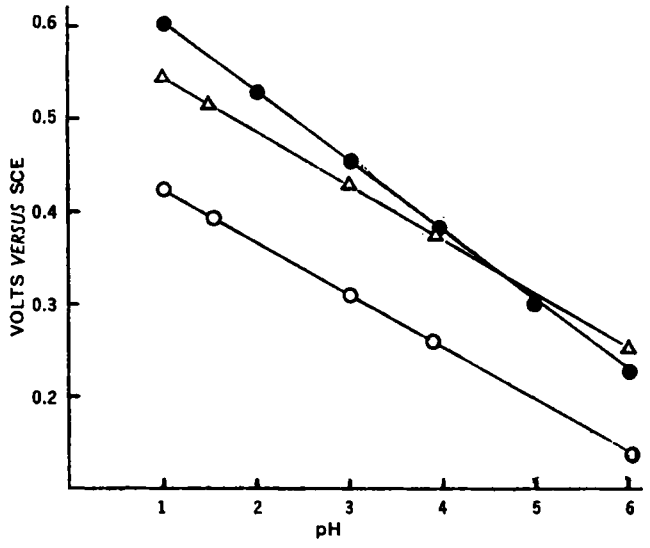

Figure 2—*Half-wave potential as a function of pH. Key:* ○, *hydroquinone;* △, *levodopa; and* ●, *3-amino-L-tyrosine.*

which agrees with theoretical considerations of the tubular electrode geometry (7).

Current–voltage scans on 10^{-4} *M* solutions of the compounds listed in Table I show only 3-amino-L-tyrosine to have a half-wave potential ($E_{1/2}$) close to that for levodopa. The other compounds listed, all of which have been suggested as possible interferences formed during synthesis of levodopa, have half-wave potentials significantly different from that of dopa or do not show an oxidation wave. From Fig. 2 it is evident, within the limits of experimental error, that the half-wave potentials for dopa and hydroquinone fit the relationship $E_{1/2} = E° - 0.059$ pH, while the pH dependence of the 3-amino-L-tyrosine oxidation is greater. Indeed, in 1.5 *M* H_2SO_4 the half-wave potential for levodopa is 0.73 v. *versus* the SCE while the oxidation of 3-amino-L-tyrosine occurs about 0.10 v. more anodic. Although a more in-depth study was not attempted, it is surmised that the oxidation of 3-amino-L-tyrosine is suppressed by protonation of the amino group on the benzene ring. Thus, samples suspected of containing this impurity may be assayed in strong acid media. Current–voltage curves for dopamine and norepinephrine were observed to have essentially the same half-wave potential as dopa. If these compounds or other 3,4-dihydroxyphenylethylamine derivatives are considered as possible contaminants in a sample, a separation procedure must precede analysis at the TCE.

The accuracy of the TCE method in determining the dopa content of solid dosage forms is shown in Table II. It is evident that the new method gives data in agreement with the colorimetric method

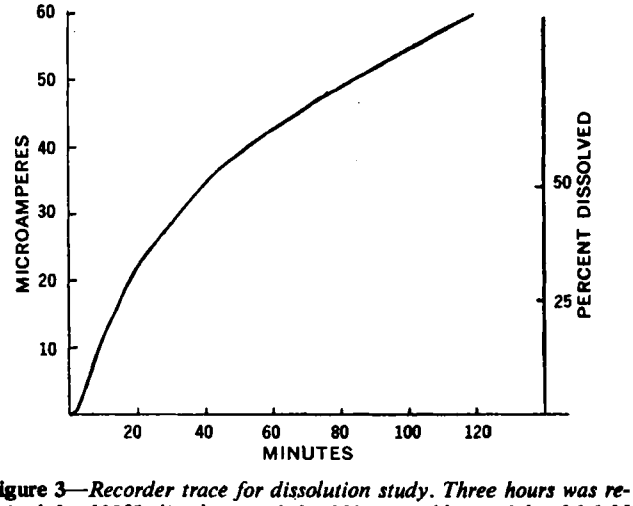

Figure 3—*Recorder trace for dissolution study. Three hours was required for 100% dissolution of the 250-mg. tablet in 1 l. of 0.1* N *HCl.*

and that comparable precision is obtained. In comparison with the colorimetric method, the TCE method is faster and simpler. Once the sample solutions were prepared, between 25 and 30 determinations could be run each hour. The reproducibility of the TCE, as determined by repeatedly assaying a single sample dilution, is indicated by a deviation of the mean ±0.90% for six determinations.

Figure 3 shows the current–time curve for the dissolution study. Once the tablet was placed in the beaker, no further manipulative steps were required to record continuously the tablet dissolution. Although not included here, the dissolution could be determined as a function of pH using the same analytical procedure.

In summary, it is concluded that the electrochemical method for the determination of dopa presented in this paper is more convenient, faster, and simpler to use than previously available methods without any significant loss in precision and accuracy. Although automation was not employed in this study, the method may readily be incorporated into automated or semiautomated systems because it employs continuous analysis on a flowing stream of sample. And, finally, the applicability of the analytical method in the direct and continuous monitoring of tablet dissolution is demonstrated.

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PHARMACEUTICAL TECHNOLOGY

Soft Gelatin Capsules I: Factors Affecting Capsule Shell Dissolution Rate

F. S. HOM▲, S. A. VERESH, and J. J. MISKEL

Abstract □ A method to study the relationship between various factors influencing the dissolution rate of the soft gelatin capsule shell is reported. The gelatin disk method makes use of the current USP rotating-basket dissolution apparatus. The effects of agitation, temperature, dissolution medium, and shell composition on the capsule shell dissolution rate are illustrated and discussed. A knowledge of these factors and their influence on dissolution should be of assistance in the formulation of soft gelatin capsule shells for various purposes.

Keyphrases □ Dissolution rate of soft gelatin capsules—effects of agitation, temperature, dissolution medium, and shell composition □ Capsules, soft gelatin—effects of agitation, temperature, dissolution medium, and shell composition on dissolution rate □ Gelatin capsules, soft—effects of agitation, temperature, dissolution medium, and shell composition on dissolution rate

Although soft gelatin capsules have been in mass production (1) since the introduction of the rotary die process (2), little or no work has been reported on the relationship between formulation design and dissolution rate of the capsule shell. Nevertheless, several formulations designed for specific uses have been patented (3–8) in this country. Recently, studies on comparisons of dissolution rates of drugs from soft gelatin capsules and tablets were reported (9, 10). The present report deals with a method to study the relationship between various factors influencing the dissolution rate of the soft gelatin capsule shell.

In the design and formulation of soft gelatin capsule dosage forms, one should consider the effects of components and other parameters on the dissolution rate of the capsule shell. A method that is simple, fast, able to differentiate minor but meaningful changes in dissolution rate, and readily reproducible is of great utility. Eckert *et al.* (11), concerned with drug availability, advocated the use of the *in vitro* initial-release rate to reflect this factor in the monitoring of soft gelatin capsule manufacture. The initial-release rate is adequate for such a purpose, but it lacks the sensitivity and simplicity based on the criteria of the present proposal. A more appropriate method was developed in this laboratory.

Many factors that influence the dissolution rate of the soft gelatin capsule shell may be adequately studied by the use of the Nelson (12) modification of the Noyes–Whitney equation. Using the Noyes–Whitney equation, one may write:

$$\frac{dW}{dt} = KS(C_s - C) \qquad \text{(Eq. 1)}$$

where W is the amount dissolved, t is the time, K is the solution rate constant with dimensions of distance/

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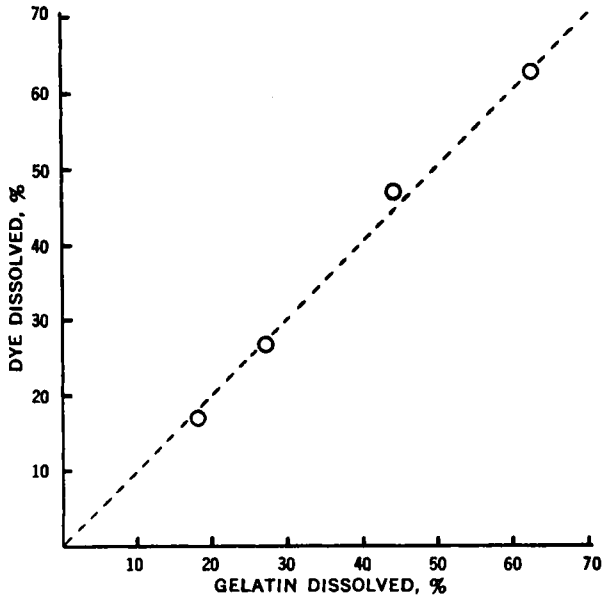

Figure 1—*Plot showing the correlation of the percent dye and gelatin dissolved in simulated gastric fluid without pepsin at 37° and 100 r.p.m. for a given dissolution time in the gelatin disk method. The dashed line is drawn for a theoretically perfect correlation.*

time, S is the surface area of the dissolving solid, C_s is the concentration of dissolving solid at the liquid–solid interface, and C is the concentration in the dissolution medium. At very low percent saturations of dissolving substance in dissolution medium, Nelson (12) showed that the C term may be ignored so Eq. 1 reduces to:

$$\frac{dW}{dt} = KSC_s \qquad \text{(Eq. 2)}$$

Integration, with S considered a constant, yields:

$$W = KSC_s t \qquad \text{(Eq. 3)}$$

By dividing both sides of Eq. 3 with S and setting $KC_s = k$, one then has:

$$\frac{W}{S} = kt \qquad \text{(Eq. 4)}$$

A plot of W/S *versus* t yields a straight line going through the origin. The slope of the line equals k. Equation 4 is valid for initial dissolution rates and at dilute solutions, which is the case in the beginning of each dissolution experiment.

EXPERIMENTAL

Materials—Gelatins used were Type A USP and Type B USP, with gel strength in Bloom grams of 264 ± 4[1], 195 ± 3[2], 27 ± 1[3], and 77 ± 2. The last was a blend of a higher and of a lower gel strength gelatin. The gelatins were sieved to obtain a particle size between U. S. Standard 20- and 40-mesh screens. The following were also used: glycerin USP[4], sorbitol solution USP[5], hexaglycerol[6], FD&C certified Blue No. 1[7], lysine hydrochloride[8], and analytical reagent grade urea[9] and chemicals. Commercial grades of additives were used where reagent grades were not available.

[1] Kind and Knox Gelatin Co.
[2] Rousselot Corp.
[3] B. Young and Co. of America, Ltd.
[4] Dow Chemical Co.
[5] Atlas Chemical Ind.
[6] Stokely-Van Camp.
[7] Hilton-Davis Chemical Co.
[8] Merck and Co.
[9] Mallinckrodt Chemical Works.

Table I—Repetitive Determinations of Dissolution Rate Constants of Gelatin A and B Type Soft Gelatin Capsule Shell Samples in Simulated Gastric Fluid T.S. without Pepsin at 37° and 100 r.p.m.

	Gelatin Type A Shell, mg./cm.² min.	Gelatin Type B Shell, mg./cm.² min.
	1.83	2.74
	2.07	2.76
	2.03	2.65
	1.98	2.81
	2.09	2.84
Average ± *SD*	2.00 ± 0.12	2.76 ± 0.08

Gel Strength—Gelatin gel strengths, measured in Bloom grams by the use of a Bloom gelometer (13), are averages of duplicate runs.

Dissolution Medium—The aqueous solution pH values were as follows: pH 1.2 simulated gastric fluid T.S. with or without pepsin (14), pH 1.2–2.0 USP standard buffer solutions (14), pH 7.5 simulated intestinal fluid T.S. without pancreatin (14), pH 2.2–5.0 McIlvaine's standard buffer solution (15), and pH 9.9 carbonate buffer solution (16).

Preparation of Sample—Soft gelatin capsule shell disks, containing about 0.25% on dry basis of FD&C Blue No. 1 with either Type A or B gelatin and varying amounts of plasticizer such as glycerin, may be made in the following manner. As an example, mix 60.25 g. gelatin with 31.50 g. glycerin in a 500-ml. filtering flask fitted with a stopcock. Add 33 ml. cold, aqueous solution of 250 mg. FD&C Blue No. 1 dye and additive, if any, and mix well. Reduce pressure of system to deaerate contents, and close off stopcock to maintain a reduced pressure. Heat system on a steam bath to dissolve contents and to obtain a flowable mass. Mix well by swirling flask contents without creating bubbles in the molten mass. Vent flask and pour flask contents on a clean glass plate. Immediately level mass with a constant-thickness doctor blade. The formed film should be homogeneous and free of bubbles. Allow film to set at room temperature for 1 hr., and then mount film between two plastic brackets to keep it flat along edges. Allow film to equilibrate for at least 2 weeks in a desiccator containing solid calcium chloride in equilibrium with a saturated solution of calcium chloride. Just before a dissolution run, cut a disk of the gelatin film with a No. 13

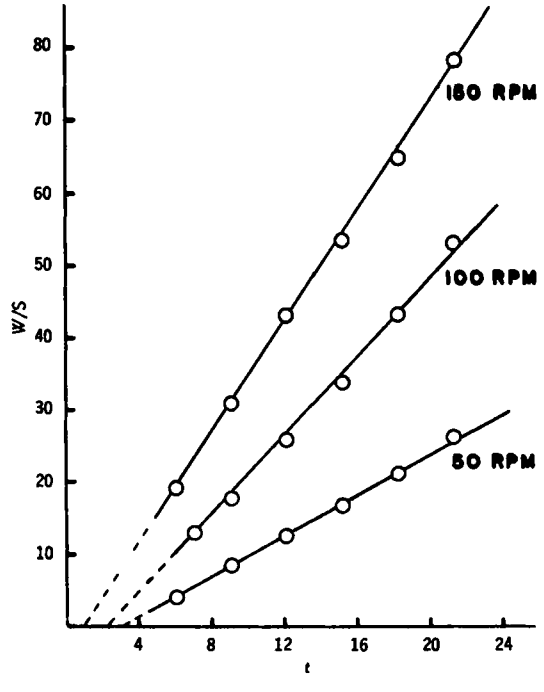

Figure 2—*Plot of the amount dissolved (mg./cm.²) versus time (minutes), showing the dissolution of soft capsule shells made from a Type B gelatin in simulated gastric fluid at 37° and three rates of agitation.*

Table II—Salt Effects on the Dissolution Rate Constants of Gelatin A and B Type Soft Gelatin Capsule Shells at 37° and 100 r.p.m.

Sodium Chloride, *M*	pH 1.2	
	Gelatin Type A, mg./cm.² min.	Gelatin Type B, mg./cm.² min.
0.00	2.09	—
0.034	2.00	2.76
0.171	1.46	2.55
0.342	1.00	2.48

Potassium Chloride, *M*	pH 1.2	pH 5.0	
	Gelatin Type B, mg./cm.² min.	Gelatin Type A, mg./cm.² min.	Gelatin Type B, mg./cm.² min.
0.00	2.78	1.08	1.45
0.05	2.84	—	—
0.15	2.39	1.24	1.38
0.30	2.35	1.13	1.44

cork borer. Determine the weight of the disk on an analytical balance, and calculate the surface area from measurements of diameter and thickness.

Dissolution Apparatus—The USP XVIII rotating-basket dissolution apparatus (14) is used. A modification of this method was described recently (17) which facilitates faster determinations of dissolution rates. Throughout this study, the temperature of the dissolution medium was kept constant at the indicated temperature ±0.1° by use of a constant-temperature circulator[10].

Dissolution Procedure—Place a weighed soft gelatin disk sample flat on the bottom of the stainless steel basket, and secure with fine stainless steel wires inserted crosswise immediately above the sample. Assemble to stirring motor and raise dissolution vessel to predetermined height by use of a laboratory jack. The temperature of the vessel and its contents is kept constant at the desired temperature ±0.1°. Add 500 ml. simulated gastric fluid T.S. (with or without pepsin as the case may be) maintained at the same desired temperature. To start dissolution, turn on stirring motor set at a predetermined revolutions per minute. At suitable time intervals, determine the absorbance of the dissolution medium at 630 nm. *versus* water in a suitable spectrophometer. A 3-ml. aliquot may be withdrawn for this purpose. However, absorbance is best determined by continuous analysis in a spectrophotometer flow cell assembly. The amount of soft gelatin film dissolved in time t, W_t, is equal to that fraction of the disk dissolved in time t. One may write:

$$W_t = \frac{A_t M}{A_\infty} \qquad \text{(Eq. 5)}$$

where M is the weight of the plug in milligrams, and A_t and A_∞ are the absorbance values at 630 nm. for time t and infinity (completed dissolution), respectively. In this manner, the term W/S may be determined for corresponding values of time. The dissolution constant, k, is obtained from the slope of the straight line in a plot of W/S *versus* t and will have the dimensions of mg./cm.² min., where S is measured in cm.² and t in minutes. The results are averages of at least duplicate runs.

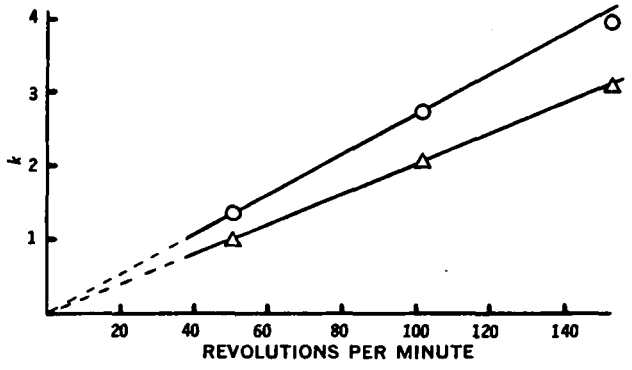

Figure 3—*Effect of agitation (r.p.m.) on the dissolution rate constant (mg./cm.² min.) of soft capsule shells made from gelatin Types A (Δ) and B (○) in simulated gastric fluid at 37°.*

[10] Bronwill model 20, VWR Scientific.

Table III—Effect of Pepsin in Simulated Gastric Fluid T.S. on the Dissolution Rate Constants of Slow Dissolving Type A Soft Gelatin Capsule Shell Samples at 37° and 100 r.p.m.

	2-Week-Old Sample, mg./cm.² min.	2-Year-Old Sample, mg./cm.² min.
With pepsin	2.22 ± 0.13	2.01 ± 0.13
Without pepsin	2.29 ± 0.08	1.50 ± 0.19

Validity of Method—The use of soft gelatin capsule shell disks in the method ensures that each test sample presents uniform and constant surface to the dissolving medium. The sample disk has the additional advantage in being relatively simple to prepare. Since it is difficult to prepare soft gelatin capsule shell films containing the same amount of water, the conditioning of the film in a constant relative humidity atmosphere before a dissolution rate determination is important because an unconditioned, freshly made sample has a relatively higher initial dissolution rate and this rate decreases to a constant value under controlled humidity storage condition. The incorporation of the water-soluble dye permits ready appraisal of dissolution of the gelatin. The relatively simple absorbance measurement of the dye in the dissolution medium renders the method amenable to the use of the present USP XVIII rotating-basket dissolution apparatus.

Apparently the water-soluble dye is distributed uniformly throughout the gelatin shell, and there is no indication that the dye diffuses from the matrix at a rate greater than the dissolution of gelatin. The diffusion of the dye out of the gelatin matrix is measurable only with nearly insoluble samples, where dissolution is extremely slow or at low temperatures and low agitations. Nevertheless, this rate is considerably less than normal dissolution and does not contribute significantly to the measurements discussed. Furthermore, visual observations indicate that the amount of dye dissolved is proportional to the amount of the gelatin mass dissolved. This was confirmed by analyses of both dye and gelatin dissolved for a given dissolution time. The percent gelatin dissolved was calculated from the relative amounts of nitrogen in solution as determined by a slightly modified USP XVIII Method II (14). The described dissolution procedure was used with the exception that 100 ml. instead of the 500 ml. of simulated gastric fluid made without pepsin was used. Figure 1 shows that the results fall on top of the theoretical, perfect correlation line.

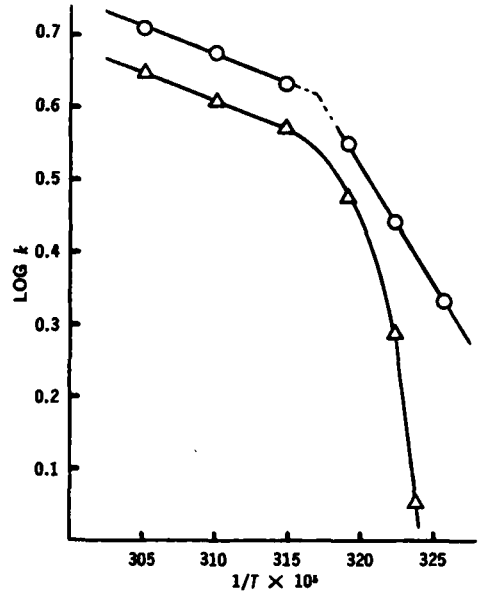

Figure 4—*Arrhenius plot showing the effect of temperature on the dissolution rate constant, k, of soft capsule shells made from gelatin types A (Δ) and B (○) in simulated gastric fluid at 100 r.p.m.*

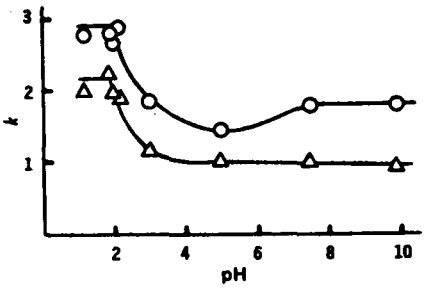

Figure 5—*Effect of pH of the medium on the dissolution rate constant (mg./cm.² min.) of soft capsule shells made from gelatin Types A (△) and B (○) at 100 r.p.m. and 37°.*

In separate experiments, the blue soft gelatin film, when dissolved in an acid dissolution medium at the concentrations of the dissolution experiments, obeyed Beer's law at the wavelength of 630 nm. The FD&C Blue No. 1 was chosen for its high absorptivity and water solubility. Although it is not the only dye that can be used in the method, the present report is limited to its use.

Gas bubbles clogging the dissolution basket wire screen were observed to slow the dissolution rate measurably, but this source of error was eliminated by the removal of dissolved gas. The addition of a small piece of stainless steel screen or a piece of boiling stone helped the dissolved gas to nucleate and to grow into larger gas bubbles. Gentle agitation under reduced pressure removed the gas bubbles. This treatment, before each dissolution rate determination, produced results that were reproducible and typical of those shown in Table I.

RESULTS AND DISCUSSION

Stirring Rate—Figure 2 shows plots of *W/S versus* time in minutes at 50, 100, and 150 r.p.m. of the rotating basket for gelatin Type B soft gelatin capsule shells in simulated gastric fluid T.S. without pepsin at 37°. It can be seen that the lines are straight, but they do not seem to extrapolate linearly to the origin as predicted by Eq. 4. These observed differences or apparent lag times may be explained by the inefficiency of mixing at lower stirring rates. This postulation is supported by the smaller apparent lag times at higher revolutions per minute values. However, these apparent initial lag times seem to have little or no effect on the values of the dissolution rate constants as shown in Fig. 3. The linear relationship as depicted in the plot of dissolution rate constant, *k*, *versus* revolutions per minute is in accord with a diffusion-controlled dissolution process (18). Both Figs. 2 and 3 show the importance of agitation on the dissolution rate of the soft gelatin capsule shell. Withey and Mainville (19) came to a similar conclusion after they tested a soft gelatin capsule sample.

Temperature—Because of the abilities of gelatin to form thermally reversible rigid gels and to hydrogen bond, temperature may be expected to have a marked effect on the dissolution rate of soft gelatin capsule shells. Indeed, this is the case, especially below about 42°, as shown by the Arrhenius plots in Fig. 4 of log *k versus* the reciprocal of the absolute temperature. In the regions of the curves below 42°, the activation energy varies from 71 to 15 kcal./mole for the gelatin Type A and Type B soft gelatin capsule shell. By contrast, the activation energy above 42° is relatively constant at 3.6–3.7 kcal./mole. Unfortunately, most of the dissolution rate determinations are at 37°. Consequently, the temperature of the medium during a dissolution rate determination must be kept precisely constant.

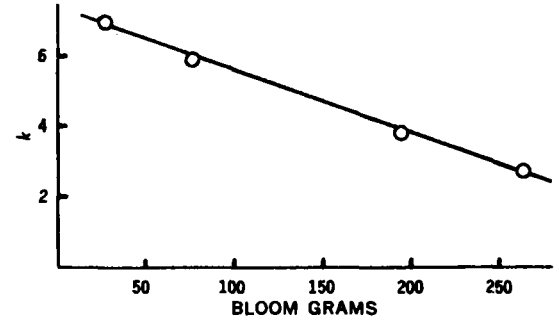

Figure 6—*Effect of gelatin gel strength in Bloom grams on the dissolution rate constant (mg./cm.² min.) of soft capsule shells made from Type B gelatins in simulated gastric fluid at 37° and 100 r.p.m.*

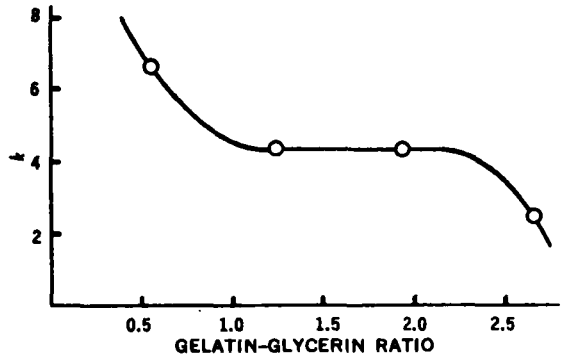

Figure 7—*Effect of gelatin-glycerin ratio in a capsule formulation on the dissolution rate constant (mg./cm.² min.) of soft capsule shells made from a Type B gelatin in simulated gastric fluid at 37° and 100 r.p.m.*

Dissolution Medium—The influence of pH of the medium on the dissolution rate constant for gelatin Type A and Type B soft gelatin capsule shells is shown in Fig. 5. The pH seems to have little effect above a value of 3 on dissolution rate constants of both types of soft gelatin capsule shells. Also, there appears to be a minimum at pH 5 for the Type B gelatin in the pH dissolution profile. The pH of the minimum seems to coincide with the isoelectric point (pH 4.7–5.1) of gelatin Type B. As the pH values decrease from 3 to 1, the dissolution rate constants seem to approach a higher plateau. This increase in dissolution rate may be due to the protonation of amino groups that have pKa values in this pH range.

Small amounts of salts such as sodium or potassium chloride may or may not have an effect on the dissolution rate constants of soft gelatin capsule shells, depending on the type of gelatin used and the pH of the medium. Both sodium and potassium chloride at concentrations from 0 to 0.30 *M* and a pH value of 5.0 seem to have no effect on the dissolution rate constant of both Types A and B soft gelatin capsule shells. However, at a pH value of 1.2, both salts seem to have a small negative effect on the dissolution rate constants of both types of gelatin shells (Table II).

In most instances the use of pepsin in the simulated gastric fluid T.S. has little or no effect on the dissolution rate constant of the soft gelatin capsule shell. This is particularly true for relatively unaged and fast dissolving samples. However, in certain aged and relatively slow dissolving samples, the use of enzyme may increase the dissolution rate. This is illustrated by the results listed in Table III for a 2-year-old sample and a relatively slow dissolving Type A soft gelatin capsule shell sample. It can be seen that in this instance there is no significant difference between the dissolution rate of a slow

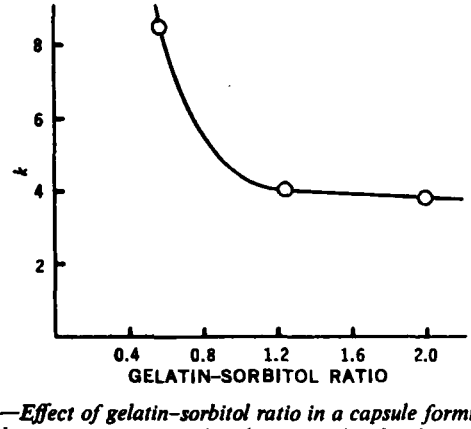

Figure 8—*Effect of gelatin-sorbitol ratio in a capsule formulation on the dissolution rate constant (mg./cm.² min.) of soft capsule shells made from a Type B gelatin in simulated gastric fluid at 37° and 100 r.p.m.*

Table IV—Normal Gelatin–Glycerin Ratio Values for Several Different Capsule Shell Formulations and Their Suggested Uses

Gelatin–Glycerin Ratio	Suggested Uses
1.5–3.0	Oral products with oil vehicles
1.0–2.0	Oral products with oil vehicles plus surfactant
1.0–2.2	Water-miscible vehicles
0.8–2.0	Organic solvents having a hardening effect on the gelatin shell
0.5–1.5	Squeeze-out-type capsules
0.4–1.0	Suppositories

dissolving sample in simulated gastric fluid T.S. with or without the use of pepsin. However, there is a small difference in the dissolution rate of the aged sample. In a case of an aged soft gelatin capsule where dissolution rate was a problem, pellicle formation was observed. A thin membrane on the inner side of the capsule shell seemed to be responsible for the delay in dissolution time. Apparently the pepsin in the simulated gastric fluid helped to accelerate the dissolution of the pellicle and the subsequent liberation of the encapsulated drug. Pellicle formation in gelatin capsules has been encountered with aldehydes and/or aldehyde-containing liquids, such as in perfumes and flavors. The use of aldehyde, *e.g.*, formaldehyde, to render gelatin less water soluble is the basis of a technique (3) to produce delayed-release soft gelatin capsules.

Gelatin Gel Strength—For a given soft gelatin capsule, there is an optimum gelatin Bloom strength to use when all factors are taken into account. One of these factors is the dissolution rate of the capsule shell. The effect of Bloom strength on the dissolution rate constant of the Type B gelatin capsule shell in simulated gastric fluid without pepsin at 37° and 100 r.p.m. is shown in Fig. 6. It is not surprising to observe a linear relationship, since the same types of gelatin may be blended to obtain a desired property, such as Bloom strength. The straight line with a negative slope indicates a reverse linear relationship: the lower the Bloom the higher the dissolution rate and the higher the Bloom the lower the dissolution rate. This relationship holds true provided other factors are constant. For example, gelatins with higher Bloom values have greater physical strength; therefore, thinner films or ribbons may be made from them. Capsules made with thinner ribbons have faster apparent dissolution. In most instances, soft gelatin capsules are made with Type B gelatin having Bloom strength values between 150 and 200 g.

Plasticizer—The plasticizer is an important part of the soft gelatin capsule shell formulation. Not too many compounds may be used to replace glycerin as the plasticizer of gelatin. Glycerin is relatively pure, commercially available in large quantities, relatively inexpensive, and nontoxic. Propylene glycol and polyethylene glycol 400 can be used, but they have no advantage over glycerin. Sorbitol has limited uses as a plasticizer for gelatin. Usually it is used to replace part of the glycerin in cases where a low glycerin content is desirable in a particular formulation. Hexaglycerol and its family of polymers are not commercially available at present. They have properties similar to glycerin as plasticizers for gelatin. The plasticizer in a

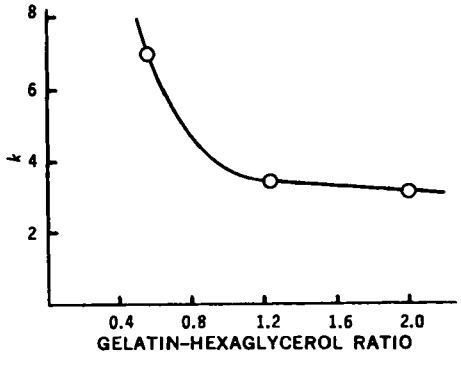

Figure 9—*Effect of gelatin–hexaglycerol ratio in a capsule formulation on the dissolution rate constant (mg./cm.² min.) of soft capsule shells made from a Type B gelatin in simulated gastric fluid at 37° and 100 r.p.m.*

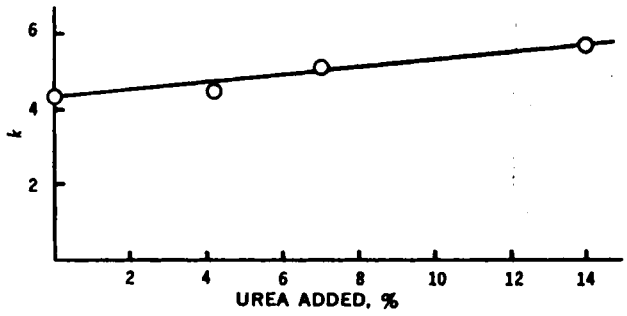

Figure 10—*Effect of percent urea added to a capsule formulation on the dissolution rate constant (mg./cm.² min.) of soft capsule shells made from a Type B gelatin in simulated gastric fluid at 37° and 100 r.p.m.*

formulation, expressed as the gelatin–plasticizer ratio, controls the consistency of the soft gelatin capsule. Hence, the gelatin–plasticizer ratio reflects the projected use of the capsule. Figures 7–9 show the influence of the gelatin–plasticizer ratio on the dissolution rate constant of the Type B gelatin capsule shell. It is surprising to see that the gelatin–plasticizer ratios for glycerin, sorbitol, and hexaglycerol have similar effects on the dissolution rate constant, in particular in the region between 1.2 and 2.0 ratios where the plasticizer seems to have little influence on dissolution. Table IV shows a tabulated list of normal gelatin–glycerin ratio values for several different capsule shell formulations and their suggested uses.

Additives—The influence of certain additives on the dissolution rate constant of the soft gelatin capsule shell in simulated gastric fluid without pepsin at 37° at 100 r.p.m. has been investigated. Figure 10 reflects the increased dissolution rate, which may be attributable to the hydrogen bond breaking effect of urea in a plot of dissolution rate constant *versus* percent urea added to the capsule shell. The increase in dissolution rate is small for the amount of urea added. A somewhat greater increase with lysine hydrochloride, probably due to acidity, is illustrated in Fig. 11. For ease of comparison, a list of additives tabulating the changes in the dissolution rate constant realized through the addition of 1% of each to a standard soft gelatin capsule shell formulation is compiled in Table V. Most of the compounds caused only a slight change in the dissolution rate constant. Acid salts and mineral and organic acids seem to cause the largest change in dissolution rate, probably due to acid hydrolysis of the polypeptide in the gelatin.

SUMMARY AND CONCLUSIONS

A simple method is described to study the effects of various factors on the dissolution rates of soft gelatin capsule shells. The method involves the use of the current USP XVIII dissolution apparatus and the spectrophotometric determination of the amount of dye dissolved from a blue, soft gelatin capsule shell disk placed in the rotating basket. The validity of the gelatin disk method was confirmed by a correlation study of the percent dye and gelatin dissolved for any given dissolution time. Dissolution rate constants may be obtained from a plot of the amount of the soft gelatin

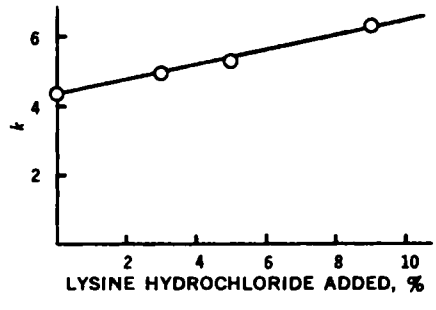

Figure 11—*Effect of percent lysine hydrochloride added to a capsule formulation on the dissolution rate constant (mg./cm.² min.) of soft capsule shells made from a Type B gelatin in simulated gastric fluid at 37° and 100 r.p.m.*

Table V—Effects of Some Additives on the Dissolution Rate of a Typical Soft Gelatin Capsule Shell

Compound Added	Change in Dissolution Rate/1% Added, mg./min. cm.[2]
Acacia	0.044
Ammonium citrate	0.030
Ammonium nitrate	0.044
Fumaric acid	0.778
Glycine	0.140
Hydrochloric acid	7.320
Lysine hydrochloride	0.215
Maleic acid	0.550
Poloxalene L44[a]	0.050
Poloxalene L64[a]	0.150
Polysorbate 80	0.460
Polyvinylpyrrolidone	0.025
Sodium acetate	0.110
Sodium carbamate	0.147
Starch, potato	0.086
Tartaric acid	0.443
Thiourea	0.155
Urea	0.096

[a] Pluronic (brand of poloxamer), BASF Wyandotte Corp.

capsule shell dissolved, which is directly proportional to the dye dissolved, divided by the dissolving solid surface area *versus* time.

The use of the gelatin disk method has permitted the present investigation of the influence of agitation, temperature, dissolution medium, gelatin gel strength, plasticizer, and certain additives on the dissolution rate of the capsule shell. Gelatin gel strength, when measured in Bloom grams, appeared to have an inverse linear relationship with dissolution rate. The plasticizer plays an important role, and its effect on dissolution rate seems to depend on the gelatin–plasticizer ratio in a particular formulation. Acid salts and mineral and organic acids seem to cause a greater change in dissolution rate, probably due to acid hydrolysis of gelatin.

In conclusion, various factors have been demonstrated to influence soft gelatin capsule shell dissolution rates. A knowledge of these relationships is helpful in the formulation of soft gelatin capsule shells for various purposes.

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4-Methanesulfonyloxybutanol: Hydrolysis of Busulfan

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Abstract □ The hydrolysis of busulfan (1,4-dimethanesulfonyloxybutane) was investigated by means of NMR spectroscopy. The final hydrolysis product was found to be tetrahydrofuran. The rather unstable intermediate, 4-methanesulfonyloxybutanol, was synthesized and proved to undergo cyclization to tetrahydrofuran by intramolecular alkylation. The half-life of this first-order reaction in aqueous solution at 37° was determined to be approximately 12 min. at pH 3 as well as at pH 7.4. From the present data, it is concluded that 4-methanesulfonyloxybutanol is unlikely to be responsible for the biological action of busulfan.

Keyphrases □ Busulfan—hydrolysis, NMR determination of hydrolysis intermediate as 4-methanesulfonyloxybutanol □ 4-Methanesulfonyloxybutanol—synthesis, identified as an unstable intermediate in the hydrolysis of busulfan, NMR □ NMR spectroscopy—identification of 4-methanesulfonyloxybutanol as an unstable intermediate in the hydrolysis of busulfan

In connection with an investigation of the genetic action of busulfan (1,4-dimethanesulfonyloxybutane), it has been found that aqueous solutions of hydrolyzed busulfan, as well as solutions of a compound assumed to be 4-methanesulfonyloxybutanol (III), induced mutations to a greater extent than busulfan itself (1). Compound III, formerly a monofunctional alkylating agent, was of potential interest as an intermediate in the hydrolysis of busulfan with reference to the discussion of the mechanism of the biological effects of busulfan. To our knowledge, the synthesis and stability of III have not been described in the literature[1]. For this reason, it seemed justified to synthesize the intermediate III and to investigate its behavior. Furthermore, the partial saponification and hydrolysis of busulfan were followed by NMR spectroscopy.

EXPERIMENTAL[2]

Synthesis—*4-Benzoyl-1-methanesulfonyloxybutane* (II)—To a stirred mixture of 4-benzyloxybutanol (I) (2) [9 g., 0.05 mole, b.p. 157–160° (12 mm.)] and methanesulfonyl chloride (6.9 g., 0.06 mole), pyridine (20 ml.) was added dropwise while keeping the temperature below 40° by cooling. After additional stirring for 1 hr., the reaction mixture was poured into a mixture of ice and 4 *N* HCl. It was extracted with chloroform and the organic layer was washed with 4 *N* HCl, dried (magnesium sulfate), and evaporated *in vacuo*. The residue was suspended in water, and the excess methanesulfonyl chloride was hydrolyzed at room temperature at pH 7 using an automatic end-point titrator (11.2 ml. of 1 *N* NaOH). It was extracted with chloroform, washed with water, dried (magnesium sulfate), and evaporated *in vacuo*, yielding crude II (12 g., 93%). Crude II (3 g.) was chromatographed on a dry silica gel column[3]. The fractions, shown by TLC[3] to be homogeneous, were combined and evaporated *in vacuo*. After drying of the residue *in vacuo* (1 mm.) for 2 hr. at 22°, II was obtained as a colorless oil (2.1 g.); NMR ($CDCl_3$, tetramethylsilane): CH_2CH_2 (m, 1.78, 4H), CH_3O_2SO (s, 2.94, 3H), CH_2CH_2O (t, 3.52, 2H), CH_2OSO_2 (*t*, 4.26, 2H), $C_6H_5CH_2O$ (s, 4.51, 2H), and C_6H_5 (s, 7.36, 5H).

Anal.—Calc. for $C_{12}H_{16}O_4S$ (mol. wt. 258.32): C, 55.80; H, 7.02; S, 12.38. Found: C, 55.67; H, 7.04; S, 11.92.

4-Methanesulfonyloxybutanol (III)—A solution of II (1 g., 3.9 mmoles) in dry ethanol (15 ml.) was hydrogenated at room temperature after addition of 10% palladium-on-charcoal (0.15 g.). After the uptake became negligible, the catalyst was removed by filtration. The filtrate was evaporated *in vacuo* in a bath not exceeding 15° to yield III as a colorless oil. All operations including analyses were performed within 6 hr., and III was kept below −10° when possible. The NMR spectrum of III is given in Fig. 1.

Anal.—Calc. for $C_5H_{12}O_4S$ (mol. wt. 168.21): C, 35.70; H, 7.20; S, 19.05. Found: C, 35.46; H, 7.59; S, 18.92.

Half-Life Studies—The half-life of III was obtained by titration of liberated methanesulfonic acid in 0.1-mmole solution of III at pH 7.4 and 3, respectively, using 0.33 *N* NaOH. The titration curve was recorded until the sodium hydroxide consumed had become negligible. The reaction order and the half-life were determined as usual.

Hydrolysis of Busulfan—*Acidic Conditions*—A suspension of busulfan (0.025 g., 0.1 mmole) in deuterium oxide (D_2O) (0.4 ml.) was kept in a sealed NMR test tube at 70° and shaken occasionally until a solution was obtained (24 hr.). The NMR spectrum was recorded to show the signals of IV. The formation of IV was verified and the absence of 1,4-butanediol was proved by NMR spectra taken after the addition of IV and the diol successively to the desealed NMR tube.

Alkaline Conditions—The experiment was performed as described under *Acidic Conditions*, except that 2.4 *N* NaOD (0.1 ml.) was added to the suspension prior heating to show IV as the reaction product.

Partial Saponification—To a mixture of busulfan (0.125 g., 0.5 mmole), D_2O (1 ml.), and $(CD_3)_2CO$ (3 ml.), 2.4 *N* NaOD (0.2 ml., 0.48 mmole) was added and the mixture was refluxed for 4 hr. The NMR spectrum of the still alkaline reaction mixture was examined, and the presence of IV along with the starting material was proved as described under *Acidic Conditions*.

RESULTS AND DISCUSSION

Experiments to provide 4-methanesulfonyloxybutanol (III) by partial saponification of busulfan, as well as total hydrolysis under acidic and alkaline conditions, were followed by NMR spectroscopy. No reaction products but tetrahydrofuran (IV) could be detected. Fast cyclization of III to IV by intramolecular alkylation was the only explanation for this result and was consistent with the described cyclization of partially mesylated or tosylated polyalcohols under acidic (3, 4) and alkaline (5) conditions. The same type of cyclization occurs during the recently reported (6) formation of

[1] The authors were not successful in obtaining the details of the original method of preparation of the compound (1) alleged to be the monomesyl derivative III.

[2] The NMR spectra were obtained on a Varian A-60 A spectrometer. The titration curves were obtained on a Radiometer titrator, Type TTT 1a, equipped with a Radiometer titrigraph, SBR 2b.

[3] HF_{254}, Merck; C_6H_{12} (three parts), ethyl acetate (one part); detection by UV.

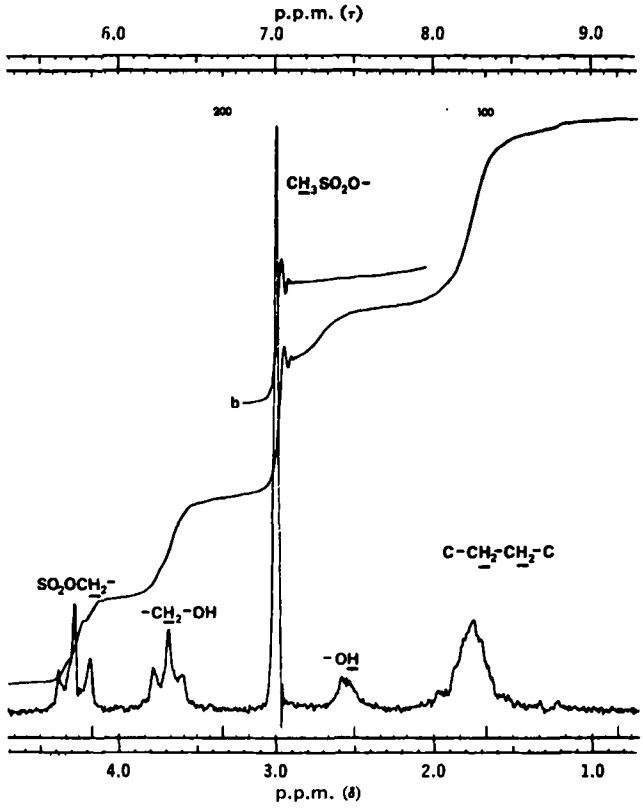
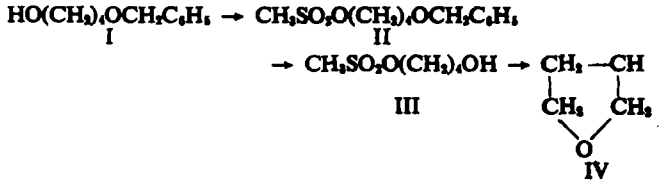


Figure 1—*NMR spectrum of III, 10% (CDCl₃, tetramethylsilane). The integral* b *is recorded after shaking the NMR solution with* D_2O.

2,5-dimethyltetrahydrofurans from their corresponding 2,5-bis-methanesulfonyloxyhexanes. For these reasons, the synthesis of III was based on a temporary protection of the hydroxyl function as outlined in Scheme I. The reductive debenzylation as the last step resulting in III was advantageous since it could be performed under mild conditions to yield III quantitatively as a colorless, analytically pure, and unstable oil. The structure was proved by its NMR spectrum in $CDCl_3$ (Fig. 1). When the NMR test tube was left for 46 hr. at room temperature, an almost quantitative for-



Scheme I

mation of IV and methanesulfonic acid was observed. In water, III has a half-life of approximately 12 min. at 37° both at pH 7.4 and 3, and it was transformed to IV both in the neutral and acidic solutions in a first-order reaction. The cyclization could conveniently be followed by titration of liberated methanesulfonic acid.

The presented results proved that the hydrolysis of busulfan leads to tetrahydrofuran (IV) through the intermediate III, which is in contrast to the reported formation of 1,4-butanediol (7). When one considers the high rate of cyclization of the monomesyl compound, III, it is unlikely that this compound is responsible for the biological action of busulfan.

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Antitumor Activity and Preliminary Phytochemical Examination of *Tagetes minuta* (Compositae)

G. R. ICKES, H. H. S. FONG, P. L. SCHIFF, Jr.*, R. E. PERDUE, Jr.†, and N. R. FARNSWORTH▲

Abstract □ A random collection antitumor screening program revealed that the whole flowering plants of *Tagetes minuta* were significantly active against the Lewis lung carcinoma *in vivo*. In the present phytochemical examination of this plant, the flavone glycosides quercetagetrin and patuletrin were isolated in addition to the monomethyl ester of fumaric acid and syringic acid.

Keyphrases □ *Tagetes minuta* (Compositae)—phytochemical study, antitumor activity □ Antitumor activity—isolation and testing of constituents of *Tagetes minuta* □ Medicinal plants—phytochemical study of *Tagetes minuta*, antitumor activity

Extracts from *Tagetes minuta*[1] were found to elicit significant activity against the Lewis lung carcinoma *in vivo*. Results of a preliminary phytochemical investigation of this plant are reported here.

Prior phytochemical studies on *T. minuta* were limited to the isolation and characterization of insecticidally active compounds such as 5-(3-buten-1-ynyl)-2,2′-bithienyl, α-terthienyl (1), 5-(but-1-chloro-2-ol-3-ynyl)-2,2′-bithienyl, *cis*-5-(1-acetoxy-but-3-enyl)-2,2′-bithienyl, 5-(but-1-en-3-ynyl)-2,2′-bithienyl, 5-(4-chloro-3-hydroxy-but-1-ynyl)-2,2′-bithienyl, and 5-(but-1-yn-4-olyl)-2,2′-bithienyl (2), in addition to the terpenes ocimene, *d*-limonene, estragole, tagetone, and α-dimethyl-Δ^{α}-octen-ε-one (3, 4). A number of rather rare flavonoids, *i.e.*, patuletin, patuletrin, quercetagetin, quercetagetrin, tagetiin, and kaempferitrin (5–8), in addition to a number of carotenoids, *i.e.*, helinine, violaxanthin, rubichrome, rubixanthin epoxide, lutein, antheraxanthin, α-cryptoxanthin, phytofluene, α-carotene, β-carotene, and helenien (9–11), have been isolated from other tagetes species but not *T. minuta*.

EXPERIMENTAL[2]

Plant Material—Originally, the aerial parts of the plant[3] *T. minuta* L. (Compositae) were collected along an open sunny bank, below the Harar-Jijiga road, near the southeastern limits of Harar, Hararge Province, Ethiopia, in December 1964. The collection was made at latitude 9° 18′ N., longitude 42° 7′ E. at an elevation of 2134 m. (7000 ft.). A second collection of plant material (PR-15256), on which the current investigation is based, was collected along the Acachi River in Shoa Province, Ethiopia, 17 km. south of Addis Ababa [elevation 2082 m. (6830 ft.), latitude 8° 52′ N., longitude 38° 50′ E.] in January 1968. This material corresponded in every detail with the original collection.

Extraction and Initial Fractionation—A 6.0-kg. sample of milled aerial parts of *T. minuta* was extracted with hexane[4] in a Lloyd extractor for 24 hr. The resulting extract was concentrated and removed from the extractor; fresh solvent was added and the extraction was continued for an additional 24 hr. This procedure was repeated two additional times, and the extracts were pooled and concentrated to a thick syrup (39.8 g., Fraction A).

The defatted plant material was then air dried, placed back into the extractor, and extracted with 95% ethanol in a similar manner as for the hexane extraction. After removing the ethanol from the extract, a fraction (B) weighing 372 g. was obtained. Fraction B was then partitioned between 2 l. each of chloroform and water, and the aqueous phase was separated and extracted with three additional 2-l. volumes of chloroform. All chloroform extracts were combined and taken to dryness *in vacuo*, and a third fraction (C) weighing 43.5 g. was obtained. Entrained chloroform was removed from the aqueous phase *in vacuo*, and the fraction was frozen and lyophilized to yield 329.5 g. of Fraction D.

Fraction D was dissolved in a convenient volume of distilled water, and a flavonoid fraction was prepared by several extractions with ethyl acetate. The ethyl acetate extraction was continued until a negative cyanidin reaction was obtained (12). In this manner, 59.3 g. of Fraction E was obtained. Further extraction of Fraction D with ethyl acetate–methanol (95:5 and 8:2) yielded only negligible quantities of residue. Finally, Fraction D was exhaustively extracted with *n*-butanol, which yielded 190 g. of Fraction F.

Fraction E, following TLC on polyamide plates using methanol as the developing solvent and 25% aqueous lead subacetate solution as the spray reagent, showed the presence of three yellow-orange spots at R_f 0.90, 0.41, and 0.59 when the chromatograms were viewed under UV light.

Isolation and Identification of Patuletrin—An aqueous slurry of polyamide (370 g.) was prepared and poured into a glass chromatographic column (4 × 7 cm.). A charge of 18.05 g. of Fraction E was absorbed on 10 g. of polyamide, and this was placed on top of the prepared column as a dry powder. Elution was initiated with water, then with water containing increasing amounts of methanol, and finally with methanol. Fifty-milliliter fractions were collected from the column, and these fractions were monitored on cellulose TLC plates, using *n*-butanol–acetic acid–water (4:1:5) as the developing solvent. Fractions were grouped and pooled on the basis of TLC patterns after spraying the plates as previously described.

Fractions 50–69, which were eluted from the column with water–methanol (1:1), were pooled and taken to dryness to yield 1.01 g. of residue. The residue was dissolved in 500 ml. of hot methanol and this was reduced *in vacuo* to 50 ml. and filtered; then the filtrate was allowed to evaporate spontaneously at room temperature. In this manner, three crops of amorphous material were harvested, which were shown to be identical by TLC. The material consisted of two major components, giving flavonoid reactions, at R_f 0.66 (major) and 0.77 (minor). The precipitate was crystallized from methanol to give 20 mg. of crystals. However, the crystals still contained a small amount of the R_f 0.77 substance, which was separated from the major component by TLC on cellulose plates. Elution of the mixture was with *n*-butanol–acetic acid–water (4:1:5). The zone at R_f 0.66 was removed by scraping, dissolved in methanol, and crystallized in the usual manner to yield microcrystals having, after drying *in vacuo* at 100° for 24 hr., m.p. 254–256°. An IR spectrum of the isolate showed absorption maxima at 3380 (s), 2940 (sh), 1660 (m), 1595 (m), 1485 (s), 1375 (m), 1200 (m), 1070 (s), 990 (s), and 700 (s) $cm.^{-1}$.

Direct comparison of the aglycone of the isolate by UV and IR analyses and by TLC analysis in three different systems showed it to be identical with patuletin. The glycone, analyzed after acid hydrolysis, was shown to be D-glycose. Attachment of the D-glucose at the 7-position was determined by UV analysis when no bathochromic shift in the UV spectrum from 259 nm. (observed) for the intact glycoside was observed after treatment with sodium acetate

[1] Collected in Ethiopia as part of the anticancer screening program of the U. S. Department of Agriculture and the National Cancer Institute.

[2] Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. The UV spectra were obtained using a Beckman model DK-2 spectrometer, and the IR spectra were determined in KBr pellets using a Beckman model IR-8 instrument.

[3] A voucher specimen is in the Herbarium of the U. S. National Arboretum and in Kew Herbarium. The original specimen was determined at Kew.

[4] Skellysolve B.

(14). It was thus concluded that the isolated flavone was 3,5,3′,4′-tetrahydroxy-6-methoxyflavone-7B-D-glucopyronoside (patuletrin).

Isolation and Identification of Quercetagetrin—TLC on cellulose plates of combined fractions 96–115 from the column, which were eluted with water–methanol (1:3), showed the presence of one major flavonoid at R_f 0.16 and five minor flavonoids. The fraction (0.74 g.) was dissolved in a minimum volume of methanol and was applied to several thick-layer cellulose plates (0.5 mm.); it was separated, using *n*-butanol–acetic acid–water (4:1:5), by triple development of the plates. The major component (R_f 0.16) was removed and eluted from the cellulose matrix with methanol to give 410 mg. of one-spot material as evidenced by TLC. After drying *in vacuo* at 100° for 24 hr., the isolate exhibited a melting point of 237–238°. An IR spectrum (KBr) of the isolate showed absorption bands at 3400 (s), 2940 (w), 1655 (s), 1605 (s), 1485 (s), 1385 (m), 1250 (w), 1070 (s), 990 (sh), 855 (w), and 825 (w) cm.$^{-1}$. The isolate exhibited an unusual dull black color under UV light on paper and thin-layer chromatograms, which is an unusual characteristic for flavonoids but which has been described previously for two rather rare flavonoids, *i.e.*, quercetagetrin and its aglycone (16). This isolate was also shown to be a flavonol as evidenced by the typical UV absorption at 363 nm. (methanol), and the *o*-dihydroxy grouping was demonstrated to be present as a result of a hypsochromic shift of 56 nm. in aluminum chloride when compared with the absorption in aluminum chloride–hydrochloric acid.

A comparison of the UV, IR, and TLC data of the isolate with those of quercetagetrin proved the two compounds to be identical. Thus, the isolate was shown to be 3,5,6,3′,4′-pentahydroxyflavone-7B-D-glucopyranoside (quercetagetrin).

Isolation and Identification of Monomethyl Fumarate—An additional portion of Fraction E (41.25 g.) was dissolved in water containing only enough methanol to effect complete solution. This solution was then extracted several times with ether; the ether fractions were pooled, dried over anhydrous sodium sulfate, and taken to dryness to give 7.11 g. of residue. This residue was dissolved in chloroform and applied to a chloroform slurry-packed column of silicic acid (400 g.), and 20-ml. fractions were collected. TLC of each fraction from the column revealed that fractions 113–118 contained a single substance. The pooled fractions (113–118) were taken *in vacuo* to a syrupy consistency (510 mg.), and the addition of benzene to this syrup resulted in a copious formation of colorless needles. The needles were recovered by filtration, in addition to a second crop from the mother liquor. A total of 35 mg. of colorless needles was obtained which, after drying *in vacuo* for 24 hr., gave a melting point of 144.5°; IR (KBr): 3100 (s), 2975 (w), 2700 (w), 2530 (w), 1720 (s), 1685 (s), 1351 (s), 1285 (sh), 1275 (s), 1265 (s), 995 (s), 920 (m), 895 (m), 765 (m), and 650 (m) cm.$^{-1}$. The UV spectrum of the isolate, in ethanol, showed only end-absorption at 208 nm. (ϵ = 7333). A mass spectrum taken at 70 ev. showed the molecular weight of the isolate to be 130. Mass tables (17) were consulted for various combinations of C, H, and O having this molecular weight. Five formulas were found: $C_5H_6O_4$, $C_6H_{10}O_3$, $C_7H_{14}O_2$, $C_8H_2O_2$, and $C_8H_{16}O$. Since the IR spectrum indicated the presence of both an unsaturated acid and an unsaturated ester, all compounds with less than four O atoms were not considered further. This left only the formula $C_5H_6O_4$. The mass spectrum showed that the compound was a methyl ester, as evidenced by the large M − 31 ion at *m/e* 99. When the IR spectrum of the isolate was compared with that of a synthetic sample of the monomethyl ester of fumaric acid, they were found to be identical. The melting point of the isolate was 144.5°, which is in agreement with the literature value for the monomethyl ester of fumaric acid (18). A mixed melting point of the isolate and a synthetic sample of the monomethyl ester of fumaric acid showed no depression. These data confirmed the identification of this isolate as the monomethyl ester of fumaric acid.

Isolation and Identification of Syringic Acid—Fractions 161–204, obtained from the silicic acid column previously described, were combined and concentrated *in vacuo* to a thick syrup and placed into a vacuum oven to dry at 50°. After several hours, colorless needles were observed projecting from the syrup. These crystals were removed and dissolved in 10 ml. of methanol for recrystallization. Three milligrams of pure, colorless needles were obtained from this solution which, after drying for 72 hr. in a vacuum desiccator at 25°, exhibited a melting point of 197–199°.

An IR spectrum of the isolate (KBr) showed absorptions at 3300 (s), 2980 (w), 2930 (w), 1695 (s), 1610 (s), 1515 (s), 1455 (s), 1415 (s), 1365 (m), 1240 (m), 1200 (s), 1025 (w), 905 (m), 860 (m), 795 (w), 765 (s), and 680 (s) cm.$^{-1}$. The carbonyl absorption at 1695 cm.$^{-1}$ was suggestive of an unsaturated aromatic acid.

The UV spectrum, in methanol, showed bands at 267 (ϵ = 4812) and 217 (ϵ = 9375) nm. On addition of a potassium hydroxide solution to the sample, a bathochromic shift to 300 (ϵ = 5937) and 224 (ϵ = 7812) nm. was observed, indicating the presence of an enolizable moiety in the isolate, perhaps due to a phenol group.

In consideration of these data, a search was made of previously isolated constituents to obtain a list of organic acids having a molecular weight of 198 (as evidenced by a mass spectrum of the isolate) and a melting-point range of 197–200°. Syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), and 2,4,5-trihydroxyphenylglyoxylic acid were the only two compounds that fulfilled these criteria. Since the IR spectrum did not show the two different carbonyl absorptions necessary for the substituted phenylglyoxylic acid, and since the literature melting point for 2,4,5-trihydroxyphenylglyoxylic acid was 193°, syringic acid was tentatively assumed to be the isolated compound.

Supporting evidence that this selection was correct is found if one examines the UV spectrum again. Comparing the theoretical maximum for syringic acid, an excellent correlation is found. If one uses a base value of 239 nm. for benzoic acid, adding 25 nm. for a *para*-substituted hydroxyl group and 7 nm. each for two *meta*-substituted methoxyl groups, one arrives at a calculated value of 269 nm. for syringic acid (19). This compares favorably with the observed value of 267 nm. for the isolate.

Mass spectra of the isolate and a reference sample of syringic acid were identical.

In addition to these data, TLC in three different solvent systems gave identical R_f values when the isolate was simultaneously chromatographed with a reference sample of syringic acid. Also, the IR spectra of the isolate and syringic acid were superimposable.

Antitumor Activity of *T. minuta*—Each crude fraction (A–D) was submitted to the Drug Research and Development Branch, National Cancer Institute, for bioassay using established protocols (23) in the PS leukemia, L-1210 leukemia, Walker muscular carcinosarcoma, and Lewis lung *in vivo* tumor systems. In addition, the fractions were evaluated for cytotoxicity using Eagle's 9 KB carcinoma of the nasopharynx in cell culture. Fractions A–C were inactive against all neoplasms and were not cytotoxic. Fraction D had marginal activity against the Lewis lung carcinoma, giving a T/C value[1] of 43% at 266 mg./kg. The monomethyl ester fumaric acid is inactive against the P-388 leukemia. It was also found to be noncytotoxic.

SUMMARY

The aqueous extract prepared from *T. minuta* L. aerial parts has shown reproducible activity against the Lewis lung carcinoma. A phytochemical investigation was initiated in an attempt to isolate and characterize the active antitumor principle(s). During the investigation, quercetagetrin, patuletrin, the monomethyl ester of fumaric acid, and syringic acid were isolated and identified. This is the first report on the occurrence of these four compounds in *T. minuta*. Only the ester was available in quantities sufficient for biological testing, and it was found to be noncytotoxic. Studies are in progress to isolate the active antitumor principle(s) from this plant.

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[1] A crude plant fraction is considered active if it gives a T/C ≤ 42% against the Lewis lung carcinoma.

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Imidazolecarbohydrazides IV: Synthesis and Biological Evaluation

J. R. NULU* and JAY NEMATOLLAHI▲

Abstract □ A series of 1-methylimidazole-4,5-dicarboxylic acid hydrazides and a bicyclic compound imidazo[4,5-*d*]pyridazine were synthesized and their structures were elucidated. The compounds were subjected to a limited pharmacological evaluation (MAO inhibitory activity) and the results are reported.

Keyphrases □ 1-Methylimidazole-4,5-dicarboxylic acid hydrazides —synthesis, structure determination, screened for MAO inhibitory activity □ Imidazo[4,5-*d*]pyridazine—synthesis, structure determination, screened for MAO inhibitory activity □ Hydrazides, 1-methylimidazole-4,5-dicarboxylic acid—synthesis, structure determination, screened for MAO inhibitory activity □ MAO inhibitors, potential—synthesis and evaluation of 1-methylimidazole-4,5-dicarboxylic acid hydrazides and imidazo[4,5-*d*]pyridazine

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As depicted in Scheme I, some analogs of III were synthesized by sodium borohydride reduction of their respective hydrazones (4). In this study, the ethylidene derivative (VI) was found to be resistant to such reduction.

The reaction product of I and II*b* could possibly be IV*b*, IV*c*, or a mixture of the two. A relatively sharp melting point of the product, together with a single spot observed on a thin-layer chromatogram and the presence of only a single NMR peak for the CH_3 protons on the pyridazine, gave convincing evidence that the compound was not a mixture of the two isomers. The elucidation of the molecular structure of the compound was attempted by the following experimental procedure. Mixing stoichiometric quantities of an aqueous solution of silver nitrate and IV*a* gave a precipitate analogous to those reported (5) for purines on whose 6-position exists an OH or NH_2 with the 7 as pyridinic N, or on the 6-position a C=O with the 7 as pyrrolic NH. Separation of the precipitate by centrifugation, its dissolution in perchloric acid, and UV spectrophotometric analysis of both the dissolved precipitate and the supernate revealed a 1:1 silver–IV*a* chelate. Similar experiments on the bicyclic reaction product of I and II*b* did not produce any precipitate. Because the purine analog of IV*c* forms a water-insoluble silver chelate (5), the reaction product was assumed to be IV*b*.

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Table I—1-Methylimidazole-4,5-dicarboxylic Acid Hydrazides or Acid Hydrazones and 1,5-Dimethyl-7-hydroxyimidazo[4,5-*d*]pyridazin-4-one

Compound	Crystallization Solvents	Yield, %	Melting Point	Analysis, % Calc.		Found
III*b*	Ethyl acetate–methanol	63	126–128°	C	42.48	42.09
				H	6.19	6.12
				N	37.17	37.42
III*c*	Ethanol	58	196–198°	C	61.70	61.33
				H	5.18	5.48
				N	23.99	24.27
IV*b*	Methanol–water	62	300°	C	46.66	46.42
				H	4.48	5.04
				N	31.10	30.79
V	Chloroform–methanol	77	226–228°	C	64.16	64.25
				H	4.85	5.07
				N	22.45	22.34
VI	Benzene–methanol	88	140–142°	C	47.99	48.13
				H	5.64	5.94
				N	33.58	33.48
VII	Benzene–methanol	85	227–229°	C	51.78	52.04
				H	6.52	6.63
				N	30.20	30.00
VIII	Ethyl acetate–methanol	75	86–88°	C	63.47	63.32
				H	5.86	5.41
				N	22.21	22.01
IX	Benzene–methanol	80	58–59°	C	51.07	50.91
				H	7.80	7.64
				N	29.80	29.40

the intermediate was excluded by recovering the compound intact after heating its butanolic solution for 24 hr.

EXPERIMENTAL[1]

1-Methylimidazole-4,5-dicarboxylic Acid Bis(2-methylhydrazide) (III*b*) and 1,5-Dimethyl-7-hydroxyimidazo[4,5-*d*]pyridazin-4-one (IV*b*)—To 2.0 g. (0.01 mole) of I was added 1.4 g. (0.03 mole) of methylhydrazine. The mixture was stirred at room temperature for 5 hr. The precipitated solid was washed with ether and then treated with a small amount of ethanol to give 1.5 g. (63%) of III*b*.

Heating of I and II*b* under reflux for 18 hr. gave 1.2 g. (62%) of IV*b*.

Compound III*c* was prepared under reaction conditions similar to those of IV*b*.

The preparation of the hydrazones and their respective hydrazides (Table I) are exemplified by the following methods described for V and VIII.

1-Methylimidazole-4,5-dicarboxylic Acid Bis(2-benzylidenehydrazide) (V) and 1-Methylimidazole-4,5-dicarboxylic Acid Bis(2-benzylhydrazide) (VIII)—Under nitrogen, a mixture of 1.9 g. (0.01 mole) of III*a* and 15 ml. of benzaldehyde was heated under reflux for 3 hr. The precipitated solid was washed with ether to give 2.9 g. (77%) of V.

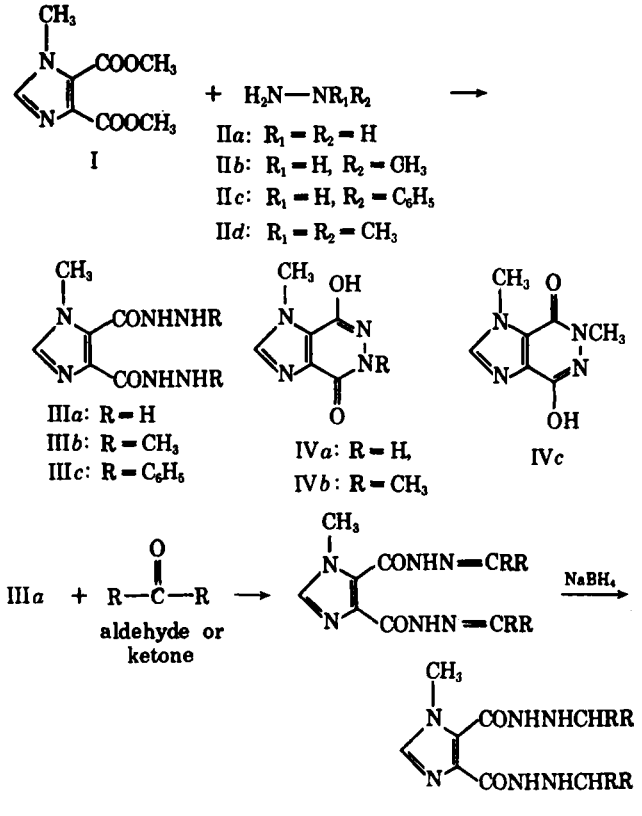
To a suspension of 1.85 g. (0.005 mole) of V in 50 ml. of water was added 0.8 g. (0.02 mole) of sodium borohydride. The mixture was heated under reflux on the steam bath for 1 hr. The solution was evaporated to 10 ml. and extracted twice with ether. The combined ether extracts was dried over sodium sulfate. After filtration, evaporation of solvent gave 1.4 g. (75%) of VIII.

Compound VI under such conditions could not be reduced.

DISCUSSION

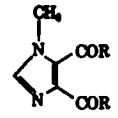
The compounds listed in Table II were evaluated for their MAO inhibitory activity by three methods which were described previously (1, 2). The results, listed in Table II, indicate that 1-methylimidazole-4,5-dicarbohydrazides possess about the same level of activity as their previously reported analogs. A similar level of activity was also detected for the bicyclic analog, IV*b*.

A broad biological evaluation of the compounds for such functions as cardiovascular effect and antitubercular activity may be justified by considering the therapeutic use of hydralazine and isoniazid. Compound IV*b* may be conceived of as an analog of purine, a system of interest in tumor research.



Scheme I

[1] Melting points were determined in open capillary tubes in a Thomas-Hoover melting-point apparatus and are not corrected. All evaporations were made *in vacuo* with rotatory evaporators. The IR spectra were determined (KBr) with a Beckman IR-8 instrument, and NMR spectra were determined with a Varian A-60 spectrophotometer at ambient temperature (tetramethylsilane as a reference and deuterated dimethyl sulfoxide as a solvent). Elemental analyses were done by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. Crystallization solvents, melting points, and elemental analysis (C, H, N) values are listed in Table I.



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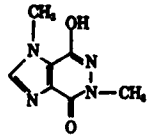

B

Table II—MAO Inhibitory Activity Evaluation of Hydrazides (Structures A and B)

Compound	R (Structure A)	Oral Dose, mmole/kg.	Activity Prior to Reserpine (2 mg./kg.)	Mean Ptotic Score	Body Temperature (± *SE*) after Reserpine (2 mg./kg.) 0 hr.	4 hr.	6 hr.	24 hr.	Hexobarbital-Induced Sleeping Time, min.[a]
Control[b]			Normal	3.75	35.8 ± 0.2°	29.9 ± 0.4°	26.3 ± 0.2°	26.6 ± 0.5°	8.3 ± 0.3
III*a*	$NHNH_2$	0.275	Increased	2.50	35.7 ± 0.3°	32.7 ± 0.3°	31.9 ± 0.4°	30.6 ± 0.5°	15.2 ± 0.2
III*b*	$NHNHCH_3$	0.275	Increased	2.90	36.1 ± 0.2°	31.5 ± 0.1°	31.0 ± 0.4°	33.6 ± 0.6°	12.7 ± 0.3
IV*b*	Structure B	0.275	Increased	2.66	35.9 ± 0.1°	32.2 ± 0.5°	30.9 ± 0.3°	28.9 ± 0.4°	13.4 ± 0.4
III*c*	$NHNHC_6H_5$	0.275	Increased	2.00	36.5 ± 0.3°	32.5 ± 0.2°	29.4 ± 0.5°	32.1 ± 0.3°	19.7 ± 0.1
V	$NHN{=}CHC_6H_5$	0.275	Increased	2.00	36.3 ± 0.3°	32.6 ± 0.4°	29.7 ± 0.6°	30.2 ± 0.5°	20.3 ± 0.3
VI	$NHN{=}CHCH_3$	0.275	Increased	2.50	35.7 ± 0.1°	32.6 ± 0.3°	31.6 ± 0.3°	32.7 ± 0.7°	14.9 ± 0.2
VII	$NHN{=}CHCH_3$	0.275	Increased	2.60	35.2 ± 0.4°	32.0 ± 0.5°	29.6 ± 0.5°	30.6 ± 0.3°	12.9 ± 0.1
VIII	$NHNHCH_2C_6H_5$	0.275	Increased	2.33	35.9 ± 0.1°	31.8 ± 0.3°	29.2 ± 0.6°	31.4 ± 0.5°	17.6 ± 0.3
IX	$NHNHCH(CH_3)_2$	0.275	Increased	1.83	35.6 ± 0.2°	32.6 ± 0.2°	30.8 ± 0.4°	32.4 ± 0.3°	22.0 ± 0.2
Isocarboxazid		0.1375	Increased	0.50	35.6 ± 0.5°	34.2 ± 0.1°	36.7 ± 0.4°	35.2 ± 0.4°	11.0 ± 0.1

[a] Hexobarbital was given intraperitoneally (5.5 mg./kg.) 2 hr. after intubation of the test compound. [b] Control implies reserpine in ptosis and hypothermia test and hexobarbital in sleeping time prolongation test.

REFERENCES

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[•] Present address: Pharma Corp., San Antonio, TX 78216

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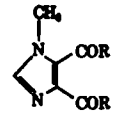
Synthesis and Anticancer Activity of 5-(Propargyloxymethyl)-2-oxazolidinones

R. B. FUGITT and L. C. MARTINELLI[▲]

Abstract □ To determine the feasibility of producing a tissue-specific anticancer agent, a series of 2-oxazolidinones bearing an alkynoxymethyl side chain in the 5-position was prepared. These compounds were submitted to the National Cancer Institute for testing against L-1210 and/or P-388 tumor test systems. None of the compounds showed significant anticancer activity. The synthetic procedures and NMR spectral properties of the title compounds are described.

Keyphrases □ 5-(Propargyloxymethyl)-2-oxazolidinones—synthesis, screened for anticancer activity □ 2-Oxazolidinones, 5-(propargyloxymethyl)—synthesis, screened for anticancer activity □ Anticancer agents, potential—synthesis and screening of 5-(propargyloxymethyl)-2-oxazolidinones

Interest in carbamates bearing an acetylenic function developed from an interest in tissue- or organ-specific anticancer agents, particularly those specific for the CNS. The investigation of the title compounds stems, in part, from the work of Dillard *et al.* (1) who first demonstrated the oncolytic action of propargyl carbamates against plasma cell tumor X-5563 and the atypical myelogenous leukemia C-1498. Among the structure–activity relationships they observed was the necessity of the ethynyl moiety for significant oncolytic



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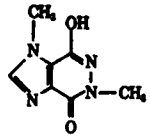

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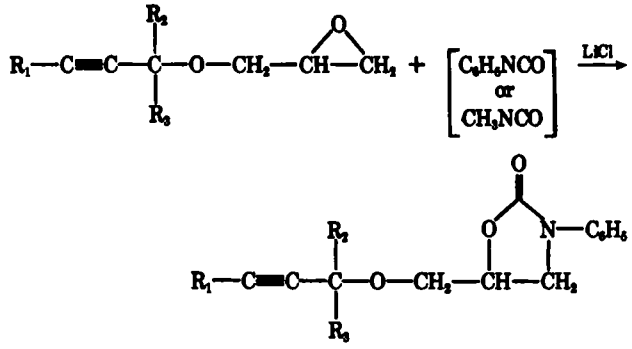
Synthesis and Anticancer Activity of 5-(Propargyloxymethyl)-2-oxazolidinones

R. B. FUGITT and L. C. MARTINELLI[▲]

Abstract □ To determine the feasibility of producing a tissue-specific anticancer agent, a series of 2-oxazolidinones bearing an alkynoxymethyl side chain in the 5-position was prepared. These compounds were submitted to the National Cancer Institute for testing against L-1210 and/or P-388 tumor test systems. None of the compounds showed significant anticancer activity. The synthetic procedures and NMR spectral properties of the title compounds are described.

Keyphrases □ 5-(Propargyloxymethyl)-2-oxazolidinones—synthesis, screened for anticancer activity □ 2-Oxazolidinones, 5-(propargyloxymethyl)—synthesis, screened for anticancer activity □ Anticancer agents, potential—synthesis and screening of 5-(propargyloxymethyl)-2-oxazolidinones

Interest in carbamates bearing an acetylenic function developed from an interest in tissue- or organ-specific anticancer agents, particularly those specific for the CNS. The investigation of the title compounds stems, in part, from the work of Dillard *et al.* (1) who first demonstrated the oncolytic action of propargyl carbamates against plasma cell tumor X-5563 and the atypical myelogenous leukemia C-1498. Among the structure–activity relationships they observed was the necessity of the ethynyl moiety for significant oncolytic



Scheme I—See Table I for R-group identification

activity. In addition, carbamates, especially ethynylmethyl carbamates, are known to be potent CNS depressants (2), thus indicating that they are readily transported across the blood–brain barrier. With the intent of screening new chemical entities with potential anticancer activity, the title compounds (Table I), bearing both an ethynyl and a carbamate function, were synthesized and subjected to anticancer screening.

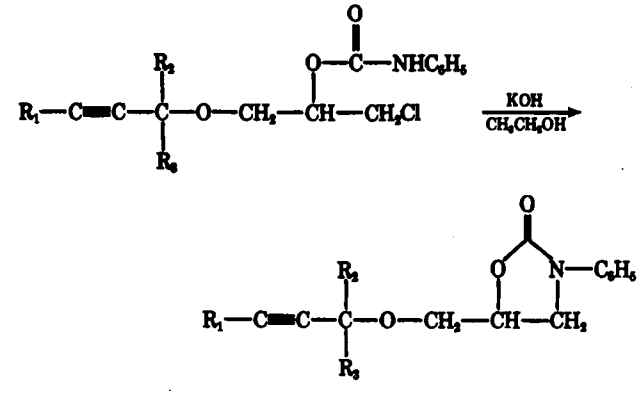
Additional impetus to study 2-oxazolidone ring compounds stems from observations that the potent anticancer agent 1,3-bis(2-chloroethyl)-1-nitrosourea decomposes in water to a Δ^2-oxazoline derivative (3). Similarly, the active *N*-acylethyleneimines readily rearrange to Δ^2-oxazolines (4).

The title compounds were synthesized by reported techniques, and their identification was achieved in part by comparison of NMR data with a compound with known ring stereochemistry. The six compounds were subjected to preliminary anticancer screening against L-1210 lymphoid leukemia and/or P-388 lymphocytic leukemia. Screening data are only available for I–IV; none showed significant activity in these systems.

CHEMISTRY

Compounds I–IV were synthesized (Scheme I) by lithium chloride-catalyzed condensation of the known epoxides (5) with phenylisocyanate or methylisocyanate in dry dimethylformamide at 100–150° (6). This method allows a high yield synthesis of *N*-alkyl- and *N*-aryl-2-oxazolidinones in one step from the epoxide. Compound II was also synthesized (Scheme II) by potassium hydroxide–ethanol cyclization (7) of the appropriate β-chloroethyl *N*-phenylcarbamate to provide a compound with known C-5 ring substitution.

Cyclization of the *N*-phenyl-β-chlorocarbamate ester resulted in the isolation of a small amount of a six-membered ring carbamate as a by-product, which arose from the isomeric γ-chlorohydrin formed in the synthesis of the starting β-chlorohydrins. Careful fractional



Scheme II—See Table I for R-group identification

distillation of the β-chlorohydrin prior to use eliminates this problem. The chlorohydrins were prepared from the reaction of the appropriate alcohol with epichlorohydrin (5).

The isocyanate–epoxide reaction is known to produce both C-4 and C-5 substitution products through breaking either the C_1—O or C_2—O epoxide bonds (8), although the C-5 isomer is generally the only product observed. From the limited study of Herweth *et al.* (6), only aryl-substituted epoxides led to the C-4 isomer regardless of the type of isocyanate (alkyl or aryl). The possibility existed, therefore, that the π-electron-rich ethynyl group may help direct C-4 isomer formation through π-interactions with the incoming isocyanate. That this did not occur is evident by comparison of the NMR data of II with the only products isolated from the isocyanate–epoxide reactions (I–IV).

The NMR spectral shift data (Table I) for H_A, H_B, and $H_{C,D}$ of I–IV are essentially identical with equivalent protons for II synthesized unambiguously. Further, these data are consistent with C-5 isomers reported in the literature (6). Compounds V and VI, the N—CH_3 analogs, are assigned as C-5 substitution since they exhibit proton NMR absorption consistent with the loss of anisotropic deshielding by a phenyl ring. The observation that the $\Delta\delta$ of H_A is larger than the $\Delta\delta$ of H_B when *N*-phenyl is replaced by *N*-methyl is not readily explainable, but it is consistent with similar findings by Herweth *et al.* (6).

BIOLOGICAL ASSAY[1]

Compounds I–IV were assayed against P-388 lymphocytic leukemia in BFD_1 mice, while only I was screened against L-1210 lymphoid leukemia (Table II). The compounds were administered at three dosages, 400, 200, and 100 mg./kg. i.p., every 4th day for three injections, and the percent increase in median survival time (percent test/control) was calculated at Day 30. Compound I was administered in hydroxypropylcellulose, while II–IV were administered in saline. The term "survivors" in Table II is a measure of toxicity and was determined at Day 5 after the first injection.

EXPERIMENTAL[2]

Melting points were obtained in open capillaries and are uncorrected. IR spectra[3] were obtained as neat oils between sodium chloride plates or in potassium bromide wafers for solids. All compounds, except III, exhibited acetylenic and carbonyl stretching frequencies at 2105–2290 and 1733–1740 $cm.^{-1}$, respectively. Compound III had no discernible acetylenic absorption but exhibited an acetylene C—H stretch at 3300 $cm.^{-1}$ as well as a carbonyl absorption at 1737 $cm.^{-1}$. All NMR spectra were obtained at 60 MHz. in deuteriochloroform containing 1% tetramethylsilane as internal standard.

1-(2-Butynyloxy)-3-chloro-2-propyl *N*-Phenylcarbamate—To a 250-ml. flask was added 25 g. (0.150 mole) of 1-(2-butynyloxy)-3-chloro-2-propanol prepared from the condensation of 2-butyn-1-ol and epichlorohydrin (5). Dried benzene (100 ml.) and 21.5 g. (0.180 mole) of freshly distilled phenylisocyanate were added and the reaction was stirred. Following a procedure similar to that of Loev and Kormendy (9), 1.5 ml. of trifluoroacetic acid was added; the reaction was topped with a calcium chloride tube and stirred for 4 hr. at room temperature. The reaction was cooled and filtered to remove diphenylurea and reduced to an oil by water aspiration. The oil was chromatographed on a 20 × 2-mm. alumina column using 1,2-dichloroethane, and the resulting oil was distilled *in vacuo* to give 22.4 g. (52%) of the desired product, b.p. 165–180° (0.1–0.2 mm.).

Anal.—Calc. for $C_{14}H_{16}ClNO_3$: C, 59.68; H, 5.72; Cl, 12.58; N, 4.97. Found: C, 59.74; H, 5.80; Cl, 12.74; N, 5.02.

The IR and NMR data were consistent with this structure. IR (neat): 3305 (N—H), 2220 and 2300 (C≡C), and 1625 (C=O) $cm.^{-1}$. NMR: 1.75 (t, 3), 3.72 (d, 4), 4.10 (d, 2), 4.30 (m, 1), 5.15 (m, 1), and 7.30 (m, 5) p.p.m.

[1] The screening was performed by Drug Research and Development, Chemotherapy, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014

[2] Elemental analyses were performed by Atlantic Microlabs, Atlanta, Ga.

[3] Recorded on a Perkin-Elmer 237B spectrometer.

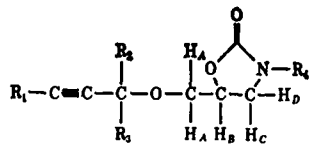


Table I—Structures and NMR Data of 2-Oxazolidinones

Compound	R_1	R_2	R_3	R_4	NMR Data (δ) H_A	H_B	$H_{C,D}$	R_2R_3	R_1	R_4
I	H	H	H	C_6H_5	3.78 (d, J_{AB} = 4.5 Hz.)	4.75(m)	3.98(m)	4.23 (d, J = 2.3 Hz.)	2.50 (t, J = 2.3 Hz.)	7.40(m)
II	CH_3	H	H	C_6H_5	3.74 (d, J_{AB} = 5.0 Hz.)	4.72(m)	3.95(m)	4.20 (q, J = 2.5 Hz.)	2.20 (t, J = 2.5 Hz.)	7.37(m)
III	H	—$CH_2(CH_2)_3CH_2$—		C_6H_5	3.85 (d, J_{AB} = 4.5 Hz.)	4.70(m)	3.96(m)	—	2.51 (s)	7.38(m)
IV	C_6H_5	H	H	C_6H_5	3.85 (d, J_{AB} = 5.0 Hz.)	4.75(m)	4.00(m)	4.45 (s)	7.35(m)	7.35(m)
V	H	H	H	CH_3	3.60 (d, J_{AB} = 4.4 Hz.)	4.65(m)	3.50(m)	4.25 (d, J = 2.3 Hz.)	2.74 (t, J = 2.3 Hz.)	2.87(s)
VI	CH_3	H	H	CH_3	3.62 (d, J_{AB} = 4.4 Hz.)	4.62(m)	3.54(m)	4.13 (q, J = 2.5 Hz.)	1.84 (t, J = 2.5 Hz.)	2.86(s)

3-Phenyl-5-[(2-propynyloxy)methyl]-2-oxazolidinone (I)—From the procedure of Herweth *et al.* (6), a 100-ml. flask equipped with a calcium chloride tube, condenser, and dropping funnel was charged with 7 g. (0.063 mole) of 1,2-epoxy-3-propargyloxypropane, 25 ml. of dry dimethylformamide, and 0.5 g. of lithium chloride and the contents were warmed at 100°. A solution of 7.5 g. of freshly distilled phenylisocyanate in 7.5 ml. of dry dimethylformamide was added dropwise (less than 1 drop/sec.), and the reaction was warmed at 150° for 4 hr. The solvent was removed *in vacuo* (pot 120–130°), and the residue was diluted with 50 ml. of carbon tetrachloride and enough methylene chloride to ensure that the liquids were miscible; the lithium chloride was removed by filtration. Upon evaporation of solvent, 11.2 g. (83%) of I was isolated and recrystallized from ethanol, m.p. 83–86° [lit. (10) m.p. 80°].

Anal.—Calc. for $C_{13}H_{13}NO_3$: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.42; H, 5.71; N, 6.06.

3-Phenyl-5-[(2-butynyloxy)methyl]-2-oxazolidinone (II)—*Procedure A: Potassium Hydroxide Method*—To a 50-ml. flask containing 12 g. (0.043 mole) of 1-(2-butynyloxy)-3-chloro-2-propyl *N*-phenylcarbamate and 35 ml. of 70% ethanol was added 29 g. (0.043 mole) of potassium hydroxide. The reaction was immediately warmed at 100° for 9 min. and then poured into 50 ml. of cold water, extracted with ether, and dried over magnesium sulfate. The ether was concentrated and the solution was cooled (5°), which afforded 5.8 g. (38%) of II. Recrystallization was from ethanol–water, m.p. 83–86°. Concentration of the mother liquor yielded 1.0 g. (7%) of a second compound, m.p. 62–65°, tentatively identified as 3-phenyl-5-(2-butynyloxy)-oxazin-2-one.

Procedure B: Epoxide–Isocyanate Method—The same procedure was followed as for I except that the oil bath was maintained at 145–150° throughout. The pot residue, after removal of dimethylformamide, was diluted with 40 ml. carbon tetrachloride; lithium chloride was removed by filtration. After concentrating to 25 ml. and cooling at 5°, 12.8 g. (85%) of II was isolated. The product was recrystallized from ethanol–water, m.p. 83–85°.

Anal.—Calc. for $C_{14}H_{15}NO_3$: C, 68.56; H, 6.16; N, 5.71. Found: C, 68.46; H, 6.23; N, 5.67.

3-Phenyl-5-{[(1-ethynylcyclohexyl)oxy]methyl}-2-oxazolidinone (III)—Following Procedure B for II, 6.1 g. (0.034 mole) of 2-{[(1-ethynylcyclohexyl)oxy]methyl}oxirane, 4.1 g. (0.034 mole) of freshly distilled phenylisocyanate, and 0.5 g. lithium chloride were reacted. Following workup in carbon tetrachloride and recrystallization from methanol, 7.3 g. of III was obtained (72%), m.p. 70–72°.

Anal.—Calc. for $C_{18}H_{21}NO_3$: C, 72.22; H, 7.07; N, 4.68. Found: C, 72.07; H, 7.12; N, 4.61.

3-Phenyl-5-{[(3-phenyl-2-propynyl)oxy]methyl}-2-oxazolidinone (IV)—Following Procedure B for II, 10 g. (0.053 mole) of 2-{[(3-phenyl-2-propynyl)oxy]methyl}oxirane, 6.55 g. (0.055 mole) of freshly distilled phenylisocyanate, and 0.4 g. of lithium chloride were reacted. Following workup and crystallization from ethanol, 12.5 g. of IV was isolated (77%), m.p. 70–73°.

Anal.—Calc. for $C_{19}H_{17}NO_3$: C, 74.25; H, 5.58; N, 4.56. Found: C, 74.37; H, 5.70; N, 4.52.

3-Methyl-5-[(2-propynyloxy)methyl]-2-oxazolidinone (V)—To a 250-ml. flask containing 20 g. (0.180 mole) of 1,2-epoxy-3-propargyloxypropane and 75 ml. dry dimethylformamide at 130° was added 1.0 g. lithium chloride, followed immediately by the slow addition (1 drop every 6–7 sec.) of a solution of 10.5 g. (0.184 mole) freshly distilled methylisocyanate in 18 ml. dry dimethylformamide. After 6 hr., dimethylformamide was removed (vacuum aspirator at 130°), the resulting oil was diluted with ethylene chlo-

Table II—Anticancer Activity

Compound	NSC Number	Tumor	Dosages[a]	Vehicle[b]	Percent T/C	Survivors
I	154295	L-1210	400	M	91	4/6
			200	M	100	6/6
			100	M	100	6/6
		P-388	400	M	100	6/6
			200	M	100	6/6
			100	M	95	6/6
II	159150	P-388	400	S	100	7/7
			200	S	92	7/7
			100	S	92	7/7
III	159151	P-388	400	S	100	7/7
			200	S	100	7/7
			100	S	100	7/7
IV	159149	P-388	400	S	92	7/7
			200	S	107	7/7
			100	S	92	7/7

[a] mg./kg. i.p. (per injection). [b] M is hydroxypropylcellulose; S is saline.

ride, and the lithium chloride was removed. After removal of the solvent, the oil was fractionally distilled to yield 22 g. (73%) of V, b.p. 122° (0.02 mm.).

Anal.—Calc. for $C_8H_{11}NO_3$: C, 56.80; H, 6.55; N, 8.27. Found: C, 56.61; H, 6.64; N, 8.21.

3-Methyl-5-[(2-butynyloxy)methyl]-2-oxazolidinone (VI)—A 50-ml. flask was equipped with a pressure-equalizing dropping funnel and a reflux condenser topped with a clamped rubber tube slit longitudinally for release of pressure. To this was added 4.2 g. (0.033 mole) of 2-[(2-butynyloxy)methyl]oxirane and 13 ml. of dry dimethylformamide. The solution was heated to 130° and 0.2 g. of lithium chloride was added, followed immediately by the slow addition (less than 1 drop every 6 sec.) of 2.0 g. (0.035 mole) of freshly distilled methylisocyanate in 3.5 ml. dry dimethylformamide. After 6 hr. at 130°, solvent was removed and the oil was diluted with methylene chloride; the lithium chloride was removed. The oil was distilled to give 4.3 g. of VI (70%), b.p. 110–125° (0.08 mm.).

Anal.—Calc. for $C_9H_{13}NO_3$: C, 59.00; H, 7.15; N, 7.64. Found: C, 58.81; H, 7.28; N, 7.57.

RESULTS AND DISCUSSION

The isocyanate–epoxide reaction provides high yield quantities of the title compounds and represents a method by which a variety of *N*-alkyl-2-oxazolidinones can be readily synthesized. Attempts to cyclize β-chloroethyl *N*-unsubstituted carbamates with several bases (potassium hydroxide–ethanol and sodium hydride–dimethylformamide) failed. Stronger bases are precluded because of the known ease of rearrangement of propargyl groups to allenes (11).

With the isocyanate–epoxide reaction, only C-5 substitution products were obtained; no C-4 products were isolated. Thus, the stereospecific nature of this reaction in the synthesis of the title compounds is apparently independent of π-electron interactions between isocyanate and acetylene π-electrons. From the work of Herweth *et al.* (6), such π-interactions might have been hypothesized for their isolation of C-4 as well as C-5 substitution products. That C-5 substitution appears exclusive in the present study is probably a result only of steric factors.

Compounds I–IV, the *N*-phenyl analogs, exhibited no anticancer activity against the L-1210 or P-388 tumor test systems employed. Based on reports preliminary to receiving testing data, the *N*-methyl Compounds V and VI are inactive as well. Activity is defined as percent T/C (test/control) of 125 or greater.

The lack of activity of the title compounds in the less sensitive L-1210 and P-388 tumor systems cannot be adequately explained at this time. The possibility exists that cyclic carbamates possess *in vivo* reactivities to nucleophiles significantly different from acyclic carbamates. Whether this or other physical–chemical and biological factors are important remains unanswered.

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Metabolism of Aspirin in Rumen and Corpus Tissues of Rat Stomach during First Four Minutes after Administration

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Abstract □ TLC and liquid scintillation spectrometry were used to determine the identities and relative quantities of aspirin and its metabolites in the gastric juice, blood plasma, rumen (nonglandular portion), and corpus (glandular portion) of the rat stomach 4 min. after the oral administration of aspirin-7-^{14}C. It was found that aspirin and salicylic acid are the major components in the gastric juice, blood plasma, and rumen and corpus tissues. Trace levels of a compound with an R_f value corresponding to that of salicyluric acid was found in the fluids and tissues analyzed. No glucuronide conjugates of salicylic acid or polyhydroxy metabolites of aspirin were found. The level of salicylic acid was fourfold higher in the corpus tissue than in the rumen tissue.

Keyphrases □ Aspirin, radiolabeled—metabolism in rumen and corpus tissues, during first 4 min. after oral administration, rats, TLC and liquid scintillation spectrometric determination of metabolites □ Salicylic acid—determination as metabolite in rumen and corpus tissues of rat 4 min. after oral administration of radiolabeled aspirin □ Metabolism—radiolabeled aspirin, determination of metabolites in rat 4 min. after oral administration, rumen and corpus tissues

It is well known that gastric irritation leading to the production of lesions and occult bleeding occurs in laboratory animals and man following oral administration of salicylates (1, 2). Aspirin has been reported (3, 4) to produce gastric lesions in the corpus portion of the albino rat when administered orally at a level of 50 mg./kg. of body weight.

The production of gastric lesions following intravenous administration of salicylates was reported by Hurley and Crandall (5), Grossman *et al.* (6), and Morris (7), but the lesions were much less severe than those produced by oral administration.

Although systematic mechanisms have been proposed to explain the production of lesions, the present authors felt that the initial mechanism of lesion formation might be a localized chemical phenomenon since previous studies (3) had shown that some rats develop gastric lesions in the corpus (glandular) portion of the stomach within 5 min. after administration of aspirin. Lesions have not been produced in the rumen (nonglandular portion) under the same experimental conditions.

It, therefore, became of interest to identify and compare the relative quantities of aspirin and/or its metabolites in the rumen and corpus tissues of the rat shortly after administration of aspirin-7-^{14}C.

MATERIALS AND METHODS

Radiochemical purity of aspirin-7-^{14}C was established by TLC in conjunction with autoradiography and liquid scintillation spectrometry. The chemical identity and purity of standards of aspirin and its metabolites were established by melting point and TLC.

Four male albino laboratory rats[1], weighing between 100 and 200 g., were fasted for 12 hr. prior to the administration of aspirin-7-^{14}C but were allowed water *ad libitum* until the time of drug administration.

Drug Administration—To achieve the most rapid and uniform absorption, 0.1 mc. (9.3 mg.) of aspirin-7-^{14}C was administered orally as the sodium salt in 1.0 ml. of citrate buffer solution (pH 4.6).

Processing of Tissue—Two minutes after administration of the labeled aspirin, the rats were anesthesized with ether and the stomachs were removed. The stomachs were opened along the line of lesser curvature and the contents were collected in a small vial. Each stomach was stretched and pinned on a large rubber stopper, and the mucosal surface was rinsed with 100 ml. of 0.9% saline. Four minutes following administration, the stomachs were rapidly frozen with dry ice. The chest cavities of the rats were opened, and blood samples were obtained by cardiac puncture using syringes which had been previously swabbed with heparin[2]. The blood was placed in a centrifuge tube and centrifuged for 10 min. at 4600×g. While frozen, the rumen and corpus portions were separated. Each portion was minced and homogenized with 5 ml. of distilled water in a ground-glass tissue grinder, which was kept in an ice bath at 4° to inhibit enzymatic action and spontaneous hydrolysis of aspirin.

Identification of Metabolites—Following homogenization, the tissue samples were centrifuged at 4600×g for 15 min. Ten microliters of the aqueous supernate was spotted on thin-layer plates and analyzed for aspirin, salicylic acid, salicyluric acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and 2,3,5-trihydroxybenzoic acid, utilizing the solvent systems described later. Ten-microliter samples of blood plasma and gastric juice were analyzed for these compounds in a similar manner. The remaining tissue was resuspended in the supernate, and the suspension was analyzed for the presence of salicyl acyl- and salicyl phenolic glucuronides using an extraction method described by Schachter (8) and Schachter and Manis (9), followed by TLC, autoradiography, and liquid scintillation spectrometry.

To determine the amount of spontaneous hydrolysis of the aspirin occurring during storage and analytical procedures, an aliquot of the dose solution was added to homogenized rumen and corpus tissue taken from four untreated animals. These samples were subsequently treated exactly as the experimental samples. An aliquot of the dose solution itself was also analyzed.

TLC—A stationary phase of standard thickness (250 μ) silica gel G mixed with fluorescent indicator on a thin aluminum backing was used for all thin-layer chromatographs[3]. The following solvent systems were used to identify aspirin and its metabolites in the gastric juice, blood plasma, and rumen and corpus tissues. Petroleum ether (boiling range 30–60°) and propionic acid (10:1 v/v) were used to separate aspirin and salicylic acid from each other and from the rest of the metabolites. A benzene, ether, glacial acetic acid, and methanol (120:60:18:1 v/v) system was used to separate salicyluric acid from the rest of the metabolites. A system of benzene, glacial acetic acid, and water (2:2:1 v/v) was used to separate the polyhydroxy metabolites from each other and from the rest of the metabolites. In all cases, unlabeled, authentic standards of the compounds were used to identify the labeled compounds found.

In the final stages of the work, two additional solvent systems were used to confirm the identities of aspirin and salicylic acid.

[1] Holtzman Rat Co., Madison, Wis.
[2] Abbot Laboratories, North Chicago, Ill.
[3] Brinkmann Instruments Co., Westbury, N. Y.

Table I—Mean Percent[a] and Ranges of Total Activity of Aspirin and Its Metabolites in Rat Tissues 4 min. after Administration of Aspirin-7-^{14}C

Tissue Analyzed	Aspirin		Salicylic Acid		Salicyluric Acid		Percentage of Total Radioactivity Identified, Mean
	Mean	Range	Mean	Range	Mean	Range	
Gastric juice	91.1	71.3–97.9	6.9	1.5–22.0	0.1	0.09–0.19	98.1
Blood plasma	65.1	60.3–73.9	18.8	0.0–31.6	11.5	3.4–19.6	95.4
Rumen tissue	91.8	87.8–94.0	6.6	0.0–10.5	0.6	0–1.19	99.0
Corpus tissue	74.0	64.7–88.2	25.4	11.3–35.0	0.5	0.0–1.59	99.9

[a] All percentages are averages taken from four animals.

These systems were hexane, glacial acetic acid, and chloroform (4:1:1 v/v) and carbon tetrachloride, ether, glacial acetic acid, and methanol (120:60:18:1 v/v).

After developing and drying the TLC plates, autoradiograms were made using no-screen medical X-ray film. Following exposure and development of the film, the R_f values of the standards (observed with UV radiation) were compared with the R_f values of the labeled compounds (observed by autoradiography). Subsequently, the areas corresponding to the R_f values of the labeled compounds, the origin, and the solvent front were removed and placed in counting vials, and the amount of radioactivity in each sample was determined by liquid scintillation spectrometry[4]. The internal method of standardization (10) was used to determine the disintegration rate of each sample. The scintillation solution consisted of 300 ml. xylene, 900 ml. dioxane, 900 ml. 2-ethoxylethanol[5], 21 g. diphenyloxazole, and 168 g. naphthalene.

RESULTS AND DISCUSSION

The results of the analyses for aspirin and its metabolites in the gastric juice, blood plasma, and rumen and corpus tissues of the rat stomachs are presented in Table I. The percentage activity reported represents the mean percentage activity calculated from individual percentage activities from four animals. Each individual percentage activity represents the percentage of the activity for the compound in question relative to the total amount of activity spotted on the thin-layer plate. No glucuronide conjugates of salicylic acid or polyhydroxy metabolites of aspirin were detected.

Trace amounts of a compound having the same R_f value as salicyluric acid were found in all samples examined for salicyluric acid, but it occurred as a significant percentage of total activity only in blood. The absolute amounts were so small (calculated to be approximately 10^{-11} g.) that further verification of identity was not warranted. In addition, very low levels of labeled but unidentified compounds were detected at the origin and solvent front in all systems studied.

The major components found in the gastric juice, blood plasma, and rumen and corpus tissues were aspirin and salicylic acid. The amounts of aspirin found (corrected for spontaneous hydrolysis), expressed as percentage of total radioactivity, were as follows: gastric juice, 91.1%; blood plasma, 65.9%; rumen, 92.1%; and corpus, 74.0%. Spontaneous hydrolysis of aspirin equaled 1.1% in the gastric juice and blood, 6% in the rumen tissue, and 8.4% in the corpus tissue. The amount of salicylic acid found (corrected for spontaneous hydrolysis of aspirin) was as follows: gastric juice, 6.9%; blood plasma, 18.8%; rumen, 6.6%; and corpus, 25.4%. The amount of salicylic acid found in the corpus tissue was fourfold higher than the amount found in the rumen tissue (25.4 *versus* 6.6%). Analysis of variance showed no significant difference between: (*a*) gastric juice and rumen tissue, (*b*) blood plasma and corpus tissue, and (*c*) animals, and it showed a significant difference between: (*a*) aspirin, salicylic acid, and salicyluric levels in all tissues, and (*b*) rumen and corpus tissue with respect to salicylic acid and aspirin levels ($p = 0.05$).

Since lesions occur only in corpus tissue under the conditions of this study, it is tempting to speculate that the higher levels of salicylic acid in that tissue may be related to lesion production. Historically, one undesirable side effect of sodium salicylate was gastric irritation, which was partly overcome by substitution on the phenolic hydroxyl group (11). It is possible to speculate that higher levels of esterase activity in corpus cells may account for the increased amounts of free salicylic acid in that tissue. The significance of this finding will be the object of future research.

[4] Tri-Carb liquid scintillation counter, model 3003, Packard Instrument Co., Downers Grove, Ill.

[5] Cellosol.

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Synthesis of Heptacosapeptide (AA_{111}–AA_{137}) of the γ-Chain of a Human Immunoglobulin (IgGl) Using *N-tert*-Butyloxycarbonyl-L-amino Acid Pentachlorophenyl Esters

BRIAN J. JOHNSON

Abstract □ The synthesis of the heptacosapeptide (AA_{111}–AA_{137}) of the γ-chain of a human immunoglobulin (IgGl) is described. The solid phase method was used, and coupling of each amino acid residue was achieved by use of the corresponding *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl ester.

Keyphrases □ Heptacosapeptide (AA_{111}–AA_{137}) of γ-chain of a human immunoglobulin—synthesis using *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters □ *N-tert*-Butyloxycarbonyl-L-amino acid pentachlorophenyl esters—used in the synthesis of heptacosapeptide (AA_{111}–AA_{137}) of the γ-chain of a human immunoglobulin □ Immunoglobulins, human—synthesis of the heptacosapeptide (AA_{111}–AA_{137}) of the γ-chain using *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters □ Peptides—synthesis of the heptacosapeptide (AA_{111}–AA_{137}) of the γ-chain of a human immunoglobulin (IgGl) using *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters

Recently the total primary structure of the human immunoglobulin IgGl Eu[1] was elucidated (4–14). The domain hypothesis (15) for immunoglobulins suggests that each region of the molecule containing disulfide linkage forms a compact domain, and that these areas are connected by a more extended peptide chain. The switch peptide, sequence AA_{111}–AA_{137}, of the γ-chain of this immunoglobulin is most probably one of these extended peptide regions bridging between two respective domains.

Some *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters were previously described (16), and they were shown to be useful intermediates for rapid peptide synthesis by the solution method (17) and also for the solid phase method of peptide synthesis (18).

This article reports the characterization of some new *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters and their application for the synthesis of the fragment AA_{111}–AA_{137} of the γ-chain of a human IgGl.

DISCUSSION

The *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters (Table I) were prepared from the corresponding free acids using the general procedure outlined in the *Experimental* section. These derivatives were used to synthesize a fragment of the γ-chain of a human IgGl comprised of 27 amino acids (I and II).

The fully protected, resin-bound peptide I was synthesized by the stepwise addition of the appropriate *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl ester to an insoluble polystyrene resin (19). The cycle for the addition of each amino acid residue consisted of the following steps: removal of the *N-tert*-butyloxycarbonyl protecting groups by treatment with excess anhydrous trifluoroacetic acid in methylene chloride, neutralization of the resulting salt with triethylamine in methylene chloride, and then double coupling the resulting free amino residue with three equivalents of the appropriate *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl ester in dimethylformamide for 4 hr., after which a further three equivalents of the same amino acid derivative was added and coupled for a further 4 hr. Where sterically hindered amino acid residues such as valine and leucine were attached to the resin, coupling times were increased to 12-hr. periods. At the end of the chain-lengthening sequence, the protected peptide was cleaved from its polymer support by liquid hydrogen fluoride (20, 21); all of the side-chain protecting groups were removed under these conditions. The peptide was purified by passage through two columns[2].

EXPERIMENTAL

General Procedure for Preparation of *N-tert*-Butyloxycarbonyl-L-Amino Acid Pentachlorophenyl Esters—The general procedure of preparation of these esters is illustrated by the preparation of *N-tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid pentachlorophenyl ester. To a solution of 9.7 g. (30 mmoles) of *N-tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid (22, 23) and 8 g. (30 mmoles) of pentachlorophenol in 100 ml. of methylene chloride, cooled to 0°, was added 6.8 g. (33 mmoles) of *N,N'*-dicyclohexylcarbodiimide. The mixture was stirred overnight at room temperature. The solid *N,N'*-dicyclohexylurea was filtered off, and the filtrate was evaporated to dryness under reduced pressure. The solid was dissolved in ethyl acetate and cooled, and a further crop of *N,N'*-dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness and the solid material was crystallized from methanol to yield the pentachlorophenyl activated ester, 13.0 g, 75%, m.p. 129–130°; $[\alpha]_D$ − 28.9° (c 2.6, dimethylformamide).

Anal.—Calc. for $C_{15}H_{15}Cl_5N_2O_5$: C, 46.2; H, 3.5; N, 2.45. Found: C, 46.0; H, 3.4; N, 2.5.

BOC-Gly-Leu-Val-O-Bzl-Thr-Val-O-Bzl-Ser-O-Bzl-Ser-Ala-O-Bzl-Ser-O-Bzl-Thr-ε-N-Z-Lys-Gly-Pro-O-Bzl-Ser-Val-Phe-Pro-Leu-Ala-Pro-O-Bzl-Ser-O-Bzl-Ser-ε-N-Z-Lys-O-Bzl-Ser-O-Bzl-Thr-O-Bzl-Ser-Gly-Resin (I)—*N-tert*-Butyloxycarbonyl-Gly resin (2 g.), containing 0.56 mmole of Gly, was prepared from Bio-Beads S-X2, 200–400 mesh, by standard procedures (24). This substituted resin was treated for the incorporation of each amino acid by the following steps:

BOC-Gly-Leu-Val-Thr-Val-Ser-Ser-Ala-Ser-Thr-Lys-Gly-Pro-Ser-Val-Phe-Pro-Leu-Ala-Pro-Ser-Ser-Lys-Ser-Thr-Ser-Gly-Polymer

Bzl Bzl Bzl Bzl Bzl Z Bzl Bzl Bzl Z Bzl Bzl Bzl

I

H-Gly-Leu-Val-Thr-Val-Ser-Ser-Ala-Ser-Thr-Lys-Gly-Pro-Ser-Val-Phe-Pro-Leu-Ala-Pro-Ser-Ser-Lys-Ser-Thr-Ser-Gly-OH

II

[1] Nomenclature recommended by the International Union of Immunological Societies (1–3).

[2] First Dowex 1-X4 and then Sephadex G-25.

Table I—*N-tert*-Butyloxycarbonyl-L-amino Acid Pentachlorophenyl Esters

Compound	Yield, %	Melting Point	Molecular Formula	Analysis, % Calc.	Found	$[\alpha]_D^{25}$
BOC[a]-β-Bzl-Asp	70	130°	$C_{22}H_{20}Cl_5NO_6$	C 46.2 H 3.5 N 2.45	46.0 3.4 2.5	−28.9°(c 2.6, DMF[b])
BOC-Asn	63	176°	$C_{15}H_{15}Cl_5N_2O_5$	C 37.25 H 3.75 N 5.8	37.1 3.7 5.7	−41.7°(c 2.1, DMF)
BOC-S-Bzl-Cys	81	154°	$C_{21}H_{20}Cl_5NO_4S$	C 45.1 H 3.6 N 2.5	45.1 3.6 2.6	−38.2°(c 1.7, DMF)
BOC-γ-OBzl-Glu	80	138°	$C_{23}H_{22}Cl_5NO_6$	C 47.1 H 3.95 N 2.4	47.0 3.9 2.4	−27.1°(c 3.0, DMF)
BOC-Gln	57	186°	$C_{16}H_{17}Cl_5N_2O_5$	C 38.9 H 3.5 N 5.7	39.0 3.7 5.6	−24.4°(c 2.25, DMF)
BOC-N^{im}-DNP-His	51	170°	$C_{23}H_{18}Cl_5N_5O_8$	C 41.2 H 2.7 N 10.5	41.4 2.9 10.6	+3.9°(c 2.7, DMF)
BOC-Met	90	139°	$C_{16}H_{18}Cl_5NO_4S$	C 38.6 H 3.6 N 2.8	38.5 3.6 2.9	−37.0°(c 2.3, DMF)
BOC-Pro	93	117°	$C_{16}H_{16}Cl_5NO_4$	C 41.45 H 3.5 N 3.0	41.2 3.6 3.1	−32.5°(c 3.9, DMF)
BOC-Ser	39	157°	$C_{14}H_{14}Cl_5NO_5$	C 37.05 H 3.1 N 3.1	37.2 3.0 3.2	−31.0°(c 1.85, DMF)
BOC-O-Bzl-Ser	63	76°	$C_{21}H_{20}Cl_5NO_5$	C 46.4 H 3.7 N 2.6	46.5 3.6 2.8	−13.4°(c 2.25, DMF)
BOC-Thr	41	196°	$C_{15}H_{16}Cl_5NO_5$	C 38.5 H 3.45 N 3.0	38.3 3.6 3.0	−29.0°(c 2.35, DMF)
BOC-O-Bzl-Thr	65	126°	$C_{22}H_{22}Cl_5NO_5$	C 47.4 H 4.0 N 2.5	47.2 4.1 2.6	−16.0°(c 2.35, DMF)
BOC-Trp	70	195°	$C_{23}H_{19}Cl_5N_2O_4$	C 47.8 H 3.5 N 5.1	47.9 3.4 5.3	−49.2°(c 1.65, DMF)

[a] BOC = *N-tert*-butyloxycarbonyl. [b] DMF = dimethylformamide.

1. Three washings with 20-ml. portions of methylene chloride.
2. Cleavage of the *tert*-butyloxycarbonyl group by the addition of 20 ml. of 20% trifluoroacetic acid in methylene chloride and shaking of the mixture for 30 min.
3. Six washings with 20-ml. portions of methylene chloride.
4. Neutralization of the trifluoroacetate salt with 15 ml. of a 10% solution of triethylamine in methylene chloride for 10 min.
5. Three washings with 20-ml. portions of methylene chloride.
6. Addition of three equivalents of *N-tert*-butyloxycarbonyl-amino acid pentachlorophenyl ester in 15 ml. of dimethylformamide–methylene chloride (1:1) and shaking for 4 hr., and then a further three equivalents of the same *N-tert*-butyloxycarbonyl-amino acid pentachlorophenyl ester in 15 ml. of dimethylformamide–methylene chloride (1:1) and shaking for a further 4 hr. Wherever *N-tert*-butyloxycarbonyl-L-valine pentachlorophenyl ester and *N-tert*-butyloxycarbonyl-L-leucine pentachlorophenyl ester were used, the coupling time of 12 hr. for each stage was used.
7. Three washings with 20 ml. of dimethylformamide.
8. Three washings with 20 ml. methylene chloride.

After 26 cycles, the resin was dried under vacuum to yield 3.3 g.

Gly-Leu-Val-Thr-Val-Ser-Ser-Ala-Ser-Thr-Lys-Gly-Pro-Ser-Val-Phe-Pro-Leu-Ala-Pro-Ser-Ser-Lys-Ser-Thr-Ser-Gly (II)—The above protected peptide resin was suspended in 30 ml. of redistilled anhydrous hydrogen fluoride containing 5 ml. of anisole. The resin was stirred for 1 hr. at 0°, after which the anhydrous hydrogen fluoride and anisole were removed under reduced pressure. The resin mixture was washed with two washes of 10 ml. of ethyl acetate. The resin was dried and the peptide was extracted into 50 ml. of 0.1 *M* acetic acid and lyophilized. The lyophilized crude peptide (0.4 g., 31%) was dissolved in 0.1 *M* acetic acid, placed on a column[3] (6.5 × 121 cm.), and eluted with pyridine–acetic acid–water solution, pH 8.0.

The lyophilized crude peptide was purified by chromatography on a column[4] (2.5 × 100 cm.) using pyridine–acetic acid–water solution, pH 6.1, as eluent. The major peak was collected and lyophilized to yield 103 mg. (8%, based on the starting substituted resin) of the free peptide. Amino acid analysis of an acid hydrolysate showed Ser, 7.8; Thr, 2.8; Gly, 3.1; Pro, 2.9; Val, 2.8; Leu, 2.0; Ala, 2.0; Phe, 0.9; and Lys, 2.0. The peptide II was found to be homogeneous by paper chromatography: R_f 0.67 (butanol–acetic acid–water–pyridine, 15:3:12:10); R_f 0.54 (pyridine–acetic acid–water, 5:3:1.5).

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[3] Dowex 1-X4.

[4] Sephadex G-25.

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LEONARD C. HOWARD, Jr., DAVID R. BROWN, and DAVID A. BLAKE▲

Abstract □ The extent of binding of halothane-1-^{14}C to mouse liver and brain 24 hr. after injection of a subanesthetic dose was measured utilizing several techniques: (*a*) exhaustive dialysis with phosphate buffer, (*b*) solvent extraction with 0.25 *M* sucrose and toluene, (*c*) protein precipitation with trichloroacetic acid, and (*d*) soxhlet extraction with methanol and benzene. A substantial portion of the radioactivity was not removed by these procedures and thus was bound both in liver and brain, the liver containing a greater amount of bound radioactivity on both an absolute basis and a protein content basis. The liver microsomal fraction, subjected to exhaustive dialysis, contained a higher specific activity (disintegrations per minute per milligram protein) than the whole homogenate, 9000×*g* pellet, or cytosol. The combination of trichloroacetic acid precipitation and soxhlet extraction with methanol and benzene (1:1) removed virtually all radioactivity from the brain, whereas 13% of the initial radioactivity remained in the liver as tightly bound material. Thus, halothane or a metabolite is tightly and probably covalently bound to a protein or other high molecular weight component in mouse liver but not in the brain.

Keyphrases □ Halothane, radiolabeled—*toxicity*, subcellular binding in mouse liver and brain □ Toxicity, halothane—subcellular binding in mouse liver and brain, radiolabeled, type of bonding □ Hepatitis—subcellular binding of halothane-1-^{14}C to mouse liver and brain, type of bonding, distribution of radioactivity

Halothane (1,1,1-trifluoro-2-bromochloroethane) possesses two properties of the ideal anesthetic: it is potent and nonexplosive. However, fatal hepatic necrosis has been reported in a small portion of exposed human subjects (1–4). While the incidence of hepatitis after halothane reportedly represents no greater a risk than surgical anesthesia itself (5), it is nevertheless a well-established untoward reaction that deserves investigation. The recurrence of hepatitis in sensitized anesthetists after exposure to only traces of halothane (6, 7) and the overall low incidence which increases with multiple exposures (5) suggest that halothane-induced hepatic damage is the result of an allergic reaction.

If halothane hepatitis is caused by a sensitization reaction, then halothane or a metabolite must form a hapten of an antigen by binding covalently to a macromolecule (8). Nonvolatile metabolites of halothane have been found to persist in animal liver (9) as well as human urine (10, 11). The presence of high molecular weight metabolites of halothane in liver and urine (12) indicates that a metabolite of halothane may be associated with a macromolecule; however, no direct evidence exists as to whether these metabolites are, in fact, covalently bound. In support of this suggestion, other halogenated hydrocarbons, such as carbon tetrachloride (13) and bromobenzene (14), do form covalent bonds with endogenous substances. Therefore, the binding character of halothane-1-^{14}C *in vivo* in mice was investigated.

EXPERIMENTAL

Dosage and Tissue Preparation—Female Swiss Webster albino mice[1] (28–30 g.) were fasted for 24 hr., and 0.5 mg. of halothane-1-^{14}C in saline[2], containing 4 μc. of radioactivity was administered by intraperitoneal injection. Twenty-four hours later, the mice were sacrificed, the livers were perfused with cold 0.25 *M* sucrose, and both the livers and brains were removed and placed in ice-cold 0.25 *M* sucrose. Ten percent homogenates of these organs were prepared in cold 0.25 *M* sucrose, using a Potter–Elvehjem tissue grinder[3]. An aliquot of these homogenates was removed for analysis of bound radioactivity and protein, and the remainder was centrifuged at 9000×*g* (average) in a refrigerated centrifuge[4] for 20 min. The pellet was removed for analysis, and the supernate was recentrifuged at

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EXPERIMENTAL

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Table I—Effect of Trichloroacetic Acid Precipitation and 24-hr. Soxhlet Extraction with Methanol–Benzene (1:1) on the Radioactivity Found in the Livers and Brains of Mice Pretreated with Halothane-1-^{14}C (0.5 mg.) 24 hr. before Sacrifice

Organ	Trichloroacetic Acid Precipitate, Percent Total Organ Disintegrations per Minute Remaining, Mean ± *SE*[a]	Liver–Brain Ratio	Extracted Protein, Percent Total Organ Disintegrations per Minute Remaining, Mean ± *SE*[a]	Liver–Brain Ratio
Liver[b]	25.3 ± 1.6	7.4	13.1 ± 0.7	21.8
Brain[b]	3.4 ± 0.4		0.6 ± 0.04	

[a] Standard error of the mean. [b] $n = 6$.

78,480×g (average) in an ultracentrifuge[5] for 160 min. to prepare microsomes and cytosol, which were also analyzed for bound radioactivity and protein.

Exhaustive Dialysis—The livers and brains from six mice were combined and separated into the fractions as previously described. Each fraction was dialyzed in a visking tubing[6] against 4000 volumes of 0.01 *M* phosphate buffer (pH 7.2), which flowed continuously for 18 hr. at 4°[7]. Protein and radioactivity contents were determined both before and after dialysis. Protein was determined using the Lowry *et al.* (15) method with bovine serum albumin as the standard. The radioactivity was measured in a liquid scintillation spectrometer[8], and disintegrations per minute were calculated using toluene-^{14}C as an internal standard.

Washing, Extraction, and Centrifugation—The 9000×g pellet and microsomal fractions were separated from the 9000×g supernate and cytosol, respectively, resuspended three times in an equivalent volume of cold 0.25 *M* sucrose, and recentrifuged to remove residual water-soluble radioactivity. The washed 9000×g and microsomal pellets were then suspended in 10 times their volume of toluene, shaken manually, and recentrifuged to extract soluble nonpolar radioactivity.

Soxhlet Extraction—Protein was precipitated by the addition of an equal volume of 1 *M* trichloroacetic acid to a homogenate and removed by centrifugation at 2000 r.p.m. for 10 min. in a refrigerated centrifuge[9]. The denatured protein was washed three times with an aqueous 5% trichloroacetic acid solution and recentrifuged. An aliquot of the washed protein precipitate was taken for the determination of bound radioactivity. The remaining protein precipitate was placed in a thimble and extracted with methanol–benzene (1:1) for 24 hr.[10] in a microsoxhlet apparatus[11]. The final extracted protein was solubilized[12] and then analyzed for radioactivity.

RESULTS AND DISCUSSION

If halothane or a metabolite binds covalently with a hepatic macromolecule *in vivo*, then a portion of the liver radioactivity would not be dialyzable. To test this hypothesis, halothane-1-^{14}C was administered to mice. Then the brains and livers were removed, homogenized, fractionated, and subjected to exhaustive dialysis. The amount of nondialyzable radioactivity was determined. The results of this experiment (Figs. 1 and 2) indicate that a significant amount of nondialyzable radioactivity remains after 24 hr. in all liver fractions. Trifluoroacetate, a known metabolite of halothane (16, 17) probably constitutes most of the dialyzable fraction. The distribution of halothane-1-^{14}C-derived radioactivity in the liver was shown to predominate in the microsomal fraction, which had the highest amount of protein-associated counts. These results show that binding occurs in the liver, the location of halothane metabolism (18), and suggest that a metabolite, possibly formed in the endoplasmic reticulum, is the moiety bound.

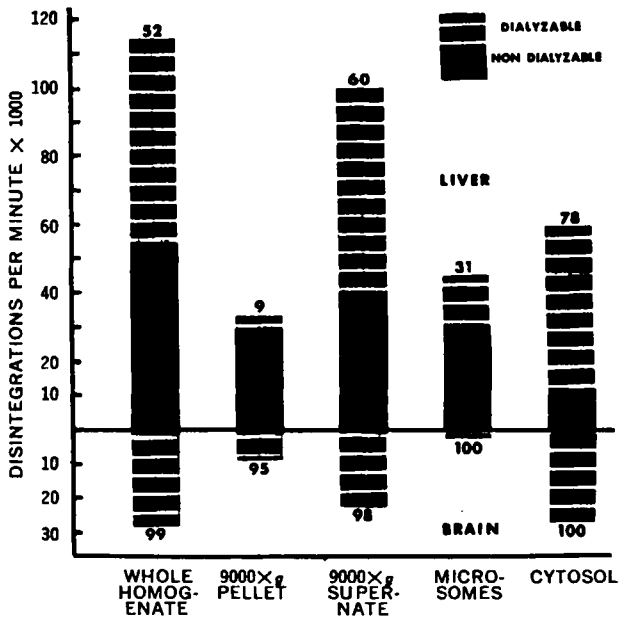

Figure 1—*Relationship between dialyzable and nondialyzable radioactivity in liver and brain subcellular fractions pooled from six mice pretreated with 0.5 mg. of halothane-1-^{14}C 24 hr. before sacrifice. (Numbers represent percentage of initial radioactivity that was dialyzable.)*

It is possible that either nonpolar halothane or a lipid-soluble metabolite is not removed by exhaustive dialysis against phosphate buffer. To test this alternative, the microsomal fraction was washed three times with 0.25 *M* sucrose and extracted with 10 volumes of toluene to remove lipid. This procedure removed 60% of the radioactivity from the microsomes, while only 31% was removed by dialysis. These results suggest that while halothane or a metabolite may be sequestered in lipid, at least 40% is bound to other tissue components. The data would also support the possibility that a portion of the radioactivity was not dialyzable against an aqueous buffer, because it was unmetabolized halothane. However, this is unlikely since dialysis did remove virtually all of the radioactivity from the brain, where small amounts of unchanged halothane would also be expected to reside under the conditions of the experiment. A more likely explanation is that part of the binding occurs on lipoprotein which, while not dialyzable, is nevertheless soluble in toluene.

To measure only protein-bound radioactivity, the liver and brain homogenates were treated with trichloroacetic acid followed by exhaustive extraction with solvent. Preliminary results of this trichloroacetic acid–soxhlet method showed that maximal extraction of radioactivity occurred at 24 hr., using a solvent system of equal amounts of methanol and benzene. Therefore, these conditions were used in all extractions. The results (Table I) show that 25.3% of the liver and 3.4% of the brain initial radioactivity were precipitated by trichloroacetic acid. The soxhlet technique extracted an additional 12.2% from the trichloroacetic acid precipitate of liver, leaving 13.1% of the initial radioactivity as tightly bound material. In contrast, soxhlet extraction of trichloroacetic acid precipitates from brain removed virtually all of the radioactivity in this tissue. The liver-to-brain ratio of radioactivity remaining after trichloroacetic acid precipitation increased from 7.4 to 21.8 after soxhlet extraction. This 22-fold higher amount of nonextractable radioactivity in the liver relative to the brain demonstrates that factors other than the initial concentration of halothane in a tissue determine the extent of binding.

[5] Model L-2, Rotor 30, Beckman Instruments, Palo Alto, Calif.
[6] No. 077-024, W. H. Curtin & Co., Rockville, Md.
[7] Dialysis for 24 additional hr. did not significantly reduce the amount of nondialyzable radioactivity, so this technique was considered to be exhaustive.
[8] Tri-Carb model 3003, Packard Instrument Co., Downers Grove, Ill. The scintillation cocktail consisted of: 2,5-diphenyloxazole, 0.55% w/v; 1,4-bis[2-(5-phenyloxazolyl)]benzene, 0.01% w/v; Triton X-100, 25% v/v; and toluene, 75% v/v.
[9] Model PR-2, International Equipment Co., Boston, Mass.
[10] These parameters were initially examined using various solvent systems (methanol, methanol–benzene (1:1), or benzene) for 6, 12, 24, or 48 hr.
[11] VWR Scientific Co., Baltimore, Md.
[12] Using hyamine hydroxide, Packard Instrument Co., Downers Grove, Ill.

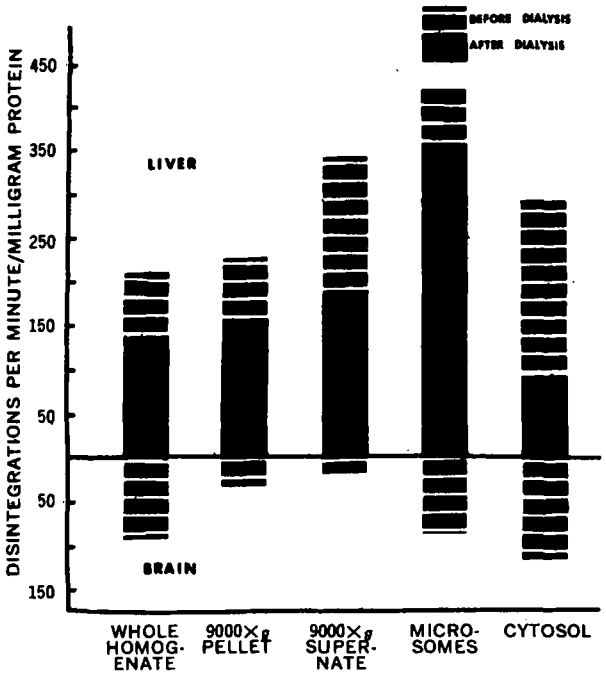

Figure 2—*Effect of dialysis on the specific activity (disintegrations per minute per milligram protein) of pooled liver and brain subcellular fractions from six mice pretreated with 0.5 mg. of halothane-1-^{14}C 24 hr. before sacrifice.*

Since exhaustive dialysis and centrifugal washing do not distinguish between reversible and irreversible binding, the method of solvent (soxhlet) extraction was employed. A similar technique has been used to demonstrate the covalent binding of bromobenzene in the liver (14). The trichloroacetic acid–soxhlet method extracted more halothane-derived radioactivity from whole homogenates of liver than either dialysis or washing, indicating that some of the radioactivity remaining after the use of the latter methods was possibly due to reversibly bound metabolites or that the labeled material may have been covalently bound to lipoprotein. Although trichloroacetic acid precipitation and solvent extraction will remove all of the radioactivity except that which is very tightly bound, it in itself does not constitute a completely rigorous proof of covalent binding. Therefore, protein digestion and the identification of amino acid-bound activity are currently being investigated.

If one proposes a hapten–antigen model for the hepatotoxicity of halothane, then the initial component of this hypothesis would require the covalent attachment of a small molecular weight moiety to some endogenous macromolecule. The altered molecule thus produced could elicit a delayed hypersensitivity *via* sensitized lymphocytes. The appearance of high levels of bound radioactivity in the membraneous fractions of the liver (*i.e.*, 9000×*g* pellet and microsomes) and the association of irreversibly and probably covalently bound radioactivity with protein in the whole liver homogenates subjected to soxhlet extraction are consistent with this suggestion that the altered macromolecule is a membrane protein. Furthermore, the protein-bound radioactivity found in the cytosol of the liver could possibly be the basis for transferral of antigenic material to sites of antibody formation.

From the results of other investigators (9, 10, 12, 17, 18), it would appear that halothane undergoes similar metabolic pathways in humans, mice, and rats. The finding of covalently bound radioactivity in hepatic tissue is consistent with the sensitivity theory and would also explain the low incidence of halothane-induced hepatitis in humans and the relative failure of investigators to reproduce hepatic injury in animals. Variability in both the immunologic and metabolic responses between and among various species may further explain these findings, and it is also possible that the binding phenomenon itself may be highly variable.

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▲ To whom inquiries should be directed.

Qualitative Method for Dantrolene and a Related Metabolite in Urine

JOHN D. CONKLIN▲ and R. J. SOBERS

Abstract □ A qualitative method is described for the determination of dantrolene, a new skeletal muscle relaxant, and a related nonreduced metabolite in human urine. The method consists of the direct extraction of the drug and the metabolite from urine into nitromethane. Addition of a hyamine hydroxide solution to the solvent extract from urines containing the drug and the metabolite results in a yellow color which is linearly related to the amount of drug and metabolite present. A yellow color is not apparent when the alkaline solution is added to solvent extracts from control urine. The method is specific for dantrolene plus the nonreduced metabolite in the presence of a reduced metabolite. Data obtained from urine specimens, collected from humans receiving sodium dantrolene and analyzed by the qualitative method, are also presented.

Keyphrases □ Dantrolene and metabolite—qualitative colorimetric determination in urine □ Colorimetry—qualitative determination of dantrolene and metabolite in urine

Sodium dantrolene[1], 1-{[5-(*p*-nitrophenyl)furfurylidene]amino}hydantoin sodium hydrate, is a new skeletal muscle relaxant (1–3). By utilizing a combination of solvent extractions and chromatography followed by fluorometric measurement, dantrolene is quantitatively determined in biological specimens (4). There still is need, however, for a qualitative method which the clinical laboratory can readily perform. This report describes a simple and rapid colorimetric method for dantrolene and a related nonreduced metabolite, designated as Metabolite A, in human urine.

EXPERIMENTAL

Reagents and Instrument—The reagents used included: crystalline dantrolene[2]; ammonium sulfate[3], special enzyme grade; *N,N*-dimethylformamide[4], reagent grade; nitromethane[5], practical grade; methanol[6], analytical reagent grade; and hyamine hydroxide[7], 1 *M* in methanol. The 1 *M* hyamine solution (2 ml.) is diluted to 25 ml. with absolute methanol to obtain a 0.08 *M* solution.

A spectrophotometer[8] was used to measure absorbances.

Procedure—To 2 ml. of urine in a tube, add 1 ml. of water and then 3 ml. of saturated ammonium sulfate solution and mix thoroughly. Add 5 ml. of nitromethane, mix the tube contents vigorously for 1 min., and centrifuge at 2000 r.p.m. for 10 min. Transfer 3 ml. of the solvent extract (top layer) to a test tube. Then add 5 drops of 0.08 *M* hyamine hydroxide solution and mix the contents.

In the qualitative test, the color of the extract from the unknown sample is immediately compared with the color of the corresponding extract from a control sample against a white background. Under these conditions, extracts from urine specimens containing dantrolene and Metabolite A exhibit a yellow color which is linearly related to the amount of drug and metabolite present. Control urine extracts do not exhibit any apparent yellow color when the hyamine reagent is added.

[1] Dantrium, Eaton Laboratories.
[2] Eaton Laboratories.
[3] Schwarz/Mann, Catalog No. 1946.
[4] Matheson, Coleman and Bell, Catalog No. DX1730.
[5] Commercial solvents.
[6] Mallinckrodt Chemical Works, Catalog No. 3016.
[7] Packard Instrument Co., Catalog No. 6003005.
[8] Hitachi model 139.

Table I—Determinations of Dantrolene and Metabolite A Standards by the Nitromethane–Hyamine Method

Sample, mcg./ml.	Absorbance[a], 400 nm. Dantrolene Water	Dantrolene Urine	Metabolite A Water	Metabolite A Urine
Control	0.009	0.033	0.006	0.037
2.5	0.079	0.083	0.078	0.077
5.0	0.157	0.162	0.154	0.158
15.0	0.478	0.483	0.464	0.469
25.0	0.780	0.799	0.759	0.765

[a] Mean based on three samples. Absorbances for standards are control corrected.

If an estimate of the drug concentration is desired, the solvent extract is then subjected to spectrophotometric measurement at 400 nm. within 1 min. after addition of the hyamine reagent. Pure nitromethane is used to set the instrument to zero absorbance. The absorbance of the control sample is subtracted from the absorbance of the unknown sample, and the estimated drug and metabolite present are calculated from dantrolene standards subjected to the method. The results are expressed as dantrolene equivalents. A dantrolene standard with a concentration of 25 mcg./ml. exhibits an absorbance of about 0.800. Dilutions are necessary when a nitromethane extract from a sample subjected to the procedure exhibits an absorbance greater than 0.800 after addition of the hyamine reagent. If this occurs, some of the remaining nitromethane extract, not reacted with the hyamine reagent, is diluted with pure nitromethane and then the hyamine reagent is added.

In preparing standard solutions, 50 mg. of crystalline dantrolene is dissolved in 50 ml. of dimethylformamide. Ten milliliters of this solution is placed in a 100-ml. volumetric flask, 40 ml. of dimethylformamide is added, and the solution is diluted to volume with water to obtain a drug concentration of 100 mcg./ml. This solution is then diluted with water to obtain the required drug concentrations. Standards[2] of Metabolite A and acetylated dantrolene[9] are prepared in an identical manner.

Urine reference standards are prepared with 2 ml. of urine, 1 ml. of standard solution, and 3 ml. of saturated ammonium sulfate solution. Aqueous standards are prepared in an identical manner except that 2 ml. of water is substituted for the 2 ml. of urine. Water or the human urine used to prepare the reference standards serves as a control.

RESULTS

The results presented in Table I show that both dantrolene and Metabolite A are susceptible to the nitromethane–hyamine procedure. Standard curves for each compound are linear from 2.5 to 25.0 mcg./ml. Drug concentrations as low as 2.5 mcg./ml. (5 mcg./tube) are visually identified by a distinct yellow color. Agreement is apparent between aqueous and human urine reference standards for both dantrolene and Metabolite A (Table I). Average recoveries of added dantrolene and Metabolite A from human urine were 102.9 ± 1.6 and 100.8 ± 1.6%, respectively, based on aqueous standards. Acetylated dantrolene, another metabolite, did not exhibit appreciable absorbance when subjected to the nitromethane–hyamine procedure.

Urine specimens collected from healthy humans who had received sodium dantrolene as an encapsulated formulation orally were subjected to the nitromethane–hyamine method. Nitromethane

[9] 1-{[5-(*p*-Acetamidophenyl)furfurylidene]amino} hydantoin.

Table II—Determinations of Urine Specimens from Humans Administered Sodium Dantrolene by the Nitromethane–Hyamine Method[a]

Subject	Absorbance[b], 400 nm.	Concentration[c], mcg./ml.
3	0.098 (0.062)	3.06
6	0.149 (0.034)	4.65
2	0.719 (0.013)	22.47
5	0.745 (0.015)	23.28
11	0.541 (0.032)	16.90

[a] An encapsulated sodium dantrolene formulation was administered orally as a single dose at 100 mg. to Subjects 3 and 6 and at 100 mg. q.i.d. to Subjects 2, 5, and 11. Urine specimens were collected from 0–24 hr., pooled, and frozen. A control urine specimen was collected just before drug administration. [b] Control corrected; figure in parentheses represents the control urine absorbance. [c] Estimated drug concentration for 0–24 hr., expressed as dantrolene equivalents, represents dantrolene plus Metabolite A.

extracts from each of the specimens, collected after drug administration, exhibited a visual yellow color following the addition of the hyamine reagent (Table II). Under these conditions, a yellow color was not apparent with extracts from corresponding control urines, collected before drug administration.

The dantrolene–hyamine complex in nitromethane exhibits an absorbance maximum near 400 nm., while the corresponding Metabolite A–hyamine complex displays a maximum near 395 nm. When subjected to the nitromethane–hyamine procedure, the human urines collected after the administration of sodium dantrolene yielded an absorbance maximum from 380 to 385 nm.

An estimate of the drug and metabolite concentration present in the urine specimens was obtained by spectrophotometric measurement (Table II). These results are expressed as dantrolene equivalents, based on dantrolene standards, and represent dantrolene plus Metabolite A.

DISCUSSION

At present, two of the major dantrolene-related metabolites recovered from human urine after the administration of sodium dantrolene are reduced acetylated dantrolene and a nonreduced metabolite designated as A (5). The amount of each of these metabolites excreted in urine is usually much greater than the corresponding amount of dantrolene (5). It is assumed that dantrolene plus Metabolite A is primarily responsible for the yellow color observed when urine specimens collected from humans receiving sodium dantrolene are analyzed by the nitromethane–hyamine procedure, since standards of acetylated dantrolene do not exhibit an appreciable absorbance when subjected to the qualitative method. A partial explanation for this specificity is that acetylated dantrolene does not contain the nitro group on the benzene ring which is present in both dantrolene and Metabolite A.

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COMMUNICATIONS

Dansyl Derivatives of Δ^9- and Δ^8-Tetrahydrocannabinols

Keyphrases □ Dansyl derivatives of Δ^9- and Δ^8-tetrahydrocannabinols—synthesis, physical and chemical properties □ Tetrahydrocannabinols—*synthesis of dansyl derivatives*, physical and chemical properties

Sir:

The use of dansyl chloride (5-dimethylamino-1-naphthalenesulfonyl chloride, I) in the fluorometric determination of amines and phenols is well documented (1). Dansylated cannabinol-related compounds were recently derived from blood, urine, and saliva samples following hashish administration (2). As a prerequisite for the development of suitable methodology for the assay of compounds and metabolites derived from *Cannabis sativa*, a series of dansyl derivatives of several cannabinoid compounds was prepared in micromolar quantities and their TLC properties were reported (3). The preparation of larger quantities of dansyl-Δ^9-tetrahydrocannabinol (II) and dansyl-Δ^8-tetrahydrocannabinol (III) was accomplished to determine the physical and chemical properties of these derivatives.

The reaction of Δ^9- or Δ^8-tetrahydrocannabinol and excess dansyl chloride was performed in acetone–water solution saturated with sodium carbonate at 40° for 2 hr. After excess dansyl chloride was hydrolyzed by treatment with base, the reaction mixture was extracted with ethyl acetate. Compounds II and III were isolated as thick oils. Crystallization could only be induced by prolonged storage at −5° in hexane. Recrystallization from hexane or heptane yielded crystalline solids which provided microanalytical values within acceptable limits and the correct molecular weight by mass spectrometry. The physical and spectral characteristics of Compounds II and III are summarized in Tables I and II.

The reaction of I would be expected to occur on the phenolic group of tetrahydrocannabinol. Examples are known with reactions similar to dansylation where the substituent does not add onto the phenolic oxygen but

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	II	III
Appearance	Greenish-yellow crystals	Greenish-yellow crystals
Melting point (uncorrected)	81–84°	105–107°
λ_{max} in ethanol	345 nm. (ε 4100) 283 s (4590) 253 (15,860)	345 nm. (ε 3780) 283 s (3880) 253 (14,800) 215 s (46,000)
λ_{max} excitation	350 nm.	350 nm.
λ_{max} emission	530 nm.	530 nm.

is substituted into the nucleophilic 2-position of the aromatic ring (4, 5). On NMR analysis in deuteriochloroform, the dansylation products of Δ^9- or Δ^8-tetrahydrocannabinol exhibit two doublets, each integrating for one proton, in the aromatic region (II, δ = 6.45 p.p.m., 6.22; III, δ = 6.50 p.p.m., 6.27) with a coupling constant of 1.5–2.0 Hz. These signals fit an *AB* system characteristic of *meta*-coupled aromatic protons. The more downfield proton corresponds to the 4-position proton and the other to the 2-position proton. On this basis, II and III are proposed to be *O*-dansyl and not C-dansyl compounds. The absence of any exchangeable protons upon the addition of D_2O provides further evidence to support the proposed structure.

Dansylated compounds were reported (1) to decompose when allowed to remain on silica gel for prolonged periods. In our experience, if pure II was left on a silica gel thin-layer plate for over 4 hr. in a dry state, the original greenish-yellow fluorescence of the spot took on a dull-orange color. Scraping and rechromatographing this spot gave at least two additional spots. This observation suggests that II undergoes significant degradation under the test conditions. The literature further documents examples of instability of dansylated phenols to UV exposure (6). Irradiation of Compounds II and III on a silica gel plate with 350- or 254-nm. UV light caused changes in TLC properties after only 10 min.

Solutions of II and III in ethanol slowly decompose regardless of storage conditions. After storage at room temperature and exposure to laboratory light, changes in color and in the UV absorption spectrum were apparent after 1 week. Refrigeration delayed these changes for several weeks. However, storage of the crystals in screw-capped vials under nitrogen, in the dark, in a desiccator produced no observable changes in TLC properties in more than 3 months.

Table II—Low-Resolution Mass Spectra of Dansyl-Δ^9- and Dansyl-Δ^8-tetrahydrocannabinols (II and III)

	II			III	
	m/e	Relative Abundance		*m/e*	Relative Abundance
M^+	547	8	M^+	547	18
$M-SO_2$	483	3	$M-SO_2$	483	14
	464	2		464	16
	412	6		412	3
	314	31		313	9
	313	100		231	3
	231	4		171	100
	171	78		170	86
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Mechanism of Phenobarbital Degradation

Keyphrases □ Phenobarbital—mechanism of degradation, products identified □ Barbiturate degradation—mechanism of ring cleavage, phenobarbital and products

Sir:

In a recent paper, Garrett *et al.* (1) further elucidated the kinetics of hydrolysis of several important barbiturates. In their studies, they discovered the rather surprising fact that diethylmalonuric acid (V) (Scheme I) in basic solution may cyclize to form the parent-substituted barbituric acid, barbital (IV). Previous workers (2, 3) assumed that the hydrolysis of the parent barbiturate to the corresponding malonuric acid was irreversible, and various degradation schemes were predicated on that assumption. Hegarty and Bruice (4) also reported a similar reaction in the cyclization of 2-ureidobenzoic acid.

We have now repeated the work relative to diethylmalonuric acid and verified by mass spectrometry that the cyclization product of diethylmalonuric acid in basic solution is barbital. The reversibility of the hydrolysis of the barbituric acid nucleus is an important discovery and may have interesting biological ramifications.

In discussing the reversibility of this reaction, Garrett *et al.* (1) challenged the mechanism of phenobarbital degradation proposed by Tishler *et al.* (3). They put forward a rather tortuous argument to explain the ex-

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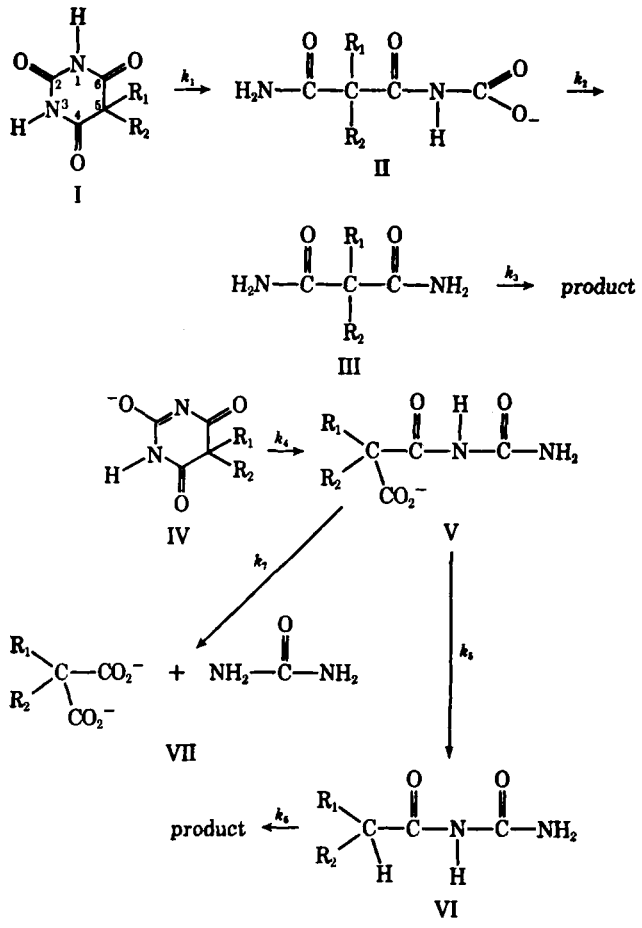
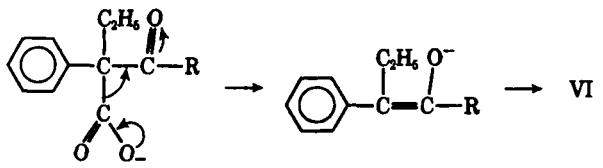


Scheme I—Degradative pathway of barbituric acid derivatives (barbital: R_1, R_2 = ethyl; phenobarbital: R_1 = ethyl, R_2 = phenyl)

perimental results of Tishler and coworkers. However, their argument depends on the reversibility of the phenylmalonuric acid formation in a manner analogous to diethylmalonuric acid. In the paper where Fretwurst (5) reported recovery of 50% of degraded barbital as the diethylmalonuric acid, he also reported finding no corresponding malonuric acid under several different conditions with phenobarbital. Several other barbiturates, substituted such that the malonuric acid derived from them contains a group that can conjugate with the ureide carbonyl in the enol intermediate postulated for the decarboxylation mechanism (Scheme II), likewise were devoid of isolatable substituted malonuric acid. If the mechanism proposed by Hegarty and Bruice (4) for the cyclization of 2-ureidobenzoic acid is correct, different malonuric acids should cyclize at about the same rate (in basic solution) since the mechanism depends upon the nucleophilicity of the terminal amino group, which would be only minimally affected by different substituents. It would, therefore, appear that substitution of one of the ethyl groups of barbital by the phenyl group results in labilization of malonuric acid, such that decarboxylation occurs at a much higher rate than in the barbital product, with little change in the cyclization rate, making the step from phenobarbital to phenylethylmalonuric acid essentially irreversible, as assumed by Tishler *et al.* (3).

Moreover, Fretwurst (5), when using two equivalents of sodium hydroxide for each equivalent of phenobarbital, identified 73% of the reaction product as



Scheme II—Postulated mechanism for decarboxylation of disubstituted malonuric acid

malonic acid (VII) instead of isolating the substituted acetylurea (VI) as the primary product, apparently indicating that the hydrolysis to malonic acid at higher base concentrations proceeds at a more rapid rate than the parallel decarboxylation reaction.

The known facts relative to barbiturate degradation would fit a scheme in which the unionized barbiturate can be cleaved at the one–two position, leading to production of the bisamide (III), or at the one–six (three–four) position, leading to the ureide (VI); the ionized barbiturate would cleave only at the one–six (three–four) position, leading to the ureide (or malonic acid) exclusively. For example, in the paper by Tishler *et al.*, (3) the equation describing the ureide pathway would be:

$$f = \frac{k_4K_a + k_u[H^+]}{k_d[K_a] + k_1[H^+]} \qquad \text{(Eq. 1)}$$

assuming that k_1, mentioned in the previous paper (3), is the sum of the rate constant relating production of the ureide, k_u, and the rate constant relative to the rate of production of the bisamide, k_d, from undissociated phenobarbital (I). In the 8.2–9.5 pH range studied by the authors, the K_a (10^{-7}–10^{-8}) of phenobarbital would be much greater than the hydrogen-ion concentration, and one could assume with Garrett *et al.* (1) that the ureide pathway for the unionized barbiturate is severely hampered through steric hindrance by the presence of the phenyl group at the five position; thus, the product k_4K_a would be much greater than $k_u[H^+]$, and the results found by Tishler *et al.* (3) are explained. On the other hand, the results also reported by Garrett *et al.* (1) relative to the hydrolysis of barbital at pH 6.15, where the ureide represents 84% of hydrolyzed barbital, is also explicable in allowing the nonsterically hindered hydroxide-ion attack at the one–six or three–four position. It is certainly clear that more work is needed on the hydrolysis of barbiturates, particularly on the degradation beyond the initial cleavage of the barbituric acid itself.

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Wilforine

Keyphrases ☐ Wilforine—duplicated nomenclature for two structurally different compounds ☐ Nomenclature duplication—wilforine used to identify two structurally different compounds

Sir:

Recently, Hayashi and Mitsuhashi (1) reported on the separation and structure determination of a new pregnane derivative from *Cynanchum wilfordi*, to which they assigned the trivial name wilforine.

An alkaloid of as yet undetermined structure was isolated by Beroza (2) in 1952 from the unrelated plant *Tripterygium wilfordii*, which he named wilforine. The same alkaloid was more recently isolated from *Maytenus senegalensis* (3).

Since these two substances are obviously dissimilar, the identical naming of them has created confusion in the literature. The product from *C. wilfordi* should be renamed by Hayashi and Mitsuhashi in subsequent publications.

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(3) M. Tin-Wa, N. R. Farnsworth, H. H. S. Fong, R. N. Blomster, J. Trojánek, D. J. Abraham, G. J. Persinos, and O. B. Dokosi, *Lloydia*, **34**, 79(1971).

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Antitumor Agents V: Effect of Epoxidation on Cytotoxicity of Helenalin-Related Derivatives

Keyphrases ☐ Helenalin derivatives—epoxidation, effect on cytotoxicity ☐ Epoxidation of helenalin derivatives—effect on cytotoxicity ☐ Cytotoxicity, helenalin derivatives—effect of epoxidation on activity ☐ Structure–activity relationships—helenalin derivatives, epoxidation and cytotoxicity

Sir:

In connection with our study of the structure–activity relationships among helenalin (I)-related sesquiterpene lactones for cytotoxic or antitumor activity (1–3), we wish to report a preliminary account of the effect of epoxidation on cytotoxicity of helenalin-related derivatives.

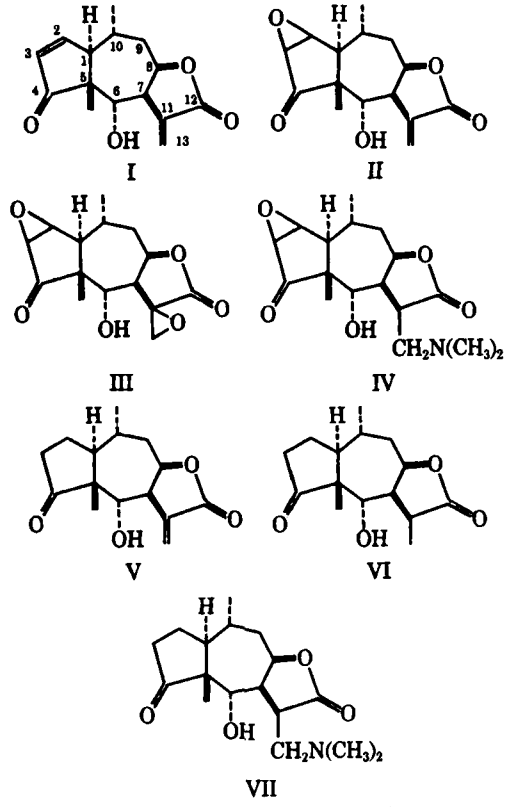
The epoxy function is a structural feature commonly found in many naturally occurring sesquiterpene lactones. It is well known that certain classes of synthetic compounds (4), as well as naturally occurring substances, owe their antitumor or cytotoxic activity to the introduction of the diepoxide or triepoxide functionality; *e.g.*, the cyclohexane diepoxide, crotepoxide (5); the sesquiterpene dilactone diepoxide, mikanolide (6, 7); and the diterpene triepoxides, triptolide and tripdiolide (8). With this in mind, it has been inferred that one important factor governing the cytotoxicity of the sesquiterpene lactones could be due to the introduction of the epoxy group. The epoxy group might act as a second alkylating function in addition to the essential alkylating center, the α-methylene-γ-lactone moiety (1, 9, 10). However, it was suggested (6) that cytotoxicity appears to be independent of the presence or absence of an epoxy group, although this conclusion was based upon only a small number of examples.

We felt that the role of the epoxide function with respect to the effect upon cytotoxicity should be further

Table I—Cytotoxicity of Helenalin-Related Derivatives

Number	Compound	Reference	ED_{50}, mcg./ml. (H.Ep.-2)
I	Helenalin	1–3	0.10
II	2,3-Epoxyhelenalin	—[a]	0.11
III	2,3,11,13-Diepoxyhelenalin	—[a]	0.50
IV	2,3-Epoxyhelenalin dimethylamine adduct	—[a]	1.36
V	2,3-Dihydrohelenalin	2	3.84
VI	2,3,11,13-Tetrahydrohelenalin	2	>40
VII	2,3-Dihydrohelenalin dimethylamine adduct	2	6.04

[a] K. H. Lee, S. H. Kim, H. Furukawa, C. Piantadosi, and E. S. Huang, unpublished data.



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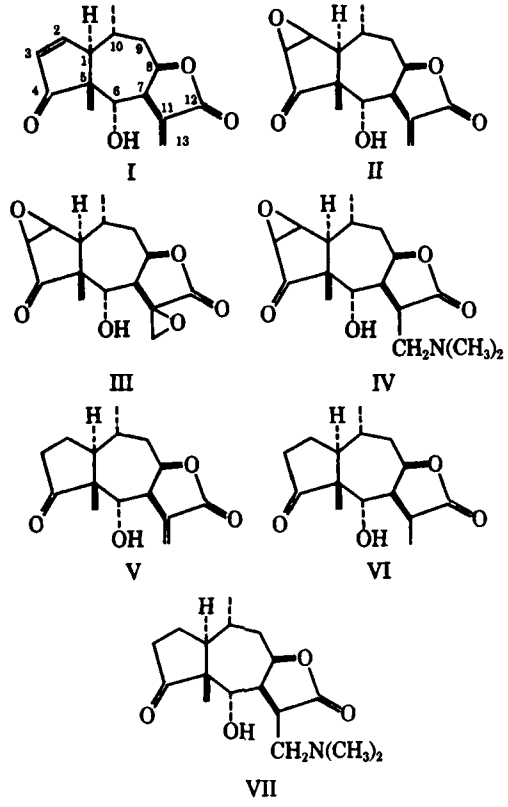

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Comparison of the ED_{50} values for the cytotoxicity of the compounds listed in Table I disclosed that both the 2–3 double bond of Compound I and the 2–3 epoxide of Compound II gave equally effective cytotoxicity. The corresponding saturated compound (V) gave a 35-fold decrease in activity. Significant cytotoxicity could also be maintained when the two alkylating centers, such as the O=C—C=CH_2 system in the ketone and the lactone of helenalin, were masked by the epoxy moiety, although the diepoxide (III) was 5 times less active in comparison with helenalin (I). However, the absence of the diepoxy functionality in Compound III resulted in more than an 80-fold diminution in cytotoxicity (compare Compounds III and VI). Similar results were seen in the case of the dimethylamine adduct of 2,3-epoxyhelenalin (IV). As the epxoy group was removed, the activity was decreased (compare Compounds IV and VII). Moreover, a comparison of the activities of Compound II to Compounds I and III further indicated that the α-epoxyketonic moiety played a more important role than the α-epoxy-γ-lactonic moiety in the contribution and maintenance of the high level of cytotoxicity.

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[1] K. H. Lee, S. H. Kim, H. Furukawa, C. Piantadosi, and E. S. Huang, unpublished data.

Commencement of Basket Rotation Time as Variable in Official Dissolution Test

Keyphrases ☐ Dissolution, tablets—basket rotation time as variable in compendial dissolution test ☐ Basket rotation time—variable in compendial dissolution testing of tablets

Sir:

The dissolution characteristics of many dosage forms are now being determined by using the procedures described in either USP XVIII or NF XIII (1, 2). NF Method I and the procedure described in the USP are based on the use of a basket–stirrer assembly. This method, although official, has been criticized by a number of researchers[1] (3, 4) who found that variable results occur because of vibrational effects within the apparatus, clogging of the 40-mesh screen, and poor stirring characteristics of the assembly. During an investigation of the dissolution characteristics of several brands of chlorpromazine hydrochloride tablets, we observed another possible variable which should be considered when using this apparatus.

The compendia state that the dosage form should be placed in the basket, immersed in the dissolution medium to a point where the bottom of the basket is 2.0 cm. from the bottom of the dissolution vessel, and rotated at the speed specified in the monograph. This procedure can be varied ("Modified USP XVIII Method" in Table I) by placing the tablet in the basket, rotating the basket at the speed specified, and then immersing the rotating basket to the required depth in the dissolution medium.

Dissolution values (Table I) for one brand of chlorpromazine hydrochloride tablets were obtained by using the USP XVIII procedure and the modification of it just described. The dissolution medium was simulated gastric fluid USP (without enzyme), the total volume of medium was 900 ml., and the basket was rotated at 50 r.p.m.

The tablets were purported to contain 25.0 mg. of active ingredient. Ten tablets were assayed individually by using the procedure described in USP XVIII. Values ranged from 24.60 to 25.75 mg./tablet and indicated that there was little variability with respect to drug content.

Table I shows that drastically different results are obtained by a seemingly minor modification in the USP procedure. For example, $T_{60\%}$ values change from 23.4 to 39.3 min. but, at the same time, the modified USP method yields more reproducible results (that is, the standard deviation values are, in general, less than those reported in column 2 of Table I).

The reason for the variation in results is not at once evident but appears to be related to the mesh size of the basket (40 mesh). Studies with a 10-mesh basket yielded relatively the same values by both methods, but the values were much less than those shown in Table I. For example, the $T_{60\%}$ value for this product was ap-

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Table I—Dissolution Values for Chlorpromazine Hydrochloride Tablets

T Value[a]	USP XVIII Method	Modified USP XVIII Method
$T_{30\%}$	4.5 ± 1.3	6.7 ± 2.2
$T_{40\%}$	12.5 ± 4.4	20.5 ± 2.3
$T_{60\%}$	23.4 ± 7.6	39.3 ± 1.8
$T_{80\%}$	35.8 ± 10.3	60.6 ± 2.7

[a] Each T value (min.) is the mean of 10 determinations ± SD.

proximately 9 min. when a 10-mesh basket was used. Furthermore, variable results were not obtained when other brands of chlorpromazine hydrochloride tablets were tested or, if observed, the degree of variability was much less than that shown in Table I. This would appear to indicate that the inherent characteristics of the tablets tested led, in part, to the type of variability reported here.

The "correct" dissolution conditions are always difficult to establish, particularly in the absence of *in vivo* data. It is important, therefore, to standardize a procedure carefully in order to evaluate accurately various lots of the same drug product. From the results reported here, it appears that the time at which the rotation of the basket is commenced is important and that any variation from a standardized approach may yield variable results.

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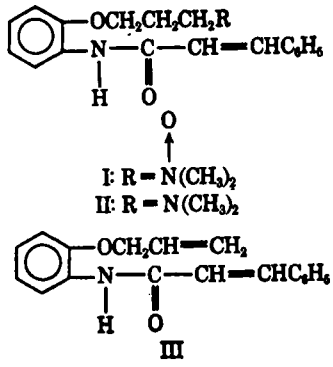
Identification of 2′-[3-(Dimethylamino)-propoxy]cinnamanilide *N*′-Oxide by Mass Spectral Thermolysis

Keyphrases □ 2′-[3-(Dimethylamino)propoxy]cinnamanilide *N*′-oxide—identified using mass spectral thermolysis □ *N*-Oxides—identification of 2′-[3-(dimethylamino)propoxy]cinnamanilide *N*′-oxide using mass spectral thermolysis □ Mass spectral thermolysis—identification, *N*′-oxide of 2′-[3-(dimethylamino)propoxy]cinnamanilide

Sir:

The occurrence of *N*-oxides as natural products or as metabolites of pharmacologically active compounds



III

was reviewed previously (1). The only mass spectral studies of *N*-oxides have been those of aromatic *N*-oxides that usually exhibit the M^+-16 ion, instead of the M^+, as the highest mass ion (2). Mass spectrometry was used to elucidate the structures of some thiopropyldimethylamino-*N*-oxide metabolites (3). In this report, these observations are extended to the thermolysis of 2′-[3-(dimethylamino)propoxy]cinnamanilide *N*′-oxide (I).

Since volatilization of the *N*-oxide base, I (mol. wt. 340), occurs very near its thermolysis point, the spectra obtained at temperatures below 150° were extremely weak and would not normally suffice to establish the structure of metabolites in the presence of the usual artifacts. Generally, the similarity in the mass spectra[1] of *N*-oxides and of free amines would make it difficult to assign structures to *N*-oxide metabolites. However, variation of the ion-source temperature does permit differentiation of amines and their *N*-oxides. At 155° or higher, I showed no M^+, and the M^+-16 ion, though still present, was diminished in intensity relative to the *m/e* 279 ion (Table I). The composition of the *m/e* 279 (M^+-61) ion corresponds to the olefin, III, formed by the Cope elimination of an *N*-oxide (5).

The *N*-oxide hydrochloride, I-HCl, shows the M^+-16 ion, the *m/e* 58 ion, and a temperature-dependent ion at *m/e* 279. At 185°, fragment ions of I-HCl are present at M^+-18 and M^+-30 (the former being a dehydration product) but are absent at higher temperatures. At temperatures >185°, the *m/e* 279 ion is more intense. However, the persistence of the M^+-16 ion, coupled with the increase in intensity of the *m/e* 58 ion, demonstrates that the thermolysis of I-HCl yields more II (mol. wt. 324) than does the thermolysis of I.

Differential thermal and thermal gravimetric analyses[2] also show the difference in the thermolysis of the hydrochloride and the free base of the *N*-oxide. The hydrochloride, I-HCl, melts in two exothermal steps at 186 and 191°, with a total loss of 7% of its weight. Since the loss of oxygen requires a weight loss of 4.3%, and the Cope elimination product requires a weight loss of 12%, the intermediate value for weight loss demonstrates that a mixed-product thermolysis can occur and substantiates the mass spectral results, which show that various thermolytic products were formed. But the free base of the *N*-oxide, I, showed an endotherm at

[1] The equipment and experimental technique for obtaining the electron-impact spectra were described in *Reference 4*.
[2] Performed on a Dupont analyzer.

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$T_{30\%}$	4.5 ± 1.3	6.7 ± 2.2
$T_{40\%}$	12.5 ± 4.4	20.5 ± 2.3
$T_{60\%}$	23.4 ± 7.6	39.3 ± 1.8
$T_{80\%}$	35.8 ± 10.3	60.6 ± 2.7

[a] Each T value (min.) is the mean of 10 determinations ± SD.

proximately 9 min. when a 10-mesh basket was used. Furthermore, variable results were not obtained when other brands of chlorpromazine hydrochloride tablets were tested or, if observed, the degree of variability was much less than that shown in Table I. This would appear to indicate that the inherent characteristics of the tablets tested led, in part, to the type of variability reported here.

The "correct" dissolution conditions are always difficult to establish, particularly in the absence of *in vivo* data. It is important, therefore, to standardize a procedure carefully in order to evaluate accurately various lots of the same drug product. From the results reported here, it appears that the time at which the rotation of the basket is commenced is important and that any variation from a standardized approach may yield variable results.

(1) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, pp. 934, 935.
(2) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, pp. 802, 803.
(3) W. F. Beyer and D. L. Smith, *J. Pharm. Sci.*, **59**, 496(1970).
(4) P. T. Shah and W. E. Moore, *ibid.*, **59**, 1034(1970).

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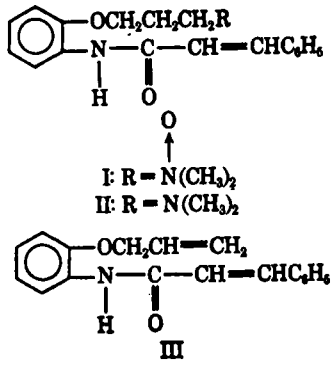
Identification of 2′-[3-(Dimethylamino)-propoxy]cinnamanilide *N*′-Oxide by Mass Spectral Thermolysis

Keyphrases □ 2′-[3-(Dimethylamino)propoxy]cinnamanilide *N*′-oxide—identified using mass spectral thermolysis □ *N*-Oxides—identification of 2′-[3-(dimethylamino)propoxy]cinnamanilide *N*′-oxide using mass spectral thermolysis □ Mass spectral thermolysis—identification, *N*′-oxide of 2′-[3-(dimethylamino)propoxy]cinnamanilide

Sir:

The occurrence of *N*-oxides as natural products or as metabolites of pharmacologically active compounds



III

was reviewed previously (1). The only mass spectral studies of *N*-oxides have been those of aromatic *N*-oxides that usually exhibit the M^+-16 ion, instead of the M^+, as the highest mass ion (2). Mass spectrometry was used to elucidate the structures of some thiopropyldimethylamino-*N*-oxide metabolites (3). In this report, these observations are extended to the thermolysis of 2′-[3-(dimethylamino)propoxy]cinnamanilide *N*′-oxide (I).

Since volatilization of the *N*-oxide base, I (mol. wt. 340), occurs very near its thermolysis point, the spectra obtained at temperatures below 150° were extremely weak and would not normally suffice to establish the structure of metabolites in the presence of the usual artifacts. Generally, the similarity in the mass spectra[1] of *N*-oxides and of free amines would make it difficult to assign structures to *N*-oxide metabolites. However, variation of the ion-source temperature does permit differentiation of amines and their *N*-oxides. At 155° or higher, I showed no M^+, and the M^+-16 ion, though still present, was diminished in intensity relative to the *m/e* 279 ion (Table I). The composition of the *m/e* 279 (M^+-61) ion corresponds to the olefin, III, formed by the Cope elimination of an *N*-oxide (5).

The *N*-oxide hydrochloride, I-HCl, shows the M^+-16 ion, the *m/e* 58 ion, and a temperature-dependent ion at *m/e* 279. At 185°, fragment ions of I-HCl are present at M^+-18 and M^+-30 (the former being a dehydration product) but are absent at higher temperatures. At temperatures >185°, the *m/e* 279 ion is more intense. However, the persistence of the M^+-16 ion, coupled with the increase in intensity of the *m/e* 58 ion, demonstrates that the thermolysis of I-HCl yields more II (mol. wt. 324) than does the thermolysis of I.

Differential thermal and thermal gravimetric analyses[2] also show the difference in the thermolysis of the hydrochloride and the free base of the *N*-oxide. The hydrochloride, I-HCl, melts in two exothermal steps at 186 and 191°, with a total loss of 7% of its weight. Since the loss of oxygen requires a weight loss of 4.3%, and the Cope elimination product requires a weight loss of 12%, the intermediate value for weight loss demonstrates that a mixed-product thermolysis can occur and substantiates the mass spectral results, which show that various thermolytic products were formed. But the free base of the *N*-oxide, I, showed an endotherm at

[1] The equipment and experimental technique for obtaining the electron-impact spectra were described in *Reference 4*.
[2] Performed on a Dupont analyzer.

Table I—Relative Intensities of Diagnostic Fragment Ions of I, II, and III

Source Temperature[a]	Type[b] of Spectrum	Intensity Relative to the *m/e* 131 Ion (1.0)					
		58	279	310	322	324	340
				Compound I			
141°	E.I.	3.6[c]	0.49	0.05	0.07	0.10	0.03
145°	E.I.	1.9[c]	0.45	0.03	0.05	0.09	0.02
147°	E.I.	1.0[c]	0.22	0.01	0.02	0.04	0.0
152°	E.I.	0.35	0.20	0.01	0.001	0.007	0.0
155°[d]	E.I.	0.25	0.21	0.005	0.0	0.02	0.0
160°[d]	E.I.	0.26	0.22	0.003	0.004	0.03	0.0
150°	C.I.	—	0.49[e]	0.03[e]	0.0	0.34[e]	0.0
				Compound I-HCl			
185°	E.I.	1.7	0.007	0.015	0.03	0.08	0.0
205°	E.I.	1.5	0.11	0.0	0.0	0.05	0.0
235°	E.I.	1.7	0.08	0.0	0.0	0.04	0.0
				Compound II-HCl			
	E.I.	7.7	0.02	0.0	0.0	0.27[f]	—
	C.I.	—	0.0	0.0	0.0	8.8[e]	—
				Compound III			
	E.I.	0.05	0.15	—	—	—	—

[a] Room temperature (25°) plus increase in source temperature. [b] E.I. = electron impact (70 e.v.); C.I. = chemical ionization (isobutane). [c] Intensity includes acetone impurity. [d] Very volatile at this temperature. [e] Chemical ionization protonated ion 1 a.m.u. greater mass than mass indicated. [f] Molecular ion of the free base.

159° and a loss of 17.3% of its weight, results that agree extremely well with the loss of 18.2% corresponding to the elimination of dimethylhydroxylamine (mol. wt. 61) from I to form III (Cope elimination). Thus, it is advisable to study the mass spectra of the free base of the *N*-oxide rather than those of the hydrochloride salt.

The use of chemical ionization[3] with thermolysis provides a further means of distinguishing between the amine and the *N*-oxide of thiopropyldimethylamino derivatives. In this series, the *m/e* 279 ion is completely absent in the chemical ionization spectrum of the oxypropyldimethylamine, II, whereas the protonated molecular ion (MH^+) of the thermolysis product (III) appears in the spectrum of I, along with the MH^+ of the thermolysis product, II, at *m/e* 325.

(1) M. H. Bickel, *Pharmacol. Rev.*, **21**, 325(1969).

[3] Obtained at the National Institutes of Health (6).

(2) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Mass Spectrometry of Organic Compounds," Holden-Day, San Francisco, Calif., 1967, pp. 328, 329.

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REVIEWS

Pharmacology of the Endocrine System and Related Drugs: Progesterone, Progestational Drugs and Antifertility Agents, Volume II. Section Editor, M. TAUSK. Pergamon Press, Inc., Maxwell House, Fairview Park, Elmsford, NY 10523, 1972. xiv + 538 pp. 16 × 24 cm. Price $36.00.

This second volume of section 48 of the International Encyclopedia of Pharmacology and Therapeutics is composed of 11 chapters by eight eminent contributors. It has accomplished, very completely, in a rather limited amount of space, what it was supposed to do—namely, provide a thorough review of the synthetic progestational drugs that will serve the purpose of a wide variety of basic and applied scientists.

The first part of the text deals with synthetic progestational agents in humans and animals, highlighting their pharmacology, metabolism, and therapeutic applications. The latter part is confined to the antifertility aspects of estrogens, progestational compounds, combinations of both, and their side effects. In addition, there is a concluding summary chapter that not only covers material in both volumes, but also includes some recent information that has come about during the 5 years this volume was in preparation.

Outstanding features of this book include separate subject and author indexes, as well as complete reference sources for this area of pharmacology. The buyer should be cautioned that, in order to really have at hand all the necessary facts for the subject under discussion, the initial volume must also be purchased. Most of the material presented is done so in as readable a manner as can be accomplished in this kind of undertaking and, for the most part, the clarity is better than most other reviews.

This book will undoubtedly be added to all medical libraries throughout the world, and it should find use among not only pharmacologists and endocrinologists, but also obstetricians, gynecologists, and steroidal research groups interested in the actions, mechanisms, and uses of the myriad of compounds described.

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Pharmaceutical Applications of Thin-Layer and Paper Chromatography. Edited by KAREL MACEK. American Elsevier, 52 Vanderbilt Ave., New York, NY 10017, 1972. xvi + 742 pp. 16 × 24.5 cm. Price $78.50.

Macek and twelve cocontributors approached the enormous literature of TLC and paper chromatography (cumulatively called "flat-bed" as the lines of demarcation have dimmed in recent years) with a single objective: to produce a volume uniquely useful to the pharmaceutical milieu. This objective is a reasonable one and long overdue. They have succeeded through diligent coverage of the chosen field and unyielding exclusion of related information from activities in agriculture, dyes, synthetic organic chemistry, and biochemistry.

Rich discussions of technique and interpretation are given so that this volume is no mere catalog of mobilities and references. Their inclusion of both paper and thin-layer data and exclusion of data not of direct pharmaceutical interest have steered the volume reasonably clear of that great and obvious danger, overlap with existing compilations. Moreover, this freshness of effort is aided by the simple fact that more recent literature is included, that is to say reasonably thorough coverage through 1970 with some 1971 work.

Twenty-one chapters, individually authored, divide the mass of information into realistic compartments with only minor discontinuity or overlap. The heart of the volume resides in 10 chapters covering pharmaceutical compounds by class: synthetic organics, steroids, glycosides, saponins, peptide and protein hormones (which suffers slightly from the exclusion of another "flat-bed" technique, electrophoresis), alkaloids, vitamins, antibiotics, plant extracts, and formulation components. One strength of this volume lies in the inclusion of isolation and sample preparation procedures within the context of the individual chapters. Each chapter is well organized and the literature coverage is excellent throughout.

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Journal of Pharmaceutical Sciences

JULY 1973
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REVIEW ARTICLE

Pharmaceutical Sciences—1972: Literature Review of Pharmaceutics I‡

ASHOK C. SHAH*▲ and ALLEN K. HERD†

Keyphrases ☐ Pharmaceutical sciences—1972 literature review of pharmaceutics ☐ Technology, pharmaceutical—sterility, dosage forms, packaging equipment ☐ Physical pharmacy—dissolution, solubility, permeability, complexation, surface phenomena ☐ Antibiotics—pharmaceutical aspects ☐ Radiopharmaceuticals—pharmaceutical aspects ☐ Biopharmaceutics—drug properties, absorption, pharmacokinetics ☐ Literature review—pharmaceutical sciences 1972

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‡*Editor's note:* Part II of this article will appear in the August 1973 issue of the *Journal of Pharmaceutical Sciences.*

This review of the literature represents a comprehensive cross section of the research and development effort in various selected disciplines of pharmaceutical sciences. As in past years, the scope of this endeavor has been limited to a review of the area of pharmaceutics because annual reviews of the literature related to other areas of pharmaceutical sciences are published elsewhere. This is the 11th annual survey of the series (1–10). To compile it, numerous journals, periodicals, and selected sections of *Chemical Abstracts* were abstracted.

The review was prepared to provide a convenient method for pharmaceutical scientists to review the literature of the past year and to supply a source of references to articles of preferred interest. To maintain continuity, the well-accepted format of last year's review was retained.

GENERAL PHARMACY

A comprehensive review of the biosynthesis, chemistry, stability, analytical methods, metabolism, and biology of the prostaglandins was presented by Oesterling *et al.* (11). Other reviews appearing over the past year in the general area of pharmacy concerned formula-

tion of aspirin (12), use of polymers for pharmaceutical applications (13), boron compounds of pharmaceutical interest (14), application of silicones in pharmacy (15), use of silicates in the pharmaceutical area (16), and pharmaceutical properties of dimethyl sulfoxide (17). Statistical data were presented on the results of quality control of raw materials in the Brazilian pharmaceutical industry between 1940 and 1968 (18). The extent of drug product recalls occurring over an 18-month period and the types of product quality defects and their significance were discussed (19). Examples for each type of product quality defect were cited. A review was presented on the chemical and physical incompatibilities in drug solutions, emulsions, suspensions, salves, and capsules and their effects within the biological system (20). As an example, streptomycin in combination with anionic compounds, such as sodium alginate, exhibited greater antimicrobial activity than that observed for the sulfate derivative.

Preservatives—In a study relating cetrimonium bromide (cetyltrimethylammonium bromide) concentrations with the ζ-potential of the bacterial cell wall, this agent completely inhibited the growth of *Streptococcus faecalis* at a concentration producing only a 10–30% decrease in the absolute value of the ζ-potential (21). These results seem to indicate that the primary site of attack of this compound is probably not the cell wall. During studies on the antimicrobial efficacy of 4-hydroxybenzoic acid esters, a direct relationship between the lipid–water partition coefficient and antimicrobial activity was indicated (22). Introduction of a hydroxy or oxo group into the alkyl ester chain increased water solubility, but it decreased antimicrobial activity. Likewise, it was shown in a study of a series of homologous aliphatic amines that inhibition increased with increasing alkyl chain length and the maximum was reached at C_{12}–C_{15} (23). The poor inhibitory effect of the longer chain molecules was thought due to micelle formation, with concomitant lowering of the solubility. In determining the antibacterial activity in a series of quaternary ammonium compounds, it was found that the length of the side chain was less determinant in the case of Gram-negative bacteria (24). This was attributed to the differences in the cell wall lipid concentration between Gram-negative and Gram-positive bacteria.

Pseudomonas aeruginosa cells grown in the presence of phenylethanol were more sensitive to the action of benzalkonium chloride, chlorhexidine, and phenylmercuric nitrate than cells grown in nutrient broth alone (25). This suggested the use of phenylethanol as a co-preservative when using these other agents. Bradshaw *et al.* (26) were able to predict the degree of inhibition of cetylpyridinium chloride in combination with a nonionic surfactant on microbial cells at higher concentrations. However, at lower concentrations of bactericide, other factors, such as changes in cell wall permeability, made this prediction less possible. It was also determined that polysorbate 80 antagonized the activity of fenticlor against exponential phase cultures of *Staphylococcus aureus*, *Proteus vulgaris*, and *Escherichia coli* (27). In the same study, it was demonstrated that phenylethanol enhanced activity against similar cultures.

The effects of glycerol and propylene glycol on microbiological contamination of some oil-in-water emulsion ointment bases were determined (28). While glycerol stimulated the growth of molds and yeasts with the simultaneous decrease of bacteria, propylene glycol gave microbial purity. However, in another study with preservation of oil-in-water emulsions, it was found that propylene glycol caused apparent partial antagonism of potassium sorbate in these systems (29). Sterilization of contaminating bacteria by solutions of phenylmercuric nitrate and thimerosal was delayed or prevented by addition of edetate (EDTA) or sodium thiosulfate, but metabisulfite reduced the sterilization times of both these agents at acid pH (30). The antibacterial activity of sodium metabisulfite appeared to be due to an interaction with bacterial DNA.

Methods for the rapid screening of preservatives for pharmaceutical and cosmetic preparations, based on instrumental monitoring of indexes of cell development that occur early in the growth cycle and that are susceptible to preservative agents, were reviewed (31). In another review, the relative sensitizing potentials of parabens, sorbic acid, phenolics, formaldehyde, mercurials, quaternary ammonium compounds, betaines, dimethoxane, and dihydroacidic acid were discussed because they are used in cosmetic emulsions and dermatological preparations (32). Combinations of benzalkonium chloride, chlorhexidine, phenylmercuric nitrate, chlorocresol, and chlorobutanol with phenylethanol were consistently effective in preserving ophthalmic solutions (33). Polymyxin and benzyl alcohol were also found to be suitable preservatives for ophthalmic drops (34). However, the use of an antibiotic as a preservative was regarded as unadvantageous.

The safety of intravenously administered benzyl alcohol and paraben preservatives was ascertained (35). The higher antibacterial activity of benzyl alcohol, shown in challenge tests, plus its comparable tolerances to parabens favor the use of benzyl alcohol as a preservative. A study was made of eight substituted phenolic preservative solutions in 2% polysorbate 80 (36). These results showed less sorption into polyethylene than from aqueous solutions, but the micellized systems were not adequately preserved. Several common preservatives that are absorbed by filter paper, rubber stoppers, and inorganic adjuvants or that react with organic macromolecular compounds were discussed (37). Preservatives preferred or considered not suitable for different pharmaceutical preparations were also listed.

Other articles of interest related to the subject of preservatives are listed in Table I.

Flavor, Aroma, and Color—Additions to the list of Generally Regarded As Safe (GRAS) substances made in 1970–1971 and their maximum use levels were tabulated (52). The concept of "toxicological insignificance" was explained, and data on total daily per capita intake of flavoring substances were presented. A review of the classification of the components of taste, methodology, choice of flavor, control, and stability of taste-masking drugs was presented (53). It was pointed out that flavoring of a drug includes two processes: the masking of the disagreeable taste and aromatization (54). Prescriptions for flavoring were given using sac-

Table I—Additional References on Preservatives

Reference	Topic
38	Evaluation of parabens for protection against fungal and bacterial contamination
39	Uses of preservatives based on esters of *p*-hydroxybenzoic acid
40	Physicochemical properties and antimicrobial efficacy of 4-hydroxybenzoic acid esters
41	Effects of pH on the multiplication of a pseudomonad in chlorhexidine and cetrimide
42	Influence of polyethylene glycol gels on the effectiveness of preservatives (ninpagin and Fenosept)
43	Studies demonstrating that the USP XVIII preservative test is adequate for the intended purpose
44	Discussion on adsorption of antiseptic substances by filtration membranes
45	Effects of antioxidants on the long-term utility of ethyl linoleate as a water-holding lipid for topical postburn treatment
46	Composition and antimicrobial properties of essential oils of pine
47	Effects of the addition of antioxidants on changes occurring in pine oil
48	Selection, control of activity, efficacy after inoculation, and determination of germicidal potency of preservatives for cosmetics
49	Choice, activity, and effectiveness levels of preservatives for cosmetics
50	Protection of cosmetics by synergistic mixtures of preservatives and bactericides
51	Fat-soluble preservatives for oil-in-water emulsions only effective with solvents that increase water solubility

charin, chocolate, vanilla, caramel, orange essence, and raisin juice in different combinations. The flavor chemist's greater reliance on the results of instrumental techniques, the faster development of new aromatics from laboratory curiosities to accepted standard items, and the increasing number of new materials and methods determine today's trend in flavors (55). Hofmann (56) reviewed and discussed the several theories explaining the relation between sweetness and chemical structure. A study of several stereoselectively sweet amino acids molecules led to a prediction of the presence and approximate location of a third structural feature, relative to the currently postulated A–H/B features in the glucophore (57). A relationship between the polarizability parameter and sweetness level in a series of substituted nitroanilines led to the hypothesis that this third binding site may be involved in dispersion bonding with an appropriate receptor feature.

An encyclopedic evaluation of the literature in the perfumery materials field was continued by Bedoukian (58) in his 28th annual review. It was pointed out that modern perfumery is no longer a question of merely adding a harmonious blend of materials in proper concentrations (59). It involves chemical interactions, research, packaging, testing, and fragrance selection, coupled with the application of perfumes to a wider range of products. It was urged that fragrance materials be examined in their use environment (60). Since it has been demonstrated that fragrance materials associate with protein, factors that influence efficacious perfumery of protein materials can be considered in a new way. Encapsulation of fragrance material was postulated as a means for overcoming difficult problems (61). A water-soluble capsule would protect the fragrance up to the time of use.

In studying odor sensations arising from the 1,2,4-triaxial arrangement of the substituents in a decalin ring system, it was determined that the position of an oxygen function within the triaxial system moves the odor sensation in the direction of different but distinct qualities (62). On the other hand, the surroundings of the center triggering the odor, as well as the chemical nature of the oxygen substituent, seemed to be of minor importance. In discussions concerning the relationships observed between odor and molecular structure, it was stated that small differences in structure can create changes in potency in some cases, changes in quality in others, and changes in both quality and potency in still others (63, 64).

Stability—The stability of neutral regular insulin was compared to conventional acid regular insulin (65). The increased stability of the former warrants its consideration as a pharmaceutical form of soluble regular insulin and as a replacement for the conventional acidic form. Seydel and Voigt (66, 67) conducted extensive studies on the stability of amino acid solutions. Stability results on individual amino acids and on combinations of amino acids alone and with various preservatives were reported. The stability of amino acids in aqueous solutions of casein hydrolysate stored at various temperatures was examined (68). Only glutamine and cysteine were rapidly degraded in this system. In a further study, these same workers determined that lyophilization significantly enhanced stability of the amino acids (69). Macromolecules such as bovine serum albumin and macrocyclon effectively enhanced the stability of aromatic nitrogen mustards, including chlorambucil, while not necessarily altering the antitumor activity of these compounds when given concurrently (70).

The causes of coloring of sulfonamide suspensions with prolonged action were investigated (71, 72). It was determined that with the proper use of antioxidants, adjustment of pH, and nitrogen flushing, coloring could be eliminated. In a study on the thermal degradation of sulfonylureas, results indicated that dissociation rather than solvolysis was the most likely mechanism by which sulfonylureas undergo breakdown in alcohols and water (73). Procaine hydrochloride was successfully stabilized by the addition of methylidene, glycerin, and propylene glycol (74). The mechanism postulated for protection was a pseudochelating action. It was shown that dipyrone degradation was not subject to pH in the 4.8–8.8 range and that the probable mechanism of degradation was oxidation (75). It was suggested that improved stability could be obtained by flushing the headspace with an inert atmosphere and by incorporating a suitable antioxidant.

A stabilized phenylephrine hydrochloride nasal drop formulation was described which contained methylparaben, propylparaben, and sodium bisulfite with a pH adjustment of 5–6 (76). The decomposition of aspirin (acetylsalicylic acid) and possibilities for its stabilization in a solid dosage form were discussed and reviewed (77). During an investigation of the decomposition of physostigmine salicylate during thermal sterilization, it was determined that the quantity of decomposition in ampuls depends on the quantity of oxygen present in the solution (78). The replacement of

Table II—Additional References on Stability

Reference	Topic
86	Hydrolysis of aspirin (acetylsalicylic acid) in a mixture of polyethylene glycol 4000 and water
87	Stability of tablets with an aspirin (acetylsalicylic acid) base in tropical environments and requirements of packaging
88	Stability of aminopyrine (aminophenazone) and allobarbital in a liquid Pabialgin preparation
89	Thermal decomposition of a mixture of aminopyrine (aminophenazone), allobarbital, and cycloadiphenine in cetyl phthalate
90	Heat stability of eight samples of anti-yellow fever vaccine from various countries
91	Determination of stability of ergotamine and ergometrine in ergot extract as injectable solutions
92	Effect of different ions on the stability of carbazochrome sodium sulfate
93	Decomposition of sorbitol in acid solution
94	Solid-state stability of anhydrous morphine hydrochloride
95	Stability of dihydrotriazine in solution and dihydrotriazine pamoate in aqueous suspension
96	Effects of surfactants on the degradation of 2α,3α-epithio-5α-androstan-17β-ol and related compounds
97	Stability of 2α,3α-epithio-5α-androstan-17β-yl 1-methoxycyclopentyl ether in tablets and capsules
98	Determination of storage conditions for 2% pilocarpine hydrochloride solutions by accelerated decomposition method
99	Stability of 5-bromo-2′-deoxyuridine in solid state and in solution at different pH values
100	Glucose stability in a concentrated plasma-replacing Ringer solution
101	Determination of stability of some benzodiazepines in pharmaceutical dosage forms
102	Effect of pH on the stability of aceclidine and oxylidine parenteral solutions
103	Incompatibilities of selected cardiovascular and psychotherapeutic agents in solutions containing sodium ethacrynate
104	Stability of amphetaminyls in aqueous suspension
105	Hydrolytic decomposition of trantelinium bromide
106	Stability of ephedrine hydrochloride solutions during steam sterilization
107	Determination of stability of seven types of insulin preparations
108	Alkaline hydrolysis of phenobarbital
109	Enzymic hydrolysis of pilocarpine
110	Instability of sulfathiazole with polyethylene glycol 600 gels
111	Compatibility of sodium lauryl sulfate with 119 various drugs
112	Incompatibility of ethacridine (Rivanol) with polyethylene glycols
113	Stability of aspirin (acetylsalicylic acid) in different suppository bases
114	Stability of aspirin (acetylsalicylic acid) in coated pills
115	Stabilization of aqueous drug forms during heat sterilization
116	Optical behavior of sucrose solutions in the inversion process
117	Method for reducing discoloration of glucose solution
118	Oxidative degradation of terbutaline in aqueous solution
119	Effects of UV radiation on drugs in hard gelatin capsules
120	Effects of UV rays on dyes used in cosmetic products
121	Evaluation of the quality of drugs from the view of their stability
122	Stabilization of buffered pilocarpine drops by freezing
123	Discussion of problems of stability of plasma substitutes
124	Stability of inclusion compounds of deoxycholic acid and β-cyclodextrins in dosage forms
125	Shelflife of some injection formulations
126	Stability predictions for pharmaceutical preparations by accelerated testing
127	Stabilization of various eyewash solutions
128	Determination of the stability of meperidine (pethidine) hydrochloride solutions in polyethylene containers
129	Stability of hexahydroadiphenine, ethionamide, and aminophylline in lyophilic suppository bases
130	General discussion of drug incompatibilities

oxygen with nitrogen decreased the decomposition threefold. Boric acid had no effect on oxidative changes of lanolin, while glycero-boric acid was very effective in this respect (79). The protective efficiency for the stabilization of oxygen-sensitive systems using gas permeation was determined by measuring the relative deaeration produced by this technique *versus* current methods for reducing dissolved oxygen concentrations in liquid systems (80). In terms of deaeration efficiency, gas permeators ranked second only to the addition of antioxidant materials in the drug system, but they did have the advantage of eliminating the addition of chemicals in the dosage form which might produce adverse reactions.

In a discussion of the advantages of preservation of medicinal solutions by freezing, emphasis was placed on the proper choice of adjuvants and construction of the containers used (81). A method was described for the rapid determination of color stability in tablet formulations (82). Data obtained in the phenometer in 24 hr., as well as results from exposure in a light cabinet for 28 days, facilitated the comparative evaluation. Rapid testing for the stability of BCG vaccine by accelerated heat testing was accomplished, along with rapid viability testing by assaying for the adenosine triphosphate content of the vaccine (83). The viability determined by adenosine triphosphate analysis correlated well with the viable count as determined by culturing. Stability programs for determining chemical and physical stability and the analytical implications were reviewed by Rehm (84). Other workers warned against too heavy a reliance on accelerated stability testing (85). As an example, they cited the case of 500-mg. ascorbic acid tablets that had strong color degradation apparent although they were chemically unchanged.

Other papers of interest related to the topic of stability are listed in Table II.

Stability Kinetics—Drug and drug system stability predictions based on kinetics of degradative reactions and Arrhenius-type relationships were reviewed by several authors. These included both isothermal and nonisothermal methods (131–135). However, one group of workers pointed out that stability based on accelerated temperature studies may lead to erroneous results because mechanisms of degradation may change at higher temperature (136). While using procaine, meperidine, homatropine, and scopolamine as examples, the hydrolytic behavior of esters in aqueous solutions was discussed (137). Such factors as the effects of pH, solvents, ionic strength, acid and base catalysis, and steric hindrance were included. Dexamethasone 21-phosphate in aqueous solution was shown to be susceptible to a reversible bisulfite addition, which was a

second-order reaction in which sulfite ion and monoanionic dexamethasone 21-phosphate were the major participants (138). The decomposition of aspirin in polyethylene glycols was shown to be a pseudo-first-order reaction (139). Different molecular weights of polyethylene glycol had no effect upon the reaction rate, which was demonstrated to be due to transesterification. In alkaline media the 2-, 3-, and 4-monohexanoates of lincomycin rapidly isomerized, each entering into facile equilibrium with the other two, accompanied by hydrolysis of each species to lincomycin. By using a digital computer and a nonlinear estimation program, Oesterling and Metzler (140) were able to estimate simultaneously the four isomerization rate constants and the three hydrolysis rate constants.

It was demonstrated that the methanesulfonic acid derivative of sulfisoxazole undergoes successively reversible hydrolysis in aqueous solution (141). The pH profiles of the logarithm of apparent hydrolysis and reverse reaction rates and those of equilibrium constants were obtained, and the effects of ionization of the derivatives and hydrolysates were explained by mathematical equations. The decomposition of a series of benzoic acid derivatives in the solid state was studied (142). It was found that for decomposition to take place below the melting point, the σ value must be less than -0.35. The decomposition then follows Bawn-type kinetics. C-21-Dideuteriohydrocortisone was shown to be more stable than hydrocortisone under oxidative conditions (143). A possible explanation for this enhanced stability was that the reaction path for these degradations involved a rate-determining enolization in the C-17-dihydroxyacetone side chain. The pharmaceutical stability of a solution of potassium canrenoate was shown to be a function of the solubility of the lactone canrenone at equilibrium and the pH-dependent thermodynamic equilibrium of the reversible lactonization and hydrolysis processes. Garrett and Hermann (144) predicted the minimum pH values for various mixed solvent solutions that would maintain unprecipitating potassium canrenoate solutions for desired injectable concentrations of the drug. The hydrolytic decomposition of intrazole obeyed first-order kinetics and was subject to both acid and base catalysis (145). The pH of maximum stability of this compound under buffer-free conditions was 3.20. The kinetics of hydrolysis of the carbamoyl group in salicylanilide *N*-methylcarbamate and 4-biphenylyl *N*-methylcarbamate showed that the reaction was first order with respect to both hydroxide ion and carbamate (146). At 37°, the hydroxide ion-catalyzed hydrolysis of the former to yield salicylanilide proceeded at a rate over 200 times that for the hydrolysis of the latter, which produced 4-biphenylol. During a study of the kinetics of dextrose degradation during autoclaving, the reaction was shown to exhibit an induction period with respect to 5-hydroxymethylfurfural production, which was due to the formation of an intermediate compound (147). A reaction mechanism was proposed that appeared consistent with experimental measurements.

In studies aimed at predicting shelflife stability of arabinosylcytosine in aqueous solutions, Notari *et al.* (148) determined that the loss of substrate in the pH 0–6 region was accompanied by formation of an intermediate which reacts further to yield arabinosyluracil. In alkaline solutions the loss of arabinosylcytosine was about 10 times more rapid than in acid and was most likely due to hydrolysis of the pyrimidine ring, since no arabinosyluracil UV absorption spectra were detected. Other workers (149) confirmed by UV spectrophotometry and TLC that cytosine and cytidine deaminate to uracil and uridine, respectively, at all pH values. These products slowly degrade further in strongly alkaline solutions. Differences in the intermediates formed in the degradation of chlorothiazide in alkaline medium and acidic medium were investigated, using a molecular orbital method to understand the hydrolysis mechanism (150). The hydrolysis pathway was explained by the electronegativity of the atoms involved in the postulated activated complex, 3-hydroxyhydrochlorothiazide. Depending upon the reaction conditions, two pathways of cleavage of butalbital were described (151): (*a*) through 1,6-ring opening to the malonuric acid, and (*b*) *via* 1,2-cleavage of the barbiturate producing the diamide. In following the hydrolysis of certain 5-aminodibenzo-[*a*,*d*]cycloheptanes, it was determined that degradation is characterized by cleavage of a carbon–nitrogen bond at the 5-position of the fused ring system (152). The process was shown to proceed possibly by a carbonium-ion intermediate or its equivalent. The hydrolytic rate of degradation of sodium sulfacetamide in aqueous solution was shown to be minimal at pH 8–8.5 (153). Hydrolysis of methyl hippurate under the influence of α-chymotrypsin and some aliphatic alcohols was investigated (154). Methanol, ethanol, *n*-propanol, *n*-butanol, and *n*-pentanol were found to inhibit the rate of hydrolysis.

Other papers related to the topic of stability kinetics are listed in Table III.

Table III—Additional References on Stability Kinetics

Reference	Topic
155	Review of kinetic approaches and limitations
156	Computer technique for calculating the expiration date of drugs based on kinetic data
157	Use of an analog computer in drug-stability studies
158	Kinetics of the oxidation of dopamine
159	Decomposition kinetics of methallibure
160	Kinetics of hydrolytic decomposition of isoindoline-nitrourea
161	Degradation of 2α,3α-epithio-5-androstan-17β-yl 1-methoxycyclopentyl ether in aqueous dioxane solutions
162	Influence of a 6-methyl substituent on the degradative rates of cytosine nucleosides
163	Decomposition rates of 6-aminopenicillanic acid adsorbed on KU-2 ion-exchange resin
164	Kinetics of degradation and stabilization of the parabens
165	Kinetic investigation of the hydrolytic decomposition of *N*-butylscopolammonium bromide
166	Alkaline hydrolysis of xylamide
167	Kinetics of hydrolysis of chloramphenicol succinate
168	Hydrolytic decomposition of oxyphenonium bromide

Antibiotic Stability—In comparing the relative stabilities of hetacillin and ampicillin solutions, Schwartz and Hayton (169) found rates of degradation inconsistent with what should be expected. An explanation of this inconsistency was offered by adopting a model reaction scheme that incorporated the concentration

Table IV—Additional References on Antibiotic Stability

Reference	Topic
184	Comparative stabilities of ampicillin and hetacillin in aqueous solution
185	Stability of ampicillin in aqueous solution
186	Stabilization of penicillin against penicillinase
187	Physicochemical properties and stability of sulfocillin
188	Effect of Cu (II)–glycine chelates on the degradation of penicillin in mildly acid solutions
189	Stability of 6-aminopenicillanic acid in solutions of low and average concentrations
190	Solid-state stability of pirazocillin
191	Modes of degradation of potassium phenoxymethyl penicillin
192	Stability of tetracycline and tetracycline–urea complex in petrolatum ointment bases
193	Stability of nystatin

Table V—Additional References on Vitamin Stability

Reference	Topic
205	Stability of vitamins A and D in multivitamin preparations
206	Comparative studies on the stability of vitamin D_2 in different ointment bases
207	Effect of cupric ion on the thermal decomposition of thiamine
208	Possibilities of stabilizing a vitamin F concentrate for cosmetic purposes
209	Mathematical model for predicting stability of ascorbic acid in food products
210	Effects of surfactants on the aerobic oxidation of ascorbic acid

dependence of both the ampicillin degradation and the hetacillin–ampicillin equilibrium. Ionic strength up to 0.5 did not affect the chemical stability of potassium penicillin G unless one of the ions contributing to ionic strength was also catalytic (170). A study to elucidate, by *in vitro* kinetic analysis, the role of cupric [Cu (II)] ions through complexation with an amino acid was undertaken (171). A mechanism was postulated that involved the rapid formation of a ternary penicillin–Cu (II)–glycine complex followed by a rate-limiting hydroxyl-ion attack. It was demonstrated that the nature of the penicillin side chain, particularly the stereospecificity, played a significant role in the stability of penicillin toward β-lactamase (172). In another study, the rate constants for the penicillinase hydrolysis of a series of phenoxymethyl penicillins substituted on the benzene ring by methyl, ethyl, chloro, bromo, formyl, nitro, or cyano were correlated with the Hammett σ-constants (173).

In studying the compatibility and stability of disodium carbenicillin in combination with other drugs and large volume parenteral solutions, Zost and Yanchick (174) reported this antibiotic to be degraded rapidly when mixed with a combination of multiple B vitamins and vitamin C (ascorbic acid). The decomposition rates of a series of semisynthetic penicillins were measured in aqueous solutions at pH 4–9 (175). Methicillin was the most labile, ampicillin and oxacillin were of intermediate stability, and cloxacillin was the most stable. Chemical evaluation of the degradation of solid tetracycline dodecylsulfamate at various temperatures indicated a pseudo-first-order reaction (176). Predictions of shelflife using Arrhenius mathematics indicated a probable shelflife of 2600 days at 25°. These results were confirmed by actual data.

In studies dealing with the stability of antibiotics in frozen systems, Larsen (177, 178) demonstrated that the rate of degradation of these drugs is dependent on buffers, absolute temperature, antibiotic concentration, and rate of freezing. Other workers reported that sodium cephalothin and cephaloridine may be stabilized for short periods by freezing (179). Hamycin was shown to exhibit its maximum stability in aqueous solutions at pH 8 (180). The influence of honey on the stability of monomycin, neomycin, streptomycin, tetracycline, oxytetracycline, and chlortetracycline was reported (181). The microbiologically determined stability of dermostatin in 12 selected ointment bases at 37° was investigated, and maximum stability was exhibited in anhydrous bases as compared with those containing water (182). The stabilities of penicillin G, tetracycline, neomycin, and chloramphenicol were determined in bentonite glycerogels (183). Additions of sorbitan monopalmitate, polysorbate 80, sorbitan monolaurate, and sodium lauryl sulfate had drastic effects on reducing degradation rates.

Other references relating to antibiotic stability can be found in Table IV.

Vitamin Stability—The effects of metal complexing agents on the stability of injectable ascorbic acid solutions were investigated (194). The optimum concentration of these agents on the stability of the drug depended on the amount of heavy metal ions in the solution. The pH–log rate profile of the rate of disappearance of ascorbic acid from an aqueous solution under aerobic conditions was determined at 67° in the pH 3.52–7.22 range and exhibited a maximum near the pKa of ascorbic acid (195). Other workers (196) reported that maximum stability of ascorbic acid solutions could be achieved by the addition of sodium thiosulfate and edetate (ETDA) after passing carbon dioxide through the solution. Microencapsulation of ascorbic acid increased its stability during storage both in the powder form and as tablets (197). Best results were achieved with cetyl alcohol as the coating agent, and the process was carried out by fluidization. An investigation of the stability of vitamin A palmitate in selected ointment bases indicated that lanolin accelerated the decomposition of this vitamin (198). The synergistic effect of the antioxidants butylated hydroxytoluene and butylated hydroxyanisole was demonstrated on the stability of vitamin A acetate in soybean oil (199). An extensive review on the oxidation of vitamin A was also presented (200).

The effects of common additives on the stability of injectable solutions of vitamin B_1 were evaluated (201). The most important factor for solution stability was shown to be the quality of the thiamine hydrochloride used. The stability of supersaturated solutions of thiamine chloride and thiamine bromide at 7 and 20° depended on the degree of supercooling (202). The addition of ethanol affected the rate of crystallization at nucleation centers, resulting from its influence on surface tension and activation energy. The stability of

multivitamin granules containing thiamine disulfide, sodium riboflavin 5′-phosphate, pyridoxine hydrochloride, niacinamide, cyanocobalamin, and calcium pantothenate was studied using accelerated tests (203). Granulation with ethanol and 5% polyvinylpyrrolidone was the recommended method of manufacture. The stability of pyridoxal 5-phosphate was determined in the presence of its degradation products and other substances with which it is commonly associated (204). It was found that the only additives increasing the degradation rate were riboflavin and cyanocobalamin.

Additional references on vitamin stability are listed in Table V.

PHARMACEUTICAL TECHNOLOGY

Recent advances in the pharmaceutical industry as they relate to pharmaceutical technology were reviewed by several authors (211–213). Use of computers in electronic data processing in the pharmaceutical technology areas was also reviewed (214, 215). The historical background, basic principles, and applications of lyophilization to pharmaceuticals were discussed (216). As an alternative to freeze drying for removal of ethanol from plasma proteins, vacuum distillation was offered (217). Both capital and operating costs were less than for freeze drying. Cookson and Morgan (218) compared the various methods for deionized water bed sterilization and recommended 0.25% formaldehyde solution, primarily because of the lower price as compared to other available agents. The various factors affecting antacid efficiency, including micromeritic properties, method of preparation, and effect of dosage form were reported (219–221). The suspension was considered the best dosage form since it has reasonable neutralizing capacity, the fastest rate for acid neutralization, and the longest sustained action within the desirable pH range. The influence of setting temperature on the organization of gelatin gels was studied (222). The gel structure and its behavior depend mainly on the first formed bonds, and a setting at a few degrees below the melting temperature promotes high rigidities. The physical and chemical properties of pharmaceutical gelatin were reviewed, with emphasis on alkaline or acid treatment and source of raw material (223). The principles and uses of microencapsulation of drugs was reviewed by Bakan and Sloan (224). Ultrasonic cleaning was reviewed from both a technological viewpoint (225) and as it can be used in the pharmaceutical industry (226).

Additional references on pharmaceutical technology are listed in Table VI.

Parenterals—Jennings (244) pointed out some problem areas in the manufacturing and administration of parenterals, including the need of a total concept of sterility, the potential hazard of particulate matter, the addition of other drugs to large-volume parenterals prior to their administration, and packaging designs for large-volume parenterals. Modes of administration, solubility, stability, and incompatibility with other drugs and their adverse effects were tabulated for 19 commonly used antibiotics including the tetracycline and penicillin derivatives (245). Procedures for the manufacture of parenteral suspensions were reviewed,

Table VI—Additional References on Pharmaceutical Technology

Reference	Topic
227	Discussion of general aspects of drug quality
228	Description of a method for optimization of sulfadimezine production
229	Sterile and ultrapure water in pharmaceutical processes—the Super-Q system
230	Use of metal stearates in pharmaceuticals and cosmetics
231	Preparation and characteristics of microcapsules containing asparaginase
232	Factors influencing microencapsulation of a waxy solid by complex coacervation
233	Complex coacervation in gelatin mixtures at temperatures below the gel melting point
234	Examples for the use of microencapsulated materials
235	Dehydration of oleandomycin phosphate by spray drying of its aqueous solutions
236	Survey of bacterial contamination in noninjectable dosage forms
237	Applications of membrane technology
238	Variations in theophylline, ephedrine hydrochloride, and phenobarbital tablets from different manufacturers
239	Influence of moisture content of the fibrous support of a nasal inhaler on the concentration of drug in the air stream
240	Review of production and control methods used for various dosage forms
241	Review of process instructions and mechanical processing techniques for the production of pharmaceuticals
242	Survival rates of microbial organisms in BPC preparations
243	Swelling of gelatin as a function of pH

with emphasis on the characteristics inherent to this dosage form not shared with other pharmaceutical suspensions (246). Perrin *et al.* (247) proposed that highly protein-bound excipients, which compete with drugs for binding sites, be added to lower the dose of drugs necessary to produce a given pharmacological response and, hence, to lower the toxicity and cost of parenteral dosage forms. Polyethylene glycol, when used as a solvent for intravenous injections of water-soluble agents, showed a relatively low toxicity (248). This solubilizing agent did not induce hemoglobinurea as was observed with glycerol, dimethyl sulfoxide, or dimethylformamide when used as solvents. The technology involved in preparing fluorocarbon emulsions as blood substitutes was reported (249). Five formulations of fat emulsions used in parenteral nutrition were reviewed as to their use in clinical medicine, absorption, and adverse effects (250). Riebe and Oesterling (251) outlined the procedures involved in the successful development of an injectable form of clindamycin. Problems encountered in the formulation of clindamycin 2-phosphate as a suitable parenteral dosage form were described.

Particulate matter in parenteral glass containers was significantly reduced by washing with either hydrofluoric acid or ammonium bifluoride solution (252). This method of washing also improved the chemical resistance of Type I glass containers to a degree equivalent to that of a good sulfur dioxide treatment. Minute crystals occurring in solutions packaged in multiple-dose vials and sealed with butyl stoppers were shown, by electron probe microanalysis, to be complexes containing zinc and calcium (253). These metal ions were believed to have originated as surface contaminants of the butyl stopper, and a procedure for washing the

stoppers to remove this contamination was described. Methods for testing of silicone coatings on ampuls and vials for injectable solutions were reported (254). Ampuls treated with a 1.5 or 2% aqueous silicone emulsion showed fewer defects of coating than those treated with a 0.5 or 1% emulsion. Hall (255) elucidated the potential problem areas in the manufacture of single-dose ophthalmic products. These included the chemicals used, the packaging materials, the water used, personnel, plant facilities, and control procedures. Use of methylcellulose and hydroxyethyl cellulose, polyvinylpyrrolidone, benzalkonium chloride, cetrimide, and thimerosal were evaluated for use in contact lens solutions (256). Tabulated data were presented to facilitate the selection of suitable combinations of these chemicals with respect to viscosity, film-forming properties, hydration, stability of solutions, and protection against microbial contamination. Ophthalmic solutions prepared with 2% aqueous polyvinyl alcohol were shown to be superior to solutions prepared with water alone (257). No side effects of polyvinyl alcohol were noticed when these solutions were administered to 30 patients with corneal lesions.

Reconstituted antibiotic intravenous products prepared by a number of companies were surveyed for foreign particulate matter (258). The results indicated a variation among companies as to the amount of particulate matter found in their products. Other workers noted that asbestos was found in approximately one-third of the samples from two sets of 17 widely used parenteral drugs (259). McGinn (260) reported that much of the nonviable particulate matter found in parenteral solutions packaged in glass vials originated from the packing material used in shipping the empty vials. He suggested that using a type of plastic film wrapping for these empty containers would reduce appreciably the rejects due to this source of particulate matter. However, all parameters involved in the manufacturing process of injectable fluids were investigated and shown to play an important role in contamination by particles (261). Summaries of the activities of a committee appointed by the Pharmaceutical Manufacturers Association (PMA) to explore the methodology for determination of particulate matter in large-volume parenterals was reported (262). A comparison of results obtained from visual inspection, Coulter counter measurements, microscopic examination, and counting of particles collected on membrane filters was made to evaluate the visual method commonly used to control the cleanliness of parenteral solutions (263). It was concluded that visual inspection data obtained during the manufacturing process must be controlled by the laboratory afterward.

Other papers of interest in the area of parenterals are listed in Table VII.

Table VII—Additional References on Parenterals

Reference	Topic
264	Conditions for the acid hydrolysis of casein which ensure the production of preparations for parenteral use
265	Parenteral and oral formulations of marijuana constituents
266	pH of drugs for parenteral use
267	pH measurements of sterilized sodium bicarbonate solutions
268	Method for the preparation of injectable cannabis extracts
269	Preparation of a solution of apomorphine hydrochloride for parenteral use
270	Review of infusion solution production
271	Preparation of fructose, sorbitol, and sodium bicarbonate parenteral infusions
272	Review of vehicles and adjuvants for the preparation of injectable solutions of steroid hormones
273	Experimental preparation of freeze-dried urea for injection
274	Physicochemical examination of glucose injection
275	Review of properties of various contact lens solutions
276	Advantages of utilization of dextrans as excipients for viscous collyria
277	Polymers used in ocular wetting agents
278	Review of colloidal injectable plasma substitutes
279	Determination of freezing-point lowering, sodium chloride equivalents, and isotonic concentrations of some drugs in aqueous solution
280	Dimethylpolysiloxane compared to sesame oil as a vehicle for progesterone injection
281	Evaluation of laminar air flow for the preparation of intravenous admixtures
282	Review of the contamination of parenteral solutions by foreign particles
283	Identification of the leached ingredients from rubber closures

Sterility—Boucher (284) reviewed the various physical and chemical methods of sterilization. He concluded that synergistic sterilization, the use of combinations of chemical and physical methods, is on its way to making a profound impact not only in the pharmaceutical field but also in chemical, food, and decontamination industries. The current status of biological indicators was discussed, and plans for future studies were outlined (285). One investigator (286) reported significant differences found among five commercial biological indicators with regard to bacterial count and performance as measured by survival and kill in 10 autoclaves at 121°. He called for further work in the improvement and standardization of these indicators.

The use of cellulase in membrane sterility testing of solutions containing carboxymethylcellulose was discussed (287). An empirical filtration rate test was developed for determining cellulase activity. A group of compounds which rapidly inactivate certain antibiotics and thus facilitate sterility testing of the antibiotic solution was reported (288). The factors necessary to consider in establishing a successful ethylene oxide sterilization cycle were discussed (289). As an example, these factors were applied in the sterilization of a production lot of plastic disposable syringes in paper wrappers. Two compounds, 2-chloroethanol (290) and chloroacetaldehyde (291), which have been associated with residual ethylene oxide in sterilized material were subjected to biological testing. Ethylene oxide sterilization was shown to have no effect on the microbiological activity of streptomycin sulfate, dihydrostreptomycin sulfate, neomycin sulfate, and paromomycin sulfate (292). A slight increase in the color of streptomycin sulfate and dihydrostreptomycin sulfate was noted.

Relative humidities of at least 50% were essential for the sterilization of spore-contaminated materials using formaldehyde (293). The history and an extensive review of the use of radiation methods for the sterilization of drugs were presented (294). Also reviewed was

the method of sterilization of air and gases used in the pharmaceutical industry (295). A survey was conducted by the Food and Drug Administration to obtain a profile of the microbial load in all antimicrobial ophthalmic ointments manufactured in the United States (296). Of the 82 batches of ointments from 27 manufacturers that were tested, 16 showed contamination. Pyrogen-free solutions of 5% glucose were obtained by sterilizing at 121–124° for only 12 min. (297). Unsterilized glucose solutions were shown to be pyrogenic by injection of 10-ml. portions into rabbits, resulting in temperature rises of 0.7–1.3°. The Limulus test method for pyrogen testing was compared with the USP XVIII pyrogen test on a variety of commercial parenteral preparations (298, 299). The correlation was excellent, providing proper allowance was made for the greater sensitivity of the Limulus method.

Other papers relating to sterility are listed in Table VIII.

Table VIII—Additional References on Sterility

Reference	Topic
300	Sterilization and disinfection and processes for their control
301	Common misconceptions relating to ethylene oxide and steam sterilization
302	Review of cautions to be observed in use of ethylene oxide
303	Changes in fatty vehicles during thermal sterilization
304	Sterilization of plastic medical equipment
305	Sterilization of an acrylic resin prothesis by hydrogen peroxide
306	Disinfection and sterilization of pharmaceutical and medical plastics
307	Method for determining the effectiveness of filter materials for sterilizing air
308	Effect of energy-rich radiation on analgesics—Part I
309	Effect of energy-rich radiation on analgesics—Part II
310	Effect of cobalt-60 γ-irradiation on tetracycline

Tablets and Capsules—Cooper and Rees (311) presented an excellent review of the literature from 1969 to 1971, including all phases of tableting research and technology. A comprehensive review article covering the various factors affecting tablet disintegration was presented by Lowenthal (312). Several other areas dealing with the general topic of tableting technology were reviewed (313–315). The other numerous publications dealing with pharmaceutical technology of tablets and capsules have been subdivided into the following classifications to facilitate a search of the literature: comminution, mixing, granulation, and drying; powder characteristics; compression; effect of excipients; and tablet coating. For a thorough review, consideration of the entire section is advised since there is an obvious overlap in the subject matter of the subclassifications.

Comminution, Mixing, Granulation, and Drying—Size reduction of drug compounds by a method involving phase conversion was reviewed (316). The rate of decrease of the mean molecular weight and molecular weight distribution of polyvinylpyrrolidone powders as affected by ball milling in nitrogen, air, and oxygen was investigated (317). The influence of the ball milling atmosphere on the probability of the polyvinylpyrrolidone molecule breakdown in a unit time and the molecular size distribution of the polymers formed by the breakdown in a unit time were discussed. The problems involved in mixing and mixing mechanisms of particulate systems were reviewed (318, 319). Mixing properties of barium sulfate, lactose, spray-dried lactose, magnesium oxide, and basic magnesium carbonate were investigated (320). The effects of the specific volume of the powders, time of mixing, and type of mixing equipment used were studied. Mixing of potato starch and sodium bicarbonate under a very high dilution ratio (2500–100,000) was studied using a radioactive tracer technique (321). The results of this study demonstrated that the state of fully random mixing does not depend on the particle-size characteristics of the diluent. However, other workers using radioactive tracer techniques determined that mixing efficiency was very dependent on the particle size of the diluent (322, 323). The dependence of the degree of mixing and the time of mixing on the speed of rotation and quantity of powder to be mixed in solid–solid mixing was investigated for industrial applications (324). To obtain the same results with scale-up of the mixing equipment, consideration must be given not only to the geometric and dynamic similarities but also to the ratio between size of sample and size of batch in both pilot and largescale equipment.

Calcium phosphate requires more granulating fluid to produce a cohesive mass during wet granulation than other tablet diluents, such as lactose or starch (325). It was postulated that the intraparticulate porosity is such in calcium phosphate that at low moisture contents the liquid becomes located within the particles, thus preventing the formation of pendular liquid bridges between adjacent particles. Lactose granules were prepared by massing and screening, using water as a binder, and the effects of initial particle size, size distribution, and binder concentration on the physical properties of the granules were studied (326). Increasing the mean initial particle size decreased the amount of binder necessary to form granules and also decreased their strength, porosity, flow, and packing at equivalent moisture contents; narrowing the size distribution increased granule porosity but decreased strength even further. Granulations made by the spheronization method showed an improved granulation flow rate and narrow particle-size distribution as compared to a conventionally processed wet granulation (327). Other testing of this same method indicated that plate rotational speed was of great importance in establishing the geometric form of final granules but that dwell time did not have an appreciable effect (328).

The optimum parameters for granulation on laboratory scale and pilot plant equipment were investigated for a fluidized-bed spray granulation process (329). An increase in the amount of binding agent increased mechanical strength and decreased wear loss, while an increase in flow rate of fluidizing air and a decrease in feeding rate of the granulating fluid resulted in a particle-size decrease. Davies and Gloor (330) reported that the physical properties of fluidized-bed granulations were strongly influenced by the binder and the binder concentration. An increase in the binder concentration increased the adhesiveness, yielding less friable granules of a greater average size. Other workers obtained fundamental information on the relationship between

the amount of a binder and several characteristics indicating granulating state and properties of the granules formed (331).

A mixture formed by heating rosin and glycerol was found to be a satisfactory anhydrous binder for the granulation of tablet preparations (332). The surfaces of granules formed by the spray drying of aqueous slurries of salicylic acid and sodium salicylate with both gum arabic and polyvinylpyrrolidone were found to contain no holes or craters as compared to slurries spray dried with other binders such as gelatin, polyvinyl alcohol, carboxymethylcellulose, methylcellulose, tragacanth, and sodium alginate (333, 334). Sodium warfarin tablets prepared by a wet granulation method were unsatisfactory as to content uniformity due to the migration of the drug substance during the drying period (335). By using a TLC method, additives were selected which demonstrated inhibition of the migration of the drug substance. The use of ultrasonics for drying pharmaceutical granulations was discussed (336). It was reported that drying time was approximately one-sixtieth that of rack drying (337).

Additional references on comminution, mixing, granulation, and drying are listed in Table IX.

Table IX—Additional References on Comminution, Mixing, Granulation, and Drying

Reference	Topic
338	Diffusion and mixing of solid particles in a tube mixer
339	Parameters involved in mixing pharmaceuticals and other fine powders
340	Mixing and grinding processes during the preparation of pharmaceutical powders using a mortar
341	Agglomeration by spray drying
342	General methodology of fluid-bed granulation and spray drying
343	Effect of granule size on the physical properties of tablets
344	Review of tablet manufacture without granulation
345	Determination of the forms of moisture bonding in tablet granulations
346	Review of principles of nodular granulation, spray coating, and coating
347	Effect of moisture and heat on the decomposition of aspirin (acetylsalicylic acid) during granulation
348	Physical characteristics of tablets prepared by dry and wet granulation
349	Effect of the movement of the suspending medium during drying in a fluidized bed

Powder Characteristics—Reviews of the physical–chemical methods of characterizing powder surfaces were presented (350, 351). Both Hiestand (352) and Pilpel (353) discussed various methods for obtaining meaningful physical data for cohesive pharmaceutical powders. The problem of solid dosage forms containing small amounts of active ingredients was treated statistically (354). Equations which included particle-size distribution were derived for the calculation of theoretical coefficients of variation of drug content for solid dosage forms. Particle size of materials had very little influence on compressed tablet properties with substances having weak particle-to-particle bonding, but it had rather considerable influence with particles having strong interactions (355).

A simple test to determine the proportion of micronized drug that can be used to prepare satisfactory mixes for tableting was reported (356). The test consisted of measuring the angle of repose of a micronized powder–coarse particle mixture as a function of the concentration of micronized material. Particle densities, packing changes, and sieving properties of five commercially available samples of magnesium stearate were investigated (357). The significance of sample variation in relation to pharmaceutical processing was discussed. Description and evaluation of various testing methods for determining powder flow and powder cohesiveness were the subjects of several papers (358–360). Results of a study investigating mechanical properties of dry powders over a range of temperatures resulted in a postulation that, under the influence of pressure, melting can occur at the points of contact between particles at temperatures below their conventional melting points (361). This hypothesis accounts for the formation of solid bonds which contribute to the strength and hardness of pharmaceutical tablets. The role of moisture in compaction of particulate material was studied (362). It was determined that moisture decreases the compact strength by reducing the strength of interparticulate bonds. The effect of moisture on tensile strength of bulk solids was determined (363, 364). At a constant state of packing, the tensile strength was shown to increase as moisture content rose. This was attributed to an increase in number and dimension of liquid pendular bonds.

Additional articles relative to powder characteristics are listed in Table X.

Compression—In a series of papers reporting studies of compression of powder-like materials, Bessho *et al.* (392–394) discussed, on a theoretical basis, stress distribution, density distribution, and energy involved. The ability to convert the load at which a tablet fails in tension under diametrical compression into a tensile strength was extended to deep concave tablets (395). It was demonstrated that sucrose tablets with an upper beveled edge capped at high applied forces unlike those tablets prepared with a flat upper face (396). This was thought due to high radial forces in the beveled top at the end of the compression cycle resulting in breakdown of interparticulate bonds formed during compression. Newton and Rowley (397) suggested that compaction force or pressure does not completely characterize the formation of a tablet. They offered the concept of force–time measurements as a possible solution. An instrumented punch and dye set was used to determine the force–displacement curve during compression of sodium chloride, boric acid, asagran, vitamin yeast powder mix, ibuprofen granulation, and acetaminophen (paracetamol) granulation (398). Although all of the materials behaved elastically following compaction, only sodium chloride was approximately linearly elastic.

A system designed to simulate the double-acting compression effect of rotary tableting presses was described (399). The double-acting compression was achieved by a controlled downward movement of the dye at a slower rate than the simultaneous downward movement of the upper punch. A 16-station high speed rotary tableting machine was fitted with pressure rolls containing piezoelectric transducers in the axles (400). The compaction force exerted at each tablet compression event produced an electrical pulse which could be fed to

Table X—Additional References on Powder Characteristics

Reference	Topic
365	Review of effects of water content of powders
366	Adhesion forces causing agglomeration of solid particles
367	Factors affecting the parameters of the flow rate equation
368	Effect of granule characteristics as related to weight variation of strip packages filled on automatic equipment
369	Cohesion of particulate solids
370	Theoretical calculations of tortuosity in a sedimentation bed
371	Significance of the form and dispersion composition of aspirin (acetylsalicylic acid) crystals in the tableting process
372	Effects of particle size, shape, and moisture content on the tensile properties of procaine penicillin powders
373	Specific surface area as a pharmaceutical control test for micronized drugs
374	Comparative study of four methods of granulometric analysis
375	Evaluation of powder surface properties by an adsorption method
376	Compactability of dispersed powders
377	Improvement of the flow properties of a chloramphenicol powder by the addition of Aerosil
378	Effects of surface tension on the tensile strength of beds of moist bulk solids
379	Compacting phenomenon of powders in the course of mixing with calcium stearate
380	Influence of particle size on the cohesion of particulate solids
381	Contribution of charge to powder particle adhesion
382	Random packing of equal and unequal spheres in two and three dimensions
383	Review of powder shear
384	Influence of particle size and tablet shape on the separation of sparingly soluble drugs from a hydrophobic matrix
385	Effect of the granulometric composition of powders on their volume weights and flow
386	Photometric process for particle-size analysis by microscopic examination
387	Electron-microscopic investigation of powders
388	Experimental studies of structural, mechanical, and thermophysical characteristics of some drug granules
389	Structural and mechanical properties of pharmaceutical molding powders
390	Flow properties of granules and powders
391	Dependence of capsule filling on the characteristics of powders

an integrated-circuit pulse-discriminating unit. An extensive review by Cavaco (401) was presented on the various aspects of tablet production by direct compaction and included physical factors influencing the cohesion of solid particles, their behavior during compression, improvement of the agglomeration by solid adjuvants, and the final characteristics of tablets. Reviews on the compression of powders (402) and the physical phenomena in tablet compression were also presented (403).

Other articles concerning compression are listed in Table XI.

Effect of Excipients—The effect of various starches such as corn, potato, rice, arrowroot, and a compressible starch on the rate of dissolution of salicylic acid tablets was investigated (415). In all instances, the dissolution of the drug was fastest from tablets containing compressible starch, whereas the rank order of the dissolution times of the other starch formulations depended on the dissolution test method used. In a

Table XI—Additional References on Compression

Reference	Topic
404	Prediction of tableting troubles such as capping and sticking
405	Theoretical consideration of several compression-state equations of powder-like materials
406	Factors affecting crushing properties of tablets
407	Effect of humidity and compression force on the retention of eucalyptol in tablets
408	Thermal processes during tableting
409	Discussion of the three stages of tablet compression
410	Pressure-process diagrams using a piezoelectric measuring method
411	Development of a formulation with the aid of force–displacement measurements
412	Compression behavior of phenacetin granulations
413	Porosity of tablets as related to compression pressure
414	Properties of tablets produced on an automatically controlled rotary press

study to determine the effect of intra- and extragranular maize starch on the disintegration of compressed tablets, intragranular starch resulted in the recovery of finer particles than when extragranular starch was used, but the disintegration time was longer with intragranular starch (416). Lowenthal (417) determined that starch grains were deformed by pressure and did not regain their original shape nor swell significantly when moistened. He concluded that the regaining of the shape of starch grains is apparently not the mechanism of action as a tablet disintegrant.

The effect of compression force on tablets containing sodium carboxymethylcellulose of two different molecular weights was measured (418). The lower molecular weight material resulted in tablets having larger specific surface area. An investigation of their disintegration properties showed that both cation-exchange resin and alginic acid were more efficient than calcium sodium alginate, sodium carboxymethylcellulose, and starch (corn) (419). Other studies determined that calcium sodium alginate compared favorably with alginic acid and starch when evaluated as tablet disintegrants (420). By using additives such as ethylcellulose, glyceryl monostearate, and sorbitol, it was possible to regulate the absorption levels for achieving a therapeutic effect with sulfosalicylic acid–polyvinyl chloride tablets (421). Tablets containing microencapsulated ferrous fumarate demonstrated that microencapsulation increased the dissolution of iron in gastric juice and reduced local irritation by preventing contact between the salt crystals and the gastric mucosa (422). Javaid and Cadwallader (423) studied 11 different compounds representing various classes of buffering agents with respect to their effect on dissolution of aspirin from tablet formulations. In general, carbon dioxide-producing agents such as sodium bicarbonate, magnesium carbonate, and calcium carbonate gave more rapid dissolution than the readily water-soluble buffering agents.

Magnesium stearate and sodium stearyl fumarate were compared as tablet lubricants in an antacid formulation and judged by punch force, distintegration time, and *in vitro* availability (424). By the three testing parameters used, sodium stearyl fumarate was superior. In another study, the pressure remaining immediately after compression was used as a measure of the lubrica-

Table XII—Additional References on Effects of Excipients

Reference	Topic
430	Mechanism of action of tablet disintegrants
431	Interpretation of the mechanism of disintegration
432	Incompatibilities of starch
433	Influence of starch, alginic acid, and Carbopol on the dissolution rate of aspirin tablets
434	Effect of binders and disintegrating agents on calcium monohydrogen phosphate-based tablets
435	Effect of incorporation of water-insoluble substances on delaying disintegration time
436	Effect of magnesium stearate and polytetrafluoroethylene as lubricants in direct compression of aspirin
437	Effect of adjuvants on the release of slightly soluble drugs from a hydrophobic tablet matrix
438	Factors involved in the formulation of soluble aspirin tablets
439	Formulation of aspirin tablets
440	Properties of powdered celluloses in tablet production
441	Ethylcellulose as a binder in meprobamate tablets
442	Tablet properties as a function of different binders
443	Determination of hydrogen bonds in tablet formation
444	Determination of the direct compressibility of phenothiazine derivatives
445	Characteristics of magnesium aluminosilicate under compression
446	Testing of directly compressible tablet bases
447	Evaluation of excipients for direct compression
448	Cellulose-type additives for direct compression
449	Excipients for tablet manufacture
450	Release of drugs from hard gelatin capsules

ting effect (425). By this measurement, both lubricants were shown to be equal. It was reported that magnesium lauryl sulfate possessed the lubricating properties of magnesium stearate but not its waterproofing liability (426). Factors affecting the direct compression of tablets using microcrystalline cellulose were reviewed (427). Twenty-four formulations of placebo tablets made from eight excipients for direct compression and three disintegrants were evaluated under various conditions of relative humidity (428). The data were evaluated statistically so that the formulations could be given a relative ranking on the basis of maximum hardness, minimum change in disintegration time, minimum moisture uptake, and minimum change in volume. Jones (429) reviewed the growing evidence indicating that formulation of the capsule fill is the rate-determining step in disintegration and dissolution.

References to additional literature on effect of excipients are listed in Table XII.

Tablet Coating—Several different films cast from solutions of ethylcellulose or polyamide resin were plasticized with varying concentrations of cetyl (hexadecyl) alcohol or butyl (tributyl) citrate (451). The permeability coefficient of ethylcellulose films was substantially greater than that of films consisting of polyamide resin, and it was also noted that the permeability coefficient was dependent upon the plasticizer used as well as its concentration. Two formulations were developed using polyvinylpyrrolidone for film coating of tablets by the pan-coating method (452). The addition of ethylcellulose and shellac eliminated the tackiness sometimes associated with polyvinylpyrrolidone due to the hygroscopicity of the film formed but did not alter disintegration or dissolution rates of the tablets. An apparatus for placing a film coat on individual tablets for film evaluation was described (453). Talc and

Table XIII—Additional References on Tablet Coating

Reference	Topic
455	Coating of pills and lozenges with polyethylene glycol and acrylate polymers
456	Film formers as coatings for solid, single-dose pharmaceuticals
457	Surface quality of film coatings
458	Acrylic resin film-coated tablets prepared by controlled spraying
459	Possibilities and problems in the automation of sugar-coated pill production
460	Description of tablet-coating processes
461	Small-scale enteric coating of hard gelatin capsules
462	Formulation of enterosoluble gelatin capsules
463	Characteristics of acidproof gelatin capsules
464	Treatment of capsules for gastric resistance

magnesium stearate were evaluated as adjuvants in enteric-coated tablets (454). Incorporation of talc was more satisfactory in providing favorable enteric coatings and served to preserve or augment the hardness of the tablets.

Additional references on tablet coating are listed in Table XIII.

Suspensions—Thickening agents for formulation of suspensions, emulsions, and gels of pharmaceutical interest were reviewed (465). Hiestand (466) developed the probable mechanism for the control of floc structure by polymeric materials from the theory of polymer stabilization of disperse systems. Although quantitative predictions were difficult to make, the curdled look after flocculation suggested that inadequate polymeric material was present and, therefore, the average coordination number was too high. On the other hand, settling to produce a very thick, noncaking sediment was probably due to excessive protection by suspending agents. Several agents were investigated for their stabilizing properties on griseofulvin suspensions (467). Among polyvinyl alcohol, polyvinylpyrrolidone, polysorbate 80, sodium carboxymethylcellulose, dry milk, and kaolin, polyvinyl alcohol was superior.

A graphical method was described for determining the effects of concentration and particle size on the sedimentation of suspensions; this method was based on traditional formulas describing the motion of particles in a liquid under the action of gravity (468). In the determination of yield values of hectorite gels, advantages of the falling sphere method over the cone-plate viscometer were discussed (469). Studies on the effects of particle charge of barium sulfate preparations indicated that charge alone contributes little to stability and cannot explain adherence of barium sulfate to mucosal linings (470). A microscopic electrophoretic method was used to study dilute aqueous barium sulfate suspensions (471). The isoelectric point for barium sulfate was reported at pH 4.2, and the ζ-potential varied from 140 mv. at pH 1 to -120 mv. at pH 10. The problem of pH measurements in suspensions was investigated (472). In the suspensions studied, the Wiegner–Pallmann suspension effect played a role which, however, was not identical with the pH-disturbing effect due to anomalous diffusion potentials.

Two types of mechanisms of particle adhesion to glass containers were found (473) by examining the adhesion

Table XIV—Additional References on Suspensions

Reference	Topic
476	Formulation and evaluation of two sulfamethazine (sulfadimidine) suspensions
477	Flow behavior of clay–water suspensions
478	Flocculation of Veegum suspensions by electrolytes
479	Evaluation of barium sulfate suspension formulations
480	Stabilization of suspensions by surfactants
481	Evaluation of the degree of dispersion of drug substances
482	Formation and properties of association colloids
483	Review of pharmaceutical suspensions

of chloramphenicol treated with benzalkonium chloride: (*a*) simple adhesion without mutual affinity or interaction and (*b*) adhesion with affinity. The ζ-potential of chloramphenicol crystals and glass powder was determined, and its influence on the adhesion and adsorption was discussed. In similar studies, but using prednisone, Runkel and Palagyi (474) determined that maximum adsorption of particles to glass containers occurred at a concentration of benzalkonium chloride that was just sufficient to neutralize the inherent negative charge of the prednisone particles. When the average particle diameter of the prednisone was increased to 50 μ, adsorption was negligible and drainage characteristics were excellent. A review of the physical and chemical characteristics of suspensions, including free energy of particles, humectation, flotation, crystal growth, sedimentation, rheology, and flocculation, as well as their pharmaceutical formulation, was presented (475).

Additional references on the subject of suspensions are listed in Table XIV.

Emulsions—Several reviews on the effects of processing and manufacturing procedures on the physical properties of emulsions were presented (484–486). The methods used in the evaluation of the physical properties and stability of emulsions were also reviewed (487, 488). Sherman (489) stated that accelerated aging tests at high and low temperatures and under centrifuge conditions are not suitable for predicting stability of emulsions. He reported that the measurement of the electrophoretic mobility better relates to the stability of oil-in-water emulsions at room temperature and at 40°. The effect of ultrasonic power on a mineral oil–surfactant–water emulsion system was reported (490). An optimum amount of energy was required to produce the best emulsion, but the effects of the hydrophilic–lipophilic balance (HLB) and surfactant parameters appeared to be more important in the emulsions studied. The time dependence of the droplet size distribution and the phase-inversion occurrence times were determined as functions of volume ratios, emulsifier concentration, and impeller speed for mixing water and carbon tetrachloride containing polysorbate 60 in a baffled, stirred tank (491). Phase inversion, which was very sensitive to emulsifier concentration, occurred even during stirring and the final specific interfacial area was independent of the emulsifier concentration.

An emulsion formulation suitable for freeze drying and reconstitution was described (492). Mannitol, urea, and glycine were used as supportive agents. In studying emulsifying agents, it was demonstrated that the critical

Table XV—Additional References on Emulsions

Reference	Topic
503	Factors affecting the formation of emulsions
504	Definition and theory of emulsions
505	Emulsifiers in cosmetic preparations
506	Water-in-oil cosmetic emulsions
507	Critical HLB and ternary diagrams of various oil phases of emulsions
508	Influence of HLB values of surfactants on drug release from emulsified systems
509	HLB for emulsifiers
510	Theory of linear HLB and its application
511	Determination of the HLB of anacardium gum
512	Theory and technology of submicron emulsions and their cosmetic applications
513	Review of the use of semisynthetic viscosity-increasing agents for emulsion preparations
514	Determination of the emulsifying properties of monoethyl polyoxyethylene esters of fatty acids
515	Copolymers of propylene and ethylene oxides as emulsifiers for organic liquids in water
516	Phase equilibrium of microemulsions, hydrotropic solutions, and emulsions
517	Role of mixed emulsifiers in emulsion stability
518	Stabilization of concentrated oil-in-water emulsions with aqueous solutions of proteins and surface-active polymers
519	Water-in-oil emulsion ointment bases with coupled emulsifiers
520	Emulsifying capacity of sodium lauryl sulfate
521	Emulsions of hydrophilic colloids in the presence of buffers
522	Physicochemical studies of the mutual breaking of emulsions
523	Wetting and emulsifying power of nonyl phenol polyglycol ethers
524	Application of the cohesive energy ratio concept to anionic emulsifiers
525	Differences in ultracentrifugal stability of various oil-in-water emulsions
526	Effect of initial concentration of emulsifying agents on stability of oil-in-water emulsions
527	Methods of production of binary emulsions
528	Properties, formation, and uses of binary emulsions
529	Influence of temperature-induced phase transitions on fat emulsions
530	Emulsion preparation by ultrasound
531	Preparation of pharmaceutical emulsions by electrical discharge

HLB numbers increased approximately linearly with the decrease of the logarithm of the oil phase dielectric constant (493). Pharmaceutical and chemical problems involved in the choice of an emulsifier for cosmetic formulations including anionic, amphoteric, cationic, and nonionic surfactants were discussed (494). Mathematical expressions for the HLB, minimum volume of emulsifier for oil-in-water and water-in-oil emulsions, and stability of the emulsions were developed. It was suggested that the optimum stability of oil-in-water emulsions stabilized by 1:1 molar ratios of sorbitan esters (Spans) and polysorbates (Tweens) is due to association between the emulsifier molecules adsorbed at the oil–water interface (495). The various analytical procedures for determining HLB values of emulsifiers do not account for interaction between emulsifiers and the aqueous and oil phases. It was, therefore, suggested that phase-inversion temperature determinations may provide more meaningful values (496), and a programmed viscometric technique was described for determining phase-inversion temperatures (497). With surfactant mixtures of polyoxyethylene ethers of fatty alcohols (Brij 92 and Brij 96), the phase-inversion temperature

was found to rise in approximately linear fashion as the HLB increased toward the optimum value.

The stability of mineral oil (Nujol) emulsions emulsified with sodium lauryl sulfate could be increased by adding lauryl alcohol (498). Speculation attributed the increase in stability to adsorption of lauryl alcohol at the interface not occupied by adsorbed sodium lauryl sulfate. Other workers concluded that combinations of emulsifying agents, as opposed to single emulsifying agents, produce more stable emulsions (499). The structure of oil-in-water emulsions stabilized by binary surfactant mixtures was represented schematically according to the oriented wedge theory (500). The stability of oil-in-water emulsions of paraffinic oils stabilized by sodium lauryl sulfate and sodium cetyl sulfate was shown to increase with increasing chain length of the paraffin (501). It was suggested that long-chain paraffin molecules are strongly held at the interface by association with alkyl sulfate molecules, thus increasing the coherence of the interfacial film. Starch treated with 4–5% glycerol monostearate formed a stable cream emulsion and was shown to be an effective emulsifier for cosmetic creams with a high water content (502).

Other references pertinent to emulsions are listed in Table XV.

Ointments and Creams—Particle dispersion as measured by the electron microscope was determined for salicylic acid in white petrolatum and polyethylene ointment bases and for a base of calcium soap and mineral oil (532). The rheological properties, as well as the consistency of the polyethylene base, were optimal and gave the best release of medication. The pharmacology, physical chemistry, and clinical actions of polyethylene glycol-based ointments containing hydrocortisone, antibiotics, iodine, potassium iodide, boric acid, sulfur, and coal tar were reviewed (533). Other reviews stressed the importance of selecting the correct vehicle for assuring greatest effectiveness of a drug applied to the skin (534, 535). Release of citric acid from ointments prepared with petrolatum or hardened soybean oil was enhanced by the addition of 1–7.5% egg lecithin (536). Phosphatidylcholine was considered to be the essential factor responsible for this effect. Ointment bases containing surfactants gave better release of salicylic acid, chloramphenicol, and tetracycline than bases without them (537). The HLB values of the surfactant incorporated into the ointment base influenced the release pattern, but the variation did not fit any general pattern and differed for different bases and drugs. When comparing hydrophilic and hydrophobic bases, it was shown that sulfathiazole and potassium iodide revealed higher release coefficients from hydrophilic bases (538).

The effect of some physicochemical properties of palmseed oil on the liberation of drugs from the vehicle was investigated, and it was found that release was more rapid and greater from palmseed oil of higher iodine numbers and also from bases that were mixtures of small-chain fatty acids (539). The release of citric acid was determined *in vitro* from ointment bases composed of white petrolatum and various natural oils and fats (540). Accelerated release was observed solely after addition of palmseed oil, and the rate of release was directly proportional to the content of palmseed oil in the base. Twelve protective creams containing silicone oil were evaluated by conductometric determination of diffusion rates for sulfuric acid and sodium hydroxide (541). The higher the diffusion rate, the lower was the protective capacity of the preparation. The technology for an anti-inflammatory ointment base for gums and oral mucosa was described (542). The penetration of crystalline particles of cosmetic and pharmaceutical formulations into the horny layer of human skin was evaluated by microscopic evaluation of stripped films of the stratum corneum in polarized light (543). Penetration was judged to be dependent on particle shape and solubility in the constituents of the horny skin layer rather than on particle size. A greater diffusion of methylene blue was obtained from an ambiphilic emulsion ointment on an agar base than from either lipophilic or hydrophilic emulsions, indicating the superior drug dispersal qualities of the ambiphilic systems (544).

Barry and Grace (545) investigated the rheological parameters operative during application of topical preparations to the skin. The information derived was used to develop a method for instrumentally determining rheological conditions in routine industrial control procedures and a spreadability screening test for application during innovative work prior to field trials. A review was presented on the use of creams in pharmaceuticals and cosmetics with examples of formulations and industrial manufacturing techniques (546). Polli *et al.* (547) described a laboratory investigation conducted to evaluate the use of conventional sterilizing techniques during manufacture of sterile topical dosage forms. Methods determined suitable for the sterilization

Table XVI—Additional References on Ointments and Creams

Reference	Topic
548	Rheological properties and composition of petrolatum
549	Use of an aliphatic sulfonate type of emulsifier as an ointment component
550	Technology and study of drug forms with dimexide
551	Rheological testing of several emollient creams
552	Effect of temperature on the consistency of hydrocarbon ointments
553	Effect of composition on consistency and bleeding of hydrocarbon salves
554	Properties and uses of sodium alginate for ointments
555	Excipients in preparations for dermatological use
556	Water absorption number of ointment bases
557	Technological and pharmaceutical characterization of dermatological preparations
558	Polyethylene glycols as bases for preparing salicylic acid ointments
559	Formulation and preparation of transparent dispersions of lanolin in liquid and gel forms
560	Dispersing capacity of dimethyl sulfoxide during preparation of white mercury and zinc ointments
561	Effects of triglycerides of saturated and unsaturated fatty acids on the liberation of active substances from hydrocarbon ointment bases
562	Review of various types of ointment bases
563	Liberation of active substances from eye ointments
564	Determination of the diffusion and distribution coefficients of active materials in salves
565	Stability testing of concentrated intermediates of suspension-type ophthalmic ointments
566	Methods for testing externally applied pharmaceuticals

of active ingredients, excipients, vehicles, and packaging materials were reported.

Other references pertaining to creams and ointments are listed in Table XVI.

Suppositories—For lipophilic bases, the homogeneity of suspensions of active agents in the bases, the rheological behavior at 37–40°, the viscosity at 40°, and the thixotropic index could be used as criteria for determining the suitability of these bases for use in suppositories (567). The index of emulsionability, the maximum percentage of water that a suppository mass may contain as a stable emulsion, was determined by the uniformity of color given to the mass by Sudan II (568). Good emulsifying power was correlated with higher dehydration tendencies. The addition of sorbitan esters (Spans) or sorbitan ester–polysorbate (Span–Tween) mixtures to suppositories containing aminopyrine (amidopyrine) decreased their disintegration time and thus accelerated the release of the active ingredient (569). Approximately twice as much sodium iodide was absorbed in rats from rectal suppositories prepared with polyethylene glycol, Witepsol S, or Witepsol E as from those prepared with cocoa butter (570). Absorption was measured by ^{131}I activity in the thyroid.

Addition of monoglycerides to suppository bases did not improve the passage of drugs through a membrane (571). As the amount of monoglycerides in the base increased, the amount of liberated drug decreased. Triglycerides gave the opposite effect. In studies on Shay butter, it was determined that the addition of 20% hard paraffin or 15% white beeswax was necessary for use in suppositories (572). Gelatin and methylcellulose used in 2 and 0.75% concentrations, respectively, were more suitable than 1% sodium alginate, 2% polyvinylpyrrolidone, or 4% yeast in the manufacture of suppositories by freeze drying (573). The choice of active ingredients present in the suppositories and their concentrations strongly affected the lyophilization properties of the base compounds. Therefore, the appropriate base must be chosen to correspond with the desired active ingredient.

Additional references on suppositories can be found in Table XVII.

Aerosols—Several aspects of aerosols were reviewed, including the development of pharmaceutical aerosols (580), the pharmaceutical uses of aerosols (581), aerosol drug therapy (582), solvent propellants (583), and technical problems of powder aerosols and aerosol propellants (584). The series of events, including product development, leading to the marketing of a new aerosol product was traced through the eyes of a cosmetic chemist (585). Sciarra *et al.* (586) investigated the possible formulation of various epinephrine salts such as malate, maleate, and fumarate for inclusion in aerosol dosage forms. The results of solubility studies of these salts in propellants indicated that the formulation of epinephrine salts as an aerosol dosage form must be accomplished through use of a cosolvent or by formulating a dispersed system. A stable aqueous suspension of a mixture of thymol, peppermint oil, and eucalyptus oil was described for application as an aerosol (587). The use of nitrogen at 6 atmospheres as the propellant gave aerosol particles of 40–50 μ in diameter. The particle-size distributions of four hair-spray formulations were measured as a function of the propellant composition (588). The mass median diameters of the sprays were found to decrease with increasing pressure of the aerosol.

A method was described for determining comparatively the proportion of the emitted dose of an aerosol formulation that penetrated a system of wet-lined tubes and arrived at a filter (589). Clinical work with bronchodilators showed that this method permitted comparisons between different formulations of the same dose of the same drug. By using a conifuge, measurements were made of the particle-size distribution of aerosols generated by three types of nebulizers intended for therapeutic use (590). After converting the size distribution to a mass distribution, the deposition of different fractions in the human respiratory tract could be predicted. The critical variables that control the movement of aerosol particles were found to be the size and density of the individual particles, their initial velocity, and the velocity of the surrounding air (591). These may be adjusted, within limits, to promote the desired deposition of the particles or to prevent an unwanted deposition.

Timed Release—A listing of recognized types of timed-release dosage forms included: barrier coating, imbedding in a slowly eroding matrix, imbedding in a plastic matrix, repeat action through layering in tablets, mixture with ion-exchange resins, imbedding in a hydrophilic matrix, epoxy resin beads, soft gelatin retard capsules, and drug complexing (592). Methods of prolonging the action of antibiotics and some other drugs by altering molecular structure so as to decrease solubility and by using coatings or capsules to delay absorption were discussed and evaluated (593). Hollister (594) suggested that although oral timed-release medications delay or slow absorption of drugs, the advantages are often slight or, at times, disadvantageous. The oral antidiabetic buformin (butylbiguanide), which has a normal half-life of approximately 2 hr., was formulated in a timed-release form for which serum concentration remained almost constant for approximately 7 hr. (595). Timed-release tablets of the insoluble matrix type containing 15 mg. phenylpropanolamine hydrochloride (norephedrine chloride) and an antihistaminic were evaluated (596). *In vivo* and *in vitro* drug release showed good agreement with all tablets except one formulation in which the *in vitro* dissolution rate depended on the pH of the dissolution medium.

Table XVII—Additional References on Suppositories

Reference	Topic
574	Review of rectal drug forms and their properties
575	Compatibility of chloral hydrate with lipophilic suppository bases
576	Mechanical and rheoviscometric parameters of oleaginous suppository bases
577	Composition of fat bases for suppositories
578	Possibility of using thyrocalcitonin in suppositories
579	Proposal for the modification of the formulation of iodochlorhydroxyquin suppositories of the Hungarian National Formulary

Surface areas and transverse surfaces of fracture from timed-release tablets manufactured by the imbedding principle were investigated (597). Pressure-dependent differences in the texture of tablets influenced the drug release rate. Granulations for tablets with prolonged action were prepared containing lauric, palmitic, and stearic acid as adjuvants, applied by coating (598). The release rate *in vitro* decreased with the increasing molecular weight of the acids. A method for preparing timed-release calcium chloride tablets in which this ingredient was suspended in a melt of cetyl alcohol, hydrogenated cottonseed oil, and stearic acid was described (599). The release rate of sodium salicylate from timed-release tablets was measured as a function of the amount of magnesium stearate in the tablet (600). Large amounts of this agent blocked the release of the active substance, while too small amounts caused a rapid disintegration of the tablets. The possible use of biodegradable polymers impregnated with the drug for timed-release preparations was discussed in terms of diffusion and permeability theories (601).

Intravenous administration of sodium barbiturates formulated in a soybean oil emulsion showed prolonged-action effects (602). It was postulated that this effect was caused by the oil droplet acting as a depot, releasing the drug to blood and brain capillaries by direct contact, and, therefore, serving as a bridge between the circulation system and the blood–brain barrier. A parenteral suspension of microspheroids consisting of microcrystals of drug surrounded by a totally enclosing semipermeable protective shell was suggested as a prolonged-action intravenous dosage form (603). Release of drugs from the circulating microspheroids markedly prolonged the apparent biological half-life of the drug quinacrine hydrochloride. The alginic acid salt of pilocarpine, when administered as an ophthalmic disk in the cul-de-sac of the eye, provided a significantly greater miotic response than was obtained from pilocarpine solutions (604). This observation suggested that the use of solid ophthalmic dosages in the treatment of glaucoma may be more effective and require less frequent drug administration to produce a prolonged physiological effect.

Additional references in the area of timed release are listed in Table XVIII.

Table XVIII—Additional References on Timed Release

Reference	Topic
605	Different methods of producing long-acting oral pharmaceuticals
606	Review of design and formulation of pharmaceuticals with sustained action
607	Review of formulation materials for gradual-release dosage forms
608	Testing methods for sustained-release preparations
609	Description of a slow-release aspirin preparation
610	Release of active substances from enteric-coated gelatin capsules
611	Method for testing ulcerogenic effect and *in vivo* release of potassium iodide from slow-release tablets
612	Preparation in pharmacies of tablets with prolonged action
613	A mathematical model for optimization of spansule production
614	Controlled dosage of testosterone through silicone rubber

Cosmetics—Microbiological contamination of cosmetic products continues to be a subject of great concern to the cosmetic industry. To facilitate a literature search relevant to this critical problem, the subject of cosmetics has been divided into the following subclassifications: microbiological contamination of cosmetics, and aspects of formulation and technology of cosmetics.

Microbiological Contamination of Cosmetics—Although contamination by Gram-negative organisms, particularly those of the pseudomonas group, is still a problem, the cosmetic industry continues to show a concerted effort to improve the quality of its products (615). Three years ago, 61 of 250 samples examined were contaminated; last year, only eight of 223 examined were reported contaminated. A review of the microbiological control of nonsterile products and raw materials was presented (616). A listing of essential oils, *e.g.*, orange and thyme oils, active against *Bacterium coli*, *Bacterium typhi*, *Corynebacterium diphtheriae*, *S. aureus*, *Streptococcus pyogenes*, and meningococcus was given (617), and other therapeutic properties of these oils were presented.

The inhibitory effect of ethanol and propylene glycol against three strains of *P. aeruginosa* was investigated in the presence and absence of 0.1% potassium sorbate (618). Results suggested that ethanol concentrations of 5% or more may exert a preservative action and that 10% propylene glycol is also suitable. The effect was augmented by addition of potassium sorbate at pH 6–7. Bruch (619) enumerated the common contaminating organisms present in nonsterile products and listed those that are most dangerous to certain types of products. The organisms of clinical significance in pharmaceutical and cosmetic preparations are "free living" types of Gram-negative bacilli and belong to the klebsiella, enterobacter, serratia, pseudomonas, and flavobacterium groups (620). Many of them have a remarkable ability to survive and even to multiply in the presence of commonly used disinfectant and bacteriostatic agents. The potential hazard to consumers from use of eye-area cosmetics contaminated with *P. aeruginosa* was evaluated (621). Experimental results suggested that corneal destruction resulted from the organism's elaboration of a collagenolytic enzyme, which may be activated *in vivo* during the infective process. A full discussion of the importance of monitoring the chemical and microbiological quality of the water used in cosmetics was presented (622).

Aspects of Formulation and Technology of Cosmetics—In suntan preparations containing a commonly used screening agent, oil-based vehicles gave better sunburn protection to human skin than oil-in-water emulsion vehicles (623). However, it was also reported that oil-in-water emulsions were superior in promoting tanning. Data were presented to indicate that substantive proteins, protein hydrolysates, may be useful moisturizing agents in skin-care products (624). Polyamino acids may have an immediate effect on the skin and/or hair, but polypeptides must be changed by enzymatic and acylatic processes to form polyamino acids (625). These processes of change often failed or were delayed, especially with unhealthy skin or hair, but polyamino acids always produced the desired results. Stratum

corneum, in the form of excised callus, was immersed in distilled water, organic solvents, and aqueous solutions of detergents, humectants, and soaps for different lengths of time (626). Weight increases were compared in an effort to determine the factors influencing the water content of skin and the conservation of this water. Although the chemical pyrrolidonecarboxylic acid has been known for many years, only recently was it reported that pyrrolidonecarboxylic acid, especially in salt form, was a naturally occurring moisturizing agent in the skin (627).

Idson (628) reviewed the factors that caused skin to retain or lose moisture, such as relative humidity, hygroscopic materials normally present in the skin, and water-binding mechanisms in the stratum corneum. In a discussion on humectants *versus* moisturizers, humectants were defined as components of cosmetic products that prevent water or moisture loss from the preparation and moisturizers as active additives which restore moisture to the stratum corneum (629).

Titanium dioxide was colored to increase its applicability in cosmetic preparations by reducing a synthetic vat dye to its leuco base followed by the addition of sufficient powdered titanium dioxide at 80° under carbon dioxide (630). Electron microscopy and X-ray analysis showed that a thin, well-adhering layer of the dye coated the particles. Tests to determine the safety of cosmetic products for infants were described. It was concluded that more attention should be paid to developing products best suited for the skin of particular age groups (631). Bell (632) predicted that, due to improvements in enzyme technology, the addition of these agents to cosmetic formulations will open a new field in the near future. Squalene was cited as a natural cosmetic raw material which could be incorporated into preparations to act as an emollient, humectant, and lubricant (633). The interrelations between moisture regulators and lipids of the dermal surface were discussed, and widely used test methods were described (634). Several formulas for modern cosmetic preparations using moisturizing factors were given.

Common ingredients used in emollient preparations and their contribution to emolliency, elegance, *etc.*, were reviewed (635). Properties of white mineral oils and their application to cosmetic preparations, differences between paraffinic and naphthenic oils, and the requirements of official compendia for these oils were discussed (636). The physical and chemical properties of various grades of white mineral oil used in cosmetic formulations were compared (637). Systems for thickening liquids using Carbopol and no alkali were described (638). The use of Carbopol as a thickening agent depended on having polyethoxy or polyhydroxy liquids present, in which case the thickening is based on hydrogen bonding. Courtney (639) presented an interesting review on the history, formulation, structure, and future of gels in cosmetics and toiletry products. Experimental results suggested that water affects the mechanical properties of single fibers of hair, and these in turn strongly influence the set-holding characteristics of hair tresses (640). The binding of water appeared to be a site binding process; *i.e.*, interactions occurred in stoichiometric ratios between the water molecules and the various hydrophilic groups of the protein (carboxylic, amino, and peptide groups) and were governed by the mass action law.

Table XIX—Additional References on Aspects of Formulation and Technology of Cosmetics

Reference	Topic
641	Origin and development of the science and technology of cosmetics
642	Review of ointments and transparent microemulsion gels
643	Review of formulations for modern hair shampoos
644	Factors influencing the formulation of suncreams
645	Review of light protection of skin
646	Discussion and definition of moisturizers
647	Behavior of moisturizers *in vitro* and *in vivo*
648	Petroleum derivatives as moisturizers
649	Review of the properties and possible use of montmorillonite in cosmetic and pharmaceutical preparations
650	Review of the role of fats and oils in cosmetics
651	Listing and possible use of biologically active compounds in cosmetics
652	Application of water-soluble resins to cosmetics
653	Summary of the uses and activities of substances commonly used in cosmetics
654	Review of alkyl sulfoacetates for cosmetic use
655	Review of cosmetic and toilet preparation research since 1968
656	Review of detergent use in cosmetics
657	Review of detergent use in cosmetics
658	Properties and use of cationic compounds in cosmetics
659	Review of biologically active substances as additives in cosmetic preparations
660	Cosmetic powder formulations and considerations in selecting a perfuming agent
661	Camelia oil and its use in cosmetics
662	Use of volatile hydrocarbons for cosmetics
663	Physicochemical properties of isopropyl myristate and palmitate and their use in cosmetic formulations
664	Review of new raw materials for cosmetic and perfume manufacturing introduced during 1971–1972
665	Use of emulsions and their behavior on the skin
666	Description of an irritation study for a feminine hygiene spray

Other papers related to aspects of formulation and technology of cosmetics are listed in Table XIX.

Packaging—The factors that should be taken into consideration when designing a "childproof" package were discussed (667). The mechanism, functioning, filling, performance, precision and constancy of dosage, compatibility with valve and solution, safety, and application of metering valves were described and discussed (668). The uses of polyethylene, polyvinyl chloride, polystyrene, and blends of these plastics and the effects of processing on their suitability for packaging of cosmetic emulsions were outlined (669). Evaluation methods for plastics that are to be in contact with medicinal preparations were described (670, 671). It was stressed that approval of a formulation is only valid for the product–plastic combination (672). Polypropylene containers were shown to be satisfactory for storing aqueous solutions containing common phenolic preservatives (673). Sorbic acid solutions in the same containers deteriorated rapidly. In evaluating disposable rubber closures for bulk irrigating solutions, it was ascertained that the amount of total extractives from the closures was influenced by the total mass of the closure as well as its surface area (674). Based on the UV absorption decrease of aqueous solutions in the presence of various plastics, the effect on the stability of local

Table XX—Additional References on Packaging

Reference	Topic
683	Teratogenicity of phthalate esters in rats
684	Review of plastics for packaging applications
685	Some improvements in packaging materials, methods, and machines
686	In-use contamination of intravenous solutions in flexible plastic containers
687	Protection of pharmaceutical solutions from catalytic effects of light by amber glass
688	Stability of sodium bicarbonate injection stored in polypropylene syringes
689	Problems involved with the development of a large-volume radiopaque diagnostic agent
690	Use of surface-treated Parasolvex glass for pharmaceutical solutions
691	Effects of water at 121° on pharmaceutical glass
692	Review of unit packaging of tablets and capsules
693	Description of eight methods for detecting poorly sealed pharmaceutical packages
694	Federal regulations regarding packaging of nitroglycerin tablets at the retail level
695	Description of a new "childproof" aerosol actuator
696	Description of a safety vent in aerosol cans which relieves pressure and prevents explosions
697	Codispensing valve system for dispensing striped aerosol foams

anesthetics in the presence of polyamides 6 and 12 and high pressure polyethylene of low crystallinity was determined (675). Polypropylene, polyvinyl chloride, polystyrene, and polycarbonate had no influence.

The biological activity and toxicity of stabilized amyl nitrate were not affected by long storage when cellular polyurethane vial closures were used (676). Effervescent granules were stored in various containers for 6 months and evaluated for carbon dioxide loss (677). Aluminum foil (2 mm.) provided the best protection for this type of product. Guidelines were presented covering the selection of syringes in process controls and procedures for prepackaging parenteral drug products in hospital pharmacies (678). The shattering of sterile vacuum-sealed fluid bottles during removal of the cap was reported (679). Apparently this can occur when the cap is removed quickly with the bottle in an inverted or partially inverted position due to a "water hammer" effect. Instructions were presented to the practicing pharmacist for the packaging of nitroglycerin tablets for preventing loss of potency during storage (680). The use of varnished aluminum tubes for packaging of ophthalmic ointments partly resolved the problem of metallic particles (681). The manufacturing process of these tubes for ointments was reviewed briefly, and the different methods used in testing were described. Leakage tests, currently in general use, for flame-sealed ampuls were demonstrated to be inadequate for determining whether passages to the outside exist in finished ampuls (682). Those containing these passageways may or may not pass existing leakage tests, depending upon the severity of the test.

Table XX contains additional references in the area of packaging.

Equipment—A European cylindrical shredder was described, and its performance was evaluated for wet and dry granulations (698). It was shown to be comparable to the Fitzpatrick mill for normally wet granulations but superior when the material was overly wetted.

Table XXI—Additional References on Equipment

Reference	Topic
707	Comparison of Erweka and Stokes hardness testers
708	Homogenizers and double-cone blenders
709	Review of fluidized-bed granulating techniques
710	Comparison of fluid-bed and hot-air drying
711	Description of a wet sieving method using *p*-hexane as the liquid dispersant
712	Cake thickness and deposition rate in plate-and-frame filter presses

The principle of sieving pharmaceutical materials by gyratory vibration was reviewed and compared with the efficiency of standard vibratory methods (699). For powder mixtures containing low concentrations of active materials, the Erweka planetary mixer, with a fixed vat, was shown to be superior to the Erweka cubical mixer in the rapidity with which homogeneity was obtained (700). The speed of dispersion of the active principle throughout the excipient was greater in the cubical mixer, but the latter was unable to reduce the agglomeration of particles. Methods for testing the friability, disintegration time, and dissolution behavior of tablets using the E-70 apparatus were reviewed, and the details of testing, the prescribed limits of disintegration times, and the performance of the test were emphasized (701).

Shah (702) reviewed the nondrying applications of spray drying including stabilization, blending, use for reaction mixtures, timed-release formulations, and waste disposal with the production of useful by-products. The design of a continuous manufacturing process for an oil-in-water cosmetic cream was described (703). In arriving at a final process design and selection of equipment, emphasis was placed upon the determination of the basic chemistry responsible for the stability and characteristic consistency of the product and the determination of the relative importance of process and ingredient variations. The fundamental microbiological aspects of modern cleaning and sterilizing equipment were discussed (704). Beard (705) discussed the variables such as speed, changeover time, and product shape and how these considerations govern the choice of tablet-counting equipment. A prototype irradiation module was built and successfully tested (706). This unit could be adapted to packaging lines for the sterilization of packages and materials prior to filling at normal line speeds.

Additional references on equipment can be found in Table XXI.

PHYSICAL PHARMACY

Methods of preparation of three polymorphs, two solvates, and an amorphous modification of sulfameter (sulphamethoxydiazine) were described (713). The interconversion of these forms under various conditions of heating, suspension in water, and grinding, as well as dissolution behavior, was studied. Thermal analysis of sulfanilamide polymorphs indicated that the α-, β-, and δ-forms are transformed to the γ-form prior to melting (714). The phase-transition temperatures and heats of transition was as follows: α-form, 108°, 358 cal./

mole; β-form, 131–141°, 347 cal./mole; and δ-form, 108°, 386 cal./mole. Shefter *et al.* (715) used X-ray crystallographic methods to determine the structures of sulfadimethoxine, sulfadoxine, and sulfisoxazole. The molecules had similar conformations about their sulfonamide linkage but had markedly different orientations of their respective heterocyclic rings relative to the sulfanilamide portion of the molecule. In a study of polymorphism in sulfonamides, it was postulated that electron-withdrawing and electron-donating groups at the *N'*-position influenced the strength of the hydrogen bonds that form and, hence, the tendency of these compounds to exhibit more than one crystalline form (716). In some cases, however, minor alterations in structure resulted in disproportionate changes in the compactness of the crystal lattice, and this fact made it difficult to develop broad generalizations applicable to large groups of compounds.

The controversy over polymorphism in aspirin continued. Schwartzman (717) attributed apparent polymorphism to different crystal habits caused by different solvents used for crystallization, and dissimilar melting points to poor transfer of heat caused by the larger crystal size or to possible crystal defects. Other workers attributed the apparent polymorphism to salicylic acid impurities present in the aspirin samples tested (718). On the other hand, Borka (719) presented evidence for the existence of two polymorphic forms of aspirin. Solution phase transformation of the metastable Form II into the stable Form I was cited as further evidence for the existence of Form II.

The properties of liquid crystals were studied by spectroscopic or electromagnetic methods such as X-ray, IR, UV, NMR, double-refraction, and dielectric constant (720). The expansion coefficient and surface tension were also measured to obtain thermodynamic information. Phase transitions in mixtures of mesomorphic cholesteryl esters were studied by visual thermal microscopy (721). Binary mixtures of saturated cholesteryl esters showed transitions from the solid phase to the mesomorphic phase at temperatures that were depressed well below the phase-transition temperatures of either pure compound. The phase-transition temperatures in the solid, liquid crystal, and isotropic liquid states of four cholesteryl esters were determined by polarization microscopy, differential thermal analysis, and measurement of the dielectric constant and dielectric loss (722). Data on transition temperatures obtained from these various methods were generally in good agreement with each other and were found to be useful for mesophase identification.

Dissolution rates of drugs in solid dispersions in polyethylene glycol 4000 were investigated, but no differences in dissolution rates were shown in drugs that were not soluble in polyethylene glycol 4000 (723). In cases where drugs were soluble in polyethylene glycol 4000, an increase in dissolution rate was noted. Surpuriya and Higuchi (724) studied the kinetics of cholesterol and desmosterol transfer from aqueous sodium lauryl sulfate solutions and from sodium taurocholate–lecithin solutions into hexadecane and vice versa by means of the multiparticulate dispersion technique. The results were discussed in terms of an interface-controlled mechanism being operative, involving solute–micelle complex or free solute in the rate-determining step.

The diffusion of ephedrine, sulfathiazole, chloramphenicol, acetaminophen (paracetemol), isoniazid, and amphetamine in solutions of polysorbate 40, polysorbate 80, and polyethylene glycol 1000 monocetyl ether (Cetomacrogol 1000) was studied (725). With the exception of isoniazid, the observed diffusion coefficients, corrected for resistance to flow, depended upon the surfactant used and both the drug and surfactant concentrations. An extensive investigation of the kinetic and thermodynamic aspects of the transfer of sulfonamides through immiscible phases was reported (726). Included was a postulated activated complex for the interphase transfer of these compounds. A linear relationship with unit slope was found between the logarithm of the intrinsic partition coefficient of *p*-alkyl pyridines and the logarithm of the extraction constants of the corresponding ion-pairs of the protonated base chlorides, as determined in chloroform–water and octanol–water systems (727). Because of methylene increments occurring distantly from the polar portion of the molecule, their influence upon the oil–water partition coefficient would be expected to be the same for the free base and ion-pair. Yalkowsky *et al.* (728) suggested that change in crystal structure as a function of chain length in alkyl *p*-aminobenzoates is somewhat analogous to micelle formation within a homologous series. The lower members of a surfactant series exist only as monomers whose properties are primarily determined by the polar group; but as the chain length is increased, the increasing amphiphilic character provides for orientation of the molecules into a particular structure, the micelle. Further increases in chain length serve to increase the stability of these structures relative to the monomeric state.

By using diffuse reflectance spectroscopy, the interaction between dextroamphetamine sulfate and spray-dried lactose and dextrose in solid–solid mixtures was studied (729). Two new absorption maxima were observed in the reflectance spectra of heated samples and were attributed to the chemisorption of the amine molecules on the surface of the sugars. In preformulation studies of a lyophilized product, melting-point–composition diagrams were constructed and percent melt values at room temperature were calculated (730). Final product physical appearance correlated well with calculated percent melt as ascertained by controlled lyophilizations. The characteristic differences between one gel and another were reviewed and traced to the variations of framework flexibility, number and nature of cross-links, attractions and repulsions between framework elements, and interactions with solvents (731). Theoretical calculations based on the Debye–Hückel theory indicated that for weak monoprotic bases that do not involve a hydroxyl group, a stoichiometric pKa determined at any ionic strength would be no different from the thermodynamic pKa (732). The pKa's of two local anesthetic amines determined at several ionic concentrations supported this theory. Terada (733) described a method for determining true partition coefficients for amphoteric acids by determining the

Table XXII—Additional References on Physical Pharmacy

Reference	Topic
737	Use of differential thermal analysis in pharmaceutical technology for prediction of stability
738	Significance of polymorphism in drug technology
739	Kinetics of polymorphic transformation of sulfathiazole Form I
740	Kinetics of interconversion of sulfameter (sulfamethoxydiazine) crystal forms
741	Determination of valinomycin crystal structure by direct methods
742	Polymorphism of mafenide (homosulfamine) by thermal analysis and IR analysis
743	Determination of polymorphic forms of chloramphenicol palmitate by IR spectrophotometry
744	Review of aspirin polymorphism
745	Preparation of aspirin (acetylsalicylic acid) studied by an image identification method
746	Effect of temperature on the physical nature of phenobarbital (phenobarbitone) produced by acid–base precipitation
747	Physicochemical properties of lyophilized aluminum hydroxide
748	Thermophysical characteristics of drugs and granular materials
749	Physicochemical properties and application to pharmaceutical science of silicate powder
750	Two new methods for the spectral determination of microdissociation constants
751	Dissociation constants and activity coefficients of Carbopols by potentiometric titration
752	Demonstration of an interfacial contact resistance during the diffusion of water in gelatin gels
753	Surface tension of polymer liquids interpreted by the hole theory
754	Calculation of electronic structure of some drugs by the Pople method
755	Molecular estimates of molar attraction constants
756	Physicochemical properties of erythromycin estolate
757	Physicochemical properties of halothane
758	Physicochemical properties of levarterenol bitartrate
759	Physicochemical properties of meperidine hydrochloride
760	Physicochemical properties of meprobamate
761	Physicochemical properties of nortriptyline hydrochloride
762	Physicochemical properties of propoxyphene hydrochloride
763	Physicochemical properties of sodium cephalothin
764	Physicochemical properties of sodium secobarbital
765	Physicochemical properties of triamcinolone
766	Physicochemical properties of triamcinolone acetonide
767	Physicochemical properties of triamcinolone diacetate
768	Physicochemical properties of vinblastine sulfate
769	Physicochemical properties of vincristine sulfate

apparent partition coefficients as a function of pH. The values obtained for *p*-aminobenzoic acid, sulfonamide, and sulfameter (sulfamonomethoxine) agreed well with the results of others.

One of the most potentially successful approaches to drug design is the attempt to place correlations between changes in molecular structure with changes in biological activity on a quantitative and predictive level (734). While qualitative efforts were made in this area by chemists and pharmacologists for over 100 years, more recent progress illustrates the potential of regression analysis, molecular orbital calculations, and parameterization using various physicochemical measurements. A review was presented dealing with basic areas of molecular orbital theory important in interpreting drug activity (735). Peradejordi *et al.* (736) investigated the relations existing between electronic structure and base strength of benzacridines and their amino derivatives using semi-empirical molecular orbital calculations. Their results indicated that the energy to protonate a nitrogen derivative of a conjugated hydrocarbon may be divided into the terms ΔE_ρ and ΔE_π for changes in localized and delocalized energies, respectively, an energy for solvation, and a term for steric hindrance to protonation.

Additional references relating to physical pharmacy are listed in Table XXII.

Dissolution—Dissolution times of some commercially available tablets of sulfamethizole were measured using four different methods, but no correlations of predictive value were obtained between dissolution and absorption parameters (770). The stationary-basket method discriminated best between formulations and was the most useful for quality control purposes. A new approach was described for increasing the dissolution rate of relatively insoluble powders (771). It was based on the concept of increasing the surface available for contact with dissolution media and was accomplished by equilibration of the drug in an organic solvent on an insoluble excipient with an extensive surface area (*e.g.*, fumed silicon dioxide). An increased rate of release from these minuscular drug delivery systems was observed in all instances, and it was suggested that a decrease in particle size was a major factor in improving the dissolution rates (772).

To minimize effects of differences in particle-surface areas which occurred from experiment to experiment and during each individual experiment, Higuchi *et al.* (773) employed a "concentration jump" technique during the determination of dissolution rates in solvents containing complexing agents. The results demonstrated the importance of the magnitudes of both the diffusion coefficient of the complexing component in the solvent and of the stability constant of the complex on dissolution kinetics. *In vitro* studies on the dissolution rate of cholesterol monohydrate crystals in micellar bile acid solutions showed that the addition of lecithin decreased the dissolution rate, even though lecithin increased the equilibrium solubility of cholesterol in these systems (774). The reduction in rates caused by lecithin was attributed to a large crystal–solution interfacial barrier.

The total solubility of benzoic acid in aqueous solutions of polysorbate 80 was shown to increase linearly as the concentration of the surfactant was increased (775). Since the concentration of polysorbate 80 was increased with a limited increase in viscosity of the micellar solution, the dissolution of benzoic acid was increased to a maximum rate, but further increases in the concentration of polysorbate 80 decreased the dissolution medium. Utilizing *in vitro* studies, Tawashi and Piccolo (776) demonstrated that very low concentrations of FD&C Blue No. 1 significantly reduced the dissolution rate of sulfathiazole crystals in 0.04 *M* sodium cholate solutions. These results were confirmed by *in vivo* absorption studies in man. The intrinsic dissolution rates of the monohydrochloride, dihydrochloride, and disulfate salts of an experimental antihypertensive were studied (777). Although there were significant differences in the dissolution rates of these salts, comparative hypotensive activity in anesthetized dogs showed that the three salts did not differ from each other.

Although the dissolution rate of powdered aspirin (acetylsalicylic acid) is inversely proportional to crystal size, the opposite was found true in tablets (778). This was shown to be due to the agglomeration of the fine particles, but this effect could be hindered by appropriate adjuvants. Under stirred and sink conditions, the dissolution of benzoic acid in aqueous solutions of polysorbate 80 was shown to occur in accord with the diffusion layer model (779). The micellar diffusion coefficient was decreased as the quantity of solubilized species in solution was increased, presumably due to changes in the size and weight of the solubilized species. Particles dissolving in a dissolution medium initially decrease in linear dimension while the number of particles remains unaltered. However, at a particular point in time, the smallest particle disappears and, from that point on, the number of particles decreases (780). These phenomena were simulated on a digital computer, and the agglomerate dissolution pattern under sink conditions was shown to follow a cube-root law.

The effect of low concentrations of an *n*-alkyl polyoxyethylene surfactant on the dissolution of hydrocortisone was investigated (781). It was shown that the plot of the dissolution rate constant *versus* the surfactant concentration exhibited a pronounced maxima at the region of the critical micelle concentration (CMC). Rates of dissolution of salicylic acid were determined at constant pH in the absence of buffer salts by using a pH-stat (782). The dissolution rate increased with increasing pH up to a maximum of pH 4. Wadke and Reier (783) described a method whereby transition temperatures and other thermodynamic parameters associated with the transitions of chloramphenicol palmitate form B, anhydrous ampicillin, and theophylline to chloramphenicol palmitate form A, ampicillin trihydrate, and theophylline monohydrate, respectively, were determined from intrinsic dissolution rates. In a series of papers, Kildsig and coworkers (784–787) investigated some parameters involved in the dissolution mechanism including concentration gradients, surface tension of solids, solid–liquid interface energies, and effective interfacial concentrations. The release of a mixture of two acidic drugs (benzoic and salicylic acids) and a mixture of two amphoteric drugs (sulfadiazine and sulfapyridine) from an inert matrix into different buffers was investigated using the physical model approach (788–790). Equations were derived describing the diffusional behavior and interactions of the various species under different conditions. The *in vitro* release of four progesterone-type steroids from a silicone polymer was studied by Roseman (791) and was found to be dependent upon the molecular structure of the steroid. Since the diffusion coefficients of the steroids were of the same magnitude, the diversity in release patterns was mainly attributed to the difference in the polymer solubilities of the steroids.

A flow visualization technique was used to compare flow patterns in several kinds of dissolution apparatus in wide use (792). The technique readily revealed the undesirable characteristics and provided a rational basis for judging an empirically developed dissolution apparatus. The shape of the dissolution flask, which is not clearly defined in USP XVIII and NF XIII, can significantly affect dissolution patterns (793). Formulators should be aware that these differences exist, and it was recommended that there be more definitive specifications for the dissolution vessel in the compendia. Tingstad *et al.* (794) described a modified, continuous flow column-type dissolution apparatus using commercially available components. Specific advantages of the flow method, especially for sublingual tablets, were cited. Dissolution studies were carried out on commercially available dosage forms of 300-mg. lithium carbonate tablets and capsules (795). Log-probability analysis of the data showed significant differences in the dissolution of the two dosage forms. The usefulness of a

Table XXIII—Additional References on Dissolution

Reference	Topic
798	Review of dissolution kinetics
799	Energy changes during the dissolution process
800	Kinetics of the dissolution process
801	Dissolution rates of poorly soluble solids in mixed organic solvents
802	Prediction of dissolution rates of slightly water-soluble powders from simple mathematical relationships
803	Influence of crystal structure on dissolution rate
804	Dissolution kinetics and thermodynamic parameters of polymorphs of an experimental antihypertensive
805	Dissolution behavior of polymorphic, pseudopolymorphic, and amorphous phases of drugs
806	Mathematical description of dissolution of a series of crystalline and amorphous drugs
807	Enhancement of dissolution rate by β-cyclodextrin inclusion
808	Dissolution rate of a monolayer of 1-monolaurin at constant area
809	*In vitro* release of active components from tablets
810	Review of factors affecting dissolution rate of oral dosage forms
811	*In vitro* dissolution rate of phenytoin in relation to particle size and specific surface area
812	Rate studies on the dissolution and degradation of phenoxymethyl penicillin at different pH values
813	Dissolution of alkyl vinyl ether–maleic anhydride copolymers and their ester derivatives
814	*In vitro* release of an amine salt by a water-soluble polyelectrolyte
815	Linearization of dissolution rate curves by the Weibull distribution
816	Influence of formulation factors and processing variables on the dissolution rate of hydrochlorothiazide tablets
817	*In vitro–in vivo* studies on commercially available nitrofurantoin tablets
818	Dissolution time of aspirin tablets as a function of the surface area
819	Critical comparison of different methods for the determination of dissolution rate
820	Review of methods for *in vitro* dissolution rates of tablets
821	Critical evaluation of the USP XVIII dissolution test for tablets
822	Technical problems of the USP–NF dissolution test
823	Review of dissolution tests, with some practical examples
824	Proposal for an *in vitro* release rate as a standard requirement for oral dosage forms
825	Modification and evaluation of a continuous flow apparatus for dissolution of tablets
826	Dissolution rate studies of some calcium phosphates using the rotating-disk method
827	Comparative study of the advantages of the modified apparatus of Warburg-type WA 0110 for control of common enteric-coated and timed-release tablets
828	Method for the study of timed-release films
829	*In vitro* dissolution of drugs from suspension ointments
830	Comparison of ointment bases by measuring *in vitro* dissolution (release) of drugs

rotating compartmentalized disk for dissolution rate determinations was demonstrated by the ease with which it discriminated between experimental capsule formulations and commercially available chlordiazepoxide hydrochloride capsules (796). An automatic multiple-channel apparatus for dissolution rate studies under sink conditions was developed, and an economical and reliable system for transmitting the experimental data to a remote computer was described (797).

Table XXIII lists additional references on dissolution.

Solubility–Solubilization Phenomena—Both Mukerjee (831) and Nelson (832) presented interesting discussions on the theoretical aspects of applying the Kelvin equation to the solubility of solids. Both refuted the concept that the effect of particle size on solubility is due to entropy changes alone. Methyl and propyl gallates were solubilized in aqueous solutions of surfactants which were polyoxyethylene ethers of cetyl alcohol, but ethyl gallate could not be solubilized in the same manner because it formed a gel-like product with the surfactants (833). The amount of the gel-like product formed was proportional to surfactant concentration and was greater when a larger number of polyoxyethylene ether units was present in the surfactant molecule. Solubility studies showed that testosterone was solubilized by glycocholate below the apparent binary CMC, although the solubilizing capacity was quite low (834). The complex association between testosterone and glycocholate suggested mixed micelle formation. Solubilities of phenobarbital, salicylic acid, and caffeine in a mixture of 12 kinds of solubilizing agents and water were measured, and the relationship between solubility and dielectric constant was examined (835). In a solvent system of water and solubilizing agents containing only a hydrophilic group, a plot of dielectric constant *versus* the logarithm of solubility, expressed as molar fraction, gave a straight line; for solubilizing solvents containing both hydrophobic and hydrophilic groups, the same plot gave one flexion in the straight line, indicating that these solubilizing agents undergo association at a specific concentration.

The solubilities of sulfadiazine, sulfisomidine, and sulfadimethoxine in several normal alcohols were determined over a limited temperature range (836). The entropy of solution was used for interpreting the results, which showed that in most cases the entropy quantity increased with decreasing solubility for any particular solute. To understand better the solubility behavior of nonelectrolytes in binary polar solvents, an equation for predicting solubility was presented and appeared to be applicable to many systems (837). Kramer and Flynn (838) examined the solubilities of two polysubstituted 1,3-dioxolanes, containing a 4-(2′-piperidyl) substituent, as a function of pH, temperature, and solvent composition. Mathematical equations describing the total solubility at any arbitrary pH in terms of the independent solubilities of the hydrochloride and free base species and the dissociation constants of the salt were derived and fitted to the data with good results. The method of sample preparation markedly influenced the rate of dissolution and attainment of supersaturated states of cholesterol in lecithin–bile salt systems (839).

Table XXIV—Additional References on Solubility–Solubilization Phenomena

Reference	Topic
840	Review of solubilization by surfactants
841	Review of solubilization by surfactants
842	Review of solubilization of soap by surface-active agents
843	Solubility parameter theory for mixed solvent systems
844	Mechanism of cosolubilization
845	Interaction of papaverine hydrochloride and sodium lauryl sulfate
846	Solubilization of steroid hormones
847	Maximization of the water solubility of vitamin D_3
848	Solubilization of griseofulvin as a function of low temperature and different nonionic surface-active agents
849	Solubility and solubilization studies on 3,4,4′-trichlorobenzenesulfonanilide (Reseptyl)

The equilibrium solubility of cholesterol studied as a function of its physical state in a model bile system was almost half that of the previously accepted values. The authors suggested that slow attainment of the equilibrium state may have acted to bias previous studies.

Other papers of interest in the area of solubility–solubilization phenomena can be found in Table XXIV.

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Spectrofluorometric Determination of Substituted Dihydroisoquinolines and Tertiary Tetrahydroisoquinolines as Their Isoquinolinium Derivatives

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Abstract □ A spectrofluorometric method was developed for the determination of a substituted dihydro- and a tertiary tetrahydroisoquinoline in biological fluids based on a chemical and/or photochemical oxidation reaction to produce the isoquinolinium derivatives which fluoresce strongly in acidic media. This principle was used for the development of assays of these compounds in blood and urine with sensitivity limits in the submicrogram range. TLC was used to establish the specificity of the assays for the intact drugs and their biotransformation products. The method was applied to the determination of blood levels and the urinary excretion of these compounds administered in the dog and in man. Both compounds were found to be rapidly absorbed and extensively metabolized by both species.

Keyphrases □ 3,4-Dihydro-1-isoquinoline acetamide—determination of blood levels and urinary excretion in dogs and man, spectrofluorometric assay developed □ 8-Methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline—determination of blood levels and urinary excretion in dogs and man, spectrofluorometric assay developed □ Isoquinoline derivatives—spectrofluorometric assay in blood and urine □ Blood levels—3,4-dihydro-1-isoquinoline acetamide and 8-methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline, dogs, man □ Excretion, urinary—3,4-dihydro-1-isoquinoline acetamide and 8-methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline, dogs, man □ Spectrophotofluorometry—analysis, dihydro- and tetrahydroisoquinoline derivatives in blood and urine

The chemistry of the isoquinoline alkaloids has been extensively investigated (1-3) and has led to the synthesis of a large number of chemical derivatives of widely different pharmacological properties (4) such as analgesic (5), antiviral (6-9), and antihypertensive (10, 11) activities. This report describes the spectrofluorometric determination of two new members of this class of compounds, one showing antiviral activity and the other antihypertensive activity. 3,4-Dihydro-1-isoquinoline acetamide hydrochloride (I-HCl)[1] was synthesized by Creighton *et al.* (12) (Scheme I and Table I) and was shown to have antiviral activity (13–15). 8-Methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline hydrochloride (II-HCl) was synthesized by Schenker *et al.* (16) (Tables I and II) and was clinically evaluated as a hypotensive agent (17).

Tertiary tetrahydroisoquinolines can be readily oxidized to their 3,4-dihydroisoquinoline derivatives, which exhibit UV absorption and fluorescence in acidic media (18). This oxidative reaction was first described by Knabe (18) for the oxidation of tertiary amines using mercuric acetate in ethylenediaminetetraacetic acid. He also found it to be a useful general reaction applicable to a variety of tertiary tetrahydroisoquinolines. Knabe's method was modified by Schwartz and Rieder (19), who omitted ethylenediaminetetraacetic acid and used mercuric acetate in acetic acid and 0.1 *N* H_2SO_4. This modification, which was reported to give more quantitative and reproducible yields of the oxidation products, was used for the determination of emetine and dehydroemetine in biological fluids (19) and for the determination of methopholine and its metabolites in man and in the rabbit (20).

The chemical oxidation step usually introduces only one double bond in the 1,2-position of the tetrahydroisoquinoline ring to form the 3,4-dihydroisoquinoline derivatives. This report describes a photochemical oxidation which introduces a second double bond in the 3,4-position of the dihydroisoquinoline to form the fully aromatized isoquinolinium derivatives (21, 22) (Table II), which are highly fluorescent in acidic media. This photochemical oxidation was used to develop sensitive assays for the quantitation of I and II in blood and urine.

EXPERIMENTAL

Determination of 3,4-Dihydro-1-isoquinoline Acetamide Hydrochloride (I-HCl) and 1-Isoquinoline Acetamide (I-A) in Blood and Urine

Reference Standards—I-HCl, mol. wt. 224.69, m.p. 195–197° dec.

Stock Solution—Weigh out 11.940 mg. of I-HCl equivalent to 10.000 mg. of free base, transfer into a 100-ml. volumetric actinic flask, and dissolve in 100 ml. of methanol. This stock solution contains 100-mcg. equivalents of free base/ml.

Working Solution—Dilute 1.0 ml. of the stock solution to 10 ml. with methanol. This solution contains 10.0-mcg. equivalents of free base/ml., suitable aliquots of which are used as internal standards added to blood for the determination of percent recovery.

Standard Solution of I-A—1-Isoquinoline acetamide, mol. wt. 186.21, m.p. 204.5–205° dec.

Stock and Working Solutions—Weigh out 10.000 mg. of I-A and follow the procedure exactly as described for I-HCl.

External Standard of I—The determination of percent recovery from blood of added internal standards requires the use of a standard curve of I in 30% trichloroacetic acid exposed to UV light under similar conditions as the internal standard.

Weigh out 11.940 mg. of I-HCl equivalent to 10.000 mg. of free base, transfer into a 100-ml. volumetric actinic flask, and dis-

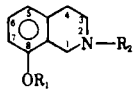
[1] The Roman numerals refer to the free base of the compound; the salts of a given compound are indicated by the appropriate abbreviations.

Table I—Chemical Names and Physical Properties of Compounds Referred to in Scheme I and Table II

Compound	Chemical Name	Molecular Weight	Melting Point	Reference Synthesis
I-HCl	3,4-Dihydro-1-isoquinolineacetamide hydrochloride	224.69	195–197° dec.	12
I-A	1-Isoquinolineacetamide	186.21 (B)[a]	204–205° dec.	—[b]
II-HCl	8-Methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline hydrochloride	213.70	215–216°	16, 21
III-HCl	8-Methoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride	199.68	261–262.5°	16, 21
IV-HCl	2-Methyl-1,2,3,4-tetrahydro-8-isoquinolinol hydrochloride	199.71 163.21 (B)	243–244° 173–174.5° (B)	16 21
V-HCl	1,2,3,4-Tetrahydro-8-isoquinolinol hydrochloride	185.68 149.18 (B)	271–272° 181–181.5° (B)	16 21
II-A-HI	8-Methoxy-2-methyl-3,4-dihydroisoquinolinium iodide	303.15	182–182.5° dec.	21
III-A-HCl	8-Methoxy-3,4-dihydroisoquinoline hydrochloride	197.70	175–175.5°	21
IV-A	2-Methyl-3,4-dihydro-8-isoquinolinol	162.18	—	N.S.[c]
V-A	3,4-Dihydro-8-isoquinolinol	147.18	156–157°	[b]
II-B-HI	8-Methoxy-2-methylisoquinolinium iodide	301.13	231–232°	16, 21
III-B-HCl	8-Methoxyisoquinoline hydrochloride	195.65 159.15 (B)	170–170.5° dec. 50.5–51.5° (B)	16, 21 16, 21
IV-B-HI	8-Hydroxy-2-methylisoquinolinium iodide	287.11	220–221° dec.	16, 21
V-B-HCl	8-Isoquinolinol hydrochloride	181.65 145.15 (B)	240–242° 210–211° (B)	22 22

[a] (B) = free base of compound. [b] F. Schenker, Department of Chemical Research, Hoffmann-La Roche Inc., Nutley, N. J., unpublished data, 1967. [c] N.S. = not synthesized.

Table II—Postulated Chemical Reactions of II and Its Desmethyl Analogs

R Group R_1	R Group R_2	Parent Compound (tetrahydroisoquinoline)	chemical oxidation → Intermediate (3,4-dihydro derivative)	photochemical oxidation → Fluorophore (isoquinolinium derivative)	Wavelength (nm.) of Activation	Wavelength (nm.) of Emission
			mercuric acetate-acetic acid; 0.1 *N* H_2SO_4 at 100° for 30 min.	irradiated for 15 min.; Pyro-Lux R-57 lamp		
$-CH_3$	$-CH_3$	II	II-A	II-B	370	458
$-CH_3$	$-H$	III	III-A	III-B	370	458
$-H$	$-CH_3$	IV	IV-A	IV-B	425	525
$-H$	$-H$	V	V-A	V-B	425	525

solve in a minimal amount of water. Add 0.6 ml. of 0.1 *N* NaOH to liberate the free base[2], which is insoluble in aqueous media, resulting in a turbid solution. Add 25 ml. of 30% trichloroacetic acid solution to dissolve the free base. Make to volume with 30% trichloroacetic acid solution. This stock solution of I contains 10 mg. free base/100 ml. or 100 mcg./ml.

Make serial dilutions of this stock solution in 30% trichloroacetic acid to give standard solutions containing 0.125, 0.25, 0.50, 1.0, and 2.0 mcg. of free base/ml. to be used in making an external standard curve.

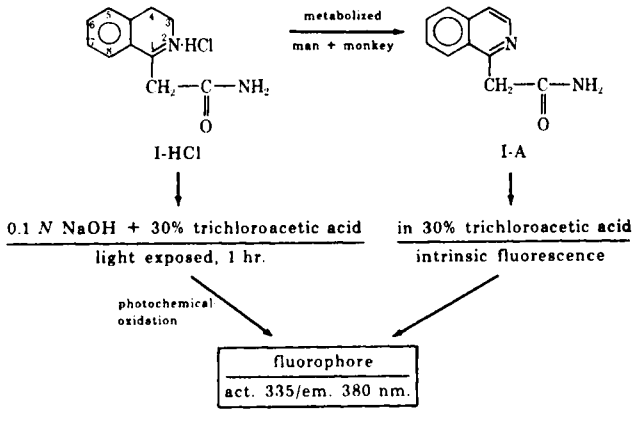
External Standards of I-A—Dissolve 10.000 mg. of compound in 100 ml. of 30% trichloroacetic acid. Make serial dilutions of this solution in 30% trichloroacetic acid to give standard solutions containing 0.125, 0.25, 0.50, 1.0, and 2.0 mcg./ml. Use these solutions to prepare an external standard curve of I-A (described later).

Reagents—The following were used: ether[3] (peroxide free), 1.0 *M* pH 9.0 borate–potassium carbonate–potassium chloride buffer solution (23), 30% trichloroacetic acid in distilled water, 5 *N* NaOH in distilled water, 1 *N* HCl in distilled water[4], and 0.1% bromthymol blue indicator in 50% alcohol.

Procedure for Blood and Urine—To 1.0 ml. of blood in a 50-ml. centrifuge tube, add 5 ml. of pH 9.0 buffer and 15 ml. of ether. Stopper the tube and shake for 15 min. on a reciprocating shaker. Along with the samples, run a 1-ml. specimen of control blood (taken prior to medication) and duplicate 1-ml. specimens of control blood containing 1.0 mcg. of both I-HCl and I-A (0.1 ml. of each working solution) added as internal standards.

To 1.0 ml. of urine, add 4 ml. of 2 *N* NaOH and 15 ml. of ether and extract as described for blood. Along with the samples, run 1.0 ml. of control urine and duplicate 1.0-ml. specimens of control urine containing 1.0 mcg. of both I-HCl and I-A added as internal standards.

Centrifuge all samples for 10 min. at 2000 r.p.m., preferably at 0–4° in a refrigerated centrifuge; then transfer the ether layer



Scheme I—Postulated chemical reactions of I and its biotransformation to I-A

[2] This procedure is necessary because only the free base (I) dissolved in 30% trichloroacetic acid gives an optimal, photochemically produced yield of I-A. When I-HCl was dissolved directly in 30% trichloroacetic acid and irradiated, it gave only 25% of the fluorescence yield as that produced by I.

[3] Mallinckrodt anhydrous analytical reagent grade diethylether from a freshly opened can may be used for up to 4 days after opening.

[4] Acculate.

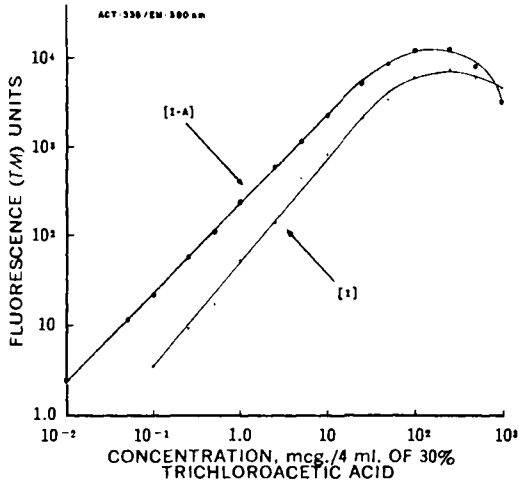


Figure 1—*Linear dynamic range of fluorescence* versus *concentration of I and I-A in 30% trichloroacetic acid.*

quantitatively into another 50-ml. centrifuge tube and evaporate to dryness. Repeat the extraction of each specimen with another 15-ml. portion of ether, combine the ether extracts, and evaporate to dryness.

Each residue is then dissolved in 100 μl. of methanol, which is transferred quantitatively onto a 20 × 20-cm. silica gel G/F_{254} thin-layer chromatoplate[6]. The chromatogram is developed in ethyl acetate–methanol–concentrated ammonium hydroxide (95:4:2), ascending for 12 cm. The intact drug (I, R_f 0.6) and the metabolite (I-A, R_f 0.3) are detected by their intrinsic blue-green fluorescence under shortwave UV light and are identified by reference to the R_f values of authentic standards run alongside the sample extracts. The areas on the plate corresponding to I and I-A are scraped off immediately after development[6] and transferred into separate 15-ml. centrifuge tubes. Both I and I-A are eluted from the silica gel with 4 ml. of 30% trichloroacetic acid by slurring vigorously on a high-speed mixer[7] for 60 sec. After centrifugation of the silica gel, the clear supernates are transferred into a fresh set of 15-ml. centrifuge tubes. At this point in the assay, a series of external standards of I and I-A in 30% trichloroacetic acid are prepared by transferring 4-ml. aliquots of each standard solution into separate 15-ml. centrifuge tubes. The external standards and biological specimens containing I *only* are then stoppered and exposed for 60 min. to high intensity light from a lamp[8] contained in an aluminum foil-lined box. The samples are placed in a single row 30.48 cm. (12 in.) away from the light source to standardize the photochemical formation of the fluorophore prior to fluorometric analysis. The intrinsic fluorescence of I-A in the external and internal standards and of the metabolite in the biological specimen is measured directly *without* prior light exposure.

Fluorometric Analysis—Read the fluorescence (TM)[9] of all the solutions in a 1-cm. path cell at 380 nm., activating at 335 nm. and using 4 ml. of irradiated 30% trichloroacetic acid as the reagent blank.

Plot external standard curves for I and I-A of the respective fluorescence units corrected for blank *versus* concentration of standard (micrograms per milliliter) (Fig. 1), from which the recovery of the internal standards of I-HCl and I-A added to blood or urine can be determined.

The fluorescence readings of the biological samples are corrected for control blood or urine blank readings, while that of the external standards of I and I-A are corrected for reagent blank (30% trichloroacetic acid) readings. The concentration of each compound in the unknown is determined by direct comparison to the fluorescence reading of the respective internal standard. The percent recovery of the added internal standards of I-HCl and I-A may be determined by direct comparison to the fluorescence reading of the respective external standards.

Calculations—*Determination of I and I-A in Blood and Urine*—

$$\frac{TM_{380} \text{ of unknown}}{TM_{380} \text{ of internal standard}} \times \frac{\text{concentration of internal standard (1.0 mcg. I or I-A)}}{\text{ml. sample assayed}} = \text{mcg. I and I-A/ml. blood or urine} \quad \text{(Eq. 1)}$$

Determination of Percent Recovery of I and I-A—

$$\frac{[TM/\text{mcg./ml.}] \text{ internal standard}}{[TM/\text{mcg./ml.}] \text{ external standard}} \times 100 = \text{percent recovery} \quad \text{(Eq. 2)}$$

The percent recovery of internal standards should be determined routinely as a check on analytical precision and reproducibility.

Determination of 8-Methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline Hydrochloride (II-HCl) and Its Metabolites in Blood and Urine

Reference Standards—II-HCl, mol. wt. 213.70, m.p. 213–214°.

Stock Solution—Weigh out 12.10 mg. of II-HCl equivalent to 10.00 mg. of free base, transfer into a 10-ml. volumetric actinic flask, and make to volume in absolute ethanol. This stock solution contains 1 mg. of II/ml.

Working Solution—Make two serial 1:10 dilutions of the stock solution with ethanol to yield a working solution containing 10 mcg./ml. Suitable aliquots of this solution are used as internal standards added to blood for the determination of percent recovery.

External Standard of II—The determination of percent recovery from blood of added internal standards requires the use of a standard curve of II in 0.1 N H_2SO_4, oxidized in mercuric acetate–acetic acid and exposed to light under the same conditions as the internal standards.

Weigh out 12.100 mg. of II-HCl equivalent to 10.000 mg. of free base, transfer into a 10-ml. volumetric actinic flask, and dissolve in a minimal amount of water (1–1.5 ml.). Add 0.6 ml. of 0.1 N NaOH to liberate the free base, which is insoluble in aqueous media, resulting in a turbid solution. Add 5 ml. of 0.1 N H_2SO_4 solution to dissolve the free base, and make to volume with 0.1 N H_2SO_4 solution. This stock solution contains 1.0-mg. equivalent of II/ml. Make serial dilutions of this solution with 0.1 N H_2SO_4 to give standard solutions containing 0.125-, 0.25-, 0.50-, 1.0-, and 2.0-mcg. equivalents of II/ml. to be used in making a standard curve (Fig 2.).

Reagents—The following were used: ether[3] (peroxide free), 0.1 N H_2SO_4 in distilled water, and 1.0 and 0.1 N NaOH solutions in distilled water. For mercuric acetate reagent (pH 6.0), dissolve 32.4 g. of sodium acetate (anhydrous), 1.0 g. mercuric acetate, and 3 ml. of glacial acetic acid/100 ml. of distilled water. This solution must be made fresh every 2–3 days due to precipitation and crystallization of the salt. For 1.0 M phosphate buffer (pH 5.5), dissolve 138.01 g. of $NaH_2PO_4 \cdot H_2O$ in distilled water and make to 1 l. Titrate to pH 5.5 with 1.0 N NaOH, and mix well by inversion.

An enzyme preparation[10] containing 100,000 units of glucuronidase and 50,000 units of sulfatase/ml. is available commercially.

Procedure—*Extraction of Blood and Urine*—To 1.0 ml. of blood or 4.0 ml. of urine in a 50-ml. centrifuge tube, add 4 ml. of 1.0 N NaOH solution and 15 ml. of ether. Stopper the tubes and shake for 15 min. on a reciprocating shaker. Along with the samples, run a 1-ml. specimen of control blood or 4 ml. of control urine (taken prior to medication) and duplicate 1-ml. specimens of control blood

[6] Brinkmann F_{254}.

[6] Compounds I and I-A are unstable to atmospheric oxidation and light exposure, especially on a thin-layer chromatoplate. Both compounds undergo extensive oxidation on exposure to the atmosphere and also when irradiated on the TLC plate by a Pyro-Lux R-57 lamp. Therefore, immediately following TLC separation of the biological extracts, the silica gel areas corresponding to I and I-A must be scraped off, eluted, and processed for fluorometric analysis as described.

[7] Vortex Super-Mix.

[8] Pyro-Lux R-57, Luxor Corp., N. Y.

[9] (TM) = transmittance (T) × meter multiplier factor (M). All fluorescence measurements were made in a Farrand spectrofluorometer equipped with a xenon arc energy source and an RCA IP-28 photomultiplier. The 10-nm. slit arrangement was used in all four positions. The monochromators were corrected for the fundamental mercury lines, but the fluorescence measurements were not corrected for energy and other instrumental artifacts. The instrument sensitivity was adjusted for maximum energy for each day, using a Pyrex reference rod and a standard solution of the compound to be analyzed.

[10] Glusulase, Endo Laboratories, Inc., Garden City, N. Y.

or 4-ml. specimens of control urine containing 0.50 mcg. II (50 μl. of the working solution evaporated to dryness under nitrogen).

Centrifuge all samples for 10 min. at 2000 r.p.m., preferably in a refrigerated centrifuge at 0–5°, and transfer the ether phase quantitatively into another 50-ml. centrifuge tube. Repeat the extraction with a second 10-ml. portion of ether, centrifuge, and combine the ether extracts. The whole blood specimen is discarded at this point, but the urine specimen is saved for further analysis of the conjugated metabolites.

Assay in Blood—The combined ether extracts of blood are back-extracted with 2 ml. of 0.1 *N* H_2SO_4 by shaking for 15 min. Centrifuge at 2000 r.p.m. at room temperature for 5 min., and aspirate off the supernatant ether layer.

The sulfuric acid extract is backwashed with two 10-ml. portions of ether by shaking for 5 min. each, centrifuging, and aspirating off the ether layer. To the ether-washed sulfuric acid extract, add 2 ml. of mercuric acetate reagent and place in a boiling water bath. Allow 5 min. for equilibration to expel all residual ether from the acid.

At this stage in the assay, pipet 2-ml. aliquots of each standard solution of the external standards of II into 15-ml. centrifuge tubes, add 2 ml. of the mercuric acetate reagent into each tube, and process these samples through the rest of the procedure along with the biological extracts. Stopper the tubes tightly (seal with a drop of distilled water), and allow the chemical oxidation step to proceed for 30 min. in a boiling water bath (100°). Then cool the samples to room temperature, wipe the tubes dry prior to irradiation, and expose all samples including the external standards of II directly to high intensity light from a lamp[8] contained in an aluminum foil-lined box. The samples must be arranged in a single row in a suitable rack and placed 30.48 cm. (12 in.) from the light source. Expose for 15 min. to complete the photochemical oxidation reaction for optimal fluorophore formation. The fluorescence of each sample is read in a 1-cm. path quartz cell at 458 nm., activating at 370 nm., within 1 hr. after light exposure. The concentrations in the unknowns are calculated by direct comparison to the corrected fluorescence of the internal standards:

$$\frac{TM_{458} \text{ of unknown}}{TM_{458} \text{ of internal standard}} \times \frac{\text{concentration of internal standard (0.5 mcg.)}}{\text{ml. sample assayed}} = \text{mcg. II/ml. blood} \qquad \text{(Eq. 3)}$$

Assay in Urine—The initial ether extract of the urine contains unconjugated metabolites in addition to the parent compound. To assay for conjugated metabolites, the ether-extracted urine specimen is transferred into a 50-ml. erlenmeyer flask, titrated potentiometrically to pH 5.5 with 2 *N* HCl, and buffered with 5 ml. of 1.0 *M* phosphate buffer (pH 5.5). The enzyme preparation is added to a final concentration of 1% (v/v), and the flask is stoppered loosely with cotton and incubated at 37° for 2 hr. in a Dubnoff incubation shaker with mild reciprocation. After incubation, the samples are cooled to room temperature, transferred into 50-ml. centrifuge tubes, and extracted at pH 5.5 with 2 × 15-ml. portions of ether to remove the deconjugated weakly acidic phenolic metabolites. The urine specimen is then made strongly alkaline (pH 14) with sufficient 10 *N* NaOH and is reextracted with 2 × 15-ml. portions of ether to remove the deconjugated basic metabolites.

The three separate ether extracts of urine are evaporated to dryness, and the residues are dissolved in 100 μl. of methanol and transferred quantitatively onto separate 20 × 20-cm. silica gel G/F thin-layer chromatoplates[5]. The TLC plates are developed in a vapor-saturated chamber containing ethyl acetate–methanol–ammonium hydroxide (90:10:1) until the solvent front has ascended 12–15 cm. The plates are air dried after development, sprayed with 0.1 *N* H_2SO_4 followed by the mercuric acetate–acetic acid reagent, and then heated for 10 min. in an oven at 100°. The parent drug (II) is located as a highly fluorescent (blue-green) spot (R_f 0.4–0.5) by reference to the R_f of an authentic standard of 1.0 mcg. of II run alongside the sample extracts. All drug-related components fluoresce with characteristically different colors when viewed under longwave UV light, especially the polar metabolites seen at the origin and slightly above it.

The major urinary metabolites, the *N*-desmethyl analog (III, R_f 0.10), the *O*-desmethyl analog (IV, R_f 0.20), and the tetrahydro-8-isoquinolinol analog (V, R_f 0.08) can be readily identified by the very distinctive colors of the fluorophores formed on the TLC plate after the initial oxidative spray treatment and by comparison with the R_f values of authentic standards run alongside the sample extracts.

Scrape the silica gel from the fluorescent areas on the plate corresponding to II into 15-ml. conical centrifuge tubes, add 3 ml. of 0.1 *N* H_2SO_4, and slurry on a supermixer[7] for 1 min. to dissolve the compound in the acid phase. Wash the acid phase with 5 ml. of ether by slurring on the supermixer for 1 min., centrifuge, and then remove the ether layer by aspiration. If the acid phase does not appear clear (water white) when viewed against the light, repeat the ether wash. Transfer a 2-ml. aliquot of the ether-washed sulfuric acid solution into another 15-ml. centrifuge tube, taking care not to disturb the silica gel at the bottom of the 15-ml. tube. Proceed as described for blood to complete the chemical and photochemical oxidation process for fluorophore formation and quantitate fluorometrically as described for blood.

Determination of Percent Recovery of II from Blood and Urine—The recovery of internal standards of II may be determined by comparison of the corrected fluorescence of the internal standard against that of an external standard as described earlier for I.

RESULTS AND DISCUSSION

Tertiary tetrahydroisoquinolines are basic compounds and are extracted into polar solvents such as ether or ethyl acetate at a pH greater than 9.0. Compounds I-HCl and II-HCl were quantitatively extracted from blood or urine into ether at pH 9.0 or above as their respective free bases and back-extracted into dilute acid (0.1 *N* H_2SO_4 or HCl). This property was used as an effective "cleanup" step prior to quantitation. Both compounds have sufficient UV absorbance in dilute acids for quantitation with sensitivity in the microgram range. However, much higher sensitivity is achieved by fluorometric determination of these compounds as their isoquinolinium derivatives.

Compound I-HCl—Studies on the *in vitro* metabolism of I-HCl by Schwartz and Taylor[11], using the 9000 × *g* microsomal fraction of liver from phenobarbital-pretreated rats, indicated a moderate conversion of I to a fluorescent metabolite. This metabolite was isolated and characterized by mass and UV spectrometry as having a structure compatible with 1-isoquinoline acetamide (I-A). An authentic reference sample was synthesized by Schenker[12] and was used to confirm the identity of the metabolite by TLC migration (R_f) values. Further metabolic studies on orally administered I in the monkey showed that the major component in the blood was not the intact drug but was a metabolite (I-A) whose fluorescent properties were similar to that of photochemically oxidized I dissolved in 30% trichloroacetic acid. Authentic I-A was also used as a reference standard in the studies on elucidating the chemical nature of the fluorophore of I.

Characterization of Fluorophore of I—The characterization of the chemical nature of the photochemically produced fluorophore of I was undertaken using UV absorption spectra, activation–emission spectra, TLC, and differential pulse polarographic techniques. It was noted initially that I-HCl dissolved in 0.1 *N* HCl or in 30% trichloroacetic acid exhibited weak intrinsic fluorescence at 380 nm., activating at 335 nm. The fluorescence yield was greatly increased by irradiating I-HCl in the high intensity light from a lamp[8]. The fluorescence yield after irradiation in 30% trichloroacetic acid was about 20 times greater than that produced in 0.1 *N* HCl. The free base I dissolved directly in 30% trichloroacetic acid after irradiation produced 2.7 times the fluorescence yield of I-HCl dissolved in the same medium. This indicated that I is more amenable to photochemical oxidation than is I-HCl.

It was also noted that I-A in 0.1 *N* HCl or 30% trichloroacetic acid exhibited strong intrinsic fluorescence at 380 nm., activating at 335 nm., and that the fluorescence yield in 30% trichloroacetic acid was about four times greater than that in 0.1 *N* HCl. These observations indicated that 30% trichloroacetic acid was the medium of choice for effecting the optimal photochemical conversion of I to its fluorophore and also for measuring the optimal fluorescence yield. The fact that the activation–emission spectral characteristics

[11] M. A. Schwartz and M. K. Taylor, Hoffmann-La Roche, Inc., Nutley, N. J., unpublished data on file, 1967.

[12] F. E. Schenker, Department of Chemical Research, Hoffmann-La Roche Inc., Nutley, N. J., unpublished data, 1967.

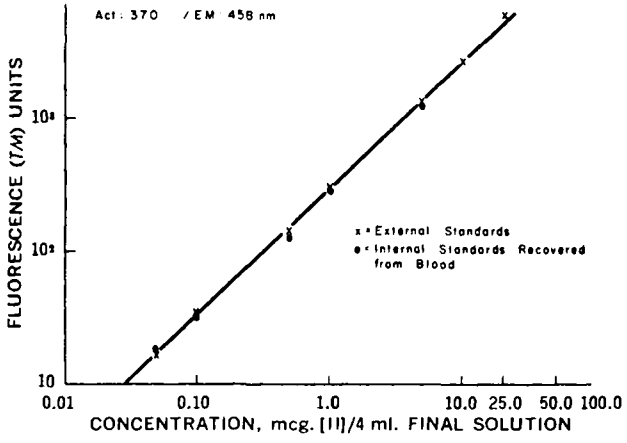


Figure 2—*Linear dynamic range of fluorescence* versus *concentration of external standards and blood-recovered internal standards of II after chemical and photochemical oxidation to their isoquinolinium derivatives.*

of I-A and the photochemically produced fluorophore of I in 30% trichloroacetic acid were identical indicated that I was oxidized (dehydrogenated) to produce I-A (Scheme I). The ratio of the fluorescence yields for equivalent (microgram) amounts of the two compounds (Fig. 1) indicated a conversion efficiency of about 20–25% at low concentrations of I (0.1–1.0 mcg./4 ml.), which increased to about 40–45% at higher concentrations (5–15 mcg./4 ml.). Although the photochemical conversion of I to I-A is not quantitative, it is sufficiently reproducible and linear to be analytically usable.

Further characterization of the fluorophore I-A was effected by a comparison of the UV absorption spectra of I, I-A, and the fluorophore produced by the photochemical oxidation of I. The UV absorption spectra of the two compounds in 0.1 *N* HCl (Fig. 3) show a single absorption maximum for I at 282 nm. (A/mcg./ml. = 0.052), whereas I-A has two absorption maxima, an intense one at 228 nm. (A/mcg./ml. = 0.260) and a weak one at 335 nm. which also corresponds to the wavelength of activation for fluorescence measurements. The reaction products of photochemically oxidized I were extracted into ether (after making the medium strongly alkaline with sodium hydroxide) and back-extracted into 0.1 *N* HCl, and its UV spectrum was determined. The UV spectrum indicated that the major peak of I at 282 nm. had decreased significantly, and absorption maxima at 228 and 335 nm. similar to those of I-A were observed. The reaction products were also analyzed by two-dimensional TLC (Fig. 4), which indicated that the major reaction product had the same R_f as I-A and also exhibited strong intrinsic fluorescence under longwave UV light when the plate was sprayed with 30% trichloroacetic acid. In addition to some unconverted parent compound, a second minor component migrating lower than I-A was also seen. This component showed weak intrinsic fluorescence in 30% trichloroacetic acid and was also present in

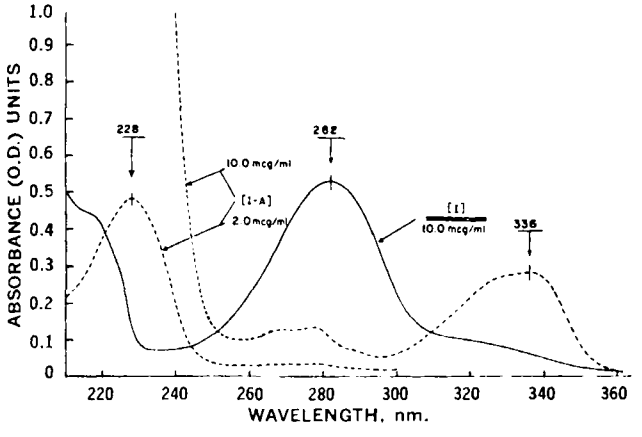


Figure 3—*UV absorption spectra of I and I-A in 0.1* N *HCl.*

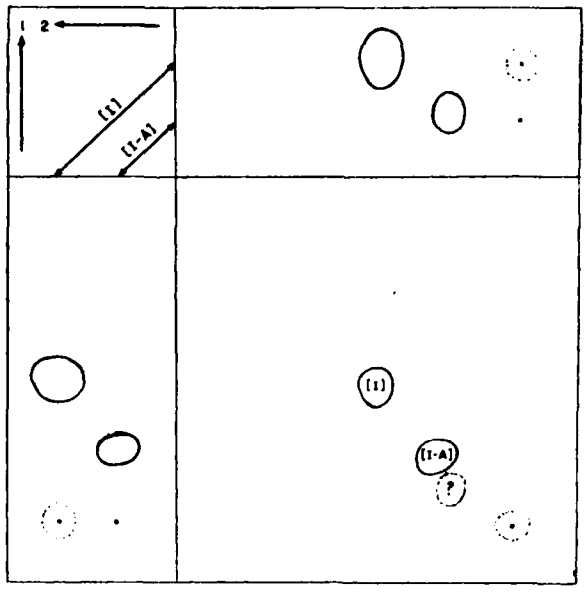


Figure 4—*Two-dimensional TLC of the photochemical oxidation products of I in 30% trichloroacetic acid. Key: 1, ethyl acetate–methanol–ammonium hydroxide (95:4:2); and 2, ethyl acetate–chloroform–ethanol (40:40:20).*

trace amounts when I-A dissolved in 30% trichloroacetic acid was irradiated for over 60 min. It appears to be a breakdown product of I-A produced by prolonged irradiation.

Electrochemical studies on the conversion of I to I-A, using differential pulse polarographic analysis (24), revealed that I-HCl gave a distinct peak at −0.700 v. *versus* standard calomel electrode (SCE) in 1.0 *N* HCl; this was attributed to the reduction of the azomethine (>C=N–) bond (25-27) in the isoquinoline ring. The peak also showed a characteristic pH shift to more negative potential with decreasing acidity from −0.700 v. *versus* SCE in 1.0 *N* HCl to −0.750 v. *versus* SCE in 0.1 *N* HCl and −0.990 v. *versus* SCE in pH 7.0 (1.0 *M* KCl/0.1 *M* PO_4) buffer solution, respectively (Fig. 5*a*). The isoquinolinium derivative (I-A), on the other hand, gave no reduction waves in any of the above media. Similar loss of electrochemical activity was reported for quinoline and isoquinoline in aqueous supporting electrolytes when compared with either nonaqueous or mixed electrolytes (28, 29) and was attributed to the adsorption of the electroinactive substance on the electrode surface. However, I-A gives a well-defined polarographic peak at −1.90 v. *versus* SCE in 95% dimethylformamide–5% water, which is about three times the height as that obtained with an equivalent concentration of I-HCl [E_p (peak potential) = −2.19 v. *versus* SCE) in the same supporting electrolyte. The E_p value for I-A is in close agreement with that reported for isoquinoline (−1.84 v. *versus* SCE) in the same electrolyte (30). Analogous electrochemical activity was noted with the products of the photochemical oxidation of I in 30% trichloroacetic acid.

These experiments demonstrated that I irradiated in 30% trichloroacetic acid, was photochemically oxidized (dehydrogenated) to 1-isoquinoline acetamide (I-A), the major fluorophore produced in this reaction.

Recovery of I and I-A—The direct assay procedure for I gave an overall recovery of 86 ± 5.0% (Table III). Since the TLC separa-

Table III—Overall Recovery Data on I-HCl, I-A, and II-HCl from Blood and Urine

Compound	Blood		Urine	
	Direct Analysis	With TLC	Direct Analysis	With TLC
	Overall Percent Recovery			
I-HCl	86 ± 5.0	63 ± 4.0	≃61	45 ± 6.0
I-A	≃57	41 ± 3.0	≃55	37 ± 5.0
II-HCl	89 ± 5.0	≃60	≃55	33 ± 5.0

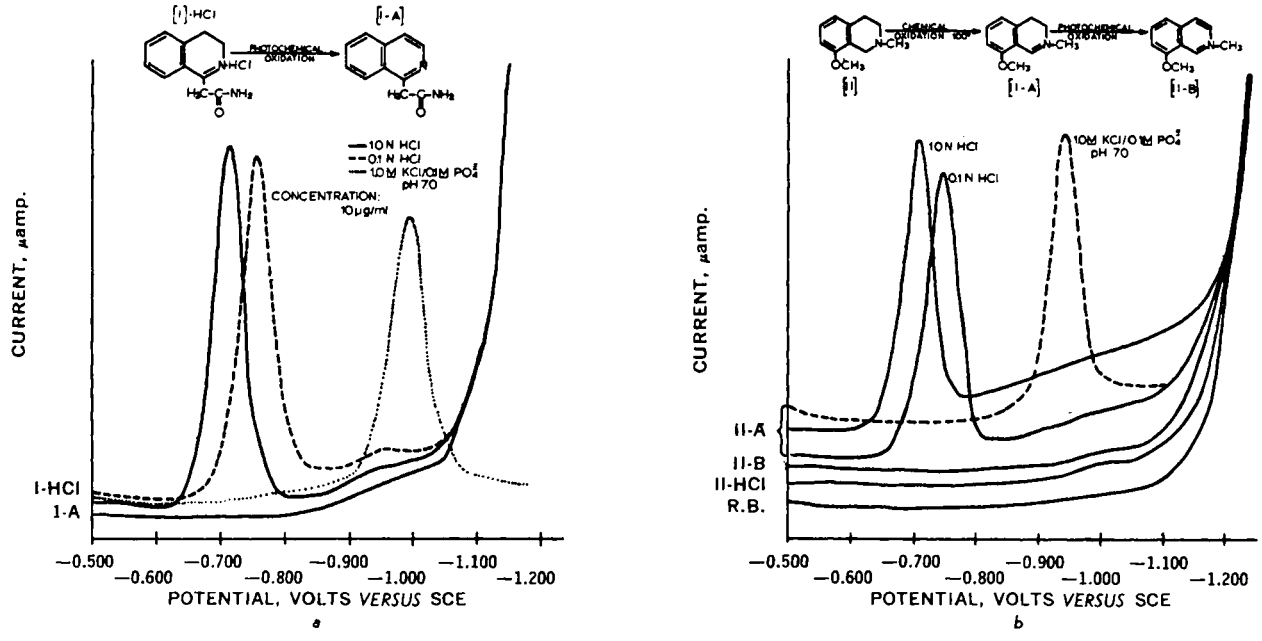


Figure 5—(a) *Differential pulse polarograms of I-HCl and I-A.* (b) *Differential pulse polarograms of II-HCl and the products of chemical oxidation (II-A) and the combined chemical and photochemical oxidations (II-B).*

tion step is essential to ensure the specificity of the assay for the intact drug (I) and its metabolite (I-A) in both blood and urine, the overall recovery of both compounds was determined after inclusion of the TLC separation step. The overall recovery from blood of I was 63 ± 4.0%, while that of I-A was 41 ± 3.0%. The overall recoveries of I and I-A from urine were 45 ± 6.0 and 37 ± 5.0%, respectively. The sensitivity limits of the assay were of the order of 0.25–0.50 mcg. I and 0.25 mcg. I-A/ml. of blood and 0.75–1.00 mcg. I and 0.50 mcg. I-A/ml. of urine, respectively.

Compound II-HCl—Compound II-HCl is a weak base (pKa = 8.2), and it is quantitatively extracted as its free base into ether from alkalinized blood or urine and is back-extracted into 0.1 *N* H_2SO_4 or 0.1 *N* HCl. The compound exhibits UV maxima at 272 and 278 nm. of about equal intensity (*A*/mcg./ml. = 0.077). Following chemical oxidation in the mercuric acetate–acetic acid–sulfuric acid mixture, a significant hypsochromic shift of the UV maxima was observed together with a significant hyperchromic effect. The UV absorption maxima shifted to 264 and 267 nm., indicative of aromatization, and the absorbance at either wavelength (*A*/mcg./ml. = 0.184) also increased twofold over that seen at 272 and 278 nm. Irradiation for 15 min. with UV light[8] produced no further shifts in the UV maxima. The absorbance, however, showed a further increase to 3.5 times that of the parent compound.

The reaction products of the chemical oxidation step and the combined chemical–photochemical oxidation process were also examined by differential pulse polarography in the same aqueous supporting electrolytes as were used for I-HCl. The polarograms shown in Fig. 5*b* indicate that the parent compound (II-HCl) has no intrinsic electrochemical activity. The product of chemical oxidation, II-A, showed peaks typical of a 3,4-dihydroisoquinoline and were almost superimposable on those shown by I-HCl (Fig. 5*a*). However, the isoquinolinium derivative (II-B) formed by the combined chemical and photochemical oxidation reaction showed no activity as did I-A. Attempts to study these reactions in 95% dimethylformamide–5% water were unsuccessful due to trace amounts of coextracted impurities from the reaction medium which interfered with the peaks in the potential region of from −1.80 to −2.00 v. *versus* SCE.

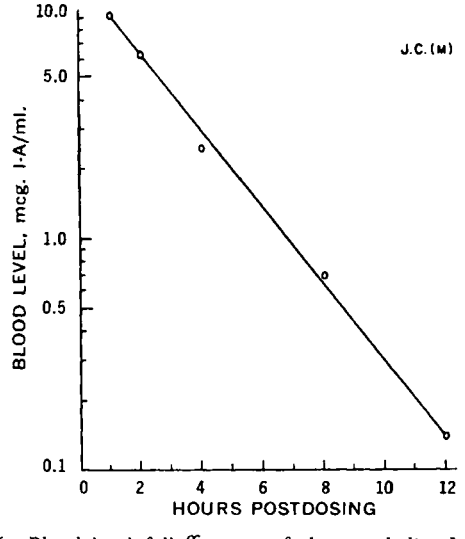


Figure 6—*Blood level falloff curve of the metabolite I-A in man following a single, 1000-mg. oral dose of I-HCl.*

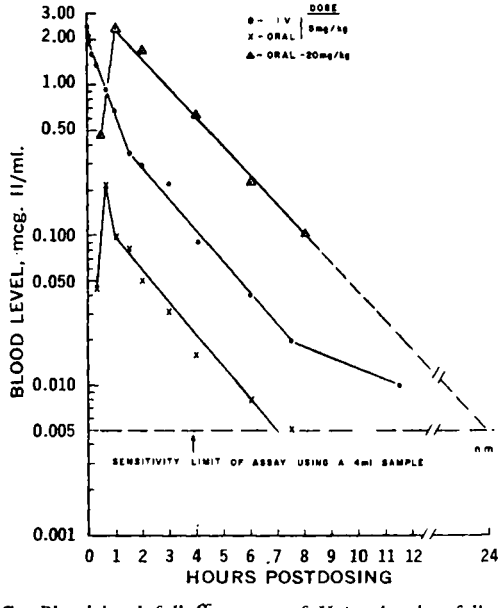


Figure 7—*Blood level falloff curves of II in the dog following intravenous and oral administration.*

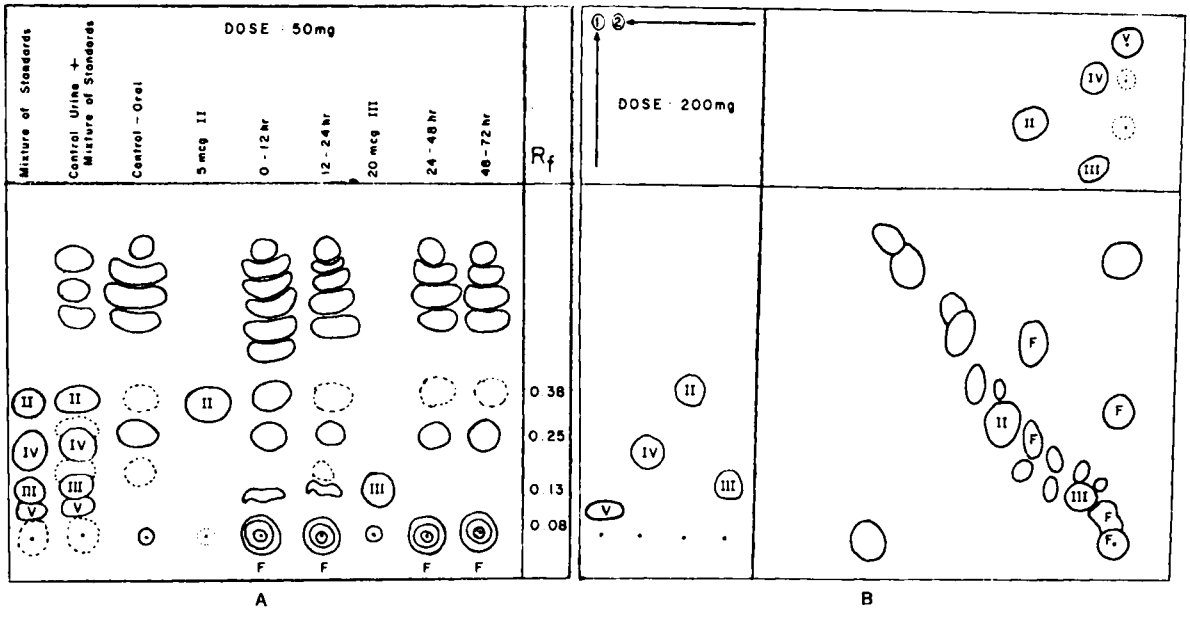


Figure 8 *Thin-layer chromatograms of the direct ether extracts of urine (made strongly alkaline, pH 14) from a dog following oral administration of II. F = fluorescent metabolites. Key: A, developed in one dimension; solvent system of ethyl acetate–methanol–ammonium hydroxide (90:10:1); B, developed in two dimensions; solvent system of (1) ethyl acetate–methanol ammonium hydroxide (90:10:1) or (2) benzene–methanol–glacial acetic acid (90:10:1).*

Polarographic and spectrophotometric studies on the analogous chemical reduction products by partial hydrogenation of isoquinoline to 3,4-dihydroisoquinoline and of 3,4-dihydropapaverine to papaverine also were reported (31) and showed similar stepwise changes in electrochemical activity.

Further characterization of the reaction products of II following either chemical oxidation or the combined chemical–photochemical oxidation reaction was obtained using TLC. The reaction products were extracted at alkaline pH into ether, analyzed by one-dimensional TLC in ethyl acetate–methanol ammonium hydroxide (90:10:1), and examined under longwave UV light. Under both sets of conditions, two fluorescent spots were present, one at the origin and the other close to the solvent front (R_f 0.90), in addition to some residual II (R_f 0.50). The fluorescence of the two spots was of about equal intensity following only chemical oxidation, whereas the fluorescence of the lower spot increased several fold in intensity following the combined oxidation procedure. The two fluorescent spots were eluted into 0.1 *N* H_2SO_4 and scanned for their spectral characteristics. The two compounds had identical UV absorption and activation–emission spectra, indicating that these reaction products are a mixture of fluorophores whose "total" fluorescence in solution is apparently reproducible and analytically acceptable.

Similar reactions were observed with the other tetrahydroisoquinoline analogs (III, IV, and V) shown in Table II. However, the fluorescence yield decreased markedly with the removal of the methyl group in either or both positions of II. Thus, the fully aromatized isoquinoline derivatives of II showed the highest fluorescence (at 370/458 nm.), the fluorescence yield being over 25 times greater than that of the *N*-desmethyl analog (III) at the same wavelengths. Of the two phenolic analogs, the isoquinoline derivative of the *O*-desmethyl analog (IV) exhibited about 2.5 times more fluorescence (at 425/525 nm.) than III (at 370/458 nm.), while the *N*- and *O*-desmethyl analog (V) had the weakest fluorescence (at 425/525 nm.) of them all. Thus, the fluorophores produced by equiv-

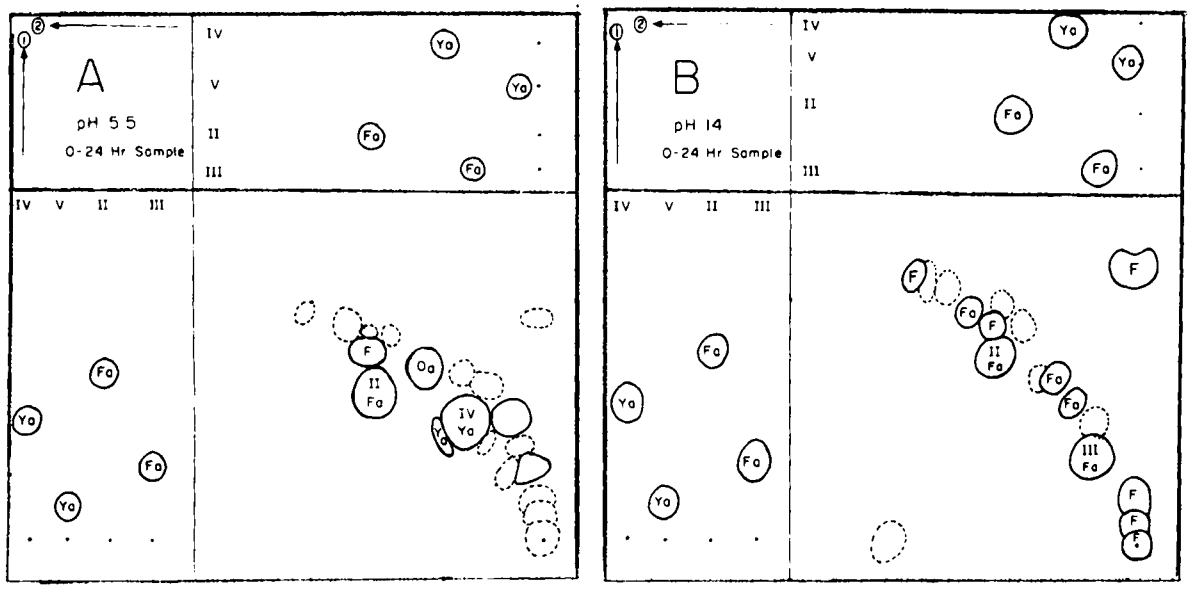


Figure 9 *Two-dimensional thin-layer chromatograms of ether extracts of dog urine after enzyme incubation following oral administration of a 20-mg./kg. dose of II. Key: solvent system: 1, ethyl acetate–methanol–ammonium hydroxide (90:10:1); and 2, benzene–methanol–glacial acetic acid (90:10:1). F = fluorescent before and after oxidation, Fa = fluorescent only after oxidation, Ya = yellow colored only after oxidation, and Oa = orange colored only after oxidation. A = acidic pH 5.5 extract, and B = basic pH 14 extract.*

alent (microgram) amounts of the respective tetrahydroisoquinolines (read at their respective wavelengths of maximum activation–emission) arranged in decreasing order of fluorescence emission are II > IV > III > V. These studies showed that in the overall oxidation process the first step (chemical oxidation) introduces a double bond in the 1,2-position of the tetrahydroisoquinoline ring while the second step (photochemical oxidation) introduces a second double bond in the 3,4-position to produce the fully aromatized isoquinolinium derivative (Table II). These derivatives exhibit high UV absorption and fluorescence in acidic media due to the increase in the aromaticity in the molecule.

Recovery of II from Blood and Urine—Recovery experiments with II from blood and urine indicated that the overall recovery from blood using the direct assay procedure was 89 ± 5.0% (Table III) while that from urine, which included the TLC separation step, was significantly lower (33 ± 5.0%).

The sensitivity limits of the assay for blood and urine are of the order of 0.05–0.10 mcg. II/ml. and can be extended to 0.005 mcg./ml. of blood using a 4-ml. specimen. The linear range of fluorescence *versus* concentration of oxidized II as external standards and as internal standards recovered from blood is of the order of 0.05–25.0 mcg. II/4 ml. of final solution (Fig. 2).

Application of Methods to Biological Specimens—*Compound I-HCl: Blood Levels in Man following a Single 1000-mg. Oral Dose*—A pilot blood level study was performed with one volunteer who was administered a single dose of 1000 mg. of I-HCl orally. Whole blood, oxalated, was drawn at 0, 1, 2, 4, 8, 12, 24, 48, 72, and 96 hr. after administration and was analyzed. No measurable levels of the intact drug (I) were seen at any time point. Measurable levels of a metabolite I-A were seen; however, the peak blood level of 9.0 mcg./ml. at 1 hr. declined rapidly within 12 hr., with an apparent half-life of about 2 hr. After 12 hr. the metabolite levels were no longer measurable (Fig. 6). The data indicate rapid absorption of I-HCl and rapid biotransformation to I-A.

Specificity of Assay for I and Characterization of Blood Metabolite I-A in Man and Monkey—Blood ether extracts from man and monkey following oral administration of I-HCl were analyzed by one-dimensional TLC in ethyl acetate–methanol–concentrated ammonium hydroxide (95:4:2). The chromatoplate did not show the presence of any intact drug (I, R_f 0.60). The major component was a metabolite seen in both man and monkey blood whose R_f 0.28 was identical with that of authentic I-A chromatographed alongside the sample extracts. This TLC step, which is also used in the assay, ensures its specificity for I and I-A. The metabolite and compound I-A, when cochromatographed, ran as one spot having the same R_f as the reference standard, indicating that the two compounds were identical. Thus, I-A was identified as the major ether-extractable metabolite of I-HCl in the blood of man and in the monkey. On-going studies[13] on the metabolism of I-HCl in man and in the dog also indicate extensive metabolism, resulting in several polar metabolites present in blood and urine which can be analyzed by selective solvent extraction and TLC separation

Compound II-HCl—Blood levels in the dog were determined in one animal following the administration of a 5-mg./kg. (total 50-mg.) dose of II-HCl by intravenous and oral routes and in another animal following a single oral 20-mg./kg. (total 200-mg.) dose. The blood level falloff curves are shown in Fig. 7. Following the 5-mg./kg. i.v. dose, a biphasic falloff pattern in the blood levels of II was seen, with the α-component (distribution phase) showing a rapid apparent half-life of 40 min., while the β-component (elimination phase) showed an apparent half-life of 1.4 hr.

Following oral administration of the 5-mg./kg. (50-mg.) dose, the blood levels of II appeared to reach a peak level (0.22 mcg./ml.) at 40 min. followed by a progressive decline, with an apparent half-life of 1.5 hr. between 1.0 and 7.5 hr. The blood level falloff data determined following a single 20-mg./kg. (200-mg.) oral dose indicated a peak blood II level of 2.33 mcg./ml. 1 hr. after dosing, which declined to nonmeasurable levels in 24 hr., again with an apparent half-life of 1.5 hr.

The recovery of the intact drug into ether from dog urine made alkaline (pH 14) was very low. After the 5-mg./kg. i.v. dose, only 1.5% of the drug was recovered in the 0–24-hr. period; following the 5- and 20-mg./kg. oral doses, only 0.21 and 3.9% of the respective administered doses were recovered in the same excretion period. Again, TLC of the direct ether extracts of urine (Figs. 8A and 8B) indicated the presence of the intact drug, the *N*-desmethyl analog (III), and several highly fluorescent, incompletely resolved compounds near the origin.

The 0–24-hr. urine sample from the dog dosed with 20 mg./kg. following enzyme incubation at pH 5.5 was extracted at pH 5.5 and 14 and analyzed by two-dimensional TLC. The chromatogram of the pH 5.5 ether extract (Fig. 9A) indicated the presence of the parent drug (II), the phenolic analog (IV), and three unidentified acidic metabolites. The pH 14 ether extract (Fig. 9B) indicated the presence of II, the *N*-desmethyl analog (III), and at least eight other discernible basic metabolites which were strongly fluorescent either intrinsically or after oxidation with mercuric acetate–acetic acid. The basic metabolites showed a typical blue-green isoquinoline-like fluorescence similar to that of oxidized II, whereas the acidic phenolic metabolites IV and V showed characteristic yellow- or orange-colored fluorescence after oxidation.

The highly fluorescent metabolites seen at the origin of the TLC plate were those present in the most significant amounts, as indicated by the intrinsic fluorescence obtained upon their elution from silica gel into 4 ml. of 0.1 *N* H_2SO_4 and measurement of the fluorescence at the same wavelengths as for oxidized II. Based on the assumption that the specific intrinsic fluorescence of these metabolites is approximately the same as that of oxidized II, the total amount of the fluorescent metabolites recovered from the chromatoplate represents about 37% of the dose. The intact drug (II) recovered from both acidic and basic extracts accounted for about 8% of the administered dose. These fluorescent metabolites appear to be chemically similar to the isoquinoline derivatives of II (oxidized) but have yet to be characterized. The percent of the dose recovered as the acidic phenolic metabolites also appears to be considerable. It is conservatively estimated that more than 50% of a given dose can be accounted for in the urine over a 24-hr. excretion period, indicating rapid and extensive biotransformation of the drug.

Blood levels of II were determined in man (17) during the clinical evaluation of the drug, when doses ranging from 50 to 300 mg. were administered orally. Peak blood II levels were seen 1–2 hr. after dosing, followed by a rapid decline in blood levels over the next 10 hr. The urinary excretion data indicated extensive biotransformation of the drug with less than 1% of the orally administered dose recovered in the 0–24-hr. period (17).

Specificity of the Assay—The blood level assay is apparently specific for the intact drug (II) after single doses in the dog, since only the intact drug was seen in the blood ether extracts analyzed by TLC. Chronic administration to dogs at high doses (20 mg./kg.), however, showed the presence of trace amounts of III and another unidentified fluorescent metabolite[14]. This would necessitate the use of TLC to establish the specificity of the assay in blood for II and its metabolites. In the direct assay in blood, small amounts of III would not seriously interfere with the assay values for II because of the 25-fold difference in the fluorescence intensities of the isoquinolinium derivatives of II over III. However, the highly fluorescent isoquinoline-like metabolites seen in urine, if present in blood following chronic administration, would have to be separated by TLC prior to the determination of II.

The urine assay requires the use of the TLC separation step to resolve the parent drug (II) from the many metabolites present, especially the highly fluorescent isoquinoline-like metabolites which have similar fluorescence properties as that of oxidized II.

CONCLUSION

The chemical oxidation of tertiary tetrahydroisoquinolines is a useful general reaction for converting these compounds to their 3,4-dihydroisoquinoline derivatives (18–20). These derivatives can be further oxidized by photochemical oxidation by exposure to UV radiation in acidic media to yield the fully aromatized isoquinolinium derivatives, which are highly fluorescent and amenable to sensitive quantitation in the nanogram range. Both principles have been effectively used in developing assays for the spectrofluorometric quantitation of these types of compounds in biological fluids. Photochemical methods are useful on the microscale for

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derivatization reactions and are also used extensively in organic synthesis (32), especially in the preparation of isoquinoline alkaloids (33).

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Serum Protein Binding of Erythromycin, Lincomycin, and Clindamycin

R. C. GORDON, C. REGAMEY, and W. M. M. KIRBY▲

Abstract □ Serum protein binding determinations for erythromycin, lincomycin, and clindamycin utilizing ultrafiltration of serum containing antibiotic in a concentration of 5 mcg./ml. and tube dilution techniques revealed a high degree of binding: erythromycin base, 73%; erythromycin propionate, 93%; lincomycin, 72%; and clindamycin, 94%.

Keyphrases □ Erythromycin and erythromycin propionate—serum protein binding □ Lincomycin—serum protein binding □ Clindamycin—serum protein binding □ Antibiotics—serum protein binding of erythromycin, lincomycin, and clindamycin □ Serum protein binding—erythromycin, lincomycin, and clindamycin

Serum protein binding of antibiotics is of potential clinical significance since bound antibiotic has been found to have no antibacterial activity and it is probable that the level of free antibiotic in the tissues is no greater than the peak level in the blood (1). The extent of protein binding of erythromycin, lincomycin, and

derivatization reactions and are also used extensively in organic synthesis (32), especially in the preparation of isoquinoline alkaloids (33).

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Serum Protein Binding of Erythromycin, Lincomycin, and Clindamycin

R. C. GORDON, C. REGAMEY, and W. M. M. KIRBY▲

Abstract □ Serum protein binding determinations for erythromycin, lincomycin, and clindamycin utilizing ultrafiltration of serum containing antibiotic in a concentration of 5 mcg./ml. and tube dilution techniques revealed a high degree of binding: erythromycin base, 73%; erythromycin propionate, 93%; lincomycin, 72%; and clindamycin, 94%.

Keyphrases □ Erythromycin and erythromycin propionate—serum protein binding □ Lincomycin—serum protein binding □ Clindamycin—serum protein binding □ Antibiotics—serum protein binding of erythromycin, lincomycin, and clindamycin □ Serum protein binding—erythromycin, lincomycin, and clindamycin

Serum protein binding of antibiotics is of potential clinical significance since bound antibiotic has been found to have no antibacterial activity and it is probable that the level of free antibiotic in the tissues is no greater than the peak level in the blood (1). The extent of protein binding of erythromycin, lincomycin, and

Table I—*In Vitro* Determination of Human Serum Protein Binding of Erythromycins Using Ultrafiltration and Bioassay (100% Serum)

Erythromycin Propionate			Erythromycin Base		
Serum, mcg./ml.	Ultrafiltrate, mcg./ml.	Percent Bound	Serum, mcg./ml.	Ultrafiltrate, mcg./ml.	Percent Bound
5.68	0.343	93.96	5.20	1.29	75.19
5.68	0.422	92.57	5.20	1.23	76.34
5.68	0.400	92.95	5.20	1.46	71.92
6.60	0.420	93.63	5.08	1.36	73.23
6.60	0.457	93.07	5.08	1.48	70.87
6.08	0.388	93.61	5.08	1.32	74.02
6.08	0.359	94.09	5.26	1.47	72.05
6.08	0.425	93.00	5.26	1.46	72.24
7.70	0.740	90.38	5.26	1.35	74.33
7.70	0.795	89.67			73.35
7.70	0.700	90.90			
6.40	0.420	93.43			
6.40	0.440	93.13			
		92.64			

Table II—*In Vitro* Determination of Human Serum Protein Binding of Lincomycin and Clindamycin Using Ultrafiltration and Bioassay (100% Serum)

Lincomycin			Clindamycin		
Serum, mcg./ml.	Ultrafiltrate, mcg./ml.	Percent Bound	Serum, mcg./ml.	Ultrafiltrate, mcg./ml.	Percent Bound
5.85	1.62	72.30	5.88	0.400	93.20
5.85	1.60	72.64	5.88	0.365	93.80
5.85	1.66	71.62	5.88	0.373	93.70
6.10	1.95	68.03	4.25	0.270	93.64
6.10	1.88	69.18	4.25	0.267	93.71
6.10	1.88	69.18	4.25	0.272	93.60
6.10	1.48	75.73	6.35	0.397	93.74
6.10	1.48	75.73	6.35	0.410	93.54
6.10	1.47	75.90			93.62
5.85	1.65	71.79			
5.85	1.67	71.45			
5.85	1.78	69.57			
		71.93			

clindamycin has been variously reported in the literature as low (2–4) to high (5–7). The present study was carried out to help delineate the protein binding characteristics of these three antibiotics using a variety of methods.

EXPERIMENTAL

Three techniques were used to study binding of these antibiotics by human serum proteins to compare the results obtained and to investigate possible causes of variation in the results reported by other workers. The antibiotic standard powders used in these studies were: erythromycin base[1], erythromycin propionate[1], ^{14}C-erythromycin[1], lincomycin[2], and clindamycin hydrochloride[2].

Ultrafiltration Method—The precise method and equipment reported by Bennett and Kirby (8) was used in these protein binding determinations involving vacuum ultrafiltration of pooled human serum from healthy volunteers containing antibiotic added in a concentration of approximately 5 mcg./ml. For these studies, a single layer of cellophane membrane[3], previously sterilized by rinsing with 90% ethanol, was utilized. All experiments were performed in a manner simulating *in vivo* conditions in that the initial serum pH was adjusted to the physiological range by bubbling of carbon dioxide, and all experiments were carried out in a 37° incubator. Ultrafiltration required less than 45 min. in every case, and post-filtration pH determinations revealed that the pH remained in the physiological range throughout the tests.

A serum sample, corresponding to 100% serum because of concentration during filtration, was obtained for antibiotic assay at the midpoint of filtration. The principle and mathematics of this were explained in the original article (8). Antibiotic concentrations in the midpoint sample and in the ultrafiltrate sample were assayed by an agar well method (9) using *Bacillus subtilus* (ATCC 6633) as the test organism for erythromycin and *Sarcina lutea* (FDA, PCI 1001) for lincomycin and clindamycin. The percent binding by 100% human serum was calculated by dividing the difference between the midpoint serum and filtrate antibiotic concentrations by the serum antibiotic concentration and multiplying by 100. Modifications of this same formula were used throughout this study for determination of protein binding. Impermeability of the membrane to unbound antibiotic as a possible cause of apparent protein binding was investigated by ultrafiltration of buffered saline solutions of the drugs.

Tube Dilution Method—A good correlation between protein binding and decrease in antibacterial activity has been observed by a number of workers (10, 11). For the present studies the minimum inhibitory concentration in tryptose phosphate broth was compared to that obtained in a series of tubes containing a fixed concentration of serum (25, 75, 90, or 100%) and small increments of antibiotic (0.02 mcg./ml.), with a range from 0.005 to 2.0 mcg./ml.

[1] Eli Lilly.
[2] Upjohn.
[3] Union Carbide, D.M. 1-7/8.

Table III—*In Vitro* Determination of Human Serum Protein Binding Using ^{14}C-Erythromycin Base (100% Serum)

Ultrafiltration			Centrifugation		
Serum, c.p.m.	Filtrate, c.p.m.	Percent Bound	Serum, c.p.m.	Filtrate, c.p.m.	Percent Bound
123,262.5	38,890.0	71.7	143,880.0	44,030.0	69.3
121,647.5	34,625.0	71.5	143,880.0	47,362.0	67.0
145,797.0	42,535.0	70.8	131,263.0	43,462.5	66.8
146,295.0	42,392.0	71.0			67.7
132,130.0	31,445.0	76.2			
129,948.0	26,440.0	79.6			
		73.5			

The test organism was a group A beta hemolytic streptococcus (clinical isolate), and hemolysis of human group O red blood cells was used as the bacteriostatic end-point. Minimal bactericidal concentrations were determined by subculture to antibiotic-free agar to establish an end-point where the hemolytic end-point was not clear cut. Percent binding was determined by dividing the difference between the minimum inhibitory concentration in serum and in tryptose phosphate broth by the minimum inhibitory concentration in serum and multiplying by 100.

Radioactive Assay Method—As an additional check on the accuracy of protein binding results, and as another way to study the possibility of membrane trapping of free antibiotic, a series of experiments was performed using labeled erythromycin. The ultrafiltration technique was modified so that counting of disintegrations per minute rather than bioassay was used to determine antibiotic concentrations. Experiments were also carried out using the centrifuge[4] cone technique, which is an ultrafiltration method using centrifugation rather than suction to effect separation of protein bound from free antibiotic. With this method, 5 ml. of serum containing the labeled antibiotic (5 mcg./ml.) was placed in a commercially prepared porous cone lined with an inner synthetic membrane and centrifuged at $100 \times g$ for 20 min. at 37°, yielding 1 ml. of protein-free filtrate. The antibiotic used in this series of experiments was ^{14}C-labeled erythromycin[5] at a specific activity of 9.45 μc./mg. All specimens were counted[6] for 20 min. in a liquid scintillation spectrometer[7] using appropriate internal standards. The concentration of erythromycin in the serum was compared with that in the filtrate or centrifugate by counting as already described, and the percent binding was calculated by dividing the difference between the counts per minute in serum and centrifugate or filtrate

[4] CF50A, Amicon Corp., Lexington, Mass.
[5] Lilly Research Laboratories.
[6] In Scintisol solution, Isolab, Akron, Ohio.
[7] Beckman.

Table IV—*In Vitro* Determination of Human Serum Protein Binding of Erythromycin Propionate, Lincomycin, and Clindamycin Using Tube Dilution Technique

Percent Serum	Erythromycin, Minimum Inhibiting Concentration			Lincomycin, Minimum Inhibiting Concentration			Clindamycin, Minimum Inhibiting Concentration		
	Broth, mcg./ml.	Serum, mcg./ml.	Percent Bound	Broth, mcg./ml.	Serum, mcg./ml.	Percent Bound	Broth, mcg./ml.	Serum, mcg./ml.	Percent Bound
25	0.06	0.08	25.0	0.40	0.8	50.0	0.04	0.10	60.0
	0.04	0.12	66.6	0.40	0.8	50.0	0.04	0.08	50.0
			45.8			50.0			55.0
75	0.02	0.12	83.3	0.40	1.8	77.7	0.008	0.08	90.0
	0.10	0.60	83.3	0.40	1.8	77.7	0.04	0.60	93.3
			83.3			77.7	0.04	0.40	90.0
									91.1
90	0.02	0.10	80.0	0.60	2.4	75.0	0.06	0.60	90.0
	0.03	0.08	62.5	0.10	0.4	75.0	0.025	0.40	93.7
			71.3			75.0			91.9
100	0.06	0.60	90.0	0.60	2.0	70.0	0.05	0.40	87.5
	0.10	0.60	83.3	0.09	0.4	77.5	0.03	0.60	95.0
			86.7			73.8			91.3

by the number of counts per minute in the serum and multiplying by 100.

Impermeability of the membranes to free antibiotic as a possible cause of high protein binding values was also studied by ultrafiltration and centrifugation of buffered saline solutions rather than serum. Possible membrane trapping was also checked in another way: the membranes used in two of the experiments with serum were carefully removed from the chambers, rinsed lightly with distilled water, and, after air drying, were burned to release carbon dioxide in a sample oxidizer[8]; radioactivity representing erythromycin adherent to the membrane was then counted.

RESULTS

Using the ultrafiltration method with 100% serum and bioassay of the specimens, binding of the four antibiotics was found to be as follows: erythromycin base, 73.4%; erythromycin propionate, 92.6%; lincomycin, 71.9%; and clindamycin, 93.6% (Tables I and II). Ultrafiltration of buffered saline solutions of the antibiotics revealed that less than 10% was trapped by the cellophane membrane, *i.e.*, did not pass through into the filtrate.

These ultrafiltration results correlated well with binding as determined by measurements of the decrease in antibacterial activity in serum by the tube dilution technique (Table III). With the use of small increments of antibiotic (0.02 mcg./ml.) from one tube to the next and bactericidal end-points, particularly for the higher serum concentrations, there was good agreement of serum binding figures obtained with the two methods.

Table IV summarizes the results of protein binding studies using ^{14}C-labeled erythromycin base. In these experiments, the mean binding of erythromycin by the ultrafiltration method was 73.5%, very close to the 73.4% observed with bioassay. Filtration of buffered saline solutions of the drug demonstrated little evidence of membrane trapping (retention <4%), again a result similar to that reported with bioassay of the specimens. Studies of membrane trapping carried out by burning the membrane after ultrafiltration of serum and counting the labeled carbon dioxide released showed that only 0.05 mcg. or less of the erythromycin was retained by the membrane. Centrifugation studies, performed with the cone technique as an alternative method of checking protein binding of ^{14}C-erythromycin, yielded a binding figure of 67.7%. With centrifugation of buffered saline solutions of the labeled antibiotic, 11.7% of the radioactivity was present in the cone.

DISCUSSION

The low degree of serum protein binding commonly attributed to erythromycin (2), lincomycin (3), and clindamycin (4) has been based chiefly upon tube dilution studies demonstrating little or no increase in minimum inhibiting concentration in serum as compared with broth. However, these measurements are subject to a large error when twofold dilutions are used and when the concentration of serum employed, often 50%, is far below that occurring in the blood. Other technical factors that may cause erroneous binding determinations include use of very high concentrations of antibiotic, far above those seen under *in vivo* circumstances, or the use of serum of different species which has been found to give binding results different for some drugs from those of human serum (1). A widely quoted ultrafiltration study said to demonstrate 18% binding of erythromycin by human serum was actually done with serum protein fractions rather than whole serum (12). A lincomycin dialysis study using 50% horse serum demonstrated no evidence of binding, while a similar study showing 20% binding did not specify the concentration of antibiotic used (3).

Recently reported high degrees of binding of erythromycin and erythromycin estolate carried out at antibiotic concentrations in the range usually found in patients and using the ultrafiltration technique revealed binding values of 81.4 and ≥95.7%, respectively (13). Other recent studies have also shown quite high binding figures for lincomycin (80–90%) (6) and clindamycin (83%) (7).

The presently reported studies, carried out in conjunction with the various controls described earlier, also indicate a high degree of serum protein binding for erythromycin and erythromycin propionate, which is the free base of erythromycin estolate. The difference in binding noted for the two erythromycins is apparently not solely due to the difference in molecular weights, since the percent binding for erythromycin base (mol. wt. 733.92) is about 20% less than that of erythromycin propionate (mol. wt. 789.96), while there is only a 7% difference in their molecular weights.

[8] Packard Tri-Carb model 305.

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Pharmacological Evaluation of Medicinal Plants from Western Samoa

T. R. NORTON▲, M. L. BRISTOL, G. W. READ, O. A. BUSHNELL, M. KASHIWAGI, C. M. OKINAGA, and C. S. ODA

Abstract □ A total of 110 plant parts from 34 different species of medicinal and toxic plants was collected for pharmacological testing in or near the Lefaga District on the island of Upolu in Western Samoa. Voucher specimens of all species were also made. From the 110 plant parts, 211 different extracts were prepared and evaluated for antiviral, antibacterial, and anti-Ehrlich ascites tumor properties and for effects on blood pressure, heart rate, respiration, electrocardiogram, electroencephalogram, and gastric motility. Twelve extracts were effective *in vitro* as antibacterial agents and six inhibited Ehrlich ascites tumor growth in mice. Ninety-one extracts showed hypotensive properties, 31 for more than 30 min.

Keyphrases □ Medicinal plants—pharmacological evaluation of 34 species from Western Samoa □ Antiviral activity—34 plants from Western Samoa screened □ Antitumor activity—34 plants from Western Samoa screened □ Antibacterial activity—34 plants from Western Samoa screened □ Antihypertensive activity—34 plants from Western Samoa screened

As part of a broad program of ethnobotanical and pharmacological studies of medicinal plants and other biota from the Pacific basin, medicinal and poisonous plants of Western Samoa were collected for pharmacological and chemical evaluation. The ethnobotanical phase was centered in Lefaga District on the island of Upolu. The flora was studied intensively, and a large collection of herbarium specimens was obtained. Ethnobotanical data concerning the names, usage, and special significance of most of the woody and some of the herbaceous flora were gathered for native and introduced plants. This information, which included a large body of medicinal lore, was gathered from many people in Lefaga District. No prominent practitioner of native medicine was located in the District, so the data obtained on medicinal plants are not those of one outstanding individual but rather reflect that body of information widely known to many Samoans. Extensive botanical collections were also made in the interior of Savaii island and to a lesser extent at many coastal points on both islands. Whenever possible, ethnobotanical data were gathered at the same time from local people.

The 34 plants chosen for inclusion in the pharmacological studies are native or naturalized species, many of which also occur on adjacent island groups. Most are of therapeutic or toxicological interest to the Samoans today and many are noted in the literature concerning Samoa. Three plants, *Ochrosia oppositifolia*, *Barringtonia samoensis*, and *Terminalia samoensis*, were included because of their close botanical relationship to plants known for their physiological activity. Herbarium voucher specimens were made for all the plants studied[1]. The plant materials analyzed were air dried in Samoa in a large rack of screen trays heated gently by kerosene lanterns. The material was sealed in polyethylene for air shipment to Honolulu, Hawaii.

A direct evaluation under clinical conditions of the use of the plants by the local Samoans was not attempted. However, it was felt that the probability of physiological and chemotherapeutic effects might be greater with plants selected by the local people for medicinal use than with plants selected at random. Whether this is so remains a moot point. No checks were carried out for possible potentiation corresponding to use of several plants in a given local remedy.

Each plant part from Western Samoa was powdered and extracted with methylene chloride and then 60% ethanol. In this manner, the extraction method gave two extracts, the methylene chloride extract containing the relatively nonpolar constituents and the aqueous ethanolic extracts containing the more polar constituents. All extracts were examined for antiviral, antitumor, and antibacterial activity as well as for systemic effects in the rat.

[1] They are deposited at the B. P. Bishop Museum, Honolulu, Hawaii; U. S. National Herbarium, Washington, D. C.; Gray Herbarium, Cambridge, England; Royal Botanic Gardens, Kew, England; and at several other herbaria.

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The 34 plants chosen for inclusion in the pharmacological studies are native or naturalized species, many of which also occur on adjacent island groups. Most are of therapeutic or toxicological interest to the Samoans today and many are noted in the literature concerning Samoa. Three plants, *Ochrosia oppositifolia*, *Barringtonia samoensis*, and *Terminalia samoensis*, were included because of their close botanical relationship to plants known for their physiological activity. Herbarium voucher specimens were made for all the plants studied[1]. The plant materials analyzed were air dried in Samoa in a large rack of screen trays heated gently by kerosene lanterns. The material was sealed in polyethylene for air shipment to Honolulu, Hawaii.

A direct evaluation under clinical conditions of the use of the plants by the local Samoans was not attempted. However, it was felt that the probability of physiological and chemotherapeutic effects might be greater with plants selected by the local people for medicinal use than with plants selected at random. Whether this is so remains a moot point. No checks were carried out for possible potentiation corresponding to use of several plants in a given local remedy.

Each plant part from Western Samoa was powdered and extracted with methylene chloride and then 60% ethanol. In this manner, the extraction method gave two extracts, the methylene chloride extract containing the relatively nonpolar constituents and the aqueous ethanolic extracts containing the more polar constituents. All extracts were examined for antiviral, antitumor, and antibacterial activity as well as for systemic effects in the rat.

[1] They are deposited at the B. P. Bishop Museum, Honolulu, Hawaii; U. S. National Herbarium, Washington, D. C.; Gray Herbarium, Cambridge, England; Royal Botanic Gardens, Kew, England; and at several other herbaria.

Table I—Pharmacological Testing

Family	Species	Plant Part[a], Solvent[b]	T/C Columbia SK Virus[c]	30-Day Survivors EA[d]	Rat Arterial Pressure[e]	Microbiology[f]
Anonaceae	*Canaga odorata* (Lam.) Hook, F. & Thomp.	BK–M	2.0, 92	—	neg.	neg.
		BK–E	2.0, 100	neg., tox.	sl. hypo.	neg.
		LV–M	2.0, 100	—	neg.	neg.
		LV–E	112	neg., tox.	v. hypo. act. i.p.	neg.
		RT–M	2.0, 138	neg., tox.	neg.	neg.
		RT–E	111	—	—	neg.
		ST–M	2.0, 112	—	hypo. neg. i.p.	neg.
		ST–E	133	1.5, neg., tox.	neg.	neg.
Apocynaceae	*Cerbera manghas* L.	FR+S–M	118	neg.	neg.	—
		FR+S–E	0.3, 100	0.5, neg., tox.	neg.	neg.
		LV–M	0.3, 100	0.5, neg.	neg.	neg.
		LV–E	0.2, 111	0.3, neg., tox.	v. hypo., neg. i.p.	neg.
		RT–CL	0.3, 100	neg.	neg.	—
		RT–E	0.6, 94	0.7, neg., tox.	sl. hypo.	—
		ST–CL	100	—	sl. hypo.	—
		ST–E	89	neg.	hypo., neg. i.p.	neg.
	Ochrosia oppositifolia (Lam.) K. Schum.	LV–M	2.0, 133	neg.	neg.	neg.
		LV–E	2.0, 88	neg.	v. hypo.	neg.
Araceae	*Amorphophallus campanulatus* (Roxb.) Bl. in Decaisne	LV+BL–M	111	—	neg.	neg.
		LV+BL–E	0.5, 67	neg.	v. hypo., act. i.p.	neg.
		PT–M	111	—	neg.	act. strep.
		RT+CRM–M	1.5, 89	—	neg.	neg.
		RT+CRM–E	100	—	sl. hypo.	neg.
	Raphidophora reineckei Engler	LV–M	2.0, 123	—	neg.	neg.
		LV–E	2.0, 100	1.5, neg.	v. hypo., act. i.p. act. oral, non-α-block.	neg.
		ST–M	2.0, 78	—	neg.	neg.
		ST–E	2.0, 154	neg.	sl. hypo.	neg.
Asclepiadaceae	*Hoya australis* R. Br.	ST+LV+RT–CL	100	—	neg.	neg.
		ST+LV+RT–E	89	—	hypo.	neg.
Barringtoniaceae	*Barringtonia asiatica* (L.) Kurz	BK–M	0.3, 100	1.3, neg., tox.	neg.	neg.
		BK–E	0.2, 124	—	v. hypo., v. tox.	—
		LV–M	128	neg., tox.	neg.	neg.
		LV–E	128	neg.	hypo.	—
	Barringtonia samoensis A. Gray	BK–M	2.0, 280[g]	—	neg.	neg.
		BK–E	352[g]	0.5, neg., v. tox.	sl. hypo.	neg.
		LV–M	100	—	neg.	neg.
		LV–E	100	—	sl. hypo.	neg.
		RT–M	2.0, 145	—	neg.	neg.
		RT–E	0.25, 150	0.5, neg.	fatal (1 ml./kg.)	neg.
		SD–M	2.0, 200[g]	—	neg.	neg.
		SD–E	—	0.4, neg., tox.	—	—
		ST–M	2.0, 280[g]	—	neg.	neg.
		ST–E	2.0, 320[g]	60% surv.[h]	hypo.	neg.
Combretaceae	*Terminalia samoensis* Rech.	BK–M	111	—	neg.	neg.
		BK–E	0.25, 111	0.2, neg., tox.	v. hypo., act. i.p., non-α-block., non-histaminergic	neg.
		LV–E	0.25, 111	0.13, 100% tox.	v. hypo., inact. i.p., act. oral.	act. shig.
		PT–M	—	—	—	—
		PT–E	1.5, 89	—	neg.	neg.
		RT–M	0.5, 111	—	neg.	neg.
		RT–E	0.5, 100	0.13, neg., tox.	v. hypo., inact. i.p., inact. oral	act. staph., strep.
Compositate	*Micania micrantha* H.B.K.	ST+RT+LV–M	—	0.50, neg.	neg.	neg.
		ST+RT+LV–E	88	neg.	sl. hypo., tox.	neg.
	Wedelia biflora (L.) DC.	FR–M	—	—	neg.	—
		LV–M	111	—	neg.	neg.
		LV–E	107	2.0, neg.	sl. hypo.	neg.
		RT–M	2.0, 73	—	neg.	neg.
		RT–E	0.25, 133	—	neg.	neg.
		ST–E	1.5, 78	2.0, neg.	sl. hypo.	neg.
Dioscoreaceae	*Dioscorea bulbifera* L.	TBR–Cl	106	neg.	neg.	—
		TBR–E	106	neg.	neg.	Act. F61, F284, F808, F1224, H37Ra, P27

Table I—(*Continued*)

Family	Species	Plant Part[a], Solvent[d]	T/C Columbia SK Virus[c]	30-Day Survivors EA[d]	Rat Arterial Pressure[e]	Microbiology[f]
Euphorbiaceae	*Bischofia javanica* Bl.	LV–M	2.0, 100	—	neg.	neg.
		LV–E	118	—	neg.	neg.
		RT–M	2.0, 128	—	neg.	neg.
		RT–E	0.3, 106	0.4, neg., tox.	fatal (1.0 ml./kg.)	neg.
		ST–M	2.0, 100	—	neg.	neg.
		ST–E	0.3, 118	neg.	fatal (0.5 ml./kg.)	neg.
	Homalanthus nutans (Forst.) Pax.	BK–M	2.0, 89	—	neg.	neg.
		BK–E	2.0, 92	neg., tox.	hypo.	neg.
		LV–M	2.0, 90	—	sl. hypo.	neg.
		LV–E	1.5, 90% tox.	0.5, neg.	sl. hypo.	neg.
		RT–M	2.0, 240[g]	—	neg.	neg.
		RT–E	2.0, 240[g]	—	sl. hypo.	neg.
		ST–M	2.0, 200[g]	—	sl. hypo.	neg.
		ST–E	2.0, 240[g]	0.5, neg.	neg.	neg.
	Macaranga stipulosa, Muell. Arg.	BK–M	112	—	sl. hypo.	neg.
		BK–E	0.2, 125	0.5, neg.	hypo.	neg.
		LV–M	2.0, 88	—	neg.	neg.
		LV–E	88	0.3, neg.	v. hypo., inact. i.p.,	neg.
		RT–M	125	—	neg.	neg.
		RT–E	88	neg.	hypo., inact. i.p.	neg.
		ST–M	67	—	neg.	neg.
		ST–E	0.3, 95	0.3, neg., v. tox.	hypo.	neg.
Flacourtiaceae	*Flacourtia rukam*, Zoll. & Morr.	BK–M	114	2.0, neg.	sl. hypo.	neg.
		BK–E	71	neg.	sl. hypo.	neg.
		FR–M	86	neg.	neg.	neg.
		FR–E	143	neg.	sl. hypo.	neg.
		LV–M	1.5, 112	neg.	neg.	neg.
		LV–E	100	neg.	v. hypo.	neg.
		ST–M	100	neg.	neg.	neg.
		ST–E	114	neg.	neg.	neg.
Guttiferae	*Calophyllum inophyllum* L.	BK–M	2.0, 67	—	—	—
		BK–E	—	—	—	—
		FR–M	2.0, 167	—	—	—
		FR–E	100	2.0, neg., tox.	fatal (1 ml./kg.)	neg.
		LV–M	—	—	neg.	—
		LV–E	107	2.0, neg.	v. hypo.	—
		RT–M	82	—	neg.	—
		RT–E	0.3, 118	—	v. hypo., inact. i.p., act. oral.	neg.
		ST–M	71	—	—	neg.
		ST–E	0.3, 78	—	v. hypo. (0.5 ml./kg.), act. i.p., act. oral, non-α block., non-histaminergic	—
Leguminosae	*Desmodium umbellatum* (L.) D.C.	LV–M	—	—	neg.	neg.
		LV–E	2.0, 143	0.5, neg.	sl. hypo.	neg.
	Erythrina fusca Lour.	BK–M	2.0, 107	—	neg.	act. strep.
		BK–E	150	2.0, neg.	v. hypo., inact. i.p.	neg.
		LV–M	2.0, 280[g]	—	neg.	neg.
		LV–E	2.0, 360[g]	neg.	neg.	neg.
		RT–M	2.0, 320[g]	—	neg.	neg.
		RT–E	240[g]	—	—	—
		ST–M	150	—	sl. hypo.	neg.
		ST–E	2.0, 360[g]	2.0, neg.	sl. hypo.	neg.
	Inocarus fagiferus (Parkins.) Fosb.	BK–M	2.0, 164	—	neg.	neg.
		BK–E	2.0, 127	—	sl. hypo.	neg.
		LV–M	1.5, 164	—	neg.	neg.
		LV–E	2.0, 94	2.0, neg.	v. hypo., inact. i.p.	neg.
		RT–M	2.0, 111	—	neg.	neg.
		RT–E	111	neg.	neg.	neg.
		SD–M	2.0, 127	—	neg.	neg.
		ST–M	2.0, 164	—	neg.	neg.
		ST–E	2.0, 182	—	neg.	neg.
		PER–M	100	—	neg.	neg.
		PER–E	0.3, 111	neg.	neg.	neg.
	Intsia bijuga (Colebr.) O. Ktze	BK–M	111	—	neg.	act. strep.
		LV–M	2.0, 107	—	neg.	neg.
		LV–E	2.0, 120	2.0, neg.	v. hypo., act. i.p., inact. oral	act. strep., P-27, P-39

(*continued*)

Table I—(*Continued*)

Family	Species	Plant Part[a], Solvent[b]	T/C Columbia SK Virus[c]	30-Day Survivors EA[d]	Rat Arterial Pressure[e]	Microbiology[f]
Leguminosae	*Intsia bijuga* (Colebr.) O. Ktze	RT–M	2.0, 67	—	neg.	neg.
		RT–E	1.5, 89	—	neg.	neg.
		ST–M	80	—	neg.	act. staph., strep.
		ST–E	2.0, 100	2.0, neg.	sl. hypo.	neg.
Loganiaceae	*Fagraea berteriana* Benth.	FR+SD–CL	100	2.0, neg.	sl. hypo.	—
		FR+SD–E	111	neg., tox.	v. hypo., act. i.p.	neg.
		ST+RT+LV–CL	111	neg., tox.	neg.	neg.
		ST+RT+LV–E	89	neg.	v. hypo., act. i.p.	neg.
Malvaceae	*Sida rhombifolia* L.	WH.PL.–M	2.0, 89	—	neg.	neg.
		WH.PL.–E	2.0, 89	—	neg.	neg.
	Thespesia populnea (L.) Solander	BK–M	111	—	neg.	neg.
		BK–E	0.75, 118	—	fatal 0.5 ml./kg., neg. 0.1 ml./kg.	neg.
		FR–M	1.5, 106	—	neg.	neg.
		FR–E	106	neg.	neg.	neg.
		LV–M	1.5, 106	—	neg.	neg.
		LV–E	106	0.5 neg.	v. hypo., act. i.p., act. oral, non-α-block., nonhistaminergic	act. salm.
		RT–M	0.5, 106	—	neg.	neg.
		RT–E	0.5, 94	20% surv.[h], tox.	v. hypo., act. i.p., act. oral	neg.
		ST–M	1.5, 94	—	neg.	neg.
		ST–E	—	0.5, 40% surv.[h], tox.	v. hypo., inact. i.p.	neg.
Meliaceae	*Dysoxylum* aff. *Maota* Rein.	BK–M	105	—	neg.	neg.
		BK–E	2.0, 105	—	hypo.	neg.
		LV–M	105	—	neg.	neg.
		LV–E	2.0, 105	neg.	sl. hypo.	neg.
		RT–M	2.0, 70	—	sl. hypo.	neg.
		RT–E	90	—	hypo.	neg.
		ST–M	2.0, 105	—	neg.	neg.
		ST–E	0.5, 105	neg.	sl. hypo.	neg.
Moraceae	*Ficus obliqua* Forst. f.	AR RT BK–M	2.0, 123	—	neg.	neg.
		AR RT BK–E	123	neg., tox.	v. hypo., inact. i.p.	neg.
		AR RT WD–M	2.0, 92	—	neg.	neg.
		AR RT WD–E	1.5, 138	neg.	neg.	neg.
		UG RT–M	2.0, 123	—	neg.	neg.
		UG RT–E	—	0.5 neg., v. tox.	v. hypo., act. i.p., act. oral	neg.
Myrsinaceae	*Maesa samoana* Meg.	BK–M	182	—	neg.	neg.
		BK–E	100% tox.	0.5, 100% tox.	v. hypo., act. i.p.	neg.
		LV–M	125	—	neg.	neg.
		LV–E	133	100% tox.	hypo.	neg.
		RT–M	75	—	neg.	neg.
		RT–E	0.3, 100	—	fatal 1 ml./kg.	neg.
		ST–M	125	—	neg.	neg.
Rhamnaceae	*Colubrina asiatica* (L.) Brongn.	BK–M	70	—	neg.	neg.
		BK–E	0.75, 80	0.5 neg., tox.	hypo.	neg.
		LV–M	2.0, 118	—	neg.	neg.
		LV–E	0.75, 59	0.5 neg., v. tox.	v. hypo. 0.1 ml./kg., act. i.p.	neg.
		RT–M	1.5, 94	—	neg.	neg.
		RT–E	0.5, 80	0.5, 40% surv., tox.	hypo.	neg.
		ST–M	2.0, 80	—	sl, hypo.	neg.
		ST–E	0.50, 100	neg.	hypo.	neg.
Rubiaceae	*Psychotria insularum* A. Gray	LV–M	114	—	neg.	neg.
		LV–E	143	—	sl. hypo. 0.1 ml./kg., tox. 1 ml./kg.	neg.
		ST–M	0.3, 71	—	neg.	neg.
		ST–E	0.3, 100	—	hypo.	neg.
Sterculiaceae	*Kleinhovia hospita* L.	BK–M	2.0, 114	—	neg.	neg.
		BK–E	2.0, 143	—	v. hypo., inact. i.p., inact. oral	neg.
		FR–E	167	—	neg.	neg.
		LV–M	2.0, 78	—	hypo., inact. i.p.	neg.
		LV–E	2.0, 128	neg.	v. hypo. 0.1 ml./kg., inact. i.p., inact. oral	neg.
		RT–M	2.0, 128	—	neg.	neg.
		RT–E	2.0, 114	0.3, neg.	sl. hypo.	neg.

Table I—(*Continued*)

Family	Species	Plant Part[a], Solvent[b]	T/C Columbia SK Virus[c]	30-Day Survivors EA[d]	Rat Arterial Pressure[e]	Microbiology[f]
Sterculiaceae	*Kleinhovia hospita* L.	ST-M	2.0, 280[g]	—	neg.	neg.
		ST-E	2.0, 128	0.5, neg.	sl. hypo.	neg.
	Melochia aristata A. Gray	BK-M	0.3, 125	—	neg.	neg.
		BK-E	0.3, 125	0.5 neg., tox.	v. hypo., fatal 0.1 ml./kg.	neg.
		LV-M	63	—	neg.	neg.
		LV-E	100	0.3 neg., tox.	hypo., tox.	neg.
		RT-M	2.0, 95	—	neg.	neg.
		RT-E	—	0.3, 20% surv.[h], tox. (15)	—	—
		ST-M	2.0, 88	—	neg.	neg.
		ST-E	2.0, 182	0.5, 73% surv.[h], tox. (15)	hypo.	neg.
Verbenaceae	*Clerodendron inerme* (L.) Gaertn.	LV-M	2.0, 145	—	sl. hypo.	act. F1224
		LV-E	2.0, 145	neg.	sl. hypo.	act. staph., strep.
		RT-M	167	—	neg.	neg.
		RT-E	2.0, 120	—	neg.	neg.
		ST-M	2.0, 145	—	neg.	neg.
		ST-E	2.0, 182	neg.	sl. hypo.	neg.
	Premna taitensis Schauer in D.C.	BK-M	2.0, 100	—	neg.	neg.
		BK-E	2.0, 114	1.5 neg.	v. hypo. 0.1 ml./kg.	neg.
		LV-M	1.5, 120	—	sl. hypo.	neg.
		LV-E	2.0, 71	2.0 neg.	v. hypo., inact. i.p.	act. F61, F284, F808, F1224, H37Ra, P27, P39
		RT-M	2.0, 100	—	neg.	neg.
		RT-E	2.0, 100	1.5 neg.	hypo.	neg.
		ST-M	0.3, 164	—	neg.	neg.
		ST-E	2.0, 118	—	sl. hypo.	—

[a] AR RT BK = aerial root bark, AR RT WD = aerial root wood, BL = blades, BK = bark, CRM = corms, LV = leaves, PER = pericarp, PT = petioles, RT = roots, SD = seeds, ST = stems, TBR = tubers, UG RT = underground root, and WH PL = whole plant. [b] M = methylene chloride extract, Cl = chloroform extract, E = 60% aqueous ethanol extract after prior M or Cl extraction. [c] The T/C number is the fraction of treated deaths divided by the fraction of diseased control deaths times 100. The occasional preceding small number indicates the concentration factor as compared to the normal 0.1-ml. dose of full-strength extract. [d] EA = Ehrlich ascites tumor. neg. = no survivors 30 days after injection of 5×10^5 Ehrlich ascites tumor cells intraperitoneally, tox. = one or two out of five mice died of drug toxicity, v. tox. = three or four out of five mice died of drug toxicity, 100% tox. = five out of five mice died of drug toxicity, and (15) = 15 mice in group instead of the usual five. The number preceding the result is the concentration factor as compared to the normal 0.1-ml. dose of full-strength extract. [e] This is based on the intravenous injection of 1 ml./kg. of extract unless stated otherwise. act. i.p. = active using intraperitoneal injection at 10 times the effective intravenous dose, act. oral = active using stomach intubation of 20 times the effective intravenous dose, hypo. = reduces arterial blood pressure by >20% for 5–30 min., neg. = no appreciable effect on arterial blood pressure, neg. i.p. = no appreciable effect when administered at 10 times the effective intravenous dose, non-α-block. = normal pressor response seen when animal was challenged with 3 mcg./kg. epinephrine while blood pressure depressed from extract, non-histaminergic = action of hypotensive extract unchanged when given after pretreatment with 10 mg./kg. diphenhydramine, sl. hypo. = reduces arterial blood pressure >20% for <5 min., and v. hypo. = reduces arterial blood pressure by >20% for >30 min. [f] neg. = no inhibition of growth of bacteria compared to no drug standard, act. = obvious inhibition of growth of bacteria, strep. = *Streptococcus pyogenes*, staph. = *Staphylococcus aureus* (penicillin resistant), shig. = *Shigella flexneri*, F61 = *Mycobacterium avium*, F284 = *M. kansasii*, F808 = *M.* sp. (Battey type), F1224 = *Nocardia* sp., H37Ra = *M. tuberculosis*, P27 = *M.* sp. scotochromogen, and P39 = *M.* sp. (Battey type). [g] Controls only 5/20 died. [h] All nonascitic.

EXPERIMENTAL

Extraction—The dried plants received by air from Western Samoa were powdered in a Wiley mill. About 100–300 g. of the powdered sample was suspended in 5–10 times its weight of methylene chloride, the amount of solvent necessary to impart fluidity to the mixture. In a few cases, chloroform was used instead of methylene chloride. After standing for about 24 hr., the mixture was filtered and the marc was washed several times with additional chlorinated solvent. The combined filtrate was evaporated to dryness at room temperature, and the residue was mixed with approximately 300 ml. of 95% ethanol. This mixture was then evaporated at ≦50° to a volume (in milliliters) equal to one-tenth of the weight (in grams) of the original sample; it was then diluted with nine volumes of water to give a 10% ethanolic emulsion, called the "methylene chloride extract."

The marc was air dried at room temperature and then suspended in 5–10 times its weight of 60% ethanol, the amount of solvent necessary to impart fluidity to the mixture. After standing for 24 hr., the mixture was filtered and the marc was washed several times with additional 60% ethanol. The combined filtrates were evaporated at ≦50° to a volume equal (in milliliters) to the original weight (in grams) of the plant sample. The resulting extract was called the "aqueous ethanol extract."

All extracts were stored frozen and thawed just before use.

Columbia SK Virus—Five-week-old Swiss-Webster mice were inoculated intraperitoneally with Columbia SK virus (brain adapted) of LD_{70-90} titer (1). The infected controls were in groups of 20 and the treated mice were in groups of 10 for each drug. Two hours after the virus inoculation, 0.1 ml. of the extract was inoculated subcutaneously. All extracts were first checked for acute toxicity at 0.1 ml. If toxic at this level, dilutions were made to achieve the maximum nontoxic dose. This latter dose was injected subcutaneously for a total of 4 days (eight doses). The mice were observed for another 5 days after termination of the treatment. The T/C listed in Table I is the percent mortality of the treated mice divided by the percent mortality of the controls, multiplied by 100. When enough sample was available, experiments that gave T/C values below 75 were rerun for confirmation.

Ehrlich Ascites Tumor—Five-week-old Swiss-Webster mice were injected intraperitoneally with 5×10^5 Ehrlich ascites tumor cells. The experiments were carried out as previously reported (2). One-tenth milliliter of extract was checked for acute toxicity. If 0.1 ml. of extract was acutely toxic, dilutions of the extract were made to determine a maximum nontoxic dose. Intraperitoneal injections of the maximum nontoxic dose to groups of five mice were started 24 hr. after Ehrlich ascites tumor cell inoculation and were continued twice daily for 10 days. The mice were observed daily for 30 days after Ehrlich ascites tumor inoculation. Groups of 10 diseased controls always died by the 21st day, with a mean survival

time of about 17 days. A drug was considered active if there were any survivors at 30 days.

Systemic Effects—Wistar-derived (MW-3) male rats (300–500 g.) were anesthetized with sodium pentobarbital[2] (50 mg./kg.) and connected to an eight-channel polygraph[3]. The following parameters were recorded: electrocardiogram (lead I), electroencephalogram (parietal skin leads), heart rate (tachograph driven by the output of the electrocardiogram channel), arterial and intragastric pressures (liquid pressure transducers[4] connected by cannulas to a femoral artery, and the stomach, *via* the esophagus, respectively), respiration (low pressure air transducer[5] connected to the trachea by a cannula), and respiratory rate (tachograph driven by the output of the respiration channel). Sodium heparin[6] (10 mg./kg.), the extracts, and drugs were given intravenously through a cannula inserted into a femoral vein.

In those cases where the extracts depressed the blood pressure, preliminary studies on the characteristics for the hypotensive action were begun. These consisted of testing the extract by other routes (oral and/or intraperitoneal) against the antihistamine diphenhydramine hydrochloride[7] (10 mg./kg.), challenging with epinephrine bitartrate[8] (3 mg./kg.), and boiling the extract for 5 min. prior to injection.

Inhibition of Bacterial Growth—The qualitative evaluation for antibacterial activity of the extracts was conducted using a modification of the small tube method of Catalfomo and Schultz (3). Cultures of mycobacteria and nocardia were maintained on Bushnell's poi agar, while all other bacteria were maintained on Bacto stock culture agar. The mycobacteria and nocardia were inoculated into Bacto dubos broth and allowed to grow for approximately 2 weeks at 37°. All other organisms were inoculated into BBL trypticase soy broth and incubated at 37° for approximatey 24 hr. These suspensions were used as the inocula for testing.

Each extract was centrifuged at approximately $30,000 \times g$ for 20 min. The supernate was then passed through a filter unit[9] equipped with a 0.45-μ filter[10] to obtain a sterile extract. Two drops of the sterile extract was added to 0.5 ml. of the desired melted medium at 45° in 17 × 60-mm. screw-capped vials. The vials were then allowed to cool in a slanted position. The mycobacteria and nocardia were grown in Bacto dubos agar, while all others were grown in BBL trypticase soy agar. After cooling, 1 drop of the liquid culture of the test bacterium was added to each vial. Aseptic technique was used throughout. The test vials were incubated at 37° for up to 2 weeks or until the control had grown. To determine growth, cultures were examined with a binocular dissecting microscope. All extracts that showed no growth were retested for confirmation of activity. In the retests, the pH of the extract was also adjusted to 7.0 to detect any effect of pH change. The organisms employed were: F21, *Mycobacterium smegmatis;* F47, *M. kansasii,* F61, *M. avium;* F284, *M. kansasii;* F808, *M.* sp., Battey type; F1224, *Nocardia* sp.; H37Ra, *M. tuberculosis;* P27, *M.* sp., scotochromogen; P39, *M.* sp., Battey type; R11, *M.* sp., from dairy; T3, *M.* sp., from patient; T122, *M.* sp., from bovine feces; OH9BA, *Escherichia coli,* pathogenic serotype; *Pseudomonas aeruginosa*[11]; *Salmonella typhimurium*[11], *Shigella flexneri*[11]; *Staphylococcus aureus,* penicillin resistant[11]; and *Streptococcus pyogenes*[11].

[2] Robinson.
[3] Grass model 7.
[4] Statham P23dc.
[5] Grass PT5A.
[6] City Chemical Corp.
[7] Tiffany Chemical Co.
[8] Winthrop Labs.
[9] Swinnex 13.
[10] Millipore HA.

RESULTS AND DISCUSSION

The results of the pharmacological tests are shown in Table I. None of the 211 extracts showed any significant inhibition of Columbia SK virus. However, 12 extracts showed inhibition of bacterial growth *in vitro* and six inhibited Ehrlich ascites tumor growth in mice. Ninety-one of the 211 extracts caused hypotensive effects; some were quite potent, lowering the blood pressure by as much as 70%, with the effects lasting for several hours. Four of the extracts produced a general systemic toxicity.

Because some species produced more than one active extract (probably because the active agent was present in more than one tissue of the plant), it is more meaningful to speak in terms of the number of species yielding active extracts. On this basis, 19 of the 34 species tested contained hypotensive substances worthy of further study (produced hypotension for more than 30 min.).

There were no clear cut correlations between local medicinal use and the pharmacological results reported here. A report of the ethnobotanical study will be made later.

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▲ To whom inquiries should be directed.

[11] Stock cultures on hand in the Tropical Medicine Department, University of Hawaii.

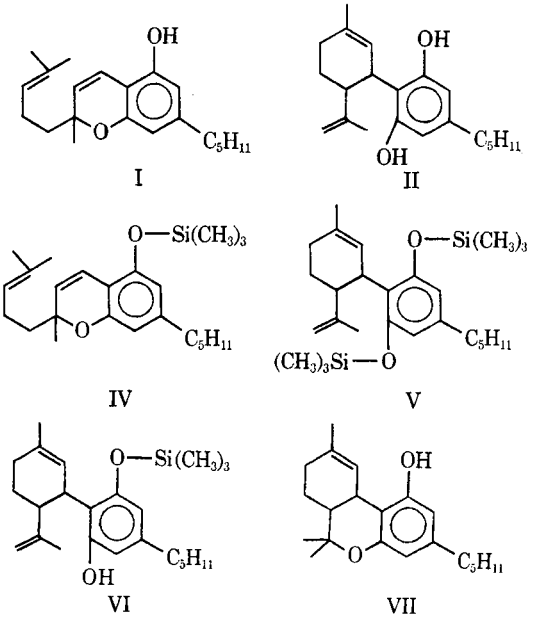
Constituents of *Cannabis sativa* L. III: Clear and Discrete Separation of Cannabidiol and Cannabichromene

CARLTON E. TURNER▲ and KATHY W. HADLEY

Abstract □ Synthetic cannabidiol and cannabichromene were discretely separated by GLC using their trimethylsilyl ether derivatives. The mono and disilylated derivatives of cannabidiol were identified. This procedure was utilized in the analysis of *Cannabis sativa* L.

Keyphrases □ *Cannabis sativa* L. constituents—separation of synthetic cannabidiol and cannabichromene using trimethylsilyl ether derivatives □ Cannabidiol—GLC separation from cannabichromene □ Cannabichromene—GLC separation from cannabidiol □ GLC—separation, cannabidiol and cannabichromene

Gaoni and Mechoulam (1) reported the separation of cannabichromene and cannabidiol by conventional GC techniques. However, this separation was based on retention times having only a 5-sec. time differentiation. With the identification of the propyl homologs by Vollner *et al.* (2), Gill *et al.* (3), and Merkus (4), there exists the possibility that, when used alone, GC analysis of cannabis constituents could lead to erroneous interpretations. Moreover, Vree *et al.* (5) showed that the propyl homolog of cannabinol, cannabivarin, was often found under the peak of a gas chromatogram normally attributed to cannabidiol or cannabichromene. These investigators were using hashish samples from Brazil and Lebanon; however, Fetterman and Turner (6) and Turner and Hadley (7) found propyl homologs in aerial parts of freshly harvested Indian *Cannabis sativa* L. grown in Mississippi. Additionally, they observed that cannabichromene was often reported as cannabidiol when GC was used as the only method for analysis (7). Accurate identification could, however, be obtained by using TLC and GC combined with a high-resolution mass spectrometer. The questioned validity of using only GC for the analysis of cannabidiol, cannabichromene, and cannabivarin prompted this investigation.



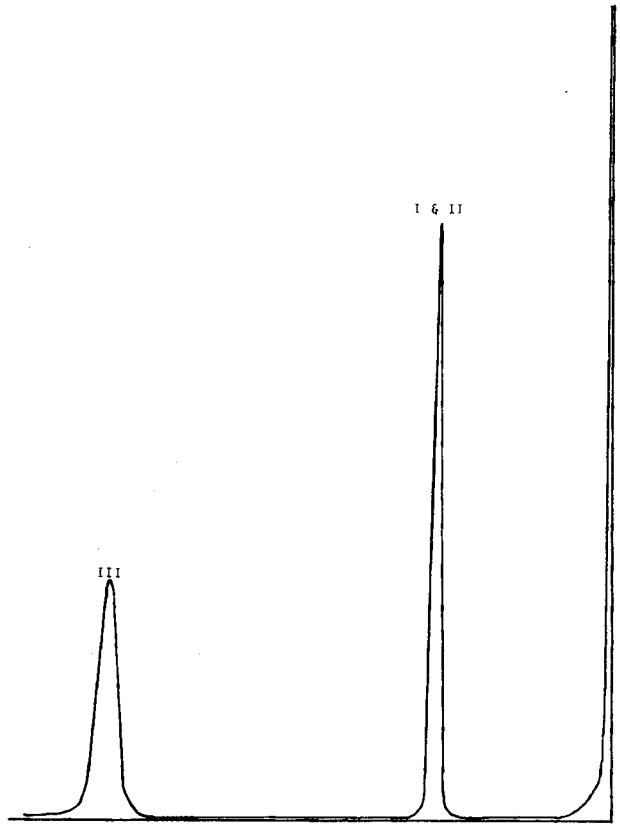


Figure 1—*Gas chromatogram of unsilylated cannabichromene (I), cannabidiol (II), and 4-androstene-3,17-dione, the internal standard (III).*

METHODS[1]

GC Analysis—Analyses were performed using gas chromatographs[2] equipped with hydrogen flame-ionization detectors and operated isothermally at 210°. The inlet temperature was 240° and the detector temperature was 260°. Glass columns [0.6-cm. (0.25-in.) o.d., 2-mm. i.d., × 2.4 m. (8 ft.)] were packed with 2% OV-17[3] on 100–120-mesh Chromosorb WHP. Nitrogen was used as the carrier gas at a flow rate of 10–16 ml./min., depending upon the instrument used.

[1] Authentic synthetic samples of cannabidiol and cannabichromene were obtained from the National Institute of Mental Health (NIMH). Relative retention times reported are relative to the internal standard 4-androstene-3,17-dione.

[2] Beckman GC-45 and GC 72-5.

[3] High purity polar phenyl methyl silicone of approximately 30,000 mol. wt. Packed by Beckman Instruments Co.

Table I—GC Relative Retention Times of Cannabinoids

Cannabinoid	Relative Retention Time
Cannabidiol bis(trimethylsilyl) ether	0.11
Cannabichromene trimethylsilyl ether	0.17
Cannabidiol mono(trimethylsilyl) ether	0.18
(−)-Δ^9-*trans*-Tetrahydrocannabinol trimethylsilyl ether	0.22
Cannabidiolic acid trimethylsilyl ester–bis(ether)	0.28
Cannabichromene	0.34
Cannabidiol	0.34
(−)-Δ^9-*trans*-Tetrahydrocannabinolic acid trimethylsilyl ester–ether	0.64
4-Androstene-3,17-dione	1.00

Silylation of Synthetic Cannabinoids—Five milligrams each of cannabidiol and cannabichromene was added to 0.2 ml. of anhydrous pyridine contained in a 50-ml. round-bottom single-neck flask. The pyridine solution was then subjected to continuous vibration from an ultrasonic vibrator until the cannabinoids were in solution. Then 0.3 mg. of the internal standard was added *via* a 10:1 pyridine–steroid solution. At this point, 0.4 ml. of *N,O*-bis(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane[4] was added. The resulting reaction mixture was heated, using a heating mantle, for approximately 10 min. at 80°. Then 0.1 μl. of the reaction mixture was injected into the gas chromatograph.

Silylation of Plant Extract—A 1-g. sample was extracted with 40 ml. of spectrograde chloroform. The resulting solution was refrigerated at 6° and shaken at 10-min. intervals for 1 hr. The plant material then was removed by filtration, and the mother liquor was concentrated *in vacuo* at ambient temperature to a greenish paste void of solvent.

Anhydrous pyridine, 0.5 ml., was added, followed by continuous vibration from an ultrasonic vibrator until all resin was in solution. At this point, 0.5 ml. of *N,O*-bis(trimethylsilyl)-trifluoroacetamide[4] with 1% trimethylchlorosilane was added. The resulting reaction mixture was then processed as described previously for the silylation of synthetic cannabinoids. Unsilylated plant samples of *C. sativa* L. were analyzed according to the literature procedure (7).

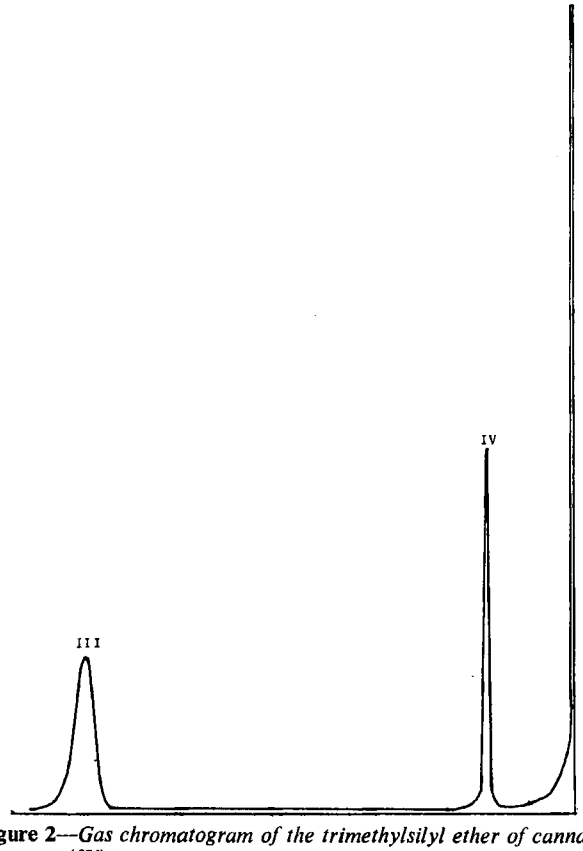


Figure 2—*Gas chromatogram of the trimethylsilyl ether of cannabichromene (IV).*

[4] BSTFA with 1% TMCS, Pierce Chemical Co.

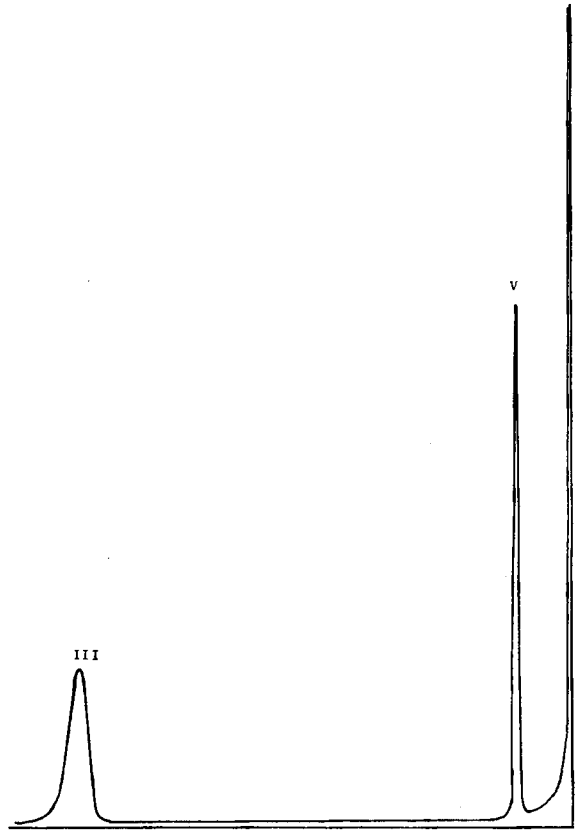


Figure 3—*Gas chromatogram of bis(trimethylsilyl) ether of cannabidiol (V).*

RESULTS AND DISCUSSION

The structures of natural and synthetic cannabichromene (I) and cannabidiol (II) are well known (1). Individual analysis of each of these cannabinoids is carried out routinely; however, when a sample contains both cannabidiol and cannabichromene (Fig. 1), no clean separation is obtained. This presents many problems to the researcher trying to ascertain if the sample contains a mixture of the two or is a pure sample of either. Claussen *et al.* (8) used the trimethylsilyl ether–ester derivatives to separate the free phenols from the carboxylic acid derivatives of some cannabinoids. Fetterman *et al.* (9) used a similar procedure to analyze for (−)-Δ^9-*trans*-tetrahydrocannabinolic acid. Claussen *et al.* (8) were unable to identify many peaks in the chromatogram obtained, whereas Fetterman *et al.* (9) used fresh marijuana having a very high concentration of (−)-Δ^9-*trans*-tetrahydrocannabinolic acid (approximately 90–97%) and a very small content of (−)-Δ^9-*trans*-tetrahydrocannabinol. Additionally, Davis *et al.* (10) described a silylated extract of cannabis. These three procedures are excellent but do not approach the problem of separating cannabidiol and cannabichromene as their free phenols.

A clean separation has been obtained by using the trimethylsilyl ethers of synthetic and natural cannabichromene and cannabidiol. Figure 1 is a chromatogram of equal parts (2 mg.) of synthetic cannabichromene and cannabidiol, analyzed as described under the *GC Analysis* section. The relative retention time of both compounds when compared to the internal standard 4-androstene-3,17-dione (III) is 0.34. Figure 2 is a chromatogram of the trimethylsilyl ether of synthetic cannabichromene (IV) having a relative retention time of 0.17, and Fig. 3 is a chromatogram of the bis(trimethylsilyl) ether of cannabidiol (V) having a relative retention time of 0.11.

Several factors affect the efficiency of silylation. The most common is incorrect handling of the sample. No solvents having an

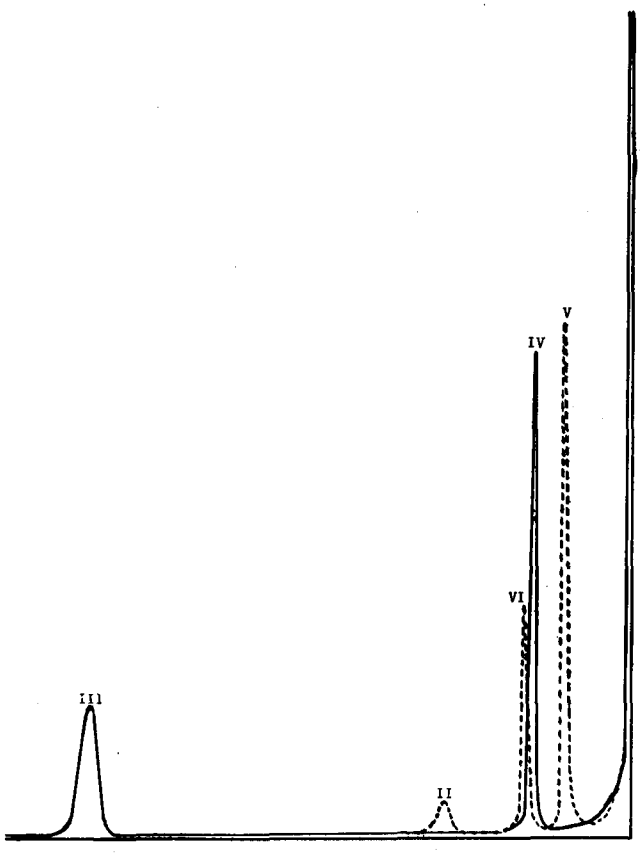


Figure 4—*Overlay of incompletely silylated cannabidiol and silylated cannabichromene, showing cannabidiol (II), mono(trimethylsilyl) ether of cannabidiol (VI), trimethylsilyl ether of cannabichromene (IV), and bis(trimethylsilyl) ether of cannabidiol (V). Key: ——, silylated synthetic cannabichromene; and - - -, products of incompletely silylated synthetic cannabidiol.*

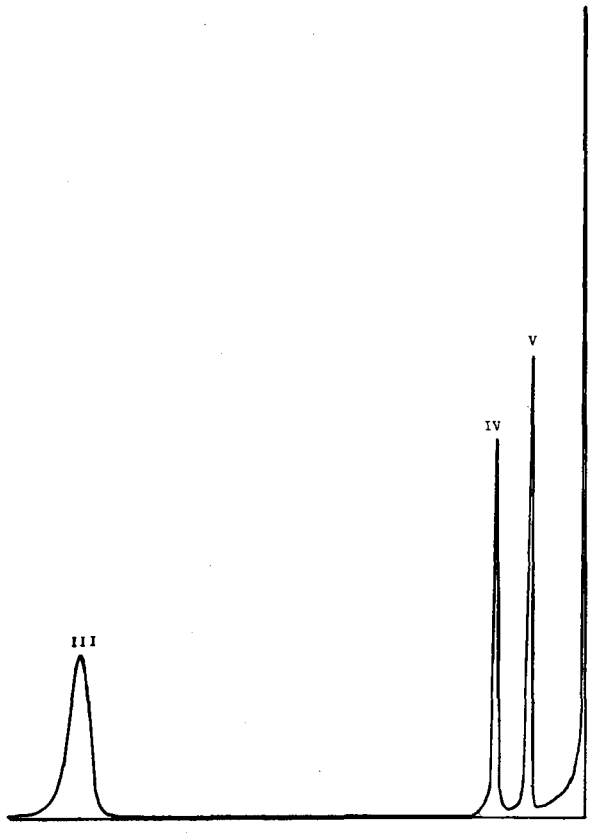
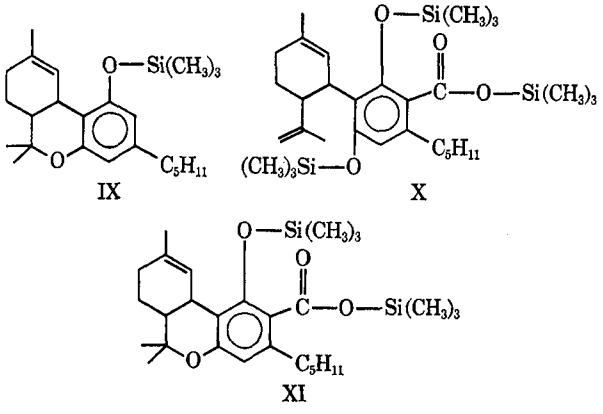


Figure 5—*Chromatogram of the trimethylsilyl ether of cannabichromene (IV) and the bis(trimethylsilyl) ether of cannabidiol (V).*

easily ionizable proton should be used. The silylation should be carried out with freshly obtained anhydrous solvents or solvents stored over drying agents such as molecular sieves.

Incomplete silylation can also be caused by an insufficient reaction time, too weak a silylating reagent, and/or an insufficient amount of silylating solution. For best results, an excess of the silylating solution should be added. Figure 4 is an overlay of an insufficient amount of a silylating solution being added to an equal weight of cannabidiol and cannabichromene. The solid line represents the trimethylsilyl ether of cannabichromene (IV), and the broken line represents silylated products of cannabidiol and free cannabidiol. The peak labeled II is unsilylated cannabidiol. The peak labeled VI is the mono(trimethylsilyl) ether of cannabidiol, and the peak labeled V is the bis(trimethylsilyl) ether of cannabidiol.

With the addition of a sufficient amount of the silylating solution, II and VI disappear. Synthetic cannabichromene and cannabidiol



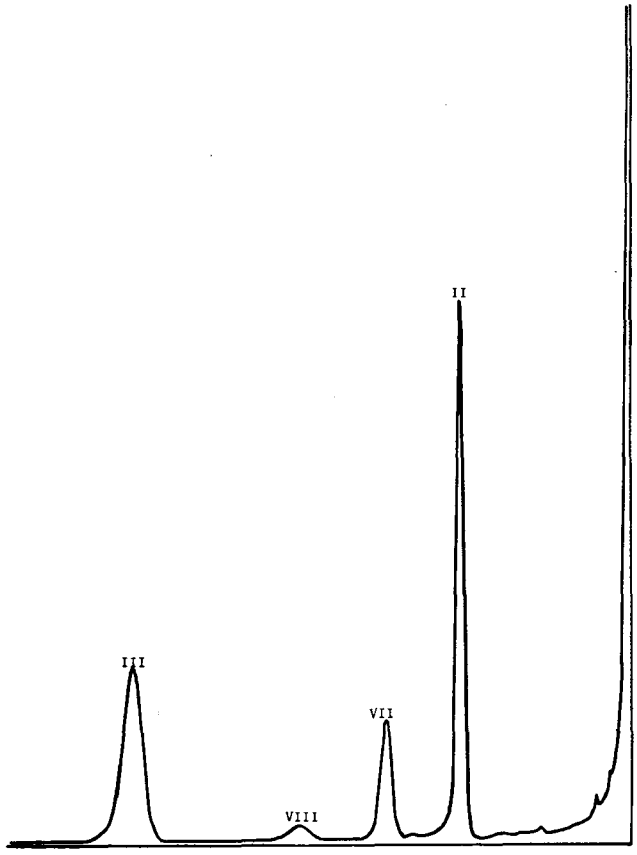


Figure 6—*Chromatogram of unsilylated Turkish (TU-A) C. sativa L., showing cannabidiol (II), (—)-Δ⁹-trans-tetrahydrocannabinol (VII), and unknown constituent (VIII).*

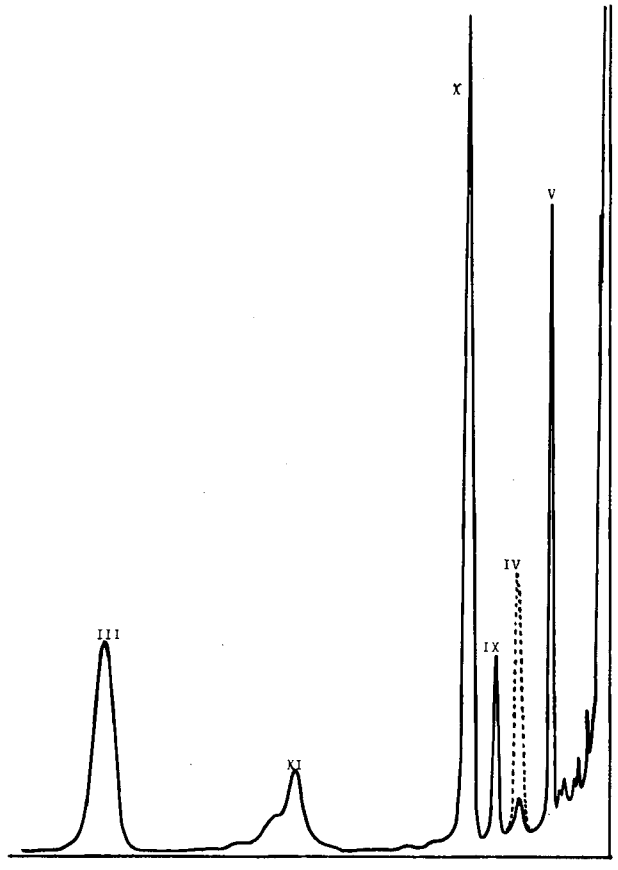


Figure 7—*Overlay of silylated synthetic cannabichromene and Turkish (TU-A) C. sativa L., showing bis(trimethylsilyl) ether of cannabidiol (V), trimethylsilyl ether of cannabichromene (IV), trimethylsilyl ether of (—)-Δ⁹-trans-tetrahydrocannabinol (IX), trimethylsilyl ester–bis(ether) of cannabidiolic acid (X), and trimethylsilyl ester–ether of (—)-Δ⁹-trans-tetrahydrocannabinolic acid (XI). Key: ——, silylated Turkish variant; and - - -, silylated Turkish variant plus synthetic cannabichromene.*

can be discretely separated when a slight excess of silylating solution is used (Fig. 5). Care must be taken, however, to ensure complete silylation since the mono(trimethylsilyl) ether of cannabidiol (Fig. 4) has a relative retention time of 0.18, very near that of the cannabichromene trimethylsilyl ether at 0.17 (Table I).

To illustrate the versatility and application of this procedure, a sample of Turkish *C. sativa* L. was analyzed (Fig. 6). Regular cannabis analysis decarboxylates cannabinoid acids to their free phenol. Thus, cannabidiol (II) and (−)-Δ⁹-*trans*-tetrahydrocannabinol (VII), along with an unknown constituent (VIII), are shown in Fig. 6. The unknown constituent is under further investigation at this time. Without close observation, VIII would normally be called cannabinol. However, cannabinol has a relative retention time of 0.63, whereas VIII is located at 0.67. Both cannabinol and VIII occur in some plant samples, particularly Mexican. Usually VIII appears as a shoulder immediately after cannabinol.

Silylated cannabinoids from a Turkish variant of *C. sativa* L. are shown in Fig. 7. The bis(trimethylsilyl) ether of cannabidiol is V. Peak IV is the trimethylsilyl ether of cannabichromene. The broken line represents a cannabichromene-enriched plant sample. Peak IX is the trimethylsilyl ether of (−)-Δ⁹-*trans*-tetrahydrocannabinol, and X is the trimethylsilyl ester–bis(ether) of cannabidiolic acid. The trimethylsilyl ester–ether of (−)-Δ⁹-*trans*-tetrahydrocannabinolic acid (XI) is followed by a shoulder of an unknown component.

Thus, trimethylsilyl derivatives of cannabinoids can be used to differentiate if a plant sample of *C. sativa* L. contains cannabidiol, cannabichromene, or a mixture of the two. However, caution must be observed to ensure complete silylation, and/or the absence of the internal standard will lead to erroneous results.

SUMMARY

Synthetic cannabidiol and cannabichromene are separated as their trimethylsilyl ethers. The mono(trimethlysilyl) ether of cannabidiol is identified. The procedure described is applicable to samples of *C. sativa* L. plant material when the defined conditions are observed. Therefore, it is possible to determine qualitatively, using GC, if a sample of cannabis contains cannabichromene, cannabidiol, or a mixture of the two.

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Kinetics and Mechanisms of Action of Drugs on Microorganisms XVI: Effect of Oleandomycin and Its Combinations with Tetracycline, Chloramphenicol, Erythromycin, Lincomycin, and 1′-Demethyl-4′-depropyl-4′-(R and S)-*n*-pentylclindamycin on Microbial Generation of *Escherichia coli*

CHONG MIN WON and EDWARD R. GARRETT▲

Abstract □ The steady-state generation of *Escherichia coli* culture is inhibited by oleandomycin to a new initial steady state with a new generation rate constant, k_{app}, which is linearly related to drug concentrations in the low concentration range. At higher concentrations, a saturation model is implied in which drug molecules bind to a limited number of receptor sites and the k_{app} asymptotically approaches zero with infinite concentration. Oleandomycin demonstrates another subsequent action in that cell division is inhibited and the oleandomycin-affected cells show increased size with generation rates that are less than the initial effect. The antimicrobial activity of oleandomycin decreases with the nutrient concentrations of Medium 3 USP or casamino acid–glucose–buffer salt medium (Anton's medium), probably because of the binding of drug molecules to the proteins and casamino acids in the broth media. The activity of the drug increases with the pH of the media. This is consistent with the postulate that only the unprotonated fraction of the drug concentration contributes to the activity. Combinations of oleandomycin or erythromycin with tetracycline are synergistic while those with chloramphenicol, erythromycin, or lincomycin are indifferent. These phenomena are evidenced by the decreased or invariant k_{app} values of *a priori* equipotent concentrations.

Keyphrases □ Antimicrobial activity—effect of oleandomycin and combinations with other antibiotics on *Escherichia coli* generation, kinetics □ Oleandomycin and combinations with other antibiotics—effect on *Escherichia coli* generation, kinetics □ *Escherichia coli* generation—effect of subinhibitory oleandomycin and combinations with other antibiotics, kinetics □ Antibiotics—effect of oleandomycin and combinations with other antibiotics on *Escherichia coli* generation, kinetics

Oleandomycin (1) is a medium-spectrum antibiotic with an antibacterial spectrum similar to that of erythromycin and other macrolides (1, 2). Its action is predominantly bacteriostatic (3) and, like other macrolide antibiotics, it inhibits the binding of radioactive chloramphenicol to bacterial ribosomes *in vitro* (4), which suggests that the antibiotic also acts on the 50S ribosomal subunit. Oleandomycin, like erythromycin and lincomycin, shows the same differential effect as chloramphenicol on proline, lysine, and phenylalanine incorporation in cell-free systems (5). This implies that its action in protein synthesis may be at the same site as chloramphenicol.

Oleandomycin has been extensively used as a 1:2 mixture with tetracycline, and synergism has been claimed for this combination (6–8). However, this opinion has been denied by others (9–13).

The effects of drugs can be quantified and insight into their mechanisms of action can be gained by monitoring the generation rate of microorganisms in the presence of varied subinhibitory concentrations (14). This paper presents the quantitative kinetic expressions for microbial generation when inhibited by oleandomycin as functions of drug concentrations, inoculum size, pH, and compositions of the nutrient media. Microbial kinetics have served as a proper tool for the appropriate classification of the actions of drug combinations (14). This paper also presents studies on the evaluation of drug combinations containing oleandomycin.

EXPERIMENTAL

Organism—Replicate slants of *Escherichia coli* (ATCC 12407) were used in all experiments. The slants were prepared from a single colony and were stored in a refrigerator at 4°.

Culture Media—Bacto Antibiotic Medium 3[1] was rehydrated to Medium 3 USP according to the specifications of the manufacturer. The media were filtered and autoclaved as described previously (14). The pH of the media was 7.05 ± 0.05 with the exception of those used to study the antimicrobial activity as a function of pH. To obtain media with a pH in the range of 6.30–7.55, various amounts of concentrated hydrochloric acid or sodium hydroxide solutions were added aseptically to the culture media. To study the effects of various components of culture medium, Anton's medium (15), which had 10% acid-hydrolyzed casein (Bacto Casamino Acids[1]) in Davis's medium, was also used.

Antibiotics—The references to concentrations of oleandomycin phosphate[2] (775 mcg. base eq. mg.$^{-1}$) throughout this paper refer to this phosphate salt. Samples of tetracycline hydrochloride USP[3], lincomycin hydrochloride[3] (895 μg. base eq. mg.$^{-1}$), 1′-demethyl-4′-depropyl-4′-(R and S)-*n*-pentylclindamycin hydrochloride[3,4] (I) (pure), erythromycin lactobionate[5] (670 μg. base eq. mg.$^{-1}$), and chloramphenicol[6] (purity grade) were also used.

Inoculation and Generation—An aliquot (5 ml.) of culture medium was inoculated from a fresh slant, and the culture was allowed to generate for 15 hr. at 37.5° in an incubator. A sample of 0.5 ml. was then diluted into 49.5 ml. broth. The generation of the culture was followed up to 2×10^7 *E. coli*/ml. This culture was then finally diluted 100-fold into a bulk amount of broth. The inoculated broth, contained in a flask fitted with 49.5-ml. delivery head, was then shaken and kept in an incubator at 37.5° for 15 min. Aliquots (49.5 ml.) of the inoculated broth were then aseptically transferred into replicate 125-ml., loosely capped conical flasks. The cultures were maintained at 37.5 ± 0.01° in a 189.27-l. (50-gal.) constant-temperature water bath with constant shaking. All pipets and media used for the dilutions of the cultures were prewarmed to prevent temperature shocks on the organisms.

Total Count Method—Samples of 1.00 or 0.50 ml. were withdrawn at 25-min. intervals and diluted into an appropriate amount (24 or 49 ml.) of diluent in such a way that the obtained counts were less than 100,000 counts/50 μl. on the Coulter counter[7], which was

[1] Difco Laboratories, Detroit, Mich.
[2] Pfizer Inc., Brooklyn, N. Y.
[3] The Upjohn Co., Kalamazoo, Mich.
[4] Referred to in previous manuscripts as U-24,729A.
[5] Abbott Laboratories, North Chicago, Ill.
[6] Thomae GMBH, Germany.
[7] Model ZBI, Coulter Electronics Inc., Hialeah, Fla.

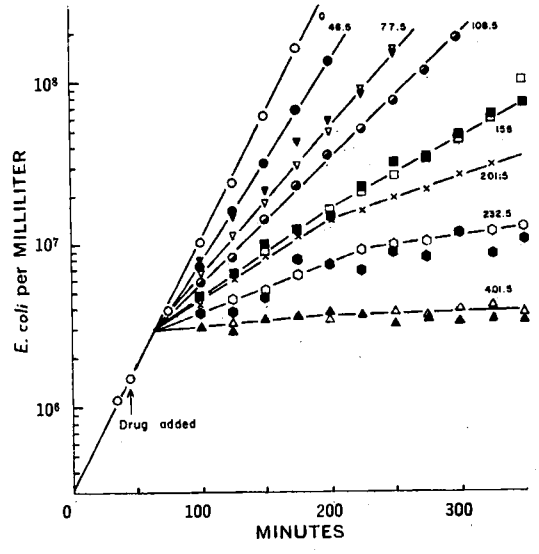

Figure 1—*Typical generation curves of* E. coli *in Medium 3 USP at pH 7.05 and 37.5° in the absence and presence of various concentrations of oleandomycin phosphate, obtained by total (○●▽○□×○△) and viable (▼■○▲) counts. The curves are labeled according to the oleandomycin phosphate concentration in micrograms per milliliter.*

equipped with a 30-μ orifice. The coincidence corrections for the counter were made by programming in a calculator[8] in accordance with the coincidence correction chart supplied in the manufacturer's manual. The diluent used was a filtered[9] aqueous solution of 0.85% NaCl and 1% formaldehyde. The total counts were also corrected for the background count of the particular batch of medium used, diluted in the same way as the sample. The average background counts were normally 400/50 μl. Semilogarithmic plots of *E. coli* per milliliter total counts against time for various oleandomycin phosphate concentrations in Medium 3 USP are given in Fig. 1.

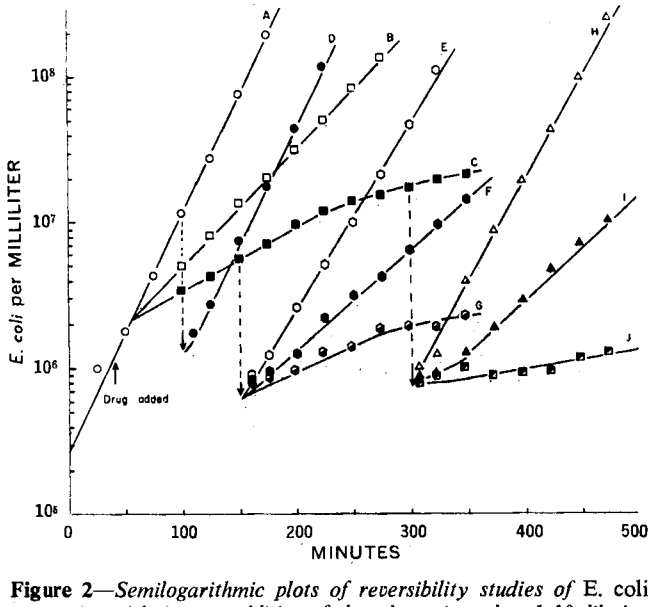

Figure 2—*Semilogarithmic plots of reversibility studies of* E. coli *generation with time on addition of oleandomycin and on 1:10 dilution of culture with broth. Curve A is drug-free culture. Curves B and C are after addition of oleandomycin phosphate to achieve final concentrations of 101 and 202 mcg./ml., respectively. Curve D is after 1:10 dilution of curve A culture. Curves E, F, and G and curves H, I, and J are after 1:10 dilution of curve C at 150 and 300 min., respectively, with broth containing oleandomycin. Curves E and H, curves F and I, and curves G and J had final concentrations of 21, 101, and 202 mcg./ml. oleandomycin phosphate, respectively.*

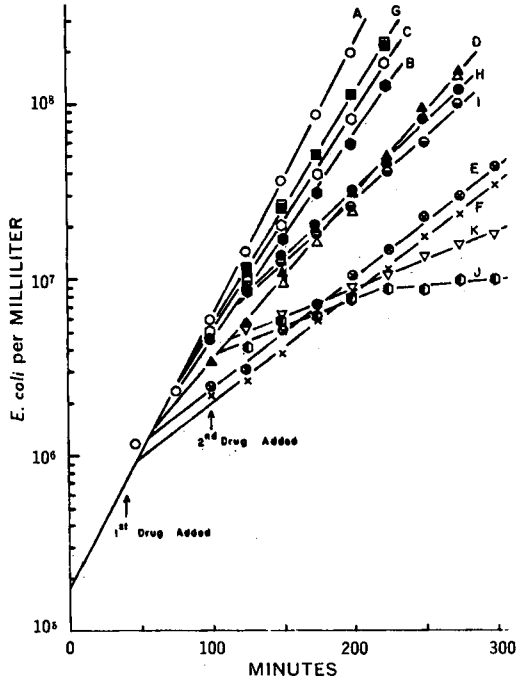

Figure 3—*Effects of order of addition of equipotent oleandomycin and tetracycline on generation rates of* E. coli *in Medium 3 USP at 37.5°. Curve A is for the generation of* E. coli *in the absence of drug. Curves B and C are for the generation in the presence of 38 mcg./ml. oleandomycin phosphate and 0.034 mcg./ml. tetracycline, respectively. Curve G is when equipotent tetracycline is added (■) to the oleandomycin-affected culture or oleandomycin is added (□) to the tetracycline-affected culture so that the final concentrations are equipotent with those of curves B and C. Curve D is for the generation in the presence of 86 mcg./ml. oleandomycin phosphate (△) or 0.076 mcg./ml. tetracycline (▲). Curves H and I are when equipotent tetracycline is added to the oleandomycin-affected culture and equipotent oleandomycin is added to the tetracycline-affected culture, respectively, so that the final concentrations are equipotent with that of curve D. Curves E and F are for the generation in the presence of 152 mcg./ml. oleandomycin phosphate and 0.15 mcg./ml. tetracycline, respectively. Curves J and K are when equipotent tetracycline is added to the oleandomycin-affected culture and equipotent oleandomycin is added to the tetracycline-affected culture, respectively, so that the final concentrations are equipotent with those of curves E and F.*

Viable Count Method—Samples of 0.50 ml. were withdrawn from the cultures and appropriately diluted into sterilized 0.85% saline solution in accordance with a preplanned dilution scheme so that 60–80 colonies per plate would result. From these dilutions, aliquots of 1.00 ml. were pipeted onto each of three replicate agar plates. The plates were incubated for 48 hr. at 37.5° and the colonies were counted on a colony counter[10]. Semilogarithmic plots of *E. coli* per milliliter viable counts for various oleandomycin phosphate concentrations in Medium 3 USP are given in Fig. 1.

Cell Size Analyses—The size-frequency distributions of the cells of *E. coli* in the absence or presence of oleandomycin were studied using the Coulter counter[7] in conjunction with a Channelyzer[11] and plotter[11]. The instruments were calibrated with polyvinyl toluene latex beads of 3.49 mean diameter. Two replicate 49.5-ml. samples of culture generating in logarithmic phase were treated with oleandomycin phosphate solution to give final concentrations of 31 and 77.5 mcg./ml. Samples were taken every 30 min., and size distributions as well as total counts were obtained. A culture generated in the absence of the drug was studied as a control.

Effect of Antibiotic Concentrations on Generation Rates—Fresh solutions of the respective antibiotics were aseptically prepared for

[8] Wang 700.
[9] Millipore 0.45-μ HA.

[10] Model C-110, New Brunswick Scientific Co., New Brunswick, N. J.
[11] Coulter Electronics Inc., Hialiah, Fla.

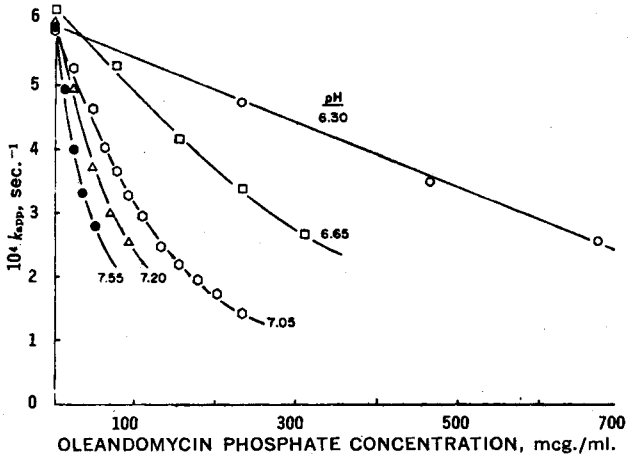

Figure 4—*Dependence of the apparent first-order generation rate constant,* k_{app}, *for* E. coli *in Medium 3 USP on oleandomycin phosphate concentration at various pH values at 37.5°.*

each experiment. They were sufficiently diluted so that aliquots of 0.50 ml. added to the 49.5-ml. culture volumes yielded the desired oleandomycin phosphate concentrations. The drug solutions were added to the cultures generating at 37.5° in the logarithmic phase at predetermined organism concentrations. Samples were withdrawn every 25 min. and counted by the Coulter counter. One culture without drug was studied in each experiment to obtain a control generation rate constant.

Effect of Inoculum Size on Drug-Affected Generation Rates—An aliquot of a culture in logarithmic phase containing 2×10^7 *E. coli*/ml. was diluted 1000-fold into a bulk amount of broth. The inoculated bulk broth was then shaken and kept in an incubator at 37.5° for 15 min. Aliquots (49.5 ml.) of the inoculated broth were aseptically transferred into one control and three sets of five replicate flasks. The drug solutions were added to each set when the organism concentrations in the control flask reached 1.8×10^6, 1.8×10^6, and 1.1×10^7/ml.

Effect of Broth Variations on Drug-Affected Generation Rates—Various strengths of Medium 3 USP and Anton's medium were prepared so that the concentrations of broth ingredients were normal, halved, and doubled. The ingredients of Anton's medium were varied in several ways to study the effects of each ingredient on the drug-affected generation rates. The ingredients studied were phosphates, magnesium sulfate, and casamino acids.

Effect of pH on Drug-Affected Generation Rates—The preparation of Medium 3 USP with different pH values was described previously (14), and the drug-affected generation rates were studied by the total count method.

Reversibility of Drug Action at 37.5°—An aliquot (5.0 ml.) of a drug-free culture generating in the logarithmic phase (curve A in Fig. 2) was added to 49.5 ml. of fresh broth so that the organism population was diluted 11-fold (curve D). Two flasks with 49.5 ml. broth were treated with 0.5 ml. drug solutions to achieve final concentrations of 101 (curve B) and 202 mcg./ml. (curve C). When the drug-affected culture of curve C was in a steady-state generation, an aliquot (5.0 ml.) of the culture was added to 49.5 ml. of fresh medium (curve E). At the same time, aliquots (5.0 ml.) of the culture of curve C were added to 50 ml. volume broth which contained enough drug solution so that the drug concentrations were restored to 101 (curve F) and 202 (curve G) mcg./ml. Similar dilutions were made after the drug-affected culture of curve C had deviated from the semilogarithmic linearity. They were made with fresh medium (curve H) and to broth containing enough drug solution to reestablish final concentrations of 101 (curve I) and 202 (curve J) mcg./ml.

Effect of Equipotent Combinations of Oleandomycin with Other Antibiotics—Equipotent mixtures of 100, 90, 80, 60, 50, 40, 20, 10, and 0% of oleandomycin and the residual percentage of tetracycline, chloramphenicol, erythromycin, lincomycin, or I were prepared. Aliquots (0.50 or 1.00 ml.) of the equipotent mixtures of drug solutions were added to replicate 49.5-ml. volumes of culture generating in logarithmic phase in Medium 3 USP. Samples were withdrawn every 25 min., and the bacterial cells were counted by the total

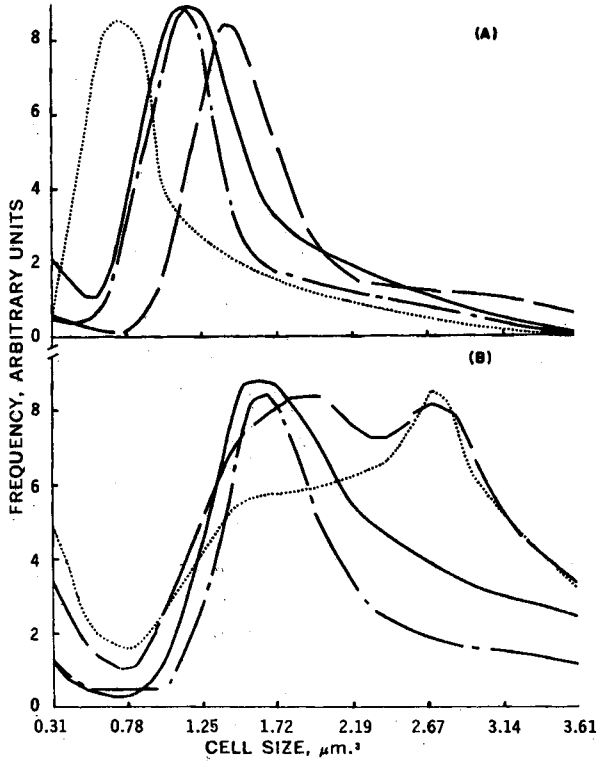

Figure 5—*(A) Size-frequency distribution of the control organisms of* E. coli *in Medium 3 USP 30–140 (——) and 170–200 (- - -) min. after inoculation and of generating organism treated with 132 mcg./ml. oleandomycin phosphate 75–230 (— —) and 260–350 (— - —) min. after the time of inoculation (see Fig. 1). (B) Size-frequency distribution of organisms treated with 233 mcg./ml. oleandomycin phosphate 75–230 (— - —), 260 (——), 290–320 (— —), and 350 (- - -) min. after the time of inoculation (see Fig. 1).*

count method. The combination studies of oleandomycin with tetracycline were repeated in Anton's medium with 0.01 and 0.1% $MgSO_4 \cdot 7H_2O$. They were also repeated in Anton's medium with the normal concentration of nutrients and with half the normal concentration.

Effect of Order of Addition of Oleandomycin–Tetracycline Combinations on Microbial Generation—Replicate 49.5-ml. volumes of a culture containing 7×10^5 *E. coli*/ml. generating in the logarithmic phase (curve A in Fig. 3) were treated with 1.0 ml. of either oleandomycin or equipotent tetracycline solution to give three different concentrations in the final Medium 3 USP media: 152 mcg./ml. oleandomycin phosphate (curve E) or 0.15 mcg./ml. tetracycline (curve F), 86 mcg./ml. oleandomycin phosphate (curve D) or 0.076 mcg./ml. tetracycline (curve D), and 38 mcg./ml. oleandomycin phosphate (curve B) or 0.034 mcg./ml. tetracycline (curve C). The cultures to which 0.5 ml. of the above tetracycline solutions had been added were treated with 0.5 ml. of the oleandomycin solutions 60 min. after the first drug addition at three different dose levels so that the mixtures and the solutions of either drug alone were equipotent *a priori*. Similarly, 0.5-ml. amounts of tetracycline were added to the cultures to which oleandomycin had been added initially.

RESULTS

In the logarithmic generation phase, the bacterial generation rate, dN/dt, is proportional to the number, N, of organisms per unit volume, so that:

$$\log N = (k_{app}/2.303)t + \log N_0 \qquad \text{(Eq. 1)}$$

where N_0 is the number of organisms per unit volume at some zero time when the new steady-state generation is achieved, and the k_{app} determined from the slopes of appropriate plots is the apparent first-order generation rate constant. The addition of graded concentrations of oleandomycin to the culture decreases the generation

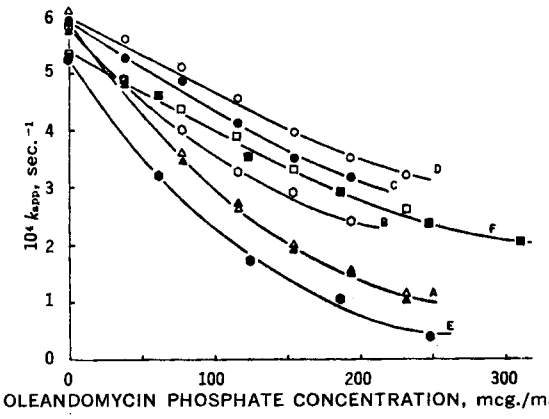

Figure 6—*Dependence of the apparent first-order generation rate constant,* k_{app}, *for* E. coli *on oleandomycin phosphate concentrations at various nutrient concentrations of Medium 3 USP (A, B, C, and D) and of Anton's medium [E and F (■)] and at various phosphate concentrations in Anton's medium [F (■) and F (□)]. The curves, the multiples of the stated concentrations, and the* 10^6 k_A*'s are: A (▲), 0.5, 3.2; A (△), 1.0, 3.2; B, 1.3, 2.3; C, 1.6, 1.5; D, 2.0, 1.3; E, 0.5, 3.4; F (■), 1.0, 1.3; and F (□), 0.5, 1.3.*

rate as a function of antibiotic concentrations, and the semilogarithmic plots of cell numbers against time show a linear relation with slopes that decrease with increasing drug concentrations (Fig. 1). Plots of the determined k_{app} values against various drug concentrations, A, of oleandomycin are given in Fig. 4. At the lower drug concentrations, the plot conforms to the expression:

$$k_{app} = k_0 - k_A A \qquad \text{(Eq. 2)}$$

where k_0 is the generation rate constant in the absence of drug and k_A is defined as the inhibitory constant.

Since the numbers of viable and total cells are coincident (Fig. 1), no kill or death of microorganisms occurs up to the highest drug concentration studied and the use of total counts to determine the generation rates of organism affected by subinhibitory concentrations of oleandomycin is justified. Prolonged contact of microorganisms to higher drug concentrations made the cells grow in size (Fig. 5) and appeared to be related to the deviation from the semilogarithmic linearity of the initial drug-affected steady state. The k_{app} values used were calculated from this initial steady state.

Culture Broth Variations—The concentrations of nutrients in the culture media, both Medium 3 USP and Anton's medium (Fig. 6), have significant effect on the drug-affected generation rate constants. There were no significant differences in drug-affected generation rate constants when the concentrations of phosphates were varied

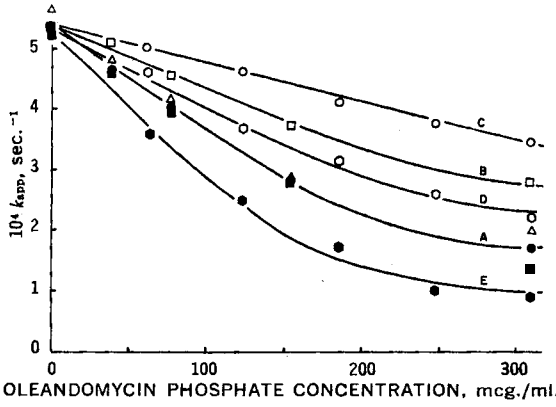

Figure 7—*Dependence of the apparent first-order generation rate constant,* k_{app}, *for* E. coli *on oleandomycin phosphate at various magnesium salt (A and B) and casamino acid (C, D, and E) concentrations in Anton's medium. The curves, the percent* $MgSO_4 \cdot 7H_2O$ *concentrations or multiples of the stated casamino acid concentrations, and the* 10^6 k_A*'s are: A (■), 0.001, 2.2; A (●), 0.005, 2.2; A (▲), 0.02, 2.2; B, 0.1, 1.1; C, 2.0, 0.65; D, 1.0, 1.4; and E, 0.5, 2.5.*

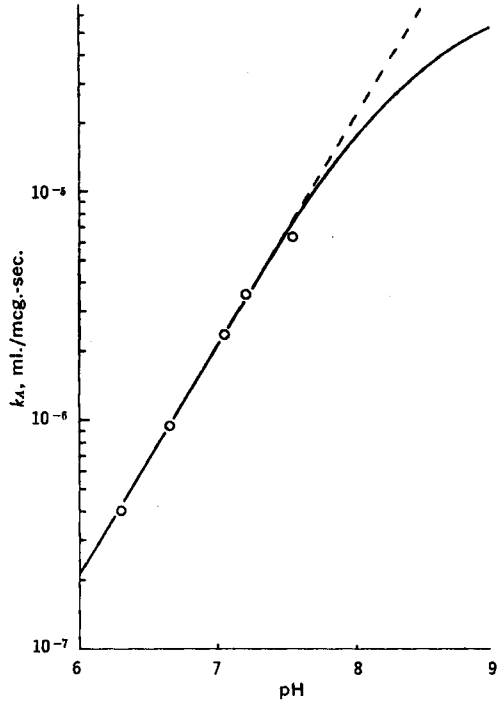

Figure 8—*Dependence of the logarithm of the inhibitory constant,* k_A, *for the action of oleandomycin phosphate on* E. coli *in Medium 3 USP at 37.5° on the pH value. The points are experimental values and the solid line is the theoretical value calculated on the basis that only the unprotonated fraction of the drug concentration contributes to the activity.*

(Fig. 6). Magnesium ion had a minor antagonistic effect only when its concentration was 10 times greater than its normal concentration in Anton's medium (Fig. 7). However, there were significant differences in k_{app} values at the same drug concentrations when the concentrations of casamino acids in Anton's medium were varied; the generation rate constants decreased with increasing nutrient strengths (Fig. 7). The drug may be bound to the casamino acids of Anton's medium and to the proteins in Medium 3 USP to decrease the amount of drug available for antibacterial activity.

Effect of Inoculum Size on Drug-Affected Generation Rates—The apparent generation rate constants were obtained at various concentrations of oleandomycin over almost a 100-fold range in inoculum size at the time of drug addition and were not significantly different. Thus, it cannot be concluded that the drug is metabolized by the organisms, adsorbed to the cells, or inactivated by excretory substances which may be functions of organism population.

Effect of pH on Drug-Affected Generation Rates—The apparent generation rate constants, k_{app}, obtained in broth at pH values 6.30–7.55 in the absence and presence of graded oleandomycin concentrations are plotted in Fig. 4. The drug-free generation rate constants were not significantly affected, but the drug-affected generation rate constants decreased markedly with increasing pH at comparable drug concentrations. The logarithms of the inhibitory constants, k_A, obtained from the slopes of the initial linear portion of plots similar to Fig. 1 appear to be a linear function of the pH of the medium in the range studied (Fig. 8).

Reversibility of Drug Action (Fig. 2)—The culture attained a new steady-state generation 20–25 min. after the drug addition to the culture generation in logarithmic phase. The reasonably short lag time would predict a reversibility of the action of oleandomycin within short time intervals. The steady-state generation affected by 202 mcg./ml. oleandomycin phosphate (curve C) reverted to a new steady state when diluted 11-fold (curve E), and the new steady state was as readily achieved as the drug-free culture (curve A to curve D). There were no significant differences between magnitudes of rate constants obtained from the generation (curve B) attained directly by addition of drug solution to the culture to give a final concentration of 101 mcg./ml. and that attained by diluting the culture of curve C into fresh medium containing enough drug

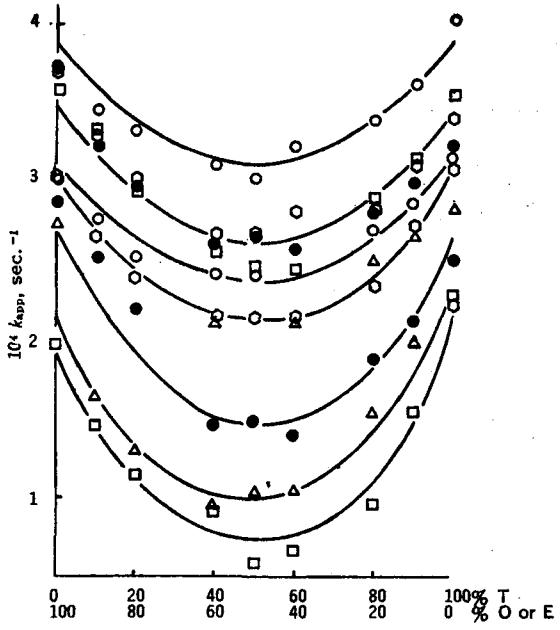

Figure 9—*Effect of varied oleandomycin (O) and tetracycline (T) fractions of* a priori *equipotent combinations at various potency levels in Medium 3 USP (□) and in Anton's medium with a normal concentration of nutrients, 0.01% $MgSO_4 \cdot 7H_2O$ (○), with half the normal concentration (△), and with a normal concentration except for 0.1% $MgSO_4 \cdot 7H_2O$ (○). The effect of varied erythromycin (E) and tetracycline fractions in Anton's medium (●) at 37.5° is also shown.*

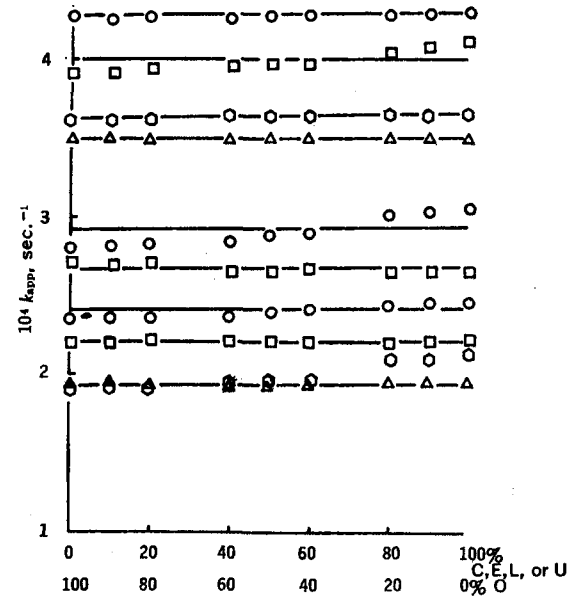

Figure 10—*Effect of varied fractions of oleandomycin with chloramphenicol [C (○)], erythromycin [E (○)], lincomycin, [L (□)], and I [U (△)] in* a priori *equipotent combinations at various potency levels on the apparent generation rate constant of* E. coli *in Medium 3 USP at pH 7.05 and 37.5°.*

solution to give the same final drug concentration (curve F). The dilution of curve C to the restored drug concentration (curve G) had no significant effect on the magnitude of the generation rate constant nor on the fact that the prolonged contact enlarges the cell size. Similarly, the 11-fold dilution of the culture of oversized cells (later portions of curve C) into fresh medium containing halved (curve I) or restored (curve J) concentrations of the drug produced new steady-state generations with predictable rate constants after longer lag periods.

Effect of Equipotent Mixtures of Oleandomycin and Other Antibiotics—The generation rate constants, k_{app}, of cultures affected by equipotent combinations of oleandomycin and tetracycline, erythromycin, lincomycin hydrochloride, chloramphenicol, or I in Medium 3 USP are given in Fig. 9. The mixtures contained 0–100% oleandomycin and the residual percentage of an equipotent amount of the other antibiotic. The mixtures were prepared so as to be *a priori* equipotent in their combined action at two or three different potency levels. The mixtures of oleandomycin and tetracycline exhibited increased activity, as indicated by lower k_{app} values than the equipotent antibiotic used alone. This unequivocally demonstrates synergism (14) between oleandomycin and tetracycline in their combined action against the generation of *E. coli*. The synergism was also observed in Anton's medium with a normal concentration (0.01%) and with a higher concentration (0.1%) of $MgSO_4 \cdot 7H_2O$. It was observed also with half the normal concentration of ingredients (Fig. 9). A similar synergism was observed with the antibiotic pair erythromycin and tetracycline (Fig. 9). However, the null slopes of plots of the k_{app} values for all of the other *a priori* equipotent mixtures demonstrate the lack of any antagonism or synergism in the subinhibitory concentration range (Fig. 10).

Effect of Order of Addition of Oleandomycin and Tetracycline in Combination on Microbial Generation—There were no significant differences between the generation inhibitions produced by the action of 152 mcg./ml. oleandomycin phosphate (curve E in Fig. 3) or 0.15 mcg./ml. tetracycline (curve F), between the inhibitions by 86 mcg./ml. oleandomycin phosphate or 0.076 mcg./ml. tetracycline (curve D), and between the inhibitions by 38 mcg./ml. oleandomycin phosphate (curve B) or 0.034 mcg./ml. tetracycline (curve C).

The addition of either 19 mcg./ml. oleandomycin phosphate (curve G) to the 0.017-mcg./ml. tetracycline-affected culture 60 min. after the first drug addition or 0.017 mcg./ml. tetracycline to the 19-mcg./ml. oleandomycin phosphate-affected culture did not inhibit the generation rate any more than the equipotent amounts of either drug alone did (curves B and C). The combined action is indifferent at the lowest dose level studied.

The addition of either 43 mcg./ml. oleandomycin phosphate (curve I) to the 0.038-mcg./ml. tetracycline-affected culture or 0.038 mcg./ml. tetracycline (curve H) to the 43-mcg./ml. oleandomycin phosphate-affected culture inhibited the generation rate more than did the equipotent amounts of either drug alone (curve D). This demonstrates that the combined action of oleandomycin with tetracycline is synergistic at higher dose levels. This pattern of response was likewise observed for combinations of 76 mcg./ml. oleandomycin phosphate with equipotent concentrations of 0.075 mcg./ml. of tetracycline, since the slopes of curve K and initial portions of curve J are lower than those of curves E and F. In both cases the sequence of addition of the antibiotics produced no significant effects on the synergistic action, since the slopes of curve I and curve K and those of curve H and the initial portions of curve J are the same, respectively.

DISCUSSION

The coincidence of viable and total counts in the subinhibitory range ($\leq$600 mcg./ml.) of oleandomycin phosphate-affected generation (Fig. 1) confirms that the action of oleandomycin is primarily bacteriostatic (3). The drug-affected generation rate constants, k_{app}, are not a linear function of oleandomycin phosphate concentration throughout the entire concentration range (Fig. 4). The shape of the curve indicates the presence of some saturable process in which drug molecules at the high concentrations bind to a limited number of receptor sites (14).

The fact that no significant changes in apparent generation rate constants were observed in the control culture when the concentrations of nutrients in Medium 3 USP or Anton's medium were varied (Fig. 6) implies that the differences in the oleandomycin-affected generation rate contants with different concentrations of Medium 3 USP and Anton's medium can be attributed to the reversible binding of oleandomycin molecules to both protein ingredients of Medium 3 USP and casamino acids in Anton's medium (Fig. 7).

The extent of generation inhibition by oleandomycin increases as the pH of the media increases (Figs. 4 and 8). Since oleandomycin has a pKa of 8.5 (16), the unprotonated fraction of the total drug concentration might be the only effective inhibitory species,

since the relative concentration of the unprotonated species increases as pH increases. The inhibitory constant k_A should be a function of the unprotonated fraction f of oleandomycin in accordance with the equation (14):

$$k_A = k_A^* f = k_A^* \left(\frac{K_a}{K_a + [H^+]}\right) \qquad \text{(Eq. 3)}$$

where k_A^* is the intrinsic inhibitory constant of the unprotonated species. The semilogarithmic plot of k_A against pH is given in Fig. 8. The intrinsic inhibitory constant k_A^* is calculated from the intercept of the plot when $[H^+] \gg K_a$; $k_A^* = 6.96 \times 10^{-5}$ ml./mcg.-sec., from which the antibacterial activity at any pH value can be estimated using Eq. 3.

At doses higher than 200 mcg./ml. of oleandomycin phosphate, a definite change in cell size distribution (Fig. 4) occurs subsequent to the inhibition of microbial generation by the drug. This results in the observed two phases of action (Fig. 1). This phenomenon was not observed with erythromycin-affected generation (17).

Both macrolides, oleandomycin and erythromycin, are synergistic in combined action with tetracycline against microbial generation (Fig. 9). Synergism of antimicrobial activity observed with a combination of two drugs (14) may be assigned to: (*a*) the allosteric modification of the receptor site of one drug by the other so that the affinity of the former increases, (*b*) sequential blocking (18) in which each drug acts on separate rate-determining steps in the sequential metabolic pathway (14) and both drugs show a saturable mechanism of binding to receptor sites, (*c*) the increase in transport to the biophase of one drug by another, and/or (*d*) the fact that one drug effectively eliminates or negates a compound or species in the nutrient media that normally acts as the inhibitor of the action of the other.

The first possible explanation is the most speculative. The second possible explanation cannot be readily reconciled with the linear dependence of k_{app} on tetracycline concentrations which demonstrates a nonsaturable process for the action that results from tetracycline binding to its receptor sites. On the basis of the similarity of structures and postulated equivalent modes of action of the macrolides, erythromycin and oleandomycin, the speculation that combinations of erythromycin and tetracycline would be also synergistic in their action was confirmed (Fig. 9).

The additivity effects for combinations of oleandomycin with chloramphenicol, erythromycin, lincomycin, or I (Fig. 10) may be expected since all of these antibiotics bind to the 50S ribosomal subunit and presumably act on a similar rate-determining step in protein biosynthesis. However, it does appear anomalous that oleandomycin is not antagonistic in combination with both lincomycin and I whereas erythromycin is antagonistic with but one (I) of the two.

The third possible explanation might lead to the demonstration of a significant variation in the degree of synergism with a change in the sequence of addition of oleandomycin and tetracycline; *i.e.*, a greater synergism may result if the antibiotic that increases the transport of the other is added first. However, there was no significant difference in the extent of synergism with a change in the sequence of addition (Fig. 3).

The fourth possible explanation is not valid with respect to inhibition by magnesium salt concentration (Fig. 9) since the degree of synergism is independent of this concentration. Thus, the explanation given for the synergistic action of novobiocin–tetracycline combinations (unpublished data) that the removal of significant amounts of magnesium ion by tetracycline chelation increased the antibacterial effect of novobiocin at low magnesium-ion concentrations is not applicable here. The demonstration of the same extent of synergism in half the normal concentration of nutrient media (Fig. 9) rejects the possibility of negation of the inhibitory action of another constituent of the media by one antibiotic of the combination.

The probable mode of action of the macrolides is on translocation (19) rather than on peptidyl transfer. Combinations of tetracycline with antibiotics such as the lincosaminides and chloramphenicol, which are presumed to act in such a manner (19), show no synergism. Thus, it is reasonable to project that these two different mechanisms of action may be manifested in combinations with tetracycline by the presence or absence of the synergism observed in these studies. It is also implied that the action of a macrolide is enhanced by tetracycline or, conversely, possibly by one increasing the affinity of the other to its respective receptor site or by one increasing the permeability of the other through the cell wall.

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Michaelis–Menten Kinetic Parameters of Renal Tubular Secretion of *para*-Alkylated Mandelic Acids and Their Use in Revealing Possible Chemical Characteristics around Receptor Site of Secretion Carrier in Rats

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Abstract □ From the determination of the Michaelis–Menten kinetic parameters (V_m and K_m) of renal tubular secretion of D-(−)-*p*-*n*-propylmandelic acid (V) and D-(−)-*p*-isopropylmandelic acid (VI) in rats, it is shown that these compounds involve the same renal tubular carrier for their secretion but the affinity of V for the carrier is approximately twice that of VI. By considering these parameters as well as the structural variations of V and VI, together with those of other homologs of D-(−)-mandelic acid (I) studied previously, inferences have been drawn regarding the chemical nature around the possible cationic receptor site of the carrier for renal tubular secretion of organic anions in rats. The utilization of the model compounds of a homologous series of I in studying the influence of alkyl groups present in the organic anions on the pharmacokinetic parameters, particularly the volume of distribution, is discussed.

Keyphrases □ Renal tubular secretion—Michaelis–Menten kinetic parameters, *para*-alkylated mandelic acids used to determine chemical nature of secretion carrier receptor site environment, rats □ Michaelis–Menten kinetic parameters—renal tubular secretion, *para*-alkylated mandelic acids used to study chemical environment of cationic receptor sites of the carrier, influence of alkyl groups, volume of distribution, rats □ Receptor sites, renal tubular secretion carrier—Michaelis–Menten kinetic parameters, *para*-alkylated mandelic acids, influence of alkyl groups, rats □ Secretion, renal tubular, rats—*para*-alkylated mandelic acids used to study chemical environment of carrier receptor site, Michaelis–Menten kinetic parameters, influence of alkyl groups, volume of distribution □ Pharmacokinetic parameters, Michaelis–Menten—renal tubular secretion of *para*-alkylated mandelic acids, use in revealing possible chemical characteristics around the receptor site of secretion carrier, rats

The Michaelis–Menten kinetic parameters (V_m and K_m) of renal tubular secretion determined in rats for D-(−)-mandelic acid (I), tropic acid (II), D(−)-*p*-methylmandelic acid (III), and D-(−)-*p*-ethylmandelic acid (IV) were reported (1, 2) recently. The analysis of these parameters indicated that these compounds are secreted in rats by involving a common carrier and that the affinity of III for the carrier is similar to that of I but that the affinity of IV or II is almost twice that of I. The data obtained in these studies in terms of V_m and K_m were further utilized to distinguish certain chemical structural features of a possible renal tubular carrier responsible for the secretion of these compounds.

Another interesting inference drawn from previous studies (1, 2) in rats was that the apparent volume of distribution (V_d) of the *para*-alkylated mandelic acids (III and IV) is approximately half of that of I, the parent compound.

Since these compounds are neither bound to plasma proteins, metabolized, nor reabsorbed from the renal tubules, in addition to being recovered completely in the urine of rats in the unchanged form, it became possible to determine the effect of introducing alkyl groups in the *para*-position of I on the V_d, initial glomerular filtration rates, and renal tubular secretion rates of these compounds in rats. Noticing such dramatic effects of *para*-alkylation of I on the pharmacokinetic parameters of the compounds, it was considered of great interest to study in rats the effect on these parameters of substituting propyl ($CH_3CH_2CH_2$—) and isopropyl [—$CH(CH_3)_2$] groups in the *para*-position of I.

Therefore, the purpose of the present study was to determine the relative magnitudes of the V_d and the Michaelis–Menten kinetic parameters of renal tubular secretion of D-(−)-*p*-*n*-propylmandelic acid (V) and D-(−)-*p*-isopropylmandelic acid (VI) from the urinary excretion kinetic studies in rats. These data will also be utilized in ascertaining certain inferences drawn from previous studies regarding the topography of the renal tubular secretion carrier receptor around its cationic site(s) which is (are) considered to form primary sites of attachment of the compounds used in the study.

EXPERIMENTAL

Materials—The following were used: D-(−)-mandelic acid, m.p. 132–133°, $[\alpha]_D^{25}$ −154°; D-(−)-*p*-*n*-propylmandelic acid, m.p. 124–125°, $[\alpha]_D^{25}$ −142°, obtained by resolving from the racemic acid synthesized by the method of Reibsomer *et al.* (3); D-(−)-*p*-isopropylmandelic acid, m.p. 159–160°, $[\alpha]_D^{25}$ −129°, obtained by resolving from the racemic acid synthesized by adapting the method of Carson *et al.* (4) for the synthesis of I; and DL-tropic acid, m.p. 117–120°.

Apparatus and Analytical Procedure—The equipment and the GC procedure used for quantitative determination of I and its homologs appearing in the urine of rats were the same as described previously (1). The procedures for specific rotation determination, pH measurements, dissociation constant determination, partition coefficient determination, protein binding determination, and absolute configuration determination of the compounds were also described previously (1). The absolute configuration of the compounds found was D.

Methodology—The procedure followed for preparing the rats and for urine collection following intravenous administration of the compounds was the same as described previously (1). Approximately 65 Sprague–Dawley male rats, weighing 170–220 g., were used repeatedly. Of the 65 rats, 30 were used in the urinary excretion studies involving VI and the rest were used in the urinary excretion studies involving V. The aqueous solutions of sodium salt of each compound used for intravenous administration were made isotonic, and the volumes of intravenous injections varied from 1 to 2 ml. The urinary excretion kinetics of V were studied over the dosage range of 72–258 μmoles/kg. (3–10 mg./rat) and that of VI were studied over the dosage of 129–387 μmoles/kg. (5–15 mg./rat). The reason for using lower doses of V is explained later.

To determine the initial glomerular filtration rates of V at the dosage levels of 72–132 μmoles/kg., the appropriate dose of the substrate compound (V) and the 3012-μmoles/kg. dose of II as an inhibitor, both as their sodium salts contained in 2 ml., were injected intravenously to the rat.

Table I—Overall Urinary Recovery of the Compounds in the Unchanged Form

Compound	Number of Rat Studied	Dose per Rat, mg.	Recovery, mg.
DL-V	3	10	6.1–6.6
DL-VI	3	10	6.5–6.8
V	3	10	9.9–10.0
VI	6	5	4.6–4.9

To determine the nature of inhibition of the renal tubular secretion of VI by II, the urinary excretion kinetic studies of VI at the intravenous dosage levels of 206, 257, and 387 μmoles/kg. in the presence of a 1355-μmoles/kg. dose of II were carried out. Both VI and II were administered as their sodium salts and were contained in the same intravenous solution.

In studies where the apparent initial glomerular filtration rates of VI and I were simultaneously determined in the same rat in the presence of 3012 μmoles/kg. of II as the inhibitor of secretion of these compounds, the equimolar doses of VI and I were used. Three different doses of these compounds were employed: 180, 258, and 387 μmoles/kg.

RESULTS AND DISCUSSION

Selection of Optical Isomers of Compounds—The selection of V and VI for the present study was based on the following ideal properties possessed by the compounds.

1. From the overall urinary recovery (Table I) of the administered intravenous doses of racemic *p*-propylmandelic acid, racemic *p*-isopropylmandelic acid, V, and VI, it becomes clear that only with the levorotatory isomers of the compounds is virtually 100% of the given doses recovered in the unchanged form. The poor recovery of the compounds (unchanged) following administration of the racemic form was due to metabolism of the dextrorotatory form of the compounds, as found previously for other homologs of I (1, 2). In view of the objectives of the study, quantitative recovery of V and VI was considered to be ideal.

2. The pKa value reported for VI is 3.64 (5) and that determined for V is 3.7. Therefore, these compounds are expected to remain in the ionized form in the biological fluids.

3. The partition coefficients ($C_{organic}/C_{buffer}$) determined for these compounds according to the procedure previously described (2) were zero, which lead to the reasonable assumption that the reabsorption of the compounds in the renal tubules is negligible, if any.

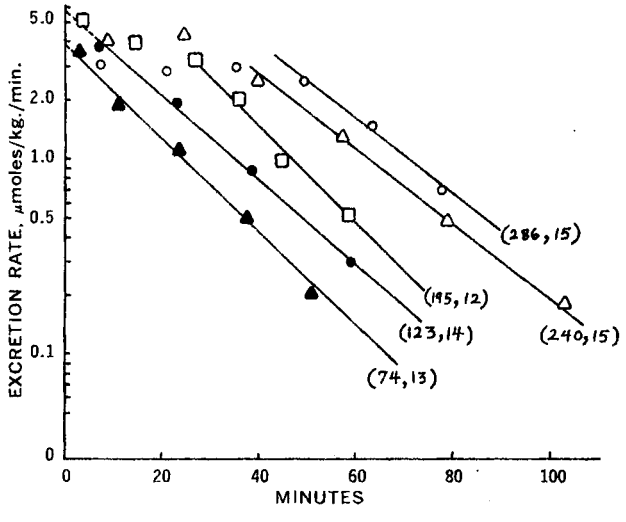

Figure 1—*Plots of log urinary excretion rate* versus *time for V following intravenous administration of various doses to rats. (The numbers in parentheses refer to intravenous dose in micromoles per kilogram and the biological half-life of the compound, respectively. At doses of 240 and 286 μmoles/kg., the biological half-life refers to the data obtained after 30 min., as explained in the text.)*

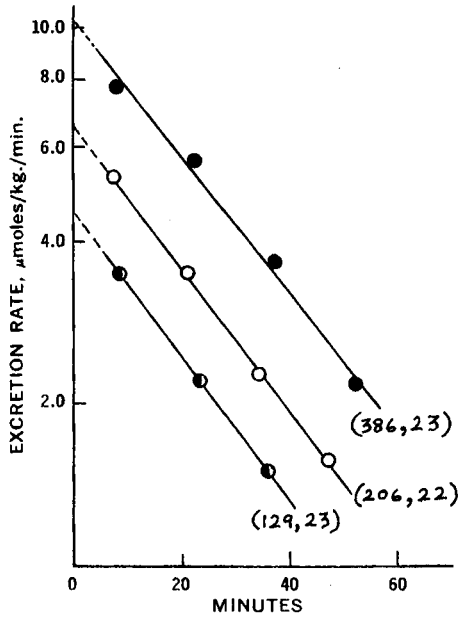

Figure 2—*Apparent first-order urinary excretion of VI following intravenous administration of various intravenous doses to rats. (The numbers in parentheses refer to the intravenous dose in micromoles per kilogram and the biological half-life of the compound, respectively.)*

4. From the equilibrium dialysis studies, it was noticed that at the equilibrium concentrations of 0.152–0.255 mg./4 ml. of V in the plasma, about 3.7–5.2% of the compound was bound. For practical purposes, this extent of binding was considered negligible. The binding of VI to plasma proteins was found to be negligible.

Urinary Excretion Kinetics of V and VI—As can be seen from the data presented in Fig. 1, two different patterns of urinary excretion of V in rats become evident. At the intravenous doses of 72–143 μmoles/kg. studied, the excretion of V occurred by an apparent first-order process and the kinetic model for the compound was indeed describable as the one-compartment open model. Since the administered dose of the compound is recovered entirely in the urine in the unchanged form, the urinary excretion data were treated according to the following equation:

$$\log \Delta Ae/\Delta t = \log kA_0 - kt/2.303 \qquad \text{(Eq. 1)}$$

where $\Delta Ae/\Delta t$ is the rate of excretion of the compound at time t, which is the midpoint of the urine collection interval; A_0 is the amount of the compound in the body at zero time, k is the apparent first-order rate constant for excretion of the compound, and kA_0 is the apparent initial excretion rate of the compound at a given intravenous dose. The rate constant k was obtained from the slope ($-k/2.303$), and the initial excretion rate was obtained from the intercept of the straight line obtained upon plotting log $\Delta Ae/\Delta t$ *versus* t. The straight line in each case was obtained by the method of least squares. The rate constant k is designated as the rate constant of urinary excretion because the administered doses of the compound are recovered entirely in the urine in the unchanged form (1). The biological half-life of Compound V was calculated from $0.693/k$. The average biological half-life of the compound determined from 23 studies over the intravenous dosage range of 72–143 μmoles/kg. was 13 min.

However, when the studies were carried out at the intravenous dosage levels of 195–294 μmoles/kg., the excretion of Compound V in almost all of the 11 studies was found to take place by an apparent first-order process (with the biological half-life of about 12 min.), but generally after 30 min. During the initial 30 min., the excretion rate of the compound declined so gradually that the rates appeared to be almost constant (Fig. 1). Although it may be expected that, since V is secreted by the renal tubules of rats as shown later, the biological half-life of the compound would tend to increase with the increase in the dose due to increasing saturation of the secretory process, such an extremely gradual decline in the excretion rate noted during the initial period cannot be accounted for

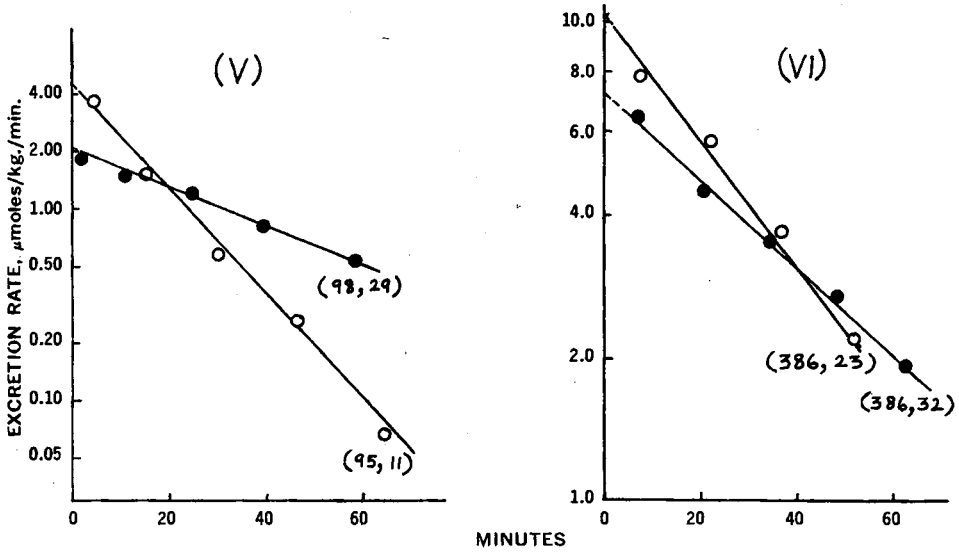

Figure 3—*Apparent first-order urinary excretion of V and VI in the absence* (○) *and in the presence* (●) *of a simultaneously administered 3020-μmoles/kg. i.v. dose of II. (The numbers in parentheses refer to the intravenous dose in micromoles per kilogram of the respective compound and its biological half-life, respectively.)*

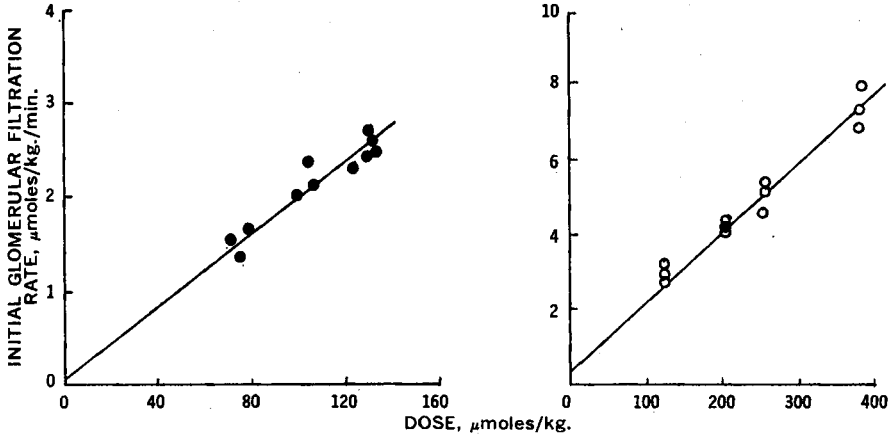

Figure 4—*Relationship between the apparent initial glomerular filtration rates and the intravenous doses for V* (●) *and VI* (○).

on this basis. The reason for this belief is that even IV, which has almost the same V_d and affinity for the renal tubular secretion carrier in rats, did not show such a saturation effect, as evidenced by the constancy of its biological half-life even up to a 885-μmoles/kg. i.v. dose (2). The reason for this phenomenon has been investigated and will be reported separately.

The facts that VI is excreted by an apparent first-order process and that its excretion is describable by the one-compartment open model become evident from the data presented in Fig. 2. The average biological half-life determined for the compound is 23 min.

Determination of Apparent Initial Glomerular Filtration Rates and Apparent Renal Tubular Secretion Rates—In previous studies (2), II was shown to inhibit competitively the renal tubular secretion of the homologs of I in rats. Therefore, II was used to inhibit the tubular secretion of 129–387-μmoles/kg. doses of VI or 72–132-μmoles/kg. doses of V. The preliminary studies indicated that the apparent initial excretion rates obtained for the compounds at these doses were the same in the presence of a 3012- or a 4500-μmoles/kg. i.v. dose of II, indicating that the dose of 3012 μmoles/kg. of II was adequate to inhibit the secretion of the compounds. Therefore, in each study, a 3012-μmoles/kg. dose of II (inhibitor), along with an appropriate dose of each compound, was administered to the rat intravenously. Both the substrate compound and the inhibitor were administered as their sodium salts contained in a 2.5-ml. isotonic solution. The overall urinary recovery of the doses of the substrate compounds in these studies was found to be quantitative and, as evidenced from the typical studies presented in Fig. 3, the excretion of the substrate compounds in the presence of II occurred by an apparent first-order process. The apparent initial excretion rates obtained in these studies were plotted against the corresponding doses of the compounds (Fig. 4). As shown in this figure, the least-squares straight lines obtained for the data almost passed through the origin, indicating that the apparent initial excretion rates determined in these studies represent the apparent initial glomerular filtration rates of the compounds at the corresponding intravenous doses.

The apparent initial secretion rates were computed by subtracting the initial glomerular filtration rates from the apparent initial excretion rates at the corresponding intravenous doses of each compound. The glomerular filtration rates used in the computation of the apparent initial secretion rates were, however, those obtained from the representative plots (Fig. 4) corrected to pass through the origin.

The composite plots of apparent initial excretion rates, initial glomerular filtration rates, and initial secretion rates *versus* intravenous doses of the compounds are shown in Figs. 5 and 6.

Determination of Michaelis–Menten Kinetic Parameters for Renal Tubular Secretion—As previously demonstrated (1, 2), the data regarding the initial secretion rates (V) and the intravenous doses (D) were treated according to the Lineweaver–Burk equation:

$$1/V = K_m/V_mD + 1/V_m \qquad \text{(Eq. 2)}$$

and the values of the apparent maximum initial secretion rate (V_m) and the intravenous dose (K_m) required to produce 1/2 V_m were calculated from the straight-line (least-squares) plot obtained for each compound upon plotting $1/V$ *versus* $1/D$ (Fig. 7). The respective

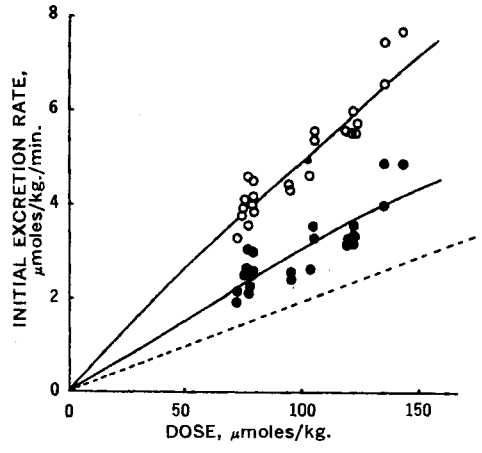

Figure 5—*Apparent initial excretion rates* (○), *initial glomerular filtration rates* (- - -), *and initial secretion rates* (●) *obtained for V following the intravenous administration of appropriate doses to rats.*

values of V_m for these compounds were calculated from the intercepts on the y-axis, and those of K_m were calculated from the slopes of the straight lines. The values of V_m and K_m are listed in Table II, along with those for I, II, III, and IV for comparison.

Although no claim can be made that the values of V_m and K_m (Table II) obtained from four different studies are precise enough to allow a distinction with respect to the precise affinity of the various compounds for the common carrier, it is reasonable to conclude that all of these compounds are secreted by the same renal tubular secretion carrier. As additional support to this conclusion, studies were carried out to show that II competitively inhibits the secretion of VI in rats. From the apparent initial excretion rates determined for VI (206–287 μmoles/kg.) in the presence of simultaneously administered 1355-μmoles/kg. i.v. doses of II, the apparent initial secretion rates were determined by subtraction from their apparent initial glomerular filtration rates at the corresponding doses. The plot of $1/V$ *versus* $1/D$ obtained for the studies is shown in Fig. 7. The apparent common y-intercept noted in this figure indicates that II competitively inhibits the secretion of VI.

Simultaneous Determination of Apparent Initial Glomerular Filtration Rates of VI and I in the Same Rats—In the present studies, it is noted that at the equimolar doses the apparent glomerular filtration rates of VI are similar (but not identical) to that of V, which in turn appear to be similar to that of III or IV reported previously (2). Randinitis *et al.* (1) showed that at the equimolar

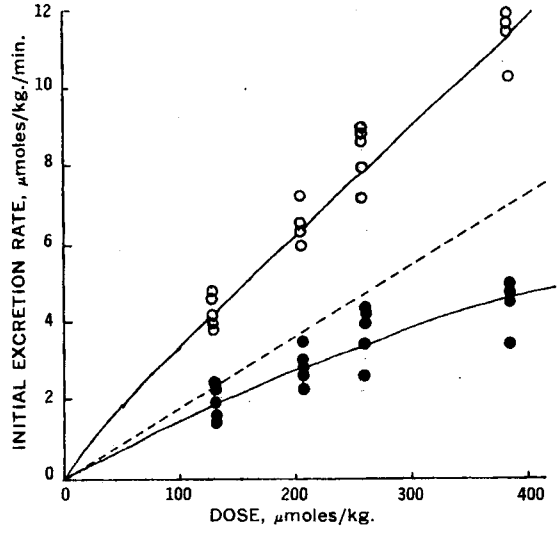

Figure 6—*Apparent initial excretion rates* (○), *initial glomerular filtration rates* (- - -), *and initial secretion rates* (●) *obtained for VI following intravenous administration of appropriate doses to rats.*

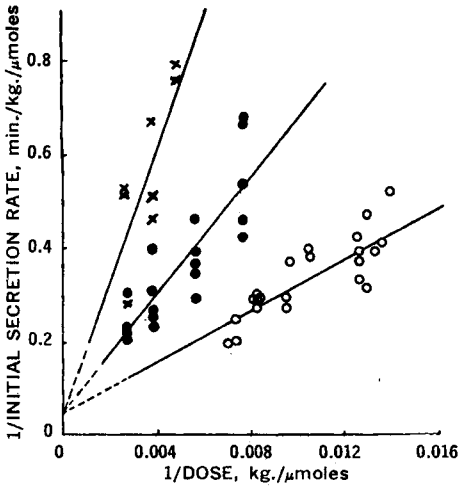

Figure 7—*Lineweaver–Burk plots of the reciprocal of intravenous doses* versus *the reciprocal of apparent initial secretion rates for V* (○) *and VI* (●) *in the absence of II and for VI* (×) *in the presence of a simultaneously administered 1355-μmoles/kg. i.v. dose of II (inhibitor).*

doses the initial glomerular filtration rates of I and its homologs obtained by introducing methylene groups in the side chain (*i.e.*, II, 4-hydroxy-4-phenylbutanoic acid, phenyllactic acid, and benzyllactic acid) are also similar. The inference (2) that the V_d in rats for III or IV is approximately half of that for I or II was based on the comparison of the apparent initial glomerular filtration rates reported in these two separate studies.

To substantiate further that the V_d of the homologs of I obtained by introducing methylene groups in the side chain is almost twice the V_d of the homologs of I obtained by *para*-alkylation, the initial glomerular filtration rates of VI and I (each representing the respective group of the homologs) were determined in the same rats by simultaneously administering the appropriate equimolar intravenous doses (180, 258, or 387 μmoles/kg.) in the presence of a 3012-μmoles/kg. dose of II. Typical data are presented in Fig. 8, and the data regarding the apparent initial glomerular filtration rates against the doses of the compounds are presented in Fig. 9. It is evident from these data that the V_d of I is almost 1.7 times greater than the V_d of VI.

Possible Interpretation of Michaelis–Menten Kinetic Parameter Data to Elucidate the Chemical Features around the Receptor Site(s) of the Renal Tubular Secretion Carrier—Since I and its various homologs (pKa 3.4–3.7) exist in the blood (pH 7.4) in ionized form, it has been tacitly assumed that the primary interaction between these compounds and the renal tubular secretion carrier present in the tubular membrane occurs through electrostatic forces. The anions of these acids are supposed to interact with the cationic receptor site(s) of the carrier.

From examination of the data in Table II, it is noted that, among the *para*-alkylated homologs of I, the affinity of V is about twice that of VI, III, and I. Although a large hydrophobic group is present, namely the isopropyl group in a VI molecule, the affinity of the molecule for the secretion "carrier" is not different from that of I or III. Therefore, it may be instinctively deduced that, among the

Table II—Michaelis–Menten Kinetic Parameters of Renal Tubular Secretion of I and Its Homologs in Rats

Compound	Apparent V_m, μmoles/kg./min.	Apparent K_m, μmoles/kg.
I[a]	18.5	11,100
II[a]	20.8	5,200
III[b]	21.6	10,200
IV[b]	18.5	4,900
V	19.6	5,640
VI	17.7	11,200

[a] Values for these compounds were obtained from *Reference 1*. [b] Values for these compounds were obtained from *Reference 2*.

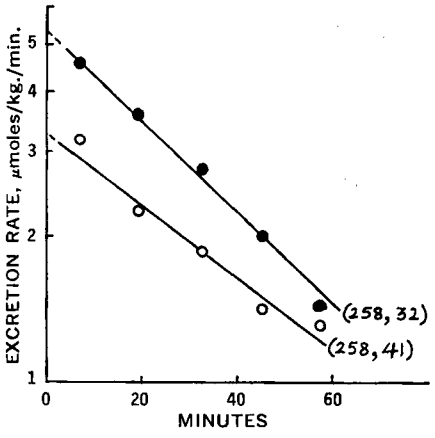

Figure 8—*Apparent first-order urinary excretion of VI (●) and I (○) in the presence of a simultaneously administered 3012-μmoles/kg. i.v. dose of II (inhibitor). (The numbers in parentheses refer to the intravenous dose in micromoles per kilogram of the respective compound and its biological half-life, respectively.)*

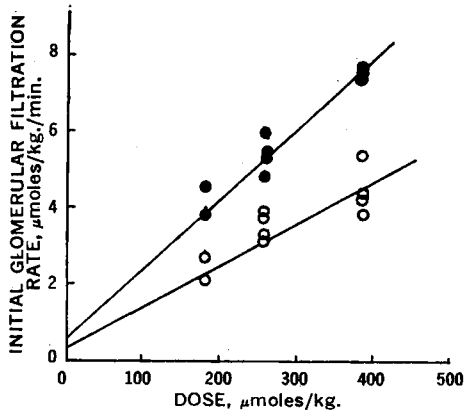

Figure 9—*Comparison of the apparent initial glomerular filtration rates observed for VI (●) and I (○) in rats.*

para-alkylated homologs of I, the length of the hydrophobic group attached in the *para*-position in the phenyl group of I determines the extent of hydrophobic interaction of the compounds with the carrier. But it is further noted that the affinity of V for the carrier is almost the same as that of IV, indicating that the length of the alkyl group in the *para*-position of the homolog molecule alone does not determine the affinity of the molecule for the carrier.

From previous studies (1) in rats involving I and its homologs [obtained by introducing methylene group(s) in the side chain of I], the possible chemical structural characteristics distinguished were that the hydrophobic region around the cationic site of the carrier molecule probably does not extend uninterruptedly beyond the hydrophobic region represented by two carbon atoms (possibly two methylene groups) and, following such hydrophobic region, there is a polar group present. This interpretation to envision the chemical nature of the receptor of the carrier was also supported by the analysis of Michaelis–Menten kinetic parameter data obtained for the *para*-alkylated homologs of I, namely, III and IV (2). Since the *para*-substitution of an ethyl group, but not that of methyl (2), resulted in increasing the affinity of the resulting molecule, as evidenced by the K_m values, it was further suggested (2) that the increased affinity of IV for the carrier is due to the additional interaction of its end methyl group with the hydrophobic portion that should be present next to the polar group of the carrier molecule.

Although it is not known exactly what component of the renal tubular membrane constitutes the carrier for the organic anions, proteins and/or phospholipid(s) have been suggested as the carrier (6, 7). Without being specific about the exact chemical entity of the membrane that is responsible for the transmembrane transport, it seems reasonable to assume that the chemical component of the membrane that serves as the carrier for the organic anions should have cationic groups. Therefore, it is believed that, because of their suitable physicochemical properties, I and its homologs used in past and present studies can serve as probes to reveal the chemical nature of the renal tubular carrier around its cationic receptor site(s). In the present study, the *para*-substitution of the *n*-propyl group in I did not result in further increase in the affinity of the resulting molecule (*i.e.*, V) for the carrier than that of IV. Owing to the additional methylene group, if the affinity of V for the carrier were to be higher than that of IV, hydrophobic interaction was expected to occur between the additional methylene (or methyl) group of V and the complementary hydrophobic group of the receptor site of the carrier. The lack of such increase in the affinity of V for the carrier may be interpreted to suggest that the end methyl group of V comes in contact not with a nonpolar group but possibly with a polar group. Thus, this study serves to distinguish a further structural characteristic of the carrier by indicating that the hydrophobic group suggested to be present next to the polar group of the carrier from the studies of (IV) (2) is probably represented by one carbon atom (possibly a methylene group), and next to this group there is present probably a polar group.

Pharmacokinetic Significance—To induce a biologial effect, it is well recognized that the drug should be present in effective concentration at the site of action and interact with the receptors in some appropriate manner. It is further recognized that there is a relationship between the physicochemical properties of the drug and its biological activity (8), because the attainment of effective concentration of the drug at the receptors is a function of several rate processes the drug is simultaneously subjected to in the body. These rate processes include the rate of access of the drug to, and the rate of disappearance of the drug from, its site of action, the rate of metabolism, the rate of glomerular filtration and renal tubular secretion, and the extent of binding of drug to plasma proteins. These processes depend on the physicochemical properties of the drug and, therefore, often rather complicated structure–activity relations are found even for compounds of a homologous series (8). It is generally believed that the introduction of hydrophobic groups in a given drug molecule tends to increase its lipid solubility, giving rise to its increased permeability into the tissue. Unfortunately, however, it does not become possible in the case of a majority of drugs to evaluate this specific effect of a hydrophobic group on the tissue permeability of the drug, since the presence of the same hydrophobic group in the drug molecule may simultaneously result in changes in its rate of metabolism, rate of excretion, and/or extent of binding to plasma proteins and thus complicate such evaluation of a single pharmacokinetic factor.

However, it is reasonable to expect that the fewer above-mentioned pharmacokinetic processes the drug is subjected to in the body the greater is the opportunity for success in correlating the effect of systematic introduction of the hydrophobic group in a drug molecule on a given pharmacokinetic process or parameter. It is in this context that I and its homologs (1, 2) are considered as the model compounds because these compounds (organic anions) are neither metabolized, bound to plasma proteins, nor reabsorbed in the renal tubules of rats. Consequently, it is possible to determine the effect of alkyl groups on the V_d, glomerular filtration rates, and renal tubular secretion rates. It is not meant to imply that the introduction of methyl or methylene groups in an organic molecule will always bring about such changes in the pharmacokinetic parameters, regardless of the nature of the organic compound. Such generalization cannot be made from the limited studies discussed here. Nevertheless, the striking effects observed of *para*-alkylation of I on the V_d, glomerular filtration rates, and tubular secretion rates are indicative of the possibility that further studies with suitable compounds would eventually provide definite insight as to the effects of alkyl substitution in the organic anions on their pharmacokinetic parameters.

The introduction of hydrophobic groups in a drug molecule is generally believed to tend to increase the lipid (tissue) solubility of the compound, consequently resulting in its increase in the V_d. But the effect of introducing alkyl groups in the *para*-position of the organic anions in the present homologous series was found to be just the opposite. The V_d of the *para*-alkylated homologs of I decreased while that of II remained practically unchanged (1), as compared to the V_d of the parent compound, I. This illustrates the influence of the position of the alkyl group in the compound on its V_d in the body.

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Chronic Isoproterenol Treatment of Neonatal Rats

GERDA I. KLINGMAN▲ and GENIE McKAY

Abstract □ The chronic subcutaneous administration of isoproterenol to neonatal and young male and female rats resulted in statistically significant reductions of the norepinephrine concentrations of the heart and submaxillary and parotid glands and increased concentrations in the stellate ganglion. However, the total norepinephrine content of these tissues was not affected. The epinephrine levels of these tissues were not significantly different from controls except for an increased total content in the submaxillary glands. Chronic treatment did not alter the norepinephrine levels of the brain, superior cervical ganglion, adrenal glands, vas deferens, uterus, spleen, kidneys, lungs, and small intestine. Growth of neonatal rats was retarded. The wet weights of the submaxillary and parotid glands were significantly greater, while the spleen, uterus, adrenal glands, and stellate ganglion weighed less in isoproterenol-treated than in control rats. The wet weights of other peripheral tissues were not affected. The ratios of the tissue weight to the body weight were significantly increased for the heart and submaxillary and parotid glands and decreased for the uterus. The first dose of isoproterenol produced hypothermia in mature rats but hyperthermia in newborn rats. Sparse hair growth in neonatal rats and hair loss in weanling rats were also observed during chronic treatment.

Keyphrases □ Isoproterenol, chronic administration—effects on tissue catecholamine levels, tissue weights, and rectal temperature in rats □ Catecholamine tissue levels—effect of chronic isoproterenol administation, rats □ Tissue weight and catecholamine levels—effect of chronic isoproterenol administration, rats

The ability of isoproterenol to produce hypertrophy and hyperplasia of the submaxillary and parotid glands of rats has been studied by various investigators (1–5). In addition, isoproterenol treatment resulted in infarct-like lesions and fatty degeneration of the heart (6, 7). Effects in other species have also been reported, *e.g.*, cats (8), hamsters (9), guinea pigs (10), and mice (11, 12). Growth stunting, premature opening of the eyes, and an increase of the ratio of heart weight to body weight have been reported following the chronic administration of isoproterenol to neonatal rats (5). Since acute and short-term treatment resulted in increased organ weights and derangements of catecholamine levels (13–15) and storage (15), and chronic isoproterenol treatment produced growth stunting and changes in organ weights in neonatal rats (5), the effects of chronic isoproterenol treatment on the tissue catecholamine levels, tissue weights, and rectal temperature were studied in neonatal rats and the results are reported in this paper.

METHODS

Animals—Two days after birth, Sprague–Dawley male and female rats from three litters were subcutaneously dosed with 0.85% sodium chloride solution (controls) or 5 mg./kg. isoproterenol twice a day for 6 days and thereafter with 15 mg./kg. twice a day for up to 43 days. Animals of two other litters received 10 mg./kg. subcutaneously twice a day for 7 days, the first dose being given on the 6th day after birth. At the end of the 1st week of drug administration, nearly 50% of the animals of this group had died and the dose was reduced to 10 mg./kg. once a day.

To obtain additional information on the hyperthermia, the frequently observed lethargy, and the sparsity of hair growth (or hair loss), animals of three other litters were treated chronically by three different dose regimens: 20 mg./kg. once a day and 5 mg./kg. twice a day for 5 days (treatment was started on the 6th day after birth) and 15 mg./kg. twice a day for 30 days (treatment was started on the 25th day after birth).

Catecholamine Analysis—For the tissue catecholamine measurements, the treated animals and their littermate controls were sacrificed by exsanguination under sodium pentobarbital anesthesia after 30–43 days of treatment. The heart, submaxillary glands, parotid glands, spleen, kidneys, lungs, small intestine, vas deferens, uterus, adrenal glands, whole brain (or brainstem[1]), superior cervical ganglia, and stellate ganglia were dissected in the cold and weighed[2]. Tissues were analyzed immediately or frozen and analyzed within 3 days after dissection. Norepinephrine and epinephrine were extracted with 1-butanol as previously described (16) and measured spectrophotofluorometrically by a modification (17, 18) of the ferricyanide oxidation method of von Euler and Floding (19).

[1] In this report the brainstem is defined as the medulla, the pons, the midbrain, and the diencephalon.

[2] On a Mettler balance or a Cahn electrobalance.

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Table I—Wet Weights of Peripheral Tissues and Ratios of Rats Chronically Treated with Isoproterenol[a]

Tissue	Control: Number of Rats	Control: Wet Weight, mg. ± SE	Control: Ratio, Tissue/Body Weight, mg./g. ± SE	Isoproterenol: Number of Rats	Isoproterenol: Wet Weight, mg. ± SE	Isoproterenol: Ratio, Tissue/Body Weight, mg./g. ± SE
Submaxillary glands	17	268 ± 22	3.0 ± 0.1	14	736 ± 138[b]	9.5 ± 0.8[c]
Parotid glands	14	235 ± 32	2.2 ± 0.2	11	843 ± 277[d]	8.6 ± 1.4[c]
Heart	18	404 ± 41	4.1 ± 0.1	14	432 ± 53	6.0 ± 0.2[c]
Spleen[e]	6	398 ± 33	4.7 ± 0.8	4	293 ± 19[d]	4.9 ± 1.0
Uterus	6	142.5 ± 32.7	0.95 ± 0.17	4	53.3 ± 14.3[d]	0.48 ± 0.02[d]
Adrenal[e] glands	8	21.3 ± 1.6	0.24 ± 0.02	6	16.5 ± 0.6[b]	0.25 ± 0.02

[a] Treatment was started 2 and 6 days after birth for 30–43 days. [b] $p < 0.01$, t test in nonpaired experiments (26). [c] $p < 0.001$, t test in nonpaired experiments (26). [d] $p < 0.05$, t test in nonpaired experiments (26). [e] Treated for 30 days.

Table II—Norepinephrine and Epinephrine Concentration and Content of Peripheral Tissues from Control and Chronic Isoproterenol-Treated Rats (Treatment 30–43 days)

Tissue	Treatment	Number of Rats	Micrograms per Gram of Tissue: Norepinephrine	Micrograms per Gram of Tissue: Epinephrine	Micrograms per Tissue: Norepinephrine	Micrograms per Tissue: Epinephrine
Heart	Control	18	0.75 ± 0.07[a]	0.15 ± 0.06	0.31 ± 0.06	0.06 ± 0.03
	Isoproterenol	14	0.54 ± 0.04	0.06 ± 0.02	0.24 ± 0.04	0.05 ± 0.02
Submaxillary glands	Control	17	1.00 ± 0.09[b]	0.23 ± 0.07	0.30 ± 0.04	0.04 ± 0.01[c]
	Isoproterenol	14	0.41 ± 0.06	0.13 ± 0.02	0.31 ± 0.06	0.08 ± 0.01
Parotid glands	Control	14	1.03 ± 0.10[b]	0.20 ± 0.02	0.26 ± 0.05	0.04 ± 0.01
	Isoproterenol	11	0.44 ± 0.07	0.18 ± 0.03	0.30 ± 0.10	0.12 ± 0.04

[a] $p < 0.02$, t test in nonpaired experiments (26). [b] $p < 0.001$, t test in nonpaired experiments (26). [c] $p < 0.01$, t test in nonpaired experiments (26).

Table III—Norepinephrine and Epinephrine Concentration and Content of Superior Cervical and Stellate Ganglia of Control and Chronic Isoproterenol-Treated Rats

Ganglia	Treatment	Number of Rats	Weight, mg./Pair	Micrograms per Gram: Norepinephrine	Micrograms per Gram: Epinephrine	Nanograms per Tissue: Norepinephrine	Nanograms per Tissue: Epinephrine
Superior cervical	Control	12	1.70 ± 0.12	21.9 ± 2.3	1.3 ± 0.2	35.9 ± 3.0	2.0 ± 0.3
	Isoproterenol	11	1.54 ± 0.12	21.8 ± 2.2	1.4 ± 0.3	33.6 ± 4.8	2.1 ± 0.4
Stellate	Control	12	2.40 ± 0.10[a]	14.9 ± 0.9[a]	0.7 ± 0.2	35.3 ± 1.7	1.7 ± 0.4
	Isoproterenol	11	1.87 ± 0.15	21.1 ± 1.5	0.8 ± 0.3	38.5 ± 3.5	1.4 ± 0.4

[a] $p < 0.01$, t test in nonpaired experiments (26).

Temperature—The rectal temperatures were measured at room temperature (23 ± 1°) with a telethermometer[3] before the isoproterenol administration and every 30 min. for up to 210 min. after dosing. An arterial probe[3] was used for measuring the rectal temperature of newborn rats and thermister probes[4] were used for older animals.

RESULTS

Body Weight—Chronic isoproterenol treatment of neonatal rats for up to 43 days resulted in growth retardation. When treatment was started 6 days after birth with 10 mg./kg., a mean difference in body weight (9%) became apparent after 5–6 days and statistically significant after 7–8 days of treatment (14%; $p < 0.05$). With continuation of the treatment for 15 days, the mean body weight of the isoproterenol-treated group amounted to 69.5% of that of control littermates ($p < 0.01$). When isoproterenol treatment with 5 mg./kg. twice a day was started on the 2nd day after birth and then increased to 15 mg./kg. twice a day, slowing of growth was first observed after approximately 8 days of treatment and a statistically significant difference was observed at 11 days. Growth retardation was still evident at the time of sacrifice and amounted to 15% when compared to control littermates.

Tissue Wet Weights and Ratios—Chronic isoproterenol treatment affected the wet weights and/or the ratios of the tissue weight to the body weight (milligrams per gram) of several organs (Table I). The wet weights of the submaxillary and parotid glands were increased while the spleen, uterus, and adrenal glands of treated rats were smaller than those of controls. The absolute weights of the hearts were similar in the treated and the control groups, but the ratios of the wet weight to body weight were elevated. These ratios were also increased for the submaxillary and parotid glands, unaltered for the spleen and adrenal glands, and decreased for the uterus.

Catecholamines—Similarity of results on the catecholamine levels from rats treated for 30–43 days permitted the pooling of these data (Table II). Significant decreases in the norepinephrine concentrations of the heart and submaxillary and parotid glands were noted after chronic isoproterenol treatment, but the norepinephrine contents of these tissues were not altered. Statistically significant changes of epinephrine concentrations of the heart, submaxillary glands, and parotid glands did not occur, but the epinephrine content of the submaxillary glands was increased. The catecholamines of other peripheral tissues such as the spleen, uterus, vas deferens, kidneys, lungs, pancreas and adrenal glands, whole brain, and brainstem did not differ from control values. Although the catecholamine concentration and content of superior cervical ganglia were not altered, the norepinephrine concentration of stellate ganglia was increased after chronic treatment (Table III); this was apparently due to a significant reduction in wet weight.

Rectal Temperature—The administration of the first dose of isoproterenol (5 or 20 mg./kg. subcutaneously) to 6-day-old rats resulted in a statistically significant increase in the rectal temperature that lasted about 90 min. Subsequent doses administered once or twice a day for several weeks did not significantly accentuate the hyperthermia (Tables IV and V). Sex difference in the temperature effect of isoproterenol was not studied in detail. However, no statistically significant difference was found in the hyperthermic

[3] Yellow Springs Instrument Co., Inc.
[4] Nos. 401 and 402, Yellow Springs Instrument Co., Inc.

Table IV—Effect of the Acute and Chronic Isoproterenol Treatment on the Rectal Temperature of Neonatal Rats

Treatment	Age, days	Number of Doses	Number of Rats	Minutes after Isoproterenol Treatment: 0	30	60	90	120	150	210
				Temperature ± *SE*						
Controls	6–9	0	12	35.3 ± 0.1°	36.3 ± 0.1°	35.9 ± 0.1°	36.2 ± 0.1°	36.1 ± 0.2°	35.9 ± 0.1°	35.8 ± 0.1°
Isoproterenol, 5 mg./kg.	6	1	6	35.5 ± 0.1°	37.2 ± 0.1°[a]	37.0 ± 0.0°[a]	37.5 ± 0.1°[a]	37.0 ± 0.2°[b]	35.3 ± 0.2°[c]	35.5 ± 0.2°
Isoproterenol, 20 mg./kg.	6	1	4	35.3 ± 0.1°	37.0 ± 0.1°[a]	37.0 ± 0.1°[a]	37.3 ± 0.2°[a]	36.7 ± 0.1°[c]	36.3 ± 0.0°[b]	35.7 ± 0.1°
Isoproterenol, 5 mg./kg.	7	3	6	35.2 ± 0.1°	37.1 ± 0.0°[a]	37.0 ± 0.2°[a]	36.9 ± 0.0°[a]	36.3 ± 0.2°	35.7 ± 0.1°	35.7 ± 0.1°
Isoproterenol, 20 mg./kg.	8	3	4	35.4 ± 0.1°	37.7 ± 0.1°[a]	37.8 ± 0.0°[a]	37.8 ± 0.1°[a]	37.7 ± 0.1°[a]	37.4 ± 0.1°[a]	35.6 ± 0.1°
Isoproterenol, 5 mg./kg.	9	7	6	35.8 ± 0.1°[b]	37.9 ± 0.1°[a]	38.0 ± 0.1°[a]	37.7 ± 0.1°[a]	37.2 ± 0.1°[a]	36.8 ± 0.2°[a]	36.6 ± 0.2°[b]

[a] $p < 0.001$, experimental *versus* control animals; *t* test in nonpaired experiments (26). [b] $p < 0.01$, experimental *versus* control animals; *t* test in nonpaired experiments (26). [c] $p < 0.02$, experimental *versus* control animals; *t* test in nonpaired experiments (26).

Table V—Effect of Acute and Chronic Isoproterenol Treatment on Rectal Temperature of Rats

Treatment	Age, days	Number of Doses	Number of Rats	Minutes after Isoproterenol Treatment: 0	30	60	90	120	150
				Temperature ± *SE*					
Control	12–13	—	6	35.1 ± 0.4°	35.0 ± 0.3°	35.3 ± 0.3°	35.7 ± 0.2°	—	—
Isoproterenol, 15 mg./kg.	12–13	12–14	7	34.1 ± 0.4°	36.0 ± 0.7°	36.6 ± 0.6°	37.1 ± 0.1°[a]	—	—
Control	17–18	—	6	36.9 ± 0.1°	36.8 ± 0.1°	36.9 ± 0.2°	36.9 ± 0.2°	—	—
Isoproterenol, 15 mg./kg.	17–18	21–23	7	36.0 ± 0.2°[b]	38.5 ± 0.4°[b]	38.0 ± 0.4°[c]	37.4 ± 0.4°	—	—
Control	24	—	6	37.6 ± 0.2°	37.6 ± 0.2°	37.6 ± 0.2°[c]	37.7 ± 0.2°	37.7 ± 0.2°	37.6 ± 0.3°
Isoproterenol[d], 15 mg./kg.	24	35	11	36.1 ± 0.2°[a]	38.4 ± 0.3°[c]	38.4 ± 0.3°[c]	38.1 ± 0.2°	37.5 ± 0.1°	37.1 ± 0.1°
Control	Adult	—	3	37.3 ± 0.4°	37.3 ± 0.4°	37.4 ± 0.2°	37.2 ± 0.3°	37.4 ± 0.6°	37.4 ± 0.3°
Isoproterenol, 15 mg./kg.	Adult	1	3	37.0 ± 0.2°	37.1 ± 0.3°	36.2 ± 0.3°[c]	35.4 ± 0.3°[e]	35.2 ± 0.4°[c]	35.1 ± 0.3°[b]

[a] $p < 0.001$, experimental *versus* control animals; *t* test in nonpaired experiments (26). [b] $p < 0.01$, experimental *versus* control animals; *t* test in nonpaired experiments (26). [c] $p < 0.05$, experimental *versus* control animals; *t* test in nonpaired experiments (26). [d] No statistically significant difference in the hyperthermic response between five male and six female rats. [e] $p < 0.02$, experimental *versus* control animals; *t* test in nonpaired experiments (26).

response between young male and female rats after 35 doses (five male and six female rats).

The hyperthermic response following the a.m. administration of the chronic dose was similar to the response of the second daily dose (p.m. administration), which was given about 8 hr. later.

The predose rectal temperature of chronically treated young rats was significantly lower than the comparable rectal temperature of control animals (Table V). In mature rats the first dose of isoproterenol (15 mg./kg.) produced hypothermia (Table V).

Other Observations—In neonatal rats, chronic treatment produced premature opening of the eyelids and scanty fur development. When treatment was started after weaning, marked hair loss was observed usually on the head, back, and/or flanks in approximately half of the treated animals. After chronic dosing, weanling and adult rats became lethargic. The lethargy was interspersed by brief periods of activity which appeared similar to that described by Ingenito and Bonnycastle (20) for *dl*-4-chloro-*N*-methylamphetamine. These authors described the activity as "a peculiar backing up and circling movement." Occasionally, the rats in the present study also exhibited a seemingly purposeless repetitious scratching motion of the wire grid with the forepaws.

Of 54 rats from five litters (14 female and 15 male treated rats and 14 female and 11 male control rats) that were dosed for 30–43 days, seven animals died (five female and two male treated rats). No deaths occurred in three other litters which were treated to obtain additional information on the hyperthermia, lethargy, and hair loss.

DISCUSSION

Growth stunting of neonatal rats treated with isoproterenol was reported by Schneyer and Shackleford (5). Despite the large difference in the doses used by these authors and the dose regimens used in the present investigation, the initial growth retardation was similar in both studies. Schneyer and Shackleford (5) administered 1.5 mg./rat twice a day; depending upon the weight of the young rats, this amounted to approximately 30–200 mg./kg. With these large doses, little effect on growth was seen during the first 7 days, but growth was retarded by 15–20% after 15 days.

According to Schneyer and Shackleford (5), animals treated for 35 days were only one-half the size of control littermates. Such profound growth retardation was not encountered with the smaller doses used in the present study.

The enlargement of the submaxillary and parotid glands and the relative increase in the heart weight (in relation to the body weight) noted in the present study were in agreement with the changes reported by Schneyer and Shackleford (5). In mice, however, the wet weights and ratios were increased for the hearts of male but not female mice (21). The smaller wet weights of the spleen and the adrenal glands observed in young chronically treated rats were in proportion to the somatic growth retardation and, therefore, the ratios were not different from those of the controls.

Since isoproterenol is a potent catecholamine closely related to epinephrine in structure and action, an endocrinological effect mediated *via* the hypothalamic–hypophysial–adrenal or ovarian axis could be responsible for the decreased uterine weight and the smaller tissue to body weight ratio after chronic treatment. A direct action on the uterine smooth muscle cannot be ruled out. In adult mice (21) and rats[5], uterine wet weights showed mean decreases, but these reductions were statistically nonsignificant. However, it is possible that chronic isoproterenol treatment during

[5] G. I. Klingman, unpublished observation.

neonatal development may have a more pronounced or even a different effect than in adult animals.

The absence of a decrease in the total norepinephrine content of the heart and submaxillary and parotid glands in the presence of decreased concentrations and increased tissue weights following chronic isoproterenol treatment could be best explained on the basis of the hypertrophy and/or hyperplasia. However, evidence presented by Mueller and Axelrod (15) indicated that short-term isoproterenol treatment resulted in an accelerated release of norepinephrine from the nerve endings of the heart. A storage defect and an increase in sympathetic activity appeared to be responsible for the decrease in cardiac norepinephrine (15). If this postulate can be extended to long-term treatment that is unaccompanied by changes in the total norepinephrine content, an additional, perhaps adaptive, mechanism must be assumed. It should be recalled at this point that only the cardiac norepinephrine concentration and the ratio (tissue weight to body weight) but not the norepinephrine content and the absolute weight were affected by the treatment.

In view of the fact that the norepinephrine content of the stellate ganglion was similar to that in control rats, the increased norepinephrine concentration cannot be readily interpreted without the acquisition of additional data. The smaller wet weight of the stellate ganglia could be explained in terms of differences in the body weights between treated and control littermates; the difference in ganglionic wet weight amounted to 23% and the difference in body weight to 26%. However, the superior cervical ganglion, the wet weight of which is very uniform among littermates, showed only a small statistically nonsignificant decrease after treatment (Table III).

The diversity of the responses of the rectal temperature following acute and chronic isoproterenol treatment in young and adult rats is difficult to interpret. Pretreatment with α- and β-adrenergic receptor blocking agents prevented or ameliorated the hyperthermia in adult rats, but these agents, when administered alone, produced hypothermia (22). The role of epinephrine and related amines on body temperature, control of body temperature, and heat production has been extensively studied and reviewed (23, 24). The direction of the change in body temperature following the administration of amines (as well as other drugs) seems to depend upon the species, route of administration, and dose. Different ambient temperatures before and after drug administration and the predrug body temperature of the experimental animal can also influence the direction of the change. The different response in adult and neonatal rats to isoproterenol observed in this study requires further exploration. Opposite responses in young and adult chickens have been reported with acute doses of epinephrine, norepinephrine, and several other amines (25). Previous investigations almost exclusively dealt with acute drug administration. Little information is available in regard to chronic drug use, body temperature, and temperature regulation.

The mortality rate encountered in the current investigation was similar to rates (5–30%) seen in several studies carried out in this laboratory when neonatal, young, and adult rats were chronically treated with relatively low doses of isoproterenol (5–15 mg./kg.)[6]. No deaths occurred in young female rats (Wistar strain, 110 ± 2.4 g.) treated for 12 days with large doses of isoproterenol: 50 mg./kg. twice a day and 400 mg./kg. once a day (2).

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Synthesis and Bioactivity of Lincomycin 2-Monoesters

W. MOROZOWICH▲, A. A. SINKULA, F. A. MacKELLAR, and C. LEWIS

Abstract □ A homologous series of straight-chain lincomycin 2-monoesters, comprised of both acyl and carbonate esters, was synthesized. Good antibacterial activity was observed with esters of chain lengths from C_4 to C_{16}. Esters with chain lengths from C_{12} to C_{16} are tasteless and highly active *in vivo* in mice infected with *Staphylococcus aureus*. The carbonate esters show essentially the same taste and bioactivity as the acyl esters. The long-chain lincomycin 2-esters appear to have the desired properties required for formulation as a tasteless pediatric preparation.

Keyphrases □ Lincomycin 2-monoesters—synthesis, antibacterial activity, tasteless long-chain esters □ Antibacterial agents, potential—synthesis of lincomycin 2-monoesters □ Pediatric formulations, potential—synthesis, antibacterial activity of long-chain lincomycin 2-monoesters □ Tasteless lincomycin derivatives—synthesis, antibacterial activity of 2-monoesters

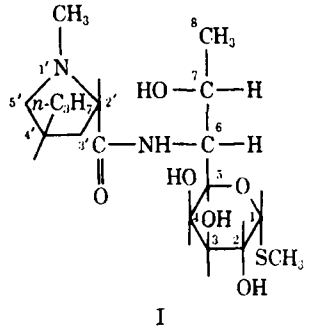
This work is part of a project designed to improve the pharmaceutical properties (*e.g.*, taste and solubility) and biological properties (*e.g.*, absorption and prolonged activity) of the antibiotic lincomycin (I) (1–3) by means of various *in vivo* reversible monoesters.

Molecular models show that each of the four hydroxyl groups in lincomycin is confronted with different steric and electronic environments. The *in vivo* rate of hydrolysis of the four possible positionally isomeric esters is probably determined by the ease of hydrolysis by esterase enzymes or by nucleophiles, such as hydroxide ion. Polyesters of lincomycin show poor antibacterial activity *in vivo*, probably because some esterified hydroxyl groups are more difficult to hydrolyze than others. In view of this possibility, monoesters at each of the four hydroxyl groups in I were synthesized in an attempt to find a hydroxyl that would provide biologically active *in vivo* reversible esters with the desired pharmaceutical or biological properties. Protective group routes were perfected for the synthesis of each of the four isomeric lincomycin monoesters. This report describes the synthesis and properties of lincomycin 2-monoesters, and future reports will deal with the 3- and 4-monoesters.

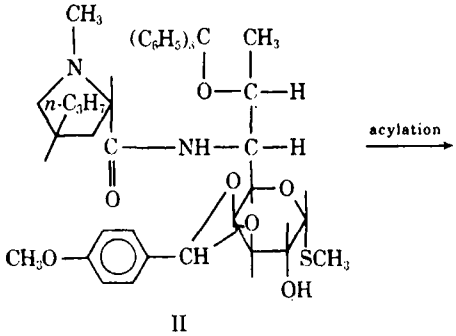
The primary objective of this work was to uncover a tasteless, bioactive lincomycin ester with low water solubility, which would permit formulation of a pharmaceutically elegant pediatric suspension. This objective was met with the long-chain lincomycin 2-monoesters.

RESULTS AND DISCUSSION

Chemistry—The lincomycin 2-monoesters were synthesized (Scheme I) by reaction of the key intermediate 7-*O*-trityl-3,4-*O*-anisylidene lincomycin (II) (4) with an acid chloride, an acid anhydride, or a chlorocarbonate in the presence of pyridine. The resulting 7-*O*-trityl-3,4-*O*-anisylidene lincomycin 2-esters (III) were treated with 80% aqueous acetic acid at 100° for 15–30 min. to remove the trityl and anisylidene protective groups. In most cases, the lincomycin 2-monoesters (IV) were maintained as the hydrochloride salts during workup and purification. The esters are un-



I



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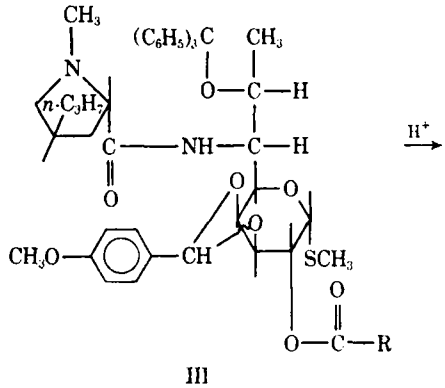

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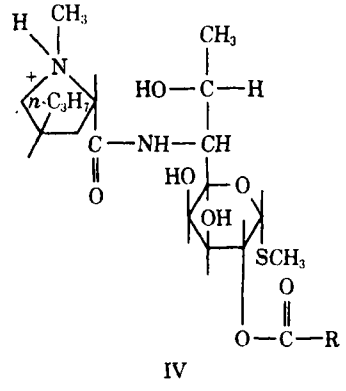

IV

Scheme I—$R = -(CH_2)_nCH_3$ or $-O(CH_2)_nCH_3$

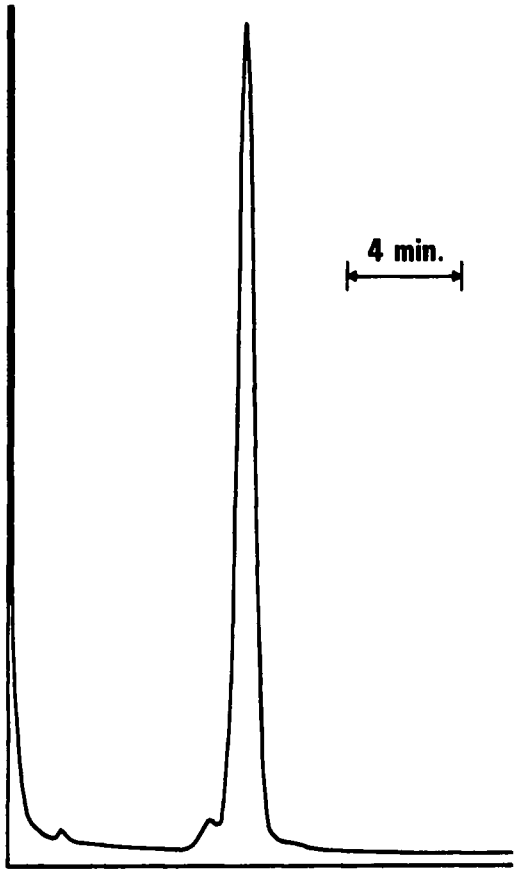

Figure 1—*Gas chromatogram of lincomycin 2-palmitate as the tris-O-trimethylsilyl derivative at 290°.*

stable under alkaline conditions; and in a few cases where crystallization failed to remove the impurities, the esters were extracted from mildly acidic, aqueous solutions with ether. The free base form of the ester in the organic phase was promptly converted to the stable hydrochloride salt.

TLC and GLC analyses of the esters showed only minor impurities in the final products. The GLC of lincomycin 2-palmitate as the *tris-O*-trimethylsilyl ether (Fig. 1) showed a trace impurity at 2 min. The small peak (7.1 min.) preceding the major peak is probably the 2-palmitate ester of lincomycin B (the 4′-ethyl analog of lincomycin), since a similar peak is seen in the GLC of the starting material (1) (5). The isomeric lincomycin 3- and 4-monopalmitates can be separated from lincomycin 2-palmitate, and these results will be reported later.

Bioactivity—The standard curve plate antibacterial assay using *Sarcina lutea* (UC 130) showed maximal activity with C_4–C_8 esters (Fig. 2). It is not clear whether these esters are intrinsically active or whether hydrolysis on the plate accounts for the activity. The effect of ester chain length on the oral CD_{50} (medium protective dose) in mice challenged with *Staphylococcus aureus* (UC 76) showed

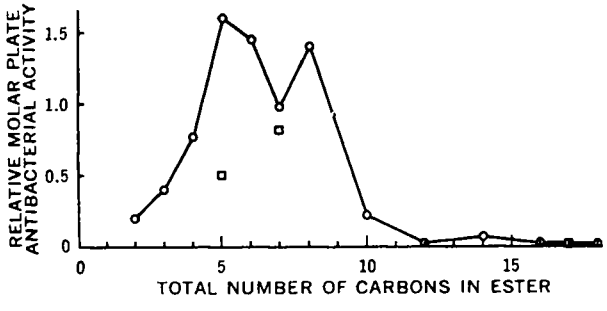

Figure 2—*Ratio of the molar plate antibacterial activity of lincomycin 2-esters to that of lincomycin using* S. lutea *(lincomycin = 1). Key: ○, acyl esters; and □, carbonate esters.*

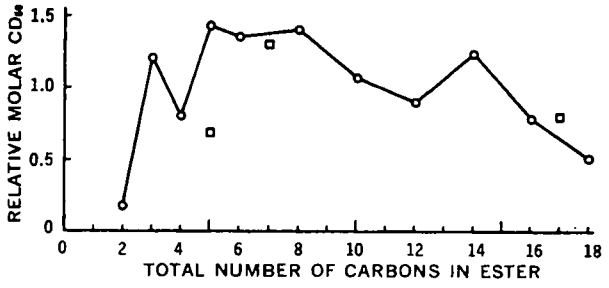

Figure 3—*Ratio of the molar oral CD_{50} of lincomycin 2-esters to that of lincomycin in mice infected with* Staph. aureus *(lincomycin = 1). Key: ○, acyl esters; and □, carbonate esters.*

that good activity was obtained with esters of chain lengths from C_4 to C_{16} (Fig. 3). The subcutaneous CD_{50} results (Fig. 4) were somewhat erratic but, in general, the results showed that the medium-chain length esters had better activity than the short-chain or the long-chain esters. The bioactivity of the carbonate esters was slightly less than the corresponding acyl esters.

Taste and Solubility—The water solubility of the hydrochloride salts of the lincomycin 2-acyl esters approaches zero above C_{12} (Fig. 5). Esters with chain lengths of C_{12} or greater are essentially tasteless. The laurate ester (C_{12}) is remarkable, since it is rather soluble and yet almost tasteless. The solubility of the esters, calculated (6) at pH 6.5, approaches zero above C_6 (Fig. 5). The lack of taste with the moderately water-soluble laurate ester can probably be explained by precipitation of the free base form of the ester in the mouth, since the pH of saliva is approximately 6.5 (7).

The 2-palmitate ester and the 2-*n*-hexadecylcarbonate ester of I have the desired properties of good CD_{50} activity and very low water solubility, indicating potentially good chemical stability in aqueous suspensions, and they are tasteless.

EXPERIMENTAL

Chromatography—TLC was conducted on silica gel G using the solvent system of hexane–ether–2-pentanone–methanol–concentrated ammonium hydroxide (60:20:20:9:1). The compounds were located by spraying the plates with ammonium sulfate followed by heating to about 180°.

GC was conducted after conversion of the lincomycin 2-acyl esters to the corresponding 3,4,7-*tris-O*-trimethylsilyl ethers, as described by Houtman *et al.* (5). A 1% solution of the ester in pyridine containing 20% hexamethyldisilazane and 20% trimethylchlorosilane was used to give the silyl ethers. After a 5-min. reaction period, 1 μl. of the solution was injected into a flame-ionization gas chromatograph[1] using a 0.61 × 33-cm. (0.125 × 17-in.) stainless steel column containing 3% OV-1 on Gas Chrom Q (100–120 mesh), with an oven temperature of 235–290°, depending on the chain length of the ester.

Synthesis—Lincomycin 2-esters were synthesized by reaction of an acid chloride, an acid anhydride, or a chlorocarbonate with II in

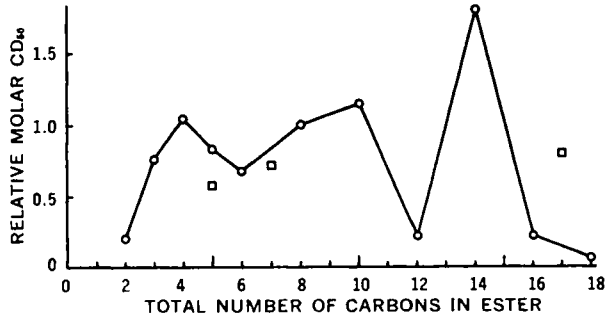

Figure 4—*Ratio of the molar subcutaneous CD_{50} of lincomycin 2-esters to that of lincomycin in mice infected with* Staph. aureus *(lincomycin = 1). Key: ○, acyl esters; and □, carbonate esters.*

[1] F & M model 700.

Table I—Analytical Data on Lincomycin 2-Monoesters Hydrochloride

2-Ester	Empirical Formula	Molecular Weight	Analytical Data[a] Calc.	Found	Water, %	Yield, %	Melting Point
n-Butyrate	$C_{22}H_{41}ClN_2O_7S$	513.10	C 51.50	51.43	4.88	45	185–187°
			H 8.05	7.93			
			Cl —	—			
			N 5.46	5.40			
			S 6.25	5.92			
n-Valerate	$C_{23}H_{43}ClN_2O_7S$	527.13	C 52.41	52.65	5.50	86	155–158°
			H 8.22	7.87			
			Cl 6.73	6.90			
			N 5.31	5.62			
			S 6.08	6.12			
n-Hexanoate	$C_{24}H_{45}ClN_2O_7S$	541.16	C 53.27	54.08	5.13	88	145–147°
				53.66	4.99		
			H 8.38	8.26			
			Cl 6.55	6.67			
			N 5.18	5.26			
			S 5.93	5.92			
n-Heptanoate	$C_{25}H_{47}ClN_2O_7S$	555.18	C 54.54	53.48	2.39	22	—[b]
			H 8.53	8.55			
			Cl 6.39	6.60			
			N 5.05	4.69			
			S 5.78	5.81			
n-Octanoate	$C_{26}H_{49}ClN_2O_7S$	569.22	C 54.86	54.83	2.93	50	—[b]
			H 8.68	8.68			
			Cl 6.23	6.56			
			N 4.92	4.97			
			S 5.63	5.86			
n-Decanoate	$C_{28}H_{53}ClN_2O_7S$	597.27	C 56.31	55.54	2.84	84	—[b]
				55.84	6.18		
			H 8.94	8.78			
			Cl 5.94	6.12			
			N 4.70	4.38			
			S 5.37	5.60			
n-Laurate	$C_{30}H_{57}ClN_2O_7S$	625.27	C 57.62	57.73	2.69	31	111–112.5°
			H 9.19	9.06			
			Cl —	—			
			N 4.48	4.67			
			S 5.13	5.13			
n-Myristate	$C_{32}H_{61}ClN_2O_7S$	653.33	C 58.83	58.32	2.89	69	139–141°
				58.67	3.22		
			H 9.41	9.08			
			Cl 5.43	5.70			
			N 4.29	4.65			
			S 4.91	4.93			
n-Palmitate	$C_{34}H_{65}ClN_2O_7S$	681.43	C 59.93	58.80	1.76	51	116–129°
				59.80	1.10		
			H 9.62	9.74			
			Cl 5.20	5.19			
			N 4.11	4.06			
			S 4.71	4.89			
n-Stearate	$C_{36}H_{69}ClN_2O_7S$	709.43	C 60.95	61.10	2.59	76	171–173.5°
			H 9.80	9.65			
			Cl —	—			
			N 3.95	4.03			
			S 4.52	4.36			
n-Butylcarbonate	$C_{23}H_{43}ClN_2O_8S$	543.14	C 50.86	51.15	3.07	54	—[b]
			H 7.98	8.10			
			Cl 6.53	6.52			
			N 5.16	5.32			
			S 5.90	6.05			
n-Hexylcarbonate	$C_{25}H_{47}ClN_2O_8S$	571.18	C 52.57	52.17	3.58	47	—[b]
			H 8.29	8.53			
			Cl 6.21	6.06			
			N 4.90	4.73			
			S 5.61	5.71			
n-Hexadecyl-carbonate	$C_{35}H_{67}ClN_2O_8S$	711.45	C 59.09	58.28	3.99	46	—[b]
			H 9.49	9.48			
			Cl 4.98	4.85			
			N 3.94	4.20			
			S 4.51	4.47			

[a] Combustion analyses and water determination (Karl Fischer) were conducted at The Upjohn Co. [b] The melting points reported were determined on a Thomas-Hoover capillary melting-point apparatus. The crystals appear to go through several stages of melting to give a wide melting range. The exact melting points are indefinite, since in most cases the compounds darken before melting. GLC showed that all compounds had a purity greater than 95% as estimated by area measurements.

pyridine. Representative examples of the synthesis of acyl esters and carbonate esters are described here, and the other esters were synthesized by similar methods. The analytical data are reported in Table I.

Method A: Esterification with Acyl Chlorides (Lincomycin 2-Stearate Hydrochloride)—A solution of 23.01 g. (0.03 mole) II in 100 ml. pyridine was treated with a solution of 12.12 g. (0.04 mole) stearoyl chloride in 40 ml. chloroform. After 15 min., 10 ml. of

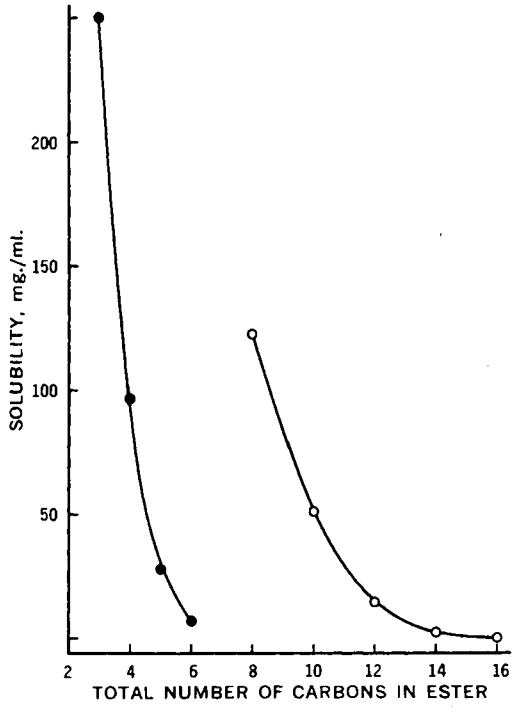

Figure 5—*Aqueous solubility of lincomycin 2-acyl esters* versus *total number of carbons in ester group. Key:* ○, *solubility of the hydrochloride salts of the esters; and* ●, *solubility of the esters calculated at pH 6.5.*

methanol was added and the solvent was removed under vacuum at 60°. The residual yellow semisolid was dissolved in 200 ml. 80% aqueous acetic acid, and the solution was heated on a steam bath for 25 min. Tritanol was removed by filtration after cooling the solution to room temperature, and the filtrate was taken to dryness under vacuum at 60°. The residue was dissolved in 120 ml. 80% aqueous acetone with heat, and the solution was diluted with 250 ml. acetone. The resulting white crystals (18.8 g., 76%) were isolated by filtration and air dried.

Method B: Esterification with Acid Anhydrides—A 2:10 mole ratio of acid anhydride to II was normally used. The reactions were conducted at room temperature or on a steam bath for 5–30 min. After removal of the protective groups, a slight excess of aqueous hydrochloric acid was added to an acetone solution of the ester to give the crystalline hydrochloride salts.

Method C: Esterification with Chlorocarbonates (Lincomycin 2-n-Butylcarbonate Hydrochloride)—A cold (−35°) solution of II (23.5 g.) in 200 ml. pyridine was treated with 5 g. *n*-butyl chlorocarbonate. The stirred suspension was allowed to warm to room temperature, then recooled to −35°, and treated with an additional 15 g. *n*-butyl chlorocarbonate. After warming to room temperature, the suspension was poured into 2 l. cold (0°) 0.01 *N* aqueous hydrochloric acid. The resulting precipitate was dissolved in 1 l. ether, and the phases were separated. The ether was removed under vacuum, and the residue was dissolved in a mixture of 1 l. methanol and 75 ml. water. The solution was adjusted to pH 1.0 with concentrated hydrochloric acid, and after 3 hr. at room temperature the solution was adjusted to pH 3 by addition of sodium bicarbonate. The solvent was removed under vacuum, and the residue was dissolved in 150 ml. chloroform. The chloroform solution was extracted twice with 150 ml. water. The combined aqueous layers were freeze dried to give 9 g. (54%) of white amorphous product.

Solubility Determinations—The solubility of the hydrochloride salts of the esters was determined at 24 ± 1° using excess compound equilibrated with water for 72 hr. Aliquots of the filtrates were evaporated to dryness at 120°, and the residue was weighed.

The solubility of the free base forms of the esters was determined by titration of aqueous solutions of the hydrochloride salts of the esters (1%) with 0.1 *N* aqueous sodium hydroxide. The solutions were filtered through a Millipore filter (1.5 μ) before titration. Titration was followed by periodically measuring the absorbance in a 1-cm. cell at 400 nm. At a certain point during titration, precipitation of the oily free base form of the ester occurred with a concomitant rapid increase in absorbance at 400 nm. Hypersaturation (8) did not occur, since there was no pH drop at the point of precipitation. The amount of base required to produce an absorbance of 0.10 divided by the volume of the solution gave the intrinsic solubility. The solubility at pH 6.5 was calculated (6):

$$S_0 = S_i[1 + \text{antilog}(\text{pKa} - \text{pH})] \qquad \text{(Eq. 1)}$$

where S_0 = solubility at pH 6.5, S_i = intrinsic solubility of the free base form, and pH = pH at which precipitation of the free base occurred.

The solubility determinations were conducted in duplicate, and the results were averaged. Repeat analyses differed by no more than 10%.

NMR Spectra at 60 MHz.—The NMR spectral features were similar to those reported previously (9). The long-chain esters such as the palmitate ester required probe temperatures of 75° to obtain well-resolved spectra.

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▲ To whom inquiries should be directed.

Chemical Modification of Clindamycin: Synthesis and Evaluation of Selected Esters

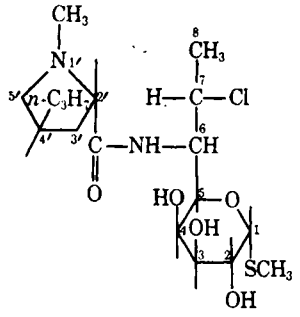
A. A. SINKULA▲, W. MOROZOWICH, and E. L. ROWE

Abstract □ Clindamycin was chemically modified to provide *in vivo* reversible derivatives that might suitably be utilized in special dosage forms. To improve taste properties, a series of 2- and 3-monoesters and some 2,3-dicarbonate esters of clindamycin were synthesized. A number of highly water-soluble salts were prepared in an attempt to provide an injectable intramuscular preparation with low incidence of pain. The long-chain clindamycin 2-monoesters (palmitate and hexadecylcarbonate) are virtually devoid of the characteristic bitter taste of clindamycin. The water-soluble salts (gluconate and lactobionate) of clindamycin showed no significant improvement over clindamycin in muscle irritation studies. Clindamycin blood levels were determined in dogs following oral administration of some esters. Clindamycin 2-palmitate and clindamycin 2-hexadecylcarbonate exhibited blood levels equivalent to clindamycin hydrochloride. The CD_{50} results using mice infected with *Staphylococcus aureus* showed that several esters possess antibacterial activity equivalent to that of clindamycin hydrochloride.

Keyphrases □ Clindamycin esters and salts—synthesis, bioactivity compared to hydrochloride salt, tasteless long-chain esters □ Antibacterial agents, potential—synthesis of clindamycin esters and salts, compared to hydrochloride salt □ Tasteless clindamycin derivatives—synthesis, blood levels in dogs and antibacterial activity in mice compared to hydrochloride salt □ Pediatric formulations, potential—synthesis, antibacterial activity of long-chain clindamycin esters

Clindamycin [7(S)-chloro-7-deoxylincomycin] (I) hydrochloride is a new, semisynthetic antibiotic having a high degree of antibacterial activity against Gram-positive organisms and a lower order of activity against Gram-negative organisms (1, 2). Its spectrum of activity approximates that of erythromycin and lincomycin (3). Clindamycin is well absorbed from the GI tract and affords serum levels approximately twice those of lincomycin (1, 4).

The fact that clindamycin is extremely bitter *per se* precludes its formulation as an acceptable pediatric syrup. Furthermore, the incidence of pain at the injection site is quite high on intramuscular administration of clindamycin hydrochloride. These factors prompted this investigation of the chemical modification of clindamycin for the purpose of enhancing its pharmaceutical acceptability. This article reports the synthesis and evaluation of selected 2- and 3-monoesters and 2,3-dicarbonate esters of clindamycin.



I: clindamycin

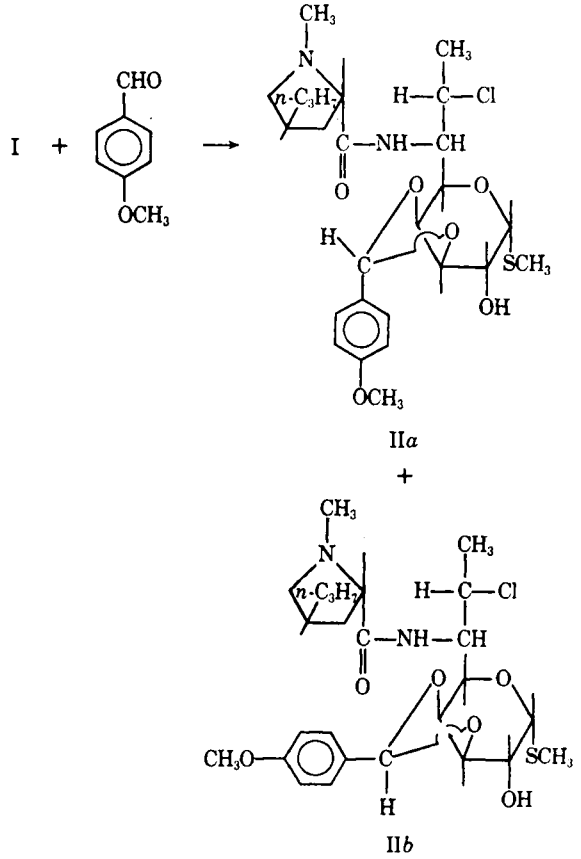
DISCUSSION

Chemistry—The synthesis of 2-esters of clindamycin was achieved by protection of the 3- and 4-hydroxyl groups of clindamycin (I) with the acid labile anisylidene moiety (5, 6). The reaction of anisaldehyde with I gave two spots by TLC, which were identified by NMR as the benzylic isomers II*a* and II*b* (Scheme I). Esterification of the 2-hydroxyl group of 3,4-*O*-anisylidene clindamycin (II), followed by treatment of the resulting ester acetal with acid, afforded pure clindamycin 2-acyl and 2-carbonate esters (Scheme II).

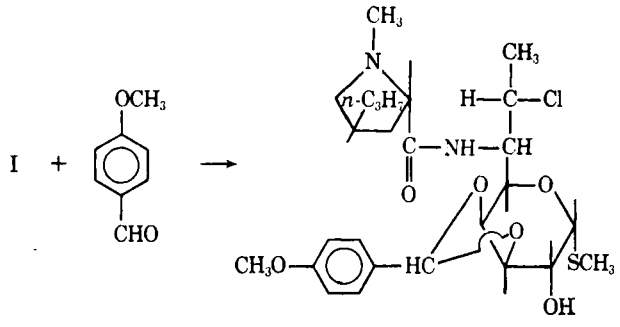
Clindamycin 3-pentylcarbonate was prepared in 54% yield by selective esterification of I in pyridine at low temperatures (−25°) (Scheme III) (7). Purification was achieved by selective solvent extraction under controlled pH conditions while purity was monitored by GLC (Figs. 1 and 2).

Clindamycin 2,3-dicarbonate esters (Scheme IV) were prepared by selective acylation of clindamycin in pyridine using excess acylating agent. The gluconate and lactobionate salts of clindamycin were prepared by mixing aqueous solutions of stoichiometric amounts of clindamycin base with either δ-gluconolactone or lactobiono-δ-lactone and then allowing them to stand for 1 hr., with subsequent removal of water *in vacuo*.

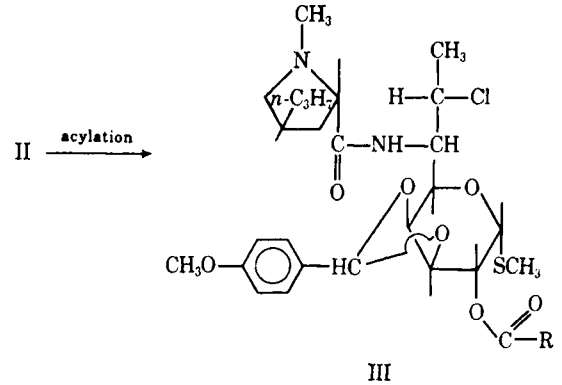
NMR—The 60-MHz. spectra of the benzylic isomers II*a* and II*b* in $CDCl_3$ showed the benzylic proton at 6.28 δ in one isomer and at



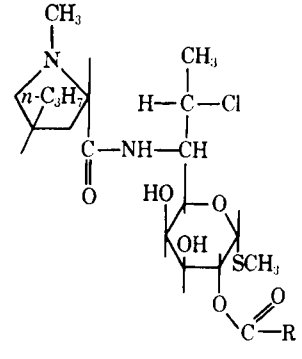
Scheme I—3,4-O-Anisylidene clindamycin



II



III



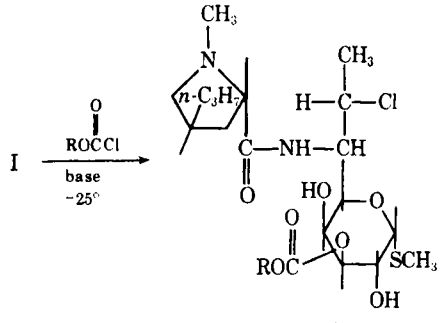
IV

Scheme II—Clindamycin 2-monoesters (R = alkyl and R = O-alkyl)

5.80 δ in the other (dimethylformamide-d_7). The isomer with a signal at 6.16 δ was assigned to II*b* since 3,4-*O*-anisylidene lincomycin shows the benzylic proton at 6.16 δ.

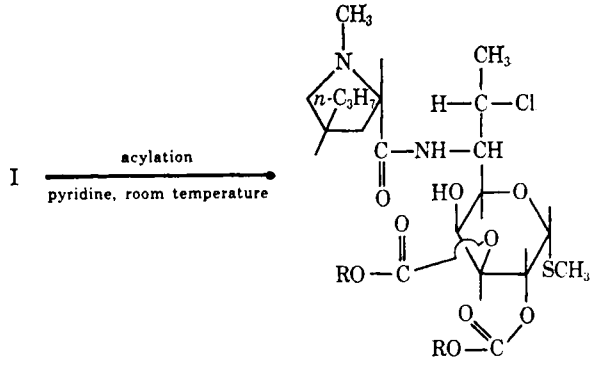
The 60-MHz. NMR spectra of the 2-clindamycin monoesters were similar to the spectra of the 2-monoesters of lincomycin and were thoroughly reviewed in previous reports (8, 9).

Bioactivity—The methods used for determining *in vitro* and *in vivo* bioactivities of clindamycin esters are the same as those used for lincomycin esters and were discussed previously (10).



54%

Scheme III—Clindamycin 3-monocarbonate esters (R = $-C_5H_{11}$)



Scheme IV—Clindamycin 2,3-dicarbonate esters

The bioactivities of the various clindamycin esters (both *in vitro* and *in vivo*) are summarized in Table I. The *in vitro* data show all esters to be less bioactive than clindamycin. This is to be expected since ester hydrolysis must occur to liberate free clindamycin. Many esterified antibiotics have been shown to be inactive until ester hydrolysis occurs (11, 12). In all probability, this is the situation with clindamycin 2-hexanoate, 2-hexylcarbonate, 2-cinnamate, and 3-pentylcarbonate. The above-mentioned short- to medium-chain length derivatives would be more soluble in the culture media than, for example, clindamycin 2-palmitate[1], 2-hexadecylcarbonate, or 2,3-dicarbonates. Increased solubility would increase the availability of enzymatic hydrolysis. This is apparent from the plate bioactivity data for the short- to medium-length chain compounds *versus* the longer chain derivatives.

The mouse subcutaneous *in vivo* data for the clindamycin 2-derivatives indicate that bioactivity is comparable to clindamycin up to and including an ester chain length of six carbons. The bioactivity decreased with esters containing 12 or more carbon atoms. Clindamycin 2-(*o*-benzoyl)benzoate bioactivity is low due probably to difficulty of hydrolysis of the sterically hindered ester. The clindamycin 2,3-dicarbonate esters possess low subcutaneous activity due to the probable low solubility of these compounds. The oral antibacterial activity of the esters roughly correlates with the subcutaneous activity pattern.

Dog serum levels for selected clindamycin esters and salts are shown in Table II. Many of the esters show an area under the blood level curve comparable to clindamycin hydrochloride, indicating high bioavailability.

Taste Studies—Four clindamycin 2-acyl esters of varying chain length (palmitate, laurate, hexanoate, and acetate) were dissolved in 30% sucrose solutions. The clindamycin ester solutions were coded A, B, C, and D, and 5-ml. samples of each were given to a taste panel of 26 people. The usual protocol was followed, with 1 hr. allowed between samples. The taste of each was rated on a scale of 1 to 9 (1 = poor, 9 = excellent). The raw score data were analyzed by computer to show any significant differences between samples.

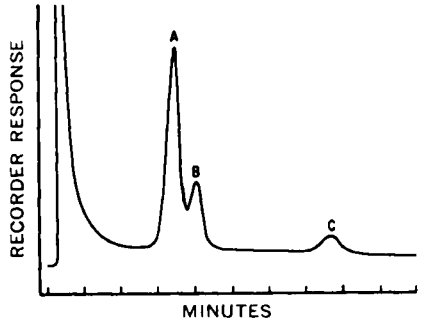


Figure 1—*Products of 7(S)-chloro-7-deoxylincomycin 3-pentylcarbonate reaction (see* Experimental *section). Key: A, 7(S)-chloro-7-deoxylincomycin 3-pentylcarbonate; B, 7(S)-chloro-7-deoxylincomycin 2-pentylcarbonate; and C, 7(S)-chloro-7-deoxylincomycin 2,3-dipentylcarbonate.*

[1] Available commercially as Cleocin Palmitate, The Upjohn Co., Kalamazoo, Mich.

Table I—Antibacterial Activity of Clindamycin 2- and 3-Monoesters and 2,3-Diesters[a]

Ester	Antibacterial Activity— *In Vitro* Activity, mcg./mg.[b]	Relative Median Protective Dose (CD_{50})[c] Subcutaneous	Oral
Clindamycin 2-hexanoate hydrochloride	690 (69)	1.31	0.67
Clindamycin 2-hexylcarbonate hydrochloride	330 (33)	1.36	0.43
Clindamycin 2-laurate hydrochloride	20 (2.0)	<0.1	<0.28
Clindamycin 2-palmitate hydrochloride	21 (2.1)	<0.3	0.21
Clindamycin 2-hexadecylcarbonate hydrochloride	<4 (<0.4)	<0.1	0.23
Clindamycin 2-(1-adamantoate) hydrochloride	9 (0.9)	1.26	0.81
Clindamycin 2-(*p*-benzoyl)benzoate hydrochloride	55 (5.5)	1.67	0.54
Clindamycin 2-(*o*-benzoyl)benzoate hydrochloride	7 (0.7)	<0.15	0.29
Clindamycin 2-cinnamate hydrochloride	260 (26)	1.73	2.33
Clindamycin 2-diphenylacetate hydrochloride	—[d]	—[d]	—[d]
Clindamycin 2-succinate	375 (37.5)	0.83	0.93
Clindamycin 3-pentylcarbonate hydrochloride	180 (18)	0.37	0.67
Clindamycin 2,3-dibutylcarbonate hydrochloride	<4 (<0.4)	0.39	0.80
Clindamycin 2,3-dipentylcarbonate hydrochloride	<4 (<0.4)	<0.1	0.20
Clindamycin 2,3-dihexylcarbonate hydrochloride	<4 (<0.4)	0.18	0.60
Clindamycin lactobionate	520 (52)	0.69	0.94
Clindamycin gluconate	620 (62)	0.63	1.05

[a] Activities calculated as clindamycin base equivalents. [b] As measured on a standard curve agar assay *versus Sarcina lutea*. Results expressed as micrograms of clindamycin base activity per milligram of ester and as percent of lincomycin base activity (in parentheses). [c] Median protective dose relative to that of clindamycin (clindamycin = 1.0) in the mouse. [d] Data not available.

The average scores shown in Fig. 3 indicate a linear trend of taste improvement with increasing chain length. The palmitate ester is significantly better than the laurate at the 5% level of confidence. The laurate, in turn, is significantly better than both the hexanoate and acetate. These latter two were ranked equally low, being very bitter-tasting compounds.

Clindamycin 2-palmitate hydrochloride is essentially tasteless, and dog blood level studies (Table II) showed that *in vivo* regeneration of clindamycin is quantitative. For these reasons, clindamycin 2-palmitate hydrochloride is being extensively tested in humans as a tasteless pediatric formulation.

EXPERIMENTAL

GLC was run on the silanized clindamycin esters. The silyl ethers were made by mixing a pyridine solution containing approximately 2% of the ester to be silanized, 20% trimethylchlorosilane, and 20% hexamethyldisilazane. One microliter of this solution was injected into a gas chromatograph[2] containing a flame-ionization detector. The column was 0.3 × 43.2-cm. (0.12 × 17-in.) stainless steel, containing 1% OV-1 on Gas Chrom Q (100–120 mesh). Oven temperatures ranged from 200 to 245° and were linearly programmed at 3°/min.

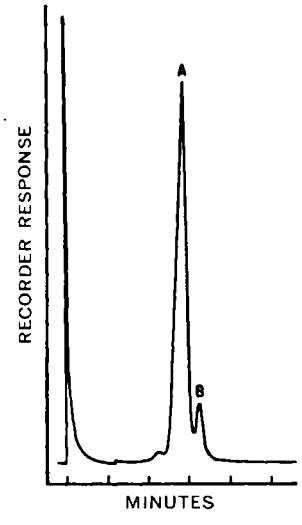


Figure 2—*Two-extraction purification of 7(S)-chloro-7-deoxylincomycin 3-pentylcarbonate. Key: A, 7(S)-chloro-7-deoxylincomycin 3-pentylcarbonate; and B, 7(S)-chloro-7-deoxylincomycin 2-pentylcarbonate.*

TLC was conducted on TLC plates[3] precoated with silica gel GF (250-μ thickness). A solvent system of hexane–ether–methyl propyl ketone–methanol–ammonia (60:20:20:9:1) (Solvent A) was used. Spots were developed with 20% aqueous ammonium sulfate, and the plate was then heated after spraying to char the organic material present.

Typical examples of reaction conditions for the synthesis of various clindamycin esters are illustrated. Analytical data are listed in Table III.

3,4-*O*-*p*-Anisylidene-7(S)-chloro-7-deoxylincomycin Hydrochloride—*Synthesis*—A solution of 65.0 g. 7(S)-chloro-7-deoxylincomycin hydrochloride hemihydrate dissolved in a mixture of 90 ml. dimethylformamide, 120 ml. anisaldehyde, and 250 ml. benzene was heated to 90°. The benzene–water azeotrope was allowed to distil at 90–95° and, upon collecting each 100 ml. distillate, an additional 100 ml. dry benzene was added. After the collection of 1000 ml. benzene, an extra 100 ml. dry benzene was added and some crystallization occurred. After 4500 ml. distillate was collected, an additional 500 ml. benzene was added. Fifty milliliters distillate was collected and the reaction mixture was allowed to cool to room temperature. The yellow reaction mixture became thick with crystals. The solids were isolated by filtration, washed with 100 ml. benzene–dimethylformamide (9:1), and finally washed with benzene. The resulting crystals were dried *in vacuo* at 45°.

Separation of Isomers by Preparative TLC—The isomers of 3,4-*O*-*p*-anisylidene-7(S)-chloro-7-deoxylincomycin were separated by preparative TLC, using silica gel G (0.25-mm. layers) with Solvent A. After development, the chromatograms were visualized with UV light and the isomers were scraped off the plate and eluted with methanol. The methanol extracts of the acetals were taken to dryness, yielding viscous tan liquids. The mass spectra of the two isomers were identical.

Clindamycin 2-Monoesters—*7(S)-Chloro-7-deoxylincomycin 2-Hexylcarbonate Hydrochloride*—3,4-*O*-Anisylidene-7(S)-chloro-7-deoxylincomycin hydrochloride (11.59 g., 0.02 mole) was dissolved in 100 ml. of analytical reagent grade pyridine, and the solution was cooled to −30°. Hexyl chloroformate[4] (6.29 g., 0.0382 mole) was added dropwise with good stirring. An additional 2 g. of hexyl chloroformate was added at 1-hr. intervals. The reaction was terminated after 3.5 hr. of stirring by pouring into 1 l. of ice water acidified to pH 2 with concentrated hydrochloric acid. The resulting aqueous suspension was extracted with three 250-ml. portions of chloroform, the chloroform extracts were combined and dried with anhydrous magnesium sulfate, and the chloroform was

[2] F & M model 700.

[3] Uniplates, Analtech, Inc.

[4] Eastman White Label.

Table II—Dog Serum Levels of Clindamycin Esters and Salts

Clindamycin Derivative	Dose, mg./kg. Clindamycin Base Equivalent	Serum Level, mcg./ml., as Clindamycin Base						Area[b] under Curve
		0.5 hr.	6 hr.	12 hr.	24 hr.	T_{50}	Peak Concentration[a]	
Clindamycin 2-hexanoate hydrochloride	25 (oral)	10.72	4.71	2.57	1.59	6.3	10.72 (0.5)	88.9
Clindamycin 2-hexylcarbonate hydrochloride	10 (i.m.)	0	0.59	0.5	<0.35	11.2	0.59 (4 and 6)	11.1
Clindamycin 2-laurate hydrochloride	25 (oral)	7.38	4.00	2.15	1.32	9.46	8.40 (2)	76.11
Clindamycin 2-palmitate hydrochloride	25 (oral)	7.19	4.05	2.21	1.34	9.39	8.29 (1)	76.09
Clindamycin 2-hexadecylcarbonate hydrochloride	25 (oral)	3.0	3.01	1.5	1.01	9.7	5.76 (2)	69.5
Clindamycin 2-(1-adamantoate) hydrochloride	25 (oral)	9.15	4.0	1.92	1.04	4.8	13.52 (1)	81.9
Clindamycin 2-(*p*-benzoyl)benzoate hydrochloride	25 (oral)	2.94	1.98	0.99	0.64	5.9	4.08 (2)	35.9
Clindamycin 2-(*o*-benzoyl)benzoate hydrochloride	25 (oral)	0	0.54	0.46	<0.38	10.5	0.65 (2)	10.5
Clindamycin 2-cinnamate hydrochloride	25 (oral)	4.1	2.33	1.58	0.97	7.1	5.44 (1)	48.4
Clindamycin 2-diphenylacetate hydrochloride	25 (oral)	1.45	3.07	1.65	1.25	8.2	4.03 (2)	50.3
Clindamycin 2-succinate	10 (i.m.)	4.11	2.0	0.96	0.7	5.4	4.69 (1)	38.8
Clindamycin 2,3-dibutylcarbonate hydrochloride	25 (oral)	3.09	3.89	2.15	1.31	6.8	7.85 (2)	71.6
Clindamycin 2,3-dihexylcarbonate hydrochloride	25 (oral)	0.5	0.38	0	0	3.55	0.67 (1)	3.95
Clindamycin lactobionate	10 (i.m.)	4.7	2.07	1.04	0.26	4.9	5.3 (1)	37.8
Clindamycin gluconate	10 (i.m.)	5.75	3.26	1.87	1.36	7.0	6.15 (1)	61.8
Clindamycin hydrochloride	25 (oral)	14.45	3.55	1.83	1.15	4.7	14.45 (0.5)	76.8
Clindamycin hydrochloride	10 (i.m.)	4.31	2.49	1.09	0.74	5.7	4.89 (2)	43.2

[a] Number in parentheses equals time in hours. [b] Area expressed as concentration × time.

removed under reduced pressure at 38°. The residue was dissolved in 100 ml. of methanol and 40 ml. of distilled water. The pH of the solution was adjusted to 1.5 with concentrated hydrochloric acid and stirred for 75 min. The pH was then lowered to 1, and the solution was stirred for 3.75 hr. Sodium bicarbonate solution was then used to adjust the solution to pH 3.5, and all solvent was removed under reduced pressure at 36°. Two hundred milliliters of chloroform was then added to precipitate the sodium chloride, and the solvent was removed at high vacuum at 60°. One hundred milliliters of acetone was added to precipitate the desired product, which was subsequently washed with two 50-ml. portions of ether. The yield was 8.85 g. (75%).

7(S)-Chloro-7-deoxylincomycin 2-Palmitate Hydrochloride—A solution of 23.18 g. (0.04 mole) of 3,4-*O*-*p*-anisylidene-7(S)-chloro-7-deoxylincomycin hydrochloride dissolved in 400 ml. dry pyridine and 40 ml. analytical reagent grade chloroform was treated with a solution of 24.38 g. (0.08 mole) of palmitoyl chloride dissolved in 50 ml. chloroform. The acid chloride solution was added dropwise to the stirred pyridine solution over 1 hr. The pale-orange solution was diluted with 100 ml. isopropyl alcohol, and the solvent was removed under high vacuum at 60°. Then the viscous orange residue was dissolved in 200 ml. hot glacial acetic acid, and the resulting solution was diluted with 40 ml. of water. The solution was heated on a steam bath for 15 min., and the solvent was removed under high vacuum at 60°. The orange residue was dissolved in 100 ml. isopropyl alcohol and evaporated as before. The orange residue was dissolved in 150 ml. hot acetone, and the solution was added dropwise to 1500 ml. acetonitrile while stirring. The resulting precipitate of 7(S)-chloro-7-deoxylincomycin 2-palmitate hydrochloride was isolated by filtration under nitrogen pressure. The cake was washed with acetonitrile and dried by passing nitrogen through the filter for 15 min. Final drying was carried out at room temperature under high vacuum. The yield was 23.5 g. (84%).

7(S)-Chloro-7-deoxylincomycin 2-(p-Benzoyl)benzoate Hydrochloride—The preparation of *p*-(benzoyl)benzoyl chloride is as follows. *p*-Benzoyl benzoate (6.78 g.), recrystallized from water, was placed in a 125-ml. conical flask. Enough thionyl chloride was then added to cover the white powder and form a thin slurry. Excess thionyl chloride was removed, along with evolved sulfur dioxide and hydrogen chloride gas, by heating on a steam bath. The pale-yellow crystals were dried overnight at 45°, and the yield was 5.5 g.

The esterification procedure used is as follows. 3,4-*O*-*p*-Anisylidene-7(S)-chloro-7-deoxylincomycin hydrochloride (5.8 g., 0.01 mole) was dissolved in 35 ml. pyridine. *p*-(Benzoyl)benzoyl chloride (2.94 g., 0.012 mole) was dissolved in 10 ml. chloroform and added dropwise to the stirred pyridine solution. About halfway through the addition, a white precipitate formed which remained until heating (~50°) caused it to redissolve. Subsequent cooling to room temperature caused no further precipitation. TLC indicated that the reaction was half complete after 4 hr. at room temperature. Another 1.5 g. *p*-(benzoyl)benzoyl chloride dissolved in 5 ml. chloroform was added dropwise, and the reaction flask was warmed to 45°. TLC showed the reaction to be 90% complete. After addition of 3 ml. water the solvents in the reaction flask were removed *in vacuo*. The residue was washed with three 100-ml. portions of isopropyl alcohol, which in turn was removed *in vacuo*. The light-yellow residue was dissolved in 40 ml. glacial acetic acid and heated on a steam bath. To this was added 10 ml. water, and the solution was heated for 40 min. The yellow solution was evaporated *in vacuo;* the residue was washed with 100 ml. isopropyl alcohol, 90 ml. isopropyl alcohol plus 15 ml. 1 *N* HCl, and then 100 ml. isopropyl alcohol again, with the solvents being removed *in vacuo*. The residue was dissolved in 50 ml. chloroform, and the clear solution was added slowly to 500 ml. of cold anhydrous ether. The white precipitate was filtered and dried under nitrogen. TLC showed essentially pure compound.

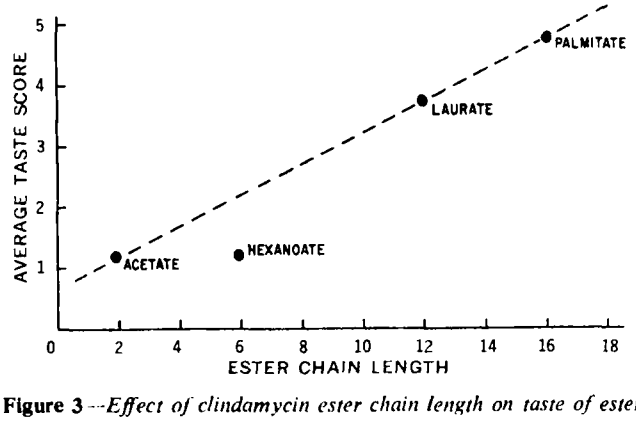


Figure 3—*Effect of clindamycin ester chain length on taste of ester in syrup.*

Table III—Analytical Data Obtained with Clindamycin Ester Hydrochlorides and Salts

Clindamycin Derivative	Empirical Formula	Molecular Weight	Analysis[a] Calc.	Found	Yield, %	Melting Point[b]
2-Hexanoate	$C_{24}H_{44}Cl_2N_2O_6S$	559.16	C 51.51	50.94	50	171–175°
			H 7.93	8.14		
			Cl 12.67	12.45		
			N 5.01	4.97		
			S 5.73	5.77		
2-Hexylcarbonate	$C_{25}H_{46}Cl_2N_2O_7S$	589.6	C 50.93	50.57	75	206–209° dec.
			H 7.86	7.84		
			Cl 12.03	12.16		
			N 4.75	4.50		
			S 5.44	5.26		
2-Laurate	$C_{30}H_{56}Cl_2N_2O_6S$	643.77	C 55.97	56.11	10	145–160°
			H 8.77	8.66		
			Cl 11.02	9.96		
			N 4.35	4.36		
			S 4.98	4.91		
2-Palmitate	$C_{34}H_{64}Cl_2N_2O_6S$	699.89	C 58.35	58.20	84	126° dec.
			H 9.22	9.15		
			Cl 10.13	9.91		
			N 4.00	3.81		
			S 4.85	4.46		
2-Hexadecylcarbonate	$C_{35}H_{66}Cl_2N_2O_7S$	729.87	C 57.60	57.34	38	190–193° dec.
			H 9.12	9.57		
			Cl 9.72	9.63		
			N 3.84	3.65		
			S 4.39	4.43		
2-(1-Adamantoate)	$C_{29}H_{48}Cl_2N_2O_6S$	623.66	C 55.85	55.62	45	159–162° dec.
			H 7.76	7.89		
			Cl 11.37	11.23		
			N 4.49	4.45		
2-(*p*-Benzoyl)benzoate	$C_{32}H_{42}Cl_2N_2O_7S$	669.68	C 57.39	56.55	57	214–216°
			H 6.32	6.11		
			Cl 10.59	10.35		
			N 4.18	4.24		
			S 4.79	4.51		
2-(*o*-Benzoyl)benzoate	$C_{32}H_{42}Cl_2N_2O_7S$	669.68	C 57.39	58.57	50	203–207°
			H 6.32	6.46		
			Cl 10.59	9.40		
			N 4.18	4.96		
			S 4.79	4.19		
2-Cinnamate	$C_{27}H_{40}Cl_2N_2O_6S$	591.0	C 54.83	54.98	85	170–172° dec.
			H 6.82	6.59		
			Cl 11.99	11.61		
			N 4.74	4.48		
			S 5.42	5.13		
2-Diphenylacetate	$C_{32}H_{44}Cl_2N_2O_6S$	655.70	C 58.62	58.40	75	206–208° dec.
			H 6.76	6.88		
			Cl 10.82	11.45		
			N 4.27	4.34		
			S 4.89	4.77		
2,3-Dibutylcarbonate	$C_{28}H_{50}Cl_2N_2O_9S$	661.69	C 50.83	50.92	38	205–207°
			H 7.62	8.32		
			Cl 10.72	11.21		
			N 4.23	4.23		
2,3-Dihexylcarbonate	$C_{32}H_{58}Cl_2N_2O_9S$	717.80	C 53.55	53.63	48	202–203°
			H 8.14	8.04		
			Cl 9.88	10.04		
			N 3.90	4.07		
3-Pentylcarbonate	$C_{24}H_{44}Cl_2N_2O_7S$	575.58	C 50.08	48.24	49	145–150°
			H 7.71	7.60		
			Cl 12.32	13.17		
			N 4.87	4.61		
3,4-*O*-*p*-Anisylidene-7(S)-chloro-7-deoxylincomycin hydrochloride	$C_{26}H_{40}Cl_2N_2O_6S$	579.61	C 53.88	53.76	75	155–161° dec.
			H 6.96	7.12		
			Cl 12.23	11.85		
			N 4.83	5.26		
			S 5.53	5.54		
Lactobionate salt	$C_{30}H_{55}ClN_2O_{17}S$	783.28	C 46.00	45.30	80	113–116°
			H 7.08	7.31		
			Cl 4.53	4.03		
			N 3.57	2.95		
Gluconate salt	$C_{24}H_{43}ClN_2O_{12}S$	621.13	C 46.41	45.54	80	73–75°
			H 7.30	7.51		
			Cl 5.71	5.26		
			N 4.51	4.16		

[a] Corrected for water content. [b] Melting points are of hydrated samples and were determined on a Thomas-Hoover melting-point apparatus and are uncorrected.

7(S)-Chloro-7-deoxylincomycin 2-(1-Adamantoate) Hydrochloride—3,4-*O*-*p*-Anisylidene-7(S)-chloro-7-deoxylincomycin hydrochloride (5.8 g., 0.01 mole) was dissolved in 35 ml. pyridine, and the solution was cooled to 4°. Then 2.4 g. (0.012 mole) adamantoyl chloride was dissolved in 10 ml. chloroform, and this was added dropwise to the stirred pyridine solution. The temperature was held between 5 and 8° during the addition and for 30 min. thereafter. TLC after 2 hr. indicated that 90% of the starting material had reacted. One gram adamantoyl chloride in 5 ml. chloroform was added dropwise to the pyridine solution (0–5°). After standing at room temperature overnight, the solvents were removed *in vacuo*.

The residue was treated with 80 ml. methanol and once with 80 ml. methanol plus 10 ml. 1 *N* HCl; each time the resulting solution was evaporated to dryness. The yellow gummy residue was dissolved in 40 ml. glacial acetic acid and heated on the steam bath for 5 min. Twenty milliliters water was added, and the solution was heated for another 30 min. The solvent was removed *in vacuo* as before, and the residue was washed three times with 80 ml. isopropyl alcohol, which was removed *in vacuo*. The product, a yellow oil, was dissolved in 40 ml. chloroform and extracted twice with 40-ml. portions of pH 2.2 buffer. The organic phase was dried (anhydrous sodium sulfate), made acidic with anhydrous hydrochloric acid (pH 1–2, pHydrion paper), and reduced to dryness. The residue was dissolved in 10 ml. chloroform and poured slowly into 500 ml. of stirred ether at room temperature. The white precipitate was isolated by filtration, washed with 75 ml. ether, and dried at 45° *in vacuo*.

Clindamycin 3-Monoesters [7(S)-Chloro-7-deoxylincomycin 3-Pentylcarbonate Hydrochloride]—A solution of 9.63 g. (0.02 mole) of 7(S)-chloro-7-deoxylincomycin hydrochloride in 100 ml. of pyridine was cooled to about −30°, and 6.02 g. (0.04 mole) of *n*-amyl chlorocarbonate was added dropwise. This solution was warmed to room temperature, stirred for about 1 hr., and cooled to about −30°. Then 2 g. of *n*-amyl chlorocarbonate was added, and the solution was stirred for an additional 2 hr. The pyridine solution was poured into 1 l. of ice water acidified to pH 2 with concentrated hydrochloric acid. The resulting aqueous suspension was extracted with two 250-ml. portions of chloroform, and the chloroform extracts were combined and dried over anhydrous magnesium sulfate. The chloroform was removed under vacuum at 38°. The resulting white precipitate was shaken with 750 ml. of anhydrous ether; the product was collected by filtration, air dried for about 1 hr., and then dried for about 16 hr. under vacuum at 45°. The crude product (6.75 g.) contained both the 3-ester and the 2-ester in about a 2:1 ratio (Fig. 1) and was equilibrated between 700 ml. of ether and 460 ml. of aqueous buffer of pH 2.2. The ether layer was removed and discarded, and the aqueous layer was extracted again with 800 ml. of ether. The aqueous layer was extracted with 780 ml. of chloroform (in two portions), and the chloroform extracts were pooled, dried with anhydrous sodium sulfate, and evaporated to dryness under vacuum at about 50°. The resulting residual light-yellow oil was taken up in 80 ml. of chloroform, which was made acidic with anhydrous hydrogen chloride, and again reduced to dryness under vacuum at about 50° to give 2.65 g. (49%) of white solid, 7(S)-chloro-7-deoxylincomycin 3-pentylcarbonate hydrochloride. The GLC[5] pattern (Fig. 2) showed only a trace of the 2-ester.

Clindamycin 2,3-Dicarbonate Esters [7(S)-Chloro-7-deoxylincomycin 2,3-Dihexylcarbonate Hydrochloride]—7(S)-Chloro-7-deoxylincomycin hydrochloride monohydrate (4.79 g., 0.01 mole) was dissolved in 75 ml. of analytical reagent grade pyridine and cooled to −30°. Hexyl chloroformate[4] (6.59 g., 0.04 mole) was added dropwise with rapid stirring. The reaction mixture was slowly warmed to room temperature and stirred for 2 hr. TLC (silica gel plates, Solvent A) indicated that the reaction was not complete, so an additional 1.5 g. of hexyl chloroformate was added to the recooled (−30°) reaction mixture. The mixture was again warmed to room temperature and stirred for 1 hr. The reaction mixture was again cooled and an additional 0.5 g. of hexyl chloroformate was added. The pyridine solution was poured into 1 l. of ice water and extracted with two 250-ml. portions of ether. The combined ether fractions were dried with anhydrous magnesium sulfate. Ether and pyridine were removed under reduced pressure, and the resulting syrup was dissolved in 100 ml. of anhydrous ether. Anhydrous hydrogen chloride gas was bubbled through the ether solution for 2 min. After standing for 10 min., a gelatinous precipitate formed. The ether was removed under reduced pressure (water aspirator), and the resulting tan syrup solidified on standing. The solidified syrup was shaken with two 75-ml. portions of anhydrous ether and filtered.

Clindamycin Salts—*7(S)-Chloro-7-deoxylincomycin Lactobionate*—Lactobiono-δ-lactone (13.61 g., 0.04 mole) was dissolved in 100 ml. of water, heated on a steam bath for 1 hr., and filtered. This solution was added slowly to 17 g. (0.04 mole) of 7(S)-chloro-7-deoxylincomycin base dissolved in 50 ml. of acetone. The resulting yellow solution was stirred for 1 hr., and then all solvent was removed *in vacuo* (200 μ) at 38°. The resulting syrupy precipitate was shaken with 100 ml. of anhydrous acetone for 1.5 hr. The resulting gum was rubbed in a mortar with a small amount of acetone until a homogeneous suspension was formed. The suspension was filtered, and the precipitate was dried *in vacuo* for 1 hr. at 55°.

7(S)-Chloro-7-deoxylincomycin Gluconate—δ-Gluconolactone (7.1 g., 0.04 mole) was dissolved in 50 ml. of distilled water, heated on a steam bath for 1.5 hr., and then cooled to room temperature. This solution was added dropwise to 17 g. (0.04 mole) of 7(S)-chloro-7-deoxylincomycin base dissolved in 25 ml. of anhydrous acetone. The resulting solution was stirred at room temperature for 1 hr. The solvent was removed *in vacuo*, and the resulting gum was treated with acetone. About 20 g. (80%) of white amorphous powder was obtained and dried *in vacuo*.

[5] Instrument, F&M 700 with flame-ionization detector; carrier gas, helium (20 ml./min.); column, 43.2 × 1.3-cm. (17 × 0.5-in.) stainless steel; support, Gas Chrom Q (100–200 mesh); stationary phase, 1% OV-1; injector port temperature, 275°; detector temperature, 275°; oven temperature, linearly programmed from 200 to 245° at 3°/min.; chart speed, 0.5 in./min.; and attenuation, $10^2 \times 5$.

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Lacrimal and Instilled Fluid Dynamics in Rabbit Eyes

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Abstract □ Drainage of an instilled drug solution away from the eye is responsible for a considerable loss of drug and, hence, affects the biological activity of drugs in the eye. The rate of this drainage is related to the volume of drug solution instilled and increases with increasing volume. A demonstration of the influence of this solution drainage on drug activity is presented using miosis studies in albino rabbits. It is shown that over a range of 5–50 μl. of instilled volumes, using various concentrations of pilocarpine nitrate so that the total amount of drug instilled at each volume is the same, there is a fourfold change in the area under the pupillary diameter–time curves. A simple isotopic technique, using radioactive technetium-adsorbed colloid, was developed and utilized in this study to determine lacrimal volume and turnover rate of lacrimal fluid as well as to evaluate drainage of instilled solutions in albino rabbits. By using both a sampling and a nonsampling technique with the technetium colloid, it is shown that unanesthetized rabbits have a lacrimal volume of 7.5 ± 2.5 μl. whereas anesthetized rabbits have a slightly larger volume of 12.0 ± 2.5 μl. In addition, lacrimal fluid turnover rate in unanesthetized rabbits is approximately 0.53 μl./min., with a range of 0.47–0.66 μl./min., while in anesthetized animals it is negligibly small or absent. Drainage loss of instilled solutions was also determined and analyzed. It is shown that the loss of instilled solution *via* drainage is first order with respect to time. The apparent first-order rate constant in unanesthetized rabbits contains a volume-dependent and a volume-independent term, whereas in anesthetized rabbits the apparent rate constant is independent of volume instilled. A discussion of the meaning of these results as related to the mechanism of fluid drainage from the eye is presented. A comparison of the present studies in rabbits to expected behavior in humans is made. It is suggested that drainage rates of instilled solutions in humans should be larger than those of rabbits; therefore, to maximize activity of drugs in humans, the drop size of ophthalmic delivery systems ought to be reduced from its present 50–75-μl. size to at most a 5- or 10-μl. drop.

Keyphrases □ Ophthalmic solutions—determination of lacrimal and instilled fluid dynamics, relationship to drug activity, rabbits □ Miosis—used to study lacrimal and instilled fluid dynamics in the rabbit eye, relationship to drug activity, rabbits □ Fluid dynamics, ophthalmic—lacrimal fluid turnover and instilled volume drainage, rabbits □ Technetium, ^{99m}Tc, colloid—used to study lacrimal volume, lacrimal fluid turnover rate, and drainage of instilled solutions, rabbits □ Lacrimal volume and fluid turnover—determined for albino rabbits

A severe problem accompanying the instillation of a drug into the eye is the loss that occurs *via* drainage into and through the drainage apparatus. This loss increases in severity as the instilled volume increases and can have a large influence on the biological activity of a drug, since it removes drug from the area of the eye, making it less available to exert a local effect or to be absorbed into the eye. The present report demonstrates that the volume of instilled dose in the eye influences the activity of drugs through drainage loss. In addition, an attempt was made to describe quantitatively the relationship between volume present and rate of drainage in albino rabbits.

Since lacrimal fluid is turned over at an appreciable rate, reported to be some 16%/min. in humans (1), high concentrations of the drug in lacrimal fluid are required for the first few minutes after instillation to maximize biological activity. Viscosity alterations of solutions, suspensions, and emulsions, as well as the use of solid delivery systems such as soft contact lenses (2, 3), are popular methods attempting to reduce or avoid drainage rate problems and, hence, to increase corneal contact time. Clearly, the volume of instilled dose must have a sizable influence on the quantity and rate of drug solution loss to the drainage apparatus.

Mishima *et al.* (1) reported that the normal lacrimal volume in humans is approximately 7 μl. and that the human eye can hold about 30 μl. without overflow or spillage at the lacrimal lake or outer angle, provided that great care is exercised and the subject does not blink. If blinking is allowed, then the eye can hold approximately 10 μl. (4). The normal dropper used in commercial ophthalmic preparations delivers approximately 50–75 μl. Apparently, when this volume of solution is instilled into the cul-de-sac, the reflex action of blinking causes a substantial portion of the drop to be sucked into the drainage apparatus while another portion may be spilled onto the cheek. Removal of the instilled solution continues until the total tear volume is back to the normal lacrimal volume of 7 μl.

Thus, as the volume of instilled dose is increased, the volume of solution lost by spillage on the cheek and drainage increases. This suggests that the optimum volume of drug solution to instill into the eye is *no volume*, since increasing the instilled volume increases the volume lost and the corresponding percentage of drug that is lost. However, an additional factor that complicates the picture is the effect of turnover rate of lacrimal fluid on the concentration and amount of drug remaining in the eye. If one assumes a constant turnover rate of lacrimal fluid of 16%/min., it seems clear that as the precorneal volume of fluid in the eye (lacrimal plus instilled) gets smaller, the turnover rate of lacrimal fluid will have a greater influence on the residual drug concentration. Thus, the rate of turnover of lacrimal fluid favors a *larger instilled volume* to maximize the bioavailability of ophthalmic drugs.

Selection of an "ideal" volume for instillation of drugs into the eye requires information on the magnitudes of the rate constants for lacrimal fluid turnover and for drainage of instilled solutions. Lacrimal fluid turnover studies have been conducted in humans (4–10), but instilled volume drainage studies have apparently not been reported. In addition, although rabbits are the main experimental animals in ophthalmic research, neither lacrimal fluid turnover nor instilled volume drainage has been studied in this animal. Thus, the problem of lacrimal and instilled fluid dynamics as it relates to drug activity seems to have been largely ignored in the ophthalmic field. The paucity of reported work in this area is somewhat surprising, since these fluid dynamics have great bearing on studies of release rates and dissolution of dosage forms and drugs as well

as the transport and general movement of drugs into the eye.

EXPERIMENTAL

Materials—Water was doubly distilled from alkaline permanganate in an all-glass distillation apparatus.

Pilocarpine nitrate USP was obtained from a commercial source[1] and used without further purification. Technetium solutions were prepared using a package[2] of solutions and equipment used to prepare technetium, ^{99m}Tc, suspensions. All other chemicals were either reagent or analytical grade.

Adult, male, albino rabbits[3] were used throughout this study. The rabbits were fed a regular diet with no restrictions on the amount of food or water consumed. All rabbits used weighed between 1.80 and 2.40 kg.

Solution Preparation—Pilocarpine nitrate solutions were prepared fresh and were discarded immediately after the experiment. The solutions were prepared by addition of pilocarpine nitrate to Sorensen's pH 7.38, 0.067 *M*, phosphate buffer. Solution pH was checked and adjusted where necessary. All pH measurements and adjustments were made on a digital pH meter[4], using a wide range glass electrode[5]. The pH meter and electrode system were standardized against phosphate buffer as described by Bates (11). No attempt was made to sterilize the solutions. The highest drug concentration used yielded a slightly hypertonic solution; the tonicities of all drug solutions were adjusted to this value.

Technetium colloidal solutions were prepared by addition of technetium, ^{99m}Tc, to solutions provided in a colloidal sulfur technetium kit[2], from which the sulfur colloid is prepared *in situ*[6]. Technetium, ^{99m}Tc, was obtained by elution off the generator column, in which it was prepared, with normal saline. The technetium solution was added to a thiosulfate–mannitol solution, and the resulting solution was treated with hydrochloric acid and heated to produce a technetium sulfide colloid. The colloid was then added to a buffer solution and again heated to produce the final product. Throughout the study the final technetium, ^{99m}Tc, suspension contained approximately 1 mc. of activity/ml. of solution.

Anesthetic solutions were composed of a mixture of sodium pentobarbital and sodium phenobarbital. Separate solutions of sodium pentobarbital and sodium phenobarbital were prepared in a vehicle containing 20% (v/v) propylene glycol and 10% (v/v) ethyl alcohol (95%). The pentobarbital solution contained 50 mg./ml. of drug, and the phenobarbital solution contained 100 mg./ml. of drug. Both solutions were refrigerated between uses and were never kept for more than 2 weeks.

Anesthesia—A combination of sodium pentobarbital and sodium phenobarbital was used to induce and maintain the desired level of anesthesia. A dose of 33 mg. of sodium pentobarbital and 100 mg. of sodium phenobarbital/kg. of animal body weight was sufficient in most cases to anesthetize the rabbits for 8–12 hr. With this dose level of drugs, the onset of anesthesia usually occurred in 15–30 min. Drugs were administered by intraperitoneal injection.

Miosis–Time Studies—Anesthetized animals were used in these experiments to minimize possible lacrimation from any stimulus, since this would influence the results. After onset of anesthesia, the test animals were placed in a restraining box with the head elevated in such a manner that the lid line, upon closure, was approximately horizontal. This position, which is not the natural posture for the rabbit, was used to facilitate pupillary diameter measurements. The unnatural posture should have the effect of decreasing the rate of lacrimal drainage, thus modifying the magnitude of the effect for which the experiment was designed. Both upper and lower eyelids were then taped open to enable accurate measurements of pupillary diameter. The lower tape was loosened at time of instillation to allow for manual mixing of drug with lacrimal fluid, after which it was replaced. Keeping the eyelids open hastens evaporation of water from the eye and again modifies the effect expected from this experiment. Since the interest was in a reproducible system giving at least a qualitative indication that volume of instilled dose affects drug activity, the experiment was conducted under these conditions. No desiccation of the cornea was observed.

Lighting in the test room was accomplished by use of one 200-w., half-silvered overhead light and remained constant throughout the study. Temperature in the room was maintained at 25 ± 1.0°. The entire room was as isolated as conditions would permit to keep audiovisual stimuli to the test animal at a minimum.

The observer was hidden from the test animal by a cardboard shield; this shield contained a vertical slit through which measurements of pupillary diameter could be made. The observer, once inside the room, was not allowed movement in or out of the room until the experiment was complete. All of these precautions were necessary to obtain reproducible results.

Measurement of pupillary diameter was made by use of a cathetometer[7], positioned approximately 50 cm. from the test animal. These measurements could be made with an accuracy of ±0.1 mm.

After positioning the animal in the restraining box, in line with the cathetometer and prior to drug instillation, pupillary diameter readings were made at 100-sec. intervals. This procedure was continued until the test animal had adapted to its environment, as judged by the constancy of pupillary diameter (±0.1 mm. for 10 min.). The adaptation period varied from 15 to 30 min., and the initial diameter ranged from 6.0 to 7.8 mm.

Pipets[8] with blowout delivery were used to instill the drug solution and were checked for accuracy of delivery prior to use by weighing the delivered solutions.

Drug solutions were instilled onto the cornea, so that they collected in the lower cul-de-sac. To prevent loss of solution during instillation, the lower eyelid was pulled slightly away from the globe of the eye to form a pocket. This particular form of solution instillation was followed at all times.

After instillation of drug solution, the lower lid was lifted back and forth over the cornea to mix the drug solution with lacrimal fluid. At no time was the lid ever massaged against the cornea, so only movement of the drug solution over the cornea occurred. To exert some degree of control in this method, the eyelid was lifted over the cornea exactly four times, during about 5 sec., in all cases. To aid in reproducibility, all procedures were performed in precisely the same manner and with the same time intervals.

After instillation of the drug solution, the lower eyelid was taped open and pupillary diameter was measured as a function of time. The other eye of the test animal served as a control. One hundred-second intervals were used for the first 50 min. of the experiment, 300-sec. intervals were used from 50 to 80 min., and 600-sec. intervals were used thereafter. Measurements were terminated when the measured diameter reached a constant value close to the initial value.

No experimental animal was used more than once, and each experiment was repeated at least four times.

Technetium Studies: Sampling Method—*Animal Preparation*—Test animals for this study were either: (*a*) anesthetized, where manual mixing of tracer solution with lacrimal fluid was employed, or (*b*) unanesthetized, where natural mixing occurred. The unanesthetized test animal was placed in a restraining box in its normal posture. The eyelids were *not* taped open and, after instillation of the technetium suspension, the lid was returned to its former position. Thus, normal movement of the eye was used to mix the colloidal suspension of technetium with lacrimal fluid. After instillation of the tracer, the animal would close its eyelids but would, in all cases, open the lids within 10–15 sec. after instillation. Closing of the lids was not due to irritation from the tracer substance, since instillation of normal saline produced the same response.

Anesthetized animals were placed in restraining boxes, and experiments were conducted with the animals in their normal posture. The eyelids were *not* taped open. Technetium suspension was instilled in the same manner as in the pilocarpine studies.

Procedures—Radioactive technetium colloidal suspensions were instilled into the rabbit eye from a syringe[9] which had been prechecked for accuracy of delivery by weighing the delivered solutions. Volumes as small as 0.1 μl. could be accurately delivered from the

[1] Mallinckrodt Chemical Works, St. Louis, Mo.
[2] Collokit, Abbott Radio-Pharmaceuticals, North Chicago, Ill.
[3] Klubertanz, Edgerton, Wis.
[4] Orion model 801.
[5] Beckman type E3.
[6] For details, see the brochure "Collokit for the Preparation of Technetium Sulfide, ^{99m}Tc, Injection," Abbott Laboratories, North Chicago, Ill., 1971, pp. 2–4.
[7] Central Scientific Co., Chicago, Ill.
[8] Scientific Products, Evanston, Ill.
[9] The Hamilton Co., Whittier, Calif.

various size syringes, although it was necessary to shake the syringe gently to discharge the drop in the case of the 0.5- and 0.1-μl. volumes.

After instillation of a suitable volume of technetium colloid, 15 sec. was allowed to elapse for either manual or natural mixing of the colloid with lacrimal fluid. A sample of tears was then obtained at appropriate time intervals by placing a 1-μl. capillary tube[10] in contact with the marginal tear strip. The capillary tube would immediately fill with tears. All samples were withdrawn from the lower marginal tear strip, approximately in the middle of the strip. The samples were counted[11] for 1 min.

Before and during the study, background readings were taken to determine the level of radioactivity in the test room. Before instillation of the technetium colloid, three readings of the activity of isotope being introduced into the eye also were made. In general, the number of counts per microliter of technetium colloid was in the range of 100,000/min. Suitable corrections for the normal decay of technetium isotope were employed wherever necessary.

Lacrimal Volume Determination—Various volumes, ranging from 0.1 to 50 μl. of technetium suspension, were instilled into the eye, 15 sec. was allowed to elapse, and then a sample was withdrawn and its radioactivity was determined. Both eyes of the animal were used, and no animal was used more than once. Knowing the initial radioactivity of the technetium colloid instilled into the eye, as well as the radioactivity after dilution, allows calculation of the lacrimal volume present as described in the *Appendix*.

Normal Lacrimal Volume Turnover Rate—Various volumes of technetium suspension, ranging from 1.0 to 50 μl., were instilled into the eye; samples were withdrawn and assayed for activity at 5-, 10-, or 20-min. intervals. No initial sample was taken after 15 sec. in this study. Sampling was continued until the isotopic activity had decreased to approximately background level, which, depending upon the volume of isotope instilled, varied from 30 min. to 2 hr. Only one eye of each experimental animal was used, and no animal was used more than once. From a plot of the logarithm of technetium concentration *versus* time, it is possible to determine the turnover rate as shown in the *Appendix*.

Drainage Rate Determination—After instillation of various volumes of technetium colloid into the eye, samples were withdrawn and radioactivity was measured at 5-, 10-, 15-, or 20-sec. intervals. The sampling technique for drainage rate determination was not pursued because of the difficulty in obtaining frequent samples without irritating the eye. More importantly, this technique measures technetium concentration. Thus, as drug is drained out of the eye, concentration does not change appreciably whereas the total *amount* of drug is altered.

Technetium Studies: Nonsampling Method—*Animal Preparation*—All procedures were the same as in the sampling method.

Procedures—The various volumes of colloidal technetium solution were instilled into the eyes of both anesthetized and unanesthetized animals, and the decline in radioactivity was monitored with a thin-probe scintillation detector[12] attached to a well counter[11]. The thin-probe detector is a cylindrical vessel, approximately 22 cm. long and 5 cm. in diameter, containing a sodium iodide crystal and capable of detecting β-emitters. Since the entire outer wall and bottom of the probe can respond to radioactivity, it was necessary to cover the entire probe, except for a 1.5-cm. opening at the bottom, with a 0.3-cm. (0.125-in.) thick lead shield. Thus, the only radioactivity picked up by the probe would be that coming through the orifice at the bottom, and this orifice was placed directly over the cornea of the test animal approximately 1 or 2 cm. away from the eye. Preliminary testing had shown that this distance from the eye, as well as the size of the orifice in the lead shield at the bottom of the probe, was a compromise to obtain maximum isotope counts from the eye as well as to exclude extraneous radiation from the surroundings or from technetium that might have passed into the drainage apparatus.

With anesthetized animals, no special precautions were followed. With unanesthetized animals, it was necessary to precondition the animals to the testing procedure so that head and eye movements were eliminated during the experiment. For preconditioning, the test animals were placed in the restraining box with the probe over their eyes once a day for 1-hr. duration on 7 successive days prior to the experiment. After this conditioning, the animals showed no head or eye movement for the duration of the experiment; if they did, it was clear from the results and the experiment was terminated at that point.

Various volumes of technetium colloidal suspension were instilled into the rabbit eye, as described in the *Sampling Method* section, and the probe was placed in front of the eye. Measurements of radioactivity in the eye were made every 5 sec., and counts were determined for periods of 5 sec. This measuring technique determines the amount of drug in the eye and *not* concentration. Measurements were made until the technetium radioactivity had decreased to approximately background level, which usually took about 60–90 min., although shorter and longer times occurred depending upon the type of experiment. Only one of the rabbit's eyes was used, and no rabbit was used more than once.

Normal lacrimal turnover rate and instilled volume drainage rate constants were obtained by computation. Derivation of the appropriate equations is shown in the *Appendix*.

Evaluation of Technetium Dilution Technique for Lacrimal and Instilled Fluid Dynamic Studies—To use a tracer substance such as isotopic technetium as a test substance for tear and instilled fluid dynamic studies, it is necessary to satisfy several criteria. These criteria are: (*a*) the test substance must be lost from the eye solely *via* the drainage apparatus, *i.e.*, no spillage; (*b*) no absorption/adsorption must occur or, if sorption occurs, its extent must be known; (*c*) the test substance must be nonirritating; and (*d*) if the substance is an insoluble solid, it must not interfere with loss of fluid into the tear drainage apparatus.

To show that neither loss nor retention of a significant portion of the technetium colloid occurred through binding to eye tissue and/or absorption, the amount of drug consumed in these possible routes was determined. Because of the differences between the two techniques (sampling and nonsampling) employed in the determination of the amount of ^{99m}Tc present, the amount shown to be present by each method could be different if any significant binding or absorption occurred. Since the ^{99m}Tc is a 142-kev. β-emitter, its presence can be detected by the probe through the conjunctiva and eyelid. Consequently, ^{99m}Tc bound to eye tissue or absorbed into the eye, with the exception of the portion that passed into the bloodstream and was swept away, would still be seen by the probe whereas ^{99m}Tc determined by the sampling technique would not show this retention. For this study, 25 μl. of technetium solution was instilled into the eyes of several anethetized animals. At various time intervals over 4-hr., representing the maximum duration of the experiments, the animals were sacrificed and isotope activity in aqueous humor, cornea, sclera, conjunctiva, and eyelids was determined. Less than 0.1% of the instilled colloid was absorbed or adsorbed into any portion of the eye tested during this time period. This small activity could have been due to contamination of the tissue samples rather than absorption/adsorption. Thus, all loss appears to be through the drainage apparatus.

Since the technetium isotope is adsorbed on a colloid, there is the possibility of blockage or interference with drainage into the nasolacrimal duct. In preliminary studies using gelatin-stabilized technetium colloid, the drainage apparatus was apparently blocked by the colloid as judged by impaired or inhibited disappearance of the technetium isotope. This problem did not appear when mannitol-stabilized technetium was used, and repetitive application of the isotope solution at various times and under various conditions gave highly reproducible results.

Insofar as irritation is concerned, the isotope, at very high concentrations, does irritate the rabbit eye as judged by animal reaction and lacrimation. Concentrations of isotope used in this study did not cause observable irritation to the test animal.

RESULTS

Miosis–Time Study to Demonstrate Effect of Dose Volume on Drug Activity—To determine the influence of dose volume on drug activity, several solutions of varying concentration of pilocarpine nitrate were prepared and instilled into the eyes of anesthetized rabbits. The volumes of solution instilled were adjusted so that the amount of drug delivered to all rabbit eyes was the same; *i.e.*, the amount of drug delivered from the 75-μl. dose was the same as that

[10] Drummond Microcap, Scientific Glass Apparatus Co., Bloomfield, N. J.
[11] Abbott model 111 well counter, Abbott Laboratories, North Chicago, Ill.
[12] Model 7498, Abbott Laboratories, North Chicago, Ill.

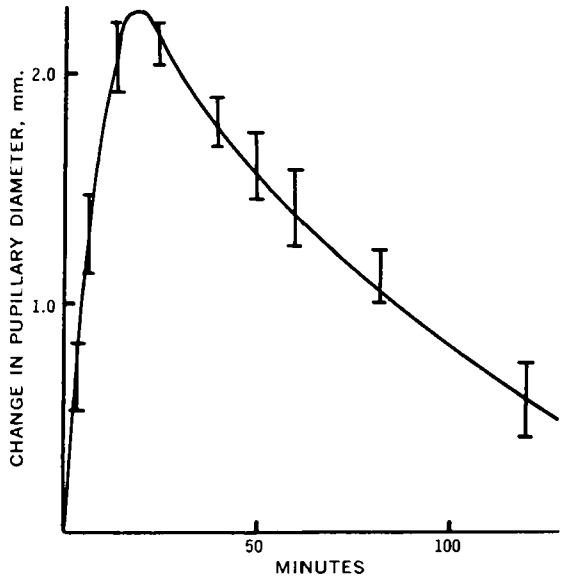

Figure 1—*Change in pupillary diameter as a function of time after instillation of 50 μl. of a 1 × 10^{-2} M pilocarpine nitrate solution. The data represent the sum of five separate runs, and the vertical lines indicate standard deviations.*

from the 5-μl. dose. If there is *no* loss of drug solution from the eye, it would be expected that the areas under the pupillary diameter–time curves would be the same, although the shape of curves for various concentrations of drug would be expected to be different.

Previous extensive work on the miotic effect of pilocarpine nitrate (12) had determined the dose-response curve.

A typical pupillary diameter–time relationship is shown in Fig. 1, along with standard deviations which are presented as vertical bars. The results shown in Fig. 1 were obtained from five animals in five different experiments and are typical of all the experiments.

Table I shows the area under the pupillary diameter–time curves for various instilled volumes. These areas are proportional to the concentration of drug reaching the target and are presumably proportional to the *amount* of drug present in the tear fluid. Since all of these solutions had identical amounts of drug, it would be expected that all would show the same area under the curve if no drug solution was lost from the application site. The ratio of productive to nonproductive drug loss by adsorption/absorption will be the same whether low volume (high concentration) or high volume (low concentration) is used. Thus, fluid loss by drainage would determine differences in area under the curve. Clearly, the increased area under the curve that is observed as the instilled volume decreases demonstrates that a substantial portion of the instilled dose is unavailable for absorption to the target area, due apparently to the drainage loss.

The range of volumes employed was restricted at the lower end by drug irritancy and at the upper end by the maximum volume the eye can hold without overflowing onto the lid and cheek. In the case of the largest dose volume, 75 μl., great care was needed in mixing the drug solution with lacrimal fluid to prevent overflow onto the cheeks.

Determination of Lacrimal Volume and Normal Lacrimal Fluid Turnover Rate by Sampling Technique—*Lacrimal Volume Determination*—Instillation of a known volume and activity of a technetium colloidal suspension, followed by sampling of the tear fluid for activity, allows determination of the volume of fluid originally present in the eye as described in the *Appendix*. This procedure assumes that good mixing takes place and that no tear production occurs from time of instillation until sampling time, which was 15 sec. in the present study.

Both anesthetized and unanesthetized animals were used, and each involved a different method of mixing the technetium suspension with lacrimal fluid as described in the *Experimental* section.

The results of the study are shown in Table II together with the range of values, mean value, and standard deviation for various instilled volumes. A broad range of volumes was used to determine if adequate mixing or loss of unmixed solution was occurring. As can be seen from Table II, the range of median values for both unanesthetized and anesthetized animals is not excessively large, considering that a 500-fold range of instilled solution was used. The standard deviation increases in the unanesthetized animals as the instilled volume increases, presumably due to incomplete mixing or loss of unmixed solution. In addition, all standard deviations are larger than desirable, pointing to the disadvantage of the sampling procedure as described by Mishima *et al.* (1).

Table I—Effect of Instilled Volume on Activity of Pilocarpine Nitrate in the Rabbit Eye

Instilled Volume, μl.	Concentration[a], *M*	Number of Animals	Peak Height, mm.[b]	Area under Curve, cm.[2c]	Activity[d], %
75	6.7 × 10^{-3}	5	2.05 ± 0.10	63.7	27.7
50	1 × 10^{-2}	5	2.40 ± 0.17	72.6	31.5
25	2 × 10^{-2}	5	2.53 ± 0.21	83.9	36.5
10	5 × 10^{-2}	4	3.53 ± 0.15	131.3	57.0
5	1 × 10^{-1}	5	4.17 ± 0.40	230.2	100.0

[a] The amount instilled is 0.136 mg. [b] Units refer to average peak height plus or minus standard deviation. [c] Units are millimeters-minute. On the vertical axis, 1 cm. = 0.25 mm.; on the horizontal axis, 1 cm. = 10 min. [d] For comparison purposes only, it was assumed that the 5-μl. instilled volume case gives 100% activity.

In unanesthetized animals, the calculated lacrimal volumes show a range of values depending on the instilled volume. For the lower instilled volumes of 0.1, 0.5, and 1.0 μl., the mean lacrimal fluid volume value of approximately 7.5 ± 2.5 μl. is essentially constant, and this value is interpreted to be the true lacrimal volume in the unanesthetized rabbit. With larger instilled volumes of 5.0, 25, and 50 μl., the calculated values for lacrimal fluid volume are considerably higher than for the smaller instilled volumes. These higher values are interpreted as being due to a loss of unmixed technetium solution or perhaps to better mixing with lacrimal fluid under the lids. The larger standard deviations with larger instilled volumes, as well as the broader range of values, suggest that loss of unmixed solution is the more likely explanation.

Anesthetized animals show a relatively constant lacrimal volume for instilled volumes ranging from 1 to 50 μl. This relatively constant value of approximately 11–12 μl. is considerably larger than the values for the lower instilled volumes in the unanesthetized animals. These higher values are probably due to relaxation of the eye musculature under anesthesia to accommodate a larger volume. Alternative explanations of loss of unmixed solutions or of irritation on mixing were considered and discarded. It is conceivable, however, that manual mixing is more efficient than natural mixing and that the true lacrimal fluid volume in both anesthetized and unanesthetized rabbits is approximately 12 μl.

Normal Lacrimal Turnover Rate Determination—Different volumes of technetium solution were instilled into the eyes of both anesthetized and unanesthetized rabbits, and samples were with-

Table II—Lacrimal Volume in Rabbits as Determined by Diluting Technetium with Tears and Sampling the Resultant Solution for Activity

Instilled Volume, μl.	Number of Determinations	Range of Calculated Lacrimal Volume, μl.	Mean, μl.	*SD*, μl.
Unanesthetized without Manual Mixing				
0.1	5	4.55–10.42	7.55	2.09
0.5	5	6.89–11.10	7.61	2.96
1.0	11	4.19–15.50	7.37	3.54
5.0	5	5.16–13.84	9.32	4.15
25.0	5	9.10–16.99	13.09	3.59
50.0	4	6.28–17.84	12.84	4.80
Anesthetized with Manual Mixing				
1.0	5	8.46–14.50	11.55	2.62
5.0	4	6.59–18.94	11.96	5.62
25.0	5	10.53–14.33	12.30	1.57
50.0	4	9.86–14.45	12.21	1.98

Table III—Turnover Rate of Lacrimal Fluid in the Rabbit by the Technetium Dilution Method Using the Sampling Technique

Instilled Volume, μl.	Sampling Time, min.	Number of Determinations	k, min.$^{-1}$, Range	k, min.$^{-1}$, Average	SD	Turnover[a] Rate, μl./min.
			Unanesthetized without Manual Mixing			
1	10	3	0.074–0.080	0.076	0.002	0.47
5	10	3	0.074–0.084	0.080	0.005	0.50
25	5	3	0.080–0.083	0.081	0.002	0.46
25	10	3	0.075–0.087	0.082	0.006	0.52
25	20	3	0.073–0.076	0.075	0.002	0.50
50	10	3	0.075–0.079	0.077	0.002	0.48
						Average = 0.49
			Anesthetized with Manual Mixing			
1	10	3	0.063–0.071	0.0656	0.005	0.69
5	10	3	0.054–0.058	0.056	0.002	0.57
25	10	3	0.0077–0.0084	0.0081	0.0003	0.00[b]
50	10	3	0.0041–0.0074	0.0053	0.0002	0.00[b]

[a] Obtained by multiplying the observed first-order rate constant times the lacrimal volume, 7.5 μl. for unanesthetized and 12.0 μl. for anesthetized rabbits, and subtracting the sampling withdrawal rate constant. [b] Subtracting the withdrawal rate gives a negative number to the turnover rate.

drawn at various time intervals. The logarithm of the decline in technetium concentration was plotted against time and the first-order rate constant was determined from the slope of the line as shown in the *Appendix*. The results of this study are presented in Table III, together with standard deviations and resultant turnover rates based on a lacrimal fluid volume of 7.5 μl. for unanesthetized rabbits and of 12.0 μl. for anesthetized rabbits.

In the case of unanesthetized rabbits, good linearity is observed when the logarithm of decline in isotope activity is plotted against time (Fig. 2). The range of observed rate constants is fairly narrow for all volumes studied (Table III). The relatively consistent results of the turnover rate under conditions of varying instilled volume as well as different sampling schedules can be seen from the last column on the right of Table III. Various sampling times were

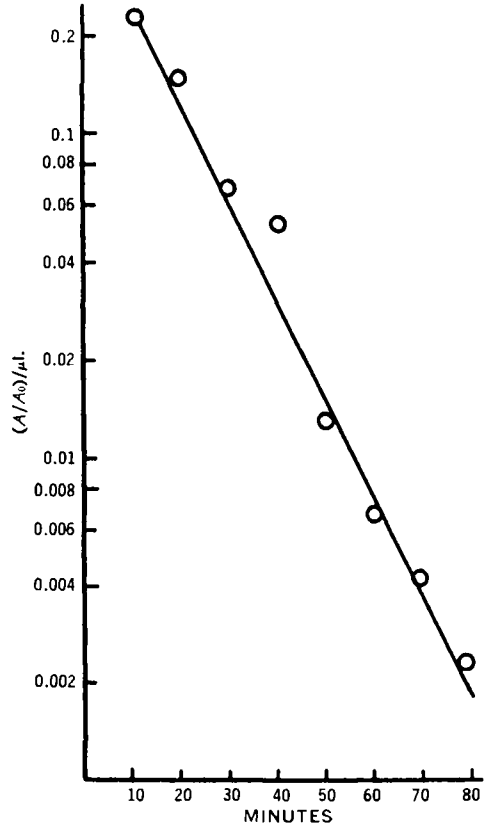

Figure 2— *Typical plot of the decline in isotopic activity of technetium* versus *time in unanesthetized rabbits. The specific run is for a 50-μl. instilled volume, in which the sampling technique was used to determine isotopic activity of technetium.*

studied to assess if the sampling technique was influencing the observed decline in activity of the isotope through irritation and lacrimation. Several different instilled volumes were used to ensure good mixing.

In the case of anesthetized animals, the turnover rate was essentially zero for the 25- and 50-μl. runs and rather large for the 1- and 5-μl. instilled volumes. It was concluded that the turnover rate in anesthetized animals is essentially zero. The large rates observed for the 1- and 5-μl. runs are possibly due to irritation from repetitive sampling, since it was difficult to obtain more than two or three samples in each experiment. Thus, for anesthetized animals it was concluded that the lacrimal turnover rate is either very small or nonexistent, which is not unusual considering that it is reported that humans do not lacrimate while sleeping (13-15). This observation has important bearing on animal studies, where time-concentration curves are generated in corneal absorption experiments. Anesthetized animals are generally used, and the results of these studies would be expected to be far different than results from unanesthetized animals considering the difference in turnover rate of lacrimal fluid.

Determination of Normal Lacrimal Fluid Turnover Rate and Instilled Volume Drainage Rate by Nonsampling Technique—*Unanesthetized Rabbits*—After instillation of technetium colloidal solution into the rabbit eye, monitoring of technetium radioactivity commenced no more than 2 or 3 sec. after instillation. A representative figure of the amount of tracer remaining as a function of time for various instilled volumes in unanesthetized animals is shown in Fig. 3. Also included in the figure are standard deviations, at random points, for the 5-μl. case to illustrate reproducibility of the procedure.

Initial inspection of Fig. 3 suggests that the data might fit a reaction sequence of the following type:

where $A \rightarrow B$ represents the rapid drainage of the instilled solution phase and $A \rightarrow C$ represents turnover of lacrimal fluid. This can be visualized as follows: After instillation of a volume of tracer solution into the eye, the total volume (instilled plus lacrimal) drains out at a given rate, removing tracer solution, until the volume is reduced to the lacrimal volume. Further decline in tracer amount is then due to turnover of lacrimal fluid.

Although the curves in Fig. 3 are depicted as smooth, uniformly changing lines, the terminal portion (turnover of lacrimal fluid) is actually oscillatory. The amplitude of the wave is rather small, and the wave has a period of approximately 400 sec. A smooth line was drawn through the wave function to simplify analysis. The cause of this unexpected behavior could be one or a combination of factors such as an intermittent input of lacrimal fluid or pulsation of the eye musculature which forces lacrimal fluid out. It is not due to either blinking or head and eye movement. Mishima *et al.* (1) did not remark upon this behavior in humans, although the scatter in

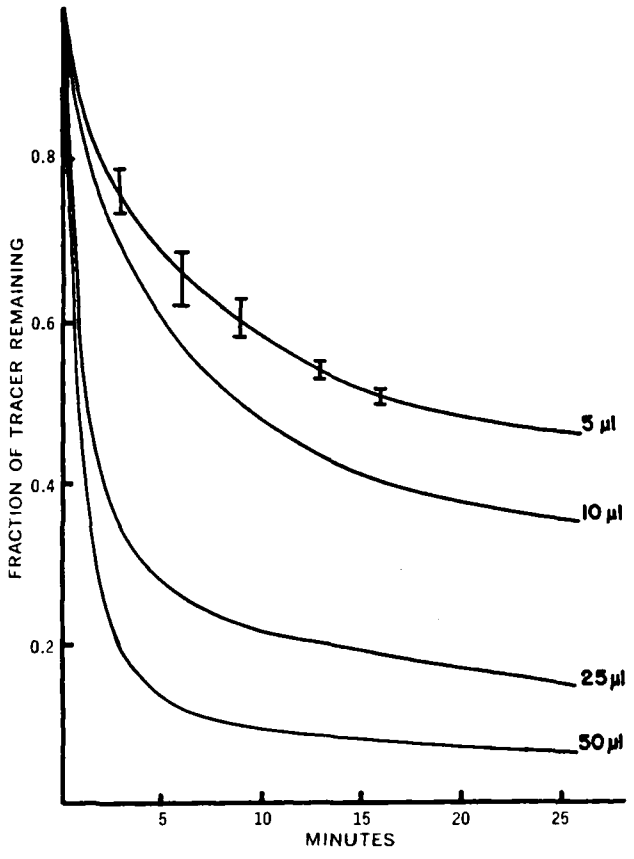

Figure 3—*Fractional amount of drug remaining as a function of time for various instilled volumes in unanesthetized rabbits. At least three separate experiments were conducted at each instilled volume, and the lines represent the mean values for these runs. Standard deviations, at selected time points, are shown only for the 5-µl. volume for the sake of clarity; the remaining runs show similar standard deviations.*

their data suggests that such a phenomenon might exist. The present authors are currently exploring this phenomenon in more detail.

It is easy to provide an underlying mechanism to explain the apparent biphasic nature of the curves in Fig. 3. The terminal portion of each curve is relatively simple to describe in that the fractional decrease in tracer substance is due entirely to the normal turnover of lacrimal fluid; *i.e.*, the tear volume, composed of lacrimal fluid and instilled volume, is back to the normal lacrimal fluid volume. The turnover rate of lacrimal fluid is easily obtained from the slopes of the lines resulting from plots of the logarithm of fractional amounts of tracer remaining *versus* time (Table IV).

The agreement between these results and results obtained from the sampling technique (Table III) is excellent. This suggests that the sampling procedure can be used with confidence to determine lacrimal fluid turnover rate, as long as great care is used to minimize irritation to the eye.

The initial portion of the curves in Fig. 3 shows a rapid decline in the amount of tracer substance due to rapid drainage of the solution present. This decline is also influenced by inflow of additional solution (normal lacrimation). If no inflow is assumed, other than

Table IV—Normal Lacrimal Turnover Rate Obtained *via* Nonsampling Technique

Instilled Volume, µl.	Number of Determinations	*F*, µl./min.	*SD*
5	4	0.42	0.12
10	3	0.60	0.18
25	3	0.66	0.06
50	3	0.60	0.12
		Average *F* = 0.57	

Table V—Volume of Solution Remaining at Various Times as a Function of Instilled Solution

Minutes	Volume Remaining[a], µl. 5[b]	10[b]	25[b]	50[b]
0[c]	12.5[d]	17.5	32.5	57.5
1	10.9	14.8	20.0	28.2
2	10.1	12.4	15.5	18.9
3	9.5	11.0	11.9	12.4
4	9.1	9.9	9.9	9.8
5	8.6	8.7	8.8	8.4
6	8.1	8.1	8.3	7.2

[a] Neglecting the inflow of lacrimal fluid which is small, approximately 0.50 µl./min., compared to instilled volume, particularly for the 25- and 50-µl. cases. [b] Instilled volume in microliters. [c] Time zero volume includes the instilled volume plus the normal lacrimal volume, which is assumed to be 7.5 µl. [d] All calculated volumes are median values with ±5% error.

the initial instilled volume solution, the mathematical treatment is much simplified as shown in the *Appendix*. This assumption is not unreasonable, since the rate of lacrimal flow is small and the time period of concern is also small, *i.e.*, the first 5 min.

To illustrate the change in volume as a function of time during the initial drainage phase, Table V shows the volume remaining at various time intervals for the different instilled volumes as calculated from equations presented in the *Appendix*. The data in Table V are mean values from a minimum of three runs.

From Eq. A15 presented in the *Appendix*, it is possible to determine the rate constant for drainage. Good linearity is obtained when the logarithm of volume remaining is plotted *versus* time (Fig. 4). This indicates that drainage rate, for various instilled volumes, is directly proportional to the volume present, *i.e.*, apparent first order.

The different slopes of the lines in Fig. 4 suggest that drainage cannot be viewed as simple flow through a conduit or that it is merely proportional to the hydrostatic head developed by the presence of a given volume of solution. A change is occurring somewhere in the drainage mechanism as the volume of instilled solution changes.

To determine the relationship between the drainage rate constant and the instilled volume, the apparent first-order rate constants were determined from the slope of the lines in Fig. 4 (Table VI). The apparent first-order rate constants were then plotted against the instilled volume (Fig. 5). The excellent correlation shown in Fig. 5 suggests that the apparent first-order rate constant contains a volume-dependent term. In addition, the nonzero intercept sug-

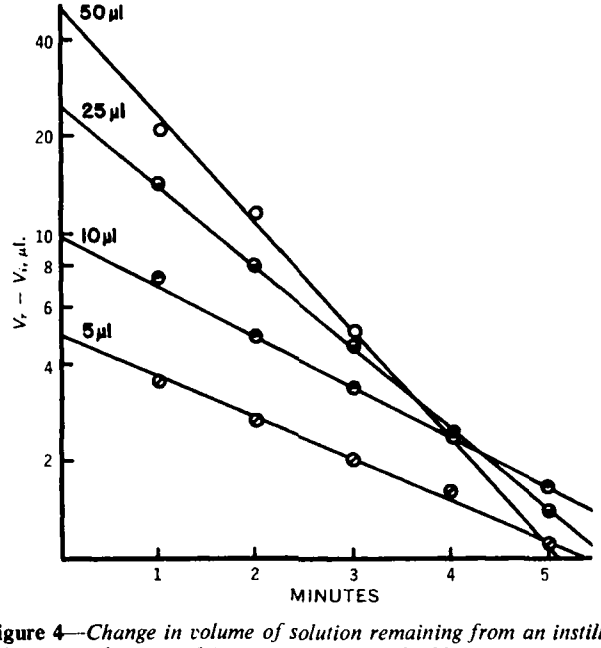

Figure 4—*Change in volume of solution remaining from an instilled solution as a function of time in unanesthetized rabbits.*

Table VI—First-Order Rate Constants for Drainage of Instilled Solution

Volume Instilled, μl.	k_{obs}, sec.$^{-1}$
5	0.308
10	0.365
25	0.545
50	0.815

gests that it also has a volume-independent term. It was expected that if the lacrimal fluid secreted under normal conditions is drained by the same mechanism as that which removes an excess of tears or an instilled solution, the intercept in Fig. 5 would represent the rate constant for normal lacrimal fluid turnover (shown by the arrow in Fig. 5). In fact, the volume-independent term is some 200 times larger than the rate constant for lacrimal fluid turnover. This suggests that removal of normal lacrimal fluid is *via* a mechanism that undergoes some modification when larger volumes are present. Two distinct mechanisms for removal of fluid from the eye, one for small volumes and one for large volumes, are in accord with the description given by Maurice (15) for tear removal in humans.

The drainage rate constant allows one to calculate the amount of tracer remaining from an instilled dose at various times, but this can be more easily seen from Figs. 2 and 4. It is observed that within 1 min. the 50-μl. instilled volume, which is slightly less than the average commercial drop, has diminished to approximately 50% of its initial volume ($V_i + V_l$). And at the end of 5 min., the eye still contains approximately 68% of the radioactivity from the 5-μl. instilled volume; whereas with 50 μl., only approximately 14% of the initial radioactivity remains. This finding corroborates the results with pilocarpine shown in Table I, namely, that instilled volume plays a substantial role in drug loss and, therefore, greatly influences drug activity.

Anesthetized Rabbits—Loss of tracer in anesthetized animals was significantly different from that in unanesthetized animals. Figure 6 illustrates the loss of tracer in both anesthetized and unanesthetized animals, using 5 μl. as the instilled volume.

Some qualitative observations on the total volume removal curve can be made. Whereas in unanesthetized animals the total volume was back to a constant volume in about 6 min., it takes considerably longer with anesthetized animals and the length of time varies

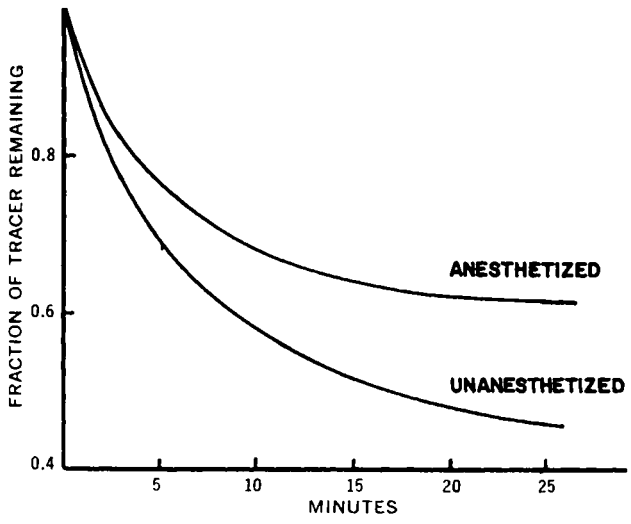

Figure 6—*Fractional amount of drug remaining as a function of time for anesthetized and unanesthetized rabbits using 5-μl. instilled volume. The lines represent the average of four separate determinations.*

with the volume instilled. This is presumably due to a lack of eye movement and a relaxation of eye muscles.

Figure 6 shows that the percentage of isotope in anesthetized animals is less than in the unanesthetized case at all times. As the instilled volume gets larger, the percentage difference in tracer remaining between anesthetized and unanesthetized animals increases. Thus, for 50 μl. instilled volume, the unanesthetized animal shows roughly 14% of tracer remaining at 5 min. whereas the anesthetized animals shows approximately 33% remaining.

As expected, the terminal portions of the percent of tracer amount *versus* time plots for anesthetized animals reach a plateau. The sampling studies of turnover of lacrimal fluid showed that no lacrimation or drainage occurred while the animal was under anesthesia. Therefore, it was expected that the decline in drug amount or concentration would cease when drain-off was complete.

The change in volume as a function of time for various instilled solutions in anesthetized rabbits is shown in Fig. 7. Drainage of an

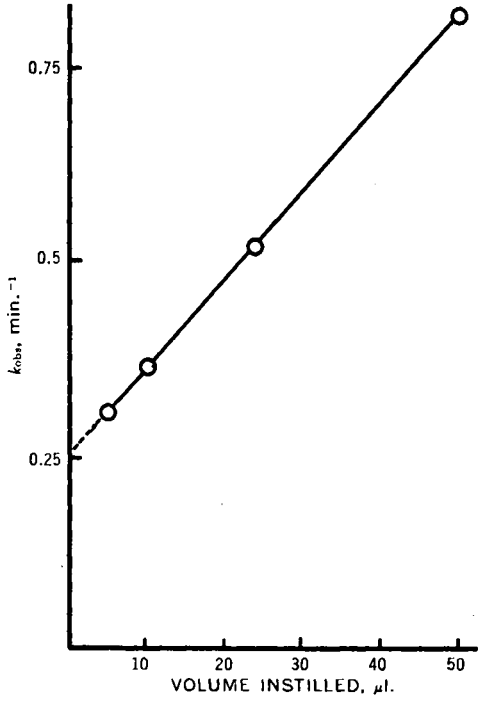

Figure 5—*Relationship between the observed rate constant for the decline in volume and volume instilled. The equation for the line is:* $k_{obs} = 0.25\,(min.^{-1}) + 0.0113\,(1\,\mu l./min.)\,(V_i)$.

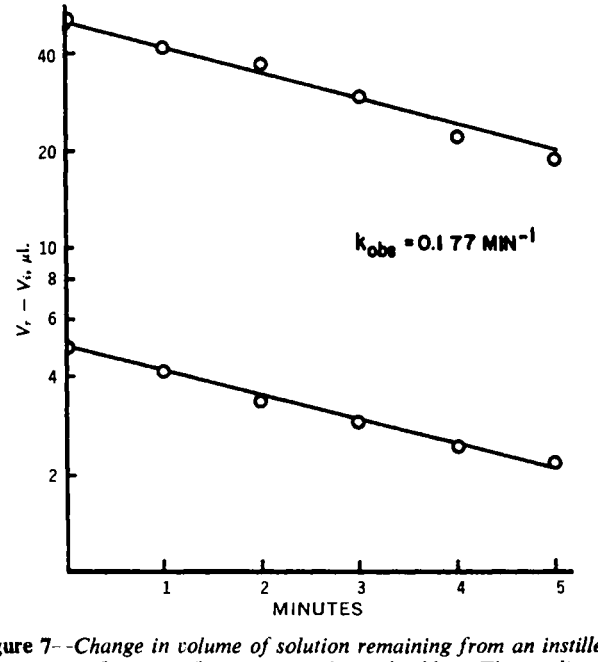

Figure 7—*Change in volume of solution remaining from an instilled solution as a function of time in anesthetized rabbits. The top line is for an instilled volume of 50 μl. and the bottom line is for 5 μl. Both lines represent three different runs, and the data points are mean values ±5%.*

instilled solution in anesthetized rabbits is strikingly different from unanesthetized animals, as can be seen by comparing Figs. 4 and 7 and can perhaps be related to the different mechanisms of drainage.

It has been suggested (16) that gravitational movement of solution is responsible for tear and instilled volume drainage in the rabbit. The data in Fig. 6 do not support this suggestion for instilled volume drainage in both anesthetized and unanesthetized rabbits. The fact that the rate of decline in radioactivity for identical instilled volumes is different in anesthetized and unanesthetized rabbits suggests that simple gravitational movement cannot be the mechanism in both cases. The conclusion with unanesthetized rabbits, based on the volume-dependent term in the apparent rate constant, was that gravitational movement was not the driving force for instilled volume removal. It can be seen from Fig. 7 that the slopes of the lines for the 50- and 5-μl. instilled solutions are identical and, therefore, the rate constants are identical. Since there is no instilled volume dependency in drainage of fluid from the eye of anesthetized animals, it was concluded that this supports the mechanism of drainage by gravitational movement in anesthetized rabbits.

DISCUSSION

It is quite clear from the pupillary diameter study in anesthetized animals that as instilled volume increases the resultant biological activity decreases for equivalent amounts of drug. This effect is expected to be much more dramatic in unanesthetized animals, since lacrimal fluid turnover is slow or absent in anesthetized animals and the drainage rate is considerably slower. Moreover, since human lacrimal fluid turnover rate (1) is twice as large as that found for rabbits and since humans blink considerably more than rabbits, a larger effect is expected in humans, assuming all other factors to be equal.

The various tear and instilled volume variables described in this report require some additional discussion.

Lacrimal Fluid Volume—The rabbit lacrimal fluid volume of 7.5 μl. is remarkably close to that of humans, *i.e.*, 7.0 μl. (1). The rabbit eye globe is somewhat smaller than the human globe, but the rabbit eye possesses a third eyelid, the nictitating membrane, which occupies some of the eye area. Based on the somewhat smaller eye and the nictitating membrane, it was expected that rabbit lacrimal fluid volume would be less than humans, but this is apparently not the case.

It is, of course, possible that the 7.5-μl. lacrimal volume in unanesthetized rabbits is actually an underestimate of the true volume. The majority of lacrimal fluid is held under the upper and lower eyelids, and the methods of mixing technetium tracer with lacrimal fluid may not have reached all of this fluid. In the case of natural mixing, the majority of mixing occurred immediately postinstillation when the rabbit closed its eyelids in response to the instilled solution. The rabbit blinks infrequently and, therefore, the first blink and eye movement were responsible for mixing. In the case of manual mixing, the upper eyelid was not actively involved in the mixing process.

Turnover Rate of Lacrimal Fluid—The turnover rate of lacrimal fluid in rabbits is approximately one-half that of humans (16%/min. in humans and 7.1%/min. in rabbits). Considering the fact that rabbits blink infrequently and that, therefore, the evaporation rate of tears would be expected to be higher in rabbits than humans, it was expected that rabbits would have a larger turnover rate of lacrimal fluid. The fact that turnover rate determinations from both the sampling and nonsampling techniques are in such good agreement lends support to the validity of the turnover rate.

Absence of, or a severe reduction in, turnover rate in anesthetized animals is shown by both the sampling and nonsampling results. Since these techniques measure both concentration and amount changes, reflecting inflow and outflow, respectively, the conclusion seems valid. Support for this was obtained by observing an absence of tears when an anesthetized rabbit was placed on its back with its head hanging down. It is possible that tears are produced under anesthesia and that tear removal by either absorption or evaporation maintains the constant volume. Neither of the techniques used would show this effect.

Drainage Studies—Movement of an instilled solution into the drainage apparatus in rabbits can be described by a first-order rate expression which contains both a volume-dependent and a volume-independent term. The volume-dependent term suggests that simple gravitational movement of the solution is not the prime mechanism for fluid removal. It is not possible to elaborate on the mechanism for fluid drainage on the basis of the results from this study.

Practical Application of Results to Clinical Therapy—From the results presented in this report, considering lacrimal volume, lacrimal fluid turnover rate, and drainage of instilled solutions, it should be possible to determine an optimum volume for drug instillation in the rabbit eye. This optimum volume would allow the maximum amount of drug to be in contact with the eye for the maximum period of time, with a minimum of drug loss.

To illustrate the advantage of an optimum volume, consider the following hypothetical example. If 50 μl. of a 1% drug solution is needed to elicit a given biological response, perhaps 5 μl. of a 2 or 3% solution will generate the same response. In essence, the volume change has been from 50 to 5, a factor of 10, whereas the concentration change has been from 1 to 3%, a factor of 3. The difference between the two factors represents loss of solution through drainage.

For unanesthetized rabbits the appropriate volume would appear to be approximately 5 μl. Smaller volumes will cause the drug to be removed too quickly *via* turnover and are difficult to introduce accurately, whereas larger volumes are washed out too quickly. In anesthetized animals, since there is no turnover of lacrimal fluid, volumes of 1 μl. or less would be optimum. However, the difficulty of manually mixing this small volume with tears must be considered.

An appropriate or optimum volume in humans is difficult to establish without precise knowledge of drainage rates, but some qualitative statements can be made. According to Maurice[13], drainage of an instilled solution in humans is considerably faster than in rabbits. Presumably the rapid rate of blinking causes an acceleration in the rate of drainage. Thus, small volumes of 1–5 μl., provided they can be accurately delivered from an ophthalmic dropper arrangement, would allow for maximum results from an instilled drug.

An important benefit of using a smaller instilled volume, in addition to improved drug activity and lower cost, is a potential decrease in side effects from ophthalmic drugs. Consider, for example, epinephrine solution used to treat glaucoma. For stability reasons, these solutions are weakly buffered to approximately pH 3–4. Instillation of 50–75 μl. of this solution causes considerable pain due at least partially to the acidity, and it can create systemic side effects (17). Using 5–10 μl. as the instilled dose should decrease the pain on instillation and minimize side effects, since less drug will drain into the drainage apparatus and be absorbed. Instillation of 10 μl. of a 2% epinephrine solution to rabbits gave the same pupillary response as 50 μl. of a 1% solution and appeared to generate less pain and lacrimation, an observation that reinforces our suggestion.

Other considerations that result from this study are:

1. Blinking of the eyes, which many people do either as a reflex action or to mix the drug solution with lacrimal fluid, should be minimized if possible. Blinking quickly squeezes solution out of the eye, which accelerates drug loss.
2. Repeated application of drops, either of the same drug or different drugs, should be reconsidered and abandoned. It can easily be shown mathematically, using the drainage rate constants, that when one drop is followed by a second drop the rate of loss of the drug solution is extremely rapid and proportional to the volume present. When two drugs are involved and each is administered separately at a short interval, the first drug will be more rapidly eliminated from the eye as compared to the second, and this loss is a function of the instilled volumes and the time between dosings. This strongly argues for administering two drugs together when combination therapy is needed rather than administering them separately.
3. The suggestion of washing the eye with normal saline or buffer solutions prior to instillation of drug (18, 19) should be viewed in light of the results of this study. The time element between washing of the eye and instillation of drug solution will greatly influence the drainage rate of drug solution since the volume of rinse solution remaining in the eye can be large.

Further Studies—The technique and approaches used in this study can be applied to evaluation of ophthalmic vehicles. Contact time and drainage rate from various vehicles as a function of vis-

[13] D. M. Maurice, Stanford University, Palo Alto, Calif., personal communication.

cosity and other parameters are currently being studied and will be reported in a subsequent publication.

It is desirable to compare these animal studies to humans, and thus drainage studies in humans ought to be more fully explored. In addition, further studies on the drainage mechanism as well as movement of tears in the eye would be helpful.

APPENDIX

Lacrimal Volume Determination *via* Sampling Technique—When a known volume, V_i, and concentration, C_0 (counts/μl.), of technetium are instilled into the eye, the solution will be diluted by the lacrimal fluid, giving rise to a new concentration, C. From the concentration of the solution after dilution it is possible to calculate the volume of lacrimal fluid, V_l, that was present by use of Eq. A1*a* or A1*b*:

$$V_l = V_i \frac{C_0}{C} - V_i \qquad \text{(Eq. A1}a\text{)}$$

$$V_l = V_i \left(\frac{C_0}{C} - 1\right) \qquad \text{(Eq. A1}b\text{)}$$

The assumptions with this method are that complete mixing occurs and that there is no loss of unmixed solution. Both of these assumptions improve as the instilled volume gets smaller.

Normal Lacrimal Fluid Turnover Rate Determination—Disappearance of an applied dose of drug or tracer substance following instillation into the eye can be described by the following equation:

$$\frac{-dC}{dt} = k(C) = \frac{F}{V_l}(C) \qquad \text{(Eq. A2)}$$

where k = first-order rate constant for the decline in tracer concentration, F = turnover rate of lacrimal fluid, and V_l is the lacrimal volume. This equation assumes that loss of applied drug is only through normal lacrimal fluid turnover. Integration of Eq. A2 and rearrangement yield:

$$\log C_0 - \log C = -\frac{F}{2.303V_l}t \qquad \text{(Eq. A3)}$$

A plot, therefore, of the log change in tracer concentration against time should be linear. The slope of the line yields the rate constant, k, for the decline in tracer concentration which, when multiplied by the lacrimal volume, V_l, yields the turnover rate. In the sampling technique, it should be remembered that if the sampling withdrawal rate constant is large with respect to the turnover rate constant, it must be subtracted from the turnover rate constant. For the nonsampling technique, activity (amount) can be substituted for concentration in Eq. A3.

Volume Remaining from Instilled Dose—After a drop of solution is instilled, the volume at various times up to 6 min. can be calculated. The assumption in the following derivation is that one must neglect the volume of inflow of lacrimal fluid, a reasonable assumption, for the first 6 min. postinstillation of solution, since this volume is small compared to the volume of tracer solution instilled.

Upon instillation of a volume of tracer solution into the eye, the concentration will change upon dilution according to a rearranged form of Eq. A1*a* or A1*b*:

$$C_0V_i = C(V_i + V_l) \qquad \text{(Eq. A4}a\text{)}$$

or:

$$\frac{C_0}{C} = \frac{V_i + V_l}{V_i} \qquad \text{(Eq. A4}b\text{)}$$

The amount of tracer substance remaining at any time is given by:

$$A = C \times V_r \qquad \text{(Eq. A5)}$$

where A = amount of drug, and V_r = volume remaining. Substituting for C in Eq. A5 from Eq. A1 yields:

$$A = \frac{V_iC_0}{V_i + V_l} \times V_r \qquad \text{(Eq. A6)}$$

and since V_iC_0 is equal to A_0, one can substitute for V_iC_0 in Eq. A6 and, upon rearrangement, obtain:

$$V_r = \frac{A}{A_0}(V_i + V_l) \qquad \text{(Eq. A7)}$$

and at time zero:

$$V_r = V_i + V_l = V_0 \qquad \text{(Eq. A8)}$$

In this study, original and subsequent radioactivity was used to represent original amount of tracer, A_0, and amount of tracer remaining, A, respectively.

Drainage Rate Constant Determination *via* Nonsampling Technique—After instillation of a known quantity of isotope solution into the eye, there will be a decline in its activity corresponding to a decrease in the volume of solution in the eye. It is possible to obtain the rate constant describing the relationship between drainage rate and volume of solution present, as shown in the following derivation.

The amount of tracer present in the instilled solution can be calculated through Eq. A9:

$$A_0 = V_iC_0 \qquad \text{(Eq. A9)}$$

where C is the concentration of tracer substance in the instilled solution. The differential equation for the change in amount of tracer with respect to time is given by:

$$\frac{dA}{dt} = \frac{C\,dV_i}{dt} + \frac{V_i\,dC}{dt} \qquad \text{(Eq. A10)}$$

Equation A10 is derived on the basis that the change in the amount of drug is due to both a continuous inflow of lacrimal fluid, which changes the concentration of tracer, as well as an outflow or drainage which changes the amount of tracer present.

A complete solution to Eq. A10 produces complex equations and will be presented at a later time. To simplify the treatment and allow solution, it was assumed that the inflow rate of lacrimal fluid is sufficiently small to be neglected for the first 5 min. postinstillation. If there is no inflow of lacrimal fluid, then dC/dt is zero and Eq. A10 reduces to:

$$\frac{dA}{dt} = C\frac{dV_i}{dt} \qquad \text{(Eq. A11)}$$

Substituting for C produces:

$$\frac{dA}{dt} = \frac{A}{V}\frac{dV_i}{dt} \qquad \text{(Eq. A12)}$$

or:

$$\frac{dA}{A} = \frac{dV_i}{V} \qquad \text{(Eq. A13)}$$

which states that the fractional amount of tracer remaining is equal to the fraction of instilled volume remaining.

Thus, it is now possible to examine the relationship between drainage rate and volume present. A first-order relationship between volume present and drainage rate was assumed:

$$dV_i/dt = -k_vV_i \qquad \text{(Eq. A14)}$$

to produce:

$$V_r - V_i = V_ie^{-k_vt} \qquad \text{(Eq. A15)}$$

which relates the change in volume to time.

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The mucosal surface of the GI tract acts as a lipoidal barrier to nutrient and drug molecules. To characterize drug absorption through such a barrier, Schanker *et al.* (1) proposed the pH–partition hypothesis, relating the degree of absorption of weak acids and bases to their lipid solubility and degree of ionization. Quaternary ammonium compounds, being fully charged at physiological pH, cannot be characterized adequately using such criteria. However, many drugs of this class are known to be pharmacologically active in very small quantities when given *via* the enteral route.

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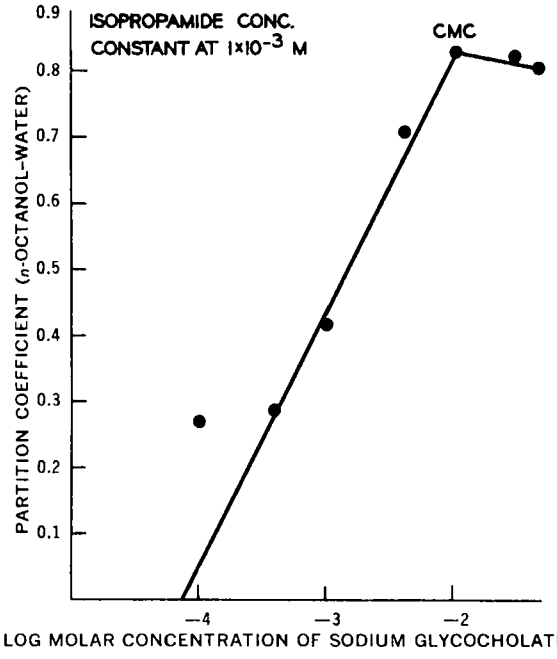

Figure 1—*Apparent partition coefficients of isopropamide iodide equilibrated between* n-*octanol and pH 7.4 potassium phosphate buffer in the presence of various concentrations of sodium glycocholate. Representative data from single experiments are shown. CMC refers to the reported CMC of sodium glycocholate.*

of which are surface active themselves, may incorporate into mixed micelles. Or ion-pairs may form between a bile salt anion and a quaternary cation at concentrations of bile salt below the CMC.

The purposes of this study were to determine if bile salts have the ability to enhance the partitioning of the monoquaternary compound isopropamide iodide across an organic lipoid barrier and to characterize the behavior of this compound in solutions of bile salt, both below and above the CMC. The *in situ* absorption of isopropamide iodide was also studied in the presence of various concentrations of sodium glycocholate.

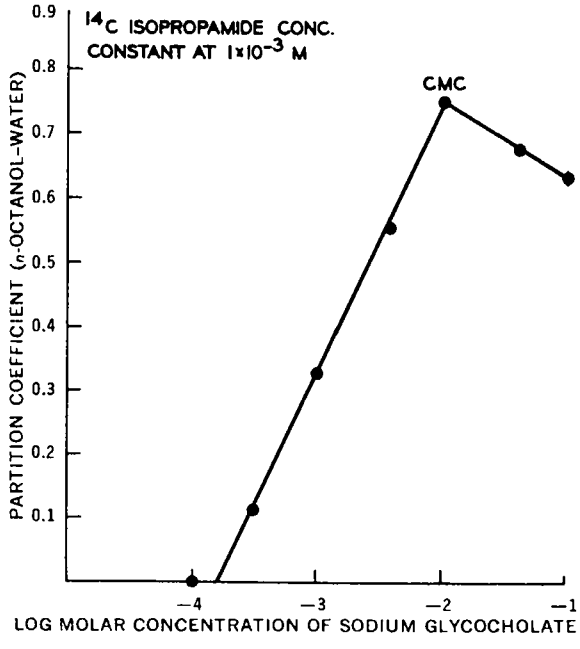

Figure 2—*Apparent partition coefficients of isopropamide iodide equilibrated for 24 hr. between* n-*octanol and pH 7.4 potassium phosphate buffer in the presence of various concentrations of sodium glycocholate. Each data point represents an average of four experimental assay values.*

EXPERIMENTAL

Materials—The following drugs were used as received: isopropamide iodide[1], ^{14}C-isopropamide iodide (specific activity 1.7 mc./mmole[2], sodium cholate[3], sodium glycocholate[4], sodium taurocholate[4], sodium taurodeoxycholate[3], sodium deoxycholate[3], and sodium dehydrocholate[3]. Methyl orange (analytical reagent grade) was used in the colorimetric assay for isopropamide iodide, and Bray's solution (4) was used as the scintillation cocktail in the assay for ^{14}C-isopropamide iodide in the partition studies. A premixed scintillation cocktail[5] was used for counting the ^{14}C-isopropamide iodide recovered from intestinal loops. Grade I (99% pure) *n*-octanol[3] was used as the organic phase in the partition studies. All other solvents and chemicals used in preparing buffers or assay solutions were of analytical reagent grade.

Procedure for *In Vitro* Partition Studies—Partitioning of isopropamide iodide from an aqueous buffer into *n*-octanol in the presence of various concentrations of bile salts was studied colorimetrically and with labeled material. In each case, the concentration of isopropamide iodide was 10^{-3} *M*, with only the bile salt concentration being varied between 10^{-4} and 10^{-1} *M*. At these concentrations of isopropamide iodide, the measured[6] specific conductance showed a linear dependence on concentration, suggesting no formation of large aggregates.

In each case, the isopropamide iodide and bile salts were dissolved together in the same volume of pH 7.4 potassium phosphate buffer[7], which was composed of 3.07 g. KH_2PO_4, 18.36 g. K_2HPO_4, and sufficient deionized water to make 1 l. All solutions were made fresh on the day of use. The pH of all buffer solutions was checked[8].

For the colorimetric assay, a modification of the method of Santoro (5) was employed. Fifteen milliliters of methyl orange buffer[9], pH 10.2, was added to a 1-ml. aliquot of the aqueous phase being assayed (containing at least 0.1 mg. of isopropamide iodide) in a 125-ml. separator. After mixing, this solution was extracted and filtered through a chloroform-saturated pledget of cotton into a 100-ml. volumetric flask, with three 25-ml. portions of chloroform. Ten milliliters of 0.5 *N* HCl in absolute ethanol was then added to develop the color. Volume was brought to a total of 100 ml. with chloroform, and a sample of the final solution was read on a spectrophotometer[10] at 525 nm. against a blank containing chloroform which had been extracted in the same manner. A standard curve was prepared with known concentrations of isopropamide iodide and was linear over the concentration range studied (0.1–0.6 mg./sample). Control experiments indicated that 98–103% of the isopropamide–indicator complex was extractable by the chloroform in the absence, or in the presence, of bile salts after vigorous shaking for 10 min.

Equilibration was with 25 ml. of each phase for 10 min. at 22 ± 1°. After separation by centrifugation, an aliquot (1.0 ml.) of the aqueous phase was assayed and a partition coefficient was calculated by difference. The organic phase, *n*-octanol, was chosen on the basis of its favorable nonpolar lipoid character as suggested by Hansch *et al.* (6).

For determination of partition coefficients using ^{14}C-isopropamide, solutions were prepared as previously described in pH 7.4

[1] Darbid, Smith Kline & French Laboratories, Philadelphia, Pa.
[2] Smith Kline & French Laboratories, Philadelphia, Pa.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Mann Research Laboratories, New York, N. Y.
[5] Insta-Gel, Packard Instrument Co., Downers Grove, Ill.
[6] Beckman conductivity bridge, model RC-18A, Beckman Instruments, Cedar Grove, N. J.
[7] At this pH the bile salts were all present primarily as ionized species, particularly taurocholic acid (pKa = 1.4) and glycocholic acid (pKa = 4.4).
[8] Beckman Zeromatic pH meter, Beckman Instruments, Cedar Grove, N. J.
[9] Prepared by saturating 1 l. of a solution containing 44 g. dipotassium hydrogen phosphate and 21 g. of sodium carbonate with approximately 2 g. of methyl orange. This solution was purified by extraction with three 50-ml. portions of chloroform and filtration through a Whatman No. 2 filter.
[10] Bausch & Lomb Spectronic-20.

potassium phosphate buffer and equilibrated against *n*-octanol for 24 hr. at 22 ± 1° by continuous shaking[11]. Equal phase volumes of 4 ml. each were placed in a 15-ml. glass centrifuge tube, and the aqueous phase was spiked with a sufficient quantity of ^{14}C-isopropamide. An aliquot (100 μl.) of the aqueous phase was taken before equilibration, counted in 15 ml. of Bray's solution, and considered as 100% of the radioactivity present before partitioning. After equilibration, 100 μl. of the aqueous phase was counted as above and partition coefficients, in the presence of each concentration of sodium glycocholate, were calculated by difference. Each partition coefficient was an average value based on four determinations.

Drug Solutions for Absorption Studies—Isopropamide iodide (10^{-3} *M*) was prepared in isotonic sodium phosphate buffer (pH 6.6) composed of 11.44 g. $NaH_2PO_4 \cdot H_2O$, 8.73 g. Na_2HPO_4, and enough deionized water to make 1 l. Ten-milliliter volumes of these solutions were prepared to contain no sodium glycocholate or 10^{-3}, 10^{-2}, or 10^{-1} *M* sodium glycocholate. Ten milliliters of each solution was spiked with 100 μl. of ^{14}C-isopropamide. A 0.5-ml. volume represented the amount of isotope present in the intestine at the beginning of the absorptive period and gave an average of 8.137×10^3 d.p.m.

Procedure for Absorption Studies—Male albino rats[12], weighing 300–350 g. and fasted overnight, were anesthesized with sodium pentobarbital (35 mg./kg. i.p.). A midline incision was made and the terminal ileum was removed from the abdominal cavity. Two ligations were made, using (00) silk thread, approximately 5 and 15 cm. proximal to the ileocecal junction. Care was taken not to tie off the mesenteric blood supply leading to the segment, and manipulation of the tissue was kept minimal. Following ligation, 0.5 ml. of the test solution was injected using a 27-gauge, 1.27-cm. (0.5-in.) needle. After injection, gentle digital pressure over the injection site for a few seconds prevented solution leakage. The ileal segment was replaced in the abdominal cavity and the incision was covered with a 5.1 × 5.1-cm. (2 × 2-in.) gauze pad saturated with 0.9% saline solution. Six periodic checks were made with an ophthalmoscope[13] to determine the presence and onset of mydriasis. Animals were randomly chosen for any given treatment (isopropamide-bile salt combination).

After a 60-min. absorption period, the animals were sacrificed with an overdose of sodium pentobarbital and the ileal segment was removed. The segment was placed in a scintillation vial and flushed with 4.5 ml. of pH 6.6 sodium phosphate buffer. After appropriate mixing, a 0.5-ml. sample of the wash was withdrawn and added to 15 ml. of premixed scintillation cocktail. The mixture was shaken and then allowed to stand several hours in the dark before counting.

After conversion of counts per minute to disintegrations per minute, the percent of ^{14}C-isopropamide still remaining in the gut was determined and the percent absorbed was calculated by difference.

To determine the extent of binding to the intestinal mucosa, tissue digests were prepared[14] by weighing a piece of the ileal segment (approximately 150 mg.) and digesting it[15] for several hours at 50°. When fully dissolved, 15 ml. of premixed scintillation cocktail was added. A separate efficiency calculation was necessary for each sample since slightly different quenching occurred due to differences in color present.

RESULTS AND DISCUSSION

Partition Experiments—Sodium glycocholate, in increasing concentration, enhanced the interphase transport of isopropamide from an aqueous buffer (pH 7.4) into *n*-octanol. Increased partitioning of isopropamide was observed until a concentration of 10^{-2} *M* bile salt was reached. This value is reported to be the approximate CMC of sodium glycocholate within the pH and counterion concentration used in this study (7). These partitioning data were obtained using both the methyl orange assay (Fig. 1) and ^{14}C-isopropamide (Fig. 2). No partitioning of isopropamide occurred in the absence of the bile salt. With ^{14}C-isopropamide at a 10-fold dilution (10^{-4} *M*), partitioning followed the same pattern (Fig. 3).

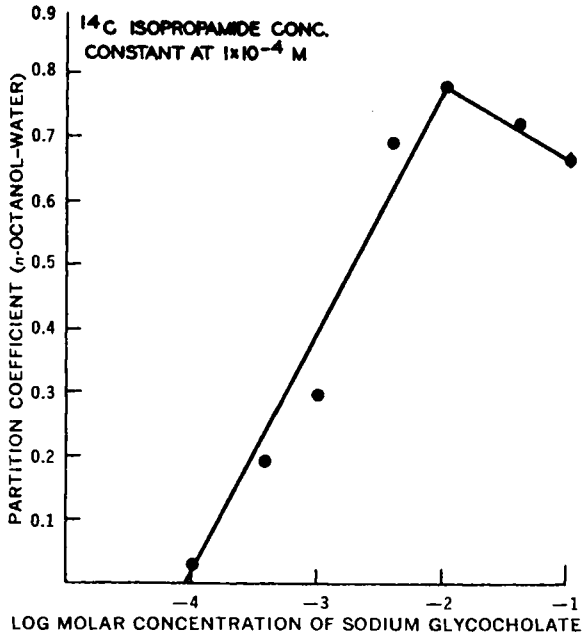

Figure 3—*Apparent partition coefficients of isopropamide iodide equilibrated for 24 hr. between* n-*octanol and pH 7.4 potassium phosphate buffer in the presence of various concentrations of sodium glycocholate.*

Several other bile salts were used in partitioning studies, but not all displayed the same behavior. Sodium taurodeoxycholate behaved very much the same as sodium glycocholate, increasing partitioning to its reported approximate CMC (8) and then exhibiting a plateau (Fig. 4). Other bile salts (Table I) showed increased partitioning of isopropamide at and above their respective CMC values; however, sodium dehydrocholate has been reported not to form micelles (9).

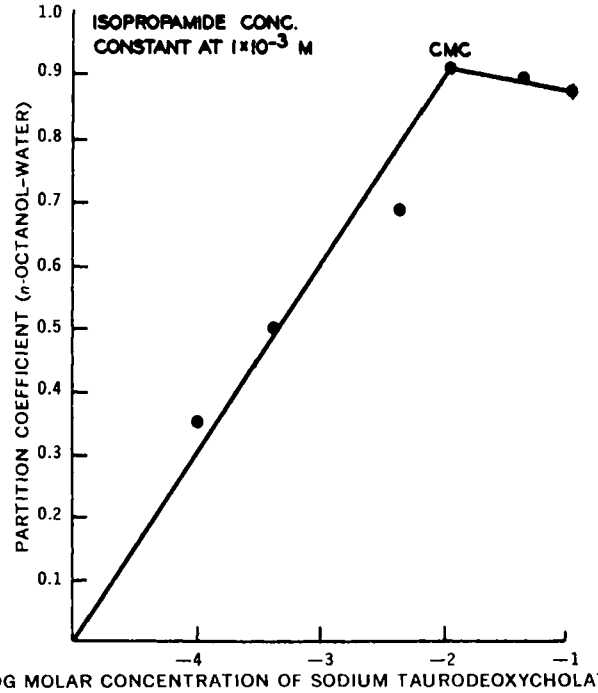

Figure 4—*Apparent partition coefficients of isopropamide iodide equilibrated between* n-*octanol and pH 7.4 potassium phosphate buffer in the presence of various concentrations of sodium taurodeoxycholate. Representative data from single experiments are shown.*

[11] Eberbach automatic shaker, Eberbach Co., Ann Arbor, Mich.
[12] Rolfsmeyer Farms, Madison, Wis.
[13] Welch Allyn, Co., Skaneateles Falls, N. Y.
[14] Only two randomly selected samples were taken from each treatment group.
[15] In 2 ml. of Soluene, Packard Instrument Co., Downers Grove, Ill.

Table I—Apparent Partition Coefficients for Isopropamide Iodide (10^{-3} *M*) in the Presence of Various Concentrations of Different Bile Salts[a]

Bile Salt	Concentration, *M*	Apparent Partition Coefficient (*n*-Octanol–Water)
Sodium cholate	1×10^{-4}	0.31
	1×10^{-3}	0.29
	1×10^{-2}	0.44
	5×10^{-2}	0.81
Sodium deoxycholate	1×10^{-4}	0.35
	1×10^{-3}	0.63
	1×10^{-2}	0.88
	5×10^{-2}	0.96
Sodium dehydrocholate	1×10^{-4}	0.29
	1×10^{-3}	0.38
	1×10^{-2}	0.52
	5×10^{-2}	0.75
Sodium taurocholate	1×10^{-4}	0.40
	1×10^{-3}	0.44
	1×10^{-2}	0.85
	5×10^{-2}	0.85

[a] Isopropamide concentration in the aqueous phase, determined after equilibration by the methyl orange assay method from duplicate extractions.

Studies by Levine and Clark (10) and by Schanker and Solomon (11) indicated that various other monoquaternary compounds can be extracted into an organic phase (ethylene dichloride) in the presence of sodium glycocholate below its CMC. Formation of ion pairs between quaternary ammonium ions and carboxylic acids (12), as well as sulfonates (13), is known to occur. Methyl orange (5) and bromthymol blue (14) also form ion-pairs with quaternary ammonium cations. Tetraheptylammonium chloride was used by Hofmann (15) to extract steriod sulfates quantitatively as well as glycine and taurine conjugates of bile acids from aqueous solvents.

Various possibilities exist for the interaction of bile salt anions *in vitro* among themselves and with quaternary cations in the aqueous phase.

Mixed micelles may form between short-chain alkyl amines and bile salts involving van der Waals' forces between the hydrocarbon chains and hydrophobic surface of the steroid nucleus (16); also, adsorption of the oppositely charged amine may occur onto the micellar surface at concentrations of bile salts above the CMC.

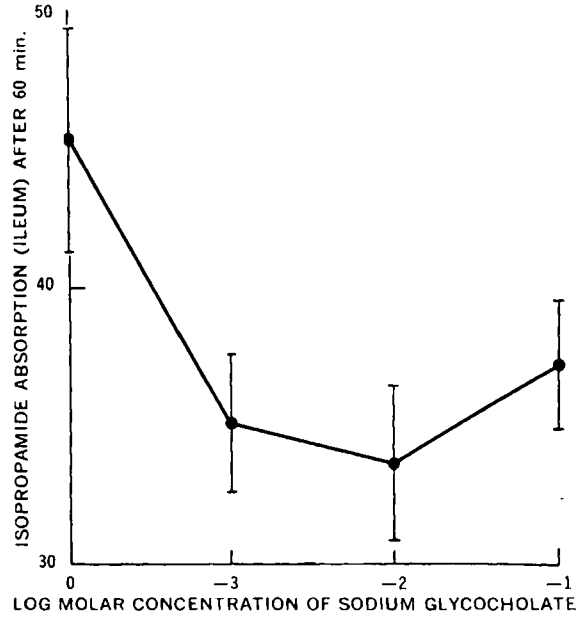

Figure 5—In vivo *absorption of isopropamide iodide from ligated segments of rat ileum. Each data point is the mean of 12 animals, and bars represent the standard error of the mean for each point. Points at zero* versus 10^{-3} *and* 10^{-2} M *were the only means significantly different* ($p < 0.05$) *from one another.*

Below the CMC, the most likely situation is the formation of ion-pairs and the partitioning of these into the organic phase. In the case of the ion-pair partitioning, the following interaction in the aqueous phase might be assumed:

$$B_a^- + Q_a^+ \overset{K_{BQ}}{\rightleftharpoons} (BQ)_a \overset{K_p}{\rightleftharpoons} (BQ)_o$$

Scheme I

Here K_{BQ} is the equilibrium constant for ion-pair formation between the bile salt anion (B_a^-) and quaternary cation (Q_a^+) in the aqueous phase, and K_p is the partition coefficient for partitioning of the neutral species $(BQ)_a$ into the organic phase.

According to the partitioning data, this would seem to be a possible mechanism for interphase transport of isopropamide below the CMC. Micellar transport probably would be inhibited by a dense negative charge surrounding the micelle.

Absorption Studies—The present studies indicate that the absorption of isopropamide iodide is not influenced by the presence of sodium glycocholate. Isopropamide iodide itself is capable of being absorbed from the rat ileum to the extent of 44% over a 60-min. absorptive period (Fig. 5). A decrease in absorption of this cation is seen in the presence of all concentrations of sodium glycocholate. Statistically, the mean value for isopropamide absorption in the absence of any bile salt was significantly different ($p < 0.05$) only from 10^{-3} and 10^{-2} *M* sodium glycocholate, as determined using analysis of variance and a Duncan's multiple-range test. Between 10^{-2} and 10^{-1} *M* bile salt concentration, there was an apparent increase in the amount of isopropamide absorbed, although this rise was still well below the value found in the absence of bile salt. Jejunal absorption followed the same pattern, with the corrected percentages for absorption being essentially the same.

Others (17) showed that above their respective CMC's, sodium glycocholate and sodium taurocholate at pH 6.5 inhibit the absorption of the amide, 2-allyloxy-4-chloro-*N*-(2-diethylaminoethyl)-benzamide hydrochloride, from the rat small intestine.

Digests of the intestinal tissue indicated that approximately 21.2 and 13.0% of the radioactivity remained in the ileal and jejunal gut wall, respectively. The value was subtracted from the graphical values (Fig. 5) and agrees well with a figure of 20% for a dose of anisotropine methylbromide reported recently (18). Corrections for volume changes due to secretion into the lumen were also made, since it appeared in early studies that 10^{-1} *M* sodium glycocholate was causing an intraluminal increase in drug solution volume. The nonabsorbable marker ^{14}C-inulin was used in control experiments involving all bile salt concentrations, and the only detectable effect was seen at the 10^{-1} *M* level. This amounted to a 15% dilution of the amount of drug remaining in the intestinal loop after 1 hr. The corrected value at this concentration of sodium glycocholate is the one expressed in Fig. 5.

During the absorptive period, observations were made to determine the onset and degree of mydriasis. This was not a good indicator of absorption, however, since only one or two animals in each group exhibited this response, and there was no relationship between time of onset of the response and bile salt concentration. Irwin *et al.* (19) suggested that ion-pair formation with trichloroacetate as the anion can enhance the partitioning of isopropamide iodide into chloroform and *n*-octanol, as well as increase the degree of mydriasis in mice given the drug orally with a large molar excess of trichloroacetate. Their conclusion that this anion increases absorption by ion-pair formation in the lumen has been criticized (20), however, since the concentrations of trichloroacetate administered could have decreased protein or tissue binding of the isopropamide, thus increasing the bioavailability of the small amount of drug that might have actually been absorbed *via* a different mechanism.

If an ion-pair or other type of complex is present in the gut at concentrations of bile salt below its CMC, several possibilities for the diffusion of this complex arise. Depending upon various equilibrium constants, the ion-pair may cross the mucosal cell barrier as the neutral species and remain unchanged within the cell, thus subsequently passing to the blood or lymph. It may, however, be disrupted by protein constituents of the membrane surface, as has been described for some dye complexes (21), and thus enter as the free cation. Furthermore, any amount of quaternary cation that is not part of an aggregate or ion-pair may complex within the intes-

tinal mucosa with anionic membrane components and be transported to the circulation as a different complex. In this regard, some related compounds (dialkyl propionamides) were recently shown to have the apparent ability to form a complex within the intestinal membrane itself and thus enhance the absorption of prednisone and prednisolone (22).

Some quaternary ammonium compounds have been found to be rapidly absorbed initially and then more slowly (23), indicating possible saturation of a carrier and a facilitated diffusion mechanism (24).

Tetracycline appears to be absorbed from the rat stomach as the charged species by such a mechanism (25), and Levine (26) postulated an endogenous carrier for quaternary compounds. Other effects, such as cooperative membrane reactions (27) or solvent drag (28), may also play a role in the absorption of quaternary drugs. Isopropamide, which possesses both some lipophilicity and hydrophilicity as do many other monoquaternary compounds, may possibly be taken up into globular micelles (29) of the absorptive cell membrane. This seems relevant in view of the ability of isopropamide to interact with bile salt micelles as reported here.

Isopropamide is absorbed to a greater extent than expected for a charged molecule, but the mechanism does not appear to involve bile salts. A facilitated transport process may be involved, but it is not proven by the data reported here.

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▲ To whom inquiries should be directed.

Steric Aspects of Adrenergic Drugs XXII: Retention of (+)- and (−)-^{14}C-Norepinephrine by Mouse Heart

B. D. GARG*, R. D. KRELL†, T. SOKOLOSKI, and P. N. PATIL▲

Abstract □ After intravenous infusion (total dose 40 mcg./kg.) of the isomers of norepinephrine to normal or reserpine-pretreated mice, the initial accumulation by the heart was the same for both isomers. The injected isomers disappeared from the normal heart in a single exponential phase. The unnatural isomer disappeared faster, $t_{1/2}$ 2.5 hr., than the natural (−)-isomer, $t_{1/2}$ 7.6 hr. In mice pretreated with iproniazid, iproniazid and guanethidine, or iproniazid and α-methyl-*p*-tyrosine, the rate constants and the $t_{1/2}$ values for the isomers were unaltered. The results indicate that retention, probably by storage vesicles, favors the natural isomer; hence, it is stereoselective. Reserpine, which inhibited the accumulation by storage vesicles, drastically reduced the accumulation of isomers, and the accumulated isomers disappeared at a similar rate constant with a $t_{1/2}$ of approximately 1 hr. Racemic norepinephrine disappeared in two phases, each having a separate rate constant which approximate the values obtained with (+)- and (−)-norepinephrine. In the iproniazid-pretreated mice, injection of equal amounts of (−)- or (+)-^{14}C-norepinephrine resulted in higher *O*-methylated products from the (+)-form.

Keyphrases □ Norepinephrine, ^{14}C-labeled—retention of (+)- and (−)-isomers by mouse heart, effects of iproniazid, guanethidine, or α-methyl-*p*-tyrosine pretreatment, steric aspects of adrenergic drugs □ Adrenergic drugs—steric aspects, retention of (+)- and (−)-isomers of ^{14}C-norepinephrine by mouse heart □ Catecholamines—retention of (+)- and (−)-isomers of ^{14}C-norepinephrine by mouse heart, steric aspects of adrenergic drugs □ Neurochemical transmitters—retention of (+)- and (−)-isomers of ^{14}C-norepinephrine by mouse heart, steric aspects of adrenergic drugs □ False neurotransmitter—retention of (+)- and (−)-isomers of ^{14}C-norepinephrine by mouse heart

Since 1963 there has been considerable interest in the fate of optical isomers of catecholamines (1–5). Lack of optically pure radiolabeled isomers, however, impeded investigations. Many, if not all, physiological processes can discriminate between the stereoisomers of catecholamines, as discussed in previous reviews (6, 7). Since the transport of isomers across the neuronal membrane shows marginal selectivity in favor of the natural isomer, it has been possible to study uptake and release of both isomers from tissues (8–10). Recently, Allen *et al.* (8) suggested that (+)-norepinephrine might be a valuable false neurochemical transmitter. In view of the large differences in adrenergic receptor-activating properties of the isomers (6, 7), the proposal appears quite attractive. However, for an unnatural substance to be an effective false neurotransmitter, the drug must be retained by adrenergic storage vesicles (11, 12). This paper reports studies on the time course of retention of the isomers by the mouse heart. Other pharmacological tools such as α-methyl-*p*-tyrosine, reserpine, 6-hydroxydopamine, and guanethidine pretreatment were used to gain information about the retention of the isomers.

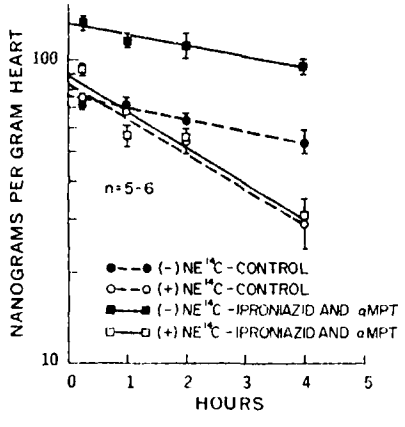

Figure 1—*Uptake and retention of (−)- and (+)-^{14}C-norepinephrines in the normal and drug-treated mouse hearts after intravenous infusion of (−)- and (+)-^{14}C-norepinephrines, 40 mcg./kg. The schedule of treatment with iproniazid and α-methyl-*p*-tyrosine was described in the* Methods *section. Key: n = number of observations, vertical bars = standard error of the mean, NE = norepinephrine, and αMPT = α-methyl-p-tyrosine.*

METHODS

Infusion of (−)- and (+)-^{14}C-Norepinephrine Isomers—Male albino ICR mice (16–26 g.) were used. Each animal was infused with either (−)- or (+)-^{14}C-norepinephrine *via* one of the lateral tail veins. The total dose infused was 0.8 mcg./20 g., which corresponded to 0.1 μc./0.1 ml./20 g. for each isomer. Infusion[1] was constant at a rate of 0.2 ml./min. and required 25–40 sec. for completion.

Mice were decapitated at varying periods postinfusion (5 min. to 24 hr.), and hearts were quickly removed, cleaned of extraneous tissue, minced with scissors, and rinsed five times with cold 0.9% sodium chloride solution. The minced material was blotted with filter paper, weighed, and transferred to 20-ml. beakers containing 5 ml. of 5% cold trichloroacetic acid containing 0.2 ml. of 10% ethylenediaminetetraacetic acid. Hearts were minced with scissors and homogenized[2] for 20 sec. at moderate speed. The homogenates were centrifuged for 10 min. in a clinical centrifuge at room temperature. The resulting supernate was filtered under suction, and the pellet was resuspended in 0.5 ml. of 0.4 *N* $HClO_4$ and recentrifuged for another 10 min. The second supernate was mixed with the first, and the final volume was made up to 8 ml. The filtrates were transferred to 20-ml. beakers containing 10 mg. ethylenediaminetetraacetic acid. The pH of these extracts was adjusted to between 6.2 and 6.5 with 2 *N* NaOH. The unchanged ^{14}C-norepinephrine was separated from the contaminating metabolites by passing the slightly acidic extracts through strong ion-exchange columns prepared[3] according to the method of Häggendal (13, 14) with a small modification. The modification made was in the use of columns 9.0 mm. in diameter and a resin height of 3.5 cm. The fractions of the eluate containing ^{14}C-norepinephrine were placed in 20-ml. beakers and evaporated to dryness. The recovery of ^{14}C-

[1] Harvard infusion pump model 901, Harvard Apparatus Co., Millis, Mass.
[2] Using an Ultra-Turrax, Brinkmann Instruments.
[3] From Amberlite CG-120 (200–400 mesh), Na^+ form, Mallinckrodt Chemical Co.

Table I—Rate Constants and Half-Lives ($t_{1/2}$) of Disappearance of ^{14}C-Norepinephrine Isomers from the Mouse Heart after Different Treatments

Treatment	Isomer	Rate Constant, hr.$^{-1}$	$t_{1/2}$, hr.
Control	(−)	0.09	7.62
	(+)	0.26	2.57
Iproniazid[a]	(−)	0.07	9.32
Iproniazid[a]	(+)	0.17	3.92
Iproniazid[a]	(±)	0.27	2.19
		0.06	10.10
Iproniazid[a] + guanethidine[b]	(−)	0.09	7.52
	(+)	0.15	4.57
Iproniazid[a] + reserpine[c]	(−)	0.64	1.07
	(+)	0.87	0.78
Iproniazid[a] + α-methyl-*p*-tyrosine[d]	(−)	0.08	8.00
	(+)	0.2	2.56

[a] Injected 100 mg./kg. i.p., 16–20 hr. before ^{14}C-norepinephrine infusion. [b] Injected 20 mg./kg. i.p., 8–10 hr. before iproniazid. [c] Injected 5 mg./kg. i.p., 8–10 hr. before iproniazid. [d] Injected in two divided doses, 200 mg./kg. each, at 3-hr. intervals and 10–12 hr. after iproniazid.

norepinephrine from the columns was 72.14 ± 0.76%. Values reported were corrected for 72% recovery. Residues for the air-dried hydrochloride eluates were taken up in 1 ml. of water and counted in 14 ml. of a liquid scintillation solution[4].

In experiments where animals were treated with iproniazid to inhibit MAO, no attempt was made to separate unchanged ^{14}C-norepinephrine from the *O*-methylated metabolites. In a few experiments, where the mice were sacrificed 10 min. after ^{14}C-norepinephrine injection, the *O*-methylated derivatives did not exceed 5% of the unchanged amine. Since metabolites do not accumulate in the tissues and are easily washed out (15), it was expected that when animals were sacrificed at longer time periods after ^{14}C-norepinephrine infusion, the amount of metabolites present would be lower still. Moreover, the minced heart was washed thoroughly to reduce contamination by *O*-methylated derivatives. The trichloroacetic acid extracts were air dried, taken up in 2 ml. of water, mixed with 13 ml. of a liquid scintillation solution, and counted[5].

Schedule of Drug Treatments in Mice—MAO inhibition was accomplished by administering iproniazid, 100 mg./kg. i.p., 16–20 hr. prior to infusion of ^{14}C-norepinephrine isomers. Effects of all other pharmacological treatments were studied in the enzyme-inhibited animals.

Guanethidine, 20 mg./kg. i.p., was given 8–10 hr. before iproniazid. ^{14}C-Norepinephrine isomers were infused 24–30 hr. after guanethidine.

Reserpine, 5 mg./kg. i.p., was given 8–10 hr. before iproniazid. ^{14}C-Norepinephrine isomers were infused 24–30 hr. after reserpine,

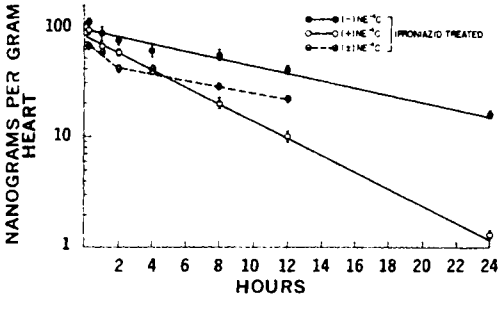

Figure 2—*Uptake and retention of (−)-, (+)-, or racemic ^{14}C-norepinephrines in the hearts of mice that received iproniazid, 100 mg./kg., 16–24 hr. before they received 40 mcg./kg. i.v. of ^{14}C-norepinephrine infusion. Each point is a mean of five to nine observations. Key: vertical bars = standard error of the mean, and NE = norepinephrine.*

[4] Aquasol, New England Nuclear.
[5] Tri-Carb liquid scintillation spectrometer.

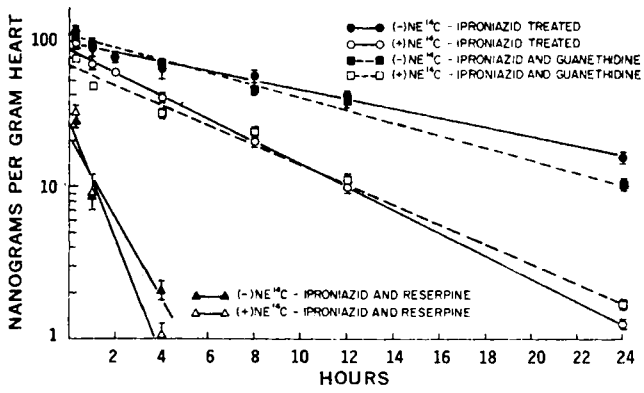

Figure 3—*Uptake and retention of (−)- and (+)-^{14}C-norepinephrines in hearts of mice treated with iproniazid and guanethidine or iproniazid and reserpine after intravenous infusion of ^{14}C-norepinephrine isomers, 40 mcg./kg. The schedule of treatments given is described in the* Methods *section. Each point is a mean of five to nine observations. Key: vertical bars = standard error of the mean, and NE = norepinephrine.*

and animals were sacrificed in the time interval from 15 min. to 4 hr. thereafter.

Tyrosine hydroxylase was inhibited by the administration of two doses of α-methyl-*p*-tyrosine, 200 mg./kg. i.p., 3 hr. apart. Iproniazid was injected 16–20 hr. before the second dose of α-methyl-*p*-tyrosine, which was given 1 hr. before ^{14}C-norepinephrine infusion.

6-Hydroxydopamine was administered in two doses of 75 mg./kg. i.p. at an interval of 1 hr., about 24 hr. before ^{14}C-norepinephrine infusion. Iproniazid was injected as already described. Animals were sacrificed 24–28 hr. after 6-hydroxydopamine treatment.

In other experiments, (−)- and (+)-^{14}C-norepinephrines were mixed in equal amounts (adjusted to equal specific activity), and the resulting (±)-^{14}C-norepinephrine was infused in the iproniazid-treated mice. Animals were sacrificed in the time interval from 15 min. to 12 hr. after (+)-^{14}C-norepinephrine infusion.

Determination of *O*-Methylated Products—Iproniazid-treated mice were given either (−)- or (+)-^{14}C-norepinephrine, 40 mcg./kg. i.v. Five hearts were pooled in one beaker and extracted in trichloroacetic acid as described earlier. *O*-Methylated derivatives were separated from the unchanged or oxidized ^{14}C-norepinephrine by ion-exchange chromatography according to the method of Häggendal (14). The eluates were air dried and counted in the manner described for ^{14}C-norepinephrine estimation.

Drugs and Chemicals—Drugs used in this study were: (−)-norepinephrine (+)-bitartrate monohydrate and (+)-norepinephrine (+)-bitartrate[6]; (−)-^{14}C-norepinephrine-7 (+)-bitartrate, specific activity 57.0 mc./mmole, and (+)-^{14}C-norepinephrine-7 (+)-bitartrate, specific activity 21.2 mc./mmole[7]; iproniazid phosphate[8]; guanethidine hemisulfate and reserpine powder[9]; 6-hydroxydopamine hydrobromide[10], and α-methyl-*p*-tyrosine methyl ester hydrochloride[10].

Reserpine was prepared as a 0.5% solution in 10% ascorbic acid. The solution of 6-hydroxydopamine was prepared in 0.1% sodium metabisulfite at 0° and was deaerated for at least 10 min. using nitrogen. Stock aqueous solutions of iproniazid and guanethidine were prepared and used as needed. Solutions of α-methyl-*p*-tyrosine were prepared fresh daily. Working solutions of labeled (−)- and (+)-norepinephrines were prepared in 1% sodium metabisulfite, protected from light and air, and stored in the refrigerator at 4°. All drugs and chemicals were dissolved in demineralized double-distilled water.

RESULTS

Accumulation and Retention of (−)- and (+)-^{14}C-Norepinephrine Isomers in Mouse Hearts—Mice were given either (−)- or (+)-

[6] Sterling-Winthrop Research Institute, Rennsselaer, N. Y.
[7] Amersham-Searle, Arlington Heights, Ill.
[8] Hoffmann-La Roche, Inc., Nutley, N. J.
[9] Ciba Pharmaceutical Co., Summit, N. Y.
[10] Regis Chemical Co., Chicago, Ill.

Table II—Accumulation and Retention of ^{14}C-Norepinephrine Isomers in Hearts of 6-Hydroxydopamine-Treated[a] Mice[b]

Time after ^{14}C-Norepinephrine Infusion (Total Dose 40 mcg./kg.)	(−)-^{14}C-Norepinephrine ng./g. Heart	(−)-^{14}C-Norepinephrine % of Control	(+)-^{14}C-Norepinephrine ng./g. Heart	(+)-^{14}C-Norepinephrine % of Control[c]
15 min.	4.90 ± 0.23	4.63	6.01 ± 0.76	6.71
4 hr.	0.81 ± 0.21	1.36	0.01	0.02

[a] Treatment with 6-hydroxydopamine was accomplished as described under *Methods*. [b] The results are mean ± standard error of margin of six independent observations. [c] Control refers to the values obtained at corresponding times in the iproniazid-treated animals.

^{14}C-norepinephrine, and the amount of ^{14}C-norepinephrine accumulated and retained in hearts at 15 min. and 1, 2, and 4 hr. was determined. The results obtained were plotted semilogarithmically against time in Fig. 1. It may be seen that at 15 min. both isomers were taken up almost to the same extent but that the (+)-^{14}C-norepinephrine loss was faster than the (−)-isomer loss. First-order rate constants and half-lives for the disappearance of the isomers were calculated (Table I). Both isomers showed a single exponential loss.

Effect of Iproniazid Treatment—(−)-Norepinephrine is a better substrate for MAO than is (+)-norepinephrine (16). Hence, it was necessary to compare accumulation after enzyme inhibition. Mice were pretreated with iproniazid before infusion of ^{14}C-norepinephrine as described in the *Methods* section. Five minutes after infusion, it was found that both isomers accumulated to the same extent (data are not illustrated). The accumulation of both isomers at 15 min. was higher than at 5 min. postinfusion. After 15 min., the concentration of both isomers began to decline. Thus, the maximum accumulation occurred between 5 and 15 min. The concentration of (+)-^{14}C-norepinephrine declined faster than that of (−)-^{14}C-norepinephrine (Fig. 2). Previously, biphasic disappearance of radioactivity was observed after intravenous infusion of (±)-^{3}H-norepinephrine. It is possible that the disappearance of (±)-^{3}H-norepinephrine in two phases may be partly due to the decline of (+)- and (−)-^{3}H-norepinephrine isomers with separate rate constants.

To investigate the disappearance of ^{14}C-norepinephrine, iproniazid-treated animals were infused with racemic ^{14}C-norepinephrine prepared by mixing equal amounts of (−)- and (+)-^{14}C-norepinephrines in the dose and infusion rate identical to those used for the pure isomers. The animals were sacrificed at different times from 15 min. to 12 hr. (Fig. 2 and Table I). Clearly, the racemic ^{14}C-norepinephrine disappeared biexponentially. After feathering the data, the first rate constant was closer to that of (+)-^{14}C-norepinephrine and the second to that of (−)-^{14}C-norepinephrine. It appears that exogenously administered norepinephrine is stored in a single kinetic pool from which it disappears as a first-order process; or if it is stored in multiple pools, they are in rapid equilibrium with each other. For subsequent experiments involving the effects of certain drugs on the accumulation and retention of ^{14}C-norepinephrine isomers, iproniazid-pretreated animals were used.

Disappearance of ^{14}C-Norepinephrine Isomers after Guanethidine or Reserpine Treatment—Both reserpine and guanethidine are known to deplete endogenous norepinephrine from tissues. Reserpine is known to produce this action by blocking uptake by vesicular storage mechanisms (17), and guanethidine is utilized to inhibit the same mechanism (18, 19). Hence, the effects of both these drugs should be similar as far as the accumulation and retention of ^{14}C-norepinephrine are concerned. The disappearance curves obtained are illustrated in Fig. 3, and the first kinetic analysis is given in Table I. The results show that guanethidine had very little influence on the disappearance of the isomers. Reserpine, on the other hand, appeared to cause marked inhibition of uptake and storage of both isomers. Both (−)- and (+)-^{14}C-norepinephrines disappeared at a much faster rate. Reserpine also abolished the stereoselectivity of efflux as observed in the control experiments. Within 4 hr. postinfusion, there was almost the complete disappearance of isomers in reserpine-treated tissues, whereas detectable amounts of ^{14}C-norepinephrine could be found even after 24 hr. in guanethidine-treated tissues.

Table III—*O*-Methylated Metabolites Present in Mouse Hearts[a] 10 min. after Intravenous Injection of (−)- and (+)-^{14}C-Norepinephrines

Isomer	^{14}C-*O*-Methylated Metabolites (c.p.m./g. heart)	Significance
(−)-Norepinephrine	1500[b] ± 221	$p < 0.01$
(+)-Norepinephrine	2672 ± 165[c]	

[a] Total dose for each isomer was 40 mcg./kg. Five hearts were pooled together. [b] Mean of nine observations ± standard error to margin. [c] Mean of eight observations ± standard error of margin.

Effect of α-Methyl-*p*-tyrosine—α-Methyl-*p*-tyrosine was given to inhibit tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of norepinephrine. By inhibiting this enzyme, endogenous norepinephrine can be significantly depleted. Accumulation and disappearance of exogenously administered ^{14}C-norepinephrine could thus be studied more clearly without being diluted or exchanged with endogenous norepinephrine. In animals pretreated with iproniazid and α-methyl-*p*-tyrosine, the accumulation and disappearance of ^{14}C-norepinephrine were observed in the interval from 15 min. to 4 hr. (Fig. 1 and Table I). There was a higher accumulation of (−)-^{14}C-norepinephrine as compared to (+)-^{14}C-norepinephrine. The disappearance rates were not significantly different from those observed in control animals.

Effect of 6-Hydroxydopamine Treatment—6-Hydroxydopamine has been shown to cause severe damage to, and specific degeneration of, the adrenergic nerves (20). In mice treated with this drug, the amount of ^{14}C-norepinephrine was determined at 15 min. and 4 hr. after infusion of each isomer of ^{14}C-norepinephrine (Table II). At 15 min., the concentration of ^{14}C-norepinephrine for either isomer was 4–6% of the control; at 4 hr., it was barely above the background.

Slight accumulation of radioactivity at 15 min. postinfusion probably represents the accumulation of ^{14}C-norepinephrine by extraneuronal uptake of *O*-methylated metabolites and, to some extent, accumulation of norepinephrine by the surviving adrenergic nerves. Thus, the accumulation of ^{14}C-norepinephrine by "uptake$_2$" (3) and *O*-methylated metabolites in the present experiments was rather small and was observed only at shorter time intervals.

Metabolism of (−)- and (+)-^{14}C-Norepinephrines by Catechol-*O*-methyltransferase—In the iproniazid-treated animals, normetanephrine formed in hearts of mice after the administration of equal amounts of (−)- or (+)-^{14}C-norepinephrine was isolated by ion-exchange chromatography. As shown in Table III, significantly more *O*-methylated metabolites formed from (+)-^{14}C-norepinephrine than from (−)-^{14}C-norepinephrine. (+)-Norepinephrine may be a better substrate than (−)-norepinephrine for the enzyme or, alternatively, more (+)-isomer may be available to the enzyme due to low vasoconstrictor activity.

DISCUSSION

The infusion of isomers in mice indicates that the accumulation of ^{14}C-norepinephrine by the heart was higher after 15 min. than after 5 min. No selectivity was observed in the initial accumulation of the isomers. Even after blockade of vesicular accumulation by reserpine treatment, the initial uptake was the same for both isomers. If the initial accumulation is considered mainly neuronal (21–23), the results suggest that the transport of the isomers across the neuronal membrane of the mouse heart lacks stereoselectivity. The interpretation is consistent with *in vitro* findings that mouse heart shows little or no stereoselectivity (24).

Neuronal uptake (3) and efflux (25) are considered active processes. The similar rates of disappearance of the isomers from the reserpine-pretreated animals indicate that neuronal efflux from the mouse heart may also be nonstereoselective. However, it could be that reserpine treatment itself destroys the sodium-dependent and optically specific amine carrier mechanism at the adrenergic neuron (26).

In vivo, the selectivity in the vesicular retention is clearly demonstrated. The manipulation of the accumulation by pharmacological tools suggests that the retention of the isomers by the vesicles is truly stereoselective in favor of the (−)-isomer:

1. Reserpine, which is known to inhibit the uptake by the vesicles, abolishes the stereoselectivity.
2. Pretreatment of mice with α-methyl-*p*-tyrosine, which depletes the vesicular stores by inhibiting the enzyme tyrosine hydroxylase, favors the accumulation of the (−)-isomer only.
3. Chemical sympathectomy with 6-hydroxydopamine drastically reduces the accumulation.
4. Almost all of the (+)-isomer is lost from the heart after 24 hr., while a considerable amount of the (−)-form can be found in the tissue.

The results favor the view that the stereochemically "correct" orientation of the β-hydroxyl group is important for the ATP–magnesium-dependent process in the adrenergic nerve granules (2, 27).

An unexpected finding of the present study is that uptake and retention of isomers were not influenced by a relatively large dose of guanethidine (Fig. 3). These observations are similar to those reported by Krell and Patil (9), who could not show the inhibition of uptake of catecholamines in vas deferens obtained from guanethidine-pretreated animals. In line with these observations, Maitre and Staehelin (28) were unable to find inhibition of uptake of catecholamine by the isolated splenic nerve granules in the presence of guanethidine. It may be that guanethidine depletes endogenous norepinephrine without affecting the vesicular uptake. The uptake of isomers would then appear normal. In the present report, the levels of endogenous norepinephrine after guanethidine were not estimated. It may be that the drug was administered at a time when guanethidine was without a depleting effect. However, as low as 8 mg./kg. of guanethidine is known to deplete 80–90% cardiac norepinephrine for 10–15 hr. after intravenous injection in rats (29). Further work is necessary to investigate the mechanism of action of guanethidine and its influence on the accumulation of exogenous norepinephrine.

In many studies, a multiphasic disappearance of labeled norepinephrine from the heart was observed. Most earlier workers used ^{3}H-labeled racemic norepinephrine as a valid tracer of the natural neurotransmitter. Since (±)-^{3}H-norepinephrine is an equal mixture of two compounds, it could be that these isomers are released from the storage sites at different rates and thus manifest the multiphasic disappearance. In this study, a biphasic decline was observed when (±)-^{14}C-norepinephrine was used. After administering pure (−)- or (+)-isomers, in control or drug-treated mice, the radioactivity disappeared in a single exponential phase (Figs. 1–3). (+)-Norepinephrine disappeared much faster than the (−)-isomer. Iversen *et al.* (23) also employed a pure ^{14}C-isomer of norepinephrine, yet they observed that norepinephrine disappeared in two phases for each isomer. Since the experimental conditions in the present study are different from those reported by Iversen *et al.*, the data cannot be critically compared.

Data on the metabolism of (−)- and (+)-norepinephrines by catechol-*O*-methyltransferase are particularly interesting. From Table III, it may be seen that there are more *O*-methylated metabolites formed from (+)-norepinephrine than from equal doses of (−)-norepinephrine. In earlier studies, this enzyme was reported to possess no stereoselectivity for either isomer of norepinephrine [review by Patil *et al.* (7)]. Iversen *et al.* (23) also reported that catechol-*O*-methyltransferase lacked any stereospecificity. Their data, similar to ours, indicated that after equal doses of (−)- or (+)-^{14}C-norepinephrine there were twice as many *O*-methylated metabolites from (+)-norepinephrine as compared to those from (−)-^{14}C-norepinephrine. Furthermore, detailed kinetic analysis of the influence of the enzyme on optical isomers is necessary for the correct interpretation of the results. As far as the false neurotransmitter status of the (+)-norepinephrine is concerned, rapid *O*-methylation and rapid loss from tissue of the substance may be undesirable properties.

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Improved, Rapid Spectrophotofluorometric Method for Assay of Epinephrine: A Comparative Study

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Abstract □ A new and improved two-step spectrophotofluorometric method for the determination of epinephrine in decomposed formulations is described. The method depends on the initial oxidation of epinephrine with iodine to "adrenochrome," which is subsequently cyclized with alkali to "adrenolutin," which is responsible for the fluorescence. The fluorescence is stabilized with alkaline ascorbate. The method is unique in that it is simple and accurate, and it has additional advantages over the official (USP XVIII and NF XIII) methods. Several samples can be accurately analyzed within a short time. Extremely dilute epinephrine solutions (1:100,000) can be analyzed, which is not possible with current official methods. The influence of the various reagents on the final fluorescence and the stability of the fluorescence as a function of time are described. The absence of interference from epinephrine decomposition products on the recovery of epinephrine from simulated formulations is also described.

Keyphrases □ Epinephrine solutions—spectrophotofluorometric analysis, compared to compendial methods □ Spectrophotofluorometry—analysis, epinephrine solutions

Numerous methods have been described for the determination of epinephrine, norepinephrine, and related catecholamines in biological material (1, 2). USP XVIII describes an assay procedure (3) for epinephrine injection which measures the optical rotation of the triacetylepinephrine derivative. This method cannot be utilized directly without modification for the estimation of very dilute solutions of epinephrine (1:10,000 or 1:100,000) or very small volumes of these extremely dilute solutions. To assay these very dilute solutions according to the official method, a large number of samples is required and quantitative extraction of a large volume of solution may lead to large errors. NF XIII describes a colorimetric method (4) for the assay of epinephrine. This method does not distinguish between the biologically active epinephrine from the inactive decomposition products. Thus, both of these methods have disadvantages in that they cannot be used directly for the assay of large numbers of samples of extremely dilute solutions or do not distinguish the active species from the inactive decomposition products.

In view of these disadvantages, the preferred assay method is the spectrophotofluorometric method, which has been extensively employed to determine catecholamine derivatives in biological fluids (5, 6). Backe-Hansen *et al.* (7) reported the application of Hellberg's (8) method for the estimation of epinephrine in injections and verified the specificity of the fluorometric method by a comparison with the biological method of elevation in the blood pressure of rats and the rat uterus method. However, neither experimental details nor effects of catecholamine decomposition products on the fluorometric method were reported.

To date the possible application of the fluorometric method to routine assay of epinephrine samples in parenteral formulation has not been investigated in depth. The various parameters including the choice of the oxidizing agent, the period of oxidation, the development and stabilization of the fluorescence, and the influence of various reagents on fluorescence in parenteral formulations have not been reported. Also, the influence of the decomposition products of epinephrine injections, stabilized with the commonly used antioxidants, on the development of fluorescence has not been investigated.

This report describes a simple, accurate, and rapid fluorometric method for the routine assay of epinephrine parenteral formulations, which is also applicable for the assay of very dilute solutions. Statistical evaluation of the fluorometric and USP XVIII methods for the assay of dilute epinephrine solutions is reported, and the advantages of the new procedure are discussed.

EXPERIMENTAL[1]

Reagents—*Potassium Ferricyanide (0.25%)*—Dissolve 250 mg. of potassium ferricyanide (Fisher certified, ACS reagent) in sufficient distilled water to make 100 ml.

Zinc Sulfate (0.25%)—Dissolve 250 mg. of zinc sulfate heptahydrate (Fisher certified, ACS reagent) in sufficient distilled water to make 100 ml.

Ascorbic Acid (2%)—Dissolve 2.0 g. of USP grade ascorbic acid in sufficient distilled water to make 100 ml. This solution must be prepared fresh daily.

Sodium Hydroxide Solution (5 N)—Dissolve 200 g. of sodium hydroxide (Fisher certified, ACS reagent) in sufficient distilled water to make 1 l.

Phosphate Buffer (0.2 N), pH 6.5—Dissolve 2.8 g. $NaH_2PO_4 \cdot H_2O$ in distilled water to make 100 ml. Dissolve 5.4 g. $Na_2HPO_4 \cdot 7H_2O$ in sufficient distilled water to make 100 ml. Titrate NaH_2PO_4 solution with Na_2HPO_4 to a pH of 6.50 using an expanded scale pH meter[2].

Citrate-Phosphate Buffer (McIlvaine's Buffer), pH 4.0—Prepare 0.1 *M* citric acid solution by weighing 21.0 g. of citric acid monohydrate (ACS certified) into a 1-l. volumetric flask and diluting to volume with distilled water. Prepare 0.2 *M* dibasic sodium phosphate solution by weighing 28.4 g. of anhydrous dibasic sodium phosphate (ACS certified) into a 1-l. volumetric flask and diluting to volume with distilled water. Prepare pH 4.0 buffer by adding

[1] An Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) was used. The excitation wavelength was 410 nm., and the emission wavelength was 530 nm. For the epinephrine concentrations described in the procedures, the Meter Multiplier was set at 0.03, with a slit width of 1.0 and sensitivity at 30.

[2] Beckman.

600 ml. of 0.1 *M* citric acid solution to 400 ml. of 0.2 *M* dibasic sodium phosphate solution.

Potassium Carbonate Solution (0.2 N)—Dissolve 1.38 g. of potassium carbonate (ACS certified reagent) in sufficient distilled water to make 100 ml.

Sodium Ascorbate Solution—Pipet 5.0 ml. of ascorbic acid solution into a 50-ml. volumetric flask and dilute to volume with 5 *N* sodium hydroxide. This solution must be prepared immediately before use. Unless this reagent is clear and colorless, it should not be used.

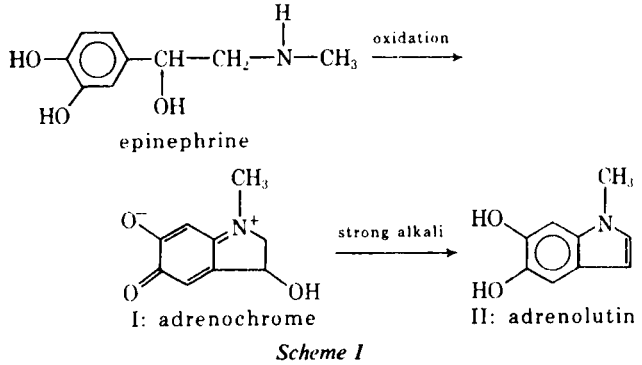
Iodine Solution (0.005 N *in 1.25% w/v Sodium Iodide)*—Weigh 5.0 g. of sodium iodide (Fisher certified, ACS reagent) into a 100-ml. volumetric flask containing 30 ml. of distilled water. Pipet 20 ml. of 0.1 *N* iodine solution and dilute to 100 ml. with distilled water. Pipet 25.0 ml. of this solution into a 100-ml. volumetric flask and dilute to volume with distilled water.

Epinephrine Standard—Accurately weigh 100 mg. of epinephrine base[3] or 182 mg. of epinephrine bitartrate[3] into a 1-l. flask. Pipet 10 ml. of 0.1 *N* HCl, swirl flask to dissolve the material in the acid, and dilute to volume with nitrogen-purged distilled water. When stored in the refrigerator, this solution is stable for several weeks.

Procedures—*Potassium Ferricyanide Method*—Dilute the unknown epinephrine solution with pH 6.5 phosphate buffer to result in approximately 10 mcg./ml. solution. Pipet 2 ml. of this solution into two test tubes, and add 1.0 ml. of distilled water. Adjust the pH of this solution to 6.50 by the addition of 0.2 *N* potassium carbonate solution. To one test tube, add 0.1 ml. of 0.25% zinc sulfate followed by 0.1 ml. of 0.25% potassium ferricyanide reagent. Mix them thoroughly and let stand for exactly 3 min. To both tubes, add 1.0 ml. of sodium ascorbate reagent (freshly made), mix well, and allow to react for 10–20 min. To the tube that did not contain any oxidant, add 0.1 ml. of 0.25% zinc sulfate and 0.1 ml. of 0.25% potassium ferricyanide reagents, in that order. Mix thoroughly and use this solution as the blank. Readings are to be taken at the wavelengths described (*Footnote 1*) on the spectrophotofluorometer 10–20 min. after the addition of the alkaline ascorbate reagent.

Prepare a standard curve using the same procedure as that described for the unknown sample. Use concentrations of 2.5, 5.0, 10.0, 15.0, and 20.0 mcg./ml. for the standard curve. Read the unknown sample from the graph.

Iodine Method—The following procedure was used to assay epinephrine solutions initially containing 100 mcg./ml. of the base. Dilute 4.0 ml. of the unknown sample solution to 100 ml. with the pH 4.0 citrate–phosphate buffer. To each of two different 100-ml. volumetric flasks, labeled Samples A and B, add 10.0 and 15.0 ml., respectively, of this diluted solution and make up to volume with the same buffer. Pipet 5.0 ml. of solution from each of these flasks (containing about 0.4 and 0.6 mcg./ml. of epinephrine base, respectively) into separately labeled 10-ml. volumetric flasks, add 1.0 ml. of 0.005 *N* iodine reagent, and shake well. After exactly 3 min., dilute to 10 ml. with freshly prepared sodium ascorbate reagent. Shake thoroughly and read the fluorescence on the spectrophotofluorometer at the specified wavelengths and settings. The readings should be taken between 10–25 min. after the addition of the sodium ascorbate reagent. The standard solution of epinephrine containing 100 mcg./ml. is also diluted in an analogous manner. Each sample and standard are diluted to two different levels and assayed simultaneously. At least two blanks are always determined, consisting of 5.0 ml. of pH 4.0 citrate–phosphate buffer to which is added 1.0 ml. of the 0.005 *N* iodine solution, and the entire procedure is carried through in the same manner as described for samples and standards.



Scheme I

Calculations—

$$\text{mcg./ml. epinephrine in Sample A} = \frac{4\,(\%\text{ transmission of sample 1} - \%\text{ transmission of blank})}{(\%\text{ transmission of Standard A} - \%\text{ transmission of blank})} \quad \text{(Eq. 1)}$$

$$\text{mcg./ml. epinephrine in Sample B} = \frac{4\,(\%\text{ transmission of sample 2} - \%\text{ transmission of blank})}{(\%\text{ transmission of Standard B} - \%\text{ transmission of blank})} \quad \text{(Eq. 2)}$$

$$\text{mcg./ml. epinephrine in unknown sample} = 12.5\,(\text{mcg./ml. epinephrine in Sample A} + \text{mcg./ml. epinephrine in Sample B}) \quad \text{(Eq. 3)}$$

Recovery of Epinephrine in Presence of Its Oxidation Products—A solution of epinephrine hydrochloride, 115 mcg./ml., was made in distilled water and the pH was adjusted to 2.50 with hydrochloric acid. This solution was kept at 56°, with no precaution taken to prevent oxidation of epinephrine, and was analyzed by the fluorometric method at the end of 6 months. A fresh solution of epinephrine, 1.0 mg./ml., containing 10 mg./ml. of sodium bisulfite was made up in 0.1 *N* hydrochloric acid. To 5.0, 5.0, 5.0, 5.0, and 10.0 ml. of the partially decomposed solution of epinephrine at 56° were added 2.0, 1.5, 1.0, 0.5, and 0 ml., respectively, of the freshly made solution of epinephrine–sodium bisulfite solution already described. The solutions were then diluted to 10 ml. with distilled water and were subsequently analyzed by the fluorometric method.

TLC—Thin-layer plates (5 × 20 cm.) coated with silica gel F_{254}[4] were spotted with 20 μl. of the following solutions: (*a*) freshly made epinephrine solution (100 mcg./ml.), (*b*) epinephrine (100 mcg./ml.) containing sodium bisulfite (10 mg./ml.) which was previously heated at 65° for 2 weeks, and (*c*) epinephrine (100 mcg./ml.) heated in the presence of air for 9 weeks. The plates were developed with a solvent system consisting of 1-butanol–100% ethanol–acetic acid–water (50:10:10:15). With the sample of epinephrine heated in the presence of air, no clearcut separation could be achieved. However, the epinephrine heated with excess sodium bisulfite resulted in another product (R_f 0.26) with excellent separation. In this system, pure epinephrine had R_f 0.45.

RESULTS AND DISCUSSION

By using epinephrine as an example of a substituted catecholamine, the principle involved in the fluorometric method is illustrated in Scheme I. With a mild oxidizing agent, epinephrine is oxidized to adrenochrome (I), which in strong alkaline solution is cyclized to the relatively unstable adrenolutin (II), which is responsible for the fluorescence (9).

Catecholamines such as epinephrine and norepinephrine that occur in biological fluids have been successfully assayed by the fluorometric method (5, 6). However, this method is lengthy and involves an initial extraction with butanol, followed by alumina adsorption to remove noncatechol fluorescent substances (5). This method also requires the addition of reagents at precise intervals, and the final fluorescence is extremely unstable in the acidic medium in which it is determined. Moreover, this method has the serious drawback of a critical oxidation time period followed by an exacting time interval between the addition of the reagents and the actual determination of the fluorescence. Due to the number of steps involved during assay, along with the very specific time intervals, it is very difficult to analyze a large number of samples at a time without seriously affecting the accuracy.

Hellberg (8) reported a method for the estimation of epinephrine and norepinephrine in solutions containing local anesthetics and sulfite as the antioxidant. This method separates the epinephrine from the local anesthetic by column chromatography, with subsequent oxidation of the sulfite with iodine in acidic solution. The excess iodine is oxidized with arsenite, and the epinephrine is then oxidized at pH 6.0 with potassium ferricyanide to adrenochrome,

[3] K & K Laboratories, Plainview, N. Y.

[4] Brinkmann Instruments Inc., Westbury, NY 11590

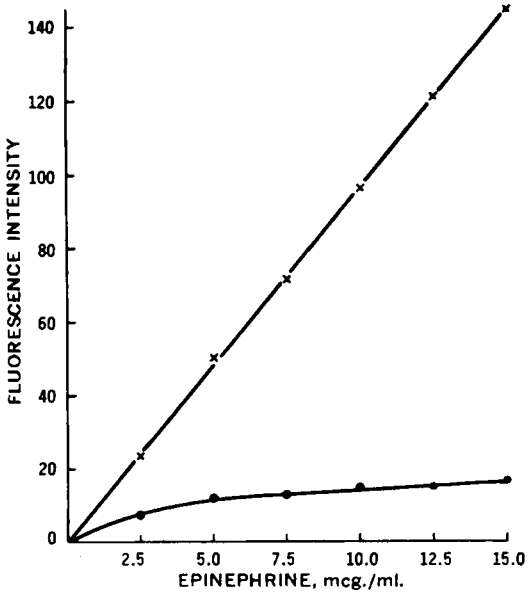


Figure 1—*Fluorometric assay of epinephrine hydrochloride by the potassium ferricyanide–zinc sulfate method. Key: ×, sodium bisulfite absent; and ●, 10-fold excess of sodium bisulfite present over that of epinephrine concentration.*

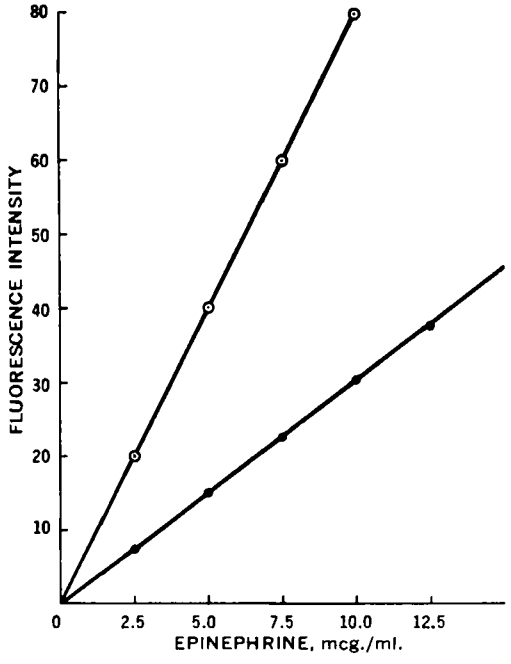


Figure 2—*Assay of epinephrine hydrochloride in the presence and absence of excess sodium bisulfite employing excess potassium ferricyanide (0.58%)–zinc sulfate (0.58%) reagent. Key: ⊙, absence of sodium bisulfite; and ●, presence of sodium bisulfite.*

which is then cyclized to adrenolutin with strong alkali. The fluorescence of these solutions is determined between 15 and 30 min. after the alkali treatment. Since this paper did not mention the wavelength at which the fluorescence was measured, the excitation and emission maxima for epinephrine were determined and found to be 410 and 530 nm., respectively.

Various oxidizing agents have been used for the initial oxidation of epinephrine to adrenochrome. The initial oxidation could be carried out with iodine (5), manganese dioxide (10), or a mixture of potassium ferricyanide and zinc sulfate (9). Manganese dioxide was eliminated from consideration as an oxidizing agent because of its insolubility and the problem of cumbersome removal from solution by centrifugal action. The use of a mixture of potassium ferricyanide–zinc sulfate and iodine solution was investigated in detail. Adrenolutin, which is responsible for the fluorescence, is quite susceptible to further oxidation, resulting in a decrease of the fluorescence. This can be prevented by the addition of a solution of an antioxidant. Of the various antioxidants suggested, the most commonly used are sodium sulfite (5) and sodium ascorbate (10). The antioxidant used not only prevents the further oxidation of adrenolutin but also reduces any excess oxidizing agent that may have been used to convert epinephrine to adrenochrome.

Potassium Ferricyanide–Zinc Sulfate Method—The majority of the commercial parenteral formulations of epinephrine contain sodium bisulfite or sodium metabisulfite as the antioxidant. Hellberg (8) recommended the initial oxidation of the antioxidant with iodine in acidic solution. However, if an excess of iodine is used, an interference with the fluorescence results. The exact amount of the antioxidant left in commercially available products is not known and, therefore, it is difficult to avoid using an excess iodine solution. Von Euler and Floding (9) used a mixture of potassium ferricyanide and zinc sulfate for the oxidation of epinephrine. A modification of this procedure was used to estimate epinephrine where the fluorescence was stabilized with alkaline sodium ascorbate. Excellent correlation between epinephrine concentration and fluorescence was observed with pure epinephrine solutions. However, for solutions containing sodium bisulfite, there was no linearity of response of fluorescence to concentration changes of epinephrine in solution (Fig. 1).

Since sodium bisulfite interferes with the development of fluorescence, the removal of sodium bisulfite by oxidation with excess potassium ferricyanide (0.58%) and zinc sulfate (0.58%) was attempted. The results show (Fig. 2) that the interference of bisulfite cannot be completely eliminated with potassium ferricyanide oxidation, as indicated by the differences in the slopes of the line in Figs. 1 and 2. In the presence of sodium bisulfite, with a lower concentration of the potassium ferricyanide–zinc sulfate reagent, epinephrine oxidation seems to be incomplete (Fig. 1). However, when the concentration of the oxidizing agent is increased, the slope of the standard curve decreases (Fig. 2) with respect to the slope of the curve obtained when a lower concentration of oxidizing agent was used (Fig. 1). This means that the fluorescence is affected by the amount of the oxidizing agent and also the presence of sodium bisulfite. These results indicate that the interference due to the presence of bisulfite cannot be eliminated by increasing the potassium ferricyanide–zinc sulfate concentration. Therefore, iodine solution containing potassium iodide was considered as an oxidizing agent.

New and Improved One-Step Method—A solution of iodine in potassium iodide was described as the oxidizing agent in the estimation of catecholamines in tissues (11). However, the initial oxidation step, the subsequent cyclization of the adrenochrome to adrenolutin, and the measurement of the fluorescence were reported to be extremely sensitive and very time dependent. Attempts to assay several samples at a time by this method failed to yield consistent results. In this method (11) the fluorescence is determined in acidic solution (pH 5.4) immediately after its development. The author also recommended the reduction of excess iodine with alkaline sodium sulfite, with the concurrent cyclization of adrenochrome to adrenolutin. The entire process should be completed within 2 min.

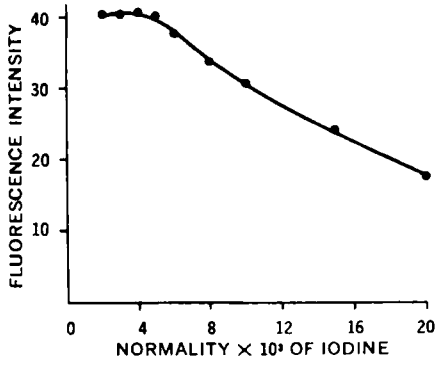


Figure 3—*Effect of iodine concentration on fluorescence of 0.6 mcg./ml. of epinephrine hydrochloride in the presence of 6 mcg./ml. of sodium bisulfite.*

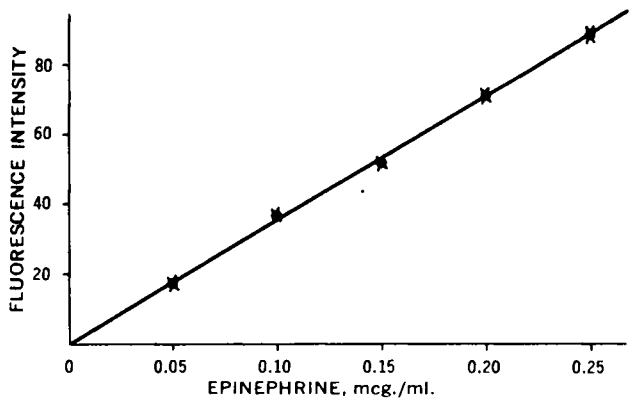


Figure 4—*Assay of epinephrine hydrochloride by fluorometric method using iodine–potassium iodide as the oxidizing agent. Key: ×, epinephrine hydrochloride alone; and ○, epinephrine hydrochloride with 20-fold excess of sodium bisulfite.*

and, subsequently, the solution is acidified with 5 *N* acetic acid to stabilize the fluorescence which must be determined immediately on the fluorometer.

To circumvent these problems and to analyze several samples at a time, the efficiency was investigated of iodine–potassium iodide solution to oxidize not only epinephrine in pH 4.0 buffer solution but also sodium bisulfite and metabisulfite, the most common antioxidants used in parenteral formulations. In the new and improved method, described in the *Experimental* section, alkaline sodium ascorbate was used to reduce the excess iodine and to stabilize the fluorescence. Various parameters such as the concentration of the oxidizing agent, the duration of the oxidation step, the fluorescence stabilizing agent and its concentration, and the duration within which the fluorescence should be determined were all studied in detail.

Effect of Oxidizing Agent on Fluorescence—The effect of iodine–potassium iodide concentration on the fluorescence of the final solution is shown in Fig. 3. Epinephrine solution was spiked with a 10-fold excess of sodium bisulfite. Iodine solution of various normalities was added such that the volumes of the final solutions were held constant, and the resulting fluorescence was measured. From Fig. 3 it is evident that the concentration of the oxidizing agent, iodine, on freshly made solutions of epinephrine containing sodium bisulfite is quite critical. The optimum concentration of the iodine reagent is 0.005 *N*, beyond which the final fluorescence is markedly affected. This concentration of iodine is sufficient to oxidize the epinephrine and the excess sodium bisulfite present in the formulation. Since formulations aged at different temperatures for various times would contain different amounts of sodium bisulfite and epinephrine, the adequacy of iodine solution as the oxidizing agent was checked by keeping the ratio of epinephrine to sodium bisulfite constant (Fig. 4). Since an excess amount of iodine quenches the fluorescence (Fig. 3), this study also indicated that the volume of iodine solution used does not affect the fluorescence in the presence or absence of excess sodium bisulfite. Concentrations of sodium bisulfite as high as 5 mcg./ml. do not interfere with the fluorometric assay of epinephrine. Many of the marketed parenteral formulations contain sodium bisulfite as much as 10 times the weight of epinephrine. Even in the presence of such high concentrations of the antioxidant, the amount of iodine described in the *Experimental* section is found to be adequate, since sodium bisulfite concentration as high as 25 times that of epinephrine does not interfere with the final fluorescence.

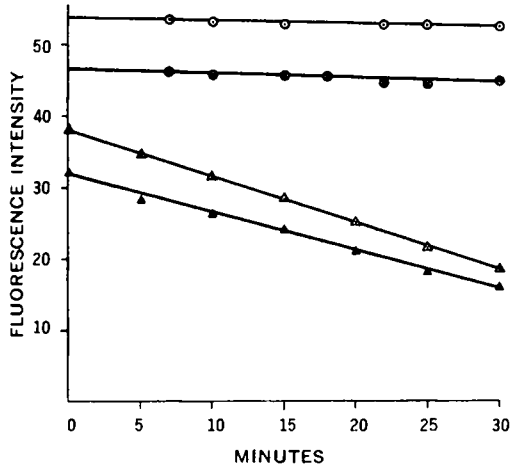


Figure 5—*Stability of fluorescence as a function of time, pH of the final solution, fluorescence stabilizer used, and final epinephrine hydrochloride concentration. Key: sodium sulfite as fluorescence stabilizer with final pH 5.0 and final epinephrine hydrochloride 0.2 mcg./ml. (▲) or 0.3 mcg./ml. (△); and sodium ascorbate as fluorescence stabilizer with final pH greater than 10 and final epinephrine hydrochloride 0.2 mcg./ml. (⊗) or 0.3 mcg./ml. (⊙).*

Table I—Effect of Iodine as Oxidizing Agent and Ascorbic Acid as Fluorescence Stabilizing Agent in the Assay of Epinephrine Hydrochloride Injections by Fluorometric Method

Ascorbic Acid, mg./ml.	Fluorescence Intensity, % Samples[a] 0.001 *N* Iodine 1	2	3	4	0.005 *N* Iodine 5	6	7	8
1.0	52.8	76.8	41.3	61.3	44.0	64.5	33.5	51.2
2.0	49.3	74.4	39.3	58.2	44.3	65.7	34.2	50.2
3.0	46.5	68.8	36.7	55.0	43.5	65.4	35.1	51.5

[a] Sample sets 1,2; 3,4; 5,6; and 7,8 were made by the appropriate dilution of four different samples such that the final concentration of epinephrine hydrochloride was in the ratio of 2:3 for each set. Final epinephrine hydrochloride concentrations were 0.2 and 0.3 mcg./ml., respectively, at these two dilutions.

The optimum pH of the solution to be oxidized was reported (6) to be 4.0. Solutions buffered with citrate–phosphate buffer at pH 4.0 also resulted in maximal fluorescence in the present investigation. Laverty and Taylor (6) recommended 1 min. as the oxidation time; however, in this study, a 3-min. oxidation period was necessary to oxidize the antioxidant normally present in formulations and also to oxidize the epinephrine to adrenochrome.

Choice of Antioxidant to Stabilize Final Fluorescence—The fluorescence of adrenolutin is quite unstable, probably due to easy oxidation, and, therefore, it needs stabilization with an antioxidant. Sodium sulfite and ascorbic acid were most often used to stabilize the fluorescence. Laverty and Taylor (6) described the use of sodium sulfite initially and subsequent acidification with glacial acetic acid, measuring the fluorescence in acidic solution (pH 5.0). Their paper failed to mention the stability of the fluorescence in the assay of epinephrine; however, the statement: "fluorescent derivatives were stable for at least 100 minutes" was made without presentation of any data to substantiate it. The stability of fluorescence in epinephrine assay was investigated in this study, using essentially the procedure of Laverty and Taylor and employing sodium sulfite as antioxidant and glacial acetic acid to make the final solution acidic. Under these conditions, the fluorescence is not as stable as was reported (6). The results according to the literature method (6) are shown in Fig. 5. When the fluorescence is determined under acidic conditions, it should be determined immediately, as was reported by Chang (5). The method reported by Laverty and Taylor (6) is identical with that reported by Chang. The relative instability of the fluorescence at pH 5.0 is evident from Fig. 5. It is also evident that the fluorescence is stable up to 25 min. when stabilized with ascorbic acid under strongly alkaline conditions.

To determine the optimum concentration to stabilize the fluorescence, ascorbic acid concentrations ranging from 1 to 3 mg./ml. were used, with parallel iodine concentrations of 0.001 and 0.005 *N* as the oxidizing agent. The results shown in Table I indicate that the readings were higher when 0.001 *N* iodine solution was used as the oxidizing agent along with 1 mg./ml. ascorbic acid as the fluorescence stabilizing agent. However, the fluorescence was less stable under the above set of conditions than when the 0.005 *N* iodine solution and 2 mg./ml. ascorbic acid combination was used. The use of 0.005 *N* iodine also assures complete oxidation of the antioxidant used in the formulation. It was also found that fluorescence with intensity readings higher than 70 units would quench faster. Fluorescence was stable for longer periods when the final readings were

Table II—Comparison of USP XVIII and Fluorometric Methods for the Assay of 1:1000 Epinephrine Solutions[a] Stored at 65° for Several Weeks

Sampling Time, Weeks	Epinephrine Hydrochloride, mcg./ml. USP XVIII Method	Fluorometric Method
Original	1000	1000
1	360	355
2	264	260
4	175	170
7	085	090

[a] These formulations contained 10 mg./ml. of sodium bisulfite, and the final pH was adjusted to 2.50.

in the range of 30–70. It is also evident from Table I that when 3 mg./ml. of ascorbic acid was used, the value of the fluorometric readings dropped sharply compared to 1 mg./ml. of ascorbic acid.

Comparison of USP XVIII and Fluorometric Methods—To determine the precision of the USP XVIII method for the assay of epinephrine, a 1:1000 stock solution was made in hydrochloric acid and stored in the refrigerator. This solution was analyzed by the USP XVIII method, which involved the conversion of the unchanged epinephrine to triacetylepinephrine, its extraction with chloroform, and subsequent determination of its optical rotation. The amount of unchanged epinephrine is calculated from the given formula. Replicate determinations of the epinephrine assay according to the USP XVIII method resulted in a standard deviation of 0.5% with a standard error of 0.2% and precision of 0.5%. Similar replicate determinations by the USP XVIII method were carried out with 1:10,000 epinephrine solution, which is 10 times more dilute than the USP solution. The solutions were assayed by a slight modification of the extraction procedure with chloroform. The standard deviation is 0.62% with the standard error being 0.28%. Simultaneous determination of epinephrine by the fluorometric method resulted in a standard deviation of 0.48% and a standard error of 0.22%. According to Hellberg's (8) method, the standard deviation of a single determination was about ±2% for epinephrine. Therefore, this new procedure is precise and reproducible.

Assay of Epinephrine Formulations—A comparison of the two analytical procedures was made using 1 mg./ml. epinephrine formulation containing 10 mg./ml. of sodium bisulfite. The final pH was adjusted to 2.50, and the samples were kept at 65° for several weeks. These samples were withdrawn at various intervals and analyzed by the USP XVIII and fluorometric methods (Table II). The excellent agreement between the two methods points to the facts that: (*a*) by the fluorometric method, only the unchanged epinephrine is measured as verified by the acetylation procedure described in USP XVIII; and (*b*) the inactivation product, namely epinephrine sulfonic acid (III), does not interfere in the fluorometric assay. In the presence of a large excess of sodium bisulfite, the only decomposition product observed by TLC is epinephrine sulfonic acid. This was reported earlier by Schroeter *et al.* (12) to be the major route of degradation.

The applicability of the fluorometric procedure to formulations containing other antioxidants was investigated. Parenteral epinephrine formulations containing up to 10 mg./ml. ascorbic acid or up to 4 mg./ml. α-monothioglycerol were made anad analyzed by the new procedure. Since ethylenediaminetetraacetic acid is often used as an antioxidant synergist, formulations containing as high as 1 mg./ml. were made and analyzed. In all instances, no interference from any of the formulation stabilizers was observed. Thus, it can be safely concluded that the new fluorometric method is of great value in analyzing epinephrine formulations containing a variety of stabilizers.

Recovery of Epinephrine from Solutions Containing Its Oxidation Products—To study the interference of oxidation products on the recovery of epinephrine, solutions at a concentration of 1:10,000 and with pH adjusted to 2.5 were allowed to degrade in the absence of antioxidant. When more than 70% of the epinephrine decomposed, a constant volume of the degraded solution was added to

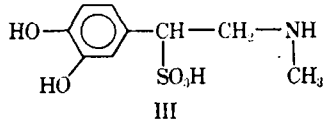


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Table III—Comparison of Biological and Fluorometric Methods for the Assay of Aged Epinephrine Injection (1:10,000) at Different Temperatures[a]

	Sample	37°, 8 Weeks	37°, 12 Weeks	25°, 8 Weeks	25°, 12 Weeks
Biological assay[b], %	A	91.8	69.7	93.4	87.4
	B	80.4	63.5	81.6	86.8
Fluorometric assay, %	A	85.6	66.7	94.5	92.6
	B	89.3	64.3	97.1	94.5

[a] Epinephrine injection (1:10,000) contained sodium chloride and sodium bisulfite, and the pH of the original solution was 2.50. Results are expressed as percentage of original activity remaining. [b] Standard error, expressed as percent of potency, ranged from 0.98 to 7.03.

known amounts of epinephrine hydrochloride in solution. The resulting solution mixtures were analyzed by the fluorometric method, with concurrent determination of pure epinephrine hydrochloride solutions. The results of such a recovery experiment showed that the fluorometric method only measures the unchanged epinephrine even in formulations containing epinephrine oxidation products. Such recovery experiments carried out according to the USP XVIII method were unsuccessful due to the interference from the oxidation products. Thus, the USP XVIII method cannot successfully estimate epinephrine when its oxidative decomposition products are also present. However, these epinephrine oxidation products do not interfere in the fluorometric method.

Further evidence of the specificity of the fluorometric method to unchanged epinephrine came when completely oxidized epinephrine solutions (as evidenced by TLC) in different amounts were added to a constant amount of epinephrine hydrochloride solution, which was later analyzed by the fluorometric method. The recoveries of epinephrine were quantitative, and the results conclusively proved that the various epinephrine oxidative degradation products, even when present in high concentrations, have no effect on the determination of epinephrine by the fluorometric method.

Comparison between Fluorometric Method and Biological Method with Epinephrine Formulations—Epinephrine injections (1:10,000) containing sodium bisulfite as the antioxidant were stored at 25 and 37° and were assayed by the fluorometric method. The rise in the mean blood pressure of dogs upon the intravenous administration of epinephrine hydrochloride injection was measured according to the method described by Noel (13). The results summarized in Table III indicate that there is a remarkable correlation between the two methods. Backe-Hansen *et al.* (7) also established such a correlation between blood pressure measurement and the fluorometric method described by Hellberg (8).

In summary, the unique feature of the described fluorometric method lies in the fact that the stabilization of the fluorescence is amenable to the routine assay of several samples at a time. The USP XVIII method is time consuming and cannot be successfully applied to the assay of a large number of samples at a time. The other major advantages of this new method are:

1. It can be used to assay dilute (even 1:100,000) epinephrine hydrochloride injections with accuracy.
2. The specificity of the new method even in the presence of epinephrine oxidation products has been established, whereas interferences were observed with the USP XVIII method.
3. The method is simple, precise, and timesaving, with the added potential for automation.
4. The fluorometric method requires a small volume of sample, whereas the USP XVIII method requires a sample containing 30 mg. of epinephrine. Thus the latter method requires a large number of samples, resulting in very large volumes to handle.
5. This new method can be used for the assay of epinephrine injections containing antioxidants other than sodium bisulfite.

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Improved, Rapid Spectrophotofluorometric Method for Assay of Isoproterenol Hydrochloride Injections: A Comparative Study

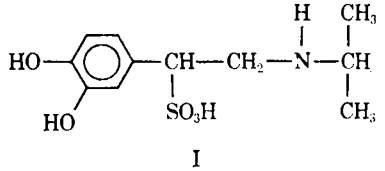
V. K. PRASAD, R. A. RICCI, B. C. NUNNING, and A. P. GRANATEK▲

Abstract □ A spectrophotofluorometric method for the determination of isoproterenol hydrochloride in parenteral formulations is described. This method is quite simple, precise, and extremely specific for unchanged isoproterenol in decomposed formulations. The method depends on the initial oxidation of isoproterenol hydrochloride with iodine–potassium iodide solution in pH 4.0 (McIlvaine's citrate–phosphate) buffer. The partially oxidized isoproterenol "chrome" derivative is cyclized to the fluorescent "lutin" derivative with strong alkali. The fluorescence is stabilized with alkaline ascorbate solution, which is subsequently determined. The influence of the various decomposition products of isoproterenol, such as isoproterenol sulfonic acid and isoproterenone, on the fluorometric procedure was investigated with concurrent determination of the same solutions by the USP XVIII method. The usefulness of the fluorometric procedure to analyze fresh as well as partially decomposed isoproterenol solutions, stabilized with other antioxidants, is evaluated. The inapplicability of the existing methods to analyze very dilute (1:50,000) solutions of isoproterenol is also described. The specific advantages of the fluorometric procedure in the routine analysis of a large number of samples with speed and accuracy are discussed. Statistical evaluation of the fluorometric method is also reported.

Keyphrases □ Isoproterenol hydrochloride solutions—spectrophotofluorometric analysis, compared to compendial method □ Spectrophotofluorometry—analysis, isoproterenol hydrochloride solutions

The official USP XVIII method (1) for the analysis of isoproterenol hydrochloride injection in specific formulations and isoproterenol hydrochloride in other formulations involves the column chromatographic separation of unchanged isoproterenol from its degradation products. This is achieved by ion-pair extraction of the intact isoproterenol with bis(2-ethylhexyl)phosphoric acid in ether solution, leaving decomposition products behind on the column (2). The major product of decomposition of isoproterenol injections containing the most commonly used antioxidant sodium bisulfite is isoproterenol sulfonic acid (I). The formation of this derivative is pH dependent, and the reaction is favored at higher pH values. This fact was demonstrated by Higuchi and Schroeter (3). However, when an insufficient quantity of sodium bisulfite is used or when other antioxidants are employed, complications arise due to the presence of other isoproterenol decomposition products such as isoproterenone (II). In such instances, the USP XVIII method may not work satisfactorily. This fact was investigated in depth, and the data are presented in this paper.

Another major disadvantage with the USP XVIII method is that it cannot be used to assay isoproterenol injections in very dilute solutions such as 1:50,000 concentrations. The official method does not work when large volumes of sample have to be eluted from the column. Other disadvantages are: (*a*) the method is lengthy and time consuming; (*b*) the method cannot be successfully used to assay several samples at a time; and (*c*) it is extremely difficult to reproduce results, since the USP XVIII does not describe any of the column chromatographic variables such as tightness of the



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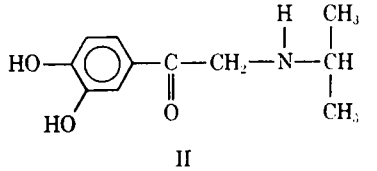

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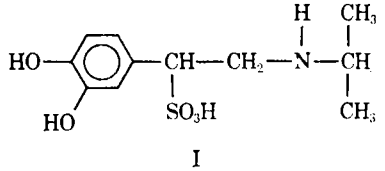

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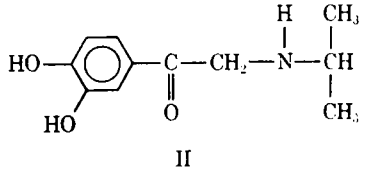

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packing of the stationary phase in the column, the rate of elution, and other pertinent factors.

Welsh and Sammul (2) reported on the analysis of aged isoproterenol solutions using a modification of Hellberg's (4) fluorometric assay for epinephrine. However, no details of the procedure were given. The purpose of this report is to present a simple, rapid, accurate, and selective fluorometric procedure for the quantitative determination of unchanged isoproterenol in aged formulations. The fluorometric method is based on the fact that isoproterenol in an acidic buffer solution can be selectively oxidized to its "chrome" derivative, which subsequently is cyclized in strong alkaline solution to the fluorescent "lutin" derivative, which is stabilized with sodium ascorbate. The described method has the additional advantage that it can be used to assay dilute (1:50,000) solutions of isoproterenol even in the presence of unusually higher concentrations of its two known decomposition products: isoproterenol sulfonic acid (I) and isoproterenone (II). The merits of the fluorometric method over that of the USP XVIII method, including a statistical evaluation of the method, are reported here.

EXPERIMENTAL[1]

Preparation of Isoproterenol Sulfonic Acid (I)—Isoproterenol hydrochloride (10.0 g., 0.04 mole) and sodium bisulfite (6.78 g., 0.04 mole) were dissolved in 50 ml. of water. The mixture was heated on a steam bath under nitrogen for 2 hr. and then was stored in the dark under nitrogen for 3 days. At the end of 3 days, 25 ml. of water was removed on a flash evaporator and the residue solution was stored in the dark at room temperature under nitrogen for a further period of 6 days. At the end of 6 days, the remaining solution was concentrated on a flash evaporator. The residue was suspended in 25 ml. of ethanol and saturated with ether. The precipitate was filtered, washed with ether, and air dried. The precipitate was suspended in 150 ml. of water, stirred for 10 min., filtered, and then crystallized from water to produce a white crystalline compound. This compound was dried at 40° under vacuum overnight to give isoproterenol sulfonic acid·1/2 H_2O. On drying the sample at 80° under vacuum over phosphorus pentoxide for 48 hr., anhydrous isoproterenol sulfonic acid was obtained, 7.6 g. (68%), m.p. 185–190° (softens), 225–230° dec.; UV (95% ethanol): λ_{max} 284 (ϵ, 3593) and 232 (ϵ, 7558) nm.; IR (KBr): 1135, 1045 (—SO_3), and 650 (—S—O) cm.$^{-1}$; NMR (dimethyl sulfoxide-d_6): δ 8.70 (br, 3, —OH + NH), 6.66 (m, 3, aromatic H), 3.80 [br, t, 1, —(—SO_3H)CH—], 2.34 (m, 3, —CH + CH_2O), and 1.20 (d, 6, —CH_3).

Anal.—Calc. for $C_{11}H_{17}NO_5S$: C, 47.97; H, 6.23; N, 5.08; S, 11.64. Found: C, 47.72; H, 6.29; N, 5.08; S, 12.13.

Preparation of Isoproterenone (II)—Ten grams (0.053 mole) of 3,4-dihydroxy-α-chloroacetophenone[2] was suspended in 100 ml. of 95% ethanol. Isopropylamine, 80 ml. (an excess), was added slowly with stirring. The mixture was refluxed for 2.5 hr., and the solvent was removed under vacuum. The solid was taken up in 400 ml. of acetone, stirred for 30 min., and filtered, and the insoluble solid was washed with more acetone. The crude isoproterenone was suspended in 200 ml. of distilled water, filtered, and washed with distilled water several times. The material was recrystallized from dimethyl sulfoxide–water, resulting in a light-tan compound which, after drying at 60° under vacuum overnight, yielded the desired material, 8.6 g. (76%), m.p. 168–169° dec.; UV (95% ethanol): λ_{max} 312 (ϵ, 7555), 280 (ϵ, 8475), and 232 (ϵ, 12,555) nm.; IR (KBr): 1655 [C_6H_5—C(=O)—CH_2—], 1600, 1550, 1495 (phenyl ring), 1470 (—CH_2—, scissoring), 1345 (CH_3—), 1250 (aromatic ketone), 1125, 1105 (the —CH— out-of-plane vibrations), and 800 (adjacent 3H) cm.$^{-1}$; NMR (dimethyl sulfoxide-d_6): δ 7.10 (m, 6, aromatic H, —OH + NH), 4.02 [S, 2, —C(=O)—CH_2—NH—], 2.85 (m, 1 ϵ H), and 1.05 (d, 6, —CH_3).

Anal.—Calc. for $C_{11}H_{15}NO_3$: C, 63.12; H, 7.23; N, 6.69. Found: C, 62.48; H, 7.12; N, 6.34.

Isoproterenol Standard—Isoproterenol hydrochloride USP, 200 mg., was placed into a 1-l. flask containing 10 ml. of 0.1 *N* hydrochloric acid. The solid was dissolved and the volume was adjusted to 1 l. with nitrogen-purged distilled water. The resulting solution is stable over a period of several weeks when stored in the refrigerator.

Reagents—The preparation of McIlvaine's citrate-phosphate buffer of pH 4.0, of the iodine solution (0.005 *N*) in 1.25% (w/v) sodium iodide, and of the 0.2% ascorbic acid in 5 *N* sodium hydroxide was previously described (5).

Procedures—Isoproterenol hydrochloride injection USP (200 mcg./ml.) is assayed exactly according to the method described for the assay of 100 mcg./ml. epinephrine injection (5). For the assay of isoproterenol hydrochloride injection USP (200 mcg./ml.), 2.0 ml. is diluted to 100 ml. with buffer. To assay the more dilute 20 mcg./ml. isoproterenol hydrochloride injection, 5 ml. of the standard and the sample is diluted to 25 ml. with the citrate–phosphate buffer. Five and 15 ml. of the diluted solutions are further diluted to 50 and 100 ml., respectively, with the citrate–phosphate buffer. The procedure from here on is the same as described for 100 mcg./ml. epinephrine injection (5).

RESULTS AND DISCUSSION

The selective determinations of isoproterenol hydrochloride in the presence of its two known degradation products, isoproterenol sulfonic acid (I) and isoproterenone (II), using the fluorometric method has not been described. Numerous modifications of the fluorometric method for the analysis of other catecholamine derivatives have been published, but none of them is useful as a routine procedure because:

1. The methods described for the initial removal of the interfering material, sodium bisulfite, are cumbersome and have to be carried out in a precise fashion (4).
2. The reported methods have not checked out the fluorometric procedure to find out whether the two above-mentioned decomposition products of isoproterenol or other unknown degradation products interfere in the assay.
3. The fluorescence produced according to the reported methods is quite unstable and necessitates precise timing between the development of fluorescence and its determination on the fluorometer. For this reason, most of the published methods recommend that the fluorescence be determined immediately.
4. Finally, the number of steps involved in the actual procedure of the older methods (4) are many and cumbersome. Fewer steps are involved in the new method described in this paper.

Effect of Various Reagents on Fluorescence—In general, the effect of the concentration of the oxidizing agent, iodine, was found to be of the same nature as was described for the assay of epinephrine (5). The optimum iodine concentration that results in maximum fluorescence was 0.005 *N*. Higher concentrations resulted in decreased fluorescence and lower concentrations were not adequate to oxidize the high concentration of bisulfite used in the simulated formulations. In general, the commercial formulations tested contained 10-fold excess by weight of sodium bisulfite as compared to the isoproterenol hydrochloride. The oxidation of isoproterenol to the chrome derivative with iodine reagent was complete at the end of 3 min. after the addition of the reagent, beyond which lowering of the fluorescence was observed. The chrome derivative of isoproterenol was then cyclized by alkali to the lutin derivative, which is responsible for the fluorescence. The lutin derivative is quite unstable and can undergo further oxidation.

Several antioxidants such as sodium sulfite and ascorbic acid have been recommended not only to stop the further oxidation with iodine but also to prevent the quenching effect of oxygen on the fluorescence. Sodium sulfite was used as the antioxidant in the fluorometric determination of epinephrine at pH 5.0 in acetic acid solution (6). When employing these conditions for the assay of isoproterenol solutions, the fluorescence was found to be extremely

[1] An Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) was used. For the isoproterenol concentrations described in the procedures, a Meter Multiplier of the instrument was set at 0.03, with slit width at 1.0 and sensitivity at 30. The excitation wavlength was 420 nm., and the emission wavelength was 510 nm.

[2] 3,4-Dihydroxy-α-chloroacetophenone was obtained from Winthrop Laboratories, Rensselaer, NY 12144

Table I—Comparison of Fluorometric and USP XVIII Methods for the Assay of Fresh Isoproterenol Solutions

Concentration of Isoproterenol Hydrochloride, mcg./ml.	USP XVIII Method		Fluorometric Method	
	Amount Recovered, mcg./ml.	% Recovered	Amount Recovered, mcg./ml.	% Recovered
180	175	97.3	178.2	99.0
150	155	103.3	149.6	99.7
100	102	102	100.5	100.5
50	49	98	50.0	100

unstable and readings had to be taken immediately after the addition of the glacial acetic acid (6). However, alkaline ascorbate solutions stabilize the fluorescence up to 30 min., thus permitting the reading of several samples at a time on the fluorometer (5). It was found that determination of the fluorescence between 15 and 20 min. after the addition of sodium ascorbate resulted in consistent recovery of isoproterenol from simulated formulations. The concentration of ascorbic acid used was between 1 and 3 mg./ml., with high and stable fluorescent readings resulting when 2 mg./ml. of ascorbic acid was employed. In general, the effects of different reagents on the stability and duration of maximum fluorescence were identical to those described in the assay of epinephrine by this method (5).

Comparison of USP XVIII Method with Fluorometric Method—To evaluate the official method and the fluorometric procedure, fresh solutions of isoproterenol hydrochloride as well as solutions after aging at higher temperatures for several months were employed. Thus, the effect of the interfering decomposition products could be checked, which was not done by an earlier reported fluorometric method (7).

Fresh solutions of different concentrations of isoproterenol hydrochloride containing sodium bisulfite were made and analyzed both by the USP XVIII and fluorometric methods (Table I). The USP XVIII method led to large errors with minor alterations in the technique. For example, the packing of the adsorbent into the column was critical. Best results were obtained when the initial water-saturated ether was allowed to pass through the column for 45–55 min. The results were consistent only when the column was eluted with bis(2-ethylhexyl)phosphoric acid–ether solution for about 50 min. Such details as the tightness of packing the adsorbent into the column and the elution times are not given in USP XVIII but are extremely important to get consistent results. Fresh solutions of isoproterenol were analyzed according to USP XVIII, taking all the above-mentioned precautions. It is evident from Table I that such results are in excellent agreement with the results of the fluorometric method. The USP XVIII method as well as the described fluorometric method gives excellent results when the initial concentration is 50–180 mcg./ml. The limitation of the USP XVIII method is that below 50 mcg./ml. of isoproterenol hydrochloride the final absorbance is less than 0.1, where the accuracy of the results was seriously affected. When a larger volume of the sample was applied to the column (to compensate for dilute solutions), the results were erratic, especially when partially decomposed solutions were analyzed. This was probably due to the excess moisture on the column, which seriously affected the partitioning of the decomposition product between the adsorbent and the bis(2-ethylhexyl)-phosphoric acid–ether solution. However, with the fluorometric method, the solutions can be appropriately diluted so that the final concentration of isoproterenol hydrochloride is between 0.2 and 0.3 mcg./ml. and estimated with accuracy.

Table II—Effect of Isoproterenol Sulfonic Acid on the Recovery of Isoproterenol from Simulated Formulations

Isoproterenol Hydrochloride, mcg./ml.	Isoproterenol Sulfonic Acid, mcg./ml.	Isoproterenol Recovered, %	
		USP XVIII Method	Fluorometric Method
180	20	95.2	101.6
150	50	94.5	101.3
100	100	94.7	101.5
50	150	97.5	100.3
0	200	—	0

Table III—Effect of Isoproterenone on the Recovery of Isoproterenol Hydrochloride from Simulated Formulations Analyzed by USP XVIII and Fluorometric Methods

Isoproterenol Hydrochloride, mcg./ml.	Isoproterenone, mcg./ml.	Recovery of Isoproterenol Hydrochloride, %	
		USP XVIII Method	Fluorometric Method
195	5	100	—
190	10	102.5	—
180	20	115.2	—
170	30	124.7	101.1
150	50	152.9	100.5
100	100	—	101.2
50	150	—	100.5
0	200[a]	—	0

[a] This sample consisted of isoproterenone only.

Comparison of USP XVIII and Fluorometric Methods for Assay of Simulated Isoproterenol Formulations—Two known decomposition products of isoproterenol are the sulfonic acid derivative (I), which is formed in solutions containing sodium bisulfite, and the oxidation product isoproterenone (II). Simulated formulations consisting of isoproterenol and its decomposition products were made to determine their effects on the recovery of isoproterenol. To evaluate the merits of the fluorometric method, the simulated formulations were analyzed by the USP XVIII and the fluorometric methods. The effect of each of the degradation products on the recovery of isoproterenol is described below.

Influence of Isoproterenol Sulfonic Acid—Results of the effect of isoproterenol sulfonic acid on the recovery of simulated isoproterenol hydrochloride formulations are shown in Table II. For the simulated formulation containing 75% isoproterenol sulfonic acid, the recovery of isoproterenol according to the USP XVIII method was always low. However, in the concentration range tested, the fluorometric method produced excellent agreement between the actual amount of isoproterenol used in the simulated formulation and its percent recovery. Even 200-mcg./ml. concentrations of isoproterenol sulfonic acid did not result in any fluorescence. Kaistha (8) mentioned that the column chromatographic method always resulted in a positive bias with formulations containing isoproterenol sulfonic acid, so he recommended a preliminary washing of the ether–bis(2-ethylhexyl)phosphoric acid layer containing the unchanged isoproterenol with the phosphate buffer solution to remove any isoproterenol sulfonic acid.

A pertinent observation made in the present study was that if any sodium bisulfite was eluted from the column with the bis(2-ethylhexyl)phosphoric acid–ether solution, the absorbance reading was higher than expected. Perhaps Kaistha's recommendation for washing the eluate initially with phosphate buffer might result in the effective removal of bisulfite or other bisulfite-derived products. In spite of this, Kaistha (8) mentioned higher recovery due to some additionally encountered decomposition products. The metaperiodate method (8) has the particular disadvantage in that higher concentrations of isoproterenol sulfonic acid, when present, adversely affect the assay method. Therefore, Kaistha recommended that the official compendia set upper and lower limits for isoproterenol sulfonic acid when the formulations contain sodium bisulfite as an antioxidant. The particular advantage with this new fluorometric method is that isoproterenol sulfonic acid, even if present in high concentrations, has no effect on the recovery of isoproterenol from formulations stabilized with sodium bisulfite or metabisulfite. When the fluorometric method is used, there is no necessity for stipulating any limits on the isoproterenol sulfonic acid concentration.

Effect of Isoproterenone on Recovery of Isoproterenol—USP XVIII describes a limit test for isoproterenone (λ_{max} 310) in isoproterenol hydrochloride. Since isoproterenone can be formed as one decomposition product of isoproterenol, the possible interference of isoproterenone on the fluorometric method was studied in detail. Simulated formulations consisting of isoproterenone and isoproterenol hydrochloride were made and analyzed both by the USP XVIII and fluorometric methods (Table III). The UV spectra of isoproterenol hydrochloride and isoproterenone when present as a mixture are

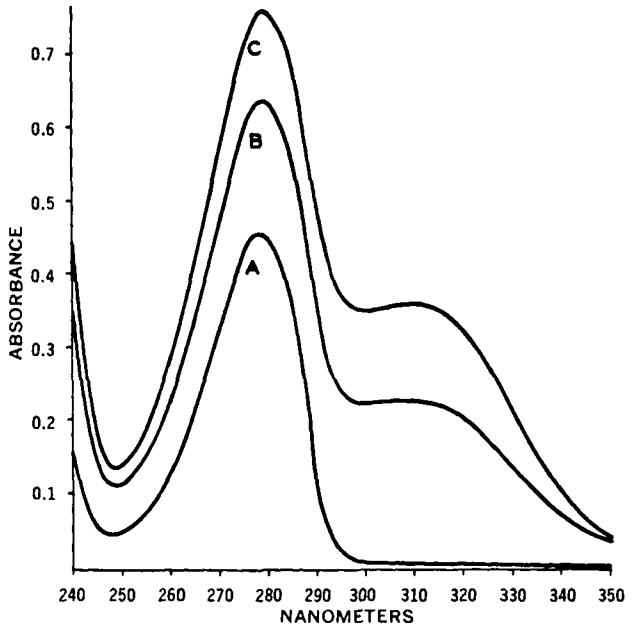

Figure 1—*UV spectra showing the effect of different amounts of isoproterenone on the USP XVIII method for determination of isoproterenol hydrochloride solutions. Key: A, isoproterenol hydrochloride, 200 mcg./ml.; B isoproterenol hydrochloride, 170 mcg./ml., and isoproterenone, 30 mcg./ml.; and C, isoproterenol hydrochloride, 150 mcg./ml., and isoproterenone, 50 mcg./ml.*

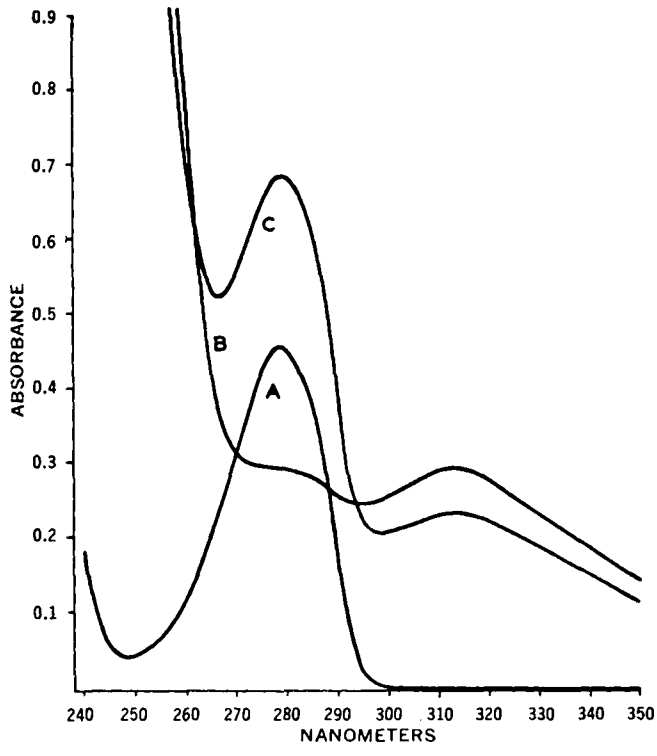

Figure 2—*UV spectra showing the effect of unknown isoproterenol decomposition products on the recovery of isoproterenol from solutions assayed by the USP XVIII method. Key: A, isoproterenol hydrochloride, 200 mcg./ml.; B, isoproterenol hydrochloride solution, 200 mcg./ml., completely decomposed in the presence of 350 mcg./ml. of sodium bisulfite; and C, mixture of A and B.*

shown in Fig. 1. Isoproterenone (molar absorptivity $\epsilon_{280}^{25} = 8053$ and $\epsilon_{310}^{25} = 6737$), when present in low concentrations, does interfere in the assay of isoproterenol (molar absorptivity $\epsilon_{280}^{25} = 2695$) when determined by the USP XVIII method. This is obvious because of the approximately threefold difference in the molar absorptivities of isoproterenone and isoproterenol at the 280-nm. absorption maximum. According to USP XVIII, the absorbance due to pure isoproterenol is determined at 278 nm. Serious errors can result if isoproterenone (λ_{max} 280 nm.) is also present in the samples of isoproterenol because both have nearly identical absorption maxima and because of the higher molar absorptivity of isoproterenone. Thus, both the USP XVIII method and the bis(2-ethylhexyl)phosphoric acid–ether extraction method (8) give false results when isoproterenone is also one decomposition product. As shown in Table III, when 200 mcg./ml. of isoproterenone was assayed by the fluorometric method, the final fluorescence was negligible. It can, therefore, be inferred that under these circumstances the new fluorometric method should be preferred over the USP XVIII method or the bis(2-ethylhexyl)phosphoric acid–ether extraction method (8) to assay isoproterenol formulations.

TLC of simulated inhalations and injections stored in the dark over a period of 4 months showed that they consisted of decomposition products other than the sulfonic acid derivative of isoproterenol (8). Although the exact nature of these additionally encountered artifacts could not be established unequivocally, it was theorized that one of them could be aminochrome–sodium bisulfite addition compound by analogy with a similar compound described by Tse and Oesterling (9) in their determination of total catecholamines in urine. According to Kaistha (8), when simulated isoproterenol formulations were analyzed by the method of Welsh and Sammul (2) (which was essentially adopted by USP XVIII), high and inconsistent values for the parent drug were obtained. This was attributed to the presence of additional decomposition products besides the sulfonic acid derivative (8). Kaistha reconciled this discrepancy by introducing a step to wash the ether effluent from the column with potassium phosphate buffer prior to acid extraction. Although this modification resulted in increased specificity for the USP XVIII method, the procedure is long and cannot be applied to very dilute solutions. The fluorometric method does not require preliminary separation of decomposition products. The fluorescence, due to the lutin derivative, results only from intact isoproterenol and not from its decomposition products.

Table IV—Comparison of USP XVIII and Fluorometric Methods for the Assay of Aged Isoproterenol Injections (1:5000)[a]

Sample[b]	USP XVIII Method, %	Fluorometric Method, %	Isoproterenol Sulfonic Acid[c], %
A	85.9	88.2	13.4
B	62.8	63.5	39.4
C	58.0	58.0	44.2
D	61.2	58.8	42.4

[a] These injections also contained a buffer consisting of lactic acid–sodium lactate, sodium chloride (7.0 mg./ml.), and sodium bisulfite (1.00 mg./ml.) as antioxidant, the final pH adjusted to 3.50. Celite 545 AW was used as the adsorbent in the USP XVIII method. [b] Sample A was stored for 2 months at 25°, while Samples B, C, and D were stored 10.5 months at 25°. [c] Isoproterenol sulfonic acid was estimated according to the method described by Kaistha (8).

Comparison of USP XVIII and Fluorometric Methods for Assay of Aged Commercial Isoproterenol Injections—Formulations of isoproterenol hydrochloride injection USP were made containing sodium lactate–lactic acid buffer, sodium bisulfite antioxidant, and sodium chloride for isotonicity. These were stored at room temperature for several months and analyzed both by the fluorometric and USP XVIII procedures. In addition, the amount of isoproterenol sulfonic acid formed was estimated according to the method described by Kaistha (8) (Table IV). The close agreement between the two methods indicates that the fluorometric method is comparable to the USP XVIII method when the method is exactly followed, as indicated previously in this report. The material balance between isoproterenol hydrochloride and isoproterenol sulfonic acid was in good agreement with the original isoproterenol hydrochloride concentration. A point worth noting here is that, in the presence of a large excess of sodium bisulfite, the isoproterenol solutions remained clear and colorless even though the solutions were completely decomposed. This suggests that the sulfonic acid derivative of isoproterenol is the only decomposition product for formulations containing high amounts of sodium bisulfite. However, as

Table V—Statistical Evaluation of Fluorometric Method to Analyze Isoproterenol Hydrochloride Solutions

	Concentration of Isoproterenol Hydrochloride, mcg./ml.	
	202.5	100.5
	200.5	100.5
	196.5	99.5
	199.5	102.0
	198.5	100.5
Mean	199.5	100.60
SD, %	1.118	0.894
SEM %	0.5	0.400
Mean error	−0.5	+0.6

pointed out earlier, if other unknown decomposition products such as isoproterenone are present, the USP XVIII method results in large errors.

Influence of Other Unknown Isoproterenol Oxidation Products on Fluorometric Method—It was stated earlier that isoproterenol decomposes to its sulfonic acid derivative in the presence of excess sodium bisulfite. However, in the presence of lower amounts of sodium bisulfite, additional decomposition products have been reported (8). The completely decomposed solutions in such cases were not clear and colorless and occasionally were accompanied with black precipitate. Therefore, it is essential to establish that the fluorometric method is specific only for the unchanged isoproterenol. Isoproterenol hydrochloride (230 mcg./ml.) in lactic acid–sodium lactate buffer (pH 3.50–3.60) stabilized with 350 mcg./ml. of sodium bisulfite was made. These solutions were stored in the dark at 56° for an extended time and were analyzed by the fluorometric method. TLC of the completely decomposed solutions indicated the presence of products other than isoproterenol sulfonic acid[3]. The completely decomposed solution was added in varying amounts to solutions of isoproterenol hydrochloride of known concentration, and the samples were analyzed by the fluorometric method. There was no fluorescence due to the completely degraded isoproterenol under the conditions tested. The results indicated that isoproterenol decomposition products other than the sulfonic acid, when present in high concentrations, do not influence the recovery of isoproterenol hydrochloride when assayed by the fluorometric method. The same decomposed isoproterenol solution was added to a known concentration of fresh isoproterenol hydrochloride solution. The decomposed isoproterenol and the mixture were separately analyzed by the USP XVIII method. The UV spectra of the results shown in Fig. 2 indicate interference from the unknown degradation products. The USP XVIII procedure requires the drawing of a baseline between the two minima (about 300 and 250 nm.) of isoproterenol hydrochloride to measure the absorbance due to unchanged isoproterenol hydrochloride. From Fig. 2, it is obvious that the minima do not occur at these prescribed wavelengths when these unknown oxidation products are present. This experiment thus proves that the fluorometric method described here is specific for the unchanged isoproterenol hydrochloride and that its decomposition products, when present, do not have any effect.

Statistical Analysis of Fluorometric Method—To check the precision and accuracy of the procedure, several samples containing isoproterenol hydrochloride of low and high concentrations were analyzed by the fluorometric method. The statistical treatment of the data is summarized in Table V. The percent standard deviation ranged from 0.89 to 1.12. However, the standard error of the mean was 0.4–0.5 at the low and high levels of isoproterenol hydrochloride. The mean error, which is the difference between the average test results and the true result, ranged from +0.6 for the lower concentration to −0.5 for the higher concentration of isoproterenol hydrochloride.

Influence of Other Antioxidants on USP XVIII and Fluorometric Methods of Isoproterenol Assay—The effect of other antioxidants, such as ascorbic acid and α-monothioglycerol, on the recovery of isoproterenol from aged solutions was investigated. Fresh solutions of isoproterenol containing ascorbic acid or α-monothioglycerol were analyzed by the fluorometric method. There was no interference from either of these two antioxidants on the fluorescence.

[3] F. Bangert, Chemical Control, Bristol Laboratories, personal communication.

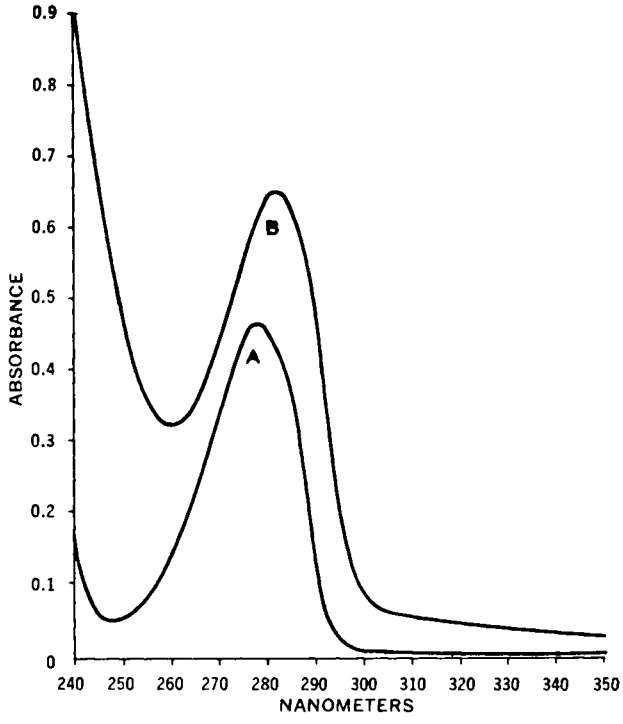

Figure 3—*UV spectra indicating the effect of isoproterenol hydrochloride decomposition products resulting from solutions stabilized with α-monothioglycerol as the antioxidant and assayed by the USP XVIII method. Key: A, isoproterenol hydrochloride, 200 mcg./ml.; and B, solution of 230 mcg./ml. isoproterenol hydrochloride reacted with 2 mg./ml. of α-monothioglycerol. This solution did not have any isoproterenol activity by the fluorometric method.*

In addition, isoproterenol formulations stabilized with either ascorbic acid or α-monothioglycerol were stored at elevated temperatures. These samples were analyzed by the fluorometric method and were shown to be completely devoid of any isoproterenol activity. The completely decomposed solution and another sample of isoproterenol were then assayed by the USP XVIII method (Fig. 3). It is obvious that the UV spectra of the intact isoproterenol hydrochloride and its degradation product in the presence of excess α-monothioglycerol are similar. It is also interesting to note that the absorbance due to this decomposition product is much higher at its maximum (about 278 nm.) when compared to the unchanged isoproterenol. Similar interferences were observed with isoproterenol solutions preserved and aged in the presence of ascorbic acid. Thus, the USP XVIII method or its modification, the bis-(2-ethylhexyl)phosphoric acid shakeout method (8), can be misleading if used to analyze solutions preserved with antioxidants other than sodium bisulfite. The reliable method in such instances is the fluorometric method, which is specific for the unchanged isoproterenol hydrochloride.

SUMMARY

The described fluorometric method is unique because of its applicability to the analysis of extremely dilute solutions of isoproterenol. The simplicity of the procedure lends itself to the assay of several samples at a time; with the official methods, it is difficult to carry out several determinations simultaneously. The fluorometric method is timesaving, whereas the USP XVIII method is lengthy and tedious. Moreover, it has been shown that the fluorometric method is unique in that it does measure only the unchanged isoproterenol; decomposition products, when present, do not interfere even in extremely high concentrations. Oxidative degradation products, if present, interfere in the USP XVIII and the bis(2-ethylhexyl)phosphoric acid shakeout methods. These latter two methods were specifically developed with the assumption that isoproterenol sulfonic acid is the only decomposition product present in the formulations.

It has been shown that the fluorometric method is the method of choice to assay isoproterenol formulations, since this procedure is based only on the intact isoproterenol and other decomposition products, even if present in significant amounts, do not influence the final fluorescence. The described fluorometric method has the potential for automation and thus can be of immense value to the pharmaceutical industry to monitor routinely the stability of isoproterenol formulations.

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Head-Space GLC Determination of Triethylamine in Pharmaceuticals

MARK A. LITCHMAN▲ and RONALD P. UPTON

Abstract □ A head-space GLC procedure is described for the quantitative determination of residual triethylamine in streptomycin sulfate and methacycline hydrochloride. This method is both rapid and accurate.

Keyphrases □ Streptomycin sulfate—head-space GLC analysis for residual triethylamine □ Methacycline hydrochloride—head-space GLC analysis for residual triethylamine □ Triethylamine residue in streptomycin sulfate and methacycline hydrochloride—head-space GLC analysis □ GLC, head-space—analysis, residual triethylamine in streptomycin sulfate and methacycline hydrochloride

Triethylamine has been determined qualitatively by GC (1–3). Umbreit *et al.* (4) decomposed aliphatic amine hydrochloride mixtures in the injection port of a gas chromatograph. They then analyzed 17–170 p.p.m. of each amine in the mixture. Pesez and Bartos (5) used derivatization and colorimetry to determine triethylamine at the 15-mcg. level. Kertes (6) used colorimetry to measure triethylamine at the 5% level. Derivative formation and TLC were employed by Baudot (7) at the 20-mcg. level. None of these methods involved the determination of triethylamine in pharmaceuticals.

Table I—Preparation of Standard Curve for Determination of Triethylamine in Streptomycin Sulfate

Stock Solution, ml.	1 *M* Sodium Hydroxide, ml.	Triethylamine per Serum Vial, mcg.
1.0	9.0	1090
3.0	7.0	3270
5.0	5.0	5450

Table II—Preparation of Standard Curve for Determination of Triethylamine in Methacycline Hydrochloride

Stock Solution, ml.	1 *M* Sodium Hydroxide, ml.	Triethylamine per Serum Vial, mcg.
0.5	9.5	535
1.0	9.0	1090
2.0	8.0	2180

This report describes a simple, rapid method for determining triethylamine in streptomycin sulfate[1] and in methacycline hydrochloride [4-(dimethylamino)-1,4,4*a*,5,5*a*,6,11,12*a* - octahydro - 3,5,10,12,12*a* - pentahydroxy - 6 - methylene - 1,11 - dioxo - 2 - naphthacenecarboxamide hydrochloride][1] to levels as low as 0.05%. The principle on which this method is based is that the head-space gas over a solution of a nonvolatile solute in a sealed container will contain amounts of any volatile substances present in the solution after equilibration at a constant temperature. The quantity of solvent vapors in the head-space gas will be proportional to the concentration of the solvents in the solution.

The method of Bassette *et al.* (8) was modified for this determination.

EXPERIMENTAL

Preparation of Standard Curve—Dilute 150 μl. triethylamine[2] to 100 ml. with 1 *M* sodium hydroxide solution. Transfer 1 ml. of this

[1] Pfizer Inc., New York, N. Y.

[2] Technical grade, Matheson, Coleman and Bell, East Rutherford, N. J.

It has been shown that the fluorometric method is the method of choice to assay isoproterenol formulations, since this procedure is based only on the intact isoproterenol and other decomposition products, even if present in significant amounts, do not influence the final fluorescence. The described fluorometric method has the potential for automation and thus can be of immense value to the pharmaceutical industry to monitor routinely the stability of isoproterenol formulations.

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▲ To whom inquiries should be directed.

Head-Space GLC Determination of Triethylamine in Pharmaceuticals

MARK A. LITCHMAN▲ and RONALD P. UPTON

Abstract □ A head-space GLC procedure is described for the quantitative determination of residual triethylamine in streptomycin sulfate and methacycline hydrochloride. This method is both rapid and accurate.

Keyphrases □ Streptomycin sulfate—head-space GLC analysis for residual triethylamine □ Methacycline hydrochloride—head-space GLC analysis for residual triethylamine □ Triethylamine residue in streptomycin sulfate and methacycline hydrochloride—head-space GLC analysis □ GLC, head-space—analysis, residual triethylamine in streptomycin sulfate and methacycline hydrochloride

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5.0	5.0	5450

Table II—Preparation of Standard Curve for Determination of Triethylamine in Methacycline Hydrochloride

Stock Solution, ml.	1 *M* Sodium Hydroxide, ml.	Triethylamine per Serum Vial, mcg.
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The method of Bassette *et al.* (8) was modified for this determination.

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[1] Pfizer Inc., New York, N. Y.

[2] Technical grade, Matheson, Coleman and Bell, East Rutherford, N. J.

Table III—Recovery of Spikes of Triethylamine in Streptomycin Sulfate

Sample Number	Added, mcg.	Recovered, mcg.	Recovery, %
1	1090	1028	94.3
2	3270	3214	98.3
3	5450	5150	94.5

stock solution to a No. 20 serum vial[3] and add 9 ml. of 1 *M* sodium hydroxide solution. Immediately cap the vial with a rubber septum[4] and metal closure[4] and swirl. Place in a water bath at 60° for 1 hr.[5] and withdraw 1 ml. of the head-space gas using a gastight syringe[6]. Immediately inject into the gas chromatograph and record the elution curve[7]. Repeat with the other standards. The preparations of standard solutions for determining the other points of the standard

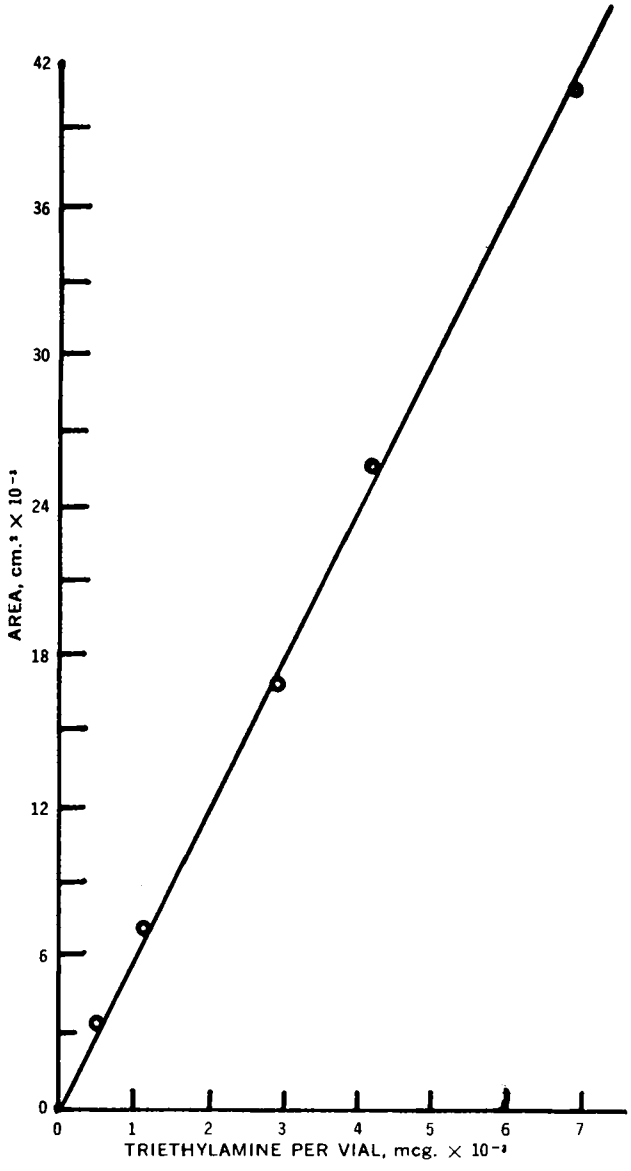

Figure 1—*Typical triethylamine standard curve.*

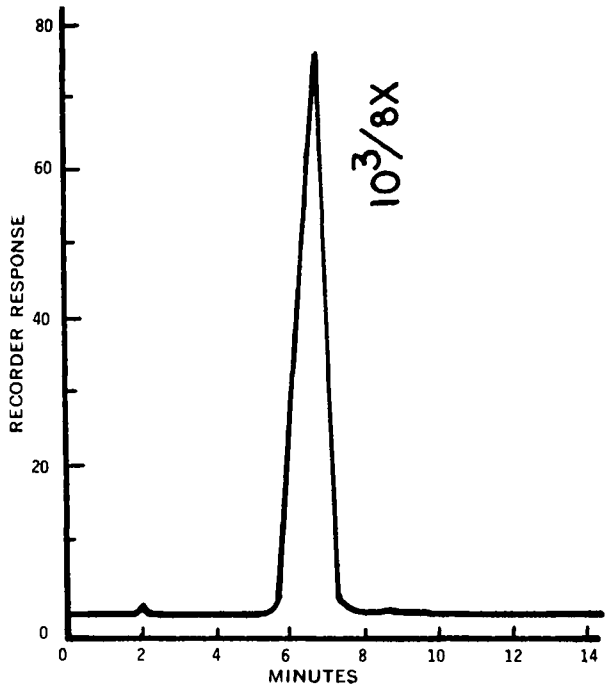

Figure 2—*Chromatogram of triethylamine in a production sample of streptomycin sulfate.*

curves for streptomycin sulfate and methacycline hydrochloride are given in Tables I and II, respectively.

Preparation of Samples—Transfer an accurately weighed 1.0-g. sample of streptomycin sulfate or 0.3-g. sample of methacycline hydrochloride to a No. 20 serum vial. Add 10 ml. of 1 *M* sodium hydroxide solution. Proceed as in *Preparation of Standard Curve*.

Gas Chromatograph—A chromatograph[8] with a hydrogen-flame ionization detector and recorder[9] was used with an electronic integrator[10] and teletype[11].

Column and Conditions—A 1.82-m. (6-ft.) stainless steel column, 0.32-cm. (0.125-in.) i.d. and 0.63-cm. (0.25-in.) o.d., was packed with polystyrene[12], 80–100 mesh, using a vibrator and conditioned at 240° with nitrogen flowing through the column for 18 hr. The operating conditions were column temperature, 160°; injector

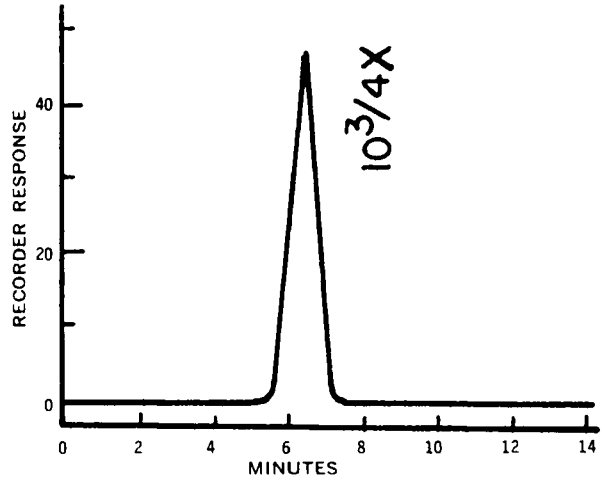

Figure 3—*Chromatogram of triethylamine in a production sample of methacycline hydrochloride.*

[3] Wheaton vial, Grand Island Biological Co., Grand Island, N. Y.
[4] Grand Island Biological Co., Grand Island, N. Y.
[5] One hour was chosen for convenience of sampling. The recovery values for triethylamine in streptomycin sulfate or methacycline hydrochloride do not change from 15 min. to 2 hr. at 60°.
[6] Precision Sampling Corp., Baton Rouge, La.
[7] The retention time of triethylamine is 6.5 min.
[8] Hewlett-Packard 5751B.
[9] Model 7127A equipped with 1-mv. span, Hewlett-Packard Corp., Avondale, Pa.
[10] Model CRS-101, Infotronics Corp., Houston, Tex.
[11] Model 33, Teletype Corp., Framingham, Mass.
[12] Porapak PS, Waters Associates., Inc., Framingham, Mass.

Table IV—Recovery of Spikes of Triethylamine in Methacycline Hydrochloride

Sample Number	Added, mcg.	Recovered, mcg.	Recovery, %
1	1090	1207	111
2	2180	2202	101
3	4360	4055	93

Table V—Determination of Triethylamine Content of Replicate Weights of a Streptomycin Sulfate Sample

Sample Number	Triethylamine Content, %
1	0.354
2	0.352
3	0.371
4	0.367
5	0.365

temperature, 235°; detector temperature, 250°; carrier gas, nitrogen, 35 ml./min.; detector gas, hydrogen, 40 ml./min.; and air, 500 ml./min.

Calculations—The area of the triethylamine peak in the samples is compared to a standard curve[13] of micrograms triethylamine per vial *versus* area found. The following formula is then used:

$$\frac{\text{micrograms triethylamine from standard curve}}{\text{weight of sample, mcg.}} \times 100 = \text{percent triethylamine} \quad \text{(Eq. 1)}$$

RESULTS AND DISCUSSION

The levels of triethylamine determined were from 0.15 to 0.36% for production samples of streptomycin sulfate, and they were from 0.06 to 0.13% for production samples of methacycline hydrochloride.

The recoveries of spikes of triethylamine in streptomycin sulfate and methacycline hydrochloride are given in Tables III and IV, respectively. The values ranged from 94.3 to 98.3% for streptomycin sulfate and from 93.0 to 111% for methacycline hydrochloride.

Table VI—Determination of Triethylamine Content of Replicate Weights of a Methacycline Hydrochloride Sample

Sample Number	Triethylamine Content, %
1	0.117
2	0.119
3	0.125
4	0.123
5	0.120

Five replicate weighings of a sample of streptomycin sulfate and methacycline hydrochloride were made, and the triethylamine content was determined (Tables V and VI). The data indicate a precision of 2 and 5%, respectively.

Examples of chromatograms for samples of streptomycin sulfate and methacycline hydrochloride are shown in Figs. 2 and 3, respectively.

The head-space method has been found to be a simple, direct, and precise method for trace volatiles component analysis. This method is currently being applied to other compounds.

[13] An example of a typical triethylamine standard curve is given in Fig. 1.

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Use of Circular Dichroism in Analysis of Mixtures of Tetracycline and 4-Epitetracycline and Its Application to Assay of Commercial Products

ROBERT F. MILLER*, THEODORE D. SOKOLOSKI▲, LESTER A. MITSCHER, ANTHONY C. BONACCI†, and BETTY-ANN HOENER

Abstract □ Mixtures of tetracycline and 4-epitetracycline were assayed utilizing the large difference in their circular dichroism spectra at 262 nm. The assay was evaluated using synthetic mixtures and applied to the analysis of commercial tetracycline hydrochloride capsules. The results obtained with the newly developed procedure compare favorably with a published spectrophotometric method. In comparison with other techniques, the proposed method has several advantages. It is specific in that it permits differentiation between tetracycline and its 4-epimer, permitting calculation of the actual level of each epimer. It is accurate and precise, having an average error of 2% for tetracycline detection and 3.3% for 4-epitetracycline. It involves a simple procedure that requires no prior separation of degradation products under usual use conditions. Furthermore, it is very rapid, requiring only the experimental measurements of ellipticity at 262 nm. and absorbance at 356 nm. The disadvantages of the assay are its inability to determine analytically the content of anhydrotetracyclines and the possible interference of these materials if they are present in large amounts. However, levels of 10% anhydrotetracyclines affect the assay for tetracycline by only 1%. In the analysis of eight commercial products, the tetracycline content varied from 92 to 111% of the labeled amount and 4-epitetracycline was present in amounts of 3–17%. When compared with a spectrophotometric assay that required prior separation of anhydro compounds, the method introduced here gave the same analytical results for tetracycline content in the various products.

Keyphrases □ Tetracycline–epitetracycline mixtures—analysis by circular dichroism, determination in commercial products, compared with spectrophotometric method □ Epitetracycline–tetracycline mixtures—analysis by circular dichroism, determination in commercial products, compared with spectrophotometric method □ Degradation products—circular dichroism analysis of tetracycline–epitetracycline mixtures, applied to commercial products □ Circular dichroism—used to analyze tetracycline–epitetracycline mixtures, applied to commercial products

Tetracyclines may degrade through at least four possible mechanisms: epimerization, dehydration, hydrolysis, and oxidation. The first two mechanisms are the most commonly encountered reactions, so their degradation products are of specific interest in this study. Epimerization at carbon-4 leads to an inactive and nontoxic drug form (1). The kinetics of this reaction were studied by several workers (2, 3), showing that epimerization follows a reversible first-order process occurring most rapidly between pH 3 and 5. Dehydration and aromatization of the C-ring of tetracycline lead to anhydro derivatives at low pH. Anhydrotetracycline appears to be inactive *in vivo*, but its 4-epimer has been implicated in the toxic effects following use of outdated tetracycline products (4). The potential severity of this hazard provided the impetus to develop practical analytical procedures to detect and quantify both inactive and toxic tetracycline derivatives.

Titrimetric, polarographic, chromatographic, spectral, and microbiological methods have been used to analyze the tetracyclines. The latter three techniques seem to have received the most attention. The most widely used assay is the official microbiological method (5, 6). The disadvantages inherent in this method are that it has an accuracy of ±15% (7) and it is of limited specificity since several tetracycline-like materials have biological activity. Furthermore, the assay is carried out under conditions where some *in situ* epimerization is possible (pH 4.5 and incubation at 32–35° for 3–4 hr.). Among the several spectral methods used, absorbance ratios at two wavelengths have received the most attention. McCormick *et al.* (8) introduced an absorbance ratio method for tetracycline and 4-epitetracycline mixtures where ratios at 254 and 267 nm. are utilized. The sensitivity of the method is expected to be low, since the absorbance ratios range from 0.86 for tetracycline to 1.07 for epitetracycline. Because anhydrotetracycline and 4-epianhydrotetracycline absorb strongly in the 250–275-nm. range, the absorbance ratio technique by itself is not useful for samples containing these decomposition products. Pernarowski *et al.* (9) used an absorbance ratio at 357 and 391 nm. to detect tetracycline in the presence of anhydrotetracycline and epianhydrotetracycline. The assay, however, does not distinguish between epimers and essentially measures total content of tetracycline and epitetracycline when epitetracycline is present, as it usually is.

The number and variety of chromatographic methods applied to the analysis of tetracyclines probably exceed those of any other general assay method investigated. The inadequate and inconsistent recovery of tetracycline and degradation products by paper chromatography (10) is improved by using column chromatography. Griffiths (11), for example, made use of a column[1] that provided reproducibility but lacked specificity; tetracycline and epitetracycline elute in one fraction and anhydrotetracycline and epianhydrotetracycline elute in another. Ascione *et al.* (7, 12) reported both column chromatographic and TLC procedures for tetracyclines. Although these methods provide separation of tetracycline and its degradation products, detailed requirements for column preparation and repeated plate development in the TLC method make the techniques tedious. An automated procedure was recently introduced based on this technique (13), but it does not provide separate analysis for decomposition products. The procedure reported by Fike and Brake (14) does apparently separate and analyze degradation products. TLC separation followed by a conversion to anhydrotetracyclines serves as the basis for

[1] Sephadex G-25.

additional methods of analysis of mixtures of tetracyclines and their degradation products (15, 16). The methods appear useful for assay of commercial samples, but success requires strict attention to procedural detail (17) and they are unsuited for routine analysis of large numbers of samples.

In studies involving the solution conformation and sites of metal-ion binding of tetracycline derivatives (18–20), note was made of the large difference in the circular dichroism spectra of acidic solutions of tetracycline and epitetracycline at 262 nm. It appeared that this large difference could be used to analyze tetracycline in the presence of epitetracycline. The approach seemed feasible because circular dichroism measurements are rapid and relatively sensitive and require small samples; moreover, they are selective for tetracycline and epitetracycline. Anticipating that such an approach would offer advantages over existing techniques and because circular dichroism has yet to realize its potential for analysis of natural product mixtures, we developed and evaluated an analytical method to determine tetracycline content in the presence of its degradation products. Eight different brands of tetracycline hydrochloride capsules were analyzed by both circular dichroism and traditional UV chromatographic methods to determine the practicality of the method and the significance and incidence of tetracycline epimerization in finished products. All of these products had passed FDA batch certification but were intercepted at the point of dispensing. Therefore, they are representative of what the patient is receiving rather than what the pharmacist receives.

EXPERIMENTAL[2]

The ellipticity and absorbance of pure tetracycline, epitetracycline, anhydrotetracycline, and epianhydrotetracycline were determined as a function of concentration at 262 nm. (ellipticity, circular dichroism), and 356, 267, and 254 nm. (absorbances, UV). UV scans were made between 450 and 240 nm. All solutions were made in 0.03 *N* HCl where epimerization and dehydration are minimized (1). The concentration range for circular dichroism measurements was generally 0.001–0.005% and, when necessary, from 2 to 5 times more dilute for UV measurements.

Known mixtures of tetracycline and epitetracycline were made where the fraction of tetracycline ranged from 0.2 to 0.9. The total concentration of the mixture was about 9×10^{-5} *M* in the circular dichroism measurements and, when necessary, from 2 to 5 times more dilute to obtain measurable UV absorbances. The blank in all cases was 0.03 *N* HCl.

The commercial samples were analyzed in the following manner. The contents of 10 capsules from each product were pooled. The samples were then analyzed for total anhydrotetracycline content using a method based on the FDA screening procedure[3]. An amount of the pooled sample containing 250 mg. of tetracycline was mixed with 50 ml. of 0.15 *N* HCl for 5 min. The mixture was then vacuum filtered, the residue was washed with water, and the filtrate was diluted to 250 ml. with water. The resulting solution contained 0.1% tetracycline in 0.03 *N* HCl. This stock solution was then used to prepare 0.04 and 0.001% solutions. The absorbance of the 0.04% solution was read at 430 nm. and the 0.001% solution at 356 nm. These two measurements were used in the calculation of total anhydro content. For application to the new method of analysis, the stock solution was diluted to 0.005% and its circular dichroism spectrum was measured. The absorbance of a 0.0014% solution (1:4 dilution of the circular dichroism solution) was measured at 356 nm.

For comparative purposes, a spectrophotometric assay was carried out on three sample products. In this method, it is necessary to separate anhydrotetracyclines from the mixture. The chromatographic technique reported by Griffiths (11) was used for this purpose. A resin column[1] preswollen in 0.03 *N* HCl was used. A portion of commercial sample containing 200 mg. of tetracycline was diluted to 10 ml. with 0.03 *N* HCl and shaken in a glass-stoppered centrifuge tube for 5 min. The suspension was centrifuged, and 0.5 ml. of the supernate was applied to the column. The column was eluted with 0.03 *N* HCl, and 5-ml. fractions were automatically collected. Fractionation was followed by measuring absorbance at 273 nm. Tubes containing the tetracycline–epitetracycline fraction (approximately the first 70–80 ml. eluted) were carefully combined and diluted to 250 ml. with 0.03 *N* HCl. The absorbance of this solution was measured at 267 and 254 nm. for spectrophotometric analysis of tetracycline and epitetracycline.

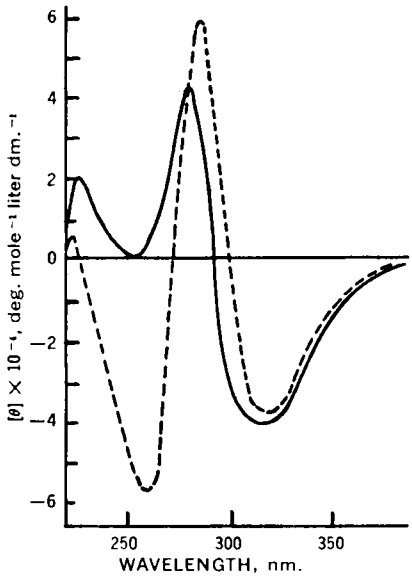

Figure 1—*Molar ellipticity* [θ] *of tetracycline hydrochloride* (- - -) *and 4-epitetracycline ammonium salt* (— —) *as a function of wavelength in 0.03* N *HCl.*

RESULTS AND DISCUSSION

The circular dichroism curves of tetracycline and epitetracycline (Fig. 1) show that there is a large difference in ellipticity between the two epimers at 262 nm. At this wavelength a mixture of the two compounds would give an ellipticity that is a sum of the ellipticities for each species:

$$\psi_{MIX} = k_{TC}C_{TC} + k_{ETC}C_{ETC} \qquad \text{(Eq. 1)}$$

where ψ_{MIX} is the observed ellipticity; and k_{TC} and k_{ETC} are the proportionality constants defining the linear relation between ellipticity and molar concentration, C_{TC} and C_{ETC}, for tetracycline and epitetracycline, respectively, where the path length of the cell

[1] Circular dichroism spectra were determined using a Durrum-Jasco ORD/UV-5 instrument with the Sprowl Scientific SS 20-2 circular dichroism modification. Samples were run in a 1-cm. path length cell at ambient temperature (29°). UV and visible light absorption were determined in a Cary model 15 or a Gilford model 240 spectrophotometer at ambient temperature. A Warner-Chilcott model 1205 D3 automatic fraction collector was used in conjunction with column chromatographic procedures.

The following materials were donated by Lederle Laboratories and used as standards: tetracycline hydrochloride (CL-13554; 4574B-1296), 4-epitetracycline ammonium salt (CL-16956; 705B-133-9), anhydrotetracycline hydrochloride (CL-17901; 8412 B97-A), and 4-epianhydrotetracycline (CL; 27212-7117B-93-5).

The several samples of commercial tetracycline capsules were acquired from the active stock of various retail and hospital pharmacies. They were collected at random without conscious regard for expiration date; however, none of the samples had expired.

Sephadex G-25, Medium (Pharmacia), was used in the chromatographic procedure. All other chemicals were of analytical grade quality.

[3] Personal communication with Dr. William W. Wright, Acting Director, National Center for Antibiotics and Insulin Analysis, Food and Drug Administration, Washington, D.C.

Table I—Circular Dichroism (CD) and UV Constants for Various Tetracyclines in 0.03 *N* HCl

Compound	Mode	Wavelength, nm.	Constant, deg./*M* (CD), *A*/*M* (UV) ± Average Percent Error	Symbol
Tetracycline hydrochloride	CD	262	−579.9 ± 3.8[a]	k_{TC}
	UV	356	14,495 ± 0.3[a]	a_{TC}
	UV	267	18,367 ± 0.42[b]	a_{TC}^{267}
	UV	254	15,571 ± 0.24[b]	a_{TC}^{254}
Epitetracycline hydrochloride	CD	262	+133.4 ± 9.2[a]	k_{ETC}
	UV	356	13,833 ± 1.2[a]	a_{ETC}
	UV	267	14,477 ± 0.39[c]	a_{ETC}^{267}
	UV	254	15,467 ± 0.32[c]	a_{ETC}^{254}
Anhydrotetracycline hydrochloride	UV	356	928 ± 5.8[b]	
	CD	262	+864 ± 8.2[d]	
Epianhydrotetracycline hydrochloride	UV	356	736 ± 4.3[c]	
	CD	262	+904 ± 1.7[d]	

[a] Average of 9–10 values. [b] Average of five values. [c] Average of four values. [d] Average of three values.

Table II—Results of the Analysis of Known Mixtures of Tetracycline and Epitetracycline by Circular Dichroism Method

Sample Number	Concentration Added, $M \times 10^5$		Ellipticity, ψ_{MIX}	Absorbance × Dilution Factor, nA_{MIX}	Concentrations Found, $M \times 10^5$		Percent Recovery	
	Tetracycline	Epitetracycline			Tetracycline	Epitetracycline	Tetracycline	Epitetracycline
1	8.32	0	−0.0488°	1.395	8.65	—	104.0	—
2	8.15	0	−0.0499°	1.180	8.52	—	104.5	—
3	7.49	0.90	−0.0433°	1.235	7.67	0.885	102.4	98.3
4	6.66	1.80	−0.0373°	1.245	6.85	1.82	102.9	101.1
5	6.24	2.25	−0.0323°	1.225	6.13	2.43	98.2	108.0
6	6.11	2.24	−0.0346°	1.190	6.40	1.89	104.7	84.4
7	4.16	4.50	−0.0180°	1.225	4.14	4.51	99.5	100.2
8	4.08	4.48	−0.0171°	1.187	3.97	4.42	97.3	98.7
9	2.08	6.75	−0.00375°	1.210	2.14	6.50	102.9	96.3
10	2.04	6.72	−0.00315°	1.198	2.04	6.52	100.0	97.0
11	0	9.00	+0.0155°	1.210	0	9.31	—	103.4
12	0	8.96	+0.0125°	1.215	0	8.90	—	99.3
					Average percent recovery		101.8	98.7
					Average percent error[a]		2.0	3.3

[a] Calculated as the average absolute difference between the percent recovered in each mixture and the average percent recovery for all 10 samples.

is 1 cm. Although it is possible to use a second equation involving ellipticity at another wavelength to calculate the content of tetracycline and epitetracycline in a sample, the greater precision that can be achieved (Table I) by using absorbance measurements to determine total tetracyclines led to the use of a second equation based on UV analysis. As discussed later, a UV analysis of a sample should be made to determine the content of anhydrotetracyclines since they can interfere with the precision of the circular dichroism measurements if they are present in significant amounts. Absorbance at 356 nm., where both tetracycline and epitetracycline show an absorption peak, is proportional to the total tetracycline content. The solutions used to measure Ψ are usually too concentrated for direct UV measurement; hence, they are diluted by a factor, n, prior to analysis. Thus, the absorbance of the solution used in determining ψ is related to the molar concentration, C, of tetracycline and epitetracycline by:

$$nA_{MIX} = a_{TC}C_{TC} + a_{ETC}C_{ETC} \qquad \text{(Eq. 2)}$$

where nA_{MIX} is the absorbance of the circular dichroism solution, diluted n-fold, using a cell with a 1-cm. path length, and a_{TC} and a_{ETC} are the molar absorbances for each species. Applying Cramer's rule (21) to Eqs. 1 and 2 gives:

$$C_{TC} = \frac{a_{ETC}\psi_{MIX} - nA_{MIX}k_{ETC}}{k_{TC}a_{ETC} - k_{ETC}a_{TC}} \qquad \text{(Eq. 3)}$$

and:

$$C_{ETC} = \frac{nA_{MIX}k_{TC} - a_{TC}\psi_{MIX}}{k_{TC}a_{ETC} - k_{ETC}a_{TC}} \qquad \text{(Eq. 4)}$$

The various constants involved in Eqs. 3 and 4 were determined from known concentrations of pure materials and are listed in Table I. In this study, we found that the constants generated for tetracycline may depend on the source of tetracycline and vary slightly from time to time; thus, they should be verified periodically to ensure the highest accuracy. Substitution of the constants from Table I into Eqs. 3 and 4 leads to:

$$C_{TC} = (103.7\psi_{MIX} - A_{MIX})(-1.340 \times 10^{-6}) \qquad \text{(Eq. 5)}$$

$$C_{ETC} = (-A_{MIX} - 25.0\psi_{MIX})(-5.824 \times 10^{-6}) \qquad \text{(Eq. 6)}$$

Analysis of known mixtures of tetracycline and epitetracycline using Eqs. 5 and 6 gave the results listed in Table II. Recovery of tetracycline averaged 101.6% with an average error of 2.0%, while epitetracycline gave a recovery of 98.7% with an average error of 3.3%. A comparison of these results with a spectrophotometric assay was made. Absorbance of these same mixtures at 267 and 254 nm. was used and the fraction of tetracycline, F_{TC}, present was calculated using the mathematical form of the equation used by Pernarowski *et al.* (22):

$$F_{TC} = \frac{a_{ETC}^{254}Q_0 - a_{ETC}^{267}}{(a_{TC}^{267} - a_{ETC}^{267}) - (a_{TC}^{254} - a_{ETC}^{254})Q_0} \qquad \text{(Eq. 7)}$$

where Q_0 is the absorbance ratio A_{267}/A_{254}; and a_{TC} and a_{ETC} are the absorbances of tetracycline and epitetracycline, respectively, at wavelengths designated by the superscripts. The values for these constants are given in Table I and their substitution into Eq. 7 gives:

$$F_{TC} = \frac{15{,}467Q_0 - 14{,}477}{3890 - 104Q_0} \qquad \text{(Eq. 8)}$$

By using the mixtures listed in Table II, the fraction of tetracycline in each was determined using Eq. 8. The results of the analysis (Table III) show that an average recovery of 102.2% with an average error of 3.4% was obtained. This compares favorably with the results using the circular dichroism method.

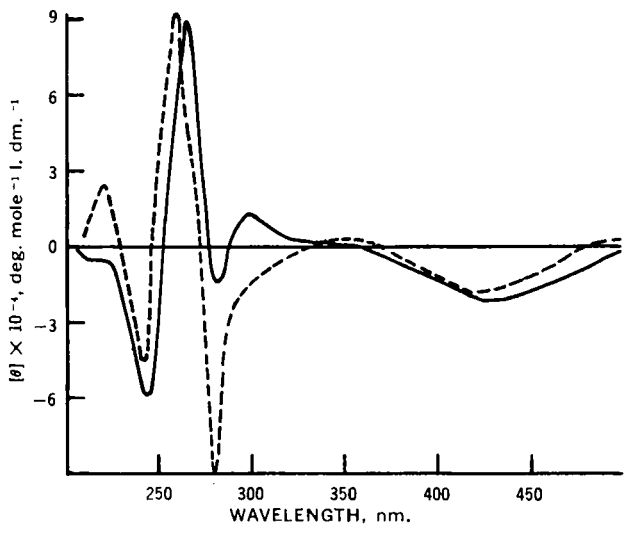

Figure 2—*Molar ellipticity [θ] of anhydrotetracycline hydrochloride (——) and 4-epianhydrotetracycline (- - -) as a function of wavelength in 0.03* N *HCl.*

Eight commercial samples of tetracycline hydrochloride capsules were then analyzed using the circular dichroism method *without* prior separation of anhydro compounds. The circular dichroism spectra of epianhydrotetracycline and anhydrotetracycline (Fig. 2) show that these compounds may interfere with the circular dichroism assay if present in significant amounts. When using the circular dichroism and UV constants for epianhydrotetracycline and anhydrotetracycline listed in Table I, it is possible to calculate the error that would be introduced in this assay if a solution of 1×10^{-4} *M* tetracyclines contained 90% tetracycline and 10% anhydrotetracycline. [Pernarowski *et al.* (9) reported that the maximum amount of anhydrotetracycline present in six commercial samples tested by them was 10%; in the present study the maximum percentage of anhydrotetracyclines in eight samples was 5.3%.] The theoretical ellipticity that would be observed at 262 nm. would be −0.0513°, and the absorbance of the solution at 356 nm. would be 1.314 for the tetracycline–anhydrotetracycline mixture. Using the new assay, Eqs. 5 and 6 would predict 88.9% tetracycline and 1.69% epitetracycline (rather than 90% tetracycline and 0% epitetracycline). Thus, the method would be low with respect to tetracycline recovery and predict the presence of a small amount of epitetracycline even though none were present. Analysis of the eight commercial products studied showed that the products contained from 2 to 5% anhydro species. This amount would not appreciably affect the tetracycline and epitetracycline calculation. Determination of the anhydro content was based on the FDA screening procedure using the formula:

$$\text{percent anhydrotetracyclines} = \frac{(A_{430} - A_{356} \times 0.0019)100}{180} \qquad \text{(Eq. 9)}$$

where A_{430} is the absorbance of the 0.04% solution at 430 nm. times 25, A_{356} is the absorbance of the 0.001% solution at 356 nm. times 1000, 0.0019 is the absorbance ratio (430 nm./356 nm.) for tetracycline, and 180 is the absorptivity (1%, 1 cm.) of anhydrotetracycline at 430 nm. The first column of Table IV lists the total percent of anhydrotetracyclines present in each product as determined by Eq. 9. Columns 2 and 3 (Table IV) give the percentage of tetracycline and epitetracycline in each product using the new assay. All samples contain tetracycline within accepted limits but also contain amounts of epitetracycline which vary from 3 to 17%. The values were not corrected for the small amount of anhydrotetracycline present. Column 5 of Table IV gives the fraction of tetracycline hydrochloride determined by the absorbance ratio assay (Eq. 7) where anhydrotetracyclines were first removed by resin column chromatography. The fraction of tetracycline present determined by the absorbance ratio method compares quite well with that obtained by the new technique (Table IV, Column 6).

Since limits have not been set on permissible levels of epitetracycline in commercial products, it is difficult to discuss the significance of the amounts found. Perhaps the most significant result of this analysis of commercial samples is the prevalence of capsule overfilling (in seven of eight products). Sample 8 would pass certification, showing 95–97% tetracycline, but it constitutes a poorer grade of drug because of the amounts of epitetracycline and anhydro compounds present. There is no specified upper limit on the amount of tetracycline that may be present in the standards set forth in the "Code of Federal Regulations," nor is any limit set for anhydrotetracycline and epianhydrotetracycline.

When Pernarowski *et al.* (9) examined six commercial tetracycline products, using absorbance ratios at 357 and 391 nm., they reported the samples as having 90–94% of the labeled tetracycline content, the remainder being only anhydro compounds as seen by TLC. Results of the present study indicate small amounts of anhydrotetracyclines and from 3 to 17% epitetracycline. There is no reason to expect that the samples used previously (9) were the same as those used in the present study, nor is it reasonable to expect large errors in epitetracycline content in the present method even in the presence of 5% anhydrotetracycline.

To determine further the accuracy with which epitetracycline could reasonably be estimated when present at low concentrations, eight known mixtures containing 4–17% epitetracycline in the presence of tetracycline were analyzed using the circular dichroism method and Eqs. 5 and 6. In this study, the concentration of epitetracycline in the samples varies from 3.82×10^{-6} to 1.58×10^{-5} *M* while the tetracycline was held constant at approximately 8×10^{-5} *M* (Table V). When the absolute difference between the percent found and the percent added is taken, the average error in detecting percent tetracycline is found to be 1.5%. Similar analyses of the results show that the percent of epitetracycline relative to the percent added was low by an average of 2.9% in all mixtures studied. Be-

Table III—Results of Known Mixtures of Tetracycline and Epitetracycline by the Spectrophotometric Method

Sample Number[a]	Q_0, A_{267}/A_{254}	Fraction Tetracycline Added	Fraction Tetracycline Found[b]	Percent Recovery
1	1.176	1.00	0.985	98.5
2	1.184	1.00	1.018	101.8
3	1.170	0.893	0.960	107.5
4	1.136	0.787	0.820	104.2
5	1.122	0.735	0.763	103.8
6	1.119	0.732	0.750	102.5
7	1.051	0.480	0.471	98.1
8	1.045	0.477	0.446	93.5
9	0.9964	0.236	0.245	103.8
10	0.9981	0.233	0.253	108.6
		Average percent recovery		102.2
		Average percent error[c]		3.4

[a] Same samples as that listed in Table II. [b] Calculated using Eq. 8. [c] Calculated as the average absolute difference between the percent recovered in each mixture and the average percent recovery (102.2%).

Table IV—Analysis of Commercial Tetracycline Capsules

Product	Percent Total Anhydrotetracycline[a]	Percent Tetracycline Hydrochloride[b]	Percent Epitetracycline Hydrochloride[b]	Fraction Tetracycline —Hydrochloride— UV Method[c]	Fraction Tetracycline —Hydrochloride— Circular Dichroism Method
C-1	2.4	92.1	8.6	—	—
C-2	2.2	97.6	8.7	—	—
C-3	2.4	84.2	9.6	—	—
C-4	2.2	92.7	10.9	—	—
C-5	2.6	95.5	11.2	—	—
C-6	3.3	111.4	3.0	0.97	0.97
C-7	3.0	104.9	3.4	0.96	0.97
C-8	5.3	95.3	17.4	0.86	0.86

[a] Determined by the FDA screening procedure for total anhydrotetracyclines. [b] Determined using the circular dichroism method. [c] Determined by the column chromatography–absorbance ratio (267/254 nm.) *via* Eq. 7.

Table V—Results of Circular Dichroism Analysis of Known Mixtures Containing Low Percentages of Epitetracycline

Percent Added[a]		Percent Found[b]	
Tetracycline	Epitetracycline	Tetracycline	Epitetracycline
82.7	17.3	84.4	15.0
84.2	15.8	84.3	13.3
86.3	13.7	88.4	11.1
87.2	12.8	86.8	9.3
90.6	9.4	93.2	6.3
91.0	9.0	91.3	5.9
95.2	4.8	99.7	2.1
95.4	4.6	96.0	1.3

[a] Calculated as: C_{TC} added or C_{ETC} added/C_{TC} added + C_{ETC} added × 100. [b] Calculated as: C_{TC} found or C_{ETC} found/C_{TC} added + C_{ETC} added × 100.

cause of the small amounts of epitetracycline present and the concomitant small changes in the ellipticity of mixtures of it and tetracycline, the assay for epitetracycline was predictably subject to error, but the error involved would not explain the amounts of epitetracycline found in commercial products in this study.

It appears that no presently existing assay will by itself completely quantify a mixture of tetracycline, epitetracycline, anhydrotetracycline, and epianhydrotetracycline. Absorbance ratios at 357 and 391 nm. permit the calculation of the fraction of total tetracycline (tetracycline and epitetracycline). Absorbance ratios at 254 and 267 nm. will predict tetracycline content providing anhydro compounds are not present. The new technique will predict tetracycline and epitetracycline levels if large amounts of anhydrotetracyclines are not present. Thus, it would appear that a recommended procedure for the analysis of tetracycline products would be to determine the total anhydro content by the FDA screening method. If more than 5% anhydrotetracyclines are present, the anhydro compounds should be separated by the described resin chromatography and the tetracycline–epitetracycline fraction should be analyzed using this method. Alternatively, mathematical corrections for the increase in epitetracycline can be made without prior chromatographic separation.

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Application of Ion-Pairing to Separation of Selected Sulfonamides by Partition Chromatography

BOBBY R. RADER

Abstract □ Methods are presented for the separation and determination of individual sulfonamides in mixtures. These procedures are based on the ion-pair formation of the sulfonamides with the tetrabutylammonium ion, followed by separation on partition chromatographic columns. One procedure separates the trisulfapyrimidines (sulfamethazine, sulfamerazine, and sulfadiazine) and a second procedure separates sulfamethizole, sulfadiazine, and phenazopyridine hydrochloride. The separated sulfonamides are then determined by UV spectrophotometry and phenazopyridine hydrochloride by visible spectrophotometry. Ten commercial and five synthetic preparations were assayed by the procedures. Recoveries from the synthetic preparations ranged from 99 to 101%.

Keyphrases □ Sulfonamide mixtures—application of ion-pairing to separation by partition chromatography, commercial samples □ Ion-pairs—used in analysis of mixtures of sulfonamides, partition chromatography, commercial samples □ Tetrabutylammonium ion—ion-pair formation with sulfonamides, separation by partition chromatography, commercial samples □ Partition chromatography—use of ion-pairs for separation of sulfonamide mixtures, commercial samples

Sulfonamide combinations are found in a number of pharmaceutical preparations. Quantitation of the individual sulfonamides in these mixtures presents a difficult analytical problem. In the USP XVIII and NF XIII procedures (1, 2), the sulfonamides are separated by paper chromatography and determined colorimetrically by the Bratton–Marshall reaction. These procedures are time consuming and require an experienced analyst to obtain consistent results.

Other procedures used for the separation of sulfonamides include TLC (3, 4), partition chromatography (5), and GLC (6). Banes and Riggleman (7) summarized problems encountered in most of these techniques. They presented a procedure for the trisulfapyrimidines in which total sulfonamides are determined colorimetrically by the Bratton–Marshall procedure. Sulfadiazine is quantitated by its specific reaction with thiobarbituric acid, and sulfamethazine is separated by column partition chromatography and determined by UV spectroscopy.

The use of partition chromatography to separate sulfonamides has been hindered by the low solubility of the sulfonamides in many water-immiscible solvents. Solubility of the sulfonamides in chloroform and methylene chloride can be greatly increased by formation of ion-pairs with the tetrabutylammonium ion. Several workers (8–10) discussed the use of ion-pairs with quaternary ammonium ions in the extraction of acidic compounds. Ion-pair extraction in conjunction with partition chromatography was presented previously by Doyle and Levine (11, 12).

In the present report, methods are given for the analysis of two drug mixtures which are commonly encountered in this laboratory. One procedure (Method I) separates sulfamethazine, sulfamerazine, and sulfadiazine. Another procedure (Method II) separates sulfamethizole, sulfadiazine, and phenazopyridine. In both procedures, the sulfonamides are eluted from the partition column as ion-pairs with tetrabutylammonium ions. Separated sulfonamides are determined by UV spectrophotometry, and phenazopyridine hydrochloride is determined by visible spectrophotometry. Ten commercial and five synthetic drug preparations were assayed.

EXPERIMENTAL

Apparatus—A recording UV–visible spectrophotometer with 1-cm. cells was used.

Solvents—Chloroform, ether, hexane, and methylene chloride (all reagent grade) were used. Wash each solvent twice with equal volumes of water. Prepare 50% chloroform in hexane by mixing 500 ml. of chloroform with 500 ml. of hexane, and filter through filter paper.

Ten Percent Tetrabutylammonium Hydroxide in Water[1] (Reagent A)—Pipet 3.0 ml. of the solution into a flask and add about 20 ml. of water. Titrate the solution with 0.1 *N* HCl, using methyl red indicator. One milliliter of 0.1 *N* acid = 25.95 mg. of tetrabutylammonium hydroxide.

Sodium Hydroxide–Tetrabutylammonium Hydroxide Solution (Reagent B)—Pipet (with a 10-ml. measuring pipet) an aliquot of Reagent A, equivalent to 800 mg. of tetrabutylammonium hydroxide, into a 25-ml. graduated cylinder. Add 10.0 ml. of 0.2 *N* NaOH to the cylinder from a pipet. Dilute to 20.0 ml. with water and mix.

Standard Preparation—USP reference standards or NF grade standards of sulfadiazine, sulfamerazine, sulfamethazine, sulfamethizole, and phenazopyridine hydrochloride were used. For each sulfonamide to be assayed, prepare a standard solution of 1 mg./ml. in 1% ammonia in methanol; pipet exactly 1.0 ml. of this solution into a 100-ml. volumetric flask and dilute to volume with 0.12 *N* HCl. Prepare a phenazopyridine hydrochloride standard solution containing 5 mcg./ml. in 1% ammonia in ethanol.

Sample Preparations—*Tablets*—Weigh a portion of finely ground sample containing about 100 mg. of each sulfonamide and transfer with methanol to a 100-ml. volumetric flask containing 1 ml. of ammonium hydroxide. Shake occasionally during 15 min., dilute to volume with methanol, and mix.

Suspensions—With a "to contain" pipet, transfer a quantity of sample containing about 100 mg. of each sulfonamide to a 100-ml. volumetric flask containing 1 ml. of ammonium hydroxide. Add about 60 ml. of methanol and shake occasionally during 15 min., dilute to volume with methanol, and mix.

Method I: Sulfamethazine, Sulfamerazine, and Sulfadiazine—Mix 5.0 g. of diatomaceous earth[2] with 3.0 ml. of Reagent B, transfer to a chromatographic column[3] in two equal portions, and tamp after each portion. Pipet 3.00 ml. of the sample solution into a 100-ml. beaker and evaporate to dryness on a steam bath, using a gentle current of air. Pipet 1.0 ml. of Reagent A into the beaker and dissolve the residue. Add 2.0 g. of diatomaceous earth, mix until uniform, quantitatively transfer to the column, and tamp. Wipe the beaker, tamping rod, and spatula with a small piece of glass wool, add it to the column, and tamp.

[1] Cat. No. 10651, Eastman Kodak Co., Rochester, NY 14650
[2] Acid-washed Celite 545, Johns-Manville Corp., New York, N. Y.
[3] Cat. No. 420300, 250 × 22-mm. i.d., Kontes Glass Co., Vineland, N. J.

Table I—Effect of Varying Sodium Hydroxide Concentration on Column Chromatographic Elution of Sulfamethazine by Various Solvents[a]

Solvent	Percent Eluted with 100 ml. of Solvent				
	1 *N* NaOH	0.5 *N* NaOH	0.25 *N* NaOH	0.1 *N* NaOH	0.05 *N* NaOH
Ether	5	5	<1	<1	<1
10% Chloroform in hexane	—[b]	—	—	—	—
30% Chloroform in hexane	30	30	10	5	2
50% Chloroform in hexane	100	100	95	80	80
Chloroform	100	100	95	90	85
Methylene chloride	100	100	100	100	90

[a] One milligram of sulfamethazine was mixed with 2 g. of diatomaceous earth and 1 ml. of 4% tetrabutylammonium hydroxide in the respective sodium hydroxide solutions and packed in the column. [b] Dashes indicate no detectable drug in 100 ml. of eluate.

Table II—Effect of Varying Sodium Hydroxide Concentration on Column Chromatographic Elution of Sulfadiazine by Various Solvents[a]

Solvent	Percent Eluted with 100 ml. of Solvent				
	1 *N* NaOH	0.5 *N* NaOH	0.25 *N* NaOH	0.1 *N* NaOH	0.05 *N* NaOH
Ether	—[b]	—	—	—	—
10% Chloroform in hexane	—	—	—	—	—
30% Chloroform in hexane	—	—	—	—	—
50% Chloroform in hexane	85	65	35	30	25
Chloroform	100	100	100	100	100
Methylene chloride	100	100	100	100	100

[a] One milligram of sulfadiazine was mixed with 2 g. of diatomaceous earth and 1 ml. of 4% tetrabutylammonium hydroxide in the respective sodium hydroxide solutions and packed in the column. [b] Dashes indicate no detectable drug in 100 ml. of eluate.

Wash the column with 50 ml. of 50% chloroform in hexane and discard the eluate. Place a 400-ml. beaker under the column and elute with an additional 250 ml. of 50% chloroform in hexane. This fraction contains sulfamethazine. Replace the receiver with a 600-ml. beaker and elute the column with 400 ml. of 50% chloroform in hexane. This fraction contains sulfamerazine.

Place a 250-ml. beaker under the column and elute with 100 ml. of methylene chloride. This fraction contains sulfadiazine.

Evaporate each fraction on a steam bath with an air stream. Dissolve the residues in 2 ml. of methanol, add 40 ml. of 0.12 *N* HCl, mix, transfer to separate 100-ml. volumetric flasks, and dilute to volume with 0.12 *N* HCl. Pipet a 15-ml. aliquot of each solution into separate 50-ml. volumetric flasks and dilute to volume with 0.12 *N* HCl. Record the UV spectrum from 400 to 220 nm., using 0.12 *N* HCl as the reference. Draw the baseline as a continuation of the curve between 400 and 360 nm., determine the corrected absorbance at the wavelength of maximum absorbance at about 242 nm., and calculate by comparison to standards.

Method II: Sulfadiazine, Sulfamethizole, and Phenazopyridine Hydrochloride—Mix 2.0 g. of diatomaceous earth with 1.0 ml. of Reagent B, transfer to the chromatographic column, and tamp. Pipet 3.00 ml. of sample solution into a 100-ml. beaker and evaporate to dryness on a steam bath, using a gentle current of air. Pipet 1.0 ml. of Reagent B into the beaker. Add 2.0 g. of diatomaceous earth, mix until uniform, quantitatively transfer to the column, and tamp. Wipe the beaker, tamping rod, and spatula with a small piece of glass wool, add it to the column, and tamp.

Place a 250-ml. beaker under the column. Wash the 100-ml. beaker with three 5-ml. portions of ether, adding each portion to the column. Elute the column with three 20-ml. portions of ether, allowing each to pass into the column before adding the next portion. Rinse the column tip with methanol. This fraction contains phenazopyridine. Replace the receiver with a second 250-ml. beaker and elute the column with 150 ml. of 50% chloroform in hexane. Rinse the column tip with methanol. This fraction contains sulfamethizole. Replace the receiver with a third 250-ml. beaker and elute the column with 100 ml. of methylene chloride. Rinse the tip with methanol. This fraction contains sulfadiazine.

For phenazopyridine hydrochloride, evaporate the first eluate on the steam bath with an air stream. Dissolve the residue in 40 ml. of ethanol, transfer the solution quantitatively to a 100-ml. volumetric flask containing 1 ml. of ammonium hydroxide, and dilute to volume with ethanol. Pipet a 15-ml. aliquot into a 50-ml. volumetric flask and dilute to volume with ethanol. Read the absorbance at 420 nm., using alcohol as the reference, and calculate by comparison to a standard.

For sulfamethizole and sulfadiazine, evaporate the respective eluates on a steam bath with an air stream. Dissolve the residues in 2 ml. of methanol, add 40 ml. of 0.12 *N* HCl, mix, transfer to separate 100-ml. volumetric flasks, and dilute to volume with 0.12 *N* HCl. Pipet a 15-ml. aliquot of each solution into separate 50-ml. volumetric flasks and dilute to volume with 0.12 *N* HCl. Record the UV spectrum from 400 to 220 nm., using 0.12 *N* HCl as the reference. Draw a baseline as a continuation of the curve between 400 and 360 nm. for sulfadiazine and between 360 and 340 nm. for sulfamethizole. Determine the corrected absorbance at the wavelength of maximum absorbance, about 242 nm. for sulfadiazine and about 268 nm. for sulfamethizole, and calculate by comparison to standards.

DISCUSSION

The extraction of the ion-pairs formed by tetrabutylammonium ions and sulfonamides from partition columns is controlled by the nature of the organic mobile solvent. Solvents with acidic protons, such as chloroform and methylene chloride, interact with the ion-pair to increase the extracting power of the organic phase. The effect of the concentration of such compounds on the partitioning of ion-pairs was studied by Higuchi and coworkers (13–15). They postulated a solvent cage of approximately two to five molecules of chloroform around the extracted ion-pair.

In the present study, the sulfonamides were readily extracted by chloroform and methylene chloride as ion-pairs with tetrabutylammonium ions from a basic partition column. The elution and separation of the ion-pairs from the partition column are influenced by the concentration of sodium hydroxide and tetrabutylammonium hydroxide in the column and the concentration of the chloroform or methylene chloride in the eluting solvent.

As the tetrabutylammonium hydroxide concentration is increased, the sulfonamides elute more rapidly. A tetrabutylammonium hydroxide concentration of about 4% was found to give the best elution times and separations for the sulfonamides studied.

Tables I and II illustrate the effect of the sodium hydroxide concentration and the solvent composition on the elution rate for the

Table III—Analysis of Sulfadiazine, Sulfamethazine, and Sulfamerazine in Commercial and Synthetic Drug Preparations by Method I

Sample	Percent of Declared			Total Sulfonamides (Bratton-Marshall), %
	Sulfamethazine	Sulfamerazine	Sulfadiazine	
Tablets 1	98.6	97.5	96.8	98.1
Tablets 2	103.0	101.3	101.0	99.6
Tablets 3	102.7	99.3	101.3	101.2
Tablets 4	93.8	100.7	103.5	98.3
Suspension 5	104.0	102.5	110.4	104.4
Suspension 6	101.7	101.8	100.4	100.9
Suspension 7	—[a]	92.8	97.4	95.1
Synthetic 1	100.1	100.0	101.0	
Synthetic 2	98.8	101.0	99.4	
Synthetic 3	99.1	98.8	101.3	

[a] Dashes indicate that these components are not present in these mixtures.

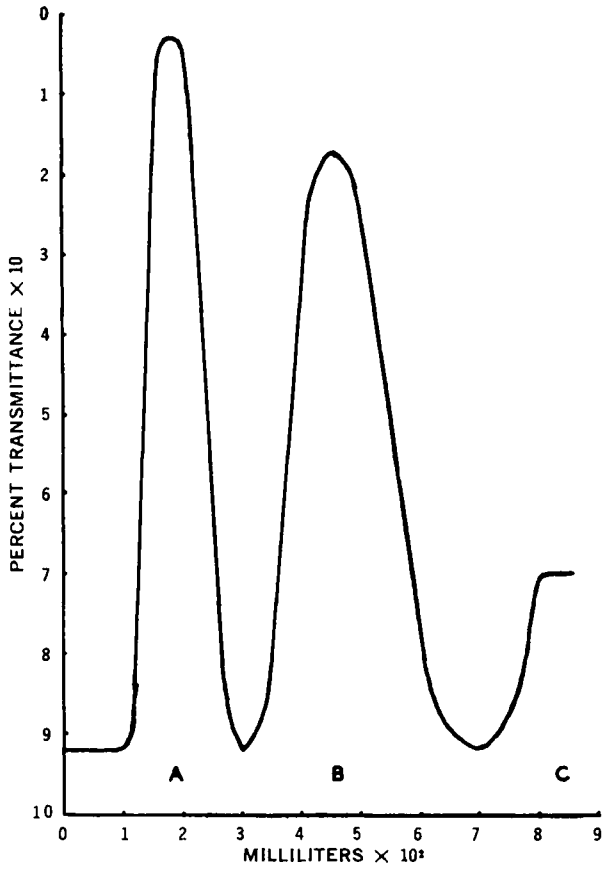

Figure 1—*Separation of sulfamethazine (A), sulfamerazine (B), and sulfadiazine (C) obtained by eluting the partition column with 50% chloroform–hexane.*

sulfamethazine and sulfadiazine ion-pairs. The increase in elution rate for the sodium hydroxide concentration range (0.05–0.2 *N*) is slight. However, the sodium hydroxide decreases the tailing of the eluting ion-pairs, resulting in a more complete separation.

In Method I, the sample is placed in a 10% tetrabutylammonium hydroxide layer. This enables the sulfonamides to reach the 0.1 *N* NaOH–tetrabutylammonium hydroxide separation layer in a narrower band and improves the separation. Figure 1 demonstrates the separation of the sulfamethazine, sulfamerazine, and sulfadiazine obtained by Method I. Also, methylene chloride is preferred to chloroform for the elution of sulfadiazine since there is less interference in the UV region.

RESULTS

Six commercial preparations of the trisulfapyrimidines and one sample containing sulfamerazine and sulfadiazine were assayed by Method I. Three commercial preparations of sulfamethizole, sulfadiazine, and phenazopyridine hydrochloride were assayed by Method II. Synthetic samples of these products were also analyzed. The results are reported in Tables III and IV. In addition, total sulfonamide content in the same commercial preparations was determined by the Bratton–Marshall procedure (7), with the results presented in Tables III and IV.

Table IV—Analysis of Sulfamethizole, Sulfadiazine, and Phenazopyridine Hydrochloride in Commercial and Synthetic Drug Preparations by Method II

Sample	Percent of Declared: Sulfamethizole	Sulfadiazine	Phenazopyridine Hydrochloride	Total Sulfonamides (Bratton–Marshall), %
Tablets 1	103.0	104.2	100.0	102.1
Tablets 2	99.6	99.9	96.8	98.4
Tablets 3	103.0	103.8	100.2	102.6
Synthetic 1	101.1	100.6	99.8	
Synthetic 2	98.7	100.8	98.8	

SUMMARY

This investigation of the separation of four sulfonamides by ion-pair partition chromatography showed that the proposed procedures were rapid and gave good recoveries (99–101%). The analytical technique might well be extrapolated to include related acidic compounds.

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Examination of Quinidine and Quinine and Their Pharmaceutical Preparations

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Abstract □ Methods for the detection and measurement of possible contaminants in quinidine and quinine and their pharmaceutical preparations were investigated to establish the actual composition of quinidine and quinine now on the market. Since no single TLC or GC procedure separated all of the compounds under consideration, multiple systems encompassing them were used to characterize the samples. Chromatographic and fluorometric techniques were applied to analysis of a wide variety of samples of quinidine and quinine and their pharmaceutical formulations. The dihydro analogs were found in all of the 75 samples examined, and the desmethoxy analogs (cinchonine or cinchonidine) were found in about half of the samples. The level of the dihydro alkaloids was higher in quinidine than in quinine (usually 5–9% in quinidine and 3–6% in quinine); the level of the desmethoxy analog was higher in quinine than in quinidine (usually 0–0.5% in quinidine compared to 1–2% in quinine). No epi-alkaloid, quininone, or quinotoxine was detected in any sample.

Keyphrases □ Quinidine, salts, and commercial formulations—composition, identification of dihydro and desmethoxy analogs as contaminants □ Quinine, salts, and commercial formulations—composition, identification of dihydro and desmethoxy analogs as contaminants □ Cinchona alkaloids—analysis of quinidine and quinine

Palmer *et al.* (1), in a recent study of the metabolites of quinidine, reported that congeneric alkaloids were present at levels of 10–30% in the quinidine samples used. They attributed the presence of these compounds to the current method of preparing quinidine (*via* base-catalyzed epimerization of quinine), which yields a complex mixture of epimers. Because of the lower therapeutic index of some epimers, a high epimer content might be undesirable. They recognized the possible presence of dihydroquinidine, which may be present in significant amounts in commercial quinidine (1, 2).

As a consequence, a study to establish the actual status of quinidine now on the market was initiated in these laboratories. Based on its source, the compounds most likely to be associated with quinidine are quinine, cinchonine, cinchonidine, epiquinidine, epiquinine, quininone, and quinotoxine, together with their dihydro analogs.

Several workers used paper chromatography for the qualitative analysis of the various cinchona alkaloids (3–5) and for study of the decomposition of quinidine solutions (6, 7). Palmer *et al.* (1) used GC but examined only the metabolites; many other workers using GC for cinchona alkaloid detection limited their studies to the four major vinyl alkaloids: quinine, quinidine, cinchonidine, and cinchonine. TLC was used first by many workers to separate these four vinyl bases and, more recently, to separate them from their dihydro analogs.

The USP XVIII monographs for quinidine and quinine salts (8) include a TLC test for "other cinchona alkaloids." This system differentiates quinine from quinidine and detects epiquinidine and epiquinine, but it does not separate dihydroquinidine from quinidine. Recently, Bohme and Bitsch (9, 10) applied two-dimensional TLC to separate quinine, quinidine, dihydroquinine, dihydroquinidine, cinchonine, and cinchonidine, but they did not investigate the presence of the epibases, quininone, and quinotoxine. They determined the quinine content of cinchona bark by measuring the UV absorbance of the material recovered from a TLC plate after a double development of the chromatogram.

Storck *et al.* (11) also separated the main alkaloids of cinchona bark by TLC and determined the quinine and cinchonidine content spectrophotometrically. Suszko-Purzycka and Trzebny (12) made a semiquantitative determination of the dihydro content of the individual alkaloids by visual comparison of the spots. Hartel and Korhonen (13) and Hartel and Harjanne (14) separated quinidine, dihydroquinidine, and quinidine metabolites isolated from biological fluids. They removed the separated zones from the TLC plate, extracted the alkaloid from each of them, and determined the fluorescence of the extracts. They reported that the quinidine preparations used in their study contained 12–13% dihydroquinidine. Gutzwiller and Uskokovic (15) used preparative TLC to isolate quinine and quinidine epibases. Vacha *et al.* (16) described TLC systems that separated epiquinidine or epiquinine from their respective analogs but did not separate the dihydro alkaloids from the parent alkaloids.

Recently, Roder *et al.* (17) reported the determination of the quinine, quinidine, cinchonidine, and cinchonine content of the extract of cinchona bark by densitometry of the fluorescent compounds on thin-layer chromatograms. They treated the developed chromatogram with a mixture of ether and concentrated sulfuric acid to activate cinchonidine and cinchonine. Although the four alkaloids were not completely resolved, the two pairs of alkaloids could be quantitated by the use of selective activation and emission wavelengths.

The scope of the present study included quinine in order to obtain a complete picture of the quality of the cinchona alkaloids now marketed as pharmaceuticals. Since no single TLC or GLC system would separate all of the compounds under consideration, multiple systems that encompassed all of them were used to characterize the samples.

EXPERIMENTAL

Materials—Cinchonine, cinchonidine, quinine, and quinidine free of their respective dihydro analogs were prepared from the commercial products *via* their mercury addition compound, essentially by the method of Thron and Dirscherl (18). Typically, a solution of 5 g. of quinidine sulfate in 50 ml. of 10% sulfuric acid is added to a solution of 7.5 g. of mercuric acetate in 75 ml. of 5%

Table I—Purified Cinchona Alkaloids

Compound	Solvent for Recrystallization	Melting Point, Observed[a]	Melting Point, Reported	Reference
Cinchonine	Alcohol	258–260°	264°	20, 21
Cinchonidine	Alcohol	216°	205,210°	20, 22
Dihydrocinchonine	Alcohol	280°	270.5–273°	23
Dihydrocinchonidine	Alcohol–water	232–234°	232°	20
Dihydroquinine	Benzene–hexane	173°	173°	20
Dihydroquinidine	Alcohol	174°	169°	20
Quinine	Benzene–hexane	176°	177°	20
Quinidine	Alcohol	176°	173°	20
Quininone	Ether	109°	107–108.5°	19

[a] Melting points were determined with a DuPont model 900 differential thermal analyzer.

Table II—hR_f Values of Cinchona Alkaloids

Alkaloid	SG Layer[a], Solvent 1[b]	SH Layer, Solvent 2	SH Layer, Solvent 3
Quininone	76	74	64
Epiquinidine	68	43	32
Epiquinine	64	41	31
Cinchonine	54	38	43
Dihydrocinchonine	48	23	30
Quinidine	45	46	52
Quinotoxine (quinicine)	45	15	12
Cinchonidine	40	40	48
Dihydroquinidine	39	34	39
Dihydrocinchonidine	37	27	35
Quinine	24	42	50
Dihydroquinine	23	32	39
Quinidine thioglycerol adduct	0	11	38

[a] SG = silica gel G or GF, 0.25 mm. SH = silica gel H, 0.25 mm. [b] Solvent 1 (8) = chloroform–acetone–diethylamine (50:40:10). Solvent 2 = chloroform–acetone–methanol–ammonium hydroxide (60:20:20:1). Solvent 3 = methanol–ammonium hydroxide (100:1).

acetic acid. The solution is heated 4 hr. at 40–50°, cooled to room temperature, and made basic with ammonium hydroxide. The dihydroquinidine is extracted with ether. The aqueous layer is acidified with dilute sulfuric acid, and the mercury addition compound is reduced by adding sodium sulfite portionwise until no more mercury is precipitated. After mercury is removed by repeated filtration, the filtrate is made basic with ammonium hydroxide and the free base is extracted with ether. The ether is evaporated under vacuum, and the quinidine is recrystallized from alcohol. The other vinyl alkaloids were prepared in an analogous manner and recrystallized with the solvent noted in Table I.

The dihydro alkaloids were prepared by catalytic hydrogenation of the corresponding vinyl alkaloid over platinum oxide in ethanol. The isolated alkaloid was then recrystallized with the solvent noted in Table I. Quininone was prepared by oxidizing quinine with benzophenone in the presence of potassium *tert*-butoxide by the procedure of Woodward *et al.* (19). It was recrystallized twice from ether.

All of the isolated recrystallized alkaloids were dried under vacuum in a drying pistol at the temperature of refluxing xylene. The solvent of crystallization is removed in this process.

Sample Preparation—*Capsules, Tablets, and Powdered Quinidine or Quinine Salts*—Dissolve a portion of the sample in enough 50% alcohol to provide a solution equivalent to about 5 mg. of alkaloid base/ml. (The dissolved excipients do not affect the subsequent procedures.) Filter if necessary.

Quinidine or Quinine Base—Prepare a solution of about 5 mg./ml. in alcohol.

Injections—Transfer an aliquot of the injection containing about 80 mg. of the alkaloid salt to a separator containing 25 ml. of water, acidify with a few drops of diluted sulfuric acid, and extract with 50 ml. of ether. Discard the ether. Make the aqueous layer alkaline with ammonia and extract with 50 ml. of ether. Wash the ether layer with 25 ml. of distilled water, transfer the ether solution to a conical flask, and evaporate just to dryness under a stream of air. Dissolve the residue in enough alcohol to make a solution of about 5 mg. of alkaloid base/ml.

Assay Preparation—Dilute an aliquot of the sample preparation with enough alcohol to make a final dilution of 0.5 mg. of alkaloid base/ml.

Preparation of Thin-Layer Plates—Spread a slurry of 30 g. of silica gel H and 70 ml. of water over five 20 × 20-cm. plates with a suitable spreader[1] to obtain a 0.25-mm. layer. Air dry the plates and store in a dust-free atmosphere. Prepare silica gel G or GF plates (0.25 mm. thick) in an analogous manner, using a slurry of 30 g. of substrate and 60 ml. of water. (Commercial silica gel G or GF plates are also suitable.)

TLC System 1 (8)—This system separates quinine, quinidine, epiquinidine, epiquinine, cinchonidine, cinchonine, and quininone. It does not separate the dihydro alkaloids from the parent alkaloids. The plates are 0.25-mm. silica gel G or GF, and the developing solvent is chloroform–acetone–diethylamine (50:40:10).

TLC System 2—This system gives the best separation of the dihydro alkaloids from their respective vinyl alkaloids. It does not separate the epi-alkaloids, nor quinine from quinidine. The plates are 0.25-mm. silica gel H, and the developing solvent is chloroform–acetone–methanol–ammonium hydroxide (60:20:20:1).

TLC System 3—This system separates the epi-alkaloids and the dihydro alkaloids from the parent alkaloids, but it does not separate quinine from quinidine. The plates are 0.25-mm. silica gel H, and the developing solvent is methanol–ammonium hydroxide (100:1).

Qualitative TLC Identification—(Use TLC Systems 1 and 3.) At a point about 2 cm. from the bottom edge of the plate, spot 2 μl. of the sample preparation and each of the standard solutions of the respective alkaloids. Add the developing solvent to a trough in the bottom of a tank, one side of which is lined with a sheet of filter paper (Whatman No. 1 or equivalent). Introduce the plate immediately after adding the developing solvent to prevent presaturation of the tank. Let the solvent migrate 10–14 cm. from the point of spotting, and air dry the developed plate. Observe the plate under long wavelength UV light to locate the quinine series. To visualize the desmethoxy series, spray the plate with either 50% sulfuric acid, which converts them into fluorescent compounds, or iodoplatinic acid, which produces colored spots with all of the alkaloids. (Because of the diethylamine used in TLC System 1, these plates must be sprayed with acid prior to visualization.)

Quantitative TLC Separation—At a point 2 cm. from the bottom of the edge of the silica gel H plate, spot three 2-μl. aliquots of each assay preparation. If the qualitative procedure shows the presence of epi-alkaloids, use TLC System 3; otherwise, use TLC System 2. Develop as described under *Qualitative TLC Identification.*

Direct Scanning Fluorescence Measurement—The following conditions were found to be optimal for the apparatus used[2]: excitation wavelength, 345 nm.; and emission barrier filter, 430 nm. Set the source slit so the maximum intensity of the observed fluorescence is obtained and the entire width of the spot is scanned. (These conditions were provided by an 8-mm. slit in the instrument.) Set the variable slit to give a maximum of 90% of the 5-mv. range of the recorder for the most intense spot when the following settings of the amplifier[3] are used: right-hand step switch on 10, sensitivity key on 1, high tension switch on 1, amplification selector switch on F, and precision setting control knob on 5.

Align the TLC plate so that the centers of the spots pass under the center of the scanning slit. Scan the plate at 50 mm./min. with a chart rate of 200 mm./min. Use baseline corrected disk integrations[4] or electronic integration to calculate the ratio of the al-

[1] Desaga standard adjustable applicator (Brinkmann Instruments Inc., Westbury, NY 11590) was used for the preparation of TLC plates.

[2] Zeiss chromatogram spectrophotometer, Carl Zeiss Inc., New York, NY 10018

[3] PMQ II, Carl Zeiss, Inc.

[4] Simpson model 2741 recorder with disk integrator, Simpson Electric Co., Chicago, IL 60644

Table III—Relative Retention Times of Trimethylsilyl Derivatives of Cinchona Alkaloids

Alkaloid	Relative Retention Time[a]
Cinchonidine	0.55
Cinchonine	0.55
Dihydrocinchonidine	0.50
Dihydrocinchonine	0.50
Epiquinidine	0.87
Epiquinine	0.95
Dihydroquinidine	0.93
Dihydroquinine	0.93
Quinidine	1.00
Quinine	1.00
Quininone	0.83
Quinotoxine (quinicine)	1.52
Thioglycerol adduct of quinidine	13.4

[a] The retention times are relative to the appearance of quinidine, with the use of a glass column 6.1 m. × 3 mm. (20 ft. × 0.125 in.) packed with 3% OV-225 on Gas Chrom Q. (Quinidine elutes at approximately 21 min. with the column at 225°.)

kaloids. The following settings on the electronic integrator[5] provided optimal background correction with the silica gel H plates (settings should be checked on each instrument with standard mixtures of alkaloids): baseline tracking up 600, down 2000 μv./min.; input noise rejection, 5; minimum peak rate, 30 sec.; slope sensitivity, 2; count rate, 4 kc./mv.; minimum peak counts, 2000/sec.; and threshold level, 100 μv./min.

Solution Fluorescence Method—View the dried chromatogram under long wavelength UV light, and mark off areas of the alkaloids on the TLC plate. Scrape off the area of the separated alkaloids and a blank area of the plate corresponding in R_f to the alkaloid spot. Quantitatively transfer each scraped portion to a 15-ml. glass-stoppered centrifuge tube, add 10.0 ml. of 0.1 *N* H_2SO_4 (prepared from fluorescent grade sulfuric acid) to each tube, stopper, shake for 30 sec., and centrifuge at 20,000 r.p.m. for 30 sec. Transfer a portion of the supernatant liquid to a 1-cm. fluorescence cell, and measure the fluorescence directly with the excitation at 350 nm. and the emission at 455 nm.[6]. Calculate the ratio of the alkaloids in each sample from the fluorescence readings corrected for the contribution of the blank.

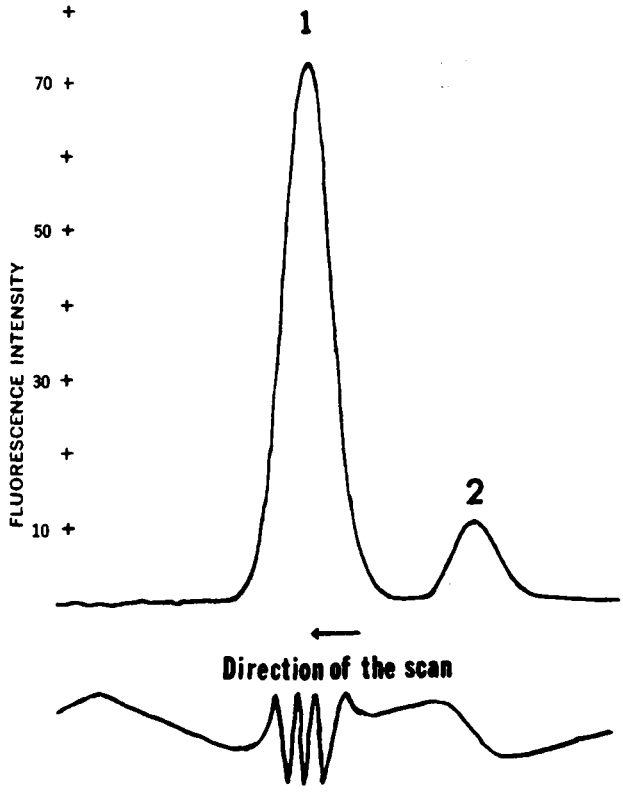

Figure 1—*TLC scan of a quinidine sample. Key: 1, quinidine; and 2, dihydroquinidine.*

[5] Infotronics model CRS-104 electronic integrator with digital readout, The Infotronic Corp., Houston, TX 77042

Table IV—Direct Scanning Fluorescence Method: Comparison of Methods of Integration of Repetitive Scans[a]

Aliquot	Electronic Integration				Disk Integration		
1	9.1	8.9	9.0	9.2	8.8	8.9	8.5
2	8.6	8.6	8.8	8.6	8.4	9.1	8.2
3	8.4	8.5	8.6	8.5	8.8	8.7	8.4

[a] All results are given in percent of dihydroquinidine.

GLC Procedure—The system described separates the vinyl alkaloids from their dihydro analogs. It does not resolve the quinidine–quinine, dihydroquinidine–dihydroquinine, cinchonidine–cinchonine, and dihydrocinchonidine–dihydrocinchonine pairs, but it does resolve these pairs from each other. The following conditions are used: column, 6.1-m. × 3-mm. (20-ft. × 0.125-in.) glass, packed with 3% OV-225 on Gas Chrom Q; injector temperature, 235°; column temperature, 225°; and carrier gas, nitrogen at 30 ml./min. A suitable gas chromatograph[7] with a flame-ionization detector and an electronic integrator[5] is used. Evaporate 0.2 ml.of the assay preparation, containing the equivalent of 0.1 mg. of alkaloid under nitrogen, in a 0.3-ml. conical vial[8] and then dry under vacuum for 30 min. Add 0.1 ml. of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide or bistrimethylsilyl-trifluoroacetamide, cover with a Teflon-lined septum secured with a screw cap, and heat at 60° for 45 min. In-

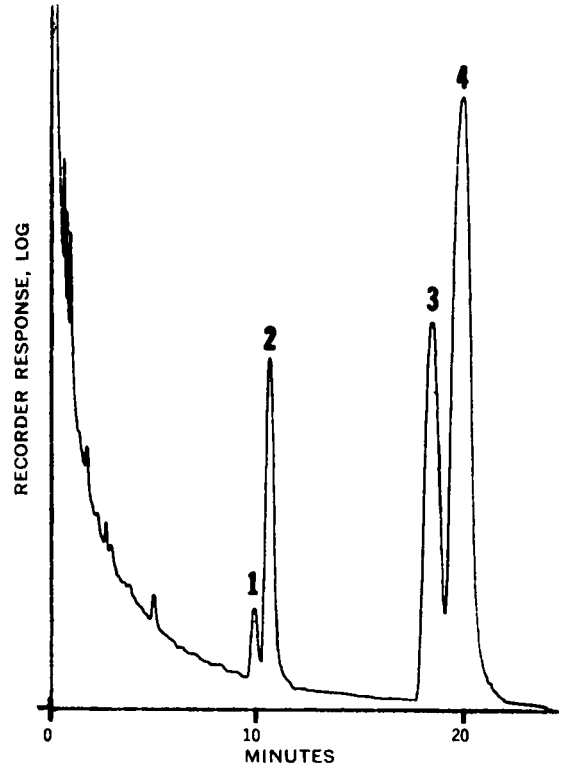

Figure 2—*GLC chromatogram of the trimethylsilyl derivatives of the alkaloids in a quinidine sulfate sample. Key: 1, dihydrocinchonidine; 2, cinchonine; 3, dihydroquinidine; and 4, quinidine.*

[6] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Silver Spring, MD 20901

[7] Varian Aerograph model 1200 gas chromatograph, Varian Aerograph, Walnut Creek, Calif.

[8] Kontes K-749000, Kontes Glass Co., Vineland, N. J.

Table V—Comparison of Methods of Determination of Dihydroquinidine in Quinidine[a]

Aliquot	Method of Determination: Electronic[b]	Disk[b]	Solution Fluorescence	GLC
1	6.8	6.5	6.3	6.3
2	7.1	6.4	6.0	6.4
3	7.1	6.5	—	6.4
4	7.0	6.5	6.9	—
5	6.8	6.7	6.5	—
Average	7.0	6.5	6.4	6.4

[a] All results are given in percent of dihydroquinidine. [b] Each value is an average of repetitive scans of the same spot.

ject 3 μl. of sample. Record the response and integrate the peak areas electronically. Calculate the ratio of the alkaloids in the samples from the integrated areas.

RESULTS AND DISCUSSION

Forty samples of quinidine and 35 samples of quinine in the form of the alkaloid, its salts, and pharmaceutical dosage forms were examined. The dihydro analogs were found in all samples, and the desmethoxy analogs (cinchonine or cinchonidine) were found in some samples. No epi-alkaloid, quininone, or quinotoxine was detected in any of the samples. The level of the dihydro alkaloids was higher in quinidine than in quinine; the level of the desmethoxy analogs was higher in quinine than in quinidine.

In preliminary work the alkaloids were extracted from the sample with methylene chloride. These solutions degraded rapidly to generate several additional TLC spots, one of which corresponded to quininone. The simple dissolution of the samples in alcohol gave stable sample preparations. No changes were observed in solutions standing in the dark for over a month.

Initial qualitative TLC is needed to verify the identity of the sample[9] and to select the quantitative TLC system to be used. TLC System 1 shows the presence of the epi-alkaloids. If they are present, TLC System 3, which resolves them from the natural bases, is used for quantitative measurement; if they are absent, TLC System 2, which gives a better resolution of the natural analogs, is preferred. The R_f values of the alkaloids are listed in Table II.

The GLC procedure permits the simultaneous quantitation of the desmethoxy alkaloids as well as the dihydro and vinyl alkaloids. The relative retention times of the trimethylsilyl derivatives of the alkaloids are listed in Table III. Since the GLC procedure does not separate the quinine series from the quinidine series, it is necessary to examine the sample by TLC, using TLC System 1 to verify the identity of the sample[9].

The most rapid and simple method for the examination of the chromatogram for the naturally fluorescent alkaloids uses the TLC scanner, preferably with a digital integrator for the quantitation. A typical scan is illustrated in Fig. 1. Since these instruments are probably not in widespread general use at this time, the method based on the fluorescence of the solution of the resolved alkaloids may be the most widely applicable procedure. The latter has greater specificity, since both the specific emission and the excitation wavelengths characteristic of these compounds can be set with the spectrophotofluorometer.

In scanning the thin-layer plates on the chromatogram spectrophotometer, occasional response was observed, indicating the presence of very minor spots not visible to the eye by their fluorescence nor after the chromatogram was sprayed with iodoplatinate reagent. These did not correspond to any of the alkaloids listed in Table II. If they were due to alkaloids having the same relative fluorescence as quinidine, they would amount to less than 0.2%.

Mixtures of known ratios of quinidine and dihydroquinidine were subjected to TLC by TLC Systems 2 and 3 and were quantitated by direct scanning and by solution fluorescence. They were also examined by the GLC procedure. Response was linear by all systems.

Table IV illustrates the results of repetitive scans of the same spot of a single sample by disk integration and electronic integration. Table V shows averages of scans of replicate chromatograms of individual solutions compared with results by the GLC procedure and the solution fluorescence of the same spot of the same chromatogram. With the disk integrator, graphic correction of the background is necessary. With the electronic integrator, accurate compensation of the background can be obtained by setting the baseline tracking characteristics with standards of known ratios of quinidine or quinine and its dihydro analog. In general, the closest agreement was found with the GLC and the solution fluorescent procedures, but on the average all methods of quantitation gave good agreement.

The amount of dihydroquinidine found in the various samples is summarized in Table VI. Dihydroquinidine was found in all samples, mostly in the 5–9% range with extremes of 3 and 22%. No epiquinidine, epiquinine, quinine, quininone, or quinotoxine was detected in any sample by the TLC systems that would detect them at 0.2%. The thioglycerol adduct of quinidine reported by Levine and Maienthal (24) was found in quinidine injections containing thioglycerol as a preservative.

Cinchonine and cinchonidine and their dihydro analogs may be detected by the qualitative TLC procedures; but because of their fluorescence properties, they do not interfere in the quantitative determination. Cinchonine was absent or was present at very low levels in the quinidine samples. It was quantitated by the GLC procedure. The results are shown in Table VI. An exception was one sample of quinidine sulfate powder which contained 7% cinchonine and 0.7% dihydrocinchonine. The GLC curve for this sample is illustrated in Fig. 2.

Table VI—Other Alkaloids in Quinidine Preparations

Sample	Dihydroquinidine, %	Cinchonine, %
Sulfate tablets	6.1	—
	6.5	0.9
	10.7	0.4
	7.6	1.3
	8.5	0.1
	6.7	0.2
	4.3	—
	6.7	0.1
	4.7	—
	6.9	0.1
	6.6	—
	6.8	—
	5.5	—
	6.5	—
	7.0	0.1
	11.8	0.5
Sulfate capsules	9.0	—
	21.7	—
	7.2	0.6
Gluconate injection	10.1	—
	4.3	—
	8.5	—
Sulfate injection	8.2	—
	10.6	—
Alkaloid powder	5.9	—
	6.1	—
	5.6	—
	7.3	0.5
Gluconate powder	6.1	—
	6.4	—
	5.5	0.6
Sulfate powder	3.0	—
	4.9	—
	5.6	—
	8.9	—
	16.5	7.1[a]
	14.2	—
	18.7	0.5
	22.1	—
	9.0	—

[a] This sample also contained 0.7% dihydrocinchonine.

[9] One sample labeled quinidine hydrochloride was found to be quinine hydrochloride, and one sample labeled quinidine was found to be cinchonidine.

Table VII—Other Alkaloids in Quinine Preparations

Sample	Dihydroquinine, %	Cinchonidine, %	Dihydrocinchonidine, %
Sulfate capsules	4.1	1.5	—
	4.8	0.9	0.2
	5.1	1.0	—
	5.2	0.7	—
	6.3	0.4	—
	5.1	—	—
	3.2	0.8	—
	8.4	0.3	0.7
	4.2	1.1	1.5
	4.1	1.1	0.6
	2.7	0.3	0.5
	5.6	1.0	0.8
	6.2	0.1	1.1
	7.9	0.3	0.1
	7.6	0.6	0.1
	3.5	1.2	0.1
	0.2	—	—
	3.2	1.2	—
	7.6	1.3	—
	4.5	—	—
	4.0	0.1	0.1
	1.9	—	—
	4.3	—	—
	8.5	—	—
Sulfate tablets	5.1	1.1	—
	4.1	1.7	0.1
Alkaloid powder	8.7	1.6	0.1
	8.1	0.6	—
Hydrobromide powder	3.3	0.7	0.1
	1.5	—	—
Dihydrochloride powder	3.4	1.6	—
Phosphate powder	6.7	0.7	—
Sulfate powder	5.1	0.1	1.3
	6.6	1.9	—
	4.8	1.9	0.1

The examination of quinine samples showed dihydroquinine contents ranging from 0.2 to 9%, with the majority of the samples containing 3–6% (Table VII). The cinchonidine content ranged from none to 2%, with most of the samples containing about 1%. The level of cinchonidine found in quinine was much higher than that of cinchonine in quinidine.

The current USP monographs have no limit tests for the dihydro analog in the natural alkaloid. In the light of the findings, it has been recommended to the USP that the standards for quinidine and quinine and their salts should be redefined to reflect their actual composition.

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▲ To whom inquiries should be directed.

Liquid and Solid Solution Interactions of Primary Certified Colorants with Pharmaceutical Gelatins

JAMES W. COOPER, Jr.▲, HOWARD C. ANSEL, and DONALD E. CADWALLADER

Abstract □ The liquid and solid solution interactions of eight FD&C dyes in 1% concentration with pharmaceutical gelatins and the effects of the dyes on the disintegration behavior of gelatins were observed. In liquid solution by visible region spectroscopy, all FD&C dyes interacted with a type A gelatin. The xanthene dye, FD&C Red No. 3 (erythrosine), interacted to the greatest extent by a combination of ionic, hydrogen, and hydrophobic types of bonds. The FD&C dyes did not appear to interact with a type B gelatin. A method for simulating a gelatin capsule shell wall was devised to examine solid-state interactions by visible and IR-attenuated total reflectance spectroscopy, dye release patterns, and the effect of the dyes on gelatin disintegration. FD&C Red No. 3 and Violet No. 1 in solid solution appeared to interact to a greater extent than the other FD&C dyes. Hydrogen bonding changes in the solid solution films of both gelatins were not observable by IR-attenuated total reflectance spectroscopy until very high (1:10 to 1:5) dye-protein ratios were attained. The same two dyes (FD&C Red No. 3 and Violet No. 1) that showed the greatest spectral changes exhibited the slowest release times from both gelatins at temperatures both below and within the temperature range at which gelatins disintegrate and go into colloidal solution. In simulated gastric fluid USP without pepsin, the FD&C Red No. 3 dye greatly diminished the average disintegration rate of both gelatins. The addition of pepsin essentially eliminated the effect of FD&C Red No. 3 on gelatin disintegration. In simulated intestinal fluid USP without pancreatin, both gelatins disintegrated slower than in simulated gastric fluid without pepsin. FD&C Red No. 3 also had a detrimental effect on gelatin disintegration in simulated intestinal fluid without pancreatin. Pancreatin was not added to the simulated intestinal fluid because of its interference with visualization of disintegration.

Keyphrases □ FD&C dyes—liquid and solid solution interactions with gelatins, effect on gelatin disintegration behavior, visible and IR-attenuated total reflectance spectroscopy □ Gelatins, pharmaceutical—effect of eight FD&C dyes on disintegration behavior, liquid and solid solution interactions with dyes, visible and IR-attenuated total reflectance spectroscopy □ Dye-protein interactions—effect on gelatin disintegration behavior □ Protein-dye interactions—effect on gelatin disintegration behavior □ Colorants, eight FD&C dyes—liquid and solid solution interactions with gelatins, effect on gelatin disintegration behavior, visible and IR-attenuated total reflectance spectroscopy □ Interactions—FD&C dyes-pharmaceutical gelatins

Gelatins used for the encapsulation of medication may be colored by the single or combined use of primary certified colorants. Several primary certified colorants were shown (1) to interact with pharmaceutical adjuvants with metallic or polyfunctional character in a chelation and/or chemisorption type of mechanism. Dyes were shown to inhibit the dissolution of sulfathiazole, sulfaguanidine, diethylstilbestrol, phenobarbital, riboflavin, and thymol (2–5) by a hypothesized preferential adsorption of dye at the primary dissolution faces in the various crystals (2–4). In addition, dyes were shown to affect the disintegration and solubility of cellulose polymers by mechanisms that vary with each dye–polymer combination (5).

While the capsule dosage form has been assumed to release its contents quickly upon administration, an article appeared that may question the reliability of capsule dosage forms (6). It stated that the use of dyes and/or opaquants in the capsule shell have no effect on disintegration rate. However, the article also stated that red-opaque and black capsules require 100–200 sec. longer to disintegrate than colorless or other colored or colored-opaque capsules which disintegrated or released their contents within 60–100 sec. All capsules were filled with sodium bicarbonate. An examination of the effects of colorants on the disintegration of gelatin was deemed necessary as the logical beginning toward a better understanding of the effects of intrinsic variables on gelatin disintegration.

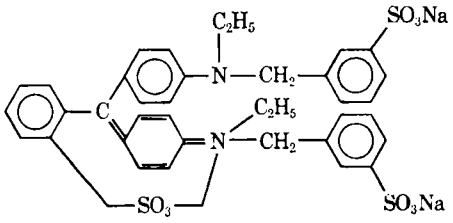
The purposes of this investigation were to define the mechanisms of dye–protein binding in the liquid and solid solution states and to relate the dye–protein interaction to its effect on the disintegration of gelatin. The objectives of this study were:

1. To prepare a solid solution film system of dye and gelatin as a simplest simulation of a capsule shell wall.
2. To examine, by spectroscopic means, interactions of dyes with both cationic and anionic gelatins in both liquid and solid solutions.
3. To observe differences in binding of dyes to the two differently charged proteins by diffusion from the protein gel at temperatures within and below the point at which gelatin "melts" and disintegrates.
4. To compare the disintegration behavior of colored with uncolored gelatins in simulated gastric and intestinal fluid environments.

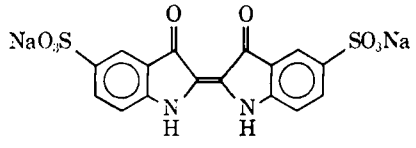
EXPERIMENTAL

Materials—The FD&C dyes (I–VIII) used in this study are listed in Table I[1], along with their synonym, color index number, and chemical class. All dyes were used as obtained. The pharmaceutical gelatins[2] utilized are presented in Table II. Gelatins were purified by the dialysis of a heterogeneous mixture of 5% gelatin and distilled water previously chilled to 5°. The gelatin served as its own dialyzing membrane. This mixture was allowed to stand overnight at 5° and was then filtered through a 20-mesh screen. The dialysis process

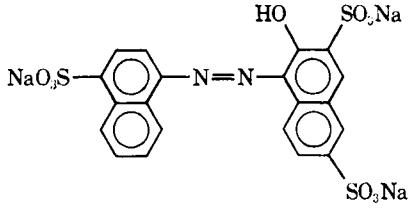
[1] Supplied by Specialty Chemicals Division, Allied Chemical Corp., Charlotte, N. C.
[2] Supplied by P. Leiner & Sons, America, Inc., St. Claire Shores, Mich.



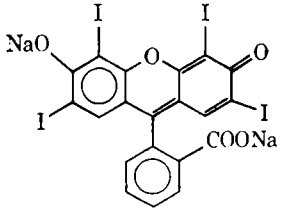
I: FD&C Blue No. 1



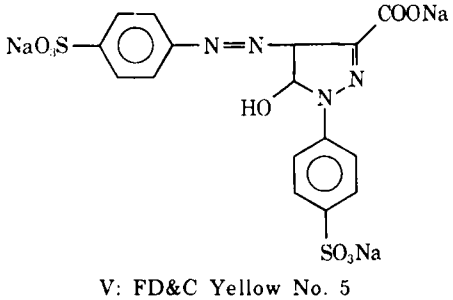
II: FD&C Blue No. 2



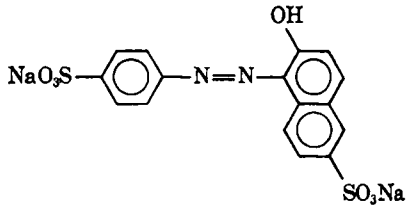
III: FD&C Red No. 2



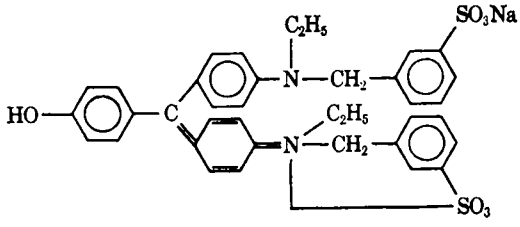
IV: FD&C Red No. 3



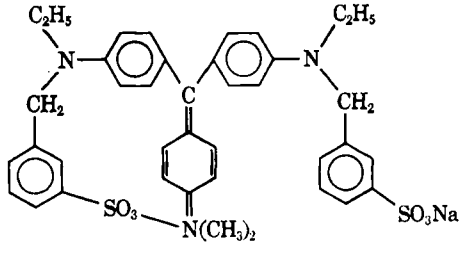
V: FD&C Yellow No. 5



VI: FD&C Yellow No. 6



VII: FD&C Green No. 3



VIII: FD&C Violet No. 1

was repeated two more times. This method appeared in the literature with little attention to details (7–9) and reportedly removes inorganic ions and polypeptide fragments with a molecular weight range from 3000 to 20,000 (9). Following the third removal of supernate, the swollen gelatin particles were again combined with distilled water and the temperature was increased to 37° to dissolve the hydrated gelatin.

Stock Solution Preparation—A freshly prepared 5% gelatin stock solution was maintained at 37 ± 0.5° in a constant-temperature bath[3], and samples were pipeted from the immersed flask. Stock solutions of the various dyes were prepared to contain 0.01, 0.1, 1, and 10 mg./ml. A 5 *M* stock solution of sodium chloride was prepared by diluting 29.22 g. of sodium chloride to 100 ml. with distilled water. A 5 *M* urea solution was prepared by diluting 30.0 g. of urea to 100 ml. Simulated gastric fluid USP was prepared by diluting 2.0 g. sodium chloride, 3.2 g. pepsin, and 7.0 ml. hydrochloric acid to 1 l. with water. Simulated intestinal fluid USP without pancreatin was prepared by dissolving 6.8 g. of monobasic potassium phosphate in 250 ml. water and adding 190 ml. 0.2 *N* sodium hydroxide and 400 ml. water. The resulting solution was adjusted to pH 7.5 ± 0.1 with 0.2 *N* sodium hydroxide and diluted to 1 l. with water.

[3] Model MW 1172SSA, Blue M Scientific Co.

Pancreatin could not be added due to its interference with the visualization of gelatin disintegration.

Liquid Solution Procedures—In a preliminary investigation, the effects of pH, ionic strength, and urea and dye concentration changes on the λ_{max} and absorbance of aqueous 0.001% dye solutions (0.01 mg./ml.) were observed at 25 ± 2°. The various pH solutions were prepared by diluting 0.1 mg. of each dye to 10 ml. with constant ionic strength (μ = 0.2) buffer solutions (10). The sodium chloride–dye solutions were prepared by adding sufficient sodium chloride solution to make the final solution 1.0 *M*. Similarly, the urea–dye solutions were made 1.0 *M* with respect to urea. Each dye solution was scanned through the UV–visible range from 190 to 750 nm., utilizing a distilled water blank.

For each liquid-state solution in which changes in dye λ_{max} spectra were to be observed, the dye–protein sample, in a 1:100 dye–protein weight ratio, was run *versus* a blank with a similar quantity of protein. Each experiment consisted of a sample containing 0.10 mg. dye and 10 mg. protein in 10 ml. distilled water *versus* a blank of 10 mg. protein in 10 ml. solvent. The sample was incubated for 30 min. at 25 ± 2° prior to the observation of the spectra in all liquid-state experiments (7). The effects of sodium chloride and urea were observed separately by the addition of sufficient respective stock solutions to make the sample and blank solutions 1.0 *M* in urea or 0.154 *M* (0.9%) in sodium chloride. All dye–protein *versus* protein spectra were observed in the visible range only and were reproducible within 1 nm.

In those liquid-state experiments in which new bands were sought, the dye–protein samples, in a 1:10 dye–protein weight ratio, were run *versus* a similar quantity of dye in the blank solution. In these cases, 5 mg. dye was combined with 50 mg. protein in 10 ml. distilled water and run *versus* a blank containing 5 mg. dye/10 ml. The dye–protein *versus* dye experiments were repeated using the same ratio of dye to protein but diluted by a factor of 10 to observe the effect of dilution on the new bands obtained. Samples consisted of 0.5 mg. dye and 5 mg. protein in 10 ml. distilled water *versus* a blank of 0.5 mg. dye in 10 ml. of solvent. Sodium chloride and urea effects were also noted by making the sample and blank solutions 0.154 and 1.0 *M*, respectively, in concentration of each reagent for each separate experiment. New bands were sought in the range of 190–750 nm.

Solid Solution Procedures—All solid-state solutions were prepared by thoroughly mixing an appropriate amount of 5% gelatin stock solution at 37° with dye and other reagents (sodium chloride

Table I—FD&C Dyes with Synonym, Color Index Number, and Chemical Class[a]

FD&C Dye	Synonym	Color Index Number	Chemical Class	Percent Dye[b]
Blue No. 1 (I)	Brilliant Blue FCF	42090	Triphenylmethane	91
Blue No. 2 (II)	Indigotine	73015	Indigoid	90
Red No. 2 (III)	Amaranth	16185	Monoazo	90
Red No. 3 (IV)	Erythrosine	45430	Xanthene	90
Yellow No. 5 (V)	Tartrazine	19140	Monoazo-pyrazolone	90
Yellow No. 6 (VI)	Sunset Yellow FCF	15985	Monoazo	89
Green No. 3 (VII)	Fast Green FCF	42053	Triphenylmethane	91
Violet No. 1 (VIII)	Wool Violet 5BN	42640	Triphenylmethane	91

[a] With the exception of FD&C Red No. 4, which is approved for use in maraschino cherries only. [b] Dyes may contain sodium chloride, sodium sulfate, leucoforms, intermediates, and subsidiary dyes.

Table II—Properties of Pharmaceutical Gelatins

Specification	Type A Gelatin	Type B Gelatin
Isoionic point	8.5	4.9
Gel strength, Bloom grams	170	255
Viscosity, millipoises	25.6	49.2
Water, %	9.8	10.2
Lot number	71058	4282

Table III—Liquid Solution Maxima for FD&C Dyes[a]

FD&C Dye	Wavelength, nm. UV	Visible
Blue No. 1	310	411, 630[b]
Blue No. 2	252, 284	610[b]
Red No. 2	217, 335	522[b]
Red No. 3	260, 312	526[b]
Yellow No. 5	258	428[b]
Yellow No. 6	237, 315	484[b]
Green No. 3	305	422, 625[b]
Violet No. 1	248, 307	545[b]

[a] As 0.001% unbuffered aqueous solutions at 25 ± 2°. [b] Major peak.

or urea). All films were prepared in this manner in a 13-cm. diameter, Teflon-coated pan suspended on the surface of the constant-temperature bath at 37°. The final liquid-state volume was sufficient to fill the pan bottom with an even distribution of solution (50 ml. or more in total volume). The liquid solutions were allowed to evaporate at room temperature in a laminar flow hood[4]. The resulting solid solution films were lifted from the pan, placed in a desiccator over anhydrous calcium chloride, and allowed to equilibrate to a constant weight. Then they were cut into strips suitable for spectrophotometric analysis (approximately 1 × 4 cm.). The blank film contained all chemicals used in the sample for the respective experiments except the dye. Sample films were examined for shifts in λ_{max} in the visible range and for changes in the Amide I (1625 cm.$^{-1}$) and Amide II (1520 cm.$^{-1}$) bands of the proteins in the IR study.

Films to be examined by visible and IR spectroscopy were prepared using 500 mg. protein and 5–100 mg. dye. Average film thickness was 25 μ. The addition of any amount of sodium chloride or urea tended to inhibit film formation. Nevertheless, up to 135 mg. sodium chloride or 900 mg. urea was added with variable effects on film formation depending on the dye. Higher amounts of either reagent made lifting any portion of film a practical impossibility.

Films prepared for dye release disintegration studies were made using 2500 mg. protein and 25 mg. dye and were equilibrated over a saturated solution of calcium chloride in a desiccator. To assess the effect of pH change, the dye–protein solutions that were to be examined by visible spectroscopy as films were adjusted to pH 7 on a pH meter[5] with dilute sodium hydroxide solution before evaporation. All films for a particular experiment were prepared simultaneously. Average thickness of films prepared for dye release and disintegration studies was 130 μ.

Dye Release Study Procedure—Disks prepared for the dye release study were made by punching out portions, approximately 1.2 cm. in diameter and 20 mg. in weight, from the 2500-mg. protein–25 mg. dye films with a No. 6 cork borer attached to a drill press. For each dye release experiment at 37°, an accurately weighed disk was placed in an NF XIII dissolution basket, which was attached by a 30-cm. stainless steel rod of 0.6-cm. diameter to a variable speed, reversible, 1/40-hp. motor[6]. The basket rotation speed was 20 r.p.m. The rotating basket plus disk was centered on a 100-ml. beaker containing 50 ml. distilled water at a temperature of 37 ± 0.5°, and the bottom edge was placed approximately 2 cm. from the bottom of the beaker after allowing the hydrated film to adhere and flatten against the bottom of the basket. In the 27° experiments, the disk was placed in the same size beaker and volume of water, and a propeller of 3.0-cm. diameter replaced the basket. The agitation speed was 40 r.p.m. but did not appear to affect dye release in the range of 20–120 r.p.m. at 27 ± 0.5°, as long as the propeller did not physically tear the swollen, freely rotating film apart.

The vessel size and volume parameters were adjusted so that an approximately 1-ml. volume could be repeatedly withdrawn at the same location and read directly within Beer's law limits of 18–82% transmittance in a 0.5-cm. microcell with a solvent blank of distilled water. The withdrawn sample was returned to the experiment within 10–15 sec. after withdrawal; the solution loss due to this process and evaporation was 2–3% over 40 min. at 37° and 1–2% at 27° over 1–200 hr. Longer experiments at 27° were sealed in aluminum foil to prevent excess evaporation and to prevent photodecomposition of the dyes. A solution of similar protein weight per unit volume (20 mg. gelatin in 50 ml. water) was scanned through the visible region in which the λ_{max} occurs and did not absorb nor scatter any portion of that spectrum when run *versus* a distilled water blank.

The study at 27° was intended to determine the relative release times of 50% of the dyes from a gelatin film in distilled water that is not simultaneously dissolving, as a relative measure of protein binding of 1% dye within the film. Gelatin films have a "melt point" of 35 ± 5°, at which point the hydrated protein passes into colloidal solution (9). The purpose of the 37° study was to determine if the differences in dye release could also be observed when polymer dissolution is occurring simultaneously with dye diffusion and dissolution and if those differences have the same rank order as when the film is merely releasing dye. All dyes, when placed in similar conditions and concentrations without protein, dissolved within 1 min. The percent dye released at various specific time intervals for each dye–protein combination from at least three separate experiments was averaged, and the 50% release times were read directly from the graphs.

Disintegration Study Procedure—Gelatin disks were prepared by pressing out portions, approximately 2 cm. in diameter and 50 mg. in weight, of the 2500-mg. protein–25-mg. dye films with a No. 14 cork borer. Each disintegration experiment consisted of placing an accurately weighed disk in the center of a round-bottom 500-ml. beaker filled with 200 ml. of simulated gastric or intestinal fluid, with or without the respective enzyme (pepsin or pancreatin), equilibrated to 37 ± 0.5°, and covering the disk with a 2.54-cm. square of 8-mesh stainless steel screen. To allow sufficient time for the gelatin to adhere firmly to the bottom of the beaker, agitation was not begun for 1 min. At 1 min. from the start of the experiment, a three-

[4] Model C 1001, Air Control, Inc.
[5] Radiometer type TTTlc.
[6] Model GT 21-18, G. K. Heller Corp.

Table IV—Protein Effect on Dye Maxima (Nanometers) and Absorbance (A)[a] from 1:100 Dye–Protein *versus* Protein Spectra in Liquid Solution at 25°

FD&C Dye	Type A Gelatin with Dye			+ Sodium Chloride		+ Urea		Type B Gelatin with Dye	
	λ_{max} Obtained	Change	A	λ_{max}	A	λ_{max}	A	λ_{max} Obtained	A
Blue No. 1	632	+2	D	630	I	NC	NC	630	NC
Blue No. 2	613	+3	D	NC	NC	NC	NC	610	NC
Red No. 2	525	+3	D	522	I	NC	NC	522	NC
Red No. 3	533	+7	D	529	I	NC	NC	526	NC
Yellow No. 5	428	0	D	—	—	—	—	428	NC
Yellow No. 6	484	0	D	—	—	—	—	484	NC
Green No. 3	626	+1	D	625	I	NC	NC	625	NC
Violet No. 1	540	−5	D	545	I	NC	NC	545	NC

[a] D = decrease, I = increase, and NC = no change.

blade, 5.0-cm. diameter, polyethylene propeller attached to a hollow rod of the same material, with a concentric 0.3-cm. diameter stainless steel rod inside, was started at 40 r.p.m. by a variable-speed motor[7] controlled by a constant-speed and torque control unit[8]. The disintegration end-point was taken to be the complete visual disappearance of the gelatin disk. All various dye–protein combinations were observed in at least three separate experiments, and their disintegration rates (as milligrams per minute) were averaged.

UV–Visible Spectroscopy Method—The visible spectra for both liquid- and solid-state solutions and the UV spectra for some liquid dye–protein *versus* dye and all dye reference solutions were determined using a spectrophotometer[9]. Matched cells with a 1-cm. path length were employed for the liquid solutions, and the various film strips (approximately 1 × 4 cm.) were placed in liquid sample holders of the spectrophotometer and held in the beam path by the clip used to secure the liquid sample cells. A scan speed of 1.6 and 3.2 nm./sec. was used in the UV and the visible region, respectively. The spectrophotometer was calibrated using a holmium oxide standard. Where absorbance and/or scattering were high, appropriate filter screens were used.

For the diffusion study, a 0.5-cm. microcell was utilized[10].

IR–Attenuated Total Reflectance Method—The IR-attenuated spectra of the various films were observed in the 2000–625-cm.$^{-1}$ region, utilizing an IR spectrophotometer[11] equipped with a attenuated total reflectance attachment[12] and a 50 × 20 × 2-mm. thallous bromide–iodide internal-reflecting crystal[13]. Film strips cut to fit the crystal dimensions were placed on both sides of the crystal. An angle of incidence of 45° and minimal plate pressure were used to obtain the spectra at a scan speed of 1 cm.$^{-1}$/sec. Since only 30–40% of the expected transmission is obtained with the KRS-5 plate (11), a variable-slit filter[14] was placed in the reference beam for all experiments. The spectrophotometer was calibrated using a polystyrene standard.

RESULTS AND DISCUSSION

UV and Visible Maxima of FD&C Dyes—Table III lists the maxima of 0.001% unbuffered (pH 6.6–7.0) aqueous dye solutions at 25 ± 2°. By varying the pH from 1 to 13 at constant ionic strength (μ = 0.2), it was found that the dyes exhibited the same maxima at pH 6–7 as in unbuffered solution. At lower and higher pH values, the dyes exhibited changes in both maxima and absorbance. Sodium chloride had no effect on unbuffered dye maxima with the exception of FD&C Violet No. 1; this dye's visible maximum wavelength shifted hypsochromically to 540 nm. Urea had no effect on unbuffered dye maxima. Both sodium chloride and urea produced a variable effect on absorbance, from no effect to less than a 5% decrease in absorbance. The spectral effect of concentration changes from 0.001 to 20% dye can be summarized with the general statement that as concentration increases the visible region bands broaden and may shift their center in a random manner.

Liquid Solution Interaction—Spectrophotometric evidence of interaction between substances may take the form of a change in maximal wavelength, absorbance, or both. The results of liquid solution dye–protein *versus* protein spectra are summarized in Table IV. The pH value of all combinations of dye and protein ranged from 6.5 to 7.0. Figure 1 illustrates the spectral interaction effect with the two dyes that appeared to interact to the greatest extent with the gelatins. Since interactions between dyes and type B gelatin appeared to be minimal, sodium chloride and urea effects were not investigated.

The masking effect of sodium chloride and the lack of urea effect lend evidence that the observed liquid solution interactions were primarily electrostatic in nature. By reversal of the overall charge on the type B gelatin (to a positive sign) by changing the solution pH to 1, a type A gelatin shift occurred when the charge-reversed type B gelatin and Blue No. 1 were combined. This finding lends further support to the hypothesis that the liquid solution interactions of at least the three triphenylmethane dyes (Blue No. 1, Green No. 3, and Violet No. 1) are probably due to electrostatic considerations. However, in the case of the Red No. 3 interaction, the addition of sodium chloride did not return the wavelength to its original position (526 nm.). Even doubling the sodium chloride concentration (0.308 M) had no further effect; the wavelength remained at 529 nm. This may indicate a degree of irreversibility, binding other than hydrogen, hydrophobic, and/or electrostatic, or both. Anderson and Boyce (12) found that erythrosine (FD&C Red No. 3) interacts in equimolar aqueous solution with polyvinylpyrrolidone, a nonionic polymer, producing a greater shift (to 538 nm.). They postulated that electrostatic and van der Waals' bonds might be responsible, but they did not investigate the mechanism(s) of binding. Nevertheless, the fact that Red No. 3 does bind with a nonionic polymer does imply a capability to bind in other than an electrovalent manner. Otto (13) and Gustavson (14) considered the overlap of π-electron clouds of dye and polymer to be the primary force responsible for

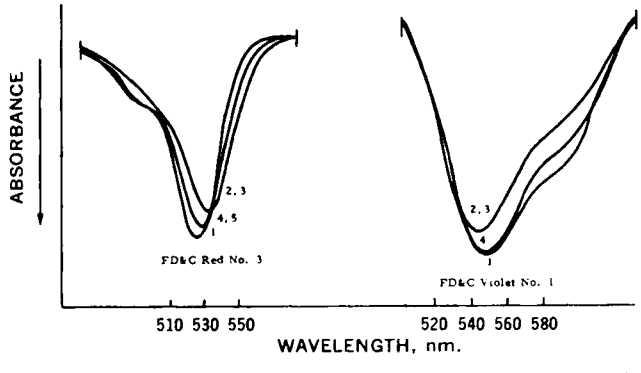


Figure 1—*Effect of type A gelatin on the absorption spectra of FD&C dyes Red No. 3 and Violet No. 1. Key: 1, dye, 0.001% in water; 2, dye with 0.1% type A gelatin; 3, dye–gelatin in 1.0 M urea; 4, dye–gelatin in 0.154 M sodium chloride; and 5, dye–gelatin in 0.308 M sodium chloride.*

[7] E. C. Motomatic model E600-013.
[8] Cole-Parmer model 4425.
[9] Perkin-Elmer model 202.
[10] With a model 9085-D Roto-Cell attachment (Arthur H. Thomas Co.). This cell holder was used in place of a standard cell holder in a Spectronic 20 photometer.
[11] Perkin-Elmer 237*b*.
[12] Model 9000, Wilks Scientific Corp.
[13] KRS-5, Wilks Scientific Corp.
[14] Barnes Engineering Co.

Table V—Bands (Nanometers) Obtained from 1:10 Dye-Protein *versus* Dye Spectra at 25° before and after 10-Fold Dilution

FD&C Dye	Before Dilution: Type A Gelatin, Dialyzed	Before Dilution: Type B Gelatin, Dialyzed	After Dilution: Type A Gelatin, Dialyzed	After Dilution: Type B Gelatin, Dialyzed
Blue No. 1	348, 430, 672	351, 432, 673	—	—
Blue No. 2	398, 408, 672	398, 408, 673	—	—
Red No. 2	590	593	—	—
Red No. 3	392, 566	380, 562	500, 547	—
Yellow No. 5	316, 497	314, 497	—	—
Yellow No. 6	542	545	—	—
Green No. 3	360, 468, 683	359, 468, 686	—	—
Violet No. 1	397, 659	400, 670	—	—

Table VI—Solid Dye-Protein Solution Visible Spectrum Maxima[a,b]

FD&C Dye	λ_{max} of Type A Gelatin Dialyzed Systems, nm.: Dye-Protein Alone	Change from Liquid Solution Dye Alone[c]	Containing: pH 7 Buffer	Containing: Sodium Chloride	Containing: Urea	λ_{max} of Type B Gelatin Dialyzed Systems, nm.: Dye-Protein Alone	Change from Liquid Solution Dye Alone[c]	Containing: pH 7 Buffer	Containing: Sodium Chloride	Containing: Urea
Blue No. 1	638	+8	630	642	647	636	+6	626	624	615
Blue No. 2	620	+10	619	613	622	617	+7	614	613	625
Red No. 2	532	+10	530	528	534	532	+10	532	557	535
Red No. 3	542	+16	539	540	543	541	+15	540	553	542
Yellow No. 5	437	+9	440	437	463	438	+10	440	490	475
Yellow No. 6	487	+3	492	487	504	487	+3	486	527	521
Green No. 3	632	+7	630	626	616	631	+5	632	620	628
Violet No. 1	557	+12	567	557	601	554	+9	554	599	600

[a] Average film thickness was 25 μ. [b] At 25°. [c] Liquid values are in Table III.

dyestuff fixation. This could lead to a hypothesis that overlapping π cloud hydrophobic bonds play a major role in binding erythrosine to type A gelatin.

Table V summarizes the band search. The purposely high (1:10) ratio of dye to protein as well as overall solution concentration of dye (0.05%) and protein (0.5%), as compared with 0.001% dye and 0.1% protein in the dye-protein *versus* protein experiments, was employed to investigate the effect of excess dye and dye aggregation on dye-gelatin interactions. As can be seen from the effect of 10-fold dilution, all bands disappeared except those with erythrosine and type A gelatin. The bands of undiluted combinations are characteristic of the dye irrespective of the gelatin type, and this could be evidence of dye-dye aggregation. Figure 2 shows the new bands obtained with the erythrosine-type A gelatin dilution combination and the effects of both urea and sodium chloride on the absorbance of the new bands. The effect of urea or sodium chloride on the 547-nm. band led to the hypothesis that the interaction represented by this new band involves both electrostatic and hydrogen bonding. The enhancement of the absorption of the 500-nm. band by sodium chloride suggests an ionic mechanism for this band, which is intensified by an increase in ionic strength.

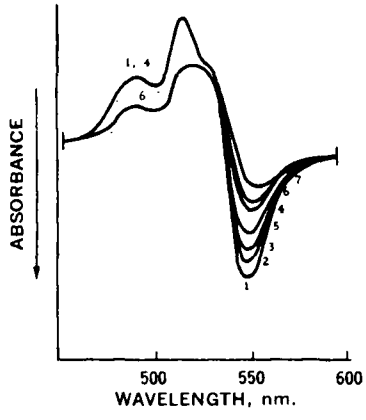


Figure 2—*Effect of sodium chloride and urea on Red No. 3–type A gelatin* versus *Red No. 3 spectra. Key: 1, 0.005% dye–0.05% protein in water* versus *0.005% dye; 2, dye-protein in 0.2* M *urea; 3, dye-protein in 0.5* M *urea; 4, dye-protein in 1.0* M *urea; 5, dye-protein in 0.077* M *sodium chloride; 6, dye-protein in 0.154* M *sodium chloride; and 7, dye-protein in 0.308* M *sodium chloride.*

Visible Spectrum Solid Solution Maxima—The visible absorption bands obtained from dyed films are shown in Table VI. The similarity of shifts with either protein suggests that the shifts may be due to solid-state dye in solution characteristics rather than solely an interaction phenomenon. While the solid solution bands for the dye-protein films without buffer, sodium chloride, or urea are as sharp as liquid solution bands, the bands obtained from dye protein films that had buffer solution, sodium chloride, or urea added before evaporation to films show variable width in their band characteristics. In addition, it should be reemphasized that any additive to the dye-protein solution seriously affected film structural integrity. Figure 3 shows bands obtained from the erythrosine-protein series with and without additional reagents and the spectra obtained for solid-state erythrosine recrystallized on a cell face.

The range of total shift from liquid solution dye without protein to solid protein-dye solution showed wide variability between dyes. Sunset yellow exhibited the least shift (+3 nm.) and erythrosine the greatest shift with either protein (+16 nm. with type A and +15 nm. with type B gelatins).

Some investigators (1, 15–18) studied solid-solid interactions of dyes and various drugs with metallic and nonmetallic adjuvants using diffuse reflectance spectroscopy. Spectra are broader and less intense in diffuse reflectance spectroscopy than solution transmittance spectra. They stated that spectral changes in diffuse reflectance spectroscopy in the order of 5–10 nm. are usually associated with physical adsorption processes. Chemisorption changes are several times this range in magnitude (18). Although the authors (1, 15–18) refer to the spectral changes observed as solid-state surface interactions, most drug-adjuvant combinations are equilibrated in aqueous suspension to facilitate maximal interaction. However, the interactions appear to occur in the solid unequilibrated states in the presence of moisture (17). The bearing that these findings have on the dye-protein solid solution spectra is debatable due to differences in spectroscopic method, method of preparation, physical state (solid particle surface interaction *versus* solid solution in this study), and substances combined with the dyes or drugs. There is basis for the belief from the results (1, 15–18) that the shifts observed in this study on the transition of dye-protein from liquid to solid solution are

Table VII—Results of IR-Attenuated Total Reflectance Study[a] at 25° as Changes in Amide I (1625 cm.$^{-1}$) and/or Amide II (1520 cm.$^{-1}$)[b]

FD&C Dye	Gelatin Type	Dye Concentration 1% I	1% II	2% I	2% II	5% I	5% II	10% I	10% II	20% I	20% II
Blue No. 1	A	NC	NC	NC	NC	+4	NC	+8	−10	+10	+7
	B	NC	NC	NC	NC	−6	+13	−5	−8	−8	−10
Blue No. 2	A	NC	+8	+8	NC	−8	−4	+5	−5	NC	+10
	B	NC	NC	NC	NC	NC	NC	−13	−12	−8	−5
Red No. 2	A	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	B	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Red No. 3	A	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	B	−10	−13	NC	−16	−5	+5	−9	−6	−10	−12
Yellow No. 5	A	NC	NC	NC	NC	−5	−5	+5	+3	+12	+13
	B	NC	NC	NC	NC	NC	NC	NC	NC	−5	NC
Yellow No. 6	A	NC	−6	+13	+10	−9	−8	+8	+7	+5	+11
	B	NC	NC	NC	NC	NC	+8	−12	NC	−8	−18
Green No. 3	A	NC	NC	NC	NC	NC	NC	+5	−7	+11	−5
	B	NC	NC	NC	NC	−5	NC	−7	−7	−8	−5
Violet No. 1	A	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	B	NC	NC	NC	NC	−6	NC	−7	−5	−17	−16

[a] NC = no change. [b] All changes are in cm.$^{-1}$.

merely due to change in state. This is further substantiated in Table VI by the fact that individual bands of each dye with either protein (without added buffer, sodium chloride, or urea) are no more than 3 nm. apart.

Carroll (19), in a discussion of sensitizing dyes used in photography, stated that dye spectra in aqueous photographic gelatin-silver halide suspensions become more complex as a result of molecular interaction and dye aggregation in liquid solution, emulsion, and dried emulsion (film). Generally, the sensitizing dye absorption bands are shifted bathochromically in emulsion as compared to a solution of the dye by itself. Polymethine dyes commonly used as sensitizing dyes may shift from 0 to 190 nm. However, to be sensitized the dye has to be first adsorbed on the surface of silver halide crystals in an aqueous suspension. Furthermore, most polymethine dyes are cationic, whereas all FD&C dyes are anionic.

IR-Attenuated Total Reflectance Study—The effects of dye concentrations (on a gelatin weight basis) of from 1 to 20% on the Amide I and Amide II bands of the protein are summarized in Table VII. Changes in hydrogen bonding between molecules are usually indicated by shifts in the area of 3100–3600 cm.$^{-1}$ (8), but since many of the dyes absorb strongly in that area also due to O—H and N—H bonds present in their molecules, the Amide I (1625 cm.$^{-1}$) and Amide II (1520 cm.$^{-1}$) bands can be used to show hydrogen bonding changes. The Amide I band is characteristic of the C=O stretching vibrations, whereas the Amide II band is characteristic of the N—H deformation mode and may be coupled with the C—N stretching mode of the peptide group (8). The shifts observed in hydrogen bonding changes may be in either direction as is evidenced in Table VII (8). Figure 4 shows typical examples of shifts (or the

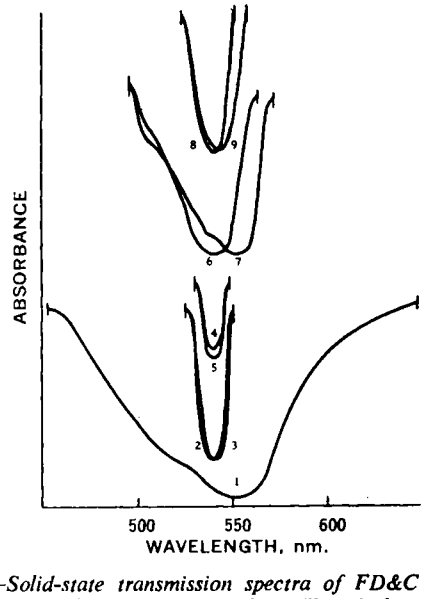


Figure 3—*Solid-state transmission spectra of FD&C Red No. 3 (1%) in type A gelatin and type B gelatin. Key: 1, dye recrystallized on cell face; 2, dye–type A gelatin dialyzed; 3, dye–type B gelatin dialyzed; 4, dye–type A gelatin dialyzed with pH 7 buffer; 5, dye–type B gelatin dialyzed with pH 7 buffer; 6, dye–type B gelatin dialyzed with urea; 7, dye–type B gelatin dialyzed with sodium chloride; 8, dye–type A gelatin dialyzed with sodium chloride; and 9, dye–type A gelatin dialyzed with urea.*

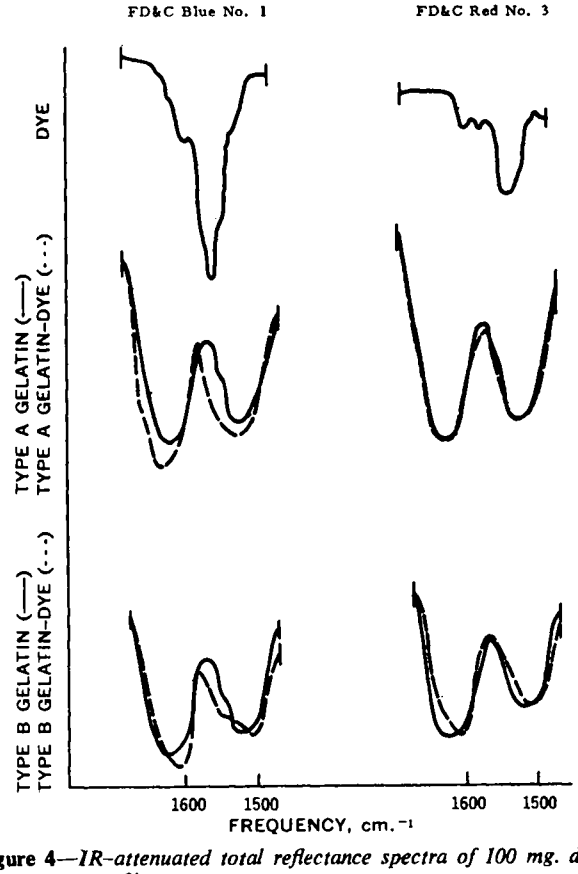


Figure 4—*IR-attenuated total reflectance spectra of 100 mg. dye–500 mg. protein films.*

Table VIII—Dye 50% Release Time (min.) into Distilled Water at 27 and 37° from Type A and Type B Gelatins[a,b]

FD&C Dye	27° Gelatin Type A	27° Gelatin Type B	37° Gelatin Type A	37° Gelatin Type B
Blue No. 1	744	10.8	15.8	6.3
Blue No. 2	792	7.5	—	—
Red No. 2	840	19.8	13.0	5.8
Red No. 3	9600	103	19.2	10.0
Yellow No. 5	1380	4.6	14.6	4.9
Yellow No. 6	588	6.8	13.8	4.8
Green No. 3	1092	18.0	12.7	7.2
Violet No. 1	6900	115	12.9	8.5

[a] Each value represents the average of three experiments. [b] Average film thickness was 130 μ; average disk weight was 20 mg.

lack of same) observed with the 20% dye spectra. Noteworthy is the lack of effect of Red No. 3 on type A gelatin hydrogen bonding. This lends support to the hypothesis that the Red No. 3-type A gelatin interaction involves primarily ionic and possibly hydrophobic types of bonding.

Nelson (20) investigated the photoelectric properties of dyed gelatin films and stated that the addition of dye to gelatin greatly increases the yield of charge carriers in a gelatin film. Charge carriers are able to mediate redox processes, which may enhance gelatin cross-linking. Erythrosine enhanced the irreversible UV "hardening" effect on gelatin in Nelson's work, but only after heavy exposures to UV light. Furthermore, Nelson's films were dehydrated by immersion in isopropanol before photoemission was observed.

From the data presented in Table VII, it can be seen that some dyes affect hydrogen bonding between polypeptide chains, but the effect is detectable for the most part only at higher ratios of dye to protein. Films of 0.1 mg. dye to 100 mg. gelatin were also examined for hydrogen bonding changes, but none was detectable.

Artemova and Usova (21) found that indigotine, tartrazine, and amaranth in 0.02% concentration in 3 and 6% gelatin gels hinder structure formation in gelation. Idson and Braswell proposed (22) that hydrogen bonding is the primary force in gel formation. Iijima and Sekido (23) found that, in the dyeing of protein, all of the *p*-hydroxy dyes and those *o*-hydroxy azo dyes, in which the OH group is not involved in an intramolecular hydrogen bond are adsorbed to the protein by a hydrogen bonding mechanism in addition to the electrostatic attraction between the sulfonic acid groups and amino groups of the protein. This has bearing on the finding that the *o*-hydroxy azo dye, amaranth, showed no effect on either gelatin's intermolecular hydrogen bonding at all concentrations. In the case of the other *o*-hydroxy dyes, tartrazine and sunset yellow,

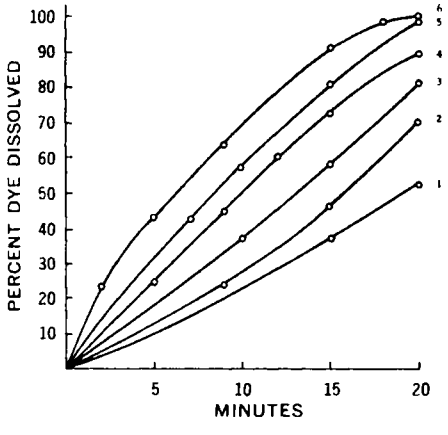


Figure 5—*Partial release curves for selected dyes from both proteins at 37°, 20 r.p.m., in NF XIII basket. Key: 1, type A gelatin dialyzed-Red No. 3; 2, type A gelatin dialyzed-Blue No. 1; 3, type A gelatin dialyzed-Violet No. 1; 4, type B gelatin dialyzed-Red No. 3; 5, type B gelatin dialyzed-Violet No. 1; 6, type B gelatin dialyzed-Blue No. 1. Each point represents the average of three experiments.*

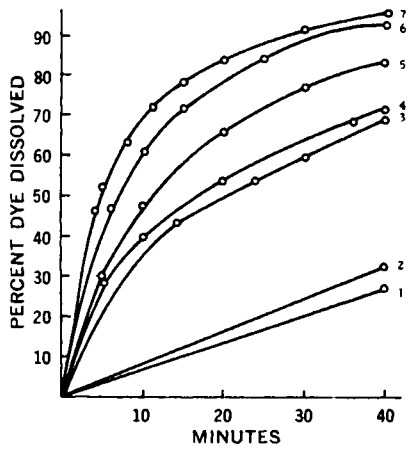


Figure 6—*Partial release curves for selected dyes from type B gelatin at 27°, 40 r.p.m. Key: 1, type B gelatin dialyzed-Violet No. 1; 2, type B gelatin dialyzed-Red No. 3; 3, type B gelatin dialyzed-Red No. 2; 4, type B gelatin dialyzed-Green No. 3; 5, type B gelatin dialyzed-Blue No. 1; 6, type B gelatin dialyzed-Yellow No. 6; and 7, type B gelatin dialyzed-Yellow No. 5. Each point represents the average of three experiments.*

effects on the hydrogen bonding of both proteins were seen. The *o*-hydroxy group of amaranth may be tied up in an intramolecular hydrogen bond, whereas the hydroxyls of the other two dyes apparently participate in intermolecular hydrogen bonding. Indigotine appeared to interact with hydrogen bonds of either type gelatin. The triphenylmethanes, with the exception of the Violet No. 1-type A gelatin combinations, appear to affect hydrogen bonding in either protein at primarily higher dye to protein ratios.

Other workers (7, 8) used IR-attenuated total reflectance to detect solid-state hydrogen bonding changes in gelatins combined with various carbohydrates.

Dye Release Results—Table VIII summarizes the times required for 50% dye release into distilled water from both proteins at 27 and 37°. Figures 5–7 illustrate representative examples of the release curves from which the data were obtained. The rank orders observed differed both between the two differently charged proteins and at the two different temperatures. Generally, the anionic FD&C dyes appeared to be more strongly bound to a cationic type A gelatin (Bloom No. 170, viscosity 25.6 millipoises) of lower Bloom strength and viscosity than to an anionic type B gelatin (Bloom No. 255, viscosity 49.2 millipoises) of higher Bloom strength and viscosity, at temperatures both within and below the "melt point" of gelatin.

At 27°, the FD&C Red No. 3 and FD&C Violet No. 1 appeared to be relatively tightly bound to both proteins. At 37°, the Red No. 3 appeared to be much more bound than any other dye. Reliable

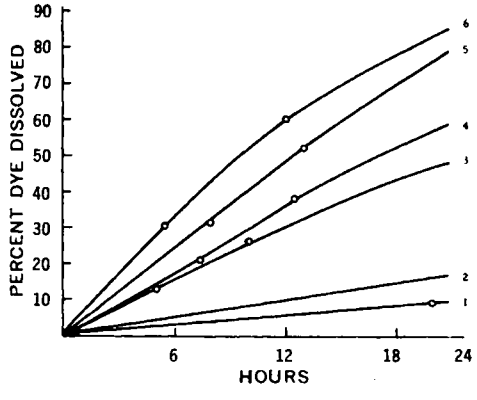


Figure 7—*Partial diffusion curves for selected dyes from type A gelatin at 27°, 40 r.p.m. Key: 1, type A gelatin dialyzed-Violet No. 1; 2, type A gelatin dialyzed-Red No. 3; 3, type A gelatin dialyzed-Yellow No. 5; 4, type A gelatin dialyzed-Green No. 3; 5, type A gelatin dialyzed-Blue No. 1; and 6, type A gelatin dialyzed-Yellow No. 6. Each point represents the average of three experiments.*

Table IX—Dyed and Undyed Gelatin Disk Average Disintegration Rates at 37°, 40 r.p.m., in 200 ml. of Simulated Milieu with or without Enzyme[a,b]

FD&C Dye	Average Disintegration Rate, mg./min.						
	Simulated Gastric Fluid				Micro-equivalents of Sodium/0.5 mg. of Dye	Simulated Intestinal Fluid —without Pancreatin—	
	Pepsin						
	Type A Gelatin, Dialyzed		Type B Gelatin, Dialyzed			Type A Gelatin, Dialyzed	Type B Gelatin, Dialyzed
	Without	With	Without	With			
Undyed	1.05	1.03	1.37	1.47	—	0.42	0.78
Blue No. 1	1.11	1.29	1.35	1.43	1.27	0.66	0.96
Blue No. 2	1.06	1.32	1.33	1.41	2.14	0.65	0.94
Red No. 2	1.07	1.44	1.34	1.44	2.48	0.61	0.87
Red No. 3	0.57	1.07	1.07	1.42	0.57	0.18	0.70
Yellow No. 5	1.09	1.37	1.34	1.52	2.81	0.66	1.02
Yellow No. 6	1.09	1.45	1.35	1.52	2.07	0.66	0.96
Green No. 3	1.06	1.37	1.35	1.28	1.23	0.65	0.93
Violet No. 1	1.09	1.17	1.36	1.34	0.68	0.60	0.83

[a] Each value represents the average of three experiments. [b] Average weight 50 mg.; average film thickness 130 μ.

spectrophotometric determination of FD&C Blue No. 2 at 37° was not possible. Others (24) found FD&C Blue No. 2 to be very unstable. Due to differences in method, revolutions per minute, and gelatin melting, comparison between the two temperatures should be approached with caution.

Disintegration Study Results—The effects of 1% dye concentrations, media, and the presence of pepsin on the average disintegration rates of both type gelatins are presented in Table IX. Although the end-point of these experiments was the disappearance of any trace of gelatin on the bottom of the beaker, the visual end-point can only be assumed to be the disintegration, dispersion, and beginning of the dissolution process or pseudodissolution rather than complete dissolution. That this was the case was confirmed by preliminary dissolution studies of undyed gelatin disks using a pH-stat[15] at pH values of 3 and 9 with a nitrogen flush to eliminate carbon dioxide alteration of the titration. Figure 8 shows that even after the gelatin had dispersed into the media, the titration of hydrated charged groups continued, indicating that the dissolution process was incomplete even though the disk was no longer visible. As a result, automation of the observation of the process would serve no useful purpose because the end-point would have to be observed visually in all experiments.

Overall, the gelatins disintegrated faster in simulated gastric fluid at pH 1.2 with or without pepsin than in simulated intestinal fluid without pancreatin at pH 7.5.

The xanthene dye FD&C Red No. 3, as might be expected from spectral and diffusion data previously given, had the most deleterious effect on average disintegration in the absence of pepsin in simulated gastric fluid or in simulated intestinal fluid. Even when pepsin was present, the type A–Red No. 3 disks in simulated gastric fluid did not disintegrate any faster than the undyed disks; *i.e.*, it appeared that pepsin did not accelerate the disintegration process as it had with other dyed disks. Xanthene dyes have been shown to inhibit the tryptic hydrolysis of casein (25). Perhaps erythrosine inhibited not only the intrinsic disintegration process by its presence in the gelatin matrix but, in addition, the diffused dye interfered with peptic action on the type A gelatin disk by complexation with enzyme. This did not appear to be the case with the type B gelatin–Red No. 3 disks in simulated gastric fluid with pepsin. However, a xanthene dye, rose bengal, has been shown to complex with trypsin (25).

The data indicate that pepsin appeared to have some effect on all disintegration rates in simulated gastric fluid with the exception of undyed type A gelatin. It was also observed that the inclusion of pepsin in simulated gastric fluid caused areas of the disk surface to be denuded within 2–5 min. into the experiment. These bare areas were irregularly shaped holes, which ranged from an estimated 20 to 50% of the disk surface. However, within several more minutes the hydrated protein reassumed a continuous circular surface with a slight convexity which increased as the experiment proceeded and the disk became smaller in diameter. Pepsin is an endopeptidase, *i.e.*, cleaves a polypeptide at peptide bonds within the chain (26). This would relieve restraints to hydration and swelling due to the intertwining of peptide chains. In addition, it would liberate progressively smaller and smaller peptide chains for the dissolution process. Results obtained in this investigation seem to agree with Czetsch-Lindenwald (27), who stated that pepsin accelerates gelatin capsule disintegration to the extent of only about 25%; type A gelatin–Red No. 2 disk disintegration rate was the most accelerated (35%) of all dye–protein combinations. On the other hand, pepsin seemed not to affect, or slightly decreased, the disintegration of undyed type A– or type B–Green No. 3 and type A gelatin–Violet No. 1 combinations.

The presence of dyes other than Red No. 3 in 1% concentration in type A gelatin in simulated gastric fluid with pepsin and both type A and type B gelatin in simulated intestinal fluid without pancreatin appeared to increase disintegration rates. This effect may be partially due to the number of microequivalents (μeq.) of sodium that are contained in the 0.5 mg. of dye in each 50-mg. disk. As can be seen in Table IX, the number of sodium microequivalents contained in each dye seems to divide the dyes into three distinct groups: those dyes with less than 1 μeq., those dyes with more than 1 and less than 2 μeq., and those dyes with greater than 2 μeq. of sodium/0.5 mg. While not absolutely correlatable, those dyes with higher microequivalent values may aid in the protein hydration process by not only pulling in water at a greater rate than those dyes with lower microequivalent values but also by diffusing outward at a greater rate (Table VII) and, in effect, allowing osmotic swelling to proceed at an increased rate. The effect may also be referred to as an internal "salting in" of the protein (28), whereby low concentrations of salts (0.05 *M* or less) solubilize the protein by breaking salt bridges and partially masking charges. The dye effect is particularly noteworthy in both type A and type B gelatins in simulated intestinal fluid. The type A gelatin disk disintegration is increased by as much as 57% by the presence of dyes; the type B gelatin disk disintegra-

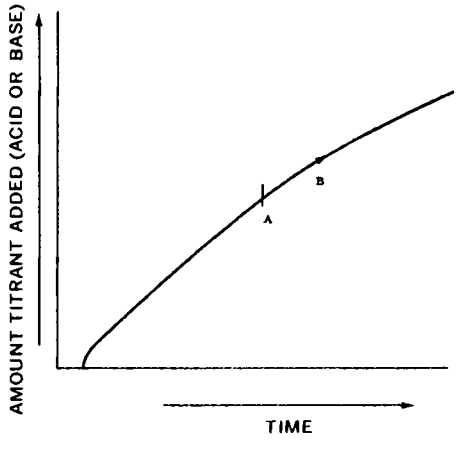


Figure 8—*Typical titration curve for type A or type B gelatin disks at pH-stat setting of 3 or 9 with acid or base titrant. Key: A, point at which disk disappeared completely and titrant continued to be added; and B, titrant may be added up to several hours beyond point A.*

[15] Radiometer, Copenhagen.

tion rate is increased by up to 31% by one dye. The pKa of sulfonic acid groups in most dyes is close to a value of 3; at a simulated intestinal fluid pH value of 7.5, the ionized form of the dyes would be predominant (23). At pH 1.2 in simulated gastric fluid, the dyes' sulfonic acid groups would be essentially (1%) unionized. In addition, the pKa of the carboxyl group of erythrosine is 4.95 (29). While approximately 1% of the dyes with sulfonic acid groups may be ionized at pH 1.2, only approximately 0.01% of the erythrosine could be ionized at pH 1.2. In addition, Red No. 3 is less soluble (9.0 g./100 ml. water, 25°) than the rest of the FD&C dyes (19.0–20.0 g./100 ml.), with the exception of Blue No. 2 (1.6 g./100 ml.) (30). At pH values of 5 or below, Red No. 3 is stated to be insoluble (30).

In summary, it appears that the effect of FD&C Red No. 3 on gelatin disk disintegration may be due to liquid and solid solution interaction with the gelatin, possible interaction with pepsin if present, and very low percent ionization and inherent insolubility at low pH values.

In vitro availability tests of capsule dosage forms, the shell of which is colored by erythrosine, may exhibit a delayed formulation release pattern as well as dye-hindered drug dissolution, particularly in acidic test media in which pepsin may have purposely been left out due to its interference with spectrophotometric determination of drug present in solution. *In vivo* effects would naturally depend on variables in stomach contents, emptying time, and drug absorption sites. Further studies on *in vitro* and *in vivo* effects with erythrosine and other xanthene dyes and dye classes are in progress.

SUMMARY AND CONCLUSIONS

1. A simple model of a gelatin shell wall was fabricated to examine dye–polymer interaction characteristics and disintegration behavior.

2. The model agreed to some extent with respect to previous findings of pepsin effect on disintegration of capsule shells.

3. In liquid solution, the anionic FD&C dyes tended to interact to a greater extent with a cationic type A gelatin than with an anionic type B gelatin.

4. All dyes interacted with both proteins in the solid state simply by virtue of the characteristics of the solid state; some dyes did affect the intermolecular hydrogen bonding of both proteins, but such effect was detectable only at relatively high concentrations.

5. The xanthene dye, FD&C Red No. 3 (erythrosine), interacted in both states of solution to the greatest extent of any FD&C class or type dye; the interaction may involve ionic, hydrogen, hydrophobic, and van der Waals' bonding, some of which may lead to a degree of irreversibility, even in the transition from solid to liquid solution state.

6. The triphenylmethane dye, FD&C Violet No. 1 (Wool Violet 5BN), interacted to some extent with the gelatins but primarily in an ionic manner that was reversible in liquid solution.

7. The FD&C dyes in 1% concentration were released at different rates from both types of proteins at temperatures both below and within the melt point of gelatins.

8. The dyes that exhibited the greatest spectral change activity (Red No. 3 and Violet No. 1) had the longest release times and adversely affected the disintegration of gelatin disks in either simulated gastric or simulated intestinal fluids; conversely, the dyes that exhibited the least spectral change activity generally appeared to be released quicker and actually may have enhanced disintegration, particularly those dyes with higher sodium microequivalent values when observed in simulated intestinal fluid.

9. It appears that, for a series of dye compounds, a spectral examination of their interactions with a polymer can yield some indication of their respective binding in liquid and solid solution. This observed indication coupled with a calculation of relative cation equivalents may lead to a presumptive hypothesis of an effect on both diffusion of the dye and the effect of the dye on the polymer's disintegration.

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Computer Optimization of Pharmaceutical Formulations I: General Procedure

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Abstract □ A technique was developed whereby a formulation with optimum properties, according to predetermined criteria, can be selected *via* computer analysis. The results of a statistically designed set of experiments based on five independent variables were used as the data input to the computer. Regression analysis of these data resulted in a set of second-order polynomial predictor equations. Restrictions placed by the pharmacist on the characteristics of interest serve to eliminate undesirable formulations. Ultimately, the formulator can trade off properties to obtain an optimum formulation. Computer-generated graphs further aid the formulator in the understanding of the particular system. An example is presented of the selection of an optimum formulation by this procedure and of its preparation in the laboratory. Predictions and experimental results show excellent agreement.

Keyphrases □ Computer optimization of pharmaceutical formulations—general procedure, examples □ Formulation of pharmaceutical preparations—computer optimization, general procedure and examples □ Pharmaceutical technology—general procedure for computer optimization of pharmaceutical formulations

The development of a pharmaceutical formulation and the associated process involves a number of variables. Mathematically, they can be divided into two groups: (*a*) the independent or formulation and process variables that are controllable factors, and (*b*) the dependent variables that are the responses or characteristics of the resultant drug delivery system. Much of the work in pharmaceutics has been in the pursuit of relationships between the two.

Table I—Experimental Design for Five Factors

Trial	Factor Level in Experimental Units				
	X_1	X_2	X_3	X_4	X_5
1	−1	−1	−1	−1	1
2	1	−1	−1	−1	−1
3	−1	1	−1	−1	−1
4	1	1	−1	−1	1
5	−1	−1	1	−1	−1
6	1	−1	1	−1	1
7	−1	1	1	−1	1
8	1	1	1	−1	−1
9	−1	−1	−1	1	−1
10	1	−1	−1	1	1
11	−1	1	−1	1	1
12	1	1	−1	1	−1
13	−1	−1	1	1	1
14	1	−1	1	1	−1
15	−1	1	1	1	−1
16	1	1	1	1	1
17	−1.547	0	0	0	0
18	1.547	0	0	0	0
19	0	−1.547	0	0	0
20	0	1.547	0	0	0
21	0	0	−1.547	0	0
22	0	0	1.547	0	0
23	0	0	0	−1.547	0
24	0	0	0	1.547	0
25	0	0	0	0	−1.547
26	0	0	0	0	1.547
27	0	0	0	0	0

Pharmaceutical research and development projects are often optimization problems. One experiments by a series of logical steps, carefully controlling the variables one at a time, until a satisfactory formulation results.

It may be necessary to trade off properties during such experimentation, to sacrifice one characteristic to improve another; *e.g.* to accept a softer tablet in order to improve the dissolution profile. Thus, the primary objective may not be to optimize absolutely but to compromise effectively and thereby to produce the best formulation under a given set of restrictions.

The techniques of optimization are well documented in the literature (1–7). Fonner *et al.* (8) demonstrated the applicability of mathematical optimization techniques to pharmaceutical systems. Their application of the Lagrangian method, while able to handle several responses or dependent variables, was generally limited to two independent variables. Experiences in these laboratories showed that a greater number of these variables are of general interest, but as the number of formulation factors is increased, the situation becomes more involved mathematically and graphically impossible. Although various multivariate techniques are available, computers are almost a necessity for their application.

The work reported here was undertaken to determine the feasibility of optimizing a pharmaceutical formulation with the aid of computers in a manner which could be used with relative ease by the development pharmacist. The procedure that resulted appears capable of being carried out by persons unfamiliar with the mathematics of optimization and with no previous computer experience.

This paper covers the general procedure followed to optimize formulations. Subsequent reports will discuss several other aspects of the optimization technique, its application to a practical situation, and the interpretation of the large quantity of data generated by this particular method.

THEORY

The type of project of concern here is one of selecting the most desirable level of ingredients or controllable process factors. That is, it is desired to *quantitate* a formulation that has been *qualitatively* determined.

The technique involves performing a set of statistically designed experiments as described by Box and Wilson (1, 2) and using the resulting data to derive a mathematical model which can be used for the optimization of a formulation. The experimental design is dependent on the number of variables involved in the study; for five independent or formulation variables, the modified half-factorial design shown in Table I requires a total of 27 experiments.

The first 16 experiments represent a half-factorial design for five factors at two levels resulting in $\frac{1}{2} \times 2^5 = 16$ trials. The two levels here are represented as +1 and −1. For the remainder of the study, three additional levels were selected: zero represented the base

Table II—Translation of Experimental Conditions into Physical Units[a]

Factor	−1.547 eu[b]	−1 eu	Base 0	+1 eu	+1.547 eu
X_1 = calcium phosphate–lactose ratio (1 eu = 10 mg.)	24.5/55.5	30/50	40/40	50/30	55.5/24.5
X_2 = compression pressure (1 eu = 0.5 ton)	0.25	0.5	1	1.5	1.75
X_3 = starch disintegrant (1 eu = 1 mg.)	2.5	3	4	5	5.5
X_4 = granulating gelatin (1 eu = 0.5 mg.)	0.2	0.5	1	1.5	1.8
X_5 = magnesium stearate (1 eu = 0.5 mg.)	0.2	0.5	1	1.5	1.8

[a] Physical quantities are rounded off only for convenience. [b] eu = experimental units.

level midway between the above-mentioned levels and the positive and negative 1.547 values represented the extreme values. The reader is referred to the literature for a discussion of the statistical design (1, 2, 5).

The proper name for this design is "a five-factor, orthogonal, central, composite, second-order design," and the type of predictor equation resulting from such a study is a second-order polynomial (with 21 terms) having the following form:

$$Y_i = a_0 + a_1X_1 \ldots + a_5X_5 + a_{11}X_1^2 \ldots + a_{55}X_5^2 + a_{12}X_1X_2 \ldots + a_{45}X_4X_5 \quad \text{(Eq. 1)}$$

where:

Y_i = level of a given response (dependent variable)
a = regression coefficients for second-order polynomial
X_j = level of independent variable

Such an equation is generated for each dependent variable relating it to the set of five independent variables.

EXPERIMENTAL

The formulation selected as a model system for the optimization program was a production formula already in existence. Briefly, the process involves a starch-paste–gelatin granulation of the drug with diluents. After drying and milling, the disintegrant, the glidant, and the lubricant are added.

The five independent formulation variables selected for this particular study were: X_1, diluent ratio; X_2, compressional force; X_3, disintegrant level; X_4, binder level; and X_5, lubricant level. With the exception of these five variables, everything else in the formulation and the processing steps remained constant throughout the study including: (*a*) the level of active ingredient; (*b*) the quantity of starch as paste; (*c*) the glidant level; (*d*) granulating, milling, drying, and blending conditions; and (*e*) the speed of the compressing machine. The translation of the statistical design (Table I) into physical units for the five variables is shown in Table II[1].

Each experiment consisted of a 30,000-tablet batch through the granulating, milling, dry mixing, and lubricating steps. From each batch, 3000 tablets were compressed on a rotary press[2] equipped with 35 sets of 0.6-cm. (0.25-in.) round, flat beveled-edge tooling run without precompression.

Table III—Values for Index of Determination from Regression Analysis

Response	R^2, %
Y_1 Disintegration time	98.08
Y_2 Hardness	98.90
Y_3 Dissolution	97.90
Y_4 Friability	75.38
Y_5 Weight	87.56
Y_6 Thickness	98.15
Y_7 Porosity	99.45
Y_8 Mean pore diameter	61.22

The responses measured on the resulting tablets were: Y_1, disintegration; Y_2, tablet hardness; Y_3, dissolution; Y_4, friability; Y_5, weight uniformity; Y_6, thickness uniformity; Y_7, tablet porosity (pore volume); and Y_8, mean pore diameter. Most of these response variables are properties of general interest to tablet formulators, but the list could be varied to fit the particular formulation.

Disintegration time was determined using the procedure and apparatus outlined in USP XVIII (9). Tablet hardness was determined using an electric hardness tester[3].

Dissolution measurements were made in 0.1 *N* HCl following the USP basket technique with a basket rotation at 100 r.p.m. The dissolution profile was followed by means of a servo-recorder[4], a spectrophotometer[5], and a flow cell.

Tablet friability was measured on a 60-tablet sample in a friabilator[6] using a 4-min. cycle (10).

Weight and thickness uniformity were calculated as the relative standard deviation of the given measurement. For tablet weight, 10 tablets were individually weighed on an analytical balance[7] and 20 tablet thicknesses were determined with a dial comparator[8].

Pore volume was determined[9], and a sample size of 10 tablets was necessary to obtain adequate readings of the mercury volume. Mean pore diameter was calculated from the porosimeter data.

RESULTS AND DISCUSSION

The responses from the 27 experiments were measured and tabulated. The large volume of data is unwieldy to report here and, for the most part, is irrelevant to the present discussion.

Statistical analysis was carried out which included the calculation of mean values for each of the eight responses in each of 27 experiments. Without repeated emphasis, it should be noted that values used for optimization must be statistically significant. The formulator must be confident that the results used as input are truly representative of the particular system.

The sets of data resulting from the statistical analysis were then subjected to computerized[10] regression analysis to determine the fit to a second-order model (Eq. 1). In most cases, the *R*-square value or the index of determination was satisfactory, as shown in Table III.

The ability of the system to predict accurately is only as good as the regression fit of the predictor equation used. The values for friability, weight, and mean pore diameter are less than desirable, and it may be that many pharmaceutical responses of interest do not follow a second-order model.

One advantage, however, of the digital type of system used is that it can be modified to accept other mathematical models, whether they be higher order polynomials, any other empirical relationships, or mathematical models based on first principles. For the present work, it was decided to restrict the model to the

[1] There are several places in the optimization procedure where the developmental pharmacist is indispensible. This is one of them, since only by formulation experience can one select reasonable ranges for those variables determined to be most important.

[2] Stokes model 580, F. J. Stokes Division of Pennwalt Corp., Warminster, Pa.

[3] Ahiba, Gubelin International Corp., Mount Kisco, NY 10549

[4] Heath model EUW-20A.

[5] Gilford model 240, Gilford Instrument Laboratories, Inc., Oberlin, Ohio.

[6] Roche.

[7] Mettler model H20T.

[8] B. C. Ames Co., Waltham, Mass.

[9] Aminco Mercury Intrusion Porosimeter, American Instrument Co., Bethesda, Md.

[10] The entire optimization system (series of programs) was written for use on a CDC 1700 (Control Data Corp., Minneapolis, Minn.).

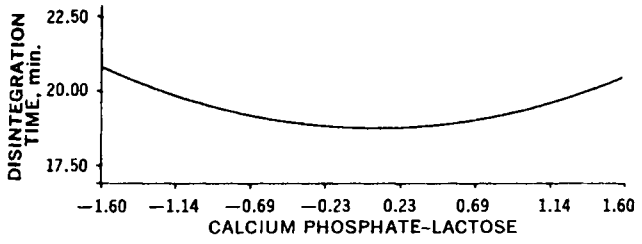


Figure 1—*Plot of disintegration time (minutes) as a function of diluent ratio generated from computer tapes and drawn by plotter. The abscissa represents the experimental range in terms of experimental units (see text).*

second-order polynomials, realizing that predictions for several variables might be inaccurate.

The remainder of the technique involves the analysis of the set of polynomial equations obtained *via* regression.

Optimization—In addition to the computerized statistical analysis and regression analysis already mentioned, programs were needed for the actual optimization. Two major steps were used: (*a*) the feasibility search, and (*b*) the grid search.

The feasibility program is used to locate a set of response constraints that is at the limit of possibilities. That is, given this particular set of restrictions, there *is* a solution (formulation), but if the constraints are tightened, there is no solution. The mechanics are to select several values for the responses of interest and slowly relax them until a possible solution is found. For example, the constraints in Table IV were fed into the computer and were relaxed one at a time until a solution was found. The first choice would have been a formulation with a disintegration time of <1 min., tablet hardness >12 kg., and dissolution >100% in 50 min.[11]. This set of constraints was expected to be unreasonable and, indeed, no solution was found until the values had been systematically relaxed to a disintegration time of 5 min., tablet hardness of 10 kg., and dissolution of 100% at 50 min. Any further relaxation of the constraints would, of course, also yield solutions.

The feasibility program is designed so that it stops after the first possibility; the formulation given may be one of many that could satisfy the constraints.

The second program, the grid search, is essentially a brute force method in which the experimental range is divided into a grid of arbitrary size and methodically searched. Based on an input of the most reasonable constraints (most likely resulting from the feasibility program), the grid search program prints out all points (formulations) in the grid that meet the criteria.

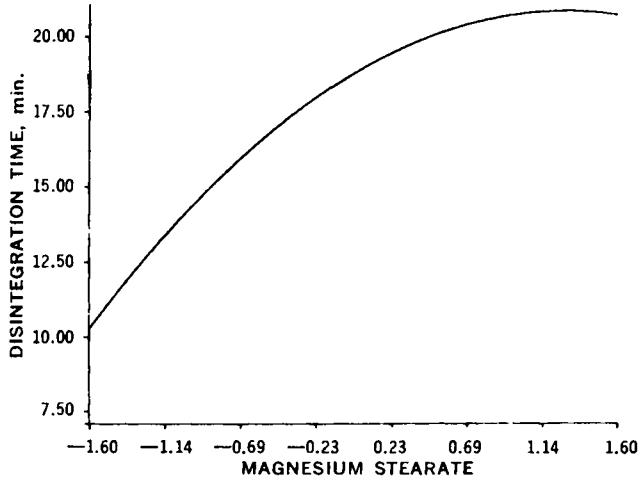


Figure 2—*Plot of disintegration time as a function of lubricant level drawn from computer tapes.*

[11] Values greater than 100%, as well as negative values, appear throughout this work. Because the second-order polynomials describe curves that may be parabolic, hyperbolic, ellipsoid, *etc.*, extrapolation may take one out of the realm of physical reality.

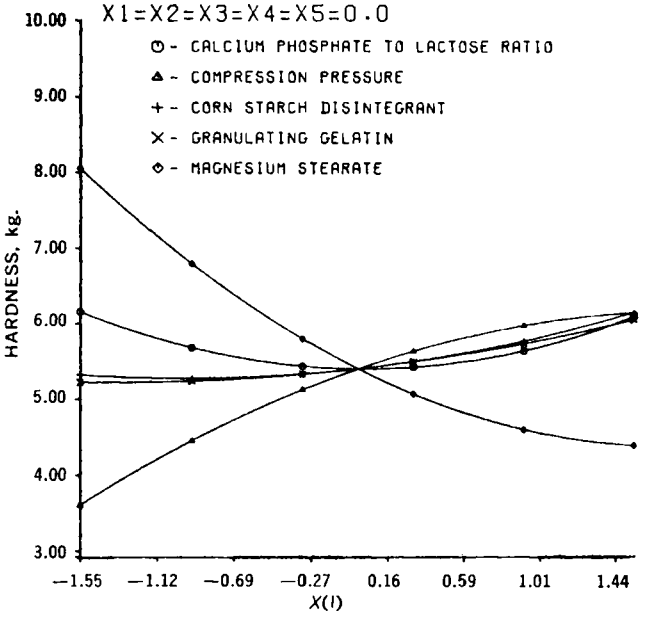


Figure 3—*Composite plot for tablet hardness as a function of each independent variable,* X_i (i = *1, 2, . . . , 5*).

The rationale for the feasibility program becomes apparent, for if one had arbitrarily selected suitable properties or constraints and made a grid search, the printout might contain hundreds of combinations that fit—or none. But, with the results of the feasibility study, it is known within reason the point at which tighter constraints yield no solutions. By running the grid search in this neighborhood, the printout will have only a few solutions from which the most suitable one can be selected.

For example, a grid search utilizing the above constraints (disintegration time ≤ 5 min., tablet hardness ≥ 10 kg., and dissolution ≥100% in 50 min.) produced eight solutions (formulations) that satisfied the requirements. A second grid search where the constraint on tablet hardness was relaxed to 8 kg. yielded 650 solutions. Of course, it is easier to choose one of eight solutions than one of 650 solutions.

In addition to providing a printout of each formulation, the grid search program also gives the corresponding values for the responses. This is the point where one can trade off one response for

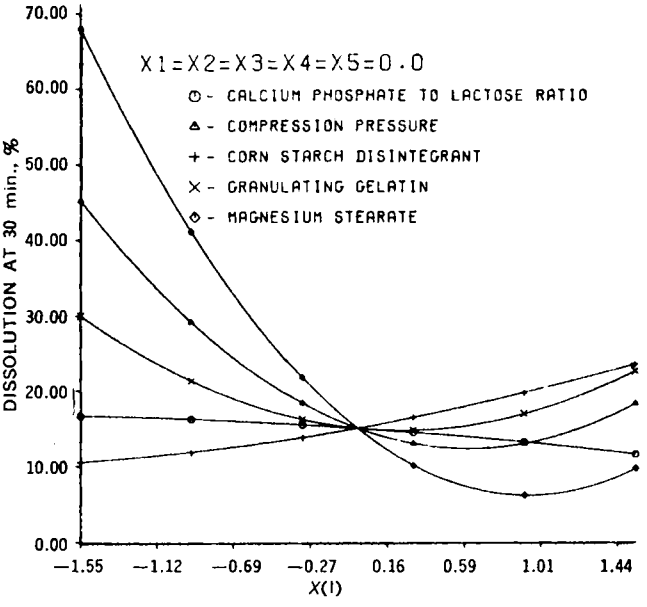


Figure 4—*Composite plot for dissolution response as a function of each independent variable,* X_i (i = *1, 2, . . . , 5*).

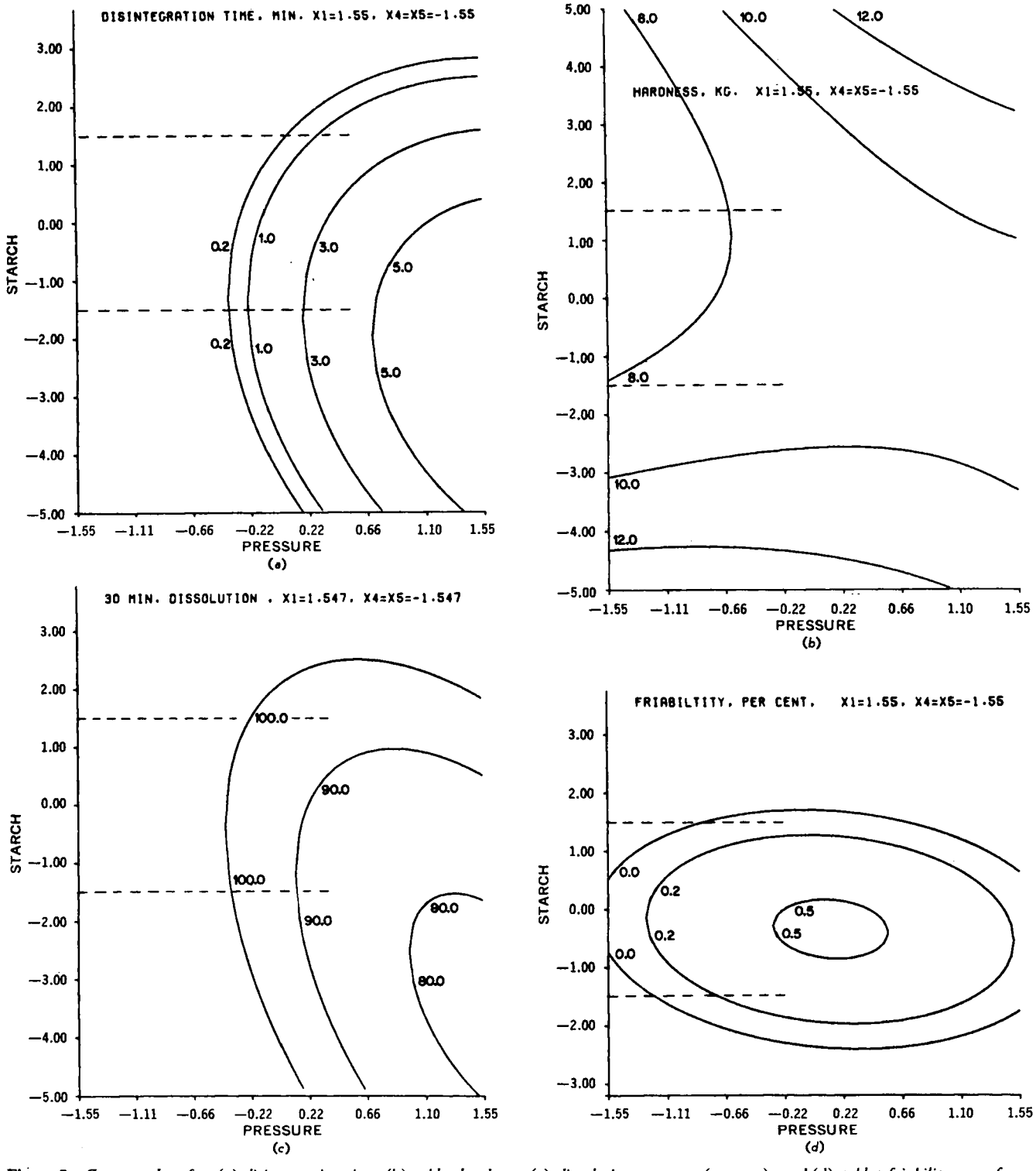


Figure 5—*Contour plots for:* (a) *disintegration time,* (b) *tablet hardness,* (c) *dissolution response (percent), and* (d) *tablet friability as a function of disintegrant level and compressional force. Dotted lines on ordinate denote limits of experimental range (−1.547 to +1.547 eu, see text).*

another or compromise as needed. Thus, the best or most acceptable formulation is selected from the grid search printout to complete the optimization.

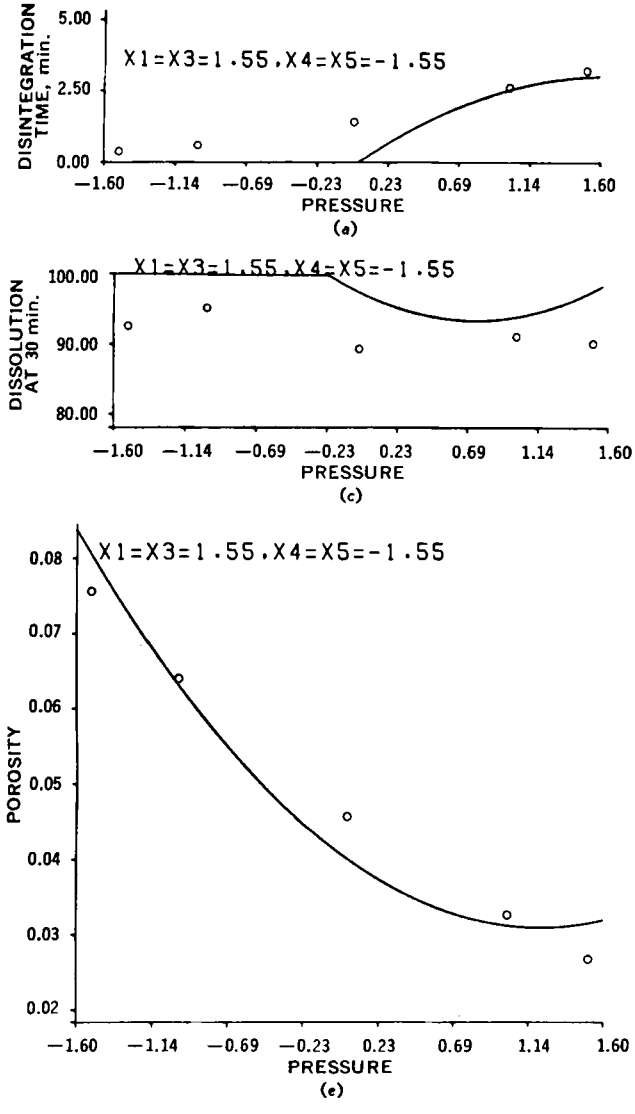
Graphical Approach—Although the computer programs discussed allow one to select the optimum formulation, certain refinements were desirable. Two graphical techniques have proven useful in the optimization procedure.

The first graphical procedure (Fig. 1) is a plot of a given response variable as a function of one of the independent variables. The responses are functions of the five independent variables and can be represented by Eq. 2:

$$Y_i = f(X_1, X_2, X_3, X_4, X_5) \qquad \text{(Eq. 2)}$$

with the full relationship given by Eq. 1. Thus, the relationship between the response and any one variable, *e.g.*, X_1, may be viewed as a partial derivative[12] of Y_i with respect to X_1 while holding all

[12] For convenience, this type of graph is called a "partial derivative" graph, although it is the slope that is actually the partial derivative.



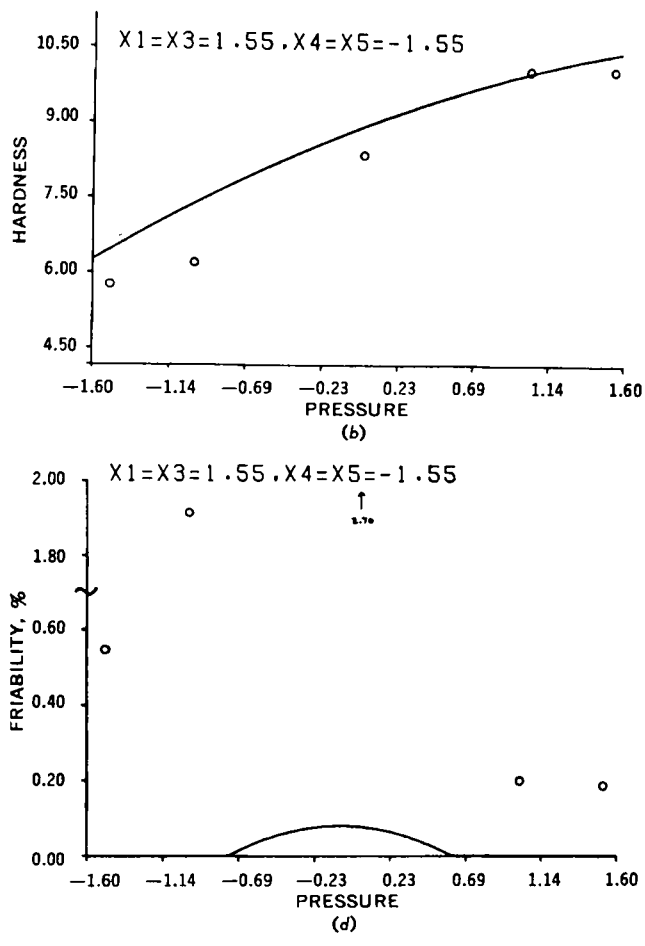


Figure 6—*Plots for several responses as a function of compressional force. The solid lines represent the computer predictions for:* (a) *disintegration time,* (b) *tablet hardness (kilograms),* (c) *dissolution response (percent),* (d) *friability, and* (e) *tablet pore volume; the circles represent experimental points.*

other X's constant. However, because Eq. 1 is a second-order equation with all possible cross-terms, the value of the partial derivatives is still a function of all the independent variables:

$$\left(\frac{\partial Y_i}{\partial X_1}\right)_{X_2X_3X_4X_5} = f(X_1,X_2,X_3,X_4,X_5) \qquad \text{(Eq. 3)}$$

For example, the full partial derivative with respect to X_1 is illustrated in Eq. 4:

$$\left(\frac{\partial Y_i}{\partial X_1}\right)_{X_2X_3X_4X_5} = a_1 + 2a_{11}X_1 + a_{12}X_2 + a_{13}X_3 + a_{14}X_4 + a_{15}X_5 \qquad \text{(Eq. 4)}$$

Thus, the values at which X_2, X_3, X_4, and X_5 are held constant are also important since a change in value will change the graph significantly.

Figure 1 is a plot of disintegration time, Y_1, as a function of the diluent ratio, X_1. In this case, X_2, X_3, X_4, and X_5 are all being held constant at zero or the base level. Note that the X axis is represented in statistical units rather than in physical units; *i.e.*, the experimental range extends from −1.547 to +1.547 experimental units (Table II). The ordinate is represented in the physical units corresponding to the dependent variable plotted. This graph, as well as the others in this series, was drawn from computer tapes[13], so that even the graphical analysis is computerized. The same type of plot can be made for each of the other independent variables, and Fig. 2 shows disintegration time as a function of X_5, the lubricant level.

In fact, one can generate five of these "partial derivative" plots for each of the responses or tablet properties. It is obvious that the paperwork could become overwhelming, so a "composite partial derivative" plot was generated (Figs. 3 and 4). This device enables one to get an overall view of any single response such as tablet hardness. Five of the previous *single* graphs were superimposed by the computer. Each of the five curves represents one of the independent variables, and the X axis represents the experimental field. Keeping the abscissa in terms of experimental units allows this superpositioning. The values at the top of the graph represent the

Table IV—Specifications for Feasibility Search

Variable	Constraint	Experimental Range[a]
Disintegration time, min.	<1 (1)[b] 3 (2) 5 (3)	1.33–30.87
Hardness, kg.	>12 (1) 10 (2) 8 (3)	3.82–11.6
Dissolution, % at 50 min.	>100 (1) 90 (2) 80 (3)	13.3–89.1

[a] It is possible to request values for a response that are more desirable than any data obtained in the set of 27 experiments. [b] (1) = first choice.

[13] By a CalComp plotter, California Computer Corp.

Table V—Optimum Formulation

Variable	Level (in Experimental Units)
Diluent ratio	+1.547
Compressional force	+1.547
Disintegrant level	+1.547
Binder level	−1.547
Lubricant level	−1.547

Table VI—Experimental and Predicted Levels of Responses for Optimum Formulation

Response	Experimental	Predicted
Disintegration time, min.	3.31	3.03
Hardness, kg.	10.08	10.38
Dissolution, % in 30 min.	89.85	98.02
Friability, %	0.18	−0.38[a]
Thickness, mm.	2.31	2.37
Porosity (pore volume), ml./g.	0.0269	0.0318
Mean pore diameter, μ	1.1042	0.5589
Dissolution, % in 50 min.	95.48	117.85[a]
Mean granule diameter, mm.	0.170	0.292
Weight uniformity, *RSD* %	0.74	0.54
Thickness uniformity, *RSD* %	1.14	2.00

[a] See *Footnote 11.*

level at which any variable is being held constant during the partial derivative operation. In this case, all are at zero or the base level.

One could generate an infinite number of these plots because the constant values noted at the top of the graphs can cover the entire experimental range for each variable; therefore, some thought must be given to the selection of the constant levels.

The second type of plot useful in optimizations is the contour plot (Fig. 5), again drawn by computer. Essentially, this allows the representation of a three-dimensional situation in two dimensions and is of special utility when the formulator knows, *a priori*, that two of the variables are of most import.

For the contour plots in Figs. 5*a*–5*d*, the specific response is noted on the graph, as are the fixed values of the three extra variables. Both axes are represented in experimental units this time.

Optimum Formula—Based on the printout of the grid search discussed in a previous section, one of the eight formulations was selected as most suitable. It was the formulation with every variable at its experimental extreme (Table V). Diluent ratio, pressure, and disintegrant were to be at high values; binder and lubricant were to be low.

One might have been able to guess these directions, but it would likely have taken many experiments to locate the exact combination of values. The particular formulation selected as the "optimum" is unlike any one of the 27 prepared as part of the experimental plan.

The formulation was prepared and tested in the same manner as the previous ones. Table VI shows the experimental results and the predicted values for the various responses. Between the initiation of this project and this experiment, several additional responses were added.

The agreement between experimental and predicted values is extremely good, especially for the responses of primary interest: disintegration, hardness, and dissolution.

Experimental *versus* Prediction—The optimum formulation was compressed at various pressures, *i.e.*, as a function of X_2. Some results are illustrated in Figs. 6*a*–6*e*, where the solid line is the computer prediction drawn by the plotter. In general, the agreement is excellent. As noted previously, the values for friability are not quantitative although qualitatively the same convex parabolic shape is present.

SUMMARY AND CONCLUSIONS

The optimization technique presented here has enabled the formulator to select a formulation exhibiting optimum properties. In addition, the large quantity of data generated by the required experimentation and the infinite possibilities of subsequent computerized experimentation and graphical analysis permit the investigator to gain an insight into his or her process.

No previous computer experience and no familiarity with computer languages are required.

The steps necessary for the developmental pharmacist to carry out the optimization program may be summarized as follows:

1. Select a system.
2. Select variables:
   a. Independent.
   b. Dependent.
3. Perform experiments and test product.
4. Submit data for statistical and regression analysis.
5. Set specifications for feasibility program.
6. Select constraints for grid search.
7. Evaluate grid search printout.
8. Request and evaluate:
   a. "Partial derivative" plots, single or composite.
   b. Contour plots.

The last step, which concerns the graphical techniques, may be requested at any time after the regression analysis has been performed and will probably be appropriate at several different stages of a project.

The choice of variables is in the hands of the formulator and there are no restrictions as long as the property can be quantitated. The ultimate goal, of course, would be to relate the formulation variables to responses of proven biological significance, *e.g.*, bioavailability, blood levels, or even therapeutic response.

It is recognized that the stability of a formulation is an important parameter. It is possible, in this method, to include the effects of time and storage conditions by using a stability-indicating property as a response variable.

The technique presented here is not meant to, and cannot, replace the developmental pharmacist. It is simply another tool which can aid in his or her search to further define the pharmaceutical system, and because it can be defined, it can be better controlled.

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Semiautomated Method for Determining Diethylstilbestrol in Low Dosage Tablet Formulations

R. L. HUSSEY▲, J. L. HALE, and D. P. HOWARD

Abstract □ A semiautomated analytical system was developed for the assay of diethylstilbestrol in tablet preparations. The automated methods are similar in principle to the USP manual procedure. The heart of the assay lies in the use of a coiled quartz capillary tubing with reflectorized backing as the irradiation cell. This method is suitable for concentrations of 0.5 mg. or greater active ingredient per tablet.

Keyphrases □ Diethylstilbestrol—analysis in low dosage tablet formulations, semiautomated method □ Tablets, low dosage diethylstilbestrol formulations—semiautomated method □ Analysis—diethylstilbestrol in low dosage tablet formulations, semiautomated method

A semiautomated method was developed for the determination of diethylstilbestrol in low dosage tablets. The method is an extension of the manual assay procedure reported by Goodyear *et al.* (1) in 1954, with the inclusion of techniques described by Banes (2, 3) and Doyle *et al.* (4). The method is similar to the procedure found in USP XVIII (5) and has been demonstrated to yield the same assay results as the USP XVIII method for tablets and raw materials. The continuous flow irradiation technique is similar to that previously reported by Skeggs and Hochstrasser (6), Grasshoff (7), Heinicke *et al.* (8), Love and McCoy (9), and Dowd *et al.* (10).

EXPERIMENTAL

Equipment—The analytical train consisted of the following modules: Technicon Liquid Sampler II, Technicon Proportionating Pump III, a spectrophotometer[1] with 5-mm. flow cell, and a recorder[2]. A 435-w. lamp[3] served as the irradiation source of UV light. The irradiation cell[4] was a quartz capillary tubing of 2-mm. i.d. and 4-mm. o.d. The capillary tubing, of 6.0-ml. liquid capacity, was glass-blower bent into a compact grid 160 mm. long per turn [15.8 cm. (6.25 in.)] (Fig. 1). The capillary was backed with an aluminum reflector to intensify the illumination. The position of the capillary was then adjusted with the lamp on and a sample flowing at steady state. The final adjustment was made so that the yellow chromophore was essentially completely produced by the time the sample passed through the first 25% of the capillary tubing. A mere visual examination, using safety glasses to avoid eye injury, proved adequate for this adjustment. A hood was also employed. The hood served to remove ozone and helped to carry heat away from the irradiation cell.

Reagents—For the 50% ethanol–water reagent, add 500 ml. of SD 3A ethanol to 500 ml. of deionized water in a 1-l. volumetric flask. Mix well but do not dilute to volume.

For the pH 9.7–9.8 buffer (as measured in 50% ethanol), dissolve 5 g. of anhydrous dipotassium hydrogen phosphate in 500 ml. of deionized water in a 1-l. volumetric flask. Add 500 ml. of SD 3A ethanol slowly and with stirring. Do not dilute to volume.

Standards—Dissolve approximately 5 mg. of the reference standard *trans*-diethylstilbestrol, weighed accurately, in 100 ml. of 50% ethanol–water in a 100-ml. volumetric flask. Use ultrasonics for 15 sec. to aid dissolution. Approximately once a month, test the linearity of the assay system. For the linearity test, use approximate weights of 7, 5, 3, and 1 mg. weighed accurately. Dissolve these in the same manner as the 5-mg. reference standard.

Sample Preparation—The individual tablet or core of enteric tablet was weighed. The formulation was then thoroughly ground with a mortar and pestle. The ground formulation was then transferred to a suitable volumetric flask so that the final concentration in the flask would be 0.5 mg./10 ml. Deionized water equal to half the labeled capacity of the flask was added by pipet. Ultrasonic vibration for 15 sec. was employed. Pure alcohol equal to half the labeled capacity of the flask was then added by pipet. The flask was diluted to volume with 50% ethanol–water and allowed to stand for 30 min. prior to assay. The contents of the flask were then filtered through filter paper[5] (medium porosity), and the filtrate was placed into sampling cups of 5-ml. capacity.

Table I—Effect of Excipients on Assay when Added to Standard Diethylstilbestrol

Excipient	Milligrams	Assay, %
Talc	52	100.2
	102	100.2
	216	100.2
Magnesium stearate	20	100.0
	48	100.0
	106	100.2
Starch	62	100.8
	125	100.0
	260	100.0
Lactose	11	99.8
	27	100.2
	51	99.8
	99	100.0

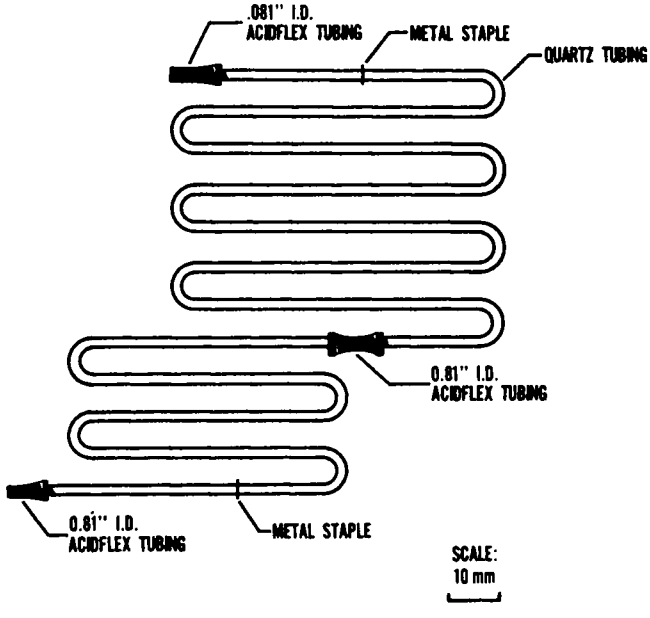

Figure 1—*Irradiation coil design for determination of diethylstilbestrol.*

[1] Turner 330.
[2] Beckman.
[3] Hanovia type 2, serial 7420, equipped with tube 727723.
[4] General Electric, Willoughby, Ohio.
[5] SS 560.

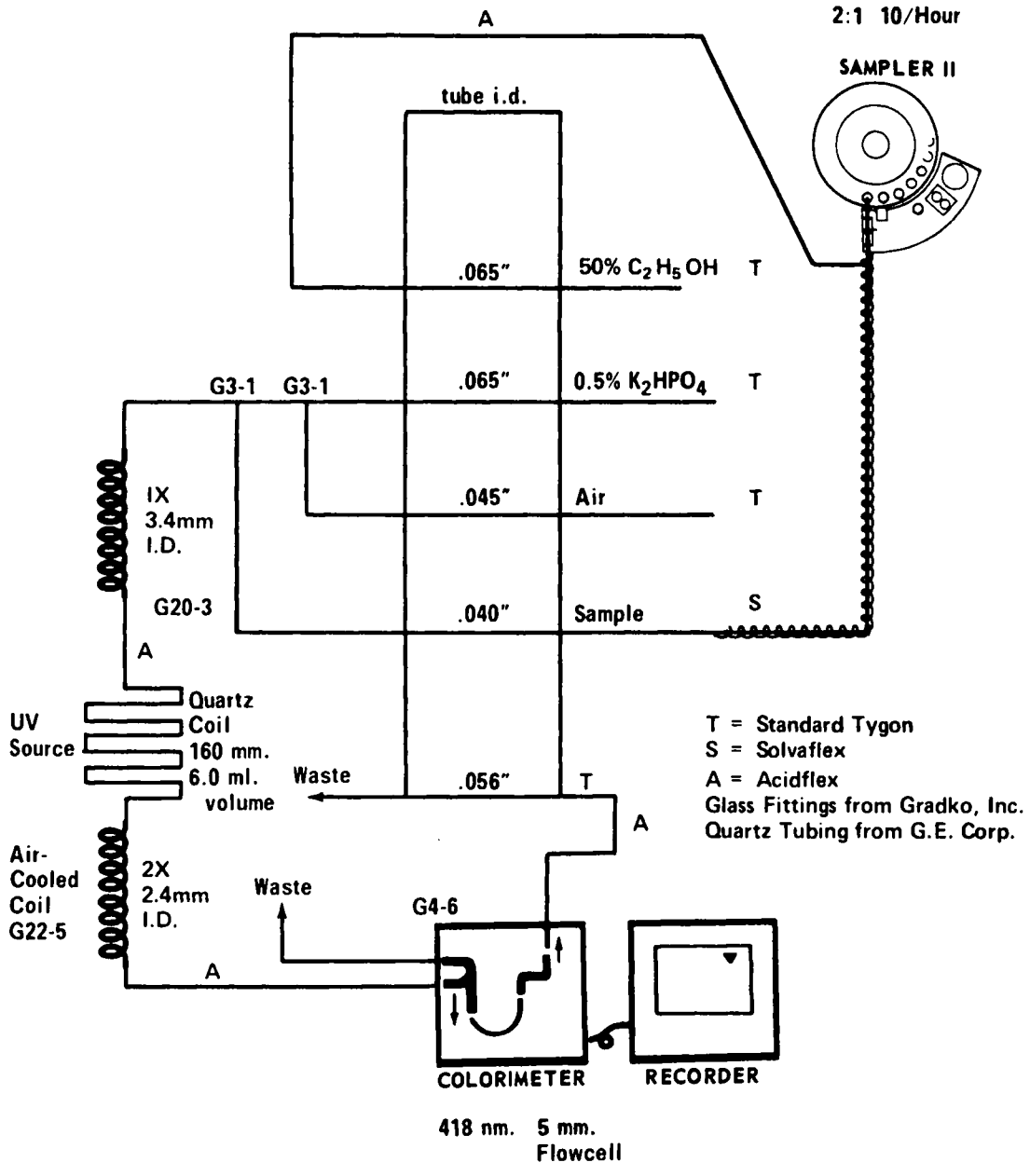

Figure 2—*Autoanalyzer schematic for determination of diethylstilbestrol.*

Procedure—A schematic diagram of the automated system is shown in Fig. 2. A typical assay run is shown in Fig. 3.

DISCUSSION

Two limitations were encountered in the development of this assay. First, tablets of dosage less than 0.25 mg. could not be assayed, owing to sample handling and volume considerations. Second, tablets containing visible dyes, such as core-coated (or finished) enterics, could not be assayed.

The introduction of water prior to alcohol in the tablet dissolution was necessary for complete diethylstilbestrol recovery. Lactose, magnesium stearate, talc, and starch were examined separately and together, both in the presence of diethylstilbestrol and alone, and were shown not to interfere (Table I).

Occasional noise found in the system was traced to aged acidflex tubing. The acidflex tubing (used for its opaqueness) was then changed every 2 weeks as a precaution.

The position of the UV radiation source was the most critical aspect of the analysis. The position needed to be arranged so that the yellow chromophore production occurred in the first 25% of the quartz irradiation coil. Hold time in the coil is approximately 3 min. Visual checking of the chromophore formation, using safety glasses, proved just as adequate as checking the rise-time to steady state and was easier.

Table II—Reproducibility of Assay, Composite Tablets[a]

Day of Assay	Formulation 1, mg./Tablet	Formulation 2, mg./Tablet
19	—	0.248
18	0.501	0.254
	—	0.254
17	—	0.254
15	0.496	—
	0.495	—
2	0.494	—
1	0.496	—
	0.495	—

[a] Relative standard deviation for Formulation 1 was 0.52%; for Formulation 2, 1.20%.

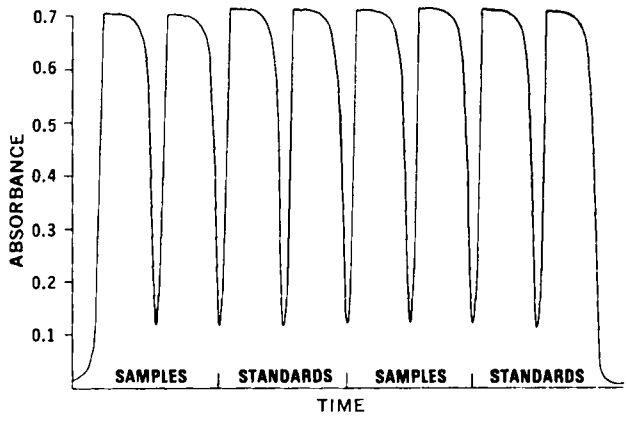

Figure 3—*Typical autoanalysis recording.*

Because of the time required for chromophore formation and wash in the assay, a sampling of 10/hr. was determined to be the maximum rate allowable.

RESULTS

Standard Curves—The absorbances, at 418 nm., of four solutions in the concentration range 0–60 mcg./ml. were measured. A plot of the four values gave a straight line passing through the origin.

Reproducibility of Standards—The reproducibility of the method was checked. The coefficient of variation was 0.005 on standards run as samples (or 0.5% expressed as relative standard deviation).

Reproducibility of Samples—Seven dosage forms, all containing magnesium stearate, starch (and/or paste), lactose, and talc, were automated. The standard deviation for all dosage forms was essentially the same. The method gave day-by-day reliability as well. Table II indicates the typical reproducibility of the assay over 19 days. Not all formulations were run on the same day. Table III compares the manual procedure of USP XVIII to the automated procedure. In this comparison, all samples equivalent to one tablet or core of enteric tablet were weighed from composite grinds to eliminate nonhomogeneity from the comparison. The method has since been employed on a unit dose basis for all seven dosage forms mentioned.

SUMMARY

A semiautomated method for the determination of diethylstilbestrol in tablet dosage forms was described. The automated procedure proved reliable and provided a threefold reduction in assay man-hours.

Table III—Correlation of Automated Method to Manual Method, Composite Tablets

Dosage Form	Strength, mg.	Lot	Manual	Automated
Tablet	0.250	1	0.250	0.242
		2	0.252	0.251
Tablet	0.500	1	0.490	0.482
		2	0.497	0.497
Tablet	1.0	—	1.00	1.04
Tablet	25.0	—	24.8	24.7
Enteric core	0.250	—	0.246	0.250
Enteric core	1.0	—	1.01	1.02
Enteric core	5.0	—	4.90	4.91

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Automated, Simultaneous Determination of Dextromethorphan Hydrobromide, Glyceryl Guaiacolate, and Phenylpropanolamine Hydrochloride in Cough Syrups

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Abstract □ An automated analytical method for the simultaneous colorimetric determination of dextromethorphan hydrobromide, glyceryl guaiacolate, and phenylpropanolamine hydrochloride in cough syrups was developed. The method significantly reduced the analysis time to 0.4 hr./sample and the relative standard deviations obtained were 1.36, 1.16, and 0.79%, respectively.

Keyphrases □ Dextromethorphan hydrobromide mixtures with glyceryl guaiacolate and phenylpropanolamine hydrochloride—automated, simultaneous colorimetric analysis □ Glyceryl guaiacolate mixtures with dextromethorphan hydrobromide and phenylpropanolamine hydrochloride—automated, simultaneous colorimetric analysis □ Phenylpropanolamine hydrochloride mixtures with dextromethorphan hydrobromide and glyceryl guaiacolate—automated, simultaneous colorimetric analysis □ Cough syrups—simultaneous analysis of dextromethorphan hydrobromide, glyceryl guaiacolate, and phenylpropanolamine hydrochloride □ Colorimetry -analysis, dextromethorphan hydrobromide, glyceryl guaiacolate, and phenylpropanolamine hydrochloride mixtures

With the wide use of an effective antitussive combined with an expectorant and a bronchodilator in cough syrups, the need for a rapid simultaneous method of analysis existed for the determination of dextromethorphan hydrobromide, glyceryl guaiacolate, and phenylpropanolamine hydrochloride. Several investigators reported on the simultaneous analysis of cough–cold preparations using GLC. Mario and Meehan (1) determined the three respective components and chlorpheniramine, Rader and Aranda (2) determined 50 compounds including the three components, and Goebbeler (3) determined dextromethorphan hydrobromide and phenylpropanolamine hydrochloride along with chlorpheniramine and salicylamide. However, the reported methods require manual sample preparation, *i.e.*, direct dilution, liquid–liquid extraction, or liquid–liquid partition chromatography, prior to injection of the sample into the gas chromatograph. An automated procedure[1] was developed by Ek *et al.* (4) for the simultaneous determination of total antihistamines by UV spectrophotometry, individual antihistamines by GC, and phenylpropanolamine hydrochloride by colorimetry using the dimethoxytetrahydrofuran reaction.

Although a wide variety of multicomponent pharmaceutical preparations have been analyzed using simultaneous automated procedures (5–7), the specific analysis of the three commonly used components in cough syrups has not been performed simultaneously using an automated system. This paper describes the simultaneous automated determination of the three active components, with initial sample dilution as the only manual step. Dextromethorphan hydrobromide is determined *via* ion-pair formation with bromcresol green, glyceryl guaiacolate is determined *via* reaction with formaldehyde in sulfuric acid–methanol, and phenylpropanolamine hydrochloride is determined *via* the ninhydrin reaction.

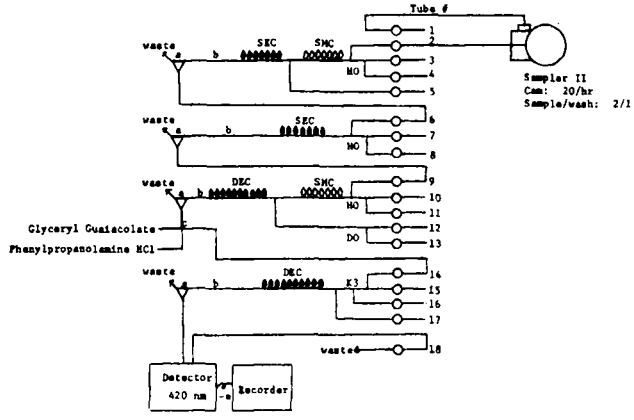

Figure 1—*Flow diagram for sample cleanup, extraction, and dextromethorphan hydrobromide determination. Key: 1, 2.50 ml./min. water (Standard); 2, 2.00 ml./min. sample (Standard); 3, 1.20 ml./min. 1 N hydrochloric acid (Standard); 4, 0.6 ml./min. air (Standard); 5, 2.42 ml./min. petroleum ether (Solvaflex); 6, 2.00 ml./min. sample (Standard); 7, 2.42 ml./min. petroleum ether (Solvaflex); 8, 0.60 ml./min. air (Standard); 9, 1.60 ml./min. sample (Standard); 10, 1.20 ml./min. 1 N sodium hydroxide (Standard); 11, 0.60 ml./min. air (Standard); 12, 13, 2.03 ml./min. chloroform (Acidflex); 14, 1.19 ml./min. sample (Acidflex); 15, 1.71 ml./min. chloroform (Acidflex); 16, 0.60 ml./min. air (Standard); 17, 2.50 ml./min. bromcresol green–buffer solution (Standard); 18, 2.03 ml./min. cell return (Acidflex); SMC, 14-turn mixing coil; SEC, 14-turn beaded coil; DEC, 28-turn beaded coil; a, B-0 separator; b, 15.2-cm. (6-in.) 0.110-i.d. Teflon tubing; and c, G-3 connector.*

EXPERIMENTAL

Reagents—The following reagents were used: 1 *N* hydrochloric acid, 1 *N* sodium hydroxide, petroleum ether[2] (low boiling, 30–60°), and chloroform[3].

pH 5.3 Buffer—Dissolve 38 g. of monobasic sodium phosphate[4], $NaH_2PO_4 \cdot H_2O$, and 3.8 g. of dibasic sodium phosphate[4], $Na_2HPO_4 \cdot 7H_2O$, in sufficient water to make 1 l. of solution. Check the pH and adjust if necessary.

Bromcresol Green–Buffer Solution—Dissolve 0.10 g. of tetrabromo-*m*-cresolsulfonphthalein sodium salt[5] in 1 l. of pH 5.3 buffer. Filter prior to use.

Sulfuric Acid–Methanol–Water Solution (60:20:20)—Cautiously mix 600 ml. of concentrated sulfuric acid with 200 ml. of methanol and 200 ml. of water in an ice bath.

Formaldehyde–Methanol Solution—Dilute 30 ml. of 38% form-

[1] Using the AutoAnalyzer, Technicon Instruments Corp., Tarrytown, N. Y.

[2] J. T. Baker Chemical Co.
[3] Certified ACS, Fisher Scientific Co.
[4] Mallinckrodt Chemical Works.
[5] Allied Chemical Corp.

Table I—Determination of Dextromethorphan Hydrobromide, Glyceryl Guaiacolate, and Phenylpropanolamine Hydrochloride in Cough Syrups

Sample	Components	Label Claim, mg./5 ml.	Manual Assay[a], mg./5 ml.	Automated Assay, mg./5 ml. Range	Automated Assay, mg./5 ml. Average	*RSD*
A	Dextromethorphan hydrobromide	15.0	15.2	15.0–15.3	15.2[b]	0.47
	Glyceryl guaiacolate	100.0	100.5	99.6–101.5	100.8[b]	0.76
B	Dextromethorphan hydrobromide	7.5	7.47	7.64–7.74	7.70[c]	0.58
	Glyceryl guaiacolate	25.0	24.3	25.6–26.1	25.9[c]	0.72
C	Dextromethorphan hydrobromide	7.5	7.56	7.44–7.64	7.54[b]	0.84
	Glyceryl guaiacolate	37.5	37.8	37.7–38.1	38.0[b]	0.83
	Phenylpropanolamine hydrochloride	8.75	8.83	8.72–8.85	8.79[b]	0.51
D	Dextromethorphan hydrobromide	5.0	5.05	5.14–5.20	5.17[c]	0.46
	Glyceryl guaiacolate	50.0	50.4	50.1–51.3	50.6[c]	0.92
	Phenylpropanolamine hydrochloride	12.5	13.4	13.1–13.2	13.1[d]	0.34

[a] Average of two manual determinations. [b] Average of 10 determinations. [c] Average of five determinations. [d] Average of four determinations.

aldehyde solution[2] to 1 l. with absolute methanol.

pH 5 Citrate Buffer—Weigh 84.0 g. of citric acid monohydrate[5] into a 2-l. volumetric flask. Dissolve in 1 l. of water, add 32 g. of sodium hydroxide pellets[6], dissolve, and dilute to volume with water. Check the pH and adjust if necessary.

Ninhydrin Reagent—Dissolve 0.0261 g. of potassium cyanide[4] in 40 ml. of water and dilute to 1 l. with 2-methoxyethanol[7] (Solution I). Dissolve 10 g. of 1,2,3-indantrione monohydrate[7] in 200 ml. of 2-methoxyethanol (Solution II). Mix the total contents of both solutions and allow the reagent to stand overnight prior to use.

Apparatus—The automated system consisted of the following modules: Liquid Sampler II[8]; two Model III proportioning pumps[8]; two variable temperature heating baths[8], each equipped with two 2.4-mm. i.d. × 12.2-m. (40-ft.) coils[8]; three spectrophotometers[9]; and three recorders[10].

Procedure—*Standard Preparation*—Prepare accurately a standard solution in water containing 0.10–0.15 mg./ml. of dextromethorphan hydrobromide, 0.50-1.0 mg./ml. of glyceryl guaiacolate, and 0.15–0.26 mg./ml. of phenylpropanolamine hydrochloride.

Sample Preparation—Dilute accurately an aliquot of the cough syrup containing 10–15 mg. of dextromethorphan hydrobromide, 50–100 mg. of glyceryl guaiacolate, and 15–26 mg. of phenylpropanolamine hydrochloride to 100 ml. with water.

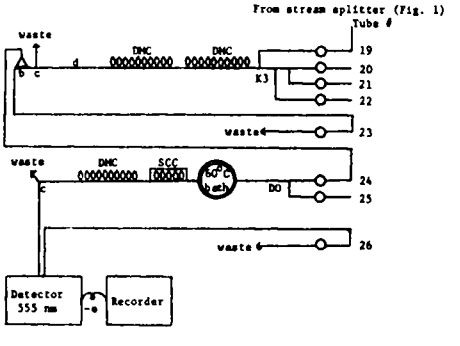

Figure 2—*Flow diagram for glyceryl guaiacolate determination. Key: 19, 1.19 ml./min. sample (Acidflex); 20, 2.03 ml./min. sulfuric acid–water–methanol reagent (Acidflex); 21, 0.6 ml./min. air (Standard); 22, 0.56 ml./min. formaldehyde–methanol reagent (Solvaflex); 23, 1.44 ml./min. waste (Acidflex); 24, 1.71 ml./min. sample (Acidflex); 25, 0.42 ml./min. air (Standard); 26, 1.19 ml./min. cell return (Acidflex); DMC, 28-turn coil; SCC, 14-turn jacketed cooling coil; a, C-1; b, B-0; c, C-3; and d, 15.2-cm. (6-in.) 0.110-i.d. Teflon tubing.*

[6] Fisher Scientific Co.
[7] Matheson, Coleman and Bell.
[8] Technicon Instruments Corp., Tarrytown, N. Y.
[9] Hitachi-Perkin-Elmer model III, Coleman Instruments.
[10] Model SRL, E. H. Sargent and Co.

Sample Testing—Place portions of the samples and standards into the 8.5-ml. polystyrene Liquid Sampler II cups. Place the samples in the odd-numbered spaces in the Liquid Sampler II turntable, interspersing the standards at periodic intervals. Into the even-numbered spaces, place cups containing water. The simultaneous automated analysis is then performed using the schematic flow diagrams shown in Figs. 1–3. Record the absorbances of the samples and standards at 420 nm. for dextromethorphan hydrobromide, at 555 nm. for glyceryl guaiacolate, and at 570 nm. for phenylpropanolamine hydrochloride.

RESULTS

The precision and accuracy for the measurement of each of the components were established by analyzing individual standard aliquots and four marketed cough syrups. For a minimum of 20 determinations, the relative standard deviations for dextromethorphan hydrobromide, glyceryl guaiacolate, and phenylpropanolamine hydrochloride were 1.36, 1.16, and 0.79% at a concentration of 0.10, 1.00, and 0.25 mg./ml., respectively. Plots of the absorbance *versus* concentration for aliquots of dextromethorphan hydrobromide, glyceryl guaiacolate, and phenylpropanolamine hydrochloride are presented in Fig. 4.

Samples of the four marketed cough syrups were analyzed using the automated colorimetric methods. The results obtained for dextromethorphan hydrobromide and glyceryl guaiacolate in cough syrups, Samples A–D, and phenylpropanolamine hydrochloride in cough syrups, Samples C and D, along with the labeled components and manual assay values, are presented in Table I.

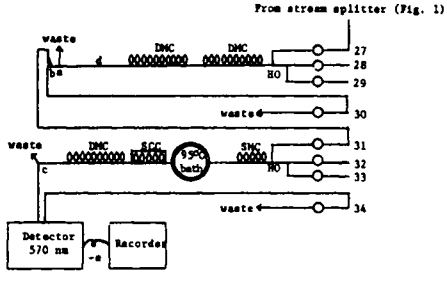

Figure 3—*Flow diagram for phenylpropanolamine hydrochloride determination. Key: 27, 1.19 ml./min. sample (Acidflex); 28, 2.50 ml./min. pH 5 citrate buffer (Standard); 29, 0.60 ml./min. air (Standard); 30, 1.44 ml./min. waste (Acidflex); 31, 2.03 ml./min. sample (Acidflex); 32, 1.44 ml./min. ninhydrin reagent (Acidflex); 33, 0.60 ml./min. air (Standard); 34, 2.42 ml./min. cell return (Solvaflex); DMC, 28-turn coil; SMC, 14-turn coil; SCC, 14-turn jacketed cooling coil; a, C-1; b, C-0; c, C-3; and d, 15.2-cm. (6-in.) 0.110-i.d. Teflon tubing.*

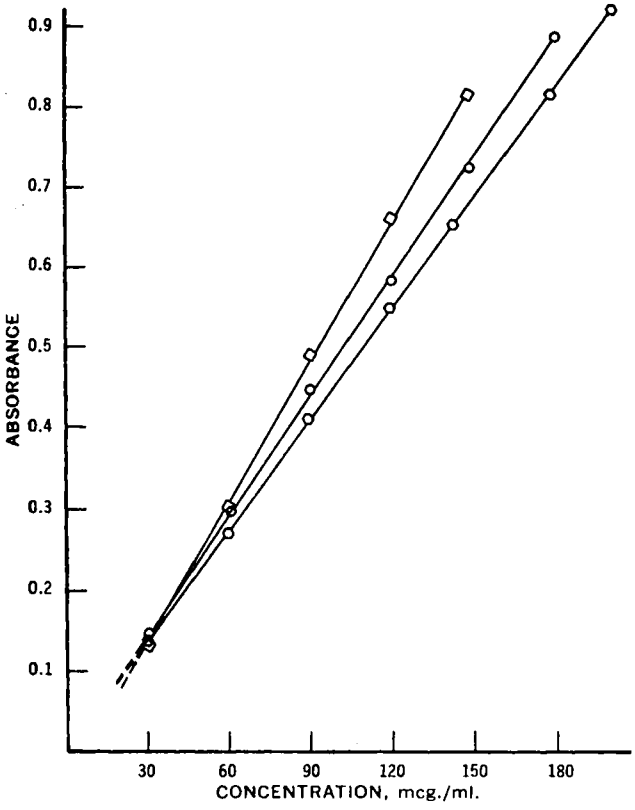

Figure 4—*Standard curves for dextromethorphan hydrobromide, glyceryl guaiacolate, and phenylpropanolamine hydrochloride. Key: O, dextromethorphan hydrobromide (micrograms per milliliter); □, glyceryl guaiacolate (micrograms per milliliter × 6.67); and ○, phenylpropanolamine hydrochloride (micrograms per milliliter × 1.67).*

Portions of the scans obtained for the sample analysis including standards are presented in Figs. 5–7.

DISCUSSION

The automated system was designed specifically for cough syrups containing only the following active components: dextromethorphan hydrobromide, glyceryl guaiacolate, and phenylpropanolamine hydrochloride. The system can be employed for the simultaneous determination of all three or any two components as well as the individual determination of any single component. The combined analytical methodologies cannot be completely applied to formulations containing other active ingredients, such as chlorpheniramine maleate, which would interfere with the colorimetric determination of dextromethorphan hydrobromide.

In the developed automated procedure, an aliquot of the sample was diluted with water and a portion was placed into the sample cups. An aliquot was automatically aspirated, acidified with 1 *N* hydrochloric acid, and extracted with petroleum ether, which was discarded after phase separation. A portion of the aqueous phase was then made alkaline with 1 *N* sodium hydroxide, and the three components were extracted into chloroform. After phase separation, the chloroform stream was divided into three segments by the stream-splitting technique.

One segment of the chloroform-extracted sample was used for formation of the dextromethorphan hydrobromide ion-pair with bromcresol green at pH 5.3, extraction of the complex into the chloroform phase, and measurement at 420 nm. The second segment was utilized for the colorimetric determination of glyceryl guaiacolate. After extraction and phase separation, the chloroform, lower phase, was discarded and the upper phase was passed through a heating bath (60°) for color development. The solution was cooled to room temperature and the resultant color was measured at 555 nm. An alternative extraction procedure was utilized instead of evaporating the chloroform extract to dryness with an in-line evaporator-digestor. Glyceryl guaiacolate was extracted into the sulfuric acid–methanol–water and formaldehyde–methanol reagents. A combination of sulfuric acid–methanol water (60:20:20) was found to yield an efficient phase separation. The third segment of the chloroform stream was used for the colorimetric determination of phenyl-

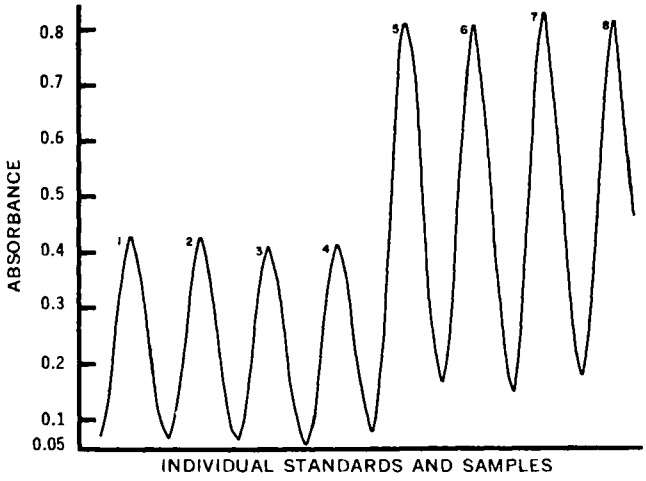

Figure 6—*Typical scans for glyceryl guaiacolate standards and samples. Key: standards, numbers 3, 4, 5, and 6; and samples, numbers 1, 2, 7, and 8.*

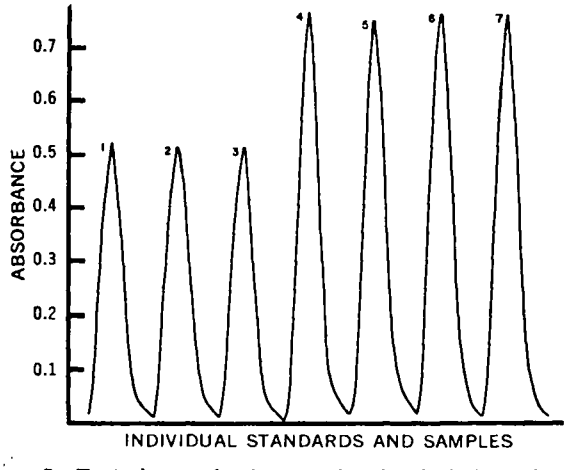

Figure 5—*Typical scans for dextromethorphan hydrobromide standards and samples. Key: standards, numbers 3, 4, and 5; and samples, numbers 1, 2, 6, and 7.*

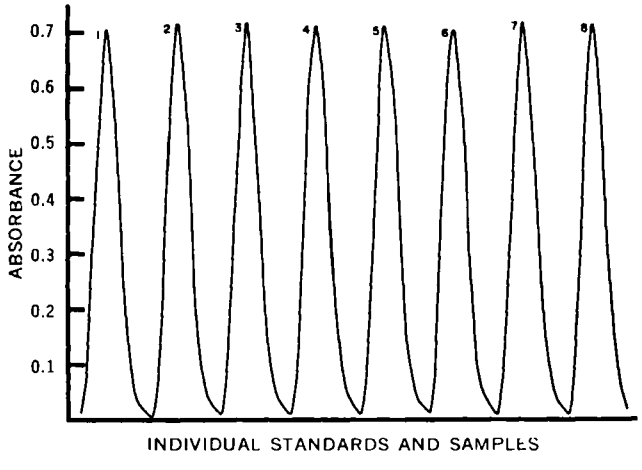

Figure 7—*Typical scans for phenylpropanolamine hydrochloride standards and samples. Key: standards, numbers 1 and 6; and samples, numbers 2, 3, 4, 5, 7, and 8.*

propanolamine hydrochloride. After extraction of the phenylpropanolamine from chloroform into pH 5 citrate buffer and separation of the phases, the chloroform was discarded and a portion of the aqueous phase was mixed with ninhydrin reagent. This mixed stream was passed through a heating bath (95°) for color development, cooled to room temperature, and measured at 570 nm.

The developed simultaneous automated system significantly reduced the analysis time. Previously, about 8 and 12 hr. were required to perform the comparable manual procedures for two-component and three-component preparations, respectively. Based on a single-lot determination with the automated procedure, the time required for analysis was 3 hr. This represented a time savings of 5–9 hr., depending on the number of components present. Since the automated system is capable of analyzing 20 samples/day in duplicate, a reduction in analytical time to 0.4 hr./sample can be realized.

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Molecular-Scale Drug Entrapment as a Precise Method of Controlled Drug Release IV: Entrapment of Anionic Drugs by Polymeric Gelation

JAMES C. BOYLAN* and GILBERT S. BANKER▲

Abstract □ A physicochemical approach to the preparation of drug-containing matrix systems is described in which a soluble anionic drug may be entrapped on a molecular scale in coagulated (gelled) polymer emulsion systems. The resultant dried product (or drug–xerogel system) was designed to provide controlled, prolonged release. The phenomenon of gelation of the polymer emulsions by the addition of a divalent cation (Mg^{+2}) was utilized for the entrapment of various drug materials. A solid, highly reproducible entrapment compound of sodium phenobarbital, magnesium sulfate, and a styrene-acrylic copolymer latex was prepared and subjected to *in vitro* and *in vivo* prolonged-release studies. The physical factors influencing both entrapment and drug release were investigated. A significantly increased duration of therapeutic effectiveness was established by the *in vivo* results. Rats, fed dry polymer powder in their diet, exhibited no toxic effects in a 27-day study. The validity and reproducibility of the entrapment procedure were demonstrated.

Keyphrases □ Polymer emulsion systems—entrapment of anionic drugs by gelation, prolonged-release rates, methods, prepared with sodium phenobarbital, tested in rats □ Timed-release formulations—molecular-scale drug entrapment as a precise method of controlled drug release, anionic drugs by polymeric gelation, prepared with sodium phenobarbital, release rates, rats □ Gelations, polymeric—entrapment of anionic drugs (sodium phenobarbital), methods of preparation, release rates, tested in rats □ Phenobarbital—prolonged-release formulation prepared by molecular-scale drug entrapment, release rates, rats □ Anionic drugs—prolonged-release sodium phenobarbital formulation prepared by molecular-scale drug entrapment, release rates, rats □ Drug release—sodium phenobarbital from a prolonged-release formulation prepared by molecular-scale drug entrapment (polymeric gelation), rats

The inclusion of soluble drugs in insoluble matrixes is well known as a means of controlling drug release rates from solid dosage forms. Diffusional models describing drug release from such systems were thoroughly described by T. Higuchi (1) and W. Higuchi (2). Systems have been designed in which "channeling agents" are added to the matrix to attract fluid into the system as well as to facilitate drug diffusion from the matrix (3). Recently, others described (4) the use of dry gels of cross-linked polymer which are charged by immersion in solutions of the drug. Charging of drugs into cross-linked polymers offers a unique method of releasing drug into the eye for very long periods from soft lens systems or other ocular inserts (5). Other drug release systems from matrixes have obvious application for intrauterine and assorted implantable devices, which might range from being totally insoluble to completely soluble or biodegradable.

In previous papers in this series (6–10), it was demonstrated that cationic drug materials could be entrapped in the solid matrix of a flocculated (linear acrylic acid–methacrylic acid copolymer) polymer emulsion system[1] in such a manner as to exhibit reproducible control of drug release and prolongation of drug action from the resultant dried material. The advantages of polymer emulsion systems for this purpose include a high solids

[1] Acrysol ASE 75, Rohm and Haas Co., Philadelphia, Pa.

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[1] Acrysol ASE 75, Rohm and Haas Co., Philadelphia, Pa.

Table I—Polymer Emulsions Studied and Their Designation

Polymer Emulsion Designation	Chemical Classification	Charge	Solids, %	pH
1	Styrene–acrylic latex[a]	Nonionic	46–48	8.0
2	Styrene–acrylic latex[b]	Anionic	46–48	9.0–9.5
3	Modified acrylic[c]	Nonionic	44–46	9.4–9.9
4	Modified acrylic[d]	Anionic	27.5–28.5	3.5
5	Modified acrylic[e]	Nonionic	19.5–20.5	3.0
6	Modified acrylic[f]	Nonionic	46	9.0–9.5
7	Modified acrylic[g]	Nonionic	45–47	9.0–10.0
8	Modified acrylic[h]	Anionic	38	9.5–10.0
9	Modified acrylic[i]	Nonionic	44.5–45.5	7.5–9.0
10	Ethyl acrylate–vinyl pyrrolidone[j]	Anionic	40	6–8
11	Styrene–vinyl pyrrolidone[k]	Anionic	40	2–4
12	Styrene–vinyl pyrrolidone[l]	Anionic	40	2–4
13	Vinyl acetate–vinyl pyrrolidone[m]	Emul. free	40	3–4
14	Polyethylene[n]	Nonionic	41	9.0
15	Acrylic copolymer[o]	Anionic	35	8.5–9.5
16	Styrene–acrylic[p]	Anionic	39–41	7–8
17	Vinyl–acrylic latex[q]	Anionic	45	4.3
18	Vinyl–acrylic copolymer[r]	Anionic	55	4.5
19	Vinyl–acetate copolymer[s]	Anionic	55	4.5
20	Acrylic copolymer[t]	—	49–51	5–6

[a] Acriflo-151, Chemical Division, The General Tire and Rubber Co., Akron, Ohio. [b] Lytron 680, Plastics Division, Monsanto Chemical Co., Springfield, Mass. [c] MC-4530, Rohm and Haas Co., Philadelphia, Pa. [d] Acrysol ASE 60, Rohm and Haas Co., Philadelphia, Pa. [e] Acrysol ASE 95, Rohm and Haas Co., Philadelphia, Pa. [f] Rhoplex AC 33, Rohm and Haas Co., Philadelphia, Pa. [g] Rhoplex AC 200, Rohm and Haas Co., Philadelphia, Pa. [h] Rhoplex B 85, Rohm and Haas Co., Philadelphia, Pa. [i] Rhoplex C 72, Rohm and Haas Co., Philadelphia, Pa. [j] Polectron 130, GAF Corp., New York, N. Y. [k] Polectron 430, GAF Corp., New York, N. Y. [l] Polectron 450, GAF Corp., New York, N. Y. [m] Polectron 845, GAF Corp., New York, N. Y. [n] Poly-Em 20017, Spencer Chemical Co., Kansas City, Mo. [o] NeoCryl W63M, Polyvinyl Chemicals Inc., Peabody, Mass. [p] NeoCryl A247H, Polyvinyl Chemicals Inc., Peabody, Mass. [q] Resyn 25-2833, National Starch and Chemical Corp., New York, N. Y. [r] Resyn 25-2243, National Starch and Chemical Corp., New York, N. Y. [s] Resyn 25-1255, National Starch and Chemical Corp., New York, N. Y. [t] Ubatol U-7001, UBS Chemical Co., Cambridge, Mass.

content (usually 40–50%) and a very low viscosity (normally 100–200 cps.).

In the previous studies (6–8), all polymer emulsion systems were observed to flocculate on being coagulated. The flocculated products were dense and nonporous and required polymer hydration and dissolution to achieve satisfactory drug release. Polymers containing carboxyl functionality had appropriate solubility and were available as anionic polymer emulsions. These emulsions were effective in the entrapment of cationic drugs but were ineffective in the entrapment of anionic drugs. Some other type of system was obviously required to achieve molecular-scale entrapment of anionic drugs. In addition, a polymer latex that would gel on coagulation rather than flocculate would provide a less dense, more porous entrapment matrix and might permit the use of less soluble or insoluble polymers as the matrix or carrier.

The purposes of this investigation were to: (*a*) study the phenomenon of gelation of polymer emulsions, as induced by anionic drugs with and without added electrolytes; (*b*) elucidate the factors affecting drug entrapment by polymeric gelation, including polymer concentration, method of preparation, and added electrolytes; (*c*) study the factors affecting drug release rate, including particle size, method of drying, and drug concentration; and (*d*) evaluate these drug–polymer systems using recognized *in vitro* and *in vivo* techniques.

EXPERIMENTAL

Materials and Equipment—The medicinal agents used were either USP or NF quality. The purity of each was checked utilizing melting-point determinations. Table I summarizes the main properties of the polymer emulsions screened and their designation in this paper.

Particle-size reduction was accomplished using a comminutor[1].

Polymer Emulsion Screening—Coagulating of the polymer emulsions of Table I in the presence of various salts was performed using a test tube method outlined by Jirgensons and Straumanis (11). Uni/univalent or bi/bivalent electrolytes (sodium chloride and magnesium sulfate) were used to coagulate the anionic and nonionic polymer emulsions studied. Sodium phenobarbital was used as an anionic model drug substance throughout the study.

Sodium Phenobarbital Assay Procedure—A method of determining the sodium phenobarbital content in the presence of polymer was developed using a differential UV spectrophotometric procedure[2] first suggested by Walker *et al.* (12) and later by others (13–16). The procedure used routinely was as follows. An accurate aliquot of supernatant solution was removed and buffered to pH 10.5 in a volumetric flask. A second identical aliquot was removed and brought to volume with 0.2 *M* NaOH solution.

By using the pH 10.5 solution in the reference side and the 0.2 *M* NaOH solution in the sample side, the differential absorbance was measured at 260 nm. A plot of sodium phenobarbital concentration *versus* absorption was found to be linear and passed through the origin, allowing the accurate determination of the drug in the presence of polymer and magnesium sulfate.

The Entrapment Procedure—The following procedure illustrates the entrapment method commonly employed. The formulation used (Formula I) was: sodium phenobarbital, 1.20 g.; magnesium sulfate, 0.80 g.; distilled water, 20.00 ml.; and Polymer Emulsion 2, 25.00 ml. (11.75 g. of solids). (The USP cathartic dose of magnesium sulfate is 15 g. Calculations show that a dose of this sustained product will give a patient a negligible 73 mg. of magnesium sulfate.)

Procedure A—Fifty milliliters of a solution of magnesium sulfate and sodium phenobarbital was prepared and transferred to a volumetric flask. A 20.0-ml. aliquot of this solution was then transferred to a 100-ml. beaker. The polymer emulsion was added to the drug solution from a freely draining 25-ml. pipet with constant mixing;

[1] Fitzpatrick model M.

[2] A double-beam, UV visible, automatic recording spectrophotometer (Bausch & Lomb Spectronic 505) was used to develop the assay procedure. A Beckman model DU spectrophotometer was used for routine UV spectral analysis. All IR spectra were obtained using a double-beam IR spectrophotometer (Perkin-Elmer model 21).

Table II—pH Gradient for the *In Vitro* Release Rate Test

Hours	Amount of Fluid Removed[a], ml.	pH of Test Fluid
0	—	1.5
0.5	50.0[b]	1.5
1.5	30.0	2.2
2.5	50.0	7.5
4.5	50.0	7.5
6.5	50.0	7.5
8	50.0	—

[a] With one exception (see *Footnote b*), the fluid removed was replaced by an equal amount of pH 7.5 buffer solution (prewarmed to 37°). [b] This sample bottle was removed after 0.5 hr. and assayed. All of the remaining samples were taken from a second identical sample bottle.

the mixture was stirred for an additional 30 sec. and was then allowed to stand for 2 min. During this interval, a solid white gel of firm semisolid consistency formed. This material was transferred to an uncovered Petri dish by means of a spatula. During the next hour, the small amount of water (usually about 3–5 ml.) that leached out of the gel due to syneresis was carefully siphoned off. The gel was then allowed to air dry for 12 hr. At the end of this time, the material was broken into small pieces by hand, oven dried at 45° for 24 hr., and air dried for an additional 24 hr. The dried material was ground in a comminuter (previously cooled by passing dry ice through the machine). This served to prevent sticking of the polymer due to the heat produced by processing. The comminuted material was then sized. Particles that passed through a 40-mesh screen were used for *in vitro* testing.

Several alternative methods of preparing entrapment products were studied to evaluate their effects on the entrapment process and release of entrapped drug. These alternative procedures were as follows.

Procedure B—The solution containing the sodium phenobarbital and the magnesium sulfate was added to Polymer Emulsion 2 (the reverse was done in *Procedure A*).

Procedure C—An aqueous solution of magnesium sulfate was added to an aqueous mixture of the sodium phenobarbital and polymer emulsion.

Procedure D—The product was vacuum dried.

Procedure E—Alcohol USP was used as the drug solvent.

Solubility being a factor, magnesium sulfate was not used and phenobarbital replaced sodium phenobarbital.

Dialysis Procedure—Dialysis studies were used as an *in vitro* technique to ascertain whether or not the sodium phenobarbital and polymer were physically or chemically bound since permanent drug binding would be expected to reduce the therapeutic effectiveness of the sodium phenobarbital. The dialysis sacs were prepared from a semipermeable cellulosic membrane[4]. The closed sac, containing 20 ml. of sample solution, was placed in a 90-ml. (3-oz.) amber glass jar containing simulated gastric fluid USP (without pepsin). The jar was capped, sealed with a water-resistant tape, and

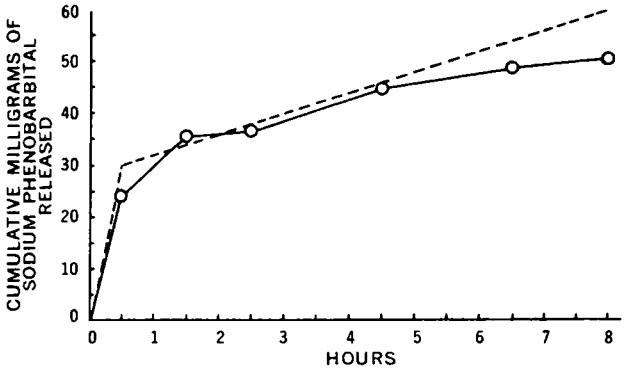


Figure 1—In vitro *release pattern—idealized* versus *experimental—for the four gels of Table VI. Key: - - -, idealized curve; and ——, experimental curve.*

[4] NoJax Casing, size 30, Visking Co., Chicago, Ill.

Table III—Oral Chronic Toxicity of Polymer Emulsion 2 Using Urine and Feces Analyses

Test[a]	Sensitivity
Proteinuria[b]	Detects less than 30 mg./100 ml. urine
Glucose[c]	Detects less than 0.5% in urine
Ketouria[d]	Detects 10 mg. of acetoacetic acid/100 ml. urine; reacts readily with acetoacetic acid but not with acetone
Bilirubin[e]	Detects 0.05 mg./100 urine
Occult blood[f]	Detects 1 part in 20,000

[a] All of the tests used are products of the Ames Division of Miles Laboratories, Elkhart, Ind. [b] Albustix test (urine). [c] Clinistix test (urine). [d] Ketostix (urine). [e] Ictotest (urine). [f] Hematest (feces).

allowed to tumble in the rotating-bottle apparatus for the specified time at 37°. Aliquot samples were taken both inside and outside the dialysis sac and assayed.

***In Vitro* Release Rate Procedure**—A rotating-bottle method (17, 18) was selected to evaluate the granular xerogel products. The comminuted samples were weighed in two individual 1.250-g. dosage units, and each sample was placed in a 90-ml. (3-oz.) amber glass jar. Fifty milliliters of simulated gastric fluid USP (without pepsin) was added, and the bottles were sealed as described earlier and were rotated at 41 r.p.m. at 37°. The first bottle was removed after 0.5 hr., the liquid was quantitatively filtered into a volumetric flask, and the solution (after proper dilutions) was assayed spectrophotometrically. The pH of the fluid in the second bottle was gradually changed from 1.5 to 7.5 over 2.5 hr. (Table II). Samples were assayed at each interval

The pH 7.5 intestinal buffer was not simulated intestinal fluid USP. The 0.2 *M* NaOH solution required in the sodium phenobarbital assay procedure precipitated the monobasic potassium phosphate of the USP fluid, but a pH 7.5 borate buffer was substituted with good results.

***In Vivo* Procedure**—*Activity Cages*—Williamson activity cages (19) located in an isolated, dark room were used to evaluate the degree and length of sedation of sodium phenobarbital administered in solution (as evidenced by a decrease in animal activity) *versus* a suspension of the sodium phenobarbital–magnesium sulfate–Polymer Emulsion 2 xerogel. The studies were performed on previously nondrugged, male, Holtzman strain, albino rats, weighing from 280 to 340 g. Male animals were selected because they demonstrate a shorter induction time as well as shorter duration of phenobarbital narcosis as compared to female rats (20). Four animals were randomly selected from the colony. Following 24 hr. of food deprivation (water was given *ad libitum*), the animals were weighed and placed in individual activity cages for a 0.5-hr. period of acclimatization. The animals were then briefly removed to a separate room for

Table IV—Coagulation of Polymer Emulsions

Polymer Emulsion Number[b]	Millimolar Coagulation Values[a]: Sodium Phenobarbital (Maximum Concentration 400 mmoles/l.)	Sodium Chloride (Maximum Concentration 1375 mmoles/l.)	Magnesium Sulfate (Maximum Concentration 325 mmoles/l.)
1	—	—	50–60[c]
2	—	—	50–60
4	180–200	350–400	65–80
5	180–200	1500	—
8	—	500	30–40
16	—	600–700	40–50
20	—	1000–1200	200–250

[a] Coagulation value is the concentration of the salt in millimoles per liter in the final system required to cause complete coagulation (gelation or flocculation) within 0.5 hr. A blank space indicates no coagulation occurred at the upper concentration stated for each salt. Coagulation, if it occurred, was usually immediate. If this rapid coagulation did not occur, the tubes were left undisturbed for 0.5 hr. and then reexamined for coagulation. [b] Polymer emulsion designations are given in Table I. [c] The concentration of the drug or electrolyte in millimoles per liter in the final coagulated system.

Table V—Dialysis Data for Sodium Phenobarbital–Polymer Emulsion 2 Xerogels in Gastric Fluid

Gel (and Replicates)	Preparation Procedure	Milligrams of Drug Entrapped/ 250 mg.[a]	Original Molarity, $M \times 10^{-3}$	Molarity Inside after 72 hr., $M \times 10^{-4}$	Molarity Outside after 72 hr., $M \times 10^{-4}$	Average Concentration Inside and Outside, $M \times 10^{-4}$	Percent Free Drug	Average Percent Free Drug
1	A	22	4.3	5.3	5.3	5.3	55	
1_1	A	22	4.3	5.4	5.6	5.5	58	
1_2	A	22	4.3	5.4	5.3	5.35	55	56.5
1_3	A	22	4.3	5.4	5.4	5.4	58	
2	C	22	4.3	3.0	4.8	3.9	46	
2_1	C	22	4.3	4.6	4.5	4.55	48	48.0
2_2	C	22	4.3	4.7	4.9	4.8	51	
3	B	22	4.3	7.1	7.1	7.1	74	
3_1	B	22	4.3	8.9	6.5	7.7	73	73.5
4	B	28	5.3	8.9	9.9	9.4	82	
4_1	B	28	5.3	9.1	9.2	9.15	77	79.5
5	C	28	5.3	6.3	7.6	6.95	62	
5_1	C	28	5.3	7.0	7.4	7.2	62	62.0

[a] Gels containing 22 mg./250 mg. were prepared by Formula I. [b] These values were obtained by dividing the total milligrams of drug found in solution after 72 hr. (inside and outside the sac) by the milligrams of xerogel entrapped drug and multiplying by 100.

oral dosing. All animals were dosed between 3:10 and 4:00 p.m. to eliminate diurnal activity variation (rats being more active at night).

The treatments were: (*a*) sodium phenobarbital, 50 mg./kg.; (*b*) sufficient suspended sodium phenobarbital–gel to release 50 mg./kg. of drug initially and a second 50 mg./kg. dose in sustained form (previously determined by the *in vitro* method already described); and (*c*) an equivalent volume of suspending agent alone (one part syrup USP and two parts water was found to be a good suspending agent for the drug-gel particles). The 50-mg./kg. dose of sodium phenobarbital was chosen from preliminary studies because of its ability to sedate the animals without inducing sleep. Malone *et al.* (20) reported that the volume of the vehicle influences the onset of sodium phenobarbital action. Consequently, fluid volume was maintained at 8 ml. After dosing, the rats were returned to the activity cages for a predetermined period.

Acute Toxicity—The purpose of this study was to determine any gross difference in acute toxicity between sodium phenobarbital and the drug-xerogel product. Since the action of sodium phenobarbital lasts much longer in the female rats as compared to the male (20), females were used. The oral LD_{50} of sodium phenobarbital in female rats reported from different laboratories was found to be quite variable (150–350 mg./kg.).

All animals used in the acute toxicity evaluation were fasted for 24 hr. prior to dosing. Starting with 150 mg./kg., rats were orally dosed with sodium phenobarbital in 25-mg. increments. The vehicle used was the same syrup–water combination previously mentioned. An approximate LD_{50} of 200 mg./kg. was established for the experimental conditions.

Twenty previously nondrugged, female, Holtzman strain, albino rats, weighing from 130 to 275 g., were used in the final acute toxicity evaluation. Ten rats received 200 mg./kg. of unmodified drug, and 10 were given an amount of drug–xerogel powder sufficient to release 200 mg./kg. of drug initially and a second 200-mg./kg. dose in sustained form (previously determined by the *in vitro* method).

Chronic Toxicity–Twenty 50-g. female rats were involved in this phase of the research. Ten animals were kept in a common cage and served as a control group, and the other 10 were kept in a separate, common cage. The control group was fed a daily measured quantity of powdered chow. The experimental group was fed in an identical manner except a quantity of solid, gelled, dried polymer (see Formula II) equivalent to 7.50 g./kg. of drug per rat was mixed daily with the rat chow powder. The rats were kept on their diets for 27 days.

The second formulation tested (Formula II) consisted of: Polymer Emulsion 2, 25.0 g.; and magnesium sulfate, 2.5 g. (The solid polymer was very tacky and difficult to work with. The presence of this small amount of magnesium sulfate made the solid polymer of Emulsion 2 much easier to handle.)

The animals were examined daily for gross physical changes. Twice weekly the animals were weighed. On these same days, they were placed in metabolism cages and their urine and feces were collected. Table III summarizes the tests run on the collected material.

At the conclusion of the 27 days, the animals were sacrificed. The kidneys, liver, and spleen were grossly examined and removed, and the net weight of each was determined. The organs were then placed in an oven (overnight) and weighed again to obtain a measure of the dry weight. In this manner, any differences in tissue weights between the control and experimental groups could be attributed to a change in either water content or tissue content.

Table VI—Reproducibility of *In Vitro* Release Rates of Sodium Phenobarbital–Magnesium Sulfate–Polymer Emulsion 2 Xerogels

Gel Replicate Number	Milligrams of Drug[a] 0.5 hr.	1 hr.	2 hr.	4 hr.	6 hr.	8 hr.
1	24.0	38.7	38.6	45.8	47.8	49.0
1_1	24.8	36.0	38.8	48.2	42.0	53.0
1_2	24.0	32.7	32.8	41.0	46.0	48.9
1_3	24.0	34.3	36.3	44.3	48.9	51.5
Mean	24.2	35.4	36.6	44.8	48.7	50.6
SD	0.40	2.56	2.79	3.02	2.52	2.00
Coefficient of variation	1.7%	7.2%	7.6%	6.7%	5.2%	4.0%

[a] Cumulative release from 1.250-g. gel sample.

RESULTS AND DISCUSSION

Polymer Screening—The results of the test tube emulsion coagulation procedure are shown in Table IV. Only those polymer emulsions that were coagulated by the drug system are shown. Four of the seven polymer emulsions were eliminated[b] because they appeared to flocculate rather than gel on coagulation and were listed only as "modified acrylics" in company literature. It was felt that the structures were too indefinite and probably very similar to the structure of the acrylic copolymer used by Goodman and Banker (6) to warrant extensive study in this project. Of the polymer emulsions left, all of which were styrene–acrylic latexes, Polymer Emulsion 2 was chosen for further investigation. The solid polymeric material precipitated from Polymer Emulsion 2 using 0.1 *N* HCl was insoluble in aqueous media over a wide pH range (from pH 1.0 to 0.2 *M* NaOH).

Dialysis Procedure—It was clearly established by spectrophotometric and gravimetric analyses that the drug could indeed pass freely through the porous membrane whereas the polymer could not cross this barrier.

[b] Polymer Emulsions 4, 5, 8, and 20 (Table I).

Table VII—Effect of Polymer–Drug Ratio on Release Rate Pattern of Sodium Phenobarbital (Cumulative Milligrams Released)[a]

Hours	Percent Drug Entrapped			
	3.1	5.9	8.7	11.3
0.5	7.0	17.5	24.2	60.0
1.5	10.2	32.7	35.4	75.0
2.5	13.7	31.6	36.6	76.6
4.5	16.8	37.0	44.8	84.1
6.5	19.2	39.5	48.7	86.1
8	20.3	40.7	50.6	87.0

[a] In all cases, a 1.250-g. sample of the drug–polymer gel was evaluated.

Drug Release from Polymer Gels—Table V summarizes the dialysis data obtained for a series of five drug–polymer xerogels. The concentration of materials in these gels and the method by which each was made are also given. Gels were prepared in at least two replicates, permitting comparison of the reproducibility of drug entrapment for a given method of preparation and concentration of drug. The effect of drug concentration on gels made by an identical method and the effect of the method of preparation on the reproducibility of gel entrapment and equilibrium dialysis for gels prepared utilizing the same concentration of drug are also shown in Table V.

Gel Series 1, 2, and 3 (Table V) contained the same initial concentration of drug. Three different methods were used to prepare the various gel products. The sodium phenobarbital available after 72 hr. ranged from 46 to 74% of the amount of drug originally entrapped (22 mg.). Gel Series 4 and 5, containing a greater amount of sodium phenobarbital but prepared by different methods, exhibited drug release varying from 62 to 82% of the original amount enclosed in the gel matrix (28 mg.). Despite this wide variance among the series (the five series in Table V), there is good agreement within each series.

***In Vitro* Release Rate Procedure**—*Rate of Release*—The *in vitro* release rate pattern was obtained initially with gels prepared using Formula I and Procedure A. Figure 1 represents a composite release curve drawn from four identically prepared gels (Gel Series 1) as shown in Tables V and VI. Except for a tailing-off between the 5th and 8th hr., the experimental data follow the "idealized" curve very closely. The idealized curve was constructed on the premise that in an oral sustained-action product involving phenobarbital, the patient should receive a dose of the drug material immediately (*i.e.*, within 0.5 hr.) and a second dose uniformly over the next 7.5-hr. period (for a total of 8 hr.). Ultimately, of course, the therapeutic response of the drug in man is the final criterion of the preparation (21).

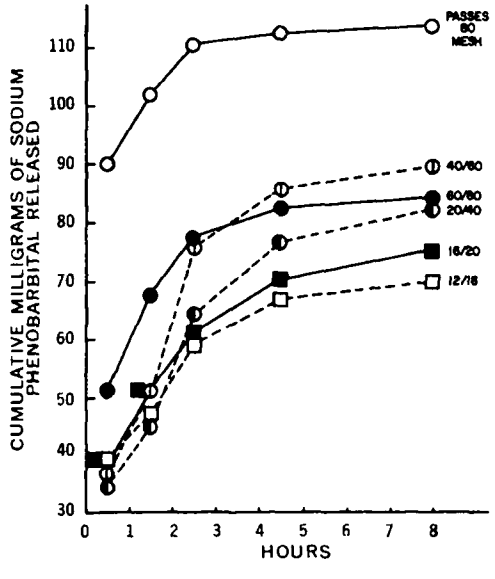


Figure 2—*Effect of particle size on release rate (Formula I and Procedure A).*

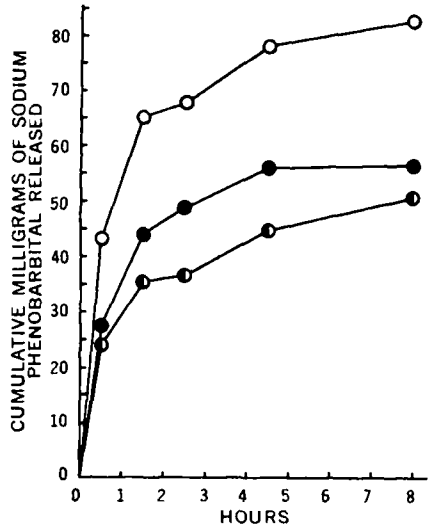


Figure 3—*Effect of method of preparation on the* in vitro *release rate of sodium phenobarbital. Key:* ◐, *Procedure A;* ●, *Procedure B; and* ○, *Procedure C. All samples contained 109 mg. of drug.*

Reproducibility—In this portion of the study, four gels[6] were prepared using Formula I and Procedure A. These gels were then subjected to *in vitro* analysis (Table VI), and excellent reproducibility of *in vitro* drug release was achieved.

Effect of Drug Concentration—This phase of the study was designed to establish limits of satisfactory drug concentrations in the Polymer Emulsion 2–magnesium sulfate gel system prepared using Procedure A. The results illustrating the effect of drug entrapment concentration on release rate are shown in Table VII.

The xerogels containing 5.9 and 8.7% sodium phenobarbital exhibited a desirable 2:1 release ratio (twice as much drug released after 8 hr. as after 0.5 hr.). The xerogel containing 3.1% drug had a 3:1 release ratio. Hence, at this concentration, the initial dose of drug might not be sufficient. At the highest drug concentration studied (11.3% entrapped), most of the drug was released initially (a 1:1.5 ratio), producing a poor release pattern. The limits of sodium phenobarbital concentration for good sustained action appear to be between 5.9 and 8.7% for this polymer system.

Effect of Particle Size—It would be reasonable to assume that the size of the final gel particles would influence the rate of drug release from the insoluble matrix. A smaller granule should release more drug and probably release it more rapidly due to greater surface exposure and a higher relative drug concentration at the xerogel surface.

The release rates of selected particle-size fractions were determined using the rotating-bottle technique. The results are summarized in Fig. 2. With minor exceptions, it can readily be seen that the smaller the particles, the more rapidly and completely the drug is released. Figure 2 also suggests the possibility of using mixed particle-size fractions to achieve a desired release rate.

Effect of Method of Preparation—In the section concerning dialysis, it was shown that, all other factors remaining constant, the method of preparing the sodium phenobarbital–magnesium sulfate–Polymer Emulsion 2 xerogel significantly altered the percent drug entrapment. As a result, an *in vitro* analysis was conducted using gels containing identical amounts of the three ingredients and varying only in the method of preparation (Fig. 3).

In Fig. 3, the shapes of curves A and C are nearly identical. However, Procedure C permits the release of approximately 30% more drug. This could result in greater economy in the manufacturing process through a savings in the amount of drug required and would produce a preparation having greater pharmacological reliability by virtue of more drug being accounted for as available drug. A possible explanation of this difference is that in Procedure A the polymer emulsion is added to a concentrated solution of the salts. As each emulsion drop enters the system, it comes into contact with a large excess of electrolyte solution, thereby gelling immediately. In Pro-

[6] These xerogels are listed as Gels 1, 1_1, 1_2, and 1_3 in Table V.

Table VIII—Effect of Drying on *In Vitro* Release Rate of Sodium Phenobarbital from Polymer Emulsion 2 Xerogels

Method of Drying	Milligrams Released after— 0.5 hr.	8 hr.	Percent Released after— 0.5 hr.	8 hr.
Air 12 hr., oven (45°) 24 hr.	24.2	50.6	22.2	46.5
Vacuum oven 24 hr., no heat	43.5	62.5	39.9	57.3
Air 1 hr., vacuum oven (60°) 12 hr.	60.0	62.5	55.0	57.3

Table IX—Effect of Polymer Concentration on Release of Sodium Phenobarbital from Polymer Emulsion 2 Xerogels

Percent Solids In Emulsion	Drug in System, mg.	Drug Released after 72 hr., mg.	Percent Drug Released
56–48	109	62.4	57
35–36	109	72.0	66
23–24	109	102.0	93

cedure C, however, the magnesium sulfate solution is slowly added to an aqueous mixture of the sodium phenobarbital and the polymer emulsion. Gelation does not occur until a sufficient concentration of electrolyte is present in the system. When this concentration is reached, the entire emulsion tends to gel simultaneously and more slowly. Consequently, the two processes result in a completely different rate of matrix formation. Of the three methods, Procedure C would be expected to produce a more porous xerogel and, in fact, resulted in the most rapid and complete dissolution release.

Another change in the method of preparing the gel involved the use of a vacuum oven to dry the product rather than conventional air and oven drying (Table VIII). Conventional drying produced a gel with the the best release rate of drug by far. One vacuum-dried product released nearly all of its unbound sodium phenobarbital within the first 0.5 hr. This was probably due to the fact that most of the water normally entrapped in the gel was rapidly drawn out of the matrix by the combination of vacuum and heat, thereby promoting drug migration to the gel surface. Sodium phenobarbital, being very water soluble, would have a greater tendency to migrate under these drying conditions. After comminution, probably very little of the unbound drug remained in the interstices of the xerogel.

When alcohol was used as the solvent in place of water, the polymer was precipitated as a solid mass. The alcohol simply "broke" the emulsion, causing the polymer to coagulate. *In vitro* analysis showed that this drug-polymer system released nearly all of its drug in the first 0.5 hr. Therefore, this product was unsuitable for sustaining the release of sodium phenobarbital.

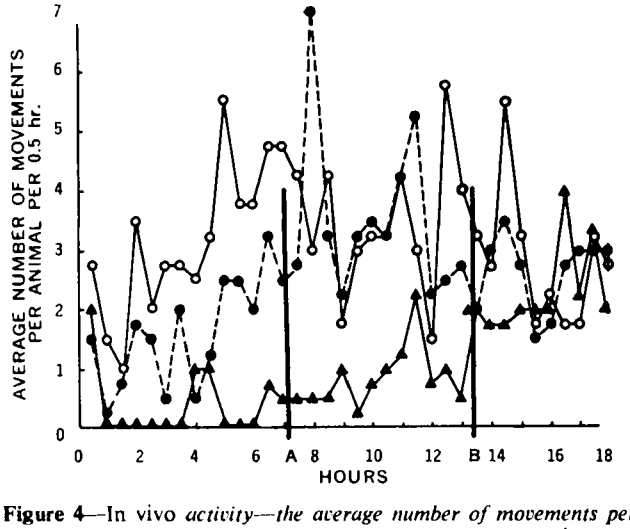


Figure 4—In vivo *activity—the average number of movements per animal per 0.5-hr. interval* versus *time. Key:* O—, *control group;* ●- - -, *sodium phenobarbital group, 50 mg./kg.; and* ▲—, *sodium phenobarbital-polymer group, 50 mg./kg.*

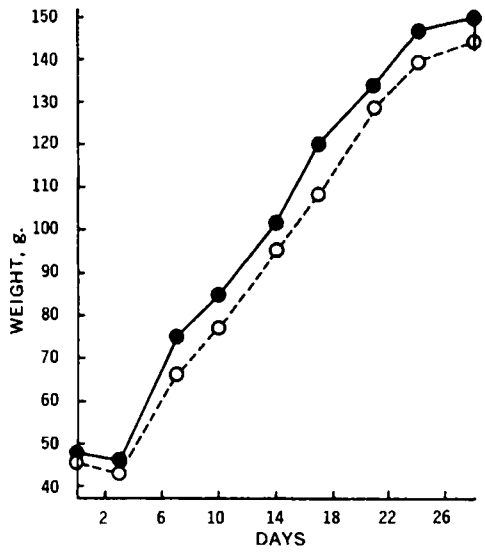


Figure 5—*Growth rate of rats on polymer feed diet* versus *control group. Key:* O- - -, *control group; and* ●—— *polymer group.*

Effect of Polymer Concentration—One potentially undesirable feature of the gel system was the "permanent" entrapment of 25–50% of the drug (depending on the procedure used). It was theorized that if the polymer emulsion were diluted and the drug-xerogel system prepared as before, the resulting matrix would be a looser and more open network. If this were the case, more drug should then be released from the interstices of the matrix.

To test this theory, the aqueous emulsion was diluted with varying amounts of distilled water. Gels were then prepared using Procedure A. The most dilute system that produced a satisfactory gel was prepared from an emulsion containing 23–24% solids (reduced from the original 46–48% solids). Table IX shows that dilution of the emulsion did, indeed, permit a substantially greater amount of the entrapped drug material to be released. This gives the formulator still another method of controlling the release of drugs from the polymer matrix.

***In Vivo* Procedures**—*Activity Cages*—The Williamson activity cages, utilized as previously described, yielded the data shown in Fig. 4. Each point represents an average value for four animals. The results clearly show a lengthening of the therapeutic effectiveness of sodium phenobarbital in the drug-polymer gel group. Furthermore, the *in vivo* drug effect observed permitted analysis of the general intensity (degree of central motor depression) of drug response throughout the test period. Over the entire period of prolonged action of the drug entrapped system, a consistent and continuous drug effect was seen. In Fig. 4, line A represents the sample

Table X—Wet Weights and Dry Weights of Various Organs of the Female Rat

Organ, g.	Polymer Group[a]	Control Group
Spleen		
Wet weight	0.502	0.569
Dry weight	0.113	0.138
Difference	0.389	0.431
Kidney		
Wet weight	1.59	1.56
Dry weight	0.42	0.41
Difference	1.17	1.15
Liver		
Wet weight	6.93	6.59
Dry weight	1.99	2.10
Difference	4.94	4.49

[a] The figures represent an average value for 10 animals (hence, a total of 20 animals was involved in this study).

Table XI—Coagulation of Polymer Emulsion 2 by Various Medicinal Agents[a]

Compound	Chemical	pH of Solution	Coagulation Type
Sodium phenobarbital	Cyclic	9.8	Gelation
Sodium citrate	Salt of an aliphatic acid	5.8	None[b]
Sodium salicylate	Salt of an aromatic acid	5.35	Gelation
Chloral hydrate	Aldehyde	4.5	Gelation
Caffeine alkaloid	Xanthine derivative	6.25	Gelation
Ephedrine sulfate	Secondary amine	5.6	Gelation
Ephedrine hydrochloride	Secondary amine	5.45	Gelation
Methapyrilene hydrochloride	Tertiary amine	6.2	Flocculation
Dextroamphetamine sulfate	Primary amine	5.9	Flocculation
Dihydrocodeinone bitartrate	Tertiary amine	1.75	Flocculation
Pyrilamine maleate	Tertiary amine	4.2	Flocculation

[a] All solutions (20 ml.) contained 1.20 g. of drug and 0.80 g. of magnesium sulfate, except the caffeine solution. This solution contained 0.80 g. of magnesium sulfate, but solubility limited the amount of caffeine to 0.40 g. [b] Gelation did occur when the concentration of magnesium sulfate was increased to 1.60 g.

mean of duration of effect for the group receiving drug alone and line B indicates the sample mean for the rats dosed with the drug–xerogel. For free drug:

$$\bar{x}_f = 7.1 \text{ hr.}$$
$$s^2 = 1.17$$
$$s = 1.08 \text{ hr.}$$
$$90\% \text{ confidence interval} = \pm 1.3 \text{ hr.}$$

where $\bar{x}_f$ is the sample mean, s^2 is the sample variance, and s is the standard deviation.

For drug xerogel:

$$\bar{x}_g = 13.4 \text{ hr.}$$
$$s^2 = 4.94$$
$$s = 2.2 \text{ hr.}$$
$$90\% \text{ confidence interval} = \pm 2.6 \text{ hr. (22)}$$

A sustained therapeutic effect was clearly achieved in that the free drug exhibited a duration of action of 7.1 ± 1.3 hr. whereas the drug–xerogel showed an effectiveness of 13.4 ± 2.6 hr. This portion of the *in vivo* work also served to substantiate the *in vitro* rotating-bottle procedure as a reliable method of predicting drug release for the systems of this study under the test conditions.

Acute Toxicity—Preliminary work suggested that, under the conditions previously described, an oral dose of 200 mg./kg. of sodium phenobarbital represented an approximate LD_{50} for female rats. Accordingly, 10 female rats were given this dose of free drug. Five of the animals died and an equal number lived. A second group of 10 animals were given the drug–polymer gel in an amount sufficient to release 200 mg./kg. of drug initially and an additional 200 mg./kg. of drug gradually. In this group, four animals died and six animals lived.

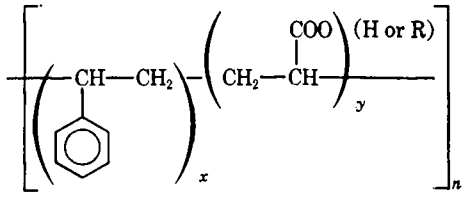
Many sustained-action preparations lower the acute toxicity of the modified drug product (23, 24). This also appeared to be true for sodium phenobarbital in the system used. The xerogel group received twice as much drug in the 8-hr. period as did the group that received unaltered sodium phenobarbital, yet one less animal receiving the sustained-release form died.

Chronic Toxicity—It was believed that the solid polymer material used in this study would not be toxic because of its apparent complete insolubility in aqueous media. It was feared, however, that contaminants (*e.g.*, catalysts, monomers, and surfactants) could be present and might themselves be toxic. Based on preliminary testing conducted in one animal species, the rat, no toxicity was noted. The growth curves of the two groups parallel each other closely (Fig. 5). Frequent urinalysis revealed no sign of any internal damage or structural malfunction (see Table III for the test procedures used). The only material detected in measurable quantity in the urine was a constant 30 mg./100 ml. of albumin in both the control and experimental groups.

The terminal study on the sacrificed animals revealed no visual abnormalities in any rats. Selected portions of the GI tract of several animals from each group revealed no visible irritation. The wet weight *versus* dry weight data of the various test organs are summarized in Table X. Considering the relatively small number of animals used, the organ weights show good experimental agreement.

Gelation Phenomenon—Physical examination and centrifugal testing established that the sodium phenobarbital–magnesium sulfate–Polymer Emulsion 2 product was a gel. It was clearly established that the magnesium ion (present as magnesium sulfate) was the coagulating ion in the system.

The best estimated structure of the copolymer latex as obtained from information from the manufacturer and an IR spectrum of a film of the emulsion is shown below. The ratio of styrene monomer



to acrylic monomer is probably not 1 to 1 (as shown), but rather 1 part styrene to between 0.5 to 0.7 part of acrylic monomer (25). Latex gels are normally highly cross-linked (11), which would contribute to their insolubility.

The gelation of selected polymer emulsions (colloidal suspensions prepared by emulsion polymerization) provides a unique physicochemical method of preparing drug–matrix and drug–xerogel systems. The xerogel entrapment technique utilizing polymer emulsions, as well as flocculation entrapment in such systems (6–8), offers physicochemical approaches to drug dispersion and entrapment with numerous applications in controlled release and drug delivery.

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NOTES

Stability of Aspirin in Liquid and Semisolid Bases I: Substituted and Nonsubstituted Polyethylene Glycols

C. W. WHITWORTH▲, H. W. JUN, and L. A. LUZZI

Abstract □ The stability of aspirin in methoxypolyethylene glycol, polyethylene glycol acetate, or a mixture of polyethylene glycols was studied at three temperatures. Degradation proceeded to the highest extent in a mixture of polyethylene glycols and to a lesser extent in methoxypolyethylene glycol. Degradation in polyethylene glycol acetate for 30 days at 45° was insignificant. The data confirm that decomposition of aspirin in polyethylene glycol is due primarily to a transesterification reaction.

Keyphrases □ Aspirin—stability in liquid and semisolid bases, polyethylene glycols □ Polyethylene glycols—stability of aspirin, liquid and semisolid bases □ Stability, aspirin—in polyethylene glycols, liquid and semisolid bases

In a previous paper (1) on the decomposition of aspirin in polyethylene glycols, these laboratories provided data showing that degradation was due at least in part to a transesterification reaction between aspirin and the polyethylene glycols. This conclusion was reached when it was found that acetic acid was not among the degradation products when a mixture of aspirin and polyethylene glycols was stored at different temperatures. Further evidence of a transesterification reaction was the degradation of aspirin in various polyethylene glycols in the apparent absence of moisture and the resultant appearance of acetylated polyethylene glycol. Both the absence of acetic acid and the presence of acetylated polyethylene glycol were established using NMR techniques (1).

The implications of these findings are far reaching and, more specifically, may be applied to cases where polyethylene glycols may be desirable as a vehicle (either liquid, semisolid, or solid) for aspirin preparation. This being the case then, the next logical step was to establish the stability of aspirin when the transesterification pathway is inhibited. It was thought that inhibition of the transesterification mechanism could be brought about by reducing the number of sites available for this reaction, namely, by blocking free hydroxyl groups on the polyethylene glycols.

Methoxypolyethylene glycol has the formula CH_3—O—CH_2—$(CH_2$—O—$CH_2)_nCH_2OH$, which has a methoxy radical at one terminal and only one remaining hydroxyl group that may enter into the reaction. Acetylation of polyethylene glycol also reduces the number of hydroxyl groups and likewise was expected to retard the decomposition of aspirin.

EXPERIMENTAL

Materials—Aspirin USP[1] and polyethylene glycols[2] 400, 1540, and 6000 were used as received. Chloroform[3] was spectroscopic grade. Monosubstituted methoxypolyethylene glycol 550[4] was purchased, and polyethylene glycol 400 acetate was prepared in these laboratories.

Analytical Method—UV spectrophotometric techniques[5] were employed to measure aspirin and salicylic acid (2). Samples were taken at specific time intervals, dissolved in 1% acetic acid–chloroform, and read at 278 nm. for aspirin and 308 nm. for salicylic acid after appropriate dilutions. Standard curves were prepared for aspirin and salicylic acid. A correction factor was obtained to account for the overlapping of absorbance at absorption maxima of each drug. The quantification was based on the standard method of simultaneous spectrophotometric determinations (3).

Procedure—The samples were prepared by the incorporation of 12% of aspirin in methoxypolyethylene glycol 550, polyethylene glycol 400 acetate, and a mixture of polyethylene glycols [polyethylene glycol 400–1540–6000 (27:31:42)] at the melting points of the individual bases. Preparations were kept in airtight amber containers and stored in a desiccator at 4, 26, and 45°. At various time intervals a weighed amount (2.5 g.) of samples was dissolved in 1% acetic acid–chloroform. Spectrophotometric readings were taken immediately after dilutions for aspirin and salicylic acid.

[1] Merck & Co., Inc., Rahway, N. J.
[2] Matheson, Coleman and Bell, Norwood, Ohio.
[3] J. T. Baker Chemical Co., Phillipsburg, N. J.
[4] Union Carbide, New York, N. Y.
[5] Beckman DU spectrophotometer.

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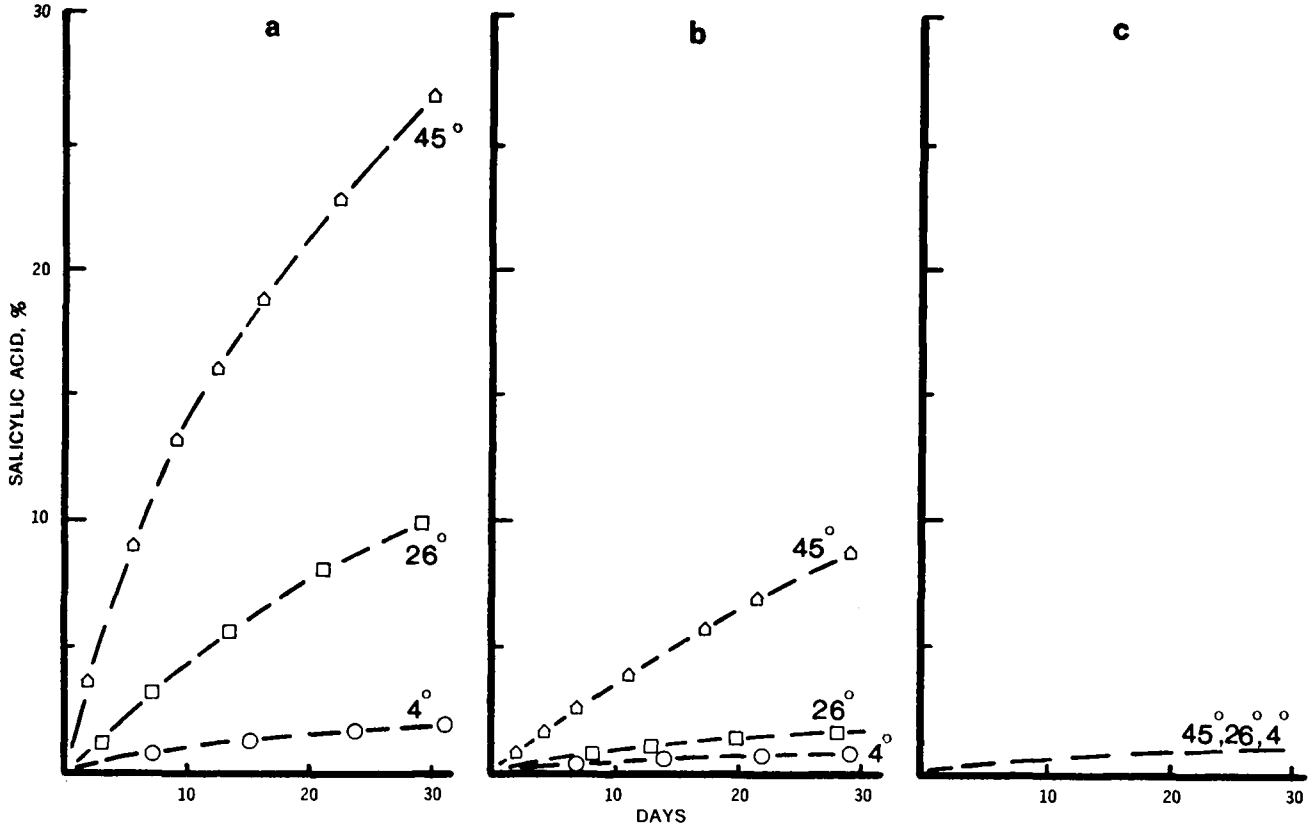

Figure 1—*Rate of appearance of salicylic acid* versus *time in a mixture of polyethylene glycols (a), in methoxypolyethylene glycol 550 (b), and in polyethylene glycol 400 acetate (c) at three temperatures.*

Acetylation of Polyethylene Glycol 400—Polyethylene glycol 400 acetate was prepared by refluxing a mixture of polyethylene glycol 400 and acetic anhydride (1:12 w/w ratio) at 139° for 4 hr. (4). Unreacted acetic anhydride and by-product were removed by vacuum evaporation.

RESULTS AND DISCUSSION

Earlier results (1) showed that degradation of aspirin in polyethylene glycols was due at least in part to transesterification (5). The present report deals with the comparison of the stability of aspirin in formulations of unsubstituted and substituted polyethylene glycols. The objectives were to investigate the possibility of blocking the transesterification route of degradation and to determine the effects on the stability of aspirin when this route is at least partially inhibited.

Table I shows the amount of aspirin remaining in three different formulations at three storage temperatures for 30 days. The percent as aspirin remaining varied widely at the three temperatures when unsubstituted polyethylene glycol was used as the base. The stability of aspirin was enhanced considerably when the monosubstituted glycol (Fig. 1b) was used. The acetylated glycol, prepared in these laboratories, was considerably more effective in preserving the stability of aspirin than the monosubstituted methoxyglycol.

Table I—Percent of Aspirin Remaining after 30 Days of Storage at Three Temperatures

Temperature	Mixture of Polyethylene Glycols (Unsubsituted)	Methoxy-polyethylene Glycol 550	Polyethylene Glycol 400 Acetate
4°	98.3	99.5	99.0
26°	90.0	98.5	99.0
45°	73.5	91.3	99.0

Figure 1 shows the overall profiles of degradation of aspirin in the three preparations at 4, 26, and 45° as indicated by the rate of the appearance of salicylic acid. A significant difference in the rate of formation of salicylic acid was seen between the substituted and unsubstituted glycols.

As shown in Fig. 1c, the stability of aspirin in the acetylated base was not temperature dependent during the 30 days of the test. From these results, it may be concluded that a transesterification reaction between aspirin and the polyethylene glycols is the predominating route for the degradation of aspirin in these bases. Speculation, at this stage, strongly favors the possibility of formulating stable preparations of aspirin in substituted polyethylene glycol bases.

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Leucine and Its Derivatives as Potential Elicitors of Insulin Secretion in Rats

VICTOR D. WARNER*▲, ANN M. WARNER*, JOSEPH R. VASSELLI†, ELISABETH DECKE†, F. XAVIER PI-SUNYER†‡, and STEPHEN C. WOODS†

Abstract □ This report describes the testing of a number of leucine derivatives for their ability to increase serum levels of glucose and immunoreactive insulin. Insulin levels increased in rats when L-leucine, DL-leucine, and L-leucineamide were administered. Esters of L-leucine caused no significant effect on glucose or insulin levels.

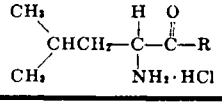
Keyphrases □ Leucine and derivatives—role in eliciting insulin secretion, rats, serum levels of glucose and immunoreactive insulin □ Insulin secretion, rats—effect of leucine and derivatives, glucose and immunoreactive insulin serum levels □ Immunoreactive insulin—role of leucine and derivatives in eliciting secretion, serum levels, rats

The amino acid, leucine, has been shown to cause hypoglycemia in normal subjects by increasing plasma levels of insulin (1, 2). Furthermore, an infusion of 10 essential amino acids, including leucine, results in a marked increase in insulin secretion, although the release of insulin by leucine appears to be *via* a different mechanism than that due to other amino acids (3, 4). In the present experiment, a number of derivatives of leucine were administered to rats, and glucose and immunoreactive insulin levels were obtained at subsequent intervals. It was found that only L-leucine (and DL-leucine to a lesser extent) had the ability to elicit insulin secretion in this preparation. Administration of L-leucineamide also caused an elevation in insulin levels, but it was secondary to a rise in blood glucose.

PROCEDURE

The compounds tested in this study are indicated in Table I. The methyl and ethyl esters of L-leucine were synthesized in the manner of Schneider and Schaeg (5), and the L-leucineamide hydrochloride and DL-leucineamide hydrochloride were synthesized from the corresponding methyl esters in the manner of Smith and Spackman (6). The drugs were all dissolved in distilled water to give a final concentration of 37.5 mg./ml. (w/v). The solutions were then coded according to Table I so that the persons injecting the rats, collecting and preparing the blood samples, and performing the biochemical determinations were unaware of the experimental conditions.

Table I—Compounds Studied



Name	R	Code on Figs. 1 and 2 and Tables II and III
—	—	A: Control solution (distilled water)
L-Leucineamide hydrochloride	NH_2	B
L-Leucine ethyl ester hydrochloride	OCH_2CH_3	C
L-Leucine methyl ester hydrochloride	OCH_3	D
DL-Leucine hydrochloride	OH	E
L-Leucine hydrochloride	OH	F
DL-Leucineamide hydrochloride	NH_2	G

A total of 118 mature albino rats of both sexes was used. All had had continuous access to both food[1] and water for at least a week prior to the experiment. For the experiment itself, all rats were deprived of food (but not water) for 24 hr. Each rat was then given an intraperitoneal injection of one of the seven solutions and returned to its individual home cage. The dose was always 150 mg./kg. of the appropriate drug or the equivalent volume of distilled water (Group A). The rats were removed from the home cage and decapitated by a guillotine at 2.5, 5, 10, or 20 min. after the injections. Uninjected rats (n = 5) were decapitated to provide baseline values for this population. Carotid blood was collected over ice and subsequently centrifuged. The serum portion was then frozen until the determinations were made.

Serum glucose determinations were done by the glucose oxidase method of O'Brien *et al.* (7), and serum insulin determinations were done by a modification (8) of the double antibody method of Morgan and Lazarow (9).

RESULTS

The results of the glucose and immunoreactive insulin determinations are summarized in Figs. 1 and 2 and Tables II and III, respectively. Baseline glucose and insulin values were 100.8 mg./100 ml. and 12.8 microunits/ml. serum, respectively. The placebo injection (Group A) caused a slight increase in glucose and a slight decrease in insulin, the magnitude and duration of which were previously observed in this laboratory (10, 11).

The administration of L-leucine hydrochloride (Group F) caused a large spike of insulin at 2.5 min., which then stabilized at a high level for the duration of the 20-min. test period. There was also a slight (nonsignificant) increase in glucose at that time. Although the small increase in glucose might have accounted for part of the initial rise in insulin in Group F, glucose levels in that group tended to

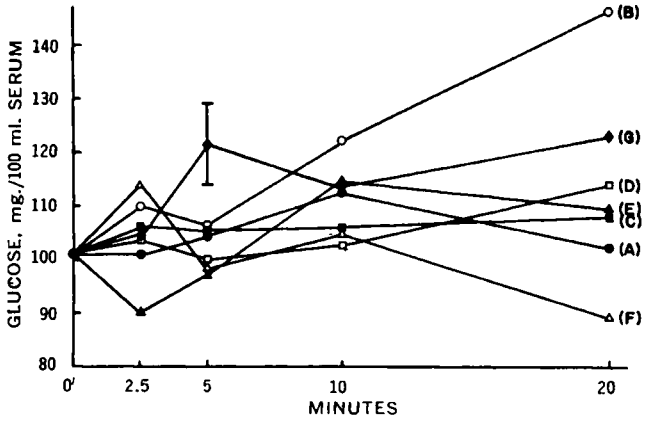


Figure 1—*Mean values of serum glucose for the different groups at the different intervals. Because there was homogeneity of variance, only the mean standard error of the mean was plotted.*

[1] Purina rat chow.

Table II—Serum Immunoreactive Insulin Values (microunits per milliliter) for the Different Groups[a]

Minutes after Injection	Group A	B	C	D	E	F	G
2.5	11.9 ± 2.8 (4)	23.2 ± 2.2 (4)	10.3 ± 4.0 (4)	11.6 ± 2.1 (4)	28.7 ± 5.2 (4)	38.3 ± 2.9 (4)	11.6 ± 3.5 (4)
5	9.8 ± 1.7 (4)	26.9 ± 5.7 (5)	10.8 ± 3.5 (4)	8.0 ± 2.3 (4)	22.5 ± 4.5 (4)	28.3 ± 3.7 (4)	9.4 ± 2.4 (4)
10	9.5 ± 2.9 (4)	28.1 ± 3.4 (4)	8.8 ± 2.2 (4)	15.4 ± 1.1 (4)	22.5 ± 2.1 (4)	32.0 ± 6.1 (4)	24.0 ± 1.9 (5)
20	9.4 ± 1.9 (4)	28.9 ± 6.1 (4)	10.3 ± 3.0 (4)	14.4 ± 2.4 (4)	23.8 ± 2.6 (4)	31.3 ± 8.1 (3)	17.2 ± 1.9 (4)

[a] Entries are expressed in the following form: mean ± standard error of the mean (number of animals).

Table III—Serum Glucose Values (mg./100 ml.) for the Different Groups[a]

Minutes after Injection	Group A	B	C	D	E	F	G
2.5	100.8 ± 1.9 (4)	109.8 ± 13.7 (4)	106.0 ± 8.7 (4)	103.4 ± 3.8 (4)	90.0 ± 5.4 (4)	114.0 ± 2.4 (4)	104.0 ± 10.0 (4)
5	104.3 ± 10.5 (4)	106.2 ± 12.4 (5)	105.5 ± 4.0 (4)	100.0 ± 10.7 (4)	96.8 ± 3.7 (4)	98.0 ± 10.4 (4)	121.8 ± 8.2 (4)
10	112.0 ± 6.4 (4)	122.0 ± 9.6 (4)	105.8 ± 6.8 (4)	103.0 ± 7.1 (4)	114.3 ± 3.5 (4)	104.0 ± 6.8 (4)	112.6 ± 15.3 (5)
20	102.0 ± 5.0 (4)	145.8 ± 6.2 (4)	108.0 ± 7.1 (4)	114.0 ± 9.2 (4)	109.0 ± 4.6 (4)	89.0 ± 5.0 (3)	123.0 ± 8.8 (4)

[a] Entries are expressed in the following form: mean ± standard error of the mean (number of animals).

decrease over the 20-min. test period while insulin values remained elevated. A similar but greatly attenuated insulin pattern was observed after the administration of DL-leucine hydrochloride (Group E); in this instance, the significant increase in insulin after 2.5 min. could not be attributed to an increase in glucose since that group showed a nonsignificant decrease in glucose at that interval.

The administration of L-leucineamide hydrochloride (Group B) also resulted in an elevation of insulin levels, although no early spike (2.5 min.) was observed. In this instance, the increased insulin levels can be attributed to a glucose level that continued to rise throughout the 20-min. period. Administration of DL-leucineamide hydrochloride (Group G) caused a similar pattern, which was again attenuated and which did not appear until 10 min. following the injection.

Administration of the methyl (Group D) and ethyl (Group C) esters had no reliable effect on either glucose or insulin values.

DISCUSSION

L-Leucine was previously shown to cause a biphasic insulin response in man (12). In that experiment, the initial peak occurred at 5 min. and the secondary peak occurred at 20 min. after the injection. The present results in rats are similar, although the initial peak appeared somewhat sooner (2.5 min.). The biphasic insulin response corresponds to the two-pool system of insulin postulated by Porte and Pupo (13) and Porte and Bagdade (14).

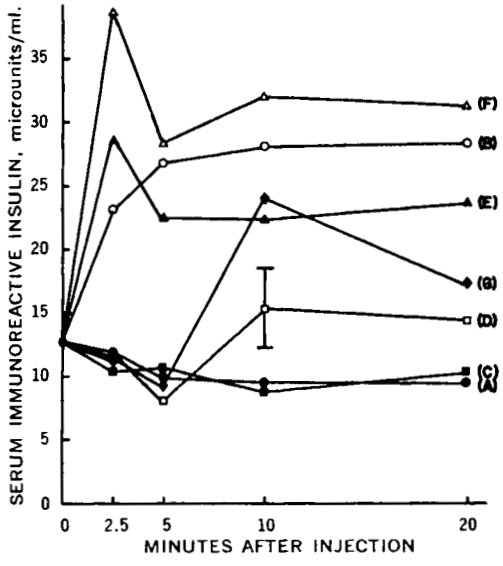


Figure 2—*Mean values of serum insulin for the different groups at the different intervals.*

The attenuated response to the DL-isomer of leucine was also reported previously (15). In that same paper, it was shown that the metabolic products of leucine have no effect in eliciting insulin secretion or hypoglycemia.

The delayed increase in insulin levels following the administration of L- and DL-leucineamide hydrochloride must be attributed to the elevated glucose levels in those rats. The mechanism for this increase in glucose is not known, although it probably is not secondary to a release of epinephrine because any epinephrine would be expected to prevent the observed increase in insulin output (16).

In summary, modification of the leucine molecule by addition of either an amide group or a methyl or ethyl ester eliminated the ability of the molecule to elicit insulin secretion. Furthermore, only the L-isomer of leucine appears to be effective because a DL-mixture responds in a manner predictable by simple dilution of the L-isomer.

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Selective Blockade of Muricidal Activity in the Rat by Anorectic Agents

S. E. BOCKNIK▲ and A. S. KULKARNI

Abstract □ The ability of several anorectics to block selectively mouse killing in the rat was examined. A significant ratio between the muricide ED_{50} and the ED_{50} of a drug to produce debilitation in rotarod performance is a measure of selectivity. The mouse-killing behavior of a rat can be blocked with certain drugs at doses that do not render the animal debilitated. This selectivity, determined by the ratio of ED_{50}'s, was found in the four anorectics examined as follows: diethylpropion > dextroamphetamine > aminorex > fenfluramine.

Keyphrases □ Anorectic agents—ability to block mouse killing in rats, determination of selectivity, correlated with decreased rotarod performance □ Muricidal activity—selective blockade by anorectic agents, rat □ Mouse-killing behavior—killer rats, effect of four anorectic agents, determination of selective blockade

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Table I—Effects of Anorexigenic Compounds on Rat Rotarod Performance and Muricide Behavior

Drug	Muricide Blockade, MKD_{50}, mg./kg.[a]	Rotarod Debilitation, NTD_{50}, mg./kg.[a]	NTD_{50}/ MKD_{50}
Aminorex	1.67 (0.97–2.86)	12.1 (6.74–21.8)	7.25
Dextro-amphetamine	0.46 (0.30–0.72)	4.64 (1.82–11.8)	10.09
Diethylpropion	3.83 (2.13–6.89)	68.1 (no limits)	17.78
Fenfluramine	3.16 (1.69–5.92)	14.7 (8.5–25.3)	4.65

[a] MKD_{50} and NTD_{50} are given with fiducial limits in parentheses.

In the present experiment, the mouse-killing selectivity of other anorectic compounds was investigated. Rotarod performance was again used as a measure of neurotoxicity.

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The anorexigenics, aminorex, dextroamphetamine, diethylpropion, and fenfluramine, were tested in rats for their muricide-blocking ability and debilitation of performance on the rotarod as described in the following procedures.

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Saline was administered intraperitoneally to condition the rats to the injection procedure and to eliminate any rats that did not kill after placebo administration. Mice were placed in the rats' cages 30, 60, and 120 min. after the saline injection. The same procedure was followed during the ensuing drug studies.

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▲ To whom inquiries should be directed.

Selective Blockade of Muricidal Activity in the Rat by Anorectic Agents

S. E. BOCKNIK▲ and A. S. KULKARNI

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VICTOR D. WARNER*▲, DALE B. MIRTH*, SAMUEL S. TURESKY†, and IRVING GLICKMAN†

Abstract □ Five straight-chain homologs of chlorhexidine, containing from two to 12 methylene groups, were synthesized and evaluated along with chlorhexidine for *in vitro* antiplaque activity. Maximum antiplaque activity was exhibited by the compounds containing six (chlorhexidine) and 12 methylene groups.

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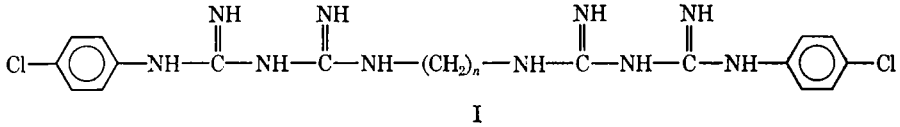

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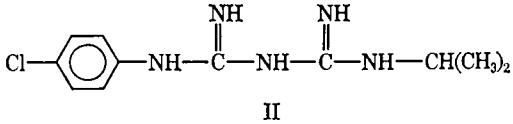

II

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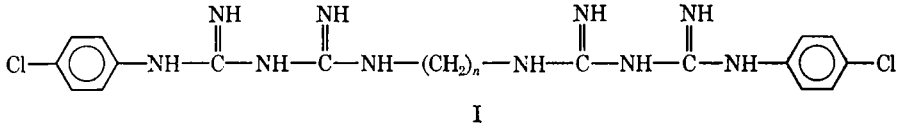

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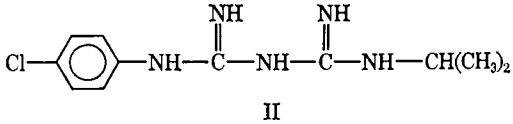


II

Table I—Bis(N^3-cyano-N^1-guanidino)alkanes

Methylene Groups, *n*	Reaction Time, hr.	Yield (Crude), %	Melting Point	Lit. (11) Melting Point	Recrystallization Solvent[a]
2	12	32	245–247°	248–250°	A
4	18	15	191–194°	197–199°	A
8	16	35	117–147°	—	A–B
10	16	10	164–182°	184–186°	A–C
12	18	46	136–169°	—	A–C

[a] A = water, B = ethanol, and C = 2-ethoxyethanol.

Table II—Bis(N^5-*p*-chlorophenyl-N^1-biguanido)alkanes

Methylene Groups, *n*	Reaction Time, hr.	Yield, %	Melting Point	Lit. (11) Melting Point	Recrystallization Solvent[a]	Formula	Analysis, %	Calc.	Found
2	6	46	255–259°	245–246°	A	$C_{18}H_{22}Cl_2N_{10}\cdot 2HCl\cdot H_2O$	C	40.02	40.04
							H	4.85	4.87
							N	25.92	25.85
4	3	40	251.5–255°	253–254°	A	$C_{20}H_{26}Cl_2N_{10}\cdot 2HCl$	C	43.65	43.50
							H	5.13	5.18
							N	25.45	25.25
8	8	9	212–227°	—	A	$C_{24}H_{34}Cl_2N_{10}\cdot 2HCl$	C	47.53	47.67
							H	5.98	6.30
							N	23.09	23.04
10	6	16	239–245°	246–248°	A–B	$C_{26}H_{38}Cl_2N_{10}\cdot 2HCl$	C	49.22	49.03
							H	6.35	6.56
							N	22.08	21.73
12	6	18	221–227.5°	—	A–B	$C_{28}H_{42}Cl_2N_{10}\cdot 2HCl$	C	50.76	50.87
							H	6.69	6.95
							N	21.14	20.91

[a] A = water and B = ethanol.

Structure–activity studies with biguanides have shown that molecules containing only one biguanide residue, *e.g.*, proguanil (II), do not inhibit plaque formation (4) and that the nature of the substituents attached to the biguanide groups in bisbiguanides has a marked effect on antiplaque (9, 10) and antibacterial activity (6). In addition, the distance between the biguanide residues in bisbiguanides affects antibacterial activity (6). These findings support the hypothesis that two properly substituted biguanide residues separated by a specific distance must be present in this type of molecule for optimal antiplaque activity.

To determine the optimal separation of the two biguanide groups, a series of chlorhexidine methylene homologs (I, n = 2, 4, 8, 10, 12) was synthesized and evaluated for *in vitro* plaque inhibition relative to chlorhexidine.

EXPERIMENTAL[1]

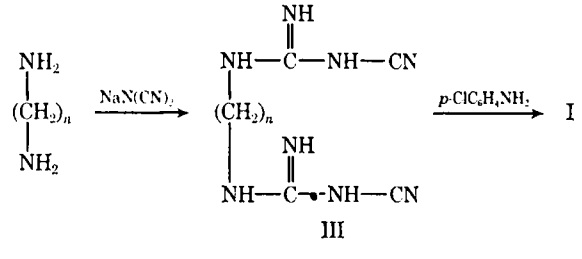
The homologs were synthesized using the method developed by Rose and Swain (11) as outlined in Scheme I. Treatment of the appropriate diaminoalkane with sodium dicyanamide gave the corresponding intermediate bis(N^3-cyano-N^1-guanidino)alkanes (III) (Table I) which, on treatment with *p*-chloroaniline, gave the desired bisbiguanides (I) (Table II).

Bis(N^3-cyano-N^1-guanidino)alkanes (III)—An intimate mixture of the appropriate diaminoalkane dihydrochloride (1.0 mole) and sodium dicyanamide (2.0 moles) was stirred in refluxing 1-butanol (15 ml./0.01 mole) for 12–18 hr. The cooled reaction mixture was filtered and, where the product was soluble (III, n = 2, 4), the filtrate was concentrated *in vacuo* and recrystallized to give the crude biscyanoguanidine which was used without further purification. Where the product was insoluble, the solid on the filter was washed with water to remove sodium chloride and recrystallized (Table I).

Bis(N^5-*p*-chlorophenyl-N^1-biguanido)alkanes (I)—A mixture of the appropriate bis(N^3-cyano-N^1-guanidino)alkane (1.0 mole) and *p*-chloroaniline hydrochloride (2.0 moles) was stirred in refluxing 2-ethoxyethanol (20 ml./0.01 mole) for 3–8 hr. Concentration *in vacuo* followed by recrystallization gave the pure bis(N^5-*p*-chlorophenyl-N^1-biguanido)alkane dihydrochlorides (Table II).

Antibacterial Activity—Antiplaque activity as displayed by chlorhexidine requires that a compound be an antibacterial agent. Therefore, the homologs were first evaluated for their *in vitro* antibacterial activity against *Streptococcus mutans* No. 6715, a pure strain of plaque-forming bacteria[2]. Chlorhexidine[3] and a solvent control were tested concurrently. One milliliter of a solution of the test compound was added to a tube containing 7.85 ml. of trypticase broth, 1 ml. of 50% sterile sucrose solution, and 0.15 ml. of a 24-hr. culture of *S. mutans* No. 6715, and this mixture was incubated under anaerobic conditions[4] for 48 hr.

Antiplaque Activity—The *in vitro* antiplaque activity of these same compounds was evaluated using the method of Turesky *et al.*



Scheme I

[1] Melting points were determined on a Thomas-Hoover capillary melting-point apparatus and are corrected. Microanalyses were performed by Scandinavian Microanalytical Laboratory, Herlev, Denmark, and by Midwest Microlab, Ltd., Indianapolis, Ind.

[2] Isolated at, and made available to us by, the National Institute of Dental Research.

[3] Supplied by Ayerst Laboratories, Inc., New York, N. Y.

[4] BBL-Gaspak, BBL, Division of BioQuest, Cockeysville, Md.

Table III—*In Vitro* Antiplaque Activity[a]

Methylene Groups, *n*	—Percent Inhibition[b] at 10^{-5} *M*— 24 hr.	48 hr.
2	20	0
4	60	0
6	100	40
8	80	20
10	20	0
12	100	20

[a] All compounds were evaluated as their hydrochloride salts in dimethyl sulfoxide solution. Solvent controls exhibited 0% inhibition. Each compound was tested on five teeth. [b] Percentage of teeth that did not show plaque formation after stated incubation period.

(12). Sterilized extracted human teeth were immersed in solutions of the test compounds for two 1-min. periods, each of which was followed by a 1-min. exposure to air. These treated teeth were washed with 250 ml. of distilled water for 5 min. and then incubated with *S. mutans* No. 6715 under anaerobic conditions. The teeth were suspended in test tubes on orthodontic wire[5] (0.71-mm. diameter) threaded through a hole in the root so that the entire tooth was completely immersed. Subjective estimates were made of adherent microbial growth on the test tube walls, wires, and the teeth and of nonadherent growth in the broth using a scale from 0 (no growth) to 4 (maximum growth). Because in an overwhelming number of tests, growth ratings were either 0 or 4 both in the broth and on the hard surfaces, the findings were expressed as either + (growth) or − (no growth). The total microbial accumulation was considered as *in vitro* plaque.

RESULTS AND CONCLUSIONS

The five homologs and chlorhexidine all inhibited bacterial growth at a concentration of 10^{-5} *M* but not at 10^{-6} *M*. As shown in Table III, antiplaque activity was greatest when the biguanide groups were separated by either six or 12 methylene groups. Since all six compounds were comparable in antibacterial activity at the concentrations tested, the observed differences in antiplaque activity may reflect better binding to the tooth surface by the more active compounds.

[5] Rocky Mount.

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Bioavailability of Dicumarol from Different Commercial Tablets in Dogs

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Abstract □ The availability of dicumarol from three different commercial tablets was determined by measuring plasma levels in dogs and by an *in vitro* dissolution rate test. The products showed significant differences in plasma levels, areas under plasma level curves, peak prothrombin time responses, and rates of dissolution even though they contained the labeled amount of dicumarol.

Keyphrases □ Dicumarol—bioavailability from three different commercial tablets, dogs □ Bioavailability—dicumarol from three different commercial tablets, dogs □ Anticoagulants—bioavailability of dicumarol from three different commercial tablets, dogs

In 1960, Losinski (1) reported that different formulations of dicumarol (bishydroxycoumarin) tablets, although chemically equivalent, exhibited large differences in therapeutic response after administration to patients requiring anticoagulation therapy. On the basis of that report, speculation has arisen that dicumarol tablets from different manufacturers may not be equivalent in terms of drug bioavailability. The availability of dicumarol from three different commercial tablets is reported here. *In vivo* studies, performed by measuring plasma levels of dicumarol in dogs, and *in vitro* dissolution rate tests indicated that significant differences in the bioavailability of the anticoagulant from commercial products do exist.

EXPERIMENTAL

Products Tested—Three commercial tablets, designated Products A, B, and C[1], were purchased in June 1971 from local pharmacies. Each was labeled to contain 25 mg. of dicumarol.

Animals—Four healthy mongrel dogs, weighing between 10 and 21 kg., were used. They were housed in stainless steel cages under controlled temperature and lighting. The animals were fed[2] daily between 8 and 11 a.m. and allowed water *ad libitum*.

Protocol—Large interanimal differences have been observed in the plasma levels of dicumarol after oral administration to dogs on a milligram per kilogram basis. The plasma levels, however, are reproducible in an individual animal (2). Similar results have been observed in humans (2). In the clinical use of the drug, the dosage is adjusted in each patient to provide the desired therapeutic response.

Similar to the clinical use of dicumarol, the dosage used in this study was adjusted in each individual animal to give a desired response. The dosage required to provide a peak plasma level of 21–25 mcg./ml. and a doubling of normal prothrombin time was determined in preliminary experiments. This was accomplished by administering different dosages of dicumarol powder to each dog and measuring drug plasma levels and prothrombin times. After the desired dosage had been determined in an animal (either 4, 5, or 7 mg./kg.), the drug was administered in tablet form. Individual dogs received the same dose of whole 25-mg. dicumarol tablets throughout the study. The animals were given Products A, B, and C on a randomized basis in a crossover design, and each dog received all three products using this procedure. At least a 2-week interval was allowed between doses of dicumarol to the same animal. During this period, the plasma levels of the drug fell to undetectable levels and prothrombin times returned to normal.

The animals were fasted from food for 8 hr. before and 24 hr. after drug administration. Blood samples were obtained from the external jugular vein at various times after the dose. The one-stage prothrombin time was determined on fresh, oxylated plasma. Plasma for dicumarol analysis was stored at −5°.

Analysis of Dicumarol—The spectrophotometric method of Weiner *et al.* (2) as revised by Nagashima *et al.* (3) was used for plasma. Tablets were analyzed for dicumarol by grinding a single tablet in a mortar and adding 25 ml. of 1 *N* NaOH. The solution was filtered and 0.1 ml. of the filtrate was added to 1.4 ml. of the citrate–phosphate buffer employed in the plasma assay for dicumarol. This solution was assayed for dicumarol by the same method used for plasma.

Rate of Dissolution—The USP XVIII method was used. One 25-mg. tablet was placed in a stainless steel basket immersed in 900 ml. of simulated intestinal fluid, pH 7.5 (without pancreatin), at 37°. The basket was about 4 cm. from the bottom of the beaker and rotated at 60 r.p.m. At appropriate times, a 5-ml. sample was withdrawn from the beaker by means of a sintered-glass filter pipet. An equal volume of fluid was added to the beaker. The sample was read directly at 373 nm. to determine dicumarol concentration.

Analysis of Data—Plasma concentrations of dicumarol, areas under plasma level curves, and peak prothrombin times were subjected to an analysis of variance randomized complete block design. Statistical differences between means were computed using the least significant difference method (4). The level of significance was chosen at $p < 0.05$.

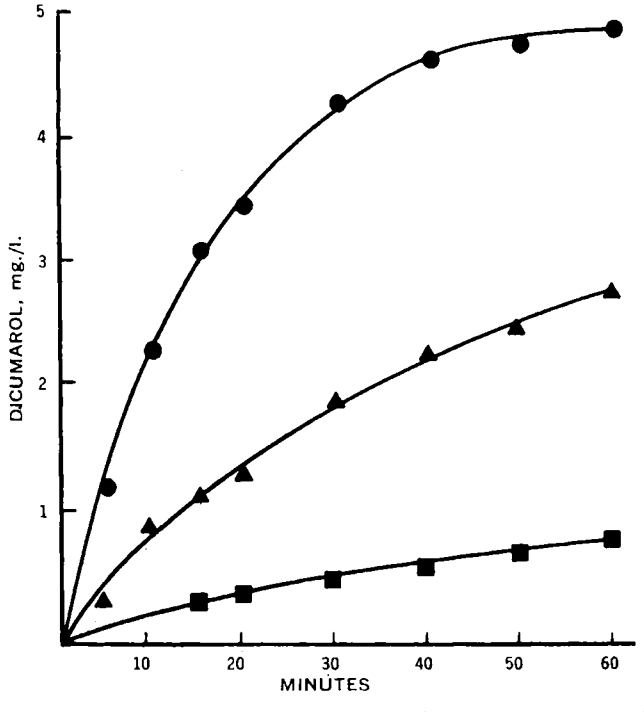

Figure 1—*Dissolution rate of dicumarol from three commercial tablets. Key: ▲, Product A; ●, Product B; and ■, Product C.*

[1] Product A was Dicoumarol, 25 mg. (Abbott Laboratories, North Chicago, Ill.), Lot No. 822-1764. Product B was Blue Cross Bishydroxycoumarin, 25 mg. (Halsey Drug Co., Brooklyn, N. Y.). No lot number was listed. Product C was Bishydroxycoumarin, 25 mg. (Columbia Medical Co., New York, N. Y.), Lot 1817 (only number visible on label).

[2] Purina dog chow.

Table I—Plasma Levels of Dicumarol (Micrograms per Milliliter) and Peak Prothrombin Times Produced by Commercial Tablets

Hours	Product B — Dog Number[a] 1	2	3	4	$\bar{X}$	Product A — Dog Number[a] 1	2	3	4	$\bar{X}$	Product C — Dog Number[a] 1	2	3	4	$\bar{X}$
1	9.5	40.3	35.2	16.6	25.4[b]	1.0	17.5	16.6	1.0	9.0	9.5	13.9	2.6	3.9	7.4
2	31.9	34.1	39.0	30.9	33.9[b]	3.4	17.5	19.7	17.2	14.5	8.6	11.8	12.2	18.4	12.7
4	28.0	34.3	39.2	29.0	32.6[b]	16.9	14.3	19.9	23.9	18.7	7.7	12.4	12.3	16.7	12.2
8	28.0	38.5	37.0	28.5	33.0[b]	23.1	13.9	19.0	23.9	19.9	11.6	13.4	12.7	15.0	13.1
12	27.2	31.3	35.9	31.0	31.3[b]	21.0	14.8	19.7	21.4	19.2[c]	9.3	12.7	13.7	12.6	12.0
24	27.7	38.5	33.1	31.8	32.7[b]	19.4	18.1	21.3	19.3	19.5[c]	9.4	14.1	13.0	12.6	12.2
48	21.0	28.2	32.5	27.1	27.2[b]	13.3	16.2	17.8	14.0	15.3[c]	5.3	9.4	12.6	9.6	9.2
72	17.2	22.0	25.5	22.8	21.8[b]	8.6	13.3	13.7	10.0	11.4	6.6	6.9	11.4	5.2	7.7
96	12.2	15.2	19.3	18.4	16.2[b]	5.6	10.8	11.1	6.1	8.4	4.1	4.1	7.2	1.7	4.2
Area under curve 0–96 hr. (mcg./ml.) (hr.) (10^2)	20.3	26.8	28.7	25.0	25.2[b]	12.6	14.2	16.1	13.6	14.1[c]	6.7	9.2	11.1	8.4	8.9
Peak prothrombin time (sec.)[d]	20.4	18.3	14.5	21.4	18.6	15.0	15.8	16.9	17.4	16.2[c]	12.4	10.8	10.9	10.9	11.2
Tablet analysis for dicumarol (% of labeled amount)			95					104					110		

[a] Doses used were: Dog 1 (10 kg.), 50 mg.; Dog 2 (18 kg.), 125 mg.; Dog 3 (21 kg.), 100 mg.; and Dog 4 (20 kg.), 100 mg. [b] Significantly different from Products A and C. [c] Significantly different from Products B and C. [d] In all cases the peak prothrombin response occurred at 48 hr. after the dose. Normal prothrombin times of 7–8 sec. were not subtracted from the values presented.

RESULTS AND DISCUSSION

Significant differences were observed among the mean plasma levels of dicumarol produced by the three different products. As shown in Table I, Product B produced significantly higher plasma levels than Product A at all time points. Product C produced the lowest mean plasma levels among the three products tested. Peak plasma levels after oral administration of Product B were almost three times higher than those produced by Product C.

The mean areas under the plasma level curves from 0 to 96 hr. also reflected product differences: Product B produced a significantly larger area than Product A or C. A semilogarithmic plot of the mean values for the 48-, 72-, and 96-hr. plasma levels showed that the mean plasma half-life for dicumarol was approximately the same after each product. The observed differences in plasma levels were probably due to differences in the availability of the drug for absorption.

Also shown on Table I are the mean peak prothrombin time responses attained after a single dose of each product. These values are directly related to the dicumarol plasma level attained with each product. Product B produced a 60% greater anticoagulant response than Product C, with the response from Product A occupying an intermediate position.

A characteristic of the dicumarol plasma concentrations observed in these experiments was the rather constant level produced between 4 and 24 hr. after the dose. The plasma levels could not be adequately fit by simple one- or two-compartment models using single zero- or first-order absorption rate constants. The absorption process was undoubtedly more complex. Since all products contained the labeled amount of drug (Table I), the different plasma concentrations but similarly shaped plasma level curves suggest that incomplete absorption may be primarily responsible for the observed inequivalency among products.

The USP dissolution rate test correlated well with the plasma level results. At the end of 1 hr., Product B allowed the dissolution of five times more dicumarol than was observed with Product C. Product A occupied an intermediate position in its availability for *in vitro* dissolution. The USP dissolution rate test, using simulated intestinal fluid, may be of use to the formulation scientist in predicting the relative bioavailabilities of experimental products.

Large differences existed in the bioavailability of the three commercial products. Only one lot of each product was tested so the reliability of the products cannot be assessed. The highly insoluble dicumarol undoubtedly represents a challenge to the formulation scientist. One would have to conclude from both the *in vivo* and *in vitro* data that the challenge had not been met for at least two of the products.

O'Reilly *et al.* (5) showed that dicumarol tablets are often poorly absorbed from the GI tract of humans. If it can be assumed that similar differences in absorption of dicumarol from the tablets studied here could occur in human patients undergoing anticoagulant therapy, then consideration of these products as biologically equivalent could lead to potentially dangerous clinical situations. It should be emphasized, however, that the present study is limited in scope and that the clinical use of dicumarol tablets should not be based on these results alone. The reporting of this data is meant to alert pharmaceutical and clinical scientists to possible differences in the commercial brands of dicumarol and to serve as an impetus for further studies in man.

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Mesomorphic Behavior of Dioctyl Sodium Sulfosuccinate in Hydrocarbon Solvents

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Abstract □ The formation of mesomorphic (liquid crystalline) systems by highly concentrated solutions of dioctyl sodium sulfosuccinate in an homologous series of five even-numbered chain length *n*-aliphatic hydrocarbon solvents was studied. It was determined that dioctyl sodium sulfosuccinate concentration, solvent chain length, and temperature significantly affect the formation of a mesomorphic phase. At constant temperature, the concentration of dioctyl sodium sulfosuccinate required to produce a mesomorphic phase increased as the solvent chain length increased from octane to dodecane and then decreased to hexadecane. At constant dioctyl sodium sulfosuccinate concentration in each of the five solvents, the mesomorphic-to-isotropic phase transition temperature reached a minimum at dodecane; however, the phase transition temperatures for dioctyl sodium sulfosuccinate systems in tetradecane and hexadecane were higher than those for decane and octane, respectively.

Keyphrases □ Dioctyl sodium sulfosuccinate—mesomorphic behavior in five hydrocarbon solvents, effects of concentration, solvent chain length, and temperature □ Mesomorphic phases—dioctyl sodium sulfosuccinate in five hydrocarbon solvents, phase transition temperatures, effects of concentration, solvent chain length, and temperature □ Phase transition, mesomorphic—dioctyl sodium sulfosuccinate in five hydrocarbon solvents

Under suitable conditions, certain solid organic compounds can exist in intermediate anisotropic states between true crystals and true isotropic liquids (1). The term mesomorphic or liquid crystal is used to describe the intermediate phase, which is a birefringent fluid of rheological properties ranging from viscous liquids to wax-like semisolids. As a result of their birefringence, mesomorphic phases may be characterized and identified by observation between crossed polarizers in an optical microscope (2). Rosevear (3) identified characteristic textures and birefringence that are related to the molecular arrangement within the phase.

Thermotropic mesomorphic phases are produced by heating a solid to a particular temperature, whereas lyotropic mesomorphic phases occur upon the addition of a solvent to a solid. Lyotropic systems can also be thermotropic in behavior, depending on the amount of solvent added and the temperature. The optical properties and sharp X-ray diffraction patterns indicate a degree of structural order in both types of mesomorphic systems (4, 5).

A number of concentrated solutions of viruses, lipids, polymers, and surfactants form lyotropic mesomorphic phases (6). Balmbra *et al.* (7) indicated that dioctyl sodium sulfosuccinate[1], a surface-active compound, is mesomorphic in the anhydrous state and in high concentration, forms lyotropic mesomorphic phases with water and toluene. At constant temperature, as the dioctyl sodium sulfosuccinate concentration in aqueous systems was increased, the systems underwent phase changes in the order: isotropic → neat phase → viscous isotropic → middle phase. The neat and middle phases were in reversed order from that seen with most other compounds except the phospholipids. The dioctyl sodium sulfosuccinate–toluene systems existed only as middle phase at high dioctyl sodium sulfosuccinate concentrations and were isotropic at lower concentrations. The middle and neat phases are both associated with the smectic mesomorphic phase, one of the three major mesomorphic structural arrangements, the others being nematic and cholesteric. The neat phase consists of a lamellar arrangement of bimolecular layers of surfactant with layers of solvent; the middle phase has an hexagonal structure of long cylindrical micelles (4). There is evidence for additional fine structure in the middle phase, indicating that the hexagonally arranged units may be linear chains of spherical micelles (5).

The purpose of this study was to investigate the formation of lyotropic mesomorphic phases in dioctyl sodium sulfosuccinate–*n*-aliphatic hydrocarbon solvent systems. The parameters evaluated included the influence of surfactant concentration, hydrocarbon solvent chain length, and temperature on the behavior of the lyotropic phases.

EXPERIMENTAL

Materials—Dioctyl sodium sulfosuccinate was synthesized and purified according to the procedure of Balmbra *et al.* (7). The final product, obtained as a hygroscopic waxy solid, gave the correct elemental analysis. Surface tension measurements indicated no minima at the CMC in water, and the CMC value was in good agreement with previously reported values (8, 9).

The hydrocarbon solvents *n*-octane[2], *n*-dodecane[2], *n*-hexadecane[2], *n*-decane[3], and *n*-tetradecane[3] were treated according to the method of Gillap *et al.* (10). Specific gravities determined for all solvents were in excellent agreement with previously reported values (11, 12).

Instrumentation—Microscopic observations were made with a polarizing microscope[4] equipped for photomicroscopy. Sample temperature was maintained on a microscope hot stage[5] regulated with a variable transformer to an approximately 2° rise in temperature per minute. Observations were made at 100×.

Procedure—The binary dioctyl sodium sulfosuccinate–hydrocarbon solvent systems were prepared on a weight/weight percentage basis. The samples, contained in Teflon-lined screw-capped culture tubes, were gently heated with agitation until they fused. The tubes were then slowly cooled to room temperature and allowed to stand for 24–48 hr. prior to observation. This latter step was necessary to ensure equilibration of the samples and thus avoid irreproducibility of the microscopic observations due to possible effects of prior thermal history.

A small portion of the sample was then placed on a microscope slide and formed into a wedge with a cover glass. The slide was

[1] Aerosol OT, American Cyanamid Co.

[2] Aldrich Chemical Co.
[3] Eastman Organic Chemicals.
[4] Nikon model S.
[5] Koeffler, A. H. Thomas Co.

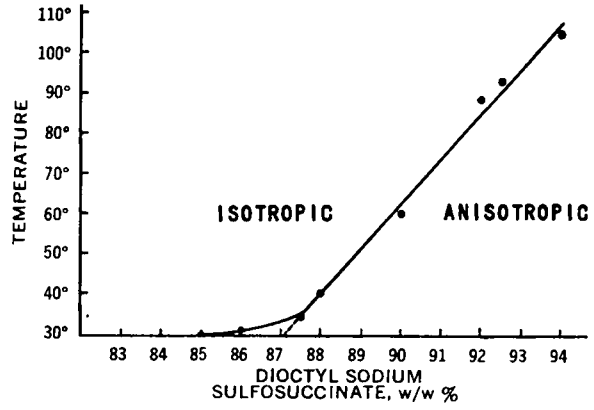

Figure 1—*Anisotropic- (liquid crystalline) to-isotropic transition temperatures for dioctyl sodium sulfosuccinate–dodecane systems.*

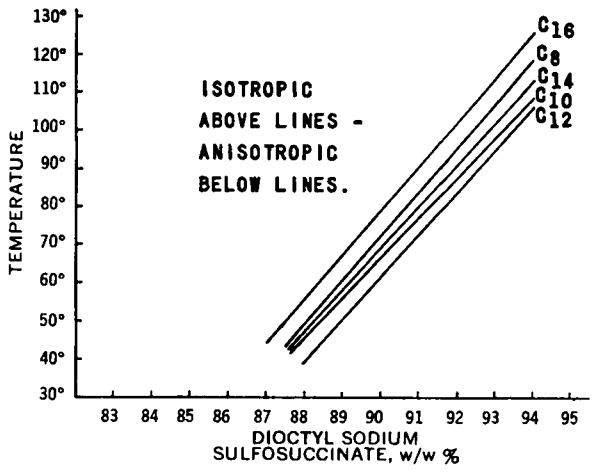

Figure 2—*Anisotropic- (liquid crystalline) to-isotropic transition temperatures for dioctyl sodium sulfosuccinate–n-aliphatic hydrocarbon solvent systems.*

placed on the hot stage at about 30°, and the center portion of the sample was observed as the stage temperature increased. The liquid crystalline systems were anisotropic and showed various birefringent colors and characteristic liquid crystal textures (9). The isotropic samples were dark with no birefringence when observed through the crossed polarizers. The anisotropic-to-isotropic transition temperature was determined to be the first significant darkening of the sample. Three samples for microscopy were taken from different portions of each mixture. Agreement between these runs was good (±0.2°), indicating good mixture homogeneity. Duplicate mixtures agreed within ±1°.

RESULTS AND DISCUSSION

In the absence of solvent, dioctyl sodium sulfosuccinate is a white, waxy, mesomorphic solid at room temperature. When observed between crossed polarizers in a microscope, it is birefringent and has a tightly packed mosaic texture. With increasing concentration of an *n*-aliphatic hydrocarbon solvent, at constant temperature, the system gradually changes to a mesomorphic, turbid, gray-white viscous liquid and finally to a clear, colorless, viscous liquid at the anisotropic-to-isotropic phase transition.

The mesomorphic behavior of dioctyl sodium sulfosuccinate–dodecane systems (Fig. 1) is typical of that of the other dioctyl sodium sulfosuccinate–*n*-aliphatic hydrocarbon solvent systems studied. In Fig. 1, anisotropic-to-isotropic phase transition temperatures are plotted as functions of the weight/weight percentage of dioctyl sodium sulfosuccinate in dodecane. The transition temperature increased as the dioctyl sodium sulfosuccinate concentration increased, consistent with the work of others (7). The lyotropic mesomorphic phase existed at higher temperatures with each increase in dioctyl sodium sulfosuccinate concentration. Below 40°, the data are nonlinear, an apparent result of the instability of the microscope hot stage at temperatures close to ambient. For this reason, the linear portion of the curve was extrapolated to the concentration axis to determine the dioctyl sodium sulfosuccinate concentration at the anisotropic-to-isotropic transition at 30°. Concentrations above 94% weight/weight were not included due to difficulty encountered in achieving adequate homogeneity of the fused binary mixtures. Due to the small amount of solvent present, it was not possible to ensure uniform solvent distribution.

The data for the five *n*-aliphatic hydrocarbon solvents are summarized in Fig. 2. As the *n*-aliphatic solvent chain length increased from octane to dodecane, the anisotropic-to-isotropic transition curves migrated to higher dioctyl sodium sulfosuccinate concentrations at a given constant temperature. For tetradecane and hexadecane, however, this trend was reversed, and at a constant temperature the transition occurred at a lower dioctyl sodium sulfosuccinate concentration with each increment in solvent chain length. Solvent chain length appears to have a significant effect on the formation of the lyotropic liquid crystalline phases in these binary surfactant–solvent mixtures.

When the anisotropic-to-isotropic transition temperature is plotted as a function of increasing *n*-aliphatic hydrocarbon solvent chain length at constant dioctyl sodium sulfosuccinate concentration (Fig. 3), the transition temperature reaches an apparent minimum at dodecane and then rises as the solvent chain length is increased to hexadecane. At constant dioctyl sodium sulfosuccinate concentration, the transition temperature for tetradecane and for hexadecane was higher than that for decane and octane, respectively. This behavior was observed for all of the surfactant concentrations studied. An alternative method of presenting these data is to plot isotherms of dioctyl sodium sulfosuccinate concentration as a function of *n*-aliphatic hydrocarbon chain length (Fig. 4). In interpreting the data in Fig. 4, it is important to remember that the systems are anisotropic above the curves and isotropic below. This is the reverse situation from that in Fig. 3. As shown in Fig. 4, at a constant temperature the dioctyl sodium sulfosuccinate concentration required to give an anisotropic liquid crystalline system increases as the solvent chain length is increased to dodecane and then decreases to hexadecane. The anisotropic system occurs at a lower dioctyl sodium sulfosuccinate concentration in hexadecane than in octane and similarly in tetradecane as compared to decane.

Balmbra *et al.* (7) determined that molecules of dioctyl sodium sulfosuccinate in the absence of solvent exist in hexagonally arranged elongated cylindrical structures. The two-dimensional hexagonal array of cylindrical structural units were generally similar to the inverse middle phase (M_2) proposed by Winsor (13) and the hexagonal II phase of Luzzati and Husson (4). The cylinders resembled elongated micelles, with the surfactant molecules oriented to give a polar core and a hydrocarbon exterior.

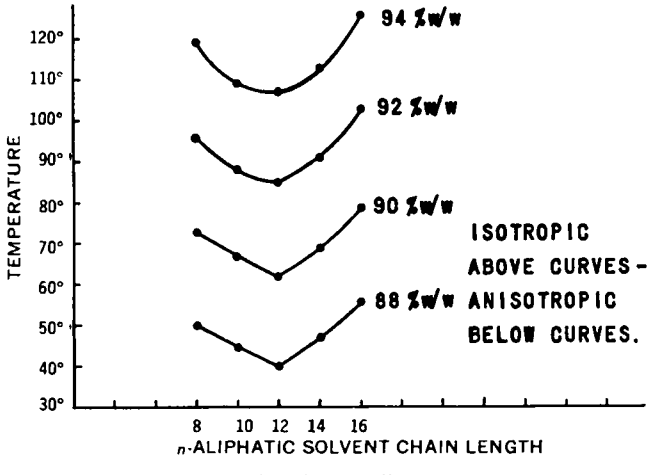

Figure 3—*Anisotropic- (liquid crystalline) to-isotropic transition temperatures for dioctyl sodium sulfosuccinate–n-aliphatic hydrocarbon solvent systems at constant dioctyl sodium sulfosuccinate concentration.*

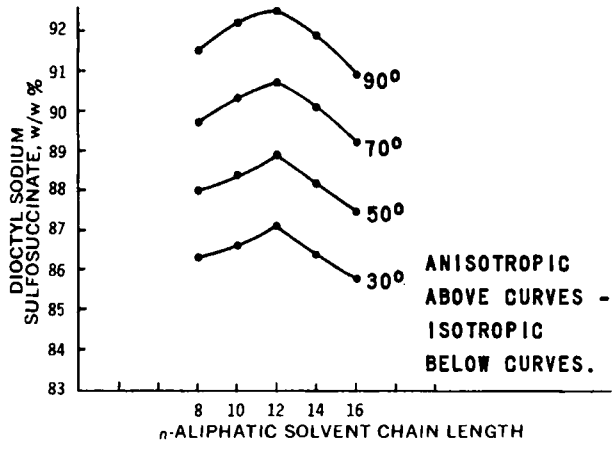

Figure 4—*Isothermal effect of dioctyl sodium sulfosuccinrte concentration on the anisotropic- (liquid crystalline) to-isotropic phase transition of dioctyl sodium sulfosuccinate*-n-*aliphatic hydrocarbon solvent systems.*

Additional fine structure was found (7), suggesting a structural unit consisting of linear chains of aggregated spherical micelles arranged in an hexagonal array. Regardless of the model selected for the structure of the elongated units, it appears that upon addition of solvent the structural arrangement is loosened by solvent orientation at the interface between the structural units (4, 5, 13). Such solvent orientation would, in effect, separate the structural units, decreasing the short-range order but maintaining a degree of long-range ordering dependent on the distance of separation of the units. If the units are composed of linearly aggregated micelles, such solvent penetration would be expected to occur between the micelles and, perhaps to a greater extent, between the linear aggregates themselves. Either of these effects, or both, would produce a weakening of the structural order of the liquid crystalline phase.

At solvent concentrations below the critical amount at a given temperature, deaggregation may be produced by elevating the temperature to the anisotropic-to-isotropic phase transition for the particular system.

Comparison of the octane and hexadecane systems indicates that the anisotropic-to-isotropic phase transition occurs at a higher temperature in hexadecane than in octane (Fig. 3). The lack of significant solvation of dioctyl sodium sulfosuccinate micelles in the longer chain *n*-aliphatic hydrocarbon solvents along with the higher cohesive energy of these solvents resulted in an increase in the micelle weight as the chain length of the solvent was increased (14). It is, therefore, reasonable to expect the dioctyl sodium sulfosuccinate micellar structural units to be more strongly bound in the longer chain *n*-aliphatic hydrocarbon solvents; hence, deaggregation occurs at a higher temperature in hexadecane than in octane. This explanation, however, does not account for the behavior of the dioctyl sodium sulfosuccinate systems in decane, dodecane, and tetradecane. As indicated earlier, the anisotropic-to-isotropic phase transition temperature decreased from octane to dodecane and then increased to hexadecane (Fig. 3). The decrease in thermal stability of the mesomorphic phase, resulting in the minimum at dodecane, indicates that the dioctyl sodium sulfosuccinate structural units are more loosely bound in this solvent than in the other solvents studied.

The "transitional" behavior of dodecane has been reported in studies of the micelle weight of dioctyl sodium sulfosuccinate in the same *n*-aliphatic hydrocarbon solvents. It was found that the micelle weight of anhydrous and hydrous dioctyl sodium sulfosuccinate–dodecane systems was larger than that expected by extrapolation of the corresponding micellar weights in the other solvents (14). In addition, studies of the solubilization of water by solutions of dioctyl sodium sulfosuccinate in the *n*-aliphatic hydrocarbon solvents also indicated the transitional behavior of dodecane systems (15). The relatively low water solubilization and phase properties resembled that of the longer chain solvents, whereas the temperature dependency of the phase transitions was similar to that of the shorter chain solvents. It is not clear at this point what interrelation may exist between these studies; however, it appears that the dioctyl sodium sulfosuccinate–dodecane systems are extremely interesting and worthy of additional study. Work is in progress to elucidate the mechanism of the transitional behavior of dodecane.

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Prototropic Reactivity in Salicylamide and Salicylanilide by Molecular Electronic Spectroscopy

STEPHEN G. SCHULMAN▲, PETER J. KOVI, and JOHN F. YOUNG

Abstract □ The acidity dependences of the electronic absorption and fluorescence spectra of salicylamide and salicylanilide were employed to demonstrate the presence of an intramolecular hydrogen bond in the ground states of the neutral molecules. Upon excitation to the lowest excited singlet state, intramolecular proton transfer from the phenolic groups to the carboxamide and phenyl carboxamide groups is observed. Hydrogen bonding and protonation of the carboxamide groups are shown to occur at the nitrogen atoms rather than at the oxygen atoms.

Keyphrases □ Salicylamide—prototropic reactivity, molecular electronic spectroscopy, presence of intramolecular hydrogen bond □ Salicylanilide—prototropic reactivity, molecular electronic spectroscopy, presence of intramolecular hydrogen bond □ Molecular electronic spectroscopy—prototropic reactivity in salicylamide and salicylanilide □ Hydrogen bonding—salicylamide and salicylanilide, prototropic reactivity, molecular electronic spectroscopy

In recent years, salicylamide and salicylanilide have become among the more notorious members of the pharmaceuticals related to salicylic acid. Nonlegend sleeping pills containing salicylamide frequently have been involved in suicide attempts, while several substituted derivatives of salicylanilide have been identified as photosensitizers. Sensitive fluorometric methods for the analysis of salicylamide are available (1–3). However, because of the similarities in the emission frequencies between the species derived from salicylamide and salicylic acid in the basic solutions on which fluorometry is carried out, there is little selectivity in fluorometric analysis and contamination by salicylate is always a problem. Little or no information about the the electronic absorption or fluorescence of salicylanilide appears to be available. As a result of our interests in the electronic structure, analytical toxicology, photochemistry, and photobiology of these compounds, the present study of the acidity dependences of the electronic spectra of salicylamide and salicylanilide was undertaken.

EXPERIMENTAL[1]

Salicylamide[2] and salicylanilide[2] were recrystallized from chloroform.

Chloroform[3] (spectroquality) and sulfuric acid[4] were used without further purification. Distilled deionized water was used to make dilutions of the sulfuric acid and to prepare acetate, phosphate, and borate buffers as well as sodium hydroxide solutions for absorption and fluorescence titrimetric studies.

[1] Absorption spectra were taken on a Beckman DB-GT spectrophotometer. Fluorescence spectra were taken on a Perkin-Elmer MPF-2A fluorescence spectrophotometer whose monochromators were calibrated against the xenon line emission spectrum and whose output was corrected for instrumental response by means of a rhodamine-B quantum counter. The pH measurements were made on an Orion model 801 pH meter with a Beckman silver–silver chloride–glass combination electrode.

[2] Eastman Organic Chemicals, Inc., Rochester, N. Y.

[3] Matheson, Coleman and Bell, Inc., Rutherford, N. J.

[4] AnalAR, Mallinckrodt Chemical Works, Inc., St. Louis, Mo.

Table I—Long Wavelength Absorption (λ_a) and Fluorescence (λ_f) Maxima[a] of Salicylamide and Salicylanilide in Aqueous Media

	Salicylamide		Salicylanilide	
	λ_a	λ_f	λ_a	λ_f
18 *M* H_2SO_4	317	383	316	364
1 *M* H_2SO_4	302	435	298	449
pH 7 phosphate buffer	302	417	298, 338	420
0.01 *M* NaOH	329	417	338	420

[a] Spectral maxima are reported in nanometers.

RESULTS

The long wavelength absorption maxima and fluoresence maxima of salicylamide and salicylanilide in representative regions of the Hammett acidity and pH scales are presented in Table I. The electronic absorption spectra of these compounds are shown in Figs. 1 and 2.

The ground-state dissociation constants for the prototropic equilibria between the protonated and neutral molecules (pKa_1) and between the neutral molecules and anions (pKa_2) derived from salicylamide and salicylanilide were estimated from the midpoints of the variations of the absorption spectra with pH and Hammett acidity (absorptiometric titrations). The excited state dissociation constants (pKa_1^* and pKa_2^*) were determined from the midpoints of the variations of relative fluorescence quantum yields with Hammett acidity and pH (fluorometric titration curves). These data are presented in Table II.

DISCUSSION

Agren (4) previously reported the pKa for the equilibrium between the neutral molecule and anion derived from salicylamide to be 8.9. However, the current spectrophotometric studies yielded a value of 8.3. Dissociation of the neutral molecule results in a shift of the long wavelength absorption maximum to longer wavelength, indicating that dissociation of the neutral salicylamide takes place from the phenolic group in the ground electronic state (5). The magnitude of the ground-state pKa for dissociation from the phenolic group of salicylamide is comparable to that of methyl salicylate (6), the only other simple derivative of salicylic acid in which the phenolic group is free to dissociate while the functional group derived from the carboxyl group of salicylic acid is uncharged.

The shift to longer wavelength of the absorption spectrum of salicylamide, accompanying protonation, is qualitatively similar to that of methyl salicylate (6) and indicates that, in the ground state, protonation of salicylamide occurs in the carboxamido group. However, while methyl salicylate can only be protonated at the carboxyl-type oxygen in concentrated acid, salicylamide can con-

Table II—Ground-State Dissociation Constants (pKa) and Lowest Excited Singlet State Dissociation Constants (pKa*) of Salicylamide and Salicylanilide.

	Salicylamide[a,b]	Salicylanilide[a,b]
pKa_1	−2.6	−4.1
pKa_1^*	−5.3	−6.6
pKa_2	8.3	6.9
pKa_2^*	2.1	—[c]

[a] The pKa values were determined by absorptiometric titrations. [b] The pKa* values were taken from the midpoints of the fluorometric titrations. [c] Equilibrium was not attained during the lifetime of the excited state of the salicylanilide zwitterion.

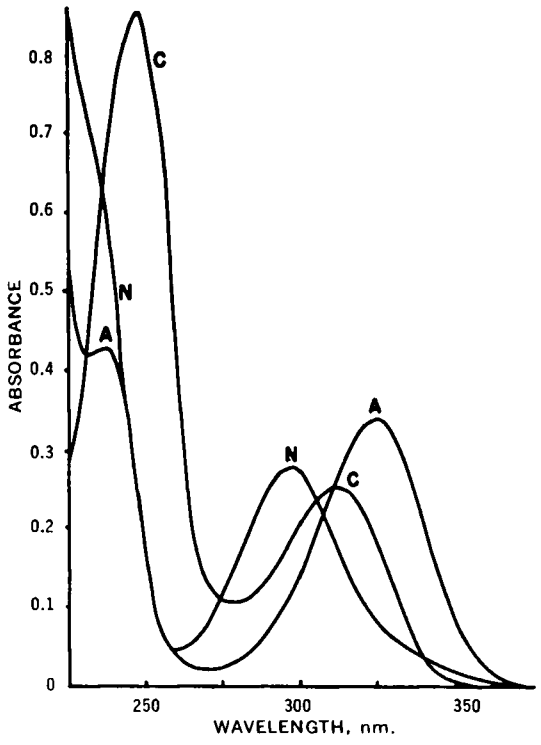


Figure 1—*Absorption spectra of the prototropic species derived from salicylamide (C) cation, $H_0 = -10$; neutral molecule (N), pH 4; anion (A), pH 12.*

ceivably be protonated at either the carbonyl oxygen atom or the amido nitrogen atom of the carbamido group. A recent study (7) showed that, in benzamide, protonation takes place in the ground state, the lowest excited singlet state, and the lowest triplet state at the amido nitrogen atom. The magnitude of the shift of the absorption spectrum of salicylamide upon protonation, which is smaller than that of methyl salicylate, and the pKa for protonation of salicylamide, which is only slightly more acidic than that of benzamide (7) and considerably less acidic than that of methyl salicylate, suggest that protonation occurs at the nitrogen atom in salicylamide. The proposed sequence of protolytic dissociations of salicylamide, beginning with the cation, is shown in Scheme I.

Upon going from concentrated acid to more dilute acid media, the fluorescence of salicylamide shifts to a longer wavelength. This indicates that the protonated molecule, present in concentrated acid,

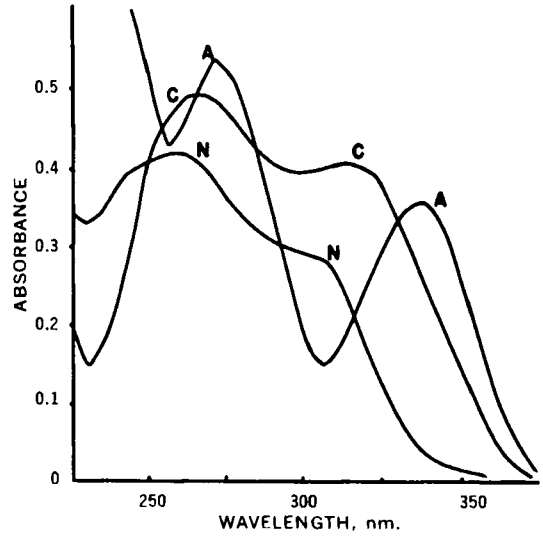
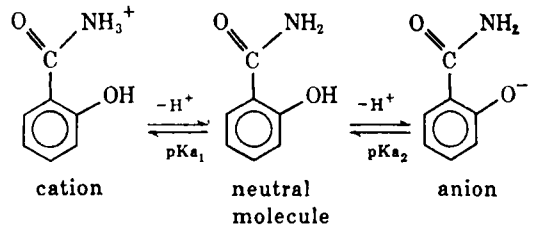


Figure 2—*Absorption spectra of the prototropic species derived from salicylanilide (C) cation, $H_0 = -10$; neutral molecule (N), pH 4; anion (A), pH 12.*



Scheme I—Protolytic reactions of salicylamide in the ground state

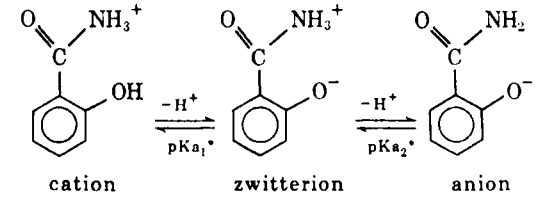
dissociates in the lowest excited singlet state from the phenolic group. In near neutral media, the fluorescence shifts to a shorter wavelength, signaling dissociation from the carboxamido group. The proposed sequence of dissociations of salicylamide in the lowest excited singlet state is represented in Scheme II.

In the lowest excited singlet state, the uncharged salicylamide is a zwitterion, while in the ground state it is a neutral molecule. As a result, no straightforward comparison between the successive ground and excited state equilibrium constants is possible.

In the region between pH 2.1 and $H_0 = -2.6$, the formation of the excited zwitterion of salicylamide must proceed entirely by phototautomerization of the neutral molecule. The pKa_1 of salicylamide is about 0.5 log unit more acidic than that of benzamide, even though the *O*-hydroxyl group might be expected to enhance the basicity of the carboxamido group. This is very much like the diminution of the basicity of the carboxyl group in salicylic acid by the presence of the hydroxyl group and suggests the presence of an intramolecular hydrogen bond between the hydroxyl group and the carboxamido group in the ground state. Presumably, the phototautomerism of salicylamide is intramolecular, consisting of readjustment of the position of the hydroxylic proton in the hydrogen bond so that the proton is essentially lost from the phenolic group and gained by the carboxamido group.

The arguments that were developed for salicylamide are all qualitatively applicable to the prototropic reactivity of salicylanilide, as seen from the similarities between the spectral shifts of the two compounds produced by protolytic dissociation. However, the presence of the phenyl group on the carboxamido nitrogen atom of salicylanilide has a substantial effect on the acid and base strengths of the various ground and excited state prototropic species derived from salicylanilide. All measurable equilibrium constants of salicylanilide are about 1.5 orders of magnitude more acidic than those of salicylamide. This observation can be rationalized in terms of the electromeric electron-withdrawing effect the phenyl group has on the lone pair of the carboxamido nitrogen atom of salicylanilide. This effect reduces the basicity of the carboxamido nitrogen, making it more difficult to protonate and thereby accounting for the lower value of pKa_1 for salicylanilide. The reduction in basicity of the carboxamido nitrogen atom also weakens the hydrogen bond between the phenolic proton and the phenyl-substituted carboxamido group. The weakening of this hydrogen bond, which stabilizes the phenolic proton in the neutral salicylamide molecule, makes the phenolic proton easier to remove. In addition, the electron-withdrawing effect of the phenyl group makes the phenyl-substituted carboxamido group more electron withdrawing than the unsubstituted carboxamido group. The combined effects of the weakening of the internal hydrogen bond and the stronger electromeric and field effects upon the hydroxyl group make pKa_2 more acidic in salicylanilide than in salicylamide.

Finally, in the lowest excited singlet state, only pKa_1^* could be measured for both compounds. Because pKa_1^* represents dissocia-



Scheme II—Protolytic reactions of salicylamide in the lowest excited singlet state

tion of the cations from the phenolic groups to form the zwitterions, hydrogen bonding is not important in this equilibrium. However, the electron-withdrawing enhancement of the carboxamido group by the phenyl group is even stronger in the lowest excited singlet state than in the ground state, as evidenced by the reduction in basicity of arylamines in the lowest excited singlet state (8). Thus, the lower pK_{a_2} of salicylanilide relative to salicylamide is attributed entirely to the greater electromeric and field effects of the phenyl-substituted carboxamido group in the lowest excited singlet state.

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Alkaloid Studies VIII: Isolation and Characterization of Alkaloids of *Tabernaemontana heyneana* Wall and Antifertility Properties of Coronaridine

WALTER E. MEYER, JOHN A. COPPOLA*, and LEON GOLDMAN▲

Abstract □ Extraction of the roots of *Tabernaemontana heyneana* Wall yielded the alkaloids coronaridine, voacangine, ibogamine, 19-oxocoronaridine, and the pseudoindoxyl of voacangine. Coronaridine was demonstrated to prevent pregnancies in adult female rats when administered orally.

Keyphrases □ *Tabernaemontana heyneana* Wall—isolation and characterization of alkaloids, antifertility properties of coronaridine □ Alkaloids—isolation and identification from *Tabernaemontana heyneana*, antifertility properties of coronaridine □ Antifertility agents, potential—isolation, characterization, and screening of coronaridine from *Tabernaemontana heyneana* □ Coronaridine—isolation from *Tabernaemontana heyneana*, screened for antifertility properties in rats

Investigation of the alkaloids of the Tabernaemontanoideae tribe of the Apocynaceae family has received considerable attention because of the interest aroused in the alkaloids isolated from *Tabernanthe iboga* of the same family.

The roots and bark of *Tabernaemontana heyneana* Wall were previously shown to contain the indole alkaloid coronaridine (I) (1) and its 20-hydroxy derivative heyneanine (II) (2). In this study, the roots of this botanical were examined, and the isolation and identification of additional indole alkaloids and some pharmacological properties of coronaridine are described.

DISCUSSION

An aqueous ethanolic extract of the roots of *T. heyneana* Wall was found to prevent fertilization of adult female rats when administered orally. The residue from this extract was treated with aqueous acetic acid, and the soluble components were then extracted into methylene chloride. Removal of the acidic components from the methylene chloride extract by washing with aqueous base gave, after evaporation of the solvent, a brown amorphous crude material (Fraction A), which retained all of the antifertility activity of the original extract. Chromatographic fractionation of a portion of Fraction A on silica gel yielded the alkaloid coronaridine and a closely associated alkaloid which was not obtained pure.

The alkaloids in Fraction A were precipitated as their hydrochloride salts, and the free bases were regenerated to afford a tan amorphous mixture of alkaloids (Fraction B). Sequential chromatography of Fraction B on silica gel, Grade I neutral alumina, and Grade III neutral alumina yielded additional coronaridine and the second alkaloid, which was identified as voacangine (III) by comparison with an authentic sample[1].

The syrup obtained from a methanol wash of the Grade III neutral alumina column was separated further by partition chromatography on diatomaceous earth to give a chromatographically pure, noncrystalline alkaloid. This alkaloid, which appeared on thin-layer chromatograms of solutions of coronaridine that had been exposed to air, was identical with the iodine–sodium bicarbonate oxidation [the procedure for conversion of ibogaine (IV) to 19-oxoibogaine (V) (3)] product of coronaridine. The appearance of a second carbonyl absorption band in the IR spectrum (6.06 μ) and a molecular-ion peak (m/e 352.1780) in the mass spectrum, which appeared 14 mass units higher than the molecular-ion peak for coronaridine, indicated this alkaloid to be 19-oxocoronaridine (VI), previously isolated from the bark of *Conopharyngia jollyana* Stapf. (4). [In *Reference 5*, this compound was characterized only by mass spectral analysis (m/e 352) and R_f values.]

Column chromatography of Fraction B on Grade II neutral alumina yielded a noncrystalline alkaloid fraction, which was further separated by partition chromatography on diatomaceous earth. A fraction containing 19-oxocoronaridine, contaminated with a highly fluorescent yellow material, was rechromatographed on silica gel to provide a crystalline yellow-green fluorescent alkaloid. The distinctive UV spectrum and mass spectrum indicated this material to be the pseudoindoxyl (VIII) (6) of voacangine (III).

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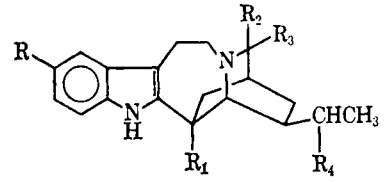
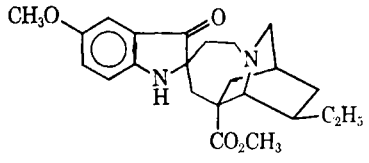


	R	R_1	$R_2 + R_3$	R_4
I	H	CO_2CH_3	H_2	H
II	H	CO_2CH_3	H_2	OH
III	OCH_3	CO_2CH_3	H_2	H
IV	OCH_3	H	H_2	H
V	OCH_3	H	=O	H
VI	H	CO_2CH_3	=O	H
VII	H	H	H_2	H



VIII

eluted were combined and subjected to partition chromatography utilizing a less polar solvent system. One fraction yielded a crystalline alkaloid which was identified as ibogamine (VII) (7–9).

Although 19-oxocoronaridine and the pseudoindoxyl of voacangine may occur naturally, the susceptibility of the iboga alkaloids to oxidation under mild conditions (10) suggests that these alkaloids may be formed by the autoxidation of the parent alkaloid. Hootele and Pecher (5) suggested the possibility of a similar origin for several 19-oxo iboga alkaloids isolated from *C. jollyana*. The possibility of the pseudoindoxyl derivatives isolated from *T. rupicola* being artifacts was suggested by Niemann and Kessel (11).

EXPERIMENTAL[2]

Extraction Procedure—Dried ground roots of *T. heyneana* Wall[3] (7.5 kg.) were extracted by percolation for 18 hr. with 67.5 l. of 5% 3A ethanol. The percolate was evaporated under reduced pressure at 25°, and the residue was stirred for 18 hr. with 3.3 l. of 10% aqueous acetic acid. The suspension was filtered and the filter cake was washed with 10% acetic acid. The combined extract and wash was extracted three times with 1-l. portions of methylene chloride. The combined extracts were washed two times with 250-ml. portions of 1 *N* sodium hydroxide and then the methylene chloride layer was dried (magnesium sulfate). Evaporation of the solution *in vacuo* yielded 26.4 g. of a brown frothy residue (Fraction A).

A 5.0-g. sample of Fraction A was dissolved in 100 ml. of ether. A solution of dry hydrogen chloride in ether was added, and the pale-yellow precipitate which formed was removed by filtration. The precipitate was distributed between chloroform and 1 *N* sodium hydroxide, and the chloroform layer was dried (magnesium sulfate) and evaporated *in vacuo* to give 3.4 g. of a tan amorphous residue (Fraction B).

Isolation of Coronaridine (I) and Voacangine (III)—Fraction A (2 g.) was chromatographed on a silica gel column (200 g.) by developing with 1 l. of benzene, followed by 200-ml. portions of benzene containing 5, 10, 20, and 40% chloroform. When a yellow band appeared at the column front, collection of 20-ml. cuts was begun. Cuts 75–150 contained 2.1 g. of a mixture of two alkaloids, a 1-g. portion of which was chromatographed on a column[4] using a mixture of chloroform and methanol (200:1); 5-ml. cuts were collected. Pure noncrystalline I (353 mg.) was obtained from the residue of cuts 30–62; $[\alpha]_D^{25}$ −35.2° (c 1.1, $CHCl_3$) [lit. (12) $[\alpha]_D^{25}$ −34° (c 1.0, $CHCl_3$)]; $\lambda_{max}^{CHCl_3}$: 2.89, 5.80, 6.84, and 7.99 nm. The hydrochloride salt was prepared in the usual manner, m.p. 213–214° dec. [lit. (12) m.p. 210–211° dec.].

Fraction B (3.4 g.) was chromatographed on 120 g. of silica gel, and the material eluted with chloroform–methanol (200:1) was purified further on a column of 200 g. of Grade I alumina which was developed with benzene–chloroform (1:1). The first fraction (125 ml.) yielded 395 mg. of coronaridine. The following fractions, which contained a mixture of coronaridine and voacangine, were rechromatographed on a column of 100 g. of Grade III alumina. The column was developed with benzene and, after collecting 17 125-ml. cuts, was washed with chloroform and methanol. The residue (245 mg. Fraction C) from the wash was reserved for further purification. Evaporation of the first cut yielded an additional 429 mg. of coronaridine, whereas the residue from the third cut yielded 122 mg. of voacangine (III), obtained as a pale-yellow syrup. The mass spectrum of this syrup was identical with that of an authentic sample of III[1]. The PMR (13), IR (14), and UV spectra (14) were in accord with published data.

Isolation of 19-Oxocoronaridine (VI)—Fraction C (245 mg.) was subjected to partition chromatography, and the effluent was monitored at 285 nm. 19-Oxocoronaridine (VI) (23 mg.) was obtained in the fourth holdback volume as a pale-yellow syrup, $\lambda_{max}^{C_2H_5OH}$: 222 (28,500), 276 (sh) (6500), 284 (7110), and 292 (6000) nm.; λ_{max}^{KBr}: 5.81, 6.06, 6.92, 8.00, 8.85, and 13.51 μ; PMR ($CDCl_3$): δ 8.20 (s, 1H, exchangeable NH), 7.15 (m, 4H, substituted aromatic), 4.51 (s, 1H, $\diagdown$C—H$\diagup$), 3.70 (s, 3H, CO_2CH_3), and 0.97 (t, 3H, CH_2CH_3); mass spectrum, molecular ion at *m/e* 352.1780 ($C_{21}H_{24}N_2O_3$, calc. 352.1786).

Oxidation of Coronaridine (I) to 19-Oxocoronaridine (VI)—Coronaridine hydrochloride (260 mg., 0.7 mmole) was dissolved in 8 ml. of 50% aqueous tetrahydrofuran containing 500 mg. of sodium bicarbonate. A solution of 360 mg. (1.4 mmoles) of iodine in 10 ml. of tetrahydrofuran was added dropwise and, after an additional 30 min. at 25°, the reaction was cooled in an ice bath and 10 ml. of water and 20 ml. of methylene chloride were added. The layers were separated and the aqueous phase was washed with 10 ml. of methylene chloride. The combined methylene chloride solutions were washed consecutively with 20 ml. of 5% sodium thiosulfate, 2 *N* sulfuric acid, and water. After drying (magnesium sulfate), the solution was evaporated to give 240 mg. of a pale-yellow syrup, which was chromatographed on a silica gel column[5] using ethyl acetate for development; 0.75-ml. cuts were collected. Coronaridine (27 mg.) was recovered by evaporation of cuts 2–8, and 19-oxocoronaridine (86 mg.) was obtained after evaporation of cuts 15–30. This material was found to be identical with the 19-oxocoronaridine isolated from *T. heyneana* by comparisons of IR and mass spectra.

Isolation of the Pseudoindoxyl VIII of Voacangine—Fraction B (12.7 g.), obtained from a 16-g. portion of Fraction A, was chromatographed on 400 g. of alumina (Grade II). After elution with 3 l. of benzene, a chloroform–methanol (4:1) wash yielded a fraction (2 l.) which, upon evaporation, gave 6.9 g. of a yellow syrup. Partition chromatography of 6.0 g. of this syrup yielded a yellow syrup (Fraction D) from the first two holdback volumes and 553 mg. of a

Table I—Antifertility Effect in Adult Female Rats

Dose, mg./kg./day	Number Rats Pregnant/ Number Rats Treated
1	3/4
5	0/4
10	0/3
20	0/4
40	0/3

[2] Melting points are uncorrected. IR spectra were determined on a Perkin-Elmer model 21 recording spectrophotometer. UV absorption spectra were determined on a model 11 MS Carey recording spectrophotometer. Mass spectra were obtained on a AEI MS 9 mass spectrometer. Proton magnetic resonance (PMR) spectra were determined on a Varian A 60 spectrometer. Davison silica gel (60–200 mesh) and Woelm neutral alumina were used as indicated. Partition chromatography was performed on Johns Mansville diatomaceous earth using a heptane–ethyl acetate–methanol–water (80:20:17:4) mixture unless otherwise specified.

[3] Collected and shipped through the courtesy of C. N. Correia, Goa, India; a voucher specimen is deposited in the Botanical Collection of Lederle Laboratories, A Division of American Cyanamid Company, Pearl River, NY 10965

[4] Quanta-Gram D-116, Quantum Industries, Fairfield, N. J.

[5] Quanta-Gram D-32.

Table II—Estrogenic Effect in Immature Female Rats

Compound	Dose, mcg./rat/day × 3	Uterine Weight Ratio (Treated/Control)
Estrone	0.37	0.92
	1.1	1.67
	3.3	2.30
	10.0	2.71
Coronaridine hydrochloride	12	0.88
	37	1.00
	111	1.35
	333[a]	1.85
	1000	2.09

[a] Approximate ED_{100} in antifertility assay.

yellow syrup (Fraction E) from the fourth holdback volume. Final purification of Fraction E was effected by chromatography on 15 g. of silica gel and eluting with ethyl acetate, followed by preparative TLC on silica gel plates[6] using ethyl acetate for development. A bright-yellow band (R_f 0.7) was eluted with methanol, and the solution was evaporated *in vacuo* to yield 175 mg. of a yellow froth. Two crystallizations from methanol gave 20 mg. of VIII as yellow-green fluorescent needles, m.p. 201–203° [lit. (6) m.p. 205–208°]; PMR ($CDCl_3$): δ 3.77 (s, 3 H, OCH_3) and 3.55 (s, 3 H, CO_2CH_3). The mass, IR, and UV spectra were in accord with published data (6).

Isolation of Ibogamine (VII)—Fraction D was fractionated by partition chromatography using a 2-methoxyethanol–heptane system. Evaporation *in vacuo* of the third holdback volume and crystallization of the residue from ethanol yielded colorless crystals of VII, m.p. 160–161° [lit. (7) m.p. 162–163°]; $[\alpha]_D^{25}$ −31.3° (c 1.1, $CHCl_3$) [lit. (7) $[\alpha]_D^{26}$ −36.4° ($CHCl_3$)]. The IR (8) and mass (9) spectra were in agreement with published data.

PHARMACOLOGY

Coronaridine was tested previously in a variety of pharmacological systems (12). Although a relatively broad spectrum of biological activity was reported, no antifertility properties were described. In the present study, the oral administration of coronaridine hydrochloride to adult female rats at levels of 5 mg./kg./day or above prevented pregnancies. Voacangine, assayed by the same procedure, did not prevent pregnancies.

Graded doses of coronaridine hydrochloride were administered orally once daily for 10 days to adult female rats (225–250 g.), which were maintained on a standard diet of laboratory rat pellets and water *ad libitum*. The dose was composed of propylene glycol and an appropriate amount of coronaridine hydrochloride, so that the desired dose was administered in a 0.25-ml. volume. Control rats were given propylene glycol without the alkaloid. From the 1st day of treatment, the females were placed with fertile males. Four days after the last dose, the females were sacrificed and uterine fetal implantation sites were counted. Estrone was used as a standard. Under these conditions, simultaneously performed vehicle control groups yielded pregnancy rates of approximately 90%, and the ED_{100} of estrone was consistently 0.5 mg./kg./day.

The results of this study are summarized in Table I.

[6] Quanta-Gram PLQ1-F.

In the uterotropic assay, which measured estrogenic activity, immature female rats (50–60 g.) received single daily oral doses in 0.25 ml. of propylene glycol for 3 days. The uteri were removed and weighed 24 hr. after the last dose. A minimum of five rats was tested at each dose level and, here also, estrone was used as reference standard. The average uterine weights of estrone- or test compound-treated rats were divided by the average uterine weight of simultaneously performed vehicle controls to estimate the degree of uterine hypertrophy. Thus, for example, a ratio of 1.25 indicated a 25% increase in uterine weight above control values. The results of this assay are summarized in Table II. The data indicated that coronaridine was weakly estrogenic in that it was only about 3×10^{-4} times as potent as estrone in eliciting uterine growth. Nevertheless, doses approximately one-third the minimally effective antifertility dose produced a 35% increase in uterine weight, and the antifertility ED_{100} was associated with an 85% increase. Therefore, the contraceptive action of coronaridine was ostensibly related to its inherent estrogenicity since the two effects were apparently inseparable.

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Influence of Lecithins on Autoxidation of Cholesterol from Aqueous Dispersions at 85°

NORMAN D. WEINER*▲, WERNER C. BRUNING*, and ALVIN FELMEISTER†

Abstract □ The autoxidative formation of 7-ketocholesterol and 7-hydroxycholesterol from aqueous cholesterol dispersions was studied in the absence and in the presence of various lecithins at 85 ± 2°. Whereas more than one-half of the cholesterol is oxidized within 8 hr. in the absence of phospholipids, less than 10% oxidation occurs in the presence of the lecithins. The *in vivo* significance of these data is discussed.

Keyphrases □ Lecithins—influence on autoxidation of aqueous cholesterol dispersions □ Cholesterol dispersions, aqueous—influence of various lecithins on autoxidation at 85°, *in vivo* significance □ Autoxidation—aqueous cholesterol dispersions, influence of various lecithins, *in vivo* significance □ Phospholipids—influence of lecithins on autoxidation of aqueous cholesterol dispersions

Cholesterol is relatively stable toward air oxidation in the macrocrystalline state, but when finely divided and exposed to excess oxygen, oxidation takes place. These conditions are met, in particular, when cholesterol, in colloidal dispersion stabilized with sodium stearate, is aerated (1–3). The reaction is rapid at 85°, and the chief products are 7-ketocholesterol and diols, principally 7α- and 7β-hydroxycholesterol (3). Oxidation also occurs when cholesterol in thin films is exposed to air and irradiated with UV light (4).

It was found that the oxidation of cholesterol under physiological conditions of pH and temperature takes place, although at a slower rate. Bergström and Wintersteiner (2, 3) suggested that a conversion of cholesterol to the 7-oxygenated sterols might occur *in vivo* and that an attack on the sensitive 7-position of the cholesterol molecule might be involved in the biological degradation of this sterol. It has also been suggested that the oxidation of cholesterol *in vivo* may be related to the development of a variety of pathological conditions such as carcinoma (5), cholelithiasis, and atherosclerosis (6). In addition, MacDougall *et al.* (7) showed a number of oxidation products of cholesterol to be toxic to organ cultures of rabbit aorta.

In a recent series of papers (8–10), the autoxidation of cholesterol in aqueous dispersions and in monomolecular films was reported. The rate of oxidation of cholesterol was much faster at the surface than in the bulk. Although more than one-half of the cholesterol was oxidized at the surface within 8 hr. at room temperature, no noticeable reaction was observed for the oxidation of cholesterol from aqueous dispersions at room temperature during this time. However, similar rates of oxidation were observed when the dispersions were maintained at 85°.

Although it is conceivable that some cholesterol oxidation does occur *in vivo* (2, 3), these reaction rates must be significantly slower than those reported for *in vitro* systems. Surface pressure–surface area data for mixed cholesterol–phospholipid films (8) did indicate that the phospholipids present in the film protected the cholesterol from attack by oxygen. The mechanism of this protection appears to be physical rather than chemical in nature. Apparently, it involves intermolecular interactions between the phospholipids and cholesterol which serve to protect the reactive groups on the cholesterol molecule from attack by oxygen. Since membrane associated cholesterol is always associated with phospholipids, it appears likely that this association could be a significant factor in the *in vivo* protection of cholesterol oxidation.

It was, therefore, of interest to quantitate the effects of phospholipids on the air oxidation of cholesterol in aqueous dispersions.

EXPERIMENTAL

Two and one-half grams of cholesterol[1] was dissolved in 100 ml. of absolute alcohol with the aid of heat. The purity of cholesterol was verified by TLC (a 250-mcg. sample yielded a single spot). Sodium stearate, 0.5 g. (purified powder), was dissolved in 500 ml.

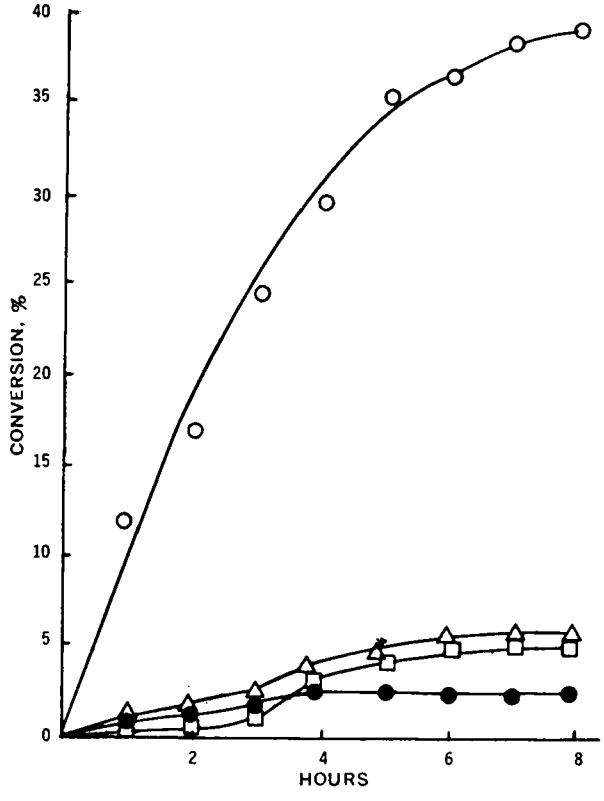

Figure 1—*Percentage conversion of cholesterol to 7-ketocholesterol for dispersions exposed to flowing air at 85 ± 2°. Key: ○, no lecithin; ●, vegetable lecithin; □, egg lecithin; and △, dipalmitoyl lecithin.*

[1] Pierce Chemical Co., Rockford, Ill.

of distilled water, which was preheated to 70°. The cholesterol solution in absolute alcohol was then poured into the sodium stearate solution with constant stirring to obtain a colloidal dispersion. Dry air was allowed to pass through a flowmeter at a rate of 100 ml./min. and was directed into the cholesterol dispersion, which was maintained at a temperature of 85 ± 2°. The air was allowed to flow for 8 hr., during which time samples of the cholesterol dispersion were removed with the aid of a micrometer syringe[2] at hourly intervals.

The amount of diols, expressed as 7-hydroxycholesterol, was determined by the method described by Bergström and Wintersteiner (3). Cholesterol dispersion, 0.03 ml., was mixed with 1 ml. of chloroform and 2 ml. of Lifschütz reagent. This color reagent was prepared by dissolving 0.1 g. of ferric chloride hexahydrate in 90 ml. of glacial acetic acid and then adding 10 ml. of concentrated sulfuric acid. After 5 min., a bluish-green color developed which remained stable for about 15 min. The absorbance was read at a wavelength of 590 nm. with a spectrophotometer[3], and the concentration of the diols was determined from the standard curve. The preparation of 7-hydroxycholesterol for use in obtaining the standard curve was described previously (9).

The amount of 7-ketocholesterol formed was determined by mixing 0.03 ml. of cholesterol dispersion and 3 ml. of ethanol. The absorbance was read at a wavelength of 256 nm. with the spectrophotometer[3]. The concentration was then determined from the standard curve of 7-ketocholesterol[4]. The purity of this standard was verified by TLC. The use of standard mixtures demonstrated that 7-hydroxycholesterol did not interfere with the analysis of 7-ketocholesterol and that the presence of 7-ketocholesterol did not interfere with the analysis of 7-hydroxycholesterol.

Similar experiments were performed for systems in which vegetable lecithin[5], egg lecithin[6], or dipalmitoyl lecithin[7] was added. In each case, the lecithin was dissolved with the cholesterol in 100 ml. of absolute alcohol to yield 1:1 cholesterol–lecithin molar ratios.

To correct for the presence of lecithins in the assays of 7-ketocholesterol and 7-hydroxycholesterol, standard curves for mixtures of the steroids and lecithins were used.

RESULTS AND DISCUSSION

Figures 1 and 2 show the percentage conversion–time curves for the conversion of cholesterol to 7-ketocholesterol and 7-hydroxycholesterol, respectively, in the absence and in the presence of the lecithins.

As can be seen, all lecithins studied are very effective inhibitors of cholesterol oxidation. Although the mechanism of inhibition cannot be stated with certainty, it appears to be physical rather than chemical in nature since: (*a*) the intermolecular interactions between cholesterol and lecithins are strong and involve the sensitive

[2] Agla, Burroughs Wellcome and Co., London, England.
[3] Beckman.
[4] Mann Research Laboratories, New York, N. Y.
[5] Ruger Chemical Co., Long Island City, N. Y.
[6] Becton Dickinson & Co., Orangeburg, N. Y.
[7] Mann Laboratories, Inc., New York, N. Y.

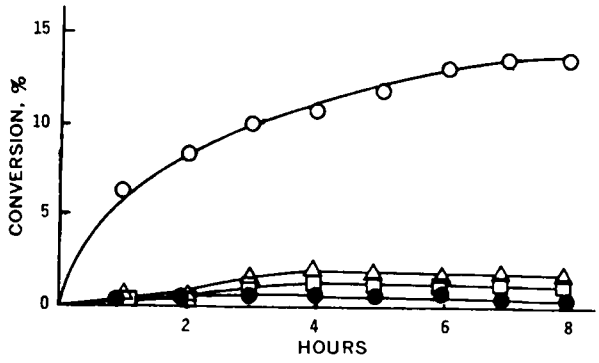

Figure 2—*Percentage conversion of cholesterol to 7-hydroxycholesterol for dispersions exposed to flowing air at 85 ± 2°. Key:* ○, *no lecithin;* ●, *vegetable lecithin;* □, *egg lecithin; and* △, *dipalmitoyl lecithin.*

3-hydroxy group of cholesterol, and (*b*) dipalmitoyl lecithin, a fully saturated phospholipid, is very effective in inhibiting oxidation.

Although the systems studied here certainly cannot be easily extrapolated to *in vivo* systems, the data indicate that a possible mechanism for the protection of membrane-bound cholesterol from oxidation may be the interactions with phospholipids.

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Synthesis and MAO Inhibitory Activity of 6-(2-Propynyl)-6,7-dihydro-5*H*-dibenz[*c*,*e*]azepine

J. RICHARD GRUNDER, LUKE SAN, and PUSHKAR N. KAUL▲

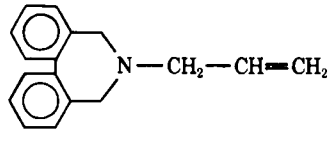
Abstract □ A propynyl dibenzazepine was synthesized with a view to design a compound that would possess the structural features essential for biological activity present in the antiadrenergic compound azapetine and in the MAO inhibitor pargyline, both of which have been used as antihypertensives. The new compound possesses MAO-inhibiting activity weaker than that of pargyline.

Keyphrases □ 6-(2-Propynyl)-6,7-dihydro-5*H*-dibenz[*c*,*e*]azepine—synthesized and screened as potential MAO inhibitor □ MAO inhibitors, potential—synthesis and screening of 6-(2-propynyl)-6,7-dihydro-5*H*-dibenz[*c*,*e*]azepine □ Dibenzazepines—synthesis and screening of 6-(2-propynyl)-6,7-dihydro-5*H*-dibenz[*c*,*e*]azepine as MAO inhibitor

Various dibenzazepine derivatives were described by Randall and Smith (1), of which azapetine[1] (I) was the most potent antiadrenergic compound. It was subsequently marketed for clinical use in hypertensive and vascular disorders. Both the allyl group and the dibenzazepine tricyclic arrangement in azapetine appear to be essential for its biological activity.

Another series of compounds that possesses strong MAO-inhibiting activity led to the clinically used antihypertensive pargyline[2] (II), the inhibiting property being not necessarily related to the hypotensive property (2). The synthesis and evaluation of several MAO inhibitors structurally related to pargyline have indicated the importance of the propargyl or 2-propynyl group to the activity of these compounds (3, 4).

The 2-propynyl or propargyl derivative of the dibenz[*c*,*e*]azepine ring system may provide a compound with desirable or even more potent antihypertensive activity than either of the two clinically tried agents, I and II. Synthesis of such a compound, 6-(2-propynyl)-6,7-dihydro-5*H*-dibenz[*c*,*e*]azepine (III), and its MAO-inhibiting activity are reported in this paper while the antiadrenergic and antihypertensive properties of the compound will be reported later.



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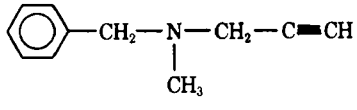

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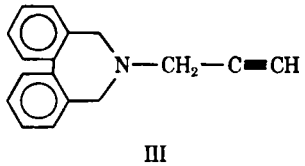

III

[1] Ilidar, Roche.
[2] Eutonyl, Abbott.

Table I—*In Vitro* Inhibition of Liver MAO by III as Compared with Other Known Inhibitors

Inhibitor	Concentration, *M*	Velocity Constant *K*, $\times 10^{-2}$ min.$^{-1}$ S_1[a]	S_2	K_i
Dibenzazepine derivative (III)	4.6×10^{-5}	6.02	4.20	4.9×10^{-5}
	9.3×10^{-5}	3.03	2.35	
	2.2×10^{-4}	2.66	1.69	
	3.5×10^{-4}	1.61	1.17	
Iproniazid	1.4×10^{-5}	8.88	6.66	1.5×10^{-5}
	2.8×10^{-5}	6.48	4.62	
	5.6×10^{-5}	5.33	3.08	
Pargyline (II)	0.7×10^{-8}	4.50	—	1.7×10^{-7}
	2.0×10^{-8}	—	31.2	
	3.9×10^{-8}	4.00	29.0	
	2.0×10^{-7}	2.70	—	
	3.9×10^{-7}	2.20	1.30	

[a] S_1 (2.0×10^{-3} *M*) and S_2 (8.9×10^{-4} *M*) are the two concentrations of tyramine used as substrate.

Compound III was prepared by the lithium aluminum hydride reduction of *N*-(2-propynyl)diphenimide. The latter was obtained by the alkylation of potassium diphenimide with 2-propynyl bromide. Diphenimide was prepared from diphenic acid by methods reported in the literature (5, 6), involving diphenic anhydride and diphenamic acid as intermediates.

EXPERIMENTAL[3]

The following were used: diphenic acid[4]; diphenic anhydride, prepared according to the method of Roberts and Johnson (5); and diphenimide, prepared according to the method of Underwood and Kochmann (6).

***N*-(2-Propynyldiphenimide)**—A solution of alcoholic potassium hydroxide (1.5 g. potassium hydroxide in 1.5 ml. water, *q.s.* to 5 ml. with absolute ethanol) was added with stirring to a hot (65–70°) solution of diphenimide (5.0 g., 22.4 mmoles) in 130 ml. of absolute ethanol. After removing the solvent *in vacuo*, the residue was triturated with anhydrous ether–acetone to yield a white solid potassium salt, which was collected by filtration and used without further purification. A solution of 2-propynyl bromide (2.7 g., 22.5 mmoles) in 10 ml. of dimethylformamide was added slowly with stirring to a solution of potassium diphenimide in 50 ml. of dimethylformamide. The mixture was heated for 4 hr. under gentle reflux and poured into water. The gray-white precipitate (5.0 g., 85%) was collected, m.p. 169–171°. Decolorization[5] and recrystallization from ethanol–water gave a colorless product, m.p. 175–176°.

[3] All melting points were determined with a Fisher melting-point apparatus and are uncorrected. IR spectra were recorded with a Perkin-Elmer model 700 grating spectrophotometer and were as expected. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn.
[4] Aldrich Chemical Co. Milwaukee, Wis.
[5] With Norite.

Table II—*In Vivo* Inhibition of Hepatic MAO in Mice by III and Iproniazid

Treatment	Inhibition of MAO at 10 min. after Incubation, %: Acute (0.1 mmole/kg. i.p., 2 hr. before Assay)	Chronic (0.01 mmole/kg. i.p., Daily for 7 days)
Dibenzazepine derivative (III)	25	10
Iproniazid	64	28

Anal.—Calc. for $C_{17}H_{11}NO_2$: C, 78.14; H, 4.24; N, 5.36. Found: C, 78.25; H, 4.34; N, 5.12.

6-(2-Propynyl)-6,7-dihydro-5*H*-dibenz[*c*,*e*]azepine (III)—*N*-(2-Propynyl)diphenimide (7.0 g., 27 mmoles) was placed in a soxhlet extraction apparatus above a stirred suspension of lithium aluminum hydride (5.5 g., 146 mmoles) in 300 ml. of anhydrous ether. Extraction was continued for 72 hr., after which time only a slight residue remained in the thimble. The excess hydride was decomposed by the cautious addition of water followed by 5 ml. of 20% sodium hydroxide. The resulting mixture was filtered and the ethereal layer was separated and dried over anhydrous sodium sulfate. The hydrochloride salt of III (5.5 g., 75%) was precipitated by mixing the dried ether layer with ethereal hydrogen chloride. The salt was recrystallized from 2-butanone, m.p. 201–203°.

Anal.—Calc. for $C_{17}H_{16}ClN$: C, 75.68; H, 5.97; N, 5.19. Found: C, 75.23; H, 6.13; N, 5.25.

MAO Inhibition—Inhibition of MAO was determined by a modification of the method of Snyder and Hendley (7), which employs tyramine as the substrate and homovanillic acid as the fluorescence generating reagent. Mouse liver was homogenized in ice-cold 0.1 *M* phosphate buffer, pH 7.8 (1:20 dilution) and centrifuged at 14,500×*g* and −5° in a refrigerated centrifuge[6] to obtain a cell-free supernate which served as the source of enzyme. Iproniazid[7] and pargyline[2] were used as standard inhibitors for comparison.

The inhibition constants (K_i) were determined by the method of Dixon (8). For *in vitro* inhibition studies, the drugs were incubated with the mouse liver enzyme prior to addition of the substrate. For acute *in vivo* inhibition studies, mice were given single intraperitoneal doses of the drugs 2 hr. prior to the removal of liver for determination of enzyme activity. In the case of chronic *in vivo* studies, however, the drugs were given once daily for 7 days and the livers were removed for the enzyme determination 4–6 hr. following the administration on the last day.

[6] Sorvall, RC2-B.
[7] Roche.

RESULTS AND DISCUSSION

Table I includes the data on the *in vitro* inhibition of the mouse liver MAO produced by varying concentrations of III and two known inhibitors, iproniazid and pargyline. Both the data and the K_i values show that III is a much weaker inhibitor of MAO than is pargyline.

The results of *in vivo* inhibition studies in both acute and chronically treated animals are shown in Table II. In these studies also, III appears to be a weaker MAO inhibitor.

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Antitumor Agents from *Jatropha macrorhiza* (Euphorbiaceae) I: Isolation and Characterization of Jatropham

R. M. WIEDHOPF, E. R. TRUMBULL, and J. R. COLE▲

Abstract □ A new lactam has been isolated from *Jatropha macrorhiza* (Euphorbiaceae) and has shown tumor-inhibitory properties against the P-388 lymphocytic leukemia test system. The compound was identified by means of NMR, UV, IR, rotary dispersion, mass spectrometry, and classical chemical procedures. It has the structure 5-hydroxy-4-methyl-3-pyrrolin-2-one and has been named jatropham.

Keyphrases □ *Jatropha macrorhiza* (Euphorbiaceae)—isolation and characterization of jatropham (5-hydroxy-4-methyl-3-pyrrolin-2-one), antitumor activity □ 5-Hydroxy-4-methyl-3-pyrrolin-2-one (jatropham)—isolation from *Jatropha macrorhiza*, characterization, antitumor activity □ Antitumor agents—isolation and characterization of jatropham (5-hydroxy-4-methyl-3-pyrrolin-2-one) from *Jatropha macrorhiza*

As a result of the continuing search for plants having tumor-inhibiting constituents, it was found that the chloroform extract of the leaves, stems, flowers, and fruits of *Jatropha macrorhiza* Benth. (Euphorbiaceae)[1] showed inhibitory activity toward the P-388 lymphocytic leukemia test system (3PS)[2]. The plant was collected in Cochise County, Ariz., in June 1968.

DISCUSSION

Solvent extractions of the chloroform extract with hexane followed by acetonitrile yielded a crystalline compound from the acetonitrile solution. This compound demonstrated activity of 125% test/control (T/C) at 45 mg./kg. in the 3PS test system. Activity in the 3PS system is defined as an increase in the survival of treated animals over that of controls resulting in a T/C $\geq$ 125% (1).

The compound was soluble in water, alcohol, and acetone but insoluble in less polar organic solvents. Elemental analysis and molecular weight (mass spectrum) indicated that the molecular formula was $C_5H_7NO_2$. The IR spectrum showed broad absorption at 3400–3450 (OH and NH) as well as 1725 (carbonyl) and 1640 cm.$^{-1}$ (double bond). The NMR spectrum showed a methyl group (δ 1.7, 3H, singlet), and the remaining four protons appeared individually at δ 4.9 (doublet), 5.4 (doublet), 6.5 (singlet), and 7.6 (singlet). When deuterium oxide was added to the solution, the doublet at δ 4.9 and the singlet at δ 7.6 disappeared and the doublet at δ 5.4 was reduced to a singlet. Apparently, in deuteroacetone, used as the solvent here, the hydrogen of the hydroxyl group does not exchange rapidly and couples with the adjacent proton. The UV spectrum showed strong end absorption and a shoulder at 230 nm.

The spectra are consistent with several pyrrolin-2-one structures with a secondary alcohol and a methyl group on the ring. In particular, they are similar to the spectra reported for a series of alkyl 5-hydroxy-3-pyrrolin-2-one derivatives (2). The location of the methyl group with respect to the carbonyl was demonstrated by reduction of the compound over platinum oxide to the known 4-methyl-2-pyrrolidone (II, Scheme I). The positions of the hydroxyl group and double bond were established by oxidation of the jatropha lactam (which was given the trivial name jatropham) with chromic acid in acetone to citraconic acid imide (III). The reactions show the structure of jatropham to be 5-hydroxy-4-methyl-3-pyrrolin-2-one (I).

Jatropham is optically active, levorotatory in the visible region, thus indicating that there is no ring tautomerism with the open-chain aldehyde-amide. This fact is consistent with the work of Scheffold and Dubs (2) who did not find evidence for ring opening with their 5-hydroxy-3-pyrrolin-2-ones, although corresponding lactones were in equilibrium with open-chain acids. The optical rotatory dispersion (ORD) curve has one peak (at 260 nm.) and two troughs (300 and 215 nm.) in the 600–200-nm. region. The higher inversion, molecular amplitude $\alpha = -22$, is centered near 270 nm. where there is no peak in the UV spectrum. The second inversion, molecular amplitude $\alpha = +208$, is centered near 235 nm. where there is a shoulder in the UV spectrum.

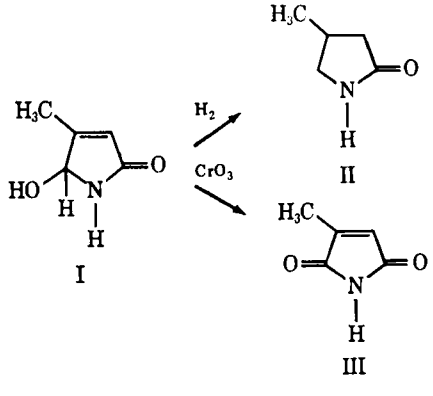
EXPERIMENTAL[3]

Isolation Procedure—Fresh-frozen leaves, stems, flowers, and fruits were placed in a lyophilizer and dried for 24 hr. The dried material (2 kg.) was ground in a Wiley mill and macerated with 12 l. of chloroform for 24 hr. The material was filtered and air dried, yielding an extract weighing 70 g. The chloroform extract was repeatedly extracted with hexane (technical) in 2-l. portions until the hexane was clear. The residue was then extracted with acetonitrile (technical) in 1-l. portions until the acetonitrile portion was clear. Evaporation of the acetonitrile solution yielded a crystalline solid material (1.3 g.).

A sample recrystallized from acetonitrile for analysis had m.p. 131–132°; $[\alpha]_{549}^{25}$ −62°; ORD data (c, 1.6 × 10^{-2}, water), $[\phi]_{589}$ −67°, $[\phi]_{300}$ −560°, $[\phi]_{260}$ +1700°, and $[\phi]_{215}$ −19,100°; IR (acetonitrile): 3550 (s), 1725 (s), 1600 (m), 1050 (s), and 870 (m) cm.$^{-1}$; UV (methanol): strong end absorption with a shoulder at 230 nm.; log ϵ 3.06.

Anal.—Calc. for $C_5H_7NO_2$: C, 53.09; H, 6.24; N, 12.38; mol. wt. 113. Found: C, 52.63; H, 6.21; N, 12.56; *m/e* 113.

Hydrogenation of Jatropham—A sample of lactam weighing 0.910 g. (8.0 mmoles) was dissolved in 70 ml. of ethanol, 0.110 g. of plat-



Scheme I

[1] Identification was confirmed by Robert J. Barr, College of Pharmacy, and Dr. Charles T. Mason, Botany Department, University of Arizona, Tucson. A reference specimen was deposited in the University of Arizona Herbarium.

[2] Drug Evaluation Branch, Drug Research and Development, Chemotherapy, National Cancer Institute.

[3] Carbon, hydrogen, and nitrogen analyses were performed by Huffman Laboratories, Inc., Wheatridge, Colo., and Chemalytics, Inc., Tempe, Ariz. Mass, NMR, UV, and IR spectra were determined using a Hitachi Perkin-Elmer double-focusing spectrometer (model RMU-6E), a Varian T-60 spectrometer, a Beckman DB-G spectrophotometer, and a Beckman IR-33, respectively. ORD measurement was obtained from a Cary 60, and melting points were determined on a Kofler hot-stage apparatus and are uncorrected.

inum oxide was added, and hydrogen was admitted to the system at atmospheric pressure. The solution absorbed 400 ml. (13.6 mmoles) of hydrogen in 30 min., after which no further uptake was observed. The reaction mixture was filtered to remove platinum and evaporated to dryness, leaving 0.851 g. of residual oil.

The crude product was separated by chromatography on 20 g. of silica gel into two main fractions, which were eluted from the column with acetone. The first of these weighed 0.451 g. and crystallized as long needles. Recrystallization from hexane gave material melting at 53–56° [lit. (4) m.p. 54–56°] and having the properties expected of 4-methyl-2-pyrrolidinone; mass spectrometry: M^+, *m/e* 99; ν_{max}^{CHF}: 3460 (m), 3240 (s), 3020 (s), and 1700 (s) cm.$^{-1}$; NMR (60 MHz.): δ 1.3 (3H, doublet), 1.8 (1H, multiplet), 2.4 (2H, unresolved), 3.4 (2H, two doublets), and 7.4 (1H, broad).

Anal.—Calc. for C_5H_9NO: C, 60.61; H, 9.09; N, 14.14; mol. wt. 99. Found: C, 60.79; H, 8.84; N, 14.33; *m/e* 99.

The second fraction, 0.364 g., did not crystallize. The IR spectrum differed from that described for the first fraction in having strong, broad absorption near 3300 cm.$^{-1}$. The NMR spectrum had a peak at δ 5.3 (1H) that was removed when deuterium oxide was added to the solution. This material, presumably a mixture of 5-hydroxy-4-methyl-2-pyrrolidinones, was not investigated further.

Oxidation of Jatropham—A solution of 0.815 g. (7.2 mmoles) of lactam in 40 ml. of acetone was cooled to 5°, and a solution of chromic acid (5) was added slowly with occasional addition of portions of anhydrous magnesium sulfate until the red-orange color of the oxidizing agent persisted. The mixture was filtered, the insoluble portion was washed thoroughly with acetone, and the solution was evaporated to dryness. The residue weighed 0.62 g. (78%). The material was recrystallized from carbon tetrachloride, giving a sample melting at 99–102°. This sample was further purified by sublimation, yielding long needles, m.p. 103–105°. The purified material was identical in IR spectrum (chloroform solution), TLC behavior, and mixed melting point (103–105°) with a sample of citraconic acid imide, m.p. 104–105°, synthesized by heating citraconic anhydride with ammonium hydroxide (6).

Anal.—Calc. for $C_5H_6NO_2$: C, 54.05; H, 4.50; N, 12.61; mol. wt. 111. Found: C, 54.35; H, 4.54; N; 12.32; *m/e* 111.

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COMMUNICATIONS

Dissolution Rates of Cholesterol Monohydrate Crystals and Human Cholesterol Gallstones in Bile Acid–Lecithin Solutions: Enhancing Effect of Added Alkyl Quaternary Ammonium Salts

Keyphrases □ Cholesterol monohydrate crystals and human cholesterol gallstones—dissolution in bile acid–lecithin solutions, effect of alkyl quaternary ammonium salts □ Cholate–lecithin dissolution media for cholesterol dissolution—effect of alkyl quaternary ammonium salts on interfacial barriers □ Benzalkonium chloride—effect on cholesterol dissolution in cholate–lecithin solutions □ Quaternary ammonium compounds—effect on cholesterol dissolution in cholate–lecithin solutions □ Bile acid–lecithin solutions—cholesterol dissolution, effect of alkyl quaternary ammonium salts □ Gallstone dissolution in bile acid–lecithin solutions—effect of quaternary ammonium salts

Sir:

Recent experiments on cholesterol dissolution in micellar bile salt solutions showed that added lecithin decreases the dissolution rates even though it increases the solubility of cholesterol monohydrate crystals in the solvent media (1, 2). From these data, effective crystal–solution interfacial barriers for the dissolution process were deduced, assuming a model based upon the interfacial barrier in series with a Nernst diffusion layer. Table I shows: (*a*) the dissolution rates of a compressed cholesterol monohydrate pellet (J/A), and (*b*) the solubilities (C_s) and diffusivities (D) of cholesterol molecules in various media determined by reported methods (2). The total transfer resistance (R) was calculated as the sum of diffusional resistance (h/D) and interfacial resistance ($1/P$). For example, in 2% cholate–1% lecithin solution, the interfacial resistance for cholesterol dissolution is nearly 20 times the diffusional resistance based upon the benzoic acid dissolution experiment. These findings are important in view of the recent clinical studies showing that *in vivo* diminution of cholesterol gallstones may be controlled by dissolution kinetics. This communication reports findings with benzalkonium chloride and other quaternary ammonium compounds in counteracting or eliminating the interfacial barriers present in the cholate–lecithin media for cholesterol dissolution.

As shown in Table I, in 2% cholate–1% lecithin and in 5% cholate–2.5% lecithin, added benzalkonium chloride significantly reduced the large R values to

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COMMUNICATIONS

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As shown in Table I, in 2% cholate–1% lecithin and in 5% cholate–2.5% lecithin, added benzalkonium chloride significantly reduced the large R values to

Table I—Dissolution Rates[a] of Cholesterol Monohydrate Pellet (J/A), Solubilities (C_s), and Diffusion Coefficients (D) Independently Determined in the Solvent Media at 37°

0.1 *M* Phosphate, pH 7.4						
Cholate, %	Lecithin, %	Benzalkonium Chloride, %	$(J/A)10^4$, mg. cm.$^{-2}$ sec.$^{-1}$	C_s, mg. cm.$^{-3}$	$D \times 10^6$, cm.^{2} sec.$^{-1}$	$R \times 10^{-3}$, sec. cm.$^{-1}$
2.0	—	—	0.67	0.53	2.17	7.91
2.0	—	0.5	4.82	0.63	1.92	1.31
2.0	1.0	—	0.16	1.05	1.49	65.63
2.0	1.0	0.5	4.82	1.37	1.50	2.84
5.0	—	—	1.95	1.34	1.90	6.87
5.0	—	1.25	8.95	1.71	1.54	1.91
5.0	2.5	—	0.56	3.0	1.30	53.57
5.0	2.5	1.25	8.33	3.27	1.10	3.93
Benzoic acid in 0.01 *N* HCl			131.0	4.70	14.0	3.59

[a] R was calculated from $J/A = C_s/R$, where $R = h/D + 1/p$ with h = Nernst diffusion layer thickness and p = effective interfacial permeability coefficient.

nearly that found with benzoic acid dissolution. Similar effects with benzalkonium chloride were also found in 2 and 5% cholate. Other dissolution studies in 2% cholate–1% lecithin showed that 0.5% cetylpyridinium chloride may have the same effect as benzalkonium chloride, giving $R = 4.85 \times 10^3$ sec. cm.$^{-1}$; 0.5% cetrimonium bromide was around 80% as effective as benzalkonium chloride, giving $R = 10.3 \times 10^3$ sec. cm.$^{-1}$. Experiments with human cholesterol gallstones were conducted by reported methods (2) in 2% cholate–1% lecithin solutions, with or without 0.5% benzalkonium chloride. Similar results as those shown in Table I for cholesterol monohydrate pellets were obtained: $R = 1.57 \times 10^3$ sec. cm.$^{-1}$ and $R = 61.76 \times 10^3$ sec. cm.$^{-1}$ with and without benzalkonium chloride, respectively.

The clinical implications of these findings with the quaternary ammonium compounds may be important in the medical treatment of the gallstone disease. Recent clinical studies of Danzinger *et al.* (3) showed that oral administration of chenodeoxycholic acid to patients with gallstones can lead to the undersaturation of the bile with respect to cholesterol and the dissolution of the stones. They found that, in six of the seven patients studied, gallstones progressively diminished in size during the 14–30 months of chenodeoxycholic acid treatment. In a different kind of study by Admirand[1] involving the dissolution of cholesterol gallstones retained in the common bile duct postoperatively *via* T-tube perfusion of 5% cholate, small stones of millimeter diameter were dissolved in 3–14 days while larger stones did not appear to diminish significantly. These studies support the idea that increasing the *in situ* gallstone dissolution rate should yield material patient benefits through reduced medical treatment times. The results and methodology reported with benzalkonium chloride and other quaternary ammonium compounds may prove valuable in finding or designing safe and efficacious agents or therapeutic regimens for the medical treatment of the gallstone disease.

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Obtaining Preliminary Estimates to Fit Two-Term Exponential Model to Blood Concentration Data

Keyphrases ☐ Absorption and elimination rate constants, preliminary estimates—obtained using exponential function curve fitting ☐ Blood concentration data—preliminary estimates for two-term exponential model, equations ☐ Rate constants, absorption and elimination—preliminary estimates for two-term exponential model, method, equations

Sir:

For a two-term exponential model, Wagner (1) discussed a graphical method of obtaining preliminary estimates of the elimination and absorption rate constants. In a later paper, Wagner and Metzler (2) concluded, by illustrating two examples, that graphical estimates (obtained by conventional feathering techniques) of the absorption rate constant and the elimination rate constant overestimate and underestimate the corresponding rate constants obtained by the least-squares solution. Wagner (1) noted that, if the starting values for the computer program are not close to the least-squares estimates, some computer programs may not necessarily converge at the global minimum because the error sum of squares may contain two minima. This communication presents another method of ob-

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2.0	1.0	—	0.16	1.05	1.49	65.63
2.0	1.0	0.5	4.82	1.37	1.50	2.84
5.0	—	—	1.95	1.34	1.90	6.87
5.0	—	1.25	8.95	1.71	1.54	1.91
5.0	2.5	—	0.56	3.0	1.30	53.57
5.0	2.5	1.25	8.33	3.27	1.10	3.93
Benzoic acid in 0.01 *N* HCl			131.0	4.70	14.0	3.59

[a] R was calculated from $J/A = C_s/R$, where $R = h/D + 1/p$ with h = Nernst diffusion layer thickness and p = effective interfacial permeability coefficient.

nearly that found with benzoic acid dissolution. Similar effects with benzalkonium chloride were also found in 2 and 5% cholate. Other dissolution studies in 2% cholate–1% lecithin showed that 0.5% cetylpyridinium chloride may have the same effect as benzalkonium chloride, giving $R = 4.85 \times 10^3$ sec. cm.$^{-1}$; 0.5% cetrimonium bromide was around 80% as effective as benzalkonium chloride, giving $R = 10.3 \times 10^3$ sec. cm.$^{-1}$. Experiments with human cholesterol gallstones were conducted by reported methods (2) in 2% cholate–1% lecithin solutions, with or without 0.5% benzalkonium chloride. Similar results as those shown in Table I for cholesterol monohydrate pellets were obtained: $R = 1.57 \times 10^3$ sec. cm.$^{-1}$ and $R = 61.76 \times 10^3$ sec. cm.$^{-1}$ with and without benzalkonium chloride, respectively.

The clinical implications of these findings with the quaternary ammonium compounds may be important in the medical treatment of the gallstone disease. Recent clinical studies of Danzinger *et al.* (3) showed that oral administration of chenodeoxycholic acid to patients with gallstones can lead to the undersaturation of the bile with respect to cholesterol and the dissolution of the stones. They found that, in six of the seven patients studied, gallstones progressively diminished in size during the 14–30 months of chenodeoxycholic acid treatment. In a different kind of study by Admirand[1] involving the dissolution of cholesterol gallstones retained in the common bile duct postoperatively *via* T-tube perfusion of 5% cholate, small stones of millimeter diameter were dissolved in 3–14 days while larger stones did not appear to diminish significantly. These studies support the idea that increasing the *in situ* gallstone dissolution rate should yield material patient benefits through reduced medical treatment times. The results and methodology reported with benzalkonium chloride and other quaternary ammonium compounds may prove valuable in finding or designing safe and efficacious agents or therapeutic regimens for the medical treatment of the gallstone disease.

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Obtaining Preliminary Estimates to Fit Two-Term Exponential Model to Blood Concentration Data

Keyphrases ☐ Absorption and elimination rate constants, preliminary estimates—obtained using exponential function curve fitting ☐ Blood concentration data—preliminary estimates for two-term exponential model, equations ☐ Rate constants, absorption and elimination—preliminary estimates for two-term exponential model, method, equations

Sir:

For a two-term exponential model, Wagner (1) discussed a graphical method of obtaining preliminary estimates of the elimination and absorption rate constants. In a later paper, Wagner and Metzler (2) concluded, by illustrating two examples, that graphical estimates (obtained by conventional feathering techniques) of the absorption rate constant and the elimination rate constant overestimate and underestimate the corresponding rate constants obtained by the least-squares solution. Wagner (1) noted that, if the starting values for the computer program are not close to the least-squares estimates, some computer programs may not necessarily converge at the global minimum because the error sum of squares may contain two minima. This communication presents another method of ob-

Table I—Lowest Serum Levels of Tetracycline Hydrochloride[a] Activity Observed in a Panel of Eight Subjects Administered 250 mg. after Specified Breakfast

Time, i	Serum Concentration of Tetracycline Hydrochloride, C_i	S_{1i}	S_{2i}
0	0.7000	0.7000	0.7000
1	1.2000	1.9000	2.6000
2	1.4000	3.3000	5.9000
3	1.4000	4.7000	10.6000
4	1.2381[b]	5.9381	16.5381
5	1.1000	7.0381	23.5762
6	0.9650[b]	8.0031	31.5794
7	0.8000	8.8031	40.3825
8	0.7312[b]	9.5343	49.9168
9	0.6000	10.1343	60.0512
10	0.5456	10.6800	70.7311
11	0.5000	11.1800	81.9111
12	0.4057[b]	11.5857	93.4968
13	0.3497[b]	11.9354	105.4322
14	0.3013[b]	12.2385	117.6707
15	0.3000	12.5385	130.2091

[a] Panmycin. [b] Indicates that these values are estimated using Eq. 7 of *Reference 1*.

taining preliminary estimates of the rate constants for a two-term exponential model. These estimates may then be used as initial starting values in a digital computer program, providing more assurance that the computer program will converge at the global minimum within a few iteration steps.

Let us suppose that we are able to observe C_t, the serum concentration of an investigational drug at time t. Furthermore, let the two-term exponential model be of the form:

$$E(C_t) = \beta_1 e^{\alpha_1 t} + \beta_2 e^{\alpha_2 t} \qquad t = 0, 1, \ldots, n \qquad \text{(Eq. 1)}$$

where E is the mathematical expectation, and α's and β's are the population parameters of the model for the drug under investigation. In clinical practice, a subject is selected at random and the values of C_t are assayed at equal intervals of time. Thus, the two-term exponential equation in the sample situation can be written as:

$$C_t = b_1 e^{\alpha_1 t} + b_2 e^{\alpha_2 t} = b_1 d_1^t + b_2 d_2^t \qquad t = 0, 1, \ldots, n \qquad \text{(Eq. 2)}$$

Because of the algebraic structure of Eq. 2, it is possible to rewrite the model in the form of a difference equation:

$$(C_{t+2} - C_{t+1}) - (C_{t+1} - C_t) = r_2 C_{t+2} - r_1 C_{t+1} \qquad \text{(Eq. 3)}$$

where:

$$d_1 + d_2 = (2 - r_1)/(1 - r_2) \qquad \text{(Eq. 4a)}$$

$$d_1 d_2 = 1/(1 - r_2) \qquad \text{(Eq. 4b)}$$

Summing Eq. 3 over the values of i from 0 to $i - 2$, we have:

$$C_i - C_{i-1} = (r_2 - r_1)S_{1i} + r_1 C_i + f \qquad \text{(Eq. 5)}$$

where $S_{1i} = \Sigma_0^i C_k$ and $f = C_0(r_1 - r_2) - r_2 C_1$.

Again, summing Eq. 5 over the values of i from 1 to i, we have:

$$C_i = (r_2 - r_1)S_{2i} + r_1 S_{1i} + if + g \qquad \text{(Eq. 6)}$$

where $S_{2i} = \Sigma_0^i S_{1k}$ and $g = C_0(1 - r_2)$.

Equation 6 is known as the internal regression (3) of Eq. 2; the values of constants, namely, $(r_2 - r_1)$, r_1, f, and g, can be obtained[1] by regressing C_i on S_{2i}, S_{1i}, and i. Having obtained these values from the data, we can then estimate d_1 and d_2 from the following equation:

$$(\hat{d_1}, \hat{d_2}) = \{1 - r_1/2 \pm \sqrt{(r_2 - r_1) + r_1^2/4}\}/(1 - r_2) \qquad \text{(Eq. 7)}$$

Theoretically, it is possible that the roots may be complex. However, if this situation does arise, then Eq. 2 can be mathematically expressed as sine, cosine functions, indicating that the two-term exponential model may not be appropriate. To illustrate the methodology, certain values of serum levels of tetracycline hydrochloride activity (Table I) from Eq. 7 (1) were interpolated to make the data equally spaced.

Using the linear regression set up by considering S_{1i}, S_{2i}, and i as independent variables and C_i as a dependent variable, we find the following regression coefficients (Eq. 6): $\hat{r}_2 - \hat{r}_1 = -0.1733$, $\hat{r}_1 = -1.2486$, $\hat{f} = 2.4522$, and $\hat{g} = 1.6671$. Now, using Eq. 7 or 4, we find $(\hat{d}_1, \hat{d}_2) = (0.862, 0.479)$ or:

$$\hat{a}_1 = \ln \hat{d}_1 = -0.14761 \qquad \text{(Eq. 8a)}$$

$$\hat{a}_2 = \ln \hat{d}_2 = -0.73697 \qquad \text{(Eq. 8b)}$$

Having obtained $\hat{a}_1$ and $\hat{a}_2$, we can obtain $\hat{b}_1$ and $\hat{b}_2$ by regressing C_t on $\exp(\hat{a}_1 t)$ and $\exp(\hat{a}_2 t)$:

$$\hat{b}_1 = 2.75726 \qquad \text{(Eq. 9a)}$$

$$\hat{b}_2 = -3.54831 \qquad \text{(Eq. 9b)}$$

Thus, the two-term exponential model (2) can be written as:

$$C_t = 2.75726e^{-0.14761t} - 3.54831e^{-0.73697t} \qquad \text{(Eq. 10)}$$

indicating that the rate constants $\hat{a}_1$ and $\hat{a}_2$ are very near to the least-squares estimates of -0.149 and -0.716.

The method does provide good starting values for a computer program. Convergence is achieved within one or two steps of iteration, indicating that the method of obtaining the estimates is nearly 100% efficient with respect to the least-squares method. The analytical comparison of efficiencies of the estimates with the standard least-squares estimate is currently under investigation.

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[1] To obtain approximation to the parameters, we may consider S_{1i} and S_{2i} as independent variables in the linear regression equation (6).

Coacervate Formation by Sodium Salicylate with Benzalkonium Chloride

Keyphrases ☐ Coacervate formation—sodium salicylate with benzalkonium chloride, density variations ☐ Sodium salicylate—coacervate formation with benzalkonium chloride ☐ Benzalkonium chloride—coacervate formation with sodium salicylate

Sir:

Colloidal solutions undergo phase transformations when subjected to changes in temperature, pH, concentration, and other factors. These phase transformations include crystallization, flocculation, and gel formation. One of the least studied of these changes is coacervation. From the definition by Bungenberg de Jong (1) and as used in this report, coacervation is the phenomenon of separation of colloidal aqueous solutions into two or more immiscible aqueous layers. One of the aqueous layers contains most of the colloid and is termed the coacervate, while the second aqueous layer is colloid poor and is termed the equilibrium liquid (2, 3).

These coacervate systems are of growing general scientific (4, 5) and biological (6) interest.

This communication reports the preparation of a new coacervate system. Also reported is a unique phenomenon of this system which occurs with changes in either temperature or composition of the system.

The coacervate system is obtained by mixing various concentrations of sodium salicylate and benzalkonium chloride in water. Figure 1 shows a diagram of this system obtained by plotting mixing concentrations at the points of coacervate formation. This diagram illustrates two main regions: (*a*) biphasic coacervate system, and (*b*) monophasic solution. The biphasic coacervate system shows two distinct regions; in one region (B), the coacervate phase has a higher density than the equilibrium liquid and settles to the bottom of the container. In the second region (T) the coacervate phase is lighter than the equilibrium liquid and floats on the top of the liquid. The transition from the biphasic to the monophasic system by increasing concentrations of benzalkonium chloride was observed to be a sharp one.

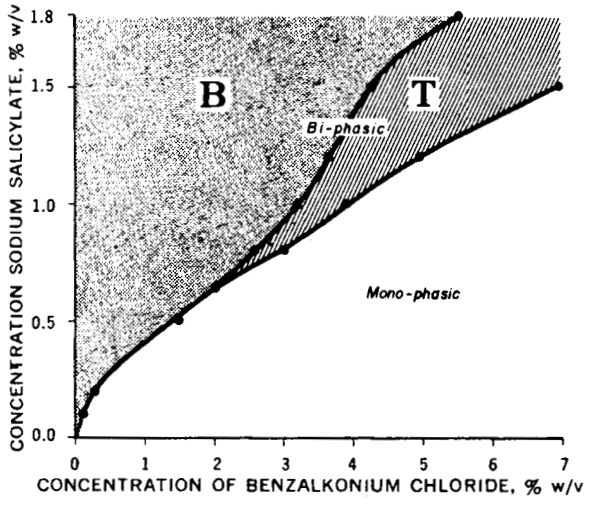

Figure 1—*Mixing concentrations of sodium salicylate and benzalkonium chloride to give coacervate systems at 24°. Dark area represents the region of coacervate formation and light area represents region of no coacervate formation. Key: B, system with coacervate at the bottom; and T, system with coacervate at the top.*

Table I—Mixing Concentrations and Relative Coacervate Density *versus* Temperature

Concentration of Sodium Salicylate, % w/v	Concentration of Benzalkonium Chloride, % w/v	Temperature	Location of Coacervate Phase in System
0.64	2.0	<26°	Bottom
		26–29°	Top
0.80	3.0	<16°	Bottom
		16–19°	Top
1.00	3.9	−2–0°	Bottom
		>0°	Top
1.12	4.0	<19°	Bottom
		19–23°	Top

Some biphasic systems at the transition line of the diagram were studied for the effect of temperature (Table I). It was observed that it is possible to change the density of a coacervate enough to make it lighter or denser than the equilibrium liquid by changing the temperature of the whole system; *i.e.*, both the equilibrium liquid and the coacervate phase change their relative densities to different degrees when undergoing the same change in temperature.

In Table I, the range in temperature for this change in relative density is reported. A more precise temperature could not be obtained, because the coacervate phase is viscous and also tends to stick to the wall of the container so an excess change in energy (temperature) is required to break it loose and make it begin to move.

The coacervate phase was observed to be isotropic when observed under a polarizing microscope.

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Picogram Analysis of Tetrahydrocannabinol and Application to Biological Fluids

Keyphrases □ Tetrahydrocannabinol in biological fluids—GLC–electron-capture analysis, picogram quantities □ GLC–electron-capture detection—analysis, tetrahydrocannabinol in biological fluids

Sir:

A major goal in marijuana research is to quantify those compounds and metabolites in biological fluids responsible for psychoactive effects (1). Clinical investigations of Δ^9-tetrahydrocannabinol (2, 3), a major active component of marijuana, and 11-hydroxy-Δ^9-tetrahydrocannabinol (4), an active metabolite, have relied on analysis by radioactive labeling. However, the study of distribution, metabolism, and excretion of the drug and its metabolites under chronic or "street" conditions demands nonradioactive analytical procedures suitable for analysis in biological fluids of the picogram quantities of Δ^9-tetrahydrocannabinol typically found under clinical conditions (2, 3).

Detection of larger amounts of tetrahydrocannabinol is possible using several procedures (5) including GLC with flame-ionization detection, but the sensitivity for the underivatized tetrahydrocannabinols does not exceed 0.8 ng. (Fig. 1). An antibody detection method was recently developed (6), and submicrogram amounts of Δ^9-tetrahydrocannabinol and metabolites may be quantified by mass fragmentography (7) or fluorometry (8).

The procedures described herein permit the quantitative analysis of the total Δ^9- and Δ^8-tetrahydrocannabinols in picogram amounts by preparing the pentafluorobenzoate ester for analysis by GLC with electron-capture detection. A gas chromatograph[1], fitted with both ^{63}Ni and flame detectors, was used. Detectors were maintained at 285°. The ^{63}Ni detector had a separate nitrogen gas flow of 35 ml./min., and the glass columns were 30 cm. long (2.0 mm. i.d.). The conditions were: column 210–230°; injection port, 245°; 3% OV-225 on Gas Chrom Q, 100–200 mesh; nitrogen gas flow, 35 ml./min.; standing current (^{63}Ni), 60–90% at 2×10^{-9}; and background noise, 2–3% at 1×10^{-10}. The minimum detectable quantity of tetrahydrocannabinol was 1.65×10^{-14} mole. The minimum detectable quantity is defined as that amount giving a signal three times the background noise at maximum sensitivity (1×10^{-10}). When tetrahydrocannabinol was extracted from various solutions prior to derivatization, the minimum detectable quantity was a function of the efficiency of the extraction processes and the nature and magnitude of the nonionizable contaminants present and ranged between 40 and 125 pg./ml. of plasma for a 5-ml. blood sample.

To extract tetrahydrocannabinol from blood, a 5-ml. blood sample was centrifuged and a 2.5-ml. aliquot of plasma was transferred into a 50-ml. glass centrifuge tube. One milliliter of 5% trichloroacetic acid was added dropwise while shaking to denature proteins. Ten

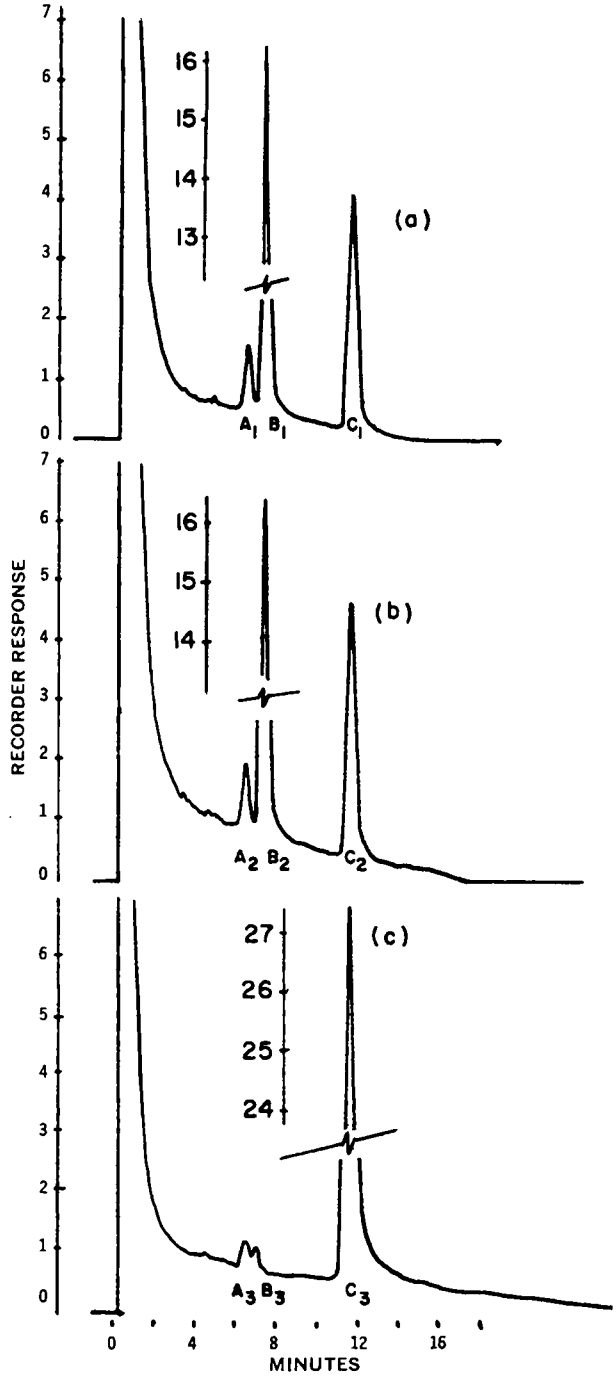

Figure 1—*(a) GLC analysis by flame-ionization detection of 1.3 μl. of a toluene solution containing 5 mg./ml. tetrahydrocannabinol and 1 mg./ml. cannabinol. Key: A_1, Δ^8-tetrahydrocannabinol (0.4 mcg.); B_1, Δ^9-tetrahydrocannabinol (6.5 mcg.); and C_1, cannabinol (1.3 mcg.). The tetrahydrocannabinols were from the same stock solution (see text), and A_1 accounted for 6.2% of the total tetrahydrocannabinols. (b) Tetrahydrocannabinol, 50 mcg., from the stock solution was added to 50 ml. water and treated as described in the text for extraction of tetrahydrocannabinol from blood. Cannabinol was added after the last step (prior to derivatization). After these various treatments, A_2 accounted for 7.0% of the total tetrahydrocannabinols. (c) GLC analysis (using flame-ionization detection) of the cannabinol used as internal standard. The amount of Δ^8- and Δ^9-tetrahydrocannabinols (A_3 and B_3, respectively) present as contaminants was approximately 1%. C_3 represents 5.2 mcg. cannabinol.*

[1] Varian model 2100.

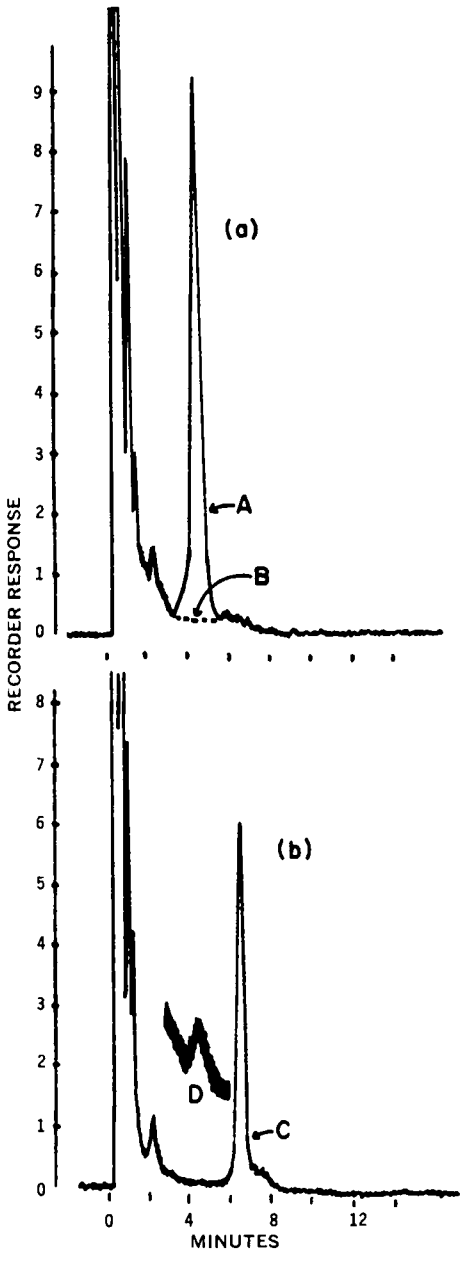

Figure 2—*(a) GLC analysis by electron-capture detection of tetrahydrocannabinol pentafluorobenzoate (A). The chromatogram is for 200 pg. and was prepared from a 5-ml. plasma sample containing 1000 pg./ml. Sensitivity = 8 × 10^{-10} (B is for a plasma blank). (b) Chromatogram of the extracted and derivatized plasma blank to which derivatized cannabinol (C) was added. The insert (D) shows the chromatographic response for the minimum detectable quantity (1.65 × 10^{-14} mole) of tetrahydrocannabinol pentafluorobenzoate at a sensitivity of 1 × 10^{-10}.*

milliliters of a 1% isoamyl alcohol solution in dichloromethane[2] was added. The mixture was shaken for 15 min. and centrifuged. The organic layer was transferred into another 50-ml. test tube and the extraction process was repeated. The combined organic layers were evaporated to approximately 5 ml., and 25 ml. pH 8.5 buffer (50 ml. 0.1 *M* tromethamine plus 14.7 ml. 0.1 *N* HCl) was added. The mixture was shaken for 5 min. and then centrifuged. The dichloromethane layer was transferred

[2] Nanograde, Mallinckrodt Chemical Works, St. Louis, MO 63160

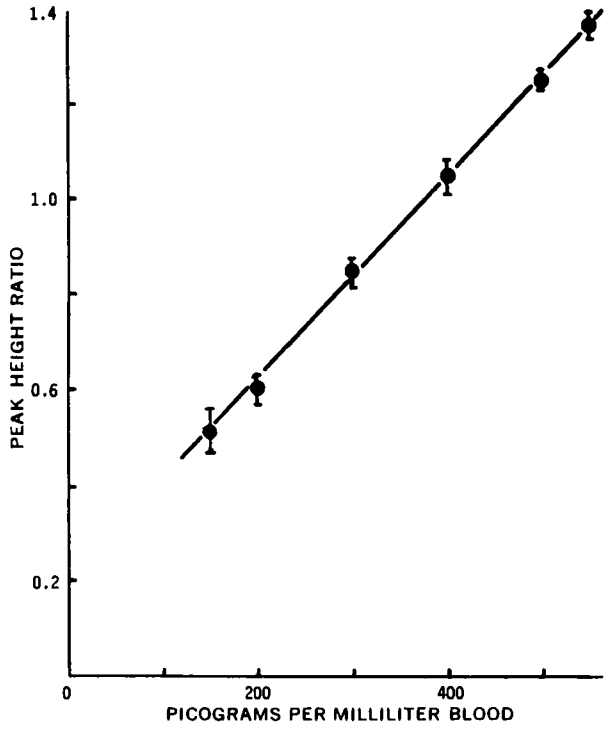

Figure 3—*Calibration curve obtained for Δ⁹-tetrahydrocannabinol added to fresh heparinized dog blood. Five-milliliter blood samples were spiked with tetrahydrocannabinol (15 mcg./ml. in ethanol) and allowed to stand 10 min. prior to extraction and derivatization. The peak height ratios are plotted as the mean of three replicate extractions, and the vertical lines represent the standard deviations.*

quantitatively to a third 50-ml. test tube containing 25 ml. of 0.1 *N* HCl. This mixture was shaken for 5 min. and centrifuged. The organic layer was transferred quantitatively to another 50-ml. test tube. The tetrahydrocannabinol solution was then ready to be derivatized.

These procedures neither isomerized Δ⁹-tetrahydrocannabinol to Δ⁸-tetrahydrocannabinol nor the converse. This was shown by adding 50 mcg. of Δ⁹-tetrahydrocannabinol [from a 5-ml. sample[3] containing 1.0 g. of (−)-Δ⁹-*trans*-tetrahydrocannabinol in 99% ethanol, assayed as 93%] to 50 ml. of water and treating in the exact manner already described. This included the addition of trichloroacetic acid and the various treatments with acid and buffer. The relative amounts of Δ⁹- and Δ⁸-tetrahydrocannabinols were compared for the material before and after these treatments.

The assay was performed by GLC analysis with flame-ionization detection in a system capable of quantifying both the Δ⁸ and Δ⁹ species. The GLC system and conditions were the same as given for the electron-capture detection, except that a 1.8-m. (6-ft.) column was used and the nonderivatized material was chromatographed and detected by flame ionization. Cannabinol (from a 1-ml. sample[4] containing 10 mg. of cannabinol in 99% ethanol, assayed as 99%) was used as the internal

[3] S.S.C. Lot 61591, furnished by the Department of Health, Education, and Welfare, Public Health Service, National Institute of Mental Health, Bethesda, MD 20014

[4] S.S.C. Lot 61656, furnished by the Department of Health, Education, and Welfare, Public Health Service, National Institute of Mental Health, Bethesda, MD 20014

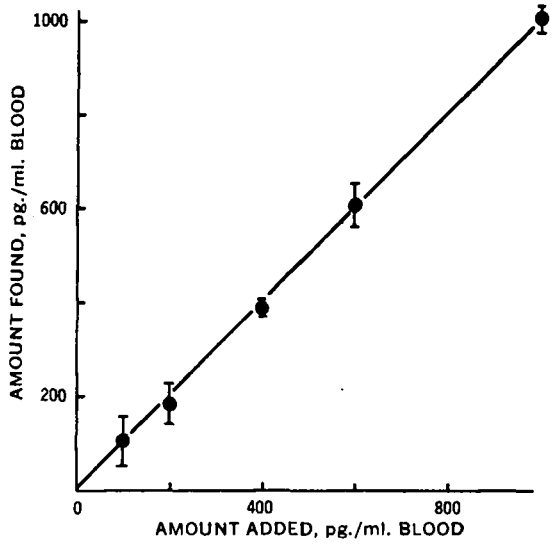

Figure 4—*Demonstration of the validity of the calibration curve and dilution technique. Sixty milliliters of fresh heparinized dog blood (same blood as used in Fig. 1) was divided into five 12-ml. samples, and one of each set was spiked with 100, 200, 400, 600, and 1000 pg./ml. with tetrahydrocannabinol (30 mcg./ml. in ethanol). The points represent the mean of three replicate extractions from 4-ml. aliquots. The vertical lines represent the standard deviations for the values shown, and the standard deviations ranged from 14.0 to 51.3 pg./ml. The slope and intercept of this plot were not statistically different from the expected values of 1.0 and zero, respectively.*

standard. The chromatograms of the Δ^9-tetrahydrocannabinol are given in Figs. 1*a* and 1*b* before and after the extraction processes, respectively. The original sample showed 6.2% Δ^8 (Fig. 1*a*); after the various treatments, there was 7.0% Δ^8, which demonstrated negligible conversion of Δ^9 to Δ^8 in the procedures. Although the cannabinol had about 1% of Δ^8- and of Δ^9-tetrahydrocannabinol impurities (Fig. 1*c*), this contribution to the peaks in Figs. 1*a* and 1*b* was below the sensitivity for these compounds at the level and conditions used with cannabinol as an internal standard.

The extractions of the tetrahydrocannabinols were made from dog blood, and the calibration curves obtained for known amounts of tetrahydrocannabinol in the blood from different dogs were significantly different. Significant differences among individuals should be assumed until shown otherwise. If electron-capture detector analysis of a derivatized blank plasma sample shows significant amounts of nonionizable compounds (steroids, *etc.*), a TLC or liquid chromatography cleanup step may be required. This was not necessary with the dog plasma used.

The solution of tetrahydrocannabinol in dichloromethane in the 50-ml. glass centrifuge tube was reduced to dryness under nitrogen and reconstituted in 0.2 ml. of dry dichloromethane containing 50 μl. of triethylamine. Ten micrograms (at least a 10-meq. excess) of pentafluorobenzoyl chloride[5] in 0.2 ml. of dry dichloromethane was added dropwise over 1 min. The solution was constantly stirred and allowed to react for 5 min. After the reaction was complete, 10 ml. of 0.1 *N* NaOH was added and the mixture was shaken for 5 min. The solution was centrifuged and the dichloromethane layer was transferred quantitatively to another 50-ml. test tube containing 10 ml. of 0.1 *N* HCl.

This mixture was shaken for 5 min. and centrifuged, and the dichloromethane layer was transferred quantitatively to a 3-ml. glass-stoppered test tube and stored overnight at 0° until time for GLC analysis.

The sodium hydroxide and hydrochloric acid washings were designed to hydrolyze and remove unreacted acid chloride and triethylamine catalyst. These removals were imperative for successful quantitative analysis.

At the time of GLC analysis, the final dichloromethane solution was dried under a stream of nitrogen and redissolved in at least 25 μl. of toluene containing the derivatized internal standard, cannabinol pentafluorobenzoate.

Although the cannabinol used had about 1% of tetrahydrocannabinol as an impurity (Fig. 1*c*), at the levels used for the addition of the derivatized cannabinol as cannabinol pentafluorobenzoate internal standard, no peaks assignable to these impurities were observable on electron-capture detection (Fig. 2*b*). Figure 2*b* shows the chromatogram for the internal standard obtained from 200 pg. of cannabinol. The chromatogram of derivatized Δ^9- and Δ^8-tetrahydrocannabinols appeared as one peak under our conditions (Fig. 2*a*) and was well separated from the peak assigned to the internal standard, cannabinol pentafluorobenzoate. This chromatogram was for the derivatized tetrahydrocannabinol obtained from 200 pg. of the original material.

Calibration curves over small concentration ranges were preferred since the ^{63}Ni detector has a small linear detectable range. Whole blood samples with known amounts of tetrahydrocannabinol added (*e.g.*, 150–550 pg./ml. plasma) were extracted and derivatized as already described. At the same time, 1 mcg. (or more) of cannabinol (internal standard) was derivatized and reconstituted in clean, dry toluene such that 1 μl. of the cannabinol pentafluorobenzoate solution contained about 10 times the minimum detectable amount, 1.65×10^{-14} mole. A volume of this internal standard solution, 25–500 μl., was added to each of the dried tetrahydrocannabinol pentafluorobenzoate samples such that the midpoint concentration of the tetrahydrocannabinol samples gave a peak height ratio of about one when 1–3 μl. of the final solution of derivatized tetrahydrocannabinol was injected. The calibration curve in Fig. 3 was obtained in this manner. A zero intercept for the linear range of the calibration curve was not necessarily expected or needed.

Once the calibration curve was obtained, a known volume of the internal standard solution (starting with 25 μl.) was added to each dried, unknown tetrahydrocannabinol pentafluorobenzoate sample prepared as described. If, on injection of 1–3 μl. of this solution the peak height ratio did not fall within the calibration curve, an additional volume of the internal standard solution was added until the peak height ratio did. The actual blood level concentration was obtained by correcting the value obtained from the calibration curve for the excess internal standard used. If a peak height ratio of 1.0 corresponded to 375 pg./ml. (X_1) on the calibration curve when 75 μl. (Y_1) of internal standard solution

[5] PCR, Inc., Gainesville, FL 32601

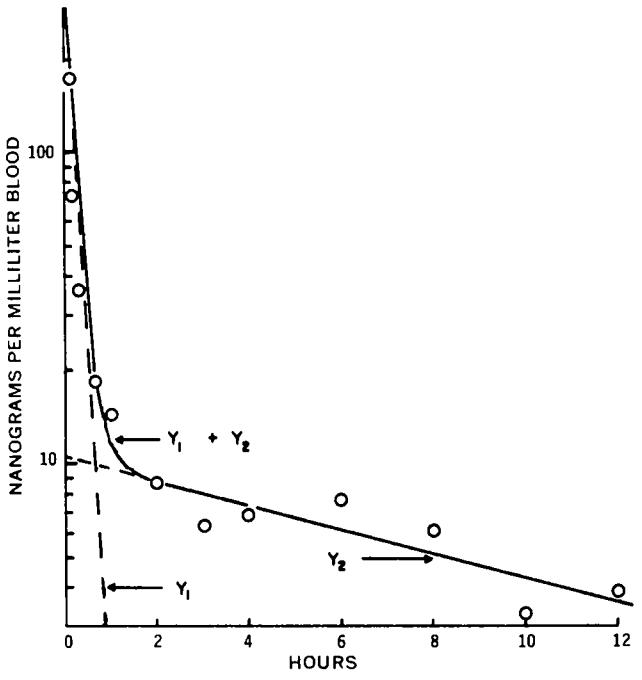

Figure 5—*Pharmacokinetic profile of tetrahydrocannabinol (0.1 mg./kg.) in blood from an 18.0-kg. dog. The calibration curve used is shown in Fig. 2. Five-milliliter blood samples were taken at 5, 10, 20, 40, and 60 min. and 2, 4, 6, 8, 10, and 12 hr. Urine was collected for 28 hr. but only a trace amount of tetrahydrocannabinol was found. The time course of this curve is fitted with the sum of two first-order exponential components,* Y_1 *and* Y_2, *where* $Y_1 = 300\ (ng./ml.)\ e^{-0.17t}$ *and* $Y_2 = 10.05\ (ng./ml.)\ e^{-1.05t}$. *The slower component,* Y_2, *has a* $t_{1/2}$ *of 8.0 hr. and the faster component,* Y_1, *has a* $t_{1/2}$ *of 7.5 min. The volume of distribution associated with* Y_2 *(equilibrated tissues) referenced to total drug in blood is 171 l. and the volume of distribution associated with* Y_1 *(central compartment) is 6.0 l.*

was used for all calibration curve values and a peak height ratio of 1.0 was obtained for an unknown amount of tetrahydrocannabinol (X_2) in blood when 25 μl. (Y_2) of internal standard was used, $X_2 = (X_1/Y_1)Y_2$. In this case, X_2 = 125 pg./ml. Figure 4 demonstrates this procedure and verifies its validity.

A number of critical variables affected the reliability and error of this procedure. Tetrahydrocannabinol bound extensively (15–40%) to glass from aqueous and organic solutions at low concentrations, and the degree of adsorption was time dependent. In the case of organic solutions, the tetrahydrocannabinol could be reincorporated from the glass into the solution on vigorous shaking. Thus, it was imperative to use vigorous shaking prior to any sampling. The degree of adsorption may be reduced if glassware is treated with an organic solution of a silyl reagent[6], followed by a solvent rinse of dichloromethane and drying. Storage times in aqueous or plasma solutions should be minimized and standardized since adsorption on storage in these media is unavoidable. Loss of tetrahydrocannabinol or a derivative from such solutions should not be assigned to instability until adsorption has been ruled out. Reagents of maximum purity must be used. Known amounts of tetrahydrocannabinol should be analyzed periodically to ensure reagent quality.

To demonstrate the applicability of this analytical technique, we determined the blood levels of tetrahydrocannabinol in a dog as a function of time. An 18.0-kg. dog was given a 1.8-mg. i.v. dose of tetrahydrocannabinol (0.1 mg./kg.) in 5 ml. of 50% aqueous ethanol. Blood samples were taken as a function of time over 24 hr. and urine was collected for 28 hr. The results are shown in Fig. 5 for tetrahydrocannabinol concentrations in blood as a function of time. Only a trace amount of unchanged tetrahydrocannabinol was found in the total urine collected when the urine was analyzed in the same manner as blood, and appropriate calibration curves were prepared. The calibration curves of Figs. 3 and 4 were prepared using blood from this dog and were used in this study. The apparent volumes of distribution of the central compartment referenced to the total concentration of the drug in blood was 6.0 l., whereas that of the rapidly equilibrated fluids was 171 l.

This pharmacokinetic profile shows an initial rapid distribution and metabolism phase (apparent half-life 7.5 min.) followed by a slow loss of tetrahydrocannabinol from blood (apparent half-life 8.0 hr.) and is consistent with the similar results reported for humans based on radiotracer studies (2).

Figure 5 demonstrates that this analytical procedure may be used to analyze blood levels of tetrahydrocannabinol. Although this procedure does not allow the separate quantitative analysis of Δ^8- and Δ^9-tetrahydrocannabinols at this sensitivity, preliminary results indicated that a similar procedure may be used to separate and quantify picogram amounts of other metabolites of tetrahydrocannabinol in blood and urine.

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[6] Rinsing glass for 30 min. with a 1% (v/v) solution of Sil-Prep (Applied Science Labs., State College, PA 16801) in dichloromethane reduces adsorption to less than 5%.

Keyphrases ☐ Moisture content of granulations—relationship to particle size ☐ Granulations—moisture content as function of particle size

Sir:

In theoretical considerations relative to the drying of granulations, whether by stationary or fluid-bed techniques, it is customary to consider: (*a*) the granulate as uniform, and (*b*) a pooled, average moisture content as the experimental variable. However, granulations have a range of granule sizes and, in the strictest sense, it is not permissible to assume that the moisture is evenly distributed during and after drying. If a granule-sieve fraction has a particle diameter a, then the following relation can be obtained (1):

$$\frac{W - W_\infty}{W_0 - W_\infty} = \frac{6}{\pi^2} \sum_{j=1}^{\infty} \frac{1}{j^2} \exp[-j^2 t/\kappa] \sim \frac{6}{\pi^2} \exp[-t/\kappa] \quad \text{(Eq. 1)}$$

where W is moisture content; the subscripts zero and infinity denote initial and final conditions, respectively; j is a running index; t is time; and $\kappa = a^2/(4\pi^2 D)$, where D is a diffusion coefficient. Hence, W depends exponentially on a^{-2}, and the smaller granules are (as is intuitively obvious) drier than the larger ones after a certain drying time, t'.

In an experimental study of a continuous process, presently being conducted, sucrose granulations originally containing 7.0 ± 0.2% moisture were subjected to countercurrent drying[1]. The original moisture quoted is composite and theoretical and is not related to any sieve fraction, since wet sieving would be distortive at this point. In the case cited, a granulation was dried for 1 hr. The air inlet temperature was 149° (300° F.), the air outlet temperature was 45° (113° F.), and the dryer was operating at 3.5 r.p.m.

The dried granulation was sieved, and the various sieve fractions were subjected to toluene moisture analysis. The percent moisture was then plotted as a function of particle size (Fig. 1). Separate experimentation showed that $W_\infty = 0.42$, and plotting of $\ln[(W - 0.42)/6.58)]$ as a function of a^{-2} then produced a straight line (Fig. 2) with a slope of $-t'4\pi^2 D$ cm.^{2} [*i.e.*, the diffusion coefficient for the particular situation would be $-(\text{slope})/(4\pi^2 t')$ cm.^{2}/sec.] and an intercept of $\ln(6/\pi^2) = -0.5$; here t' is 3600 sec. It is seen from Fig. 2 that: (*a*) the data adhere fairly well to linearity and (*b*) the intercept is −0.56, in good agreement with the theoretical. These two facts substantiate the view that granules dry by a diffusional process and that, therefore, moisture content is a function of particle size. In cases where granules are not case hardened, the moisture levels equilibrate and the granulation is uniform when it reaches the tablet press. Most wet granulations, however, exhibit case hardening to a greater or lesser degree, and the above phenomena must be accounted for in processing.

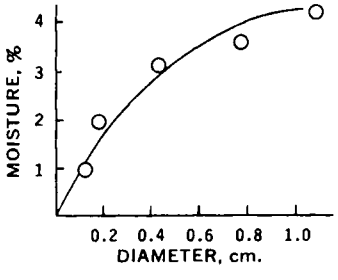

Figure 1—*Moisture content (percent) as a function of particle diameter. Diameters represent the average of the sieve fraction in question.*

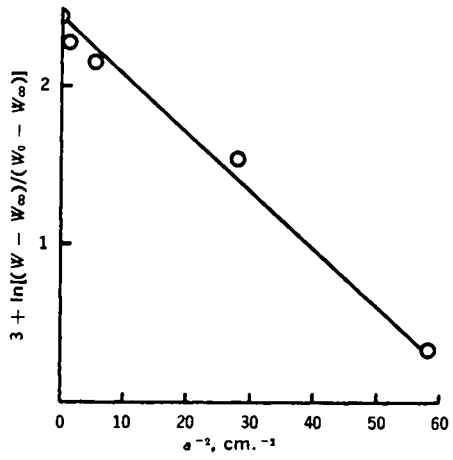

Figure 2—*Data from Fig. 1 plotted as shown in Eq. 1. The slope is -3.68×10^{-2} cm.^{2}, and the intercept is −0.56, i.e., 2.44 − 3.*

The diffusion coefficient for the curve in Fig. 2 is $(3.68\ 10^{-2})/(4\pi^2 3600) = 3 \times 10^{-7}$ cm.^{2}/sec., which is of the right order of magnitude for diffusion through capillary void space (2, 3).

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(3) K. Ridgway and J. A. B. Callow, *J. Pharm. Pharmacol.*, **19**, 155S(1967).

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[1] Bartlett Snow Dryer Style J, 7.9 m. (26 ft.) long and 137.2 cm. (54 in.) in diameter.

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Methods in Pharmacology, Volume 2 Physical Methods. Edited by C. F. CHIGNELL, Appleton-Century-Crofts, 440 Park Ave. S., New York, NY 10016, 1972. 499 pp. 16 × 23 cm. Price $29.95.

For those who see the pharmacologist as a tabulator of rat tail flicks, the topics covered in this text should be an eye opener: fluorescence and phosphorescence spectroscopy, circular dichroism and optical rotatory dispersion, Mössbauer effect spectrometry, nuclear magnetic and electron spin resonance, scattered light measurement, spectrophotometry of enzyme suspensions, flow and relaxation methods in the measurement of fast reactions, X-ray diffraction, mass spectrometry, oscillographic polarography, and heatburst microcalorimetry. Although aimed at the pharmacologist, this volume would be equally at home in the libraries of biochemists, physiologists, and medicinal chemists, illustrating that when disciplines converge, distinctions vanish.

Dr. Chignell's purpose in assembling (and substantially contributing to) this book was to acquaint the researcher with newer physical techniques in a way that would enable him or her to judge their applicability to his or her own problems. In this, the book succeeds. Each chapter is lucidly written, presumes a minimum of ignorance, and is well illustrated and referenced. There may be disagreement, in some instances, with the amount of detail included or omitted. In the chapter on mass spectrometry, for instance, those of us returning to the use of stable isotopes could wish for a more explicit treatment of the mass spectrometer as a quantitative analytical tool. But these are only matters of personal leaning and do not detract from the main aim of the book. If a serious complaint is to be raised against the book, it will come loudest from those quarters where the pharmacologist, physiologist, or biochemist works under tight budgets and without access to the costly instrumentation described. For those so deprived, agonizing over the good fortune of their plusher colleagues will be little assuaged by a theoretical knowledge of techniques which are passing them by.

Reviewed by S. S. Walkenstein
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Philadelphia, PA 19101 ■

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REVIEW ARTICLE

Pharmaceutical Sciences—1972: Literature Review of Pharmaceutics II‡

ASHOK C. SHAH*▲ and ALLEN K. HERD†

Keyphrases ☐ Pharmaceutical sciences—1972 literature review of pharmaceutics ☐ Technology, pharmaceutical—sterility, dosage forms, packaging equipment ☐ Physical pharmacy—dissolution, solubility, permeability, complexation, surface phenomena ☐ Antibiotics—pharmaceutical aspects ☐ Radiopharmaceuticals—pharmaceutical aspects ☐ Biopharmaceutics—drug properties, absorption, pharmacokinetics ☐ Literature review—pharmaceutical sciences 1972

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‡ *Editor's note:* Part I of this article appeared in the July 1973 issue of the *Journal of Pharmaceutical Sciences*.

PHYSICAL PHARMACY (*continued*)

Membrane Permeation—Phenomena associated with membrane permeability are important pharmaceutically in drug transport modeling and in the design of effective drug delivery systems. Flynn and his coworkers studied the influence of solvent composition and permeant solubility on membrane transport (850) and the influence of alkyl chain length on flux-determining properties of the barrier and diffusant (851), and they derived equations describing the diffusion layer control flux of a permeant through a membrane sandwiched between two liquid phases (852). It was shown that, under certain conditions, the steady-state transfusion through dimethylpolysiloxane membranes by *p*-aminoacetophenone from binary solvent mixtures was essentially controlled by the thermodynamic activity of the compound in the applied phase (850). A theoretical expression was presented correlating the effect of alkyl chain length on partition coefficient, solubility, and maximum steady-state flux through a model membrane. This expression, predicting a parabolic dependency of the flux with respect to alkyl chain length, was experimentally verified by determining transport rates of an homologous series of esters across the dimethylpolysiloxane membrane (851). Equations derived for the diffusion layer control flux of a permeant through a membrane sandwiched between two liquid phases confirmed that the steady-state flux in such cases is only dependent on the applied phase concentration, the diffusivity within the diffusion layers, and the reciprocal of the sum of the diffusion layer thicknesses. Furthermore, a lag time expression was derived which relates the duration of the nonsteady state to the membrane and diffusion layer

thicknesses, the membrane–solvent partition coefficient, and the reciprocal of diffusivity within the solvent. The significance of the lag time effect as the limiting factor in the structure–activity profile for a series of organic homologs was discussed (852).

The changes in *in vitro* permeability rates of benzocaine through silicone rubber implants by introduction of copermeant plasticizers into the implant were investigated. Solubility, diffusivity, molecular volumes and mobilities of the copermeants, hydrogen-bonding capabilities, and potential for internal segmental motion of polymeric plasticizers were considered as the factors influencing drug transmission rates (853). Studies on permeation of aniline and substituted anilines (854), aromatic acids, alcohols, amines, esters, aldehydes, and ketones (855) were performed to ascertain the effect of chemical structure on the permeation of these agents through polyethylene. The permeability constants of these compounds were shown to correlate with their hexane–water partition coefficients by an empirical equation (855). A thermogravimetric analysis method was used to evaluate the diffusion and solubility of a series of aliphatic alcohols in a specific polyurethane film at several different temperatures. In general, the diffusion coefficients of the straight-chain alcohols increased with temperature and decreased with molecular weight. By knowing solubility and diffusion coefficients, the permeability constants were estimated (856).

The effects of changes in the polymer ratio and environmental conditions on the water vapor transmission properties of plasticized films containing combinations of hydroxypropyl methylcellulose and ethylcellulose were evaluated. The findings of this study demonstrated the presence of another mechanism of vapor transmission, in addition to diffusion, which is apparently related to the hydrophilic character of the film (857). Water vapor transmission through cellulose acetate phthalate and *n*-butyl methacrylate polymer films showed significant differences in permeability behavior with the cellulose acetate phthalate films, depending on whether moisture was present on one or both sides of the film during permeation; however, this effect did not occur with the *n*-butyl methacrylate films. The dehydration of the distal cellulose acetate phthalate film surface when moisture is present only on the proximal surface was provided as an explanation for the permeability behavior of the membrane (858). A method was described for the study of free unsupported polymer films; its applicability was demonstrated by testing effects of spray rate and film substrate on mechanical strength and water permeability of cellulose acetate films (859).

The hindrance of solute diffusion was measured through microporous membranes of known pore geometry with pore radii ranging from 2.5 to 300 Å. The membranes were prepared by bombardment of mica sheets with fission fragments from a ^{235}U source and subsequent etching with hydrofluoric acid. It was concluded that in most membrane operations there is a considerable resistance to diffusion due to the presence of a liquid film boundary layer along the surface of the membrane. However, this boundary layer resistance was not inversely proportional to the solute diffusivity, as has been often assumed in the unstirred layer theory,

Table XXV—Additional References on Membrane Permeability

Reference	Topic
868	Review emphasizing physicochemical properties of drugs and cell membranes that control or modify transmembrane movement
869	Review of theories and characteristics of monolayer permeation of water, gases, and ions
870	Review of theoretical considerations and experimental studies of vapor and gas permeability of commonly used plastics
871	Review of the permeability of polymeric packaging materials to gases and organic vapors
872	Review emphasizing aspects of selective permeability of biological and synthetic membranes
873	Ion transport across biological membranes
874	Evaluation of water vapor transmission, hardness, drug release rates, and other physical properties of polymeric films in the formulation of an aerosol occlusive dressing
875	Influence of hydrophobic interactions on the electrochemical selectivity ratios of liquid membranes responsive to organic ions
876	Sodium- and chloride-ion permeabilities measured across both charged and uncharged unmodified phospholipid bilayers
877	Diffusion of small nonelectrolytes across liposome membranes indicating structure of such biological membranes similar to smectic mesophases and hydrophobic polymer networks
878	Permeability of alkali metal and ammonium salt electrolytes through polyphthalamide microcapsule membranes suggesting formation of a stable diffusion layer of electrolytes in the interior of the microcapsules

but was proportional to the −0.6 power of the solute diffusivity (860). A new theory based on absolute reaction rates was proposed to explain diffusion of polystyrene spheres through a thin membrane with cylindrical pores (861). Two different models for the diffusion of a binary solution through a membrane were used to derive relations between the solute permeability coefficient and the reflection coefficient. The theoretical considerations were shown to agree with previously reported data for highly porous membranes (862).

The transport rates of sodium and potassium chlorides through the bovine eye-lens capsule, insoluble gelatin membrane, and collagen membrane were measured to estimate the permeability coefficient, the rate constant of permeation at the interface, the apparent membrane constant, and the interface constant. The permeability of the bovine eye-lens capsule was greater than the gelatin membrane but was comparable to the collagen membrane (863). In a subsequent study, ionic diffusion and permeability of potassium hydrogen aspartate and magnesium hydrogen aspartate across the vitreous bovine eye-lens capsule was investigated (864). The permeability of potassium chloride through two-component membranes, composed of gelatin and chondroitin sulfate C, was measured. The membrane permeability was decreased with an increase of total weight of the membrane and with an increase of the amount of chondroitin sulfate C (865).

The effect of salicylate on the relative cation permeability of a membrane was investigated in large, identified molluscan neurons. Salicylate caused a reversible, dose-dependent decrease in the permeability of rubidium, cesium, sodium, and lithium ions relative to

that of potassium ions, probably due to the adsorption of salicylate anions to the membrane with a subsequent increase in the density and field strength of anionic sites in the membrane (866). Salicylate, benzoate, and their analogs reversibly increased the membrane potential and conductance of identified molluscan neurons by increasing potassium conductance and decreasing chloride conductance. The relative potencies of these compound's were closely correlated with their octanol–water partition coefficients and their pKa values (867).

Additional membrane permeability studies are listed in Table XXV.

Complexation—The studies related to complexation phenomena are categorized into: (*a*) interactions of drugs with biological substances, and (*b*) interactions of drugs with nonbiological substances.

Interactions of Drugs with Biological Substances—Computer programs were used to compute and plot theoretical data in which the binding capacities, association constants, and level of protein were varied in a system of dicumarol (bishydroxycoumarin) and human serum albumin. In general, for any one drug level the amount of unbound drug was inversely proportional to the binding capacity, association constant, and protein content (879). The *in vitro* binding of a number of sulfa drugs to human serum albumin was studied as a function of pH, and the results were correlated with pKa, octanol–water partition coefficient, and electron distribution of the sulfa drug. It was concluded that the primary effect in sulfa drug binding with human serum albumin may be an electrostatic attraction between the anionic drug molecule and a cationic site of human serum albumin, while hydrogen bonding and attraction between the tryptophan residue of human serum albumin and the sulfa drug play a secondary role (880–882).

Interaction between 5-dimethylamino-1-naphthalenesulfonamide and human serum albumin suggested that the binding of the drug to the protein is hydrophobic in nature, and it occurs near the tryptophan residue of the protein (883). The binding constants of various sulfonamide drugs with bovine serum albumin determined as a function of pH were analyzed with five energy-related physicochemical parameters, taking into account the correction for ionization of the drugs. It was postulated that the binding equilibrium depends on the hydrophobicity of the drug and binding occurs between the neutral drug molecule and the hydrophobic fraction of the protein surface (884). The binding of 33 organic compounds to bovine serum albumin was studied at pH 7.4 and 37°. The binding affinity of these compounds correlated with their octanol–water partition coefficients when more than 50% of the compound existed as unionized molecules at pH 7.4. It was shown that the octanol–water and isobutanol–water reference systems for defining hydrophobic character yield comparable results (885).

The interaction of tritiated prostaglandin E_1 with various constituents of human blood was examined by equilibrium dialysis and incubation experiments. Prostaglandin E_1 apparently exhibited weak interactions with serum albumin but did not interact with blood cells and plasma globulins (886). Following incubation of tritiated digoxin with human blood, all the radioactivity was found associated with the plasma, with 25% bound to the serum albumin and the rest free in solution. Addition of cortisol, progesterone, or cholesterol to the serum decreased digoxin binding (887). However, analysis of the serum obtained from human subjects 2 hr. after receiving tritiated digoxin orally or by intravenous injection showed only 1–10% of the drug bound to serum protein (888). The plasma protein binding of diphenylhydantoin in adult patients with epilepsy, in normal subjects, and in hyperbilirubinemic newborn infants was investigated. Competition was indicated between the drug and bilirubin for albumin binding sites (889). The binding of estrone sulfate by human serum albumin appeared to involve one strong binding site and several weaker binding sites. Estradiol 3-sulfate, androsterone sulfate, and similar other compounds were shown to compete for estrone sulfate binding (890).

The binding of salicylic acid to human serum albumin was studied by equilibrium dialysis and frontal gel chromatography. The reproducibility of the data provided by chromatography was far superior to that obtained with equilibrium dialysis (891). An ultrafiltration method, using membranes that quantitatively separate small molecules from plasma proteins, was employed to measure plasma binding of salicylate. The results obtained by this method were comparable to those obtained by equilibrium dialysis (892). The proportion of salicylate bound to serum proteins of rats *in vitro* increased with the increasing age of the rats, from 30% at 7 days to 53.2% at 60 days, due to increased serum albumin concentrations in the older rats. These results may partially explain the greater drug sensitivity of newborn animals (893). Three sulfonylureas, acetohexamide, chlorpropamide, and tolbutamide, were investigated in terms of their affinity for the major human serum proteins. At pH 6.5, 7.4, and 8.4 and normal human serum protein concentrations, these drugs were primarily bound to albumin. Inhibition of sulfonylurea–albumin binding by drugs that are known to potentiate activity of these sulfonylureas was studied (894). The serum albumin binding capacity of doxycycline, metoclopramide, sulfaethylthiazole, and progesterone was increased approximately 40% in the presence of physiological concentrations of urea. Disorganization of the protein structures by a reduction of intramolecular hydrophobic forces was suggested as a probable reason for the urea-induced increase of the drug–protein binding (895). Antileprous drugs and thyroxine bind to human serum proteins at a common site. This was demonstrated by mutual interference between thyroid hormones and antileprous drugs in binding to human serum (896). On the basis of affinity for serum proteins, antileprous drugs were classified into: the albumin-affinitive type, the globulin-affinitive type, the nonadsorbing type, and those having moderate affinity to serum proteins (897).

In lecithin–water model membrane systems, polymyxin B interacted with the polar and nonpolar regions of the phospholipid, while gramicidin S interacted only with the polar region. The implication of these observations with respect to lipid–protein interactions and

antibiotic activity was discussed (898). At equimolar concentrations, chlorothricin formed a strong complex with lecithin and prevented the phospholipid from swelling in water. This was ascribed to nonpolar association of the antibiotic with the hydrocarbon chains of the phospholipid (899). Phospholipid, cholesterol, protein, and mixed lipid–protein monolayers were used as models to study interactions between 3,4-benzpyrene, a carcinogenic hydrocarbon, and cell membranes. The carcinogen was shown to interact strongly with phospholipid, protein films, and mixed lipid–protein films. The biological significance of these data was discussed (900). A similar study dealt with interactions between dimethyl sulfoxide and these monolayer model films. The results indicated that the remarkable penetration abilities of dimethyl sulfoxide may be due to some alteration of protein structure as a result of dehydration at the biomembrane (901). The interactions of some oral contraceptive steroids with cholesterol and lecithin monolayer films were studied to determine their role in platelet aggregation. The data support the hypothesis that steroids may adsorb at platelet surfaces and alter the surface properties or the penetrability of the platelet membrane to cause increased platelet aggregation (902).

Additional references pertaining to interactions of drugs with biological substances are given in Table XXVI.

Interactions of Drugs with Nonbiological Substances— A theoretical study dealt with the calculation of heats of complexation and the structures of charge-transfer complexes of chloranil, fluoranil, and tetrachlorophthalic anhydride with various methylbenzenes in carbon tetrachloride solvent. The theoretical interaction energies were calculated from the monopoles-bond polarizabilities approximation, and the ground-state charge distributions were taken from CNDO/2 calculations[1]. The solvent contribution to the heat of complexation was estimated using the cavity model (927). The effects of water on the charge-transfer transition energy and the equilibrium constant were investigated for complexation of chlorpromazine hydrochloride with *p*-xyloquinone and of sodium *N*,*N*-dimethyl-*p*-aminobenzoate with sodium anthraquinone-2-sulfonate (928). In a subsequent study, the influence of water on the rate of charge-transfer interaction between aniline and chloranil was reported. It was concluded that water promotes the charge-transfer complexation presumably due to the enhancement of the solvent polarity (929).

Metal complexes of thiouracils were studied by potentiometric titration and polarographic techniques in an attempt to determine stability constants and rationalize the mechanistic bases of the relative complex stabilities, thiouracil structures, and biological activities. The relative tendency of metal ions to complex with thiouracils, $Cu^{+2} \gg Pb^{+2} > Cd^{+2} \gg Ni^{+2} \sim Zn^{+2}$, was found consistent with the "natural order" of such transition metals, their relative ionic radii, their electronic structure, and the concept that "soft acids" prefer to complex with "soft bases" (930). Stability constants were determined for a series of imidazole, pyrimidine, and related heterocyclic thiones with Cu^{+2}, Al^{+3}, and Fe^{+3} ions. The relative magnitude of these constants indicated formation of four-membered chelate rings involving nitrogen and sulfur atoms (931). Metal complexes of *ortho*-, *meta*-, and *para*-aminobenzoic acids were examined by atomic absorption, IR, differential thermal analysis, and thermal gravimetric analysis techniques. These complexes consisted of 2:1 ratios of metal to ligand, with amino and carboxyl groups assumed to participate in the complex formation (932). Based on electron microscopy and gel chromatography studies of an iron–dextran complex, a structural model was presented for the complex, with dextran attached by the terminal metasaccharinic acid units to the FeOOH component (933).

The interactions of alkyl trimethylammonium bromides with amaranth were investigated by microscopy, microelectrophoresis, phase solubility, and other physi-

[1] Complete neglect of differential overlap/second parameterization.

Table XXVI—Additional References on Interactions of Drugs with Biological Substances

Reference	Topic
903	Review of the binding of iodinated radiocontrast agents to blood plasma proteins
904	Review of drug binding to plasma proteins
905	Review of drug binding to plasma and tissue proteins, encompassing influence of physicochemical properties of drug on binding and methods for protein-binding study
906	Review describing biological role of metal complexes with drugs and their effects on membrane permeability
907	Review of drug interactions with nucleic acids, proteins, and membranes studied by fluorescence spectroscopy
908	Binding of phenothiazines to bovine serum albumin and related phenomena
909	Binding of ^{99m}technetium to human serum albumins
910	Binding of sulfobromophthalein (bromsulphthalein) sodium by human serum albumin
911	Binding of pentylenetetrazol to plasma proteins
912	Binding of edetate (EDTA), histidine, and aspirin (acetylsalicylic acid) to zinc–protein complex in intestinal content, intestinal mucosa, and plasma
913	Binding of aluminum by hair shown as diffusion control process, with activation energy of 11 kcal. mole^{-1}
914	Protein binding of nikethamide (cordiamine) as reason for its action on tissue respiration processes
915	Niacin (nicotinic acid) bound to human proteins in amounts of 20.1% to α-globulin, 17% to γ-globulin, 5.3% to albumin, and 2.6% to β-globulin
916	Interaction of penicillin and tetracycline with microbic polysaccharides
917	Dermal binding of testosterone and benzyl alcohol to human autopsy skin, and its significance to percutaneous absorption
918	Effects of dilution, specific ions, and inhibitors on thyroxine binding of serum proteins
919	Fluorescent dye 1,8-anilinonaphthalenesulfonate competition with thyroxine for binding to globulin
920	Tetracycline–bovine serum albumin interactions with 2–3 kcal. mole^{-1} enthalpy change seeming to involve formation of noncovalent bonds
921	Relation between the structure of tetracycline derivatives and the extent of binding to albumin
922	Reversible binding of oxacillin, nafcillin, and cephalothin to human serum protein
923	Complexation of chlorpromazine with adenosine and adenosine phosphates
924	Extent of *in vitro* plasma protein binding of amphetamine in different species found independent of drug concentration, similar in normal and uremic plasmas, and significantly different among the species
925	Binding of amobarbital, phenobarbital, and pentobarbital to human serum albumin at various pH's
926	Binding of phenylbutazone and sodium warfarin to bovine serum albumin

cal techniques. The dye–surfactant complexation was shown to be a coacervation phenomenon, and the results were interpreted using general coacervation theories (934). Crystalline 1:1 complexes of acetaminophen with caffeine and theophylline were isolated from aqueous solution. The caffeine–acetaminophen complex was found to exist in three forms, each differing only in the degree of solvation (935). Acetaminophen was shown to form a stable 1:1 complex with antipyrine; the complexation involved amino and hydroxyl groups of acetaminophen and the carbonyl group of antipyrine (936). The formation of a complex between phenylmercuric nitrate and sodium metabisulfite, which is more active in acidic media but less active in alkaline media than phenylmercuric nitrate alone, provided an explanation for the observed antibacterial activity of the drug–metabisulfite mixture (937). A series of six 2,6-diamino-8,9-disubstituted purines was shown to form cyclic 2:1 hydrogen-bonded complexes with phenobarbital in dilute chloroform solution at room temperature, whereas 9-phenbutyladenine formed a 1:1 complex with phenobarbital under similar conditions (938). The effects of the CNS active drugs pentobarbital, bemegride, and trimethadione on the NMR spectrum of formanilide showed that the *cis*-isomer of the amide forms a doubly hydrogen-bonded complex with the drugs, but the *trans*-isomer favors formation of a single hydrogen bond. Implications of results in terms of the mechanistic basis of CNS active drugs were discussed (939).

The formation of inclusion complexes of barbiturate drugs with cycloheptaamylose was examined by solubility analysis and by circular dichroism measurement. These complexes existed in 1:1 ratios with the relative strength of interaction in the order: phenobarbital > pentobarbital > amobarbital > barbital (940). The interactions of β-cyclodextrin with six barbituric acid derivatives and thiopental were studied by solubility analysis. Barbituric acid derivatives having a cyclic substituent at the 5-position formed a more stable complex than those having an acyclic substituent. The physical stability of aqueous solutions of sodium barbiturates was improved in the presence of β-cyclodextrin (941).

The interaction of sodium lauryl sulfate with polyvinylpyrrolidone and polyvinyl alcohol in aqueous solutions was studied by intrinsic viscosity and membrane equilibrium measurements. The intrinsic viscosity and membrane equilibrium yielded a bell-shaped profile with increasing concentration of sodium lauryl sulfate (942). It was shown that monolayers of polyvinylpyrrolidone copolymers interact more strongly with dissolved benzoic acid than with *p*-hydroxybenzoic acid. However, in bulk solutions of polyvinylpyrrolidone, the latter is more strongly bound. This difference was ascribed to the highly oriented structure which polymers assume at interfaces (943). Aqueous mixtures of papaverine hydrochloride and atropine sulfate with various surfactants and polymer excipients were examined by TLC. An interaction between the drugs and sodium lauryl sulfate, as well as a tendency toward complexation of the drugs with methylcellulose, was evidenced (944). Equilibrium constants and coordination numbers were determined for complexes obtained by coupling methacrylic acid and acrylic acid polymers with various ammonium salts of drugs. The experimental data were interpreted by a modified Bjerrum-Gregor theory (945). Complexation of aspirin, salicylic acid, and thimerosal with methacrylate copolymers was indicated by equilibrium dialysis measurements (946).

Additional references related to interactions of drugs with nonbiological substances are listed in Table XXVII.

Table XXVII—Additional References on Interactions of Drugs with Nonbiological Substances

Reference	Topic
947	Review of interactions of diphenylhydantoin, phenobarbital, and other antiepileptics with other drugs of various activities
948	Complex formation between nicotinic acid diethylamide and sulfanilamide and the solubility function of the 1:1 complex in water and acetone
949	NMR studies of interactions between oxyethylene–oxypropylene, macrogol, and phenol
950	Intramolecular hydrogen bonding in pamaquine studied by fluorescence spectroscopy
951	Aggregation of (+)-propoxyphene hydrochloride in aqueous solution examined by circular dichroism measurements
952	Reversible thermal aggregation of vinblastine sulfate and its interactions with hydrophobic groups
953	Ionic associations of amphetamine isomers with mandelic and tartaric acids studied by conductometric measurements
954	Molecular associations of amphetamine isomers with optically active and racemic acids studied by thin-layer electrophoresis
955	Association of tetracycline ions in solution and in complexes with polyelectrolytes
956	Stability and solubility of tetracycline complexes with pyrocatechuic acid and gentisic acid determined as a function of pH
957	Association constants of 14 aminoketones with phenol and their biological activity
958	Amines capable of forming dissociable complexes with aldehyde group of pyridoxal postulated to have radioprotective activity
959	Binding of metal ions and aspartic acid measured by electric conductivity found to decrease in the order of magnesium, calcium, strontium, and barium
960	Structure and bonding of water molecules in α-cyclodextrin hexahydrate
961	Survey of interactions between surfactants and macromolecules (proteins and polymers)
962	Interaction between dextroamphetamine sulfate and spray-dried lactose in solid–solid mixture studied by diffuse reflectance spectroscopy
963	Solid–solid interaction between sulfamethoxazole and lactose examined by thermal analytical methods

Surface Phenomena—The publications dealing with surface phenomena are divided into four major categories: interface studies, adsorption studies, general properties of surfactants, and micelle studies. However, because of the obvious overlap among these categories, the reader with a special interest in this field is advised to consider the entire section.

Interface Studies—Equations correlating the free volume and zero energy of interaction with the surface tension of liquids are based upon the assumption that each particle moves independently in its potential well. Since this concept does not account for the interaction resulting from particle oscillation, the free volume term in these equations was replaced by the free phase space, which can be calculated from the free energy and entropy of interaction among particles (964). Surface tension calculation for simple liquids using the Kirk-

wood–Buff theory, assuming a smooth variation of density between liquid and vapor, and the estimation of the thickness of the interface were shown to be in fair agreement with experimental results (965). The surface energy, surface entropy, and latent heat of surface formation were calculated for water, benzene, *n*-butanol, and six other similar liquids from the information on surface tension as a linear function of temperature (966). The surface tensions of oleic acid, sodium oleate, and a mixture of both in aqueous media were determined as a function of temperature and concentration. Occurrence of intermolecular attraction between these compounds was indicated from the surface energies calculated for the mixture (967). A thermodynamic description of the interfacial layers was presented by treating interfacial layers as a collection of molecular planes (968). This model, at equilibrium, was shown to be in agreement with the Gibbs formula (969).

The thermodynamic properties of the film compression process were evaluated for a series of liquid-condensed and liquid-expanded lipid films. It was shown that, for the liquid-condensed and liquid-expanded monolayers, the hydrocarbon region is energetically similar to bulk hydrocarbon liquids. Entropies of compression for the polar groups suggested that characteristic changes in water structure are likely to occur in the formation of each condensed monolayer state (970). In a subsequent study with mixed lipid films, it was concluded that: (*a*) in the absence of specific interactions, the liquid-condensed films of octadecanol, stearic acid, and dipalmitoyl lecithin are immiscible with cholesterol; and (*b*) cholesterol forms nonideal mixtures with liquid-expanded lipid films (971). Surface pressure–area curves were obtained for mixed monolayers of myristic acid, an expanded film-forming acid, with condensed film-forming acids of chain lengths from C_{17} to C_{26}. The change in characteristics of the curves with chain length indicated the dominant effect of hydrophobic interactions (972). An empirical equation was given relating the average carbon chain length of a molecule in films spread at the air–water interface with the pH of the aqueous phase, and it was applied for films of palmitic acid, stearic acid, arachidic acid, and behenic acid (973). Measurements of equilibrium spreading pressure for various saturated fatty acid triglycerides revealed that the spreading pressure decreases while the collapse pressure increases with the increase in fatty acid chain length. Successive stages leading to collapse of the film were discussed (974). IR spectra of stearic acid films spread on aqueous aluminum chloride solutions were studied by the attenuated total reflectance (ATR) technique to correlate the molecular structure of the film with the film stability. The stability of this film was attributed to the formation of an adsorption layer of dihydroxoaluminum stearate (975).

The surface pressure–area and oxygen permeation behavior of stearic acid, dipalmitoyl lecithin, and lung alveolar surfactant films were investigated to elucidate the mechanism of surface hysteresis exhibited by the lung surfactant. Mechanisms for the hysteresis phenomenon were proposed based on surface relaxation and compression rates of these films. The results of oxygen permeation studies indicated that only the films with the abilities both to pack efficiently in the condensed state and to regenerate themselves from the collapsed state significantly reduce oxygen permeation (976). The interaction of cetyl sulfate and cetrimonium (cetyltrimethylammonium) ions with dipalmitoyl glycerol monolayers at the air–water interface was followed by variation of the surface pressure and surface potential. The kinetics and mechanism of these monolayer interactions and their implications to microstates of biomembranes were described (977). The free energy changes, activation energies, and activation entropies associated with material transfer through liquid–liquid interfaces were experimentally determined for propionic acid, *n*-butyric acid, and *n*-valeric acid in the benzene–water system. The increase in the interface resistance with increasing alkyl chain length was attributed to the change in activation entropy (978). Thermodynamic parameters for the transfer of *n*-alkanols at the air–solution interface were obtained from surface pressure measurements. In accordance with Traube's rule, free energy values decreased linearly with increasing alkyl chain length and the enthalpy values were independent of the chain length. Approximately a twofold difference was observed between the theoretical and experimental entropy values and was presumed to be due to hydrogen bonding between alcohol and water (979). Equations were derived characterizing diffusion and viscous-type propagation of a liquid on solids (980).

Additional references on interface studies are listed in Table XXVIII.

Adsorption Studies—Gibbsian thermodynamics of adsorption from liquid mixtures on solids were formulated in terms of excess properties. A general expression was derived for the temperature coefficient of the surface excess in terms of heat of immersion of the adsorbent. Experimental data on nonisothermal adsorption and the heats of immersion were reported for the benzene–cyclohexane system adsorbed on silica gel (1001). A new isotherm for adsorption of a monolayer on a smooth surface, which is reducible in limiting cases to the Langmuir, Freundlich, or Temkin isotherm, was proposed. Immobile and mobile adsorbed molecules were considered, the number of the latter being determined by a Boltzman expression (1002). The influence of counterion concentration on the thermodynamic parameters of dodecylpyridinium chloride at the free surface of solution was investigated. The free energy, enthalpy, and entropy of adsorption values were correlated with the temperature and counterion concentration of the solution (1003). Adsorption of five steroids was determined at the oil–water interface in an effort to test a localized and a nonlocalized model for the adsorbed layer. Since neither model provided an accurate description of the adsorption kinetics, it was concluded that both the energy barrier and diffusion play a role in adsorption kinetics (1004).

Carstensen and Su (1005) described the effect of solvent intercalation in adsorption isotherms of montmorillonite from solvents with a high dielectric constant. It was shown that the number of solvent layers in the interlaminar spacing is higher when the clay is equilibrated with liquid than with the solvent vapor. These

Table XXVIII—Additional References on Interface Studies

Reference	Topic
981	Review describing effective surface-layer thickness, thermodynamics of surface phenomena, surface-layer thickness at two-phase boundary, *etc.*
982	Structural properties and biological significance of organic films; specific data for film structure of stearic acid esters
983	Review of the influence of interfacial tension-driven instabilities on rates of mass transfer in liquid–liquid systems
984	Review of the theory of heat of wetting and its application to solid surface studies
985	Review of structural theories of surface tension of liquids, with emphasis on molten salts
986	Comparison of liquid–liquid interfaces and solid–vacuum interfaces
987	Review of intermolecular energies in lipid monolayers, use of film balance for measuring thermodynamic properties of monomolecular films, FORTRAN computer programs, *etc.*
988	Review of surface and interfacial tension contact angle
989	Surface tensions of organic solids—amides, substituted acids, quinones, caffeine, and camphor
990	Contact angle and wettability of packaging materials and of human skin
991	Autoxidation of cholesterol in aqueous dispersions and in monomolecular films
992	Description of nondifferential equations relating surface tension to concentration, temperature, and other properties that depend on the size and energy characteristics of the particles
993	Surface pressure–area behavior of several fatty acid monolayers, dipalmitoyl lecithin monolayers, and mass transfer across a liquid–liquid interface
994	Equation involving cohesive force for the surface pressure of long-chain ampholytes at oil–water interface
995	Monolayers of poly(organosiloxane) on water; structure of siloxane skeleton without decisive influence on spreading phenomena
996	Dispersion forces and surface tension of liquids described in terms of cavity formation theory
997	Role of the diffusion layer in water-in-oil microemulsions; Adamson's model for microemulsions modified to allow for the diffusion layer in the aqueous droplet interior
998	Influence of surface area variation on kinetics of hydration of tricalcium silicate
999	Automated drop volume apparatus described for surface tension measurement
1000	Surface properties and intermolecular interaction in liquid media

authors (1006) also presented data supporting the hypothesis that the adsorption of unionized organic compounds on montmorillonite is a surface adsorption phenomenon mediated *via* an ion–dipole interaction. Dissolution and dialysis studies were reported showing that cationic drugs and some nonionic drugs adsorb very strongly to montmorillonite clay. A two-step process involving a cation-exchange reaction followed by strong surface chemisorption was proposed as the mechanism for cationic drug–clay binding. The initial urine levels of amphetamine after oral administration of the drug–clay complex were significantly lower than those for the pure drug (1007). The adsorption of benzidine on montmorillonite suspended in aqueous solutions was studied as a function of pH and electrolyte concentration. The interaction between benzidine and the clay was considered to be a cation-exchange process (1008).

The liquid and vapor phase adsorption of primary aliphatic alcohols (C_1–C_{10}) on calcium and sodium montmorillonite was investigated by X-ray techniques. Molecular configurations of the adsorbed molecules consistent with the experimental data were suggested (1009). The adsorption of salicylic and gallic acids by sodium and calcium forms of kaolinite, montmorillonite, and other clays was determined in aqueous media. The results were discussed in terms of the differing crystal structures of the clays (1010). In aqueous solutions, Langmuir-type isotherms were observed for the adsorption of crystal violet on the H-forms of bentonite and kaolinite. Both clays sorbed crystal violet in amounts greater than their cation-exchange capacities, indicating that unionized molecules of the dye were adsorbed in the interlamellar spaces of the clay and that dimerization of the dye occurred on the clay surfaces (1011). Adsorption of benzoic acid by kaolin was studied as a function of pH. For effective preservative action, when both benzoic acid and kaolin are present in a formulation, the use of either high benzoic acid concentrations at pH > 5 or low concentrations at pH < 5 was recommended (1012).

Adsorption of tetracycline on calcium fluoride was investigated in an attempt to develop a physical–chemical model capable of describing the adsorption of specific tetracycline species from aqueous solution onto model adsorbents, both in the presence and absence of added electrolytes and over wide concentration and pH ranges. In the absence of other electrolyte species, Langmuirian-type adsorption isotherms were observed when the equilibrium concentrations of the adsorbate were calculated to reflect the activity of the negatively charged tetracycline species. The data obtained in the presence of fluoride or sulfate anions were explained on the basis of competitive binding by these anions for the same surface site. In the presence of calcium or magnesium cations, it was proposed that other drug species adsorb simultaneously with the negatively charged tetracycline species (1013). Determination of adsorption isotherms for four sulfanilamides on activated carbon, as a function of pH, showed an adsorption maximum between pH 4.5 and 5.5, corresponding to the isoelectric points of the molecules (1014). The influence of surface area of activated carbon upon the adsorption of coccinine and picric acid from granular and other forms was investigated. A mathematical interpretation of the data showed that the adsorbing power of the carbon can be represented by an exponential equation (1015, 1016).

Additional references on adsorption studies are listed in Table XXIX.

General Properties of Surfactants—Surface activity and emulsifying properties of quaternary ammonium salts were correlated with the structure of the surfactant molecule and its orientation at the toluene–water interface. Differences between the predicted and experimental values of adsorption energies suggested that about 40% of the methylene groups present in the surfactant molecule were submerged in the aqueous phase. The maximum emulsifying ability was observed for the alkyl trimethylammonium-type compounds containing 14–16 carbon atoms (1031). The surfactant properties of a water-soluble phosphated nonylphenol ethoxylate in aqueous media were examined as a function of pH, temperature, and surfactant concentration. From the measurements of the CMC in buffer solutions

Table XXIX—Additional References on Adsorption Studies

Reference	Topic
1017	Review of adsorption properties of hydrophobic surfaces, including discussion of adsorbate interaction models and heats of adsorption
1018	Review of theories of monolayer adsorption, isoenergetic and homotactic adsorbents, and adsorption isotherms of simple molecules
1019	Review of ionic double layers and adsorption
1020	Diffuse reflectance spectra as a source of information about the absorption spectra of adsorbed molecules
1021	Adsorption kinetics at a solution–air interface of a surface-active substance
1022	Role of hydrophobic interactions during adsorption of simple diphilic molecules studied by the dependence of adsorption on temperature
1023	Adsorption of sodium and calcium lauryl sulfates at the surface of their aqueous solutions
1024	Heats of reversible adsorption measured for *n*-heptanol, *n*-nonanol, and secondary straight-chain nonanols
1025	Evaluation of sorption–desorption processes between polymers and low molecular weight compounds by GC
1026	Sorption of preservatives by plastic packaging materials relative to polarity of the plastic and the preservatives
1027	Adsorption of a nonionic surfactant on different clays limited by formation of a monomolecular layer
1028	Adsorption of sodium salicylate on clay minerals
1029	Possible use of Estonian clays in pharmaceutical preparations evaluated by their adsorption characteristics
1030	Adsorption kinetics of anionic surfactants at air–water interface in the presence of electrolytes

Table XXX—Additional References on General Properties of Surfactants

Reference	Topic
1046	Review of solubility and micelle formation properties of surfactants in nonaqueous solutions
1047	Review of solubilization properties of surfactants in nonaqueous solutions
1048	Review of physicochemical properties of polyoxyethylene alcohols
1049	Review of application of surfactants to improve drug yield and formulation stability
1050	Review of surfactant classification and selection, emulsion polymerization, and surfactant coating problems
1051	Review of properties, classification, evaluation, industrial application, *etc.*, of nonionic surfactants
1052	Review of physical properties of surfactants including surface tension, film formation, micelles, HLB, and rheological properties
1053	Review discussing concept of HLB, coalescence of emulsion particles, and partition coefficient of surfactants in terms of chemical potential
1054	Review of characteristics of oil-soluble surfactants in nonaqueous media, solubilization
1055	Review describing effects of chain length and branching in sodium alkyl benzenesulfonate surfactants upon their interfacial properties
1056	Review of relations between chemical structure and surface activity discussed in terms of mechanism of surface activity, role of different functional groups, molecular configuration, and chain branching
1057	Interfacial activity of sodium lauryl sulfate in the presence of alcohols and its effect on emulsion stability
1058	Influence of surfactants on liquid absorbability of various powders of pharmaceutical interest

over the 4.0–9.2 pH range, it was suggested that the surface-active properties of the compound were unaffected by the state of ionization of the polar phosphate group. The group interfacial cross-sectional area of the molecule varied from about 0.92 nm.^{2} at 15° to 0.82 nm.^{2} at 50°, which was consistent for the system having nonionic portions of the molecule oriented at the interface (1032). A thermodynamic treatment for the occurrence of a minimum on the surface tension isotherm suggested that the presence of an impurity that lowers the CMC causes a minimum on the isotherm, but an impurity that increases the CMC may not always yield a minimum (1033). The literature data for surface pressures of mixtures of anionic and cationic surfactants were found consistent with theoretical predictions based on the surface behavior of the separate constituents (1034). An ultrasound method was described for the determination of the degree of hydration of nonionic surfactant molecules in an aqueous solution (1035).

The association equilibrium and micelle formation by polyethylene glycol *n*-nonylphenyl ether (1036) and β-D-*n*-octyl glucoside (1037) were determined by vapor pressure, osmometric, light scattering, ultracentrifugation, and sedimentation methods. For both surfactants, the data agreed with the model of closed association, and a linear relationship existed between the enthalpy and entropy of association. A subsequent publication dealt with the theoretical estimation and experimental measurements of viscosity of the two surfactants (1038). The effects of a polymer interacting with a surfactant upon the solubilization of the surfactant, surface tension, and viscosity were discussed. Interactions of polymers were more pronounced with anionic surfactants than with nonionic and cationic surfactants (1039). In a similar study, viscometric and conductometric measurements showed that the interactions of polyvinyl alcohol with anionic surfactants occurs below the CMC's (1040).

The HLB values of fluorocarbon and ionic hydrocarbon surfactants were related to their CMC values. A modified equation, derived from Davies' theory, was employed for the estimation of cohesive free energy change per methylene group in the surfactant molecule (1041, 1042). Application of NMR as a direct measure for the determination of HLB values for nonionic surfactants was demonstrated. Estimation of the relative proportion of lipophilic and hydrophilic protons by this technique provided the basis for the calculation of the HLB characteristics of a surfactant (1043). The HLB values of surfactants were correlated with the ratio of the work of surfactant adsorption from two liquid phases at their interphase (1044) and with the spreading coefficient determined from surface and interfacial tension measurements (1045).

Additional studies on general properties of surfactants are listed in Table XXX.

Micelle Studies—The definition and phenomenological interpretation of the CMC in multicomponent systems were discussed. The CMC was considered as the transition point for a second-order phase transition in a multicomponent system. Expressions were given for the mutual dependencies of temperature, pressure, and composition of a system at its CMC (1059). Mukerjee (1060) applied procedures for dealing with multiple

equilibria in stepwise self-association when all species are in a rapid association-dissociation equilibrium to micellar systems. The possibility of determining the number average degree of association by utilizing experimental weight average values was demonstrated. The self-association model, along with some approximate nonideality corrections, was stated to reproduce the shapes of some observed reduced turbidity data showing minima (1060). A model describing the kinetics of micelle formation from chemical relaxation studies predicted a single relaxation time and a correlation between the relaxation time and overall surfactant concentration. The model, consisting of an associated state of micelles of various sizes and a nonassociated state of monomers, assumed that the enthalpy change for the micellization was independent of the size of the micelle (1061). Some consequences of the equilibrium model of closed association between monomeric and polymeric molecules for micellar systems were discussed. In some cases, the Debye method of determining monomer concentration suggested the existence of a premicellar equilibrium (1062).

The changes in molecular motion of ions and their hydration layers during adsorption from the bulk solution into the Stern layer of a detergent micelle were studied by NMR. The results suggested that the rate of rotation of hydrated sodium ions is unlikely to alter by >35% on adsorption from the bulk to the Stern layer of sodium lauryl sulfate micelles; however, the symmetry of the hydrated layer may be altered by the charged head groups of the micelle (1063). The kinetics of micelle dissociation determined by temperature-jump and NMR methods did not agree with the mechanism involving a single, slow step dissociation process. The results, when treated on the basis of many sequential steps for dissociation, were compatible with rate constants 10^5–10^6 $sec.^{-1}$ for micelles having aggregation numbers greater than 50 (1064).

Yalkowsky and Zografi (1065) calculated the partial molal volume of a large number of surfactants in the micellar state by adding partial atomic values and accounting for the hydrocarbon liquid-like nature of the micelle interior. This approach provides a means of determining relatively accurate values by simple calculation (1065). In response to a previous publication questioning the existence of spherical-shaped micelles of common single-chain surfactants, Zografi and Yalkowsky (1066) concluded that the exact shape of micelles is still an open question but, in general, previous arguments presented against the spherical micelle shape were considered invalid. The authors proposed that until more definitive evidence is available, a spherical or near-spherical micelle model can be considered useful in the estimation of molecular area and other micelle dimensions. Tanford (1067) concluded, on the basis of simple geometric considerations, that most of the common small soluble micelles must be ellipsoidal rather than spherical in shape. It was suggested that amphiphiles with a single hydrocarbon chain per polar head group form micelles with a distribution of sizes with spherical and ellipsoidal shapes, while those with two hydrocarbon chains per head group prefer extended bilayer or vesicle-shaped micelles.

The number average micellar molecular weight for a purified sample of polysorbate 80 in aqueous systems was determined by membrane osmometry. This method was also employed to study the effect of micellar solubilization on the molecular weight of the micelle (1068). The surface tension measurements of aqueous polysorbate 20 solutions from 0.001 to 10 mg./ml. concentrations suggested that the CMC of polysorbate 20 is in the vicinity of 0.06 mg./ml. surfactant concentration (1069). The CMC of homogeneous polyethylene glycol lauryl ethers was determined from the spectral change produced by the charge-transfer solubilization of 7,7,8,8-tetracyanoquinodimethan. The values obtained by this method were consistent with those obtained by surface tension measurements (1070). The aggregation of an uncharged surfactant in aqueous solution was described in terms of a multiple equilibrium model. Average aggregation numbers and diffusion coefficients for *n*-alkyl hexaoxyethylene glycol monoethers were calculated from light scattering and free boundary experiments (1071).

The temperature dependence of the CMC of several cationic surfactants in the presence of glucose or sucrose was reported. Thermodynamic parameters were calculated using the uncharged phase-change model. The changes in CMC values in glucose or sucrose solutions were interpreted as the effects of modifications in water structure and the reduction of dielectric constant (1072). Micellization behavior of four antihistamines in aqueous solutions was studied by light scattering to estimate the effective micellar charge, CMC, and degree of ionization. It was pointed out that since the effective charge refers to the equivalent charge under ideal conditions, which is generally lower than the true value at the shear surface, the value for the degree of ionization, therefore, is likely to be an underestimation of the extent of ionization of the micelles (1073). Aggregation numbers and effective charges of two cationic surfactant micelles were determined at various ionic strengths and temperatures. From the micellar parameters, the hydrophobic and electrostatic contributions to the free energy and enthalpy of micellization were calculated and the enthalpy values were compared with calorimetric values (1074).

Additional references on micelle studies are given in Table XXXI.

Dispersion Stabilization—The quantitative investigation of coalescence kinetics in dilute oil-in-water emulsions was presented. Experimental data were in agreement with the flocculation model, but they significantly deviated from the classical coalescence model. However, incorporation of the hydrodynamic effect into the coalescence model yielded reasonable agreement between the experimental coalescence data and the theoretical results (1089, 1090). The effect of nonlagging molecular forces on the coagulation and condensation growth of highly dispersed aerosol particles was studied as a function of the size of the impinging particles. It was shown that molecular forces have less effect on coagulation of particles of different sizes than of identical sizes (1091). The changes in interfacial tension, particle size, and stability of an oil–water–nonionic surfactant emulsion system were studied as

Table XXXI—Additional References on Micelle Studies

Reference	Topic
1075	Review of micelle formation of surfactants
1076	Review of micellization of surfactants in nonaqueous solutions
1077	Review of CMC, size, shape, and solvation of micelles
1078	CMC determination of nonionic surfactants by interference refractometry
1079	Decrease in CMC of polyethylene glycol alkyl ethers with increasing hydrocarbon chain; increase in CMC with increasing number of oxyethylene groups
1080	Surface tension, conductivity, and CMC data for aqueous rifamycin SV solutions
1081	Use of electron spin resonance (EPR) spectra for estimation of the local counterion concentrations at the micellar surface of copper lauryl sulfate
1082	Fluorescence absorption and emission studies of solubilization of pyrene in long-chain cationic micelles
1083	Second CMC of the aqueous solution of sodium lauryl sulfate shown by conductivity studies
1084	Polarographic determination of CMC of anionic surfactants
1085	Effect of temperature on the CMC of decylpyridinium chloride
1086	Degree of dissociation and CMC of some cationic surfactants
1087	Micelle formation of sodium lauryl and sodium tetradecyl sulfates in water–organic solvent mixtures evaluated by conductivity and viscosity measurements
1088	Influence of polar additives (*e.g.*, caproic acid, hexanol, and aniline) on CMC of sodium alkyl phosphonates

functions of temperature and ethylene oxide units of the surfactant. In systems containing surfactants with more than 10 ethylene oxide units, the particle size was decreased and the stability improved with increasing emulsification temperature (1092). The factors influencing coalescence in liquid–liquid dispersions, including the effect of mass transfer, shape of droplets, agitation, and coalescence at a liquid or solid surface, were discussed (1093).

Expressions for the work and heat of wetting were derived and applied to heptane–water–powdered glass systems containing variable amounts of a surface-active agent (1094, 1095). Addition of lauryl alcohol to a mineral oil (Nujol) oil-in-water emulsion had a stabilizing effect, while the stability of an olive oil-in-water emulsion was unaffected by the addition of lauryl alcohol. These observations were explained in terms of adsorption of lauryl alcohol at the mineral oil–water interface (1096). The effect of micelle formation and the nature of the oil phase on the distribution of a nonionic surfactant in three- and four-component emulsions was investigated. The results were related to mechanisms responsible for the formation and stability of emulsions (1097). The stability of a liquid petrolatum–water emulsion containing 0.2% sodium lauryl sulfate decreased upon storage up to 25 days and thereafter increased with time. This was rationalized in terms of an initial desorption of the surfactant upon increase in the droplet size, followed by readsorption of the surfactant at the oil–water interface (1098). The stability and phase dispersion of petrolatum emulsions were studied as functions of surfactant and water concentrations. The stability was enhanced by increasing the proportion of water, while the stability was not greatly influenced by the surfactant concentration (1099).

The role of adsorption in the interaction of water-soluble polyelectrolytes with colloids and highly dispersed systems was discussed. The reversible adsorption was considered as an important phenomenon governing stability of relatively dilute systems, while irreversible adsorption causing alteration of the solid surface was considered important in the stabilization of concentrated dispersions (1100). Theoretical calculations analyzing the effect of polydispersity of nonattracting spherical particles upon sedimentation volume were presented. In a polydisperse mixture, small particles occupy interspaces between large particles so that the sedimentation volume becomes smaller than for a monodisperse mixture (1101). The colloidal stability of polydisperse systems was calculated for particles differing in size and surface potential. According to these calculations, dispersions of particles with Gaussian distributions of radii or surface potential were shown to be less stable than the corresponding monodisperse systems with constant surface potential (1102). An asymptotic solution to a simplified equation for the kinetics of coagulation was applied to aerosol systems. The simplified coagulation model with a constant collision parameter was shown to approach a self-preserving form, which is independent of the initial distribution (1103).

Sedimentation kinetics of flocculated silica suspensions were studied to elucidate the role of ζ-potential in sedimentation mechanisms. In the initial time periods, the sedimentation rate constant was shown to be a function of both Stokes and aggregational forces; in the final phase, the reciprocal of the smaller of the two contraction rate constants was linearly related to the reciprocal of the ζ-potential (1104). The structural stability of montmorillonite and palygorskite suspensions in organic liquids was studied in relation to their electrokinetic properties. No direct correlation between the ζ-potential and stability of these suspensions was observed (1105). A kaolin suspension containing sodium caprylate was stabilized by the addition of caprylic acid. A phase diagram was presented for the water–sodium caprylate–caprylic acid liquid crystal system (1106). The effect of pH on the flocculation of bentonite sols by an acrylamide–acrylate copolymer was studied. The results demonstrated the existence of a flocculation threshold at the pH where the macromolecular chain exists in the completely extended form (1107).

Additional references on dispersion stabilization are given in Table XXXII.

Rheology—The structure and rheological properties of emulsions stabilized by the mixed emulsifier, polyethylene glycol 1000 monocetyl ether (cetomacrogol 1000) and cetostearyl alcohol, were investigated. The mixed emulsifier exhibited a self-bodying network phenomenon showing an increase in consistencies with increasing emulsifier concentration and with increasing temperature up to a maximum. At higher temperatures, consistency decreased as the network weakened and finally dissolved to form an isotropic solution. The mechanisms responsible for the formation of nonionic

Table XXXII—Additional References on Dispersion Stabilization

Reference	Topic
1108	Review of the behavior of colloidal particles in the presence of ions, surfactants, polyelectrolytes, and polymers
1109	Review describing current state of colloid science and areas of future research
1110	Review of the theory of lyophobic colloid stability
1111	Review describing force constants for molecular interactions involving hydrophobic surfaces
1112	Review of theories of dispersion and emulsion stability in nonaqueous media
1113	Review of ζ-potential, electrical double layer, and measurements of electrophoretic mobilities
1114	Analogy between Smoluchowski's equation for slow coagulation and Bhattacharya's equation
1115	Kinetics of emulsion stability dependent on the size of the dispersed phase
1116	Effect of electrolytes on the stability and breaking of emulsions
1117	Thermal transport process shown as major factor in the attenuation of sound in suspensions and emulsions
1118	Electrical interaction of colloidal particles with surface charge of constant density
1119	Flocculation and dispersion stability of mineral particles in relation to ζ-potential
1120	Physical structure of foam, and role of adsorption in foam stability
1121	Temperature stability of silver iodide and antimony sulfide hydrophobic sols stabilized by water-soluble polymer additives
1122	Theory of flocculant systems, and contributions of electrostatic and nonelectrostatic forces in relation to flocculation by water-soluble polymers
1123	Review of mechanism of solid suspension flocculation by synthetic polymers

networks were discussed and compared with mechanisms that function in gels formed by ionic surfactant–long-chain alcohol mixed emulsifiers (1124, 1125). The rheological behavior of 20% water-in-oil emulsions and dispersions of glyceryl tristearate crystals in oils was interpreted by the network model for a system of dispersed particles where particles tend to aggregate into random chains due to van der Waals' forces (1126). Rheological parameters were correlated with stability of liquid paraffin emulsions prepared with carboxymethylcellulose and with a mixture of gum acacia and gum tragacanth. The thixotropy of the carboxymethylcellulose preparation was practically constant during storage, while that of the gum emulsion increased and then decreased (1127). A similar study dealt with the rheological aspects of liquid paraffin, castor oil, and cod liver oil emulsions prepared with acacia and methylcellulose (1128).

The effect of wetting and surface-active agents on the rheological properties of various ointment gels was studied. Viscosity varied inversely with the degree of wetting, especially upon addition of surface-active agents (1129). Some rheological properties of hectorite dispersions such as viscosity, plastic flow, and static yield values were related to the particle shape, particle interactions, and addition of uni-univalent electrolytes (1130). Some anionic surfactants were found to increase the viscosity of the salicylic acid–cetrimide system to a maximum and then decrease it on further addition of the surfactant. The results were explained in terms of interaction between the anionic surfactant and free cetrimide molecules (1131). Quantitative measurements

Table XXXIII—Additional References on Rheology

Reference	Topic
1135	Review describing behavior in combined shear and electrical fields of dispersions, emulsions, polymer suspensions, *etc.*
1136	Review of rheological properties of monomolecular films—basic concepts and experimental methods
1137	Review of rheological properties of monomolecular films—experimental results, theoretical interpretation, and applications
1138	Elementary concepts of rheology relevant to food texture studies
1139	Rheological study of certain polyethylene glycol gels used in ointments
1140	Critical shear stress and estimation of the energy of contacts in lyophilic dispersed gelatin gel systems
1141	Interfacial shear viscosity at fluid–fluid interfaces containing surfactants or macromolecules
1142	Kinetics and mechanism of thixotropy of aqueous bentonite suspensions
1143	Surface viscosity measurements by an improved rotational–torsion method
1144	Effect of nonionic emulsifiers on the structural rheological properties of gels
1145	Influence of metallic soaps on the rheological properties of lipophilic bases

of thixotropic characteristics by a weight-actuated viscometer were demonstrated. This approach eliminated the problems involved in obtaining the classical hysteresis loop, since it provided data on the actual rates of breakdown and buildup of the systems (1132). Elastic, retarded elastic, and viscous components of the total deformation of gels were derived by plotting the penetration of a cone against the logarithm of time. The results were in agreement with those obtained from creep measurements with coaxial cylinders (1133). Rheological properties of Newtonian (silicone) fluids and a viscoelastic material (cetrimide cream BPC) were studied by a modified single-pan chemical balance method. Equations were given for the calculation of viscosities and compliances using this method (1134).

Additional references on rheology are provided in Table XXXIII.

PHARMACEUTICAL ASPECTS

Antibiotics—Water-soluble polymer derivatives of penicillin with vinylpyrrolidone copolymers were prepared. These derivatives retained their antimicrobial activity and were inactivated by penicillinase at a slower rate than penicillin (1146). Studies on combined precipitation of nystatin with polyvinylpyrrolidone from dimethylformamide solution included dynamics of precipitation, solvent effects, and the composition of the complexes (1147). Gramicidin was shown to aggregate in aqueous solution and form monolayer films at the air–water interface. The observed first-order rates of lecithin monolayer penetration by gramicidin were consistent with the proposed intramembrane dimerization of gramidicin to form ion-conducting channels (1148). Semipermeable membrane sacs containing *E. coli* were placed in the peritoneal cavity of rabbits to study the effects of proteins on the antibiotic activity of gentamicin. It was concluded that the serum protein inhibited, while immune serum enhanced, the action of gentamicin (1149).

Table XXXIV—Additional References on Pharmaceutical Aspects

Reference	Topic
	Antibiotics
1159	Review of chemical properties, pharmacokinetics, antibiotic activity, and clinical uses of erythromycin, oleandomycin, and troleandomycin
1160	Review of antimicrobial properties, pharmacokinetics, toxicity, and clinical uses of polymyxins, colistin, bacitracin, ristocetin, and vancomycin
1161	Review of erythromycin derivatives (esters, oximes, and quaternary salts) and their antibacterial activity
1162	Review of antibacterial activity and chemical stability of penicillin derivatives
1163	Review of biopharmacy of chloramphenicol
1164	Review of chemical and antimicrobial properties and mechanisms of action of streptomycin
1165	Review of mechanism of action, binding to ribosomes, and bacterial resistance of tetracycline analogs
1166	Effect of microbic polysaccharides and nonionic surfactants on the activity of some antibiotics
	Radiopharmaceuticals
1167	Review of radioactive compounds (*e.g.*, $^{59}FeCl_3$, insulin-^{131}I, and $Na_2{}^{51}CrO_4$) used in medical diagnosis
1168	Review of radioactive pharmaceuticals for medical diagnosis
1169	Review of therapeutic uses of radiopharmaceuticals
1170	Physical and biological half-life, radiation type, and purity of radiopharmaceuticals
1171	Preparation of high-purity carrier-free ^{123}I-iodine monochloride for iodination of radiopharmaceuticals

Heman-Ackah and Garrett (1150) reported on the effect of erythromycin on the action of lincomycin (phase II) and the 7(*S*)-chloro analogs of lincomycin against *E. coli*. Combinations of erythromycin and the 7(*S*)-chloro analogs of lincomycin demonstrated *a priori* antagonism of effects because of possible allosteric interactions which decrease the effects of one drug in the presence of the other at their site of action. It was emphasized that the dose–response relationship over a wide concentration range, as well as the kinetics and mechanisms of the separate drug action, must be considered in the quantification and prediction of combined action of antibiotics (1150). Addition of sodium edetate (EDTA) to sodium penicillin G or potassium phenoxymethyl penicillin (penicillin V) failed to enhance *in vitro* the antibacterial activity of either antibiotic against several bacterial species. Oral administration of edetate (EDTA) in combination with potassium phenoxymethyl penicillin or tetracycline hydrochloride also failed to increase antibiotic absorption in pigs (1151). Combinations of disodium carbenicillin and gentamicin showed an additive effect on *E. coli* and prevented its regrowth. Clinical use of this and other antibiotic combinations and development of resistance to these combinations were discussed (1152). An ophthalmic preparation of chloramphenicol containing methylcellulose remained in the conjunctival sac of the eye for several hours due to a viscosity effect, while one without the methylcellulose remained in the sac for only 10 min. (1153).

Radiopharmaceuticals—The type of theoretical background and practical experience required by a pharmacist in the preparation of radiopharmaceuticals in hospitals was discussed (1154). Pharmacological, pathological, and radiation hazards associated with the use of radioactive pharmaceuticals were reviewed. These hazards were considered slight with the use of modern radiopharmaceuticals, especially in view of their diagnostic value (1155). Factors influencing the stability of radioiodinated insulin including pH, concentrations of insulin and ^{125}I, and the addition of bovine serum albumin were examined. Insulin-^{125}I retained its immunoreactivity and gel filtration characteristics best when stored at <0.2 mcg./ml., −20°, in borate buffer (pH 8.6) with the addition of 1% bovine serum albumin (1156). Metal chelate complexes of ^{153}Sm and ^{169}Yb were prepared by exchange reaction for use as radiopharmaceuticals. Electrophoresis provided the most suitable method for controlling the radiochemical purity of the complexes (1157). Values for the equilibrium absorbed dose constants of ^{111}In and ^{169}Yb were calculated, and the metabolic behavior of indium and ytterbium chelates was reviewed (1158).

Additional references on pharmaceutical aspects are listed in Table XXXIV.

BIOPHARMACEUTICS

The various publications dealing with biopharmaceutics were subdivided according to the areas of special interest. However, because of the obvious overlap in subject matter, the reader seeking a thorough review should consider the entire section.

Current concern over generic inequivalence among various drug products was discussed by several workers (1172–1175). Smith (1176) stressed an urgent need to pursue clinical and pharmacokinetic measurements of relative absorption efficiency of those drugs where consistent potency is essential. Official action to introduce better *in vitro* standards and to implement quality control that will monitor any batch-to-batch variation in bioavailability was also recommended. Wurster (1177) discussed the influence of drug particle size, polymorphism, tablet hardness, formulation composition, dissolution rate, and other factors upon the efficacy of drug delivery systems. Similar aspects of factors influencing drug bioavailability were reviewed in several other publications (1178–1182).

An *in vitro* diffusion model for the transport rates of solutes in a three-phase system was applied to theoretical and experimental exploration of the pH–partition theory of drug absorption. Experimental rates for a variety of conditions reasonably correlated with computer predictions obtained by independent evaluation of physicochemical parameters in the system (1183, 1184). A three-phase model cell, employing a liquid lipid barrier, was used to establish correlations between reported *in vivo* absorption data and experimentally determined *in vitro* transport rates for a series of *N*-substituted heterocyclic sulfanilamides. These correlations suggested utility of this model to simulate passive drug absorption (1185).

Physical models for the diffusional transport of drugs across membranes of cells in culture suspensions were introduced by Ho *et al.* (1186) to describe the nature of the principal transport barrier, the kind of drug species

being transported, solute binding, and the effect of pH and partition coefficients on the drug transport. Mathematical expressions were derived for the quasi-steady-state diffusion through the membrane followed by distribution of drug in the heterogeneous cell interior (1186). A subsequent study dealt with the development of some baseline procedures for cell culture–drug transport experiments, consisting of cholesterol uptake by Burkitt lymphoma cells, and the methods of quantitative mechanistic interpretation of the experimental results (1187). A physical model for the adsorption of drugs applicable to situations in which the diffusional flux of the drug may be influenced by the bulk fluid flow and surface pH was described. Theoretical computations were made for different cases in simulation of the *in situ* absorption of drugs in animals with varying tonicity of the drug solution (1188). Previously reported results on human buccal absorption were quantitatively analyzed by the physical model approach using a two-phase compartmental diffusion model. Theoretical estimations of the absorption rate–buffer pH profiles were in good agreement with experimental results (1189).

A two-phase series model for the permeability behavior of the fully hydrated stratum corneum was examined using previously reported data on steroids. In general, reasonable self-consistencies were found among the various experimental results and parameters of the model (1190). Penetration of drugs through skin and the dependence of penetration rate on various parameters subject to vehicle influence were reviewed by Poulsen (1191). A systematic approach in the design of a topical corticoid dosage form, which provides good physical stability, patient acceptability, and optimum drug absorption through the skin, was presented (1192). Drug absorption through the stratum corneum of humans and the effects of hydration, temperature, humidity, solvents, and racial variations in drug penetration were reviewed (1193). The properties of the stratum corneum as a reservoir for topically applied drugs in humans were discussed. Data showing the effects of temperature and humidity on the reservoir content of topically applied sodium fusidate and fusidic acid were presented (1194). Application of the attenuated total reflectance IR spectroscopy technique to study the uptake of dodecylbenzenesulfonate by human skin *in situ* was demonstrated. The reproducibility of the technique was better than 5% for the drug permeation systems that are not influenced by moisture and/or hydrogen bonding (1195). General test methods for measuring percutaneous absorption of drugs in rabbits and guinea pigs were described, along with a discussion of various factors that affect percutaneous absorption (1196).

Engineering control systems analysis and optimization techniques were described for the evaluation and control of the therapeutic performance of drugs, drug products, and interacting drug combinations; for the design of dosage forms with optimum drug release patterns; and for setting optimum dosage regimens for individual patients. The feasibility of the approach was demonstrated by computer simulation of an ideally sought level of therapeutic drug response intensity (1197). A detailed basis for the use of experimentally determined dose–effect curves to transform observed intensities of pharmacological response into biphasic drug levels at all times following dosing by any route was presented. These principles were graphically demonstrated through the construction of dose–response–time surfaces (1198). An electronic circuit, which simulates theoretical curves associated with multiple-dosing kinetics, was described. Plasma level–time curves can be generated by this circuit, assuming a one-compartment open-model system and rapid intravenous injection (1199). A digital computer program was developed to calculate safe and effective doses of kanamycin for individual patients with renal insufficiency. The validity of the program was demonstrated by its ability to predict serum concentrations of kanamycin measured by microbiological assay. The principles of the program were considered potentially applicable to many other drugs (1200). Computer estimations of dosage regimens for digitalis, kanamycin, gentamicin, and lidocaine were discussed in a review article (1201). Several other reviews considered the application of computers to simulate pharmacokinetic models and to determine various pharmacokinetic parameters (1202–1205). The use of dosage regimen calculations as a facet of drug evaluation that can be implemented by the pharmacist was exemplified by tetracycline and penicillin antibiotic dosage regimens (1206).

Additional references on biopharmaceutics are listed in Table XXXV.

Effects of Physicochemical Properties—Various physicochemical parameters including solubility, surface tension, partition coefficient, vapor pressure, activity parachor, R_f values, molecular orbital calculations, and Hammett and Taft constants were correlated with the biological activity of a number of sulfonamides (1241). Rational physicochemical approaches in drug design to modulate biological action were discussed for various types of drugs (1242, 1243). A comprehensive review by Hansch and Dunn (1244) dealt with the linear relationships between lipophilic character and biological activity of drugs, a linear free energy model for structure–activity relationships, and hydrophobically sensitive and insensitive free energy relationships (1244). Determination of hydrophobicity constants and their application to structure–activity correlations of systems such as penicillin serum binding, 2-haloalkylamine adrenergic blocking activity, and *m*-nitroaniline sweeteners were reviewed. Tables of hydrophobic, electronic, and steric constants for a number of biologically active compounds were presented (1245). An interactive searching program of a structure and biological activity file, coupled with a program that scans a file of herbicidal activity of compounds, was presented. These programs were considered useful in providing background knowledge for further testing and synthesis of active drugs (1246). Utilization of operational schemes for the stepwise approach in the design of a most potent drug analog was suggested. These schemes were based on the considerations of hydrophobic, electronic, and steric effects by a particular substituent upon biological activity of the molecule (1247).

The methylene group contribution to the thermodynamics of solutions of drug molecules was obtained

Table XXXV—Additional References on Biopharmaceutics

Reference	Topic
1207	Review describing role of biopharmaceutics in the design of drug products
1208	Review of drug administration, interaction with physiological milieu, absorption, and validity of animal and *in vitro* test models
1209	Review of physicochemical and pharmacokinetic bases for the biopharmaceutical evaluation of drug bioavailability from drug formulations
1210	Review of usefulness of biopharmaceutical concepts in formulation of drugs
1211	Review discussing requirements for drugs with sustained action based on biological considerations
1212	Use of confidence intervals in analysis of comparative bioavailability data
1213	Three-phase apparatus for *in vitro* drug absorption studies
1214	Review of membrane surface and drug actions
1215	Review describing membrane transport of molecules and ions, molecular diffusion, transport across fixed-charge membranes, active transport, *etc.*
1216	Review of bioavailability as influenced by dissolution rate, and enteral absorption as a function of physicochemical properties of drugs
1217	Skin dehydration and decreased emotional arousal considered as reasons for the slower and more consistent percutaneous absorption upon repeated tests
1218	General principles of percutaneous absorption and permeation of a bioflavonoid
1219	Review discussing action of local anesthetics on excitable membranes, structure-activity correlations, and penetration through mucous membranes and skin
1220	Review of structure-activity relations and molecular pharmacology of receptors
1221	Review of drug structure and kinetics of drug-receptor interactions
1222	Review of kinetics of drug action in man, and design of safe and effective dosage regimens
1223	Review of pharmacokinetics, chemical composition, and pharmacological activity of essential oils
1224	Processes involved in drug absorption, distribution, metabolism, and excretion and their relationship to onset, duration, and intensity of drug action
1225	Equation to estimate the fraction of drug remaining in the body after intravenous injection
1226	Application of salivary salicylate data to biopharmaceutical studies of salicylates
1227	Review of drug-protein interactions as they affect drug distribution, dosage schedule, and drug use in disease states
1228	Review of drug interactions in clinical medicine
1229	Review of drug interactions as they alter pharmacokinetic parameters of a drug
1230	Review describing drug interactions during intestinal absorption, protein binding, metabolism, and excretion
1231	Review of drug incompatibility due to chemical reaction, induction of degradative enzymes, interference with absorption process, *etc.*
1232	Comparative depressant activity and interaction of ethanol and pentobarbital in goldfish
1233	Review of interactions of alcohol with CNS and cardiovascular agents
1234	Carcinogenic nitrosamines formed by interaction of nitrite with drugs containing tertiary amino groups
1235	Bioaggregational aspects of erythrocytes evaluated in terms of coagulation-flocculation theory
1236	Inhibition of platelet aggregation by bisulfite-sulfite
1237	Electrokinetic properties of bacteria studied as a function of ionic strength and pH
1238	Effect of aspirin and ethanol on the gastric mucosa of the rat
1239	Surface area term in Myhill-Piper equation for antacids
1240	Adsorption of pepsin by commercially available antacids measured *in vitro* as a function of pH

from activity coefficient, Henry's constant, and partition data. The free energy of transfer of the methylene group from water to nonpolar organic solvent was estimated as -850 cal. mole^{-1}, but for polar solvents values ranged from -712 to -452 cal. mole^{-1}. Application of these thermodynamic concepts to structure-activity relations was discussed (1248). Adoption of a thermodynamic standard state for comparing drug molecules in structure-activity studies was suggested. This standard state, acting as if it were infinitely dilute where the solvent is a suitable aliphatic hydrocarbon such as cyclohexane or isooctane, could be a 1 molal, 1 molar, or 1 mole-fraction solution (1249).

The two most frequently used regression models for structure-activity analyses, the additive and the linear multiple regression models, were shown to be fundamentally interrelated. Each approximation used in reducing the additive model to its linear multiple regression counterpart provided useful insight into how these regression methods can be best applied (1250). Statistical significance of structure-activity correlations using multiple regression analysis and the potential problems in obtaining chance correlations when many variable parameters are involved were examined. The data were presented to assess the probable degree of chance correlation as a function of the number of observations and the number of variables (1251). Suitability of the calculated electron population parameters for multiple regression analysis of biological activity was determined. It was concluded that both the CNDO/2 and *ab initio* methods of Pople *et al.*, but not extended Hückel theory, may be suitable for use in the multiple regression analysis (1252).

Quantitative relationships between physical properties and antibacterial activity of erythromycin esters were evaluated by the Free-Wilson and extrathermodynamic techniques. Both analyses indicated that esterification decreases activity; this decrease increases with increasing chain length of the ester. The extrathermodynamic equations suggested that the partition coefficient is an important determinant of activity (1253). Antibacterial activity of rifamycins was shown to correlate with the chromatographic substituent constant R_m (1254). The structure-activity relations of penicillins were discussed in several reviews (1255–1257). Analgesic activity of *p*-substituted acetanilides showed better correlation with the chromatographic substituent constant R_m than with the Hansch hydrophobic substituent constant π (1258). The structure-activity relationships in the anesthetic action of aliphatic ethers were found to be parabolic functions of their octanol-water partition coefficients. An optimum lipophilic character (log P_0) of about 2 was determined for general anesthetics, which is comparable to that obtained for barbiturates, ureas, alcohols, *etc.* (1259).

Correlations between steric, electronic, and hydrophobic parameters and biological activity were estab-

lished for 2-phenylquinoline-4-carbinol antimalarials (1260), 1,3-dioxolane cholinergics (1261), tricyclic neuroleptics and antidepressants (1262), anti-inflammatory 3-(5-aryl-2-tetrazolyl)alkanoic acid derivatives (1263), hemolytic activity of polyene antibiotics (1264), decahydroisoquinolines possessing antiarrhythmic activity (1265), veratrum alkaloids (1266), and a series of 2*H*-1,2,4-benzothiadiazine 1,1-dioxide antihypertensive compounds (1267, 1268). The steric and electronic parameters of styrylpyridine analogs were correlated with their choline acetyltransferase inhibitory activity in an effort to elucidate the nature of interactions of this type of inhibitor with the enzyme. Hydrophobic and electron-donor contributions by the aryl moiety and electron-acceptor characteristics of the pyridinium moiety were believed to be involved in the inhibitor–enzyme binding (1269). A similar approach to structure–activity correlation was applied to study interactions of acetylcholinesterase with substituted phenylammonium iodide inhibitors (1270). The parts of the inhibitor molecule that contribute to the hydrophobic bonding with the enzyme might only be the parts that are in contact with the enzyme. Such a directional nature of hydrophobic bonding was demonstrated in the case of phenylethanolamine *N*-methyltransferase inhibitors (1271). The lipophilicity of some hexamethonium analogs was compared with their ganglionic blocking activity. The compounds more lipophilic than hexamethonium were less active than hexamethonium, but the lipophilic character of a highly active analog was comparable to that of hexamethonium (1272, 1273).

Theoretical prediction of the influence of complex formation on the transport rate of a drug and computation methods were described by Vaidhyanathan (1274, 1275). It was shown that complexation of a drug with a substance of lower diffusion coefficient leads to enhancement of the transport rate of the drug; complexation with substances having similar diffusion coefficients, however, results in reduction of the transport rate (1274). Fluxes of drugs across membranes affected by the presence of a complexing agent in the membrane were represented by a generalized form of Fick's law, which accounts for the existence of coupling between fluxes of different species (1275). A series of publications by Hayton and coworkers (1276–1281) dealt with the subject of drug complexation and its effect on drug absorption. The effects of certain *N,N*-dialkyl propionamides on the permeability of the rat small intestine and of a synthetic lipoid barrier to prednisone and prednisolone were investigated. Enhancement in the intestinal absorption of these drugs by *N,N*-dibutyl- and *N,N*-dipropylpropionamide appeared to be due to the formation of a steroid–amide complex in the intestinal barriers. However, the intestinal absorption of several nonsteroid drugs which complex with the amides was not significantly affected by these amides. This indicated that the absorption-enhancing effect of the amides was relatively specific to a drug. Some structural features of the steroid molecule required for the enhancement of drug absorption by its complexation with an amide were considered (1276–1281). The transfer process of various acidic drugs from water into chloroform containing phospholipids was examined using a nonemulsifying system. The ionized forms of the drugs with little lipid solubility showed a marked increase in transfer to the chloroform phase containing lecithin at the physiological pH of the small intestines. These results indicated the possible role of phospholipids in the intestinal absorption of ionized acidic drugs (1282).

Relationships between surface activity and biological activity of drugs were reviewed for local anesthetics, sedatives and hypnotics, antibacterial agents, hormones, fat-soluble vitamins, and miscellaneous drugs (1283). Iso-branched fatty acids, with fungistatic and bacteriostatic properties, were found to enhance the effect of conventional antimicrobial agents acting inside the cell membrane. A molecular mechanism for this effect was proposed on the basis of surface film studies (1284). Molecular interactions between quaternary ammonium surfactant bactericides and bacterial cell wall constituents were studied by means of surface film balance and conductometric methods. The results suggested that in Gram-positive organisms, the bactericide first becomes associated with the protein in the cell wall; subsequent penetration leads to disruption of the cell membrane. In Gram-negative organisms, the phospholipid present in the cell wall affords the organism a degree of protection from the bactericide (1285, 1286).

Additional studies on the effects of physicochemical properties are listed in Table XXXVI.

Effects of Formulation—Two crystal forms of the drug sulfameter were orally administered to man. The absorption rate was about 1.4 times greater with the more energetic metastable crystal form than with the stable crystal form; the results were discussed in relation to the free energy differences between the two crystal forms and their *in vitro* dissolution rates (1316). The rate and extent of reserpine absorption in rats were significantly enhanced upon oral administration of reserpine–polyvinylpyrrolidone coprecipitates. The fraction of the oral dose absorbed was 2–3 times greater with the coprecipitate than with either the pure drug or physical mixtures of drug with polyvinylpyrrolidone. The *in vivo* absorption data were consistent with the *in vitro* dissolution characteristics of the tested drug forms (1317, 1318). Crystalline and amorphous preparation of asparaginase were found comparable with regard to toxicity and capacity to induce remission of acute leukemia in children (1319).

The absorption of orally administered riboflavin-5′-phosphate by healthy male subjects was increased significantly when it was given in 50 ml. of 2% sodium alginate solution rather than in the aqueous vehicle without sodium alginate. Retention of the vitamin at specialized absorption sites for prolonged time periods because of the slow movement of the highly viscous sodium alginate solution was proposed as the rationale for the absorption data (1320). Oral administration of sodium *p*-aminosalicylate with methylcellulose and gum arabic to rats produced higher but delayed absorption, probably due to a viscosity effect; administration with surface-active agents in concentrations below the CMC considerably increased the drug absorption (1321).

Table XXXVI—Additional References on Effects of Physicochemical Properties

Reference	Topic
1287	Review of the Free-Wilson method and Hansch multiple regression analysis method for structure-activity correlations
1288	Review of quantitative structure-activity correlations by using Hammett and Taft constants and partition coefficient parameters
1289	Review of various factors that may affect the interpretation of drug structure-activity relations
1290	Role of partitioning phenomena in drug absorption distribution; review of response and elimination
1291	Review of physicochemical and biological factors influencing concentration of drugs at body sites
1292	Structure-activity relationship in the taste effect of ribonucleotide derivatives
1293	Review of molecular orbital approach to quantitative drug design illustrated by benzothiadiazines
1294	Review of application of molecular orbital theory to drug-receptor mapping
1295	Influence of electron-donating properties on the psychotropic activity of phenothiazine derivatives
1296	Conformational and structural relationships among antipeptic ulcer compounds
1297	Review of physicochemical properties of dextran solutions and their pharmacological effects
1298	Comparative physicochemical studies of plasma substitutes
1299	Review of modulation of drug pharmacokinetics by alterations in biofunctional moieties
1300	Review of relation between partition coefficients and drug action
1301	pK values and their significance in enteral resorption
1302	Physicochemical properties of polyene macrolide esters and salts of amphotericin and their biological activity
1303	Review of structure-activity relationships for digitalis glycosides
1304	Review of structure-activity relationships between acetylcholine and quaternary ammonium neuromuscular blocking agents
1305	Review of correlation between lipid solubility and biological action of homologous local anesthetics
1306	Structure-activity correlation for substrates of phenylethanolamine *N*-methyltransferase
1307	Microbiological determination of drug partitioning and protein binding in complex coacervate model systems
1308	Relationship between antibacterial activities in rabbit serum and protein binding of mitomycin derivatives
1309	Influence of protein binding of chlorpromazine upon pharmacokinetic parameters in man, dog, and rats
1310	Inhibition of folate synthesis by sulfonamides correlated with their antibacterial activity and physicochemical parameters
1311	Relation between biological activity and the degree of resolution of optical isomers of phenylcyclohexylglycolloylcholine and benzhexol
1312	Association constants of various amphetamine molecular complexes correlated linearly with the threshold hallucinogenic dose in humans
1313	Influence of organic phase composition on distribution coefficient of weak polar bases
1314	Review of structure-activity relationships between drugs and bioreceptors in terms of three-dimensionally defined molecular entities
1315	Correlations between kinetics of drug-receptor interaction and chemical structure of cholinomimetic agents

Partition coefficients of ionized and unionized salicylic acid molecules between water and the micelles of several nonionic surfactants were determined. The results were discussed in terms of their significance to the process of drug absorption from surfactant solutions (1322). The influence of macromolecular nonionic agents such as polyethylene glycol, methylcellulose, and polyvinyl alcohol on the enzymatic activity of pepsin, amylase, invertase, and lipase was investigated. Most of the high viscosity polymers tested decreased to a varying degree the activity of these enzymes (1323–1325). Samples of dicumarol (bishydroxycoumarin), equilibrated with various tablet excipients, were administered to dogs to determine the excipient effects on the drug absorption. Administration of the drug with talc, colloidal magnesium aluminum silicate (Veegum), aluminum hydroxide, or starch resulted in significantly lower plasma levels of the drug; but with magnesium oxide and hydroxide, plasma levels of the drug were higher than for the drug administered alone. In further work, *in vitro* dissolution characteristics of three commercial brands of dicumarol (bishydroxycoumarin) tablets were shown to correlate positively with the *in vivo* plasma levels of the drug. As a second phase of the study, application of diffuse reflectance spectroscopy in evaluating physical stability of simple emulsion bases and chemical stability of various drugs within an emulsion was demonstrated (1326).

Blood salicylate levels after administration of aspirin preparations of varying particle sizes to rabbits indicated that the absorption rate was inversely proportional to the particle size; however, the absorption rates of aspirin particles coated with 10% precirol were approximately the same regardless of particle size (1327). Four buffered aspirin tablets were tested for their *in vitro* dissolution rate, buffer capacity, absorption rate, and induced GI bleeding in normal human subjects. An inverse rank-order correlation was found between buffer capacity and GI bleeding (1328). In a comparative study with four commercial enteric-coated aspirin tablets, disintegration times in pH 6 buffer solution correlated with the drug absorption rates in humans (1329). Significant differences were observed in the absorption characteristics of three commercial tetracycline preparations. These differences were observed after both single-dose and multiple-dose administration (1330). The *in vitro* dissolution rates of tablet and capsule dosage forms of diazoxide in 0.1 *N* hydrochloric acid and of griseofulvin in simulated intestinal fluid were shown to correlate linearly with the blood levels of the respective drugs (1331). Tablet, capsule, and suspension formulations containing trimethoprim and sulfamethoxazole were considered bioequivalent in humans on the basis of blood level data (1332).

Timed-release tablet formulations of sulfamethizole consisting of a lipase–lipid system as the release-controlling vehicle were studied for their *in vitro* release rates and *in vivo* absorption patterns in dogs. Uniform blood levels over 12 hr. were observed following oral administration of the tablets. Lipolytic digestion of the lipid was considered as the primary factor in the timed-

release rates of the drug (1333). Three slow-release preparations of isoniazid were studied in man for their potential use as once-a-week dosage forms in the treatment of tuberculosis; a matrix-type formulation was considered therapeutically effective (1334). The cancer chemotherapeutic agents sodium methotrexate, vincristine sulfate, and cytosine arabinoside provided timed-release activity in mice when administered in multiple-emulsion injectable formulations (1335). A timed-release multiple emulsion was used to extend the radioprotective action of cysteamine in mice (1336). Determination of blood phenobarbital levels in minipigs or dogs, during long-term administration of the drug in conventional or three timed-release dosage forms, failed to show any advantage with the timed-release formulations (1337).

Pharmaceutical aspects of corticosteroids in dermatological preparations, such as uptake of steroids from ointments by the skin, physical and chemical modification of the steroids during manufacture, and modification of the carrier, were reviewed (1338). Selection of a particular base vehicle for topical formulation of each individual drug to obtain optimum therapeutic activity was demonstrated by comparing activities of fluocinonide and fluocinolone acetonide in the same vehicle with intrinsic activities of the drugs. Fluocinolone acetonide was readily released from the base and exhibited significant clinical effectiveness; however, fluocinonide activity was considerably reduced because of poor drug release from the base (1339). Ointment preparations of betamethasone 17-benzoate in white soft paraffin and propylene glycol or in white paraffin and isopropyl myristate showed higher vasoconstrictor activity in humans than five other ointments tested, including water-miscible ointments based on polyethylene glycols (macrogols) (1340).

The rate of water desorption of untreated and surfactant-treated depilated rabbit skin was determined to supplement previous data of surfactant-induced changes in biological membranes. The water desorption rate was always greater with the surfactant-treated than with the control skin. The results confirmed earlier findings that the tested nonionic surfactants affect membrane structure, thereby increasing skin permeability (1341). Various sunscreening, deodorant, antiperspirant, and insect repellent preparations were studied to determine the effect of formulation variables on the skin retention of the medicament and efficacy of the formulation. The retention of the drug on human skin was correlated with the solubility and partition coefficient of the drug (1342). The delayed rate of onset of erythema on the human skin induced by methyl nicotinate in aqueous glycerol solutions appeared to be related to the decreased partition coefficient and diffusivity of the drug in these solutions (1343). A correlation was established between the diffusion of ditetracycline from ointment bases of various chemical nature through agar and its permeation through rabbit conjunctiva (1344). Relative rates of percutaneous absorption from hydrophilic and lipophilic ointment bases were evaluated for 4-bromophenyl isothiocyanate-^{35}S (1345) and salts of 8-hydroxyquinoline and procaine (1346). The anti-inflammatory effect of ichthammol (Ichthyol) ointments was shown to correlate with the dielectric constant of the preparation (1347).

Properties of ophthalmic formulations, such as pH, isotonicity, hydrophilic and lipophilic bases, and medicament, that affect absorption through the cornea were discussed by Silverman (1348). The adjuvant effect on intramuscular drug absorption was investigated by *in vivo* absorption studies in rats and *in vitro* diffusion model experiments. The absorption rate correlated with the viscosity of the injectable preparation for adjuvants having relatively small molecular size such as propylene glycol, glycerin, and polyethylene glycol 400. From *in vitro* diffusion studies, it was concluded that the contribution of the diffusion process through the pores of the capillary wall was dominant compared with diffusion through muscle fiber space (1349). The degree of tissue lesion in rat quadriceps femoris muscles following intramuscular injections of aqueous formaldehyde was studied as a function of duration, concentration, solvent composition, and volume of the injected solution. The extent of tissue lesion was proportional to the concentration and postinjection time, and it was twofold larger with ethanol than with glycerol formaldehyde (1350, 1351). A method for clinically evaluating pain of injection for parenteral preparations was described (1352).

Additional references on the effects of formulation are listed in Table XXXVII.

Absorption Control and Alteration—This section of biopharmaceutics is comprised of studies related to control and alteration of drug absorption by coadministration of drugs or a drug with other chemical agents, disease, blood flow, food, route of administration, and age.

Parallel *in vitro* and *in vivo* determinations of adsorption characteristics of activated charcoal were carried out with drugs having different pKa's: aspirin, salicylamide, and phenylpropanolamine. Adsorption and desorption characteristics of these drugs, evaluated by *in vitro* tests, were predictive of the effect of dose and mode of charcoal administration on its antidotal effectiveness in humans (1387, 1388). Prompt administration of a sufficient quantity of activated charcoal had a significant inhibitory effect on the absorption of aspirin from solution, conventional tablets, enteric-coated tablets, and timed-release tablets. Aspirin absorption can be reduced even if activated charcoal is administered 3 hours after aspirin ingestion, provided that the drug is still in the GI tract at that time (1389). Drug adsorption efficacy of commercial activated charcoal tablets and activated charcoal powder was compared by *in vitro* tests and in man, using phenylpropanolamine as the test drug. The rate and extent of drug adsorption by charcoal tablets *in vitro* were much lower than by the powder. Equal doses of charcoal tablets and powder reduced phenylpropanolamine absorption in man by 48 and 73%, respectively (1390). Similar inhibitory effects of activated charcoal were also observed for the absorption of sulfamethazine (sulfadimidine) and sulfaguanidine (1391). The absorption of diazepam in man from a suspension formulation containing colloidal magnesium aluminum silicate (Veegum) was slower than from a solution preparation

Table XXXVII—Additional References on Effects of Formulation

Reference	Topic
1353	Review of factors influencing physiological availability of drugs from tablets
1354	Comparative bioavailability study for three commercial brands of oxytetracycline hydrochloride
1355	Variation in bioavailability of digoxin tablets attributed to tablet disintegration
1356	Review emphasizing importance of chemical and physical formulation factors in drug absorption process
1357	Review discussing influence of formulation on drug action
1358	Comparison of *in vitro* dissolution rates of phenobarbital tablets in relation to possible effects on drug absorption
1359	Comparison of capsule, tablet, and intravenous infusion of quinine in an open, multidose crossover study; toxicity effects
1360	Effects of antacids on oral absorption of sulfadiazine and quinidine
1361	Review discussing effect of other drugs and surfactants on the activity of local anesthetics
1362	Use of hydantoic acids, hydantoic acid esters, and *N*-acyl phthalimides as water-soluble prodrugs
1363	Increased resorption of anesthetics and antibiotics by addition of hyaluronidase in suppositories and ointments; effect of trypsin and chymotrypsin on the resorption of penicillin and tetracycline
1364	Lack of correlation between physiological availability of commercial triple sulfa preparations and *in vitro* dissolution rates
1365	Digestive action of enzyme substitutes shown to be dependent on the composition, method of preparation, and proper enzyme release rates
1366	Variation in bioavailability among seven commercial brands of phenylbutazone tablets sold in Finland
1367	Plasma concentrations of proxyphylline following repeated administration of timed-release tablets similar to conventional tablets after 4th day of administration
1368	Selective review of the methods and principles used to assess effects of excipients on drug absorption
1369	Effectiveness of rifamycin derivatives as inhibitors of polymerase activity reduced in presence of nonionic surfactants
1370	Combined effects of surfactants with germicidal compounds; activity of butylparaben reduced in presence of nonionic surfactants
1371	Comparison of four vehicles for intraperitoneal administration of Δ^1-tetrahydrocannabinol
1372	Clindamycin palmitate flavored granules with rapid absorption and excretion and good tolerance to multiple dosing in children
1373	Clindamycin palmitate flavored granules equally absorbed before or after meals
1374	Delayed onset of action obtained upon intraperitoneal or subcutaneous administration of barbituric acids in emulsion formulation
1375	Anti-inflammatory action of phenylbutazone in a polyethylene glycol ointment base enhanced by the presence of a chymotrypsin complex
1376	Evaluation of antiseptic effect of five topical anesthetic ointments
1377	Percutaneous absorption of estradiol benzoate from polyethylene glycol vehicles
1378	Comparative evaluation of percutaneous absorption of drug through rabbit, dog, and guinea pig skin to develop an animal model for dermatological research
1379	Effect of vaselinum flavum and different batches of cera liquida ointment bases on epidermal width and mitotic activity
1380	Rate of absorption of salicylic acid through animal skin faster from a nonaqueous ointment base than from a hydrophilic base
1381	Sodium lauryl sulfate enhancement of the rate of penetration of glycol salicylate administered cutaneously and rectally
1382	Effects of excipients and surfactants on rectal absorption of erythromycin
1383	Formulation factors influencing rectal absorption of *N*-pyrrolidinomethyltetracycline guaiacolsulfonate
1384	Polysorbate 80 in concentrations of less than 10% considered suitable for intravaginal formulations
1385	Liposomes as carriers of enzymes or drugs—an approach to the treatment of storage diseases
1386	Review of factors influencing release and absorption of drugs from ointment bases

without it. This was attributed to the desorption of diazepam from the emulsifier as a rate-limiting factor in the drug absorption process (1392).

Cholestyramine, an anion-exchange resin with hypocholesterolemic activity, was shown to interact strongly and rapidly with the antibiotic sodium fusidate. As suggested by the *in vitro* results, concurrent oral administration of the resin and sodium fusidate to rats yielded statistically significant reductions in serum antibiotic levels at all experimental time intervals. *In vitro* adsorption studies also demonstrated that physiological bile salt and fatty acid anions effectively compete with fusidate anions for the available binding positions on the resin (1393, 1394). Inhibition of iron and vitamin B_{12} absorption by cholestyramine was considered the reason for the observed iron and vitamin B_{12} deficiency in rats following prolonged administration of the resin (1395). Various factors influencing the absorption of vitamin B_{12} (*e.g.*, food, charcoal, and pentagastrin) were studied in healthy subjects and in patients with pernicious anemia. The drug absorption in patients was decreased by simultaneous administration of charcoal (1396). The oral absorption of ^{57}Co-labeled vitamin B_{12} at low doses in cats was enhanced by coadministration of dog or human pancreatic juice (1397). Effects of sugars, polyalcohols, and organic acids on hydrolytic polymerization of iron were examined. The results suggested that these compounds enhance the absorption of iron across the intestine by formation of low molecular polymers of iron (1398).

The effect of tetracyclines on the intestinal absorption of sulfonamides was investigated by the recirculation and loop methods in the rat. At pH 6 and 8, demeclocycline (demethylchlortetracycline) and methacycline (methyleneoxytetracycline) enhanced the absorption of sulfaguanidine, while tetracycline enhanced the sulfa drug absorption only at pH 8 (1399). In a subsequent study, the amount of intravenously administered sulfa drug exsorbed into the intestine was examined with the intestine either perfused with tetracycline solution or isotonic buffer. The effect of tetracycline on the absorption and exsorption of sulfa drugs was attributed to changes in the small intestinal tissue by tetracycline (1400). Specific inhibition of cholesterol absorption in rats and mice was shown to occur with sulfaguanidine but not with other structural analogs of the sulfa drug (1401). The influence of polybasic antibiotics and sulfaguanidine on the absorption and excretion of

cholesterol and bile salts was discussed in a review article (1402). Heparinic acid and its acid salts were readily absorbed from the intestine to provide systemic anticoagulant activity in rabbits, monkeys, and dogs. Increased absorbability, however, was accompanied by a decrease in stability. Weak nitrogenous bases such as nicotinamide were complexed with heparinic acid to give compounds with high absorbability coupled with good stability (1403). The absorption of streptomycin from uterine and salpingeal mucosa was significantly greater when the drug was applied with hyaluronidase; the absorption was further enhanced when galvanic current was introduced during intrauterine electrophoresis (1404).

Oral administration of the anticholinergic drug propantheline bromide to healthy adult males 30–60 min. before an oral dose of riboflavin phosphate delayed the absorption of the vitamin but caused a pronounced increase in total amount absorbed. This was attributed to delayed gastric emptying and slowed transit of riboflavin solution through the small intestinal lumen due to the anticholinergic effect of propantheline (1405). However, oral administration of aminopyrine with barbital produced significantly higher initial plasma levels of aminopyrine, which were attributed to the increased rate of gastric emptying induced by barbital (1406). Magnesium hydroxide and aluminum hydroxide given by gastric intubation to the rat prior to, or simultaneously with, sodium pentobarbital decreased GI absorption of pentobarbital and delayed the onset of sleep. Magnesium hydroxide, by raising the gastric pH, allowed the drug to remain in the ionized unabsorbable form while aluminum hydroxide acted by delaying gastric emptying time. Although these antacids decreased the absorption rate of pentobarbital, the total amount absorbed remained unaltered (1407). Enhanced absorption of pseudoephedrine by concurrent administration of aluminum hydroxide gel was ascribed to the increase in GI pH caused by the antacid (1408). Intestinal absorption of tritiated folic acid in human subjects with a triple lumen tube positioned in the proximal jejunum was decreased by about 44% after perfusion with diphenylhydantoin. This inhibition of folic acid absorption was considered as a likely reason for the folate deficiency that occurs with diphenylhydantoin therapy (1409).

Rapid penetration of gentamicin in the aqueous humor of rabbit eyes with corneal ulcers occurred when the antibiotic was administered by subconjunctival injection, topical drops, or continuous lavage. Concomitant serum levels of gentamicin were lowest when topical drops were used (1410). In a review paper, calculation of multiple-dosage schedules for maintenance of desired drug levels in anuric patients and elimination rate constants of several drugs in such patients were presented (1411). Acetaminophen (paracetamol), taken orally after overnight fasting, was absorbed up to five times faster but to the same extent as when it was taken after a high carbohydrate breakfast. Subjects taking the drug immediately before sleep excreted up to 36% less than after taking it in the morning (1412). The presence of food in the intestine decreased the *in vivo* uptake rate of anisotropine methylbromide in the rat but had no effect on the total uptake. The sum of drug bound to intestinal tissue and that taken into the circulation was 48% of the dose administered (1413). Absorption of ampicillin and nalidixic acid by infants and children with acute shigellosis was determined. Five of the 16 patients receiving nalidixic acid had a pronounced delay in absorption, while 10 of 21 ampicillin-treated patients showed depressed plasma levels and prolonged half-lives (1414). A mathematical model was described to explain the apparent difference in the rate of increase of alveolar halothane concentration between children and adults. Concentration of the anesthetic used, cardiac output, and metabolic rate were considered as reasons for the observed difference (1415). Since intestinal absorption of tritiated 25-hydroxycholecalciferol was about the same in both normal children and those with osteopathies, it was concluded that the resistance of osteopathies to the drug therapy is not because of malabsorption of the drug (1416).

Oral doses required for a drug are sometimes larger than are needed by the parenteral route of administration. This might be the case when a large fraction of the oral dose is eliminated on first passage through the liver. An equation derived based on these considerations provided an estimation of the availability of a completely absorbed, orally administered drug. The importance of clearance concepts in bioavailability studies, drug design, and enzyme induction studies was discussed (1417). The antagonism of isoproterenol-induced tachycardia by orally and intravenously administered propranolol was measured in five subjects and related to the plasma propranolol concentration. The degree of β-blockade at a given plasma drug concentration observed 2 hr. after oral administration of a single dose of propranolol was greater than with intravenous administration. This was explained by the formation of an active metabolite, 4-hydroxypropranolol, only after a single oral dose; this metabolite has a shorter half-life than the parent drug (1418).

Polyinosinic acid–polycytidylic acid was given to rabbits by intravenous, intraocular, and topical ocular routes. A pyrogenic response was achieved by all three routes. The minimum pyrogenic doses were 0.1, 1.0, and 1000 mcg./kg. for intravenous, intraocular, and topical ocular administrations, respectively. Ocular responses of the drug paralleled the pyrogenic responses (1419, 1420). Streptomycin concentration in lung tissue and bronchii of tuberculosis patients was three to four times higher for 12 hr. after intratracheal–bronchial administration than after intramuscular or intravenous injection. Clinical effectiveness of the drug administered by the intratracheal route was increased by simultaneous general antibacterial therapy (1421). Inhalation by asthma patients of beclomethasone dipropionate from a pressurized aerosol exerted a significant topical action on the airways, with the expected clinical response (1422). A more lasting and effective clearing of airways in asthmatic patients was obtained by administering rimiterol in aerosol form in frequent small inhalations rather than in a single larger dose (1423).

Additional references on absorption control and alteration are listed in Table XXXVIII.

Table XXXVIII—Additional References on Absorption Control and Alteration

Reference	Topic
1424	Administration of two or more related drugs to study the effect of molecular modification and formulation on drug absorption, metabolism, and excretion
1425	Influence of vitamin D on intestinal calcium transport in normal rats
1426	Effect of phenobarbital on intestinal calcium transport in rats
1427	Intestinal absorption of the radiographic contrast agent iopanoic acid accelerated with increase in GI pH
1428	Ocular absorption of catecholamines altered by ionic composition of the vehicle influencing corneal epithelium permeability
1429	Effect of chemical form of orally administered zinc on absorption and metabolism in cattle
1430	Aluminum complex of dextran sulfate better absorbed from small intestine than sodium salt of the polymer
1431	Drug therapy dependence upon the route of administration, plasma concentration, rate of elimination, and other body conditions
1432	Blood levels of morphocycline and neomycin lowered in dogs by concurrent intravenous administration of heparin
1433	Dioctyl sodium sulfosuccinate acceleration of peritoneal dialysis of urea and phosphate but not of creatinine in rabbits
1434	Effects of ε-aminocaproic acid on radiation-induced increase in capillary permeability of the skin
1435	Effects of nonionic surfactants that modify experimental tuberculosis on lipase activity of macrophages; correlation with surfactant chain length
1436	Salicylic acid absorption and excretion rates after oral administration of salicylic acid–choleic acid inclusion compound in suspension form
1437	Longer duration of drug action for catecholamines covalently bound to porous glass particles
1438	Changes in aspirin potency in the presence of protein-bound dye
1439	Review of analgesic synergism of aspirin and binding sites of salicylates on plasma proteins
1440	Effects of riboflavin on boric acid toxicity; riboflavin depletion induced by borate
1441	Comparison of the chemical stability and oral absorption in rabbits of ascorbic acid complexes with organic bases
1442	Conjugates of nitrogen mustards with proteins, polypeptidyl proteins, and polypeptides as cytotoxic agents with higher therapeutic indexes
1443	Nitrogen mustard–serum protein complexes with higher antitumor activity than the drug alone
1444	Slower absorption of benzoic acid and hydroxybenzoic acids through *in situ* rat gut from solutions containing polyvinylpyrrolidone
1445	Review of local and systemic factors controlling human sebaceous activity
1446	Normal absorption of vitamin B_{12} in chronic pancreatitis patients in nonfasting state
1447	Enteral absorption of drugs in rats of different ages
1448	Role of modes and sites of administration of cortisol and cortisone upon drug metabolism
1449	Effects of high altitude exposure on the rate of ingesta flow in the GI tract of the rat
1450	GI calcium absorption in nephrolithiasis
1451	Inhibition of glucose absorption by prostaglandins E_1, E_2, and $F_{2\alpha}$
1452	Effect of dicumarol (bishydroxycoumarin) on intestinal absorption of D-mannose and D-xylose
1453	Analysis of the inhibitory effect of biguanides on glucose absorption
1454	Effects of tolbutamide and glyburide on intestinal glucose absorption
1455	Site of intestinal absorption of sodium taurocholate and its consequence on drug absorption in the rat
1456	Chloramphenicol plasma levels in the dog after multiple oral and intramuscular administration
1457	Chloramphenicol plasma levels in the dog after oral, subcutaneous, and intramuscular administration
1458	Comparison of intradermal and subcutaneous routes of cholera vaccine administration
1459	Significantly slower absorption of ephedrine analogs after percutaneous than after oral administration
1460	Review of relations between the mode of administration and the pharmacodynamics of a drug and its plasma levels
1461	Decreased plasma levels of dicumarol (bishydroxycoumarin) after hypnotic doses of phenobarbital, glutethimide, or methaqualone administered orally to rats

Absorption Mechanisms—Mucosal-to-serosal flux of a number of solutes across the rat everted small intestine was determined as a function of time. The transfer rates of certain solutes progressively increased during the experiment, which was attributed partially to a loss of functional integrity of the intestine with respect to passive transfer (1462). Histological changes in the rat small intestine induced by edetate (EDTA), tetracycline, and sodium lauryl sulfate were examined using light and scanning electron microscopes. The observations provided further insight into the possible mechanism for the facilitative absorption of poorly absorbed drugs by these agents (1463). Concurrent absorption of water from the *in situ* rat small intestine during drug absorption experiments indicated that approximately 4 ml. of water was lost from the intestine during 60 min. The importance of considering the rate of water absorption to estimate accurately a drug absorption rate constant was emphasized (1464). The influence of net water transfer on drug absorption in an *in situ* rat intestinal preparation was studied using hypotonic, hypertonic, and isotonic intestinal lumenal solutions. The results indicated that the intestinal water loss or gain which occurred with the different tonicity solutions did alter the apparent rate constant for sulfaethidole absorption. When the apparent rate constants were corrected for the liquid volume and relative available surface area, permeability constants of sulfaethidole were comparable for the isotonic and hypotonic solutions, but a reduced permeability constant was obtained with the hypertonic solution (1465). Intestinal absorption of drug was positively correlated with the blood flow by the determination of absorption rates from perfused rat jejunal loops at different levels of blood flow. A mathematical model for intestinal drug absorption was discussed (1466).

The principal mechanisms of drug transport through the skin, the respiratory tract, and the GI tract were discussed. Two kinds of absorption regions were described: (*a*) the lipid protein matrix for the penetration of lipophilic substances, and (*b*) intercellular pores for hydrophilic substances (1467). Permeation of ions through *Necturus* gallbladder epithelium was shown to occur mainly through the intercellular junctions, thus bypassing the epithelial cells (1468). The mechanism by which surfactants affect drug absorption was studied by

measuring the rate of permeation of chloramphenicol through model membranes in the presence of surfactants. The rate was related to the extent of drug binding to the bovine serum albumin transport barrier and was controlled by competitive binding of the surfactant molecules (1469). The diffusion of hydrocortisone across a cellulose acetate membrane and the effects of *n*-alkyl polyethylene surfactants on the drug transport were explained by the thermodynamics of the test system (1470). A protein of about 12,000 molecular weight, which binds long-chain fatty acids and certain other lipids, was identified in cytosol of intestinal mucosa, liver, myocardium, and other tissues. The presence of this protein was regarded as a possible reason for the previously observed differences in intestinal absorption among fatty acids (1471).

The absorption of quinine and chlorpheniramine through the rat rectum, small intestine, and stomach was enhanced in the presence of anionic agents. Although enhancement of absorption was related to partition behavior, organic solvent, and surface activity of ion-pair complexes, these properties alone failed to provide a complete explanation for the observed effect (1472). The intestinal absorption of two poorly absorbed quaternary ammonium drugs, hexamethonium chloride and pralidoxime chloride, in the presence of various organic and inorganic anions was investigated using a modified *in situ* gut technique. Enhanced absorption of these drugs by cholate and phoscholate ions was attributed to the effect of these anions on the structural integrity of the membrane tissue. A kinetic model was suggested for the possible mechanism of absorption of quaternary ammonium drugs (1473). Kakemi and coworkers (1474–1476) investigated the intestinal absorption of sulfapyridine, salicylamide, acetanilide, and methyl orange from oil solutions and oil-in-water emulsions in rats, using *in situ* recirculation and loop techniques. In the absorption of drugs having partition coefficients greater than one, the amount of drug in the aqueous phase of the emulsion was the critical factor in the absorption process. Two possible mechanisms for methyl orange transport were proposed: passive transport and an active-like process involving enzymatic reduction of methyl orange on the mucosal side of the membrane (1474–1476). Major pathways involved in the GI absorption of sodium nitrite and its concentration in the mouse stomach 10 min. after oral administration were determined. The major pathway for the nitrite absorption was directly from the stomach into the bloodstream (1477).

In a comparative study, percutaneous absorption of haloprogin, *N*-acetylcysteine, cortisone, testosterone, caffeine, and butter yellow was shown to occur at a decreasing rate in the order: rabbit, rat, miniature swine, and man, with the swine having the closest permeability characteristics to human skin (1478). Absorption profiles of intramuscularly administered cationic drugs, protonated at physiological pH, were studied using the rat thigh muscle clearance method and *in vitro* uptake experiments. The storage or affinity of these drugs for the muscle tissue was proposed as one factor responsible for the low absorptive nature of cationic drugs (1479).

Subcutaneous absorption of a series of local anesthetics, chemically related to lidocaine, was investigated in anesthetized rats using the system of Ballard and Menczel. The results concurred with the proposed mechanism for subcutaneous absorption involving two processes: (*a*) spreading into surrounding connective tissue, and (*b*) absorption through capillaries (1480, 1481). Absorption of saccharides and urea from the rat lung appeared to be mainly by the process of diffusion (1482). A subsequent study dealt with the absorption of various drugs (*e.g.*, antipyrine, pentobarbital, and salicylic acid) from the rat lung. Comparison of absorption rate constants with chloroform–water partition coefficients and aqueous diffusion coefficients of the drugs suggested that these drugs are absorbed by diffusion across a lipoid–pore membrane. Absorption of drugs was significantly faster from the lungs than from the small intestine (1483). Solubilization as a mechanism of gas transport in biological systems by aggregation through associated micelle formation was investigated. Halothane, ether (ethyl), trichloroethylene, and nitrous oxide were used with surfactant systems of polysorbate 80, dioctyl sodium sulfosuccinate, and bovine albumin; a modified tonometer was used as an absorption chamber (1484).

Additional references on absorption mechanisms are listed in Table XXXIX.

Drug Absorption—Oral absorption characteristics of a systemic anti-inflammatory agent, naproxen, was studied in beagle dogs. Complete absorption of the drug was observed, with the absorption occurring at a significantly faster rate from the sodium salt and calcium salt of the drug than from the unmicronized form of the drug. No significant differences were noticed in the rate and extent of drug absorption in man between tablet and capsule formulations of this drug (1510). The absorption, distribution, metabolism, and excretion of naproxen were studied in rats, dogs, monkeys, and human subjects after either oral or intravenous administration. The drug administered orally was rapidly absorbed in all species; all except the dog excreted naproxen plus its metabolites predominantly in the urine (1511). When propantheline labeled with ^{14}C in its xanthene carboxylic moiety was administered orally or intrajejunally to healthy subjects, the absorption of the intact drug after oral administration was estimated to be less than 50% (1512). Transformation of 1-β-D-arabinofuranosylcytosine to 1-β-D-arabinofuranosyluracil by the intestinal microflora of the rat seemed to be partially responsible for the poor absorption of the orally administered drug. This was further evidenced by a twofold increase in the drug absorption by coadministration with antibiotics (1513). Salicylazosulfapyridine was also extensively metabolized in the human digestive tract, presumably by the action of the gut flora (1514).

The oral absorption of mepenzolate bromide in humans was studied by administration of the drug in solution or capsule dosage forms. In either case, an average of 14% of the total administered drug was eliminated in the urine, thus indicating substantial oral absorption of this quaternary onium-type drug (1515). Kinetics of absorption and elimination of pralidoxime

Table XXXIX—Additional References on Absorption Mechanisms

Reference	Topic
1485	Review of physiology and biochemistry of intestinal absorption; factors affecting drug absorption
1486	Review of absorption of drugs through oral mucosa
1487	Review describing active transport and mechanisms of membrane permeation including drug effects, diffusion, transfer of ions, and macromolecules
1488	Ionic movement across the gastric mucosa of man; reproducibility and effect of intravenous atropine
1489	Mediative role of sodium in intestinal calcium transport considered to involve activation of an enzyme complex
1490	Mechanism of absorption of salicylic acid and its isomers from rat jejunum
1491	Use of a cannulated everted intestinal sac for screening the permeability parameter of drugs
1492	Protein absorption by the intestine of the fetal rat
1493	Absorption of pronase and lysozyme through intestinal mucosa of rabbits
1494	Mechanisms of GI absorption of simifibrate ester in relation to its rate of hydrolysis
1495	Decarboxylation of orally administered levodopa (L-dopa) in the human digestive tract
1496	Transport mechanism of hadacidin and hydroxyurea (carcinostatic compounds) in rat small intestine
1497	Mode of iopanoic acid absorption through the portal venous system of the GI tract of rats
1498	Active transport of thiamine from chick intestine, and competitive inhibition by chloroethylthiamine
1499	Isolation of human epidermal components soluble in dimethyl sulfoxide; possible mechanism of dimethyl sulfoxide action in percutaneous absorption
1500	Mechanism of action of xylene, dimethyl sulfoxide, and di(2-ethylhexyl)amine in skin penetration of drugs
1501	Comparison of drug potency by dermal tissue permeability test on a filter paper implanted subcutaneously in the ventral region of rats
1502	Keratin layer regarded as the major barrier against penicillin penetration through the skin
1503	Penetration of salicylic acid through epidermal barrier of the skin
1504	Time course of cutaneous reservoir in percutaneous absorption of drugs
1505	Corneal endothelium and Descemet's membrane permeability by solutes with varying molecular weights
1506	Influence of various drugs and surfactants on corneal permeability
1507	Biliary excretion of riboflavin found negligible in man
1508	Absorption of sulfobromophthalein (bromsulphthalein) from the biliary system of the rat
1509	Absorption of iodipamide from the biliary system of the rabbit

chloride, a quaternary ammonium drug, were investigated in the dog. Apparent absorption rate constants from the isolated jejunum and ileum were found to be 7.89×10^{-3} and 1.75×10^{-2} min.$^{-1}$, respectively. After oral administration of the drug in solution, detectable plasma levels were obtained in unanesthetized dogs but not in anesthetized dogs. A three-compartment model was employed to describe plasma levels of the drug after intravenous administration (1516).

Methods commonly employed to calculate absorption rate constants from the time course of drug in the blood were shown to yield "apparent" rate constant values when the drug at the site of administration is simultaneously lost to an extravascular compartment(s). It was demonstrated that the true rate constant for absorption in such a case can be calculated by determining the fraction of the dose actually absorbed, provided that all of the initial dose can be accounted for by other simultaneous rate processes (1517). A biokinetic method for the analysis of temporal pharmacological data was described, which permits the computation of the amount of drug absorbed from a site of administration at any time following dosing by any route. This pharmacological approach obviates the need for the periodic sampling of body fluids to determine drug absorption rates (1518). Application of the pharmacological method was demonstrated for two mydriatically active drugs, tropicamide and tridihexethyl chloride, administered ophthalmically, intravenously, intramuscularly, and orally to rabbits (1519). The time course of indomethacin plasma levels in rabbits after intravenous or intraduodenal administration followed a two-compartment model system, but the rate constants estimated by this system increased as the dose was increased, which was ascribed to the saturable binding of the drug to protein (1520). In a subsequent study, the mechanism of intestinal absorption in the rat and drug bioavailability in man after oral administration of micronized indomethacin powder were investigated. Major conclusions derived from the study were: (*a*) indomethacin was bound strongly to the intestinal tissue, (*b*) both the ionized and unionized forms of the drug were absorbed, and (*c*) 96.2% of the drug dose was absorbed in man from capsules containing micronized drug powder (1521).

The absorption of iron was not influenced by concurrent oral administration of ascorbate, succinate, dioctyl sulfosuccinate, fumarate, aspartate, or folate in normal humans or subjects with iron deficiency. Slow-release preparations of ferrous sulfate were only half as active compared to conventional rapid-release dosage forms (1522). Following oral administration of 1–10 mg. sodium fluoride to humans, the peak salivary fluoride concentrations occurred about 45 min. later, with the rate of the subsequent decline being a function of the dose (1523). Copper sulfate has been regarded as a rapidly acting emetic for use in the immediate treatment of poisoning by ingestion. However, in view of the significant oral absorption of copper sulfate observed in rats, its use as an emetic was not recommended (1524). The peak serum levels of lithium in human subjects, after oral administration of a single standard dose of lithium acetate or timed-release tablets of lithium carbonate or lithium sulfate, were attained after 1, 2, and 3 hr., respectively (1525).

The kinetics and equilibrium of hexachlorophene sorption and desorption by human epidermis were studied *in vivo*, utilizing a bioelectrometric technique and direct drug analysis. An average of 41.6% of hexachlorophene was recovered by elutriation from the contents of three apparent kinetic compartments (1526). The *in vitro* penetration of methotrexate through human and hairless mouse skin was determined by scintillation counting and by determining the inhibition of dihydrofolate reductase. Significant absorption of the drug through the skin evidenced by both methods suggested efficacy of methotrexate in dermatology (1527). The fate of disodium cromoglycate in asthmatic patients was examined after oral, inhaled, and intravenous administration. Although maximum plasma concentrations

were obtained within 15 min. of inhaling the drug, which suggests rapid drug absorption from the lung, most inhaled drug was swallowed and excreted in the feces (1528, 1529). In a similar study, absorption and excretion characteristics after oral and inhaled administration of salbutamol to asthmatic patients were investigated (1530).

Additional studies on drug absorption are listed in Table XL.

Pharmacokinetics—General principles of pharmacokinetics, mathematical aspects of single- and multicompartmental models, and application of these concepts to absorption, distribution, metabolism, and excretion studies of a drug as well as to establishment of an optimal dosage scheme were discussed in a number of review articles (1558–1567). In addition, many other reviews focused on pharmacokinetics of specific drugs—*viz.*, niacin (nicotinic acid) and its derivatives (1568); morphine and morphine-like analgesics (1569); hydrochlorothiazide, ioglycamate, and antibiotics (1570); magnesium, plutonium, and iodine (1571); aspirin (1572); penicillin (1573); pentobarbital and penicillin G (1574); and antibiotics (1575). Quantitative and qualitative aspects of pharmacokinetics in relation to biological membrane permeability (1576) and the skin as an organ of absorption from a pharmacokinetic viewpoint (1577) were discussed in two separate reviews.

Earlier pharmacokinetic methods generally assumed the body as a single compartment, with drug absorption and other kinetic processes occurring at first-order rates. A two-compartment model has been proposed, which assumes a central and a peripheral body compartment. An equation for the two-compartment model was derived, which provides interrelationships among absorption, distribution, and elimination processes following oral administration of a drug. These concepts were further applied to study the effect of a solid drug dispersion on the absorption of highly water-insoluble steroids (1578). The two-compartment model assumes that the amount transferred to the peripheral compartment is directly proportional to the dose. A nonlinear model was considered in which the amount of drug transferred to the peripheral compartment is a curvilinear function of dose due to limited drug storage capacity of the peripheral compartment. It was emphasized that several different doses of a drug need to be administered intravenously to elaborate the appropriate pharmacokinetic model (1579). General treatments were presented by which linear mammillary models with elimination from any compartment can be described, utilizing the method of partial fractions for solving Laplace transforms and a multiple-dosing function. The approach was considered relatively simple and applicable in deriving equations for any linear mammillary compartment model with any first-order, zero-order, or impulse input process (1580). A statistical program for the calculation of pharmacokinetic parameters was described. The results of intravenous injection and drug retention experiments were analyzed by this program according to monoexponential or biexponential relationships (1581).

Many therapeutic agents can be applied more predictably and effectively if a constant plasma concentration can be maintained. With the use of a two-compartment open-system model, a method of combining intravenous bolus and continuous infusion was described to calculate the regimen required to reach a predetermined plateau concentration. Application of the method was demonstrated by administration of selected bolus and continuous infusion of theophylline dose regimens to humans (1582). Attainment of desired peak tissue levels of griseofulvin and aspirin required significantly higher peak plasma levels with a multiple intravenous

Table XL—Additional References on Drug Absorption

Reference	Topic
1531	Review describing chemistry, absorption, distribution, and excretion of digitoxin, digoxin, and ouabain
1532	Absorption and metabolism of tritium-labeled factor M_1 of staphylamycin (virginiamycin) by oral and parenteral administration
1533	Comparison of intestinal absorption, lymph and plasma transport, and tissue uptake of α- and γ-tocopherols in the rat
1534	Review describing absorption of folic acid in man
1535	Absorption, distribution, and excretion of levodopa (L-dopa) in mice and uptake into the cat corpus striatum
1536	Absorption, distribution, and excretion of chlorophenothane in rats after oral and intraperitoneal administration
1537	Measurement of enteric absorption rate using a double tracer technique
1538	Plasma levels of nitroglycerin in man after buccal administration
1539	Absorption, distribution, and excretion of the antiparkinsonian drug 6,6,9-trimethyl-9-azabicyclo-[3.3.1]non-3β-yl-α,α-di(2-thienyl)glycolate hydrochloride monohydrate in mice
1540	Uptake of anti-Rh immunoglobulin after intramuscular and subcutaneous injection
1541	Absorption of sodium hydroxybutyrate in man faster from suppositories than from oral dosage forms
1542	Comparable serum levels of sulfathiazole (norsulfazole) obtained by intravenous injection or rectal suppository
1543	Absorption of potassium phenoxymethyl penicillin in man following oral administration of tablet or solution dosage
1544	Human serum and tissue concentrations of erythromycin stearate and erythromycin estolate after oral administration
1545	Comparison of absorption and excretion after oral administration of erythromycin stearate and erythromycin estolate
1546	Absorption, transport, and biotransformation of methyl salicylate in the dog
1547	GI absorption and excretion of aspirin in rats from ethylcellulose-coated tablets
1548	Absorption and physiologial disposition of fenoprofen in man after oral and intravenous administration
1549	Dose-dependent elimination of propranolol during oral absorption in man
1550	Absorption of kanamycin from respiratory organs
1551	Percutaneous absorption of zinc oxide ointment through rabbit skin
1552	Review of pharmacodynamics of absorption and elimination of inhalation anesthetics
1553	Percutaneous absorption of salicylic acid and carbinoxamine; time course of cutaneous reservoir of drugs
1554	Ocular uptake and kinetics of orally administered osmotic agent, isosorbide, in rabbits
1555	Ascorbic acid absorption in man; pharmacokinetic implications
1556	Absorption kinetics of aspirin in man following oral administration of an aqueous solution
1557	Absorption, distribution, and elimination of fenfluramine and its main metabolite in man

Table XLI—Additional References on Pharmacokinetics

Reference	Topic
1602	Plasma levels and pharmacokinetics of pralidoxime methanesulfonate after oral administration to man
1603	Pharmacokinetics and metabolism of diphenoxylate in man
1604	Effect of renal failure and hemodialysis on cephacetrile pharmacokinetics
1605	Kinetics of some pyrazole derivatives in the rat
1606	Pharmacokinetic study of fatty acid esters of polyethylene glycols
1607	Pharmacokinetics of iopanoic acid metabolism
1608	Comparative pharmacokinetics of daunomycin and adriamycin in several animal species
1609	Pharmacokinetics of colloidal gold-198 after intraarticular and intravenous administration in rabbits and rats
1610	Intravascular retention, dispersal, excretion, and breakdown of gelatin plasma substitutes
1611	Comparative studies on the velocity of the elimination of plasma substitutes by the rabbit
1612	Pharmacokinetics of fentanyl in rabbits in view of the importance for limiting the effect
1613	Pharmacokinetics of escin in mice and rats
1614	Biological half-life of 47.1 hr. for phenobarbital in human babies (about 50% greater in adults)
1615	Pharmacokinetic studies of minoxidil in hypertensive patients
1616	Pharmacokinetics of theophylline; application to adjust clinical dose of aminophylline
1617	Correlation of plasma levels of 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile with antiinflammatory activity in polyarthritic rats
1618	Volume of distribution and half-life of heparin after intravenous administration
1619	Pharmacokinetic characteristics of 14-hydroxydaunomycin, an antitumor drug
1620	Comparative pharmcokinetic studies on fenoterol hydrobromide in the rat, dog, and man
1621	Effect of volume of distribution on plasma levels of total radioactivity
1622	Methods of *in vitro* pharmacokinetic investigation of drugs classified as static and dynamic methods
1623	Dynamic *in vitro* method used to study pharmacokinetic characteristics of drugs
1624	Review concerning the effect of distribution, complexing, and pH on pharmacokinetic aspects of drugs
1625	Correlations of pKa and lipid solubility of some tobacco alkaloids with urine excretion in man
1626	Pharmacokinetics of a mixture of chloramphenicol and rifampin (rifampicin) in rats
1627	Relation between the biliary excretion behavior and the elimination from plasma of xanthene dyes and sulfobromophthalein (bromsulphthalein) in the rat
1628	Relation between the renal elimination rate constant of a drug and the urinary flow rate described by a theoretical model
1629	Pharmacokinetics, metabolism, and excretion of alphaprodine in dogs
1630	Plasma urea kinetics in patients treated with an infusion of 30% urea–10% invert sugar solution
1631	Use of hydrolytic rate constants to study glutethimide and its metabolite in human serum
1632	*In vitro* hydrolysis rates of methanesulfonic acid derivatives of sulfonamides compared with pharmacokinetic parameters in rabbits
1633	Integral coefficients of multicompartmental pharmacokinetic models related to chemotherapy
1634	Influence of replacement of *N*-ethyl group by cyanoethyl group on pharmacokinetic parameters of ethylamphetamine in man
1635	Individual differences in the plasma half-lives of lipid-soluble drugs in man
1636	Determination of pharmacokinetic parameters for rapid and slow acetylators of sulfamethazine (sulfadimidine)
1637	Review of the biopharmaceutical applications and factors affecting the biological half-life of drugs
1638	Estimation of plasma concentration following oral administration of a timed-release preparation utilizing a one-compartment open-model system
1639	Pharmacokinetic profile of sulfisoxazole following intravenous, intramuscular, and oral administration to man
1640	Effect of protein binding on the pharmacokinetics of sulfanilamides in rats
1641	Disappearance rate of exogenous gastrin in dogs analyzed by a nonlinear curve-fitting computer program
1642	Simulation of pharmacokinetics of cyproterone acetate by analog computer
1643	Pharmacokinetic models for methotrexate and 1-β-D-arabinofuranosylcytosine
1644	Mathematical models simulating pharmacokinetic parameters of methotrexate and of DNA biosynthesis
1645	Plasma half-lives of the enantiomers of warfarin in drug-resistant and drug-susceptible rats
1646	Plasma half-lives and pharmacological effects of the enantiomers of warfarin in rats
1647	Effects of protein binding of drugs on areas under plasma concentration–time curves
1648	Effect of halofenate on protein binding of other drugs and on plasma half-life of antipyrine in monkeys
1649	Pharmacokinetics of phenylbutazone, pentobarbital, antipyrine, sulfanilamide, quinine, and tolazoline in the goat

dosing regimen than with continuous infusion. Thus, infusion therapy was preferred, especially with relatively toxic drugs, when appearance of the drug in a peripheral compartment was a desired consequence of therapy (1583). Relationships between the dose and plateau levels of drugs eliminated by parallel first-order and capacity-limited kinetics were derived by Tsuchiya and Levy (1584). The authors concluded that repetitive administration at constant time intervals of fixed doses of drugs that are eliminated by apparent first-order kinetics usually results in the eventual attainment of a drug level plateau in the body. If a drug is eliminated solely by capacity-limited kinetics in the therapeutic dose range, it accumulates in the body without limit when the dose exceeds a certain amount (1584). Derivation of theoretical relationships useful for the characterization of multiple-dosing pharmacokinetics of hypoprothrombinemic anticoagulant drugs and the computation of optimum dosing regimens for individual patients was presented (1585).

Various parameters of the two-compartment open model were classified into three groups, depending upon how these parameters are influenced by a change induced in the elimination constant. At a given dose of drug, certain parameters are independent of the elimination constant, others change exactly in proportion to the elimination constant, while a third group is nonlinear or hybrid parameters (1586). A relationship between drug distribution and elimination in multicompartment systems was presented, which suggested that under certain conditions a decrease in the elimination rate constant results in a significant decrease in the apparent volume of distribution. The implication of a change in the apparent volume of distribution with respect to dose–response relationships, estimation of bioavailability, absorption rate, *etc.*, was considered (1587). An unusual

example of nonlinear, dose-dependent pharmacokinetics was observed in the case of riboflavin absorption and excretion in the rat. The elimination of the vitamin was shown to involve at least two nonlinear processes occurring simultaneously. One process involved biliary excretion which increased disproportionately with increasing body levels of riboflavin; the other process appeared to be a binding of the vitamin to tissues which function kinetically as deep compartments. Over the dose range studied, the nonlinear tissue-binding phenomenon was predominant over the nonlinear biliary excretion in riboflavin-deficient rats. The converse was true in the normal, nondeficient animal (1588).

The biliary excretion of intravenously administered methyl orange and its metabolites by rats was used to illustrate a one-compartment model in which a drug underwent two successive metabolic reactions. Data were analyzed graphically and with a computer program to estimate successive metabolic rates and the rate of biliary excretion (1589). Graphical analysis of the curves of biliary concentration as a function of time and the rate constants obtained after intravenous injection of fenpentadiol in rats indicated that the system may be represented by a two-compartment open model. Comparison of the pharmacokinetic parameters of biliary excretion with those of the enterohepatic cycle showed that the total elimination period is shortened if excretion occurs without the cycle. Half of the injected dose was observed to undergo total elimination within 1 hr., and 47% was excreted by the biliary route within 2.5 hr. (1590).

A pharmacokinetic model for the distribution and elimination of tubocurarine in man was presented, which represented the body as a three-compartment linear system with the site of drug action located in the central compartment. The decline in neuromuscular blocking effect with increasing dose elicited by tubocurarine was in accordance with the pharmacokinetic characteristics of a multicompartment system (1591, 1592). The distribution kinetics of inulin and urea were studied in the rat to test the validity of a proposed compartmental model. An open two-compartment model provided valid parameters for inulin data, while a three-compartment model fit the urea data (1593). The time course of rifampin (rifampicin) concentration in the serum, bile, and urine was determined in patients after oral administration of the drug. The results were interpreted on the basis of a four-compartment model consisting of intestine, blood, bile, and urine compartments (1594).

The pharmacokinetics of methylene blue, representative of highly ionized drugs, was investigated in the rat, dog, and man by DiSanto and Wagner (1595–1597). The authors concluded that methylene blue is well absorbed in man but poorly absorbed in the dog after oral administration. Plasma concentration–time data obtained from intravenous injection of methylene blue to a dog seemed to fit a linear two-compartment open model; the results of drug concentrations in the lung, liver, kidney, and heart tissues of rats after intravenous injection, with respect to dose, were shown to fit the nonlinear heterogeneous one-compartment open model (1595–1597). The blood level–time relationships for sulfamethazine, sulfisomidine, and sulfathiazole were determined in rabbits after intravenous injection. The results were fitted to a two-compartment open model, and the model parameters were obtained. Differences between drugs, animals, and treatments were studied in terms of urinary and metabolic rate constants, intercompartment diffusion constants, and clearance values (1598). In a similar study comparing pharmacokinetic parameters of sulfanilic acid and 1-naphthylamine-4-sulfonic acid, it was concluded that the latter compound is actively secreted into the renal tubule while the former is not (1599).

Computer simulations were used to determine suitable experimental conditions for distinguishing between a recently developed pharmacokinetic model for salicylate elimination (involving saturable salicyl phenolic glucuronide formation) and a previously proposed model (based on the assumption that the syntheses of salicyl phenolic and acyl glucuronides are apparent first-order processes). Studies in man according to the computer-simulated experimental design confirmed the new pharmacokinetic model, which provided further evidence that man has a limited capacity to form salicyl phenolic glucuronide (1600, 1601).

Additional references on pharmacokinetics are listed in Table XLI.

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RESEARCH ARTICLES

Carbocyclic Analogs of 6-Substituted Purine Ribonucleosides and of Adenosine Ribonucleotides

Y. FULMER SHEALY▲ and JOE D. CLAYTON

Abstract □ New analogs of 6-substituted purine ribonucleosides in which the furanose ring is replaced by a cyclopentane ring were synthesized. The cyclopentane (carbocyclic) analog of 6-chloropurine ribonucleoside was obtained in pure form and was used to prepare analogs having a methylamino, dimethylamino, hydroxylamino, or methoxy group at position 6 of the purine ring. The availability of the pure 6-chloropurine derivative also permitted improved syntheses of the adenosine and 6-(methylthio)purine ribonucleoside analogs, which had been prepared previously. The ribonucleoside analogs having the chloro, methylamino, hydroxylamino, or methoxy group at position 6 were cytotoxic to neoplastic cells (human epidermoid carcinoma No. 2) in culture, but these compounds were less active than the adenosine analog. In tests against leukemia L-1210 in mice, all of the new and the previously synthesized 6-substituted purine ribonucleoside analogs were administered in a single dose (Day 1 or 2), and most were also administered daily (*q.d.* 1–9) at several dose levels. There was no evidence of activity in these tests. The racemic carbocyclic analogs of adenylic acid and of 3′,5′-adenosine monophosphate (cyclic) were prepared from the adenosine analog. These nucleotide analogs were also cytotoxic.

Keyphrases □ Cyclopentane analogs of 6-substituted purine ribonucleosides and adenosine ribonucleotides—synthesis, cytotoxicity □ Carbocyclic analogs of 6-substituted purine nucleosides and adenosine ribonucleotides—synthesis, cytotoxicity □ Purine ribonucleosides, 6-substituted—synthesis of cyclopentane analogs, cytotoxicity □ Adenosine ribonucleotides, 6-substituted—synthesis of cyclopentane analogs, cytotoxicity □ Cytotoxicity—synthesis, evaluation of cyclopentane analogs of 6-substituted purine ribonucleosides and adenosine ribonucleotides

Nucleoside analogs in which a cyclopentane ring replaces the furanose ring may be termed carbocyclic analogs. The racemic[1], carbocyclic analog (II, C-Ado[2]) of adenosine (I) has been synthesized (1) by a multistep route from norbornadiene. The carbocyclic analogs (III–V) of inosine and of the anticancer agents 6-mercaptopurine ribonucleoside and 6-(methylthio)purine ribonucleoside were also prepared (2) from the precursors of II. The carbocyclic analogs of 2′- and 3′-deoxyadenosine were synthesized by similar routes (3); the analog of a pyrimidine deoxyribonucleoside, thymidine, had been prepared earlier by a different method (4).

Carbocyclic analogs, having a stable carbon–nitrogen bond instead of a glycosidic bond at position 9 of the purine ring, should not be subject to cleavage by purine nucleoside phosphorylases or hydrolases, but the similarity of their structures to nucleosides endows the carbocyclic analogs with the potential to function either as substrates for, or as inhibitors of, other enzymes that metabolize nucleosides. Biochemical studies show that this potential can, in fact, be realized. Bennett and co-

[1] Structures II–XVI depict one enantiomer of the racemic form that was actually obtained.

[2] (±)-*trans*-3-(6-Amino-9*H*-purin-9-yl)-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol. For the sake of brevity, the term C-Ado was employed in reports of biochemical studies (5, 6). In earlier publications (1, 2), C-Ado was designated (±)- or DL-9-[β-(2α,3α-dihydroxy-4β-(hydroxymethyl)cyclopentyl)] adenine.

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Carbocyclic Analogs of 6-Substituted Purine Ribonucleosides and of Adenosine Ribonucleotides

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Abstract □ New analogs of 6-substituted purine ribonucleosides in which the furanose ring is replaced by a cyclopentane ring were synthesized. The cyclopentane (carbocyclic) analog of 6-chloropurine ribonucleoside was obtained in pure form and was used to prepare analogs having a methylamino, dimethylamino, hydroxylamino, or methoxy group at position 6 of the purine ring. The availability of the pure 6-chloropurine derivative also permitted improved syntheses of the adenosine and 6-(methylthio)purine ribonucleoside analogs, which had been prepared previously. The ribonucleoside analogs having the chloro, methylamino, hydroxylamino, or methoxy group at position 6 were cytotoxic to neoplastic cells (human epidermoid carcinoma No. 2) in culture, but these compounds were less active than the adenosine analog. In tests against leukemia L-1210 in mice, all of the new and the previously synthesized 6-substituted purine ribonucleoside analogs were administered in a single dose (Day 1 or 2), and most were also administered daily (*q.d.* 1–9) at several dose levels. There was no evidence of activity in these tests. The racemic carbocyclic analogs of adenylic acid and of 3′,5′-adenosine monophosphate (cyclic) were prepared from the adenosine analog. These nucleotide analogs were also cytotoxic.

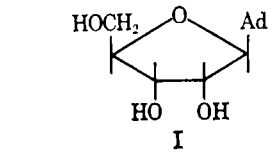
Keyphrases □ Cyclopentane analogs of 6-substituted purine ribonucleosides and adenosine ribonucleotides—synthesis, cytotoxicity □ Carbocyclic analogs of 6-substituted purine nucleosides and adenosine ribonucleotides—synthesis, cytotoxicity □ Purine ribonucleosides, 6-substituted—synthesis of cyclopentane analogs, cytotoxicity □ Adenosine ribonucleotides, 6-substituted—synthesis of cyclopentane analogs, cytotoxicity □ Cytotoxicity—synthesis, evaluation of cyclopentane analogs of 6-substituted purine ribonucleosides and adenosine ribonucleotides

Nucleoside analogs in which a cyclopentane ring replaces the furanose ring may be termed carbocyclic analogs. The racemic[1], carbocyclic analog (II, C-Ado[2]) of adenosine (I) has been synthesized (1) by a multistep route from norbornadiene. The carbocyclic analogs (III–V) of inosine and of the anticancer agents 6-mercaptopurine ribonucleoside and 6-(methylthio)purine ribonucleoside were also prepared (2) from the precursors of II. The carbocyclic analogs of 2′- and 3′-deoxyadenosine were synthesized by similar routes (3); the analog of a pyrimidine deoxyribonucleoside, thymidine, had been prepared earlier by a different method (4).

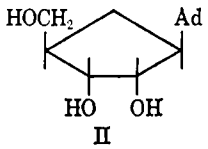
Carbocyclic analogs, having a stable carbon–nitrogen bond instead of a glycosidic bond at position 9 of the purine ring, should not be subject to cleavage by purine nucleoside phosphorylases or hydrolases, but the similarity of their structures to nucleosides endows the carbocyclic analogs with the potential to function either as substrates for, or as inhibitors of, other enzymes that metabolize nucleosides. Biochemical studies show that this potential can, in fact, be realized. Bennett and co-

[1] Structures II–XVI depict one enantiomer of the racemic form that was actually obtained.

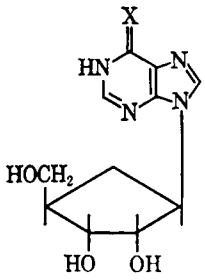
[2] (±)-*trans*-3-(6-Amino-9*H*-purin-9-yl)-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol. For the sake of brevity, the term C-Ado was employed in reports of biochemical studies (5, 6). In earlier publications (1, 2), C-Ado was designated (±)- or DL-9-[β-(2α,3α-dihydroxy-4β-(hydroxymethyl)cyclopentyl)] adenine.



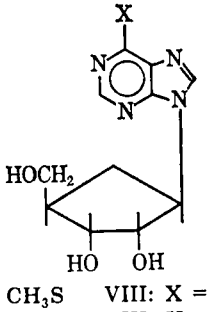
I



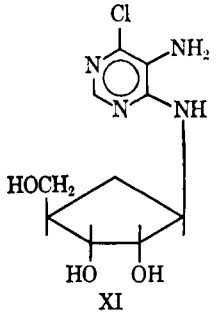
II



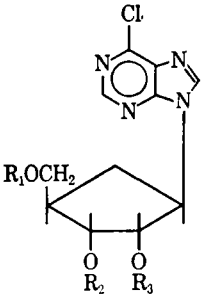
III: X = O
IV: X = S



V: X = CH_3S
VI: X = Cl
VII: X = CH_3NH
VIII: X = $(CH_3)_2N$
IX: X = HONH
X: X = CH_3O

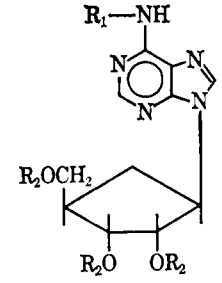


XI

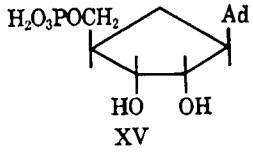


XIIa: R_1 = CHO, R_2 + R_3 =
XIIb: R_1 = H, R_2 + R_3 =
XIIc: R_1 + R_2 + R_3 = H + 2CHO
XIId: R_1 + R_2 + R_3 = 2H + CHO

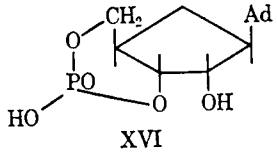
workers (5, 6) showed that II can serve as a substrate for adenosine kinase and for adenosine deaminase and as an inhibitor, presumably after it is phosphorylated, of an early step in the biosynthesis *de novo* of inosinic acid. Hill *et al.* (7) found that II-phosphate (XV), the preparation of which is reported here, is a potent inhibitor of guanylic acid kinase. Furthermore, an analog of vitamin B_{12} coenzyme prepared from II can act as a coenzyme in the reaction catalyzed by dioldehydrase (8). Of added interest are the findings that II is a moderate inhibitor of the protozoan *Tetrahymena pyriformis* (9) and that aristeromycin (10), an antibiotic for certain plant pathogens, is one enantiomeric form of II (11, 12). These biochemical and biological studies show that alteration of the nucleoside structure by replacement of the furanose oxygen atom with a methylene group can produce biologically active compounds. The preparation of carbocyclic analogs of other bio-



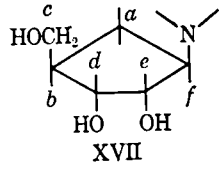
XIII: R_1 = R_2 = C_3H_7CO
XIV: R_1 = H, R_2 = C_3H_7CO



XV



XVI



XVII

chemically significant or biologically active 6-substituted purine ribonucleosides and ribonucleotides is now described.

CHEMISTRY

(±)-*trans*-3-(5-Amino-6-chloropyrimidin-4-yl)-*trans*-(5-hydroxymethyl)-*cis*-1,2-cyclopentanediol (XI) is the immediate precursor of the purine ring of carbocyclic analogs of 6-substituted purine ribonucleosides (1, 2). The pyrimidine (XI) was treated with triethyl orthoformate and concentrated hydrochloric acid to form the 6-chloropurine ring (VI), and the crude syrup obtained from the acid-catalyzed reaction was converted with anhydrous hydrogen chloride to a solid consisting of a mixture of hydrochlorides of VI and its derivatives (2). Such crude 6-chloropurine hydrochloride preparations, used previously (2) for the preparation of II and the inosine carbocyclic analog (III), furnished the 6-(methylamino)-purine (VII) and the 6-(dimethylamino)purine (VIII) derivatives by nucleophilic displacement of the 6-chloro substituent.

However, acid-catalyzed reactions of ribonucleosides with *ortho*-esters produce 2',3'-alkoxymethylene derivatives (13–16), which may be hydrolyzed under mildly acidic conditions to formate esters (14–16). Mass spectral studies of crude VI-hydrochloride specimens showed, as expected, the presence of derivatives of these types in addition to VI. Strongly acidic (and subsequent basic) conditions employed during the isolation and purification of II, VII, and VIII and during the preparation of the inosine analog (III) ensured liberation of the cyclopentyl hydroxyl groups. However, strongly acidic or basic conditions are detrimental to some 6-substituted purines, including 9-substituted 6-chloropurines. For this reason and because VI was desired as a target compound for biological evaluation, the preparation and purification of VI were investigated further. By omitting hydrochloride formation after the reaction of XI with triethyl orthoformate and by treating the reaction product successively with aqueous acetic acid and methanolic ammonia, pure VI was obtained. Mass spectral analyses at each stage indicated that: (*a*) the initial reaction product obtained from XI and triethyl orthoformate contained predominantly the formylethoxymethylene derivative (XII*a*), together with small amounts of XII*b* (or XII*c*) and VI; (*b*) the product after the aqueous acetic acid treatment consisted principally of a mixture of VI, monoformate esters (XII*d*), and diformate esters (XII*c*); and (*c*) the product after methanolic ammonia treatment was almost pure VI. TLC or the mass spectrum indicated that small amounts of II, III, X, and the 6-ethoxypurine analogous to X were formed by the sequence of treatments that began with XI. Pure VI could be obtained by recrystallization.

The 6-(hydroxyamino)purine derivative (IX) was obtained by replacement of the chloro group of purified specimens of VI with hydroxylamine by a procedure similar to that used for the ribonucleoside (17). The 6-methoxypurine (X) was prepared by treating pure VI with sodium methoxide in methanol. Previously (2), the 6-(methylthio)purine derivative (V) had been prepared by methyla-

Table I—Evaluation of Carbocyclic Analogs of 6-Substituted Purine Ribonucleosides against Neoplastic Cells *In Vitro* and *In Vivo*

Compound X	Number	H.Ep.-2[a] or KB Cells, ED_{50} (mcg./ml.)	Leukemia L-1210[b] Single-Dose Treatment[c] Dose, mg./kg.	Average Weight Change, *T/C*	*T/C*, %	*q.d.* 1–9 Dose, mg./kg./Day	Average Weight Change, *T/C*	*T/C*, %
Cl	VI	3	400	+3.7/+3.4	96	200	−2.7/+2.9[d]	93
						150	−1.4/+1.3	92
						100	−0.3/+1.3	101
						75	+0.6/+1.3	107
NH_2	II	0.7[e]	400[f]	−4.7/+1[d]	88	50	−2.1/+1.5	95
			200	−3.2/+1[d]	91	50	−1.5/+0.9	82[d]
			100	−0.4/+1	102	25	−0.4/+0.9	89
			50	+1.5/+1	93	12.5	+0.7/+0.9	97
						6.3	+1.0/+0.9	98
CH_3NH	VII	4.5	400	+1.4/+1.7	100	400	−3.7/+1.6[d]	76[d]
						200	−2.6/+1.3	84[d]
						100	−0.3/+1.3	100
$(CH_3)_2N$	VIII	>100	400	+1.5/+1.6	94			
HONH	IX	9	400	−4.6/+2.1[d]	72[d]	100	−2.9/+2.9[d]	89
			200	+0.9/+1.8	100	75	−2.1/+1.3	86
						50	−1.5/+1.3	92
						25	+0.5/+1.3	103
CH_3O	X	20 (KB)	400	+0.3/+1.4	98	200	−2.2/+2.9[d]	84[d]
						100	−1.3/+0.2	91
						50	−1.5/+0.2	94
						25	−0.3/+0.2	102
CH_3S	V	92	400	+3.0/+2.8	91	200	+0.5/+1.3	101
O	III	>100	400[f]	+2.3/+1.9	97	400	−2.4/+1.6[d]	68[d]
			200	+2.1/+1.9	92	200	0/+1.0	87
			100	+2.9/+1.9	101			
S	IV	>100	400[f]		t[g]	200	−0.2/+1.1	101
			200	+1.5/+1.0	96			
			100	+1.6/+1.0	96			
			50	+1.7/+1.0	96			
NH_2, phosphate	XV	0.7	400	−3.3/+2.0[d]	69[d]	150[h]	+1.8/+2.5	100
			300	−2.9/+1.9[d]	79[d]	75[h]	+2.2/+2.5	110
			150	−0.7/+1.9	87	37[h]	+1.4/+2.5	105
			75	+1.5/+1.9	107			
NH_2, cyclic phosphate	XVI	3.6[i]				50	+0.7/+1.8	115
						25	+1.5/+0.4	103

[a] ED_{50} determined for H.Ep.-2 cells unless otherwise indicated. H.Ep.-2 = human epidermoid carcinoma cells, No. 2, growing in cell culture. KB = Eagle's KB cells growing in cell culture and derived from a carcinoma of the human nasopharynx. ED_{50} = concentration of a compound that inhibits cell growth, measured by protein determinations, to 50% of the growth of untreated cells. [b] Mice were implanted intraperitoneally on Day 0 with 10^6 L-1210 cells. For single-dose treatment, a compound was injected intraperitoneally on Day 1 (about 24 hr.) or on Day 2 (about 48 hr.) after implantation; *q.d.* 1–9 means that daily injections at the specified dose were initiated on Day 1 and continued through Day 9 or until the death of the animal. Average weight change = average weight change of host animals. Weight changes were determined 4 days after the first (for daily treatment) or only injection. *T* = treated mice; *C* = control mice. [c] Treatment on Day 1 unless otherwise indicated. [d] $T/C < 85\%$ and a difference in weight change ($T-C$) greater than 4 g. are criteria of toxicity to the host animals. [e] Approximate average of several tests. ED_{50} determined by the clone-colony method = 0.7 μmole/ml. (6). [f] Single-dose treatment with this compound was performed on Day 2. [g] Half of the treated animals died on or before Day 5. [h] The treatment schedule was Days 1, 5, and 9 rather than *q.d.* 1–9. [i] Average of three tests.

tion of the purine-6-thione (IV). The availability of pure VI permitted the synthesis of V by methylmercaptide ion generated from sodium methoxide and hydrogen sulfide in methanol (18). Proton magnetic resonance analysis of recrystallized V showed that it contained no more than 1% of the 6-methoxypurine (X). The 6-(methylamino)purine (VII) and the adenine (II) derivatives were also obtained more conveniently and in superior yields from purified VI.

The following derivatives of II were synthesized: the tetrabutyryl (XIII) and tributyryl (XIV) derivatives; the 5-dihydrogen phosphate (XV), which is the (±)-analog of adenylic acid; and the cyclic 1,5-phosphate (XVI), which is the (±)-analog of cyclic 3′,5′-adenosine monophosphate. The phosphate (XV) was obtained in 67% yield by phosphorylating isopropylidene II with phosphorus oxychloride in trimethyl phosphate (19, 20), isolating the product as the ammonium salt, and converting the salt to the free acid. After this work had been completed, the preparation of aristeromycin phosphate was reported by Imai *et al.* (21). The cyclic phosphate (XVI) was synthesized from XV by the method of Smith *et al.* (22) for the preparation of cyclic adenosine monophosphate; a simplified isolation procedure afforded a good yield of XVI.

BIOLOGICAL EVALUATION

Most purine ribonucleosides are not active *per se;* observed biological effects are caused by ribonucleotides formed from the ribonucleosides (23). Since it is generally accepted that nucleotides do not penetrate cell walls, activity is dependent on intracellular activation by kinases or by the sequential action of phosphorylases and phosphoribosyl transferases (23). Some new derivatives (VI–VIII and X) were selected, therefore, because the analogous ribonucleosides are good substrates for adenosine kinase from human epidermoid carcinoma (H.Ep.-2) cells (24), and these derivatives might also be phosphorylated by this enzyme. Additionally, the 6-(hydroxyamino)purine derivative (IX) is the analog of a nucleoside having anticancer activity (17, 25), and the cyclic phosphate (XVI) is the analog of cyclic adenosine monophosphate which may, in some degree, be transported intact into cells (26, 27).

The adenine derivative (II) is a highly cytotoxic compound (5, 6). The 6-chloropurine (VI), 6-(methylamino)purine (VII), and 6-(hydroxyamino)purine (IX) derivatives and the two nucleotide analogs (XV and XVI) also proved to be cytotoxic to neoplastic cells growing in culture (Table I), and the 6-methoxypurine (X) was modestly inhibitory. There is no evidence to indicate whether XV and XVI are cytotoxic as such or whether the observed activity is due to cleavage to II. As mentioned in the introduction, XV was found to be a potent inhibitor of guanylic acid kinase (7).

The results shown in Table I summarize tests of carbocyclic analogs of 6-substituted purine ribonucleosides against leukemia L-1210 in mice. All of the nucleoside analogs (II–X) were tested on a single-dose schedule (Day 1 or 2) at 400 mg./kg. If toxic symptoms appeared at this dose, testing was continued at lower doses. The 6-chloropurine (VI), 6-aminopurine (II), 6-(methylamino)purine (VII), 6-(hydroxyamino)purine (IX), and 6-methoxypurine (X) derivatives and the hypoxanthine (III) were administered at several

doses on a *q.d.* 1–9 schedule. These doses ranged from an upper, toxic dose (indicated by host weight loss or T/C <85%) to lower doses that caused little, or no, weight loss. The remaining compounds shown in Table I received more limited testing *in vivo*. None of the tests performed gave evidence of activity. The results in Table I indicate the degree of toxicity of these compounds *in vivo* (to leukemia-bearing mice) and show the scope of the evaluation.

EXPERIMENTAL[3]

(±)-*trans*-3-(6-Chloro-9*H*-purin-9-yl)-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol (VI)—A solution of 500 mg. of XI, 15 ml. of triethyl orthoformate, and 0.15 ml. of 12 *N* hydrochloric acid was stirred at room temperature for 19 hr. The solution was concentrated *in vacuo* at 25° to a syrup, and several portions of toluene were similarly evaporated from the residue to aid in the removal of traces of volatile components. The mass spectrum (direct-probe inlet temperature 395°) of the residue indicated that it was composed principally of derivatives of VI formed by the reaction of triethyl orthoformate with the hydroxyl groups, along with trace amounts of VI and other components[4,5]: *m/e* 368 (M^+ of XII*a*, weak), 340 (M^+ of XII*b* or XII*c*, very weak), 339 (XII*a* − CHO), 323 (XII*a* − OC_2H_5, very strong), 295 (XII*b* − OC_2H_5 or 323 − CO), 284 (M^+ of VI, very weak), 235 (XII*a* − $OCHOC_2H_5$ − CH_2OCHO, very strong), 155 (P + 2H), 154 (P + H), and 153 (P); relative peak heights above 150 mass units, *m/e* 155 > 323 > 235 > 153. The fact that the peak of *m/e* 323 was the second strongest in the spectrum indicates that the crude product was mostly XII*a*; the strong peak at *m/e* 235 is consistent with this interpretation, but it also might have arisen partly from XII*b* (340 − $OCHOC_2H_5$ − CH_2OH).

To hydrolyze the ethoxymethylene group of XII*a* and XII*b* and other *ortho*-ester groups that might be present, a solution of the crude product in 10 ml. of 50% acetic acid was stirred at room temperature for 5.5 hr. and then concentrated to a syrup. To remove residual acetic acid, a solution of the syrup in water was lyophilized, yielding 553 mg. An IR spectrum showed strong ester absorption at 1710 $cm.^{-1}$ (—OCHO), and the mass spectrum[4,5] (direct-probe inlet temperature 390°) indicated that the product was chiefly a mixture of VI and its formate esters: *m/e* 340 (M^+ of XII*c*), 312 (M^+ of XII*d*, stronger than 284 and 340), 311 (XII*c* − CHO), 294 (XII*d* − H_2O), 284 (M^+ of VI), 283 (XII*d* − CHO), 253 (VI − CH_2OH or XII*d* − CH_2OCHO), 235 (XII*d* − CH_2OCHO − H_2O or VI − CH_2OH − H_2O), 181 (P + C_2H_4), 155 (P + 2H, base peak), 154 (P + H), and 153 (P); relative peak heights above 100 mass units, 155 > 157 > 181 > 154 ~ 235 > 156 > 119 > 183. The peak of *m/e* 235 must have been derived chiefly from XII*d*, because it was much stronger than the molecular ion peak of VI and it was much weaker than *m/e* 284 in the spectra of pure specimens of VI. Weak peaks of *m/e* 368 (XII*a*), 358, 330, 323 (XII*a* − OC_2H_5), and 302 showed the presence of other minor components.

A solution of 353 mg. of the gummy solid in 10 ml. of 10% ammonia–methanol was kept at room temperature for 4 hr. to remove the formyl groups. After evaporation of the solvent and of several portions of ethanol from the reaction product, it was stirred with a mixture of ethyl acetate (7 ml.) and ethanol (3 ml.) until solidification occurred, yielding 227 mg. (68% yield from XI), m.p. 152–170° dec. UV data and the mass spectrum (compared with spectra of pure specimens of VI) indicated that this material was almost pure VI; TLC showed that it was VI contaminated with small amounts (less than 3–5%) of both II and the hypoxanthine derivative (III). These impurities must have been introduced by displacement of the 6-chloro group by ammonia and *via* acidic hydrolysis, respectively. Also, weak peaks of *m/e* 280 and 294 in the mass spectrum correspond to the molecular ions of the 6-methoxypurine (X) and the analogous 6-ethoxypurine derivative, respectively, and suggest that slight displacement of the 6-chloro group by methanol and ethanol occurred. Recrystallization of such specimens from a small volume of ethanol–ethyl acetate or from ethanol–hexane (1:1) gave pure VI, m.p. 171–175° dec.; TLC, 1 spot [20 mcg., chloroform–methanol (4:1)]; UV: λ_{max} 265 (ϵ 9400) at pH 1 and 7; mass spectrum[4,5] (direct-probe inlet temperature 390°): *m/e* 284 (M^+), 267 (M − OH), 266 (M − H_2O), 256 (M − CO), 255, 253 (M − CH_2OH), 237, 235 (M − CH_2OH − H_2O), 225 (M − CH_2OH − CO), 219, 209, 197, 181 (P + C_2H_4), 167, 155 (P + 2H), 154 (P + H), and 153 (P). The peak of *m/e* 155 was the base peak in the spectra of purified specimens of VI. The peaks of *m/e* 157 (due to the ^{37}Cl-fragment analogous to *m/e* 155) and *m/e* 181 were the next strongest; other strong peaks above 100 mass units were 156, 154, 183, 119, 284, and 209.

Anal.—Calc. for $C_{11}H_{13}ClN_4O_3$: C, 46.40; H, 4.60; Cl, 12.45; N, 19.75. Found: C, 46.35; H, 4.69; Cl, 12.72; N, 19.51.

Crude 6-chloropurine (VI) hydrochloride specimens were used for preparing some 6-substituted purine derivatives. These very hygroscopic, white specimens were prepared by the previously described procedure (2) from XI, triethyl orthoformate, and concentrated hydrochloric acid, followed by treatment of the crude syrup with hydrogen chloride to obtain a solid product. Several specimens of the crude mixture of hydrochlorides gave similar (weak) mass spectral patterns. Dissolution of such specimens in water to dissociate the hydrochloride salts, followed by lyophilization, increased the volatility of the crude products; mass spectra were improved but not appreciably changed. Typical mass spectra showed peaks corresponding to the molecular ions of VI, XII*b* or XII*c*, XII*d*, and fragments derived from these compounds. Since the crude hydrochloride preparations are very hygroscopic, hydrolysis of some ethoxymethylene and formyl groups may have occurred during handling. Weak molecular ion peaks of *m/e* 358, 348 and 330, and 302 in the spectra correspond to by-products in which one or two hydroxyl groups of XII*b* or XII*c*, XII*d*, and VI, respectively, are replaced by chloro groups. These minor by-products may have been introduced during the preparation of the crude mixture of hydrochlorides or during one of the two preceding steps (2) and, because of their greater volatility, may have been detectable in trace amounts.

(±)-*trans*-3-(Hydroxymethyl)-*trans*-5-[6-(methylamino)-9*H*-purin-9-yl]-*cis*-1,2-cyclopentanediol (VII)—A solution of 1.0 g. of crude VI hydrochloride (2) and 60 ml. of liquid methylamine was heated in a glass-lined, stainless steel bomb at 70° for 18 hr. and then concentrated to a syrup. A solution of the syrup, 30 ml. of water, 30 ml. of ethanol, and 3 ml. of 12 *N* HCl was heated at 45° for 0.5 hr. The solvents were evaporated *in vacuo*, and several portions of water were evaporated from the residue. A solution of the residue in 150 ml. of water was stirred with 10 g. of an anion-exchange resin[6] for 0.5 hr., the resin was removed by filtration and washed several times with 50-ml. portions of warm water, and the residue remaining after evaporation *in vacuo* of the combined filtrate and wash solutions was dried further by evaporating several portions of ethanol from it. A solution of the amorphous residue in 50 ml. of ethanol deposited 693 mg. (80%) of crude VII free base (m.p. 103–106° dec.). A solution of 670 mg. of the crude free base, 25 ml. of ethanol, and 15 ml. of ether was treated with anhydrous hydrogen chloride. A white precipitate, with the approximate composition of a dihydrochloride, was recrystallized from methanol–ether, yielding 555 mg. of the monohydrochloride of VII, m.p. 177–

[3] UV spectra were recorded with a Cary model 17 or 14 spectrophotometer. UV maxima are in nanometers; sh = shoulder. Solutions for UV determinations were prepared by diluting a 5-ml. aliquot of a water or ethanol solution of the compound to 50 ml. with 0.1 *N* HCl, phosphate buffer (pH 7), or 0.1 *N* NaOH; absorption maxima are reported at pH 1, 7, and 13. IR spectra were recorded with a Perkin-Elmer model 621 or 521 spectrometer from samples in KBr disks. Mass spectral data were taken from low-resolution spectra determined with a Hitachi-Perkin-Elmer RMU-7 double-focusing instrument; the peaks listed are those due to the molecular ion (M^+), those attributable to the loss of certain fragments from the molecular ion (M − a fragment), and other prominent peaks due to fragments heavier than the purine moiety (minus the cyclopentyl group). Proton magnetic resonance (PMR) spectra were determined with a Varian model XL-100-15 spectrometer for observing proton resonance at 100 MHz. Chemical shift data (δ) are in parts per million downfield from tetramethylsilane, the internal reference; s = singlet, and m = multiplet. Melting temperatures were determined in capillary tubes heated in a Mel-Temp apparatus. Unless otherwise indicated, TLC was performed on plates of silica gel, and spots were detected by UV light (254 nm.) after spraying the chromatogram with an optical whitening agent (Ultraphor WT, BASF Colors and Chemicals, Inc., Charlotte, N. C.) and by spraying with a basic solution of potassium permanganate. The quantity applied and the developing solvent are shown parenthetically at the appropriate places in the procedures.

[4] The chlorine-containing fragments listed contain ^{35}Cl; the corresponding fragments (heavier by 2 mass units) containing ^{37}Cl are not listed unless the peaks were stronger than expected from the isotope ratio.

[5] P = the purine ring fragment without a 9-substituent.

[6] Dowex 1-X8, OH^- form.

179° dec.; UV: λ_{max} 263 (ε 17,200) at pH 1 and 267 (ε 16,800) at pH 7 and 13.

Anal.—Calc. for $C_{12}H_{17}N_5O_3 \cdot HCl$: C, 45.65; H, 5.74; N, 22.17. Found: C, 45.74; H, 5.79; N, 22.26.

Pure VI (500 mg.) was treated with 30 ml. of methylamine according to the procedure already described for crude VI hydrochloride. Concentration of the reaction mixture to dryness left a white solid. The product was recrystallized twice from ethanol and dried *in vacuo* at 78°, yielding 440 mg. (77%), m.p. 110–112° dec.; TLC, 1 spot [60 mcg., chloroform–methanol (3:1)]; mass spectrum (direct-probe inlet temperature 240°): *m/e* 279 (M^+), 262 (M − OH), 248 (M − CH_2OH), 232, 230 (M − CH_2OH − H_2O), 220 (M − CH_2OH − CO), 204, 192, 176 (P + C_2H_4), 150 (P + 2H, base peak), 149 (P + H), and 148 (P).

Anal.—Calc. for $C_{12}H_{17}N_5O_3 \cdot C_2H_5OH$: C, 51.68; H, 7.12; N, 21.53. Found: C, 51.72; H, 7.43; N, 21.36.

(±)-*trans*-3-[6-(Dimethylamino)-9*H*-purin-9-yl]-*trans*-(5-hydroxymethyl)-*cis*-1,2-cyclopentanediol (VIII)—A solution of 350 mg. of crude VI hydrochloride (2) in 12 ml. of 25% aqueous dimethylamine was heated under reflux for 3 hr., allowed to stand at room temperature for 5 days, and evaporated *in vacuo* to a syrup. By a procedure similar to that described for the preparation of VII from crude VI, the dihydrochloride of VIII was obtained from a chilled mixture of ethanol, ether, and dry hydrogen chloride, yielding 130 mg. (32%), m.p. 98–105° with premature softening; UV: λ_{max} 268 (ε 17,700) at pH 1 and 277 (ε 18,000) at pH 7 and 13.

Anal.—Calc. for $C_{13}H_{19}N_5O_3 \cdot 2HCl$: C, 42.63; H, 5.78; N, 19.12. Found: C, 42.78; H, 5.52; N, 18.84.

trans-3-[6-(Hydroxyamino)-9*H*-purin-9-yl]-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol (IX)—A solution of 854 mg. of pure VI, 6 ml. of methanol, 6 ml. of ethanol, and 2.5 g. of hydroxylamine (prepared from the hydrochloride and sodium methoxide in methanol) was heated under reflux for 3 hr. and then allowed to stand at room temperature for 18 hr. A white precipitate was separated by filtration, washed with ethanol, and dried *in vacuo* at 78°, yielding 655 mg., m.p. 204–220° dec. The crude product was dissolved in 50% aqueous ethanol, the solution was concentrated to a syrupy consistency, ethanol was added, and the solution was chilled. The first crop (357 mg.) of IX was stirred with water, washed with ethanol, and dried *in vacuo* at 78°, yielding 313 mg., m.p. 225° dec.; TLC, 1 spot [40 mcg., butanol–water–acetic acid (5:3:2)]; UV: λ_{max} 266 (ε 16,700) at pH 1 and 267 (ε 13,700) at pH 7.

Anal.—Calc. for $C_{11}H_{15}N_5O_4$: C, 46.97; H, 5.37; N, 24.90. Found: C, 46.64; H, 5.65; N, 24.89.

A second crop of IX, obtained by concentrating the filtrate and cooling it to −20°, was recrystallized from aqueous ethanol, yielding 73 mg., m.p. 224° dec.; UV: λ_{max} 266 (ε 16,500) at pH 1.

(±)-*trans*-3-(Hydroxymethyl)-*trans*-5-(6-methoxy-9*H*-purin-9-yl)-*cis*-1,2-cyclopentanediol (X)—A solution prepared from 600 mg. of pure VI, 14 ml. of anhydrous methanol (dried with molecular sieves), and 6 ml. of 1 *N* sodium methoxide in methanol was heated under reflux for 2 hr., allowed to stand at room temperature for 48 hr., and evaporated to dryness *in vacuo*. A solution of the residue in 10 ml. of water was acidified with 1 ml. of 6 *N* HCl and then neutralized with solid sodium bicarbonate. The mixture, containing a precipitate, was allowed to stand at 5° and was then filtered to separate white crystals, yielding 490 mg., m.p. 203–210° dec. The crude product was recrystallized from water and dried *in vacuo* at 78°, yielding 387 mg. (65%), m.p. 210–212° dec.; TLC, 1 spot [40 mcg., chloroform–methanol (3:1)]; UV: λ_{max} 252 (ε 10,800) and 262 (sh) at pH 1 and 250–254 (ε 11,400) and 262 (sh) at pH 7 and 13; mass spectrum[5] (direct-probe inlet temperature 250°): *m/e* 280 (M^+), 263 (M − OH), 262 (M − H_2O), 252 (M − CO), 249 (M − CH_2OH), 233, 231 (M − CH_2OH − H_2O), 221 (M − CH_2OH − CO), 205, 193, 177 (P + C_2H_4), 163, 151 (P + 2H, base peak), 150 (P + H), and 149 (P); PMR[7] (dimethyl sulfoxide-d_6): δ 1.6–about 2.5 (m, H*a*H*a*H*b*), 3.4–3.63 (m, H*c*H*c*), 3.78–3.96 (m, H*d*), 4.1 (s, CH_3O), 4.24–4.5 (m, H*e*), 4.56–4.98 (m, H*f* + 3OH), 8.44 (s, H_8 of purine ring), 8.50 (s, H_2 of purine ring), and 4.62–5.04 (after addition of D_2O, m, H*f*). A specimen for analysis was dried at 110°.

Anal.—Calc. for $C_{12}H_{16}N_4O_4$: C, 51.40; H, 5.75; N, 19.99. Found: C, 51.15; H, 5.48; N, 19.60.

(±)-*N*-{9-[*trans*-2,*trans*-3-Dihydroxy-*cis*-4-(hydroxymethyl)cyclopentyl]-9*H*-purin-6-yl}butyramide Tributyrate (XIII)—An anhydrous mixture of 500 mg. of II, 10 ml. of pyridine, and 7.5 ml. of butyric anhydride was stirred at room temperature for 2 days and then concentrated *in vacuo* to a solid residue. White crystalline XIII was obtained by triturating the residue with ethanol–hexane (1:9) and recrystallizing the crude product (821 mg.) from ethyl acetate–hexane (4:15), yielding 723 mg. (70%), m.p. 116–117°; UV: λ_{max} 282 (ε 17,800) at pH 1.

Anal.—Calc. for $C_{27}H_{39}N_5O_7$: C, 59.43; H, 7.21; N, 12.84. Found: C, 59.25; H, 7.24; N, 12.87.

(±)-*trans*-3-(6-Amino-9*H*-purin-9-yl)-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol Tributyrate (XIV) Picrate—A solution of 190 mg. of XIII, 229 mg. of picric acid, and 11 ml. of methanol was heated under reflux for 45 min. A yellow precipitate was collected by filtration, washed with methanol, and recrystallized from an ethanol–acetone mixture, yielding 180 mg. (73%), m.p. 190–195°.

Anal.—Calc. for $C_{23}H_{33}N_5O_6 \cdot C_6H_3N_3O_7$: C, 49.43; H, 5.17; N, 15.92. Found: C, 49.25; H, 5.03; N, 15.96.

(±)-*trans*-3-(6-Amino-9*H*-purin-9-yl)-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol 5-Dihydrogen Phosphate (XV)—A solution of 12 ml. of trimethyl phosphate (19, 20), 0.732 ml. (8 mmoles) of phosphorus oxychloride, and 1.188 g. (3.9 mmoles) of the isopropylidene derivative (2) of II was prepared at −10°, stirred for 3 hr. at from −5 to −10°, and poured into 350 ml. of water. The resulting solution was allowed to stand at 25° for 1 hr., neutralized with 2.5 *N* aqueous ammonia, and passed through a column of a cation-exchange resin[8] (20 g.). The column was washed with 300 ml. of water and then with 400 ml. of 1 *N* aqueous ammonia. The ammonia effluent was concentrated *in vacuo* to a low volume (about 10 ml.), and ethanol (100 ml.) was added and the evaporation continued. Then additional ethanol (100 ml.) was added, and a white precipitate was collected by filtration and dried *in vacuo* over phosphorus pentoxide, yielding 1.036 g. (70%, calculated as a monohydrate of the ammonium salt of XV[9]). A solution of 1 g. of this material in 2 ml. of water was neutralized with 0.5 ml. of 6 *N* hydrochloric acid, diluted with 5 ml. of ethanol, and cooled to −20°. The white precipitate was dried *in vacuo* at 78° over phosphorus pentoxide, yielding 901 mg. (adjusted yield 67%), m.p. 250–252° dec.; TLC, 1 spot [60 mcg. on cellulose, butanol–water–acetic acid (5:3:2), detection by UV light after spraying[10]]. A specimen was recrystallized from water–ethanol and dried as before, m.p. 251–253° dec.; UV: λ_{max} 258 (ε 14,500) and 212 (ε 21,000) at pH 1 and 261 (ε 15,100) and 204 (ε 21,900) at pH 7.

Anal.—Calc. for $C_{11}H_{16}N_5O_6P \cdot {}^1/_2H_2O$: C, 37.29; H, 4.84; N, 19.77; P, 8.74. Found: C, 37.43; H, 4.60; N, 19.56; P, 8.64.

(±)-*trans*-3-(6-Amino-9*H*-purin-9-yl)-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol Cyclic 1,5-Hydrogen Phosphate (XVI)—A solution of 353 mg. of II-phosphate (XV), 293 mg. of 4-morpholino-*N*,*N*′-dicyclohexylcarboxamidine, 25 ml. of pyridine, and 5 ml. of water was evaporated to dryness *in vacuo*, and three 15-ml. portions of pyridine were added and evaporated separately *in vacuo* to remove water. A solution of the residue in 100 ml. of anhydrous pyridine was added dropwise during 3 hr. to a refluxing solution of 412 mg. of dicyclohexylcarbodiimide in 100 ml. of anhydrous pyridine, and the mixture was heated under reflux for an additional hour and then evaporated to dryness *in vacuo*. A mixture of the residue and water (250 ml.) was allowed to stand at room temperature for about 2 hr. and then concentrated *in vacuo* to dryness. Equal volumes of water and ether (100 ml.) were added, the mixture was shaken and filtered to remove dicyclohexylurea (m.p. 222–227°), and the aqueous layer was evaporated *in vacuo* to a yellow syrup. A solution of the syrup in 25 ml. of water was stirred with 3 g. of a strongly basic ion-exchange resin[11] for 15 min., the resin was separated by filtration and washed with water (10 × 3 ml.), and the product was recovered by stirring the resin with 175 ml. of 25% acetic acid. The aqueous acetic acid filtrate was concentrated *in vacuo* to a small volume (10 ml.), diluted with ethanol (10 ml.), chilled, and filtered to remove the white precipitate which was dried *in vacuo* at 78°, yielding 259 mg. (79%). The cyclic phosphate (XVI) was recrystallized from water (40 ml.) and dried *in vacuo* at 110°, yielding 198 mg. (61%), m.p. 268–272° dec.; UV: λ_{max} 258

[7] The positions of the protons are shown in Structure XVII.

[8] Amberlite CG-120, H^+ form.

[9] Analytical data from a similar product of another experiment were in approximate agreement with the monoammonium salt monohydrate.

[10] With Ultraphor.

[11] Rexyn 201, OH^- form.

(ε 13,500) and 211 (ε 20,600) at pH 1 and 261 (ε 14,100) and 205 (ε 20,400) at pH 7; TLC [silica gel, detection by UV and spraying[10], propanol–water (3:1)], XVI moved similarly to II and faster than XV; TLC [butanol–water–acetic acid (5:3:2)], XVI moved slower than II and slightly faster than XV.

Anal.—Calc. for $C_{11}H_{14}N_5O_5P$: C, 40.37; H, 4.31; N, 21.44; P, 9.46. Found: C, 39.98; H, 4.47; N, 21.71; P, 9.37.

(±)-*trans*-3-(6-Amino-9*H*-purin-9-yl)-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol (II)—This compound was obtained in better yield and by a simpler procedure by beginning with purified VI (*cf.* 2). A mixture of 1.215 g. of VI and 20 ml. of liquid ammonia was heated in a glass-lined, stainless steel bomb at 60° for 20 hr. and then at 80° for 1 hr. The residue obtained by evaporating the ammonia with a current of nitrogen was stirred with 20 ml. of warm 90% ethanol. Compound II (885 mg., m.p. 238–247° dec.) was separated by filtration, recrystallized from water (35 ml.), and washed with ethanol, yielding 768 mg. (white crystals), m.p. 245–247° dec. A second crop (113 mg., m.p. 231–240° dec.) of II from the 90% ethanol filtrate was recrystallized from water, yielding 86 mg., m.p. 244–248° dec. TLC and UV data showed that both portions (total yield 75%) were pure II.

(±)-*trans*-3-(Hydroxymethyl)-*trans*-5-[6-(methylthio)-9*H*-purin-9-yl]-*cis*-1,2-cyclopentanediol (V)—This compound was prepared by an improved procedure (*cf.* 2). A solution consisting of 780 mg. of purified VI, 30 ml. of anhydrous methanol (dried with molecular sieves), and 5.5 ml. of a 1 *N* methanol solution of sodium methoxide was saturated with methanethiol at 5°. The solution was heated at 100° in a glass-lined, stainless steel bomb for 18 hr. and then concentrated to dryness *in vacuo*. A water solution (25 ml.) of the residue was acidified to pH 3 with 1 *N* HCl, neutralized by the addition of solid sodium bicarbonate[12], and evaporated to dryness *in vacuo*. The residual solid was leached repeatedly (10 times) with a boiling ethyl acetate–acetone (4:1) mixture. The total extract was concentrated *in vacuo* to about 10 ml., and the precipitate (630 mg.) was separated by filtration. Recrystallization of the crude product from ethanol gave 605 mg. (74%) of white crystals, m.p. 179–181° dec.; mass spectrum (direct-probe inlet temperature 270°): *m/e* 296 (M^+), 279 (M − OH), 278 (M − H_2O), 265 (M − CH_2OH), 249, 247 (M − CH_2OH − H_2O), 237 (M − CH_2OH − CO), 231, 222, 221, 209, 193 (P + C_2H_4), 179, 167 (P + 2H, base peak), 166 (P + H), and 165 (P); PMR[7] (dimethyl sulfoxide-d_6): δ 1.64–about 2.5 (m, H*a*H*a*H*b*), 2.68 (s, CH_3S), 3.42–3.64 (m, H*c*H*c*), 3.8–3.96 (m, H*d*), 4.24–4.52 (m, H*e*), 4.58–5.06 (m, H*f* + 3OH), 8.53 (s, H_8 of purine ring), 8.7 (s, H_2 of purine ring), and 4.66–5.06 (after addition of D_2O, m, H*f*). A very weak singlet at 4.1 p.p.m. indicated that this sample contained about 1% of the 6-methoxypurine derivative (X).

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[12] If the solution is not neutralized after acidic decomposition of the excess sodium methylmercaptide, residual acid may catalyze the reaction of V with the leaching solvents; mass spectra of the products of earlier experiments in which sodium bicarbonate was not added prior to evaporation of solvents showed the presence of small amounts of a monoacetate of V (formed by transesterification) and the isopropylidene derivative of V.

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Toxicological and Pharmacological Actions of Methacrylate Monomers II: Effects on Isolated Guinea Pig Ileum

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Abstract □ Methacrylic acid and 12 methacrylate esters were tested for their effects upon contraction of the isolated guinea pig ileum. Each compound was employed at three concentration levels, ranging from 1:500 to 1:100,000 (v/v), depending upon activity and solubility of the compounds. Most of the compounds, except dimethylaminoethyl methacrylate, produced an inhibition of spontaneous contractions of the isolated ileum which antagonized the stimulant actions of both acetylcholine and barium chloride. Although most of the methacrylate effects were readily washed out, 1,3-butylene dimethacrylate produced a relatively irreversible response. Dimethylaminoethyl methacrylate stimulated the isolated ileum, an effect that was not blocked by 0.1–1 mcg. of atropine/ml. of tissue bath fluid. Those compounds producing inhibition were ranked in order of their relative activities in antagonizing acetylcholine- or barium-induced contraction by determining the approximate molar ratio (*i.e.*, molar concentration of methacrylate per molar concentration of acetylcholine or barium chloride) required to produce 50% inhibition of the stimulant effect of acetylcholine or barium chloride.

Keyphrases □ Methacrylate monomers—effects on isolated guinea pig ileum, acetylcholine- and barium-induced contractions □ Smooth muscle relaxation—effects of methacrylate monomers on isolated guinea pig ileum, acetylcholine- and barium-induced contractions

The polymerized acrylates, particularly methyl methacrylate, find a number of applications as biomedical materials, especially in dentistry and orthopedic surgery. Acute lethality of the various monomers shows considerable variability. The LD_{50} values in mice for the specific samples employed in this study were presented in a previous paper (1). Deichmann (2) and Spealman *et al.* (3) reported that the acute lethal effect in laboratory animals was due to respiratory depression for monomeric methyl methacrylate and some related esters. Various aspects relating to other potential toxicity problems with methacrylates were presented by a number of workers (4–9). Previous work on the methacrylate monomers conducted in these laboratories included investigations into the use of mathematical models to predict acute toxicity (10), alteration of drug biological half-life by methacrylate inhalation (11), embryo–fetal toxicity and teratogenic effects in rats (12), and effects upon the isolated, perfused rabbit heart (1).

MATERIALS AND METHODS

Materials—Methacrylic acid and 12 methacrylate esters were tested on the isolated guinea pig ileum. The following compounds were included: methacrylic acid[1], 1,3-butylene dimethacrylate[1], and the methyl[1], ethyl[1], *n*-butyl[1], isobutyl[1], isodecyl[1], lauryl[1] (dodecyl), hydroxyethyl[1], *tert*-butylaminoethyl[1], dimethylaminoethyl[1], *n*-propyl[2], and 2-ethylhexyl[2] methacrylates.

Methods—Guinea pigs (300–500 g.) of either sex were sacrificed and exsanguinated, and the intestine was promptly exposed *via* a midline incision. An actively contracting loop of ileum, 4–6 cm. in length, was removed and placed in oxygenated Tyrode's solution. A 1.5–2-cm. section of ileum was suspended in oxygenated (95% oxygen + 5% carbon dioxide) Tyrode's solution in a 25-ml. chamber of an isolated organ-tissue bath[3], which was maintained at a constant temperature of 37 ± 0.3°. One end of the intestine was attached to a glass holder and oxygenator; the other end was attached to a force-displacement transducer[4], which was electrically connected to a polygraph[5] to produce a permanent record of the response. To maintain uniform sensitivity, the polygraph was calibrated to produce a 1-cm. pen deflection/g. of tension applied to the transducer.

In all cases the spontaneous activity of the intestine in Tyrode's solution was recorded; the methacrylate was then added to the bath and thise response was recorded. The methacrylate was administered by forceably dispensing the measured quantity of test compound from a syringe with a small bore needle, the tip of which was placed below the liquid surface of the chamber. Preliminary experiments were conducted to determine the concentration of methacrylate that would produce a measurable effect upon the isolated ileum; the other concentrations employed were two and four times as great as the initially determined one.

RESULTS

The responses of the guinea pig ileum to treatment with nine of these 13 compounds are presented in Table I. Three of the other four compounds, isodecyl, 2-ethylhexyl, and lauryl methacrylates, were rather insoluble in Tyrode's solution; it was, therefore, not possible to obtain solutions sufficiently concentrated to produce an observable response. The other compound, 1,3-butylene dimethacrylate, tended to produce relaxation of the smooth muscle but, unlike the majority of these compounds, the effect was very slow in onset. This slow, relatively mild effect did not lend itself well to quantitative measurement.

Concentrations of 1:1000 and 1:500 (v/v) of 1,3-butylene dimethacrylate exhibited a latent period of about 5 min., which was followed during the next 5 min. or so by a gradual decrease in pendular movements and a slow relaxation of the muscle. After the maximum effect developed, it appeared to be irreversible since the muscle would not return to control levels of activity even after repeated washing with Tyrode's solution. The activity of 1,3-butylene dimethacrylate partially antagonized neurogenic stimulation by acetylcholine (0.1 mcg./ml.) and myogenic stimulation by barium chloride (30 mcg./ml.).

Eight of the other nine compounds produced prompt (within 15–30 sec.) and qualitatively rather uniform effects, consisting of inhibition of pendular movements and relaxation of the muscle. The effect of each compound could be terminated by promptly washing the ileum with fresh Tyrode's solution. All of these compounds produced a concentration-dependent inhibition of the usual effects of acetylcholine and barium chloride upon the isolated ileum.

Dimethylaminoethyl methacrylate acted differently, both qualitatively and quantitatively, from the other compounds tested. While the other compounds either produced an inhibition of smooth muscle activity or had no effect on its activity, dimethylaminoethyl methacrylate produced contraction of the ileum. This smooth

[1] Rohm & Haas, Philadelphia, Pa.
[2] K&K Laboratories, Inc., Plainview, N.Y.
[3] Phipps & Bird, Richmond, Va.
[4] FT03C, Grass Instrument Co., Quincy, Mass.
[5] Grass model 7.

Table I—Effects of Methacrylates on the Isolated Guinea Pig Ileum and upon Acetylcholine- and Barium Chloride-Induced Contractions

Concentration in Tissue Bath (v/v)	Response to Methacrylate, mm.[a]	Response to Acetylcholine[b], mm.[a]	Response to Methacrylate and Acetylcholine, mm.[a]	Inhibiton of Acetylcholine Response, %	Response to Barium Chloride[b], mm.[a]	Response to Methacrylate and Barium Chloride[a], mm.	Inhibition of Barium Chloride Response, %
Methacrylic acid		+31.6 (3)[c]			+30.0 (3)		
1:10,000	−8.2 (5)		+25.5 (2)	19.3		+26.5 (2)	11.6
1:5000	−13.6 (5)		+16.5 (2)	47.7		+16.5 (2)	45.0
1:2500	−22.0 (5)		+2.0 (2)	93.6		+5.5 (2)	81.6
Methyl methacrylate		+35.6 (3)			+31.6 (3)		
1:2000	−9.4 (5)		+29.5 (2)	17.1		+26.5 (2)	16.1
1:1000	−15.4 (5)		+16.0 (2)	55.0		+16.0 (2)	49.3
1:500	−30.2 (5)		+4.5 (2)	87.3		+3.0 (2)	90.5
Ethyl methacrylate		+30.6 (3)			+32.6 (3)		
1:2000	−7.6 (5)		+29.5 (2)	5.2		+29.0 (2)	11.0
1:1000	−14.6 (5)		+15.5 (2)	49.3		+16.5 (2)	49.3
1:500	−23.0 (5)		+2.5 (2)	91.8		+3.5 (2)	89.2
n-Propyl methacrylate		+29.6 (3)			+32.0 (3)		
1:2000	−7.2 (5)		+23.2 (2)	21.6		+27.5 (2)	15.6
1:1000	−16.2 (5)		+15.0 (2)	44.3		+17.5 (2)	45.3
1:500	−23.6 (5)		+4.5 (2)	84.8		+6.0 (2)	81.2
n-Butyl methacrylate		+35.6 (3)			+31.3 (3)		
1:2000	−10.6 (5)		+29.0 (2)	18.5		−29.2 (2)	7.3
1:1000	−18.0 (5)		+19.5 (2)	45.2		+15.5 (2)	50.4
1:500	−24.0 (5)		+4.0 (2)	88.7		+3.5 (2)	88.8
Isobutyl methacrylate		+34.6 (3)			+31.0 (3)		
1:2000	−14.4 (5)		+28.5 (2)	17.6		+25.5 (2)	17.7
1:1000	−18.8 (5)		+17.5 (2)	49.4		+15.5 (2)	50.0
1:500	−25.6 (5)		+2.5 (2)	92.7		+2.0 (2)	93.5
"Substituted" methacrylates							
Hydroxyethyl methacrylate		+38.3 (3)			+33.3 (3)		
1:10,000	−11.2 (5)		+27.5 (2)	28.1		+23.5 (2)	36.4
1:5000	−17.8 (5)		+16.5 (2)	56.9		+17.5 (2)	52.7
1:2500	−26.0 (5)		+0.0 (2)	100.0		+0.0 (2)	100.0
tert-Butylaminoethyl methacrylate		+37.6 (3)			+33.3 (3)		
1:10,000	−11.8 (5)		+31.0 (2)	17.5		+27.5 (2)	17.4
1:5000	−19.2 (5)		+16.5 (2)	56.1		+15.0 (2)	54.9
1:2500	−26.4 (5)		+0.0 (2)	100.0		+0.0 (2)	100.0
Dimethylaminoethyl methacrylate		n/a[d]			n/a		
1:100,000	+23.5 (5)		n/a	n/a		n/a	n/a
1:50,000	+32.4 (5)		n/a	n/a		n/a	n/a
1:25,000	+35.2 (5)		n/a	n/a		n/a	n/a
			(This contraction was not blocked by 1×10^{-7} or 10^{-6} atropine sulfate)				

[a] + = contraction; − = relaxation. 10 mm. ≈ 1 g. tension. [b] Acetylcholine concentration = 1:10,000,000; barium chloride concentration = 3:100,000 (w/v). [c] Number of observations in parentheses. [d] n/a = not applicable.

muscle stimulation was not blocked by 0.1 mcg./ml. of atropine sulfate. Of the other compounds tested, hydroxyethyl and *tert*-butylaminoethyl methacrylates were the most potent, inhibiting ileum contraction by about 11 mm. at a 1:10,000 dilution, while dimethylaminoethyl methacrylate produced about 23 mm. contraction at a dilution of 1:100,000 (v/v).

DISCUSSION

Most of the methacrylates examined produced a depressant effect upon spontaneous motility of the isolated guinea pig ileum, and generally a good dose-response (concentration-dependent) relationship was observed (eight of the nine compounds listed in Table I). These eight compounds also demonstrated a good concentration-dependent antagonism of the neurogenic and myogenic stimulant effects of acetylcholine and barium chloride upon the isolated ileum. With the concentrations used, from 5 to 100% of the stimulant effect of acetylcholine was blocked by prior administration of the methacrylate, and from 7 to 100% of the stimulant effect of barium chloride was blocked. These data suggest that the inhibitory effects of these methacrylates upon the isolated ileum are myogenic in origin. The effects of these eight methacrylates may be terminated by promptly washing the intestine with fresh Tyrode's solution.

1,3-Butylene dimethacrylate [1:1000 and 1:500 (v/v)] also produced a reduction in pendular movements and a relaxation of the intestine. Unlike those already discussed, where the effect occurred within 15–30 sec., this compound showed a latent period of about 5 min. before any effect could be observed, and then the effect developed very slowly over the next 5–10 min. When the effect had developed, it antagonized the stimulant effect of acetylcholine (0.1 mcg./ml.) and barium chloride (30 mcg./ml.) upon the muscle. Unlike the previously discussed compounds, the depressant effects of 1,3-butylene dimethacrylate were not dissipated by repeated washings with Tyrode's solution. This may be due to either a stronger binding of this compound to the receptor tissue of the muscle or a greater physical penetration (solubility related) of the compound into the tissue, since the onset of action was much slower with greater difficulty in "washing out" the effect.

The three least soluble members of the series, 2-ethylhexyl, isodecyl, and lauryl methacrylates, did not elicit a demonstrable effect by this procedure. Since the effects produced by a compound of limited solubility are dependent upon the quantity that will go into solution to reach its target site and the inherent activity of the compound at its target site, the combination of solubility and inherent activity must not have reached critical values in these experiments for the isolated ileum; thus, no effect was observed. Use of a more sensitive system for detection of the action of the methacrylates may lead to quantitative results for these compounds;

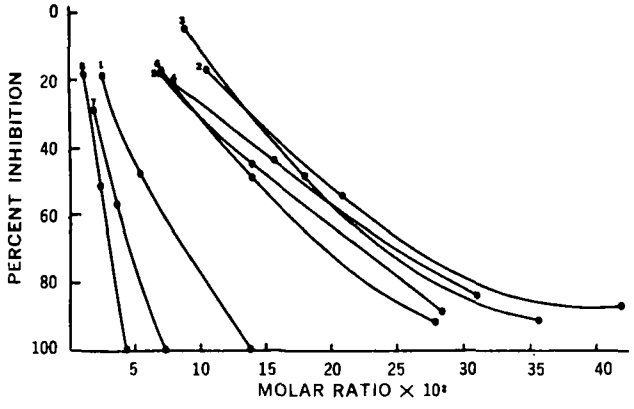

Figure 1—*Methacrylate inhibition of acetylcholine-induced contraction. Key: 1, methacrylic acid; 2, methyl methacrylate; 3, ethyl methacrylate; 4,* n*-propyl methacrylate; 5,* n*-butyl methacrylate; 6, isobutyl methacrylate; 7, hydroxyethyl methacrylate; and 8,* tert*-butylaminoethyl methacrylate.*

in the isolated heart, these compounds did demonstrate a measurable response (1).

These eight methacrylates may be grouped into two general orders of activity: (*a*) those in which the highest dilution used was 1:2000 (methyl, ethyl, *n*-propyl, *n*-butyl, and isobutyl methacrylates), and (*b*) those in which the highest dilution used was 1:10,000 (methacrylic acid and the "substituted" methacrylates, hydroxyethyl and *tert*-butylaminoethyl).

Figure 1 shows the antagonistic effect of the methacrylates upon acetylcholine-induced contraction of the isolated ileum. In this figure the molar ratio (molar concentration of the methacrylate per molar concentration of acetylcholine) is plotted against percent inhibition of the acetylcholine response. The standard concentration of acetylcholine bromide (0.1 mcg./ml.) employed in these studies represents 4.422×10^{-7} mole/l.

Figure 2 presents a similar graph showing the antagonistic effect of the methacrylates upon barium chloride-induced contraction of the isolated ileum. Again, the molar ratio (molar concentration of methacrylate per molar concentration of barium chloride) is plotted against percent inhibition of the barium chloride response. The standard concentration of barium chloride (30 mcg./ml.) used in these studies represented 1.228×10^{-4} mole/l.

The approximate molar ratios required for the compounds to produce 50% inhibition of the acetylcholine and barium chloride responses were estimated from Figs. 1 and 2, arranged in order of decreasing potency, and listed in Table II. The lower the molar ratio required to provide 50% inhibition, the more active is the compound in inhibiting contractions produced by the stimulant agent. Since these curves are constructed from only three data points, they must be considered as estimates; however, they do clearly represent the order of magnitude involved in the antagonism.

The activity of dimethylaminoethyl methacrylate was atypical of the series of compounds investigated by differing both in terms of

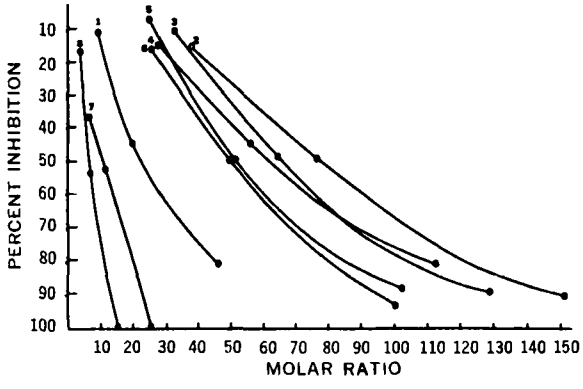

Figure 2—*Methacrylate inhibition of barium chloride-induced contraction. For key, see Fig. 1.*

Table II—Molar Ratio[a] to Produce 50% Inhibition of Induced Contraction

Compound	Acetylcholine	Barium Chloride
tert-Butylaminoethyl methacrylate	2,250	6.5
Hydroxyethyl methacrylate	3,250	10.0
Methacrylic acid	5,750	22.0
Isobutyl methacrylate	14,125	50.0
n-Butyl methacrylate	15,500	51.0
n-Propyl methacrylate	17,375	61.0
Ethyl methacrylate	18,125	65.0
Methyl methacrylate	19,375	76.5

[a] Molar ratio = molar concentration of methacrylate per molar concentration of stimulating agent at point where contraction is 50% of control contraction.

relative potency and type of action. Unlike the others, this compound produced contraction of the isolated ileum, and it was active in more dilute solutions than the others. At a concentration of 1:100,000 (v/v) (0.000059 mole/l.), dimethylaminoethyl methacrylate produced a significant contraction of the ileum. Pretreatment of the guinea pig ileum with atropine sulfate [1×10^{-7} and 10^{-6} (w/v) in the tissue bath] did not block or inhibit the contraction produced by this dimethylaminoethyl derivative. This would imply a myogenic effect, as suggested for the other homologs; but unlike the others, dimethylaminoethyl methacrylate stimulated the ileum to contract, while the other methacrylates inhibited such contractions.

SUMMARY AND CONCLUSIONS

The effects observed in this study, in which methyl methacrylate and most of the other monomers tested produced relaxation of the smooth muscle of the guinea pig ileum, are not inconsistent with the reports of Homsy *et al.* (9), who reported a fall in blood pressure of the dog by various concentrations of methyl methacrylate, or of Mir *et al.* (1), who observed a relative increase in coronary flow in the isolated rabbit heart following perfusion with methyl methacrylate and most of the other homologs included in this study. This latter response would suggest a direct vasodilator effect of the compounds upon the coronary circulation, although cardiac slowing *per se* may contribute to this effect by allowing more complete filling of coronary vessels prior to contraction. The work of Mir *et al.* (1) also showed that the pacemaker of the isolated heart was slowed and that the force of myocardial contraction was reduced when the heart was perfused with a solution containing the methacrylate monomer. While the hypotensive effect of methyl methacrylate on blood pressure in the dog (9) and possibly some hypotensive responses reported from the clinical use of self-curing acrylic cement (methyl methacrylate) in orthopedic surgery (8, 13, 14) may be due to a combination of effects, the reduction in tone of the smooth muscle of the arterioles, and hence vasodilation, must be considered as a possible primary or contributory factor in such hypotensive responses.

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Tetracycline Binding to Bovine Serum Albumin Studied by Fluorescent Techniques

JOSEPH K. H. MA, H. W. JUN, and LOUIS A. LUZZI▲

Abstract □ Binding of demeclocycline and oxytetracycline to bovine serum albumin was studied using fluorescent methods. The mechanism of binding for tetracyclines is shown to be hydrophobic. The number of binding sites and the equilibrium constants were calculated over a range of protein concentrations. Two strong binding sites, at or near the tryptophan residues of bovine serum albumin, were found for both tetracyclines. The equilibrium constants for tetracyclines increased with increasing protein concentration, and the number of binding sites on the protein decreased with increasing protein concentration. These findings suggest the possibility of a sharing of one tetracycline molecule by more than one protein molecule at relatively high protein concentrations.

Keyphrases □ Demeclocycline—mechanism of binding to bovine serum albumin studied using fluorescent techniques □ Oxytetracycline—mechanism of binding to bovine serum albumin studied using fluorescent techniques □ Tetracyclines, demeclocycline and oxytetracycline—binding to bovine serum albumin, fluorescent techniques □ Serum protein binding—demeclocycline and oxytetracycline, fluorescent techniques □ Fluorescent techniques, probe and quenching titration—used to study mechanism of binding of demeclocycline and oxytetracycline to bovine serum albumin

The binding of drugs by plasma proteins has been recognized as an important factor in drug availability, drug efficacy, and drug transport for many years (1). Many experimental techniques have been employed to study drug–protein interactions (2–4). The fluorescence probe technique (5, 6) has recently been employed to study the mechanism of drug–protein binding and the nature of binding sites. This technique has also been very useful in biochemical research related to binding (7–9). The number of probes available at this time is limited; to explore the use of probes in binding studies more fully, it is necessary to search for new probe compounds. Therefore, the fluorescence studies of tetracyclines and bovine serum albumin were carried out in this laboratory.

Tetracyclines are fluorescent compounds with the excitation and emission maxima in the range of 390 and 520 nm., respectively. Ibsen and Urist (10) reported that the excitation maximum of tetracyclines varies with the presence of metal ions. They found that increasing metal concentration produced a bathochromic shift, indicating the formation of metal complexes. The quantum yields of several tetracyclines in various solvents and of their protein mixtures were reported by Popov *et al.* (11, 12). In a recent paper (13), they also studied the interaction of tetracyclines with bovine serum albumin using a fluorescence technique. The increase of the fluorescence of tetracyclines accompanied by an auxochromic shift of the fluorescence maxima in the presence of protein and in nonpolar solvents suggests that the tetracyclines exhibit the main features of fluorescence probes.

Fluorescence quenching titration is another sensitive method for the study of drug–protein molecular interactions. The procedure has been applied to many systems (14–16) and was best described by Chignell (17). It was found that the native fluorescence of bovine serum albumin was quenched by most of the tetracyclines. To examine the possible use of tetracyclines as probe compounds for drug–protein binding studies, the nature of binding of demeclocycline and oxytetracycline to bovine serum albumin was studied by both methods.

EXPERIMENTAL

Materials—Demeclocycline hydrochloride[1], oxytetracycline hydrochloride[2], and crystalline bovine serum albumin[3] were obtained from commercial sources. Methanol[4] was spectroscopic grade, and all other chemicals were reagent grade or of special purity. All chemicals were used without further purification.

Apparatus—Fluorescence measurements were made with a spectrophotofluorometer[5] equipped with a 150-w. xenon lamp and 1P21 photomultiplier tube. The relative fluorescence intensities were recorded directly from fluorometer readings.

Fluorescent Titration—The fluorescence and quenching titrations were performed manually with microsyringes[6]. Two milliliters of

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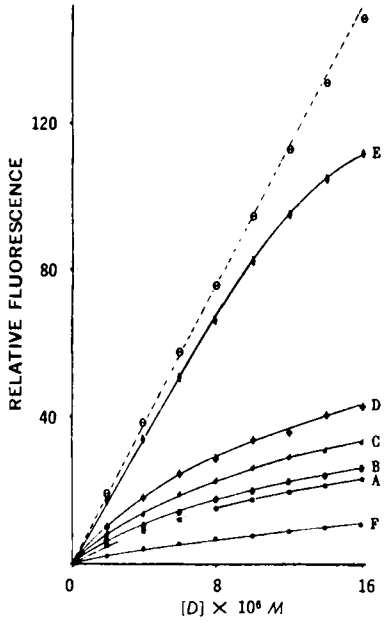

Figure 1—*Plots of demeclocycline fluorescence intensities as a function of demeclocycline concentrations,* [D], *at (A) 5.095 × 10⁻⁶ M, (B) 7.99 × 10⁻⁶ M, (C) 13.61 × 10⁻⁶ M, (D) 19.55 × 10⁻⁶ M, and (E) 136.1 × 10⁻⁶ M bovine serum albumin, and (F) without bovine serum albumin. The values of* I_b *are plotted in dashed line.*

each protein solution was titrated with successive additions of 2 μl. of drug solution (2×10^{-3} *M*) in methanol. Methanol did not affect the binding of the drug in the concentration range used. The protein solutions were prepared in pH 7.4 phosphate buffer. Concentrations of protein solutions were determined by measuring the absorbance at 280 nm. and using $a = 0.66$ (18); the molecular weight of 69,000 was used to determine the molar concentrations. All titrations were carried out at 25 ± 0.1°. The temperature was controlled by the use of thermostated cell compartments.

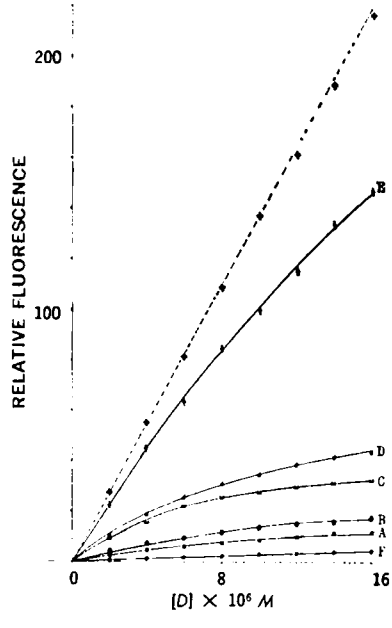

Figure 2—*Plots of oxytetracycline fluorescence intensities as function of oxytetracycline concentrations,* [D], *at (A) 1.436 × 10⁻⁶ M, (B) 7.436 × 10⁻⁶ M, (C) 14.18 × 10⁻⁶ M, (D) 21.62 × 10⁻⁶ M, and (E) 143.6 × 10⁻⁶ M bovine serum albumin, and (F) without bovine serum albumin. The values of* I_b *are plotted in dashed line.*

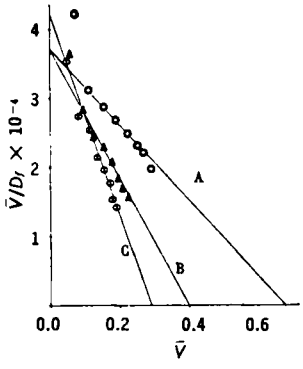

Figure 3—*Plots of* $\bar{V}/D_f$ versus $\bar{V}$ *for the demeclocycline-bovine serum albumin system in the demeclocycline concentration range of 2–16 × 10⁻⁶ M at (A) 5.095 × 10⁻⁶ M, (B) 7.99 × 10⁻⁶ M, and (C) 13.61 × 10⁻⁶ M bovine serum albumin.*

Treatment of Data—The fluorescence enhancement of the drug, upon addition to bovine serum albumin, was determined, and these data were used to calculate the binding constants for the albumin–drug complexes. The fraction of bound drug, X, was calculated from the following equation (7):

$$X = (I - I_f)/(I_b - I_f) \qquad \text{(Eq. 1)}$$

where I and I_f refer to the fluorescence intensities of a given concentration of drug in solutions of low protein concentration and in solutions without protein, respectively; I_b refers to the fluorescence intensity of the same concentration of drug in solutions of high protein concentration, which gives the fluorescence intensity of the drug in the presence of excess binding sites. In this study, the fluorescence titrations were carried out at several different protein concentrations, and the values of I_b were taken to be the extrapolated values of the plots of $1/I$ *versus* $1/[P]$, where $[P]$ represents the concentration of protein. After the value X is found for each point along the titration curve, the Scatchard equation (19) may be applied to determine the association constant of the drug:

$$\bar{V}/D_f = nK_a - \bar{V}K_a \qquad \text{(Eq. 2)}$$

where $\bar{V}$ is the number of moles of bound drug per mole of protein, D_f is the concentration of free drug, n is the number of binding sites on the protein, and K_a is the association constant of drug. The $\bar{V}$ is determined by multiplying the value for X by the ratio of the total drug concentration to the total protein concentration in solution. When $\bar{V}/D_f$ is plotted against $\bar{V}$, a straight line is obtained with a slope equal to $-K_a$. The ordinate and abscissa intercepts of this line give nK_a and n, respectively. The Scatchard equation assumes that the intrinsic association constants for all binding sites are equal.

In the fluorescence quenching titration method, titrations are made over a range of protein concentrations. The intensities, expressed as a percentage of the initial fluorescence of the protein, are then plotted as a function of the drug–protein ratio. Above a certain critical protein concentration, the titration curves are superimpos-

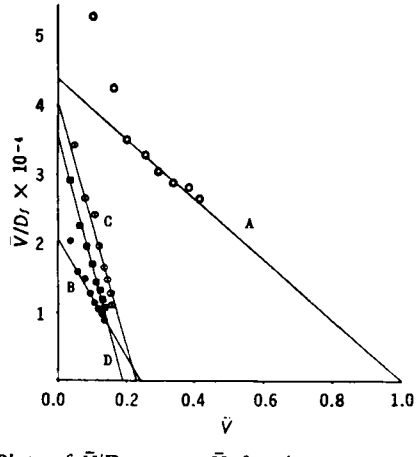

Figure 4—*Plots of* $\bar{V}/D_f$ versus $\bar{V}$ *for the oxytetracycline-bovine serum albumin system in the oxytetracycline concentration range of 2–16 × 10⁻⁶ M at (A) 1.436 × 10⁻⁶ M, (B) 7.436 × 10⁻⁶ M, (C) 14.18 × 10⁻⁶ M, and (D) 21.62 × 10⁻⁶ M bovine serum albumin.*

Table I—Equilibrium Constants and n Values for Binding of Demeclocycline and Oxytetracycline to Bovine Serum Albumin

$[P] \times 10^6$ M	$[D] \times 10^6$ M	K_a	n
Demeclocycline–Bovine Serum Albumin System			
5.095	2–16	5.456×10^4	0.68
7.99	2–16	9.200×10^4	0.40
13.61	2–16	14.450×10^4	0.29
1.377[a]	8–80	0.781×10^4	2.00
11.02[a]	8–80	0.858×10^4	2.00
13.77[a]	8–80	0.858×10^4	2.00
27.54[a]	8–80	0.858×10^4	2.00
40.65[a]	8–80	1.130×10^4	1.96
68.87[a]	8–80	1.620×10^4	1.80
Oxytetracycline–Bovine Serum Albumin System			
1.436	2–16	4.390×10^4	1.00
7.436	2–16	8.360×10^4	0.24
14.180	2–16	17.320×10^4	0.23
21.620	2–16	18.740×10^4	0.19
1.377[a]	8–80	0.743×10^4	2.00
14.060[a]	8–80	0.775×10^4	2.00
25.340[a]	8–80	0.775×10^4	2.00
44.330[a]	8–80	0.950×10^4	1.79

[a] Calculated from quenching data.

able since all the drug is bound. The concentration of bound drug (D_b) for a solution containing the protein below this critical concentration can be calculated by the equation:

$$D_b = (D_t)(R_h)/(R_l) \qquad \text{(Eq. 3)}$$

where D_t is the total drug concentration of the solution, and R_h and R_l are the drug–protein ratios at the same intensity for high and low protein solutions, respectively. The results can then be graphically treated using the Scatchard equation.

RESULTS AND DISCUSSION

The excitation and emission maxima, λ_{ex} and λ_{em}, for demeclocycline and oxytetracycline were found to be 382 and 520 nm. and 375 and 515 nm., respectively, in the concentration range used in this study. In the presence of bovine serum albumin, an auxochromic shift of about 20 nm. for the emission maxima was observed for both tetracyclines. Increasing protein concentration also produced a bathochromic shift for the excitation maxima. The fluorescence titrations were carried out over a range of protein concentrations, and the excitation and emission maxima used for demeclocycline and oxytetracycline were 390 and 504 nm. and 384 and 490 nm., respectively. The fluorescence data of the demeclocycline–bovine serum albumin and the oxytetracycline–bovine serum albumin systems, in which the fluorescence intensities are plotted as a function of drug concentration, are shown in Figs. 1 and 2, respectively. The dashed lines represent the I_b values.

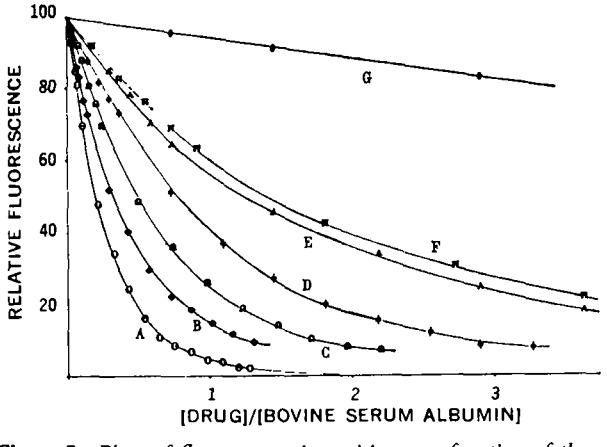

Figure 5—*Plots of fluorescence intensities as a function of the concentration ratios of demeclocycline and bovine serum albumin, where the bovine serum albumin concentrations for curves A, B, C, D, E, F, and G are 137.7 × 10⁻⁶ M, 68.87 × 10⁻⁶ M, 40.65 × 10⁻⁶ M, 27.54 × 10⁻⁶ M, 13.77 × 10⁻⁶ M, 11.02 × 10⁻⁶ M, and 1.377 × 10⁻⁶ M, respectively.*

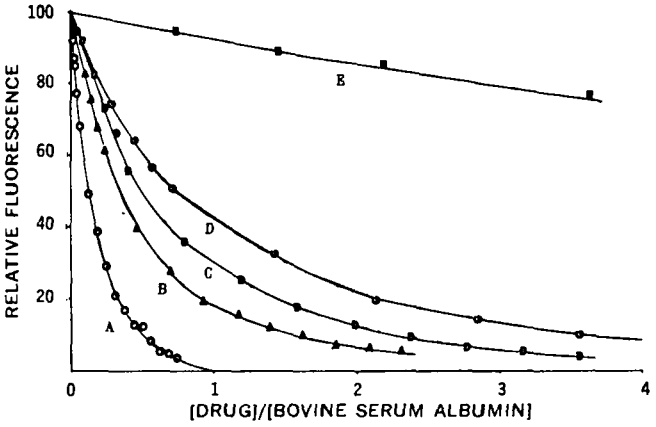

Figure 6—*Plots of fluorescence intensities as a function of the concentration ratios of oxytetracycline and bovine serum albumin where the bovine serum albumin concentrations for curves A, B, C, D, and E are 162.5 × 10⁻⁶ M, 44.33 × 10⁻⁶ M, 25.34 × 10⁻⁶ M, 14.06 × 10⁻⁶ M, and 1.377 × 10⁻⁶ M, respectively.*

The Scatchard plots for the binding of demeclocycline to bovine serum albumin are shown in Fig. 3. At different protein concentrations, the Scatchard plots gave straight lines corresponding to different n and K_a values. As shown in Table I, the equilibrium constant increased consistently with increasing protein concentration, whereas n decreased with increasing protein concentration. The same effect has also been observed in the binding of oxytetracycline to bovine serum albumin. The Scatchard plots of the oxytetracycline–bovine serum albumin system are shown in Fig. 4, with the corresponding n and K_a values listed in Table I.

The native fluorescence of bovine serum albumin was quenched by the addition of demeclocycline and oxytetracycline. The titration curves are shown in Figs. 5 and 6. The intensities, expressed as a percentage of the initial fluorescence of protein, are plotted as a function of the drug–protein ratio. The excitation and fluorescence maxima for all solutions were taken to be 285 and 354 nm., respectively. The concentration of bound drug and other parameters used for the Scatchard plot were calculated by the method previously described.

The plots of $\bar{V}/D_f$ *versus* $\bar{V}$ for the demeclocycline–bovine serum albumin system are shown in Fig. 7. Again, the equilibrium constant increased with increasing protein concentration, whereas the number of binding sites decreased with increasing protein concentration. The one exception is that a group of straight lines is superimposed

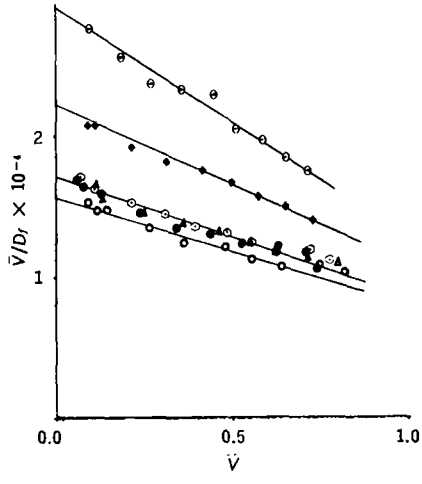

Figure 7—*Plots of* $\bar{V}/D_f$ versus $\bar{V}$ *for the demeclocycline–bovine serum albumin system in the demeclocycline concentration range of 8–80 × 10⁻⁶ M at various bovine serum albumin concentrations:* ○, *1.377 × 10⁻⁶ M;* △, *11.02 × 10⁻⁶ M;* ○, *13.77 × 10⁻⁶ M;* ○, *27.54 × 10⁻⁶ M;* Φ, *40.65 × 10⁻⁶ M; and* ⊙, *68.87 × 10⁻⁶ M.*

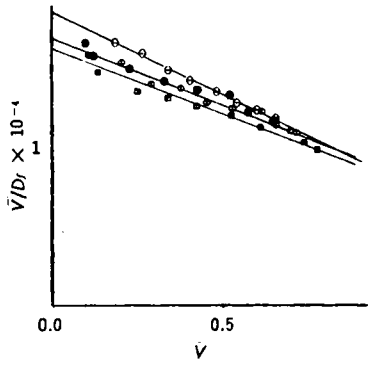

Figure 8—*Plots of* $\bar{V}/D_f$ versus $\bar{V}$ *for the oxytetracycline–bovine serum albumin system in the oxytetracycline concentration range of 8–80* $\times$ 10^{-6} M *at various bovine serum albumin concentrations:* ⊡, *1.377* $\times$ 10^{-6} M; ⊕, *14.06* $\times$ 10^{-6} M; ○, *25.34* $\times$ 10^{-6} M; *and* ⊙, *44.33* $\times$ 10^{-6} M.

upon each other in the protein concentration range of 11.02–27.54 $\times$ 10^{-6} *M*. Similar results were also found in the binding of tetracycline to bovine serum albumin. The values of n and K_a calculated from Figs. 7 and 8 are also shown in Table I; Fig. 8 shows the plots of $\bar{V}/D_f$ *versus* $\bar{V}$ for the binding of oxytetracycline to bovine serum albumin.

The binding information obtained from this study and the results recently reported by Popov *et al.* (13) show that the mechanism of binding for tetracyclines to bovine serum albumin is of a hydrophobic nature. The number of binding sites varies with varying tetracycline and protein concentrations, and the maximum number of strong or high affinity binding sites appears to be two. A quenching of tryptophan fluorescence, which is primarily responsible for the fluorescence of bovine serum albumin, by tetracyclines was also observed. Since bovine serum albumin is known to have two tryptophan binding sites per molecule (20), the quenching studies indicate that the binding sites are at or near the tryptophan residues. This is possible, since previous investigators (21, 22) showed that serum albumin has binding sites for small organic molecules which are located in the vicinity of the tryptophan residue. It is claimed that this is due to a clustering of hydrophobic apolar amino acids in the environment of tryptophan residues of these protein molecules.

The fact that the equilibrium constant is increasing with increasing protein concentration, together with the fact that n is concentration dependent, may suggest a sharing of one tetracycline molecule by more than one protein molecule in the binding. This observation is further substantiated by examination of Figs. 5 and 6, which show that at a certain drug–protein concentration ratio, the native fluorescence of protein is quenched more strongly at high protein concentration than at low protein concentration. In other words, the ratio of bound drug and total drug concentrations at high protein concentration is larger than that at low protein concentration.

The present studies show that the tetracyclines represent a class of compounds that can be used as probes for both techniques used in this investigation. The results also show that investigations at different protein concentrations are necessary to understand further the nature of binding.

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Metabolism of Clindamycin I: Absorption and Excretion of Clindamycin in Rat and Dog

F. F. SUN▲ and R. S. P. HSI

Abstract □ Clindamycin hydrochloride, randomly labeled with tritium, was synthesized and used for animal metabolism studies. The drug was administered to rats (oral and intraperitoneal routes) and dogs (oral and intramuscular routes) at a dose of 50–150 mg./kg. In both species, after the oral or parenteral drug administration, the excretion of radioactive materials in the urine (approximately one-third of the dose) and in the feces (approximately two-thirds of the dose) was independent of the route of administration. The initial rate of excretion for the oral dose was significantly faster than the parenteral dose. In the dog, peak plasma radioactivity concentrations were observed at 2 and 4 hr. after oral and intramuscular administration, respectively. The slower rate of absorption and excretion following parenteral compared to oral administration was believed due to precipitation of the drug at the injection site or a difference in transport processes. Based upon the identical areas under the plasma total radioactivity *versus* time curves, plus the fact that the distribution of radioactivity excreted in urine and feces was independent of the route of administration, it was concluded that after oral administration the absorption of this drug into the bloodstream was almost complete and that little appeared to pass through the GI tract for direct elimination.

Keyphrases □ Clindamycin hydrochloride, radiolabeled—absorption and excretion after oral and parenteral administration, rats, dogs □ Absorption, radiolabeled clindamycin hydrochloride—after oral and parenteral administration, rats, dogs □ Excretion, radiolabeled clindamycin hydrochloride—after oral and parenteral administration, rats, dogs

Clindamycin[1] (I), a new antibacterial agent produced by chlorination of lincomycin, exhibits approximately four- to eightfold the *in vitro* antibacterial activity of lincomycin against Gram-positive organisms. *In vitro* clindamycin gave significantly lower CD_{50} values than lincomycin with a number of organisms tested. In addition, this drug has been shown to be a promising antimalarial agent in both mice and monkeys. The chemistry and biological activities were reported previously by Birkenmeyer (1), Magerlein *et al.* (2), and Birkenmeyer and Kagan (3).

Studies on the absorption, excretion, and half-life of clindamycin administered orally (4) or intramuscularly (5) to human subjects were also reported previously. Microbiological assays were used for end-point measurement. It was found that an orally administered dose was absorbed quantitatively and extremely fast in man. The maximal serum activity was achieved within 45 min. postadministration, with a clearance half-life of 2.38 hr. On the other hand, the intramuscularly injected dose was absorbed rather slowly. The maximal serum activity was achieved 2 hr. after administration, with an elimination half-life of 4.76 hr. Local precipitation of the drug at the injection site was believed to be the cause of this absorption delay. Since the bioassay measures only the algebraic sum of the antibacterial activities of the drug and its metabolites, the present study was undertaken to provide an overall understanding of the excretion of this antibiotic.

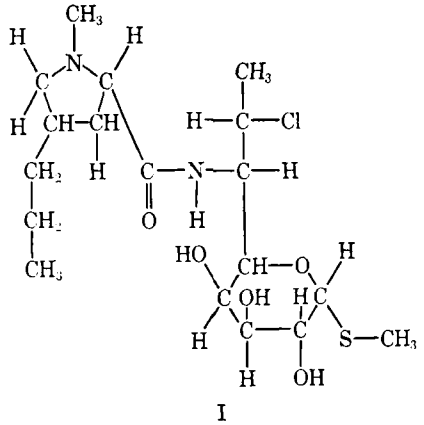
EXPERIMENTAL

Synthesis of Clindamycin-^{3}H Hydrochloride—Since extensive work had already been done on the tritiation of lincomycin hydrochloride (6), it was decided to prepare tritium-labeled lincomycin hydrochloride according to previously described methods and to use it as the starting material for conversion to clindamycin hydrochloride. Although the catalytic gas-exposure tritiation method gives a product of higher radiochemical purity and specific activity, it results in tritiation predominately in the propyl hygric acid portion of the molecule (6). Therefore, to achieve a more random distribution of the tritium label in the molecule, the Wilzbach gas-exposure tritiation method in the absence of catalyst was used.

Tritiation of Lincomycin—Three grams of lincomycin free base was submitted[2] for tritiation. The sample was exposed to 15 c. of carrier-free tritium gas for 2 weeks at 27° and 660 mm. Hg pressure. The crude material was twice dissolved in 25 ml. of water and freeze dried to give 2.922 g. of material with a specific activity of 19.26 μc./mg.

Purification of Lincomycin-^{3}H—A 2.8 × 57-cm. column of 200–400-mesh Dowex 50WX8 was washed with water, 2 *N* NH_4OH, water, 2 *N* HCl, and 0.1 *N* HCl in that order. The freeze-dried crude material was loaded onto the column, which was then eluted with 1.4 l. of 0.1 *N* HCl and 1.5 l. of water. Elution was continued with 1.5 l. of 2 *N* NH_4OH, which was collected in 17-ml. fractions at 12 min./fraction. Monitoring the radioactivity of the ammonium hydroxide eluate indicated that the desired product appeared in fractions 34–47. These fractions were combined and concentrated at water-aspirator pressure and 36°. The residue was recrystallized by dissolving in 1 *N* HCl (1 g./ml.) followed by the addition of 10 volumes of acetone. The resulting crystalline material was repeatedly recrystallized until constant specific activity was achieved on successive recrystallizations.

Two final samples were obtained. One sample, weighing 882 mg. with a specific activity of 2.45 μc./mg., was analyzed by paper chromatography with a solvent mixture of *n*-butanol–acetic acid–water (2:1:1). It was also analyzed by TLC on silica gel with the following solvent systems: I, methanol; II, *n*-butanol–acetic acid–water (2:1:1); and III, methyl ethyl ketone–acetone–water (3:1:1). Radioactivity analyses of the papergram and thin-layer plates showed that the material was radiochemically pure.



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[1] Cleocin, The Upjohn Co., Kalamazoo, Mich.

[2] To New England Nuclear Corp., Boston, Mass.

Table I—Recovery of Radioactivity of Clindamycin-^{3}H from Female Rats

	Percent of Administered Dose — Rat Number				
	1	2	3	4	Mean ± *SD*
Oral Administration					
Urine	20.76	33.30	26.93		27.04 ± 5.12
Feces	74.06	62.26	69.10		68.47 ± 4.84
^3H_2O in urine	0.91	1.06	1.63		1.20 ± 0.31
Total	94.96	95.56	96.03		95.51 ± 0.44
Intraperitoneal Injection					
Urine	31.37	21.49	29.30	28.56	27.73 ± 3.7
Feces	66.82	73.95	73.43	66.87	67.76 ± 3.5
^3H_2O in urine	1.20	0.61	0.86	1.42	0.89 ± 0.34
Total	98.40	85.44	102.73	95.43	95.50 ± 6.36

The second sample, weighing 447 mg. with a specific activity of 2.59 μc./mg., was shown to be identical to the first sample by TLC on silica gel with Solvent System III. These two samples were combined for use in the subsequent step.

Preparation of Clindamycin-^{3}H—Twenty milliliters of carbon tetrachloride was added to a stirred slurry of 1.315 g. (2.94 mmoles) of tritiated lincomycin hydrochloride, 3.86 g. (14.72 mmoles) of triphenylphosphine, and 16 ml. of acetonitrile. The mixture was stirred at room temperature under nitrogen for 18 hr. The resulting clear solution was concentrated at reduced pressure, and the residue was dissolved in 20 ml. of water. The insoluble triphenylphosphine oxide was removed by filtration. The filtrate was basified with 6 *N* NaOH solution, and the liberated free base was extracted into 2 × 15 ml. of chloroform. The combined extracts were washed and saturated with sodium chloride solution and concentrated at reduced pressure. The residual oil was dissolved in 20 ml. of methanol, and the solution was refluxed for 1 hr. and concentrated at reduced pressure. The residue was dissolved in about 20 ml. of 0.1 *N* HCl. The insoluble solids were removed by filtration, and the filtrate was extracted with carbon tetrachloride. The aqueous phase was then basified with 6 *N* NaOH and extracted with 2 × 15 ml. of chloroform. The combined chloroform extracts were washed with saturated sodium chloride solution and dried over magnesium sulfate. The dried chloroform solution was concentrated at reduced pressure, and the residual oil was dissolved in a mixture of 2 ml. of ethanol and 7 ml. of ethyl acetate and treated with anhydrous ethanolic hydrochloric acid.

Cooling the mixture in a refrigerator overnight gave the crystalline product, which was dried in a vacuum desiccator over phosphorus pentoxide. The product obtained, 488 mg. (37.2% yield), had

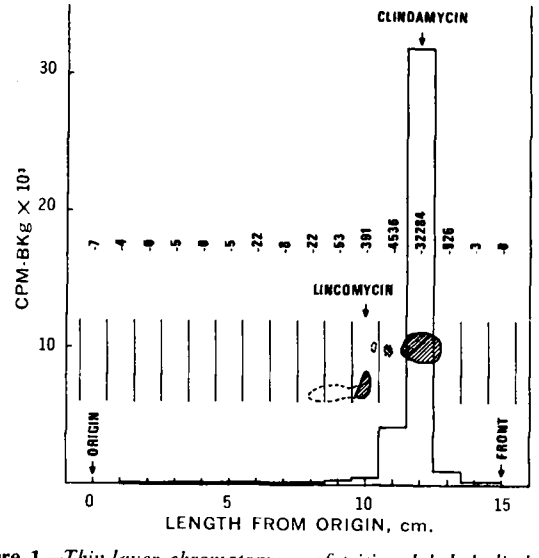

Figure 1—*Thin-layer chromatogram of tritium-labeled clindamycin on silica gel with a solvent system of methyl ethyl ketone–acetone–water (3:1:1).*

Table II—Urinary and Total Excretion Half-Times[a] of Clindamycin-^{3}H Radioactivity in Female Rats

Rat Number	Urine, $t_{1/2}$, hr.	Total, $t_{1/2}$, hr.
Oral Administration		
1	4.1	19.2
2	4.9	25.0
3	4.0	20.6
Average	4.3 ± 0.4	21.60 ± 2.5
Intraperitoneal Injection		
1	7.9	38.0
2	11.0	14.4
3	10.0	42.7
4	8.9	30.0
Average	9.45 ± 1.17	31.28 ± 10.8

[a] Half-times are measured graphically from the plots of $U_\infty - U_t$ *versus* time.

a specific activity of 2.386 μc./mg. This material was chromatographed on a silica gel thin-layer plate with lincomycin hydrochloride and clindamycin hydrochloride standards in Solvent System III (Fig. 1). Analyses of radioactivity on the thin-layer plate indicated that the material was 98.64% radiochemically pure, while 0.90% of the total radioactivity appeared in the same region as lincomycin hydrochloride. Its IR spectrum was identical to form I clindamycin hydrochloride standard.

Anal.—Calc. for $C_{18}H_{34}Cl_2N_2O_5S$ (mol. wt. 461.45): C, 46.85; H, 7.43; Cl, 15.37; N, 6.07; S, 6.95. Found: C, 46.92; H, 7.79; Cl, 14.98; N, 5.68; S, 7.10.

Dosage and Collection of Samples—*Absorption and Excretion Studies in Rats*—Three female Sprague–Dawley (Upjohn strain) rats, with an average weight of 181 g., were used in the oral administration experiments. These rats were fasted for 12 hr. and loaded with 10 ml. of water before dosing. Each rat accepted, through a stomach tube, 1.5 ml. of the solution containing 1.54 mg. of the tritium-labeled clindamycin hydrochloride and 18.7 mg. of the carrier drug. The radioactivity dose was 3.6 μc./rat. The rats were then placed in stainless steel metabolism cages with free access to food and water, and urine and feces samples were collected.

In the intraperitoneal injection experiments, four female Sprague–Dawley rats (average weight 190 g.) were used. Each rat was loaded with 5 ml. of water before being injected with 0.5 ml. of an aqueous solution containing 1.85 mg. of the tritium-labeled drug and 28.40 mg. of the cold drug. The radioactivity dose was 4.42 μc./rat. Urine and feces samples were collected for 14 days in the same way as in the oral experiment.

Absorption and Excretion Studies in Dogs—Three male beagle dogs, weighing 11.7, 8.9, and 8.2 kg., respectively, were used. The labeled drug was given orally in hard filled capsules or intramuscularly as a solution. The intramuscular experiment was carried out 8 weeks after the oral experiment to ensure that the dogs retained no residual radioactivity from the first treatment.

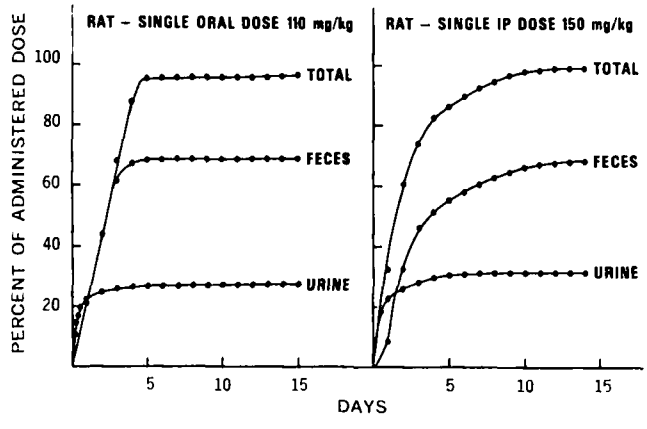

Figure 2—*Cumulative excretion of clindamycin-^{3}H radioactivity by female rat.*

Table III—Recovery of Clindamycin-^{3}H Radioactivity from Male Beagle Dogs

Route of Administration	Mode	Percent of Administered Dose: Dog B67-104 (11.7 kg.)	Dog G67-305 (8.9 kg.)	Dog G67-501 (8.2 kg.)	Mean ± *SD*
Oral administration	Urine	26.70	32.83	31.26	30.29 ± 2.56
	Feces	57.57	53.19	54.42	55.06 ± 1.85
	Total	84.36	86.02	85.67	85.35 ± 0.72
Intramuscular injection	Urine	21.73	27.06	34.42	27.74 ± 5.20
	Feces	71.99	67.50	52.13	63.87 ± 8.50
	Cage Wash	0.10	—	0.64	—
	Total	93.82	94.56	87.19	91.86 ± 3.31

Table IV—Parameters of Urinary and Total Excretion of Clindamycin-^{3}H Radioactivity in Male Beagle Dogs

Parameter	Dog B67-104 Oral	Dog B67-104 Intramuscular	Dog B67-305 Oral	Dog B67-305 Intramuscular	Dog G67-501 Oral	Dog G67-501 Intramuscular	Mean ± *SD* Oral	Mean ± *SD* Intramuscular
Urinary excretion half-time, hr.	4.0	9.12	4.5	8.16	3.8	8.64	4.1 ± 0.29	8.64 ± 0.39
Time required to excrete 90% of the urinary radioactivity, hr.	24	36	24	36	36	36	28	36
Combined urinary and fecal excretion half-time, hr.	7.68	11.04	8.16	10.08	8.16	12.48	8.00 ± 0.23	11.20 ± 0.98
Time required to excrete 90% of the total radioactivity, hr.	24	48	36	48	36	48	32	48

In the oral experiment, both labeled and carrier drugs were weighed into hard-gelatin capsules individually for each dog. Ten milligrams of the labeled drug was also weighed into a volumetric flask for standardization of the radioactivity and correction for decay losses. Dogs were fasted for 18 hr. before dosing and were fed 4 hr. after dosing. Food[3] was given once a day and water was given *ad libitum* throughout the 2-week period. On the 1st and 2nd days, food wetted with water was given to increase the urine output.

Blood samples were collected from the jugular vein immediately before dosing and 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, 36, and 48 hr. after dosing. Samples were obtained by using heparinized tubes[4], and plasma was prepared by centrifugation. Urine samples were collected every 4 hr. for the first 12 hr. by catheterization and once a day in stainless steel metabolism cages for the next 2 weeks. Fecal samples were collected as soon as they were seen or at least once a day. They were weighed and homogenized with water to make 20% homogenates. All samples were frozen immediately until analysis for radioactivity.

In the intramuscular experiment, 500 mg. of the drug containing 47.78 μc. of the labeled drug was dissolved in 2 ml. of sterile normal saline and the solution was injected as two split doses of 1 ml. each into the two hindlegs. The dogs were fed 1 hr. after dosing. The rest of the feeding schedules and the collections of blood, urine, and feces were the same as for the oral experiment.

Counting of Radioactivity—A counting medium[5] was used for direct counting of synthetic samples, urine, and tritiated water. A toluene–methanol–4% 2,5-diphenyloxazole/0.04% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene mixture (9.25:5:0.72) was used for combusted samples. All solvents were reagent grade and were used without further purification.

Radioactivity in all samples was counted by the liquid scintillation technique, with the internal standard method to correct for quenching losses. Synthetic samples were dissolved in appropriate volumes of methanol, and 1.0-ml. aliquots were mixed with 15 ml. of counting solvent. Urine samples were counted directly after mixing 0.5-ml. aliquots with 15 ml. of counting solvent. Blood plasma (0.5-ml.) samples were dried in cellophane bags and combusted by the Schöniger oxygen flask procedure (7). The tritiated water generated from the combustion was collected in 17 ml. of counting solvent, and 15 ml. of the mixture was counted. Aliquots of fecal homogenate were weighed, dried, and combusted in the same way as the plasma samples. Tritiated water in the urine was separated by lyophilization, and 0.5 ml. of the condensate was counted directly as described for the urine samples.

All samples were analyzed in duplicate and counted twice to minimize any error in handling.

[3] Purina dog chow.
[4] Vacutainer.
[5] The Diotol counting solvent used is a mixture of 300 ml. of toluene, 210 ml. of methanol, 350 ml. of 1,4-dioxane, 73 g. of recrystallized naphthalene, and 50 ml. of 4% 2,5-diphenyloxazole and 0.04% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene.

RESULTS

Absorption and Excretion of Clindamycin-^{3}H Radioactivity in Rats—Table I lists the total recovery from both experiments. When the drug was orally administered, a mean of 27.04% of the total radioactivity was excreted in the urine and 68.47% in the feces. The mean total recovery after 2 weeks was 95.51%. A very small amount of tritiated water was found in the urine (1.2%). Assuming that the same amount of tritiated water was eliminated in the expired air, the total quantity of tritiated water produced would be 2.4% of the dose.

The same excretion pattern was obtained when the drug was injected intraperitoneally. A mean of 27.73% of the total radioactivity was excreted through the kidney and 67.76% in the feces. The mean total recovery was 95.5%. Only 0.89% of the total radioactivity was found in the urine as tritiated water, corresponding to 1.78% if the expired air is included.

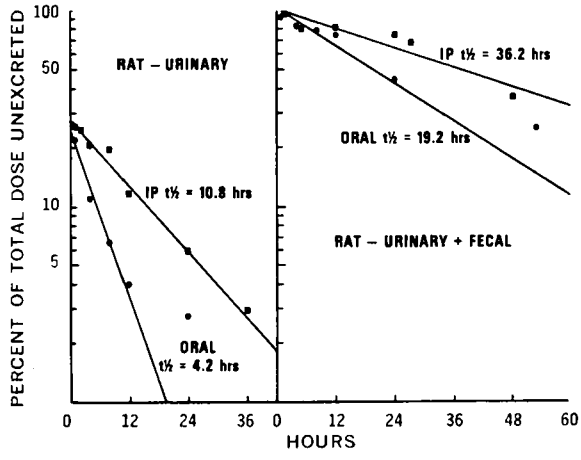

Figure 3—*Urinary and combined urinary and fecal excretion of clindamycin radioactivity by female rat.*

Table V—Parameters of Plasma Clindamycin-^{3}H Radioactivity in Male Beagle Dogs

	Dog B67-104		Dog G67-305		Dog G67-501		Mean ± *SD*	
	Oral	Intramuscular	Oral	Intramuscular	Oral	Intramuscular	Oral	Intramuscular
Maximum concentration (uncorrected), mg./ml.	43.577	18.70	61.245	43.26	63.178	41.01	56.00	34.32
Maximum concentration (corrected for dose level), (mcg./ml.)/(mg./kg.)	0.861	0.438	0.921	0.770	0.875	0.672	0.886 ± 0.026	0.627 ± 0.140
Time of maximum, hr.	2	5	2	5	3	4	2.33 ± 0.47	4.67 ± 0.47
Disappearance half-time, hr.	4.00	5.65	4.40	5.37	3.20	5.50	3.87 ± 0.50	5.55 ± 0.39
Area under the curve (uncorrected), mcg.-hr./ml.	292.48	250.91	521.80	473.75	482.83	564.69	432.30	429.78
Area under the curve (corrected for dose level), (mcg.-hr./ml.)/(mg./kg.)	5.78	5.87	7.84	8.43	6.68	9.26	6.77 ± 0.85	7.85 ± 1.44
Relative absorption efficiency, %	98.45	100	93.03	100	72.21	100	87.90 ± 11.3	100

Since the intraperitoneally injected dose was presumably absorbed quantitatively, the identical distribution of radioactivity excreted after the two routes of administration suggested that oral absorption was also quantitative.

Figure 2 shows cumulative urinary, fecal, and total excretion of radioactivity after both routes of administration. Excretion was rapid in the urine but slow in the feces. Eighty-six percent of the radioactivity excreted in the urine was excreted in the first 48 hr. Fecal excretion was relatively slow and irregular. With both routes of administration, it took 120 hr. before 90% of the total fecal radioactivity was recovered.

During the first 24 hr. after administration, the percent urinary excretion of radioactivity from the oral (22.40 ± 4.0) and intraperitoneal (22.20 ± 2.37) doses was equivalent. During the same period, fecal excretion eliminated 29.68 ± 8.86% of the oral dose but only 8.79 ± 6.0% of the intraperitoneal dose. The difference apparently indicated that the transport of this drug to the bile was substantially slower after intraperitoneal than oral administration.

Although the different routes of drug administration did not appear to affect the distribution of total radioactivity in urine and feces, they did affect the initial rate of excretion (Fig. 3). The semilogarithmic plots clearly show that the initial rate of urinary excretion of the orally administered clindamycin radioactivity was twice as fast as the intraperitoneally injected dose. Table II lists the estimated half-lives for appearance of radioactivity in the excretions of each rat in this experiment. The average half-time for urinary excretion was 4.3 hr. for the oral dose in contrast to 9.45 hr. for the intraperitoneal dose. The half-times for the total elimination of the drug, as obtained by combining the urinary and fecal excretions of radioactivity at each time interval, also showed that the orally administered dose was excreted faster than the injected dose.

Absorption and Excretion of Clindamycin-^{3}H Radioactivity in Dogs—Two weeks after the administration of clindamycin-^{3}H, the average recoveries of radioactivity from three dogs in the oral experiment were 30.29 ± 2.56% from urine, 55.06 ± 1.85% from feces, and 85.35 ± 0.72% total. On the other hand, 27.74 ± 5.2% of the intramuscular dose appeared in the urine and 63.85 ± 8.5% in the feces. The total recovery in this case was 91.86 ± 3.31%. Recovery data for each individual dog are listed in Table III.

Figure 4 shows cumulative urinary, fecal, and total excretion of radioactivity after each route of administration. The identical distribution of radioactivity between urine and feces again suggested quantitative oral absorption. In all cases, only trace amounts of tritiated water were found in the urine. This indicated good stability of the radioisotope label in the drug during metabolic transformations.

Semilogarithmic plots for excretion of clindamycin-^{3}H radioactivity are presented in Fig. 5. The patterns in both urinary and overall excretion appear to be similar to that of the rat (Table IV). Both plots show that the dose of orally administered clindamycin was eliminated faster than the intramuscularly injected dose.

In the initial phase of the urinary excretion, the average elimination half-life after intramuscular administration was 8.64 ± 0.39 hr. compared with 4.10 ± 0.29 hr. after oral administration, which indicated that the drug and metabolites were eliminated 2.1 times more rapidly through the kidney after oral dosing. On the other hand, in the initial phase of the total excretion, the average elimination half-life after intramuscular administration was 11.20 ± 0.98 hr. compared to 8.0 ± 0.23 hr. *via* the oral route. This implies that the drug was eliminated 1.4 times more rapidly following oral than intramuscular administration. The delayed excretion following intramuscular administration was believed due to drug precipitation at the injection site or a difference in the transport process.

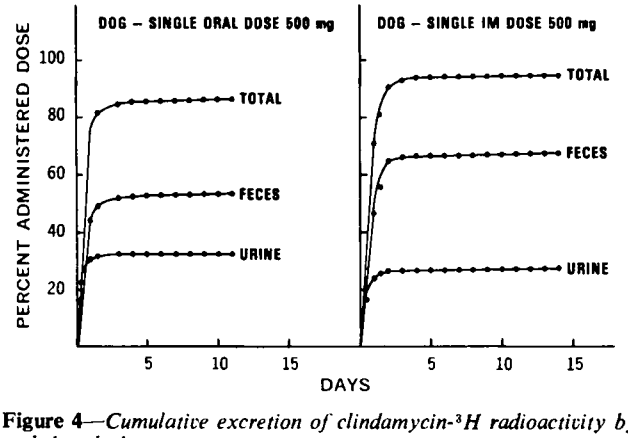

Figure 4—*Cumulative excretion of clindamycin-^{3}H radioactivity by male beagle dog.*

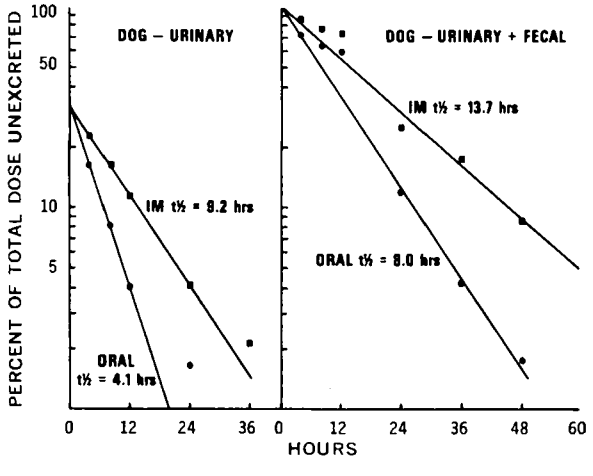

Figure 5—*Urinary and combined urinary and fecal excretion of clindamycin radioactivity by male beagle dog.*

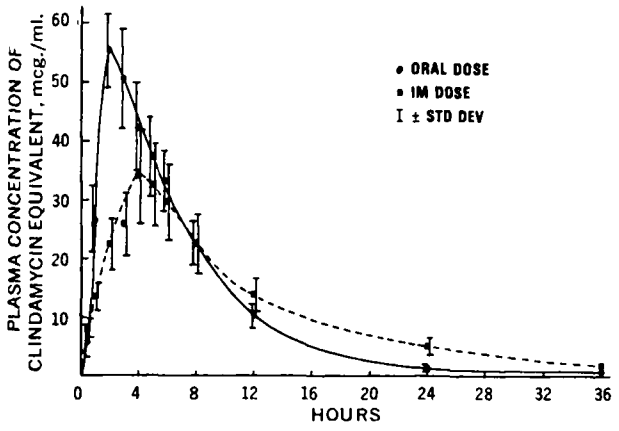

Figure 6—*Plasma concentration of clindamycin and metabolites (expressed as micrograms of clindamycin equivalent per milliliter of plasma) in dogs after oral (●) or intramuscular (■) administration of tritium-labeled clindamycin hydrochloride.*

Figure 6 shows the plasma levels of clindamycin radioactivity in dogs after two different routes of drug administration. When drug was administered orally, a peak concentration of 56-mcg. drug equivalents/ml. was observed at 2–3 hr. with a disappearance half-time of 3.8 hr. When drug was given intramuscularly, a peak concentration of 34-mcg. drug equivalents/ml. was observed at 4–5 hr. The disappearance half-time was estimated to be 5.5 hr.

Table V lists the parameters of plasma clindamycin concentration curves obtained for the three dogs. After the dosage levels were corrected to account for the slight differences in body weight, the mean areas under the plasma concentration curve per milligram dose per kilogram body weight were 6.77 ± 0.85 and 7.85 ± 1.44 mcg./hr./ml. for the oral and intramuscular doses, respectively. The ratio of the areas indicated that an average of 87.9 ± 11.3% of the drug was absorbed orally.

The plasma drug disappearance half-times were estimated graphically to be 3.87 ± 0.50 hr. for the orally administered dose and 5.55 ± 0.39 hr. for the intramuscularly injected dose. These values only represent the initial drug and/or metabolite disappearance in the first 12-hr. period since the curves deviate from the first-order kinetics after that period. A small secondary peak occurred after the animals were fed, strongly suggesting enterohepatic recirculation of drug and/or metabolites.

DISCUSSION

Based upon the described data it appears that orally administered clindamycin hydrochloride is rapidly and nearly quantitatively absorbed by rats and dogs. The drug is also rapidly excreted through both kidney and bile. However, the injected dose is absorbed and excreted much slower than the oral dose. These observations are consistent with the results of Novak *et al.* (5) in their study of absorption and excretion of intramuscularly administered clindamycin in man.

The absorption efficiencies of orally administered drug for each individual dog, as estimated by urinary excretion and areas under the plasma concentration curve, were not identical but were consistent. Both estimations show that orally administered drug was absorbed nearly quantitatively in Dogs B67-104 and G67-305 but only 70–90% in Dog G67-501. The difference is not considered significant.

The delayed absorption of the injected dose was suggested by Novak *et al.* (5) as being caused by the precipitation of the drug at the injection site. Since most of the drug at pH 7.4 (extracellular fluid pH) exists as the free base with low aqueous solubility and since the fluid volume in the injection site is limited, it is possible that the injected dose acts as if the drug were given in aqueous suspension. In addition, local swelling and irritation were observed at the injection site and persisted for 5–6 hr. This finding would be inconsistent with this postulation.

The lower plasma level and the slower initial rate of excretion of clindamycin radioactivity after intramuscular administration than after oral administration in dogs could also be caused by a difference in transport processes. The oral dose was absorbed and went through the portal system to the liver, where the entire dose was exposed to metabolism. On the other hand, the parenterally administered dose was absorbed into the venous system and delivered to the heart. Only a fraction of the cardiac output passes through the liver in each circulation.

However, the same argument cannot be used to explain the delayed excretion of the intraperitoneally administered clindamycin in the rat. Lukas *et al.* (8) recently showed that drugs administered intraperitoneally are absorbed primarily through the portal circulation and pass through the liver before reaching other organs. Therefore, the delay of excretion must be related to the slow absorption process through the peritoneal membrane, either due to local precipitation of the drug or other unknown reasons.

A similar case was reported by Burns *et al.* (9) in their study of phenylbutazone absorption. This drug was rapidly and completely absorbed from the GI tract in man but very slowly absorbed after intramuscular injection. The peak plasma concentration was reached 2 hr. after oral administration; but if an intramuscular dose was given, the peak was not observed until 6–10 hr. afterward. In this case, however, Burns *et al.* (9) proved that the cause of the delayed absorption was drug precipitation at the site of injection.

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Correlation of Dissolution, Disintegration, and Bioavailability of Aminosalicylic Acid Tablets

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Abstract □ Rank-order correlations are demonstrated among dissolution, disintegration, and several measures of bioavailability for tablets of aminosalicylic acid and its salts. The correlation between disintegration time and the percent of the dose excreted in the urine is examined quantitatively using both linear and quadratic models. The results support the use of either disintegration or dissolution tests to control the availability of this drug and suggest control limits for these tests. The suggested limits on the disintegration tests are shorter than those currently specified in USP XVIII monographs on these items.

Keyphrases □ Aminosalicylic acid and aminosalicylate tablets—rank-order correlations among dissolution, disintegration, and bioavailability □ Dissolution, disintegration, and bioavailability of aminosalicylic acid and aminosalicylate tablets—rank-order correlations □ Disintegration, dissolution, and bioavailability of aminosalicylic acid and aminosalicylate tablets—rank-order correlations □ Bioavailability, dissolution, and disintegration of aminosalicylic acid and aminosalicylate tablets—rank-order correlations

In 1956, Chapman *et al.* (1) reported a direct relationship between *in vitro* disintegration times and the biological availability of sodium aminosalicylate tablets. More recently, Middleton *et al.* (2) compared disintegration times and dissolution rates with the biological availability of tablets of aminosalicylic acid and sodium, potassium, and calcium aminosalicylate. Middleton *et al.* concluded, however, that disintegration times did not correlate with availability while dissolution rates gave good correlation. Neither paper supported its claims with a statistical evaluation of the data.

The doubt Middleton *et al.* (2) cast upon the value of disintegration tests for monitoring drug availability from aminosalicylate tablets, plus the fact that they did not use the USP–NF dissolution apparatus and presented only very limited data on tablets of aminosalicylic acid itself (our primary concern), led to this reevaluation of the relationship between *in vivo* and *in vitro* data for this product. Data available in the literature were used along with data available in this laboratory on a variety of aminosalicylic acid formulations exhibiting a wide range of dissolution and disintegration times. Significant rank-order correlations were found between several *in vivo* and *in vitro* parameters of availability, but only the data compiled on disintegration times and urinary excretion of drug were adequate for quantitative correlation.

EXPERIMENTAL

Tablets—Uncoated tablets and tablets with various enteric coatings were used. These tablets exhibited a wide range of dissolution and disintegration behavior. Suspensions of aminosalicylic acid powder were also administered to several subjects.

Dissolution Studies—Dissolution rates for uncoated tablets were measured in the USP–NF dissolution apparatus, using 900 ml. of pH 7.5 buffer (USP) in each vessel and a stirring speed of 165 r.p.m. Enteric-coated tablets were washed with pH 1.2 buffer for 1 hr. before placing them in the dissolution apparatus. Samples were collected at 5-min. intervals for rapidly dissolving tablets and at 20-min. intervals for all other tablets. The dissolution samples were analyzed for aminosalicylic acid using a modification of the automated analysis procedure reported by Wrightman *et al.* (3).

The results of the dissolution tests are reported as the average time required for 20% of the drug to go into solution from four or five tablets (T_{20}). This time was determined by a straight-line interpolation between the data points; in the case of enteric-coated tablets, it does not include the time spent in pH 1.2 buffer prior to the dissolution test. T_{20} was chosen rather than the more common T_{50} because several of the tablets tested dissolved so slowly that T_{50} could not be determined in a reasonable length of time; several T_{20} values were not determined for this same reason.

Disintegration—Disintegration times were determined using the USP apparatus. The disintegration times for enteric-coated tablets were determined in simulated intestinal fluid USP following 1 hr. in simulated gastric fluid USP. The disintegration times for nonenteric tablets were determined in simulated gastric fluid USP. Disks were used in both cases. The procedure differed from the USP procedure in that the tablets were observed continuously and a disintegration time for each tablet was recorded. The average of the observed disintegration times is reported rather than the time required for all the tablets to disintegrate, as specified in USP XVIII. This modification was expected to give better correlation with the clinical results, which are also reported as average plasma or urine concentrations from several subjects, each of whom ingested several tablets. In addition, a measure of tablet uniformity is available from data recorded in this manner.

Clinical Studies—All subjects were fasted overnight before receiving six 500-mg. tablets of aminosalicylic acid, which constitutes a normal therapeutic dose of this drug substance. Blood samples

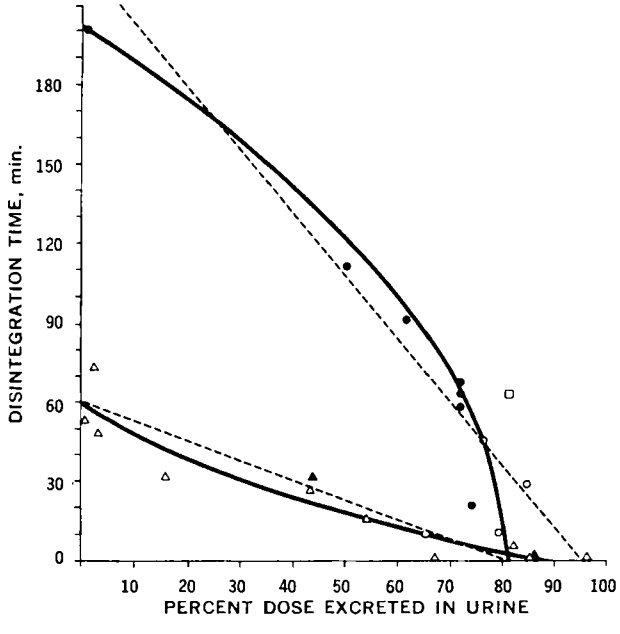

Figure 1—*Correlation between disintegration times and the percent of the dose excreted in the urine. The solid and dashed lines represent the quadratic and linear regression lines, respectively. Key: △, aminosalicylic acid data from this paper; ▲, aminosalicylic acid data (2); ○, sodium aminosalicylate data (2); □, calcium aminosalicylate data (2); and ●, sodium aminosalicylate data (1).*

Table I—Individual Levels of Drug in Plasma and Urine

Formulation	Subject	Plasma Level, mcg./ml. 0 hr.	1 hr.	2 hr.	3 hr.	4 hr.	6 hr.	8 hr.	10 hr.	12 hr.	Urine Recovery, g./12 hr.
A	1	0.0	65.0	54.0	22.0	10.2	0.6	0.0	—	0.0	2.81
	2	0.0	42.0	84.0	55.0	28.0	4.7	1.7	—	0.0	3.11
	3	0.0	4.4	2.7	2.7	34.0	19.0	5.3	—	1.0	2.32
	4	0.0	20.0	11.5	64.0	63.0	22.0	6.0	—	1.0	2.80
	5	0.0	84.0	40.0	14.7	4.7	0.0	0.0	—	0.0	2.85
	6	0.0	43.0	46.5	25.0	10.5	1.7	0.0	—	0.0	2.75
	7	0.0	67.0	47.0	22.0	7.0	1.0	0.0	—	0.0	3.19
	8	0.0	8.0	34.7	56.0	41.0	13.0	1.7	—	0.0	2.43
	9	0.0	2.0	57.0	64.0	43.5	8.0	1.7	—	0.0	2.38
	10	0.0	47.0	65.0	34.5	13.7	2.3	0.0	—	0.0	4.17
B	1	0.0	—	53.1	—	9.0	1.3	0.0	0.0	0.0	2.58
	2	0.0	—	43.5	—	16.3	3.3	2.3	0.0	0.0	2.90
	3	0.0	—	14.3	—	0.7	0.0	0.0	0.0	0.0	1.79
	4	0.0	—	52.0	—	31.4	14.0	5.0	0.0	0.0	3.00
C	1	0.0	—	43.0	—	13.3	1.6	0.0	0.0	0.0	2.57
	2	0.0	—	25.7	—	34.0	9.0	1.6	0.0	0.0	2.68
	3	0.0	—	15.7	—	5.0	12.3	0.0	0.0	0.0	1.57
	4	0.0	—	57.0	—	43.0	0.7	3.0	1.0	0.0	2.97
D	1	0.0	—	44.7	—	11.7	1.5	0.0	0.0	0.0	2.95
	2	0.0	—	39.0	—	25.5	4.3	0.0	0.0	0.0	2.26
	3	0.0	—	23.4	—	2.3	0.0	0.0	0.0	0.0	0.47
	4	0.0	—	64.0	—	32.0	8.3	2.0	0.0	0.0	2.34
E-2	1	0.0	0.0	0.0	16.0	28.0	7.3	2.0	—	0.0	2.86
	2	0.0	0.0	0.0	58.5	46.0	16.0	4.5	—	0.0	2.44
	3	0.0	0.0	0.0	4.5	13.0	2.0	1.0	—	11.5	1.02
	4	0.0	0.0	0.0	16.0	33.0	13.0	6.5	—	1.0	1.38
	5	0.0	0.0	0.0	29.0	32.0	7.5	1.0	—	0.0	2.70
	6	0.0	0.0	0.0	8.0	14.7	7.0	2.0	—	0.0	1.18
	7	0.0	0.0	0.0	45.5	48.7	7.3	1.0	—	0.0	2.70
	8	0.0	0.0	0.0	0.0	2.0	0.0	0.0	—	0.0	0.05
	9	0.0	0.0	0.0	4.0	4.0	4.0	3.3	—	1.5	1.41
	10	0.0	0.0	0.0	19.7	35.0	4.5	2.0	—	0.0	1.71
E-1	1	0.0	—	0.0	—	36.7	9.0	1.3	0.0	0.0	1.89
	2	0.0	—	20.3	—	31.3	20.0	4.3	1.2	0.0	2.56
	3	0.0	—	0.0	—	0.0	2.8	2.8	1.4	0.0	0.66
	4	0.0	—	0.0	—	12.0	16.7	2.8	0.7	0.7	1.35
F	1	0.0	—	0.0	—	0.0	2.0	6.0	—	4.0	1.20
	2	0.0	—	0.0	—	0.0	0.0	2.0	—	2.0	0.73
	3	0.0	—	8.0	—	16.0	4.0	2.0	—	0.0	1.60
	4	0.0	—	0.0	—	0.0	0.0	8.0	—	11.0	1.35
	5	0.0	—	0.0	—	24.0	30.0	6.0	—	0.0	1.60
G	1	0.0	—	0.0	—	0.0	0.0	1.6	—	0.6	0.37
	2	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.26
	3	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.21
	4	0.0	—	0.0	—	0.0	0.0	0.0	—	0.6	0.27
	5	0.0	—	0.0	—	0.0	0.3	5.7	—	7.0	1.30
H	1	0.0	—	0.0	—	0.0	0.0	1.5	—	0.6	0.18
	2	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.01
	3	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.26
	4	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.02
	5	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.03
I	1	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.04
	2	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.02
	3	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.02
	4	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.02
	5	0.0	—	0.0	—	0.0	0.0	1.5	—	2.7	0.03
J	1	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.02
	2	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.01
	3	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.02
	4	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.02
	5	0.0	—	0.0	—	0.0	0.0	0.0	—	0.0	0.05

were drawn at 0, 2, 4, 6, 8, and 12 hr. in early studies. In later studies, 1-, 3-, and 10-hr. samples were also drawn. Urine was collected for 12 hr. following drug administration.

Plasma and urine samples were analyzed by procedures adapted from Way *et al.* (4). The basic procedure consists of diazotization of the aminosalicylic acid followed by reaction with *N*-1-naphthylethylenediamine to form a rose-colored adduct whose absorbance is measured at 540 nm. This procedure measures the primary aromatic amines in the sample.

To precipitate plasma proteins, plasma samples were treated with trichloroacetic acid prior to developing the color. The reported plasma concentrations represent the sum of aminosalicylic acid and its metabolites that have a free amino group; aminosalicyluric acid, for example, is found in plasma in low but significant amounts (4–6) and would be included in the reported concentration. The major metabolite (4–7), *N*-acetyl-*p*-aminosalicylic acid, would not be included in the reported concentrations. However, the kinetics of elimination of aminosalicylic acid and its metabolites are very nearly linear up to a plasma concentration of approximately 110 mcg./ml. (8). Since the plasma concentrations encountered in this study did not exceed 85 mcg./ml. (Table I), which is well within the linear region, the area under the plasma level–time curve as determined by this method is a suitable measure of drug availability.

Urine samples were subjected to an acid hydrolysis before

Table II—*In Vivo* and *In Vitro* Availability of Aminosalicylic Acid from Tablets[a]

Tablet	Number of Subjects	Time of Plasma Peak, hr.	Plasma Peak, mcg./ml.	Area under Plasma Curve, mcg.-hr./ml.	Percent Recovered in Urine[b]	Mean Disintegration Time, min.	T_{20}, min.
A Uncoated	10	2 ± 1	63 ± 5	177 ± 14	96 ± 18	<1	<4
B Powder	4	<2	41 ± 18	123 ± 72	85 ± 19	—	<4
C Uncoated	4	2.5 ± 1	37 ± 17	133 ± 61	82 ± 20	4.6 ± 1.3	<5
D Uncoated	4	<2	43 ± 17	129 ± 67	67 ± 35	0.5 ± 0.7	<4
E-2[c] Enteric	10	4 ± 0	27 ± 6	82 ± 16	58 ± 30	—	—
E-1[c] Enteric	4	5 ± 1	22 ± 15	82 ± 58	54 ± 27	15 ± 5	16 ± 9
F Enteric	5	8 ± 3	13 ± 11	55 ± 45	43 ± 13	26 ± 5	36 ± 11
G Enteric	5	—[d]	—[d]	—[d]	16 ± 15	32 ± 8	96 ± 32
H Enteric	5	—[d]	—[d]	—[d]	3 ± 4	48 ± 15	—[e]
I Enteric	5	—[d]	—[d]	—[d]	2 ± 4	73 ± 13	—[f]
J Enteric	5	—[d]	—[d]	—[d]	0.8 ± 0.5	53 ± 5	—[f]

[a] Ranges are ±1 *SD*. [b] Calculated as the percent of labeled potency of the tablet. [c] Two independent clinical trials were conducted on Tablet E. [d] Plasma level parameters could not be calculated because the plasma concentration had not passed its maximum at the time the last blood sample was drawn. The mean area under the plasma level curve up to 12 hr. was less than 8 mcg.-hr./ml. [e] A T_{20} value was not calculated because one out of four tablets showed no dissolution after 2 hr. in the pH 7.5 buffer. [f] There was no measurable dissolution after 3 hr. in the pH 7.5 buffer.

Table III—Rank-Order Correlation Coefficients for *In Vivo* and *In Vitro* Availability of Aminosalicylic Acid[a]

	Peak Plasma Level	Area under Plasma Level–Time Curve	Percent of Dose Excreted in Urine	Mean Disintegration Time	Dissolution, T_{20}
Time of plasma peak level	−0.77	−0.49	−0.71	+0.94 (0.02)	+1.00 (0.01)
Peak plasma level		+0.83 (0.1)	+0.83 (0.1)	−0.83 (0.1)	−0.77
Area under plasma level–time curve			+0.83 (0.1)	−0.60	−0.49
Percent of dose excreted in urine				−0.96 (0.01)	−0.93 (0.01)
Mean disintegration time					+0.95 (0.01)

[a] For correlations significant at the 0.1 level or below, the approximate confidence level is given in parentheses.

analysis. This procedure frees the amino group in the *N*-acetyl metabolite, so that the reported values are a measure of total aminosalicylic acid and metabolites and provide a good basis for comparing the relative bioavailability of aminosalicylic acid from various formulations.

DISCUSSION

The results of the *in vivo* and *in vitro* experiments are summarized in Table II. The entries in Table II are arranged in order of decreasing percentage of drug recovered in the urine, and a comparison of the columns suggests several rank-order correlations among the variables. The data suggest that longer disintegration times and T_{20}'s are associated with a greater delay before the peak plasma concentration is reached, a lowering of the peak concentration, a smaller area under the plasma concentration–time curve, and reduced amounts of drug excreted in the urine. The correlation coefficients and corresponding significance levels derived from the distribution of sums of squares of rank differences (9) are presented in Table III for these data. Several correlations are, in fact, significant, the most notable being the correlations between time of plasma peak, the percent of the dose excreted in the urine, and the disintegration and dissolution times.

Table IV summarizes the published data relating tablet disintegration time to the amount of drug excreted in the urine following the administration of tablets of aminosalicylic acid and its salts. A significant correlation between the *in vivo* and *in vitro* test results is also found with these data. However, the correlation coefficient calculated for the disintegration times and urine recoveries of the salts was only −0.72, while the correlation coefficient calculated from the data on tablets of the acid was −0.96. The lower correlation coefficient found for the salts is due to the existence of a range of disintegration times over which the bioavailability remains essentially at its maximum value (Fig. 1). A better evaluation of the correlation in this case is obtained from the regression analysis discussed below.

Although a rank-order correlation between *in vivo* and *in vitro* tests may be sufficient for establishing manufacturing controls to assure a lower limit on the availability of drug from manufactured lots of tablets, a more quantitative correlation is desirable because it allows the data to be summarized in a simple algebraic relation and also because it allows one to estimate the reliability of predictions made from the model equation. Therefore, the correlation between disintegration time (X) and percent of the dose excreted in the urine (Y) was examined using both a linear and a quadratic model, *i.e.*, ($Y = a_1 + b_1X$) and ($Y = a_2 + b_2X + c_2X^2$), respectively.

Table IV—Summary of Availability Data on Aminosalicylate Tablets from the Literature[a]

Tablet	Percent Recovered in Urine[a]	Mean Disintegration Time, min.	Reference
Sodium salt:			
A	74	19.5	1
B	72	58.0	1
C	72	67.0	1
D	72	63.5	1
E	61.5	91.1	1
F	50.0	111.5	1
G	2.1	200.4	1
H	78.3	10	2
I[b]	84.5	28	2
J[b]	76.4	45	2
Calcium salt:			
K	81.2	63	2
Free acid:			
L	85.9	1	2
M[b]	43.7	31	2

[a] Calculated as the percent of labeled potency of the tablet. The correlation between the tabulated urine recoveries and disintegration times for the sodium and calcium salts is significant at the 0.02 level when calculated from the distribution of sums of squares of rank differences (9). The correlation coefficient is −0.720. [b] Enteric-coated tablets.

Table V—Relation of Percent of Dose Excreted in the Urine (Y) to Tablet Disintegration Time (X)

	Linear Model	Quadratic Model
Aminosalicylic acid:		
Equation	$Y = 80.8 - 1.37X$	$Y = 87.1 - 2.34X + 0.0153X^2$
Multiple correlation coefficient	−0.94	−0.96
Standard error of estimate	13.1	10.6
Aminosalicylate salt:		
Equation	$Y = 94.1 - 0.412X$	$Y = 80.5 - 0.009X - 0.00194X^2$
Multiple correlation coefficient	−0.94	−0.98
Standard error of estimate	8.3	4.8

Only disintegration times and urine levels were used in this analysis, because these parameters covered a wider range of values than the other parameters, and more data pairs were available (Tables II and IV). The quadratic model was included in the analysis, because a plot of disintegration time against the percent drug recovered in the urine (Fig. 1) suggested a curvilinear relation between the variables. A curvilinear relation would be expected on theoretical grounds as well. If the rate of release of drug from the dosage form were much faster than the rate of absorption, the drug would be maximally available; a further increase in the rate of release would have no effect on the availability of the drug. Similarly, when the release is so slow that essentially no drug is absorbed, a further decrease in the rate of release would not affect the availability. These regions of maximal and minimal availability would be expected to be joined by some smooth function, $Y = Y(X)$. In the absence of a theory defining the functional form of $Y(X)$ more precisely, a simple polynomial is a reasonable choice for use in a study of the correlation between Y and X. The results of the analyses using these models are presented in Table V, and the regression curves are plotted in Fig. 1.

As indicated in Table V, the regression analyses of the data on aminosalicylic acid and its salts were carried out separately. The experimental points in Fig. 1 suggest that the acid and its salts do not fall on the same regression curve, and subsequent statistical treatment confirmed that the difference is significant. This difference in behavior is probably not an artifact generated by interlaboratory assay differences, because the data of Middleton *et al.* (2) on aminosalicylic acid are consistent with the present results, while their data on the salts are consistent with the results of Chapman *et al.* (1). Three possible sources of this difference are: (*a*) differences between the acid and its salts with respect to physical-chemical properties, such as solubility or rate of dissolution of the crystals; (*b*) the fact that most of the slow disintegrating tablets of aminosalicylic acid were enteric coated, whereas most tablets of the salts were not; or (*c*) the difference in the disintegration tests used for enteric tablets as opposed to nonenteric tablets (see *Experimental* section). The available data are not sufficient to decide among these alternatives.

The multiple correlation coefficients in Table V show that 92 or 96% of the variance in the urinary excretion of drug following the administration of aminosalicylic acid or an aminosalicylate salt, respectively, can be ascribed to the effect of tablet disintegration time when the quadratic model is assumed. Only 88% of the variance can be ascribed to tablet disintegration time when the linear model is assumed.

The standard error of the estimate for the quadratic model indicates that 95% of the time the experimental urine recovery of aminosalicylic acid should lie within ±21% of the value predicted by the regression line. The 95% confidence limits for urine recovery values predicted for the salts of aminosalicylic acid are ± 10%. The confidence limits for the linear model are broader than those for the quadratic model for both groups of tablets. While the confidence limits are not as tight as one would like, they are reasonable in view of the large intersubject variability normally encountered in clinical studies.

The constant terms in the equations in Table V represent the maximum availability of the drug predicted by the model. The values of 87.1 and 80.5% obtained for the quadratic model are in good agreement with the values reported in the literature, which range from 70 to 85% (1, 2, 4–6, 10, 11).

CONCLUSIONS

The correlations between disintegration times and bioavailability (Table V) show that it is possible to use disintegration tests to control the availability of drug from aminosalicylate tablets. Tablets of aminosalicylic acid and sodium and potassium aminosalicylates are official in USP XVIII. The USP requires that uncoated tablets disintegrate within 30 min. and that enteric-coated tablets disintegrate within 150 min. As can be seen from Fig. 1, only the limits set for uncoated tablets of the sodium and potassium salts are adequate to ensure drug availability; tablets of the salts disintegrating within 30 min. are as available as a suspension of the drug (80.5%). The USP tests would pass uncoated tablets of aminosalicylic acid, which were only 30.4% available, and enteric-coated tablets of aminosalicylic acid and aminosalicylates, which were 0.0 and 35.5% available, respectively. The results indicate that the time limits for enteric-coated tablets should be the same as the time limit for uncoated tablets and that the limit on the disintegration time for aminosalicylic acid tablets should be reduced from 30 to approximately 10 min.

The data in Tables II and III, as well as the data of Middleton *et al.* (2), show that dissolution tests may also be used to control drug availability from tablets of aminosalicylic acid and its salts. In principle, dissolution tests are more likely to give valid comparisons of drug availability from different formulations than are disintegration tests, because availability could be limited by the rate of dissolution from the tablet fragments rather than by the rate of disintegration of the tablet. The available data indicate, however, that the possible discrepancy between dissolution and disintegration times does not, in fact, occur for typical formulations of tablets of aminosalicylic acid and its salts. Middleton *et al.* (2) probably failed to find a correlation between disintegration times and bioavailability because they studied tablets of both aminosalicylic acid and its salts, and these fall on different correlation lines. Their data were not sufficient to make the existence of two lines obvious, and the resulting distribution of points was interpreted as a lack of correlation. The open symbols in Fig. 1 represent the data of Middleton *et al.* and clearly show the apparent lack of correlation.

In the present study, a problem was encountered in attempting to correlate dissolution time with urinary excretion of drug because T_{20}'s for the least available tablets were too long to be conveniently measured, whereas the T_{20}'s for the most available tablets were too short to be measured accurately. This situation made a full regression analysis of the data impossible, although it did not obscure the rank-order correlation existing between these variables. The data in Table II show that T_{20} must be limited to 15 min. or less, when the test is conducted as described in the *Experimental* section, to ensure at least 50% drug availability.

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GLC Determination of 7-Trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione in Plasma

LEONARD C. BAILEY and ARVIN P. SHROFF▲

Abstract □ A GLC assay method is described for the determination of 7-trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione in the plasma or serum of dogs, rabbits, or humans. After extraction with hexane from acidified plasma or serum, the compound is analyzed by GLC using a flame-ionization detector. Quantitation is accomplished using a standard curve, which was found to be linear over the 0.002–3.5-mcg. range. The analysis is sensitive to amounts of the drug above 0.1 mcg./ml. Recovery of the title compound added to human plasma averaged 98.7%, with a variation of approximately 7.1% (*RSD*). Plasma data are presented to demonstrate the utility of the method.

Keyphrases □ 7-Trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione—GLC analysis in plasma □ 1,5-Benzodiazepine compounds—GLC analysis of 7-trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione in plasma □ GLC—analysis, 7-trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione in plasma

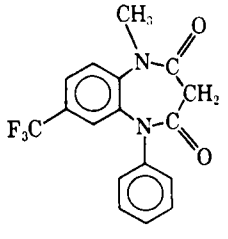
A recently synthesized member of the benzodiazepine class of compounds, 7-trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione[1] (I), is currently being evaluated for its activity as a minor tranquilizer. It differs significantly from other benzodiazepine psychotropic agents in that the carbon and nitrogen atoms in the 4- and 5-positions of the diazepine ring are reversed, giving rise to the 1,5-benzodiazepine structure. The pharmacological action of Compound I on the CNS of various test animals has been reported (1, 2), as well as the metabolism of the drug in animals and man (3).

To assist in correlating pharmacological activity with blood levels of Compound I, it was necessary to develop a sensitive, specific method of analysis that would permit processing of a large number of samples in a relatively short time. Various methods have been described for the determination of benzodiazepines, notably diazepam and chlordiazepoxide, in physiological fluids. In some cases, quantitation is achieved by making use of the UV-absorbing properties of the compound (4), while in others it depends upon hydrolysis of the drug followed by colorimetric determination of a Bratton–Marshall adduct (5–8). Cathode ray polarography has been successfully applied to plasma determinations (9). The most sensitive and specific methods use GLC, either of the intact compounds or of their hydrolysis products, and electron-capture detection (4, 10–12). However, these methods require extensive, time-consuming manipulations to produce an extract clean enough for chromatography.

The method described here used GLC followed by flame-ionization detection for the determination of Compound I in plasma or serum to levels of 0.1 mcg./ml. The analysis is specific for Compound I in the presence of its known metabolites and is applicable to human or animal samples after administration of therapeutic doses.

EXPERIMENTAL

Reagents—The purity of Compound I used was 100% as determined by GLC and TLC. All other materials were analytical reagent quality. The hexane was purified further by shaking with sulfuric acid, washing with water until neutral, and redistilling



I

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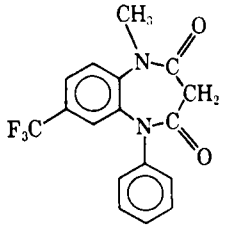

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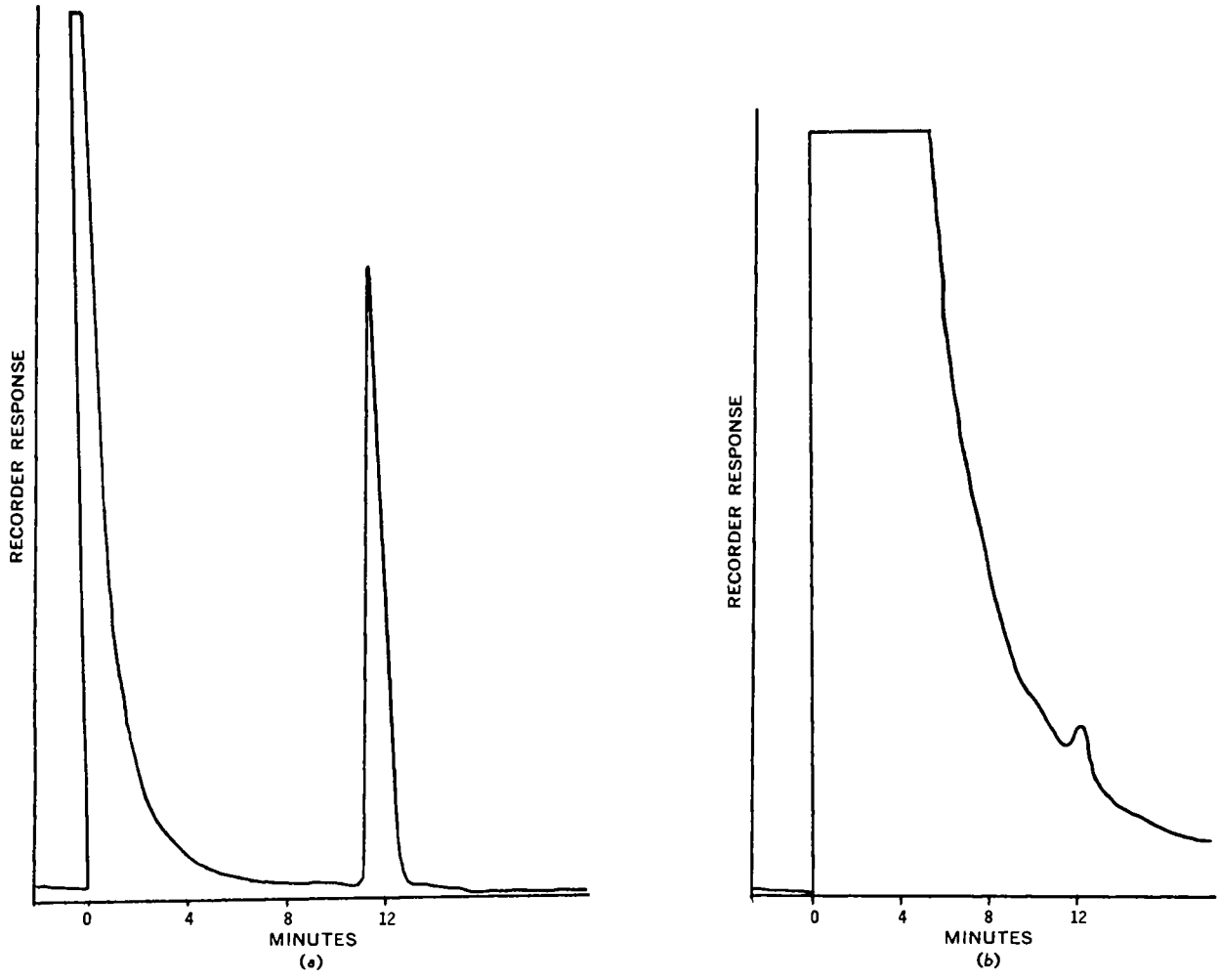


Figure 1—*Representative chromatograms of Compound I:* (a) *0.27 mcg. (attenuation* × *16), and* (b) *0.8 ng. (attenuation* × *1).*

twice through a 30.48-cm. (12-in.) Vigreux column. This was necessary to eliminate interferences which became significant after concentration of the hexane.

Apparatus—A gas chromatograph[2] with a dual flame-ionization detector was used. The output of the chromatograph was recorded on a recorder[3] with a full-scale range of 1.0 mv.

Analyses were performed using a glass column, 0.61 m. (2 ft.) × 4 mm. i.d., packed with 6% Carbowax 20M on Gas Chrom Q (100–120 mesh). The column was conditioned for 24 hr. at 250° with a helium flow of 60 ml./min. The inner surface of the column and the glass wool used to retain the packing were previously deactivated by treatment with dichlorodimethylsilane (13).

The analytical parameters used were: oven temperature, 240°; injector and manifold temperature, 230°; hydrogen inlet pressure, 1 kg./cm.^{2}; oxygen inlet pressure, 2 kg./cm.^{2}, and helium flow, 60 ml./min. With these conditions, Compound I was eluted as a symmetrical peak at 12.03 min. (Fig. 1*a*). The minimum detectable quantity of standard is less than 1 ng. (Fig. 1*b*).

Procedure—The glassware was washed with detergent and water, rewashed with chromic acid cleaning solution, rinsed with water and methanol, and dried overnight at 110°.

Blood samples were routinely collected in heparinized tubes, and the plasma was separated by centrifugation. However, in the case of human samples, it was found that less contamination occurred and greater sensitivity was possible if serum was analyzed instead of plasma. Either plasma or serum samples could be stored at 5° for at least 2 months without adversely affecting the analysis.

Exactly 1 ml. of serum was pipeted into a 25-ml. glass-stoppered extraction tube, and 0.05 ml. of concentrated hydrochloric acid was added. The tube was agitated to redissolve the small precipitate, 7 ml. of hexane was added, and the sample was extracted with vigorous shaking for 5 min. The layers were allowed to separate for 5 min., and the hexane layer was transferred with a Pasteur pipet to another extraction tube containing 2 ml. of 10% aqueous sodium carbonate. This was shaken for 1 min. and then allowed to separate for 1 min.; the hexane layer was transferred to a 12-ml. centrifuge tube for evaporation to dryness at 50° under a gentle stream of nitrogen.

The sample was extracted twice more with 6-ml. portions of hexane. These, in turn, were back-extracted with the carbonate solution and transferred to the centrifuge tube. After evaporation of the hexane, the residue was concentrated at the tip of the centrifuge tube with the aid of small portions of hexane–methylene chloride (50:50). The residue was quantitatively transferred to a tube[4] and concentrated in the tip. Exactly 20 μl. of hexane was added, and an accurately measured quantity (~2.5 μl.) was injected into the chromatograph. Figures 2*a* and 2*b* show representative chromatograms of control blood and of a sample containing 0.3 mcg./ml., respectively.

A calibration curve prepared by injecting known amounts of standard Compound I was found to be linear over the range of interest (0.002–3.5 mcg.) and to pass through the origin. To minimize the effects of variations in instrument parameters, standards were run on a daily basis.

Quantitation of the samples was achieved by drawing the baseline and determining the area by multiplying the height times the width at half-height. Sample areas were normalized by dividing by the volume injected, and the quantity of Compound I per microliter

[2] Perkin-Elmer model 900.
[3] Honeywell Electronik 194 or Hewlett-Packard 7123A.

[4] Kontes Microflex.

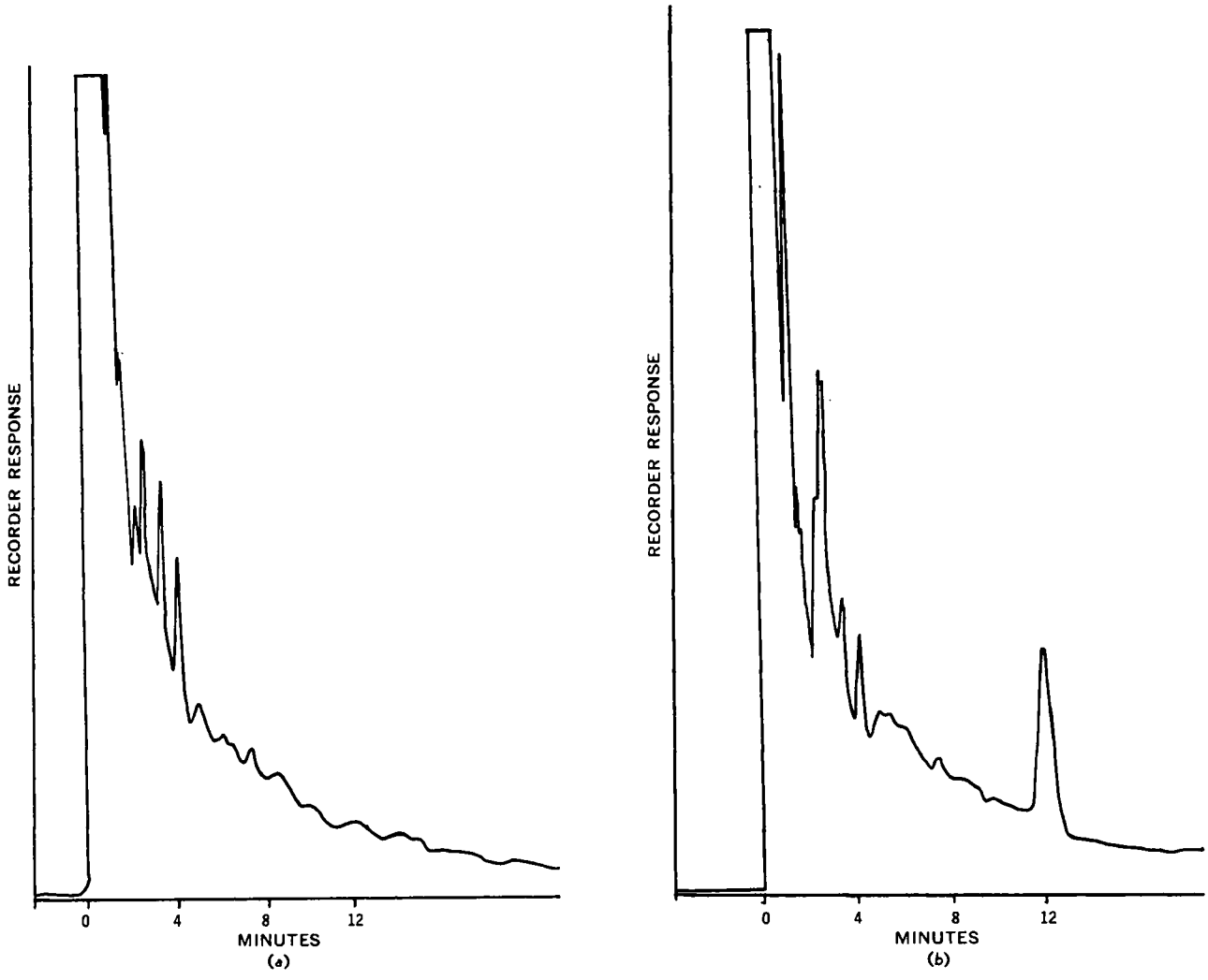


Figure 2—*Typical chromatograms of human plasma extracts:* (a) *control plasma (attenuation ×8), and* (b) *plasma containing 0.30 mcg./ml. Compound I.*

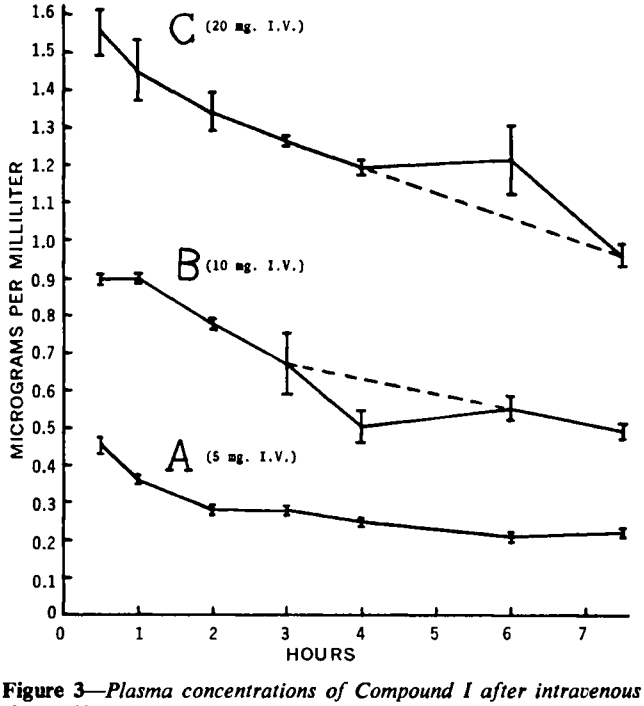


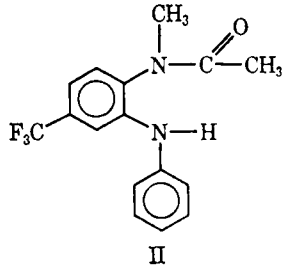
Figure 3—*Plasma concentrations of Compound I after intravenous doses of (A) 5, (B) 10, and (C) 20 mg. to rabbits.*

injected was determined by interpolation of the standard values. This result was multiplied by 20 to arrive at the final concentration in micrograms per milliliter.

RESULTS AND DISCUSSION

Sadée and Van der Kleijn (14) reported that certain diazepine compounds are decomposed during GLC as indicated by GLC–mass spectrometry. To verify the identity of the species being analyzed, mass spectra were obtained on a solid sample of Compound I and on the material eluted from the GLC column. Both mass spectra were identical, indicating the stability of Compound I to GLC.

Although Compound I does not form salts with mineral acids, it was determined in this investigation that recoveries of the drug were enhanced if the plasma or serum was acidified before extraction. Interfering substances that are also extractible from acid solution are removed by the carbonate back-extraction.



II

Table I—Accuracy and Precision of GLC Analysis of Compound I Added to Human Plasma

Added, mcg./ml.	Number of Analyses	Recovered, $\bar{x}$ (Range)	Recovery, %	*RSD*[a], %	*RE*[b], %
10	2	10.15 (9.8–10.5)	101.5	5.29	+1.5
5	13	5.06 (4.7–5.8)	101.2	5.89	+1.2
2	15	2.00 (1.8–2.2)	100.0	7.05	0
1	9	0.96 (0.9–1.1)	96.0	5.31	−4.0
0.5	13	0.48 (0.44–0.56)	96.0	9.79	−4.0

[a] *RSD* = relative standard deviation. [b] *RE* = relative error.

Table II—Plasma Levels[a] of Compound I in Humans

Dose, mg./day	Patient					
	R.E.B.	R.H.	R.B.	J.D.	M.K.	P.A.
10	0.169	0.182	0.099	0.176	0.107	0.193
20	0.286	0.300	0.300	0.414	0.214	0.375
30	0.371	0.376	0.403	0.442	0.328	0.470

[a] Values are micrograms per milliliter.

It was reported[5] that Compound I undergoes hydrolytic degradation in strong acid to form N^1-acetyl-N^1-methyl-N^2-phenyl-(4-trifluoromethyl)-2,1-phenylenediamine (II). However, the rate constants indicate that a negligible amount of hydrolysis should occur at room temperature during the time necessary to extract the plasma. Residues from sample analyses were chromatographed on silica gel thin-layer plates using various solvent systems. Comparison with concurrently chromatographed authentic Compound II indicated no degradation of Compound I. Elution of the sample zone and subsequent GLC confirmed this observation.

The accuracy and precision of the analysis, determined by adding known amounts of Compound I to plasma or serum, are shown in Table I.

To demonstrate the suitability of the method for actual biological samples, rabbits were dosed intravenously with 5, 10, or 20 mg. of Compound I and plasma samples were taken at appropriate intervals for analysis. The plasma concentrations (mean ± *SE*) shown in Fig. 3 illustrate the applicability of the method.

Blood levels were also determined in human plasma samples obtained from a group of patients participating in a clinical study. Plasma concentrations ranging from 0.099 to 0.47 mcg./ml. were found in samples taken 24 hr. after successive daily doses of 10, 20, and 30 mg. (Table II). These results confirm the utility of the GLC analysis for clinical samples.

[5] R. E. Huettemann, Ortho Pharmaceuticals, Raritan, NJ 08869, personal communication.

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▲ To whom inquiries should be directed.

Intraamniotic Prostaglandin $F_{2\alpha}$ Dose–Twenty-Four-Hour Abortifacient Response

W. E. BRENNER[A], C. H. HENDRICKS, J. I. FISHBURNE, Jr., L. STAUROVSKY, J. BRAAKSMA, and R. TAFT

Abstract □ Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was administered intraamniotically to 132 midtrimester gravidas by one of six dose schedules to determine the dose–24-hr. abortifacient response. Single doses between 15 and 50 mg. and multiple doses between 15 and 25 mg. were administered to three groups each, the latter regimen at 6-hr. intervals. Dose–response relationships with both the single- and multiple-injection methods were different in patients of different parities and gestational ages. Interpretation of the dose–response relationships indicated that: (*a*) no one-dose schedule will obtain maximum rates of abortion in all types of patients; (*b*) single injections of greater than 50 mg. of $PGF_{2\alpha}$ are indicated in multiparas of greater than 16 weeks' gestational age, (*c*) in patients at 16 weeks' gestation or less, the low dose multiple-injection schedule attains high abortion rates that probably cannot be achieved by higher multiple-injection methods or any single-injection method; (*d*) it is unlikely that any significant increase in effectiveness can be obtained by multiple injections in excess of 25 mg. initially and repeating an identical dose in 6 hr. in patients greater than 16 weeks' gestation; and (*e*) it is unlikely that any increase in effectiveness will result from single doses in excess of 25 mg. in multiparas of 16 weeks' gestation or less. Complications were not of such severity or frequency as to limit the practicability of these schedules.

Keyphrases □ Prostaglandin $F_{2\alpha}$—intraamniotic dose–24-hr. abortifacient response □ Intraamniotic prostaglandin $F_{2\alpha}$ administration—dose–24-hr. abortifacient response □ Abortion—24-hr. response after intraamniotic prostaglandin $F_{2\alpha}$ administration □ Dose–response relationships—prostaglandin $F_{2\alpha}$ intraamniotic administration and 24-hr. abortifacient response

Although the intraamniotic administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been demonstrated to be an effective means of aborting pregnancies after 14 weeks' gestation (1, 2), the dose schedule safely resulting in the greatest frequency of abortion within the shortest period of time remains to be demonstrated. In an effort to make the search for this dose less arduous for both the subjects and investigators, the dose–24-hr. abortifacient response relationships were studied.

MATERIALS AND METHODS

One hundred thirty-two physically healthy gravidas between 10 and 23 menstrual weeks' gestation received $PGF_{2\alpha}$ intraamniotically by one of six dose schedules to induce therapeutic abortion[1]. There were no significant differences in the compared characteristics between the groups (Table I).

Prostaglandin $F_{2\alpha}$ in a concentration of 5 mg./ml. of diluent was injected through a polyethylene catheter[2] inserted into the amniotic cavity *via* an 18-gauge, thin-wall needle by either the transabdominal or transvaginal approach. The initial 5 mg. $PGF_{2\alpha}$ was injected over 5-min., while the remainder of the dose was injected over the following 5-min. period.

One of six $PGF_{2\alpha}$ dose schedules was used. The single-dose schedules were:

Group I (15 mg.)—Twenty gravidas were injected with 15 mg.

Group II (25 mg.)—Thirty-one gravidas were injected with 25 mg.

Group III (50 mg.)—Twenty-four gravidas were injected with 50 mg. at the initiation of the study, with no further $PGF_{2\alpha}$.

The multiple-dose schedules were:

Group IV (15–15 mg.)—Ten gravidas were injected with 15 mg. at the initiation of the study and an identical dose 6 hr. later.

Group V (25–25 mg.)—Twenty-two gravidas were injected with 25 mg. at the initiation of the study and an identical dose 6 hr. later if abortion had not occurred.

Group VI (25–25–25 mg.)—Twenty-five gravidas were injected with 25 mg. at the initiation of the study with an identical dose 6 and 12 hr. later if abortion had not occurred.

For this study, all trials were declared a failure if abortion (either complete or incomplete) did not occur within 24 hr. of the initial injection. Abortion was classified complete when both the fetus and placenta were completely expelled from the uterus; it was considered incomplete when the placenta was not completely expelled from the uterus. Abortion interval was the period of time from the initial injection to expulsion of the fetus from the uterus. Gestational age was calculated as the nearest number of whole weeks from the first day of the patient's last normal menstrual period. A subject was classified as multiparous if she had one previous delivery after 20 weeks of gestation. Differences at the $p < 0.05$ level were considered statistically significant.

RESULTS

Single-Dose Schedules—Although there was an increase in effectiveness when the dose was raised from 15 mg. (Group I) to 25 mg. (Group II), there was an insignificant increase when the dose was raised from 25 to 50 mg. (Group III) (Fig. 1 and Table II). Increases in frequency of abortion (9%) and in the mean abortion time (3.1 hr.) and differences in the rates of incomplete abortions noted when the dose was raised from 25 to 50 mg. were statistically insignificant.

Different dose–response relationships were noted for patients of different parities and gestational ages (Fig. 2). As the dose was progressively increased in nulliparous patients, increasing rates of abortion were observed. In contrast, as the dose was progressively increased in multiparous patients, decreasing rates of abortion were observed.

Differences were also observed between patients of greater than 16 weeks' gestation (>16 weeks) and 16 weeks' gestation or less (≤16 weeks). In gravidas >16 weeks' gestation, significantly higher rates of abortion were observed with each increase in dose. Higher

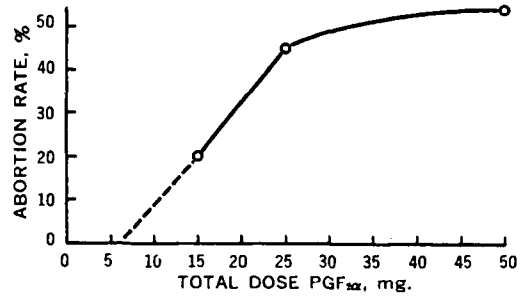

Figure 1—*The 24-hr. abortion rate in percent with three single-injection dose schedules of intraamniotic prostaglandin $F_{2\alpha}$ detailed in the text. The dashed line is extrapolated as the best fitting line between existing frequencies.*

[1] At the Clinical Research Unit of the North Carolina Memorial Hospital, University of North Carolina.
[2] VX020, Becton Dickinson and Co., Rutherford, N. J.

Table I—Characteristics of 132 Gravidas Treated by One of Six Intraamniotic Prostaglandin $F_{2\alpha}$ Dose Schedules

	Group					
	I	II	III	IV	V	VI
	Dose Abbreviation					
	15 mg.	25 mg.	50 mg.	15–15 mg.	25–25 mg.	25–25–25 mg.
Patients, number	20	31	24	10	22	25
Race, number						
Negroid	6	14	5	6	11	13
Caucasian	14	17	18	4	11	12
Mongoloid	0	0	1	0	0	0
Parity, number						
Nulliparas	12	19	17	6	13	16
Multiparas	8	12	7	4	9	9
Gestation, weeks						
Mean	16.8	16.9	16.5	16.5	16.1	16.0
(Range)	(14–20)	(14–21)	(12–23)	(14–20)	(10–23)	(14–20)
≤16 Weeks, number	9	16	13	5	15	16
>16 Weeks, number	11	15	11	5	7	9
Marital status, number						
Single	16	21	21	7	15	21
Married	4	10	3	3	7	4
Weight, kg.						
Mean	51.7	61.0	57.8	54.0	63.2	64.9
(Range)	(44–79)	(48–80)	(45–90)	(43–107)	(41–102)	(48–118)
Height, in.						
Mean	64.6	64.0	64.0	63.7	63.3	65.0
(Range)	(52–70)	(59–69)	(59–71)	(58–68)	(59–67)	(60–72)

abortion rates were not obtained with single injections in excess of 25 mg. in patients ≤16 weeks' gestation. In nulliparous patients and in patients >16 weeks' gestation, the abortifacient response was higher when the dose was increased from 25 to 50 mg. In patients ≤16 weeks' gestation, the 50-mg. dose was no more effective than the 25-mg. dose, and in multiparas the 50-mg. dose was less effective.

Multiple-Dose Schedules—Although the overall results indicated no increase in the frequency of abortion within the 24-hr. period for dosage levels higher than 15 mg. repeated in 6 hr. (Group IV) (Fig. 3), differences in abortion rates were noted for patients of different parities and gestational ages (Fig. 4). Nulliparas and patients ≤16 weeks' gestation had significantly lower rates of abortion when the dose was increased above that of Group IV. However, in multiparas and patients ≤16 weeks' gestation, higher rates of abortion were noted to the Group V level (25 mg. repeated in 6 hr.). Although the mean abortion time of Group V was shorter and the rates of incomplete abortion were higher for Group VI, these differences were not statistically significant.

Single-Dose Compared to Multiple-Dose Schedules—All multiple-dose schedules resulted in higher abortion rates than the single-dose schedules (Fig. 5). The multiple-injection schedule of Group VI, however, did not result in a statistically significant increase over the single injection of 50 mg. (Group III).

When the differences in dose schedules were analyzed for patients of different parities and gestational ages (Fig. 6), the multiple-injection schedules were as effective, if not more effective, in comparable total doses. In both nulliparas and patients ≤16 weeks' gestation, the Group IV dose schedule was the most effective. In contrast, in multiparas and patients >16 weeks' gestation, the Group V (25–25 mg.) schedule was the most effective. Single doses of 50 mg. (Group III) appeared equally as effective as the 50-mg. dose administered in two separate 25-mg. doses (Group V) in nulliparas and patients >16 weeks' gestation.

Complications—Although the minor complications were frequent, no serious complications were observed (Table III). The frequency

Table II—Twenty-Four-Hour Abortion Results of 132 Gravidas Treated by One of Six Intraamniotic Prostaglandin $F_{2\alpha}$ Dose Schedules

	Group					
	I	II	III	IV	V	VI
	Dose Abbreviation					
	15 mg.	25 mg.	50 mg.	15–15 mg.	25–25 mg.	25–25–25 mg.
Patients, number	20	31	24	10	22	25
Abortions, number (%)	4 (20)	14 (45)	13 (54)	7 (70)	15 (68)	15 (60)
Complete	2 (10)	9 (29)	6 (25)	6 (60)	14 (63)	10 (40)
Incomplete	2 (10)	5 (16)	7 (29)	1 (10)	1 (05)	5 (20)
Mean time, hr.	19.0	14.5	17.6	17.4	13.8	15.2
(Range)	(15.6–21.5)	(4.8–21.0)	(8.5–23.9)	(8.5–23.0)	(6.5–23.8)	(4.0–22.5)
Nulliparas, number	12	19	17	6	13	16
Abortions, number (%)	0 (0)	7 (37)	11 (65)	5 (83)	9 (69)	9 (56)
Complete	0 (0)	6 (32)	4 (24)	5 (83)	9 (69)	8 (50)
Incomplete	0 (0)	1 (05)	7 (41)	0 (0)	0 (0)	1 (6)
Multiparas, number	8	12	7	4	9	9
Abortions, number (%)	4 (50)	7 (58)	2 (29)	2 (50)	6 (67)	6 (67)
Complete	2 (25)	3 (25)	2 (29)	0 (0)	5 (56)	2 (22)
Incomplete	2 (25)	4 (33)	0 (0)	2 (50)	1 (11)	4 (44)
≤16 Weeks' gestation						
Patients, number	9	16	13	5	15	16
Abortions, number (%)	2 (22)	6 (38)	5 (38)	5 (100)	10 (67)	9 (56)
Complete	1 (11)	4 (25)	2 (15)	4 (80)	10 (67)	7 (44)
Incomplete	1 (11)	2 (13)	3 (23)	1 (20)	0 (0)	2 (12)
>16 Weeks' gestation						
Patients, number	11	15	11	5	7	9
Abortions, number (%)	2 (18)	8 (53)	8 (73)	2 (40)	5 (71)	6 (67)
Complete	1 (9)	5 (33)	4 (36)	1 (20)	4 (57)	3 (33)
Incomplete	1 (9)	3 (20)	4 (36)	1 (20)	1 (14)	3 (33)

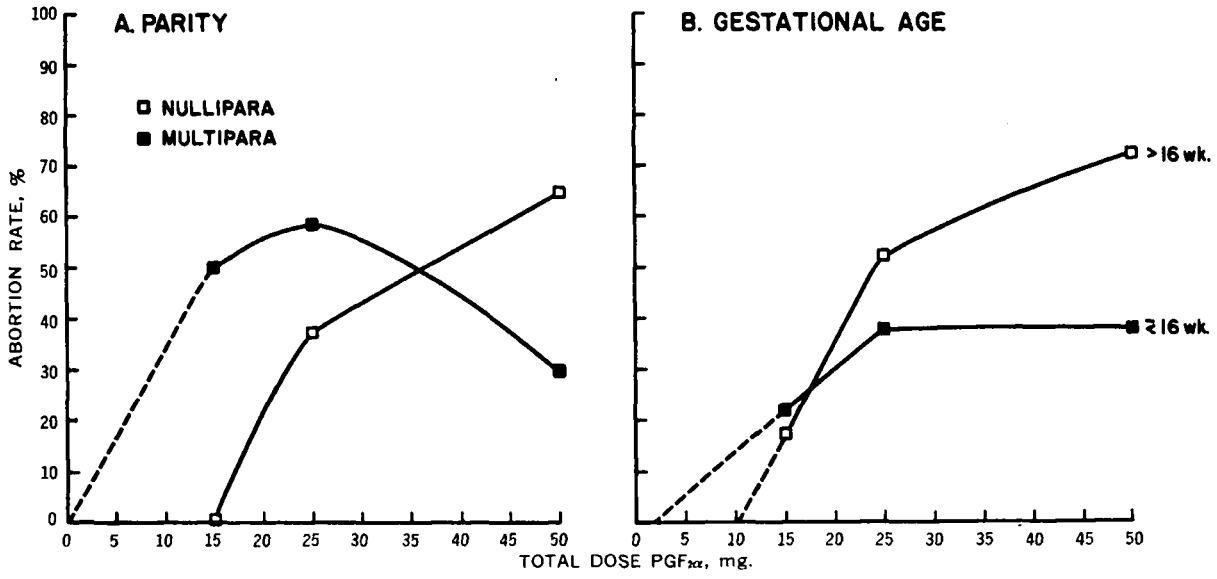

Figure 2—*The 24-hr. abortion rate in percent with three single intraamniotic prostaglandin $F_{2\alpha}$ dose schedules (detailed in the text) for: A, nulliparas and multiparas; and B, patients 16 weeks' gestation or less (≤16 wk.) and patients greater than 16 weeks (>16 wk.). The dashed line is extrapolated as the best fitting line between existing points.*

of complications did not appear to correlate with dose, parity, or gestational age.

DISCUSSION

In an effort to develop practicable methods of midtrimester abortion, investigators have pursued both multiple- and single-intraamniotic $PGF_{2\alpha}$ injection techniques (3–7). If intraamniotic injection is as effective as intravenous infusion, high abortion rates within 24 hr. are possible (8). Search for the most effective dose schedules has been conducted by administering progressively increasing amounts in an increasing number of injections. Results from these studies indicate that more than 50% of the patients can be aborted without serious complications within 24 hr. by a variety of unaugmented $PGF_{2\alpha}$ dose schedules. Initially, only small groups of patients were necessary to demonstrate differences in effectiveness between these schedules because there were large differences in the rates of abortion. However, single- and multiple-injection dose schedules with smaller differences in rates of abortion have been developed which require large numbers of patients to demonstrate differences with any statistical certainty. Therefore, the progressively-increasing-the-dose method is no longer a practical means for determining the dose schedule with the highest rates of abortion and tolerable side effects.

Because most therapeutic agents have certain characteristics in common, by determining one of these characteristics, the dose–response relationship, one should be able to use it to make the search for the most effective dose schedule less arduous for patients and investigators. Although dose–response relationships are constant for a drug under identical conditions (9), in this study the midtrimester patients differ from each other in many variables, some of which greatly influence the dose–response relationships. Two of these variables are parity and gestational age.

Single-Injection Techniques—Although doses of greater than 25 mg. of $PGF_{2\alpha}$ do not appear warranted from the results in the whole group (Fig. 1), parity and gestational age are important variables in

Table III—Complications in 132 Gravidas Treated by One of Six Intraamniotic Prostaglandin $F_{2\alpha}$ Dose Schedules

	Group					
	I	II	III	IV	V	VI
	Drug Abbreviation					
	15 mg.	25 mg.	50 mg.	15–15 mg.	25–25 mg.	25–25–25 mg.
Patients, number	20	31	24	10	22	25
Vomiting						
Patients, number (%)	8 (40)	8 (26)	15 (62)	7 (70)	10 (45)	14 (56)
Mean number episodes	1.6	0.7	1.3	1.1	1.0	1.0
(Range)	(0–4)	(0–8)	(0–5)	(0–3)	(0–5)	(0–4)
Antiemetics						
Patients, number (%)	2 (10)	5 (16)	2 (8)	1 (10)	3 (14)	5 (20)
(Range)	(0–3)	(0–2)	(0–2)	(0–1)	(0–1)	(0–3)
Pain						
Patients, number (%)	14 (70)	11 (35)	23 (96)	7 (70)	16 (73)	19 (76)
Mean doses meperidine	1.6	0.8	2.8	1.5	1.2	1.8
(Range)	(0–5)	(0–4)	(0–9)	(0–6)	(0–4)	(0–6)
Diarrhea						
Patients, number (%)	0 (0)	1 (3)	6 (25)	2 (20)	1 (5)	0 (0)
Fever >100°F						
Patients, number (%)	1 (5)	1 (3)	3 (13)	0 (0)	1 (5)	6 (24)
≤16 Weeks' gestation, number	9	16	13	5	15	16
Vomiting, number (%)	4 (44)	4 (25)	7 (54)	4 (80)	7 (47)	9 (56)
>16 Weeks' gestation, number	11	15	11	5	7	9
Vomiting, number (%)	4 (36)	4 (27)	8 (73)	3 (60)	3 (43)	5 (56)
Nullipara, number	12	19	17	6	13	16
Vomiting, number (%)	5 (42)	5 (26)	13 (76)	5 (83)	7 (54)	10 (62)
Multipara, number	8	12	7	4	9	9
Vomiting, number (%)	3 (38)	3 (25)	2 (29)	2 (50)	3 (33)	4 (44)

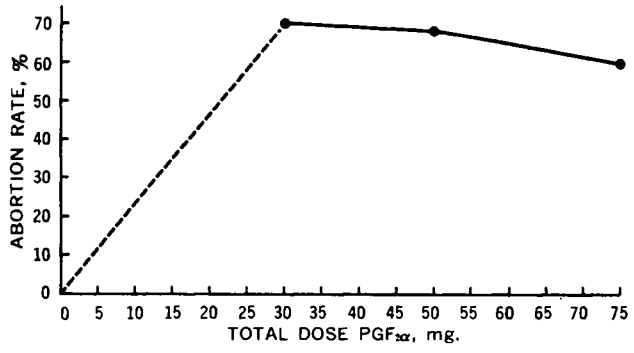

Figure 3—*The 24-hr. abortion rate in percent with three intraamniotic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) multiple-injection dose schedules (detailed in the text). The dashed line is an extrapolation to zero abortion rate at zero dose of $PGF_{2\alpha}$.*

significant variable, two conclusions appear justified: (*a*) doses greater than 15 mg. initially with an identical dose repeated 6 hr. later probably will not increase the 24-hr. abortion rate in nulliparas ≤16 weeks' gestation, and (*b*) doses greater than 25 mg. repeated in 6 hr. will probably not improve the 24-hr. abortion rate in multiparas ≤16 weeks' gestation.

Comparison of Single- and Multiple-Dose Schedules—Overall, these multiple-dose schedules achieved higher 24-hr. abortion rates than the single-injection schedules (Fig. 5). However, when parity and gestational age were considered, the single-dose abortifacient response curves had not reached a plateau (no further increase in frequency of abortion with further increase in dose) at the 50-mg. (Group III) dose in nulliparas and patients >16 weeks' gestation. Rates in multiparas receiving a single injection of 25 mg. (Group II) were not significantly different from those in multiparas receiving multiple injections of 25 mg. (Groups V and VI), and rates greater than about 65% probably cannot be achieved for all multiparas with either type of dose schedule. The multiple-injection technique appears definitely superior for patients ≤16 weeks' gestation. Although the single-dose schedule curve reached a plateau after 25 mg. for these patients at less than a 50% abortion rate, the multiple-injection schedule used in Group IV (15–15 mg.) resulted in a 100% abortion rate. Whether the dose–response curve for single doses higher than 50 mg. in nulliparas and patients >16 weeks' gestation will reach a plateau or surpass the rates achieved with multiple injections remains to be demonstrated. Finally, it does not appear that any one dose, whether it is administered by single-injection or multiple-injection methods, will be maximally effective in patients of all gestational ages and parities.

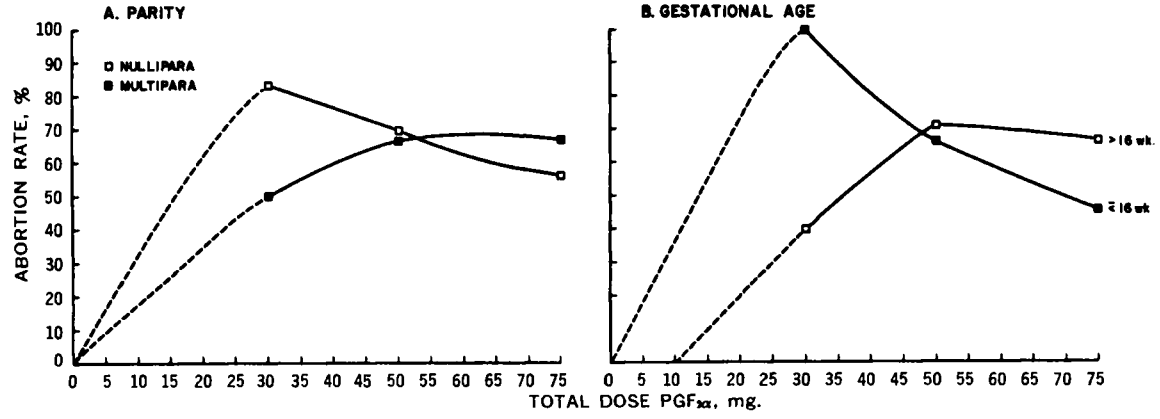

Figure 4—*The 24-hr. abortion rate in percent with three multiple intraamniotic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) dose schedules (detailed in the text) for: A, nulliparas and multiparas; and B, patients 16 weeks' gestation or less (≤16 wk.) and patients greater than 16 weeks' gestation (>16 wk.). The dashed line is the extrapolation of either the best fitting line between existing points or to zero abortion rate at zero dose of $PGF_{2\alpha}$.*

the dose–response relationship (Fig. 2). Two conclusions appear warranted as a means of increasing the 24-hr. abortifacient efficiency: (*a*) doses in excess of 25 mg. in multiparas ≤16 weeks' gestation probably will not increase the rate, and (*b*) investigation of single injections greater than 50 mg. in nulliparas >16 weeks' gestation may improve the rate.

Multiple-Injection Schedules—Within the limits of these dose schedules, it appears from the overall results that there is no benefit to increasing the dose schedule above an initial injection of 15 mg. and repeating an identical dose in 6 hr. However, parity and gestational age are significant variables (Fig. 4). Interpretation of multiple-injection dose–response relationships is even more limited than that of single-dose schedules because of the many dose combinations possible. Although there were not enough patients in this series to determine whether parity or gestational age was the more

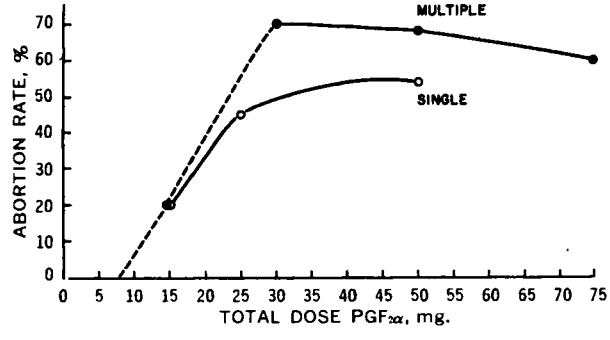

Figure 5—*The 24-hr. abortion rate in percent for three single and three multiple intraamniotic prostaglandin $F_{2\alpha}$ dose schedules (detailed in the text). The dashed lines are extrapolations.*

Complications—Because the dose schedules investigated were not associated with any serious complications and minor complications were of minimal severity, the effective schedules can be used clinically. Chlorpromazine and diphenoxylate hydrochloride/atropine sulfate[3] were demonstrated to be effective in decreasing the frequency and severity of vomiting and diarrhea, respectively (1, 10). One can only speculate upon whether premedicating patients to prevent minor complications will be advisable with the most effective dose schedule when it is identified.

Even though one can make certain hypotheses from dose–response curves, comparative series of different dose schedules will be necessary to verify these hypotheses. Dose–response relationships are absolutely reproducible only when all conditions are equal —a situation unimaginable in clinical medicine. However, once the significant variables are identified, the dose–response relationships are within ranges useful for clinical purposes. Two variables significantly modifying the dose–response relationship have been identified: parity and gestational age. As the information expands, the relative importance of these and other conditions should be specified.

[3] Lomotil, Searle. (Each tablet contains 2.5 mg. diphenoxylate hydrochloride and 0.025 mg. atropine sulfate.)

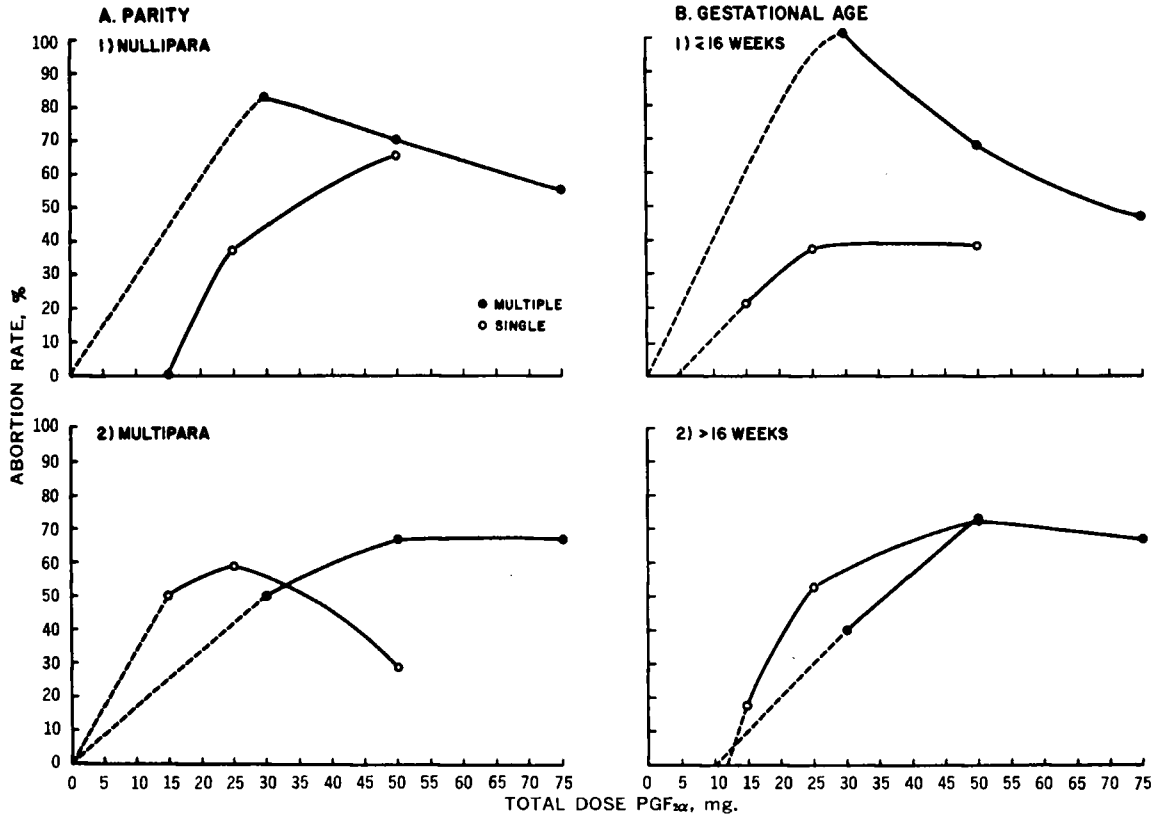

Figure 6—*The 24-hr. abortion rates in percent with three single- and three multiple-dose intraamniotic prostaglandin $F_{2\alpha}$ schedules (detailed in the text) for: A, patients of different parities (nulliparas and multiparas); and B, patients of different gestational ages [16 weeks' gestation or less (≤16) and greater than 16 weeks' gestation (>16)]. The dashed lines are extrapolations.*

Interpretation of the dose-response relationships indicates that further study of the following is desirable to improve the abortion rates: (*a*) single doses greater than 50 mg. in nulliparas and patients >16 weeks' gestation, (*b*) single doses in the range of 25 mg. in multiparas and patients <16 weeks' gestation, (*c*) multiple doses in the range of 15 mg. repeated in 6 hr. for nulliparas and patients >16 weeks' gestation, and (*d*) multiple doses in the range of 25 mg. repeated in 6 hr. for multiparas and patients >16 weeks' gestation. Although these interpretations of the dose-response relationships appear to be logical, extensive clinical trials will be necessary to confirm these hypotheses.

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Nonsolvolytic Acetylation of Homatropine by Aspirin

E. SHAMI[▲], J. DUDZINSKI, L. LACHMAN, and J. TINGSTAD*

Abstract □ A nonsolvolytic (solid-state) reaction between aspirin and homatropine, yielding acetylhomatropine and free salicylic acid, is reported. A GC assay for homatropine is described. In the presence of excess aspirin, the kinetics are pseudo first order with respect to homatropine. The apparent heat of activation is estimated and a possible mechanism of the reaction is discussed.

Keyphrases □ Homatropine—kinetics and possible mechanism of nonsolvolytic (solid-state) acetylation by aspirin □ Aspirin—kinetics and possible mechanism of nonsolvolytic (solid-state) acetylation of homatropine □ Acetylhomatropine and salicylic acid—products of solid-state acetylation of homatropine by aspirin, kinetics □ Acetylation, nonsolvolytic (solid state)—reaction of aspirin and homatropine □ Solid-state (nonsolvolytic) acetylation reactions—aspirin and homatropine

Compared to the literature on aqueous solutions, there are relatively few publications on kinetics and mechanisms of reactions in the solid state. The nonsolvolytic (solid-state) acetylation of drugs containing alcoholic, aminic, or phenolic groups by aspirin has been reported (1–3). This article describes the kinetics and possible mechanism of a nonsolvolytic reaction between aspirin and homatropine, yielding acetylhomatropine and free salicylic acid.

EXPERIMENTAL

Aspirin USP (containing less than 0.05% free salicylic acid) was used. The homatropine base employed assayed 100.3% by nonaqueous titration. The two drugs were mixed in a 12:1 (aspirin–homatropine) molar ratio and were allowed to react at the desired temperatures. The 12:1 ratio was chosen because of assay convenience and because it would simplify reaction kinetics by eliminating aspirin concentration as a significant factor (4). It was felt that this experimental procedure was valid because the ratio of the two in the tablet formulation under study was even greater than 12:1.

Homatropine Assay—A gas chromatograph[1] utilizing a flame-ionization detector was used with a 1.2-m. (4-ft.) × 3-mm. i.d. glass column, using Diatoport S (80–100 mesh) coated with UC-W-98 silicone rubber (3.8%). The column temperature was 215°, and both the detector temperature and the injection port temperature were 270°. Gas flow rates were: helium, 75 ml./min.; hydrogen, 35 ml./min.; and air, 350 ml./min. Codeine was used as the internal standard.

About 1.4 g. of the aspirin–homatropine mixture was accurately weighed and dissolved in 100.0 ml. of chloroform. A 25.0-ml. aliquot was taken and extracted three times with an equal volume of distilled water adjusted to pH 11–12 with ammonia. A 20.0-ml. aliquot of the chloroform layer was pipeted into a 250-ml. distilling flask, the internal standard was added, and the solution was evaporated to dryness *in vacuo*. A standard homatropine solution was similarly treated. Ten milliliters of chloroform was pipeted into each of the sample and standard flasks and, after solution was complete, 1.8 μl. was injected into the gas chromatograph with a 1.0-μl. solvent flush. The peak height of homatropine relative to codeine was determined in both the sample and reference standard.

Free Salicylic Acid Assay—Approximately 1.4 g. of the aspirin–homatropine mixture was accurately weighed and dissolved in 100.0 ml. of methanol. An aliquot was taken and assayed by the standard ferric nitrate procedure (5).

Tropine Assay—The same procedure was used as for homatropine except that the column temperature was 115° and the detector and flash heater were both at 190°.

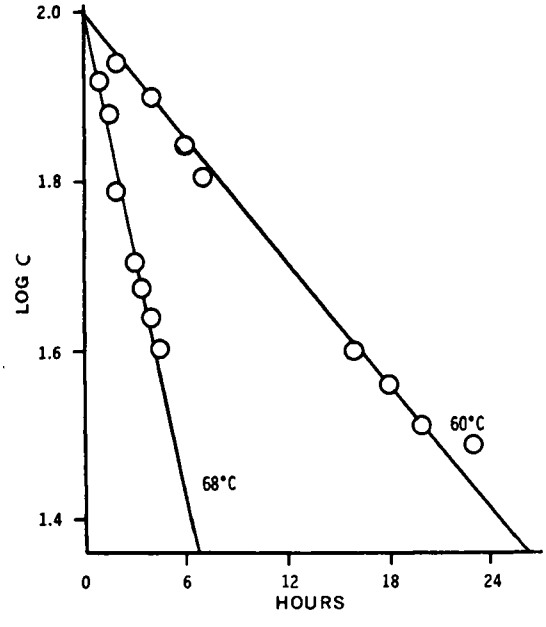

Figure 1—*Disappearance of homatropine at 60 and 68°.*

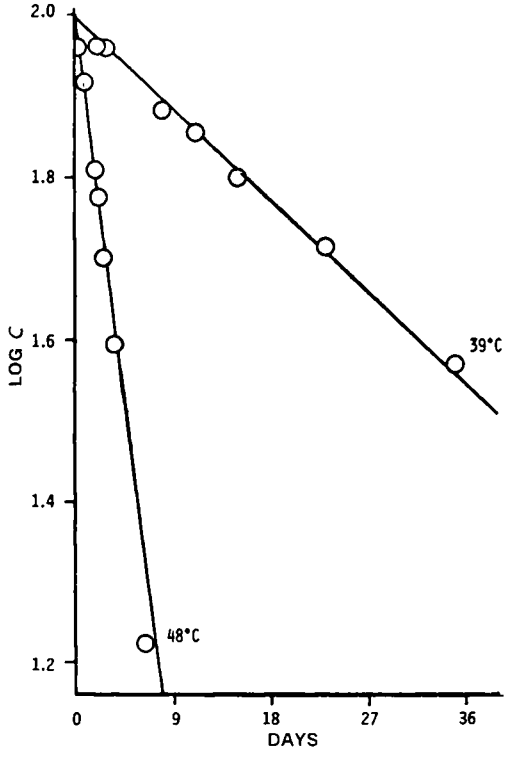

Figure 2—*Disappearance of homatropine at 39 and 48°.*

[1] F & M 402.

Table I—Reaction Rate Constants and Half-Lives for Homatropine Disappearance

Temperature	Reaction Rate Constant, hr.$^{-1}$	Half-Life, hr.
68°	0.209	3.32
60°	0.0531	13.0
48°	0.0105	65.8
39°	0.00120	577

Table II—Reaction Rate Constants and Half-Lives for Free Salicylic Acid Formation

Temperature	Reaction Rate Constant, hr.$^{-1}$	Half-Life, hr.
60°	0.0532	13.0
48°	0.0105	65.8

Kinetic Studies—Aspirin and homatropine base (12:1) were gently mixed in a glass mortar and placed in preweighed glass ampuls. Weights were accurately determined and the ampuls were sealed. Samples were stored at 39, 48, 60, and 68°, periodically removed, and assayed. Because a eutectic forms after a period of time, the entire contents of the ampul were used for each assay to ensure no loss of material. The 68° samples were maintained in a constant-temperature bath (±0.2°) while the rest were stored in hot air ovens (±1°). To ensure proper temperature control in the ovens, a recording thermal probe was placed near the samples.

Synthesis of Acetylhomatropine—Homatropine base was dissolved in a 1:1 mixture of pyridine and acetic anhydride and stored at room temperature (in the dark to prevent color formation) for 24 hr. Methanol was added to destroy the acetic anhydride; the solution was then evaporated on a flash evaporator until there was no odor of pyridine. The resulting oil was difficult to purify; consequently, the picrate salt was made. One-half gram of the oil was dissolved in 5 ml. of methanol, 5 ml. of a saturated solution of picric acid was added, and the resulting precipitate was recrystallized from boiling methanol.

Anal.—Calc. for $C_{15}H_{21}NO_4$: C, 52.75; H, 4.80; N, 10.25. Found: C, 52.79; H, 4.80; N, 10.23.

TLC System—A chamber[2] with a saturation pad was used for all chromatograms. Precoated aluminum oxide plates[3] were activated for 0.5 hr. at 100° prior to use. The developing solvent system was chloroform–methanol–diethylamine (200:10:1). For color development the plates were sprayed with Dragendorff's reagent followed by an overspray of 5% sodium nitrite.

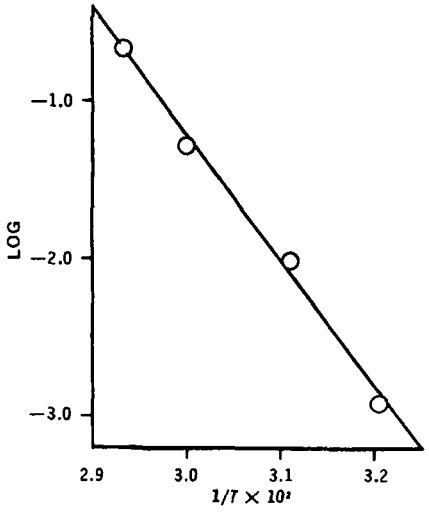

Figure 3—*Arrhenius plot for homatropine disappearance.*

[2] Gelman.
[3] Brinkmann T250μ.

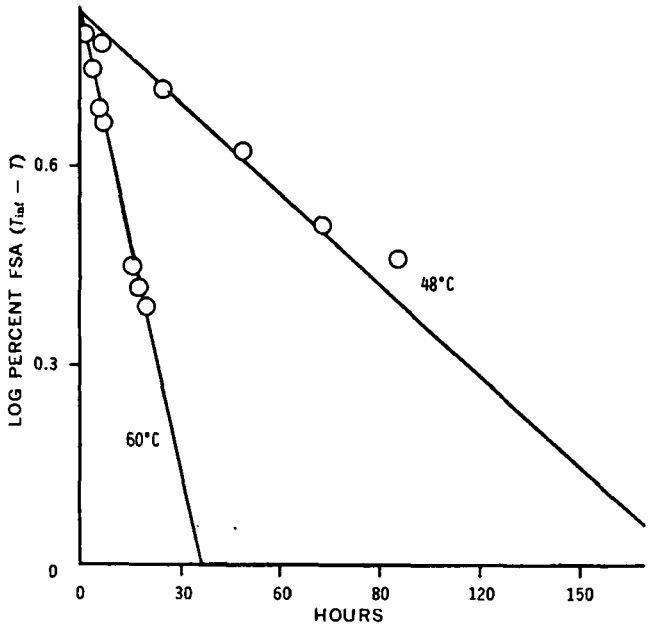

Figure 4—*Appearance of free salicylic acid (FSA) at 48 and 60°. See text for explanation.*

RESULTS

TLC and GLC analyses indicated that the partially reacted mixtures contained only homatropine, aspirin, salicylic acid, and acetylhomatropine, with trace amounts of acetic acid. The completely reacted mixtures (at time infinity) contained only aspirin, salicylic acid, and acetylhomatropine, with trace amounts of acetic acid. No tropine was found in any of the reaction mixtures; consequently, hydrolysis of homatropine can be ignored (6, 7).

Disappearance of Homatropine—Based on the kinetic plots shown in Figs. 1 and 2, the reaction appears to be pseudo first order. An Arrhenius plot (Fig. 3) yields an activation energy of 36.4 kcal./mole. The reaction rate constants and half-lives at the various temperatures are given in Table I.

Appearance of Free Salicylic Acid—The rates of appearance of free salicylic acid, plotted as percentage free salicylic acid at time infinity minus percentage at time t for 48 and 60°, are shown in Fig. 4. The experimentally determined values of the free salicylic acid infinity term (6 days, 60° = 6.93% and 8 weeks, 48° = 6.50%) are in good agreement with the theoretical value of 6.39%. Based

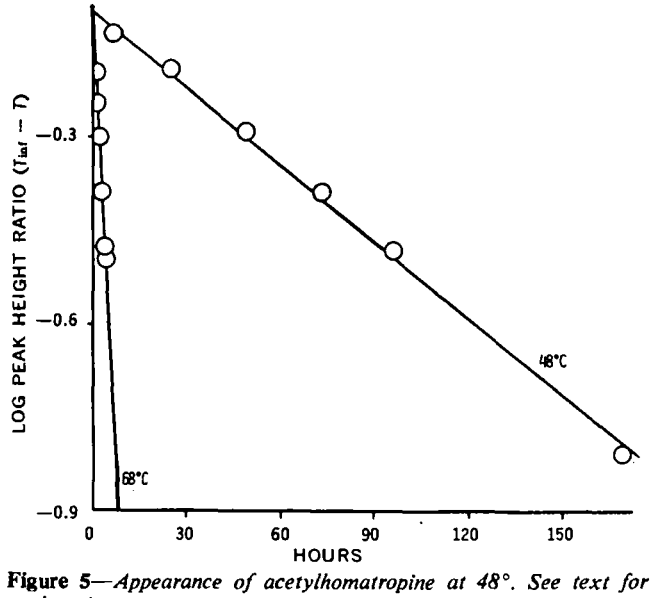

Figure 5—*Appearance of acetylhomatropine at 48°. See text for explanation.*

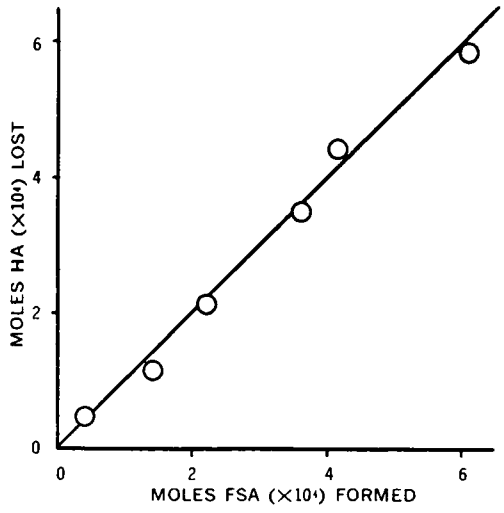

Figure 6—*Correlation of disappearance of homatropine (HA) with appearance of free salicylic acid (FSA) at 48°.*

upon the appearance of acetylhomatropine and the disappearance of homatropine, the reaction seemed to be complete at 48° in at least 5 weeks. The fact that 3 additional weeks at this temperature did not produce significant amounts of excess free salicylic acid indicates that degradation of aspirin to salicylic acid apart from its reaction with homatropine was not a significant factor in these studies. This is further confirmed by the fact that the reaction rate constants and half-lives for the disappearance of homatropine (Table I) are in good agreement with those found for the appearance of salicylic acid (Table II).

Appearance of Acetylhomatropine—The rate of acetylhomatropine formation, plotted as the peak height ratio (acetylhomatropine–codeine after normalizing all sample weights to 1 g.) at time infinity minus that at time t for 48 and 68°, is shown in Fig. 5. The infinity value was taken as the average normalized peak height ratio at 5 and 8 weeks at 48°, and the two values agreed within 3%. These figures were confirmed by the fact that at 5 weeks, at 48°, no homatropine could be detected in the reaction mixture. The reaction rate constants and half-lives for acetylhomatropine appearance are given in Table III; they are in good agreement with those of Tables I and II.

Correlation of Homatropine Loss and Free Salicylic Acid Formation—The tabular data indicate a 1:1 molar relationship in the reaction. This is further emphasized in Fig. 6 where, at 48°, the moles of free salicylic acid formed is plotted against moles of homatropine lost; the correlation coefficient is 0.99. Similar plots of 39 and 60° data yield coefficients of 1.06 and 0.96, respectively. The coefficient for 68° is 0.87, indicating some possible variations in the reaction not observed at lower temperatures.

Confirmation of Acetylhomatropine Synthesis—NMR analysis of the synthesized homatropine picrate showed the disappearance of the alcohol proton and the appearance of three methyl protons on the acetate group. TLC results for the "known" acetylhomatropine base (the oil resulting from the reaction mixture) and unknown samples (the heat-treated mixtures of aspirin and homatropine base) were identical (no tropine present in the unknown). The following R_f values were obtained: acetylhomatropine, 0.64; homatropine, 0.42; and tropine, 0.20.

Table III—Reaction Rate Constants and Half-Lives for Acetylhomatropine Formation

Temperature	Reaction Rate Constant, $hr.^{-1}$	Half-Life, hr.
68°	0.202	3.43
48°	0.00946	73.3

DISCUSSION

There are two probable mechanisms for the reaction. In the first version, there is liquefaction followed by subsequent reaction. The low water content (less than 0.05%) and the absence of excess free salicylic acid in the reaction mixture eliminate water as a factor, either in liquefaction or in the reaction kinetics. The eutectic contains no solid homatropine (as shown by differential scanning calorimetry); aspirin is present both in the liquid phase and as a crystalline solid. The "saturated" solution with respect to aspirin (with excess solid phase present) accounts for the pseudo-first-order character of the reaction.

The second version of the proposed mechanism is identical to the first except that after liquefaction the reaction (probably intramolecular transesterification) is preceded by salt formation. A similar "solid-state" salt formation between aspirin and antipyrine has been reported (8). The second version, involving salt formation, seems more likely for the following reasons:

1. If a 1:1 molar mixture of aspirin and homatropine is dissolved in a chloroform–methanol mixture and evaporated to dryness, the resulting oil is soluble in water.
2. When a 1:1 molar mixture of the two is gently heated, an oily liquid results which does not solidify upon cooling. IR scans of this oil and of a solid 1:1 mixture indicate salt formation.
3. The relatively high heat of activation (36.4 kcal./mole) points toward a possible contribution from heat of ionization and/or fusion.

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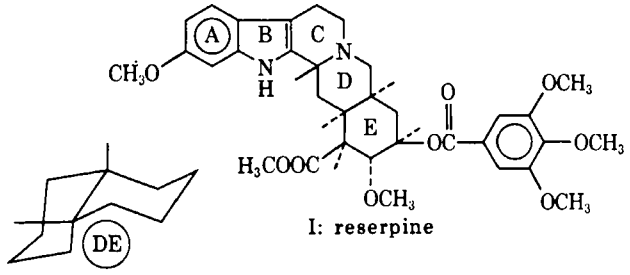
Cardiovascular Effects of Reserpine, Yohimbine, and Reserpine–Yohimbine Mixtures on Intact Anesthetized Dog

MARK M. LUCKENS[▲] and MARVIN H. MALONE*

Abstract □ Cardiovascular and respiratory parameters plus responses to periodically injected epinephrine were monitored for 5 hr. in the anesthetized dog after single intravenous injections of saline, reserpine, yohimbine, or reserpine–yohimbine mixtures. Whole root *Rauwolfia serpentina* contains reserpine-like and yohimbine-like alkaloids and is widely used and classified as a reserpine-like agent. However, qualitative results in the present study indicate interactions between reserpine and yohimbine when administered concurrently, with end results incorporating the time–response profiles of both agents when given singly.

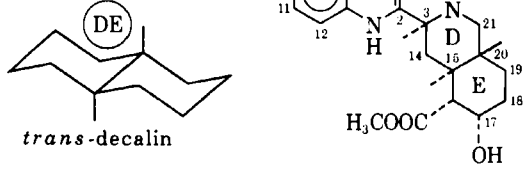
Keyphrases □ Reserpine, yohimbine, and reserpine–yohimbine mixtures—cardiovascular effects on intact anesthetized dog □ Yohimbine, reserpine, and yohimbine–reserpine mixtures—cardiovascular effects on intact anesthetized dog □ Cardiovascular effects—reserpine, yohimbine, and reserpine–yohimbine mixtures, dogs □ Rauwolfia whole root preparations—cardiovascular effects of reserpine, yohimbine, and reserpine–yohimbine mixtures, dogs

Reserpine (I) and yohimbine (II) are naturally occurring pentacyclic tertiary alkaloids whose basic ring systems present a number of similarities. In the former, the D and E rings have a *cis*-decalin conformation; in the latter compound, these rings are *trans*-decalin. Reserpine is esterified with a trimethoxybenzoic moiety on the E ring. The methyl ester grouping at position 16 on the E ring is in a *trans*-position in the reserpine compound, while it is in a *cis*-position in yohimbine. Both compounds possess the α-configuration at the C-15 position. Since the proton at this site does not appear to exhibit a facile epimerization, it may well be that some pharmacological properties of these alkaloids may be due partly to this configuration. Cursory examination indicates that "flipping" with respect to the D and E rings could exist in reserpine, but this would not be the case for the yohimbine molecule. This may also contribute to the different pharmacological profiles of these compounds.

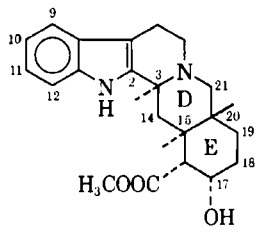


cis-decalin

I: reserpine



trans-decalin



II: yohimbine

Both compounds occur together in many rauwolfia species of the family Apocynaceae. The isolation of reserpine from *Rauwolfia serpentina* Benth may be considered a landmark in the development of modern psychopharmaceuticals. Its isolation also spurred productive investigations in antihypertensive therapy and provoked a general revival of interest in plants as a source of useful and potent therapeutic agents. Our interest in studying selected cardiovascular effects of reserpine, yohimbine, and mixtures of these alkaloids in the intact animal stemmed from the fact that they are concurrently administered therapeutically in medicinals prepared from, or containing, whole root *R. serpentina*. Such whole root preparations antagonize the pressor response to epinephrine (1).

Yohimbine is a classic α-adrenergic blocking agent. It blocks the pressor response to epinephrine and, in sufficient doses, induces "epinephrine reversal" (2). Reserpine is presumed to have no effect on adrenergic receptors, but it depletes central and peripheral stores of catecholamines. This depletion has been reported to hypersensitize adrenergic receptors and potentiate the effect of exogenously administered norepinephrine (3, 4). Yohimbine does not deplete either serotonin or epinephrine centrally (5), but there is evidence that it reduces or depletes norepinephrine in the brain (5). It has also been reported (6) to be an antimetabolite of serotonin. Malone and Roth (7) reported that yohimbine potentiates reserpine-induced blepharoptosis in mice without altering the slope of the dose–response curve. Blepharoptosis is a manifestation of hypothalamic depression and is seen in Horner's syndrome in man (8).

A number of clinical and animal studies demonstrated that preparations made from the whole root of rau-

Table I—Experimental Treatments and 5-hr. Lethality

Treatment at Zero Hour	Dose[a], mg./kg. i.v.	Mean Weight, kg.	Quantal Survival (>300 min.)	Mean Time of Death, min.
0.9% saline	—	14.0	4/4	—
Reserpine	1.0	12.4	4/4	—
Yohimbine	0.1	18.9	4/4	—
Yohimbine	1.0	16.2	4/4	—
Reserpine + yohimbine	1 + 0.1	20.0	3/4	195 (1)
Reserpine + yohimbine	1 + 0.25	14.9	3/4	165 (1)
Reserpine + yohimbine	1 + 1.0	14.1	0/4	26 (4)
Reserpine + yohimbine	0.5 + 0.5	16.6	1/4	129 (3)

[a] Constant dosage of 5 ml. was used.

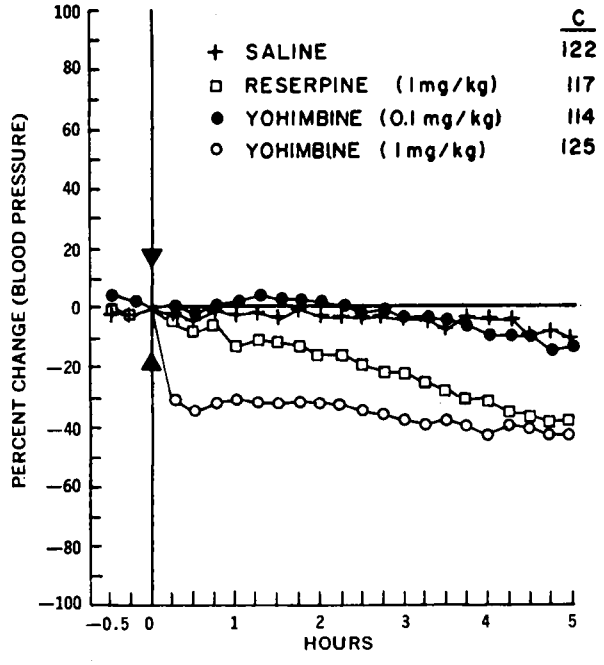


Figure 1—*Blood pressure changes after intravenous administration of isotonic saline, reserpine, or yohimbine expressed as percent change from control (C) values (mm. Hg).*

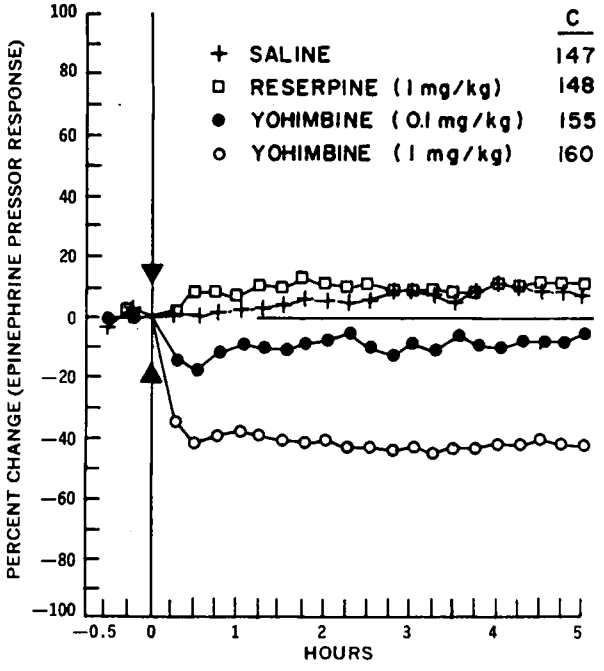


Figure 3—*Changes in epinephrine pressor response after intravenous administration of isotonic saline, reserpine, or yohimbine expressed as percent change from control (C) values (mm. Hg).*

wolfia exhibit greater sedative–hypotensive potency than would be expected from reserpine alone or simply the additive effects of a combination of the various reserpine-like alkaloids (9–15). To date, no experimental study has investigated the cardiovascular dynamics of reserpine–yohimbine mixtures in the intact animal. The reserpine content of *R. serpentina* Benth, collected in the Himalayan foothills, was documented at 0.14% (16). The yohimbine content of such roots has not been reported to date. The ratios of reserpine–yohimbine tested in this cardiovascular study varied from 1.0:1.0 to 1.0:0.1 based on the assumption that the actual content of yohimbine in rauwolfia whole root neither exceeds that of reserpine nor is less than one-tenth that of reserpine.

MATERIALS AND METHODS

Thirty-two mongrel dogs of both sexes, weighing 9.5–40.0 kg., were randomly divided into eight experimental groups (Table I).

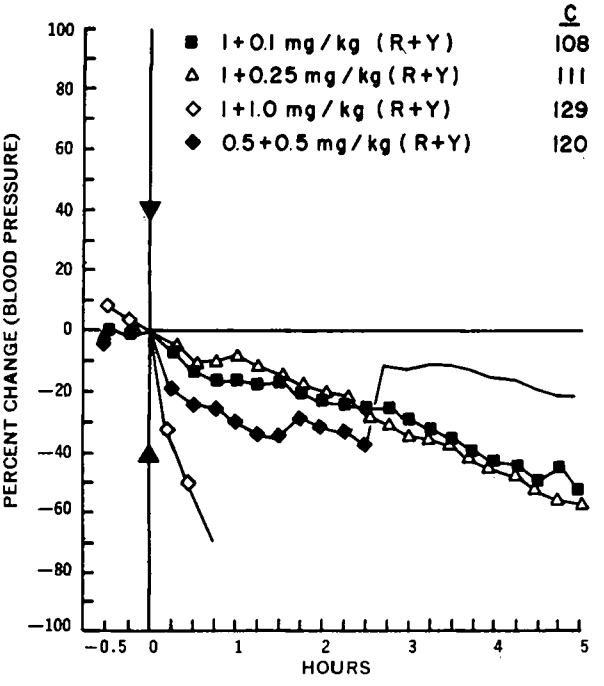


Figure 2—*Blood pressure changes after intravenous administration of nonhypotensive doses of reserpine and yohimbine (R + Y) mixtures expressed as percent change from control (C) values (mm. Hg).*

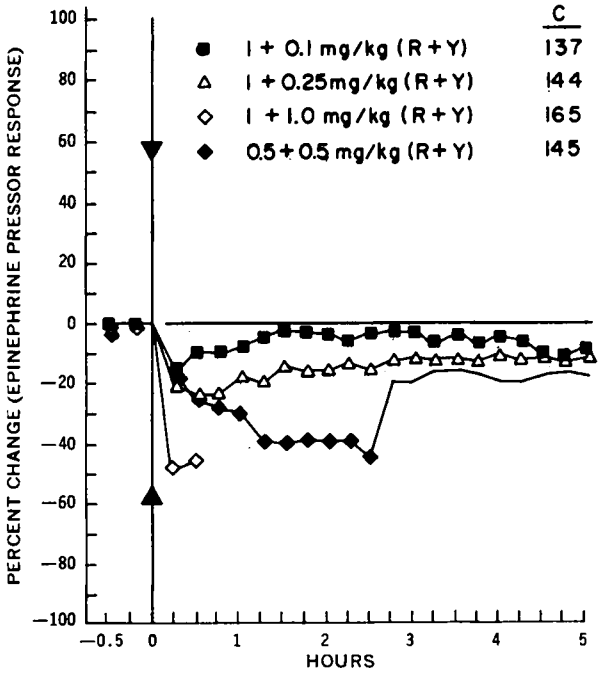


Figure 4—*Changes in epinephrine pressor response after intravenous administration of reserpine and yohimbine (R + Y) mixtures expressed as percent change from control (C) values (mm. Hg).*

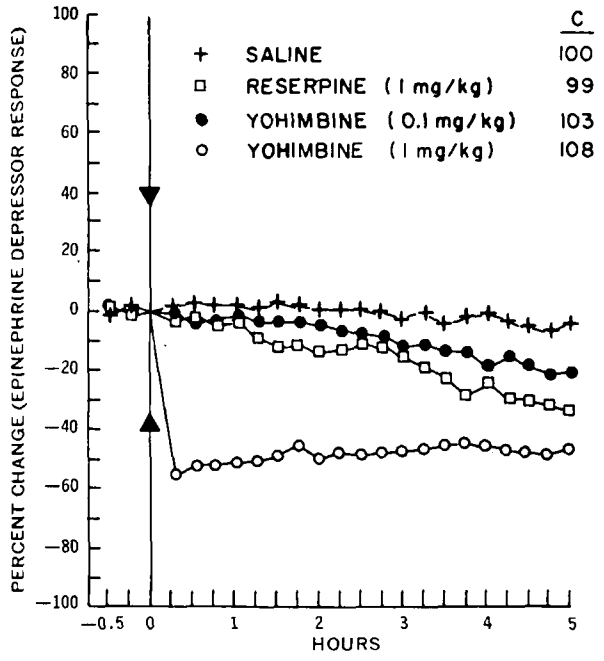


Figure 5—*Changes in epinephrine depressor response after intravenous administration of isotonic saline, reserpine, or yohimbine expressed as percent change from control (C) values (mm. Hg).*

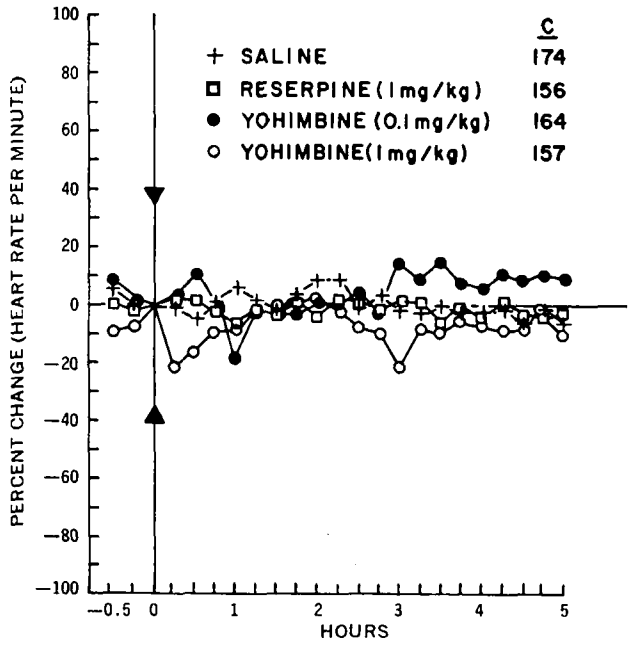


Figure 7—*Heart rate changes after intravenous administration of isotonic saline, reserpine, or yohimbine expressed as percent change from control (C) values (mm. Hg).*

Test animals were taken off feed 12 hr. prior to the start of the experiment with water allowed *ad libitum*. Each animal was anesthetized to the point of blockade of the corneal reflex by slow intravenous titration of a 30% solution of urethan, at a rate of approximately 1 ml./min., *via* the cephalic vein in the left foreleg. Direct femoral arterial blood pressure, heart rate, and respiratory rate were recorded kymographically. After a stabilization period of 15–30 min., a standard dose of epinephrine was determined, intravenously, for each animal. The standard injection (30-sec. duration, with saline wash) was defined as one that would be reproducible at 15-min. intervals and would show a clear biphasic response of pressor–depressor activity. After two control injections of the standard dose of epinephrine, one of the eight treatments was administered at a uniform rate *via* the femoral vein at "zero time." Each injection was of 5-min. duration, followed by a 3-ml. wash of isotonic saline, over 3 min. The total injection time was 8 min.

Reserpine and yohimbine combinations were not administered as a single solution. Reserpine acetate was injected first and washed in with saline. The yohimbine hydrochloride was then injected, followed by a saline wash. The total injection time, for this procedure, also totaled 8 min. Recording of blood pressure, other physiological parameters, and the response to periodic injections of epinephrine was continued throughout the 300-min. observation period unless the test animal died. The control dose of reserpine used (1 mg./kg.) has been shown in this laboratory to produce unequivocal and characteristic effects without lethality.

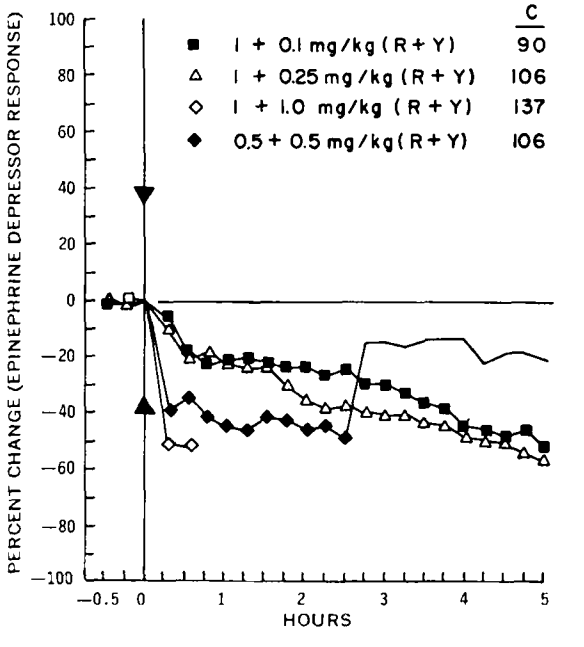


Figure 6—*Changes in epinephrine depressor response after intravenous administration of reserpine and yohimbine (R + Y) mixtures expressed as percent change from control (C) values (mm. Hg).*

RESULTS AND DISCUSSION

Figures 1 through 11 summarize the percent change from control values (preinjection levels of the parameters measured) for each set of treatments. Figure 1 clearly shows the percent change in blood pressure seen after the administration of 1.0 mg./kg. of reserpine as compared to isotonic saline. This dose elicited a slowly developing, progressive hypotension which amounted to a 37% fall at the end of the 5-hr. observation period. This may be contrasted with the absence of such an effect in the salinized animals or those receiving 0.1 mg./kg. of yohimbine. A dose of 1.0 mg./kg. of yohimbine, however, elicited a prompt 32% fall in mean blood pressure. This hypotensive response was maintained throughout the 5-hr. observation period, reaching a maximum of 43% of the control values at the end of the 5th hr.

Concurrent administration of nonhypotensive doses of yohimbine combined with reserpine accentuates the hypotensive effect of reserpine and may cause death. Figure 2 illustrates the effects of such combinations on the percent change in blood pressure as compared to control levels. Injections of 0.1 or 0.25 mg./kg. of yohimbine combined with 1 mg./kg. of reserpine induced essentially equivalent responses, which appeared reserpine-like in onset. However, the effects seen at the end of 5 hr. (54–58%) were considerably lower than the 37% reduction seen with reserpine only (Fig. 1). One animal died during each of these treatments, both in the latter half of the experiment. The combination of 1 mg./kg. each of reserpine and yohimbine was uniformly fatal for two dogs. Both died of respiratory arrest shortly after injection. In the two surviving animals, the resting blood pressure fell to 50% of the control values at the end of 30 min. and respiratory arrest occurred shortly thereafter. Of the four animals treated with 0.5 mg./kg. of each of the two alkaloids, one died after 75 min. and two expired after 150 min.

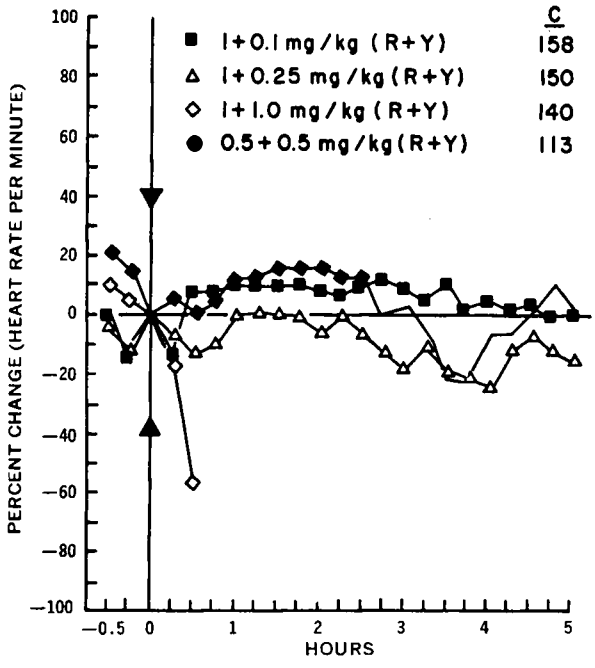


Figure 8—*Heart rate changes after intravenous administration of reserpine and yohimbine (R + Y) mixtures expressed as percent change from control (C) values (mm. Hg).*

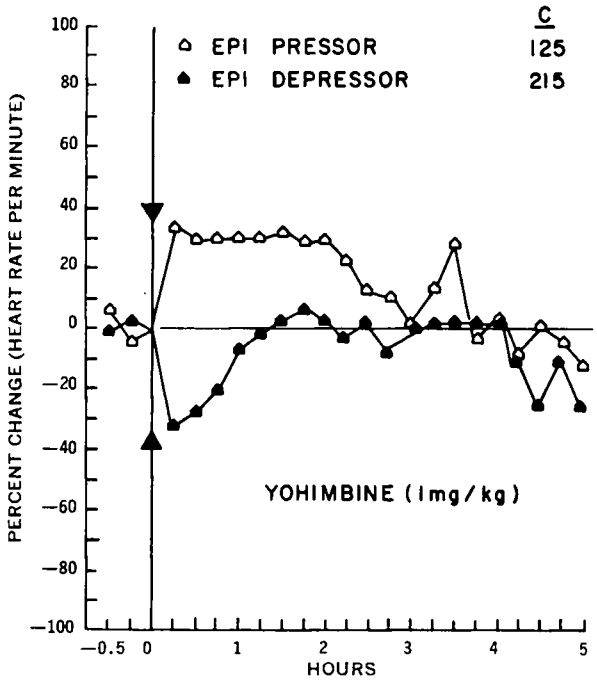


Figure 9—*Changes in epinephrine (EPI) pressor and depressor responses after intravenous administration of yohimbine expressed as percent change from control (C) values (mm. Hg).*

Only one dog survived the full 5-hr. observation period. Its tracing is shown in Fig. 2 as a line without markers. In this test group, the fall in blood pressure was rapid (30% of control levels at 1 hr. and 41% of the controls at the end of 2 hr.).

The effect of the test treatments on exogenous epinephrine is illustrated in Fig. 3. These data indicate that both physiological saline and reserpine enhance the pressor effects of epinephrine about 10% over control values. Bein (17) and Bein *et al.* (18) originally reported a significant enhancement of the epinephrine pressor response after the administration of 0.1 mg./kg. i.v. of reserpine under allobarbital[1] anesthesia. The quantitatively modest results of the present study may be due to species differences or, more probably, the continued repetitive challenges of intravenous epinephrine every 15 min. Such repetitive challenges may prevent the sensitization of adrenergic receptors suggested by Bein. In any event, a dose of 1 mg./kg. of yohimbine has a prompt, unequivocal α-adrenolytic effect which was sustained throughout the 5-hr. experimental period. A dose of 0.1 mg./kg. produced a similar but lesser effect.

Figure 4 illustrates the effects elicited by combinations of reserpine and yohimbine upon the epinephrine pressor response. In all treatments, the effect appears to be what one would expect from yohimbine alone. The effect of the combination of 0.5 mg./kg. reserpine and 0.5 mg./kg. yohimbine is essentially the same as that seen at the lower levels. Even when the lethal combination of 1.0 mg./kg. reserpine and 1.0 mg./kg. yohimbine was used, the α-adrenolytic effect of the combination matched that of yohimbine alone.

Figure 5 illustrates the effect of reserpine on epinephrine's secondary phase. An attenuation of this depressor phase slowly developed over the 5-hr. observation period, at the end of which time a mean value of −34% had been documented. Yohimbine, at a dosage of 1 mg./kg., rapidly attenuated the depressor response by −53% within 15 min. This dramatic effect was sustained in a plateau-like fashion throughout the rest of the observation period. This could be interpreted as β-blocking activity; however, when one considers that the response curves seen here parallel the effects previously presented for resting blood pressure, another conclusion presents itself. From this work and other studies in this laboratory, one can generalize that nonspecific and nonmusculotropic agents which lower the resting blood pressure tend to dampen epinephrine's depressor effect and accentuate the pressor response. Conversely, a nonspecific and nonmusculotropic agent which raises the resting blood pressure tends to accentuate epinephrine's depressor effect and dampen the pressor response.

Figure 6 also shows a direct and passive correlation between the percent decrease in the epinephrine depressor response and the percent decrease in resting blood pressure. Therefore, it was concluded that neither reserpine, yohimbine, nor mixtures of these alkaloids pharmacologically affect the depressor response to exogenous epinephrine.

Figure 7 is concerned with the effects of these alkaloids on the resting heart rate. Treatment with 1 mg./kg. of reserpine did not produce a significant bradycardic effect during the 5-hr. observation

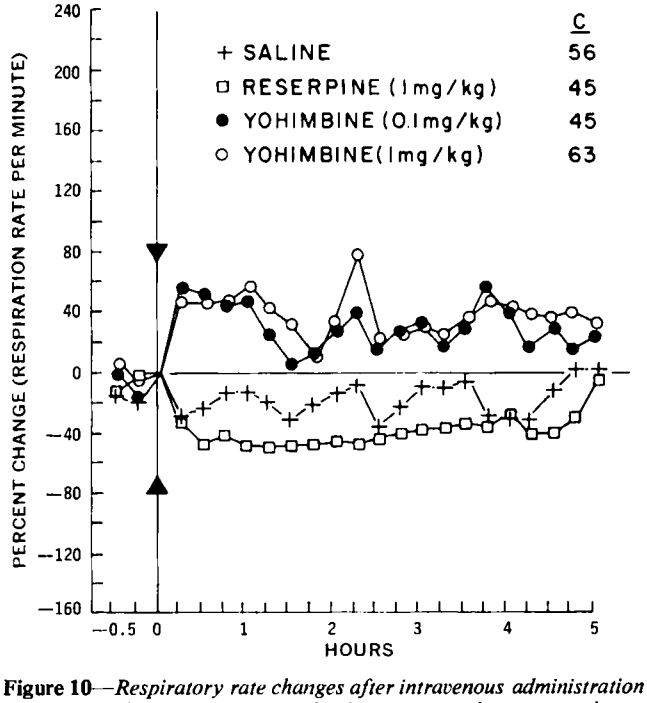


Figure 10—*Respiratory rate changes after intravenous administration of isotonic saline, reserpine, or yohimbine expressed as percent change from control (C) values (mm. Hg).*

[1] Dial.

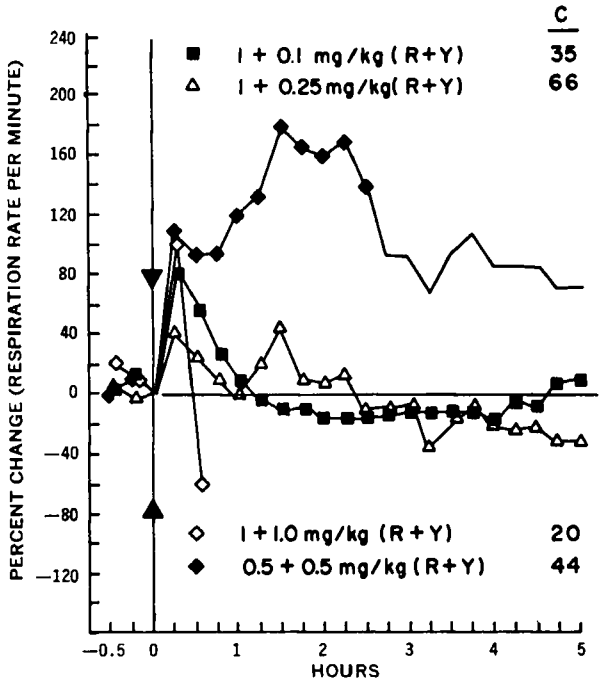


Figure 11—*Respiratory rate changes after intravenous administration of reserpine and yohimbine (R + Y) mixtures expressed as percent change from control (C) values (mm. Hg).*

period. However, 1 mg./kg. of yohimbine produced a prompt 22% slowing of the heart rate, with recovery at the end of the 1st hr. At the 3-hr. interval of the experiment, there was a second period of bradycardia.

Figure 8 exhibits no distinct dose–response pattern. However, a profound and unequivocal bradycardia was seen with the lethal combination of 1.0 mg./kg. of reserpine and 1.0 mg./kg. of yohimbine.

The combination of 1.0 mg./kg. of reserpine and 0.25 mg./kg. of yohimbine elicited a somewhat cyclical bradycardia, which one would not expect considering the effects of reserpine alone (Fig. 7). The effects produced by the combination of 0.5 mg./kg. each of reserpine and yohimbine is shown by the line without markers and is characterized by some equivocal tachycardia seen between 1 and 2 hr. and the bradycardia seen between 3 and 4 hr. Except for the steepness of parts of the first portion of the curve, it bears some similarity to the response seen with the combination of 1.0 mg./kg. reserpine and 0.25 mg./kg. yohimbine.

Figure 9 clearly shows that yohimbine (1 mg./kg.) blocks the vagal slowing seen during the pressor phase of the response to epinephrine. Likewise, it tends to block the tachycardia seen during the depressor phase. This phenomenon was observed with all reserpine–yohimbine combinations and is pharmacologically most interesting. In 1930, Heymans and Bouckaert (19) reported that yohimbine could suppress carotid sinus vasomotor reflexes.

Percentage shifts in respiration were more pronounced than the other parameters measured. Isotonic saline appeared to produce some cyclic depression of the resting respiratory rate, whereas 1 mg./kg. of yohimbine was uniformly depressant (Fig. 10). This depression appeared to be overcome at the end of the 5-hr. observation period. There was some evidence of cyclic stimulation of the respiratory rate in the animals treated with either 1 mg./kg. of reserpine or 0.1 mg./kg. of yohimbine. From Fig. 11, it can be seen that all alkaloid combinations produced a transient stimulation of respiratory rate at the 15-min. interval. Subsequently, the combination of 1 mg./kg. reserpine and 1 mg./kg. yohimbine induced a profound respiratory depression, resulting in the death of all test animals before the end of the 1st hr. of observation. Although it appears in Fig. 11 that treatment with the combination of 0.5 mg./kg. each of reserpine and yohimbine produced only respiratory stimulation, three test animals plunged into respiratory depression during the middle of the observation period and died. Only one dog in this group survived the entire 5-hr. period (its response is noted by the line without markers). After the initial stimulation, the treatment consisting of 1 mg./kg. reserpine and 0.1 mg./kg. yohimbine elicited respiratory responses much like predrug levels. The pattern for the combination of reserpine and yohimbine (1 and 0.25 mg./kg., respectively) showed some evidence of cyclization during most of the observation period.

SUMMARY

It is evident from the data presented that preparations made from the whole root of *R. serpentina* cannot be considered as just other "reserpine-like" drugs. It appears that yohimbine and/or yohimbine-like alkaloids can contribute significantly to the pharmacological–therapeutic profile of rauwolfia whole root.

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Opium Alkaloids XIV: Biosynthesis of Aporphines—Detection of Orientaline in Opium Poppy

E. BROCHMANN-HANSSEN▲, C. H. CHEN, H.-C. CHIANG*, C.-C. FU†, and H. NEMOTO

Abstract □ Orientaline was detected in the opium poppy by an isotope dilution method based on its biosynthesis from norlaudanosoline. Administration of labeled orientaline revealed that this alkaloid was not a precursor of isoboldine, and the experimental results provided no evidence for a pathway involving norprotosinomenine. No conclusion was possible relative to the origin of magnoflorine in the opium poppy.

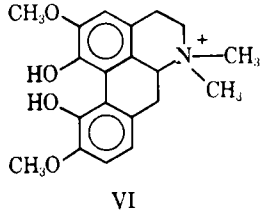
Keyphrases □ Opium alkaloids—biosynthesis of aporphines, detection of orientaline in opium poppy □ Orientaline—detection in opium poppy by isotope dilution method, biosynthesis from norlaudanosoline □ Biosynthesis of opium alkaloids—orientaline from norlaudanosoline □ Aporphine biosynthesis—detection of orientaline in opium poppy □ Isotope dilution technique—determination, orientaline in opium poppy

An earlier paper in this series (1) reported that isoboldine (III) is biosynthesized in the opium poppy by direct oxidative coupling (*ortho–para*) of reticuline (II), whereas a direct pathway could not be ascertained for magnoflorine (VI). At the same time, it was pointed out that an alternative pathway *via* orientaline (IV) and orientalinone (V) might also exist for isoboldine (Scheme I). Orientaline and orientalinone are present in *Papaver orientale* and *P. bracteatum* (2, 3), where they are involved in the biosynthesis of the aporphine isothebaine (2, 4, 5), but they have not yet been isolated from opium or the opium poppy. Biosynthesis of aporphines by direct *ortho–ortho* coupling of reticuline has been demonstrated for bulbocapnine in *Corydalis cava* (6, 7) and for magnoflorine in Columbine plants (aquilegia, McKana hybrid) (8). Battersby *et al.* (9) were unable to show either type of direct coupling for the biosynthesis of corydine (X), glaucine (XI), and dicentrine (XII) in *Dicentra eximia*. Instead, they found evidence of an indirect mechanism involving norprotosinomenine (VII) and the dienones VIII and IX. This opened a new possibility for the biosynthesis of isoboldine and magnoflorine in the opium poppy.

The present study was undertaken to discover alternative pathways for the biosynthesis of these alkaloids.

RESULTS AND DISCUSSION

Enzymes catalyzing biosynthetic reactions show a great deal of configurational specificity. Yet, they will often interact with substances foreign to the organism and transform them along normal metabolic routes, provided these foreign compounds are sufficiently similar to the normal metabolites. Thus, codeine 6-methyl ether has been converted to codeine by the opium poppy (10) and 1,3-dimethyl-1-pyrrolinium chloride to 3′-methylnicotine by the tobacco plant (11). Incorporation of a potential precursor into an alkaloid is, therefore, not sufficient evidence of its participation in the normal biosynthesis. It is also necessary to demonstrate that the proposed precursor actually exists in the living plant.

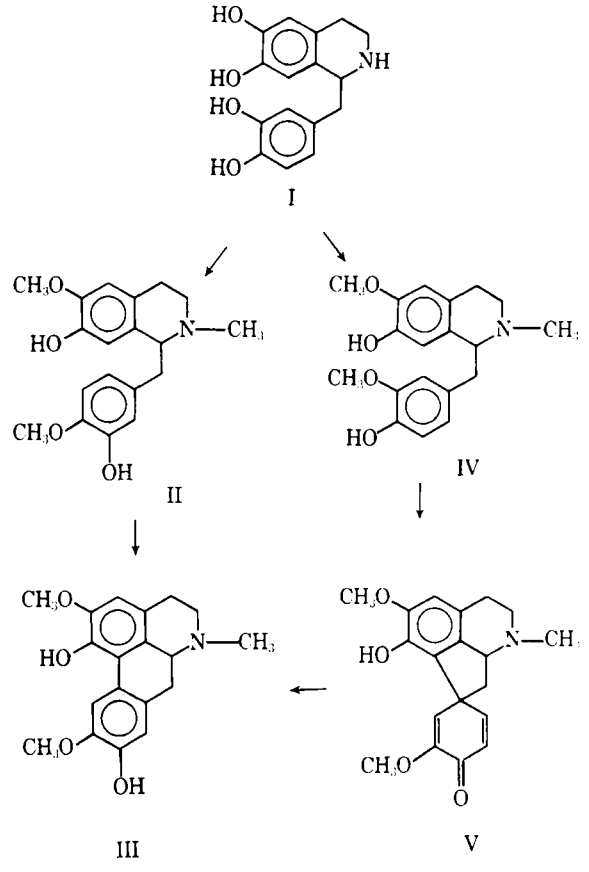


VI

In the anticipation of an alternative pathway for biosynthesis of isoboldine by way of orientaline, a scheme was developed for detection of orientaline based on its biosynthesis from norlaudanosoline (I). (±)-Norlaudanosoline-3-^{14}C was administered to opium poppies, and (±)-orientaline was added as cold carrier during the extraction. Isolation and purification of orientaline, first as the perchlorate and then as *O,O*-diacetylorientaline picrate, gave a radioactive compound. Based on the amount of orientaline added as a carrier, the incorporation of radioactivity amounted to 0.54%.

Orientaline (IV) and reticuline (II) are position isomers and difficult to separate. It was, therefore, conceivable that the radioactivity might be due to a small amount of reticuline present as an impurity. This possibility was tested by diluting a sample of the purified, radioactive diacetylorientaline picrate with 2.5 times its weight of diacetylreticuline picrate. Fractional crystallization of the mixture gave diacetylreticuline picrate of negligible specific activity.

Feeding experiments with (±)-orientaline-*N*-$^{14}CH_3$ gave no incorporation of radioactivity into isoboldine. In view of the apparent role of norprotosinomenine in the biosynthesis of apor-



Scheme I—Potential pathways for biosynthesis of isoboldine

phines (9), (±)-norprotosinomenine-3-^{14}C-7-*O*-methyl-^{3}H was administered to opium poppies, and isoboldine and magnoflorine were isolated after dilution with "cold" carriers. Neither alkaloid showed any incorporation of radioactivity.

It may be concluded that orientaline is a normal alkaloid constituent of the opium poppy. However, it does not participate in the biosynthesis of isoboldine. No definite conclusions may be drawn from these experiments about the biosynthesis of magnoflorine in the opium poppy, since both reticuline and norprotosinomenine have given negative results. There are great quantitative variations in the alkaloid composition of different varieties of the opium poppy. It is, therefore, quite possible that the variety used in these experiments (Noordster) may not have contained detectable amounts of magnoflorine.

EXPERIMENTAL

The methods used for the determination of radioactivity, cultivation of plants, and administration of labeled precursors were described previously (12). *Papaver somniferum*, Noordster variety, was used in these experiments. The radioactive purity of labeled, potential precursors was determined by radioactivity scanning of thin-layer chromatograms[1].

Alkaloids for Carrier Dilution—*Magnoflorine Iodide*—This alkaloid was used as supplied.

(±)-*Isoboldine*—This compound was synthesized as described by Kametani *et al.* (13). The product was identified by TLC and by comparison of its UV and NMR spectra with those of natural isoboldine (14).

(±)-*Orientaline*—This compound was synthesized by conventional methods (15, 16). It was purified as the perchlorate, m.p. 127° [lit. (16) m.p. 127°]. The base was further characterized by NMR (17) and mass spectrometry (18).

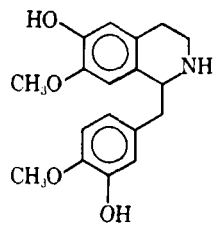
Synthesis of Labeled Precursors—(±)-*Norlaudanosoline-3-^{14}C Hydrochloride*—This compound was prepared by demethylation of (±)-tetrahydropapaverine-3-^{14}C as described by Battersby *et al.* (19), m.p. 278–281° [lit. (19) m.p. 278–281°], specific activity 0.36 mc./mmole.

(±)-*Orientaline*-N-$^{14}CH_3$—This compound was synthesized in the same way as the "cold" material, except that radioactive methyl iodide was distilled into the reaction vessel in a vacuum manifold system as described previously for (±)-reticuline-*N*-$^{14}CH_3$ (12). The intermediates in the synthesis and the final product were compared with the corresponding nonradioactive compounds by TLC on silica gel with two solvent systems (20) and by GLC on silicon rubber OV-1 and OV-225. The specific activity of (±)-orientaline-*N*-$^{14}CH_3$ was 1.68 mc./mmole.

(±)-*Norprotosinomenine-3-^{14}C-7-O-methyl-^{3}H Hydrochloride*—

1. (±)-*O,O*-Dibenzylnorprotosinomenine-3-^{14}C was prepared by standard methods as described for (±)-*O,O*-dibenzylnorreticuline-3-^{14}C (21), specific activity 0.24 mc./mmole.

2. (±)-*O,O*-Dibenzylnorprotosinomenine-7-*O*-methyl-^{3}H was prepared by dissolving catechol (55 g.) and benzyl chloride (63.3 g.) in 120 ml. of absolute ethanol. A solution of 11.5 g. of sodium in 170 ml. of absolute ethanol was added, and the mixture was refluxed for 2 hr. under a stream of nitrogen. The reaction mixture was allowed to cool and was then acidified with hydrochloric acid and placed in a refrigerator overnight. Sodium chloride and dibenzylcatechol crystallized out and were removed by filtration. The filtrate was evaporated to an oil, which was extracted with ether. The combined ether extracts were washed with water and evaporated to dryness. The oily residue was distilled under reduced pressure (10 mm. Hg). Unchanged catechol distilled over first, followed by the monobenzyl ether as a pale-yellow oil, boiling range 168–173°. To a well-stirred solution of *O*-benzylcatechol (5 g.) and potassium hydroxide (9 g.) in ethanol (40 ml.) and water (20 ml.) was added dropwise 10 ml. of chloroform while the solution was kept under an atmosphere of nitrogen. After 2 hr. at room temperature, the reaction mixture was refluxed for 30 min. Ethanol and unreacted chloroform were removed under reduced pressure. The residue was acidified with hydrochloric acid and extracted with ether. The combined ether extracts were washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The brown,



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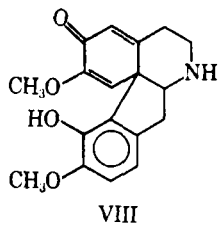

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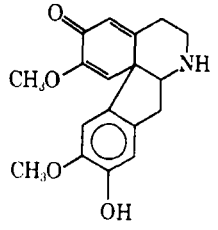

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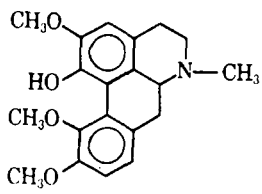

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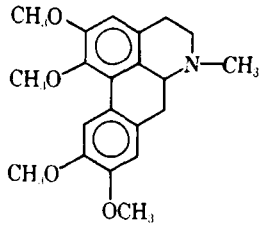

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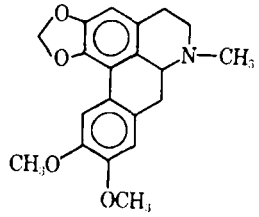

XII

oily residue was chromatographed on silica gel[2], first with chloroform and then with chloroform containing 3% of methanol. The fractions containing 3-benzyloxy-4-hydroxybenzaldehyde were combined, evaporated to dryness, and crystallized from benzene, m.p. 113–114°, yield 0.288 g. NMR data agreed well with the structure. Mixed melting point with 4-benzyloxy-3-hydroxybenzaldehyde (m.p. 121–123°) showed great melting-point depression.

3-Benzyloxy-4-hydroxybenzaldehyde (22.8 mg., 0.1 mmole) was dissolved in 3 ml. of anhydous benzene, and 0.095 mmole of freshly prepared sodium ethoxide solution in 0.5 ml. of absolute ethanol was added. Radioactive iodomethane-^{3}H (10 mc., 0.01 mmole)[3] was distilled into the solution in a vacuum transfer system (21). The tube containing the reaction mixture was sealed and left at room temperature for 3 days with frequent shaking. The contents were transferred to a flask and evaporated to dryness. The residue was mixed with 330 mg. of 3-benzyloxy-4-methoxybenzaldehyde (*O*-benzylisovanillin) and dissolved in chloroform; the solution was washed with 10% potassium hydroxide solution (2 × 10 ml.) and then with water, dried over potassium carbonate, and evaporated to dryness. The residue was crystallized from ethanol.

O-Benzylisovanillin-*O*-methyl-^{3}H was converted to *O,O*-dibenzylnorprotosinomenine-7-*O*-methyl-^{3}H by conventional methods (21), specific activity 4.45 μc./mmole.

A mixture of 100 mg. of (±)-*O,O*-dibenzylnorprotosinomenine-3-^{14}C and 200 mg. of (±)-*O,O*-dibenzylnorprotosinomenine-7-*O*-methyl-^{3}H was debenzylated with hydrochloric acid (12) and purified by crystallization of the hydrochloride. Specific activity: ^{14}C, 80 μc./mmole; ^{3}H, 2.97 μc./mmole; and a ratio ^{14}C/^{3}H = 26.9.

Detection of Orientaline—(±)-Norlaudanosoline-3-^{14}C hydrochloride (10 mg.) was dissolved in 5 ml. of water, and the solution was administered to 10 plants. Two weeks later the plants were extracted by maceration with methanol in a high-speed blender[4]. (±)-Orientaline perchlorate (265 mg.) was dissolved in 100 ml. of 70% methanol and mixed with the plant material. The suspension was poured into a glass percolator and extracted with methanol until the extract gave negative tests for alkaloids. The extract was concentrated to about 500 ml. in a rotary vacuum evaporator at a temperature maintained below 40° and shaken in a separator with

[1] Berthold Radio Scanner, Varian Aerograph Co.

[2] Woelm, Activity I.

[3] Most of the iodomethane decomposed before use.

[4] Waring Products Co.

2 × 150 ml. of ethyl acetate. The ethyl acetate extract was back-extracted with 3 × 50 ml. of 0.5 *N* hydrochloric acid, and the washings were combined with the original aqueous solution (total alkaloids). The aqueous, acidic solution of total alkaloids was extracted with chloroform to remove weakly basic alkaloids, the pH was adjusted to 9 with ammonium hydroxide, and the solution was extracted with a mixture of chloroform and isopropyl alcohol (3:1). The extract was evaporated to dryness under reduced pressure, and the residue (2.2 g.) was chromatographed on a column of silica gel[5] with a mixture of chloroform and methanol (97:3). The fractions containing orientaline, as indicated by micro-TLC, were combined and evaporated to dryness. The residue (63 mg.) was converted to the perchlorate (16) and crystallized from ethanol. The specific activity decreased slowly with each recrystallization and was not yet constant after six crystallizations (930 d.p.m./mg.). The remainder of the sample (about 30 mg.) was converted to the base, dissolved in about 1 ml. of a mixture of pyridine and acetic anhydride (1:1), and left at room temperature. After 2 days, 1.05-mole equivalents of picric acid in a minimum volume of absolute ethanol were added gradually to the reaction mixture and the picrate was allowed to crystallize in a refrigerator. The crystals were collected and recrystallized from 75% ethanol to constant radioactivity, 338 d.p.m./mg., m.p. 216–217°.

To prove that the radioactivity was not due to an impurity of reticuline, 3 mg. was mixed with 7.5 mg. of *O,O*-diacetylreticuline picrate and the mixture was crystallized from 75% ethanol. The crystals, consisting mainly of *O,O*-diacetylreticuline picrate, had a specific activity that was considerably lower than that calculated for the mixture, and it decreased with each recrystallization. After six recrystallizations, when the sample was exhausted, the specific activity was 13 d.p.m./mg.

Isolation of Isoboldine and Magnoflorine—The aqueous, acidic solution of total alkaloids from the plants to which (±)-isoboldine and magnoflorine iodide had been added as cold carriers was extracted with chloroform to remove the weakly basic alkaloids. The aqueous phase was basified to pH 8–9 with sodium bicarbonate and extracted with several portions of ether. The combined ether extracts were washed with water and evaporated to dryness. The residue was chromatographed on silica gel[2] with chloroform–methanol (100:1). The fractions containing isoboldine were combined, evaporated to dryness, and crystallized from methanol to constant radioactivity; (±)-orientaline-*N*-$^{14}CH_3$ feeding: 3 d.p.m./mg.; norprotosinomenine feeding: 5 d.p.m./mg.

The aqueous, alkaline solution remaining after extraction with ether was acidified with glacial acetic acid. Magnoflorine was isolated as the iodide as described by Doskotch and Knapp (22) and crystallized from methanol to constant radioactivity; norprotosinomenine feeding: 2 d.p.m./mg.

[5] Woelm, Activity II.

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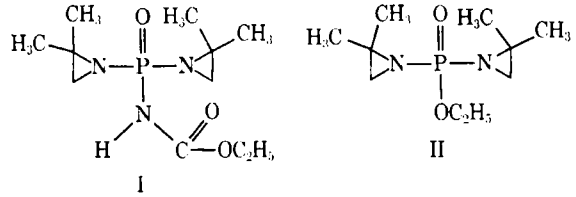
Reactions of 2,2-Dimethylaziridine-Type Alkylating Agents in Biological Systems I: Colorimetric Estimation and Stability in Physiological Media

DAVID LALKA and T. J. BARDOS▲

Abstract □ Procedures were developed for the quantitative estimation of 2,2-dimethylaziridine containing alkylating agents in blood and urine. Rapid alkalinization of the biological samples, followed by protein precipitation and extraction of the compounds at high salt concentration into acetophenone, resulted in good recoveries. The use of a nonaqueous solvent (acetophenone) and the adoption of *N*-phenylpiperazine as the proton-accepting reagent significantly improved the sensitivity and reproducibility of the colorimetric assay with 4-(*p*-nitrobenzyl)pyridine. These improvements appear to be generally applicable to the quantitative determination of aziridine-type alkylating agents. The 2,2-dimethylaziridine agents were shown to undergo extremely rapid hydrolysis under neutral or acidic conditions and were stable only in alkaline solution. The half-lives of tris(2,2-dimethyl-1-aziridinyl)phosphine oxide, bis(2,2-dimethylaziridinyl)phosphinylurethan, and ethyl bis(2,2-dimethyl-1-aziridinyl)phosphinate in whole blood, at 37°, pH 7.38, were 7, 11, and 18 min., respectively; the corresponding half-lives in plasma were approximately four times longer.

Keyphrases □ 2,2-Dimethylaziridine-type alkylating agents—stability and colorimetric estimation in blood and urine, half-lives □ Aziridine-type alkylating agents, 2,2-dimethyl analogs—stability and colorimetric estimation in blood and urine, half-lives □ pH dependence—hydrolysis rates of 2,2-dimethylaziridine-type alkylating agents □ *N*-Phenylpiperazine—used as base reagent for colorimetric estimation of 2,2-dimethylaziridine-type alkylating agents in urine and blood □ Colorimetry—analysis and stability of 2,2-dimethylaziridine-type alkylating agents in blood and urine

Bis(2,2-dimethylaziridinyl)phosphinylurethan[1] (I), one of a series of "dual antagonists" synthesized and studied in this laboratory (1–8), has been undergoing extensive clinical trials[2] as an anticancer agent (9–18). It appears that this drug potentiates the antitumor effects of X-rays clinically (14–19) as well as in animal experiments (20). In man, relatively small doses of X-rays produced short-term remissions of solid tumors when administered with Compound I (14–17). Furthermore, a recently concluded study of the treatment of bronchogenic carcinoma with this compound in combination with radiotherapy showed some encouraging long-term therapeutic effects (18, 19). These results suggest continuing interest in the clinical study of this agent. In the meantime, several other compounds containing the 2,2-dimethylaziridine moiety were synthesized and found to be effective against a spectrum of transplanted tumors in rodents (21–26). Two of these agents, ethyl bis(2,2-dimethyl-1-aziridinyl)phosphinate (II) (23, 24) and tris(2,2-dimethyl-1-aziridinyl)phosphine oxide (III) (25), which showed particularly potent activities against leukemia L-1210 in mice, passed through the preclinical pharmacology stage (24) and are presently undergoing preliminary evaluation against solid tumors in man.



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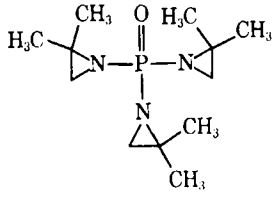
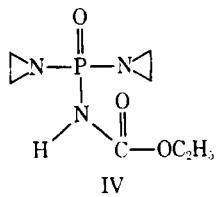


III

In view of the unique chemical and pharmacological properties (2, 7, 8, 22, 26) of these 2,2-dimethylaziridine derivatives as compared to their ring-C-unsubstituted aziridine analogs [*i.e.*, the conventional "ethyleniminetype" alkylating agents, bis(1-aziridinyl)phosphinylurethan (IV), triethylenemelamine (V), tris(1-aziridinyl)-phosphine oxide (VI), *etc.*] and in view of their apparently selective chemotherapeutic effects (26), it appeared worthwhile to investigate the reactions of these compounds in biological systems. Such studies may lead to a better understanding of the chemical, metabolic, and pharmacokinetic processes influencing the chemotherapeutic activities of these unusually "water-labile" alkylating agents. Therefore, the results of these studies may facilitate the design of optimally effective agents and may aid in establishing optimal conditions for their therapeutic application. The present paper describes studies to determine the *in vitro* stabilities of these agents in various aqueous and physiological media and to establish an assay methodology applicable to subsequent studies of their metabolism and distribution *in vivo*.



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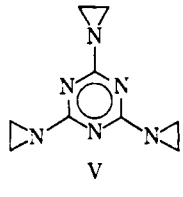

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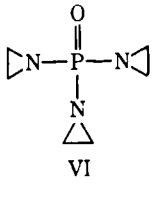

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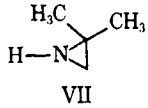


VII

[1] The following designations have been used in the literature for the agents discussed in this paper: I, AB-132, dimethylurethimine, meturedepa, NSC-51325; II, AB-163, NSC-108878; III, TEPA-132, NSC-118436; IV, AB-100, NSC-37095; V, TEM, tretamine, NSC-9706; and VI, TEPA, NSC-9717.

[2] At the Roswell Park Memorial Institute.

Table I—TLC of 2,2-Dimethylaziridine Derivatives

Compound	Solvent[a]	Absorbent	R_f Value
I	B	Alumina	0.04
II	B	Alumina	0.35
III	B	Alumina	0.24
I	B	Silica gel	0.19
II	B	Silica gel	0.24
III	B	Silica gel	0.14
VII	B	Silica gel	0.02
I	A	Silica gel	0.86
II	A	Silica gel	0.83
III	A	Silica gel	0.91
VII	A	Silica gel	0.58

[a] A, chloroform–ethanol (1:1); and B, ethyl acetate.

4-(*p*-Nitrobenzyl)pyridine (N, Scheme I) (27) is the most widely used analytical reagent for the assay of alkylating agents. Many procedures have been reported for the determination of various alkylating agents in blood and tissues using this reagent (28–35). However, the unusually rapid hydrolysis of the 2,2-dimethylaziridine derivatives in acidic or neutral aqueous media, as well as their relatively low chemical alkylating activities, necessitated the development of procedures that would provide: (*a*) rapid alkalinization followed by efficient extraction into nonaqueous media and (*b*) increased sensitivity of the colorimetric assay.

EXPERIMENTAL

Materials—Acetophenone and acetic acid were distilled before use. 4-(*p*-Nitrobenzyl)pyridine[3] and *N*-phenylpiperazine[4] were used as supplied. Compound V[5] and 2,2-dimethylaziridine[6] (VII) were purified before use. Compounds I, II, III, and VI were synthesized in this laboratory[7]. Since some of the aziridine derivatives may hydrolyze or polymerize during storage, IR and NMR spectra were taken periodically. TLC examination was also performed to check the purity and identity of the samples.

Colorimetric Determination of Alkylating Agents Using Nonaqueous Medium (Procedure A)—Reagent solutions were prepared by adding 1.0 ml. of glacial acetic acid (for I, III, VI, and VII) or 1.6 ml. of glacial acetic acid (for II and V) to 20 ml. of 25% 4-(*p*-nitrobenzyl)pyridine in acetophenone. These reagent solutions should be used within 30 min. after the addition of the acetic acid because the acetate salt precipitates upon standing longer. One milliliter of the reagent solution was added to 1.0 ml. of the sample, *i.e.*, an acetophenone solution containing the alkylating agent. The mixture was heated for 35 min. on an oil bath at 85° (55° in the case of the volatile Compound VII). To terminate the reaction, the tubes were placed into cold tap water. The color was developed by addition of 0.50 ml. of *N*-phenylpiperazine. The volume was then made up to 3.0 ml. with acetophenone. This mixture was transferred to a cell, and the absorbance at 575 nm. was measured spectrophotometrically against a blank prepared exactly as the sample except that the alkylating agent was omitted. The absorbance of the colored product is stable for at least 5 min.

Extraction of Alkylating Agents from Fresh Heparinized Blood (Procedure B)—Eight milliliters of human or dog blood was drawn into a 10-ml. syringe containing about 0.1 ml. of heparin injection USP (1000 units/ml.). The contents of the syringe were quickly emptied into a centrifuge tube containing the given amount of alkylating agent. After a few seconds of mixing[8], the centrifuge tubes were placed into an ice water bath. Then 0.5 ml. of 0.5 *M* NaOH was slowly added while vigorously shaking the tube on a mixer[8] (excessively high local concentrations of sodium hydroxide cause hemolysis). The tubes were kept in an ice water bath until they were centrifuged for the separation of the plasma. This was carried out at 1500×*g* for 10 min. at 10°. The preceding steps should be accomplished in less than 15 min. from the time the blood is drawn, and the temperature of the samples should be maintained at 0–10°.

Subsequently, 1.5-ml. aliquots of plasma were further alkalinized by the addition of 0.25 ml. of 50% NaOH. After this alkalinization, the tubes can be kept in ice water for at least 2 hr. without significant loss of activity. To each aliquot was added, in the following sequence, 1.5 ml. of 5% $ZnSO_4$, 1.5 ml. of 0.3 *N* $Ba(OH)_2$, 4.5 ml. of saturated ammonium sulfate, and 1.5 ml. of acetophenone. At least 30 sec. of vigorous agitation on a mixer[8] was applied after the addition of each reagent. This mixture was centrifuged (1500×*g*) for 35 min. at 10°, and about 1.1 ml. of an acetophenone phase was obtained. The colorimetric assay already described was then employed to determine the amount of alkylating agent in 1.0 ml. of the acetophenone phase. Contaminating traces of the aqueous phase or precipitated protein do not significantly affect the accuracy of the procedure.

Extraction of Alkylating Agents from Cellular Fraction of Blood—*Procedure C-1*—After separation of the plasma from formed elements as described for *Procedure B*, the cell fraction was kept in ice water except when otherwise stated. An aliquot of the cell fraction (1.5 ml.) was extracted with 2.0 ml. of 1.5% $NaHCO_3$ (adjusted to pH 8.4 with sodium hydroxide). The cells were resuspended by shaking on a mixer[8] and then centrifuged again. A 1.5-ml. aliquot of the supernate was treated in the same manner as was the plasma in *Procedure B*.

Procedure C-2—The cell fraction (1.5 ml.) was alkalinized with 0.2 ml. of 0.5 *M* NaOH. To this (under thorough mixing) was added, in rapid succession, 1.5 ml. of 5% $ZnSO_4$, 1.5 ml. of 0.3 *N* $Ba(OH)_2$, 0.2 ml. of 50% NaOH, 4.5 ml. of saturated ammonium sulfate, and 1.5 ml. of acetophenone. The sample was then centrifuged (1500×*g*, 10°) for 55 min. and about 1.1 ml. of an acetophenone phase was obtained. A 1.0-ml. aliquot of this phase was then treated as described in *Procedure A*.

Extraction of Alkylating Agents from Aqueous Buffers and Urine (Procedure D)—Buffer solution or urine (1.5 ml.) containing the alkylating agent was alkalinized with 0.2 ml. of 50% NaOH; then 1.5 ml. of saturated ammonium sulfate solution and 1.5 ml. of acetophenone were added. The mixture was agitated for about 30 sec. on a mixer[8] and then centrifuged (1500×*g*) for 10 min. at 10°. A 1.0-ml. aliquot of the acetophenone phase was spectrophotometrically assayed according to *Procedure A*. When the concentration of drug was too low, it was possible to extract 20 ml. of urine with 3.0 ml. of acetophenone by alkalinizing the urine with 2.0 ml. of 50% NaOH, adding 20 ml. of saturated ammonium sulfate (to salt-out drug and precipitated protein), and then adding 3.0 ml. of acetophenone. After mixing and centrifugation, about 2.5 ml. of an acetophenone phase was obtained. Duplicate 1.0-ml. aliquots

Table II—Determination of Alkylating Agents by *Procedure A*

Compound	Concentration Range Studied, mcg./ml. of Sample	Slope[a] Absorbance/ mcg./ml. of Sample	Slope[a] Absorbance/ μmoles/ml. of Sample
I	0.64–32.3	0.021	5.79
II	0.63–62.5	0.024	5.59
III	0.43–42.6	0.031	7.69
V	0.22–11.1	0.054	11.02
VI	0.13–13.1	0.038	6.61
VII	0.30–30.0	0.066	4.69

[a] The "slopes" of the Beer's law plots were calculated from quadruplicate absorbance measurements taken at five different concentrations in the range indicated, utilizing an Olivetti Programma 101 (least-squares fit through the origin). The precision and accuracy of the absorbance measurements were comparable to the values shown in Table III.

[3] 4-(*p*-Nitrobenzyl)pyridine was purchased from the Aldrich Chemical Co.

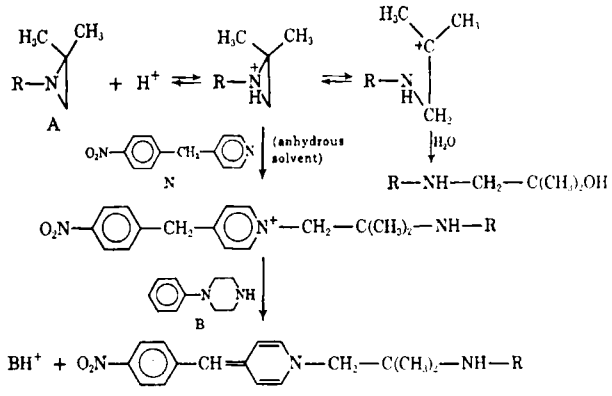
[4] *N*-Phenylpiperazine was obtained at 99% purity from the Aldrich Chemical Co. A practical grade (95% purity) was purchased from Eastman Organic Chemicals. Both products were equally satisfactory in the assay.

[5] Product of the American Cyanamid Co., purified by recrystallization from chloroform.

[6] Purchased from Fike Chemicals (through Columbia Organic) and redistilled before use.

[7] Thanks are due to Dr. Z. F. Chmielewicz and Dr. Y. Y. Hsiao for preparation of these materials.

[8] Vortex.



Scheme I—A = 2,2-dimethylaziridine-type alkylating agent (I–III, VII), N = nucleophilic reagent [4-(p-nitrobenzyl)pyridine], and B = base reagent (N-phenylpiperazine)

of this phase were then spectrophotometrically assayed according to *Procedure A*.

Chromatography (Procedure E)—TLC[9] was conducted, using two solvent systems: A, chloroform–absolute ethanol (1:1); and B, ethyl acetate. The alkylating agents were located on the chromatogram with a spray reagent. The most satisfactory reagent was a mixture of 9 ml. of 15% 4-(*p*-nitrobenzyl)pyridine in acetone and 1 ml. of glacial acetic acid (which were combined less than 5 min. before use). After spraying, the chromatogram sheets were heated to 95° in a hot air oven for 15 min. Color development was achieved by spraying with a 15% solution of *N*-phenylpiperazine in acetone (this solution is stable for about 2 days). The alkylating agents appeared as dark-blue spots on a yellow background. The R_f values are listed in Table I. Alkylating agents were extracted from plasma by adding, with vigorous shaking, in this order: 20 ml. of plasma, 3.0 ml. of 50% NaOH, 20 ml. of saturated ammonium sulfate, and 5.0 ml. of methylene chloride. Centrifugation (1500×*g*) for 35 min. at 10° yielded 1–2 ml. of a methylene chloride phase, which was then suitable for spotting.

This method permits the detection of 1–2 mcg. of alkylating agent.

RESULTS AND DISCUSSION

Assay Procedures—By using *Procedure A*, linear absorbance *versus* concentration plots (colorimetric assay standards) were obtained for 2,2-dimethylaziridine (VII), for the three agents (I–III) containing the 2,2-dimethylaziridine moiety, and for the two "conventional" alkylating agents (V and VI) containing C-unsubstituted aziridine functions. For all these compounds, good agreement with Beer's law was observed throughout the entire concentration range studied. The "slopes," given in Table II, reflect the sensitivity of the colorimetric assay. On a molar concentration basis, the slopes of the 2,2-dimethylaziridine derivatives are comparable to those of the corresponding analogous unsubstituted aziridine agents (see III *versus* VI) and are related to the number of alkylating moieties in the molecule. Thus, on a molar basis, the slopes obtained for the two bifunctional agents (I and II) are almost identical but are smaller than those obtained for the analogous trifunctional agents (III and VI) and are larger than that of the monofunctional agent (VII).

By using a nonaqueous solvent for the reaction with 4-(*p*-nitrobenzyl)pyridine, *Procedure A* eliminates the competing hydrolysis reactions for the aziridines. These reactions were particularly significant with the 2,2-dimethylaziridine derivatives (Scheme I). Rapid hydrolysis probably caused the unsatisfactory results obtained when previously published methods (using 50% aqueous buffers, usually at pH 4.5) were applied to the determination of Compound I. Acetophenone was previously employed as a solvent (36) for the colorimetric assay of alkyl halides with 4-(*p*-nitrobenzyl)pyridine; however, in the latter case, no acid was needed for the reaction to occur. In contrast, the reactions of aziridines require an appreciable

[9] On Eastman chromagram sheets (No. 6061 silica gel and No. 6062 alumina, without fluorescent indicator).

Table III—Statistical Evaluation of Results of *Procedure B* for Compounds I–III

Compound	Micrograms Added per Milliliter of Blood	Mean Absorbance[a]	Standard Deviation	Mean Error	Slope[b]
I	1.05	0.011	0.0045	−0.002	0.0127
	4.20	0.060	0.0047	+0.007	
	13.05	0.164	0.0138	−0.002	
	52.20	0.665	0.0340	+0.006	
II	0.93	0.029	0.026	+0.007	0.023
	3.70	0.089	0.017	+0.002	
	15.45	0.345	0.015	−0.017	
	61.80	1.450	0.012	−0.007	
III	0.47	0.030	0.016	+0.032	0.030
	4.04	0.150	0.009	+0.030	
	16.60	0.534	0.024	+0.011	
	66.40	2.052	0.059	−0.041	

[a] Based on four determinations. [b] Absorbance per microgram per milliliter of sample. Calculated as described in footnote of Table II.

acid concentration (see *Procedure A*), because protonation of the aziridine ring is the rate-determining step. The addition of acid to the reagent solution increases the color (absorbance) of the "blank" tubes (after alkalinization). This may be due to stabilization of the 4-(*p*-nitrobenzyl)pyridinium salt of the acid (*via* formation of a charge-transfer complex), which could favor the removal of a benzyl proton upon addition of the base. The nature of the base was a critical factor. Among a large series of bases studied, *N*-phenylpiperazine was unique in producing the maximal amount of colored product from the alkylated 4-(*p*-nitrobenzyl)pyridine in the sample tubes and, at the same time, causing very little color formation in the blanks.

Because of its overall structural similarity to 4-(*p*-nitrobenzyl)-pyridine, it is possible that the *N*-phenylpiperazine is capable of "discharging" the acid-salt complex; alternatively, *N*-phenylpiperazine is a sufficiently strong base to serve as an efficient proton

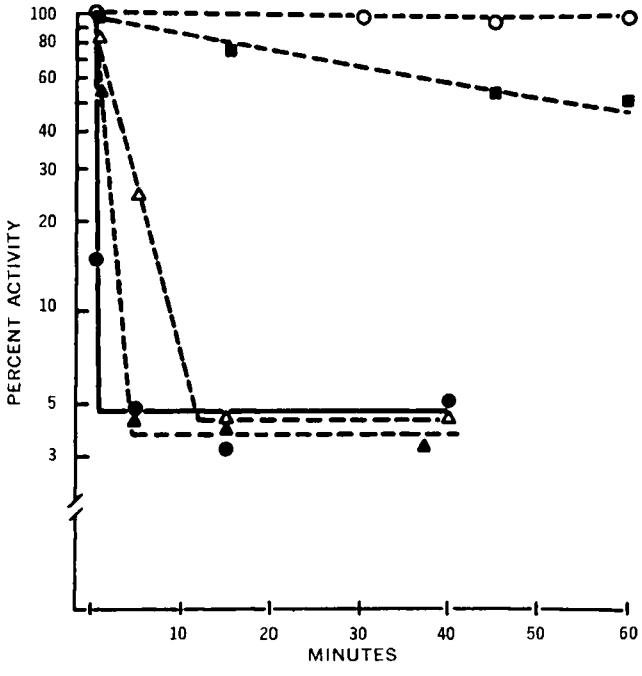


Figure 1—*Rate of hydrolysis of III in buffers of various composition (at 25°). Key: ○, pH 10.9 (0.6 M Na_2CO_3); ■, pH 7.5 (0.6 M $NaHCO_3$); △, pH 6.0 (10^{-3} M potassium hydrogen phthalate); ▲, pH 6.0 (10^{-1} M potassium hydrogen phthalate); and ●, pH 5.0 (10^{-3} M potassium hydrogen phthalate). The ordinate is the remaining alkylating activity, expressed as a percent of the t = 0 alkylating activity; t = 0 activity was calculated on the basis of an absorbance of 0.030/mcg. of III (Table III).*

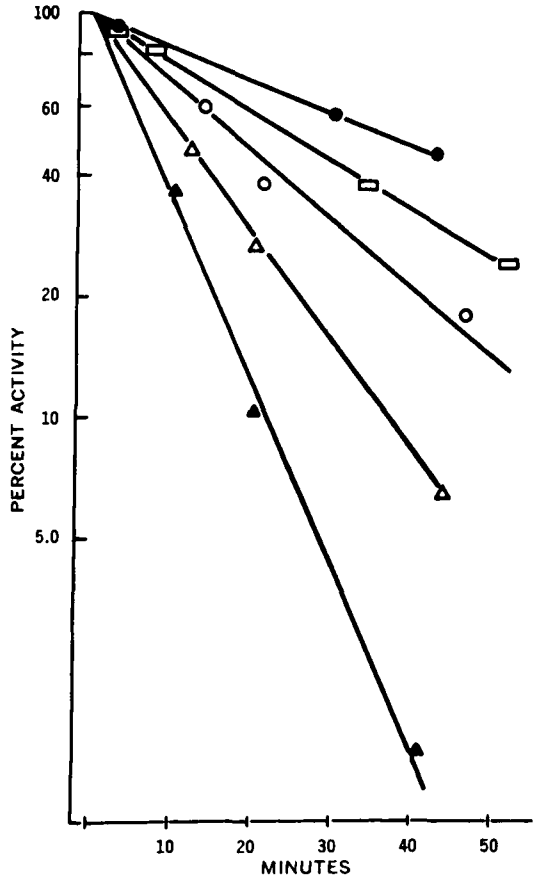


Figure 2—*Disappearance of reactivity with 4-(p-nitrobenzyl)pyridine in fresh human heparinized blood and plasma (pH 7.36). Key: ▲, III in blood at 37°; △, I in blood at 37°; ○, II in blood at 37°; □, III in plasma at 37°; and ●, III in blood at 25°.*

acceptor for a benzyl proton of the alkylated reagent but not for that of its acetate salt. The adoption of *N*-phenylpiperazine as the base reagent greatly increases the sensitivity of the colorimetric assay (Scheme I).

For the determination of 2,2-dimethylaziridine derivatives in blood samples according to *Procedure B*, it is advantageous to use another set of standard curves that already include the "recovery factor" of each agent, *i.e.*, the yield of extraction into the nonaqueous phase. Data for such curves were determined for Compounds I–III (Table III), and these data show good adherence to Beer's law. In other words, the absorbance measurements obtained in the acetophenone phase show a linear relationship to the theoretical concentrations (*i.e.*, the amount of drug per milliliter of blood added immediately before extraction) for each agent. This means that the percent recovery from blood (based on micrograms per milliliter concentration) for any given agent is constant throughout the concentration range studied, its value being dependent on the partition of the agent between the aqueous (salt) phase and the acetophenone phase (see *Procedure B*). Comparison of the data in Table III with those in Table II gives the "recovery factors": 60% for I, 95% for II, and 95% for III. The data in Table III also give some indication of the reproducibility and sensitivity of the assay procedure.

These recoveries were obtained by adding the compounds to whole blood immediately prior to separation of the plasma phase according to *Procedure B*. Virtually identical recoveries (absorbance per microgram per milliliter of biological fluid) and standard curves were obtained when the drugs were added directly to plasma. This suggests that the drugs rapidly equilibrate between the cellular fraction and plasma.

When the drugs added to the whole blood were extracted from the cell fraction, according to *Procedure C*, the percent recoveries (per milliliter of cell fraction) were somewhat lower (Table IV). This may be due to increased contact time of these reactive agents with the cells, resulting in binding and/or degradation.

Table IV—Recovery of Compounds I–III from Blood

Compound	Concentration Prepared in Whole Blood, mcg./ml.[a]	Recovery from Plasma, mcg./ml.[b,e]	Recovery from Cellular Fraction, mcg./ml.[c,e]	Recovery from Cellular Fraction, mcg./ml.[d,e]
I	78	71	49	38
II	88	76	64	39
III	68	63	46	27

[a] The alkylating agent was added to blood at 25°. [b] Results obtained utilizing *Procedure B* after the blood and alkylating agent had been incubated for 10 min. at room temperature. [c] Using *Procedure C-1*. [d] Using *Procedure C-2*. [e] The values in these columns were corrected for the 60% recovery of Compound I and the 95% recovery of Compounds II and III.

The recoveries of Compounds I–III, either from urine or from aqueous buffers, by *Procedure D* were virtually identical with the corresponding recovery factors obtained by the application of *Procedure B* to whole blood or plasma. This is significant, because it shows that the different proteins present in these biological fluids do not affect the results obtained with these assay procedures.

Stability Studies—Figure 1 shows the rates of hydrolysis of III in buffers and infusion vehicles at various pH values. The corresponding hydrolysis rates of I and II are slightly lower but show the same pH dependence. It is evident that III is stable in alkaline solution (pH 10.9) but that it loses 50% of its alkylating activity[10] in 1 hr. at pH 7.5 and in 1–3 min. at pH 6.0. At pH 5.0, about 85% of the drug is hydrolyzed in 30 sec. A 100-fold increase of the buffer concentration (at pH 6) further increases the rate of hydrolysis. Dependence of the rates of hydrolysis upon both hydrogen-ion and buffer concentrations has also been observed in the case of unsubstituted aziridine (37) and its derivatives (V and VI) (38). However, the corresponding hydrolysis rates are more than an order of magnitude lower for the latter compounds (*e.g.*, VI has a half-life of 15 hr. at pH 7.0) than for the 2,2-dimethylaziridine-containing agents. Therefore, the vehicle and rate of administration are much more critical factors in the clinical application of I–III than in the case of the conventional alkylating agents.

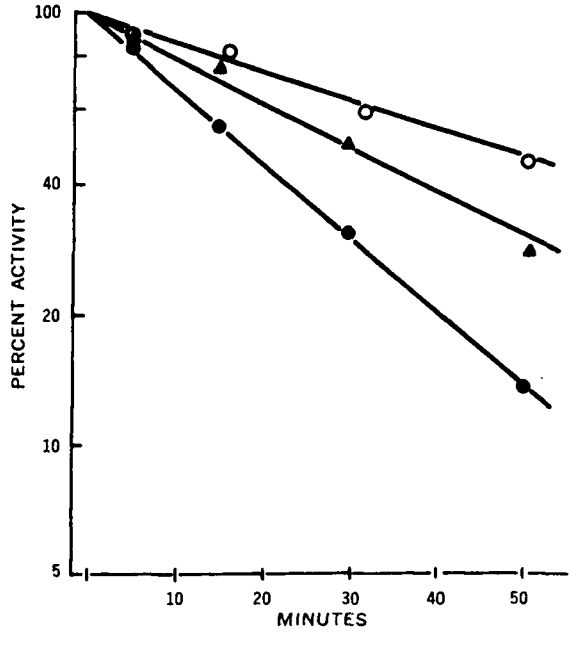


Figure 3—*Hydrolysis of Compounds I (▲), II ○, and III (●) in fresh human urine adjusted to pH 7.8 at 37°.*

[10] That is, reactivity with 4-(*p*-nitrobenzyl)pyridine, usually referred to in the literature as "NBP-reactivity."

It can be observed in Fig. 1 that about 4.5% of the zero-time alkylating activity remains after a time interval corresponding to many times the half-life of the parent compound. This suggests that the hydrolysis products contain some reactive material which is relatively stable to further hydrolysis. TLC (*Procedure E*) shows the presence of two such components, one having the same R_f values as Compound VII (Table I).

Figure 2 shows the rate of disappearance of the alkylating activities of Compounds I–III upon incubation with human blood or plasma. (Similar results were obtained with canine blood.) It is apparent that solutions of all three agents lose their alkylating activities very rapidly; the half-lives, in whole blood, at 37° are 11, 18, and 7 min. for Compounds I, II, and III, respectively. Comparison of the rates of loss of the alkylating activity for Compound III in whole blood and in plasma (half-life of 7 *versus* 26 min., respectively, at 37°) indicates that the blood cells contribute significantly to the loss of reactive material. The rate of loss was much lower at 25° than at 37° (half-life of 39 *versus* 7 min., respectively, in whole blood) (Fig. 2).

TLC analysis (*Procedure E*) of samples taken at various time points confirmed that at least 95% of the substances reacting with 4-(*p*-nitrobenzyl)pyridine were, in each case, identical with the parent compounds. Trace amounts of the two above-mentioned hydrolysis products (VII and unknown) were also detected.

The rates of hydrolysis of these agents in urine (Fig. 3) are comparable to those in blood plasma; however, substantial variations may be expected if such studies are conducted with urine samples of different compositions and pH's.

CONCLUSIONS

These studies demonstrated that the alkylating agents containing 2,2-dimethylaziridine moieties show a very rapid loss of recoverable alkylating activity when incubated with whole blood under physiological conditions. Since the half-lives of these agents in blood plasma are significantly longer, it appears that the blood cells provide effective binding sites and/or reactive nucleophilic targets for these agents. However, the possibility that some cellular enzymes (*e.g.*, phosphoramidases) may catalyze the degradation of these agents cannot be ruled out entirely.

For clinical chemotherapy, it is important to consider the extreme pH dependence of the hydrolysis rates of 2,2-dimethylaziridine-containing alkylating agents (Fig. 1). These drugs should be administered in an alkaline vehicle; otherwise, the actual dose of active drug introduced into the patient cannot be controlled.

In view of: (*a*) the relatively high and reproducible recovery of these agents from body fluids after rapid alkalinization, (*b*) the increased sensitivity of the modified colorimetric assay using nonaqueous solvent and *N*-phenylpiperazine as the base reagent, and (*c*) the relatively large doses employed clinically with the 2,2-dimethylaziridine agents, the application of these assay procedures to the study of the pharmacokinetics of these agents is feasible.

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Cellophane as a Dialysis Membrane for Studying Nonionic Surfactants

S. J. A. KAZMI and A. G. MITCHELL▲

Abstract □ The permeability of cellophane membranes to the nonionic surfactant cetomacrogol 1000 was investigated using equilibrium dialysis, dynamic dialysis, and an ultrafiltration technique. Cellophane and silicone rubber membranes were compared in an equilibrium dialysis study of the interaction of chlorocresol with cetomacrogol. The permeability and interaction studies showed that the cellophane membranes were permeable to the surfactant. In addition, errors were introduced into the calculation of the binding constants unless changes in volume and in surfactant concentration, which occur as a result of the osmotic differential across the membrane, were taken into account.

Keyphrases □ Cellophane dialysis membranes—permeability to nonionic surfactant, used to study interaction of chlorocresol with cetomacrogol, compared to silicone rubber membranes □ Dialysis membrane permeability to nonionic surfactant, interaction with preservative—cellophane and silicone rubber compared □ Surfactant (nonionic) interaction with preservatives—studied using cellophane dialysis membranes, effect of osmosis on binding constants

Cellophane membranes are widely used in equilibrium (1–7) and dynamic (7–9) dialysis studies involving the interaction of preservatives and drugs with nonionic surfactants. Cellophane has also been used to study the effects of surfactants on the diffusion of drugs across membranes (8–12). Ideally, such investigations require that the membrane be impermeable to the nonionic surfactant while allowing diffusion of the drug and that the osmotic differential across the membrane is negligible.

There is some controversy concerning the permeability of cellophane dialysis membranes to nonionic surfactants. Patel and Kostenbauder (13) reported that Visking cellophane membrane was permeable to polysorbate 80, and they considered this membrane unsatisfactory for equilibrium dialysis work. This observation was supported by Nishida *et al.* (14). Breuninger and Goettsch (2) found that, although Visking cellophane membrane was permeable to polysorbate 80, Fisher cellophane membrane was impermeable to the same surfactant. Matsumoto *et al.* (4) studied the permeability of polysorbate 80 and a polyoxyethylene lauryl ether through Visking cellophane membrane, using dynamic dialysis (without stirring the solutions) under sink and nonsink conditions. They found that Visking cellophane membrane was practically impermeable to the nonionic surfactants and that only impurities, such as low molecular weight polyethylene glycols, passed through it.

Patel (5) compared Fisher cellophane membrane with nylon and rubber membranes in equilibrium dialysis studies, involving the interaction of several preservatives with cetomacrogol 1000 and polysorbate 80. Since close agreement was found between data obtained using Fisher cellophane membrane and those obtained using the rubber or nylon membranes, it was assumed that the cellophane membrane was impermeable to cetomacrogol and polysorbate 80. Ikeda *et al.* (7) used Visking cellophane membrane in equilibrium and dynamic dialysis techniques to study the interaction of barbiturates with a polyoxyethylene ether surfactant. Although the surfactant permeated through the cellophane membrane, the amount passed during 48 hr. was below the CMC of the surfactant.

Short *et al.* (10) reported that Visking cellophane membrane was impermeable to polyoxyethylene surfactants and that only small traces of nonsurface-active impurities passed through it.

Although the permeability of cellophane to polyethylene glycols has been studied less extensively than the nonionic surfactants, it has been shown that cellophane is permeable to polyethylene glycols with a molecular weight less than 20,000 (3, 15). Polyethylene glycols permeate cellophane membranes more readily than nonionic surfactants of similar molecular weight.

Rubber (16) and nylon (17) membranes were used in equilibrium dialysis studies of the interaction of various preservatives with the nonionic surfactant cetomacrogol, since qualitative tests showed that cellophane is permeable to the nonionic surfactant and that volume changes occurred as a result of osmosis. Apart from Matsumoto *et al.* (4), few workers using cellophane appear to have corrected for the volume changes. In view of the continued use of cellophane, it seemed desirable to make quantitative measurements of the permeability of cellophane membranes to cetomacrogol and to assess the effects of surfactant permeation and osmosis on the binding constants for the interaction between the surfactant and a preservative determined using the equilibrium dialysis technique.

EXPERIMENTAL

Materials—Cetomacrogol 1000 BPC[1] of the general formula $CH_3(CH_2)_m(OCH_2CH_2)_nOH$, where m may be 15 or 17 and n may be 19–23, was used. The molecular weight was taken as 1300. The preservative selected was chlorocresol, reagent grade[2]. Three membranes were used: Membrane 1, cellophane, dialysis tubing 3.6-cm. ($1^{47}/_{64}$-in.) flat width[3]; Membrane 2, cellophane, dialysis tubing 2.4-cm. ($^{15}/_{16}$-in.) flat width[4]; and Membrane 3, silicone rubber (silastic sheeting, nonreinforced 0.01 cm. (0.005 in.)[5]. All membranes were washed thoroughly with distilled water before use. Deionized glass-distilled water was used in all studies.

Analysis of Cetomacrogol—The presence of surface-active material in the "surfactant-free" compartment of the dialysis cell was readily detected by the formation of foam on agitation and by the

[1] Glovers Chemicals Ltd., Leeds, England.
[2] British Drug Houses, Poole, England.
[3] Fisher cellophane, Fisher Scientific Co.
[4] Visking cellophane, Union Carbide Ltd.
[5] Dow Corning Corp.

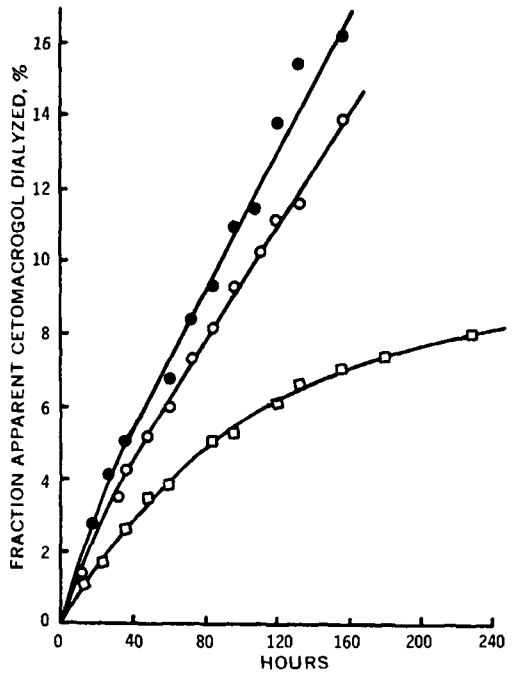

Figure 1—*Permeability of cellophane membranes to cetomacrogol in equilibrium dialysis. Initial cetomacrogol concentrations were:* □, *38.46* × *10*$^{-3}$ M; *and* ○, *76.92* × *10*$^{-3}$ M. *Open and closed symbols represent Membranes 1 and 2, respectively. The fraction dialyzed was not corrected for changes in surfactant concentration due to osmosis.*

precipitation of a phosphomolybdic acid complex in a barium chloride–hydrochloric acid medium (18).

Quantitative analysis of surfactant was made using a polarograph[6]. The analysis was based on the suppression of the oxygen maximum by surfactants. Potassium chloride solution (0.002 *N*) gives a very pronounced oxygen maximum. A comparison of the heights of the maxima in the presence and absence of cetomacrogol and reference to a calibration curve permitted the determination of surfactant down to 1.0 mg./l. (19, 20). Since the method is not specific for cetomacrogol, the results are expressed as apparent cetomacrogol concentrations.

Permeability of Membranes to Cetomacrogol—*Equilibrium Dialysis*—Dialysis cells similar to those described by Patel and Foss (21) were used. The two chambers of the cell were separated by a cellophane or silicone rubber membrane. Twenty milliliters of cetomacrogol solution was pipeted into one chamber, and 20 ml. of distilled water was pipeted into the other. The cells were tumbled in a temperature-controlled water bath; at 12-hr. intervals, equal volumes were pipeted from both chambers and analyzed for cetomacrogol.

Dynamic Dialysis—Twenty-five milliliters of 10% cetomacrogol was transferred to membrane 1 suspended in 200 ml. of distilled water in a jacketed beaker. The solution in the jacketed beaker was stirred with a magnetic stirrer, while the cetomacrogol solution in the cellophane bag was stirred with a glass stirrer. One hundred milliliters of the solution was pipeted from the jacketed beaker at 12-hr. intervals and analyzed for cetomacrogol. The volume of solution in the jacketed beaker was immediately made to 200 ml. with fresh distilled water to maintain sink conditions.

Ultrafiltration—Membrane 2 was cut and fitted into an ultrafiltration cell[7]. Thirty-five milliliters of 1.8% cetomacrogol solution was placed in the cell, and a pressure of 18.2 kg. (40 lb.)/in.^{2} was applied until complete filtration of the liquid was attained. The filtrate was analyzed for cetomacrogol.

Interaction of Chlorocresol with Cetomacrogol—The equilibrium dialysis technique was used to study the interaction of chlorocresol with cetomacrogol. The dialysis membrane was either Membrane 1 or 3. An aqueous solution of chlorocresol in cetomacrogol was placed in one side of the cell, and water or water plus chlorocresol was placed in the other. Two glass beads were added to each compartment to ensure continuous mixing, and the cells were rotated in the water bath at 25°. The cholorocresol in both chambers was analyzed spectrophotometrically at 280 nm. after 4 days, and the volume of solution in each chamber was measured.

[6] Radiometer Polariter PO$_4$.
[7] Amicon Corp.

Table I—Change of Surfactant Concentration in Equilibrium Dialysis with Cellophane as a Semipermeable Membrane

Cetomacrogol Concentration, $M \times 10^3$			
M_1, Initial	M_2, Calculated from Volume Change[a]	M_3, Calculated Using Eq. 2[b]	M_4, Permeated at 96 hr.[c]
38.46	33.88	31.60	2.19
76.92	62.06	55.42	7.23

[a] Each value represents the mean of five readings. [b] Values were obtained by substituting n_1K_1 and n_2K_2, calculated from the binding curve obtained using the silicone rubber membrane (Fig. 3), and $[D_b]$ and $[D_f]$, obtained using the cellophane membrane (Fig. 3), into Eq. 2. Each value represents the mean of five readings. [c] From Fig. 1.

RESULTS AND DISCUSSION

Permeability of Membranes to Cetomacrogol—Figure 1 shows the fraction of cetomacrogol dialyzed through Membranes 1 and 2 as a function of time in equilibrium dialysis. Both membranes are permeable to cetomacrogol at approximately the same rate. The silicone rubber membrane, Membrane 3, was completely impermeable to the nonionic surfactant. Figure 2 shows the permeability of Membrane 1 to cetomacrogol using dynamic dialysis under sink conditions. Increases in the volume of solution in the surfactant chamber of the equilibrium dialysis cell and the cellophane bag used in the dynamic dialysis technique showed that osmosis had occurred.

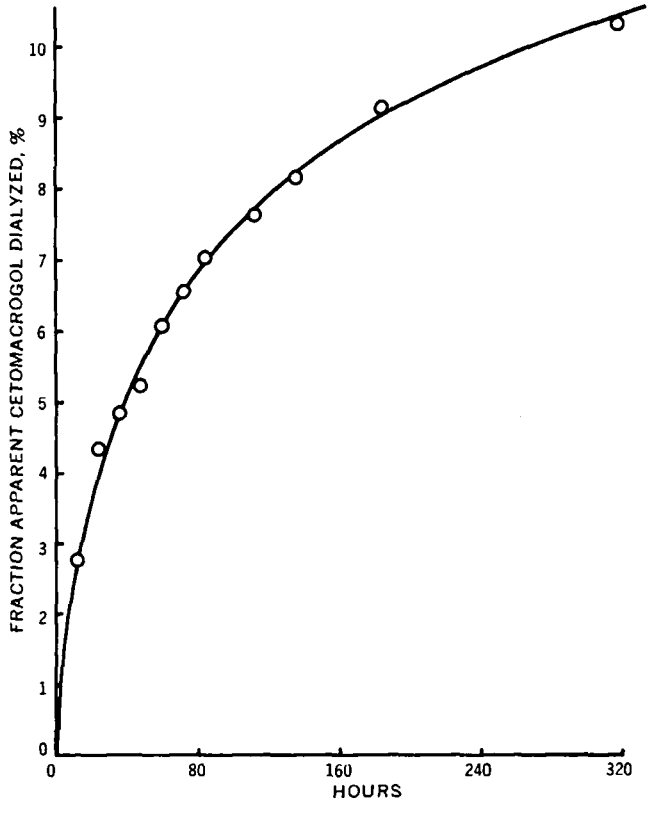

Figure 2—*Permeability of Membrane 1 to cetomacrogol in dynamic dialysis under sink conditions. Initial cetomacrogol concentration was 76.92* × *10*$^{-3}$ M. *The fraction dialyzed was not corrected for changes in surfactant concentration due to osmosis.*

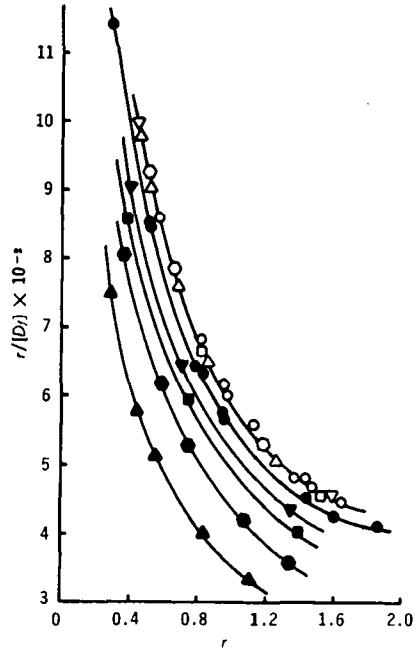

Figure 3—*Scatchard plot for the interaction of chlorocresol with cetomacrogol. Initial cetomacrogol concentrations were:* ○, *7.69* × *10⁻³* M; ▽, *15.3* × *10⁻³* M; □, *23.07* × *10⁻³* M; ○, *38.46* × *10⁻³* M; *and* △, *76.92* × *10⁻³* M. *Closed and open symbols represent data obtained using Membranes 1 and 3, respectively.*

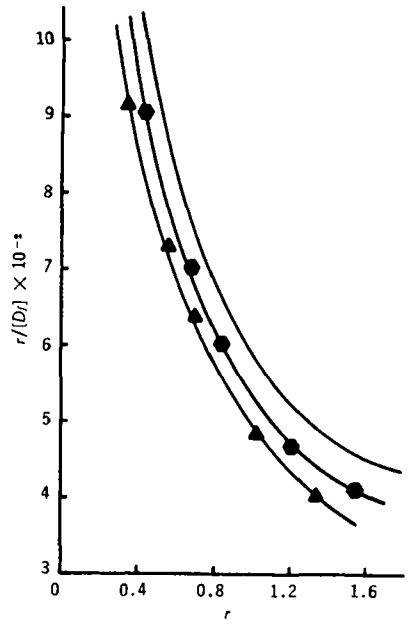

Figure 4—*Scatchard plot for the interaction of chlorocresol with cetomacrogol. The upper binding curve was obtained using the silicone rubber membrane as in Fig. 3. Cellophane membrane data were corrected for changes in surfactant concentrations due to osmosis. Initial concentrations were as listed in Fig. 3; corrected concentrations are shown in Table I.*

Ultrafiltration of cetomacrogol solution (1.8%) through Membrane 2 showed that 2.7% of the total surfactant passed through the membrane.

Interaction of Chlorocresol with Cetomacrogol—The binding results in the form of a Scatchard plot (22) are shown in Fig. 3. The binding of chlorocresol with cetomacrogol is independent of surfactant concentration when silicone rubber is used as the dialysis membrane. Similar independence of surfactant concentration was reported previously for the binding of several other preservatives with cetomacrogol when nylon was used as the dialysis membrane (17). The binding constants were estimated from the slope of the curve in the region of interest. In this work, however, the curve was characterized according to Eq. 1 on the assumption that two classes of binding sites are involved in the interaction:

$$\frac{[D_b]}{[M]} = r = \frac{n_1K_1[D_f]}{1 + K_1[D_f]} + \frac{n_2K_2[D_f]}{1 + K_2[D_f]} \qquad \text{(Eq. 1)}$$

where:

$[D_f]$ = concentration of free preservatives in the aqueous phase
$[D_b]$ = concentration of preservative bound to surfactant
$[M]$ = concentration of surfactant
n_1 = number of independent binding sites of Class I on the surfactant molecule
n_2 = number of independent binding sites of Class II on the surfactant molecule
K_1 = intrinsic association constant for the binding of a molecule of preservative to one of the binding sites of Class I
K_2 = intrinsic association constant for the binding of a molecule of preservative to one of the binding sites of Class II

The experimental data are indicated by points, while the solid lines were fitted according to the n and K values computed from Eq. 1 using a nonlinear regression program.

Figure 3 shows that when a cellophane dialysis membrane was used, the binding is apparently not independent of cetomacrogol concentration and a series of curves are obtained. Theoretically, such results indicate that an increase in the concentration of cetomacrogol results in a decrease in the number of binding sites on the cetomacrogol molecule. However, since the results obtained with the silicone rubber membrane are independent of surfactant concentration, it is suggested that changes in cetomacrogol concentration due to dilution as a result of osmosis and/or to permeation of surfactant through the cellophane membrane provide a more likely explanation. Permeation of sufficient cetomacrogol into the surfactant-free chamber of the dialysis cell would lead to an increase in the apparent value of $[D_f]$ due to micellar interaction with the chlorocresol and, therefore, decrease the ratio $r/[D_f]$ for a given r value. Moreover, loss of surfactant by permeation through the membrane would also decrease r due to the uncorrected decrease in the value of $[M]$ and the decrease in $[D_b]$. Thus, a plot of $r/[D_f]$ *versus* r for different concentrations of cetomacrogol would result in a series of curves, each representing a given surfactant concentration. Dilution of cetomacrogol as a result of osmosis would produce a similar displacement of the binding curves.

The surfactant concentration required to produce the observed displacement of the binding curves was calculated from a rearrangement of Eq. 1:

$$[M] = \frac{[D_b](1 + K_1[D_f] + K_2[D_f] + K_1K_2[D_f]^2)}{[D_f](n_1K_1 + n_2K_2 + n_1K_1K_2[D_f] + n_2K_1K_2[D_f])} \qquad \text{(Eq. 2)}$$

where $[D_b]$ and $[D_f]$ are the experimental values for the cellophane membrane (Fig. 3) and n_1, K_1, n_2, and K_2 are the binding constants obtained using the silicone rubber membrane. The data in Table I show that the changes in surfactant concentration (*i.e.*, differences between the initial and calculated cetomacrogol concentrations, $M_1 - M_3$) were considerably greater than could be accounted for solely by loss of surfactant as a result of permeation through the cellophane membrane, M_4. However, when the binding curves are replotted using surfactant concentrations corrected for volume changes in the chambers of the dialysis cell, M_2, the discrepancies between the curves determined using the silicone rubber membrane and the cellophane membrane are markedly reduced (Fig. 4).

Since the difference between M_2 and M_3 is of the same order of magnitude as M_4, the residual displacement of the binding curves can be attributed to the decrease in $[M]$ due to permeability of the cellophane membrane to cetomacrogol.

Hence, it can be concluded that the observed displacement of the binding curves obtained in the equilibrium dialysis study using cellophane membranes is due both to osmosis and to permeability of the membrane to the surfactant, with dilution of surfactant as a result of the osmotic differential across the membrane being the major factor. The use of cellophane as a membrane will introduce appreciable errors into interaction and transport studies involving nonionic surfactants unless corrections are made for osmosis.

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Tremor Induction by Intracaudate Injections of Bretylium, Tetrabenazine, or Mescaline: Functional Deficits in Caudate Dopamine

P. M. LALLEY*‡, G. V. ROSSI*, and W. W. BAKER†▲

Abstract □ Tremor responses were evoked by intracaudate injections of tetrabenazine, bretylium, or mescaline in cats with chronically implanted recording electrodes and microinjection cannulas. The characteristics of the maximal tremors with each agent closely resembled those induced by increased levels of cholinergic activity in the caudate (previously reported). These maximal tremors, like the cholinergic tremors, were suppressed by local injections of catecholamines (dopamine and epinephrine), scopolamine, or hemicholinium but were intensified by intracaudate serotonin. Although local acetylcholine had no effect on established tremor activity, tremors abolished by hemicholinium were reestablished by small doses of acetylcholine. These results suggested that interference with local dopamine inhibitory mechanisms ("functional dopamine deficiency") was the basis for the tremorgenic actions of bretylium, tetrabenazine, and mescaline; the findings also indicated that sustained endogenous acetylcholine activity in the caudate was a necessary condition for the development and maintenance of tremor activity. These data lend support to the hypothesis that an imbalance in the caudate between dopamine inhibition and acetylcholine excitation in favor of the latter results in tremors.

Keyphrases □ *Dopamine*—functional deficiency in caudate tremor mechanisms, role in tetrabenazine-, bretylium-, and mescaline-induced tremor □ Tetrabenazine—tremor evoked by intracaudate injection □ Bretylium—tremor evoked by intracaudate injection □ Mescaline—tremor evoked by intracaudate injection □ Catecholamine—inhibition of tetrabenazine-, bretylium-, and mescaline-induced tremor □ Scopolamine—inhibition of tetrabenazine-, bretylium-, and mescaline-induced tremor □ Hemicholinium—inhibition of tetrabenazine-, bretylium-, and mescaline-induced tremor □ Serotonin—intensification of tetrabenazine-, bretylium-, and mescaline-induced tremor

Resting tremors have been induced consistently by carbachol or anticholinesterases injected directly into the head of the caudate nucleus in unanesthetized cats prepared with permanently implanted recording electrodes and microinjection cannulas. These tremors were inhibited by intracaudate injection of agents that interfere with either postsynaptic receptor actions (atropine and scopolamine) or presynaptic synthesis (hemicholinium-3) of acetylcholine, or they were suppressed by intracaudate injection of catecholamines (epinephrine and dopamine) (1, 2). From these findings and from a consideration of the high concentrations of both acetylcholine and dopamine in the caudate, it has been postulated that the functions of acetylcholine (excitatory) and dopamine (inhibitory) are critically balanced in the caudate so as to comprise a local tremor regulatory mechanism (3–5).

Clinically, the involuntary movement disorders associated with Parkinson's disease have been linked to deficiencies in caudate dopamine (6). Also, the extra-

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Table I—Characteristics of Tremor Produced by Intracaudate Injections of Tetrabenazine, Bretylium, and Mescaline

Tremor Parameters[a]	Tremorgenic Agents		
	Tetrabenazine	Bretylium	Mescaline
Effective dose, mcg.[b]	161.2 ± 8.9	112.0 ± 0.5	64.5 ± 5.4
Latency, min.	18.1 ± 1.7	4.0 ± 0.3	6.0 ± 1.2
Maximal tremor characteristics:			
Onset of peak effect, min.	58.6 ± 6.6	13.0 ± 1.7	20.1 ± 3.1
Duration of maximal tremor, hr.	>5[c]	>6[d]	>5
Amplitude, μv.	269.7 ± 32.8	261.3 ± 20.2	311.7 ± 47.1
Amplitude, graded intensity[e]	+++	+++	+++
Tremor bursts per minute	20.7 ± 1.4	23.1 ± 2.0	23.0 ± 2.1
Tremor frequency, c.p.s.	23.6 ± 0.3	24.8 ± 1.1	21.3 ± 0.9
Tremor time, %	59.7 ± 2.8	68.2 ± 3.8	68.2 ± 1.7

[a] Values obtained from no less than six different cats. [b] Mean dose of base (±standard error). [c] Duration of maximal tremor extended beyond 5 hr. in nine of 10 trials. [d] Duration of maximal tremor always extended at least 6 hr.; actual duration beyond this determined in only two trials. [e] Graded against physostigmine tremor (++) as the standard (2).

Table II—Effects of Locally Injected Dopamine, Epinephrine, or Serotonin on Maximal Tremors Evoked by Intracaudate Tetrabenazine, Bretylium, and Mescaline

Locally Injected Agents	Activity Parameters	Tremors Evoked by		
		Tetrabenazine	Bretylium	Mescaline
Dopamine	Effect on tremor[a]	(−)	(−)	(−)
	Dose, mcg.[b]	81.0 ± 5.2	107.3 ± 7.9	168.0 ± 2.3
	Latency, min.	5.3 ± 0.7	5.0 ± 1.1	6.3 ± 1.4
	Duration, min.	46.2 ± 4.1	60.3 ± 5.1	60.7 ± 8.6
	Activity ratio[c]	6/6	6/6	6/7
Epinephrine	Effect on tremor	(−)	(−)	(−)
	Dose, mcg.	12.3 ± 0.9	4.5 ± 0.4	14.7 ± 1.2
	Latency, min.	6.7 ± 0.4	3.3 ± 0.3	5.7 ± 1.0
	Duration, min.	44.3 ± 3.8	53.2 ± 1.4	54.0 ± 6.9
	Activity ratio	6/6	6/6	6/6
Serotonin	Effect on tremor	(+)	(+)	(+)
	Dose, mcg.	64.5 ± 6.0	44.6 ± 4.6	52.0 ± 7.5
	Latency, min.	8.2 ± 1.6	4.3 ± 1.2	5.8 ± 1.3
	Duration, min.	27.3 ± 2.2	42.0 ± 5.0	45.8 ± 11.3
	Activity ratio	6/6	6/6	6/7

[a] (−) = tremor activity abolished; (+) = tremor activity intensified. [b] Doses of dopamine, epinephrine, or serotonin represent their respective bases (mean ± standard error). [c] Activity ratio = number of tremor inhibitions (or intensifications)/total number of trials.

pyramidal symptoms developing after systemic administration of neuroleptic drugs have been attributed to reduced dopamine functioning (7, 8).

The present investigation was undertaken to explore the relationship between dopamine and acetylcholine in tremor, utilizing tetrabenazine, bretylium, and mescaline as pharmacological tools. The purposes were to ascertain whether interference with caudate dopamine mechanisms was tremorgenic and to determine whether acetylcholine was involved in this tremorgenic process.

EXPERIMENTAL

Thirty-two male cats (3.0–4.5 kg.) were used in these studies. Under pentobarbital anesthesia, permanent bipolar assemblies[1], consisting of microinjection cannulas and recording electrodes, were implanted stereotaxically in both caudate nuclei (R. and L. Caud.) at Horsley-Clarke coordinates A_{15}, R_5, and L_5, D + 5 (9). Bipolar twisted electrodes were also positioned in the cortex and other subcortical recording sites. All electrodes were externalized to a 15-pin plug[2], which was fixed to the skull with acrylic dental cement and hard wax. Details of the assembly and microinjection technique were described previously (2, 10, 11).

After a recovery period of at least 2 weeks, the unanesthetized cat was suspended in a canvas hammock and positioned so that its hindlimbs were extended. Motor and electrographic responses were monitored on an oscilloscope and recorded on an electroencephalograph. Tremor activities were detected by a phonocartridge transducer[3] affixed to the dorsal surface of one hindfoot. Tremor responses were analyzed in terms of amplitude, percent tremor time, tremor frequency, and number of tremor bursts per minute, which provided an accurate assessment of relative tremor intensity (1, 10). The maximal tremor response is characterized in terms of the amplitude of the recorded involuntary movement oscillations (microvolts) for any given dose of drug.

All microinjections were made into the head of the right or left caudate through the bipolar assemblies[1] by way of a delivery cannula coupled to a calibrated micrometer-driven hypodermic syringe. Only one caudate nucleus was utilized for drug injection in any given experiment. Concentrations (0.1–1.5%) of drug solutions for intracaudate injections were adjusted so that microinjected volumes usually did not exceed 10 μl. Pilot experiments showed that alterations in pH and tonicity of drug solutions over a wide range produced no discernible effect on tremorgenesis or on other drug responses. Solutions of the following compounds prepared in distilled water were employed: bretylium tosylate, mescaline hydrochloride, tetrabenazine methanesulfonate, acetylcholine chloride, physostigmine sulfate, epinephrine bitartrate, dopamine hydrochloride, serotonin creatinine sulfate, hemicholinium-3 hydrobromide, and scopolamine hydrochloride. Drug quantities specified in the text refer to those of their respective bases.

Animals were rested at least 1 week between experiments. At the end of the study, after each brain was fixed in formalin, placements of the electrodes and permanent bipolar assemblies[1] were verified by examination of their tracts and the electrolytic lesions at the electrode tips.

[1] Injectrode.
[2] Amphenol.
[3] Astatic 13TB.

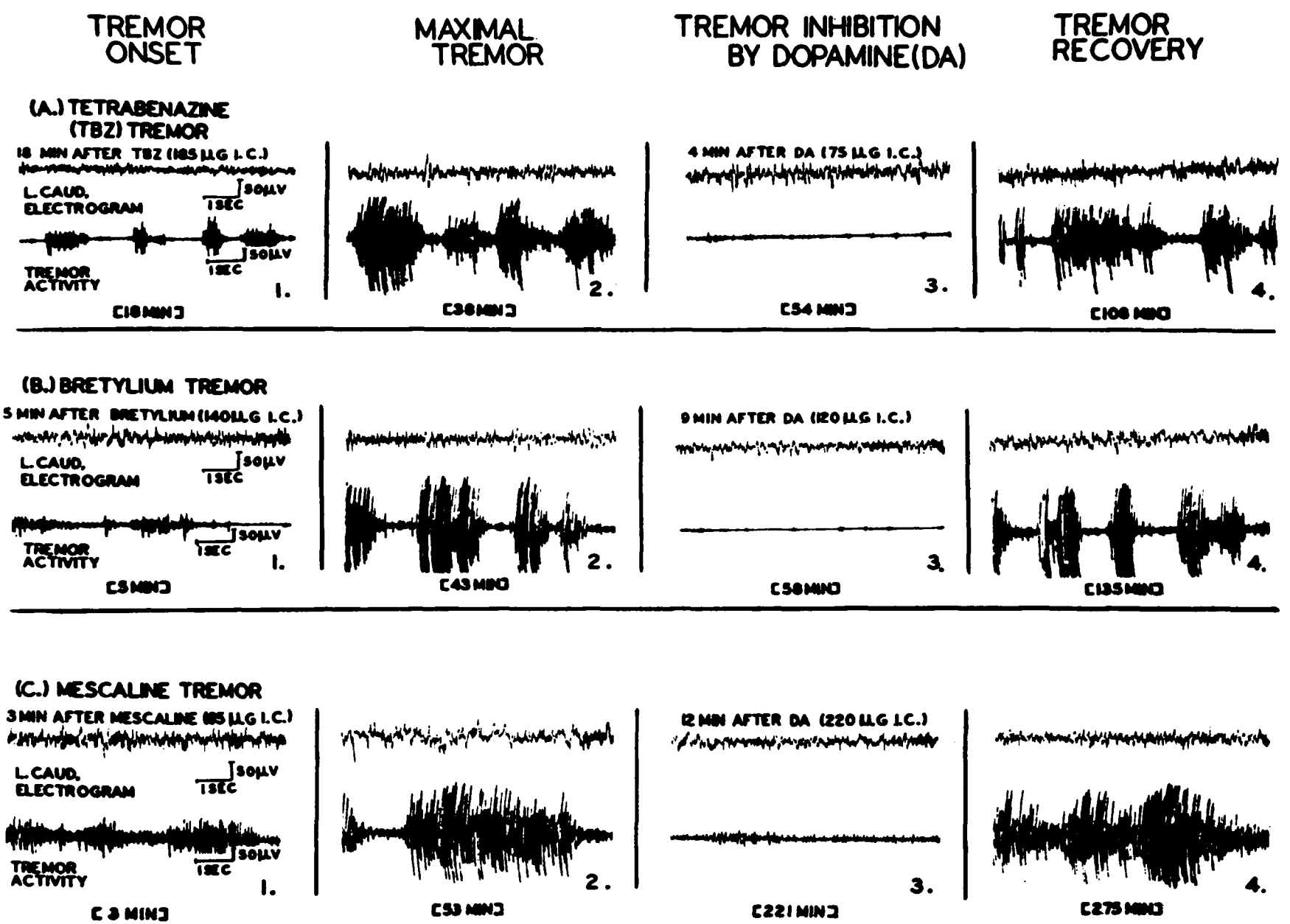

Figure 1—*Suppression of maximal tetrabenazine (TBZ), bretylium, and mescaline tremors by intracaudate injections of dopamine (DA). In each panel, upper tracings are caudate electrograms and lower tracings are tremor recordings. Numbers in brackets indicate the time sequence in minutes, continuous from the injection of each tremorgenic agent. Onset of significant tremor activity after intracaudate injections of tremorgenic agents (column 1) and maximal tremor activities (column 2) are shown. Tremors are abolished after intracaudate injections of DA (column 3) but recover to previous intensities (column 4). Doses of the tremorgenic agents (in parentheses) indicate their respective bases; those of DA represent its hydrochloride salt.*

Table III—Effects of Locally Injected Acetylcholine, Hemicholinium, or Scopolamine on Maximal Tremors Evoked by Intracaudate Tetrabenazine, Bretylium, and Mescaline

Locally Injected Agents	Activity Parameters	Tremors Evoked by—Tetrabenazine	Bretylium	Mescaline
Acetylcholine	Effect on tremor[a]	(0)	(0)	(0)
	Dose, mcg.[b]	32.2	32.2	32.2
	Activity ratio[c]	0/6	0/6	0/6
Hemicholinium	Effect on tremor	(−)	(−)	(−)
	Dose, mcg.	124.3 ± 4.3	140.0 ± 10.0	120.0
	Latency, min.	43.4 ± 1.1	35.0 ± 1.7	42.4 ± 2.0
	Activity ratio	7/7	6/6	7/7
	Duration[d]			
Scopolamine	Effect on tremor	(−)	(−)	(−)
	Dose, mcg.	78.1 ± 4.2	102.2 ± 9.2	178.8 ± 29.9
	Latency, min.	25.7 ± 3.4	19.0 ± 3.8	19.0 ± 3.9
	Activity ratio	6/6	6/6	7/7
	Duration[e]			

[a] (−) = tremor activity abolished; (0) = no effect on tremor activity. [b] Doses of acetylcholine or scopolamine represent their respective bases (mean ± standard error). [c] Activity ratio = number of tremor inhibitions/total number of trials. [d] Duration not followed; tremors reestablished by intracaudate injection of acetylcholine chloride (15–30 mcg.). [e] Duration of inhibition not followed; higher doses of scopolamine, after initially blocking tremors, evoked behavioral effects and involuntary movements, distinguishable from the test tremors elicited by tetrabenazine, bretylium, or mescaline.

RESULTS

Tremor Characteristics—Tetrabenazine, bretylium, and mescaline were tremorgenic over a range of doses (50–175 mcg.); the intensity and duration of tremor responses were proportional to the dose. Only the tremor responses to the higher doses of each drug were quantified; only in these higher doses was maximal tremor maintained throughout the entire experimental period of 5–6 hr.

As indicated in Table I, the tremors generated by these agents were more intense and of longer duration than the cholinergic tremors evoked by 150 mcg. intracaudate physostigmine (2). Bretylium had the most rapid onset of action, mescaline was intermediate, and tetrabenazine exhibited the longest latencies for onset and development of maximal tremor. The mean intracaudate doses required for comparable maximal tremor responses and duration were: tetrabenazine, 161 mcg.; bretylium, 112 mcg.; and mescaline, 64 mcg. Tremor characteristics (bursts per minute, tremor frequency, and percent tremor time) were similar for these three compounds and did not differ appreciably from those recorded during physostigmine (cholinergic) tremors. Neither autonomic nor behavioral changes were observed with any of the drugs while the animals were tremoring.

Effect of Intracaudate Monoamines on Tremors—The effects of microinjected dopamine, epinephrine, or serotonin when superimposed on ongoing maximal tremors induced by intracaudate tetrabenazine, bretylium, or mescaline are summarized in Table II. Intracaudate injections of the catecholamines (dopamine and epinephrine) temporarily abolished all three types of maximally developed tremors. Figure 1 illustrates the progressive changes in the development of tremors to maximal intensities, their subsequent inhibition by dopamine, and recovery with the return of tremor activity. Based on the doses required for maximal tremor suppression, epinephrine was six to seven times more potent than dopamine, but their respective latencies and durations of tremor inhibition were comparable. Mescaline tremors required appreciably higher doses of the catecholamines than the other two tremorgenic agents for complete suppression. Tetrabenazine tremors, although the most susceptible to suppression with dopamine, recovered to maximal intensities more rapidly than those of bretylium or mescaline.

In contrast to the catecholamines, intracaudate injection of serotonin, even at relatively low doses, intensified all three types of tremors, as evidenced by significant increases in essentially all tremor parameters (*i.e.*, amplitude, percent tremor time, and duration of tremor). However, these tremor intensities declined steadily to preserotonin levels within 25–60 min.

Intracaudate Cholinergic Agents on Tremors—Intracaudate acetylcholine, at a dose of 32 mcg., produced no discernible changes in tremor activity or electrograph patterns when injected during periods of maximal tremors induced by each of the three tremorgenic agents (Table III); this dose of acetylcholine is substantially below that (>500 mcg.) shown to be tremorgenic in the caudate (10). Comparable small doses of acetylcholine injected during the declining phases of these tremors also failed to alter existing tremor intensities. By contrast, local injections of hemicholinium, an agent known to interfere with acetylcholine synthesis, abolished all three types of tremors within 33–45 min., with no evidence of spontaneous recovery with the reestablishing of tremor activity during the experimental period. This inhibition of tremor was accompanied by bilateral synchronous slow waves in the caudate nuclei and postcruciate (frontal) cortex (Fig. 2). Parallel changes in behavior, autonomic functioning, or respiration were not evident. In all experiments, tremor suppressed by hemicholinium was readily restored (within 8–20 min.) by minimal supplemental doses (12–24 mcg.) of acetylcholine. This reestablished tremor activity usually persisted for less than 30 min. before declining. Nontremorgenic doses (6–13 mcg.) of physostigmine, when added to the acetylcholine injection, extended the duration of the reestablished tremor well beyond 1 hr.

Scopolamine, when microinjected into the caudate, also abolished the maximal tremors evoked by each of the three tremorgenic agents within 13–30 min. (Table III). The relatively high doses of scopolamine (>120 mcg.) required in the present study to suppress tremor (contrasting with scopolamine doses of less than 25 mcg. that suppress cholinergic tremors) produced another type of involuntary motor activity. These movements were slower (10–20 c.p.s.) rotational excursions of the hindlimb and were readily distinguished from the characteristic maximal tremor (21–25 c.p.s.).The involuntary movements generated by large doses of scopolamine were accompanied by pronounced autonomic (mydriasis), behavioral (apparent disorientation and distress), and electrographic (caudate-localized and projected synchronous slow waves) changes. In contrast to the inhibitory action of hemicholinium, tremor suppression by scopolamine was not reversed by intracaudate acetylcholine.

DISCUSSION

Although significant differences exist in their neuropharmacological actions (12), several lines of evidence support the position that tetrabenazine, bretylium, and mescaline share a common basis for their local tremorgenic actions in the caudate, namely interference with the level of dopamine functioning. The characteristics of their tremor responses (frequencies, bursts per minute, and tremor time) were strikingly similar; however, there were appreciable differences in their tremorgenic potencies (mescaline > bretylium > tetrabenazine) and in their onset and time sequences for reaching peak activity. It is significant that their tremor activities were quite comparable to the tremor type characterized previously during increased cholinergic activity in the caudate (10) and also that the catecholamines (dopamine and epinephrine) and hemicholinium were highly effective in suppressing cholinergic tremor (1, 2) as well as "dopamine-deficiency" tremors described in the current investigation. Because of its putative neurotransmitter role (13), attention

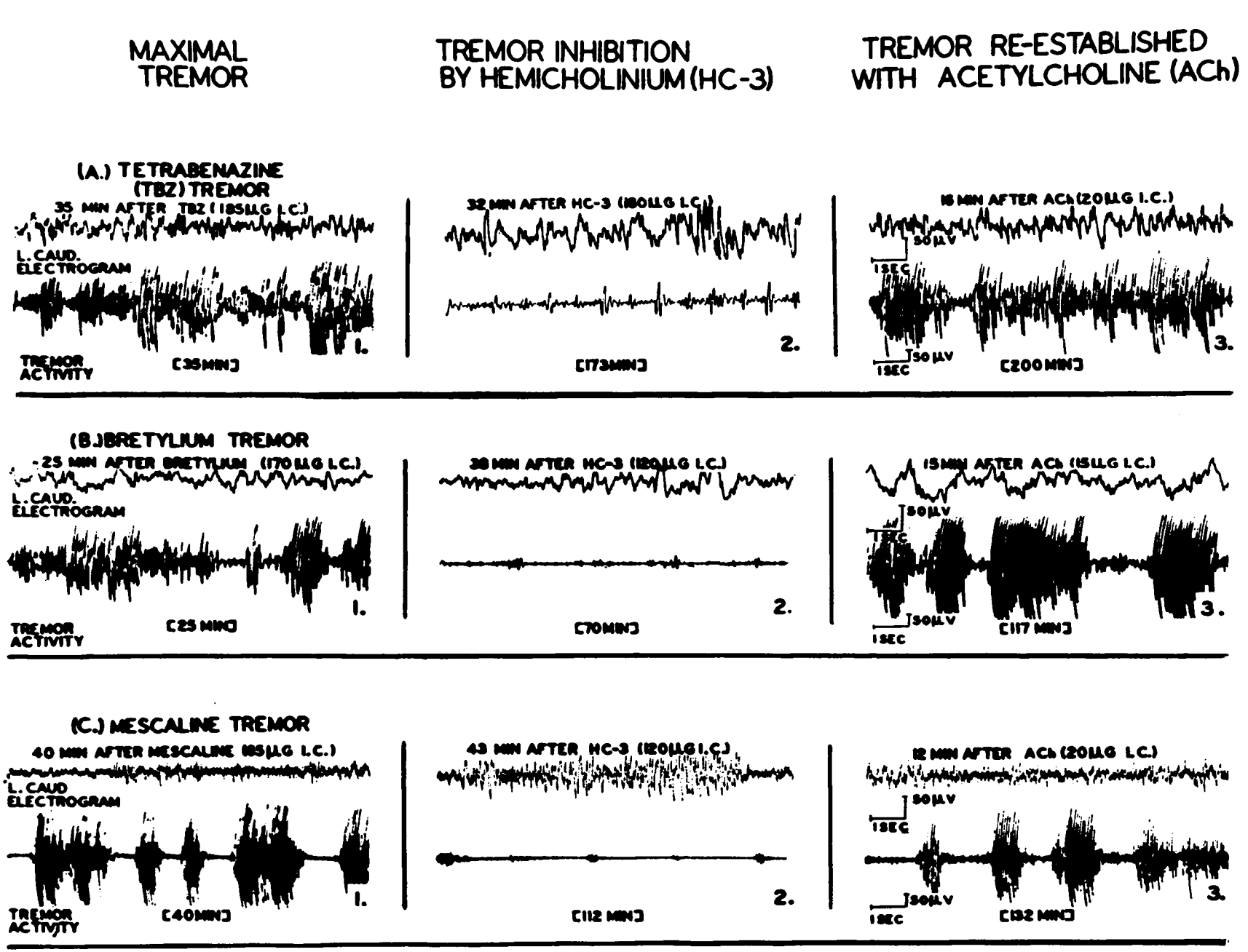

Figure 2—*Inhibition of maximal tetrabenazine (TBZ), bretylium, and mescaline tremors by intracaudate injections of hemicholinium-3 (HC-3). Tremors were reestablished temporarily by intracaudate supplemental injections of acetylcholine (ACh). Doses of acetylcholine represent its chloride salt.*

was directed to the functional status of dopamine (rather than epinephrine) in the caudate. The interpretation that seems most consistent with the present data and previous findings with cholinergic tremor is that interference with local dopamine functioning results in a relative increase (or imbalance) in favor of acetylcholine activity, which then causes tremors. In keeping with this is the proposal that the effects of acetylcholine in the caudate are excitatory and that, under stable conditions, these effects are offset by the local inhibitory action of dopamine (3, 14).

Since levels of dopamine in the caudate were not determined chemically, evidence is lacking to substantiate this interpretation directly. However, a consideration of the neuropharmacology of the three agents may establish a relationship to their ability to impair the functioning of endogenous dopamine. Bretylium, by virtue of its quaternary structure, is limited in its access to the brain; but when administered systemically, bretylium is taken up by tissues having a relatively high concentration of dopamine (15). Thus, it would be reasonable to expect that microinjected bretylium might be localized in dopamine-containing axon terminals of the caudate nucleus and, by analogy with its peripheral antiadrenergic actions (16), that it may elicit tremors by interfering with the release of intracaudate dopamine. Along parallel lines, tetrabenazine, like reserpine, depletes catecholamines (as well as serotonin) in the CNS by blocking ATP-dependent uptake of monoamines into storage granules (17). Of the monoamines in question, a deficiency of serotonin is ruled out as the basis for the tremors in these studies, since supplemental microinjected serotonin intensified tetrabenazine tremor responses whereas dopamine suppressed them. It thus appears that tetrabenazine tremor can be linked more appropriately to a deficiency of dopamine.

Mescaline and other *O*-methylated derivatives of dopamine were reported (18, 19) to produce tremors experimentally. Tremorgenic effects of intracaudate mescaline in cats demonstrated in this study essentially parallel the findings by Little and Dill (19) of dyskinesias after injection of mescaline into the caudate–putamen complex in rats. Dill (14) attributed mescaline dyskinesias in part to blockade of adrenergic inhibitory receptors, which in turn upsets a hypothesized local acetylcholine–dopamine balance. Results of the present studies tend to support this general proposition. Apparently, mescaline also exerts an "intrinsic," nonadrenergic, tremorgenic action since various other adrenergic receptor blocking agents (chlorpromazine and propranolol) neither suppress the dyskinetic excitatory effects of mescaline (14) nor are themselves tremorgenic when injected into the caudate (1). Significantly, on brainstem neurons, microinjected mescaline blocked only the inhibitory actions of the catecholamines without affecting the excitatory ones (20). The higher doses of dopamine required to suppress mescaline tremors, in comparison to doses that suppressed bretylium or tetrabenazine tremors, might be explained by the effective dopamine receptor blocking action of mescaline.

The data also underscore the key role of local acetylcholine in the development and maintenance of tremor activities with all three agents. This is evidenced not only by hemicholinium suppression of tremor and by restoration of this activity with acetylcholine but also by antagonism with scopolamine. However, since neither tetrabenazine nor bretylium nor mescaline had an apparent anticholinesterase action (no potentiation of low doses of microinjected acetylcholine), the involvement of acetylcholine as a necessary condition for these tremors may be attributed to a resulting predominance of endogenous cholinergic mechanisms. This appears to have a bearing on the increased cholinergic hyperactivity secondary to decreased dopamine functioning noted in clinical Parkinsonism (21).

It is concluded that tetrabenazine, bretylium, and mescaline are tremorgenic because they each produce, by somewhat different mechanisms, a functional dopamine deficiency in the caudate. Yet, for the development and maintenance of these tremors, a sustained endogenous acetylcholine activity in the caudate is essential. The data thus add support to the hypothesis that the excitatory effects of acetylcholine and the inhibitory actions of dopamine in the caudate are critically balanced and, as such, represent an important local system for regulating motor activity.

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Use of Thermal Gravimetric Analysis, GLC, and Mass Spectrometry in Sorption Studies III: Evaluation of Clustering Functions of Ethanol–Water–Polyurethane System

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Abstract □ An equilibrium sorption study on the ethanol–water–polyurethane system over an extended ethanol concentration range was conducted by means of thermal gravimetric analysis, GLC, and mass spectrometry. From a single dynamic experiment, the equilibrium solubility in and the activation energy of desorption from the polymer matrix were evaluated simultaneously. The equilibrium sorption behavior of ethanol and water in the polymer matrix was interpreted in terms of the modified Zimm–Lundberg clustering theory applied to the liquid phase. The results indicate that the activation energy for desorption of ethanol decreases to a minimum and then increases with an increasing amount of sorption. The clustering function suggests that there is a greater tendency for ethanol molecules to cluster in the polymer matrix at lower ethanol activity but that there is no tendency for water molecules to cluster together. Water molecules show ideal sorption behavior at high water content and localized sorption on specific polymer sites at lower water content. Below 43% by weight or 0.51 activity, ethanol demonstrates ideal behavior; but at higher ethanol compositions, localized sorption takes place. There is also a great tendency for ethanol and water molecules to cluster together in the polymer matrix. The mean cluster size for ethanol is 1.4–2.5 molecules, while that for ethanol–water is 2.5–3.9 molecules.

Keyphrases □ Ethanol–water–polyurethane system—clustering functions evaluated using thermal gravimetric analysis, GLC, and mass spectrometry □ Polyurethane–water–ethanol system—clustering functions evaluated using thermal gravimetric analysis, GLC, and mass spectrometry □ Polymer–solute (solvent) interactions—clustering functions of ethanol–water–polyurethane system □ Clustering of penetrant molecules in polymer matrix—studied using thermal gravimetric analysis, GLC, and mass spectrometry, Zimm–Lundberg theory □ Sorption of solvent molecules in polymer matrix—clustering functions of ethanol–water–polyurethane system □ Thermal gravimetric analysis—determination of clustering functions for ethanol–water–polyurethane system □ GLC—determination of clustering functions for ethanol–water–polyurethane system □ Mass spectrometry—determination of clustering functions for ethanol–water–polyurethane system

Previous studies in these laboratories demonstrated the usefulness of thermal gravimetric analyses in sorption studies of selected liquid compounds with nylon (1) and with the diffusion and solubility of a series of aliphatic alcohols in polyurethane (2). This report continues these studies, exploring the clustering effects of sorbed molecules in a specific polyurethane using thermogravimetry, GLC, and mass spectrometry. Since polyurethanes have a wide range of applications in medicine, pharmacy, and dentistry, the information presented here should be of fundamental value in understanding aspects of solute (or solvent)–polymer interactions.

The tendency for penetrant molecules or solvent molecules to cluster in the polymer matrix can be interpreted by the Zimm–Lundberg clustering theory. Zimm (3) and Zimm and Lundberg (4) modified the formulas for the theory of solutions proposed by Kirkwood and Buff (5) and applied them to solvent vapor–high polymer systems.

The Zimm–Lundberg clustering theory was developed on the basis of statistical mechanical considerations employing molecular distribution functions. This theory has one advantage over others in that it gives a direct measurement of nonrandom mixing of penetrant molecules in the polymer phase without the use of any preconceived model. It thus shows how the sorption process changes with increasing solvent content from one of sorption on a few highly specific sites to a diffuse swelling phenomenon. It has been found useful and applicable to the treatment of vapor phase sorption data of solvent–high polymer systems (4, 6–9) in molecular terms at isothermal conditions. However, the present report is concerned with equilibrium sorption measurements of the ethanol–water–polyurethane system in the liquid phase as a function of widely varying concentrations or activities of ethanol at 25 ± 0.1°. The possible mechanism of sorption behavior of penetrant molecules in the polymer matrix is also discussed.

The amount of equilibrium sorption of penetrant molecules (included are ethanol and water molecules) in the polyurethane film was measured by thermal gravimetric analysis. From a single dynamic (nonisothermal) thermal gravimetric analysis experiment, the solubility (equilibrium sorption value) of penetrant molecules in and the activation energy for desorption of penetrant molecules from the polyurethane film were determined simultaneously at a specific ethanol activity. The amount of sorption of individual water molecules was obtained from thermal gravimetric analysis sorption data by assuming a constant amount of water sorbed in the polyurethane film independent of the amount of ethanol sorbed in the film. Mass spectrometry was also used to estimate the ethanol–water composition of the sorbed material. The activity of ethanol in the vapor phase at equilibrium with the ethanol–water–polyurethane system was measured by GLC. Then the clustering functions for penetrant molecules in the polyurethane film at each different ethanol activity were evaluated from the activity *versus* volume fraction sorption isotherms of the ethanol–water–polyurethane system.

THEORETICAL

Mathematical Derivatives of Clustering Functions—The original equation of Kirkwood and Buff (Eq. 19 of *Reference 5*) for a two-component system is:

$$G_{11}C_1 = kT\left(\frac{\partial C_1}{\partial P}\right)_{T,\mu_2} - 1 \qquad \text{(Eq. 1)}$$

Table I—Physical Properties of Ethanol and Water at 25°

Compound	Formula Weight	Density[a], g./ml.	Molal[b] Volume, ml./mole	Boiling[a] Point	Dipole[c] Moment, debyes	Heat[d] of Vaporization, kcal./mole	Energy[e] of Hydrogen Bonding, kcal./mole	Activation Energy[f] of Desorption, kcal./mole
Ethanol	46.07	0.78505	58.37	78.40°	1.73	9.40	5.64	8.50
Water	18.00	0.99704	18.05	100.00°	1.82	9.71	9.00	9.83

[a] Values obtained from "Handbook of Chemistry and Physics," 44th ed., The Chemical Rubber Publishing Co., Cleveland, Ohio, 1963. [b] Values calculated from the relationships $d = M/V$ and $V = M/d$. [c] Values obtained from "Tables of Experimental Dipole Moments," A. L. McClellan, Ed., W. H. Freeman, San Francisco, Calif., 1963. [d] Values from "Lange's Handbook of Chemistry," 8th ed., McGraw-Hill, New York, N. Y., 1956. [e] Values from L. Pauling, "The Nature of the Chemical Bond," 2nd and 3rd eds., Cornell, Ithaca, N. Y., 1940 and 1960. [f] Activation energy of desorption of solvent or solute from the polyurethane film determined by thermogravimetry.

Table II—Physical Properties of Dry Polyurethane

Sample Size and Shape		Initial Dry Film Weight Range, mg.	Average Weight, mg.	Density, g./ml.	Temperature of Initial Thermal Degradation	Activation Energy of Thermal Degradation, kcal./mole
Diameter, cm.	Thickness, cm.					
0.640	0.0770–0.0778	25.5–25.7	25.5	1.1274	204.56°	17.8
		25.8–26.0	25.9	1.1329		
		26.7–26.9	26.9	1.1319		
		27.0–27.2	27.1	1.1246		
				1.1292 (av.)		

where G_{11} and C_1 are the cluster integral and molecular concentration for type 1 molecules, respectively, k is the Boltzmann constant, T is the absolute temperature, P is the vapor pressure, and μ_2 is the chemical potential for component 2.

On the basis of some thermodynamic properties and relationships and by further mathematical modifications, Zimm (3) transformed Eq. 1 into:

$$\frac{G_{11}}{V_1} = -\phi_2\left[\frac{\partial(a_1/\phi_1)}{\partial a_1}\right]_{P,T} - 1 \qquad \text{(Eq. 2)}$$

or:

$$\frac{G_{11}}{V_1} = -(1-\phi_1)\left[\frac{\partial(a_1/\phi_1)}{\partial a_1}\right]_{P,T} - 1 \qquad \text{(Eq. 3)}$$

where a_1, ϕ_1, and V_1 are the activity, volume fraction, and partial molecular volume of type 1 molecules, respectively, and ϕ_2 is the volume fraction of component 2 (polymer material); G_{11}/V_1 is called the clustering function. It denotes a tendency for type 1 molecules to cluster in the polymer matrix. Three physical meanings may be considered:

Case I—If values of $G_{11}/V_1 > -1$, there will be a tendency for type 1 molecules to cluster together. In other words, the concentration of type 1 molecules is higher than average in the neighborhood of a given type 1 molecule. This is due to the fact that the first type 1 molecule enters the polymer structure, loosens the structure, and makes it easier for subsequent molecules to enter in the neighborhood of the first one than to go elsewhere.

Case II—If values of $G_{11}/V_1 = -1$, this shows ideal solution behavior. A particular type 1 molecule in such a system excludes its own volume to the other molecules but otherwise does not affect the distribution.

Case III—If values of $G_{11}/V_1 < -1$, this is interpreted in terms of localized sorption of type 1 molecules on specific sites.

Equation 3 is very useful, since the right-hand side of the equation is in terms of experimentally measurable quantities, activity (a_1), and volume fraction (ϕ_1) of type 1 molecules.

The quantity $\phi_1 G_{11}/V_1$ is a measure of the mean number of type 1 molecules in the neighborhood of a given type 1 molecule in excess of the mean concentration of type 1 molecules. The mean size of a cluster is given by $(\phi_1 G_{11}/V_1 + 1)$. This cluster size represents an overall average of the penetrant species (type 1 molecules).

Thermodynamic Activity of Type 1 Molecules—Thermodynamically, it is always true that at equilibrium at constant temperature and pressure the chemical potential of component 1 in the liquid phase, $\mu_1(L)$, is equal to the chemical potential of the same component in the vapor phase, $\mu_1(V)$. That is:

$$\mu_1(L) = \mu_1(V) \qquad \text{(Eq. 4)}$$

However, at pressures of a few atmospheres or less, the vapor phase obeys the ideal gas law, so that the chemical potential of component 1 in the vapor phase can be represented by Eq. 5:

$$\mu_1(V) = \mu_1^\circ + RT \ln P_1 \qquad \text{(Eq. 5)}$$

where μ_1° is the chemical potential of pure component 1 at the standard state of pure gas at $P = 1$ atm. and temperature T; R is the universal gas constant.

If component 1 in the liquid phase obeys Raoult's law, it is true that:

$$P_1 = P_1^\circ N_1 \qquad \text{(Eq. 6)}$$

and Eq. 5 can be rewritten as:

$$\mu_1(V) = \mu_1^\circ + RT \ln N_1 \qquad \text{(Eq. 7)}$$

where N_1 is the mole fraction of component 1 in the liquid phase, and μ_1° is the chemical potential of pure component 1 at the standard state of pure liquid at $N_1 = 1$ and temperature T.

If component 1 in the liquid phase deviates from Raoult's law, then:

$$P_1 \neq P_1^\circ N_1 \qquad \text{(Eq. 8}a\text{)}$$

but:

$$P_1 = P_1^\circ a_1 \qquad \text{(Eq. 8}b\text{)}$$

Therefore, Eq. 5 may be rewritten as:

$$\mu_1(V) = \mu_1^\circ + RT \ln a_1 \qquad \text{(Eq. 9)}$$

where a_1 is the activity of component 1 (that is, type 1 molecules) in the vapor phase in terms of the relative pressure, and μ_1° now represents the chemical potential at the standard state of pure liquid at $a_1 = 1$ or $N_1 = 1$ and temperature T. In this study, pure liquid ethanol at the mole fraction $N_E = 1$ or $a_E = 1$ and pure liquid water at the mole fraction $N_W = 1$ or $a_W = 1$ were taken as the standard states for type 1 molecules at a temperature of 25.0°.

Therefore, the activity of type 1 molecules in the liquid phase may be evaluated from measurements of the relative pressure, P_1/P_1°; these data, in turn, can be used in Eq. 3 to calculate the clustering functions.

Table III—Sorption Data and Activation Energy of Desorption for Ethanol–Water–Polyurethane System at 25 ± 0.1°

Initial Concentration of Aqueous —Ethanol Solutions— wt. %	moles/l.	Total Amount of Sorption, g. Solute/g. Film	Activation Energy of Desorption, kcal./mole
0.	0.	0.0190	9.83 (pure water)
0.78	0.170	0.0219	9.13
1.57	0.341	0.0232	8.69
3.16	0.682	0.0262	8.64
4.76	1.02	0.0296	8.49
6.36	1.36	0.0318	7.87
7.98	1.70	0.0377	7.12
12.9	2.73	0.0471	7.32
16.2	3.41	0.0551	7.57
33.2	6.92	0.1170	7.27
52.1	10.2	0.1757	8.41
73.4	13.6	0.2574	8.43
100.0	17.0	0.2702	8.50 (pure ethanol)

EXPERIMENTAL

Materials—The solvent was reagent grade absolute ethanol, obtained commercially, and it was used without further purification. Doubly distilled water was used in all equilibrium sorption measurements. Table I summarizes some physical properties of ethanol and water.

The polyurethane was obtained[1] as a thin film. It is a thermoset solid polyurethane prepared by reacting a polyurethane prepolymer with 4,4′-methylenebis(2-chloroaniline). Its general properties are those of an elastomer (Table II).

Preparation of Samples—Test samples were punched from the polyurethane film with a paper punch, No. 1, 0.63-cm. diameter. Cylindrical-shaped samples were cut of 0.62-cm. diameter, 0.0770–0.0778-cm. thickness, and 25.5–27.2-mg. weight. All test samples for each different ethanol concentration or activity were as uniform as possible, and variation never exceeded 0.5% (thickness or weight) from sample to sample. The test samples were soaked in 95% ethanol for 48 hr. and then rinsed repeatedly with distilled water. An additional soaking in 50% ethanol for 24 hr., with subsequent rinsing with distilled water, was performed. Finally, the test samples were rinsed with acetone, dried to constant weight, and stored in a desiccator until ready for use.

Thirteen different aqueous ethanol solutions were prepared volumetrically. The concentrations of these solutions varied from 0 to 100% ethanol by volume. Eight pieces of approximately the same initial dry weight of polyurethane film were placed in an all-glass sorption tube containing 20.0 ml. of ethanol solution. The tubes were then stoppered (screw cap with Teflon-faced rubber liner) and immersed in a thermally controlled water bath adjusted to 25.0 ± 0.1°.

Preliminary measurements indicated that the equilibrium sorption occurred within several weeks. For uniformity, the sorption tubes with samples were kept in the water bath for exactly 30 days. After this time, the plastic sample was removed from the test ethanol solution and excess liquid was blotted with tissue paper. Immediately (within 70 sec.) the plastic sample was introduced into the thermal gravimetric analysis instrument, the sample weight was recorded, and a thermogram was run.

Thermal Gravimetric Analysis for Desorption Studies—Descriptions of the thermal gravimetric analysis instrument and the method of running a desorption experiment were presented previously (2); in the present study the desorption runs were made in a dynamic manner by increasing the temperature at a constant rate.

A stream of nitrogen was passed through concentrated sulfuric acid and then into the chamber containing the test sample, at a constant flow rate of 200 ml./min., to flush and remove any adhering gases or decomposed gaseous products evolved during the desorption experiment. The nitrogen gas, in turn, was led out of the chamber into a series of gas-washing bottles. After the sorbed plastic sample was placed in the thermal gravimetric analysis system, the instrument run was initiated and the weight loss *versus* temperature (and time) was recorded on chart paper until a constant weight was obtained. This thermogram was used for evaluation of the solubility of solvent molecules in and the activation energy for desorption of solvent molecules from the polymer film. The solubility was determined from the difference between the initial reading (equilibrium sorption value) and the constant weight reading on the thermogram after complete desorption. The activation energy for desorption was evaluated from the corresponding thermogram by employing Broido's method (1, 10). To ensure reproducibility, at least two test samples for each different group of ethanol solutions were run. The relative precision of the calculated solubility for each group of test samples was 2.5% or less. The reproducibility for activation energy of desorption was about 1.0–3.0%.

[1] Code No. MP950, Molded Products Co., Easthampton, Mass.

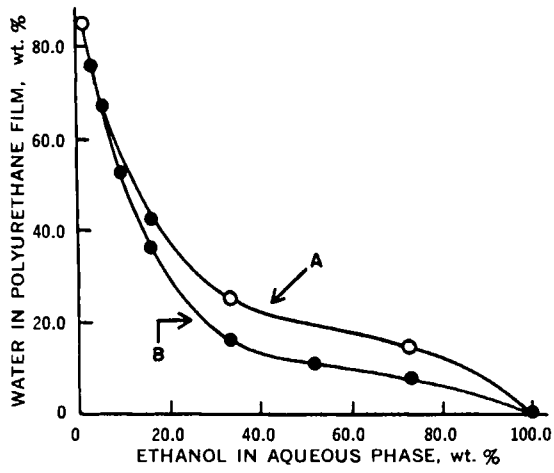

Figure 1—*Weight percent water in polyurethane film* versus *weight percent ethanol in aqueous phase at 25°. Key: A, mass spectrometry; and B, thermal gravimetric analysis.*

Determination of Ethanol Activity in Vapor Phase—The activity of ethanol, a_1, in the vapor phase above various concentrations of ethanol solutions can be determined in terms of relative pressure, P_1/P_1°, by GLC. Furthermore, at equilibrium at constant temperature and pressure, the following relations are true:

$$a_1 = \frac{P_1}{P_1^\circ} = \frac{\text{mole}_1}{\text{mole}_1^\circ} = \frac{(\text{mass/mol. wt.})_1}{(\text{mass/mol. wt.})_1^\circ} = \frac{\text{mass}_1}{\text{mass}_1^\circ} \quad \text{(Eq. 10)}$$

However, the area of the peak in GLC with a flame-ionization detector (FID) is:

$$\text{area}_1 = K_{\text{FID}}\text{mass}_1 \quad \text{(Eq. 11)}$$

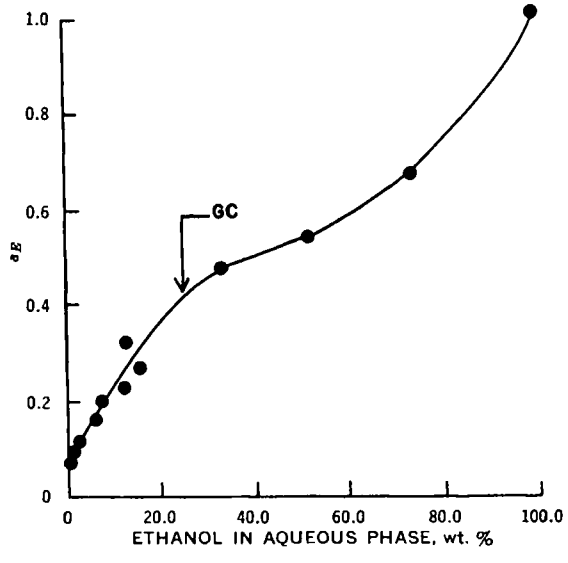

Figure 2—*Ethanol activity in vapor phase as a function of aqueous phase ethanol composition at 25°.*

Table IV—Volume Fraction Data for Ethanol–Water–Polyurethane System at 25 ± 0.1°

Initial Concentration of Aqueous —Ethanol Solutions— wt. %	moles/l.	ϕ_E	ϕ_W	ϕ_P
3.16	0.682	0.0066	0.0224	0.971
16.2	3.41	0.0468	0.0212	0.932
33.2	6.92	0.123	0.0190	0.858
52.1	10.2	0.192	0.0180	0.790
73.4	13.6	0.241	0.0160	0.743
100.0	17.0	0.271	0.0000	0.729

where K_{FID} is a proportionality constant for type 1 molecules.

Equation 11 shows that for a detector responding to the mass flow rate, the peak area is proportional to the total mass of the eluted component (11).

Similarly, for a pure component of type 1 molecules:

$$\text{area}_1^\circ = K_{FID}\text{mass}_1^\circ \qquad \text{(Eq. 12)}$$

Dividing Eq. 11 by Eq. 12, one gets:

$$\frac{\text{area}_1}{\text{area}_1^\circ} = \frac{K_{FID}\text{mass}_1}{K_{FID}\text{mass}_1^\circ} = \frac{\text{mass}_1}{\text{mass}_1^\circ} \qquad \text{(Eq. 13)}$$

Comparison of Eqs. 10 and 13 gives:

$$a_1 = \frac{P_1}{P_1^\circ} = \frac{\text{area}_1}{\text{area}_1^\circ} \qquad \text{(Eq. 14)}$$

Therefore, by simply measuring GLC peak areas for type 1 molecules in the vapor phase and then dividing by the peak area of pure type 1 molecules in the vapor phase, the activities of type 1 molecules were determined.

Determination of Volume Fraction of Type 1 Molecules in Polymer Film—Experimentally, the volume fraction, ϕ_1, for type 1 molecules can be evaluated from the following:

$$\phi_1 = \frac{V_1}{V_1 + V_p} = \frac{V_1}{V_{\text{total}}} \qquad \text{(Eq. 15)}$$

But:

$$V_1 = \frac{\text{wt.}_1}{D_1} \qquad \text{(Eq. 16a)}$$

$$V_p = \frac{\text{wt.}_p}{D_p} \qquad \text{(Eq. 16b)}$$

$$\phi_1 + \phi_p = 1 \qquad \text{(Eq. 16c)}$$

where V_1, wt.$_1$, and D_1 are equilibrium sorption volume, equilibrium sorption weight, and density, respectively, of type 1 molecules at the same temperature; V_p, wt.$_p$, and D_p are volume, weight, and density of the polymer film in that order; and ϕ_1 and ϕ_p are volume fractions of type 1 molecules and the polymer film, respectively.

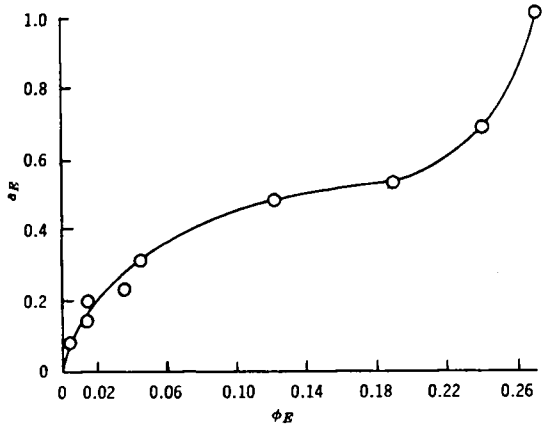

Figure 3—*Ethanol activity as a function of volume fraction of sorbed ethanol in polyurethane film at 25°.*

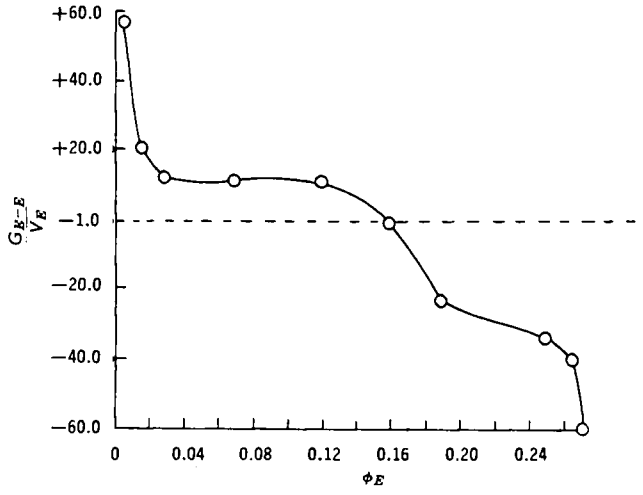

Figure 4—*Clustering function for ethanol in ethanol–water–polyurethane system at 25°.*

The equilibrium sorption weight of type 1 molecules was determined by thermal gravimetric analysis, and the density of type 1 molecules in the liquid state at the same temperature was obtained from the literature. The density of the polymer film was measured by the water or solvent displacement method with a pycnometer. Finally, ϕ_1 was calculated from the measured V_1 and V_p. By knowing ϕ_1, ϕ_p can be computed from Eq. 16c.

RESULTS AND DISCUSSION

Table III summarizes the sorption data and activation energy of desorption for the ethanol–water–polyurethane system at 25°. The total amount of sorption increases with increasing concentration of ethanol. The amount of sorption of pure water is about 7.0% of the amount of sorption of pure ethanol. The activation energy of desorption of solvent molecules from the polymer film decreases with an increasing amount of total sorption to a minimum and then increases again. This suggests a possible change in the sorption mechanism. The activation energy of desorption of pure water from the polymer film is a little higher than that of pure ethanol.

Figure 1 shows the plot of weight percent of water in the polyurethane film *versus* weight percent of ethanol in the liquid phase. The weight percent of water in the polyurethane film was differentiated from the total amount of sorbed solvent molecules in the film by mass spectrometry (open circles in Fig. 1). The other curve of Fig. 1 (black circles) was obtained from thermal gravimetric analysis sorption data by assuming a constant amount of water

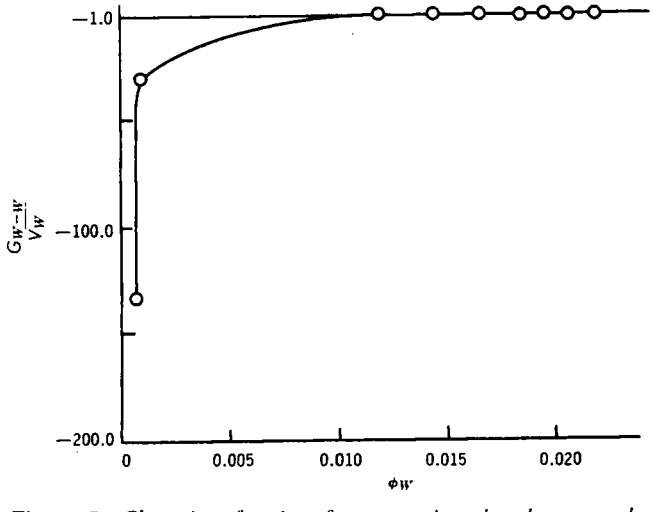

Figure 5—*Clustering function for water in ethanol–water–polyurethane system at 25°.*

Table V—Clustering Functions for Ethanol in Ethanol–Water–Polyurethane System at 25 ± 0.1°

Initial Concentration of Aqueous Ethanol Solutions						
wt. %	moles/l.	a_E	ϕ_E	$\frac{G_{E-E}}{V_E}$	$\frac{\phi_E G_{E-E}}{V_E}$	$1+\frac{\phi_E G_{E-E}}{V_E}$
3.16	0.682	0.13	0.0066	+57.3	0.4	1.4
16.2	3.41	0.31	0.0468	+13.5	0.7	1.7
33.2	6.92	0.48	0.123	+12.3	1.7	2.5
43.4	8.71	0.51	0.163	−1.0	0.2	0.8
52.1	10.2	0.53	0.192	−23.0	—	—
73.4	13.6	0.68	0.241	−23.0	—	—
100.0	17.0	1.00	0.271	−60.0	—	—

Table VI—Clustering Functions for Water in Ethanol–Water–Polyurethane System at 25 ± 0.1°

Concentration of Aqueous Ethanol Solutions						
wt. %	moles/l.	a_W	ϕ_W	$\frac{G_{W-W}}{V_W}$	$\frac{\phi_W G_{W-W}}{V_W}$	$1+\frac{\phi_W G_{W-W}}{V_W}$
3.16	0.682	0.96	0.0224	−1.0	0.0	1.0
16.2	3.41	0.89	0.0212	−1.0	0.0	1.0
33.2	6.92	0.79	0.0190	−1.0	0.0	1.0
43.4	8.71	0.78	0.0189	−1.0	0.0	1.0
52.1	10.2	0.75	0.0180	−1.0	0.0	1.0
73.4	13.6	0.62	0.0160	−1.0	0.0	1.0
93.4	16.3	0.30	0.0099	−30.1	—	—
96.4	16.6	0.18	0.0074	−133.0	—	—

sorbed in the polyurethane film independent of the amount of ethanol sorbed in the film. It is obvious that the two curves are very close. At higher concentrations of ethanol, the amount of sorption of water estimated by mass spectrometry is higher than that determined by thermal gravimetric analysis. Possibly, ethanol molecules may enhance the sorption of water molecules in the polymer film by breaking the hydrogen bonds in liquid water clusters and carrying more free water molecules into the polymer matrix.

Figure 2 shows the plot of ethanol activity as a function of weight percent of ethanol in the aqueous phase for the ethanol–water–polyurethane system. The activity of ethanol is directly proportional to the concentration of ethanol in the liquid phase. However, the activity of water, although not shown here, was computed from the partial vapor pressure of water in the ethanol–water binary system at 25.0° obtained from the literature (12).

Table IV lists some volume fraction data for the ethanol–water–polyurethane system at 25.0°. In general, the volume fraction of ethanol in the polyurethane film, ϕ_E, increases with an increasing concentration of ethanol in solution. The volume fraction of the polymer film, ϕ_P, shows opposite behavior, while ϕ_W for water does not vary significantly.

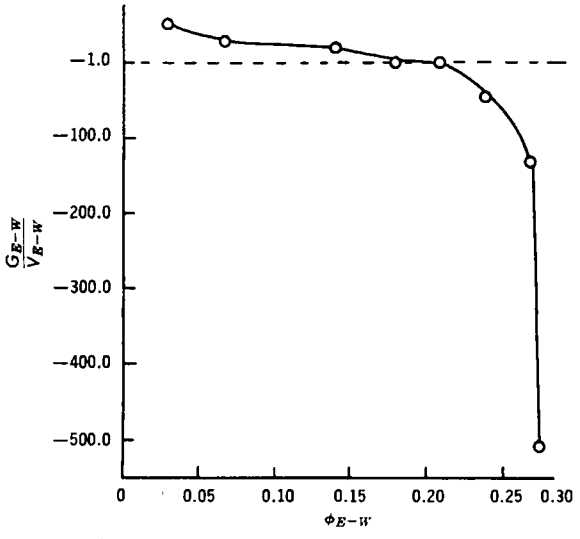

Figure 6—*Clustering function for ethanol and water mixtures in ethanol–water–polyurethane system at 25°.*

A typical plot of ethanol activity, a_E, *versus* volume fraction of ethanol, ϕ_E, is shown in Fig. 3. From this sorption isotherm, the clustering functions for ethanol molecules in the polyurethane film were evaluated. Similar treatments were performed for the evaluation of clustering functions of water molecules and of ethanol–water molecules in the polymer matrix.

The clustering function for ethanol molecules in the polyurethane film is plotted in Fig. 4 (numerical values are listed in Table V). It is obvious that there is a greater tendency for ethanol molecules (compared to water molecules) to cluster together at the smaller volume fractions of sorption (or at the lower concentrations or activities of ethanol, as can be seen from Table V), then pass through ideal behavior at a volume fraction of 0.16 (or activity of 0.51), and finally demonstrate localized sorption on specific polyurethane segment sites. The mean number of clustering is from 0.4 to 1.5, whereas the mean cluster size is about 1.4–2.5 molecules.

Figure 5 shows the clustering function for water molecules. Water molecules would not cluster together in this particular system. As can also be seen from Table VI, water molecules show a wide range of ideal sorption behavior from a low to a high ethanol concentration of 73.4% by weight and then show a localized sorption phenomenon.

By assuming that ethanol and water molecules may also cluster together in this particular system, the clustering functions for ethanol–water molecules were evaluated (Fig. 6 and Table VII). However, the activity of ethanol–water molecules, a_{E-W}, was taken from the square root of the product of the activities of ethanol and water molecules, and additive properties of the volume fractions were assumed. In general, the clustering phenomena for ethanol–water molecules are very similar to those of ethanol molecules themselves, except that the ideal behavior of ethanol–water molecules shifts from 43.4 to 52.1% by weight. The mean cluster number ranges from 1.5 to 2.9, and the mean cluster size ranges from 2.5 to 3.9 molecules.

In summary, the Zimm–Lundberg clustering theory was applied to the treatment of sorption data of the ethanol–water–polyurethane system in the liquid phase at 25 ± 0.1°. The results indicate that there is a greater tendency for ethanol molecules to cluster together than for water molecules. Water molecules in this particular system do not appear to cluster together, even though the evaluation of clustering functions for water molecules is only an approximation approach. The tendency for ethanol–water molecules to cluster together is high.

The mechanism or sorption behavior of ethanol and water molecules in the polyurethane film may be interpreted as follows. At the lower ethanol concentration or activity, the first ethanol or water

Table VII—Clustering Functions for Ethanol–Water in Ethanol–Water–Polyurethane System at 25 ± 0.1°

Concentration of Aqueous Ethanol Solutions, wt. %	Concentration of Aqueous Ethanol Solutions, moles/l.	a_{E-W}	ϕ_{E-W}	$\frac{G_{E-W}}{V_{E-W}}$	$\frac{\phi_{E-W}G_{E-W}}{V_{E-W}}$	$1 + \frac{\phi_{E-W}G_{E-W}}{V_{E-W}}$
3.16	0.682	0.354	0.0287	+51.9	1.5	2.5
16.2	3.41	0.525	0.0676	+30.0	2.0	3.0
33.2	6.92	0.616	0.142	+20.5	2.9	3.9
43.4	8.71	0.631	0.182	−1.0	−0.2	0.8
52.1	10.2	0.631	0.210	−1.0	−0.2	0.8
62.7	12.1	0.647	0.238	−46.7	—	—
85.2	15.2	0.566	0.270	−132.4	—	—
89.3	15.7	0.505	0.273	−509.9	—	—

molecule enters the polyurethane chain structure, loosens the structure, and makes it easier for subsequent ethanol or water molecules to enter in the neighborhood of the first one than to go elsewhere; therefore, they cluster together. As the concentration or activity of ethanol increases, the amount of sorption increases. However, because the polyurethane film cannot swell enough for accommodation of excess penetrant molecules, ethanol and water molecules diffuse out from the initial position and become a homogeneously localized sorption phenomenon under the driving forces of concentration or chemical potential gradient. The binding forces for the ethanol–water–polyurethane system are through hydrogen-bond formation between ethanol and/or water molecules and the polar groups (such as urethane groups) of polyurethane segments. However, the hydrophobic properties of ethanol molecules play an important role in the interpretation of stronger affinity for ethanol molecules toward the polyurethane chain structure. The formation of water clusters (13) between liquid water molecules in the liquid phase gives a consistent explanation for the lower content of water sorbed in the polyurethane film.

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Acceleration of Peritoneal Dialysis with Minimal *N*-Myristyl-β-aminopropionate

EMAD A. EL-BASSIOUNI* and ALBERT M. MATTOCKS▲

Abstract □ A single short-term exposure of the peritoneal membrane to *N*-myristyl-β-aminopropionic acid solution (0.25%) gave an accelerating effect on the peritoneal clearance of salicylate, barbiturate, and diphenylhydantoin, which lasted throughout several subsequent exchanges with standard dialysis fluid. A brief kinetic study on *N*-myristyl-β-aminopropionate was done, and its intraperitoneal absorption and urinary and peritoneal clearances are reported. The LD_{50} of this wetting agent in mice and its hemolytic threshold were also estimated.

Keyphrases □ Peritoneal dialysis of salicylate, barbiturates, and diphenylhydantoin—acceleration with *N*-myristyl-β-aminopropionate □ Salicylate, peritoneal clearance—acceleration with *N*-myristyl-β-aminopropionate □ Barbiturates, peritoneal clearance—acceleration with *N*-myristyl-β-aminopropionate □ Diphenylhydantoin, peritoneal clearance—acceleration with *N*-myristyl-β-aminopropionate □ *N*-Myristyl-β-aminopropionate—intraperitoneal absorption, urinary and peritoneal clearances, used to accelerate peritoneal dialysis of salicylate, barbiturates, and diphenylhydantoin

Previous work in this laboratory (1, 2) showed that the wetting agent *N*-myristyl-β-aminopropionic acid[1], when added to a standard dialysis fluid, accelerated the peritoneal removal of barbiturates, diphenylhydantoin, and salicylate. Since absorption of *N*-myristyl-β-aminopropionate from the peritoneum might occur during its use in dialysis and a hazard of added toxicity from this chemical might arise, an effort was made to attain its beneficial effects with minimum exposure. A priming lavage technique, devised in this laboratory and proven successful in accelerating peritoneal removal of urea (3), was used.

EXPERIMENTAL

Materials—Radioactive drugs were used to facilitate measurement of drug concentration. ^{14}C-Diphenylhydantoin was synthesized and purified by a published method (4). Solutions for injection were freshly prepared to contain 10 mg. diphenylhydantoin as the sodium salt and 1 μc./ml. and were injected at the level of 10 mg./kg. ^{14}C-Phenobarbital was obtained commercially[2], and the injection was prepared to contain 50 mg. phenobarbital as the sodium salt and 0.1 μc./ml. It was injected at the level of 50 mg./kg. ^{14}C-Pentobarbital was obtained commercially[2], and the injection contained 18 mg. pentobarbital as the sodium salt and 4 μc./ml. The pentobarbital dose was 18 mg./kg. ^{14}C-Amobarbital and ^{14}C-butabarbital were synthesized from ^{14}C-urea and the appropriate malonic esters (5). Their identity and purity were checked by IR spectrometry and paper chromatography, and their injections were prepared and used in the same manner as pentobarbital. ^{14}C-Salicylic acid was obtained commercially[2], and the injection contained 50 mg. salicylic acid as the sodium salt and 1 μc./ml. It was injected at the level of 100 mg./kg.

The control dialysis fluid was a typical formula with 377 milliosmoles/l. It contained dextrose (1.5%), sodium lactate (0.5%), sodium chloride (0.56%), calcium chloride (0.025%), and magnesium chloride hexahydrate (0.015%). *N*-Myristyl-β-aminopropionic acid dialysis fluid was prepared simply by dissolving 0.25% of the commercial solution in the control fluid. The only exception was in phenobarbital experiments where the concentration of the surfactant was increased to 0.5%. This change came as a result of preliminary work in which weaker solutions were used and the clearances obtained showed high variations.

Intermittent Dialysis—Healthy, mature, male albino rabbits were used as the experimental animals. They were administered ^{14}C-tagged drug intravenously *via* the marginal ear vein, and 1 hr. was allowed for drug distribution in the body before beginning dialysis. At that time a blood sample was taken and 60 ml./kg. of dialysis fluid was rapidly injected intraperitoneally *via* a pediatric-size catheter. At the end of the prescribed dwell time, the dialysate was drained by gravity, over 10 min., through the same catheter into a graduated cylinder. At the end of drainage, another blood sample was taken and the next exchange of fluid was rapidly injected. The volume of return dialysate was measured for each exchange, it was mixed thoroughly, and a sample was taken for measurement of drug concentration.

Preliminary work showed that a single short-term exposure of the peritoneum to a 0.25% *N*-myristyl-β-aminopropionate solution gave an accelerated dialysis effect which lasted throughout several subsequent exchanges with ordinary control fluid. A similar short-term exposure to control fluid had no significant effect on subsequent exchanges. On this basis, the test animals were given a priming lavage with *N*-myristyl-β-aminopropionate fluid, in which the dialysis fluid was allowed to remain in the peritoneum for 5 min. and then was drained for 10 min., giving a 15-min. first cycle. Then five 30-min. exchanges were run with control fluid, each consisting of a 20-min. dwell period and a 10-min. drainage. Control tests consisted of the same procedure except for elimination of the 15-min. exposure to *N*-myristyl-β-aminopropionate fluid.

Measurement of Drug Concentration—For salicylate and phenobarbital, since the amount of drug metabolized in the 3-hr. experiment is negligible (1, 2), 0.5-ml. samples of plasma and dialysate were added directly to the counting fluid [0.01% 1,4-bis-2-(phenyloxazolyl)benzene and 0.4% 2,5-diphenyloxazole in a solvent of 33% nonionic surfactant[3] and 67% toluene]. Samples thus prepared were counted in a liquid scintillation spectrometer[4] using the channels ratio method for quench correction.

A new procedure was developed for pentobarbital assay in which a sample of 1.0 ml. plasma or 2.0 ml. dialysate was transferred to a centrifuge tube, acidified with 0.4 ml. of 0.5 *N* HCl, and extracted four times with 3-ml. portions of ether. The ether extracts, containing all the unchanged pentobarbital and small amounts of metabolites, were evaporated to dryness. The residue was dissolved in about 0.05 ml. of ether and quantitatively spotted on a 20 × 20-cm. sheet of Whatman No. 2 chromatography paper. The chromatogram was developed in a chromatography chamber[5] with *n*-amyl alcohol–concentrated ammonium hydroxide (90:10 v/v). This system gave average R_f values of 0.90 for pentobarbital and 0.10 for metabolites. After drying, the chromatograms were viewed under UV light to locate the pentobarbital spots. The spots were cut out, placed in counting vials containing 10 ml. scintillation cocktail, shaken well, and counted. Application of this method to samples of known concentrations gave recoveries of 97% or better.

[1] Deriphat 170-C, a mixture of 70% *N*-myristyl- and 30% *N*-lauryl-β-aminopropionic acids as their sodium salts, marketed in 50% solution by General Mills, Kankakee, Ill. Percentages used in this work are of 50% solution as supplied unless otherwise indicated.

[2] Tracerlab, Waltham, Mass.

[3] Triton X-100, Rohm & Haas, Philadelphia, Pa.

[4] Packard 3320 scintillation spectrometer, Packard Instrument Co., LaGrange, Ill.

[5] Eastman.

Table I—Effect of 15-min. Lavage with *N*-Myristyl-β-aminopropionic Acid on Peritoneal Clearances

Drug	Clearance[a], ml./min. Without Lavage	With Lavage	Drug	Clearance, ml./min. Without Lavage	With Lavage
Pentobarbital	0.94[b]	1.65	Amobarbital	0.70[b]	2.31
	0.77[b]	1.60		0.48[b]	1.67
	1.07[b]	2.17		1.06[b]	1.26
	0.98[b]	2.08		1.25[b]	Av. 1.75 ± 0.31
	1.16[b]	2.19		1.05[b]	
	1.09[b]	1.91		Av. 0.91 ± 0.14	
	1.10	Av. 1.93 ± 0.11	Salicylate	0.36[d]	0.84
	Av.[c] 1.02 ± 0.05			0.43[d]	1.01
Phenobarbital	0.86[b]	1.25		0.37[d]	1.05
	1.37[b]	2.05		Av. 0.39 ± 0.02	1.15
	1.06[b]	1.76			1.00
	0.93[b]	1.60			Av. 1.01 ± 0.05
	1.12[b]	Av. 1.67 ± 0.17	Diphenylhydantoin	0.33	1.00
	1.01[b]			0.34	0.70
	Av. 1.06 ± 0.07			0.47[b]	0.68
Butabarbital	1.66[b]	2.58		0.39[b]	Av. 0.79 ± 0.10
	1.94[b]	2.74		0.89[b]	
	1.79	1.92		0.36[b]	
	1.01	1.83		Av. 0.46 ± 0.09	
	Av. 1.60 ± 0.20	Av. 2.27 ± 0.23			

[a] Each entry represents the average clearance for five exchanges on one animal. [b] From previous work (1). [c] Average ± standard error of the mean. [d] From previous work (2).

Amobarbital and butabarbital were extracted completely from acidified samples with 4 × 5 ml. ether. Contamination by metabolites was less than 3%, as determined by paper chromatography. Extracts were evaporated in counting vials, scintillation fluid was added, and they were then counted.

For diphenylhydantoin, the samples were acidified with 0.5 *N* HCl, extracted with 2 × 5 ml. chloroform, which was evaporated in counting vials, and counted in the usual manner.

In each case, an exact dilution of radioactive injection was counted with the samples as a standard.

Studies on *N*-Myristyl-β-aminopropionic Acid—Absorption of the wetting agent *N*-myristyl-β-aminopropionate from the peritoneum might occur during its use in dialysis. High blood levels coupled with low excretion rate might prove hazardous to the health of the patient undergoing dialysis as a result of accumulation of this chemical in the body. Therefore, it was thought pertinent to determine whether this agent is absorbed during its passage across the peritoneum and, if so, to what degree and how rapidly it would be removed in subsequent exchanges. From these data, it would be possible to estimate the likelihood of toxicity.

Since a reliable method for quantitative determination of *N*-myristyl-β-aminopropionic acid could not be found, ^{14}C-tagged compound was used to measure its concentration in body fluids. The synthesis was performed in this laboratory. The first step involved the preparation of ^{14}C-tetradecanitrile by the method of Friedman and Shechter (6), using 1-bromotridecane and ^{14}C-sodium cyanide in dimethyl sulfoxide. The nitrile was reduced to tetradecylamine-1-^{14}C by sodium butoxide in toluene (7). *N*-Myristyl-β-aminopropionate was then prepared by reacting the amine with 20% excess of methyl acrylate. The product was saponified with an equivalent amount of 50% NaOH solution after the excess methyl acrylate was removed at 100° under vacuum (8). The identity and purity of each intermediate compound and the final product were checked with TLC and IR spectrometry. The overall yield of *N*-myristyl-β-aminopropionate was 42%, with a specific activity of 0.373 μc./mg.

To estimate the amount of wetting agent that might be absorbed in a single peritoneal lavage, absorption was measured over a longer period than just a 15-min. cycle. This afforded measurements useful even if lavages of different cycle times were used. The rabbits were prepared for the experiment in the same manner as mentioned previously. The dialysis fluid, containing 0.125% radioactive *N*-myristyl-β-aminopropionate (equivalent in concentration to 0.25% of the commercial solution) was injected at the same level and using the same procedure as before, except that the fluid was left in the peritoneum for 3 hr. Samples of blood and dialysate were taken 5 min. after injection, at 10-min. intervals for the 1st hr., at 20-min. intervals for the 2nd hr., and at 30-min. intervals for the 3rd hr. The samples were counted directly for total radioactivity.

To determine the rate of disappearance from plasma and the urinary clearance of *N*-myristyl-β-aminopropionate, two rabbits were given an intravenous dose of 30 mg. radioactive compound in normal saline. Plasma and urine samples were collected every 10 min. for the 1st hr. and every 20 min. for the following 2 hr. The samples were counted directly.

To determine the peritoneal clearance of *N*-myristyl-β-aminopropionate, a rabbit was injected intravenously with ^{14}C-labeled compound at the level of 10 mg./kg. A 30-min. period was allowed for distribution in the body before commencing dialysis. Five dialysis cycles with control fluid were carried out, each consisting of a 20-min. dwell time and a 10-min. draining period. Plasma, dialysate, and urine samples were collected and directly counted in the liquid scintillation spectrometer.

To determine the hemolytic threshold and the concentration of *N*-myristyl-β-aminopropionate producing 50% hemolysis, a series of dilutions of the surfactant in normal saline, ranging from 2 to 74 mcg./ml., was prepared. Normal saline was used as the negative control (no hemolysis), and a 1% solution of sodium carbonate in saline was used as a positive control (100% hemolysis). Ten milliliters of each solution was placed in a centrifuge tube and kept in a water bath at 37° for 30 min. to provide temperature equilibration. Then 0.1 ml. of heparinized rabbit whole blood was added to each tube and incubated at the same temperature. At the end of 1 hr., the tubes were centrifuged for 10 min. at 60,000 r.p.m. and the supernatant solutions were carefully removed and measured in a spectrophotometer at 540 nm.

To obtain an estimate of the LD_{50} of *N*-myristyl-β-aminopropionate in mice, young, healthy, male albino animals (15–28 g.) were used. They were divided into five groups, each containing five mice.

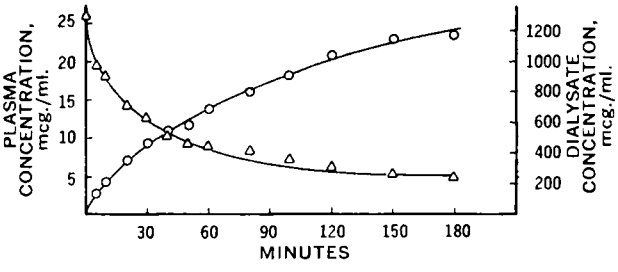


Figure 1—*Intraperitoneal absorption of* N-*myristyl-β-aminopropionate. Key:* ○, *plasma; and* △, *dialysate.*

Table II—Dialysis of *N*-Myristyl-β-aminopropionic Acid with Control Fluid[a]

Average Plasma Concentration, mcg./ml.	Dialysate Concentration, mcg./ml.	Amount Removed, % Dose	Clearance, ml./min.	Accumulated Amount in Urine, mcg.
14.32	0.53	0.23	0.14	77.53
12.77	0.51	0.32	0.21	292.81
14.11	0.45	0.25	0.15	646.87
13.76	0.47	0.25	0.15	1089.17
13.72	0.44	0.25	0.15	1367.87
	Total	1.30	Av. 0.16	

[a] Experiment on Animal E-27, which weighed 2.30 kg. The dialysis cycle time was 30 min.

Four doses of commercial *N*-myristyl-β-aminopropionate, ranging from 250 to 1000 mg./kg., were selected due to a pilot experiment. These doses were administered intraperitoneally as a 1% solution in normal saline to four test groups, and the control group received 2 ml. normal saline. The animals were put under observation for 24 hr.

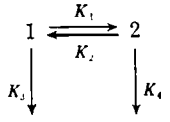
RESULTS

Results of intermittent dialysis of the drugs tested are shown in Table I, where each entry represents the average clearance of five exchanges on one animal. Previously reported results for control fluid were added for comparison. It can be seen that a 15-min. lavage with *N*-myristyl-β-aminopropionate fluid accelerated the peritoneal removal of all drugs tested. The highest increases in clearance were observed with salicylate and diphenylhydantoin, where clearance values about 2.5 times control were obtained. With the barbiturates, clearances ranged between 1.4 and 1.9 times control.

The high affinity of diphenylhydantoin for plasma protein appears to be the underlying cause for its low clearance. If the unbound diphenylhydantoin is considered to be 14% of the total plasma concentration over the range studied (1), the calculated average dialysate–plasma ratio of free drug is found to be 0.50 with control dialysis and 0.99 with the priming lavage technique. In this case, the protein binding of diphenylhydantoin seems to be the rate-limiting step during dialysis and equilibrium between drug in plasma and dialysate is rapidly attained. Therefore, peritoneal removal of diphenylhydantoin could be further accelerated by much shorter dwell periods.

Typical plasma and peritoneal fluid curves demonstrating intraperitoneal absorption of *N*-myristyl-β-aminopropionate are illustrated in Fig. 1. The plasma level did not exceed 2.7 mcg./ml. in the first 5 min., corresponding to the dwell period in a lavage. After 15 min., a period that would allow absorption to continue during drainage, the level did not exceed 7 mcg./ml. The absorption of wetting agents was quite rapid, as reflected in the dialysate curve, with about 30–50 mg./kg. being absorbed in a 15-min. period.

The plasma curves following intravenous injection were biexponential, indicating a two-compartment model. Of several two-compartment models possible, only one gave an acceptable fit to the urine curve as shown in Scheme I, where 1 is the central compartment including the plasma, 2 is a peripheral tissue compartment (possibly largely the liver), K_1 and K_2 are distribution constants between the two compartments, K_3 is the urinary excretion constant, and K_4 represents direct elimination from the tissue compartment (possibly elimination *via* the bile). The constants may be found by solving a biexponential equation obtained from the plasma curve alone, but it was considered preferable to solve it with an analog computer which would utilize the cumulative urine curve as well



Scheme I

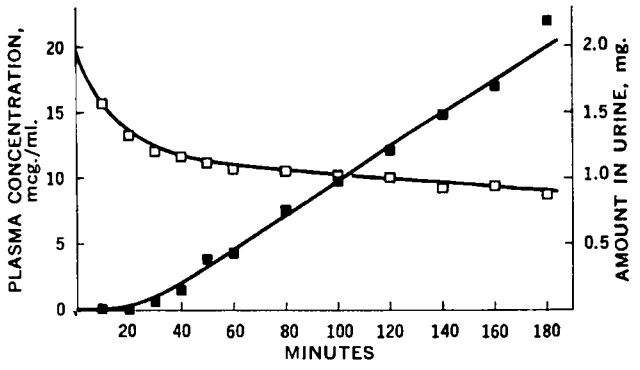


Figure 2—*Plasma and urine curves following intravenous administration of* N-*myristyl-β-aminopropionate to Animal E-24. The lines are computer drawn. Key:* □, *plasma; and* ■, *urine.*

as plasma data. This was done with the data of Animal E-24, as illustrated in Fig. 2, and the rate constants were estimated to be as follows: $K_1 = 1.60 \times 10^{-2}$ min.$^{-1}$, $K_2 = 2.08 \times 10^{-2}$ min.$^{-1}$, $K_3 = 1 \times 10^{-5}$ min.$^{-1}$, and $K_4 = 1.25 \times 10^{-3}$ min.$^{-1}$. These constants demonstrate the relatively low rate of urinary excretion as compared to the presumed biliary excretion. Also, the high distribution volumes, 152% for the central compartment and 380% for the equilibrium distribution volume, explain the relatively low plasma concentrations found in these experiments. The model fitted to these data should not be accepted as an established one for this compound, since it is based on limited data, but it was the only one that satisfactorily fit the data available.

The removal of *N*-myristyl-β-aminopropionate from the body by peritoneal dialysis with control fluid is summarized in Table II. Once absorbed, the wetting agent is slowly removed by peritoneal dialysis. The peritoneal clearance was only 0.16 ml./min., as compared to a urinary clearance of 0.58 ml./min. measured in the same experiment.

The data for the hemolysis experiment are illustrated in Fig. 3, where the results, as percentage hemolysis, were plotted against the concentrations of *N*-myristyl-β-aminopropionate used, to give a dose-response curve. The concentration giving 50% hemolysis was estimated to be 63.2 mcg./ml. It was also found that below 15 mcg./ml. no hemolysis occurred. The plasma level of *N*-myristyl-β-aminopropionate remained below 7 mcg./ml. for the first 15 min. during the intraperitoneal absorption experiment, thus being well below the hemolytic threshold.

The results of the LD_{50} experiment were plotted (Fig. 4) as the logarithms of the doses administered against the probit effects according to the method of Miller and Tainter (9). The best fitting line was calculated by the least-squares method, and the LD_{50} of *N*-myristyl-β-aminopropionate in mice was estimated to be 620 mg./kg. with a standard error of ±136.

DISCUSSION

The data obtained in this study clearly confirm the earlier findings in this laboratory (1, 2) that *N*-myristyl-β-aminopropionic acid has an accelerating effect on the peritoneal clearance of salicylate, barbiturates, and diphenylhydantoin. Of particular interest is the

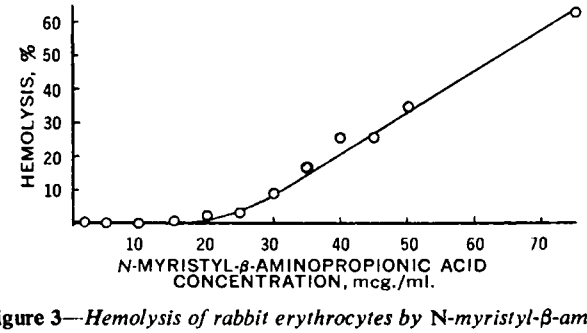


Figure 3—*Hemolysis of rabbit erythrocytes by* N-*myristyl-β-aminopropionate.*

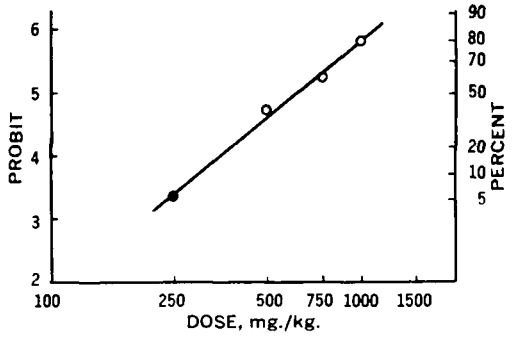


Figure 4—*LD_{50} of N-myristyl-β-aminopropionate in mice.*

finding that a single short-term exposure of the peritoneal membrane to *N*-myristyl-β-aminopropionate solution gave an accelerated dialysis effect which lasted throughout at least five subsequent exchanges with standard dialysis fluid. This would make possible the effective use of this accelerator with minimum exposure to the patient. The absorption tests also demonstrated that, although *N*-myristyl-β-aminopropionate is rapidly taken up from the dialysis fluid, the resulting plasma levels are quite low and appear to be well below concentrations that would result in acute toxicity. With its comparatively short 5-hr. biological half-life (as calculated from the terminal phase of the blood curves), practically all surfactant absorbed into the circulation should be eliminated from the blood in less than 48 hr. These findings should enhance the potential for the clinical use of *N*-myristyl-β-aminopropionate as an accelerator during peritoneal dialysis. Further toxicological studies are essential, of course, before this substance might be tested in man.

The mechanism of action of the surfactant is not yet known. Studies to determine the mechanism are being conducted but are not yet conclusive.

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Survival of *Staphylococcus aureus* on Pharmaceutical Oral Solid Dosage Forms

ROBERT F. WATERMAN, EDWARD D. SUMNER▲, JACK N. BALDWIN, and FLYNN W. WARREN

Abstract □ The relationship of temperature, relative humidity, size of inoculum, and duration of storage on survival of *Staphylococcus aureus* inoculated onto surfaces of 17 commercial tablets and one gelatin capsule was determined. The data were analyzed using a two-way factorial analysis of variance. Decreased survival was associated with an increase in each of the variables, within limits of the experiment; within those limits, size of inoculum and storage time were the least significant factors and storage environment appeared to be the most significant.

Keyphrases □ *Staphylococcus aureus* inoculation—surface of commercial tablets and capsules, effects of temperature, relative humidity, size of inoculum, and storage time on survival □ Tablet and capsule surface inoculated with *Staphylococcus aureus*—effects of temperature, relative humidity, size of inoculum, and storage time on survival □ Sterility testing, tablets and capsules inoculated with *Staphylococcus aureus*—effects of size of inoculum, storage time, and environmental conditions on survival

The necessity for sterility of parenteral products has long been recognized. NF V was the first official compendium in the United States to require sterility for such products (1). With respect to distribution and use of nonsterile pharmaceuticals, it is now recognized that microbial contamination must be minimal; with respect to certain species, it is totally unacceptable. Kallings *et al.* (2) focused attention on microbial content of nonsterile products when they reported infections resulting from the use of contaminated medications. The Swedish National Board of Health is restricting levels of microbes that may be present in liquid and tableted preparations (3), and a policy of not allowing an excess of 100 colonies/g. for practically all medical preparations was adopted (4). Czechoslovakia adopted statutory microbiological standards for tablets (5).

Numerous studies (6–12) confirmed existence of microbial contamination in some products. Hirsch *et al.* (13) reported that 82% of 57 oral liquid products tested contained microorganisms, and other researchers (14) found more than 100 microorganisms/ml. in 40 of 489 samples of such preparations. USP XVIII now includes a statement regarding microbial contamination of nonsterile pharmaceutical products (15).

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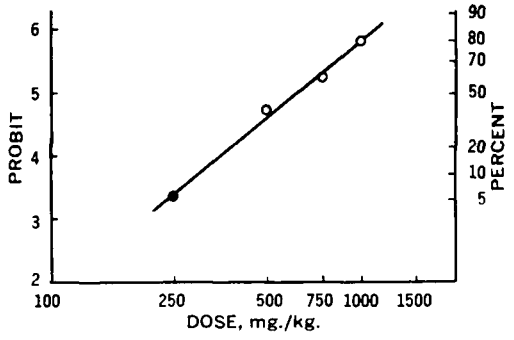

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The purposes of this study were to: (*a*) determine the ability of solid pharmaceutical dosage forms to carry bacteria; and (*b*) investigate the effect of temperature, relative humidity, size of inoculum, and storage time on

Table I—Effect of Temperature, Relative Humidity, and Time of Storage on the Survival of *S. aureus* on Solid Dosage Forms[a]

Sample	Percentage of Cells Surviving					
	3 Days			7 Days		
	Group 1[b]	Group 2	Group 3	Group 1	Group 2	Group 3
Aspirin	0[c]	0	0	0	0	0
Buffered aspirin	0	0	0	0	0	0
Sodium butabarbital	0.11	0.05	0	0.008	0.0004	0
Chewable multiple vitamins	0.12	0.08	0.04	0.01	0.001	0
Digitalis USP	5.83	1.06	0.08	2.42	0.26	0.0001
Chlorothiazide	3.51	1.26	0.25	1.75	0.45	0.10
Nasal decongestant	0	0	0	0	0	0
Meprobamate	2.12	0.53	0.003	0.93	0.073	0
Barbiturate–analgesic combination	0	0	0	0	0	0
Gelatin capsule	24.33	17.01	11.02	17.50	14.86	8.15
Magnesia hydroxide	0	0	0	0	0	0
Tolbutamide	2.25	0.73	0	1.10	0.10	0
Pentaerythritol tetranitrate	0	0	0	0	0	0
Quinidine sulfate	0	0	0	0	0	0
Magaldrate	27.67	19.12	12.16	18.33	16.23	5.07
Methocarbamol with aspirin	0	0	0	0	0	0
Antiasthmatic	0.17	0.05	0	0.01	0.003	0
Ethinamate	0.201	0.062	0	0.033	0.0001	0
Filter paper disk control	0.195	0.063	0.002	0.030	0.0045	0

[a] Each value represents the average from counts of three tablets or capsules inoculated with 3.78×10^6 bacteria. [b] Group 1, 28°, 24% relative humidity; Group 2, 33°, 10% relative humidity; and Group 3, 37°, 20% relative humidity. [c] 0 indicates no viable organisms detected.

the survival of *Staphylococcus aureus* on the surfaces of commercial products.

EXPERIMENTAL

Materials and Methods—The products (and their strength if applicable) were as follows: aspirin[1] (325 mg.), buffered aspirin[2] (325 mg.), sodium butabarbital[3] (100 mg.), chewable multiple vitamins[4], digitalis USP[5] (100 mg.), chlorothiazide[6] (500 mg.), a nasal decongestant[7], meprobamate[8] (400 mg.), a barbiturate–analgesic combination[9], a gelatin capsule[10], magnesia hydroxide[11] (325 mg.), tolbutamide[12] (500 mg.), pentaerythritol tetranitrate[13] (20 mg.), quinidine sulfate[14] (200 mg.), magaldrate[15] (400 mg.), methocarbamol with aspirin[16] (400 mg.), an antiasthmatic[17], and ethinamate[18] (500 mg.).

Survival tests were performed with *S. aureus* FDA 209[19] as the test organism, cultured in trypticase soy broth or on trypticase soy agar as appropriate. Relative humidity in the test chambers was maintained at 33, 52, or 85% (at 25°) by means of saturated solutions of magnesium chloride, magnesium nitrate, or potassium chloride, respectively (16). Potassium thiocyanate was employed as an indicator (17).

Sterilization—Products to be tested were placed in cotton-plugged erlenmeyer flasks (25 or 50 ml.) or culture tubes (25 × 130 mm.) and exposed to ethylene oxide[20] in a sterilizer[21] for 6 hr. at 55°. The efficiency of the sterilization cycle was determined by means of *Bacillus subtilis* indicator strips[22]. Sterility was rechecked after 1 week at ambient temperature by incubating control samples in 10 ml. sterile broth for 24 hr. at 37°.

Inoculation of Samples—Following the rechecking of sterility, samples were placed in humidity chambers and were inoculated after 1 week with 50 μl. of a suspension of the test organism prepared as follows:

1. Incubate broth culture 20 hr. at 37°.
2. Centrifuge.
3. Wash cells in 10 ml. sterile water.
4. Recentrifuge.
5. Resuspend in 10 ml. sterile water.

Capsules were inoculated by placing inoculum into one of the two parts, and tablets were inoculated by distributing the inoculum on the surface. All procedures were conducted in a laminar flow hood.

One, 3, and 7 days after inoculation and storage under the respective conditions, samples were placed in sufficient sterile water to make a final volume of 10 ml. They were permitted to stand for 1 hr. and then shaken. Then 50 μl. of the dispersion was streaked on agar plates; these plates were incubated at 37° for 48 hr., at which time colonies were counted.

Maintenance of sterility was verified by inoculating tablets (digitalis, chlorothiazide, and magaldrate) with sterile saline and subjecting them to the experimental conditions used in the study. To determine the basal survival rate of the microorganisms, filter paper disks[23] (12.7 mm. diameter) were inoculated because they are porous and nonbactericidal.

Contamination in Commercial Products—Commercial preparations were tested to determine the presence of microbial contaminants by transferring them aseptically from the original packages to tubes containing 10 ml. of broth. Six samples of each product were prepared. Following incubation at 37° for 24 hr., the tubes were examined for evidence of bacterial growth.

Effect of pH on Survival—To study the effect of pH on survival rates, tubes containing 10 ml. of distilled water, adjusted with acid or base to pH values of 2.5–10, were sterilized and inoculated with *S. aureus* (17.18×10^6 organisms). After 1 hr., 0.1-ml. samples were removed and streaked on agar plates. The plates were examined after incubation at 37° for 24 hr.

pH of Aqueous Dispersions of Tablets—Triplicate samples of products were placed in sufficient water to make 30 ml. After the tablets disintegrated, the pH of the resulting dispersions was measured.

Statistical Analysis—The data were analyzed using a two-way factorial analysis of variance. The variables involved were time

[1] Lot No. 216, Bayer Co.
[2] Bufferin.
[3] Lot No. ML 7667, Butisol.
[4] Lot No. 7629 ML, Chocks.
[5] Lot No. 293-209, Lederle.
[6] Lot No. 693, Diuril.
[7] Lot No. 1366-26, Dristan.
[8] Lot No. 1710839, Equanil.
[9] Lot No. 116 R 1359, Fiorinal.
[10] Lot No. LC 6011 C, Eli Lilly & Co.
[11] Lot No. 8M575, Squibb.
[12] Lot No. 1H P35 IO, Orinase.
[13] Lot No. 0277 PO 29 B, Peritrate.
[14] Lot No. 5DD53A, Eli Lilly & Co.
[15] Lot No. A3503LL, Riopan chewable.
[16] Lot No. 71 1723 4, Robaxisal.
[17] Lot No. 0174P1049A, Tedral.
[18] Lot No. 50D72A, Valmid.
[19] Supplied by the Department of Microbiology, University of Georgia.
[20] PennGas, Pennsylvania Engineering Co., Philadelphia, Pa.
[21] Cryotherm Gas Sterilizer, American Sterilizer Co., Erie, Pa.
[22] Spordex, American Sterilizer Co., Erie, Pa.
[23] No. 740-E, Carl Schleicher and Schuell Co., Keene, N. H.

Table II—Effect of Size of Inoculum, Relative Humidity, and Time of Storage on the Survival[a] of *S. aureus* on Solid Dosage Forms Stored at 25°

Sample		Percentage of Cells Surviving								
		1 Day			3 Days			7 Days		
		Group 4[b]	Group 5	Group 6	Group 4	Group 5	Group 6	Group 4	Group 5	Group 6
Sodium butabarbital	(L)	2.11	0.96	0.415	0.18	0.087	0.022	0.012	0.003	0.0004
	(H)	1.30	0.61	0.29	0.105	0.048	0.007	0.008	0.0007	0.0001
Chewable multiple vitamins	(L)	1.11	0.522	0.22	0.175	0.081	0.017	0.015	0.003	0
	(H)	0.77	0.371	0.160	0.115	0.051	0.009	0.009	0.0006	0.0001
Digitalis USP	(L)	12.67	7.025	3.221	5.73	3.41	0.842	2.28	0.717	0.093
	(H)	6.31	4.09	1.93	3.57	1.83	0.47	1.23	0.256	0.010
Chlorothiazide	(L)	6.51	3.51	1.17	5.27	1.33	0.42	1.93	0.09	0.007
	(H)	3.22	1.84	0.81	2.70	0.617	0.15	0.998	0.025	0.001
Meprobamate	(L)	10.36	6.12	2.317	2.67	1.71	0.622	1.052	0.123	0.047
	(H)	4.171	3.09	1.23	1.53	0.81	0.42	0.875	0.044	0.008
Gelatin capsule	(L)	30.26	19.11	6.05	25.81	8.93	2.12	16.33	2.49	0.295
	(H)	14.39	8.12	3.11	12.57	4.735	0.875	9.425	0.745	0.091
Tolbutamide	(L)	6.83	3.94	1.27	2.41	1.11	0.37	1.26	0.07	0.004
	(H)	8.54	2.11	0.92	1.33	0.42	0.124	0.834	0.013	0.0009
Magaldrate	(L)	34.57	20.33	7.35	32.18	9.42	2.45	17.45	1.625	0.32
	(H)	18.71	9.94	4.285	15.45	3.77	0.937	11.375	0.641	0.105
Antiasthmatic	(L)	0.83	0.49	0.193	0.215	0.093	0.024	0.023	0.005	0.0004
	(H)	0.65	0.30	0.117	0.119	0.06	0.009	0.011	0.0009	0.0001
Ethinamate	(L)	0.717	0.422	0.15	0.238	0.125	0.028	0.035	0.009	0.0007
	(H)	0.59	0.26	0.09	0.145	0.074	0.02	0.015	0.002	0.0002
Filter paper disk control	(L)	1.13	0.64	0.33	0.235	0.105	0.025	0.035	0.007	0.0005
	(H)	1.025	0.572	0.191	0.133	0.06	0.01	0.015	0.001	0.0001

[a] Each value reported represents the average from counts of two tablets or capsules. (L) = 1.47×10^6 bacteria/sample. (H) = 13.57×10^6 bacteria/sample. [b] Group 4, 33% relative humdity; Group 5, 52% relative humidity; and Group 6, 85% relative humidity.

(three categories), level of inoculum concentration (two categories), and environmental storage conditions (three categories). The *F*-distribution was used as the test of significance in the analysis of variance.

RESULTS

The survival of *S. aureus* on solid dosage forms under conditions where storage time, temperature, and relative humidity were varied is reported in Table I. The data reveal decreased survival with increased temperature and storage time. *S. aureus* did not survive on eight samples (aspirin, the nasal decongestant, the barbiturate–analgesic combination, methocarbamol with aspirin, magnesia hydroxide, buffered aspirin, quinidine, and pentaerythritol tetranitrate tablets). There was a high frequency of survival on the gelatin capsules (8%) and magaldrate (5%) after 7 days under conditions that resulted in no survivors on 15 of the products.

Table II shows the percent survival of *S. aureus* after storage at a constant temperature of 25° when relative humidity and size of inoculum varied. Decreased survival rates were noted when relative humidity, storage time, and inoculum concentration were increased.

Tests on unsterilized pharmaceuticals revealed contamination of 12. Contaminants were not identified. Bacterial growth was not observed in the tubes containing aspirin, buffered aspirin, sodium butabarbital, the nasal decongestant, magnesia hydroxide, and methocarbamol with aspirin.

The pH of aqueous dispersions of the tablets ranged from 2.7 to 9.8. *S. aureus* did not survive in water outside the pH range of 4.0–8.6. Maximum survival was observed in the pH range of 7.0–8.6.

DISCUSSION

Sterility is not a requirement in official compendia for oral pharmaceutical dosage forms. However, contamination may occur during manufacturing, packaging, and handling by the consumer. This causes concern, since some dosage forms, if stored in favorable environments, can serve as substrates for microorganisms. Not all of the dosage forms studied supported bacterial (staphylococcal) contamination under the experimental conditions, suggesting that some pharmaceuticals are less likely than others to support bacterial survival (or at least survival of staphylococci).

Many factors determine whether a microorganism will survive on a solid dosage form. Active ingredient and formulation additives may have a profound effect on bacteria; in certain formulations, they may act as bactericidal agents. Environmental conditions such as humidity, temperature, and absence or presence of oxygen may give rise to unfavorable conditions for survival of bacteria. Other factors involved may be size of inoculum and length of storage (18).

Using aqueous solutions adjusted to varying pH's with acid or base, it was found that *S. aureus* did not survive except in the pH range of 4.0–8.6. Because the pH of aqueous dispersions of aspirin, the nasal decongestant, the barbiturate–analgesic combination, methocarbamol with aspirin, and magnesia hydroxide was outside the range of 4.0–8.6, pH was considered a major factor for *S. aureus* not surviving. However, lack of survival cannot be explained solely on the basis of pH since the other three tablets (buffered aspirin, quinidine, and pentaerythritol tetranitrate) did not permit survival even though aqueous dispersions were in the favorable pH range of 4.0–8.6. The formulation ingredients may be the reason for zero survival of *S. aureus* on these tablets, since bacteria survived on the filter paper disk control under the same experimental conditions.

At high relative humidities, the survival rate was reduced (Table II). A thin aqueous film may form on the surface of the solid dosage forms and, as the humidity increases, this aqueous film formation should increase. Not only would more water be available for the organism, but additional quantities of the soluble ingredients would be dissolved in the film and a greater bactericidal action would be exerted. The existence of a critical moisture content at which a bacterial cell becomes more susceptible to toxic agents has been postulated (19).

Table I shows that there were fewer survivors at 37° than at 28° where the relative humidities were similar. This may be due in part to increased solubility of tablet ingredients in the surface film at the higher temperature and the fact that the bactericidal action of chemical agents is greater at higher temperatures (18). Consequently, there would be a greater bactericidal effect on *S. aureus*.

The frequency of survivors on nine samples after storage for 7 days at 25° and 33% relative humidity was 100–1000-fold higher than on the control filter paper disks, indicating that ingredients in the samples protected the *S. aureus*.

High and low concentrations of inoculum were used to determine the effect on survival rates, because some medications may be more contaminated than others. Differences between percent survivals were noted; in general, the highest survival rate was observed with the lower inoculum concentrations. The differences were not statis-

tically significant, and no explanation can be offered for these results.

The size of inoculum and storage time (within the limitations of the experiment) were less significant factors. Storage conditions appeared to be the most significant factor.

Numerous variables were inherent in the study, and the data are inconclusive. Future studies may provide data to explain the differences in survival rates of *S. aureus* on solid dosage forms.

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PATRICK P. DeLUCA▲, LEON LACHMAN*, and HANS G. SCHROEDER

Abstract □ The tetramethyl-substituted amides of pimelamide, suberamide, azelamide, and sebacamide markedly enhance the solubility of glutethimide in aqueous solution. Partition studies, surface tension measurements, and light-scattering measurements strongly suggest that the amides are associating at infinitely dilute concentrations and further aggregation of these associated molecules occurs with the possible formation of micelles at concentrations slightly higher than that observed for surfactants. The CMC's were identified at 0.41, 0.20, 0.031, and 0.11 *M* for pimelamide, suberamide, azelamide, and sebacamide, respectively. The solubility of glutethimide was increased significantly above the critical concentrations and, from the nature of the solubility curves, a micellar type of solubilization appears to be dominant.

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The formulation of a suitable dosage form for administration *via* the oral, parenteral, and topical routes is often a problem with drugs that have only limited water solubility. For oral administration, a solution would preclude the requirement for dissolution as in the case of suspensions or solid dosage forms and facilitate passage of the drug across the lipid membrane. For topical preparations, a homogeneous system would facilitate percutaneous absorption; for parenteral administration, a solution is generally desirable, especially *via* the intravenous route.

Nonaqueous solvents are often combined with water to dissolve a sparingly soluble drug even for injectable preparations (1), and the solvent selected must be free of toxic and irritating effects and not give rise to pharmacological responses. Two-component solvent systems

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Nonaqueous solvents are often combined with water to dissolve a sparingly soluble drug even for injectable preparations (1), and the solvent selected must be free of toxic and irritating effects and not give rise to pharmacological responses. Two-component solvent systems

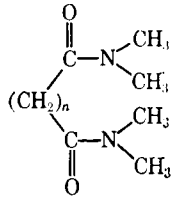
and hydrotropic agents have been employed and, more recently, surfactants have been used extensively.

The subject of micellar solubilization was dealt with thoroughly in two recent reviews by Mulley (2) and Swarbrick (3); and while hydrotropic solubilization (4) has received less attention, the literature on this type of solubilization has increased. Hydrotropes differ from surface-active agents in that they presumably do not possess surface activity or do not form micelles at low concentrations. Monosubstituted and disubstituted α-hydroxy acid amides, which are effective solubilizers, have been classified as hydrotropes.

N,N-Dimethylacetamide has been employed in the preparation of many dosage forms containing poorly soluble drugs (5). Specifically, the solubilities of tetracycline and oxytetracycline have been increased in nonaqueous systems by the addition of *N,N*-dimethylacetamide (6) and other amides of β-hydroxybutyric acid, succinic acid, adipic acid, tartaric acid, glycolic acid, and salicylic acid (7). A comprehensive study of the solubility of a number of drugs in acid amide solutions was undertaken by Samejima (8). The increase in solubility of the drugs studied was of relatively low order.

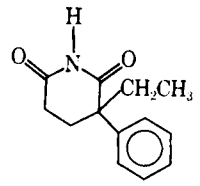
The excellent solvent properties of amides can be attributed in part to the strong dipole of the amide function and to the good electron-donating properties of the carbonyl group. Kostenbauder and Higuchi (9, 10) studied the molecular complexing tendencies of *N,N,N',N'*-tetramethylphthalamide series and some lower dicarboxylic acid amides and found increased solubility for chloramphenicol and *p*-hydroxybenzoic acid derivatives.

In light of these findings and on the basis of preliminary data for *N,N*-dimethylacetamide, *N*-methylpyrrolidone, and *N*-methylacetamide as solubilizing agents for poorly soluble drugs[1], it was felt that the higher homologs of *N,N,N',N'*-tetramethyl-substituted dicarboxylic acid amides were worthy of study. The general structure for the compounds selected is shown here. Hereafter these compounds will be referred to periodically simply as "amides."



pimelamide: $n = 5$
suberamide: $n = 6$
azelamide: $n = 7$
sebacamide: $n = 8$

The higher homologs were preferred because the inductive effect of the terminal amide groups upon each other would be negligible beyond succinamide where the functional groups are separated by three or more methylene groups. This type of structure would provide two, essentially independent, electron-donating groups



glutethimide

on a single molecule. Alkylation of the nitrogen groups was necessary to increase the solubility of the amides.

In this investigation, the properties of the amides in aqueous solution were studied and the potential solubilizing action of the amides was evaluated. Since the carbonyl group of an amide is a good electron donor, attraction for electron-accepting groups exists. Compounds possessing acidic hydrogen(s) such as alcohols, phenols, amines, xanthines, sulfonamides, and barbiturates should interact with the substituted amides. Glutethimide (2-ethyl-2-phenylglutarimide) is soluble to the extent of only 1 mg./ml. in water and it was selected for study.

Although glutethimide is a weak acid, having a pKa of approximately 11.8, it was felt that the hydrogen of the imide grouping would be sufficiently acidic to accept electrons. The tendency of the amides to associate or aggregate in aqueous solution was determined by partition studies and light-scattering measurements, and the surface activity of the amides was determined by surface tension measurements.

EXPERIMENTAL

Preparation of Amides[2]—The tetramethyl-substituted amides were prepared by reacting the corresponding acid chlorides with dimethylamine in alkaline media. Since only the azelaoyl and sebacoyl chlorides were commercially available, pimeloyl and suberoyl chlorides were prepared by reacting thionyl chloride with the dicarboxylic acids. Purification of the amides involved either vacuum distillation for the odd-carbon amides, pimelamide and azelamide, or recrystallization from acetone and hexane for the even-carbon amides, suberamide and sebacamide.

The purity of the amides was determined by vapor phase chromatography[3] and checked by differential scanning calorimetry[4] and elemental (C,H,N) analysis[5]. In all cases, the amides were found to be greater than 99% pure.

Solubility of Amides—The solubility of the amides was determined in distilled water at 30° in a constant-temperature water bath using the phase solubility technique (11). The amides were added to 20 ml. of distilled water in 30-ml. glass-stoppered bottles and agitated for 24 hr. The amide concentration in solution was determined by reaction with hydroxylamine (12); where excess amide was employed, the amount undissolved was weighed. The effect of added electrolyte on solubility was determined by adjusting the ionic strength of the solutions to 0.1 and 0.75 with potassium chloride.

Partitioning Studies—The amides were partitioned between organic and aqueous phases using chloroform–isooctane (12.5:87.5 v/v) as the organic phase and water as the aqueous phase. Twenty milliliters of the water phase of known amide concentration was placed in a special shaker tube (13), and 20 ml. of the organic phase was added. The tubes were agitated for 10 hr. at ambient condition at a rate of 1 c.p.m. The amount of amide partitioning into the organic phase was determined by GC employing the

[1] Solubilities of glutethimide, reserpine, griseofulvin, and hexestrol were increased in the presence of *N,N*-dimethylacetamide, *N*-methylpyrrolidone, and *N*-methylacetamide.

[2] The authors greatly appreciate the help of Dr. John Nelson, Ciba-Geigy Pharmaceutical Co., for developing the method of synthesizing the amides.

[3] Model 1740-10 Varian gas chromatograph.

[4] DSC-1B, Perkin-Elmer.

[5] Model 185, Hewlett-Packard analyzer.

Table I—Molar Solubility of *N,N,N',N'*-Tetramethyl-Substituted Amides

Compound	Distilled Water	—Potassium Chloride— 0.1 *M*	0.75 *M*
Tetramethylpimelamide	Mixes in all proportions	Mixes in all proportions	Mixes in all proportions
Tetramethylsuberamide	2.52	2.50	1.14
Tetramethylazelamide	3.90	3.85	3.72
Tetramethylsebacamide	0.527	0.344	0.170

following conditions: 1.25% OV-225 on Chromosorb G, 1.8-m. × 0.635-cm. (6-ft. × 0.25-in.) column, column temperature 225°, detector temperature 280°, and injector temperature 285°.

Surface Tension Measurements—Surface tension measurements were made with a surface tensiometer[6] employing a platinum–iridium ring. The measurements were made at ambient conditions using 10 ml. of solution contained in a 5.1 × 1.3-cm. (2 × 0.5-in.) Petri dish. The ring had a circumference of 6.060 cm. and was thoroughly rinsed with 95% alcohol and heated to incandescence in a bunsen flame between measurements. Readings were allowed to stabilize, and an average of three readings was recorded for each concentration. The effect of sodium chloride and urea on the surface tension measurements was determined by adding sodium chloride or urea to the amide solutions. No correction was made for volume changes with sodium chloride, but with urea it was necessary to account for the volume change to correct the amide concentration.

Light-Scattering Measurements—A photometer[7] was utilized for light-scattering measurements at a wavelength of 436 nm. While the instrument measures absolute turbidity, the calibration was checked periodically with a polystyrene fraction of known molecular weight (105,000). The scattering cell was a sinter-fused optical glass cell which permitted readings at angles of 0, 45, 90, and 135° to the direction of the transmitted beam. The method employed was essentially the same as that used by Brice *et al.* (14) for the determination of absolute turbidity. The scattering intensities at an angle of 90° were used to determine the absolute turbidity. The turbidity due to the solvent was subtracted from the total values, and the difference represented the excess turbidity. It was necessary to filter the solutions several times through a 0.45-μ cellulose ester membrane filter[8] and to allow sufficient time for stabilization of the readings to occur.

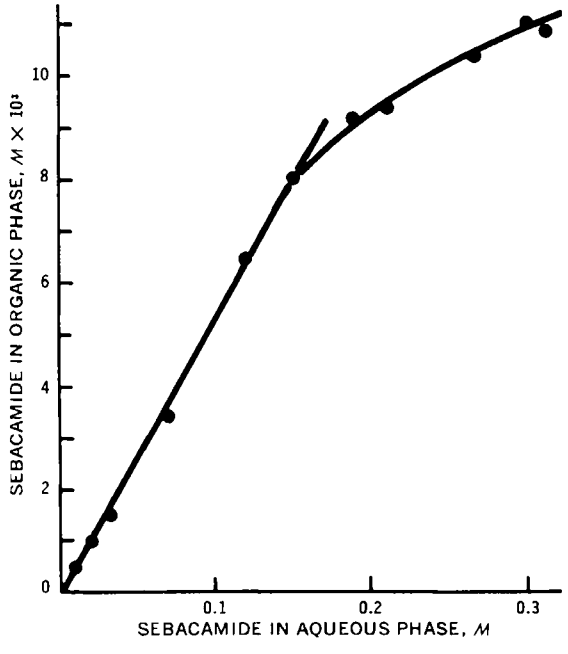


Figure 1—*Partitioning of tetramethylsebacamide between an organic phase of 12.5% chloroform in isooctane and an aqueous phase of water at room temperature.*

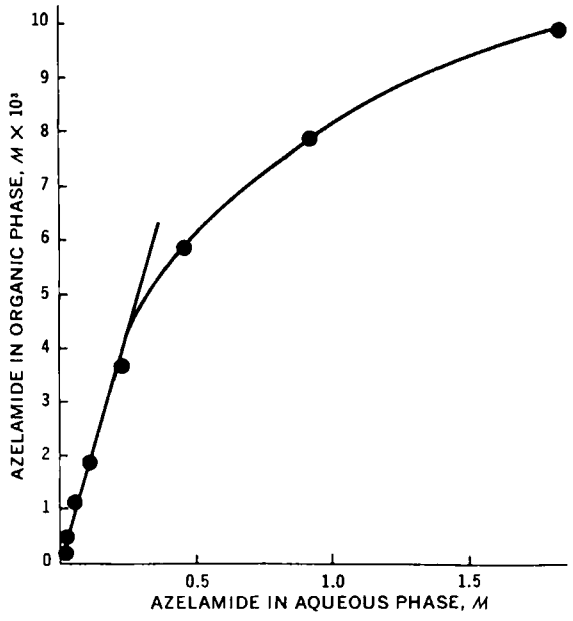


Figure 2—*Partitioning of tetramethylazelamide between an organic phase of 12.5% chloroform in isooctane and an aqueous phase of water at room temperature.*

The specific refractive index increments, *dn/dc*, were determined with a differential refractometer[9] at a temperature of 25° and a wavelength of 436 nm., using water as the solvent at five concentrations between 0 and 2.5%. Potassium chloride was used as a reference standard.

Solubilization of Glutethimide—Excess quantities of glutethimide[10], m.p. 84°, were placed into 30-ml. glass-stoppered bottles, and 20 ml. of solution of varying amide concentration was added. The bottles were tightly sealed and agitated in a constant-temperature water bath at 30° for at least 24 hr. until equilibration occurred. Equilibrium was determined by repetitive sampling, and it was found that 24 hr. of agitation was sufficient. The glutethimide concentration was determined spectrophotometrically using a spectrophotometer[11] with a recorder attachment at a wavelength of 257.6 nm. Anhydrous reagent methanol was used as the diluent for the drug samples.

RESULTS AND DISCUSSION

Aqueous Solubility—The tetramethyl-substituted amides were found to be extremely soluble in water. Table I lists the aqueous solubility values of the amides and the influence of ionic strength at a temperature of 30°. The solubility was not strictly a function of the chain length but was greater for the odd-carbon chain length amides. The C_5 compound, tetramethylpimelamide, is a liquid and mixes with water in all proportions. The C_7 compound, tetramethylazelamide, was soluble to the extent of 3.9 *M*.

The ionic strength of the solution appears to have a greater influence on the solubility of the even-carbon amides. At an ionic strength of 0.1, the solubility of tetramethylsuberamide was not altered; at an ionic strength of 0.75, the solubility was reduced by more than one-half. The solubility of tetramethylsebacamide was lowered by one-third at 0.1 and by two-thirds at 0.75. For the odd-carbon amides, tetramethylpimelamide and tetramethylazelamide, there was no apparent lowering of the solubility at either concentration of potassium chloride.

[6] DuNouy.
[7] Brice-Phoenix model 2000, Phoenix Precision Instrument Co., Philadelphia, Pa.
[8] Millipore Corp., Bedford, Mass.
[9] Brice-Phoenix model 430, Phoenix Precision Instrument Co., Philadelphia, Pa.
[10] 2-Ethyl-2-phenylglutarimide, supplied by the Ciba Pharmaceutical Co., Summit, N. J.
[11] Beckman DB.

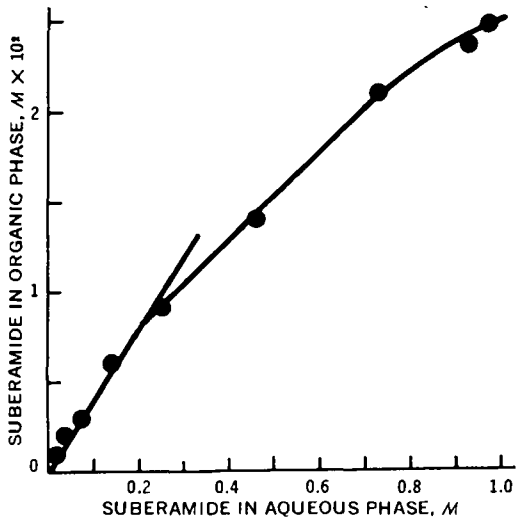


Figure 3—*Partitioning of tetramethylsuberamide between an organic phase of 12.5% chloroform in isooctane and an aqueous phase of water at room temperature.*

The salting-out effect of potassium chloride on the even-carbon amides indicates that solutions of these compounds might be less polar than the odd-carbon amides. However, to make a comparative assessment of polarity, dielectric constants of the amide solutions would have to be determined. Closer packing of the even-carbon chain has been demonstrated for dicarboxylic acids using X-ray analysis (15). Such behavior with the amides may give rise to a less polarizable compound, and the decrease in solubility of the even-carbon amides might be attributed to a preferential orientation of the polarizable water molecules around the ions of the added electrolyte.

Construction of molecular models shows that the odd-carbon amides favor a *cis*-configuration while the even-carbon compounds prefer a *trans*-orientation. The carbonyl groups for the odd-carbon amides are *cis* and in a parallel plane, while the nitrogen groups are *cis*-antiparallel; this type of orientation gives rise to a compound with a high dipole moment. The even-carbon amides have *trans*-antiparallel carbonyl groups and *trans*-parallel nitrogen groups; this symmetrical type of orientation results in a lower dipole and lower solubility.

Partition Studies—Partition isotherms for the amides are illustrated in Figs. 1–3. A linear relationship exists at low concentrations of the amides, but deviation from linearity occurs at higher concentrations, with more amide partitioning into the aqueous phase. Deviation of the partition isotherms at higher amide concentrations indicates increased association between amide molecules in aqueous solution. The break in the curve occurred at 0.15, approximately 0.25, and 0.22 *M* for sebacamide, azelamide, and suberamide, respectively.

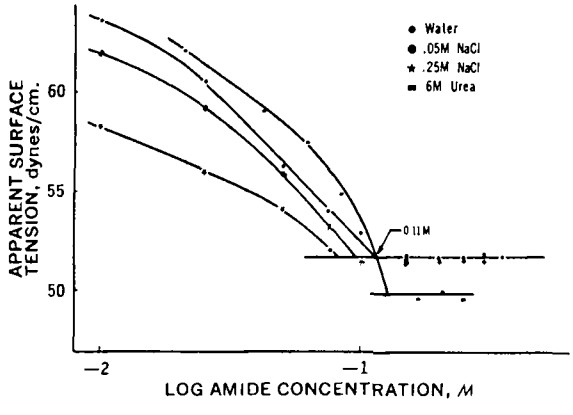


Figure 4—*Apparent surface tension as a function of tetramethylsebacamide concentration in water, 0.05* M *NaCl, 0.25* M *NaCl, and 6* M *urea.*

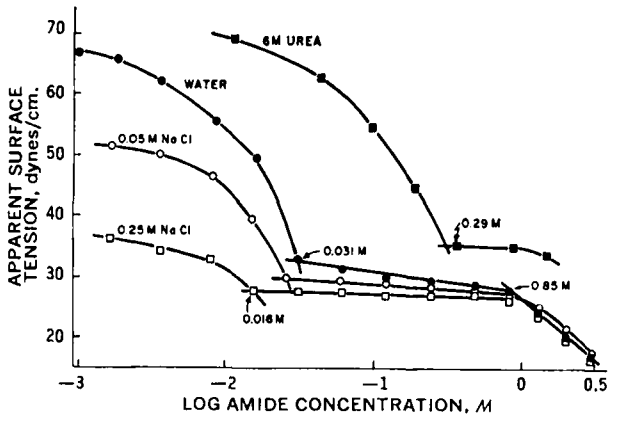


Figure 5—*Apparent surface tension as a function of tetramethylazelamide concentration in water, 0.05* M *NaCl, 0.25* M *NaCl, and 6* M *urea.*

Surface Tension Measurements—The results of the surface tension measurements are represented in Figs. 4–7 as plots of apparent surface tension *versus* the logarithm of the molar concentration. The curves show that the amides possess surface activity and that a critical concentration is associated with each amide.

For sebacamide, the break in the surface tension curve (Fig. 4) occurred at approximately 0.11 *M*. The addition of electrolyte suppressed the critical concentration only slightly. This effect is consistent with the findings of previous workers who studied the effect of electrolytes on nonionic association colloids (16–19) and found a decrease in the CMC.

Urea was included in the surface tension studies because of its ability to disaggregate nonpolar units within a molecule by weakening hydrophobic bonding (20, 21). Aside from the occurrence of a slight lowering of surface tension at the critical concentration, 6 *M* urea had no significant effect on the measurements. Due to the limited solubility of sebacamide, it was not possible to follow the surface tension beyond 0.5 *M* amide.

For azelamide, the break in the surface tension curve (Fig. 5) occurred at 0.031 *M*. In the presence of electrolyte, the surface tension was lowered and the break in the curve occurred at slightly lower concentrations. Urea at 6 *M* concentration had a profound effect on the surface tension, delaying the lowering and shifting the break in the curve to a concentration 10-fold higher than that in water. A second break in the surface tension curve was observed at a concentration of approximately 0.85 *M* amide, and a further lowering of the surface tension occurred with an increase in amide concentration. Much research has been done on the properties of

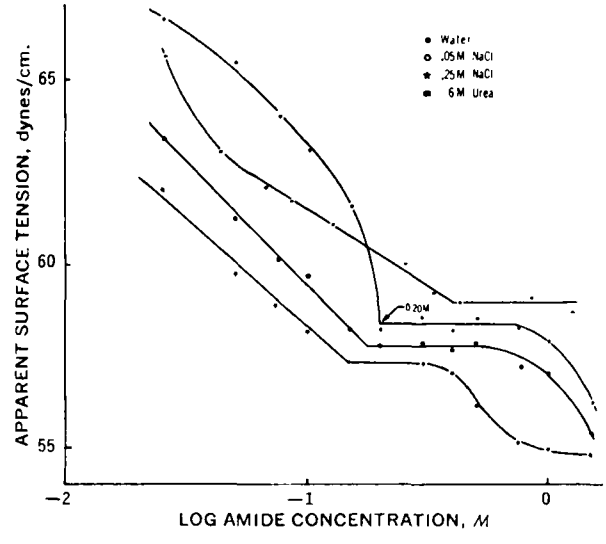


Figure 6—*Apparent surface tension as a function of tetramethylsuberamide concentration in water, 0.05* M *NaCl, 0.25* M *NaCl, and 6* M *urea.*

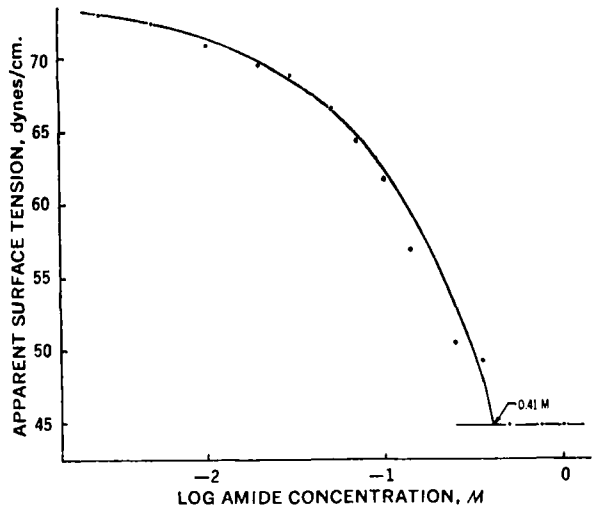


Figure 7—*Apparent surface tension as a function of tetramethylpimelamide concentration in water.*

surfactant solutions above the CMC, and several workers have identified a second CMC (22–24). While little has been done to explain this phenomenon, it has been postulated that an increase in size and asymmetry was associated or responsible for the second CMC.

Figure 6 shows the surface tension curves for tetramethylsuberamide, with the initial break in the curve occurring at 0.2 *M* amide in water; the lowering of the surface tension with suberamide is not as pronounced as with the other amides. The surface tension was found to be stable up to approximately 0.9 *M*, where the second break in the curve occurred. Added electrolyte enhanced the lowering of the surface tension and caused the break in the curve to occur at slightly lower concentrations. Urea delayed the break in the curve.

In the surface tension curve for pimelamide (Fig. 7), the break in the curve occurred at approximately 0.41 *M* amide concentration. Because of the limited amount of pimelamide available, no attempt was made to study the effect of urea or electrolyte, but it is

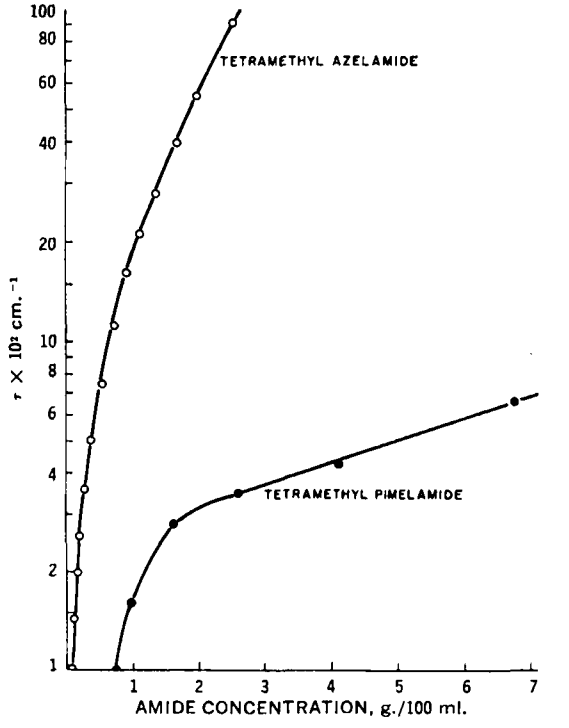


Figure 8—*Excess turbidity as a function of amide concentration for tetramethylazelamide and tetramethylpimelamide in water.*

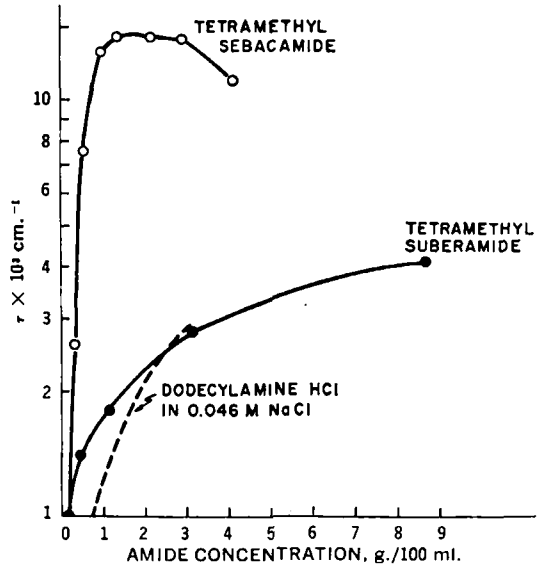


Figure 9—*Excess turbidity as a function of amide concentration for tetramethylsuberamide and tetramethylsebacamide in water. The dotted line is for dodecylamine hydrochloride in 0.046 M NaCl (27).*

believed that the effects would be the same as those observed for the other amides.

The surface tension lowering with the increase in amide concentration indicates that the amides have surface activity, and the break in the curve with a subsequent plateau suggests that saturation at the surface has been achieved in a manner closely resembling that for surfactants. Such behavior is well recognized for surfactant or detergent molecules; but to our knowledge, nothing has been reported on the possibility of a compound with two similar polar groups, one on each end of the molecule, such as the substituted dicarboxylic acid amides, behaving in this manner.

The apparent formation of micelles of the amides is not as spontaneous as one would expect for ionic surfactants. Additionally, it is suspected that some premicellar aggregation is occurring because of the necessity to "age" the solution to obtain stable surface tension readings. The effect of electrolyte in lowering the CMC has been well documented for ionic and nonionic surfactants. In addition to a simple dehydrating effect, Schick (18) postulated a salting-out effect for the lowering of CMC's by electrolytes.

Urea, by virtue of its two bonding centers on each molecule, undergoes hydrogen bonding with water; at high enough concentrations, it reduces the structure of water. This effect was studied with dodecylpyridinium salts (20), proteins (25), and alkyltrimethylammonium alkyl sulfates (21). As a result of the lowering of the interfacial energy between the hydrocarbon and water, the free energy differences between the hydrophobic groups and the water would be reduced and, therefore, the CMC would increase. Urea was found to have a profound effect with azelamide, elevating the CMC 10-fold, while with suberamide and sebacamide the CMC was increased only slightly.

Light-Scattering Measurements—To obtain estimates of weight average molecular weights of associated molecules and aggregates, *i.e.*, polymers and micelles, light-scattering measurements have been employed (26). Light scattering depends largely upon the microscopic heterogeneity of the solution due to fluctuations in concentration. The extent of these spontaneous fluctuations in concentration is a measure of the osmotic work; therefore, a relationship exists between the intensity of the scattered light and the osmotic pressure of the solution. Light scattering offers the advantage that the property being measured increases with an increase in molecular weight, whereas the reverse is true for osmotic pressure.

In Figs. 8 and 9, the excess turbidity at 90° of the amide solutions over that of water is plotted on a log scale as a function of amide concentration. The turbidity was found to increase rapidly from zero concentration in a manner resembling the behavior of high polymer solutions (27).

The odd-carbon amides, azelamide and pimelamide, showed unusually high scattering intensities, and the values obtained for

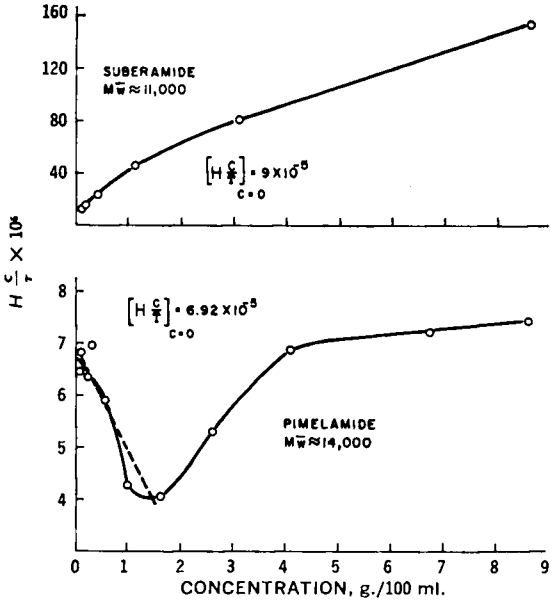


Figure 10—*Specific reciprocal turbidity as a function of amide concentration for tetramethylsuberamide and tetramethylpimelamide in water.*

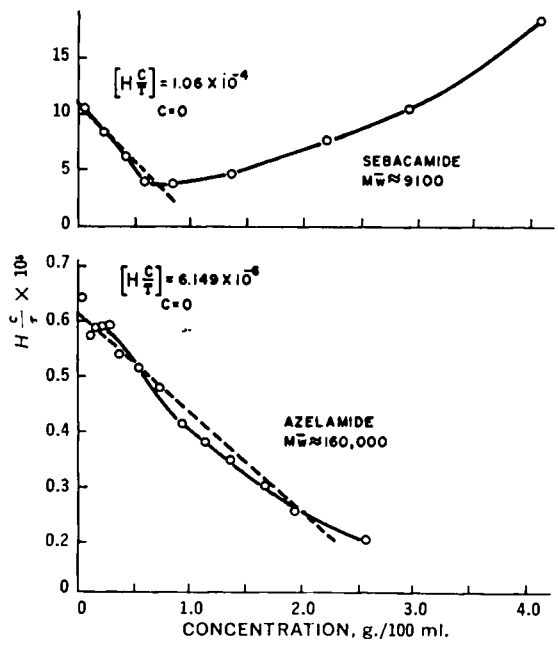


Figure 11—*Specific reciprocal turbidity as a function of amide concentration for tetramethylazelamide and tetramethylsebacamide in water.*

azelamide are in the order of at least 10 times those found for polymer solutions at similar concentrations. It was difficult to carry light-scattering measurements beyond 2 g./100 ml. because a high amount of scattering occurred at 0°. The turbidity of the pimelamide solution was lower than that for azelamide but was still higher than that found for most polymer solutions.

The intensity of scattering for the even-carbon amides was approximately one order of magnitude less than that for the previous odd-carbon amides. The turbidity of the sebacamide solution increased rapidly and reached a plateau at about 1.5 g./100 ml. Herrmann *et al.* (28), in their studies on the micellar properties of alkyl dimethylphosphine oxide solutions, observed maxima in the turbidity–concentration curves. The curve for suberamide closely resembles that found for polymers but is still of higher magnitude than that found for soap solutions above the CMC. The dotted line represents the curves for dodecylamine hydrochloride in 0.046 *M* sodium chloride solution above the CMC (26). It was not possible to plot the curves for the detergent in water on the graph since the turbidity reached a maximum value at approximately 0.3×10^{-3} cm.$^{-1}$. The influence of chain length on the turbidity is clearly evident for the even- and odd-carbon amides, with the higher homologs in each case showing greater scattering.

To determine average molecular weights from turbidity measurements, the refractive index increment must be determined since the refractive index of the solution is also a function of concentration. Debye (29) transformed Van't Hoff's law for high polymer solutions into the following equation:

$$H\frac{c}{\tau} = \frac{1}{MW} + 2Bc + \ldots \qquad \text{(Eq. 1)}$$

which, at high dilutions, would be a good linear approximation for the reciprocal specific turbidity as a function of concentration; τ is the experimentally measured excess turbidity of the solution, c is the concentration in grams per milliliter, and MW is the average molecular weight. The constant B depends on the solvent, and H can be calculated from refraction measurements using the following equation:

$$H = \frac{32\pi^3}{3}\frac{\eta_0^2}{N\lambda^4}\left(\frac{\eta - \eta_0}{c}\right)^2 \qquad \text{(Eq. 2)}$$

where η_0 and η are the refractive indexes of the solvent and solution, respectively; N is Avogadro's number (6.023×10^{23}); and λ is the wavelength of light employed for the light-scattering measurements. A plot of $H(c/\tau)$ *versus* concentration generally produces a straight line at low concentrations, where the Van't Hoff equation is valid. The intercept is equal to the reciprocal of the average molecular weight.

In Figs. 10 and 11, $H(c/\tau)$ is plotted as a function of the amide concentration. As shown in Fig. 10, the curve for pimelamide decreases, passes through a minimum, and then increases. Such nonideal behavior was reported by Robins and Thomas (24) for dodecylaminoethanol salts and by Herrmann *et al.* (28). Extrapolation of the data below 1 g./100 ml. using least-squares treatment results in an average molecular weight of approximately 14,000. For suberamide, the curve closely resembles that obtained for polymers and extrapolation of the data gives an average molecular weight of 11,000.

As shown in Fig. 11, the plot for azelamide has a negative slope with considerable fluctuations in the turbidity values at dilute concentration. The curve resembles that for bovine serum albumin in water (30), with the curvature and "scattering" of points at dilute concentrations considered to be routine occurrences and attributed to the activity of the solvent and various charge factors. The average molecular weight for azelamide appears to be in the range of 160,000.

For sebacamide, extrapolation of the data below 1 g./100 ml. gives a molecular weight of 9100, which is similar to that for suberamide at infinite concentration. However, unlike suberamide, further association of aggregation appeared to occur as the concentration of sebacamide was increased. An initial negative slope was found, with a minimum in the curve occurring at approximately 0.8 g./100 ml. The results of the light-scattering measurements are listed in Table II.

The deviation from linearity of the light-scattering curves was discussed previously for polymers (27, 31, 32) and can be attributed to one or more of the following:

1. Interparticulate interference, giving rise to dissymmetry; *i.e.*, the ratio of the intensity scattered at two angles symmetrical about 90° (33).
2. A distribution of molecular weights, giving rise to depolarization (34); *i.e.*, the molecules are no longer isotropic.
3. The presence of an impurity.
4. Continued association of polymer molecules.
5. The equation is valid only at very dilute concentrations.

Dissymmetry became a problem above a concentration of 1.5–2.0 g./100 ml. Because of the disproportionate increase of scattering intensities at angles of 45 and 135° as the amide concentrations were increased, it was not possible to extend the light-scattering measurements to a concentration where the breaks in the apparent surface tension curves, partition isotherms, and glutethimide solubility profiles occurred.

Table II—Light-Scattering Results for Tetramethyl-Substituted Amides at 436 nm.

	dn/dc[a]	$\left[H\frac{c}{\tau}\right]_{c=0}$	$\overline{MW}$	N[b]
Pimelamide	0.162	6.9×10^{-5}	14,000	65
Suberamide	0.164	9.0×10^{-5}	11,000	48
Azelamide	0.164	6.2×10^{-6}	160,000	660
Sebacamide	0.137	1.1×10^{-4}	9,100	36

[a] Refractive index increment. [b] Number of monomers comprising the aggregate.

It was felt that, at the dilute concentrations, dissymmetry would be negligible and the depolarization caused by large molecules, a mixture of different molecular sizes, or concentration effects would not be significant. While it may not be possible to estimate average molecular weights by such treatment, the significance of the light-scattering data is that the amides appear to exist as associated molecules at very dilute solutions. To gain insight into the orientation and size of these aggregates, the influence of time, temperature, and electrolytes on the light-scattering measurements must be considered. Without such measurements, it is possible only to conjecture when trying to explain the light-scattering curves. The presence of maxima, minima, and curvature in the light-scattering curves was reported by several researchers (24, 28, 30, 32, 35). Robins and Thomas (24) showed that, for the iodide and nitrate salts of 2-dodecylaminoethanol, maxima in the curves occurred at a salt concentration of approximately 1.5% followed by a broad minimum at approximately 3% concentration. The hypothesis proposed for this behavior was that, at just above the CMC, the micelles were spherical in shape and the positive slope was due to solvent–solute interactions. At the inflection point in the curve, the micelles became more asymmetrical in shape and the negative slope was attributed to solute–solute interaction, *i.e.*, growth of the micelles. The increase in turbidity over this range was greater than the solute–solvent interaction effect and, consequently, the slope was negative. The upward curvature in the curves following the minima was believed to be due to a less rapid growth in the size of the micelles to the extent that the solute–solvent interaction had greater influence.

Curves for sebacamide, azelamide, and pimelamide show negative slopes, with the sebacamide and pimelamide curves exhibiting minima at approximately 1.0% concentration followed by an increase in the specific reciprocal turbidity function. On the basis of this hypothesis, it is possible that association of the amides continues to occur at dilute amide concentration due to strong solute–solute interaction. The inflection in the curve occurs when the solute–solvent interaction becomes greater than the affinity of the amide molecules to associate further.

No attempt was made in this study to correct for dissymmetry or depolarization, even though the data show that dissymmetry was occurring and became significant as the concentration of amide was increased. Consequently, the data in this study are subject to error, but this should be of a low order of magnitude at the dilute concentration. It is unlikely that this behavior can be attributed to the presence of an impurity since GC, thermal, and elemental analyses show the amides to be at least 99% pure.

Table III—Solubilization of Glutethimide with Tetramethyl-Substituted Amides at 30°

Amide Concentration, M	—Glutethimide Solubility ($M \times 10^3$) in Presence of— Pimelamide, $M \times 10^3$	Suberamide, $M \times 10^3$	Azelamide, $M \times 10^3$	Sebacamide, $M \times 10^3$
0.0[a]	4.60	4.60	4.60	4.60
0.04	5.20	4.60	4.85	4.60
0.10	6.06	4.60	5.70	4.60
0.25	8.23	4.70	7.50	8.56
0.40	11.3	11.9	—	17.62
0.50	—	16.6	18.0	22.22
1.0	29.1	34.5	39.5	
2.0	103.4	138.0	230.0	
2.4	—	330.0	—	
2.8	318		—	
3.0	399		700	
3.9	1343		1472	

[a] Solubility of 2-ethyl-2-phenylglutarimide in water (1.0 mg./ml.).

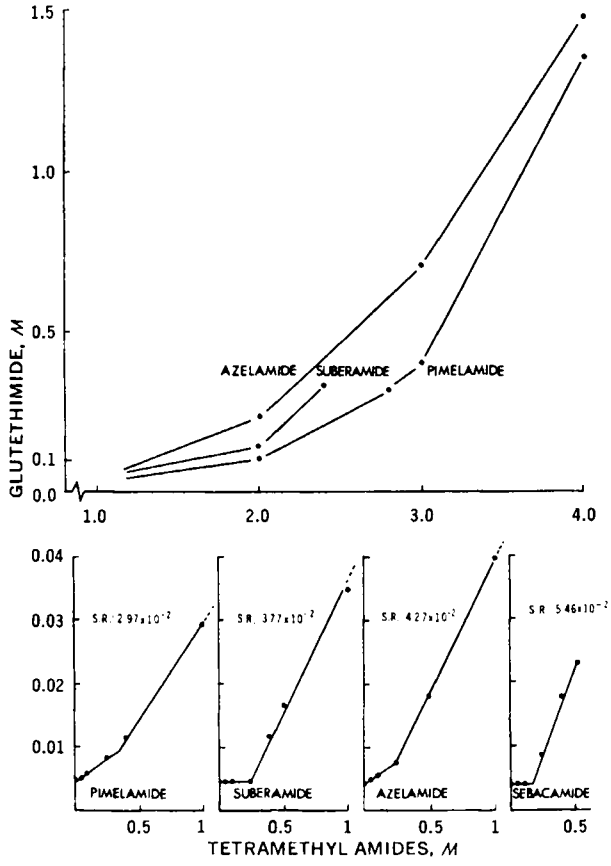


Figure 12—*Solubility curves for glutethimide with tetramethyl-substituted amides at 30°. Lower curves show the solubility over an amide concentration of 0–1* M, *and the upper curves show the solubility above 1* M.

Solubilization of Glutethimide—The data presented in Table III illustrate the influence of the substituted amides on the solubility of glutethimide. For the even-carbon amides below a concentration of 0.25 *M*, there appeared to be no significant increase in glutethimide solubility; with the odd-carbon amides, interaction did occur. However, a substantial increase in the solubility of the drug was not observed until above 0.4 *M* concentration of the amides. The increase in solubility of glutethimide parallels the increase in chain length of the amides but, because of the greater solubility of the odd-carbon amides, it is possible to attain higher levels of glutethimide with pimelamide and azelamide.

The data in Table III are represented graphically in Fig. 12, where the amount of glutethimide solubilized is plotted as a function of amide concentration. To permit the full range of glutethimide levels reached to be plotted on a single graph, the *y* axis is broken. The solubilization at low amide concentrations is shown by the lower curves for the four amides, while the upper curves depict what occurs at higher amide concentrations. With the possible exception of sebacamide, it is evident from the nonlinearity of the plots that glutethimide solubility is not directly proportional to the amide concentration over the entire solubility range. However, for all the amides there appears to be a linear relationship over an amide concentration of 0.2–1.0 *M*.

The solubilizing capacity of the amides for glutethimide is represented on the lower graphs by the saturation ratios, *i.e.*, moles of glutethimide per mole of amide, over the concentration range from the break in the curve up to 1.0 *M* amide. It was not possible to calculate saturation ratios above 1 *M* amide concentration due to the nonlinearity of the curves. Above 1 *M* amide concentration, the amount of glutethimide solubilized increased significantly with an increase in amide concentration. At 3.9 *M* azelamide, a concentration of 320 mg./ml. of glutethimide was attained, representing

a 320-fold increase from that in water; for pimelamide at the same concentration, 292 mg. of glutethimide was solubilized per milliliter.

The shape of the solubility curves suggests that two types of interactions are involved; one type occurs below some critical concentration of amide and a second, more pronounced interaction, predominates above the critical concentration. The interaction that occurs between glutethimide and azelamide and pimelamide prior to the break in the curve was found to be of low order. The fact that the even-carbon amides, sebacamide and suberamide, do not interact with glutethimide below the critical point might be attributed to either a preferred orientation of the odd-carbon amides or, as indicated by light-scattering data, the existence of larger aggregates for the odd-carbon amides giving rise to a greater hydrophobic center.

Of greater significance is the interaction with glutethimide occurring at higher amide concentrations. The nature of the interaction and the break in the surface tension curves suggest micelle formation with higher order complexes forming above the critical point. It appears that at higher amide concentrations, above 1.0 *M*, the drug may be participating in or influencing the aggregate structure or that further aggregation of the amides occurs which gives rise to larger or more densely oriented hydrophobic centers. In any event, the solubility of glutethimide in azelamide and suberamide was enhanced significantly at a concentration where the second break in the surface tension curves occurred.

Preliminary toxicity screening of the amides revealed that the toxicity by the intravenous route for pimelamide and suberamide was similar or slightly less than that for dimethylacetamide and *N*-methylacetamide, while azelamide and sebacamide appeared to be slightly more toxic. However, due to the existence of large associated molecules at low concentration, passage across the lipid membrane may be restricted and the amides may find application for solubilizing drugs for oral and topical administration. Absorption studies are presently being contemplated, but an oil–water partition study revealed that the oil–water partition coefficient is extremely low and both the toxicity and oil partitioning appear to increase with an increase in chain length. Further toxicity and tissue tolerance experimentation will be conducted.

SUMMARY AND CONCLUSIONS

The tetramethyl-substituted amides of sebacamide, azelamide, suberamide, and pimelamide appear to possess the potential of solubilizing hydrophobic molecules due to their strong association tendencies, as indicated by partitioning studies, surface tension measurements, light-scattering measurements, and solubility studies. The nature of the partition isotherms, surface tension curves, and solubility curves for glutethimide suggests the existence of aggregates or micelles of the amides, and possibly the solubilization of glutethimide can be attributed to a micellar type of interaction.

While it is conceivable, as indicated by surface tension measurements, that a micellar type of aggregation occurs at some critical concentration, the light-scattering measurements strongly suggest the existence of large associated molecules at very dilute concentrations. The scattering intensity was of a magnitude similar to that for polymer solutions, and the intensity increased significantly with an increase in amide concentration. The odd-carbon amides, azelamide and pimelamide, show greater light-scattering intensity, greater lowering of surface tension, and higher solubility in water than the even-carbon amides, suberamide and sebacamide. Additionally, unlike the solubility observed for the even-carbon amides, the solubility of the odd-carbon amides was not reduced by an increase in ionic strength and they interacted slightly with glutethimide at low concentration. The further lowering of surface tension following a plateau area in the surface tension curves for two of the amides, azelamide and suberamide, suggests either that the aggregates formed are surface active or that a reorientation at the surface is occurring.

If the amides are of a size that hinders penetration of semipermeable membranes, they may find application in drug delivery systems as implants as well as being used as dialysis solutions in cases of glutethimide intoxication. Considerably more work is needed to determine the potential value of the amides as solubilizing agents and vehicles in drug delivery systems.

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Assay of Quaternary Ammonium Compounds in Various Dosage Forms by Acid-Dye Method

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Abstract □ An organic dye salt partition technique was employed to estimate quantitatively some medicinally important quaternary ammonium compounds. Methyl orange, bromthymol blue, and orange IV were used as color-forming agents for the quaternary ammonium agents. The intensity of the color was measured spectrophotometrically. Linear relationships between concentration and absorbance were obtained for the majority of quaternary ammonium substances investigated. By using the calibration curves obtained, a quantitative procedure was developed for pharmaceutical dosage forms. Satisfactory results were obtained with the majority of dosage forms investigated. The tablet excipients studied had negligible effects on the procedure. The results obtained with this method were compared to results by an official procedure or the manufacturer's method, where possible.

Keyphrases □ Quaternary ammonium compounds (16) in 23 dosage forms—analysis, acid-dye technique □ Acid-dye technique—analysis, 16 quaternary ammonium compounds in 23 dosage forms □ Colorimetry—use of acid dyes as color-forming agents for analysis of quaternary ammonium compounds □ Complex formation—acid dyes–quaternary ammonium compounds, spectrophotometric analysis in dosage forms

In 1943, Auerbach (1) described a colorimetric method of analysis in which buffered solutions of quaternary ammonium compounds were treated with bromphenol blue and the ion-pair was extracted with ethylene dichloride. Colichman (2) analyzed two surfactants by a direct photometric titration, while Ballard *et al.* (3) determined several quaternary ammonium compounds by colorimetric measurement of the salt that the drugs formed with bromthymol blue.

Two photometric methods were presented by Helgren *et al.* (4) in 1957 for the analysis of hexocyclium methosulfate. Bromthymol blue was used as the color-producing agent in one instance, and ammonium cobaltothiocyanate was used in the other. Santoro (5) selectively determined isopropamide iodide in the presence of amine bases by treating it with methyl orange in a buffer at pH 10.2.

The most intensive work in this field has been by Schill and his coworkers. They published a series of papers on the photometric determination of amines and quaternary ammonium compounds with bromthymol blue (6–8). Results also were published on the determination of quaternary ammonium compounds with hexanitrodiphenylamine (9) and by their picrates (10). However, these methods were nonspecific because amines would react under the same conditions. In a more recent investigation, Irving and Markham (11) utilized bromcresol green as a color-forming agent in the analysis of long-chain, tertiary alkylamines and quaternary ammonium salts. Unfortunately, the blank is dependent both on pH and on concentration of the excess reagent.

UV spectroscopy has been employed by several authors as a means of analyzing certain quaternary ammonium salts and some of their dosage forms (12–15). In addition, the BP (16), USP XVIII (17), and NF XIII (18) employ this technique for a limited number of quaternary compounds.

In 1954, Carkhuff and Boyd (19) assayed cetylpyridinium chloride by titrating it with sodium lauryl sulfate in a two-phase system. NF XIII (18) describes a similar titrimetric method for benzethonium and cetylpyridinium chlorides, which also employs a two-phase system with sodium tetraphenylboron as titrant and bromphenol blue as indicator. NF XIII (18) also extensively employs nonaqueous titrimetry in glacial acetic acid as a means of assaying a substantial number of quaternary ammonium salts. In addition, a variety of titrimetric techniques are employed for various dosage forms. Billow and Baker (20) proposed a procedure for benzethonium chloride which is similar to that of NF XIII but which was carried out in a one-phase system. Eksborg *et al.* (21) reported the determination of emepronium bromide in urine by an ion-pair extraction method.

Although several methods have been employed in the analysis of quaternary ammonium substances, disadvantages were found to exist in all of them. The UV method is sensitive and rapid; however, interferences from tablet excipients occur in many instances. Most volumetric and gravimetric methods require large samples and cannot be applied to many dosage forms.

Therefore, the purpose of this investigation was to employ a sensitive and rapid method of analysis that could be applied to a large number of dosage forms whose active ingredient contains the quaternary ammonium functional group. A colorimetric method involving reaction of the medicinal agents with various acid dyes was selected.

EXPERIMENTAL[1]

Chemicals—The following were used: chloroform, methylene chloride, glacial acetic acid, ether, hydrochloric acid, potassium chloride, potassium acid phthalate, sodium hydroxide, potassium phosphate, and boric acid. All were ACS grade.

[1] A Beckman model B spectrophotometer, a Beckman model DB spectrophotometer, a Beckman Zeromatic II pH meter, magnetic stirring apparatus, and conventional laboratory glassware were used.

Table I—Conditions for Quantitative Assay of Commercial Quaternary Ammonium Dosage Forms by the Organic Dye Salt Partition Method

Quaternary Ammonium Compound	Molecular Weight	Amount of Compound in Assay Solution Aliquot, μmoles	Dye Used	Optimum pH	Optimum Wavelength for Measurement, nm.	Procedure Used	Modification of General Procedure for Dosage Forms
Ambenonium chloride	608.5	0.24	Orange IV	6.0	410	B (tablets)	None
Benzethonium chloride	466.1	0.36	Methyl orange	8.0	415	A (solution)	See text
Cetylpyridinium chloride	358.0	0.42	Methyl orange	7.0	405	A (solution)	See text
Demecarium bromide	716.6	0.21	Orange IV	10.0	415	B (solution)	None
Domiphen bromide	414.5	0.36	Methyl orange	8.0	415	A (tablets)	Lozenges assayed by tablet method, powder treated as calibration curve
Edrophonium bromide	246.2	0.60	Bromthymol blue	7.0	410	C (solution)	None
Hexocyclium methosulfate	428.0	0.35	Methyl orange	8.0	410	A (tablets)	None
Isopropamide iodide	480.4	0.31	Methyl orange	8.0	415	A (tablets)	None
Methantheline bromide	420.3	0.35	Methyl orange	8.0	415	A (tablets)	None
Oxyphenonium bromide	428.4	0.35	Methyl orange	8.0	415	A (tablets)	None
Penthienate bromide	420.4	0.35	Orange IV	6.0	405	B (tablets)	None
Penthienate bromide			Orange IV	6.0	405	B (solution)	None
Pentolinium tartrate	538.6	0.27	Bromthymol blue	7.0	410	C (tablets)	See text
Pentolinium tartrate			Bromthymol blue	7.0	410	C (solution)	See text
Pipenzolate bromide	434.4	0.34	Orange IV	8.0	410	B (tablets)	None
Propantheline bromide	448.4	0.33	Methyl orange	8.0	410	A (tablets)	See text
Propantheline bromide			Methyl orange	8.0	410	A (solution)	See text
Pyridostigmine bromide	259.1	0.57	Bromthymol blue	7.0	410	C (tablets)	None
Trimethidinium methosulfate	490.7	0.30	Bromthymol blue	7.0	410	C (tablets)	None

Reagents—The following were used: 0.0001 *N* methyl orange[2], 0.0001 *N* orange IV[2] (tropaeolin OO), 0.0001 *N* orange II[2], 0.0001 *N* bromphenol blue[2], 0.0001 *N* bromthymol blue[2], 0.05 *N* perchloric acid in dioxane (standardized against potassium acid phthalate), 0.02 *M* sodium tetraphenylboron, 6% mercuric acetate, crystal violet indicator (0.5% in glacial acetic acid), methyl red indicator (0.1% in glacial acetic acid), and bromphenol blue solution (1 in 2000).

Quaternary Ammonium Substances—See Table I.

Analysis of Crystalline Quaternary Ammonium Materials—The purity of most of the crystalline materials was determined by a well-recognized, nonaqueous titrimetric technique.

The following substances were analyzed by a method similar to that employed in USP XVIII (17) for demecarium bromide: bethanechol chloride, demecarium bromide, isopropamide iodide, mepenzolate bromide, penthienate bromide, pentolinium tartrate, and pipenzolate bromide. For edrophonium bromide, methyl red was a more suitable indicator than crystal violet.

For the following substances, chloroform was the solvent and methyl red the indicator: benzethonium chloride, cetylpyridinium chloride, domiphen bromide, hexocyclium methosulfate, methantheline bromide, oxyphenonium bromide, propantheline bromide, pyridostigmine bromide, and valethamate bromide.

Ambenonium chloride and trimethidinium methosulfate were assayed by their respective NF XIII (18) procedures.

Selection of Color-Forming Agent—Solutions of 0.0001 *N* were prepared from each dye and were buffered at pH values of 2, 4, 6, 8, and 10, respectively. Five milliliters (0.5 mmole) of the quaternary ammonium substance was added to 10 ml. of each buffered dye solution contained in a 250-ml. separator. The ion–ion colored species was extracted with 10 ml. of methylene chloride, and the organic layer was drawn into a cell. The cells were stoppered, and absorbance measurements were taken at regular intervals for 120 min., at the wavelength of maximum absorbance. This wavelength was employed for all subsequent determinations of that particular substance.

[2] These dye solutions were buffered with Clark and Lubs phosphate buffers to the appropriate pH.

Calibration Curves—*General Procedure*—Fifty milligrams of the quaternary ammonium compound was accurately weighed into a 100-ml. volumetric flask and dissolved in distilled water. The solution was made up to volume with distilled water, and 10 ml. of it was diluted to 100 ml. to produce a 0.05-mg./ml. solution.

To five 250-ml. separators, each containing 10 ml. of a 0.0001 *N* solution of the dye, were added 1, 2, 3, 4, and 5 ml. of the 0.05-mg./ml. solution of the quaternary ammonium compound. Sufficient distilled water was added to make the volume constant in each separator. Twenty-five milliliters of methylene chloride was then added to each separator from a 50-ml. buret. The separator was shaken vigorously for 1 min., and the system was allowed to equilibrate for 10 min. The inside of the stem was wiped clean with adsorbent paper. The organic phase was then drawn into a cell, and the absorbance was measured at the optimum wavelength, using a methylene chloride blank. The procedure was repeated five times and the calibration curve was constructed from the average.

Procedure A—The general procedure was employed at pH 8.0 with methyl orange as the color-forming agent for those substances so designated in Table I. For cetylpyridinium chloride and propantheline bromide, only 20 ml. of methylene chloride was used to extract the colored species.

Procedure B—This procedure employed orange IV as the color-forming agent and was followed for those substances so designated in Table I. For ambenonium, 20 ml. of orange IV at pH 6.0 was used. The equilibration time, however, was 30 min. and the organic phase was drawn into a 25-ml. volumetric flask and the contents were shaken.

Demecarium required a 20-min. equilibration time and also 10 min. to stabilize the color in the cell prior to taking absorbance measurements. The general procedure was followed for penthienate, and again a 10-min. period was allowed for color stabilization prior to measuring the absorbance.

Procedure C—The general procedure was employed with 20 ml. of bromthymol blue at pH 7.0 as the color-forming agent for the substances so designated in Table I.

Analysis of Pharmaceutical Dosage Forms—*General Procedure for Tablets*—Ten tablets were weighed and finely powdered. A sample of powder equivalent to 5 mg. of the quaternary ammonium compound was weighed into a 150-ml. beaker and stirred magnetically

for 15 min. with 40 ml. distilled water. This solution was suction filtered through Whatman No. 1 paper into a 125-ml. flask. The beaker and residue were washed with a further 30 ml. of distilled water, and the filtrate was made to volume in a 100-ml. volumetric flask. Three milliliters of this solution (theoretically 0.05 mg./ml.) and 2 ml. of distilled water were added to the dye solution employed in the preparation of its calibration curve. The remainder of the procedure was identical to that described for the calibration curve of each particular substance.

General Procedure for Injections and Solutions—A suitable volume of the injection was diluted to yield a theoretical concentration of 0.05 mg./ml. Three milliliters of this solution was added to the appropriate dye solution. The remainder of the procedure was identical to that described for the calibration curve of each particular substance.

For many of the dosage forms, the general procedures, as previously outlined, can be followed. The conditions regarding dye, pH, wavelength, *etc.*, are presented in Table I. The following are modifications required for the assay of certain pharmaceuticals.

Benzethonium Chloride—Ten milliliters of benzethonium chloride tincture (1:500) was pipeted into a 150-ml. beaker containing 10 ml. of distilled water. This solution was heated on a water bath to evaporate the acetone and alcohol. After cooling, this solution was made to 100 ml. in a volumetric flask. Twenty-five milliliters of the resulting solution was diluted to 100 ml., yielding a theoretical concentration of 0.05 mg./ml. of benzethonium. Three milliliters of this solution and 2 ml. of distilled water were added to 10 ml. of 0.0001 *N* methyl orange (pH 8.0). The remainder of the procedure was identical to that described for the calibration curve beginning with: "Twenty-five milliliters of methylene chloride. . . ."

Cetylpyridinium Chloride—

1. Ten milliliters of cetylpyridinium chloride solution (1:2000) was pipeted into a 150-ml. beaker and heated over a steam bath to evaporate the ethanol. The solution was cooled to room temperature and then made to 100 ml. in a volumetric flask. Three milliliters of this solution and 2 ml. of distilled water were added to 10 ml. of 0.0001 *N* methyl orange at pH 8.0 in a 250-ml. separator. The remainder of the procedure was identical to that described for the calibration curve beginning with: "Twenty-five milliliters of methylene chloride. . . ."

2. Five cetylpyridinium chloride lozenges were weighed and finely powdered. A sample equivalent to 2.5 mg. of cetylpyridinium chloride was weighed in a beaker and stirred magnetically for 30 min. with 25 ml. distilled water. The solution was made to volume in a 50-ml. volumetric flask. Three milliliters of this solution and 2 ml. of distilled water were added to 10 ml. of 0.0001 *N* methyl orange at pH 8.0 in a separator. The remainder of the procedure was identical to that described for the calibration curve beginning with: "Twenty-five milliliters of methylene chloride. . . ." A blank was also determined, replacing the dye solution with 10 ml. of distilled water.

Pentolinium Tartrate—

1. The general procedure for tablets was used for pentolinium tartrate tablets (40 mg.), except a solution of theoretical concentration 0.5 mg./ml. was prepared first.

2. Five milliliters of pentolinium tartrate injection was pipeted into a 250-ml. volumetric flask and made to volume with distilled water. To 20 ml. of 0.0001 *N* bromthymol blue at pH 7.0 were added 1.5 ml. of the diluted injection and 3.5 ml. of distilled water. The remainder of the procedure was identical to that described for the calibration curve beginning with: "Twenty-five milliliters of methylene chloride. . . ."

Propantheline Bromide—

1. Ten propantheline bromide tablets (7.5 mg.) were weighed and finely powdered. Fifty milligrams of the powdered material was weighed in a 150-ml. beaker and stirred magnetically for 30 min. with 25 ml. of methylene chloride. The solution was filtered through Whatman No. 1 paper, and the residue was washed with a further 10 ml. of methylene chloride. The filtrate was evaporated to dryness and the residue was dissolved in water and made to volume in a 100-ml. volumetric flask. The remainder of the procedure was identical to that described for its calibration curve.

2. The procedure for 15-mg. propantheline bromide tablets was identical to that described for the 7.5-mg. tablets, except that only 25 mg. of the powdered tablets was used.

3. The general procedure for tablets was used for 30-mg. propantheline bromide tablets.

Table II—Analysis of Commercial Pharmaceutical Dosage Forms

Compound	Average Recovery, %	Average Recovery, % Comparative Method
Ambenonium chloride tablets		
10 mg.	102.9 ± 0.9	95.20[a]
25 mg.	100.2 ± 1.3	94.52[a]
Benzethonium chloride:		
Solution	98.9 ± 0.9	101.26[b]
Tincture	98.8 ± 0.7	95.00[a]
Cetylpyridinium chloride:		
Solution	97.2 ± 1.0	Not available
Lozenge	102.7 ± 1.9	
Demecarium bromide ophthalmic solution	115.3	
Domiphen bromide:		
Lozenge	97.1 ± 0.9	103.33[a]
Powder	100.2 ± 1.1	99.81[c]
Edrophonium bromide injection	100.8 ± 1.1	99.57[b]
Hexocyclium methosulfate tablets with phenobarbital	102.0 ± 1.3	101.45[a]
Methantheline bromide tablets, 50 mg.	102.9 ± 0.9	Not available
Oxyphenonium bromide tablets		
5 mg.	100.1 ± 1.2	98.80[a]
10 mg.	100.5 ± 1.1	100.00[b]
Penthienate bromide:		
Tablets, 5 mg.	102.1 ± 0.8	102.40[b]
Elixir	98.4 ± 0.9	108.00[a]
Pentolinium tartrate:		
Tablets, 40 mg.	101.4 ± 0.7	Not available
Injection	102.8 ± 1.4	100.76[a]
Pipenzolate bromide tablets, 5 mg.	95.8 ± 1.3	Not available
Propantheline bromide tablets		
7.5 mg.	96.4 ± 1.0	Not available
15 mg.	101.7 ± 1.4	
30 mg.	99.4 ± 0.7	
Injection	103.7 ± 0.1	
Pyridostigmine bromide tablets, 60 mg.	97.8 ± 0.4	96.22[b]
Trimethidinium methosulfate tablets, 20 mg.	86.5 ± 2.0	Not available

[a] Analytical data supplied by manufacturer. [b] Analysis by official procedure. [c] Analysis by nonaqueous titration.

4. The contents of the propantheline bromide injection ampul were transferred to a beaker and weighed. Distilled water was added to dissolve the powder, and the solution was transferred to a 100-ml. volumetric flask and made to volume. Appropriate dilutions were made to ensure that the absorbance would fall within the limits of the calibration curve. The remainder of the procedure was identical to that described for its calibration curve.

RESULTS AND DISCUSSION

The results for the analyses of the crystalline materials indicated that they were of high purity, and all were used without further purification.

The dyes chosen for the investigation were bromphenol blue, bromthymol blue, orange IV, orange II, and methyl orange. In most instances, the wavelength of maximum absorbance was the same for all ion-ion-pairs involving one particular dye. Methyl orange was found generally to give colored species with high absorbances and greatest stability. Therefore, it was employed where possible.

Methylene chloride was found to be a more suitable solvent than chloroform. In most instances, 25 ml. was found to be a sufficient volume to extract completely the ion-ion-pair species. Blank determinations on the dyes alone indicated negligible absorbances.

When the most suitable dye for each quaternary compound was selected, the optimum wavelength for each colored species and the optimum pH of the aqueous media were determined (Table I). Linear calibration curves were obtained for all pure substances except bethanechol, isopropamide, and mepenzolate.

Table III—Molar Absorptivities of Quaternary Ammonium Compounds

Compound	Molecular Weight	$M \times 10^{-5}$	Absorbance	Absorptivity ($\times 10^4$)
Benzethonium[a]	466.09	1.28	0.408	3.19
Cetylpyridinium[a]	357.99	2.09	0.593	2.84
Domiphen[a]	414.46	1.45	0.470	3.24
Hexocyclium[a]	428.61	1.40	0.330	2.36
Isopropamide[a]	480.42	1.25	0.321	2.57
Methantheline[a]	420.34	1.43	0.394	2.76
Propantheline[a]	448.42	1.67	0.504	3.02
Oxyphenonium[a]	428.41	1.40	0.346	2.47
Echothiophate[b]	383.23	2.09	0.264	1.26
Edrophonium[b]	246.15	3.25	0.704	2.17
Pentolinium[b]	538.58	1.49	0.597	4.01
Pyridostigmine[b]	261.14	3.06	0.457	1.49
Trimethidinium[b]	490.67	1.63	0.695	4.26
Ambenonium[c]	608.50	1.31	0.497	3.79
Pipenzolate[c]	434.39	1.84	0.483	2.63
Penthienate[c]	420.41	1.90	0.557	2.93
Demecarium[c]	716.61	1.12	0.541	4.83

[a] Reacted with methyl orange. [b] Reacted with bromthymol blue. [c] Reacted with orange IV.

Before the pharmaceutical dosage forms were analyzed, a study on the effect of common tablet excipients was performed. In this investigation, a quantity of the tablet excipient was added to 10 ml. of the appropriate dye and extracted with methylene chloride. The absorbance of the organic phase was measured, and the results showed that the absorbance due to these tablet excipients was negligible. Other common ingredients used as tablet excipients are kaolin, starch, magnesium stearate, and talc. However, all of these compounds are water insoluble and would be filtered out in the procedure. One of the products contained phenobarbital as an active constituent. This drug was also treated with the appropriate dye (methyl orange) and extracted with methylene chloride. The result produced negligible absorbance.

The results for the analysis of the dosage forms are presented in Table II. Where official procedures existed, they were used as a comparative means of analysis. Good results were obtained with the majority of dosage forms, and generally good agreement was noted with the comparative method.

A general procedure could be employed for most analyses, but slight modifications were required in some instances. For the majority of tablets, extraction of the powdered material was made by distilled water. For 7.5- and 15-mg. propantheline bromide tablets, which are sugar coated, methylene chloride was utilized as the extracting solvent, since extraction with distilled water gave poor recoveries.

In some analyses, blank determinations of the sample without the dye were required because the sample was colored. Both benzethonium chloride tincture and penthienate bromide elixir gave no absorbance when extracted with methylene chloride. This indicated that the absorbance would be entirely due to the colored species formed between the quaternary ammonium and the dye in these instances.

Satisfactory results could not be obtained with the dosage forms of demecarium, isopropamide, and trimethidinium. The high results obtained for the dosage forms of isopropamide may be attributed to the calibration curve, which was not entirely linear, or to decomposition of the drug.

The high recoveries obtained for demecarium bromide ophthalmic solution (0.25%) can be explained by the fact that this preparation contained benzalkonium chloride as a preservative[3]. Since this method cannot differentiate between quaternary ammonium compounds, the benzalkonium would also react with the dye. The result would be overestimation.

An explanation for the low recoveries obtained with trimethidinium methosulfate tablets is not apparent, because the calibration curve for the crystalline material was linear and reproducible.

[3] D. F. Solometo, Merck Sharp & Dohme Laboratories, Philadelphia, Pa., personal communication.

A study of the molar absorptivities of the quaternary ammonium compounds shown in Table III gave more indication of the stoichiometry of the reaction. In those instances where methyl orange was the dye of choice, the cationic portion contains one quaternary nitrogen and shows approximately the same molar absorptivity. With bromthymol blue and orange IV, the bisquaternary compounds show molar absorptivities that are approximately twice that of the monoquaternary compounds. This would, therefore, indicate a dye–quaternary stoichiometry of 2:1 for the bisquaternary compounds.

SUMMARY AND CONCLUSIONS

1. A study on various acid dyes as color-forming agents for quaternary ammonium compounds was presented. Methyl orange, orange IV, and bromthymol blue were the most satisfactory.

2. Linear and reproducible calibration curves were obtained for 16 quaternary compounds. Of the compounds investigated, only bethanechol, isopropamide, and mepenzolate failed to show a linear relationship.

3. The effect on the procedure by common tablet excipients was found to be negligible.

4. A quantitative method of analysis was developed for 23 pharmaceutical dosage forms containing a quaternary ammonium compound. The procedure is very sensitive and was adaptable to the various types of dosage forms investigated.

5. On the basis of the present investigation, it is concluded that the proposed method is generally superior to existing methods. It is recognized that the UV method is equally satisfactory except that it is more prone to error by interferences than is the colorimetric technique.

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Microdetermination of Hyoscyamine and Scopolamine in Mixtures

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Abstract □ Following the acid-dye technique, hyoscyamine (or atropine) could be selectively determined in the presence of scopolamine, using bromcresol purple at pH 6.6. Total alkaloids were determined using bromthymol blue at pH 5.6. By referring to calibration curves of the two alkaloids with the appropriate dye, the concentration of each alkaloid in the mixture could be computed. The suggested procedure was adopted to analyze the two alkaloids in synthetic mixtures. As low as 0.05 mg. of each alkaloid could be estimated, with average percentage recoveries of 98.6 and 101.5 for hyoscyamine and scopolamine, respectively. Tincture of belladonna was assayed following a modification of the suggested method. The results obtained were comparable with those obtained following the BP method.

Keyphrases □ Hyoscyamine mixtures with scopolamine—acid-dye analysis without prior separation, effect of pH and dye type □ Scopolamine mixtures with hyoscyamine—acid-dye analysis without prior separation, effect of pH and dye type □ Tropane alkaloid mixtures—acid-dye analysis of hyoscyamine and scopolamine □ Acid-dye technique—analysis of hyoscyamine and scopolamine in mixtures

Most reported methods for the determination of tropane alkaloids present in mixtures necessitate separation of the individual alkaloids (1–3). The acid-dye technique reported for the estimation of microquantities of atropine (4, 5) can be adopted to determine the individual alkaloids, without preliminary separation, if a proper choice is made regarding the dye used and the pH of the medium. Khalil and El-Masry (6) found that tropine did not interfere in the assay of atropine when using bromthymol blue at pH 3. The present article examines the effects of pH and selection of the dye on the assay of microquantities of hyoscyamine and scopolamine without prior separation.

EXPERIMENTAL

Materials—Both hyoscyamine sulfate[1] and scopolamine hydrobromide[1] were BP quality. For the dye solutions, bromcresol purple and bromthymol blue were separately dissolved in chloroform to produce 2×10^{-4} *M* solutions. For the buffer solutions, McIlvaine's buffer solutions of various pH values (3–7.5 ± 0.05) were used. Chloroform was reagent grade[1] and freshly distilled. Two lots[2] of belladonna tincture BP were used.

Method—The procedure described by Khalil and El-Masry (6) was followed. Into a 50-ml. separator containing 10 ml. of the buffer solution, a volume of the sample corresponding to about 0.05–0.1 mg. of either alkaloid was added. Then 10 ml. of the chloroformic dye solution was added. After manual shaking for 1 min., the layers were left for complete separation and the chloroformic layer was transferred into a 25-ml. volumetric flask. The aqueous layer was further extracted with 10 and 5 ml. of chloroform, and the color intensity of the combined chloroform extracts was measured at 420 nm. using a spectrophotometer[3] and a blank similarly prepared.

RESULTS AND DISCUSSION

Figure 1 shows the effect of pH on the extractability of the alkaloid–bromcresol purple complex. At all pH values studied, the hyoscyamine–dye complex gave comparatively higher absorbance values at 420 nm. compared with scopolamine. The latter was not assayable at pH values higher than 6.6 since the chloroformic phase was colorless. Due to the relatively high absorbance readings obtained at pH 6.6, hyoscyamine was thus selectively determined at this pH using bromcresol purple. A linear relationship was obtained over the concentration range used (Fig. 2). When using bromthymol blue, both alkaloids were complexed at all pH values studied. However, the calibration curve for hyoscyamine using bromthymol blue at pH 5.6 and that with bromcresol purple at pH 6.6 were almost identical. The slopes were 2.564 and 2.578, respectively (Fig. 2). Therefore, to simplify calculation, pH 5.6 was chosen for the

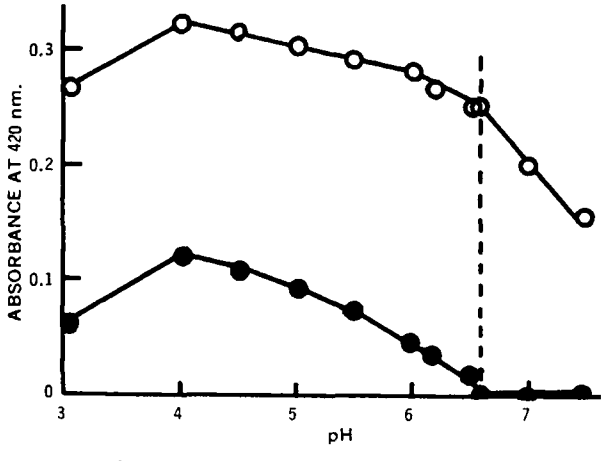

Figure 1—*Effect of pH on extraction of the alkaloid–bromcresol purple complex. Key: ○, hyoscyamine base; and ●, scopolamine base. The amount of the alkaloidal base used was 0.1 mg.*

[1] British Drug Houses Ltd., Poole, England.
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▲ To whom inquiries should be directed.

Microdetermination of Hyoscyamine and Scopolamine in Mixtures

SAWSAN EL-MASRY and SALEH A. H. KHALIL▲

Abstract □ Following the acid-dye technique, hyoscyamine (or atropine) could be selectively determined in the presence of scopolamine, using bromcresol purple at pH 6.6. Total alkaloids were determined using bromthymol blue at pH 5.6. By referring to calibration curves of the two alkaloids with the appropriate dye, the concentration of each alkaloid in the mixture could be computed. The suggested procedure was adopted to analyze the two alkaloids in synthetic mixtures. As low as 0.05 mg. of each alkaloid could be estimated, with average percentage recoveries of 98.6 and 101.5 for hyoscyamine and scopolamine, respectively. Tincture of belladonna was assayed following a modification of the suggested method. The results obtained were comparable with those obtained following the BP method.

Keyphrases □ Hyoscyamine mixtures with scopolamine—acid-dye analysis without prior separation, effect of pH and dye type □ Scopolamine mixtures with hyoscyamine—acid-dye analysis without prior separation, effect of pH and dye type □ Tropane alkaloid mixtures—acid-dye analysis of hyoscyamine and scopolamine □ Acid-dye technique—analysis of hyoscyamine and scopolamine in mixtures

Most reported methods for the determination of tropane alkaloids present in mixtures necessitate separation of the individual alkaloids (1–3). The acid-dye technique reported for the estimation of microquantities of atropine (4, 5) can be adopted to determine the individual alkaloids, without preliminary separation, if a proper choice is made regarding the dye used and the pH of the medium. Khalil and El-Masry (6) found that tropine did not interfere in the assay of atropine when using bromthymol blue at pH 3. The present article examines the effects of pH and selection of the dye on the assay of microquantities of hyoscyamine and scopolamine without prior separation.

EXPERIMENTAL

Materials—Both hyoscyamine sulfate[1] and scopolamine hydrobromide[1] were BP quality. For the dye solutions, bromcresol purple and bromthymol blue were separately dissolved in chloroform to produce 2×10^{-4} *M* solutions. For the buffer solutions, McIlvaine's buffer solutions of various pH values (3–7.5 ± 0.05) were used. Chloroform was reagent grade[1] and freshly distilled. Two lots[2] of belladonna tincture BP were used.

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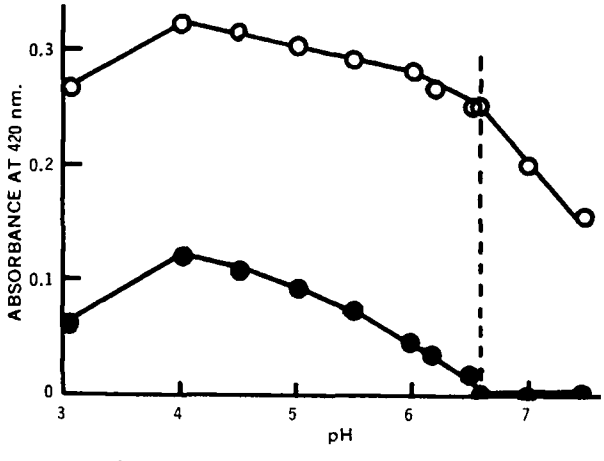

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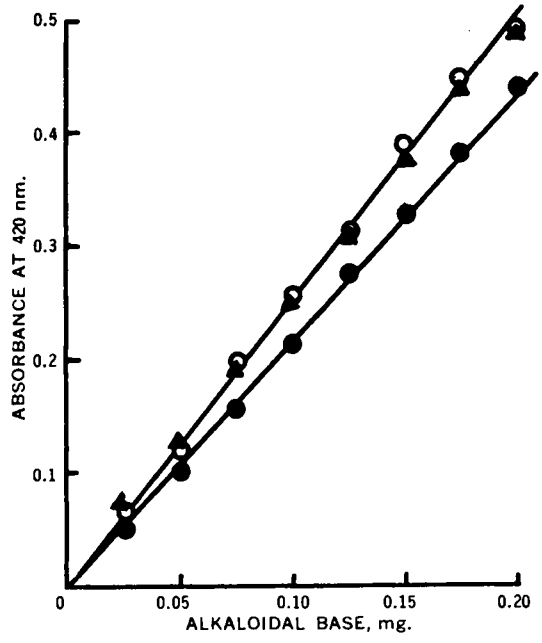

Figure 2—*Calibration curves of hyoscyamine (▲) and scopolamine (●) using bromthymol blue at pH 5.6 and of hyoscyamine (○) using bromcresol purple at pH 6.6.*

analysis of both alkaloids using bromthymol blue. From the slopes of the calibration curves shown in Fig. 2 for the two alkaloids, and since the absorbance values obtained for hyoscyamine complex were almost identical when using either dye, the following equations can be derived:

$$\text{milligrams hyoscyamine in mixture} = A/2.57 \quad \text{(Eq. 1)}$$

$$\text{milligrams scopolamine in mixture} = B - (A/2.18) \quad \text{(Eq. 2)}$$

where:

A = absorbance at 420 nm. of hyoscyamine-bromcresol purple complex at pH 6.6

B = absorbance at 420 nm. of complexes of both alkaloids with bromthymol blue at pH 5.6; 2.57 and 2.18 are the slopes of the curves (Fig. 2) for hyoscyamine and scopolamine, respectively, as determined by the method of least squares

The validity of Eqs. 1 and 2 was tested by assaying synthetic mixtures containing known amounts of hyoscyamine sulfate and scopolamine hydrobromide (Table I). As low as 0.05 mg. of either alkaloid could be determined, with percentage recoveries of 98.6 and 101.5 for hyoscyamine and scopolamine, respectively.

Determination of Hyoscyamine Content of Tincture of Belladonna—An improved procedure, suitable for the determination of hyoscyamine content in tincture of belladonna, was found. About 0.1–0.2 ml. of the tincture was added to a 50-ml. glass-stoppered separator containing 10 ml. of buffer solution (pH 6.6). A 10-ml.

Table I—Results of Analysis of Synthetic Mixtures of Hyoscyamine and Scopolamine[a]

Mixture Number	Compositions, mg. %[b] Hyoscyamine	Scopolamine	Found, mg. % Hyoscyamine	Found, mg. % Scopolamine
1	0	75	0	74.30
2	5	50	4.93	49.29
3	20	20	19.60	19.71
4	40	15	40.81	15.21
5	60	10	59.30	9.89
6	80	5	80.72	5.05
7	100	5	98.84	5.10
8	100	0	99.11	0

[a] Average of four replicates (±3%). [b] As alkaloidal base.

Table II—Results of Analysis of Two Batches of Belladonna Tincture BP by the BP Procedure and the Suggested Method

Batch	BP Method[a], mg. %	Suggested Method, Hyoscyamine, mg. %
A	30.1	29.6
B	28.8	28.2

[a] Total alkaloids calculated as hyoscyamine.

portion of chloroformic solution of bromcresol purple was added, and the mixture was manually shaken for 1 min. and left for complete separation (15 min.). After separation of the chloroformic phase, the aqueous layer was further extracted with 10 and 5 ml. of chloroform. The combined chloroformic extracts were treated with 0.1 *N* NaOH, and the liberated dye was measured at 580 nm. In this way, sensitivity was increased by a factor of about 2.4 (Fig. 3). Interference due to chlorophyll was also eliminated through the measurement of the violet color of the liberated dye. Tropine, one of the hydrolytic products of hyoscyamine, did not interfere in the assay following the above-mentioned procedure. This is an advantage over the procedure of Durick *et al.* (7), who used bromcresol purple at pH 5.3. Under this condition, it was found that tropine (6) and scopolamine (Fig. 1) interfered.

Two batches of belladonna tincture BP were assayed following the suggested method. The results obtained are compared in Table II with the data obtained following the BP procedure for the assay of belladonna tincture. The hyoscyamine contents of both batches, as determined by the suggested method, are in good agreement with the results obtained following the BP procedure for the total alkaloidal content. The application of Eqs. 1 and 2 failed to show any scopolamine in the tinctures, apparently because of the relatively minute amounts of scopolamine in the two tinctures studied; TLC testing[4] of the tinctures revealed no spots corresponding to scopolamine. The suggested method for the determination of hyoscyamine content in tincture of belladonna couples both simplicity and sensitivity, and only a small volume (0.1–0.2 ml.) is required.

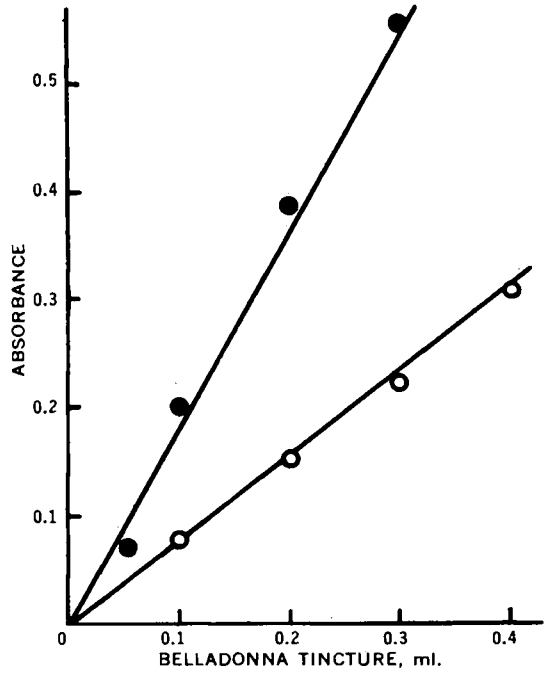

Figure 3—*Calibration curves of belladonna tincture (Batch A): (○) absorbance at 420 nm. of the yellow complex, and (●) absorbance at 580 nm. of the liberated dye after treatment with 0.1 N NaOH. The dye used was bromcresol purple at pH 6.6.*

[4] Using silica gel H as the adsorbent and a system of methanol (100 ml.) and ammonia 0.88 (1 ml.).

SUMMARY

1. Bromcresol blue at pH 6.6 forms a chloroform-extractable complex with hyoscyamine (atropine); tropine and scopolamine do not interfere.

2. Both hyoscyamine and scopolamine form chloroform-extractable complexes using bromthymol blue at pH 5.6. In this way, mixtures of both alkaloids can be determined without preliminary separation.

3. The hyoscyamine content in belladonna tincture can be accurately and selectively determined using as little as 0.1–0.2 ml. of the tincture. Bromcresol purple is used at pH 6.6 and the complex formed is treated with 0.1 *N* NaOH and measured at 580 nm. Neither tropine nor scopolamine interferes.

REFERENCES

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NMR Quantitative Analysis of Quinidine in Mixtures of Quinidine and Hydroquinidine

THO HUYNH-NGOC and GERARD SIROIS▲

Abstract □ A new procedure for the rapid quantitative analysis of quinidine in mixtures of quinidine and hydroquinidine is described. The method is based upon measurement of the NMR spectrum of these compounds in deuterated chloroform, using 2,3,5-tribromothiophene as an internal standard. The signal chosen is from the vinyl group of quinidine. The great advantage of this procedure is that it permits the determination of the percentage of hydroquinidine in commercial bulk quinidine.

Keyphrases □ Quinidine in mixtures with hydroquinidine—NMR analysis □ Hydroquinidine as impurity in quinidine bulk—NMR analysis □ NMR spectroscopy—analysis, quinidine and hydroquinidine mixtures

Commercial bulk quinidine, the base and its salts, contains variable amounts of hydroquinidine. The USP identification test for quinidine by TLC (1) does not separate these two cinchona alkaloids (2). Many analytical procedures for quinidine have been reported, including UV spectrophotometric (3, 4), colorimetric (5), fluorometric (6), titrimetric (1), and gravimetric (7, 8) determinations. All of these procedures determine the total mixture of alkaloids: quinidine and hydroquinidine. Work in this laboratory indicates that the UV and visible absorptivity and the quantum efficiency of fluorescence are the same for quinidine and hydroquinidine.

A method for their separation and identification by TLC was described previously (2, 9). NMR spectroscopy affords a suitable method of distinguishing between the $-CH{=}CH_2$ and $-CH_2{-}CH_3$ groups present in quinidine and hydroquinidine, respectively. The quinidine spectrum (10) in deuterated chloroform possesses signals that are absent in the hydroquinidine spectrum and are thus available for use in quantitative analysis. A specific NMR quantitative analytical method for quinidine is described here.

EXPERIMENTAL

Spectra were obtained at 60 MHz. using an analytical NMR spectrometer[1]. All spectra were taken at 8 Hz., and a chart width of 72 Hz. was used for all integrals. Tetramethylsilane in deuterated chloroform[2] was used as an internal reference to measure chemical shifts.

Assay Procedure—Pure quinidine base was obtained from quinidine sulfate[3] by eliminating hydroquinidine by the method of Thron and Dirscherl (11).

Approximately 60 mg. of quinidine and 65 mg. of the internal reference 2,3,5-tribromothiophene[4] were accurately weighed and dissolved in a volumetric flask of 1 ml. of deuterated chloroform containing tetramethylsilane. The NMR spectrum was obtained and integrated five times through the region of interest. The mean of the integrated values was used for quantitative calculations.

Calculation—

$$W_{quin} = \frac{EW_{quin}}{EW_{std}} \times \frac{A_{quin}}{A_{std}} \times W_{std} \times F \qquad \text{(Eq. 1)}$$

where:

W_{quin} = weight of quinidine in milligrams
EW_{quin} = molecular weight of quinidine divided by the number of protons in the signal chosen, 324.45/1 = 324.45
EW_{std} = molecular weight of standard divided by the number of protons in the signal chosen, 320.84/1 = 320.84
A_{quin} = integral value of quinidine signal at 5.16 p.p.m.
A_{std} = integral value of standard signal at 6.93 p.p.m.

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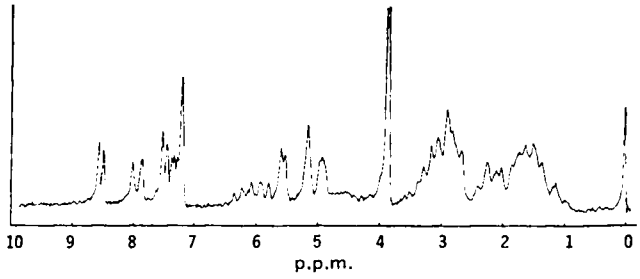


Figure 1—*NMR spectrum of quinidine in $CDCl_3$.*

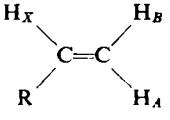
W_{std} = weight of standard in milligrams
F = correction factor

RESULTS AND DISCUSSION

The NMR spectra of quinidine and hydroquinidine[4] in deuterochloroform solutions are shown in Figs. 1 and 2, respectively. The solvent contains about 1% tetramethylsilane, which produces the reference signal at zero on the parts per million or δ scale on the charts. The concentrations of quinidine and hydroquinidine are 50.72 and 57.60 mg./ml., respectively.

The positions of the observed chemical shifts are located at lower fields than reported by Dega-Szafran and Mitura (10). The results were confirmed by recording these spectra on another spectrometer[5]. Peaks at 5.88 and 6.13 p.p.m. (δ) for quinidine and hydroquinidine, respectively, were also reported in saturated solutions and attributed to the hydroxylic proton (10). In this study, saturated solutions at room temperature also presented these peaks at δ values of 6.24 and 6.70 p.p.m. for quinidine and hydroquinidine, respectively. The rise in concentration of these solutes in $CDCl_3$ increases the extent of intermolecular hydrogen bonding. Hydrogen bonding decreases the electron density around the proton and thus moves the proton absorption to lower field. For instance, when the concentration of hydroquinidine is weak, the proton shift is presumably located at a δ value of 5.60 p.p.m. However, when this concentration is increased above 125 mg./ml., work in this laboratory shows that the peak starts moving gradually to lower field.

Quinidine is an *ABX* system:



in which the protons H_A, H_B, and H_X are presumably located in Fig. 1 at 4.90, 5.14, 5.76, 5.90, 6.05, 6.20, and 6.32 p.p.m. (10). The 2,3,5-tribromothiophene (12) was chosen as an internal standard for quantification of quinidine, because its NMR spectrum in deuterated chloroform presents a singlet at 6.90 p.p.m. and does not interfere with the spectrum obtained from a mixture of quinidine and hydroquinidine (Fig. 3).

The singlet at 5.16 p.p.m. was chosen for quantification of quinidine because of its high intensity of integration and its absence in the hydroquinidine spectrum. A correction was made on the integral value of this peak, because a resonance peak (or peaks) of some protons on the substance studied having a chemical shift in the close proximity of the chosen resonance signal for integration interferes in a constant ratio with this signal. The relative intensity of the integrals of each peak depends on the couplage of the protons (13). In the NMR analysis of mixtures of aspirin, phenacetin, and caffeine, a correction was made by Hollis (14) for the carbon-13 sideband from the aspirin peak which falls beneath the analytical methyl peak of caffeine at 3.4 p.p.m. Such carbon-13 sidebands could also contribute to the interference with the integral of the singlet at 5.16 p.p.m. Furthermore, in some cases, spinning sidebands can interfere with integration. In this work, the spinning rate of the sample tube was adjusted between one-fifth and three-fifths of the maximum speed position of the spinner air control valve on the spectrometer. For this range of spinning rate, the integral value of the chosen signal is constant. It does appear then that, in these conditions, spinning sidebands do not interfere with the integral of the chosen signal, because the intensity and displacement of these sidebands from a resonance peak are recognized to be proportional to the spinning speed (15). The correction factor that must be applied to the integral of the chosen analytical resonance signal is determined experimentally with the internal standard.

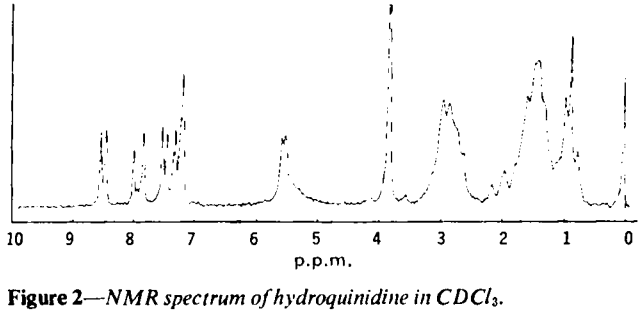


Figure 2—*NMR spectrum of hydroquinidine in $CDCl_3$.*

[5] Varian A-60.

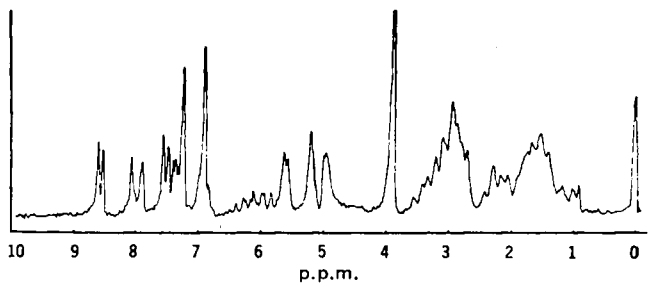


Figure 3—*NMR spectrum of quinidine and hydroquinidine mixture in $CDCl_3$.*

The results obtained from the analysis of 11 known samples of purified quinidine are shown in Table I. The correction factor, F, was calculated from Eq. 1 for each sample. The mean value of F was then used to evaluate the amount of quinidine found by Eq. 1. The mean of percent found is 100.04 and the standard deviation from the mean is 0.972. Synthetic mixtures of purified quinidine and hydroquinidine were made to detect the influence of hydroquinidine on the quinidine spectrum, particularly on the singlet at 5.16 p.p.m. Table II shows five different mixtures containing up to 40.97% of hydroquinidine. The mean of percent found and the standard deviation from the mean are not statistically different from those of Table I. For Student's t test and Fisher's F test, the level of significance, p, is less than 0.01 for differences of the means and standard deviations from the mean, respectively.

Table I—Analysis of Quinidine by NMR

Amount Weighed of Quinidine, mg.	Amount Weighed of Standard, mg.	F	Amount Found, mg.	Percent of Amount Found	Deviation from Mean, %
41.51	83.42	0.925	42.29	101.88	1.84
49.46	73.75	0.947	49.18	99.44	0.60
52.82	83.82	0.954	52.11	98.66	1.38
52.99	80.18	0.946	52.75	99.55	0.49
54.96	53.02	0.948	54.60	99.34	0.70
57.55	54.24	0.947	57.25	99.49	0.55
59.15	66.09	0.936	59.55	100.68	0.64
61.78	64.74	0.944	61.66	99.81	0.23
61.87	55.50	0.943	61.80	99.89	0.15
73.15	83.31	0.928	74.28	101.54	1.50
78.10	81.40	0.940	78.28	100.23	0.19
Mean F: 0.942			Mean percent found:		100.04
SD: 0.0079			*SD:* 0.972		
Average deviation from mean: 0.0063			Average deviation from mean: 0.75%		

Table II—Analysis of Quinidine by NMR in Synthetic Mixtures of Quinidine and Hydroquinidine

Amount Weighed of Quinidine, mg.	Amount Weighed of Hydroquinidine, mg.	Percent Hydroquinidine in Mixtures	Amount Weighed of Standard, mg.	Amount Found of Quinidine, mg.	Percent of Amount Found	Deviation from Mean, %
52.72	6.00	10.22	73.77	52.14	98.89	1.21
49.60	14.29	22.37	55.77	50.00	100.81	0.71
65.71	20.83	24.07	89.03	65.93	100.33	0.23
55.08	26.06	32.12	77.61	54.77	99.44	0.66
47.24	32.79	40.97	58.85	47.73	101.04	0.94
					Mean percent found: 100.10%	
					SD: 0.912	
					Average deviation from mean: 0.748%	

The precision, reproducibility, and specificity of this NMR method make it useful as a rapid procedure for the determination of quinidine in the absence or presence of hydroquinidine. However, because of the displacement of the chemical shift of the hydroxyl proton in the region of the internal standard at very high concentration, the weight of the sample must be kept as low as possible. Excellent results were obtained for samples of 40–80 mg./ml. Preliminary work indicates that this method can also be applied to the analysis of quinidine in mixtures of the salts of these two alkaloids.

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Complete Analysis of Levodopa Capsules and Tablets by GLC

B. L. CHANG, B. F. GRABOWSKI▲, and W. G. HANEY, Jr.

Abstract □ A GLC assay was developed for the determination of levodopa in both capsule and tablet preparations. The procedure also allows for the quantification of closely related amino acids that might be present, as a consequence of either the natural or synthetic production of levodopa, in these dosage forms. The method is based on the assay of the trimethylsilyl derivatives of the constituents of the dosage form, using α-methyldopa as the internal standard for the analysis of levodopa and α-methyldopate as the internal standard for the related amino acid contaminants. This procedure has been applied to the analysis of several commercial capsule and tablet preparations containing levodopa with reproducible results ranging from 97.6 to 101.8% of the labeled amount of levodopa. In addition, several closely related amino acids in these commercial preparations were identified and quantified.

Keyphrases □ Levodopa capsules and tablets—GLC–flame-ionization analysis after silylation, quantitation of companion amino acid impurities □ Amino acids as impurities in levodopa formulations—GLC analysis after silylation □ α-Methyldopa and α-methyldopate—used as internal standards in GLC analysis of levodopa formulations containing amino acid impurities □ GLC–flame-ionization detection—analysis, levodopa and amino acid impurities in levodopa capsules and tablets

Interest in the analysis (1–3) of levodopa [(−)-3-(3,4-dihydroxyphenyl)-L-alanine] has increased considerably since the initial demonstration of its effectiveness in the treatment of some patients afflicted with Parkinson's disease (4). The development of a reliable procedure for the analysis of levodopa has been complicated because of the wide range of possible contaminants that might be present in the drug as a result of the numerous methods used for its preparation (5–7). In addition, the large daily dose of the drug suggests that a patient may be exposed to a significant daily intake of these impurities even when they are present in relatively small amounts.

DISCUSSION

Since it is the immediate precursor of dopamine, levodopa occupies an important position in the biosynthesis of bioactive aromatic amines. Its metabolism, through dopamine, results in the production of 3-methoxytyramine, norepinephrine, or dihydroxyphenylacetic acid (8), and each of these products may, in turn, be further metabolized in the body. Likewise, amino acids closely related to levodopa, such as tyrosine and 3-aminotyrosine, follow qualitatively similar routes of metabolism.

The metabolism of levodopa and of closely related amino acids, therefore, gives rise to a multiplicity of products, each with its own pharmacological activity. While the metabolism of levodopa has been implicated in its beneficial biological activity (9), the metabolism of closely related amino acids in a pharmaceutical preparation containing levodopa may introduce needless therapeutic complexities into the activity of the drug preparation. Therefore, it is important to identify and quantify these amino acids in the preparation.

While these considerations may apply to the analysis of most drugs and drug products, they are particularly important in the case of levodopa because of its large daily dose (6–8 g.). As a result of this large dose, an impurity that comprises only 1% of the preparation would result in an intake of 60–80 mg./day of extraneous material. Hence, the large therapeutic dose of levodopa increases the likelihood that impurities present in the preparation will give rise to untoward effects and makes their determination critical.

Some assay procedures proposed for levodopa do not possess sufficient specificity to differentiate levodopa from products closely related to it. For example, the spectrophotometric assay designed by Nedergaard (10) does not allow for the unequivocal determination of the drug but determines the presence of tyrosine, 3-methoxytyrosine, and 3,4,6-trihydroxyphenylalanine in addition to levodopa.

A specific assay for levodopa would, however, not be satisfactory if the procedure gave information pertaining only to the quantity of levodopa in the drug preparation. The data from an assay of levodopa must allow for the identification of related amino acids and their quantification, even when they are present in relatively small quantities (0.1–1%). Unless such data are available for the drug preparation, it is impossible to make an intelligent judgment as to its therapeutic efficacy.

While a series of different assays might give a range of information, analysis time for such a scheme would, of necessity, be substantial. Hence, a single assay procedure would be desirable. The procedure should give reliable data for substances present in concentrations ranging over several orders of magnitude. Also, it should be specific in the sense that no one component can interfere with the determination of any other component, and it should be nonselective in the sense that each component in the sample should be identified and quantified.

Currently, chromatographic procedures are the only methods capable of providing the needed information; one can isolate, identify, and quantitate many components in a single rapid procedure. In particular, the use of a flame-ionization detector in conjunction with GLC offers an appealing combination of both linear range and nonspecificity of response. Hence, a procedure for the complete characterization of levodopa-containing pharmaceutical preparations was developed based on the flame-ionization detection of levodopa and related amino acids separated by GLC.

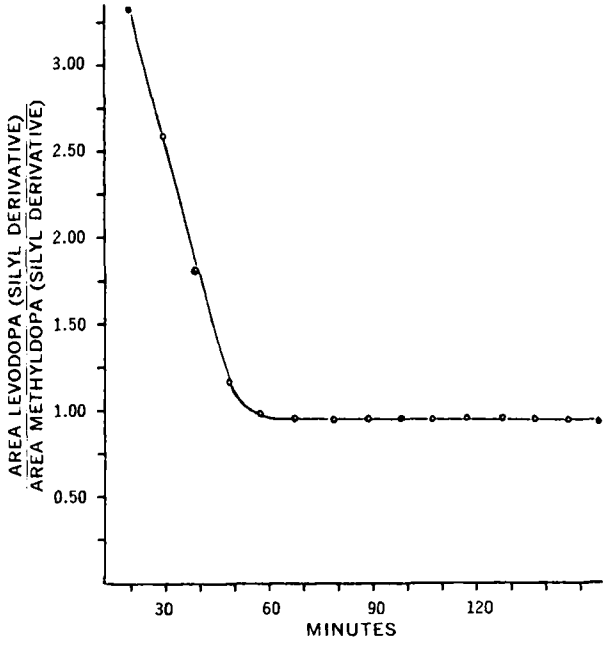

Figure 1—*Relative silylation rate of levodopa and internal standard at 80°.*

Table I—Analysis of Standard Mixtures of Levodopa and Related Amino Acids

	Tyrosine	3-Methoxytyrosine	Levodopa	3-Aminotyrosine	3,4,6-Trihydroxyphenylalanine	n
Standard mixture A:						
Calculated, %	1.60	3.40	92.30	1.50	3.20	
Found[a], %	1.54	3.31	91.74	1.51	3.22	
SD, %	4.54	3.02	1.54	5.29	2.68	9
Standard mixture B:						
Calculated, %	0.25	0.75	95.25	3.50	0.25	
Found[a], %	0.23	0.76	96.17	3.44	0.25	
SD, %	5.29	2.75	1.79	4.36	3.16	10
Standard mixture C:						
Calculated, %	3.50	0.75	92.00	0.25	3.50	
Found[a], %	3.27	0.77	90.86	0.26	3.35	
SD, %	4.89	3.22	2.33	3.47	0.017	10

[a] Mean of n determinations.

Because of their low volatility, amino acids have chromatographic characteristics unsuitable for precise quantification. Prior to analysis, therefore, it is necessary to form derivatives of the amino acids. While several procedures are available (11, 12), almost all rely on two or more reaction steps. Since the formation of trimethylsilyl derivatives is rapid and a one-step procedure, silylation was chosen for derivatization of the amino acids. In addition, the reaction process leading to these trimethylsilyl derivatives is relatively nonspecific, giving products with acids, amines, alcohols, *etc.* (13).

EXPERIMENTAL

Materials—*N,O*-Bis(trimethylsilyl)acetamide[1] was used as the silylating solvent. 3-Methoxytyrosine[2], 3,4,6-trihydroxyphenylalanine[2], levodopa[2], α-methyldopa[2], and α-methyldopate[2] were obtained. Alanine[3], phenylalanine[4], and *l*-tyrosine[4], each of which melted at literature values after recrystallization, were used. Commercial capsules and tablets containing levodopa were used in the analysis.

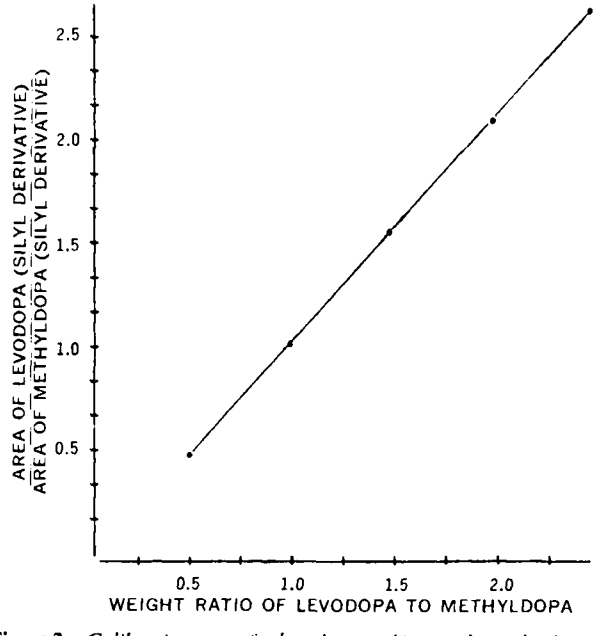

Figure 2—*Calibration curve for levodopa and internal standard.*

[1] Pierce Chemical Co., Rockford, Ill.
[2] United States Pharmacopeial Convention, Inc.
[3] Certified, Fisher Scientific Co., Fair Lawn, N. J.
[4] Standardized, Nutritional Biochemical Corp., Cleveland, Ohio.

Table II—Levodopa and Related Substances in Commercial Levodopa Capsules and Tablets

Sample Number	Dosage Form	Levodopa[a]	*SD*	Related Substances[a]	*SD*	n
I	Capsule	98.42	2.64	1.27[b]	0.04	8
II	Capsule	101.86	2.66	—	—	10
III	Capsule	97.56	1.82	1.07[c]	0.027	10
				0.57[d]	0.018	
IV	Tablet	98.24	2.37	0.27[b]	0.007	8
				0.78[e]	0.011	
V	Tablet	99.47	1.56	0.57[e]	0.022	12
VI	Tablet	98.56	1.87	1.26[c]	0.025	8
				0.54[d]	0.008	
				—[f]		

[a] Expressed as percent of labeled amount of levodopa. [b] 3-Aminotyrosine. [c] 3-Methoxytyrosine. [d] 3,4,6-Trihydroxyphenylalanine. [e] Tyrosine. [f] Phenylalanine.

Standard Solution—α-Methyldopate was used as a solution (1.0 mg./ml.) in 0.1 *N* HCl.

Apparatus—A gas chromatograph[5] with isothermal control and a flame-ionization detector was used for the analysis. The columns were 3.04 m. (10 ft.) in length, spiral-shaped, borosilicate glass tubing of 0.61 cm. (0.125 in.) inner diameter and packed with 2% methyl silicone and 6% phenyl methyl silicone on 80–100-mesh, silanized, acid-washed, flux-calcined diatomite[6]. Prior to use, the column was conditioned at 250° for 30 hr. The injector port (fitted with a glass injector sleeve), column, and detector temperatures were 225, 198, and 245°, respectively. The gas flow rates were: hydrogen, 40 ml./min.; compressed air, 226 ml./min.; and nitrogen, as the carrier gas, 40 ml./min. Column peak areas were determined using a fully automatic electronic digital integrator with printout[7].

Determination of Reaction Time and Product Stability—Ten milligrams each of 3-methoxytyrosine, levodopa, 3-aminotyrosine, 3,4,6-trihydroxyphenylalanine, and the internal standards α-methyldopa and α-methyldopate was placed in cone-shaped vials[1] fitted with Teflon septums. A 0.6-ml. sample of the silylating agent, *N,O*-bis(trimethylsilyl)acetamide, and 0.6 ml. of the silylating solvent, dry pyridine, were added to each vial. The vials were maintained in a water bath at 80°, and aliquots were taken for analysis from each vial at 10-min. intervals. After 3 hr., heating was discontinued, and the vials were protected from light and moisture. Aliquots for analysis were then taken at 4-hr. intervals for 24 hr.

Preparation of Calibration Curves—*Levodopa*—α-Methyldopa (20.0 mg.) was placed in each of six separate cone-shaped vials fitted with Teflon septums. A weight of levodopa calculated to give weight ratios of levodopa to reference standard over the range 0–2.5 at 0.5 increments was then added to the respective vials. Dry pyridine (0.6 ml.) and the silylating agent (0.6 ml.) were added to each vial, which was then heated in a water bath at 80° for 1 hr. The reaction mixture was then cooled to room temperature, and 1.0 μl. of the mixture was injected into the chromatographic system. The peak area ratios of the trimethylsilyl derivative of levodopa to the trimethylsilyl derivative of the α-methyldopa derivative were plotted *versus* the weight of levodopa, and the line of best fit was determined.

Related Amino Acids—Solutions (2.0 mg./ml. in 0.1 *N* HCl) of each amino acid included in the study were prepared. A volume of the standard solution of α-methyldopate (internal standard) equivalent to 0.2 mg. of α-methyldopate was added to each of six vials. A volume of each amino acid solution calculated to give weight ratios over the range 0–2.5 (related amino acid to internal standard) at 0.5 increments was added to the vials. Each sample was then evaporated to dryness at 60° with a stream of dry nitrogen and treated as described under *Levodopa*, beginning: "Dry pyridine . . ."

Analysis of Commercial Products—Tablets containing levodopa were sampled by selecting four tablets at random from each sample lot. These tablets were then weighed and finely powdered. In the

[5] Varian model 575.
[6] OV-1 (2%) and OV-17 (6%) on Chromosorb G, A-W, DMCS, from Varian Aerograph, Walnut Creek, Calif.
[7] Varian model 475.

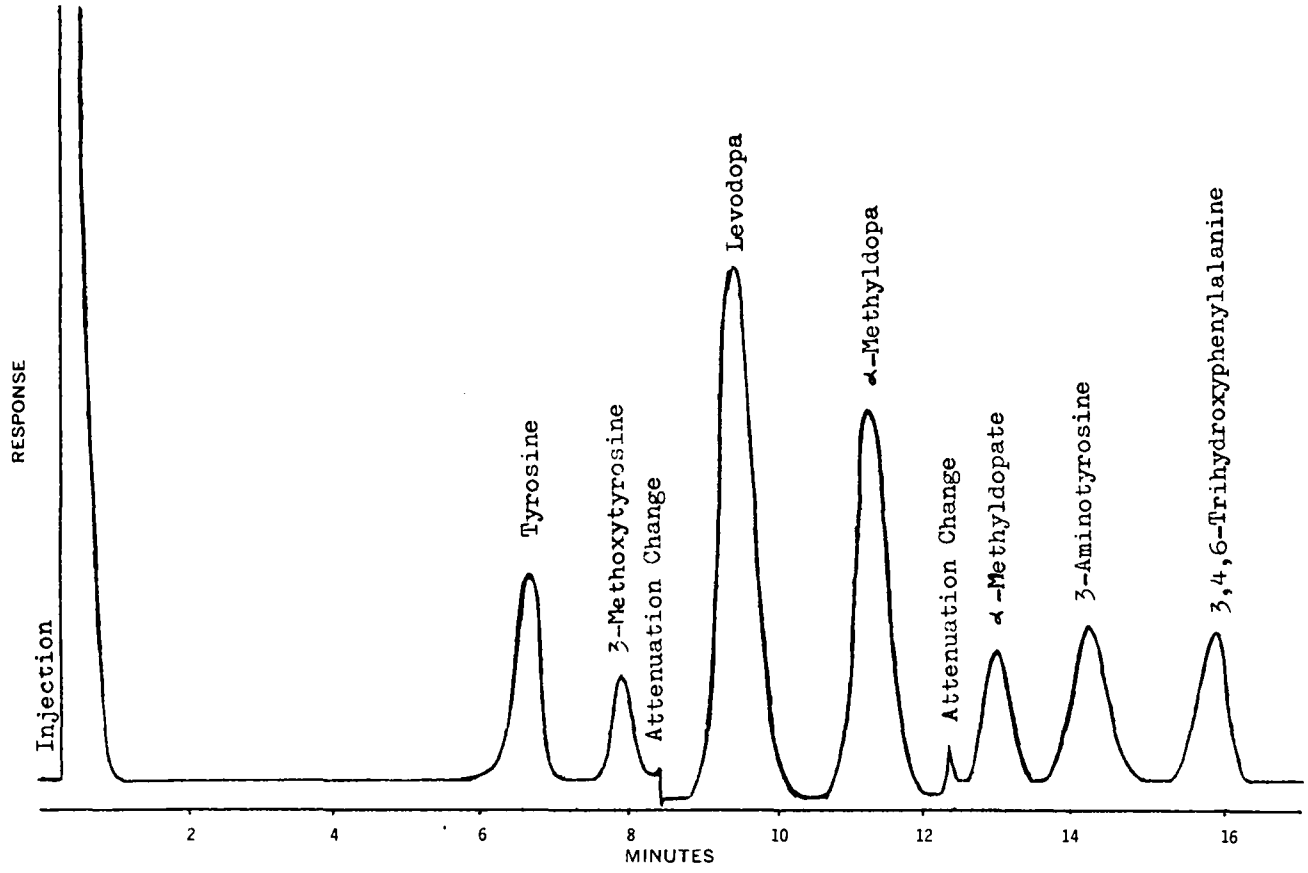

Figure 3—*Chromatographic trace of the trimethylsilyl derivatives of a synthetic mixture of levodopa, related substances, and internal standards. See text for conditions.*

case of the capsules, four capsules were weighed and emptied and their contents were weighed and then thoroughly mixed.

A volume of the α-methyldopate standard solution equivalent to 0.2 mg. of α-methyldopate was added to a vial, and the solution was evaporated to dryness at 60° with a stream of dry nitrogen. A sample of dosage form equivalent to about 25 mg. of levodopa was weighed accurately and placed in the vial along with 20.0 mg. of α-methyldopa. Dry pyridine (0.6 ml.) and the silylating agent (0.6 ml.) were added to the mixture, and the vial was capped and heated in a water bath at 80° for 1 hr. A volume of the solution (1.0–3.0 μl.) was injected into the chromatographic system, and the peak area ratio of the trimethylsilyl derivative of levodopa to the trimethylsilyl derivative of α-methyldopa was determined. The quantity of levodopa in the sample was then determined graphically from the calibration curve. Any related amino acids present in the sample were quantified, using the trimethylsilyl derivative of α-methyldopate as the internal standard. The percentage of levodopa or related amino acids in the product was calculated as the percentage of the claim of levodopa.

RESULTS AND DISCUSSION

Results of the study of the relative silylation rate of levodopa and the internal standard at 80° are illustrated in Fig. 1. From the curve, it can be seen that a stable ratio of the trimethylsilyl derivatives of levodopa to α-methyldopa is obtained after 40 min. For the related amino acids, a qualitatively similar curve showed the stable ratio of trimethylsilyl derivatives of related amino acids to α-methyldopate to occur after 30 min. However, alanine does not appear to give a response in this system. While no explanation for this anomaly is offered, a similar result was pointed out by Gehrke *et al.* (14).

Generally, trimethylsilyl derivatives are known to be sensitive to moisture (15). In these studies, however, the ratios of the trimethylsilyl derivatives of levodopa or related amino acids to their internal standards were stable for at least 24 hr. if the trimethylsilyl derivatives were protected from light and moisture. This implies that degradation of the derivatives does not occur or that it occurs at the same rate for all substances in the study.

The calibration curve for the trimethylsilyl derivatives of levodopa and α-methyldopa is shown in Fig. 2. This relationship was linear over the concentration range studied, and the slope was computed from the formula for least squares to be 1.086, with a coefficient of variation of ±1.16%. However, continued use of the chromatographic system over 2 months gave rise to a decreased slope, which was 0.31% in excess of the mean slope error. This decrease occurred despite the fact that the detector was cleaned weekly. Hence, it is necessary to prepare calibration curves periodically and to calculate the quantity of levodopa in the sample graphically.

Calibration curves for the trimethylsilyl derivatives of α-methyldopate and the related amino acids were linear and passed through the origin. One exception to this generality was *l*-tyrosine, which gave a calibration curve having negative deviation from linearity at ratios greater than 1.5. Nonlinearity might be expected in this case due to the rather large difference between the retention time of the trimethylsilyl derivatives of *l*-tyrosine and α-methyldopate. However, the precision and accuracy of the assay were not greatly affected as long as calibration curves were used.

The precision and accuracy of the assay procedure were examined by assaying prepared standard mixtures composed of levodopa and the related substances in varying ratios (Table I). Even when the ratio of levodopa to related amino acid was greater than 300:1, the method gave reliable data for both levodopa and the related amino acid. While the deviation for *l*-tyrosine was larger than that for the other substances, the deviation was still within the range required for an acceptable assay.

The selectivity of the method is exemplified in Fig. 3, which illustrates a chromatographic trace of the trimethylsilyl derivatives of levodopa, related amino acids, and the internal standards. Although levodopa and α-methyldopa are very closely related in physicochemical properties, the resolution factor for their trimethylsilyl derivatives (16) is 1.68. In addition, each peak from the trimethylsilyl derivatives of the related amino acids is relatively symmetrical and well separated. The chromatographic parameters used in the deter-

mination are necessary for the complete separation of all the components used in the study. However, once an initial determination of the constituents present in the sample has been made, the column temperature may be increased to shorten the analysis time without affecting the precision and accuracy of the results.

Table II illustrates the results of the application of this procedure to the analysis of several commercial capsule and tablet preparations. The route of preparation of levodopa (synthetic or natural) may be determined by examining the related amino acids present. TLC (17) was used to identify the amino acid phenylalanine found in Tablet VI.

Since the procedure did not employ any cleanup or extraction procedures prior to analysis, diluents and binders used to formulate the dosage forms of levodopa could offer a potential source of interference. However, since there were no extraneous peaks observed in the chromatograms of any dosage form analyzed in this study, these materials apparently do not interfere with the assay procedure.

In conclusion, a GLC determination of levodopa has been developed and is proposed as an assay for the drug contained in both capsules and tablets. Related amino acids which were present as possible contaminants were also separated, identified, and quantified. In addition, these results indicate that analysis of α-methyldopa by the same or a closely related procedure may be feasible.

This procedure was applied to several commercial tablet and capsule preparations containing levodopa, with satisfactory results.

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GLC Determination of Meclizine Hydrochloride in Tablet Formulations

C. K. WONG, J. R. URBIGKIT, N. CONCA, D. M. COHEN, and K. P. MUNNELLY▲

Abstract □ A method was developed for the quantitative determination of meclizine hydrochloride in tablet formulations by GLC after a suitable separation technique.

Keyphrases □ Meclizine hydrochloride tablets—GLC analysis after chloroform extraction □ GLC—analysis, meclizine hydrochloride tablets

Meclizine hydrochloride is an antihistamine which shows marked protective activity against nebulized histamine. It is indicated in the management of nausea, vomiting, and dizziness associated with motion sickness, and it has been found useful in the management of vertigo associated with diseases affecting the vestibular system. Various methods for the determination of meclizine hydrochloride have been reported, including TLC (1–4), polarography (5), NMR (6), fluorometry (7), nonaqueous titration (8), and UV spectroscopy (9–11). However, these methods do not have the rapidity, simplicity, and degree of sensitivity found in GLC methods.

Papers (12–15) published on the GLC determination of meclizine hydrochloride lack quantitative data or include chromatograms showing serious peak tailing. This paper describes a simple and direct GLC procedure for the quantitative determination of meclizine hydrochloride after extraction from a tablet formulation with chloroform.

EXPERIMENTAL

Equipment—A gas chromatograph[1], equipped with a dual flame detector and an electronic integrator[2], was used throughout this study. The column was 1.82-m. × 4-mm. (6-ft. × 0.25-in.) stainless steel tubing packed with diatomaceous earth[3], 80–100 mesh, coated with 3% OV-17. The operating temperatures were: column, 290°; de-

[1] Hewlett-Packard 5750.
[2] Hewlett-Packard 3370A.
[3] Chromosorb W, AW-DMCS, Supelco Inc., Bellefonte, Pa.

mination are necessary for the complete separation of all the components used in the study. However, once an initial determination of the constituents present in the sample has been made, the column temperature may be increased to shorten the analysis time without affecting the precision and accuracy of the results.

Table II illustrates the results of the application of this procedure to the analysis of several commercial capsule and tablet preparations. The route of preparation of levodopa (synthetic or natural) may be determined by examining the related amino acids present. TLC (17) was used to identify the amino acid phenylalanine found in Tablet VI.

Since the procedure did not employ any cleanup or extraction procedures prior to analysis, diluents and binders used to formulate the dosage forms of levodopa could offer a potential source of interference. However, since there were no extraneous peaks observed in the chromatograms of any dosage form analyzed in this study, these materials apparently do not interfere with the assay procedure.

In conclusion, a GLC determination of levodopa has been developed and is proposed as an assay for the drug contained in both capsules and tablets. Related amino acids which were present as possible contaminants were also separated, identified, and quantified. In addition, these results indicate that analysis of α-methyldopa by the same or a closely related procedure may be feasible.

This procedure was applied to several commercial tablet and capsule preparations containing levodopa, with satisfactory results.

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GLC Determination of Meclizine Hydrochloride in Tablet Formulations

C. K. WONG, J. R. URBIGKIT, N. CONCA, D. M. COHEN, and K. P. MUNNELLY▲

Abstract □ A method was developed for the quantitative determination of meclizine hydrochloride in tablet formulations by GLC after a suitable separation technique.

Keyphrases □ Meclizine hydrochloride tablets—GLC analysis after chloroform extraction □ GLC—analysis, meclizine hydrochloride tablets

Meclizine hydrochloride is an antihistamine which shows marked protective activity against nebulized histamine. It is indicated in the management of nausea, vomiting, and dizziness associated with motion sickness, and it has been found useful in the management of vertigo associated with diseases affecting the vestibular system. Various methods for the determination of meclizine hydrochloride have been reported, including TLC (1–4), polarography (5), NMR (6), fluorometry (7), nonaqueous titration (8), and UV spectroscopy (9–11). However, these methods do not have the rapidity, simplicity, and degree of sensitivity found in GLC methods.

Papers (12–15) published on the GLC determination of meclizine hydrochloride lack quantitative data or include chromatograms showing serious peak tailing. This paper describes a simple and direct GLC procedure for the quantitative determination of meclizine hydrochloride after extraction from a tablet formulation with chloroform.

EXPERIMENTAL

Equipment—A gas chromatograph[1], equipped with a dual flame detector and an electronic integrator[2], was used throughout this study. The column was 1.82-m. × 4-mm. (6-ft. × 0.25-in.) stainless steel tubing packed with diatomaceous earth[3], 80–100 mesh, coated with 3% OV-17. The operating temperatures were: column, 290°; de-

[1] Hewlett-Packard 5750.
[2] Hewlett-Packard 3370A.
[3] Chromosorb W, AW-DMCS, Supelco Inc., Bellefonte, Pa.

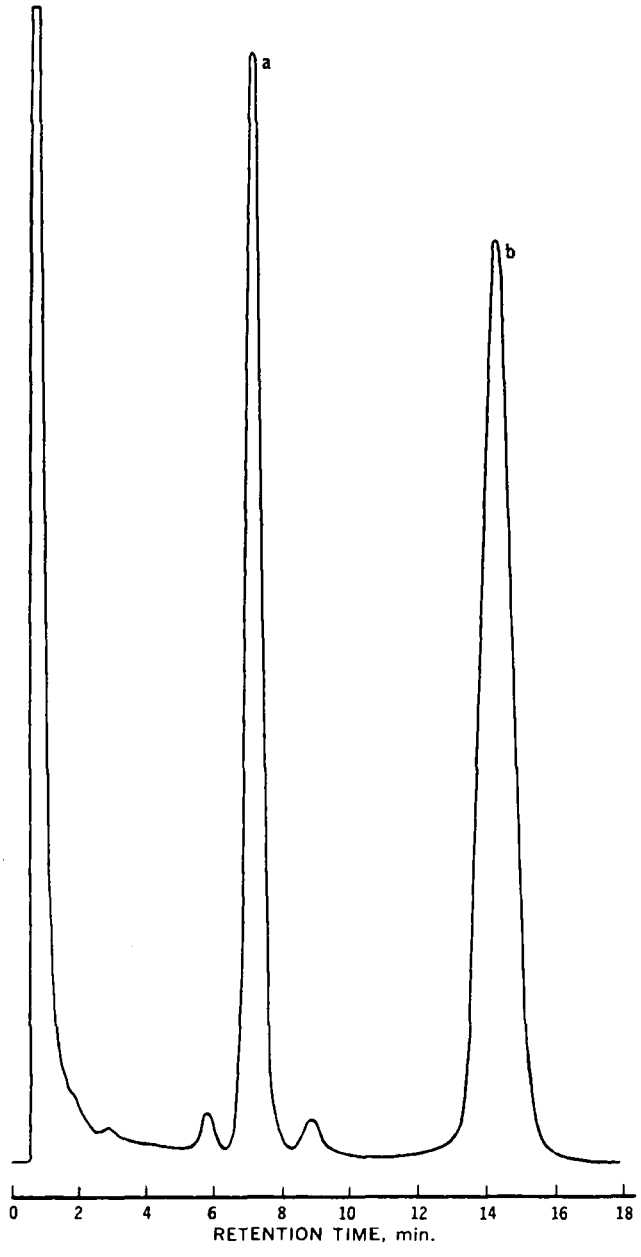

Figure 1—*Representative chromatograms. Key: a, dinonyl phthalate; and b, meclizine.*

tector, 330°; and injection port, 320°. Helium, with a flow rate of 75 ml./min., was the carrier gas. The hydrogen flow rate was adjusted to optimum sensitivity without causing high noise background in the electronic integrator. Oxygen was maintained at 30 psig. head pressure.

Reagents—Meclizine hydrochloride USP XVIII reference standard was dried at 100° for 2 hr. before use. Dinonyl phthalate[4], practical grade, was the internal standard. Chloroform[5] was GC spectrophotometric quality.

Preparation of Standard Solution—Dissolve approximately 500 mg. of dinonyl phthalate in 500 ml. chloroform. Accurately weigh approximately 100 mg. of meclizine hydrochloride standard and transfer to a 125-ml. conical flask. Pipet 50 ml. of the dinonyl phthalate solution into the conical flask containing the meclizine hydrochloride and swirl to make a uniform solution.

Preparation of Sample Solution—Determine the average tablet weight of 20 tablets. Pulverize the tablets, weigh an amount equivalent to 100 mg. of meclizine hydrochloride, and transfer the powder to a 125-ml. conical flask. Pipet 50 ml. of the dinonyl phthalate solution into the conical flask and shake for 30 min. Centrifuge the solution and use the supernate for the GLC determination.

[4] Eastman Kodak.
[5] J. T. Baker Chemical Co.

Table I—Percent Recovery of Meclizine Hydrochloride

Day	Weight Number	Injection 1	Injection 2	Injection 3
1	1	100.9	102.8	102.9
	2	99.5	100.2	100.1
2	3	99.0	100.8	101.1
	4	100.9	101.6	101.7
3	5	99.4	98.4	99.2
	6	99.0	99.0	100.7
		Overall average	100.4%	

Table II—Estimates of Precision for Determination of Meclizine Hydrochloride in a Pharmaceutical Formulation

Number of Days	Number of Weights per Day	Number of Injections per Weight	Estimates of Precision[a], %
1	1	1	±2.8
1	1	3	±2.4
1	2	3	±1.9
2	1	3	±1.7
Injection to injection within a weight on a day			±1.7

[a] Ninety-five percent of individual results or average of three or six results will not vary from each other by more than the percentages quoted. These estimates include variability due to days, weights, aliquots, and injections.

Chromatography—After conditioning the column with four injections of the sample solution, approximately 5 μl. each, inject 5 μl. of the standard solution followed by three 5-μl. injections of the sample solution and one 5-μl. injection of the standard solution. The peak areas obtained from the electronic integrator are used for the calculation.

Calculations—Calculate the potency of meclizine hydrochloride in milligrams per tablet according to the following formula:

$$\text{milligrams per tablet} = \frac{R_{spl} \times \text{standard weight (mg.)} \times \text{average tablet weight (mg.)}}{R_{std} \times \text{sample weight (mg.)}} \quad \text{(Eq. 1)}$$

where R_{spl} is the ratio of meclizine peak area to dinonyl phthalate peak area in the sample solution, and R_{std} is the average ratio of

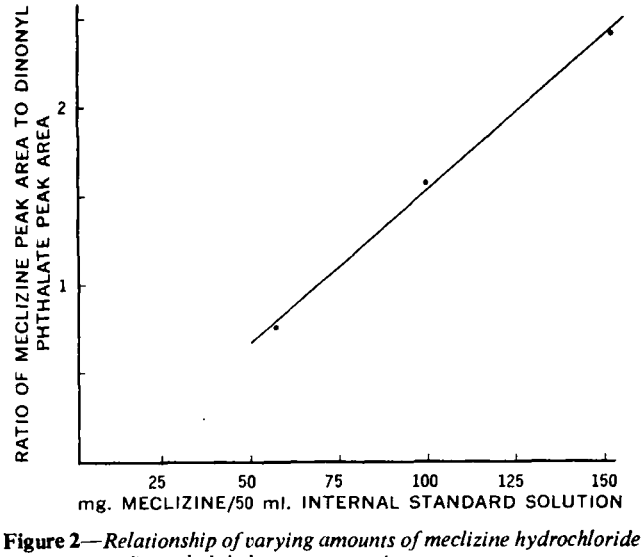

Figure 2—*Relationship of varying amounts of meclizine hydrochloride to a constant dinonyl phthalate concentration.*

the meclizine peak area to the dinonyl phthalate peak area in the standard solution before and after sample injections.

RESULTS AND DISCUSSION

A typical chromatogram of meclizine and dinonyl phthalate is shown in Fig. 1. Figure 2 shows the linear relationship of various amounts of meclizine to a constant amount of dinonyl phthalate.

The accuracy and precision of the method were tested by the following experiment. Two weights of a placebo blend in which known quantities of meclizine hydrochloride had been added were assayed per day for 3 consecutive days. The average recovery (Table I) was 100.4%. The estimate of precision (Table II) for injection to injection within a weight on a day, excluding variability due to days and weights, was ±1.7%. The standard error for the average of three injections is ±2.4%.

Meclizine hydrochloride yields a symmetrical peak, identical with meclizine base. A possible explanation for this phenomenon is that two HCl molecules are thermally removed from the meclizine molecule inside the injection port (16). By taking advantage of this effect, sample preparation is simplified since a two-phase liquid extraction step is eliminated.

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Spectrophotometric Determination of Thyroxine Iodine in Thyroid Preparations

DAVID L. SONDACK

Abstract □ An improved quantitative determination of thyroxine iodine in USP thyroid preparations is described. The iodine-containing proteins are dissolved in 8 *M* urea and separated from cell debris and excipient materials by centrifugation and filtration. One aliquot of the clear yellow filtrate is adjusted to pH 9.0, and a second aliquot is adjusted to pH 5.0. The difference in absorbance between the two is observed at 325, 350, and 360 nm. These values are inserted into an equation utilizing the appropriate absorptivities of the iodoamino acids and dilution factors, and the amount of thyroxine iodine is calculated. The relative standard deviation of the method is ±6.0%. Data are presented to demonstrate the application of this method to several thyroid formulations, thyroid powder USP, tablets, and enteric-coated tablets. Correlation with biological data is shown.

Keyphrases □ Thyroxine iodine—UV titrimetric analysis in thyroid preparations □ Thyroid preparations—UV titrimetric analysis of thyroxine iodine □ UV spectrophotometric titration—analysis, thyroxine iodine in thyroid preparations

Thyroxine contained in pure proteins can be determined by spectrophotometric titration, taking advantage of the unique ionization and absorption properties of the iodoamino acids (1, 2). Various other methods to measure chemically both thyroxine and triiodothyronine (liothyronine) in pharmaceutical thyroid preparations have been described (3–13). These methods involve: chemical or enzymic hydrolysis of the thyroproteins; extraction of the released iodoamino acids; separation of these acids by paper chromatography, TLC, or gel permeation chromatography; and quantitation by iodometry. The methods are time consuming and the stability of the iodoamino acids in solution also has been a problem (14–16). The USP

Table I—Precision of Measurement of Thyroxine in Thyroid Preparations

Preparation	Average Value	Range	*RSD*, %
Powder USP, mcg./g.	207.0	191.0–222.0	±6
120-mg. tablets USP, mcg./tablet	24.2	22.7–25.3	±5
200-mg. enteric-coated tablets USP, mcg./tablet	32.8	30.2–33.9	±6

the meclizine peak area to the dinonyl phthalate peak area in the standard solution before and after sample injections.

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200-mg. enteric-coated tablets USP, mcg./tablet	32.8	30.2–33.9	±6

Table II—Thyroxine Iodine and Total Organic Iodine Contents of Thyroid Preparations from the Same Starting Material

Preparation	Thyroxine Iodine, mcg./Tablet Spectrophotometric	Bioassay[a]	Total Organic Iodine, mcg./Tablet
120-mg. enteric-coated tablets USP	22.7	30.8	253.0
60-mg. enteric-coated tablets USP	12.0	14.9	120.0
120-mg. tablets USP	22.5	29.5	242.0
60-mg. tablets USP	11.5	14.9	120.0
30-mg. tablets	6.1	7.2	61.5
Precursors to above preparations:			
Powder USP	209.0 mcg./g.	258.0 mcg./g.	2120.0 mcg./g.
Desiccated crude material	NA[b]	914.0 mcg./g.	7500.0 mcg./g.

[a] Bioassay values were calculated by reducing the value obtained for the desiccated crude material in proportion to the total organic iodine. [b] NA = not available.

XVIII method measures the total iodine content (17), which is not truly reflective of the physiological activity of the preparation (10, 18, 19).

This report presents the application of spectrophotometric titration to pharmaceutical thyroid preparations. The data are compared with data obtained by bioassay. The rapidity of the method and the parallel data to bioassay should make this a useful method for control of thyroid preparations in conjunction with biological or chemical evaluation of the raw material.

EXPERIMENTAL

Thyroxine Iodine—An amount of the thyroid preparation, equivalent to 500–1000 mg. of USP material, is transferred to a centrifuge tube. The material is suspended in 10 ml. 8 *M* urea containing 1 *M* guanidine hydrochloride, as described by Covelli *et al.* (2), and is mixed intermittently for 1 hr. The tubes are then centrifuged for 15 min. at 2000 r.p.m. The yellow, slightly turbid supernate is filtered with gentle suction through a Whatman glass fiber pad on a Gooch crucible with the aid of a bell jar microfiltration assembly. The clear yellow liquid is collected in a small conical flask. Equal amounts (2 ml.) of the filtrate are pipeted into each of two test tubes. To one tube an equal volume of 8 *M* urea–1 *M* guanidine hydrochloride, buffered to pH 5.0 with 0.2 *M* acetate, is pipeted. To the other tube an equal volume of 8 *M* urea–1 *M* guanidine hydrochloride, buffered to pH 9.0 with 0.2 *M* tromethamine, is similarly added. After mixing, the solutions are transferred to matched silica cells and the difference in absorbance (ΔA) between them is measured at 325, 350, and 360 nm.

The concentration of thyroxine iodine in the cell is given by the following equation[1]:

$$\text{mcg. thyroxine iodine/ml.} = \frac{(\Delta A_{350} - \Delta A_{360})5960 - \Delta A_{325} \times 280}{10.6 \times 10^3} \times 507.6 \frac{\text{mg. iodine}}{\text{mmole thyroxine}} \quad \text{(Eq. 1)}$$

The amount of thyroxine iodine per unit of the thyroid preparation is obtained by applying the appropriate dilution factors.

The thyroid preparations tested were: desiccated crude thyroid glands, thyroid powder USP, ground tablets, and enteric-coated tablets (decoated with dilute acid and then cracked to expose the interior).

[1] For the derivation of this expression, see *Reference 2.*

Table III—Thyroxine Iodine and Total Organic Iodine of Thyroid Preparations at Various Stages of Manufacture

Preparation	Thyroxine Iodine—Spectrophotometric	Thyroxine Iodine—Bioassay	Total Organic Iodine
A:			
200-mg. enteric-coated tablets USP, mcg./tablet	33.0	44.0	380.0
Powder USP, mcg./g.	178.0	232.0	2000.0
Desiccated crude material, mg./g.	680.0	927.0	8000.0
B:			
200-mg. enteric-coated tablets USP, mcg./tablet	35.0	51.5	380.0
Powder USP, mcg./g.	213.0	292.0	2200.0
Desiccated crude material, mcg./g.	NA[a]	983.0	7400.0
C:			
Powder USP, mcg./g.	209.0	333.0	2100.0
Powder USP, mcg./g.	198.0	333.0	2100.0
Desiccated crude material, mcg./g.	NA	1250.0	7900.0

[a] NA = not available.

Bioassay—Bioassay was performed by either a mouse anoxia method (20) or a rat goiterogenic method (21).

Total Iodine—Total iodine was determined by the USP XVIII method (17).

RESULTS AND DISCUSSION

To obtain the precision of the method, five replicate analyses were run on composite samples of the various thyroid formulations. The results are shown in Table I.

Tablets and enteric-coated tablets of various dose sizes, prepared from the same lot of desiccated crude thyroid, were assayed (Table II). As expected, the spectrophotometric results are consistently lower than biological activity calculated from the value measured for the desiccated crude material in proportion to the total iodine content of the tablet or enteric-coated tablets.

Table III also displays the relation between thyroxine iodine and bioassay values. The first line in each section shows the values obtained or calculated for the preparation. The material represented on the first line was made from the powder represented on the second line which, in turn, was made from the desiccated crude material shown on the third line.

The presence of triiodothyronine is ignored by this method, since it is not measured spectrophotometrically (1, 2). This compound is reported to be at least five times as active as thyroxine (22). The deviation between the spectrophotometric and bioassay values in Tables II and III can thus be accounted for by an amount of triiodothyronine representing 5–10% of the thyroxine level.

This method may prove useful as a control assay in the manufacture of thyroid formulations. It is probably best used in conjunction with biological or chemical assay of the desiccated crude thyroid glands entering the process as raw material.

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GLC of Cycloserine

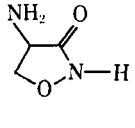
DAVID L. SONDACK▲, FRANK E. GAINER, and HAROLD J. WESSELMAN

Abstract □ A GLC assay is described for cycloserine (D-4-amino-3-isoxazolidinone). The sample is dissolved in pyridine–water (2:1), an aliquot is evaporated, and the residue is treated with *N,O*-bis(trimethylsilyl)acetamide and trimethylchlorosilane. After heating for a few minutes, chromatography is performed using a column containing methyl vinyl silicone, a relatively nonpolar liquid phase. Favorable quantitation is achieved by using hexamethylbenzene as the internal standard. The observed peak is the *N,N'*-bistrimethylsilyl derivative of cyloserine. The procedure eliminates interference from the cycloserine dimer, which does not chromatograph under these conditions.

Keyphrases □ Cycloserine—GLC analysis using trimethylsilyl derivatives □ GLC—analysis of cycloserine trimethylsilyl derivatives □ Trimethylsilyl derivatization—GLC analysis of cycloserine

Cycloserine (D-4-amino-3-isoxazolidinone, I) equilibrates with its dimer, (+)-*cis*-3,6-bis(aminoxymethyl)-2,5-piperazinedione (II) even in the solid state (1, 2). In solution, pH extremes cause degradation of the dimer to 3,6-dimethylene-2,5-piperazinedione and to β-aminoxyalanine (3, 4). At or near neutral pH, dimerization predominates (4).

Quantitative determination of cycloserine in pharmaceutical preparations has been done by the nitroprusside method (5), titrimetrically and by UV spectrophotometry (6), or microbiologically against suitably susceptible microorganisms. The chemical treatment and solvents required for these assays cause formation of degradation products (1–4), which may interfere with the assay or give inaccurate results. This report presents a more specific GLC method for the quantitative analysis of cycloserine.



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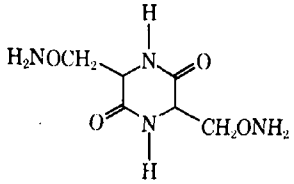

II

EXPERIMENTAL

Equipment—A gas chromatograph[1], equipped with a flame-ionization detector, was used. The detector signal was fed to a computer[2] for peak integration and to a 1-mv. recorder[3] with a chart speed of 15 in./hr. and a 1-sec. fullscale response. Samples were injected with a 10-μl. syringe[4]. Helium was used as the carrier gas, and electrolytic hydrogen and oxygen were used in the detector. The stationary phase was 3.8% UC W98 on Diatoport S[5] (80–100 mesh) packed in borosilicate glass columns 0.91-m. × 0.64-cm. o.d. All chemicals used were reagent grade or the best quality available.

Operating Conditions—The column was operated isothermally at 115° with the detector block and injection port at 150°. The helium flow rate was 55 ml./min. with an inlet pressure of 40 p.s.i. The electrometer range was 100 with an attenuation of 32. Sample injections of between 1 and 5 μl. were made.

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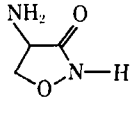

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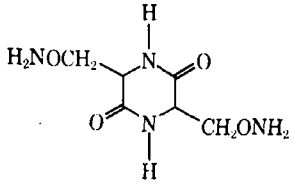

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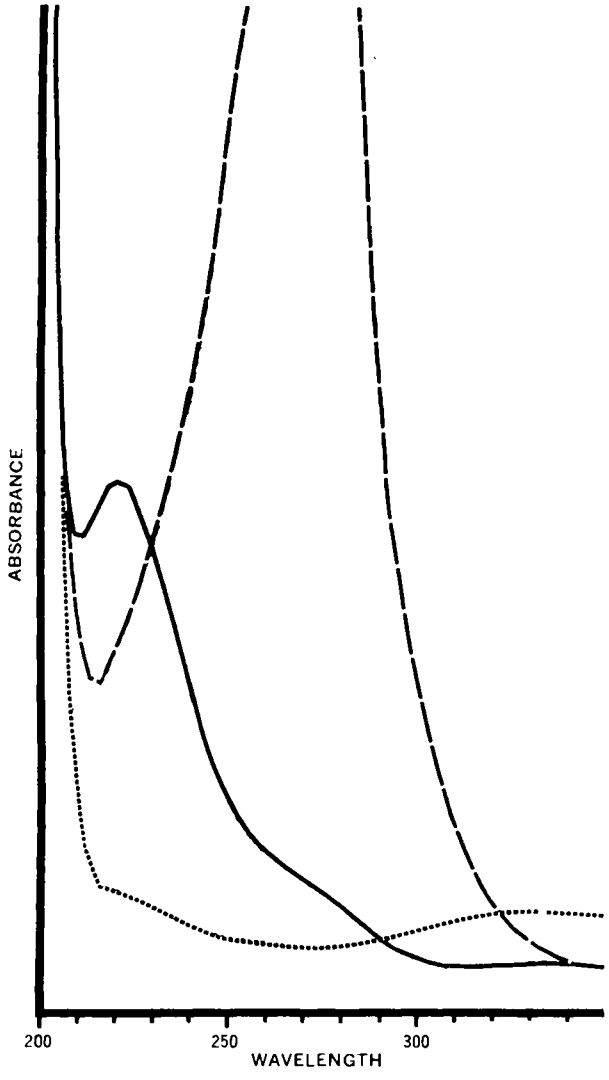

Figure 1—*UV spectra of cycloserine (——) and of 3,6-bis(aminomethyl)-2,5-piperazinedione base treated (- - -) and untreated (. . .).*

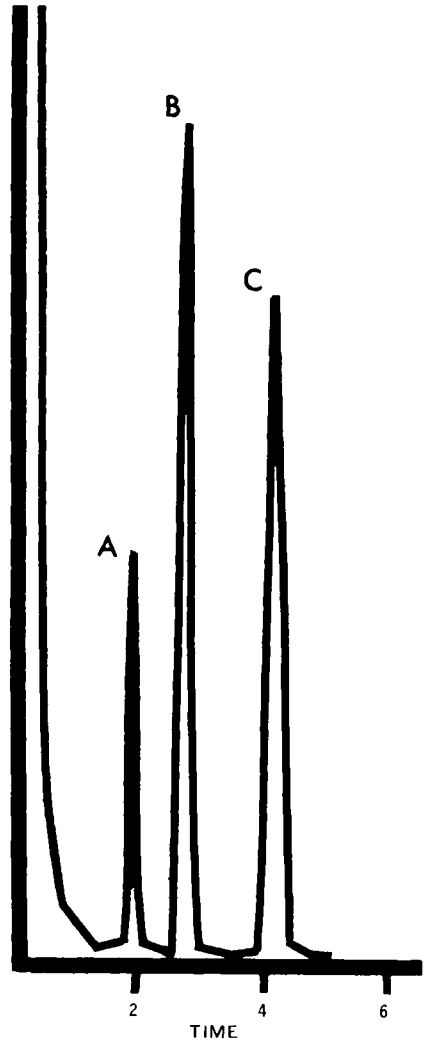

Figure 2—*Typical gas chromatogram of trimethylsilyl-derivatized cycloserine. Key: A, derivatization by-product; B, N,N'-bis-(trimethylsilyl)-cycloserine; and C, hexamethylbenzene.*

according to the procedure described in the British Pharmacopoeia (6). Since this treatment converts II to 3,6-bismethylene-2,5-piperazinedione, solutions of II were also prepared in 0.1 *N* hydrochloric acid. UV spectra were obtained on a recording spectrophotometer[7].

GLC–Mass Spectrometry—Mass spectra of the peak observed on GLC were obtained on a mass spectrometer[8] coupled with a gas chromatograph[9] by means of a separator[10]. The sample was ionized under the influence of 70 ev.

RESULTS AND DISCUSSION

The UV spectra of cycloserine monomer (I) and dimer (II), after treatment with sodium hydroxide as described in the British Pharmacopoeia (6), are presented in Fig. 1. Obviously, a mixture of the two would not yield valid data for the assay of cycloserine using the 219-nm. reading. Also shown is the spectrum of an equivalent concentration of II not treated with base. Although significantly less absorbance at 219 nm. is exhibited, readings taken on mixtures of monomer and dimer would not be completely free of error.

A typical gas chromatogram is presented in Fig. 2. The first peak is a by-product of silylation. The second peak is the trimethylsilyl derivative of cycloserine, which was identified as *N,N'*-bis-(trimethylsilyl)-cycloserine by GLC–mass spectrometry. The fragmentation pattern was analogous to that described for various cycloserine derivatives (7). The third peak in the chromatogram is hexamethylbenzene, the internal standard. Relative to hexamethylbenzene, the retention time of cycloserine is 0.67.

Accuracy and precision were determined by analyses of five replicate samples. A residual standard deviation of ±0.5% and a relative error of +2.0% were obtained.

Samples of II and of 3,6-bismethylene-2,5-piperazinedione were taken for GLC under conditions identical to those for I. An insoluble residue remained after treatment with silylating reagents, and no peaks were detectable on chromatography even after extended time periods and temperature elevation. These compounds interfere with UV analysis of I.

A sample of β-aminoxyalanine, treated in the same manner as I, gave a peak with a relative retention time of 4.0 compared to hexamethylbenzene. Thus, the known degradation products of I have been shown not to interfere with GLC analysis.

Aged and degraded samples of cycloserine capsules were evaluated by this method. The assay values showed a decrease *versus* the age of the sample and were in agreement with microbiological data. No interferences or extraneous peaks were observed on the chromatograms.

SUMMARY

A GLC procedure for the specific determination of cycloserine was developed. The method is precise to within ±0.5%. The determination of cycloserine in a pharmaceutical preparation and in

[7] Cary model 14.
[8] Hitachi Perkin-Elmer RMN-6D.
[9] Hewlett-Packard 402 F&M.
[10] Biemann-Watson.

the presence of degradation products was performed. The method may also find application in kinetic studies of cycloserine degradation.

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Selective Determination of Belladonna Alkaloids by GLC

RALPH S. SANTORO▲, PATRICIA P. PROGNER, EDWARD A. AMBUSH, and DAVID E. GUTTMAN

Abstract □ Atropine/hyoscyamine and scopolamine, in dosage forms in which the scopolamine represented less than 9% of the total belladonna alkaloids, were isolated as free bases from phenylpropanolamine, chlorpheniramine, and interfering excipients by a series of pH- and solvent-controlled extractions and were quantitated by GLC. Homatropine was used as the internal standard. Precision and accuracy data are represented, along with assay results on three commercial capsule lots.

Keyphrases □ Belladonna alkaloid mixtures with phenylpropanolamine and chlorpheniramine, low scopolamine content—GLC analysis, atropine/hyoscyamine and scopolamine □ Atropine/hyoscyamine and low-content scopolamine—GLC analysis in mixtures with phenylpropanolamine and chlorpheniramine □ Scopolamine (low content) and atropine/hyoscyamine—GLC analysis in mixtures with phenylpropanolamine and chlorpheniramine □ GLC—analysis, atropine/hyoscyamine and scopolamine in mixtures with phenylpropanolamine and chlorpheniramine

Because of its specificity, sensitivity, and resolving properties, GLC has been increasingly used for belladonna alkaloid analysis (1–6). The USP collaborative study for the assay of atropine and scopolamine dosage forms by GC (7) indicated that these two substances, when found individually in tablet dosage forms, could be assayed in this manner with an acceptable degree of precision.

However, the case becomes less clear, even with the high resolving power of GC, when belladonna is found in the presence of a preponderant concentration of other amines and/or in dosage forms with high background contributions. The problem is further complicated when both atropine and scopolamine must be determined together with, as is many times the case, the scopolamine only a small fraction of the atropine.

This paper describes a GLC procedure in which hyoscyamine sulfate, atropine sulfate, and scopolamine hydrobromide in dosage forms are determined in the presence of chlorpheniramine maleate and phenylpropanolamine hydrochloride, and also where the scopolamine content is less than 9% of the total belladonna present.

EXPERIMENTAL

Materials—*Buffer*—Dissolve 34.8 g. of dibasic potassium phosphate in 900 ml. of water, adjust to pH 9.0 with 1 *N* hydrochloric acid or sodium hydroxide, and dilute to 1 l. with water.

Methylene Chloride—Distill reagent grade methylene chloride and store in a tightly closed container.

Atropine Sulfate Standard Solutions—Prepare solutions of USP Atropine Sulfate Reference Standard (or NF Hyoscyamine Sulfate Reference Standard), dried at 105° for 4 hr., in 0.01 *N* sulfuric acid with concentrations of 0.70, 0.65, and 0.60 mg./ml.

Scopolamine Hydrobromide Standard Solutions—Prepare solutions of USP Scopolamine Hydrobromide Reference Standard, dried at 105° for 4 hr., in 0.01 *N* sulfuric acid with concentrations of 0.070, 0.065, and 0 060 mg./ml.

Internal Standard Solution—Prepare a solution of USP Homatropine Hydrobromide Reference Standard, dried at 105° for 4 hr., in 0.01 *N* sulfuric acid with a concentration of 0.50 mg./ml.

Procedure—For this study, the contents of six capsules, each containing 50 mg. of phenylpropanolamine hydrochloride, 4 mg. of chlorpheniramine maleate, and 0.25 mg. of combined belladonna alkaloids (0.0375 mg. atropine sulfate, 0.0219 mg. scopolamine hydrobromide, and 0.1906 mg. hyoscyamine sulfate) were triturated with 0.01 *N* sulfuric acid. The slurry was quantitatively transferred to a 50-ml. volumetric flask, 2.0 ml. of internal standard was added, and it was diluted to volume with 0.1 *N* sulfuric acid. The contents of the flask were transferred to a 50-ml. centrifuge bottle and centrifuged at 2000 r.p.m. for 15 min., and 25.0 ml. of clear supernatant solution was transferred to a 150-ml. separator.

The solution was extracted twice with 25-ml. portions of chloroform, and the chloroform extracts along with any emulsion were discarded. Vigorous shaking was avoided in this step to prevent excessive emulsion formation, so losses of belladonna alkaloids and internal standard were minimized. The pH of the solution was adjusted to 8.0 ± 0.1 by the addition of 25 ml. of pH 9.0 buffer and the dropwise addition of 1 *N* sodium hydroxide; then the solution was extracted with three 25-ml. portions of cyclohexane, and the cyclohexane extracts were discarded. The solution was further

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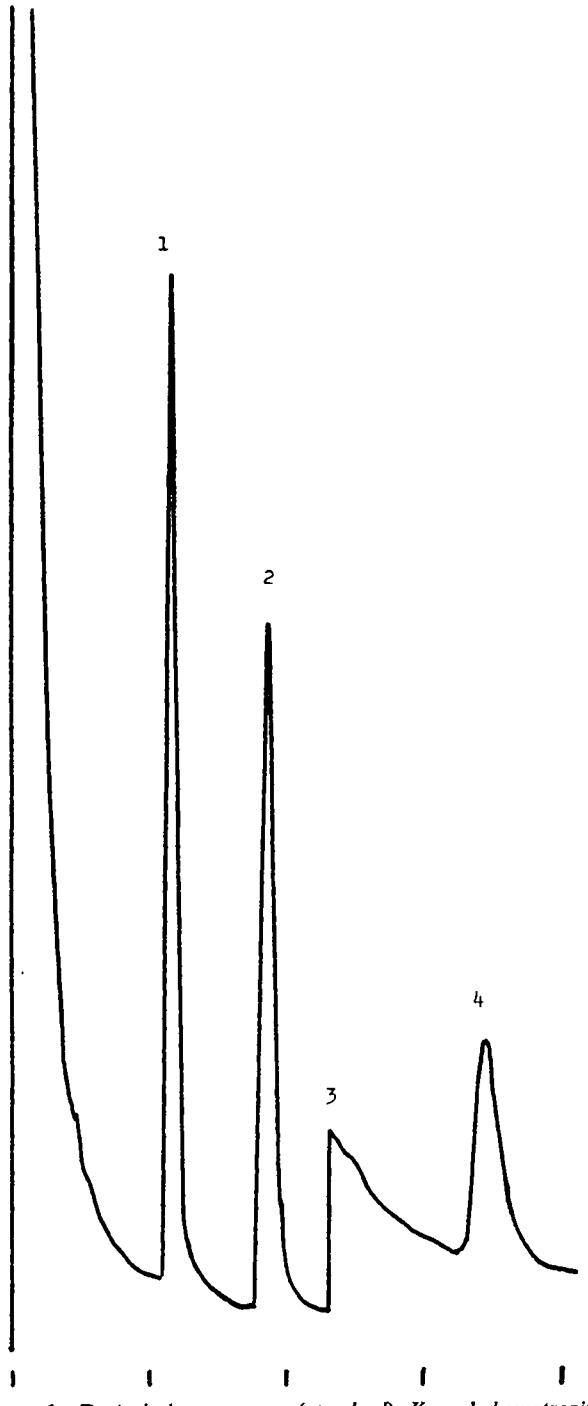

Figure 1—*Typical chromatogram (standard). Key: 1, homatropine; 2, atropine/hyoscyamine; 3, attenuation change; and 4, scopolamine.*

adjusted to pH 9.0 ± 0.1 with 1 *N* sodium hydroxide and extracted with two 10-ml. portions of methylene chloride. Then the methylene chloride extracts were combined and allowed to stand to permit as much of the emulsion to break as practical. The clear extract was filtered through a cotton–anhydrous sodium sulfate filter, previously wetted with methylene chloride into a 25-ml. graduated cylinder, transferred to a 19 × 36-mm. glass vial equipped with a plastic liner cap, and evaporated to dryness under nitrogen and mild heat. The residue was dissolved in 0.9 ml. of methylene chloride and used for GLC. A series of combined standards was prepared by pipeting 1.0 ml. of each atropine and scopolamine standard solution into each of three 150-ml. separators containing 25 ml. of 0.1 *N* sulfuric acid, so that combined standards of 0.70 and 0.070, 0.65 and 0.065, and 0.60 and 0.060 mg. of atropine and scopolamine, respectively, were obtained. A 1.0-ml. of aliquot of the internal standard solution was pipeted into each separator, and the combined standards were then carried through the procedure as already described, beginning with: "The solution was extracted twice with 25-ml. portions of chloroform. . . ."

GLC—The analysis was performed on a gas chromatograph[1] equipped with a 1.22-m. (4-ft.) × 4-mm. glass column packed with 3% (w/w) methyl phenyl silicone oil on 100–120-mesh silanized, acid-washed, flux-calcined diatomite[2] and also equipped with a flame-ionization detector. The column was conditioned at 250° for 1 hr. with helium flowing to remove oxygen and solvents, stopping the flow of helium and heating at about 340° for 4 hr., and finally lowering the temperature to 250° and conditioning with helium flowing until a stable baseline was obtained (7, 8). Weekly injections[3]

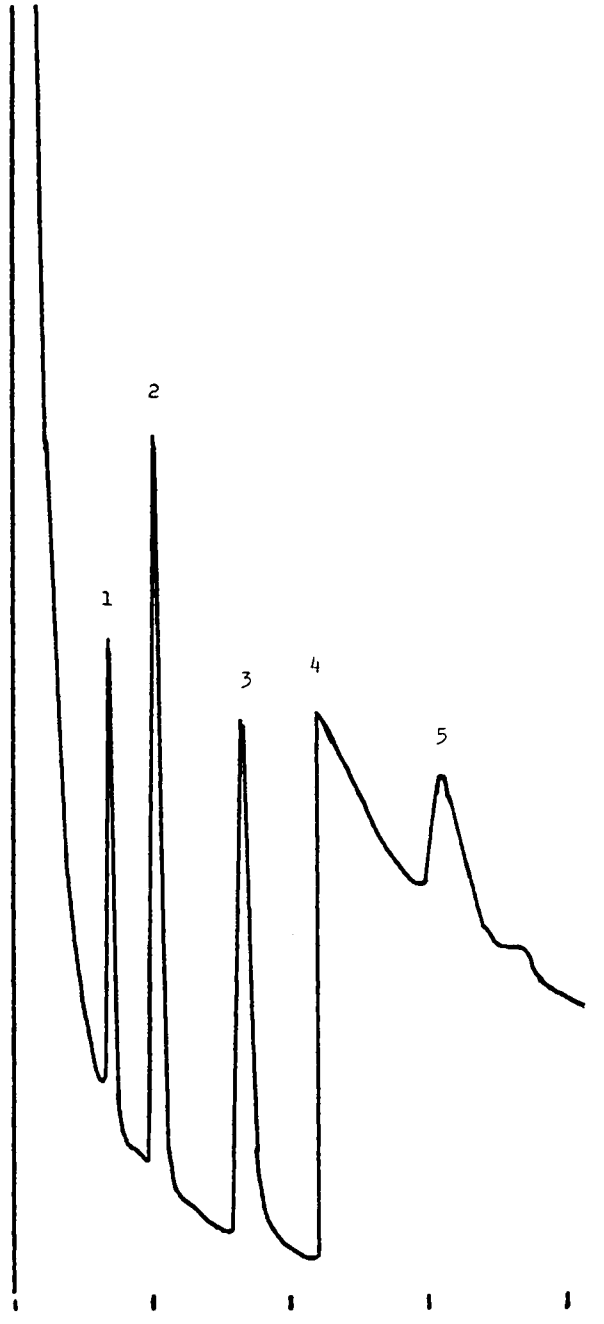

Figure 2—*Typical chromatogram (capsule). Key: 1, chlorpheniramine; 2, homatropine; 3, atropine/hyoscyamine; 4, attenuation change; and 5, scopolamine.*

[1] Perkin-Elmer 900.
[2] 3% OV-17 on 100–200-mesh Gas Chrom Q, Applied Science Laboratories.
[3] Silyl-8 GLC column conditioner, Pierce Chemical Co.

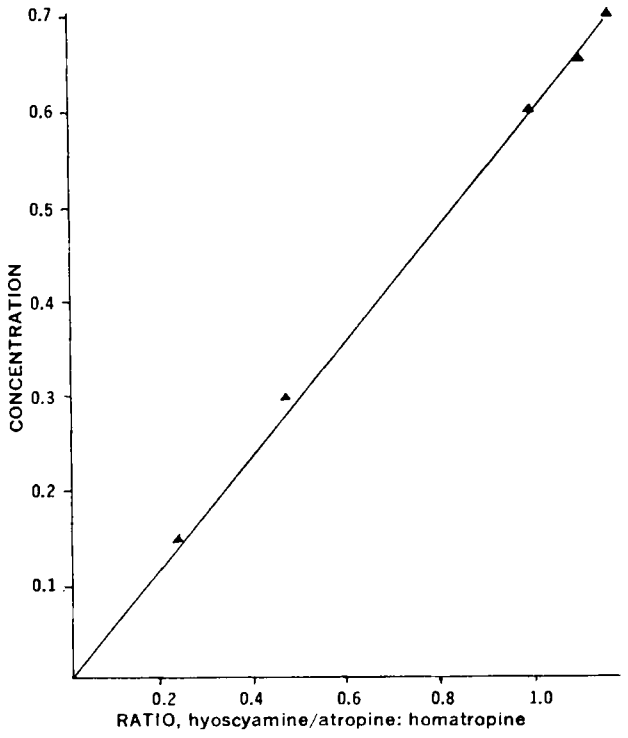

Figure 3—*Representative standard curve for atropine/hyoscyamine dissolution studies.*

maintained column efficiency. The analysis was carried out isothermally at 210°, with the helium carrier gas flow rate at about 50 ml./min. and the temperature of the injection port not more than 25° above that of the column.

Appropriate volumes of the standard and sample preparations were injected into the chromatograph, and the peaks were obtained at a convenient attenuation. The attenuation was decreased by a factor of 8 about halfway between the hyoscyamine/atropine peak and the scopolamine peak to increase the height of the scopolamine peak. The heights were measured for the first (homatropine), second (hyoscyamine and atropine), and third (scopolamine) components for each standard injection, and the values were recorded as P_1, P_2, and P_3, respectively. The standard hyoscyamine/atropine ratio, R_a, and standard scopolamine ratio, R_s, were calculated by:

$$R_a = P_2/P_1 \qquad \text{(Eq. 1)}$$

$$R_s = P_3/P_1 \qquad \text{(Eq. 2)}$$

The average factors for hyoscyamine/atropine, F_a, and scopolamine, F_s, were determined by dividing each R_a and R_s into its respective standard concentration and averaging the individual factors obtained. The peak heights were measured for the sample preparations in the same manner as above and recorded as p_1, p_2, and p_3, respectively. The hyoscyamine/atropine ratio, r_a, and scopolamine ratio, r_s, were calculated by:

$$r_a = p_2/p_1 \qquad \text{(Eq. 3)}$$

$$r_s = p_3/p_1 \qquad \text{(Eq. 4)}$$

The hyoscyamine/atropine (A_1) and scopolamine (A_2) assay was calculated by:

$$A_1 = \frac{F_A \times r_a \times 1.023}{3} \qquad \text{(Eq. 5)}$$

$$A_2 = \frac{F_s \times r_s \times 1.12}{3} \qquad \text{(Eq. 6)}$$

The factors 1.023 and 1.12 convert anhydrous hyoscyamine/atropine and scopolamine to the USP hydrated form.

Table I—Belladonna Alkaloid Recovery

Sample	Percent Recovered[a] Hyoscyamine/Atropine	Scopolamine
1	99	95
2	101	102
3	100	102
4	100	105
5	101	105
6	100	100
Average	100	102
Sigma	0.8	3.7
Standard error	0.3	1.5

[a] Each recovery sample contained 150 mg. phenylpropanolamine hydrochloride, 12 mg. chlorpheniramine maleate, and 0.75 mg. belladonna alkaloids (atropine sulfate, 0.1125 mg.; hyoscyamine sulfate, 0.5700 mg.; and scopolamine hydrobromide, 0.0675 mg.) plus placebo.

Table II—Belladonna Alkaloid Assay of Commercial Capsule Lots

Lot	Percent Claim[a] Hyoscyamine/Atropine	Scopolamine
1	97	97
2	100	97
3	100	102

[a] Claim per capsule: 50 mg. phenylpropanolamine hydrochloride, 4 mg. chlorpheniramine maleate, and 0.25 mg. belladonna alkaloids (atropine sulfate, 0.0375 mg.; hyoscyamine sulfate, 0.1906 mg.; and scopolamine hydrobromide, 0.0219 mg.).

RESULTS AND DISCUSSION

Figures 1 and 2 are typical chromatograms obtained from standards and samples with a 10-μl. injection, preliminary attenuation of 256, and a final attenuation for scopolamine of 32. The system does not differentiate the isomers, hyoscyamine and atropine, which are eluted as one peak. The peak before the internal standard, homatropine, in the sample chromatogram (about 3 4 min.) is a trace of chlorpheniramine not removed by the cyclohexane extraction at pH 8.0. The dissolution of atropine/hyoscyamine in some dosage forms was followed, and Fig. 3 is a representative standard curve obtained in these experiments. The curve shows good linearity throughout the required range.

Table I lists recoveries of known amounts of the belladonna alkaloids mixed with placebo, which contained phenylpropanolamine hydrochloride and chlorpheniramine maleate in the ratio of 200 and 16 times, respectively, the concentration of the total belladonna content. Recoveries were quantitative, with the standard deviation for scopolamine significantly higher than that for hyoscyamine/atropine, reflecting the fact that its peak was measured at a much lower attenuation because it represented less than 9% of the total belladonna present. Furthermore, a small error in measurement produced a proportionately greater error in the final calculation.

Three different commercial lots of capsules were assayed (Table II). The reproducibility was checked in Lot 1, and the standard deviations were found to be 1.4 and 3.4 atropine/hyoscyamine and scopolamine, respectively. These values were of the same order of magnitude as reported in the recovery experiments.

Control of pH was important for obtaining correct results and acceptable precision limits. The initial extraction with chloroform from acid was to remove interfering wax excipients that chromatograph with similar retention times as the internal standard and belladonna alkaloids. The adjustment to pH 8.0 for the cyclohexane extraction was carefully controlled, since a higher pH would entail significant loss of belladonna while a lower value would leave too much chlorpheniramine behind. The chlorpheniramine would be extracted in the next step and interfere with the internal standard peak. Similarly, careful adjustment to pH 9.0 for the methylene chloride extraction of the belladonna was required because phenylpropanolamine would begin to partition at a higher pH. Distilled methylene chloride was necessary for extraction because reagent grade and spectrograde material contained additives that chromatographed with a similar retention time as that of scopolamine.

Throughout the extractions, vigorous shaking was avoided to control emulsion formation.

SUMMARY

A GLC procedure was presented that is capable of determining atropine/hyoscyamine and scopolamine in the presence of phenylpropanolamine and chlorpheniramine, and also where the scopolamine is less than 9% of the belladonna present. Interfering excipients were removed with chloroform from acid, adjustment to pH 8.0 and extraction with cyclohexane removed chlorpheniramine, and further adjustment to pH 9.0 with methylene chloride extraction isolated the belladonna alkaloids. The final extract was concentrated and gas chromatographed. Homatropine was used as the internal standard. The method was sensitive, highly specific, and reasonably precise.

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PHARMACEUTICAL TECHNOLOGY

Sensory Assessment of Spreadability of Hydrophilic Topical Preparations

B. W. BARRY▲ and M. C. MEYER

Abstract □ The rheological conditions operative during spreading of topical preparations on the skin were determined for series of aqueous gels and oil-in-water emulsions. Master curves were derived from the rheological data obtained using a sensory test with a panel of 24 members. Rates of shear varied from 350 to 10,000 sec.$^{-1}$, depending on the consistency and the type of preparation being spread. Two preference scoring techniques were used to assess the series of preparations in terms of spreadability. From the preference data, the shearing conditions with optimum patient acceptance potential were derived. An indication is given for the use of such master curves with their preferred regions for the determination of instrumental rheological conditions for use in routine and innovative industrial control procedures. Where relevant, all results were compared with previously reported work on lipophilic formulations.

Keyphrases □ Spreadability—sensory tests correlated to rheological conditions, hydrophilic topical preparations □ Rheological master curve—shear rate variation, spreadability of hydrophilic topical preparations, sensory tests □ Skin, sensory perception of spreadability of hydrophilic topical preparations—correlation of rheological conditions, master curves derived

In the pharmaceutical and cosmetic industries, it is important that formulations, besides possessing the correct physicochemical and biopharmaceutical properties, should have maximum patient and consumer acceptability. Spreadability plays an important role in the patient's assessment of a topical product. The correct consistency for such a preparation helps to ensure that a suitable dose is applied to the skin. This is particularly important with vehicles that incorporate potent drugs such as corticosteroids; excessive doses may lead to collagen atrophy and other undesirable side effects. Previous authors have assessed spreadability in terms of shear rate, either by a correlation with non-Newtonian viscosities (1–4) or by assuming that the spreading procedure can be likened to plane laminar flow between parallel plates (5–7) and thus can be described by the equation:

$$\dot{\gamma} = V/d \qquad \text{(Eq. 1)}$$

where $\dot{\gamma}$ is the rate of shear, V is the relative velocity of the plates or skin surfaces, and d is the distance between them. *Estimates* of V and d were used to find the shearing conditions operative during spreading.

In contrast, Wood (8) developed a method which involved comparing Newtonian liquids with pseudoplastic liquids to determine the approximate rate of shear developed in the mouth. This procedure, which has the advantage that V and d need not be known to determine the shearing conditions occurring *in vivo*, was modified by Barry and Grace (9) to investigate the rheological conditions that operate during the spreading of lipophilic preparations on the skin.

In the present work, the techniques used by Barry and Grace (9) were extended to investigate the shearing con-

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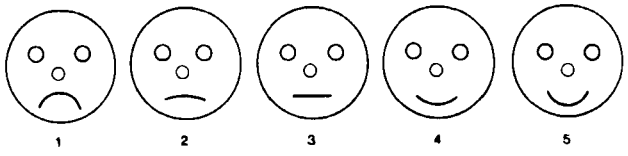

Figure 1—*Preference Scoring System 2. Faces represent degrees of agreeableness during spreading, with numerical scores as shown.*

ditions that operate during the spreading of aqueous gels. Oil-in-water emulsions were similarly tested to ascertain whether an internal lipophilic phase affected these conditions.

Preference scaling techniques were used to obtain the optimum consistency for spreading and, thus, the maximum patient acceptability potential for a preparation.

The formulations investigated varied in consistency from mobile liquids to stiff and, in some cases, highly elastic semisolids and, therefore, encompassed the range of consistencies found in hydrophilic topical applications.

EXPERIMENTAL

In general, the theory used and experimental procedures applied were as reported previously (9). Only the essential details and variation in procedure are reported here.

Preparations Used—Fifteen gels, ranging in consistency from mobile liquids to elastic semisolids, were prepared by neutralizing aqueous solutions of carboxypolymethylene 940 or 941[1] with triethanolamine (Table I). Sample 1 was a mixture of starch and Carbopol gel. Samples 17–22 were liquid paraffin in water emulsions stabilized by the mixed emulsifier cetrimide–cetostearyl alcohol in various concentrations. All materials were of BP or BPC quality, except the Carbopols which were commercial samples. Prior to use, samples were stored at 25 ± 1°.

Instrumental Rheology—A range of silicone oils [viscosities determined[2] by Barry and Grace (9) at 34 ± 0.2°] were used as standards. A cone and plate viscometer[3] with automatic flow curve recorder unit and *X–Y* plotter was used in two modes to obtain rheograms for the test preparations at 34 ± 0.2° (approximate skin temperature).

Instrumental Method 1—The samples were continuously sheared from 0.0 to 1754.0 to 0.0 sec.$^{-1}$ in 120 sec. When necessary, the shear rate range was changed either by alteration (by a factor of 10) of the gear train in the cone drive unit or by using cones of different angles.

Instrumental Method 2—The viscometer (with *X–Y* plotter measuring torque as a function of time) was used to determine shear stresses at various constant rates of shear. The "fast-up" control applied the rate of shear within about 1 sec., and the "hold speed" control maintained the shear rate. From the plot obtained at each shear rate, the shear stresses at zero time and after 20 sec. [estimate of average time of spreading (9)] were determined.

Instrumental Method 2 is relevant when the material being tested exhibits time-dependent behavior on shearing. For Carbopol gels, time-dependent effects were negligible (programmed shearing provided negligible hysteresis loops); thus, the data obtained by Method 2 agreed with those from Method 1 and are not included. Since the oil-in-water emulsions provided large hysteresis loops, both sets of data are shown.

Sensory Tests—From a panel of 24, a minimum of 14 and a maximum of 24 persons were selected for any one test. The untrained panel contained both sexes, with an age range of 18–50 years. The panel members were asked to assess the preparations critically, according to the relevant scaling procedure, while spreading the materials onto the inner surface of the forearm. In all sensory tests, three or four samples were presented to each panel

[1] Carbopol, Honeywill and Stein Ltd.
[2] Using a Haake-Rotovisko.
[3] Ferranti-Shirley.

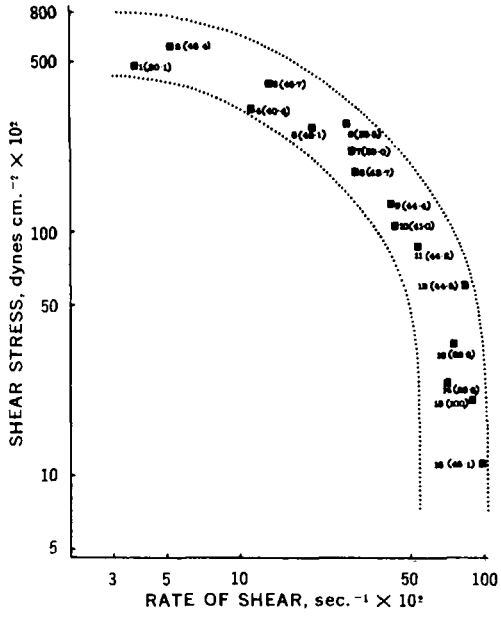

Figure 2—*Master curve of rheological conditions that operate during spreading of aqueous gels on the skin. The data are numbered to identify the samples; in parentheses are the coefficients of variation of the Newtonian viscosity values used to obtain the data.*

member at each session according to a scheme designed to eliminate the sample sequence affecting the results. The reliability of the panel members was confirmed using the method of circular triads (10–12) with Samples 5–12.

Shear Conditions during Spreading—Panel members were asked to indicate for each sample the Newtonian silicone oil that appeared

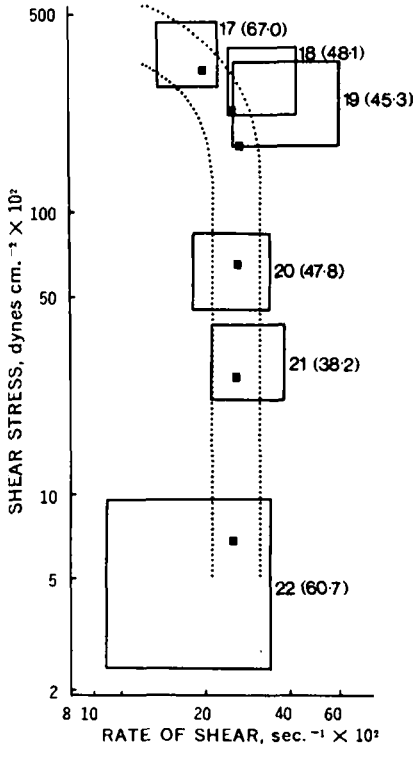

Figure 3—*Master curve of rheological conditions that operate during spreading of oil-in-water emulsions on the skin. The black squares indicate data derived from Instrumental Method 1, and the open rectangles indicate data derived from Instrumental Method 2. The data are numbered to identify the samples; in parentheses are the coefficients of variation of the Newtonian viscosity values used to obtain the data.*

Table I—Formulations of Preparations Used (by Weight)

Sample Number	Carbopol 940 or 941	Starch	Cetrimide	Cetostearyl Alcohol	Water	Liquid Paraffin
1	5	50	—	—	45	—
2–16	20–0.025	—	—	—	80–99.975	—
17	—	—	6.4	57.6	100	300
18	—	—	4.8	43.2	100	300
19	—	—	4.0	36.0	100	300
20	—	—	3.2	28.8	100	300
21	—	—	2.4	21.6	100	300
22	—	—	0.8	7.2	100	300

most similar while spreading on the skin. The shearing conditions operative during spreading are assumed to be those located at the intersection of the rheogram obtained by Instrumental Method 1 or 2, and the flow curve for the Newtonian silicone oil, judged by the panel to be of similar spreadability to the sample under test.

Preference Testing—Panel members were asked to evaluate Samples 4–22 taken in groups of three or four according to two scoring systems (only one scoring system was used per session).

System 1 [Five-Point Semantic Hedonic Scale (5, 8, 9)]—

Score	Sensation during Spreading
1	Too fluid, disagreeable
2	Fluid but all right
3	Agreeable
4	Stiff but all right
5	Too stiff, disagreeable

System 2 [Five-Point Facial Hedonic Scale (13, 14)]—Panel members were asked to indicate the face that most closely agreed with their feeling with regard to the spreadability of each sample. Scores were allocated to their decision (Fig. 1). The faces depict the degree of agreeableness or disagreeableness experienced by the subject, the neutral face being the median interval. System 2 was developed (13) to overcome problems in semantics, which can arise with the use of descriptive rating scales such as System 1. A score of 1 or 5 in System 1 corresponds approximately to a score of 1 in System 2. A score of 2 or 4 in System 1 corresponds approximately to a score of 2 in System 2, and a score of 3 is approximately the same for both systems. Scores of 4 and 5 in System 2 represent degrees of agreeableness not present in System 1; hence, System 2 was considered by one worker to be more sensitive than System 1 when used for tasting tests (13).

Data Analysis[4]—A computer program was used to fit the best straight line or curve to the data obtained from the two preference scoring methods, according to the least-squares criterion. A second program was used to calculate the means, standard deviations, coefficients of variation, and other statistical data used in this work.

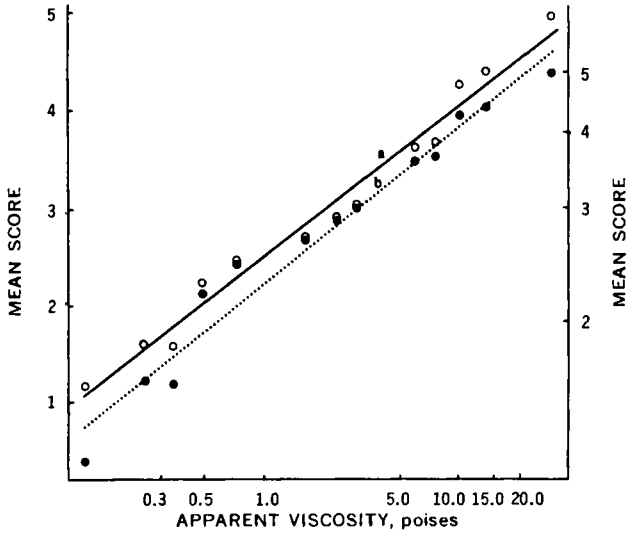

Figure 4—*Data from Preference Scoring System 1 for gels. Key: —, O, a, single-logarithmic plot; and . . . , ●, b, double-logarithmic plot.*

[4] Using an I.C.L. 4130 computer with computer instrumentation plotter C4600.

RESULTS

The rheological conditions operative during spreading of the aqueous gels were similar whether derived by Instrumental Method 1 or 2. These conditions, when used in a double-logarithmic plot of shearing stress against shear rate, showed a distinct trend (dotted lines in Fig. 2). The double-logarithmic plot of the oil-in-water emulsions showed a similar trend to the gels but, in general, the oil-in-water emulsions were assessed at lower rates of shear (Fig. 3). The Newtonian viscosity, used to determine the rheological conditions for any sample, was the arithmetic mean of the silicone oil viscosities considered by each panel member to be similar to the topical product during spreading. The coefficients of variation of the panel data are indicated in Figs. 2 and 3.

The data derived from the two preference scaling procedures are listed in Table II. The apparent viscosities quoted were obtained from Instrumental Method 1 data at the rate of shear indicated approximately by the master curve. The mean panel scores were compared graphically with their respective instrumental viscosities. For Scoring System 1, linear plots were obtained using semi- and double-logarithmic axes; for Scoring System 2, parabolic plots were obtained using the same axes (Figs. 4–7). The equations for these lines are listed in Table III. The use of higher order polynomials in the computer analysis gave better fitting curves but were not used owing to the limited accuracy of the experimental data.

The data from Scoring System 2 for Samples 4 and 16 were not included in the computer analysis, because these points would have

Table II—Data Derived from Preference Scoring Procedures[a]

Sample Number	Apparent Viscosity, poises	Preference Scoring—System 1[b]			Preference Scoring—System 2[b]		
		S	*SD*	*CV*	*S*	*SD*	*CV*
4	29.5	4.9	0.2	4.7	1.4	0.6	41.5
5	13.5	4.4	0.4	10.0	1.9	0.7	37.4
6	10.1	4.3	0.5	11.9	2.3	0.9	37.4
7	7.6	3.7	0.6	17.1	2.9	0.9	32.1
8	6.0	2.9	0.5	14.9	3.5	0.8	24.4
9	3.2	3.0	0.3	11.0	4.1	0.8	19.1
10	2.5	2.9	0.4	13.0	4.0	0.8	19.6
11	1.6	2.7	0.5	19.5	3.7	1.0	26.6
12	0.72	2.4	0.6	24.9	3.3	1.0	32.3
13	0.48	2.2	0.6	29.4	3.5	1.3	36.5
14	0.35	1.6	0.6	36.5	2.4	1.1	47.1
15	0.23	1.6	0.8	47.8	2.0	1.0	47.3
16	0.11	1.8	0.4	33.4	1.7	0.9	54.1
17	15.8	4.2	0.5	13.0	2.4	0.9	39.2
18	9.2	3.7	0.5	14.8	3.0	1.0	33.3
19	6.44	3.4	0.4	12.6	3.6	0.9	24.6
20	2.5	2.9	0.4	12.5	4.5	0.6	14.5
21	1.0	2.5	0.5	18.7	3.7	1.1	29.9
22	0.27	1.5	0.4	27.1	2.0	0.8	38.1

[a] Samples arranged in decreasing order of apparent viscosity, grouped as gels and emulsions. [b] *S* is the arithmetic mean of the data, *SD* is the standard deviation (determined using $n - 1$ degrees of freedom), and *CV* is the coefficient of variation expressed as a percentage.

Table III—Equations[a] for the Plots Shown in Figs. 4 and 5 of the Type $y = bx + c$ and in Figs. 6 and 7 of the Type $y = ax^2 + bx + c$

	Mean Score, y	Log Mean Score, y'	Coefficient, a, of (Log Apparent Viscosity)^2, x^2	Coefficient, b, of Log Apparent Viscosity, x	Constant, c
Figure 4	y	—	—	1.54	2.49
	—	y'	—	0.25	0.36
Figure 5	y	—	—	1.43	2.37
	—	y'	—	0.24	0.35
Figure 6	y	—	−2.71	1.37	3.82
	—	y'	−0.41	0.21	0.59
Figure 7	y	—	−2.60	1.83	3.82
	—	y'	−0.37	0.28	0.57

[a] Equations for Figs. 4 and 5 are derived from Preference Scoring System 1; those for Figs. 6 and 7 are derived from Preference Scoring System 2.

unrealistically distorted the curve. This situation arises because the minimum preference score is 1, whereas apparent viscosities may range from near zero to infinity. If such extreme data are added to the graph, the resulting extended tails seriously widen the curve apex. This makes the curve a less sensitive means for assessing the range of acceptable apparent viscosities. For Scoring System 1, the optimum apparent viscosity for spreading corresponded to a score of 3 and the range of acceptable apparent viscosities was given by the score of 2–4. For Scoring System 2 the optimum was given by the highest score obtained and corresponded to the maximum of the parabola; the range was given by a score of 3 or greater. These values were obtained from the plots (Figs. 4–7) and checked by substituting the appropriate scores in the respective equations (Table III). The optimum viscosities were plotted, in terms of shear rate–shear stress, on the master curves, and the intersection of the two plots defined the shearing conditions with maximum patient acceptability for the class of preparations under consideration (Fig. 8). The range of acceptable values were similarly plotted (shaded areas in Fig. 8).

DISCUSSION

Figure 2 shows the approximate rheological conditions that operate during the spreading of aqueous gels on the skin. The master curve obtained for these hydrophilic preparations approximates to a mirror image, about the figure's diagonal axis, of the master curve for lipophilic materials (9). This suggests that there is a large general region defining the shear stress–shear rate conditions which operate during spreading of topical preparations and that exact conditions depend on the class of preparations applied.

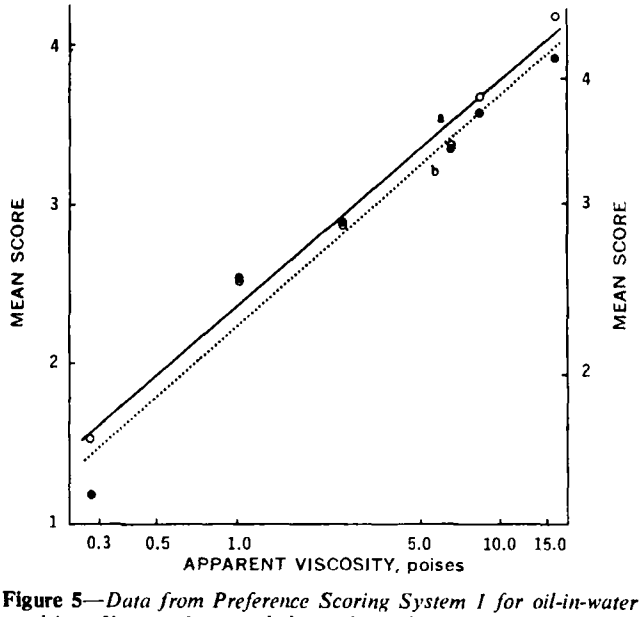

Figure 5—*Data from Preference Scoring System 1 for oil-in-water emulsions. Key: —, ○, a, single-logarithmic plot; and . . . , ●, b, double-logarithmic plot.*

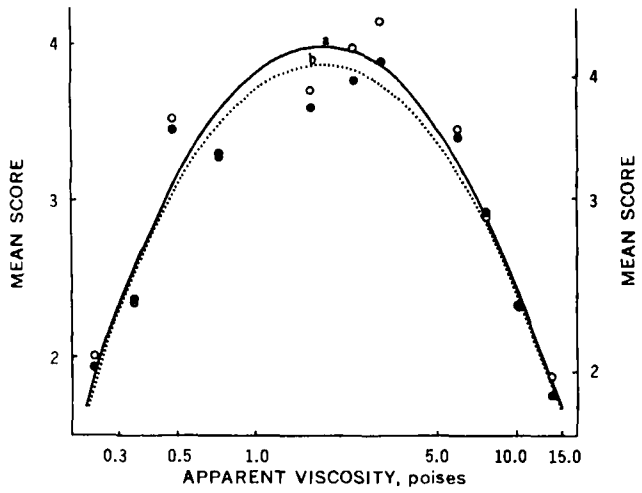

Figure 6—*Data from Preference Scoring System 2 for gels. Key: —, ○, a, single-logarithmic plot; and . . . , ●, b, double-logarithmic plot.*

The Carbopol gels containing more than 5% polymer (Samples 2–4) were highly elastic semisolids, which tended to roll up rather than to spread when applied to the skin. It was considered possible that this dominant elasticity was mainly responsible for the shape of the curve at high stress values and that very viscous preparations which lacked substantial elastic properties would not fit on the curve. To resolve this point, it was necessary to prepare and test a hydrophilic preparation of high consistency but minimal elastic recoil. This was achieved by blending starch with gel (Sample 5) to provide Sample 1. The position of the experimental point for this sample (Fig. 2) confirms the general curvature of the graph. In addition, gels containing 5% polymer or less (Samples 5–12), although they exhibited little rolling-up behavior, still defined the curved portion of the graph. Thus, it was considered valid to use elastic gels (Samples 2–4) as aids in defining the general shape of the curve, which then would be applicable to hydrophilic, elastic, and nonelastic gels.

The master curve for aqueous gels is approximately vertical at high shear rates, indicating that mobile materials are applied to the skin at a constant high rate of shear, with shear stress being the variable assessed subjectively by the patient. However, as consistency increased, the applied shear rate decreased so that both rheological

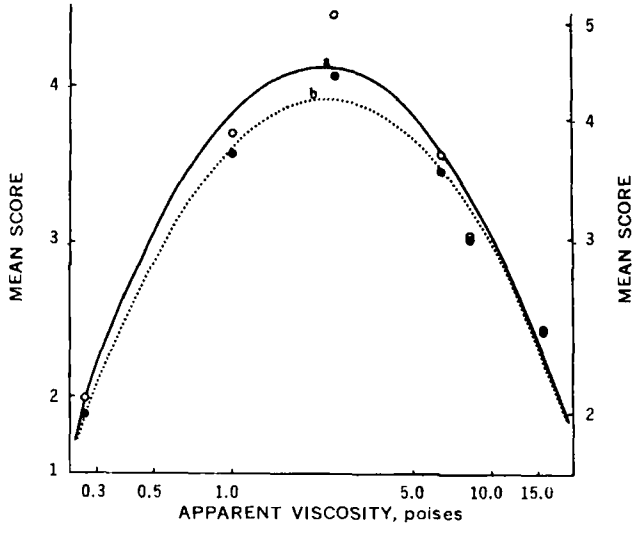

Figure 7—*Data from Preference Scoring System 2 for oil-in-water emulsions. Key: —, ○, a, single-logarithmic plot; and . . . , ●, b, double-logarithmic plot.*

Table IV—Optimum and Range of Acceptable Apparent Viscosities (poises) Derived from Single- (A) and Double- (B) Logarithmic Plots of Preference Scoring Data

Type of Material	Preference Scoring System 1				Preference Scoring System 2			
	A		B		A		B	
	Optimum[a]	Range[b]	Optimum[a]	Range[b]	Optimum[c]	Range[d]	Optimum[c]	Range[d]
Gels	2.1	0.5–9.5	3.0	0.6–9.4	1.8	0.4–7.3	1.8	0.5–6.8
Oil-in-water emulsions	2.8	0.6–13.9	3.4	0.5–11.7	2.2	0.5–10.7	2.3	0.6–9.9
Lipophilic materials[e]	6.8	3.9–11.8	7.4	3.8–11.8	—	—	—	—

[a] Corresponding to a preference mean score of 3 (Figs. 4 and 5). [b] Corresponding to a preference mean score of 2–4 (Figs. 4 and 5). [c] Corresponding to parabola maximum (Figs. 6 and 7). [d] Corresponding to a preference mean score of 3 (Figs. 6 and 7). [e] Derived from *Reference 9*.

parameters varied from gel to gel. The shear rate variation was caused by two factors: (*a*) the more mobile the gel, the faster the rubbing cycle used (V increases in Eq. 1); and (*b*) the stiffer the material, the thicker the layer applied (d increases in Eq. 1).

The master curves for lipophilic and hydrophilic preparations diverge widely over much of their lengths. This suggests that panel members consciously or unconsciously consider the extent of oiliness of a preparation when assessing its spreadability. This phenomenon is less important at the extremes of the consistency range where the master curves converge at high and low stress values; *i.e.*, at these consistencies, rheological parameters are much more important than a tactile stimulus such as oiliness. If it is true that, for the major portion of the consistency range, patients are influenced in a judgment of spreadability by the proportion of lipophilic material present, it follows that if an oil phase is introduced into a preparation but its overall nature is maintained as hydrophilic, the resulting master curve should lie between the lipophilic and gel curves. As can be seen from Fig. 3, the curve for such materials formulated as oil-in-water emulsions has the same general shape as the gel curve but it is shifted towards the lipophilic curve, thus confirming this view.

When master curves such as those shown in Figs. 2 and 3 have been constructed, it is possible to define preferred areas of acceptability by using the data derived from preference testing (Table IV). Optimum apparent viscosities for spreading, together with the acceptable ranges, are plotted on the master curves. (Figure 8 illustrates arithmetic average values for the two preference scoring methods calculated from both semi- and double-logarithmic plots.) As can be seen from Table IV, both preference scoring procedures gave similar results. However, compared to lipophilic preparations, the preferred regions for gels and oil-in-water emulsions were enlarged, caused mainly by the low limit corresponding to the "fluid but all right" rating. The difficulty that the panel experienced in scoring hydrophilic preparations as "too fluid, disagreeable" was possibly because they were conditioned from birth to accept very mobile, aqueous solutions for spreading on the skin (*i.e.*, washing with soap and water). Such conditioning would not significantly affect the rating for oily preparations; hence, a definite, relatively high, limit was obtained for lipophilic preparations. This conditioning effect is also illustrated in the way in which the optimum apparent viscosity for spreading increases in the order of: gels, oil-in-water emulsions, and lipophilic preparations, *i.e.*, in the order of decreasing hydrophilicity (Table IV). The upper limits for lipophilic and hydrophilic formulations were similar, although shearing conditions were higher in the latter case (*i.e.*, higher shear stresses and shear rates operate for hydrophilic materials).

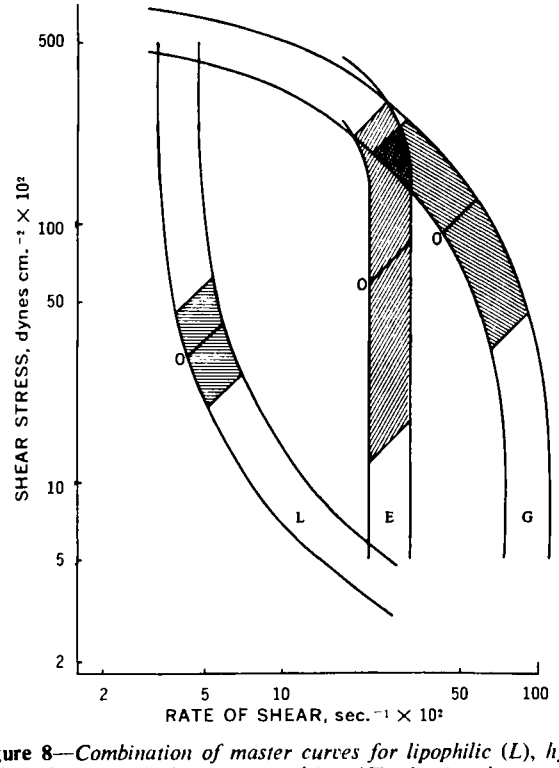

Figure 8—*Combination of master curves for lipophilic (L), hydrophilic gels (G), and oil-in-water emulsions (E), showing the acceptable range of viscosities for spreading on the skin (shaded area) and the optimum values (solid line 0).*

In the context of this conditioning effect, it was thought possible that there might be a difference in the location and extent of the preferred regions, based on whether these were derived using an all male or all female panel. For example, it might be that women, due to their experience with cosmetics, would be more critical than men of the spreadability of a preparation and would report a more condensed preferred region. Therefore, the preference results were divided into two subsets, male and female; the data were computed and graphed as before; and the subsets were compared with each other and with the total panel. However, any differences based on sex were found to be nonsignificant when compared by an F test and, thus, only total panel results have been reported here.

The data obtained from Scoring System 1 for hydrophilic preparations are similar to those obtained for lipophilic preparations (9), in that the regression lines derived from the data can equally well be represented by either single- or double-logarithmic equations as proposed by Weber and Fechner (15) and Stevens (16). In addition, the data obtained by Scoring System 2 (represented by second-order polynomials) provide similar curves when plotted on either single- or double-logarithmic axes (Figs. 6 and 7). According to Stevens' definition of stimuli (16), preference testing should be metathetic (qualitative—agreeable or disagreeable) and should thus be represented by a single logarithmic equation of the type:

$$\theta = K \log S \qquad \text{(Eq. 2)}$$

where θ is the sensory response to a stimulus, S, and K is a constant. Thus, as pointed out by Barry and Grace (9), the classification proposed by Stevens is probably invalid for the type of test considered in this paper. Treisman (17, 18) questioned Stevens' work and claimed that no experiment can distinguish between the two equations and that the relationship obtained will be a function of the method of scaling.

The correlation coefficients and the other data quoted tend to suggest that individual panel members react similarly when evaluating spreadability. However, the data used in the diagrams are arithmetic means. The coefficients of variation (Figs. 2 and 3 and Table II) show the variability of a sensation produced by the same stimulus on different people. One main disadvantage of preference testing in this case is that only a limited number of samples can be tested on a single occasion and no really effective means of cross-linking the data exists (20). The types and causes of other such variable factors were fully discussed by other authors (15, 20, 21).

The present work gives a broad picture of the type of laboratory screening procedure that may be utilized in innovative work on topical preparations prior to field trials. The new formulation, whether it be lipophilic (anhydrous or water-in-oil emulsion) or hydrophilic (aqueous gel or oil-in-water emulsion) can be tested using Instrumental Method 2 to obtain its rheogram. The instrumental data are then plotted onto the combined master curves (Fig. 8). Depending on the type of formulation under test, the new product curve should intersect the appropriate master curve in the preferred region. To achieve the maximum patient acceptance potential, this intersection should occur at the optimum value of the preferred region.

In addition, manufacturers often wish to standardize a product by a simple rheological test involving a single shear rate. At present, this parameter is often selected arbitrarily. It would be an improvement if selection were made relevant to the individual consistency of the product, taking into account its nature as an ointment, cream, gel, *etc*. This could be done as discussed here, and a shear rate between 350 and 10,000 sec.$^{-1}$ would be obtained. The particular shear rate, selected from such a wide range, would at least be relevant as a quality control parameter in that it would approximate that used by the patient when applying the medicament.

SUMMARY

The work reported here is an extension of that performed by Barry and Grace (9) on the rheological conditions operative during spreading of topical preparations. A master curve for aqueous gels was obtained, which was an approximate mirror image of that obtained previously for lipophilic preparations. To investigate the effect of the introduction of a lipophilic phase into an aqueous preparation, a master curve was similarly obtained for oil-in-water emulsions. This curve, although similar in shape to the gel master curve, was displaced toward the lipophilic plot.

The gels and emulsions were assessed for spreadability by two preference scoring techniques. These data gave the optima and ranges of acceptable apparent viscosities for spreading of the topical preparations used. These values were superimposed on the master curves to show the "preferred regions" (shearing conditions with maximum patient acceptance potential). A combination of these master curves and preferred regions may be used in the design of instrumental tests for industrial quality control procedures concerned with preparations for topical use.

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Automated Assay of Single Tablets of Methdilazine and Methdilazine Hydrochloride

RHYS BRYANT▲, F. J. BURGER, D. E. MANTLE, D. L. TIMMA, F. B. TRENK, and D. S. YODER

Abstract □ An automated analytical system was used to determine the amount of methdilazine base or methdilazine hydrochloride in a single tablet. Tablets were sampled at a rate of 20/hr., with observed coefficients of variation of 2.8 and 3.4% for methdilazine base and methdilazine hydrochloride, respectively. Blending of methdilazine hydrochloride tablets was improved by presoaking the tablets in the sample cups. Interference from ascorbic acid was overcome by first blending the tablet in a basic solution containing copper (II) ion followed by acidification to solubilize the methdilazine hydrochloride prior to sampling.

Keyphrases □ Methdilazine and methdilazine hydrochloride single tablets—automated UV analysis □ UV spectrophotometry—analysis, automated, single methdilazine and methdilazine hydrochloride tablets

Methdilazine[1] (1), as the base or as the hydrochloride (I), is a potent long-acting antipruritic agent, reportedly effective in relieving a variety of itching conditions (2, 3). In this study, it was determined in two different formulations by an automated analysis system.

EXPERIMENTAL[2]

Reagents—The following were used:

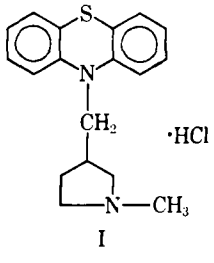
Phosphoric Acid Solution—Phosphoric acid[3], 0.1 and 2 *M* in distilled water.

Cupric Chloride Solution—Cupric chloride dihydrate[3], analytical reagent grade, 0.001% in 0.1 *M* aqueous ammonia[3], analytical reagent grade.

Alcohol Reagent—Methanol[3], analytical reagent grade, and ethanol[3], analytical reagent grade, mixed in the ratio of 3:1 v/v.

Standards—For methdilazine tablets, solutions of methdilazine base were prepared in 0.1 *M* phosphoric acid at three levels of concentration (0.333, 0.392, and 0.450 g./l.). Ten-milliliter aliquots of each solution were equivalent to 3.03, 3.56, and 4.09 mg. of methdilazine, respectively, when diluted in the Solidprep sampler.

For methdilazine hydrochloride tablets, aqueous solutions of methdilazine hydrochloride were prepared at three levels of concentration (0.748, 0.880, and 1.012 g./l.). Ten-milliliter aliquots of each solution were equivalent to 6.8, 8.0, and 9.2 mg. of methdilazine hydrochloride, respectively, when diluted in the Solidprep sampler.



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Samples—Production lots of tablets were used in this study. Theoretical tablet content was 3.6 mg. for the methdilazine tablets and 8.0 mg. for the methdilazine hydrochloride tablets.

Procedure—Schematics of the automatic analyzer systems are shown in Figs. 1 and 2.

Methdilazine tablets were disintegrated in the presence of 0.1 *M* phosphoric acid, sampled, and dialyzed. The intensity of the absorbance at 252 nm. was then measured and recorded (Fig. 3).

Methdilazine hydrochloride tablets, after pretreatment with alcohol, were disintegrated in the presence of basic cupric chloride. After 45 sec., the sample was acidified with 2 *M* phosphoric acid. This resultant solution was then sampled and dialyzed, and the drug content was measured and recorded as previously indicated. The steady-state curve of the system is shown in Fig. 4.

Tablets were sampled at a rate of 20/hr. Standard solutions were analyzed several times during the day to check for any variation in absorbance. No significant variation was observed.

Calculation—The average absorbance for each standard was plotted *versus* tablet equivalent to give a standard curve. Reference to the appropriate standard curve for each sample yielded the milligrams of methdilazine or methdilazine hydrochloride per tablet.

DISCUSSION

Methdilazine tablets contained no interfering excipient. Disinte-

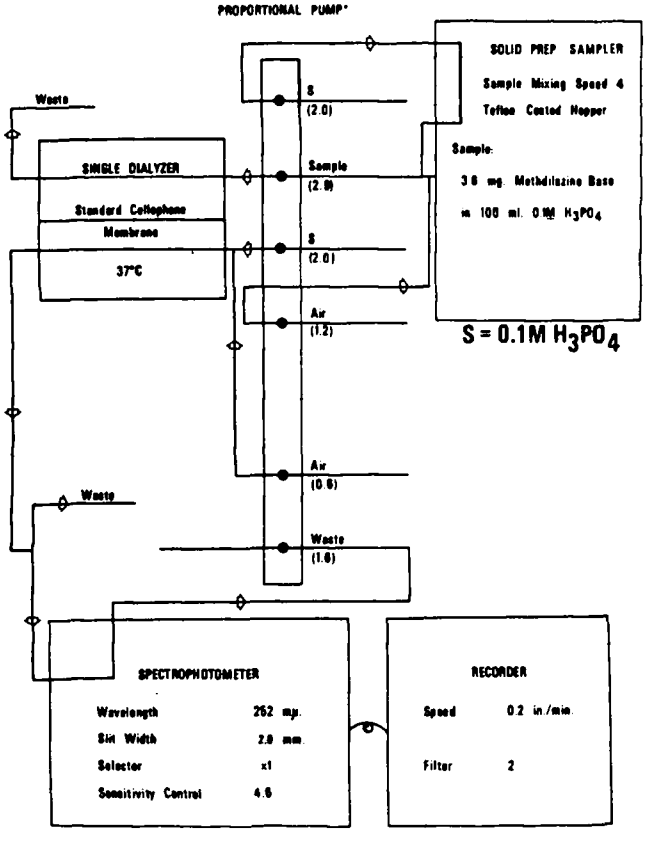

Figure 1—*Schematic for methdilazine tablets. (*Values in parentheses are flow rates in milliliters per minute. All pump tubing is Tygon.)*

[1] Tacaryl Chewable and Tacaryl Hydrochloride Tablets, Mead Johnson Laboratories, Division of Mead Johnson and Co.

[2] The analytical train consisted of the following Technicon modules (Technicon Corp., Tarrytown, N. Y.): (*a*) Solidprep sampler, (*b*) proportioning pump (model I), and (*c*) dialyzer (model I), in conjunction with a Hitachi Perkin-Elmer model 139 spectrophotometer using a microflow cell, 9120 No. 5, a Sargent model TR-05 recorder, and a Brewer model 40 automatic pipeting machine, equipped with a 50-ml. syringe (BBL, Division of BioQuest, Cockeysville, Md.).

[3] Mallinckrodt.

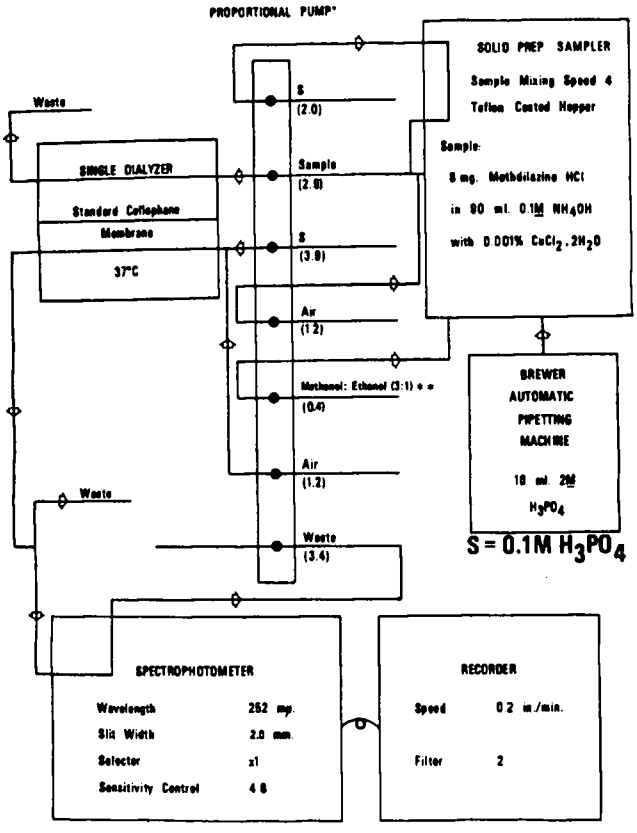

Figure 2—*Schematic diagram for methdilazine hydrochloride, 8 mg. tablets. (*Values in parentheses are flow rates in milliliters per minute. **Solvaflex tubing; all other tubing is Tygon.)*

gration in phosphoric acid followed by dialysis was sufficient to measure the drug absorbance at 252 nm.

Methdilazine hydrochloride tablets gave somewhat erratic results when assayed by the procedure developed for methdilazine tablets. Investigation indicated two factors contributing to this observation: (*a*) incomplete disintegration of the tablet in the Solidprep sampler, and (*b*) a tablet excipient that interfered in the UV measurement of drug.

Methdilazine hydrochloride tablets are manufactured as coated tablets, with a center drug-containing core. Pretreatment of individual tablets at position one on the sampler tray with 1.2 ml. of

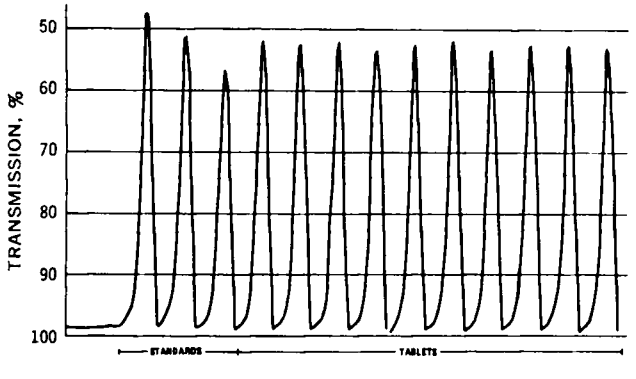

Figure 3—*Typical curve for methdilazine tablets and standards.*

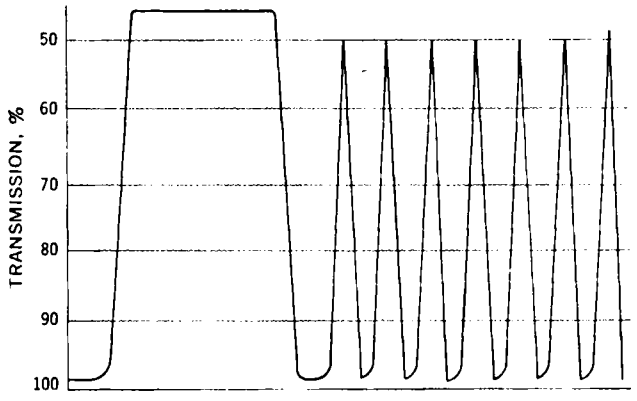

Figure 4—*Steady-state response of methdilazine hydrochloride.*

alcohol for 3 min. prior to disintegration in the sampler was sufficient to loosen the outer tablet coating.

The presence of ascorbic acid (2 mg./tablet) in methdilazine hydrochloride tablets resulted in assay values 13% above theory. This was overcome by oxidative destruction of the ascorbic acid using a cupric chloride–ammonia solution. However, methdilazine precipitated in basic solution, necessitating the addition of a sufficient amount of phosphoric acid to redissolve the methdilazine.

This requirement was met by using an automatic pipet in conjunction with the Solidprep sampler. Utilization of the cam system in the sampler allowed the addition of 2 *M* phosphoric acid 45 sec. into the analysis cycle.

Using the described analytical systems, linearity of absorbance values obtained for standards was satisfactory from 0 to 200% of declared tablet potency for both methdilazine and methdilazine hydrochloride.

RESULTS

Standard Curve—The average absorbance for each of the three standards was determined. A plot of the values gave a straight line in accordance with Beer's law.

Reproducibility of Standards—The reproducibility of the method was checked by the analysis of 30 standards (three levels of concentration) for both methdilazine and methdilazine hydrochloride. The coefficient of variation ranged from 0.3 to 0.9%.

Accuracy and Precision—The accuracy and precision of the method were ascertained by spiking a product placebo with the theoretical amount of drug prior to sampling. Data generated in this manner indicated the accuracy to be 1% with a coefficient of variation of 2.4%.

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Synthesis and Biological Activity of 9-Substituted Acridines

JAMES T. STEWART

Abstract □ Various 9-substituted acridine derivatives, *i.e.*, carbamic and thiocarbamic acid esters, urea, and thioureas, were synthesized and tested for potential biological activity. Some compounds were found active in antibacterial, metabolic, parasitologic, and GI screening procedures. The screening results indicate that acridine derivatives of these types possess biological activity similar to other known acridines, especially in the areas of antibacterial action and parasitology. The antisecretory action of one carbamate is a new potential use of acridines which has not been reported previously.

Keyphrases □ Acridines, 9-substituted—synthesis and biological screening □ Biological screening—9-substituted acridine derivatives

Interest in this laboratory in the use of acridines as potential medicinal agents resulted in the synthesis of some 9-substituted acridines, *i.e.*, carbamic acid esters (I), urea (II), thiourea (III), and thiocarbamic acid esters (IV). The antineoplastic activity of acridine derivatives of types I, II, and III was reported previously (1). A search of the scientific literature revealed that little or no information was available concerning additional biological activity of these types of structures. The carbamate, thiocarbamate, urea, and thiourea derivatives described are of special interest since some were found active in antibacterial, metabolic, parasitologic, and GI screening procedures. Compounds I*a*, I*c*, and II were also active in a preliminary virology screen involving a nonspecific nucleic acid polymerase, but the results are only qualitative at this time.

The syntheses of I*a*, I*b*, II, and III*a* were reported previously (1). The benzyl (I*c*) and 2-methylpropyl (I*d*) esters of 9-acridinecarbamic acid were prepared in one-step, high yield reactions starting with 9-aminoacridine. Compounds III*b* and IV also were not reported previously. Synthetic steps leading to these compounds are described in the *Experimental* section.

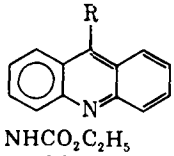
SCREENING RESULTS[1]

Compounds III*a* and III*b* were evaluated for antibacterial activity by a medicated agar dilution technique. In this test, the compounds are added to the agar and the test organisms are inoculated on the agar surface. The end-point is the level of compound that prevents emergence of visible growth. At 400-mcg./ml. levels, Compounds III*a* and III*b* were effective in inhibiting strains of *Staphylococcus aureus* (ATCC 6538), *Proteus vulgaris*[2], *Salmonella pullorum*[2], *Salmonella typhimurium*[2], *Escherichia coli*[2], *Klebsiella pneumoniae*[2], and *Diplococcus pneumoniae*[2]. Compound III*b* also inhibited *Proteus mirabilis*[2], *Streptococcus pyrogenes*[2], and *Pasteurella multocida*[2]. In comparison, 9-aminoacridine is effective at 40-mcg./ml. levels (2). Compound IV was also tested in an antibacterial screen (3) involving a variety of Gram-positive and Gram-negative bacteria and fungi of industrial importance. It was active against *Aerobacter aerogenes* (IPC 500)[3], *Bacillus mycoides* (IPC 509), and *S. aureus* (ATCC 6538) at 100-p.p.m. level, but it failed against these organisms at 5 p.p.m. 9-Aminoacridine is active against these organisms at 6–7-p.p.m. levels (4).

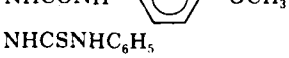
The activity of Compounds I*b* against certain other organisms was assessed by measuring inhibition of metabolic acitivity. One such test for anaerobic microorganisms is an assessment of reduction in formation from carbon dioxide and hydrogen by *Methanobacterium ruminantium*[2] at 39° for 2.5 hr. The level of methane is quantitated by passing a sample of the gas phase through a gas partitioner. Compound I*b* inhibited methane formation completely at 250 mcg./ml. and by 50% at 5 mcg./ml. However, under these same conditions, a 29.4-mcg./ml. level exhibited only a 20% inhibition after a 48-hr. incubation. This loss of effectiveness on continued incubation seems to be characteristic of a number of acridines[4].

Compound IV was active in an *in vitro* parasitology screen. Larvae or eggs of trichostrongyle nematodes were exposed to the test drug *in vitro*, initially at 100-mcg./ml. concentration. Efficacy was determined by direct microscopic inspection of the conditions of the parasites. Active compounds were retested in 10-fold serial dilutions to determine the end-point (5). The thiocarbamate (IV) was active against the eggs at 0.01 mg./ml., but it was inactive in the *in vivo* parasitology screen (6). In contrast, 9-aminoacridine has only shown effectiveness against parasites in *in vitro* testing at 2.5-mg./ml. levels (7). It is also inactive *in vivo*.

Compound I*a* was effective in inhibiting gastrin-stimulated acid secretion in an animal with a chronic gastric fistula. The carbamate (I*a*) in 50 ml. of aqueous methylcellulose (1%) was administered directly into the stomach of an unanesthetized chronic gastric fistula dog. One hour later, the gastric contents were drained and gastrin tetrapeptide (0:064 mg./kg. s.c.) was administered. Gastric secretions were collected over 2 hr., and the volume of secretions and total acid (meq./l.) were measured. The percent inhibition of volume and acid response were calculated using control values obtained in the same animal in an earlier experiment. Compound I*a*,



I*a*: R = $NHCO_2C_2H_5$
I*b*: R = $NHCO_2$-*n*-C_4H_9
I*c*: R = $NHCO_2CH_2C_6H_5$
I*d*: R = $NHCO_2CH_2CH(CH_3)CH_3$



II: R = NHCONH— —OCH_3

III*a*: R = $NHCSNHC_6H_5$
III*b*: R = $NHCSNHCH_2C_6H_5$
IV: R = $NHCSOC_2H_5$

[1] Tests for the various biological activities were carried out at Merck Sharp & Dohme Research Laboratories, Rahway, N. J. Only those acridine derivatives that showed activity in the testing are discussed.

[2] Identified by Dr. Eugene L. Dulaney, Merck Sharp & Dohme Research Laboratories, Rahway, N. J.

[3] IPC, Institute of Paper Chemistry, Appleton, Wis.

[4] H. B. Woodruff, Merck Sharp & Dohme Research Laboratories, personal communication, 1972.

at a dose of 16.1 mg./kg., reduced the volume of secretions and total acid content by approximately 35%.

The screening results indicate that acridine derivatives of the I, II, III, and/or IV types possess biological activity similar to 9-aminoacridine, especially in the areas of antibacterial action and parasitology. The antisecretory action of Compound Ia is a new potential use of acridine derivatives which has not been reported previously.

EXPERIMENTAL[5]

9-Acridinecarbamic Acid Esters (Ic and Id)—9-Aminoacridine (0.02 mole) and 0.02 mole of the necessary chloroformate ester were refluxed for 1 hr. in 300 ml. of acetone in the presence of 4 g. of sodium bicarbonate. The hot suspension was filtered, followed by evaporation of the acetone to yield a residue. The residue was recrystallized from ethanol or ethanol–water to give a solid, 80% yield.

The melting point for Ic was 210–213°.

Anal.—Calc. for $C_{21}H_{16}N_2O_2$: C, 76.81; H, 4.91; N, 8.53. Found: C, 76.91; H, 4.95; N, 8.46.

The melting point for Id was 152–154°.

Anal.—Calc. for $C_{18}H_{18}N_2O_2$: C, 73.44; H, 6.16; N, 9.51. Found: C, 73.29; H, 6.13; N, 9.59.

1-(9-Acridinyl)-3-benzyl-2-thiourea (IIIb)—*Method A*—9-Aminoacridine (0.02 mole) and 0.02 mole of benzyl isothiocyanate were refluxed for 1 hr. in 300 ml. of acetone.The acetone was evaporated, and the residue was recrystallized from ethanol–water to give a yellow solid, m.p. 178–180°, in 90% yield.

Method B—9-Isothiocyanatoacridine (0.02 mole) and 0.02 mole of benzylamine in 100 ml. of ethanol were heated on a water bath for 30 min. When the reaction mixture cooled to room temperature, the precipitate was collected and dried. Two recrystallizations from 25% ethanol–water gave a yellow powder, m.p. 178–180°, in 80% yield.

Anal.—Calc. for $C_{17}H_{17}N_3S$: C, 73.44; H, 4.99; N, 12.23. Found: C, 73.15; H, 5.15; N, 12.05.

Ethyl-N-(9-acridinyl)thiocarbamate (IV)—9-Isothiocyanatoacridine (0.02 mole) and 50 ml. of absolute ethanol were refluxed for 10 hr. The ethanol was evaporated to yield a residue, which was recrystallized from ethanol–water to give an orange solid, m.p. 160–162°, in 90% yield.

Anal.—Calc. for $C_{16}H_{14}N_2OS$: C, 68.06; H, 4.99; N, 9.92. Found: C, 68.16; H, 5.02; N, 10.04.

[5] Melting points were taken in capillary tubes on a Thomas-Hoover apparatus and are uncorrected. Elemental analyses were obtained from Atlantic Microlabs, Atlanta, Ga. IR spectra were recorded on a Perkin-Elmer model 237B spectrophotometer and were as expected. 9-Isothiocyanatoacridine was obtained from Eastman Organic Chemicals.

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Keyphrases □ *Couroupita amazonica* (Lecythidaceae)—isolation, identification of β-amyrin and ellagic acid □ β-Amyrin—isolated and identified from *Couroupita amazonica* □ Ellagic acid—isolated and identified from *Couroupita amazonica*

The Amazonian plant *Couroupita amazonica* was selected for investigation because no phytochemical studies have as yet been reported on this genus of plants. These studies resulted in the isolation of β-amyrin and ellagic acid, which are commonly encountered plant principles.

EXPERIMENTAL

Plant Material[1]—The trunk bark of *Couroupita amazonica* Kunth. (Lecythidaceae) was collected during July 1969 in the vicinity of Iquitos, Perú.

Extraction—The coarsely milled plant material (1.1 kg.) was extracted continuously for 24 hr. in a soxhlet apparatus with petroleum ether (b.p. 30–60°). The petroleum ether extract yielded 6 g. of a semicrystalline residue. The defatted plant material was then air dried and extracted with 95% ethanol, which afforded 67 g. of residue following evaporation of the solvent. This residue was partitioned between equal volumes of chloroform and water, and the aqueous fraction was extracted four times with ethyl acetate.

[1] Voucher specimens (2331 and 2342) were identified by Dr. J. Wurdack, Smithsonian Institution, Washington, D. C., and are deposited at this address.

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A 30-mg. sample of the impure ellagic acid was heated under reflux with an excess of acetic anhydride for 15 hr. The resulting product was crystallized from dioxane to obtain colorless crystals, which began to sinter at 331° and melted at 338–341° [lit. (3) m.p. 343–346°]. A second 30-mg. sample was methylated in the usual manner with diazomethane. The product was crystallized from dioxane to yield pale-yellow crystals, m.p. 338–340° [lit. (4) m.p. 342–344°]. The IR spectrum (KBr) corresponded with published data for the tetramethyl ether of ellagic acid (4).

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Isolation of Daphnetin-8-β-glucoside from *Daphne papyracea*

RITEN NASIPURI and EGIL RAMSTAD▲

Abstract □ Daphnetin occurs free and bound as β-glucoside in the roots of *Daphne papyracea* Wall. ex Steud. The bound form, however, is not in the form of daphnin, as found in several Thymelaceae, but in the form of daphnetin-8-β-glucoside.

Keyphrases □ Daphnetin-8-β-glucoside—isolated from *Daphne papyracea* □ *Daphne papyracea*—isolation of daphnetin-8-β-glucoside

The isolation of three flavone glycosides was reported by Basu and Nasipuri (1) in their preliminary investigation of the roots of *Daphne papyracea* Wall. ex Steud., collected from Ranikshet, India. Later, Sharma *et al.* (2) reported the isolation of daphnin from the roots of the same plant. The present authors found that daphnetin occurs free and bound as 8-β-glucoside in the roots of the same plant[1] collected in Nepal.

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DISCUSSION

Two crystalline compounds were isolated: A, m.p. 255–256° dec.; and B, m.p. 229–230°, $[\alpha]_D^{25}$ −92° (solvent: 50% alcohol).

Acid hydrolysis of Compound B provided A_1, identical to Compound A, as shown by its chromatographic behavior, no depression of melting point by admixture with Compound A, and identical UV and IR spectra. The hydrolysate of B contains glucose, identified by chromatography and chromogenic tests. Emulsin hydrolyzed B into A and glucose, showing B to be a β-glucoside of A.

Compound A, giving color reactions for phenols, has the elementary composition of $C_9H_6O_4$ (mol. wt. 178.0282; theoretical 178.0266), and the UV spectral characteristics of a coumarin: λ_{max}^{EtOH} 224 (log ε 4.2), 262 (log ε 3.9), and 326 nm. (log ε 4.05) [literature (3) data for 7,8-dihydroxycoumarin: λ_{max}^{EtOH} 260 (log ε 3.8) and 327 nm. (log ε 4.1)].

Compound A was identified as 7,8-dihydroxycoumarin (daphnetin) from the mass spectrometry, IR, and NMR data.

Mass spectral findings can be rationalized as follows (Scheme I). The fragmentation pattern and relative mass peak abundance are very similar to those of umbelliferone (4), each corresponding *m/e* having an additional value of 16 (for the added oxygen).

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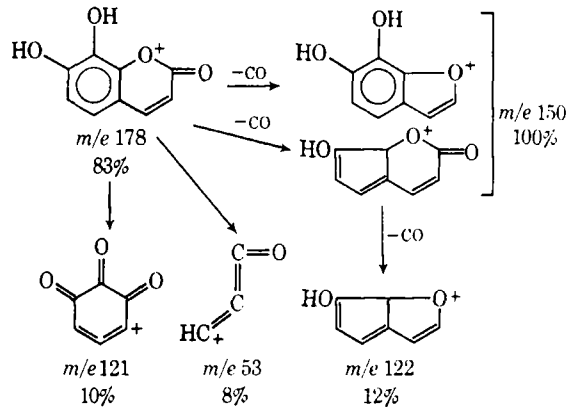

Scheme I

three coumarins reported in the literature supports the structure as being dihydroxycoumarin (Table I).

The splitting of two aromatic protons into doublets (J = 9.5 Hz.) in the NMR spectrum shows them to be *ortho* to each other. Proton 4 in coumarins without substitution at C-5 resonates at δ7.65–7.77 [umbelliferone (5), herniarin (6), 7-geranyl-8-hydroxycoumarin (7), and scoparone (8)]. Substitution at C-5 with oxygen would have caused a downfield shift of proton 4 as in limettin (δ7.95) (9) and sibricin (δ7.95) (9); since the two aromatic protons of A (5 and 6) are coupled, the only positions left for the two hydroxyl groups are 7 and 8. Compound A is, therefore, 7,8-dihydroxycoumarin (daphnetin).

Several species of daphne have been found to contain daphnin, which is 7-daphnetin-β-glucoside (10, 11). Compound B, however, melts sharply at 229–230° compared to 215–217° (12) or 223–224° dec. (13, 14) for daphnin. Also, $[\alpha]_D^{24}$ is −92° compared to $[\alpha]_D^{22}$ −123.9° for daphnin (13). It must be concluded that in B the glucosidic linkage is at C-8 rather than at C-7. For verification, Compound B was peracetylated. The NMR spectrum of the acetate showed five acetyl groups and a downfield shift of proton C-6 by δ0.35, identical in magnitude to the shift experienced by the same proton in salicylic acid upon acetylation.

EXPERIMENTAL[2]

Isolation of A and B—Powdered dry root of *D. papyracea* was extracted with alcohol (95%), the extract was concentrated under reduced pressure, and the concentrate was poured in a thin stream into a large volume of water. The precipitated mass was separated by filtration and the filtrate was concentrated under reduced pressure. A 10% solution of neutral lead acetate was added to the concentrated liquid. A yellow precipitate (a) was filtered from the solution (b). Substance A was isolated from the yellow precipitate (a) by treatment of its aqueous alcoholic suspension with hydrogen sulfide gas, concentration of the resulting solution, and subsequent crystallization.

Filtrate (b) was treated with a 10% solution of basic lead acetate, yielding a pale-yellow precipitate (c) from which Compound B was isolated in the same way as was A from precipitate (a).

Hydrolysis of Compound B—Fifty milligrams of B was refluxed for 1 hr. with 20 ml. of 1% sulfuric acid, and the solution was then neutralized with barium carbonate and filtered.

TLC of the filtrate revealed a phenolic compound identical to that of Compound A. The adsorbent was silica gel G. The solvent systems were: (*a*) ethyl acetate–pyridine–water (3.6:1.0:1.15), (*b*) chloroform–methanol (60:40), and (*c*) butanol–acetic acid–water (4:1:1). The detectors were: (*a*) ferric chloride solution and (*b*) Pauly's reagent (15).

Chromatograms of the same filtrate gave spots of identical chromatographic behavior and color reactions as those of glucose. Two systems were used: (*a*) absorbent: silica gel G; solvent system: chloroform–methanol (60:40); and (*b*) absorbent: silica gel G buffered with sodium acetate; solvent system: acetone–water (90:10). The detector was aniline–diphenylamine–phosphoric acid (15). The nonaromatic portions of the NMR spectra of peracetylated Compound B and peracetylated glucose were virtually identical except for the presence of three protons at δ2.36 (aromatic acetyl group) in Compound B and three protons at δ2.10 (C-1 acetyl group) in peracetylated glucose.

Compound A was isolated from the filtrate. Admixture of this and A originally isolated from the plant gave no melting-point difference.

The presence of the β-glucosidic linkage in Compound B was shown by hydrolyzing B with emulsin at 24° for 2 hr. and identifying the released glucose.

Table I—NMR Spectral Data

Compound	Chemical Shift (δ) and Splitting Pattern H-3	H-4	H-5	H-6
Compound A ($CDCl_3$ + 30% dimethyl sulfoxide) (100 MHz.)	6.18 d	7.67 d	6.92 d	6.88 d
Umbelliferone ($CDCl_3$) (5)	6.26 d	7.77 d	7.42 m	6.89 m
Herniarin ($CDCl_3$) (6)	6.23 d	7.65 d	7.40 m	6.87 m
7-Geranyl-8-hydroxycoumarin ($CDCl_3$) (7)	6.25 d	7.65 d	7.38 m	6.88 m

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▲ To whom inquiries should be directed.

[2] IR spectra were determined in Nujol on a Perkin-Elmer 137, NMR spectra were determined in $CDCl_3$ on a Varian HA-100 spectrometer (Varian AG, Switzerland) and a Varian A 56/60 spectrometer (University of Ibadan, Nigeria), and mass spectra were determined on a Hitachi RMV 6 H mass spectrometer.

Simplified Method for Determining Chemical Stability of Drug Substances in Pharmaceutical Suspensions

J. TINGSTAD▲, J. DUDZINSKI, L. LACHMAN, and E. SHAMI

Abstract □ A simplified method for determining the chemical stability of drug substances in pharmaceutical suspensions is described. The applicable theory is discussed, and a suitable example from the literature is presented. The value of the method is confirmed experimentally using aspirin.

Keyphrases □ Stability, chemical—drugs in suspensions, simplified method, calculations using aspirin □ Suspensions—simplified method for determining chemical stability of drug substances □ Drug stability in pharmaceutical suspensions—simplified method for determination of chemical stability □ Preformulation studies—simplified method for determining chemical stability of drug substances in pharmaceutical suspensions, example using aspirin, equations

One of the most common and important preformulation studies is the determination of a drug substance's chemical stability in aqueous systems. The usual procedure is to dissolve the compound in suitably buffered water or, if necessary, in a mixed solvent system and then to perform classical kinetic experiments. However, many drug substances have low aqueous solubilities, and it is often desirable to determine the chemical stability of the compound suspended in water. In addition, it is often important to perform accelerated chemical stability studies on suspension formulations. Relatively few such experiments have been reported (1–5), primarily because: (*a*) kinetic studies on drug substances in solution are easier to perform and lend themselves to more sophisticated treatment, and (*b*) with suspensions the kinetic calculations are complicated by the change in drug solubility with temperature. This report describes a simplified method for determining the chemical stability of drug substances in aqueous suspension, one that does not require solubility determinations and yet lends itself to classical mathematical treatment.

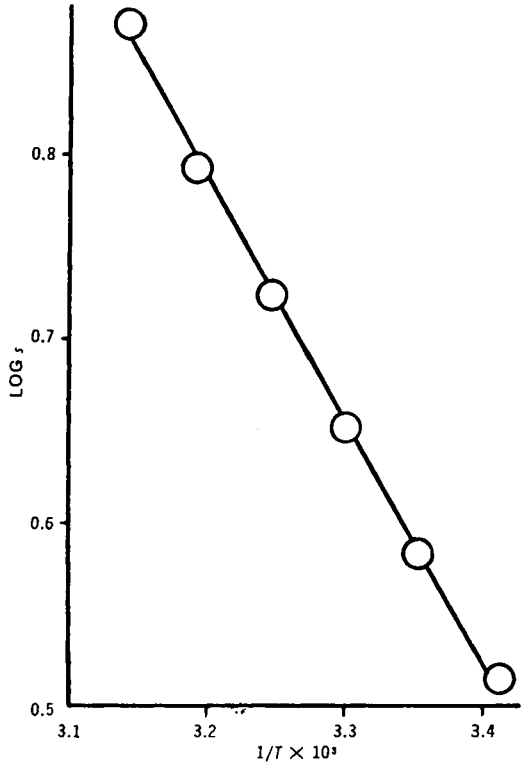

Figure 1—*Plot of log solubility of undissociated aspirin* versus *the reciprocal of the absolute temperature from data of Edwards* (6).

THEORY

Many drug substances in solution degrade according to first-order kinetics:

$$-dc/dt = kc \qquad \text{(Eq. 1)}$$

where c is the concentration of reactant, t is time, k is the rate constant, and dc/dt is the rate at which the concentration decreases. When the system involves a drug substance in suspension, the concentration in solution usually remains constant because, as the reaction proceeds, solid drug substance dissolves and the solution remains saturated with respect to undegraded reactant. Equation 1 then becomes:

$$-dc/dt = k_0 \quad \text{or} \quad -dc/dt = ks \qquad \text{(Eq. 2)}$$

where k_0 is the apparent zero-order rate constant (equal to ks), k is the first-order rate constant of Eq. 1, and s is the solubility of the

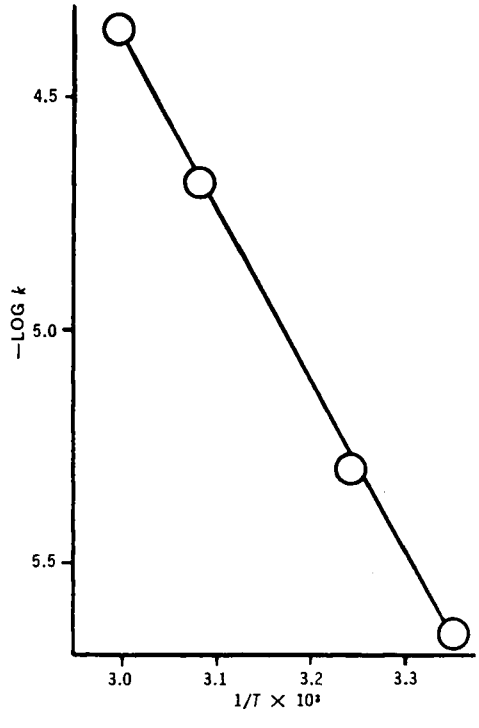

Figure 2—*Plot of log reaction rate constant of undissociated aspirin* versus *the reciprocal of the absolute temperature from data of Garrett* (7).

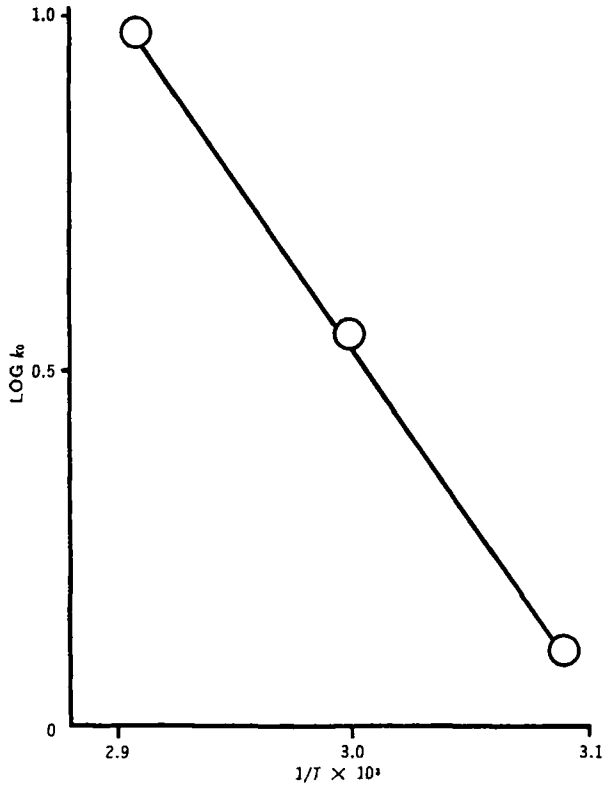

Figure 3—*Plot of log (apparent) zero-order reaction rate constant* versus *the reciprocal of the absolute temperature from data of James (2) for aspirin suspension.*

drug substance at the given temperature (and equal to c of Eq. 1).

When the reaction is studied at various temperatures, the apparent zero-order rate constant (k_0) will vary because of the temperature effect on the first-order rate constant and on the solubility of the drug substance in the solvent. One way to proceed is to determine its solubility at the various reaction temperatures, calculate k from the experimentally determined values of k_0 and s, and then estimate the room temperature stability from the heat of activation and the solubility at 25° (1).

Under certain conditions, room temperature stability can be estimated without determining the solubility of the drug substance at any temperature. These conditions are: (*a*) drug substance degradation takes place only in solution and according to first-order kinetics, (*b*) the drug substance–solvent system obeys the classical relationships to temperature with respect to solubility and rate of reaction, and (*c*) the dissolution–degradation kinetics are not dissolution rate limited.

From Eq. 2 it can be said that:

$$k_0 = ks \qquad \text{(Eq. 3)}$$

Taking logs of both sides yields:

$$\log k_0 = \log k + \log s \qquad \text{(Eq. 4)}$$

The classical relationship between reaction rate and temperature is:

$$\log k = -\frac{\Delta H_a}{2.303RT} + \text{a constant} \qquad \text{(Eq. 5)}$$

where k is the reaction rate constant, ΔH_a is the molar energy of activation, R is the gas constant, and T is the absolute temperature. Similarly, the relationship between solubility and temperature is:

$$\log s = -\frac{\Delta H_f}{2.303RT} + \text{a constant} \qquad \text{(Eq. 6)}$$

where s is the solubility at absolute temperature T, and ΔH_f is the molar heat of fusion.

Substituting from Eqs. 5 and 6 into Eq. 4 gives:

$$\log k_0 = -\frac{\Delta H_a + \Delta H_f}{2.303RT} + \text{a constant} \qquad \text{(Eq. 7)}$$

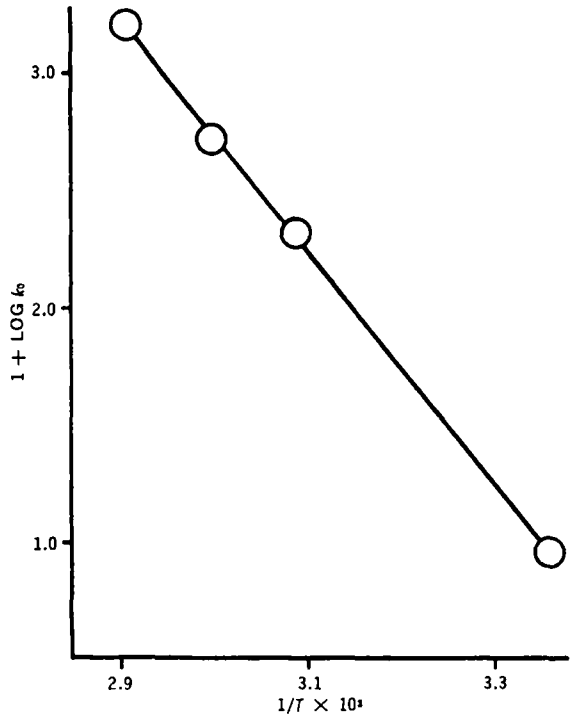

Figure 4—*Plot of log (apparent) zero-order reaction rate constant* versus *the reciprocal of the absolute temperature for experimental aspirin suspension.*

Thus, a plot of log k_0 *versus* $1/T$ should yield a straight line with a slope of $-(\Delta H_a + \Delta H_f)/2.303R$. By this method, room temperature stability can be estimated from studies at elevated temperatures without determining solubilities or first-order rate constants for the drug substance in solution.

EXAMPLE

An example from the literature, involving several unrelated sources, will help clarify the method and indicate its usefulness. Edwards (6) determined the solubility of aspirin as the undissociated acid (pH 1) at various temperatures, and a plot of log s *versus* $1/T$ (Fig. 1) yields a straight line with a slope of -1327. Garrett (7) determined the first-order rate constants for aspirin degradation in solution at pH 1.1 at various temperatures, and a plot of log k *versus* $1/T$ (Fig. 2) gives a straight line with a slope of -3698. Thus, $(\Delta H_a + \Delta H_f)/2.303R$ for this system is estimated to be $-(1327 + 3698)$ or -5025 kcal./mole. Therefore, a plot of log k_0 *versus* $1/T$ (see Eq. 7) for a suspension of aspirin at pH $\sim$ 1 should result in a straight line with a slope of approximately -5000 kcal./mole. Plotting the data of James (2) in such a manner (Fig. 3) results in a straight line whose slope is -4817 kcal./mole. Only data from 50, 60, and 70° were used because only those data were obtained on the same suspension formula.

EXPERIMENTAL

Because only three points were used to determine the slope in Fig. 3, a limited stability study on aspirin in suspension was performed to confirm the usefulness of the abbreviated method. Twelve hundred milligrams of aspirin USP (80 mesh) was placed in 50-ml. vials containing 20 ml. of 0.1 *N* sulfuric acid, which had been preheated to the experimental temperature. The vials were stoppered and stored at 70, 60, 50, and 24.5°. Duplicate samples were removed at appropriate times and quenched in an ice bath, and the entire contents were assayed for salicylic acid by the method of Tinker and McBay (8).

RESULTS AND DISCUSSION

The pH of both the fresh and degraded suspensions was 1.4. Plotting the stability data according to Eq. 7 yields a straight line

(Fig. 4) with a slope of −5002 kcal./mole, confirming the usefulness of the method.

Four points should be emphasized concerning kinetic studies on drug substances in suspension.

1. Data should be reported as "amount degraded" rather than "amount remaining" or "percentage lost," since (for example) for a given drug substance a suspension having a potency of 20 mg./ml. will degrade at the same rate as one containing 10 mg./ml. but the "percentage lost" is one-half as much.

2. If the drug substance under study is acidic or basic, it will often act as the primary buffer in the system, since solid drug substance is always in excess.

3. Because the solvent system is saturated with drug substance, concentrations of degradation products may reach a point where they affect the reaction rate, causing deviations from zero-order kinetics as the reaction proceeds.

4. In those cases where the simplified method fails because the system is dissolution rate limited, it may help to: (*a*) use a batch of drug substance with smaller particle size, and (*b*) agitate the stored samples. Estimations of room temperature stability based upon such conditions will err on the safe side.

CONCLUSION

Using the simplified procedure (for systems obeying the underlying assumptions stated under *Theory*), the room temperature stability of a drug substance in suspension can be estimated from elevated temperature studies (measuring amount degraded at various times) without determining solubilities or first-order rate constants. When the assumptions are not valid for a particular system, the abbreviated method will not work. The procedure is being presented here so that its applicability can be tested by investigators concerned with evaluating the stability of drug substances in suspension or suspension formulations.

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Hydrindene Derivatives as Potential Oral Hypoglycemic Agents: *N*-Alkyl 1,2,3,3a,4,8b-Hexahydroindeno[1,2-*b*]pyrroles

A. U. DE▲ and B. P. SAHA

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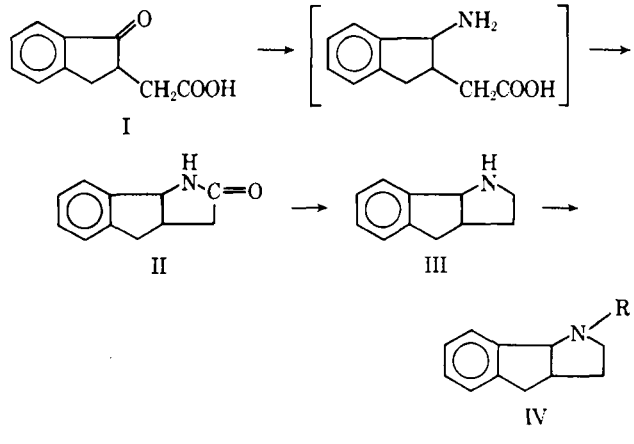
Keyphrases □ Hydrindene derivatives—*N*-alkyl 1,2,3,3a,4,8b-hexahydroindeno[1,2-*b*]pyrroles synthesized and screened as potential oral hypoglycemic agents □ *N*-Alkyl 1,2,3,3a,4,8b-hexahydroindeno[1,2-*b*]pyrroles—synthesized and screened as potential oral hypoglycemic agents □ Hypoglycemic agents, oral, potential—synthesis and screening of *N*-alkyl 1,2,3,3a,4,8b-hexahydroindeno-[1,2-*b*]pyrroles □ Heterocyclic compounds—synthesis of new class of hydrindene derivatives

In continuation of an investigation on hydrindene derivatives as potential oral hypoglycemic agents (1), it was considered worthwhile to synthesize isomeric indeno[1,2-*b*]pyrroles and test their activity. This new type of heterocyclic compound, although prepared earlier by different routes without any alkyl substitution at the nitrogen atom (2–4), was synthesized, in high yield (Scheme I), by a novel route by way of reductive amination.

1-Keto-2-indanylacetic acid (I) (5, 6), on being subjected to reductive amination with hydrogen and dry ammonia gas in absolute ethanol in the presence of Raney nickel catalyst, yields the corresponding pyrrolidone (II) in one step. Pyrrolidone (II), after reduction with lithium aluminum hydride in dry tetrahydrofuran, gives indeno[1,2-*b*]pyrrole (III). The pyrrolidone (II) shows the characteristic C=O and N—H stretching vibrations, while the indenopyrrole (III) shows only the N—H stretching vibrations. The indenopyrroles were



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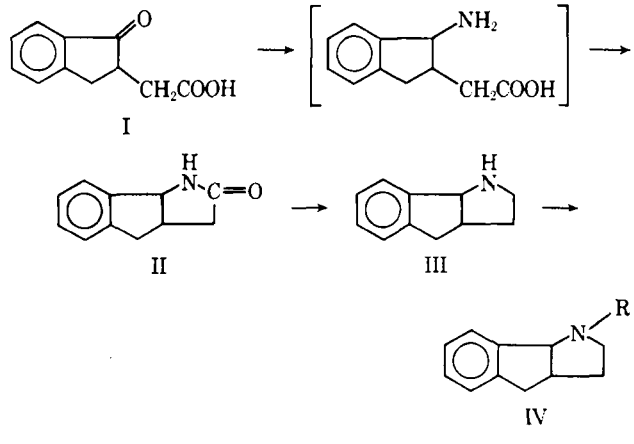
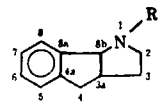


Scheme I

Table I—*N*-Alkyl 1,2,3,3a,4,8b-Hexahydroindeno[1,2-*b*]pyrroles (IV)



Compound Number	R	Boiling Point	Formula	Analysis, % Calc.	Found	Melting Point of Picrate
1	CH_3[a]	140°/1.2 mm.	$C_{12}H_{15}N$	C 83.26 H 8.67 N 8.09	82.94 8.92 7.94	158–160°
2	C_2H_5[a]	83–85°/0.45 mm.	$C_{13}H_{17}N$	C 83.43 H 9.09 N 7.49	83.21 9.38 7.13	141–143°
3	*n*-C_3H_7[a]	92°/0.5 mm.	$C_{14}H_{19}N$	C 83.58 H 9.45 N 6.97	83.71 9.51 6.62	115–117° dec.
4	*n*-C_4H_9[b]	82–85°/0.3 mm.	$C_{15}H_{21}N$	C 83.73 H 9.77 N 6.51	84.02 9.92 6.18	103–105°

[a] Iodide was used. [b] Bromide was used.

treated with the appropriate alkyl halide and purified by tosylation to furnish the respective *N*-alkyl indenopyrroles (IV).

ACTIVITY

Four compounds were synthesized in this series; only *n*-propylhexahydroindeno[1,2-*b*]pyrrole (IV: R = *n*-propyl) caused a 12% blood sugar lowering at a dose level of 250 mg./kg. body weight in normal, overnight fasted rabbits while the butyl derivative (IV: R = *n*-butyl) caused a 10% lowering. The blood sugar was estimated at 0, 2, and 4 hr. Tolbutamide was a standard in this test, and it caused a reduction of about 40% of the blood sugar at a dose of 250 mg./kg.

In another test, inhibition of epinephrine biosynthesis *in vitro* from norepinephrine, the hydrochloride of 1,2,3,3a,4,8b-hexahydroindeno[1,2-*b*]pyrrole (III), caused a 73.1% inhibition of phenylethanolamine-*N*-methyltransferase activity (7) at a concentration of 1×10^{-4} *M*.

EXPERIMENTAL[1,2]

2-Oxo-1,2,3,3a,4,8b-hexahydroindeno[1,2-*b*]pyrrole (II)—1-Keto-2-indanylacetic acid (I, 0.3 mole) was subjected to reductive amination in the presence of absolute ethanol (300 ml.) containing dry ammonia gas (16%) and Raney nickel (10 g.) at a hydrogen pressure of 150 atm. at 125°. After the reaction, which required nearly 8 hr., the mass was separated from the catalyst by filtration, ethanol was removed by distillation, and the residual mass was repeatedly washed with water and finally crystallized from hot water as shining white crystals, m.p. 191–193°; ν_{max} 1652 (C=O) and 2975 (NH) $cm.^{-1}$.

Anal.—Calc. for $C_{11}H_{11}NO$: C, 76.32; H, 6.36; N, 8.09. Found: C, 76.02; H, 6.58; N, 7.88.

1,2,3,3a,4,8b-Hexahydroindeno[1,2-*b*]pyrrole (III)—Pyrrolidone (II, 1 mole) was reduced with lithium aluminum hydride (0.6 mole) in dry tetrahydrofuran for 10–12 hr. and worked up in the usual way. The indenopyrrole distilled at 99–101°/0.6 mm. as a colorless liquid; ν_{max} 2950 $cm.^{-1}$ (NH). Picrate darkens at 182° and melts at 224–226° dec.

Anal.—Calc. for $C_{11}H_{13}N$: C, 83.04; H, 8.18; N, 8.80. Found: C, 82.87; H, 8.42; N, 9.03.

***N*-Alkyl 1,2,3,3a,4,8b-Hexahydroindeno[1,2-*b*]pyrrole (IV)**—Indenopyrrole (III, 1 mole) was heated in a sealed tube with the appropriate alkyl halide (1.1 moles) on a water bath for 8–12 hr. and then poured into water and extracted with benzene. It was then tosylated with *p*-toluenesulfonyl chloride (1 mole) in benzene solution under stirring at 40° with simultaneous addition of sodium hydroxide (10%) to keep the mass alkaline. The benzene layer was separated out, and the tertiary amine was repeatedly extracted with hydrochloric acid (6 *N*). The combined acid extracts were basified with sodium hydroxide under cooling, extracted with ether, and dried over anhydrous sodium sulfate, and the amine was distilled under reduced pressure to furnish colorless liquids (Table I).

[1] All melting points are uncorrected.
[2] IR spectra were recorded in mineral oil mulls using a Perkin-Elmer spectrophotometer.

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Kinetics of Acid-Catalyzed Hydrolysis of Dexoxadrol

LEO W. BROWN and ARLINGTON A. FORIST▲

Abstract □ The kinetics of the hydronium-ion-catalyzed hydrolysis of the ketal group in dexoxadrol hydrochloride [*d*(+)-2,2-diphenyl-4-(2′-piperidyl)-1,3-dioxolane hydrochloride], an orally active analgesic agent, were determined as functions of acid concentration, ionic strength (μ), and temperature. Pseudo-first-order kinetics were observed. Specific reaction rate constants increased with increasing μ; log $k'/[H^+]$ was linearly related to $\sqrt{\mu}$ at $\mu \leq 0.03$. The energy (ΔE_a), enthalpy ($\Delta H^{\ddagger}$), and entropy ($\Delta S^{\ddagger}$) of activation were 16.8 kcal./mole, 16.2 kcal./mole, and −18.8 e.u., respectively. The rate of dexoxadrol hydrolysis was about 2% of that reported in the literature for 2,2-diphenyl-1,3-dioxolane, indicating a marked retardation due to the protonated piperidyl moiety. For a 60-min. residence time at pH 2.0 in the stomach, it was estimated that more than 91% of orally ingested dexoxadrol would remain intact.

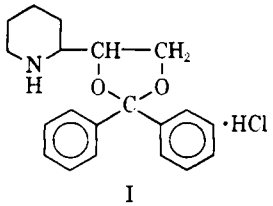
Keyphrases □ Dexoxadrol—kinetics of acid-catalyzed hydrolysis □ Hydrolysis, acid catalyzed—dexoxadrol, kinetics □ Kinetics of hydrolysis—dexoxadrol, acid catalyzed

Dexoxadrol hydrochloride [*d*(+)-2,2-diphenyl-4-(2′-piperidyl)-1,3-dioxolane hydrochloride, I][1] is a potent, orally active analgesic agent in man (1–4). The synthesis (5), physicochemical properties (6), and pharmacology (7–9) of I were reported previously. The known instability of ketals in acid solution made it important to determine the extent of possible degradation of orally administered I in the stomach. Therefore, the kinetics of the acid-catalyzed hydrolysis of I were determined and are the subject of this report.

EXPERIMENTAL

Acid hydrolysis of I yields *l*-2-(2-piperidyl)-1,2-ethanediol (II) (10) and benzophenone (III). Production of III was confirmed in the present study by: (*a*) the UV spectra of reaction mixtures at completion of hydrolysis which were identical to the spectrum of III, and (*b*) TLC of a chloroform extract of the reaction mixture which showed only one UV-absorbing product, identical to III [TLC used silica gel G and the plates were developed with isooctane–chloroform (80:35), $R_f \sim 0.3$].

In the kinetic studies, aqueous solutions of I and of hydrochloric acid were preequilibrated to the desired temperature and rapidly mixed to yield the final concentrations shown in Table I. Each reaction mixture was placed in a UV spectrophotometer equipped with a constant-temperature cell compartment. Absorbances (A_t) were determined at appropriate times at 247 nm., the absorption maximum of III (ϵ = 11,570), until no further absorbance increases occurred (A_∞). Pseudo-first-order reaction rate constants, k', were obtained from the slopes of plots of log $(A_\infty - A_t)$ *versus* time. The analytical wavelength chosen was highly sensitive to the production of III, since neither I (λ_{318}, ϵ = 10,700; λ_{247}, ϵ < 300) nor II absorbs significantly at 247 nm. Based on observed A_∞ values and the molar absorptivity of III, determined under the same conditions, hydrolysis of I was complete (mean yield of III in all studies at 37° was 102.7%).



I

RESULTS AND DISCUSSION

The generally accepted mechanism for the acid-catalyzed hydrolysis of acetals and ketals involves a rapid protonation of the substrate molecule followed by a unimolecular rate-determining decomposition to an alcohol and a resonance-stabilized carbonium ion (11, 12, and references cited therein). Thus, the reaction should show first-order dependence on the acetal or ketal and hydronium ion.

In the present study, plots of log $(A_\infty - A_t)$ *versus* time were strictly linear, confirming first-order dependence on I (Fig. 1). Pseudo-first-order reaction rate constants, k', obtained from the slopes of these plots are shown in Table I along with the resulting values of $k'/[H^+]$. Hydronium-ion concentrations were converted to activities by interpolation of the data of Harned and Owen (13) and were used to calculate the specific reaction rate constants, k_H - (Table I).

Inspection of the data shows that $k'/[H^+]$ and k_{H^+} increased with acid concentration and, therefore, with ionic strength (μ), since the latter was permitted to vary with the acid concentration. For hydrolysis of a neutral species, k_{H^+} should be essentially independent of μ. However, the pKa of I is approximately 8.9 (6). As a consequence, I was completely in the cationic form in the systems studied, and the hydronium-ion-catalyzed hydrolysis involved a reaction between like-charged ions. In such a case, an increase in the rate constant with increasing μ would be predicted, with log $k'/[H^+]$ linearly related to $\sqrt{\mu}$ at low values for μ (14). To test this relationship for the present system, linear least-squares regression of log $k'/[H^+]$ *versus* $\sqrt{\mu}$ (for $\mu \leq 0.03$) was conducted with predetermined theoretical slopes of 1.00, 0.97, and 0.93 at 30, 37, and 44°, respectively (14). The resulting curves (Fig. 2) provided convincing evidence for the predicted effects of μ on the rate constants. Accord-

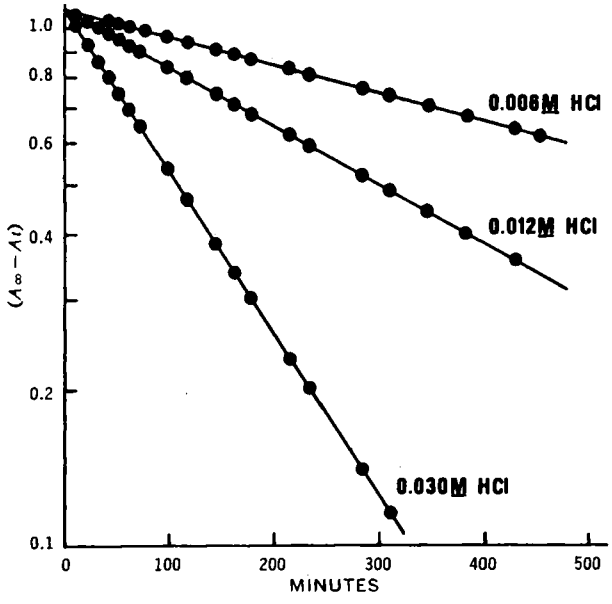


Figure 1—*Pseudo-first-order reaction rate curves for dexoxadrol hydrolysis at 44°.*

[1] Dexoxadrol hydrochloride has also been referred to in the literature as U-22,559A (The Upjohn Co.) and CL-911-C (Cutter Laboratories). USAN chemical name is (+)-2-(2,2-diphenyl-1,3-dioxolan-4-yl)piperidine hydrochloride.

Table I—Reaction Conditions and Kinetic Constants for Dexoxadrol Hydrolysis

Temperature	Dexoxadrol, $M \times 10^5$	Hydrochloric Acid, $M \times 10^2$	k', sec.$^{-1}$	$k'/[H^+]$, M^{-1} sec.$^{-1} \times 10^3$	k_{H^+}[a], M^{-1} sec.$^{-1} \times 10^3$	k_0[b], M^{-1} sec.$^{-1} \times 10^3$
30°	8.67	0.60	5.90×10^{-5}	0.983	1.07	0.818
30°	8.67	1.20	1.28×10^{-4}	1.07	1.19	
30°	8.67	3.00	3.68×10^{-4}	1.20	1.41	
37°	8.67	0.15	2.38×10^{-5}	1.59	1.70	1.59
37°	8.67	0.60	1.17×10^{-4}	1.95	2.13	
37°	9.26	0.60	1.14×10^{-4}	1.90	2.07	
37°	8.67	1.20	2.58×10^{-4}	2.15	2.41	
37°	9.26	1.20	2.45×10^{-4}	2.04	2.29	
37°	9.26	3.00	6.80×10^{-4}	2.27	2.66	
37°	14.46	3.00	6.77×10^{-4}	2.26	2.64	
37°	14.46	3.00	6.70×10^{-4}	2.23	2.62	
37°	8.67	3.00	7.37×10^{-4}	2.46	2.88	
37°	8.67	4.80	1.17×10^{-3}	2.44	2.94	
37°	8.67	4.80	1.15×10^{-3}	2.40	2.89	
37°	8.67	7.20	1.80×10^{-3}	2.50	3.09	
37°	8.67	7.20	1.87×10^{-3}	2.60	3.21	
37°	8.67	9.60	2.65×10^{-3}	2.76	3.48	
37°	8.67	9.60	2.60×10^{-3}	2.71	3.41	
44°	8.86	0.60	2.07×10^{-4}	3.45	3.76	2.80
44°	8.86	1.20	4.26×10^{-4}	3.55	3.98	
44°	8.86	3.00	1.10×10^{-3}	3.97	4.67	

[a] $k_{H^+} = k'/a_{H^+}$. [b] Intercept from log $k'/[H^+]$ *versus* $\sqrt{\mu}$ for $\mu \leq 0.03$ (Fig. 2).

ingly, k_0 (specific reaction rate constant at infinite dilution) was obtained from the intercept of each curve in Fig. 2 and is shown in Table I.

Parameters of activation for the hydrolysis of I were determined from k_0 at 30, 37, and 44° and appropriate linear regressions (Fig. 3). Similar determinations were made utilizing k_{H^+} values obtained at the three temperatures in 0.006, 0.012, and 0.030 M HCl solutions. From the summary in Table II, it may be seen that the classical Arrhenius activation energy (ΔE_a) is in good agreement with the value of 17.2 kcal./mole reported by Ceder (15) for the hydrolysis of 2,2-diphenyl-1,3-dioxolane under similar conditions. The enthalpy of activation ($\Delta H\ddagger$) is somewhat larger than that reported by De Wolfe *et al.* (16) for the hydrolysis of 2,2-diphenyl-1,3-dioxolane in 30% dioxane ($\Delta H\ddagger$ = 14.6 kcal./mole). Fife and Hagopian (12) suggested that steric inhibition of resonance in the incipient carbonium ion should make $\Delta H\ddagger$ more positive. The differences in $\Delta H\ddagger$ may, therefore, reflect such an inhibition by the piperidyl moiety present in I. The entropy of activation ($\Delta S\ddagger$) is considerably more negative than values reported for the hydrolysis of 2,2-diphenyl-1,3-dioxolane in 30% dioxane ($\Delta S\ddagger$ = −8.3 e.u.) (16) and for a series of 2-phenyl-2-alkyl-1,3-dioxolanes in 50% dioxane ($\Delta S\ddagger$ = −8.6 to −8.9 e.u.) (12). Although the magnitude of $\Delta S\ddagger$ is typical of those observed for bimolecular reactions (17), there is no reason to conclude that the mechanism of reaction for I differs

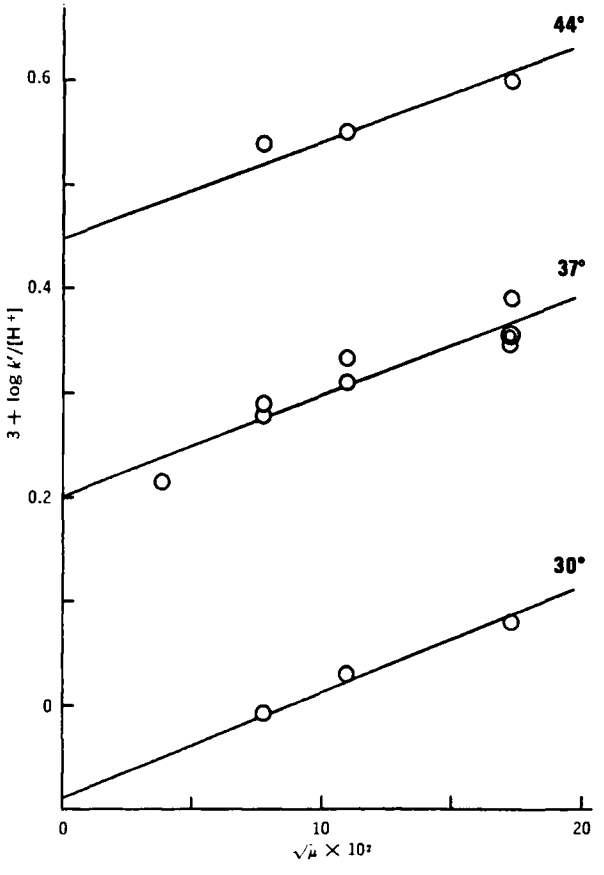


Figure 2—*Linear least-squares regression of log* k'/[H^+] versus $\sqrt{\mu}$ *with predetermined theoretical slopes.*

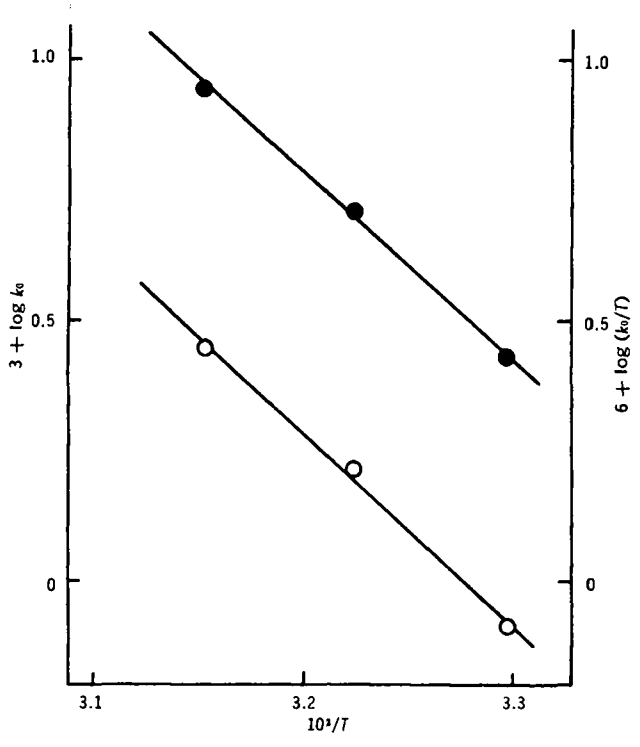


Figure 3—*Linear least-squares regression of log* k_0 versus 1/T (○) *and log* (k_0/T) versus 1/T (●).

Table II—Activation Parameters for Dexoxadrol Hydrolysis

Basis for Calculation	ΔE_a, kcal./mole	$\Delta H‡$, kcal./mole	$\Delta S‡$, e.u.
k_0	16.9 ± 0.6	16.3 ± 0.6	−19.1 ± 1.9
k_{H^+}, 0.006 *M* HCl	17.2 ± 0.5	16.6 ± 0.5	−17.3 ± 1.7
k_{H^+}, 0.012 *M* HCl	16.6 ± 1.0	15.9 ± 1.0	−19.3 ± 3.2
k_{H^+}, 0.030 *M* HCl	16.4 ± 0.8	15.8 ± 0.8	−19.4 ± 2.7
Mean	16.8 ± 0.8	16.2 ± 0.8	−18.8 ± 2.5

from that generally accepted for ketal hydrolysis. Rather, it appears that I represents a case where steric hindrance in the transition state produces an increase in $\Delta H‡$ and a decrease in $\Delta S‡$ (17).

The data of De Wolfe *et al.* (16) allow a comparison of the hydrolysis rates for I and 2,2-diphenyl-1,3-dioxolane. These investigators found $k_{H^+} = 7.50 \times 10^{-2}\ M^{-1}$ sec.$^{-1}$ for the latter compound at 30° (0.0197 *M* HCl in 1.3% dioxane–98.7% water). Interpolation of the data for I from Fig. 2 gave $k_{H^+} = 1.29 \times 10^{-3}\ M^{-1}$ sec.$^{-1}$ at 30° (0.0197 *M* HCl in water). Presence of the protonated piperidyl group in I, therefore, produced a greater than 98% reduction in hydrolysis rate, reflecting the less favorable entropy of activation.

De Wolfe *et al.* (16) found that hydrolysis of a number of benzophenone ketals was susceptible to general acid catalysis. The catalytic constants for acids other than the hydronium ion were small but significant. For example, dichloroacetic acid (HA) catalyzed the hydrolysis of 2,2-diphenyl-1,3-dioxolane (30°, 20% dioxane) such that $k_{H^+}/k_{HA} = 40$. No attempt was made in the present studies to determine the role of general acid catalysis in the hydrolysis of I. However, it may be concluded from the data in Table I (37°, 0.03 *M* HCl) that the protonated substrate (a Bronsted acid) did not make a significant catalytic contribution to its own hydrolysis, since observed rate constants were independent of initial I concentration.

Oral doses of I in man have ranged from 10 to a maximum of 150 mg. (1–4). Since the solubility of I is high (11.66 mg./ml. at 23°) (6), rapid and complete dissolution should occur in the stomach. Gastric pH and emptying times vary widely. To provide a reasonable estimate of the stability of I during its transit through the stomach, a pH of 2.0 (corresponding to the data for 0.012 *M* HCl in Table I) and a residence time of 60 min. (approximately three half-lives for the stomach emptying process) (18) were assumed. Under those conditions, more than 91% of orally ingested I would survive hydrolysis. This estimate is consistent with the high order of oral activity observed in clinical studies.

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Colorimetric Assay of Disulfiram

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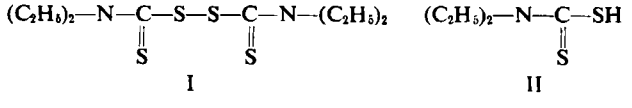
Abstract □ A quantitative assay for disulfiram was developed based on color formation during the interaction of disulfiram, ethanol, and cyanide. A linear curve is obtained over a wide range of disulfiram concentrations. Metabolites of disulfiram do not interfere in this assay. The color reaction is suitable for identification of small aliphatic alcohols and ketones.

Keyphrases □ Disulfiram—colorimetric assay using ethanol and cyanide, used to identify small aliphatic alcohols and ketones □ Alcohols and ketones, aliphatic—identification, colorimetric reaction with disulfiram and cyanide □ Colorimetry—analysis of disulfiram using ethanol and cyanide, used to identify aliphatic alcohols and ketones

During a study of the metabolic effects of disulfiram[1] [bis(diethylthiocarbamoyl) disulfide, I], attention was directed to a color reaction, quoted in the manual of drug identification of Clarke (1). This reaction is found in older manuals and its original discoverer is not known[2]. The color produced by mixing an alcoholic disulfiram solution with aqueous cyanide was listed as a colorimetric spot test for disulfiram. Further investigation showed that this assay could be suitably adapted to a sensitive quantitative method for disulfiram determination. The mechanism and specificity of this reaction were examined further, since the strong blue color, produced rapidly at room temperature, was very puzzling. An examination of a large list of organic compounds proved that the reaction with cyanide and disulfiram could be used as a sensitive class reaction for aliphatic alcohols and ketones and that it may serve as a tool for identification of unknown compounds. Some of the present results were presented as a preliminary communication (2).



I

II

EXPERIMENTAL

Color was developed by mixing a freshly prepared solution of disulfiram (50 mg./5 ml.) in ethanol or other solvents with 1 ml. of 1 *M* NaCN at room temperature. For quantitative determinations, the reaction mixture was let stand for 30 min. at room temperature. TLC was carried out on silica gel GF[3] (3). Disulfiram showed R_f 0.67 in chloroform and R_f 0.24 in benzene, and it could be identified by quenching UV light and by a yellow spot when sprayed with ethanol and cyanide.

Absorption and IR spectra were obtained in spectrophotometers[4]. GC was carried out[5] using stainless steel columns, 1.8 m. × 1.3 cm. (6 ft. × 0.5 in.), with Chromosorb W (100–120 mesh) and 0.5% SE-30 as the liquid phase between 70 and 190°. The reaction mixture with ethanol was investigated further and was tested for diethyl dithiocarbamate (II) and carbon disulfide by colorimetric methods (4, 5). Sulfur was identified as barium sulfate after wet-ashing with concentrated nitric acid, and thiocyanate was identified by reaction with ferric chloride (6).

RESULTS AND DISCUSSION

An ethanolic solution of disulfiram treated with 1 *M* sodium cyanide rapidly forms an intense yellow color, which quickly passes to green and blue, with a broad maximum between 520 and 580 nm. and a minimum at 450–460 nm. (Fig. 1). The blue color fades on standing, reverting to yellow; neither the intensity of colors nor the rate of color formation is affected by the order of addition. The blue color can be extracted into ether but does not pass into petroleum ether.

When the graded levels of disulfiram are used for color development, a straight line is obtained over a wide concentration range. This can be used as a convenient colorimetric assay for disulfiram (Fig. 2). Eventual interference by other colored compounds, as found in urine, can be eliminated by extracting the blue color into ether and determining the color of the organic phase. Diethyl dithiocarbamate (II) and carbon disulfide, important metabolites of disulfiram (7–11), do not give a color reaction with cyanide, nor are they formed in the reaction of disulfiram with cyanide. The present method is, therefore, suitable for studies of metabolic transformations of disulfiram.

Color formation only takes place with oxygenated compounds that act not only as solvents but also participate in the reaction (Table I). Best results are obtained with small primary aliphatic alcohols, of which butanol forms an intense blue color at the interphase. The blue color is most stable and intense when methoxyethanol or *tert*-butanol is used as a reactant. Acetaldehyde could

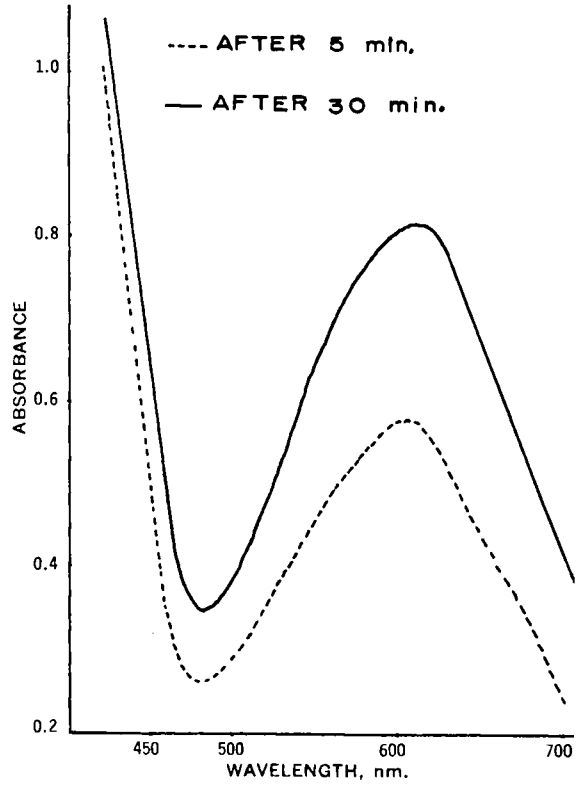


Figure 1—*Absorption spectrum of reaction product of the cyanide–disulfiram ethanol condensation. Key: - - -, after 5 min. at 25°; and ——, after 30 min. Thirty milligrams of disulfiram in 5 ml. of ethanol was used.*

[1] Antabuse.
[2] E. G. C. Clarke, personal communication.
[3] Merck.
[4] Beckman DB and IR-8 spectrophotometers, respectively.
[5] In a Beckman GC 2A.

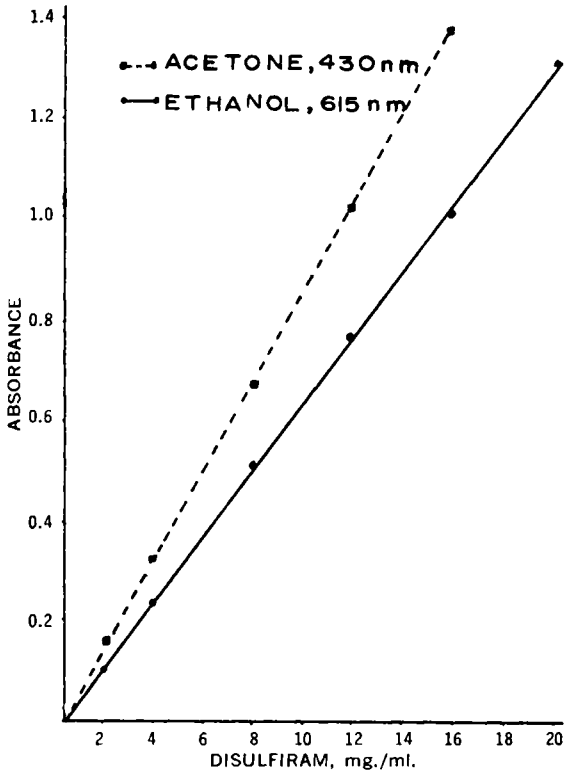


Figure 2—*Standard curve for disulfiram, with graded levels of disulfiram in 5 ml. of solvent, and 1.0 ml. of 1 M NaCN added. Absorbance was read after 30 min. at 25°.*

not be tested, because acetaldehyde and cyanide alone (in absence of disulfiram) form an intense brown color in a strongly exothermic reaction. An intense yellow color is formed by acetone, dioxane, dimethyl sulfoxide, and methylcellulose. Contrary to the reaction with alcohols, this color is stable and does not pass to blue nor fade on standing.

Acetone also serves as a suitable reagent for quantitative determination of disulfiram (Fig. 2). Dimethyl sulfoxide gives an exothermic reaction in which gas and precipitate formation are observed. When the reaction is carried out in this reagent, the yellow color is not converted to blue on addition of ethanol.

To identify the products of this reaction, a large batch was prepared with ethanol using the same proportions of reagents as for the routine color development. Several compounds were isolated, and the presence of others was detected by TLC. The new reaction products were found not to be disulfiram or its known derivatives by using TLC, GC, and UV and IR spectrophotometry. In view of the rapid formation and great intensity of color, a clarification of the reaction mechanism would be of considerable theoretical interest.

A proposed mechanism (12, 13) may be pertinent, in which a disulfide bridge is split by cyanide, with the formation of a thioether and free thiocyanate. However, no color formation has been reported (13). Thiocyanate could not be detected in the present study by reaction with ferric chloride; the reasons for these discrepancies are not clear. Similar interactions of cyanide with disulfide bridges occur not only with simple organic compounds but also with biological compounds. Cyanide causes cleavage of protein chains at the cystine bridges (14, 15) with the liberation of thiocyanate (16). Xanthine oxidase is irreversibly inactivated by cyanide, which forms a covalent bond with the enzyme (17, 18), and thiocyanate is liberated from the persulfide group, which is essential for enzyme activity (19).

The present procedure was used to determine disulfiram in urine of chronic alcoholics receiving disulfiram therapy. The drug could not be identified in urine or urine extracts, which is in agreement with previous reports (4, 20, 21).

SUMMARY

The interaction of disulfiram, cyanide, and organic solvents was studied. A simple colorimetric assay of disulfiram was developed, which gives a linear response over at least a 20-fold range. The reaction can be used as a type reaction for alcohols.

Table I—Color Reaction of Disulfiram with Organic Reagents[a]

Reagent	Color	Reaction Rate[b]
Methanol	Blue	Fast
Ethanol	Blue	Fast
Isopropanol	Green	Fast
1-Butanol	Blue	Fast
Methoxyethanol	Blue	Fast
2-Butanol	Light green	Slow
tert-Butanol	Blue	Fast
Acetaldehyde[c]	(Brown)	(Fast)
1-Octanol	Light orange	Very slow
Isoamyl alcohol	Yellow	Slow
Acetone	Lemon yellow	Fast
Dioxane	Brown	Fast
Formamide	Yellow	Slow
Dimethylformamide	Yellow	Slow
Dimethyl sulfoxide[c]	Yellow	Fast

[a] Fifty milligrams of disulfiram was dissolved in 5.0 ml. of solvent, and 1.0 ml. of 1 *M* NaCN was added. [b] Negative reactions were obtained with cyclohexanol, benzyl alcohol, phenol, acetic acid, ethyl acetate, butyraldehyde, hexane, benzene, ether, chloroform, carbon tetrachloride, and carbon disulfide. [c] See text.

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Fluorometric Determination of Primary and Secondary Aliphatic Amines by Reaction with 9-Isothiocyanatoacridine

ANDRÉ DeLEENHEER*, J. E. SINSHEIMER▲, and J. H. BURCKHALTER

Abstract □ An improved procedure was developed for the analysis of primary and secondary aliphatic amines based upon the fluorescence of a cyclized product derived from isothiourea derivatives of amines and 9-isothiocyanatoacridine. Initial isothiourea formation as well as its cyclization is carried out in toluene, which results in the formation of highly fluorescent 2-alkylamino-1,3-thiazino-[4,5,6-*kl*]acridine with a minimum of by-products. The fluorescence of these thiazinoacridine compounds is determined either directly in solution after dilution with acidified alcohol or after TLC separation and direct scanning of the TLC plate. For example, amphetamine could be determined with a sensitivity of 0.12 nl./ml. in solution or at a 3-pl. level by the TLC procedure.

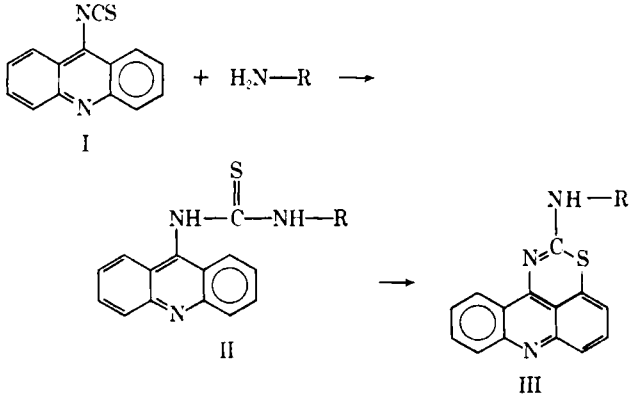
Keyphrases □ Amines, primary and secondary aliphatic—fluorescent analysis with 9-isothiocyanatoacridine □ Fluorescent analysis—primary and secondary aliphatic amines using 9-isothiocyanatoacridine, TLC fluorometry □ Amphetamine—fluorescent analysis using 9-isothiocyanatoacridine □ 9-Isothiocyanatoacridine—used as a reagent in fluorometic determination of primary and secondary aliphatic amines

The use of 9-isothiocyanatoacridine (I) as a reagent for the fluorometric determination of primary amines was previously reported from these laboratories (1). In that reaction (Scheme I), the thiourea (II) that formed was found to undergo a photochemical oxidation to yield a cyclized product (III), whose fluorescence can be related to the concentration of the original amine present (2). Knowledge of this cyclization has led to modification of the original analytical procedure (1), which has increased its sensitivity as well as permitted the analysis of secondary aliphatic amines.

This paper describes the modified procedure and its application to primary and secondary amines, with special emphasis on amphetamine analysis.

EXPERIMENTAL

Fluorometric Procedure—Solutions of 10–50 nl. (nanoliters) of liquid amines or of 10–50 mcg. of *p*-anisidine in 5.00 ml. of toluene were allowed to react with 2 ml. of 9-isothiocyanatoacridine reagent (80.0 mg. of 9-isothiocyanatoacridine in 100.0 ml. of toluene). Liquid amines were distilled over zinc dust, and *p*-anisidine was recrystallized from *n*-hexane prior to use. These solutions were allowed to react in 15-ml. glass-stoppered centrifuge tubes overnight (15 hr.) on the laboratory desk. These conditions permitted exposure to indirect morning sunlight after an initial reaction period in the dark. Fluorometric measurements[1] were made directly on the toluene solutions as well as on dilutions of 0.5 ml. of the toluene solution with 4.5 ml. of alcohol and of 0.5 ml. of the toluene solution with 4.5 ml. of alcohol and 0.1 ml. of concentrated hydrochloric acid. Reagent solutions without amine present were allowed to stand under the same conditions and were measured as controls.



Scheme I

TLC Separation and Fluorometry—Aliquots (15 μl.) of the toluene reaction mixtures from butylamine, amphetamine, and the reagent control described under *Fluorometric Procedure* were chromatographed on commercial[2] 250-μ precoated silica gel G plates (20 × 20 cm.), which were scored in 0.5-cm. channels prior to use. Development was in benzene–methanol (140:40) with detection under UV light. Quantitative fluorometric measurements were made directly from thin-layer plates with the aid of a scanner[3] as previously described (2).

RESULTS AND DISCUSSION

Reaction of 9-isothiocyanatoacridine with amines in alcoholic solutions leads to a mixture of fluorescent compounds (1). The fluorescence of one of these products could be related to the amount of amine present. It was subsequently recognized (2) that this fluorescent product was a cyclized compound (III) produced by photooxidation of thiourea II. Therefore, a study was undertaken to develop an analytical procedure with an increased yield of cyclized product in a minimal background of fluorescent by-products. The use of toluene in place of alcohol produced a more stable reagent solution and eliminated the interaction of reagent and solvent. Experiments to establish a maximum yield of cyclized product by exposure of the initial reaction mixture of amine and excess reagent to UV light in a photochemical reactor[4] were unsuccessful. While individual thioureas such as *N*-butyl-*N'*-(9-acridinyl)thiourea could be cyclized under these conditions, the procedure could not be applied to the analytical reaction mixture. Under these conditions, the required excess of the reagent, 9-isothiocyanatoacridine, was readily converted into acridone [λ (activation) 320 nm.; λ (emission) 440 nm.] by exposure to UV radiation. This resulted in excessive background fluorescence.

The most convenient reaction condition for the present study was to allow the amine and a 10–70-fold molar excess of reagent to react overnight at room temperature in the dark. This was followed by a limited morning exposure to indirect sunlight. An alternative procedure was to allow the amine sample and excess reagent to react in the dark at 60° before exposure to sunlight. In either case, care was taken to expose all sets of reaction mixtures containing increasing concentrations of amines and control reagent solutions to the same conditions.

Progress of the reaction can be monitored by TLC as described in the *Experimental* section. For example, a 15-μl. aliquot of a typical overnight reaction mixture of 0.05 ml. of *n*-butylamine and 1.6 mg. of 9-isothiocyanatoacridine in 7 ml. of toluene showed fluorescent spots corresponding to the R_f values and conditions of the following reference compounds: 0.02, blue, trace (9-aminoacridine);

[1] The Aminco-Bowman spectrophotofluorometer model 4-8106 was employed.
[2] E. Merck AG.
[3] Aminco 4-8221A.
[4] Rayonet, The Southern N. E. Ultraviolet Co.

Table I—Analysis of Amines

	Fluorescence		
	Activation, nm.	Emission, nm.	Intensity/ Concentration[a]
n-Butylamine	300	525	14.0
Cyclohexylamine	300	515	14.4
Benzylamine	300	520	12.2
β-Phenylethylamine	300	520	12.8
Amphetamine	300	520	8.6
Diethylamine	300	520	17.6
N-Methylpiperazine	310	525	12.8

[a] Slope of linear range: given as the difference in fluorescence over the reagent blank (calculated for a meter multiplier reading of 1 at a sensitivity of 25) and divided by concentration of amine (in nanoliters per milliliter). The average value for the acidic, alcoholic dilution of reagent blank was 60 at 465 nm. at a meter multiplier reading of 0.1. This value was reduced to about 12 in the wavelength range of maximum fluorescence of the test compounds.

0.16, yellow-green (cyclized product: 2-butylamino-1,3-thiazino[4,-5,6-*kl*]acridine); and 0.36, blue (excess reagent: 9-isothiocyanatoacridine). While a reagent control solution showed the spot at R_f 0.66 and those corresponding to trace quantities of aminoacridine and acridone, there was no fluorescence in the region of the thiourea or the cyclized compound.

When the final reaction mixture was measured in an alcoholic solution, especially an acidic alcoholic solution, there was a pronounced increase in the difference between fluorescence of the amine reaction solution and the control solution. Thus, while alcoholic solutions were avoided in original reaction mixtures, they could be employed to advantage just before determination of fluorescence. However, there is a decrease in stability of such solutions, with a change in fluorescence appearing in about 6 hr., as opposed to no appreciable change in fluorescence of the more concentrated reaction mixtures in toluene after 3 days.

The final analytical procedure was performed as outlined in the *Experimental* section. This included the acidic, alcoholic dilution of the original toluene reaction mixture just prior to measurement of fluorescence. Of particular importance in this procedure are the facts that the final reaction product has fluorescent characteristics that permit its direct determination in the presence of excess reagent and that this fluorescence is proportional to the amount of amine originally present.

Table I is a summary of compounds that were successfully analyzed by the modified technique. Included are the wavelengths of activation and emission used to obtain a maximum difference in fluorescence between the reaction mixture and its corresponding reagent blank. Table I also includes a summary of the intensity of this difference in fluorescence.

In addition to the compounds listed in Table I, aniline, *p*-toluidine, *p*-anisidine, and *N*-ethylaniline were subjected to the procedure but did not yield satisfactory results. Failure to achieve positive results with these compounds, which is consistent with the nucleophilicity of the amines involved, suggests that the rate of thiourea formation (Compound II) is a critical consideration. Thus, by using the basicity of amines as a measure of nucleophilicity, it was found that all amines that were successfully determined have a pKa $\geq$ 9.33 (benzylamine), while no compound with a pKa $\leq$ 5.34 (*p*-anisidine) (3) could be measured.

The procedure is applicable to amines of biological significance such as amphetamine. A detection limit down to a concentration of 0.12 nl./ml. was established for quantitative results for amphetamine solutions. The results for six samples in the range from 0.2 to 0.5 nl./ml. gave a mean recovery of 97% with a standard deviation of 3.3%. The sensitivity of the solution procedure could be greatly enhanced by combination with TLC followed by direct fluorometric scanning of the plates. The fluorescence scanning procedure was conducted as previously described (2) after chromatography on silica gel G with benzene–methanol (140:40). Measurement of the cyclized product derived from amphetamine was at an activation of 355 nm. and an emission of 518 nm. The recorder response was linear to the original volume of amphetamine over a range of samples of 3, 6, 9, 20, and 25 pl. (picoliters). The results for eight samples over this range gave a mean recovery of 98.9% with a standard deviation of 9.9%. The TLC scanning procedure thus results in a loss in precision but a great increase in sensitivity and it eliminates the interfering fluorescence of products and excess reagent.

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Stability of Aspirin in Liquid and Semisolid Bases II: Effect of Fatty Additives on Stability in a Polyethylene Glycol Base

C. W. WHITWORTH[A], L. A. LUZZI, B. B. THOMPSON, and H. W. JUN

Abstract □ Aspirin decomposes rapidly when incorporated into mixtures of polyethylene glycols, but aspirin incorporated into fatty-type suppository bases is much more stable. Fats of vegetable origin were incorporated into a polyethylene glycol base in an attempt to inhibit the decomposition of aspirin in this type of mixture. The process is temperature dependent and refrigeration of the mixture greatly slows decomposition. Degradation was retarded at 26°, but little effect was noted at 4 and 45° within the time limits of this study.

Keyphrases □ Aspirin—effect of fatty additives on stability of polyethylene glycol base suppositories □ Fatty additives—effect on decomposition of aspirin in polyethylene glycol base suppositories □ Degradation—effect of fatty additives on aspirin stability in polyethylene glycol base suppositories □ Polyethylene glycol–aspirin formulations—effect of fatty additives on aspirin stability □ Additives, fatty—effect on aspirin stability in polyethylene glycol base suppositories

Aspirin has been found to degrade in polyethylene glycol mixtures. The process has been shown to be due in part to transesterification (1). The process is greatly temperature dependent, with refrigeration at 4° markedly slowing the rate of decomposition.

Aspirin is commercially available in suppository form in a cocoa butter base. Under refrigeration, these suppositories are sufficiently stable, although an expiration date is necessary. Aspirin is likewise much more stable in other fatty vehicles than in the polyethylene glycols. A product composed of distilled acetylated monoglycerides was tested as a vehicle for aspirin and found to inhibit greatly the degradation process (Fig. 1). It was then decided that fatty additives might have an inhibiting effect on aspirin decomposition in a polyethylene glycol base. The purpose of this study was to formulate a stable aspirin suppository using a polyethylene glycol base without altering the desirable characteristics of the base. A 10% concentration of additive was used in the study, and no compatibility problems were encountered with the formulation. Six different additives were utilized.

Polyethylene glycol mixtures serve as excellent suppository bases for some drugs (2). Their main advantages are water solubility, an innocuous nature, the ability to release a drug for absorption (3), and firmness at room temperature. Since the polyethylene glycols dissolve in the body secretions rather than melting, they do not require refrigeration with most drugs; in this respect, they have a great advantage over the fatty-type bases such as cocoa butter.

Unfortunately the polar and perhaps also the hygroscopic nature of these glycols tends to allow certain drugs such as aspirin to degrade when incorporated into this type of base.

One aspect of the study was to determine if packaging and storage procedures contributed significantly to the degradation process. Commercially, polyethylene glycol base suppositories are packaged unwrapped in jars and stored at room temperature until dispensed. In this project, the control batch of aspirin suppositories was divided into groups and some were wrapped in aluminum foil and stored both with and without a desiccant in the container. The degradation of these suppositories was compared with another batch stored without either wrapping or desiccant. There were no appreciable differences in the degradation curves for these batches. Throughout the study, however, a desiccant was present in all containers to reduce the effect of moisture on the decomposition process.

EXPERIMENTAL

Materials—The formula for the polyethylene glycol base used in the study was as follows:

polyethylene glycol 400[1]	24.8%
polyethylene glycol 1540[1]	30.4%
polyethylene glycol 6000[1]	40.0%
polysorbate 60[2]	4.8%

The additives used in the project were:

1. Hard butter containing lauric fats[3]. Free fatty acids, 0.1% max. Iodine value, 3 max. Used in confections and candies as coatings.
2. A coating butter made from domestic vegetable oils[4]. Free fatty acids, 0.1% max. Iodine value, 58–63. Used in confections and candies.
3. Hard butters obtained from lauric oils[5]. Free fatty acids, 0.05% max. Used in candies and confections.
4. Distilled, fully acetylated monoglycerides[6]. Acid value, 4. Iodine value, 44. Made from edible fats. Used in cosmetics as emulsifiers.
5. Oil expressed from roasted seeds of *Theobroma cacao*[7]. A mixture of glycerides. Iodine value, 35–43. Used as a suppository base.
6. Hydrogenated glycerides of fatty acids from palm oils[8]. Acid value, 1. Iodine value, 2. Used as suppository base.

Procedure—The additives were added in quantities equivalent to 10% of the amount of base used. The ingredients were then melted at 55° with stirring, and aspirin equal to 12% of the base–additive mixture was stirred into the melted mixture. The mixture was poured with stirring into suppository molds and allowed to congeal. The batch of suppositories was divided into three quantities for storage at the temperatures reported in this study. All suppositories were stored in airtight containers containing a desiccant.

Aspirin was added with stirring to the acetylated monoglyceride product in the same ratio as with all the other formulations and stored under similar conditions.

Analytical Method—Assays were performed at short intervals for the first part of the study, and the intervals gradually lengthened

[1] Union Carbide.
[2] Atlas Chemicals.
[3] Paramount H, Glidden-Durkee.
[4] Kaomel, Glidden-Durkee.
[5] Pinnacle 18, Humko Products.
[6] Myvacet 9-45, Eastman Chemicals.
[7] Theobroma oil USP, Fisher Scientific.
[8] Suppocire AM, A and S Corp.

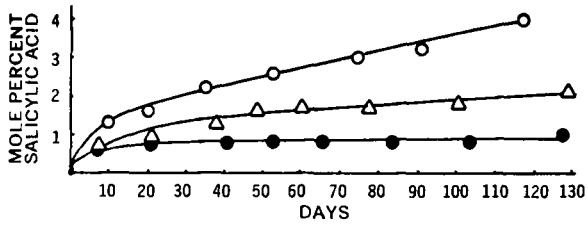

Figure 1—*Mole percent decomposition of aspirin with time in a vehicle composed of acetylated monoglycerides. Key: ○, 45°; △, 26°; and ●, 4°.*

as decomposition slowed down. When the percent decomposition reached 30–35%, assaying was discontinued. The assay procedure consisted of dissolving each suppository (approximately 2.4 g.) in 100 ml. of a solution of chloroform containing 1% acetic acid. A 1:50 dilution was made, and the absorbance of the dilution was read at 278 nm. for aspirin and 308 nm. for salicylic acid on a spectrophotometer[9]. From a standard curve the moles of each drug were determined and mole percent of salicylic acid (percent decomposition) was calculated.

RESULTS AND DISCUSSION

Figure 1 shows the degradation of aspirin in acetylated monoglycerides. The graphs are all plots of mole percent salicylic acid against time. The ordinates likewise represent mole percent decomposition of aspirin. Figure 2 represents the control, which was the polyethylene glycol mixture without additives. Since all additives yielded essentially identical results, only one curve at each temperature is shown in Fig. 3. For purposes of comparison, the control curves appear as broken lines in Fig. 3.

A comparison of Figs. 1 and 2 shows a drastic difference in the rate of decomposition of aspirin in a fatty-type vehicle compared to a polyethylene glycol base. In acetylated monoglycerides the decomposition after about 130 days at 45, 26, and 4° had reached

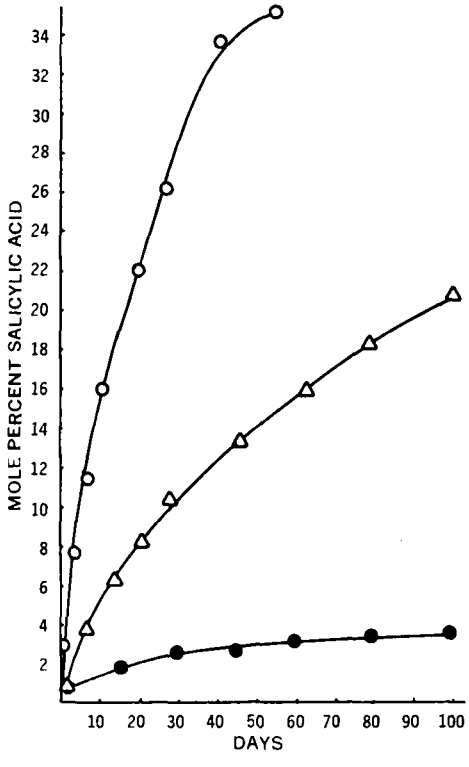

Figure 2—*Mole percent decomposition of aspirin with time in a polyethylene glycol base (control). Key: ○, 45°; △, 26°; and ●, 4°.*

[9] Beckman DU.

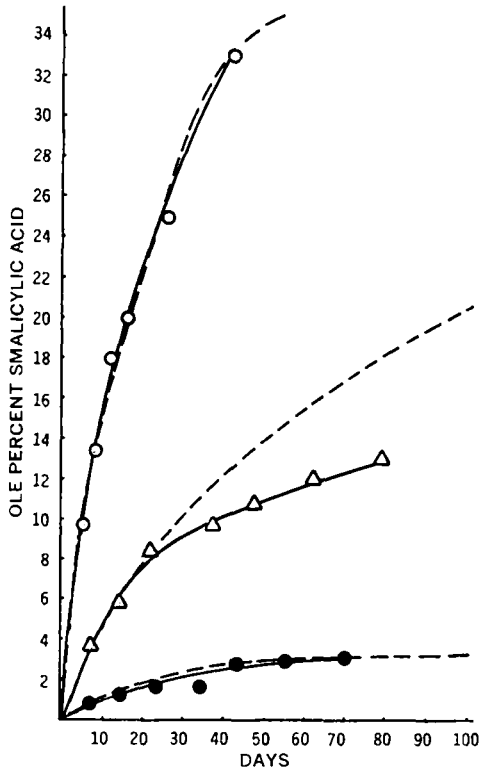

Figure 3—*Mole percent decomposition of aspirin with time in a polyethylene glycol base containing 10% acetylated monoglycerides. Broken lines represent control. Key: ○, 45°; △, 26°; and ●, 4°.*

only approximately 5, 2, and 1%, respectively; in the polyethylene glycol base, the decomposition was much greater.

The effect of temperature on aspirin degradation in many different types of vehicles is well documented. However, no study heretofore has dealt with aspirin stability in a polyethylene glycol-type suppository base. In practice, polyethylene glycol-type suppositories are stored at room temperature. This study shows this procedure should not be followed with aspirin.

As could be expected and as may be seen from Fig. 3, degradation was drastically inhibited in all the mixtures by reducing the temperature of storage. At 45°, both the control and the bases with additives reached the range of 30–35% decomposition in about 40–50 days, at which time sampling was discontinued. It is readily apparent that the fatty additives used in this study had no significant effect on aspirin degradation in the polyethylene glycol base at 45°. This is probably due to the rapid rate of the reaction at this temperature.

Figure 3 also shows that storage of suppositories at 26° drastically reduced the rate of decomposition of both the control and additive bases. For comparable time periods, the percent decomposition at 26° was less than half that at 45°. It may be seen that the additives had an inhibiting effect on aspirin degradation at this temperature. The difference in stability becomes apparent after about 20 days when the curves diverge.

Refrigeration of the suppositories at 4° reduced the decomposition of aspirin to less than 4% within the time limits of this study, for both the control and the additive bases. There was no apparent difference between the results from the control and the treated bases. It is possible that the additive would show an inhibiting effect on the degradation of aspirin after a much longer storage period.

The aims of this project were to study aspirin decomposition in polyethylene glycol mixtures and to devise means of stopping or reducing this degradation. As previously reported, the degradation is partly due to a transesterification mechanism (1).

It was felt that fatty additives might render a polyethylene glycol suppository base suitable as a vehicle for aspirin. From this study, it is concluded that while the fatty additives have an inhibiting effect at a room temperature of 26°, the percent of decomposition is still prohibitively high. Furthermore, the results showed temperature

effect to be much more pronounced than additive effect. Refrigeration dramatically slows the rate of decomposition.

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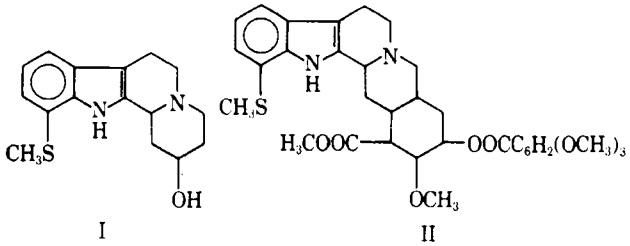
Synthesis of (±)-*trans*-11-Methylthio-1,2,3,4,6,7,12,12b-octahydrohydroxyindolo[2,3-*a*]quinolizine

GARY E. DUGAN* and KARL A. NIEFORTH†▲

Abstract □ (±)-*trans*-11-Methylthio-1,2,3,4,6,7,12,12b-octahydrohydroxyindolo[2,3-*a*]quinolizine was synthesized as a cardiovascular agent. Biological evaluation of the title compound and several precursors (7-methylthiotryptamine formate, 7-methylthiotryptamine formamide, 8-methylthio-3,4-dihydro-β-carboline, *trans*-11-methylthio - 1,2,3,4,6,7,12,12b - octahydro - 2 - oxoindolo[2,3 - *a*] quinolizine) showed no activity.

Keyphrases □ (±)-*trans*-11-Methylthio-1,2,3,4,6,7,12,12b-octahydrohydroxyindolo[2,3-*a*]quinolizine—synthesis, pharmacological evaluation □ Thioindole derivatives—synthesis and pharmacological evaluation of (±)-*trans*-11-methylthio-1,2,3,4,6,7,12,12b-octahydrohydroxyindolo[2,3-*a*]quinolizine

The title compound, (±)-*trans*-11-methylthio-1,2,3,4,-6,7,12,12b - octahydrohydroxyindolo[2,3 - *a*]quinolizine (I), was prepared as an extension of the authors' interest in thioindole derivatives and the reported (1) activity of methiopidine (II). Unsubstituted octahydroindolo[2,3-*a*]-quinolizine has been synthesized (2) and reported to be a hypotensive agent. Synthesis (Scheme I) of I was achieved by first preparing 7-methylthiotryptamine through a sequence described by Abramovitch and Shapiro (3). Preparation of the phenylhydrazone (III) was carried out *via* the Japp-Klingemann reaction of S-methyl - 2 - aminobenzenethiol and 3 - carbethoxy - 2-piperidone. Cyclization of III into the β-carboline (IV) was smooth in hot acetic acid–hydrogen chloride gas. Hydrolysis of the β-carboline (IV) to the amino acid (V) was quantitative in 5% alcoholic potassium hydroxide. Decarboxylation to 7-methylthiotryptamine (VI) was accomplished with difficulty in refluxing 2 *N* hydrochloric acid–acetic acid. Synthesis of the octahydroindolo[2,3-*a*]quinolizine (X) followed a procedure described previously (4–6). Addition of ethyl acetate–90% formic acid (1:1) to an ethyl acetate solution of 7-methylthiotryptamine afforded the formate salt (VII) which, upon heating, was converted to the formyl derivative (VIII). The *N*-formyl derivative was treated under Bischler–Napieralski conditions to form the Schiff base (IX), which was converterted to the *trans*-indolo[2,3-*a*]-quinolizine (X) by warming with methyl vinyl ketone and a catalytic amount of ethanol saturated with hydrogen chloride gas. Assignment of the *trans*-configuration was based on the presence of a Bohlman band between 2700 and 2800 cm.$^{-1}$ (KBr) on the IR spectrum (7). Reduction of X with lithium aluminum hydride afforded the title compound, I.



I II

PHARMACOLOGICAL RESULTS

The compounds were tested[1] in a variety of *in vitro* and *in vivo* assays: antibacterial, antifungal, antiparasitic, and antiviral assays. In some cases they were checked for activities of potential interest in the cardiovascular and behavioral fields. A total of 114 individual bioassays was run.

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effect to be much more pronounced than additive effect. Refrigeration dramatically slows the rate of decomposition.

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Synthesis of (±)-*trans*-11-Methylthio-1,2,3,4,6,7,12,12b-octahydrohydroxyindolo[2,3-*a*]quinolizine

GARY E. DUGAN* and KARL A. NIEFORTH†▲

Abstract □ (±)-*trans*-11-Methylthio-1,2,3,4,6,7,12,12b-octahydrohydroxyindolo[2,3-*a*]quinolizine was synthesized as a cardiovascular agent. Biological evaluation of the title compound and several precursors (7-methylthiotryptamine formate, 7-methylthiotryptamine formamide, 8-methylthio-3,4-dihydro-β-carboline, *trans*-11-methylthio - 1,2,3,4,6,7,12,12b - octahydro - 2 - oxoindolo[2,3 - *a*] quinolizine) showed no activity.

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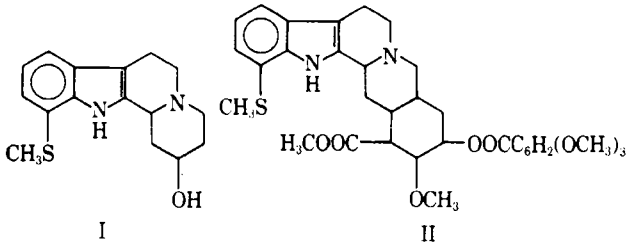

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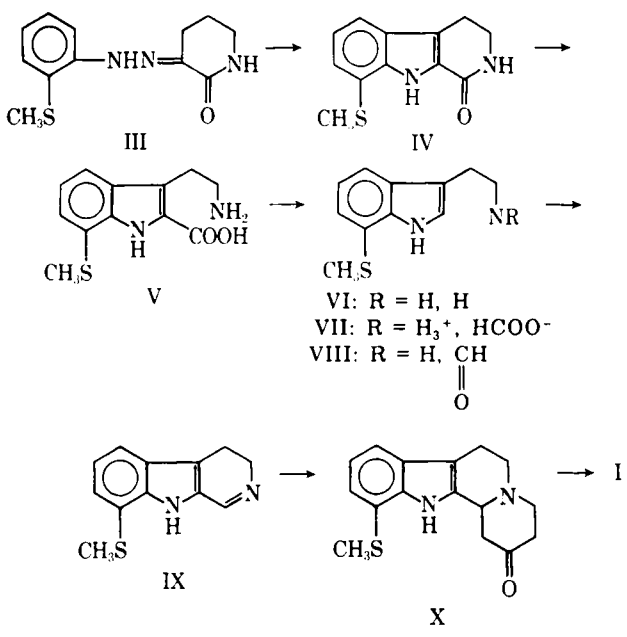

Scheme I

acid. The solid was collected and recrystallized from ethanol to give 19.5 g. (82%) of white crystals, m.p. 152–153°.

Anal.—Calc. for $C_{12}H_{16}N_2O_2S$: C, 57.10; H, 6.39; N, 11.11; S, 12.69. Found: C, 56.80; H, 5.21; N, 11.43; S, 12.71.

***N*-Formyl-7-methylthiotryptamine (VIII)**—A test tube containing 7 g. (0.038 mole) of the formate salt VII was heated in an oil bath at 185° for 45 min. After cooling, a small portion of ethanol was added to the thick gummy residue. Vigorous stirring of the mixture yielded white crystals; 5.35 g. (82%) was collected and recrystallized from a minimal amount of hot water, m.p. 109–110°.

Anal.—Calc. for $C_{12}H_{14}N_2OS$: C, 61.51; H, 6.02; N, 11.95; S, 13.68. Found: C, 61.51; H, 6.14; N, 11.97; S, 13.45.

8-Methylthio-3,4-dihydro-β-carboline (IX)—A mixture of 7 g. (0.03 mole) of VIII and of anhydrous benzene (126 ml.) was heated until solution was nearly complete. The solution was allowed to cool slightly and then 21 g. (0.137 mole) of freshly distilled phosphorus oxychloride was added dropwise. When spontaneous boiling ceased, the solution was heated for 30 min. on an oil bath. Upon cooling, the hydrochloride salt of the desired product precipitated. The hydrochloride, 6.8 g. (90.5%), m.p. 242–243°, upon neutralization yielded the free amine as white crystals, m.p. 87–88°.

Anal.—Calc. for $C_{12}H_{11}N_2S \cdot HCl$: C, 57.02; H, 5.18; N, 11.08; S, 12.86. Found: C, 56.72; H, 5.33; N, 10.84; S, 12.68.

***trans*-11-Methylthio-1,2,3,4,6,7,12,12b-octahydro-2-oxoindolo-[2,3-*a*]quinolizine (X)**—To a warmed solution of 34 g. (0.136 mole) of IX in absolute ethanol (75 ml.) were added 38 g. (0.544 mole) of methyl vinyl ketone and a catalytic amount of absolute ethanol saturated with hydrogen chloride. The solution was allowed to reflux for 6 hr. and then to cool overnight, and the solvent was removed *in vacuo*. The residue, on crystallization from ethanol, gave 4.5 g. (9.9%) of the crude ketone X. A pure sample of the ketone X, m.p. 164–166°, was obtained *via* preparative TLC using chloroform–methanol (1:9) as the solvent. The 2,4-dinitrophenylhydrazone derivative had a melting point of 148–150°.

Anal.—Calc. for $C_{22}H_{22}N_6O_4S$: C, 56.64; H, 4.75; N, 18.01; S, 6.87. Found: C, 56.75; H, 4.91; N, 17.78; S, 6.93.

(±)-*trans*-11-Methylthio-1,2,3,4,6,7,12,12b-octahydro-2-hydroxyindolo[2,3-*a*]quinolizine (I)—A solution of 0.9 g. (0.003 mole) of crude X in 100 ml. of anhydrous dioxane–anhydrous ether (1:1) was slowly added to 0.12 g. (0.003 mole) of lithium aluminum hydride in 100 ml. of the same solvent. After the addition was completed, the solution was allowed to reflux overnight and then to cool, and the solvent was removed *in vacuo*. The residue, on crystallization from chloroform–acetone, yielded 0.4 g. (43%) of the crude I, m.p. 250–252° dec. Preparative TLC using chloroform–acetic acid (95:5) as solvent afforded an analytical sample of the alcohol IX, m.p. 262–264°.

Anal.—Calc. for $C_{16}H_{20}N_2OS$: C, 66.63; H, 6.99; N, 9.72. Found: C, 66.48; H, 7.24; N, 9.51.

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GLC Determination of 1-(2,6-Dimethylphenoxy)-2-aminopropane in Urine

NAOMI SZINAI*, ROBERT J. PERCHALSKI*, RICHARD H. HAMMER†▲, B. JOE WILDER*, and RICHARD R. STREIFF*

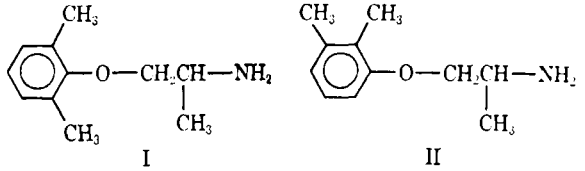
Abstract □ A rapid, sensitive, and accurate GLC method of analysis of a new anticonvulsant drug, 1-(2,6-dimethylphenoxy)-2-aminopropane, was developed, utilizing an acid hydrolysis procedure to increase its degree of recovery from urine and employing one of its isomers, 1-(2,3-dimethylphenoxy)-2-aminopropane, as an internal standard.

Keyphrases □ 1-(2,6-Dimethylphenoxy)-2-aminopropane—GLC analysis in urine □ GLC—analysis, 1-(2,6-dimethylphenoxy)-2-aminopropane in urine

A new anticonvulsant drug, 1-(2,6-dimethylphenoxy)-2-aminopropane[1] (I), is presently undergoing clinical testing and metabolic studies in humans (1, 2). Significant anticonvulsant activity has been demonstrated in patients with psychomotor seizures or temporal lobe epilepsy when receiving divided daily doses of 4–8 mg./kg. (1). To evaluate its rate of elimination and other kinetic parameters, a rapid and accurate method of analysis in urine of I was sought. Due to the structural similarity of I to amphetamine and its congeners, several GLC methods of analysis of amphetamine in biological fluids were evaluated from the literature (3–7). The method of Ramsey and Campbell (7) was found to be a simple, accurate, and rapid GLC method which was modified to accommodate the urinary analysis of I. This paper describes a fast (50 samples/day/technician), sensitive, and accurate GLC method of analysis for measuring urinary levels of I in humans.

EXPERIMENTAL

Extraction Procedure—Two milliliters of patient or standard urine was accurately measured and pipeted into a 15-ml. ground-glass conical centrifuge tube, and 1.0 ml. hydrochloric acid solution was added. The tube was stoppered and the mixture was heated in a constant-temperature bath at 100° for 1 hr. After cooling to room temperature, the pH was adjusted to 12 (Hydrion E paper) with a saturated solution of sodium hydroxide. To the alkaline solution, 0.5 ml. of internal standard (II)[2] solution (300 mcg./ml. water) and 0.1 ml. chloroform were accurately measured and added. The free bases were extracted into the chloroform layer by vigorous shaking for 1 min., followed by centrifugation for 4 min. After separation of the two layers, 1.0 μl. of the lower chloroform phase was injected into the gas chromatograph.



GLC—GLC experiments were performed on a dual-channel instrument[3] equipped with four hydrogen flame detectors. The 1.83-m. (6-ft.) long, U-shaped column (2-mm. i.d.) was packed with 3% OV-17 on Gas Chrom Q, 100–120 mesh. Operating temperatures were: injector port, 340°; column, 125° (isothermal); and detector, 260°. Flow rates (milliliters per minute) were: nitrogen, 60; air, 300; and hydrogen, 50.

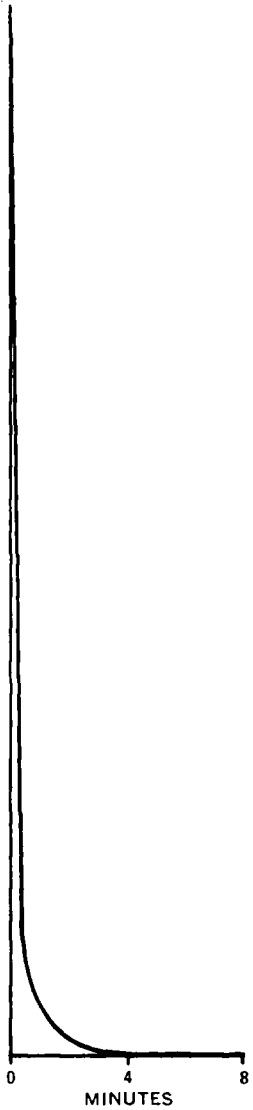


Figure 1—*Chromatogram of hydrolyzed normal urine.*

[1] Designated as KÖ 1173 and supplied as the hydrochloride salt by C. H. Boehringer Sohn, Ingelheim, Germany. Amounts and levels of KÖ 1173 and KÖ 1398 are designated throughout the paper in milligrams of free base rather than hydrochloride salt (1.0 g. KÖ 1173-HCl or KÖ 1398-HCl = 0.83 g. KÖ 1173 or KÖ 1398 free base, respectively).

[2] Designated as KÖ 1398 (II) [1-(2,3-dimethylphenoxy)-2-aminopropane] (isomer of KÖ 1173-I) and furnished as the hydrochloride salt by C. H. Boehringer Sohn, Ingelheim, Germany.

[3] Varian 2100.

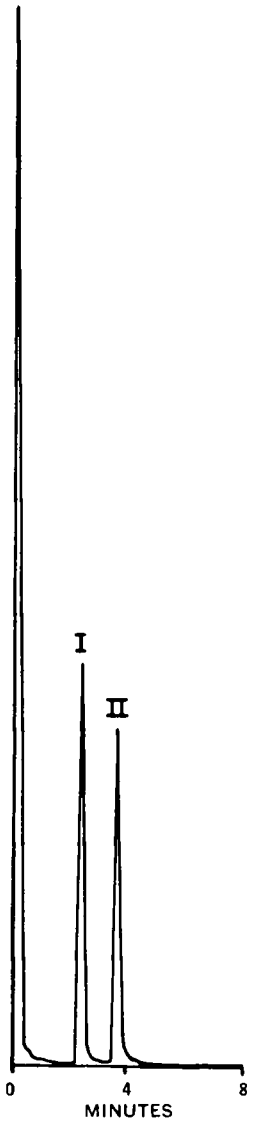


Figure 2—*Chromatogram of hydrolyzed normal urine with drug (I) and internal standard (II) added.*

The standard calibration curve was prepared by measuring the peak height at seven different concentration levels of I (5.06–150.90 mcg./ml. urine) and II (75 mcg./ml. urine) *via* GLC in normal urine after acid hydrolysis and extraction (Figs. 1–3). The mean of the peak height ratio of I:II of six determinations at each concentration level was plotted *versus* its respective concentration level of I (micrograms per milliliter urine). The least-squares method, employed to obtain the best fit for the seven concentration levels, indicated that the peak height ratios of I:II were linear with respect to the concentration of I (Fig. 4).

RESULTS AND DISCUSSION

GLC analysis of urine of epileptic patients who were taking I showed an increase in the chromatographic peak height of I when the urine was hydrolyzed in acid media *versus* the peak height of I measured in nonhydrolyzed urine. Apparently, I exists partly conjugated in urine and acid hydrolysis released the free drug, producing a larger peak height on the chromatogram. The optimum length of time for the urinary acid hydrolysis step was 1 hr. Additional hydrolysis time did not increase the amount of free unconjugated I in urine.

Amphetamine, 2,3-II, and 2,4- and 2,5-dimethyl isomers of I were evaluated as internal standards. Amphetamine had an undesirable short retention time, with its chromatographic peak occurring in front of I close to the solvent front. The most favorable

Table I—Typical Urinary Levels of I in Humans

Patient	Daily Dose, mg.	24-hr. Urine: Volume, ml.	24-hr. Urine: Concentration, mcg./ml.	24-hr. Urine: Total Amount Excreted, mg.
L.J.F.	100	3260	13.5	44.0
J.W.	100	1600	29.7	47.5
L.F.	200	1500	42.3	63.5
F.C.	200	1930	13.5	26.1
L.S.	300	1900	24.1	45.8
T.S.	300	1360	29.8	40.6

marker appeared to be II. It was completely separated from I and possessed a longer retention time than I. Both drugs (I and II) gave sharp, well-defined peaks with no tailing (Figs. 2 and 3). Initially, II was added as an internal standard before acid hydrolysis and then was hydrolyzed along with I for 1 hr. However, the results were erratic and inconsistent, and the standard calibration curve was not reproducible. GLC analysis of I added to standard urine before and after hydrolysis indicated that no acid degradation had taken place in either case. Therefore, the erratic quantitative results were not

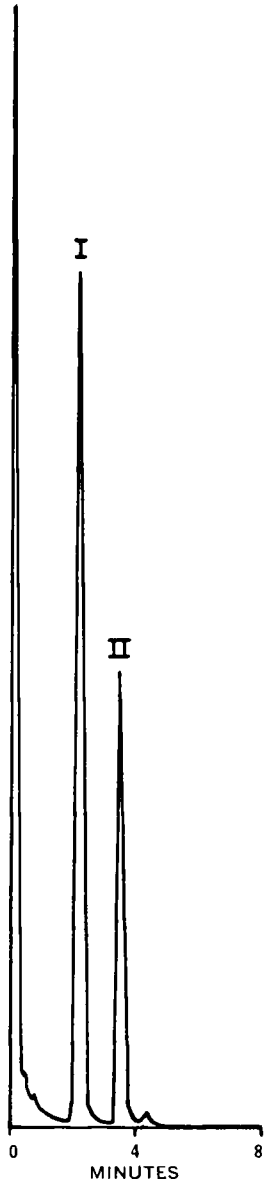


Figure 3—*Chromatogram of hydrolyzed patient urine with internal standard (II) added.*

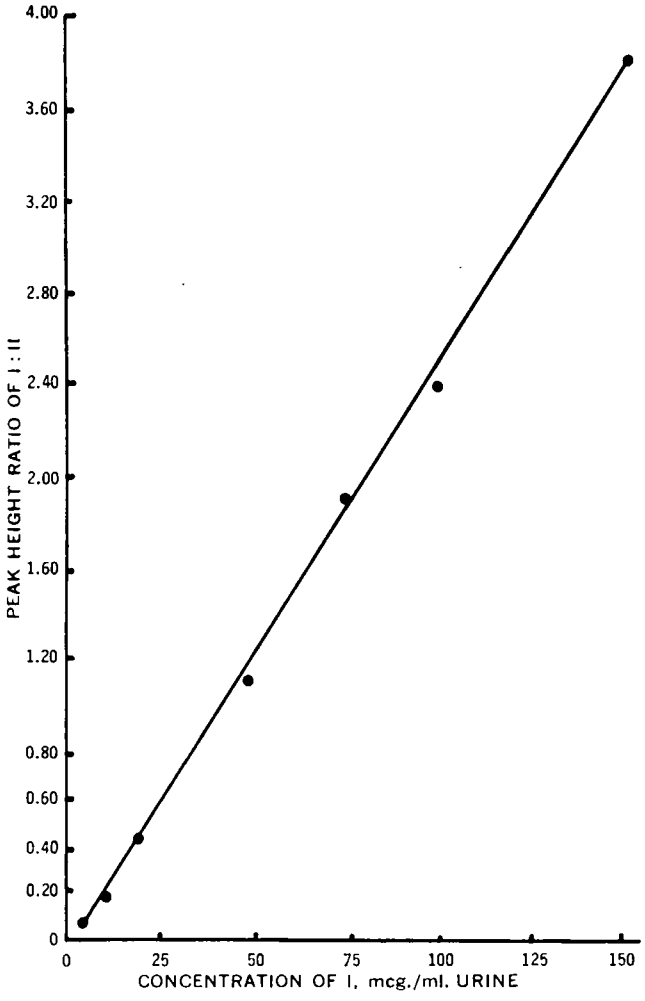


Figure 4—*Standard calibration curve.*

due to degradation of I. Similar studies of II revealed some acid degradation when it was added before hydrolysis. The problem of erratic quantitative results was, therefore, traced to acid degradation of II during the hydrolysis step.

One can postulate that I is stable to acid degradation through steric hindrance imposed by the 2,6-dimethyl groups shielding the ether oxygen to protonation. However, II, with its 2,3-dimethyl group substitution, cannot provide the same degree of steric hindrance as I and would be expected to degrade partially in acid media. In the case of amphetamine, the quantitative results were the same whether it was added before or after the acid hydrolysis, indicating acid stability. When II was added after the acid hydrolysis step, the results were consistent with the values obtained when using amphetamine either before or after hydrolysis. Therefore, the 2,3-dimethyl isomer (II) of I was chosen as the internal standard rather than amphetamine or one of the other isomers of I. The addition of II after the acid hydrolysis step led to reproducible and accurate results, as indicated by the linearity of the standard calibration curve (Fig. 4, mean standard deviation = ±5.9%).

The advantage in using small volumes of extracting solvent (*e.g.*, 0.1 ml. chloroform) *versus* larger volumes is in the reduction of impurities that would normally be extracted with larger volumes (Figs. 2 and 3; note absence of impurities). An additional advantage is that only one centrifuge tube is used in the extraction.

Several examples of urinary levels of I in humans are cited in Table I. The complete results of the clinical trial and kinetic studies in humans will be published at a later date.

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▲ To whom inquiries should be directed.

Synthesis and Pharmacological Activity of 1-(Arylsulfonyl)-3,5-dialkyl-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-triones

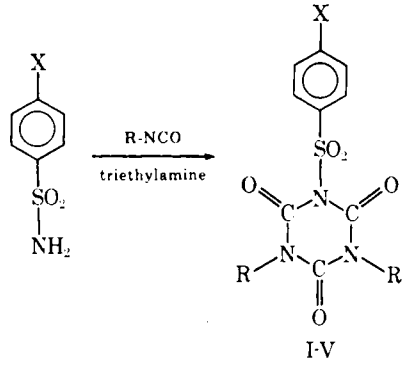
GEORGE N. HOLCOMB*▲, LORRAINE A. KLEMM*, THOMAS J. SILHAVY*‡, and RAYMOND E. COUNSELL†

Abstract □ A series of five 1-(arylsulfonyl)-3,5-dialkyl-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-triones was synthesized by the base-catalyzed reaction of arylsulfonamides with alkyl isocyanates. The compounds were tested for apomorphine antagonism in mice, diuretic activity in fasted rats, antiviral activity in mice, antiviral activity in infected cell cultures, CNS stimulant activity in mice, and hypoglycemic activity in rats. The only biological activity exhibited by these compounds was a slight tendency to lower blood sugar levels in glucose-primed, fasted rats. This activity may be attributable to their structural similarity to the sulfonylurea hypoglycemics.

Keyphrases □ 1-(Arylsulfonyl)-3,5-dialkyl-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-triones—synthesized and screened as potential hypoglycemic agents □ Triazine derivatives—synthesis as potential hypoglycemic agents and screening of 1-(arylsulfonyl)-3,5-dialkyl-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-triones □ Hypoglycemic agents, potential—synthesis and screening of 1-(arylsulfonyl)-3,5-dialkyl-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-triones

Recently it was reported that 1-(*p*-iodobenzenesulfonyl)-3,5-di-*n*-propyl-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-trione (I) is formed when *p*-iodobenzenesulfonamide reacts with an excess of *n*-propyl isocyanate in the presence of triethylamine (1). This represents the first example of a 1-(arylsulfonyl)-3,5-dialkyl-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-trione; however, this compound has certain structural features in common with a number of sulfonamides with known biological activity. Of particular interest in this regard is the structural similarity between I and the sulfonylurea hypoglycemics. In fact, I actually contains a sulfonylurea moiety within its structure.

The sulfonylureas are known to exert a wide variety of extrapancreatic effects (2). However, most investigators currently feel that the primary mode of action of these compounds is the stimulation of insulin release from pancreatic β-cells (3). Because of the structural similarities between I and the sulfonylureas, it seemed quite possible that I could interact with β-cell receptors in much the same way as the sulfonylureas and, thereby, it could be an active hypoglycemic.



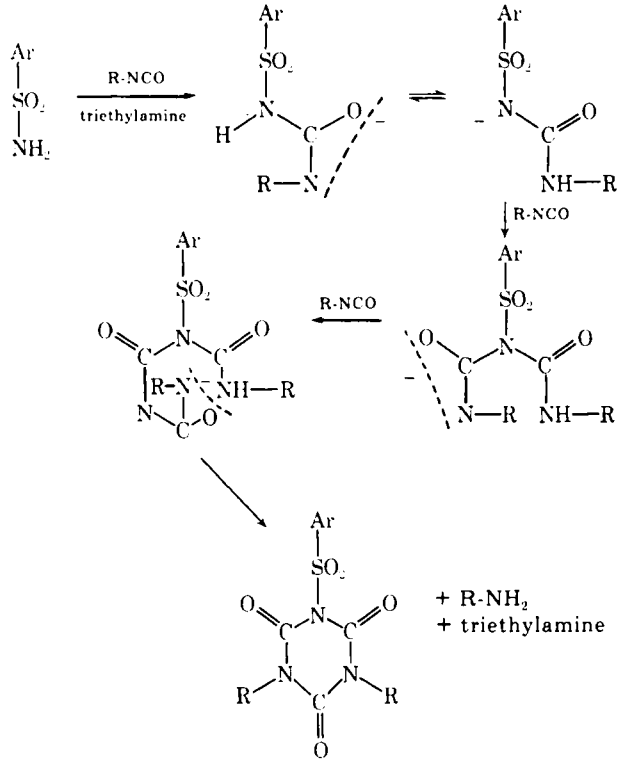
Scheme I

In addition to their hypoglycemic effects, sulfonamides are known to have various other pharmacological activities (4, 5). Some biological effects that have been attributed to sulfonamides are bacteriostatic (6), diuretic (7), hypotensive (8), anti-inflammatory (9), and CNS depressant effects (10).

The present studies were designed to determine if I and certain of its analogs possess sulfonylurea-like hypoglycemic activity and also to ascertain whether these compounds exert other significant biological effects.

The arylsulfonamides were prepared by treating the appropriate sulfonyl chloride with ammonium hydroxide as described in the *Experimental* section. The arylsulfonyl-*s*-triazine derivatives (I–V) were synthesized (Scheme I) by treating the arylsulfonamides with an excess of the appropriate alkyl isocyanate, with triethylamine as the catalyst.

Attempts to synthesize *s*-triazine derivatives from several isocyanates (*tert*-butyl, cyclohexyl, isopropyl, and isobutyl isocyanates) were unsuccessful, thus suggesting that the reaction is subject to steric hindrance. This is in agreement with a previous proposal that the *s*-triazines are formed by the base-catalyzed reaction of the arylsulfonamide with a threefold excess of the alkyl



Scheme II

Table I—Physical Data

Compound	R	X	Melting Point	Formula	Analysis, % Calc.	Found	Yield, %
I	n-C_3H_7	I	186–187°	$C_{15}H_{18}IN_3O_5S$	C 37.59 H 3.79 N 8.77	37.75 3.83 8.83	44
II	n-C_3H_7	Cl	223–224°	$C_{15}H_{18}ClN_3O_5S$	C 46.45 H 4.65 N 11.68	46.49 4.68 11.53	33
III	n-C_3H_7	Br	212–213°	$C_{15}H_{18}BrN_3O_5S$	C 41.67 H 4.17 N 9.72	41.40 4.14 9.88	14
IV	C_2H_5	Cl	215–217°	$C_{13}H_{14}ClN_3O_5S$	C 43.39 H 3.89 N 11.68	43.23 3.81 11.61	26
V	n-C_4H_9	Cl	176–177°	$C_{17}H_{22}ClN_3O_5S$	C 49.10 H 5.29 N 10.11	49.02 5.46 10.14	13

isocyanate, with the subsequent elimination of an alkyl amine (1). These observations are consistent with the mechanism proposed in Scheme II. The transient existence of the anionic intermediates can be justified by the delocalization of the developing charge as proposed by Ulrich (11) for similar reactions.

EXPERIMENTAL[1]

Arylsulfonamides—The appropriate arylsulfonyl chloride (0.085 mole) was dissolved in ether (300 ml.), and ammonium hydroxide (100 ml.) was added to the reaction dropwise over 20 min. The mixture was stirred for 30 min., and 1 *N* NaOH (250 ml.) was added. The aqueous layer was filtered, and the pH of the filtrate was adjusted to 6 with 1 *N* HCl. The arylsulfonamide precipitated on cooling and was isolated by suction filtration. Yields and melting points were as follows: *p*-iodobenzenesulfonamide, 91.4%, 191–192° [lit. (12) m.p. 190–191°]; and *p*-bromobenzenesulfonamide: 92.4%, 169–170° [lit. (12) m.p. 165°].

*1-(p-Iodobenzenesulfonyl)-3,5-di-*n*-propyl-*s*-triazine-2,4,6-(1H,3H,5H)-trione (I)*—This compound was prepared as described previously (1).

*1-(Arylsulfonyl)-3,5-dialkyl-*s*-triazine-2,4,6-(1H,3H,5H)-triones (II-V)*—A mixture of the appropriate arylsulfonamide (0.02 mole), the corresponding alkyl isocyanate (0.20 mole), and triethylamine (1 ml.) was heated at 70° with stirring for 120 hr., and the excess alkyl isocyanate was removed under vacuum. The residual solid was washed with ether (25 ml.) and 1 *N* NaOH (20 ml.) and then recrystallized from ethanol–water. Yields and physical constants are reported in Table I.

Biological—The animals used for hypoglycemic testing were glucose-primed, fasted, pathogen-free, male rats[2]. The test compounds were administered orally in 0.5 ml. of carboxymethylcellulose vehicle. Immediately prior to administration of the test compound, the animals were injected subcutaneously with 100 mg. of glucose. The rats were bled *via* the jugular vein 2 hr. after the injection of the test compound. Blood glucose concentrations were determined[3] by a modification of the method of Hoffman (13).

RESULTS AND DISCUSSION

Although significant hypoglycemic activity was detected in some compounds, they were not as potent as tolbutamide (Table II). Compound I (X = I, R = n-C_3H_7) exhibited the greatest degree of hypoglycemic activity. However, this compound only depressed blood glucose levels by 10.1% when it was administered at a dose of 100 mg./kg., whereas tolbutamide produced a 34.0% decrease in blood sugar when administered at a dose of only 25 mg./kg. Compounds II (X = Cl, R = n-C_3H_7) and IV (X = Cl, R = C_2H_5) exhibited a slight tendency to lower blood sugar levels in glucose-primed, fasted rats. Compounds III (X = Br, R = n-C_3H_7) and V (X = Cl, R = n-C_4H_9) did not exhibit any significant hypoglycemic activity in this test system.

Table II—Hypoglycemic Activities of 1-(Arylsulfonyl)-3,5-dialkyl-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-triones

Treatment	Dose, mg./kg.	Number of Animals	Blood Glucose, mg. %, 2 hr. ±*SE*[a]	Percent Change
Control		8	71.6 ± 1.5	
Tolbutamide	25	4	47.3 ± 0.8	−34.0
I	100	4	64.4 ± 1.6	−10.1
Control		7	70.4 ± 0.8	
Tolbutamide	25	4	49.0 ± 1.0	−30.4
II	100	4	65.8 ± 2.3	−6.6
Control		8	72.1 ± 1.7	
Tolbutamide	25	4	45.3 ± 2.5	−37.1
III	100	4	72.9 ± 2.9	1.2
Control		7	70.4 ± 0.8	
Tolbutamide	25	4	49.0 ± 1.0	−30.4
IV	100	4	65.2 ± 1.5	−7.4
Control		7	74.0 ± 1.5	
Tolbutamide	25	4	49.1 ± 3.3	−33.6
V	100	4	72.4 ± 2.1	−2.2

[a] Standard error of the mean.

The hypoglycemic activity exhibited by some compounds may be attributable to their structural similarity to the sulfonylurea hypoglycemics. No definite reason can be stated for their low order of potency, although it could be due to the presence or absence of certain structural features which interfere with their ability to interact with pancreatic β-cell receptors and stimulate insulin release. Alternatively, the diminished activity of these compounds could be a result of altered absorption and/or distribution characteristics arising from their extremely low aqueous solubility. The limited aqueous solubility observed in these compounds may be attributable to the fact that, unlike most other sulfonamides with demonstrated biological activity, they do not contain an acidic hydrogen.

All five compounds were also tested for apomorphine antagonism in mice, diuretic activity in fasted rats, antiviral activity (Influenza A_2 virus) in mice, antiviral activity in infected cell cultures, CNS stimulant activity in mice, and CNS depressant activity in mice. They did not exhibit significant activity in any of these tests.

[1] All melting points were determined on a Fisher-Johns melting-point apparatus and are corrected. The structures of all compounds were supported by IR and NMR spectra. IR spectra were taken on a Perkin-Elmer 337 spectrophotometer using KBr pellets. NMR spectra were determined on a Varian A-60 spectrometer in $CDCl_3$ at a concentration of 10%, with trimethylsilane as an internal reference. Elemental analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich. Chromatogram strips (K301R) were used for TLC, and the spots were detected with UV light.

[2] Upjohn Sprague-Dawley.

[3] By Auto-Analyzer.

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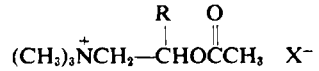
Ketoxime Acetates: Substrates for Cholinesterases

GEORGE M. STEINBERG▲, NORMAN C. THOMAS, MORTON L. MEDNICK, and VERNON BALTHIS

Abstract □ Ketoxime acetates function as typical substrates for acetylcholinesterase. Several 3-pyridinium ketoxime esters have potential utility for spectrophotometric studies with the enzyme. Each is highly water soluble and gives a large change in absorbance upon hydrolysis. With each, the acylation step is rate limiting. They bind to the enzyme strongly; the lowest $K_{m(app)}$ observed is 1.5×10^{-5} *M*. The substrates are hydrolyzed by butyrylcholinesterase. Several show great selectivity for butyrylcholinesterase over acetylcholinesterase.

Keyphrases □ Ketoxime acetates—as cholinesterase (acetyl and butyryl) substrates, binding constants, hydrolysis, absorbance changes, selectivity □ Cholinesterase substrates, acetyl and butyryl —ketoxime acetates, binding constants, hydrolysis, absorbance changes, selectivity □ Acetylcholinesterase—1-alkyl 3-pyridinium ketoxime halide esters as substrates □ Butyrylcholinesterase—1-alkyl 3-pyridinium ketoxime halide esters as substrates

A recent publication (1) reported the activity of aldoxime acetates as substrates for acetylcholinesterase (acetylcholine hydrolase, E.C. 3.1.1.7). The two principal purposes of that study were: (*a*) to gain information on the kinetic specificity and selectivity of the enzyme, and (*b*) to widen the range of spectrophotometrically useful substrates for studying enzyme properties. This note extends the work to ketoxime esters.



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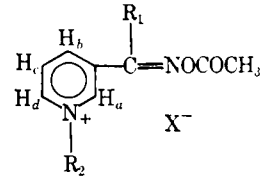
Acetylcholinesterase is generally insensitive to bulking on the alcohol portion of the ester molecule. For example, methacholine (II) is hydrolyzed nearly as rapidly as acetylcholine (I) (2). The reverse pattern is observed with butyrylcholinesterase (acylcholine arylhydrolase, E.C. 3.1.1.8), where bulking near the ester linkage in a test substrate may markedly reduce or even completely abolish response to the enzyme (2). This study was initiated to learn if these characteristics extend to the oxime esters and to determine whether appreciable changes in K_m could be provided by the simple expedient of changing the size (bulk) of the alkyl group attached to the pyridine nitrogen atom.

EXPERIMENTAL

The oxime acetates (III–VII) were prepared in conventional manner by oximation of the corresponding aldehydes or ketones, acetylation with acetic anhydride, and finally alkylation of the pyridine nitrogen atom with methyl iodide or benzyl bromide (3–7).



	R_1	R_2
III:	CH_3	CH_3
IV:	CH_3	$C_6H_5CH_2$
V:	C_2H_5	CH_3
VI:	H	$C_6H_5CH_2$
VII:	acetophenone oxime acetate	

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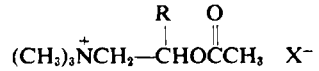

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EXPERIMENTAL

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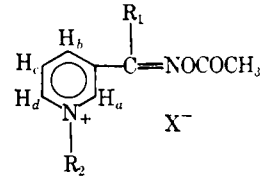

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IV:	CH_3	$C_6H_5CH_2$
V:	C_2H_5	CH_3
VI:	H	$C_6H_5CH_2$
VII:	acetophenone oxime acetate	

Table I—Elemental Analyses

Compound		Analysis, % C	H	Br	I	N	O
III	Calc.	37.52	4.09	—	39.64	8.75	10.00
	Found	37.4	4.2	—	39.5	8.7	10.0
IV	Calc.	55.03	4.91	22.89	—	8.02	9.16
	Found	54.9	5.2	22.8	—	8.2	9.1
V	Calc.	39.53	4.52		37.97	8.38	9.58
	Found	39.7	4.5	—	37.7	8.4	9.4
VI	Calc.	53.74	4.51	23.84	—	8.36	9.54
	Found	53.5	4.5	23.9	—	8.1	9.7
3-Acetylpyridine oxime acetate							
	Calc.	60.66	5.65	—	—	15.72	17.96
	Found	60.4	5.5	—	—	15.7	17.8

NMR spectra[1] of the oxime acetates were determined in approximately 10% solutions containing internal standards. For the aromatic compounds, the solvent was $CDCl_3$ and the standard was tetramethylsilane. For the pyridine derivatives, D_2O was used as the solvent and sodium 3-trimethylsilyl-1-propanesulfonate was the standard. The chemical shifts (δ) reported are in parts per million.

The integration of peaks together with the sharpness of spectra indicates the absence of impurities. Hence, the purity of each compound is greater than 96–97% and *each exists as a single isomer* (1). For several compounds, elemental analyses were performed (C, H, N, O, and halogen, Table I).

Pertinent data[2] on the studied compounds are as follows: acetophenone oxime acetate, m.p. 56 57° [lit. (6) m.p. 55°], with NMR chemical shifts at 2.23 and 2.34 (acetate and C-methyl, respectively); *syn*-benzaldoxime acetate, m.p. < room temperature [lit. (7) m.p. 21°], with an NMR chemical shift at 2.12 (acetate methyl); IV, glass, with NMR chemical shifts at 2.43 (acetate methyl), 5.99 (C-methyl), 7.59 (benzene), 9.48 (H_a), 8.28 (t or 2d, H_c), 8.99 (d), and 9.13 (d, one is H_b and the other H_d); 3-acetylpyridine oxime acetate, m.p. 37.8°, with NMR chemical shifts at 2.36 and 2.39 (acetate methyl and C-methyl, respectively), 7.55 (m, H_c), 8.18 (d, H_b), 8.69 (d, H_d), and 8.85 (H_a); III, m.p. 116–119° dec. [lit. (3) m.p. 115°], with NMR chemical shifts at 2.39 (acetate methyl), 2.60 (C-methyl), 4.55 ($^+$N-methyl), 8.25 (m, H_c), 9.00 (m, H_b and H_d), and 9.35 (H_a); V, m.p. 112–115° dec., with NMR chemical shifts at 1.04 (C-methyl), 2.38 (acetate methyl), 3.08 (m, methylene), 4.54 ($^+$N-methyl), 6.95 (m, H_c), 7.64 and 7.77 (H_b and H_d, respectively), and 8.06 (H_a); and VI, m.p. 126–128°, with NMR chemical shifts at 2.40 (acetate methyl), 6.02 (methylene), 7.65 (benzene), 8.30 (m, H_c), 8.90 (aldehydic proton), 9.10 (m, H_b and H_d), and 9.46 (H_a).

Absorption Spectra[3]—All measurements were made at 25.0 ± 0.1°, pH 6.61, in 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid, using buffer solution in the reference compartment (Table II). The oximes were obtained by enzymatic hydrolysis of the corresponding esters. Oxime configuration probably remains unchanged.

For the difference spectra, the ketoxime esters were placed in the reference beam. Hence, the positive values of $\Delta\epsilon$ represent an increase in absorbance upon hydrolysis. With *syn*-benzaldoxime acetate, the reverse is true; hydrolysis results in a reduction in absorbance. At pH 6.61, the oximes listed in Table II exist in the protonated form, since all have pKa values greater than 9 (3, 4).

Enzyme Kinetics—*Acetylcholinesterase*—Measurements were made spectrophotometrically as previously described (1). The enzyme source[4] and its storage were identical. Runs were made at 25 ± 0.1° in 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid, pH 6.61. Computation of $K_{m(app)}$ and V_{max} (or k_{cat}) was made by computer using the Wilkinson (8) weighted regression method. Estimates of the 95% confidence limits of the computed constants were calculated from their standard errors using the Student *t* table ($n - 2$ degrees of freedom). The number of experimental points taken varied from seven to 15, using a minimum of seven different concentrations of substrate. In each case, double-reciprocal plots (Lineweaver–Burk) gave good straight lines. With Compounds V and VI, the Beer–Lambert relationship was examined and the plots of both substrate and products, in each case, were linear (Table III).

[1] Varian A-60D.
[2] d = doublet, t = triplet, m = multiplet.
[3] Cary model 14.
[4] Worthington Biochemicals, Code ECHP.

Table II—Spectral Characteristics of Oxime Acetates and the Corresponding Oximes

Compound	Acetate λ_{max}, nm.	Acetate ϵ_{max}, M^{-1} cm.$^{-1}$ × 10^{-3}	Oxime λ_{max}, nm.	Oxime ϵ_{max}, M^{-1} cm.$^{-1}$ × 10^{-3}	Difference Spectrum[a] λ_{max}, nm.	Difference Spectrum[a] $\Delta\epsilon_{max}$, M^{-1} cm.$^{-1}$ × 10^{-2}
III	254	5.17	258	6.04	260	8.5
					295	16.8
IV	241	10.2	<240	—	263	11.0
			254	8.87	300	16.9
V	<240	—	<245	—	259	8.0
	268	4.9			297	18.1
VI	248	11.4	252	11.0	263	15.9
					305	15.9
VII	239	13.3	240	14.6	—	—
syn-Benzaldoxime acetate	257	5.8	253	4.8	262	−12.4

[a] Ester is reference beam, $\Delta\epsilon = \Delta A/[\text{substrate}]$.

Butyrylcholinesterase[5]—A standard solution of the enzyme was prepared in potassium chloride-gel (1), which gave one-seventieth the activity of the acetylcholinesterase concentrate (E_w) when assayed with phenyl acetate, 7.71×10^{-3} *M*, at pH 6.61 in the standard buffer. Comparative hydrolysis rates were determined for the two enzymes using appropriately diluted solutions to give the same hydrolysis rate with phenyl acetate.

Phenyl acetate was chosen as the basis of comparison instead of acetylcholine because its hydrolysis could be measured spectrophotometrically. However, the hydrolysis rates of the two substrates by these enzymes are closely similar, so extrapolation to the more conventional acetylcholine can be made[6].

Measurements were made at pH 6.61 in the standard buffer using the concentrations of the substrates indicated in Table IV.

RESULTS AND DISCUSSION

Comparison of the kinetic constants of the 1-methylpyridinium Compounds III and V (Table III) with those of *syn*-3-formyl-1-methylpyridinium iodide oxime acetate, VIII (1), indicates that the bulking methyl and ethyl groups have little effect on either $K_{m(app)}$ or V_{max} for the enzyme acetylcholinesterase. Elimination of the positive charge, as in VII, resulted in an increase in $K_{m(app)}$ without change in V_{max}. Replacement of the 1-methyl group by the larger 1-benzyl group, as in Compounds IV and VI, decreased $K_{m(app)}$ by one order of magnitude and produced a corresponding reduction in V_{max}. The decrease in $K_{m(app)}$ may be attributed to increased hydrophobic binding in the region just beyond the anionic binding site (9). The decrease in V_{max} cannot clearly be attributed to any specific parameters. Speculation on its cause at this point seems unwarranted.

The hydrolytic stability of the pyridinium ketoxime acetate, III, is approximately twice that of the corresponding aldoxime ester, VIII (Table III). The hydrolysis rates of the pairs III and IV and VI and VIII are probably very similar, since the effect of increased bulk at the remote *meta*-position should be very small. Similarly, Compound V, with the bulkier ethyl group in place of the methyl group in Compound III, may be expected to be equal to or even a bit more resistant to hydrolysis than III. Elimination of the charge, as in VII, considerably reduces the hydrolysis rate of this oxime ester.

[5] Worthington horse serum cholinesterase, code CHE.
[6] H. Michel, unpublished. Using acetylcholine, pH 7.4, 0.224 *M* KCl, 0.1% gelatin, 25°, titrimetrically, acetylcholinesterase had a $K_m = 3 \times 10^{-4}$ *M* and $V_{max} = 1.63 \times 10^{-4}$ meq./min. and horse serum cholinesterase had a $K_m = 1.85 \times 10^{-3}$ *M* and $V_{max} = 1.4 \times 10^{-4}$ meq./min. Using phenyl acetate, pH 7.2, 0.01 *M* morpholinopropanesulfonic acid, 0.01 *M* magnesium sulfate, 0.01% gelatin, 10^{-3} *M* Versene, 25°, spectrophotometrically, acetylcholinesterase had a $K_m = 1.8 \times 10^{-3}$ *M* and $V_{max} = 1.4 \times 10^{-4}$ meq./min. and horse serum cholinesterase had a $K_m = 1.6 \times 10^{-3}$ *M* and $V_{max} = 3.2 \times 10^{-4}$ meq./min.

Table III—Ester Hydrolysis by Eel Acetylcholinesterase[a]

Substrate	K_m, $M \times 10^4$	$\frac{V_{max(s)}}{V_{max(p)}} \times 100$[b]	Concentration Range, $M \times 10^4$	λ, nm.	Δε, M^{-1} cm.$^{-1}$ $\times 10^{-3}$	k_{OH^-}, M^{-1} sec.$^{-1}$
III	1.7 (0.54–2.9)	5.0	2.5–22	302.5	1.6	42[c]
IV	0.16 (0.11–0.20)	0.60	0.2–2.6	300	1.69	—
V	2.5	5.1	5–35	300	1.75	—
VI	0.15 (0.10–0.20)	0.76	0.2–4.7	305	1.59	—
VII	25 (11–39)	4.4	7–25	295	0.355	2.46[d]
VIII[e]	1.55	10	—	305	2	80[c]

[a] 25.0°, 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid, pH 6.61. [b] $V_{max(s)}$ and $V_{max(p)}$ refer, respectively, to V_{max} values of the individual substrates and of phenyl acetate. [c] *Reference 3.* [d] *Reference 4.* [e] *Reference 1.*

Table IV—Comparative Hydrolysis Rates: Butyrylcholinesterase *versus* Acetylcholinesterase[a]

Substrate	v_{ChE}/v_{AChE}	Concentration of Substrate, $M \times 10^3$
Phenyl acetate	1.0	7.71
III	20.8	8.35
IV	15.4	6.0
V	6.7	9.03
VI	57	5.89

[a] v_{ChE} and v_{AChE} refer, respectively, to the substrate hydrolysis rates catalyzed by horse serum cholinesterase and eel acetylcholinesterase.

Following the discussion in *Reference 1* for the three-step mechanism involved in substrate hydrolysis by acetylcholinesterase, the data in Table III lead to the conclusions that acylation is the rate-limiting step and that the $K_{m(app)}$ values are closely similar to K_s for the compounds examined here. A comparison of the reaction rates of Compounds III and VII shows that VII, the uncharged ester, reacts equally rapidly with acetylcholinesterase while its reaction rate with hydroxide ion is only one-twentieth that of III. With Compound VII, kinetic specificity is reflected in loss of affinity (increased K_m) and with a relative increase in reaction rate.

The pyridinium ketoxime esters have similar kinetic constants to the corresponding pyridinium aldoxime esters. They are more resistant to aqueous hydrolysis. Furthermore, experience has shown that the ketoximes are substantially more stable during storage than the aldoximes, and it is assumed that this property extends to the oxime esters. Hence, in the absence of other considerations, the ketoxime esters would be preferred.

Table IV indicates the comparative ease of hydrolysis of Compounds III–VI by butyrylcholinesterase and acetylcholinesterase, using phenyl acetate as the reference standard. The oxime acetates are considerably more selective than phenyl acetate as substrates for butyrylcholinesterase. Compound VI is particularly outstanding in this regard, and the corresponding butyrate might be even more useful as a highly selective substrate for serum (butyryl) cholinesterase.

syn-Benzaldoxime acetate is hydrolyzed by acetylcholinesterase. However, the spectra of ester and oxime are quite similar. Thus, at 262 nm., the wavelength of maximum absorbance change, the ester absorbance is very high, $\epsilon = 5.7 \times 10^3$. At longer wavelengths where the ester absorbance falls, $\Delta\epsilon$ falls correspondingly. Thus, this compound is not particularly useful for spectrophotometric purposes and its properties were not studied further.

SUMMARY

1. 3-Substituted 1-alkyl pyridinium ketoxime acetates are useful substrates for acetylcholinesterase.

2. Among the 1-alkyl pyridinium compounds, replacement of the methyl group by the benzyl group decreases $K_{m(app)}$ approximately 10-fold and reduces V_{max} (k_{cat}) to approximately the same extent.

3. The oxime acetates are good substrates for butyrylcholinesterase. 1-Benzyl-3-formylpyridinium halide oxime acetate is hydrolyzed considerably more rapidly by butyrylcholinesterase than by acetylcholinesterase. This compound or the corresponding butyrate might be useful as a highly selective substrate for butyrylcholinesterase.

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Biochemical Interactions of Dimethyl Sulfoxide II: Lactate Dehydrogenase-Catalyzed Reactions

ROBERT L. BEAMER▲, JAMES E. WYNN, JANET R. ROLLINS, and RAFAEL E. LEDESMA*

Abstract □ The effect of dimethyl sulfoxide upon the reactions catalyzed by lactate dehydrogenase was studied. Both the forward (lactate to pyruvate) and the reverse (pyruvate to lactate) reactions were investigated using dimethyl sulfoxide concentrations lower than those used by previous investigators. An aldol-type condensation of dimethyl sulfoxide with pyruvate is proposed as an explanation for the observation that the forward reaction is accelerated and the reverse reaction is slowed by the presence of dimethyl sulfoxide in the reaction medium. A product of the aldol-type condensation was identified in the enzyme reaction medium. The aldol condensation product was synthesized and determined to be 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid.

Keyphrases □ Dimethyl sulfoxide—effect on lactate dehydrogenase-catalyzed reactions, aldol-type condensation with pyruvate proposed, synthesis and identification of condensation product □ Lactate dehydrogenase-catalyzed reactions—effect of dimethyl sulfoxide, 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid proposed as dimethyl sulfoxide–pyruvate aldol-type condensation product and synthesized □ 2-Hydroxy-2-methyl-3-methylsulfinylpropanoic acid—synthesis, proposed as aldol-type condensation product of dimethyl sulfoxide–pyruvate in lactate dehydrogenase-catalyzed reactions □ Enzyme systems—effect of dimethyl sulfoxide on lactate dehydrogenase-catalyzed reactions (lactate to pyruvate and pyruvate to lactate)

In the previous paper of this series, the inhibition of the respiration of rabbit liver homogenates by dimethyl sulfoxide was reported (1). The effects of dimethyl sulfoxide upon isolated enzyme systems have received little attention in the literature. Ramler (2) studied the effects of high concentrations of dimethyl sulfoxide upon glutamate dehydrogenase- and lactate dehydrogenase-catalyzed reactions. He concluded that the observed inhibition resulted from a change in the secondary and tertiary structures of the enzyme protein.

L-Glutamic acid oxidation, catalyzed by glutamate dehydrogenase, was not affected by dimethyl sulfoxide concentrations of 10–20% (2), but there was a rapid lowering of reaction rate with increasing dimethyl sulfoxide concentrations above 10–20%.

L-Alanine also was reported (3) to be a substrate for glutamate dehydrogenase. L-Alanine oxidation, catalyzed by glutamate dehydrogenase, was observed to increase with increasing dimethyl sulfoxide concentrations (2).

The work described in this paper concerns the effects of dimethyl sulfoxide upon lactate dehydrogenase-catalyzed reactions. Ramler (2) showed an increase in the forward reactions (lactate to pyruvate) with dimethyl sulfoxide concentrations up to 30%. Dimethyl sulfoxide concentrations above 30% produced a marked decrease in the reaction rate.

Ramler did not investigate the effects of dimethyl sulfoxide upon the reverse reaction (pyruvate to lactate), nor did he study the effects of dimethyl sulfoxide at the concentration levels usually employed for enzyme inhibitors. His studies were conducted at pH 9 rather than at pH 7.4.

Therefore, it was decided to study the effects of dimethyl sulfoxide upon the lactate dehydrogenase-catalyzed reactions in less concentrated dimethyl sulfoxide solutions (10^{-3}–10^{-1} *M*) than those employed by Ramler. Both the forward and the reverse reactions were studied at pH 7.4. A product study was conducted on the enzyme reaction mixture to help establish the mechanism of the interaction of dimethyl sulfoxide with the lactate dehydrogenase-catalyzed reaction.

EXPERIMENTAL[1]

Reagents—The following were used: experimental drug grade dimethyl sulfoxide[2], redistilled under reduced pressure (100°/2 torr.); sodium lactate parenteral solution[3]; sodium pyruvate[4]; lactate dehydrogenase[5]; nicotinic adenine dinucleotide[6]; and methyl-*n*-propyl sulfide[7].

Enzyme Rate Studies—These studies were carried out using a spectrophotometer[8] equipped with a linear recorder[9] and a constant-temperature chamber set at 37°. The lactate dehydrogenase-catalyzed reactions were studied by assessing the rate of appearance or disappearance of nicotinic adenine dinucleotide by measuring the change in absorbance at 340 nm.

Lactate to Pyruvate Reaction—Studies of the forward reaction were conducted in a 0.06 *M* sodium phosphate buffer at pH 7.4. The initial reaction mixture was 8.0 m*M* in sodium lactate and 2.4 m*M* in nicotinic adenine dinucleotide. The dimethyl sulfoxide concentrations ranged from 0.00 to 10^{-1} *M*. In all studies, a constant lactate dehydrogenase concentration (0.001 mg. protein/ml., representing 0.147 unit of lactate dehydrogenase activity) was maintained. All materials, except the coenzyme, were placed in a cell and allowed to equilibrate to 37°. After 3 min., the reaction was initiated by adding the coenzyme. The coenzyme solution was added to the bottom of the cell and air was introduced to ensure mixing. The volume of each cell was 3 ml. Reaction rates were determined by comparing the absorbance at the end of 1 min. with standards of known concentrations.

Pyruvate to Lactate Reaction—The reverse reaction was run in a similar manner to that described for the forward reaction. The reaction was carried out in a 0.06 *M* sodium phosphate solution buffered at pH 7.4 and was 2 m*M* in sodium pyruvate and 0.6 m*M* in reduced nicotinic adenine dinucleotide; the enzyme concentration was 0.008 mg. protein/ml. (0.840 unit of lactate dehydrogenase activity).

Product Study—*Isolation of Aldol-Type Condensation Product from Enzyme Reaction*—In each of five 50-ml. conical flasks were placed 0.0032 ml. of an 8 m*M* sodium lactate solution, 5.27 ml. (0.1 *M*) of dimethyl sulfoxide, 0.001 ml. (0.003 mg. protein) of lactate dehydrogenase, and 5.63 ml. of 0.06 *M* sodium phosphate

[1] IR spectra were determined with a Beckman IR 20A. NMR spectra were determined using a Varian A60 NMR spectrometer. Carbon–hydrogen analyses were determined using a Hewlett-Packard model 185 C, H, and N analyzer. GC data were obtained using a Beckman GC-2A gas chromatograph. All temperatures are uncorrected.
[2] Crown Zellerbach Corp.
[3] Abbott Laboratories, Lot 825-0808.
[4] Nutritional Biochemicals Corp., Lot 2171.
[5] Worthington Biochemical Corp.
[6] Nutritional Biochemicals Corp.
[7] Aldrich Chemical Co.
[8] Beckman DB.
[9] Bausch & Lomb VOM 7.

Table I—Rates of the Lactate to Pyruvate Reaction

Dimethyl Sulfoxide Concentration, *M*	Mean Rate, mmoles/min.	Standard Error[a] (±)	*p*[b]
0.00	4.8×10^{-2}	7.8×10^{-4}	—
10^{-3}	5.0×10^{-2}	5.5×10^{-4}	0.038
10^{-2}	5.5×10^{-2}	7.5×10^{-4}	<0.0005
10^{-1}	5.9×10^{-2}	1.2×10^{-3}	<0.0005

[a] Degrees of freedom ≥ 40. [b] A one-tailed *t*-test was used.

Table II—Rates of the Pyruvate to Lactate Reaction

Dimethyl Sulfoxide Concentration, *M*	Mean Rate, mmoles/min.	Standard Error[a] (±)	*p*[b]
0.00	3.72×10^{-4}	1.88×10^{-5}	—
10^{-3}	1.90×10^{-4}	1.25×10^{-5}	<0.0005
10^{-2}	1.01×10^{-4}	4.10×10^{-6}	<0.0005
10^{-1}	0.31×10^{-4}	5.50×10^{-6}	<0.005

[a] Degrees of freedom ≥ 40. [b] A one-tailed *t*-test was used.

buffer, pH 7.4. The flasks were placed in a shaker water bath at 37° and allowed to equilibrate for 5 min. The reaction was started by adding 16 mg. of nicotinic adenine dinucleotide from a 2.4 m*M* solution. The reaction was run for 4 days, after which the flask contents were pooled, cooled in an ice bath, and adjusted to pH 1.4 with cold 15% sulfuric acid. The acidified mixture was extracted with ether in a continuous extractor for 48 hr. The extract was dried over anhydrous magnesium sulfate and the ether was removed *in vacuo*, leaving a small quantity of liquid residue which was subjected to TLC.

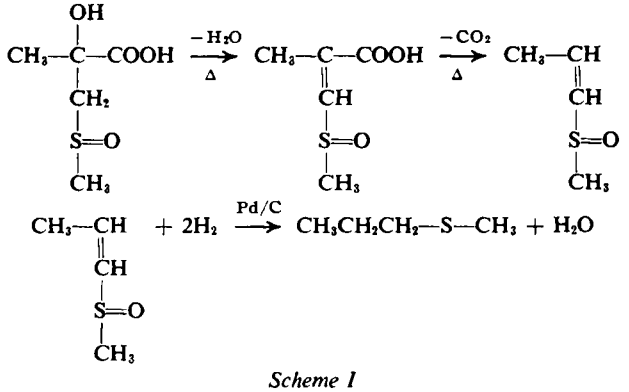
TLC—Ten microliters of the lactate to pyruvate reaction mixture or the residue from the continuous ether extraction was removed and applied to TLC plates[10]. The plates were developed using methanol–chloroform (75:25). The dried plates were treated with iodine vapors for visualization of the spots.

Synthesis of Proposed Intermediate (2-Hydroxy-2-methyl-3-methylsulfinylpropanoic Acid)—To 0.073 mole of potassium pyruvate or sodium pyruvate in 60 ml. of distilled water was added 9.33 ml. (10.30 g., 0.132 mole) of dimethyl sulfoxide. The pH of the mixture was adjusted to 7.5 using a 0.4 *N* sodium hydroxide solution, and the mixture was stirred at room temperature for 24 hr. Following the reaction period, the pH of the mixture fell to 6.5–7.0. The reaction mixture was cooled in an ice water bath to 0°, acidified to pH 2 with cold 15% sulfuric acid, and extracted with ether in a continuous extractor for 48 hr. The extract was dried over anhydrous magnesium sulfate for 48 hr. The ether was removed *in vacuo*, leaving 9.10 g. (75%) of a colorless to light-yellow corrosive liquid possessing a sour odor, b.p. 58°/0.6 torr. IR (neat on sodium chloride cells): 3400 (OH), 2900 [CH(sh)], 1650 (COOH carbonyl), and 1025 [—(O=)S—] cm.$^{-1}$; NMR (60 MHz., dimethyl sulfoxide-d_6): δ 2.6 (s, 3), 4.35 (m, 5), 1.9 (broad, 1), and 7.35 (broad, 1).

Anal.—Calc. for $C_5H_{10}O_4S$: C, 36.14; H, 6.07. Found: C, 36.10; H, 6.04.

The 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid was subjected to TLC as described, and the chromatogram was compared with that from the enzyme reaction mixture.

Chemical Degradation of 2-Hydroxy-2-methyl-3-methylsulfinylpropanoic Acid—Chemical degradation of the product of the aldol-type condensation was studied according to Scheme I. A solution of 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid (0.02 mole in toluene) was placed in a flask equipped with a Dean-Starke trap and a condenser. The top of the condenser was fitted with a delivery tube leading to a saturated solution of barium hydroxide. The toluene solution was refluxed overnight. A copious precipitate of barium carbonate formed in the barium hydroxide solution, and 0.5 ml. (0.0278 mole) of water was collected in the Dean-Starke trap.



Scheme I

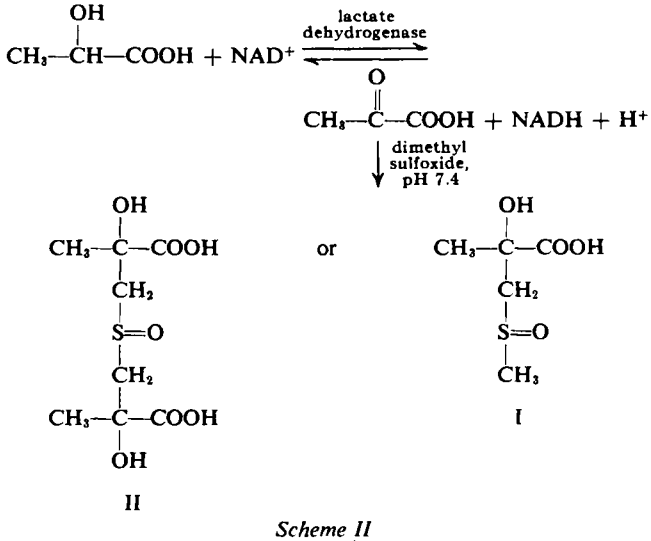
A second degradation was conducted using 0.06 mole of the 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid in 25 ml. of absolute ethanol. To this solution was added 2 drops of concentrated sulfuric acid. The solution was refluxed for 14 hr. under a nitrogen atmosphere, and the reaction was followed using the barium hydroxide solution described previously.

Following the reaction, the total mixture was hydrogenated on a low-pressure Parr hydrogenator at an initial hydrogen pressure of 65 p.s.i. and using 1 g. of palladium-on-charcoal (100 mg. palladous chloride/1 g. of charcoal) as the catalyst. After 12 hr. of hydrogenation, an additional 1 g. of catalyst was added and the hydrogenation was continued for another 12 hr. The catalyst was removed by filtration and the solution was distilled. The impure methyl-*n*-propyl sulfide fraction was collected at 32°/20 torr.

The methylpropyl sulfide fraction was subjected to vapor phase chromatography using a 1.8-m. (6-ft.) metal column packed with 1.15% silicone rubber SE-30 on Gas Chrom P (100–140 mesh). A thermal conductivity detector was employed. The helium carrier gas was maintained at a flow rate of 20 ml./min., and the column temperature was 70°. The sample volume was 10 μl. The retention time of the peak identified as methyl-*n*-propyl sulfide was 6.5 min. and corresponded to that of a commercial sample determined under identical conditions. An IR spectrum of the hydrogenation product was identical with that of commercial methyl-*n*-propyl sulfide containing 1 ml. of 95% ethanol/100 ml. of sulfide.

RESULTS

The rates of the forward reaction in the presence of varying concentrations of dimethyl sulfoxide are presented in Table I. The rates of the reverse reaction in the presence of varying concentrations of dimethyl sulfoxide are concentration dependent. This concentration dependence is statistically significant (Table II).



Scheme II

[10] Absorbasil-3, Applied Science.

TLC showed the presence of a compound in the enzyme reaction mixture and the ether extract which was neither lactate nor pyruvate nor dimethyl sulfoxide. The R_f value for this compound was identical with that for 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid. No intermediate spot could be detected in the absence of dimethyl sulfoxide in the reaction medium.

DISCUSSION

Baker *et al.* (4) showed that the lactate dehydrogenase reaction sites are the same. Therefore, inhibition at the reaction site should decrease the rates of both the forward and the reverse reactions. The increase in the forward reaction rate and the decrease in the rate of the reverse reaction could be explained by the presence of a concentration gradient on the reversible reaction in a direction favoring pyruvate formation. Such a concentration gradient could result from a complexation or condensation of dimethyl sulfoxide with pyruvic acid, thus removing that species from the reaction medium. A possible reaction that could remove effectively the keto acid salt (pyruvate) is an aldol-type condensation between the methyl groups of dimethyl sulfoxide and the carbonyl function of the pyruvate. Such a condensation may involve either a single methyl group or both methyl groups of the dimethyl sulfoxide (Scheme II). The effect on the enzyme-catalyzed reaction would be concentration dependent (Tables I and II).

The presence of 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid in the enzyme mixture leads to the conclusion that dimethyl sulfoxide interferes with the lactate dehydrogenase-catalyzed reaction by condensing with pyruvate in an aldol-type condensation.

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Cinchona Alkaloids on Ion-Exchange Resins V: Ammonium Form of Resins

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Abstract □ Equilibrium exchange of cinchona alkaloid sulfates and column runs of cinchona bark were carried out with the ammonium form of sulfonic acid cation-exchange resins. The results indicate that, for 100 meq. of the resin capacity, the amounts of the alkaloid-containing material extracted from cinchona bark are 15 and 8 g. for the ammonium forms of two commercially available ion-exchange resins. Dilute aqueous sulfuric acid was used as the extraction solvent, and ammonical ethanol was used as the eluant.

Keyphrases □ Cinchona alkaloids—separation from cinchona bark using ion-exchange resins in ammonium form, comparison of two commercially available resins, separation capacity □ Alkaloids, cinchona—separation from cinchona bark using ion-exchange resins in ammonium form, comparison of two commercially available resins, separation capacity □ Resins, ion exchange, in ammonium forms—comparison of separation capacities of two commercially available resins, cinchona alkaloids □ Ion-exchange chromatography—comparison of separation capacities of two commercially available resins, cinchona alkaloids □ Chromatography, ion exchange—comparison of separation capacities of two commercially available resins, cinchona alkaloids

A previous paper (1) reported the effect of the particle size of styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins on equilibrium exchange and column studies for cinchona alkaloid sulfates. The work suggested that a fine mesh resin with a relatively low degree of cross-linking (Resin I[1]) or with an expanded structure (Resin II[2]) in ammonium form could be suitable for recovery of cinchona alkaloids, with aqueous sulfuric acid as the solvent for the exchange run and ammoniacal ethanol as the solvent for the elution run. The present work includes a study of equilibrium exchange of cinchona alkaloid sulfates and column runs of cinchona bark with the ammonium form of sulfonic acid cation-exchange resins.

EXPERIMENTAL

Resins—The resins were conditioned and converted into hydrogen form. The experimentally estimated values of percent moisture content and capacity in milliequivalents per gram of air-dried resin were: Resin I (50–100), 24.0, 3.89; Resin I (100–200), 22.0, 3.72; and Resin II (20–50), 26.0, 3.53.

The ammonium form of the resin was prepared by treating the hydrogen form with excess aqueous ammonia. The moisture content of the air-dried resin in the ammonium form was estimated, and its capacity was calculated by taking the oven-dried capacity of the resin in hydrogen and ammonium forms to be the same.

Chemicals and Materials—Chemicals were from earlier stocks. Cinchona bark[3] was powdered below 60 mesh, and samples were estimated for alkaloid content (2). The value was 6.5%. Samples of extracted alkaloid material were analyzed (3), and the mixture of quinine and cinchonidine obtained was estimated for each by UV absorption (4). The percent analysis was: quinine, 18.0; quini-

[1] Dowex 50W-X4 (50–100, 100–200).

[2] Amberlite-200 (20–50).

[3] Obtained from the Government Quinine Factory, West Bengal, India.

TLC showed the presence of a compound in the enzyme reaction mixture and the ether extract which was neither lactate nor pyruvate nor dimethyl sulfoxide. The R_f value for this compound was identical with that for 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid. No intermediate spot could be detected in the absence of dimethyl sulfoxide in the reaction medium.

DISCUSSION

Baker *et al.* (4) showed that the lactate dehydrogenase reaction sites are the same. Therefore, inhibition at the reaction site should decrease the rates of both the forward and the reverse reactions. The increase in the forward reaction rate and the decrease in the rate of the reverse reaction could be explained by the presence of a concentration gradient on the reversible reaction in a direction favoring pyruvate formation. Such a concentration gradient could result from a complexation or condensation of dimethyl sulfoxide with pyruvic acid, thus removing that species from the reaction medium. A possible reaction that could remove effectively the keto acid salt (pyruvate) is an aldol-type condensation between the methyl groups of dimethyl sulfoxide and the carbonyl function of the pyruvate. Such a condensation may involve either a single methyl group or both methyl groups of the dimethyl sulfoxide (Scheme II). The effect on the enzyme-catalyzed reaction would be concentration dependent (Tables I and II).

The presence of 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid in the enzyme mixture leads to the conclusion that dimethyl sulfoxide interferes with the lactate dehydrogenase-catalyzed reaction by condensing with pyruvate in an aldol-type condensation.

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Cinchona Alkaloids on Ion-Exchange Resins V: Ammonium Form of Resins

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Abstract □ Equilibrium exchange of cinchona alkaloid sulfates and column runs of cinchona bark were carried out with the ammonium form of sulfonic acid cation-exchange resins. The results indicate that, for 100 meq. of the resin capacity, the amounts of the alkaloid-containing material extracted from cinchona bark are 15 and 8 g. for the ammonium forms of two commercially available ion-exchange resins. Dilute aqueous sulfuric acid was used as the extraction solvent, and ammonical ethanol was used as the eluant.

Keyphrases □ Cinchona alkaloids—separation from cinchona bark using ion-exchange resins in ammonium form, comparison of two commercially available resins, separation capacity □ Alkaloids, cinchona—separation from cinchona bark using ion-exchange resins in ammonium form, comparison of two commercially available resins, separation capacity □ Resins, ion exchange, in ammonium forms—comparison of separation capacities of two commercially available resins, cinchona alkaloids □ Ion-exchange chromatography—comparison of separation capacities of two commercially available resins, cinchona alkaloids □ Chromatography, ion exchange—comparison of separation capacities of two commercially available resins, cinchona alkaloids

A previous paper (1) reported the effect of the particle size of styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins on equilibrium exchange and column studies for cinchona alkaloid sulfates. The work suggested that a fine mesh resin with a relatively low degree of cross-linking (Resin I[1]) or with an expanded structure (Resin II[2]) in ammonium form could be suitable for recovery of cinchona alkaloids, with aqueous sulfuric acid as the solvent for the exchange run and ammoniacal ethanol as the solvent for the elution run. The present work includes a study of equilibrium exchange of cinchona alkaloid sulfates and column runs of cinchona bark with the ammonium form of sulfonic acid cation-exchange resins.

EXPERIMENTAL

Resins—The resins were conditioned and converted into hydrogen form. The experimentally estimated values of percent moisture content and capacity in milliequivalents per gram of air-dried resin were: Resin I (50–100), 24.0, 3.89; Resin I (100–200), 22.0, 3.72; and Resin II (20–50), 26.0, 3.53.

The ammonium form of the resin was prepared by treating the hydrogen form with excess aqueous ammonia. The moisture content of the air-dried resin in the ammonium form was estimated, and its capacity was calculated by taking the oven-dried capacity of the resin in hydrogen and ammonium forms to be the same.

Chemicals and Materials—Chemicals were from earlier stocks. Cinchona bark[3] was powdered below 60 mesh, and samples were estimated for alkaloid content (2). The value was 6.5%. Samples of extracted alkaloid material were analyzed (3), and the mixture of quinine and cinchonidine obtained was estimated for each by UV absorption (4). The percent analysis was: quinine, 18.0; quini-

[1] Dowex 50W-X4 (50–100, 100–200).

[2] Amberlite-200 (20–50).

[3] Obtained from the Government Quinine Factory, West Bengal, India.

Table I—Equilibrium Exchange[a] of Aqueous Quinine Sulfate and Cinchonidine Sulfate with Cation-Exchange Resins in Ammonium Form

Resin	Quinine Sulfate			Cinchonidine Sulfate		
	$[\bar{A}]_e$	P_A	P_R	$[\bar{A}]_e$	P_A	P_R
Resin I (50–100)	0.49	20.5	49.0	0.45	21.0	68.1
	0.79	33.5	50.1	0.80	37.5	68.8
	1.30	55.0	51.4	1.26	59.0	69.3
	1.74	73.5	52.1	1.62	76.0	69.6
Resin II (20–50)	0.63	28.5	26.3	0.52	25.0	29.0
	0.89	40.5	25.3	0.76	37.0	27.4
	1.29	58.5	23.9	1.10	53.5	25.1
	1.76	80.0	22.6	1.56	76.0	22.3

[a] $[\bar{A}]_e$ = Milliequivalents of alkaloid in the resin phase per liter of solution, at equilibrium; P_A = percent exchange of alkaloid sulfate at equilibrium; and P_R = percent resin capacity exchanged at equilibrium. Effective capacity = (capacity of the resin) (average value of P_R).

dine, 17.5; cinchonine, 29.0; cinchonidine, 9.0; and the rest (by difference), 26.5.

Procedure—The procedure for equilibrium exchange of cinchona alkaloid (quinine, quinidine, cinchonine, and cinchonidine) sulfates with ammonium forms of Resin I (50–100) and Resin II (20–50) was same as given earlier (5, 6).

Columns, each containing 100 meq. of the resin in the ammonium form, were set up (1).

The values of bed length (cm.) and bed volume (ml.) were: for Resin I (100–200), 44.1, 75.0; and for Resin II (20–50), 35.0, 59.5.

The procedure for carrying out a run was as follows. A weighed amount of the powdered cinchona bark was stirred with 0.01 *N* aqueous sulfuric acid solution (4 l.), allowed to stand overnight, and filtered. The filtrate was transferred to an overhead aspirator and passed through the column at a flow rate of 10 ml./min. The effluent was collected, and its acid strength was adjusted to about 0.01 *N* by pH measurement; then it was returned to the container of bark, stirred well, and filtered. The filtrate was transferred to the overhead aspirator and thus the run was continued. The run was discontinued when the alkaloids were almost completely extracted from cinchona bark, as indicated by absence of fluorescence, or when the alkaloid content of the effluent and influent was almost same, as indicated by fluorescence when exposed to UV radiation. Then the column was washed with distilled water and backwashed to remove extraneous materials from the resin bed. Fifty percent aqueous ethanol (50 ml.) was passed through the column.

The alkaloid material was eluted with 0.1 *N* ammoniacal ethanol at a flow rate of 1.5 ml./min. The effluent containing alkaloids was concentrated by distillation, evaporated on a water bath, dried at 100 ± 1°, weighed, and analyzed.

The resin in the column was then replaced by an equivalent amount of resin from stock, backwashed, and allowed to settle under gravity. The column then was ready for the next run.

RESULTS AND CONCLUSIONS

Equilibrium Exchange Study—The equilibrium exchange study was carried out for the cinchona alkaloid (quinine, quinidine, cinchonine, and cinchonidine) sulfates with Resin I (50–100) and Resin II (20–50) in the ammonium form. Table I gives the results for quinine sulfate and cinchonidine sulfate. From the results obtained, it may be concluded that:

Table II—Data for Column Runs with Cinchona Bark

	Resin I	Resin II
Weight, g.[a]	1000	357
Amount of acid[b] solution passed, l.	44	24
Amount of eluant[c] used, l.	6	3
Weight of extracted material, g.	15	8

[a] Weight (grams) = weight (grams) of cinchona bark taken for the run. [b] Acid = 0.01 *N* sulfuric acid. [c] Eluant = 0.1 *N* ammoniacal ethanol.

1. The value of P_R (the percent resin capacity exchanged at equilibrium) for the optical isomers (quinine and quinidine or cinchonine and cinchonidine) is not measurably different.
2. The value of P_R for quinine sulfate is lower than that of cinchonidine sulfate.
3. The value of P_R for Resin II decreases as the value of P_A (the percent exchange of alkaloid sulfate at equilibrium) increases.

These results are similar to those obtained earlier (5, 6).

Column Study—Runs were carried out starting with different amounts of powdered cinchona bark. From the results obtained, it is concluded that:

1. When the alkaloid content (milliequivalents) of the powdered cinchona bark is lower than the capacity (milliequivalents) of the resin in the column, the alkaloid material present in the bark is extracted.
2. When the alkaloid content of the powdered cinchona bark is much greater than the capacity of the resin in the column, the amount of extracted alkaloid-containing material is 15 g. for Resin I and 8 g. for Resin II (capacity of the resin in the column being 100 meq.). Thus, the amount of the alkaloid-containing material obtained is close to the effective capacity of the column (7). Table II gives the data for runs with excess powdered cinchona bark.

The results indicate that for the extraction of alkaloids from cinchona bark with the ammonium form of the resin, dilute aqueous sulfuric acid may be used as the extraction solvent and ammoniacal ethanol may be used as the eluent. It would be desirable to assess the stability of the resins for repeated use over time.

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Clemmensen Reduction of Mannich Bases: Potential Ganglionic and Nicotinic Agents

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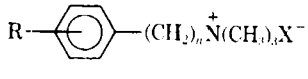
Abstract □ An attempt was made to prepare *p*-aminophenylpropyltrimethylammonium iodide (a potential ganglionic stimulant) by a route involving the Clemmensen reduction of 3-dimethylamino-1-*p*-nitrophenylpropan-1-one. The main product was the *trans*-isomer of 1-*p*-aminophenyl-3-dimethylaminoprop-1-ene, but this could be converted to the required trimethylammonium derivative by hydrogenation and methylation. The Clemmensen reduction of other Mannich bases also was investigated. 3-Dimethylamino-1-arylprop-1-enes are of particular interest because of the reported nicotinic activity of the methosalts. Several products were formed, however, including an azeotropic mixture of the aminoalkane and the aminoalkene. The azeotrope could be converted smoothly to the pure 1-aryl-3-dimethylaminopropane by catalytic hydrogenation. This route can, therefore, be used to prepare 1-arylpropyltrimethylammonium halides, which are potentially nicotine-like. Other methods for reducing the carbonyl group of Mannich bases completely are discussed.

Keyphrases □ *p*-Aminophenylpropyltrimethylammonium iodide—synthesis and screening as a potential ganglionic stimulant □ Ganglionic stimulants, potential—synthesis and screening of *p*-aminophenylpropyltrimethylammonium iodide, Clemmensen reduction of Mannich bases □ Clemmensen reduction of Mannich bases—synthesis of potential ganglionic and nicotinic agents □ Mannich bases—use of Clemmensen reduction to synthesize potential ganglionic and nicotinic agents

Some aralkyltrimethylammonium salts (I) were highly active on the isolated frog rectus abdominis muscle (1). The most active agonists were *m*-hydroxyphenethyltrimethylammonium iodide (leptodactyline iodide) (I*a*, $n = 2$), *m*-hydroxyphenylpropyltrimethylammonium bromide (I*a*, $n = 3$), and *p*-aminophenethyltrimethylammonium iodide (I*b*, $n = 3$), which were 20–50 times as potent as nicotine on this preparation. *p*-Aminophenethyltrimethylammonium iodide was found later to be the most specific as a ganglionic stimulant (2), and it was decided to prepare *p*-aminophenylpropyltrimethylammonium iodide for pharmacological evaluation. A number of arylpropenyltrimethylammonium halides (VI), which are structurally similar to the aralkyltrimethylammonium series (I), also were reported (3, 4) to possess high nicotinic activity.

DISCUSSION

A three-stage synthesis was designed (II*a* → III*b* → I*b*, $n = 3$) to prepare *p*-aminophenylpropyltrimethylammonium iodide (Scheme I). The first step involved the Clemmensen reduction (5) of 3-dimethylamino-1-*p*-nitrophenylpropan-1-one (II*a*) to the corresponding *p*-aminophenylpropylamine (III*b*). This produced the *trans*-*p*-aminophenylpropenylamine (IV*b*) in 24% yield, together with a large quantity of intractable material. The product (IV*b*) was identified by its PMR spectrum, which showed a vinylic multiplet (ABX_2, $J_{AB} = 16$ Hz.) characteristic of *trans*-1-aryl-3-tertiary-aminoprop-1-enes (6). Catalytic hydrogenation of IV*b* gave 1-*p*-aminophenyl-3-dimethylaminopropane (III*b*), from which *p*-aminophenylpropyltrimethylammonium iodide (I*b*, $n = 3$) was prepared.

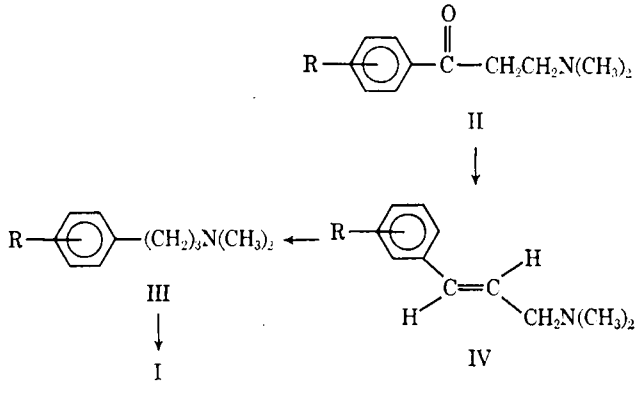


I*a*: R = *m*-OH
I*b*: R = *p*-NH$_2$

The formation of olefinic by-products in the Clemmensen reaction is well established (7), but there appears to be no report relating to the reduction of β-aminoketones (II). The isolation of *trans*-1-*p*-aminophenyl-3-dimethylaminoprop-1-ene (IV*b*) from the reaction, however, was of much interest, because the quaternary methiodide derivatives (VI) of such aminoalkenes have been reported (3, 4) to be highly active nicotinic agents. It was, therefore, decided to see whether aminoalkenes were consistently formed with other Mannich bases (Table I), three of which (II*c*, II*d*, and II*e*) were studied in detail. The Clemmensen reduction gave two products in low yield[1]: (*a*) an azeotropic mixture of the appropriate aminoalkane[2] (III) and aminoalkene[2] (IV), and (*b*) a high boiling liquid which could be crystallized. The solid product appeared to be a mixture of the *meso*- and *dl*-forms of the dimer[3] (V) and was probably formed by the addition of two electrons and protons from the Clemmensen reagent (8) to the Mannich base.

With the three other β-aminoketones (II*f*, II*g*, and II*h*), no attempt was made to isolate the corresponding dimers. Only the low boiling distillate was collected, and it was again found to be a mixture of aminoalkane and aminoalkene[2]. The formation of pure aminoalkene (IV*b*) in the initial reaction seems to depend on the presence of the nitro group, which may prevent complete reduction. The exact mechanism of the Clemmensen reduction of β-aminoketones is obscure, but the formation of aminoalkenes may involve a monomeric alcohol[4] intermediate (7).

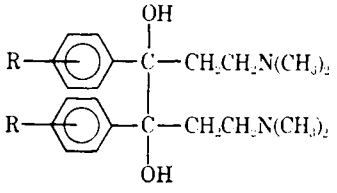
The carbonyl group in Mannich bases appears to be very difficult to reduce to methylene; treatment of both the phenyl and *o*-tolyl aminoketones with the lithium aluminum hydride–aluminum



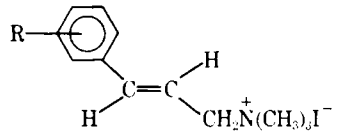
II–IV *a*: R = *p*-NO$_2$
b: R = *p*-NH$_2$
c: R = H
d: R = *p*-F
e: R = *p*-Cl
f: R = *m*-CF$_3$
g: R = *o*-CH$_3$
h: R = *p*-CH$_3$

Scheme I

[1] A large amount of intractable resinous material was evident after distillation of each crude Clemmensen product.
[2] Proton magnetic resonance evidence.
[3] Mass spectral total ion-current evidence.
[4] When one reaction with II*c* was terminated by basification after 3 hr., a sample of 3-dimethylamino-1-phenylpropan-1-ol hydrochloride (3%) was isolated, m.p. 135°, identical to authentic material (6), but the ratio of the other products remained unchanged.



V



VI

chloride reagent, which has been suggested as an alternative to the Clemmensen reduction (15), gave only the corresponding secondary alcohols. Both the carbonyl and the *p*-nitro groups of the Mannich base (II*a*) could be reduced, however, when the compound was suspended in ethanolic hydrogen chloride and hydrogenated at room temperature and atmospheric pressure for 14 days with palladium-on-carbon as catalyst[5]. This method provided another route to *p*-aminophenylpropyltrimethylammonium iodide (I*b*, n = 3).

PHARMACOLOGY

p-Aminophenylpropyltrimethylammonium iodide showed no hypotensive effects in the pithed rat and acted as a weak blocker[6]. It was only one-tenth as active as *p*-aminophenethyltrimethylammonium iodide (I*b*, n = 2) when tested for ganglionic stimulant activity on the isolated guinea pig ileum.

EXPERIMENTAL[7]

β-Aminoketone Hydrochlorides (II)—The hydrochloride salts of II*a*–II*h* were prepared by the usual method (16) from the appropriately substituted acetophenones. The melting points of II*a* (17), II*c* (9), II*d* (10), II*e* (11), II*f* (12), II*g* (13), and II*h* (14) were in agreement with literature values.

***trans*-1-*p*-Aminophenyl-3-dimethylaminoprop-1-ene (IV*b*)**—A solution of mercuric chloride (5 g.) in 10% HCl (100 ml.) was shaken (5 min.) with granulated zinc (60 g.) as previously described (5), and the amalgam was rinsed with water. 3-Dimethylamino-1-*p*-nitrophenylpropan-1-one hydrochloride (17) (II*a*) (38 g., 0.147 mole) was dissolved in 35% HCl (150 ml.), and the solution was added dropwise to the amalgam. The vigorous reaction was kept under control by cooling under the tap before a further portion of 35% HCl (100 ml.) was added and the mixture was refluxed overnight. The solution was basified with enough ammonium hydroxide to redissolve the zinc hydroxide precipitate and was then extracted with ether and dried (sodium sulfate). Evaporation of the extracts gave an oil (14 g.) which, when distilled *via* a Vigreux head, gave 5.4 g. (24%) of the product, b.p. 121° (0.35 mm.), n_D^{25} = 1.5960. The proton magnetic resonance (PMR) spectrum ($CDCl_3$) agreed well with that of the parent alkene (IV*c*) (6). The product formed an unstable *methiodide*, which formed a light-sensitive *hydrogen iodide*, m.p. 215–217° (methanol–ether), λ_{max} 282 nm. (ε 21,900).

Anal.—Calc. for $C_{12}H_{22}I_2N_2$: C, 32.04; H, 4.69; N, 6.28. Found: C, 32.30; H, 4.51; N, 6.28.

***p*-Aminophenylpropyltrimethylammonium Iodide (I*b*, n = 3)**—The aminoalkene (IV*b*) (1 g.) was dissolved in a mixture of ethanol and 10% palladium-on-carbon (0.4 g.) and hydrogenated overnight at room temperature and atmospheric pressure. The reaction mixture was filtered (kieselguhr), and the filtrate was evaporated to a small volume. After the addition of methyl iodide (2 ml.) and a few drops of ether, the product separated as gold needles (1.2 g.), m.p. 204–205°; PMR (D_2O): vinylic signals absent.

Anal.—Calc. for $C_{12}H_{21}IN_2$: C, 45.01; H, 6.61. Found: C, 44.79; H, 6.83.

Other Clemmensen Reductions—These were carried out in the manner described for IV*b*. Distillation of the crude products derived from II*c*, II*d*, and II*e* gave two fractions (Table I) with the following characteristics:

A mixture of III*c* and IV*c*, b.p. 62° (0.20 mm.), and the isomeric *dimer* V*c*, b.p. 168° (0.25 mm.), m.p. 148–150° (ethanol); M^+ at *m/e* 356 ($C_{22}H_{32}N_2O_2$) (both isomers).

Anal.—Calc. for $C_{22}H_{32}N_2O_2$: C, 74.12; H, 9.05. Found: C, 74.22; H, 8.89.

A mixture of III*d* and IV*d*, b.p. 66–68° (0.20 mm.), and the isomeric *dimer* V*d*, b.p. 168–169° (0.30 mm.), m.p. 170–172° (ethanol); M^+ at *m/e* 392 ($C_{22}H_{30}F_2N_2O_2$) (both isomers).

Anal.—Calc. for $C_{22}H_{30}F_2N_2O_2$: C, 67.32; H, 7.70. Found: C, 67.58; H, 7.48.

A mixture of III*e* and IV*e*, b.p. 80–82° (0.10 mm.), and the isomeric *dimer* V*e*, b.p. 230° (0.20 mm.), m.p. 198–200° (ethanol); M^+ at *m/e* 425/427 ($C_{22}H_{30}Cl_2N_2O_2$) (both isomers).

Anal.—Calc. for $C_{22}H_{30}Cl_2N_2O_2$: C, 62.11; H, 7.11. Found: C, 62.22; H, 7.09.

The aminoalkene–aminoalkane mixtures from the *m*-CF_3, *o*-CH_3, and *p*-CH_3 Mannich bases (II*f*, II*g*, and II*h*, respectively) were collected at ~70° (0.20 mm.) in various ratios (Table I).

In all the reductions specified, the PMR spectra ($CDCl_3$ and tetramethylsilane) of the low boiling products were compatible with mixtures of the aminoalkene (IV) and the aminoalkane (III). In most cases, direct comparison was possible with the PMR spectra of authentic aminoalkenes and aminoalkanes prepared by standard methods. The aminoalkanes showed $N(CH_3)_2$ singlets at higher field (~0.08 p.p.m.) than the corresponding signals for the aminoalkenes. The integral ratios of the $N(CH_3)_2$ peaks were used to assess the product ratios (Table I).

Lithium Aluminum Hydride–Aluminum Chloride Reductions—Treatment of 3-dimethylamino-1-phenylpropan-1-one (II*c*) with ethereal lithium aluminum hydride–aluminum chloride in the usual manner (15) gave 3-dimethylamino-1-phenylpropan-1-ol, identical to authentic material (6).

Similarly, lithium aluminum hydride–aluminum chloride reduction of 3-dimethylamino-1-*o*-tolylpropan-1-one (13) gave 3-dimethylamino-1-*o*-tolylpropan-1-ol, which formed a *methiodide*, m.p. 152–154° (ethanol–ether).

Anal.—Calc. for $C_{13}H_{22}INO$: I, 37.86. Found: I, 37.88.

Catalytic Reduction of 3-Dimethylamino-1-*p*-nitrophenylpropan-1-one (II*a*)—II*a* hydrochloride (17) (7.7 g.) was suspended in ethanol (100 ml.), and the mixture was saturated with hydrogen chloride gas. Most substrate went into solution and, after cooling, 10% palladium-on-carbon (0.5 g.) was added and the reaction mixture was hydrogenated at room temperature and atmospheric pressure until hydrogen absorption ceased (14 days). The filtered solution (kieselguhr) was evaporated to dryness, the residual solid was treated with ammonium hydroxide, and the liberated base was extracted into ether and dried (magnesium sulfate). Evaporation of the extracts gave an oil (5 g.), which was distilled to give 1-*p*-aminophenyl-3-dimethylaminopropane (III*b*), b.p. 120° (0.1 mm.), n_D^{20} = 1.5430, which formed the *methiodide* (I*b*, n = 3), m.p. 203–

Table I—Reaction Products from Clemmensen Reduction of Mannich Bases

R-$C_6H_4COCH_2CH_2N(CH_3)_2$ (II)	Aminoalkene (IV)–Aminoalkane (III), % Yield[a]	Dimer (V)
II*c*, R = H (9)[b]	13 (1:1)	28
II*d*, R = *p*-F (10)	17[c]	18
II*e*, R = *p*-Cl (11)	29 (1:1)	9
II*f*, R = *m*-CF_3 (12)	27 (1:1)	—
II*g*, R = *o*-CH_3 (13)	27 (3:2)	—
II*h*, R = *p*-CH_3 (14)	26 (1:2)	—

[a] Product ratio from proton magnetic resonance integral data in parentheses. [b] Reference. [c] Greater than 90% aminoalkene.

[5] The authors thank Dr. J. M. Osbond, Roche Products Limited, Welwyn Garden City, Herts., U.K., for suggesting these reaction conditions.

[6] D. S. McQueen, Department of Pharmacology, University of Edinburgh, Edinburgh EH89JZ, U. K., personal communication.

[7] Melting points were recorded on a Mettler FP1 instrument connected to a pen recorder. The NMR spectra were recorded on Perkin-Elmer R10 and Varian HA-100 instruments. Mass spectrometry was carried out with an LKB-9000 (ev. 27.5).

204°, identical (PMR, mixed melting point) to the material prepared previously by the Clemmensen route.

CONCLUSIONS

Clemmensen reduction of Mannich bases leads to a variety of products, but the reaction can still be used to prepare members of the arylpropylamine series. Catalytic hydrogenation of the low boiling fraction distilled from the crude product ensures complete saturation of the side chain and the isolation of pure aminoalkane (III). These derivatives can then be converted to phenylpropyltrimethylammonium derivatives (I, n = 3), which have potential nicotinic properties (1).

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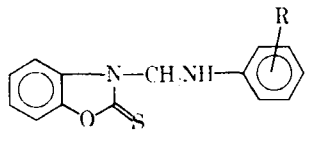
Potential Biologically Active Agents V: Synthesis and Pharmacological Screening of Substituted 3-Aminomethylbenzoxazolin-2-thiones

RAJENDRA S. VARMA

Abstract □ A series of substituted 3-aminomethylbenzoxazolin-2-thiones was synthesized and evaluated for antibacterial activity.

Keyphrases □ 3-Aminomethylbenzoxazolin-2-thiones—synthesized and screened as potential antibacterial agents □ Antibacterial agents, potential—synthesis and screening of 3-aminomethylbenzoxazolin-2-thiones □ Benzoxazolin-2-thiones, substituted—synthesized and screened as potential antibacterial agents

Benzazoles have been reported to exhibit diverse biological properties. The 2- and 3-substituted benzoxazolinone analogs have shown anticonvulsant (1) and antimicrobial (2, 3) activities. Antitubercular (4), antispasmodic (5), and antibacterial (6–9) activities have been observed for benzothiazolin-2-thione and 2-alkylmercaptobenzothiazoles. Substituted 3-aminomethylbenzothiazolin-2-thiones have displayed antimicrobial (10, 11) activity. Further antimicrobial (12, 13), diuretic (14), cancerostatic (15), antitubercular (15), hypoglycemic (16), parasympatholytic (17), and herbicidal (18) properties have been associated with benzoxazolin-2-thiones. In view of these reports, it was of interest to synthesize substituted 3-aminomethylbenzoxazolin-2-thiones (I) for biological screening. The synthesis of I was achieved by condensing benzoxazolin-2-thione (II), formaldehyde, and an amine.



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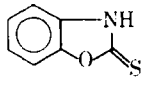

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The compounds listed in Table I were screened for their inhibitory effect against *Escherichia coli* and *Staphylococcus aureus*[1] by the agar diffusion technique (19). Four compounds (I, VII, XII, and

[1] Bacterial cultures maintained at Central Drug Research Institute, Lucknow, India, were used.

204°, identical (PMR, mixed melting point) to the material prepared previously by the Clemmensen route.

CONCLUSIONS

Clemmensen reduction of Mannich bases leads to a variety of products, but the reaction can still be used to prepare members of the arylpropylamine series. Catalytic hydrogenation of the low boiling fraction distilled from the crude product ensures complete saturation of the side chain and the isolation of pure aminoalkane (III). These derivatives can then be converted to phenylpropyltrimethylammonium derivatives (I, n = 3), which have potential nicotinic properties (1).

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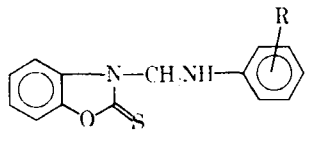

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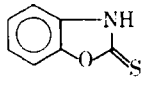

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Table I—Substituted 3-Aminomethylbenzoxazolin-2-thiones (I)

Compound	R	Melting Point	Molecular Formula	Analysis, % Calc.	Found	Yield, %
I[a]	4-COOH	226–228°	$C_{15}H_{12}N_2O_3S$	C 59.99	59.50	65
				H 4.02	4.30	
II[b]	4-$COOCH_3$	205–206°	$C_{16}H_{14}N_2O_3S$	C 61.13	61.50	60
				H 4.48	4.70	
				N 8.91	8.54	
III[c]	4-$COOC_2H_5$	176°	$C_{17}H_{16}N_2O_3S$	C 62.18	62.00	70
				H 4.91	5.08	
				N 8.51	8.38	
IV[d]	3-COOH	213°	$C_{15}H_{12}N_2O_3S$	C 59.99	59.70	65
				H 4.02	4.40	
				N 9.33	9.49	
V[b]	3-$COOCH_3$	182°	$C_{16}H_{14}N_2O_3S$	C 61.13	61.40	55
				H 4.48	4.74	
				N 8.91	9.10	
VI[e]	3-$COOC_2H_5$	178°	$C_{17}H_{16}N_2O_3S$	C 62.18	62.25	75
				H 4.91	5.13	
				N 8.51	8.65	
VII[f]	2-COOH	188° dec.	$C_{15}H_{12}N_2O_3S$	C 59.99	60.20	50
				H 4.02	4.38	
				N 9.33	8.89	
VIII[b]	2-$COOCH_3$	145°	$C_{16}H_{14}N_2O_3S$	N 8.91	8.30	60
IX[b]	2-$COOC_2H_5$	128–129°	$C_{17}H_{16}N_2O_3S$	C 62.18	62.80	55
				H 4.91	5.51	
X[g]	2-Thiazolyl	136–137°	$C_{11}H_9N_3OS_2$	C 50.17	50.65	45
				H 3.44	3.85	
XI[b]	4-Phenyl	186–188°	$C_{20}H_{16}N_2OS$	C 72.26	72.60	80
				H 4.85	5.15	
				N 8.42	8.01	
XII[b]	2-Phenyl	145–146°	$C_{20}H_{16}N_2OS$	N 8.42	8.04	75
XIII[h]	4-I; 2-COOH	165–166°	$C_{15}H_{11}IN_2O_3S$	C 42.27	42.45	70
				H 2.60	2.90	
				N 6.57	6.15	
XIV[i]	4-Br; 2-COOH	195–196°	$C_{15}H_{11}BrN_2O_3S$	C 47.51	47.30	60
				H 2.92	3.22	
				N 7.38	7.19	
XV[b]	2-OCH_2CH_3	130°	$C_{16}H_{16}N_2O_2S$	C 63.98	64.20	80
				H 5.37	5.59	
				N 9.33	9.25	
XVI[b]	4-COO-*n*-C_4H_9	148°	$C_{19}H_{20}N_2O_3S$	C 64.03	63.82	65
				H 5.65	5.82	
				N 7.86	7.49	
XVII[b]	4-COO-*n*-C_3H_7	178°	$C_{18}H_{18}N_2O_3S$	C 63.14	63.10	70
				H 5.29	5.52	
				N 8.18	7.78	

[a] Recrystallized from ethanol. IR (μ): 2.95 (NH), 3.4 (OH, broad), and 5.92 (C=O). [b] Recrystallized from ethanol. [c] Recrystallized from ethanol. IR (μ): 2.95 (NH) and 5.92 (C=O). [d] Recrystallized from ethanol. IR (μ): 2.95 (NH), 3.4 (OH, broad), and 5.90 (C=O). [e] Recrystallized from ethanol. IR (μ): 2.95 (NH) and 5.85 (C=O). [f] Recrystallized from acetone. [g] Recrystallized from methanol. The compound is 3-(*N*-2-thiazolylaminomethyl)-benzoxazolin-2-thione. IR (μ): 3.12 (NH). [h] Recrystallized from methyl ethyl ketone. IR (μ): 2.97 (NH), 3.3 (OH, broad), and 5.97 (C=O). [i] Recrystallized from ethyl acetate.

XIII) showed inhibition against both organisms. Compounds III, V, VI, VIII, X, and XV were only effective against *E. coli*.

EXPERIMENTAL[2]

Benzoxazolin-2-thione was prepared by a published procedure (20). Esters of aminobenzoic acids were obtained by refluxing a mixture of appropriate alcohol, thionyl chloride, and the aminobenzoic acid for several hours.

The synthesis of the substituted 3-aminomethylbenzoxazolin-2-thiones (I) was conducted as follows. Compound II (1.51 g., 0.01 mole) and the amino compound (0.01 mole) were suspended in 20 ml. of boiling ethanol. To this suspension, 2 ml. of 37% formalin was added. The reaction mixture was warmed on a steam bath for 5 min. and then set aside at room temperature overnight. The solid product thus deposited was filtered, washed with petroleum ether (60–80°), and recrystallized from a suitable solvent.

The analyses, melting points, and other pertinent data are recorded in Table I.

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New Compounds: Synthesis of 3,4,5-Trimethoxybenzenesulfonamides

GIORGIO PIFFERI▲ and RICCARDO MONGUZZI

Abstract □ Some new 3,4,5-trimethoxybenzenesulfonamides were synthesized for biological screening. The intermediate 3,4,5-trimethoxybenzenesulfonic acid was unequivocally prepared from 3,4,5-trimethoxyaniline according to the procedure of Meerwein.

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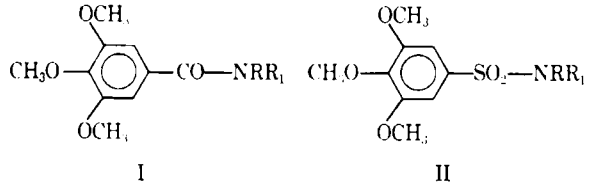
In connection with pharmacological research on new heterocyclic analogs of 3,4,5-trimethoxybenzamide (I) (1), it was interesting to synthesize a series of 3,4,5-trimethoxybenzenesulfonamides (II) because of their steric and electronic similarity[1].

DISCUSSION

Although numerous derivatives of 3,4,5-trimethoxybenzoic acid are of biological interest (1, 3), the SO_2 analogs (II) are unknown in the literature. The present authors ascertained that the structure of the sulfonic acid derivative VII*b* was incorrectly assigned by Alimchandani (4); in fact, pyrogallol trimethyl ether (III) reacts with sulfuric acid in the experimental conditions reported (4) to give the vicinal isomer IV*b*. The same product was also prepared (Scheme I) by treating III with chlorosulfonic acid at room temperature and subsequent hydrolysis of the intermediate IV*a* to 2,3,4-trimethoxybenzenesulfonic acid (IV*b*). For the unequivocal synthesis of the isomer VII*b*, 3,4,5-trimethoxybenzoic acid (V) was converted *via* VI*a* into the aniline derivative VI*b* (5); the latter was diazotized and the diazonium salt was decomposed with sulfur dioxide according to the method of Meerwein *et al.* (6) to afford the sulfonyl chloride VII*a*. Subsequent hydrolysis gave 3,4,5-trimethoxybenzenesulfonic acid (VII*b*), which was easily distinguished from its isomer IV*b* by comparison of the NMR coupling constants of the two aromatic protons in the *meta*- and *ortho*-positions, respectively (7).



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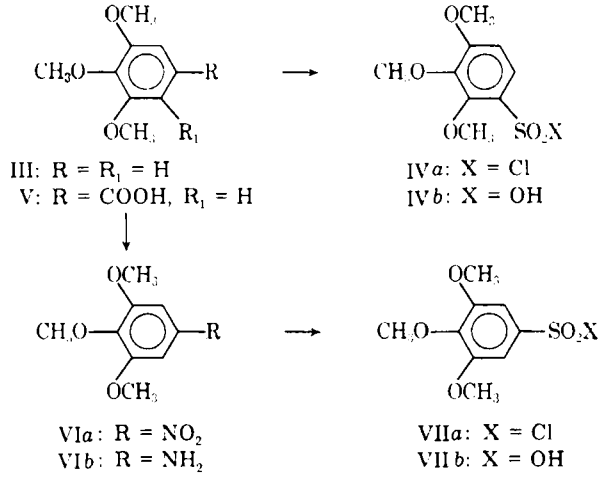

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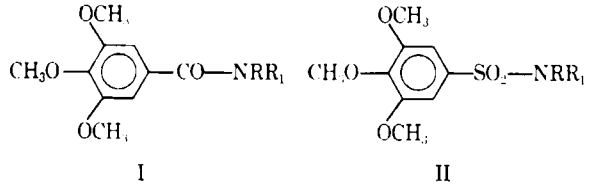

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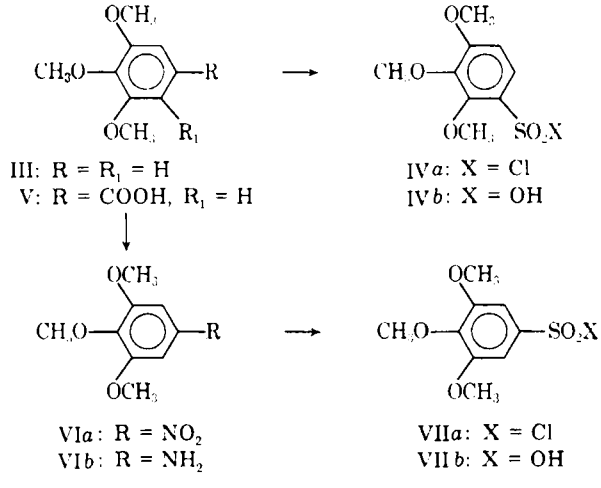

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[1] Actually, carbonyl and sulfonyl groups may be considered as "nonclassical isosteres" (2).

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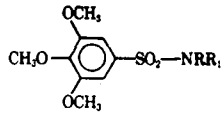
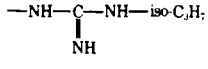
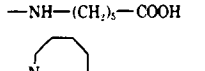


Table I—3,4,5-Trimethoxybenzenesulfonamides (II)

Compound	NRR_1	Method	Melting Point[a]	Yield, %	Spectral Data, $cm.^{-1}$ νSO_2 asym.	νSO_2 sym.	Formula	Analysis, % Calc.	Found
IIa		B	112–113°	82	1355	1160	$C_{12}H_{17}NO_6S$	N 4.62 S 10.57	4.39 10.65
IIb		A	109–110°	83	1340	1160	$C_{13}H_{19}NO_6S$	N 4.41 S 10.10	4.26 10.39
IIc		A	147–149°	25	1310	1150	$C_{13}H_{21}N_3O_5S$	N 12.68 S 9.68	12.64 10.07
IId		B	158–159°	42	1320	1150	$C_{15}H_{23}NO_7S$	N 3.86 S 8.87	3.68 8.73
IIe		A	108–111°	48	1330	1150	$C_{16}H_{25}NO_5S$	N 4.08 S 9.34	3.88 9.88

[a] From ethanol.

chloroform was treated dropwise at 0° with 11.5 g. (98 mmoles) of chlorosulfonic acid. The reaction mixture was stirred for 30 min. at room temperature and poured into 150 g. of crushed ice. The organic layer was separated and the mother liquor was extracted two times with 30 ml. of chloroform. The combined extracts were washed with water and dried over sodium sulfate, the solvent was removed, and the oily residue was distilled through a Claisen head to yield 1.32 g. of IV*a*, b.p. 165° (1.5 mm.), which crystallized from hexane to a solid, m.p. 38–39°; IR: 1350 and 1170 (SO_2 asym. and sym.) and 815 (two adjacent aromatic hydrogens) $cm.^{-1}$.

Anal.—Calc. for $C_9H_{11}ClO_5S$: Cl, 13.30; S, 12.02. Found: Cl, 13.86; S, 11.95.

2,3,4-Trimethoxybenzenesulfonic Acid (IV*b*)—A solution of 10 g. (59.5 mmoles) of pyrogallol trimethyl ether (III) in 25 ml. of 96% sulfuric acid was stirred at 25–30° for 3 hr. After cooling, water (25 ml.) was added dropwise and the solution was kept overnight in a refrigerator. The white hygroscopic precipitate was filtered and crystallized from ethyl acetate to yield 8.45 g. (57%) of IV*b*, m.p. 96° [lit. (4) m.p. 95–97°; IR: 3600–2150 (OH), 1190 and 1145 (SO_3 group), and 815–805 (two adjacent aromatic hydrogens) $cm.^{-1}$; NMR: δ(p.p.m.) 3.90 s and 4.00 s (methyl protons of methoxy groups) and 6.88 d and 7.59 d (two *ortho* aromatic protons: J = 9.0 Hz.).

2,3,4-Trimethoxybenzenesulfonic Acid (IV*b*) from IV*a*—A stirred suspension of 0.3 g. (1.13 mmoles) of 2,3,4-trimethoxybenzenesulfonyl chloride (IV*a*) in 9 ml. of water was refluxed until a solution was formed. After concentration *in vacuo*, the residue was crystallized from ethyl acetate to yield 100 mg. (36%) of IV*b*, identical (by mixed melting point and IR comparison) with the sample described above.

3,4,5-Trimethoxynitrobenzene (VI*a*)—Compound VI*a* was prepared from 3,4,5-trimethoxybenzoic acid (V) and 70% nitric acid according to the method of Hughes *et al.* (5), m.p. 96–98° (from ethanol).

3,4,5-Trimethoxyaniline (VI*b*)—Compound VI*b* was obtained by catalytic reduction at room temperature of 3,4,5-trimethoxynitrobenzene (VI*a*) in the presence of 5% palladium-on-charcoal, m.p. 111–112° [lit. (5) m.p. 113–114°].

3,4,5-Trimethoxybenzenesulfonyl Chloride (VII*a*)—To 320 ml. of concentrated hydrochloric acid was added, at 70° with stirring, 40 g. (0.218 mole) of 3,4,5-trimethoxyaniline (VI*b*). The mixture was stirred at the same temperature for 10 min. and, after cooling to 0°, a solution of 15.2 g. (0.22 mole) of sodium nitrite in 30 ml. of water was added dropwise. The solution was then stirred at 0° for 15 min. and added dropwise with caution to a saturated solution of sulfur dioxide in 480 ml. of glacial acetic acid containing 4.8 g. of cuprous chloride. At the end of addition, the mixture was stirred at room temperature for 3 hr., poured into cold water, and extracted four times with 1 l. of dichloromethane. The combined organic extracts were washed with cold water and dried over calcium chloride, and the solvent was evaporated. The solid residue was recrystallized twice from hexane to yield 11.84 g. (20.5%) of VII*a*, m.p. 82–85°; IR: 1370 and 1175 (SO_2 asym. and sym.), and 835 (two *meta* aromatic hydrogens) $cm.^{-1}$.

Anal.—Calc. for $C_9H_{11}ClO_5S$: C, 40.55; H, 4.16; Cl, 13.30; S, 12.02. Found: C, 40.59; H, 4.29; Cl, 13.46; S, 12.22.

3,4,5-Trimethoxybenzenesulfonic Acid (VII*b*)—A stirred suspension of 0.9 g. (3.37 mmoles) of 3,4,5-trimethoxybenzenesulfonyl chloride (VII*a*) in 30 ml. of water was refluxed until solution. After evaporation *in vacuo*, the solid residue was crystallized from nitroethane to yield 0.5 g. (60%) of VII*b* as a very hygroscopic material, m.p. 147–149°. The mixed melting point with IV*b* was depressed. IR: 3700–2100 (OH), 1160 and 1130 (SO_3 group), and 835 (two *meta* aromatic hydrogens) $cm.^{-1}$; NMR: δ(p.p.m.) 3.83 s and 3.95 s (methyl proton of methoxy groups) and 7.20 s (two *meta* aromatic protons).

Anal.—Calc. for $C_9H_{12}O_6S$: C, 43.54; H, 4.87; S, 12.91. Found: C, 42.80; H, 5.21; S, 13.50.

3,4,5-Trimethoxybenzenesulfonamides (II)—*Procedure A*—A solution of 12 mmoles of 3,4,5-trimethoxybenzenesulfonyl chloride (VII*a*) in 15 ml. of dichloromethane was added at room temperature to a stirred solution of 27 mmoles of the appropriate base in 80 ml. of the same solvent. The mixture was refluxed for 3 hr., cooled at room temperature, and washed successively with diluted hydrochloric acid, a saturated sodium bicarbonate solution, and water. The organic extracts were dried over sodium sulfate and evaporated *in vacuo*, and the solid residue crystallized (Table I).

Procedure B—A solution of 15 mmoles of 3,4,5-trimethoxybenzenesulfonyl chloride (VII*a*) in 15 ml. of dichloromethane was added at room temperature to a stirred solution of 15 mmoles of the appropriate base hydrochloride and 34 mmoles of triethylamine in 80 ml. of the same solvent. The mixture was stirred for 1 hr. at room temperature and refluxed for 3 hr. After cooling, it was washed with diluted hydrochloric acid and water and dried over sodium sulfate. The solvent was evaporated and the residue was crystallized (Table I).

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COMMUNICATIONS

Sulfamate Sweeteners: A Reappraisal

Keyphrases □ Sulfamates, alicyclic—hydrolysis rate constants determined from polar and steric parameters, equation □ Cyclamates, alicyclic—hydrolysis rate constants determined from polar and steric parameters, equation □ Sweeteners, alicyclic sulfamates—hydrolysis rate constants determined from polar and steric parameters, equation

Sir:

There is evidence from feeding experiments that *in vivo* conversion of the nonnutritive sweeteners, the cyclamates [sodium cyclohexylsulfamate (I) and calcium cyclohexylsulfamate (II)] gives cyclohexylamine (1–4), which is considered to be responsible for the induction of tumors (4). These and other experiments (5–7) resulted in a ban since 1970 on the use of cyclamates in foodstuffs, beverages, and pharmaceuticals. No work appears to have been published on the nature of the metabolic conversion of cyclamate to cyclohexylamine. However, it is possible that the ease of metabolism to primary amine may be correlative with the hydrolytic stability of the sulfamate.

We derived a Taft–Pavelich-type equation (Eq. 1) which correlates the hydrolytic rate constants (measured under identical conditions) for cleavage of the nitrogen–sulfur bond with polar (σ^*) and steric (E_s) parameters for a large number of aromatic, aliphatic, and alicyclic sulfamates (8) and is of the form:

$$\log k = 2.35\Sigma\sigma^* - 1.0037\Sigma E_s + 0.6971 \qquad \text{(Eq. 1)}$$

Further tests on Eq. 1 indicate that it reproduces log k values to within an average of 12%. The k values are reproduced to within 26% on average. For Compound I, the k value was reproduced with an accuracy of 5%.

By using Eq. 1, it is possible to predict the hydrolytic stability of unsynthesized sulfamates (whose σ^* and E_s values are known). Equation 1 could thus act as a guide to the choice of sulfamates that might be more resistant to *in vivo* conversion to primary amines than are I and II. Such sulfamates may lack carcinogenic or cocarcinogenic activity because of the possibility of a lower conversion to amine. A second criterion in choosing sulfamates is that they have a comparable degree of sweetness to I and II. Audrieth and Sveda (9) showed that the retention of the sulfamate function, $-NHSO_3^-$, and the presence of a reduced ring (*e.g.*, cyclohexyl) appear to be essential for sweetness in sulfamates. Later results substantiated these findings and extended them to exclude ring substitution in the reduced ring since such compounds tend to be tasteless or to leave a bitter aftertaste (10–12). These criteria for sweetness should be met by Compounds III. In fact, sodium cyclopentylsulfamate (III, $n = 3$) (10), sodium cycloheptylsulfamate (III, $n = 5$), and sodium cyclooctylsulfamate (III, $n = 6$) (11) are extremely sweet.

Equation 1 predicts that the cycloheptyl compound (estimated $\sigma^* = -0.10$, $E_s = -1.10$)[1] will hydrolyze almost 3 times more rapidly than I or II, the cyclopentylsulfamate ($\sigma^* = -0.20$, $E_s = -0.51$) will hydrolyze 2.5 times more slowly, and the unprepared cyclobutyl (III, $n = 2$) compound (estimated $\sigma^* = -0.28$, $E_s = -0.06$) will hydrolyze 11 times more slowly. Compound IV, *N*-(α-cyclohexylmethyl)sulfamate ($\sigma^* = -0.06$, $E_s = -0.98$) is predicted to hydrolyze 2.5 times more rapidly than I or II. Cyclopropylsulfamate (III, $n = 1$) was prepared (10), but its sweetness was not assessed. Polar and steric parameters are not available for this compound or for cyclooctylsulfamate.

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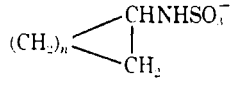

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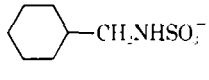

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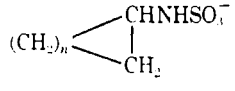

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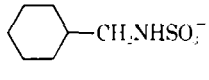

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Determination of Trimethoprim and Its *N*-Oxide Metabolites in Urine of Man, Dog, and Rat by Differential Pulse Polarography

Keyphrases ☐ Trimethoprim and *N*-oxide metabolites—analysis in urine of man, dog, and rat, differential pulse polarography ☐ Polarography, differential pulse—analysis, trimethoprim in urine of man, dog, and rat

Sir:

An analytical method was recently reported for the determination of trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] in blood and urine of man and dog by differential pulse polarography (1). It was qualitatively noted that a portion of the dose was excreted as the unconjugated N_1-oxide, N_3-oxide, and hydroxy-methyl metabolites of trimethoprim, all of which are resolved by TLC. However, due to low recoveries, the measurement of these metabolites was not previously reported. The purposes of this work were to quantitate the *N*-oxide metabolites and to determine if a species difference existed regarding the formation of the *N*-oxides. Since recovery of the hydroxy-methyl metabolite remained low with this modified assay; its measurement is not reported.

A modification of the above-mentioned assay is reported, which includes a "salting-out" procedure to facilitate the extraction of the *N*-oxides into chloroform. In the modified procedure, 1 ml. urine and 4 ml. distilled water are added to a 50-ml. centrifuge tube to which 6 g. anhydrous potassium carbonate is then added. The method from this point onward is identical to the previously reported one (1), with the exception that polarography is performed in 1 *M* pH 3.0 phosphate buffer rather than in 0.1 *N* H_2SO_4. Five and ten micrograms of trimethoprim and the *N*-oxides added to urine as internal standards should be processed with the unknowns. The choice of pH 3.0 phosphate buffer for the urinary assay is dictated by the need for maximal separation in potential of the two *N*-oxide peaks for qualitative identification. This supporting electrolyte was completely free of background interferences in the region of the polarographic reduction of the *N*-oxides.

Polarography is a useful means of identifying and quantitating the *N*-oxides (Fig. 1). The *N*-oxides of trimethoprim possess two distinct polarographic peaks at −1.095 and −0.950 v. *versus* a saturated calomel electrode (SCE) in 1 *M* pH 3 phosphate buffer due to the reduction of the N_1- and N_3-oxide functional groups, respectively. These peaks are easily distinguished from the reduction peak of the azomethine bond in the pyrimidine ring of all three compounds, which occurs at −1.190 v. *versus* SCE in the pH 3.0 phosphate buffer. Although each *N*-oxide possesses a distinct functional group, the *N*-oxide compounds cannot be determined without prior separation because large quantities of trimethoprim in the urine result in a large analytical peak that masks the *N*-oxide peaks. Thus, TLC separation, in which the compounds are identified by comparison with the R_f values of the authentic compounds

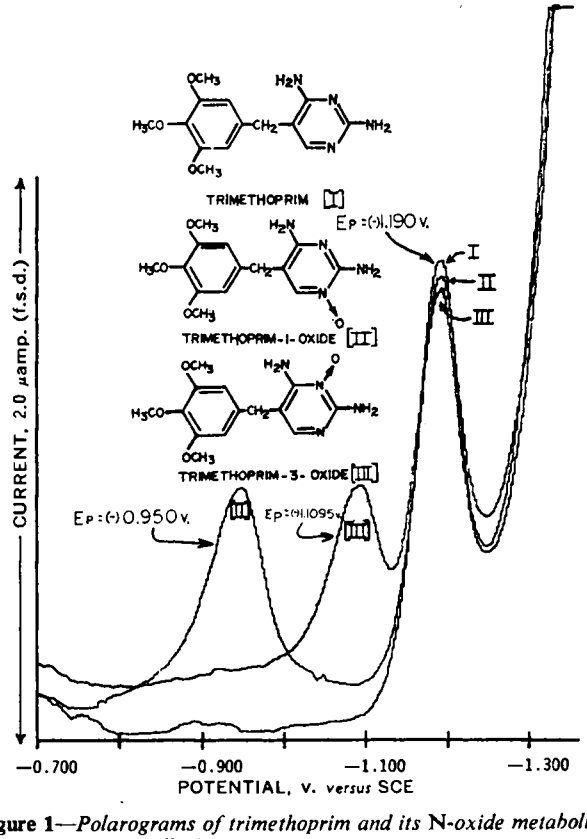

Figure 1—*Polarograms of trimethoprim and its* N*-oxide metabolites in 1* M *phosphate buffer (pH 3).*

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Determination of Trimethoprim and Its *N*-Oxide Metabolites in Urine of Man, Dog, and Rat by Differential Pulse Polarography

Keyphrases ☐ Trimethoprim and *N*-oxide metabolites—analysis in urine of man, dog, and rat, differential pulse polarography ☐ Polarography, differential pulse—analysis, trimethoprim in urine of man, dog, and rat

Sir:

An analytical method was recently reported for the determination of trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] in blood and urine of man and dog by differential pulse polarography (1). It was qualitatively noted that a portion of the dose was excreted as the unconjugated N_1-oxide, N_3-oxide, and hydroxy-methyl metabolites of trimethoprim, all of which are resolved by TLC. However, due to low recoveries, the measurement of these metabolites was not previously reported. The purposes of this work were to quantitate the *N*-oxide metabolites and to determine if a species difference existed regarding the formation of the *N*-oxides. Since recovery of the hydroxy-methyl metabolite remained low with this modified assay; its measurement is not reported.

A modification of the above-mentioned assay is reported, which includes a "salting-out" procedure to facilitate the extraction of the *N*-oxides into chloroform. In the modified procedure, 1 ml. urine and 4 ml. distilled water are added to a 50-ml. centrifuge tube to which 6 g. anhydrous potassium carbonate is then added. The method from this point onward is identical to the previously reported one (1), with the exception that polarography is performed in 1 *M* pH 3.0 phosphate buffer rather than in 0.1 *N* H_2SO_4. Five and ten micrograms of trimethoprim and the *N*-oxides added to urine as internal standards should be processed with the unknowns. The choice of pH 3.0 phosphate buffer for the urinary assay is dictated by the need for maximal separation in potential of the two *N*-oxide peaks for qualitative identification. This supporting electrolyte was completely free of background interferences in the region of the polarographic reduction of the *N*-oxides.

Polarography is a useful means of identifying and quantitating the *N*-oxides (Fig. 1). The *N*-oxides of trimethoprim possess two distinct polarographic peaks at −1.095 and −0.950 v. *versus* a saturated calomel electrode (SCE) in 1 *M* pH 3 phosphate buffer due to the reduction of the N_1- and N_3-oxide functional groups, respectively. These peaks are easily distinguished from the reduction peak of the azomethine bond in the pyrimidine ring of all three compounds, which occurs at −1.190 v. *versus* SCE in the pH 3.0 phosphate buffer. Although each *N*-oxide possesses a distinct functional group, the *N*-oxide compounds cannot be determined without prior separation because large quantities of trimethoprim in the urine result in a large analytical peak that masks the *N*-oxide peaks. Thus, TLC separation, in which the compounds are identified by comparison with the R_f values of the authentic compounds

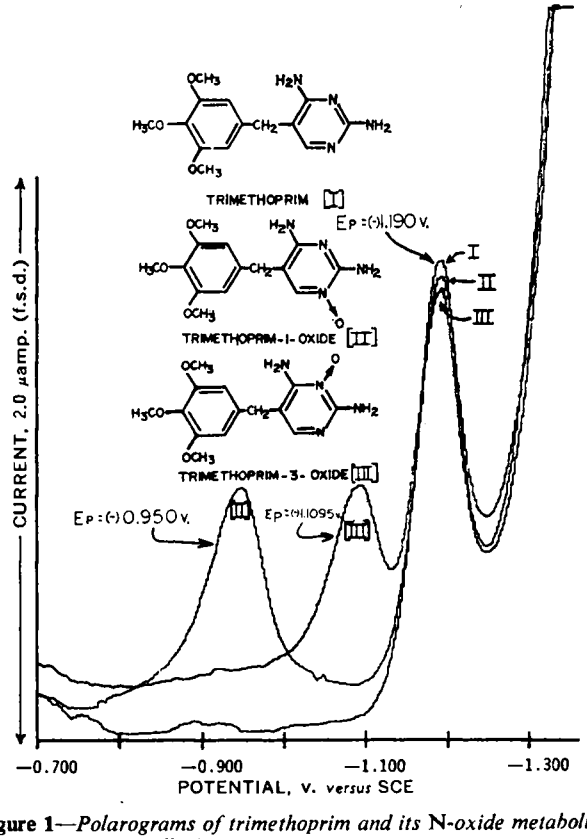

Figure 1—*Polarograms of trimethoprim and its* N*-oxide metabolites in 1* M *phosphate buffer (pH 3).*

Table I—Urine Levels of Trimethoprim and Trimethoprim *N*-Oxides in Man following Oral Administration[a]

	Trimethoprim			Trimethoprim N_1-Oxide			Trimethoprim N_3-Oxide			
	0–24 hr.	24–48 hr.	48–72 hr.	0–24 hr.	24–48 hr.	48–72 hr.	0–24 hr.	24–48 hr.	48–72 hr.	Total
Subject 1:										
Total milligrams equivalent to trimethoprim	118.4	122.0	16.9	3.6	6.0	n.m.[b]	9.1	n.m.	n.m.	276.0
Percent dose	29.6	30.5	4.2	0.9	1.5	—	2.3	—	—	69.0
Subject 2:										
Total milligrams equivalent to trimethoprim	178.9	34.9	s.n.t.[c]	6.6	n.m.	s.n.t.	4.1	n.m.	s.n.t.	224.5
Percent dose	44.7	8.7	—	1.6	—	—	1.0	—	—	56.0

[a] Total dose was 400 mg. trimethoprim (5.7 mg./kg.). Five tablets were given orally (each tablet contained 400 mg. sulfamethoxazole and 80 mg. trimethoprim). [b] n.m. = nonmeasurable. [c] s.n.t. = sample not taken.

Table II—Urine Levels of Trimethoprim and Trimethoprim *N*-Oxides in the Dog following Oral Administration[a]

	Trimethoprim		Trimethoprim N_1-Oxide		Trimethoprim N_3-Oxide		
	0–24 hr.	24–48 hr.	0–24 hr.	24–48 hr.	0–24 hr.	24–48 hr.	Total
Dog 1:							
Total milligrams equivalent to trimethoprim	32.7	5.6	0.8	n.m.[b]	6.5	1.1	46.7
Percent dose	16.3	2.7	0.4	—	3.2	0.6	23.2
Dog 2:							
Total milligrams equivalent to trimethoprim	48.8	9.5	0.6	n.m.	10.0	1.8	70.7
Percent dose	24.4	4.7	0.3	—	5.0	0.9	35.3

[a] Total dose was 200 mg. trimethoprim (16 mg./kg.). Two and one-half tablets were given orally (each tablet contained 400 mg. sulfamethoxazole and 80 mg. trimethoprim). [b] n.m. = nonmeasurable.

run alongside, is required before quantitative determination by polarography.

The modified method gives recoveries of the N_1- and N_3-oxide of 36.3 ± 8.4 and 59.1 ± 7.5 (*SD*), respectively, in the concentration range of 5.0–30.0 mcg. However, due to the salting out, large amounts of biological interferences are coextracted with the compound of interest. Since the biological interferences tend to make recognition of the zones on the TLC plate difficult, zones are chosen by R_f, *i.e.*, 0.16, 0.25, and 0.45 for N_1-oxide, N_3-oxide, and trimethoprim, respectively. To ensure that no overlap of the *N*-oxide zones occurs, the plates may be redeveloped using the same solvent system. Thus, this modified method is recommended only when metabolite information is required. For the determination of trimethoprim, the previous method (1) is preferred.

Trimethoprim was administered to man, dog, and rat, and the amounts of intact drug and *N*-oxides were measured as already outlined. The urines from two human subjects receiving tablets containing a total of 2000 mg. sulfamethoxazole and 400 mg. trimethoprim (5.7 mg. trimethoprim/kg. body weight) were analyzed for trimethoprim and its *N*-oxide metabolites (Table I).

Table III—Urine Levels of Trimethoprim and Trimethoprim *N*-Oxides in the Rat following Intraperitoneal Administration[a]

	Trimethoprim		Trimethoprim N_1-Oxide		Trimethoprim N_3-Oxide		
	0–24 hr.	24–48 hr.	0–24 hr.	24–48 hr.	0–24 hr.	24–48 hr.	Total
Rat 1:							
Total milligrams equivalent to trimethoprim	2.4	0.07	0.7	n.m.[b]	n.m.	n.m.	3.2
Percent dose	8.5	0.3	2.7	—	—	—	11.4
Rat 2:							
Total milligrams equivalent to trimethoprim	1.4	0.08	0.3	n.m.	n.m.	n.m.	1.8
Percent dose	5.0	0.3	1.0	—	—	—	6.3
Rat 3:							
Total milligrams equivalent to trimethoprim	0.55	0.04	0.10	n.m.	n.m.	n.m.	0.69
Percent dose	5.5	0.4	1.0	—	—	—	6.9
Rat 4:							
Total milligrams equivalent to trimethoprim	1.09	0.08	0.06	n.m.	n.m.	n.m.	1.23
Percent dose	10.9	0.7	0.6	—	—	—	12.3

[a] Total dose for Rats 1 and 2 was 28 mg. trimethoprim (140 mg./kg.). For Rats 3 and 4, it was 10 mg. trimethoprim (50 mg./kg.). The dosage form was 2 ml. of a suspension in 5% gum arabic. [b] n.m. = nonmeasurable.

In the two subjects, more than 50% of the administered dose was excreted mainly as intact drug in the 0–48-hr. period. Both the N_1- and N_3-oxides were found in the urine of man in approximately equal quantities, totaling 4.7 and 2.6% of the dose administered in Subjects 1 and 2, respectively. The urines from two dogs receiving tablets containing a total of 1000 mg. sulfamethoxazole and 200 mg. trimethoprim (16 mg. trimethoprim/kg. body weight) were analyzed for trimethoprim and its *N*-oxide metabolites (Table II). In the two dogs, 19.0 and 29.1% of the dose were excreted as intact drug. A significant amount of the N_3-oxide was excreted by both dogs in the 0–48-hr. period, while only a trace amount of the N_1-oxide was excreted. The urines of four rats receiving 28 mg. (140 mg./kg. body weight) and 10 mg. (50 mg./kg. body weight) trimethoprim i.p. in 5% gum arabic were analyzed for trimethoprim and its *N*-oxide metabolites. Between 5 and 12% of the dose was excreted as intact drug at both dose levels. Only the N_1-oxide metabolite was found in the 0–48-hr. rat urine, totaling between 0.6 and 2.7% of the administered dose (Table III).

Schwartz *et al.* (2) studied the metabolism of trimethoprim in man, dog, and rat and found that each species excreted the N_1-oxide as a urinary metabolite; this finding was confirmed in the present study. Meshi and Sato (3) detailed the metabolism of trimethoprim in the rat and reported levels of an *N*-oxide, but they were unable to determine the point of attachment of the oxygen to either nitrogen of the pyrimidine ring. Additional information on the *N*-oxidation of trimethoprim is presented in this study. Although limited numbers of subjects and animals of each species were studied, the results suggest that the human converts trimethoprim to the N_1-oxide and N_3-oxide with equal facility, whereas N_3-oxide formation is favored by the dog and N_1-oxide formation is favored by the rat.

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BOOKS

REVIEWS

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Reviewed by Govind J. Kapadia and S. N. Dutta
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The subject index has been abolished in this volume. However, this hardly reflects on the ease with which the proper subject reference can be located. This has been achieved by providing a detailed table of contents. This volume is a must for the desk of the scientist working in the areas of amino acids, peptides, and proteins.

Reviewed by A. Kapoor
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Chemical and Biological Aspects of Drug Dependence. Edited by S. J. MULE and Henry BRILL, M.D. CRC Press, 18901 Cranwood Pkwy., Cleveland, OH 44128, 1972. 561 pp. 18 × 26.5 cm.

The purpose of this publication is to provide, in one reference source, the basic knowledge and background information that currently define the chemical and biological basis of drug dependence. To obtain a better understanding of drug dependence and drug abuse, the characteristics of drug dependence of various pharmacological classes of drugs are described. Theories related to tolerance development and physical dependence are then discussed. Finally, the biochemical mechanisms associated with biological strata, *e.g.*, enzymes, proteins, and hormones, are explained in relationship to drug dependence. Twenty-five separately authored discussions are divided into five sections covering significance and characteristics of drug dependence, criteria for their evaluation, chemical aspects, physiological and pharmacological aspects, and biochemical aspects.

Staff Review ■

Modern Analytical Methods. By D. BETTERIDGE and H. E. HALLAM. The Chemical Society, Burlington House, London W1V OBN, England, 1972. 220 pp. 14 × 21.5 cm. Price £2.00.

The objective of this publication is to acquaint teachers with the most important methods of modern analysis. To aid in understanding the analytical methods, modern methods of testing complex equilibria are also included. Although the discussion of individual techniques is condensed, an attempt has been made to include the fundamental theory necessary to understand the method or technique being discussed.

Staff Review ■

Handbook of Commercial Scientific Instruments, Volume 1: Atomic Absorption. By CLAUDE VEILLON. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1972. 174 pp. 15 × 23 cm. Price $11.75.

Commercially available instruments, by type, will be detailed in this new multivolume series. In volume 1, the instrumentation available for atomic absorption spectroscopy is covered. Specifications, descriptions, schematic drawings, photographs, approximate prices, and accessories are given in separate discussions for each manufacturer, and in summary tables given at the end of the book for quick comparisons.

The information is essentially that provided by the manufacturer. The volume authors are engaged in research in the area covered by their respective books and are familiar with the available instrumentation. The authors do not represent any instrument manufacturer.

This volume begins with a general discussion of atomic absorption spectroscopy requirements and instruments, followed by sections on each manufacturer and his instruments. A summary section focusing on cost considerations and tables comparing the discussed instruments complete the book.

This book and, presumably the rest of the series, bring together information available but from many diverse sources. The book should be useful in preliminary considerations of equipment acquisitions.

Staff Review ■

NOTICES

Medicaments Organiques de Synthese, Chimie et Pharmacologic, Volume V. Edited by Leon VELLUZ. Masson et Cie, 120 Boulevard Saint-Germain, Paris VIe, France. 270 pp. 15 × 21.5 cm. Price Fr. 165. (*French*)

Molecular Mechanisms of Antibiotic Action on Protein Biosynthesis and Membranes, Proceedings of a Symposium held at the University of Granada (Spain), June 1–4, 1971. Edited by E. MUNOZ, F. GARCIA-FERRANDIZ, and D. VAZQUEZ. American Elsevier, 52 Vanderbilt Ave., New York, NY 10017, 1973. 804 pp. 16 × 24 cm. Price $27.50.

Carbohydrate Chemistry, Volume V, A Specialist Periodical Report. Senior Reporter, J. S. BRIMACOMBE. The Chemical Society, Burlington House, London, W1V OBN, England, 1972. 434 pp. 13 × 21.5 cm. Price £8.00.

Philosophy of Medicine and Science: Problems and Perspectives. Compiled by Department of Philosophy of Medicine and Science, Institute of History of Medicine and Medical Research, New Delhi 62, India. 289 pp. 16 × 24 cm.

Developmental Physiology and Aging. By P. S. TIMIRAS. Macmillan, 866 Third Ave., New York, NY 10022, 1972. 692 pp. 17.5 × 25 cm. Price $19.95.

Glucagon: Molecular Physiology, Clinical and Therapeutic Implications. Edited by PIERRE J. LEFEBVRE and ROGER H. UNGER. Pergamon Press, Inc., Maxwell House, Fairview Park, Elmsford, NY 10523, 1972. 370 pp. 17 × 25 cm. Price $37.50.

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REVIEW ARTICLE

High Pressure Liquid Chromatography

A. F. MICHAELIS▲, D. W. CORNISH, and R. VIVILECCHIA

Keyphrases □ High pressure liquid chromatography—review of theory, column design, instrumentation, methods, performance measurements, pharmaceutical applications □ Drug analysis—use of high pressure liquid chromatography, review □ Chromatography, high pressure liquid—review, use in drug analysis □ Column chromatography—review of high pressure liquid chromatography, theory, design, instrumentation, methods, performance, pharmaceutical applications

Prior to 1967, liquid column chromatography (LCC) separations were not normally carried out in an optimum manner; for this reason, the technique was viewed as slow and inefficient (1–3). Consequently, other techniques such as thin-layer chromatography (TLC) and gas chromatography (GC) found favor. Within the last 5 years, LCC has undergone dramatic changes and now rivals GC in speed of analysis and column efficiency. The modernized technique is commonly known as high pressure liquid chromatography (HPLC).

Table I roughly compares HPLC with other commonly used chromatographic techniques in terms of support particle size and speed of analysis, N_{eff}/t (see *Measurement of Column Performance*). A maximum N_{eff}/t means the shortest analysis time. Classical LCC and TLC are seen to be slow, although a new form of high speed TLC, thin-film chromatography, was recently described (4). GC and HPLC are seen to be comparable in speed and efficiency. A common misconception in comparing these two techniques is that HPLC is less efficient. Rather, with GC, longer columns and, hence, many more theoretical plates can be used. Because of the much lower permeability of HPLC columns, the apparatus places a limitation on the column length. The lower permeability results from the smaller particle sizes and the higher viscosities of liquids. However, even with relatively short columns, very difficult separations can be accomplished with HPLC due to its greater selectivity. Greater selectivity is achieved through choice of the mobile phase, solvent programming, and variation of the separation mechanism, *i.e.*, adsorption, ion exchange, or partition.

Much early work in HPLC involved the development of high efficiency and high speed systems according to theoretical guidelines. With a knowledge of modern chromatographic theory (5, 6), a better understanding of the basic principles underlying HPLC and the deficiencies of classical LCC techniques is achieved.

THEORY

General Considerations—Improvement in LCC has been achieved primarily through the use of more efficient columns and of higher mobile phase velocities. Column efficiency, or band broadening, is described by the height equivalent to a theoretical plate, H, concept, which is a measure of solute band broadening per unit length of the column. The height H is defined as:

$$H = \frac{\sigma_l^2}{L} = \frac{L}{N} \qquad \text{(Eq. 1)}$$

where σ_l is the standard deviation of the Gaussian function (the solute band profile) in length units, L is the column length, and N is the number of theoretical plates. The latter is given by:

$$N = 16(L/W)^2 \qquad \text{(Eq. 2)}$$

Table I—Comparison of Chromatographic Performance

Technique	Particle Diameter (d_p), μ	N_{eff}/t^a	Reference
TLC	2–20	<0.08	3
Classical LCC	150	<0.05	3
HPLC			
Silica gel	20	2	3
Porous layer beads			
Zipax	30	8	114
Corasil	28–37	2	114
Silica microspheres	5	23	51
GC			
Classical packed	130	10	150
Porous layer beads	90	50	150

[a] Effective plates per second, where $N_{eff} = N[k'/(1 + k')]^2$, N = number of theoretical plates, and k' = capacity factor.

where W is the peak width determined by tangents to the Gaussian profile. In the construction and operation of columns, it is desirable that H be as small as possible.

Until recently, the Van Deemter equation:

$$H = A + B/V + CV \qquad \text{(Eq. 3)}$$

had been used to represent the chromatographic band broadening mechanism. In Eq. 3, the A term arises from complex flow processes (eddy diffusion), the B term describes solute dispersion by longitudinal molecular diffusion, and the C term relates to nonequilibrium resulting from resistance to mass transfer in the stationary and mobile phases. There is now considerable evidence to show that Eq. 3 is generally inadequate (3, 5–7).

Huber and Hulsman (8) and Huber (9) developed an equation, in which H is the sum of four terms, which takes into account four independent processes: mixing by longitudinal molecular diffusion, H_{MD}; mixing by convection, H_{MC}; resistance to mass transfer in the stationary phase, H_{ES}; and resistance to mass transfer in the moving phase, H_{EM}. Hence:

$$H = H_{MD} + H_{MC} + H_{ES} + H_{EM} \qquad \text{(Eq. 4)}$$

The longitudinal molecular diffusion, H_{MD}, is given by:

$$H_{MD} = 2D_m/T_mV \qquad \text{(Eq. 5)}$$

Since H_{MD} is inversely proportional to mobile phase velocity, V, and since the diffusion coefficients, D_m, in liquids are 10^4–10^5 smaller than those in gases, this term contributes significantly only at very low velocities in LCC. Figure 1 illustrates this point and compares *H versus V* curves of LCC and GC. Consequently, at the velocities employed in HPLC, this term is negligible (5, 6).

The most complex dispersion mechanism is convective mixing in the mobile phase. This term is related to point-to-point velocity inequalities within the packed bed (eddy diffusion) and the compensating effects of transverse molecular diffusion and stream splitting (5, 6). Giddings (5, 10) stressed the fact that these terms are not independent but are coupled. An expression similar to the theoretically derived equation (5, 10) was developed (8, 9). The mobile phase mixing term, H_{MC}, is given by:

$$H_{MC} = \frac{2\lambda_1 d_p}{1 + \lambda_2(D_m/Vd_p)^{1/2}} \qquad \text{(Eq. 6)}$$

At high velocities, H_{MC} approaches a value equal to $2\lambda_1 d_p$ (classical eddy diffusion term). Thus, at the higher velocities, lateral diffusion has a smaller effect in decreasing the magnitude of this term. In addition, this term is independent of the capacity factor, k'.

The magnitude of the H_{MC} term is dependent upon the bed structure, the column-to-particle diameter ratio (d_c/d_p), the particle shape, and the packing technique (11, 12). Columns of uniform, dense packing structure give the best column efficiencies (6, 13, 14*a*). Such columns are most readily prepared from dense, spherical particles (13). In addition, Eq. 6 predicts that decreasing the particle diameter should increase efficiency. This effect is experimentally found to be true for particles less than 100 μ only if the correct packing technique is employed (4, 9, 14*a*). Furthermore, theory predicts that increasing the column-to-particle diameter ratio will increase band spreading due to the increasing importance of transcolumn flow inhomogeneities (5). However, the optimum column diameter was found to be between 2 and 4 mm. i.d. for regular packed columns ($d_c/d_p > 10$) (13, 14*b*). Below 2 mm., efficiencies are significantly poorer (13, 15). Apparently, this discrepancy is associated with the difficulty of packing small particles homogeneously into narrow bore tubes. With columns greater than 4 mm., distorted and doublet peaks have been noted (15). This distortion is probably associated with the different packing structure in the vicinity of the column wall (wall effects) (5), where the velocity is greater than the average. Since solute diffusion is slow in liquids, the solute in the vicinity of the walls does not have enough time to diffuse back to the center before elution; hence, bands elute unsymmetrically. However, Knox and Parcher (16) proposed a theory where slow diffusion leads to improved chromatographic performance. These authors calculated that, with a 5-mm. i.d. column of length <0.33 m. containing particles with a diameter of 30 μ, the solute never reaches the wall area before elution. Consequently, the band is not distorted by wall effects. This type of column is known as the "infinite diameter" column and was successfully demonstrated with columns of 7.94 and 10.9 mm. i.d. (15). A column is judged to be of infinite diameter if the column diameter, d_c, is greater than $(2.4Ld_p)^{1/2}$.

At velocities above the minimum in *H versus V* curves (Fig. 1) for GC or LCC, H_{ES} and H_{EM} terms become increasingly important as the velocity increases. These terms relate to nonequilibrium resulting from resistance to mass transfer in the stationary phase, H_{ES}, and the mobile phase, H_{EM} (5, 6). The extent of broadening from nonequilibrium is a function of the rate of flow of the mobile phase relative to the rate of mass transfer of the solute between the mobile and stationary phases. Rate-limiting processes that contribute to this type of broadening in LCC include desorption kinetics and diffusion in the mobile phase which is stagnant in pores of the support[1]. General

[1] For a detailed discussion of these terms, see *Reference 5*.

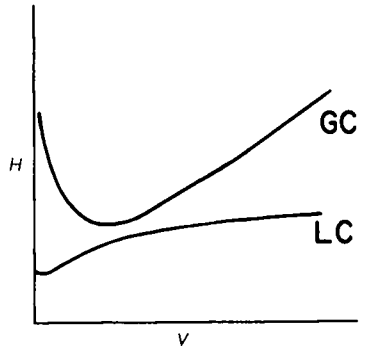


Figure 1—*Typical shapes of* H (*height*) versus V (*velocity*) *curves for GC and LC.*

equations (8, 9) that describe these processes are:

$$H_{EM} = \frac{1}{5.7} \frac{\epsilon_\alpha - \epsilon_m + K\epsilon_\beta}{\epsilon_\alpha + K\epsilon_\beta} \frac{\epsilon_m^{1/2} d_p^{1/2} \eta^{1/6} V^{1/2}}{(1 - \epsilon_m) D_m^{1/2}} \quad \text{(Eq. 7)}$$

and:

$$H_{ES} = \frac{1}{30} \frac{\epsilon_\alpha - \epsilon_m + K\epsilon_\beta}{(\epsilon_\alpha + K\epsilon_\beta)^2} \frac{\epsilon_m(1 - \epsilon_m)}{\epsilon_s} \frac{T_s d_p V}{D_{ts}} \quad \text{(Eq. 8)}$$

H_{ES} is more strongly dependent than H_{EM} upon the mobile phase velocity, V, and the particle diameter, d_p. However, both terms are minimized by decreasing d_p. Many workers have noted, however, that for $d_p < 100\ \mu$, H increases (4, 17, 18). This discrepancy from theory is associated with the difficulty of packing homogeneously small particles, especially those of a low density, nonspherical type (15, 18). However, with special packing techniques, the use of small diameter particles was impressively demonstrated by Hamilton (19), Scott *et al.* (20), and Burtis (21) using roughly 10–20-μ particle diameter ion-exchange resins. Piel (22) successfully used a variety of supports in the submicron region, and Huber and Hulsman (8) and Huber (9) packed efficient columns with 20–30-μ diatomaceous earth. The successful use of small diameter particles to reduce mass transfer effects requires dense uniform packing, *i.e.*, minimal contribution from H_{MC}. Recently, Kirkland (23) demonstrated the superiority of small particles with 5-μ porous silica microspheres (Table I).

Equations 7 and 8 indicate that the mass transfer term can also be reduced by maximizing solute diffusion coefficients, D_m and D_{ts}, in the mobile and stationary phases, respectively. Since the liquid diffusion coefficient is inversely proportional to liquid viscosity, the stationary and mobile phases with the lowest possible viscosities should be chosen (2, 24, 25).

Resistance to mass transfer from diffusional processes in the stationary phase can also be reduced by minimizing the diffusion path length (*i.e.*, minimizing stationary film thickness) (5, 11). In addition, a stagnant mobile phase in the pores of the support further contributes to slow mass transfer (5, 6, 11). These effects have been decreased by the reduction in particle size, since specific pore volume decreases with decreasing particle size (8, 9), and by the development of porous layer beads, which will be described.

Equation 8 can be simplified if it is assumed that the pores of the support are completely filled with β (stationary phase). In such a case:

$$H_{ES} = \frac{1}{30} \frac{k'}{(1 + k')^2} \frac{T_s d_p^2 V}{D_s} \quad \text{(Eq. 9)}$$

where k' is the capacity factor equal to $K(\epsilon_\beta/\epsilon_\alpha)$.

The capacity factor function reaches a maximum at $k' = 1$ and then decreases strongly with increasing k'. If mobile phase effects predominate over stationary phase effects, H is essentially independent of k' (6). However, since the magnitude of k' is related also to sample resolution and since low k' values are desirable for fast analysis, there is an optimum k' value (6). Snyder (3) calculated the optimum value of k' to be 5 for a given column length and any desired column pressure and 2.9 for a given column pressure and any column length.

Speed and Optimization in Liquid Chromatography—A number of recent papers dealt with the theoretical treatment of the optimization of chromatographic parameters for fast separations (1, 8, 17, 22, 26–28). Since the time of separation is a function of a number of parameters, mathematical optimization with respect to all relevant parameters is virtually impossible (28). The problem is simplified by holding a number of parameters fixed (26, 28). However, all treatments of the resolution of a two-component system start with the following equation:

$$t_r = (1 + k') \frac{L}{V} = N(1 + K') \frac{H}{V} \quad \text{(Eq. 10)}$$

Resolution is related to N, the number of theoretical plates necessary to achieve the separation. The difficulty of the optimization problem arises from the complexity of the H function, which is in itself a function of the other parameters in Eq. 10. Equation 10 does, however, show that the retention time, t_r, can be reduced by minimizing the H/V ratio. It follows that the optimum conditions for fast analysis correspond to the minimization of the H/V factor. However, the H/V factor initially decreases sharply with increasing V but then decreases slowly at higher velocities (6, 9, 26). The point at which the slope of the H/V *versus* V plot stops changing significantly can be considered as the optimum velocity, especially where pressure drop is a limitation. The optimum velocity for 2–3-mm. i.d. columns is about 2–3 cm./sec. (6). Beyond the optimum velocity, a decrease in analysis time is afforded only by a large increase in pressure drop, ΔP, in the column. Pressure drop is related to velocity through the column permeability, K^0:

$$K^0 = \frac{V\eta L f}{\Delta P} \quad \text{(Eq. 11)}$$

The importance of pressure drop and permeability in regard to analysis time can be seen by substituting the expression for V from Eq. 11 into Eq. 10:

$$t_r = \frac{(1 + k')\eta L^2 f}{K^0 \Delta P} \quad \text{(Eq. 12)}$$

For constant values of k', η, L, f, and ΔP, the more permeable the column the faster is the analysis time. Equation 12 also indicates that the analysis time can be

decreased by lowering the solvent viscosity, η, and the column length, L.

Equation 10 indicates that t_r can be reduced by optimizing factors that minimize H. These factors were discussed previously, but, in the main, a decrease in particle size (8, 9, 26) and the development of support materials that give flat H *versus* V curves will give the fastest analysis time.

The equations used previously to describe analysis time neglected the effects of extracolumn band broadening. The shorter the column length, the smaller must be the contributions from injection and detection (26, 28). Smuts and Pretorius (28) showed that, for simple separations, the minimum separation time is limited by extracolumn effects.

COLUMN DESIGN

Column Packings—The use of the relevant theories has led to the development of two methods to achieve high speed analysis by LCC: the development of porous layer beads of 30–50-μ diameter and the use of supports with particle diameters less than 10 μ. Because both supports have shallow pore depths, slow mass transfer in the stagnant mobile phase held within the pores is reduced.

A porous layer bead has a hard, nonporous core surrounded by a thin, porous shell. Golay (29) and Bohemen and Purnell (30) first suggested, from a theoretical standpoint, the advantages of porous layer beads for fast analysis. Other authors (31, 32) suggested the use of such supports for LCC. Rapid mass transfer is possible, since diffusion occurs only in a thin, porous layer. As a result, flat H *versus* V curves are produced; consequently, high carrier velocities can be used with small loss in efficiency. The various types of porous layer beads were described (6, 33, 34). Two commercially available supports, Zipax[2] (26, 35) and Corasil I and II[3] (36), have siliceous, porous layers of roughly 1-μ thickness. Zipax is a relatively inert support (35) and is used primarily in liquid–liquid chromatography (LLC). The Corasils are much more active and, therefore, can be used for both LLC and liquid–solid chromatography (LSC) (36). Supports with a porous layer of alumina have also been prepared (11, 37). A new type of support, the surface-textured bead having 30–40-μ diameter and surface-etched pores with 2–4-μ depths, has potential in high speed LLC (24). However, surface-textured beads have even a lower sample capacity than the porous layer beads. Porous layer adsorbents also have been developed for ion-exchange chromatography. A pellicular ion-exchange bead[4] in which the ion-exchange resin is polymerized on the surface of a spherical, solid glass bead has been introduced (11, 38*a*). Kirkland (39, 40) developed similar materials.

In addition to the fast mass transfer properties of porous layer beads, their spherical shape, high density, and rigid structure yield dense, regular packed beads which are stable at high pressures. However, because of their low surface areas, small sample sizes and very sensitive detectors must be used.

A possible solution to the capacity problem is to use porous supports of small particle diameters (<10 μ). As already noted, the use of supports with such small particle sizes has been successfully demonstrated. Supports that have been used are diatomaceous earth (8, 9, 41), silica gel (4, 22), alumina (22), carbon black (22), silica microspheres (23), and ion-exchange resins (20, 42). Huber and Hulsman (8), Huber (9), and Deelder *et al.* (41) used diatomaceous earth of 28–32 and 9 μ. The efficiency of these columns for retained or unretained solutes was comparable to efficiencies of porous layer beads. However, with the exception of ion-exchange resins, for which special packing techniques have been developed (43, 44), practical difficulties arise in preparing homogeneous beds of these small particles (4). Very narrow size fractions are a definite must in preparing satisfactory columns (8). Batch separation into narrow size fractions by elutrification was described for diatomaceous earth (41) and ion-exchange resins (42). With the development of reproducible packing techniques, 2–5-μ porous particles will become commonplace in the future. Some supports available for HPLC are shown in Table II.

Column Tubing and Shape—Kirkland (39) found significantly poorer efficiencies for solutes chromatographed in columns of seamless stainless steel relative to precision glass[5] and precision bore stainless steel. Karger *et al.* (24) also found precision glass tubing to give better efficiency than seamless stainless steel; however, the difference between the two column tubings was not as great as was found by Kirkland (39). Karger *et al.* (24) recommended that the seamless stainless steel be washed as follows: chloroform, acetone, water, 50% phosphoric acid, 10% nitric acid, water (until washings are neutral), acetone, and chloroform. Kirkland (45) recommended scrubbing the interior with laboratory detergent solution.

Columns filled with packings and then bent have shown a significant decrease in efficiency in comparison to straight columns (39, 46). Straight columns 50 or 100 cm. in length are generally used. To obtain more theoretical plates, columns can be connected in series with low dead volume connectors. Kirkland (33) obtained a 5–6% loss in efficiency for two connected columns and a 10% loss for three connected columns.

Column Packing Techniques—Column packing is one of the most difficult and most important experimental operations in high efficiency LCC. The various packing techniques were reviewed (4, 47). Columns are packed by either dry-fill (14, 45, 48) or wet-fill (14, 44, 49) techniques.

The standard dry-fill technique involves small incremental additions of dry packing, with horizontal tapping of the column at the level of the packing and with simultaneous vertical tapping of the column on the floor or benchtop. Sie and van den Hood (14*a*) found H to decrease by a factor of 5 or more for relatively large column diameters (d_c = 1.0 cm.) when a special dis-

[2] Dupont, Wilmington, DE 19898
[3] Waters Associates, Framingham, MA 01701
[4] Available from Northgate Laboratories, Hamden, Conn.
[5] Truborc.

Table II—Supports for High Pressure Liquid Chromatography

Type	Particle Size, μ	Use
	Porous Layer	
Silica (active)	37–50[a]	LLC, LSC
	37–44[b]	LLC, LSC
	30–40[c]	LLC, LSC
	30–44[d]	LLC, LSC
Silica (inactive)	44–53[e]	LLC
	25–37[f]	LLC
Alumina	37–44[g]	LLC, LSC
	Porous	
Silica	20–44[h]	LSC, LLC
	37–75[i]	LSC, LLC
	<40[j]	LSC, LLC
	36–45[k]	LSC
	10,20,30[l]	LSC, LLC
Alumina	18–30[m]	LSC, LLC
	<74[n]	LSC, LLC

[a] Corasil I and II (Waters Associates). [b] Pellosil (Northgate Laboratories). [c] Perisorb A [Varian, E. Merck (Germany), E. M. Laboratories]. [d] Vydac (Applied Science Laboratories, Separations Group). [e] Liqua-Chrom (Applied Science Laboratories). [f] Zipax (Dupont Instruments). [g] Pellumina (Northgate Laboratories). [h] BioSil (Varian, Bio Rad Laboratories). [i] Porasil (Waters Associates). [j] Spherosil [Pechiney-Saint-Gobain (France)]. [k] Sil-X (Nester-Faust). [l] Li Chrosorb [Varian, E. Merck (Germany), E. M. Laboratories]. [m] Woelm (Waters Associates). [n] Bio Rad AG (Bio Rad Laboratories).

tributor head for feeding the adsorbent evenly into the column, plus rapid rotation and tapping of the column, is used. These workers also found that smooth particles gave consistently better columns by the dry-fill technique. They found H to decrease in the following order: silica beads > silica gel > alumina > sil-o-cel. In addition to vibration, Stewart *et al.* (50) recommended gentle tamping (weight of rod on top of the packed adsorbent) for the dry fill of silica gel. The efficiencies of tamped columns improved with decreasing particle size down to about d_p equal to 50 μ. The loss in efficiency for smaller particle sizes is probably due to the difficulty of obtaining dense, regular bed structure with irregularly shaped particles of alumina or silica gel (4). Conversely, the dense, spherical porous layer beads can be efficiently packed with the standard dry-fill techniques for particle sizes down to 20–30 μ (33). Kirkland (45) described an optimum procedure for packing Zipax. Column efficiency was found to improve with a decrease in particle size down to at least 37 μ. For 1% β,β'-oxydipropionitrile on Zipax (<37 μ), plate heights of 0.23 mm. ($k' = 2$) were found at 1 cm./sec. mobile phase velocity. Although other porous layer packings were not included in this study, similar results are expected.

Other workers (8, 9, 41) were able to pack good liquid partition columns with diatomaceous earth with d_p down to 10 μ. Columns were packed by small incremental additions of the support, with tamping between increments using a closely fitting rod. However, such packings have the disadvantages of bed compression and lower permeability at pressures exceeding 1 atm./cm. (41).

For supports of particle sizes less than about 20 μ, the slurry packing technique is often used (14, 44, 49). The balanced slurry technique (49), where the support particles are suspended in a solvent of equal density, is probably the best method. Sie and van den Hood (14*a*) recommended the use of calcium sulfate hemihydrate as a binder in the slurry. Silica[6] with a mean particle size of 10 μ, when packed by the balanced slurry technique, gave columns of high efficiency (51). On the other hand, Stahl *et al.* (52) found poorer efficiencies by the slurry technique (probably not balanced slurry) than by a dry-fill technique for silica gel particles of less than 15 μ. More recently, Kirkland (23) introduced silica microspheres (5-6-μ particle diameter) and generated 23 effective plates/sec. (see *Measurement of Column Performance*) with these particles packed by a slurry technique in a column 35 × 0.32 cm. at 600 p.s.i. pressure drop.

INSTRUMENTATION

Many papers have been written on the overall design of LCC systems (53–61). Scheme I shows a block diagram of a simplified liquid chromatograph. Although many manufacturers supply complete systems, a liquid chromatograph can be built easily from individual components which are readily available. In general, all components should be constructed of corrosion-resistant materials such as stainless steel, glass, or Teflon.

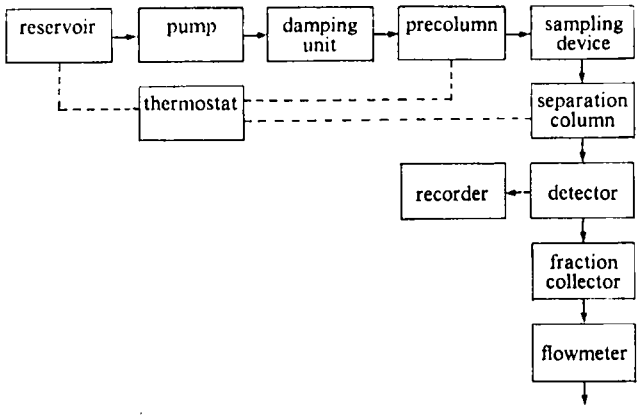
Flow System

Pumps can be classified into two major types: mechanical and gas displacement. Mechanical pumps are constant flow rate devices such as the screw-driven syringe pump and the reciprocating piston or diaphragm pumps. The former are pulse free, whereas the latter type requires pulsation dampening for detector stability. Several methods for pulsation dampening have been described (57, 60, 61). Karger and Berry (62) constructed a simple device for producing pulseless flow with inexpensive reciprocating piston pumps. Mechanical pumps can be purchased which operate to pressures of about 5000 p.s.i.

Of the gas-displacement pumps, the gas pressurized reservoir (2, 54) is the least expensive and is capable of pulseless flow. The reservoir can be a stainless steel container or a coil of stainless steel tubing, pressurized by nitrogen from a gas cylinder. A second type of gas-displacement pump is the gas-driven piston pump (55), which amplifies the gas pressure by utilizing a large area gas piston pushing against a smaller area liquid piston. The prime disadvantage of gas-displacement pumps is that the flow rate changes with varying back pressure.

The design of the injection port[7] is critical to minimize band spreading, especially for solutes of low k'. Inlet bandwidths must be restricted to submillimeter values. The major factors that describe band spreading in the injection port are: (*a*) distance between syringe needle and the column inlet, (*b*) mobile phase linear velocity, (*c*) sample volume, and (*d*) rapidity of radial mass transfer (63). Direct on-column injection gives minimum band spreading (63). Mobile phase swept injection ports (including sampling valves) are satisfactory for solutes with $k' > 1$ (53). Smuts *et al.* (63) designed

[6] Li Chrosorb, E. M. Laboratories, Inc., Elmsford, NY 10523
[7] For design of injection ports, see *References 53–61.*



Scheme I—Diagram of a liquid chromatograph

a unique solvent swept inlet system giving bandwidths as small as 0.3 mm.

Detectors

A number of excellent papers have been published on the various detectors available for LCC (64–66). Munk (67) and Huber (68) compared the most commonly used detectors. LCC detectors can be categorized as solute sensitive or bulk sensitive.

Solute-Sensitive Detectors—*UV and Visible Absorption*—The UV photometer is most commonly used since it is relatively insensitive to variations in flow rate or temperature and is adaptable to solvent programming. A number of workers (69–71) described the construction of low dead volume UV detectors. These detectors and the commercially available detectors are single wavelength units (254 or 280 nm.). Such detectors are capable of sensing less than 10^{-9} g./ml. (55). Commercial spectrophotometers have also been used successfully as high performance detectors (68, 72–74). The obvious advantage is wavelength selectivity. In addition, in some cases the number of solvents to choose from is increased. In cases where solutes have little or no UV absorption, the use of color-forming reactions can be of great value for increasing sensitivity and selectivity. Stahl *et al.* (52) described the construction of a high performance system for the analysis of carboxylic acids in the nano-equivalent scale, using the sodium salt of *o*-nitrophenol as the indicator. The authors suggested the use of umbelliferone, whose protonated form is highly fluorescent, as an alternative indicator. Such a technique can be of extreme value in trace analysis and metabolism studies.

Fluorescence—Although no work has been published using a fluorescence detector in HPLC, a high performance, dual-beam fluoro monitor[8] is on the market. In addition, filter fluorometers[9] can be converted with suitable flowthrough cells (75). The technique has the advantages of specificity and high sensitivity (about 10^{-9} g./ml.). In addition, fluorescent derivatives can be prepared from many compounds lacking fluorescent capabilities.

Flame Ionization—To obtain a suitable response in a flame-ionization detector, the solvent must be removed. This is done by coating part of the column effluent on a moving wire which then passes through a heated chamber. Currently, two general methods of getting the sample into the flame are used: direct combustion in the flame (76–81) and pyrolysis of the residue on the wire prior to detection in the flame (82–85). The former technique is noisier due to the movement of the wire through the flame and the intermittent misalignment of the wire in the flame. Scott and Lawrence (83) modified the pyrolysis detector in such a way as to reduce significantly the noise level. Instead of pyrolyzing the solute, combustion of the solute in an oxygen atmosphere to carbon dioxide is used. The carbon dioxide is then hydrogenated catalytically over nickel to methane which is then passed into the flame-ionization detector. This detector's response is directly proportional to the carbon content, and the sensitivity is about 2 mcg./ml. of mobile phase. The moving wire detector can be used with all modes of column chromatography including gradient elution. However, it has the disadvantages of: (*a*) poor sensitivity since only part of the effluent is detected, (*b*) markedly reduced sensitivity as solute volatility increases, and (*c*) imprecise quantitation if the sample is not homogeneously dispersed over the column cross section (68).

Polarographic—The polarographic detector is specific and highly sensitive. The detector is sensitive to solutes that can be oxidized or reduced at the mercury electrode. Additional specificity results from varying the half-wave potential. The only other requirement is that the mobile phase must have sufficient conductivity.

A detector based upon the dropping mercury electrode (DME) suitable for high speed LCC was described by Koen *et al.* (86). The cell volume was only a few cubic millimeters. The detector was successfully applied to the chromatographic analysis of methyl parathion and parathion at concentrations down to 10^{-8} mole/l., with a standard deviation less than 2%. The major problem of this design is dissolved oxygen in the mobile phase and the sample solution, and damping is required to reduce the noise associated with the DME. The sensitivity of the detector depends upon the electrochemical valency, the solute diffusion coefficient, the flow rate and drop time of the mercury, and the flow rate of the eluant.

Joynes and Maggs (87) described a simpler system based upon a carbon-impregnated silicone membrane as the electrode. The advantages over the DME are low residual currents, decreased proneness to mercury oxide films, simple construction, low noise level (no damping required), no atmospheric exposure of mercury, and 100 times lower interference from oxygen in the mobile phase. The linear dynamic range is of the order 1×10^5, with a detection limit of about 2×10^{-9} mole/l. (organics), and the reproducibility is about 0.25%.

Radioactivity—This detector is extremely useful for metabolic studies because of its specificity and high sensitivity. The utilization of β-radiation using suspended scintillators in continuous-flow systems was described (88). A detector suitable for high speed LCC

[8] Laboratory Data Control.
[9] American Instrument Co., G. K. Turner Associates.

was also described (89). The detector cell consists of a Teflon tube, 36 mm. in length × 0.38 mm. i.d., in a continuous-flow Geiger-Muller counter.

The following expression for detector sensitivity was derived:

$$\text{peak area} = \frac{S_A V_D}{\mu} A \qquad \text{(Eq. 13)}$$

where S_A is the sensitivity of the detector, V_D is the volume of the detector cell, μ is the volume flow rate, and A is the total activity of the sample component. The sensitivity is seen to be inversely proportional to the flow rate but directly proportional to the cell volume. A compromise must be made between the cell volume size and the tolerable band spreading.

Davies and Mercer (90) described an automated system for the analysis of labeled, unsaponifiable lipids. The scintillator flowthrough cell was packed with europium-activated calcium fluoride crystals.

Bulk-Sensitive Detectors—*Refractive Index*—The refractive index detector monitors the difference in refractive index between the reference and analytical column effluents, and sensitivity is determined by this difference. This detector is close to being a universal detector, the exception being when the solute and solvent have the same refractive index. Two types are commercially available: the deflection type[3] and the reflection type[10] (91). The sensitivity is about 10^{-7} refractive index unit full scale. However, to achieve such sensitivity, the temperature and flow rate must be controlled precisely (64, 67). The temperature coefficient is about 10^{-4} refractive index unit/°C. Heat exchanger tubing is commonly used. To minimize band spreading and to improve temperature stability in the heat exchanger, Deininger and Halasz (61) recommended that the free cross section of the heat exchanger be squeezed alternately at 90° angles. Thus, high radial mass transport in the heat exchanger tube is achieved through turbulent flow. The peak broadening is minimized to that of the high efficiency UV detectors, and the noise levels are reduced to less than 3×10^{-8} refractive index unit.

Solvent programming is not feasible unless solvents of the same refractive index are used (92). A new detector (93) based upon continuous evaporation of the eluate and redissolution of the residue in a fixed solvent gives the maximum response in a refractive index detector.

Heat of Adsorption—The heat of adsorption detector or microadsorption detector (94–100) is a universal detector which measures minute temperature changes accompanying the sorption of the eluent and the solute on an active solid surface, usually the support held in the chromatographic column. The detector can be used with all modes of chromatography. However, this detector is extremely flow and temperature sensitive. In addition, it requires frequent calibration, and the peak shape (97, 100) is unusual in that a positive component and negative component result. The real value of this detector is in qualitatively visualizing the elution pattern (64).

[10] Laboratory Data Control.

Other Detectors—The conductivity detector is sold by a number of manufacturers and is commonly used in amino acid analyzers and in gel permeation techniques (65). Other techniques that have been applied to HPLC are the gas density balance (101), the mass (102), the electron-capture (103), the interferometer (104), and the capacitance (105–107) detectors.

LIQUID CHROMATOGRAPHY METHODS

Selectivity—The ability of any chromatographic technique to perform separations depends upon the efficiency and selectivity of the chromatographic system. The former was discussed previously while the latter is expressed in terms of the relative retention, α, that is equal to the ratio of retention volumes of two solutes. Selectivity is determined by solute–solvent or solute–adsorbate interactions and, to a lesser extent, by their molecular size and shape (108).

Although chromatographic systems are primarily chosen by trial and error, some theoretical and empirical treatments have been developed which can be of aid. Snyder (109) discussed the role of the mobile phase in LCC. Solvent polarities are measured in terms of the Hildebrand solubility parameter, δ, and the solvent strength parameter, ϵ^0. In addition, Keller and Snyder (110) discussed the relationship between δ and ϵ^0.

Martire and Locke (111) considered the thermodynamic basis for selectivity in terms of both the athermal (size) and the thermal (energy) factors in LLC. Locke (112) measured the infinite dilution activity coefficients of hydrocarbons in the acetonitrile–squalene system.

Huber (113) reviewed the methods for prediction of partition coefficients in liquid–liquid systems. Recently, Huber *et al.* (114) developed a new method of predicting partition coefficients. The partition coefficient (K_i) is described as a function of P parameters, each having a solute factor (A_{ip}) and a liquid–liquid system factor (X_{kp}). The equation has the form:

$$\log K_i = \sum_P A_{ip} X_{kp} \qquad \text{(Eq. 14)}$$

The method was tested for 28 steroids in six ternary liquid–liquid systems composed of water, ethanol, and isooctane. For $P = 3$, a precision of about 4% was obtained.

Liquid–Liquid Partition—*Liquid–Liquid Systems*—The types of supports available for partition chromatography are shown in Table II. The various porous layer beads were evaluated by Karger *et al.* (115), and a similar comparison was made by Kirkland (33) and Majors (116). On the basis of the data given, Zipax appears to be the best porous layer support for liquid–liquid chromatography. The Corasils are much more active than Zipax since Corasil I, when packed dry, adsorbs 1.1% (w/w) β,β'-oxydipropionitrile from eluent saturated with β,β'-oxydipropionitrile. On the other hand, Zipax adsorbs only 0.25% (115). Majors (116) recommended that Corasil be deactivated by heating to 350° for LLC. Tailing was partially eliminated upon deactivation. Huber *et al.* (117, 118) successfully used small particles of ground Porasil (2–5 μ) and diatomaceous earth (5–10 μ, 20–30 μ) as LLC supports.

To produce columns of high capacity, Halasz *et al.* (119) proposed the use of heavily loaded columns. Columns packed with Porasil A (38–53 μ) coated with 50% (w/w) β,β'-oxydipropionitrile exhibited high capacity factors which were independent of carrier linear velocity. Such columns have low bleed rate and large sample capacity (at least 1 mg. in 2-mm. i.d. column). Heavily loaded columns should perform well in preparative applications, providing the stationary phase can be separated easily from the solutes. Kirkland (23) recently introduced the use of 5 6-μ silica microspheres. This packing is not yet commercially available.

A fluorocarbon support[11] coated with 23% Amberlite LA-1 gave excellent separation of steroids by reverse phase chromatography (120). Zipax and diatomaceous earth supports were also used in this study.

Karger *et al.* (24) introduced surface-textured beads, small particle diameter fractions of GLC-100 support[12]. Although very efficient columns (5500 plates/m., 1 cm./sec.) were produced, the capacity of the columns was poor.

Kirkland (33, 48) reviewed the various packed columns and the experimental techniques of LLC.

The choice of stationary phase in LLC is, at this stage, largely trial and error. Commonly, binary two-phase systems are used. For polar solutes, polar mobile phases such as β,β'-oxydipropionitrile, 1,3-propanediol, ethylene glycol, and Carbowax are used with nonpolar carriers saturated with stationary phase. To decrease retention times, polar modifiers such as chloroform, tetrahydrofuran, or alcohol can be added to the carrier up to about 10% (v/v). Binary, ternary, or quaternary two-phase systems are also useful.

Huber *et al.* (114, 118) described six ternary two-phase systems of water, ethanol, and isooctane for the analysis of steroids. With such systems, the relative polarities of the two phases can be varied to achieve optimum k' and α values. Other possible ternary systems are chloroform cyclohexane nitromethane, benzene–water–alcohol, and water ethyl acetate–*n*-butanol. Commonly, the polar phase is used as the stationary phase; however, for reverse phase applications, the less polar phase can be coated onto surface-deactivated supports. Commonly used nonpolar, stationary phases are squalene (112), hydrocarbon, or cyano ethyl silicone polymers (121). A series of liquid–liquid partition systems which may find use in LLC was described by Metzsch (122).

Experimental techniques necessary to obtain precise quantitative analysis with high speed LLC were described by Leitch (123).

Permanently Bonded Stationary Phases—LLC has such disadvantages as the tendency of the stationary phase to bleed and the inability to solvent program. To overcome these disadvantages, column packings with chemically bonded, organic, stationary phases have been developed. Two different types of chemically bonded phases are commercially available.

1. Esterified Siliceous Supports (Brushes)—Esterification of surface-active silanol groups (Si—O—H) of silica with alcohols creates the Si—O—C bond at the silica surface. Halasz and Sebestian (124) bonded 3-hydroxypropionitrile to the surface of Porasil C and demonstrated the usefulness of "brushes" for fast gas and liquid chromatographic separations. Durapaks[3] are commercially available silicate ester packings. A more efficient packing is made by the esterification of Corasil. The disadvantage of these packings is that the Si—O—C bond is not hydrolytically stable. In addition, extreme caution is necessary if the eluant contains proton donor liquids such as isopropanol (125). With such solvents, a good proportion of the bonded material elutes (126).

A more promising brush-type bonded phase is based upon the much more hydrolytically and thermally stable Si—C bond. Locke *et al.* (127) produced naphthyl-Porasil C by chlorinating the surface of Porasil with silicon tetrachloride or titanium tetrachloride followed by coupling with naphthylmagnesium bromide. Organic contents of 4.0 ± 0.5% were found. The reactive, bonded naphthalene ring can undergo such reactions as bromination or sulfonation. Repeated bromination of naphthyl-Porasil C and combination with naphthylmagnesium bromide provided a polynaphthyl-Porasil containing as much as 24% by weight of organic material. Both cation and anion exchangers were prepared from these substituted Porasils.

2. Chemically Bonded Silicone Polymers—Bonded silicone polymers are prepared by the reaction of organochlorosilanes with the active surface hydroxyls to form silicone polymers of the type Si—O—(R_2 Si—O—)$_n$, where R is an organic radical bonded by carbon to the siloxane chain. Aue and Hastings (128) described the means of preparation and chromatographic applications of surface-bonded silicones to diatomaceous earths, silica gels, silica beads, and glass beads. These materials were thermally and hydrolytically stable.

Similarly, Kirkland (49) and Kirkland and De Stefano (129) introduced two permanently bonded silicone polymer phases, where R = $C_6H_{12}O_2$, R′ = O—(ether bonded phase) and R = C_2H_4CN, R″ = CH_3 or —O— (nitrile bonded phase), for HPLC. Schmit *et al.* (130) introduced a bonded phase where R = $C_{18}H_{37}$ (octadecyl silicone) for reverse phase chromatography. Both the ether and nitrile bonded phases show selectivity toward proton donors (129). The octadecyl phase is selective for samples that are sparingly soluble in water and fairly soluble in alcohols and/or alkanes (130). The retention mechanism on these columns is not clearly understood; however, there is evidence that the mechanism is a combination of both partition and adsorption (33, 49, 130). Unlike conventional liquid–liquid systems, the temperature, the modifier, or the percent modifier can be varied at will without any column deterioration. Undoubtedly, many future developments in LCC will involve synthesis of many new bonded phases of varied polarities.

Liquid–Solid Chromatography For reviews and for detailed discussion of the experimental technique and selectivity in liquid–solid chromatography (LSC), consult the works of Snyder (1–3, 109, 110, 131–133).

[11] Plaskon CTFE-2300, Allied Chemical.
[12] Corning Glass.

LSC is best suited to samples of intermediate molecular weight (<1000), particularly those that are oil soluble (131). Any sample type that has been successfully chromatographed by TLC can be separated by LSC. Separations are usually carried out on polar adsorbents such as silica, alumina, or other inorganic solids[13].

Both porous layer materials and porous supports are used in high performance LSC. The porous layer materials (alumina and silica) produce faster and more efficient separations than porous support materials. Little *et al.* (36) found pellicular silica (Corasil I and II) to give substantially less peak broadening than conventional porous silica beads (Porasil) of the same particle diameter (37–50 μ). However, the disadvantage of porous layer beads is their low sample capacity. Recently, Majors (134) showed that columns packed with 10-μ diameter silica gel[14], when packed by the balanced slurry technique, can give 10^4 theoretical plates/m. Sample sizes of 1 mg./g. of adsorbent can be injected on such columns.

Snyder (2, 3) reviewed the factors affecting column efficiency in LSC.

Stewart *et al.* (50) compared high pressure LSC with TLC and found comparable separations; however, LSC produced better resolution and shorter analysis times.

Lebedeva *et al.* (135) studied the effect of the geometrical structure and the surface chemistry of silica gels on separations in LSC. They concluded that, for analytical purposes, it is preferable to use macroporous silica gels with specific surface areas of 80–300 $m.^2/g.$ and an average pore radius greater than 80 Å.

The need to control carefully the water content of the adsorbent and the mobile phase has been emphasized (131, 132). The addition of water to the adsorbent increases linear sample capacity and efficiency. For porous silica or alumina, 0.02–0.04 g. of water should be added for each 100 $m.^2$ of surface area (131).

The use of adsorbents impregnated with complexing agents can greatly improve separations of isomers and compounds within a homologous series. On the other hand, classical LSC is most selective for compound classes. Snyder (132) reviewed the use of complexing agents in LSC. For example, adsorbents impregnated with silver nitrate are selective for olefins. Recently, Vivilecchia *et al.* (136) used Zipax impregnated with silver oxide as a specific adsorbent for the rapid separation of polynuclear aza aromatic hydrocarbons. Selectivity is based upon complexation between the aromatic nitrogen atom and the silver ions.

Ion-Exchange Chromatography– The speed of ion-exchange chromatography was significantly improved by the development of pellicular ion exchangers by Horvath and coworkers (38). Pellicular ion exchangers consist of a nonporous, central core and a thin, porous outer shell of the ion-exchange resin. Similar materials were introduced by Kirkland (39, 40). Both anion and cation exchangers are available commercially[15]. These materials have small ion-exchange capacities in the order of 8–10 μeq./g. Dupont's cation exchanger exhibits an exchange capacity of 3.5 μeq./g. (40). Although sample capacity is low, these exchangers exhibit high efficiency.

Spherical, porous ion-exchange resins of small particle sizes in the range of 10 μ can be used as a high efficiency, high capacity packing (20, 43). A tabulation of the various ion exchangers for HPLC was compiled (134).

The performance of ion exchange is a complex function of many variables, including temperature, pH, buffer type, and ionic strength. Increasing temperature generally reduces retention volumes and improves column efficiency (38*a*, 40). Column performance improves with increasing temperature due to increased solute diffusivity in both phases. At present, no good guide exists for the optimization of the mobile phase. However, resolution is primarily affected by the pH and ionic strength. Temperature is of less importance in this respect.

To achieve optimum resolution of adenine and its metabolites, Gere *et al.* (137) reported that it is best to keep the temperature as high as possible while varying the pH and ionic strength. Since columns packed with pellicular ion-exchange resins are characterized by a large ratio of mobile phase to stationary phase, eluent strengths of the mobile phase can be much lower than in conventional ion-exchange columns.

Gel Chromatography– Steric-exclusion chromatography is most commonly used for the analysis of biological macromolecules and synthetic polymers. The large number of publications describing the principles and applications of gel chromatography are far beyond the limits of this paper. General reviews (138–140) and a monograph (141) were published on this subject.

Programming—The broad variance in band migration rates of a multicomponent sample under fixed experimental conditions (normal elution) often leads to poor separation of the first eluted components and/or excessive separation times with concomitantly poor detectability. This "general elution problem" can be handled by several techniques: flow or pressure programming, solvent programming, temperature programming, or coupled column operation (stationary phase programming).

Flow programming (142, 143) involves the continuous increase of mobile phase velocity to increase band migration rates of the later eluting peaks. In addition, slow mobile phase velocity at the beginning of elution can lead to increased resolution for the weakly retained components due to an increase in column efficiency at lower flow rates. However, resolution at the back end of the chromatogram is sacrificed (higher mobile phase velocity) as compared to normal elution (143).

The other techniques involve changes in capacity factors (k' programming). Of these techniques, solvent programming (gradient elution) is the most common. Snyder (144) reviewed gradient elution, and Henry (53) described several commercial gradient devices. Other gradient devices were recently described (92, 145, 146). Snyder and Saunders (147) theoretically derived optimum conditions for solvent programming

[13] See *Reference 134* for a complete compilation of LSC adsorbents.
[14] Available from E. M. Laboratories and Varian.
[15] Northgate Laboratories and Dupont Instruments.

with LSC. Under optimum conditions, gradient elution can provide marked improvement in resolution at all points in the chromatogram, particularly at the front end (143).

Temperature programming in LCC has not found much use. Two types of temperature programming have been used: "normal" programming where the temperature is raised during the separation (135) and "inverse" programming where the temperature is lowered (142, 148). Inverse programming is restricted to LSC with mobile phases containing a "moderator," a very strongly adsorbing compound at low concentration (*e.g.*, 0.15% isopropanol in *n*-heptane). As the temperature is lowered, the moderator covers more of the adsorbent active sites, thereby reducing the k' values of the solutes. The major problem with this technique is the long times required to reequilibrate the column to the starting condition (142).

Coupled-column operation involves the use of two or more columns, each with a different stationary phase or with the same stationary phase but different column lengths (143). The latter does not involve change in k' values. Snyder (143) described an LSC system where adsorbent strength is varied by water deactivation. Recently, Scott *et al.* (20) coupled anion- and cation-exchange columns for the analysis of urine constituents. With parallel gradient elution of the columns after injection, 16 new peaks (total >100) were resolved and analysis time was reduced from 24 to 14 hr.

Snyder (143) compared normal elution, coupled columns, solvent programming, flow programming, and temperature programming under time normalization. He found that resolution decreased in the order solvent programming > coupled columns > temperature programming > flow programming > normal elution. Solvent programming is especially useful for unknown samples but of less value for routine or repetitive samples since reequilibration time is long. Temperature programming has the same disadvantage. Coupled columns and flow programming are more amenable to repetitive analysis.

MEASUREMENT OF COLUMN PERFORMANCE

Since capacity factors (k') in HPLC are generally small, the use of the number of theoretical plates (N) as a measure of the separating power of a column is unsatisfactory since resolution is also a function of k'. A more suitable parameter is the number of effective plates, N_{eff} (6, 149), where:

$$N_{eff} = \left(\frac{k'}{1 + k'}\right)^2 N = 16\left(\frac{t_r - t_0}{w}\right)^2 \qquad \text{(Eq. 15)}$$

Effective plates is related to resolution by:

$$R_S = {}^1/_4\left(\frac{\alpha - 1}{\alpha}\right)N_{eff}^{1/2} \qquad \text{(Eq. 16)}$$

The number of effective plates per unit time, N_{eff}/t, can tell directly how long a separation must take; N_{eff}/t is related to k' by the following expression:

$$N_{eff}/t = \frac{V}{H}\frac{k'^2}{(1 + k')^3} \qquad \text{(Eq. 17)}$$

If H is assumed to be independent of k' (which is often true in HPLC), the optimum k' for fast separations is about 2 (6).

Some workers prefer the reduced parameters (5) for comparing column efficiency of different chromatographic systems as a function of mobile phase velocity. Reduced plate height (h), equal to H/d_p, and the reduced velocity (v), equal to Vd_p, D_m, are used instead of H and V. The reduced plate height normalizes H for particle diameter, and the reduced velocity normalizes for diffusion over a particle diameter distance. Halasz (125) and Snyder (3) pointed out the dangers of using reduced parameters for comparing different HPLC columns.

A semiquantitative method for comparing *H versus V* curves of different columns was described (3, 143); *H versus V* curves were found to obey the empirical relationship:

$$H = DV^n \qquad \text{(Eq. 18)}$$

where D is a constant for a given column and set of experimental conditions (*e.g.*, type of column packing, particle size, and packing technique), and n is 0.4 for LSC columns. This equation affords a simple basis for comparing column efficiencies obtained by different workers, using any set of experimental conditions.

The value of D for silica is found to decrease with decreasing d_p (4). Kirkland (45) found D to decrease with decreasing d_p down to at least 37 μ with Zipax coated with 1% stationary liquid.

The importance of extracolumn band broadening from the injector and detector should not be neglected in the determination of column performance since the variance of the peak measured by the detector is a summation of all the variances in the chromatographic system. Deininger and Halasz (150) considered the effect of the equipment on measured column efficiency and resolution.

PHARMACEUTICAL APPLICATIONS

To date, a relatively limited number of practical applications of HPLC has been reported in the literature. This paucity of publications can be expected to change very rapidly with the ever increasing availability of commercial instrumentation, with improved column technology, and with the realization that many separations, which had heretofore been both tedious and complicated, may now be effected quite readily by means of this powerful analytical technique. Many applications that have been developed are illustrative of this method's immense value for pharmacological studies of drug substances and their metabolites and for clinical investigations of metabolic disorders.

Schmit *et al.* (130) described the use of high speed reverse phase liquid chromatography for the separation of natural products and also biochemicals. The biologically active constituents of hashish were isolated and chromatographed by gradient elution (water to methanol) on an octadecylsilane permaphase column. Both water-soluble and oil-soluble vitamins have been separated on an octadecylsilane permaphase column, using a water to methanol gradient.

General—In a brief survey of the applicability of high speed liquid chromatography (HSLC) to pharmaceutical analysis, Williams *et al.* (151) described procedures for the rapid determination of different classes of drugs.

Using a Zipax controlled-surface porosity support coated with a 1% polyamide stationary phase, the separation of such antibiotics as penicillin and its derivatives, myosins, and rifampin was achieved. A suitable mobile phase was hexane–ethanol (75:25), which was also capable of separating impurities from these antibiotics.

Steroids and a variety of their derivatives have been successfully chromatographed from a number of matrixes using reverse phase chromatography on a hydrocarbon column, with a water–methanol solution as the eluant. Efficient separations of some steroids have also been obtained by normal phase chromatography on an ethylene glycol column, using hexane–chloroform as the eluting solvent.

A single-tablet analysis of thiabutazine was presented as an example of a rapid separation for a diuretic. The analysis was performed on an oxypropionitrile column, using *n*-hexane for the elution. The total time for elution was 4 min., with the minimum detectable limit being 10 ng. of the diuretic.

The antihistamine chlorpheniramine maleate was chromatographed in 2 min. on a trimethylene glycol column using 10% chloroform–*n*-hexane. Analyses were carried out on single-component and multicomponent tablets with no interferences from excipients or the other actives.

Barbiturates and Alkaloids—The applicability of high speed chromatography for the analysis of barbiturates, diphenylhydantoin, and their hydroxylated metabolites was reported (152). Separations were performed without the necessity of any derivatization, using a pellicular anion-exchange column and eluting with either a nongradient or gradient mode of operation. Column inlet pressures ranged from 700 to 1600 psig., and flow rates ranged from 26 to 36 ml./hr. The eluting peaks were detected at the microgram level with the aid of a fixed wavelength (254 nm.) UV detector.

Optimal conditions for the separation of diphenylhydantoin and its phenolic metabolite were established to be analogous to those conditions necessary for the separation of phenobarbital and the hydroxy derivative of phenobarbital. A nongradient mode of elution, performed at 80° using a 20.0 m*M* phosphate solution, buffered at pH 3.5, produced the most rapid and satisfactory resolution for these compounds. These conditions were unsatisfactory for other barbiturates, however. Successful separation of these parent compounds and their metabolites required the use of a linear sodium chloride gradient at 80°. The sensitivity of the method could be increased by working with neutral solutions due to the increased absorptivity of the barbiturates in more alkaline media.

Alkaloids in plant extracts have been separated on oxypropionitrile and cyanoethyl silicone columns, using either *n*-heptane or *n*-heptane with a polar modifier as the eluant (121). The choice of eluant depended upon how strongly the alkaloids were retained on the columns.

Urine and Blood Analysis—Burtis *et al.* (153) reported a high pressure anion-exchange chromatographic technique for the separation and identification of two metabolites of phenacetin in urine. These metabolites were ultimately shown to be 4-hydroxyacetanilide and 3-methoxy-4-hydroxyacetanilide. The system employed was a UV analyzer[16]; this system consisted of a 200-cm. small bore column filled with Dowex 1-X8 resin and a spectrophotometer, which was used to monitor the column effluents at various wavelengths. The mobile phase was an ammonium acetate–acetic acid buffer.

The UV analyzer (154–158) was able to separate 140 UV-absorbing compounds in urine and should prove to be invaluable in many pharmacological studies. In the present example, the two metabolites of phenacetin were separated on the anion-exchange column and subsequently identified by their UV, mass spectrometric, and IR spectra.

Recently, Scott *et al.* (20) described modifications of their original system to increase resolution and also to decrease analysis time. By using coupled anion- and cation-exchange chromatography and gradient elution (in which the concentration of the acetate ion in an ammonium acetate–acetic acid solution buffered at pH 4.4 was gradually increased from 0.015 to 6 *M*), 16 additional peaks were separated from urine samples. The anion-exchange resin used was Aminex A-27, with quaternary ammonium active sites, while the cation-exchange resin employed was Aminex A-6, with sulfonic acid active sites. The eluant was pumped at a flow rate of about 10.5 ml./hr. through the 150-cm. long column at pressures up to 3500 p.s.i., and the eluting peaks were detected at two wavelengths in the UV (254 and 280 nm.). With this system, the time of analysis was decreased from 24 to 14 hr.

Although the UV analyzer is capable of resolving a large number of components in complex biological mixtures, the qualitative identification of the eluting peaks, for the most part, has been extremely difficult. By using the chromatographic technique proposed by Scott *et al.* (154), Burtis (21) reported that reproducible chromatograms of urinary samples could be accomplished on a liquid chromatograph[17] by high pressure anion-exchange chromatography. Detection of nanogram amounts of substances was possible using an efficient microvolume UV photometer. It was proposed that the resultant chromatograms or "urinary profiles" could be used to great advantage in the diagnosis of certain abnormal metabolic or pathological conditions.

At present, only 50 of the molecular constituents that have been reported have been identified by a combination of analytical techniques (159).

Scott *et al.* (157) also reported the development of a companion carbohydrate analyzer, which has successfully separated and detected 48 different carbohydrates in urine and blood serum. This system also utilizes a high pressure anion-exchange column. Qualitative and

[16] Developed at the Oak Ridge National Laboratory.
[17] Varian Aerograph model LCS-1010.

quantitative detection of peaks was accomplished by use of a colorimeter, the color being produced through reaction of the eluting carbohydrates with a phenol–sulfuric acid reagent. Sodium borate–boric acid buffer concentration gradient solutions served as the eluant. The most effective anion-exchange resin was reported to be a Type 1 resin of small particle size, with a final cross-linkage of 7–10%.

The use of the carbohydrate analyzer in the clinical laboratory in the investigation of various carbohydrate metabolism disorders was proposed (160). The detection of excreted sugars and some of their derivatives was possible without interferences from other eluting substances such as vitamins, aliphatic and aromatic hydroxy and keto acids, and polysaccharides.

The automation of a system was described for rapid separations of saccharides by high pressure ion-exchange chromatography, using a neutral pH boric acid–2,3-butanediol buffer solution to minimize alkaline rearrangement reactions (161).

An improved automated system with more rapid analysis times was developed by Floridi (162). This system utilizes from one to three different borate buffers adjusted to pH 8.40 or 8.80. The analysis of the eluting mono-, di-, and trisaccharides was accomplished by the orcinol colorimetric method[18]. The one-buffer elution system allowed rapid and effective separations of monosaccharides, the two-buffer elution system produced high resolution of di- and trisaccharides, while the three-buffer solution effected a separation of 17 different saccharides in 10 hr. In all cases, the analyses were carried out on Dowex ion-exchange resin at a column temperature of 55°. Flow rates were varied according to the particular system employed.

A high speed ion-exchange chromatographic method was devised for the direct analysis of glucuronide and sulfate conjugates of drug substances in biological samples (163, 164). The analyses were carried out at 80° in narrow bore stainless steel columns, 250 cm. in length, containing a pellicular anion-exchange resin. Column inlet pressures were maintained between 800 and 1000 psig., giving rise to a flow rate of 0.5 ml./min. A fixed-wavelength (254 nm.) UV detector was employed to monitor the column effluent.

Both nongradient and gradient elution methods were required. The use of pH 3.0 formic acid solutions of high ionic strength (1.0 *M* potassium chloride) and a linear gradient solution of potassium chloride permitted the successful separation and quantitative determination of sulfate and glucuronide conjugates in urine in less than 40 min.

Benzodiazepines—To avoid the undesirable task of acid hydrolysis or derivatization, which is necessary for the GC determination of benzodiazepines, Scott and Bommer (126) utilized HSLC for the separation of these pharmacologically important compounds. The stationary phase was Durapak "OPN"—the oxypropionitrile group chemically bonded to porous glass beads—which was dry packed in a narrow bore stainless steel column 1 m. in length. Elution of the column was effected at a solvent flow rate of 1 ml./min. using either: (*a*) a constant composition hexane–isopropanol mixture or (*b*) an initial elution with 100% hexane followed by a stepwise change to a 70:30 mixture of hexane–isopropanol. The latter solvent system served to sharpen the elution pattern of the slowest eluting component without sacrificing the resolution of the more rapidly eluting substances. A fixed-wavelength (254 nm.) UV microvolume photometric cell detected the eluting substances at the microgram level.

This procedure not only effectively separated mixtures of benzodiazepines but also was useful for the separation and quantitative determination of the parent compounds and their metabolites in a pharmacological study of urine samples.

Steroids—High resolution liquid chromatography (HRLC) at moderate pressures, using small bore borosilicate glass columns, 50 cm. in length, was shown to be effective for the rapid analysis of steroid hormones (120). A representative number of polar and moderately polar steroids was separated by reverse phase chromatography, and separation conditions for the various classes of steroid hormones were described.

Three types of solid supports were investigated: (*a*) Anakrom AB diatomaceous earth, (*b*) Plaskon CTFE-2330 terpolymer, and (*c*) Zipax controlled-surface porosity silica beads. In all cases, Amberlite LA-1, a water-insoluble amine, was used as the stationary phase; each support was coated at a level giving optimal results for that material. Water or a water–methanol mixture was used as the eluant.

The separation patterns observed for the four classes of steroids studied (adrenal corticosteroids, androgens, progestins, and estrogens) indicated that the main factor governing the chromatography of these compounds was actually a competition between liquid–liquid partitioning with the stationary liquid phase and liquid–solid adsorption with the solid support. It was further demonstrated that the relative effect of each separation mechanism was dependent upon the percentage of liquid loading used. Therefore, optimal resolutions for various pairs of compounds could be obtained by merely varying the level of liquid loading.

Henry *et al.* (165) surveyed numerous applications of HSLC to the analysis of steroids and steroid conjugates. The column effluent was continually monitored with both a fixed-wavelength (254 nm.) UV photometer and a refractive index detector. The advantages and disadvantages of each detector were noted. For UV-absorbing steroids, the UV detector had a lower detection limit of about 10 ng., while the refractive index detector had a 1-ng. lower detection limit whether or not the steroid absorbed in the UV. The formation of a strongly absorbing 2,4-dinitrophenylhydrazine derivative of the nonabsorbing carbonyl-containing steroids was proposed as a useful and attractive technique for increasing sensitivities for these compounds.

Examples of separations of all major types of steroids (including estrogens, androgens, progestogens, and adrenocortical hormones) by conventional liquid–liquid partition chromatography, reverse phase chromatography, and ion-exchange chromatography were presented, and useful column packing materials, stationary phases and mobile phases were described.

[18] Performed with a Technicon Auto-Analyzer.

Mollica *et al.* (166) described a suitable stability-indicating assay for the steroid flumethasone pivalate. A quantitative determination could be effected by using a controlled-surface porosity support coated with β,β′-oxydipropionitrile as the stationary phase and eluting with a solvent system of 5% ethyl acetate and 0.2% acetonitrile in hexane. Other chromatographic studies were reported for the steroids dexamethasone, prednisolone, and hydrocortisone.

A rapid and precise method for the quantitative determination of estrogens in urine was developed and applied to the determination of estriol in pregnancy urine (118). Detection of the estrogens was accomplished with a UV spectrophotometer operated at a fixed wavelength of 281 nm., corresponding to the absorption maximum of this class of compounds. Separation was effected with the use of two coexistent phases of a water–ethanol–2,2,4-trimethylpentane system (molar ratios 0.229:0.680:0.09 and 0.019:0.177:0.804) as the stationary and mobile phases, respectively. The water-rich phase was coated on diatomaceous earth (28–32 μ in diameter), which was then packed into a 2.7-mm. i.d. borosilicate glass column (50 cm. in length) through which the water-poor phase eluant was pumped. All analyses were performed at a constant temperature of 22°.

Analgesics—Henry and Schmit (167) described the analyses of the active ingredients in analgesics. The analyses were performed on Zipax controlled-surface porosity supports coated with an anion-exchange resin, using small bore stainless steel columns 1 m. in length. The mobile phase was distilled water buffered at pH 9.2. To lower the retention times of the more highly retained components, the ionic strength of the mobile phase was increased by the addition of ammonium nitrate. Detection of the eluting peaks at the nanogram level was accomplished by means of a fixed-wavelength (254 nm.) UV photometer. By using benzoic acid as the internal standard, both single- and multicomponent tablets were determined quantitatively without any interference from excipients. A routine time of analysis was 10 min., using a flow rate of 1.5 ml./min. at a column inlet pressure of 1200 psig. Since all separations were performed at ambient temperature, no problems of thermal degradation occurred, as had been encountered in GC. In addition, by merely increasing the ionic strength of the mobile phase, the determination of the decomposition product, free salicylic acid, could be performed quantitatively, with pyromellitic acid employed as the internal standard.

In a similar study, the successful quantitative determination of the active ingredients in 12 commercial analgesic tablets was reported, using an anion-exchange column 300 cm. in length (168). The eluant was a 1.0 *M* aqueous solution of tromethamine buffered at pH 9.0. The flow rate was 8.6 ml./hr. at 925–1000-p.s.i. pressure and a temperature of 60°. The eluting peaks were detected with a fixed-wavelength UV detector. This chromatographic system permitted baseline separation of acetaminophen and phenacetin, which had previously eluted at the same time (1); therefore, a qualitative identification of the components in a single tablet could be made by comparison of retention times.

In an extension of this study, Wolford *et al.* (169) reported the separation of oxypurines and the determination of caffeine in pharmaceutical separations. By using 3 *M* NH_4OH as the eluant at a flow rate of 3 ml./min., caffeine was eluted as a sharp peak from a 1 × 20-cm. column in less than 10 min.; qualitative results were obtained. Phenacetin, which is strongly basic, appeared between 15 and 30 min. while such compounds as aspirin, salicylamide, tetracycline, and barbiturates were effectively retained.

Amino Acids, Nucleotides, and Nucleic Acid Bases—High speed ion-exchange chromatography on small bore columns has been shown to be amenable to the analysis of amino acids using a modified sequential multisample amino acid analyzer[19] (170).

By eluting the column with citrate buffers at varying pH's, a flow rate of 0.5 ml./min., and a pressure of 600 p.s.i., satisfactory resolutions were obtained for the acid, neutral, and basic amino acids in approximately 60 min. A photometer equipped with a high efficiency flow cell detected the eluting peaks, the areas of which were found to be directly proportional to the amount of amino acid present in the sample.

Numerous authors have used HSLC to great advantage for the separation and identification of nucleotides, nucleosides, and nucleic acid bases.

The ribonucleoside mono-, di-, and triphosphates of cytidylic, uridylic, adenylic, and guanylic acids were separated on anion-active and cation-active resins coated on glass beads, producing pellicular ion exchangers (38*a*). Rapid separations of nanomole quantities of the substances were achieved by using a linear gradient elution with a phosphate or an ammonium formate buffer solution. The eluting peaks were detected by means of a spectrophotometer at a wavelength of 260 nm. using a microvolume flow cell. Analyses could be accomplished in all cases in less than 90 min.

The four major bases (uracil, guanine, adenine, and cytosine) and nucleosides (uridine, guanosine, adenosine, and cytidine) formed by the hydrolysis of RNA were successfully separated and detected at subnanomole levels using a pellicular cation-exchange resin in small bore stainless steel columns (38*b*). Acidic potassium or ammonium phosphate solutions served as the mobile phase, and detection at these levels was afforded by a micro-UV detector operating at a fixed wavelength of 254 nm. The elapsed time for an analysis was 6 min. The optimal conditions for these separations were investigated and described. It was found that the pH of the sample and of the eluant had the most pronounced effect on the resolution.

A similar separation of the nucleotides and nucleic acid bases and other less commonly encountered purines and pyrimidines was successfully performed on controlled-surface porosity supports coated with strong cation- and/or anion-exchange resins (40). A 0.01 *N* nitric acid solution was utilized for the elution. The total time of analysis for the major hydrolysates of RNA was 5 min. By employing an ammonium formate solution first at 3000 p.s.i. with a flow rate of 50 ml./hr.

[19] Technicon.

and then at 4600 p.s.i. with a flow rate of 80 ml./hr., the elution times for these alkaline hydrolysis products were reduced to 4 and 2 min., respectively, by Burtis (171), who also suggested the obvious applicability of the method for the determination of the base composition of DNA following an enzymatic, rather than alkaline, hydrolysis reaction. The elution times and the conditions for many "unusual bases" and common ribo- and deoxynucleosides were also reported. Quantitation and detection at the nanomole to picomole level were possible. These high speed separations, with no derivative formation required, rival those observed for GC separations.

The use of citrate and acetate buffers as effective eluants for the chromatography of these compounds on a cation-exchange resin also was noted (172).

To eliminate the interferences and anomalous peaks caused by UV-absorbing substances present as contaminants in buffer solutions described by Horvath and Lipsky (38*b*), Shmukler (173) devised a purification system for potassium dihydrogen phosphate. Due to the relatively low solubility of potassium dihydrogen phosphate in water, the non-UV-absorbing salt, potassium chloride, was added to increase the total salt content of the eluant. With this buffer system, the chromatography of adenine nucleotides and ^{32}P-labeled compounds in a deproteinized mitochondrial reaction mixture was studied (174).

Sample preparation methods and elution conditions for the separation of mononucleotides, ribomononucleotides, deoxyribomononucleotides, nucleoside diphosphates, and dinucleotides were reported and their *R* values were tabulated (175). The chromatographic separation was carried out on an anion-exchange column, maintained at 70°, using a linear or exponential gradient elution technique and "purified" potassium phosphate solutions.

The qualitative and quantitative determination of nucleotide profiles of cell extracts was characterized (176), and optimal conditions for the rapid separation of the mono-, di-, and triphosphates of the naturally occurring ribosides, as well as for the sulfur analogs of some of these compounds, were noted. The method was developed with the aid of a high pressure chromatograph[20] equipped with an anion-exchange column and a double-beam UV detector operated at 254 nm. A potassium phosphate–potassium chloride gradient served as the eluant, and the eluting peaks were identified by one or more of the following methods: (*a*) spectrophotometric or chemical means, (*b*) injection of known standards, (*c*) comparison with chromatograms of known substances, and (*d*) use of known enzymatic reactions. "Enzymatic peak shifts" not only served to identify peaks but also aided in unmasking a chromatogram and allowing the detection of other smaller peaks to be made. A further use of this system combined with isotopic labeling to measure the radiospecific activities of nucleotides was proposed.

The four common 5′-deoxyribonucleotides were successfully analyzed on a Zipax strong anion-exchange column eluted with 0.008 *M* phosphate buffer at pH 4.40 (177).

Nucleotides, nucleosides, and nucleic acid bases were also successfully separated by ligand-exchange chromatography using a cation-exchange resin, Chelex 100, impregnated with copper (II) ion (178). The flow rate of the eluting agent (water and/or ammonium hydroxide, depending upon the substances being separated) was maintained at 0.75 ml./min., and the effluent was continually monitored with a UV spectrophotometer. The nucleotides were not retained on the column; the weakly basic nucleosides were separated by elution with water, the more basic nucleosides were resolved with 1 *N* ammonium hydroxide, and the nucleic acid bases required 2.5 *N* ammonium hydroxide to be displaced from the column.

The quantitative determination of enzymatically formed adenyl cyclase at the microgram level was reported (179), using high pressure anion-exchange chromatography following an initial zinc–barium precipitation step which removed interfering substances. The elution was effected by a nongradient mode of operation with a pH 2.20 hydrochloric acid solution as the eluant. A flow rate of 12 ml./hr. with an inlet pressure of 450 550 p.s.i. was employed, while the column temperature was controlled at 80°. Detection of the peaks was accomplished with a sensitive microvolume UV flow cell. The total time for an analysis was about 10 min.

Qualitative identification and quantitative detection of adenine and its metabolites in body fluids were described, and an empirical means of optimizing the separation conditions was discussed, presenting a systematic and logical approach to a HSLC problem (137).

Aza Aromatic Hydrocarbons—Snyder (133) developed a rapid liquid–solid chromatographic method for the analysis of hydrogenated quinoline mixtures, using alumina as the adsorbent and a 60% water-saturated 35% dichloromethane–pentane mixture as the eluting solvent. Qualitative and quantitative analyses could be performed over a long period with no changes in the efficiency of the column being observed, provided that the water level of the alumina was maintained at a constant level and the column was regenerated occasionally to remove strongly adsorbed components. Routine analyses of quinoline mixtures could be performed in less than 30 min., with the detection of the eluting substances being realized with a UV spectrophotometer equipped with a microvolume flow cell. Problems inherent in such procedures and methods of solving them were also presented.

A high speed liquid–solid column chromatographic procedure for the separation of polynuclear aza heterocyclic compounds was reported (136). The adsorbent was Zipax, a high efficiency chromatographic support, the surface of which was impregnated with silver ions. Separation was achieved by means of formation of donor–acceptor complexes between the silver ion and the heterocyclic nitrogen. The order of elution was found to be dependent upon both the basicity of the nitrogen and the steric accessibility of the lone pair of electrons.

A 1% (v/v) acetonitrile in *n*-hexane solution produced

[20] Picker Nuclear.

highly efficient separations. Unlike many liquid–solid chromatographic methods, no water deactivation of the column nor careful control of the solvent water content was required for this method to give consistently good results.

A high speed liquid chromatographic method has been developed for the separation and quantitative determination of isomeric mixtures of the cyano-, carboxamido-, and carboxy-substituted pyridines. A 1-m. × 2.1-mm. i.d. column filled with a strong cation-exchange resin (1% sulfonated fluorocarbon) coated on Zipax was employed. Elution was effected with an aqueous solution of 0.1 *N* sodium nitrate and 0.1 *N* phosphoric acid at a flow rate of 1.70 ml./min. at an inlet pressure of 1500 p.s.i. Detection of the eluting substances was accomplished with a fixed-wavelength (254 nm.) UV detector.

The analysis of trisulfapyrimidines by cation-exchange liquid chromatography was accomplished using a phosphate buffer solution for the mobile phase; an application for the quantitative determination of sulfadiazine, sulfamerazine, and sulfamethazine was presented (180).

Miscellaneous—A liquid–solid adsorption chromatographic technique, using microbore glass columns packed with dry silica gel, was developed by Gordon and Peters (181) to separate 4,4′-diaminodiphenylsulfone from its monosubstituted and disubstituted acetylated derivatives. 4,4′-Diaminodiphenylsulfone has had a great amount of pharmacological interest since it has been shown to be a highly effective antileprotic agent as well as an antimalarial drug.

The procedure was adapted from a TLC separation, with anhydrous ethyl acetate as the eluting solvent. A satisfactory separation of all three compounds was obtained in less than 30 min. by pumping the mobile phase through a 30-cm. column at a pressure of 100 p.s.i. and a flow rate of 0.25 ml./min. Detection of the compounds at the microgram level was accomplished by measuring the UV absorption at 280 nm. A linear relationship existed between the peak areas obtained and the amount of substance, thereby providing a rapid quantitative estimation of each compound.

N,*N*-Dimethyl-*p*-aminobenzeneazobenzoyl chloride reacts with a wide range of organic compounds to form colored products with differing polarities. This reaction was used in conjunction with HPLC, using small bore glass columns, and the effects of the various parameters were discussed (182). Either high speed cation-exchange chromatography using a strongly acidic sulfonated styrene–divinylbenzene resin or high speed liquid–solid adsorption chromatography on silica columns permitted satisfactory separations. A microvolume flow-through photometric detector continuously monitored the column effluent stream. The effect of the hydrochloric acid concentration in aqueous methanolic or aqueous ethanolic solutions and also the effect of the alcohol concentration in the eluting solutions were determined.

The acidic and neutral catabolites from catecholamines were quantitatively and rapidly separated on a cation-exchange resin, PA-28 (183). A scheme for the elution of the catabolites formed *in vivo* from dopamine, noradrenaline, and adrenaline was outlined: the column was successively eluted with sodium citrate buffers, pH 3.28 for 15 min., followed by a sodium citrate–boric acid buffer, pH 4.53, for 100–235 min., and, finally, with 0.2 *N* sodium hydroxide for 60 min. The flow rate was maintained at 50 ml./hr. with a back-pressure of 140–200 p.s.i. in the 60-cm. × 9 mm. column. All analyses were performed at a constant temperature of 55°. The eluting peaks were detected with a spectrophotometer[21] at 280 nm.

In a similar manner, Lange and Hempel (184) demonstrated that a wide range of aromatic acids, aldehydes, and alcohols could be separated. The eluting substances were determined by: (*a*) measuring the absorbance of the eluate at 280 nm. or (*b*) determining the fluorescence at 315 nm. of the effluent fractions by means of a spectrofluorometer[22]. The elution times for 50 aromatic compounds were tabulated, with the order of elution generally being dependent upon the polarities of the substances.

Efficient separations of aromatic monosulfonates and disulfonates and of isomeric and homologous aromatic sulfonates were achieved (185) by use of high speed anion-exchange chromatography, using narrow bore columns packed with a cross-linked polyalkylene amine resin. Elution of the sorbed substances was accomplished with an aqueous organic solution of water–acetonitrile–methanol (a 1:1:1 mixture provided the best performance) and a linear salt gradient of lithium chloride. A monochromator was utilized for the absorbance measurements over the UV range from 220 to 400 nm., which permitted the maximizing of general or selective sensitivities for the sulfonates being determined.

Linear gradient elution chromatography has provided an efficient means of separation of aromatic hydrocarbons having widely different boiling points (186). Analyses were carried out on small bore glass columns packed with alumina, using a pentane–ether gradient at a flow rate of 45 ml./hr. The eluting peaks were detected with the aid of a fixed-wavelength photometer at 260 nm., and identification was made either by comparison of retention times or by addition of pure standards to the mixture being analyzed.

Beyer (187) developed a quantitative HPLC analytical method for sulfonylurea antidiabetic agents. A column of 1% hydrocarbon polymer on Zipax eluted with citrate or borate buffers separated glyburide, chlorpropamide, tolazamide, tolbutamide, and acetohexamide.

HPLC was used for the analysis of *N*,*N*-dimethyl-2,2-diphenylacetamide and its metabolites in soybean plants (188). The analysis was obtained on a 1-m. hydrocarbon polymer column eluted with a water–methanol (4:1) mixture.

APPENDIX: LIST OF SYMBOLS

A = eddy diffusion term in the Van Deemter equation

B = longitudinal molecular diffusion in the Van Deemter equation

[21] Beckman DB.
[22] Aminco-Bowman.

C = mass transfer term in the Van Deemter equation
D_m = diffusion coefficient of solute in the mobile phase
D_{is} = diffusion coefficient of solute in the stationary phase
d_c = internal diameter of the column
d_p = particle diameter of the packing
f = total porosity in the column
h = reduced plate height (H/d_p)
H = height equivalent to a theoretical plate
K = distribution coefficient
K^0 = specific permeability of a column
k' = capacity factor = $K(V_s/V_m)$
L = column length
N = number of theoretical plates
N_{eff} = number of effective plates
N_{eff}/t = effective plates per second
ΔP = column pressure drop
R_s = resolution
T_m = interparticle tortuosity factor
T_s = intraparticle tortuosity factor
t = time of analysis
t_r = retention time of peak measured from start
t_0 = retention time of a nonsorbed species
V = mobile phase velocity
V_m = column mobile phase volume (dead volume)
V_s = column stationary phase volume
W = peak width
ϵ_α = fraction of column volume occupied by phase α
ϵ_m = fraction of column volume occupied by the moving phase
ϵ_s = fraction of column volume occupied by the static phase
ϵ = fraction of the column volume occupied by the two phases
$1 - \epsilon$ = fraction of column volume occupied by the support material
η = mobile phase viscosity
$\lambda, \lambda 2$ = dimensionless constants
σ_l = length-based standard deviation of the Gaussian band
μ = micron (micrometer)

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RESEARCH ARTICLES

Absorption and Distribution of Naloxone in Rats after Oral and Intravenous Administration

S. H. WEINSTEIN▲, M. PFEFFER, J. M. SCHOR, L. FRANKLIN, M. MINTZ, and E. R. TUTKO

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Keyphrases □ Naloxone hydrochloride—absorption and distribution after oral and intravenous administration, rats □ Absorption and distribution, naloxone hydrochloride—after oral and intravenous administration, plasma levels, liver metabolism, rats

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Abstract □ The effect of route of administration on the absorption and distribution of naloxone, a narcotic antagonist, was investigated in rats. Plasma levels were determined by GLC. Five minutes after intravenous administration of 1 mg./kg., the plasma concentration was 258 ng./ml. Plasma levels after low oral doses were undetectable; but after 100 mg./kg. orally, the peak level of unchanged drug was almost 5000 ng./ml. In terms of percent of administered dose, the maximum amount of naloxone in the calculated plasma volume is 1.04% of the intravenous dose *versus* 0.19% of the oral dose. Pharmacokinetic parameters were generated with a computer program; the models constructed are of a rapidly absorbed and rapidly excreted and/or metabolized drug. These results, together with results from absorption studies with ligated intestinal loops, indicate that poor absorption of naloxone is not the cause of its relatively low oral potency. *In vitro* metabolic studies with rat liver slices confirmed rapid naloxone metabolism, suggesting that the lower potency of oral naloxone compared to parenteral naloxone is due to rapid first-pass liver metabolism.

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Naloxone [(−)-*N*-allyl-14-hydroxynordihydromorphinone] is a potent narcotic antagonist upon parenteral administration to laboratory animals (1, 2) or man (3), but it is approximately one-fiftieth as po-

tent when administered orally in human subjects (3). A similar relationship was reported for rats and mice (4).

The reduced oral effectiveness of a drug may be due to poor or incomplete absorption from the GI tract or to rapid first-pass metabolism in the liver since orally administered drugs enter the systemic circulation *via* the hepatic portal system. To determine the extent of absorption of naloxone from the intestine, *in vivo* intestinal loop experiments were performed in rats. This technique has been used to study the absorption of many compounds including quaternary ammonium compounds (5) and digitalis glycosides (6).

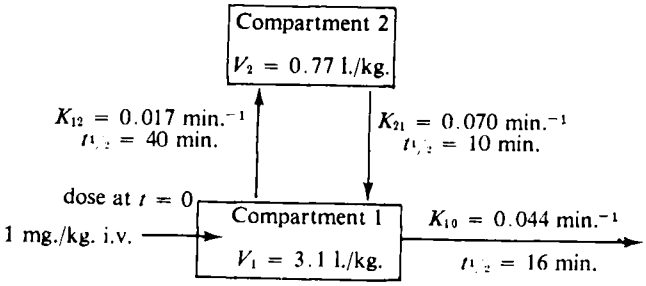
In vitro rat liver slice experiments were performed to determine the extent of naloxone metabolism in the liver. This method was previously applied to the study of morphine metabolism (7). Naloxone has been reported to undergo glucuronidation, *N*-dealkylation, and reduction of the 6-oxo group (8, 9).

Plasma levels of naloxone in rats were determined following oral and intravenous administration. Absorption and elimination rate constants, as well as other pharmacokinetic parameters, were calculated to gain insight into the fate of naloxone following administration by the two routes.

EXPERIMENTAL

Analytical Methods—Extraction of naloxone from rat plasma was performed as described by Mulé (10), except that chloroform containing 1% isopropanol was used as the solvent and was back-extracted with 1.3 ml. of 0.1 *N* HCl. A 1-ml. portion of the final hydrochloric acid extract was evaporated to dryness[1]. An internal standard, 500 ng. of tetraphenylethylene (25 μl. of a 20 mcg./ml. methanol solution), was then added. The samples were again evaporated to dryness, and 25 μl. of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane[2] was added. Wilkinson and Way (11) demonstrated the efficacy of bis(trimethylsilyl)trifluoroacetamide as a silylating agent for morphine, and it was equally useful for naloxone. The tubes were flushed with dry nitrogen and closed with ground-glass stoppers, and the silylation reaction was carried out for 30 min. at 60–65° in a dry heating block[3]. One-microliter aliquots of the silylated samples were injected into a gas chromatograph[4], with carbon tetrachloride as a solvent flush. Naloxone was measured by comparing the peak height ratio of naloxone-tetraphenylethylene in the experimental samples to the peak height ratio obtained from standards extracted from plasma.

Naloxone was extracted from the liver and intestine by homogenizing the tissue in a total of 4 ml. of water (including the volume of water used to rinse the homogenizer), and the protein was precipitated by addition of 0.5 ml. of 10% $ZnSO_4$ and 0.5 ml. of 0.5 *N* NaOH. This was centrifuged 15 min. at 3000×*g*, and 3 ml. of the protein-free supernate was diluted to 15 ml. with 0.5 *M* phosphate buffer, pH 7.4. The diluted supernate was then sequentially extracted with three 15-ml. portions of chloroform. The pooled chloroform extracts were evaporated to dryness in a rotary evaporator[5], and the residue was quantitatively washed out of the evaporating flask with 5 ml. of chloroform into a conical centrifuge tube. This chloroform was removed by evaporation to dryness[1]. The residue was redissolved in 0.1 ml. chloroform containing 300 ng. of *N*-cyclobutylmethyl-7,8-dihydro-14-hydroxynormorphine (nalbuphine, 3 mcg./ml.) as the internal standard. A portion of this chloroform solution was injected into the gas chromatograph, and the naloxone was measured by comparing the peak height ratio of naloxone–nalbuphine to the peak height ratio obtained for standards also extracted from intestinal loops or rat liver slices. Metabolites such as noroxymorphone and naloxone glucuronide are not extracted under these conditions.



Scheme I—*Two-compartment open pharmacokinetic model for intravenous naloxone*

The gas chromatograph was equipped with a flame-ionization detector. The column was 3.8% UC-W98 on high performance Chromosorb W, 80–100 mesh. Conditions used were: oven, 245°; detector, 310°; flash heater, 300°; hydrogen flow rate, 37 ml./min.; helium, 75 ml./min.; and air, 350 ml./min.

Animal Studies—Three to five fasted, male, CFN rats (average weight 397 g.) were used for each time interval of the intravenous study. Naloxone hydrochloride (1 mg./ml., aqueous solution) was administered *via* the tail vein at a dose of 1 mg./kg. while the rats were under light ether anesthesia. At 5, 10, 15, 22, 30, 38, 45, 50, and 60 min. after dosing, blood was obtained by cardiac puncture with heparinized syringes. The plasma was separated by centrifugation at 3000×*g* for 20 min.

For the oral studies, naloxone hydrochloride was administered to fasted rats (average weight 375 g.) in aqueous solution (100 mg./ml.) *via* stomach tube at a dose of 100 mg./kg. At 2, 3.5, 5, 15, 22, 30, 35, 45, 50, and 60 min. after dosing, blood was obtained as already described. Four to 11 rats were used for each time interval.

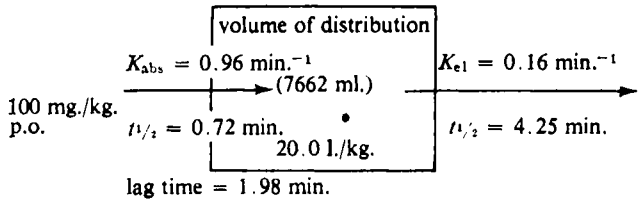
Intestinal loops were prepared, while fasted rats were under ether anesthesia, by exposing the intestine and locating a section supplied by three blood vessels, approximately 20–22 cm. from the pyloric sphincter. One end of a segment approximately 2.54 cm. (1 in.) in length was securely ligated with thread. The other end of the segment was ligated with a 22-gauge needle entering the end through the center of the tightened loop of thread. Five hundred micrograms of the drug in 0.5 ml. of saline solution was injected and, as the needle was removed, the ligature was completed. The midline incision made to expose the intestine was then closed. At 30, 60, or 90 min. after treatment, the animals were again anesthetized and the previously tied section of gut was removed for analysis as already described. Controls for these intestinal loop experiments consisted of the incubation of 500 mcg. of naloxone in gut sections *in vitro* at 37° in normal saline for a time interval equal to that of the *in vivo* incubation. Loss of drug by absorption was determined by comparing the naloxone recovered from *in vivo* intestinal loops to the naloxone recovered from *in vitro* loops. Loss of drug due to metabolism by intestinal tissue itself was determined by comparing the naloxone recovered after incubation *in vitro* in intestinal loops to the naloxone recovered from *in vitro* loops that were not incubated but were homogenized and extracted immediately after the drug was placed within the loop.

For liver slice experiments, ether-anesthetized rats were killed and the livers were immediately removed. The livers were washed with ice-cold normal saline solution and sliced with a hand microtome[6]. Three hundred milligrams of liver slices was placed in 25-ml. conical flasks containing 1.5 ml. of Krebs–Henseleit solution. Then 0.5 mg. of naloxone hydrochloride in 0.5 ml. of Krebs–Henseleit solution was added, giving a total incubation volume of 2.0 ml. Prior to use, the Krebs solution was saturated with 95% O_2–5% CO_2 (7).

The incubations were carried out in duplicate on a shaking incubator[7] at 37° under a 95% O_2–5% CO_2 atmosphere delivered at a rate of 5 ft.^{3}/hr.

After 30, 60, and 120 min., duplicate incubation mixtures were homogenized. Extraction of naloxone from the homogenized in-

[1] Evaporation performed in 5-ml. conical centrifuge tubes with a Buchler Evapo-mix.
[2] Regisil, Regis Chemical Co.
[3] TEMP-BLOK Heater, Lab-Line Instruments.
[4] Hewlett-Packard, F&M 402.
[5] Rinco rotary evaporator.
[6] Stadie-Riggs hand microtome.
[7] Dubnoff.



Scheme II—One-compartment open model for orally administered naloxone

cubation mixture was carried out as previously described. The extracts were analyzed for unmetabolized naloxone by GLC. The peak height ratio (naloxone–nalbuphine) of naloxone obtained from extracts of incubation mixtures was compared with peak height ratios obtained from extracts of unincubated liver–naloxone mixtures. Duplicate determinations were performed for each sample.

As controls, to determine whether loss of naloxone was an assay artifact or due to liver metabolism, incubations were also carried out with boiled liver slices.

Pharmacokinetic Calculations—A computer program (COMPT) for optimizing the solution of integral nonlinear compartmental models of drug distribution, written in extended BASIC for use in time-sharing computer systems, was used to generate pharmacokinetic parameters (12).

The mathematical formulations for compartmental models (Schemes I and II) reduce, in their most general form, to sums of exponential terms:

$$f(T) = \sum_{i=1}^{M} N_i \exp(-a_i T) \qquad \text{(Eq. 1)}$$

The formulations for the one- and two-compartment models are, respectively:

$$C = \left(\frac{D}{V}\right)\left(\frac{K_{abs}}{K_{abs} - K_{el}}\right)[\exp(-K_{el}T) - \exp(-K_{abs}T)] \qquad \text{(Eq. 2)}$$

$$C_1 = \frac{D}{V_1(a_1 - a_2)}[(K_{21} - a_2)\exp(-a_2T) - (K_{21} - a_1)\exp(-a_1T)] \qquad \text{(Eq. 3)}$$

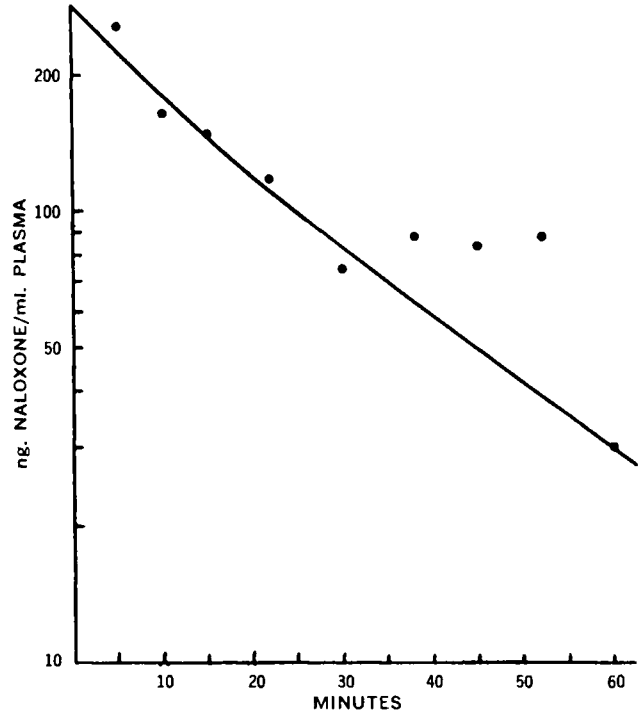


Figure 1—*Semilog plot of naloxone plasma concentrations in rats following 1 mg./kg. i.v.*

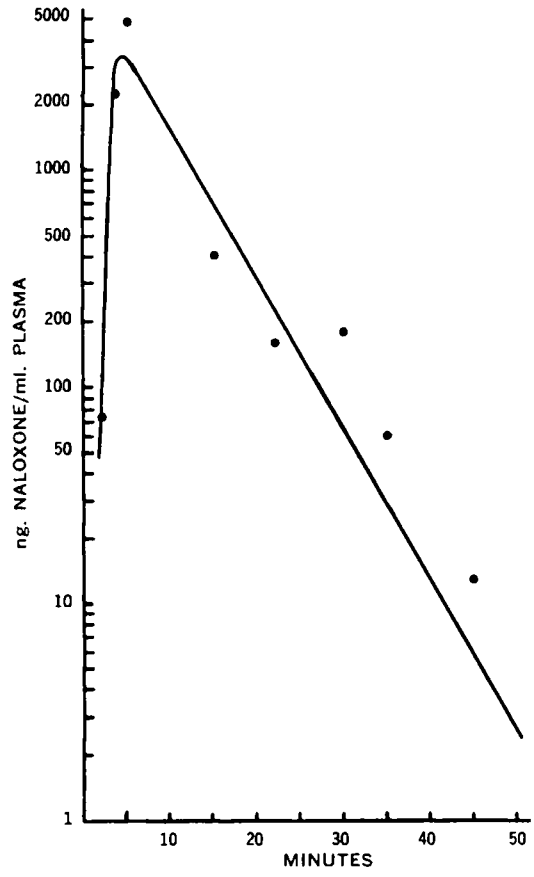


Figure 2—*Semilog plot of naloxone plasma concentrations in rats following 100 mg./kg. p.o.*

N_i and a_i are obtained from the line projected from the terminal portion of the log C *versus* T curve. Calculation of pharmacokinetic parameters for these models was extensively discussed by Wagner (13).

RESULTS

Gas Chromatography and Extractions—Under the described chromatographic conditions, tetraphenylethylene had a retention time of 2.2 min. and the trimethylsilyl derivative of naloxone had a retention time of 4.5 min. Underivatized naloxone and nalbuphine had retention times of 3.6 and 7.8 min., respectively.

The use of parallel extraction standards as controls was necessary because the recovery of naloxone from plasma was 50–60%. There was a linear, reproducible relationship between GLC response and plasma concentration of naloxone.

Preliminary experiments showed that naloxone was recovered from intestinal loops and liver slices in quantities sufficient for GLC analysis without silylation and that there was a linear relationship between naloxone concentration and GLC response.

Experiments in which naloxone was incubated *in vitro* in sections of gut showed that the gut itself did not metabolize naloxone. Therefore, disappearance from *in vivo* loops cannot be attributed to destruction within the loop but must be ascribed to absorption from the loop. When naloxone was incubated with boiled liver slices, the naloxone was recovered intact, indicating that the disappearance found with unboiled slices was due to metabolism. Metabolites such as naloxone glucuronide or noroxymorphone are not extracted under the conditions used.

Plasma Levels of Naloxone—Figure 1 is a plot of log of rat plasma levels of naloxone *versus* time after intravenous administration. The increases in drug levels between 38 and 52 min. may be due to biliary recycling. The curve shown is the best fit computed with the COMPT program, neglecting those points attributable to biliary recycling.

Initially, an oral dose of 10 mg./kg. was administered, but naloxone was not detectable in plasma at this level. At a dose of

Table I—Absorption of Naloxone from *In Vivo* Intestinal Loop in Rat[a]

Minutes	Number of Animals	Naloxone Remaining in Loop, % ± *SD*
30	5	55.5 ± 17.9
60	8	18.8 ± 12.5
90	4	4.73 ± 3.6

[a] Five hundred micrograms injected into ligated intestinal loop *in vivo* and assayed by GLC after indicated time. See text for details.

Table II—Percent Naloxone Remaining after Incubation with Rat Liver Slices

Minutes	Naloxone Remaining, %	
	Experiment 1[a]	Experiment 2
30	48.5	73.5
60	23.6	42.2
120	13.5	13.4

[a] Experiments 1 and 2 represent two separate experiments, each performed in duplicate.

100 mg./kg., detectable levels were obtained. Figure 2 is a plot of log of rat plasma levels of naloxone *versus* time after oral administration. The curve shown is the best fit computed with the COMPT program.

Absorption from Ligated Intestinal Loop—The percent of administered naloxone absorbed from *in situ* ligated intestinal loops at various time intervals is presented in Table I. Naloxone is well absorbed; 95.3% was absorbed at 90 min.

Metabolism by Rat Liver Slices—Table II presents the results of the incubation of naloxone with rat liver slices. After 2 hr., almost 90% of the added naloxone was metabolized. Earlier time intervals showed some variations; but in 30 min., 25–50% of the naloxone was metabolized.

Pharmacokinetics—The plasma level data obtained for intravenously administered naloxone (Fig. 1) fit a two-compartment open model (Scheme I). The data for orally administered naloxone (Fig. 2) fit a one-compartment model with first-order absorption (Scheme II).

DISCUSSION

The pharmacokinetic model (Scheme I) for intravenous naloxone yields a very rapid elimination rate ($t_{1/2}$ = 16 min.). The relatively large volume of distribution in Compartment 1 could indicate either extensive tissue binding or rapid metabolism, because the model is simply attempting to account for the absence of a large fraction of the administered dose as unchanged drug in the plasma. The small values of K_{12} and V_2 and the larger K_{21} value indicate slow entry into, little binding in, and rapid removal from a peripheral compartment, respectively.

The one-compartment model for naloxone plasma levels after oral administration (Scheme II) satisfies the available data. If a more sensitive analytical method were available, a leveling off of plasma concentrations probably would have been observed after 45 min., and the use of a two-compartment model would be indicated. The observed terminal straight-line portion of the curve in Fig. 2 is probably really the distributive phase. The computer-fitted curve does, however, show a very rapid absorption rate ($t_{1/2}$ = 0.72 min.) for naloxone. The large volume of distribution may indicate either extensive tissue binding or rapid metabolism. The pharmacokinetic models of naloxone are, therefore, those of a rapidly absorbed and rapidly excreted and/or metabolized drug.

Figures 1 and 2 show the plasma concentrations of naloxone after intravenous and oral administration. The peak concentration after oral administration, 4856 ng./ml., occurs at 5 min. In terms of percent of the administered dose, calculated from the average plasma volume of rats (40.4 ml./kg.) and the average weight of the rats used, the maximum amount of naloxone found in plasma is 0.19% of the oral dose. Five minutes after intravenous administration, the plasma concentration is 258 ng./ml. or 1.04% of the dose. This could indicate that a large portion of the oral dose is either not absorbed or is metabolized before reaching the systemic circulation.

The rapid absorption rate observed for orally administered naloxone is in agreement with the results of the ligated intestinal loop experiments. At 30 min., 45% of the drug is absorbed and after 90 min. absorption is virtually complete (Table I). In these experiments the entire intestinal loop was analyzed for naloxone, eliminating the possibility of naloxone loss through binding to some component of intestinal tissue, as reported by Levine *et al.* (5) for quaternary ammonium compounds.

These results lead to the conclusion that the lower therapeutic effectiveness of orally administered naloxone is not the result of poor absorption from the intestine. The remaining factor, rapid first-pass liver metabolism, therefore, appears to be the mechanism by which efficacy of orally administered naloxone is diminished. This conclusion is strongly supported by the rapid metabolism of naloxone by rat liver slices (Table II). To be sure, plasma concentrations of naloxone in rats are erratic after oral administration (coefficient of variation 1.10 compared to 0.25 for intravenous administration), but this may be due to the well-known biological variation in drug-metabolizing enzymes rather than to erratic absorption from the GI tract.

The data presented show that low oral effectiveness of naloxone cannot be attributed to poor absorption. Pentazocine's low oral effectiveness (compared to parenteral dosing) has been attributed to poor absorption (14), but this is now questionable since Berkowitz *et al.* (15) reported finding an additional metabolite in plasma following oral administration which has not been found after intramuscular administration. This indicates route-dependent metabolism of pentazocine.

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Statistical Approach to Evaluating Effect of Physical and Chemical Factors on Fecal Excretion of Chlorophenothane

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Abstract □ A study using ^{14}C-labeled chlorophenothane was statistically designed to evaluate the effect of five treatments (cold, fasting, levarterenol bitartrate, prediphenylhydantoin, and postdiphenylhydantoin) upon the fecal elimination of the pesticide from rats. To 24-hr.-fasted rats, 7 μc. of ^{14}C-chlorophenothane was administered intraperitoneally. The pesticide was allowed to distribute into the tissues of the animals for 5 days prior to initiation of the treatments which were continued for 3 days. The cold group was kept at 2–4°. The fasted rats were deprived of food but had water *ad libitum*. Levarterenol bitartrate and diphenylhydantoin were administered twice a day at 1.0 and 75.0 mg./kg. body weight, respectively. Diphenylhydantoin was administered before chlorophenothane (prediphenylhydantoin) to one group of rats and after administration of the pesticide (postdiphenylhydantoin) to a second group. The ^{14}C-activity in the feces was measured by liquid scintillation. Thirty-six rats were assigned in a randomized complete block design of three replicates with two rats per treatment, five treatments, and a control per replicate. Homogeneity of variance tests indicated that the variances between rats were homogeneous. Two-way analysis of variance tests showed that replicates could be pooled and the data reanalyzed as if a completely randomized design were run. Hence, a one-way analysis of variance and the Newman-Keuls sequential range tests were performed. The latter tested for significant differences between all pairs of treatments. The statistical design allowed for minimum expenditure of animals and radioisotope and provided reliable data. Cold treatment was the only treatment that increased the fecal elimination of chlorophenothane. Preadministration of diphenylhydantoin may have stimulated hepatic enzymes to alter deposition and increase apparent elimination of chlorophenothane.

Keyphrases □ ^{14}C-Chlorophenothane fecal excretion—effects of cold, fasting, and levarterenol bitartrate and diphenylhydantoin administration, statistical analysis, rats □ Pesticide removal from tissues—effects of cold, fasting, and levarterenol bitartrate and diphenylhydantoin administration on ^{14}C-chlorophenothane fecal excretion, rats □ Excretion, fecal, ^{14}C-chlorophenothane—effects of cold, fasting, and levarterenol bitartrate and diphenylhydantoin administration, rats

Although some countries and several states in the United States have banned the use of chlorophenothane[1] (1), the fact remains that the pesticide has been with us for more than 30 years and is still with us today. For most persons, residues in food represent the greatest source (90%) of exposure to persistent pesticides (2–5). Therefore, recent research activity on the accelerated removal of pesticides from animal and poultry products has increased (6). Many workers demonstrated the extensive accumulation of chlorophenothane in the fat depots of the body (7–20). The chlorophenothane concentrations found in adipose tissue were approximately 10 times those of liver and 100 times the concentration found in the kidney, gonads, and brain (21). The storage of chlorophenothane in body lipids was suggested as an effective detoxifying mechanism for such a lipophilic insecticide (22). However, this adipose tissue storage was nonpermanent, especially during accelerated lipid mobilization induced by starvation or increased energy expenditure due to exposure to cold (17, 22–26). Mobilization of fat eliminated some lipid-soluble pesticide from animals, and the remaining residues were further diluted as depot fat was replaced. Induction of drug-metabolizing enzymes of the liver microsomes involved in the metabolic degradation of many lipoid-soluble chemicals was another approach recommended for increasing the rate of removal of persistent pesticides from animals (6). The anticonvulsant drugs, specifically diphenylhydantoin and phenobarbital, are potent inducers of drug-metabolizing enzymes in the rat as well as man (1, 27–32).

The objectives of this work were to: (*a*) study the effect of some physical (cold and fasting) and chemical (levarterenol bitartrate and diphenylhydantoin) treatments on the fecal excretion of ^{14}C-chlorophenothane, and (*b*) find a treatment that would quickly deplete the residues from rats following a measured exposure to the pesticide. Cold, fasting, and endogenous norepinephrine (and the comparable drug entity, levarterenol bitartrate) stimulate the mobilization of fatty tissues (17, 19, 23, 25, 26), the main storage site of chlorophenothane. On the other hand, diphenylhydantoin stimulates certain hepatic microsomal enzymes that are involved in the breakdown of chlorophenothane (1, 27). A simple and efficient statistical design was used to minimize expenditure of animals, radioisotopes, and human efforts and to evaluate the treatment effects, replicability, and the interaction between replicates and treatment factors. For these purposes, randomized complete block and completely randomized designs (33) were applied.

Previous results (34) regarding the respiratory excretion of ^{14}C-chlorophenothane by rats indicated negligible (0.007% of the administered activity) $^{14}CO_2$ in the exhaled air after 24 hr. The cumulative ^{14}C urinary and fecal excretion of the same animals after 5 days were 1.8 and 12.4%, respectively, of the administered dose. Therefore, it was decided to study the fecal radioactivity as a reflector of the influence of the defined treatments on the elimination of chlorophenothane.

EXPERIMENTAL

^{14}C-Chlorophenothane Dosing Solution—The radiochemical purity of ^{14}C-chlorophenothane (*p,p'*-DDT), ring uniformly labeled[2] (specific activity 3.85 mc./mmole), was greater than 98.7%, as shown by TLC, autoradiography, and liquid scintillation counting techniques. The radiolabeled pesticide was dissolved in peanut oil.

[1] 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDT).

[2] Mallinckrodt-Nuclear, St. Louis, Mo.

Each milliliter of this solution contained 12.9 μc. of ^{14}C and 20 mg. of chlorophenothane. The administered radioactivity ranged from 6.78 to 7.32 μc./rat.

Treatments—The physical treatments investigated were cold and fasting. The cold was provided by a walk-in environmental room[3] maintained at 2–4°. Animals exposed to this treatment are referred to as the "cold group." The fasted rats were deprived of food, but water was allowed *ad libitum*. This group is referred to as the "fasted group." The chemical treatments evaluated were diphenylhydantoin[4] and levarterenol bitartrate[5]. The rats were treated with diphenylhydantoin either prior to administration of chlorophenothane (prediphenylhydantoin group) or after receiving the pesticide (postdiphenylhydantoin group). The dosage (30, 31) of diphenylhydantoin was 75 mg./kg. body weight i.p. twice a day. The animals receiving levarterenol bitartrate are referred to as the levarterenol group. The dosage (23) of levarterenol bitartrate was 1 mg./kg. body weight s.c. twice a day. The doses of diphenylhydantoin and levarterenol bitartrate were calculated as the base of the drug, and the necessary corrections were made to account for the difference due to the salts (sodium diphenylhydantoin and levarterenol bitartrate hydrate). The amount of diphenylhydantoin or levarterenol bitartrate administered to the animals was calculated individually, depending upon the rat body weight. The radioactivity in the feces was measured as a reflector of chlorophenothane excretion.

Thirty-six male Sprague–Dawley strain descendent rats[6], weighing 180–210 g. at the beginning of the experiment, were acclimated to laboratory conditions for 2 days. Each animal was kept in a 20 × 11.5-cm. stainless steel metabolism cage[7]. The rats were assigned to the six treatments in a randomized complete block design (33) of three replicates with two rats per treatment. The six treatments included control animals receiving only ^{14}C-chlorophenothane and the cold, fasted, prediphenylhydantoin, postdiphenylhydantoin and levarterenol bitartrate treatments previously defined. All groups of the first replicate, except prediphenylhydantoin, were fasted for 24 hr. prior to intraperitoneal dosing (on Day 1) with ^{14}C-chlorophenothane. The pesticide was allowed to distribute into the tissues of the animals for 5 days prior to initiation of treatments. On Day 6 following administration of the ^{14}C-chlorophenothane, each group was subjected to the assigned treatment, which was continued for 3 days. On Day 9 after dosing with the pesticide, the animals were sacrificed by decapitation using a small guillotine[8]. The prediphenylhydantoin group received diphenylhydantoin treatment for 3 days prior to the ^{14}C-chlorophenothane administration and then followed the same scheme as the other groups. The rats of the prediphenylhydantoin group were also fasted 24 hr. prior to dosing with the pesticide. The other two replicates were started on different days to allow subsequent replicate evaluation. The feces were collected daily, weighed, and stored frozen until the day of analysis.

Radioanalysis—The ^{14}C-radioactivity in all fecal samples was measured with an internal liquid scintillation spectrometer[9] equipped with bialkali phototubes. The scintillation solution consisted of 0.4% 2,5-diphenyloxazole in an equal volume of toluene and 2-ethoxyethanol. The percent counting efficiency of each sample was determined by adding ^{14}C-toluene internal standard and recounting. Whenever practical, the samples were counted with less than 5% counting error at the 95% confidence level. The feces pellets were homogenized by grinding in a glass mortar, and aliquots were digested as described by Bishara *et al.* (34). To about 20 mg. of the homogenized feces in a counting vial, 0.2 ml. of perchloric acid (70%) was added and mixed well to ensure thorough wetting. Two-tenths milliliter of hydrogen peroxide (30%) was then added and the contents were mixed. The vials were put in the oven for 15 min. at 70–75° and then cooled to room temperature. Another 0.5 ml. of hydrogen peroxide solution was added and the samples were bleached for 30 min. in the oven. The vials were allowed to cool to room temperature, and 15 ml. of the scintillation solution was added, mixed, and counted. Most fecal samples (72%) were assayed in quintet and the rest were assayed in triplicate. The results were averaged to get a mean value per sample of feces. The weights of the rats were recorded at the beginning and end of the experiment.

Table I—Daily Fecal ^{14}C-Activity (Chlorophenothane and/or Metabolites)

Treatment	Excreted Percentage of Administered Dose[a] Day 1	Day 2	Day 3	Day 4
Control	1.22	2.04	2.60	3.19
Postdiphenylhydantoin[b]	—	—	—	—
Prediphenylhydantoin	2.38	3.44	4.21	4.64
Cold[b]	—	—	—	—
Fasted	1.40	1.74	2.52	2.98
Levarterenol bitartrate[b]	—	—	—	—
Standard error	0.27	0.28	0.39	0.54

	Excreted Percentage of Administered Dose[a] Day 5	Day 6	Day 7	Day 8
Control	3.67	3.81	4.16	3.58
Postdiphenylhydantoin[b]	—	2.51	2.91	4.16
Prediphenylhydantoin	4.08	3.57	3.71	3.60
Cold[b]	—	5.29	4.85	5.07
Fasted	2.84	1.75	1.55	1.91
Levarterenol bitartrate	—	2.32	2.37	2.65
Standard error	0.51	0.45	0.48	0.79

[a] Data expressed as mean ± *SE* for six rats. [b] Fecal samples were not analyzed for Days 1–5.

Statistical Analysis of Data[10]—Homogeneity of variance was tested for the 18 cells (six treatments × three replicates) using the Bartlett (35) and/or Burr–Foster (36) tests for all days. A two-way analysis of variance (33) was conducted to investigate the effects of treatments, replicates, and the interaction between replicates and treatment for each day. Since almost all of the analyses showed no significant effect at the 0.25 level (37) between replicates (no significance at the 0.17 level the 1st day) or interaction between replicates and treatments, the data were then pooled and analyzed as if the experiment were a completely randomized design (33). For the analysis of the completely randomized design, the pooled data were then subjected to a one-way analysis of variance (33), and the resultant *F*-ratio for testing the differences between the means of groups was compared with the corresponding critical *F*-value at the 0.05 probability level. Whenever a significant *F*-ratio was obtained, the data were tested by a Newman–Keuls sequential range test (38) to locate the treatment means that were significantly different by pairs.

RESULTS AND DISCUSSION

During the first 5 days of the experiment, analysis of daily fecal samples for ^{14}C-activity was conducted on the control, prediphenylhydantoin, and fasted groups. Analysis of the feces of the fasted and the control groups was to test the hypothesis that all animals could be considered as controls in the Day 1–5 period except the prediphenylhydantoin group. Analysis of the feces of the latter group allowed comparison of the effect of diphenylhydantoin on the excretion of chlorophenothane in relation to the control group since diphenylhydantoin has been reported as a hepatic enzyme activator (1, 27, 32, 39).

All daily fecal samples collected during Days 6 through 8 were analyzed to monitor the effect of exposing the animals to the five treatments.

Daily Excretion—The daily fecal raw data from the randomized complete block design of the groups and days defined previously were accepted to have homogeneous variances. Interpretation of the two-way analysis of variances showed that there was no effect

[3] Hotpack, Philadelphia, Pa.
[4] Dilantin (sterile sodium diphenylhydantoin USP); Parke, Davis and Co., Detroit, Mich. Solution (S-V 105) contained 50 mg. Dilantin/ml.
[5] L-Arterenol bitartrate hydrate, CNE-E-grade, Calbiochem, Los Angles, Calif. Solution in saline contained 1 mg. norepinephrine/ml.
[6] Laboratory Supply Co., Indianapolis, Ind.
[7] Acme Metal Products, Chicago, Ill.
[8] Harvard Apparatus Co., Dover, Mass.
[9] Tri-Carb model 2002, Packard Instrument Co., Downers Grove, Ill.
[10] All statistical analyses were performed using the facilities of the Purdue University Computer Sciences Center and the standard computer programs for the statistical tests available at the facility.

Table II—Newman-Keuls Sequential Range Test of Daily Fecal ^{14}C-Activity (Chlorophenothane and/or Metabolites)[a]

Treatment	Day 1	2	3	6	7	1	2	3	6	7	1	2	3	6	7	1	2	3	6	7	1	2	3	6	7
	Fasted					Levarterenol Bitartrate					Postdiphenyl-hydantoin					Prediphenyl-hydantoin					Control				
Cold				**	**				**	**				**	*				*						*
Control				*	**																				
Prediphenylhydantoin	*	**	*	*	*																*	**	•		
Postdiphenylhydantoin																									
Levarterenol bitartrate																									

[a] * = significant at 5% level, and ** = significant at 1% level.

Table III—Cumulative Fecal ^{14}C-Activity (Chlorophenothane and/or Metabolites) Starting from Day 1

Treatment	Excreted Percentage of Administered Dose[a] Day 1	Day 2	Day 3	Day 4
Control	1.22	3.26	5.87	9.06
Prediphenylhydantoin	2.38	5.82	10.04	14.68
Fasted	1.40	3.15	5.67	8.65
Standard error	0.27	0.45	0.72	1.15

Treatment	Excreted Percentage of Administered Dose[a] Day 5	Day 6	Day 7	Day 8
Control	12.73	16.54	20.70	24.28
Prediphenylhydantoin	18.76	22.33	26.04	29.64
Fasted	11.49	13.25	14.79	16.70
Standard error	1.59	1.92	2.28	2.76

[a] Data expressed as mean ± *SE* for six rats.

of replicates nor treatment by replicate interaction. This indicated that repeating the experiment at different time intervals had no effect on the observed results; *i.e.*, the time interaction did not have any effect. Therefore, the data of each treatment were pooled and analyzed as if the experiment were a completely randomized design.

The pooled data were again statistically analyzed as described in the *Statistical Analysis of Data* section. Table I shows the mean daily excretion results of the pooled data for all groups from Days 6 to 8 and from Days 1 to 5 for the control, prediphenylhydantoin, and fasted groups.

Analysis of variance (ANOVA) was performed on the daily means to evaluate the differences due to the treatment effects. Significant *F*-ratios at the 0.05 level were obtained for Days 1, 2, 3, 6, and 7, indicating that the treatments affected the rats differently.

To locate the specific treatment effect, the data were then subjected to the Newman-Keuls sequential range test. The results are reported in Table II with the corresponding level of significance.

No significant differences were observed between the control and the fasted groups, indicating that for Days 1–3 the two groups could be considered as controls. Further elaboration on the fasted and control groups will be made later. The rats of the prediphenylhydantoin group excreted more ^{14}C-activity (chlorophenothane and/or metabolites) than the control groups for Days 1–3. The high excretion of ^{14}C-activity in the feces by the prediphenylhydantoin group may be the result of inducing the liver microsomal enzymes that metabolized chlorophenothane (39).

On Day 6, the cold treatment was significantly different from all other treatments. This indicated that the cold treatment has the strongest stimulating effect on the fecal excretion of chlorophenothane from the rats. There were also differences between the cold treatment and the fasted, levarterenol bitartrate, and postdiphenylhydantoin groups on Day 7. Since cold has been reported to mobilize fat (23, 25, 26), the high ^{14}C fecal activity, which was significantly different from other treatments, may be due to the availability of more chlorophenothane to be metabolized and excreted. However, the effect of the cold treatment seemed to wear off with time, since less differences were noticed on Day 7 and none was noticed on Day 8 between the cold group and the other groups. This may be due to the adaptability of the rats to the cold environment. Dirksen (40) reported that a cold environment affected rats most (detected by low adrenal ascorbic acid) at 6 and 18 hr. of exposure and that the animals adapted to the cold stress after this period.

The fasted group differed from the control group at the 0.05 and 0.01 levels of significance for Days 6 and 7, respectively. Although the fasted group was expected to mobilize its fat and the associated chlorophenothane, the low fecal excretion (due to no food ingestion) may explain the low activity excreted by the fasted group. There was a significant difference between the prediphenylhydantoin and the fasted groups at Days 6 and 7, which again reflected the low fecal excretion of the fasted group.

Cumulative Excretion—The daily means reported in Table I were then expressed as cumulative activity and were subjected to the same statistical tests. These were performed on data of Days 1–8 for the control, prediphenylhydantoin, and fasted groups. The mean cumulative fecal ^{14}C-activity for these three groups are reported in Table III. The statistical evaluation of the cumulative data clearly showed a significant difference (Table IV) between the fecal excretion of the prediphenylhydantoin group and each of the control and fasted groups for the Days 1–6 period. These results favorably backed the assumption that all rats were controls until the treatments started on Day 6. Further differences were observed between the fasted and prediphenylhydantoin groups for Days 7 and 8, thus indicating low fecal excretion of chlorophenothane and/or its metabolites by the fasted group.

When comparing the control and fasted groups based upon the cumulative data (Table III), no significant differences between them were noticed (Table IV). During the first 5 days of the experiment, these results were expected since it has been previously shown that both groups could be considered as controls. Due to the effect of the treatment starting on Day 6, a difference of chlorophenothane fecal excretion between the fasted and control groups was anticipated. However, the expected increased elimination was not observed. This may be due to the masking effect of the first 5 days of cumulative excretion.

The cumulative means starting from Days 6 to 8 for all six groups are reported in Table V. The data of prediphenylhydantoin are included in the table for completeness. It has already been shown (Table IV) that by Day 6 the prediphenylhydantoin group could be considered as a control group since the effect of the drug has been

Table IV—Newman-Keuls Sequential Range Test of Cumulative Fecal ^{14}C-Activity (Chlorophenothane and/or Metabolites) Starting from Day 1[a]

Treatment	Day 1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
	Control								Fasted							
Prediphenylhydantoin	*	**	**	**	*	*			*	**	**	**	*	*	**	*
Fasted																

[a] * = significant at 5% level, and ** = significant at 1% level.

Table V—Cumulative Fecal ^{14}C-Activity (Chlorophenothane and/or Metabolites) Starting from Day 6

Treatment	Excreted Percentage of Administered Dose[a]		
	Day 6	Day 7	Day 8
Control	3.81	7.96	11.55
Postdiphenylhydantoin	2.51	5.42	9.58
Prediphenylhydantoin	3.57	7.28	10.88
Cold	5.29	10.14	15.21
Fasted	1.75	3.30	5.21
Levarterenol bitartrate	2.32	4.68	7.33
Standard error	0.45	0.84	1.48

[a] Data expressed as mean ± *SE* for six rats.

dissipated. The cumulative data clearly reflect that there is no statistical difference between the prediphenylhydantoin and control groups on Days 6–8 (Table VI).

The cumulative chlorophenothane fecal excretion starting from Day 6 (Table V) of the cold group was always the highest and was followed by the control, prediphenylhydantoin, postdiphenylhydantoin, levarterenol bitartrate and fasted groups in descending order. The cold group was statistically different from all other groups on Day 6. Also, significant effects (Table VI) of the cold treatment existed between this group and each of the fasted and levarterenol bitartrate groups for Days 7 and 8. On Day 7, the cold group differed from the postdiphenylhydantoin group. This difference was previously explained in the discussion of daily excretion data.

The fasted group differed from the control group on Days 6–8 while the levarterenol bitartrate group differed from the control only on Day 7. The latter case may be explained by the observation that the rats of the levarterenol group reduced their food consumption after administration of the compound, which resulted in less fecal excretion as was the case with the fasted group. Another explanation of the significant difference between the control and levarterenol bitartrate groups may be due to the fact that this drug inhibited some metabolizing enzymes (28, 41, 42). This may have resulted in low excretion of chlorophenothane and/or its metabolites.

The mean initial and final body weights of the rats used in this experiment are reported in Table VII. The final body weight means were all higher than the initial weights, except for the fasted group which was the same. The Newman–Keuls sequential range test (Table VIII) for these data showed a significant difference of the body weights between the fasted and all the other treatments at the 0.01 level. Postdiphenylhydantoin and cold groups differed from the control group at a significance level of 0.01 and 0.05, respectively. In the case of the postdiphenylhydantoin group, the animals did not consume as much food after dosing. On the other hand, the animals exposed to cold may have lost some of their fat that had been mobilized for energy during cold exposure.

In conclusion, the cold treatment was the most effective of all studied treatments in stimulating fecal excretion of ^{14}C-chlorophenothane and/or metabolites. However, the efficiency of this treatment diminished after the first 24 hr. due to adaptation.

The prediphenylhydantoin treatment may have stimulated the hepatic microsomal enzymes that metabolize chlorophenothane. This was clearly shown in the cumulative data starting from Day 1 (Table III) and is in agreement with previously published data (1, 27, 32, 39).

Table VII—Initial and Final Body Weight of the Rats[a]

Treatment	Initial Body Weight, g.[b]	Final Body Weight, g.[c]
Control	192.3	268.2
Postdiphenylhydantoin	186.2	225.0
Prediphenylhydantoin	191.7	258.7
Cold	197.7	242.6
Fasted	193.4	191.7
Levarterenol bitartrate	194.2	252.1
Standard error	3.8	5.9

[a] Data expressed as mean ± *SE* for six rats. [b] Body weight at 1st day of adaptation. [c] Body weight at day of sacrifice.

The postdiphenylhydantoin treatment was not effective in enhancing the fecal excretion of chlorophenothane and/or its metabolites. Similar results have been reported in the case of humans taking daily doses of diphenylhydantoin to determine the effect on chlorophenothane residue reduction. No reduction was detectable at 3 weeks, and the study was to be continued for 3 months (43). Recently, Davies *et al.* (44) reported that oral sodium diphenylhydantoin (300 mg. daily for up to 9 months) reduced the chlorophenothane and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE) residues in human volunteers by 75 and 61% of pretreatment levels, respectively.

Although several authors used diet restriction and fasting to mobilize fat (17, 19, 25) together with its pesticide content, the redistribution of the pesticide in other tissues was always the end result (19, 22, 24, 25, 45). In this study, fasting, which is a form of diet restriction, had the least effect on the excretion of chlorophenothane from rats.

The statistical designs (randomized complete block and completely randomized) used in this work complied with the basic principles of experimental design, namely replication, randomization, and local control (33). The designs also supplied statistically reliable data which lead to valid inferences with respect to the results obtained using minimum effort and expense.

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Table VI—Newman–Keuls Sequential Range Test of Cumulative Fecal ^{14}C-Activity (Chlorophenothane and/or Metabolites) Starting from Day 6[a]

Treatment	Day														
	6	7	8	6	7	8	6	7	8	6	7	8	6	7	8
	Fasted			Levarterenol Bitartrate			Postdiphenyl-hydantoin			Prediphenyl-hydantoin			Control		
Cold	**	**	**	**	**	**	**	**		*			*		
Control	*	**	*		*										
Prediphenylhydantoin	*	*	*												
Postdiphenylhydantoin															
Levarterenol bitartrate															

[a] * = significant at 5% level, and ** = significant at 1% level.

Table VIII—Newman–Keuls Sequential Range Test of Final Body Weight of the Rats[a]

Stress	Fasted	Postdiphenyl-hydantoin	Cold	Levarterenol Bitartrate	Prediphenyl-hydantoin
Control	**	**	*		
Prediphenylhydantoin	**	**			
Levarterenol bitartrate	**	**			
Cold	**	*			
Postdiphenylhydantoin	**				

[a] * = significant at 5% level, and ** = significant at 1% level.

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Cardiovascular Effects of Isoxsuprine and Sotalol

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Abstract □ Isoxsuprine, a vasodilating agent, and sotalol, a β-adrenergic receptor blocking agent, were administered separately and in combination to normotensive rats and hypertensive dogs. In rats, sotalol produced a hypotensive response only in low doses whereas isoxsuprine produced a significant hypotension at all doses used. Administration of either agent was followed by a significant decrease in heart rate. The combination of isoxsuprine with sotalol decreased the heart rate more than either drug alone, but the hypotensive response did not follow this pattern. In hypertensive dogs, isoxsuprine and sotalol significantly decreased blood pressure. In contrast to the rat, the hypotensive response to isoxsuprine in the dog was accompanied by an increase in heart rate. The combination of isoxsuprine with sotalol produced a greater hypotensive response than either drug alone in medium and high doses but not in low doses. Sotalol prevented the increase in heart rate that accompanied the hypotensive response to isoxsuprine. The data suggest that the combination of isoxsuprine and sotalol may be useful in the treatment of hypertension.

Keyphrases □ Isoxsuprine and sotalol—cardiovascular effects after separate and concurrent administration in rats and dogs, potential antihypertensive therapy □ Sotalol and isoxsuprine—cardiovascular effects after separate and concurrent administration in rats and dogs, potential antihypertensive therapy □ Antihypertensive activity—concurrent administration of isoxsuprine and sotalol evaluated, rats, dogs □ Cardiovascular effects—isoxsuprine and sotalol, separate and concurrent administration, rats, dogs

The treatment of essential hypertension has centered in recent years on the use of various agents that interfere with adrenergic neuronal transmission. Although this approach has been generally successful in reducing blood pressure, it is not without problems. Hypotensive agents that interfere with sympathetic nervous function commonly cause postural hypotension, weakness, lethargy, and sexual dysfunction (1–6).

Since an elevation of peripheral vascular resistance appears to be the major hemodynamic abnormality present in essential hypertension (7), a new treatment based on the concurrent administration of vasodilating and β-receptor blocking drugs was recently introduced (8, 9). In previous investigations, drugs such as hydralazine were used to produce vasodilation (9). Hydralazine was reported to decrease peripheral resistance by a direct relaxing action on arteriolar smooth muscle (10–12). Its antihypertensive effectiveness is limited, however, by a reflex action that increases heart rate and cardiac output (12, 13). Even though the reflex cardiac augmentation produced by hydralazine can be prevented by propranolol (9), other side effects, such as immunologic disturbances occurring during chronic therapy (14), make the use of different vasodilating drugs more desirable.

Isoxsuprine[1], an agent that produces vasodilation through a direct relaxing action on vascular smooth muscle (15–17) as well as by activation of β-adrenergic receptors (18, 19), appears to be more suitable for this purpose since it has been reported to be free of undesired effects (20). The hypotensive effect produced by isoxsuprine may be useful in the treatment of hypertension if the positive chronotropic and inotropic effects accompanying the hypotensive response can be prevented. In the present investigation, sotalol[2], a β-receptor blocking agent, was utilized in an attempt to block selectively the cardiac augmentation produced by isoxsuprine while retaining the peripheral vasodilating and hypotensive effects.

[1] Vasodilan, Mead Johnson.

EXPERIMENTAL

Measurement of Blood Pressure and Heart Rate in Anesthetized Rats—Wistar albino rats of both sexes, weighing approximately 200–250 g., were anesthetized with 1.25 g./kg. of urethan administered intraperitoneally. The animals were secured ventral side up on an animal board. A midline incision was made over the tracheal region; the trachea was exposed and cannulated with polyethylene tubing[3] to ensure freedom of respiration. A common carotid artery was isolated and cannulated with polyethylene tubing[4], which was connected to a pressure transducer[5]; all recordings were made on a polygraph[6]. One femoral vein was isolated for injection purposes, and the blood pressure was allowed to stabilize before administration of any drug. In each case the experimental compounds, dissolved in saline, were administered in volumes of 0.2 ml. or less. Groups of three male and three female rats were administered intravenously either 2, 3, or 4 mg./kg. of isoxsuprine, sotalol, or equal combinations of both. The heart rate and systolic blood pressure were monitored for 90 min. following drug administration. The changes obtained after drug administration were compared to the control values obtained before drug administration and analyzed by the Student t test. Values of $p \leq 0.05$ were considered significant.

Measurement of Blood Pressure and Heart Rate in Unanesthetized Hypertensive Dogs—Two male and two female mongrel dogs were made hypertensive by the method of Grollman (21). In this study the method was modified in that both kidneys were ligated, thus increasing the amount of residual, normally functioning renal tissue and minimizing the possibility of uremia developing. Two mongrel dogs who were received from the supplier and found to be hypertensive were used as well. Systolic blood pressure was determined before surgery and at weekly intervals thereafter until a stable pressure of 180 mm. Hg or higher was attained. All dogs were given normal saline for drinking water *ad libitum*. The dogs were trained to lie quietly in a hammock, which allowed all four limbs to be freely suspended. The blood pressure and heart rate were recorded from the brachial artery by means of an infant sphygmomanometer cuff which contained a sensitive microphone[7]. The cuff was connected to an electrosphygmomanometer, and recordings were made on a physiograph[8]. Ten clear readings of the systolic blood pressure were recorded at each time interval, and an average value was obtained. Heart rate was determined from the pulse wave by partially occluding the artery for 15 sec.

Systolic blood pressure and heart rate were determined 5–10 min. before administration and at 30, 90, 180, and 360 min. after administration. Each dog served as its own control. All drugs were administrated orally as a powder in gelatin capsules. Each dog received nine drug dosages, and the schedule of administration and

[2] 4′-[1-Hydroxy-2-(isopropylamino)ethyl]methanesulfonanilide monohydrochloride, MJ-1999, Mead Johnson.
[3] PE 240.
[4] PE 90.
[5] Statham.
[6] Grass 5D.
[7] Korotkoff
[8] E&M Mark IV console.

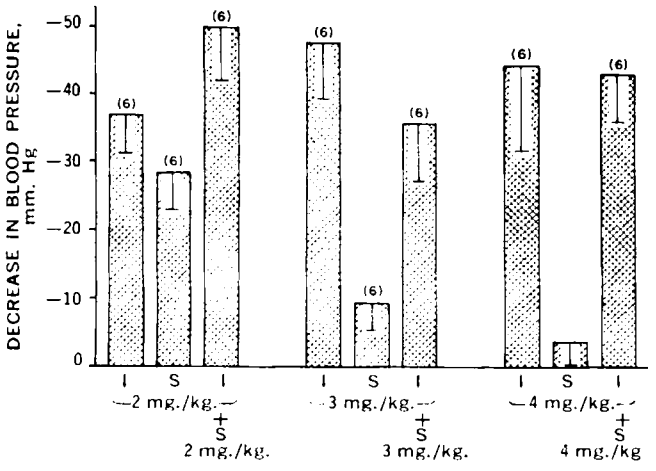

Figure 1—*Effect of isoxsuprine (I) and sotalol (S) on blood pressure in rats. Isoxsuprine and sotalol were administered intravenously alone and in combination (I + S). The number of observations is indicated in parentheses. The hypotensive response produced by I is not significantly different from I + S at any dose. The response to I + S is significantly greater than that to S at the 3- and 4-mg./kg. doses* ($p < 0.05$).

dosage was as follows: isoxsuprine at 6, 12, and 18 mg./kg.; sotalol at 10, 20, and 30 mg./kg.; and a simultaneously administered combination of 6, 12, and 18 mg./kg. of isoxsuprine with 10, 20, and 30 mg./kg. of sotalol, respectively. These doses of sotalol were reported by Lish *et al.* (22) to produce β-receptor blockade in dogs as measured by blockade of isoproterenol-induced tachycardia. A control series was performed in which lactose was administered in place of the active drugs, and the heart rate and blood pressure were followed over a time course identical to that of the drug studies. Each dog was given at least a 3-day rest between drug administrations. The effects of the drugs administered separately and in combination were analyzed by the Student *t* test. Values of $p \leq 0.05$ are considered significant.

RESULTS

Effect of Isoxsuprine and Sotalol on Blood Pressure and Heart Rate in Anesthetized Rats—The intravenous administration of isoxsuprine in rats produced a significant decrease in blood pressure ($p < 0.001$; Fig. 1). There was no significant difference in the magnitude of the hypotensive response between the three doses of isoxsuprine ($p > 0.05$). The decrease in blood pressure produced by isoxsuprine was not accompanied by a reflex increase in heart rate (Fig. 2). In fact, in all but two instances (2/18), the rats responded with a bradycardia, the magnitude of which was not significantly different between the three doses of isoxsuprine ($p > 0.05$).

The administration of the low dose (2 mg./kg.) of sotalol resulted in a significant decrease in blood pressure ($p < 0.05$), while the medium (3 mg./kg.) and high (4 mg./kg.) doses did not produce a significant depressor effect ($p > 0.05$; Fig. 1). The administration of sotalol produced a significant decrease in heart rate at all three doses employed ($p < 0.05$; Fig. 2). There was no significant difference in the magnitude of the effect at the different doses.

The combination of isoxsuprine and sotalol produced a significant hypotensive effect at all three dose levels ($p < 0.05$; Fig. 1). Although the hypotensive response produced by the combination was significantly greater than that produced by sotalol alone at the same dose ($p < 0.05$), it did not differ from that produced by isoxsuprine alone at the same dose ($p > 0.05$). Thus, there was no evidence to suggest that sotalol potentiated the hypotensive response produced by isoxsuprine. The combination of isoxsuprine with sotalol produced a greater negative chronotropic effect than the administration of either compound alone in the low and medium doses ($p < 0.05$) but not at the high dose ($p > 0.05$).

Effect of Isoxsuprine and Sotalol on Blood Pressure and Heart Rate in Unanesthetized Hypertensive Dogs—The oral administration of lactose to six unanesthetized hypertensive dogs did not produce any significant change in blood pressure or heart rate. The oral administration of isoxsuprine alone at low (6 mg./kg.), medium (12 mg./kg.), and high (18 mg./kg.) doses to hypertensive dogs produced a significant reduction in blood pressure of approximately 18–23 mm. Hg ($p < 0.05$; Fig. 3). There was no significant difference in the magnitude of the hypotensive effect between the three doses of isoxsuprine ($p > 0.05$). At each dose, a significant increase in heart rate accompanied the hypotensive response, the pattern being such that the high dose of isoxsuprine produced the greatest increase in heart rate (Fig. 4). The progressive elevation in heart rate produced by increasing the dose of isoxsuprine may explain the lack of a greater hypotensive response when the higher doses of isoxsuprine were administered.

The administration of sotalol produced a significant reduction in blood pressure, the magnitude of which was not different between the doses or from that produced by isoxsuprine at any dose (Fig. 3). However, the hypotensive response to sotalol, in contrast to isoxsuprine, was not accompanied by an increased heart rate but by a significant reduction in rate at each dose (Fig. 4). The magnitude of the effect was not different between doses of sotalol ($p > 0.05$). Diminution of sympathetic influences to the heart probably explains the hypotensive response to sotalol and the lack of any reflex adjustments.

The simultaneous adminstration of isoxsuprine and sotalol produced a consistent drop in blood pressure (Fig. 3). The maximum hypotensive response was achieved with the combination of 12 mg./kg. isoxsuprine and 20 mg./kg. sotalol (medium dose); increasing the dose of either drug did not result in a greater hypotensive response. Since the medium and high doses of the combination produced a decrease in blood pressure that was the same as the total of both drugs alone, the hypotensive effects of isoxsuprine and sotalol seem to be additive. It is possible that isoxsuprine in low doses (6 mg./kg.) produces vasodilation primarily through stimulation of β-receptors whereas higher doses produce a direct vasodilation which is independent of β-receptor activation. The β-receptor-mediated hypotensive response produced by low doses can be prevented by sotalol, but the direct vasodilation produced by high doses is not affected by sotalol.

The simultaneous administration of sotalol with isoxsuprine prevented the increase in heart rate produced when isoxsuprine was administered alone (Fig. 4). In the low and medium doses of the combination, sotalol converted the increase in heart rate to a decrease of approximately 16 beats/min. With the high dose of the combination, however, although there was an overall decrease in heart rate for the group, three of the six animals responded to the combination with an increase in heart rate which was, on the average, approximately 12 beats/min. It is possible, therefore, that the increase in heart rate in these animals may explain the lack of an increased hypotensive response with the high dose of the combination compared to the medium dose in which all dogs responded with a decrease in heart rate.

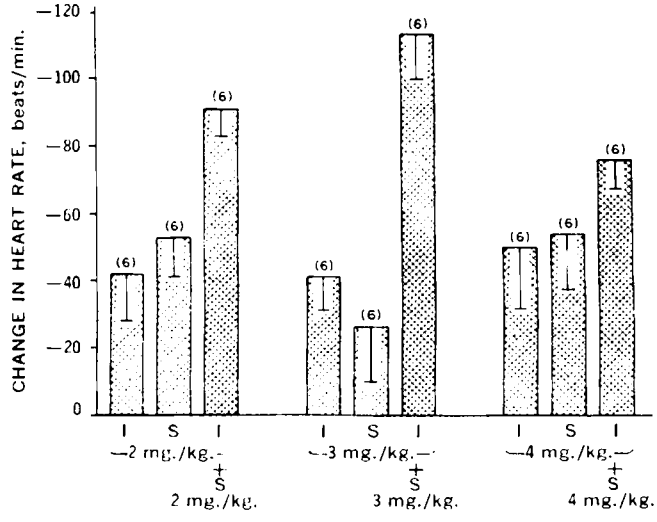

Figure 2—*Effect of isoxsuprine (I) and sotalol (S) on heart rate in rats. The response to I + S is significantly greater than the response to I or S alone at the 2- and 3-mg./kg. doses* ($p < 0.05$) *but not at 4 mg./kg. See Fig. 1 for details to legend.*

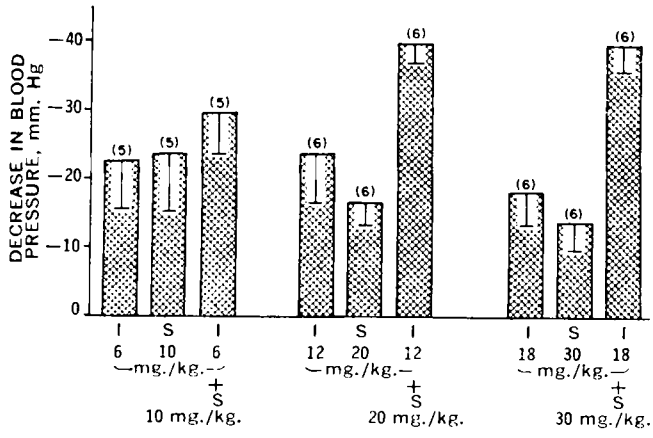

Figure 3—*Effect of isoxsuprine (I) and sotalol (S) on blood pressure in hypertensive dogs. Isoxsuprine and sotalol were administered orally alone and in combination (I + S). The number of observations is indicated in parentheses. The response to I + S is significantly greater than the response to I or S alone at the medium and high doses ($p < 0.01$). The response to I + S is the same as that to I or S at the low dose.*

DISCUSSION

The results of this study demonstrated that isoxsuprine is an effective hypotensive agent. Furthermore, the increase in heart rate produced by either a direct or reflex action of isoxsuprine could be prevented by the concurrent administration of sotalol whereas the hypotensive response was unaffected.

Marked differences were found in the heart rate effects of isoxsuprine in the conscious dogs and anesthetized rats. In the conscious dogs, the fall in arterial blood pressure produced by isoxsuprine was accompanied by an increase in heart rate, whereas in the rats the fall in blood pressure was always associated with bradycardia. Similar results were reported by Brunner *et al.* (23) using hydralazine as the vasodilating agent. These authors reported that the depressor response to hydralazine was accompanied by an increase in heart rate in conscious rats but a decrease in heart rate in anesthetized rats. It is concluded from the results of the present study that anesthetized rats must be used with caution in the evaluation of antihypertensive compounds where reflex cardiovascular changes may result since the anesthetized rat may obscure these changes. The present results in the unanesthetized dogs are not in agreement with Brunner *et al.* (23, 24), who reported that the administration of pronethalol prevented the direct vasodilating response to hydralazine in unanesthetized dogs. However, these results are in agreement with the findings of Gilmore *et al.* (25), Sonnerstedt *et al.* (26), Gottlieb *et al.* (8), and Zacest *et al.* (9), who found that the administration of β-blocking agents along with direct acting peripheral vasodilating agents prevents the reflex cardiac effects in response to a decrease in blood pressure but maintains or potentiates the hypotensive response. This study represents the first report of the use of isoxsuprine and sotalol for this purpose.

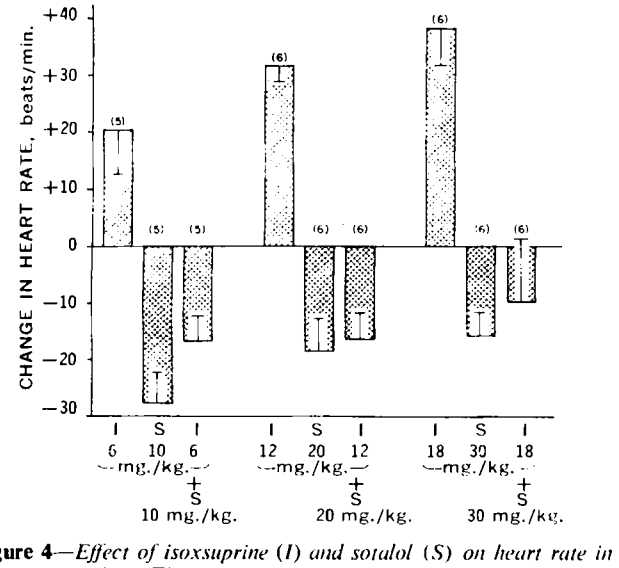

Figure 4—*Effect of isoxsuprine (I) and sotalol (S) on heart rate in hypertensive dogs. The response to sotalol alone is not significantly different from the response to I + S at any dose. See Fig. 3 for details to legend.*

Hydralazine is the only direct acting vasodilating agent currently used in the treatment of hypertension. Despite the capacity of this agent to lower blood pressure, its antihypertensive effectiveness is limited by the reflex increase in sympathetic discharge that produces an undesirable increase in both heart rate and cardiac output. Glick and Braunwald (27) showed that the reflex cardiovascular adjustment initiated by a fall in blood pressure consists of increased sympathetic discharge rather than a decrease in vagal activity. The cardiac augmentation produced by this means may often compromise the depressor response to this agent (12, 13). Several investigators showed that the cardiac augmentation resulting from the hypotensive action of hydralazine can be prevented by the concurrent administration of β-blocking agents (9, 26), but other side effects associated with this agent, *e.g.*, lupus-like reaction, suggest that vasodilating agents such as isoxsuprine and nylidrin would be more valuable adjuncts to β-blockade therapy.

Propranolol is the only β-blocking agent currently available in clinical practice in the United States that can be used in combination with vasodilating drugs. In some respects, sotalol is more desirable than propranolol for this purpose. The use of propranolol with a vasodilating agent may not result in as great a hypotensive response as would result with the use of other β-blockers since it has been reported that propranolol causes an increase in peripheral resistance (16, 28, 29). It is possible, therefore, that the increase in vascular resistance produced by propranolol may directly counteract the decrease in arteriolar resistance produced by the vasodilating drug. There have been no reports to date that sotalol produces such an increase in peripheral vascular resistance. In addition, propranolol has been reported to possess a direct myocardial depressant action (30, 31), which may limit its usefulness in patients with hypertensive heart disease. Sotalol, on the other hand, has been reported to be a "safer" β-blocking agent (32) since it does not directly reduce myocardial function at doses that produce β-blockade (30, 33).

Since at least part of the vasodilation produced by isoxsuprine is the result of stimulation of β-receptors (22) and since propranolol and sotalol block both cardiac and vascular β-receptors, these agents may reduce to some extent the hypotensive effect elicited by isoxsuprine. In this regard, β-blockers, such as practolol, that exert a specificity for blockade of the cardiac β-receptors more so than vascular β-receptors (34) should prove to be even more useful hypotensive drugs when combined with vasodilating agents such as isoxsuprine and nylidrin.

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Detection of Prostaglandin $F_{2\alpha}$ as Pentafluorobenzyl Ester by Electron-Capture GLC

J. A. F. WICKRAMASINGHE▲, W. MOROZOWICH, W. E. HAMLIN, and S. R. SHAW

Abstract □ The pentafluorobenzyl ester of prostaglandin $F_{2\alpha}$ was synthesized on a preparative scale and was gas chromatographed as the tris(trimethylsilyl) ether. The latter was found to be stable during GLC and highly sensitive to electron-capture detection. The lower limit of detection was 12.5 pg. of the ester, injected on-column as the silylated product. The nanogram scale conversion of prostaglandin $F_{2\alpha}$ to the ester, under conditions amenable to electron-capture GLC detection, was developed. The electron-capture GLC response was linear over the 0.03–0.84-ng. range of the ester, injected as the tris(trimethylsilyl) ether.

Keyphrases □ Prostaglandin $F_{2\alpha}$—electron-capture GLC analysis as pentafluorobenzyl ester □ GLC, electron-capture detection—analysis, prostaglandin $F_{2\alpha}$ as pentafluorobenzyl ester

Prostaglandins are biologically important compounds that are active at very low concentrations, usually in the nanogram range. To study the physiological role and the mechanisms of action, as well as the absorption, metabolism, and excretion of these prostaglandins, simplified analytical methodology of high sensitivity and specificity is required. Among the many methods considered for the quantitation of prostaglandins are those based on biological responses (1, 2), enzymatic assay (3), fluorescence and UV spectroscopy (4–6), radioimmunoassay (7–11), and chromatography. The last includes: (*a*) high-pressure liquid chromatography for the detection of low levels of prostaglandins after conversion to the C-1-*p*-nitrobenzyl esters (12), and (*b*) GLC utilizing flame-ionization detection (13–15), mass spectrometric detection (16–22), or electron-capture detection (23–26). GLC methods utilizing flame ionization usually lack adequate sensitivity for the detection of low levels of prostaglandins incident in biological fluids. The GLC–mass spectrometric methods and the electron-capture GLC methods, which afford high sensitivity, also offer a high degree of specificity when combined with a preliminary TLC separation. In terms of general utility, the widely used and elegant GLC–mass spectrometric method has the disadvantage of requiring expensive and usually inaccessible instrumentation, as well as the synthesis of the isotopically labeled (deuterated) prostaglandins for internal standards and carriers.

Reported electron-capture GLC methods based on the inherent electron-capturing properties of prostaglandin B (PGB) compounds (23, 24) have drawn considerable attention. This approach, however, is limited to the PGB compounds or to prostaglandins that can be readily and quantitatively converted to the PGB's. The most common derivatives of the prostaglandins examined for electron-capture detection involve derivatization of the hydroxy groups as, for example, conversion to a heptafluorobutyrate ester (25, 26) or bromomethyl–dimethylsilyl ether (24). The thermal instability of the heptafluorobutyrate derivative, resulting in the formation of multiple GLC peaks, renders it unsuitable for electron-capture GLC analysis (26).

Since all naturally occurring prostaglandins contain a C-1 carboxyl group that has been shown to undergo facile reaction with benzylic halides, the C_1-penta-

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Detection of Prostaglandin $F_{2\alpha}$ as Pentafluorobenzyl Ester by Electron-Capture GLC

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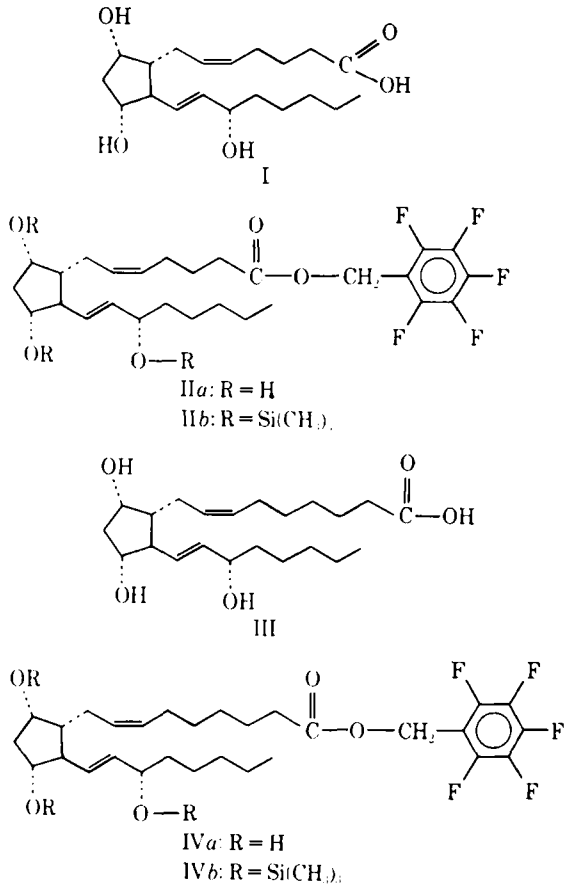
Abstract □ The pentafluorobenzyl ester of prostaglandin $F_{2\alpha}$ was synthesized on a preparative scale and was gas chromatographed as the tris(trimethylsilyl) ether. The latter was found to be stable during GLC and highly sensitive to electron-capture detection. The lower limit of detection was 12.5 pg. of the ester, injected on-column as the silylated product. The nanogram scale conversion of prostaglandin $F_{2\alpha}$ to the ester, under conditions amenable to electron-capture GLC detection, was developed. The electron-capture GLC response was linear over the 0.03–0.84-ng. range of the ester, injected as the tris(trimethylsilyl) ether.

Keyphrases □ Prostaglandin $F_{2\alpha}$—electron-capture GLC analysis as pentafluorobenzyl ester □ GLC, electron-capture detection—analysis, prostaglandin $F_{2\alpha}$ as pentafluorobenzyl ester

Prostaglandins are biologically important compounds that are active at very low concentrations, usually in the nanogram range. To study the physiological role and the mechanisms of action, as well as the absorption, metabolism, and excretion of these prostaglandins, simplified analytical methodology of high sensitivity and specificity is required. Among the many methods considered for the quantitation of prostaglandins are those based on biological responses (1, 2), enzymatic assay (3), fluorescence and UV spectroscopy (4–6), radioimmunoassay (7–11), and chromatography. The last includes: (*a*) high-pressure liquid chromatography for the detection of low levels of prostaglandins after conversion to the C-1-*p*-nitrobenzyl esters (12), and (*b*) GLC utilizing flame-ionization detection (13–15), mass spectrometric detection (16–22), or electron-capture detection (23–26). GLC methods utilizing flame ionization usually lack adequate sensitivity for the detection of low levels of prostaglandins incident in biological fluids. The GLC–mass spectrometric methods and the electron-capture GLC methods, which afford high sensitivity, also offer a high degree of specificity when combined with a preliminary TLC separation. In terms of general utility, the widely used and elegant GLC–mass spectrometric method has the disadvantage of requiring expensive and usually inaccessible instrumentation, as well as the synthesis of the isotopically labeled (deuterated) prostaglandins for internal standards and carriers.

Reported electron-capture GLC methods based on the inherent electron-capturing properties of prostaglandin B (PGB) compounds (23, 24) have drawn considerable attention. This approach, however, is limited to the PGB compounds or to prostaglandins that can be readily and quantitatively converted to the PGB's. The most common derivatives of the prostaglandins examined for electron-capture detection involve derivatization of the hydroxy groups as, for example, conversion to a heptafluorobutyrate ester (25, 26) or bromomethyl–dimethylsilyl ether (24). The thermal instability of the heptafluorobutyrate derivative, resulting in the formation of multiple GLC peaks, renders it unsuitable for electron-capture GLC analysis (26).

Since all naturally occurring prostaglandins contain a C-1 carboxyl group that has been shown to undergo facile reaction with benzylic halides, the C_1-penta-



fluorobenzyl ester of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was prepared to examine its electron-capture detection properties.

EXPERIMENTAL

Preparative Scale Synthesis of $PGF_{2\alpha}$ Pentafluorobenzyl Ester (IIa)—A solution of $PGF_{2\alpha}$ (I) (40 mg.) in acetonitrile (2 ml.) was treated with pentafluorobenzyl bromide (0.2 ml.) and diisopropylethylamine (0.2 ml.). TLC examination [on silica gel in ethyl acetate–acetic acid (97:3)] indicated quantitative formation of the derivative (II*a*) (R_f 0.4) in 1 hr. with little evidence of side-products. The reaction mixture was diluted with 0.1 *M* citric acid (50 ml.) and extracted with ethyl acetate (50 ml.). The organic phase was washed with water (50 ml.) and dried with anhydrous sodium sulfate. The crude product was chromatographed[1], and the ester was eluted with ethyl acetate-acetone (6:4). Removal of the solvent gave a colorless oil (42 mg.) which showed a single spot with TLC and a single peak with GLC (flame-ionization detection) as the trimethylsilyl derivative (II*b*).

Submicrogram Scale Conversion of $PGF_{2\alpha}$ to IIa—$PGF_{2\alpha}$ (I) was treated with a solution of pentafluorobenzyl bromide in acetonitrile [10 μl. containing 5.8 mg. (0.022 mmole) of pentafluorobenzyl bromide] and a solution of diisopropylethylamine in acetonitrile [10 μl. containing 0.886 mg. (0.007 mmole) of diisopropylethylamine]. The reaction mixture was heated at 40° for 5 min. and then evaporated to dryness under nitrogen. The recovered sample was treated with the reagents again in a manner identical to that indicated previously and was evaporated to dryness under nitrogen.

Tris(trimethylsilyl) $PGF_{2\alpha}$ Pentafluorobenzyl Ester (IIb)—Electron-capture GLC analysis was carried out on the tris(trimethylsilyl) ether of the $PGF_{2\alpha}$ pentafluorobenzyl ester (II*a*).

On a preparative scale, II*a* (10 mg.) was dissolved in *N,O*-bis-(trimethylsilyl)acetamide–pyridine (1:1) (1 ml.), and the solution was heated at 60° for 15 min. Excess reagent was removed with a stream of nitrogen, and the residue was dissolved in tetrahydrofuran for GLC analysis. Appropriate dilutions of this stock solution were made with the same solvent. The gas chromatograms did not show any additional peaks or significant decrease in concentration on standing for 24 hr. at room temperature.

In a submicrogram scale analysis, II*a* residues from the esterification were treated with *N,O*-bis-(trimethylsilyl)acetamide (10 μl.) at room temperature (~23°) for 5 min. Aliquots (1 μl.) of the solution were directly analyzed by electron-capture GLC.

Internal Standard for GLC—The tris(trimethylsilyl) pentafluorobenzyl ester of 9-{3α,5α-dihydroxy-2β-[(3*S*)-3-hydroxy-*trans*-1-octenyl]-1α-cyclopentyl}-*cis*-7-nonenoic acid or 2*a*,2*b*-dihomo-$PGF_{2\alpha}$ (III) was used as an internal standard for GLC.

*Pentafluorobenzyl Ester of 9-{3α,5α-dihydroxy-2β-[(3S)-3-hydroxy-*trans*-1-octenyl]-1α-cyclopentyl}-cis-7-nonenoic Acid (III)*—A solution of III (31.91 mg.) in acetonitrile (2 ml.) was treated with pentafluorobenzyl bromide (0.2 ml.) and diisopropylethylamine (0.2 ml.) at room temperature. After stirring for 1 hr., the reaction mixture was concentrated (to about 0.5 ml.) by evaporation under nitrogen, diluted with 0.1 *M* citric acid (10 ml.), and extracted with ethyl acetate (2 × 10 ml.). The combined organic phase was washed with water (10 ml.), dried with anhydrous sodium sulfate, filtered, and evaporated. The crude product was purified by sequential preparative TLC on silica gel in solvent systems of: (*a*) ethyl acetate–acetic acid (97:3), and (*b*) ethyl acetate–acetic acid (95:5). The pentafluorobenzyl ester (IV*a*) zone was located by exposure of the reference spots on discrete lanes to iodine vapor in the manner indicated previously. Then IV*a* was extracted from the silica gel using ether (2 × 5 ml.). The purified ester (IV*a*) (9.48 mg.) was observed to be homogeneous by TLC on silica gel in ethyl acetate–acetic acid (95:5).

Tris(trimethylsilyl) Ether of Pentafluorobenzyl Ester (IVa)—An aliquot of IV*a* (2.04 mcg.) was converted to the tris(trimethylsilyl) derivative (IV*b*) by treatment with *N,O*-bis-(trimethylsilyl)acetamide (1 ml.) and examined by electron-capture GLC. A GLC peak corresponding to a minor impurity (retention time ~12 min.) was observed in addition to the peak due to the major product tris-(trimethylsilyl) pentafluorobenzyl ester (IV*b*). The identity of the latter was confirmed by GLC–mass spectrometry.

A solution of IV*b* in the silylating agent (65.174 mcg./ml.) was employed as the internal standard for GLC analysis. Aliquots (10 μl.) of this solution were added to submicrogram quantities of II*a* and mixed well[2]. Samples (1 μl.) of this solution were utilized for electron-capture GLC analysis.

Electron-Capture GLC—*Method A*—A chromatograph[3], equipped with a ^{63}Ni electron-capture detector, was operated with a constant (d.c.) polarizing voltage. Spiral glass columns [0.61 m. (2 ft.) × 0.28 cm. (0.125 in.) i.d.] packed with 3% OV-1 on 80–100-mesh Gas Chrom Q were utilized. High purity nitrogen was used as the carrier gas at a flow rate of 40 ml./min. The column oven was operated isothermally at 255°, the flash heater at 275°, and the detector at 290°. Samples of 1 μl. were chromatographed. Under these conditions, II*b* and the internal standard (IV*b*) had retention times of 8.9 and 16 min., respectively.

Method B—A second chromatograph[4] was equipped with a ^{63}Ni electron-capture detector (pulse interval of 5 μsec.). A glass column [0.77 m. (2.5 ft.) × 0.28 cm. (0.125 in.) i.d.] packed with 1% OV-1 on 100–120-mesh Gas Chrom Q was arranged for on-column injection. Argon (95%)–methane (5%) was used as the carrier gas at a flow rate of 60 ml./min. The column oven was operated isothermally at 250°, the flash heater at 250°, and the detector at 280°. Samples of 1 μl. were chromatographed. Under these conditions, the tris(trimethylsilyl) $PGF_{2\alpha}$ pentafluorobenzyl ester (II*b*) had a retention time of 4 min.

GLC–Mass Spectrometry—GLC–mass spectra were recorded on a gas chromatograph–mass spectrometer[5], utilizing a column [0.77 m. (2 ft.)] packed with 3% OV-1 on Diatoport S (60–80 mesh). The column was maintained at a temperature of 225° and the flash heater at 270°.

Tris(trimethylsilyl) $PGF_{2\alpha}$ Pentafluorobenzyl Ester (IIb)—A sample of $PGF_{2\alpha}$ (I) was converted to the pentafluorobenzyl ester (II*a*) on a microscale. The product was treated with *N,O*-bis-(trimethylsilyl)-acetamide, and an aliquot was analyzed.

[1] On Silicar CC-4, 50 g., prepacked in ethyl acetate.

[2] Using a Vortex mixer.
[3] Varian Aerograph model 1740.
[4] Hewlett-Packard model 5750.
[5] LKB-9000.

A GLC peak with a retention time of ~2.2 min. was observed. A mass spectrum of this material showed an M^+ ion at *m/e* 750 and prominent peaks at: 735 ($M^+ - 15$) due to loss of CH_3, 679 ($M^+ - 71$) due to loss of C_5H_{11}, 660 ($M^+ - 90$) due to loss of $(CH_3)_3$—Si—OH, 645 ($M^+ - 105$) due to loss of $(CH_3)_3$—Si—OH + CH_3, 589 ($M^+ - 161$) due to loss of C_5H_{11} + $(CH_3)_3$—Si—OH, 570 ($M^+ - 180$) due to loss of 2× [$(CH_3)_3$—Si—OH], 237 ($M^+ - 513$), and 181 ($M^+ - 569$) due to CH_3—C_6F_5.

Tris(trimethylsilyl) Pentafluorobenzyl Ester (IVb)—A sample of the pentafluorobenzyl ester (IV*a*) prepared was treated with *N,O*-bis-(trimethylsilyl)acetamide, and an aliquot was analyzed.

A GLC peak with a retention time of ~16.5 min. was observed. A mass spectrum of the material showed an M^+ ion at *m/e* 778 and prominent peaks at: 763 ($M^+ - 15$) due to loss of CH_3, 707 ($M^+ - 71$) due to loss of C_5H_{11}, 688 ($M^+ - 90$) due to loss of $(CH_3)_3$—Si—OH, 673 ($M^+ - 105$) due to loss of $(CH_3)_3$—Si—OH + CH_3, 617 ($M^+ - 161$) due to loss of C_5H_{11} + $(CH_3)_3$—Si—OH, 598 ($M^+ - 180$) due to loss of 2× [$(CH_3)_3$—Si—OH], 237 ($M^+ - 541$), and 181 ($M^+ - 597$) due to CH_3—C_6F_5.

RESULTS AND DISCUSSION

The preparative scale synthesis of $PGF_{2\alpha}$ C_1-pentafluorobenzyl ester (II*a*) was conducted in acetonitrile using excess pentafluorobenzyl bromide (0.2 ml.), diisopropylethylamine (0.2 ml.), and $PGF_{2\alpha}$ (40 mg.). Under these conditions, the reaction was virtually quantitative in less than 1 hr. at room temperature on the basis of TLC examination. The ester was purified by silica gel column chromatography[6], and GLC-mass spectroscopy of the tris(trimethylsilyl) derivative confirmed the structure.

Only two reports appear in the literature for the preparation of pentafluorobenzyl esters of carboxylic acids, and the reported methods are slower than the method employed herein. Kawahara (27) refluxed carboxylic acids in acetone with potassium carbonate and excess pentafluorobenzyl bromide for 3 hr. to effect ester formation. Ehrsson (28) developed an ion-pair extraction process employing methylene chloride and an aqueous alkaline solution of tetrabutylammonium sulfate. However, only long-chain esters gave quantitative reaction in less than 20 min. Formation of the *n*-butyrate and *n*-valerate esters was incomplete after 2 hr. The use of diisopropylethylamine in the present study allowed homogeneous reaction conditions, and the high steric requirement of the amine served to minimize quaternary ammonium salt formation with pentafluorobenzyl bromide.

The $PGF_{2\alpha}$ C_1-pentafluorobenzyl ester (II*a*) was converted to the tris(trimethylsilyl) derivative (II*b*) prior to GLC.

Electron-capture GLC was conducted on one of two chromatographs[3,4]. Under the GLC conditions employed (see *Experimental* section), the lower limit of detection was 12.5 pg. of II*a* injected on-column in one instrument[3]. The lower limit of detection on the second instrument[4] was not determined. Typical chromatograms at the 1- and 10-ng. levels of II*a* are shown in Fig. 1. The GLC peak height response due to II*b* was linear for the 0.5–10-ng. range of II*a* injected on-column on the first instrument[3] (Fig. 2) and for the 0.03–0.84-ng. range of II*a* on the second[4].

The reaction conditions for the submicrogram scale conversion of $PGF_{2\alpha}$ (I) to the pentafluorobenzyl ester (II*a*) were explored. Since poor yields of the pentafluorobenzyl ester were obtained on attempting to derivatize nanogram quantities of $PGF_{2\alpha}$ adopting the conditions described earlier, the influence of reagent concentrations, temperature, and reaction time on the submicrogram pentafluorobenzyl esterification of $PGF_{2\alpha}$ was studied. The formation of the $PGF_{2\alpha}$ pentafluorobenzyl ester (II*a*) was monitored by electron-capture GLC analysis of the trimethylsilyl ether of the isolated product. Maximum conversion with minimum background interference was achieved when a picogram or nanogram quantity of $PGF_{2\alpha}$ (or the tromethamine salt of $PGF_{2\alpha}$) was treated with pentafluorobenzyl bromide (0.022 mmole) in acetonitrile (10 μl.) and diisopropylethylamine (0.007 mmole) in acetonitrile (10 μl.) and heated at 40° for 5 min. Maximum consistency of reaction was realized when the product was treated with the reagents again under identical conditions.

Initial attempts to isolate the pentafluorobenzyl ester product from the acidified reaction mixture by solvent extraction presented difficulties. Traces of citric acid used for the acidification caused high backgrounds on electron-capture analysis, probably as a result of pentafluorobenzyl esterification. Furthermore, under these extraction conditions, recoveries of the submicrogram quantities of the ester II*a* were erratic. Isolation of the product by direct evaporation of the solvent and excess reagents under nitrogen at room temperature was found to be ideal. Electron-capture detection background interference was minimal, and no sample loss was involved. Avoiding a conventional "workup" has the obvious advantages of saving time and effort. The $PGF_{2\alpha}$ pentafluorobenzyl ester product (II*a*) was converted to the tris(trimethylsilyl) ether (II*b*) by treatment with *N,O*-bis-(trimethylsilyl)acetamide. Hydrolysis of the trimethylsilyl groups of the submicrogram quantity of the derivative by traces of moisture was prevented, and maximum consistency of sample response was achieved by injection of the samples directly in the silylating agent. Typical gas chromatograms of the reagent blank and of II*b* formed by the reaction of a nanogram quantity of $PGF_{2\alpha}$ (I) are illustrated in Fig. 3. Although conversion of the $PGF_{2\alpha}$ ester (II*a*) to a bromomethyldimethylsilyl ether of enhanced electron-capture response is feasible, the excess halogen-containing silylating reagent would have to be completely eliminated, since even traces may cause "swamping" of the electron-capture detector (or high backgrounds). Attempts to remove rigorously all traces of the excess halogen-containing reagent prior to dissolving in a dry solvent (*e.g.*, ether) invariably result in partial hydrolysis of the timethylsilyl groups of the submicrogram sample and a consequent loss in sample response. To select a suitable "internal standard" for GLC, several prostaglandin analogs were converted to their pentafluorobenzyl esters on a microscale, and the GLC properties of the tris(trimethylsilyl) ethers of these esters were examined. Among those investigated, the tris(trimethylsilyl) pentafluorobenzyl ester of 9-{3α,5α-dihydroxy-2β-[(3*S*)-3-hydroxy-*trans*-1-octenyl]-1α-cyclopentyl}-*cis*-7-nonenoic

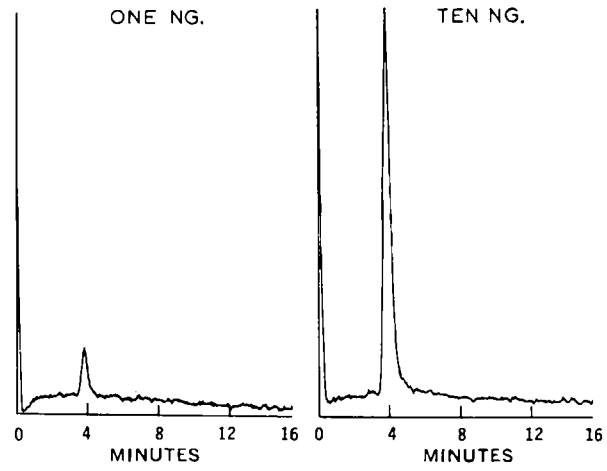


Figure 1—*Typical gas chromatograms of tris(trimethylsilyl) $PGF_{2\alpha}$ pentafluorobenzyl ester using electron-capture detection.*

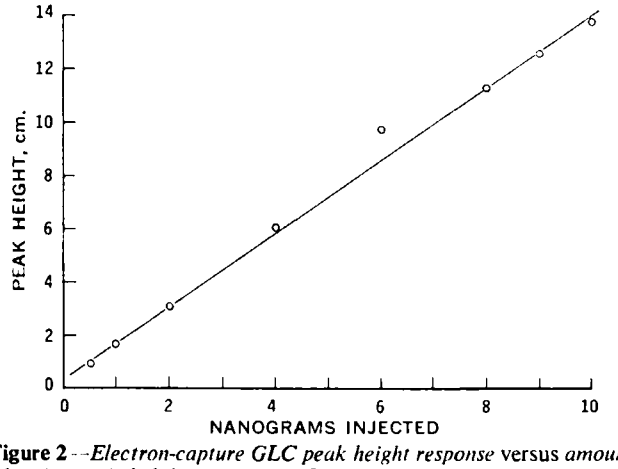


Figure 2—*Electron-capture GLC peak height response* versus *amount of tris(trimethylsilyl) $PGF_{2\alpha}$ pentafluorobenzyl ester injected.*

[6] Silicar CC-4, ethyl acetate–acetone (1:1).

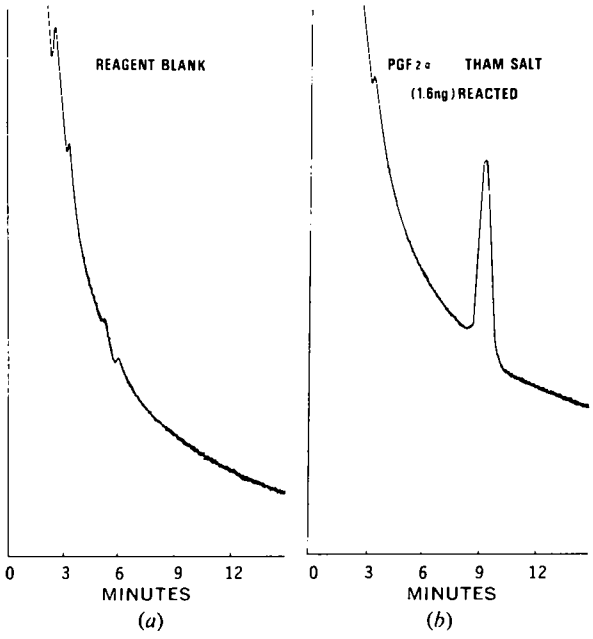


Figure 3—*Typical gas chromatograms of:* (a) *the reagent blank, and* (b) *$PGF_{2\alpha}$-tromethamine salt (1.6 ng.) reacted to the tris(trimethylsilyl) pentafluorobenzyl ester.*

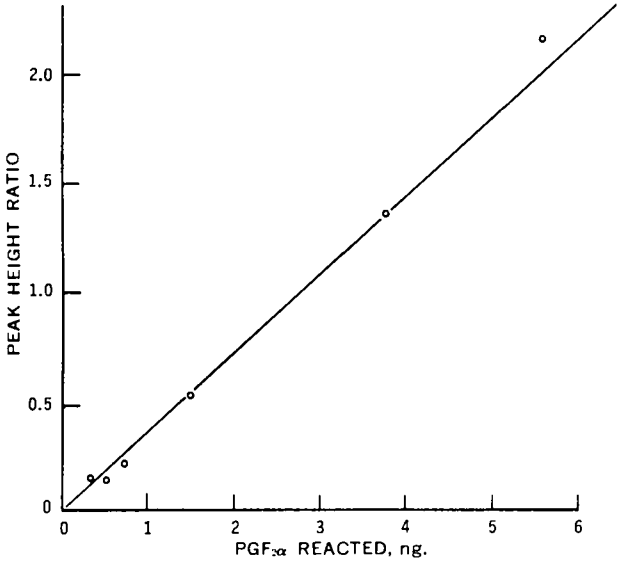


Figure 4—*Electron-capture GLC response of the tris(trimethylsilyl) $PGF_{2\alpha}$ pentafluorobenzyl ester* versus *quantity of $PGF_{2\alpha}$ (in nanograms) reacted (10% of each sample injected on-column).*

acid (III) proved to be the most suitable. This compound (IV*b*) had a retention time of 16.0 min. Compound II*b* had a retention time of 8.9 min. under the same GLC conditions on the first instrument[3]. The pentafluorobenzyl ester (IV*a*) was synthesized on a preparative scale by reaction of III (31.9 mg.) in acetonitrile with pentafluorobenzyl bromide and diisopropylethylamine (see *Experimental* section). The product was isolated from the acidified reaction mixture using ethyl acetate and was purified by sequential preparative GLC. Compound IV*b* was characterized by GLC–mass spectrometry (see *Experimental* section). Microgram and submicrogram scale syntheses of IV*a* were carried out by treatment with pentafluorobenzyl bromide and diisopropylethylamine in acetonitrile in the manner described for the derivatization of $PGF_{2\alpha}$ (I). Excess reagents were removed by evaporation under nitrogen at room temperature. The pentafluorobenzyl ester product (IV*a*) was dissolved in the silylating agent *N,O*-bis-(trimethylsilyl)-acetamide and added to the $PGF_{2\alpha}$ pentafluorobenzyl ester (II*a*) prior to electron-capture GLC analysis. The electron-capture GLC response due to II*b* resulting from the submicrogram scale reaction of $PGF_{2\alpha}$ (I) was found to be linear over the concentration range of 0.2–5.6 ng. $PGF_{2\alpha}$ (Fig. 4) (determined on the first chromatograph[3]).

Thus, the pentafluorobenzyl ester of $PGF_{2\alpha}$ (II*a*) is easily formed, and the corresponding trimethylsilyl derivative (II*b*) allows quantitation of $PGF_{2\alpha}$ (I) in the picogram and nanogram range using electron-capture detection. The application of this methodology to the quantitation of $PGF_{2\alpha}$ in biological fluids is currently being developed.

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Carbocyclic Analogs of Guanosine and 8-Azaguanosine

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Abstract □ (±)-9-[*trans*-2,*trans*-3-Dihydroxy-*cis*-4-(hydroxymethyl)-cyclopentyl]guanine, the carbocyclic analog of guanosine, was synthesized by a five-step route beginning with (±)-*trans*-3-amino-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol and 2-amino-4,6-dichloropyrimidine. Preparation and reduction of a 5-(*p*-chlorophenylazo)pyrimidine derivative introduced the prerequisite 5-amino substituent. Formation of the imidazole ring from this 5-amino derivative with triethyl orthoformate and acidic hydrolysis of the resulting mixture of 2-amino-6-chloropurine derivatives furnished the guanosine analog. The same 5-aminopyrimidine intermediate was diazotized to the 2-amino-6-chloro-8-azapurine, and the latter derivative was hydrolyzed to the carbocyclic analog of 8-azaguanosine. Neither of these analogs was cytotoxic to human epidermoid carcinoma cells *in vitro*. In tests using leukemia L-1210, the guanosine analog was quite toxic but was inactive at nontoxic doses; the 8-azaguanosine analog was not active in initial (single-dose) tests.

Keyphrases □ Guanosine, cyclopentyl analog—synthesis, cytotoxicity □ 8-Azaguanosine, cyclopentyl analog—synthesis, cytotoxicity □ Carbocyclic analogs of purine nucleosides—synthesis and cytotoxicity of cyclopentyl derivatives of guanine and 8-azaguanine □ Cyclopentyl derivatives of guanine and 8-azaguanine—synthesis, cytotoxicity □ Purine nucleosides, carbocyclic analogs—synthesis, cytotoxicity

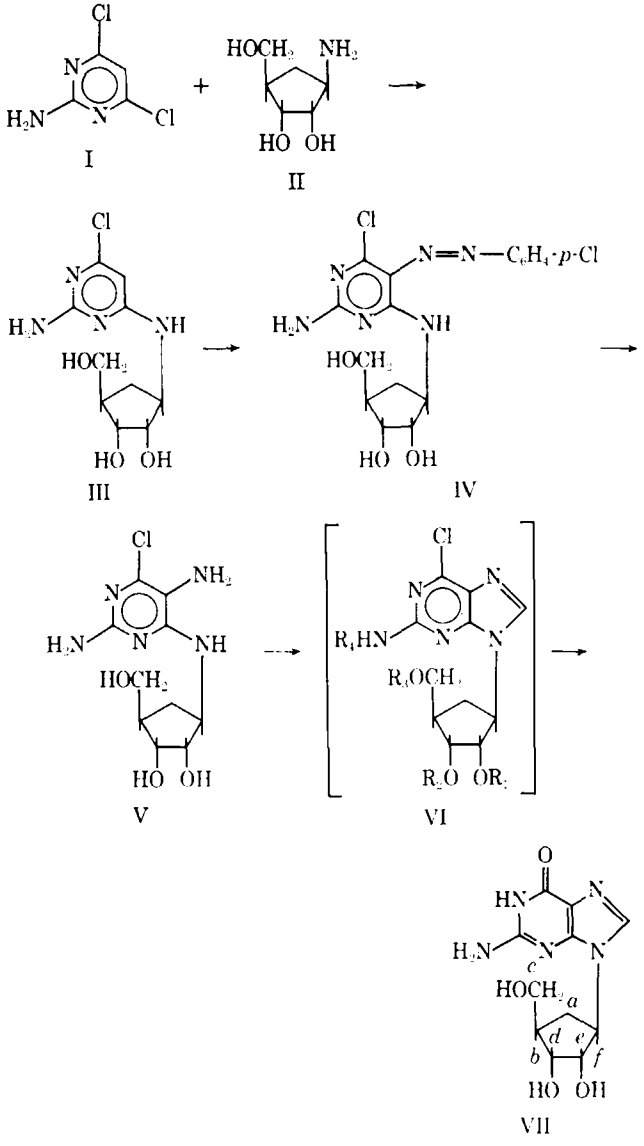
Syntheses of racemic, carbocyclic (cyclopentane) analogs of adenosine, 2′- and 3′-deoxyadenosine, adenylic acid, adenosine cyclic 3′,5′-monophosphate, and other 6-substituted purine nucleosides were described previously (1–4). Some of these nucleoside analogs, which have a stable carbon nitrogen bond at position 9 of the purine ring in place of the glycosidic bond, are cytotoxic to cells in culture (4–6), function as substrates for or inhibitors of purine-metabolizing enzymes (5–7), or display antimicrobial activity[1] (8). In the previously described analogs, the only substituent on the purine ring, other than the 9-cyclopentyl group, is at position 6. This report deals with the carbocyclic analogs of guanosine and 8-azaguanosine, which are 2-amino-6-substituted purines.

DISCUSSION

The synthesis of the racemic guanosine analog is outlined in Scheme I. Prolonged treatment of 2-amino-4,6-dichloropyrimidine (I) with *trans*-3-amino-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol (II) (2) gave the pyrimidinylaminocyclopentane derivative (III). Triethylamine was employed as a proton acceptor, rather than an excess of II, because of the difficulty in obtaining large amounts of II. Coupling of III with *p*-chlorobenzenediazonium chloride in aqueous acetic acid buffered with sodium acetate gave good yields of the 5-(*p*-chlorophenylazo)pyrimidine (IV). The required 5-amino group was introduced by reducing the *p*-chlorophenylazo group with zinc in acetic acid, a method previously found (9) to effect this type of reduction of simpler derivatives without significantly affecting the chloro substituent on the pyrimidine ring.

The purine ring was formed by an acid-catalyzed reaction of triethyl orthoformate with the tetrasubstituted pyrimidine (V). Reactions of the hydroxyl groups and the 2-amino group with the reagent should produce derivatives (formyl, orthoester, cyclic orthoester, or polymeric) of the expected 2-amino-6-chloropurine (VI, $R_1 = R_2 = R_3 = R_4 = H$). The isolation of pure derivatives (VI) was not attempted; instead, the material obtained from V and triethyl orthoformate was subjected to acidic hydrolysis to hydrolyze the 6-chloro substituent and to liberate the functional groups simultaneously. That purine ring formation had proceeded satisfactorily was shown by the 78% overall yield of the guanosine analog (VII) from V. The proton magnetic resonance (PMR) and the UV absorption spectra were in accord with Structure VII.

Treatment of pyrimidine V (Scheme II) with sodium nitrite and aqueous acetic acid produced a high yield of the 5-amino-7-chloro-3*H*-*v*-triazolo[4,5-*d*]pyrimidine (VIII). Either acidic or basic hydrolysis of VIII gave the 8-azaguanosine analog (IX).



Scheme I—Letters a–f *identify protons with proton magnetic resonance chemical shifts* (*see* Experimental)

[1] R. F. Pittillo, Southern Research Institute, Birmingham, Ala., personal communication.

The guanosine analog (VII) was tested against leukemia L-1210 in mice on three treatment schedules[2]. When administered on Day 1 at doses of 400, 300, 150, and 75 mg./kg., VII displayed acute toxicity at the two higher doses. The difference (ΔW) between the average weight changes of the treated (T) and the untreated (C) mice indicated that the two lower doses were also toxic (ΔW = −5.6 and −3.9 g., respectively). Administered on Days 1, 5, and 9, doses of 38 and 19 mg./kg. were nontoxic and inactive; a shortened survival time (T/C = 70%) of treated animals indicated that a dose of 75 mg./kg. on this schedule is toxic. Doses of 38 and 19 mg./kg./day given *q.d.* 1–9 were toxic, as evidenced by shortened survival times (T/C = 71 and 83%, respectively) relative to untreated animals and by weight differences (ΔW = −5.1 and −2.3 g., respectively), whereas doses of 9.5 and 5 mg./kg./day were nontoxic and inactive. These data, especially the results of daily treatment, indicate that the toxicity of VII *in vivo* is approximately the same as that of the adenosine analog (4). In contrast to the similarity of the toxicity of the two analogs *in vivo*, the guanosine analog (VII) is not cytotoxic (ED_{50} >100 mcg./ml.) to human epidermoid carcinoma cells (H.Ep.-2) in culture, whereas the adenosine analog is highly cytotoxic[3] (ED_{50} = 0.7 mcg./ml.) (4).

The 8-azaguanosine analog (IX) was not cytotoxic (ED_{50} >100 mcg./ml.) to H.Ep.-2 cells in culture and was not active against L-1210 leukemia at doses of 100, 200, or 400 mg./kg. administered on the Day 1 schedule. The observed ΔW (−2.3 g.) at the highest dose was indicative of chronic toxicity.

EXPERIMENTAL[4]

(±)-*trans*-3-[(2-Amino-6-chloro-4-pyrimidinyl)amino]-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol (III)—A mixture of 3.68 g. (22.4 mmoles) of 2-amino-4,6-dichloropyrimidine (I), 3.3 g. (22.4 mmoles) of *trans*-3-amino-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol (II) (2), 2.26 g. (22.4 mmoles) of triethylamine, and 200 ml. of absolute ethanol was heated under reflux for 140 hr. The white, crystalline product was separated by filtration from the cooled reaction mixture, yielding 3.9 g. (63%), m.p. 234–238° dec. A specimen was recrystallized from water, m.p. 236–239° dec.; UV: λ_{max} 302 (sh), 287 (infl), 274 (ε 9600), 240 (ε 12,600), and 214 (ε 19,800) at pH 1; 286 (ε 10,200) and 237 (ε 11,400) at pH 7; and 287 (ε 10,200) and 238 (ε 11,200) at pH 13.

Anal.—Calc. for $C_{10}H_{15}ClN_4O_3$: C, 43.72; H, 5.51; N, 20.40; Cl, 12.90. Found: C, 43.53; H, 5.49; N, 20.20; Cl, 12.9.

(±)-*trans*-3-{[2-Amino-6-chloro-5-[(*p*-chlorophenyl)azo]-4-pyrimidinyl]amino}-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol (IV)—A cold (0–5°) solution of *p*-chlorobenzenediazonium chloride, prepared from 5.90 g. (46.3 mmoles) of *p*-chloroaniline, 12.8 g. of 12 *N* HCl, 52 ml. of water, and 3.5 g. (50.5 mmoles) of sodium nitrite in 42 ml. of water, was added dropwise during 1 hr. to a well-stirred mixture of 11.53 g. (42 mmoles) of III, 84 g. of sodium acetate trihydrate, 200 ml. of acetic acid, and 200 ml. of water. The diazonium salt solution was maintained at 0–5° by adding it from a jacketed addition funnel kept cold with circulating ice water; the pyrimidine (III) solution was at room temperature. The mixture was stirred for 18 hr. at room temperature, cooled in an ice bath, and filtered to remove the yellow precipitate, which was washed with cold water and dried *in vacuo* over phosphorus pentoxide, yielding 14.1 g. (81%), m.p. 249–250° dec. (inserted at 230°); homogeneous by TLC [10 mcg., chloroform–methanol (5:1)]. After several days at room temperature, the filtrate deposited a second crop, weighing 1.25 g., m.p. 245–246° dec. A specimen of IV for analysis was recrystallized from dimethylformamide–water, m.p. 258–259° dec. (inserted at 240°, 2°/min.) and m.p. 262–263° dec. (inserted at 250°, 2°/min.); UV: λ_{max} 372 (ε 26,600), 280 (ε 8800), and 240 (ε 18,300) at pH 1; and 385 (ε 28,600), 280 (ε 10,700), and 227 (ε 16,300) at pH 7 and 13.

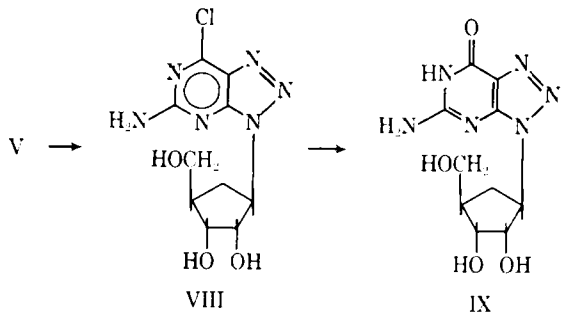
Anal.—Calc. for $C_{16}H_{15}Cl_2N_6O_3$: C, 46.50; H, 4.39; Cl, 17.16; N, 20.34. Found: C, 46.33; H, 4.29; Cl, 17.3; N, 20.32.

(±)-*trans*-3-[(6-Chloro-2,5-diamino-4-pyrimidinyl)amino]-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol (V)—A mixture of 7.2 g. of the *p*-chlorophenylazopyrimidine (IV), 140 ml. of ethanol, 172 ml. of water, and 14 ml. of acetic acid was heated to 70° with vigorous mechanical stirring under a nitrogen atmosphere. Zinc dust (11.2 g.) was added in small portions during 45 min., and the mixture was heated at 70° with vigorous stirring for an additional 1.25 hr. Excess zinc was filtered from the hot mixture and washed with three portions of ethanol; the filtrate, combined with the ethanol washings, was concentrated *in vacuo* to about 50 ml.; and the resulting mixture was diluted with ethanol (50 ml.) and stored at 5° overnight. The tan crystalline precipitate was separated by filtration, washed with ethanol–water (3:1), and dried *in vacuo* at 78°, yielding 2.46 g. (49%), m.p. 245–247° dec. (inserted at 230°); UV: λ_{max} 298 (ε 8000), 237 (ε 15,500), and 210 (ε 16,200) at pH 1; 303 (ε 9000), 240 (sh), 225 (sh), and 204 (ε 19,200) at pH 7; and 303 (ε 9000), 240 (sh), and 225 (sh) at pH 13.

Anal.—Calc. for $C_{10}H_{16}ClN_5O_3$: C, 41.45; H, 5.57; Cl, 12.24; N, 24.18. Found: C, 41.22; H, 5.70; Cl, 12.4; N, 24.00.

The filtrate was concentrated *in vacuo*. A water (100 ml.) solution of the residual oil was washed with ether (3 × 75 ml.), concentrated *in vacuo* to about 30 ml., and kept at 5° overnight. Recrystallization of the brown crystalline precipitate from water gave 320 mg. of V (total yield 55%), m.p. 235–243° dec.

(±)-9-[*trans*-2,*trans*-3-Dihydroxy-*cis*-4-(hydroxymethyl)cyclopentyl]guanine (VII)—A solution consisting of 1.45 g. of V, 20 ml. of dimethylformamide, 40 ml. of triethyl orthoformate, and 1 ml. of 12 *N* HCl was kept at room temperature for 1.5 hr., at 5° for 16 hr., and at room temperature for 8 hr. The reaction solution was concentrated *in vacuo* at 30°. A mixture of the residual syrup and 200 ml. of 2 *N* HCl was heated under reflux for 5 hr. and then concentrated *in vacuo* at 50°. Several portions of water were evaporated *in vacuo* from the residue before it was dissolved in water (10 ml.). The pH of the aqueous solution was adjusted to 5 with 6 *N* NaOH, the mixture was chilled (5°) for 2 hr., and the solid precipitate (1.285 g.) was separated by filtration and washed with water. The precipitate was recrystallized from water (15 ml.) and dried *in vacuo* over phosphorus pentoxide at 78° for 18 hr., yielding 1.154 g. (78% as a 0.75 hydrate), m.p. 279–282° dec. (softened and resolidified in 250–260° range); purity 96–97% by UV (calculated as a 0.75 hydrate). A sample for analysis [white needles (150 mg.) obtained by recrystallizing 225 mg. of crude VII from 10 ml. of water] was dried at 100° over phosphorus pentoxide for 20 hr., m.p. 278–284° dec. (inserted at 230°, softened and darkened at 250–255°, resolidified);



Scheme II

[2] In the standard L-1210 leukemia test, mice are innoculated intraperitoneally on Day 0 with 10^5 L-1210 cells. Administration of a compound is also by intraperitoneal injection. Weight changes are determined 4 days after the first (or only) injection. Weight differences in excess of 4 g. or values of $T/C \lesssim 85\%$ are accepted criteria of chronic toxicity. Since the carbocyclic analogs have generally shown a good correlation between dose and weight difference (ΔW), a weight difference of 2–3 g. also appears to indicate chronic toxicity.

[3] ED_{50} = concentration of a compound that inhibits growth, measured by protein determinations, to 50% of the growth of untreated cells.

[4] Melting temperatures were determined in capillary tubes heated in a Mel-Temp apparatus and are not corrected. UV spectra were recorded with a Cary model 17 or 14 spectrophotometer. UV maxima are in nanometers; sh = shoulder, and infl = inflection. Solutions for UV determinations were prepared by diluting a 5-ml. aliquot of a water or ethanol solution (0.1 *N* HCl for III) to 50 ml. with 0.1 *N* HCl, phosphate buffer (pH 7), or 0.1 *N* NaOH; absorption maxima are reported at pH 1, 7, or 13. IR spectra were recorded with a Perkin-Elmer model 621 or 521 spectrometer from samples in KBr disks. PMR spectra were determined with a Varian model XL-100-15 spectrometer for observing proton resonance at 100 MHz. Chemical shift data (δ) are in parts per million downfield from tetramethylsilane, the internal reference; s = singlet, d = doublet, and m = multiplet. Unless otherwise indicated, TLC was performed on plates of silica gel, and spots were detected by: (*a*) UV light (254 nm.) after spraying the chromatogram with an optical whitening agent (Ultraphor WT, BASF Colors and Chemicals, Inc., Charlotte, N. C.) and (*b*) spraying with a basic solution of potassium permanganate. The quantity applied and the developing solvent are shown parenthetically at the appropriate places in the procedures.

IR: 1725, 1675, 1625, 1605 (sh), 1565, 1545, 1480, 1170, 770 (sharp), and 680 cm.$^{-1}$ and other bands (broad or weak); PMR[5] (dimethyl sulfoxide-d_6): δ about 1.3–1.7 (m, H*a*), about 1.8–2.4 (m, H*a*H*b*), 3.28–3.6 (m, H*c*H*c*), 3.37 (s, water), 3.72–3.9 (m, H*d*), 4.08–4.34 (m, H*e*), 4.36–4.78 (m, H*f* + 2OH), 4.82–4.98 (d, OH), 6.39 (s, NH_2), 7.79 (s, H_8 of purine ring), and 10.58 (s, NH of purine ring); UV: λ_{max} (ϵ calculated for 0.75 hydrate) at 278 (ϵ 8000) and 255 (ϵ 11,900) at pH 1; 268 (sh) and 253 (ϵ 13,000) at pH 7; and 268 (ϵ 11,100) and 257 (sh) (ϵ 10,500) at pH 13.

Anal.—Calc. for $C_{11}H_{12}N_5O_4 \cdot {}^3/_4H_2O$: C, 44.81; H, 5.64; N, 23.76. Found: C, 44.75; H, 5.87; N, 23.44.

Specimens of VII dried at 100° for about 20 hr. apparently rehydrated readily since they were observed to increase in weight during weighing for analysis. The melting point was evidently influenced by the degree of hydration as well as the heating rate. A specimen recrystallized from dimethylformamide ethanol melted at 298–300° dec. without prior softening (inserted at 100°, 2°/min. at 260°). Recrystallization of this specimen from water (including treatment with activated carbon) changed the melting point to 284–286° dec. (softened and darkened at 260°, resolidified; inserted at 230°, 2°/min. at 260°); homogeneous by TLC [silica gel or cellulose, 10 mcg., propanol–water (3:1); detection by UV, UV and spraying[6], and potassium permanganate spray]. The exact values of ϵ are also determined by the degree of hydration at the time the sample is weighed for UV determination.

(±)-*trans*-3-(5-Amino-7-chloro-3*H*-*v*-triazolo[4,5-*d*]pyrimidin-3-yl)-*trans*-5-(hydroxymethyl)-*cis*-1,2-cyclopentanediol (VIII) A solution of 240 mg. (3.48 mmoles) of sodium nitrite in 6 ml. of water was added dropwise to a cold (0°) mixture of 870 mg. (3.0 mmoles) of V, 9 ml. of water, and 3 ml. of acetic acid. The mixture was stirred at 0° for 1 hr., and the product was separated by filtration and washed with 5 ml. of cold water, yielding 828 mg. Recrystallization of the crude VIII from ethanol–hexane gave a white solid, which was dried *in vacuo* over phosphorus pentoxide at room temperature for 18 hr. and then at 78° for 0.5 hr., yielding 766 mg. (85%), m.p. 172–174° dec.; TLC: 1 spot [20 mcg., chloroform–methanol (4:1)]; UV: λ_{max} 226 (ϵ 21,600), 245–260 (infl), and 316 (ϵ 7500) at pH 1; 226 (ϵ 21,900), 245–260 (infl), and 316 (ϵ 7500) at pH 7; and 246 (ϵ 4700) and 286 (ϵ 9900) at pH 13.

Anal.—Calc. for $C_{10}H_{13}ClN_6O_3$: C, 39.94; H, 4.36; N, 27.95. Found: C, 39.75; H, 4.55; N, 27.71.

(±)-5-Amino-3,6-dihydro-3-[*trans*-2,*trans*-3-dihydroxy-*cis*-4-(hydroxymethyl)cyclopentyl]-7*H*-*v*-triazolo[4,5-*d*]pyrimidin-7-one (IX)— A mixture of 250 mg. of VIII and 20 ml. of 1 *N* HCl was heated under reflux for 4 hr. and concentrated *in vacuo* at 40° to a syrup. Water (2 × 20 ml.) was added to the residue and evaporated *in vacuo*, and ethanol (10 ml.) was then added. A gelatinous solid was separated by filtration, washed with acetone, and dried *in vacuo* over phosphorus pentoxide at 78°, yielding 75 mg. The filtrate and the acetone washings were combined and evaporated *in vacuo*, the residue was triturated with ethanol–hexane (1:1, 10 ml.), and a gelatinous solid was filtered away and dried *in vacuo* over phosphorus pentoxide at 78°, yielding 99 mg. (total yield 74%). Both portions were homogeneous according to TLC [20 mcg., ethanol–15 *N* aqueous ammonia (4:1)]. A 6 *N* HCl solution of the two portions was diluted with ethanol, and the gelatinous precipitate was dried as before, yielding 88 mg. (38%), m.p. 252–254° dec. (inserted at 240°, 2°/min.); IR: 1700, 1635, 1595 (sh), 1580, 1525, 780, and 680 cm.$^{-1}$; UV: λ_{max} 253 (ϵ 11,800) and 270 (sh) at pH 1, 253 (ϵ 11,700) and 270 (ϵ 8600) at pH 7, and 279 (ϵ 11,000) and 260 (sh) at pH 13.

Anal.—Calc. for $C_{10}H_{14}N_6O_4 \cdot {}^2/_3H_2O$: C, 40.81; H, 5.25; N, 28.56. Found: C, 40.99; H, 5.22; N, 28.60.

Hydrolysis of VIII with refluxing 1 *N* NaOH also yields IX.

[5] The positions of protons *a–f* are shown in Structure VII.
[6] Ultraphor WT.

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Effects of Polyelectrolytes on Drug Transport I: Diffusion

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Abstract □ The capillary method was used to determine the diffusive transport properties of radioactive salicylate ion in the presence of a commonly used polyelectrolyte, sodium carboxymethylcellulose. In a solution of this polymer, the self-diffusion coefficient of salicylate was found to decrease only moderately, even though the bulk macroscopic viscosity increased about two to three orders of magnitude. These data were evaluated with two theories from the literature. The tracer drug release rate out of the polymer solutions was also studied and was more rapid than if the polymer were not present. Increases of up to 40% were observed.

Keyphrases □ Diffusion properties, radiolabeled salicylate as co-ion in sodium carboxymethylcellulose—determined using capillary method □ Carboxymethylcellulose, sodium—effect on co-ion diffusion of radiolabeled salicylate, capillary method □ Salicylate, radiolabeled, diffusion coefficient and tracer release rate in sodium carboxymethylcellulose—determined using capillary method, co-ion effect □ Polyelectrolytes—effect of carboxymethylcellulose on salicylate co-ion diffusion □ Drug transport—effect of carboxymethylcellulose on salicylate co-ion diffusion

Hydrophilic polymers that are commonly added to pharmaceutical preparations can influence the transport properties of the system and, hence, the rate of drug absorption. Levy and Jusko (1) demonstrated that the increase in viscosity due to methylcellulose decreased the absorption rate of ethanol and salicylic acid in rats. Other investigators (2, 3) also reported similar viscosity–absorption effects.

The most widely known relationship between diffusive transport and viscosity is the Stokes–Einstein equation. This equation is based partly on the movement of a spherical solute through a continuous medium. With high molecular weight polymer solutions, however, the physical conditions are somewhat different. The relatively low concentration of polymer imparts a substantial increase in the bulk or "macroscopic" viscosity. The microscopic environment of a diffusion species, however, is largely solvent, and only infrequently does the diffusant encounter a polymer segment along its diffusion path. Thus, the Stokes–Einstein equation would not be expected to hold for diffusion in dilute polymer solutions.

Reports have appeared involving studies of diffusion in dilute polymer solutions. Florence *et al.* (4) measured the diffusion coefficients of sodium chloride and potassium chloride in aqueous solutions of polyethylene oxide (polyethylene glycol) and polyvinylpyrrolidone. Using these diffusion coefficients and the Stokes–Einstein equation, they calculated the "effective" or "microscopic" viscosities. These values were smaller than the corresponding relative bulk viscosities. Block and Lamy (5) also observed a difference between the bulk viscosity and that apparently experienced by a diffusant. Li and Gainer (6), working with organic solvents, developed equations relating the diffusivity in these systems to such parameters as heat of mixing, viscosity, and diffusivity (in the pure solvent). Taking another approach, Wang (7) developed an equation for the self-diffusion of water in protein solutions based on the physical obstruction by the protein and the hydration effect.

The self-diffusion of the counterion in dilute polyelectrolyte solutions was studied by Huizenga *et al.* (8) and Pefferkorn and Varoqui (9). They found that diffusion coefficients decrease as the degree of neutralization increases. Rinando *et al.* (10) also found that diffusivity decreases as the degree of substitution on carboxymethylcellulose increases. These results were explained in terms of electrostatic effects of the polyelectrolyte on the counterion.

The use of polyelectrolytes to enhance the absorption of drugs that are co-ions of the polymer was suggested by Higuchi *et al.* (11). The experimentally observed increased transport rate was attributed to the Donnan membrane effect. Diffusion properties in the solution were not considered.

The present studies were undertaken to determine the diffusional properties of drugs that are present as co-ions in polyelectrolyte solutions and the characteristics of their transport out of these solutions across a cellophane membrane. This article reports the results of the diffusion studies.

EXPERIMENTAL

The capillary method (12) was employed to determine the diffusion coefficient of salicylate in a solution of sodium carboxymethylcellulose. Briefly, this method is based on the diffusion of a radioactive tracer out of a capillary tube into a tracer sink, which has the

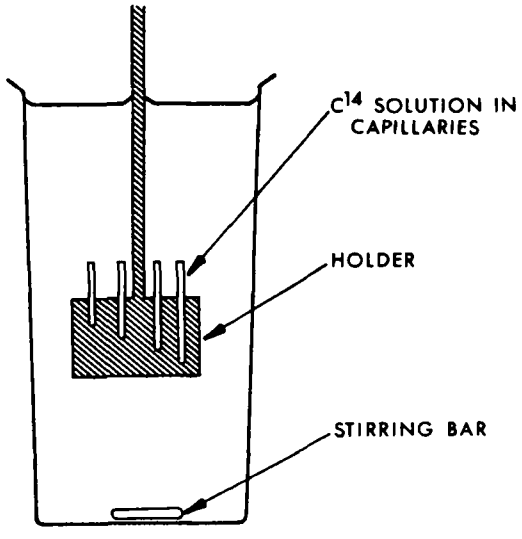

Figure 1—*A set of four capillaries in a tall beaker containing 250 ml. sink solution stirred by magnetic bar.*

Table I—Diffusion Coefficients of Salicylate (8 m*M*) in Sodium Carboxymethylcellulose Solutions (w/v %) and in Sodium Fluoride (1 *M*) at 37°, (Average ± *SD*) × 10^5 cm.²/sec.

Medium		Concentration					
		0.5	0.75	1.25	1.5	2.0	2.5
Sodium carboxymethylcellulose (7MXF)	D[a]	1.10 ± 0.04	1.07 ± 0.04	1.12 ± 0.01	1.03 ± 0.04	1.09 ± 0.08	1.09 ± 0.09
	D'[b]	1.35 ± 0.02	1.35 ± 0.01	1.39 ± 0.02	1.44 ± 0.05	1.55 ± 0.01	1.55 ± 0.02
		0.5	0.75	1.0	1.25	1.5	2.0
Sodium carboxymethylcellulose (7M27SF)	D	1.11 ± 0.01	1.08 ± 0.06	1.11 ± 0.02	1.06 ± 0.03	1.09 ± 0.04	1.04 ± 0.08
	D'	1.33 ± 0.03	1.32 ± 0.06	1.42 ± 0.03	1.35 ± 0.05	1.42 ± 0.07	1.42 ± 0.02
		0.5	0.85	1.25	1.5	2.0	2.5
Sodium carboxymethylcellulose (12M8P)	D	1.04 ± 0.07	0.98 ± 0.03	1.03 ± 0.05	1.03 ± 0.09	1.01 ± 0.06	0.99 ± 0.04
	D'	1.23 ± 0.04	1.29 ± 0.06	1.42 ± 0.05	1.47 ± 0.06	1.48 + 0.01	1.48 ± 0.07
		1.0	2.0	3.0	4.0	5.0	6.0
Sodium carboxymethylcellulose (7LXF)	D	1.08 ± 0.06	1.03 ± 0.02	1.00 ± 0.03	0.94 ± 0.05	0.91 ± 0.03	0.86 ± 0.05
	D'	1.32 ± 0.03	1.42 ± 0.03	1.46 ± 0.03	1.49 ± 0.07	1.45 ± 0.02	1.27 ± 0.04
		0.01	0.02	0.04	0.1	0.2	0.4
Sodium fluoride	D	1.08 ± 0.03	1.18 ± 0.04	1.18 ± 0.04	1.15 ± 0.03	1.12 ± 0.03	1.09 ± 0.01
	D'	1.09 ± 0.06	1.18 ± 0.04	1.13 ± 0.02	1.17 ± 0.04	1.12 ± 0.12	1.11 ± 0.02

[a] Self-diffusion coefficient (see Case A in text). [b] Apparent diffusion coefficient (see Case B in text).

same chemical composition as the solution inside the capillary. The solution to Fick's second law corresponding to these boundary conditions is:

$$\frac{C}{C_0} = \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp\left[-(2n+1)^2 \pi^2 \frac{Dt}{4l^2}\right] \quad \text{(Eq. 1)}$$

where C_0 and C are the tracer activities in the capillary initially and at time t (seconds), l is the length of the capillary in centimeters, and D is the self-diffusion coefficient in square centimeters per second. Four capillaries were used for each run; they had an inside diameter of 0.5 mm. and nominal lengths of 2.4, 2.7, 3.1, and 3.5 cm. The accurate length of each capillary was determined with a projection device[1], and the volume was calculated from the weight of mercury required to fill the capillary at a known temperature. The capillary holder was so constructed that each opening of the set of four capillaries was at about the same level below the surface in a 250-ml. tall beaker, which held the sink solution (Fig. 1). The effect of stirring at 100 r.p.m. on the diffusion path length was negligible (13, 14).

Sodium salicylate[2] solutions (8 m*M*) were prepared containing the appropriate amount of sodium carboxymethylcellulose[3]. An appropriate amount of neutralized ^{14}C-salicylic acid[4] was added to about 0.3 ml. of the solution to yield an initial activity of about 5000 counts/min./μl. The tracer solution was degassed and then put into the capillary with a 33-gauge needle. The precaution about temperature equilibrium and immersing effects was followed as suggested by Wang (13). After 96–120 hr. of diffusion, the remaining activity in each capillary was washed into a counting vial with water (0.2 0.3 ml.) from a syringe. Most of the water in the vial was evaporated by heating in an oven to increase the counting efficiency. The radioactivity was obtained using the standard preset ^{14}C window opening[5]. The external standard ratio-efficiency curve was constructed to correct the quenching effect; background was also corrected. The range of C/C_0 was about 0.1–0.4. The diffusion coefficients were calculated from Eq. 1, and the average values and standard deviations are listed in Table I.

RESULTS AND DISCUSSION

The effect of sodium carboxymethylcellulose on salicylate diffusion was studied in two cases: Case A, polymer was both inside and outside of the capillary; and Case B, polymer was inside the capillary only. In both cases, the initial sodium salicylate concentration of 8 m*M* was the same inside and outside the capillary; *i.e.*, there was no salicylate concentration difference. The diffusion coefficient D obtained in Case A is the true self-diffusion coefficient since there is no chemical potential gradient at the capillary opening. The diffusion coefficient obtained in Case B is not the true self-diffusion coefficient, even though it was evaluated through Eq. 1, because there is a polyelectrolyte concentration gradient at the boundary.

Case A: Self-Diffusion—The relative viscosity of bulk solution deduced from the manufacturer's data booklet (15) is listed in Table II. It is apparent that the Stokes–Einstein equation does not hold since the relative viscosity increases over 1000-fold while the diffusion coefficient decreases less than 10% (Table I).

Mackie and Meares (16) proposed the following equation to calculate the mobility of an ion, U, in a polymer matrix:

$$U = U_0 \left(\frac{1 - V_p}{1 + V_p}\right)^2 \quad \text{(Eq. 2)}$$

where U_0 is the mobility of the ion in water, and V_p is the volume fraction of polymer. Since the diffusion coefficient, D, is proportional to U, one can write (4):

$$D = D_0 \left(\frac{1 - V_p}{1 + V_p}\right)^2 \quad \text{(Eq. 3)}$$

Since the density of a 2% carboxymethylcellulose solution at 25° is 1.0068 g./cm.³ (15), assuming weight to volume concentration equal to volume to volume concentration, the diffusivity in the polymer solutions can be calculated with Eq. 3 if D_0 is known. The experimental value of the diffusion coefficient for salicylate (8 m*M*) in water at 37° was found to be 1.11 × 10^{-5} cm.²/sec. Others have reported values of 1.00 × 10^{-5} (17), 1.33 × 10^{-5} (18), and 0.925 × 10^{-5} cm.²/sec. (19), all determined under somewhat different experimental conditions. The experimentally determined diffusivities, shown as the ratio D/D_0, are given in Fig. 2 (Case A). The line generated from Eq. 3 is included and it can be seen that the agreement is rather good.

Taking a somewhat different approach, Wang (7) developed an equation for self-diffusion of water in a protein solution. Neglecting the hydration effect, *i.e.*, the association between polymer and diffusant, the proposed equation is:

$$D = D_0(1 - \alpha V_p) \quad \text{(Eq. 4)}$$

where α is the shape factor of protein. This equation predicts a linear dependence of D on the volume fraction. At low values of volume fraction, *i.e.*, in the range of the present study, it can be shown that Eq. 3 reduces to:

$$D = D_0(1 - 4V_p) \quad \text{(Eq. 5)}$$

Thus, at low concentrations of polymer, Eqs. 3 and 4 have the same functional relationship. The current data thus appear to obey either equation because of the low polymer concentration.

Data from the literature are available to evaluate these two equations further. By using two sets of diffusion coefficients reported by Florence *et al.* (4), one set given by Wang (7), and the set deter-

[1] Shadowgraph, Nippon Kogaku K. K. Japan.
[2] Sodium salicylate analytical reagent, Mallinckrodt Chemical Works, St. Louis, Mo.
[3] Cellulose gum, various types, Hercules Inc., Wilmington, Del.
[4] ^{14}C-Salicylic acid, Amersham/Searle Corp., Arlington Heights, Ill.
[5] Liquid scintillation system, LS-100, Beckman, Fullerton, Calif.

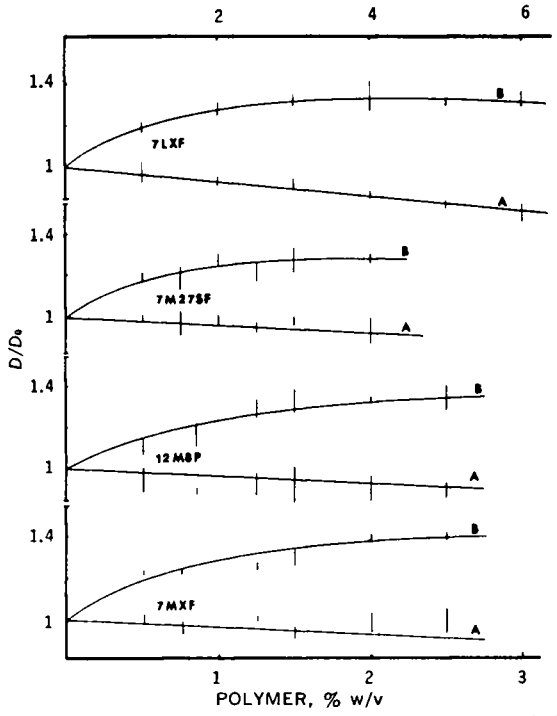

Figure 2—D/D_0 *of sodium salicylate* versus *concentration (w/v %) of sodium carboxymethylcellulose of various types: 7MXF, 12M8P, 7M27SF, and 7LXF. Upper scale is for 7LXF only. Case A = self-diffusion in polymer solution; Case B = diffusion* out of *polymer solution.*

mined for sodium carboxymethylcellulose (7LXF) in this work, the equations can be tested for higher concentrations of polymer. With these diffusion coefficients, V_p was calculated from Eq. 3 and plotted against polymer concentration (Fig. 3). By assuming the actual volume fraction of polymer to be directly proportional to the polymer concentration, if Eq. 3 is the correct equation, Fig. 3 should display linear relationships. It can be seen, however, that all data display positive curvature.

A similar test can be made for Eq. 4. Because of the constant factor α, the quantity αV_p calculated from Eq. 4 is plotted against polymer concentration (Fig. 4). Again, if the equation truly describes the phenomenon, this plot should be linear. It can be seen that Fig. 4 displays much better linear relationships than Fig. 3.

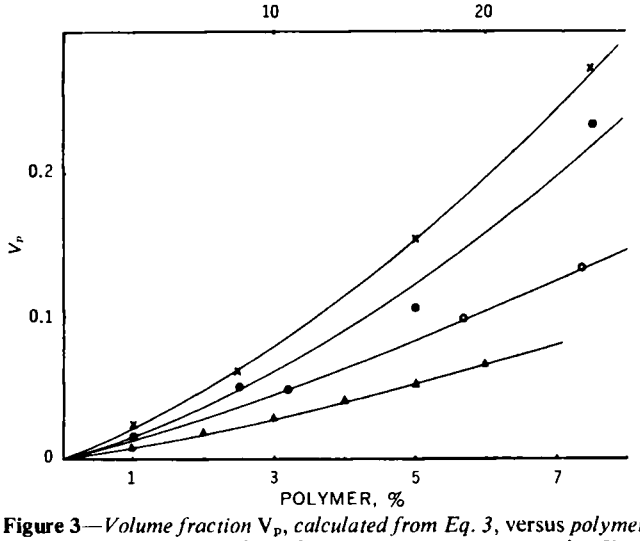

Figure 3—*Volume fraction* V_p, *calculated from Eq. 3,* versus *polymer concentration. Upper scale is for water-protein system only. Key:* ●, *sodium chloride-polyethylene glycol 6000* (Reference 4); ×, *potassium chloride-polyethylene glycol 6000* (Reference 4); ○, *water-protein* (Reference 7); *and* △, *sodium salicylate-sodium carboxymethylcellulose 7LXF (this work).*

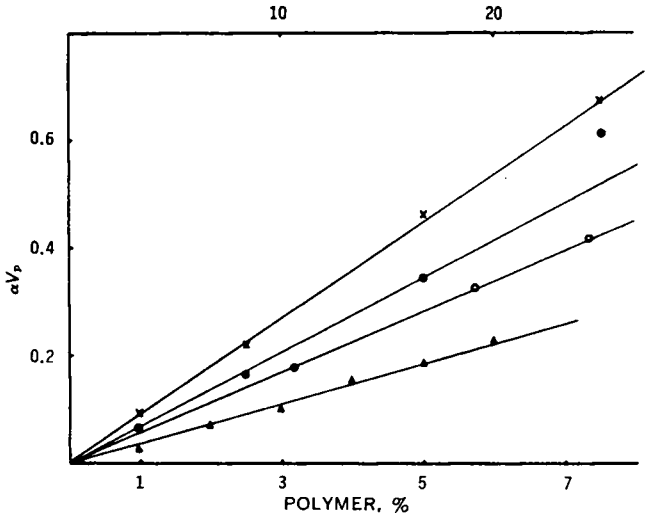

Figure 4—αV_p, *calculated from Eq. 4,* versus *polymer concentration. Upper scale is for water-protein system only. Key:* ●, *sodium chloride-polyethylene glycol 6000* (Reference 4); ×, *potassium chloride-polyethylene glycol 6000* (Reference 4); ○, *water-protein* (Reference 7); *and* △, *sodium salicylate-sodium carboxymethylcellulose 7LXF (this work).*

Therefore, Eq. 4 appears to be a better representation for the experimental data at higher polymer concentrations.

A practical consideration based on this information is that the diffusive transport of a drug in a dilute polymer solution is dependent on αV_p. One can define $\overline{\alpha V_p}$ as the value of αV_p per unit concentration, so $\overline{\alpha V_p}$ can be evaluated experimentally. The slopes of the lines in Fig. 4 were $\overline{\alpha V_p}$ in units of volume fraction per percent. Their values are listed along with the standard errors in Table III, and these factors are different for different polymers. A polymer having a relatively large $\overline{\alpha V_p}$ decreases diffusive transport more than one having a small $\overline{\alpha V_p}$. This factor $\overline{\alpha V_p}$ may be a more useful term than viscosity to characterize a polymer relative to its effect on the diffusive properties of small molecules in dilute solutions of the polymer.

Case B—To study further the effect of the polyelectrolyte on the diffusive transport of a drug as co-ion of the polymer, release rates *out of* the polyelectrolyte solutions were determined. Experiments similar to those of Case A were performed, except that the polyelectrolyte was present only inside the capillary and not in the sink. Thus, the diffusion coefficient calculated from the data by Eq. 1 is not the true self-diffusion coefficient because of the existence of a polyelectrolyte gradient at the boundary. However, the calculated apparent diffusion coefficient, D', does give an indication of the polyelectrolyte effect on the co-ion transport. The D' values are reported in Table I and, for comparison with Case A, the results are presented in Fig. 2 (Case B) as a ratio of D' to D_0. It can be seen that the radioactive salicylate ion is released faster than in Case A.

The polymer contains a net ionic charge, although it does not correspond exactly to the degree of substitution. These concentrated charges create a rather strong electric field which influences the distribution of ions around the polymer. Since the polyelectrolyte is present inside the capillary only, the electrochemical potential of the co-ion is changed relative to that outside the capillary. In addition to the Brownian diffusive transport, this electric field gradient gives another driving force for the salicylate transport. This additional driving force increases the apparent diffusion coefficient.

The increase in D' may also arise from other sources. The polymer diffusion out of the capillary may carry with it some salicylate ion. However, this probably would be small because the diffusion coefficient of a high molecular weight polymer is relatively small. Also, the osmotic pressure arising from the polyelectrolyte may induce water to flow into the capillary, forcing out the salicylate ion. Experiments were performed using the strong electrolyte sodium fluoride in place of the polyelectrolyte to establish an osmotic pressure difference. Using sodium fluoride up to a concentration of 0.4 M inside the capillary did not change D' significantly from D,

Table II—Relative Viscosity of Various Types of Sodium Carboxymethylcellulose Solutions (15)

Type of Polymer	Degree of Substitution	Concentration (% by Weight) 1.0	1.5	2.0	2.5	3.0	4.0	5.0	6.0
7MXF, 12M8P	0.7, 1.2	50	1.8×10^2	4.5×10^2	1.2×10^3	—	—	—	—
7M27SF	0.7	1.6×10^2	6×10^2	1.5×10^3	3×10^3	—	—	—	—
7LXF	0.7	11	—	20	—	1×10^2	3×10^2	8×10^2	2×10^3

Table III—Factor $\overline{\alpha V_p}$ for Various Polymer Systems

Polymer System	$(\overline{\alpha V_p} \pm SE) \times 10^2$, Volume Fraction/%	Reference
Sodium chloride–polyethylene glycol 6000	8.31 ± 0.73	4
Potassium chloride–polyethylene glycol 6000	9.08 ± 0.20	4
Sodium chloride–polyvinylpyrrolidone	48.1 ± 0.42	4
Water–protein	1.73 ± 0.03	7
Sodium salicylate–sodium carboxymethylcellulose (7LXF)	3.80 ± 0.12	This work

which was determined with sodium fluoride present both inside the capillary and the sink (Table I). Therefore, it can be inferred that the osmotic effect of the polymer is negligible.

From a practical point of view, the release rate of a medication from a preparation is an important factor to be considered by the formulator. The present work shows that high molecular weight polymers commonly used to increase the viscosity in liquid preparations decrease the diffusion of drugs *within* the solution only slightly (Eq. 4). Also, if the drug is present as a co-ion of the polyelectrolyte, it will be transported *out of* the polymer solution faster than if the polymer were not present. Increases of up to 40% were observed (Fig. 2).

The use of polyelectrolytes to enhance drug absorption was suggested by Higuchi *et al.* (11) nearly 2 decades ago. The present work substantiates this proposal by means of a different experimental approach and sheds light on the diffusive transport properties of small molecules in these dilute polymer solutions.

For sodium carboxymethylcellulose (degree of substitution 0.7), the release of co-ion increases as the concentration of polyelectrolyte increases initially and then levels off at about 2–3% (w/v), regardless of the molecular weight of sodium carboxymethylcellulose. Thus, when using this polyelectrolyte, the viscosity needed determines the molecular weight of the polymer, whereas the optimum polymer concentration for the drug release is in the range of 2–3%.

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Influence of Temperature and Nature of Hydrophobic Groups on Thermodynamic Parameters of Hydrophobic Bonding in a Model Polymeric System and Their Implications in Drug-Biopolymer Interactions

JANARDAN B. NAGWEKAR▲ and NUALTA MUANGNOICHAROEN

Abstract □ By using a model polymeric system, the thermodynamic parameters of hydrophobic bonding between the alkyl groups as well as between two methylene groups were determined. This was accomplished by studying the binding of the anions of fatty acids, propionate through caproate, by the hexyl copolymer from 28 to 45°. The hexyl copolymer was synthesized by quaternizing with hexyl bromide the vinylpyridine units of a copolymer of an overall composition of 1 unit of vinylpyridine to 22 units of vinylpyrrolidone. The hexyl vinylpyridinium units of the copolymer are shown to constitute the definite sites for hydrophobic bonding. The results of the present study, together with those of a previous study, revealed the influence of temperature and the nature of hydrophobic groups on the thermodynamic parameters of hydrophobic bonding. The significance of these characteristic properties of hydrophobic bonding in drug-biopolymer interactions is discussed. A GLC method, developed for quantitative determination of the fatty acids, is described.

Keyphrases □ Hydrophobic bonding between alkyl and between methylene groups—temperature effect, implications in drug-biopolymer interactions □ Drug-biopolymer interactions—temperature and group effects on hydrophobic bonding □ Thermodynamic parameters, hydrophobic bonding—effects of temperature and length of alkyl group □ Temperature effect—thermodynamic parameters of hydrophobic bonding, alkyl fatty acids and vinylpyridine–vinylpyrrolidone polymer □ Vinylpyridine–vinylpyrrolidone copolymer—used to study hydrophobic bonding between alkyl and between methylene groups

In addition to the noncovalent forces such as ionic attraction and hydrogen bonding, the importance of hydrophobic bonding is well recognized in drug-receptor interactions (1). Hydrophobic bonding is the interaction between hydrophobic groups in an aqueous medium, and such an interaction is characteristically accompanied by positive enthalpy and entropy effects (2). In view of the biopharmaceutical significance of hydrophobic bonding phenomena, a model water-soluble polymeric system was devised and studied (3); it served to demonstrate certain properties of hydrophobic bonding between the phenyl and alkyl groups. An interesting feature noted in this study, carried out at 15, 22, 30, 37, and 45°, was that the binding between the hydrophobic groups increased up to 37° but decreased at 45°. Furthermore, when the corresponding binding constant was plotted against the alkyl copolymer of the homologous series, an ascending zigzag curve was obtained.

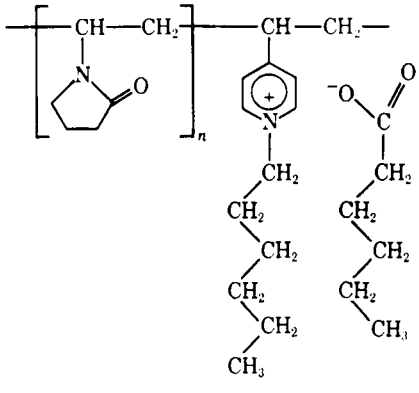
Based on a physical model, Nemethy and Scheraga (4) theorized that hydrophobic bond formation becomes stronger up to 42° between the aromatic hydrocarbons but that it becomes stronger up to 58° between the aliphatic groups. Therefore, the purposes of the present study were: (*a*) to determine the thermodynamic parameters of hydrophobic bonding (28–45°) between the alkyl groups, as well as between two methylene groups, using a model water-soluble alkyl copolymer containing known sites of hydrophobic bonding; and (*b*) to determine if the hydrophobic bonding between the alkyl groups in the model system continues to be stronger up to 45°, as well as with the increase in chain length at a given temperature.

MODEL SYSTEM

The present model system consists of the study of the binding of the fatty acid anions, propionate through caproate, by the hexyl copolymer. The procedures for synthesis and overall composition determination of the hexyl copolymer were previously reported (3). The overall composition of the copolymer was 1:23 (1 unit of quaternized vinylpyridine to 23 units of vinylpyrrolidone). The fatty acids (pKa 4.81–4.87) were expected to remain in solution in the ionized form since the interactions were carried out at pH 7.4. The binding energy involved in these interactions is due to both electrostatic binding and hydrophobic bonding. Scheme I depicts how the electrostatic binding facilitates the approach of the fatty acid anions sufficiently close to the quaternized units so that hydrophobic bonding can occur between the alkyl group of the carboxylate ion and the hexyl group of the hexyl copolymer. Evidence has been obtained (as explained later) to indicate that the sites on the alkyl copolymers for binding of fatty acid anions are indeed the vinylpyridinium units. The suitability of such a model system in determining the thermodynamic parameters of hydrophobic bonding between the interacting hydrophobic groups was explained previously (3).

EXPERIMENTAL

Materials—Hexyl copolymers (1:23, 1:27, and 1:42) were synthesized according to the method previously described (3). Sodium propionate[1] and sodium butyrate[1] were recrystallized from



Scheme I—Representation of the interaction of a caproate ion with a hexyl copolymer unit. The iceberg structures around the methylene and methyl groups are not shown. In the diagram, n represents the average 23 units of vinylpyrrolidone to 1 unit of quaternized vinylpyridine.

[1] Matheson, Coleman and Bell, East Rutherford, N. J.

Table I—Retention Times Observed for the Fatty Acids[a]

Compound	Column Temperature	Retention Time, min.
Propionic acid	120°	6.0
Butyric acid	140°	4.5
Valeric acid	150°	5.0
Caproic acid	160°	5.0

[a] The conditions used were: sensitivity, range 10, attenuation 8–32×; injection port temperature, 210°; detector temperature, 210°; and helium flow rate, 30 ml./min.

an acetone–water (5:1) mixture according to Childers and Struthers (5). Sodium valerate and sodium caproate were prepared from *n*-valeric acid[2] and *n*-caproic acid[1], respectively, according to the methods described by Childers and Struthers (5). Other chemicals used were reagent grade.

Apparatus—The fatty acids were quantitatively analyzed with the aid of a gas chromatograph[3] equipped with a flame-ionization detector. The column employed was a 1.21-m. long and 0.63-cm. o.d. copper tube packed with 80–100-mesh diatomite[4] coated with 15% diethylene glycol adipate. Helium was used as the carrier gas. A 10-μl. standard syringe[5] was used for injecting the sample. A recording spectrophotometer[6] was used for the copolymer analysis. An expandomatic pH meter[7] was used for the potentiometric determination of bromide content of the hexyl copolymer and for pH determinations of the solutions. Plexiglas dialysis cells[8] of 2-ml. capacity were used in the equilibrium dialysis studies.

Solution of Hexyl Copolymer—A 4% (w/v) solution of hexyl copolymer (1:23) was prepared in a pH 7.4 phosphate buffer of 0.1 ionic strength. Similar solutions of hexyl copolymers (1:27 and 1:42) were also prepared. The ionic strength of all solutions was adjusted to 0.114 with sodium chloride.

Solution of Sodium Salt of Fatty Acid—The solutions of each acid (0.004–0.014 *M*) were prepared in pH 7.4 phosphate buffer. The ionic strength of each solution was adjusted to 0.114 with sodium chloride.

Dialysis Procedure—Each dialysis cell consisted of two Plexiglas halves, 5 × 5 × 2 cm., each with a cavity having 1-ml. capacity. The dialysis cells were set up in the same manner as previously described (3). The solutions were transferred into each cavity of the dialysis cell by means of 1-ml. tuberculin syringes. The cells were allowed to shake in a water bath maintained at 28, 32, 37, or 45 ± 0.3°. Although the system attained equilibrium in 60 hr., the cells were allowed to shake in the bath for 84 hr. To ensure accuracy of the results, the standard dialysis cells were set up along with the sample cells. The standard cells were set up in the same manner as the sample cells, except that 1 ml. solution of pH 7.4 phosphate buffer of appropriate ionic strength was used in place of 1 ml. solution of the hexyl copolymer.

After 84 hr., the cells were removed from the bath and promptly the contents from the nonpolymer side and polymer side of each cell were quantitatively transferred to 2.5-ml. graduated tubes by means of Pasteur pipets. The graduated tubes were stoppered and allowed to stand until the contents of the tubes attained room temperature. The volume of solution obtained from the nonpolymer side of each cell generally measured 0.9 ml. and that obtained from the polymer side of each cell generally measured 1.1 ml. Therefore, the appropriate correction was made for the concentration of hexyl copolymer in the polymer solution in each dialysis set-up. All studies were carried out in duplicate at each concentration of the fatty acid.

Quantitative Determination of Fatty Acids by GLC—The quantitative determinations of the fatty acids in the solutions obtained from the nonpolymer side of each cell were carried out by GLC, using 15% diethylene glycol adipate liquid phase coated on diatomite[4]. Immediately prior to GLC analysis, 0.01 ml. concentrated hydrochloric acid was added to each solution to convert the salt of the fatty acid to the free fatty acid. The pH of such solutions was found to be about 1. Four microliters of the acidified solution was then injected into the GLC column. The conditions for analysis are given in Table I. A typical chromatogram of fatty acids is shown in Fig. 1. The area under the curve of each chromatogram was calculated by the trapezoidal rule. The calibration curves relating the area under the curve to the concentration were prepared for each fatty acid by analyzing gas chromatographically the acid solutions of known concentration. A typical calibration curve obtained for valeric acid is shown in Fig. 2.

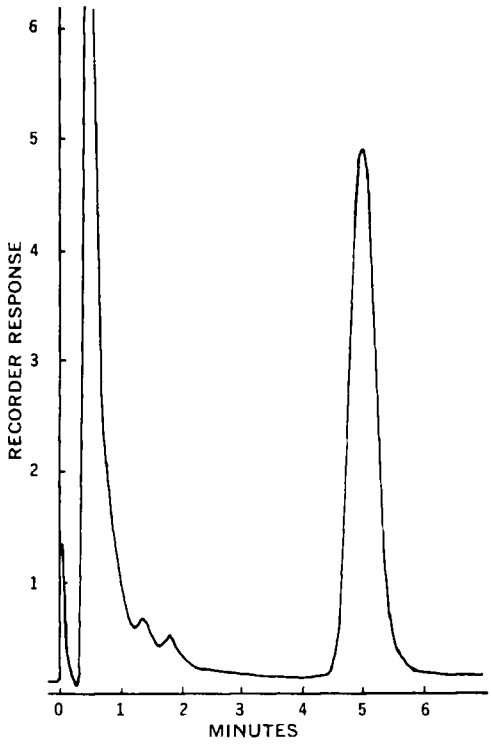


Figure 1—*A typical gas chromatogram obtained for valeric acid.*

Binding of Anions of Valeric Acid by 1:27 and 1:42 Hexyl Copolymers—To determine if binding of the fatty acid anions by the hexyl copolymer was solely due to the quaternized vinylpyridine units of the latter, the binding of the anions of valeric acid (0.006 *M*) by the hexyl copolymers (1:27 and 1:42) was studied at 37° and 0.114 ionic strength in the usual manner.

RESULTS AND DISCUSSION

Quantitative Determination of Fatty Acids by GLC—The use of 15% diethylene glycol adipate as the liquid phase in the quantitative GLC analysis of the fatty acids was found to be satisfactory (Fig. 2). With reference to such a standard plot as in Fig. 2, the amounts of the fatty acid contained in the solutions from the nonpolymer side of the sample cells were calculated. To ascertain the accuracy of such quantitative analyses, the standard plots were constructed for the fatty acid contained in the standard cells of each run. The amount of fatty acid bound by hexyl copolymer in each dialysis cell was calculated in the usual manner. It was generally observed that the difference between the quantity of fatty acid calculated from each duplicate sample was about 1–2%. None of the fatty acids was bound to the cellophane membrane or the dialysis cells.

Identification of Binding Sites in Hexyl Copolymer—As noted in Fig. 3, the extent of binding of valerate ions was directly proportional to the molar concentration of the hexyl vinylpyridinium bromide content of the hexyl copolymer, indicating that the hexyl vinylpyridinium groups constituted the binding sites for the fatty acid anions. Further support to this conclusion was obtained by demonstrating that a negligible amount of valerate ions is bound by nonquaternized 1:42 copolymer (4% w/v) at 37° and 0.114 ionic strength.

Binding Constants—The binding data obtained for the anions of

[2] Eastman Kodak Co., Rochester, N. Y.
[3] F&M model 810R-19.
[4] Diatoport S.
[5] Hamilton.
[6] Beckman model DK-2A.
[7] Beckman.
[8] The Chemical Rubber Co., Cleveland, Ohio.

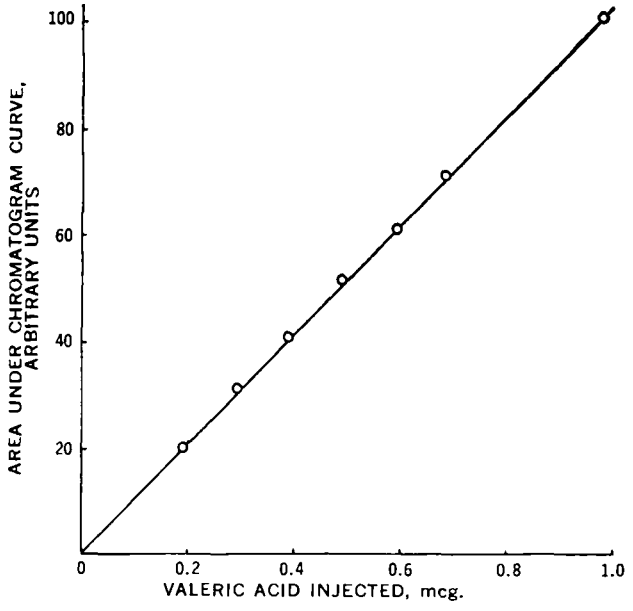


Figure 2—*Calibration curve for valeric acid.*

each fatty acid were treated according to the Klotz equation (6):

$$1/r = 1/n + 1/nKa \qquad \text{(Eq. 1)}$$

where r is the number of moles of fatty acid anion bound per mole of hexyl copolymer, n is the average number of binding sites per mole of the hexyl copolymer, a is the molar concentration of free fatty acid anion at equilibrium, and K is the binding constant (liters per mole). As shown in Fig. 4, typical Langmuir plots of $1/r$ *versus* $1/a$ were constructed for the binding data of the anion of each fatty acid, and the values of n and K were determined from the intercept ($1/n$) and the slope ($1/nK$) of the straight line obtained by the least-squares method. The binding constants are listed in Table II. The straight-line plots in Fig. 4 indicate that the fatty acid anions bind to identical sites present in the hexyl copolymer. Also, out of every 10 binding sites, the number of sites available on the hexyl copolymer for the binding of the fatty acid anion was about 7–9.7. This may be due to some coiling of the hexyl copolymer molecules, making a few sites unavailable for binding (3).

As seen in Table II, not only the extent of binding of the fatty acid anion increased with the increase in the chain length at each temperature but also the extent of binding of the anion of each fatty acid increased with the increase in temperature from 28 to 45°.

Thermodynamic Parameters for Binding of Fatty Acid Anion—The free energy of binding (ΔF_b) of the fatty acid anion was calculated from the following equation:

$$\Delta F_b = -RT \ln K \qquad \text{(Eq. 2)}$$

where R and T have the usual meanings. Since the plots of log K *versus* $1/T$ (Fig. 5) were reasonably straight lines (least squares), the enthalpy of binding is considered to be constant over the 28–45° range. The enthalpy of binding (ΔH_b) was calculated from the slope of the straight line obtained according to the van't Hoff equation:

$$\log K = \frac{-\Delta H}{2.33RT} + \text{constant} \qquad \text{(Eq. 3)}$$

The values of entropy of binding (ΔS_b) were calculated from the following relationship:

$$\Delta S_b = \frac{\Delta H_b - \Delta F_b}{T} \qquad \text{(Eq. 4)}$$

The thermodynamic constants thus determined are listed in Table III.

Since the binding of the fatty acid anion by the hexyl copolymer was found to be associated with the positive enthalpy and entropy effects (Table III), it is concluded that the polymeric system used in the present study exhibits the properties of hydrophobic bond formation. Despite the fact that the positive enthalpy observed in the study is unfavorable for the binding process, the ΔF_b becomes increasingly negative (and, therefore, favorable) with the increase in temperature because of the gain in entropy. The gain in entropy occurs due to hydrophobic interactions in the aqueous medium between the alkyl groups of the hexyl copolymer and those of the fatty acid resulting in the release of water molecules from the "icebergs" around the alkyl groups and, consequently, in the release of configurational entropy of the alkyl groups (3).

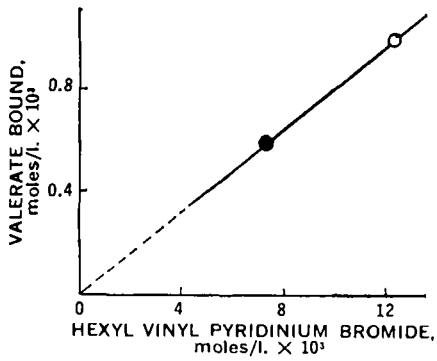


Figure 3—*Binding of valeric acid anion (6 μmoles/dialysis cell) by 1:42 (●) and 1:27 (○) hexyl copolymers at 37° and 0.114 ionic strength.*

The fact that the ΔF_b in the present system increased with the increase in chain length of the fatty acid further substantiates that the present polymeric system displays the characteristics of hydrophobic bonding.

Determination of Thermodynamic Parameters of Hydrophobic Bonding—Since the hexyl vinylpyridinium units of the hexyl copolymer constitute the sites for binding of fatty acid anions, it is apparent that the observed ΔF_b is mainly a combination of the free energy of binding due to electrostatic forces (ΔF_e) and the free energy of binding due to hydrophobic bonding between the side chains ($\Delta F_{H\phi}{}^S$):

$$\Delta F_b = \Delta F_e + \Delta F_{H\phi}{}^S \qquad \text{(Eq. 5)}$$

Furthermore, the ΔF_e of the anions of all fatty acids can be considered to be the same. Therefore, from the data in Table III, the $\Delta F_{H\phi}{}^S$ due to the alkyl groups of the copolymer and the corresponding fatty acid anions can be calculated by subtracting the ΔF_b of the appropriate fatty acid anion from those of the higher homologs at each temperature. It is recognized that there is a possibility

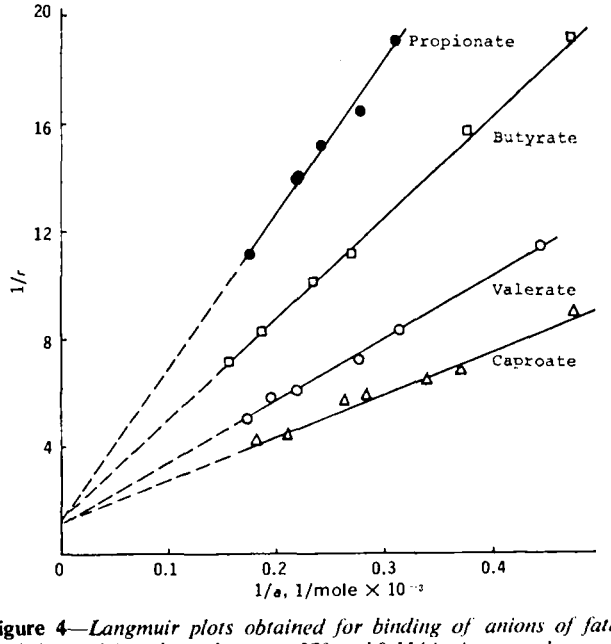


Figure 4—*Langmuir plots obtained for binding of anions of fatty acids by 1:23 hexyl copolymer at 37° and 0.114 ionic strength.*

Table II—Binding Constants Determined for the Interaction of the Fatty Acid Anion with the Hexyl Copolymer (1:23) at 28, 32, 37, and 45° and 0.114 Ionic Strength

Fatty Acid Anion	Temperature	Binding Constant, K, l./mole
Propionate	28°	15.92
	32°	16.67
	37°	23.57
Butyrate	28°	22.35
	37°	37.03
	45°	57.48
Valerate	28°	30.82
	32°	43.76
	37°	46.20
	45°	98.26
Caproate	28°	49.64
	32°	58.97
	37°	75.61
	45°	170.73

that the alkyl groups attached to the charged groups (such as the quaternary nitrogen in the hexyl copolymer and the carboxylate group of the fatty acid anion) may influence the structure of water around them differently than when they exist in water as aliphatic hydrocarbons. Therefore, in this context, the appropriate fatty acid anion in the homologous series refers to that anion of sufficient chain length whose end methyl group is just beyond the sphere of electrostatic effect of its charged group. This would then make it possible to determine the thermodynamic parameters of hydrophobic bonding between two methylene groups if, indeed, the iceberg structure around the methylene groups of the alkyl groups is uniform beyond the sphere of the electrostatic influence of the charged group. However, it is recognized that in a homologous series such uniformity in the iceberg structure around the methylene groups can be expected only up to a limited chain length of the alkyl group, since the alkyl groups beyond a certain chain length are subject to curling to reduce their surface exposure to water.

In view of the enthalpy of solution data in water reported by Snell and Greyson (7) for sodium formate (−0.269 kcal./mole), sodium acetate (−3.973 kcal./mole), sodium propionate (−3.015 kcal./mole), and sodium butyrate (−3.385 kcal./mole) at 25°, it

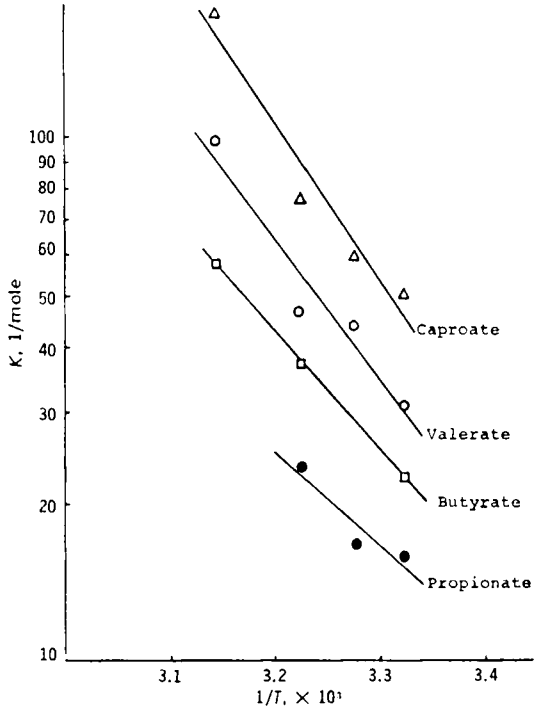


Figure 5—*Van't Hoff plots obtained for binding of anions of fatty acids by 1:23 hexyl copolymer over the temperature range of 28–45° and 0.114 ionic strength.*

Table III—Thermodynamic Parameters of Binding of the Anions of Fatty Acids by the Hexyl Copolymer (1:23) in pH 7.4 Phosphate Buffer at 0.114 Ionic Strength and 28–45°

Fatty Acid Anion	$-\Delta F_b$, cal./mole 28°	32°	37°	45°	ΔH_b, cal./mole	ΔS_b, e.u.
Propionate	−1655	−1705	−1946	—	8,211	32.65
Butyrate	−1858	...	−2225	−2560	10,560	41.25
Valerate	−2051	−2290	−2361	−2899	12,250	47.40
Caproate	−2336	−2471	−2665	−3247	13,817	53.40

becomes apparent that there exists a nonuniformity in the iceberg structure around the alkyl groups of short-chain fatty acid anions. Unfortunately, such thermodynamic data are not available for sodium salts of valeric and caproic acids to suggest that the nonconformity is minimized in the iceberg structure around the tail-end methylene groups of the alkyl chains of these salts. However, Everett (8) observed that the singly charged ions such as the quaternary nitrogen and carboxylate group exert a significant influence on the solvent (water) structure only over a distance of 5 Å. Since the C—C bond length is about 1.54 Å (8), this would then suggest that the influence of the singly charged groups of the copolymer and fatty acid anions on the structure of water around the methylene groups, which are separated from them by at least three methylene groups, should be minimal. Therefore, it is considered appropriate to obtain the data for free energy of hydrophobic bonding between the alkyl groups by subtracting the ΔF_b of butyrate ions from those of the higher homologs at corresponding temperatures. In this manner the free energy of hydrophobic bonding between the CH_2 group of valerate ion and the CH_2 group of the hexyl chain of the copolymer and between the CH_2—CH_2 group of caproate ion and the CH_2—CH_2 group of the hexyl chain of the hexyl copolymer can be estimated. The values of free energy of hydrophobic bonding thus obtained for the portions of the alkyl groups of the reactants are designated as $\Delta F_{H\phi}{}^{PA}$ (Table IV).

As shown for ΔF_b in Eq. 5, the two types of forces that contribute to the enthalpy of binding as well as to the entropy of binding of the fatty acid anions by the hexyl copolymer can be described as follows:

$$\Delta H_b = \Delta H_e + \Delta H_{H\phi}{}^{S} \qquad \text{(Eq. 6)}$$

$$\Delta S_b = \Delta S_e + \Delta S_{H\phi}{}^{S} \qquad \text{(Eq. 7)}$$

The contribution of ΔH_e and ΔS_e due to electrostatic interaction can be considered to be the same for all of the fatty acid anions. Therefore, the enthalpy ($\Delta H_{H\phi}{}^{PA}$) and the entropy ($\Delta S_{H\phi}{}^{PA}$) due to hydrophobic bonding between the portions of the alkyl groups of the reactants involved are calculated by subtracting the ΔH_b and ΔS_b of the butyrate ions from those of the higher homologs. The values thus calculated are presented in Table V.

It is interesting to note that the values of $\Delta H_{H\phi}{}^{PA}$ and $\Delta S_{H\phi}{}^{PA}$ (Table V) obtained for the interaction between CH_2—CH_2 (copolymer) and CH_2—CH_2 (caproate) are almost twice those obtained for the interaction between CH_2 (copolymer) and CH_2 (valerate). Although these data are limited, it appears that the enthalpy and

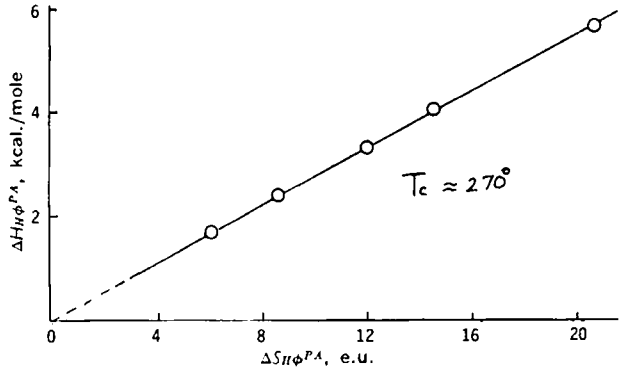


Figure 6—*Plot showing a linear relationship between* $\Delta H_{H\phi}{}^{PA}$ *and* $\Delta S_{H\phi}{}^{PA}$ *(from the data in Table V and that obtainable from Table III, as explained in the text).*

Table IV—Free Energy ($\Delta F_{H\phi}^{PA}$) Determined by Hydrophobic Bond Formation between the Corresponding Portions of the Alkyl Groups of the Hexyl Copolymer and the Fatty Acid Anions at 0.114 Ionic Strength

Temperature	Interacting Portions of Alkyl Groups of Hexyl Copolymer	Fatty Acid Anion	$\Delta F_{H\phi}^{PA}$, cal./mole
28°	CH_2	CH_2 (valerate)	−193
	CH_2—CH_2	CH_2—CH_2 (caproate)	−478
37°	CH_2	CH_2 (valerate)	−136
	CH_2—CH_2	CH_2—CH_2 (caproate)	−440
45°	CH_2	CH_2 (valerate)	−339
	CH_2—CH_2	CH_2—CH_2(caproate)	−687

Table V—Enthalpy ($\Delta H_{H\phi}^{PA}$) and Entropy ($\Delta S_{H\phi}^{PA}$) Determined for Hydrophobic Bonding between the Corresponding Portions of the Alkyl Groups of the Hexyl Copolymer and the Fatty Acid Anions at 0.114 Ionic Strength

Interacting Portions of Alkyl Group of Hexyl Copolymer	Fatty Acid Anion	$\Delta H_{H\phi}^{PA}$, kcal./mole	$\Delta S_{H\phi}^{PA}$, e.u.
CH_2	CH_2 (valerate)	1.69	6.15
CH_2—CH_2	CH_2—CH_2 (caproate)	3.26	12.15

entropy of hydrophobic bonding between two methylene groups can be expected to be uniform as long as these groups of the alkyl chain are not influenced by the charged groups and are fully exposed to water. This finding also corroborates the observation made by Everett (8) that the singly charged ions significantly influence the structure of water only over a distance of 5 Å. This may also explain why the $\Delta H_{H\phi}^{PA}$ and $\Delta S_{H\phi}^{PA}$ values calculated by subtracting the ΔH_b and ΔS_b values of propionate ions from those of the higher homologs do not appear to be as uniform as those obtained by subtracting the ΔH_b and ΔS_b values of butyrate ions. This becomes evident by considering, for instance, the $\Delta H_{H\phi}^{PA}$ values of 2.34, 4.04, and 5.61 kcal./mole obtainable from the data in Table III (upon subtracting ΔH_b of propionate ions) for hydrophobic bonding between CH_2 (copolymer) and CH_2 (butyrate), CH_2—CH_2 (copolymer) and CH_2—CH_2 (valerate), and CH_2—CH_2—CH_2 (copolymer) and CH_2—CH_2—CH_2 (caproate), respectively. However, the extrathermodynamic analysis (Fig. 6) of the $\Delta H_{H\phi}^{PA}$ and $\Delta S_{H\phi}^{PA}$ data obtainable from Table III by subtracting the ΔH_b and ΔS_b values of propionate ion, or those data presented in Table V, reveals that a single interaction mechanism (in this case the hydrophobic bonding) is operative in the binding of the various alkyl groups of the anions of fatty acids with the alkyl group of the hexyl copolymer. This conclusion is drawn not only from the linear relationship observed between $\Delta H_{H\phi}^{PA}$ and $\Delta S_{H\phi}^{PA}$ according to the theory advocated by Leffler and Grunwald (9) but also from the compensation temperature (T_c) of 270° (10) noted from the slope of the straight line passing practically through the origin.

Comparison of Nature of Binding Constant Data Obtained for Hydrophobic Bonding between Phenyl and Alkyl Groups and between Alkyl Groups—It was observed in the present study that the binding constant of the fatty acid anions increased with the increase in the chain length. In the previous study (3), however, an ascending zigzag pattern in the binding constant was observed. The zigzag pattern was explained in terms of probable closer orientation of the terminal methyl group of the propyl and pentyl chain of the alkyl copolymers to the phenyl group of the target sulfonate ion than the terminal methyl group of the butyl and hexyl chain of the alkyl copolymers. In the present model system, both interacting groups involved in hydrophobic bonding are alkyl groups. Therefore, having brought these alkyl groups closer to each other through electrostatic forces, it does not seem unreasonable to expect the alkyl groups to align against each other in such a way that each methylene group and methyl group of the fatty acid would approach the methylene groups of the hexyl chain of the hexyl copolymer within the same van der Waals radius. Such alignment of the alkyl groups would result in the release of water molecules from the icebergs around the methylene groups in increasing numbers, thereby increasing the binding constants, with the increase in the chain length of the fatty acid anion.

Comparison of Temperature Effects of Hydrophobic Bonding between Phenyl and Alkyl Groups and between Alkyl Groups—In the previous study (3), it was observed that the binding of *p*-toluenesulfonate ions by the alkyl copolymers, ethyl through hexyl, increased with an increase in temperature up to 37° but decreased at 45°. However, it is noted in the present study that binding of fatty acid anions by the hexyl copolymer increased with an increase in temperature up to 45°. The decrease in binding of the sulfonate ions by the alkyl copolymers was attributed to the breakdown of the icebergs around these ions at 45°. Therefore, a gain in entropy due to hydrophobic interaction between the alkyl groups of the alkyl copolymer and the phenyl group of the sulfonate ions at 45° was substantially lower than that at 37°. The finding of the present study then suggests that the icebergs around the alkyl groups are not disrupted at 45°. Consequently, a gain in entropy due to hydrophobic interaction between the alkyl groups of the hexyl copolymer and those of the fatty acid anions would be expected to continue at 45°. These findings are in agreement with the theoretical expectation by Nemethy and Scheraga (4). It is, therefore, reasonable to conclude further that the polymeric system employed in the present study is successful in revealing experimentally the additional characteristic of hydrophobic bonding with regard to its temperature dependency.

SIGNIFICANCE

The interesting feature of the polymeric system employed in this study is that the hexyl copolymer contained the binding sites (to interact with fatty acid anions) whose chemical nature was known, thereby making it possible to determine the apparent thermodynamic parameters of hydrophobic bonding between two methylene groups.

The previous (3) and present studies, both of which involved essentially the same polymeric system, demonstrated that the strengths of hydrophobic bonds depend on the nature (aliphatic or aromatic) of the interacting hydrophobic groups. For instance, on comparison, it becomes clear that the hydrophobic bonding noted in the previous study between the toluyl group and the hexyl group is much weaker than that between the pentyl group and the hexyl group noted in the present study. It was also observed that the strength of the hydrophobic bond formed between the toluyl group and each of the alkyl groups (ethyl through hexyl) did not increase with the increase in chain length of the alkyl groups but varied in a zigzag manner, reflecting the importance of the odd or even number of carbon atoms contained in the alkyl groups. In the present study, however, the strength of hydrophobic bonding of the hexyl group of the hexyl copolymer with the alkyl groups (propyl through hexyl) of the fatty acid anions increased with the increase in chain length of the alkyl groups. Therefore, it is conceivable that these features of hydrophobic bonding can provide a useful basis for revealing the presence of polar or nonpolar (aliphatic or aromatic) groups in the vicinity of a binding site (or a receptor) of an enzyme or a similar biopolymer responsible for the action of a drug, by appropriately evaluating the thermodynamic parameters of the binding of a drug and its suitable homologs with the biopolymer. Such an approach has been the basis of the macromolecular perturbation theory of drug action developed by Belleau (11).

A similar approach can also be useful in detecting the hydrophobic groups present in the vicinity of the charged binding site(s) of the plasma proteins for a variety of drugs that remain in the charged form in the plasma. Such approaches will by no means provide instant knowledge of the chemical nature of the binding or receptor sites on the macromolecules, but they will serve to focus attention on the more likely chemical entities or residues of macromolecules that possess positive or negative charges in the plasma. For instance, one would consider evaluating the amino acid residues such as lysine and arginine as the possible binding sites if the binding of anionic drugs to proteins is being investigated, or one would evaluate the amino acid residues such as aspartic acid and glutamic acid as the possible binding sites if the binding of cationic drugs to proteins is concerned.

The effect of temperature on the icebergs around the phenyl group as noted in the previous study (3), in contrast to those around the alkyl groups in the present study, is noteworthy. This phenomenon, in case of hydrophobic bonding between a drug molecule and a biopolymer, should offer additional means of revealing if the hydrophobic bonding is due to the interaction between the phenyl

groups, between the alkyl groups, or between the alkyl and phenyl groups of the interacting species. Also, in this context, the degree of effect of higher body temperature (fever) on the tertiary structure of vital biopolymers (enzymes), and consequently on their physiological activities, could be expected to depend on the nature of hydrophobic groups involved in hydrophobic bonding responsible for maintaining the tertiary structure of the biopolymers.

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Preparation and *In Vitro* Evaluation of Cellulose Acetate Phthalate Coacervate Microcapsules

H. P. MERKLE and P. SPEISER▲

Abstract □ Microcapsules of phenacetin were prepared by coacervation of aqueous cellulose acetate phthalate solutions. Appropriate solutions were made by dissolving cellulose acetate phthalate in an equivalent concentration of disodium hydrogen phosphate. A triangular phase diagram of the coacervation system was elaborated by using sodium sulfate as the coacervating agent, and the coacervation and encapsulation conditions were optimized. The amount of drug encapsulated has no significant effect on the particle-size distribution of the capsules; however, it influences the release rates of the drug, indicating that drug diffusion through the shells is the controlling step. When the shells are plasticized by imbibition with glycerol, the release rate is no longer controlled by drug diffusion through the shells but by the dissolution of phenacetin in the microcapsules.

Keyphrases □ Cellulose acetate phthalate coacervate microcapsules—formulation, *in vitro* drug (phenacetin) release rates, effect of glycerol plasticizer □ Encapsulation—formulation and *in vitro* testing of cellulose acetate phthalate coacervate microcapsules of phenacetin □ Coacervation of cellulose acetate phthalate—formulation of microcapsules, *in vitro* phenacetin release rates, plasticizer effect □ Microcapsules, phenacetin- formation from cellulose acetate phthalate coacervate system, release rates, plasticizer effect

The preparation of microcapsules by coacervation can be applied to various products (1). Different coating materials, suitable for coacervate encapsulation, were reported by Ranney (2). In pharmacy, however, the most common preparation technique is either simple coacervation of gelatin with ethanol or sodium sulfate as dehydrating agents (3–9) or complex coacervation of gelatin-acacia mixtures (10–12). Coacervation methods with pure gelatin and mixtures are rather complicated and difficult to keep under control, particularly with regard to the hardening of shells and the recovery technique of the microcapsules. The suitability of other coacervation systems for pharmaceutical purposes has not yet been studied.

Cellulose acetate phthalate was used by Jensen and Wagner (13) as a film-forming agent for encapsulation of a herbicide by simple coacervation. However, the encapsulation procedure presented in this patent is not practicable, owing to the insolubility of the cellulose ester in pure water and its total hydrolysis at pH 9.7, which is proposed as the encapsulation pH. Due to acidic groups, the cellulose ester is insoluble in strong acids but soluble in weak acids (>pH 5.5), neutral electrolytes, and bases. It is a suitable encapsulation material for timed-release dosage forms. Due to its very low acute and chronic toxicity, cellulose acetate phthalate was chosen as the encapsulating material in this study.

The present article reports a relatively uncomplicated process for the encapsulation of solid drugs by the simple coacervation of the cellulose ester, which is dissolved in appropriate disodium hydrogen phosphate solutions. The coacervation is induced by the dehydrating effect of the added sodium sulfate solution. Furthermore, the optimal coacervation and encapsulation conditions and the properties of the resulting microcapsules are examined with regard to drug content, size distribution, and release and release mechanisms of drugs.

EXPERIMENTAL

Materials and Methods—The cellulose acetate phthalate used was of Ph. Helv. VI quality, containing 34.1% phthalic acid and 2.7% free acid, calculated as phthalic acid.

Phenacetin (*N*-acetyl-*p*-aminophenetidin), of Ph. Helv. VI quality (m.p. 134–135°), was chosen as the drug model for the encapsula-

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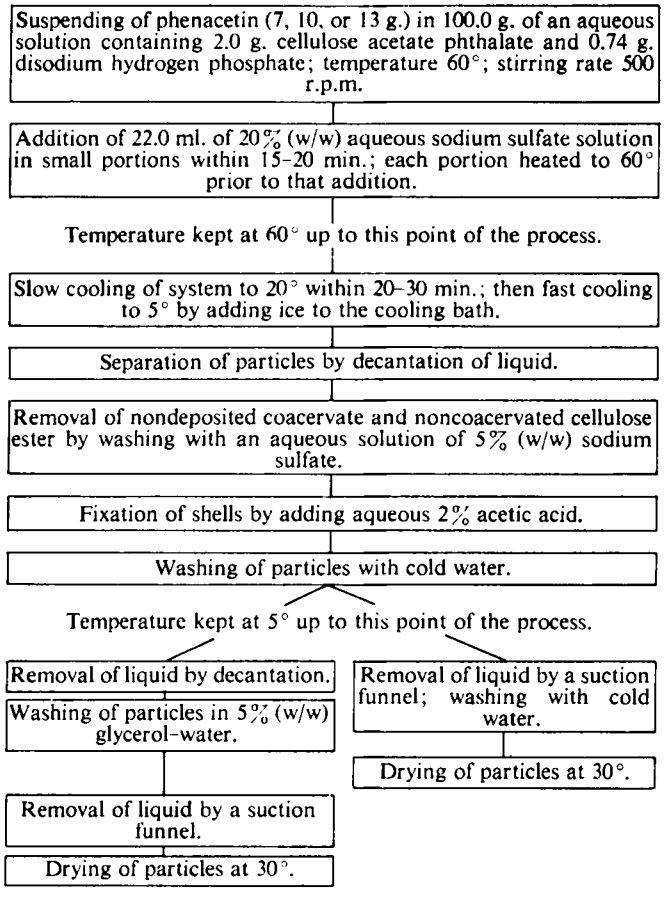
Phenacetin (*N*-acetyl-*p*-aminophenetidin), of Ph. Helv. VI quality (m.p. 134–135°), was chosen as the drug model for the encapsula-



Scheme I—Preparation of cellulose acetate phthalate microcapsules

tion, primarily because of its sufficient stability and insolubility in aqueous cellulose ester solutions. These properties are the fundamental requirements for good core materials. Sieve fractions of 0.10–0.50-mm. diameter were used.

Sodium sulfate (reagent grade) in a 20% (w/w) aqueous solution was used as a coacervating agent. All other compounds used were reagent grade.

Preparation of Cellulose Acetate Phthalate Solutions—A certain amount of cellulose ester was agitated in an aqueous solution of stoichiometrically equivalent quantities of disodium hydrogen phosphate (Na_2HPO_4) (calculated on the basis of the acidity of cellulose ester amounts, including free and bound acid, with 1.0 g. of cellulose ester being equivalent to 0.37 g. Na_2HPO_4) by means of a high speed stirrer (Polytron type) at 60°. After 1 min., most of the cellulose ester suspended was dissolved. The remaining solid cellulose ester was dissolved by low speed stirring using a magnetic stirrer at 60°. The dissolution was completed in about 15–20 min. The resulting solutions were filtered and stored at room temperature. The solutions proved to be stable for at least 1 week and showed no variation in coacervation behavior during that time.

Preparation of Microcapsules—As a preliminary proceeding, a triangular diagram was elaborated for the three phases participating in the cellulose ester coacervating system, *i.e.*, water, the cellulose ester, and salt. The latter consisted of disodium hydrogen phosphate (solvating agent) and sodium sulfate (dehydrating and coacervating agent). Portions of 100.0 g. of the cellulose ester solution were placed in a flask, which was maintained at 60° in a thermostated water bath and equipped with a reflux condenser. The solutions were stirred by a two-blade propeller at 500 r.p.m. For the coacervation of the cellulose ester, a 20% (w/w) aqueous sodium sulfate solution was added in parts. The supply of the sodium sulfate solution was constantly maintained at 60° with the help of a thermostated heating mantle. The start of coacervation was optically observed under the microscope. Solutions with 0.5 and 1.0–10.0% (1.0% interval) of cellulose ester were tested.

For microencapsulation, the same procedure was repeated by adding 10.0 g. of phenacetin to each concentration of the cellulose ester tested. The encapsulating and coacervating behavior of the slurries was observed microscopically. An optimum of coacervation and encapsulation was reached at a minimum agglomeration of the particles, a maximum deposition of coacervate on particles, and a minimum deformation of the liquid shells during encapsulation.

Following this procedure, all batches of microcapsules were produced within the field of optimum coacervation and encapsulation conditions. The method of preparation is shown in Scheme I. The encapsulations were made in a three-necked, 250-ml. beaker, equipped with a reflux condenser and a thermostated water bath for heating and cooling. The mixtures were agitated by a two-blade propeller-stirrer at a constant stirring rate of 500 r.p.m. The position of the stirrer was kept constant in all experiments. Special attention was given to the position of the stirrer, which must produce efficient mixing without nonconvected regions, an important requirement for preventing agglomeration of the particles. As may be seen from Scheme I, gelling of liquid shells was caused by cooling to 5°. The cellulose ester, which was not coacervated or deposited on the particles, was removed by washing and decanting with a 5% (w/w) aqueous sodium sulfate solution at 5°. Thereafter, the shells were fixed by addition of 2% acetic acid to the final pH 3.5. This process converted the solvatized cellulose ester gel to the nondissociated insoluble cellulose ester acid. If desired, the shells of washed particles can be treated with water-soluble plasticizers by washing the particles, for example, with a 5% (w/w) aqueous glycerol solution for 15 min. at 5°. The plasticizer treatment of the microcapsules should prevent rupture of the shells during drying.

Assay of Phenacetin Content—Microcapsules, 100.0 mg., were thoroughly triturated in a mortar. The resulting powder was transferred to a 600-ml. beaker. After a thorough rinsing of mortar and pestle with water, the powder was suspended in approximately 250 ml. of water (including the water used for rinsing). For complete rupture of the shells, the suspension was treated with ultrasonics for 2 min.[1]. The completeness of rupture by this treatment was observed previously. Heating to 40° and stirring with a magnetic stirrer for about 30 min. provide a complete dissolution of the phenacetin crystals. The resulting solution was filtered through a glass suction funnel (G3) to separate the shell fragments. After thorough rinsing of all equipment, the solution was transferred into a 500.0-ml. volumetric flask. More water was used for rinsing and to reach the total volume of 500.0 ml. Samples of this solution were diluted at a ratio of 1 to 20 with aqueous pH 1.2 buffer (0.1 *M* hydrochloric acid, 0.1 *M* sodium chloride) and assayed spectrophotometrically[2] at 245 nm.

Particle-Size Analysis—Before analyzing the particle-size distributions, the microencapsulated phenacetin was put through a 0.63-mm. analytical sieve to separate coarse, agglomerated particles exceeding this size. The following method for the determination of particle size and particle-size distribution was developed. Microphotos of pure and microencapsulated drug were enlarged on photographic paper. The reproduced particles were counted and measured with a semiautomatic particle-size analyzer[3]. For each analysis, a minimum of 500–600 particles was evaluated from several microphotos. The particle-size distributions were calculated by means of a computer program (ALGOL).

Release—The release of drug from microcapsules was followed by a flow cell column method, similar to that described by Langenbucher (14) and Tingstad and Riegelman (15). Column height was 3.0 cm. and cross-sectional area was 3.14 cm.^{2}. The solvent used was 0.1 *M* hydrochloric acid, which was brought to an ionic strength of 0.2 by addition of sodium chloride. Solvent was pumped with a two-roller silicone tube pump through the flow cell. The quantity of the solvent and the flow rate were kept constant for all experiments (500.0 ml. of solvent; flow rate of 50 ml./min.). Before passing through the flow cell, the solvent was thermostated at 37 ± 0.5° by a heat exchanger. An amount of microcapsules, equivalent to a mass load of 65.0 mg. of total drug, was taken for all experiments. To analyze the phenacetin content of the solution, samples of 2.0 ml. were taken out of the solution reservoir after appropriate intervals. After diluting with the same solvent, the samples were analyzed spectrophotometrically at 245 nm. The maximum drug concentration in the solution did not exceed 10% of the saturation

[1] Elgasonic TS-100 ultrasonics generator, 25 kHz., Elgasonic SA, Biel, Switzerland.
[2] Zeiss PMQ II spectrophotometer.
[3] Zeiss TGZ 3 Analysator.

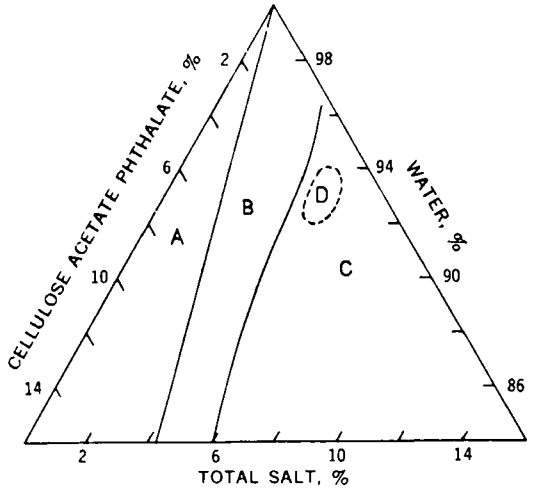


Figure 1—*Triangular phase diagram of the cellulose acetate phthalate coacervate system. Key: A, cellulose ester partially dissolved; B, clear solution of the cellulose ester; C, coacervate region (coacervate and equilibrium phase); and D, optimum encapsulation conditions. (Temperature 60°; pH 5.3-5.4; within field A, the salt consists of disodium hydrogen phosphate; within fields B, C, and D, the salt consists of mixtures of disodium hydrogen phosphate and sodium sulfate.)*

concentration of the drug, which guaranteed sufficiently good sink conditions.

RESULTS AND DISCUSSION

Triangular Phase Diagram of the Coacervate System—The isothermal triangular diagram of phase boundaries of the coacervate system of cellulose acetate phthalate, water, and salt is shown in Fig. 1. The salt phase consists of two components: disodium hydrogen phosphate and sodium sulfate as solvating agent and coacervating agent, respectively. The values on the total salt axis in Fig. 1 are the additive percentages of the individual salt species in the system. A procedure to calculate these individual concentrations from this graph is given. The combination of both salt species to one phase was necessary to reduce the actual four-component system to a three-component system which can be visualized graphically by a usual triangular phase diagram.

The phase diagram presented is divided into three fields: A, B, and C. In field A, the cellulose ester is only partially dissolved. At the boundary between fields A and B, the ratio between the amounts of disodium hydrogen phosphate and the cellulose ester is constant. At this ratio, *i.e.*, 0.37, the cellulose ester is completely dissolved. Within field A, the salt phase consists only of disodium hydrogen phosphate. The salt species additionally added to the system in fields B and C is sodium sulfate. The coacervation starts at the boundary separating fields B and C. Field C indicates the region of coacervate and equilibrium phases. The explanations demonstrate the recalculation procedure of the individual salt species in the system for any given point. Within field A, the salt consists of the phosphate only. Within fields B, C, and D, the point of intersection of the boundary A B and the line of a given constant concentration of the cellulose ester mark the phosphate concentration for that given cellulose ester concentration. For example, a coacervate mixture containing 6% cellulose ester and 86% water obviously contains 8% total salt. The line of 6% cellulose ester has an intersection point with the boundary A–B. This point indicates about 2% disodium hydrogen phosphate. Two percent phosphate subtracted from 8% total salt gives a 6% concentration of sodium sulfate for the mixture discussed.

Table I—Effect of Drug Amount Encapsulated on Drug Content of Microcapsules[a]

Amount of Drug, g.[b]	Drug Content of Microcapsules, %[c] Without Plasticizer	With Plasticizer (Glycerol)
7 I	88.5	82.0
7 II	88.5	81.5
10 I	91.7	85.5
10 II	92.5	84.7
13 I	93.2	88.5
13 II	93.2	88.5

[a] Encapsulation conditions: 60°, 100.0 g. 2% cellulose ester solution, 22.0 ml. 20% (w/w) sodium sulfate solution, and 500 r.p.m. [b] Each batch made twice (I and II). [c] Two determinations were run on I and II at each drug amount; the data presented are the means of the two determinations.

Table II—Effect of Drug Amount Encapsulated[a] on Deposition of Shell Material

Amount of Drug, g.[b]	Shell Material Deposited, g.[c] Cellulose Ester	Cellulose Ester and Glycerol
7 I	0.91	1.55
7 II	0.91	1.59
10 I	0.90	1.77
10 II	0.81	1.79
13 I	0.94	1.69
13 II	0.94	1.69
Means of all formulations	0.90	1.68
Mean of glycerol deposited	0.78	

[a] Encapsulation conditions: 60°, 100.0 g. 2% cellulose ester solution, 22.0 ml. 20% (w/w) sodium sulfate solution, and 500 r.p.m. [b] Each batch made twice (I and II). [c] Data calculated from corresponding data in Table I.

Microencapsulation—After addition of the sodium sulfate solution, part of the total cellulose ester available is coacervated, separating in the form of viscous liquid drops. These drops tend to deposit on the surface of phenacetin particles suspended in the solution. The subsequent cooling of the system causes a continually passing coacervation of further cellulose ester. During the cooling

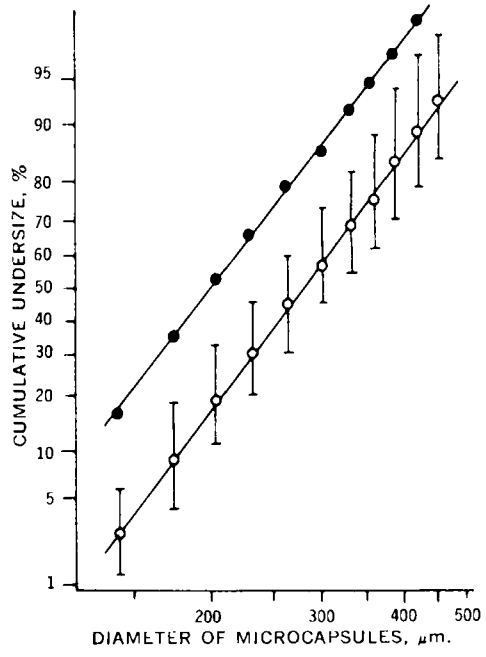


Figure 2—*Cumulative percent undersize plots of encapsulated and nonencapsulated phenacetin. Key: ●, nonencapsulated phenacetin; and ○, encapsulated phenacetin. [Encapsulation and coacervation conditions were constant; amounts of drug to encapsulate varied (7, 10, and 13 g.); batches made with and without glycerol treatment; batches made twice; points are means of all formulations; bars indicate range of values.]*

Table III—Effect of Drug Amount and Plasticizer Treatment on the Mean Cumulative Percentage Undersize Diameter of Microcapsules[a]

Amount of Drug, g.[b]	Mean Diameter of Microcapsules at 50% Cumulative Undersize, μm.[c] With Plasticizer (Glycerol)	Without Plasticizer
7 I	300	320
7 II	300	285
10 I	280	245
10 II	270	240
13 I	265	280
13 II	295	275

[a] Encapsulation conditions: 60°, 100.0 g. 2% cellulose ester solution, 22.0 ml. 20% (w/w) sodium sulfate solution, and 500 r.p.m. [b] Each batch made twice (I and II). [c] Data graphically determined from their individual logarithmic probability plots of the percent cumulative undersize distributions of all the different formulations.

process, the coacervate phase is gradually deposited on already formed walls of the cellulose ester coacervate.

Optimum encapsulation conditions were found at about 1–3% cellulose ester and 5% total salt concentration (D in Fig. 1). Higher concentrations of the cellulose ester (5–6%) produced filiform bulks of coacervate surrounding the core particles. A higher total salt concentration than 6% caused agglomeration of particles and deformation of the coacervate films still in the liquid state. The deposition of coacervate onto the particles (examined microscopically) was nearly complete at the optimum encapsulation conditions. The encapsulation technique was simple in the region of optimum coacervation and encapsulation. The agglomeration of particles and the deformation of the liquid coacervate films by the shearing forces of the stirring occurred only to a small extent.

For better utilization of the cellulose ester available in solution, it is desirable to work at higher sodium sulfate concentrations, which produce higher portions of coacervate. But this is not possible by simply increasing the sodium sulfate concentration owing to the increased agglomeration and deformation tendency at high salt concentrations. Therefore, a technique was developed for the continuous addition of sodium sulfate during the cooling of the system. Suitable cooling with addition of sodium sulfate produced thicker coacervate films than the coventional method. These films were found to be laminated, and the inner layers were heavily vacuolized. However, after washing and drying, no significant increase of the cellulose ester content of the microcapsules would be noticed.

Drug Content of Microcapsules—Table I shows the drug content of several formulations of microcapsules. The amounts of the cellulose ester available in solution and the coacervation conditions were kept constant, whereas the amount of drug to encapsulate and the use of glycerol as shell plasticizer were varied. The data show good reproducibility of the encapsulation process with regard to the drug content. When glycerol was used to plasticize the shells, it was incorporated in the shell structure and so decreased the drug percentage of the microcapsules as compared with the corresponding nonplasticized formulations. Table II shows the amounts of shell material deposited on the drug particles in the encapsulation process. The figures in this table were deduced from Table I using the following formula:

$$\text{shell material deposited} = \frac{\text{amount of drug encapsulated}\left(100\% - \text{percent drug content}\right)}{\text{percent drug content}} \quad \text{(Eq. 1)}$$

The mean amount of glycerol deposited was calculated from the difference of the arithmetic means of the shell material deposited in both procedures. Table II indicates that the amounts of the cellulose ester deposited on the phenacetin crystals and the glycerol uptake by the cellulose ester shells are almost constant. The data clearly show that the described encapsulation process is able to produce microcapsules of varying drug-to-shell ratios in a reproducible way. This is a fundamental requirement for controlling release rates by shell thickness. Other information about the encapsulation process is provided by the calculation of the utilization

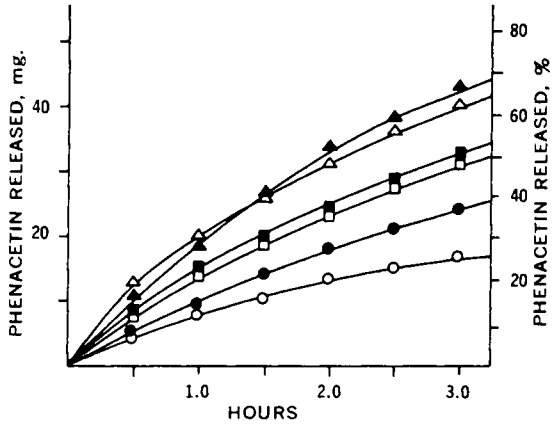


Figure 3—*Release plots of microencapsulated phenacetin, nonplasticized formulations. Key: ●, 7 g. I; ○, 7 g. II; □, 10 g. I; ■, 10 g. II; ▲, 13 g. I; and △, 13 g. II. [Temperature 60°; 100.0 g. 2% cellulose ester solution; 22.0 ml. 20% (w/w) sodium sulfate solution; 500 r.p.m.; each batch made twice (I and II); points on plot indicate mean of two determinations; amounts of drug varied.]*

factor of the cellulose acetate phthalate in the system used to prepare the microcapsules. Out of the 2.0 g. cellulose ester available in the system, about 0.9 g. was deposited on the drug crystals; therefore, the utilization factor of the ester was about 0.45.

Particle Size of Microcapsules—The amounts of coarse and agglomerated particles larger than 0.63 mm. in diameter, as determined by sieve analysis, never exceeded 3% of the total mass of the final encapsulated product. The mean geometric diameters of different formulations, which were graphically determined from the individually obtained logarithmic probability plots of the percent cumulative undersize curves, are shown in Table III. The data show small variations between the different formulations, which are, however, within the confidence limit of the determination method. It is, therefore, assumed that the amount of drug to encapsulate, as well as the treatment with glycerol as plasticizer, has no significant influence on the particle size. The mean logarithmic probability plot of percent cumulative undersize is shown in Fig. 2. The mean plot of all encapsulated formulations is transformed to a greater diameter than compared with the nonencapsulated phenacetin.

Drug Release—The formulations of microcapsules previously assayed for drug content were examined for drug release. The re-

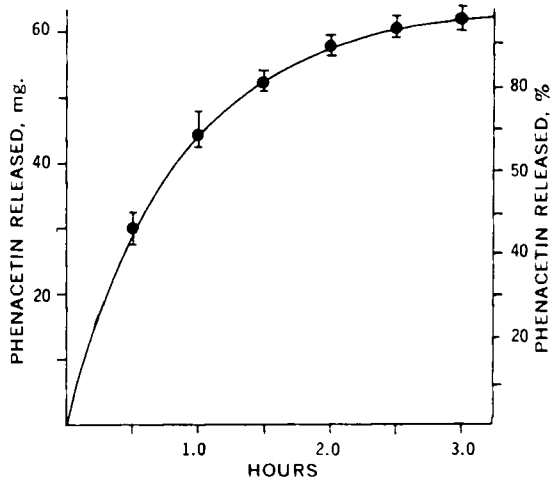


Figure 4—*Release plots of microencapsulated phenacetin, plasticized formulations. [Temperature 60°; 100.0 g. 2% cellulose ester solution; 22.0 ml. 20% (w/w) sodium sulfate solution; 500 r.p.m.; each batch made twice (I and II); amounts of drug varied (7, 10, and 13 g.); two determinations were run on I and II at each drug amount; points on plot are means of all determinations, batches, and drug amounts; bars indicate range of values obtained.]*

lease plots for nonplasticized formulations shown in Fig. 3 demonstrate a good correlation between the drug content of microcapsules and the release rate, *i.e.*, between the shell thickness and the release rate. The release of parallel batches with the same drug content can be considered as reproducible, because the noticeable differences between two parallel batches are inevitable due to individual technological variations. In contrast, all batches of plasticized capsules showed identical release curves in spite of different drug contents (Fig. 4).

To explain this obvious difference in the release mechanisms of plasticized and nonplasticized formulations, the individual steps of drug release from microcapsules are illustrated. In the first step, the film of cellulose ester takes up solvent by imbibition. In the second step, the solvent diffuses into the free space within the capsules. In the third step, the dissolution of drug takes place; and in the fourth, the dissolved drug is released by diffusion through the coacervate film, whereas further drug is continually dissolved. In the case of plasticized films, the porosity of these films is supposed to be so high that the dissolved drug can easily diffuse through the pores, which are formed by washing out glycerol, without any hindrance. Under this assumption, drug release depends only on the dissolution rate of the encapsulated phenacetin. In the case of pure, nonplasticized films of cellulose ester, the data show that the film thickness influences the release rates of a drug, indicating that diffusion of a drug through the shells is the controlling factor of release. Along with analogous results for ethylcellulose-coated microcapsules (16), it is indeed reasonable to assume that this is the only way release might occur for the capsules tested; leaching of the drug out of the microcapsules is, therefore, regarded to be negligible.

In contrast to the typical release curves of Nixon *et al.* (5) and Nixon and Walker (9), which show an extremely rapid initial release from gelatin coacervate microcapsules, the release of drug in the present studies was more regular and the release curves are smoother. This may be due to the more complete encapsulation of the core material. Furthermore, in contrast to the findings of these authors, the release of drug from microcapsules can be controlled significantly by the ratio of the amounts of drug to shell substance, *i.e.*, the technological formulation.

CONCLUSIONS

The results of this investigation show that cellulose acetate phthalate coacervate encapsulation is a more suitable encapsulation technique, less complicated and less difficult to control than other methods of coacervate encapsulation. The data demonstrate clearly that simple variations of the encapsulation process can significantly affect drug release rates. The importance of these accurately defined release rates on the therapeutic application of drugs is well known and has often been described. Owing to the broad variety of film-forming materials, which are not yet tested for this purpose, more research is needed in the field of preparation and evaluation of coacervate microcapsules.

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▲ To whom inquiries should be directed.

Adenosine Analogs: Structure–Activity Relationships in Vascular and Intestinal Smooth Muscle

S. W. LESLIE▲, J. L. BOROWITZ, and T. S. MIYA

Abstract □ The relaxant effects of a series of adenosine analogs were compared on isolated intestinal and aortic smooth muscle of the guinea pig. Blood pressure-lowering effects of these compounds were also compared in the guinea pig and dog. Analogs with a modified purine ring system (1-methyladenosine and toyocamycin) were more effective in relaxing intestinal than vascular smooth muscle. Substituents that decreased the basicity of the N_1-position or interfered with hydrogen bonding in the 6-position decreased activity in both vascular and intestinal smooth muscle. Deoxyadenosine, which lacks an oxygen in the 2′-position of the ribose portion of the molecule, was ineffective in intestinal but still relaxed vascular smooth muscle. It was concluded that intestinal smooth muscle interacts significantly with the 2′-position of the ribose portion of the adenosine molecule whereas vascular smooth muscle requires an intact purine ring system in the molecule for maximal activity.

Keyphrases □ Adenosine analogs, structure–activity relationships—relaxant effects on isolated ileal and aortic smooth muscle, blood pressure depression compared in guinea pig and dog □ Smooth muscle relaxation, isolated ileal and aortic muscle—effects of adenosine analogs, structure–activity relationships □ Relaxation of isolated ileal and aortic smooth muscle—effects of adenosine analogs, structure–activity relationships □ Blood pressure depression—adenosine analogs, guinea pig and dog □ Structure–activity relationships—adenosine analogs, smooth muscle relaxation and blood pressure depression

Adenosine is thought to be a breakdown product of the purinergic transmitter (1) and is found circulating in the blood in a concentration of 4×10^{-8} *M* (2). It is known to be released from intestinal (3), cardiac (4, 5), and adrenal (6) tissue. Adenosine relaxes bronchiolar (7), intestinal (8), and certain vascular (9) smooth muscle.

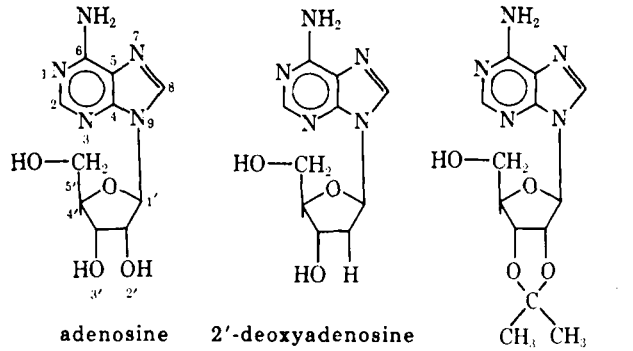
The interaction between adenosine and adenosine deaminase was studied at the molecular level (10), but the structure–activity relationships for adenosine's relaxant effects on intestinal and vascular smooth muscle are not fully known (11–14). Therefore, structural characteristics of a series of adenosine analogs important for relaxation of these smooth muscles were determined.

METHODS

A series of purine ribosides was tested *in vitro* on longitudinal smooth muscle of the guinea pig ileum and on circular smooth muscle of guinea pig descending thoracic aorta. *In vivo* blood pressure studies were also performed in guinea pigs and dogs to determine whether analogs that were inactive *in vitro* in the descending thoracic aorta were also inactive within the vascular bed as a whole.

Fasted (18–24 hr.) male guinea pigs (300–600 g.) were sacrificed by cervical dislocation, and a segment of ileum 20 cm. from the ileocecal junction was excised. Ileal strips 2–4 cm. in length were suspended in an oxygenated bath with Tyrode's solution maintained at 37°.

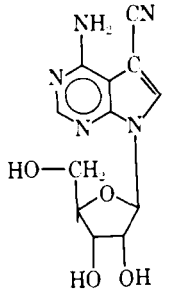
The descending thoracic aorta was also excised and prepared as described by Furchgott and Bhadrakom (15). The spirally cut segments were suspended in an oxygenated bath with the Krebs-Henseleit solution maintained at 37°.



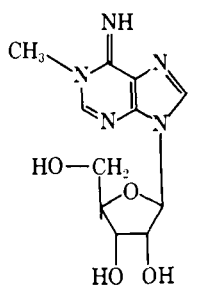
adenosine

2′-deoxyadenosine

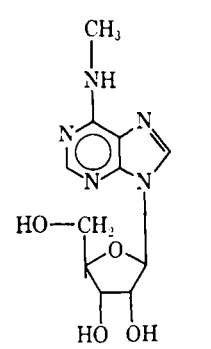
2′,3′-isopropylidene adenosine



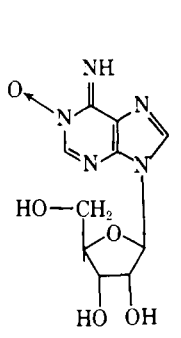
toyocamycin



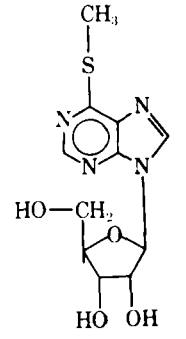
1-methyladenosine



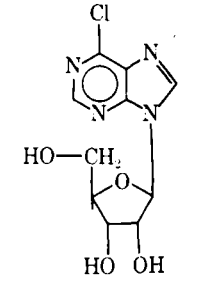
N-6-methyladenosine



adenosine *N*-oxide



6-methylmercapto-purine riboside



6-chloropurine riboside

One gram of tension was placed on both the longitudinal muscles of the ileum and the circular muscles of the aorta, and contraction was recorded[1]. The aortic strips were allowed to equilibrate in the bath for 2 hr. and the ileal strips for 1 hr.

Nicotine was used as the agonist in the ileum, and epinephrine was used as the agonist in the aorta. The concentrations of nicotine or epinephrine were the same for all experiments (ranging from 10^{-9} *M* to 3×10^{-6} *M* or from 10^{-7} *M* to 3×10^{-5} *M*, respectively). Drugs were pipeted directly into the baths and did not dilute the baths by more than 1%.

The purine ribosides used in this study were adenosine[2], 2′-deoxyadenosine, 6-methylmercaptopurine riboside, 2′,3′-isopro-

[1] Using a Grass force-displacement FTO 3C transducer on a model 5 polygraph.

[2] Sigma Chemical Co., St. Louis, Mo.

Table I—Inhibitory Effects of Adenosine and Its Analogs on Isolated Guinea Pig Ileum and Thoracic Aorta

Compound	Concentration, *M*	Tissue	Inhibition[a] of Contraction, %	*N*
Adenosine	10^{-5}	Ileum	49	6
Adenosine	10^{-5}	Aorta	30	6
N-6-Methyladenosine	10^{-5}	Ileum	35	5
N-6-Methyladenosine	10^{-5}	Aorta	39	5
Isopropylidene adenosine	5×10^{-5}	Ileum	43	6
Isopropylidene adenosine	5×10^{-5}	Aorta	0	5
Isopropylidene adenosine	10^{-4}	Aorta	53	5
Deoxyadenosine	10^{-4}	Ileum	0	5
Deoxyadenosine	10^{-4}	Aorta	59	7
1-Methyladenosine	10^{-4}	Ileum	43	8
1-Methyladenosine	10^{-4}	Aorta	0	4
Toyocamycin	10^{-5}	Ileum	34	6
Toyocamycin	10^{-6}	Aorta	0	6

[a] Percent inhibition produced by adenosine and adenosine analogs was calculated for concentrations of 1×10^{-6}, 3×10^{-6}, and 5×10^{-6} *M* nicotine in ileal tissue and for concentrations of 4×10^{-9}, 1.4×10^{-8}, 4.4×10^{-8}, and 1.4×10^{-7} *M* epinephrine in the aorta. The values obtained were then added and a mean percent depression was calculated.

pylidene adenosine, adenosine *N*-oxide[3], 1-methyladenosine, *N*-6-methyladenosine[4], and toyocamycin (a pyrrolo [2,3-*d*]pyrimidine nucleoside antibiotic)[5].

Male guinea pigs (300–600 g.) or healthy mongrel dogs (8–13 kg.) were anesthetized with pentobarbital (35 or 50 mg./kg., respectively) and carotid blood pressures were recorded. Drugs were injected *via* the external jugular vein in the guinea pig and *via* the femoral vein in the dog. A control series of five doses (25–400 mcg./kg.) of adenosine was first injected into each animal, followed by a series of doses of the adenosine analogs (25 mcg./kg.–5 mg./kg.). A test dose of adenosine (25 mcg./kg.) was injected after each series of doses of each analog to ensure that no potentiation or desensitization of the blood pressure response had occurred.

RESULTS

Effects of Adenosine Analogs on Isolated Ileum and Thoracic Aorta—A summary of the inhibitory effects of adenosine and adenosine analogs on isolated intestinal and arterial smooth muscle is presented in Table I. Adenosine and *N*-6-methyladenosine had inhibitory effects in both tissues (Table I). These compounds were previously reported to inhibit vascular smooth muscle contractions (14), and adenosine was reported to relax intestinal smooth muscle (8).

At a concentration of 1×10^{-4} *M*, isopropylidene adenosine significantly depressed contractions of the aorta in response to 1.4×10^{-7} *M* ($p < 0.001$) and 4.4×10^{-7} *M* ($p < 0.025$) epinephrine. At a lower concentration, 5×10^{-5} *M* isopropylidene adenosine depressed ($p < 0.05$) nicotine- (3×10^{-6} *M*) induced contractions in the ileum.

Deoxyadenosine depressed contractions in arterial tissue (Fig. 1) but not in the ileum at a concentration of 10^{-4} *M*. By contrast, 1-methyladenosine and toyocamycin (Figs. 2 and 3, respectively) depressed contractions in the ileum but not in isolated aorta at the concentrations used. 6-Chloropurine riboside, 6-methylmercaptopurine riboside, and adenosine *N*-oxide were each tested (10^{-4} *M*) in at least three ileal and three aortic preparations and were found to be ineffective in inhibiting the contractile responses.

Since toyocamycin and 1-methyladenosine appear to produce a rather selective relaxation of ileal contractions induced by an indirect-acting agonist (nicotine), further experiments were performed with these two analogs ($N = 6$ in each case) against acetylcholine, a direct-acting agonist. Results were similar to those with nicotine, although these analogs were somewhat less potent in depressing acetylcholine-induced contractions. Toyocamycin (1×10^{-4} *M*) produced a mean depression of 44%, while 1-methyladenosine (1×10^{-4} *M*) resulted in a 16% mean depression of acetylcholine-induced contractions (mean percent depression was calculated by averaging percent depressions produced by 1×10^{-7}, 3×10^{-7}, 1×10^{-6}, and 3×10^{-6} *M* acetylcholine).

Concentration–response curves of the guinea pig ileum to nicotine were consistently reproducible. However, a decrease in sensitivity of the aorta to epinephrine was seen, as previously reported (15). No further desensitization was noted after the first concentration–response curve (four experiments). Therefore, in the guinea pig ileum, the responses obtained in the presence of the inhibitors were compared statistically with the mean value of the control and recovery responses, while in the aorta the responses obtained in the presence of the inhibitors were compared statistically only with the recovery responses.

Effects of Adenosine Analogs on Guinea Pig and Dog Blood Pressure—Adenosine (Fig. 4) was found to produce a transient blood pressure depression in the guinea pig, resulting in a 27-mm. Hg decrease after the highest dose of 400 mcg./kg. Depressor responses were generally short lived, and pressure returned to control levels in about 30 sec. The effect was more prolonged (about 3 min.) when doses of 1 mg. or more of the analogs were given. *N*-6-Methyladenosine (Fig. 4) also depressed guinea pig blood pressure but was only one-fourth to one-half as potent as adenosine.

Although 1-methyladenosine was approximately 10 times less

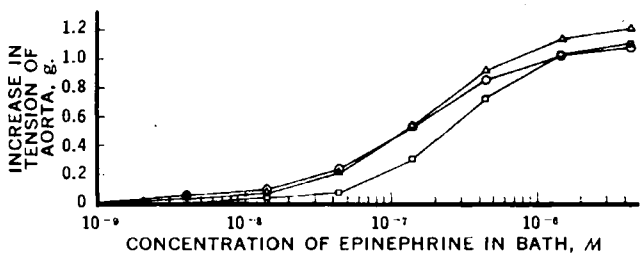


Figure 1—*Effect of deoxyadenosine (1×10^{-4} M) on epinephrine-induced contractions in the guinea pig descending thoracic aorta. Each point represents the mean response of seven aortic strips. Deoxyadenosine significantly depressed the contractile response to 1.4×10^{-7} M ($p < 0.01$) and 4.4×10^{-7} M ($p < 0.01$) epinephrine. The paired difference form of the Student t test was used to compare the recovery response with the responses obtained in the presence of the adenosine analog in all experiments involving the aorta. Key: ○, control; □, deoxyadenosine, 1×10^{-4} M; and △, recovery.*

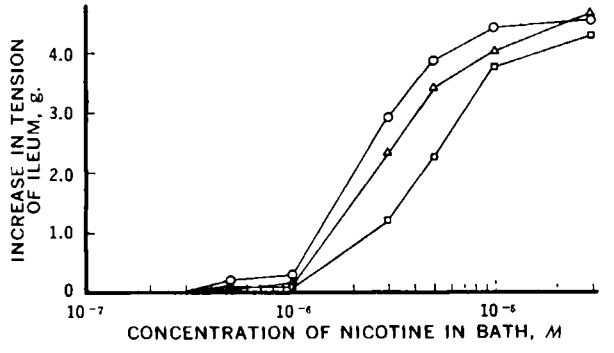


Figure 2—*Effect of 1-methyladenosine (1×10^{-4} M) on nicotine-induced contractions in the guinea pig ileum. Each point represents the mean response of eight ilea. 1-Methyladenosine significantly depressed the contractile response to 3×10^{-6} M ($p < 0.025$) and 5×10^{-6} M ($p < 0.05$) nicotine. The paired difference form of the Student t test was used to compare the mean of control and recovery responses with the responses obtained in the presence of the adenosine analog in all experiments involving the ileum. Key: ○, control; □, 1-methyladenosine, 1×10^{-4} M; and △, recovery.*

[3] Nutritional Biochemicals Corp., Cleveland, Ohio.
[4] Terra-Marine Bioresearch, La Jolla, Calif.
[5] Obtained from Dr. Suhadolnik, Albert Einstein Medical Center, Philadelphia, Pa.

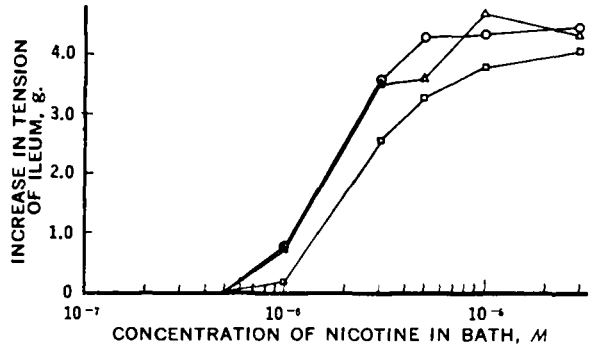


Figure 3—*Effect of toyocamycin (1×10^{-5} M) on nicotine-induced contractions in the guinea pig ileum. Each point represents the mean response of six ilea. Toyocamycin significantly depressed the contractile response to 1×10^{-6} M ($p < 0.01$) and 3×10^{-6} M ($p < 0.005$) nicotine. Key: ○, control; □ toyocamycin, 1×10^{-5} M; and △, recovery.*

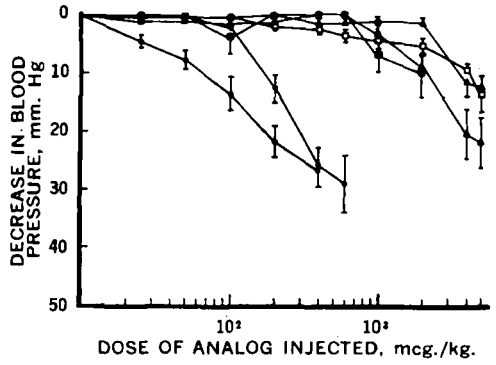


Figure 4—*Effect of adenosine and adenosine analogs on the diastolic blood pressure in the guinea pig. Each point represents the mean response ± SE in from five to seven male guinea pigs. Mean control blood pressure was 42.3 mm. Hg, which is comparable to reported values for the guinea pig (16). Key: ●, adenosine; ○, N-6-methyladenosine; □, deoxyadenosine; △, isopropylidene adenosine; ■, toyocamycin; and ▲, 1-methyladenosine.*

potent than adenosine in relaxing ileal smooth muscle, it was 25–50 times less potent in depressing blood pressure. Thus, 1-methyladenosine was about four times more potent in ileal smooth muscle than in vascular smooth muscle.

Toyocamycin, at a dose of 1×10^{-5} *M*, was shown to be approximately equal to adenosine in inhibiting contractions of smooth muscle of the guinea pig ileum but was not effective in relaxing the descending thoracic aorta nor in depressing guinea pig blood pressure unless a large dose (1 mg./kg.) was used. The effect of toyocamycin in ileal smooth muscle was about 50 times that in vascular smooth muscle. This analog was peculiar in that, at doses of 500 mcg./kg. or greater, it markedly potentiated the vasodepressor effects of adenosine in the guinea pig. No other analog produced this effect.

Adenosine and each adenosine analog were also tested on the blood pressure of two to five mongrel dogs. The vasodepressor action of these compounds was similar relative to one another in this species compared to the activity in the guinea pig. However, toyocamycin did not potentiate the vasodepressor response to adenosine in the dog.

DISCUSSION

Molecular changes that increase the electron density and thus the basicity of the N_1-position of adenosine are known to increase its affinity for adenosine deaminase (10). This study shows that a certain degree of basicity of the N_1-position is also important for adenosine's interaction with smooth muscle. Addition of an oxygen to the N_1-position of adenosine to form the *N*-oxide decreases the basicity of this area and results in loss of activity in both vascular and ileal smooth muscle.

The aromaticity of the purine ring system appears to influence the selectivity of action of adenosine analogs on smooth muscle. Replacing the nitrogen in the 7-position of the purine riboside with a cyano-carbon group, as in toyocamycin, alters electronic and steric factors which affect the electron distribution of the whole purine ring. Toyocamycin is approximately 50 times more active in intestinal muscle relative to arterial smooth muscle. Addition of a methyl group to the N_1-position of adenosine also tends to disrupt the aromaticity of the ring system and again results in a more selective activity on intestinal musculature. This methyl group also increases the basicity of the N_1-position, which may explain the increased activity remaining in vascular smooth muscle as compared to toyocamycin and reinforces the assumption that basicity of this area of the purine ring is important for activity in both tissues. Apparently, the usual purine ring system is not rigidly required for relaxation of intestinal smooth muscle by adenosine analogs, but basicity of the N_1-position is required in both tissues.

It was suggested (10) that hydrogen bonding occurs between adenosine and adenosine deaminase before removal of the amino group in the 6-position is completed. If a similar hydrogen bonding is required at the adenosine receptor to produce smooth muscle relaxation, replacement of the 6-amino group with moieties that modify hydrogen bonding should reduce or eliminate activity. Addition of a methyl group to the 6-amino group decreases the hydrogen-bonding capabilities and/or increases steric hindrance and, as shown in this study, results in a general reduction of activity. Replacement of the 6-amino group with a methylmercapto or chloride group results in elimination of hydrogen-bonding capabilities and, as shown in this study, also eliminates the effect on smooth muscle. Thus, hydrogen bonding through the *N*-6-position of adenosine may be important for the adenosine effect.

This study also showed that an intact ribose moiety is essential for complete activity of adenosine. The 2′-hydroxyl group seems to play an important role in the activity of the compound. The 2′-hydroxyl and 3′-hydroxyl groups contain electrons which are available for hydrogen bonding. If hydrogen bonding from the 2′-hydroxyl group to a site on the receptor is required for activity, it would be expected that removal of the 2′-hydroxyl group would result in loss of effectiveness. Studies with deoxyadenosine showed that a decrease in activity did in fact occur. It appears, however, that this hydrogen bonding may be more necessary in ileal smooth muscle than in vascular smooth muscle since some activity remained in the latter tissue after removal of the 2′-hydroxyl group.

Attachment of the 2′,3′-isopropylidene radical would be expected to decrease the proton-accepting power of the oxygen atoms involved. Thus, hydrogen bonding with the ribose part of the molecule is reduced but not eliminated in the isopropylidene derivative of adenosine. In this case, a relative reduction of the relaxant effect was obtained in both tissues, which suggests that some hydrogen-bonding capability of the 2′,3′-positions of the ribose moiety of adenosine is required for activity.

This study showed that the basicity of the N_1-position and hydrogen-bonding capabilities of the N_6-position of adenosine are important for activity in both intestinal and vascular smooth muscle. An intact purine ring system favors activity in vascular smooth muscle but is not required for maximal activity in intestinal smooth muscle. Hydrogen-bonding capabilities of the 2′-hydroxyl group are more important for relaxation of vascular than intestinal smooth muscle.

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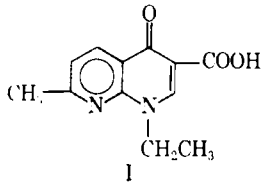
Keyphrases □ Nalidixic and hydroxynalidixic acids—analysis in human plasma and urine, high pressure liquid chromatography □ Hydroxynalidixic and nalidixic acids—analysis in human plasma and urine, high pressure liquid chromatography □ High pressure liquid chromatography—analysis, nalidixic and hydroxynalidixic acids in human plasma and urine

Nalidixic acid[1] (I) is currently used as an antibacterial agent in urinary tract infections (1–3). A major metabolite is hydroxynalidixic acid (II). This metabolite has an *in vitro* antibacterial spectrum similar to that of nalidixic acid (4). Both compounds have similar chemical structures and fluorescent spectra.

The original fluorescent method for the assay of nalidixic acid and hydroxynalidixic acid (4) involved differential extraction, which provided only partial separation of nalidixic acid and hydroxynalidixic acid. Thus, simultaneous equations were employed for the determination of the two drugs. Furthermore, blank values were occasionally high and erratic, giving unsatisfactory values for nalidixic acid.



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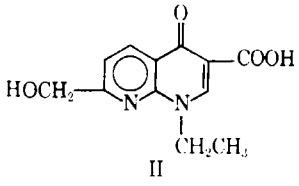

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EXPERIMENTAL

Materials—Nalidixic acid[2] and hydroxynalidixic acid[2] were routinely prepared in the same standard solution of 0.03 *N* NaOH. Chloroform, analytical reagent grade[3], was used for all extractions. All other reagents were purchased from commercial sources.

Plasma Assay—To a 15-ml. glass-stoppered centrifuge tube were added 1.0 ml. of plasma[4] and 1.0 ml. of a standard solution containing both nalidixic acid and hydroxynalidixic acid in concentrations ranging from 0 to 40 mcg./ml. For unknown plasma samples, 1.0 ml. of sample was added to 1.0 ml. of 0.03 *N* NaOH. To this mixture was added 0.5 ml. of 0.6 *N* HCl (final pH approximately 1.0) and 5 ml. of chloroform. The tubes were stoppered, shaken 5 min., and centrifuged. The upper aqueous phase was then aspirated. A 4.0-ml. aliquot of the chloroform phase was transferred to a clean 15-ml. glass-stoppered centrifuge tube, and 1.0 ml. of 0.03 *N* NaOH was added. The tubes were shaken for 5 min. and centrifuged. The aqueous phase containing nalidixic acid and hydroxynalidixic acid was then subjected to high pressure liquid chromatography.

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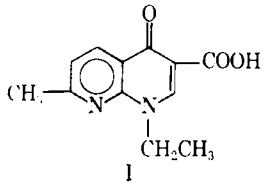

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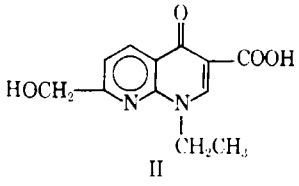

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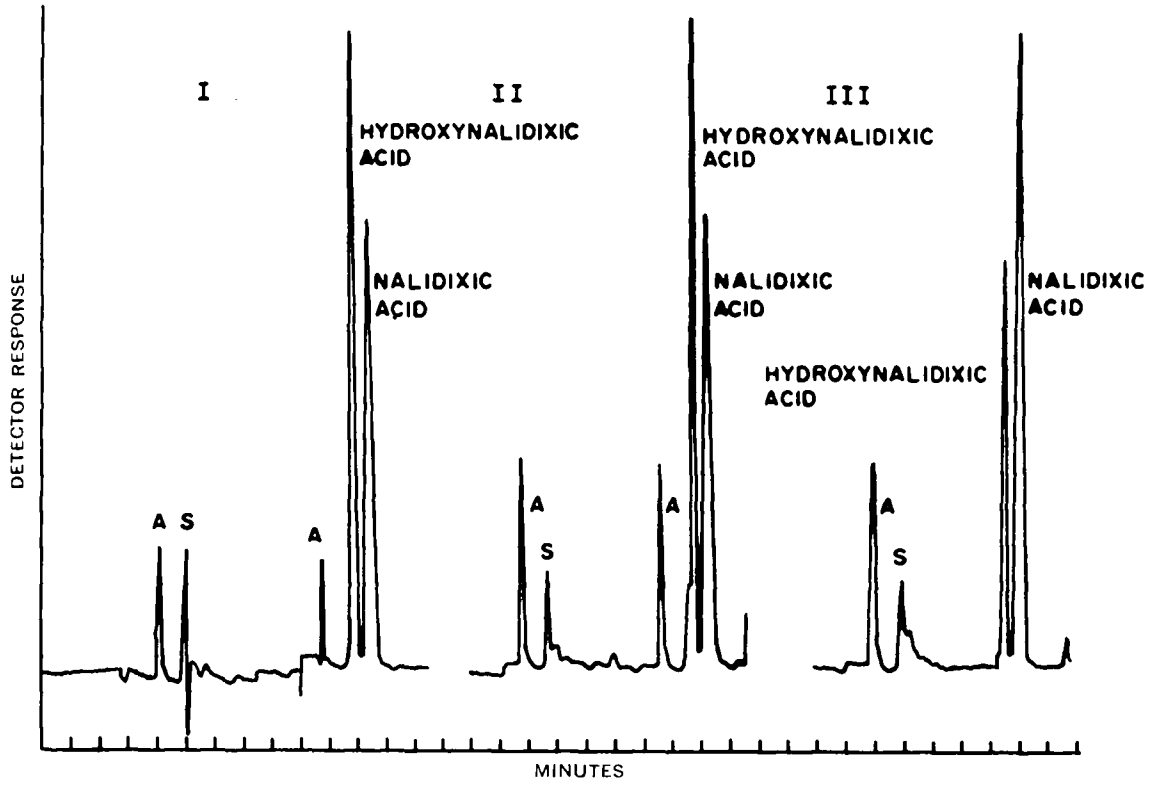

Figure 1—*Liquid chromatograms of processed standards of nalidixic acid and hydroxynalidixic acid in plasma and urine. Key: I, plasma blank (attenuation 0.02), and plasma containing 10 mcg./ml. each of nalidixic acid and hydroxynalidixic acid (attenuation 0.02); II, nonhydrolyzed urine blank (attenuation 0.02), and nonhydrolyzed urine containing 25 mcg./ml. each of nalidixic acid and hydroxynalidixic acid; III, hydrolyzed urine blank (attenuation 0.02), and hydrolyzed urine (attenuation 0.16) containing 280 mcg./ml. nalidixic acid and 125 mcg./ml. hydroxynalidixic acid; A, sample injection; and S, solvent front. Liquid chromatographic conditions are described in the* Experimental *section.*

ynalidixic acid were hydrolyzed by adding 0.5 ml. of 0.6 *N* HCl (final pH approximately 1.0) and heating the extraction tube on a steam bath for 30 min. The mixture was then neutralized with 0.5 ml. of 0.6 *N* NaOH. To the extraction tubes containing nonhydrolyzed urine samples was added 0.5 ml. of 0.6 *N* NaOH to keep the ionic strength and pH constant.

To both the hydrolyzed and nonhydrolyzed urine mixtures were added 1 ml. of a 0.2 *N* sodium phosphate buffer, pH 6.0, and 1.0 ml. of water. Nalidixic acid and hydroxynalidixic acid were extracted into 25 ml. of chloroform. The tubes were shaken for 5 min., followed by centrifugation. The aqueous upper phase was aspirated, and a 4.0-ml. aliquot of the lower chloroform phase was placed into a clean 15-ml. glass-stoppered centrifuge tube containing 1.0 ml. of 0.03 *N* NaOH. The tube was shaken for 5 min. and centrifuged. Then the aqueous phase containing nalidixic acid and hydroxynalidixic acid was subjected to high pressure liquid chromatography.

Liquid Chromatography[5]—Solutions were chromatographed at room temperature on a 0.5-m. stainless steel column (2.1 mm. i.d.) packed with Zipax SAX[6] strong anion-exchange resin. The eluting mobile phase was a solution containing 0.05 *M* sodium sulfate and 0.02 *M* boric acid (pH 9). Column pressure was maintained at 600 p.s.i. by compressed nitrogen gas. The flow rate of the eluate was 0.8 ml./min.

A 10-μl. aliquot of the extracted urine or plasma sample was chromatographed by a modified stop-flow procedure[7]. Direct standards of nalidixic acid and hydroxynalidixic acid were made in 0.03 *N* NaOH and chromatographed similarly. Recovery of nalidixic acid and hydroxynalidixic acid was calculated by comparing the peak heights of the processed standards to standards made directly in base. Concentrations of the unknown urine or plasma samples were calculated by comparing the peak heights of the extracted samples to processed standards.

Correlation with Microbiological Assay—Two tablet formulations containing 1000 mg. of nalidixic acid[8], but differing in tablet excipients, were administered orally to 16 adults of both sexes. The drug was administered with water after an 8-hr. fast. A premedication urine sample and a 0–24-hr. postmedication urine sample were obtained. After measurement of the volume and pH, the urine samples were frozen until analyzed for free and conjugated nalidixic and hydroxynalidixic acids by the liquid chromatographic assay. Only nonconjugated, biologically active nalidixic acid and hydroxynalidixic acid were analyzed by the microbiological assay.

Microbiological Assay—Urinary levels of nalidixic and hydroxynalidixic acids were determined by a modified, four-point, agar-diffusion method (5). Nalidixic acid standards were prepared in pooled, normal human urine to give final concentrations of 10 and 20 mcg./ml. Urine samples from medicated subjects were diluted appropriately to give estimated concentrations corresponding to those of the standards. By means of forceps, 1.3-cm. (0.5-in.) filter paper disks were saturated by immersion into the standard or sample solutions and excess fluid was removed. Saturated disks from the standard and sample solutions were placed on the surface of each agar plate seeded with the test organism, *Klebsiella pneumoniae* (ATCC 10872). Five plates containing standard and sample disks at constant dilution intervals were employed for each assay.

Following a prediffusion period of 2 hr. at 4°, all plates were incubated at 37° for 16–18 hr. and the zones of inhibition (diameters in millimeters) were recorded. The levels of biologically active drug (nalidixic acid and hydroxynalidixic acid) in the samples were calculated by extrapolation from the standard dose responses (6).

[5] Performed on a duPont model 840 liquid chromatograph equipped with a UV (254 nm.) photometric detector and a Hewlett-Packard model 7128A strip-chart recorder.

[6] Instrument Product Division, E. I. duPont de Nemours & Co., Wilmington, Del.

[7] The modified stop-flow procedure is as follows. The liquid chromatograph is maintained at 600 p.s.i. The valve is turned to the "Flow Off" position. After 30 sec., 10 μl. of the sample is injected on the column. The valve is then placed on the "Flow On" position and the sample is chromatographed. This method provided sharper peaks, possibly due to less diffusion of the drug prior to being chromatographed through the column and less "blowback" of sample from the syringe.

[8] NegGram Caplet, Winthrop Laboratories, New York, N. Y.

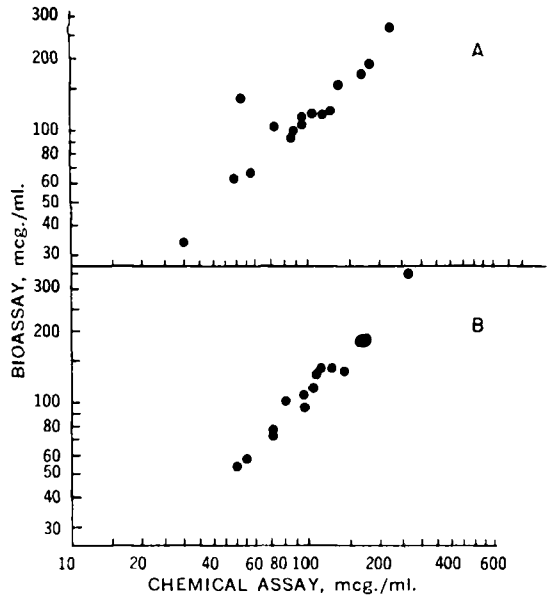

Figure 2—*Correlation of the liquid chromatographic and microbiological assays for nalidixic acid and hydroxynalidixic acid in urine. Human volunteers were administered 1000 mg. of nalidixic acid contained in tablet Formulation A or Formulation B. Nalidixic acid and hydroxynalidixic acid in the 24-hr. urine samples were assayed by both methods. Correlation coefficients for Formulations A and B were 0.83 and 0.94, respectively.*

RESULTS

Nalidixic acid and hydroxynalidixic acid were measured simultaneously by liquid chromatography. The retention times for hydroxynalidixic acid and nalidixic acid were 1.2 and 1.6 min., respectively. Chromatograms from control plasma and control urine yielded a small blank which did not interfere with the chromatograms from processed standards (Fig. 1). Plasma extracts demonstrated an "on-column" lower detection limit of 2 ng. each of nalidixic acid and hydroxynalidixic acid, corresponding to a plasma concentration of 0.25 mcg./ml. for each drug. Urine extracts demonstrated an on-column lower detection limit of 4 ng. each of nalidixic acid and hydroxynalidixic acid, corresponding to a urine concentration of 2.5 mcg./ml. for each drug. The sensitivity of this assay was comparable to that of the fluorescent method (4). Processed standards in plasma and urine were linear in concentrations up to 250 mcg./ml. One advantage of this method is that high urine levels need not be reprocessed; the sensitivity of the instrument is attenuated upward and the sample is reinjected.

Recoveries of processed standards of nalidixic acid from plasma and urine were 99.2 and 101%, respectively; the recoveries of processed standards of hydroxynalidixic acid were 91.0 and 88.8%, respectively (Table I). The lower recovery of hydroxynalidixic acid is possibly due to partitioning between the aqueous and chloroform phases. The small standard errors shown for the recovery data in Table I demonstrate that these compounds can be measured with high precision.

Urine from patients medicated with two different oral tablet formulations of nalidixic acid were subjected to both the microbiological and liquid chromatographic assays (Fig. 2). The mean ($\pm SE$) urine levels of nalidixic acid and hydroxynalidixic acid after administration of Formulation A were 121 ± 55.1 mcg./ml. (biological) and 109 ± 53.2 mcg./ml. (liquid chromatographic). The mean ratio of biological to liquid chromatographic values was 1.20. Only one major discrepancy was noted between the two assays (ratio 2.50). The mean ($\pm SE$) urine levels of nalidixic acid and hydroxynalidixic acid after administration of Formulation B were 129 ± 71.9 mcg./ml. (biological) and 110 ± 55.6 mcg./ml. (liquid chromatographic). The mean ratio of biological to chemical values was 1.04. Correlation coefficients for Formulations A and B were 0.83 and 0.94, respectively.

Table I—Recovery of Nalidixic Acid and Hydroxynalidixic Acid from Plasma and Urine[a]

Concentration, mcg./ml.	Percent Recovery	
	Nalidixic Acid	Hydroxynalidixic Acid
	Plasma	
1.0	98.9	76.9
2.5	95.0	91.1
5.0	101	94.4
10	102	93.8
20	98.9	94.2
30	99.4	94.5
Mean ± *SE*	99.2 ± 1.0	91.0 ± 3.1
	Urine	
12.3	101	89.8
25	101	89.7
50	100	87.7
100	99.7	87.2
150	104	89.5
Mean ± *SE*	101 ± 0.6	88.8 ± 0.5

[a] Standards were made in 0.03 *N* NaOH. Dilutions were added to human urine or plasma and processed according to the procedure described in the *Experimental* section.

DISCUSSION

The advantages of the liquid chromatographic assay of nalidixic acid and hydroxynalidixic acid include: (*a*) simple, rapid, and reproducible analyses of a multicomponent system without the need for an involved separation and purification method; and (*b*) more precision and specificity than the fluorescent method used previously. Moreover, 30–40 samples/day (8 hr.) can be determined by a single technician compared to 6–8 samples/day by the fluorescent method.

Microbiological assays of antibacterial agents such as nalidixic acid and hydroxynalidixic acid have been used frequently in the clinic. Although the bioassay is not specific (both nalidixic acid and hydroxynalidixic acid have similar antibacterial activity), the high degree of correlation between these methods demonstrates that either assay can be used routinely in the clinical laboratory.

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Quantitative Effect of Topically Applied Anti-Inflammatory Agents on External Ocular Inflammation in Rats

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Abstract □ A new quantitative approach to investigate the effect of topically applied anti-inflammatory agents is proposed. An external ocular inflammation was produced in the rat by topical instillation of ocular irritants. The method requires a short time and is based upon quantitatively determining the degree of edema by weighing the eyelid. This end-point can be evaluated objectively, in contrast to the subjective results obtained by many widely used methods. Hydrocortisone, triamcinolone acetonide, triamcinolone, fluocinolone acetonide, and dexamethasone were tested. Dose-response relationships were obtained, and relative potencies were determined by parallel-line assay, using hydrocortisone as a reference standard. Fluocinolone acetonide, the most potent steroid studied, had a topical relative potency of 2745; triamcinolone, the least potent, had a topical relative potency of 4.9. The systemic relative potency was also investigated. The results obtained show that the experimental model of external ocular inflammation appears to be a reliable method for quantitative assay of topical anti-inflammatory agents.

Keyphrases □ Anti-inflammatory steroids, topical—quantitation of relative effects using external ocular inflammation technique in rats □ Ocular inflammation technique—used to quantitate effects of topical anti-inflammatory steroids, rats □ Steroids, anti-inflammatory—quantitation of relative effects using external ocular inflammation technique in rats

Several methods have been reported for provoking ocular inflammation to study the effect of anti-inflammatory substances. The most frequently used procedures are the lamellar graft technique, consisting of the insertion of a pig corneal button intralamellarly into the rabbit cornea (1); the immuno uveitis model (2–4); and methods based upon the topical instillation (5, 6) or injection (7) of ocular irritants. Opacity and vascularization of the cornea, edema, and hyperemia of the conjunctiva and iris and exudation in the anterior chamber are the parameters used for determining the degree of ocular inflammation produced. However, scoring is subjective and arbitrary due to the lack of criteria that can be evaluated objectively. Furthermore, some methods are too time consuming for routine screening.

Therefore, the development of a method to produce temporary ocular inflammation in the rat having an end-point that could be evaluated objectively was desirable. In these experiments, the quantitative determination of the weight of the inflamed eyelid was the end-point. By using this method, dose-response rela-

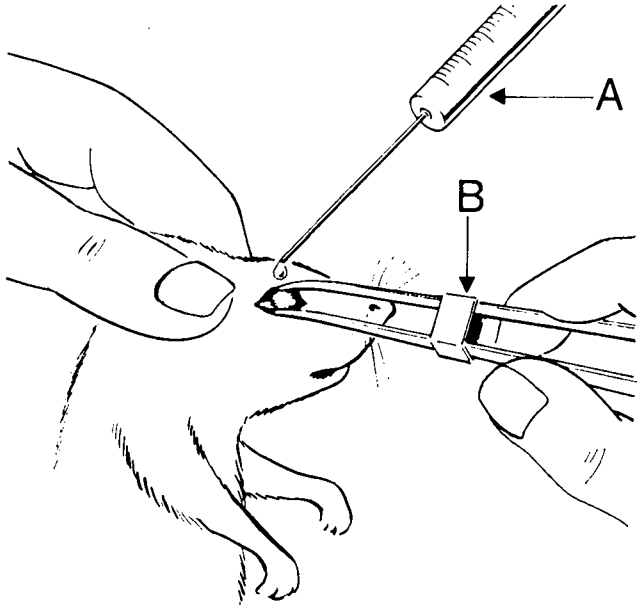

Figure 1—*Technique for instilling substances into the eye of the rat. Key: A, 25-μl. syringe clamped at a 45° angle relative to the horizontal plane; and B, arrest of the curved forceps to allow a maximum spread of 5 mm. at the tip.*

Table I—Effect of Topical Administration of Corticoids on Rat Palpebral Edema

Experiment	Compound	Total Dose, mcg./Animal	Eyelid Weight, mg. (Mean ± *SE*)[a]	Deviation from Control Value, %
A	Control	—	44.61 ± 1.79	
	Hydrocortisone	73.2	38.33 ± 1.88[b]	−14
		131.8	36.65 ± 2.30[c]	−18
		237.2	32.16 ± 1.18[c]	−28
		427	27.44 ± 0.95[c]	−38
	Dexamethasone	0.83	37.83 ± 1.98[b]	−15
		1.5	35.88 ± 2.11[c]	−20
		2.7	29.61 ± 1.18[c]	−34
B	Control	—	49.12 ± 3.99	
	Hydrocortisone	73.2	39.78 ± 1.77[c]	−19
		131.8	34.37 ± 1.13[c]	−30
		237.2	31.66 ± 2.17[c]	−35
		427	24.94 ± 0.68[c]	−49
	Triamcinolone	30	34.26 ± 1.54[c]	−30
		60	27.35 ± 1.39[c]	−44
		120	24.92 ± 1.37[c]	−49
C	Control	—	45.90 ± 2.37	
	Hydrocortisone	73.2	39.11 ± 2.56[b]	−15
		131.8	37.40 ± 1.82[c]	−18
		237.2	33.51 ± 2.14[c]	−27
		427	24.51 ± 0.61[c]	−47
	Fluocinolone acetonide	0.034	34.90 ± 2.28[c]	−24
		0.067	34.15 ± 1.61[c]	−26
		0.135	29.48 ± 1.70[c]	−36
		0.27	26.16 ± 1.16[c]	−43
D	Control	—	44.04 ± 2.73	
	Hydrocortisone	73.2	37.34 ± 2.43[b]	−15
		131.8	33.35 ± 1.39[c]	−24
		237.2	29.31 ± 1.41[c]	−33
		427	25.54 ± 0.99[c]	−42
	Triamcinolone acetonide	0.150	37.15 ± 1.68[b]	−16
		0.312	35.93 ± 2.42[c]	−18
		0.625	34.40 ± 1.29[c]	−22
		1.25	29.52 ± 1.34[c]	−33
		2.5	29.34 ± 1.33[c]	−33

[a] Nine rats per group. [b] $p < 0.05$ when compared with the control. [c] $p < 0.01$ when compared with the control.

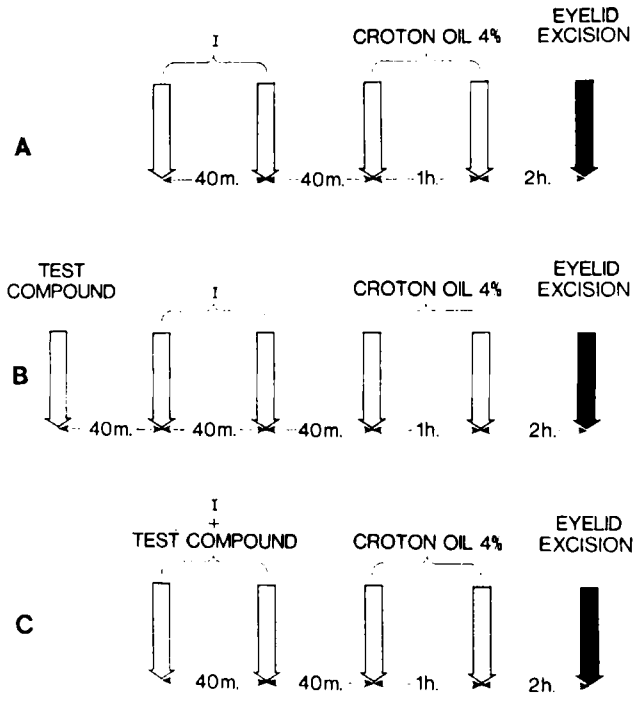

Figure 2—*Treatment schedule for producing ocular inflammation and for testing the effect of anti-inflammatory steroids administered topically or systemically. (A) Production of ocular inflammation. Two doses of 2.5 μl. each of I were instilled into the right eye 40 min. apart, followed 40 min. later by two doses of 4% croton oil in I 60 min. apart. Two hours after the last dose of croton oil, the eyelid was excised. (B) Test of anti-inflammatory potency by systemic administration of the test compound prior to producing inflammation. (C) Test of anti-inflammatory activity by topical instillation of the test compound into the right eye. Note that the test compound is dissolved in I and administered as in A.*

tionships and the relative potency of several anti-inflammatory substances were determined after topical and systemic administration.

MATERIALS AND METHODS

Experimental Animals—Female Sprague Dawley rats, weighing 60–70 g., were randomly divided into groups of nine or 10 animals. The animals were fed standard rat chow and water *ad libitum* up to sacrifice.

Induction of Palpebral Edema—A combination of two irritants, 2-(2-ethoxyethoxy)ethanol[1] (I) and croton oil, was selected to produce a satisfactory external ocular inflammation. Both substances were instilled in the conjunctival sac of the right eye of the rat. 2-(2-Ethoxyethoxy)ethanol was administered as such, while croton oil was administered as a 4% solution in 2-(2-ethoxyethoxy)-ethanol.

Figure 1 illustrates the handling of the animal for drug instillation. Particular care was taken to prevent mechanical damage to the ocular tissue by the needle; a 25-μl. syringe[2] was fixed in a clamp at about 45° to the horizontal plane to prevent spontaneous leakage of the drop of solution. The eyelids were spread apart with curved forceps which were prevented from opening more than 5 mm. at the tips. The rat was then delicately moved toward the syringe until a drop was applied to the center of the cornea, from where it spread onto the entire conjunctival sac. The lids were kept apart for 2–4 sec. to assure adequate diffusion of the substance in the conjunctival sac. The volume of instillation was 2.5 μl.

External ocular inflammation was obtained by instilling two doses of I followed by two doses of croton oil (Fig. 2A). The steroids used for the study of anti-inflammatory activity were administered both systemically and topically. For systemic administration, the drugs were given orally by stomach tube in 1 ml./100 g. body weight of an aqueous vehicle containing 0.9% sodium chloride, 0.5% sodium carboxymethylcellulose, 0.4% polysorbate 80, and 0.9% benzyl alcohol. The treatment schedule is shown in Fig. 2B. For topical administration, the compounds were dissolved in I, which is an optimal solvent for steroidal substances (8), and administered by instillation into the conjunctival sac in two doses of 2.5 μl. each. The treatment schedule is shown in Fig. 2C.

The effect of the irritants and the anti-inflammatory activity of the products tested were evaluated by isolating and weighing the inflamed upper eyelid. Two hours after the last administration (Fig. 2), the animal was killed by cervical fracture and the upper eyelid was isolated, using straight and sharply honed scissors (Fig. 3). After making an incision in the skin of the cranium, the skin was peeled away toward the eyelids (Fig. 3A). When arriving at the palpebra, the skin was cut along the palpebral edge (Fig. 3B). The

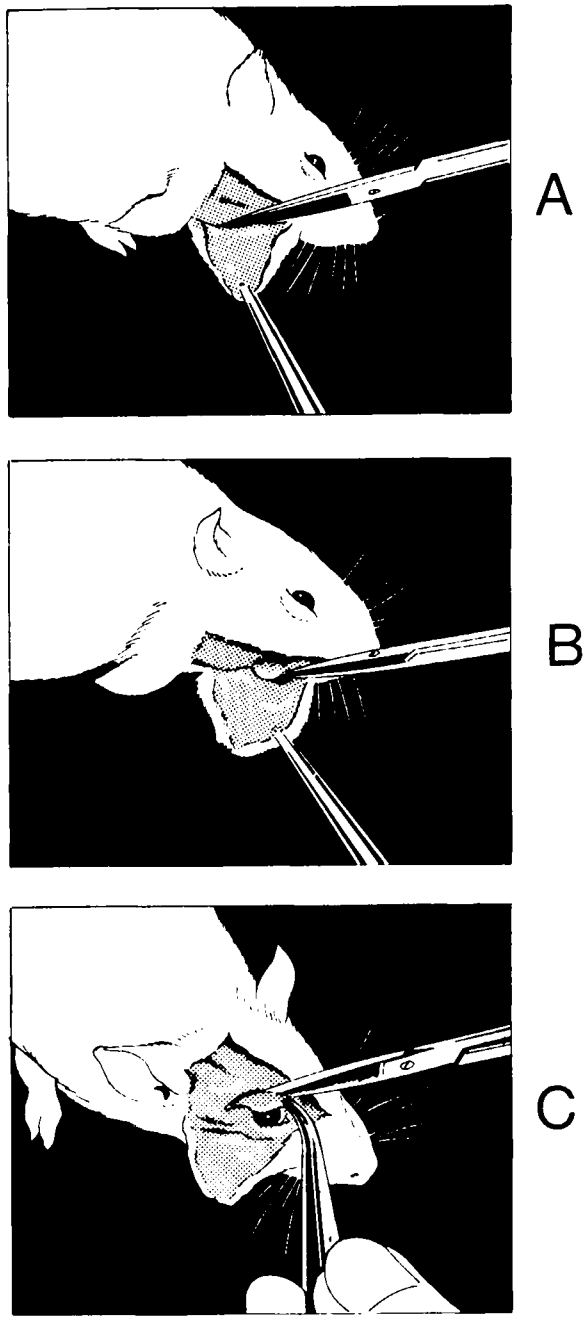

Figure 3—*Procedure for isolation of the rat eyelid. Key: A, peeling off of the cranial skin toward the eyelid; B, cutting along the palpebral edge; and C, removal of the eyelid.*

[1] Carbitol, Union Carbide Chemicals Co., New York, N. Y.
[2] Hamilton.

Table II—Effect of Systemic Administration of Corticoids on Rat Palpebral Edema

Experiment	Compound	Total Dose, mcg./kg.	Eyelid Weight, mg. (Mean ± *SE*)[a]	Deviation from Control Value, %
E	Control	—	42.14 ± 1.41	
	Hydrocortisone	9,250	35.44 ± 1.77[b]	−16
		18,500	31.71 ± 1.03[b]	−25
		37,000	25.71 ± 0.73[b]	−39
	Dexamethasone	45	34.61 ± 0.84[b]	−18
		90	29.58 ± 0.82[b]	−30
		180	25.76 ± 0.76[b]	−39
F	Control	—	44.93 ± 1.96	
	Hydrocortisone	12,650	34.60 ± 1.80[b]	−23
		22,800	32.00 ± 1.22[b]	−29
		41,000	25.57 ± 1.32[b]	−43
	Triamcinolone	1,250	37.20 ± 1.48[c]	−17
		2,500	32.35 ± 1.30[b]	−28
		5,000	26.43 ± 0.88[b]	−41
G	Control	—	46.18 ± 2.17	
	Hydrocortisone	12,650	29.20 ± 1.29[b]	−37
		22,800	26.04 ± 1.20[b]	−44
		41,000	22.66 ± 0.53[b]	−51
	Fluocinolone acetonide	15	39.03 ± 1.21[b]	−15
		30	36.18 ± 1.10[b]	−22
		60	26.93 ± 1.52[b]	−42
		120	23.06 ± 0.58[b]	−50
H	Control	—	47.26 ± 2.00	
	Hydrocortisone	7,030	33.74 ± 1.39[b]	−29
		12,650	33.05 ± 1.79[b]	−30
		22,800	27.82 ± 1.01[b]	−41
		41,000	22.24 ± 0.71[b]	−53
	Triamcinolone acetonide	25	40.82 ± 1.33[b]	−14
		50	35.95 ± 1.69[b]	−24
		100	30.11 ± 1.19[b]	−36
		200	23.13 ± 0.71[b]	−51

[a] Nine rats per group. [b] $p < 0.01$ when compared with the control. [c] $p < 0.05$ when compared with the control.

skin-free palpebra was then removed by cutting along the orbital arc (Fig. 3C). No bleeding was evident during the isolation procedure. The excised eyelid tissue was then immediately weighed on a torsion balance[3] to the nearest 0.2 mg. without blotting. The whole procedure can be carried out in 40–45 sec. after some practice.

Drugs—The anti-inflammatory activity of the following substances was tested: hydrocortisone, triamcinolone (alcohol), triamcinolone acetonide, dexamethasone, and fluocinolone acetonide.

Statistical Analysis—Relative potencies were determined by the parallel-line assay (9). The precision index of the test (λ) was obtained according to Bliss (10).

RESULTS

Time Course of Palpebral Edema Formation—For this experiment, 150 rats were subdivided into 15 groups of 10 animals each. The animals were killed at various times after beginning treatment with the irritants. Increase in eyelid weight began immediately after the first instillation of irritant and reached a peak 2 hr. after the last instillation (Fig. 4). At the peak, the eyelids weighed 50.6 ± 1.5 mg. (mean value ± standard error) compared to the controls weighing 15.5 ± 0.46 mg.—an increase of 226%. The edema remained elevated for 2 hr. more and then began to decline slowly, reaching normal values 10 hr. after the last instillation of irritant.

Anti-Inflammatory Bioassay—The anti-inflammatory effect of various compounds was investigated in eight different experiments. The substances were administered topically in four of these experiments (A, B, C, and D of Table I) and systemically in the other experiments (E, F, G, and H of Table II). In each experiment, the rats were subdivided into various groups of nine animals each,

[3] Sauter.

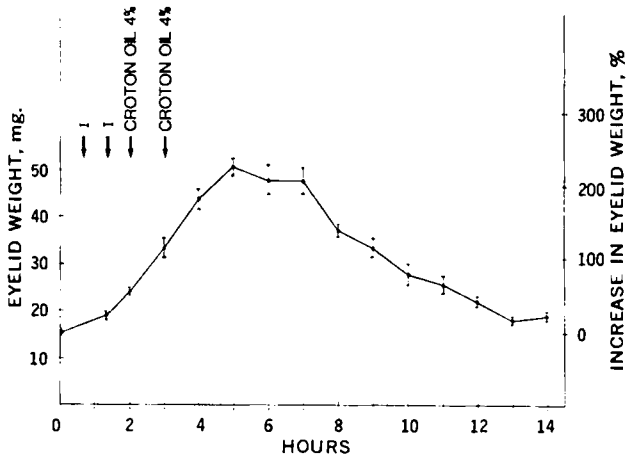

Figure 4—*I–croton oil palpebral edema as a function of time. I and croton oil were administered as described in Fig. 2A. Points and vertical bars indicate the mean and* SE.

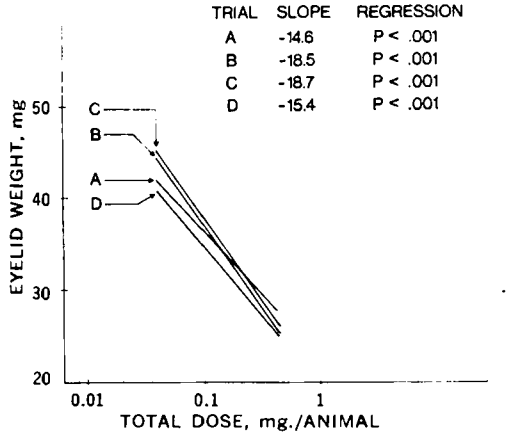

Figure 5—*Regression lines of hydrocortisone, administered topically, obtained in several experiments.*

arranged as indicated in Tables I and II. The animal treatment schedule employed was that shown in Fig. 2; 2 hr. after the last instillation of croton oil in I, the animals were killed and the eyelids were excised and immediately weighed.

Hydrocortisone was used as a reference standard in each test, and at least three doses of the test compounds were used. All compounds significantly reduced palpebral edema, and good linear log dose–response regressions were obtained.

To demonstrate the reproducibility of the method, the regression lines for the topically applied hydrocortisone standard of Table I are shown in Fig. 5. The slopes of the lines were only slightly varied, and the regressions were highly significant by statistical analysis.

The dose–response data for the various assays are plotted in Figs. 6–9. Each figure gives the results of a single steroid applied

Table III—Relative Potencies of Corticoids after Topical and Systemic Administration

Compound	Topical Activity Potency	Topical Activity λ[a]	Systemic Activity Potency	Systemic Activity λ[a]
Hydrocortisone	1		1	
Dexamethasone	103 (67–160)	0.35	238 (185–310)	0.21
Triamcinolone	4.9 (3.6–7.0)	0.25	7.7 (5.6–10.3)	0.23
Fluocinolone acetonide	2745 (1813–4512)	0.38	267 (203–338)	0.19
Triamcinolone acetonide	180[b]	—	157 (122–197)	0.22

[a] Precision index. [b] Graphical estimation (40% inhibition).

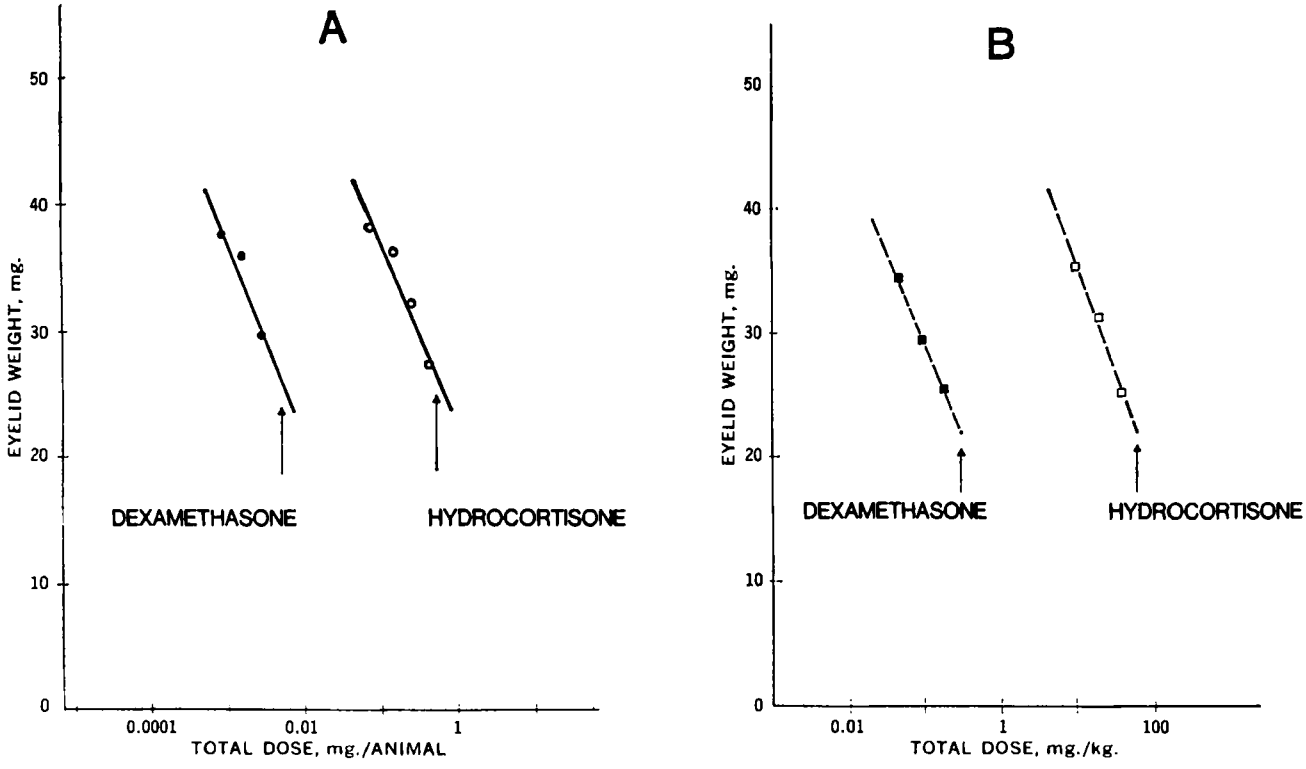

Figure 6—*Anti-inflammatory activity of dexamethasone* versus *hydrocortisone after topical (A) and systemic (B) administration.*

topically and systemically. The regression lines for each test compound, applied either topically or systemically, showed no significant dèparture from linearity. With the exception of triamcinolone acetonide applied topically, the regression line for each compound was parallel to the hydrocortisone standard administered by the same route. Thus, in each case, the relative potency could be determined by the parallel-line assay method. Table III reports the relative potencies of these compounds, with their 95% confidence limits and the precision index (λ).

Triamcinolone acetonide, applied topically, presented a significantly different and less steep regression than hydrocortisone. Thus, a precise relative potency estimate could not be made. However, a graphical estimate was made by comparing the doses producing a 40% inhibition in palpebral edema. Fluocinolone acetonide, applied topically, was the most active compound, already producing a significant reduction in edema at a total dose of 0.034 mcg./animal. The relative potency was 2745. The activity of fluocinolone acetonide was lower after oral administration, having a relative

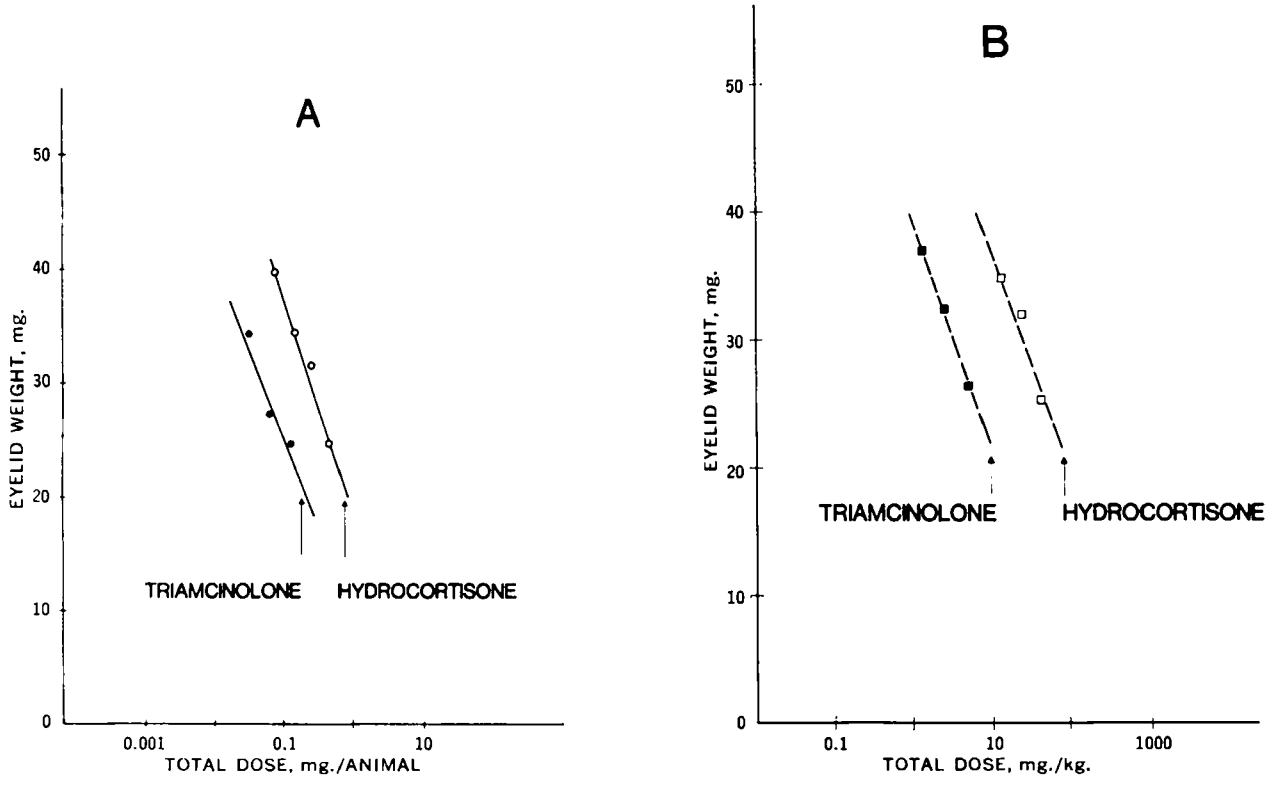

Figure 7—*Anti-inflammatory activity of triamcinolone* versus *hydrocortisone after topical (A) and systemic (B) administration.*

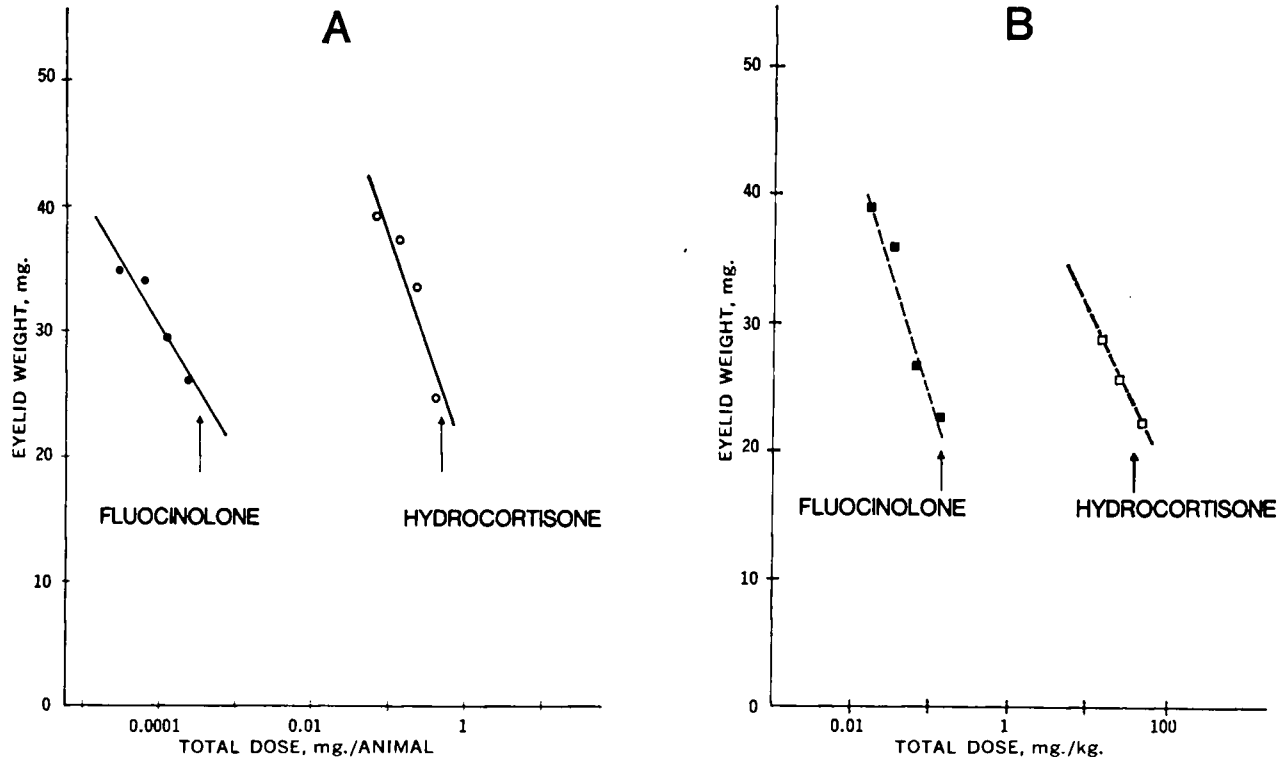

Figure 8—*Anti-inflammatory activity of fluocinolone acetonide* versus *hydrocortisone after topical (A) and systemic (B) administration.*

potency of 267. The least active product was triamcinolone, having relative potencies after topical and systemic administrations of 4.9 and 7.7, respectively. The relative potencies of dexamethasone were 103 (topical) and 238 (systemic).

The graphical estimate of topical relative potency (TRP) for triamcinolone acetonide was 180, while the systemic relative potency (SRP) for this compound was 157. A smaller quantity of the given substance was required to produce an anti-inflammatory effect when applied topically rather than systemically.

DISCUSSION

Preliminary experiments indicated that it is relatively simple to produce inflammation by instilling various types of irritants into

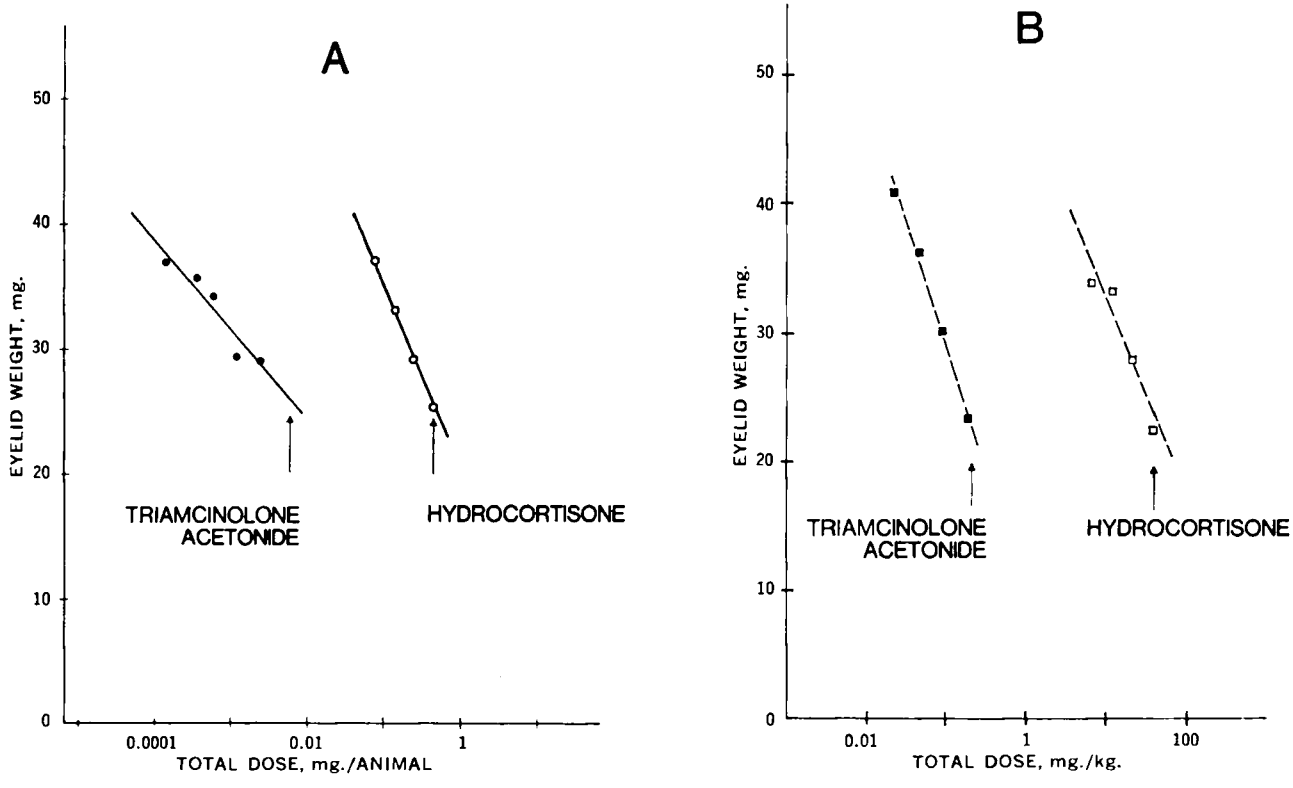

Figure 9—*Anti-inflammatory activity of triamcinolone acetonide* versus *hydrocortisone after topical (A) and systemic (B) administration.*

the conjunctival sac; the inflammation is also reduced significantly by treatment with anti-inflammatory steroids (11). The difficulty is increased considerably, however, when statistically valid and reproducible dose–response relationships are desired. The described scheme for producing irritation was adopted after considerable trial and error and is the only one that permitted quantitative determinations of relative potency within statistically acceptable limits.

Previously described methods on various types of ocular inflammation have permitted only an approximate ranking of the products or have yielded relative potency values with high confidence limits due to great biological variations (4). Moreover, it appears arbitrary to transfer results obtained from topical cutaneous tests or other general tests such as the carrageenin edema assay and the pellet granuloma inhibition assay to the eye.

The differences observed between the topical relative potency and systemic relative potency values demonstrate that it is not possible to ignore the importance of the conjunctiva as a biological membrane on the activity of anti-inflammatory substances. With triamcinolone acetonide, the dose–response relationship of the topically administered compound tended to be flatter than the dose–response curve of the same product given orally.

Regarding the characteristics of inflammation produced in these experiments, it was observed that they pertain to the anterior segment of the eye. The most evident symptoms were exudation, conjunctival hyperemia, and edema. The edemal component of inflammation was used as the sole parameter, since it is an anatomical part easily isolatable and measurable by objective criteria. This model of inflammation, although limited to the anterior segment of the eye, is of interest since the most frequent ocular diseases and the use and abuse of the glucocorticoids involve the lid edge and conjunctiva (12).

The method requires a short time, similar in this respect to methods producing inflammation in the rat paw by injection of irritating substances such as carrageenin and egg white.

The results of the method are highly reproducible. The reproducibility is more notable when compared with the method most frequently used, carrageenin-induced paw edema, which shows great variability from experiment to experiment and from day to day (13, 14).

The method is sensitive, particularly to substances administered topically. Therefore, only small quantities of substances are required for testing.

The use of rats rather than rabbits, which are generally used in ocular inflammation methods, is advantageous in bioassay experiments where large groups of animals are required.

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Tetracycline Degradation Products in Commercially Available Tetracycline-7-^{3}H

ROBERT C. LANMAN▲, THOMAS M. LUDDEN, and LEWIS S. SCHANKER

Abstract □ The radiochemical purity of three commercially available tritium-labeled tetracycline products was investigated using TLC and spectrophotometry. All three products contained, in addition to tetracycline, substantial amounts of radioactively labeled 4-epitetracycline and anhydrotetracycline. Two products also contained an unidentified component which remained at the origin.

Keyphrases □ Tetracycline-7-^{3}H—radiochemical purity and identification of degradation products in three commercial samples □ Radiolabeled tetracycline—radiochemical purity and identification of degradation products in three commercial samples □ 4-Epitetracycline, radiolabeled—identified as degradation product in commercial tetracycline-7-^{3}H □ Anhydrotetracycline, radiolabeled—identified as degradation product in commercial tetracycline-7-^{3}H □ TLC—analysis, radiochemical purity of tetracycline-7-^{3}H

The chemical purity of commercially available tetracycline products was the subject of several recent investigations (1–5). In these studies, various analytical techniques revealed that virtually all products investigated contained, in addition to tetracycline (I), varying small amounts of tetracycline degradation products such as 4-epitetracycline (II), anhydrotetracycline (III), and 4-epianhydrotetracycline (IV). In view of these findings, the question arose whether commercially available, radioactively labeled I might also contain tetracycline-like impurities. The presence of such radiolabeled contaminants could result in serious errors in biological studies in which the antibiotic is assayed by nonspecific radioisotopic methods.

The purity of tetracycline-7-^{3}H obtained from two commercial suppliers was investigated using TLC and radiochromatographic scanning of TLC plates. These methods allowed estimation of the relative amounts of I, II, and III.

EXPERIMENTAL

Reagents—Analytical grade reagents used were disodium ethylenediaminetetraacetate, methanol, glycerin, polyethylene glycol 400, succinic acid, citric acid, *n*-butanol, and methyl ethyl ketone.

Solvent Systems—Two solvent systems were used: A, methyl ethyl ketone saturated with McIlvaine's buffer (pH 4.7)(6); and B, *n*-butanol–methanol–10% citric acid (4:1:2). It was previously reported (6) that I does not undergo degradation during TLC with Solvent System A.

Thin-Layer Plates—Kieselguhr G[1] and kieselguhr N[2] TLC plates were prepared as described by Gyanchandani *et al.* (6). Acid-washed silica gel[3] and silica gel[4] plates were pretreated by soaking for 1 hr. in a 0.1 *M* solution of disodium ethylenediaminetetraacetate and then were air dried for at least 16 hr. before use.

[1] Merck.
[2] Macherey, Nagel and Co.
[3] Acid-washed silica gel (ITLC-SA) instant thin-layer plates, Gelman Instrument Co.
[4] Silica gel (ITLC-SG) instant thin-layer plates, Gelman Instrument Co.

Table I—TLC Analysis of Nonlabeled Reference Compounds[a]

Compounds	R_f Values—Acid-Washed Silica Gel—Solvent System A[b]	R_f Values—Acid-Washed Silica Gel—Solvent System B[c]	R_f Values—Silica Gel—Solvent System B
I	0.51	0.52	0.87
II	0.30	—	—
III	0.95	0.85	0.99

[a] Compounds dissolved in Gomori's succinate buffer, pH 4.65. [b] Methyl ethyl ketone saturated with McIlvaine's buffer, pH 4.7. [c] *n*-Butanol–methanol–10% citric acid (4:1:2).

Nonlabeled Reference Compounds—I-Hydrochloride (I-HCl)[5] was used as the tetracycline reference standard. Compound II, the carbon-4 isomer of I, was prepared according to McCormick *et al.* (7). The III-base was prepared by the method of Simmons *et al.* (8).

Radioactive Materials—Two samples of I-HCl, labeled with tritium at C_7 (V and VI)[6], and a single lot of I-base, similarly labeled (VII)[7], were studied.

Detection—Nonlabeled reference compounds (I, II, and III) were visualized on TLC plates by their fluorescence in longwave UV light[8]. Tritium-labeled V, VI, and VII, along with radiolabeled degradation products, were detected using a radiochromatogram scanner[9]. All radiochromatogram scans were quantitated by tracing onto graph paper, cutting out the tracing, and expressing the weight of individual peaks as a percentage of the weight of the entire tracing.

RESULTS

Nonlabeled Reference Compounds—Twenty-five-microliter aliquots of 0.06% solutions of I, II, and III, dissolved in pH 4.65 Gomori's succinate buffer, were manually streaked in a band 2 cm. from the bottom and 1 cm. from each edge of 5 × 20-cm. acid-washed silica gel[3] or silica gel[4] plates, using one compound per plate. Immediately after drying, the plates were allowed to develop 14–15 cm. (45–50 min.) in Solvent Systems A and B at room temperature. The R_f values obtained are given in Table I. It can be seen that I, II, and III applied to the acid-washed silica gel plates[3] were well separated by Solvent System A. Solvent System B provided good separation of I and III but failed to distinguish these compounds from the C_4-epimer, II. Good separation of I and III was also obtained using the silica gel plates[4] and Solvent System B, but again II was not resolved.

Product V—One millicurie (1.2 mg.) of this product was received in the dry state. The material was dissolved in 1 ml. of methanol, appropriate volumes (50–100 μl.) of solution were transferred to siliconized[10] 1-dram vials, and the vial contents were taken to dryness *in vacuo* at room temperature. Vials were then sealed, protected from light, and stored at room temperature.

For TLC, a vial containing 50–100 μc. of Product V was dissolved in Gomori's succinate buffer (pH 4.65), distilled water, or methanol containing sufficient I-HCl to give a final concentration of 48 mcg./ml. Twenty-five-microliter amounts of these solutions were ap-

[5] Lot No. 174-009, 96.9% pure, Lederle Laboratories.
[6] Lot No. 268-238, specific activity 400 mc./mmole (V), and Lot No. 566-135, specific activity 500 mc./mmole (VI), New England Nuclear Corp.
[7] Lot No. WR-1159, specific activity 9 c./mmole, Schwarz/Mann.
[8] Chromato-vue, Ultra-violet Products, Inc.
[9] Model 7201, Packard Instrument Co.
[10] Siliclad, Clay Adams.

Table II—R_f Values and Percent Composition[a] of Product V as Determined by TLC[b]

Support Medium	Solvent	Unknown R_f	Unknown %	II R_f	II %	I R_f	I %	III R_f	III %	Total[c], %
Acid-washed silica gel	Buffer[d]	0	6.6	0.31	18.8	0.53	59.6	0.94	10.1	95.1
Acid-washed silica gel	Water	0	4.8	0.28	16.2	0.53	60.7	1.00	11.1	92.8
Acid-washed silica gel	Methanol	0	4.4	0.28	20.1	0.53	57.2	0.96	18.1	99.8
Kieselguhr G	Buffer	0	13.7	0.26	11.9	0.62	57.6	1.00	11.9	95.1
Kieselguhr N	Buffer	0	7.0	0.41	19.7	0.75	53.8	1.00	10.4	90.9

[a] Components identified by reference to nonlabeled reference compounds (Table I) and quantitated as described under *Experimental*. [b] Solvent System A (see Table I). [c] Total area of peaks on basis of weight and exclusive of background. [d] Gomori's succinate buffer, pH 4.65.

plied to 5 × 20-cm. acid-washed silica gel[3], kieselguhr G, and kieselguhr N plates by streaking, and the plates were developed to 14–15 cm. in Solvent System A. As shown in Table II, Product V was resolved by Solvent System A and acid-washed silica gel[3] plates into four areas of radioactivity. Whether Gomori's succinate buffer, water, or methanol was used as the initial solvent, the resulting chromatograms were qualitatively similar. Three radioactive peaks were identified as I, II, and III by reference to chromatograms of nonlabeled reference compounds. The results suggest that Product V is composed of 57–61% I, 16–20% II, 10–18% III, and 4–7% unknown material with zero R_f.

Results obtained with kieselguhr plates and Solvent System A compared favorably with those given above, except that there was a higher percentage of unknown material at the origin with kieselguhr G.

Since the tetracyclines have the property of forming chelates with certain metal ions, the material at the origin might be a complex of this nature. However, despite the presence of disodium ethylenediaminetetraacetate, a peak at the origin was consistently observed, even with kieselguhr N, which lacks a binding agent. If the radioactivity localized at the origin were a bound form of Product V, under- or overstreaking with nonlabeled I should reduce or eliminate this peak. However, spotting 0.3 mcg. of V in methanol on acid-washed silica gel, alone and before or after applying 100 mcg. of nonlabeled I, resulted in virtually identical chromatograms when developed in Solvent System A. The resulting chromatograms all contained four areas of radioactivity representative of 51–54% I (R_f 0.49–0.52), 17–21% II (R_f 0.25–0.28), 14–18% III (R_f 0.93–0.95), and 3–5% unknown material which failed to migrate.

The nature of this material at the origin has not been determined, but it is either firmly bound or very polar since it could not be eluted with methanol. Although elution was accomplished using Solvent System B, rechromatography of the eluted material on acid-washed silica gel with Solvent System A produced a single radioactive peak which remained at the origin.

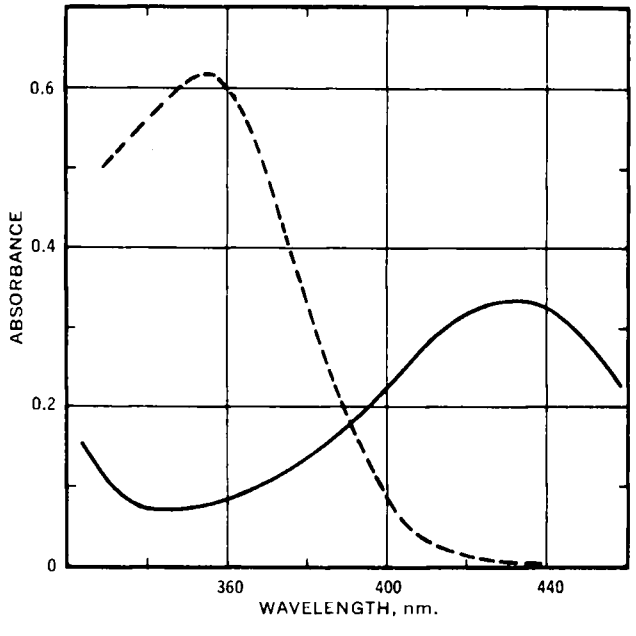

Figure 1—*Spectrophotometric curves for I-HCl (- - -) and Product VI (——). Each solution contained 20 mg. of compound/l. of 0.1 N hydrochloric acid.*

Product VI—One millicurie (1 mg.) of VI, as received in the dry state, was dissolved in 1 ml. of methanol, and 50 μl. of a 1:200 dilution in methanol was streaked on acid-washed silica gel and chromatographed in Solvent System A. One-tenth-milliliter aliquots of the remaining initial methanol solution were pipeted into 1-dram vials and treated as described previously for Product V. The vial contents were reconstituted by adding 1 ml. of methanol, and chromatography was accomplished by spotting 5 μl. (0.5 μc.) of this solution on acid-washed silica gel and silica gel and developing in Solvent Systems A and B, respectively. Methanol solutions of I-HCl and III (1 mg./ml.) were spotted in 5-μl. aliquots on the same plates.

Chromatograms of the initial solution of Product VI (before it was divided and taken to dryness) produced three areas of radioactivity (Table III). Two radioactive peaks were identified as I (R_f 0.50) and III (R_f 0.90) by reference to results obtained simultaneously with nonlabeled reference compounds. The third peak of radioactivity remained at the origin. The major radioactive component had the most rapid migration rate and was localized at an R_f value corresponding to that obtained with III.

Chromatograms of Product VI taken to dryness and reconstituted with methanol also produced three areas of radioactivity (Table III). As with the initial solution, a minor peak was present at the origin; the remaining peaks were identified as I (R_f 0.51) and III (R_f 0.94). Once again, the major radioactive component was localized at an R_f value corresponding to that obtained with III.

When reconstituted Product VI was chromatographed on silica gel with Solvent System B (Table III), a single radioactive peak was obtained with an R_f value equal to 0.98, the value expected with III in this system. Thus, TLC of both initial and reconstituted methanol solutions of Product VI on acid-washed silica gel and silica gel with Solvent Systems A and B suggested that this product is mainly III rather than I.

To confirm the chromatographic results, Product VI was assayed spectrophotometrically[11] using the absorbance ratio method described by Pernarowski *et al.* (9). Seven and one-half milliliters of 0.1 *N* hydrochloric acid was added to a vial of Product VI containing 150 mcg. of material. The spectral characteristics of this solution were determined by scanning from 320 to 460 nm., using 0.1 *N* hydrochloric acid as a blank. The spectral characteristics of I-HCl (20 mg./l. of 0.1 *N* hydrochloric acid) were determined in the same manner (Fig. 1). All scans were completed within 3 hr. after solution preparation.

The spectrophotometric curve obtained with I-HCl indicated an absorption maximum at 357 nm., in agreement with results of Pernarowski *et al.* (9). In contrast, the absorption maximum obtained with Product VI was at 434 nm., the wavelength of maximal absorption for III and IV (9). Furthermore, the absorbance of solutions of I-HCl and Product VI were equal (A_s = 0.178) at 391 nm., the wavelength at which I, III, and IV have identical absorptivity values (a_s) (9).

The absorbance of the solution of Product VI at 357 and 391 nm. was used with the following equation (9) to calculate the percentage of I present in the solution:

$$\%\ \mathrm{I} = \frac{Q{:}357{:}391 - 0.23}{0.0367} \qquad \text{(Eq. 1)}$$

in which Q:357:391 represents the absorbance ratio value, while

[11] Beckman DU-2.

Table III—TLC Analysis of Product VI

	R_f Values					
	Acid-Washed Silica Gel—Solvent System A[a]				Silica Gel Solvent System B[a]	
Product VI (initial solution)[b]	0.01	—	0.50	0.90[c]	—	—
Product VI (reconstituted)[d]	0.02	—	0.51	0.94[c]	—	0.98[c]
Nonlabeled reference compounds[e]						
I		—	0.51	—	0.87	—
II	—	0.29	—	—	—	—
III	—	—	—	0.91	—	0.99

[a] As described in Table I. [b] Product VI, as received in the dry state, dissolved in methanol. [c] Major radioactive component. [d] 1:10 methanol dilution of initial solution of Product VI. [e] Methanol solutions, 1 mg./ml.

0.0367 and 0.23 are the slope and y-intercept, respectively, of the equation for the linear relationship between Q values and percent I in various synthetic mixtures. By using Eq. 1, the relative concentration of I in the solution of Product VI was calculated to be only 6.1%, while application of Eq. 1 to the absorbance values obtained with a solution of nonlabeled I-HCl indicated the presence of 88% I.

Product VII—This product was received as a solution containing 1 mc. (53 mcg.) in 2 ml. of ethanol. The solution was stored in a sealed vial at 0° and samples were taken as needed.

For chromatography, 50 μl. of Product VII was diluted to 1 ml. with methanol or Gomori's succinate buffer containing nonlabeled I. Twenty-five-microliter quantities of these solutions were applied to acid-washed silica gel plates and developed in Solvent System A (Table IV). Product VII was resolved into three areas of radioactivity. The R_f of the major peak was the same as that of I, and the R_f values of the two minor peaks were the same as those of II and III (Table I). Unlike Products V and VI, Product VII did not show a radioactive peak at the origin when chromatographed in an identical manner.

DISCUSSION

All three samples of commercially available tritiated tetracycline were found to contain substantial amounts of radioactive degradation products. In fact, chromatographic and spectrophotometric studies suggested that Product VI is almost entirely a mixture of III and IV. Products V and VII contained 50–60% I together with smaller amounts of II and III. In addition, Products V and VI contained labeled material that remained at the origin after TLC. The amount of this material was not significantly altered by understreaking or overstreaking the site of application with a solution of nonlabeled I, as might be expected if the material were a bound form of I. It is possible that this material is chemically different from bound I or is a decomposition product of I other than those resulting from epimerization.

Gyanchandani *et al.* (6) reported that when I and its degradation products were chromatographed on kieselguhr G using Solvent System A, I and IV migrated at nearly the same rates with R_f values of 0.53 and 0.47, respectively. In the present study with acid-washed silica gel plates and Solvent System A, I and IV were not resolved into separate radioactive peaks. Therefore, the radioactive peaks identified in this study as I probably represent a summation of the percentages of I and IV, in which case the percentages of I listed in the tables would be maximal values. Since epimerization results in an equilibrium mixture (7), the presence of relatively small proportions of III means that a correspondingly small fraction of IV will be present also. Conversely, when larger proportions of III are present, the proportion of IV should be correspondingly greater. The latter situation appears to exist in the case of Product VI.

Table IV—R_f Values and Percent Composition[a] of Product VII as Determined by TLC[b]

Diluent	II		I		III		Total[c], %
	R_f	%	R_f	%	R_f	%	
Methanol	0.27	17.5	0.51	51.4	0.94	23.7	92.6
Buffer	0.31	15.9	0.53	52.5	0.95	24.9	93.3

[a] Components identified by reference to TLC of nonlabeled reference compounds and quantitated as described under *Experimental.* [b] Original ethanol solution diluted with methanol or Gomori's succinate buffer, pH 4.65, and chromatographed on acid-washed silica gel with Solvent System A (see Table I). [c] Total area of peaks on basis of weight and exclusive of background.

Whereas chromatographic analysis of reconstituted Product VI indicated the presence of about 25% I, the calculated amount of I present as determined spectrophotometrically was 6.1%. Apparently about 19% of the material in Product VI that has an R_f value corresponding to I is actually IV. With only 6.1% of I present, it is unlikely that detectable amounts of II would be formed by epimerization; this could account for the apparent absence of II from Product VI.

Biological tracer studies with labeled compounds that contain radioactive degradation products may be misinterpreted if the investigator is unaware of the presence of such contaminants. For example, the labeled isomeric forms of I present in Products V, VI, and VII cannot be assumed to be handled by the body in a manner identical with I. Therefore, the use of such a product in a pharmacodynamic study would necessitate using a method of assay that could quantitatively distinguish among the various components of the radioactive product. Such a requirement greatly reduces the advantages of using labeled I over nonlabeled I.

Results of this study underline the importance of verifying the radiochemical purity of commercially available labeled compounds using analytical systems that have been demonstrated to resolve isomers and degradation products adequately.

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Isolate from the Annelid, *Reteterebella queenslandia* (Australia), Active against Ehrlich Ascites Tumor

TED R. NORTON[A], MIDORI KASHIWAGI, and RONALD J. QUINN

Abstract □ A fraction, isolated by dialysis and gel permeation chromatography from an aqueous ethanolic extract of the tentacles of *Reteterebella queenslandia*, controlled and cured Ehrlich ascites tumor in mice in microgram doses. It is effective by either intraperitoneal or subcutaneous injection.

Keyphrases □ *Reteterebella queenslandia*—isolation, antitumor activity against Ehrlich ascites tumor in mice □ Annelids, extract of *Reteterebella queenslandia*—isolation, antitumor activity against Ehrlich ascites tumor in mice □ Antitumor activity—fraction isolated from annelid *Reteterebella queenslandia* □ Gel permeation chromatography—isolation of extract from *Reteterebella queenslandia*

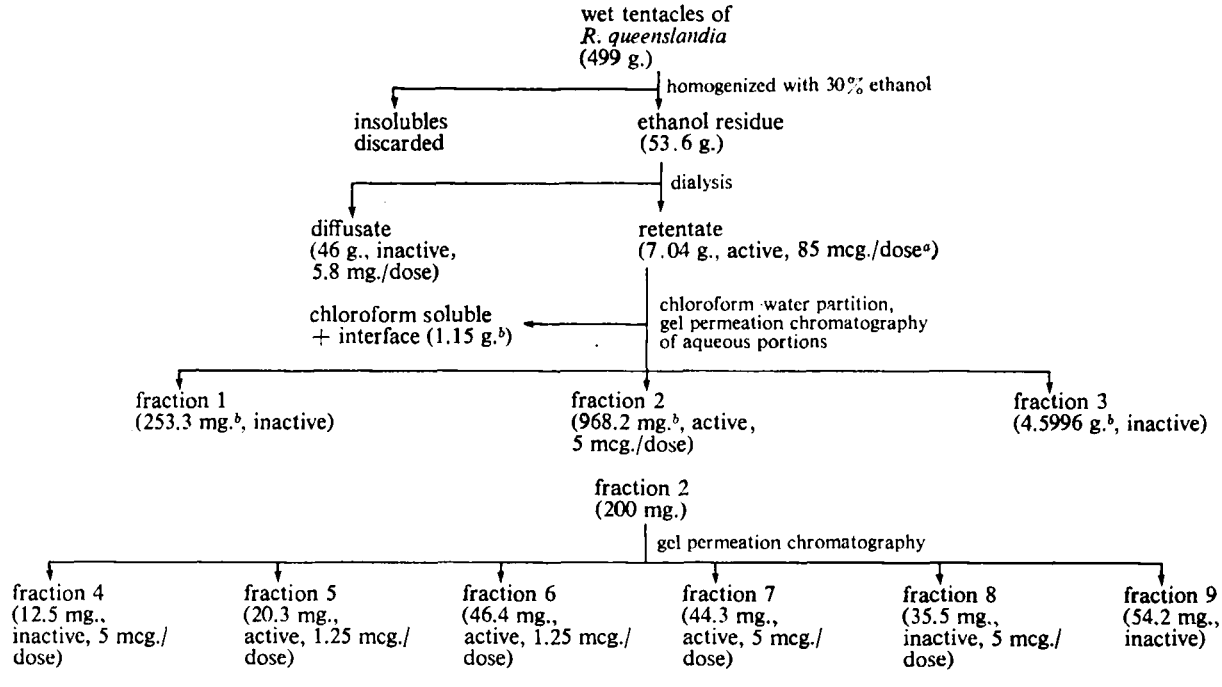
During the pharmacological evaluation of ancient Polynesian medicines, the polychaetous annelids *Lanice conchilega* and *Reteterebella queenslandia* were studied. The Kaunaoa (*L. conchilega*) was reported by Tabrah *et al.* (1) to have been used by the Hawaiians for cancer control in humans. Aqueous ethanolic extracts of both related annelids showed significant antitumor activity against Ehrlich ascites tumor in mice.

Although *L. conchilega* is found widely in the Hawaiian Islands coastal waters and tidepools, it is not found in large numbers. Because of the great difficulty in collecting, and the possible danger of depleting, *L. conchilega*, the more plentiful and closely related *R. queenslandia* from the Great Barrier Reef was chosen for purification and administration method studies.

RESULTS AND DISCUSSION

The purification of the antitumor agent is summarized in Scheme I. The indications of activity are based on >50% survivors at 30 days using 20 doses over 10 days. Controls had a mean survival time of about 17 days and never survived for 30 days, based on more than 2000 diseased control mice used during the past 4 years. Intraperitoneal drug inoculation was started 20 hr. after intraperitoneal inoculation of 5×10^6 Ehrlich ascites tumor cells. The details of the Ehrlich ascites tumor testing were described previously (1).

The fresh tentacles of the *R. queenslandia* were preserved in ethanol and shipped by air from Heron Island[1] to Honolulu where they were kept at −18° until processed. The tentacles were homogenized with 30% ethanol, and the mixture was centrifuged. The crude extract was dialyzed and the active retentate was defatted with chloroform before gel permeation chromatography[2]. The active fraction was eluted[3] at void volume, indicating a molecular weight above 30,000 (if globular protein), so that G-75 was used. All of the antitumor activity was found in fraction 2 (V_e/V_0 = 1.34-2.34). A small sample of fraction 2 was chromatographed on both anionic and cationic cellulose at pH 7.0 with 0.02 *M* phosphate buffer. The active material was not retarded by either ion-exchange column. Fraction 2 was rechromatographed[2] with peak antitumor activity being found



Scheme I—Procedure for purification of the antitumor agent in R. queenslandia. (*[a] Since 25-g. mice were used, 1 mcg./dose was equivalent to 40 mcg./kg./dose. Doses were 0.1 ml. in volume. [b] These weights are extrapolated (1.637 factor) from the somewhat smaller scale run described in the* Experimental *section.*)

[1] Supplied by Reginald McMahon, Heron Island Marine Station, Queensland, Australia.
[2] Sephadex G-75, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
[3] Sephadex G-50, Pharmacia.

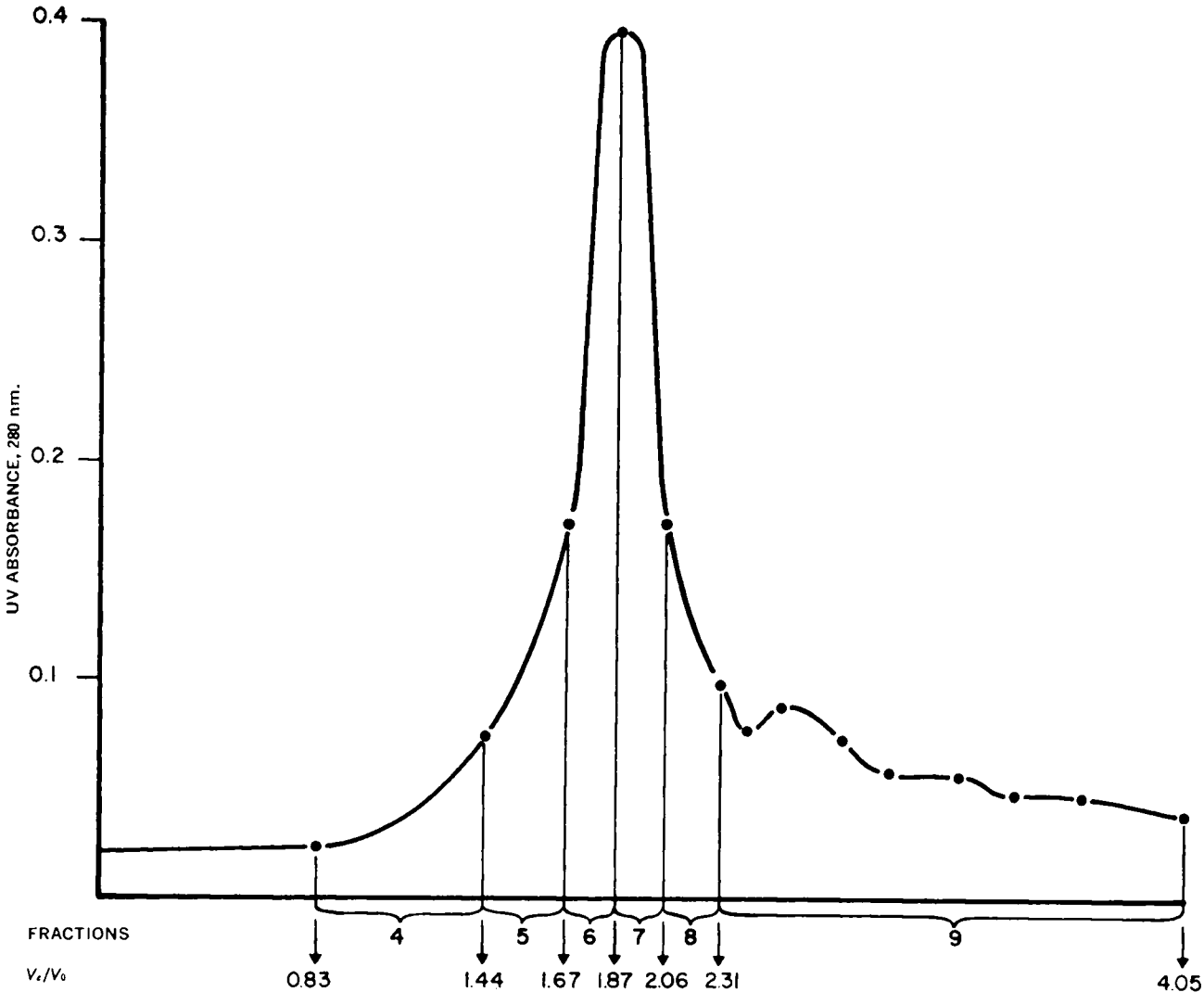


Figure 1—*Gel permeation chromatography of fraction 2 (on Sephadex G-75).*

in fractions 5 and 6 (V_e/V_0 = 1.44–1.87), which did not coincide with the UV absorption peak at 280 nm. (V_e/V_0 = 1.87). Figure 1 illustrates the UV absorption curve, and Table I lists the bioassay data.

In an attempt to correlate the antitumor activity of the gel permeation fractions with some physical or chemical parameter, 17 15-ml. fractions from V_e/V_0 = 0.90–2.47 were assayed for UV absorption at 254 nm., chromate oxidizables, foam stability (height at 48 hr.), dry weight, and anti-Ehrlich ascites tumor activity. The data are plotted in Fig. 2 and described in the *Experimental* section. The activity parameter peak did not correlate with any of these properties.

A sample of fraction 2 run through both cationic and anionic cellulose gave a 37% recovery of nonretarded material, showing a 75% cure rate using Ehrlich ascites at 0.36-mg./kg. doses. A sample of the latter, hydrolyzed for 90 hr. at 110° in 12 *N* HCl, showed an array of amino acids by a ninhydrin test[4] and showed negative tests for sugars. Since the fraction was still impure, this is only an indication that the active substance may be a protein.

Gel electrophoresis of fractions 5 and 6 at pH 4.3 with 15% cross-linked polyacrylamide (5 ma./tube, 200 v., 105 min.) showed two major bands when stained with naphthol blue black at R_f 0.16 and 0.73 (basic fuchsin at R_f 1.00) and six minor bands at R_f 0.41, 0.45, 0.52, 0.57, 0.61, and 0.66. Fraction 5 had a somewhat intensified R_f 0.73 band and a considerably diminished R_f 0.16 band. Fractions 7 and 8 had a considerably diminished R_f 0.73 band and an intense R_f 0.16 band. Based on the bioassays of these fractions, the band at R_f

[4] On Celplate F-22, Brinkmann Instruments, Inc., New York, N. Y.

Table I—Bioassay of Fractions 4–8

Fraction	Dosage[a], mcg. in 0.1 ml.	Number of Mice[b] Inoculated with Ehrlich Ascites Tumor	Number Developing Ascites	Number of Survivors at 30 Days	Percent of Survivors at 30 Days
4	5.0	5	5	0	0
5	5.0	5	0	5 (5[c])	100
	2.5	9	0	9 (9[c])	100
	1.25	10	3	7 (7[c])	70
	0.63	10	10	1	10
6	5.0	5	0	5 (5[c])	100
	2.5	10	0	10 (10[c])	100
	1.25	10	10	5	50
	0.63	10	10	0	0
7	5.0	5	2	4 (3[c])	80
	2.5	10	5	7 (5[c])	70
	1.25	10	10	1	10
	0.63	10	10	1	10
8	5.0	5	5	0	0
Controls	—	10	10	0	0

[a] Twenty of these doses given intraperitoneally over 10 days, starting 20 hr. after 5 × 10^5 Ehrlich ascites tumor cells inoculated intraperitoneally. [b] Swiss-Webster female mice weighing 25 g. [c] Number of "nonascitic" mice at 30 days. Nonascitic means no abdominal distension. See *Reference 3*.

Table II—Effectiveness of Different Modes of Drug Administration Using Fraction 5

Experiment	Experiment Number	Administrative Route	Treatment	Drug, mcg.[a]	Number of Doses	Number of Mice Inoculated with EA[b]	Number developing Ascites	Number of Survivors at 30 Days	Percent of Survivors at 30 Days[c]
Minimum number of doses required	1	EA, 5×10^5 cells, intraperitoneally; drug intraperitoneally[d]	Control	—	—	5	5	0	0
			Drug	2.5	20 (10 days)	9	0	9	100
	2	EA, 5×10^5 cells, intraperitoneally; drug intraperitoneally[d]	Control	—	—	4	4	0	0
			Drug	25.0	4 (2 days)	8	2	8 (6)[e]	100
	3	EA, 5×10^5 cells, intraperitoneally; drug intraperitoneally[d]	Control	—	—	4	4	0	0
			Drug	25.0	1	8	6	5 (2)[e]	62.5
Pretreatment with drug	4	EA, 5×10^6 cells, intraperitoneally; drug intraperitoneally in three doses over 1.5 days, last dose *36 hr. prior to EA cells* inoculation	Control	—	—	5	5	0	0
			Drug	5.0	3 (1.5 days)	10	10	2	20
Drug absorption	5	EA, 1×10^6 cells, intraperitoneally; drug *subcutaneously*	Control	—	—	5	5	0	0
			Drug	10.0	10 (10 days)	9	9	2	22
							Number Developing Solid Tumor	Tumor Weight, mg., Mean (Range) at 9 Days	
	6	EA, 1×10^6 cells, *subcutaneously;* drug intraperitoneally	Control	—	—	10	10	199 (74–388)	
			Drug	10.0	20 (10 days)	10	5	37 (0–98)	

[a] Mice used weighed 25 g., so 1 mcg./ dose was equivalent to 40 mcg./kg./dose. [b] EA = Ehrlich ascites tumor. [c] Mean survival time 17 days. [d] Drug administered 20 hr. after Ehrlich ascites tumor cells inoculated. [e] All nonascitic. The eight nonascitic survivors were kept for 3 months with no signs of abdominal distension.

Table III –Gel Permeation Chromatographic Fractions from the Retentate

Fraction	V_e/V_0	V_e/V_0 Peak Absorbance, UV at 280 nm.	Weight, g.
1	0.85–1.35	1.03	0.1574
2	1.35–2.30	1.95	0.5914
3	2.30–3.70	3.09	2.8094

Table IV –Gel Permeation Chromatographic Fractions from Fraction 2

Fraction	V_e/V_0	UV Absorbance at 280 nm.	Weight, mg.
4	0.83–1.44	0.04–0.08	12.5
5	1.44–1.67	0.08–0.18	20.3
6	1.67–1.87	0.18–0.41 (max.)	46.4
7	1.87–2.06	0.41–0.18	44.3
8	2.06–2.31	0.18–0.10	35.5
9	2.31–4.05	0.10–0.05	34.2

0.73 (high positive charge to mass ratio) appears to be the active basic protein.

Fraction 5 was used to study the effectiveness of various modes of administration of the drug. It was found to be 100% effective with 20 doses over 10 days at 100 mcg./kg. and 62.5% effective with only one dose of 1.0 mg./kg. The drug given 36 hr. prior to inoculation with Ehrlich ascites tumor cells showed 20% control. Systemic activity was indicated by some control of intraperitoneally induced tumor by subcutaneous injection of drug and by good control of subcutaneously induced solid tumors by intraperitoneally administered drug, with 50% of the mice developing *no* solid tumors (Table II). Antitumor evaluations were carried out using the method described previously (1), with exceptions as noted in Table II.

The drug (fraction 2) showed no activity against P-388 mouse leukemia[5]. Tests on other systems are still in progress.

EXPERIMENTAL

Extraction and Purification—A 499-g. wet drained weight sample of the tentacles of *R. queenslandia*, preserved in 95% ethanol for air shipment, was kept on arrival at −18° until use. One liter of 30% ethanol was used to homogenize the sample at top speed in a blender[6] for 5 min. The solids were removed by centrifugation at 30,000-$\times g$ for 1 min. and then triturated with 200 ml. of 30% ethanol and recentrifuged; the combined supernates were evaporated at ≦50° to 400 ml. volume. Some solid that separated was removed by filtration using a büchner funnel and a small amount of diatomaceous earth[7] filter aid. The solid was washed with 50 ml. of water, and the filtrate was adjusted to 500 ml. (dry weight 53.6 g.).

The 500 ml. of crude aqueous extract was dialyzed using a 1.0-m.[2] hollow-fiber artificial kidney[8]. The extract was passed through the fibers at 10 ml./min. concurrently with 1 l./min. of tap water flowing outside the fibers through the jacket (total of 50 l. of water). After flushing the kidney, the retentate was lyophilized to give 7.0385 g. of solids.

A 4.30-g. sample of this retentate was dissolved in 150 ml. water and thoroughly shaken with 150 ml. chloroform. The emulsion formed was centrifuged for 5 min. at 20,000$\times g$, and the aqueous layer was removed using a pipet. The chloroform and interface were dried and weighed 0.70 g. The clear aqueous layer was evaporated at ≦50° to 50 ml. and chromatographed in four 12.5-ml. batches (total dry weight 3.60 g., 900 mg./batch).

[5] These tests were carried out at the Southern Research Institute with the cooperation of Dr. Jonathan Hartwell, National Cancer Institute.

[6] Waring.

[7] Celite.

[8] Functional Products & Systems Dept., Dow Chemical Co., Midland, Mich.

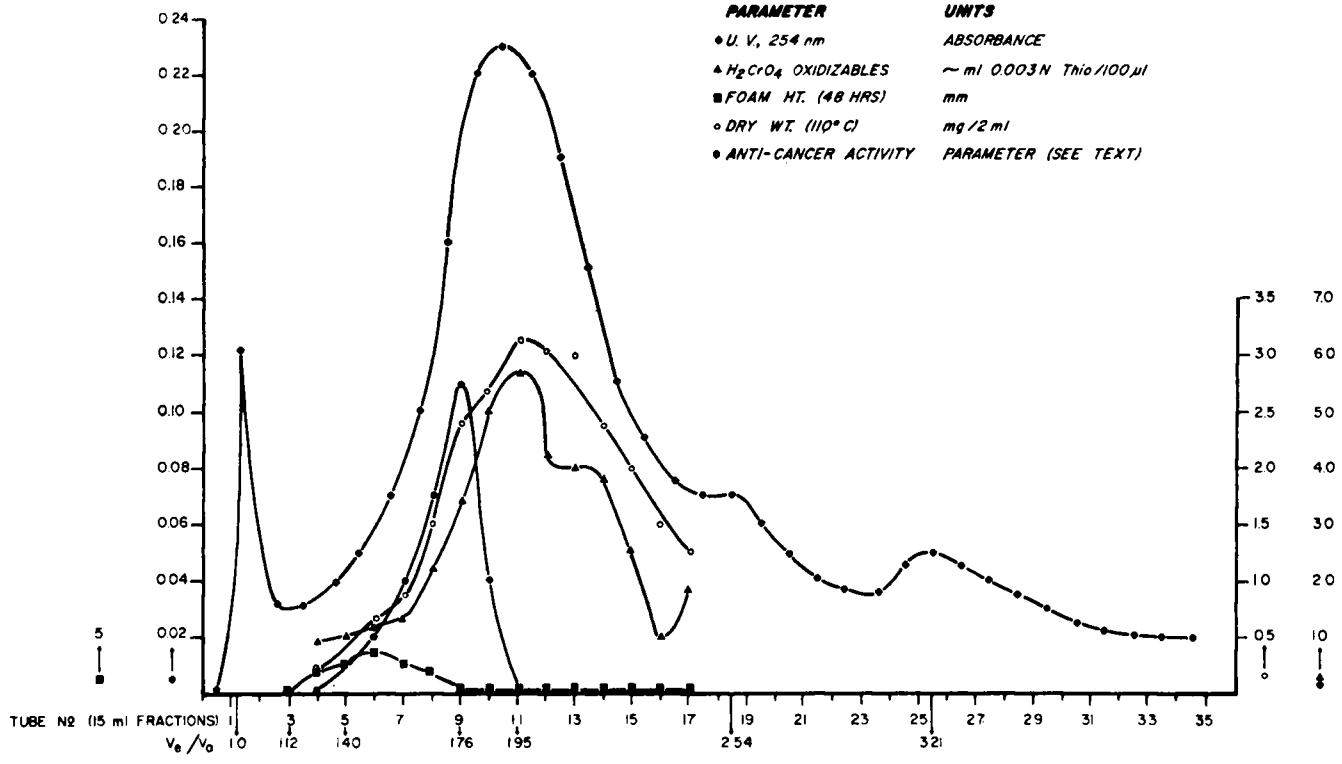


Figure 2—*Gel permeation chromatography of fraction 2, parameter correlations.*

Table V—Antitumor Assay of Tubes 4–11 from Gel Permeation Chromatography of Fraction 2

Tube Number	Number of Survivors at 30 Days		Parameter
	Nonascitic	Ascitic	
4	0	0	0
5	0	1	0.5
6	1	0	1.0
7	2	0	2.0
8	3	1	3.5
9	5	1	5.5
10	2	0	2.0
11	0	0	0

The 12.5-ml. batches were each chromatographed on 550 ml. (wet) resin[9] in a column (44 × 4 cm.) using distilled water for elution at about 3 ml./min. The void volume (V_0) was determined to be 170 ml. using 12.5 ml. of a 0.15% solution of blue dextran of mol. wt. 200,000. The fractions were collected as shown in Table III. Each fraction was lyophilized, weighed, and stored under refrigeration.

A 200-mg. sample of fraction 2 dissolved in 5 ml. water was chromatographed on 585 ml. (wet) resin[9] in a column (47 × 4 cm.) equilibrated with 0.030 *N* NaCl saturated with chloroform and eluted with the same solvent. The void volume was 174 ml. The fractions collected were desalted using a 0.1-m.[2] hollow-fiber beaker dialyzer[8], passing distilled water through the fibers at 80 ml./min. for 31 min. (2.5-l. total), and then lyophilized to give the dried fractions (Table IV).

Behavior on Ion Exchange—A 50-mg. sample of fraction 2 was dissolved in 1.0 ml. water and put on a 25 × 1-cm. column of previously conditioned cation-exchange packing[9] (5.0 g. dry and 0.73-meq./g. capacity), which had been equilibrated with 0.02 *M* phosphate at pH 7.0, and was eluted with the same solvent. The peak 280-nm. UV absorption was at V_0 (0.94 absorbance). The fraction from 13–31 ml. effluent (18 ml.) was desalted[10] (sample at V_0, phosphate retarded to V_e/V_0 = 1.8–2.2) and lyophilized to give 51.8 mg. (104% recovery, probably contains a trace of phosphate).

The same procedure was used on this 51.8-mg. sample with the anion-exchange packing[11] (capacity 0.93 meq./g.), providing 18.4 mg. (fraction 10) in the void volume fraction at pH 7 and 0.02 *M* phosphate. Under these conditions the active substance was not retarded by either the anionic or cationic cellulose.

Hydrolysis of Fraction 10—A 1.0-mg. sample of fraction 10 and 1.0 ml. of 12 *N* HCl was sealed in a Pyrex tube under nitrogen and heated at 110° for 90 hr. The acid was removed from the colorless hydrolysate by evacuation over sodium hydroxide pellets in a desiccator. The dry residue was dissolved in 0.5 ml. water, and 1 µl. was spotted[12]; using an *n*-butanol–water–acetic acid (3:1:1) solvent, strong ninhydrin positive spots were observed at R_f values of 0.054, 0.127, 0.17, 0.27, 0.34, and 0.38 with the solvent front 15.75 cm. from the origin.

A test of the hydrolysate for reducing sugars with Fehling's solution was negative.

Correlation of Physical and Chemical Properties with Antitumor Activity—A 241-mg. sample of fraction 2 equivalent (prepared in a different run from above) was dissolved in 10 ml. water and spun at 20,000 × *g* for 5 min. to remove a trace of insolubles. The supernate was put on 550 ml. of resin[9] in a column (44 × 4 cm.) and eluted with water. The void volume (V_0) was 165 ml. A total of 32 fractions of approximately 15 ml. each was collected. The exact total volume eluted (V_0) was noted at the end of each fraction, and the UV absorption at 254 nm. was recorded continuously. Dry weights were determined using 2 ml. of the cut heated at 130° to constant weight. The foam height was determined by giving each tube 30 sharp raps by hand, allowing the samples to stand for 48 hr., and measuring the height of the foam.

To determine chromate oxidizables, a modification of the method of Märki and Witkop (2) was used. The procedure involved carefully adding exactly 2.00 ml. of the chromic acid solution (0.02013 *N* $Na_2Cr_2O_7 \cdot 2H_2O$ in 98% H_2SO_4) to 100 µl. of the fractions and to control blanks and heating for 20 min. in a steam bath. The solution was then diluted to 100 ml., 1 ml. of 5% KI was added, and the solution was allowed to stand no less than 10 nor more than 20 min. (less time gave incomplete I^- to I_2 conversion and more than 20 min. gave a gradual drop in titer). The liberated iodine (I_2) was titrated with the 0.00303 *N* thiosulfate using a starch indicator; the end-point was taken when the starch–iodine color was first completely extin-

[9] Cellex CM, Bio-Rad Labs., Richmond, Calif.
[10] Using Sephadex G-25.
[11] Cellex D, Bio-Rad Labs., Richmond, Calif.
[12] Celplate F-25.

guished (on standing the color reappeared). The blanks required 13.40 ml. thiosulfate (theory = 13.29 ml.).

The activity parameter was determined as follows. A 5-ml. portion of the fraction was diluted to 60 ml., and a 0.1-ml. dose was given intraperitoneally twice daily for 10 days to eight mice starting 20 hr. after inoculation of 5×10^6 Ehrlich ascites tumor cells. At 30 days, the number of ascitic (abdominal distension) and nonascitic survivors was counted. The activity parameter is the sum of the nonascitic survivors and one-half the ascitic survivors out of the group of eight mice used for each bioassay. Only tubes 5–10 had any survivors at 30 days. The bioassays gave the results shown in Table V. The data on all of the parameters obtained are plotted in Fig. 2.

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Nonsink Dissolution Rate Equation

PAKDEE POTHISIRI and J. THURØ CARSTENSEN▲

Abstract □ An equation was developed which describes the dissolution of monodisperse particles beyond the point where concentrations are small compared to solubility. If it is assumed that a stagnant layer model applies, the thickness of these layers is of the same order of magnitude as calculated *via* the Hixson–Crowell treatment but dissolution rate constants are 1.5–2 times as large. The application of the equation to dissolution of hydrocortisone, levodopa, and *p*-hydroxybenzoic acid is shown.

Keyphrases □ Dissolution rates, monodisperse particles—equation developed for nonsink conditions, applied to hydrocortisone, levodopa, and *p*-hydroxybenzoic acid □ Particles, monodisperse—nonsink dissolution rate equation developed, applied to hydrocortisone, levodopa, and *p*-hydroxybenzoic acid

The equation by Hixson and Crowell (1) has been employed for many years [*e.g.*, Wurster and Taylor (2)] for the purpose of describing the dissolution rates of monodisperse powders. Higuchi and Hiestand (3), Carstensen and Musa (4), and Brooke (5) extended its use to describe the dissolution kinetics of polydisperse powders. The treatment relies on an assumption of sink conditions, a condition that frequently—but not always (particularly for sparingly soluble compounds)—applies. Therefore, it was considered appropriate to seek a solution not relying on sink conditions.

THEORY

When Fick's law (6) is applied to dissolution of a spherical particle under laminar flow conditions, allowing for an adsorbed surface film, it takes the form:

$$dm/dt = (L)\,dC/dt = \frac{DO}{h}[S - C] = LkO[S - C] \quad \text{(Eq. 1)}$$

where m is the mass dissolved, L is the volume of the dissolution medium, C is the concentration in the dissolution medium, t is time, ρ is the density of the solid, D is the diffusion coefficient, O is the surface area, h is the thickness of the adsorbed liquid film, S is solubility, k is the dissolution rate constant[1], n is the number of particles, r is the diameter of each particle, and subscript zero denotes initial magnitudes.

The Hixson–Crowell treatment emanates from the Noyes–Whitney equation, where it is assumed that $C \ll S$; this leads to the well-known cube root equation:

$$m_0^{1/3} - m^{1/3} = Kt \quad \text{(Eq. 2)}$$

where:

$$K = 1.61LkS(n^{1/3})/(\rho^{2/3}) \quad \text{(Eq. 3)}$$

If sphericity is assumed as above, S is assumed to be independent of r, and sink conditions are not invoked, one has the following expression for the concentration in the medium at time t:

$$C = \frac{n\rho}{L}\frac{4\pi}{3}[r_0^3 - r^3] = \alpha[r_0^3 - r^3] \quad \text{(Eq. 4)}$$

where:

$$\alpha = \frac{n\rho}{L}\frac{4\pi}{3} \quad \text{(Eq. 5)}$$

Equations 1 and 4 may be combined in the form:

$$dC = -\frac{n\rho}{L}\frac{4\pi}{3}3r^2\,dr = k(n^{1/3}\pi r^2)[S - \alpha(r_0^3 - r^3)]\,dt \quad \text{(Eq. 6)}$$

or:

$$-\frac{\rho}{L}dr = k[\beta + \alpha r^3]\,dt \quad \text{(Eq. 7)}$$

where:

$$\beta = S - (m_0/L) \quad \text{(Eq. 8)}$$

is positive when:

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The assumption is made in the following that the amount of

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The assumption is made in the following that the amount of

[1] The dimension of k is cm.$^{-2}$ sec.$^{-1}$. Some authors denote kL (cm./sec.) as the dissolution rate constant.

Table I—Data by Hussain (7) Fitted by Eqs. 2 and 13

Hydrocortisone			Levodopa		
Time t, min.	$\psi_1(r)$, $\frac{cm.^4}{g.}$	$y_1 = \sqrt[3]{m_0} - \sqrt[3]{m}$	Time t, min.	$\psi_2(r)$, $\frac{cm.^4}{g.}$	$y_2 = \sqrt[3]{m_0} - \sqrt[3]{m}$ (g.$^{1/3}$)
0	42.44	0.00000	0	0.211	0.0000
8.5	42.24	0.00768	1	0.206	0.0191
23	40.25	0.02009	1.3	0.202	0.0252
26	39.31	0.02676	2	0.183	0.0406
40	35.93	0.04387	3	0.178	0.0635
49.6	32.12	0.05902	4	0.161	0.0926
70.1	30.15	0.06722			
Slope[a]	−0.197 ± 0.003[b]	0.00105 ± 0.00003[b]		−0.014 ± 0.004[b]	0.023 ± 0.003[b]
Intercept[a]	43.61 ± 0.07[b]			0.218 ± 0.009[b]	

[a] Least-square fits of the equations in the form $\psi(r) = -kt + \psi(r_0)$ and $y = Kt$. For goodness of fit, see text. [b] The 95% confidence values based on the stated equations.

Table II—Dissolution Rate Data for *p*-Hydroxybenzoic Acid in 0.1 *N* HCl

Time t, min.	C_1[a], mg./ml. Calculated	C_1[a], mg./ml. Found	$\Delta_1^2 = [C_1 - C_1]^2$	C_2[b], mg./ml., Calculated	$\Delta_2^2 = [C_2 - C_1]^2$
0	0.000	0.000	0.000	0.089	7.8×10^{-3}
2	0.179	0.511	0.110	0.479	1.6×10^{-3}
6	0.529	1.036	0.257	0.921	13.2×10^{-3}
10.4	0.901	1.533	0.400	1.573	1.5×10^{-3}
13	1.115	1.781	0.443	1.814	1.1×10^{-3}
16.5	1.398	2.074	0.457	2.127	2.9×10^{-3}
20	1.672	2.397	0.526	2.374	0.6×10^{-3}
55	4.026	4.633	0.368	4.705	5.2×10^{-3}
105	6.308	6.201	0.011	6.264	0.3×10^{-3}
120	6.782	6.562	0.049	6.551	0.1×10^{-3}
			$\frac{\Sigma\Delta^2}{9-1} = 0.328$		$\frac{\Sigma\Delta^2}{10-2} = 4.29 \times 10^{-3}$

[a] Calculated *via* cube root equation corresponding to $\sqrt[3]{m_0} - \sqrt[3]{m} = 0.0049t$. [b] Calculated *via* Eq. 13 corresponding to $\psi(r) = 3.136 - (5.714 \times 10^{-3}) \times t$ (where $q = 0.00683$ cm. and $\beta = 2.2173$ mg./ml.).

powder used is insufficient to saturate completely the dissolution medium or just suffices to do so. Since the term $(\beta + \alpha r^3)$ is positive, Eq. 7 may be rewritten:

$$\frac{dr}{\beta + \alpha r^3} = -\frac{kL}{\rho}\,dt \qquad \text{(Eq. 10)}$$

The integral of the left-hand side is:

$$\psi(r) = \frac{q}{6\beta} \ln\left[\frac{(r+q)^2}{r^2 - qr + q^2}\right] + \frac{q}{\beta\sqrt{3}} \operatorname{arctg} \frac{r\sqrt{3}}{2q - r} \qquad \text{(Eq. 11)}$$

where:

$$q = (\beta/\alpha)^{1/3} \qquad \text{(Eq. 12)}$$

Equation 10, when integrated and subjected to initial conditions, therefore becomes:

$$\psi(r) = \psi(r_0) - \frac{kL}{\rho}t \qquad \text{(Eq. 13)}$$

$\psi(r)$, when plotted *versus* t, should then give a straight line with intercept $\psi(r_0)$ and slope $-kL/\rho = -(DL)/(h\rho)$.

RESULTS AND DISCUSSION

To test whether Eq. 13 gives results significantly different from Eq. 2 (and Eq. 3), the data reported by Hussain were plotted (7), primarily because the dissolution rates reported were carried into nonsink concentration ranges (30% saturation) and because Hussain rigorously reported all values of parameters necessary for computations of the cited nature.

Furthermore, the range of dissolution was extended in the following manner, using *p*-hydroxybenzoic acid as a test substance. *p*-Hydroxybenzoic acid was recrystallized from water, dried *in vacuo*, and screened. Material finer than 60 mesh but coarser than 80 mesh (USP) was used; this material had an average "diameter" of 212 μm., *i.e.*, $r = 106$ μm. The density of the material was determined pycnometrically to be 1.2626 g./ml. at 25 ± 0.1°. Three hundred milliliters of 0.1 *N* HCl was placed in a 1000-ml. conical flask and agitated by a magnetic stirrer at 58 r.p.m. The temperature was maintained at 25 ± 0.5°, and 2.485 g. of the *p*-hydroxybenzoic acid was added at zero time. Samples were withdrawn by pipet through glass wool at various time intervals and assayed at the UV absorption peak at 255 nm.

Results are listed in Tables I and II and shown graphically in Figs. 1–3. The calculated parameters in the tables are least squares fitted. It is noted from Figs. 1 and 2 that Eqs. 2 and 13 are adhered to well. How well can be estimated by the variances of the fits in

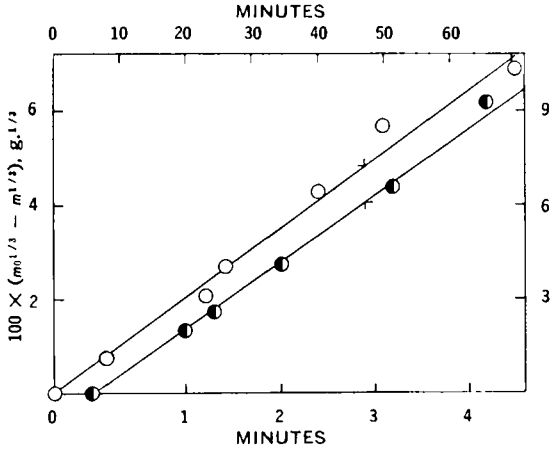

Figure 1—*Data by Hussain (7) plotted according to Eq. 2. The upper and left scales refer to hydrocortisone* (○) *and the lower and right scales refer to levodopa* (◐).

Table III—Dissolution Rate Constants (k) Calculated from Eqs. 2 and 13

	I, cm.$^{-2}$ sec.$^{-1}$, Eq. 2	k, cm.$^{-2}$ sec.$^{-1}$, Eq. 13	D, cm.^{2}/sec.	L, cm.[3]	h, cm., Eq. 13
Hydrocortisone	4.4×10^{-6}	8.4×10^{-6}	2.47×10^{-6}	500	0.6×10^{-3}
Hydrocortisone	4.4×10^{-6}	8.4×10^{-6}	6×10^{-6} [a]	500	1.4×10^{-3}
Levodopa	4.1×10^{-6}	7.1×10^{-6}	4.6×10^{-6} [a]	500	1.3×10^{-3}
p-Hydroxybenzoic acid	0.19×10^{-6}	0.40×10^{-6}	6.21×10^{-6} [a]	300	5.2×10^{-3}

[a] By Wilke's method (8).

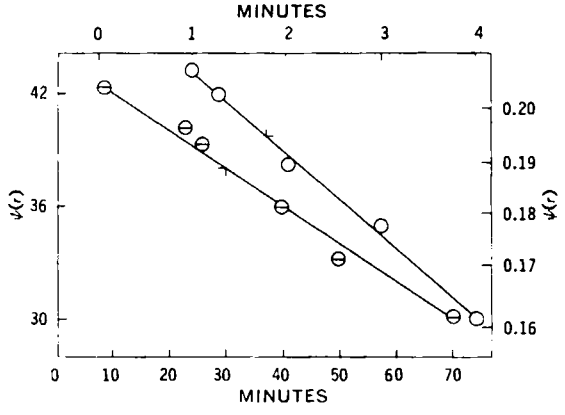

Figure 2—*Data by Hussain (7) plotted according to Eq. 13. The upper and right scales refer to levodopa (○) and the lower and left scales refer to hydrocortisone (●).*

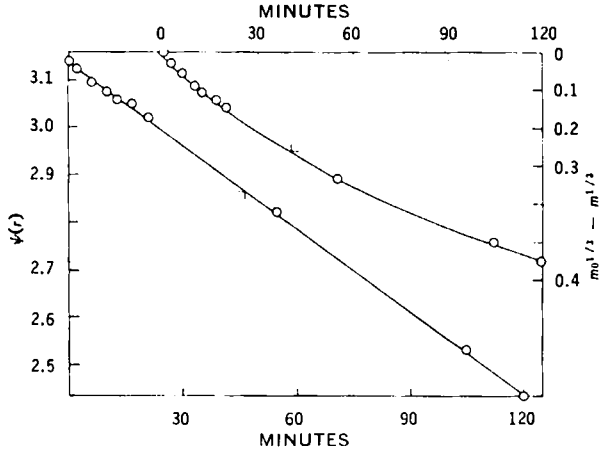

Figure 3—*Dissolution rate data of p-hydroxybenzoic acid plotted according to Eq. 13 (lower curve) and to Eq. 2 (upper curve).*

linear form, *i.e.*, $C = f(t)$. The variance is $\Sigma \Delta^2/\nu$, where $\Delta = \hat{C} - C$, ν denotes degrees of freedom, and $\hat{C}$ is the concentration calculated from the least-squares fit. For example, at time $t = 40$ in hydrocortisone dissolution (Table I), $\sqrt[3]{0.022} - \sqrt[3]{m} = 0.00105 \cdot 40$, so $m = 0.0135$ or $\hat{C} = 0.0135/500 = 27 \times 10^{-3}$ mg./ml. as compared to $C = 23 \times 10^{-3}$ mg./ml. found experimentally. The calculation of $\hat{C}$ corresponding to Eq. 13 is more cumbersome but can be done either graphically or by aid of a computer. It is noted from Table II (Fig. 3) that for p-hydroxybenzoic acid under nonsink conditions, Eq. 13 affords a significantly better fit than Eq. 2 [$F = 76$ as compared to $F_{0.01}$ (critical) = 5.5]. In the case of hydrocortisone and levodopa (where nonsink conditions were not so prevalent), both equations afford comparable fits.

Although statistical comparisons are worthwhile, the main point is that Eq. 13 gives a theoretically more correct solution than Eq. 2 under nonsink conditions and the data support this by exhibiting reasonable fits.

From the slope of the data plotted *via* Eq. 13, k can be found through knowledge of L and ρ. Similarly, knowledge of L, S, n, and ρ allows calculation of k from the slopes of data plotted *via* Eq. 2. These k values are listed in Table III. It is noted, as a general trend, that k values calculated from Eq. 2 are about half as large as those calculated from Eq. 13. Diffusion constants can be estimated *via* the Stokes-Einstein equation either *via* radius estimates from crystallographical parameters or *via* Wilke's method (8). The data by Haner and Norton (9) were used to obtain a diffusion coefficient for hydrocortisone of 2.47×10^{-6} cm.^{2}/sec.; by Wilke's method a value of 6×10^{-6} cm.^{2}/sec. results. By employing the relation $k = D/(hL)$, the value of h (the thickness of the adsorbed layer of liquid) can be estimated and is found to be 0.6×10^{-3} cm. = 60 μm. when using $D = 2.47 \times 10^{-6}$ cm.^{2}/sec. and 140 μm. when using $D = 6 \times 10^{-6}$ cm.^{2}/sec. Other values of this type are listed in Table III. The values are of the same order as those estimated by Hussain (7), Levy (10), and Cressman *et al.* (11).

The dimension of q is obtained from Eqs. 9 and 5 and was found to be $\{(\text{g./ml.})/[\text{g./(ml.)}^2]\}^{1/2}$ = cm. The polynomial $r^2 - qr + q^2$ has no rational roots. The term in the denominator of the arctg function in Eq. 11 must be larger than zero; *i.e.*, cubing the inequality $2q > r$ (which is allowable since $\beta/\alpha > 0$), one gets: $(\beta/\alpha) = \{[S - (m_0/L)]/(n\pi 4\rho/3L)\} > (r^3/8)$ or $(LS - m_0) > (m/8)$, so that there is the added requirement that $LS > (9m_0/8)$ (since m can be at most m_0). This requirement is slightly more stringent than the one expressed in Eq. 9 and was adhered to in the experiment dealing with p-hydroxybenzoic acid (the initial amount being 80% of solubility). Not all of the p-hydroxybenzoic acid was dissolved at the end of the experiment, and the particle-size range was sufficiently narrow so that the number of particles had not decreased (4).

The arctg function is not single valued; $x = r\sqrt{3}/(2q - r)$ is here expressed in radians between 0 and $\pi/2$. Equation 11 could equally well have been expressed with values of x between 2π and $5\pi/2$, but this would simply increase both $\psi(r)$ and $\psi(r_0)$ by $2\pi q/(\beta\sqrt{3})$ and Eq. 13 would still apply. Hence, using $0 < x < \pi/2$ does not cause a loss in generality.

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Bridged D-Ring Steroid Analogs XII: Effect of D-Ring Substituents on Chemical Shift of Angular Methyl Protons

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Abstract □ The chemical shifts of the C_{18}- and C_{19}-hydrogens of 30 14α,17α-bridged 20-ketopregnanes and of four 14α,17α-bridged 17-cyanoandrostanes were correlated by means of additive substituent constants. Because the bridged D-ring of these steroids is a substituted bicyclo[2.2.1]heptane system, attempts were made to explain the magnitude of some of these substituent constants by analogy to empirical effects in other such systems as well as by reference to more general theory. The data indicate that the acetyl side chain of the bridged 20-ketopregnanes exists in one rotomer form if the D-ring bears no extra substituents or a 16α-substituent and that it exists in a second form if a 16β-substituent or a 16-substituent on a double bond is present. In the latter case, the carbonyl group deshields the C_{18}-hydrogens by an extra 0.11 p.p.m.

Keyphrases □ Steroids, bridged D-ring -effect of D-ring substituents on chemical shifts of angular methyl protons, NMR spectroscopy □ Pregnanes, 14α,17α-bridged—effect of D-ring substituents on chemical shifts of angular methyl protons, NMR spectroscopy □ 5-Androstenes, 3β-acetoxy-14α,17α-ethano—effect of D-ring substituents on chemical shifts of angular methyl protons, NMR spectroscopy □ Bridged D-ring steroids—effect of D-ring substituents on chemical shifts of angular methyl protons □ NMR spectroscopy—substituent effect on chemical shifts of angular methyl protons in bridged D-ring steroids

During initial investigations of the 14α,17α-etheno-20-ketopregnane system, the use of NMR for the determination of the stereochemistry of the bridged D-system was attempted. A comparison of the NMR spectra of some of the first compounds prepared in that series (*e.g.*, X and XI in Table I), if considered according to the theory of that time (1), would have resulted in assignment of wrong stereochemistry for these compounds. Fortunately, internal inconsistencies in the NMR data, together with the fact that other evidence (2) tended to favor the correct structures, recommended the use of other methods. Since a large group of compounds of rigorously proved structure (3–5) is now available for this series, it seemed appropriate to reconsider the spectra of these compounds in the hope of correlating the chemical shifts of their C_{18}- and C_{19}-hydrogens and of explaining the origin of the shifts in terms of current theory.

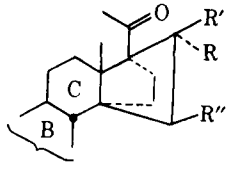
DISCUSSION

Table I lists a variety of 14α,17α-ethanopregnane derivatives and the positions of their angular methyl groups in the NMR. Six pairs of these compounds (I–II, V–VI, VII–VIII, IX–X, XII–XIII, and XIV–XV) differ only in the presence or absence of a double bond in the 14α,17α-bridge. These compounds show that such a double bond has essentially no effect on the C_{18}-hydrogens, even though it causes a shielding of the more distant C_{19}-hydrogens by 0.04 ± 0.005 p.p.m. In contrast, conversion of the saturated bridged D-ring to the doubly unsaturated system (compound pairs XI–XVI, XVIII–XIX, XXII–XXIII, XXIV–XXV, and XXVI–XXVII) results in a deshielding of the C_{18}-hydrogens by 0.30 ± 0.042 p.p.m. while shielding the C_{19}-hydrogens by 0.03 ± 0.008 p.p.m.

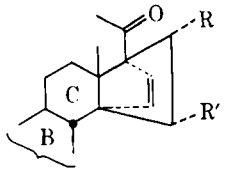
Recently, by synthesizing and determining the NMR of 3β-acetoxy-14α,17α-ethano-5,15-pregnadien-20-one (Compound XXX in Table I) the introduction of a 15,16-double bond was demonstrated to have almost no effect on the chemical shift of either the C_{18}- or C_{19}-hydrogens[1] (6).

Since introduction of a single double bond into either bridge of these D-rings has almost no effect on the chemical shift of the C_{18}-hydrogens, the shift of 0.30 p.p.m. which occurs on introducing double bonds into both bridges was considered to be anomalously large. This paper attempts to assess a variety of factors for their possible contributions to this effect.

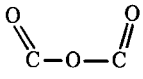
Orientation of 17-Acetyl Side Chain—Examination of the pairs of compounds that were compared to determine the effect of introducing a single double bond into the bridged D-ring system reveals that, within each pair, the configuration of all substituents on the molecule (hydrogens excepted) remains constant. In contrast, in each pair in which two double bonds were introduced into the bridged D-ring system, a C_{16}-substituent or C_{15},C_{16}-disubstituents

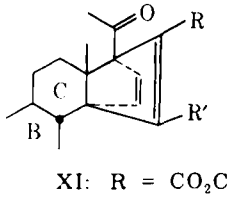


I, III, IV, V, VII, XXI: R = R′ = R″ = H
IX, XII, XIV, XX: R = CO_2CH_3, R′ = R″ = H
XVI: R = R″ = H, R′ = CO_2CH_3
XVII: R = CO_2H, R′ = R″ = H
XVIII: R = H, R′ = R″ = CO_2CH_3
XXII, XXIV, XXVI: R = H, R′ = R″ = CF_3
XXVIII, XXIX: R = I, R′ = R″ = H



II, VI, VIII: R = H, R′ = H
X, XIII, XV: R = CO_2CH_3, R′ = H

XXXV: R, R′ = 



XI: R = CO_2CH_3, R′ = H
XIX: R = R′ = CO_2CH_3
XXIII, XXV, XXVII: R = R′ = CF_3

[1] Both Tillieu's and Pople's models (7) lead to a prediction that this double bond should shield the C_{18}-hydrogens but by less than 0.1 p.p.m. Calculations made according to the method of ApSimon *et al.* (8) indicate that such a double bond should deshield the methyl hydrogens by 0.01 p.p.m.

Table I—Chemical Shifts[a] of Angular C_{18}- and C_{19}-Methyl Protons of 14α,17α-Bridged Pregnanes

Compound	Compound Name	Observed C_{18}	Observed C_{19}	Calc. C_{18}	Calc. C_{19}
I	3β-Acetoxy-14α,17α-ethano-5-pregnen-20-one[b]	0.90	1.03	0.91	1.05
II	3β-Acetoxy-14α,17α-etheno-5-pregnen-20-one[c]	0.90	1.00	0.91	1.00
III	3β-Acetoxy-14α,17α-ethano-5α-pregnan-20-one	0.87	0.85	0.87	0.85
IV	3β-Hydroxy-14α,17α-ethano-5α-pregnan-20-one[d]	0.88	0.83	0.87	0.83
V	3β-Hydroxy-14α,17α-ethano-5-pregnen-20-one[e]	0.91	1.03	0.91	1.04
VI	3β-Hydroxy-14α,17α-etheno-5-pregnen-20-one[c]	0.92	1.00	0.91	0.99
VII	14α,17α-Ethano-4-pregnene-3,20-dione[e]	0.94	1.22	0.94	1.22
VIII	14α,17α-Etheno-4-pregnene-3,20-dione[c]	0.92	1.17	0.94	1.17
IX	3β-Acetoxy-14α,17α-ethano-16α-carbomethoxy-5-pregnen-20-one[d,f]	0.96	1.04	0.96	1.04
X	3β-Acetoxy-14α,17α-etheno-16α-carbomethoxy-5-pregnen-20-one[g]	0.96	1.00	0.96	0.99
XI	3β-Acetoxy-14α,17α-etheno-16-carbomethoxy-5,15-pregnadien-20-one[f]	1.29	1.01	1.29	1.01
XII	3β-Hydroxy-14α,17α-ethano-16α-carbomethoxy-5-pregnen-20-one[f]	0.97	1.03	0.96	1.03
XIII	3β-Hydroxy-14α,17α-etheno-16α-carbomethoxy-5-pregnen-20-one[g]	0.96	0.98	0.96	0.98
XIV	14α,17α-Ethano-16α-carbomethoxy-4-pregnene-3,20-dione[h]	1.00	1.22	1.00	1.21
XV	14α,17α-Etheno-16α-carbomethoxy-4-pregnene-3,20-dione[g]	0.98	1.18	1.00	1.16
XVI	3β-Acetoxy-14α,17α-ethano-16β-carbomethoxy-5-pregnen-20-one[f]	0.90	1.03	0.90	1.03
XVII	3β-Acetoxy-14α,17α-ethano-16α-carboxy-5-pregnen-20-one[d]	0.95	1.04	0.96	1.04
XVIII	3β-Acetoxy-14α,17α-ethano-15β,16β-dicarbomethoxy-5-pregnen-20-one	1.10	1.07	1.10	1.07
XIX	3β-Acetoxy-14α,17α-etheno-15,16-dicarbomethoxy-5,15-pregnadien-20-one[f]	1.33	1.03	1.33	1.03
XX	19-Nor-14α,17α-ethano-16α-carbomethoxy-4-pregnene-3,20-dione[h]	1.00	—	1.01	—
XXI	19-Nor-14α,17α-ethano-4-pregnene-3,20-dione[h]	0.97	—	0.96	—
XXII	3β-Acetoxy-14α,17α-ethano-15β,16β-di(trifluoromethyl)-5-pregnen-20-one[i]	0.99	1.05	1.00	1.07
XXIII	3β-Acetoxy-14α,17α-etheno-15,16-di(trifluoromethyl)-5,15-pregnadien-20-one[i]	1.30	1.02	1.29	1.03
XXIV	3β-Hydroxy-14α,17α-ethano-15β,16β-di(trifluoromethyl)-5-pregnen-20-one[i]	1.02	1.06	1.00	1.06
XXV	3β-Hydroxy-14α,17α-etheno-15,16-di(trifluoromethyl)-5,15-pregnadien-20-one[i]	1.30	1.01	1.29	1.02
XXVI	14α,17α-Ethano-15β,16β-di(trifluoromethyl)-4-pregnene-3,20-dione[i]	1.03	1.26	1.04	1.24
XXVII	14α,17α-Etheno-15,16-di(trifluoromethyl)-4,15-pregnadiene-3,20-dione[i]	1.31	1.23	1.32	1.20
XXVIII	3β-Acetoxy-14α,17α-ethano-16α-iodo-5-pregnen-20-one[d]	0.92	1.03	0.92	1.03
XXIX	3β-Hydroxy-14α,17α-ethano-16α-iodo-5-pregnen-20-one[d]	0.92	1.02	0.92	1.02
XXX	3β-Acetoxy-14α,17α-ethano-5,15-pregnadien-20-one[b]	0.89	1.04	0.89	1.04

[a] Measured in parts per million downfield from internal tetramethylsilane in deuteriochloroform solution. [b] *Reference 6.* [c] *Reference 9.* [d] *Reference 3.* [e] *Reference 10.* [f] *Reference 4.* [g] *Reference 11.* [h] *Reference 12.* [i] *Reference 13.*

were oriented beta in the saturated member of the pair but shifted to the nodal plane of the 15,16-double bond in the unsaturated member of the pair.

Cross and coworkers (14, 15) demonstrated that interaction between substituents in 16,17-disubstituted steroids can lead to conformational changes in the D-ring and to changes in the preferred rotomer form of substituents, especially of a C_{17}-acetyl group. These changes have been shown to result in appreciable changes in the chemical shift of the C_{18}-hydrogens and numerous warnings have been given against too ready use of additive substituent constants for calculation of chemical shifts in cases where the substituents may interact sterically, by hydrogen bonding, or by dipole-dipole effects (15, 16).

In the present case, the rigidity of the bridged D-ring prevents any major conformational change in the C- or D-ring of the steroid. However, the possibility does remain that substituents, particularly those at C_{16}, may cause a change in the preferred orientation of the 17-acetyl group and thereby cause an additional change in the chemical shift of the C_{18}-hydrogens.

Previously, an X-ray structure determination on 3β-acetoxy-14α,17α-ethano-16α-iodo-5-pregnen-20-one (XXVIII) was reported (3). It showed that, in the crystalline state, the acetyl group of XXVIII is oriented with the oxygen beta and at an angle of 38° with respect to the plane containing atoms C_{14}, C_{15}, C_{16}, and C_{17}.

In an attempt to find a structural abnormality that could explain the deshielding of the C_{18}-hydrogens of the 15-dehydro-14α,17α-etheno compounds, an X-ray structure determination of XXVII was made. That study (5) disclosed no structural anomalies, but it did show that the vicinal angle defined by the acetyl group and the plane containing atoms C_{14}, C_{15}, C_{16}, and C_{17} is 11° larger in XXVII than in XXVIII.

This change in orientation of the acetyl group is similar to the change postulated (15) to occur in 20-ketopregnanes when a 16β-substituent is introduced. Cross and Beard (15) assigned a ψ-equatorial conformation to the 16β-substituent; in that form, the relative orientation of the 13β-methyl, the acetyl group, and the 16-substituent is very similar to that of the corresponding groups in XXVII. If this analogy is valid, one could argue that the deshielding observed going from a 16α-substituted 14α,17α-ethenopregnan-20-one to the 15-dehydro analog (from X to XI) is, at least in part, the expected result of reorientation of the acetyl side chain. To the extent that this is true, it changes the task to that of explaining the relative lack of deshielding by the 16β-substituents.

Cross and Beard's estimate (15) of the long-range shielding effect of different acetyl rotomers was qualitative and was based on Jackman's (1) early view regarding the shape of the carbonyl shielding cones. Since that time, more refined views of the long-range shielding effect of the carbonyl group have emerged (17–19) and the importance of also considering the shielding effects of carbon-carbon single bonds and of carbon-hydrogen bonds has been emphasized (8, 19). Accordingly, equations of ApSimon *et al.* (8, 18) were used to estimate the shielding effect exerted by the acetyl group, in its various rotomer forms, on the C_{18}-hydrogens. These calculations showed a larger shielding for the 16α-substituted compound, XXVIII (−0.09 p.p.m.), than for the unsaturated compound, XXVII (−0.16 p.p.m.)[2]. As shown in Fig. 1, the rotomer that points the acetyl oxygen toward the center of rotation of the C_{18}-hydrogens would be maximally deshielded while the rotomer in which the carboxyl bond axis has been rotated by 180° would be maximally shielded. These results demonstrate that reorientation of the acetyl group may account for at least a small portion of the anomalous deshielding observed for the C_{18}-hydrogens of the 14α,17α-etheno-15-pregnen-20-ones.

17-Cyano Compounds—Because the calculation of the shielding of the C_{18}-hydrogens by the acetyl group involves many assumptions, it was necessary to determine experimentally the magnitude of this effect. This was done by studying compounds in which the acetyl group was replaced by the axially symmetrical cyano group. Accordingly, 3β-acetoxy-17-cyano-5,14,16-androstatriene (2) was reacted with dimethyl acetylenedicarboxylate to afford 3β-acetoxy-17-cyano-15,16-dicarbomethoxy-14α,17α-etheno-5,15-androstadiene (XXXI)[3]. Hydrogenation of XXXI over palladium-on-charcoal afforded 3β-acetoxy-17-cyano-15β,16β-dicarbomethoxy-14α,17α-ethano-5-androstene, XXXII.

[2] These calculations do not pertain to the compounds studied by Cross and Beard (15) since those compounds could undergo conformational changes in the D-ring as well as rotation of the acetyl group. Moreover, the torsional angle formed by the C_{13}–C_{18} and C_{17}–C_{20} bonds is greater in the present case than in the compounds of Cross and Beard (15).

[3] Stereochemistry was assigned by analogy to that which was demonstrated in the corresponding 20-ketopregnane system (3, 4).

Table II—Chemical Shifts[a] of C_{18}- and C_{19}-Protons of 3β-Acetoxy-14α,17α-ethano-5-androstenes

Substituents	17-Cyano Compound Number	17-Cyano Observed C_{18} H's	17-Cyano Observed C_{19} H's	17-Cyano Calculated C_{18} H's	17-Cyano Calculated C_{19} H's	17-Acetyl Compound Number	17-Acetyl Observed C_{18} H's	17-Acetyl Observed C_{19} H's	17-Acetyl Calculated C_{18} H's	17-Acetyl Calculated C_{19} H's
15β,16β-Dicarbomethoxy	XXXII	1.12	1.06	1.13	1.07	XVIII	1.10	1.07	1.10	1.07
15,15′-Didehydro-15,16-dicarbomethoxy	XXXI	1.36	1.02	1.36	1.03	XIX[c]	1.33	1.03	1.33	1.03
15′-Dehydro-15α,16α-dicarboxyanhydride	XXXIII[b]	1.17	1.03	1.16	1.02	XXXV	1.02	1.02	1.02	1.02
15′-Dehydro-16α-carbomethoxy	XXXIV[b]	1.09	1.00	1.10	0.99	X[b]	0.96	1.00	0.96	0.99

[a] Parts per million downfield from tetramethylsilane in deuteriochloroform. [b] *Reference 2.* [c] *Reference 4.*

In Table II, the chemical shifts of the angular methyl hydrogens of four 17-cyano-14α,17α-bridged steroids are compared to those of the corresponding 17-acetyl compounds. The C_{18}-hydrogens of the 16α-substituted 17-acetyl compounds, XXXV and X, are shielded by 0.14 ± 0.01 p.p.m. relative to those of the cyano steroids, XXXIII and XXXIV. By contrast, both in the case of 16β-substituted steroids and in the case of 16-substituted 15,15′-didehydrosteroids, the acetyl compounds are shielded by only 0.03 ± 0.01 p.p.m. relative to the cyano compounds. These data can be rationalized by postulating a change in orientation of the acetyl group on going from the 16α-substituted compounds, XXXV and X, to the 15,15′-didehydro-16-substituted or 16β-substituted compounds, XIX and XVIII. This change in rotomer form would be accompanied by a decrease in shielding of 0.11 ± 0.01 p.p.m. Such a change in shielding is in reasonable agreement with that calculated (0.07 p.p.m.) to occur going from the 16α-substituted compound, XXVIII, to the unsaturated compound, XXVII. The unique geometry of the 16β-substituted series would be expected to lead to a new shielding curve similar to those in Fig. 1. However, the similarity of the curves in Fig. 1, for the range of interest, leads to the expectation that the similar shielding effect of the acetyl group in the 16β-substituted series and in the doubly unsaturated bridged series reflects a similar orientation of the group in the two series.

While, as discussed previously, the data in Table II do confirm[4] that a change in acetyl rotomer form is responsible for a small part of the increased deshielding of the C_{18}-hydrogens of 16-substituted 15,15′-didehydropregnan-20-ones as compared to those of the corresponding 16α-steroids, they also clearly show that some additional factor must contribute to the deshielding of the C_{18}-hydrogens of the didehydrosteroids as compared to those of either the 16α- or the 16β-substituted steroids. Alternatively, the data could be explained by assuming that some factor causes the C_{18}-hydrogens of the 16α- and/or the 16β-steroids to be excessively shielded.

Substituent Constants—As a further test for unusual factors affecting the shielding of the angular methyl groups of these bridged steroids, an attempt was made to correlate their chemical shifts by the use of additive substituent constants after the manner of Zurcher (20). By using the values shown in Table III together with Zurcher's values (20) for substituents in the A- or B-ring, one can obtain the values shown as "calculated" in Tables I and II.

Shielding by Substituents at C_{15} and C_{16}—The constants in Table III clearly illustrate that 16β-trifluoromethyl and 16β-carbomethoxy groups cause little deshielding of the C_{18}-hydrogens, and correlations with the cyano steroids show that this effect is not caused by changes in orientation of the 17-acetyl group. Initially, these results were surprising because it was known that, in accord with theory (21), substituents such as halogen and hydroxyl, located 1–3 to a methyl on a cyclopentane ring, generally cause a greater deshielding of the methyl group when they are *cis* to it than when they are *trans* to it (20, 22–24). Of particular pertinence, because the bridged D-ring of these steroids constitutes a 7-methylbicyclo-[2.2.1]heptane system (where the 7-methyl is C_{18} of the steroid), is the fact that this effect has been confirmed in a variety of 7-methylbicyclo[2.2.1]heptanes (25). In the latter series, *exo*-substituents deshield the 7-*syn*-methyl group by 0.16–0.31 p.p.m., which is 2–6 times more than that caused by the corresponding *endo*-substituents. Calculations show that such substituents, when located at the 2-position of a 7-*syn*-methylbicyclo[2.2.1]heptane, should deshield the methyl hydrogens least when the substituent is *endo*, slightly more in the 2-dehydro compound, and most when the substituent is *exo* (21).

In view of this dicussion, the 16β-substituted compounds listed in Table I appear to show surprisingly little deshielding of the C_{18}-hydrogens by the 16β-substituents. For example, in Compounds IX, X, XII, XIII, XIV, and XV the *endo*-carbomethoxy group causes a deshielding of the C_{18}-hydrogens, which averages 0.06 p.p.m., but the *exo*-carbomethoxy group of Compound XVI has no apparent effect on them. Also, an alternative estimate of the effect of such a carbomethoxy group can be made by assuming that the effect of a 2-substituent on the 7-*syn*-methyl in the bornane system is a linear function of Taft's σI value (26) for such a substituent; such a relationship has been shown to hold for hydrogens on the carbon beta (27) or alpha (28) to the substituted one. Extrapolation

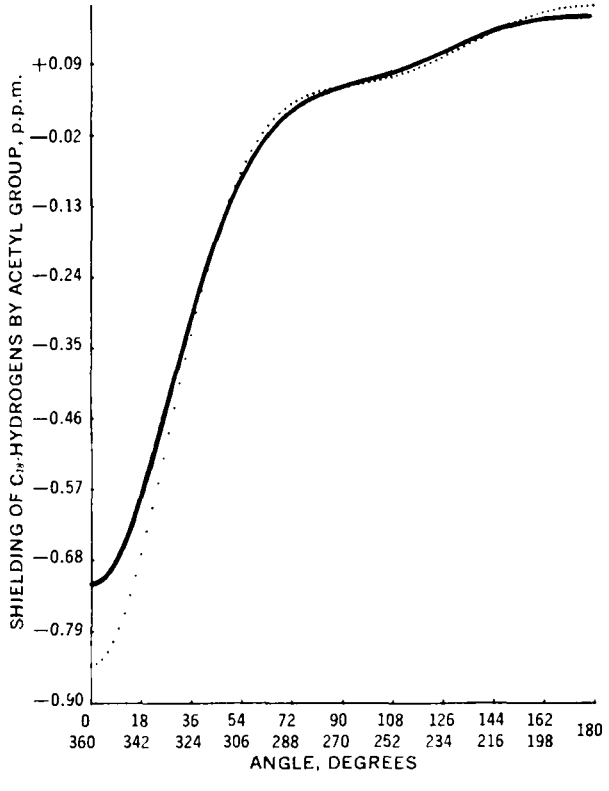


Figure 1—*Shielding of C_{18}-hydrogens by acetyl group* versus *the angle defined by the C_{20}-oxygen bond axis and the vector that is perpendicular to the C_{17}–C_{20}-bond axis and that passes through the center of rotation of the C_{18}-hydrogens. The solid line was calculated for XXVII, and the dotted line was calculated for XXVIII.*

[4] In response to a referee's suggestion that the restricted conformational freedom, postulated for the 17-acetyl compounds, should be partly cancelled by an increase in temperature, the effect of temperature on the chemical shift of the C_{18}-hydrogens of progesterone and of Compounds III, XIV, and XXII was studied. The C_{18}-hydrogens of all of these compounds appear to be less shielded at higher temperatures. While this could be interpreted as supporting the present position, it was decided not to attribute any significance to these data because: (*a*) in carbon tetrachloride or deuteriochloroform, the shifts are so small as to be only slightly greater than the probable error of the measurements; (*b*) the shifts are solvent dependent (larger in dioxane and much larger in dimethyl sulfoxide than in carbon tetrachloride or deuteriochloroform; and (*c*) other peaks such as that of the C_{18}-hydrogens also shift with temperature. Effects (*b*) and (*c*) could result from temperature-dependent solvent–solute interactions of sufficient magnitude as to obscure the observation of the rotational isomerism.

from the known effect of 2-substituents in the bornane system (25) leads to the prediction that a 16α-carbomethoxy group, in the bridged steroids, should cause a deshielding of the C_{18}-hydrogens of 0.06 p.p.m., as observed, and that a 16β-carbomethoxy group should cause a deshielding of 0.20 ± 0.05 p.p.m. By a similar process, one can estimate that the effect of a 16β-trifluoromethyl group on the C_{18}-hydrogens should be to deshield them by 0.24 ± 0.05 p.p.m.

In the discussion of the effects on the shielding of the C_{18}-protons resulting from changes in configuration of 16-substituents, calculations were based on the geometry that would be obtained if the substituent were a halide or hydroxyl group. However, the results of those calculations might not be valid for the trifluoromethyl or carboxy ester groups. To avoid the problems introduced by possible hindered rotation of the carbomethoxy group, calculations were based on the trifluoromethyl group and were further simplified by considering only the effects caused by the net dipole of the trifluoromethyl group. These calculations (21) indicate that, to the extent that deshielding of the C_{18}-protons by the trifluoromethyl group is caused by a direct electrostatic effect rather than by a classical inductive effect, the deshielding should be greatest when the trifluoromethyl group is *endo* (alpha). The change in geometry caused by introduction of a 15,16-double bond should cause the deshielding to decrease by 50%, and a change to an *exo*- (beta) orientation of the trifluoromethyl group should result in a *shielding* twice as great as the *deshielding* caused by the field effect of the *endo*-isomer. While these results are based on crude approximations[5], they do correlate exactly with the chemical shifts found for the 16α- and 16β-carboxy ester substituted compounds. The possibility of undefined vicinal interactions in the 15,16-disubstituted compounds makes them a less reliable test of the validity of this approach. While the deshielding caused by the 15-dehydro-16-carbomethoxy moiety is clearly excessive in terms of the predictions, a number of factors, including resonance interactions which put a partial positive charge at C_{13}, could easily account for the discrepancy. This is unfortunate in the sense that the plurality of possible explanations could mask a small but anomalous deshielding peculiar to the 7-methylbicyclo-[2.2.1]heptadiene system[6].

EXPERIMENTAL[7]

3β-Acetoxy-14α,17α-ethano-5α-pregnan-20-one (III)—A solution of 291 mg. of II in 100 ml. of ethanol and 4 ml. of water was reduced over 52 mg. of 10% palladium-on-charcoal, at an initial pressure of 3.71 kg./cm.², for 18 hr. at room temperature. Standard workup of the reaction mixture afforded 235 mg. of III as white flakes from methanol, m.p. 177–179°.

Anal.—Calc. for $C_{23}H_{38}O_3$: C, 77.68; H, 9.91. Found: C, 77.76; H, 9.85.

3β-Acetoxy-14α,17α-ethano-15β,16β-dicarbomethoxy-5-pregnen-20-one (XVIII)—A solution of 1.0 g. of XIX in 200 ml. of methanol was hydrogenated over 150 mg. of 10% palladium-on-charcoal at an initial pressure of 3.71 kg./cm.² for 13 hr. at room temperature. Standard workup afforded XVIII, in a yield of 698 mg., as white rods from ethanol, m.p. 176–177°; ν (mineral oil): 1735 (sh), 1730, 1690, and 1625 cm.⁻¹. The NMR spectrum has singlets at δ 2.06 [OC(=O)CH_3], 2.22 (C_{21} H's), 3.75, and 2.85 [C(=O)OCH_3].

Anal.—Calc. for $C_{29}H_{40}O_7$: C, 69.58; H, 8.05. Found: C, 69.53; H, 7.85.

3β-Acetoxy-17-cyano-15,16-dicarbomethoxy-14α,17α-etheno-5,15-androstadiene (XXXI)—A mixture of 314 mg. of 3β-acetoxy-17-cyano-5,14,16-androstatriene (2), 5 mg. of hydroquinone, and 1.0 ml. of dimethyl acetylenedicarboxylate was heated at 128–130°, in a sealed tube, for 148 hr. The crude product was chromatographed over 12 g. of acid-washed alumina[8]. Elution with benzene afforded XXXI as a foam (from ether) in a yield of 285 mg. (68%); ν (mineral oil): 2242 and 1730 cm.⁻¹. The NMR spectrum had singlets at δ 2.02 [OC(=O)CH_3] and 3.82 (OCH_3), a multiplet at δ 5.35 (C_6 H), and doublets at δ 6.83 and 6.88 (bridge vinyl hydrogens).

Anal.—Calc. for $C_{28}H_{33}NO_6$: C, 70.13; H, 6.94; N, 2.92. Found: C, 70.36; H, 7.26; N, 2.76.

3β-Acetoxy-17-cyano-15β,16β-dicarbomethoxy-14α,17α-ethano-5-androstene (XXXII)—A solution of 151 mg. of XXXI in 200 ml. of methanol and 5 ml. of water was reduced over 22 mg. of 10% palladium-on-charcoal for 18 hr. at room temperature under an initial pressure of 3.65 kg./cm.². The product was chromatographed over 15 g. of neutral activity III alumina[9]. Elution with mixtures of ethyl acetate and benzene afforded XXXII as a glass, in a yield of 96 mg.; ν ($CHCl_3$): 2335 and 1722 cm.⁻¹. The NMR spectrum had singlets at δ 2.04 [OC(=O)CH_3] and 3.84 [C(=O)-OCH_3] and a multiplet at δ 5.35 (C_6 H).

Anal.—Calc. for $C_{28}H_{37}NO_6$: C, 69.54; H, 7.71; N, 2.90. Found: C, 69.49; H, 7.64; N, 2.80.

3β-Acetoxy-14α,17α-etheno-15α,16α-dicarboxyanhydride-5-pregnen-20-one (XXXV)—A mixture of 0.35 mg. of 3β-acetoxy-5,14,16-pregnatrien-20-one, 0.45 g. of maleic anhydride, and 15 mg. of hydroquinone was heated at 95–100° for 25 hr. in a sealed glass tube under nitrogen. The unreacted maleic anhydride was removed by distillation under reduced pressure at 100°. The residue was chromatographed over 15 g. of silica gel. Benzene containing up to 10% ethyl acetate eluted XXXV as a foam in a yield of 0.32 mg.; ν (mineral oil): 1830, 1765, 1720, and 1685 cm.⁻¹. The NMR spectrum has singlets at δ 2.04 [OC(=O)CH_3] and 2.31 (C_{21} H's), doublets at δ 3.50, 4.45 [J = 8 Hz., CC(=O)H], 6.39 and 6.49 (J = 6.5, CH=CH), and a multiplet at 5.46 (C_6 H).

Anal.—Calc. for $C_{27}H_{32}O_6$: C, 71.66; H, 7.13. Found: C, 71.57; H, 7.20.

SUMMARY

Correlation of the chemical shifts of 30 14α,17α-bridged 20-ketopregnanes and of four similarly bridged 17-cyanoandrostanes, by means of additive substituent constants, requires the assumption that, depending upon the substitution pattern at C_{16}, the acetyl group of the pregnanes exists in one of two possible rotomer forms. Thus, introduction into these bridged pregnanes of a 16β-substituent or of a 16-substituent on a 15,16-double bond presumably causes the β-oriented carbonyl group to rotate toward C_{18}, which results in a 0.11 p.p.m. deshielding of the C_{18}-hydrogens. Support for this assumption is provided by X-ray crystal structures which were determined for 3β-acetoxy-14α,17α-ethano-16α-iodo-5-pregnen-20-one and for 14α,17α-etheno-15,16-di(trifluoromethyl)-4,15-pregnadiene-3,20-dione. Calculation, by the method of ApSimon *et al.* (8, 18), of the shielding effect of the acetyl group for each of the above structures indicates that the C_{18}-hydrogens of the latter compound should be deshielded by 0.07 p.p.m. relative to those of the former.

The finding that carboxy ester and trifluoromethyl groups substituted at the 15β- and/or 16β-positions of these bridged steroids cause little deshielding of the C_{18}-hydrogens contrasts sharply with the known tendency of 2-*exo*-halide functions to deshield 7-*syn*-methyl groups strongly in bicyclo[2.2.1]heptane systems. However, the dipoles of the former substituents bear a different geometrical relationship to the methyl than do those of the latter. Calculation of the expected field effects for these compounds shows that the observed chemical shifts are normal. Calculations also reveal that

[5] Approximations because: (*a*) the calculated values show large changes for relatively small variations in molecular geometry, (*b*) the geometry shown in the Dreiding models for the β-isomer is not correct in that it does not compensate for the crowding between the trifluoromethyl and methyl groups, (*c*) the authors did not correct for any partial charge transfer from the trifluoromethyl group to C_{16} or for the interaction of the 16-substituent with the double bond in the 15-dehydro compound, and (*d*) the method of calculation may be invalid for the β-isomer because of th proximity of the trifluoromethyl and methyl groups.

[6] Introduction of double bonds into norbornanes has been found to cause the chemical shifts of the C_7-hydrogens to change anomalously (7, 29, 31). While a partial explanation for this phenomenon has been advanced (28, 30, 31), recent data raise doubts as to its adequacy (32), and no satisfactory explanation has yet been put forward for the unusual deshielding of the corresponding hydrogens of norbornadiene (29). A similar effect of reduced magnitude seems to be shown by the methyl hydrogens of 7-methylbicyclo[2.2.1]heptadiene (33) which are more deshielded than the corresponding hydrogens of either of the 7-methylbicyclo[2.2.1]heptenes (7). In contrast, the *tert*-butyl hydrogens of the corresponding 7-*tert*-butyl compounds appear to have normal chemical shifts (34).

[7] Melting points were determined in capillary tubes on a Mel-Temp apparatus and are uncorrected. Elemental analyses were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn. NMR spectra were determined in $CDCl_3$ on a Varian A-60 spectrometer and are reported in parts per million downfield from a tetramethylsilane internal standard.

[8] Merck.

[9] Woelm.

Table III—NMR Substituent Constants[a] for 14α,17α-Ethano Bridged Steroids

Substituent	C_{18}-Hydrogens, p.p.m.	C_{19}-Hydrogens, p.p.m.
14α,17α-Ethano-5α-pregnan-20-one	0.865	0.80
17α-Cyano-14α,17α-ethano-5α-androstane	1.005	0.80
15-Dehydro	−0.02	−0.01
15′-Dehydro	0.0	−0.05
16β-Substituent or Δ15-16-substituent in pregnane series	0.11	0
16α-Carboxy	0.055	−0.01
16α-Carbomethoxy	0.055	−0.01
16β-Carbomethoxy	−0.12	−0.02
15α,16α-Dicarboxyanhydride	0.11	0.02
15β,16β-Dicarbomethoxy	0.08	0.02
15β,16β-Di(trifluoromethyl)	−0.015	0.02
16α-Iodo	0.015	−0.02
19-Nor	0.015	---
15-Dehydro-16-carbomethoxy	0.27	0.01
15-Dehydro-15,16-dicarbomethoxy	0.31	0.03
15-Dehydro-15,16-di(trifluoromethyl)	0.27	0.03

[a] For solution in deuteriochloroform. A positive shift indicates a shift downfield from tetramethylsilane.

the strong deshielding associated with a substituted 15,16-double bond could be a normal consequence of a field effect arising from the dipole created by electron donation from the double bond to the substituent.

While it appears possible to explain the NMR observations on these compounds, many discussed effects could not have been anticipated. Because of this situation and because of continuing reports of anomalies in related small bridged ring compounds (35, 36), caution should be used in interpreting the NMR spectra of such substances.

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Biopharmaceutics of Drugs Administered in Lipid-Containing Dosage Forms I: GI Absorption of Griseofulvin from an Oil-in-Water Emulsion in the Rat

PHILIP J. CARRIGAN and THEODORE R. BATES▲

Abstract □ The GI absorption characteristics of micronized griseofulvin from an oil-in-water emulsion dosage form, an oil suspension, and an aqueous suspension were assessed in the rat. Although there was a slight delay in the time of occurrence of the peak plasma concentration of griseofulvin following its oral administration in the emulsion, this dosage form produced a mean peak plasma antibiotic level 1.5 and 2.3 times higher than the oil and aqueous suspensions, respectively. In addition, the emulsion dosage form significantly increased the time over which plasma concentrations of drug were maintained above 1 mcg./ml. All six rats dosed with the emulsion attained this 1-mcg./ml. plasma level or greater (mean duration of 7.5 hr.), while only two out of six rats dosed with the aqueous suspension (duration of 3.7 and 4.0 hr., respectively) and four out of six rats dosed with the oil suspension (mean duration of four rats of 3.9 hr.) ever achieved this plasma concentration. Based on area under the plasma concentration *versus* time curve measurements, it was established that the bioavailability of micronized griseofulvin can be increased 2.5-fold, as compared to an aqueous suspension, by orally administering this relatively water-insoluble antibiotic in an oil-in-water emulsion dosage form.

Keyphrases □ Griseofulvin (micronized) GI absorption profiles—oil-in-water emulsion compared to oil suspension and aqueous suspension, rats □ Absorption, GI, micronized griseofulvin—plasma concentration *versus* time curves, oil-in-water emulsion compared to oil and aqueous suspensions □ Lipid-containing dosage forms—GI absorption of micronized griseofulvin from oil-in-water emulsion, oil suspension, and aqueous suspension

Griseofulvin is a relatively water-insoluble, systemic, antifungal antibiotic, whose oral absorption has been shown to be dissolution rate limited (1). As a result, its absorption from the GI tract of lower animals (2, 3) and man (4–6) has been characterized as being erratic and incomplete. The rate and extent of absorption of this drug in man have also been shown to be dependent on particle size (7, 8), dosage form (1, 9, 10), and formulation factors (11, 12).

Several investigators demonstrated that the absorption of both regular and micronized griseofulvin in rats (13), guinea pigs (14), and man (5, 7, 15) could be markedly enhanced if coadministered with meals high in fat or triglyceride content. The application of these findings to the clinical treatment of fungal infections offers obvious advantages. However, to date, there are no commercially available dosage forms containing the drug and lipid. Since poor palatability and patient acceptance of an oil suspension dosage form is anticipated, it appears that an oil-in-water emulsion would be the best delivery system for this drug.

The GI absorption of drugs orally administered to intact animals in two- or three-phase, lipid-in-water emulsion dosage forms has received limited attention in the literature (16–21), and no studies have explored the various factors affecting drug release and absorption from such dosage forms. The emulsion dosage form is unique in that it represents the only conventional dosage form containing appreciable quantities of triglycerides or other lipids. The potential influence of the presence of lipid in the GI tract on the absorption of drugs (22) prompted the present preliminary investigation, which was designed to assess the absorption profiles of micronized griseofulvin following its oral administration to rats in the form of an aqueous suspension, a corn oil suspension, and a three-phase corn oil-in-water emulsion dosage form.

EXPERIMENTAL

Materials—The griseofulvin[1] employed in this study was the micronized form (specific surface area 1.32 $m.^2/g.$). Polysorbate[2] 60, mono- and diglycerides of edible fats[3] (I), and corn oil USP[4] were used as received. Anhydrous, reagent grade ether was distilled, immediately prior to use, from an all-glass still having a 47-cm. vigreaux column. Reagent grade anhydrous methanol was treated with activated charcoal[5] to remove fluorescent impurities; water was double distilled.

Dosage Forms—Three dosage forms were subjected to *in vivo* bioavailability testing. The first was an aqueous suspension containing 10 mg. of micronized griseofulvin and 10 mg. of polysorbate 60/ml. The second preparation was a corn oil suspension containing 25 mg. of micronized griseofulvin and 25 mg. of polysorbate 60/ml. The third dosage form was a three-phase oil-in-water emulsion containing 10 mg. of micronized griseofulvin, 10 mg. of polysorbate 60, 10 mg. of I, 0.4 ml. of corn oil, and a sufficient quantity of distilled water to make 1 ml. The emulsion was refined in a blender[6] and finally passed twice through a hand homogenizer[7]. The mean globule size of the internal (oil) phase of the resultant emulsion, as determined by a modification of the photomicrographic method of Singiser (23), was 5.2 μ in diameter (3.8 μ < 90% of globules < 9.7 μ).

Each test dosage form was assayed in duplicate for drug content by dissolving an aliquot of the preparation in ethanol and subsequently measuring its absorbance at 292 nm. in a recording spectrophotometer[8]. The resultant absorbance values were converted to concentration units with the aid of a Beer's law plot constructed over the 0–10-mcg./ml. range. The constituents of the three dosage forms, in the quantities present in the assay samples, were found not to interfere with the determination of griseofulvin.

***In Vivo* Absorption Studies**—Adult, male, Sprague–Dawley rats[9], weighing 250–300 g., were fasted for 20 hr. prior to, and for a 12-hr. period subsequent to, the initiation of the absorption experiments. However, they were allowed free access to water throughout the experiment. Each freshly prepared dosage form was administered by oral intubation to six rats at a constant griseofulvin dosage level of 50 mg./kg. body weight. The dosing volume employed for

[1] Supplied by Schering Corp., Bloomfield, NJ 07003
[2] Supplied as Tween 60 by ICI America, Inc., Atlas Chemical Division, Wilmington, DE 19899
[3] Supplied as Atmul 84 by ICI America, Inc., Atlas Chemical Division, Wilmington, DE 19899
[4] Amend Drug and Chemical Co., New York, NY 10010
[5] Norit, Fisher Scientific Co., Rochester, NY 14624
[6] Waring blender, model PB-5A, with Polytron Rotor-Stator, VWR Scientific, Rochester, NY 14603
[7] Model 33998-001, VWR Scientific, Rochester, NY 14603
[8] Beckman DB-G, Beckman Instruments Inc., Fullerton, CA 92634
[9] Obtained from Blue Spruce Farms, Altamont, NY 12009

Table I—Plasma Levels of Griseofulvin (Micrograms per Milliliter) following its Oral Administration as a Single 50-mg./kg. Dose in Three Test Dosage Forms

Dosage Form	Rat	Hours 1	2	4	6	8	12	24
Aqueous suspension	1	0.508	1.08	2.04	0.941	0.650	0.450	0.079
	2	0.263	0.527	0.714	0.213	0.276	0.196	0.109
	3	0.162	0.271	0.361	0.199	0.168	0.126	0.027
	4	0.822	1.31	1.08	0.940	0.661	0.469	0.112
	5	0.195	0.389	0.781	0.595	0.307	0.201	0.064
	6	0.237	0.427	0.383	0.245	0.130	0.215	0.058
	Mean (*SE*)[a]	0.364 (0.10)	0.667 (0.17)	0.892 (0.25)	0.522 (0.14)	0.365 (0.10)	0.276 (0.06)	0.075 (0.01)
Oil suspension	7	0.283	0.169	0.645	0.754	0.631	0.178	0.078
	8	0.250	0.359	0.782	0.886	0.681	0.554	0.107
	9	0.416	0.855	2.00	0.491	0.557	0.488	0.043
	10	0.211	0.442	1.08	0.973	0.616	0.246	0.086
	11	0.625	1.39	2.04	2.24	1.70	0.488	0.037
	12	0.363	0.904	1.25	0.779	0.646	0.536	0.031
	Mean (*SE*)[a]	0.358 (0.06)	0.686 (0.18)	1.30 (0.24)	1.02 (0.25)	0.805 (0.18)	0.415 (0.06)	0.063 (0.01)
Oil-in-water emulsion	13	0.313	0.606	1.73	1.84	1.51	0.761	0.055
	14	0.405	0.680	2.96	3.11	2.60	0.494	0.033
	15	0.265	0.737	1.65	1.75	1.78	1.40	0.100
	16	0.438	1.39	2.68	1.99	0.733	0.197	0.035
	17	0.389	1.10	2.15	1.90	0.974	0.315	0.066
	18	0.316	0.619	1.23	1.32	1.19	0.790	0.083
	Mean (*SE*)[a]	0.354 (0.03)	0.855 (0.13)	2.07 (0.27)	1.98 (0.24)	1.46 (0.27)	0.660 (0.18)	0.062 (0.01)

[a] Standard error of the mean in parentheses.

the aqueous suspension and emulsion dosage forms was 5.0 ml./kg. body weight. To maintain the volume of oil administered in the form of the corn oil suspension consistent with that of the emulsion dosage form, a dosing volume of 2.0 ml./kg. body weight was used. The animals were subsequently placed in restraining cages, and approximately 0.5-ml. blood samples were collected from the tail vein into heparinized tubes at 1, 2, 4, 6, 8, and 12 hr. postadministration. Twenty-four-hour samples were obtained *via* decapitation. The blood samples were then centrifuged, and a 0.2–0.3-ml. aliquot of the plasma obtained was accurately measured directly into glass-stoppered extraction vessels. A 2.0-ml. plasma sample was obtained at the 24-hr. time period. All plasma samples were immediately frozen solid and assayed for intact drug content within 48 hr. of collection.

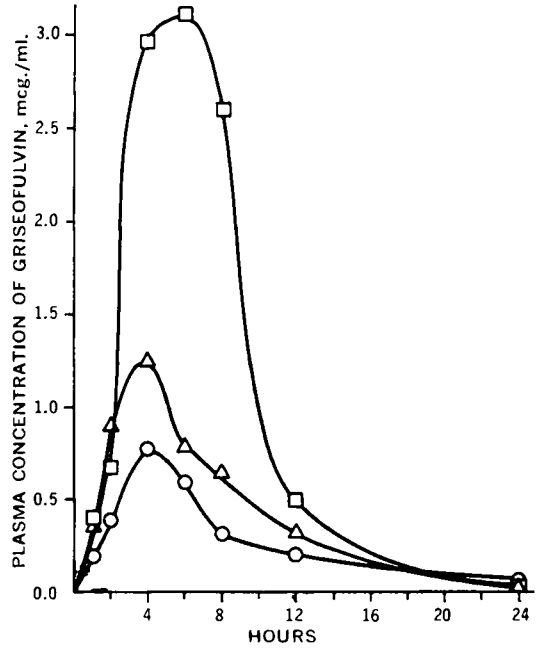

Figure 1—*Representative plasma concentrations of griseofulvin as a function of time following the oral administration of a 50-mg./kg. dose of micronized griseofulvin to rats in the form of an aqueous suspension* (○), *a corn oil suspension* (△), *and a corn oil-in-water emulsion* (□) *dosage form.*

Assay Procedure for Griseofulvin in Plasma—The specific spectrofluorometric procedure of Shah *et al.* (24) for plasma griseofulvin was appropriately modified for handling small plasma samples. The method essentially involves extracting griseofulvin from the plasma samples under neutral pH conditions into 7 ml. of freshly distilled ether and subsequently evaporating to dryness an aliquot (3–5 ml.) of the organic phase. The residue is then reconstituted with 2 ml. of a 1:1 (v/v) mixture of water and methanol and washed with 2 ml. of reagent grade hexane; the fluorescence of the hydroalcoholic phase was measured on a spectrofluorometer[10] using activation and emission wavelengths of 303 and 428 nm. (uncorrected), respectively. Subsequently, 4 drops of concentrated sulfuric acid was added to the sample and the fluorescence was redetermined.

On each assay day, a linear calibration curve of net fluorescence intensity (sample minus quench reading) *versus* concentration, over the range of 0.0071–0.143 mcg./ml. of hydroalcoholic solution, was constructed by carrying standard solutions of griseofulvin through the entire extraction procedure. The net fluorescence values of the extracted plasma samples were first corrected for the small positive intercept value of the calibration plot and then converted to concentration units by dividing the corrected net values by the least-squares slope value of the calibration plot.

Recovery studies performed with plasma samples containing 0.040–0.800 mcg. of griseofulvin indicated that the extraction procedure employed was capable of quantitatively removing the drug from plasma [mean recovery $\pm SD$ ($n = 6$) was $105 \pm 2.4\%$].

RESULTS AND DISCUSSION

To provide a basis for valid comparisons, control dosage forms employed in this *in vivo* absorption study (*i.e.*, aqueous and oil suspensions) were designed to incorporate certain components present in the emulsion dosage form. For example, both control dosage forms contained the same amount of nonionic surfactant (polysorbate 60) and the same particle-size fraction of drug. In addition, animals dosed with either the oil suspension or emulsion dosage form received an equivalent amount of triglyceride (corn oil).

Based on the results of equilibrium solubility studies performed at 25°, it was established that at the time of dosing a maximum of only 0.5, 2.4, and 2.7% of the orally administered dose of drug was

[10] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Silver Spring, MD 20910

Table II—Peak Plasma Levels (C_{max}) and Its Time of Occurrence (T_{max}) following Oral Administration of Micronized Griseofulvin (50 mg./kg.) in Three Test Dosage Forms

	Aqueous Suspension		Oil Suspension		Oil-in-Water Emulsion	
	C_{max}, mcg./ml.	T_{max}, hr.	C_{max}, mcg./ml.	T_{max}, hr.	C_{max}, mcg./ml.	T_{max}, hr.
	1.31	2.0	2.24	6.0	3.11	6.0
	0.36	4.0	0.75	6.0	1.78	8.0
	0.71	4.0	1.25	4.0	2.15	4.0
	2.04	4.0	2.00	4.0	2.68	4.0
	0.78	4.0	1.08	4.0	1.84	6.0
	0.43	2.0	0.89	6.0	1.32	6.0
Mean (SE)[a]	0.94 (0.26)	3.3	1.37 (0.25)	5.0	2.15 (0.27)	5.7
Statistical significance[b]	N.S.		$p < 0.05$			
	$p < 0.02$					
Coefficient of variation, %	67.8	—	44.7	—	30.4	—

[a] Standard error of the mean in parentheses. [b] Determined by Student's t test.

in solution in the aqueous suspension, oil suspension, and emulsion dosage forms, respectively. Hence, differences encountered among the absorption characteristics of the drug from the three dosage forms cannot be attributed to this potential variable.

The results of *in vivo* absorption studies are summarized in Table I. Figure 1 depicts representative plasma griseofulvin concentration *versus* time plots obtained following the oral administration of a single 50-mg./kg. dose of micronized griseofulvin to individual rats in the aqueous suspension, oil suspension, and oil-in-water emulsion dosage forms. Examination of this figure, together with the mean data for the six animals dosed with each test preparation (Table II), reveals that a delay existed in the attainment of peak plasma drug levels following oral administration of both the oil suspension and emulsion as compared to the aqueous suspension (*i.e.*, 5–6 hr. *versus* 3 hr.). However, the magnitude of the mean peak plasma level of griseofulvin from the emulsion was approximately 1.5 and 2.3 times higher than from the oil and aqueous suspensions, respectively (Table II). These differences are highly significant as determined by Student's t tests performed on the mean data.

Griseofulvin blood levels greater than 1 mcg./ml. are required to effect a cure in human *Tinea capitis* infections (25). The same minimum effective blood level was also reported for dogs (26). If it is assumed that a similar value exists for the rat, an indication of differences among the dosage forms as to the duration of therapeutic levels may be realized. An interesting finding was that all six rats receiving the emulsion maintained a plasma level of 1 mcg./ml. or greater for approximately 6–11 hr. (mean duration of 7.5 hr.), while only two out of six rats dosed with the aqueous suspension (duration of 3.7 and 4.0 hr., respectively) and four out of six rats dosed with the oil suspension (mean duration of four rats of 3.9 hr.) ever achieved this proposed plasma level.

By using the trapezoidal rule, the area under the plasma griseofulvin level *versus* time curve from time zero to 24 hr. was determined for each animal and test preparation. The results of these area calculations, which reflect the amount of griseofulvin absorbed in 24 hr., are summarized in Table III. A one-way analysis of variance performed on the area under the plasma level–time curve for the three dosage forms yielded an F value of 10.4 (df 2,15) with $p < 0.005$. Individual differences between two treatments were tested statistically using Student's t test (Table III). As can be seen from an examination of the mean area under plasma level-time curve values, there is no statistical difference between the fraction of the oral dose of griseofulvin absorbed from the aqueous and oil suspensions. However, there is a highly significant 1.6- and 2.5-fold increase in the amount of drug absorbed from the emulsion dosage form as compared to the oil and aqueous suspensions, respectively. As reflected by the magnitudes of the coefficients of variation listed in Table II, the uniformity of absorption of micronized griseofulvin decreased in the following order: emulsion > oil suspension > aqueous suspension.

Table III—Bioavailability of Micronized Griseofulvin following Its Oral Administration as a Single 50-mg./kg. Dose in Three Test Dosage Forms

	Area under Plasma Level–Time Curve from Time Zero to Twenty-Four Hours (mcg.-hr./ml.)		
	Aqueous Suspension	Oil Suspension	Oil-in-Water Emulsion
	14.1	20.5	25.5
	3.36	9.32	16.0
	5.96	12.2	19.3
	13.2	12.5	25.3
	6.44	11.2	16.8
	4.59	7.12	15.9
Mean (SE)[a]	7.95 (1.87)	12.1 (1.86)	19.8 (1.85)
Statistical significance[b]	N.S.	$p < 0.02$	
	$p < 0.005$		
Coefficient of variation, %	57.4	37.6	22.8

[a] Standard error of the mean in parentheses. [b] Determined by Student's t test.

The physicochemical and/or physiological mechanism(s) by which the presence of triglyceride in the emulsion dosage form markedly enhances the absorption characteristics of griseofulvin, and possibly other poorly absorbed drugs, is currently under intensive investigation in these laboratories. Several possible physiological mechanisms being explored involve the influence of unemulsified and emulsified triglyceride (corn oil) on the rate of gastric emptying, GI motility, and bile flow. The results of these studies will be subsequently reported.

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Photolytic Decomposition of *N*-(2,6-Dichloro-*m*-tolyl)anthranilic Acid (Meclofenamic Acid)

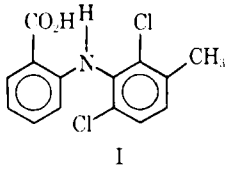
JOSE PHILIP▲ and DALE H. SZULCZEWSKI

Abstract □ Exposure of dilute solutions of *N*-(2,6-dichloro-*m*-tolyl)anthranilic acid to visible or UV light results in fairly rapid decomposition with concurrent formation of approximately equimolar amounts of 8-chloro-7-methylcarbazole-1-carboxylic and 8-chloro-5-methylcarbazole-1-carboxylic acids.

Keyphrases □ *N*-(2,6-Dichloro-*m*-tolyl)anthranilic acid (meclofenamic acid)—isolation, identification of photolytic decomposition products □ Meclofenamic acid [*N*-(2,6-dichloro-*m*-tolyl)anthranilic acid]—isolation, identification of photolytic decomposition products □ Photolysis, *N*-(2,6-dichloro-*m*-tolyl)anthranilic acid (meclofenamic acid)—isolation, identification of decomposition products

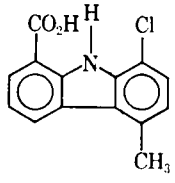
N-(2,6-Dichloro-*m*-tolyl)anthranilic acid (meclofenamic acid)[1], I, is an investigational new drug being evaluated for use as an anti-inflammatory agent. Results from previous studies in these laboratories indicated that one possible route of decomposition involved decarboxylation to the corresponding substituted diphenylamine in accord with the general chemistry of anthranilic acids. This reaction, however, proceeds at an appreciable rate only at elevated temperatures (100° for strongly acidic solutions or above the melting point, 258.5–259.5° in the case of the solid state) and would have little relevancy to dosage form shelflife at near ambient temperatures.

As part of continuing preformulation studies, the

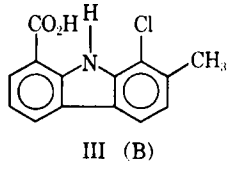


I

[1] Parke, Davis & Co.



II (A)



III (B)

influence of light, as another condition of stress, was studied.

DISCUSSION

Sequential UV spectra obtained during exposure of a dilute solution of I (c 0.00426%, methanol) to visible light[2] are shown in Fig. 1. There was a fairly rapid spectral change during the initial period of exposure, after which the spectra become essentially constant. Spectra obtained from aqueous alkaline solutions exposed in the same manner were qualitatively similar, whereas like solutions stored in the dark gave spectra invarient with time.

A thin-layer chromatogram obtained on a methanolic solution of I (R_f 0.7) exposed to the point of spectral redundancy showed the formation of at least two reaction products (1, R_f 0.4; and 2, R_f 0.31), one of which predominated. Available data indicate that I undergoes a facile reaction in the presence of light, but the nature of this reaction is unknown. Relevant information from the literature is rather scarce; however, the work of Bowen and Eland (1) indicated that diphenylamine is converted to carbazole in the presence of light.

To determine the nature of this reaction, reaction products were isolated for identification. A large volume of a more concentrated methanolic solution of I was exposed to UV light[3] (c 0.1%), and the solvent was removed under vacuum. The residue was recrystallized to remove residual I and the minor decomposition product. During the recrystallization, fractions were obtained whose R_f values were the same as the major decomposition product but whose

[2] Light cabinet (a steel cabinet equipped with several 100-w. incandescent bulbs).
[3] Hanovia Letheray germicidal lamps.

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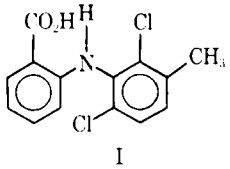
JOSE PHILIP▲ and DALE H. SZULCZEWSKI

Abstract □ Exposure of dilute solutions of *N*-(2,6-dichloro-*m*-tolyl)anthranilic acid to visible or UV light results in fairly rapid decomposition with concurrent formation of approximately equimolar amounts of 8-chloro-7-methylcarbazole-1-carboxylic and 8-chloro-5-methylcarbazole-1-carboxylic acids.

Keyphrases □ *N*-(2,6-Dichloro-*m*-tolyl)anthranilic acid (meclofenamic acid)—isolation, identification of photolytic decomposition products □ Meclofenamic acid [*N*-(2,6-dichloro-*m*-tolyl)anthranilic acid]—isolation, identification of photolytic decomposition products □ Photolysis, *N*-(2,6-dichloro-*m*-tolyl)anthranilic acid (meclofenamic acid)—isolation, identification of decomposition products

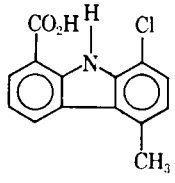
N-(2,6-Dichloro-*m*-tolyl)anthranilic acid (meclofenamic acid)[1], I, is an investigational new drug being evaluated for use as an anti-inflammatory agent. Results from previous studies in these laboratories indicated that one possible route of decomposition involved decarboxylation to the corresponding substituted diphenylamine in accord with the general chemistry of anthranilic acids. This reaction, however, proceeds at an appreciable rate only at elevated temperatures (100° for strongly acidic solutions or above the melting point, 258.5–259.5° in the case of the solid state) and would have little relevancy to dosage form shelflife at near ambient temperatures.

As part of continuing preformulation studies, the

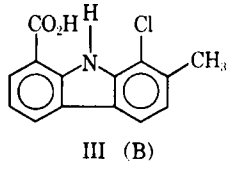


I

[1] Parke, Davis & Co.



II (A)



III (B)

influence of light, as another condition of stress, was studied.

DISCUSSION

Sequential UV spectra obtained during exposure of a dilute solution of I (c 0.00426%, methanol) to visible light[2] are shown in Fig. 1. There was a fairly rapid spectral change during the initial period of exposure, after which the spectra become essentially constant. Spectra obtained from aqueous alkaline solutions exposed in the same manner were qualitatively similar, whereas like solutions stored in the dark gave spectra invarient with time.

A thin-layer chromatogram obtained on a methanolic solution of I (R_f 0.7) exposed to the point of spectral redundancy showed the formation of at least two reaction products (1, R_f 0.4; and 2, R_f 0.31), one of which predominated. Available data indicate that I undergoes a facile reaction in the presence of light, but the nature of this reaction is unknown. Relevant information from the literature is rather scarce; however, the work of Bowen and Eland (1) indicated that diphenylamine is converted to carbazole in the presence of light.

To determine the nature of this reaction, reaction products were isolated for identification. A large volume of a more concentrated methanolic solution of I was exposed to UV light[3] (c 0.1%), and the solvent was removed under vacuum. The residue was recrystallized to remove residual I and the minor decomposition product. During the recrystallization, fractions were obtained whose R_f values were the same as the major decomposition product but whose

[2] Light cabinet (a steel cabinet equipped with several 100-w. incandescent bulbs).
[3] Hanovia Letheray germicidal lamps.

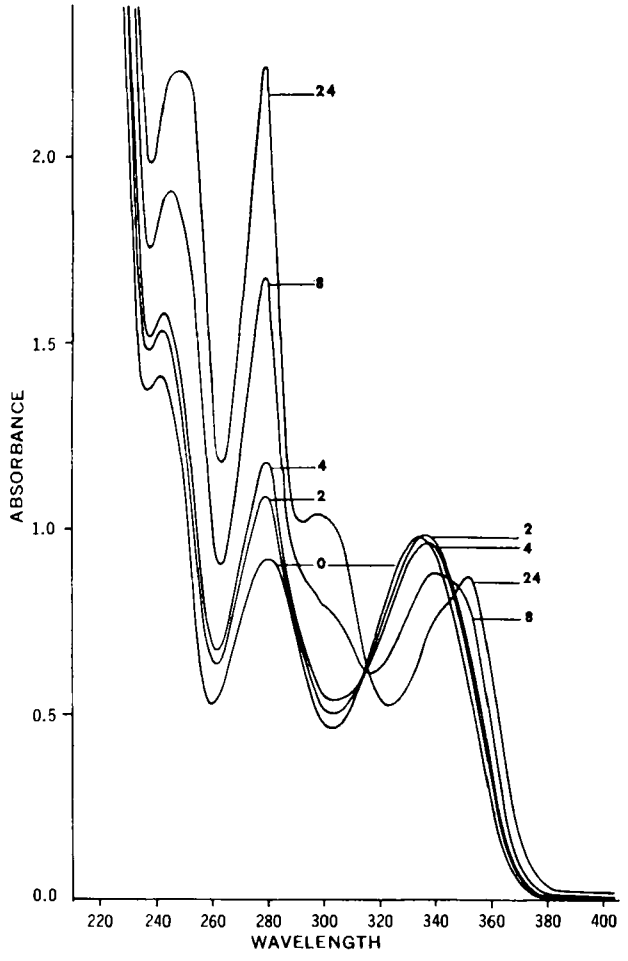


Figure 1—*Sequential UV spectra of the photolysis of I in methanol. Exposure time (hours) is noted.*

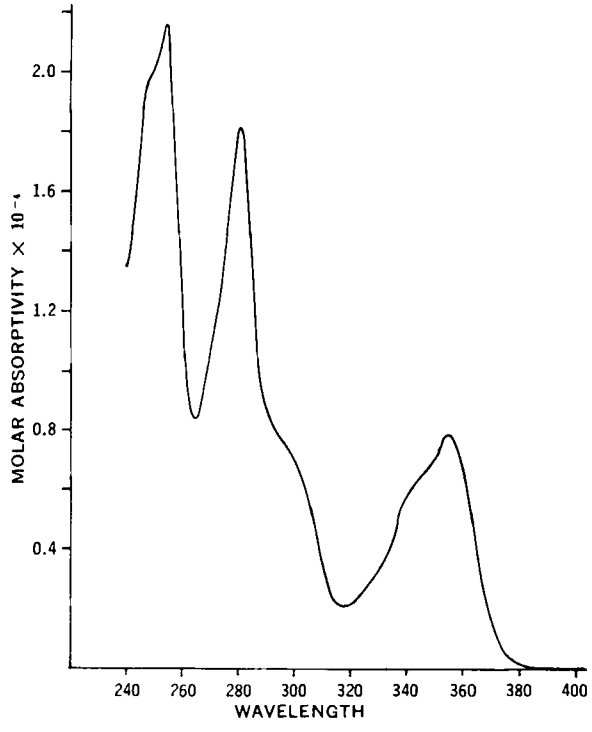


Figure 2—*UV spectrum of II in 95% methanol–0.01* N *hydrochloric acid.*

Table I—IR Spectral Data for Fractions A and B (KBr Dispersion)

Group	Wave Number, $cm.^{-1}$ A		B	
—NH	3450	(sharp)	3460	(sharp)
—CO_2H	3300–2400	(broad)	3300–2400	(broad)
	1665	(strong)	1680	(strong)
CO_2H dimer	928		920	

UV spectra differed. In particular, absorption near 300 nm. varied, taking the form of a shoulder in some and a distinct maximum in others (Figs. 2 and 3). The crystalline materials (A and B) giving these two spectra were investigated to determine structure.

The empirical formula, $C_{14}H_{10}ClNO_2$, as determined by elemental analysis and the molecular weight, 259, determined by mass spectroscopy for both A and B indicated that they were isomeric compounds, each differing from I by the elements of HCl. The presence of amino and carboxylic acid moieties in both was established from corresponding IR spectra (Table I). NMR spectra (Figs. 4 and 5 and Table II) were definitive and, together with preceding data and information, established the dominant photolytic products to be 8-chloro-5-methylcarbazole-1-carboxylic acid (II) and 8-chloro-7-methylcarbazole-1-carboxylic acid (III).

The relative amounts of II and III formed during photolysis were determined from the terminal UV spectrum of the spent reaction mixture. The observed absorbance ratio ($R_{obs} = A_{\lambda 302}/A_{\lambda 351}$) was 1.18, which indicated that the product distribution was 1 : 1.

EXPERIMENTAL[4]

Initial Photolysis Studies—Solutions of I, approximately 0.004%, were prepared in methanol and pH 9.0 phosphate buffer. Volumetric flasks containing 100-ml. aliquots of these solutions were placed in the light cabinet and sampled periodically for UV determination.

Preparation and Isolation of Major Photolysis Products—Two liters of a 0.1% methanolic solution of I was placed in a flat tray

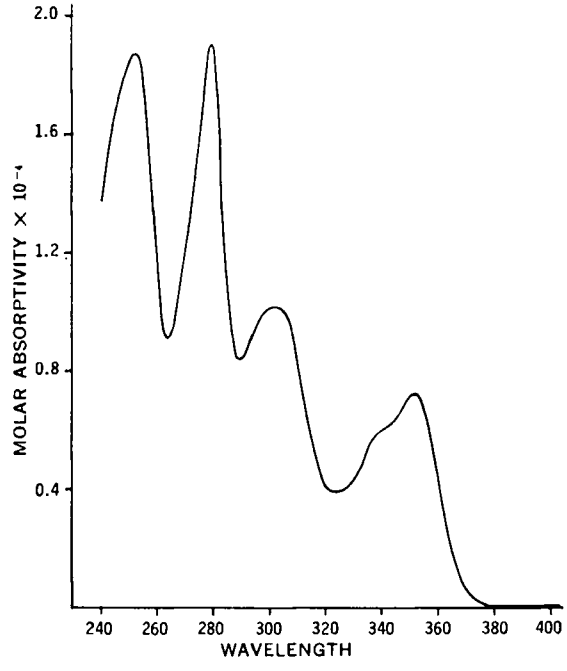


Figure 3—*UV spectrum of III in 95% methanol–0.01* N *hydrochloric acid.*

[4] UV spectra were determined using a Cary 14 spectrophotometer. NMR spectra were recorded on a Varian A-60 spectrophotometer. IR spectra were taken in potassium bromide disks using a Perkin-Elmer 457 grating IR spectrophotometer. Thin-layer chromatograms were run using silica gel plates (Quantum Industries, QIF, 250 μ).

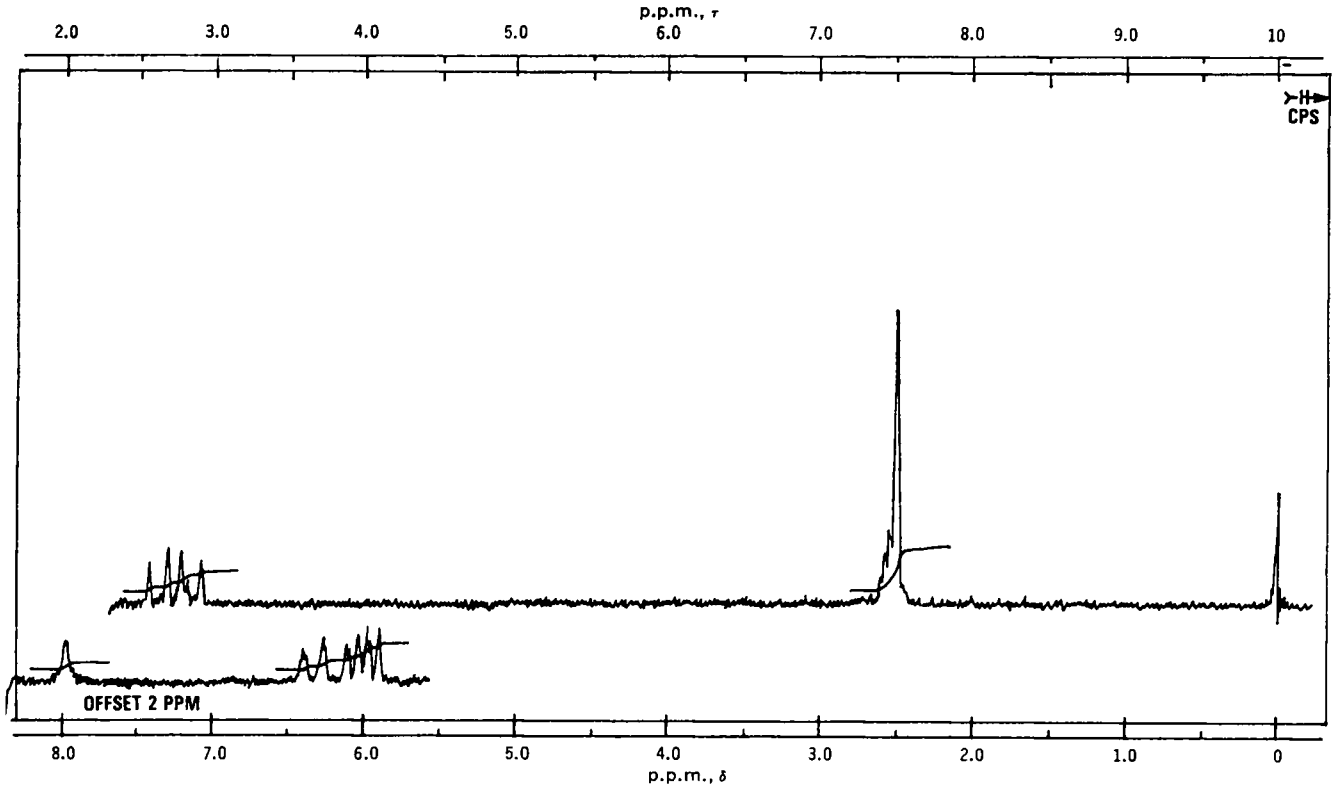
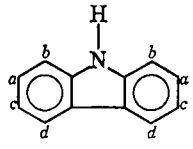


Figure 4—*NMR spectrum of III in dimethyl sulfoxide-*d_6. *For carbazole (acetone as solvent):*



the following assignments are made (2) (p.p.m.): a, *7.17;* b, *about 7.45;* c, *7.49; and* d, *8.12.*

and irradiated with three germicidal lamps[3] resting on the rims of the tray. After the reaction was essentially complete (monitored by TLC and UV spectroscopy), the solvent was removed and the residue was recrystallized from ethyl acetate–isooctane to free it of residual I and other minor products (TLC).

A second recrystallization from ethyl acetate yielded a crystalline precipitate (A) whose UV spectrum (Fig. 2) differed from the terminal spectrum (Fig. 1) obtained during the reaction in that there was no maximum in the 302-nm. region. Material obtained from the mother liquor of this recrystallization (B) showed a distinct maximum in this region. Furthermore, A showed an absorption in its NMR spectrum at 2.70 p.p.m., whereas B showed two peaks in this region (2.70 and 2.50 p.p.m.). Fraction B was then repeatedly recrystallized until the compound contained in A was removed to yield crystalline material whose NMR spectrum showed only a single peak at 2.50 p.p.m. Both compounds had identical behavior with regard to TLC [silica, chloroform–ethyl acetate–acetic acid (75:25:1)]; *i.e.*, they both had the same R_f (0.40) and matched the major decomposition product seen in the TLC of the spent reaction mixture. Neither A nor B melts below 290°.

Table II—NMR Data[a] Obtained for Fractions A and B

Group or Proton	Chemical Shifts, p.p.m.[b] 5-Methyl Isomer, II	7-Methyl Isomer, III
CH_3	2.70	2.50
H-2[c]	8.27 (d)	8.33 (d)
H-3[c]	7.30 (t)	7.30 (t)
H-4[c]	8.05 (d)	8.05 (d)
H-5[c]	—	7.97 (d)
H-6[c]	6.96 (d)	7.15 (d)
H-7[c]	7.37 (d)	—

[a] Chemical shifts are in parts per million from external tetramethylsilane; d = doublet, t = triplet. [b] Dimethyl sulfoxide as solvent. [c] Center of multiplet.

Anal.—Calc. for $C_{14}H_{10}ClNO_2$: C, 64.80; H, 3.86; Cl, 13.70; N, 5.41. Found (A): C, 64.74; H, 3.93; Cl, 13.61; N, 5.44; (B): C, 64.71; H, 4.03; Cl, 13.89; N, 5.35.

As seen in Figs. 4 and 5, whereas protons *a*, *b*, and *c* show only small shift differences, a large difference exists between the *d* protons and the remaining protons. In this case, the isomer having protons in the 5,6-positions, *i.e.*, III, would be expected to exhibit a larger shift difference than the isomer having protons in the 6,7-positions, *i.e.*, II. Protons in the 2-, 3-, and 4-positions are common to both structures. Furthermore, the anisotropic effect (3) of this aromatic system would likewise cause the signal arising from the methyl protons in the 5-methyl isomer to be shifted downfield to a greater extent than would the chlorine atom (position 8), which is adjacent to the methyl group in the 7-methyl isomer.

Absorptions characteristic of —NH— and —CO_2H were observed in the IR spectra (KBr dispersion) of both A and B (Table I).

UV spectra were determined in solvents as noted (Figs. 1–3). The UV properties for II, λ_{max} ($\epsilon \times 10^{-3}$), were 355 (7.98), 280 (18.4), and 253 (22.0) nm. For III, λ_{max} ($\epsilon \times 10^{-3}$), they were 351 (7.21), 302 (10.09), 278 (18.90), and 252 (18.65) nm.

The relative amounts of II and III as produced by photolysis were determined from the UV spectrum of the spent reaction mixture, with the assumption that the observed spectrum was the result of significant absorption contribution from only II and III.

Compounds II and III have equal molar absorptivities at 351 nm. whereas at 302 nm. absorptivity differs. The absorbance ratio, $R = A_{\lambda 302}/A_{\lambda 351}$, then reflects relative composition and is independent of concentration. The R values for pure II and III are 0.94 and 1.435, respectively. The fraction of the total carbazole photolysis

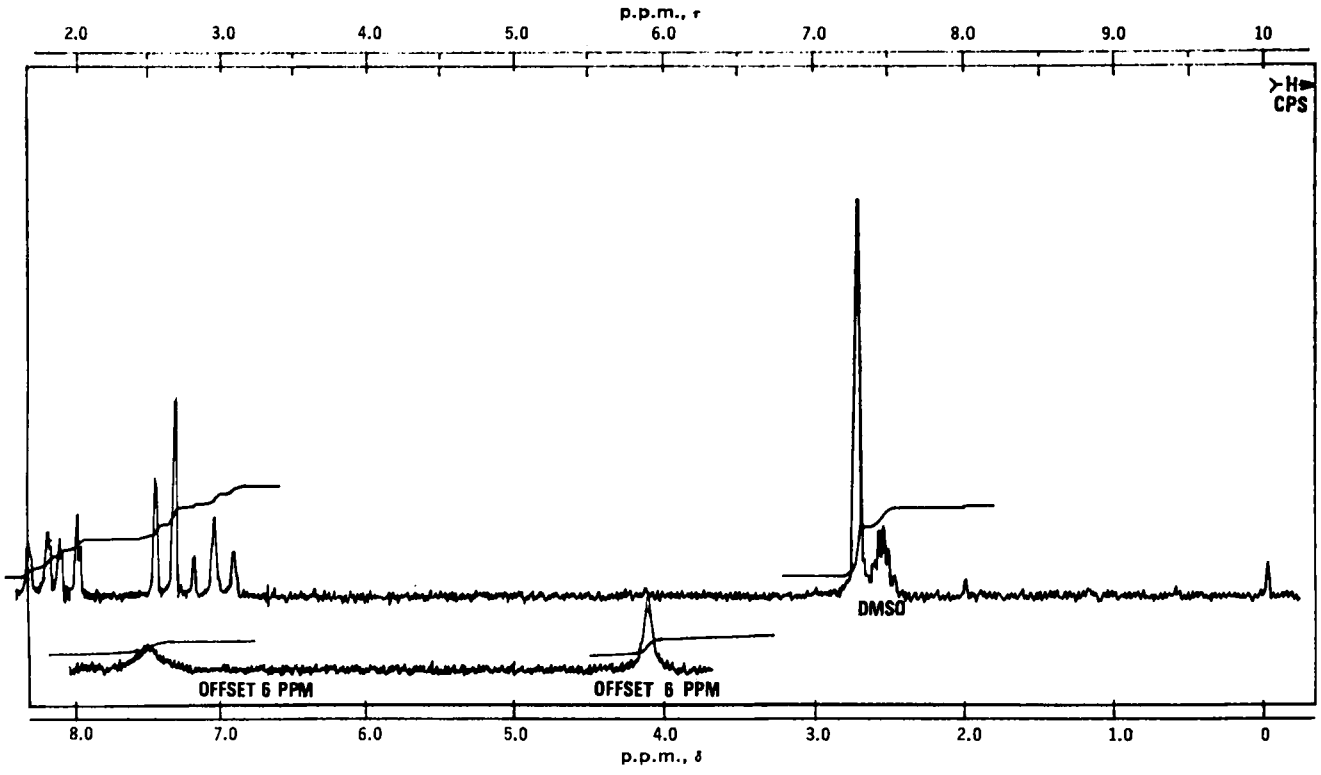
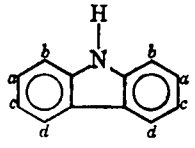


Figure 5—*NMR spectrum of II in dimethyl sulfoxide-*d_6. *For carbazole (acetone as solvent):*



the following assignments are made (2) (p.p.m.): a, *7.17;* b, *about 7.45;* c, *7.49; and* d, *8.12.*

products present as III is calculable from the following equation:

$$f_{III} = \frac{R_{obs} - R_{II}}{R_{III} - R_{II}} \qquad \text{(Eq. 1)}$$

The value of R_{obs} was 1.175, which then leads to an f_{III} value of 0.475 and an f_{II} value of 0.525. An equimolar mixture would have $f_{II} = f_{III} = 0.50$.

CONCLUSIONS

Irradiation of solutions of *N*-(2,6-dichloro-*m*-tolyl)anthranilic acid results in decomposition to 8-chloro-5-methylcarbazole-1-carboxylic acid and 8-chloro-7-methylcarbazole-1-carboxylic acid (and HCl). The photolysis products are formed in approximately equimolar amounts.

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Renal Contribution to Overall Metabolism of Drugs IV: Biotransformation of Salicylic Acid to Salicyluric Acid in Man

B. VON LEHMANN*, S. H. WAN†, S. RIEGELMAN▲, and C. BECKER

Abstract □ Salicylic acid was infused at three different steady-state levels to study its possible metabolism to salicyluric acid within the kidney in man. Concomitant with the salicylic acid infusion, radiolabeled salicyluric acid was infused at a constant rate to define its intrinsic renal clearance characteristics. Thereby, the rate of formation of salicyluric acid from salicylic acid, within the kidney, was calculated to be approximately 60–70% of the total amount formed.

Keyphrases □ Salicylic acid—renal metabolism to salicyluric acid in man □ Salicyluric acid—biotransformation after salicylic acid administration in man, renal clearance characteristics determined using salicyluric acid-^{14}C □ Renal metabolism—biotransformation of salicylic acid to salicyluric acid, man

The liver is usually considered the primary organ involved in the metabolism of drugs. Nevertheless, conjugation of aromatic acids with glycine in the kidney has been shown to take place both *in vitro* (1–7) and *in vivo* (8–10). Wan and coworkers (11–13) showed extensive *in vivo* conjugation in animals and reviewed the earlier studies in this field. In some species studied, the specific enzyme activity in the kidney has been found to be higher than that of the liver (1, 5–7, 10). However, Schachter and Manis (14) found no evidence for the renal synthesis of salicyluric acid from salicylic acid in man. Their statement is based on the observation that the clearance of salicyluric acid did not exceed the approximate renal plasma flow. The latter was calculated from the endogenous creatinine clearance. This conclusion is not in agreement with the results reported here.

Previous studies from these laboratories on the conversion of benzoic acid to hippuric acid in the rabbit (11), aminobenzoic acid to *p*-acetamidobenzoic acid and *p*-aminohippuric acid in the rabbit (12), and salicylic acid to salicyluric acid in the monkey (13) showed clearly that the kidney plays a major role as a metabolizing organ for these compounds. A summary of these findings is shown in Table I. These results encouraged the investigation of the renal rate of formation of salicyluric acid in man. A survey of the literature revealed that the apparent half-lives for the plasma disappearance of salicylic acid in man increased with dose (15–17), with values ranging from 2.4 to 19 hr. in adults. Similar results are evident from studies of the urinary excretion of salicylates as a function of dose (18), which show that the percentage of the dose excreted at a given time decreases with increasing dose. Practically all of an administered dose can be recovered in the urine as free, unaltered salicylic acid and as four major metabolites. At low doses, the main metabolite is salicyluric acid. The percent of salicyluric acid excreted in the urine has been observed to range from 0 to 91% of the dose of salicylic acid administered (14, 18, 19), decreasing as the total dose of salicylic acid increases. The remainder of the dose is excreted as salicyl phenolic glucuronide (12–30%), salicyl acyl glucuronide (0–10%), and gentisic acid (1%) (19) and its conjugates. The dose-dependent kinetics of salicylic acid have been attributed to the saturation of the metabolic process, which leads to the formation of salicyluric acid. This has been shown to be the reason for the observed wide variation in the plasma half-lives (18, 20–23). Since the rhesus monkey has been shown (12) to metabolize salicylic acid to salicyluric acid in similar amounts as man, it became increasingly interesting to examine if the human kidney functions similarly.

Table I—Renal Contribution to Metabolism of Benzoic Acid Derivatives and Salicylic Acid in Animals

Compound	Animal	Percent of Metabolite Formed in— Kidney	Liver[a]
Benzoic acid–hippuric acid	Rabbit	79	21
	Rabbit	28	72
	Rabbit	55	45
Aminobenzoic acid–*p*-acetamidobenzoic acid	Rabbit	52	48
Aminobenzoic acid–*p*-aminohippuric acid	Rabbit	70	30
Salicylic acid–salicyluric acid	Monkey	100	0

[a] Metabolism within the body as a whole, other than that which takes place in the kidney, is referred to in this table as "liver" metabolism.

EXPERIMENTAL

Subject One healthy male adult with no history of liver or kidney dysfunction or salicylate idiosyncrasy was studied.

Materials—Salicyluric acid-carboxy-^{14}C was synthesized, m.p. 170°, from salicylic acid-carboxy-^{14}C[1], following the method of Froemming and Vollenberg (24). The specific activity, as determined by liquid scintillation counting, was 34.9 μc./mmole. The efficiency was calculated using the internal standard method. A purity of >99% was established by radioscan of the TLC silica gel plate. The solvent system was butanol–acetic acid–water (4:1:1). Salicylic acid and salicyluric acid have R_f values of 0.49 and 0.62, respectively. No trace of radioactive starting material was observed in the radioscan. The absence of glycine was confirmed by NMR, which is capable of detecting low concentrations of glycine (2% or more).

The salicylic acid infusion solution was prepared by dissolving varying amounts of sodium salicylate in normal saline, whereas salicyluric acid was only soluble in a 5% sterile solution of sodium bicarbonate. All intravenously administered solutions were prepared under aseptic techniques and were bacteriologically filtered shortly before use.

[1] Tracerlab.

Infusion Procedures—A 10-μc. sample of salicyluric acid-carboxy-^{14}C, isotopically diluted in 300 mg. total salicyluric acid and dissolved in 5% sodium bicarbonate solution, was infused at a rate of 27.4 mg./hr. throughout the experiment. An intravenous priming dose of 200 mg. (8 μc.) salicyluric acid-carboxy-^{14}C was administered 20 min. prior to the start of the infusion. This initial infusion was done to define the renal clearance characteristics of salicyluric acid in man. The salicyluric acid infusion was continued through the experiment to ascertain the constancy of the salicyluric acid renal clearance. Two hours later, an intravenous priming dose of 250 mg. salicylic acid was given, followed by infusion of salicylic acid at a dose level of 14.2 mg./hr. (level 1). This procedure was repeated with an intravenous priming dose of 50 mg. salicylic acid and an infusion rate of 28.4 mg./hr. (level 2) and another intravenous priming dose of 100 mg. salicylic acid and an infusion rate of 56.8 mg./hr. (level 3). The infusion was maintained at each level for 2 hr. At these doses, the calculated plasma levels of salicylic acid were 5–20 mcg./ml. All infusions were made into a fast flowing intravenous drip of normal saline–5% dextrose.

Sampling Time—Five-milliliter blood samples were obtained at each sampling period. Blood and urine blanks were taken before the start of the experiment. Four blood samples were taken at 15-min. intervals during the 2nd hr. of the infusion at each level. (A total of 16 blood samples was taken.) Urine was voided in 15-min. intervals and collected between the blood sampling times for a total of 20 urine samples. Due to the amount of fluids infused into the test subject, the amount of urine voided in 15 min. averaged 111 ml.

ANALYTICAL METHODS

Plasma and urine samples were assayed for salicylic acid and salicyluric acid spectrophotometrically after elution from a column[2] according to the method described by Wan and Riegelman (12). Amounts of salicylic acid and salicyluric acid greater than 0.5 mcg./ml. of plasma or urine could be detected. The calibration curve showed linearity to at least 20 mcg./ml. and allowed accurate analysis even if one substance was in a 20-fold excess. None of the other metabolites of salicylic acid interfered with the assay. For the detection of the lowest levels of salicyluric acid, 0.5 ml. plasma was treated with 0.5 ml. isopropyl alcohol to precipitate the protein. A 0.6-ml. sample of the supernate was placed on the column and the salicyluric acid and salicylic acid fractions were collected as described previously (12). The effluents were directly measured in a spectrophotofluorometer[3]. A 50–100-μl. sample of urine was placed on the column and eluted with buffer (0.7 *M* phosphate buffer, pH 7.0). The fractions were measured at an activation wavelength and emission wavelength of 305 and 400 nm., respectively. The slit width was 6 mm.

The radioactivity in the urine samples was determined in a liquid scintillation counter[4]. A 0.5-ml. sample of urine was mixed in a polyethylene vial with 10 ml. modified Bray's cocktail having a composition of 8 g. 2,5-diphenyloxazole, 0.6 g. 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (or 6 g. of an alternate cocktail[5]), and 150 g. reagent grade naphthalene, dissolved in 100 ml. ethoxyethanol and 20 ml. ethylene glycol, and was made up to 1 l. with scintillation grade dioxane. The window was set on 50–1000 and the gain was set on 50 ± 1%. The efficiency was determined by the internal standard method (25) using a toluene-^{14}C standard with an activity of 3.48×10^6 d.p.m./ml. The efficiency ranged between 94 and 97% with a maximum standard deviation of 0.7%.

RESULTS AND DISCUSSION

Studies by Rowland and Riegelman (26) elucidated some pharmacokinetic parameters. When a 250-mg. i.v. dose of salicylic acid was administered, the half-life for the slow disposition phase was found to range from 120 to 170 min. and the volume of distribution at steady state ($V_{d_{ss}}$) was 9.6 ± 0.8 l. From these parameters, the priming dose as well as the rate of infusion was calculated for each level. During the first infusion period (level 1), the concentration of salicylic acid did not reach steady state. The rate of excretion of ^{14}C-labeled salicyluric acid stayed constant throughout the experiment except for the first level (Fig. 1). This indicates that no changes in the clearance took place for levels 2 and 3, in spite of the increasing doses of salicylic acid.

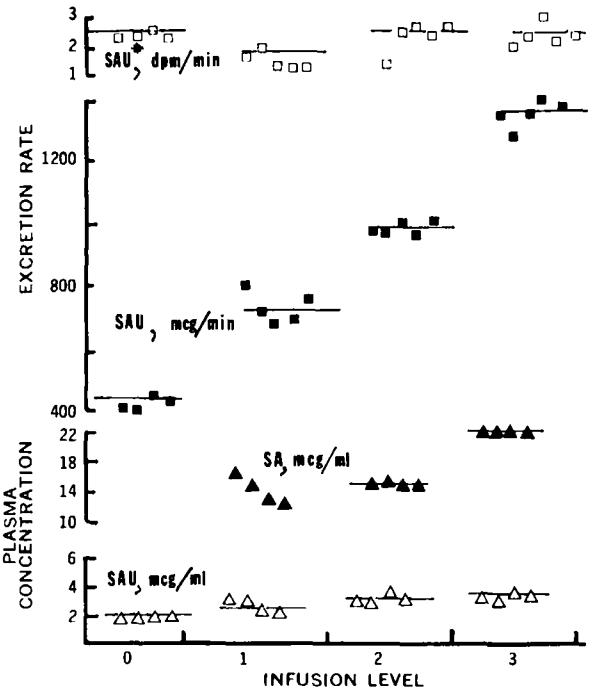

Figure 1—*Plasma concentration and urinary excretion rates during salicyluric and salicylic acid infusion in man. Each horizontal line represents 1-hr. infusion interval. (SAU = salicyluric acid, and SA = salicylic acid.)*

The renal clearance of salicyluric acid remained essentially constant at 227 ml./min., uncorrected for protein binding and body surface. Since the assessment of this value is critical to the remaining calculation of renal metabolism, comparison of this value with literature values is warranted. Corrected renal clearance values of salicyluric acid were reported by Schachter *et al.* (14) to be 444 ± 112 ml./min./1.73 m.[2] body surface. Unfortunately, the data of Schachter *et al.* (14) did not permit an estimate of the free fraction of salicyluric acid. Knoefel *et al.* (27), in 1962, reported data for salicyluric acid binding; the data indicate a rather large change in binding of the drug with changes in total plasma concentration[6]. Schachter *et al.* (14) used a complex assay procedure[7]. Although there are potential errors in the procedure due to the number of steps, the assay is essentially based on the separation of salicylic acid from the insoluble salicyluric acid when carbon tetrachloride is used. In our studies of this system, salicyluric acid was found to be slightly, but significantly, soluble in carbon tetrachloride. For example, 2- and 4-mcg./ml. solutions of salicyluric acid in 0.1 *N* hydrochloric acid were extracted with 15 volumes of carbon tetrachloride. The organic phase was reextracted with 1 *N* sodium hydroxide and as-

[2] Sephadex G-10.
[3] Hitachi MPF-2A fluorescence spectrophotometer.
[4] Packard model 3375.
[5] Omnifluor, New England Nuclear.

[6] When using the Freundlich absorption isotherm, log-log plots result in the following data. They include two data points of Knoefel *et al.* (27) and two data points obtained by extrapolation to lower total plasma concentration of salicyluric acid.

Total Plasma Concentration, mcg./ml.	Free Concentration, mcg./ml.	Bound Concentration, mcg./ml.	Percent Free
82.4 [experimental (27)]	71	11.4	86
5.0 [experimental (27)]	32	18	64
1.9 (extrapolated)	10	9	53
0.92 (extrapolated)	4	5.2	44

[7] The assay included a formation of salicylic hydroxamate from the acylglucuronide, precipitation of plasma protein, extraction of salicylic acid and salicyluric acid with ether, and evaporation to dryness. The residue, equivalent to 1.6 ml. plasma, was then extracted with 18 ml. carbon tetrachloride to remove salicylic acid, presumably leaving the salicyluric acid for the final fluorometric assay.

Table II—Steady-State Plasma Concentrations and Excretion Rates of Salicylic Acid and Salicyluric Acid at Different Infusion Levels

Level of Infusion	Cp, mcg./ml.		dA/dt, mcg./min.[a]	
	Salicylic Acid	Salicyluric Acid	Salicylic Acid	Salicyluric Acid
0	0	1.9 ± 0.15	0	431 ± 44
1	14.4 ± 1.8[b]	2.3 ± 0.1	0	717 ± 56
2	13.4 ± 0.9	2.8 ± 0.4	0	981 ± 32
3	21.7 ± 0.2	3.1 ± 0.3	54	1352 ± 108

[a] This refers to the urinary excretion of salicylic acid or salicyluric acid in micrograms per minute. [b] This plasma salicylic acid level was still declining from a high of 16.2 to a low of 12.6 mcg./ml. during the experimental period of infusion.

sayed on a spectrophotofluorometer (excitation 320 nm., emission 420 nm.). The results indicate that 25% of the salicyluric acid was extracted into carbon tetrachloride. Additional studies of salicylic acid in the presence of salicyluric acid indicate incomplete extraction of the salicylic acid by the carbon tetrachloride, leading to further potential error.

Smith *et al.* (28), in 1945, estimated the corrected clearance of salicyluric acid in two subjects. Their results for the two subjects were 269 and 487 ml./min./1.73 m.^{2} body surface. They infused salicylic acid and estimated the values using a reaction of the phenolic group of salicyluric acid with *N*-2,6-trichlorobenzoquinonimine. Our studies indicated that the Smith *et al.* reaction leads to high and variable plasma blank values, and once again there may be some question as to the method of correction for protein binding since the results are not specified.

Levy and coworkers (29, 30) also utilized the carbon tetrachloride method for the separation of salicylic acid and salicyluric acid. The exact method is not clearly identified in the reference; however, it undoubtedly included a solvent extraction and fluorometric analysis. They (29) reported the partitioning characteristics of salicyluric acid at 250 mcg./ml. in the presence of 500 or 1000 mcg./ml. of salicylic acid. These concentrations in the extraction procedure are probably appropriate for urine, so, their data are probably valid for urine studies. However, they do not verify separation of the two compounds at plasma levels, which usually are 1–3 mcg./ml. of salicyluric acid in the presence of 1–40 mcg./ml. of salicylic acid.

One can attempt to estimate the renal clearance of salicyluric acid from the data of Levy *et al.* (30) by estimating the excretion rate of salicyluric acid at the appropriate equivalent plasma concentrations. The resultant (corrected) value appeared to possibly exceed the renal plasma flow rate.

It is suggested, therefore, that the available literature data on renal clearance of salicyluric acid are ambiguous. The data of the present study were derived with concomitant infusions of the salicyluric acid-^{14}C. The renal excretion of this compound remained fairly constant throughout the study, which indicated that the excretion remained virtually constant. The excretion rate determined from the amount of salicyluric acid-^{14}C in the urine was found to be 426 mcg./min. This value agrees well with the value of 431 mcg./min. found from the cold assay. At an infusion rate of 456 mcg./min., the value of 431 mcg./min. represents a 94.5% urinary recovery of salicyluric acid. Unfortunately, the plasma radioactivity levels were in the range of background and, therefore, could not be used to estimate clearance. The plasma concentrations and rates of excretion found for salicylic acid and salicyluric acid at each infusion level are included in Table II and Fig. 1. At level 1, the plasma concentration of salicylic acid did not reach steady state. The same problem was observed with the plasma level of salicyluric acid, where only the last two data points of the first level could be considered to be at steady state. In the urine, salicylic acid could only be detected in the terminal infusion level, indicating that only negligible amounts of salicylic acid were excreted unchanged over this range of infusion.

The true and apparent clearance values for salicyluric acid (Table III) were calculated from the observed plasma levels and excretion rates of salicyluric acid. In spite of the declining plasma concentration of salicylic acid during the first infusion period, one can calculate an apparent clearance of salicyluric acid since the salicyluric acid plasma data do not reflect the magnitude of difference seen in the salicylic acid data. However, the apparent clearance for the first infusion period (Table III) was calculated only from the last two data point times (Fig. 1). With increasing amount of salicylic acid being infused, the percent of the dose excreted as salicyluric acid decreased (column 2 of Table IV). The mass balance for the initial infusion of salicyluric acid (level 0) shows that 94.5% of the input was recovered in the urine. This value agrees well with the value reported by Levy *et al.* (30). The method of calculating the contribution of kidney metabolism to overall metabolism was recently published (1). Equation 1 describes the rate of formation of salicyluric acid by the kidney:

$$\left(\frac{dM}{dt}\right)_{\text{kidney}} = \left(\frac{dM}{dt}\right)_{\text{total}} - \left(\frac{dM}{dt}\right)_{\text{liver}} \qquad \text{(Eq. 1)}$$

At steady state, (dM/dt) liver[a] is given by the equation:

$$\left(\frac{dM}{dt}\right)_{\text{liver}} = (\dot{V}_{cl})_{ex} \times Cp(\text{SAU}) \qquad \text{(Eq. 2)}$$

where $(\dot{V}_{cl})_{ex}$ is the renal clearance of salicyluric acid (SAU) as calculated from the level 0 data (Table III). By multiplying this value by the plasma concentration of salicyluric acid at each infusion level, one obtains the rate of formation of metabolite at any time. At steady state, the total rate of metabolism, (dM/dt) total, equals the rate of excretion. This definition is valid only if no portion of metabolite synthesized by the kidney is reabsorbed back into the blood.

Table IV includes an estimate of the rate of formation of salicyluric acid by the kidney and extrarenal processes (liver) and also as a percent of the total rate of metabolism. The resultant data indicate that an average of 67 ± 4% of salicyluric acid was formed in the kidney. To our knowledge, no similar study of the contribution of renal metabolism has been reported for man. While this study was done in only one subject, the results indicate clearly the importance of the kidney as a site of salicylate metabolism in man.

Table III—True and Apparent Renal Clearances of Salicyluric Acid in Man

Compound $(\dot{V}_{cl})_{ex}$	$(\dot{V}_{cl})_{app}$ at Levels		
	1	2	3
Salicyluric acid 227	312	350	436

Table IV—Contribution of Renal Metabolism to Overall Metabolism of Salicylic Acid in Man

Level of Infusion	Percent of Dose Excreted as Salicyluric Acid[a]	Excretion Rate (dM/dt), mcg./min.		Percent Salicyluric Acid Formed	
		Kidney	Liver	Kidney	Liver
1	85	195	91	70	30
2	81.5	346	204	63	37
3	68.3	649	272	71	29

[a] The values were estimated by calculating the excretion rate of salicyluric acid resulting from salicylic acid metabolism (by correcting for the steady-state excretion rate for salicyluric acid of 431 mcg./min.), multiplying by the time interval, and correcting for the molecular weight.

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[a] It should be emphasized that (dM/dt)liver is the sum of the rates of metabolism of all sites in the body other than the kidney.

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DONALD PERRIER▲ and MILO GIBALDI

Abstract □ Among the most commonly used methods for studying intestinal drug absorption are the *in vitro* everted rat gut and the *in situ* rat intestinal loop. The methods were compared employing various penicillins, cephalosporins, and tetracyclines. Steady-state and, particularly, initial drug clearances across the everted rat gut and the *in situ* absorption data were not always in agreement. A comparison of these results with antibiotic absorption data in man demonstrated rank-order agreement between absorption from the *in situ* intestinal loop and human GI absorption. Based on present observations, the *in situ* intestinal loop of the rat appears suitable as an animal model for predicting human drug absorption.

Keyphrases [ ] Absorption, intestinal- *in vitro* everted rat gut and *in situ* rat intestinal loop drug permeability data, compared to antibiotic absorption data in man □ Antibiotic (penicillins, cephalosporins, and tetracyclines) intestinal absorption *in vitro* everted rat gut and *in situ* rat intestinal loop data compared to absorption data in man □ Drug permeability across intestinal membrane data from *in vitro* everted rat gut and *in situ* rat intestinal loop techniques compared to antibiotic absorption in man

A variety of *in vitro* and *in situ* animal techniques have been employed to study drug permeability across the intestinal membrane. Many of these techniques were recently discussed by Bates and Gibaldi (1). The present investigation was undertaken to compare the data obtained from two such techniques, the *in vitro* everted gut and the *in situ* intestinal loop, and to evaluate these techniques as possible models for human drug absorption. Several groups of antibiotics were studied, since this class of compounds offers a wide range in the degree to which absorption occurs in man.

METHODS

Male, Sprague–Dawley strain rats[1], weighing approximately 250 g., were fasted 14–20 hr. prior to the experiment. Water was allowed *ad libitum*.

Drug Transfer across Isolated Everted Rat Intestine—Intestinal transfer rates were determined using a modification of the method of Crane and Wilson (2). The method for preparing the everted intestine preparation was described previously (3). After severing the intestine at the pyloric junction, the first 15 cm. of intestine was discarded, the gut was everted, and the proximal portion was divided into two 10-cm. segments. The initial, proximal segment was designated Segment 1 and the distal portion was designated Segment 2.

Both segments were placed into test tubes containing approximately 100 ml. of mucosal drug solution, previously equilibrated at 37° and continually gassed with oxygen–carbon dioxide (95:5 v/v). Two milliliters of a modified physiological Krebs bicarbonate

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buffer[2] (pH 7.4) was placed inside each sac (serosal solution). In any given experiment, the initial mucosal and serosal solutions were identical except for the presence of drug in the mucosal solution; the mucosal concentration of drug remained essentially constant throughout each experiment due to its large volume. Sink conditions were maintained at all times, and the maximum serosal concentration obtained in any one experiment was less than 20% of the mucosal drug concentration. The serosal compartment was sampled every 30 min. for 2 hr. At each sampling time, the entire serosal volume was removed and then 2 ml. of buffer solution was introduced into the serosal compartment as a rinse, immediately removed, and added to the previous sample. Finally, another 2 ml. of buffer was placed into the serosal compartment for withdrawal at the next sampling interval.

A loss of functional and structural integrity of the everted gut preparation with time was reported previously (4, 5). To account for these changes, experiments were conducted where the everted intestinal preparation was incubated in drug-free buffer solution for up to 90 min. During incubation, sham samples of serosal solution were taken at 30-min. intervals and discarded. After incubation, the preparation was immediately transferred to a mucosal drug solution. The serosal compartment was sampled in the usual manner for 30 min.

In another experiment, the everted intestinal sac was incubated for 30 min. in drug-free buffer solution, at which time sham samples of serosal solution were taken and discarded. The mucosal surface was then scraped with forceps to remove the epithelial layer from the muscularis. The resulting preparation was transferred to the mucosal solution containing drug and sampled in the usual manner after 30 min.

Drug transfer rates across the everted rat intestine were expressed as mucosal-to-serosal clearances (milliliters per minute) and calculated from Eq. 1:

$$\text{clearance} = \frac{\text{amount transferred in 30 min.}}{\text{mucosal drug concentration} \times 30} \quad \text{(Eq. 1)}$$

Clearances corrected for loss of functional integrity of the everted gut preparation were calculated for sampling periods ending at 60, 90, and 120 min., denoted as Periods II, III, and IV, respectively. The clearance for the first 30 min., Period I, was used as the standard. Employment of Period I as a standard assumes that essentially no change in the functional integrity of the everted gut occurs within the first 30 min. This assumption appears valid, since Levine *et al.* (4) observed that everted gut preparations from ether-anesthetized rats show only a 10–15% loss in structural integrity in the first 60 min.

The clearance values obtained from the studies involving incubations in buffer for 30, 60, and 90 min., followed by 30 min. in drug solution, are represented by II_i, III_i, and IV_i, respectively. The increase in clearances II_i, III_i, and IV_i over Period I is attributed to a loss of functional integrity of the everted gut; when this increase is subtracted from the respective clearances for Periods II, III, and IV, the corrected clearances result. For example, to calculate the corrected clearance for Period III, III_c, Eq. 2 could be employed:

$$III_c = III - (III_i - I) \quad \text{(Eq. 2)}$$

Similarly for Periods II and IV:

$$II_c = II - (II_i - I) \quad \text{(Eq. 3)}$$

and:

$$IV_c = IV - (IV_i - I) \quad \text{(Eq. 4)}$$

When the corrected clearances for two consecutive periods were within 10% of each other, the achievement of steady-state clearance was assumed. The average of these two clearances will be referred to as the steady-state clearance.

Absorption from *In Situ* and *In Vitro* Intestinal Loops—The method of Levine *et al.* (6) was utilized to study *in situ* absorption from the rat small intestine. The animals were anesthetized with urethan, 1.3 g./kg. i.p., for the duration of the experiment. A midline incision was made, the small intestine was located, and two loops, approximately 5 cm. long, were formed. The first loop was approximately 15 cm. from the pylorus, with 1 cm. of intestine separating the two consecutive loops. One milliliter of a modified Krebs buffer solution containing drug was injected into a loop, through a ligature, by means of a syringe and blunt needle. After 30 min., the loops were excised and rinsed with normal saline. The sac was cut open at both ends, and the drug solution remaining inside the sac was permitted to run out. Approximately 25 ml. of buffer was then used to rinse out the inside of each sac.

In vitro experiments were conducted where two consecutive loops were prepared as before but, immediately prior to injection of drug solution, the loops were excised from the animal. Each loop containing drug solution was then suspended in 40 ml. of buffer solution, which was stirred at a constant rate and maintained at 37° in a jacketed beaker. At the end of 30 min., these loops were treated in the same manner as the *in situ* loops.

Before assaying the solutions obtained from the intestinal loops, each solution was passed through a 0.45-μ filter[3] to remove all solid material. Control experiments indicated that no drug was removed by this filtration step.

Drugs—The drugs examined were sodium dicloxacillin[4], sodium ampicillin[5], sodium benzylpenicillin[6], tetracycline hydrochloride[7], doxycycline hyclate[8], cephaloridine[9], and cephalexin monohydrate[10].

Assay—Cephaloridine and cephalexin were assayed polarographically using a dropping mercury electrode polarograph[11], based on the procedure reported by Benner (7). Five milliliters of 1.5 *N* sulfuric acid or 1.5 *N* sodium hydroxide was added to an equal volume of drug solution containing cephaloridine or cephalexin, respectively. The cephalexin–sodium hydroxide solution was heated at 50° for 1 hr. To both solutions, 5 drops of a nonionic detergent[12] was added as a maximum suppressor. The solutions were then deoxygenated by bubbling nitrogen through them for 5 min. Cephaloridine samples were then scanned from −0.7 to −1.2 v., while cephalexin samples were scanned from −0.5 to −1.1 v. The concentration of cephaloridine and cephalexin was related to the wave intensity, in microamperes, at −1.05 and −0.9 v., respectively.

The tetracyclines, doxycycline and tetracycline, were assayed spectrophotometrically with a spectrophotometer[13] by the method of Chatten and Krause (8). Equal volumes of 5% thorium nitrate in 0.012 *N* hydrochloric acid and tetracycline solution were combined, producing a pH 6.05 solution. The absorbance of the complex formed was measured at 395 nm.

Spectrophotometric assays were also employed for dicloxacillin, ampicillin, and benzylpenicillin. Benzylpenicillin was assayed by the method of Brandriss *et al.* (9) under the conditions outlined by Tutt and Schwartz (10). Equal volumes of a pH 2.5 glycine–hydrochloric acid buffer (0.4 *M*) containing mercuric chloride (1.6×10^{-2} *M*) and drug–buffer solution were combined, producing a final pH of 2.7. This solution was assayed at 325 nm., after standing at 25° for 100 min.

The method of Smith *et al.* (11) was employed for assaying ampicillin. A pH 4.9 citric acid (0.2 *M*)–dibasic sodium phosphate (0.4 *M*) buffer containing copper (30 mcg./ml.) was prepared. Five milliliters of pH 4.9 buffer and an equal volume of drug solution were combined, yielding a final pH of 5.2, and heated at 75° for 30 min. Absorbance was measured at 320 nm. A modified ampicillin assay was used to quantitate dicloxacillin. When equal volumes of drug solution and pH 2.2 citric acid–phosphate were mixed, a pH 2.45 solution resulted. This solution was heated at 75° for 40 min. and then assayed at 340 nm.

Microbiological assays were employed for ampicillin and dicloxacillin in the *in situ* loop experiments due to the presence of unidentified material which interfered with the chemical method. It was also decided to employ a microbiological method to check

[2] KCl, 5 m*M*; KH_2PO_4, 1 m*M*; $NaHCO_3$, 26 m*M*; and NaCl, 122 m*M*.

[3] Millipore.
[4] Bristol Laboratories, Syracuse, N. Y., Lot No. 71F1311 (D1734).
[5] Bristol Laboratories, Syracuse, N.Y., Lot No. 71F1312 (LO530).
[6] E. R. Squibb and Son, Inc., New Brunswick, N. J., Lot No. 6K877(4980).
[7] Pfizer Inc., Brooklyn, N. Y., Lot No. 0Y158–71000.
[8] Pfizer, Inc., Brooklyn, N. Y., Lot No. 11640-58002.
[9] Eli Lilly and Co., Indianapolis, Ind., Lot No. 4WM36.
[10] Eli Lilly and Co., Indianapolis, Ind., Lot No. 5BX82.
[11] Heath Built, model EUA-19-6, Heath Co., Benton Harbor, Mich.
[12] Triton X-100, 0.2%.
[13] Hitachi-Perkin-Elmer model 139, Perkin-Elmer Corp., Palo Alto, Calif.

Table I—Mucosal-to-Serosal Clearance of Various Antibiotics across the Everted Isolated Rat Jejunum

Antibiotic	Clearance, ml./min. × 10^6, ± SE^a Period I	Steady State
Benzylpenicillin (250)[b]	229 ± 36	219 ± 47
Ampicillin (500)	395 ± 54	373 ± 45
Dicloxacillin (1000)	445 ± 45	791 ± 72
Tetracycline (500)	184 ± 39	615 ± 78
Doxycycline (5000)	199 ± 32	912 ± 38
Cephaloridine (2000)	454 ± 44	749 ± 50
Cephalexin (2000)	454 ± 43	719 ± 57

[a] Mean ± standard error, five determinations. [b] Parenthetic values denote mucosal concentration in micrograms per milliliter.

Table II—Absorption of Various Antibiotics from the *In Situ* Loop of the Rat

Antibiotic	Percent Absorbed ± SE^a
Benzylpenicillin (250)[b]	15.8 ± 1.9 (6)[c]
Ampicillin (500)	22.3 ± 2.9 (5)
Dicloxacillin (1000)	30.3 ± 2.1 (6)
Tetracycline (500)	41.7 ± 0.3 (5)
Doxycycline (5000)	66.1 ± 1.6 (6)
Cephaloridine (7500)	19.4 ± 1.8 (4)
Cephalexin (7500)	53.4 ± 1.2 (3)

[a] Percent absorbed = 100 × (amount injected into loop − amount remaining in loop after 30 min.)/amount injected into loop. Mean ± standard error. [b] Parenthetic values denote initial concentration of drug in the *in situ* loop in micrograms per milliliter. [c] Parenthetic values denote number of determinations.

the *in situ* cephaloridine results obtained with the polarographic method. This procedure was followed because, following oral administration of cephaloridine to rats, virtually all of the absorbed dose was eliminated as degradation products which were believed to have been formed in the GI tract (12). It is conceivable that the polarographic method may not differentiate between cephaloridine and such degradation products. Excellent agreement was obtained between these two methods. An additional assay was not felt to be necessary for cephalexin because, following oral administration to rats, cephalexin was shown to be totally eliminated unchanged (13). For all three antibiotics, the agar well method of Bennett *et al.* (14) was used with *Bacillus subtilis* as the organism. Assays were performed in triplicate.

RESULTS

For all of the antibiotics studied, Period I clearances for the second segment of the everted gut were consistently less than the corresponding clearances for the first segment. The same general trend was observed with steady-state clearance values. This variation in clearance can probably be related to surface area differences between the two segments. However, little qualitative difference in the overall behavior of the two segments was observed. Accordingly, only data from the second segment are presented.

Mucosal-to-serosal clearances across the isolated everted gut during Period I indicate that dicloxacillin is the most permeable of the three penicillins studied, followed by ampicillin and then by benzylpenicillin (Table I). However, the differences in clearance are not very substantial. Penicillin clearances at steady state were of the same rank order as was observed for Period I, although the steady-state dicloxacillin clearance was significantly greater than Period I dicloxacillin clearance. The relative extent of penicillin absorption from the *in situ* loop (Table II) was in the same rank order as steady-state and Period I clearances.

Very little difference was seen between the Period I clearance values for doxycycline and tetracycline (Table I). However, there was approximately a 50% difference in their steady-state clearances, with doxycycline being cleared across the everted gut at a more rapid rate. The same rank order was observed in the *in situ* absorption studies (Table II) as was seen in steady-state clearance across the everted gut. In initial clearance studies with doxycycline, a steady-state clearance was not reached when a mucosal doxycycline concentration equal to the tetracycline concentration (0.5 mg./ml.) was employed. This inability to reach steady state was possibly due to continual uptake and accumulation of doxycycline in the everted gut. As a result, a doxycycline mucosal concentration significantly greater than the concentration used for tetracycline was employed to reach a steady-state doxycycline clearance.

Period I clearance values for cephaloridine and cephalexin were identical (Table I). At steady state, cephaloridine had a slightly higher clearance than did cephalexin. The everted gut clearance data for these two cephalosporins are not in agreement with *in situ* absorption results (Table II). In the latter case, cephalexin was absorbed much more extensively than cephaloridine.

In an attempt to explain the discrepancy in cephalosporin results obtained with the everted gut and *in situ* loop techniques, further studies were undertaken; the results are presented in Tables III and IV. Cephalexin traversed the intestinal membrane much more readily than did cephaloridine *in situ* (Table III). However, little difference existed between these two antibiotics in the extent to which they permeated the intestine *in vitro*, as measured by employing isolated intestinal loops. Also, there was no significant difference in the mucosal-to-serosal clearance of cephalexin and cephaloridine across the everted gut in either the presence or absence of the epithelial layer (Table IV).

DISCUSSION

Results of the present investigation demonstrated rank-order agreement between Period I and steady-state clearances across the everted gut and the *in situ* absorption data for the penicillins. Dicloxacillin was cleared at a faster rate and was absorbed more extensively than ampicillin. Of the penicillins studied, benzylpenicillin was the most slowly cleared and poorly absorbed. Period I clearance values did not differentiate either the cephalosporins or tetracyclines. However, *in situ* loop absorption results did show significant differences between doxycycline and tetracycline and between cephalexin and cephaloridine, with doxycycline and cephalexin being more extensively absorbed in their respective groups. The steady-state rank-order clearances between the two tetracyclines agreed with *in situ* absorption data. However, no difference in steady-state clearance was observed between the cephalosporins, which is contrary to the *in situ* cephalosporin results. As can be seen, *in vitro* and *in situ* methods do not agree with respect to the relative intestinal permeabilities of the various antibiotics studied.

To evaluate these two animal techniques, the *in vitro* everted gut and the *in situ* intestinal loop, with respect to their ability to serve as possible models for human drug absorption, the results obtained were compared to human absorption data from the literature. The extent to which the various antibiotics studied are absorbed from the human GI tract was calculated, employing literature urinary excretion data, using an intravenous dose as the standard.

A number of factors are involved in determining the extent to which an orally administered drug is absorbed from the GI lumen. Since the vast majority of orally administered drugs are supplied as solid dosage forms, the extent to which the drug is released from its supporting matrix becomes of prime importance. Once released, the drug's solubility and stability in solution must be considered. Resistance to degradation and lack of interaction with components present in the GI tract are desirable. The drug must also possess suitable physical properties that enable it to permeate the GI membrane. One or more of these factors appears responsible for rendering the majority of the antibiotics studied in the present investigation incompletely absorbed from the GI lumen.

In most literature reports surveyed, human GI absorption was studied employing solid dosage forms. This raises the problem of potential dissolution rate-limited absorption rather than permeability rate-limited absorption. However, in our view, the antibiotics studied would be expected to be permeability rather than dissolution rate limited in their absorption since they are freely soluble in water.

Benzylpenicillin is very susceptible to acid hydrolysis and attack by penicillinase in the GI tract (15, 16). Both processes compete for drug at the absorption site. Ampicillin, which is relatively stable in acid and neutral solutions (15, 17), would on a stability basis be expected to be and, in fact, is more extensively absorbed than benzylpenicillin (Table V) even though it does not appear to be

Table III—Transfer of Cephalosporins out of Rat Jejunum Loops

Cephalosporin	Percent Transferred ± SE[a]	
	In Situ	*In Vitro*
Cephaloridine (7500)[b]	19.4 ± 1.8 (4)[c]	18.2 ± 2.3 (4)
Cephalexin (7500)	53.4 ± 1.2 (3)	24.1 ± 1.6 (3)

[a] Percent transferred = 100 × (amount injected into loop − amount remaining in loop after 30 min.)/amount injected into loop. Mean ± standard error. [b] Parenthetic values denote initial concentration of drug in loop in micrograms per milliliter. [c] Parenthetic values denote number of determinations.

Table IV—Effect of Epithelial Layer Removal on Clearance of Cephalosporins across the Everted Isolated Rat Jejunum

Cephalosporin	Clearance, ml./min. × 10^6, ± SE[a]	
	Control	Epithelial Layer Removal
Cephaloridine (2000)[b]	712 ± 23	832 ± 24
Cephalexin (2000)	748 ± 22	761 ± 47

[a] Mean ± standard error, five determinations. [b] Parenthetic values denote mucosal concentration in micrograms per milliliter.

resistant to penicillinase (18). However, it should not be implied that if a particular penicillin derivative is not subject to GI degradation it will be 100% absorbed. For example, dicloxacillin appears resistant to both acid-catalyzed hydrolysis and degradation by penicillinase (19), but considerably less than 100% of this drug appears to be absorbed (Table V).

The rank order obtained with respect to the steady-state penicillin clearance across the everted gut and absorption from the *in situ* intestinal loops is in agreement with the relative extent of penicillin absorption in humans (Tables I, II, and V). Benzylpenicillin had the lowest clearance and percent absorbed of the three penicillins studied, followed by ampicillin and then dicloxacillin. The everted gut studies (notably steady-state studies) as well as *in situ* studies suggest that the penicillins do indeed have different membrane permeability characteristics. At normal intestinal pH as well as the pH of the everted gut studies, the carboxyl group of the penicillins would be essentially totally dissociated, which may partly explain the generally poor permeability of the penicillins. With ampicillin, the negative charge associated with the carboxyl group would be partially balanced by the positive charge associated with the amino group, which has a pKa of 7.25 (20), resulting in an ampicillin molecule with a net partial negative charge rather than a total negative charge. This difference in overall polarity of these two penicillins may aid in explaining why ampicillin is more permeable than benzylpenicillin. Dicloxacillin, even though totally dissociated, appears to have a substantially more lipophilic substituent than the α-aminophenyl and phenyl substituents of ampicillin and benzylpenicillin, respectively. Such an increase in lipophilicity may account for the increased permeability of dicloxacillin over the other two penicillins.

The cephalosporins are structurally very similar to the penicillins but are much more stable than the penicillins (17). Cephalexin and ampicillin are identical in structure except for the basic differences in the cephalosporin and penicillin nuclei, but cephalexin is essentially totally absorbed on oral administration (21). Higher steady-state clearance values and increased absorption from the *in situ* intestinal loops were observed for cephalexin as compared to ampicillin (Tables I and II). These permeability differences can be attributed to the variation in the basic nuclei of the penicillin and cephalosporin molecules.

Although cephalexin is extensively absorbed, cephaloridine is very poorly absorbed (1–2%) in human subjects (22). This lack of absorption has been attributed to the presence of the pyridinium group (23). *In vitro* clearance values suggest that there is no significant difference in the permeability characteristics of cephalexin and cephaloridine (Table I). This is totally inconsistent with *in situ*[14] rat and human absorption results (Tables II and V), and an attempt was made to explain this anomaly. Initially, the extent to which the cephalosporins were transported across *in vitro* intestinal loops was studied. Cephalexin and cephaloridine permeated the *in vitro* loop to comparable degrees, even though substantial differences were observed *in situ* (Table III).

In vivo and *in situ* the epithelial cell of the intestinal villus constitutes the only anatomical barrier of significance controlling the movement of drug from the lumen of the gut into the body. Once past this barrier, the transferred drug gains ready access to either blood or lymph capillaries. Therefore, *in vivo* and *in situ* the process of intestinal absorption is governed by properties of the epithelial barrier and the rates of blood and lymph flow. However, *in vitro* a drug must traverse the epithelium plus the underlying connective and muscle tissue. As a result, the rate-limiting barrier for *in vitro* transport might be the intestinal musculature, a barrier that would not be encountered in *in vivo* and *in situ* transfer. The *in situ* and *in vitro* intestinal loop results in the present investigation suggest that the muscle and connective tissue serve as significant barriers to cephalexin transfer resulting in both cephalosporins being cleared or absorbed to similar extents *in vitro*. Further support for this postulate are the results of a study where clearance measurements were made across the everted gut from which the epithelial layer had been removed, leaving only the underlying muscle layer and connective tissue. There is relatively little change in the mucosal-to-serosal clearance after removal of the epithelial layer, and no significant difference was observed in the clearance values either before or after the everted gut was stripped of the epithelial layer (Table IV). These results suggest that the epithelial layer is probably not the primary barrier to cephalosporin transport *in vitro* and that the muscle layer serves as an equal barrier to both cephalexin and cephaloridine *in vitro*. Based on these results, the everted gut technique appears unsuitable as a model for human drug absorption in the case of the cephalosporins.

The initial clearance values (Period I) of doxycycline and tetracycline are essentially identical. However, steady-state clearances suggest that doxycycline is considerably more permeable than tetracycline (Table I). Furthermore, this increase in permeability of doxycycline over tetracycline is in agreement with absorption data from the *in situ* loop studies (Table II). The difference in permeability of these two tetracyclines is consistent with observed differences in their chloroform–water partition coefficients. Doxycycline was shown to have a significantly higher partition coefficient than tetracycline over the 2–8 pH range (24). It is generally accepted that doxycycline is better absorbed than tetracycline in man (25), and studies in the literature support such claims. An analysis of oral and intravenous plasma level data, presented recently by Leibowitz *et al.* (26), suggests that 85% of an oral dose of doxycycline is absorbed. This study supports the data of Fabre *et al.* (27), where 93% of an oral doxycycline dose was reported to be absorbed. These findings can be compared to 77% for tetracycline (Table V). Differences in absorption are further supported by studies where doxycycline and tetracycline have been administered with food. Food appears to have little influence on doxycycline absorption (28–30) while significantly depressing the oral absorption of tetracycline (31). Furthermore, dissolution studies[15] conducted in this laboratory indicate that doxycycline hyclate has approximately a 3.5 times greater dissolution rate than tetracycline hydrochloride. This finding may also contribute to better absorption of doxycycline.

CONCLUSIONS

Based on the results of the present investigation, *in vitro* methods, where the drug must traverse both the epithelial and underlying connective tissue and muscle layers rather than just the epithelial layer to permeate the intestinal membrane, should be employed as models for GI absorption only with extreme caution. Period I clearances across the everted gut yield very limited insight into human GI absorption. Although the rank order in Period I clear-

[14] Due to limitations in assay sensitivity, initial cephalosporin concentrations in the *in situ* loop were substantially greater than the initial mucosal concentrations employed in the everted gut studies, 7.5 *versus* 2 mg./ml. Based on one run at a mucosal concentration of 7.5 mg./ml., the clearances of both cephalosporins across the everted gut were the same as the clearances obtained when a mucosal concentration of 2 mg./ml. was employed.

[15] Rotating-disk method as described by Levy and Sahli (32) using a precision speed control apparatus (33), modified Krebs bicarbonate buffer, 37°, and a rotation speed of 60 r.p.m.

Table V—Absorption of Various Antibiotics following Oral Administration to Humans

Antibiotic	Percent Absorbed[a]	Reference[b]
Benzylpenicillin	18	31, 34
Ampicillin	35	35, 36
Dicloxacillin	64	34, 37
Tetracycline	77	38
Doxycycline	93	27, 30
Cephaloridine	2	22
Cephalexin	83	21, 39

[a] Amount eliminated in urine after oral administration as a percent of amount eliminated in urine after an equal intravenous dose. [b] Literature reference from which human data was obtained.

ances of the penicillins agrees with human absorption data, relatively small differences in clearances were observed as compared to human studies. Moreover, Period I clearance values did not differentiate either the cephalosporins or the tetracyclines. Steady-state clearance measurements did yield a reasonable rank-order estimate of the relative extent to which the penicillins and tetracyclines were absorbed in man. However, steady-state clearances seriously failed with the cephalosporins. Even where appropriate, steady-state clearance studies require much more time and are more difficult to run than are *in situ* loop studies.

The *in situ* loop technique proved to be a very suitable animal model for predicting antibiotic absorption in man. Within each group of antibiotics, the same rank order was obtained between the extent of absorption from the *in situ* loop and the human GI lumen. The *in situ* intestinal loop technique also serves as a reasonable model for absorption even when all of the antibiotics studied are considered as a single group. With the exception of cephaloridine, excellent rank-order agreement was obtained between *in situ* loop and human studies. The *in situ* loop studies indicate more extensive absorption of cephaloridine than would be expected. This may be due to an inherent error in the technique since the amount of drug leaving the loop lumen is measured and not the amount of drug that actually reaches the blood supply. Therefore, extensive accumulation of cephaloridine in the intestinal tissue may account for the apparent overestimate in the extent of cephaloridine absorption from the intestinal loop.

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Application of Quantitative GC-Mass Spectrometry to Study of Pharmacokinetics of Amphetamine and Phentermine

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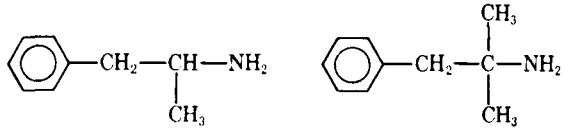
Abstract □ Quantitative GC–mass spectrometric assays were used to determine plasma and brain levels of amphetamine and phentermine. Deuterium-substituted variants of the two amines were used as internal standards in the procedures, which were sensitive to 10 pmoles/ml. The levels in rats given doses of 0.5 mg./kg. i.v. were examined in terms of a two-compartment open model. Levels of amphetamine were also examined after bilateral nephrectomy.

Keyphrases □ Amphetamine plasma and brain levels—GC-mass spectrometric analysis, rats □ Phentermine plasma and brain levels—GC-mass spectrometric analysis, rats □ GC-mass spectrometry—analysis, amphetamine and phentermine, plasma and brain, rats

The phenylisopropylamines are an interesting class of pharmacological agents because of their similar behavioral effects and their differing actions on the biogenic amines. For example, *p*-chloroamphetamine [1-(4-chlorophenyl)isopropylamine] and amphetamine (1-phenylisopropylamine, I) are qualitatively similar in their ability to cause central stimulation and anorexia. Various related compounds exhibit similar behavioral actions (1). The actions of these compounds on the biogenic amines are more diverse and, while amphetamine reduces steady-state levels of brain norepinephrine (2), *p*-chloroamphetamine reduces levels of 5-hydroxytryptamine (3).

An additional example of the differences observed within this class of compounds is the multiple pathways of metabolism, reflecting both chemical and species differences. In general, there are three initial sites of metabolism: the aromatic ring, the α-carbon, and the amino nitrogen. Metabolites resulting from oxidation at each position have been described, and the relative proportions of each metabolite differs with species (4). In the rat, the major metabolite of amphetamine *in vivo* is the *p*-hydroxy compound; in the rabbit, phenylacetone and benzoic acid resulting from deamination are the major metabolites. The role of divergent metabolic pathways in the pharmacology of these compounds has been studied by numerous investigators (2, 5–7).

Phentermine (2-methyl-1-phenylisopropylamine, II) is another structural variant of amphetamine which has similar anorexic action; but like *p*-chloroamphetamine, it has reduced central nervous system (CNS) stimulatory activity (1). The structural differences between phentermine and amphetamine are reflected in their chemical and biochemical differences. The base strength and partition coefficient of phentermine are higher than those for amphetamine, and the α-methyl group should prevent the α-oxidation–deamination pathway of metabolism (8). These differences could also result in differences in the distribution of the two drugs which, in turn, might account for some pharmacological differences. In the present study, the pharmacokinetics of these two compounds in rats were examined after intravenous administration.



I

II

The compounds were estimated by quantitative GC–mass spectrometry using procedures developed in these laboratories. The procedure for amphetamine was described elsewhere (9), and the procedure for phentermine was developed during these studies using analogous techniques. The assays involve extraction of the amines from the biological fluid, derivatization, and GC, using a mass spectrometer to monitor the effluant. The spectrometer is set to monitor mass fragments that are characteristic for the substance to be analyzed and for an isotopically enriched variant of the substance, which is the internal standard. Quantitation is based on the ratio of spectrometer response to unknown and to internal standard. The data collected were analyzed according to the two-compartment open kinetic model described for plasma decay curves (10).

EXPERIMENTAL

Extraction and Sample Preparation—The amines were extracted from the biological fluids and converted to their *N*-trifluoroacetyl derivatives for analysis by GC–mass spectrometry. Thus, a 2-ml. volume of plasma or a 4-ml. volume of brain extract was charged with 500 pmoles of deuterium-labeled amine as internal standard, alkalinized with 0.5 ml. of 5 *M* NaOH, and extracted with 6 ml. of benzene by shaking for 20 min. on a mechanical shaker. After centrifugation to separate layers, 5 ml. of the benzene layer was transferred to another extraction tube and shaken with 0.6 ml. of 1 *N* HCl. A 500-μl. aliquot of the aqueous phase was then transferred to another extraction tube containing 1.2 ml. of benzene and 0.2 ml. of 5 *M* NaOH. The resulting mixture was again shaken, 1 ml. of the benzene layer was transferred to a microcentrifuge tube, and 20 μl. of trifluoroacetic anhydride was added. The acetylation was allowed to proceed overnight at 4° and then the benzene was evaporated under a stream of nitrogen. The overnight acetylation time is not necessary but was carried out for convenience in scheduling the mass spectrometer. The residue from the evaporation step was dissolved in 20 μl. of acetonitrile for introduction into the GC–mass spectrometry system.

A standard curve was routinely prepared by adding 50–500 pmoles of amine to a series of extraction tubes containing 2 ml. of a 5% solution of plasma proteins[1] and 500 pmoles of internal standard. These tubes were then carried through the extraction and quantitation procedure.

Analysis—The GC–mass spectrometry system[2] utilized an ionizing energy set at 70 ev. with the emission current at 250 μamp. The GC column was a 2-m. × 2-mm. (i.d.) silynized glass column packed with 3% OV-17 on Gas Chrom Q (80–100 mesh), operated at 130°; the injection port and separator were operated at 180 and 185°,

[1] Plasmanate, Cutter Laboratories, Berkeley, Calif.
[2] EAI Quad 300 quadrupole mass spectrometer coupled to a Varian 1400 gas chromatograph with a glass frit separator.

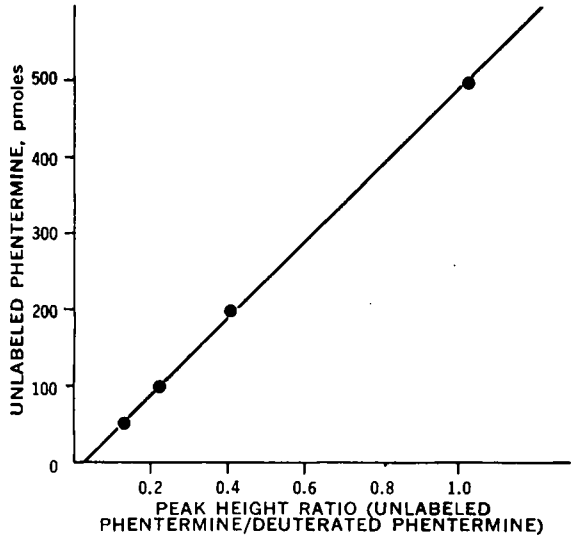


Figure 1—*Standard curve for phentermine determination.*

respectively. The mass spectrometer was operated in its specific ion detection mode (11) focused on masses 140 and 143 for amphetamine and 154 and 157 for phentermine. Quantitation was achieved by comparing the mass spectrometer responses at 140 and 143 (internal standard) for amphetamine and 154 and 157 (internal standard) for phentermine. The GC retention times for the trifluoroacetyl derivatives of amphetamine and phentermine were approximately 2.3 and 1.9 min., respectively. The internal standards used in the assays were 1-phenyl[3,3,3-$^2H_3$]isopropylamine (12) and 2-methyl-1-phenyl[3,3,3-$^2H_3$]isopropylamine (13) of 99.9 and 99.8% isotopic purity, respectively.

Animal Procedures—Male Sprague–Dawley rats, weighing 250–300 g., were given an intravenous injection of amine salt equivalent to 0.5 mg. free base/kg. at 0.25 ml./100 g. The animals were lightly anesthesized with ether, and blood was collected by cardiac puncture with a heparinized syringe. The brains were then removed and immediately frozen. The whole blood was centrifuged (3000 × *g*), and aliquots of plasma were taken for analysis. The brain was homogenized in four volumes of 0.1 *N* HCl, and the protein was precipitated by the addition of 0.2 ml. of 60% perchloric acid and centrifugation. A 4-ml. aliquot of the supernate was then extracted as described for plasma.

Bilateral nephrectomized animals were prepared by ligation of both renal arteries and veins, followed by excision of both kidneys under ether anesthesia. The animals were allowed to recover overnight before administration of the drug[3].

RESULTS AND DISCUSSION

A typical standard curve for phentermine is shown in Fig. 1. The standard deviation of the slopes from three curves was 1.3%. The standard deviation for the average slopes of five amphetamine curves was 0.3%. The 95% confidence limits calculated from these standard curves was 2–5 pmoles/ml. sample for amphetamine and 3–8 pmoles/ml. for phentermine.

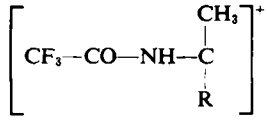
The specificity of the extraction procedure was established for phentermine by focusing the specific ion detector on three major mass peaks of the spectrum of the compound. Thus, the GC peak at 1.9 min. in the extracts from phentermine-treated animals was examined for the relative intensities at *m/e* 154, 132, and 59, major peaks in the spectrum of phentermine. As shown in Table I, the relative abundances of these fragments were identical to those of authentic compounds. A similar experiment was reported previously for amphetamine (9).

In quantitative GC–mass spectrometry, the spectrometer is alternately focused on two mass numbers, one representing a major fragment in the spectrum of the compound to be analyzed and the other representing the corresponding fragment from the internal standard. For these quantitative analyses, the specific ion detector was focused on *m/e* values corresponding to the base peak for each compound. The mass spectra of the trifluoroacetyl derivatives of amphetamine and phentermine have base peaks at *m/e* 140 and 154, corresponding to the fragment (III) that results from cleavage between the α- and

Table I—Relative Abundances of Key Fragments in the Mass Spectrum of Phentermine-Trifluoroacetamide in Standard and Extract[a]

	m/e		
	154	132	59
Standard	1.000	0.164	0.959
Plasma Extract	1.000	0.169	0.959
Brain Extract	1.000	0.163	0.947

[a] A comparison of the relative abundances of key fragments of phentermine-trifluoroacetamide in extracts of biological fluids from phentermine-treated animals and in a standard solution of phentermine-trifluoroacetamide. The values represent relative ion current at each mass integrated over the appropriate GC peak.



III

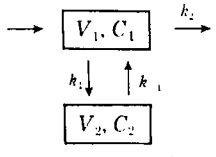
amphetamine: R = H
phentermine: R = CH_3

β-carbons (14). The internal standard is labeled with three deuterium atoms at the side-chain methyl group and the corresponding fragments for the labeled variants occur at *m/e* 143 and 157. The use of the base peak is desirable for maximal sensitivity, but it is more important that the peak chosen for detection is relatively isolated in the spectrum (9).

The internal standard serves to quantitate the spectrometer response so that the unknown concentration can be obtained by the ratio of responses. In addition, by its presence at levels of 500 pmoles, it acts as a "carrier" to minimize losses of the compound to be analyzed by absorption into glass columns, *etc.*, during extraction and chromatography. These losses become significant when picogram levels of amines are being handled but are minimized with this technique, since the addition of internal standard is equivalent to operating at nanogram levels where the losses are not significant.

The changes in brain and plasma levels of amphetamine and phentermine with time are plotted in Figs. 2*a* and 2*b*. Brain levels are consistently higher than plasma for both drugs, with the ratio of brain to plasma level gradually decreasing with time. Early (<1 hr.) levels of the two amines were very close, but plasma phentermine declined more rapidly after 3 hr. Results (Table II) of an analysis of the data are based on a two-compartment open model (Scheme I), where V_{dist} is the volume of distribution; V_1 and V_2 are the volumes of the central and second compartments, respectively; and the *k*'s refer to the rate constants for movements indicated by the arrows. The $t_{1/2}$ values are derived from the β term in the two-exponential equation: $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ (10). The data indicate that the transfers out of the different compartments were comparable but that the apparent volumes were different. The volume of distribution of phentermine was higher than that of amphetamine, suggesting that greater localization occurs with the more lipid-soluble amine.

The decay curves for plasma and brain were not single exponentials and were analyzed according to the two-compartment model discussed by Riegelman *et al.* (10), using a nonlinear regression analysis procedure (Table II). In the case of amphetamine, the differ-



Scheme I

[3] The amphetamine sulfate used in these experiments was obtained from Dr. Glenn Ullyot of Smith Kline & French Laboratories, and the phentermine was obtained from Dr. Aldo P. Truant of Pennwalt Corp.

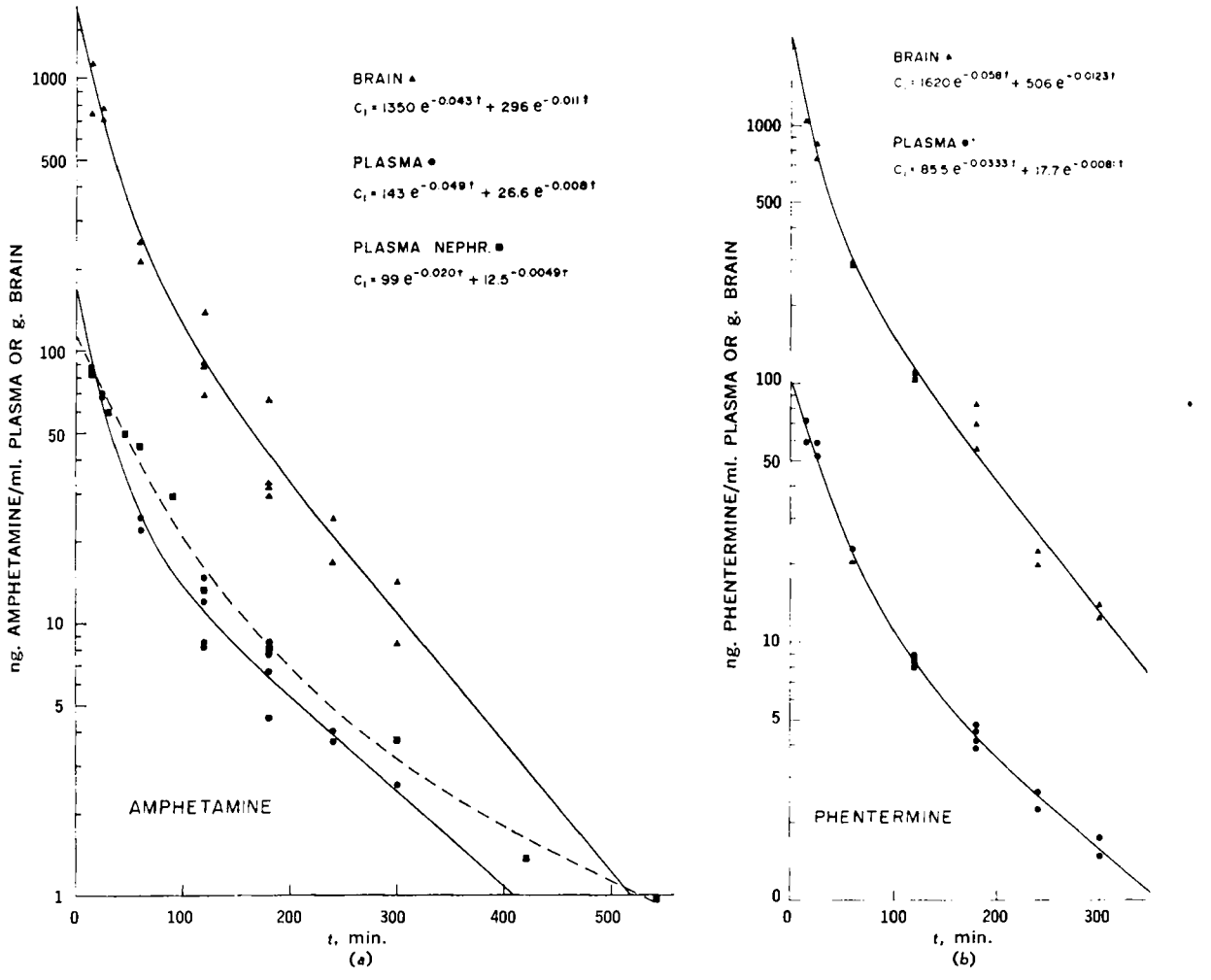


Figure 2—*Plasma and brain levels of amphetamine* (a) *and phentermine* (b). *The data are superimposed on curves representing the double-exponential equation shown in the upper right corner of each figure. The dotted lines in* 2a *represent the corresponding curves for data from nephrectomized animals.*

ing volumes (V_1 and V_2) calculated for the two compartments reflect the differences in tissue concentrations, with the plasma volume about 10-fold greater. The rate constants are comparable for the two tissues, indicating that they are both part of the central compartment. This would be expected from the high perfusion rate of the brain and the ease with which amphetamine would penetrate the blood–brain barrier.

Analysis of the phentermine data gives similar results, with the ratios of the plasma and brain volumes differing by a factor of 20. The values of the rate constants were somewhat different, perhaps reflecting the greater deviation of the 3- and 4-hr. brain levels from the calculated curve representing two-compartment kinetics (Fig. 2*b*).

The plasma volume of distribution calculated for phentermine was larger than that for amphetamine, reflecting the difference in the volume of V_1, the central compartment. The difference is consistent with the higher organic–aqueous partition coefficient (1) for phentermine, since highly lipid-soluble compounds tend to be localized and give larger volumes of distribution. Another example of this phenomenon is the larger volume of distribution of thiopental compared to its more polar analog, pentobarbital (10). However, in contrast to the amines, it is the volume of the second compartment that reflects the differing volumes of distribution of these weakly acidic compounds.

The role of excretion in controlling the volume of distribution and half-life of amphetamine was examined in nephrectomized rats. The changes in plasma level are shown in Table III and are compared to the levels from control animals.

Nephrectomy caused a small increase in the plasma half-life as calculated from β, the disposition rate constant (10). This increase, from 87 to 141 min., reflected differences in plasma levels at later time points (Fig. 2*a*). In contrast, inhibition of metabolism increased brain levels (2) and the brain half-life (15) of amphetamine by a factor of 5. A corresponding increase in the plasma half-life of the drug can be assumed since both brain and plasma belong to the central compartment. These data indicate that metabolism is considerably more important than renal excretion as the route of elimination of amphetamine.

Table II—Analysis of Phentermine and Amphetamine Levels[a]

	—Amphetamine—		—Phentermine—	
	Plasma	Brain	Plasma	Brain
V_{diat}, l.	0.81	0.084	1.34	0.065
V_2, l.	0.85	0.088	0.78	0.046
V_1, l.	1.66	0.172	2.12	0.111
k_1, min.$^{-1}$	0.015	0.010	0.007	0.016
k_{-1}, min.$^{-1}$	0.016	0.017	0.012	0.023
k_2, min.$^{-1}$	0.028	0.025	0.022	0.029
$t_{1/2}$, min.	86.7	62.3	85.6	56.3

[a] The plasma and brain levels were fitted to the two-exponential function describing the two-compartment model of Riegelman *et al.* (10). A Wang desk computer programmed for nonlinear regression analysis was used.

Little information is available on the *in vivo* metabolism of phentermine. Vree *et al.* (16) reported that phentermine is not metabolized in man but is excreted unchanged in the urine. In preliminary experiments with rat liver microsome preparations, we found little if any *p*-hydroxylation occurring but observed *N*-hydroxylation in the rabbit (17). However, it is difficult to demonstrate *p*-hydroxylation of amphetamine *in vitro* (18) and a similar situation may exist

Table III—Plasma Levels of Amphetamine and Phentermine

t, min.	Amphetamine[a], ng./ml.	Amphetamine[a] (Nephrectomized), ng./ml.	Phentermine[a], ng./ml.
15	86.5 (1.8)[b]	83.8	65.1 (2.1)
25	69.4 (2.1)	59.5	55.7 (6.9)
45	— —	49.0	— —
60	23.0 (2.5)	44.6	21.1 (1.9)
90	— —	29.4	— —
120	10.8 ± 3.1[c]	12.0	8.5 ± 0.4[c]
180	6.8 ± 1.8[c]	7.8	4.4 ± 0.4[c]
240	3.8 (0.9)	—	2.4 (0.4)
300	2.5	3.62	1.6 (0.2)
420	— —	1.35	— —
540	— —	0.95	— —

[a] All animals were given 0.5-mg./kg. i.v. doses of drug and lightly anesthetized with ether; blood was collected as described in the *Experimental* section. [b] Numbers in parentheses are the range of two values, and the numbers given ± *SD* are the mean of four values. [c] Standard deviation.

with phentermine. Experiments examining this possibility are in progress.

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Novel Method for Bioavailability Assessment

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Keyphrases □ Bioavailability—determination with flexible, model-independent method based on estimates of renal clearance, plasma concentration, and urinary excretion of unchanged drug □ Plasma concentration and urinary excretion data—used in flexible, model-independent method for assessing bioavailability □ Urinary excretion and plasma concentration data—used in flexible, model-independent method for assessing bioavailability

There are many ways to estimate bioavailability; each one entails a set of assumptions, some of which can be experimentally verified. Oser *et al.* (1) assumed that the same fraction of the absorbed compound from different preparations is excreted unchanged in the urine. Hence, the ratio of the urinary recovery following a test preparation to that following the same dose administered as a solution is taken to be a measure of relative absorption, which they defined as "physiologic availability."

Pharmacokinetic methods of assessing bioavailability from blood plasma concentrations or urinary excretion data were summarized by Wagner (2). In essence, methods based on plasma concentration data are predicated on comparisons of the products of plasma clearance, $\dot{V}_{cl,p}$, and the total area under the plasma concentration curve, $(AUC)_\infty$. If one assumes $\dot{V}_{cl,p}$ to be constant for a given subject from one test dose to another, then the ratio of $(AUC)_\infty$ is a measure of relative absorption. But if there are intrasubject variations in elimination, the assumption of constant $\dot{V}_{cl,p}$ will not hold and an adjustment is indicated. The nature of such an adjustment depends on one's ability to estimate the terminal plasma $t_{1/2}$ in a region free from the influence of continued absorption and on one's conception of the pharmacokinetic model (3, 4). In any event, a proper

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Table I—Simulated Plasma Concentration and Urinary Excretion Data following Single 500-mg. Intravenous and Oral Doses of a Drug to the Same Subject on Three Separate Occasions

Treatment	Plasma Concentration, mcg./ml., in Minutes											
	5	10	20	30	40	60	120	180	240	300	360	720
Intravenous	17.99	15.12	11.21	8.78	7.14	5.05	(2.09)	(0.89)	(0.38)	0.16	0.07	—
Oral No. 1	—	—	1.43	—	2.06	2.32	(2.22)	(1.69)	(1.17)	0.77	0.49	0.02
Oral No. 2	—	—	4.50	—	5.42	5.15	(3.15)	(1.65)	(0.82)	0.40	0.20	0

	Urinary Recovery, mg., in Hours								
	−1–0	0–1	1–2	2–3	3–4	4–6	6–12	12–24	0–24
Intravenous	0	171.97	56.30	(23.77)	(10.14)	6.18	1.37	0	(269.73)
Oral No. 1	0	21.45	31.25	(26.27)	(19.02)	21.08	12.64	0.59	(132.30)
Oral No. 2	0	50.81	48.08	(26.91)	(13.74)	10.12	3.10	0.20	(152.96)

estimate of the terminal plasma $t_{\frac{1}{2}}$ is usually required to calculate $(AUC)_\infty$ by extrapolation beyond the last data point; in some cases, this extrapolation process may also be model dependent (5).

Methods based on urinary excretion data for unchanged drug or metabolites embody the same assumptions as those of Oser *et al.* (1). In pharmacokinetic terms, the assumptions are that component rates of total elimination are individually constant or that the ratio of the rate of urinary excretion to the overall rate of elimination of that which is absorbed is constant. Intrasubject variations in the rate of urinary excretion are manifested as changes in the slopes of the amounts remaining to be excreted *versus* time plots. Compensations are subject to the same considerations as with plasma $t_{\frac{1}{2}}$ and are, therefore, also model dependent.

Depending on the biological disposition of the drug, bioavailability studies are designed to utilize one or more of these methods. Such studies should include means for testing some of the assumptions. Where it is safe and experimentally feasible, it is generally desirable to include at least one intravenous dose as a reference of maximum bioavailability. The purpose of this report is to describe a different experimental strategy which utilizes plasma concentration and urinary excretion data together so as to permit greater flexibility in the design of bioavailability protocols.

THEORETICAL

Plasma and renal clearances are physiological phenomena which should not be model dependent. Estimates of plasma and renal clearances become model dependent when their values are inferred from pharmacokinetic parameters. Observed plasma concentrations are manifestations of the net effect of absorption, distribution, metabolism, and excretion; hence, sets of plasma concentration data points by themselves are not model dependent. But mathematical reconstructions of plasma concentration data may be model dependent or uncertain when projections are made concerning AUC beyond the last data point. The proposed strategy for the assessment of bioavailability is based on determinations of renal clearance and assumptions about the constancy of plasma clearance or parts thereof following test doses of a drug.

Suppose the bioavailability of an oral dosage form is to be assessed by comparing it with an intravenous injection of the same drug. From the total urinary recovery of unchanged drug following the intravenous dose:

$$Ff = \frac{U_\infty}{D} \qquad \text{(Eq. 1)}$$

For an intravenous dose, $F = 1.0$; hence:

$$f^{iv} = \frac{U_\infty{}^{iv}}{D^{iv}} \qquad \text{(Eq. 2)}$$

where F is the fraction of the dose D that reaches the general circulation unchanged, f is the fraction of F that is excreted in the urine unchanged, U_∞ is the total amount of unchanged drug recovered in the urine, and the superscripts refer to the treatment. Renal clearance, $\dot{V}_{cl,r}$, of unchanged drug can be estimated from plasma samples taken over intervals coinciding with one or more fractional urine collection periods, *i.e.*:

$$\dot{V}^{iv}_{cl,r} = \frac{U_{t_2} - U_{t_1}}{\int_{t_1}^{t_2} Cp\, dt} \qquad \text{(Eq. 3)}$$

where U_{t_1} and U_{t_2} are the cumulative amounts of unchanged drug excreted in the urine at times t_1 and t_2, respectively; and $\int_{t_1}^{t_2} Cp\, dt$ is the area under the plasma curve from t_1 to t_2, which can be estimated by the trapezoidal rule or other suitable means. Plasma clearance is estimated by:

$$\dot{V}^{iv}_{cl,p} = \frac{\dot{V}^{iv}_{cl,r}}{f^{iv}} = \frac{D^{iv}\dot{V}^{iv}_{cl,r}}{U_\infty{}^{iv}} \qquad \text{(Eq. 4)}$$

Following oral administration, the observed fraction of the unchanged drug excreted in the urine is the product of the fraction absorbed and the fraction of the compound that is absorbed and excreted unchanged, *i.e.*:

$$F^{po}f^{po} = \frac{U_\infty{}^{po}}{D^{po}} \qquad \text{(Eq. 5)}$$

If plasma clearance is assumed to be constant between test doses, $\dot{V}^{iv}_{cl,p} = \dot{V}^{po}_{cl,p}$, then:

$$f^{po} = \frac{\dot{V}^{po}_{cl,r}}{\dot{V}^{po}_{cl,p}} = \frac{\dot{V}^{po}_{cl,r}}{\dot{V}^{iv}_{cl,p}} \qquad \text{(Eq. 6)}$$

and the fraction absorbed:

$$F^{po} = \frac{F^{po}f^{po}}{f^{po}} = \frac{U_\infty{}^{po}\dot{V}^{iv}_{cl,p}}{D^{po}\dot{V}^{po}_{cl,r}} \qquad \text{(Eq. 7)}$$

The assumption of constant plasma clearance may be reassessed by comparing observed values for $\dot{V}^{po}_{cl,r}$ and $\dot{V}^{iv}_{cl,r}$. If they are equal, it may be reasonable to accept the hypothesis of constant plasma clearance in the absence of contrary evidence. This is tantamount to saying that the ratio of fractional urinary recovery, Ff, is a measure of bioavailability when the ratio of $\dot{V}_{cl,r}$ to $\dot{V}_{cl,p}$ is constant between treatments. However, if they are not equal, several obvious alternatives can be considered. First, the observed differences may be ignored on the premise that the nonrenal components of plasma clearance would be compensating. Second, one may assume that only the renal components change but that the nonrenal components remain constant, in which event:

$$\dot{V}^{po'}_{cl,p} = \dot{V}^{po}_{cl,r} + \dot{V}^{iv}_{cl,p} - \dot{V}^{iv}_{cl,r} \qquad \text{(Eq. 8)}$$

and:

$$f^{po} = \frac{\dot{V}^{po}_{cl,r}}{\dot{V}^{po'}_{cl,p}} \qquad \text{(Eq. 9)}$$

Third, the nonrenal component of $\dot{V}_{cl,p}$ may be thought to vary in

Table II—Sample Calculation of Bioavailability Using the Intravenous Dose as Reference

Treatment	Clearance, ml./min. $\dot{V}_{cl,r}^{iv}$[a]	Clearance, ml./min. $\dot{V}_{cl,p}^{iv}$[b]	Clearance, ml./min. $\dot{V}_{cl,r}^{po}$[a]	Clearance, ml./min. $\dot{V}_{cl,p}^{po'}$[c]	Fraction of Dose Excreted in Urine, fF^d	Fraction of Dose Absorbed, F^e
Intravenous	266.2 (282.1)[f]	493.9 523.4			0.539	
Oral No. 1			223.1 (222.9)	450.8 (464.2)	0.265	0.536 (0.552)
Oral No. 2			186.6 (192.4)	414.3 (433.7)	0.306	0.680 (0.690)

[a] By Eq. 3. [b] By Eq. 4. [c] By Eq. 8. [d] By Eq. 1. [e] By Eq. 7. [f] See text.

Table III—Sample Calculation of Relative Bioavailability Using Oral Dose No. 2 as Reference

Treatment	Clearance, ml./min. $\dot{V}_{cl,r}^{s}$[a]	Clearance, ml./min. $[\dot{V}_{cl,p}^{s}]_{ex}$[b]	Clearance, ml./min. $\dot{V}_{cl,r}^{x}$[a]	Clearance, ml./min. $[\dot{V}_{cl,p}^{x}]_{ex}'$[c]	Fraction of Dose Excreted in Urine, fF^d	Relative Absorption, F^xF^{se}
Oral No. 1			223.1	646.3	0.265	0.768
Oral No. 2	186.6	609.8			0.306	

[a] By Eq. 3. [b] By Eq. 13. [c] By Eq. 17. [d] By Eq. 1. [e] By Eq. 16.

direct proportion to changes in the renal clearance, so that:

$$\dot{V}_{cl,p}^{po''} = \frac{\dot{V}_{cl,r}^{po}}{\dot{V}_{cl,r}^{iv}} \cdot \dot{V}_{cl,p}^{iv} \qquad \text{(Eq. 10)}$$

and:

$$^{po} = \frac{\dot{V}_{cl,r}^{po}}{\dot{V}_{cl,p}^{po''}} \qquad \text{(Eq. 11)}$$

Finally, one may take the view that none of the first three alternatives is reasonable; hence, no estimate of bioavailability is warranted. The choice of alternatives may be dictated by one's understanding of the physicochemical and pharmacological properties of the drug and prior knowledge of its disposition in animals and in man. For example, changes in cardiac output would influence $\dot{V}_{cl,p}$ and $\dot{V}_{cl,r}$ and the other excretory components of $\dot{V}_{cl,p}$ proportionately, whereas changes in urinary pH may affect only $\dot{V}_{cl,r}$ of some drugs.

The proposed strategy applies even when an intravenous dose is not included. In such cases, one treatment is usually designated as the standard (s) to which all other treatments (x) are compared. Accordingly:

$$\frac{U_\infty^s}{D^s} = F^s f^s \qquad \text{(Eq. 12)}$$

and:

$$[\dot{V}_{cl,p}^{s}]_{ex} = \frac{\dot{V}_{cl,r}^{s}}{F^s f^s} \qquad \text{(Eq. 13)}$$

The quantity $[\dot{V}_{cl,p}^{s}]_{ex}$ denotes an experimentally derivable value which is only proportional to the true plasma clearance, $\dot{V}_{cl,p}^{s}$, in that:

$$\dot{V}_{cl,p}^{s} = F^s[\dot{V}_{cl,p}^{s}]_{ex} \qquad \text{(Eq. 14)}$$

For $\dot{V}_{cl,r}^{s} = \dot{V}_{cl,r}^{x}$:

$$f^x = \frac{\dot{V}_{cl,r}^{x}}{\dot{V}_{cl,p}^{x}} = \frac{\dot{V}_{cl,r}^{s}}{\dot{V}_{cl,p}^{x}} = \frac{\dot{V}_{cl,r}^{x}}{F^s[\dot{V}_{cl,p}^{s}]_{ex}} \qquad \text{(Eq. 15)}$$

Relative absorption is given by:

$$\frac{F^x}{F^s} = \frac{F^x f^x}{F^s f^x} = \frac{U_\infty^x \cdot [\dot{V}_{cl,p}^{s}]_{ex}}{D^x \dot{V}_{cl,r}^{x}} \qquad \text{(Eq. 16)}$$

Corrective measures, when indicated, should be applied to $[\dot{V}_{cl,p}^{s}]_{ex}$. Thus, Eqs. 8 and 10 are revised to read as follows:

$$[\dot{V}_{cl,p}^{x}]_{ex}' = \dot{V}_{cl,r}^{x} + [\dot{V}_{cl,p}^{s}]_{ex} - \dot{V}_{cl,r}^{s} \qquad \text{(Eq. 17)}$$

and:

$$[\dot{V}_{cl,p}^{x}]_{ex}'' = \frac{\dot{V}_{cl,r}^{x}}{\dot{V}_{cl,r}^{s}} \cdot [\dot{V}_{cl,p}^{s}]_{ex} \qquad \text{(Eq. 18)}$$

DISCUSSION

Experimentally, the proposed method calls for the complete collection of urine in planned increments. Plasma sampling times should at least coincide with selected incremental urinary collection periods. Intermediate plasma samples may also be indicated to permit accurate determinations of the incremental areas under the plasma curve, which is essential to the calculations of renal clearance. Assays of plasma and urine specimens should be specific for unchanged drug. The number of plasma points may be minimized by choosing relatively short urine collection periods at times when plasma concentrations do not change rapidly. It is also prudent to include more than one estimate of $\dot{V}_{cl,r}$ per treatment. In this regard, it might be advantageous to make estimates of $\dot{V}_{cl,r}$ from contiguous urine collection periods. This is so in respect both to economy in the required number of plasma data points and to the modulation of experimental difficulties in voiding completely and on time. (See Appendix for sample calculations.)

The description of the proposed method is confined to the case where bioavailability is defined as the amount, or relative amount, of drug reaching the general circulation unchanged. In some cases, such as with prodrugs, it may be more appropriate to define bioavailability as that fraction, or relative fraction, of the dose reaching the general circulation as a specific metabolite. The proposed strategy accommodates these situations with the following provisions. Estimates of $\dot{V}_{cl,r}$ and $\dot{V}_{cl,p}$ are those of the metabolite. Equation 4 applies only if the intravenous dose is administered as the metabolite; otherwise, only estimates of relative absorption would be possible, and Eq. 13 should be used regardless of the route of administration. Because the method relies on estimates of $\dot{V}_{cl,p}$ or $[\dot{V}_{cl,p}]_{ex}$, it may be considered only when bioavailability can be appropriately defined in terms of drug or metabolite(s) reaching the general circulation. Thus, if metabolism is route dependent, then, by definition, drug metabolized on the first pass through the liver is nonavailable. For this reason, this method is generally inappropriate if estimates of bioavailability of a drug are attempted through measurements of metabolite(s).

In summary, the proposed method requires estimates of U_∞ and $\dot{V}_{cl,r}$, from which $\dot{V}_{cl,p}$ or $[\dot{V}_{cl,p}]_{ex}$, f, and F can be calculated. Some salient features are:

1. It is model independent. The only assumptions are those related to plasma clearance or components thereof. It should be recalled that the same assumptions concerning $\dot{V}_{cl,p}$ and/or $\dot{V}_{cl,r}$ are implicit in all pharmacokinetic methods; however, the proposed method does not entail further assumptions concerning individual

or hybrid model parameters. Since estimates of $\dot{V}_{cl,p}$ and $\dot{V}_{cl,r}$ are not influenced by the nature of the absorption process, this method should apply even when absorption is inordinately slow or defies mathematical description. At the same time, the techniques are not only compatible with but also may be applied advantageously in conjunction with pharmacokinetic techniques.

2. A complete definition of the time course of change in plasma concentrations is not needed; thus, the required number of plasma data points is flexible. It is, therefore, particularly well suited in situations where large volumes of plasma are required per sample, such as when the assay method lacks sensitivity or when the dosages are low. If necessary, several doses may be given repetitively so that plasma levels will be sufficiently high before estimates of $\dot{V}_{cl,r}$ are made. In this event, one would obtain an assessment of average bioavailability upon repetitive dosing, but one does not need to wait for the attainment of steady state before terminating the study.

3. Since $\dot{V}_{cl,p}$ is an estimate of the net result of physiological disposition, the method should yield valid assessments of bioavailability even when enterohepatic recirculation exists or is suspected.

4. Even though the present discussion on intrasubject variations, manifested as changes in $\dot{V}_{cl,r}$, considers only alternative assumptions, concomitant measurements of metabolic turnover rates and renal clearances of endogenous substances, particularly those with both renal and extrarenal components of elimination, are seen as interesting possibilities as independent indicators of change in $\dot{V}_{cl,r}$ and $\dot{V}_{cl,p}$. This would entail tracer doses of the indicator, which should be selected among candidates having elimination patterns resembling those of the drug being studied. Various applications of the proposed method will be discussed in future reports.

APPENDIX

Sample calculations using the proposed method are performed on a set of simulated data for a subject who received the same dose of a drug intravenously once and orally on two other occasions. Except for the renal clearance of unchanged drug, all other aspects of drug disposition remained constant for each of the three test doses. Absorption was arbitrarily fixed at 55.0 and 68.8% for the two oral doses. Typical plasma concentration and urinary excretion data for unchanged drug simulated according to the prescribed conditions are shown in Table I.

Application of the proposed method does not require all of the data in Table I. An assessment of bioavailability can be attempted from just a few selected points, such as those shown in parentheses. Sample calculations using the intravenous dose as the standard reference are shown in Table II. Renal clearances are calculated from incremental urinary recoveries at 2–3 and 3–4 hr. and trapezoid areas under the plasma curve for the corresponding times. Since they are different for each test dose, estimates of $\dot{V}_{cl,p}^{po\prime}$ are made with the aid of Eq. 8. The fractions absorbed, F^{po}, are then calculated from $\dot{V}_{cl,r}^{po}$, $\dot{V}_{cl,p}^{po\prime}$, and U_{∞}^{po} using Eq. 7. The difference between the calculated and the true fraction absorbed is due to the trapezoid approximation of areas under the plasma curve. Had the true areas been used, the answers would have been exact; these are shown in parentheses.

With the assumption that there were no data from an intravenous dose, calculations of relative absorption from the two oral doses are shown in Table III in which the second oral dose is designated as the standard. The answer, $F^2/F^1 = 0.767$, is slightly different from the ratio of $F^1/F^2 = 0.788$ obtained in Table II using the intravenous dose as the standard. This is because the appropriate expressions for estimating $\dot{V}_{cl,p}^{po\prime}$ (Eq. 8) and $[\dot{V}_{cl,p}^{2}]_{ex}{}'$ (Eq. 17) in this example are not linearly related to $\dot{V}_{cl,p}^{iv}$ and $[\dot{V}_{cl,p}^{1}]_{ex}$, respectively; hence, an exact proportionality is not expected when different standards are used. Conversely, when linear expressions, such as Eqs. 9 and 18, are appropriate, exact correspondence between ratios should result regardless of how the standards are chosen.

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Effect of Concurrent Administration of Choline Salicylate and Acetaminophen on Their Mutual Biotransformation in the Rat

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Abstract □ Rats were administered either choline salicylate (2 mg./kg.) or acetaminophen (10 mg./kg.) or a combination of both by gastric intubation. Plasma levels of salicylic acid and acetaminophen were measured at various time intervals by suitable modifications of reported analytical methods. Concurrent administration of the two drugs did not alter the plasma salicylic acid levels but resulted in a slight increase of the plasma acetaminophen values at 5 and 10 min. Differing degrees of glucuronidation of the two drugs may explain this interaction which, however, appears too low to be of clinical significance.

Keyphrases □ Choline salicylate and acetaminophen, concurrent administration—effect on individual plasma levels, rats □ Acetaminophen and choline salicylate, concurrent administration—effect on individual plasma levels, rats □ Analgesics, concurrent administration of choline salicylate and acetaminophen—effect on individual plasma levels, rats □ Plasma levels—choline salicylate and acetaminophen after concurrent administration, rats

Mutual inhibition of the metabolism of drugs sharing a common biotransformation pathway is well documented (1–3). Among the nonprescription analgesics, such interactions have been reported to occur between salicylamide and acetaminophen (4) and salicylic acid and salicylamide (5). In man, salicylic acid is metabolized to salicyluric acid, salicyl acylglucuronide, phenolic glucuronide, and gentisic acid (6). The main metabolic pathway of acetaminophen is conjugation to the glucuronide and sulfate (7). It is conceivable that salicylic acid and acetaminophen would compete for sites on the enzyme systems responsible for their biotransformation, this competition leading to decreased glucuronide formation which, in turn, might result in increased blood levels of the free drug(s). Levy and Regårdh (8) observed little interaction in the biotransformation of acetaminophen and salicylic acid in human volunteers. In their study, acetaminophen and sodium salicylate were given at different time intervals, and the urinary excretion rates of the metabolites were studied. Amsel and Davison (9) administered acetaminophen with single and multiple doses of aspirin and observed no effect on acetaminophen metabolism in man.

The present study was initiated to determine if the plasma levels of acetaminophen and salicylic acid are affected by concurrently administering the former with choline salicylate to rats. This experimental design was chosen because: (*a*) choline salicylate has been reported to be absorbed much faster, giving rise to higher blood levels of salicylic acid than an equivalent dose of aspirin in man (10, 11); and (*b*) in experimental animals like the rat, it is more convenient and amenable to measure quantitatively plasma drug levels at various intervals like 5, 10, and 15 min. than urinary excretion rates.

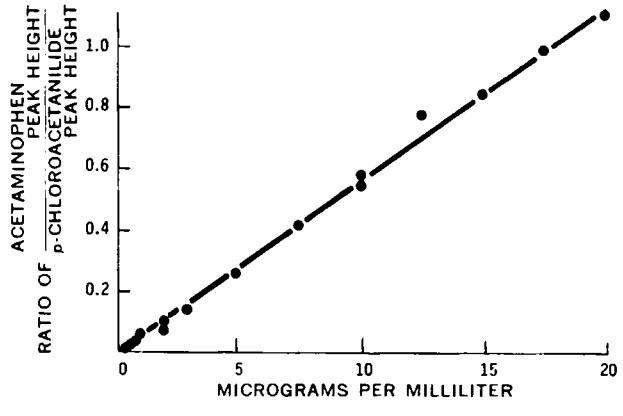

Figure 1—*Calibration curve of acetaminophen added to rat plasma.*

EXPERIMENTAL

Materials—The following were used: dichloroethane, spectroquality[1]; ethyl acetate, spectroquality[1]; phosphate buffers, 1.0 *M*, pH 8.0, and 0.1 *M*, pH 7.0; sodium sulfate, anhydrous, reagent grade[2]; *p*-chloroacetanilide[3]; nitrogen gas, high purity[4]; bis(trimethylsilyl)trifluoroacetamide[5]; pyridine[6]; choline salicylate, 51% (w/w) aqueous solution[6]; a column conditioner containing trimethylsilyl donors[7]; phenyl methyl silicone, 50% phenyl (OV-17)[8]; and Gas Chrom Q[8].

Methods—*Determination of Acetaminophen in Rat Plasma*—The method of Prescott (12) was modified and adopted as follows. Rat plasma (2.0 ml.) was adjusted to pH 8.0 by the addition of 1.0 ml. phosphate buffer, 1.0 *M*, pH 8.0, and then extracted by shaking for 10 min. with 6.0 ml. ethyl acetate. The mixture was centrifuged for 5 min. at 6000 r.p.m. The organic layer was separated and dried over 1.0 g. anhydrous sodium sulfate. A 4.0-ml. aliquot of the extract was transferred to a screw-capped conical tube containing 15 mcg. *p*-chloroacetanilide in ethyl acetate. The solvent was evaporated by heating at 60° under a stream of dry nitrogen. The sides of the tube were washed with 1.0 ml. ethyl acetate, and the solvent was again removed by evaporation. The residue was dissolved in 15 μl. pyridine, reacted with 15 μl. bis(trimethylsilyl)trifluoroacetamide at 60° for 30 min., and cooled; then 1.0 μl. was injected into a gas chromatograph[9] equipped with a flame-ionization detector.

The chromatographic conditions were: glass column, 0.63 cm. (0.25 in.) o.d., 1.82 m. (6 ft.), containing 10% phenyl methyl silicone (50% phenyl) on Gas Chrom Q (100–120 mesh); carrier gas (nitrogen) at a flow rate of 45 ml./min.; column temperature, 160°; injection block temperature, 210°; and detector temperature, 220°. The retention times of *p*-chloroacetanilide and acetaminophen were 4.0 and 7.8 min., respectively.

Determination of Salicylic Acid in Rat Plasma—The spectrofluorometric method of Rowland and Riegelman (13) was modified as follows. Plasma (2.0 ml.) was acidified to pH 2.0 with 3.0 *N* HCl and extracted with dichloroethane (25 ml.). Salicylic acid from

[1] Matheson, Coleman and Bell, Norwood, Ohio.
[2] J. T. Baker Co., Phillipsburg, N. J.
[3] Eastman Organic Chemicals, Rochester, N. Y.
[4] SOS Gases Inc., Kearny, N. J.
[5] Regisil, Regis Chemical Co., Chicago, Ill.
[6] RSA Corp., Ardsley, N. Y.
[7] Silyl-8, Pierce Chemical Co., Rockford, Ill.
[8] Applied Science Laboratories Inc., State College, Pa.
[9] Perkin-Elmer model 900.

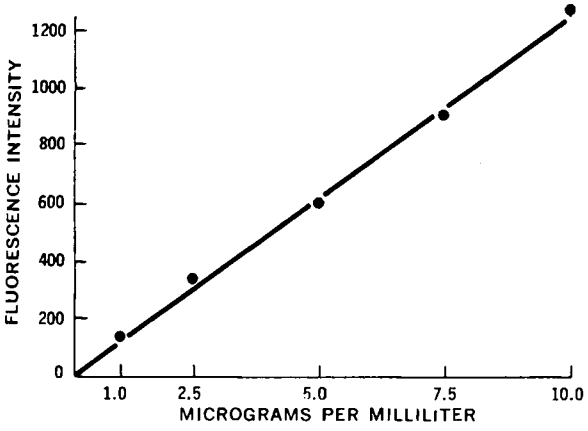

Figure 2—*Calibration curve of salicylic acid added to rat plasma.*

a 20-ml. aliquot of the organic layer was reextracted into 5.0 ml. of phosphate buffer, 0.1 *M*, pH 7.0, and the fluorescence was measured in a spectrophotofluorometer[10]. Activation and emission wavelengths were 302 and 406 nm., respectively, and both slit dials were set at 4.0 nm.

Separation and Estimation of Acetaminophen and Salicylic Acid from Rat Plasma in the Presence of Each Other—By employing the two procedures already outlined, a method was developed for the separation and estimation of acetaminophen and salicylic acid in plasma.

Two milliliters of plasma was extracted by shaking with ethyl acetate and analyzed for acetaminophen by the GLC procedure already described. The aqueous layer (2.5 ml.) was then reextracted with 6.0 ml. of ethyl acetate to remove residual acetaminophen in solution. The organic layer was discarded, and 2.0 ml. of the aqueous phase was acidified with 0.2 ml. of 6 *N* HCl and extracted with 25 ml. of dichloroethane. The salicylic acid was taken into phosphate buffer, 0.1 *M*, pH 7.0, and the fluorescence was measured as described earlier.

Plasma Levels of Acetaminophen and Salicylic Acid in Rats—Groups of 10 female Sprague–Dawley rats, 220–260 g., were fasted for 18 hr. They then received, by stomach tube, aqueous solutions of: (*a*) acetaminophen, 10 mg./kg.; (*b*) choline salicylate, 2 mg./kg.; or (*c*) a combination of acetaminophen, 10 mg./kg., and choline salicylate, 2 mg./kg. Blood samples were withdrawn into heparinized tubes by cardiac puncture immediately prior to and at 5, 10, 15, 30, 45, 60, 90, and 120 min. after treatment. Ten rats were used per time period. The plasma was separated by centrifugation and analyzed for acetaminophen and/or salicylic acid according to one of the three described procedures.

RESULTS

Determination of Acetaminophen in Rat Plasma—A linear relationship was observed between the concentration of acetaminophen (0.05–20 mcg./ml.) and the peak height ratio (peak height of acetaminophen/peak height of *p*-chloroacetanilide) when known amounts were added to normal rat plasma and assayed by the analytical procedure (Fig. 1). The recovery of added drug was 67%. It was found that the column efficiency could be maintained better by periodic injections of five 10-μl. portions of column conditioner[7] at 60° and programming the column to 200° at 2°/min. with its detector end disconnected after every 20–30 analyses.

Known amounts of salicylic acid (1.0–10 mcg./ml.) were added to rat plasma and assayed. A linear relationship was observed between the salicylic acid concentration and the fluorescence (Fig. 2). The recovery was 100%.

Varying concentrations of acetaminophen (1–20 mcg./ml.) were added to rat plasma in the presence of 20 mcg. of salicylic acid, as were known concentrations of salicylic acid (0.5–20 mcg./ml.) in the presence of 20 mcg. of acetaminophen. Then the samples were assayed differentially according to the procedure already outlined. There was no interference by either drug on the recovery or measurement of the other. Consequently, the calibration curves shown in Figs. 1 and 2 were employed to determine the concentrations of the two drugs in plasma samples containing the two in unknown proportions.

[10] Perkin-Elmer model MPF-3.

Table I—Plasma Levels[a] of Salicylic Acid (Micrograms per Milliliter) after Oral Administration of Choline Salicylate Either Alone or in Combination with Acetaminophen

Time Postdrug, min.	Choline Salicylate, 2.0 mg./kg.	Choline Salicylate, 2.0 mg./kg., plus Acetaminophen, 10 mg./kg.
0	0	0
5	6.9 ± 0.6	6.7 ± 0.6
10	6.1 ± 0.3	6.7 ± 0.4
15	6.6 ± 0.2	6.8 ± 0.6
30	5.8 ± 0.3	5.5 ± 0.4
45	5.3 ± 0.3	5.9 ± 0.4
60	4.9 ± 0.5	5.4 ± 0.4
90	4.6 ± 0.2	4.9 ± 0.5
120	4.4 ± 0.3	4.6 ± 0.5

[a] Average of 10 individual experiments ± *SE*.

Plasma Levels of Acetaminophen and Salicylic Acid in Rats—The average plasma levels of salicylic acid and acetaminophen attained after administration of either drug alone or in combination to rats are shown in Tables I and II, respectively.

Oral administration of choline salicylate (2 mg./kg.) to rats resulted in peak salicylic acid plasma levels (6.9 mcg./ml.) within 5 min., which slowly decreased over 2 hr. to 4.4 mcg./ml. at 120 min. Concurrent administration with acetaminophen (10 mg./kg.) resulted in similar levels of salicylic acid, which were 6.7 mcg./ml. at 5 min., 6.8 mcg./ml. at 15 min., and 4.6 mcg./ml. at 120 min.

Acetaminophen (10 mg./kg.) administered orally to rats produced plasma levels that rose quickly to a peak (11.3 mcg./ml.) at 15 min. and then declined sharply to 0.1 mcg./ml. by 120 min. However, concurrent administration of acetaminophen (10 mg./kg.) with choline salicylate (2 mg./kg.) resulted in higher plasma acetaminophen levels at 5 and 10 min. ($0.05 < p < 0.1$ by a two-tail *t*-test).

DISCUSSION

Derivatizing acetaminophen by heating with bis(trimethylsilyl)-trifluoroacetamide under controlled conditions resulted in quantitative and more reproducible results. Employing dichloroethane in place of ether for extracting salicylic acid rendered the operation more facile and less hazardous. Therefore, the methodology appeared to be an improvement over existing ones (12, 13).

Levy and Regårdh (8) observed no interaction in man in the biotransformation of acetaminophen and salicylate on the basis of urinary excretion data. They did not rule out, however, the possibility of the inhibition of glucuronide and/or sulfate formation under forced or different conditions. Amsel and Davison (9) suggested that the two drugs might be metabolized by different systems in man.

The interaction between acetaminophen and salicylates in experimental animals has not been studied before. In the present study, concurrent administration of these two drugs to the rat resulted in slightly elevated plasma acetaminophen concentrations at 5 and 10 min. while the salicylate levels remained unaffected. The dose (per body weight) of acetaminophen given to rats compares favorably with that given to humans by earlier workers (8, 9). Salicylate was administered at a much lower dose because: (*a*) choline salicylate given orally resulted in peak plasma levels by 5 min. which slowly declined up to 120 min., and (*b*) too high plasma salicylate levels could so saturate the enzyme systems as to make it difficult to discern any alterations caused by acetaminophen. The interaction in rats, although it may be too low to be of any clinical significance, is possibly due to differing degrees of glucuronidation undergone by the two drugs in the rat. This is analogous to the inhibitory effect of benzoic acid on the formation of salicyluric acid from salicylate in man (14). Salicylic acid, on the other hand, was without an effect on hippuric acid formation from benzoic acid. The latter is almost completely biotransformed to hippuric acid, whereas only 50–70% of the former is conjugated with glycine in man. If it is assumed that a substrate or a drug with a greater affinity to the glucuronide-

Table II—Plasma Levels[a] of Acetaminophen (Micrograms per Milliliter) after Oral Administration of Acetaminophen Either Alone or in Combination with Choline Salicylate

Time Postdrug, min.	Acetaminophen, 10 mg./kg.	Acetaminophen, 10 mg./kg., plus Choline Salicylate, 2.0 mg./kg.
0	0	0
5	9.5 ± 0.9	11.4 ± 1.0[b]
10	9.0 ± 0.6	11.6 ± 1.3[b]
15	11.3 ± 2.8	8.5 ± 1.2
30	4.4 ± 0.3	4.5 ± 0.6
45	2.5 ± 0.3	3.5 ± 0.7
60	1.5 ± 0.2	1.5 ± 0.2
90	0.6 ± 0.1	0.6 ± 0.1
120	0.1 ± 0.03	0.3 ± 0.1

[a] Average of 10 individual experiments ± *SE*. [b] $0.05 < p < 0.1$ (by two-tail *t*-test).

forming enzyme system would be metabolized to a greater extent than one with a lower affinity, then salicylate appears to have a greater affinity and extent of glucuronide formation than acetaminophen in the rat.

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DRUG STANDARDS

Individual Tablet Analysis for Codeine and Caffeine in Codeine-Aspirin-Phenacetin-Caffeine Tablets

THERON JAMES

Abstract □ A procedure is reported for determining the codeine and caffeine content of individual codeine-aspirin-phenacetin-caffeine tablets. Codeine is determined fluorometrically after extraction into dilute sulfuric acid; caffeine is extracted from a chloroform solution of the remaining ingredients with phosphoric acid and determined by UV spectroscopy. Average recoveries with a synthetic mixture were 99.7 and 98.9% for codeine and caffeine, respectively. Assay results are reported for codeine [1–65 mg. (1/65–1 grain)/tablet] and caffeine [32 mg. (1/2 grain)/tablet] in several different commercial samples. The proposed procedure is also compared with official methods.

Keyphrases □ Codeine with aspirin-phenacetin-caffeine tablets—individual tablet analysis for codeine and caffeine, compared to official methods □ Aspirin-phenacetin-caffeine with codeine tablets—individual tablet analysis for codeine and caffeine, compared to official methods □ Spectrophotofluorometry—analysis, codeine in aspirin-phenacetin-caffeine with codeine tablets □ UV spectrophotometry—analysis, caffeine in aspirin-phenacetin-caffeine with codeine tablets □ Analgesic formulations—analysis of codeine and caffeine in individual aspirin-phenacetin-caffeine with codeine tablets

The NF XIII monograph (1) for tablets containing codeine and aspirin-phenacetin-caffeine (1) requires content uniformity tests for codeine and caffeine. The methods involve a GC determination for codeine, while caffeine is determined by UV spectroscopy after column chromatographic separation. Utilization of these procedures for content uniformity necessitates two separate analyses. Consequently, a single tablet is not tested for *both* drugs (*i.e.*, codeine is determined in one group of tablets while caffeine is determined in

Table II—Plasma Levels[a] of Acetaminophen (Micrograms per Milliliter) after Oral Administration of Acetaminophen Either Alone or in Combination with Choline Salicylate

Time Postdrug, min.	Acetaminophen, 10 mg./kg.	Acetaminophen, 10 mg./kg., plus Choline Salicylate, 2.0 mg./kg.
0	0	0
5	9.5 ± 0.9	11.4 ± 1.0[b]
10	9.0 ± 0.6	11.6 ± 1.3[b]
15	11.3 ± 2.8	8.5 ± 1.2
30	4.4 ± 0.3	4.5 ± 0.6
45	2.5 ± 0.3	3.5 ± 0.7
60	1.5 ± 0.2	1.5 ± 0.2
90	0.6 ± 0.1	0.6 ± 0.1
120	0.1 ± 0.03	0.3 ± 0.1

[a] Average of 10 individual experiments ± *SE*. [b] $0.05 < p < 0.1$ (by two-tail *t*-test).

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Individual Tablet Analysis for Codeine and Caffeine in Codeine-Aspirin-Phenacetin-Caffeine Tablets

THERON JAMES

Abstract □ A procedure is reported for determining the codeine and caffeine content of individual codeine-aspirin-phenacetin-caffeine tablets. Codeine is determined fluorometrically after extraction into dilute sulfuric acid; caffeine is extracted from a chloroform solution of the remaining ingredients with phosphoric acid and determined by UV spectroscopy. Average recoveries with a synthetic mixture were 99.7 and 98.9% for codeine and caffeine, respectively. Assay results are reported for codeine [1–65 mg. (1/65–1 grain)/tablet] and caffeine [32 mg. (1/2 grain)/tablet] in several different commercial samples. The proposed procedure is also compared with official methods.

Keyphrases □ Codeine with aspirin-phenacetin-caffeine tablets—individual tablet analysis for codeine and caffeine, compared to official methods □ Aspirin-phenacetin-caffeine with codeine tablets—individual tablet analysis for codeine and caffeine, compared to official methods □ Spectrophotofluorometry—analysis, codeine in aspirin-phenacetin-caffeine with codeine tablets □ UV spectrophotometry—analysis, caffeine in aspirin-phenacetin-caffeine with codeine tablets □ Analgesic formulations—analysis of codeine and caffeine in individual aspirin-phenacetin-caffeine with codeine tablets

The NF XIII monograph (1) for tablets containing codeine and aspirin-phenacetin-caffeine (1) requires content uniformity tests for codeine and caffeine. The methods involve a GC determination for codeine, while caffeine is determined by UV spectroscopy after column chromatographic separation. Utilization of these procedures for content uniformity necessitates two separate analyses. Consequently, a single tablet is not tested for *both* drugs (*i.e.*, codeine is determined in one group of tablets while caffeine is determined in

another). In addition, the assay procedures have several disadvantages when they are used for individual tablet analysis:

1. The original method for codeine (1) uses propoxyphene hydrochloride as an internal standard. Erratic results are often obtained because of the thermal decomposition of this compound.

2. The revised method for codeine (2) uses another internal standard but includes a lengthy *system suitability* procedure.

3. Assay results on tablets containing less than 30 mg. codeine are questionable because errors inherent in multiple extractions, transfers, and evaporations are greatly magnified when the active ingredient is at a low concentration.

4. Applying the assay procedure (1) for I to individual tablet analysis requires the acquisition and preparation of at least 10 chromatographic columns. With the accessory glassware needed as well, the method becomes both lengthy and cumbersome.

Obviously, a utilitarian method for determining both drugs in the same tablet would have more appeal. Heuermann and Levine's (3) method for determining I with other drugs has been used to determine codeine and caffeine. Codeine is trapped on a strong acid column and subsequently eluted with triethylamine in chloroform. Incomplete elution of codeine is common because of difficulty in completely basifying the strong acid column. Furthermore, the triethylamine must be completely removed prior to the determinative step and be of sufficient purity so as not to leave any UV-absorbing residues. Compound I passes through this acid column, and caffeine is determined as previously described by Levine (4). A procedure reported by Siegmund (5) is also capable of assaying both drugs. Its application to individual tablet analysis, however, is unwieldy because it requires *two* chromatographic columns per analysis. Parenthetically, Siegmund also reported incomplete separation of codeine and caffeine if the sample is not ground sufficiently.

The sensitivity and separation properties inherent in GLC offer another plausible alternative; however, reported procedures (6–9) are not apposite for individual tablet analysis. The methods of Hoffman and Mitchell (6) and Haefelfinger *et al.* (7) do not include codeine, and the method reported by Dechene *et al.* (8) involves a preliminary separation and temperature programming. Retention data reported by Rader and Aranda (9) indicate good separation between codeine and I. However, most parameters in their procedure (*e.g.*, sample extraction, instrument operating conditions, and retention times) need modification for individual tablet analysis.

The quest for a more practical approach generated this investigation. It was determined experimentally that caffeine can be quantitatively extracted from chloroform and effectively separated from phenacetin by using phosphoric acid. This discovery, coupled with the easy extractability of codeine by dilute acids, led to the development of a practical, facile method for individual tablet analysis for codeine and caffeine in common analgesic tablets. In the proposed method, a single tablet is dispersed in dilute sulfuric acid and extracted with chloroform. The acid layer retains codeine, which is determined fluorometrically after filtration and proper dilution. Caffeine is extracted from a portion of the chloroform solution with phosphoric acid and subsequently determined by UV spectroscopy.

EXPERIMENTAL

Apparatus—Fluorescence measurements were made using a recording spectrophotofluorometer[1] with 1-cm. cells. The following instrument parameters were employed: xenon lamp; meter multiplier, 0.03; sensitivity, 40–50; 1P21 photomultiplier; slit arrangement No. 4; excitation wavelength, about 285 nm.; and emission wavelength, about 355 nm.

UV spectra were obtained using a recording spectrophotometer[2] with 1-cm. cells.

Reagents—Reagent grade chloroform and ether and approximately 0.1 *N* ether-saturated sulfuric acid were used. A phosphoric acid solution was prepared by mixing equal volumes of distilled water and reagent grade phosphoric acid.

Standard Solutions—The following were used:

1*a*. Codeine phosphate stock solution, 0.13 mg./ml. in 0.1 *N* ether-saturated sulfuric acid.

1*b*. Codeine phosphate working solution, 13 mcg./ml. Dilute 10.0 ml. stock solution to 100 ml. with 0.1 *N* ether-saturated sulfuric acid.

2*a*. Caffeine stock solution, 1 mg./ml. in chloroform.

2*b*. Caffeine working solution, 0.01 mg./ml. Pipet 1.0 ml. stock solution into a 100-ml. volumetric flask and evaporate to dryness. Then add 30 ml. phosphoric acid solution to the residue, swirl to dissolve, and dilute to volume with distilled water.

Sample Preparation—Place one tablet in a separator containing about 30 ml. 0.1 *N* ether-saturated sulfuric acid and allow to disintegrate by swirling or standing. Extract with four 20-ml. portions of chloroform, collecting the extracts in a 100-ml. volumetric flask. Dilute to volume with chloroform and save for the caffeine determination.

Codeine Determination—Wash the acid solution in the separator with one 50-ml. portion of ether, and filter acid layer through a wetted filter into a 100-ml. volumetric flask. Rinse separator and filter with several portions of 0.1 *N* ether-saturated sulfuric acid, adding rinsings to flask. Finally, dilute to volume with 0.1 *N* ether-saturated sulfuric acid and mix. Using 0.1 *N* ether-saturated sulfuric acid, dilute an appropriate aliquot to a codeine phosphate concentration of 10–13 mcg./ml.

With the codeine phosphate working standard, at an excitation wavelength of 285 nm., adjust the spectrophotofluorometer to about 70% relative fluorescence intensity at 355 nm. Scan the sample and standard solutions from 285 to 375 nm., reading the maximum at about 355 nm. Use 0.1 *N* ether-saturated sulfuric acid as a blank.

Caffeine Determination—Pipet 3.0 ml. of the chloroform solution from the sample preparation into a separator containing 20 ml. chloroform. Add 30 ml. of the phosphoric acid solution and extract by shaking vigorously for 2 min. Allow the layers to separate and discard the chloroform. Backwash the acid layer with another 20 ml. of chloroform and discard wash. Rinse the stopper with distilled water and add about 25 ml. distilled water to the acid solution. Mix and filter through a wetted filter into a 100-ml. volumetric flask. Rinse separator and filter with several portions of distilled water, and finally dilute to volume with distilled water. Compare the absorbance of this solution to that of the caffeine working standard at 269 nm., using 30% phosphoric acid solution in distilled water as a blank.

RESULTS AND DISCUSSION

Caffeine is sparingly soluble in aqueous solutions, while phenacetin is only very slightly soluble. However, because phenacetin is present in relatively large amounts and because of its high absorptivity, an essentially complete separation is necessary for an ac-

[1] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Silver Spring, Md.

[2] Cary model 15 spectrophotometer, Applied Physics Corp., Monrovia, Calif.

Table I—Recovery of Codeine and Caffeine from a Synthetic Mixture of Codeine–Aspirin–Phenacetin–Caffeine

Codeine Recovery, %	Caffeine Recovery, %
100.4	99.1
100.0	98.2
100.0	99.4
99.3	98.2
98.8	97.6
99.8	97.9
99.4	98.2
99.6	100.3
99.5	99.4
100.0	100.3
Average = 99.7	Average = 98.9
SD = 0.46	*SD* = 0.98

Table II—Comparison of Assay Methods for Codeine and Caffeine in Commercial Tablets of Codeine–Aspirin–Phenacetin–Caffeine

Sample	Codeine Found, mg.[a] Proposed Method	Codeine Found, mg.[a] AOAC Method[b]	Caffeine Found, mg.[a] Proposed Method	Caffeine Found, mg.[a] NF XIII Method[c]
1	1.04	1.03	33.24	33.11
2	7.91	7.91	33.44	33.37
3	17.43	17.50	32.79	33.05
4	62.73	62.53	31.56	31.88

[a] Each assay result is the average of four determinations. [b] *Reference 12.* [c] *Reference 1.*

curate caffeine determination. Previously, it was generally thought impossible to separate caffeine from phenacetin by liquid–liquid partitioning in a separator. The organic phase must be chloroform to prevent significant amounts of phenacetin from extracting into the aqueous phase, but sulfuric acid, the commonly used aqueous phase, will not quantitatively extract caffeine from chloroform. The switch to phosphoric acid proved eminently successful. Under the experimental conditions used, caffeine is completely recovered in a *single* phosphoric acid extraction. Traces of phenacetin in the acid extract are effectively removed with an additional chloroform wash, thereby permitting UV determination of caffeine.

Although the natural fluorescence of codeine was previously reported (10, 11), it was thought to be too nonspecific and insensitive for clinical or biological work and its application to pharmaceutical analysis has been largely overlooked. Fluorescence was chosen in this instance because the UV spectra for codeine in some commercial samples showed interferences and because the sensitivity of the fluorescence permitted easy assay of low level codeine preparations [1 and 8 mg. ($^1/_8$ grain)]. However, at the sensitivity used, it was determined empirically that ether-saturated aqueous solvents contribute to the fluorescence of the solute. Consequently, all codeine solutions were prepared in ether-saturated sulfuric acid.

A synthetic mixture was prepared to contain the usual combination of I [*i.e.*, 227 mg. ($3^1/_2$ grains), 162 mg. ($2^1/_2$ grains), and 32 mg. ($^1/_2$ grain), of aspirin, phenacetin, and caffeine, respectively] plus 16 mg. ($^1/_4$ grain) codeine, about 16% starch, and 2% magnesium stearate. Ten sample weights, each approximating one theoretical tablet, were assayed by the proposed procedure (Table I). The average recoveries were 99.7 and 98.9% for codeine and caffeine, respectively.

The proposed procedure was compared to official methods for codeine (12) and caffeine (1) by assaying composites of four different commercial preparations (Table II). The method shows excellent agreement with the existing methods.

Table III shows individual tablet data. The samples represent five different manufacturers. The time required for analysis of five tablets was about 1.5 hr.

With minor modification, the described procedure may be adaptable to the determination of aspirin and phenacetin also. The chloroform washes from the caffeine determination contain aspirin and phenacetin. A single extraction with 5% sodium bicarbonate will remove aspirin. The chloroform solution can then be evaporated, and the phenacetin residue can be determined by UV spectroscopy after dissolution in chloroform and isooctane. Aspirin can be reextracted into chloroform after acidifying the bicarbonate solution and also determined by UV spectroscopy. Cursory experiments indicate that this approach is quite feasible, but an extensive investigation was not undertaken.

Table III—Individual Tablet Analysis for Codeine and Caffeine in Commercial Samples

Sample	Codeine, mg. Declared	Codeine, mg. Found	Caffeine, mg. Declared	Caffeine, mg. Found
A	1	0.964	32.40	31.88
		1.11		33.44
		1.06		30.65
		1.07		34.34
		1.08		33.44
B	16.20	16.07	32.40	32.72
		15.16		31.10
		15.55		32.14
		16.07		31.95
		15.55		32.66
C	16.20	15.68	32.40	31.95
		16.20		32.21
		15.55		32.40
		15.62		31.49
		15.68		32.53
D	32.40	31.04	32.40	33.70
		32.33		34.67
		32.01		29.81
		30.33		27.54
		30.97		33.44
E	32.40	30.84	32.40	30.52
		31.69		31.30
		30.91		30.07
		31.43		32.21
		32.66		31.49
F	64.80	59.94	32.40	29.09
		57.87		29.87
		57.87		29.55
		58.71		29.48
		64.35		29.68

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Spectrophotometric Determination of Methenamine and Its Salts by 2-Hydrazinobenzothiazole

JAMES K. LIM▲ and CONNIE C. C. CHEN

Abstract □ A rapid and sensitive method is described for the assay of the pure powder form and the tablet dosage form of methenamine and its salts. The procedure is shown to be capable of measuring as little as 0.005 μmole of material and is highly reproducible. The proposed method is based on the color development of formaldehyde released from the acid hydrolysis of methenamine with a dye, 2-hydrazinobenzothiazole. Beer's law is obeyed in the concentration range of 1–8 μ*M* of methenamine at 510-nm. wavelength. This method offers greater versatility over the official assay methods, which have been designed primarily for determining specifically either methenamine or its mandelate salt. Data comparing the assays obtained by the official methods with this new technique are presented.

Keyphrases □ Methenamine and salts in powders and tablets—colorimetric analysis using 2-hydrazinobenzothiazole, compared to compendial methods □ 2-Hydrazinobenzothiazole—used as color-forming agent in spectrophotometric analysis of methenamine and its salts □ Colorimetry—analysis, methenamine and salts in powders and tablets

The NF XIII (1) assay for methenamine involves its acid hydrolysis by reflux until the "odor of formaldehyde is no longer perceptible," followed by back-titration with an alkali. The procedure proved to be not only tedious but also subject to inaccuracies arising from human error in obtaining the "perceptible end-point," *i.e.*, absence of formaldehyde odor. The USP XVIII (2) method for determining methenamine mandelate, on the other hand, represents an indirect method of analysis. It essentially involves nesslerization followed by an iodometric titration of the mandelic acid moiety; therefore, this method cannot be employed for determining other salts of methenamine.

Other methods also have been developed for the quantitation of this compound, including those based on chromotropic acid reactions (3–5), the Hantzsch reaction between acetylacetone, ammonia, and formaldehyde (6), and fluorometric techniques (7). This report presents a method for determining methenamine and its salts through the formation of xanthylinum dicationic dyes with formaldehyde and formaldehyde-releasing compounds. Measurements based on such a reaction are reported (8, 9) to be more sensitive than those by chromotropic acid.

EXPERIMENTAL

Apparatus—Absorbance measurements were made on a spectrophotometer[1].

Reagents and Chemicals—2-Hydrazinobenzothiazole[2] was the dye-forming reagent used. Powdered samples of methenamine[3–5], methenamine mandelate[6], and methenamine hippurate[7] were used as received. All other chemicals and reagents used were USP or the highest grade of commercial materials. Several commercial brands of either the plain or enteric-coated tablets of methenamine, methenamine mandelate, and methenamine hippurate were employed for the tests comparing the official assays with the proposed method. The dye reagent consists of a 0.5% solution of 2-hydrazinobenzothiazole in 10% hydrochloric acid. It is stable for 2 weeks.

Assay Procedure—Aqueous solutions of methenamine or the methenamine salt in the concentration range of 0.01–0.08 μmole/ml. were made up in 100-ml. volumetric flasks. A 0.5-ml. volume of each solution was placed in a test tube and mixed with 0.5 ml. of 10 *N* sulfuric acid. The samples were refluxed in a boiling water bath for 15 min. to obtain complete hydrolysis and were allowed to cool to room temperature before the addition of 0.5 ml. of the 2-hydrazinobenzothiazole reagent. After 5 min., 0.5 ml. of a 1% aqueous solution of potassium ferricyanide was added and this reaction was allowed to proceed another 20 min. before the final admixture of 3 ml. of dimethylformamide solvent. The color thus formed was allowed to stabilize for 20 min. before absorbance readings were made at a 510-nm. wavelength against a reagent blank.

Table I—Absorbance by Different Methenamine Concentrations

Determination Number	Concentration, μmole/5 ml. 0.005	0.01	0.02	0.04
1	0.075	0.141	0.297	0.598
2	0.084	0.161	0.290	0.598
3	0.084	0.161	0.276	0.584
Average	0.081	0.154	0.288	0.593
± *SD*	0.004	0.009	0.008	0.007

Table II—Assay of Commercial Methenamine Powder by the 2-Hydrazinobenzothiazole and NF Methods

Powder	Percent Found—2-Hydrazinobenzothiazole Method	NF Method
A	98.5[a] ± 1.08[b]	98.8
B	96.3 ± 2.07	93.5

[a] Average of three determinations. [b] Standard deviation.

RESULTS AND DISCUSSION

Absorbance of the color obtained of known standards in the concentration range of 0.005–0.04 μmole/5 ml. of methenamine was seen to obey Beer's law. The calculated linear correlation coefficients were: methenamine, 0.9996; and methenamine mandelate and methenamine hippurate, 0.9969 and 0.9975, respectively. Initial studies showed that the slopes might vary from time to time, probably due to aging of the reagents employed with the 2-hydrazinobenzothiazole reaction. However, the experimental values were reproducible within the normal analysis period (Table I). It is, therefore, recommended that a fresh standard curve be prepared with each analysis.

Table II compares the percentages of methenamine ($C_6H_{12}N_4$) found in two different commercial brands of methenamine powder as assayed by the 2-hydrazinobenzothiazole and the NF methods.

[1] Bausch and Lomb Spectronic 70.
[2] Eastman Organic Chemicals.
[3] Baker Chemical Co.
[4] Amend Drug Chemical Co.
[5] Fisher Scientific Co.
[6] K & K Laboratories, Inc.
[7] Riker Laboratories, Inc.

Table III—Assay of Commercial Methenamine Products by the 2-Hydrazinobenzothiazole Method

Determination Number	Percent Found: Powders: Methenamine Mandelate	Powders: Methenamine Hippurate	Tablets: Methenamine	Tablets: Methenamine Hippurate	Tablets: Methenamine Mandelate, Enteric Coated
1	96.6	96.6	103.8	96.6	95.2
2	96.6	101.4	99.0	101.4	91.0
3	96.6	99.0	99.0	99.0	91.0
Average	96.6	99.0	100.6	99.0	92.4
± *SD*	0.0	2.0	2.3	2.0	2.0

Only 0.02 μmole of material was needed per determination in this proposed procedure compared to approximately 1 g. or 7.1 mmoles in the NF method. Table III presents the results of assays on methenamine mandelate and methenamine hippurate powders and those on their tablet dosage forms. Tablets were crushed, dissolved, and filtered, if necessary, before an aliquot representing approximately 0.02 μmole was withdrawn for the determination. There appeared to be no significant interference in absorbance where color was formulated in the tablets because of the high dilutions involved in obtaining the extremely small amount of material.

It is clear from these data that the proposed 2-hydrazinobenzothiazole procedure possesses several distinct advantages over the present official methods for the analysis of methenamine or methenamine mandelate. It offers the convenience of a single method for the analysis of either the free base or salts. It has been found to be simple, sensitive, and less time consuming. It does not require prior standardization of a number of titrants, as for nessleriza-tion with the official assay of methenamine mandelate. Furthermore, the proposed method can be used for the assay of other salts of methenamine, unlike the USP procedure. With respect to the NF method, the lengthy period needed for the acid hydrolysis of methenamine using 1 *N* sulfuric acid as well as the uncertain end-point of the hydrolysis can be eliminated with the 2-hydrazinobenzothiazole procedure.

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Chromatographic Separation and Assay of Morphine in Injectables

I. J. HOLCOMB, R. B. LUERS, Jr., and S. A. FUSARI▲

Abstract □ A method specific for the determination of morphine sulfate in injectables was developed. Morphine is separated from preservatives, pseudomorphine, and other degradation products by a column chromatographic method. The eluate containing morphine is extracted into an acid solution and determined spectrophotometrically. Recovery from standard solutions is better than 99%. The method presented is more specific for the separation and determination of morphine in injectables than present official methods.

Keyphrases □ Morphine sulfate injection—separation from preservatives and degradation products, UV analysis □ Column chromatography—separation, morphine from preservatives and degradation products, morphine sulfate injection □ UV spectrophotometry—analysis, morphine sulfate injection

Morphine has been well established as an effective analgesic and sedative since its isolation in 1805 (1, 2). Due to the limited solubility of morphine base in aqueous media, the more soluble hydrochloride and sulfate salts have been used in pharmaceutical preparations. The dosage form commonly employed since 1941 has been morphine sulfate as an injectable aqueous solution, since it is the most stable of the morphine salts in solution (3). When stored for a long time or exposed to heat, morphine sulfate injections develop a light-brown color.

The discoloration is not desirable, since decomposition of the dosage form is suggested and is, in fact, taking place; however, the extent of the discoloration is not an indication of the extent of decomposition. The cause has been attributed to the decomposition of morphine sulfate to pseudomorphine and further oxidation products (4–8). Foster *et al.* (5) and later Lach and Yeh (9–11) demonstrated that morphine injections decomposed first to pseudomorphine and then to oxidation products of pseudomorphine. Foster *et al.* (5) also showed that rubber closures contributed to the discoloration of the morphine injection and possibly to the decomposition of morphine sulfate.

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[4] Amend Drug Chemical Co.
[5] Fisher Scientific Co.
[6] K & K Laboratories, Inc.
[7] Riker Laboratories, Inc.

Table III—Assay of Commercial Methenamine Products by the 2-Hydrazinobenzothiazole Method

Determination Number	Percent Found: Powders: Methenamine Mandelate	Powders: Methenamine Hippurate	Tablets: Methenamine	Tablets: Methenamine Hippurate	Tablets: Methenamine Mandelate, Enteric Coated
1	96.6	96.6	103.8	96.6	95.2
2	96.6	101.4	99.0	101.4	91.0
3	96.6	99.0	99.0	99.0	91.0
Average	96.6	99.0	100.6	99.0	92.4
± *SD*	0.0	2.0	2.3	2.0	2.0

Only 0.02 μmole of material was needed per determination in this proposed procedure compared to approximately 1 g. or 7.1 mmoles in the NF method. Table III presents the results of assays on methenamine mandelate and methenamine hippurate powders and those on their tablet dosage forms. Tablets were crushed, dissolved, and filtered, if necessary, before an aliquot representing approximately 0.02 μmole was withdrawn for the determination. There appeared to be no significant interference in absorbance where color was formulated in the tablets because of the high dilutions involved in obtaining the extremely small amount of material.

It is clear from these data that the proposed 2-hydrazinobenzothiazole procedure possesses several distinct advantages over the present official methods for the analysis of methenamine or methenamine mandelate. It offers the convenience of a single method for the analysis of either the free base or salts. It has been found to be simple, sensitive, and less time consuming. It does not require prior standardization of a number of titrants, as for nessleriza-tion with the official assay of methenamine mandelate. Furthermore, the proposed method can be used for the assay of other salts of methenamine, unlike the USP procedure. With respect to the NF method, the lengthy period needed for the acid hydrolysis of methenamine using 1 *N* sulfuric acid as well as the uncertain end-point of the hydrolysis can be eliminated with the 2-hydrazinobenzothiazole procedure.

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Chromatographic Separation and Assay of Morphine in Injectables

I. J. HOLCOMB, R. B. LUERS, Jr., and S. A. FUSARI▲

Abstract □ A method specific for the determination of morphine sulfate in injectables was developed. Morphine is separated from preservatives, pseudomorphine, and other degradation products by a column chromatographic method. The eluate containing morphine is extracted into an acid solution and determined spectrophotometrically. Recovery from standard solutions is better than 99%. The method presented is more specific for the separation and determination of morphine in injectables than present official methods.

Keyphrases □ Morphine sulfate injection—separation from preservatives and degradation products, UV analysis □ Column chromatography—separation, morphine from preservatives and degradation products, morphine sulfate injection □ UV spectrophotometry—analysis, morphine sulfate injection

Morphine has been well established as an effective analgesic and sedative since its isolation in 1805 (1, 2). Due to the limited solubility of morphine base in aqueous media, the more soluble hydrochloride and sulfate salts have been used in pharmaceutical preparations. The dosage form commonly employed since 1941 has been morphine sulfate as an injectable aqueous solution, since it is the most stable of the morphine salts in solution (3). When stored for a long time or exposed to heat, morphine sulfate injections develop a light-brown color.

The discoloration is not desirable, since decomposition of the dosage form is suggested and is, in fact, taking place; however, the extent of the discoloration is not an indication of the extent of decomposition. The cause has been attributed to the decomposition of morphine sulfate to pseudomorphine and further oxidation products (4–8). Foster *et al.* (5) and later Lach and Yeh (9–11) demonstrated that morphine injections decomposed first to pseudomorphine and then to oxidation products of pseudomorphine. Foster *et al.* (5) also showed that rubber closures contributed to the discoloration of the morphine injection and possibly to the decomposition of morphine sulfate.

Numerous assay methods have been developed for

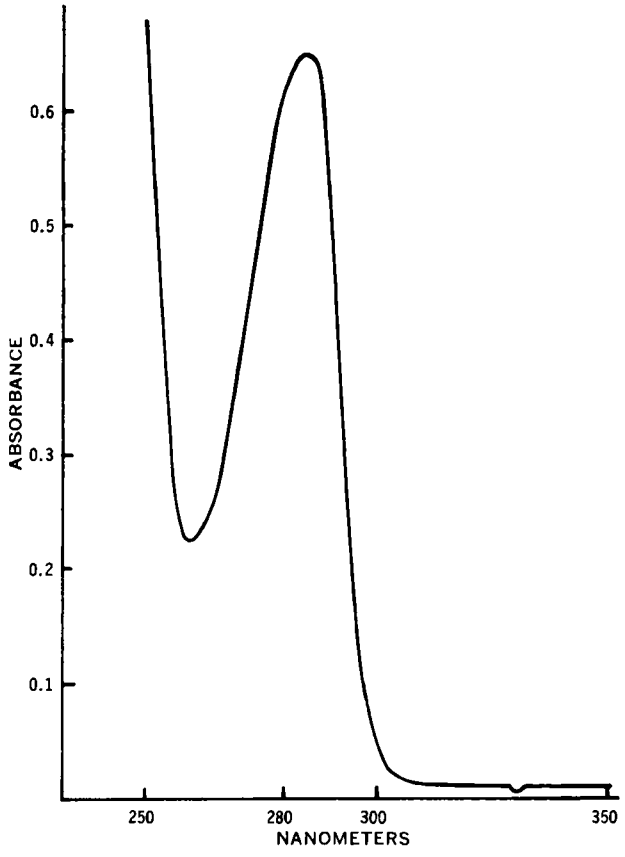

Figure 1—*UV spectrum of morphine sulfate, 0.1622 mg./ml., in 0.05* N *hydrochloric acid.*

the determination of morphine, using gravimetric (12, 13), colorimetric (14–19), fluorometric (20, 21), titrimetric (22, 23), and spectrophotometric (24) procedures. These methods are satisfactory for freshly prepared solutions of morphine sulfate but are not always specific for the determination of morphine in the presence of its degradation products. Ion-exchange (25–27) and partition chromatographic (9, 28–30) methods have been used to separate morphine in pharmaceutical preparations. Most of these procedures are time consuming and are not applicable to routine analysis. A partition chromatographic method was developed (9,

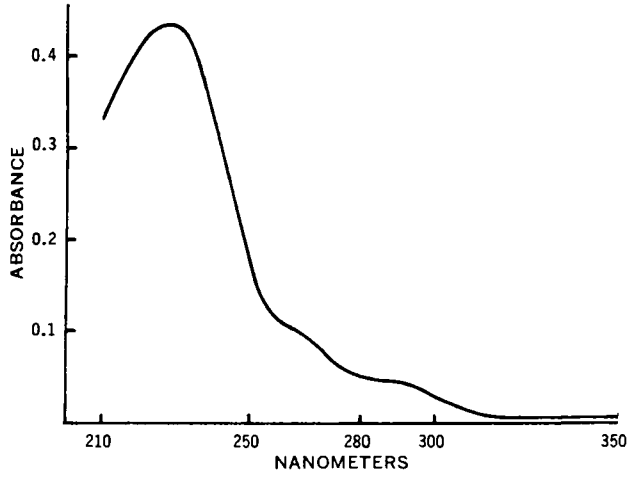

Figure 2—*UV spectrum of pseudomorphine, 0.00746 mg./ml., in 0.05* N *hydrochloric acid.*

Table I—Recovery of Morphine Sulfate from Chromatographic Column

Theoretical, mg./ml.	Recovered, mg./ml.	Recovery, %
13.76	13.45	99.2
15.90	15.83	99.5
15.95	15.95	100.0
17.00	16.95	99.7
17.95	17.88	99.6

Table II—Recovery of Morphine Sulfate from a Standard Formulation

Morphine Sulfate in Standard Formulation, mg./ml.	Recovered, mg./ml.	Recovery, %
15.10	15.03	99.6
15.10	15.06	99.7
15.10	15.09	99.8

28) for the separation of intact morphine from degradation products; this method was employed in studies on the stability of morphine in aqueous solution (9–11). This procedure was also time consuming. The present USP extraction–titrimetric method (23) extracts small amounts of decomposition products as well as morphine from discolored solutions of morphine injection, giving false, high assay values. When the extracts from the USP procedure were examined by TLC, both morphine and pseudomorphine were found.

The purpose of the present investigation was to provide a simple, rapid chromatographic method specific for the separation of morphine from its degradation products. The same procedure may be employed to separate morphine from such alkaloids as atropine and codeine. The separation of morphine and

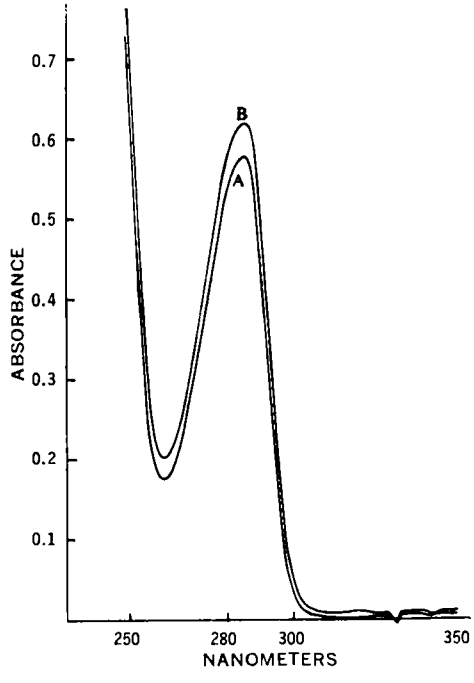

Figure 3—*UV spectra of: A, morphine sulfate, 0.1486 mg./ml., in 0.05* N *hydrochloric acid; and B, morphine sulfate, 0.1486 mg./ml., and pseudomorphine, 0.0075 mg./ml., in 0.05* N *hydrochloric acid.*

Table III—Recovery of Morphine Sulfate from a Standard Formulation and Other Added Substances

Morphine Sulfate in Standard Formulation, mg./ml.	Substance Added, mg./ml.	Recovered, mg./ml.	Recovery, %
15.10	Pseudomorphine (1.65)	15.06	99.7
15.10	Codeine phosphate (16.60)	15.21	100.7
15.10	Atropine sulfate (2.14)	15.19	100.6

Table IV—Assay Results on Morphine Injection USP [Sulfate], 15 mg./ml.

Manufacturer	Morphine Sulfate Found, mg./ml.	Percent Label Claim
A	14.27	95.4
	14.32	95.5
B	15.35	102.3
	15.47	103.1

atropine is significant because of the use of these alkaloids in combination for preanesthetic injection.

The procedure involves a release of morphine as the base with the use of sodium bicarbonate. Chloroform elution through an acidic trap layer removes neutral substances such as the parabens and bases such as codeine and atropine. Morphine, which is partially eluted by chloroform, is retained by the acidic trap layer and is finally eluted from the column using isobutanol in chloroform. The decomposition products are retained on the column.

EXPERIMENTAL

Reagents and Apparatus—*USP Grade Chemicals*—The following were used: morphine sulfate, codeine phosphate, atropine sulfate, methylparaben, and propylparaben.

Reagent Grade Chemicals—Reagent grade acid-washed diatomaceous earth[1], sodium bisulfite, isobutanol, chloroform, and anhydrous ether were used. Pseudomorphine base was prepared by oxidation of morphine in a basic solution with potassium ferricyanide (31). The product was washed with methanol and dried. TLC showed only one zone, confirming the purity of the base.

Apparatus—The following were used: 25 mm. i.d. × 15 cm. long chromatographic column[2] with a coarse fritted disk in the base of the column, 1-cm. quartz cells, and a spectrophotometer[3] with a recorder[4].

Separation—In a 100-ml. beaker, 2.0 ml. of 1 *M* pH 6.5 potassium phosphate buffer solution was mixed with 3 g. of reagent grade, acid-washed diatomaceous earth. The mixture was transferred to the chromatographic column and packed firmly. To 1.0 ml. of the test solution in a 150-ml. beaker, 0.5 g. sodium bicarbonate was added and mixed well. Then 3 g. of reagent grade, acid-washed diatomaceous earth was added and mixed well. The mixture was transferred to the chromatographic column and packed firmly. The beaker was wiped with a pledget of glass wool, and the glass wool was placed on the top of the packed column. The column was eluted with 25 ml. of reagent grade chloroform, and the eluate was discarded. The column was then eluted with 55 ml. of 15% (v/v) isobutanol in chloroform, and the eluate was collected in a 250-ml. separator. Water-washed ether, 100 ml., was added to the eluate in the separator, and the contents were carefully mixed. The solvent mixture was extracted with three 25-ml. portions of 0.05 *N* hydrochloric acid. The acid phases were collected in a 100-ml. volumetric flask, and 0.05 *N* hydrochloric acid was added to volume and mixed. The spectrum of the acid solution was obtained from 350 to 250 nm. (Fig. 1). The absorbance maximum obtained at 285 nm. was used to calculate the quantity of morphine sulfate.

[1] Celite 545, Johns-Manville Co.
[2] Chromaflex, Kontes Glass Co.
[3] Perkin-Elmer model 124.
[4] Perkin-Elmer model 165.

Table V—Separation of Morphine Sulfate from Degradation Products

Theoretical, mg./ml.	Conditions of Treatment	Recovery, mg./ml.	Recovery, %
17.00	Initial assay	16.95	99.7
	After 17 days at 105°	15.94	93.8

By employing this chromatographic procedure, aqueous solutions of morphine sulfate and of morphine sulfate in combination with other substances present were assayed.

Morphine Sulfate Solutions—Aqueous solutions of morphine sulfate USP, ranging in concentration from 13.76 to 17.95 mg./ml., were prepared and assayed (Table I).

Morphine Sulfate Standard Formulation—A morphine sulfate aqueous solution was prepared using methylparaben and propylparaben as preservatives and sodium bisulfite as an antioxidant. The standard formulation consisted of the following (mg./ml.): morphine sulfate USP, 15.10; methylparaben USP, 1.69; propylparaben USP, 0.21; and sodium bisulfite, reagent grade, 1.03.

Assay results for the standard formulation are given in Table II.

The standard formulation was modified by the addition of other substances (pseudomorphine and atropine sulfate) commonly associated with morphine sulfate in injectables and with the chemi-

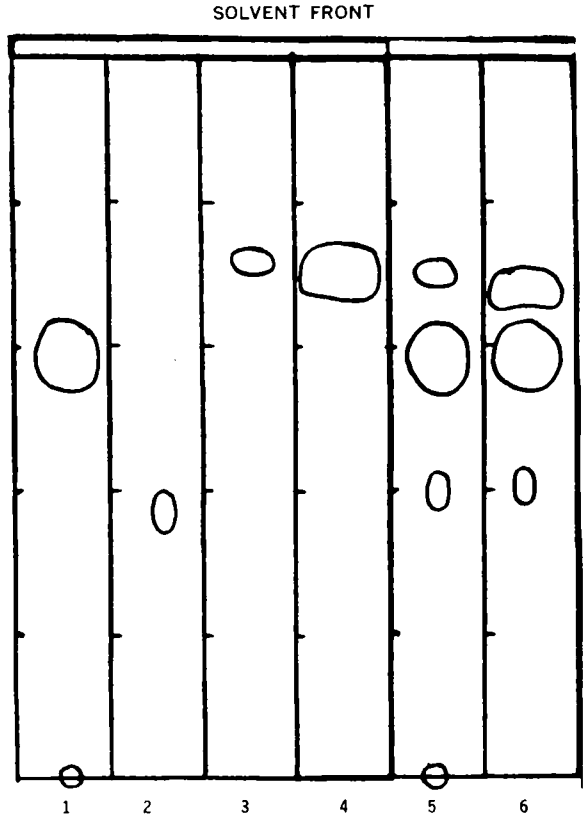

Figure 4—*Chromatogram of reference compounds used in the thin-layer examination study of morphine sulfate, standard formulation, and decomposition products. Key: 1, 150 mcg. of morphine sulfate; 2, 3 mcg. of pseudomorphine; 3, 3 mcg. of morphine* N*-oxide; 4, 18 mcg. of methylparaben and propylparaben mixture; 5, mixture of morphine sulfate, pseudomorphine, and morphine* N*-oxide; and 6, mixture of morphine sulfate, pseudomorphine, and parabens.*

cally related substance codeine. Morphine was successfully separated and quantitatively assayed in the presence of these substances (Table III). These separations were confirmed by TLC. To demonstrate the applicability of this assay method to formulations, samples of morphine injection USP [sulfate], 15 mg./ml., were obtained and assayed (Table IV).

UV Spectra: Morphine and Pseudomorphine—The UV curves of morphine sulfate (Fig. 1) and pseudomorphine (Fig. 2) in 0.05 *N* hydrochloric acid clearly show the spectral differences of the two compounds. The curves in Fig. 3 show the effect of pseudomorphine sulfate at the maximum at 285 nm. Application of the column chromatographic method separates morphine from pseudomorphine and other degradation products.

Separation from Degradation Products—To show the separation of morphine sulfate from degradation products, a solution of morphine sulfate was submitted to conditions to accelerate the decomposition. A solution of morphine sulfate was sealed in a glass ampul and placed in an oven at 105° for 17 days. The solution developed a distinct brown color. The ampul was opened and the contents were assayed. The morphine content definitely decreased, as indicated by the data in Table V. The brown color originally present was retained on the chromatographic column. The morphine eluate and subsequent acid extract were clear and colorless. Thus, the chromatographic system is capable of separating intact morphine from its colored degradation products.

Additional work on the aged product involved assay of room temperature samples which were 4–10 years old. These samples were assayed by both the column chromatographic procedure and GLC (Table VI).

TLC—Complete separation of morphine in the preceding standard mixtures was monitored by TLC. Following the chloroform and chloroform–isobutanol elutions, the chromatographic columns were eluted with 0.01 or 0.05 *N* hydrochloric acid in methanol to remove the remaining decomposition products.

Precoated silica gel GF plates[5] (10 × 20 cm.) were used as the stationary phase. The mobile phase consisted of chloroform–methanol–diethylamine (80:15:5).

Eluates from the chromatographic columns were concentrated to 1 ml. prior to application to the stationary phase. Twenty microliters of each concentrate was applied, and the plates were developed to a height of 15 cm. from the origin. Plates were air dried and examined under long and short wavelength UV light.

In every instance, complete and clean separation of intact morphine was obtained from the other substances present or added (Figs. 4–8).

Table VI—Assay Values on Sterile Morphine Injection in Rubber-Stoppered Amber Glass Vials

Sample	Date of Manufacture	Milligrams Morphine Sulfate Found per Milliliter (Percent Label Claim) Column Separation	GLC[a]
1	1962	14.26(88.0)	14.00(86.4)
2	1963	14.42(89.1)	13.91(85.9)
3	1964	14.62(90.3)	13.89(85.9)
4	1966	14.82(91.5)	14.72(90.8)
5	1968	14.84(91.6)	14.92(92.1)

[a] The GLC assays were performed by Mr. V. G. Davies.

RESULTS AND DISCUSSION

The recovery data in Table I indicate that quantitative recovery of morphine sulfate was obtained from the morphine sulfate solutions assayed. No morphine was detected in the initial chloroform eluate from the chromatographic column. Recovery of morphine sulfate from the morphine sulfate standard formulation was quantitative (Table II). The other ingredients in the formulation did not

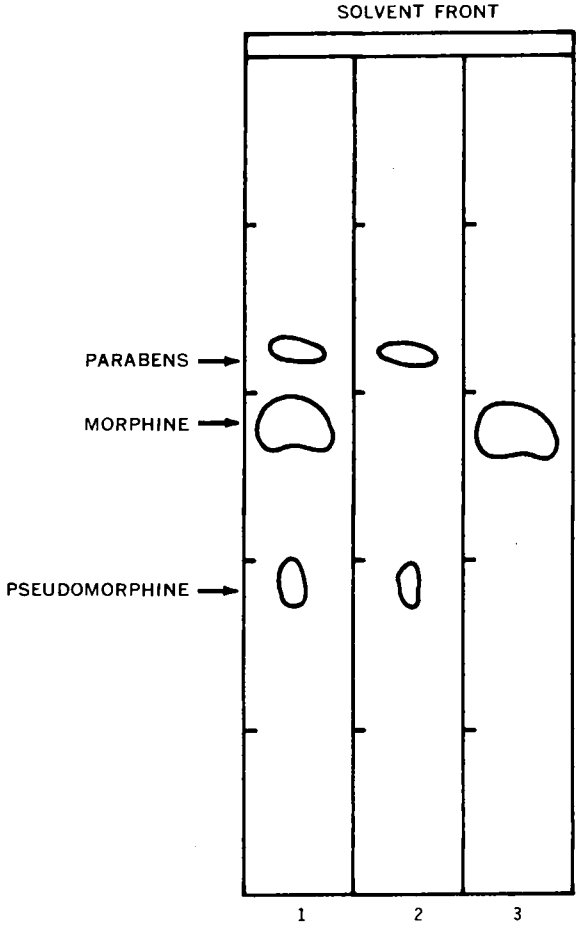

Figure 5—*Chromatogram demonstrating the column separation of morphine from pseudomorphine and other components in the standard formulation. Key: 1, 20 μl. of the standard formulation; 2, 20 μl. of the chloroform eluate; and 3, 20 μl. of the chloroform–isobutanol (85:15) eluate.*

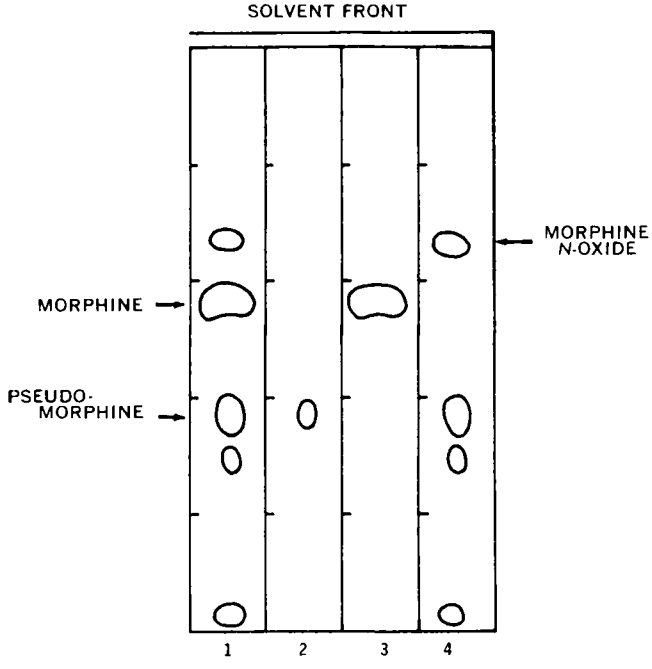

Figure 6—*Chromatogram showing the column separation of morphine from decomposition products. Key: 1, 20 μl. of degraded morphine sulfate solution; 2, 20 μl. of the chloroform eluate; 3, 20 μl. of the chloroform–isobutanol (85:15) eluate; and 4, 20 μl. of the 0.01* N *hydrochloric acid eluate in methanol.*

[5] Analtech, E. Merck.

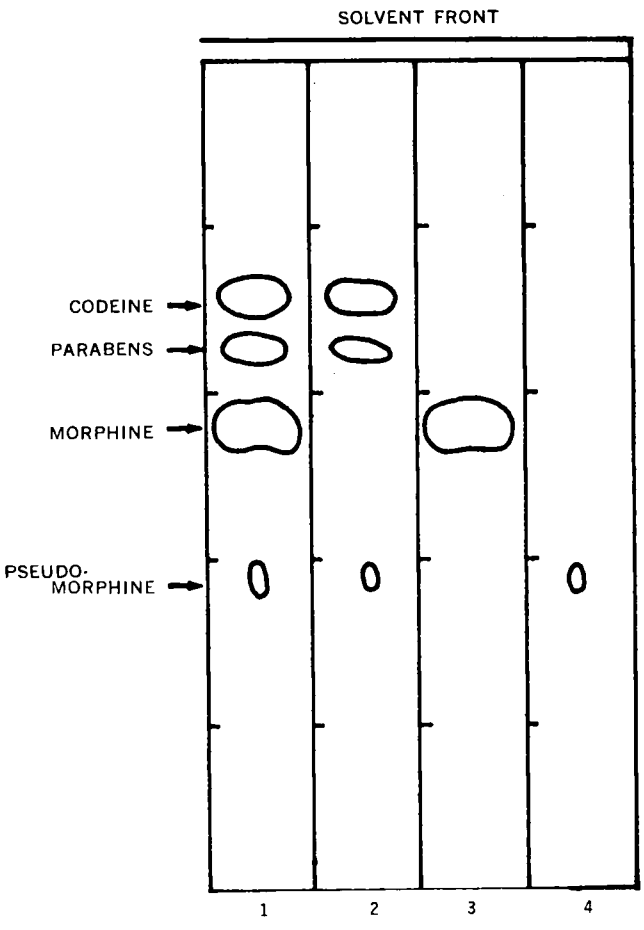

Figure 7—*Chromatogram showing the column separation of morphine in the standard formulation with codeine phosphate. Key: 1, 20 μl. of the standard formulation with codeine phosphate; 2, 20 μl. of the chloroform eluate; 3, 20 μl. of the chloroform–isobutanol (85:15) eluate; and 4, 20 μl. of the 0.01* N *hydrochloric acid eluate in methanol.*

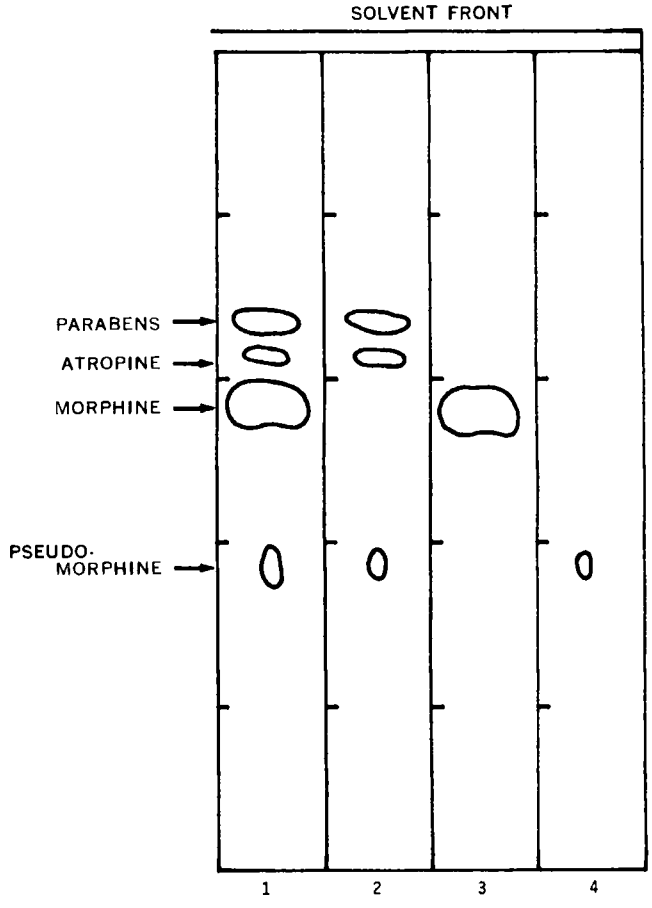

Figure 8—*Chromatogram showing the column separation of morphine in the standard formulation with atropine sulfate. Key: 1, 20 μl. of the standard formulation with atropine sulfate; 2, 20 μl. of the chloroform eluate; 3, 20 μl. of the chloroform–isobutanol (85:15) eluate; and 4, 20 μl. of the 0.05* N *hydrochloric acid eluate in methanol.*

interfere with the assay of morphine. The separation of morphine sulfate from the standard formulation with pseudomorphine added was complete and quantitative (Table III). The column chromatographic separation of morphine and pseudomorphine was confirmed by TLC (Fig. 5). Morphine sulfate was quantitatively recovered from the standard formulation with codeine phosphate added (Table III). The codeine was quantitatively recovered in the initial chloroform eluate from the chromatographic column; therefore, it did not interfere with the determination of morphine sulfate. Finally, morphine sulfate was completely and quantitatively separated from the standard formulation with atropine sulfate added (Table III). The atropine was found in the initial chloroform eluate from the chromatographic column. All of the preceding tabulated data indicated quantitative recovery of morphine sulfate from the solutions and mixtures.

CONCLUSIONS

By using this chromatographic procedure, morphine sulfate was separated quantitatively from its degradation products and from other substances present in an aqueous formulation of morphine sulfate for injection and from other alkaloidal salts which might be employed with morphine sulfate in injectables. The method is simple, rapid, and stability indicating for measuring actual morphine content in aged or discolored morphine injections.

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Purity Profiles for Heroin, Morphine, and Morphine Hydrochloride

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Abstract □ Purity profiles were established for samples of heroin (diacetylmorphine), morphine, and morphine hydrochloride in order to define the scope of utility of each as qualitative and/or quantitative analytical standards. Systems for solubility analysis and high-pressure liquid chromatography were investigated. A heroin synthesis affording a product of high purity is described.

Keyphrases □ Purity profiles—heroin, morphine, and morphine hydrochloride, phase solubility analysis, high-pressure liquid chromatography □ Heroin—synthesis, purity profile, phase solubility analysis, high-pressure liquid chromatography □ Morphine and hydrochloride salt—purity profiles, phase solubility analysis, high-pressure liquid chromatography □ Phase solubility analysis—purity profiles for heroin, morphine, and morphine hydrochloride □ High-pressure liquid chromatography—analysis, purity profiles, heroin, morphine, and morphine hydrochloride

Increased concern in recent years has given rise to demands for greater specificity and accuracy of analytical procedures for various abuse drugs, notably morphine and heroin. Along with this arises a need for standards for these drugs, having known scopes of utility for known qualitative and quantitative applications. The purposes of this report are to identify analytical procedures of value in determining the purity profiles (1) of these drugs and to define the analytical utility of given samples of the drugs. A synthesis of heroin is described which has yielded material suitable for use as an analytical standard.

METHODS AND MATERIALS

Morphine and its hydrochloride salt were obtained commercially. All reagents used were USP, NF, or ACS grade, except as noted.

Phase Solubility Analysis (2)—The method described in NF XIII (3) was followed. Nine 10-ml. tared glass ampuls were flushed with nitrogen, charged with an appropriate progression of sample weights and a fixed volume (3 ml. by pipet) of solvent, and flame sealed. All solvents were freshly distilled and degassed, starting with spectroquality or commercial distilled-in-glass grades. The bath temperature was 25°, the time allowed for equilibration varied with the drug, and rotation was 28 r.p.m. The residues obtained were dried to constant weight at 130°, except for heroin which was dried at 105°. All samples were kept in the dark when possible or in subdued light. The appropriate Student's t value, two-tailed, was used in calculating (2) the 95% confidence limits. In computing and graphing the system composition, the sample weights used were converted to the anhydrous basis.

Titrations—Commercial equipment[1] was used for all potentiometric titrations (n = 3, averages given in Table I) and the dead-stop end-point titration (duplicates).

Nonaqueous Titration (*4a*, *5*)—The sample was dissolved in 5 ml. of glacial acetic acid and titrated to the potentiometric end-point with 0.1 *N* acetous perchloric acid, using a glass indicating electrode and a calomel reference electrode filled with 0.02 *N* lithium chloride in glacial acetic acid. The titrant was standardized against dried potassium biphthalate. Five milliliters of glacial acetic acid served as a blank.

Chloride—The sample was dissolved in 5 ml. of 1.5 *N* sulfuric acid and was titrated to the potentiometric end-point with 0.1 *N* silver nitrate, using a silver indicating electrode and a mercurous sulfate reference electrode. The titrant was standardized with dried sodium chloride, and 5 ml. of 1.5 *N* sulfuric acid served as a blank.

Moisture (*4b*, *6*, *7*)—An aliquot of sample was dissolved in anhydrous methanol, injected into a vessel containing methanol titrated to the end-point, and titrated with Karl Fischer reagent[2], using the dead-stop end-point technique and a 20-sec. delay. An aliquot of methanol was titrated as a blank. The titrant was standardized by titrating known volumes of water, delivered by gravimetrically calibrated microsyringe. Loss on drying to constant weight was determined on 300-mg. samples at 130°, except for heroin which was dried at 105°.

Spectroscopy—Recording UV[3] and IR[4] spectrophotometers were used to obtain spectra of the samples, and rotation data were obtained using an automatic polarimeter[5]. Absorptivity data in Table I are the averages of triplicates and rotational data are the averages of duplicates.

GLC—The GLC system[6] was as follows: on-column injection; 1.2-m. × 4-mm. i.d. glass columns; methylsilicone gum on silanized, acid-washed, flux-calcined diatomite; cured, conditioned, and tested as described previously (8); helium carrier gas at 50 ml./min.; flame-ionization detector; and automatic digital integrator[7] for area normalization. Temperature programs were 150–250°, and isothermal purity determinations were at 210°. A nonlinear adsorption graph was prepared for 0.5–100-mcg. sample injections of morphine, codeine, heroin, acetylcodeine, and both acetylmor-

[1] Radiometer automatic titrator (TTT-11), pH meter (PHM-26), recorder (SBR-2), 2.5-ml. automatic buret (ABU-1), titration assembly (TTA-31), and Karl Fischer assembly (TTA/KF); all electrodes were of the same manufacture.
[2] Stabilized reagent, Fisher Chemical Co., diluted fivefold prior to use.
[3] Cary model 14.
[4] Perkin-Elmer model 21.
[5] Perkin-Elmer model 141.
[6] Hewlett-Packard 5750B detector, oven, control, and electrometer modules with strip-chart recorder; 3% OV-1 on Gas Chrom Q, 100–120 mesh, was from Applied Science Labs.
[7] Infotronics CRS-204.

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Purity Profiles for Heroin, Morphine, and Morphine Hydrochloride

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Abstract □ Purity profiles were established for samples of heroin (diacetylmorphine), morphine, and morphine hydrochloride in order to define the scope of utility of each as qualitative and/or quantitative analytical standards. Systems for solubility analysis and high-pressure liquid chromatography were investigated. A heroin synthesis affording a product of high purity is described.

Keyphrases □ Purity profiles—heroin, morphine, and morphine hydrochloride, phase solubility analysis, high-pressure liquid chromatography □ Heroin—synthesis, purity profile, phase solubility analysis, high-pressure liquid chromatography □ Morphine and hydrochloride salt—purity profiles, phase solubility analysis, high-pressure liquid chromatography □ Phase solubility analysis—purity profiles for heroin, morphine, and morphine hydrochloride □ High-pressure liquid chromatography—analysis, purity profiles, heroin, morphine, and morphine hydrochloride

Increased concern in recent years has given rise to demands for greater specificity and accuracy of analytical procedures for various abuse drugs, notably morphine and heroin. Along with this arises a need for standards for these drugs, having known scopes of utility for known qualitative and quantitative applications. The purposes of this report are to identify analytical procedures of value in determining the purity profiles (1) of these drugs and to define the analytical utility of given samples of the drugs. A synthesis of heroin is described which has yielded material suitable for use as an analytical standard.

METHODS AND MATERIALS

Morphine and its hydrochloride salt were obtained commercially. All reagents used were USP, NF, or ACS grade, except as noted.

Phase Solubility Analysis (2)—The method described in NF XIII (3) was followed. Nine 10-ml. tared glass ampuls were flushed with nitrogen, charged with an appropriate progression of sample weights and a fixed volume (3 ml. by pipet) of solvent, and flame sealed. All solvents were freshly distilled and degassed, starting with spectroquality or commercial distilled-in-glass grades. The bath temperature was 25°, the time allowed for equilibration varied with the drug, and rotation was 28 r.p.m. The residues obtained were dried to constant weight at 130°, except for heroin which was dried at 105°. All samples were kept in the dark when possible or in subdued light. The appropriate Student's t value, two-tailed, was used in calculating (2) the 95% confidence limits. In computing and graphing the system composition, the sample weights used were converted to the anhydrous basis.

Titrations—Commercial equipment[1] was used for all potentiometric titrations (n = 3, averages given in Table I) and the dead-stop end-point titration (duplicates).

Nonaqueous Titration (*4a*, *5*)—The sample was dissolved in 5 ml. of glacial acetic acid and titrated to the potentiometric end-point with 0.1 *N* acetous perchloric acid, using a glass indicating electrode and a calomel reference electrode filled with 0.02 *N* lithium chloride in glacial acetic acid. The titrant was standardized against dried potassium biphthalate. Five milliliters of glacial acetic acid served as a blank.

Chloride—The sample was dissolved in 5 ml. of 1.5 *N* sulfuric acid and was titrated to the potentiometric end-point with 0.1 *N* silver nitrate, using a silver indicating electrode and a mercurous sulfate reference electrode. The titrant was standardized with dried sodium chloride, and 5 ml. of 1.5 *N* sulfuric acid served as a blank.

Moisture (*4b*, *6*, *7*)—An aliquot of sample was dissolved in anhydrous methanol, injected into a vessel containing methanol titrated to the end-point, and titrated with Karl Fischer reagent[2], using the dead-stop end-point technique and a 20-sec. delay. An aliquot of methanol was titrated as a blank. The titrant was standardized by titrating known volumes of water, delivered by gravimetrically calibrated microsyringe. Loss on drying to constant weight was determined on 300-mg. samples at 130°, except for heroin which was dried at 105°.

Spectroscopy—Recording UV[3] and IR[4] spectrophotometers were used to obtain spectra of the samples, and rotation data were obtained using an automatic polarimeter[5]. Absorptivity data in Table I are the averages of triplicates and rotational data are the averages of duplicates.

GLC—The GLC system[6] was as follows: on-column injection; 1.2-m. × 4-mm. i.d. glass columns; methylsilicone gum on silanized, acid-washed, flux-calcined diatomite; cured, conditioned, and tested as described previously (8); helium carrier gas at 50 ml./min.; flame-ionization detector; and automatic digital integrator[7] for area normalization. Temperature programs were 150–250°, and isothermal purity determinations were at 210°. A nonlinear adsorption graph was prepared for 0.5–100-mcg. sample injections of morphine, codeine, heroin, acetylcodeine, and both acetylmor-

[1] Radiometer automatic titrator (TTT-11), pH meter (PHM-26), recorder (SBR-2), 2.5-ml. automatic buret (ABU-1), titration assembly (TTA-31), and Karl Fischer assembly (TTA/KF); all electrodes were of the same manufacture.
[2] Stabilized reagent, Fisher Chemical Co., diluted fivefold prior to use.
[3] Cary model 14.
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[6] Hewlett-Packard 5750B detector, oven, control, and electrometer modules with strip-chart recorder; 3% OV-1 on Gas Chrom Q, 100–120 mesh, was from Applied Science Labs.
[7] Infotronics CRS-204.

Table I—Purity Profile Data[a]

Test	Heroin	Morphine	Morphine Hydrochloride
Phase solubility analysis	99.9 ± 0.7%	99.6 ± 0.4%	See *Discussion*
Titrations			
Acetous perchloric acid	100.5%	100.2%	103.1%
Chloride	—	—	102.9%
Moisture (Karl Fischer)	0.03%	(6.1%)[b]	(14.4%)[b]
Loss on drying	0.03%	(6.1%)[b]	(14.3%)[b]
Chromatography			
GLC	99.8% *a/a* (two minor peaks)	99.9% *a/a* (two minor peaks)	99.9% *a/a* (one minor peak)
HPLC	≥99.8% *a/a*	≥99.5% *a/a*	≥99.8% *a/a*
TLC	One spot	One trace impurity	One trace impurity
Differential scanning calorimetry	<0.01 mole % eutectic impurity; ΔH = 7430 cal./mole; C.F. = 0.4%; T_0 = 446.9 °K.	Decomposes	Decomposes
IR	0.5% in KBr	0.5% in KBr	0.5% in KBr
UV absorption			
$A_{1\,cm.}^{1\%}$ (max.)	47.8 at 278 nm.	52.7 at 285 nm.	46.5 at 285 nm.
Ratio (max./min.)	5.22 (in 0.1 *N* HCl)	3.38 (in 0.1 *N* HCl)	3.33 (in 0.1 *N* HCl)
Specific rotation			
$[\alpha]^{25}$°, 589 nm.	−147.2°	−140.0°	—
578 nm.	−154.0°	−146.9°	—
546 nm.	−175.3°	−167.6°	—
436 nm.	−303° (0.5% in 0.015 *N* HCl–methanol)	−292° (1% in methanol)	—

[a] Values reported on anhydrous basis. [b] Morphine was the monohydrate (theoretical = 5.9%) and the hydrochloride was the trihydrate (theoretical = 14.4%), with moisture values on the as is basis.

phines. Morphine exhibited a trailing factor of 1.5 at 5% of peak height and efficiency, n = 1500; greater efficiency, n = 2600, was observed for heroin which, in contrast, did not trail. At the 100-mcg. injection level, heroin showed overload effects.

High-Pressure Liquid Chromatography (HPLC)—The HPLC system[8] was as follows: low-pressure mercury, 254-nm., UV detector; electronic integrator[7] for area normalization; and 1.0-m. × 2.2-mm. i.d., prepacked, stainless steel columns. The conditions tried were: (*a*) 40% methanol in water–chemically bonded ether on porous silica, 45°, 600 p.s.i.; and (*b*) pH 8.6 phosphate buffer–octadecylsilane on porous silica, 50°, 850 p.s.i. Strong cation-exchange experiments used a chemically bonded sulfonated fluorocarbon polymer. All solvents were filtered through suitable membrane filters and degassed prior to use.

TLC—Each undried sample was dissolved in methanol and 200, 100, and 20 mcg. were applied with micropipets (Drummond) to glass plates precoated commercially[9] with: (*a*) a 0.25-mm. layer of silica gel containing a fluorescent indicator, or (*b*) a 0.10-mm. layer of microcrystalline cellulose containing a fluorescent indicator. Sample application was made in subdued light, and the spots were dried in a stream of nitrogen and developed to a height of 10 cm. in the dark in glass chromatographic tanks containing one of the seven mobile phases listed. Spots were visualized by one or more of the five agents. Due to the low abundances of organic impurities in these samples and the lack of a common visualization agent, high-low TLC as described elsewhere (1) was not applicable. Some systems (II, III, V, VIII, and IX) were obtained from general references (9, 10). Relative mobility, R_f, of the main spots only are given here.

Systems for Morphine and Its Salt—The systems used were:

I. Glacial acetic acid–ethanol–water (3:6:1), R_f 0.52
II. Methanol–25% ammonium hydroxide (200:3), R_f 0.32
III. Ethanol–pyridine–dioxane–water (10:4:5:1), R_f 0.22
IV. Methanol, R_f 0.30 (cellulose)
V. Benzene–dioxane–ethanol–25% ammonium hydroxide (10:8:1:1), R_f 0.10
VI. Dimethylformamide–ethyl acetate (1:3), R_f 0.25

Systems for Heroin—The systems used were:

VII. Glacial acetic acid–ethanol–water (3:6:1), R_f 0.44
VIII. Methanol–25% ammonium hydroxide (200:3), R_f 0.38
IX. Benzene–dioxane–ethanol–25% ammonium hydroxide (10:8:1:1), R_f 0.46
X. Methanol, R_f 0.38
XI. 2-Propanol–water–glacial acetic acid (8:1:1), R_f 0.72 (cellulose)

Visualization (All Samples)—The following were used: long wavelength UV light (366 nm.), short wavelength UV light (254 nm.), iodine in chloroform spray (0.5%), acidified iodoplatinate spray (0.25 g. of platinic chloride and 5 g. of potassium iodide dissolved in sufficient water to make 100 ml. with 2 ml. of hydrochloric acid added), and acidified iodoplatinate spray followed by exposure to ammonia vapor.

Differential Scanning Calorimetry—Thermograms were recorded[10] from 80° to beyond the melting or decomposition points using microsamples in nitrogen-flushed, open pans.

Heroin Synthesis—Morphine monohydrate (the lot analyzed herein, 350 g., 1.154 moles) and acetic anhydride (99+%; 1095 ml., 1185 g., 11.6 moles) were placed in a 2-l. conical flask, and the resulting suspension was placed on a steam bath and kept at a temperature of 90° for 3 hr. The dark red-brown solution obtained was allowed to stand at room temperature for 3 days. All liquid (acetic acid and acetic anhydride) was removed with a rotary evaporator over a boiling water bath. Sufficient water was added to the solid residue to give an aqueous suspension with a volume of about 2.4 l. The aqueous suspension was cooled in an ice bath and made basic by slow addition, with stirring, of 5% aqueous sodium carbonate.

The pinkish-tan solid which precipitated was collected by filtration, washed twice with water, air dried, and powdered to give 419.4 g. of crude heroin base. Two recrystallizations from acetone afforded 117.9 g. of heroin base as a white crystalline powder, m.p.[11] 173.8–174.7°; molecular ion[12] m/e 369.

Second and third crops of heroin base were isolated from the combined recrystallization filtrates. The second crop consisted of 197.8 g. of a cream-colored crystalline powder, m.p. 172.5–174.1°, and the third crop consisted of 35 g. of a pale-tan crystalline powder,

[8] DuPont model 820 fitted with gradient elution accessory. Column packings were of the same manufacture; an ethereal polymer (ETH), octadecylsilane (ODS), and SCX Permaphase on Zipax support.
[9] E. Merck adsorbents from Brinkmann.
[10] DuPont model 990 thermal analysis unit fitted with the DSC head.
[11] Mettler FP2 apparatus with an FP21 microfurnace.
[12] Hitachi Perkin-Elmer RMU-6L mass spectrometer, 70 ev. ionizing potential.

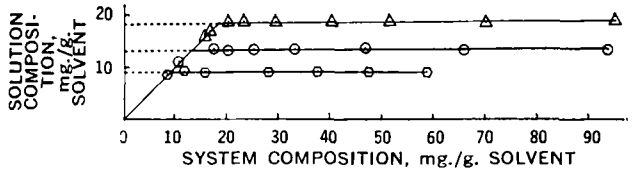

Figure 1—*Phase solubility diagrams of: △, morphine monohydrate in 2:1 methanol–ethanol, solubility (intercept) = 18.4 mg./g., slope (soluble impurity) = 0.38 ± 0.37%, undissolved phase was the monohydrate; ○, heroin in 3:1 hexane–dioxane, solubility = 13.3 mg./g., slope = 0.07 ± 0.71%; and ○, morphine hydrochloride (anhydrous) in ethanol, solubility = 9.2 mg./g., slope = −0.07 ± 0.85%, undissolved phase was the anhydrous.*

m.p. 171.6–173.5°. A total of 350.7 g. (82.3%) of heroin base was obtained.

RESULTS AND DISCUSSION

Heroin (Diacetylmorphine)—Heroin was prepared by acetylation of morphine with acetic anhydride in a manner similar to reported syntheses (11–16). Morphine and acetic anhydride were reacted in direct mixture to form the acetate salt of heroin. The heroin base was then isolated from aqueous sodium carbonate and purified by two recrystallizations from acetone. Product identification was verified by reference to published IR, UV, and rotation data (9, 17–19), in addition to the molecular ion and melting point. Literature melting points vary over a range of 169–173° (12–16).

Solubility analysis was achieved, after several attempts, with 3-day equilibration in a dry hexane–dioxane mixture (Fig. 1). GLC analysis of the undissolved crystals and the residue from the supernate showed that hydrolytic decomposition had not occurred. It is important that the dioxane used be freshly treated with iron dust (20) followed by activated charcoal to remove any peroxides and then distilled to give a dry, residue- and peroxide-free fraction. Solubility analysis in diluted ethanol, 55% in degassed water, failed due to hydrolytic decomposition: 8% after 1 day as determined by GLC. Insoluble impurities were not present in either solubility system. Analyses by GLC of the two phases of the latter system showed that the hydrolysis products did not form solid solutions.

Titrimetric and calorimetric purity data were consistent with the assay by solubility analysis. TLC did not reveal any impurities. Calculation of apparent eutectic impurities was done by the usual Van't Hoff relationship discussed elsewhere (1). Remelt of a sample showed, by the appearance of a probable eutectic endotherm at 4° less than the melting point, that some thermal decomposition had occurred on melting the sample, even under nitrogen.

UV-absorbing impurities were not detected (Fig. 2) by HPLC, using gradient elution in the adsorption–cation-exchange mode. Morphine, codeine, and both acetylmorphines are eluted more rapidly than heroin in this system, the most desirable situation for purity evaluations. These impurities (≤ 0.2%) were ruled out for this sample. The order of elution is that of decreasing polarity of substituents and, presumably, decreasing solubility in the aqueous salt solution. Heroin, 200 mcg., gave 0.065 absorbance unit peak height, with overload effects apparent. Calibration mixtures of O^6-acetylmorphine in heroin exhibited a rectilinear relationship in the peak height ratio (0.05 height ratio unit per 1% impurity). Because HPLC is in an early stage, it was deemed prudent to test the reliability of this system. Three other packed columns, from other laboratories, were tested in this laboratory with similar results. Figure 2 represents intermediate performance.

Acetylcodeine, an important heroin impurity, eluted just prior to heroin and can be estimated only when present in abundance, as is the case for the mixture of authentic drug substances in Fig. 2. Acetylcodeine was completely overlapped by the heroin peak when present to the extent of a few percent, so that GLC would be preferred for the measurement of this impurity in the usual illicit samples of heroin.

Cashman and Thornton (21) reported an adsorption–liquid chromatographic separation of heroin, O^6-acetylmorphine, and morphine, in that order of elution. Unfortunately, heroin eluted first and was poorly separated from acetylmorphine, rendering the system less useful for purity or stability work.

GLC revealed two unresolved peaks (total area 0.15%) at 0.65–0.70 relative retention, which were the two acetylmorphines. This system almost separated them[13] [resolution factor (8) ~0.7]. This level is too low for reasonable correction for nonlinear adsorptive losses. No acetylcodeine was evident in the sample, as would be expected for a sample prepared from morphine of defined purity.

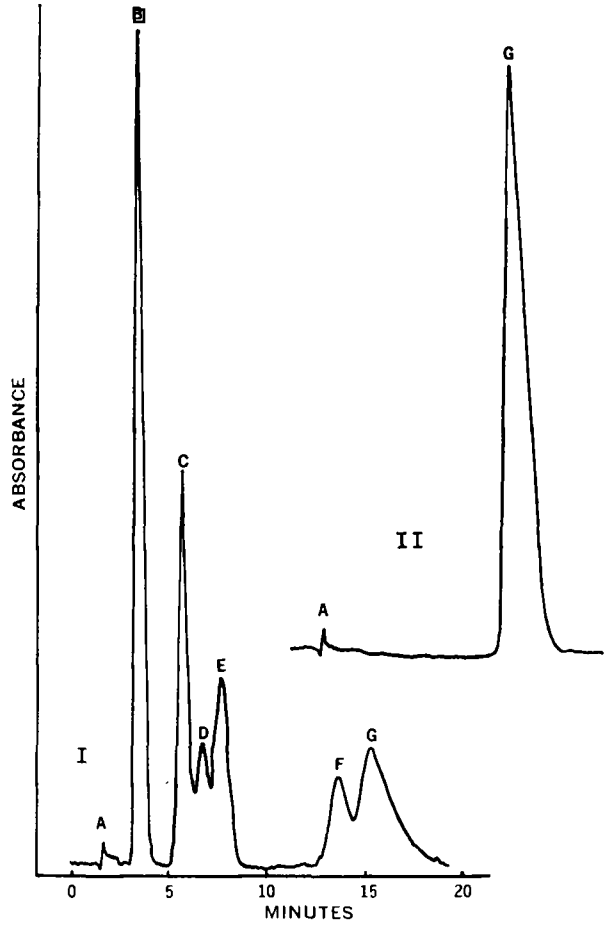

Figure 2—*High-pressure liquid chromatograms of: I, authentic mixture; and II, analytical sample. Key: A, solvent; B, morphine; C, O^6-acetylmorphine; D, O^3-acetylmorphine; E, codeine; F, acetylcodeine; and G, heroin. Gradient elution 0.4–1.4 M sodium perchlorate in 0.01 M pH 6.8 aqueous phosphate buffer containing 10% ethanol; 35°; SCX, 1000 p.s.i.*

The HPLC system is to be preferred over GLC for determining the morphine and acetylmorphine contents of heroin unless several percent of impurities is present so that reasonable correction for nonlinear adsorptive losses can be made. Such losses are characteristic of phenols and commonplace for compounds with several polar functional groups, *i.e.*, morphine and its polar derivatives. It is also characteristic that individual chromatographic systems vary widely in this regard. Because any derivatization step can be the occasion either of purification or decomposition, purity values by such methods (except where a separately defined standard is examined concomitantly) are not considered reliable, even though they may be the basis of entirely satisfactory assay procedures once a standard is otherwise established.

All these purity profile data showed this sample of heroin to be satisfactory for use as an analytical standard in qualitative and quantitative applications.

Morphine Monohydrate—Phase solubility analysis (Fig. 1), using 6-day equilibration, indicated that this sample of morphine was essentially single-component material. Nonaqueous titration results (Table I) were consistent with this indication. The base and hydrochloride were characterized by UV (17) and IR spectropho-

[13] O^3-Acetylmorphine at 0.66, acetylcodeine at 0.68, and O^6-acetylmorphine at 0.71. For comparison, on OV-17/Q these relative retentions (heroin = 1.0) are 0.58, 0.56, and 0.59, respectively, but higher temperatures make this even less desirable as a phase. Silylation (bistrimethylsilylacetamide) does not improve this situation.

tometry (9, 18) and optical rotation (19). The spectra were found to be similar to published spectra and identical to those of another dried sample of morphine.

TLC showed the material to be nearly homogeneous using six systems. One small but distinct impurity was detected which appeared as a yellow fluorescent spot having the approximate mobility of papaverine (orange fluorescence), indicating the possible presence of such a benzylisoquinoline. Semiquantitation (1) was not feasible due to the lack of a common visualization agent. Within the morphine spot was an area of blue fluorescence not separable from the main spot by any solvent system tried; this was interpreted to be pseudomorphine, an artifact (22) of on-plate oxidation, particularly in the presence of ammonia. Two-dimensional TLC did not reveal any additional artifacts.

Temperature-programmed GLC revealed no impurities of greatly different vapor pressures in the morphine sample. Isothermal purity determination showed the two impurities (Table 1) at relative retentions of 0.94 and 4.2, respectively. The retentions of the two impurities resembled those of papaverine and codeine. Spiked samples indicate that the larger impurity may be codeine and the same experiment tends to exclude papaverine. It is not possible at such low levels of impurities to exclude firmly or identify likely impurities without mass spectrograms of the eluted peaks; for example, reagent rather than spectroquality chloroform gives a peak at the same locus as codeine.

In the HPLC of morphine, three packings[14] were tried in both normal and reverse phase modes. Although these packings are among the most reliable available, none yields ideal peak shapes, and substantial adsorptive trailing precluded exact quantitation. Exchange resins also were investigated. Only the reverse phase systems yielded interpretable chromatographic purity data. Electronic area integration did not give useful data because of the contribution of trailing-in from earlier peaks. The single impurity peak had the same mobility as codeine and it is estimated that this is less than 0.5%.

Samples of both dried and undried material were examined by differential scanning calorimetry. Due to decomposition, however, no purity estimate can be made. The undried sample showed a substantial endotherm in the 110–140° region, which represents the removal of water, and a sharp endotherm at about 260° with severe decomposition. A sample dried at 50° *in vacuo* showed only traces of the lower endotherm, and a sample dried at 130° lacked this endotherm completely. No attempt was made to prepare or identify different polymorphs, although polymorphism has been observed for this drug[15].

Drying of morphine monohydrate at 130° was shown by differential scanning calorimetry and Karl Fischer titrimetry to remove all moisture from the sample, and TLC of the dried material indicated no decomposition. Drying of this material, in small amounts for quantitative use, at 130° for 2 hr. in static air is, therefore, recommended.

The purity profile afforded by these data allowed the recommendation of this sample of morphine monohydrate as an analytical standard for both qualitative and quantitative applications.

Morphine Hydrochloride Trihydrate—This sample was found to be nearly homogeneous by TLC with six solvent systems, and these results were essentially the same as those reported for morphine.

Prior to GLC, the hydrochloride salt was converted to the free base by extraction from pH 9.5 buffer into a 10% solution of 2-propanol in chloroform. This same solvent mixture also was used for morphine. Isothermal operation revealed one impurity with a relative retention of 0.94, and temperature programming revealed no additional impurities. Spiked samples suggested that this impurity was codeine, but at such low abundances that positive identification is difficult. HPLC located one minor impurity, similar to that in the morphine sample.

Nonaqueous titration (Table 1) of morphine hydrochloride required that mercuric acetate be added to the titration medium to convert all chloride salts to titratable bases; however, in this system any alkali metal or ammonium ions present also titrate as bases (5), contributing by a disproportionately large amount to the total titration value. Taken with the high chloride content, titrimetry clearly indicates lower molecular weight impurities. The moderate pH value (4.7 using a 2% aqueous solution) excluded excess hydrochloric acid as a significant factor. Chromatographic analyses showed a lack of substantial organic impurity. Inorganic chlorides were indicated, and some sodium was shown to be present by a classic flame test.

Samples of both dried and undried material were examined by differential scanning calorimetry. Due to decomposition, however, no purity estimate was possible. In the 60–110° range, two endothermic peaks were found. No evidence was found of a main melt in the 50–260° range by this technique. A scan of material dried at 50° *in vacuo* showed a slight trace of the two endotherms, whereas material dried at 130° lacked these peaks completely. When taken together with the titrimetric moisture and comparative TLC results, these results substantiate the complete removal of moisture and lack of significant degradation at 130°. The moisture content was found to be consistent with the expected trihydrate form, but the material should be dried prior to use as a semiquantitative standard.

An initial solubility analysis in alcohol (10-day equilibration) gave 2.2 ± 0.4% slope; however, anhydrous drug was isolated as the undissolved phase whereas the trihydrate was used as the sample, indicating that solvent composition was not constant. Coupled with common-ion effects of the inorganic chlorides, this rendered the slope uninterpretable[16] (23), and this slope value was ignored in constructing the purity profile of the sample. An 8-day equilibration of previously dried sample gave a −0.1% slope. Because titrimetry revealed lower equivalent weight chloride salts in the sample, this slope also cannot directly reflect sample composition because the excess chloride equivalent can exert a common-ion effect on the slope that is opposite to the mass impurity effect. Titrimetric values and this latter slope are consistent with the equivalent of 0.5–0.6% sodium and ammonium chlorides.

These data are interpreted as representing an organically pure sample of morphine as the trihydrated hydrochloride salt which contains inorganic material. This would not impair the qualitative utility of this standard. The morphine monohydrate, because of its single-component composition, would be required for *quantitative* use.

SUMMARY

Samples of heroin, morphine, and morphine hydrochloride were evaluated by phase solubility analysis, titrimetric moisture, loss on drying, nonaqueous titration, chloride titration, UV and IR absorption, differential scanning calorimetry, optical rotation, GLC, TLC, and HPLC.

Systems for solubility analysis and HPLC were developed in support of this work. A synthetic method yielding heroin of a high degree of purity is reported.

The purity profiles established for each sample allowed definition of the scope of utility of each as an analytical standard in either qualitative or quantitative applications.

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PHARMACEUTICAL TECHNOLOGY

Shear Cell Measurements of Powders: Determination of Yield Loci

E. N. HIESTAND▲, S. C. VALVANI, C. B. PEOT, E. P. STRZELINSKI, and J. F. GLASSCOCK, Jr.

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It is postulated that the flow of powder occurs when the forces acting on a powder bed cause the resultant shear force just to exceed the powder bed's shear strength in any given direction. Therefore, the flow characteristics of the powder can be described in terms of an orthogonal set of principal forces. Only two, the major and minor forces, have a significant effect on the location of the shear plane. The magnitude of these forces depends upon the state of consolidation of the powder bed. For many applications, it is adequate to describe the changes of unconfined powder bed properties with the change in the state of consolidation. This description is in the form of a failure function, a plot of the major stress necessary to cause failure of an unconfined bed *versus* the major stress that produced the given state of consolidation.

The failure function can be estimated from shear cell data (1) or a triaxial test method (2); both methods usually fail to provide completely accurate evaluations. The shear cell methods are the most popular. This article briefly discusses the theory relating to these concepts and reports results of studies with a simple shear cell. Also included is a limited comparison of the data obtained with one sample using two different shear cells.

CONCEPTS

Figure 1 shows the direction of forces acting in both the triaxial test and the shear cell. It also shows the Mohr semicircle used to describe the stress balance at failure. With the triaxial method, the major stress, σ_m, and the minor stress, σ_s, are the applied stresses and τ and σ_n are the resultant stresses. Moreover, the angle α is observed, so the corresponding Mohr semicircle can be constructed. With the shear cell, σ_m, σ_s, and α are not determined; only τ and σ_n are known. Therefore, a single measurement does not provide enough information to permit construction of the corresponding Mohr semicircle. When σ_m and σ_s are of the correct magnitude to produce failure, *i.e.*, shearing of the powder bed, then τ and σ_n appear on the circle. As can be seen from Fig. 2, the principal stress, σ_i, which is intermediate in magnitude, does not influence the failure. The resultant τ and σ_n values represented by the Mohr semicircles involving σ_i are always less than any τ, σ_n values on the semicircle between the major and minor principal stresses. There-

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CONCEPTS

Figure 1 shows the direction of forces acting in both the triaxial test and the shear cell. It also shows the Mohr semicircle used to describe the stress balance at failure. With the triaxial method, the major stress, σ_m, and the minor stress, σ_s, are the applied stresses and τ and σ_n are the resultant stresses. Moreover, the angle α is observed, so the corresponding Mohr semicircle can be constructed. With the shear cell, σ_m, σ_s, and α are not determined; only τ and σ_n are known. Therefore, a single measurement does not provide enough information to permit construction of the corresponding Mohr semicircle. When σ_m and σ_s are of the correct magnitude to produce failure, *i.e.*, shearing of the powder bed, then τ and σ_n appear on the circle. As can be seen from Fig. 2, the principal stress, σ_i, which is intermediate in magnitude, does not influence the failure. The resultant τ and σ_n values represented by the Mohr semicircles involving σ_i are always less than any τ, σ_n values on the semicircle between the major and minor principal stresses. There-

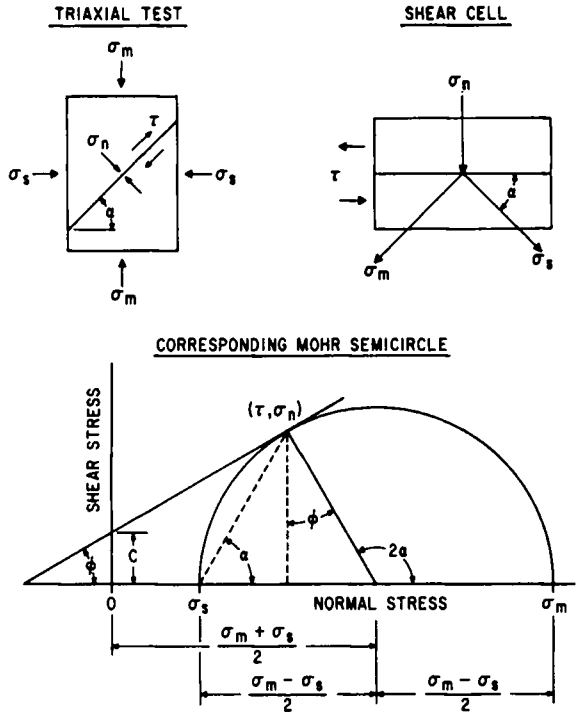

Figure 1—*Relationship of the triaxial test, the shear cell, and the Mohr semicircle. The stress equations are:*

$$\tau = \tfrac{1}{2}(\sigma_m - \sigma_s) \sin 2\alpha$$
$$\sigma_n = \tfrac{1}{2}(\sigma_m + \sigma_s) + \tfrac{1}{2}(\sigma_m - \sigma_s) \cos 2\alpha$$

Their relationship to the Mohr semicircle is obvious.

fore, the two-dimensional representation of the Mohr semicircle is adequate where the precision required is not outside the limits of the simple shear failure model.

Figure 3 is developed from the triaxial diagram, but it applies also to the shear cell. It shows the relationship between the angle α and the internal friction coefficient, which for this discussion is designated μ and is assumed to be a constant. By using any given combination of σ_m and σ_s that produces failure, the resultant magnitudes of the normal and shear stresses at any angle, α, may be calculated. These resultant stresses are σ_α and τ_α, respectively; τ_s is the shear strength of the powder bed. It is calculated from the friction coefficient, μ, the normal stress, σ_α, and an apparent cohesion, C. When τ_α is equal to τ_s, the angle of failure in shear is determined. At this point, $\mu = \tan \phi$.

A series of combinations of σ_m and σ_s can be found that produce shear failure with any given state of consolidation of a powder bed. When the corresponding τ and σ_n values are plotted, a yield locus may be drawn through the points. Each respective Mohr circle will be tangent to the yield locus, and it is this property that permits the shear cell to be used to determine α. A series of values obtained with

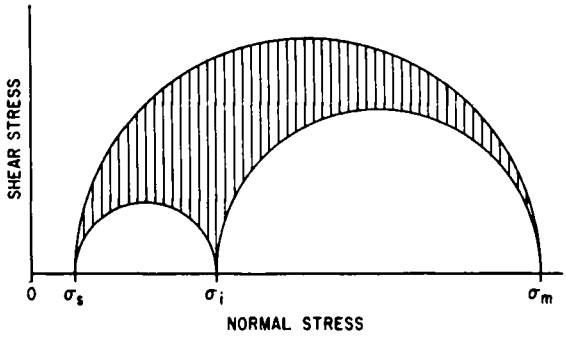

Figure 2—*The Mohr semicircles show that the largest shear stresses are produced as a resultant of the major and minor principal stresses, σ_m and σ_s, and not as a resultant of the intermediate principal stress, σ_i, with either σ_m or σ_s.*

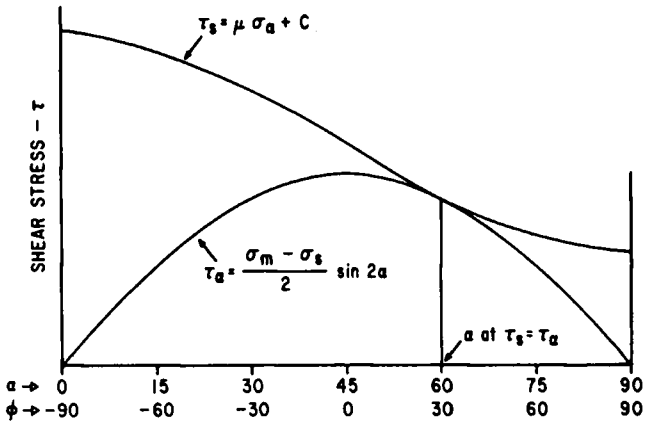

Figure 3—*Failure occurs at the angle where the resultant shear stress, τ_α, is equal to the shear strength, τ_s. Note that the shear strength results from the internal friction and cohesion. The change of both τ_s and τ_α with change in ϕ or α is shown; ϕ is the angle of friction and is equal to $2\alpha - 90°$.*

the shear cell determine a yield locus. Any corresponding combination of σ_m and σ_s can be obtained by drawing a Mohr circle tangent to the yield locus through the τ, σ_n point of interest.

SHEAR CELL DESIGN AND TREATMENT OF DATA

Probably the most popular shear cell design is that of Jenike (1). Schwedes (3) showed that the shear region in the cell has a lenticular shape. However, Williams *et al.* (2) claimed that the results are consistent with the triaxial test. Schwedes used a shear cell similar to that of Roscoe (4) and a very low, constant rate of shear. His choice of cells was, in part, based on the conclusion that the stress conditions in the Jenike cell cannot be determined.

In the authors' laboratory, the choice of cells was based on the need to work with small amounts of powders and to accumulate useful data rapidly. It was hoped that the stress distributions would be more uniform and easily defined in a thin layer of powder because the shear region would be restricted. A modification of a cell described by Nash *et al.* (5) was adopted, and a method of obtaining reproducibly a given state of consolidation was developed. The shear cell (Fig. 4) consists of a thin layer of powder between two sandpaper-covered surfaces (see *Appendix* for details). To determine a yield locus, the powder is brought to a reproducible state of consolidation by repeatedly initiating shear in the powder bed while keeping the normal load constant. The shear stress is returned to zero as soon as possible so that minimal movement occurs. After a series of shear initiations, the observed shear force becomes constant. This is called the plateau condition and is the reference state of consolidation for the powder under that specific applied load. The cell and procedures were described previously (6, 7). The plateau load is the maximum load in a given yield locus. Other points on the yield locus are obtained by reducing the load after a plateau condition has been reached; then shear is initiated just once after the load is reduced. The reduced load and resultant shear force provide one point on the yield locus, and this procedure is repeated to obtain additional points. Different yield loci are obtained by

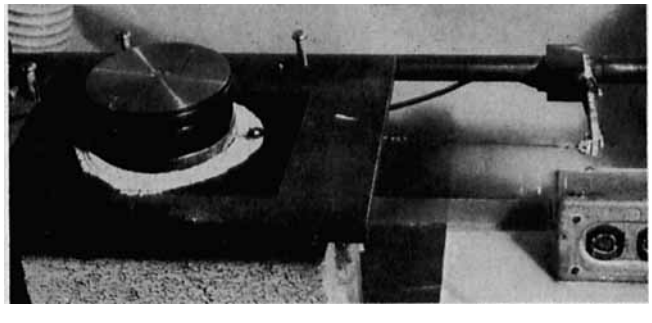

Figure 4—*Simple shear cell.*

Table I—Results of Shear Cell Studies of β-Sitosterol (40 Mesh)

	35-Point Data	Replicate 14-Point Data	14 Points from 35-Point Data
Estimate of tan ϕ	0.660	0.657	0.666
95% confidence interval for tan ϕ	0.635, 0.685	0.616, 0.698	0.626, 0.706
Estimate of tan β	0.167	0.166	0.162
95% confidence interval for tan β	0.146, 0.188	0.134, 0.198	0.131, 0.193
Multiple-correlation coefficient	0.997	0.997	0.992
Standard error of estimate of τ_r[a]	0.713	0.708	0.695
Estimate of b[a]	0.544	0.472	0.552
f_c *versus* σ_{pm} slope	0.245	0.243	0.237
f_c *versus* σ_{pm} intercept[a]	1.773	1.538	1.819

[a] Kilodynes/cm.^{2}.

using a different normal load to attain the plateau condition and then reducing the load from that state of consolidation.

Reducing the load after reaching a state of consolidation leads to partial elastic recovery of the powder bed. Thus, the yield locus is determined under conditions analogous to those used in evaluating other solids, *i.e.*, yield conditions under various elastic stresses; this differs from the dynamic steady-rate condition used with the other cells already discussed. This simple cell can be criticized in several ways. Obviously, it is difficult to demonstrate that the shear plane is in the powder and not at the sandpaper surface. Also, edges of the cell may not have powder under identical stress and consolidation conditions as the interior regions of the cell. However, the thin layer of powder helps to minimize these effects and, of course, requires less powder, an important consideration when working with expensive medicaments.

Powders are more complex than ordinary solids because powders have a broad range of consolidations. The shear strength changes with the state of consolidation. Thus, instead of one yield locus characterizing the powder, a family of yield loci is necessary, one for each state of consolidation. With the procedure used in this study, the plateau load is the principal factor in determining the state of consolidation. Empirically, it has been found that the family of yield loci corresponds to a simple relationship:

$$\tau_r = \sigma_r \tan \phi + \sigma_p \tan \beta + b \qquad \text{(Eq. 1)}$$

where subscript r denotes the reduced load values, subscript p denotes the plateau or consolidation condition, and tan ϕ is the internal friction coefficient; $\sigma_p \tan \beta + b$ describes the apparent cohesion (C in Fig. 1), and, therefore, tan β is the rate of change and b is the intercept of the apparent cohesion function.

These relationships and related equations were described previously (7). Equation 1 is the classical empirical law of friction, with additional terms to account for the different states of consolidation.

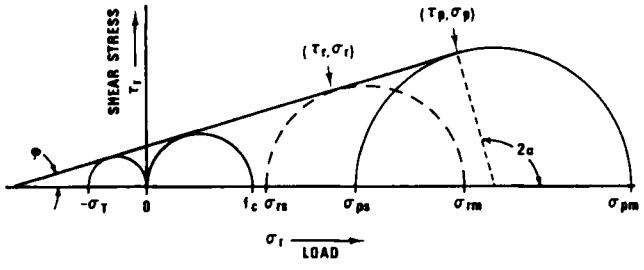

Figure 5—*Mohr semicircles, drawn tangent to a linear yield locus, that represent the plateau condition between* σ_{ps} *and* σ_{pm}, *a reduced load condition between* σ_{rs} *and* σ_{rm}, *the unconfined yield stress* f_c, *and the ultimate tensile strength* σ_T.

Table II—Loads Used on an 8.8-cm. (3.5-in.) Diameter Cell in Shear Cell Studies[a]

Plateau Load	Reduced Load				
3352	3109	2372	1392	902	412
(2862)	(2770)	(2170)	(1420)	(920)	(412)
(2372)	2274	1882	1392	902	(412)
(1980)	1882	(1587)	1196	804	412
(1588)	(1490)	1294	902	706	412
(1196)	1147	1000	(804)	608	412
(902)	(853)	(706)	(608)	(510)	(412)

[a] All values are in kilodynes. The 14-point data are indicated by parentheses.

The individual yield locus is associated with only one state of consolidation. Therefore, it is not surprising that it is linear[1] since the conditions become identical to those between two large pieces of solid, where the linear relationship is the accepted and expected result. Different equations have been used with data obtained with the Jenike shear cell. The Warren Springs equation[2] is said to provide adequate flexibility in describing nonlinear yield loci such as those obtained in the dynamic conditions used with the Jenike cell (8). However, it would have to be modified to incorporate the large tensile strength values observed here. The simplicity of a linear relationship permits other interesting relationships (*e.g.*, Eqs. 2 and 3) to be derived readily (7).

In the authors' laboratory, values of τ_r, σ_r, and σ_p are used in a multiple-regression analysis[3] to obtain estimates of tan ϕ, tan β, and b. These values may be used to determine the failure function and the ultimate tensile strength function. Equation 2 is the former and Eq. 3 is the latter:

$$f_c = \left[\frac{2 \cos \phi \tan \beta}{1 + \cos \phi \tan \beta}\right]\sigma_{pm} + \frac{2b(1 + \sin \phi)}{\cos \phi (1 + \cos \phi \tan \beta)} \qquad \text{(Eq. 2)}$$

$$\sigma_T = \left[\frac{1 - \sin \phi}{1 + \sin \phi}\right]f_c = \left[\frac{2(1 - \sin \phi)^2 \tan \beta}{\cos \phi(1 + \cos \phi \tan \beta)}\right]\sigma_{pm} + \frac{2b(1 - \sin \phi)}{\cos \phi(1 + \cos \phi \tan \beta)} \qquad \text{(Eq. 3)}$$

Equations 2 and 3 are derived from Eq. 1 and the geometric relationships shown in Fig. 5; σ_{pm} is the major consolidation stress, f_c is the unconfined yield stress (the major principal stress when the minor one is zero); and σ_T is the ultimate tensile strength. The ultimate tensile strength is the strength a powder bed would have in tensile failure if conditions comparable to those in the shear cell could be

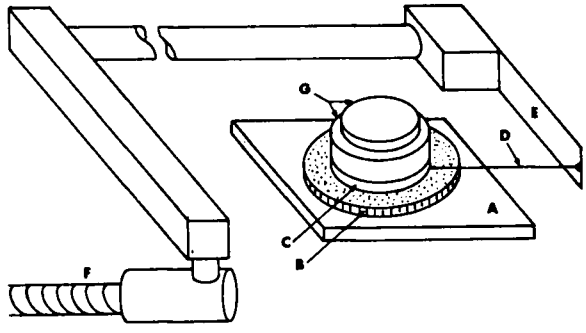

Figure 6—*Arrangement of apparatus. Key: A, bottom plate of shear cell; B, powder bed; C, top plate of shear cell; D, tow line; E, strain gauges; F, jack (motor driven); and G, weights.*

[1] Some deviation from linearity is observed. However, for many materials (*e.g.*, β-sitosterol) the tensile strength is too large to conform to the curvilinear model. Therefore, the linear model has been proposed.

[2] The Warren Springs equation is of the form $(\tau_r/C)^n = (\sigma_r/S) + 1$ where τ_r, σ_r, and C have the same meaning as already given, S is the tensile strength, and n is an empirical constant.

[3] The point (τ_p, σ_p) usually is below the corresponding yield locus. Therefore, it is not used in the multiple-regression analysis. A procedure analogous to that used by Jenike in a similar situation (1) is used here; *i.e.*, a $f(\tau_r, \sigma_r)$ point is determined where σ_r is approximately 95% of σ_p. This is the largest value used in evaluating an *individual* yield locus.

Table III—Tan ϕ Calculated by Least Squares for Each Individual Yield Locus[a]

Plateau load	3352	2862	2372	1980	1588	1196	902
tan ϕ	0.665	0.653	0.654	0.642	0.660	0.582	0.575

[a] Based on 35 load conditions shown in Table II.

Table IV—Comparison of Two Different Lots of the Same Chemical Produced during the "Scale-Up" of a New Drug

Function	Ambient Conditions —(as Received)— Lot A	Lot B	Equilibrated with a 50% Relative Humidity —Environment— Lot A	Lot B
tan ϕ	0.761	0.688	0.714	0.689
tan β	0.206	0.155	0.238	0.120
b[a]	1.90	0.795	1.562	0.792
f_c *versus* σ_{pm} slope	0.283	0.175	0.324	0.180
f_c *versus* σ_{pm} intercept[a]	6.602	2.721	5.088	2.738

[a] Kilodynes/cm.^{2}.

realized in a tensile strength measurement. Of course, the value also is based on the assumption that the yield locus is linear into the tension region so that the extrapolation is valid; this is not easily demonstrated. However, comparison of the tensile strengths calculated from shear cell measurements with values obtained using the diametral compression method of evaluating compacts provides strong evidence that, at least in some cases, the extrapolation is not grossly in error (7).

RESULTS AND DISCUSSION

To illustrate the reproducibility of evaluations of powders with this cell, some experimental results are included in the tables. Table I shows the observed properties of a β-sitosterol sample, and Table II shows the normal load forces used in this study. Column 1 of Table I shows the results of using a 35-point determination. The middle column is for a duplicate determination in which only 14 points were collected. The normal loads for these 14 points are shown in parentheses in Table II. The last column of Table I shows the results when the corresponding 14 data points (those in parentheses) are selected from the 35-point data. The 35 points defined seven yield loci. Table III shows the values of these slopes, tan ϕ, for each individual yield locus. As illustrated by this example, tan ϕ often is less for small conditioning loads. Note that the multiple-regression analysis yields a value for tan ϕ characteristic of the yield loci for larger conditioning loads. The slope and intercept values for the corresponding failure functions, Eq. 2, are given at the bottom of Table I. These variations are larger than desired but they are believed to be respectable when one allows for the great difficulties experienced in evaluating powders.

Table IV illustrates the usefulness of the shear cell data. Two different, small production lots of a new drug were compared. Shear cell measurements were not necessary to reveal that the flow properties of the two lots were different. Lot A would not fall through a coarse screen without agitation, but Lot B fell through unless carefully placed on the screen. Obviously, both lots had poor—but not equally poor—flow properties. Both, the slope and intercept of the failure function, f_c *versus* σ_{pm}, indicate poorer flow characteristics for A than for B. Thus, the shear cell study measured quantitatively the differences. It also established that these differences were not due just to the equilibration with different atmospheres. Obviously, it is important to determine what causes variations such as those in Table IV, but the ability to measure meaningful flow properties must come first. Unfortunately, small differences in measured values are not always significant. The determinations are not accurate to the three significant figures shown in the tables, but the multiple-regression analysis yields the associated confidence limits to serve as guides to the significance of the values.

Table V shows the values obtained in two different equilibrium humidity environments for sucrose, starch, and a mixture of the two powders. It is obvious that the starch is a less cohesive powder than the sucrose, especially at the lower humidity. Furthermore, the starch is effective at the 25% level in imparting low cohesiveness to the mixture when the humidity is low. At higher humidity, the influence is less. Again, these data are included to illustrate the shear cell method of evaluation. No explanation of the observed effects are established at this time.

The only data available to the authors are inadequate for a thorough comparison of the results with the simple shear cell method described here and the Jenike method. However, the available data are included to provide a limited comparison and to illustrate some problems in evaluating powders. A powder mixture was submitted to a commercial laboratory, one that uses the Jenike cell and procedures. They made only a six-point determination, three points on each of two yield loci, and reported the failure function graphically. It was equivalent to $f_c = 0.186\ \sigma_{pm}$. The values of f_c and σ_{pm} were obtained by drawing Mohr semicircles tangent to the yield loci. For comparison, the multiple-regression analysis method was applied to their data (Table VI). In the authors' laboratory, the simple shear cell was used to obtain a 40-point determination on the same lot of material (Table VI). It is difficult to compare 40-point and 6-point data except through statistical values, such as in Table VI. It is obvious that improved precision would be desirable. The simple cell used in this study was only 5.1 cm. (2 in.) in diameter. Later, the diameter was increased to 8.8 cm. (3.5 in.) and significant improvement in precision resulted. The other data reported in this paper were obtained with the larger cell.

The graphs submitted by the commercial laboratory showed that one data point had been omitted in their analysis. The last column of Table VI shows the results of the multiple-regression analysis when the same point is omitted. Since only three points were available to determine a yield locus, rejection of one left only two. The criterion for such a selection is not obvious, but certainly it improves the multiple-correlation coefficient. Also, the calculated failure function corresponds much more closely to the one reported graphically.

CONCLUSION

The examples discussed demonstrate that the simple shear cell can be used to provide characterization of the unconfined yield strengths of powders. It is not anticipated that the results will be identical to those obtained by different procedures. However, the simplicity of the system and the use of small quantities of powder make this approach attractive. In time, definitive work may determine which procedure and cell yield the most accurate data.

Table V—Effect of Mixing Two Powders

	Sucrose	Starch[a]	75% Sucrose–25% Starch
Relative Humidity 25%			
tan ϕ	0.672	0.704	0.665
tan β	0.162	0.054	0.132
b[b]	1.058	9×10^{-3}	5.6×10^{-2}
f_c *versus* σ_{pm} slope	0.237	0.0838	0.199
f_c *versus* σ_{pm} intercept[b]	3.49	3.2×10^{-2}	0.187
Relative Humidity 50%			
tan ϕ	0.663	0.655	0.650
tan β	0.216	0.081	0.167
b[b]	1.024	0.187	0.794
f_c *versus* σ_{pm} slope	0.305	0.127	0.246
f_c *versus* σ_{pm} intercept[b]	3.237	0.645	2.560

[a] StaRx 1500. [b] Kilodynes/cm.^{2}.

Table VI—Comparison of Jenike Cell Data with Data from a Simple Shear Cell

	40 Points, Simple Cell	6 Points, Jenike Cell	5 Points, Jenike Cell
Estimate of tan φ	0.653	0.784	0.720
95% confidence interval for tan φ	0.640, 0.667	0.492, 1.076	0.520, 0.868
Estimate of tan β	0.068	0.054	0.111
95% confidence interval for tan β	0.056, 0.080	−0.105, 0.213	0.021, 0.201
Estimate of b[a]	5×10^{-3}	23.2	4.46
Multiple-correlation coefficient	0.999	0.989	0.999
Standard error of estimate of τ_r[a]	1.11	18.0	6.21
f_c *versus* σ_{pm} slope	0.108	0.082	0.165
f_c *versus* σ_{pm} intercept[a]	2×10^{-2}	91.5	16.0

[a] Kilodynes/cm.^{2}.

APPENDIX: SHEAR CELL AND POWDER BED PREPARATION

A 12- by 16-cm. piece of 50-grit X-weight aluminum oxide emery cloth is bonded (using double-sided pressure sensitive tape) to a level, heavy piece of steel plate to form the bottom element of the shear cell. A 12.4-cm. diameter disk of the same material is bonded to the bottom of a 12.4-cm. diameter brass disk to form the upper element. A small steel hook is silver soldered to the bottom of the edge of the brass disk to provide a connection for pulling as near to the shear plane as possible.

A hole of 10.5-cm. diameter is made in a piece of aluminum plate to serve as a template for forming the powder bed. Plates of various thicknesses are available, permitting a choice in powder bed thickness. The initial thickness of the powder bed usually is 0.31 cm. (0.125 in.) with coarse materials and 0.15 cm. (0.06 in.) with fine materials. The template is placed over the bottom emery cloth, and powder is sifted into the hole of the template. A spatula is used to scrape across the plate to level the powder while avoiding packing of the bed. Excess powder is pushed out of the way and the template is removed. The top element of the shear cell is placed gently onto the powder bed, and the steel tow line from the strain gauge is placed over the hook. Care is exercised to assure that the pulling motion is parallel to the plane of the powder bed (Fig. 6).

A series of thin brass weights are made from brass cylinders to facilitate stacking and removal of selected weights. After placing the desired weights on the top element of the cell, shear is induced by moving a cantilever-type strain gauge. The elastic deflection of the gauge beam is 6.2 mm./kg. The magnitude of this deflection influences the movement of the top element of the shear cell each time shear is initiated. Excess movement affects the magnitude of stress on the next shear initiation.

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Computer Optimization of Pharmaceutical Formulations II: Application in Troubleshooting

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Abstract □ The computer optimization technique presented in a previous paper was applied to a practical situation. The data generated by the set of statistically designed experiments and the subsequent computer analysis were used to indicate the directions necessary to improve various characteristics of a production tablet. Data from experimental size and production size batches are compared with computer predictions.

Keyphrases □ Computer optimization of pharmaceutical formulations—application in troubleshooting production tablet characteristics □ Formulation of pharmaceutical preparations—computer optimization, troubleshooting production tablet characteristics □ Pharmaceutical technology—application of computer optimization to troubleshooting characteristics of a production tablet

The previous paper in this series (1) described the general procedure used for computer optimization developed in these laboratories. The ability of the program to aid in the selection of a formulation with optimum properties was demonstrated.

The optimization technique can be useful in the development of a product by virtue of its capacity to generate a large amount of information and thus help the investigator understand his or her system. The present article reports on the practical application of such a program to another problem area in the pharmaceutical industry—troubleshooting.

The model system originally selected for the optimization study was a product formula. During the development of the optimization technique, it was learned that this product was experiencing some rejections in the production area for failure to meet a dissolution specification. Therefore, dissolution became the response of primary interest, but the objective was to increase the dissolution response without sacrificing other tablet properties.

For practical purposes, in this case a product that was already in production, the experimental range used to collect data for the optimization study was too large. That is, many possible solutions were located where the level of a given ingredient was outside the limits specified in the New Drug Application (NDA) for the product (2). Nevertheless, the results of the optimization procedure were useful because they pointed in the direction of an improved formulation.

Table I—Directions for Improvement from First Grid Search

Independent Variable		Qualitative Level
X_1	Diluent ratio	High
X_2	Compressional force	High[a]
X_3	Disintegrant level	High
X_4	Binder level	Low
X_5	Lubricant level	Low

[a] Maximum pressure set by punch specification.

Table II—Comparison of Tablet Responses

Response	Tablets from Production	Tablets from Experimental Lot (Formula at NDA Limits)
Disintegration time, min.	13.96	8.58
Hardness, kg.	3.80	5.40
Dissolution, % at 30 min.	35.18	67.16
Dissolution, % at 50 min.	60.00	82.02
Friability, %	0.14	2.065

Table III—Responses for Production Granulation Lubricated on Laboratory Scale (Only Levels of Disintegrant and Lubricant Vary)

Response	Production Levels for X_3 and X_5	NDA Limits for X_3 and X_5
Disintegration time, min.	16.04	10.12
Hardness, kg.	8.15	7.45
Dissolution, % dissolved at 30 min.	23.04	67.70
Dissolution, % dissolved at 50 min.	37.13	84.33
Friability, %	0.29	2.44

EXPERIMENTAL

The data utilized here are the same data discussed previously (1). The results of the 27 optimization experiments were analyzed according to the procedures outlined, and the predictions in this paper are based on the second-order regression equations generated[1]. Any additional measurements were carried out by the methods previously reported (1).

RESULTS AND DISCUSSION

The "grid search" program discussed previously (1) resulted in eight choices for an optimum formulation. Although they all contained ingredient levels unacceptable to the present very constrained problem, a trend was noted (Table I).

To test these predictions, it was decided to prepare a formulation with each variable set at its NDA limit in the direction specified by Table I. The method of preparation was identical to that for the 27 optimization experiments, *i.e.*, different from the production method. The initial interest was primarily in trends or qualitative results so that differences in batch size, equipment, lot numbers of material, and test methods were not of immediate concern.

The responses of primary interest for this formula as well as for a sample from production are shown in Table II. The dissolution response had indeed been improved by the changes.

For obvious reasons, as few changes as possible should be made in a production formula. The disintegrant level and the lubricant level, since they are added in the dry state at the end of the process, would be changed most easily.

[1] The predicting ability of individual equations is discussed in *Reference 1*.

Table IV—Computer-Predicted and Experimental Values for Tablet Responses

Response	Production Formula —Prepared on Laboratory Scale— Predicted	Experimental	Recommended Change —Prepared on Laboratory Scale— Predicted	Experimental
Disintegration time, min.	11.45	15.79	7.08	12.50
Hardness, kg.	5.97	5.00	7.30	5.48
Dissolution, % in 30 min.	37.80	26.34	66.26	64.99
Friability, %	0.91	0.46	1.06	1.07
Thickness, mm.	2.44	2.37	2.43	2.36
Porosity, ml./g.	0.0427	0.0418	0.0443	0.0440
Mean pore diameter, μ	0.7135	0.9926	0.6502	0.8488
Dissolution, % in 50 min.	57.18	47.51	82.96	82.57
Mean granule diameter, mm.	0.338	0.327	0.329	0.327
Weight uniformity, *RSD*, %	1.08	0.61	1.40	1.52
Thickness uniformity, *RSD*, %	0.78	0.42	0.79	0.59

Table V—Computer-Predicted and Experimental Values for Responses for Tablets Prepared in Production

Response	—Production Formula— Predicted	Prepared in Production	—Recommended Change— Predicted	—Prepared in Production— Batch 1	Batch 2
Disintegration time, min.	11.45	13.96	7.08	5.79	5.29
Hardness, kg.	5.97	3.80	7.30	6.60	6.42
Dissolution, % in 30 min.	37.80	35.18	66.26	72.30	72.68
Friability, %	0.91	0.14	1.06	0	0.076
Thickness, mm.	2.44	2.40	2.43	2.43	2.42
Porosity, ml./g.	0.0427	0.0878	0.0443	0.0827	0.0877
Mean pore diameter, μ	0.7135	0.9508	0.6502	0.7336	1.3518
Dissolution, % in 50 min.	57.18	60.00	82.96	89.18	87.48
Mean particle diameter[a], mm.	0.338	—	0.329	—	—
Weight uniformity, *RSD*, %	1.08	2.67	1.40	0.96	1.22
Thickness uniformity, *RSD*, %	0.78	1.18	0.79	0.71	0.58

[a] Samples not available for measurement.

Unlubricated granulation was obtained from the production area and lubricated with the dry "adds" at two levels: (*a*) at the production level and (*b*) with the disintegrant and lubricant levels changed to NDA limits. The responses for the two sets of tablets are shown in Table III. Since the production material responded in the same manner as the experimental material (*i.e.*, dissolution improved with the changes in lubricant and disintegrant), such changes were recommended, although of a lesser magnitude.

As a test, two formulations were prepared in the laboratory. One was prepared with the production formula and one with the recommended changes; the changes involved an increase in disintegrant of 0.4 mg./tablet and a decrease in lubricant level of 0.35 mg./tablet.

By using the appropriate coding in statistical units, the computer was asked to perform the same experiments. The results are shown in Table IV. It is important to note that the predicted change is *qualitatively* correct for each response listed. That is, the predicted change and the experimental change are always in the same direction. This in itself makes the optimization program useful. It *can* point in the direction of an improved formulation.

In addition, many computer predictions in the table are quantitatively matched by experimental results. This, of course, is the ultimate goal of the optimization technique and an ideal situation.

As a final test, since the recommended formula performed satisfactorily in the laboratory, a production sample was necessary. Table V represents predicted and experimental values for the responses for tablets prepared totally in production. These data were obtained using the testing procedures previously mentioned (1). For the improved formulation, the dissolution value obtained by the quality control procedure (increased rotation) is approximately 100%. The results were further substantiated by many subsequent production batches.

CONCLUSIONS

The optimization technique is useful in troubleshooting as well as in the development of a new formulation. Because the statistical design and the computerized calculations result in an understanding of variable interactions, more than one variable can be changed at a time with predictable results. Very small changes in ingredient levels result in significantly improved tablet properties, which are adequately reflected in the computer predictions.

It is unlikely that one would carry out an optimization study if faced with the specific problem presented here. An improved formulation would eventually have been found by trial and error; but, if the optimization information is available, its use allows the investigator to reduce the time necessary to accomplish this objective.

The point to be emphasized is that once the optimization data have been generated (usually during development), it can be of great utility in a troubleshooting situation.

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Performance of Tablet Breaking Strength Testers I: Intralaboratory Comparison and Prediction

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Abstract □ Two motorized and two manually powered instruments were used to measure the breaking strength of tablets of three different shapes, each of two sizes and each at three different target breaking strength values. The experimental results were statistically analyzed. The uniformity among breaking strength testers was compared and these results are presented. A high correlation among devices was observed, allowing for the derivation of regression equations which could be used to predict precisely the breaking strengths from one instrument to another. Manually operated instruments tended to yield lower and more variable values than those of their electrically powered counterparts. Tablet shape and size were also found to affect the uniformity and consistency of tablet breaking strengths.

Keyphrases □ Tablet breaking strength—comparison among six different tablets and four hardness testers, effect of tablet tester, tablet size and shape, and target breaking strength □ Instruments for testing tablet breaking strength—characterization, comparison, effect of tablet shape and size, regression equations to predict breaking strength values □ Breaking strength testers for tablets—comparison among six different tablets and four hardness testers

Resistance to breaking, more commonly referred to as hardness, is one of the most important parameters measured as indexes of a pharmaceutical tablet quality. Available to the development pharmacist is a wide and ever increasing variety of instruments[1] designed for measuring this property.

Frequently during the development of a pharmaceutical tablet formulation, the researcher, either out of personal preference or because of availability limitations, uses one instrument for making breaking strength measurements on the formulation he or she is developing. In the interest of consistency, this instrument is usually used throughout the development program. The assignment of specifications for the final product is then based on the specific tablet tester used. It has been observed that the breaking strength of the same product can be different when a tablet tester other than the one specified is used for measurement. Such occurrences frequently arise in multinational organizations where more than one manufacturing site exists. Presently, there are no known procedures available to the pharmacist for converting specifications based on one device to those of another.

A resolution of this problem was, in part, the purpose of the work described here. More specifically, an attempt was made to: (*a*) characterize a selected number of tablet breaking strength testers with respect to their uniformity, consistency, and average values for a number of selected target breaking strengths; (*b*) compare the testers with respect to their averages and variances; (*c*) study the effect of tablet shape and size on tablet breaking strength values associated with each tester; (*d*) study the combined effect of the three factors (tablet testers, shape and size, and target breaking strength) on values; and (*e*) estimate the correlation between breaking strength values associated with the tablet testers and develop regression equations to predict breaking strength values of one instrument from those of another.

[1] A partial list would include the Stokes (formerly Monsanto) tester (Stokes Compacting Division, Pennwalt Corp., Warminster, Pa.), the Heberlein tester (Cherry-Burell, Park Ridge, Ill.), the Ahiba tester (Gubelin International Corp., Mount Kisco, N. Y.), the Pfizer tester (Chas. Pfizer & Co., Brooklyn, N. Y.), the Strong-Cobb tester (Strong-Cobb Inc., Cleveland, Ohio), and the Erweka tester (Chemical and Pharmaceutical Industry Co., New York, N. Y.).

HISTORICAL

In marked contrast to the past, a large number of instruments for measuring tablet breaking strength has been made available recently to the pharmaceutical industry. It was not until the early 1930's that the first instrument for measuring tablet breaking strength, the Monsanto (now Stokes) tester, became available. Since, then, elaborate studies on tablet breaking strength and related equipment have been completed and reported. Nutter Smith (1–5) for example, studied and discussed conditions that influence the resistance of tablets to normal "wear and tear"; two devices, one each for testing the breaking point of tablets and effects of abrasion; and two instruments for measuring the surface hardness of tablets. None of the equipment Nutter Smith studied, however, other than the Stokes tester has received wide acceptance in the industry.

In the decade of 1940–1950, the Strong-Cobb testing device became available and continues to enjoy wide appeal. Subsequently, Fairchild and Michel (6) described and evaluated the Pfizer tablet tester and compared it against the Stokes tester for ease of operation, accuracy, and reliability. In 1961, Endicott *et al.* (7) reported on an instrument they developed and evaluated which produced values of tablet breaking strength expressed in terms of fracture resistance. De Lonca *et al.* (8) reported on work with two devices (the Dynestat and Erweka) for measuring tablet breaking strength, and in 1968 Brook and Marshall (9) pointed out that variations observed on four devices involved in their studies were partly due to inaccuracies in equipment mechanisms and emphasized the need for calibration when comparing the results of one machine to another. In none of this work, however, was there an attempt to relate values obtained on one instrument to those of another.

EXPERIMENTAL

Tablets evaluated in this study contained the following: hydrous dibasic calcium phosphate NF, 32%; lactose USP, 32%; microcrystalline cellulose NF, 32%; starch USP, 3.5%; and magnesium stearate USP, 0.5%. The formulation was manufactured in one batch large enough to conduct all experiments.

All tablets were compressed on a single-punch press[2] operated at a rate of 90 tablets/min. Tablets of three different shapes, each in two sizes at target breaking strengths of 4, 8, and 12 kg., were prepared. The tablet sizes and shapes selected for the study were: Tablet I, round, standard concave, 7.14 mm. (0.28 in.) in diameter; Tablet II, round, standard concave, 10.3 mm. (0.40 in.) in diameter; Tablet III, capsule shaped, deep concave, 3.97 × 9.52 mm. (0.15 × 0.37 in.); Tablet IV, capsule shaped, deep concave, 4.76 × 11.9 mm.

[2] Model E, Stokes Compacting Division, Pennwalt Corp., Warminster, Pa.

Table I—Comparison of Averages[a] of Tablet Breaking Strength Testers

Tablet	Target Hardness, kg.	Statistical Intercomparison[b] T-3	T-4	T-2	T-1
I	4	3.90 ± 0.10[c]	4.90 ± 0.12[a]	4.66 ± 0.11[a]	4.25 ± 0.10[b]
II	4	4.22 ± 0.08[c]	5.32 ± 0.10[a]	5.25 ± 0.10[a]	4.49 ± 0.09[b]
III	4	4.53 ± 0.18[c]	6.18 ± 0.25[a]	5.95 ± 0.24[a]	5.61 ± 0.23[a]
IV	4	4.95 ± 0.19[b]	6.38 ± 0.24[a]	5.86 ± 0.22[a]	5.99 ± 0.22[a]
V	4	3.91 ± 0.13[b]	5.20 ± 0.17[a]	5.14 ± 0.17[a]	5.76 ± 0.19[c]
VI	4	3.89 ± 0.11[c]	5.13 ± 0.15[a]	5.16 ± 0.15[a]	5.67 ± 0.17[b]
I	8	8.15 ± 0.10[a]	10.07 ± 0.13[b]	9.72 ± 0.12[b]	9.37 ± 0.12[d]
II	8	8.96 ± 0.12[c]	11.25 ± 0.15[b]	10.89 ± 0.15[b]	9.89 ± 0.13[a]
III	8	9.01 ± 0.24[b]	11.51 ± 0.30[c]	10.69 ± 0.28[a,c]	9.97 ± 0.26[a]
IV	8	8.86 ± 0.22[a]	12.00 ± 0.30[b]	10.88 ± 0.27[c]	9.65 ± 0.24[d]
V	8	8.80 ± 0.38[b]	11.31 ± 0.49[a]	9.99 ± 0.43[c]	11.33 ± 0.49[a,c]
VI	8	7.89 ± 0.11[c]	10.31 ± 0.14[a]	9.37 ± 0.13[b]	10.57 ± 0.15[a]
I	12	13.02 ± 0.16[c]	14.86 ± 0.18[a]	14.44 ± 0.18[a,b]	13.94 ± 0.17[b]
II	12	12.16 ± 0.19[a]	14.73 ± 0.24[b]	13.46 ± 0.22[c]	12.74 ± 0.20[d]
III	12	12.24 ± 0.29[a]	14.62 ± 0.35[b]	13.23 ± 0.31[c]	12.47 ± 0.30[a,c]
IV	12	12.64 ± 0.25[a]	15.30 ± 0.30[b]	13.93 ± 0.28[c]	11.59 ± 0.23[d]
V	12	12.54 ± 0.16[a]	15.15 ± 0.20[b]	13.11 ± 0.17[c]	15.88 ± 0.21[d]
VI	12	12.59 ± 0.20[a]	15.29 ± 0.24[b]	13.23 ± 0.21[c]	14.07 ± 0.22[d]

[a] The values are the geometric average and its standard error, respectively, based on Cochran's approximation (12). [b] Two testers' averages are not statistically different (at $p = 0.05$) if they have at least one superscript in common. Two testers' averages are statistically different (at $p < 0.05$) if they have different superscripts. Note: These intercomparisons in this section are designated with superscript letters.

(0.18 × 0.46 in.); Tablet V, oval, deep concave, 5.56 × 10.3 mm. (0.21 × 0.40 in.); and Tablet VI, oval, deep concave, 7.14 × 12.7 mm. (0.28 × 0.50 in.).

The following four testers were evaluated: T-1, a manually powered, spring-loaded tester[3]; T-2, a motorized tester[4] whose function is derived from a counterweight principle; T-3, a manually operated tester[4] whose function is derived from a counterweight principle; and T-4, a motorized, spring-loaded tester[5].

To achieve the target breaking strengths desired, an arbitrary tablet weight for each size and shape was selected. This weight of powder mix was introduced into the corresponding die and pressure was exerted onto the mass. The resulting tablet was then measured for breaking strength on tester T-3. If the target value was not achieved, a new charge of powder mix of the same weight was introduced into the die and a different pressure was exerted. The resulting tablet was again measured for breaking strength. This procedure was repeated, if necessary, until the correct pressure to achieve the desired tablet breaking strength was reached. Once the correct set of machine adjustments was obtained, 500 tablets were compressed, keeping tablet weights ±4% of target and tablet thicknesses ±1% of target. When this operation was completed, a new size or shape was chosen and the same procedure was repeated until all of the different sizes, shapes, and target breaking strengths were manufactured.

The design protocol called for obtaining at random, for each shape and size, samples of four tablets each for testing on the four testers (involving random assignment of tablets to testers). The four breaking strength values for each occasion were determined concurrently. This procedure was repeated sequentially until 10 such samples of four were collected at each of the target values of 4, 8, and 12 kg. Only one operator was involved in the completion of all operations.

DISCUSSION

The discussion presented here is based upon the results of the statistical analysis of the 720 breaking strength values obtained from the randomized design of experiment described in the previous section. To compare the breaking strength values (Table I) generated by the testers in this study, both the ANOVA and Duncan's multiple range test (10) were used. The Levene (11) test of homogeneity of average absolute mean deviations (Table II) was employed for comparing the variability of instruments. The results of the regression analysis are presented in Table III; linear regressions were the best fit for the data and polynomial regressions were tried but did not contribute substantially to the fit.

[3] Stokes tester.
[4] Heberlein tester.
[5] Ahiba tester.

Using known breaking strength values experimentally obtained, one can now predict the breaking strength value from one tester to another using the following equation:

$$Y = A + BX_0 \qquad \text{(Eq. 1)}$$

where Y is the predicted breaking strength value, A and B are the numerical coefficients from Table III, and X_0 is the experimentally obtained breaking strength value associated with a given tester.

For instance, if one wishes to predict breaking strength values for T-4 from an experimentally obtained breaking strength value associated with T-3, Eq. 1 has the following form:

$$\text{(T-4)} = A + B\text{(T-3)} \qquad \text{(Eq. 2)}$$

The 95% confidence limits of the predicted breaking strength value can be computed by inserting the appropriate X_0 value into the formula given in the last column of Table III. The explicit expression of the confidence limits formula for the predicted values

Table II—Comparison of Variability[a] (Uniformity) among the Testers

Tablet	Target Hardness, kg.	Statistical Intercomparison[b] T-3	T-4	T-2	T-1
I	4	0.12[b]	0.16[b,c]	0.30[a,c]	0.42[a]
II	4	0.23[a]	0.19[a]	0.15[a]	0.15[a]
III	4	0.39[b]	0.50[b]	0.48[b]	0.81[a]
IV	4	0.42[a]	0.38[a]	0.42[a]	0.70[a]
V	4	0.25[a]	0.65[b]	0.25[a]	0.42[a,b]
VI	4	0.22[b]	0.38[a,b]	0.37[a,b]	0.51[a]
I	8	0.23[a]	0.29[a]	0.33[a]	0.37[a]
II	8	0.33[a]	0.25[a]	0.32[a]	0.36[a]
III	8	0.42[b]	0.43[b]	0.45[b]	1.20[a]
IV	8	0.75[a]	0.62[a]	0.51[a]	0.54[a]
V	8	1.02[a]	1.07[a]	1.50[a]	1.02[a]
VI	8	0.33[a]	0.41[a]	0.17[a]	0.27[a]
I	12	0.13[a]	0.42[b]	0.56[b]	0.51[b]
II	12	0.47[a]	0.60[a]	0.47[a]	0.45[a]
III	12	0.67[a]	0.82[a]	0.55[a]	0.97[a]
IV	12	0.40[a]	0.82[a]	0.69[a]	0.75[a]
V	12	0.40[a,b]	0.25[a]	0.45[a,b]	0.63[b]
VI	12	0.35[a]	0.39[a]	0.55[a,b]	0.78[b]

[a] The values are the average absolute mean deviations based on 10 observations. [b] Two testers' average absolute mean deviations are not statistically different (at $p = 0.05$) if they have at least one superscript in common. Two testers' average absolute mean deviations are statistically different (at $p < 0.05$) if they have different superscripts. Note: These intercomparisons in this section are designated with superscript letters.

Table III—Regression Equation, Prediction Efficiency (RSQ, %), and Formula for 95% Confidence Limits of Predicted Values

Tablet	(X)	(Y)	A	B	RSQ, %[a]	Formula for[b] 95% Confidence Limits
I	T-3	T-2	0.718	1.065	97.8	$\pm[0.053990 + 0.003873 (X_0 - 8.35833)^2]^{1/2}$
	T-3	T-4	0.812	1.087	98.4	$\pm[0.039932 + 0.002865 (X_0 - 8.35833)^2]^{1/2}$
	T-3	T-1	0.403	1.052	97.4	$\pm[0.062581 + 0.004489 (X_0 - 8.35833)^2]^{1/2}$
	T-2	T-4	0.245	1.009	98.3	$\pm[0.043070 + 0.002665 (X_0 - 9.61667)^2]^{1/2}$
	T-2	T-1	−0.195	0.977	97.3	$\pm[0.065084 + 0.004027 (X_0 - 9.61667)^2]^{1/2}$
	T-4	T-1	−0.338	0.959	97.0	$\pm[0.070593 + 0.004215 (X_0 - 9.95000)^2]^{1/2}$
	T-2	T-3	−0.473	0.918	97.8	$\pm[0.046570 + 0.002882 (X_0 - 9.61667)^2]^{1/2}$
	T-4	T-3	−0.647	0.905	98.4	$\pm[0.033239 + 0.001985 (X_0 - 9.95000)^2]^{1/2}$
	T-1	T-3	−0.153	0.925	97.4	$\pm[0.055006 + 0.003468 (X_0 - 9.20000)^2]^{1/2}$
	T-4	T-2	−0.073	0.974	98.3	$\pm[0.041563 + 0.002482 (X_0 - 9.95000)^2]^{1/2}$
	T-1	T-2	0.458	0.996	97.3	$\pm[0.066321 + 0.004182 (X_0 - 9.20000)^2]^{1/2}$
	T-1	T-4	0.638	1.012	97.0	$\pm[0.074544 + 0.004700 (X_0 - 9.20000)^2]^{1/2}$
II	T-3	T-2	1.168	1.029	96.0	$\pm[0.071829 + 0.006600 (X_0 - 8.45833)^2]^{1/2}$
	T-3	T-4	0.562	1.168	96.8	$\pm[0.074404 + 0.006836 (X_0 - 8.45833)^2]^{1/2}$
	T-3	T-1	0.320	1.032	97.2	$\pm[0.049175 + 0.004518 (X_0 - 8.45833)^2]^{1/2}$
	T-2	T-4	−0.521	1.110	96.5	$\pm[0.081118 + 0.006753 (X_0 - 9.87500)^2]^{1/2}$
	T-2	T-1	−0.589	0.976	96.0	$\pm[0.071472 + 0.005950 (X_0 - 9.87500)^2]^{1/2}$
	T-4	T-1	−0.032	0.870	97.4	$\pm[0.046970 + 0.003061 (X_0 - 10.44170)^2]^{1/2}$
	T-2	T-3	−0.752	0.933	96.0	$\pm[0.065085 + 0.005419 (X_0 - 9.875)^2]^{1/2}$
	T-4	T-3	−0.192	0.828	96.8	$\pm[0.052774 + 0.003439 (X_0 - 10.44170)^2]^{1/2}$
	T-1	T-3	−0.069	0.942	97.2	$\pm[0.044891 + 0.003765 (X_0 - 9.05000)^2]^{1/2}$
	T-4	T-2	0.801	0.869	96.5	$\pm[0.063498 + 0.004138 (X_0 - 10.44170)^2]^{1/2}$
	T-1	T-2	0.975	0.983	96.0	$\pm[0.072005 + 0.006039 (X_0 - 9.05000)^2]^{1/2}$
	T-1	T-4	0.311	1.119	97.4	$\pm[0.060452 + 0.005070 (X_0 - 9.05000)^2]^{1/2}$
III	T-3	T-2	2.075	0.917	92.7	$\pm[0.102592 + 0.009850 (X_0 - 8.61667)^2]^{1/2}$
	T-3	T-4	1.685	1.057	91.7	$\pm[0.157331 + 0.015105 (X_0 - 8.61667)^2]^{1/2}$
	T-3	T-1	2.098	0.850	81.7	$\pm[0.251962 + 0.024191 (X_0 - 8.61667)^2]^{1/2}$
	T-2	T-4	−0.337	1.116	92.6	$\pm[0.140218 + 0.014855 (X_0 - 9.97500)^2]^{1/2}$
	T-2	T-1	0.490	0.896	82.2	$\pm[0.245761 + 0.026037 (X_0 - 9.97500)^2]^{1/2}$
	T-4	T-1	1.166	0.765	80.6	$\pm[0.267204 + 0.021067 (X_0 - 10.79170)^2]^{1/2}$
	T-2	T-3	−1.474	1.012	92.7	$\pm[0.113207 + 0.011994 (X_0 - 9.97500)^2]^{1/2}$
	T-4	T-3	−0.749	0.868	91.7	$\pm[0.129197 + 0.010186 (X_0 - 10.79170)^2]^{1/2}$
	T-1	T-3	−0.444	0.961	81.7	$\pm[0.284846 + 0.030917 (X_0 - 9.42500)^2]^{1/2}$
	T-4	T-2	1.016	0.830	92.6	$\pm[0.104348 + 0.008227 (X_0 - 10.79170)^2]^{1/2}$
	T-1	T-2	1.326	0.918	82.2	$\pm[0.251785 + 0.027329 (X_0 - 9.42500)^2]^{1/2}$
	T-1	T-4	0.861	1.054	80.6	$\pm[0.367857 + 0.039928 (X_0 - 9.42500)^2]^{1/2}$
IV	T-3	T-2	1.387	1.002	89.0	$\pm[0.190114 + 0.018582 (X_0 - 8.84167)^2]^{1/2}$
	T-3	T-4	1.470	1.106	88.3	$\pm[0.248939 + 0.024331 (X_0 - 8.84167)^2]^{1/2}$
	T-3	T-1	3.059	0.686	80.2	$\pm[0.177767 + 0.017375 (X_0 - 8.84167)^2]^{1/2}$
	T-2	T-4	0.098	1.089	96.4	$\pm[0.075905 + 0.006583 (X_0 - 10.24170)^2]^{1/2}$
	T-2	T-1	2.302	0.666	85.3	$\pm[0.132575 + 0.011497 (X_0 - 10.24170)^2]^{1/2}$
	T-4	T-1	2.445	0.594	83.3	$\pm[0.150480 + 0.010613 (X_0 - 11.25000)^2]^{1/2}$
	T-2	T-3	−0.259	0.889	89.0	$\pm[0.168681 + 0.014628 (X_0 - 10.24170)^2]^{1/2}$
	T-4	T-3	−0.137	0.798	88.3	$\pm[0.179625 + 0.012668 (X_0 - 11.25000)^2]^{1/2}$
	T-1	T-3	−1.829	1.169	80.2	$\pm[0.302970 + 0.050469 (X_0 - 9.12500)^2]^{1/2}$
	T-4	T-2	0.279	0.886	96.4	$\pm[0.061730 + 0.004354 (X_0 - 11.25000)^2]^{1/2}$
	T-1	T-2	−1.436	1.280	85.3	$\pm[0.254659 + 0.042421 (X_0 - 9.12500)^2]^{1/2}$
	T-1	T-4	−1.547	1.402	83.3	$\pm[0.355431 + 0.059208 (X_0 - 9.12500)^2]^{1/2}$
V	T-3	T-2	1.874	0.899	89.2	$\pm[0.190797 + 0.014619 (X_0 - 8.45000)^2]^{1/2}$
	T-3	T-4	1.382	1.090	89.4	$\pm[0.275469 + 0.021106 (X_0 - 8.45000)^2]^{1/2}$
	T-3	T-1	1.414	1.137	94.0	$\pm[0.162287 + 0.012434 (X_0 - 8.45000)^2]^{1/2}$
	T-2	T-4	0.105	1.108	83.6	$\pm[0.426812 + 0.036136 (X_0 - 9.46667)^2]^{1/2}$
	T-2	T-1	0.076	1.157	87.9	$\pm[0.324653 + 0.027486 (X_0 - 9.46667)^2]^{1/2}$
	T-4	T-1	0.902	0.956	88.2	$\pm[0.318191 + 0.018346 (X_0 - 10.59170)^2]^{1/2}$
	T-2	T-3	−0.949	0.993	89.2	$\pm[0.210832 + 0.017850 (X_0 - 9.46667)^2]^{1/2}$
	T-4	T-3	−0.237	0.820	89.4	$\pm[0.207299 + 0.011952 (X_0 - 10.59170)^2]^{1/2}$
	T-1	T-3	−0.659	0.826	94.0	$\pm[0.117882 + 0.006561 (X_0 - 11.02500)^2]^{1/2}$
	T-4	T-2	1.476	0.754	83.6	$\pm[0.290667 + 0.016759 (X_0 - 10.59170)^2]^{1/2}$
	T-1	T-2	1.084	0.760	87.9	$\pm[0.213412 + 0.011877 (X_0 - 11.02500)^2]^{1/2}$
	T-1	T-4	0.420	0.923	88.2	$\pm[0.307132 + 0.017093 (X_0 - 11.02500)^2]^{1/2}$
VI	T-3	T-2	1.794	0.919	97.3	$\pm[0.045277 + 0.003546 (X_0 - 8.13333)^2]^{1/2}$
	T-3	T-4	0.870	1.154	97.9	$\pm[0.055393 + 0.004338 (X_0 - 8.13333)^2]^{1/2}$
	T-3	T-1	2.406	0.949	93.3	$\pm[0.123906 + 0.009703 (X_0 - 8.13333)^2]^{1/2}$
	T-2	T-4	−1.132	1.229	96.3	$\pm[0.095999 + 0.008662 (X_0 - 9.26667)^2]^{1/2}$
	T-2	T-1	0.619	1.026	94.6	$\pm[0.099812 + 0.009006 (X_0 - 9.26667)^2]^{1/2}$
	T-4	T-1	1.674	0.824	95.7	$\pm[0.079378 + 0.004566 (X_0 - 10.25830)^2]^{1/2}$
	T-2	T-3	−1.677	1.059	97.3	$\pm[0.052167 + 0.004707 (X_0 - 9.26667)^2]^{1/2}$
	T-4	T-3	−0.564	0.848	97.9	$\pm[0.040687 + 0.002340 (X_0 - 10.25830)^2]^{1/2}$
	T-1	T-3	−1.820	0.983	93.3	$\pm[0.128344 + 0.010411 (X_0 - 10.12500)^2]^{1/2}$
	T-4	T-2	1.228	0.784	96.3	$\pm[0.061199 + 0.003520 (X_0 - 10.25830)^2]^{1/2}$
	T-1	T-2	−0.070	0.922	94.6	$\pm[0.089731 + 0.007279 (X_0 - 10.12500)^2]^{1/2}$
	T-1	T-4	−1.504	1.162	95.7	$+[0.111941 + 0.009080 (X_0 - 10.12500)^2]^{1/2}$

[a,b] Formulas for calculating RSQ(%) and 95% confidence limits can be found in the text.

Table IV—Comparison of Averages[a] of Size-Shape Groups[b]

Target Hardness, kg.	Device	Tablet I	Tablet II	Tablet III	Tablet IV	Tablet V	Tablet VI
4	T-3	3.9[a]	4.22[b]	4.53[b]	4.95[c]	3.91[a]	3.89[a]
4	T-2	4.66[a]	5.25[b]	5.95[c]	5.86[c]	5.14[b]	5.16[b]
4	T-4	4.90[a]	5.32[a]	6.18[b]	6.38[b]	5.20[a]	5.13[a]
4	T-1	4.25[a]	4.49[a]	5.61[b]	5.99[b]	5.76[b]	5.67[b]
8	T-3	8.15[a]	8.96[b]	9.01[b]	8.86[b]	8.80[b]	7.89[a]
8	T-2	9.72[a]	10.89[c]	10.69[b,c]	10.88[c]	9.99[a,b]	9.37[a]
8	T-4	10.07[a]	11.25[b]	11.51[b,c]	12.00[c]	11.31[b,c]	10.31[a]
8	T-1	9.37[a]	9.89[a,b]	9.97[a,b]	9.65[a]	11.33[c]	10.57[b,c]
12	T-3	13.02[b]	12.16[a]	12.24[a]	12.64[a,b]	12.54[a,b]	12.59[a,b]
12	T-2	14.44[c]	13.46[a,b]	13.23[a]	13.93[b,c]	13.11[a]	13.23[a]
12	T-4	14.86[a]	14.73[a]	14.62[a]	15.30[a]	15.15[a]	15.29[a]
12	T-1	13.94[c]	12.74[b]	12.47[b]	11.59[a]	15.88[d]	14.07[c]

[a] The values are the geometric averages based on 10 observations. [b] Two groups are not statistically different ($p = 0.05$) if they have at least one superscript in common. Two groups are statistically different ($p < 0.05$) if they have different superscripts. Note: These intercomparisons in this section are designated with superscript letters.

has the following form (10):

$$\hat{Y}_0 \pm t_{0.05} S_{yx} \sqrt{\frac{1}{n} + \frac{(X_0 - \bar{X})^2}{[X^2]}} \qquad \text{(Eq. 3)}$$

Comparisons of the averages and variabilities of breaking strengths among size-shape groups were also made and are presented in Tables IV and V, respectively.

The results given in Table I indicate that, in most cases, the two motorized testers yielded breaking strength values significantly higher than those of the manually operated instruments. T-4 generally gave higher readings than T-2, and these differences were significant for the five cases in the 12-kg. group. Breaking strength values generated with T-3 were significantly lower than those of the other three testers in 17 of the 18 possible cases. In the 12-kg. group, all devices were significantly different from one another in four out of six cases; that is, the two manually operated and the two motorized testers were significantly different from each other in this group.

With the data in Table IV, it is possible to make 15 comparisons derived from the combination of six categories taken two at a time within any given row. Such an exercise reveals that there are significant differences among many size-shape groups. The two sizes associated with the round shape (Tablets I and II) had significantly different breaking strength values in eight of the 12 possible cases. This result is somewhat surprising and unexplainable. Size made a difference in three out of 12 comparisons in each of the capsule-shaped (Tablets III and IV) and oval-shaped (Tablets V and VI) groups, but the effect of size was not pronounced in the capsule- and oval-shaped groups for the target values of 8 and 4 kg., respectively.

An analysis of the data presented in Table II shows that T-1 maintained a higher level of variability than the other testers in more than half of the possible cases. However, there was no significant difference in variability when all testers were compared in 10 out of 18 cases. Furthermore, there was no significant difference among T-3, T-4, and T-2 as far as their variabilities in 16 out of 18 cases.

Table V reveals that, except for the oval shape (Tablets V and VI) at the 8-kg. target, there were significant differences in variability related to a size-shape grouping in only two cases.

The use of the equation for predicting breaking strength values from one instrument to another is evident from Table III. The correlation between any two testers under consideration can be obtained by taking the square root of the RSQ value [RSQ(%)/100] noted in Table III. RSQ(%) is calculated as follows:

$$\text{RSQ}(\%) = \frac{B^2[X^2]100}{[Y^2]} \qquad \text{(Eq. 4)}$$

where B is the linear regression coefficient, $[X^2]$ is the corrected sum of squares for X's, and $[Y^2]$ is the corrected sum of squares for Y's.

The values of the correlation coefficients ranged from 0.90 to 0.99 in this study (the theoretical range here is from 0.0 to 1.0). One must be aware, however, that accurate predictions can be obtained only if: (*a*) the X_0 value is the average of exactly 10 breaking strength values experimentally derived, (*b*) the X_0 value lies between 3.0 and 15.75 kg., and (*c*) the experimental conditions are as close to those described in this paper as possible.

CONCLUSION

The characterization and comparison of tablet breaking strength

Table V—Variability[a] Comparison of Size-Shape Groups[b]

Target Hardness, kg.	Device	Tablet I	Tablet II	Tablet III	Tablet IV	Tablet V	Tablet VI
4	T-3	0.12[a]	0.23[a,b]	0.39[b,c]	0.42[c]	0.26[a,b,c]	0.22[a,b]
4	T-2	0.30[a,b,c]	0.15[a]	0.48[c]	0.42[b,c]	0.25[a,b]	0.37[b,c]
4	T-4	0.16[a]	0.19[a]	0.50[b,c]	0.38[a,b,c]	0.65[c]	0.38[a,b]
4	T-1	0.42[a,b]	0.15[a]	0.81[b]	0.70[b]	0.42[a,b]	0.51[a,b]
8	T-3	0.23[a]	0.33[a]	0.42[a,b]	0.75[b,c]	1.02[c]	0.33[a]
8	T-2	0.33[a]	0.32[a]	0.45[a]	0.51[a]	1.50[b]	0.17[a]
8	T-4	0.29[a]	0.25[a]	0.43[a]	0.62[a]	1.07[b]	0.41[a]
8	T-1	0.37[a]	0.36[a]	1.20[b]	0.54[a]	1.02[b]	0.27[a]
12	T-3	0.13[a]	0.47[b,c]	0.67[c]	0.40[a,b,c]	0.40[a,b,c]	0.35[a,b]
12	T-2	0.56[a]	0.47[a]	0.55[a]	0.69[a]	0.45[a]	0.55[a]
12	T-4	0.42[a]	0.60[a,b]	0.82[b]	0.82[b]	0.25[a]	0.39[a]
12	T-1	0.51[a]	0.45[a]	0.97[a]	0.75[a]	0.63[a]	0.78[a]

[a] The values are the average absolute mean deviations based on 10 observations. [b] Two groups are not statistically different ($p = 0.05$) if they have at least one superscript in common. Two groups are statistically different ($p < 0.05$) if they have different superscripts. Note: These intercomparisons in this section are designated with superscript letters.

testers offered considerable insight into the performance capability of the instruments studied. The characterization information is fundamental to any developmental work in this area. The study also revealed a sufficiently high correlation among the tablet testers to permit precise prediction of breaking strength values. It is possible now to expand the study to evaluate breaking strength testers of the same kind in interlaboratory crossover experiments.

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Semiautomated UV Analysis of Caffeine in Aspirin–Phenacetin–Caffeine Tablets

MACK W. OVERTON▲, LARRY L. ALBER, and RAYMOND S. VALENTINE

Abstract □ A semiautomated individual tablet assay was developed for caffeine in aspirin–phenacetin–caffeine formulations. Caffeine and phenacetin are extracted from the bicarbonate tablet solution with chloroform. The caffeine and some phenacetin are extracted from the chloroform with acid, and an additional chloroform wash of the acid phase removes any phenacetin. The caffeine is determined by UV spectroscopy in the acid at 266 nm. at a rate of 20 tablets/hr. The coefficient of variation for 10 determinations of one sample solution was 1.72. The difference in results between the NF XIII method and the proposed method, expressed as percent of declared, did not exceed 2.6% when 10 different products were analyzed.

Keyphrases □ Aspirin–phenacetin–caffeine tablets—semiautomated UV analysis of caffeine □ Caffeine in aspirin–phenacetin–caffeine tablets—semiautomated UV analysis □ UV spectrophotometry—analysis, caffeine in aspirin–phenacetin–caffeine tablets

Aspirin, phenacetin, and caffeine in pharmaceutical preparations are determined quantitatively by the official NF procedure (1). Since the monograph employs a partition chromatographic separation on a diatomaceous earth[1] column (2), the content uniformity requirement for caffeine in the tablets is extremely time consuming.

An automated method for caffeine in blood converts the caffeine to an aromatic amine, with subsequent diazotization for color determination (3). This procedure could not be used in the presence of phenacetin.

Caffeine has been reported to fluoresce in chloroform, where the excitation at 280 nm. causes emission that can be measured at 310 nm.[2]. With filter fluorometer equipment normally applied to an automated analyzer[3], excitation energy from the source lamp is rather weak at 280 nm. In addition, the fluorescence is not strong and linearity may be difficult to achieve.

In the method reported here, the caffeine and phenacetin are extracted from the bicarbonate solution with chloroform. The caffeine is extracted from the chloroform with dilute sulfuric acid, which is washed with chloroform to remove any phenacetin from the acid phase. The caffeine is determined by measuring its UV absorbance at 266 nm.

EXPERIMENTAL

Apparatus—The following were used: Liquid sampler II[4] (20/hr.), proportioning pump[4] I, and a spectrophotometer[5] equipped with a 10-mm. flow cell[6].

Reagents—The following were used: 4 *N* sulfuric acid; 0.1 *M* $NaHCO_3$; and chloroform, reagent grade, free of UV-absorbing impurities.

Standard Preparation—Place 82 mg. of caffeine in a 250-ml. volumetric flask with 25 ml. of 0.1 *M* $NaHCO_3$, and heat on a steam bath 4–5 min. to dissolve. Cool and dilute to volume with 0.1 *M* $NaHCO_3$. The concentration of the standard solution is 0.328 mg./ml.

Sample Preparation—For tablets declared to contain 30 mg.

[1] Celite, Johns-Manville Corp., New York, N. Y.

[2] A. Gillespie, Food and Drug Administration, Detroit, Mich., Dec. 1969, personal communication.

[3] Technicon AutoAnalyzer, Technicon, Tarrytown, N. Y.

[4] Technicon, Tarrytown, N. Y.

[5] Beckman DK 2A, Beckman Instruments, Fullerton, Calif.

[6] A. H. Thomas Co., No. 9120-NO 5, Philadelphia, Pa.

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In the method reported here, the caffeine and phenacetin are extracted from the bicarbonate solution with chloroform. The caffeine is extracted from the chloroform with dilute sulfuric acid, which is washed with chloroform to remove any phenacetin from the acid phase. The caffeine is determined by measuring its UV absorbance at 266 nm.

EXPERIMENTAL

Apparatus—The following were used: Liquid sampler II[4] (20/hr.), proportioning pump[4] I, and a spectrophotometer[5] equipped with a 10-mm. flow cell[6].

Reagents—The following were used: 4 *N* sulfuric acid; 0.1 *M* $NaHCO_3$; and chloroform, reagent grade, free of UV-absorbing impurities.

Standard Preparation—Place 82 mg. of caffeine in a 250-ml. volumetric flask with 25 ml. of 0.1 *M* $NaHCO_3$, and heat on a steam bath 4–5 min. to dissolve. Cool and dilute to volume with 0.1 *M* $NaHCO_3$. The concentration of the standard solution is 0.328 mg./ml.

Sample Preparation—For tablets declared to contain 30 mg.

[1] Celite, Johns-Manville Corp., New York, N. Y.

[2] A. Gillespie, Food and Drug Administration, Detroit, Mich., Dec. 1969, personal communication.

[3] Technicon AutoAnalyzer, Technicon, Tarrytown, N. Y.

[4] Technicon, Tarrytown, N. Y.

[5] Beckman DK 2A, Beckman Instruments, Fullerton, Calif.

[6] A. H. Thomas Co., No. 9120-NO 5, Philadelphia, Pa.

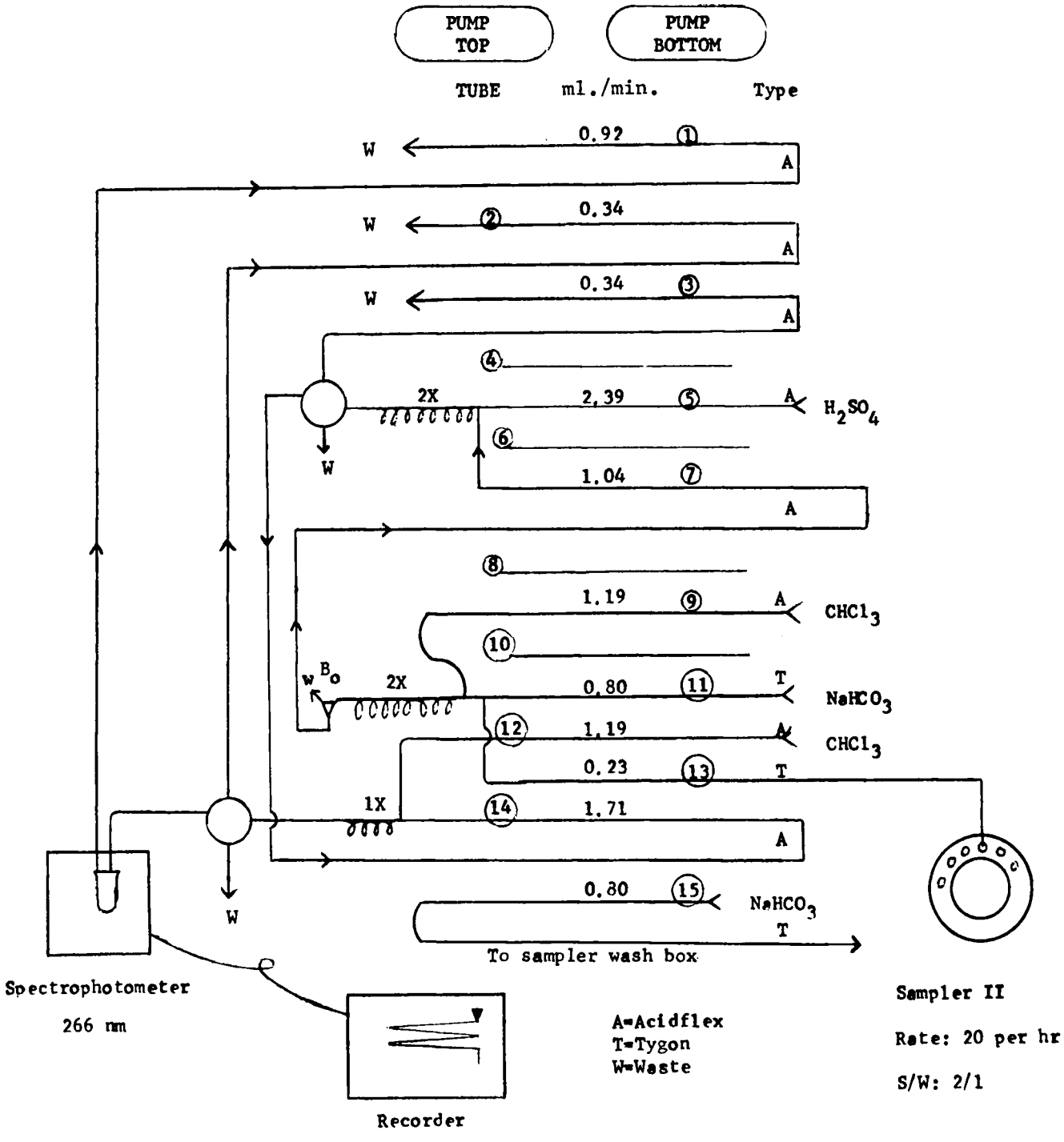

Figure 1—*Automated analyzer flow diagram for caffeine in aspirin–plenacetin–caffeine tablets.*

(1/2 gr.) caffeine, place them separately in a series of 100-ml. volumetric flasks. Add 25 ml. of 0.1 *M* $NaHCO_3$, heat on the steam bath 4–5 min., cool, dilute to volume with 0.1 *M* $NaHCO_3$, and mix (much of the phenacetin will not dissolve, even in warm 0.1 *M* $NaHCO_3$). For tablets declared to contain 15 mg. (1/4 gr.) caffeine, place them separately in a series of containers and add 25.0 ml. of 0.1 *M* $NaHCO_3$ from an automatic delivery pipet. Heat 4–5 min., cool, add another 25.0 ml. of 0.1 *M* $NaHCO_3$, and mix. Filter all tablet solutions through filter paper before placing in sample cups.

Automated Sample Assay—The flow system and the manifold settings are shown in Fig. 1. Coils labeled 1X are 14 turns, 2 mm. i.d.; those labeled 2X are 28 turns, 2 mm. i.d.

Special Connector for Automated System—To eliminate gas bubbles which formed at the interface of two immiscible liquids in the automated system, a special connector was developed (Fig. 2). The two immiscible liquids are pumped into the connector where the heavier liquid settles to the bottom (D) and is pushed to waste by the excess liquid being pumped in. Some less dense liquid is pumped out the top (B) through the pump to waste, along with any gas released from the interface. This eliminates air bubbles from the flow cell compartment. In addition, if any droplets of the denser liquid are attached to the gas bubble, it falls through D to waste. The liquid, free of gas bubbles, goes out the connector at C.

RESULTS AND DISCUSSION

A warm bicarbonate solution was selected for tablet preparation because caffeine dissolved completely, while much of the phenacetin

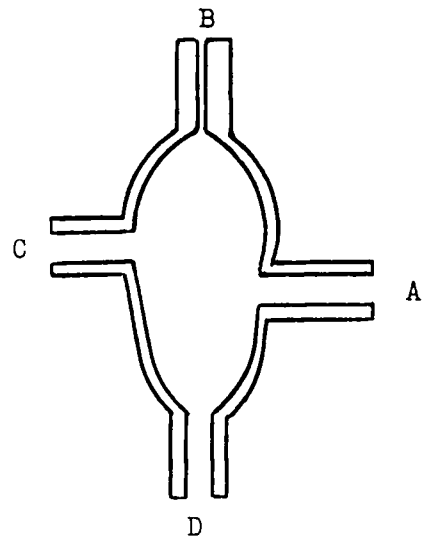

Figure 2—*Special connector for automated analyzer system. Scale, 2 mm. = 1 mm. A, C, and D = 2 mm. i.d., 4 mm. o.d. B = 0.5 mm. i.d., 4 mm. o.d.*

remained at the bottom of the flask. This greatly reduced the interference of phenacetin in the automated UV procedure. Heating on the steam bath seemed to effect solution more completely than did the sonic vibrator.

During this study, the tubing employed in the automated system seemed particularly subject to fatigue and failed after approximately

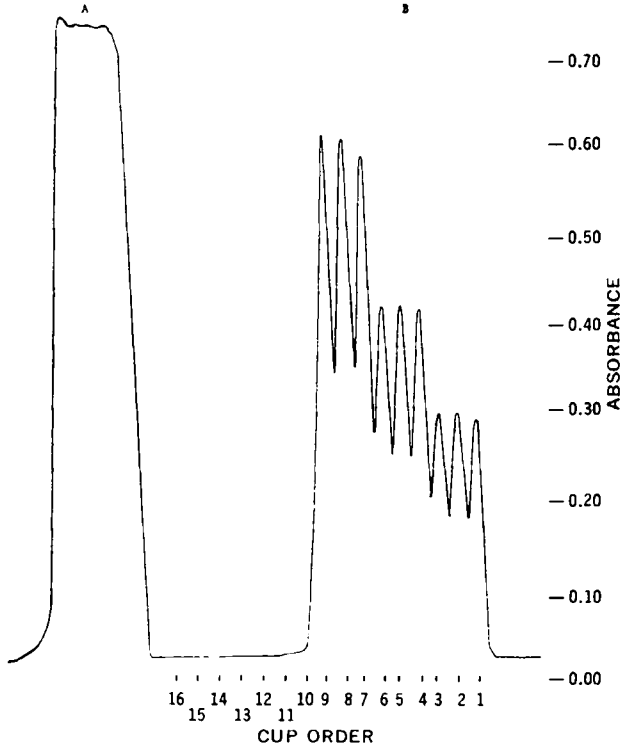

Figure 3—*Automated analyzer recordings of: (A) steady state of caffeine, 0.582 mg./ml.; and (B) linearity for caffeine at 266 nm. at 20 cups/hr.*

Cups	*Contents*
1, 2, 3	*caffeine, 0.2910 mg./ml.*
4, 5, 6	*caffeine, 0.4074 mg./ml.*
7, 8, 9	*caffeine, 0.5820 mg./ml.*
10, 11, 12	*$NaHCO_3$, 0.1 M*
13, 14	*aspirin, 2.27 mg./ml.*
15, 16	*phenacetin, 1.62 mg./ml.*

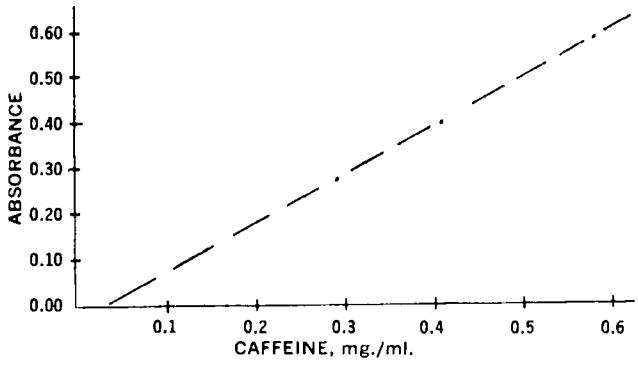

Figure 4—*Automated analyzer linearity of caffeine at 266 nm.*

30–40 hr. of use. This is most readily detected by running at steady state on a daily basis.

Figure 3 demonstrates the automated analyzer response for caffeine, phenacetin, and aspirin standards. Figure 4 shows the linearity of the caffeine in the range investigated. The lack of a zero intercept, however, indicates that some error could be introduced if sample and standard absorbances were significantly different.

The precision of the procedure was determined by analyzing one

Table I—Standard Deviation of One Solution of Aspirin–Phenacetin–Caffeine

Cup Order	Contents	Net Absorbance	Milligrams per Tablet Found	Percent of Declared
1	Standard	—[a]	—	—
2	Standard	—[a]	—	—
3	Standard	0.384	—	—
4	Sample	0.378	32.4	100.0
5	Sample	0.379	32.4	100.0
6	Sample	0.385	33.0	101.8
7	Sample	0.363	31.0	95.8
8	Sample	0.384	32.9	101.6
9	Standard	0.383	—	—
10	Sample	0.381	32.7	100.8
11	Sample	0.384	32.8	101.4
12	Sample	0.383	32.8	101.4
13	Sample	0.381	32.6	100.6
14	Sample	0.382	32.7	101.0
15	Standard	0.384	—	—
16	Standard	—[a]	—	—
Average (10)			32.5	100.4
SD		0.009		
CV		1.720		

[a] The absorbances of the first two cups and the last cup are not used in the calculations.

Table II—Comparison of Official and Automated Procedures for Caffeine in Aspirin–Phenacetin–Caffeine Tablets

Product	Composite Results, Percent of Declared: NF XIII	Composite Results, Percent of Declared: Automated[a]	Composite Results, Percent of Declared: Difference	ITA[b] by Automated, Average (10)	Coefficient of Variation
A	101.7	99.6	−2.10	97.3	3.399
B	97.0	97.5	+0.50	98.8	2.738
C	99.5	100.6	+1.10	98.2	2.612
D	102.8	100.7	−2.10	102.5	4.434
E	98.0	100.6	+2.60	100.6	1.991
F	98.3	96.3	−2.00	98.0	1.727
G	96.0	98.5	+2.50	98.3	3.420
H	99.2	97.4	−1.80	97.5	3.295
I	100.1	98.1	−2.10	99.9	3.793
J	98.0	98.3	+0.30	101.6	5.213

[a] Technicon AutoAnalyzer assay. [b] Individual tablet analysis.

composite solution in 10 separate cups. The usual sequence used in this laboratory is as follows: three standards, five tablets, standard, five tablets, *etc.*, composite, and two standards. The composite is a weighed portion of 20 tablets ground to fine powder, equivalent to one tablet weight. The coefficient of variation for 10 determinations of one composite solution was 1.72 (Table I).

The accuracy of the procedure was studied by comparing the automated assay with the NF column method on 10 different product composites. Both analyses were made on the same mixed powdered composites. The results of the automated method were in general agreement with those by the NF procedure (Table II). The deviation, expressed as percent of declared, was less than ±2.6%.

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(2) J. Levine, *J. Pharm. Sci.*, **52**, 1015(1963).

(3) H. M. Ederma, "Advances in Automated Analysis," vol. II, Technicon International Congress 1969, Mediad Inc., New York, N. Y., 1970, pp. 179–181.

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▲ To whom inquiries should be directed.

Dissolution Rate Studies IV: Solvent Flow Patterns in a Column-Type Apparatus

J. TINGSTAD▲, J. DUDZINSKI, L. LACHMAN, and E. SHAMI

Abstract □ Solvent flow patterns in a column-type, continuous flow apparatus were determined using a flow visualization technique. A supporting bed of glass spheres in the dissolution chamber ensures laminar flow, and this is preferred over the complex, poorly defined flow found with static beaker methods.

Keyphrases □ Dissolution rate studies—solvent flow patterns and laminar flow conditions, column-type equipment □ Tablet dissolution—solvent flow patterns and laminar flow conditions, column-type equipment □ Solvent flow in column-type dissolution equipment—flow patterns, laminar flow conditions □ Column-type dissolution equipment—solvent flow patterns, laminar flow conditions

The advantages of the column-type, continuous flow method for determining the dissolution characteristics of solids have been repeatedly emphasized (1–4). One important advantage is that solvent flow is columnar as opposed to the complex, poorly defined flow found with static beaker methods (5). However, some problems have been encountered with columnar flow because of turbulence (4), problems that would be minimized under conditions of laminar flow. This report describes, for a column-type apparatus, (*a*) solvent flow patterns under various experimental conditions, and (*b*) the necessary conditions for laminar flow.

EXPERIMENTAL

Equipment—The dissolution apparatus was the column type described previously (3). The light sources were a common laboratory microscope light and an electronic tachometer and motion analyzer[1]. The latter was calibrated with respect to the powerline frequency according to the instruction manual. The reflecting spheres were polystyrene base beads[2] (diameter 90–125 μ; specific gravity 1.06).

[1] Strobotac, General Radio Co., West Concord, Mass.

[2] Duke Standards Co., Palo Alto, Calif.

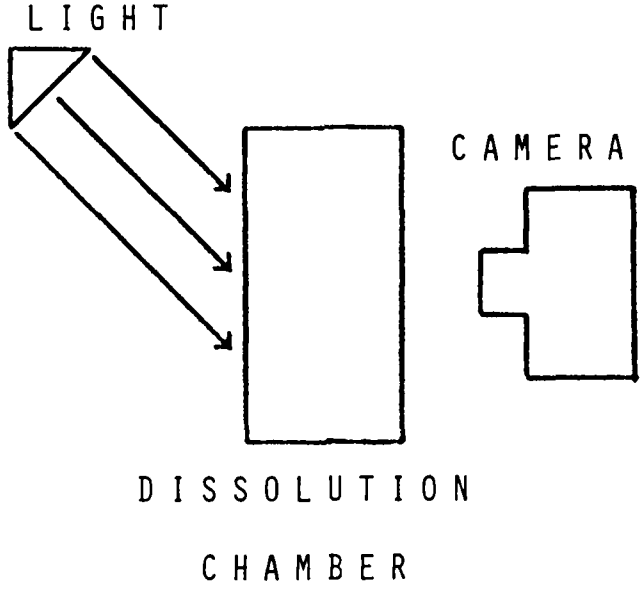

Figure 1—*Arrangement of equipment for photographic and television work.*

A lens hood was used to reduce flare with the still camera[3]. Depending on the tachometer flashing rate, apertures varied between f-11 and f-22 and exposure times between 0.5 and 0.25 sec. All photographs were taken at one-half life size image.

The television camera[4] had a 75-mm. f-1.9 lens and a video tape recorder[5].

Procedure—The experimental procedure was similar to that described by Withey and Bowker (5) but was appropriately modified for the column-type apparatus. The basic arrangement of the equip-

[3] A Nikon FTN with a 105-mm. Novoflex short-mount lens on a Novoflex bellows. The film was Kodak 2475 recording film developed in Acufine to an ASA index of 2400.

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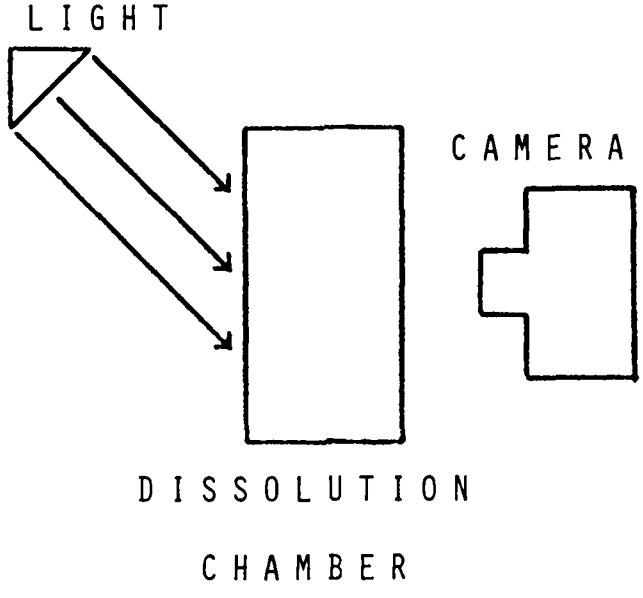

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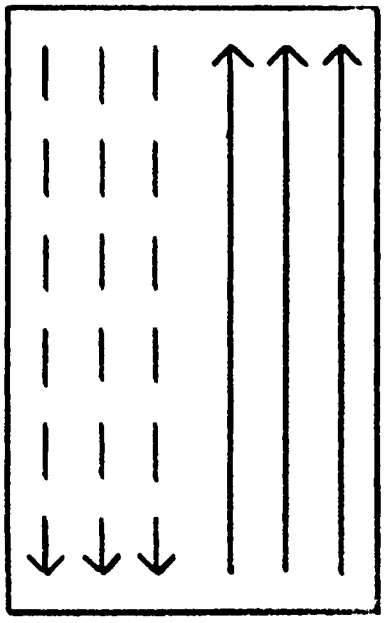

Figure 2 *Diagram of flow patterns with 25-mm. dissolution chamber at a flow rate of 9 ml./min.*

ment for the photography and television work is shown in Fig. 1. The angle between the tachometer and the camera was approximately 135°. The microscope light was used for making simple visual observations.

The polystyrene spheres reflect the light and, since their specific gravity is close to that of water, their flow patterns are essentially representative of the solvent molecules. The tachometer, properly pulsed for a given set of experimental conditions, reveals the paths of the particles as a series of images (usually streaks) on photographic film or video tape. However, the most accurate method for determining flow patterns is simple visual observation.

The solvent reservoir was a 150-ml. beaker containing 100 ml. of a 1-mg./ml. suspension of the polystyrene spheres in distilled water, with 0.05% polysorbate 80 added to reduce air entrapment. After determining that flow patterns were essentially identical with or without filters, all experiments were performed without filters (but with screens) at ambient temperature. The suspension could then

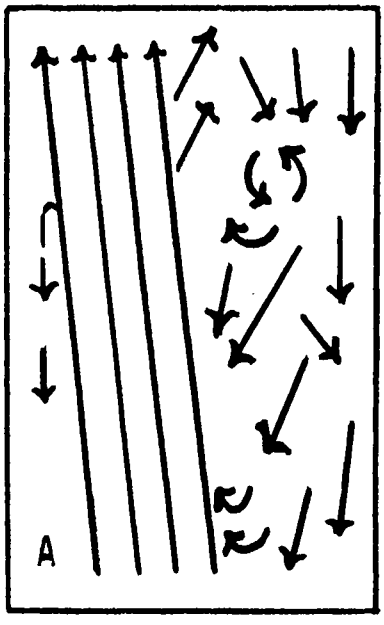

Figure 3—*Diagram of flow patterns with 25-mm. dissolution chamber at a flow rate of 55 ml./min. "A" is an area of low solvent velocity.*

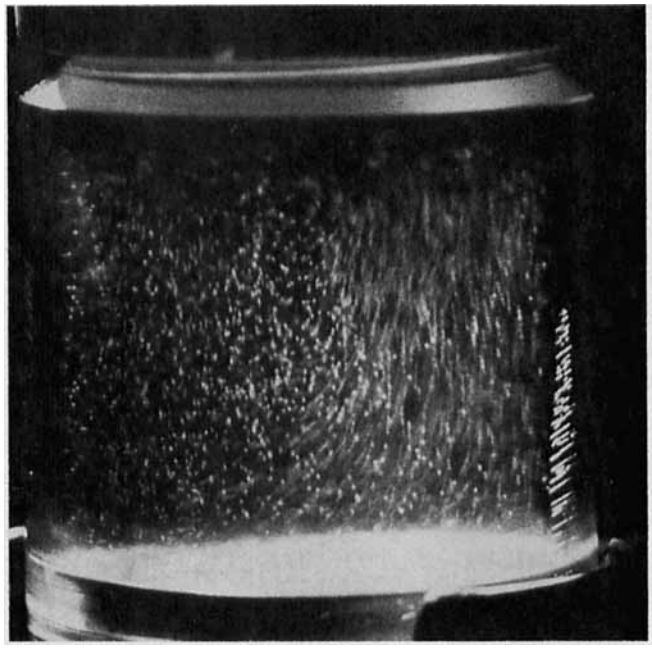

Figure 4—*Photograph of flow patterns with 25-mm. dissolution chamber at a flow rate of 48 ml./min. The tachometer flashing rate was 6000/min., the aperture was f-11, and the exposure time was 0.25 sec.*

be pumped through the dissolution chamber and recycled to the reservoir. The flow rate was varied, and the flow patterns in the 13- and 25-mm. chambers were observed and (when desirable) photographed or recorded on video tape.

RESULTS AND DISCUSSION

With the 25-mm. chamber at relatively low flow rates (5–9 ml./min.), the flow patterns shown in Fig. 2 was observed. While the solvent flow coming into the chamber (solid arrows) had essentially laminar characteristics, solvent was dispersed as it struck the upper screen and filter holder, and some returned to the bottom of the cell, still with the characteristics of laminar flow. This two-directional flow is, of course, undesirable. At intermediate flow rates (14–34 ml./min.), the same general pattern held except that turbulence increased and, in general, downward flow was much slower than upward flow. The degree of turbulence progressed with an increasing flow rate until (*e.g.*, at 48–55 ml./min.) a column of solvent moved rapidly upward and randomly dispersed after striking the upper screen and filter holder. Turbulence was much more wide-

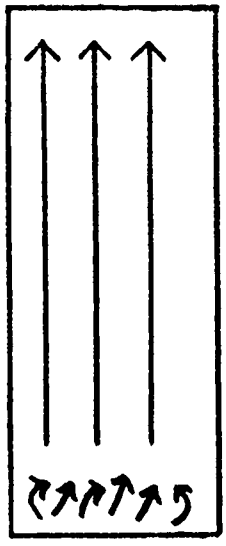

Figure 5—*Diagram of flow patterns with 13-mm. dissolution chamber at a flow rate of 13 ml./min.*

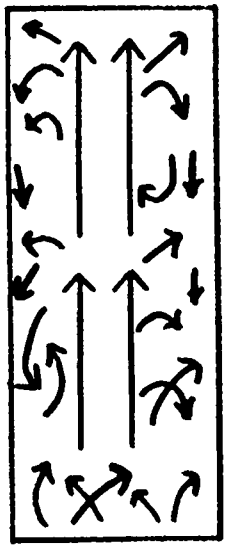

Figure 6—*Diagram of flow patterns with 13-mm. dissolution chamber at a flow rate of 36 ml./min.*

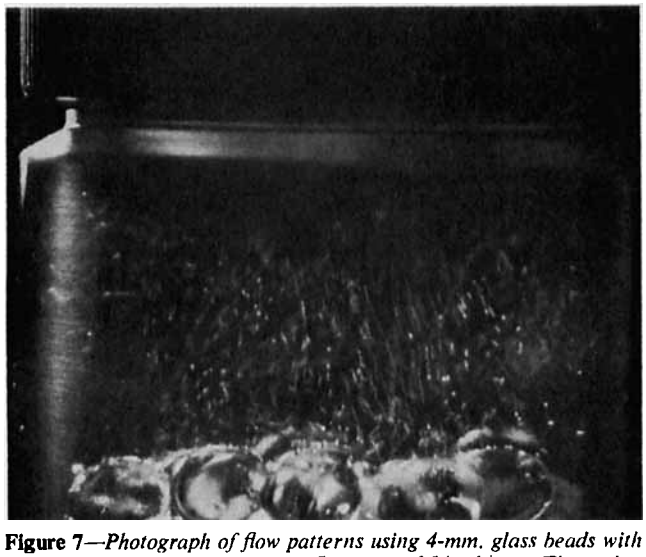

Figure 7—*Photograph of flow patterns using 4-mm. glass beads with 25-mm. dissolution chamber at a flow rate of 54 ml./min. The tachometer flashing rate was 6000/min., the aperture was f-16, and the exposure time was 0.5 sec.*

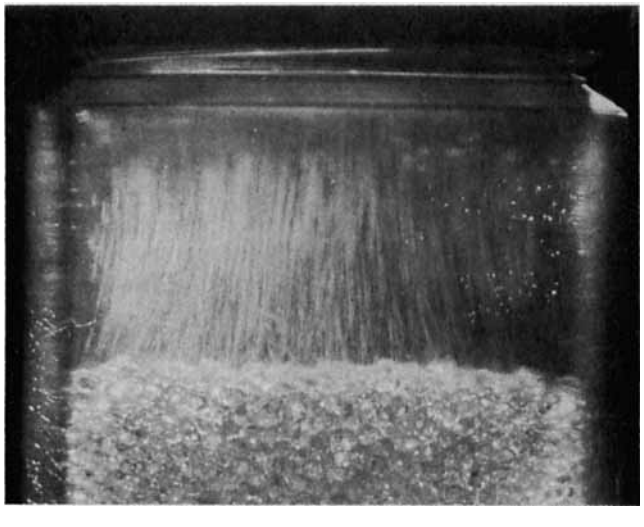

Figure 8—*Photograph of flow patterns using 1-mm. glass beads with 25-mm. dissolution chamber at a flow rate of 73 ml./min. The tachometer flashing rate was 12,000/min., the aperture was f-16, and the exposure time was 1 sec.*

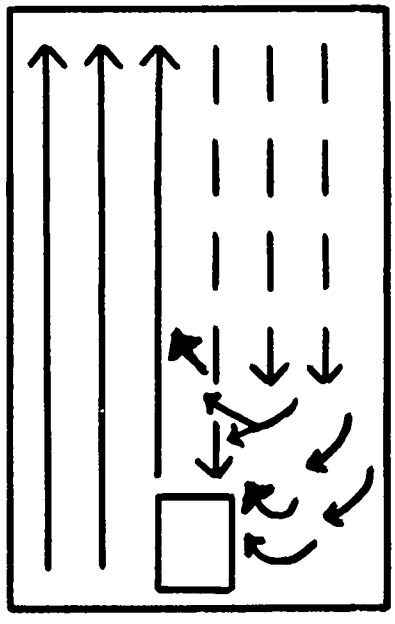

Figure 9—*Diagram of flow patterns with constant-surface pellet in the center of the lower screen of 25-mm. dissolution chamber. Flow rate was 34 ml./min.*

spread and dominant, and areas of low solvent flow developed (Figs. 3 and 4).

With the 13-mm. chamber at low flow rates (5–13 ml./min.), the flow was essentially laminar except for some turbulence in the first 0.5–1 cm. of the cell (Fig. 5). This is also undesirable because the area of turbulence would surround the tablet. At 36 ml./min., the flow was quite turbulent throughout the cell (Fig. 6). The spheres moved in relatively straight lines up the center of the chamber, but there was considerable turbulence on either side and at the bottom. It was not possible to obtain acceptable photographs with the 13-mm. cell because of excessive reflection and glare.

During these investigations, it was noted that flow patterns would vary considerably from one experiment to another (under the same set of conditions) if excessive air was trapped within the lower filter holder and beneath the lower screen. To minimize this variation and attain more laminar flow over a wider range of solvent velocities, a

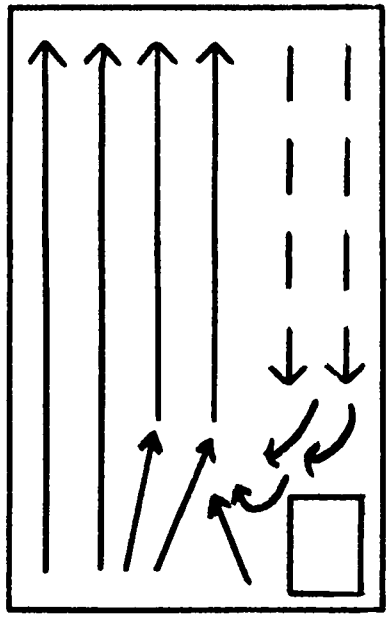

Figure 10—*Diagram of flow patterns with constant-surface pellet at the side of the lower screen of 25-mm. dissolution chamber. Flow rate was 34 ml./min.*

bed of glass beads was added to both cells, occupying about half of the chamber volume. The beads acted as dampers, and the 4-mm. size improved the flow characteristics considerably (Fig. 7 *versus* Fig. 4). However, it was necessary to use 1-mm. beads before proper laminar flow was attained (Fig. 8). Laminar characteristics were observed up to 80 ml./min., the highest flow rate used.

Because the photographs and drawings are two dimensional and static, they do not accurately represent the total, dynamic, three-dimensional situation. The video tapes from the television work provide a better record of solvent flow patterns because the actual motion is recorded. As mentioned earlier, visual observation is best.

In a previous investigation (4), constant-surface pellets of salicylic acid were used with the column apparatus. To gain more insight into the results of those experiments, flow patterns were observed in the 25-mm. chamber with the pellet in the center of the lower screen (Fig. 9) and at the side (Fig. 10). The results confirmed that, for a given flow rate, the lowering of the dissolution rate when the pellet was moved to the side of the chamber was due to the lower rate of solvent flow in that region.

SUMMARY

These results indicate that using a bed of 1-mm. glass beads with the column apparatus helps ensure laminar flow over a wide range of liquid velocities. This type of solvent flow is much preferred over the poorly defined, random-type flow characteristic of all beaker methods. Because surface tension slows solvent movement near the sides of the chambers, it is preferable to place the tablet well away from the sides of the cell.

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Dustiness of Pharmaceutical Formulations I: Instrumentation

G. GOLD▲, R. N. DUVALL, B. T. PALERMO, and R. L. HURTLE

Abstract □ Instrumentation for evaluating the dustiness of tableting materials is reported. The sample is dropped into a dust chamber where a controlled flow of air carries the resulting dust particles through a beam of light. The scattered light is received by a photodetector which converts light pulses into electrical pulses. The instrumentation was precalibrated in particle-size ranges of 0.5–10, 11–50, and >50 μ, and the number of particles in each size range is displayed on a digital counter. The particle size refers to the equivalent diameter of the dust particle that generates the same electrical response in the photodetector as the reference particle used to calibrate the instrumentation. Eleven commonly used tableting aids were classified into three categories based on their relative dustiness. Data obtained with the dust counter were shown to correlate with the dustiness resulting from the bulk handling of larger quantities of materials.

Keyphrases □ Dustiness of tableting materials—determination using light-scattering method and equipment □ Pharmaceutical technology—method and equipment for determining dustiness of tableting materials □ Tableting materials—measurement of dustiness □ Light-scattering method and equipment—determination of dustiness of tableting materials

Dust of any kind in a pharmaceutical plant is a serious matter. Areas of particular concern are: (*a*) cross-contamination of other products, and (*b*) potential hazards to the health of workers. There is always a risk of cross-contamination occurring with potentially serious consequences in pharmaceutical plants that utilize the same manufacturing facility to produce a variety of pharmaceutical products. Cross-contamination of a product with penicillin, for example, is capable of causing a serious reaction or even death to a person sensitive to penicillin (1). It is essential, therefore, that dust always be kept under control.

In regard to the workers' health, dust may be classified as a lung-depositing dust, toxic dust, primary irritant dust, sensitizing dust, or nuisance dust. A lung-depositing dust is deposited and retained in the lungs, where it may be benign or cause lung pathology. Toxic dust is a systemic poison which enters the circulation by absorption from the respiratory tract or after being swallowed. A primary irritant is limited largely to the mucous membranes of the eyes, nose, and throat. Prolonged exposure to low-grade irritants is capable of producing disturbances in respiratory function and secondary infection (2). A sensitizing dust elicits the antigen–antibody response in susceptible individuals as a result of either inhalation, skin contact, or ingestion. A nuisance dust is primarily discomforting to the worker and is often associated with increased colds and bronchitis. In general, the inhalation of any kind of dust in sufficient amounts for long periods of time can lead to disturbances in pulmonary function (3).

Particle size, frequency of exposure, quantity of concentration, and chemical action on the body tissue and fluids are important factors that influence whether a

bed of glass beads was added to both cells, occupying about half of the chamber volume. The beads acted as dampers, and the 4-mm. size improved the flow characteristics considerably (Fig. 7 *versus* Fig. 4). However, it was necessary to use 1-mm. beads before proper laminar flow was attained (Fig. 8). Laminar characteristics were observed up to 80 ml./min., the highest flow rate used.

Because the photographs and drawings are two dimensional and static, they do not accurately represent the total, dynamic, three-dimensional situation. The video tapes from the television work provide a better record of solvent flow patterns because the actual motion is recorded. As mentioned earlier, visual observation is best.

In a previous investigation (4), constant-surface pellets of salicylic acid were used with the column apparatus. To gain more insight into the results of those experiments, flow patterns were observed in the 25-mm. chamber with the pellet in the center of the lower screen (Fig. 9) and at the side (Fig. 10). The results confirmed that, for a given flow rate, the lowering of the dissolution rate when the pellet was moved to the side of the chamber was due to the lower rate of solvent flow in that region.

SUMMARY

These results indicate that using a bed of 1-mm. glass beads with the column apparatus helps ensure laminar flow over a wide range of liquid velocities. This type of solvent flow is much preferred over the poorly defined, random-type flow characteristic of all beaker methods. Because surface tension slows solvent movement near the sides of the chambers, it is preferable to place the tablet well away from the sides of the cell.

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Dustiness of Pharmaceutical Formulations I: Instrumentation

G. GOLD▲, R. N. DUVALL, B. T. PALERMO, and R. L. HURTLE

Abstract □ Instrumentation for evaluating the dustiness of tableting materials is reported. The sample is dropped into a dust chamber where a controlled flow of air carries the resulting dust particles through a beam of light. The scattered light is received by a photodetector which converts light pulses into electrical pulses. The instrumentation was precalibrated in particle-size ranges of 0.5–10, 11–50, and >50 μ, and the number of particles in each size range is displayed on a digital counter. The particle size refers to the equivalent diameter of the dust particle that generates the same electrical response in the photodetector as the reference particle used to calibrate the instrumentation. Eleven commonly used tableting aids were classified into three categories based on their relative dustiness. Data obtained with the dust counter were shown to correlate with the dustiness resulting from the bulk handling of larger quantities of materials.

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Dust of any kind in a pharmaceutical plant is a serious matter. Areas of particular concern are: (*a*) cross-contamination of other products, and (*b*) potential hazards to the health of workers. There is always a risk of cross-contamination occurring with potentially serious consequences in pharmaceutical plants that utilize the same manufacturing facility to produce a variety of pharmaceutical products. Cross-contamination of a product with penicillin, for example, is capable of causing a serious reaction or even death to a person sensitive to penicillin (1). It is essential, therefore, that dust always be kept under control.

In regard to the workers' health, dust may be classified as a lung-depositing dust, toxic dust, primary irritant dust, sensitizing dust, or nuisance dust. A lung-depositing dust is deposited and retained in the lungs, where it may be benign or cause lung pathology. Toxic dust is a systemic poison which enters the circulation by absorption from the respiratory tract or after being swallowed. A primary irritant is limited largely to the mucous membranes of the eyes, nose, and throat. Prolonged exposure to low-grade irritants is capable of producing disturbances in respiratory function and secondary infection (2). A sensitizing dust elicits the antigen–antibody response in susceptible individuals as a result of either inhalation, skin contact, or ingestion. A nuisance dust is primarily discomforting to the worker and is often associated with increased colds and bronchitis. In general, the inhalation of any kind of dust in sufficient amounts for long periods of time can lead to disturbances in pulmonary function (3).

Particle size, frequency of exposure, quantity of concentration, and chemical action on the body tissue and fluids are important factors that influence whether a

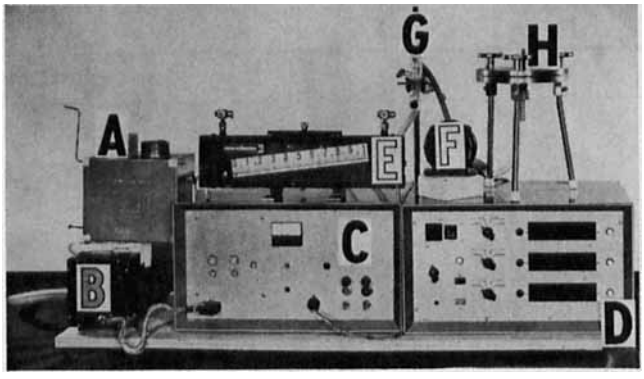

Figure 1—*Photograph of instrumentation showing sample chamber (A), detection chamber (B), pulse height discriminator (C), three-channel counter (D), inclined draft gauge (E), spiral exhauster (F), rotometer (G), and filter assembly (H).*

dust is an industrial health hazard (4). Particles between 1 and 2 μ have the greatest probability of being deposited in the pulmonary air spaces, and particles >10 μ are essentially removed in the nasal chamber and have little probability of penetrating to the lungs (5). Silica dust particles reaching and remaining in the alveoli are believed to be largely limited to the size ranging between 0.1 and 3 μ (6). The published threshold limit values (7) for several hundred materials refer to airborne concentrations of substances and represent conditions under which it is believed that nearly all workers may be repeatedly exposed day after day without adverse effect. The threshold limit values for nuisance dusts are 5 mg./m.^{3} for the respirable fraction and 15 mg./m.^{3} for total dust.

With the enactment of the Williams–Steiger Occupational Safety and Health Act of 1970, there will be more stringent governmental control of the concentration of dust in manufacturing plants; the formulator now has the added responsibility of preparing products that generate minimal dust. Unfortunately, little information is available for selecting low dust-yielding ingredients. Often the formulator is not aware of a dust problem until a production scale-up run is made, at which time it is extremely costly and time consuming to reformulate the product or to design a manufacturing system to control the dust. Furthermore, the present method for evaluating dustiness by simply observing the workroom environment is empirical and may lead to erroneous conclusions. Since the harmful respirable particle size is below the normal limit of visibility, work atmospheres that appear to be only slightly dusty often contain extremely hazardous concentrations of dust (8), and several cases were reported (9) where visible dust was practically absent from the air but the workers still contracted pulmonary disease.

The purpose of the first phase of this study was to develop a practical, objective method for measuring the relative dustiness of pharmaceutical powders. To meet this objective, instrumentation was developed based on light-scattering principles for sizing and counting airborne particles in the harmful respirable range. This report describes the instrumentation and indicates how it may be used to evaluate the relative dustiness of raw materials.

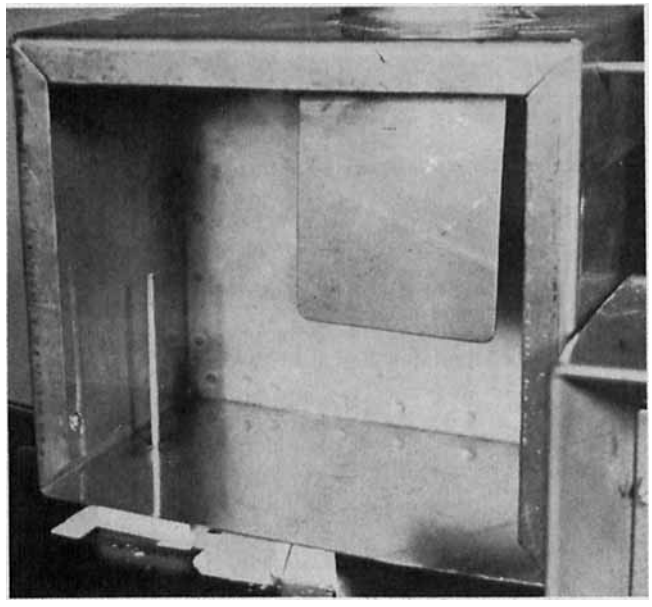

Figure 2—*Photograph of sample chamber (front removed) showing the inlet tube and platform (tripped position).*

EXPERIMENTAL

Instrumentation—The instrumentation (Fig. 1) consists of a sample chamber (A), a detection chamber (B), a pulse height discriminator (C), a three-channel counter (D), an air-flow system which includes a draft gauge[1] (E), a spiral exhauster[2] (F), a rotometer[3] (G), and a filter assembly[4] (H).

The sample is placed on the platform of the sample chamber (Fig. 2) and dropped by tripping the platform lever. The resulting dust particles enter the inlet tube of the sample chamber where they are carried by a controlled flow of air to the detection chamber. In the detection chamber, the dust particles are surrounded by filtered air entering from the filter and passed through a beam of light emanating from a light source[5] within the chamber. Scattered light is picked up by a photomultiplier[6] located within the chamber at a 40° angle from the light beam and converted to an electrical pulse. A preamplifier intensifies the electrical pulse from the photomultiplier, and a pulse height discriminator using stearing logic and pulse height detectors transfers the pulse to one of three channels of the counter. The counter is constructed with digital integrator circuits using wire-wrapped panels. Three separate scalers are used with a common time base generator and three 4.5-digit displays and overrange indicators. Three decades of prescaling are provided for each channel with a range selector switch. The timer can be adjusted in minute intervals up to 10 min. with an expired time indicator light.

Calibration—The instrument was calibrated using reference latex spheres[7] having particle diameters of 0.091, 0.481, 6–14, and 50–100 μ. The reference spheres were packaged as aerosols in which the particles were kept separated from the propellent and sprayed into one opening of a 1-l., three-necked distilling flask. Heat was blown into the second opening, and the third opening was connected to the inlet tube of the detection chamber. For each reference sphere size, the average pulse height was measured at the input of the pulse height discriminator. A linear relationship was found to exist between the size of the reference sphere and the pulse height. The three discriminator circuits were then adjusted so that one channel of the counter measured particle-diameter ranges of 0.5–10 μ, the second channel 11–50 μ, and the third channel >50 μ. The particle size refers to the

[1] Ellison Draft Gage Co., Chicago, Ill.
[2] Model SE2A-1, Rotron Inc., Woodstock, N. Y.
[3] F. W. Dwyer Manufacturing Co., Michigan City, Ind.
[4] GSWP 14200, 0.22-μ filter, Millipore Corp., Bedford, Mass.
[5] Tungsten lamp back-plate assembly from Beckman DU spectrophotometer, model 2400, Beckman Instruments, Inc., Fullerton, Calif.
[6] Type IP 28, RCA, New York, N. Y.
[7] Dow Chemical Co., Midland, Mich.

Table I—Dust Counts in the 0.5–10-μ Range for Several Commonly Used Tableting Aids

Material	Minutes	Mean[a]	*SD*	Mean Limits[b]
Direct compression dextrose	1	25.8	15.1	14.2–37.4
	5	26.9	15.8	
Direct compression sucrose formulation	1	109.8	44.5	76–144
	5	112.1	45.5	
Spray-dried lactose	1	253.9	91.0	184–324
	5	254.7	90.9	
Terra alba	1	429.9	163.3	304–555
	5	443.3	176.1	
Direct compression sucrose	1	1301.7	293.3	1076–1527
	5	1374.8	342.2	
Unmilled dicalcium phosphate dihydrate	1	2269.6	530.7	1862–2676
	5	2409.0	619.7	
Starch	1	4475.9	1562.0	3275–5677
	5	4743.1	1689.5	
Direct compression starch	1	6592.9	1804.1	5206–7980
	5	6837.9	1899.2	
Microcrystalline cellulose	1	8028.8	816.1	7401–8656
	5	8335.8	972.2	
Magnesium stearate	1	8743.9	2299.3	6976–10,511
	5	11,682.3	3130.5	
Talc	1	10,362.1	2478.5	8457–12,267
	5	12,991.2	3903.3	

[a] Mean of nine cumulative counts. [b] The 95% confidence limits about the mean.

Table II—Dust Counts in the 11–50-μ Range for Several Commonly Used Tableting Aids

Material	Minutes	Mean[a]	*SD*
Direct compression dextrose	1	1.3	1.4
	5	1.4	1.5
Direct compression sucrose formulation	1	3.0	2.1
	5	3.0	2.1
Spray-dried lactose	1	8.8	4.1
	5	8.8	4.1
Terra alba	1	11.0	8.6
	5	11.0	8.6
Direct compression sucrose	1	25.9	5.5
	5	25.9	5.5
Unmilled dicalcium phosphate dihydrate	1	71.4	26.3
	5	71.6	26.2
Starch	1	283.0	206.9
	5	283.4	207.2
Microcrystalline cellulose	1	605.2	102.1
	5	605.2	102.0
Direct compression starch	1	666.3	264.9
	5	667.8	265.2
Talc	1	1145.6	445.5
	5	1152.8	449.7
Magnesium stearate	1	2287.4	592.4
	5	2312.2	588.7

[a] Mean of nine cumulative counts.

Table III—Classification[a] of Relative Dustiness of Several Commonly Used Tableting Aids

Group I (0–1000 Counts)	Group II (1001–6000 Counts)	Group III (>6000 Counts)
Direct compression dextrose	Direct compression sucrose	Microcrystalline cellulose
Direct compression sucrose formulation	Dicalcium phosphate	Magnesium stearate
	Starch	Talc
Spray-dried lactose	Direct compression starch[b]	
Terra alba		

[a] Based on mean of nine measurements after 1 min. in the 0.5–10-μ range. The 95% confidence limits about the mean for each tableting aid are within the assigned group's range. [b] Classified between Groups II and III.

equivalent diameter of the particle that generates the same electrical response in the photodetector as the reference particle used to calibrate the instrument (10). Using the soap film method of calibration, the air flow through the inlet tube of the sample chamber was 870 ml./min. at a reading of 0.13[8]. For checking the counting and light circuitry, a secondary calibration system was incorporated in the instrumentation which consists of a d.c. μ ammeter to measure the dark current of the photomultiplier and a 60-Hz. line frequency check for the prescaling, scalers, time base, and display circuitry.

Measurements—A 15-g. sample was placed on a platform located 15 cm. above the floor of the sample chamber. The platform lever was then tripped and the timer was started. Particle counts for each size range were recorded in 1-min. intervals up to 5 min. Three measurements were made for each sample and were repeated on 2 different days for a total of nine measurements. Measurements were made in a temperature- and humidity-controlled room using conventional engineering equipment in which the temperature varied from 23 to 27° and the relative humidity did not exceed 20%.

Materials—The following commercially available USP, NF, or pharmaceutical grade tableting ingredients were evaluated for dustiness: direct compression dextrose[9], unmilled dicalcium phosphate dihydrate[10], spray-dried lactose[11], magnesium stearate, microcrystalline cellulose[12], starch, direct compression starch[13], direct compression sucrose[14], direct compression sucrose formulation[15], talc, and terra alba[16].

RESULTS AND DISCUSSION

One- and 5-min. dust counts in the 0.5–10-μ range (for several commonly used tableting aids) are tabulated in Table I. One-minute dust counts varied from a low mean of 25.8 for direct compression dextrose to a high of 10,362 for talc. Five-minute dust counts varied from 26.9 for direct compression dextrose to a high of 12,991 for

[8] On the Ellison inclined draft gauge.

[9] Celutab, Penick and Ford, Ltd., Cedar Rapids, Iowa.
[10] Stauffer Chemical Co., Chicago, Ill.
[11] Foremost Dairies, Inc., San Francisco, Calif.
[12] Avicel PH 105, FMC Corp., Marcus Hook, Pa.
[13] STA-Rx 1500, Colorcon Co., West Point, Pa.
[14] Di-Pac, American Sugar Co., New York, N. Y.
[15] Nu-Tab, SuCrest Corp., New York, N. Y.
[16] U. S. Gypsum Co., Chicago, Ill.

Table IV—Dust Measurements with the Laboratory Dust Counter and Commercial Air Sampler on Three Selected Tablet Formulations

	Dust Counter[a] Number of Particles		Air Sampler[b] Weight Gain, g.	
	Mean[c]	*SD*	Mean[d]	*SD*
Direct compression dextrose formulation[e]	222.0	38.6	0.0075	0.0020
Dicalcium phosphate formulation[e]	4137.1	775.6	0.729	0.293
Microcrystalline cellulose formulation[e]	12,950.0	1458.9	1.958	0.425

[a] A 15-g. sample, 0.5–10 μ, 1 min. [b] Unico sampler, Gelman type A filter, 30-kg. sample dropped from a 3-ft.^{3} twin-shell dry blender. [c] Mean of nine counts. [d] Mean of four counts. [e] Contains 0.1% FD&C Red No. 2 and 0.5% magnesium stearate.

talc. A paired comparison between the 1- and 5-min. data using the *t* statistic indicated that the 5-min. data were significantly higher than the 1-min. data. Dust particles in the 0.5–10-μ range have the highest probability of being deposited in the pulmonary air spaces and represent the greatest hazard to health.

Although particles in the 11–50-μ range have little probability of penetrating to the lungs, they may be swallowed to produce systemic activity. Dust counts in the 11–50-μ range are listed in Table II. The 1-min. dust counts varied from a low mean of 1.3 for direct compression dextrose to a high mean of 2287.4 for magnesium stearate. Five-minute dust counts varied from a low of 1.4 for direct compression dextrose to a high of 2312.2 for magnesium stearate. The lower dust counts in the 11–50-μ range and the absence of a significant difference between the 1- and 5-min. data are indicative of the faster settling-out rates of the larger size particles.

A classification of relative dustiness was devised based on placing the 1-min. mean counts of the 0.5–10-μ range particles into one of three groups. Group I was assigned a mean count range of 0–1000; Group II, 1001–6000; and Group III, >6000. The 95% confidence limits about the mean (Table I) were used in classifying the tableting aids into the various groups. The tableting aid was classified in the group whose range included the limits about the mean. As indicated in Table III, Group I consists of direct compression dextrose, direct compression sucrose formulation, spray-dried lactose, and terra alba. Group II contains direct compression sucrose, unmilled dicalcium phosphate, and starch. Direct compression starch is classified between Groups II and III. Group III contains microcrystalline cellulose, magnesium stearate, and talc.

An experiment was designed to determine if the data obtained with the dust counter correlated with dust generated during the bulk handling of larger quantities of the same materials. Direct compression dextrose, dicalcium phosphate, and microcrystalline cellulose were selected from the classification as representing various levels of dustiness. To each material, 0.1% FD&C Red No. 2 and 0.5% magnesium stearate were added. Dust counts for each of these three formulations were made using the dust counter as already described. Dustiness of the same three formulations was also measured using an air sampler[17] by dropping 30 kg. of each formulation from a 3-ft.^{3} twin-shell dry blender. The results (Table IV) indicate a high correlation coefficient (r = 0.998) between the dust counts obtained with the laboratory dust counter and the dustiness resulting from the bulk handling of larger quantities of the same material.

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[17] Unico 600, Unico Industrial Equipment Corp., Fall River, Mass.

Formula Modifications in a Solvent-Free Tablet Film Coat

PAUL A. TUERCK and DUNCAN E. McVEAN▲

Abstract □ Solvent-free formulations suitable for use as film coatings are described. Coatings are applied by spraying molten mixtures onto tablets in a conventional coating pan. The effects of a series of 17 additives on two representative basic formulations are evaluated. Results are presented relative to appearance, disintegration, friability, and uniformity. Desirable characteristics imparted by the additives may be easily selected from tables and used in the formulation of subsequent coating compositions. The concentration of shellac in the basic formulations was found to govern the rapidity of disintegration of the coatings regardless of additives. Of the additives evaluated, only castor oil, cocoa butter, and isopropyl myristate improved the basic formulations.

Keyphrases □ Tablet coating—solvent-free shellac polyethylene glycol 4000 formulations, effects of 17 additives, molten mixture spraying □ Shellac-polyethylene glycol 4000 solvent-free tablet film coatings—formulation, effects of 17 additives, molten mixture spraying □ Film coatings—formulation of solvent-free shellac-polyethylene glycol 4000 films for molten mixture spraying of tablets, effect of 17 additives

The use of coatings on tablets and pills is well documented. For example, "Remington's Pharmaceutical Sciences" lists a bibliography of 322 references pertaining to the subject (1). Those familiar with the art of tablet coating are acquainted with the reasons for coating and advantages and disadvantages of the application methods.

Until recently, sugar coating was overwhelmingly preferred and it still provides tablets of exceptional esthetic qualities. But for reasons of economy, both with respect to labor and material costs, other methods have gained popularity. Film-type coatings, in particular, offer reduced labor and material costs, are thin enough that embossed emblems are retained, mask tastes and odors, offer improved stability over sugar coatings, provide increased resistance to abrasion, and provide increased luster when compared to compressed tablets (1).

Unfortunately, it is necessary that most newer film coatings be applied with the film-forming material dissolved or suspended in volatile solvents such as acetone, chloroform, and alcohol. While these solvents evaporate quickly and make it possible to complete the coating operation in a short period, they also have many disadvantages. These solvents evaporate quickly, necessitating the use of large quantities which are either lost completely during the drying cycle or are partially recovered with expensive recovery systems. Elaborate precautions are sometimes necessary to protect against their inherent toxicity. The associated hazards of explosion and fire from local concentrations of vapors also must not be ignored. Furthermore, it is sometimes necessary to alternate the coating applications with drying cycles, since the use of solvent tends to dissolve portions of the coating already applied, resulting in sticking and clumping of tablets in the coating pan.

Film coating may also be accomplished by other methods, such as the Wurster air-suspension technique. This unique method also involves volatile solvents as vehicles for the film-forming materials (2, 3).

Pan coating remains one of the most widely used methods for applying film coats. Numerous modifications of methods exist for applying the coating materials and include the time-honored method of pouring on solutions in frequent and divided portions with intermittent drying cycles; spraying, using air pressure for atomization; and airless spraying, using hydraulic pressure alone for atomization. All these methods have been automated in part or *in toto* (4).

A recently issued patent (5) is concerned with some basic, novel coating compositions of dry shellac with polyethylene glycol. It is possible to apply these combinations as molten solutions to tablets with the aid of steam-jacketed pneumatic spray heads.

The purpose of this experimental work was to conduct screening studies to determine the effect of various additives on two basic film coating compositions containing 10 and 20% shellac in polyethylene glycol 4000 (I). The method and formulas described obviate the use of volatile organic solvents and the attendant hazards of toxicity or explosion.

EXPERIMENTAL

Preliminary feasibility work indicated that satisfactory coatings could be expected from application of any one of several basic formulas described in the patent (5). Two basic formulations containing pharmaceutical grade shellac (10 and 20%) and I were selected. Since little is known concerning the solvent properties of the molten I–shellac mixtures, selection of materials for screening could not be made on this basis. Therefore, approximately 50 materials were selected with different properties, *i.e.*, hydrophobic, hydrophilic, or surface active. Each was also believed not to represent a toxicological hazard should it be considered for future use.

Each additive was first prepared as 1 and 10% (by weight) solutions or dispersions in the molten mixture to determine miscibility. Approximately 60% of the additives were miscible at both levels. This group, in general, included alcohols, acids, esters, and glycols. Saccharides as a group were insoluble. Only 17 additives are included in this report. The others were eliminated, since, during later trials, their inclusion made application of satisfactory film coats impossible.

Materials that appeared soluble in the basic coating mixture at 110–140° included cocoa butter, castor oil, stearic acid, oleic acid, abietic acid, I, polyethylene glycol 6000, glycerin, propylene glycol, glyceryl monostearate, acetylated monoglycerides[1], tristearin, benzyl benzoate, polyoxyethylene-polyoxypropylene block polymers of molecular weight 2500[2] (II) and 8350[3] (III), polyoxyethylene lauryl ether[4], and polysorbate 80. Carnauba wax, cetyl alcohol, and stearyl alcohol were soluble at 140° but insoluble at 110°. Materials insoluble at 100–140° included paraffin, mineral oil, corn oil, beeswax, solid petrolatum, mannitol, carboxypolymethylene,

[1] Myvacet, Type 7-00, Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N. Y.
[2] Pluronic L 62, Wyandotte Chemical Corp., Wyandotte, Mich.
[3] Pluronic F 68, Wyandotte Chemical Corp., Wyandotte, Mich.
[4] Brij 35, Atlas Chemical Ind., Wilmington, Del.

Table I—Effects of Additives to 10% Shellac–90% I Base on Tablet Film Coatings

Additive (10% w/w)	—Disintegration, sec.— Median of Range[a]	Range[b]	Coating Weight[c]	$(SD)_c$[d]	SD[e]	Friability[f]	α[g]	Disintegration Factor[h]	Initial Appearance[i]
Abietic acid	380	80	29.2	4.57	3.67	0	13	13	3
Acacia	415	330	39.6	8.17	7.70	0	19	10	18
Acetylated monoglycerides	385	90	50.3	4.13	3.11	0.025	6	8	17
Benzyl benzoate	360	60	35.5	4.01	2.95	0	8	10	1
Polyoxyethylene lauryl ether	345	90	32.7	4.59	3.70	0	11	11	12
Castor oil	445	150	55.7	4.63	3.75	0.005	7	8	9
Cocoa butter	425	90	37.5	5.02	4.22	0 (1)	11	11	6
Glycerin	365	70	24.4	3.43	2.09	0.01	9	15	4
Isopropyl myristate	465	170	48.0	4.46	3.53	0 (1)	7	10	1
Oleic acid	580	200	74.5	9.44	9.04	0.008	12	8	16
Polyethylene glycol 400	320	40	47.3	4.00	2.93	0	6	7	6
Polyethylene glycol 6000	410	60	27.8	7.25	6.72	0 (1)	24	15	5
II	385	150	48.1	6.04	5.39	0.01	11	8	9
III	340	40	25.2	4.64	3.76	0	15	13	6
Polysorbate 80	378	55	43.3	5.52	4.80	0.01	11	9	13
Propylene glycol	438	105	38.8	5.08	4.29	0.01	11	11	9
Stearic acid	545	50	66.3	6.69	6.11	0.03	9	8	14
Coated control	375	130	29.9	3.74	2.57	0	9	13	15
Uncoated control	323	75	—	2.72	—	2.8 (1)	—	—	—

[a] Median of the range of 12 tablets disintegrated as described in USP XVII. [b] Range encountered in disintegration of 12 tablets as described. [c] Average coating weight in milligrams based on an average of 30 tablets as compared to an average weight of 30 uncoated control tablets. [d] Standard deviation in milligrams based on 30 individual weights of coated tablets (or an uncoated control). [e] Standard deviation in milligrams of the coat; obtained from $SD = \sqrt{(SD)_c^2 - (SD)_u^2}$, where $(SD)_c$ = standard deviation of coated tablets, and $(SD)_u$ = standard deviation of uncoated tablets. [f] Percent loss—20 tablets rotated for 4 min. in a Roche friabilator. (1) signifies splitting. [g] Coefficient of variation (of coat) = $\sqrt{(SD)_c^2 - (SD)_u^2}/W_c \cdot 100$, where W_c = weight of coat. [h] A number indicating the delay in disintegration contributed by a unit weight of applied coating. Units of this number are seconds per milligram. The lower the number, the faster the coat will disintegrate. [i] Average point-score rating of six people familiar with tablet dosage forms (1 = excellent and 18 = very poor). Tablets were inspected for smoothness, mottling, shine, and general appearance.

lactose, sucrose, dextrose, corn syrup solids, sorbitol, ethylcellulose (50 cps.), microcrystalline cellulose, sodium alginate, sodium carboxymethylcellulose, sodium lauryl sulfate, hydroxypropyl methylcellulose, polyvinylpyrrolidone, cetylpyridinium chloride, urea, sorbitan tristearate[6], hydroxypropyl cellulose[6], and gelatin. Isopropyl myristate and acacia, while insoluble, were readily suspendable and sprayable. Glyceryl monostearate and tristearin, while soluble, were eliminated because they imparted poor spraying characteristics to the basic formulations.

EQUIPMENT, MATERIALS, AND METHODS

A rotary tablet machine[7] (16 station) equipped with 0.95-cm. (0.375-in.) deep cup punches was used to compress placebo tablets. Tablets were prepared by direct compression from anhydrous lactose USP, direct tableting grade[8], and lubricated with 1% magnesium stearate. Tablets were compressed at a weight of 250 mg. each.

Two basic coating mixtures were prepared. One consisted of a 10% solution, by weight, of pharmaceutical grade dry shellac[9] with 90% I. The second was a mixture of 20% shellac, by weight, with 80% I. Approximately half the required amount of I was melted, shellac was added with constant stirring, and heat was applied until solution was complete. Heat was then discontinued, and the remainder of I was added and mixed. The molten mass was cast into paper cups and allowed to harden. The resulting ingots were later remelted and mixed with other ingredients. At this time, 0.5% of D&C Yellow No. 10 was added to define more easily areas of application and to evaluate the uniformity of the coat during the spraying operation.

During the coating process, the tablets were rotated in a stainless steel coating pan having a diameter of approximately 16.51 cm. (6.5 in.) at a rate of 43 r.p.m.

The coating compositions were applied to the rotating tablets by spraying molten material at 110–140° through a steam-jacketed pneumatic spray head[10]. Low pressure (12 psig.) steam was introduced into the steam jacket to keep the spray head hot during the operation. In each instance, the spray coat was applied to batches of 175 g. of tablets for 5 min. Air pressure for atomization was regulated at 15 psig.

Within 10 days after they were coated, the tablets were evaluated initially for friability, appearance, disintegration, and tablet-to-tablet uniformity of coat. Since these were screening studies, factors such as the additives being capable of assuming more than one crystalline form on cooling were not considered. These factors could be defined later should an additive appear promising as a result of this preliminary evaluation. Weights of tablets were obtained using a balance[11], and friability was determined[12] (6). Disintegration times were determined using the Stoll–Gershberg apparatus, with disks, and 37° distilled water as described in the USP XVII (7). The appearance of the coats before and after storage for 9 months at ambient room temperature was checked visually. In addition, the tablets were inspected for sticking (clumping), fading, and general appearance.

Tables I and II record disintegration times (in seconds) presented as a median of the range of 12 tablets for each formula modification presented, the range of disintegration times (in seconds), the coating weight, friability, appearance, coefficient of variation of the coat, and a factor derived from the other physical parameters.

The range of disintegration times, in general, may reflect uniformity or nonuniformity of the coat, with the more uniform coat exhibiting the narrowest range.

The weight of the coat is an index of the sprayability of the coating material. Since the time of application is uniform, the increase in coating weight over that of the coated control indicates increasing sprayability and vice versa. Since the weight of the coat varied from additive to additive and with the coated control, a normalizing

[6] Arlacel 60, Atlas Chemical Ind., Wilmington, Del.
[6] Klucel, Hercules Powder Co., Wilmington, Del.
[7] Stokes B-2 model 512.
[8] Sheffield Chemical Co., Division of National Dairy Products Corp.
[9] Mantrolac R 49, Mantrose Co., New York, N. Y.

[10] 1/4 JBCJ spray head equipped with a No. 2850 fluid nozzle and a No. 70 air nozzle supplied by Spraying Systems Co., Bellwood, Ill.
[11] Type H6T, Mettler Instrument Corp., Hightstown, N. J.
[12] Using a Roche Friabilator.

Table II - Effects of Additives to 20% Shellac–80% I Base on Tablet Film Coatings

Additive (10% w/w)	—Disintegration, sec.— Median of Range[a]	Range[b]	Coating Weight[c]	$(SD)_c$[d]	SD[e]	Friability[f]	α[g]	Disintegration Factor[h]	Initial Appearance[i]
Abietic acid	800	200	39.5	4.24	3.25	0	8	20	17
Acacia	525	170	38.2	3.88	2.77	1.0	7	14	18
Acetylated monoglycerides	515	90	39.5	5.06	4.27	0	10	13	5
Benzyl benzoate	720	360	41.9	4.21	3.21	0	8	17	11
Polyoxyethylene lauryl ether	483	115	36.7	5.14	4.36	0	12	13	8
Castor oil	530	40	31.8	4.45	3.52	0	11	17	4
Cocoa butter	515	150	31.6	4.02	2.96	0 (1)	9	16	9
Glycerin	438	185	21.1	2.79	0.20	0	1	21	7
Isopropyl myristate	570	160	39.2	5.03	4.23	0	11	15	1
Oleic acid	603	145	35.3	5.05	4.26	0	12	17	14
Polyethylene glycol 400	485	110	44.8	5.05	4.26	0	10	11	15
Polyethylene glycol 6000	355	50	16.7	3.44	2.11	0 (1)	13	21	13
II	405	150	30.4	5.42	4.69	0	15	13	6
III	480	200	39.6	5.10	4.32	1.0	11	12	13
Polysorbate 80	370	40	27.8	3.70	2.51	0	9	13	4
Propylene glycol	550	160	34.1	3.36	1.97	0	6	16	2
Stearic acid	470	80	20.9	3.94	2.95	1.0	14	22	16
Coated control	530	100	31.1	3.57	2.31	0 (1)	7	17	11
Uncoated control	323	75	—	2.72		2.8 (1)	—	—	—

[a–i] See footnotes to Table I.

factor, called a disintegration factor (D.F.), was developed to aid comparison among modified coatings as follows:

$$\text{D.F.} = \frac{t}{W_c} \qquad \text{(Eq. 1)}$$

where t = median of the disintegration time of 12 tablets (seconds), and W_c = average weight of the coat (milligrams). A comparison of the disintegration factor for a modified coating material with that of the coated control indicates the effect of the additive upon the apparent rate of solution of the basic coating formulation.

DISCUSSION

Throughout the experiment, attempts were made to keep variables at a minimum. The objective was to determine the effects of individual additives on the coating. Although certain additives such as carnauba wax, cetyl alcohol, and stearyl alcohol were miscible with the basic coating formulations, these materials raised the melting points of the formulations to such an extent that acceptable coats could not be achieved when these additives were used in 10% concentrations.

Other additives such as isopropyl myristate and acacia were not miscible with the coating compositions. However, they were suspendable to some degree and were included since they did not interfere with the sprayability of the coats. Isopropyl myristate formed a temporary emulsion and greatly improved the performance of the spraying operation as well as the appearance of the finished tablets.

It was generally observed that the series consisting of 10% shellac–90% I could be applied much more easily and resulted in tablets of a more elegant appearance than the series containing 20% shellac–80% I (last column, Tables I and II).

Examination of tablets stored 6 weeks at 45° revealed that all tablets had faded except those containing cocoa butter, castor oil, oleic acid, or glycerin. The mechanism of the stabilizing effect of these additives on the D&C Yellow No. 10 is not clear at this time. Tablets stored 9 months at room temperature did not exhibit the same degree of color instability.

Tablets stored 6 weeks at 45° containing benzyl benzoate, polyethylene glycol 400, propylene glycol, or II had softened to the point where they had clumped together. Here, again, tablets stored 9 months at room temperature presented a slightly different picture.

In general, the 9-month room temperature evaluation was more revealing than the high temperature study. The coated control

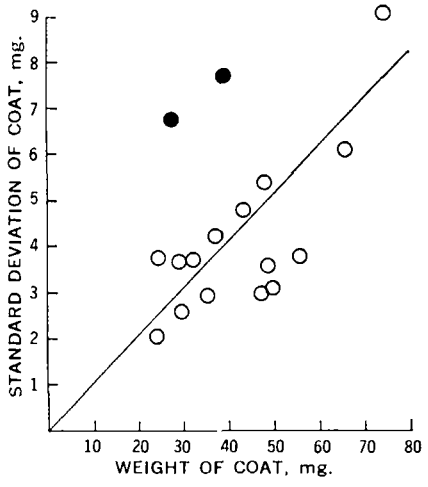

Figure 1—*Dependence of the standard deviation of the coat upon the weight of the coat illustrated for the 10% shellac–90% I system. Key: ●, parameters of additives that are statistically different from the parameters of the other additives;* i.e., *the process is out of control.*

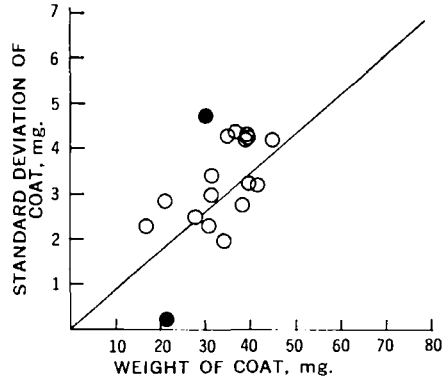

Figure 2—*Dependence of the standard deviation of the coat upon the weight of the coat illustrated for the 20% shellac–80% I system. Key: ●, parameters of additives that are statistically different from the parameters of the other additives;* i.e., *the process is out of control.*

Table III—Comparative Rank of Acceptable Additives According to Descriptive Factors

Additive	Disintegration Factor		Coefficient of Variation		Appearance		Row Total
	A[a]	B[b]	A[a]	B[b]	A[a]	B[b]	
Castor oil	1	3	1	3	4	2	14
Cocoa butter	3	2	4	2	3	3	17
Isopropyl myristate	2	1	1	3	1	1	9
Polyethylene glycol 6000	5	5	5	5	2	5	27
Coated control	4	3	3	1	5	4	20

[a] A = 10% shellac–90% I base. [b] B = 20% shellac–80% I base.

tablet exhibited a slight degree of clumping, and tablets could easily be separated with one or two gentle shakes of the container. This was also the case with all tablets found to exhibit clumping, with the exception of those containing benzyl benzoate, polyethylene glycol 400, polysorbate 80, propylene glycol, II, abietic acid, and acetylated monoglycerides, which required slightly more vigorous shaking.

Coatings composed of the 10% shellac–90% I base with cocoa butter, polyethylene glycol 6000, or isopropyl myristate additives exhibited no clumping or fading after 9 months of storage at room temperature.

Coatings composed of the 20% shellac–80% I base with castor oil, polyethylene glycol 6000, isopropyl myristate, or cocoa butter additives performed in a like manner. With respect to clumping and fading, these additives provide an improvement over the coated control.

As a result of the 9-month room temperature evaluation, only four of the 17 miscible additives were found to be useful as modifying agents to the basic formulations. These additives are castor oil, cocoa butter, isopropyl myristate, and polyethylene glycol 6000.

Regarding weight, the coefficients of variation (α) of the coats are listed in Tables I and II and were calculated by:

$$\alpha = 100 \cdot \frac{\sqrt{(SD)_c^2 - (SD)_u^2}}{W_c} \qquad \text{(Eq. 2)}$$

where $(SD)_c$ = standard deviation of coated tablets, $(SD)_u$ = standard deviation of uncoated tablets, and W_c = average weight of coating. An F test shows acacia and polyethylene glycol 6000 to be significantly different from the remainder when the data in Table I are considered; glycerin and II are likewise significantly different from the remainder when the data in Table II are considered. The coefficient of variation *for the process* can be obtained from a plot of standard deviation of coats, SD, *versus* W_c (with the constraint of zero intercept), and the coefficient of variation for the process emerges in the slope of the line (Figs. 1 and 2).

A least-squares fit yields $\alpha = \Sigma SD \cdot W_c / \Sigma W_c^2 = 0.0952$ or 9–10% for Fig. 1 and 0.0988 or 9–10% for Fig. 2. These values are, of course, close to the coefficient of variation for the coated control, showing that, within statistical limits, the substances tested (other than acacia and polyethylene glycol 6000 in Table I and glycerin and II in Table II) are not detrimental to the uniformity of the coat.

The disintegration factor, coefficient of variation of the coat, and appearance rating for each additive were ranked in relation to the others and to those of the coated control. The order of rank was assigned on the basis of the lowest number in each category being assigned first order in rank, *etc.* The results of this ranking are in Table III. Note that each additive is ranked for each criterion in each of the two basic formulations.

To select an order of preference for the use of these additives, the ranks which each additive held in each category and for each basic formulation were summed ("Row Total" column of Table III). Again, the lower the sum the more desirable is the effect of the additive upon the basic formulation. From Table III, it can be seen that isopropyl myristate, castor oil, and cocoa butter all improved the basic formulations while polyethylene glycol 6000 ranks lower than the coated control.

The disintegration factor for each additive to the 10% shellac–90% I basic formulation was divided by the corresponding factor in the 20% shellac–80% I basic formulation. The quotients were then summed and averaged, and their standard deviation was computed. When this average, 0.66 ($SD = \pm 0.15$), is compared to the quotient obtained from a like division of the disintegration factors for the coated controls, 0.76, the conclusion can be made that shellac is the controlling factor with regard to disintegration of this type of coating composition.

A similar treatment was applied to the coefficients of variation. The ratio for glycerin was aberrent to those obtained for the other additives. When the ratio for glycerin is eliminated, the average of the ratios for the remaining additives, 1.17 ($SD = \pm 0.58$), was, within statistical limits, consistent with that for the coated controls, 1.3. Examination of the quotients for individual additives reveals that glycerin, acacia, polyethylene glycol 6000, and propylene glycol effect a considerable increase in uniformity with increasing concentration of shellac. These agents probably act as plasticizers for the shellac and should be considered as such when used in combination with shellac. Since only one concentration of each additive was evaluated with each of two concentrations of shellac, a more comprehensive factorial analysis of the data was not attempted. However, the results of these screening studies did elucidate additives suitable for future work.

SUMMARY AND CONCLUSIONS

1. A novel method was described for application of tablet coatings. Tables citing the effect of various additives to the two basic coating compositions described were presented.

2. A disintegration factor was developed to aid comparison of basic coating formulations and the effect of additives upon them in conjunction with the use of the coefficient of variation. Use of these factors resulted in the selection of three additives that favorably modify the basic formulations described herein. Based on an overall appraisal, improvements in the basic coats were attained by addition of 10% (w/w) castor oil, cocoa butter, or isopropyl myristate to a shellac–polyethylene glycol 4000 mixture. These additives not only improved stability with respect to clumping and fading but also with respect to disintegration, friability, uniformity of coat, and appearance.

3. The concentration of shellac in the basic formulation appears to control disintegration regardless of additives.

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Physical and Chemical Characteristics of Water-Soluble, Semisolid, Anhydrous Bases for Possible Ophthalmic Use

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Abstract □ Following a preliminary review of pertinent literature citations, a total of 84 semisolid, water-soluble, anhydrous bases for possible ophthalmic use was formulated. Of these 84 exploratory bases, five were designated evaluatory bases for further study on the basis of apparent pH and/or desirable physical spreadability characteristics over the temperature range of 0–50°. Four of the five bases were further characterized by rotational viscometer studies, and an organogel was selected on the basis of several of its listed attributes as the best attempt in this investigation to formulate the type of semisolid base desired for possible ophthalmic use.

Keyphrases □ Semisolid bases—formulated, evaluated for use as potential ophthalmic ointments, pH and viscosity considerations □ Ophthalmic ointments, potential—formulation, evaluation of semisolid, water-soluble, anhydrous bases, pH and viscosity considerations □ Organogels—formulated for use as possible ophthalmic ointments

In USP XVIII (1), four classes of ointment or semisolid bases are recognized on the basis of physical characteristics: hydrocarbon or grease-type bases, absorption bases, water-removable bases, and water-soluble bases. Although polyethylene glycol ointment USP is the only official nonaqueous, water-soluble base, the more recent organogel-type bases comprise a little investigated supplement to these classes.

In the past, a hydrocarbon-type base was the most frequent choice when an anhydrous base was required; however, the nonaqueous organogels may offer advantages over the hydrocarbons and other traditional bases because of some of the following undesirable attributes of the latter bases:

1. Spreadability or extrudability over only a narrow incidence of temperatures.
2. Irritation to eyes and mucous membranes.
3. Inability to release effectively active medicinal ingredients.
4. Incompatibility with body tissue at site of application.
5. Instability of active ingredient(s) in base, *e.g.*, physical separation and/or chemical decomposition upon storage, especially because of improper pH, oxidation-reduction reaction, hydrolysis, *etc.*
6. Lack of cosmetic or esthetic appeal, *e.g.*, greasiness or stickiness, which could result in improper application of the base by the patient.
7. Lack of a completely dependable sterilization method.
8. Difficulty in removal of contaminating particulate matter from the base because of inherent physical, chemical, and/or viscosity characteristics.

The purpose of this investigation was to formulate, by reproducible methods, semisolid, anhydrous, water-soluble bases spreadable and/or extrudable over the general temperature range of 0–50° and with an apparent acidic pH. Furthermore, the bases should be: (*a*) compatible with a variety of drugs for ophthalmic use, *e.g.*, antibiotics, alkaloids, dimercaprol, and corticosteroids; (*b*) clinically nonirritating to the eyes; and (*c*) able to release medicinal agents in a desirable therapeutic manner.

DISCUSSION

A gel (2) is a solid or semisolid system of two or more ingredients, including a matrix that encloses and is interpenetrated by a liquid vehicle. Gels that are formed by aggregates or floccules of small particles represent two-phase systems; upon standing, they may exist as semisolids but, upon stirring or agitation, they liquefy and thus are termed thixotropic or shear thinning. Gels may also consist of entangled and/or matted strands of long-chain polymeric macromolecules, which may be joined by van der Waals forces and may align in a parallel-type sequence to maintain a gel matrix within a vehicle. These gels of macromolecules are usually one-phase systems and may be termed organogels.

The main disadvantage of certain anhydrous organogel-type systems is a tendency toward syneresis, which was defined (3) as: "The spontaneous separation of an initially homogeneous colloid system into two phases, a coherent gel and a liquid. The liquid is actually a dilute solution whose composition depends on the original gel. When the liquid appears, the gel contracts, but there is no net change in volume."

USP XVIII (4) requires only that ophthalmic bases be free from contamination with viable microorganisms and palpable particulate matter, that they be nonirritating, that they permit effective release of medicinal agents, and that they maintain the activity of the agents for a reasonable time during storage.

Unfortunately, the ophthalmic applications and characteristics of water-soluble, anhydrous, organogel-type bases have not received much study. However, cosmetic applications are numerous and at least one topical base (5) containing the antifungal agent tolnaftate[1] exists. Moreover, some industrial pharmacists recently recommended use of the polyethylene glycol polymers in water-soluble ophthalmic bases and emphasized the importance of their cosmetic appeal with concomitant medicinal efficacy (6).

A variety of factors influence the ophthalmic use of drugs and/or semisolid bases. The primary site of drug action in the eye is the cornea. Although recent studies (7, 8) indicated precorneal tear film to be lipid in nature, most drugs probably penetrate to the cornea from semisolid bases with little influence from this thin precorneal tear film.

One study (9) involving rabbits indicated that particles greater than 50 μ in size are capable of causing significant mechanical eye irritation. Furthermore, application of excess amounts of hydrocarbon bases to postsurgical eyes resulted in the entry of visible globules of base into the anterior chamber, and in one case a globule of petrolatum persisted innocuously for 3 years (10, 11).

The pH values of normal human tears and of ophthalmic pharmaceuticals have received some attention; the pH of normal tears is about 7.4. Tears are reported to have a great buffer capacity and are able to alter almost immediately a drop of unbuffered solution between pH 3.5 and 10.5 to neutrality or slight alkalinity; for this reason, buffering of some ophthalmic products is not recommended by some authorities (12, 13). The importance of pH adjustment rather than buffering in some ophthalmic bases includes prevention of excess lacrimation (and subsequent drug loss from the eye) and maintenance of drug stability. As a general rule, most ophthalmic

[1] Tinactin Cream, Schering Corp., Bloomfield, N. J.

drugs are 100 times more stable at pH 5.0 than at pH 7.0, contingent upon dissociation characteristics of the compound and upon relative stability of the free base and cation (14).

Isotonicity is another factor of importance to ophthalmic products. Rosenberg (13) reported that tears directly in contact with the cornea are slightly hypertonic. A second source (15) indicated 0.93% rather than the classical 0.90% as the true NaCl equivalent of normal tears and suggested that irritation to the eyes occurred only when the NaCl equivalent extremes of less than 0.6% and more than 2.0% existed.

Nearly all eye irritation tests involve the use of animals, usually albino rabbits, and clinical observation of their eyes periodically following instillation of the test compound. The standard for these tests is the Draize eye irritation test (16). The Draize test, when compared with several other similar tests in a recent comprehensive study (17), indicated as high as 100% error for a given test compound between it and the test normally used by the participating laboratory. Thus, results such as these support the need to delineate between irritation such as definite physical damage (*e.g.*, lesions, tissue swelling, and excess erythema) to the eyes of animals and the transitory discomfort irritation experienced subjectively by humans.

Viscosity (18), *i.e.*, resistance to flow of a fluid under stress, has been directly related to both the diffusion rate of a drug from a semisolid base and the therapeutic efficacy of the drug in the base (19–21). However, one study (22) of some organogel-type bases made from carboxy vinyl polymers[2] and involving ocular absorption of several drugs from the bases showed no correlation between viscosity of the base and drug release. A second study (23) indicated no change in viscosity of some carboxy vinyl polymer gels after 4 weeks of exposure to sunlight or repeated autoclaving at 121° and 15 p.s.i. for 15 min.

NF XIII (24) suggested that medications including antibiotics may be more effective in water-soluble rather than in hydrocarbon-type bases. This contention was corroborated for some polyethylene glycol-type bases by some investigators (25, 26). One study (27) involving incorporation of sulfa drugs, coal tar, and ichthammol into semisolid carboxy vinyl polymer bases indicated good stability of the drugs in the bases. However, the disadvantage of complexation of certain drugs with polyethylene glycols was established in other studies (28, 29). Some researchers (23, 30) indicated contaminants within the polyethylene glycols and carboxy vinyl polymers as a source of drug and/or semisolid base degradation.

A final important factor in the ophthalmic use of semisolid bases is sterility. The cornea and anterior chamber of the eye offer an optimum environment for growth of many types of pathogenic microorganisms, since these tissues lack the immunological defenses ambient in the bloodstream (31). Therefore, there is a stringent requirement for sterility in the manufacture and product life of semisolid bases for ophthalmic use. Several different studies (22, 32–34) of the sterility of commercially prepared ophthalmic ointments each indicated that some portion of the sample population was contaminated with viable microorganisms.

EXPERIMENTAL[3]

Materials—Materials used in this investigation were selected on the basis of previous similar research[4] (35) and with consideration of water-solubility, official and unofficial reports of low incidence of eye irritation and/or acute toxicity, ability to form semisolid bases or organogels in selected combinations, and commercial availability. All materials were of USP grade unless otherwise specified.

Table I—Percent (Weight) of Materials and Methods of Preparation of Evaluatory Bases

Base	Matrix Solids	Percent	Vehicles	Percent	Method of Preparation
E-1	Micronized, fumed silica, M-5[a]	7.3	Glycerin	91.2	A
	Nonionic surfactant polyol, F68[b]	1.5			
E-2	Poly(methyl vinyl ether–maleic anhydride)-139[c]	2.6	Polyethylene glycol 200[d]	97.4	B
E-3	Carboxy vinyl polymer 940[e]	1.36	Polyethylene glycol 200	29.24	D
			Polyethylene glycol 400[f]	68.83	
			Polyoxyethylene (15) cocoamine[g]	0.57	
E-4[h]	Polyethylene glycol 4000[i]	16.0	Polyethylene glycol 400	24.0	C
			1,2,6-hexanetriol	60.0	
E-5	Carboxy vinyl polymer 940	1.50	Polyethylene glycol 200	38.40	D
			Polyethylene glycol 400	58.12	
			Polyoxyethylene (15) cocoamine	0.53	
			Hexadecyl[j] alcohol	1.44	

[a] Cab-O-Sil, M-5, Cabot Corp., New York, NY 10017 [b] Pluronic F68, Wyandotte Chemical Corp., Wyandotte, MI 48192 [c] Grantrex AN-139, GAF Corp., New York, NY 10020 [d] Carbowax 200, Union Carbide Corp., New York, NY 10017 [e] Carbopol 940, B.F. Goodrich Chemical Co., Cleveland, OH 44115 [f] Carbowax 400, Union Carbide Corp., New York, NY 10017 [g] Ethomeen C/25, Armour Industrial Chemical Co., Chicago, IL 60690 [h] Base E-4 was not formulated in sufficient quantity for viscometer studies because of lack of 1,2,6-hexanetriol, previously generously supplied as a sample. [i] Carbowax 4000, Union Carbide Corp., New York, NY 10017 [j] Cosmetic grade, Enjay Chemical Co.

Formulation and Evaluation of Bases—Initial investigation in this study involved formulation of approximately 100 g. of each of 84 exploratory bases produced by one of the following methods:

Method A—The liquid vehicle was heated to 90° on a water bath with stirring at medium speed. Then the matrix polymer (solid) was added with continued heating and stirring until either a homogeneous dispersion or gelation resulted.

Method B—The matrix polymer was added to the liquid vehicle with stirring at medium speed at 22–25° for 48 hr. Then the dispersion was heated carefully to 125° with stirring and maintained at 125° for 1 hr., after which a homogeneous gel resulted.

Method C—The liquid vehicle and polymer solids were combined with stirring at medium speed at 65° until homogeneously mixed. Heat was then removed and the mixture was allowed to congeal or thicken with continuous stirring until its temperature reached 30°.

Method D—The matrix polymer was added to the liquid vehicle(s) with stirring at medium speed at 22–25°; stirring was continued for 1–2 hr. until a homogeneous dispersion resulted. This dispersion was then heated to 85–95° with stirring at medium speed and neutralized with an appropriate amine to yield a collapsed or viscous organogel.

Each of the 84 exploratory bases was developed incrementally by the addition of small portions of matrix solids to the balance of the liquid vehicle(s). After each addition, the physical characteristics of each base were observed at 0, 30, and 50°, and increments of matrix ingredients were added until a desirable base resulted or the amount

[2] Various Carbopols, B. F. Goodrich Chemical Co., Cleveland, Ohio.

[3] All liquids not measured in standard graduated cylinders were weighed on either an Ohaus "Dial-O-Gram" 1600 series balance (which reads to 0.1 g.) or a Mettler B-5 analytical balance. Bases were mixed in glass beakers of appropriate size using a model F "Lightnin' Mixer" equipped with a three-bladed marine-type impeller. Heat was provided by a Corning model PC-35 hot plate. Determinations of pH were conducted on a Corning model 10 pH meter with standard Corning electrodes; a Corning model PC-353 magnetic stirrer was used to mix all samples constantly for pH determinations. Samples of bases were placed into 90-ml. (3-oz.), general purpose (NP), amber glass ointment jars fitted with metal screw caps with waxed cardboard liners; they were stored in Thelco model 6M ovens adjusted to 30 and 50° and in a Herrick refrigerator adjusted to 0°. Viscosity studies were conducted on a Haake Rotovisko (model 67-369) rotational viscometer equipped with six-bladed star-shaped paddle (FL-10).

[4] At the University of Florida, College of Pharmacy.

Table II—Physical Characteristics of Evaluatory Bases at Selected Temperatures[a]

Base	0°	30°	50°
E-1	tr, sp, ss	c, f, tk, sp, l	tr, f, tk, sp, l
E-2	tr, sp, ss	tr, sp, s	tr, sp, ss
E-3	tr, sp, ss	c, sp, ss	c, sp, ss
E-4	o, sp, ss	o, sp, ss	o, sp, ss
E-5	c, sp, ss	c, sp, ss	o, sp, ss

[a] Abbreviations are as follows: c, clear or transparent; f, flowing; tr, translucent; o, opaque; tk, thick; sp, spreadable; l, liquid (a base that flows to some extent at temperature specified); and ss, semisolid (a base that is spreadable and/or extrudable at temperature specified).

Table III—Apparent pH values for Diluted Evaluatory Bases

Base	pH of 10 g. Base Diluted to 50 ml. with Distilled Water[a]
E-1	5.4
E-2	3.3
E-3	4.4
E-5	4.2

[a] The pH of the distilled water used to dilute all samples was 7.0.

of solids approached 15% by weight (because of limited amounts of some ingredients) and a semisolid base did not result.

On the basis of physical characteristics at 0, 30, and 50° and of apparent pH, five of the 84 exploratory bases were designated evaluatory Bases E-1 through E-5. The formulas and physical characteristics of Bases E-1 through E-5 are listed in Tables I and II, respectively.

Determinations of apparent pH of the bases studied were conducted as follows. Exactly 10 g. of the base was diluted to 50 ml. with distilled water and thoroughly dispersed. The pH value of the dispersion was then observed. The pH values of E-1, E-2, E-3, and E-5 are listed in Table III. In an attempt to determine arbitrarily if these four bases were buffer-type systems, 10 g. of each was thoroughly dispersed in distilled water to make 100 ml. and this dispersion was titrated with 0.1 *N* NaOH solution until pH 7.0 was reached. The results of these titrations are listed in Table IV.

Viscosity studies[5] were conducted at 30° on 100-g. samples of E-1, E-2, E-3, and E-5 by immersing the star-shaped paddle to a standard depth in the sample, allowing it to remain in place for 1 min., and then engaging the clutch at a selected *U* value. Upon clutch engagement, the initial *S* value, S_0, was read; the viscometer was then allowed to operate for 2 min. until a second *S* value, S_2, was recorded. An undisturbed sample of each base was used for each separate determination at each given *U* value.

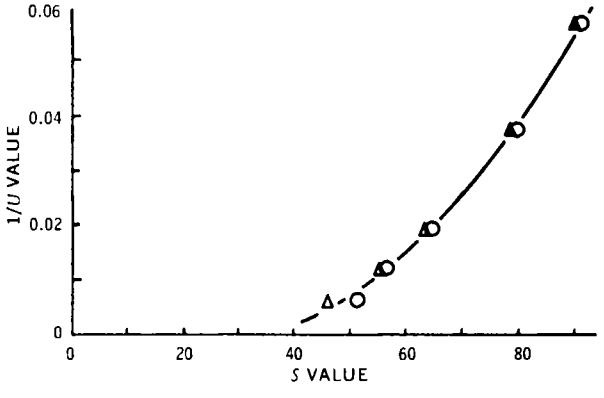

Figure 1—*Characteristic viscosity curve for Base E-5 at zero time and observed at 30°. Key:* ○, S_0; *and* △, S_2.

Table IV—Amount of 0.1 *N* NaOH Solution Required to Neutralize 10 g. of Base Diluted to 100 ml. with Distilled Water[a,b]

Base	pH of 10 g. Base Diluted to 100 ml.	Amount 0.1 *N* NaOH Required, ml.
E-1	6.1	0.1
E-2	3.5	11.9
E-3	4.0	—[c]
E-5	3.9	—[c]

[a] The pH of the distilled water used was 7.0. [b] All readings were taken at 30°. [c] Addition of approximately 2.5 ml. of 0.1 *N* NaOH solution yielded a thick, collapsed semisolid gel of pH 5.2, which thickened more with further addition of 0.1 *N* NaOH and prevented pH determination concurrent with titration by 0.1 *N* NaOH.

Following viscometer evaluations of each base at five different *U* values at zero time (0–96 hr. after formulation of the base), five tightly sealed samples of each base were stored in glass containers for 1 month in two ovens adjusted to 30 and 50°, respectively. Following this storage, viscometer studies on E-1, E-2, E-3, and E-5 were completed as previously described and at 30°.

RESULTS

The findings after storage of samples of Bases E-1, E-2, E-3, and E-5 at 30 and 50° for 1 month and subsequent viscosity studies and attempts to describe rheological[6] (36) characteristics of these bases are summarized as follows.

1. Storage of E-1 at 50° for 1 month resulted in syneresis of the normally thixotropic system; storage of E-1 at 30° for 1 month resulted in a viscous liquid-type system, which appeared nearly Newtonian in rheological behavior.

2. Storage of E-2 at 50° for 1 month resulted in a two-layered system (liquid upper phase and semisolid lower phase). Storage of E-2 at 30° for 1 month resulted in a homogeneous organogel system, which indicated development of some thixotropy.

3. Storage of E-3 at 50° for 1 month resulted in an apparent dilatent (shear-thickening) system, probably as a result of redispersion of microscopic transparent lumps of matrix polymer by the agitation of the viscometer paddle. Storage of E-3 at 30° for 1 month resulted in plots of S_0 and S_2 that were nearly coincidental and were very similar to plots for E-3 at zero time.

4. Storage of E-5 at 50° for 1 month resulted in an organogel containing some small dense lumps, whereas storage of this base at 30° for 1 month resulted in a thick homogeneous organogel. A comparison of plots of S_0 and S_2 in Fig. 1 with those in Figs. 2 and 3 indicates that storage of E-5 for 1 month at 30 and 50°, respectively, resulted in some thixotropy of the base. However, for given *U* values, plots of S_0 are in approximately the same position along the abscissas of all three figures; this suggests acceptable stability

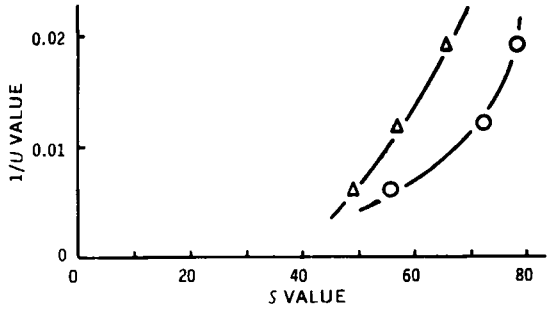

Figure 2—*Characteristic viscosity curve for Base E-5 after storage for 1 month at 30° and observed at 30°. Key:* ○, S_0; *and* △, S_2.

[5] The viscometer is fitted with an *S* scale of 0–100 arbitrary units, corresponding to shearing stress, and with a *U* scale of 10 arbitrary clutch values, corresponding to rates of shear. The viscometer is also equipped with a sensitivity switch adjustable for "fine" or "normal" and with a head setting switch with the settings 50 and 500 (the 500 setting is theoretically 10 times as sensitive as the 50 setting and is used for highly viscous samples).

[6] Rheology may be defined as the study of deformation of solids and flow of liquids.

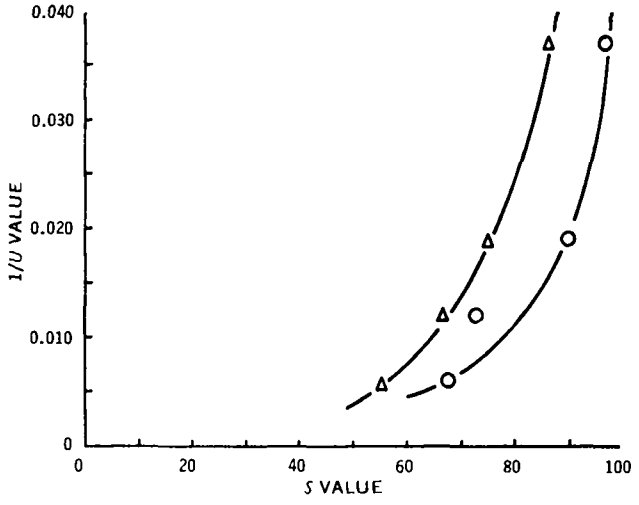

Figure 3—*Characteristic viscosity curve for Base E-5 after storage for 1 month at 50° and observed at 30°. Key: O, S_0; and Δ, S_2.*

of E-5 regarding resistance to changes in viscosity resulting from storage at 30 and 50° for at least 1 month.

Preliminary analyses[7] of E-5 (for dimercaprol content) containing approximately 6% dimercaprol and stored at selected temperatures in Type I glass containers are reported in Table V. Dimercaprol was selected as the initial test medicament for incorporation into Bases E-1 through E-5. It is employed ophthalmically as an antidote for heavy metal poisons and certain vesiccants, and its stability is a function of pH, temperature, and oxidation.

Formulation of E-2 (without dimercaprol) by Method B resulted in the appearance of a dark-pink color as the dispersion reached 125°. This is a probable result of a chemical reaction of poly(methyl vinyl ether-maleic anhydride) 139 with polyethylene glycol 200, since such reactions are known to occur (37); the reaction product (probably a chromophore compound) is possibly only an intermediate since the pink color disappeared after storage of the base for 1–2 weeks at 30°. This possible reaction could explain large variations in the physical consistency of E-2 at zero time as compared to that when the base is stored for 1 month at 30 and 50°.

The appearance of small, dense lumps in E-3 and E-5 after storage for 1 month at 30 and 50° may be the result of coalescence of microscopic particles of partially dissolved carboxy vinyl polymer 940; appearance of the lumps could also be suspected to be a function of prolonged storage at higher temperatures.

A serious disadvantage of the methods of preparation of the bases in this investigation is the entrainment of large amounts (in some cases) of small air bubbles, especially in organogels formulated from carboxy vinyl polymers. This entrainment of air could result in a nonhomogeneous rheological system, degradation of the base upon storage, and/or decomposition of potentially active medicinal ingredients. It is possible that these air bubbles could have been eliminated by preparing the bases under partial vacuum or that they could have been removed from the finished bases by partial evacuation of the system. Vacuum treatment, however, was not investigated. Furthermore, one investigator (38) studied the use of ultrasonic homogenizers in preparing organogels of carboxy vinyl polymers which were virtually free of entrained air.

CONCLUSIONS

In this investigation, Base E-5 represented the best attempt to formulate a semisolid, anhydrous, water-soluble base for ophthalmic use as determined by the following parameters:

[7] Analyses of E-5 containing approximately 6% dimercaprol were conducted as follows. Three 0.1–0.3-g. samples of E-5, weighed to the nearest 0.1 mg., were placed in nitrogen-flushed, 125-ml., rubber-stoppered conical flasks. Then 30–40 ml. distilled water and 3 ml. of 0.30 *M* phosphate buffer (pH 7) were added, the samples were thoroughly dispersed, and the dimercaprol was titrated with fresh iodide solution using freshly prepared starch solution as the end-point indicator.

Table V—Results of Preliminary Analyses of Approximately 6% (w/w) Dimercaprol in Base E-5 Stored in Type I Glass Vials[a,b]

Time Stored	Storage Temperature	Dimercaprol Remaining, %	Degradation of Dimercaprol, %
Zero time	30°	5.73	0.0
2 weeks	30°	5.76	0.0
1 month	30°	5.71	0.3
2 months	30°	5.75	0.0
3 months	30°	5.67	1.0
Zero time	50°	5.73	0.0
2 weeks	50°	5.63	1.7
1 month	50°	5.63	1.7
2 months	50°	5.57	2.8
3 months	50°	5.40	5.8

[a] Differences of 0.15 (or less) unit regarding percent dimercaprol remaining were considered insignificant and probably due to human variation in the precision of the analyses. [b] Results for bases other than E-5 and for other temperatures and containers other than Type I glass will be reported in subsequent studies.

1. Desirable physical spreadability characteristics over the temperature range of 0–50°.
2. An apparent pH value in the acidic range (which may be important to the stability of many ophthalmic drugs).
3. Desirable lubricity.
4. The cosmetic appeal of greaselessness and possible esthetic and technical attributes of transparency.
5. Least observed change in physical characteristics of all bases studied after storage at 30 and 50° for 1 month.
6. Apparent lack of strong buffer capacity and thickening due to viscosity of a 10% (w/v) aqueous dispersion with addition of small amounts of dilute alkali which, in the near neutral environment of the eye, could result in advantageous prolonged contact of a medicated base with the eye.
7. Content of the maximum amount[8] of the most desirable (in this investigation) vehicle, polyethylene glycol 400; polyethylene glycol 400 is the least hygroscopic (39) of the solvents used, its known eye irritation and acute toxicity are low, it is official in USP XVIII, and it has desirable lubricity and viscosity characteristics.
8. Known low eye irritation by the primary matrix polymer, carboxy vinyl polymer 940, as reported for a 1% aqueous solution (40), resistance of some gels of carboxy vinyl polymer 940 to support fungal and bacterial growth (41); and the possibility that some gels of carboxy vinyl polymer 940 may be sterilized by autoclaving without significant change in viscosity (23).
9. Optimistic preliminary reports of tolerable rates of degradation of dimercaprol incorporated in E-5 when stored in Type I glass vials at various temperatures for selected time (Table V).

Further research concerning ophthalmic bases such as those formulated for this investigation will attempt to elucidate eye irritation properties of the bases through animal testing, compatibility of the bases with several types of containers designed for unit dose-type application, compatibility and stability of various ophthalmic drugs in the bases, and therapeutic efficacy of the bases in antidoting the deleterious effects of selected compounds through a program of clinical diagnosis of the eyes of intentionally inoculated animals.

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[8] Since polyethylene glycol 400 freezes to a solid from 4 to 10°, it must be "plasticized" with a minimum of 25–30% of its close congener polyethylene glycol 200.

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Differential Thermal Analysis as Screening Technique for Candidate Adjuvants in a Parenteral Formulation: Cephradine for Injection

HAROLD JACOBSON▲ and IRWIN GIBBS

Abstract □ Differential thermal analysis (DTA) was used to study parenteral formulations of cephradine. Such studies showed that cephradine interacted with *N*-methylglucamine, tromethamine, and trisodium phosphate dodecahydrate, but it did not react with anhydrous sodium carbonate. The presence of an interaction does not necessarily indicate incompatibility, but it is an early warning of such a possibility. Finally, because DTA showed no new characteristic peaks, the absence of eutectics or of other specific phase entities could be presumed. Chemical analyses of various test formulations that had been stored at 23 and 50° gave excellent correlations with the results obtained earlier by DTA and showed that the interactions had been ones of incompatibility.

Keyphrases □ Adjuvants, parenteral formulations—use of differential thermal analysis as a screening technique, cephradine □ Parenteral formulations—drug–adjuvant interactions screened using differential thermal analysis □ Cephradine—suitability of adjuvants for parenteral formulation screened using differential thermal analysis □ Differential thermal analysis—use in determining suitability of adjuvants for cephradine parenteral formulation

Jacobson and Reier (1) discussed the application of differential thermal analysis (DTA) to problems of compatibility and stability that occur with mixtures of solid-state drugs and excipients, notably combinations of penicillin and stearic acid. The method is here extended to a study of parenteral formulations of cephradine, a new antibiotic of the cephalosporin class, coincidentally emphasizing the general utility of this rapid screening method.

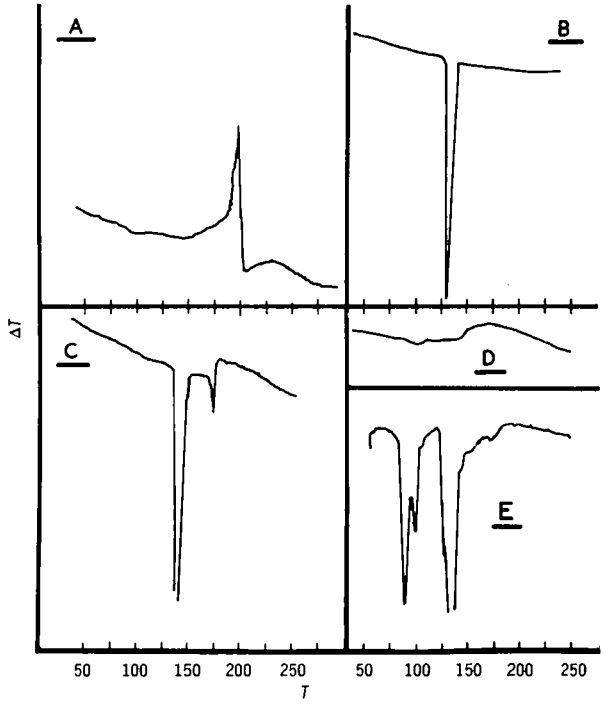

Figure 1—*Thermograms of pure materials. Key: A, cephradine; B, N-methylglucamine; C, tromethamine; D, anhydrous sodium carbonate; and E, trisodium phosphate dodecahydrate.*

In the formulation of a parenteral dosage form of cephradine, a stable, dry powder for reconstitution was sought which could be readily dissolved just prior to injection. Dürsch[1] suggested the use of a solubilizing adjuvant for the parenteral form of cephradine.

Several physiologically acceptable solubilizing adjuvants exist, and it became necessary to establish that the dry powder mixture of each adjuvant with cephradine exhibited stability under the usual storage conditions. Among the possible solubilizers, *N*-methylglucamine, tromethamine, trisodium phosphate dodecahydrate, and anhydrous sodium carbonate appeared useful.

EXPERIMENTAL

Blends of cephradine with the appropriate solubilizer were prepared, using the minimum amount of solubilizer that would give a complete solution in 1 min. or less when 250 mg. of cephradine was reconstituted with 1.2 ml. of water.

The tromethamine and trisodium phosphate dodecahydrate employed were reagent grade, the *N*-methylglucamine was USP grade (2), and the anhydrous sodium carbonate conformed to specifications in the British Pharmacopoeia (3).

The mole fractions of solubilizer in the respective blends were: *N*-methylglucamine, 0.445; trisodium phosphate dodecahydrate, 0.374; tromethamine, 0.564; and anhydrous sodium carbonate, 0.500.

Each solubilizer was passed through a 40-mesh screen before being blended with cephradine in a mortar and pestle. Containers for storage were 7.5-ml. siliconed, type III glass vials with butyl rubber stoppers. Samples were stored at 23 and 50°.

The thermograms of the freshly prepared mixtures were obtained on a thermal analyzer[2], using 2-mm. microsample tubes. A heating rate of 15°/min. was used, with a differential sensitivity of 1°/2.54 cm. The thermograms were obtained in the presence of static air.

The stability of cephradine in each blend was determined by an iodometric assay method (4).

[1] F. Dürsch, Squibb Institute for Medical Research communication, Oct. 16, 1970.

[2] Du Pont, model 900.

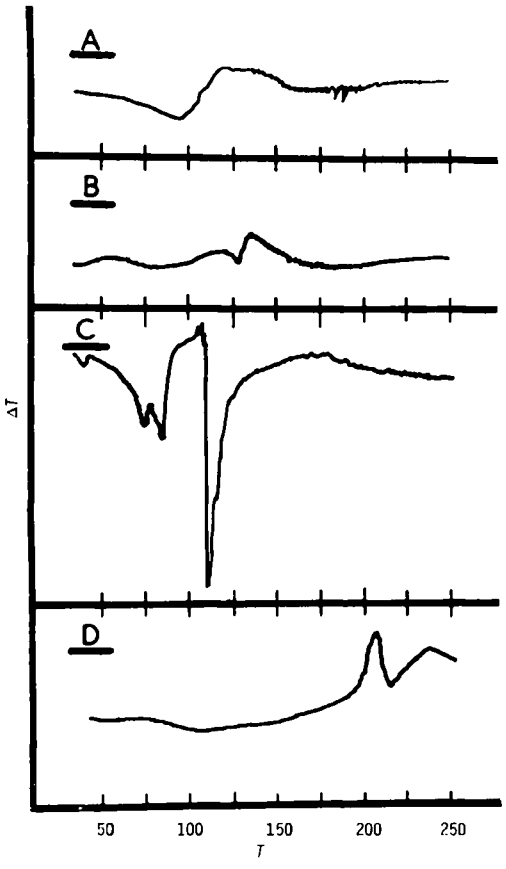

Figure 2—*Thermograms of mixtures of cephradine with: (A) N-methylglucamine, (B) tromethamine, (C) trisodium phosphate dodecahydrate, and (D) anhydrous sodium carbonate.*

RESULTS AND DISCUSSION

Thermograms of the pure materials are shown in Fig. 1. The exotherm observed with cephradine between 175 and 200° was due to an oxidative degradation. The endotherm exhibited by *N*-methylglucamine at about 129° was due to melting; those in tromethamine were due to a solid-solid phase transition (5) and to melting. Sodium carbonate had no characteristic features at temperatures of interest (up to about 350°). The first two endotherms of trisodium phosphate dodecahydrate (Fig. 1E) were due to desolvation, and the third was due to the boiling of released water.

Thermograms of the various mixtures are shown in Fig. 2. It was evident that interactions had taken place when cephradine was mixed with *N*-methylglucamine, tromethamine, or trisodium phosphate dodecahydrate. With the former two compounds, the features of both components of the mixture had been changed or obliterated. With trisodium phosphate dodecahydrate, only the features of cephradine were obliterated; those of the phosphate were largely intact and unchanged. In the thermogram of the mixture of cephradine and anhydrous sodium carbonate, the most significant feature is the retention of the cephradine exotherm at about 200°. It can be surmised that no interaction had taken place.

The presence of a physical or chemical interaction does not necessarily indicate incompatibility. It may indicate the formation of a eutectic mixture or the occurrence of a new phase with different thermal characteristics which are not necessarily deleterious to stability. However, the fact of an interaction is undeniable and gives an early warning for a more rational approach to the design of formulations.

In the examples examined in this work, DTA showed no new characteristic thermal peaks. Consequently, it could be presumed that eutectic mixtures or other specific phase entities, such as the formation of a new compound, were absent.

Figures 3 and 4 illustrate the potencies of the various blends as a function of time at 23 and 50°. The least stable blend (that with trisodium phosphate dodecahydrate) showed marked instability after storage at room temperature for 1 month. The other blends did not show a decline in potency by chemical assay when stored at room temperature for 6 months. At 50°, the mixture of cephradine with sodium phosphate dodecahydrate exhibited complete loss of potency as determined by chemical assay after 2 weeks of storage, while testing for at least 1 month was required to make a judgment of instability for the other mixtures. By use of DTA, the cephradine

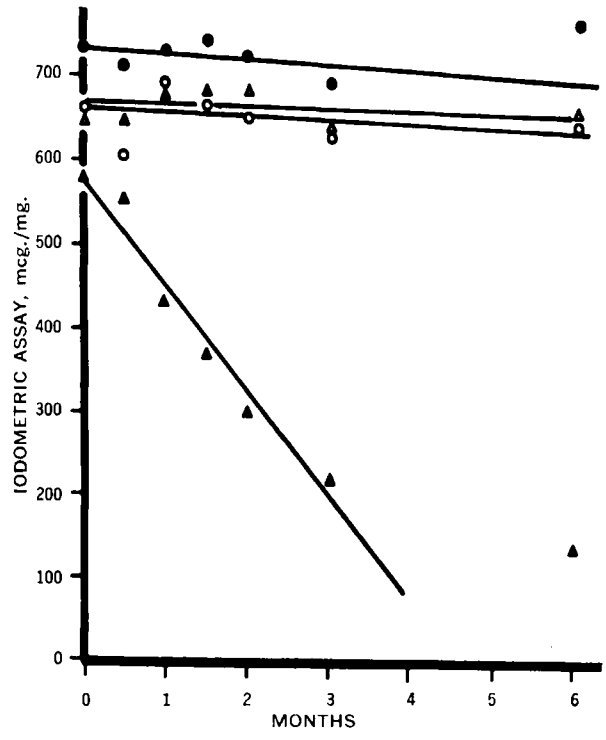

Figure 3—*Potencies of blends of cephradine as a function of time at 23°. Key for adjuvants: ▲, trisodium phosphate dodecahydrate; O, tromethamine; △, N-methylglucamine; and ●, anhydrous sodium carbonate.*

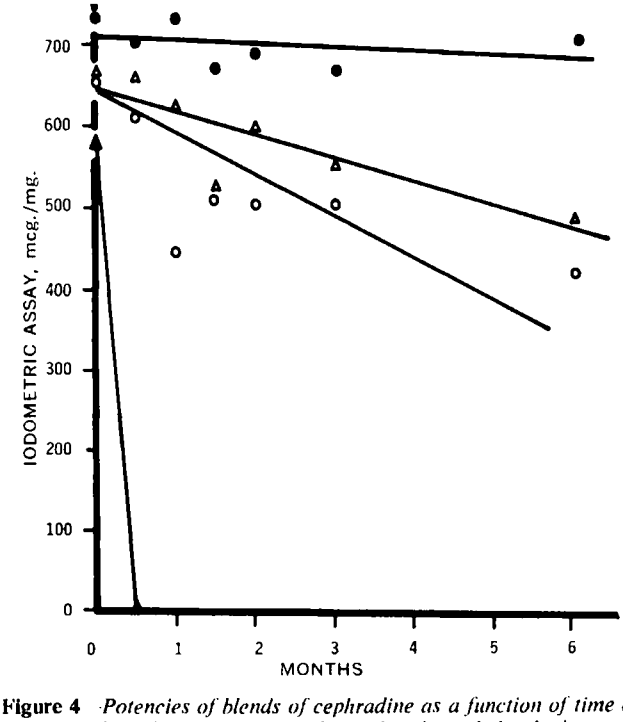

Figure 4—*Potencies of blends of cephradine as a function of time at 50°. Key for adjuvants: ▲, trisodium phosphate dodecahydrate; O, tromethamine; △, N-methylglucamine; and ●, anhydrous sodium carbonate.*

sodium carbonate mixture could be predicted to be the most stable blend after 1 day of testing. The prediction was clearly confirmed by results of a classical stability study requiring up to 6 months of storage at 50°.

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▲ To whom inquiries should be directed.

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J. BLANCHARD*, T. N. TOZER▲, D. L. SORBY, and L. DALLAS TUCK

Abstract □ Three spin labels were used to monitor possible drug-induced conformational changes in bovine serum albumin. The electron spin resonance spectra of labeled bovine serum albumin in all cases consisted of two subspectra corresponding to labels in a partially immobilized environment and a strongly immobilized environment. Only bovine serum albumin with the triazine spin label was suitable for quantitation of data. The strongly protein-bound acidic drug, phenylbutazone, caused a conversion of strongly immobilized sites to partially immobilized sites, an effect that was proportional to the amount of drug added. This probably was due to the initial drug binding inducing a conformational change in the bovine serum albumin, thereby exposing additional binding sites.

Keyphrases □ Drug–albumin interactions—use of three spin labels to monitor drug-induced protein conformational changes, electron spin resonance spectra □ Albumin–drug interactions—use of three spin labels to monitor drug-induced protein conformational changes, electron spin resonance spectra □ Spin labeling—monitoring drug-induced conformational changes in bovine serum albumin, three spin labels studied

The technique of spin labeling was recently applied to a number of macromolecular systems where conformational changes were suspected (1, 2). This report describes, for the first time, an attempt to monitor drug-induced changes in bovine serum albumin utilizing this method. Although various analogous systems have been studied similarly (3–5), the pharmacodynamically important drug–albumin system (6, 7) has not been investigated; preliminary data are reported here.

EXPERIMENTAL

Materials and Methods—The spin labels *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)bromoacetamide and *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide were purchased[1]. The spin label 2,2,6,6-tetramethyl-4-dichlorotriazineaminopiperidine-1-*N*-oxyl was synthesized according to a reported procedure (8). These spin labels are referred to here as bromoacetamide, maleimide, and triazine, respectively.

Prior to use, the crystalline bovine serum albumin[2] was dialyzed against several changes of glass-distilled water at 4° followed by lyophilization. Protein concentration was checked by the biuret assay (9) or by measurement of absorbance at 279 nm., using a = 0.667 (10). Spin-labeled derivatives of bovine serum albumin were prepared using techniques similar to those previously reported (8, 11). All solutions were prepared and labeled in phosphate buffer of pH 6.9 (μ = 0.0527). Electron spin resonance spectra were recorded on an *X*-band spectrometer, utilizing 100-kHz. field modulation and Fieldial control of the magnetic field. The titration of triazine spin-labeled bovine serum albumin with increasing amounts of phenylbutazone was performed at ambient temperature using a quartz flat cell and a syringe pump set up similarly to one described elsewhere (3). Each spectrum could be recorded under identical instrumental conditions, thereby facilitating direct comparison of successive spectra.

RESULTS AND DISCUSSION

The electron spin resonance spectra obtained with both maleimide and bromoacetamide spin-labeled bovine serum albumin did not change appreciably when interacted with phenylbutazone. This could be due to the fact that these two sulfhydryl group-directed labels were located at a site far removed from the binding locus for phenylbutazone. Another possibility is that these labels may have been present in too low a concentration to have their spectra detectably altered by the binding process. It is also possible that the sulfhydryl grouping may be in a region of the protein that results in the attached labels being too strongly immobilized for their spectra to be adequately perturbed by the binding process.

In contrast, the amino group-directed triazine spin label, which at pH 6.9 is linked solely to histidine residues (8), appears to contain a ratio of strongly immobilized sites amenable to quantitation. Figure 1A shows that the spectrum of triazine spin-labeled bovine serum albumin actually consists of two types of signals, a sharp three-line spectrum corresponding to a label at a partially immobilized site and a superimposed broadened spectrum characteristic of the label in a strongly immobilized environment. Figure 1B shows the effect of adding 24 moles of phenylbutazone/mole of labeled bovine serum albumin. This spectrum represents the endpoint in the titration, because this was the maximum amount of phenylbutazone added. Spectra of several intermediate ratios of phenylbutazone to labeled bovine serum albumin were also recorded. Figure 2 shows a plot of the relative intensities of the high field peak of the partially immobilized spectrum (peak 4) *versus*

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[2] A grade, Calbiochem.

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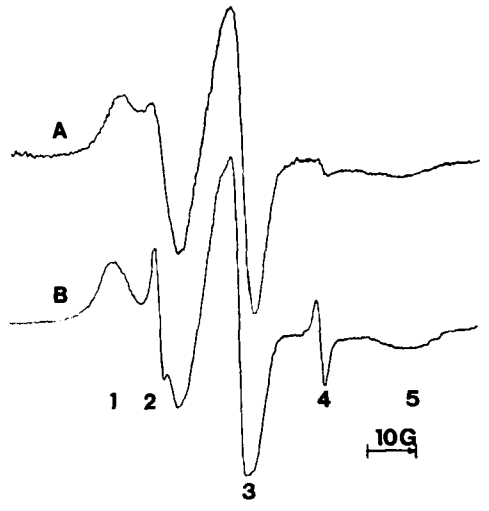

Figure 1 *Electron spin resonance spectrum of triazine spin-labeled bovine serum albumin before (A) and after (B) addition of 24 moles of phenylbutazone. Peaks 1, 3, and 5 correspond to strongly immobilized sites and peaks 2, 3, and 4 correspond to partially immobilized sites.*

increasing drug concentration. The increase in this peak was taken as a measure of the conversion of strongly immobilized sites to partially immobilized sites as a result of the interaction of the phenylbutazone with bovine serum albumin. This parameter was chosen since it appeared to be little affected by overlap of the strongly immobilized spectrum and, therefore, was a relatively accurate measure of the number of partially immobilized sites actually present.

The fact that this effect seems to increase monotonically up to molar ratios of 20:1 or more before leveling off seems to indicate that there is indeed more than one binding site for phenylbutazone on bovine serum albumin. This could possibly be due to the initial binding of the drug inducing a conformational change in the albumin (reflected by the triazine spin label as a conversion of strongly immobilized sites to partially immobilized sites), thereby enabling the macromolecule to accommodate successive drug molecules. The possibility of these secondary binding sites becoming available only after occupation of the primary binding site without a conformational alteration of the bovine serum albumin seems unlikely.

Chignell (12) and Chignell and Starkweather (13), using circular dichroism spectrometry, correlated the molar ellipticity of the complex between phenylbutazone and human serum albumin with the binding of phenylbutazone. The molar ellipticity increased up to a limit of 2.6 moles of drug bound/mole of albumin. This extrinsic Cotton effect is a result of a perturbation of the carbonyl chromophore of phenylbutazone when bound to the albumin. In the present studies, it is the effect of binding on the environment of the spin label attached to the albumin that is being observed. These two different approaches to the study of phenylbutazone-albumin interactions yield different kinds of information about the binding process. in these studies the environment of the spin label is apparently altered at drug to albumin concentration ratios above which the molar ellipticity seems to have a limiting value.

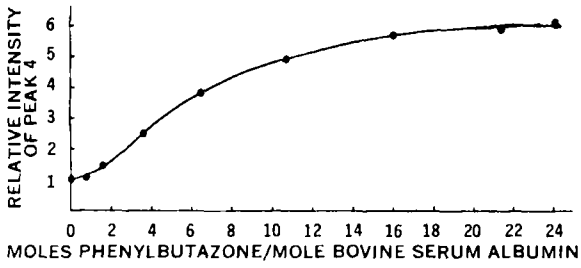

Figure 2—*Plot of the relative spectral intensity of peak 4 against the molar ratio of phenylbutazone–bovine serum albumin.*

It would be instructive to know the extent to which the observed spectral changes correspond to the actual degree of binding of the drug molecule. In addition, some data on the universality of this effect with other strongly binding drugs are desirable. These areas are being investigated further, and complete details of this work will appear shortly.

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New Schistosomicidal and Carcinostatic Agents of the Thiaxanthone Type III

I. NABIH[▲] and A. ZAYED

Abstract □ Compounds structurally related to cyclohexenothiaxanthone were synthesized. In biological testing, a representative compound, 1-(β-diethylaminoethylamino)-3,4-cyclohexenothiaxanthone, showed pronounced schistosomicidal and antitumor activity.

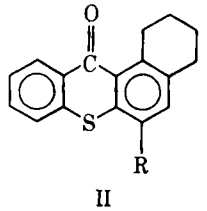
Keyphrases □ Thiaxanthone derivatives—synthesized and screened as potential schistosomicidal and carcinostatic agents □ 1-(β-Diethylaminoethylamino)-3,4-cyclohexenothiaxanthone—synthesized and screened as potential schistosomicidal and carcinostatic agent □ Schistosomicidal agents, potential—synthesis and screening of cyclohexenothiaxanthone analogs □ Carcinostatic agents, potential—synthesis and screening of cyclohexenothiaxanthone analogs

The thiaxanthone derivatives are considered to be the first nonmetallo-organic compounds of biological activity in the chemotherapy of schistosomiasis (1). They have received increasing interest since the discovery of their carcinostatic activity (2, 3). The most promising member in this group is 1-(β-diethylaminoethylamino)-4-methylthiaxanthone (I), but this agent is poorly tolerated by patients and usually induces severe unfavorable side effects during treatment (4). Because of these considerations, efforts were made to synthesize and develop new related structures suitable for application in both schistosomiasis and cancer.

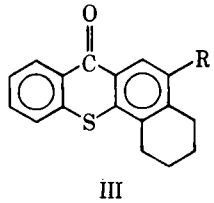
DISCUSSION

In a previous article[1] (5), the total syntheses and biological activity of two new agents related to cyclohexenothiaxanthone, namely, 1,2-cyclohexeno-4-(β-diethylaminoethylamino)thiaxanthone (II) and 2-(diethylaminoethylamino)-3,4-cyclohexenothiaxanthone (III), were described. The rationale for including the reduced tetralin system in these structures was also discussed.

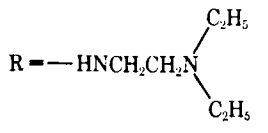
The approach to the synthesis of both II and III began with sulfuric acid condensation of thiosalicylic acid with the corresponding chlorotetralin compound. However, attempts to introduce the desired diamino side chain by replacing the chlorines in both condensation products failed. The chlorine atoms in both products, 2-chloro-3,4-cyclohexenothiaxanthone and its isomer 4-chloro-1,2-cyclohexenothiaxanthone, were resistant to any further substitution. This could be explained by the absence of the activating effect of the carbonyl groups on the lability of both these chlorine atoms. Such an effect suggested preferential condensation of the diamino side chain with 1-chloro-4-methylthiaxanthone, as with the synthesis of I (1).



II



III



Alternatively, both II and III were obtained through the condensation of the corresponding acetaminotetralin with thiosalicylic acid in the presence of sulfuric acid, followed by subsequent hydrolysis and condensation of the free amine with the desired side chain. Generally, in this type of condensation of thiosalicylic acid with aromatic components by sulfuric acid, the sulfide function forms first. The configuration of the condensation products depends upon the nature of these components (6). In the case of 5-chlorotetralin or 5-acetaminotetralin, the sulfide linkage initially formed at the 8-position, since this is activated due to the presence of the chlorine located *para* and the cyclic methylene group located *ortho*. In electrophilic substitution, *e.g.*, sulfonation, 5-chloro-8-sulfonic acid is the exclusive product (7). With 6-chlorotetralin and 6-acetaminotetralin, the 7-position is preferred for sulfide linkage formation.

In this work, the synthesis of another isomer of II and III, namely, 1-(β-diethylaminoethylamino)-3,4-cyclohexehothiaxanthone (IV), was of interest. This compound represents a direct structural analogy to I, where the cyclic methylene stands for the 4-methyl group located *para* to the diamino side chain. The synthesis of IV by any described approach using sulfuric acid condensation with chlorotetralin or acetaminotetralin was not successful, so other routes were tried. Diazotized 5-amino-7-nitrotetralin (8) reacted with thiosalicylic acid to give the intermediate, 2',3'-cyclohexeno-5'-nitrodiphenylsulfide-2-carboxylic acid (V). Reactions of the diazotized amines with thiophenols led to the formation of the sulfide linkage (9). Reduction of V gave the free amine VI which, upon cyclization with polyphosphoric acid, gave 1-amino-3,4-cyclohexenothiaxanthone (VII). Condensation of VII with β-chlorodiethylaminoethylamine gave the desired Compound IV.

The acidic intermediate V could also be obtained in better yield through the reaction of 5-iodo-7-nitrotetralin (10) with thiosalicylic acid in the presence of sodium carbonate. Furthermore, this route of synthesis was applied as an alternative for the preparation of II. For this purpose, diazotized 6-amino-7-nitrotetralin (11) reacted with thiosalicylic acid to build the acid intermediate, 2'-nitro-4',5'-cyclohexenodiphenylsulfide-2-carboxylic acid (VIII). Similarly, this was obtained through the reaction of 6-iodo-7-nitrotetralin (12) with thiosalicylic acid in the presence of sodium carbonate. Upon cyclization of VIII, 1,2-cyclohexeno-4-nitrothiaxanthone (IX) was obtained. This compound was reduced by hydrochloric acid and iron powder to give the free amine, 1,2-cyclohexeno-4-aminothiaxanthone (X). This product was identical to that obtained from the previously described synthesis of II (5).

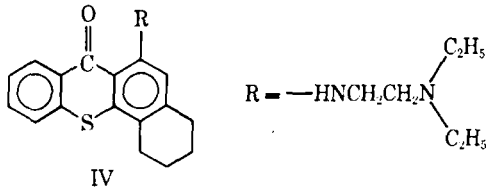
Compound III also was prepared by this route. Diazotized 5-amino-8-nitrotetralin (13) was reacted with thiosalicylic acid; the product, 2',3'-cyclohexeno-4'-nitrodiphenylsulfide-2-carboxylic acid (XI), was reduced to give 2',3'-cyclohexeno-4'-aminodiphenylsulfide-2-carboxylic acid (XII). Compound XII, upon cyclization with polyphosphoric acid, gave the amine, 2-amino-3,4-cyclohexenothiaxanthone (XIII). This product was identical to that obtained from the previously described synthesis of III (5).

BIOLOGICAL TESTING

The biological data for Compounds II and III in schistosomiasis and cancer were reported previously (5). In this work the isomer IV was submitted for testing as follows.

Schistosomiasis—Two groups of six mice, weighing 20–24 g. each, were infected with cercaria of *Schistosoma mansoni* obtained from a number of snails to ensure that both sexes were in the infecting cercaria. Ten weeks after infection, both groups showed viable eggs in their stools. The six members of one group were given

[1] Part I of this work. Part II will be published in *J. Chem. UAR.*



IV

daily oral doses (in water solution) of 80 mg./kg. body weight of IV as the hydrochloride, through a polyethylene feeding tube, for 12 consecutive days. The six mice in the other group were controls. After 1 week from the end of treatment, regular examination for ova excretion (every other day) was carried out for 3 weeks. In the treated group, a gradual decrease in the ova count was observed and ova excretion ceased by the end of the observation period (3 weeks). In the control group, ova excretion continued without remarkable change. Upon sacrificing the animals in both groups, living worms were found distributed in the liver and mesenteric venules. In the treated group, dead worms were present in the liver.

Experimental Tumors—*ELD (Ehrlich Ascites Carcinoma)*—Two groups of four mice each (ABYXdba strain), weighing 20–25 g., were inoculated intraperitoneally with 10^6 living tumor cells of the ELD type. In one group, Compound IV (as the hydrochloride in water solution) was injected intraperitoneally daily at doses of 80 mg./kg. body weight on the 3rd day after inoculation and for 6 consecutive days. The other group was kept as the control. Observation and weight control continued for 6 weeks. After 10 days, the control group showed clear tumor development with a remarkable increase in weight. In the treated group, no signs of tumor development were observed and there was no appreciable change in weight. Upon sacrificing the animals in both groups, living tumor cells were found in the controls; in the treated group, no tumor cells were found.

TA_3 (Mammary Carcinoma)—Two groups of four mice, weighing 16–18 g. each, were inoculated with 10^6 cells of the TA_3 type. On the 3rd day after inoculation and for 6 consecutive days, one group of four mice was treated intraperitoneally with 80 mg./kg. of Compound IV (as the hydrochloride in water solution). The other group was left untreated as a control. After 10 days, the control animals showed development of the transplanted tumor accompanied by an increase in weight (20–22 g.). In the treated mice, no remarkable change in weight was observed. After sacrificing the animals in both groups, no tumor cells were found in treated animals; in the controls, living tumor cells were present.

Regression of well-established experimental tumors by purely chemical means is seldom seen (14). Thus, testing the compound's effect on established tumors was of interest.

Treatment of established ascites tumors of the ELD type (14–18 days postinoculation with 2×10^6 living tumor cells) by daily doses of 80 mg./kg. for 4 consecutive days showed marked regression in the tumor sizes and cell counts, *e.g.*, the number counted before treatment was 900×10^6 cells/ml., and after treatment the number found was 26×10^6 cells/ml. After sacrificing the treated animals, masses of dead cells were found on the wall of the abdominal cavity.

Established mammary tumors, TA_3 type (8–10 days postinoculation with 10^6 living tumor cells) were treated with 80 mg./kg. of the compound for 4 days. Marked regression in tumor size and cell count was observed. For example, a mouse bearing a tumor cell count of 171×10^6/ml. showed a posttreatment cell count of 15×10^6/ml. The treated cells showed vacuolization and dispersion. This was contrary to control, nontreated cells which usually aggregate in colonized groups. This observed intracellular dispersion could be attributed to the electrostatic interaction between the polarized thiaxanthone molecule [due to electron delocalization (15), measured dipole moment for the compound 5.8 Debye units] and the tumor cells which carry high electronegative charges (16). This property provides a clue for the selective attack on tumor cells.

Preliminary studies in human leukemia (myeloblastic and monocytic types) *in vitro* showed pronounced effect of the compound toward the leucocytes.

The observed antitumor activity of the compound could be rationalized on the basis that the thiaxanthone system serves as a target-seeking device or carrier to bring the tetralin part of the molecule to the tumor cells, since tetralin tends to fix molecular oxygen in an oxygenation process (17). This would affect the glycolytic rate in the cell through oxidation of the sulfhydryl (—SH) enzymes engaged in this metabolic process. If cell division depends on the presence of small quantities of sulfhydryl, then the division could be inhibited by keeping the sulfhydryl in an oxidized condition by the presence of such oxygen carriers. Furthermore, the tetrahydronaphthalene system could undergo biological transformation to a naphthoquinone-type structure which depresses cell division. Also, the thiaxanthone system could act through complexing with DNA strands in the tumor cells, thus inhibiting DNA-dependent RNA synthesis. Biochemical studies are underway to determine the possibility of complex formation with this compound.

EXPERIMENTAL[2]

2′,3′-Cyclohexeno-5′-nitrodiphenylsulfide-2-carboxylic Acid (V)—*Method A*—To a mixture of 5-amino-7-nitrotetralin (10 g.) and sulfuric acid (40 ml., 50%) at 0° was added dropwise a solution of sodium nitrite (5 g. in 30 ml. water). The diazonium solution was then poured into a stirred solution of thiosalicylic acid (7 g. in 100 ml. 35% NaOH), heated on a steam bath under stirring for 3 hr., and cooled, and the supernate was then decanted. The residue was boiled with water and filtered while hot, and the filtrate was acidified with acetic acid. The separated product was purified by redissolving it in warm ammonium hydroxide solution and filtering. The filtrate was acidified with acetic acid and filtered. The product was dried to give 11 g. of V (69%), and it was recrystallized from benzene–alcohol, m.p. 210–212°.

Anal.—Calc. for $C_{17}H_{15}NO_4S$: C, 62.07; H, 4.59; N, 4.25; S, 9.73. Found: C, 62.18; H, 4.63; N, 4.42; S, 10.12.

Method B—A mixture of 5-iodo-7-nitrotetralin (2.4 g.), thiosalicylic acid (1.2 g.), sodium carbonate (1 g.), amyl alcohol (15 ml.), and 0.2 g. copper powder was refluxed for 20 hr. Five milliliters of sodium hydroxide solution was then added and the alcohol was steam distilled. The residue was boiled with excess water (25 ml.) and filtered, and the filtrate was acidified after cooling with dilute hydrochloric acid. The formed product weighed 1.90 g. (74%) after drying. Upon recrystallization from benzene–alcohol, it had a melting point of 210–212°, with no depression in the melting point when mixed with the product obtained by Method A.

2′,3′-Cyclohexeno-5′-aminodiphenylsulfide-2-carboxylic Acid (VI)—The nitro Compound V (3 g.) was stirred on a water bath with acetic acid (30 ml.), and then iron powder (3 g.) was added portionwise. After addition of half of the iron, 10 ml. acetic acid was added. Heating was continued for 2 hr. The mixture was filtered while hot, and the residue was washed with additional hot 50% acetic acid. The combined filtrate was poured into ice water. The formed precipitate was collected, washed with water, and dried to give 2.10 g. (74%) of VI. It was recrystallized from ethanol, m.p. 214–216°.

Anal.—Calc. for $C_{17}H_{17}NO_2S$: C, 68.27; H, 5.73; N, 4.68. Found: C, 68.08; H, 5.69; N, 4.65.

1-Amino-3,4-cyclohexenothiaxanthone (VII)—Sixteen grams of phosphoric acid was heated at 190°; then 2 g. of VI was added portionwise, and heating at this temperature was continued for 2 hr. Then the mixture was cooled and poured into ice water. The formed precipitate was collected, washed with hot ammonium hydroxide solution and then water, and dried. It gave 1.7 g. (90%) of VII. Upon recrystallization from benzene, it melted at 198–200°.

Anal.—Calc. for $C_{17}H_{15}NOS$: C, 72.56; H, 5.37; N, 5.37. Found: C, 72.78; H, 5.43; N, 5.46.

1-(β-Diethylaminoethylamino)-3,4-cylohexenothiaxanthone (IV)—A mixture of VII (2 g.) and β-chlorodiethylaminoethylamine (1.5 ml.) was heated at 180° for 4 hr. It was boiled with 25 ml. water for 0.5 hr., and then 4 ml. of concentrated hydrochloric acid was added; it was heated and filtered while hot. The filtrate was basified with sodium hydroxide solution; the formed precipitate was collected, washed with water, and then dried. It gave 2.26 g. of V (82%). Upon recrystallization from ethanol, it melted at 94–96°; IR: 1630 (C=O), 3340 (—NH—), 3017 (cyclohexyl), and 680 (—S—) $cm.^{-1}$.

Anal.—Calc. for $C_{23}H_{28}N_2OS$: C, 72.29; H, 7.41; N, 7.36; S, 8.43. Found: C, 72.34; H, 7.48; N, 7.39; S, 8.51.

The hydrochloride was prepared by dissolving the base in dry ether and allowing a stream of hydrogen chloride gas to pass through the solution, whereby it separated as a deep-orange product, m.p. 202–204°. It was highly hygroscopic and soluble in water.

[2] All melting points were taken in open capillaries using a Gallenkamp melting-point apparatus and are uncorrected. Microanalyses were performed at the National Research Center (microanalytical unit) and Spang Microanalytical Laboratory, Ann Arbor, Mich.

2′-Nitro-4′,5′-cyclohexenodiphenylsulfide-2-carboxylic Acid (VIII)—*Method A*—A mixture of 6-amino-7-nitrotetralin (6 g.) and concentrated hydrochloric acid (20 ml.) in 10 ml. of water was stirred at 0°, and then a solution of sodium nitrite (2.5 g. in 10 ml. water) was added. The diazonium solution was added portionwise to a stirred solution of thiosalicylic acid (3.5 g. in 50 ml. 20% NaOH solution) and then heated on a steam bath for 3 hr. After cooling, the supernatant layer was decanted and the residue was boiled with water. This was filtered while hot, and the filtrate was acidified after cooling with acetic acid. The product was redissolved in hot ammonium hydroxide solution, filtered, and then acidified with acetic acid. The collected product was dried to give 5.83 g. of VIII (64%). Upon recrystallization from benzene–alcohol, it melted at 162–164°.

Anal.—Calc. for $C_{17}H_{15}NO_4S$: C, 62.07; H, 4.59; N, 4.25; S, 9.73. Found: C, 62.32; H, 4.68; N, 4.37; S, 9.92.

Method B—A mixture of 6-iodo-7-nitrotetralin (3 g.), thiosalicylic acid (1.5 g.), amyl alcohol (30 ml.), sodium carbonate (1.3 g.), and 0.3 g. of copper powder was refluxed for 17 hr., and then 6 ml. of 50% NaOH was added. The alcohol was removed through steam distillation. The residue was boiled with 50 ml. water and filtered, and the filtrate was acidified with dilute hydrochloric acid. The formed product was collected, washed with water, and dried to give 2.4 g. of VIII. Upon recrystallization from benzene–alcohol, it melted at 162–164°, with no depression in melting point when mixed with the product prepared by Method A.

1,2-Cyclohexeno-4-nitrothiaxanthone (IX)—A mixture of VIII (2 g.) and phosphorus oxychloride (10 ml.) was refluxed for 1 hr. Then the excess of the oxychloride was removed under reduced pressure, and the residue was refluxed with 1 *N* HCl (50 ml.) for 30 min. The formed precipitate was filtered, washed with hot ammonia solution and then with water, and dried to give 1.5 g. of IX (78%). Upon recrystallization from benzene, it melted at 167–169°.

Anal.—Calc. for $C_{17}H_{13}NO_3S$: C, 65.58; H, 4.20; S, 10.29. Found: C, 65.67; H, 4.26; S, 10.38.

1,2-Cyclohexeno-4-aminothiaxanthone (X)—Compound IX (1 g.) and concentrated hydrochloric acid (5 ml.) in 20 ml. of ethyl alcohol were refluxed on a steam bath, and 1 g. of iron powder was added portionwise. Reflux continued for 4 hr. Then the mixture was cooled and the formed product was filtered. Sodium hydroxide solution (50%) was added to the filtrate to give 0.6 g. of X. It was recrystallized from benzene, m.p. 182–183°. The compound was identical to that previously prepared (5) as an intermediate in the synthesis of II.

2′,3′-Cyclohexeno-4′-nitrophenylsulfide-2-carboxylic Acid (XI)—A mixture of 5-amino-8-nitrotetralin (3 g.) and 50% sulfuric acid (20 ml.) was stirred at 0°. A solution of sodium nitrite (1 g.) in 15 ml. of water was added dropwise. Then the diazonium solution was added gradually to a solution of 1.6 g. of thiosalicylic acid in 50 ml. of 50% NaOH and heated on a water bath. After 2 hr., the mixture was cooled and the supernate was decanted. The residue was boiled with water and filtered, and the filtrate was acidified with acetic acid. The obtained product was dissolved in dilute ammonia and filtered. The filtrate was acidified, and the product was dried to give 2.8 g. of XI. Upon recrystallization from benzene–alcohol, it melted at 220°.

Anal.—Calc. for $C_{17}H_{15}NO_4S$: C, 62.07; H, 4.59; N, 4.25. Found: C, 61.78; H, 4.82; N, 4.51.

2′,3′-Cyclohexeno-4′-aminodiphenylsulfide-2-carboxylic Acid (XII)—A solution of IX (2 g.) in 25 ml. of acetic acid was stirred on a steam bath, and 2 g. of iron powder was added portionwise. Then 20 ml. of water was added. Heating was continued for 3 hr. The hot mixture was filtered and washed with dilute acetic acid. The filtrate was cooled and poured into ice water; the formed product was collected, washed with water, and dried to give 1.2 g. of XII. After recrystallization from benzene, it melted at 217–218°; IR: 3010 (cyclohexyl) and 690 (—S—) $cm.^{-1}$.

Anal.—Calc. for $C_{17}H_{17}NO_2S$: C, 68.34; H, 5.73; N, 4.69. Found: C, 68.42; H, 5.78; N, 4.76.

2-Amino-3,4-cyclohexenothiaxanthone (XIII)—To 10 g. of polyphosphoric acid heated at 170° was added 1 g. of XII; then heating was continued for 1 hr. After cooling, the mixture was poured into ice water. An excess of ammonia solution was added, and the solution was filtered. The formed product was washed with hot water and dried to give 0.70 g. of XIII, m.p. 235–237° [lit. (5) m.p. 239–240°].

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Spectrofluorometric Determination of Carbidopa [L-(−)-α-Hydrazino-3,4-dihydroxy-α-methylhydrocinnamic Acid] in Plasma

S. VICKERS▲ and E. K. STUART

Abstract □ A spectrofluorometric assay is described for carbidopa utilizing *p*-dimethylaminobenzaldehyde. The fluorophore is dimethylaminobenzalazine which fluoresces intensely in chloroform saturated with trichloroacetic acid. The assay was applied to the analysis of carbidopa in monkey plasma.

Keyphrases □ Carbidopa—spectrofluorometric determination, monkey plasma □ L-(−)-α-Hydrazino-3,4-dihydroxy-α-methylhydrocinnamic acid—spectrofluorometric determination, monkey plasma □ Spectrophotofluorometry—analysis of carbidopa in monkey plasma

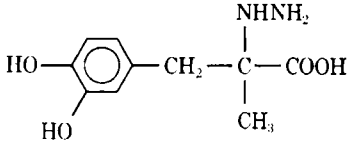
Carbidopa [L-(−)-α-hydrazino-3,4-dihydroxy-α-methylhydrocinnamic acid] is an inhibitor of aromatic amino acid decarboxylase, and it is able to potentiate levodopa in the treatment of Parkinson's disease (1–3). Carbidopa decreases the peripheral decarboxylation of dopa but not the conversion of dopa to dopamine in the brain, since carbidopa does not penetrate the blood–brain barrier (4).

To measure levels of carbidopa in plasma after oral administration of the drug in therapeutic doses, a sensitive assay was required. A fluorometric method was developed which depends upon the formation of dimethylaminobenzalazine, chloroform solutions of which fluoresce in the presence of trichloroacetic acid.

EXPERIMENTAL

Analytical Method—Trichloroacetic acid[1], chloroform[2], *p*-dimethylaminobenzaldehyde[3], and anhydrous magnesium sulfate[1] were used in these studies.

Plasma (2 ml.) was mixed with aqueous 10% trichloroacetic acid (3 ml.) and centrifuged (15 min.). The supernate was removed (as much as possible) and shaken with chloroform (2 ml.) for 15 min.; the aqueous layer was removed (as much as possible) and mixed with 0.4% ethanolic *p*-dimethylaminobenzaldehyde (2 ml.). The solution was heated at 70° for 45 min., cooled, and shaken with chloroform (3 ml.) for 10 min. The chloroform layer was removed following centrifugation, and anhydrous magnesium sulfate (10 mg.) was added. After centrifugation, the solution was analyzed spectrophotometrically in a spectrophotofluorometer[4] using quartz cells (10-mm. lightpath). Carbidopa analyzed in this manner exhibited a maximum value of fluorescence at an excitation wavelength of 466 nm. and an emission wavelength of 546 nm.



I: carbidopa

[1] Baker Analyzed.
[2] Fisher Analytical.
[3] Fisher Certified.
[4] Aminco-Bowman.

Table I—Reproducibility of the Fluorescence Assay for Carbidopa[a]

Carbidopa, mcg./2 ml.	Arbitrary Fluorescence Units ± *SD*
2	10.5 ± 0.7
1	4.9 ± 0.4
0.5	2.3 ± 0.3
0.1	0.4 ± 0.05

[a] Four experiments.

Identification of Fluorophore—Carbidopa (0.55 g.) was dissolved in the least amount of 1 *N* HCl possible, and ethanol (10 ml.) was added. This solution was added to an ethanolic solution (10 ml.) of *p*-dimethylaminobenzaldehyde (0.74 g.). After 30 min. at 70°, solid sodium acetate was added until precipitation occurred. The precipitate was filtered and recrystallized from dimethylformamide to yield 346 mg. material, m.p. 274–275°.

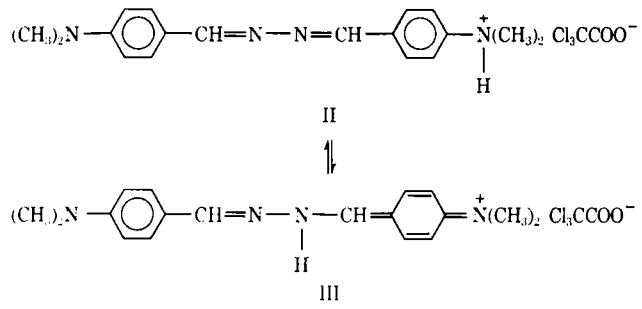
Animal Experiments—Five female rhesus monkeys (3–4 kg.) were dosed orally with an aqueous solution of carbidopa. Three received a dose of 20 mg./kg. and two received a dose of 2 mg./kg. Plasma was drawn at 1, 2, and 4 hr., and levels of carbidopa were measured with a calibration curve constructed by using normal plasma spiked with known amounts of carbidopa and plotting the fluorescence of the chloroform solution (after correcting for the blank value) *versus* the concentration of carbidopa in plasma.

RESULTS

Eighty nanograms of carbidopa/2 ml. of plasma could be detected by this method. The sensitivity of the method could be increased by using larger volumes of plasma since this caused no concomitant increase in background fluorescence. To test the reproducibility of the method, plasma samples (2 ml.) containing carbidopa (1, 0.5, 0.25, and 0.05 mcg./ml.) were analyzed (Table I). The intensities of the fluorescence obtained in these experiments were compared to those of standard solutions of the azine in chloroform saturated with trichloroacetic acid. It was determined that the reaction and the extraction of the product were essentially complete (93.5% ± 4.4 *SD*) over the 0.1–2-mcg. range of carbidopa/ml. of plasma. A water bath temperature of 70° and a 45-min. incubation were optimum conditions for maximum intensity of fluorescence. The latter was reduced when ethyl acetate or butanol was substituted for chloroform. Magnesium sulfate, unlike sodium chloride or hydrochloric acid, did not decrease the sensitivity of the assay. Trichloroacetic acid served a dual purpose: precipitating

Table II—Identification of Dimethylaminobenzalazine by Comparative TLC

Solvent System	R_f of Fluorescent Product	R_f of Authentic Dimethylaminobenzalazine
Benzene–acetic acid (3:2)	0.05	0.05
Benzene–acetic acid (2:3)	0.1	0.1
Benzene–acetic acid (4:1)	Origin	Origin
Benzene–acetone–water–acetic acid–butanol (equal parts)	0.92	0.92
Ethanol–acetic acid (1:1)	0.81	0.78



Scheme I—Ion-pair formation between dimethylaminobenzalazine and trichloroacetic acid

protein and increasing the fluorescence intensity of the product. There was no advantage in using trichloroacetic acid concentrations greater than 10%.

Carbidopa was shown to form dimethylaminobenzalazine (mixed melting point 274°) when reacted with dimethylaminobenzaldehyde. The structure of the product was confirmed by the use of mass spectroscopy and comparative TLC (Table II), which also did not indicate the presence of another product.

Anal.—Calc. for $C_{18}H_{22}N_4$: C, 73.45; H, 7.48; N, 19.05. Found: C, 73.52; H, 7.85; N, 18.98.

The recovery of the product and the reproducibility of the assay at levels anticipated after clinical doses[5] of carbidopa in man were found to be satisfactory.

Interfering Substances—No interference was produced by dopa, dopamine, phenylhydrazine, tryptamine, or tyrosine at levels of 3, 1, 1, 10, and 10 mcg./ml. of plasma, respectively. The 3-*O*-methyl derivative of carbidopa will interfere, but this was not detected as a metabolite of carbidopa. Hydrazine will also interfere, and the method forms the basis for a very sensitive assay for hydrazine. However, hydrazine has not been identified as a metabolite of carbidopa[6].

Plasma Levels of Carbidopa in Monkeys—Levels of carbidopa were measured in the plasma of monkeys dosed with carbidopa (see *Experimental*). Maximum levels were usually achieved within 2 hr. (Table III). These doses gave plasma levels which were in the range described by the calibration curve, *i.e.*, 0.1–2 mcg./2 ml. plasma (Table I).

[5] Plasma levels of radioactivity in man (nine subjects) after administration of a single 50-mg. oral dose of carbidopa-^{14}C when expressed as equivalents of carbidopa ranged from 0.05 to 0.9 mcg./ml. (unpublished results of the authors).

[6] Unpublished observations of the authors.

Table III—Levels of Carbidopa[a] in Monkey Plasma after an Oral Dose

Monkey	Dose, mg./kg.	Carbidopa in Monkey Plasma—Hours 1	2	4
1	20	0.7	2.0	0.3
2	20	1.6	0.5	0.2
3	20	2.6	1.8	0.8
4	2	0.15	0.05	<0.03
5	2	<0.03	0.03	0.18

[a] Expressed as micrograms of carbidopa per milliliter of plasma.

DISCUSSION

In the present investigation, carbidopa formed the corresponding azine with dimethylaminobenzaldehyde without preliminary hydrolysis; it was not possible to detect the hydrazone. Structure III (5) may be the isomeric form of II that is responsible for the fluorescence properties of dimethylaminobenzalazine (Scheme I). This follows a similar suggestion made by Glazko *et al.* (6) to explain the observation that solutions of reserpine in ethylene dichloride become strongly fluorescent in the presence of trichloroacetic acid.

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Effect of Stimulation and Acetylcholine on ^{32}P and ^{14}C Incorporation into Phospholipids of Eel Electroplax

PHILIP ROSENBERG

Abstract □ Incorporation of ^{32}P and ^{14}C into phosphatidylinositol and phosphatidylcholine was increased when electroplax of the Sachs organ of the electric eel was exposed to 10^{-4} *M* concentrations of acetylcholine or physostigmine or when the cells were stimulated. In contrast, there was no significant effect on the specific activities of sphingomyelin, phosphatidylserine, phosphatidylethanolamine, or phosphatidic acid. The concentration of total phospholipids was unaltered. These results support the proposals that the neurotransmitter action of acetylcholine is associated with an increased turnover of phospholipids.

Keyphrases □ Acetylcholine—effects on ^{32}P and ^{14}C incorporation into eel electroplax phospholipids □ Electroplax, eel—stimulation and acetylcholine effects on ^{32}P and ^{14}C incorporation □ Radiolabeled phosphorus and carbon—effects of stimulation and acetylcholine on incorporation into eel electroplax phospholipids □ Stimulation—effects on ^{32}P and ^{14}C incorporation into eel electroplax phospholipids

Various functions have been proposed for phospholipids in bioelectrically excitable tissues including their possible involvement in the control of membrane resistance and capacitance, in the genesis of bioelectricity, as receptors for various drugs, and in active transport (1–7). Earlier studies from this laboratory, however, showed that axonal conduction and normal permeability were maintained in the squid giant axon even after extensive splitting of its phospholipids by phospholipases A and C (8–10). It was of interest to extend these studies to a synaptic-containing preparation since some investigators related the action of synaptic transmitters to an interaction with membrane phospholipids (6, 11–14). The isolated single electroplax used in the present study has acetylcholine as the neurotransmitter at the thousands of synapses present on each cell, and the conducting membrane can be stimulated either directly or indirectly *via* the nerves. The phospholipid composition of this preparation was recently reported (15) from this laboratory, and the postsynaptic and action potentials were noted to be blocked when more than one-third of the phosphatidylcholine of the cell was hydrolyzed by phospholipase A. As a further test of the possible involvement of phospholipids in the bioelectrical functioning of the electroplax, the incorporation of ^{32}P and ^{14}C into the phospholipids of control, stimulated, and acetylcholine- and physostigmine-treated electric tissue was measured.

MATERIALS AND METHODS

Cells from the Sachs organ were incubated in 7 ml. of eel Ringer's solution (pH 7) of the following composition (m*M*): NaCl, 160; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; NaH_2PO_4, 0.3; Na_2HPO_4, 1.2; and glucose, 10. In addition, each milliliter of the solution contained 25 μc. of ^{32}P-H_3PO_4[1] (>99% radiochemical purity) and 2.5 μc. of U-^{14}C-D-glucose[1] (>95% radiochemical purity, U = uniformly labeled). For the stimulation experiments, slices of Sachs organ, 1 cell thick and 15–30 cells long (1–2 g.), were incubated in this solution for 1 hr., after which the rows were stimulated with supramaximal voltages 5–10 times/sec. for 2 hr. with a 10-min. rest period every 20 min. In control experiments, the action potentials decreased no more than 20% during this 2-hr. period. Extracellular silver–silver chloride electrodes inserted into each end of the row were used for stimulating and recording electrical activity. Depending on the direction of the current, the innervated (conducting) membrane of the cell was either stimulated directly or indirectly *via* the nerve endings. Control slices were incubated for 3 hr. without stimulation.

In other experiments, 30 single isolated cells (~2 g.) from the Sachs organ were exposed for 6 hr. to eel Ringer's solution containing acetylcholine and physostigmine (10^{-4} *M* of each) or physostigmine (10^{-4} *M*) in addition to the radioactive compounds. This concentration of physostigmine decreases the action potential very slowly, taking several hours to block conduction, whereas acetylcholine plus physostigmine blocks electrical activity within 1 or 2 min. Control cells were exposed for the same length of time to a similar solution not containing acetylcholine or physostigmine. After incubation, lipid extracts of the tissues were prepared using chloroform–methanol (1:3, followed by 2:1) and were spotted on a two-dimensional thin-layer chromatogram used for the separation of the individual phospholipids (8–10, 15). Each phospholipid spot was visualized with iodine vapors, scraped off of the chromatogram, eluted with 4 ml. of chloroform–methanol (1:1), and divided into two equal samples. Both samples were evaporated to dryness, after which phosphorus was determined in one sample (16) and 10 ml. of scintillator fluid[2] was added to the other sample. This latter sample was counted in a scintillation spectrometer[3] with the windows set to count ^{14}C and ^{32}P simultaneously. An automatic external standard was used to monitor counting efficiency, and all samples were corrected for background, for radioactive decay of ^{32}P, and for the approximately 15% of the ^{32}P counts that appeared in the ^{14}C channel. A small portion (5%) of the original lipid extract was used for the determination of total lipid phosphorus. Elution of phospholipids from silica gel with chloroform–methanol may not completely extract the phospholipids (17–19); therefore, it is essential that all results be reported as specific activities, that is, counts per minute per micromole of phosphorus.

RESULTS

All treatments caused a significant increase in ^{32}P and ^{14}C incorporation into phosphatidylinositol and phosphatidylcholine (Tables I and II). No significant changes were seen in the specific activities of sphingomyelin, phosphatidylserine, phosphatidylethanolamine, or phosphatidic acid. Although there appeared to be a tendency toward an increase with phosphatidic acid, these results are probably the least reliable because of the very small amounts of phosphatidic acid present. In the initial experiments, it appeared that a large incorporation of ^{14}C into sphingomyelin was occurring, which would have been most unusual considering the known metabolic stability of this phospholipid in other membrane systems. Later it was found that this radioactivity was associated with a nonphosphorus-containing compound, which plated out on the sphingomyelin spot and adjacent area. By careful separation of supernates and phases during each step of the lipid extraction, especially at the stage of washing with pure solvent upper phase, it was possible to decrease markedly the amount of this contaminant, although the sphingomyelin values still probably represent maximum values.

[1] New England Nuclear Corp., Boston, Mass.

[2] Aquasol, New England Nuclear Corp., Boston, Mass.

[3] Packard model 3375.

Table I—^{32}P Incorporation into Phospholipids of the Sachs Organ of the Electric Eel[a]

Tissue Condition	c.p.m. (hundreds)/μmole P[b]					
	SM	PC	PI	PS	PE	PA
Single cells						
Control	3.1 ± 0.8	4.5 ± 0.2	43 ± 6	11 ± 4	9.7 ± 1.0	19 ± 7
ACh + Physo	2.2 ± 0.6	8.7 ± 0.4**	164 ± 9**	18 ± 6	9.2 ± 1.1	31 ± 3
Physo	3.7 ± 0.2	5.8 ± 0.5*	83 ± 4**	18 ± 4	10.3 ± 1.0	26 ± 6
Rows of cells						
Control	4.0 ± 1.1	5.2 ± 0.7	76 ± 10	4.3 ± 1.2	13 ± 5	13 ± 2
DS	3.0 ± 0.5	21 ± 3**	190 ± 19**	3.8 ± 0.8	14 ± 4	19 ± 2
IS	3.0 ± 0.7	29 ± 2**	217 ± 17**	2.4 ± 0.7	15 ± 3	13 ± 2

[a] SM = sphingomyelin, PC = phosphatidylcholine, PI = phosphatidylinositol, PS = phosphatidylserine, PE = phosphatidylethanolamine, PA = phosphatidic acid, ACh = 10^{-4} *M* acetylcholine, Physo = 10^{-4} *M* physostigmine, IS = indirect stimulation, and DS = direct stimulation. Single cells were exposed to 25 μc. ^{32}P-H_3PO_4/ml. incubation mixture for 6 hr., while rows of cells were exposed for 3 hr. [b] Each value (mean ± standard error) is based upon three experiments. ** = $p < 0.01$ compared to controls and * = $p < 0.05$ compared to controls.

Table II—^{14}C Incorporation into Phospholipids of the Sachs Organ of the Electric Eel[a]

Tissue Condition	c.p.m. (hundreds)/μmole P[b]					
	SM	PC	PI	PS	PE	PA
Single cells						
Control	17 ± 4	46 ± 4	130 ± 20	33 ± 2	42 ± 5	76 ± 45
ACh + Physo	21 ± 5	132 ± 11**	840 ± 50**	41 ± 9	31 ± 8	162 ± 50
Physo	24 ± 5	61 ± 5*	370 ± 90*	50 ± 10	28 ± 6	98 ± 30
Rows of cells						
Control	10 ± 3	24 ± 10	110 ± 20	14 ± 3	5 ± 2	44 ± 14
DS	7 ± 2	202 ± 23**	520 ± 40**	21 ± 7	7 ± 1	93 ± 23
IS	10 ± 3	111 ± 19**	320 ± 10**	14 ± 3	2 ± 1	89 ± 21

[a] Single cells were exposed to 2.5 μc. U-^{14}C-glucose/ml. of incubation mixture for 6 hr., while rows of cells were exposed for 3 hr. Abbreviations are given in Table I. [b] Each value (mean ± standard error) is based upon three experiments. ** = $p < 0.01$ compared to controls, and * = $p < 0.05$ compared to controls.

In four experiments, single cells were exposed to 2.5 μc. *N*-methyl-^{14}C-choline[1]/ml. of incubation mixture. More than 90% of the radioactivity incorporated into the phospholipids was found in phosphatidylcholine, a small amount was found in sphingomyelin, and phosphatidylethanolamine and phosphatidylserine remained nonradioactive. This is in agreement with earlier findings that choline is incorporated as a unit into lecithin (20–23).

Studies similar to those in the Sachs organ were also carried out on rows of cells from the Main organ, but the results were more variable and the ^{32}P incorporation was very low.

Total lipid phosphorus values of the tissues were not affected by stimulation or exposure to acetylcholine (Table III). The values for the Sachs organ are slightly higher and the values for Main organ are slightly lower than those previously found (15). The absolute values will, however, vary considerably, depending on the area of the Sachs and Main organs used. The closer to the head end of the eel the tissue samples are taken from, the more tightly packed are the cells and, therefore, the higher are the values for phosphorus per unit weight.

DISCUSSION

All experimental conditions studied caused depolarization of the innervated membrane of the electroplax. It is, however, unlikely that the results are due to an increased penetration and, therefore, an increase in the specific activity of ^{32}P-H_3PO_4 or U-^{14}C-glucose within the cell. If that were the case, one would expect to see increased specific activity of ^{32}P and ^{14}C in all phospholipids, whereas only an increased incorporation into phosphatidylcholine and phosphatidylinositol was found.

Acetylcholine, physostigmine, and stimulation cause an influx of sodium into the cell and an efflux of potassium, which may then be followed by an increase in active transport of sodium. The effects of these treatments on phospholipid metabolism may be associated with ionic changes occurring as a result of depolarization. Depending on conditions, ionic alterations may either decrease or increase ^{32}P incorporation into brain phospholipids (24, 25). The application of acetylcholine and physostigmine to a wide variety of tissues increases incorporation into both phosphatidic acid and phosphatidylinositol and may be due to a stimulation of active transport processes (26–31). In the electric organ, however, it has been reported (32) that phospholipids do not act as intermediates in the transport adenosine triphosphatase system.

The Main organ of the electric eel showed very low ^{32}P incorporation, probably because of the permeability barriers to the penetration of phosphorus in this tissue. The cells are much closer together in the Main organ than in the Sachs organ, so that it is not possible to dissect out single cells. By comparing the results shown in Tables I and II, one can see that even in the Sachs organ the incorporation of ^{14}C is much greater than the incorporation of ^{32}P, even though the specific activity of ^{32}P used in the incubation mixture was much higher than that of ^{14}C. Phosphorus does not readily penetrate through neuronal membranes (33–36), which could explain these findings.

Table III—Total Lipid Phosphorus Values for Sachs and Main Electric Organ[a]

Treatment	Sachs Organ		Main Organ
	Single Cells	Rows of Cells	Rows of Cells
Control	0.80 ± 0.05 (3)	1.33 ± 0.04 (3)	2.85 ± 0.15 (12)
ACh + Physo	0.78 ± 0.02 (3)	—	2.80 ± 0.19 (3)
Physo	0.73 ± 0.06 (3)	—	2.72 ± 0.19 (3)
DS	—	1.26 ± 0.05 (3)	2.91 ± 0.16 (6)
IS	—	1.20 ± 0.04 (3)	—

[a] All results are presented as micromoles per gram wet weight (means ± standard errors), with numbers of experiments shown in parentheses. ACh = 10^{-4} *M* acetylcholine, Physo = 10^{-4} *M* physostigmine, DS = direct stimulation, and IS = indirect stimulation.

The results with phosphatidylinositol resemble those seen by Larrabee and coworkers (13, 14) on the sympathetic ganglia. An increased incorporation of ^{32}P and ^{14}C into the phospholipids of the electroplax was observed irrespective of whether one stimulated the conducting membrane directly or *via* the nerve endings, which would indicate a site of action not exclusively localized to the synaptic region. In contrast, the increased incorporation observed previously (13, 14) appeared to be due exclusively to the transmitter, acetylcholine, acting on the postsynaptic cell. Acetylcholine acts only at the synapses of the electroplax; however, depolarization of these thousands of synapses causes a short-circuiting and depolarization of the entire conducting membrane.

The increased turnover of phosphatidylinositol in the electroplax, as a result of stimulation or the action of acetylcholine, is in agreement with suggestions that both excitation and the action of acetylcholine are mediated *via* an increase in phosphatidylinositol of polyphosphoinositide metabolism (6, 12). The increased turnover of phosphatidylcholine is of special interest in relationship to the proposal of Watkins (11) that acetylcholine, due to its structural similarity to the polar head group of phosphatidylcholine, may compete with this phospholipid for binding to a receptor protein. In fact, DeRobertis (37) suggested that the acetylcholine receptor of the electric organ is a proteolipid. If acetylcholine displaces phosphatidylcholine from a receptor proteolipid, the phospholipid may be more readily available to metabolic enzymes, thereby giving rise to the increased ^{32}P and ^{14}C incorporation values found in this study.

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Chemical Investigation of *Alhagi pseudalhagi* (Bieb.) Desv.: β-Phenethylamine and Tetrahydroisoquinoline Alkaloids

S. GHOSAL▲ and R. S. SRIVASTAVA

Abstract □ From the stems of *Alhagi pseudalhagi* (Bieb.) Desv. (Leguminosae), five β-phenethylamine and one tetrahydroisoquinoline alkaloids were isolated and characterized. On the basis of chemical transformation and spectral (UV, IR, PMR, and mass) evidence, the alkaloids were identified as β-phenethylamine (I), *N*-methyl-β-phenethylamine (II), hordenine (III), 3,4-dihydroxy-β-phenethyltrimethylammonium hydroxide (IV), *N*-methylmescaline (V), and salsolidine (VI). This is the first time that the occurrence of a 3,4,5-trioxygenated β-phenethylamine such as V has been demonstrated outside the family Cactaceae. The roots of this plant contain essentially the same alkaloids but in poorer yields. Preliminary pharmacological studies of the total alkaloids indicated that some curative properties of the plant extract are due to these entities.

Keyphrases □ *Alhagi pseudalhagi*—isolation, identification of five β-phenethylamine and one tetrahydroisoquinoline alkaloids □ 3,4-Dihydroxy-β-phenethyltrimethylammonium hydroxide—isolation, identification from *Alhagi pseudalhagi* □ Salsolidine—isolation, identification from *Alhagi pseudalhagi* □ Medicinal plants—*Alhagi pseudalhagi*, chemical investigation and pharmacological screening

Alhagi pseudalhagi (Bieb.) Desv. (syn. *A. camelorum* Fisch., *A. maurorum* Baker) (Leguminosae: Lotoidae) is a low erect shrub, widely distributed in the gangetic plains in India. The plant is bitter and acrid with a distinct flavor and finds uses in the indigenous system of medicine for a variety of purposes (1, 2). Pharmacological screening with a 50% alcoholic extract of the whole plant showed (3) antiprotozoal, spasmolytic, cardiotonic, and anticancer (sarcoma 180 in mice) activities. Previous reports (4, 5) indicated only the presence of an alkaloid in this plant. A recent investigation (6) in the authors' laboratory resulted in the isolation of a considerable amount of an alkaloid mixture from its fresh stems. Separation of these alkaloids into individual entities and identification of the major alkaloids have now been achieved.

RESULTS AND DISCUSSION

The stems and roots of *A. pseudalhagi* were separately processed for alkaloids. Gradient pH extraction (7), fractionation into phenolic and nonphenolic bases over a resin[1] column (8), precipitation of the reineckate salts under basic and acidic conditions, and their regeneration over ion-exchange resins (7, 8) resulted in the isolation of five β-phenethylamine and one tetrahydroisoquinoline alkaloids from the stems of *A. pseudalhagi*. The alkaloids are β-phenethylamine (I), *N*-methyl-β-phenethylamine (II), hordenine (III), 3,4-dihydroxy-β-phenethyltrimethylammonium hydroxide (IV), *N*-methylmescaline (V), and salsolidine (VI). This is the first demonstration of the occurrence of a 3,4,5-trioxygenated β-phenethylamine (V) outside the family Cactaceae.

In addition to the mentioned alkaloids, two partially characterized β-phenethylamine bases (one secondary and one quaternary) and a liberal amount of choline were obtained from the stems of this plant. The roots contained essentially the same alkaloids but in poorer yields.

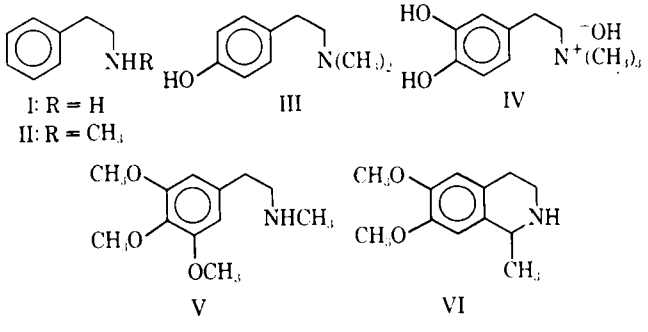
Cooccurrence of aryl-unsubstituted β-phenethylamines (I and II), the oxygenated analogs (III–V), and the cyclized compound (VI) has not been encountered before in nature. In the members of the Cactaceae, which are known to elaborate liberally 3,4,5-trioxygenated β-phenethylamines and their cyclized analogs, occurrence of mono- and dioxygenated compounds is a rare event (9). Also, aryl-unsubstituted compounds (I and II) have not been reported in the Cactaceae. These observations seem to indicate that *A. pseudalhagi* is a phylogenetically complex species, and further studies with related species in this genus are warranted.

Preliminary pharmacological studies with the total alkaloids of *A. pseudalhagi* indicated that the stimulant and expectorant properties of the plant extracts (1, 2) are due to these entities. Details of these findings will be reported soon.

EXPERIMENTAL

Extraction of Alkaloids—The principle of isolation and separation of the alkaloids involved utilization of differential solubility in solvents of graded polarity (petroleum ether, chloroform, and ethanol) in the presence and absence of fat, difference in base strengths, and phenolic and nonphenolic characters. The petroleum ether extract was kept aside for further examination for less basic constituents. The alkaloid mixtures from the ethanol extract were broadly divided into three groups (7): chloroform-soluble acetates, chloroform-soluble strong bases, and water-soluble bases. Subsequently, the alkaloids were separated into phenolic and nonphenolic bases in the usual way (8). The individual alkaloids were obtained by chromatography of the mixtures over neutral alumina[2] and preparative TLC[3] (2-mm. thickness). Monitoring of the column chromatographic runs was accomplished at each stage of purification by TLC. Two solvent systems were used as developers: Solvent 1, chloroform–methanol–concentrated ammonia (80:20:1); and Solvent 2, chloroform–methanol–acetic acid (75:15:10). Dragendorff's, tetrazotized *o*-dianisidine (8), and α-nitroso-β-naphthol–nitrous acid (10) reagents were used for staining. The identity of the homogeneous alkaloids was established by their co-TLC behavior with authentic markers (10–12), by chemical transformation into suitable derivatives where possible, and from correspondence of melting point, optical rotation, and UV, IR, PMR, and mass spectra of the alkaloids with those reported in the literature (9, 13, 14). Satisfactory combustion analyses were obtained for all reported compounds.

In a typical experiment, air-dried and milled stems of *A. pseudal-*



[1] Amberlite IRA-400.

[2] Brockmann, activity grade about III.

[3] Silica gel G, E. Merck.

hagi[4] (10.3 kg.) were processed for alkaloids. The description of the alkaloids is given here in the order of their isolation.

β-Phenethylamine (I)—Column chromatography and preparative TLC (Solvent 2) of the chloroform-soluble acetates afforded (from the upper preparative TLC zone) β-phenethylamine as a pale-brown liquid (0.18 g.) (identified by co-TLC, IR, melting point, and mixed melting point of the picrate).

***N*-Methyl-β-phenethylamine (II)**—The lower zone of the previously mentioned preparative chromatogram gave *N*-methyl-β-phenethylamine as a brown oil (72 mg.) (identified by co-TLC, IR, mass spectrometry, melting point, and mixed melting point of the picrate).

Salsolidine (VI)—Preparative TLC (Solvent 1) of the nonphenolic fraction from the chloroform-soluble strong bases furnished (from the middle zone) salsolidine as a semisolid mass (42 mg.) (identified by co-TLC, UV, IR, mass spectrometry, PMR, melting point, and mixed melting point of the base hydrochloride; $[\alpha]_D^{25}$ 0°.

***N*-Methylmescaline (V)**—From the preparative chromatogram, the lower zone of the nonphenolic alkaloid fraction afforded *N*-methylmescaline as a brown liquid (9 mg.) (identified by co-TLC, UV, and mass spectrometry). No solid derivative could be prepared due to the lack of sample. However, correspondence of spectral data (9, 13) and co-TLC behavior (Solvents 1 and 2) with an authentic synthetic sample, prepared from mescaline by treatment with formaldehyde and reduction of the anil with sodium borohydride in methanol (15), established its identity as *N*-methylmescaline.

Hordenine (III)—Column chromatography and preparative TLC (Solvent 1) of the phenolic alkaloid fraction afforded hordenine as light-brown needles (38 mg.) (identified by melting point, mixed melting point, co-TLC, UV, and melting point and mixed melting point of the methiodide).

3,4-Dihydroxy-β-phenethyltrimethylammonium Chloride/Hydroxide (IV)—The water-soluble bases, after separation of choline in the usual way (7, 16), gave two phenolic quaternary chlorides: R_f 0.11 (major) and 0.20 (minor) (Solvent 2). These were separated by preparative TLC using the same solvent system. The hydroxide of the major component, prepared by passing an alcoholic solution of the chloride through De-Acidite FF (7), readily suffered aerial oxidation. The quaternary chloride (18 mg.) showed UV λ_{max} 218, 230 (sh), and 283 nm., and $\lambda_{max}^{C_2H_5OH-NaOC^1OCH_2\text{-}H_2BO_3}$ 242 and 310 nm., characteristic of *o*-dihydroxy-β-phenethylamine chromophore (14, 17). The PMR spectrum of the quaternary chloride (in D_2O) showed signals at δ 7.1 6.3 (3H, m, unsymmetrical aromatic protons), 3.5–2.7 (4H, m, methylenes), and 3.2 (9H, s, quaternary *N*-methyl) p.p.m., which were consistent with the assigned structure. Co-TLC with an authentic sample of 3,4-dihydroxy-β-phenethyltrimethylammonium chloride, prepared from homoveratrylamine (18), established the correctness of this conclusion.

[4] The identity of the plant material was confirmed by Dr. C. S. P. Rao, Department of Botany, Banaras Hindu University. A voucher specimen has been kept at the Department of Pharmaceutics, Banaras Hindu University.

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Glycyrrhetic Acid Derivatives with Modified Ring A

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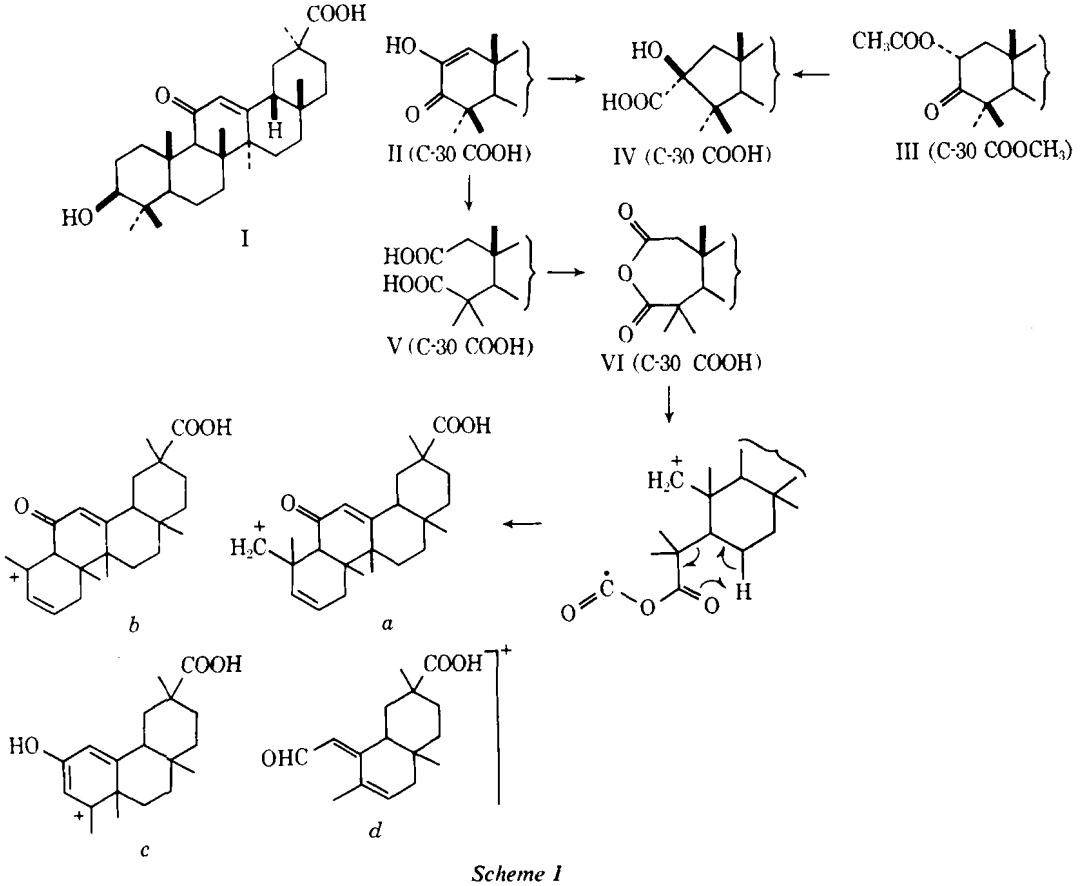
Abstract □ Ring A in glycyrrhetic acid (a therapeutically useful triterpenoid) was modified by contraction to an A-norhydroxy-acid derivative and by cleavage to a *seco*-A-2,3-dicarboxylic acid variant.

Keyphrases □ Glycyrrhetic acid derivatives—modification of ring A □ Triterpenoid aglycones—modification of ring A in glycyrrhetic acid

Glycyrrhetic acid (I), the principal triterpenoid aglycone obtained by acid hydrolysis of licorice (*Glycyrrhiza glabra* L.) root saponins, is perhaps the only triterpenoid with an established therapeutic utility (as an anti-inflammatory agent). Of the numerous studies made to modify this compound's structure, with the object of modifying its activity, very few have been concerned with ring A of the pentacyclic system. During studies in this area, the dicarboxylic and tricarboxylic acids were obtained by ring A contraction and cleavage, respectively, and are described (Scheme I). The first resulted by benzilic acid rearrangement of the diosphenol system in 2-hydroxy-18β-oleana-1,12-dien-3,11-dion-30-oic acid (II) (1) through reaction with boiling alcoholic potassium hydroxide and by a similar treatment of methyl 2α-acetoxy-18β-olean-12-ene-3,11-dion-30-oate (III) (2). The produced A-norhydroxy-acid is tentatively assigned Structure IV, in which the indicated stereochemistry of the ring A oxygen functions follows from the mechanism of the rearrangment where attack by hydroxyl ion at either C-2 or C-3 of the diketone form of II leads to a β-orientation of the resulting hydroxyl group (3).

Oxidative cleavage of ring A between C-2 and C-3 in glycyrrhetic acid, found not to be readily manageable by treatment of the appropriate glycol systems (1) with periodate, was accomplished by reaction of the diosphenol (II) with alkaline hydrogen peroxide. The resulting 2,3-*seco*-2,3,30-tricarboxylic acid (V), giving fitting spectral data, yielded a trimethyl ester and, upon heating in acetic anhydride, the corresponding anhydride (VI). The mass spectrum of V exhibited a very weak M^+ (*m/e* 516), and all fragmentations appear to result from the $M^+ - H_2O$ ion (*m/e* 498, VI) formed as a primary product. These include ions at *m/e* 483, 465, and 437 (losses of CH_3, $H_2O + CH_3$, and $H_2O + CH_3 + CO$, respectively, from the ion *m/e* 498). Collapse of the anhydride ring A with losses of $C_5H_7O_3$ and $C_6H_9O_3$ gives ions (*m/e* 383 and 369, respectively), which may have Structures *a* and *b*, respectively, produced by a mechanism involving transfer of the hydrogen atom on C-6. The base peak of the spectrum (at *m/e* 315) appears to be the product of a dubious reaction type. The



Scheme I

familiar cleavages (4) across ring B (involving a McLafferty transfer of the C-1 hydrogen atom) and across ring C (by a retro-Diels–Alder type of reaction) are both observed giving ions at *m/e* 303 (species *c*) and 262 (species *d*), respectively; the relatively low abundance of the first may be evidence that the original 18β stereochemistry is retained.

EXPERIMENTAL

Melting points are uncorrected. Optical rotations were measured in chloroform unless otherwise stated. UV spectra were taken in ethanol. The R_f values were taken on silica gel G chromatoplates, developed with toluene ethyl acetate–acetic acid (12:4:0.5).

2β-Hydroxy-A-nor-18β-olean-12-en-11-one-2α,30-dicarboxylic Acid (IV)– *Method A*—A solution of methyl 2α-acetoxy-18β-olean-12-ene-3,11-dion-30-oate (III) (2) (100 mg.) in ethanolic potassium hydroxide solution (10%, 5 ml.) was refluxed for 5 hr. After acidification, the precipitated solid matter (shown by TLC to be a mixture of two products, R_f 0.20 and 0.43) was separated by fractional crystallization from acetone–benzene into: (*a*) 2-hydroxy-18β-oleana-1,12-dien-3,11-dion-30-oic acid (II) (1), obtained after purification from ethanol–benzene as needles (20 mg., R_f 0.43), m.p. and mixed m.p. 320–323°; and (*b*) the A-norhydroxy-acid (IV, R_f 0.20) obtained as light-yellowish needles (35 mg., from acetone–hexane), m.p. 250°, $[\alpha]_D$ + 176°, giving one UV maximum at 250 nm. (ϵ = 10,800) and IR (KBr) bands near 3430, 3300, 1720, 1650, and 1630 cm.$^{-1}$.

Anal.—Calc. for $C_{30}H_{44}O_6$: C, 71.97; H, 8.86. Found: C, 71.75; H, 8.83.

Method B—A solution of the free acid of the diosphenol (II, 50 mg.) in ethanolic sodium hydroxide (10%, 5 ml.) was refluxed for 5 hr. followed by the usual workup. The isolated product, m.p. 250°, was shown to be identical (mixed melting point and co-TLC) with the previously described A-norhydroxy-acid (IV).

2,3-*seco*-18β-Olean-12-en-11-one-2,3,30-tricarboxylic Acid (V)– A refluxing solution of the free acid of the diosphenol (II, 1 g.) in ethanol (50 ml.) was treated with hydrogen peroxide solution (100 vol., 110 ml.) and potassium hydroxide solution (10%, 35 ml.), both being added in four equal portions at intervals of 15 min. After acidification, the precipitated product was washed with water and crystallized from *n*-hexane to give hexagonal plates of V (R_f 0.30, 880 mg.), m.p. 205–208°, $[\alpha]_D$ + 172.5° (pyridine). The UV spectrum showed one maximum at 249 nm. (ϵ = 12,700), and the IR spectrum (mineral oil) contained bands near 3510 (broad), 1710, and 1655 cm.$^{-1}$.

Anal. Calc. for $C_{30}H_{44}O_7$: C, 69.74; H, 8.58; mol. wt. 516. Found: C, 69.90; H, 8.64; mol. wt. (by mass spectrometry) 516.

Trimethyl Ester of V—Methylation of V (200 mg.) in chloroform (50 ml.) solution with ethereal diazomethane (from 4 g. *N*-nitrosomethylurea) afforded, after crystallization from chloroform–methanol, 150 mg. of the trimethyl ester (R_f 0.57), m.p. 187–188°; $[\alpha]_D$ +37.6°; UV maximum at 250 nm. (ϵ = 12,800); IR (mineral oil) bands near 1730 and 1650 cm.$^{-1}$.

Anal.—Calc. for $C_{33}H_{50}O_7$: C, 70.93; H, 9.02. Found: C, 70.77; H, 9.11.

Anhydride of V—After refluxing a solution of the triacid (V, 160 mg.) in acetic anhydride for 1 hr., the residue remaining upon evaporation was crystallized from *n*-hexane to give the anhydride (VI) (R_f 0.50, 60 mg.), m.p. 218–220°, $[\alpha]_D$ +180°; UV maximum at 250 nm. (ϵ = 9900); IR (KBr) bands near 3370, 1730, and 1660 cm.$^{-1}$.

Anal.– Calc. for $C_{30}H_{42}O_6$: C, 72.26; H, 8.49. Found: C, 71.86; H, 8.59.

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Alkaline Degradation Product of Cephradine

ALLEN I. COHEN▲, PHILLIP T. FUNKE, and MOHINDAR S. PUAR

Abstract □ Cephradine yields an unique alkaline degradation product, which has been identified by proton magnetic resonance, ^{13}C-NMR, and mass spectrometry as 2-[6-(1,4-cyclohexadine-1-yl)-2,5-dioxo-3-piperazinyl]-5,6-dihydro-5-methyl-2*H*-1,3-thiazine-4-carboxylic acid, sodium salt.

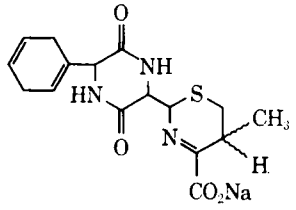
Keyphrases □ Cephradine—structure determination of alkaline degradation product as 2-[6-(1,4-cyclohexadine-1-yl)-2,5-dioxo-3-piperazinyl]-5,6-dihydro-5-methyl-2*H*-1,3-thiazine-4-carboxylic acid, sodium salt □ 2-[6-(1,4-Cyclohexadine-1-yl)-2,5-dioxo-3-piperazinyl]-5,6-dihydro-5-methyl-2*H*-1,3-thiazine-4-carboxylic acid, sodium salt—cephradine alkaline degradation product, structure determination □ Cephem derivatives—structure determination of cephradine alkaline degradation product

The alkaline hydrolysis of penicillins and, more recently, of ampicillin (α-aminobenzylpenicillin) was investigated extensively (1). Similar studies into the aminolysis products of cephalosporins were also reported (2). The structure of the alkaline degradation product of cephradine, a new semisynthetic cephem derived from D-2-(1,4-cyclohexadienyl)glycine and desacetoxy-7-aminocephalosporanic acid (3), is reported here.

EXPERIMENTAL[1]

Materials—Cephradine[2], 7-[D-2-amino-2-(1,4-cyclohexadien-1-yl)acetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid hydrate, was prepared by the procedure of Dolfini *et al.* (3). The sample was characterized by NMR and by mass spectrometry as its trimethylsilyl derivative (Fig. 1). Anhydrous sodium carbonate (reagent grade), sodium hydroxide, or 20% sodium deuteroxide in deuterium oxide was used in the degradation studies. Either *N,O*-bis(trimethylsilyl)acetamide[3] or trimethylchlorosilane[3] was employed as the silylating agent.

Preparation of Degradation Product—Cephradine (3.0 g.) and sodium carbonate (9.0 g.) were mixed thoroughly. Then 1.6 g. of the mixture was transferred to a vial, 5 ml. of water was added, and the vial was capped and agitated. The vial was stored at 5° for 1 week. The white precipitate that formed was collected on a sintered-glass funnel. The filter cake was washed with five 2-ml. portions of cold water and then with three 1-ml. portions of 95% ethanol and was dried by suction. The crystals were further dried in a vacuum oven at 40° for 3 hr. The mass spectrum of the trimethylsilyl derivative of the isolated products shows a molecular ion of *m/e* 565.2297, corresponding to the addition of three trimethylsilyl groups, $C_{25}H_{43}N_3O_4SSi_3$ (calc. 565.2280). PMR (dimethyl sulfoxide-d_6 and tetramethylsilane): δ 1.06 (3H, d, J = 7 Hz.), 2.95 (2H, m), 5.05 (1H, m, J = 3 Hz.), 2.6 (5H, m), 4.19 (1H, t, J = 3 Hz.) [which, on deuterium exchange, becomes a doublet (J = 3 Hz.)], 4.93 (1H, $w_{1/2}$ = 3 Hz.) (which has a $w_{1/2}$ = 2 Hz. after deuterium exchange),



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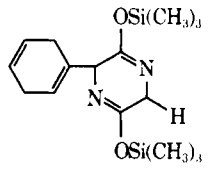


II: *m/e* 335

5.72 (1H), 5.67 (2H), 8.18 (1H, exchangeable), and 8.10 (1H, d, J = 3 Hz., exchangeable); ^{13}C-NMR (dimethyl sulfoxide-d_6; parts per million from tetramethylsilane, δ = 0): δ 16.8, 23.7, 25.7 (2C), 28.7, 39.6 (dimethyl sulfoxide), 58.8, 59.4, 63.8, 122.6, 123.3, 124.2, 130.2, 166.2, 166.7, and 167.7. There are at least 15 carbon atoms; the formula requires 16.

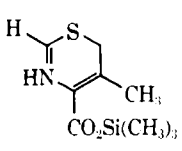
RESULTS AND DISCUSSION

The PMR assignments of the isolated degradation product, dissolved in dimethyl sulfoxide-d_6, are: C-3 methyl δ 1.06 (d, 7 Hz.), vinyl protons δ 5.72 (1H) and δ 5.67 (2H), methylene and C-3 cephem protons δ 2.62 (5H), α-proton of glycyl δ 4.93, and —NH— δ 8.10 and 8.18. The respective chemical shifts of the C-6 and C-7 cephem protons are δ 5.05 (d, 3 Hz.) and 4.19 (t, 3 Hz.); the latter peak becomes a doublet of 3 Hz. coupling on deuterium exchange. The C-2 protons near δ 3 appear as an *ABX* system coupled to the C-3 methine proton. The presence of a methyl doublet and the methine proton at C-3 coupled to the vicinal methylene protons indicates that the double bond has migrated to the C-4,5 position.

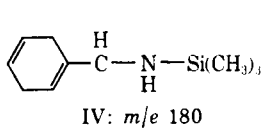
The ^{13}C-NMR spectrum was also used to establish the structure of the degradation product. There are 14 peaks (one has an intensity for two carbons), accounting for 15 of the 16 carbons. Because it is a tertiary carbon of an azomethine group, the 16th carbon occurs as a broad resonance due to a longer relaxation time which results from nuclear quadrupole coupling and π-electron polarization. The pulse interval used in the Fourier experiment did not permit sufficient time for complete relaxation. Additionally, the nitrogen quadrupole coupling can give rise to broadened resonances. The three ^{13}C resonances at δ 166.2, 166.7, and 167.7 clearly demonstrate the presence of three carbonyl groups in the alkaline degradation product (4). The resonances at δ 122–130 account for four of the other five carbon atoms bearing double bonds. The five carbon resonances between δ 16 and 29 are the alkyl carbons bearing two allylic double bonds or one electronegative group, whereas the three resonance lines between δ 59 and 64 account for three carbon atoms bearing two electronegative groups and/or two allylic double bonds.

Structure I for the alkaline degradation product is consistent with the NMR data and the formula derived from the mass spectral data. While the NMR data do not permit assignment of the configuration of the C-3 methyl group, the appearance of a single doublet suggests that one product is formed.

The mass spectrum provides additional support for the structural assignment. Trimethylsilylation with chlorotrimethylsilane resulted in the precipitation of sodium chloride. The molecular ion of *m/e* 565.2297 agrees with the formula $C_{25}H_{43}N_3O_4SSi_3$ (calc. 565.2280) (Fig. 2). The most significant fragment ion is the *m/e* 335 ion, II, which demonstrates the presence of a disilyl moiety resulting from the direct cleavage of the C_6—C_7 bond. Cephradine, on the other



III: *m/e* 230



IV: *m/e* 180

[1] Proton magnetic resonance (PMR) spectra were obtained on a Varian Associates XL-100 spectrometer employing the deuterium field-frequency lock system. The ^{13}C-magnetic resonance (^{13}C-NMR) spectrum was taken on a JEOL PS-100 equipped with a Fourier transform pulser and a Nicolet computer. At 25.1 MHz., the effective sweep range was 5000 Hz. (200 p.p.m.). Low- and high-resolution mass spectra were taken on an AEI MS-902 mass spectrometer. Data were acquired *via* a frequency-modulated analog magnetic tape, which was subsequently processed on a Digital Equipment Corp. PDP-11 computer, employing Squibb programs.

[2] Batch NNO11ND, E.R. Squibb and Sons, New Brunswick, N. J.

[3] Pierce Chemical Co., Rockford, Ill.

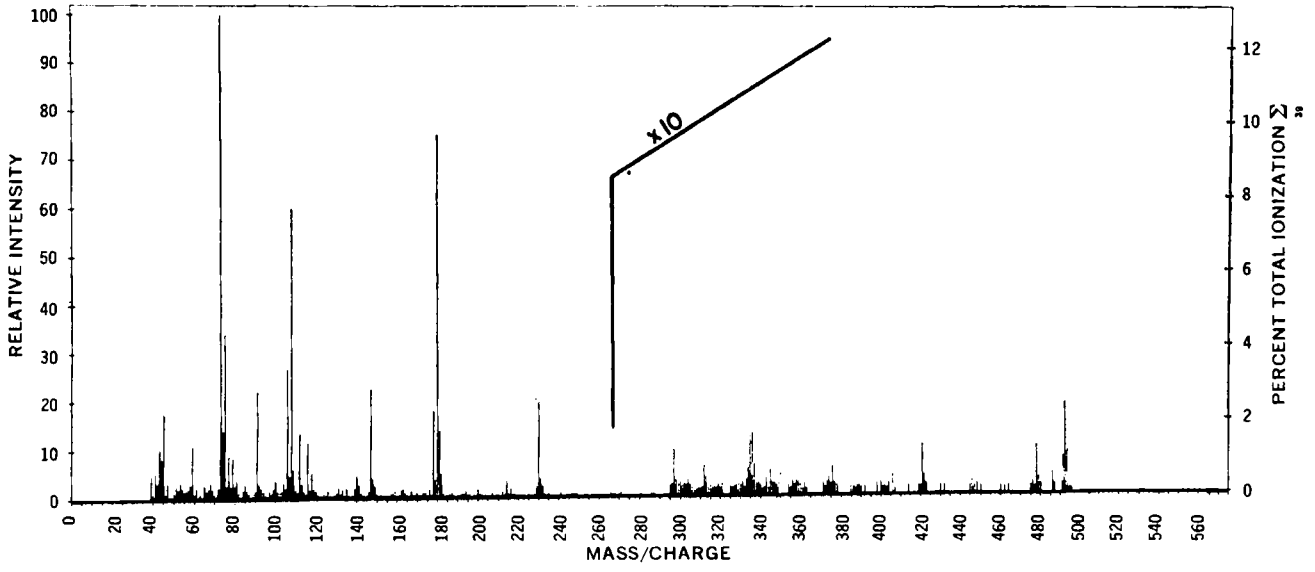


Figure 1—*Low-resolution mass spectrum of the trimethylsilyl derivative of cephradine.*

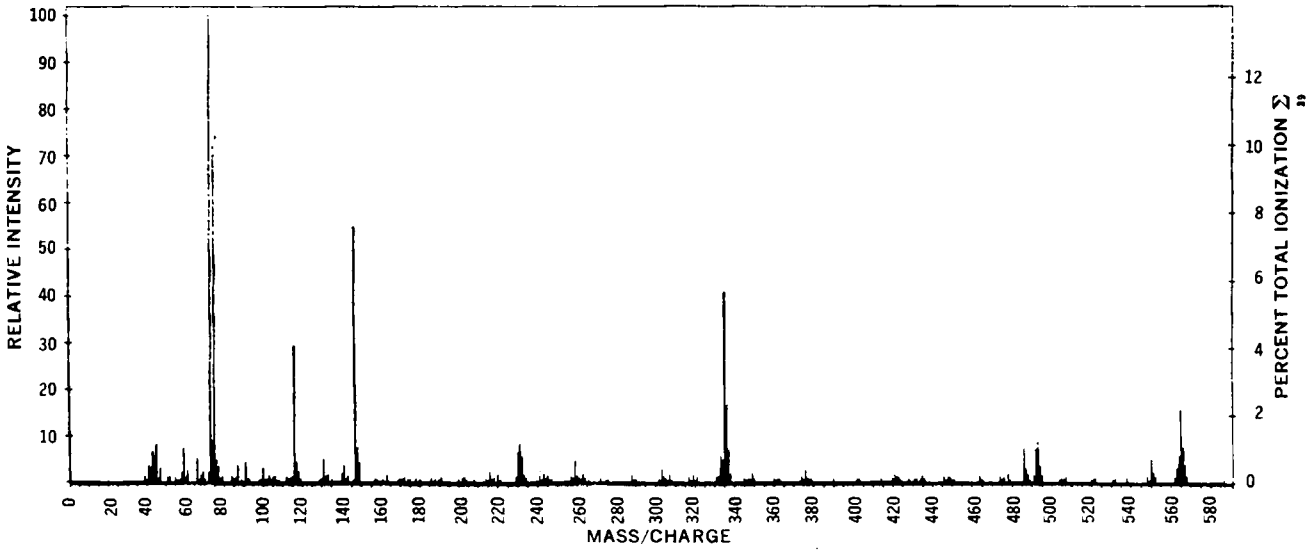


Figure 2—*Low-resolution mass spectrum of the trimethylsilyl derivative of the alkaline degradation product of cephradine.*

hand, was disilylated primarily by *N,O*-bis(trimethylsilyl)acetamide, as demonstrated by the *m/e* 493 ion (Fig. 1), and was also partially trisilylated due to addition to the amide group under the forcing conditions of the trimethylsilylation. Trimethylsilylated cephalosporins yield a *m/e* 230 ion, III, arising through the β-lactam cleavage, proton addition, and cleavage of the C_6—C_7 bond.

The other significant ion in cephradine, at *m/e* 180, produced by the cleavage of the α-carbonyl bond of the trimethylsilylated glycyl portion of the molecule, IV, is absent in the spectrum of the alkaline degradation product. The degradation product exhibits an $M^+ - 79$ ion at *m/e* 486, arising from the cleavage of the dihydrophenyl moiety.

In one continuing program, NMR spectrometry is employed in the determination of stability of drugs in acidic, neutral, and basic aqueous media. The NMR spectrum of a 10-mg./ml. D_2O–NaOD solution of cephradine was determined for 12 hr. at 30° or for 5 hr. at 60°. A progressive decrease in the C-3 methyl proton resonance at δ 1.82 was observed, with the corresponding appearance of a new singlet proton at δ 1.24. When the hydrolysis was performed in water, the methyl proton resonance appeared as a doublet with a coupling constant, *J*, of 7 Hz. That the migration does not occur in the C-2,3 position was demonstrated by the retention of the C-2 methylene proton resonances and, when water was used as the solvent, by the additional vicinal coupling of these protons to the newly introduced methine proton. Thus, double-bond migration to the C-4,5 position occurs with the concurrent introduction, depending on the solvent used, of a proton or of deuterium at C-3 after the cleavage of the β-lactam bond. It appears that the major product[4] of this degradation is identical to the product isolated after treatment with sodium carbonate. A partial tautomerization to the C-3,4 position was demonstrated by redissolving the product isolated from water in D_2O–NaOD and observing the rapid decrease in the C-3 methine proton signal in the PMR spectrum.

The alkaline degradation product represents a unique structure in the penam and cephem series. For example, the glycyl penicillin analog, ampicillin, is reported to yield a penicilloic acid and the decarboxylated derivative (1). Indelicato *et al.* (5) prepared a similar compound, with the retention of the double bond at the C-3,4 position, by refluxing cephalexin in benzene overnight. In a basic aqueous medium, cephradine yields a product that has undergone migration of the double bond to the C-4,5 position and protonation at C-3. Under the same conditions, ampicillin does not yield a cyclic product (1); a cyclic product is not obtained in refluxing benzene either (5). The failure of the cyclization of ampicillin has been attributed to steric effects of the C-2 dimethyl and C-3 methine

[4] Additional studies are being conducted to separate the degradation products formed in acid, neutral, and alkaline media and to elucidate their structures.

groups (5). In cephalosporin studies (2), a double-bond migration was reported only when accompanied by dehydroacetoxylation; otherwise, the double bond remained at the C-3,4 position.

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▲ To whom inquiries should be directed.

Phytochemical Investigation of *Virola peruviana*, A New Hallucinogenic Plant

A. LAI, M. TIN-WA, E. S. MIKA, G. J. PERSINOS*, and N. R. FARNSWORTH▲

Abstract □ Although *Virola peruviana* has been described in ethnobotanical studies as being used as a hallucinogen, no proof of the presence of chemical agents explaining this activity existed previously. The present study resulted in the isolation of 5-methoxy-*N*,*N*-dimethyltryptamine as well as the identification of *N*,*N*-dimethyltryptamine and 5-methoxytryptamine, thus confirming the hallucinogenic aspect of *V. peruviana*. In addition, myoinositol and the lignans lirioresinol-A dimethyl ether and lirioresinol-B dimethyl ether were isolated and identified. A mixture of *n*-alkanols was isolated and shown to consist of octacosanol, triacontanol, and dotriacontanol, whereas a mixture of sterols was isolated and shown to consist of β-sitosterol, campesterol, and stigmasterol.

Keyphrases □ *Virola peruviana*—phytochemical investigation, hallucinogenic constituents isolated and identified □ Hallucinogens—isolation and identification of hallucinogenic constituents of *Virola peruviana* □ Phytochemistry—isolation and identification of hallucinogenic constituents of *Virola peruviana*

In the Rio Apaporis, Puinave Indians refer to *Virola peruviana* as yá-kee, suggesting its possible utilization in preparing the hallucinogenic snuff by the same name (1). Schultes and Holmstedt (1) suggested that this species may be used as a hallucinogen and determined that it contained alkaloids (1). Other virola species, *i.e.*, *V. theiodora*, *V. rufula*, *V. multinervia*, *V. venosa*, *V. calophylloidea*, *V. calophylla*, and *V. sebifera*, are well documented as hallucinogenic plants, with their active principles being determined as *N*,*N*-dimethyltryptamine, 5-methoxy-*N*,*N*-dimethyltryptamine, and/or *N*-methyltryptamine (2–5).

The purpose of this investigation was to determine whether *V. peruviana* contained chemical constituents that would explain its use as a hallucinogen.

Table I—Chromatographic Separation of Fraction A

Fraction Number	Eluent	Isolate	Yield, mg.
1–34	Benzene	—	—
35–39	Benzene	*n*-Alkanols	26
40–64	Benzene	—	—
65–81	Benzene–acetone (9:1)	Phytosterols	205
82–154	Benzene–acetone (9:1)	—	—
155–169	Benzene–acetone (6:1)	—	—
170	Benzene–acetone (6:1)	Lirioresinol-A dimethyl ether	185
171–174	Benzene–acetone (6:1)	Lirioresinol-B dimethyl ether	216
175–200	Benzene–acetone (6:1)	—	—
201–234	Benzene–acetone (3:1)	—	—
235–245	Acetone	—	—
246–280	Methanol	—	—

EXPERIMENTAL[1]

Plant Material—The plant material[2] used represented the bark of *Virola peruviana* (A.DC.) Warburg (Myristicaceae), collected near Leticia, Peru, during October 1968.

Extraction and Fractionation—Coarsely milled bark (4.7 kg.) of *V. peruviana* was defatted with 35 l. of petroleum ether (b.p. 30–60°) using a soxhlet apparatus for 24 hr. The combined extracts were filtered and evaporated to dryness to yield 4.0 g. of Fraction A. The defatted plant material was then exhaustively extracted in the soxh-

[1] All chemicals used were of reagent grade quality. Melting points were determined by means of a Thomas-Hoover apparatus or a Kofler hot plate and are uncorrected. Optical rotations were measured in a Carl Zeiss optical polarimeter. The UV spectra were recorded in methanol using a Beckman model DB-G spectrophotometer. IR spectra were taken using a Beckman model IR-18A instrument. Mass spectral analyses were made using the Hitachi Perkin-Elmer model RMD-6D single-focusing mass spectrometer. NMR spectra were taken using a Bruker model HFX-5 magnetic resonance spectrometer. GC analyses were carried out by means of a Perkin-Elmer model 881 linear programmed temperature gas chromatograph, equipped with a hydrogen flame-ionization detector and a Sargent model SR,S-72180-20 1-mv. recorder; a 1-sec. full-scale response was used. A borosilicate coiled glass column, 1.8 m. (6 ft.) × 2.0 mm. (i.d.), was packed with either 3% OV-1 or 5% OV-101 on 100–120-mesh Gas Chrom Q. Silica gel GF_{254} plates were used for monitoring the chromatographic separation of Fraction A; the solvent system used for development of plates was the same as the eluent used to elute the fractions. The same type plates were used for monitoring the chromatographic fractions from Fraction D, but development was with benzene–ethyl acetate–diethylamine (7:2:1). Spots were visualized after spraying the plates with sulfuric acid (70%) or Ehrlich reagent and heating at 110° for 5 min.

[2] A voucher specimen (2245) was authenticated by Dr. J. Wurdack, Smithsonian Institution, Washington, D. C. A specimen has been deposited at that address.

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Extraction and Fractionation—Coarsely milled bark (4.7 kg.) of *V. peruviana* was defatted with 35 l. of petroleum ether (b.p. 30–60°) using a soxhlet apparatus for 24 hr. The combined extracts were filtered and evaporated to dryness to yield 4.0 g. of Fraction A. The defatted plant material was then exhaustively extracted in the soxh-

[1] All chemicals used were of reagent grade quality. Melting points were determined by means of a Thomas-Hoover apparatus or a Kofler hot plate and are uncorrected. Optical rotations were measured in a Carl Zeiss optical polarimeter. The UV spectra were recorded in methanol using a Beckman model DB-G spectrophotometer. IR spectra were taken using a Beckman model IR-18A instrument. Mass spectral analyses were made using the Hitachi Perkin-Elmer model RMD-6D single-focusing mass spectrometer. NMR spectra were taken using a Bruker model HFX-5 magnetic resonance spectrometer. GC analyses were carried out by means of a Perkin-Elmer model 881 linear programmed temperature gas chromatograph, equipped with a hydrogen flame-ionization detector and a Sargent model SR,S-72180-20 1-mv. recorder; a 1-sec. full-scale response was used. A borosilicate coiled glass column, 1.8 m. (6 ft.) × 2.0 mm. (i.d.), was packed with either 3% OV-1 or 5% OV-101 on 100–120-mesh Gas Chrom Q. Silica gel GF_{254} plates were used for monitoring the chromatographic separation of Fraction A; the solvent system used for development of plates was the same as the eluent used to elute the fractions. The same type plates were used for monitoring the chromatographic fractions from Fraction D, but development was with benzene–ethyl acetate–diethylamine (7:2:1). Spots were visualized after spraying the plates with sulfuric acid (70%) or Ehrlich reagent and heating at 110° for 5 min.

[2] A voucher specimen (2245) was authenticated by Dr. J. Wurdack, Smithsonian Institution, Washington, D. C. A specimen has been deposited at that address.

Table II—Chromatographic Separation of Fraction D

Fraction Number	Eluent	Isolate	Yield, mg.
1–20	Petroleum ether	—	—
21–44	Petroleum ether–benzene (9:1)	—	—
45–55	Petroleum ether–benzene (1:1)	5-Methoxy-*N,N*-dimethyltryptamine (as methiodide)	35
56–434	Petroleum ether–benzene (1:1)	5-Methoxy-*N,N*-dimethyltryptamine (as picrate)	259
435–633	Benzene	5-Methoxy-*N,N*-dimethyltryptamine (as picrate)	530
634–705	Benzene–ether (6:1)	—	—
706–936	Ether	—	—
937–1030	Chloroform	—	—
1031–1066	Chloroform–methanol (9:1)	—	—
1067–1087	Methanol	—	—

let apparatus with methanol until the final methanol extract no longer gave a positive alkaloid test with Mayer's reagent. Concentration of the methanol extract *in vacuo* gave 232 g. of a dark-brown syrupy liquid, which was redissolved in 2 l. of boiling methanol and filtered. A nonalkaloidal precipitate (Fraction B), which formed on standing, was removed by filtration (22.7 g.). The filtrate was poured into 6 l. of 2% acetic acid, and the methanol was removed by evaporation *in vacuo*. Suspended impurities were removed by filtration; the filtrate was extracted with chloroform (6 × 2 l.), and, after evaporation *in vacuo*, gave 11 g. of Fraction C.

The aqueous layer was made alkaline with 28% ammonium hydroxide and extracted with chloroform (9 × 2 l.). The combined chloroform extracts were evaporated to dryness *in vacuo* to yield 3.4 g. of Fraction D.

Chromatographic Separation of Fraction A—Fraction A (4.0 g.) was chromatographed over a column (5.5 × 62 cm.) of silica gel PF_{254} (300 g.) and eluted with solvents having increasing polarity (Table I). Each fraction (50 ml.) was monitored by means of TLC, and fractions were combined on the basis of TLC patterns.

Isolation and Identification of *n*-Alkanols—Combined fractions 35–39 from the column, which were eluted with benzene, formed a white precipitate when concentrated *in vacuo*. The precipitate was removed by filtration and crystallized from acetone several times. The IR spectrum of this isolate was typical of that seen with *n*-alkanols. GLC analysis (3% OV-1, 280° isothermal) of this isolate showed it to be a mixture of octacosanol (55%), triacontanol (35%), and dotriacontanol (15%).

Isolation and Identification of Phytosterols—Fractions 65–72 from the column, which were eluted with benzene–acetone (9:1), were combined and evaporated to dryness *in vacuo*. The residue (340 mg.) was crystallized from a benzene hexane mixture to give white plates. GLC analysis (5% OV-101, 250° isothermal) of this material showed it to be a mixture of campesterol (9.9%), stigmasterol (33.9%), and β-sitosterol (57.3%).

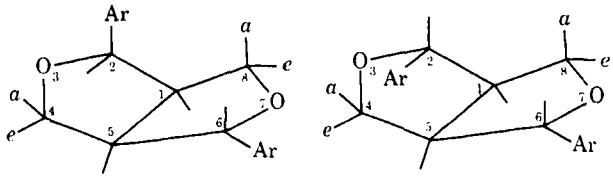
Isolation and Identification of Lirioresinol-A Dimethyl Ether—Concentration of fraction 170 from the column, which was eluted with benzene acetone (6:1), resulted in the formation of crystals. Recrystallization from acetone–hexane yielded 185 mg. of crystals having a melting point of 116° [lit. (6) m.p. 118–120°). The UV spectrum of the isolate showed absorptions at λ_{max} 208 (log ε 4.88) and 271 nm. (log ε 3.48), with a shoulder at 230 nm. (log ε 4.19). IR absorptions were found at 2900, 2800, 1590, 1510, and 810 $cm.^{-1}$. A molecular ion was observed in the mass spectrum at *m/e* 446, in agreement with a formula of $C_{24}H_{30}O_8$. Other prominent peaks were noted at *m/e* 416, 250, 249, 224, 207, 196, and 181, the fragmentation pattern of which is similar with that of lirioresinol-B dimethyl ether (7–9).

Since the presence of six methoxy groups was observed in the NMR spectrum at δ 3.85, the remaining two oxygen atoms were assigned as ether linkages in a lignan of the 3,7-dioxabicyclo[3.3.0]-octane type, considering the lack of hydroxy, phenolic, and carbonyl groups in the IR spectrum. However, the compound was not symmetrical as is the case with lirioresinol-B dimethyl ether. One benzylic hydrogen appeared as a doublet centered at δ 4.89 (J = 3 Hz.), while the other benzylic proton was at δ 4.47 (d, J = 3 Hz.). One methylene proton was seen upfield in the δ 3.38–3.47 region as a multiplet, which corresponded with the axial proton due to the shielding effect of the aryl group; another methylene proton was observed downfield in the δ 4.13–4.24 region, corresponding with the other axial proton affected by the equatorial aryl group. Two additional methylene protons appeared between δ 3.8 and 3.97. Furthermore, two nonequivalent methine protons appeared at δ 2.94 and 3.30, indicating that its NMR spectrum was similar to that reported for lirioresinol-A (10). The identity of this isolate as lirioresinol-A dimethyl ether was confirmed by measurement of the specific rotation $[\alpha]_D^{26}$ + 116 (c 0.65 in $chloroform_3$) [lit. (6) + 119] and preparation of the dibromo derivative (11), m.p. 122° [lit. (6) m.p. 124–126°].

Isolation and Identification of Lirioresinol-B Dimethyl Ether—When the pooled fractions 171–174 were eluted from the column with benzene–acetone (6:1) and concentrated, a crystalline material formed which was removed by filtration. This material was recrystallized from acetone containing a trace of hexane to give 216 mg. of a pure sample, m.p. 118–119° [lit. (6) m.p. 121–123°]. The UV spectrum showed absorption maxima at λ_{max} 208 (log ε 4.88) and 271 nm. (log ε 3.48), with a shoulder at 230 nm. (log ε 4.19). The IR spectrum was very similar to that of the lirioresinol-A dimethyl ether but not identical. The mass spectrum indicated a molecular ion at *m/e* 446, in agreement with a formula of $C_{24}H_{30}O_8$, suggesting that it might be an isomer of lirioresinol-A dimethyl ether. Its fragmentation pattern was in agreement with previously published data for lirioresinol-B dimethyl ether (7–9). This was further supported by the NMR spectrum, which indicated that the isolate was symmetrical; *i.e.*, it gave a proton count of 30 only if the apparent integrals were doubled. The assignments of the signals as indicated below is in agreement with those reported for lirioresinol-B dimethyl ether (9, 10), *i.e*, methine protons at C_1 and C_5 at δ 3.11 (m, 2H), benzylic protons at C_2 and C_6 at δ 4.78 (d, J = 3 Hz., 2H), axial methylene protons at δ 4.24 (m, 2H) and equatorial methylene protons at δ 4.00 (m, 2H), six methoxy protons at δ 3.87 (18H), and four aromatic protons at δ 6.60 (s, 4H).

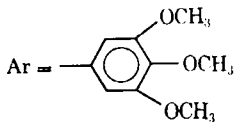
The identity of this compound as lirioresinol-B dimethyl ether was confirmed by measurement of the specific rotation $[\alpha]_D^{26}$ + 49 (0.65 in chloroform) [lit. (6) + 46.2] and preparation of the dibromo derivative (11), m.p. 153° [lit. (6) m.p. 152–155°].

Isolation and Identification of Myoinositol—Fraction B (22.7 g.) was dissolved in 70% ethanol and, on standing, formed crystals which were removed by filtration. Recrystallization from 70% ethanol afforded an analytical sample having a melting point of 220°, which was not depressed on admixture with a reference sample



lirioresinol-A dimethyl ether

lirioresinol-B dimethyl ether



of myoinositol. The IR spectrum of the isolate was identical with that of a reference sample of myoinositol, with $[\alpha]_D^{26} \pm 0$; paper chromatographic comparison of the isolate and myoinositol showed them to be identical (12). The identity of the isolate was further established by preparation and comparison of the acetates, m.p. 216° [lit. (13) m.p. 216°] and the benzoates (14), m.p. 260° [lit. (15) m.p. 258°] of the isolate and reference myoinositol.

Chromatographic Separation of Fraction D—Fraction D (3.0 g.) was chromatographed over a column (2.2 × 27 cm.) of neutral alumina[3] (100 g.), activity grade III. Fractions consisting of 15 ml. each were collected. Initially the column was eluted with petroleum ether (b.p. 30–60°), followed by solvents of increasing polarity (Table II).

Isolation and Identification of 5-Methoxy-*N*,*N*-dimethyltryptamine—Fractions 45–55 from the column were combined and taken to dryness *in vacuo* to yield 50 mg. of residue. This residue was shown by TLC to contain one major component, which gave a pale-yellow fluorescence under UV light and a positive reaction with the Ehrlich reagent. Since the material was resistant to crystallization as the free base, preparative TLC was employed to obtain the base in pure form. The zone at R_f 0.38 was removed from the plates and extracted with chloroform. The combined extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. A mass spectrum of this residue indicated a molecular ion at *m/e* 218, which corresponded with a molecular formula of $C_{13}H_{18}N_2O$. Other prominent peaks at *m/e* 174, 173, 160, 159, 145, and 58 were all in agreement with published data for 5-methoxy-*N*,*N*-dimethyltryptamine (16). The UV spectrum of the isolate showed absorptions at λ_{max} 224, 277, and 295 nm., which compares favorably with that found in the literature for 5-methoxy-*N*,*N*-dimethyltryptamine (16).

Reaction of the alkaloid residue (40 mg.) with ethanol (10 ml.), 80% sodium hydroxide (2 drops), and 8 ml. of methyl iodide at room temperature for 8 hr. gave the methiodide (17), m.p. 182–183° [lit. (16) m.p. 181–182°]. The IR spectrum was superimposable with that of a synthetic sample of 5-methoxy-*N*,*N*-dimethyltryptamine methiodide, prepared from 5-methoxy-*N*,*N*-dimethyltryptamine as described previously.

Pooled fractions 56–434 (304 mg.) and 435–633 (550 mg.) from the column were processed by the preparation of picrate derivatives. The concentrated residues were dissolved in methanol and mixed with an equimolar solution of picric acid dissolved in methanol. Orange-yellow picrates were obtained and recrystallized from methanol–acetone to give pure samples having melting points of 172° [lit. (16) m.p. 172°]. The IR spectra of these isolates were superimposable with those of a reference sample of 5-methoxy-*N*,*N*-dimethyltryptamine picrate.

Detection of 5-Methoxytryptamine and *N*,*N*-Dimethyltryptamine—GLC (5% OV-101, with a temperature of 150° initially for 2 min. followed by a 10° rise/min. from 150 to 300°) of Fractions C and D indicated the presence of small amounts of 5-methoxytryptamine in Fraction C, while *N*,*N*-dimethyltryptamine, 5-methoxytryptamine, and 5-methoxy-*N*,*N*-dimethyltryptamine were identified in Fraction D.

[3] Brockmann.

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Specificity of Antibodies Produced by Linear Antigenic Polypeptides of a Known Primary Structure: Synthesis and Use of the Polymers Poly(L-phenylalanyl-L-glutamyl-L-valylglycyl)glycine Methyl Ester and Poly(L-phenylalanyl-L-aspartyl-L-valylglycyl)glycine Methyl Ester

BRIAN J. JOHNSON

Abstract □ The syntheses of the polypeptides poly(L-phenylalanyl-L-glutamyl-L-valylglycyl)glycine methyl ester and poly(L-phenylalanyl-L-aspartyl-L-valylglycyl)glycine methyl ester are described. Using these materials, it was found by cross-reaction and absorption studies that the antibodies to the antigen poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester show specificities for the phenolic hydroxyl group of the tyrosyl residue and the γ-carboxyl group of the glutamyl residue.

Keyphrases □ Polypeptides, linear—synthesis of poly(L-phenylalanyl-L-glutamyl [and L-aspartyl]-L-valylglycyl)glycine methyl ester, immunochemical properties □ Antibodies, polypeptide produced—specificity, synthesis and testing of poly(L-phenylalanyl-L-glutamyl [and L-aspartyl]-L-valylglycyl)glycine methyl ester □ Immunochemical properties—synthesis and testing of poly(L-phenylalanyl-L-glutamyl [and L-aspartyl]-L-valylglycyl)glycine methyl ester □ Antigenic polypeptides, linear—synthesis and use of poly(L-phenylalanyl-L-glutamyl [and L-aspartyl]-L-valylglycyl)glycine methyl ester

It was previously reported that antibodies produced by rabbits against the polypeptide poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I) (1–3) are most probably dependent upon the conformation of the antigen (4–7). By the use of the polypeptides poly(L-phenylalanyl-L-glutamyl-L-alanylglycyl)glycine (II) (8), poly(L-tyrosyl-L-aspartyl-L-alanylglycyl)glycine (III) (9), and poly(L-tyrosyl-L-glutamyl-L-valylglycyl)-glycine (IV) (10), it was shown that these rabbit antibodies possess specificities for the phenolic hydroxyl group of the tyrosyl residue (8) and the γ-carboxyl group of the glutamyl residue (9). All these specificities were deduced by the use of polymers in which only one amino acid residue was modified at a time. It was, therefore, of interest to investigate the ability of these antibodies to cross-react with polypeptides possessing two or more modified residues. For this purpose, the following polymers were prepared and used: poly(L-phenylalanyl-L-glutamyl-L-valylglycyl)glycine methyl ester (V) and poly(L-phenylalanyl-L-aspartyl-L-valylglycyl)glycine methyl ester (VI).

Table I—Amounts[a] of Protein Nitrogen Precipitated by Homologous and Heterologous Polypeptides

Polypeptide	Protein Nitrogen Precipitated at Equivalence Point, mcg.	Protein Nitrogen Precipitated by I after Absorption, mcg.	Protein Nitrogen Precipitated by Polypeptide, %
(Tyr-Glu-Ala-Gly)$_n$Gly (I)	106	0	100
(Phe-Glu-Ala-Gly)$_n$Gly (II)	61	45	57
(Tyr-Asp-Ala-Gly)$_n$Gly (III)	26	78	25
(Tyr-Glu-Val-Gly)$_n$Gly (IV)	106	0	100
(Phe-Glu-Val-Gly)$_n$Gly (V)	63	43	60
(Phe-Asp-Val-Gly)$_n$Gly (VI)	11	94	10

[a] Per milliliter of anti-poly(Tyr-Glu-Ala-Gly)$_n$Gly-1-^{14}C ethyl ester serum.

DISCUSSION

The syntheses of both polymers, V and VI, were performed as detailed in the *Experimental* section. The respective polymerization units were activated by use of the pentachlorophenyl ester (11). The high dilution procedure of polymerization was employed in both cases, since this method has been shown to produce linear high molecular weight polypeptides (1, 2, 4–10). Each polymer was purified by extensive dialysis and then fractionated by successive diafiltrations through membranes[1] into four different molecular weight fractions: $>5 \times 10^4$, $2\text{–}5 \times 10^4$, $1\text{–}2 \times 10^4$, and $<1 \times 10^4$.

Eight rabbits were immunized against I of molecular weight >50,000, using the previously reported protocol (3). It was found that each serum gave a positive precipitin reaction with the homologous polymer I. The serum from each animal was pooled since it was assumed that each rabbit had responded to the same antigenic determinants in this time interval (12). Using the polypeptides V and VI, each of molecular weight >50,000, incremental amounts of these polymers were added to 1-ml. aliquots of this pooled antiserum; cross-reactions occurred with both polymers. However, neither heterologous polymer precipitated as much antibody as the homologous polypeptide I. To quantitate the amounts of antibody not precipitated by either heterologous polymer, two separate experiments were performed. Quantities equal to the equivalence point amounts of each heterologous polypeptide, V and VI, were reacted with the pooled serums. After removal of the precipitates, 30 mcg. of the homologous polypeptide I was added to the two resulting supernatant liquids. Further precipitation was obtained and quantitated by analysis for nitrogen (Kjeldahl) (Table I).

It has been found that the heterologous polypeptides, V and VI, cross-react with anti-I-serum but precipitate less antibody than the homologous antigen I. It appears from these cross-reactions that the conformations of polymers V and VI are similar to that of the homologous antigen I. A similar situation has been shown to exist for the polypeptides II (8), III (9), and IV (10) (included in Table I for comparative purposes). By using the rationale that the determinants of these heterologous polypeptides are in the same orientation as those of the antigen I, it is suggested that observed differences in the binding abilities of these heterologous polymers are due only to those specifically modified amino acid residues. Comparison of the percentage of protein nitrogen precipitated by polymers I and IV has caused the inference to be made that anti-I-serum does not possess a specificity for the alanyl residue (10). Thus, since the percentages of protein nitrogen precipitated by the polymers II and V are similar, their lower abilities to precipitate with anti-I-serum

[1] Diaflo.

may be due only to their deficiency of phenolic hydroxyl groups. Comparison of the percentage of protein nitrogen precipitated by polymers I and III suggested that antibodies to I have a high affinity for the γ-carboxyl group of the glutamyl residue (9). The small amount of precipitate produced by polymer VI is considered as further evidence that antibodies to I have specificities for the phenolic hydroxyl group of the tyrosyl residue and the γ-carboxyl group of the glutamyl residue.

EXPERIMENTAL[2]

***N*-Benzyloxycarbonyl-γ-*tert*-butyl-L-glutamyl-L-valylglycine Methyl Ester (VII)**—To a solution of 2.05 g. (0.005 mole) of γ-*tert*-butyl-L-glutamyl-L-valylglycyl methyl ester hydrochloride (13) and 0.51 g. (0.005 mole) of triethylamine in 200 ml. of methylene chloride was added 2.74 g. (0.005 mole) of *N*-benzyloxycarbonyl-L-phenylalanine pentachlorophenyl ester. The mixture was stirred overnight at room temperature and concentrated; the product was dissolved in ethyl acetate, washed with 1 *N* HCl and water, and then dried (sodium sulfate). Concentration under reduced pressure gave an oil, which was chromatographed on a column[3] using chloroform–ethyl acetate (1:1) as eluent to give the fully blocked tetrapeptide. Crystallization from ethyl acetate–hexane yielded 3.0 g. (88%), m.p. 158–160°; $[\alpha]_D^{24}$ −21.6° (c 1.50 in dimethylformamide).

Anal.—Calc. for $C_{34}H_{46}N_4O_9$: C, 62.36; H, 7.08; N, 8.56. Found: C, 62.51; H, 6.95, N, 8.28.

***N*-Benzyloxycarbonyl-L-phenylalanyl-γ-*tert*-butyl-L-glutamyl-L-valylglycine (VIII)**—To a solution of 3.0 g. (0.00458 mole) of VII in 200 ml. of methanol was added 4.6 ml. of 1 *N* KOH, and the solution was stirred for 90 min. at room temperature and then concentrated under reduced pressure. The residue was flooded with water, acidified with 10% citric acid solution, and extracted into ethyl acetate. The ethyl acetate solution was dried (sodium sulfate) and concentrated under reduced pressure to give the tetrapeptide free acid. This was crystallized from ethyl acetate–hexane to yield 2.7 g. (95%), m.p. 185–186°; $[\alpha]_D^{24}$ −11.4° (c 1.26 in dimethylformamide).

Anal.—Calc. for $C_{33}H_{44}N_4O_9$: C, 61.84, H, 6.92; N, 8.75. Found: C, 61.63; H, 6.80; N, 8.90.

***N*-Benzyloxycarbonyl-L-phenylalanyl-γ-*tert*-butyl-L-glutamyl-L-valylglycine Pentachlorophenyl Ester (IX)**—To a solution of 2.7 g. (0.00422 mole) of VIII in 200 ml. of methylene chloride were added 1.12 g. (0.00422 mole) of pentachlorophenol and 2.0 g. (0.0047 mole) of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide methotoluene-*p*-sulfonate. The mixture was shaken for 2 days at room temperature and then concentrated under reduced pressure to give a solid. This material was washed with water and crystallized from methanol to yield 1.7 g. (45%), m.p. 223°; $[\alpha]_D^{24}$ −19.7° (c 3.04 in dimethylformamide).

Anal.—Calc. for $C_{39}H_{43}Cl_5N_4O_9$: C, 52.69; H, 4.87; N, 6.30. Found: C, 52.43; H, 4.90; N, 6.24.

L-Phenylalanyl-γ-*tert*-butyl-L-glutamyl-L-valylglycine Pentachlorophenyl Ester Hydrochloride (X)—A suspension of 1.7 g. (0.00191 mole) of the tetrapeptide active ester IX and 0.5 g. of 10% palladium-on-carbon in 200 ml. of methanol was treated with 0.07 g. (0.00192 mole) of dry hydrogen chloride in methanol, and the suspension was hydrogenated for 2 hr. The reaction mixture was filtered and the filtrate was concentrated. The residue was crystallized from methanol–ether to give 1.1 g. (73%), m.p. 210–212° dec.; $[\alpha]_D^{24}$ −25° (c 0.60 in dimethylformamide).

Anal.—Calc. for $C_{31}H_{38}Cl_6N_4O_7$: C, 47.03; H, 4.84; N, 7.08. Found: C, 46.83; H, 4.91; N, 7.22.

Poly(L-phenylalanyl-L-glutamyl-L-valylglycyl)glycine Methyl Ester (V)—To a solution of 0.42 g. (0.00415 mole) of triethylamine and 0.5 mg. of glycine methyl ester hydrochloride in 5 ml. of dimethyl sulfoxide was added a solution of 1.1 g. (0.00139 mole) of the polymerizing unit X in 8.3 ml. of dimethyl sulfoxide. The mixture was shaken for 1 week and then centrifuged to yield the fully protected polymer. This material was washed with three 35-ml. portions of water and three 35-ml. portions of ether and dried to give the blocked polymer. Then this material was treated with 30 ml. of 90% trifluoroacetic acid, stirred for 50 min., and concentrated under reduced pressure to yield the crude polypeptide V. This polypeptide was suspended in 15 ml. of water and dissolved by the addition of 1 *N* NaOH to pH 7.5. The solution was dialyzed against distilled water overnight, acidified with 6 *N* HCl to pH 2.5, and dialyzed against distilled water for a day. The precipitated polypeptide V was collected by centrifugation and then lyophilized to yield 0.13 g. (21%); amino acid ratios of an acid hydrolysate were: $Phe_{1.0}$ $Glu_{1.0}$ $Val_{0.9}$ $Gly_{1.0}$.

***N*-Benzyloxycarbonyl-β-*tert*-butyl-L-aspartyl-L-valylglycine Methyl Ester (XI)**—To a solution of 9.0 g. (0.04 mole) of L-valylglycine methyl ester hydrochloride and 4.1 g. (0.04 mole) of triethylamine in 230 ml. of methylene chloride was added 22.8 g. (0.04 mole) of *N*-benzyloxycarbonyl-β-*tert*-butyl-L-aspartic acid pentachlorophenyl ester. The mixture was stirred overnight at room temperature and concentrated, and the product was dissolved in ethyl acetate, washed with 10% citric acid solution and water, and then dried (sodium sulfate). Concentration under reduced pressure yielded an oil, which was chromatographed on a column[3] using chloroform–ethyl acetate (1:1) as eluent. The tripeptide was obtained and crystallized from ethyl acetate–hexane to give 10.0 g. (50%) of the product, m.p. 128°; $[\alpha]_D^{24}$ −25.4° (c 1.18 in dimethylformamide).

Anal.—Calc. for $C_{24}H_{33}N_3O_8$: C, 58.40; H, 7.14; N, 8.51. Found: C, 58.21; H, 7.23; N, 8.50.

***N*-Benzyloxycarbonyl-L-phenylalanyl-β-*tert*-butyl-L-aspartyl-L-valylglycine Methyl Ester (XII)**—A suspension of 6.5 g. (0.0132 mole) of XI and 0.8 g. of 10% palladium-on-carbon in 200 ml. of methanol was treated with 0.482 g. (0.0132 mole) of dry hydrogen chloride in methanol, and the suspension was hydrogenated for 2 hr. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The residue was dissolved in 200 ml. of methylene chloride and 1.35 g. (0.0132 mole) of triethylamine, and then 7.2 g. (0.0132 mole) of *N*-benzyloxycarbonyl-L-phenylalanine pentachlorophenyl ester was added. The reaction mixture was stirred overnight at room temperature and concentrated under reduced pressure. The product was dissolved in ethyl acetate, washed with 1 *N* HCl and water, and then dried (sodium sulfate). Concentration under reduced pressure yielded a solid, which was chromatographed on a column[3] using chloroform–ethyl acetate (1:1) as eluent to give the fully blocked tetrapeptide. Crystallization from ethyl acetate–hexane yielded 6.5 g. (76%), m.p. 183°; $[\alpha]_D^{24}$ −30.3° (c 3.80 in dimethylformamide).

Anal.—Calc. for $C_{33}H_{44}N_4O_9$: C, 61.84; H, 6.92; N, 8.74. Found: C, 61.73; H, 7.01; N, 8.82.

***N*-Benzyloxycarbonyl-L-phenylalanyl-β-*tert*-butyl-L-aspartyl-L-valylglycine Pentachlorophenyl Ester (XIII)**—To a solution of 6.0 g. (0.00936 mole) of XII in 250 ml. of methanol was added 9.36 ml. of 1 *N* KOH, and the solution was stirred for 90 min. at room temperature and then concentrated under reduced pressure. The residue was flooded with water, acidified with 10% citric acid solution, and extracted into ethyl acetate. The ethyl acetate solution was dried (sodium sulfate) and concentrated under reduced pressure to give 5.9 g. (100%) of the tetrapeptide free acid. This material was dissolved in 250 ml. of methylene chloride, and then 2.49 g. (0.00936 mole) of pentachlorophenol and 4.4 g. (0.0135 mole) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methotoluene-*p*-sulfonate were added. The mixture was shaken for 3 days at room temperature and then concentrated under reduced pressure to give a solid. This solid was washed with water and crystallized from methanol to yield 3.5 g. (43%) of the product, m.p. 164–166°; $[\alpha]_D^{24}$ −22.2° (c 2.48 in dimethylformamide).

Anal.—Calc. for $C_{38}H_{41}Cl_5N_4O_9$: C, 52.17; H, 4.72; N, 6.41. Found: C, 52.04; H, 4.62; N, 6.36.

Poly(L-phenylalanyl-L-aspartyl-L-valylglycyl)glycine Methyl Ester (VI)—A suspension of 3.5 g. (0.004 mole) of the tetrapeptide active ester XIII and 0.5 g. of 10% palladium-on-carbon in 250 ml. of methanol was treated with 0.146 g. (0.004 mole) of dry hydrogen chloride in methanol, and the suspension was hydrogenated for 2 hr. The reacting mixture was filtered, and the filtrate was concentrated under reduced pressure to yield 3.1 g. (100%) of the tetrapeptide hydrochloride. This material was dissolved in 35 ml. of dimethyl sulfoxide and added to a solution of 1.21 g. (0.012 mole) of triethylamine and 0.7 mg. of glycine methyl ester hydrochloride in 5 ml. of dimethyl sulfoxide. The mixture was shaken for a week and then centrifuged to yield the fully protected polymer. This

[2] Melting points were taken with a Mel-Temp apparatus and are uncorrected.

[3] Silicar CC-7.

material was washed with three 35-ml. portions of water and three 35-ml. portions of ether and then dried to give the blocked polymer. This material was treated with 30 ml. of 90% trifluoroacetic acid, stirred for 50 min., and then concentrated under reduced pressure to yield the crude polypeptide VI. This material was suspended in 30 ml. of water and dissolved by the addition of 1 *N* NaOH to pH 7.5. The solution was dialyzed against distilled water overnight, acidified with 6 *N* HCl to pH 2.5, and then dialyzed against distilled water for a day. The precipitated polypeptide VI was collected by centrifugation and then lyophilized to yield 0.1 g. (6%) of the polymer; amino acid ratios of an acid hydrolysate were: $Phe_{1.0}$-$Asp_{1.0}Val_{1.0}Gly_{1.0}$.

Molecular Weight Determinations—The polypeptide V (0.12 g.) was dissolved in 50 ml. of water by the addition of 1 *N* NaOH to pH 7.4. This solution was separated into four molecular weight ranges by successive diafiltrations through membranes[4]. Each fraction was acidified with 6 *N* HCl, dialyzed, and lyophilized to yield 0.06 g. (mol. wt. >50,000), 0.02 g. (mol. wt. 20,000-50,000), 0.02 g. (mol. wt. 10,000-20,000), and 0.015 g. (mol. wt. <10,000).

A similar process was used to fractionate 0.1 g. of the polypeptide VI, which was comprised of the following: 0.02 g. (mol. wt. >50,000), 0.01 g. (mol. wt. 20,000-50,000), 0.03 g. (mol. wt. 10,000-20,000), and 0.03 g. (mol. wt. <10,000).

Immunochemical Procedures—Eight rabbits were treated with poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I), molecular weight >50,000, at weekly intervals, using the immunization schedule previously described (3); 25 days after the last injection, all rabbits were bled using the standard heart puncture technique. Serum from each rabbit was tested for the precipitin reaction with the homologous antigen I, and serum from each animal gave a positive precipitin reaction. The serum from each animal was pooled, and this combined serum was used for the following experiments. It was assumed that the antibodies produced by each rabbit after the same time interval was directed against the same antigenic determinants of the antigen I.

Quantitative Precipitin Reactions—To 1-ml. aliquots of the pooled rabbit serum were added incremental amounts of the polypeptide I. Each tube was made up to 2 ml. with buffer (0.1 *M* NaCl-0.05 *M* $NaHCO_3$), incubated for 1 hr. at 37°, and then kept at 4° for 48 hr. The tubes were centrifuged in the cold and the precipitates were washed twice with 1 ml. of buffer (0.05 *M* K_2HPO_4-NaOH), pH 7.0. The total amount of protein precipitated was estimated by analysis for nitrogen (Kjeldahl). For V and VI, both of which possessed a molecular weight >50,000, quantitative precipitin reactions were performed using the pooled rabbit serum. These reactions were identical to and run simultaneously with those used for the polypeptide I (Table I).

Absorption Studies—The pooled rabbit serum was treated with the equivalence point amount of the heterologous polypeptides V and VI, each possessing a molecular weight >50,000. The corresponding precipitates were centrifuged out, and the supernatant liquids were poured off into separate tubes. To each supernatant liquid was added 30 mcg. of the homologous antigen, poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I). The tubes were incubated at 37° for 1 hr. and then stored at 4° for 48 hr. The precipitates were collected by centrifugation and washed twice with 1 ml. of buffer solution (0.05 *M* K_2HPO_4-NaOH), pH 7.0. The amount of protein was estimated by analysis for nitrogen (Kjeldahl) (Table I). Controls in which the serum was first absorbed with the homologous antigen I ascertained that the homologous antigen precipitated all of the antibody, since the supernatant liquid gave no further precipitin reaction when 30 mcg. of I was added.

[4] Diaflo, XM 50, UM 20E, and UM 10, Amicon Corp., Lexington, Mass.

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Solubility of Cholesterol and Hormone Drugs in Water

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Abstract □ The aqueous solubilities of cholesterol, testosterone, progesterone, and diethylstilbestrol at 30° were determined using a radioactive assay procedure.

Keyphrases □ Cholesterol—radioactive determination of water solubility □ Testosterone—radioactive determination of water solubility □ Progesterone—radioactive determination of water solubility □ Diethylstilbestrol—radioactive determination of water solubility □ Solubility, water—cholesterol, testosterone, progesterone, diethylstilbestrol, radioactive assay procedure

A search of the literature indicated that solubility values for hormone drugs in water varied considerably and in some cases were not available (1–11). Scattered data reported in the literature for the aqueous solubilities of steroid hormones are presented in Table I. No values were found for diethylstilbestrol. As part of a study on the effects of macromolecules on the solubility of cholesterol and some steroid hormones (12), it was necessary to consider the solution behavior of these compounds.

EXPERIMENTAL

Materials—Progesterone[1], diethylstilbestrol[2], and testosterone[3] were recrystallized from alcohol or diluted alcohol, and their melting points were in good agreement with published values. Cholesterol[4] was purified by recrystallizing from acetic acid. The crystals were dried at 90° in an oven. The sample was then recrystallized from a 70% alcohol–water mixture and dried in a vacuum desiccator. The melting point was found to be 148.5°, and the IR spectrum was in good agreement with reported spectra.

Solubility Experiments—A stock solution of cholesterol in benzene was prepared containing 1.0 mg. of untagged cholesterol and 10 μc. of cholesterol-26-^{14}C[5]. Stock solutions of testosterone and progesterone were prepared to contain 200 mg. of untagged compound and 5 μc. of the labeled compound (testosterone-4-^{14}C and progesterone-4-^{14}C)[5] in 100 ml. of benzene, and a similar stock solution of diethylstilbestrol [using diethylstilbestrol (monoethyl-1-^{14}C)][6] was prepared in methanol. A known amount of a particular stock solution was transferred into a 125-ml. iodine flask, and the solvent was evaporated under a mild stream of nitrogen with constant shaking. A 50-ml. volume of dissolution medium (distilled water or 0.9% saline) kept at 30° was added to the flask. Dissolution studies were carried out at 30 ± 0.5° using a controlled environment unit[6] to maintain the temperature. The solution was stirred at approximately 250–300 r.p.m. using a magnetic stirrer and a 2.7 × 1-cm. Teflon-coated spin bar.

For each dissolution study, the amount of compound used was approximately 10 times in excess of its determined aqueous solubility. At predetermined intervals, 5 ml. of sample was withdrawn from the system and filtered immediately through a filtration assembly[7] containing a 0.45-μ filter. Additional samples containing cholesterol were also filtered through 0.22-μ filters.

[1] Sigma Chemical Co.
[2] Matheson, Coleman and Bell.
[3] Shering Corp.
[4] Fisher Scientific.
[5] Nuclear Chicago.
[6] Hot Pack model 1278-8.
[7] Millipore.

Table I—Solubility of Steroids in Water

Compound	Solubility in Water, mcg./ml. This Work (30°)	Literature
Cholesterol	0.09[a]	2600 (20°) (1), 52 (25°) (2)
	0.041[b]	0.025 (30°) (3), 2 (25°) (8), 0.06 (30°) (4)
Progesterone	11.0[c]	9 (25°) (5), 6.6 (25°) (6), 16.8 (37°) (1)
Diethylstilbestrol	25.0[d]	No values found
Testosterone	30.0[e]	24 (25°) (5), 27 (25°) (6), 36 (37°) (6), 29 (25°) (7), 25 (25°) (9–11), 28 (37°) (14), 48 (25°) (2), 125 (37°) (3)

[a] Mean of eight experiments (range 0.07–0.12) using 0.45-μ filter. [b] Mean of eight experiments (range 0.022–0.050) using 0.22-μ filter. [c] Mean of eight experiments (range 8–12). [d] Mean of six experiments (range 21–28). [e] Mean of eight experiments (range 26–33).

Dissolution experiments were carried out in water and 0.9% NaCl solution for cholesterol, testosterone, progesterone, and diethylstilbestrol. A 100-ml. volume of dissolution medium in a 250-ml. iodine flask was used for testosterone studies.

Radioactive Assay Procedure—A 0.20-ml. volume of the particle-free filtrate was pipeted into a liquid scintillation vial. To this was transferred 15 ml. of dioxane–naphthalene scintillation cocktail (13). The scintillation vial was tightly capped and shaken for about 30 sec. to ensure thorough mixing of the experimental solution and the phosphore. At the same time, a blank was prepared in the same manner except that the compound was omitted from the solution. The standard was prepared by pipeting 0.10 ml. of the standard stock solution of the experimental compound into a scintillation vial. After the solvent (benzene or methanol) was evaporated, 0.20 ml. of water and 15 ml. of dioxane–naphthalene scintillation cocktail were added and the vial was shaken. The samples were counted directly using a liquid scintillation system[8]. Sufficient time was allowed for the samples to cool to 4.0° to minimize quenching. The samples were counted for 10 min. and the counts per minute (c.p.m.) were obtained by dividing the 10-min. counts by 10. The solubility values were obtained from the ratio of the counts per minute of the internal standard and the sample.

RESULTS AND DISCUSSION

By using a radioactive assay procedure, the aqueous solubilities of cholesterol, testosterone, progesterone, and diethylstilbestrol were determined at 30° (Table I). Solubilities were also determined in 0.9% NaCl (Table II). Solubilities of hormone compounds were determined as they approached equilibrium and after equilibrium had been established. The solubility values reported in Tables I and II are the solubility values at equilibrium, and the complete solution behavior of these steroids is presented in Figs. 1 and 2.

Variations in water solubility of cholesterol, progesterone, and testosterone were in general accord with the principle that solubility increases with the number of polar groups. As expected, cholesterol

[8] Unilux II, Nuclear Chicago.

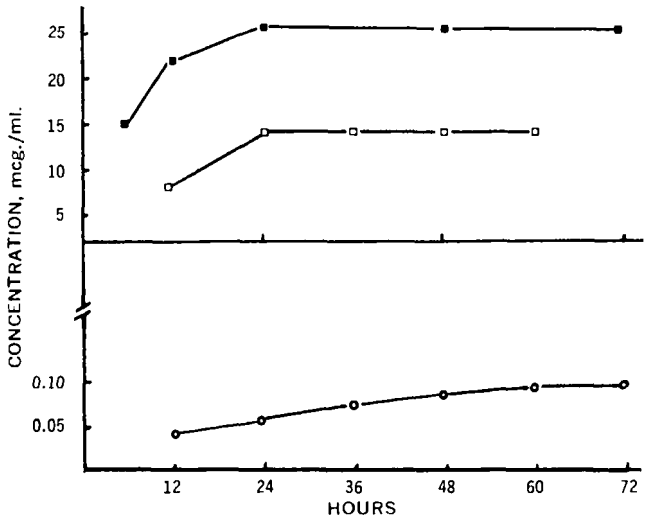

Figure 1—*Aqueous solubility of cholesterol* (○), *progesterone* (□), *and diethylstilbestrol* (■) *at 30°.*

was the least water soluble since it was the most nonpolar and bulkiest of the compounds studied. Testosterone, with one α,β-unsaturated ketone group and a free hydroxyl group, had the highest water solubility of the hormones studied.

Although diethylstilbestrol has a lower molecular weight (268) than testosterone (288), its aqueous solubility was lower; however, its melting point is considerably higher (170°) than testosterone (154°). Therefore, it is quite conceivable that due to its symmetric nature, it may form crystals of high lattice energy as compared to the relatively less symmetrical molecule of testosterone.

Using ^{14}C-labeled cholesterol, Gemant (8) studied the solubilization of cholesterol using a partition chromatographic method. He assumed that, in a solution that solubilized cholesterol, the cholesterol would travel with the moving phase; the stronger the solubilizing action, the farther it would travel. Determination of cholesterol distribution along the paper was accomplished by a radioactive tracer method. ^{14}C-Labeled cholesterol was applied to the paper and, after a suitable time of development, the strip was cut into sections and the activity of each section was measured. He found the solubility of cholesterol to be 0.2 mg./100 ml. of water (= 2 mcg./ml.), more than 20 times the solubility found in the current study.

In more recent work, Saad and Higuchi (3) found the solubility of cholesterol to be 0.025 mcg./ml. in both water and 0.9% NaCl. They equilibrated the cholesterol-water or cholesterol–0.9% NaCl for 30 days and used 0.10- and 0.22-μ filters to clarify the solutions before assay. When 0.45-μ filters were used, significantly higher results (1.5 and 2 times greater) were obtained. The results obtained

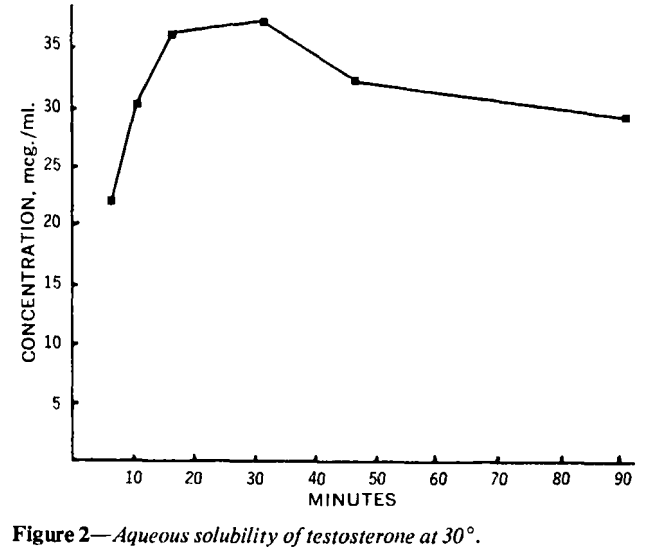

Figure 2—*Aqueous solubility of testosterone at 30°.*

Table II—Solubility of Steroids in 0.9% NaCl

Steroid	Solubility, mcg./ml.[a] This Work (30°)	Literature
Cholesterol	0.017	0.025 (30°) (3)
Progesterone	10.0	6.6 (25°) (6)
Diethylstilbestrol	24.5	No values found
Testosterone	29.5	36 (37°) (6), 27 (25°) (6)

[a] Average of two experiments.

in the present study using 0.22-μ filters (0.041 mcg./ml. water and 0.017 mcg./ml. 0.9% NaCl) are in good agreement with the findings (0.025 mcg./ml.) of Saad and Higuchi (3) considering the extremely low solubility levels of cholesterol. When 0.45-μ filters were used in the present study, the aqueous solubility was more than 2 times higher than the solubility data for 0.22-μ filters.

Several experiments were carried out in which sodium chloride was added to saturated solutions of cholesterol (0.09 mcg./ml.) to give a 0.9% salt concentration. The cholesterol solubility values for these solutions decreased to 0.017 mcg./ml. The marked decrease in solubility indicated the possibility that cholesterol crystal nuclei were salted out of solution by the addition of sodium chloride, leaving a true solution of cholesterol. These experiments appear to substantiate that the Coulter counter method used by Saad and Higuchi (3) was able to ascertain the true solubility of cholesterol by detecting crystal nuclei formation.

The solubility values for progesterone, testosterone, and diethylstilbestrol were essentially the same in 0.9% sodium chloride as in water (Tables I and II). There was no significant decrease in hormone concentration when 0.9% sodium chloride was added to saturated solutions of these compounds.

The solution behavior of testosterone is presented in Fig. 2. The equilibrium was approached from supersaturation and not from undersaturation. The decrease in high initial solubility and its subsequent leveling off suggest the conversion of a metastable crystalline form possessing a higher solubility to a form of lower solubility. Thakkar and Hall (9–11) examined the aqueous solution behavior of anhydrous and hydrous testosterone and observed an initial supersaturation with a subsequent leveling for the anhydrous form. The anhydrous form dissolved faster than the hydrous form. They also compared the X-ray diffraction patterns of the original anhydrous form and the residue remaining after the equilibration of the anhydrous form and found that an alteration in the crystalline structure did take place.

In 1954, Bischoff and Stauffer (6) studied the solution behavior of testosterone and found that short equilibration periods gave rise to supersaturated solutions. They also observed that if the solvent was changed after 5 hr. of equilibration, the residue did not produce supersaturated solutions. Heating this residue to slightly below the melting point restored it to the form that produced supersaturated solutions. They hypothesized that this difference in the solution behavior could be due to a difference in the particle sizes of the two samples of testosterone, *i.e.*, one that gave rise to supersaturated solution and one that did not. Actual microscopic examination revealed, however, that there was no difference in the particle size of the two samples. These workers postulated that an explanation of the anomolous solution behavior of testosterone may lie in the existence of polymorphic forms.

Recently, Thakkar and Hall (9–11) attempted to explain the observation of Bischoff and Stauffer (6) by stating that the testosterone sample which produced a supersaturated solution was clearly an anhydrous form whereas the water equilibrated residue which did not was a hydrated form. As might be expected, this hydrate lost its water of hydration and reverted to the anhydrous form when heated to slightly below the melting point. That such a conversion does really occur was shown by the similarity of the X-ray diffraction pattern of the heat-dehydrated hydrate sample to that of the anhydrous form.

Other workers (15, 16) also observed similar solution behavior differences in various solvents for a number of organic compounds including steroids, cholesterol, and fluorohydrocortisone acetate. The decrease in solubility with time once the peak solubility is attained has been explained as being due to nucleation and crystallization of the more stable hydrate.

The peak solubility value displayed during the dissolution of the anhydrous form of testosterone (Fig. 2) may correspond to a short-term steady-state situation involving equal rates of dissolution of the anhydrous form and crystallization of the stable hydrate. On the other hand, the peak solubility could also correspond to the solubility of the anhydrous form. However, the solubilities of both forms of testosterone appear to approach the same value with time (11).

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Central Cholinomimetic Actions of 3,3-Dimethyl-1-butanol Carbamate

LYNN WECKER* and C. Y. CHIOU▲

Abstract □ The action of 3,3-dimethyl-1-butanol carbamate on the CNS was studied to determine the mechanism of its convulsant activity. Dimethylbutanol carbamate penetrated into the brain with maximum levels at 15 min. after intraperitoneal injection. The LD_{50} of dimethylbutanol carbamate in mice was 21 mg./kg., and atropine pretreatment protected animals against the toxic effects of dimethylbutanol carbamate, whereas physostigmine enhanced dimethylbutanol carbamate toxicity. Dimethylbutanol carbamate potentiated nicotine-induced convulsions in mice and had no effect on brain cholinesterase activity. Therefore, it appears that dimethylbutanol carbamate is a centrally active agent that produces convulsions *via* a mechanism similar to that postulated for nicotinic agents, although it produces antinicotinic actions peripherally.

Keyphrases □ 3,3-Dimethyl-1-butanol carbamate—mechanism of convulsant activity, central cholinomimetic actions, mice □ Cholinomimetic actions—3,3-dimethyl-1-butanol carbamate, mice □ Convulsant activity—3,3-dimethyl-1-butanol carbamate, mice

3,3-Dimethyl-1-butanol carbamate, a synthetic carbachol analog containing a carbon in place of the quaternary nitrogen atom, is an unusually interesting compound with a potent peripheral antinicotinic effect (1, 2) as well as a central convulsant action in mice, producing tonic-clonic convulsions (3, 4). The effects of this agent on cholinergic-induced drinking behavior in rats have been studied, and it was reported (5) that dimethylbutanol carbamate, when implanted into the lateral hypothalamic region of the rat brain, initiated a response similar to those of acetylcholine and carbachol. These results suggested a central cholinomimetic action of dimethylbutanol carbamate. It has been hypothesized that alterations in brain levels of acetylcholine may be correlated with behavioral changes and that there is a decrease in the acetylcholine content of the brain during convulsions (6–8). When an increase in cortical activity is evident, there is a release of acetylcholine from its storage sites in nerve terminals, resulting in an overall decrease in brain acetylcholine content (6–8).

Dimethylbutanol carbamate was found to cause a significant increase in the release of acetylcholine from the minced guinea pig cerebral cortex (1). Therefore, the present study investigated the possibility that the CNS actions of dimethylbutanol carbamate are due to a direct-releasing action of acetylcholine from storage sites inside nerve endings, a mechanism similar to that reported for the action of nicotinic agents (9). The unusual character of this compound could be used as a tool to elucidate cholinergic mechanisms.

EXPERIMENTAL

ICR Charles River mice, weighing 20–35 g., were used for all studies. To determine the extent of brain penetration of dimethylbutanol carbamate, mice were injected intraperitoneally with 20 mg./kg. ^{14}C-dimethylbutanol carbamate (0.7 μc./mg.). Dimethylbutanol carbamate was synthesized in this laboratory according to the method described previously (1). The animals were sacrificed by cervical dislocation at various times, and their brains were removed, weighed, homogenized (1 ml. water/1 g. tissue), and solubilized[1] at 50° for 12–15 hr. The samples were allowed to cool, and

[1] NCS solubilizer, Amersham Searle, Arlington Heights, Ill.

The peak solubility value displayed during the dissolution of the anhydrous form of testosterone (Fig. 2) may correspond to a short-term steady-state situation involving equal rates of dissolution of the anhydrous form and crystallization of the stable hydrate. On the other hand, the peak solubility could also correspond to the solubility of the anhydrous form. However, the solubilities of both forms of testosterone appear to approach the same value with time (11).

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Central Cholinomimetic Actions of 3,3-Dimethyl-1-butanol Carbamate

LYNN WECKER* and C. Y. CHIOU▲

Abstract □ The action of 3,3-dimethyl-1-butanol carbamate on the CNS was studied to determine the mechanism of its convulsant activity. Dimethylbutanol carbamate penetrated into the brain with maximum levels at 15 min. after intraperitoneal injection. The LD_{50} of dimethylbutanol carbamate in mice was 21 mg./kg., and atropine pretreatment protected animals against the toxic effects of dimethylbutanol carbamate, whereas physostigmine enhanced dimethylbutanol carbamate toxicity. Dimethylbutanol carbamate potentiated nicotine-induced convulsions in mice and had no effect on brain cholinesterase activity. Therefore, it appears that dimethylbutanol carbamate is a centrally active agent that produces convulsions *via* a mechanism similar to that postulated for nicotinic agents, although it produces antinicotinic actions peripherally.

Keyphrases □ 3,3-Dimethyl-1-butanol carbamate—mechanism of convulsant activity, central cholinomimetic actions, mice □ Cholinomimetic actions—3,3-dimethyl-1-butanol carbamate, mice □ Convulsant activity—3,3-dimethyl-1-butanol carbamate, mice

3,3-Dimethyl-1-butanol carbamate, a synthetic carbachol analog containing a carbon in place of the quaternary nitrogen atom, is an unusually interesting compound with a potent peripheral antinicotinic effect (1, 2) as well as a central convulsant action in mice, producing tonic-clonic convulsions (3, 4). The effects of this agent on cholinergic-induced drinking behavior in rats have been studied, and it was reported (5) that dimethylbutanol carbamate, when implanted into the lateral hypothalamic region of the rat brain, initiated a response similar to those of acetylcholine and carbachol. These results suggested a central cholinomimetic action of dimethylbutanol carbamate. It has been hypothesized that alterations in brain levels of acetylcholine may be correlated with behavioral changes and that there is a decrease in the acetylcholine content of the brain during convulsions (6–8). When an increase in cortical activity is evident, there is a release of acetylcholine from its storage sites in nerve terminals, resulting in an overall decrease in brain acetylcholine content (6–8).

Dimethylbutanol carbamate was found to cause a significant increase in the release of acetylcholine from the minced guinea pig cerebral cortex (1). Therefore, the present study investigated the possibility that the CNS actions of dimethylbutanol carbamate are due to a direct-releasing action of acetylcholine from storage sites inside nerve endings, a mechanism similar to that reported for the action of nicotinic agents (9). The unusual character of this compound could be used as a tool to elucidate cholinergic mechanisms.

EXPERIMENTAL

ICR Charles River mice, weighing 20–35 g., were used for all studies. To determine the extent of brain penetration of dimethylbutanol carbamate, mice were injected intraperitoneally with 20 mg./kg. ^{14}C-dimethylbutanol carbamate (0.7 μc./mg.). Dimethylbutanol carbamate was synthesized in this laboratory according to the method described previously (1). The animals were sacrificed by cervical dislocation at various times, and their brains were removed, weighed, homogenized (1 ml. water/1 g. tissue), and solubilized[1] at 50° for 12–15 hr. The samples were allowed to cool, and

[1] NCS solubilizer, Amersham Searle, Arlington Heights, Ill.

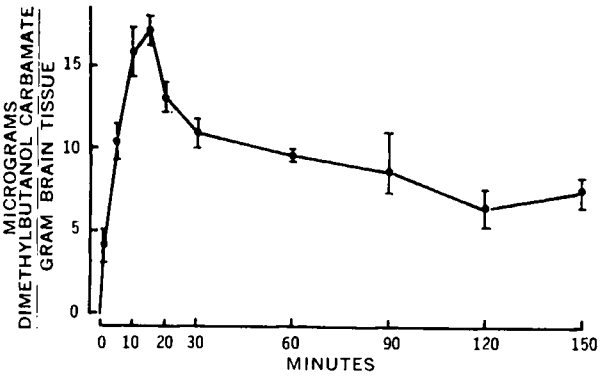

Figure 1—*Uptake of dimethylbutanol carbamate by mouse brain. Mice were injected with 20 mg./kg. ^{14}C-dimethylbutanol carbamate and sacrificed at time intervals shown on the abscissa. The brains were analyzed for radioactivity, and results are expressed as micrograms dimethylbutanol carbamate per gram brain tissue. Each point is a mean of three values, and the bars represent standard errors.*

then 20 ml. scintillation cocktail (6 g. 2,5-diphenyloxazole/l. toluene) was added. The samples were analyzed for radioactivity on a liquid scintillation system[2], with an error of ±1.0%.

The effects of atropine and physostigmine on the toxicity of dimethylbutanol carbamate were determined as follows. Mice were divided into three groups. The LD_{50} of dimethylbutanol carbamate was determined as the control using the first group. The second group of mice was pretreated with atropine (100 mg./kg.) 30 min. prior to dimethylbutanol carbamate administration. It was reported (10) that the effect of this atropine pretreatment is to decrease significantly brain levels of acetylcholine. The third group of mice was pretreated with 0.2 mg./kg. physostigmine 30 min. prior to dimethylbutanol carbamate injection. This physostigmine pretreatment has been found to produce significant behavioral alterations in mice. The LD_{50} and limits at the 95% confidence level for each group were determined by a log dose probit plot (11).

To determine the effects of dimethylbutanol carbamate on nicotine-induced convulsions, mice were divided into two groups. The first group was injected with nicotine intraperitoneally, and the number of animals that developed convulsions was noted. The second group was injected with a subconvulsant dose of dimethylbutanol carbamate (1 mg./kg.) intraperitoneally 30 min. prior to the nicotine injection, and the occurrence of convulsions was once again noted. The dose-response curves were constructed, and the ED_{50}'s to produce convulsant activity and potency ratios were calculated (11).

The effect of dimethylbutanol carbamate on intact brain cholinesterase was determined radioisotopically (12). Mice were sacrificed by cervical dislocation, and their brains were removed immediately, frozen, and weighed. The brains were homogenized for 30 sec. at 10,000 r.p.m.[3], in phosphate buffer (pH 8, 0.1 *M*), yielding a homogenate of 0.1 mg. brain/ml. phosphate buffer. A 0.1-ml. aliquot of the homogenate was added to centrifuge tubes containing phosphate buffer with or without dimethylbutanol carbamate (10^{-4} or 10^{-3} *M*). The tubes were preincubated for 15 min. at 37° before addition of ^{14}C-acetylcholine (1.2×10^{-5} *M*). The reaction mixtures were incubated for various periods, and 500 mg. cation-exchange resin[4] and 5 ml. absolute ethanol were added to stop the reaction. The mixture was shaken for 15 sec. and centrifuged at $1470 \times g$ for 5 min. Aliquots of the supernate (4.0 ml.) were sampled and added to 10 ml. scintillation cocktail (7 g. 2,5-diphenyloxazole and 100 g. naphthalene/l. dioxane). The samples were counted in a liquid scintillation system. Values for autohydrolysis of ^{14}C-acetylcholine were determined from incubation mixtures in the absence of homogenate. Initial rates of hydrolysis were determined during the first 30 min., and the rates were expressed as Δ c.p.m./min. Levels of statistical significance were determined with Student's *t* test at $p \leq 0.05$.

The drugs studied included 3,3-dimethyl-1-butanol carbamate, atropine sulfate, physostigmine sulfate, nicotine tartrate, and acetyl-1-^{14}C-choline iodine (4.53 mc./mmole)[5].

Table I—Effects of Atropine and Physostigmine on the Toxicity of Dimethylbutanol Carbamate in the Mouse

Treatment	Number of Animals	LD_{50} of Dimethylbutanol Carbamate, mg./kg.
Dimethylbutanol carbamate alone	150	21.0 (18.3–24.2)[a]
Atropine plus dimethylbutanol carbamate[b]	80	49.0 (29.7–80.9)[c]
Physostigmine plus dimethylbutanol carbamate[d]	80	17.0 (15.2–19.0)[c]

[a] Limits at the 95% confidence level determined by the method of Litchfield and Wilcoxon (11). [b] Treatment with atropine (100 mg./kg.) 30 min. prior to dimethylbutanol carbamate administration. [c] Significantly different from control at $p \leq 0.05$, determined by the method of Litchfield and Wilcoxon (11). [d] Treatment with physostigmine (0.2 mg./kg.) 30 min. prior to dimethylbutanol carbamate administration.

[2] Beckman liquid scintillation system, model 1650, Beckman Instruments, Fullerton, Calif.

RESULTS

To determine whether dimethylbutanol carbamate penetrated into the CNS, mice were injected intraperitoneally with ^{14}C-dimethylbutanol carbamate. Maximum brain levels were reached at approximately 15 min., and the concentration at that time was 17.1 mcg. dimethylbutanol carbamate/g. wet weight brain tissue (1.3×10^{-4} mole/kg.) (Fig. 1). After 15 min., the concentration of dimethylbutanol carbamate decreased steadily until a fairly constant level was apparent (8.5 mcg. dimethylbutanol carbamate/g. brain tissue) for the following 60 min.

When mice were injected with a lethal dose of dimethylbutanol carbamate, they exhibited initial ataxia followed by hyperactivity leading to tonic-clonic convulsions approximately 30–45 min. after injection. The duration of convulsions was approximately 5 min. before death occurred. At the time of death, the chest cavity of the animal was opened and heart function was inspected. Respiratory paralysis appeared to be the cause of death, but death was probably not due to a peripheral neuromuscular blockade since sciatic nerve gastrocnemius muscle blockade in rabbits was not achieved at doses that produced death. The LD_{50} for the mouse was 21 mg./kg. (Table I). When animals were pretreated with atropine, there was significant protection from dimethylbutanol carbamate toxicity and the LD_{50} was increased to 49 mg./kg. dimethylbutanol carbamate. Conversely, when mice were pretreated with physostigmine, dimethylbutanol carbamate toxicity was enhanced and the LD_{50} was lowered to 17 mg./kg.

The ED_{50} of nicotine to cause convulsion was lowered from 3.8 to 2.7 mg./kg. with dimethylbutanol carbamate pretreatment (Table II). This was a significant potentiation of convulsant activity, with a potency ratio of 1.4. The slopes of the dose-response curves indicate a parallelism with a slope ratio of 1.1.

The effect of dimethylbutanol carbamate on brain cholinesterase was studied to determine whether cholinesterase inhibition was involved with the central actions of dimethylbutanol carbamate. When mouse brain homogenates were incubated with dimethyl butanol carbamate (10^{-4} or 10^{-3} *M*), there was no effect on the rate

Table II—Effects of Dimethylbutanol Carbamate Pretreatment on Nicotine-Induced Convulsions in the Mouse

Treatment	Number of Animals	ED_{50} of Nicotine, mg./kg.	Slope
Nicotine alone	55	3.8 (3.4–4.2)[a]	1.3 (1.1–1.5)
Dimethylbutanol carbamate plus nicotine[b]	59	2.7 (2.3–3.1)[c]	1.4 (1.1–1.7)

[a] Limits at the 95% confidence level determined by the method of Litchfield and Wilcoxon (11). [b] Treatment with dimethylbutanol carbamate (1.0 mg./kg.) 30 min. prior to nicotine administration. [c] Significantly different from control at $p \leq 0.05$, determined by the method of Litchfield and Wilcoxon (11).

[3] Willems Polytron, Brinkmann Instruments, Westbury, N. Y.
[4] Amberlite CG120, Mallinckrodt Chemical Works, St. Louis, Mo.
[5] New England Nuclear, Boston, Mass.

Table III—Effects of Dimethylbutanol Carbamate on Rates of Acetyl-1-^{14}C-choline Hydrolysis by Mouse Brain Cholinesterases

Concentration of Dimethylbutanol Carbamate, *M*	Rate of Hydrolysis[a], Δ c.p.m./min.
Control[b]	96.1 ± 7.8
10^{-4}	105.3 ± 6.8
10^{-3}	108.0 ± 6.0

[a] Values expressed as the mean ± *SE* (n = 6). [b] Dimethyl sulfoxide (0.3% by volume) used as solvent control to prepare stock solutions of dimethylbutanol carbamate due to the limited solubility of dimethylbutanol carbamate in aqueous solutions.

of ^{14}C-acetylcholine hydrolysis (Table III), supporting a direct acetylcholine-releasing action by dimethylbutanol carbamate.

DISCUSSION

Dimethylbutanol carbamate has been shown to possess central cholinomimetic effects, although it has cholinolytic actions peripherally (1, 2). This agent has been shown to release acetylcholine from the minced guinea pig cerebral cortex (1) at concentrations that did not affect brain cholinestease activity, suggesting a direct acetylcholine-releasing action. From the studies on dimethylbutanol carbamate toxicity, it has been shown that atropine is able to protect mice from the lethal effects of dimethylbutanol carbamate, while physostigmine potentiates dimethylbutanol carbamate toxicity. Therefore, it appears that dimethylbutanol carbamate toxicity may be due to an increased transmitter release. This idea is supported by the observation that protection of cholinergic actions by atropine is able to decrease dimethylbutanol carbamate toxicity. Although precise action mechanisms of atropine in the CNS are not well established, it is clear that atropine reduces central acetylcholine levels (10), which are probably responsible for the reduction of central cholinergic actions of dimethylbutanol carbamate. Likewise, enhancement of toxicity has been shown to be evident in the presence of a cholinesterase inhibitor.

Dimethylbutanol carbamate was found to enhance nicotine toxicity. It has been shown that nicotine causes the release of acetylcholine in rat cerebral cortex (9), and nicotine-induced convulsions have been postulated to be due to an action on the CNS (13). Thus, it appears that the central actions of dimethylbutanol carbamate and nicotine may be quite similar. It was reported (14) that atropine was the most effective agent against nicotine-induced convulsions. It was also shown that atropine was very effective in protecting animals from dimethylbutanol carbamate toxicity, supporting a similar mechanism for the central actions of dimethylbutanol carbamate and nicotine.

In conclusion, it appears that dimethylbutanol carbamate is a potent central cholinomimetic agent whose action is due to the direct release of endogenous acetylcholine from nerve terminals. This may result in an overall decrease in brain acetylcholine to produce central convulsant activity in animals.

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Interaction between DNA and Coralyne Acetosulfate, an Antileukemic Compound

Keyphrases ☐ Coralyne acetosulfate—interaction (complexation) with calf thymus DNA in water ☐ DNA (calf thymus)—interaction (complexation) with coralyne acetosulfate in water

Sir:

The alkaloid coralyne displayed confirmed antileukemic activity against leukemias L-1210 and P-388 in laboratory animals. A practical preparation of the acetosulfate (sulfoacetate) and the chloride salts of coralyne was recently reported (1); the availability of this method has permitted an easy access to this alkaloid, thus permitting unhampered the continuation of biological, pharmacological, and clinical evaluation. Although the exact mode of action of coralyne is not yet understood, a comparative study of UV absorption characteristics revealed an *in vitro* interaction between coralyne and calf thymus DNA in aqueous solution. This phenomenon may be associated with the biological activity of coralyne.

When coralyne acetosulfate (I*a*) was dissolved in water, the UV absorption of the aqueous solution at pH 7 changed rapidly. The major absorption peak of the solution moved from 297 to 262 nm. within 1 hr. This hypsochromic shift was practically complete after 2 days (Fig. 1; the same phenomenon was observed with the less water-soluble chloride salt I*b*). However, when a 1:4 (weight ratio) mixture of I*a* and the sodium salt of calf thymus DNA[1] was dissolved in water at pH 7, the UV spectrum of this complex, which is different from that of either component under the same controlled conditions, showed no change on standing. Even after the solution was allowed to stand at 25° for 4 weeks, the shape of the UV spectrum remained practically unchanged (Fig. 2). Under similar conditions, when the thymus DNA was dissolved in water at pH 7, the UV spectrum showed a single peak at 258 nm. (Fig. 1) which slowly decreased in peak height on standing. A

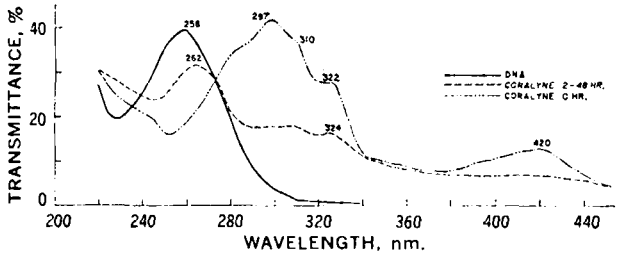
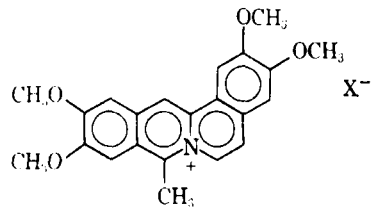


Figure 1—*UV absorption of coralyne and DNA in water at pH 7, 25° [coralyne acetosulfate, 5 mg./l.; and DNA (calf thymus, sodium salt), 20 mg./l.].*

[1] Calbiochem.



I*a*: X = $CH_3CO_2SO_3^-$
I*b*: X = Cl

precipitate formed after the aqueous DNA solution was stored at 25° for 4 weeks.

Coralyne acetosulfate apparently undergoes a rapid hydration process in aqueous solution. Presumably this is due to multiple bond hydration (2–4) across the C=N region, which results in breaking of the original conjugative system and, hence, alters the electronic transitions. The hydration is reversible; when an aqueous solution containing I*a* was kept at 25° for 2 hr. followed by subsequent lyophilization, the resulting solid showed an identical melting point and IR and UV spectra in methanol (Fig. 3) as the original material, I*a*. Under similar conditions, methanol was not added across the C=N bond, since the UV absorption peak remained unchanged even after long standing.

These results revealed that an interaction between coralyne and thymus DNA in water existed and that the resulting complex blocked the breaking of aromatic conjugation of coralyne by covalent hydration. These observations are significant since complex formation between many biologically active compounds with DNA double helix has been suggested to account for their activity (5–13). Also, a common N—O—O— triangulation feature, which was found among a number of antileukemic compounds including coralyne, has been proposed as a possible pharmacophore, which may contribute to the *in vivo* binding to some biologically pertinent sites (14). Detailed studies on hydration of coralyne as well as certain related problems are being actively performed.

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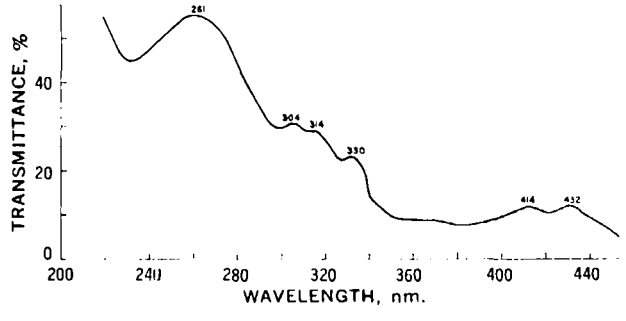

Figure 2—*UV absorption of DNA–coralyne in water at pH 7, 25° [coralyne acetosulfate, 5 mg./l.; and DNA (calf thymus, sodium salt), 20 mg./l.].*

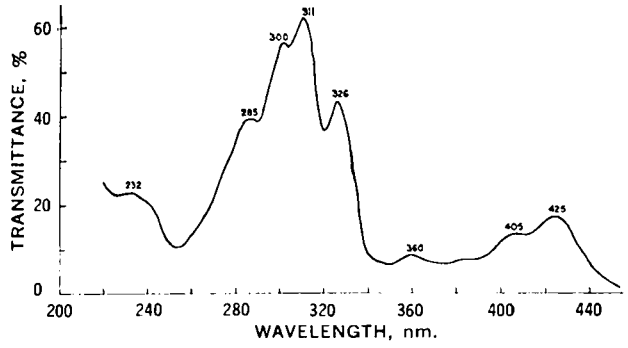

Figure 3—*UV spectrum of coralyne (acetosulfate) in methanol (5 mg./l.).*

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Effect of Formulation on Dissolution of Sodium Warfarin Tablets

Keyphrases □ Sodium warfarin tablets—effect of pregranulation dissolving on dissolution rate □ Dissolution rate, sodium warfarin tablets—effect of dissolving drug prior to granulation

Sir:

Numerous publications have shown that formulation of a solid dosage form can influence dissolution of the active drug. These studies have discussed the effect of binder concentration and tablet hardness (1), types of binders and particle size (2), types of starches (3), lubricants (4), and disintegration agents (5).

Table I—Dissolution Rate Constants (k) of Various Sodium Warfarin Tablet Formulations and Surface Tension of Solutions Used to Granulate

Formulation	k, min.$^{-1}$	Surface Tension[a], γ, dynes/cm.
A. Drug incorporated in dry form	0.060	—
B. Drug and polyvinylpyrrolidone dissolved in alcohol[b]	0.074	26.1
C. Drug dissolved in alcohol[c]	0.113	25.6
D. Drug and polyvinylpyrrolidone dissolved in water[d]	0.154	57.4
E. Drug dissolved in water[e]	0.246	59.4
	Absolute alcohol	24.4
	Water, deionized	74.1

[a] Of solution used to granulate. Measured by a Du Noüy interfacial tensiometer. [b] Solution ratio of drug–polyvinylpyrrolidone–alcohol = 1:1:6. [c] Solution ratio of drug–alcohol = 1:6. [d] Solution ratio of drug–polyvinylpyrrolidone–water = 1:1:4. [e] Solution ratio of drug–water = 1:4.

The purpose of this communication is to report that dissolving sodium warfarin prior to granulating can influence the dissolution rate.

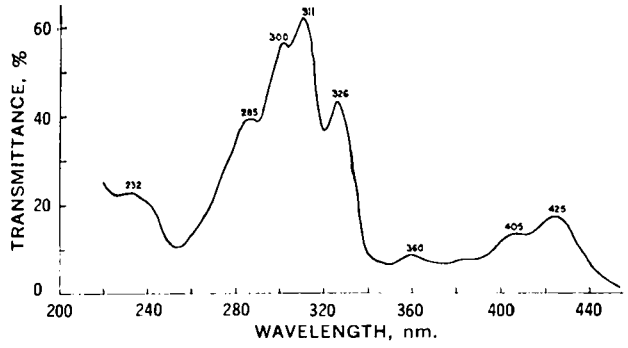
Sodium warfarin was incorporated into a microcrystalline cellulose–lactose (3:1) mixture such that 25 mg. of sodium warfarin was contained/275-mg. tablet. The drug was either added into the formulation in the dry state or dissolved in an aqueous or alcoholic media, with or without an equal ratio of polyvinylpyrrolidone–sodium warfarin. The solutions (Table I) were used to granulate the microcrystalline cellulose–lactose mixture. Magnesium stearate (0.1%) was added to either the powder mixture or the dried, 20-mesh screened granulation. Tablets were compressed on a compressing machine[1]. All tablets disintegrated within 1 min. Dissolution tests were performed in a round-bottom flask, containing 500 ml. of distilled water, maintained at 37°. A half-moon (2.5 cm. in diameter) stirring blade was used at a speed of 50 r.p.m. Assays were performed spectrophotometrically.

The pseudo-first-order rate constants obtained from this study are shown in Table I. As can be seen, the slowest rates are obtained when the drug is in the dry state or is dissolved in an alcoholic polyvinylpyrrolidone solution. Dissolution can be increased when the drug is dissolved in water—with or without polyvinylpyrrolidone—or in alcohol alone prior to granulating.

By dissolving the drug prior to granulating, it is possible to increase the available surface area when this solution is poured onto a powder mixture. This would result in increased dissolution (Table I).

The difference in the rate of solution between Formulations C and E appears to be a function of the surface tension of the solution used to pour onto the lactose–microcrystalline cellulose mixture. It is known that the high surface area of microcrystalline cellulose is due to fissures and crevices found abundantly in the granules. Since the alcoholic solution (Formulation C) has a

[1] Colton SX, four station.



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Effect of Formulation on Dissolution of Sodium Warfarin Tablets

Keyphrases □ Sodium warfarin tablets—effect of pregranulation dissolving on dissolution rate □ Dissolution rate, sodium warfarin tablets—effect of dissolving drug prior to granulation

Sir:

Numerous publications have shown that formulation of a solid dosage form can influence dissolution of the active drug. These studies have discussed the effect of binder concentration and tablet hardness (1), types of binders and particle size (2), types of starches (3), lubricants (4), and disintegration agents (5).

Table I—Dissolution Rate Constants (k) of Various Sodium Warfarin Tablet Formulations and Surface Tension of Solutions Used to Granulate

Formulation	k, min.$^{-1}$	Surface Tension[a], γ, dynes/cm.
A. Drug incorporated in dry form	0.060	—
B. Drug and polyvinylpyrrolidone dissolved in alcohol[b]	0.074	26.1
C. Drug dissolved in alcohol[c]	0.113	25.6
D. Drug and polyvinylpyrrolidone dissolved in water[d]	0.154	57.4
E. Drug dissolved in water[e]	0.246	59.4
	Absolute alcohol	24.4
	Water, deionized	74.1

[a] Of solution used to granulate. Measured by a Du Noüy interfacial tensiometer. [b] Solution ratio of drug–polyvinylpyrrolidone–alcohol = 1:1:6. [c] Solution ratio of drug–alcohol = 1:6. [d] Solution ratio of drug–polyvinylpyrrolidone–water = 1:1:4. [e] Solution ratio of drug–water = 1:4.

The purpose of this communication is to report that dissolving sodium warfarin prior to granulating can influence the dissolution rate.

Sodium warfarin was incorporated into a microcrystalline cellulose–lactose (3:1) mixture such that 25 mg. of sodium warfarin was contained/275-mg. tablet. The drug was either added into the formulation in the dry state or dissolved in an aqueous or alcoholic media, with or without an equal ratio of polyvinylpyrrolidone–sodium warfarin. The solutions (Table I) were used to granulate the microcrystalline cellulose–lactose mixture. Magnesium stearate (0.1%) was added to either the powder mixture or the dried, 20-mesh screened granulation. Tablets were compressed on a compressing machine[1]. All tablets disintegrated within 1 min. Dissolution tests were performed in a round-bottom flask, containing 500 ml. of distilled water, maintained at 37°. A half-moon (2.5 cm. in diameter) stirring blade was used at a speed of 50 r.p.m. Assays were performed spectrophotometrically.

The pseudo-first-order rate constants obtained from this study are shown in Table I. As can be seen, the slowest rates are obtained when the drug is in the dry state or is dissolved in an alcoholic polyvinylpyrrolidone solution. Dissolution can be increased when the drug is dissolved in water—with or without polyvinylpyrrolidone—or in alcohol alone prior to granulating.

By dissolving the drug prior to granulating, it is possible to increase the available surface area when this solution is poured onto a powder mixture. This would result in increased dissolution (Table I).

The difference in the rate of solution between Formulations C and E appears to be a function of the surface tension of the solution used to pour onto the lactose–microcrystalline cellulose mixture. It is known that the high surface area of microcrystalline cellulose is due to fissures and crevices found abundantly in the granules. Since the alcoholic solution (Formulation C) has a

[1] Colton SX, four station.

lower surface tension, it is able to penetrate into fissures and crevices which water cannot readily enter, resulting in a decreased rate of solution.

The addition of polyvinylpyrrolidone into Formulations B and D (Table I) does not alter the surface tension. Therefore, the decrease in dissolution when compared to Formulations C and E, respectively, must be a function of the rate of solution of polyvinylpyrrolidone, causing a decrease in the rate of solution of a rather soluble drug in water.

The results of this study show that the method used to incorporate drug into a solid dosage form can affect the dissolution rate of that drug.

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NMR Spectroscopy: Chloro-(tetra-*p*-methylphenylporphinato)indium (III)

Key phrases □ Chloro(tetra-*p*-methylphenylporphinato)indium (III)—NMR spectra □ Porphine complexes with indium—NMR spectra of chloro(tetra-*p*-methylphenylporphinato)indium (III) □ Indium (III), chloro(tetra-*p*-methylphenylporphinato)—NMR spectra □ NMR spectroscopy—identification, chloro(tetra-*p*-methylphenylporphinato)indium (III)

Sir:

Porphine complexes are important biologically and chemically. Structural studies of these complexes may provide information relative to their mechanisms of action. We wish to report NMR studies on the complex chloro(tetra-*p*-methylphenylporphinato)indium (III).

Chloro(tetra-*p*-methylphenylporphinato)indium (III) was prepared and purified as previously reported (1, 2). NMR spectra were obtained using a spectrometer[1] equipped with a variable-temperature probe and operated at power levels below saturation. Temperatures were measured with a thermocouple mounted in the probe; temperature calibration was done with ethylene glycol. Spectra were obtained in 1,1,2,2-tetrachloroethane which had been purified by distillation from phosphorus pentoxide under nitrogen. Spectra were obtained with the spectrometer locked on the solvent resonance, and chemical shifts were reported relative to the solvent.

[1] Varian-HA-100.

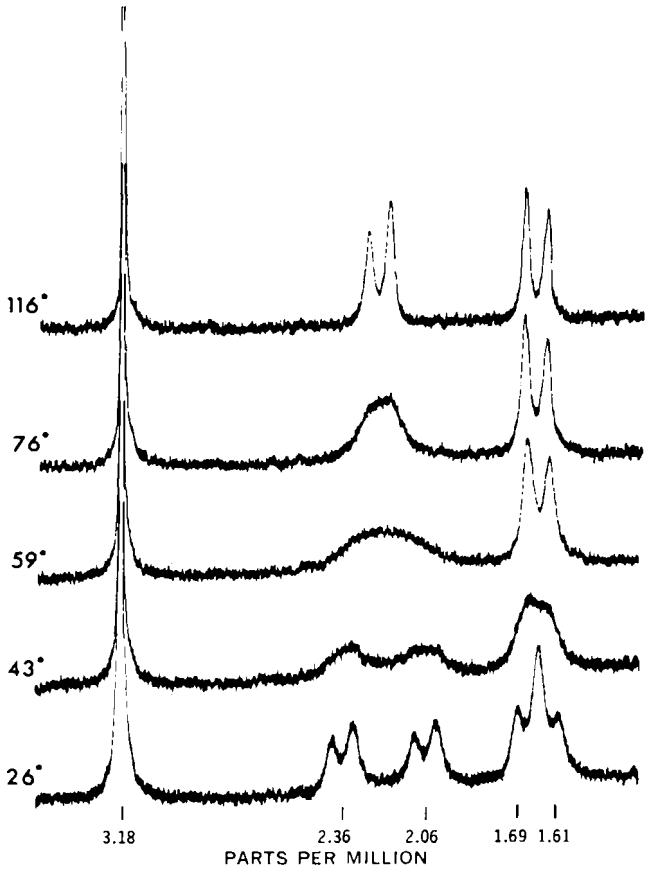

Figure 1—*NMR spectra (100 MHz.) of chloro(tetra-*p*-methylphenylporphinato)indium (III) at various temperatures.*

It has been shown that the NMR spectra of indium–tetraphenylporphine complexes at room temperature exhibit nonequivalence of the phenyl ring protons (2). The NMR spectrum of chloro(tetra-*p*-methylphenylporphinato)indium (III) in Fig. 1 shows that as the temperature rises, the phenyl signals broaden and finally collapse into a pair of doublets at approximately 100°. Spectral changes were reversible with temperature. The resonance at 3.18 p.p.m. resulted from the porphine pyrrole protons. Resonances at 2.06 and 2.36 p.p.m. were assigned to the *ortho*-protons; resonances at 1.61 and 1.69 were assigned to the *meta*-protons (2).

The room temperature spectrum has been assigned an *AA'BB'* pattern in which the two different *ortho*- and *meta*-protons are nonequivalent due to restricted rotation about the *meso*-carbon to phenyl (carbon carbon) bond. As the temperature was elevated, the rotation became fast on the NMR time scale, appearing to yield equivalence of *ortho*- and the *meta*-protons. No attempt was made to obtain accurate kinetic parameters for this highly coupled spin system; the rate of averaging can be estimated (3) as 66/sec. at the coalescence temperature of approximately 60° for the *ortho*-protons: ($\Delta G^{\ddagger}_{333}$ = 16.8 kcal./mole).

The concept of restricted rotation about the carbon-to-carbon bond is strongly supported by X-ray diffraction studies on porphines and porphine complexes (4). Such studies have indicated that the *meso*-phenyl rings are tilted at an angle to the mean plane of the porphine ring (5–7). This angle was found to be approximately

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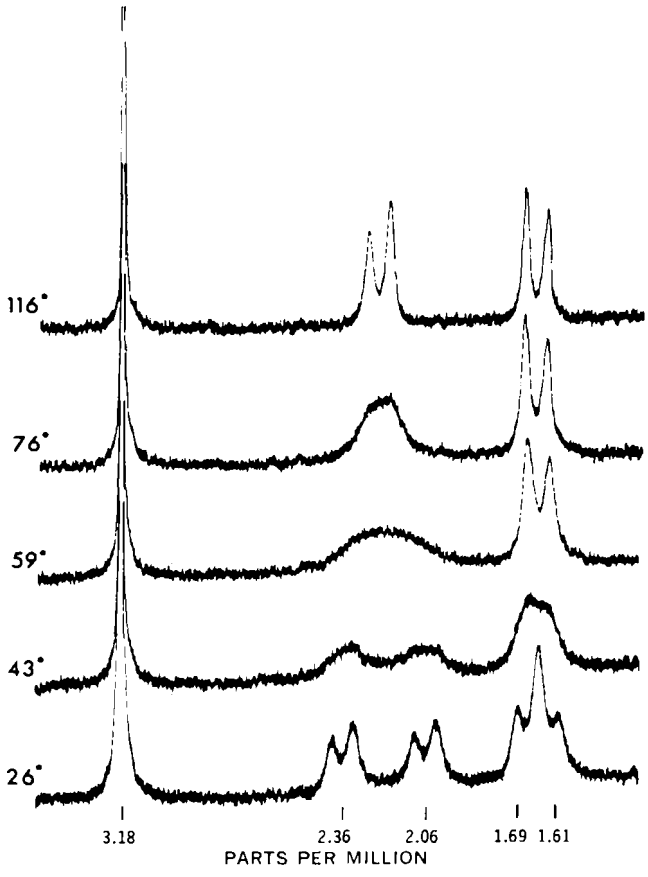

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90° in many metal complexes of tetraphenylporphine (4, 8), although angles as small as 69 (9) and 76° (10) were found in certain metal complexes. Steric interactions between pyrrole hydrogen and phenyl hydrogen atoms have been estimated to require a dihedral angle of 60 (10) or 70° (4).

The sequence of spectral changes given in Fig. 1 is nearly identical to those reported for tetrakis[(*p*-isopropylphenyl)porphinato]ruthenium carbonyl complexes (8, 11). The averaging process for the indium complex occurs at slightly lower temperatures than for the ruthenium complex.

Phenyl ring rotation has been observed in tetra-*o*-hydroxylphenylporphine and its copper complex (12). The rate constant for rotation of the free porphine at 23° in methanol was 1.5×10^{-5}/sec. ($\Delta G^{\neq} = 24$ kcal./mole); rotation was about 10 times slower for the copper complex. Isomers of tetra-*o*-tolylporphine and its nickel complex have also been studied (13). The NMR signals for the methyl resonances of this compound did not appreciably broaden up to 180°, indicating that $\Delta G^{\neq}$ for rotation exceeds 26 kcal./mole (13). The present studies of chloro(tetra-*p*-methylphenylporphinato)indium (III) confirm earlier observations on ruthenium complexes (II); there appears to be a more rapid phenyl group rotation when the *ortho*-substituent is hydrogen than when it is hydroxyl or methyl.

Alternative interpretations of the NMR spectrum for chloro(tetra-*p*-methylphenylporphinato)indium (III) appear less plausible. Since *AA'* and *BB'* coupling was not resolved for indium (III) tetraphenylporphine, it is possible to attribute the room temperature spectra to rapidly rotating phenyl groups in nonequivalent positions with accidental degeneracy of nonequivalent pyrrole and methyl resonances. The temperature dependence of such a spectrum would result from the interchange of nonequivalent phenyl sites. However, it has been demonstrated that phenyl rotation is slow in the ruthenium complexes at room temperature (8, 11); it is not likely that rotation would be fast in the chloro(tetra-*p*-methylphenylporphinato)indium (III) complex at the same temperature. If the chloride in the indium complex were dissociated, ion migration or inversion of a "dished" ring conformation could explain the NMR results; however, there are no data to substantiate such an interpretation.

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Specificity of Binding to Human Serum Albumin

Keyphrases ☐ Drug-human serum albumin binding—specificity, lipophilic dependency, equations ☐ Binding, drug-human serum albumin—specificity, lipophilic dependency, equations

Sir:

Whitehouse *et al.* (1) suggested the need for reevaluation of the widely held concept (2, 3) that the fraction of certain acidic drugs bound to plasma protein is pharmacologically inactive. In support of this suggestion, data were presented indicating that a correlation exists between the ability of certain anti-inflammatory agents and uricosuric agents to displace uric acid from its primary binding site on human serum albumin *in vitro* (1) and their ability to reduce uric acid binding capacity *in vivo*. The results of other studies were cited to support this thesis (4, 5). One cited study showed that fatty acids displace warfarin and phenylbutazone from their binding sites on human serum albumin *in vitro* (5).

A relationship was recently shown (6) between the ability of certain acidic drugs to displace albumin-bound uric acid and their affinity for the primary binding site of 5-dimethylaminonaphthalene-1-sulfonamide on human serum albumin. Analysis of these affinity data revealed that for certain members of two classes of nonsteroid anti-inflammatory and uricosuric agents—*viz.*, (*a*) carboxylic acids, and (*b*) benzenesulfonamides and

90° in many metal complexes of tetraphenylporphine (4, 8), although angles as small as 69 (9) and 76° (10) were found in certain metal complexes. Steric interactions between pyrrole hydrogen and phenyl hydrogen atoms have been estimated to require a dihedral angle of 60 (10) or 70° (4).

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benzenesulfonylureas, a highly significant relationship exists between the displacing ability of the two structure types and the lipophilicity of these agents as measured by their partition coefficients in the 1-octanol–water system (7). These relationships are given in Eqs. 1 and 2 (7).

50% Displacement of 5-Dimethylaminonaphthalene-1-sulfonamide from Human Serum Albumin by Carboxylic Acids—

$$\log 1/C = 1.06\,(\pm 0.67) + 1.26\,(\pm 0.42)\log P - 0.12\,(\pm 0.06)\log^2 P \quad \text{(Eq. 1)}$$
$$n = 29 \quad R = 0.91 \quad SD = 0.36$$
$$\log P_0 = 5.48\,(4.69\text{–}7.80)$$

50% Displacement of 5-Dimethylaminonaphthalene-1-sulfonamide from Human Serum Albumin by Benzenesulfonamides and Benzenesulfonylureas—

$$\log 1/C = 3.64\,(\pm 0.21) + 0.74\,(\pm 0.28)\log P - 0.34\,(\pm 0.10)\log^2 P \quad \text{(Eq. 2)}$$
$$n = 13 \quad R = 0.91 \quad SD = 0.21$$
$$\log P_0 = 1.10\,(0.89\text{–}1.29)$$

In these equations, C is the concentration (moles/liter) required to displace 50% of 5-dimethylaminonaphthalene-1-sulfonamide from its binding site on human serum albumin, P is the 1-octanol–water partition coefficient, n represents the number of agents in the set, R is the correlation coefficient, SD is the standard deviation of the regression, and P_0 is the partition coefficient associated with optimum affinity for the 5-dimethylaminonaphthalene-1-sulfonamide binding site. The terms in parentheses are the 95% confidence intervals for the appropriate regression coefficients. The analysis of additional data (8) for the displacement of 5-dimethylaminonaphthalene-1-sulfonamide from its primary binding site on human serum albumin by various sulfonamides substantiates the nonlinear relationship between the displacing ability and lipophilicity (Eq. 4).

Percent Decrease in Fluorescence of 5-Dimethylaminonaphthalene-1-sulfonamide–Albumin Complex by Various Sulfonamides—

$$\log \%D = 1.56\,(\pm 0.20) + 0.18\,(\pm 0.14)\log P \quad \text{(Eq. 3)}$$
$$n = 13 \quad SD = 0.27 \quad R = 0.63$$

$$\log \%D = 1.62\,(+0.09) + 0.42\,(\pm 0.10)\log P - 0.13\,(\pm 0.04)\log^2 P \quad \text{(Eq. 4)}$$
$$n = 13 \quad R = 0.94 \quad SD = 0.13$$
$$\log P_0 = 1.60\,(1.32\text{–}2.05)$$

Including the nonlinear term in Eq. 3 is significant at the 99% level of confidence, $F_{1,13} = 43.76$, $F_{1,13,\alpha\,0.01} = 9.07$.

On comparing the analyses of the data for the sulfa drugs (Eqs. 2 and 4), it is significant to note the similarities in log P_0's from the two relationships. The data used to derive Eq. 4 are given in Table I. The agreement is very good considering that the data were obtained from different laboratories. The difference in the two values may be due to the fact that the data used to derive Eq. 2 were obtained at 37° while those used for Eq. 4 were obtained at 24°. The dependence of log P_0 on temperature is not understood, however. The difference in intercepts of Eqs. 2 and 4 may be due to the fact that the dependent variable in the former case is a standard response while that of the latter case is a relative biological response.

Table I—Influence of Various Sulfa Drugs on the Fluorescence of 5-Dimethylaminonaphthalene-1-sulfonamide–Human Serum Albumin Complex

Sulfa Drug	log P	log Percent Decrease in Fluorescence		
		Observed	Calculated[a]	$\lvert\Delta \log \%D\rvert$
Sulfadimethoxine	1.56[b]	1.99	1.96	0.03
Sulfamethoxypyridazine	0.40[b]	1.98	1.77	0.21
Sulfamethazine	0.27[b]	1.91	1.73	0.18
Sulfaphenazole	1.57[b]	1.90	1.96	0.06
Sulfisoxazole	1.15[b]	1.90	1.94	0.04
Sulfadoxine	1.56[c]	1.89	1.96	0.07
Sulfamerazine	0.13[b]	1.71	1.68	0.03
Sulfathiazole	0.35[b]	1.65	1.76	0.11
Sulfamethoxazole	0.54[b]	1.61	1.81	0.20
Sulfanilamide	−0.83[b]	1.22	1.18	0.04
Sulfaguanidine	−1.22[b]	0.84	0.91	0.07
Probenecid	3.21[d]	1.63	1.62	0.01
Tolbutamide	2.34[d]	1.96	1.89	0.07

[a] Equation 4. [b] *Reference 9.* [c] log P estimated to be equivalent to that for sulfadimethoxine. [d] Experimental value, this laboratory.

The parabolic nature of Eqs. 1, 2, and 4 is unusual in that recent studies (10–12) of the dependence of the binding of drugs to serum albumin on lipophilicity led to linear relationships between log P and an appropriate binding parameter. This report is at variance with these studies in that the dependent variable in this case is a measure of the affinity of the displacing agent, relative to that of 5-dimethylaminonaphthalene-1-sulfonamide, for a specific site on human serum albumin. In the cases where linear relationships were found between binding and log P, the binding parameter was an equilibrium binding constant or a related parameter which could give no information about binding specificity.

The nonlinearity found in this and the prior study (7) can be explained by proposing a secondary binding site with an affinity for the displacing agents. As the lipophilicity becomes greater than log P_0 for the series of the displacing agents, affinity for this secondary site increases and the effective concentration of the drug decreases.

There is theoretical justification for the nonlinearity we have found and this lies in the pseudoequilibrium model developed by Higuchi and Davis (13). They found that for systems in equilibrium or quasiequilibrium, nonlinear relationships can result between relative affinities for binding to specific sites or for receptor sites and lipophilicity.

In summary, the findings of this report indicate that drug–human serum albumin interactions can be highly specific and dependent on lipophilicity. It may be possible to take advantage of this specificity and, using Eqs. 1, 2, and 4 as guides in design, synthesize more effective uricosuric agents.

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Such broad coverage leads necessarily to deficiencies in detail, as in the chapter dealing with inhibitors of protein synthesis, but these are balanced by expanded discussions on antibiotics of more current interest. In the chapter dealing with drugs affecting the cytoplasmic membrane, present membrane models are briefly and critically discussed. The surveys noting the influences of antibiotics on membrane structure and on energy-dependent transport processes are stimulatory and cause one to reflect on how similar effects might be involved more generally in drug transport and other types of drug action. Inhibitors of nucleic acid synthesis, particularly those which function by intercalation, are discussed in considerable detail. In view of the diversity of biological effects manifested by intercalating agents such emphasis is understandable, even though the specificity of action of these compounds is more generally attributable to other factors governing drug action such as differential permeability. A discussion of the genetic basis for antibiotic resistance is incorporated into the chapter terminating the overall development to support earlier descriptions of resistance mechanisms.

There is sufficient content to this book for it to be of interest to students and researchers in nearly all areas of the basic health sciences. This reviewer can recommend it highly.

Reviewed by Arthur Cammarata
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Fundamentals of Chemotherapy. By WILLIAM B. PRATT. Oxford University Press, 200 Madison Ave., New York, NY 10016, 1973. 332 pp. 14 × 20.5 cm. Price $6.95.

Primarily intended as a textbook, this book's chapters are organized by grouping drugs according to their biochemical site of action. In general, the biochemical process affected by a particular drug is described as is its mechanism of action. Drug resistance and drug interactions are also emphasized. The four major sections are drugs employed in the treatment of bacterial and fungal infection, parasitic disease, viral infection, and neoplastic disease.

Staff Review ■

Hypersensitivity to Drugs, Volume I. International Encyclopedia of Pharmacology and Therapeutics, Section 75. Edited by M. SAMTER and C. W. PARKER. Pergamon Press. Inc., Maxwell House, Fairview Park, Elmsford, NY 10523, 1973. xii + 439 pp. 14.5 × 23 cm. Price $36.00.

It is now recognized that the clinical symptoms which are the result of hypersensitivity to drugs are the final step in a long and often complicated sequence. This volume of the well-regarded *International Encyclopedia of Pharmacology and Therapeutics* series identifies the known conditions which must be met by the drug and by the host before immunological reactions can occur. The wide variety of responses which drugs can induce in organs and systems are covered. Precise definition of the requirements to be met by drug and host will eventually allow prediction of probable hypersensitivity development and will permit the development of measures to prevent it.

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NOTICES

Polymerization in Biological Systems. Ciba Foundation Symposium 7. Associated Scientific Publishers, 52 Vanderbilt Ave., New York, NY 10017, 1972. 314 pp. 17 × 25 cm.

Physiology, Emotion & Psychosomatic Illness. Ciba Foundation Symposium 8. Associated Scientific Publishers, 52 Vanderbilt Ave., New York, NY 10017, 1972. 421 pp. 17 × 25 cm.

Intrauterine Infections. Ciba Foundation Symposium 10. Associated Scientific Publishers, 52 Vanderbilt Ave., New York, NY 10017, 1973. 219 pp. 17 × 25 cm.

Actualités Pharmacologiques. By RENÉ HAZARD. Masson & Cie, Editeurs, 120 Boulevard Saint-Germain, Paris, France, 1972. 268 pp. 16 × 24 cm. Price 105 fr. (*French*)

A Depolarization–Hyperpolarization Cycle (A Molecular Model). By CLARA TORDA. Torda, 101 W. 12th St., New York, NY 10011, 1972. 100 pp. 15.5 × 23.5 cm.

Contamination in the Manufacture of Pharmaceutical Products. Proceedings of the Stockholm meeting, April 1972. Secretariat of the European Free Trade Association, Rue de Varembe 9-11-1211, Geneva 20, Switzerland, 1973. 64 pp. 15 × 21 cm.

The Quality of Pharmaceutical Products: The Role of the Inspector. Proceedings of a Seminar held at the Ecole nationale de la sante publique, Rennes, France, Sept. 1972. Secretariat of the European Free Trade Association, Rue de Varembe 9-11-1211, Geneva 20, Switzerland, 1973. 131 pp. 15 × 21 cm.

Protein Turnover. Ciba Foundation Symposium. Associated Scientific Publishers, 52 Vanderbilt Ave., New York, NY 10017, 1973. 319 pp. 17 × 24.5 cm.

Enzyme Inhibitors of Microbial Origin. By HAMAO UMEZAWA. University Park Press, Chamber of Commerce Building, Baltimore, MD 21202, 1972. 114 pp. 15.5 × 23.5 cm. Price $9.50.

Agonist and Antagonist Actions of Narcotic Analgesic Drugs. Edited by H. W. KOSTERLITZ, H. O. J. COLLIER, and J. E. VILLARREAL. University Park Press, Chamber of Commerce Building, Baltimore, MD 21202, 1973. 290 pp. 15.8 × 24 cm. Price $19.50.

Advances in Antimicrobial and Antineoplastic Chemotherapy, Volume I/1. University Park Press, Chamber of Commerce Building, Baltimore, MD 21202, 1972. 677 pp. 17.3 × 24.4 cm. Price $24.50.

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REVIEW ARTICLE

Use of Statistical Methods in Evaluation of *In Vivo* Performance of Dosage Forms

WILFRED J. WESTLAKE

Keyphrases ☐ Statistical methods used in evaluating *in vivo* performance of dosage forms—review ☐ Blood level data, *in vivo* evaluation of dosage forms application of statistical methods, review ☐ Analysis of variance review of applications to evaluating *in vivo* performance of dosage forms ☐ Confidence intervals—review of applications to evaluating *in vivo* performance of dosage forms ☐ Dosage forms—review of statistical methods used to evaluate *in vivo* performance ☐ Drugs, evaluation of *in vivo* performance of dosage forms—statistical methods of analysis, review

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Interest in the evaluation of the *in vivo* performance of drug dosage forms has increased greatly in the last few years, as an examination of recent volumes of this Journal will readily attest. The pharmacokinetic aspects of dosage form performance have received particular emphasis. In a typical experiment, the dosage form is administered to a group of subjects and blood samples are drawn at various sampling times following administration. Assay of the blood samples for drug content gives a sequence of drug concentrations which may be used to characterize the performance of the formulation. Such questions as how much of the drug does the formulation make available and how rapidly are of obvious interest and importance. When assay methods for blood drug levels are not feasible, an alternative approach consists of collecting and assaying urine specimens.

Methods of statistical analysis are becoming more widely used in the evaluation of such experiments, and there appear to be two main areas of application. The first is in the characterization of the behavior of the formulation in the biological organism by some kind of mathematical model; the second is in the comparison of the behavior of different dosage forms. Both types of application lead naturally to the use of certain statistical techniques. The first leads to estimation problems, particularly those of estimating the various parameters in a mathematical model. The second leads to problems in hypothesis testing, that is, in testing the hypothesis that no difference exists between two formulations. As discussed later in this article, a different approach to the problem of comparison of formulations—*via* confidence intervals—was recently suggested.

Olson and Lee (1) previously reviewed the application of statistical methods to quality control in the pharmaceutical industry. The purpose of the present article is to provide a similar review of the evaluation of *in vivo* performance of dosage forms. No attempt is made to refer to every article in which statistical methods were used. Rather, this article provides a critical review of proposed methods, some of which are now in wide use, with reference to selected papers. The author's opinion is that many statistical techniques have been applied

uncritically, albeit enthusiastically, to this field. The main aim of this review is, therefore, to expose the statistical problems and proposed methods of solution in a critical examination.

CHARACTERIZATION OF A FORMULATION

To examine the *in vivo* behavior of a formulation, one administers the formulation to a subject, draws blood samples at various times following administration, and obtains a sequence of blood concentrations, or blood levels as they are often called. The sequence of blood levels is then used to characterize the formulation. A number of methods of characterization are possible and have been used by various workers. The most popular method, by far, is that of characterizing the formulation by one or more estimated parameters (*e.g.*, an absorption rate constant) obtained from fitting a biologically meaningful mathematical model to the blood level data. In the great majority of cases, compartmental models are employed in which the biological organism is represented as a series of connected compartments (*e.g.*, blood compartment and tissue compartment) and transfers of drug among compartments are usually first order.

A second, purely empirical, method of characterization consists of fitting some mathematical function to the blood levels. In this case, the mathematical function is not intended to be a meaningful model of the biological organisms but only to give a good fit to the observed blood levels. However, the possibility exists—and, indeed, has been utilized—of choosing some parameter of the mathematical function to characterize the formulation. Other methods of characterization of the performance of the formulation, such as choosing some feature of the blood level sequence (*e.g.*, peak blood level or time of peak level) or some computed characteristic of the blood level sequence (*e.g.*, area under the blood level curve as a measure of amount of drug absorbed) are possible and are widely used. The statistical methodology that has been employed in utilizing these methods of characterization will be discussed, and some of the statistical problems will be examined.

Compartmental Models—The overwhelming choice of most workers who are faced with the problem of using a biologically meaningful model to characterize blood levels produced by a drug formulation is the compartmental model with linear transfers. One-, two-, and three-compartment models [see, for example, Chapter 38 of *Reference 2*, Riegelman *et al.* (3), and Nagashima *et al.* (4)] figure prominently in the pharmaceutical literature. Taking the one-compartment open model as an example, one can derive the expression:

$$C(t) = \frac{Dk_a}{V(k_a - k_e)}(e^{-k_e t} - e^{-k_a t}) \qquad \text{(Eq. 1)}$$

for the concentration of drug in the blood at time t. The symbol D represents the amount of drug absorbed, V is an apparent volume of distribution of the blood, and k_a and k_e are absorption and elimination rate constants, respectively. The salient feature of this expression, as it is in all compartmental models, is that it is the sum of a number of exponential terms.

The statistical problem is that of finding the values of the unknown parameters D/V, k_a, and k_e which, in some way, best "fit" the observed data. Again, in the overwhelming majority of cases, the technique of fitting used is that of "least squares," *i.e.*, the estimation of the parameters such that they minimize the sum of squares of differences between observed and predicted blood level values. The method has a number of desirable statistical features but is not the only method possible. Determination of this minimum sum of squares results in three simultaneous equations to be solved for the three parameters. If these equations were linear in the parameters, one could fall back on a well-developed theory for their solution; but in the case of the present example and all compartmental models, the equations are nonlinear in the parameters and little general theory is available for solution of the equations. Therefore, the equations are usually solved by iterative methods. That is, an initial rough estimate of the parameters is obtained and one then proceeds, iteratively, to find a sequence of solutions for which the residual sum of squares becomes progressively smaller. When no further substantial diminution in the sum of squares appears possible, the process is said to have converged to the required least-squares solution.

The initial estimates of the pharmacokinetic parameters are usually obtained by the familiar process of "peeling off" the exponential terms one at a time from a semilogarithmic plot of the blood levels. The technique is frequently mentioned in the pharmaceutical literature; for a systematic approach that has been programmed for a digital computer, reference may be made to Foss (5). Once initial tentative estimates of the pharmacokinetic parameters have been obtained, several techniques of proceeding to the least-squares solution are available. Most of these are based, at least in part, on the so-called Gauss Newton method, which involves linearizing the problem in the neighborhood of the initial estimates and computing by linear least-squares techniques the solution giving the "minimum" sum of squares. One then proceeds, iteratively, from the new solution through further improved solutions until convergence is attained.

Hartley (6) gave a modified version of this technique and Marquardt (7) proposed a method based both on the Gauss Newton method and the method of "steepest descent." Numerous programs based on these methods [*e.g.*, Metzler (8) and Marquardt (9)] are available and are widely used in fitting compartmental models to blood level data. Smith and Shanno (10) produced an improved version of Marquardt's original method, and Ackerman *et al.* (11) proposed a method of solution termed "iterative guessing." However, they gave no reasons for considering their method superior to the other methods already discussed. Lawton and Sylvestre (12) suggested the possibility of taking advantage of any linearities that occur in the functional equations. Since only one term, the D/V introduced earlier, appears linearly in compartmental models, their suggestion would appear to have little utility in the solution of the problems discussed here.

Other methods of fitting sums of exponentials to data are available. Two examples are Cornell's (13) method, which requires equal spacing of the observations in time, and Foss's (14) method, which gives a noniterative

scheme for sums of two or three exponentials not restricted to equal spacing.

An important concern is the reliability of the estimates of the parameters given by the least-squares solution. The usual way of approaching this statistical problem is through the construction of a "confidence region" for the parameter estimates. Briefly, a 95% confidence region, for example, is a region in the parameter space that is so constructed that in repeated trials the true values of the parameter will lie in the confidence region in 95% of the cases. As an example, consider the two-compartment open model in which the five parameters, D/V, k_a, k_{12}, k_{21}, and k_e—or, equivalently, D/V, k_a, k_{21}, α, and β (where $\alpha\beta = k_e k_{21}$ and $\alpha + \beta = k_e + k_{12} + k_{21}$)—are to be estimated. In this case, the confidence region is a five-dimensional ellipsoid and the practical difficulties of interpreting such regions lead, in most programs, to a series of confidence statements for the individual parameter estimates based on the concepts of tangent planes to the ellipsoid. In addition, one can make the straightforward confidence interval statements for each parameter estimate separately, as was done by Maas and Patel (15), but these statements ignore the substantial correlations that usually exist between parameter estimates whenever one is fitting a sum of exponential terms. One result of the strong correlation existing between the parameter estimates is that confidence ellipsoids or, equivalently, the ellipsoids corresponding to the contours of constant residual sum of squares are greatly elongated. In practical terms, this means that one may be able to change the parameter estimates substantially without changing the residual sum of squares or, in other words, that many very different sets of estimates of the pharmacokinetic parameters give about equally good fits of the model to the data.

It should be apparent from this brief discussion that, in most cases in which the equation for blood concentration obtained from a compartmental model is fitted to experimental data, very little faith should be placed in the estimated pharmacokinetic parameters *per se*. The problems inherent in fitting sums of exponential terms to data have been recognized by mathematicians and statisticians for a long time, and the well-known book on numerical analysis by Lanczos (16, chap. IV) contains an excellent discussion of the subject. In view of its great significance to would-be fitters of compartmental models, it is worth spending a little time on the example given by Lanczos.

Lanczos gives a set of 24 data points (accurate to the second decimal place) generated by a function that is the sum of a number of exponentials; the data are error free except for the round-off to the second decimal place. An exact method for estimating the coefficients (amplitudes) of the exponential term and the values of their exponents is used. Lanczos starts tentatively with a three-term exponential model but finds that the series of observations needs only two exponential terms to describe it. He then finds the values of the parameters of the two-exponential term model that best fit the data and is able to generate a near-perfect fit. When the residuals (the differences between the actual data value and the value predicted by the fitted model) are examined, only one of them exceeds 0.005—it is, in fact, 0.006—and the square root of the residual mean square error is 0.0026. One could hardly expect a more perfect fit. Then Lanczos drops his bombshell: not only is the fitted model incorrect, but it does not even have the correct number of terms! In fact the data were generated from an expression that was the sum of *three* exponential terms. Not only was the exponential term with the smallest coefficient lost in the fitting process, but the other two terms of the original function were very different from the two exponentials as actually fitted. Lanczos decides that the result is thus completely unsatisfactory and his brief summation deserves to be quoted in full (p. 279).

> *It would be idle to hope that some other modified mathematical procedure could give better results, since the difficulty lies not with the manner of evaluation but with the extraordinary sensitivity of the exponents and amplitudes to very small changes of the data, which no amount of least square or other form of statistics could remedy. The only remedy would be an increase of accuracy to limits which are far beyond the possibilities of our present measuring devices.*

This quotation might serve as a suitable warning to all who embark on the task of fitting compartmental models to blood level data, especially since the pharmacokineticist is faced with a far more severe problem than that illustrated by Lanczos in his example. Not only is round-off error present, but assay error is also inevitable in determining the blood samples. However, there is worse to follow in view of the fact that no biological organism can be expected to behave as a theoretically perfect compartmental model. One should be prepared to think in terms of another component of error, namely, lack of fit of the actual organism to the theoretical model.

The pharmaceutical literature abounds with examples of blood level data to which the authors have fitted compartmental models by the method of least squares. The foregoing discussion suggests that rather than confining their attention solely to a least-squares solution, authors would be well advised to give confidence limits, alternative solutions with almost equal sums of squares and, additionally, solutions for alternative models having almost equally good fits. Only in this manner would it be possible to form some idea of the validity of the proposed solution from a purely statistical standpoint. Naturally, if extrastatistical arguments (*e.g.*, physiological) can be adduced, the nature of the problem changes.

Methods other than least squares have been proposed for fitting sums of exponentials to blood level data. Gardner *et al.* (17), for example, proposed a method based on Fourier transforms. To date, however, methods other than least squares have not been widely used in the characterization of blood levels by compartmental models. Ackerman *et al.* (11), discussing briefly the problems already raised in this paper and referring to the literature on multicompartment analysis of tracer experiments (18), stated: "Complex transform methods have been attempted but none appear to have proved especially helpful with real biological data."

One aspect of the characterization of a drug formulation by compartmental models that has received little emphasis to date is the possibility of arranging the blood sampling times so as to estimate only certain specified parameters with high precision. For example, the essential characteristic of an orally administered formulation may lie in one parameter only, namely, an absorption rate constant. If one is allowed only a certain number of blood samples from each subject, the best procedure might be to arrange them so as to give the maximum precision in the estimation of this absorption rate constant at the expense of poorer precision for the other parameters. The problem of designing experiments where the underlying model is nonlinear in the model parameter has attracted some attention in the statistical literature but little, as yet, in pharmaceutical applications. Box and Lucas (19) wrote perhaps the first paper on the topic, and since then a number of papers by associates of Box have appeared. The paper by Box (20) extended the theory and also provided useful additional references. A characteristic requirement for the construction of an optimum design in a nonlinear situation is the need for some idea of the magnitude of the parameters that one is attempting to estimate. In practice, pilot studies would normally be carried out from which such rough estimates could be obtained, and this field appears potentially ripe for further investigation.

Normally, one estimates the parameters in a compartmental model by fitting the model to blood level data since this is the only compartment that can be sampled at varying points in time. An interesting possibility is that of obtaining data from other compartments of the model by indirect means. Wagner *et al.* (21) demonstrated that for subjects receiving the drug LSD-25, there appears to be a simple linear relation between the scores obtained by the subjects on arithmetic tests and the predicted concentration of drug in the so-called tissue compartment of a two-compartment open model. Metzler (22) used this idea to fit a two-compartment model to both blood level and test score data simultaneously. In a two-compartment model, there are five parameters (D/V, k_a, k_{12}, k_{21}, and k_e) to be estimated. If the assumption is made that the arithmetic test scores are of the form found in Eq. 2:

$$S(t) = 100 - F_1 C_T(t) \qquad \text{(Eq. 2)}$$

where $S(t)$ is the score at time t, $C_T(t)$ is the tissue concentration at time t, and F_1 is an unknown parameter, then there are six parameters to be estimated but the number of data points available is now the sum of the number of blood levels and the number of test scores. The model is fitted by the usual least-squares techniques, and no new statistical principles are involved. This procedure, when valid, may be viewed as a means of increasing the amount of experimental data available for the fitting process.

In this discussion of compartmental models, attention should be drawn to the paper of Rodda *et al.* (23) which breaks new ground in several aspects of parameter estimation. Their paper dealt, however, only with one of the simplest possible cases, namely, the one-compartment model with oral drug administration. By simulation techniques, they examined the effects of assay error and of random variation in the pharmacokinetic parameters (physiological variation) on the least-squares estimates of the parameters. Their results suggest that the least-squares estimates do, in fact, give unbiassed estimates of the true underlying parameters. However, their estimates of the sampling variance of these parameters are sufficiently different from those obtained by the usual linearization techniques used in most nonlinear least-squares programs to suggest that the confidence intervals given by such programs may be substantially in error. This paper also examined a favorite device of workers in this field, namely, "curve averaging"—the practice of fitting a compartmental model to blood level data that are taken not from one trial but are the mean values of a number of trials. Here, they found, not surprisingly, a distinct tendency for the estimates of the parameters to be biassed.

Finally, the authors (23) discuss an alternative estimation technique which appears to be less sensitive to "outliers" than the usual least-squares estimation. An outlier is an observation subject to considerably greater error than most of the other observations. As should be only too apparent fom the earlier discussion, an outlier in a series of blood level values can have a disastrous effect on the least-squares estimates of the pharmacokinetic parameters. Outright discarding of apparent outliers is open to the objection of arbitrariness and the charge of attempting to make the data fit preconceived theories; the outliers may, in fact, be legitimate observations.

The contents of this paper have been discussed in detail because it is one of the few attempts so far made to examine some of the severe statistical and computational problems that beset the user of compartmental models. However, since it dealt only with one of the simplest possible compartmental models, much statistical examination yet remains to be done to counter the uncritical manner in which workers in this field have embraced compartmental models and nonlinear least-squares programs that fit them to their data. In view of Lanczos's sobering example, an interesting simulation study would be that of fitting two-compartment models to data generated from one-compartment models (and vice versa), with an accompanying examination of the relationship between the true and estimated pharmacokinetic parameters. By using intravenous infusion to mimic known absorption rates, Loo and Riegelman (24) demonstrated that the choice of the wrong model can lead to substantial errors in the estimates of the absorption rate constant. The clear implication is that blood level data must be fitted to the "correct" model for accurate characterization of a dosage form. However, the earlier discussion and, in particular, the example given by Lanczos imply that statistical and mathematical manipulation of the data may provide little guidance in determining this model. To demand that a nonlinear least-squares program decide on the appropriate model and estimate its parameters solely on the basis of statistical criteria is, demonstrably, unrealistic. Moreover, even if nonstatistical criteria (for example, physiological factors) can be adduced to support the validity of a particular model, the severe problems associated with the fitting of the model

parameters to the blood level data remain. The question of the sensitivity of exponential curves to their parameters was discussed in a recent paper by Julius (25).

Empirical Characterization—A feature of the characterization of blood level data by the compartmental model is that the model is intended to represent, however imperfectly and approximately, the biological organism and its handling of the drug. One can, of course, characterize the blood level data in a purely empirical manner by any type of mathematical function that gives a good fit to the data. Westlake (26) suggested that if the characterization is required only to predict future blood levels (*e.g.*, from various regimens), then a simple mathematical representation is adequate. Wold (27) proposed a more elaborate, but still empirical, characterization based on spline functions. Wold used a polynomial spline function to characterize blood levels of a group of subjects receiving various modifications of a semisynthetic penicillin. Briefly, a polynomial spline function is a function that is, piecewise, a polynomial of specified degree. The pieces join each other in points called knots and are subject at the knots to various continuity conditions on the function itself and its derivatives. After a logarithmic transformation of the blood levels, Wold was able to obtain a good fit to blood level data for the 60 subjects in his experiment using a spline function of two pieces, although he encountered numerical difficulties in trying to fit the sum of two exponential terms to the same data sets. Even though the use of spline functions is an empirical approach, Wold suggested taking this idea one step further and using certain features of the function (such as time of peak and final slope) as representative of "meaningful" biological parameters (such as absorption rate and elimination rate). He cited the greater stability of these estimates within subject groups (on the same modification of the drug formulation) in comparison with estimates of the traditional pharmacokinetic parameters as a great advantage. And he considered the disadvantage, namely, the lack of any direct relationship to the parameters of a compartmental model, to be unimportant and mainly a question of the experimenter's familiarization with this new idea.

COMPARISON OF FORMULATIONS

The previous section dealt with the problem of characterizing the *in vivo* performance of a formulation of a drug. In many cases, however, one is less concerned with characterizing a formulation *per se* than with comparing its performance with that of another formulation. This is particularly the case of an abbreviated new drug application (NDA) submitted to the Food and Drug Administration (FDA), where a part of the submission is a comparative blood level trial in which the new formulation is compared with an already approved "standard" formulation. The general problem is that of comparing the *in vivo* behavior of two or more formulations of the same drug by conducting trials in which the formulations are administered to subjects and blood samples (or urine samples) are taken at various times following administration.

A major problem in comparing the performance of the formulations is deciding which characteristics of the blood level sequence should be compared. The following list of possibilities is suggested:

1. Comparison of the formulations with respect to estimated parameters (*e.g.*, an absorption rate constant or area under the curve as a measure of the amount of drug absorbed) in a biologically meaningful model, such as a compartmental model.

2. Comparison of the formulations with respect to some empirical, but presumably meaningful, characteristic of the blood level sequence (*e.g.*, peak blood level achieved or time of peak blood level).

3. Comparison of the formulations solely with respect to the sequences of blood levels that they generate. Essentially, the comparison of formulations is treated as a "repeated-measurements" experiment (the statistical terminology for a sequence of observations—in this case, blood levels at various sampling times—on each item in the trial). The essential feature of such a method of analysis is that one does not pick out any "meaningful" feature of the blood level sequence for special treatment.

Trials in which the *in vivo* performance of formulations are compared have come to be known as (comparative) bioavailability trials. The term bioavailability denotes different things to different workers. For a limited definition as "relative absorption efficiency," see *Reference 2* (chap. 25). Metzler (28) suggested that, in its broadest use, "the term includes the study of the factors which influence and determine the amount of active drug which gets from the administered dose to the site of pharmacologic action as well as the rate at which it gets there." Metzler gave an excellent, brief summary of the statistical problems arising in comparative bioavailability trials. Underlying all comparative studies of the *in vivo* performance of different formulations lies a question that is of the utmost importance: If statistical analysis detects "significant" differences between formulations, are these differences of any clinical or therapeutic importance? Without an adequate answer to the question of what differences in blood level characteristics or estimated pharmacokinetic parameters correspond to significant clinical or therapeutic differences, all comparative blood level trials must, to a certain extent, be academic exercises.

Design of Comparative Blood Level Trials—The design of comparative blood level trials is based, at least in its statistical aspects, on the well-established principles laid out by Cochran and Cox (29), for example. Designs may employ parallel groups in which each subject of a group receives a given formulation, or they may be of the crossover type in which each subject receives all formulations, in some sequence, with a suitable wash-out period between administrations of the different formulations. The crossover design has achieved almost universal acceptance because, as in much biological experimentation, subject-to-subject variation is considerable. Use of the crossover design, as contrasted with the design in parallel groups, for example, enables one to eliminate subject-to-subject variation in the analysis, thereby achieving a more sensitive test of the formulations. The various ways of laying out the assignment of formulations to subjects were reviewed by Westlake (30). Briefly, one can assign all formulations to

each subject in a random sequence (randomized blocks), or the administration of formulation to subjects can be made in sequences that form a series of Latin squares. A further possibility, not widely used to date, is that of assigning formulations in a balanced incomplete block design. In this case, each subject does not receive all of the formulations (if more than two are involved in the study) but receives at least two of them. The analysis is only slightly more complicated than the more usual Latin-square design, and the crossover concept, which enables one to isolate the subject-to-subject variation, is preserved.

Once the appropriate design has been selected, the remaining problem is determining the appropriate number of subjects for the trial. Again, there is a standard statistical methodology for achieving this end. (See, for example, chap. II, *Reference 29.*) One needs: (*a*) an estimate of the minimum difference between two formulations with respect to some characteristic that one wishes to detect, (*b*) a probability of detecting this difference should it occur, (*c*) a significance level (commonly 0.05) at which the test of the null hypothesis of no difference between the formulations is to be made, and (*d*) an estimate of the residual error standard deviation, which is a measure of the inherent variability in the data. Usually, such an estimate will be available from preliminary work or from a pilot experiment. An elementary discussion of the problem of determining the number of subjects required in the trial was given by Westlake (30).

Analysis of Variance in Comparative Blood Level Trials—The basic method of analyzing a crossover trial in a Latin-square design is an analysis of variance (ANOVA). For example, suppose that it is desired to analyze a characteristic for which there is one value per administration of a formulation to a subject. Examples are an estimated pharmacokinetic parameter, an area under the blood level curve, and a peak blood level. Then the basic ANOVA partitions the total sum of squares of the observations about their mean into a component due to the subjects, a component due to the different days of administration, a component due to the formulations, and a component termed residual error. The formulation sum of squares is tested against the residual error sum of squares to check for differences among formulations, and, of course, similar tests are available to test for differences between individual pairs of formulations.

Variations on the basic ANOVA are possible and correspond to various factors in the assignment of subjects to the respective sequences of formulations that they receive. Cochran and Cox (29, chap. IV) summarized the variations on the basic crossover design and its analysis. Wagner (31) mentioned three of these variations in connection with blood level trials. The variation that he designated as Type I is the same as that discussed above with a further component, due to sequence of administration, extracted from the sum of squares due to subjects. Type II, on the other hand, contains one essential point of difference from the basic design. Subjects are assigned to Latin-squares in a nonrandom manner; for example, one might split them up into fairly homogeneous groups with respect to weight and assign each group to a Latin-square. One can then extract from the subjects' sum of squares a component due to Latin-squares which enables one to test whether the weight of the subjects was a factor of importance. One feature of the analysis is that instead of extracting one overall "day of administration" effect, a day effect for each Latin-square is generated. This has the effect of reducing the number of degrees of freedom available for the error term and it might be questioned whether one would really expect the day effect, if present, to vary from square to square. If the day effects are collapsed into one overall day effect, Type II becomes essentially the same as Type I. The Type III crossover design can be analyzed as in Type I, but it can also be subjected to a much more elaborate form of analysis which adjusts for potential "residual effects," that is, the possible carryover from one administration to the following administration. This type of design has potential application in certain clinical trials, but for blood level trials there is already a very direct check on residual effects, namely, the zero-hour blood level, which is customarily taken at the time of administration of the formulation.

Another variation based on an analysis given in Lindquist (32) was used by MacLeod *et al.* (33) in their study of the bioavailability of three brands of ampicillin. Their analysis extracts from the error term a component that can only be described as a "within subject interaction between formulations and days." One would not, perhaps, be greatly interested in testing this component, but its removal from the error term could possibly affect the test on formulations, since the error term would now be different. This component can only be obtained if the number of formulations compared in the Latin-square design is greater than two. The "among subjects interaction between formulations and days," which the authors extracted from the subjects' sum of squares, is the same thing as the sequence component referred to earlier. Finally, the analysis of a crossover trial arranged as a balanced incomplete block design was given by Westlake (30).

Validity of Analysis of Variance and Transformations—Although the basic analysis of the crossover design has been discussed, the question now arises of whether the data should be transformed in some manner before analysis. To answer this question, an understanding of the underlying structure implicit in the analysis is necessary, namely, that any observation is made up of the sum of an overall mean, a component due to the particular subject, a component due to the day, a component due to the formulation, and a random error term. The error terms for the various administrations are assumed independent and normally distributed with zero mean and uniform variance. A characteristic that is often analyzed in a crossover trial is an estimated pharmacokinetic parameter (*e.g.*, an absorption rate constant), the object being to determine if the formulations differ with respect to this parameter. In numerous examples in the literature, the analyses were carried out in terms of the basic ANOVA. If each experiment yielded an observed value of the parameter, the analysis might be appropriate since it could reasonably be assumed that the observed value was equal to the true value for that subject–week–formulation combination plus a random

error term. In actual practice, of course, the parameter has not been observed but estimated from data; consequently, a second error term applies to the estimated parameter. This can be expressed as: estimated parameter value equals true mean parameter value plus e_1 plus e_2, where e_1 is the error term expressing random fluctuation of the parameter value about its mean value for that subject–day–formulation combination, and e_2 is a sampling error term which is dependent on the goodness-of-fit of the model to the data. An estimate of the variance of e_2 can be obtained directly when the parameter is estimated, and frequently this variance is far from uniform.

The foregoing discussion suggests that the use of ANOVA may not be appropriate and that a better course of action lies in analyzing the data with a test that does not require the assumption of uniform variance. A number of nonparametric tests are possible candidates. Westlake (30) suggested that, in a crossover trial, the simple sign test would be suitable for testing estimated pharmacokinetic parameters. Bernard *et al.* (34), in comparing half-lives and peak serum concentrations of minocycline in different groups of patients, used another nonparametric test, the Mann–Whitney U-test. An elementary reference to these and several other nonparametric tests is Siegel (35). An interesting possibility[1], which does not yet appear to have been studied, is that of comparing formulations with respect to the variance of some estimated pharmacokinetic parameter—not with respect to the mean value of the parameter as is usually done. A formulation that provided a much more variable value of the absorption rate constant, k_a, for example, might well be regarded as an inferior, because more variable, product.

The area under the blood level curve from zero to infinity is one of the most frequently analyzed characteristics of the blood level curve. Under the assumptions that elimination of the drug occurs only from the blood compartment and that the elimination is first order, Eq. 3 can be readily demonstrated:

$$\text{area} = FD/k_eV \qquad \text{(Eq. 3)}$$

where F is the proportion of the dose D of drug absorbed, while k_e and V are the elimination rate constant and apparent volume of distribution, respectively. Wagner (2, chap. 25) discussed several ways of transforming the areas prior to analysis. He suggested that, in a crossover design, one can obtain the ratio of the proportions of drug absorbed by taking the ratios of the areas. In this case, D cancels out of the ratio, as does the product k_eV if this is assumed constant for a given subject. However, the ensuing analysis must then ignore the "day effect," since this was absorbed into the ratio. An alternative approach, suggested by Westlake (30), is to take the logarithms of the areas which gives the relation found in Eq. 4:

$$\log \text{area} = \log F + \log D - \log (k_eV) \qquad \text{(Eq. 4)}$$

The log of the area may now be analyzed by the standard ANOVA, since the term $\log (k_eV)$ can be treated as an additive effect due to the subject. It is not essential that k_eV remains exactly constant for a given subject but only that it be distributed around a mean value for that subject with either a relatively small variance or with a standard deviation proportional to the mean.

Wagner (2) suggested performing the analysis of the data after a transformation which involves correction for the volume of distribution and elimination rate constant. My understanding of this method is as follows. The area is multiplied by the product of W (body weight) and the decay constant, as estimated from the final exponential decay of the blood levels, and then divided by the dose. For a one-compartment model, this transformation results in Eq. 5:

$$\frac{(\text{area})(Wk_e)}{D} = \frac{(FD/Vk_e)(Wk_e)}{D} = \frac{F}{V/W} \qquad \text{(Eq. 5)}$$

Thus, on the assumption that V/W is constant and k_e has been correctly estimated, the transformed area can be analyzed as a measure of fraction of dose absorbed. However, if the behavior of the subject is more closely modeled by a two-compartment model, one sees that the transformation gives not $F/(V/W)$ but $F/(V/W)$ multiplied by k_{21}/α or, equivalently, by β/k_e. The approximate constancy of the mean value of the factor k_{21}/α in the group of subjects does not, of course, imply that this factor therefore cancels out in the analysis since it is multiplicative and not additive. In fact, the situation seems quite similar to the analysis of unadjusted areas proposed first. In the former case, one is analyzing FD/k_eV; with a logarithmic transformation and the assumption that k_eV for a given subject is approximately constant or distributed with relatively small variance, one can proceed to the standard ANOVA. In the case where areas are adjusted in the manner just described, one is analyzing $[F/(V/W)](k_{21}/\alpha)$ and, again, a logarithmic transformation and the assumption that both V/W and k_{21}/α are distributed with a relatively small variance about a mean value allow one to proceed to the ANOVA. It is thus difficult to see, on statistical grounds, what the adjustment of areas has achieved, especially since one is also making the assumption that β (the final exponential decay constant) has been adequately estimated from the data. However, the adjustment of areas might be an appropriate procedure if it could be demonstrated that the product k_eV were considerably more variable in a given subject than the quotient of the pharmacokinetic parameters k_{21}/α.

Analysis of the area under the blood level curve, when feasible, is likely to be of major importance in any comparative blood level trial in view of its direct relation to the amount of drug absorbed. Other parameters that are not dependent on any model of the drug distribution, such as peak blood level and time at which it occurs, are sometimes studied since they characterize the performance of the drug formulation. One either picks the peak blood level occurring among the samples or attempts to interpolate it in the sequence of sample values. Little appears to have been written on the problems associated with interpolation of maximum blood levels, although the spline-function approach, suggested by Wold (27), should be of value in this regard.

In addition, the question can be asked: Should such

[1] Suggested by Dr. K. R. Heimlich, personal communication.

data be transformed before subjecting them to an analysis of variance? Again, little seems to have been done on this subject, although Westlake (30) tentatively suggested that *any* concentration data can be regarded as possible candidates for a logarithmic transformation. The reason put forward is that for any pharmacokinetic model the apparent volume of distribution will appear in the denominator of the expression for drug concentration in the blood. Thus, if volume of distribution is roughly constant for a subject and is to be treated as an additive effect in the ANOVA, then the data cannot be expected to conform to the ANOVA model without some prior transformation. Logarithmic transformation will certainly have the effect of bringing the volume of distribution into the model additively, as required, but the question is a complicated one and no completely satisfactory answer appears forthcoming.

Analysis of Comparative Blood Level Trials as "Repeated-Measurements" Experiments—In the final type of analysis of blood level data to be considered, no particular features of the sequence of blood levels are picked out for special treatment. For each subject–day–formulation combination—in other words, for each administration of the drug—one is simply faced with a sequence of blood levels taken at various sampling times. The statistical term for such an experiment is a repeated-measurements experiment, the repeated measurements, of course, being the sequence of blood levels taken at repeated times. Numerous methods of statistical analysis are available and are based on no assumptions (implicit or otherwise) concerning the nature of the biological organism or the relative importance of features of the blood level sequence. Such methods have a certain appeal, particularly inasmuch as it usually is not clear which features of the blood level sequence are important or have a direct bearing on the therapeutic effect of the drug formulation.

The method most often used is that of performing the basic ANOVA at each sampling time, that is, a series of n ANOVA's where n is typically in the 6–12 range. Countless literature references could be given so that there is little point in singling out any one as an example. The problem, statistically speaking, is that one is forced to adjust the significance level to make this series of tests meaningful. For example, to test a null hypothesis (the hypothesis of no difference between formulations) at the 0.05 level means that one is accepting a 0.05 probability of deciding that the formulations are different when they are, in fact, the same. However, if there are, say, 10 sampling times, then it can be shown that if one rejects the null hypothesis on the grounds that any individual ANOVA shows a significant difference between the formulations at the 0.05 level, the probability of rejecting the null hypothesis even when true is about 0.40 (assuming independence of sampling times). The reason is, of course, that there are 10 opportunities to reject the null hypothesis; thus, to make sense of the series of tests, one must scale the significance level for individual tests down to a very much lower level. This procedure also has some unsatisfactory features, and the problem was discussed at length by Westlake (30).

A method of analyzing comparative blood level trials, which is in many ways more satisfactory, consists of viewing the trial as a "split-plot" design or, more accurately stated, as a "strip-plot" design. Details of the analysis of variance of a repeated-measurements design treated in this way were given by Winer (36). Briefly, the ANOVA comprises two sections, each with its appropriate error term for testing the various effects. The so-called "main plot" part of the analysis is, essentially, an analysis of mean blood levels (the mean being taken over all sampling times) and has the same structure as that given earlier for the basic ANOVA, namely a partitioning into components due to days, subjects, formulations, and residual. The so-called "subplot" is comprised of a partitioning into components due to sampling times and the interactions of sampling times with days, subjects, and formulations, respectively, and a subplot error term. Two tests, one in the main plot and one in the subplot, are of particular interest in the comparison of formulations. The test on the formulation mean square in the main plot tests whether there are differences among formulations with respect to their mean blood levels over the sampling times. The test on the (sampling times × formulations) interaction in the subplot tests whether there are differences among formulations with respect to the *patterns* of blood levels to which they give rise. Tests for difference between mean blood levels and patterns of blood levels for any given pair of the formulations studied in the design can also be made.

This method of analysis has a number of advantages, not least of which is that it produces one simple test on the mean blood levels and one test on the blood level patterns over time. Another advantage is that the concepts of mean blood level and blood level pattern are readily grasped by the nonstatistical scientists who must make the final decision concerning equivalency. There is an important restriction in that the method of analysis is only valid if the variance–covariance matrix of residuals has a particular form known as "uniform" (that is, the variances at the different sampling times are equal and all covariances between different sampling times are equal). A practical consequence of this is that the correlation between blood levels at different sampling times must be independent of the distance in time between the samples. In his book, Winer (36) gave details of a test, first devised by Box (37), to be carried out to determine whether the assumption of uniform variance–covariance holds. The author's experience (see *Reference 38*) with many orally administered drugs—in which samples are usually taken at intervals approximately an hour apart—is that the assumption is often valid, especially when a transformation of the data is made to ensure equal variances. This paper (38) also gives an extension of the analysis, including Box's test, to the case of comparative blood level trials arranged as balanced incomplete block designs. For the case where the uniform variance–covariance matrix requirement is not satisfied, Greenhouse and Geisser (39) gave an approximate test which could be useful in blood level trials.

Other more complex methods of analysis are, of course, available. Cole and Grizzle (40) discussed an example in which the effects of intravenous morphine sulfate and intravenous trimethaphan on blood hista-

mine levels of dogs were studied. Sampling times were at 0, 1, 3, and 5 min. and, perhaps not surprisingly with samples taken so close together in time, the uniform variance–covariance assumption was not valid. They discussed the application of a more complex method of multivariate analysis, namely the multivariate analysis of variance (MANOVA). Apart from their example, few applications of these techniques to blood level data appear to have been published in the literature. One drawback to routine application of MANOVA to typical blood level trials in which formulations are to be compared is the difficulty of interpreting these more sophisticated concepts so that they are meaningful to the nonstatistical scientists who must make the decisions based on the analysis.

Another possible method of analysis, described by Church (41), is based on principal components. The sequence of, say, n blood levels over the sampling times is treated as a vector of n observations. One then seeks a linear transformation of these n variables into a new set of n orthogonal (uncorrelated) variables such that the first variable accounts for the maximum amount of variability in the data, the second component for the maximum amount of the remaining variability, and so on. The advantage is that the new variables are uncorrelated and can be ranked in order of importance in accounting for the variability of the data. The dimension of the vector may also be effectively reduced if it is found that fewer than n principal components account for the bulk of the variability. Redman (42) discussed the application of principal components to blood level experiments. Snee (43) proposed a modification of Church's method in which the variation of the data is partitioned into two parts, the first connected with the mean of the blood levels and the second with the pattern of the blood levels. Principal components are then used to analyze the pattern component. A possible problem with the use of methods based on principal components is, as with MANOVA, the potential difficulties of conveying the statistical ideas to a nonstatistical user of the results. A second problem is that, instead of one simple test for pattern difference among formulations, there are a number of tests dependent on how many principal components are thought to be sufficient to account for the variation of the data. Thus, there is a greater likelihood that some difference among patterns will be detected as statistically significant, even though it may not have any meaningful relation to clinical or therapeutic significance. So at the end of this discussion of possible methods of analysis, one returns to the problem that plagues the whole subject of comparative bioavailability, namely, the relationship, if any, of differences between the blood level characteristics of the formulations to differences in their therapeutic effects. Until much more is known about this fundamental aspect of *in vivo* testing, unsatisfactory features will be connected with most statistical analysis.

Use of Confidence Intervals—All of the methods of analysis discussed so far have been formulated in terms of the statistical theory of hypothesis testing. To illustrate the simplest possible case, suppose that a crossover trial is conducted in which two formulations are to be compared with respect to some single parameter, such as area under the blood level curve as a measure of drug absorbed. If the basic ANOVA is used to analyze the data, one is essentially testing the null hypothesis that $\mu_1 = \mu_2$, where μ_1 denotes the mean amount of drug absorbed when the first formulation is administered to an essentially infinite population of subjects and μ_2 has the same meaning for the second formulation. Now it appears reasonable to assert that, in actual fact, μ_1 and μ_2 will never be equal since there will always be some differences, however slight, in the composition and methods of manufacture of the formulations. Consequently, if a sufficiently large number of subjects is employed in the crossover trial, one should be able to detect a statistically significant difference between any pair of formulations. This observation suggests that the idea of testing a simple null hypothesis of the type $\mu_1 = \mu_2$ may not be a very realistic approach to testing whether two formulations provide equivalent amounts of drug absorbed. The key lies in the word "equivalent" which, in a practical situation, means therapeutically or clinically equivalent. This might mean, for example, that the second formulation could be considered equivalent to the first if it resulted in an area under the blood level curve (taken to be a measure of absorption) that was within $n\%$ of that achieved with the first formulation. How small n must be to indicate therapeutic equivalence is, of course, a decision for the clinician to make; and as Levy (44) pointed out, the nature of n depends on the particular drug under consideration.

Suppose that for a given drug the value of n is taken to be 20; that is, the mean area under the blood level curve for the second formulation must be within 20% of the mean area for the first formulation in order to be judged "practically" equivalent. Then a suitable null hypothesis to test would be $(0.80\ \mu_1 \leq \mu_2 \leq 1.20\ \mu_1)$ rather than simply $\mu_1 = \mu_2$. However, construction of a test poses considerable problems and an alternative approach is to abandon hypothesis testing methods completely and replace them with the concept of confidence intervals. A 95% confidence interval statement, for example, resulting from the analysis of the example under discussion would be of the type $(\mu_1 - k_1 \leq \mu_2 \leq \mu_1 + k_2)$. Note that this gives an indication of where μ_2 is located in relation to μ_1; when the result of the analysis is stated in this form, it gives the physician or clinical pharmacologist information in a form from which he or she can make a judgment as to the practical equivalence of the formulations. Metzler (28) discussed the use of confidence intervals as a means of summarizing a comparative bioavailability trial and gave a number of examples, for each of which various criteria of acceptance are proposed based on a confidence interval approach. He then showed how certain formulations meet the acceptance criteria while some do not. Some that meet the acceptance criteria would be rejected as being statistically significantly different from the standard formulation against which comparison was made; some that would pass the hypothesis testing do not meet the acceptance criteria based on confidence intervals.

Many instructive examples can be constructed to

show how the confidence interval approach, which can be made the basis of a decision by the physician or clinical pharmacologist, leads to quite different results from the hypothesis testing approach, which is based only on the statistician's more-or-less arbitrary selection of significance levels. Defenders of the hypothesis testing approach will probably reply that, in planning the trial, the number of subjects is selected to allow detection of a certain minimum difference between the bioavailability of the formulations based on the error variance (estimated from pilot studies), significance level of the test, and required probability of detecting the minimum difference. This is true, but much of this careful planning may be negated if the variability turns out to be different from that estimated from the pilot experiments. In this case, the trial may be either incapable of detecting the minimum difference as statistically significant or, on the other hand, may show supposedly unimportant differences to be statistically significant. The confidence interval approach thus appears to provide a more satisfactory answer.

One disadvantage of constructing conventional confidence intervals, which are symmetrical about the difference of the population means of the two formulations, is that when expressed in the form given earlier, $(\mu_1 - k_1 \leq \mu_2 \leq \mu_1 + k_2)$, they are *not* symmetrical; that is, k_1 and k_2 are not equal. Many clinical pharmacologists would, however, make their equivalence statements in a symmetrical form, for example: the second formulation is clinically equivalent to the first if its bioavailability is within 20% of that of the first formulation. This implies that k_1 and k_2 are equal in a confidence statement of the type just given. Westlake (45) suggested that the traditional method of constructing confidence intervals could be modified so as to ensure a symmetrical statement in which k_1 and k_2 are equal, and he gave a method for carrying out this procedure.

The author's opinion is that confidence intervals currently afford the best means of summarizing the results of a comparative bioavailability trial since, unlike hypothesis tests, they portray the information in a manner that can form the basis for an informed judgment by a physician or clinical pharmacologist. Unfortunately, this only seems to be true when one is discussing a simple quantity such as amount of drug absorbed or mean blood level over time. More complex concepts, such as the difference between the blood level patterns over time, would not seem to be amenable to a confidence interval treatment at present. Theoretically, there is no difficulty; the problem lies in the practical interpretation of confidence interval statements concerning more complex functions of blood levels.

SUMMARY

The discussion of the statistical methods used in the evaluation of *in vivo* performance of dosage forms has covered the characterization of dosage forms by biologically meaningful models and purely empirical means and the comparison of formulations. The subject seems to have resulted in very few novel developments in statistical theory and, by and large, existing statistical methods seem more than adequate to cope with the problems that arise in this particular field. It seems questionable whether new statistical techniques could improve matters in the estimation of pharmacokinetic parameters in compartmental models. Rather, as Lanczos (16) so elegantly demonstrated, only an immense refinement in measuring techniques can improve the estimation of parameters in a function that is the sum of exponential terms. Even if this were possible, one would still face the fact that any compartmental model is a grossly simplified model of the biological reality. For the comparison of dosage forms or formulations, numerous methods of analysis are available but it has been suggested that a confidence interval approach, rather than the more usual hypothesis testing, may offer more hope for a realistic appraisal of comparative dosage form performance. Eventually, it seems that the major problem in the use of statistical methods is a nonstatistical one. That problem is the elucidation of the relationship of blood level characteristics to therapeutic use of the drug. It is only with improved knowledge in this area that we can hope to determine accurately those blood level characteristics which are of really critical importance. Statisticians can perform many types of analyses on blood level sequences; some of them are of considerable sophistication and complexity. But the value and significance of such analyses can hardly be assessed until a clear idea of the relation between blood levels and therapeutic use of the drug is available.

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RESEARCH ARTICLES

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J. V. CHAMPION and G. H. MEETEN▲

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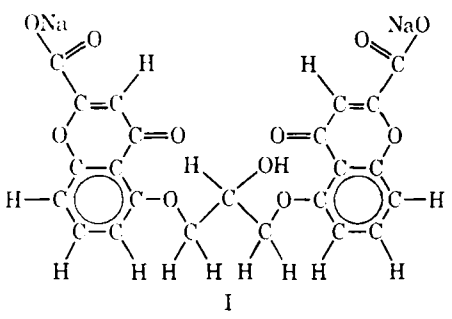
Keyphrases □ Sodium cromolyn (disodium cromoglycate)—molecular conformation in aqueous solution, light-scattering and magnetic birefringence techniques □ Molecular conformation, sodium cromolyn—determination in aqueous solution, light-scattering and magnetic birefringence techniques □ Light-scattering techniques—determination of sodium cromolyn conformation in aqueous solution □ Magnetic birefringence techniques—determination of sodium cromolyn conformation in aqueous solution

The conformation of the molecule of sodium cromolyn[1] (disodium cromoglycate) is of particular interest because the compound has been used as a new pharmacological approach to the treatment of bronchial asthma. The solid-state chemistry of sodium cromolyn was recently described (1), and the lattice parameters of the crystalline solid solution at high (90%) relative humidity indicate that the molecule is in a planar conformation. In the concentrated solution state, the formation of liquid crystals occurs.

The aim of this study was to determine the conformation of the molecule in aqueous solution. This may be important in assessing the activity and specificity of biological action at a membrane surface.

The chemical structure of sodium cromolyn in the planar conformation is shown here (I), and the molecule may be approximated to a pair of rigid chromones joined by a glycerol bridge [—O—CH_2—CH(OH)—CH_2—O—], the hydroxy, carbonyl, and ether groups giving many possibilities of hydrogen bonding, especially in the presence of water molecules. Simple molecular model building shows that numerous different conformations are possible by rotation about the bonds of the glycerol bridge, many of these having a small



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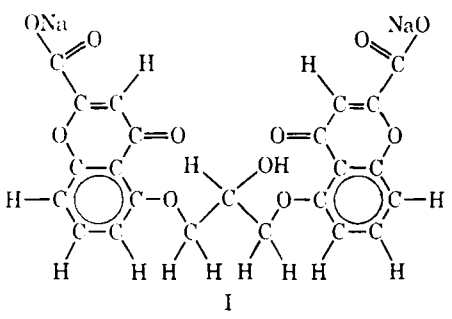
The chemical structure of sodium cromolyn in the planar conformation is shown here (I), and the molecule may be approximated to a pair of rigid chromones joined by a glycerol bridge [—O—CH_2—CH(OH)—CH_2—O—], the hydroxy, carbonyl, and ether groups giving many possibilities of hydrogen bonding, especially in the presence of water molecules. Simple molecular model building shows that numerous different conformations are possible by rotation about the bonds of the glycerol bridge, many of these having a small



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energy barrier. Hence, in dilute aqueous solution, the molecule may exist in a form in which the two chromone structures either are randomly oriented with respect to each other or are in a fixed orientation due to intramolecular or intermolecular hydrogen bonding.

Precursory measurements, using viscometric and light-scattering techniques whereby the hydrodynamic volume was determined, showed that aggregation or strong solute–solute interaction occurred at concentrations greater than 0.5 g./100 ml. in aqueous solution. Above 0.8 g./100 ml., the aggregation was time dependent.

The conformation of the molecule can be related to the molecular optical and magnetic anisotropy, which can be measured using depolarized light scattering and magnetic birefringence, respectively. The necessity of using very dilute aqueous (conducting) solutions of relatively low molecular weight material (516) limits the techniques available for observing molecular anisotropy to the two described here.

OPTICAL AND DIAMAGNETIC ANISOTROPY

It will be shown in the following section that the optical and diamagnetic anisotropies of the sodium cromolyn molecule are sensitive functions of its conformation, hence the use of light-scattering depolarization and magnetic birefringence techniques.

Calculation of the sodium cromolyn molecule optical anisotropy was made using bond polarizability tensor additivity (2). In this calculation, each bond is assigned a polarizability tensor α. For single bonds, α is diagonal in coordinates x, y, and z, where x is along the bond and y and z are perpendicular to it. Thus:

$$\alpha = \begin{pmatrix} \alpha_x & 0 & 0 \\ 0 & \alpha_y & 0 \\ 0 & 0 & \alpha_y \end{pmatrix}$$

For double bonds:

$$\alpha = \begin{pmatrix} \alpha_x & 0 & 0 \\ 0 & \alpha_y & 0 \\ 0 & 0 & \alpha_z \end{pmatrix}$$

where the x axis lies along the bond and the y axis is perpendicular to the π-electron plane.

The polarizability of a molecule with many bonds is then calculated by choosing axes 1, 2, and 3 fixed to the molecule and calculating the molecular polarizability tensor:

$$\begin{pmatrix} \alpha_{11} & \alpha_{12} & \alpha_{13} \\ \alpha_{21} & \alpha_{22} & \alpha_{23} \\ \alpha_{31} & \alpha_{32} & \alpha_{33} \end{pmatrix} = \alpha_m$$

The components are:

$$\alpha_{jk} = \alpha_{kj} = \sum_b \sum_u \alpha_u \gamma_{uj} \gamma_{uk} \qquad u = x, y, z \qquad \text{(Eq. 1)}$$

where γ_{uj} is the direction cosine of the angle between the u ($u = x$, y, or z) and the j axes, and j and k take value 1, 2, or 3. The Σ_b denotes a summation over all bond types in the molecule. In this scheme, benzene rings are considered as bonds having x axes perpendicular to the ring plane.

Calculation of the molecule diamagnetic susceptibility tensor χ_m was made in an exactly similar way as for the optical polarizability tensor, with bond susceptibilities χ_x, χ_y, and χ_z replacing α_x, α_y, and α_z.

Two conformations for sodium cromolyn were chosen as representing extreme examples of its conformation: (*a*) the planar conformation, as suggested by X-ray measurements on the crystal (1); and (*b*) the perpendicular conformation, obtained when the benzene rings of the molecule lie in perpendicular planes. This perpendicular conformation is possible due to internal rotation around O—C and C—C bonds in the O—C_3—O bridge connecting the rings. Experiments on a Courtalds space-filling molecular model showed no steric hindrance to a perpendicular conformation. Light-scattering and magnetic birefringence theories require principal polarizabilities such that:

$$\alpha_m = \begin{pmatrix} \alpha_1 & 0 & 0 \\ 0 & \alpha_2 & 0 \\ 0 & 0 & \alpha_3 \end{pmatrix} \quad \text{and} \quad \chi_m = \begin{pmatrix} \chi_1 & 0 & 0 \\ 0 & \chi_2 & 0 \\ 0 & 0 & \chi_3 \end{pmatrix}$$

Since $\alpha_{jk} = \chi_{jk} = 0$ for molecular axes of symmetry, these are also the principal polarizability or susceptibility axes. For the planar conformation, the 1 axis is coplanar with the benzene rings and parallel to a line joining their centers, the 2 axis is perpendicular to the benzene ring planes, and the 3 axis lies in the plane containing the planes of the benzene rings.

The perpendicular conformation is the result of rotation about the 1 axis of each of the molecular halves such that the 2 axis of one-half of the molecule becomes parallel to the 3 axis of the other half. A large number of —OC_3O— bridge conformations may attend this rotation, and the average —OC_3O— bridge conformation is unknown. However, the optical and diamagnetic anisotropies of the —OC_3O— bridge are small compared with the whole molecule since most anisotropy arises from the ring structures. Thus, to a good approximation:

$$\alpha_1(\text{perpendicular}) = \alpha_1(\text{planar}) \qquad \text{(Eq. 2)}$$

$$\chi_1(\text{perpendicular}) = \chi_1(\text{planar}) \qquad \text{(Eq. 3)}$$

$$\alpha_2(\text{perpendicular}) = \alpha_3(\text{perpendicular}) = {}^1/_2\alpha_2(\text{planar}) + {}^1/_2\alpha_3(\text{planar}) \qquad \text{(Eq. 4)}$$

$$\chi_2(\text{perpendicular}) = \chi_3(\text{perpendicular}) = {}^1/_2\chi_2(\text{planar}) + {}^1/_2\chi_3(\text{planar}) \qquad \text{(Eq. 5)}$$

Vuks' (3) comprehensive and well-tested set of bond polarizabilities was used; relevant values are listed in Table I. The benzene ring value in Table I was derived from light-scattering measurements on gaseous benzene, using Vuks' values for the CH bond polarizabilities. Since further data were lacking, no distinction was made between aliphatic and aromatic carbon atoms, the latter denoted by C′. Thus, C′X and CX are assumed to have the same optical anisotropy.

Bond diamagnetic susceptibility anisotropy data for CC, CH, C′H, and CO in Table I are taken from Bothner-By and Pople (4). The other values in Table I were calculated by the present authors from the measured molecular susceptibility anisotropies of benzene (4), hexamethylbenzene (4), resorcinal (4), and formaldehyde (5).

Models of sodium cromolyn molecules in the planar and perpendicular conformation were constructed using stick models, and the bond angles were measured relative to the symmetry axes. The molecular anisotropies (Table II) were calculated.

Mean values of optical molecular polarizability $\bar{\alpha}$ were calculated from the sum of mean bond values over the molecule. Tables of bond average polarizabilities were published by several workers for commonly encountered bonds, and there is close agreement on values between tables. No O—Na bond mean polarizability has been reported. Its value was estimated for present purposes by plotting $\bar{\alpha}^{0-z}$ against the atomic weight of element X, where X was C, H, P, and As as given by LeFevre (6) and Vuks (3), and interpolating for

Table I—Optical and Diamagnetic Anisotropies

Bond	Å^3			10^{-6} e.m.u./mole		
	$\alpha_x - \alpha_y$	$\alpha_y - \alpha_z$	$\alpha_z - \alpha_x$	$\chi_x - \chi_y$	$\chi_y - \chi_z$	$\chi_z - \chi_x$
CC	1.43	0	−1.43	1.21	0	−1.21
CH	0.09	0	−0.09	0.9	0	−0.9
CO	1.23	0	−1.23	3.0	0	−3.0
C=O	1.11	0	−1.11	10.3	5.9	−16.2
OH	0	0	0	1.62	0	−1.62
Benzene ring	−5.35	0	−5.35	−58.3	0	58.3
C′H	0.09	0	−0.09	0.49	0	−0.49
C′C	1.43	0	−1.43	2.12	0	−2.12
C′O	1.23	0	−1.23	−0.3	0	0.30

Table II—Molecular Anisotropies

Conformation	Å³ $\alpha_1 - \alpha_2$	Å³ $\alpha_2 - \alpha_3$	Å³ $\alpha_3 - \alpha_1$	10^{-6} e.m.u./mole $\chi_1 - \chi_2$	$\chi_2 - \chi_3$	$\chi_3 - \chi_1$
Planar	2.2	21.9	−24.1	5.6	169.6	−175.3
Perpendicular	13.5	0	−13.5	91.7	0	−91.7

X = Na. Table III shows Denbigh's (2) and Vuks' (3) values with $\bar{\alpha}^{0-Na}$ inserted as estimated previously. The mean molecular polarizabilities according to the table do not differ significantly from 44 Å, and this value was adopted for future calculation. Estimates of mean molecular polarizability may also be made from the crystalline refractive indexes 1.488, 1.66, and 1.705 measured (1) at 40% relative humidity (16.2% water by weight). Assuming additivity of molar refractivities for sodium cromolyn and water in the crystal gives 44.7 Å³. The Vuks (7) modification to the molar refractivity scheme gives 44.9 Å³. In both cases, a water molecule mean polarizability of 1.59 Å³ was used (8).

LIGHT SCATTERING

The scattering of light by a transparent medium arises from the inhomogeneities of the medium and may be used to characterize them. In a liquid or solution, the scattering is due to the local statistical fluctuations in the polarizability of the medium, arising from density and concentration fluctuations and, for anisotropic molecules, orientational fluctuations. The total scattering power of such a medium is usually expressed in terms of its Rayleigh ratio, $R(\theta)$, defined as the intensity of light, I_θ, scattered in a direction θ with respect to the incident beam, per unit solid angle per unit scattering volume, divided by the incident light intensity, I_0.

The total Rayleigh ratio can be separated into an isotropic part and an anisotropic part:

$$R = R_{is} + R_{an} \qquad \text{(Eq. 6}a\text{)}$$

and:

$$R = KI_\theta \qquad \text{(Eq. 6}b\text{)}$$

for a given scattering system with I_0 constant and with the angular dependence omitted if I_θ is in a direction perpendicular to I_0. The scattered light contributing to R_{is} is polarized perpendicularly to the plane determined by the direction of I_0 and the direction of observation of I_θ and is termed vertically polarized. The anisotropically scattered light has a horizontally (H) as well as a vertically (V) polarized component. Hence, Eqs. 6*a* and *b* become:

$$R = R_{is,V} + R_{an,V} + R_{an,H} = R_V + R_H \qquad \text{(Eq. 7)}$$

and the depolarization of the scattered light for unpolarized incident light is given by:

$$\rho_u = R_H/R_V = I_H/I_V \qquad \text{(Eq. 8)}$$

where $I_H + I_V = I_\theta$.

It has been shown at liquid densities that the isotropic scattering per molecule is strongly reduced (to 5–10%) in comparison with its vapor due to pronounced positional correlations and has a much larger influence on the isotropic than on the anisotropic part of the scattering. The latter, due to orientational correlations, is largely independent of density or concentration fluctuations. Therefore, observations of the anisotropic scattering can lead to information regarding the optical anisotropy of the molecules.

Experimental—A light-scattering photometer, developed for depolarization measurements, was used, whereby the sensitivity and signal/noise ratio were maximized by using a 5.08-cm. (2-in.) o.d. low noise photomultiplier tube[2]; the output was fed to a low noise, high gain d.c. amplifier. The amplifier output was averaged with a time constant of 10 sec. and then recorded on a chart. The source was a 250-w. compact arc mercury lamp, giving an output brightness of 20,000 stilb. The smallest signal, the scattered intensity of the horizontally polarized component of pure water (I_H^w), had a signal/noise ratio of 8:1, and the presence of dust could be readily detected by an increase in signal/noise ratio and the occurrence of large spurious peaks in the recorder trace. A high quality polarizing prism[3] was mounted in front of the photomultiplier entrance pupil, enabling I_V and I_H to be separately determined.

A rectangular Pyrex glass scattering cell was used. The length of the cell was 25 times the length of the observed scattering volume, removing the scattering volume as far as possible from the exit and entrance windows of the incident beam and thus considerably reducing the intensity of the spurious multiple reflections along the cell walls seen by the detector.

The distilled water was prepared by double distillation over potassium permanganate to remove dust and organic matter. The pure water or solution was then centrifuged at 80,000×g for 1 hr. and transferred to the scattering cell *via* a 50-nm. filter[4] under dust-free atmospheric conditions.

All scattered intensities were measured at the mercury green (5461 Å) and mercury blue (4358 Å) wavelengths using narrow band isolating filters.

All concentrations of sodium cromolyn solutions used were corrected for water content of the solid by weighing at known humidity and using the data of Cox *et al.* (1).

Theory and Results—It has been shown (9) that for a system of independent scatters in the liquid state, assuming a Lorentz internal field:

$$R_{is} = \left(\frac{8\pi^4 kTN^2\beta_T\bar{\alpha}^2}{\lambda^4}\right)\left(\frac{n^2+2}{3}\right)^4 \qquad \text{(Eq. 9)}$$

and:

$$R_{an} = \left(\frac{8\pi^4 N\gamma^2}{\lambda^4}\right)\left(\frac{13}{90}\right)\left(\frac{n^2+2}{3}\right)^2 \qquad \text{(Eq. 10)}$$

where k is Boltzmann's constant, T is the absolute temperature, N is the number of scatterers per milliliter, β_T is the isothermal compressibility, λ is the wavelength *in vacuo*, n is the refractive index, and $\bar{\alpha} = (\alpha_1 + \alpha_2 + \alpha_3)/3$ (the mean polarizability of the particle), and $\gamma^2 = (\alpha_1 - \alpha_2)^2 + (\alpha_2 - \alpha_3)^2 + (\alpha_3 - \alpha_1)^2$ (the optical anisotropy of the particle).

The apparatus is calibrated for the distilled water–sodium cromolyn scattering system from Eq. 7, whereby $R^w = k$; $I_s = k(I_H^w + I_V^w) = 0.87 \times 10^{-6}$ cm.$^{-1}$ at λ = 5461 Å and $R^w = 2.32 \times 10^{-6}$ cm.$^{-1}$ at λ = 4360 Å, as given by Cohen and Eisenberg (10). Thus, k can be determined in terms of the instrumental values of $(I_H^w + I_V^w)$. The superscripts w, s, and d represent pure water, solution of sodium cromolyn-water, sodium cromolyn, respectively.

For dilute solutions, the excess horizontally polarized anisotropic scattering of sodium cromolyn molecules is:

$$R_{an,H}^d = k(I_H^s - I_H^w) \qquad \text{(Eq. 11)}$$

Table III—Mean Bond and Molecular Polarizabilities

Bond	Number of Bonds per Sodium Cromolyn Molecular	Å³ per Bond, Denbigh (2)	Å³ per Bond, Vuks (3)
C—C	10	0.64	0.536
C_{ar}—C_{ar}	12	1.07	1.07
C—H	17	0.65	0.65
C—O	11	(0.571)[a]	0.571
C=O	4	1.16	1.32
O—H	1	(0.74)	0.74
O—Na	2	1.1	1.1
Average polarizability per molecule		44.3 Å³	43.9 Å³

[a] Values in parentheses were not given by Denbigh, and Vuks' (3) values were used.

[2] E.M.I. 9502S.

[3] Glan-Thompson.

[4] Millipore VMWP.

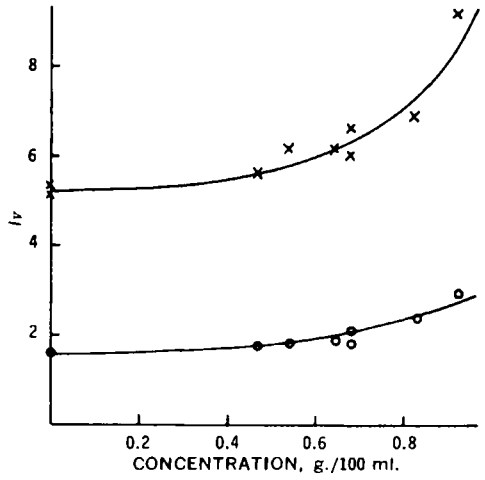

Figure 1—*Vertically polarized scattered intensities as a function of sodium cromolyn concentration and wavelength. Key:* O, *5461 Å; and* ×, *4358 Å.*

giving $R^d_{an} = R^d_{an,V} + R^d_{an,H} = K13/6\ (I_H^s - I_H^w)$ making use of the result $R_{an,V}/R_{an,H} = 7/6$ obtainable from the Cabbannes (11) depolarization factors. By using Eqs. 10 and 11 then:

$$I_H^s - I_H^w = \left(\frac{8\pi^4\gamma^2(I_H^w + I_V^w)}{15\lambda^4 R^w}\right)\left(\frac{n^2+2}{3}\right)^2\left(\frac{N_0}{M}\right)c \quad \text{(Eq. 12)}$$

where N_0 is Avogadro's number, M is the molecular weight of sodium cromolyn, c is the concentration in grams per milliliter, and n is the refractive index of the medium surrounding the sodium cromolyn molecules (*i.e.*, water).

It was found from the experiment that I_H^s and I_V^s were time dependent. For concentrations below 1.0 g./100 ml., this time dependence was relatively slow. No change was observable within the first few hours of making up a solution, while 50% changes were found after several days. Also I_H^s was markedly less sensitive to time than I_V^s. Hence, all the observations reported here were made within 3 hr. of making up the solutions, and low (<1 g./100 ml.) concentrations were used.

The scattered intensities I_H and I_V and the depolarization ratio ρ_u^s as a function of concentration are shown in Figs. 1–4. The scattered intensities (I's) are in arbitrary instrument units, calibration being obtained from the known scattering power of water (10) as described previously. A graph of $I_H^s - I_H^w$ as a function of concentration is shown in Fig. 5. From the slopes of these curves and Eq. 12, values of γ^2 at the two wavelengths are $\gamma^2_{5461} = 1390$ Å^6 and $\gamma^2_{4360} = 1430$ Å^6, using a molecular weight of 516 for sodium cromolyn. The refractive index correction $[(n^2+2)/3]^2$ in Eq. 12 converts the independent particle (gas) value of γ^2 to the value appropriate to the molecule being surrounded by water. These values of γ^2 are directly comparable to those derived from the polarizability anisotropy tensor additivity scheme.

An alternative approach is to obtain the depolarization ratio

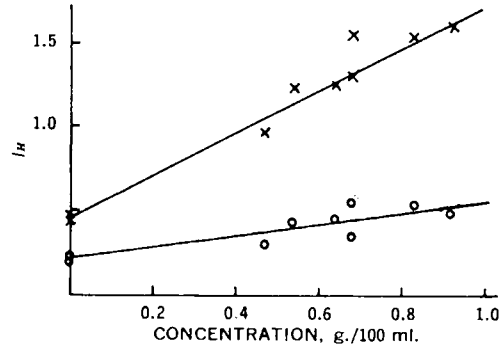

Figure 2—*Horizontally polarized scattered intensities as a function of sodium cromolyn concentration and wavelength. Key:* O, *5461 Å; and* ×, *4358 Å.*

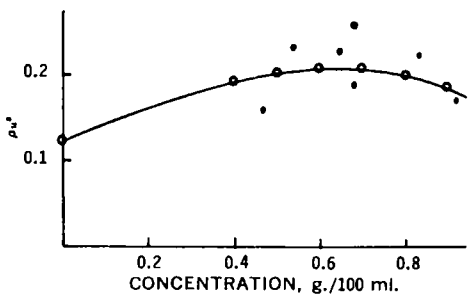

Figure 3—*Depolarization ratio as a function of sodium cromolyn concentration at 5461 Å. Key:* O, *interpolated points from Figs. 1 and 2.*

(ρ_u^d) of the sodium cromolyn molecule. According to Sicotte and Rinfret (12) and using Eq. 8:

$$\frac{I_s^s\rho_u^s}{I_s^w(1+\rho_u^s)} = \rho_u^d\left(\frac{I_s^s}{I_s^w(1+\rho_u^s)}\right) - \left(\frac{\rho_u^d - \rho_u^w}{1+\rho_u^w}\right)\phi_w \quad \text{(Eq. 13)}$$

where ϕ_w is the volume fraction of water in the solution. For dilute solutions in the concentration range 0 → 1.0 g./100 ml., ϕ_w varies from 1.00 to 0.99 and may be assumed constant, the variables being ρ_u^s and I_s^s ($= I_H^s + I_V^s$). Figure 6 shows a plot of $\{(I_s^s/I_s^w)[\rho_u^s/(1+\rho_u^s)]\}$ *versus* $[I_s^s/I_s^w(1+\rho_u^s)]$. The slope of the linear part of the curve, below concentrations of 0.6 g./100 ml., gives a value of $\rho_u^d = 0.31$. It is expected that values for both wavelengths should fall on the same curve due to the negligibly small variation of polarizability with wavelength.

It was shown (10) that the depolarization ratio of a particle in a surrounding medium (sodium cromolyn in water) is given by:

$$\rho_u^d = \frac{6\left(\frac{n^2+2}{3}\right)^2\gamma^2}{\frac{90N_0kT\beta_T d}{M_w}\left(\frac{n^2+2}{3}\right)^4\bar{\alpha}^2 + 7\left(\frac{n^2+2}{3}\right)^2\gamma^2} \quad \text{(Eq. 14)}$$

where the density d, β_T, M_w, and n relate to the medium surrounding the sodium cromolyn molecule. The terms containing these quantities convert the independent particle value of optical anisotropy γ^2 and the average polarizability $\bar{\alpha}$ to a value appropriate to the molecule in a surrounding medium.

Using the values $\beta_T = 4.5 \times 10^{-11}$ cm.^{2}/dyne (10), $d = 1.0$ g./ml., $M_w = 18$, $n = 1.33$, and $\bar{\alpha} = 44$ Å^3 with $\rho_u^d = 0.31$, one gets $\gamma^2 = 1100$ Å^6.

From the bond polarizability data in the preceding section, $\gamma^2_{planar} = 1060$/Å^6 and $\gamma^2_{perpendicular} = 365$ Å^6. The average experimental value is 16% higher than that calculated for the planar conformation and 300% higher than the calculated perpendicular conformation of the sodium cromolyn molecule. With the large (20%) error in individual experimental observations, this must give very strong evidence for the existence of the planar conformation in very dilute solutions.

MAGNETIC BIREFRINGENCE

The Cotton–Mouton constant C is defined as $\Delta n/\lambda B^2$, where Δn is the birefringence induced in a specimen by a magnetic field B, measured using light of wavelength λ. The effect is due to the small

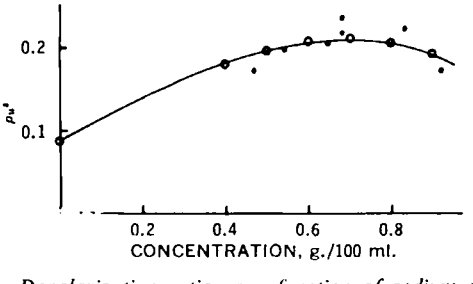

Figure 4—*Depolarization ratio as a function of sodium cromolyn concentration at 4358 Å. Key:* O, *interpolated points from Figs. 1 and 2.*

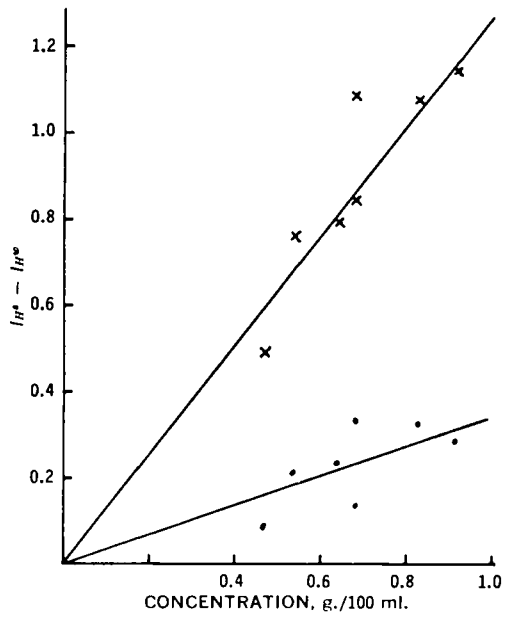

Figure 5—*Horizontally polarized excess scattering of sodium cromolyn solution. Key:* ×, *4358 Å; and* ○, *5461 Å.*

degree of nonrandom alignment of molecular optic axes caused by interaction between the magnetic field and the anisotropy of molecular diamagnetism.

In the measurement of C, it is necessary to pass a beam of monochromatic linearly polarized light through length L of the specimen and traverse to a known field B. The birefringence $\Delta n = C\lambda B^2$ produces ellipticity in the emergent beam. This ellipticity is a maximum when the incident beam polarization azimuth is $\pm 45°$ to B, the phase difference δ between the principal vibration components being $(2\pi/\lambda)L\,\Delta n$.

Experimental—The apparatus was constructed around an electromagnet[5] having a maximum field of about 23 kOe. The solution was contained in standard 20-cm., fused quartz polarimeter tubes with the glass windows replaced by spectrosil fused quartz. Practically monochromatic light was produced by a 250-w. mercury arc lamp, and a filter[6] was used to isolate the 5461 Å green line. Light from the lamp, after being collimated by means of a pinhole and two lenses, passed through a polarizer[7]. After traversing the specimen, the emergent beam's ellipticity was analyzed using a quarter-wave plate specially constructed to be free from optical strain and another polarizer[8] as an analyzer. Intensity minima were observed using a telescope focused for infinity on the pinhole and a filter[9] fitted to the eyepiece to remove a small amount of red light not removed by the other filter[6]. The whole apparatus was used in a dark room at ambient temperatures between 21 and 25°.

Since the field was not constant over the optical path length in the solution, the average value of B^2 (denoted by $\bar{B}^2$) is necessary to calculate C. This was measured for various magnet currents using nitrobenzene, which has $C = 262 \times 10^{-13}$ Oe.$^{-2}$ cm.$^{-1}$ (13) at 5461 Å and 23°. Point-to-point measurement of B along the optical path using a gaussmeter and calculation of:

$$\bar{B}^2 = \frac{1}{L}\int_0^L \beta^2(dL) \qquad \text{(Eq. 15)}$$

agreed to within ±1% with the calibration using nitrobenzene.

The experimental procedure was thus to rotate the quarter-wave plate and analyzer for an intensity minimum with the specimen between the magnet pole-pieces, the polarizer azimuth having been previously set at ±45° to the direction of B. Energizing the magnet then produced a weak illumination in the field of view. The intensity minimum could be restored by adjusting the analyzer azimuth by a rotation $\Delta\theta$, whereupon $\delta = \pm 2(\Delta\theta)$.

Sources of error were Faraday rotation in the specimen and residual birefringence in the polarimeter tube windows. These were reduced to negligible proportions by accurate alignment and selection of low strain windows, respectively.

The solutions were prepared by shaking the sodium cromolyn powder with distilled water and using 50- or 100-nm. filters[10] before making the measurements. Weighing tests had shown that these filters did not remove any of the dissolved sodium cromolyn. No glass was allowed to contact the solutions at any stage. Aged or unfiltered solutions often showed a nonlinearity of Δn with $\bar{B}^2$. This dependence of C upon B is typical of solutions of very large particles or liquid crystals. Solutions exceeding about 2% concentration invariably showed this behavior, which varied with time in a nonreproducible manner. Accordingly, all measurements were made immediately after filtering at less than 1.5% concentration. Any result showing a field dependence of C was rejected. Figure 7 shows a typical graph of Δn *versus* $\bar{B}^2$. Each point is the average of between 3 and 10 analyzer readings for each value of $\bar{B}^2$. The concentration dependence of C is shown in Fig. 8, where the intercept on the C axis (pure water) was taken from the Landolt-Bornstein

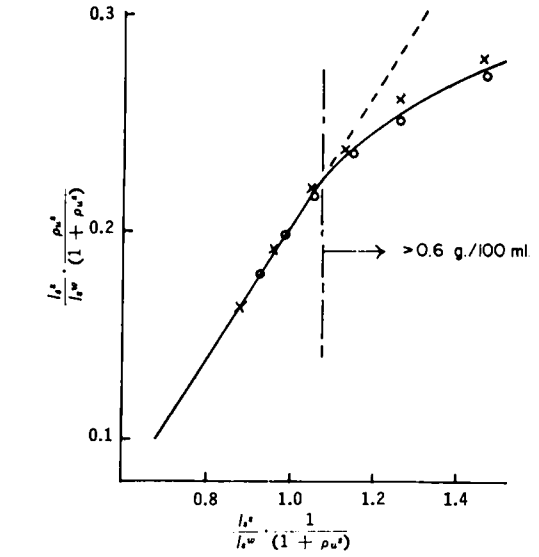

Figure 6—*Interpolated data from Figs. 1–4. Key:* ○, *5461 Å; and* ×, *4358 Å.*

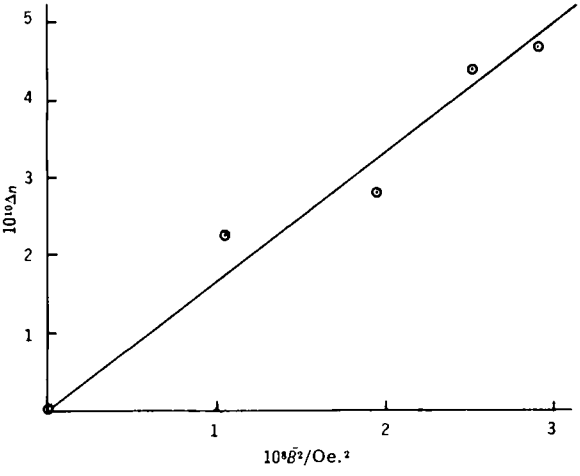

Figure 7—*Birefringence* Δn *as a function of mean square field* $\bar{B}^2$ *for 0.82 g./100 ml. sodium cromolyn solution. Filter pore size was 100 nm.; immediate use after filtration.*

[5] Newport type E.
[6] Wratten 77A.
[7] Polarex, type Ko-MIK, Kasemann Ltd.
[8] Polarex.
[9] Ilford Tricolour, type 404.
[10] Millipore, type VMWP and VCWP.

Table IV—Summary of Experimental Results

Technique	Parameter	Value of Parameter: Experiment	Planar (Calculated)	Perpendicular (Calculated)
Depolarized light scattering	$\gamma^2/Å^6$	1100	1060	365
Magnetic birefringence	$10^{12}(dC/dc)/$ Oe.$^{-2}$ cm.$^{-1}$ g.$^{-1}$ ml.	3.6	2.26 4.2 (relative method)	0.71 1.3 (relative method)

tables. Errors in C, judged from the scatter of experimental Δn values, are shown as vertical bars in Fig. 8. The error in concentration C is negligible. The line through the points in Fig. 8 is a line of best fit, assuming the value of C for water to have zero error. This was done since the value of C for water is of higher precision than the values of C for the measured solutions.

Values of incremental refractive index dn/dc and density $d\rho/dc$ were measured using a high precision Abbé refractometer and weighing bottle, respectively. The values at 23°, 5461 Å, were found to be $dn/dc = 0.233 \pm 0.005$ ml. g.$^{-1}$ and $d\rho/dc = 0.49 \pm 0.02$.

A relative method, not requiring absolute values of α and χ, is to measure the Cotton-Mouton constant for a compound similar to sodium cromolyn but having a known conformation. Such a compound is 9C in which the —O—C_3—O— bridge connecting the benzene rings in sodium cromolyn is replaced by a —O—C_9—O— bridge. Due to the increased bridge length (therefore more possibilities for internal rotation about C—C bonds), the bridge is thought to be flexible. Additional evidence for flexibility comes from Raman spectra of linear carbon chains in *n*-alkanes. The spectra show (16) that there are no rigid all-*trans*-isomers present when the chain exceeds nine carbon atoms in length. Thus, the orientation of one end of the 9C molecule by the applied magnetic field may take place without affecting the other end. When neglecting the flexible bridge, which has a low optical and diamagnetic anisotropy, this lack of correlation between the molecule ends means that each 9C molecule contributes to the Cotton-Mouton constant as though it were two free halves of a sodium cromolyn molecule.

Theory and Results—Bottcher (14) gave an expression for the refractive index of a mixture in terms of the number densities N and effective polarizabilities α^* of each component:

$$\frac{(n^2 - 1)(2n^2 + 1)}{12\pi n^2} = N_1\alpha_1^* + N_2\alpha_2^* \qquad \text{(Eq. 16)}$$

where $\alpha_i^* = \bar{\alpha}_i/(1 - f_i\bar{\alpha}_i)$, and $f_i = (1/a_i^3)[(2n^2 - 2)/(2n^2 + 1)]$; a_i is the effective radius of the particles of the ith sort and $\bar{\alpha}_i$ is their mean polarizability. According to the Langevin theory (15) of the Cotton-Mouton effect of diamagnetic molecules, an applied field B in the x direction produces an average polarizability difference:

$$\bar{\alpha}_x - \bar{\alpha}_y = \frac{3}{2}\theta_1 B^2 \qquad \text{(Eq. 17)}$$

where $\theta_1 = (1/45kT)[(\alpha_1 - \alpha_2)/(\chi_1 - \chi_2) + (\alpha_2 - \alpha_3)(\chi_2 - \chi_3) + (\alpha_3 - \alpha_1)(\chi_3 - \chi_1)]$. Differentiating Bottcher's equation for small changes in α^* of both components and in the limit of low concentrations of Component 1 gives:

$$\Delta n = \frac{(18n^2\pi N_1)(\Delta\bar{\alpha}_1)}{(2n^2 + 1)^2(1 - f_1\bar{\alpha}_1)^2} + \Delta n_2 \qquad \text{(Eq. 18)}$$

where $\Delta\alpha_1 = 3/2\theta_1 B^2$ using Langevin's theory. Bottcher's equation may also be used to calculate dn/dc, if low concentrations are again assumed:

$$\frac{dn}{dc} = \frac{18\pi n^3 N_0}{(2n^2 + 1)^2}\left[\frac{\alpha_1^*}{M_1} - \frac{\alpha_2^*}{M_2}\left(1 - \frac{d\rho}{dc}\right)\right] \qquad \text{(Eq. 19)}$$

where N_0 is Avogadro's number. By assuming, as $c \to 0$, that $\alpha_2^* = \alpha_{water}^*$, then:

$$\alpha_1^* = \frac{M_1(2n^2 + 1)}{36\pi n^3 N_0}\left[2(2n^2 + 1)\frac{dn}{dc} + 3n(n^2 - 1)\left(1 - \frac{d\rho}{dc}\right)\right] \qquad \text{(Eq. 20)}$$

Thus $(1 - f_1\bar{\alpha}_1) = \bar{\alpha}_1/\alpha_1^* = 0.815$, using the bond polarizability value of 44 Å^3 for $\bar{\alpha}_1$, and measured values of dn/dc and $d\rho/dc$.

Putting $M_1 = 516$ for sodium cromolyn and $n = 1.335$ gives absolute dC/dc values as follows:

$$\left(\frac{dC}{dc}\right)_{abs} = 2.26 \times 10^{-12} \text{ Oe.}^{-2} \text{ cm.}^{-1} \text{ g.}^{-1} \text{ ml.}$$

(planar conformation) (Eq. 21)

$$\left(\frac{dC}{dc}\right)_{abs} = 0.71 \times 10^{-12} \text{ Oe.}^{-2} \text{ cm.}^{-1} \text{ g.}^{-1} \text{ ml.}$$

(perpendicular conformation) (Eq. 22)

Neglecting the optical and diamagnetic anisotropy of the 9C flexible bridge and assuming complete flexibility give θ_1 (planar sodium cromolyn) $= 2\theta_1$(9C). Bond polarizability calculations predict a ratio of 3.3 between the values of $\theta_{1\,\text{sodium cromolyn}}$ in the planar and perpendicular conformation; thus, it may be assumed that:

$$\theta_1(\text{perpendicular sodium cromolyn}) = \frac{2}{3.3}\theta_1(9C) = 0.61\theta_1(9C) \qquad \text{(Eq. 23)}$$

Only a small quantity of 9C was available, and two experiments were made on one solution of concentration 0.625%. These gave 0.66×10^{-14} and 0.85×10^{-14} Oe.$^{-2}$ cm.$^{-1}$ for the Cotton-Mouton constant (Fig. 9). Adopting $(0.75 \pm 0.1) \times 10^{-14}$Oe.$^{-2}$ cm.$^{-1}$ as a mean value and using the Landolt-Bornstein value of C for water allow an estimate of dC/dc for the two sodium cromolyn conformations after allowing for the molecular weight difference between sodium cromolyn (516) and 9C (600). This gives, for sodium cromolyn:

$$\left(\frac{dC}{dc}\right)_{rel} = 4.2 \times 10^{-12} \text{ Oe.}^{-2} \text{ cm.}^{-1} \text{ g.}^{-1} \text{ ml.} \quad \text{(planar)} \qquad \text{(Eq. 24)}$$

$$\left(\frac{dC}{dc}\right)_{rel} = 1.3 \times 10^{-12} \text{ Oe.}^{-2} \text{ cm.}^{-1} \text{ g.}^{-1} \text{ ml.} \quad \text{(perpendicular)}$$

(Eq. 25)

Comparing the dC/dc values, both absolute and relative, with the

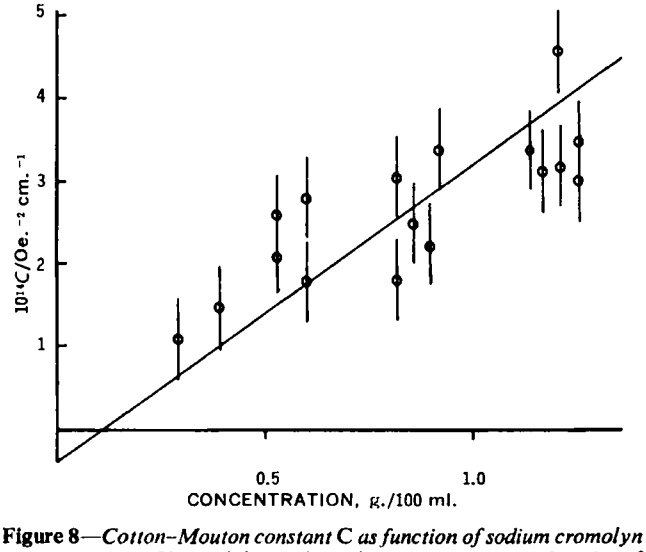

Figure 8—*Cotton-Mouton constant C as function of sodium cromolyn concentration. Vertical lines through points represent domain of measurement error.*

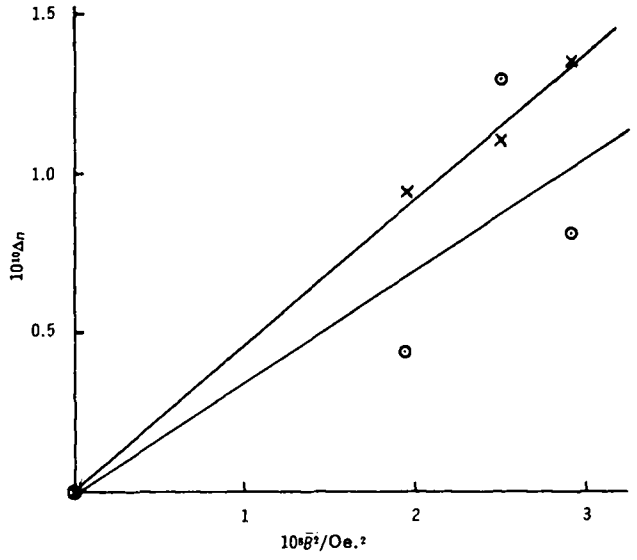

Figure 9—*Birefringence* Δn *of 0.625 g./100 ml. 9C solution. Key:* O, *lower line, first experiment; and* ×, *upper line, second experiment. Both lines are least-square fits to the experimental points.*

experimental value of 3.6×10^{-12} Oe.$^{-2}$ cm.$^{-1}$ g.$^{-1}$ ml. from Fig. 8 shows that the planar conformation is favored.

CONCLUSION

The summary of experimental and calculated data relating to the conformation is shown in Table IV. Both techniques show conclusively that the planar rather than the perpendicular or random conformation exists in very dilute solution within the estimates of error discussed in the text. The larger errors apparent in the magnetic birefringence comparisons are thought to arise from uncertainties in some of the bond magnetic anisotropy values.

Due to flexibility in the —O—C_3—O— bridge between the two identical halves of the sodium cromolyn molecule, a planar conformation of the isolated molecule is unexpected. The molecule's affinity for water (1) provides an explanation in terms of water molecules bridging the chromone groups by hydrogen bonding and thus forming a rigid structure. Experiments using scale space-filling molecular models show that several water molecules can be accommodated to form this bridge. Due to their low optical and diamagnetic anisotropy, the water molecules would not greatly affect the values of γ^2 and dC/dc and thus not show up in the experimental measurements.

Both techniques are sensitive indicators of molecular association or liquid crystal formation, and the time-dependent and nonlinear behavior found at about > 1g./100 ml. shows that these phenomena are occurring at much lower concentrations than previously thought (1).

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Embryonic-Fetal Toxicity and Teratogenic Effects of Adipic Acid Esters in Rats

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Abstract □ Seven adipates (dimethyl, diethyl, dipropyl, diisobutyl, di-*n*-butyl, di-2-ethylhexyl, and dicyclohexyl) were evaluated for their embryonic-fetal toxicity and teratogenic effects in rats, and the results were compared to those of a previous study with phthalate esters. The data suggest that the adipates are less teratogenic than the comparable phthalates when administered during gestation in equivalent fractions of the respective LD_{50} doses.

Keyphrases □ Adipic acid esters—embryonic fetal toxicity and teratogenic effects in rats □ Embryonic fetal toxicity and teratogenic effects—adipic acid esters, rats □ Teratogenicity and embryonic fetal toxicity—adipic acid esters, rats □ Plasticizers, adipic acid esters—embryonic-fetal toxicity and teratogenic effects, rats

Adipates, esters of adipic acid and various alcohols, are employed as plasticizers for polyvinyl chloride, particularly for low temperature applications. Although no teratological information is available on these materials, there are some reports concerning their acute and subacute toxicities. Data on the acute oral toxicity of some of these adipates have been reported (1–3). A subacute study was conducted on di-2-ethylhexyl adipate, in which rats were fed a diet containing 0.5, 2.0, and 5.0% of the adipate, representing approximately 0.25, 1.0, and 2.5 g./kg./day, respectively. The only abnormality observed was a retardation of growth at the 5.0% level (1). Furthermore, dogs were given 2 g./kg./day of the compound, and only a transient loss of appetite was noted (1). In another study (2), di-2-ethylhexyl adipate was fed to rats; no adverse effect was noted at dietary levels equivalent to 0.16 g./kg./day, but an increased mortality was seen in those receiving 4.74 g./kg./day.

The metabolism and toxicity of adipic acid have also been studied, showing that this compound is oxidized *in vitro* by isolated rat liver mitochondria. However, its *in vivo* metabolism is incomplete, as evidenced by the presence of varying amounts of unchanged acid in the urine (4). When using ^{14}C-labeled adipic acid, it was found that 70% of the radioactivity in a 50-mg. dose administered to rats appeared in the expired carbon dioxide within 24 hr.; such carbon dioxide was produced more rapidly from acid labeled in the first rather than in the second carbon position (5). Low residual activity was confined mainly to the kidneys and liver, and ^{14}C-labeled urea, as well as glutamic, lactic, adipic, β-ketoadipic, and citric acids, was identified in the urine. Further studies showed that the urea and citric acid were not direct metabolites of adipic acid but were formed from the radioactive carbon dioxide. Demonstration that the ^{14}C-label of adipic acid can be incorporated into glycogen, with acetic and succinic acids as intermediates, and the identification of β-ketoadipic acid in the urine indicate that the metabolism of adipic acid is by β-oxidation (5).

Table I—Acute Toxicity of Adipic Acid Esters in Rats

Compound	Acute LD_{50}, ml./kg.	95% Confidence Limits, ml./kg.	Slope (b)[a]
Dimethyl adipate	1.8086	1.5735–2.0789	16.24
Diethyl adipate	2.5119	2.1859–2.8871	16.24
Dipropyl adipate	3.7858	3.3279–4.2087	19.55
Diisobutyl adipate	5.9500	4.6903–7.5482	4.69
Di-*n*-butyl adipate	5.2441	4.9023–5.6096	39.41
Di-2-ethylhexyl adipate	>50	-	—
Dicyclohexyl adipate	5.1007[b]	4.7038–5.5313	16.24

[a] Probit slope (b) = probits/log of dose. [b] Compound warmed to 37–38° to liquefy it for injection.

The acute LD_{50} of adipic acid in mice was found to be 1900 mg./kg. p.o. and 680 mg./kg. i.v. In rats, the intraperitoneal LD_{50} was 275 mg./kg. (6). These workers also fed adipic acid at dietary levels of 0.1–5.0% (representing an intake of 50–2500 mg./kg./day) to rats for 2 years, and the only abnormality they detected was reduced growth at levels of 3.0% (1500 mg./kg./day) or more. In another study, increased mortality, diarrhea, and reduced growth were noted in rats given a dietary level equivalent to 16 or 11.4 g./kg./day for 5 or 33 weeks, respectively (7). These effects were observed during the first 2 weeks, after which surviving animals recovered but histological changes in the liver, kidneys, and intestine were reported. However, none of these effects was evident at a dose level of 5.7 g./kg./day (7)[1].

Gaunt *et al.* (9) reported acute toxicity studies of dialkyl 79 adipate (adipate esters in which alcoholic portions contained seven–nine carbon atoms) in mice and rats and short-term feeding studies in rats. They found it to be most toxic to mice by the oral route (LD_{50} = 8–12 g./kg.), while rats tolerated 20 g./kg. without any mortalities. There were no deaths in either mice or rats at intraperitoneal doses up to 20 g./kg. These doses of dialkyl 79 adipate produced piloerection and diarrhea in both species along with reduced spontaneous activity; the diarrhea was more pronounced in the animals receiving the compound orally. In 98-day feeding studies with rats, they found the "no-effect" level to be 0.25% in the diet, representing approximately 125 mg. adipate/kg. rat/day. Some alterations in values obtained for nonlethal parameters (*e.g.*, weight gain, hemoglobin concentration, and relative kidney weight) were noted when the diet contained 0.5 or 1.0% dialkyl 79 adipate; however, no deaths were attributed to the adipate.

As the adipates comprise an important group of

[1] The Joint FAO/WHO Expert Committee on Food Additives allocated a conditional acceptable daily intake for man of 0–5.0 mg./kg. for adipic acid (8).

Table II—Embryonic-Fetal Toxicity of Adipate Esters on Rat Fetuses[a]

Treatment Groups	Volume Injected, ml./kg.	Number of Corpora Lutea	Number of Resorptions	Dead Fetuses	Live Fetuses	Mean Weight of Fetuses, g.[b]
Blunt needle (injection)	—	69	4 (6.0)	0	63 (94.0)	3.91 ± 0.02
Distilled water	10.00	59	4 (6.8)	0	55 (93.2)	4.40 ± 0.33
Normal saline	10.00	62	7 (11.5)	0	54 (88.5)	4.10 ± 0.13
Cottonseed oil	10.00	71	5 (7.5)	0	62 (92.5)	3.89 ± 0.09
Dimethyl adipate	0.0603	60	4 (6.8)	0	55 (93.2)	3.94 ± 0.05
	0.1809	71	9 (14.1)	0	55 (85.9)	3.79 ± 0.08
	0.3617	60	1 (1.8)	0	56 (98.2)	3.90 ± 0.08
	0.6028	62	3 (5.7)	0	50 (94.3)	3.85 ± 0.08
Diethyl adipate	0.0837	55	1 (1.9)	0	51 (98.1)	4.12 ± 0.12
	0.2512	58	4 (7.0)	0	53 (93.0)	4.01 ± 0.08
	0.5024	59	5 (9.3)	0	49 (90.7)	3.93 ± 0.04
	0.8373	58	6 (10.7)	2 (3.6)	48 (85.7)	3.95 ± 0.07
Dipropyl adipate	0.1262	66	2 (3.2)	0	61 (96.8)	3.90 ± 0.09
	0.3786	61	6 (10.9)	0	49 (89.1)	3.77 ± 0.17
	0.7572	64	6 (9.8)	0	55 (90.2)	3.63 ± 0.11*
	1.2619	56	9 (20.0)	0	36 (80.0)	3.61 ± 0.22
Diisobutyl adipate	0.1983	62	2 (3.6)	0	54 (96.4)	3.91 ± 0.07
	0.5950	64	1 (1.6)	0	62 (98.4)	3.93 ± 0.05
	1.1900	65	3 (4.8)	1 (1.6)	59 (93.6)	3.69 ± 0.03**
	1.9833	63	2 (3.2)	1 (1.6)	60 (95.2)	3.65 ± 0.08*
Di-*n*-butyl adipate	0.1748	52	2 (3.8)	0	50 (96.2)	3.91 ± 0.09
	0.5244	61	3 (4.9)	0	58 (95.1)	3.88 ± 0.05
	1.0488	72	2 (2.9)	1 (1.4)	66 (95.7)	3.71 ± 0.07*
	1.7480	65	6 (9.4)	2 (3.1)	56 (87.5)	3.72 ± 0.20
Di-2-ethylhexyl adipate	1.00	62	3 (5.3)	0	54 (94.7)	3.90 ± 0.09
	5.00	65	2 (3.1)	0	63 (96.9)	3.83 ± 0.03*
	10.00	60	4 (7.0)	0	53 (93.0)	3.49 ± 0.14*
Dicyclohexyl adipate	0.1700	68	9 (14.5)	2 (3.2)	51 (82.3)	3.70 ± 0.22
	0.5100	59	10 (19.6)	1 (2.0)	40 (78.4)	3.50 ± 0.22
	1.0201	63	12 (19.4)	2 (3.2)	48 (77.4)	3.50 ± 0.34
	1.7002	66	13 (20.0)	5 (7.7)	47 (72.3)	3.06 ± 0.23**

[a] Five pregnant female rats were injected in each group on Days 5, 10, and 15 of pregnancy. [b] Numbers represent the average values (grams) ± the standard error of the mean for each group. *$p \leq 0.05$, **$p \leq 0.01$, significantly different from blunt needle-injected controls.

plasticizers, it was decided to initiate embryonic-fetal toxicity and teratogenic tests in rats with seven adipic acid esters. A parallel study (10) on some phthalic acid esters provided data with which to make a comparison between certain members of the two series.

EXPERIMENTAL

Materials—The adipates included in this study were: dimethyl[2], diisobutyl[2], diethyl[3], dipropyl[3], di-2-ethylhexyl[3], di-*n*-butyl[3], and dicyclohexyl[3]. Test animals were adult, virgin female, Sprague-Dawley rats[4], weighing 175–225 g. Adult, male rats of this strain were utilized as the "stud pool."

Procedure—Acute toxicity of the adipate esters was determined by administering graded doses of these specific samples by intraperitoneal injection to rats and observing the animals for mortality for 7 days. Acute LD_{50} values, 95% confidence limits, and slopes were calculated by Cornfield and Mantel's modification of Karber's method (11).

Female rats were selected for experimentation only after observation of at least two complete 4- or 5-day estrus cycles. Occurrence of estrus was determined by daily vaginal smears, obtained by introducing 0.2 ml. of fresh, clean tap water into the vagina with a smooth, clean, sterile medicine dropper, withdrawing a part of the liquid, and transferring it to a clean slide. The slide was examined microscopically while fresh, and the stage of estrus (proestrus, estrus, or diestrus) was determined according to cell types found in the vaginal smear.

Five female rats were housed with one male in a large cage at room temperature (22–27°) with food[5] and fresh tap water provided *ad libitum*. The onset of gestation was established by the presence of sperm in the vaginal smear and was designated as Day 0, with the following day being Day 1 of the gestation period. At this time, the female rats were moved to individual cages, where they were kept undisturbed except for specified injections. There were 31 groups, each composed of five female rats.

All treatments were administered by intraperitoneal injection on the 5th, 10th, and 15th days of gestation. The six more toxic adipates were injected intraperitoneally at four dosage levels: one-thirtieth, one-tenth, one-fifth, and one-third of their acute LD_{50} values in each injection. Di-2-ethylhexyl adipate was administered at three dosage levels of 1, 5, and 10 ml./kg. Distilled water, normal saline, and cottonseed oil were used at a dose of 10 ml./kg., corresponding to the largest volume of adipate injected (di-2-ethylhexyl adipate), although no diluent was used with the adipates. A "blunt-needle-injected" group[6] was also included for comparative purposes.

On the 20th day of gestation, 1 day prior to expected parturition, the rats were sacrificed by ether inhalation. The uterine horns and ovaries were surgically exposed to permit counting and recording of the numbers of corpora lutea, resorption sites, and viable and dead fetuses. Fetuses were removed, blotted dry, and weighed[7] to the nearest tenth of a milligram. All fetuses, both viable and nonviable, were examined for gross abnormalities. A randomly selected number (usually 50% of the total, excluding whenever possible those showing gross evidence of deformities) were prepared as transparent specimens to permit skeletal visualization for evaluating malformations. In a few instances, in which the incidence of resorption was very high, all fetuses were included. Skeletal visualization was accomplished by the procedure of Staples and Schnell (12). The remaining fetuses were examined for visceral abnormalities.

[2] Organic Chemical Division, Monsanto, St. Louis, Mo.
[3] Eastman Chemical Products, Kingsport, Tenn.
[4] Sprague-Dawley, Inc., Madison, Wis.
[5] Purina laboratory chow.
[6] In this group of controls, a dull needle was inserted in the same manner as used for intraperitoneal injections, but no substance was actually injected.
[7] Using a Mettler H6T balance.

Table III—Gross, Skeletal, and Visceral Malformations Produced by Adipic Acid Esters

Treatment Groups	Volume Injected, ml./kg.	Resorptions[a] (%)	Abnormalities— Gross[b]	Skeletal[c]	Visceral[d]
Blunt needle (injection)	—	4 (6.0)	0	1 (3.0%)	0
Distilled water	10.00	4 (6.8)	0	0	—
Normal saline	10.00	7 (11.5)	1 (1.9%)	4 (14.3%)	—
Cottonseed oil	10.00	5 (7.5)	1 (1.6%)	2 (6.3%)	0
Dimethyl adipate	0.0603	4 (6.8)	0	0	0
	0.1809	9 (14.1)[e]	1 (1.8%)	2 (7.4%)	0
	0.3617	1 (1.8)	2 (3.6%)[e]	4 (13.8%)[e]	0
	0.6028	3 (5.7)	4 (8.0%)[e]	5 (19.2%)[e]	2 (8.3%)
Diethyl adipate	0.0837	1 (1.9)	0	0	0
	0.2512	4 (7.0)	1 (1.9%)	0	1 (4.0%)
	0.5024	5 (9.3)	1 (2.0%)	1 (4.0%)	0
	0.8373	6 (10.7)	2 (4.2%)[e]	2 (8.0%)	0
Dipropyl adipate	0.1262	2 (3.2)	0	0	0
	0.3786	6 (10.9)	1 (2.0%)	0	0
	0.7572	6 (9.8)	2 (3.6%)	0	0
	1.2619	9 (20.0)[e]	2 (5.6%)[e]	1 (5.3%)	0
Diisobutyl adipate	0.1983	2 (3.6)	0	0	0
	0.5950	1 (1.6)	2 (3.2%)[e]	2 (6.3%)	0
	1.1900	3 (4.8)	1 (1.7%)	3 (10.0%)	0
	1.9833	2 (3.2)	5 (8.3%)[e]	3 (9.7%)	1 (3.4%)
Di-*n*-butyl adipate	0.1748	2 (3.8)	0	0	0
	0.5244	3 (4.9)	1 (1.7%)	0	1 (3.7%)
	1.0488	2 (2.9)	2 (3.0%)	0	0
	1.7480	6 (9.4)	3 (5.4%)[e]	2 (6.7%)	1 (3.8%)
Di-2-ethylhexyl adipate	1.00	3 (5.3)	0	1 (3.6%)	0
	5.00	2 (3.1)	1 (1.6%)	3 (9.4%)	1 (3.2%)
	10.00	4 (7.0)	2 (3.8%)[e]	2 (7.1%)	1 (4.0%)
Dicyclohexyl adipate	0.1700	9 (14.5)[e]	0	0	0
	0.5100	10 (19.6)[e]	4 (10.0%)[e]	0	0
	1.0201	12 (19.4)[e]	3 (6.3%)[e]	1 (3.8%)	0
	1.7002	13 (20.0)[e]	4 (8.5%)[e]	1 (4.0%)	0

[a] Percent resorptions are based on total number of resorptions and dead and live fetuses. [b] Percent gross abnormalities are based on total number of fetuses. [c] Percent skeletal abnormalities are based on total number of stained fetuses (50% of total fetuses). [d] Percent visceral abnormalities are based on total number of unstained fetuses. [e] Values greater than the 95% confidence interval of the "pooled volume control."

The following parameters of adverse effects were investigated: (*a*) embryonic–fetal toxicity, as evidenced by resorptions and stillbirths, (*b*) gross (external) malformations of fetuses, (*c*) skeletal malformations, (*d*) visceral abnormalities, and (*e*) fetal size (weight). In all evaluations, both viable and nonviable fetuses were considered.

RESULTS

The observed number of corpora lutea ranged from 52 to 72 per group, with a mean of 62.5. Of the 1938 corpora lutea observed in this study, 1840 or 94.9% were accounted for as live fetuses, dead fetuses, or resorption sites.

The acute intraperitoneal LD_{50} values and the 95% confidence limits for the adipate esters are presented in Table I. As shown, LD_{50} values were obtained for six of the adipates used in this study, but the sample of di-2-ethylhexyl adipate used failed to produce 50% mortality at doses considerably in excess of 50 ml./kg. Dimethyl adipate is the most acutely toxic, while the other adipate esters showed a decreased toxicity, generally as the size of the alcoholic portion increased.

Compound-dependent and dose-related effects upon embryonic and fetal growth and development were generally observed with these adipates. The results of developmental changes are presented in Tables II and III for the distilled water, normal saline, cottonseed oil, blunt-needle-injected, and all experimental groups. Table II presents data concerning the number of corpora lutea, resorption sites, dead fetuses, live fetuses, and fetal weights according to treatment groups. Table III shows a comparison of the number and percent of resorptions and gross, skeletal, and visceral abnormalities.

Resorptions—All control and treatment groups exhibited one or more resorptions. These ranged from 1.6% for one-tenth of the acute LD_{50} of diisobutyl adipate to 20.0% for one-third of the LD_{50} dose level of dicyclohexyl and dipropyl adipates. The highest incidences were seen with the four dose levels of dicyclohexyl adipate (20.0, 19.4, 19.6, and 14.5%), and the highest dose of dipropyl adipate (20.0%). The lowest incidences of resorptions occurred in the groups treated with one-tenth of the LD_{50} of diisobutyl adipate (1.6%), one-fifth of the LD_{50} of dimethyl adipate (1.8%), and one-thirtieth of the LD_{50} of diethyl adipate (1.9%). Only one resorption site was observed in each of these last three experimental groups.

Fetal Deaths—Fetal deaths were observed with much less frequency and in smaller numbers than the other criteria considered. Although most experimental groups revealed some resorption sites, dead fetuses were found only in groups treated with four of the compounds: diisobutyl, diethyl, di-*n*-butyl, and dicyclohexyl adipates. The two high doses of diisobutyl adipate produced one dead fetus (1.6%) for each dose level; two dead fetuses (3.6%) were found in the group receiving the high dose of diethyl adipate; and two (3.1%) dead fetuses and one (1.4%) dead fetus were found in groups treated with one-third and one-fifth of the acute LD_{50} dose levels of di-*n*-butyl adipate, respectively. Dicyclohexyl adipate produced fetal toxicity at all four dose levels employed. It revealed the largest number of fetal deaths in this study. Two (3.2%), one (2.0%), two (3.2%), and five (7.7%) fetal deaths were noted with one-thirtieth, one-tenth, one-fifth, and one-third of the acute LD_{50} dose, respectively.

Teratogenicity—*Gross Abnormalities*—A significant number of malformations were observed throughout the treatment groups, and these tended to show a dose-related incidence when considered as percentage of malformations. No gross abnormalities were seen in the blunt-needle (without injection) or distilled water control groups; neither were there any with one-thirtieth of the acute LD_{50} dose of any adipates employed nor with 1 ml./kg. of the di-2-ethylhexyl adipate. One malformed fetus was seen in each group treated with normal saline, cottonseed oil, dimethyl adipate (0.1809 ml./kg.), diethyl adipate (0.5024 and 0.2512 ml./kg.), di-*n*-butyl adipate (0.5244 ml./kg.), diisobutyl adipate (1.1900 ml./kg.), dipropyl adipate (0.3786 ml./kg.), and di-2-ethylhexyl adipate (5 ml./kg.), representing from 1.6 to 2.0% of the fetuses within the various groups. The greatest number of gross malformations seen was five per group, which occurred with the high dose of diisobutyl adipate; this represented 8.3% of the total fetuses. A complete tabulation of the

number and percentage of fetuses in which gross abnormalities were noted is presented in Table III.

The most common gross abnormalities observed were hemangiomas on various parts of the fetus, twisted hindlegs, and compact head and neck (some were monsters). One-fifth the acute LD_{50} of dimethyl and dipropyl adipates produced one fetus each without a tail. Most adipates studied produced a number of poorly developed and very small fetuses.

Skeletal Abnormalities—The number of skeletal abnormalities observed for each group and its percentage of visualized fetuses are presented in Table III. The abnormalities most often seen were elongated anterior ribs, often fused to the sternebrae, as well as elongated and fused posterior ribs (bilateral or unilateral); less frequently, one or two ribs were missing.

The incidences of skeletal abnormalities ranged from 0% for the distilled water, the lowest dose of dimethyl and diisobutyl adipates, the two lower doses of diethyl and dicyclohexyl adipates, and the three lower doses of di-*n*-butyl and dipropyl adipates up to a high of 19.2% for the high dose of dimethyl adipate.

Visceral Abnormalities—Visceral abnormalities were observed with much less frequency and in smaller numbers than were the gross and skeletal abnormalities. No visceral abnormalities were seen in the blunt-needle control group, cottonseed oil group, or any groups treated with dipropyl and dicyclohexyl adipates. The highest incidence of visceral abnormalities seen was two per group, which occurred with the high dose of dimethyl adipate. The most common visceral abnormalities observed were umbilical hernia, angulated anal opening, and absence of one kidney.

Fetal Size—Fetuses from most of the treated groups of rats were smaller, based upon weight, than those from the blunt needle-injected control group. However, these differences were not statistically significant except with the two higher dose levels of diisobutyl, the two higher dose levels of di-2-ethylhexyl, the one-fifth LD_{50} dose levels of dipropyl and di-*n*-butyl, and the highest dose level of dicyclohexyl adipates. The control fetuses averaged 3.91 g. each, while those from the adipate ester-treated rats had a mean fetal weight from 3.06 to 4.12 g. (Table II).

DISCUSSION

Administration of these adipate esters to pregnant rats on the 5th, 10th, and 15th days of gestation generally produced a compound-dependent and dose-related increase in resorptions and gross and skeletal abnormalities and a reduction in fetal weight at birth. The types of abnormalities found with these compounds were quite similar to those reported by Singh *et al.* (10) for a group of phthalate esters; however, the incidences of abnormalities were less in the adipate-treated animals. Although visceral abnormalities were observed less frequently than other types, when they occurred they were generally in the groups receiving the highest dose or doses. The two exceptions seen were with 0.2512 ml./kg. of diethyl adipate and 0.5244 ml./kg. of di-*n*-butyl adipate. In the former group, one fetus had an umbilical hernia and, in the latter group, one fetus exhibited an incomplete vascular development, particularly in the area of the shoulders.

As shown in Tables II and III, the blunt-needle-injected rats produced no dead fetuses or gross or visceral abnormalities, and there were only four (6%) resorption sites, one (3%) skeletal abnormality, and 63 (94%) live fetuses from 69 corpora lutea. Thus, if these findings are used as the baseline, most treatments produced an effect in one or more of the parameters studied except the lowest dose level, one-thirtieth of the acute LD_{50} of each adipate ester.

A more conservative evaluation could be made between the effects produced by the adipic acid esters when compared to the results of a "volume control" to compensate for any effects resulting from the physical or mechanical trauma of injection and handling. Since no diluent was used when these compounds were injected and the injected volume ranged from 0.0603 to 10.00 ml./kg., it was decided to employ distilled water, normal saline, and cottonseed oil in a quantity equivalent to the largest dose of the test compound (10.00 ml./kg.). Since the results obtained from these three "controls" were not uniform and there was no justification for selecting one of these in preference to the others, the data were combined to calculate a 95% confidence interval (13) for the "pooled volume control" in regard to resorptions and gross and skeletal abnormalities. The confidence intervals thus obtained were as follows: resorptions, 3.7–12.3%; gross abnormalities, 0.7–3.1%; and skeletal malformations, 1.0–12.6%. A significant increase in resorptions was observed for the 0.1809-ml./kg. level of dimethyl adipate, for 1.2619 ml./kg. of dipropyl adipate, and for all four dose levels of dicyclohexyl adipate. The incidence of gross abnormalities was increased significantly in groups receiving the following treatments: 0.3617 and 0.6028 ml./kg. of dimethyl adipate, 0.8373 ml./kg. of diethyl adipate, 0.7572 and 1.2619 ml./kg. of dipropyl adipate, 0.5950 and 1.9833 ml./kg. of diisobutyl adipate, 1.7480 ml./kg. of di-*n*-butyl adipate, the high dose (10.00 ml./kg.) of di-2-ethylhexyl adipate, and the three highest doses of dicyclohexyl adipate. Concerning skeletal malformations, only the two highest dose levels of dimethyl adipate showed an incidence greater than the 95% confidence interval (Table III).

Dicyclohexyl adipate was interesting due to the marked effect this compound had upon the developing embryo and fetus. Quite a number of fertilized ova were detected only as resorption sites in all four dose levels, indicative of an early embryotoxic effect; dead fetuses were found with all four dose levels employed, indicating a later toxic action as well. In spite of its embryonic-fetal toxicity, a number of gross abnormalities were noted in the three higher dose groups. Most abnormalities produced by this compound were hemangiomas of different parts of the body, and twisted hindlegs were often observed. After visualizing and staining the skeleton, one of the 25 visualized fetuses of the high dose level had two ribs less than normal; one of the 26 in the next highest dose group had elongated and fused posterior ribs, while those in the two lower dose groups did not show any skeletal malformation. No visceral abnormalities were seen in the groups receiving this adipate.

Another compound, dimethyl adipate, produced considerable embryotoxic and teratogenic effects. In the high dose group, one fetus had hemangiomas of the neck, one on the shoulder, one of the foreleg, and one on the right thigh. Skeletal examinations of this group revealed five of 26 fetuses had elongated frontal ribs fused to the sternebrae. This group also contained two visceral abnormalities: one fetus did not have the left kidney and one fetus had an angulated anal opening. In the second highest dose group, one fetus did not have a tail; two of the 29 stained fetuses had a few elongated and fused posterior ribs, and two fetuses had elongated anterior ribs fused to the sternebrae. The 0.1809-ml./kg. dose level of this compound produced only one fetus with hemangiomas of the right hindquarter, while two of the 27 stained fetuses had elongated anterior ribs fused to the sternebrae. The lowest dose level did not produce any gross, skeletal, or visceral abnormalities.

Diisobutyl adipate produced a number of gross and skeletal abnormalities in offspring of treated rats; however, only one fetus demonstrated a visceral abnormality (perforated anal opening) in the high dose group. Also within this group, one fetus had a hemangioma of the chin and another had one on the back; three had twisted hindlegs. Skeletal examinations of this group revealed three of 31 fetuses had elongated and fused posterior ribs. In the second highest dose group, one fetus had a hemangioma of the chin; two of 30 stained fetuses had elongated and fused posterior ribs, and one fetus had elongated anterior ribs fused to the sternebrae. In the group receiving 0.5950 ml./kg., one fetus had a hemangioma of the shoulder, and another fetus had a hemangioma of the neck. One of 32 stained fetuses had elongated and fused posterior ribs, and one had elongated anterior ribs fused to the sternebrae. The lowest dose group of this compound did not show any gross, skeletal, or visceral abnormalities.

Diethyl, dipropyl, di-*n*-butyl, and di-2-ethylhexyl adipates produced few gross, skeletal, or visceral abnormalities, and these occurred mainly at the higher dose levels. In very few of these groups were the incidences greater than the pooled volume control. Two dead fetuses were found in the high dose group of diethyl adipate, and two dead fetuses were observed in the high dose group and one dead fetus in the second highest dose group of di-*n*-butyl adipate.

One-thirtieth of the acute LD_{50} dose of the six adipic acid esters used in this study for which there was an acute LD_{50} (dimethyl, diethyl, dipropyl, diisobutyl, di-*n*-butyl, and dicyclohexyl adipates) were completely tolerated by rat fetuses after intraperitoneal administration on the 5th, 10th, and 15th days of pregnancy, since this dose level of each compound did not produce any gross, skeletal, or visceral abnormalities. Therefore, these compounds may be considered not teratogenic under these conditions at this dose level. Therefore, from the data obtained, it would appear that the "no effect" level for these adipates under the conditions of this test would be equal to or greater than one-thirtieth but less than one-tenth

Table IV—Comparison of Embryonic–Fetal Toxicity and Teratogenicity of Adipates and Phthalates[a]

Fraction of Acute LD_{50} Dose of Ester Administered	Dead Fetuses, %		Resorptions, %		Abnormalities, %: Gross		Abnormalities, %: Skeletal	
	Adipate	Phthalate	Adipate	Phthalate	Adipate	Phthalate	Adipate	Phthalate
Dimethyl								
0.10	0	0	14.1	33.3	1.8	9.5	7.4	25.0
0.20	0	1.9	1.8	0	3.6	7.5	13.8	35.3
0.33	0	9.4	5.7	32.1	8.0	11.1	19.2	75.0
Diethyl								
0.10	0	0	7.0	3.6	1.9	0	0	26.3
0.20	0	0	9.3	0	2.0	0	4.0	47.1
0.33	3.6	0	10.7	44.4	4.2	0	8.0	81.3
Diisobutyl								
0.10	0	0	1.6	9.6	3.2	0	6.3	14.8
0.20	1.6	3.6	4.8	5.5	1.7	3.9	10.0	17.2
0.33	1.6	0	3.2	25.8	8.3	0	9.7	33.3

[a] Phthalate ester data taken from Singh *et al.* (10).

of their respective acute LD_{50} values. Di-2-ethylhexyl adipate, which failed to produce 50% mortality at doses in excess of 50 ml./kg. i.p., did not produce gross or visceral abnormalities at the 1-ml./kg. dose level; only one skeletal abnormality was noted in this group.

A previous study with phthalic acid esters (10) indicated a range of embryonic–fetal toxicity from 0 to 98.2% in rats treated with one-tenth, one-fifth, and one-third of the acute LD_{50} doses of the phthalates, whereas similar dose levels of adipates resulted in an embryonic fetal toxicity of 1.6–27.7%. It would appear, therefore, that the adipic acid esters possess a lower degree of toxicity to the developing embryo and fetus than was seen with the phthalic acid esters.

Fetal malformations varied from 0 to 100%, both for gross and skeletal abnormalities, with skeletal defects generally being more common with phthalate ester-treated rats; fetal malformations in adipate-treated rats ranged from 1.6 to 10% for gross abnormalities and from 0 to 19.2% for skeletal deformities. Thus, the comparison suggests the phthalates exert a more potent teratogenic effect than that noted for the adipates.

Three adipates included in this study were comparable analogs to phthalates reported earlier (10). When comparable doses, based upon the fraction of an acute LD_{50} dose of each compound, were administered to rats at the same time intervals, marked differences were noted in the abnormalities seen. Examination of Table IV reveals differences between the esters; in general, the phthalates tended to produce the greatest effect. This is especially noticeable for skeletal abnormalities.

The mean fetal weight on the 20th day of gestation ranged from 1.75 to 4.0 g. in rats treated with phthalic acid esters; in those treated with adipic acid esters, the mean fetal weight was 3.06–4.12 g. This indicates that adipic acid esters exert less growth-retarding effect than the phthalic acid esters. Overall, the data suggest that the embryonic–fetal toxicity and teratogenic effects of adipic acid esters were less than those produced by comparable doses of the phthalic acid esters.

SUMMARY AND CONCLUSIONS

All of the adipic acid esters studied exerted some degree of deleterious effect upon the developing embryo and fetus. At one or more of the dose levels employed, each compound produced some or all of the following effects: resorptions; gross, skeletal, and visceral abnormalities; fetal death; and decreased fetal size. Blunt-needle-injected animals were used as controls to determine the "normal" fetal size and a baseline of normal values; within this group there were no gross or visceral abnormalities or fetal deaths, and only 6% resorptions and one (3%) skeletal deformity were observed. However, seven experimental groups contained fetuses of significantly lighter weights than these controls. A secondary comparison was made using a 95% confidence interval obtained by pooling data from volume controls of distilled water, normal saline, and cottonseed oil. When a comparison was made using the 95% confidence limits of the pooled volume controls, 12 of the adipate-treated groups showed a significant incidence of gross abnormalities, six groups had a significant increase in resorptions, and two showed a definite increase in skeletal malformations.

Although this regimen of adipic acid ester administration did not elicit as great an effect as did the phthalates upon embryonic and fetal development, the incidences of adverse effects noted were generally dose related and compound dependent, perhaps with the more soluble compounds tending to be more active. The "no effect" level established in this study for adipic acid esters was approximately one-thirtieth of the acute LD_{50} dose.

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Constituents of *Cannabis sativa* L. IV: Stability of Cannabinoids in Stored Plant Material

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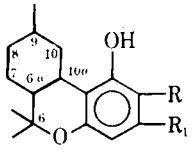
Abstract □ The (−)-Δ^9-*trans*-tetrahydrocannabinol content of *Cannabis sativa* L. stored at −18, 4, and 22 ± 1° decomposed at a rate of 3.83, 5.38, and 6.92%, respectively, per year, whereas the material stored at 37 and 50° showed considerable decomposition. *C. sativa* L. stored in the absence of direct light at −18, 4, and 22 ± 1° was more stable than cannabis stored under nitrogen. These data indicate that for normal research use, storage under nitrogen at 0° is not mandatory. Cannabinol is not the only decomposition product of (−)-Δ^9-*trans*-tetrahydrocannabinol. Tentative evidence supports the possible formation of hexahydrocannabinol as a decomposition product in stored *C. sativa* L.

Keyphrases □ *Cannabis sativa* L.—stability of cannabinoid constituents in stored plant material □ Cannabinoids—stability in stored *Cannabis sativa* plant material □ GLC—analysis, cannabinoid content in stored *Cannabis sativa* plant material

Eckler and Miller (1), using the dog ataxia assay, reported that certain preparations[1] of *Cannabis sativa* retained their biological activity after 5 years, whereas dry storage for the same period resulted in loss of biological activity. Moreover, it was reported (2) that a 43-year-old sample of cannabis fluidextract showed the presence of cannabidiol (II), (−)-Δ^9-*trans*-tetrahydrocannabinol (V), and cannabinol (VII) when analyzed by TLC and GLC. These two reports on fluidextracts are important but insufficient, since most cannabis currently used for research is stored dry under a myriad of conditions. Therefore, the limited data available on the effects of dry storage on a well-defined sample of *C. sativa* L. plant material prompted an investigation of II, V, and VII in stored plant material. GLC was employed as the principal analytical tool.

To duplicate as nearly as possible those conditions normally used by researchers for storage of cannabis and to determine the percent decomposition of V to VII or other products, the Mexican[2] variant normally supplied to researchers by the National Institute of Mental Health (NIMH) was selected. The study began in 1970 and terminated in 1972 after 104 weeks.



Number	Cannabinoid	Other	R	R_1
I	(−)-Δ^9-*trans*-tetrahydrocannabivarin	$\Delta^{9,10}$	H	C_3H_7
IV	(−)-Δ^8-*trans*-tetrahydrocannabinol	$\Delta^{8,9}$	H	C_5H_{11}
V	(−)-Δ^9-*trans*-tetrahydrocannabinol	$\Delta^{9,10}$	H	C_5H_{11}
VII	cannabinol	$\Delta^{8,9}$; $\Delta^{10,10a}$ $\Delta^{6a,7}$	H	C_5H_{11}
XI	(−)-Δ^9-*trans*-tetrahydrocannabinolic acid	$\Delta^{9,10}$	COOH	C_5H_{11}
XIII	hexahydrocannabinol		H	C_5H_{11}

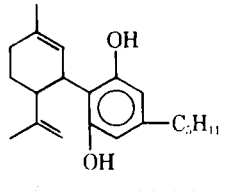
[1] Fluidextracts and granulated drug moistened with alcohol.

METHODS

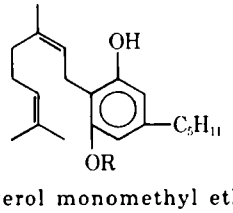
Mexican *C. sativa* L. grown from seed stock ME-A[3] was utilized. The plant material was grown in the Mississippi Delta in 1969 and was harvested at flowering[4]. Female plants were dried for 2 days at ambient temperature and humidity and subsequently manicured[5]; the plant material then was weighed and stored in 1-g. amber, large-mouth bottles. Each bottle was sealed with an aluminum cap containing an inserted paper lining, but the seal was not absolutely airtight. No sample was stored under nitrogen as proposed by Liskow (3). Five storage conditions were employed: (*a*) freezer at −18°; (*b*) refrigerator at 4°; (*c*) ambient temperature 22 ± 1°, with limited exposure to light; (*d*) oven at 37°; and (*e*) oven at 50°. Analyses were performed at 10-week intervals. Major cannabinoids were graphed as percent cannabinoids *versus* their neutral phenol and trimethylsilyl ethers or trimethylsilyl ester-ether derivatives.

GLC Analysis—The extraction procedure is basically that described by Lerner (4) and modified by Fetterman *et al.* (5) and Turner and Hadley (6). Two 1-g. samples were extracted simultaneously with 40 ml. of spectrograde chloroform. The resulting solutions were refrigerated at 4° and shaken for approximately 30 sec. at 10–12-min. intervals for 1 hr.

The plant material was removed by filtration, and the mother liquor was concentrated *in vacuo* at ambient temperature to a greenish paste void of solvent. This concentrate was dissolved in approximately 1 ml. of 95% ethanol. The ethanolic solution was subjected to continuous vibration from an ultrasonic vibrator until



II: cannabidiol



III: cannabigerol monomethyl ether, R = CH_3
VI: cannabigerol, R = H

[2] Seed Code ME-A.
[3] Origin: Acapulco, Guerrero, Mexico, 1968. Obtained through NIMH.
[4] Herbarium specimens of Mexican *C. sativa* L. are stored in the Herbarium, Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677
[5] Manicured material is devoid of fruits and large stems. This was accomplished by passing the material through a 14-mesh sieve.

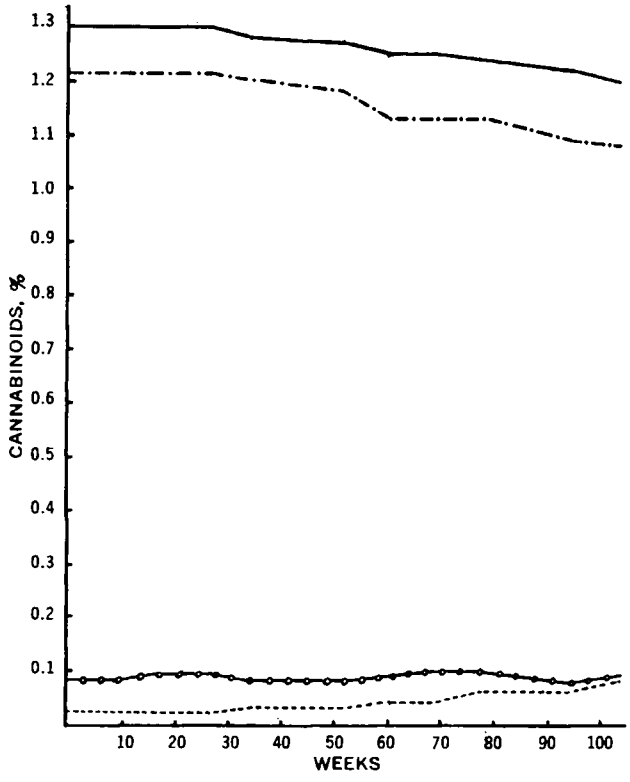


Figure 1—C. sativa *L. plant material stored at −18°. Key:* ——, (−)-Δ^9-trans-*tetrahydrocannabinol;* -·-, (−)-Δ^9-trans-*tetrahydrocannabinolic acid;* -O-, *cannabidiol; and* - - -, *cannabinol.*

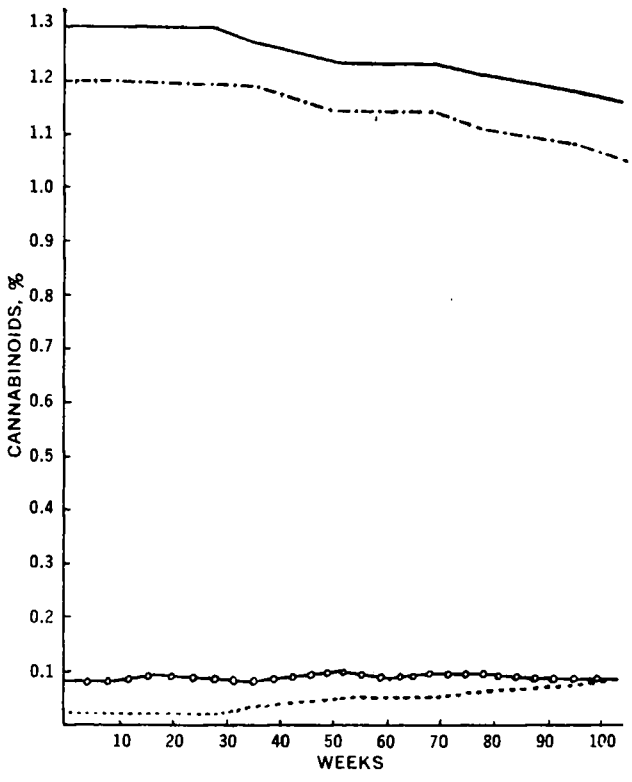
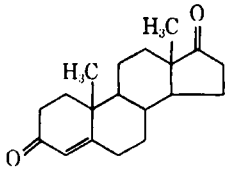


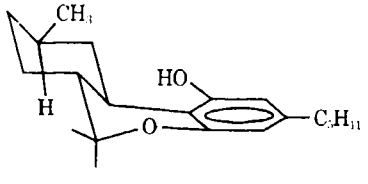
Figure 2—C. sativa *L. plant material stored at 4°. Key:* ——, (−)-Δ^9-trans-*tetrahydrocannabinol;* -·-, (−)-Δ^9-trans-*tetrahydrocannabinolic acid;* -O-, *cannabidiol; and* - - -, *cannabinol.*

all resin was in solution. At this point, an additional 0.5 ml. of an ethanolic solution containing a known concentration of 4-androstene-3,17-dione (VIII) as the internal standard was added. The concentration of VIII varies depending upon the plant sample. Usually from 0.05 to 0.5 μl. of this solution is injected into the chromatograph.

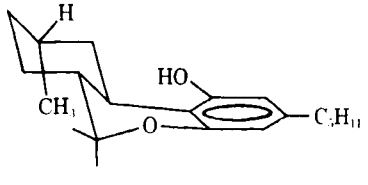
Analyses were performed using gas chromatographs[6] equipped with hydrogen flame-ionization detectors and operated isothermally at 210°. The inlet temperature was 240°, and the detector temperature was 260°. Glass and stainless steel columns [0.63 cm. (0.25 in.) o.d.; 2 mm. i.d. × 2.43 m. (8 ft.)] were packed[7] with 2% OV-17[8] on 100–120-mesh Chromosorb WHP or Gas Chrom Q. Nitrogen was used as the carrier gas at a flow rate of 10–30 ml./min., depending on the instrument used. Peak area measurements were made using the method of peak height times width at half height. Peak area of each cannabinoid was compared with the peak area of the internal standard and, using the appropriate relative response factors, the cannabinoid concentration was determined. Synthetic cannabinoids used in determining the relative response factors were obtained through NIMH.



VIII: 4-androstene-3,17-dione



XIV: C-9 equatorial hexahydrocannabinol



XV: C-9 axial hexahydrocannabinol

Silylation of Plant Extract—A 1-g. sample was extracted with 40 ml. of spectrograde chloroform. The resulting solution was refrigerated at 4° and shaken for approximately 30 sec. at 10-min. intervals for 1 hr. Then the plant material was removed by filtration, and the mother liquor was concentrated *in vacuo* at ambient temperature to a greenish paste void of solvent.

Anhydrous pyridine, 0.5 ml., was added followed by continuous vibration from an ultrasonic vibrator until all resin was in solution. The internal standard in a 10:1 pyridine to steroid solution was then added. The amount of internal standard varies with different plant samples. At this point, 0.5 ml. of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane was added[9]. The resulting reaction mixture was then heated, using a heating mantle, for 10–12 min. at 80°, and 0.1 μl. was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Lerner (4) reported that V converted to VII at the rate of approximately 5%/month. However, as Lerner pointed out, this was only an indication since no quantitative analysis was available of the original sample. Schou and Nielsen (7) reported that one sample of cannabis was examined twice with an interval of 4 years. The content of V had dropped to less than one-fourth of the original,

[6] Beckman GC-5, GC-45, and GC 72-5.

[7] By Beckman Instruments.

[8] High purity polar phenyl methyl silicone, mol. wt. approximately 30,000.

[9] BSTFA with 1% TMCS, Pierce Chemical.

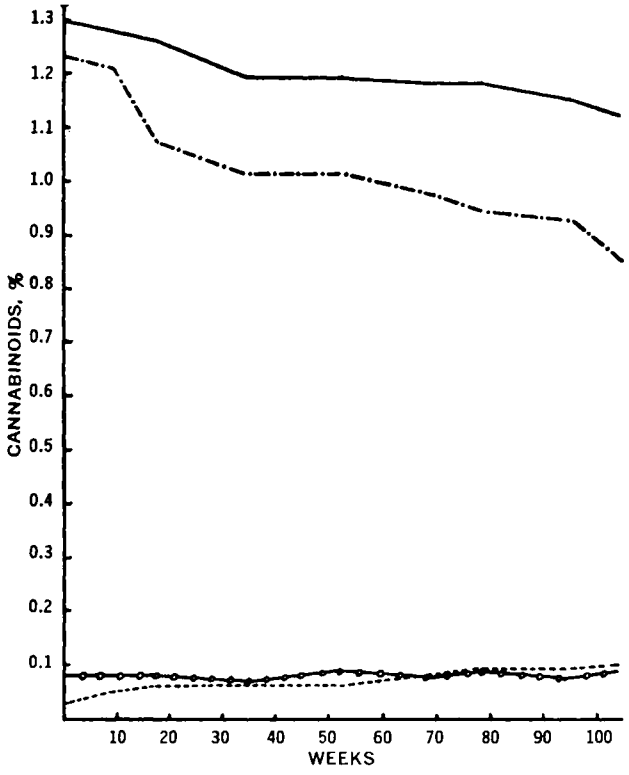


Figure 3—C. sativa *L. plant material stored at ambient temperature 22 ± 1°. Key:* ——, (−)-Δ^9-trans-*tetrahydrocannabinol;* --, (−)-Δ^9-trans-*tetrahydrocannabinolic acid;* -O-, *cannabidiol; and* - - -, *cannabinol.*

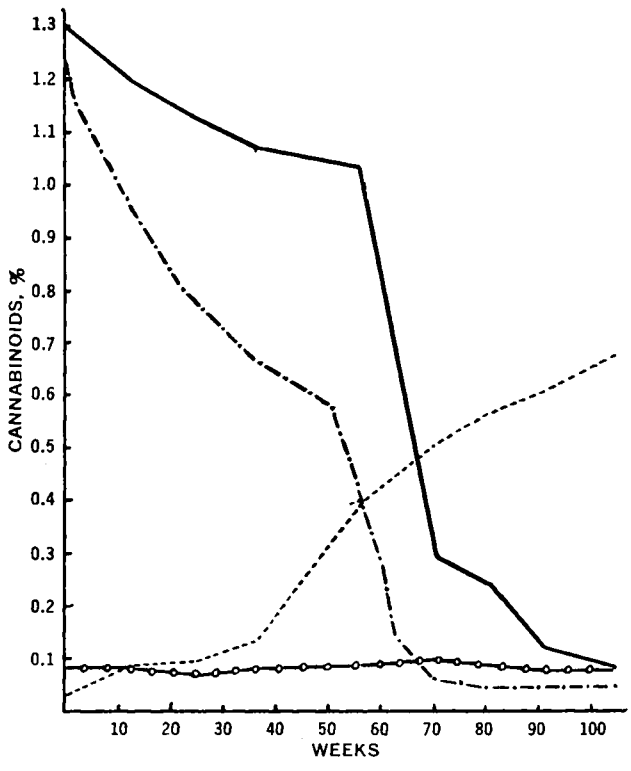


Figure 4—C. sativa *L. plant material stored at 37°. Key:* ——, (−)-Δ^9-trans-*tetrahydrocannabinol;* --, (−)-Δ^9-trans-*tetrahydrocannabinolic acid;* -O-, *cannabidiol; and* - - -, *cannabinol.*

but the contents of II and VII were relatively unchanged. This sample was stored under nitrogen at room temperature. This study indicated that V is not necessarily oxidized to VII under storage as previously proposed by Levine (8). Turk *et al.* (9) reported that a sample of 100% synthetic V decomposed to VII in the ratio of 97.1 : 2.9%, indicating that V is converted only to VII. Additionally, Razdan *et al.* (10) reported that synthetic V oxidized to VII at the rate of 10%/month at 25°. Thus, these reports seem to indicate that V in stored plant material probably decomposes by a different route than does stored synthetic V.

The Mexican variant chosen for this study contained 1.30% V, 0.02% VII, and 0.08% II. The percent of (−)-Δ^9-*trans*-tetrahydrocannabinolic acid (XI) as its trimethylsilyl ester–ether derivative (11) was 95%.

At the end of this study (104 weeks), the samples stored at 4 and −18° contained 1.16 and 1.20% of V, respectively; 0.08 and 0.07% of VII, respectively; 0.08 and 0.08% of II, respectively; and 91 and 90% of XI, respectively (Figs. 1 and 2). Although II was 0.08% at the start and 0.09% after 104 weeks in the material stored at −18°, this change is within experimental error for concentrations of such small percentages.

Figure 3 is a graph of plant material stored at 22 ± 1°. Percentages of the cannabinoids in the beginning were the same as those reported for 4 and −18°. However, after the end of 2 years, V was 1.12% and XI was 76%. The content of VII and II was 0.10 and 0.09%, respectively. These data do not correlate with data obtained previously (4, 7). However, it is obvious that conditions greatly determine the stability of cannabinoids in stored plant material. Under the conditions set forth in this experiment, the decomposition of V was 6.92%/year when stored at 22 ± 1°; material stored at −18 and 4° decomposed at 3.83 and 5.38%, respectively.

Plant material stored at 36° showed a steady decline in V and XI through Week 60 (Fig. 4). Between Weeks 60 and 70, there was a marked decrease in V from 1.03 to 0.29%. The increase of VII was from 0.40 to 0.50%. From Week 70, there was a steady decline in V and a steady rise in VII. At the termination date, the composition of V and VII was 0.08 and 0.67%, respectively. Thus, there was a material balance deficit of 0.57% between these two cannabinoids (1.30 + 0.02 = 1.32% at the start and 0.08 + 0.67 = 0.75% at termination).

The material deficit and decomposition rate between V and VII were more pronounced when the material was stored at 50°. Pars

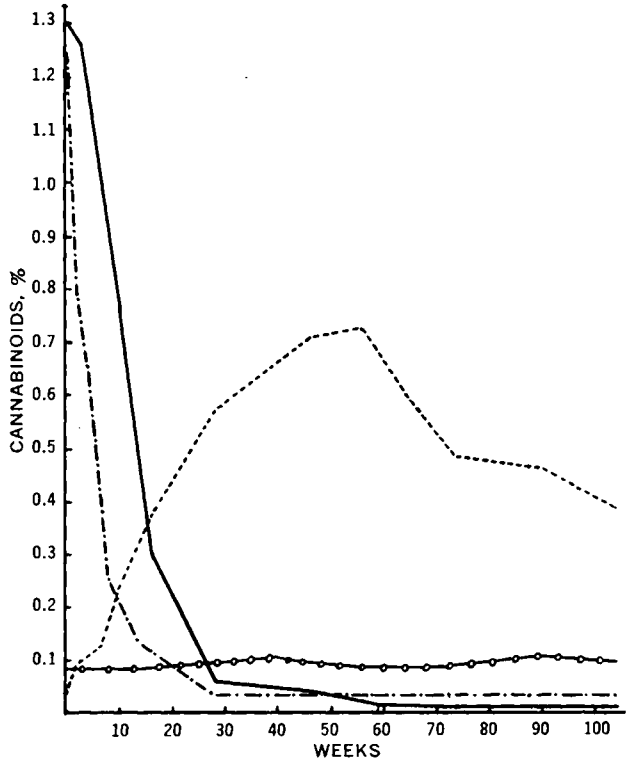


Figure 5—C. sativa *L. plant material stored at 50°. Key:* ——, (−)-Δ^9-trans-*tetrahydrocannabinol;* --, (−)-Δ^9-trans-*tetrahydrocannabinolic acid;* -O-, *cannabidiol; and* - - -, *cannabinol.*

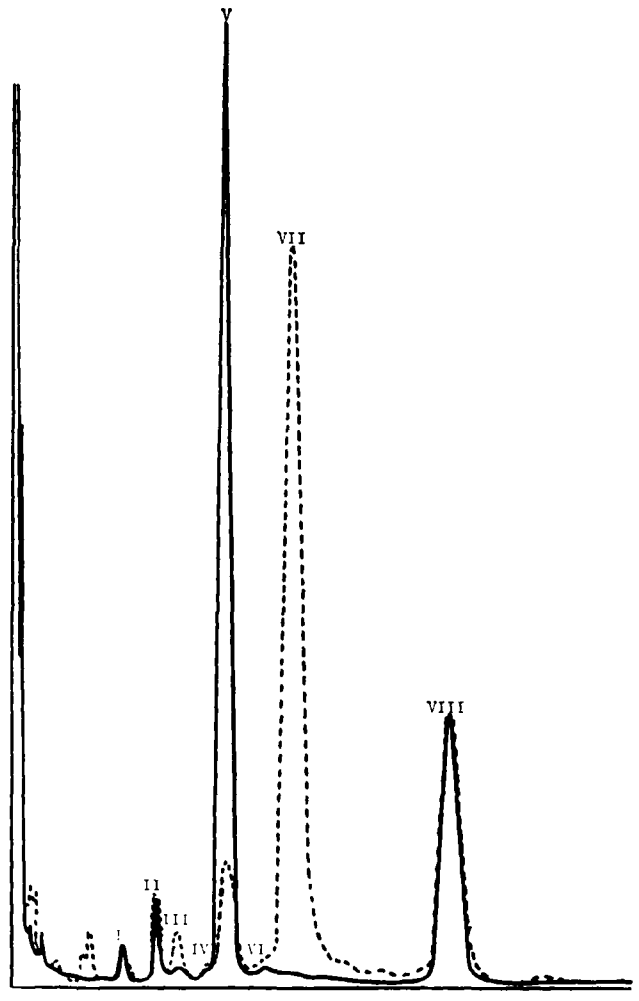


Figure 6—*Overlay of original plant sample* (——) *and plant sample stored at 37° for 2 years* (- - -). *Key: I,* (−)-Δ⁹-trans-*tetrahydrocannabivarin; II, cannabidiol; III, cannabigerol monomethyl ether; IV,* (−)-Δ⁸-trans-*tetrahydrocannabinol; V,* (−)-Δ⁹-trans-*tetrahydrocannabinol; VI, cannabigerol; VII, cannabinol; and VIII, 4-androstene-3,17-dione.*

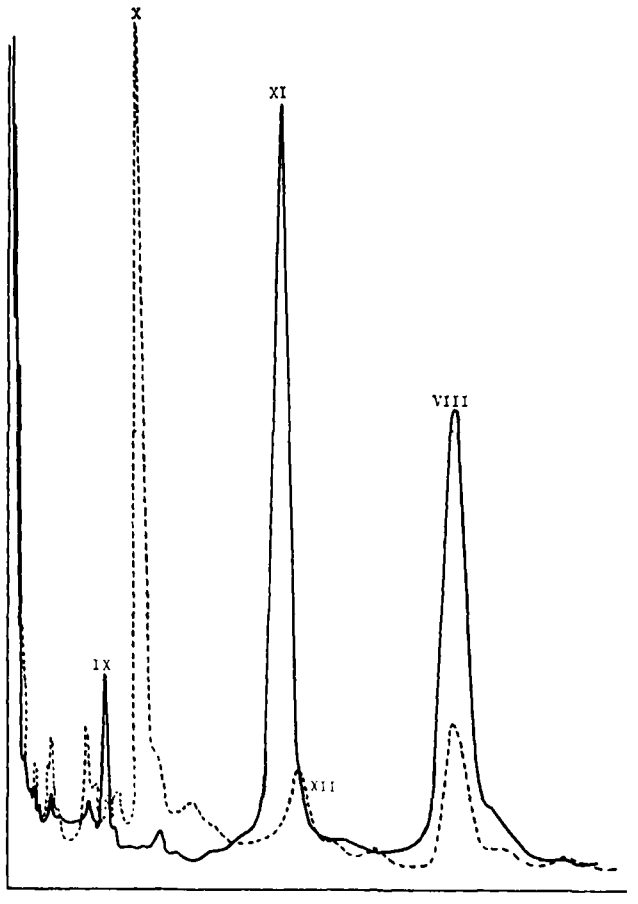


Figure 7—*Overlay of original plant material silylated* (——) *and silylated plant material stored at 37° for 2 years* (- - -). *Key: IX,* (−)-Δ⁹-trans-*tetrahydrocannabinol trimethylsilyl ether; X, cannabinol trimethylsilyl ether; XI,* (−)-Δ⁹-trans-*tetrahydrocannabinolic acid trimethyl ester-ether; XII, cannabinolic acid trimethylsilyl ester-ether; and VIII, 4-androstene-3,17-dione.*

and Razdan (12) observed that synthetic V heated at 80° for 7 days disappeared with concomitant formation of VII, whereas IV showed no change in composition. Data in Fig. 5 indicate that V in stored plant material under thermal conditions did not decompose entirely to VII as was observed with synthetic V. Moreover, the concentration of VII decreased after 50 weeks. The deterioration of V in plant material stored at 37 and 50° could possibly be initiated by an autocatalytic state.

Since V possesses a benzylic proton which is also allylic, the formation of a radical stabilized by resonance would be highly possible under thermal conditions; however, a radical formation would not be as stabilized by delocalization in IV. Thus, the concentration of IV would be expected to remain fairly constant even under thermal conditions, but the concentration of V would decrease precipitously (Fig. 6).

If, indeed, a radical is formed at carbon-10*a* in V, a disproportionation reaction would be possible. It is known that certain heterocyclic systems readily undergo disproportionation, with three molecules giving one hexahydro and two aromatic levels (13). The relative retention time for the aromatic cannabinoid level (VII) is well documented, but no relative retention time is available for hexahydrocannabinol (XIII) using VIII as the internal standard. Thus, XIII was prepared by catalytic hydrogenation of V as described by Archer *et al.* (14). GLC analysis of the reaction mixture produced one peak having a relative retention time of 0.37. This is close to cannabigerol monomethyl ether (III), which has a relative retention time of 0.38. Although the concentration of III increased slightly on storage (Fig. 6), it was impossible to determine if this increase was due exclusively to the formation of hexahydrocannabinol since the amount of plant material allocated for this study was limited. This will be the subject of a future article.

Since, to the authors' knowledge, no GLC data are available in the literature on the trimethylsilyl ether of XIII, this derivative was produced. Silylation of XIII followed by GLC analysis yielded relative retention times of 0.17 and 0.22, respectively. Two peaks were expected since there existed the possibility of a mixture of C-9 equatorial (XIV) and axial (XV) methyl isomers (Fig. 7). (See Fig. 7 for silylated plant material.)

Archer *et al.* (14) reported an axial-to-equatorial ratio of 3:1 as determined by PMR analysis. In these laboratories, the ratio was 3:2 as determined by GLC analysis of the trimethylsilyl ethers. Therefore, since the C-9 methyl axial isomer was the major product, it was assigned the relative retention time of 0.17; the equatorial isomer was assigned the relative retention time of 0.22.

Decomposition of V in *C. sativa* L. produces many products not yet identified; some are probably polymerization products. Moreover, biological data obtained on *C. sativa* L. will remain contradictory due to variations in chemical constituents. Although the cannabinol content of cannabis does, indeed, increase with storage, the suggestion that V plus VII would approximate the V content, irrespective of degradative changes (5), must be revised.

The cannabidiol content of all stored material remained relatively constant with no detectable decomposition. Additionally, a sample of *C. sativa* L. plant material of 1930 vintage[10] was analyzed in 1970 and 1972. The content of II (0.28%) was constant over this period, whereas V and VII changed from 0.05 and 0.65 to 0.04 and 0.66, respectively.

[10] From a pharmacy in Wesson, Miss.

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▲ To whom inquiries should be directed.

Kinetics and Mechanisms of Monolayer Interactions II: Effect of Chain Length of Alkyl Ionic Surfactants on Their Interaction with Dipalmitoyl Glycerol, Dipalmitoyl Phosphatidylethanolamine, and Dipalmitoyl Lecithin

FEDERICO A. VILALLONGA▲ and EDWARD R. GARRETT

Abstract □ The energies of activation of the interaction of alkyl trimethylammonium surfactants with dipalmitoyl glycerol, dipalmitoyl phosphatidylethanolamine, and dipalmitoyl lecithin monolayers spread at the air–water interface were estimated from the increase of the surface pressure with the concentration of the injected surfactant. The energies of activation are a linear function of the chain length of the injected surfactant for the six-, eight-, 12-, and 16-carbon chains studied. The ionic groups of the polar hydrophilic moiety of dipalmitoyl lecithin were not equivalent in the perturbation that an attached surfactant ion produces in the surface pressure of the monolayer. The energies of activation per methylene group of the hydrocarbon chain and per polar ionic head of the injected surfactant were calculated and were compared with those that correspond to the energies of adsorption of these groups at hydrocarbon–water and air–water interfaces.

Keyphrases □ Alkyl sulfates and alkyl trimethylammonium ions—interactions with dipalmitoyl glycerol, dipalmitoyl phosphatidylethanolamine, and dipalmitoyl lecithin monolayers, kinetics, mechanisms □ Monolayers, dipalmitoyl glycerol, dipalmitoyl phosphatidylethanolamine, and dipalmitoyl glycerol—interactions with alkyl sulfate and alkyl trimethylammonium ions, kinetics, mechanisms □ Phospholipid monolayers—interactions with long-chain surfactants, kinetics, mechanisms □ Surfactants, long chain—interaction with phospholipid monolayers, kinetics, mechanisms □ Chain length effect—interaction of alkyl surfactants with phospholipid monolayers

The energies of interaction of cetyl sulfate and cetrimonium ions with dipalmitoyl glycerol and dipalmitoyl lecithin monolayers spread at the air–water interface were estimated recently (1) from the variation of the equilibrium surface pressure with varying concentrations of subphase-injected surfactants on the premise that the entropy factor calculated on the basis of collision theory was constant for all species.

The energies of adsorption at the oil–water and air–water interfaces of homologous series of alkyl surfactants are provided in the literature (2–11). These data permit the estimation of the contributions of the hydrocarbon moiety per methylene group and that of the polar head to the total energy of adsorption at a "clean" interface. A clean interface is defined here as a liquid–liquid or air–liquid interface without any spread monolayer.

These studies were designed to determine the dependence of the maximum obtainable changes in surface pressure and energies of interaction of monolayers of dipalmitoyl glycerol, dipalmitoyl phosphatidylethanolamine, and dipalmitoyl lecithin with subphase-injected surfactants on the numbers of methylene groups and on the nature of the charged polar head in the injected surfactant.

EXPERIMENTAL

Reagents—Dipalmitoyl glycerol[1], dipalmitoyl lecithin[1], cetyl sodium sulfate[1], and cetrimonium bromide[2] were the same samples

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[2] Eastman Kodak, Rochester, N. Y.

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These studies were designed to determine the dependence of the maximum obtainable changes in surface pressure and energies of interaction of monolayers of dipalmitoyl glycerol, dipalmitoyl phosphatidylethanolamine, and dipalmitoyl lecithin with subphase-injected surfactants on the numbers of methylene groups and on the nature of the charged polar head in the injected surfactant.

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Table I—Estimated Energies of Activation of the Interaction of Alkyl Sulfate and Alkyl Trimethylammonium Ions with Dipalmitoyl Glycerol, Dipalmitoyl Phosphatidylethanolamine, and Dipalmitoyl Lecithin

Interaction		ψ, kcal. mole^{-1}				Energy of Activation, kcal. group^{-1} mole^{-1}			Percent of Methylene Groups of C_n Interacting[a]
		C_n							
Lipidic Monolayer	Surfactant Ion	6	8	12	16	$-CH_2-$	$-SO_4^-$	$-N^+-(CH_3)_3$	
Dipalmitoyl glycerol	$C_{n(2n+1)}\cdot SO_4^-$	−4.8	−6.1	−8.2	−10.7	−0.58	−1.3	—	58
	$C_nH_{(2n+1)}\cdot N^+(CH_3)_3$	−4.4	−5.8	−8.2	−10.8	−0.63	—	−0.64	63
Dipalmitoyl phosphatidylethanolamine	$C_nH_{(2n+1)}\cdot SO_4^-$	−5.1	−5.7	−8.6	−10.9	−0.60	−1.2	—	60
	$C_nH_{(2n+1)}\cdot N^+(CH_3)_3$	−5.1	−6.3	−9.1	−11.3	−0.63	—	−1.4	63
Dipalmitoyl lecithin	$C_nH_{(2n+1)}\cdot SO_4^-$	−4.2	−5.4	−8.4	−9.6	−0.56	−1.0	—	56
	$C_{n(2n+1)}\cdot N^+(CH_3)_3$	−4.7	−5.9	−10.6	−12.0	−0.78	—	−0.1	78

[a] Calculated on the premise that 1 kcal. mole^{-1} is the energy of interaction per pair of methylene groups in close contact (4.2 Å).

described previously (1). Dipalmitoyl phosphatidylethanolamine[1] was chromatographically homogeneous by TLC (12). Sodium lauryl sulfate[2], sodium octyl sulfate[1], sodium hexyl sulfate[1], and dodecyltrimethylammonium bromide[3] gave no minima in the curves of surface tension against the logarithm of the concentration. The octyltrimethylammonium and hexyltrimethylammonium bromides were prepared[4] by the reaction of *n*-dimethyloctylamine with methyl bromide and *n*-hexyl bromide with trimethylamine and were chromatographically homogeneous by TLC (13). The hexanes[2,5] used to prepare the lipidic spreading solutions and the distilled water used as subphase fulfilled the requirements described previously (1).

Instruments and Methods—Surface tension was measured with a Wilhelmi platinum plate attached to an electrobalance[6]. Surface potential was measured with an americium-241 air electrode and an electrometer[7]. The output of the electrobalance and the electrometer were fed into a dual-pen recorder[8].

The experimental assembly and the methods for the measurement of the increase of the surface pressure after the injection of the alkyl surfactant ion were described previously (1). The concentration of the surfactant solution to be injected was kept below the critical micelle concentration (CMC) in all cases. The average values of three different experiments for each of three different final concentrations of injected surfactant ions were used for the estimation of the slope of the plot of the reciprocal of the change of the surface pressure at equilibrium ($1/\Delta\pi_{eq}$) against the reciprocal of the number of surfactant ions per cubic centimeter ($1/n$) (1). The reproducibility of the changes of the surface pressure was within ±0.5 dyne cm.$^{-1}$. All experiments were performed at an initial surface pressure of the lipidic monolayer of 5 dynes cm.$^{-1}$ (±0.1 dyne cm.$^{-1}$) and at 22° (±1°).

RESULTS

The values of the maximum change of the surface pressure ($\Delta\pi_m$) were calculated from the intercept, $1/\Delta\pi_m$, of the plot of the reciprocal of the surface pressure change at equilibrium ($1/\Delta\pi_{eq}$) against the reciprocal of the number of subphase surfactant ions per cubic centimeter ($1/n$) on the assumption of the continuity of the straight-line relationship for surfactant concentrations above those used in the experiments in accordance with the equation: $1/\Delta\pi_{eq} = 1/\Delta\pi_m + B/\Delta_m \cdot 1/n$. They are plotted in Fig. 1 for dipalmitoyl glycerol, dipalmitoyl phosphatidylethanolamine, and dipalmitoyl lecithin monolayers against the chain length of the injected alkyl sulfate anions and alkyl trimethylammonium cations.

The maximum change of the surface pressure ($\Delta\pi_m$) is not a linear function of the chain length of the injected surfactant ion. The plots present distinct *maxima* in $\Delta\pi_m$ at the eight-carbon atom chain for the observed cases of the interaction of the positively charged alkyl trimethylammonium cations with the monolayers. The plots present *minima* at this same chain length for the interaction of the negatively charged alkyl sulfates with dipalmitoyl glycerol and dipalmitoyl lecithin monolayers. The minimum may be at 12-carbon atoms with dipalmitoyl phosphatidylethanolamine monolayers.

The energies of activation (ψ) were calculated on the premise of a collision model and a constant entropy factor (1) for the interace tion of the subphase-injected surfactant ion with the moleculer that form the monolayer at the air-water interface. They ahe plotted in Fig. 2*a* for dipalmitoyl glycerol monolayers against ts-chain length of the injected alkyl sulfate anions and alkyl trimethylammonium cations. The calculated energy of activation for a given chain length of the injected surfactant was independent of the

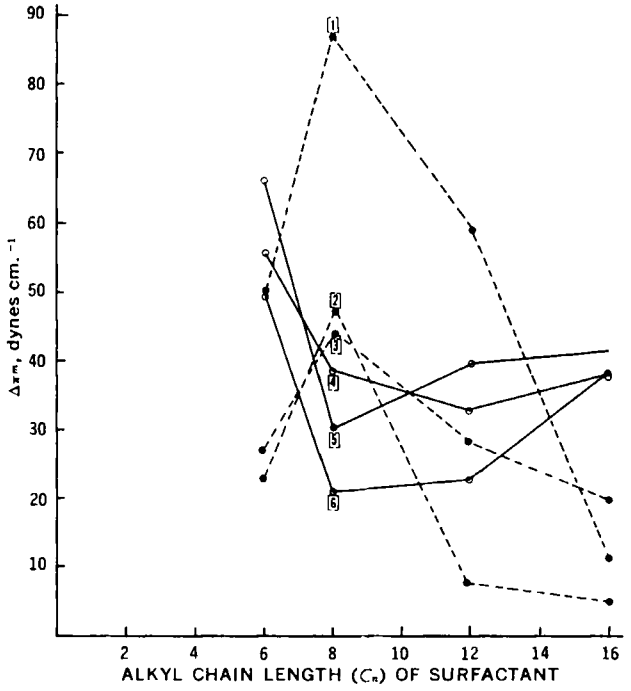

Figure 1—*Effect of chain length of alkyl surfactants* (C_n) *on the maximum surface pressure* ($\Delta\pi_m$). *Key: ---, alkyl trimethylammonium surfactants interacting with: (1) dipalmitoyl glycerol, (2) dipalmitoyl lecithin, and (3) dipalmitoyl phosphatidylethanolamine; and ——, alkyl sulfate surfactants interacting with: (4) dipalmitoyl phosphatidylethanolamine, (5) dipalmitoyl glycerol, and (6) dipalmitoyl lecithin.*

[3] Pfaltz and Bauer, Flushing, N. Y.
[4] Prepared by K. V. Rao.
[5] Spectroquality, Matheson, Coleman and Bell, East Rutherford, N. J.
[6] Cahn Division, Ventrom Instruments Corp., Paramount, Calif.
[7] Keithley Instruments (610c), Cleveland, Ohio.
[8] Speedomax w/1, Leeds and Northrup, North Wales, Pa.

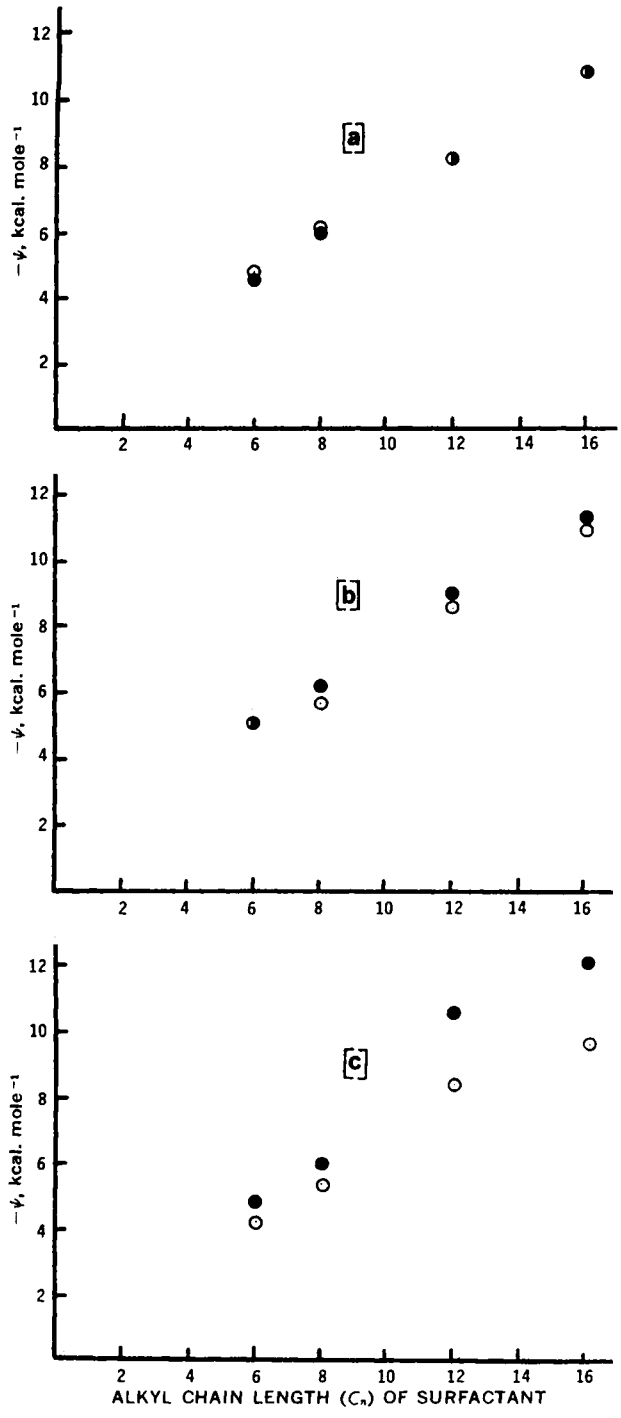

Figure 2—*Effect of chain length of alkyl sulfate* (○) *and alkyl trimethylammonium* (●) *surfactants on the energy of activation of their interactions with: (a) dipalmitoyl glycerol, (b) dipalmitoyl phosphatidylethanolamine, and (c) dipalmitoyl lecithin monolayers.*

nature of their polar heads within the limits of experimental error (±0.1 kcal. mole^{-1}) and was a linear function of the chain length between the studied six- and 16-carbon atoms.

The energies of activation (ψ) for the interaction of dipalmitoyl phosphatidylethanolamine monolayers were plotted against the chain lengths of the same series of alkyl sulfate and alkyl trimethylammonium ions in Fig. 2*b*, and the results were similar.

The energies of activation (ψ) for the interaction of dipalmitoyl lecithin monolayers with both series of surfactant ions are given in Fig. 2*c*. In these cases, there was a difference between the energy of activation of the interaction with the surfactant with the positively charged polar head compared with the interaction with the surfactant with the negatively charged head at a given chain length. This was more pronounced at the higher chain lengths. The same general increase in the energy of activation with the number of carbon atoms in the alkyl chain of the injected surfactant ion was observed as with the two other monolayers.

The energy of activation (Table I) per methylene group of the surfactant hydrocarbon chain was calculated from the regression coefficient of the best straight lines through the experimental points of Fig. 2. The energies of activation for the sulfate or trimethylammonium groups were calculated from the intercept on the assumption that the extrapolation of the straight line to $C_n = 0$ (C_n represents alkyl chain length of surfactant) gives a numerical value related to the energy of activation of the polar head group of the surfactant ion. The values of the energy of activation per methylene group were in the range of from −0.56 to −0.77 kcal. mole^{-1}, with an overall average value of −0.63 kcal. mole^{-1}. The values of the energy of activation per polar head of the injected surfactant ion were in the range of from −0.10 to −1.4 kcal. mole^{-1}.

DISCUSSION

Interfacial Phase—It has been suggested (1, 14) that the molecules that form these lipidic monolayers at a surface pressure of 5 dynes cm.$^{-1}$ are oriented with the long hydrocarbon chains perpendicular to the plane of the air-water interface. The total length of the dipalmitoyl glycerol molecule is roughly 26 Å measured from space-filling models. The length of dipalmitoyl phosphatidylethanolamine and dipalmitoyl lecithin molecules, assuming a configuration of the phosphatidylethanolamine polar group parallel to the interface (see Fig. 9, *Reference 1*), is roughly 30 Å. If the molecules are completely submerged in the water phase, the volume of the interfacial phase is determined by the length of the molecule times the total surface area of the air-water interface, which was 42.54 cm.^{2} in all of the experiments. The concentrations (in moles per liter) in this thin crust of water were calculated (Table II) for each lipid on this basis, since the total number of molecules necessary to form the monolayer was known.

The percentage of water in this interfacial phase (Table II) can be estimated from the total volume of the interfacial phase and from the volume occupied by the lipidic molecules calculated from the length of the molecule, the cross-sectional area of two hydrocarbon chains, and the total number of lipidic molecules. According to the values of Table II, the interfacial phase can be considered to be predominantly hydrocarbon.

Surfactant-Ion Hydrocarbon Chain—Van der Waals' forces between methylene groups of neighboring hydrocarbon chains can be calculated from Salem's equation (15):

$$W_{disp} = A\left(\frac{9.42}{8 \times l}\right)\left(\frac{n}{d^6}\right) \qquad \text{(Eq. 1)}$$

where W_{disp} is the energy of interaction in kcal. mole^{-1} for each pair of methylene groups of neighboring chains, A is a constant (−1.340 kcal. mole^{-1}), l is the effective distance between carbon atoms along a chain (1.253 Å), n is the number of methylene pairs, and d is the distance between the central axes of the hydrocarbon chains. The equation is valid for $d \geq 4.0$ Å. The energy of interaction becomes appreciable when d is less than 7 Å and increases exponentially with shorter distances as the hydrocarbon chains approach each other.

The minimum possible distance between the central axes of two neighboring hydrocarbon chains is 4.2 Å. From Eq. 1, the energy of interaction per pair of methylene groups will be 1.0 kcal. mole^{-1} for that distance.

The contribution to the total energy of activation that corresponds to the hydrocarbon chain for the interaction of the surfactant ions with the lipidic monolayers can be estimated by subtracting the contribution that corresponds to the polar ionic head from the total energy of activation (Table I). On the assumption that each methylene group of the surfactant ion that interacts with a methylene group of the hydrocarbon chains of the molecules that form the monolayer is in close contact (*i.e.*, at 4.2 Å with 1.0 kcal. mole^{-1} per pair of methylene groups), these values would numerically approximate the number of methylene groups of the surfactant ion that interacts with the hydrophobic moiety of the monolayer.

It was suggested (1) that the first step of the interaction process was the attachment of the polar charged head of the injected surfactant ion to the polar or the ionic attraction centers of the hydro-

Table II—Average Values of the Surface Parameters of Dipalmitoyl Glycerol, Dipalmitoyl Phosphatidylethanolamine, and Dipalmitoyl Lecithin before Injection of the Surfactant

	Dynes cm.$^{-1}$	Å^2 molecule^{-1}	ΔV, mv.	μ, mD.	Total Number of Molecules at Interface (42.54 cm.^{2})	Molarity at Interfacial Phase, mole l.$^{-1}$	Molecular Volume, Å^3	Volume of Water in Interfacial Phase, %
Dipalmitoyl glycerol	5(±0.1)	46(±2)	+340(±20)	500	9.3×10^{15}	1.4[a]	1014	14.7
Dipalmitoyl phosphatidyl-ethanolamine	5(±0.1)	40(±2)	+500(±20)	530	10.7×10^{15}	1.4[b]	1170	1.9
Dipalmitoyl Lecithin	5(±0.1)	55(±2)	+460(±20)	560	7.7×10^{15}	1.0[b]	1170	29.4

[a] Calculated from an estimated 1106 Å^3 volume of the interfacial phase. [b] Calculated from an estimated 1276 Å^3 volume of the interfacial phase.

philic moiety of the molecules that form the monolayer. The subsequent increase of the surface pressure could then be rationalized as reflecting the subsequent interactions of the hydrocarbon chains with the hydrophobic moiety.

The hydrophilic region of the dipalmitoyl glycerol molecule does not have an ionic charge. However, the two fatty acid ester linkages and an alcoholic group give a high electronic density to this region. It can be assumed that the positively charged or negatively charged surfactant-ion polar head tends to be attached by ion-dipole interactions to this region. It is noted that the percentage of methylene groups in close contact with the hydrophobic region of the monolayer apparently is independent of the charge of the surfactant ion (64 and 58%) (Table I).

The hydrophilic zwitterionic group of dipalmitoyl phosphatidylethanolamine is fully charged at the experimental conditions (pH 6.0–6.5). Its coplanarity with the plane of the interface (18–21) produces an attractive potential between vicinal molecules which keeps the monolayer closely packed (Table II). The strong P^-–N^+ electrostatic interaction jointly with the attractive potential between neighboring molecules should produce a rigid structuring of the monolayer in this case where any net charge is essentially negligible. Thus, it is understood that the calculated percentage of methylene groups in close contact with the hydrophobic region of this monolayer is similar to that of the nonionic dipalmitoyl glycerol (Table II).

The hydrophilic zwitterionic group of dipalmitoyl lecithin is similarly charged in the experimental conditions and should show a similar rigid structure. [The studies on the conformation of this group have resulted in much disagreement. The zwitterion has been considered oriented with its axis parallel to the plane of the interface (1, 14, 16–19), perpendicular (19, 20), or at some unlocalized intermediate position (23). On the basis of the apparent dipole moment measured (1, 14) on monolayers spread at the air–water interface, it will be assumed in the following discussion that the zwitterionic group of dipalmitoyl lecithin is oriented with its axis *parallel* to the plane of the interface.]

However, it can be argued that the dipalmitoyl lecithin monolayer would be less closely packed at the air–water interface than that of dipalmitoyl phosphatidylethanolamine (Table II) because of the shielding effect of the positively charged amino group by the three methyl groups that decreases the attractive potential between neighboring molecules or because of the lack of hydrogens that would promote hydrogen bonding. Accordingly, a less rigid structural organization of this monolayer can be expected as compared with the dipalmitoyl phosphatidylethanolamine and has been shown experimentally as indicated by the comparison of the Å^2 molecule^{-1} in Table II.

The calculated percentage of methylene groups of the surfactant hydrocarbon chain supposedly in close contact with the hydrophobic region of the dipalmitoyl lecithin monolayer differs markedly with the nature of the charge of the surfactant polar head. This contrasts with the other two monolayers. It can be assumed that the first step of the interaction involves the attachment of the negatively charged polar head of the surfactant to the positively charged trimethylammonium group of the P–N ionic dipole. Such an attachment could produce a change in the dipole orientation toward the perpendicular position that would displace the trimethylammonium group of the phosphatidylcholine moiety 5 Å farther below the hydrophobic region of the dipalmitoyl lecithin molecules.

The phosphate group of the P–N dipole constitutes the hinge for this change or orientation (Fig. 9 of *Reference 1*). Consequently the attachment of a positively charged polar head to the hinge should not readily produce rotation about the hinge. However, the attachment of a negatively charged polar head to the extreme end of the lever could readily cause rotation about the hinge to 5 Å below the hydrophobic portion of the dipalmitoyl lecithin molecule. The greater rigidity of the dipalmitoyl phosphatidylethanolamine molecule and its greater affinity for its neighboring molecules in the monolayer, as already stated, could rationalize the greater resistance to such a change in the steric position of its P–N axis. The fact that space-filling models show that a 5-Å lowering of the trimethylammonium group subtracts two to three pairs of methylene groups of an attached surfactant hydrocarbon chain from the hydrophobic region of the dipalmitoyl lecithin molecule is consistent with this explanation.

These phenomena strongly suggest that entropic factors associated with the configuration of the molecules that form the monolayer *do* contribute to the energies of interaction. Thus, the simplified assumption of a common entropy factor for such interactions based on collision theory on which the calculations of energies of activation were based (1) is most probably an oversimplification of a complex process.

Surfactant-Ion Polar Head—The contribution of the sulfate group to the total energy of adsorption of an alkyl sulfate surfactant has been estimated to be −1.8 kcal. mole^{-1} at a "clean" hydrocarbon–surfactant solution interface (8) and 2.4 kcal. mole^{-1} at a "clean" air–surfactant solution interface (11). The positive value for the latter interface has been attributed to the strongly hydrophilic nature of the sulfate group (11).

The values estimated for the contribution of the sulfate group to the total energy of activation of the interaction of alkyl sulfates with the three lipidic monolayers studied in this work are negative in all cases (Table I), which seems to indicate that the interaction of this group takes place at a hydrocarbon environment.

The contribution of the trimethylammonium group to the total energy of adsorption of an alkyl trimethylammonium surfactant has been estimated to be −0.95 kcal. mole^{-1} at a clean hydrocarbon–surfactant solution interface (8). From literature data (9, 10), a value of 2.9 kcal. mole^{-1} for the adsorption at a clean air–surfactant solution interface can be calculated. Again the negative estimated values of the contribution of the trimethylammonium group to the total energy of activation of the interaction of alkyl trimethylammonium surfactants suggest the interaction of this group with a hydrocarbon environment.

The adsorption of the polar heads of the surfactant ions to a hydrocarbon–water interface would be in accord with the postulated hydrocarbon nature of the interfacial phase (Table II). However, there is a discrepancy in regard to the hydrocarbon chains of the surfactant ion. The free energy of adsorption per methylene group of a hydrocarbon chain that adsorbs at a clean hydrocarbon–surfactant solution interface has been estimated to be between −0.80 and −0.82 kcal. mole^{-1} (6–8). At a clean air–surfactant solution interface, the estimated value is between −0.600 and −0.625 kcal. mole^{-1} (2, 8, 9, 11). With the exception of the value that corresponds to the alkyl trimethylammonium–dipalmitoyl lecithin

interaction (−0.77), the energy of activation per methylene group in all cases (Table I) has been found to be much closer to that which corresponds to the adsorption of the methylene group to an air-water interface.

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KATHLEEN S. PELZMANN

Abstract □ The GI absorption of chlormadinone acetate and norethindrone was studied in a rat *in situ* preparation. The data evaluated with respect to the absorption half-life in the ligated stomach and intestine suggest that chlormadinone acetate is absorbed slightly more rapidly than norethindrone and that both steroids are absorbed to a greater extent in the intestine. The effects of bile duct cannulation, ethanol, and exogenous bile salts were investigated. The presence of bile significantly altered the absorption and metabolism of chlormadinone acetate. Ethanol, which was used as the drug vehicle, did not improve or depress the absorption of either steroid. Tissue accumulation experiments indicated that the jejunum of the intestine and the pyloric and cardiac area of the stomach accumulate the steroids to a greater extent than other GI tissues. Both compounds exhibited biexponential absorption with significant membrane accumulation.

Keyphrases □ Chlormadinone acetate and norethindrone—absorption from *in situ* rat gut □ Norethindrone and chlormadinone acetate—absorption from *in situ* rat gut □ Absorption, GI—norethindrone and chlormadinone acetate, *in situ* rat gut

Although the oral administration of steroids is common today, relatively little is known about their GI absorption characteristics. Most published studies measure absorption indirectly by plasma or urine levels of a steroid and its metabolites. The present investigations were undertaken to study the GI absorption of two progestins, chlormadinone acetate and norethindrone, and to develop an animal model for these studies.

Some investigators have used the *in vitro* rat intestine preparation (1, 2) or the everted intestinal sac method (3, 4). The absorption rates measured in these experiments are not influenced by blood and blood pressures, lymph, nerves, and GI secretions. Levine *et al.* (5) reported the loss of structural integrity and cellular death occurring in the intestinal mucosa within 10–15 min. after the preparation of an everted intestine. Consequently, the only observation that can be made is the movement of drug across a semiviable membrane.

Searching for a less traumatic intestinal preparation, Schanker *et al.* (6) used *in situ* perfusion techniques consisting of a single pass or a recycling of the drug solution through the cannulated intestine. Doluisio *et al.* (7) described an *in situ* technique with duodenal and ileal ends of the intestine cannulated with L-shaped cannulas. Stopcocks and syringes were attached to both cannulas for instilling and sampling the drug solution. Although Doluisio *et al.* reported very reproducible results, this preparation was found cumbersome by the present author.

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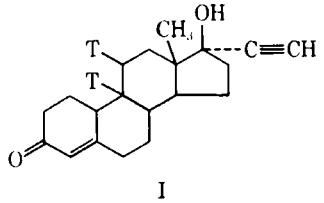
et al. (8), using in-dwelling intestinal cannulas, studied the absorption of betamethasone in various sections of the dog gut. Meli *et al.* (9) studied the possible active transport of estrogens by the rat small intestine, and Collins and Forist (10) inferred the absorption properties of hydrocortisone in the dog from plasma levels. The most comprehensive study was reported by Schedl and Clifton (11) on the absorption of 22 androgens, estrogens, and corticosteroids. Their data were obtained with the rat gut intestinal perfusion technique; the percentage of absorbed steroid was independent of concentration over a wide range. It was concluded that steroids seem to be absorbed by passive diffusion.

In this paper, the absorption characteristics of chlormadinone acetate and norethindrone from the GI tract are presented in detail. The animal preparation described is a simple and an improved *in situ* method. Intestinal and gastric absorption rates can be described by sampling the lumen contents as well as the bile and plasma with a minimum of apparatus and trauma to the animal.

EXPERIMENTAL

Animal Preparation—Sprague–Dawley female rats, weighing 200–250 g., were fasted 16–18 hr. with water *ad libitum* prior to surgery. They were housed in false-bottom cages to prevent coprophagy. The animals were anesthetized with pentobarbital, 50 mg./kg. i.p., and the viscera were exposed by midline abdominal incision. To prevent enterohepatic circulation, the bile duct was cannulated with 11 cm. of polyethylene tubing[1]. The portal vein was cannulated to permit blood sampling (12), and the pylorus was closed with clamps or sutures. In some experiments, the jejuno-ileal junction was ligated. In experiments in which the jejunum was not restricted, 10 ml. of the drug solution was instilled into the duodenum with a 23-gauge needle and a disposable syringe at the rate of 5 ml./min. In experiments that were restricted to certain ligated sections, the amount of solution varied, depending on the volume required to fill, but not distend, the intestinal section, usually 3–5 ml. The gut was not rinsed previous to the instillation. To sample the intestinal contents, a 27-gauge needle was inserted through the intestinal wall along the ligated section, and at each time point a 0.1-ml. sample was withdrawn into a tuberculin syringe. Intralumenal bleeding was not a problem, because care was taken not to sample in the vascular lateral regions of the intestine. The bile samples, approximately 0.1 ml., were an accumulation of flow over 5-min. intervals. Blood samples of 0.3 ml. were taken from the portal vein at specific times, and 0.1 ml. of plasma was used for the drug assay.



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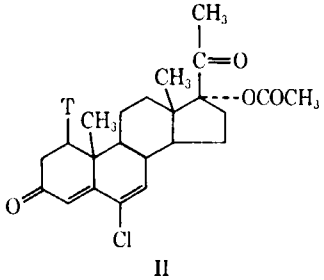

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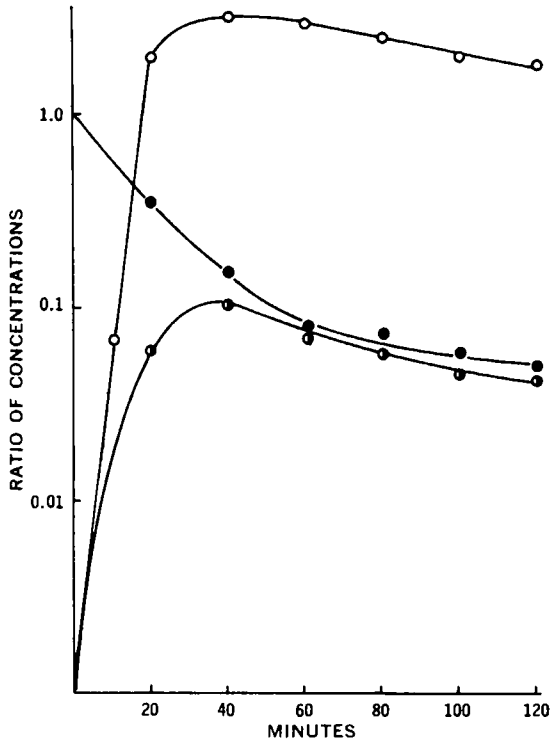

Figure 1—*Time course of norethindrone absorption in the intestine* (●) *and its appearance in the plasma* (◑) *and the bile* (○). *Ratio of concentrations is calculated in terms of* (*drug at* t_n/*0.1 ml. sample*)/(*drug at* t_0/*0.1 ml. sample*). *Norethindrone is highly concentrated by the liver and, consequently, the rise above 1.0 is not unreasonable.*

For gastric absorption studies, the stomach was exposed. The muscular area was made ready for fistulation by purse string sutures in the least vascular pyloric area. An incision was made through the muscle, and a polyethylene cannula was inserted and secured by purse string sutures. The pylorus was clamped securely, the animal's head was raised slightly, and 2.5 ml. of the drug solution was instilled through the cannula into the stomach. Samples of 0.1 ml. were taken through the cannula at regular intervals. Throughout the experiments, the viscera were kept warm with a high-intensity lamp and moist with gauze and saline.

Drug Solutions—The compounds studied were the tritiated progestogens, norethindrone (I) and chlormadinone acetate (II). Tritiated steroid was coprecipitated with unlabeled steroid in cold acetone and water to make a specific activity of 4 mc./mmole. The dose per rat was 0.4 mg./kg., approximately 30 times the normal human dose. Because of the low aqueous solubility of these steroids, the 100-mcg. dose was dissolved in 1 ml. absolute ethanol. Just prior to the experiment, the alcohol solution was diluted with 9 ml. of Sørensen's buffer, pH 6.3, or 9 ml. of Clark and Lub's potassium chloride–hydrochloric acid buffer, pH 1.6, and made isotonic.

Water diffusion out of the intestine has a considerable effect after 45–60 min. Phenol red was used as a standard to correct for changes in steroid concentration resulting from water absorption (13).

The intestinal drug solution volume was 10 ml., although solutions of 8, 5, and 3 ml. were also used depending on the length of the intestinal section studied. The final ethanol concentration was 10%. In gastric studies, 2.5-ml. solutions of varying ethanol concentration were used. A double-tracer experiment was used to follow the absorption of ^{14}C-ethanol and ^{3}H-steroid simultaneously.

All samples of lumen contents, bile, and plasma were added to Bray's solution (14) and counted on a scintillation counter[2]. Several bile samples were pooled and extracted with ether. The drug remaining in the water phase was considered to be metabolite. No further determination of the metabolites was made. All data reported are total radioactivity. To account for quenching, a "spike" of ^{3}H-toluene was added to each vial and the vial was recounted. The

[1] Clay-Adams Intramedic PE 50 polyethylene tubing.

[2] Packard Tri-Carb scintillation counter, model 3365.

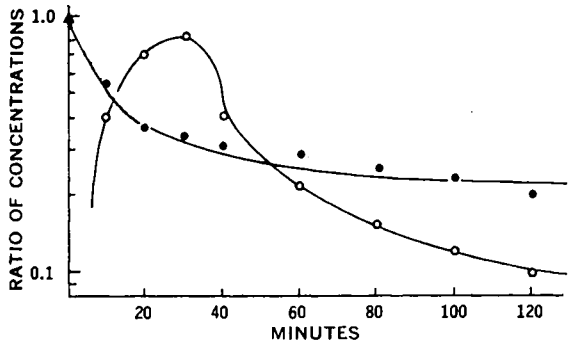

Figure 2—*Time course of norethindrone in the stomach* (●) *and its appearance in the bile* (○).

disintegrations per minute and amount of drug recovered from each sample were calculated and used to determine the percent dose remaining in the gut or entering the bile.

Site of Accumulation—Experiments were done to determine if steroids are absorbed uniformly in all sections of the GI tract. Drug solutions (25 mcg./2.5 ml. or 100 mcg./5 ml.) were instilled into the stomach or the ligated duodenum, jejunum, or ileum. The solutions remained in the stomach 30 min. before the rat was sacrificed. The stomach was removed, washed, and divided into four parts: cardiac, pyloric, and two fundus portions. In the intestinal experiments, the animals were sacrificed 1 hr. after the drug solution was instilled into the ligated section. The intestine was excised, washed with saline, and cleaned of mesentery and fat. The intestinal sections were divided into 1-cm. pieces.

All tissue sections were dried overnight at 60° and combusted using a tissue oxidizer[3]. The samples were assayed for tritium by scintillation counting.

Bile Effect—The intestinal absorption of each steroid was studied with and without bile duct cannulation. To investigate the effects that bile might have in drug absorption, 1 ml. of the exogenous bile salt, sodium dehydrocholate (100 m*M*), was used to stimulate bile flow in uncannulated animals. The bile salt was administered by placing the solution in the exposed peritoneal cavity beneath the portal vein immediately prior to injecting the drug solution. Drug was then instilled into the duodenum; bile and lumen samples were taken as previously described.

To extend the bile study, dual bile duct cannulation experiments were devised. Two rats were prepared as described in the previous section and both bile ducts were cannulated. Rat 1 supplied fresh bile to Rat 2 by placing the first cannula into the duodenum of Rat 2. In the first experiment, drug solutions were instilled into the duodenum of Rat 2 with bile samples collected from Rat 2. In the second experiment, drug solutions were placed in the duodenum of Rat 1 with a few bile samples from Rat 1 and continuous bile sampling from Rat 2.

Binding Affinities of Steroids to Gastric Juices—Human gastric juice and gastric mucin[4] were used to determine the possible effect of steroid binding on absorption characteristics. Human gastric juice (pH 1.6) was obtained from hospital patients undergoing surgery for other than GI ailments. Simulated gastric fluid was made from 2.6 g./l. mucin, 2.0 g./l. NaCl, and 80 ml./l. concentrated HCl. The final pH was 1.3. By using a microequilibrium dialysis apparatus, 100 μl. of drug solution in Sørensen's buffer was placed in one well-half and 100 μl. of binding substance was placed in the other, separated by cellulose dialysis membrane. The drug solutions ranged from 10^{-10} to 10^{-6} *M*.

The dialysis apparatus was shaken for 24 hr. at 37°. Samples of 50 μl. were taken from each well-half with a syringe[5]. The samples were placed in Bray's solution for scintillation counting. The binding constants and bound-to-free ratios were determined by a computer program written from Scatchard's original binding calculations (15). Human serum albumin was used as a standard.

Partition Coefficient—The partition coefficients of ^{3}H-chlormadinone acetate and ^{3}H-norethindrone were determined in a two-phase system of 10% ethanol–Sørensen's buffer at pH 6.3 and isopropyl myristate. The phases were first equilibrated at 37° to ensure saturation of each phase with the other. The drug solution (0.1 μc. in 1 μl. ethanol) was added to the ethanol–buffer solution. The phases were shaken 5 min. and stored overnight at 37°. Duplicate 1-ml. samples were taken from each phase and assayed by scintillation counting. Four separate determinations were made.

[3] Packard Tri-Carb oxidizer.
[4] Nutritional Biochemical Corp., Cleveland, Ohio.
[5] Hamilton.

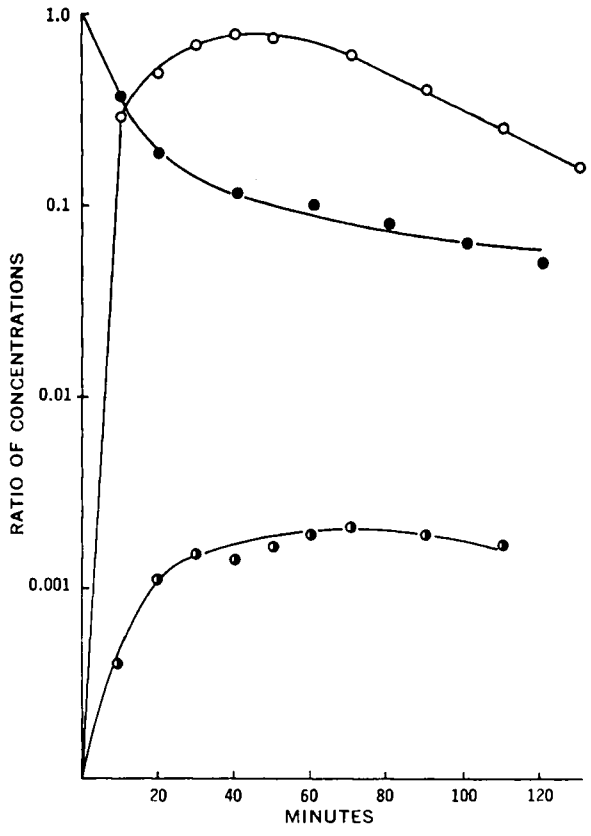

Figure 3—*Time course of chlormadinone acetate in the intestine* (●) *and its appearance in the plasma* (◑) *and the bile* (○).

RESULTS

In the present experiments, the absorption of two steroids from the stomach and small intestine of the rat was studied under physio-

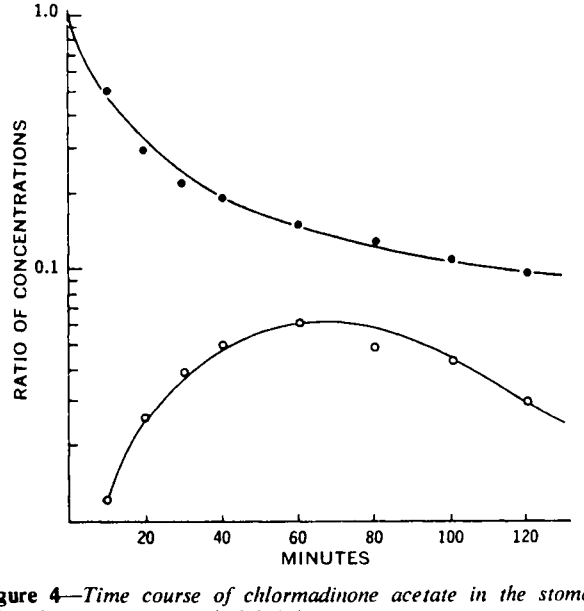

Figure 4—*Time course of chlormadinone acetate in the stomach* (●) *and its appearance in the bile* (○).

Table I—Intestinal and Gastric Absorption Data

Drug and Vehicle	Animal Number	$t_{1/2}L$, min.	$t_{1/2}B$, min.	Time to Apparent Second Phase, min.	Time to Bile Peak, min.	Plasma Maximums, mcg./ml. (min.)
			Intestinal			
Norethindrone and 10% ethanol-buffer	1	5	8	21	20	0.95 (20)
	2	6	8	24	15	1.1 (20)
	3	5	7	23	21	0.92 (15)
	4	8	8	24	20	0.98 (20)
Chlormadinone acetate and 10% ethanol-buffer	2	4	—	10	—	0.31 (30)
	5	4	10	15	15	0.29 (30)
	6	3	12	10	20	0.25 (35)
	8	4	10	11	18	0.48 (33)
	10	3	11	13	18	0.20 (35)
	11	4	10	14	15	0.19 (35)
			Gastric			
Norethindrone:						
10% ethanol-buffer	5	6	11	30	15	
	2	11	14	30	30	
	3	13	14	30	30	
5% ethanol-buffer	1	10	—	30	40	
	4	13	36	45	45	
0% ethanol-buffer	6	11	15	15	15	
Chlormadinone acetate:						
10% ethanol-buffer	1	16	—	45	—	
	2	7	na[a]	45	55	
	5	10	na[a]	45	30	
20% ethanol-buffer	6	6	na[a]	45	60	
30% ethanol-buffer	8	6	60	45	60	

[a] na = not attained.

logical conditions. The absorption curves showed that the absorption of these progestins was not described by simple first-order kinetics but was biexponential in all sections of the gut. This absorption was not only dependent on the concentration of drug in any one tissue compartment but was also regulated by the membrane structure, apical and basal; by the mesentery blood flow, pressure, and volume; and by tonicity of the instilled solution.

Chlormadinone acetate and norethindrone were readily absorbed from both the intestine and the stomach, as measured by the appearance in the plasma and bile and by the disappearance from lumen and gastric contents. Both steroids showed biexponential disappearance from the gut.

As shown in Fig. 1, when 0.1 mg. norethindrone in 10% ethanol-buffer solution was instilled into the intestinal lumen, the time to reach the apparent second phase of absorption was approximately 20–25 min. The maximum concentration of drug in the bile was also found within this time range. The time required for the original drug concentration to decrease to half in the lumen ($t_{1/2}L$) was less than 10 min.; 8–10 min. was required for one-half the original drug concentration to appear in the bile ($t_{1/2}B$). These data were corroborated by experiments conducted in another laboratory using the Doluisio technique[6]. The $t_{1/2}L$ was 10 min. in those intestinal absorption experiments.

In the stomach, the absorption of norethindrone was not as rapid or as complete as from the intestine (Fig. 2); $t_{1/2}L$ ranged from 10 to 13 min., and $t_{1/2}B$ was about 15 min. The time to reach the apparent second phase of lumenal absorption was 20–30 min., and the peak drug concentration in the bile appeared at 30–40 min.

Chlormadinone acetate was absorbed slightly more rapidly than norethindrone (Figs. 3 and 4). In the intestine, the $t_{1/2}L$ was less than 5 min. and the time to reach the apparent second phase was 10–15 min. The bile concentration peaked at 15–20 min., with one-half the original drug concentration appearing in the bile from 9 to 12 min. Chlormadinone acetate absorption from the stomach was considerably less than from the intestine. The $t_{1/2}L$ ranged from 6 to 16 min. The drug concentration in the bile reached a maximum value at 50–60 min., but the biliary concentration did not reach one-half the original lumen concentration. This low response of the liver to the clearance of chlormadinone acetate from the blood is quite remarkable when compared to the rapid $t_{1/2}B$ reported for norethindrone. Table I is a summary of the absorption data.

From the lumenal and plasma data, rate constants were determined. The feathering method was used to determine the rate constants from graphical estimations as shown in Fig. 5.

There is apparently no unique site of absorption for these steroids

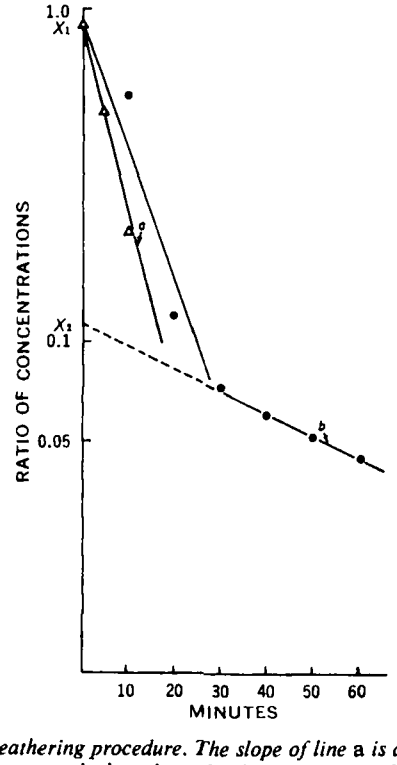

Figure 5—*Feathering procedure. The slope of line* a *is determined by extending the second phase* b *and subtracting second phase values from the first phase values.*

[6] J. T. Doluisio, College of Pharmacy, University of Kentucky, Lexington, Ky., personal communication.

Table II—Site of Accumulation

Section	Disintegrations per Minute Ratio[a]
Intestinal	
Duodenum	4–9
Jejunum	2–3
Ileum	1
Gastric	
Cardiac	538–563
Pyloric	473–491
Fundus	1

[a] The ratios include values for ^{3}H-norethindrone and ^{3}H-chlormadinone acetate obtained from eight rats. Both steroids behave similarly with respect to the location of accumulation.

in the stomach or in the intestine, but there is a possibility that the stomach is more selective. Compared to the fundus sections, the pyloric and cardiac regions of the stomach exhibited considerable affinity to norethindrone and chlormadinone acetate. The large amount of drug absorbed by the pyloric region is easily understood because of the ample supply of blood and the binding properties of both drugs to the gastric fluids produced there. This does not explain the high accumulation observed in the less vascular area of the cardiac region. In the intestine, the duodenum showed greater affinity for the steroids than did the jejunum, and the ileum absorbed drugs to the least extent. Along with the tissue combustion data and the biexponential disappearance of the steroids from the lumen, these results led to the conclusion that accumulation or storage of the drugs in the intestinal or gastric membranes was occurring. This can account for the rapid distribution of drugs from the lumen solution into the membrane, with a slower appearance of the drug into the plasma (Table II).

The binding studies (Table III) were designed to show any correlation between absorption rates and affinities of steroids to gastric juice and gastric mucin. It is obvious from these data that chlormadinone acetate bound much more to the gastric mucin and natural gastric juice than did norethindrone. Binding of norethindrone appears to occur mainly with gastric mucin, whereas the binding of chlormadinone acetate is only partially accounted for by mucin. Chlormadinone acetate possibly binds to other components of

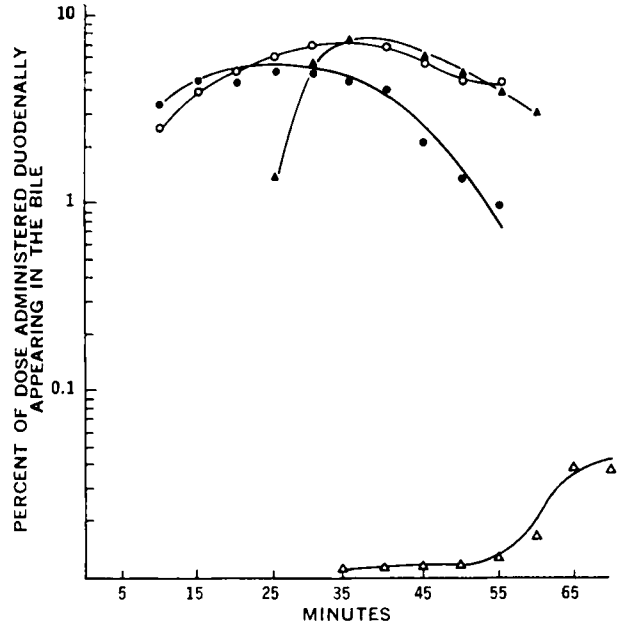

Figure 6—*Metabolite appearance in the bile. No change was seen in metabolite output when norethindrone was instilled into the duodenum with and without bile (●, dual cannulation; ▲, bile cannulation). When chlormadinone acetate was instilled in the presence of bile, the drug was metabolized to a great extent (○, dual cannulation; △, bile cannulation).*

Table III—*In Vitro* Binding Properties of Chlormadinone Acetate and Norethindrone

Drug	Binder	Ratio Bound/ Free	Percent Bound
Norethindrone	0.4% Human serum albumin	0.92	48.0
		1.09	52.2
		1.38	58.1
	Natural gastric juice	0.07	6.6
		0.10	9.2
		0.13	11.4
	Synthetic gastric juice	0.08	7.0
		0.04	3.8
		0.18	14.9
Chlormadinone acetate	0.4% Human serum albumin	1.19	54.3
		1.31	56.7
		1.43	58.8
	Natural gastric juice	0.68	40.6
		0.69	41.0
		0.83	45.6
		1.01	50.3
	Synthetic gastric juice	0.34	25.5
		0.41	28.9
		0.42	29.4

natural gastric juice such as chondroitin sulfate or other low molecular weight mucoproteins which serve as the "intrinsic factor."

Extending the *in vitro* comparison of chlormadinone acetate and norethindrone in GI absorption, the partition coefficients of the two steroids were found to be 65 for chlormadinone acetate and 48 for norethindrone. The partition coefficient data support results previously given in this paper which imply that chlormadinone acetate is absorbed to a greater extent and at a faster rate than norethindrone in gastric preparations. The *in vivo* absorption rates did not indicate such a large difference as might be expected by the differences in the partition coefficients and binding affinities. Consequently, the estimation of *in vivo* absorption rates from the partition coefficient and other *in vitro* methods should be made with caution.

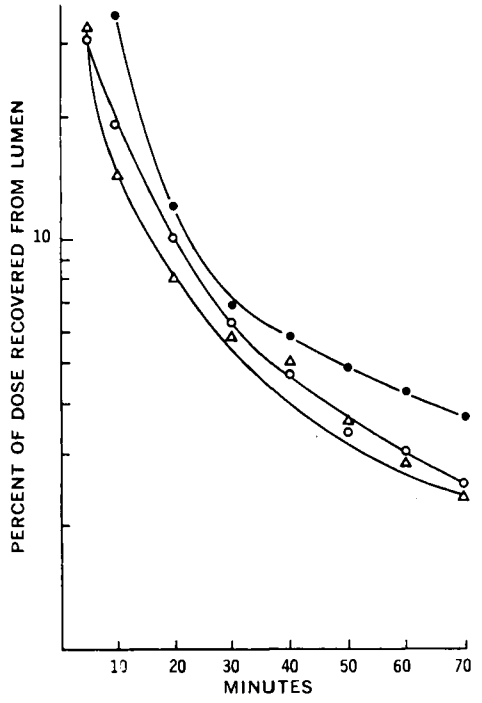

Figure 7—*Intestinal absorption of norethindrone under various conditions. Key: ○, no bile cannulation or stimulation; ●, animals with bile duct cannulation; and △, animals with exogenous sodium dehydrocholate stimulation. The intestinal absorption is affected only slightly.*

It is recognized that bile salts play an important role in the intestinal absorption of lipids and other substances and that they themselves are absorbed by active transport in the ileum. Because of the bile cannulation in the preparations, absorption was unaided by bile salts in most experiments. However, several experiments were devised to ascertain the effects of bile on chlormadinone acetate and norethindrone absorption. The bile appeared to have a significant effect on the absorption and metabolism of chlormadinone acetate, but it did not appear to influence norethindrone. As seen in Fig. 6, bile greatly enhanced the output of chlormadinone acetate or its metabolite from the liver. In cannulated animals, less than 1% radioactivity was recovered in the bile as compared to 40% recovered after the intestine received fresh bile from the donor rat. Rates of lumenal absorption were not altered significantly by the presence or absence of bile. Bile flow stimulation through the use of exogenous sodium dehydrocholate did have an effect on the lumenal absorption rates of chlormadinone acetate and norethindrone, but the enhanced absorption was not significant (Fig. 7).

These results are in contrast with data obtained previously (16) using sulfadiazine, which showed the exogenous stimulation greatly increased the bile acidity and consequently the solubility of sulfadiazine. This result possibly increased the absorption of the drug. Kakemi *et al.* (17) found from perfusion experiments that unionized and poorly absorbed drugs are hardly affected by bile salts. This is consistent with the findings on the two progestins. Kakemi *et al.* also suggested that any enhanced absorption of poorly absorbed drugs may be due to the bile salt action on the mucosal membrane and not to the direct drug–bile salt interaction.

Because the steroids were administered in an alcoholic solution, experiments were conducted to determine the effect of the vehicle. The percent of ethanol in the drug solutions was varied, and simultaneous ^{14}C-ethanol–^{3}H-drug solution absorption curves were plotted. In the gastric preparations, the variation of ethanol in the drug solutions did not have an effect on the drug absorption; the "solubilizing function" proposed by Tappeiner (18) in 1880 was ruled out. In some instances, however, there appeared to be a delay in the appearance of metabolites in the bile. Ethanol is known to cause irritation to organic tissue. In concentrations exceeding 15% in the stomach, the solution was so strong that marked congestion and ulceration often occurred. It is possible, therefore, that the delay in metabolite appearance was the result of ethanolic irritation of the liver and interaction with drug metabolism as well. In the few experiments done, no evidence was found to indicate any difference whatsoever in the intestinal absorption of chlormadinone acetate and norethindrone using ethanol concentrations of 0–15%. Magnussen (19) reported that ethanol aids gastric absorption of only a few drugs, these drugs being already well absorbed without ethanol.

The absorption of the drugs is not affected by the presence or absence of ethanol or bile. Chlormadinone acetate does show greater binding properties, with higher partition coefficient values and greater general intestinal absorption, than norethindrone. According to Schedl and Clifton (20), the absorption is predictable from the chemical structure: the less polar the steroid, the greater is the absorption rate. The wall of the intestinal wall is lipoidal and substances that are lipid soluble pass into the wall more readily than do some hydrophilic compounds. The structures of norethindrone and chlormadinone acetate are so similar that this comparison is difficult to make. However, chlormadinone acetate is slightly more hydrophobic, resulting in the small differences seen in the GI absorption of the two steroids.

The data from the intestinal absorption experiments show a biexponential profile which suggests that the drug is rapidly distributed between the gut lumen and the intestinal wall, followed by a slower release of the drug from the membranes into the blood. The initial absorption of the drugs into the intestinal membranes and the consequent distribution into the blood is not simple first-order kinetics. The absorption did appear to be *via* diffusion without an energy requirement or the mediation of an enzyme system.

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Determination of Bioavailability of Digitoxin Using the Radioimmunoassay Procedure

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Abstract □ A commercial digitoxin radioimmunoassay procedure, normally used to evaluate equilibrium-state plasma levels, was modified such that the sensitivity was increased 20-fold. Due to the extensive metabolism of digitoxin in man, the specificity of the assay procedure for digitoxin was determined against several known metabolites. These studies indicated that the assay procedure was more sensitive to digoxin and the monodigitoxoside and bisdigitoxoside of digitoxigenin than to digitoxin itself. It also had a lesser degree of sensitivity to the aglycone digitoxigenin. For the bioavailability study, single 0.1-mg. doses of digitoxin were administered orally, as one of two commercial brands of tablets, to a panel of eight subjects in a crossover study with 56 days separating the two doses. Plasma levels were measured for 15 days following dosing. Significant differences in the plasma levels between brands, as determined by analysis of variance, were found at 0.5, 1, 72, and 240 hr. The peak plasma levels were found to differ significantly. No statistical difference in the relative bioavailability, as measured by the area under the plasma level curve, was found between the two tablets tested.

Keyphrases □ Digitoxin plasma levels—radioimmunoassay determination, two commercial tablets, man □ Bioavailability, two commercial digitoxin tablets—determination of plasma levels, man □ Radioimmunoassay technique—determination, digitoxin plasma levels, two commercial tablets, man

During the past few years, this laboratory has been involved in a continuing effort to study the generic equivalence or inequivalence of marketed drug products. Past studies have included ephedrine (1), warfarin (2), and propoxyphene (3).

The importance of uniform dosage and bioavailability is perhaps epitomized by the cardiac glycosides as with no other series of therapeutic agents, because these agents produce such a strong inotropic effect on the heart and frequently represent the difference between life and death for many patients suffering from congestive heart failure. Since digitoxin is known to have a long half-life, it is particularly important that uniform dosage and bioavailability limits are maintained. These difficulties are further complicated by the fact that a fairly low dose of the cardiac glycoside is used to achieve the desired therapeutic results, and manufacturing tablets with uniform drug content apparently is difficult when such low levels of medication are required. The manufacturing difficulties are evidenced by the number of tablets of various cardiac glycosides that have been recalled during a 1-year period. As determined from the "Drug Recall" section of the *Journal of the American Pharmaceutical Association* during the period of June 18, 1970, to June 16, 1971, approximately 25 million tablets containing cardiac glycosides were recalled by the Food and Drug Administration (FDA).

Assay methodology to perform adequate bioavailability studies on digitoxin tablets was lacking, since assay sensitivity was usually not sufficient to follow plasma levels long enough to measure bioavailability. With the advent of the red blood cell ^{86}Rb-uptake assay (4) and the Na,K-ATPase inhibition procedure (5), new levels of assay sensitivity for digitoxin were achieved. Nonetheless, both procedures are too impractical for routine assays as are needed for a bioavailability study. The development of the radioimmunoassay procedure for digitoxin (6) unquestionably provided the researcher with an assay technique that could be made sufficiently sensitive to measure the levels of drug for an adequate length of time to determine the bioavailability of a single dose of digitoxin.

This report is the first in a series on the bioavailability of digitoxin and digoxin commercial tablets. The commercial radioimmunoassay[1] procedure, normally used to determine equilibrium-state plasma levels for patients receiving the medication chronically, was modified and the sensitivity was increased at least 20-fold to achieve the necessary assay sensitivity for a single-dose bioavailability study. Thus, a reproducible sensitivity of 0.2 ng./ml. was achieved. In the study reported here, plasma levels of digitoxin and its metabolites were measured in eight normal volunteers in a crossover study for 15 days following the administration of a 0.1-mg. dose of the drug. The innovators' product was compared to one other commercial digitoxin tablet USP.

EXPERIMENTAL

The radiotracer, antiserum, standard digitoxin solution, and buffer components used for this radioimmunoassay procedure were all obtained from the radioimmunoassay kit[1]. The dextran-coated charcoal[2] suspension was prepared from laboratory materials. The assay samples were counted in a liquid scintillation spectrometer[3], using a liquid scintillation cocktail[4]. The reference standard used for preparation of a quench curve was standard tritiated water[5], and the most suitable quenching agent for the scintillation cocktail was found to be chloroform. All other chemicals used were reagent grade and were used as received.

Study Conditions and Subjects—Both brands of tablets used conformed to the USP XVIII requirements with respect to identity, disintegration time, content, content uniformity, and other digitoxosides. Individual tablets of Treatment A[6] were assayed by FDA and were found to contain an average of 101.3% of label, with a range of 98.6–104.9%. Treatment B[7] tablets were also assayed at FDA laboratories and were found to contain an average of 96.5% of label, with a range of 89.7–103.5%.

The screening method used for this study was previously reported by Wagner *et al.* (2). The subjects selected were eight normal males from 22 to 30 years of age. The subjects were arranged into two groups of four as follows: Group I, Subjects 1–4, weight 54.4–78 kg., age 22–29 years; and Group II, Subjects 5–8, weight 81.2–93 kg., age 24–30 years.

[1] Tritiated-digitoxin Radioimmunoassay Kit, Schwarz/Mann, Orangeburg, NY 10962

[2] Dextran T70, Pharmacia, Uppsala, Sweden; and Norit A charcoal, Sigma Chemical Co., St. Louis, Mo.

[3] Packard Tri-Carb, model 3320.

[4] Unogel, Schwarz/Mann, Orangeburg, N. Y.

[5] New England Nuclear, Boston, Mass.

[6] Treatment A was one tablet of Purodigin, 0.1 mg., Wyeth, Lot 1693870.

[7] Treatment B was one tablet of digitoxin USP, 0.1 mg., Squibb, Lot 9L695.

The protocol was as previously reported by Wagner *et al.* (2) with some modifications. The changes in protocol were related to the changes in medication, sampling times, and period of treatments. First, samples of whole blood were taken from a forearm vein at time zero, 0.5, 1, 2, 4, 6, 12, 24, 72, 120, 240, and 360 hr. after dosing. The two doses administered in the crossover study were separated by 56 days or approximately 9–10 half-lives of elimination.

During Phase I of the study, Subjects 1–4 received a dosage form designated as Treatment A[6], while Subjects 5–8 received Treatment B[7]. Fifty-six days later, Subjects 1–4 received Treatment B and Subjects 5–8 received Treatment A.

***In Vitro* Dissolution Studies**—The dissolution of individual tablets was conducted in a 1-l. three-necked flask containing 500 ml. of distilled water maintained at 37°. A single-bladed, Teflon stirrer was rotated at 50 r.p.m., and 10-ml. samples were removed at predetermined time periods with immediate replacement with fresh solvent. At least four dissolution trials were conducted for each brand of tablets.

The samples were analyzed by the fluorometric method of Wells *et al.* (7) in a spectrophotofluorometer[8], using an activation wavelength of 395 nm. and an emission wavelength of 570 nm. The procedure was used to analyze quantitatively aqueous solutions containing 0.01–0.25 mcg./ml. of digitoxin. With different sets of reagents, the slope varied slightly; therefore, a separate calibration curve (0.2–8.0 ng./ml.) was run with each individual dissolution trial. All assays were carried out at least in duplicate.

Radioimmunoassay—The procedure used for the analysis of total digitoxin derivatives in plasma was a modification of the one described in the commercially available digitoxin radioimmunoassay kit[1]. The commercial procedure essentially was that reported earlier by Smith (6).

Whole blood, obtained by venipuncture, was collected from patients in vacutainers containing sodium citrate as an anticoagulant. The plasma samples were stored in capped polystyrene test tubes at −15° for up to 2 weeks prior to assay. It had been previously determined that these storage conditions did not adversely affect the assay of digitoxin.

Two significant changes were made in the recommended procedure. The modifications involved changing the ratio of the tracer to antiserum to obtain the necessary assay sensitivity in the concentration range of 0.2–8.0 ng./ml. Yalow and Berson (8) discussed the factors involved in the antigen–antibody binding process and gave an excellent background discussion for the radioimmunoassay procedure. Also, since it was necessary to change the quantity of plasma used in the assay procedure to 1 ml., the concentration of charcoal in the dextran-coated charcoal suspension had to be changed. The factors that determine the amount and type of adsorbent used in radioimmunoassay procedures were discussed by Herbert *et al.* (9). The charcoal suspension used in the experiment described in this paper consisted of 250 mg. dextran and 10 g. charcoal in 100 ml. of pH 7.4 phosphate-buffered saline. The phosphate-buffered saline was prepared from the kit material, and the pH was adjusted as described in the commercial kit.

The assay procedure involved the following steps. To 1 ml. of patient plasma or plasma spiked with digitoxin standard solution, 10 μl. of antiserum and 5 μl. of tracer were added. To the blank plasma sample, only 5 μl. of tracer was added. Calibrated Lang Levy constriction pipets[9] were used to measure all microliter quantities, because automatic pipeting devices were found to be too inaccurate. The material was then gently mixed on a vortexer and allowed to incubate at ambient temperature for 30 min. Following the incubation, 0.5 ml. of the dextran-coated charcoal suspension was rapidly added to each sample, the material was vortexed gently, and after 5 min. the material was centrifuged at 1500×*g* for 20 min. The clear supernate was then decanted into 15 ml. of liquid scintillation-solubilizer fluid and counted in a liquid scintillation counter.

A quench correction for all samples was made through use of an automatic external standard, and a standard curve was prepared from reference standard tritiated water[5]. The data obtained were then corrected to disintegrations per minute, and the percent of tritiated digitoxin bound to the antiserum could be calculated. The percent bound was then plotted against the concentration of unlabeled digitoxin in solution to obtain the standard curve shown in Fig. 1. In addition to the patient samples and the standards determined with each assay group, it was also necessary to determine the appropriate blanks (consisting of all components except antiserum), total counts for the tracer added (consisting of adding 5 μl. of the tracer directly to the liquid scintillation flask), and the total amount of tracer bound by the antiserum in the absence of unlabeled drug, which in this case was 30–35%. The total count value was used as a control for the number of counts added to each sample, and the blanks were used as a background value for correction of all counts obtained for the assayed samples.

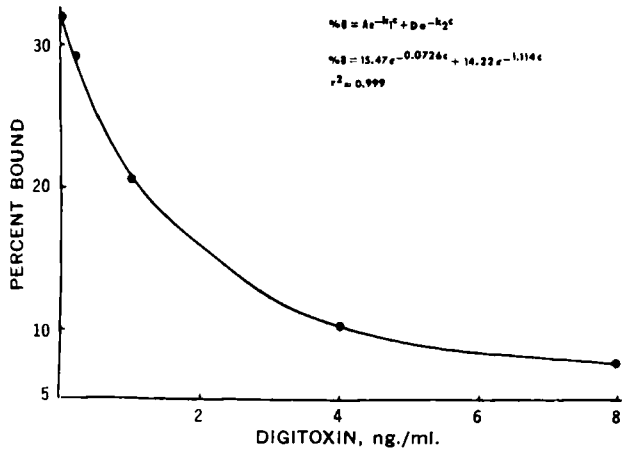


Figure 1 – *Example of typical calibration curve determined for each set of samples analyzed.*

An example of a typical calibration curve is shown in Fig. 1. Each curve was fitted to a biexponential equation using the program NONLIN[10] on a computer[11]. In all cases, the fits were essentially perfect, giving a coefficient of determination[12] of at least 0.999. An example of the equation used for the calibration curves and the parameter estimates for the sample calibration curve shown are found inset in Fig. 1. Since all samples were assayed in duplicate, it was possible to calculate a mean normalized difference between duplicates in terms of percentages. In all, 352 samples (not including calibration curves) were analyzed. The mean normalized difference between duplicates was 12.3% with a standard deviation of 14.1%. This value represents the actual differences found from day to day for all patient samples and at all concentrations levels.

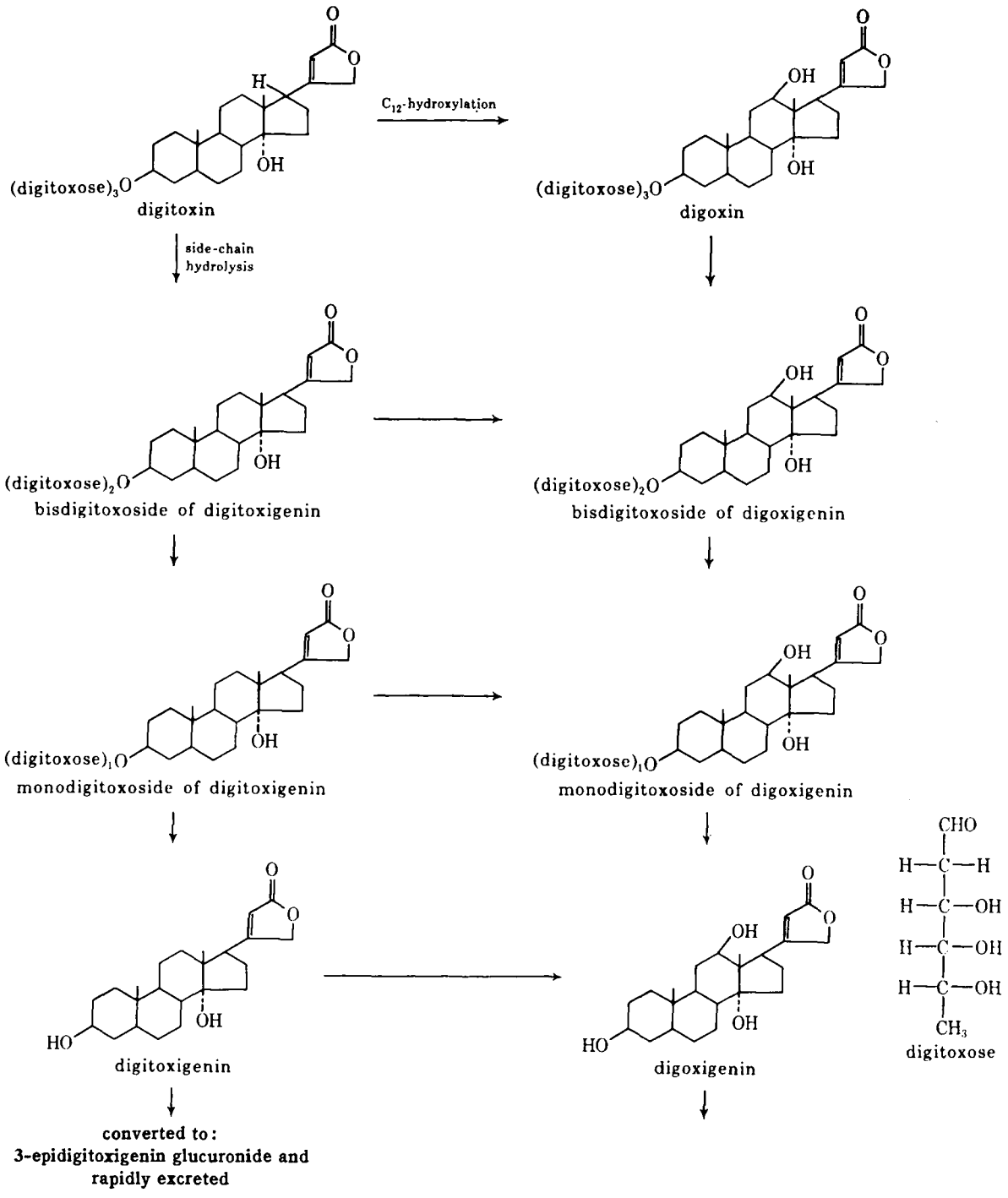
Cross-Reactivity Studies Since it has been noted that digitoxin is extensively metabolized in man and other species (10–13), it was deemed necessary to evaluate the binding of the major digitoxin metabolites with the antiserum under the assay conditions. Thus, the degree of binding of digoxin[1], the bisdigitoxoside[13] and the monodigitoxoside[13] of digitoxigenin, and digitoxigenin[13] with the antiserum was studied. Since these compounds interact with the antiserum on a molecular basis, the comparison of their reactivity with the antiserum was made on a molar basis. Previous studies (6, 14) showed that most endogenous steroid compounds produce virtually no interference with the digitoxin antiserum. Usually, levels of 1000 10,000 ng./ml. of progesterone or testosterone were necessary to inhibit antibody binding of tritiated digitoxin. In the present studies, approximately 20% of the blank serum samples produced small false-positive responses. The "apparent" concentration of drug in the blanks was usually calculated to be less than 0.1–0.2 ng./ml. For those subjects where such apparent positive blank values occurred, the percent tritiated digitoxin bound was corrected or normalized for all samples.

RESULTS

The scheme for the metabolism of digitoxin (Scheme I) was proposed by Lauterbach and Repke (15) following *in vitro* and *in vivo* studies with rats. A similar pattern for the metabolism of digitoxin

[8] Aminco-Bowman, model 4-8166, American Instrument Co., Silver Spring, Md.
[9] A. H. Thomas Co., Philadelphia, PA 19105

[10] The program NONLIN was supplied by Dr. C. M. Metzler, The Upjohn Co., Kalamazoo, Mich.
[11] IBM 360/67.
[12] Coefficient of determination = $r^2 = (\Sigma obs^2 - \Sigma dev^2)/\Sigma obs^2$.
[13] Boehringer Mannheim Corp., New York, N. Y.



Scheme I—Proposed metabolic scheme for digitoxin by Lauterbach and Repke (15)

in man has evolved primarily through the works of Okita and coworkers (12, 13), Ashley *et al.* (11), Hermann and Repke (16), and Repke (17). Therefore, the effects of these various metabolites on the analysis of digitoxin in plasma were determined.

Figure 2 shows the results of the studies comparing the effect of nonradiolabeled digitoxin and digoxin on the binding of ^{3}H-digitoxin with the antiserum. It is apparent that, on an equimolar basis, digoxin displaces the tracer from the antiserum to a larger extent than digitoxin itself. This was not too surprising since the antiserum used was prepared by challenging rabbits with a digoxin-albumin conjugate; thus, such cross-reactivity was anticipated. Although the relative proportions of digitoxin to digoxin necessary to produce the same percent of tracer bound to antiserum are variable, for most of the curve it takes approximately four times as much digitoxin as digoxin to produce the same decrease in the percent tracer bound.

A separate study was undertaken to determine the sensitivity of the radioimmunoassay to the various digitoxigenin derivatives of digitoxin, and all of the metabolites interacted to a varying degree with the antiserum (Fig. 3).

Figure 4 is a plot of the *in vitro* dissolution data for the two brands of tablets used in the *in vivo* studies. The curves for each brand were obtained from the average data of four dissolution trials. The vertical bars represent 1 standard deviation on either side of the mean. The results of the analysis of variance for the dissolution of the two dosage forms are shown in Table I.

Table II presents the average plasma levels of digitoxin and its metabolites obtained at each indicated time period. The values reported are the mean plasma levels of the eight subjects. Also shown are the average peak plasma concentrations and the average elimination half-lives. The area under the plasma level–time curve, shown

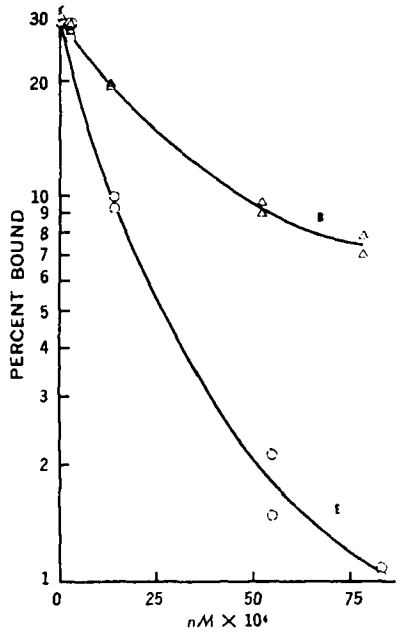


Figure 2—*Effect of digoxin on digitoxin radioimmunoassay. Key: B, △, digitoxin; and E, ○, digoxin.*

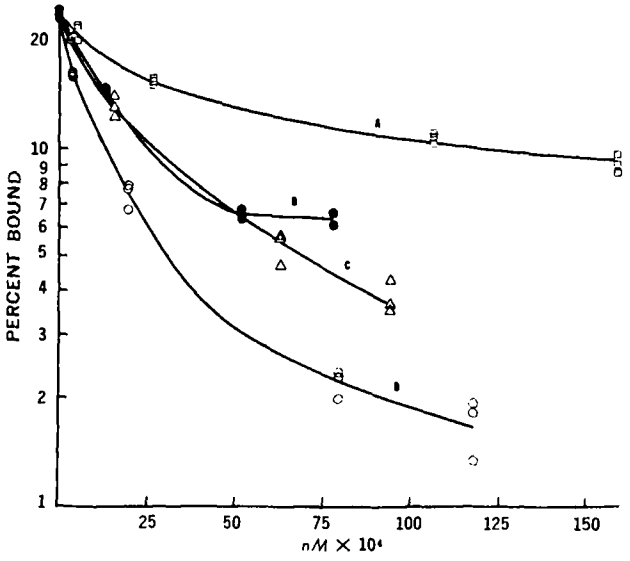


Figure 3—*Affinity of various digitoxigenin metabolites of digitoxin for antiserum in commercial radioimmunoassay procedure. Key: A, □, digitoxigenin; B, ●, digitoxin; C, △, bisdigitoxoside of digitoxigenin; and D, ○, monodigitoxoside of digitoxigenin.*

as average area in this table, is an indication of the relative availability of each dosage form. Data for the 0–360-hr. period and the estimated area from zero to time infinity are also reported.

DISCUSSION

The metabolic pathway shown in Scheme I was developed primarily from urinary excretion data. Very little information is available on the blood levels of these various metabolites, primarily because of the extremely low levels of drug and metabolites obtained following normal doses of digitoxin. This problem has not been satisfactorily resolved to date. However, urinary excretion studies have indicated that digitoxin is extensively metabolized in man and, as reported by Okita (12), only approximately 8% of an administered dose of ^{14}C-digitoxin was recovered as unchanged drug in the urine over a 3-week period; approximately 70% of the dose was recovered as metabolic products over the same time interval (12). A major component of the metabolic products was determined to be digoxin (12).

Since the kit[1] used in this study had an antiserum that was prepared by challenging rabbits with a digoxin albumin conjugate, it was apparent that some cross-reactivity would exist between digitoxin and its metabolite digoxin. Since all of the other metabolites shown in Scheme I have also been detected in urine, the possibility of their existence in the blood should be considered. As shown in Fig. 2, the antiserum exhibited a much greater affinity for digoxin than for digitoxin.

On an equimolar basis, the ability of each of the digitoxigenin derivatives to interfere with the ^{3}H-digitoxin antiserum binding process was found to be the monodigitoxoside of digitoxigenin > the bisdigitoxoside of digitoxigenin ≃ digitoxin > digitoxigenin (Fig. 3). Thus, all metabolites of digitoxin tested so far would in a very real sense interfere with the analysis of digitoxin, depending on the relative proportions of each in the blood. It would appear that the level of digitoxigenin would be extremely small since it has been reported that this material is rapidly conjugated and excreted. Also, since this compound has the least affinity for the antiserum, it would contribute very little in the analysis of digitoxin in plasma. However, both the monodigitoxoside and bisdigitoxoside of digitoxigenin would markedly affect the radioimmunoassay of digitoxin in plasma. The digoxigenin derivatives (Scheme I) were not studied in this experiment. However, recent studies in these laboratories with the digoxin radioimmunoassay procedure indicated that these derivatives have essentially the same affinity for the antiserum as digoxin itself. Thus, again depending upon their plasma levels in man, these components could potentially interfere with the analysis of digitoxin. Having verified that many of these substances have at least the same or greater affinity for the antiserum as digitoxin itself, it can be concluded that the digitoxin radioimmunoassay procedure used in this study was not specific for just digitoxin and most likely was actually a measure of total drug and metabolites in the plasma.

It is felt that this information in no way detracts from the clinical usefulness of the radioimmunoassay procedure. It is still the most sensitive, convenient, and economical procedure available at present for measuring cardiac glycosides in the blood. It has been shown (18) that all of the metabolites are cardioactive to varying degrees in both the guinea pig and the cat. Digitoxin and the monodigitoxoside and bisdigitoxoside of digoxigenin were approximately equipotent. Digitoxigenin itself was only about one-half as potent as digitoxin. The same is true for digoxin and the monodigitoxoside and bisdigitoxoside of digoxigenin, which have about the same potency, with digoxigenin having only approximately one-tenth the activity of digoxin. Nonetheless, all important metabolites of digitoxin ap-

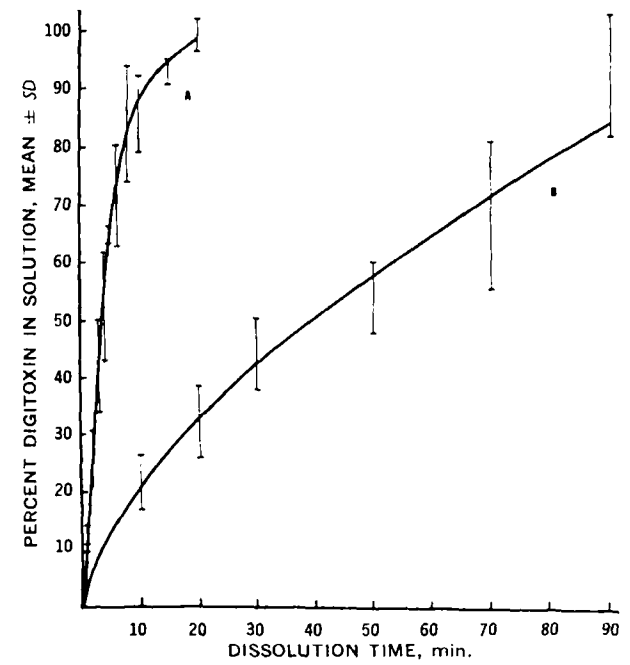


Figure 4—In vitro *dissolution of two commercial digitoxin tablets in distilled water at 37°, Treatments A and B.*

Table I—Summary of Analyses of Variance of $t_{25\%}$, $t_{50\%}$, and $t_{75\%}$ Values for Two Brands of Digitoxin Tablets Supplied by FDA

Brand	Brand[b] Average $t_{25\%}$[a], min.	$t_{50\%}$[a], min.	$t_{75\%}$[a], min.
Treatment A	1.9	3.9	6.7
Treatment B	14.6	46.5	74.1
F-value from ANOVA	26.17[c]	119.96[c]	141.50[c]
Significance level	$0.025 > p > 0.01$	$0.055 > p > 0.001$	$0.005 > p > 0.001$

[a] $t_{25\%}$, $t_{50\%}$, and $t_{75\%}$ are the times in minutes necessary for dissolution of 25, 50, and 75% of the total tablet contents, respectively. [b] Each average is based on four tablets drawn randomly from the same bottle. [c] Significant.

Table II—Summary of Average Plasma Concentrations of Digitoxin

Hours	Average Plasma Concentration, ng./ml. Treatment A	Treatment B	—From Analysis of Variance for Crossover Design— Coefficient of Variation[a]	Significant Level of Differences between Averages[b]
0.5	5.4	2.3	52.5	Sig. ($p < 0.001$)
1.0	5.4	4.1	24.8	Sig. ($0.01 > p > 0.005$)
2.0	3.3	3.0	19.0	N.S. ($p > 0.25$)
4.0	2.2	2.4	16.7	N.S. ($p > 0.25$)
6.0	1.9	1.9	20.5	N.S. ($p > 0.25$)
12.0	1.4	1.4	17.4	N.S. ($p > 0.25$)
74.0	1.5	1.3	22.2	N.S. ($0.25 > p > 0.10$)
72.0	1.3	1.0	22.1	Sig. ($0.05 > p > 0.025$)
120.0	1.0	0.9	27.4	N.S. ($0.25 > p > 0.10$)
240.0	0.7	0.5	31.2	Sig. ($0.025 > p > 0.01$)
360.0	0.5	0.5	42.2	N.S. ($p > 0.25$)
Average peak plasma concentration	6.6	4.6	30.1	Sig. ($0.005 > p > 0.001$)
Average area, 0–360 hr.	338.0	291.0	27.9	N.S. ($p > 0.25$)
Average estimated area, zero to infinity	467.0	446.0	41.5	N.S. ($p > 0.25$)
Average elimination $t_{1/2}$, days	8.03	9.85	32.9	N.S. ($p > 0.25$)

[a] Coefficient of variation (%) = $\frac{\sqrt{\text{residual mean square}}}{\text{grand average}} \times 100$. [b] Sig. = significant, and N.S. = not significant.

pear to be cardioactive and thus the assay procedure is a measure of the active drug in the blood following any given dose of digitoxin. However, pharmacokinetic analysis of digitoxin data cannot be carried out using this assay procedure unless the metabolites are separated from the parent compounds prior to analysis.

Tablets of two different manufacturers were subjected to *in vitro* dissolution tests to determine which tablets would be used for the *in vivo* bioavailability studies. The dissolution rate curves for the two tablets used in the studies are shown in Fig. 4. The dissolution of the Treatment B tablet was significantly slower than that of the Treatment A tablet, but in all cases dissolution was found to be complete within 2 hr. Table I shows the data for the average time required to dissolve 25, 50, and 75% of the digitoxin from the two tablet forms studied. As determined by analysis of variance, the differences between the average dissolution time at 25, 50, and 75% dissolved were highly significant.

Table II presents the average plasma levels of digitoxin and its metabolites obtained at each indicated time period. The differences between the average plasma levels of digitoxin during the first two sampling times of 0.5 and 1.0 hr. were highly significant. This finding correlates with the significant differences in the *in vitro* dissolution rate of the two tablets. Also, the average peak plasma concentration for the two tablets was found to be significantly different by analysis of variance for crossover design. However, the difference between the average areas under the plasma level curve following the two tablets was not statistically significant for either the 0–360-hr. period or the estimated area from zero to infinite time.

The data indicate that the differences in *in vivo* dissolution rates for the two tablets were not of sufficient magnitude to depress the relative bioavailability of the tablet that dissolves more slowly *in vitro*. The slower absorption rate and the consequent lower peak level, as noted in these studies, correlated well with the *in vitro* results.

As indicated in Table II, the average half-life, estimated from terminal plasma concentrations, was found to be 8.03 days for Treatment A. The range in half-lives for this dosage form for all eight patients varied between 6.5 and 9.9 days. In the case of Treatment B, the mean half-life was 9.84 days with a range of 5.0–13.7 days. Again, since the assay is nonspecific, this value is a measure of the total drug in the plasma. These values should, therefore, be comparable to the half-life of digitoxin determined with radiolabeled digitoxin, a procedure that also is nonspecific. Okita and coworkers (13, 19), measuring the urinary excretion of digitoxin and its metabolites, found the half-life to be 9.0 days for both a 1.2- and a 0.5-mg. dose of ^{14}C-digitoxin. Caldwell *et al.* (20) measured the levels of radioactivity in the blood following a 1.2-mg. oral dose of ^{3}H-digitoxin in hydroalcoholic solution. In their study, the mean half-life was 11.5 ± 2.3 days. However, in subjects who received a 4-g. dose of cholestyramine 4 hr. after dosing with digitoxin, there was a significant change in the mean plasma half-life to 6.6 ± 1.9 days. The apparent drop in the plasma half-life of the drug following treatment with cholestyramine, a compound that binds digitoxin, was due to the interruption of the enterohepatic circulation of the drug. Okita (21) showed that, in man and several other species, digitoxin and its metabolites are excreted in bile into the small intestine and subsequently reabsorbed. Okita also was able to relate the species differences in the half-life of digitoxin to differences in the enterohepatic circulation. There is little doubt that enterohepatic circulation plays an important role in regulating the time course of digitoxin and its metabolites in the body. It is also quite apparent that this effect can cause considerable variability in the apparent half-life of digitoxin from one subject to another or conceivably within the same subject. This would be expected since the compounds excreted in the bile are concentrated in the gallbladder and gallbladder emptying is dependent upon the time at which food or fatty materials are consumed. Nevertheless, the half-life values obtained in this study generally agree well with the reported literature values.

In summary, one main problem associated with the determination of digitoxin plasma levels in a single-dose study is sensitivity. The only procedures available with the required sensitivity are the radioimmunoassay, the ^{86}Rb-uptake assay, and the Na,K-ATPase

inhibition procedures. All of these procedures are plagued with the same lack of specificity and, as stated earlier, in our opinion the radioimmunoassay is the most convenient and readily available procedure of these three.

For bioavailability studies, when unlabeled drug must by necessity be used, a separation of unchanged digitoxin and its metabolites by a chromatographic procedure is virtually impossible. This is because of: (*a*) the extremely low plasma levels of drug following a single oral dose, and (*b*) the number of samples that must be processed for a bioavailability study. Furthermore, the problem of assay specificity is in reality more involved than just separating the drug from its metabolites. Studies have shown that the metabolites of digitoxin also are cardioactive in animals. Since this is the case, metabolism in the intestinal wall and in the GI tract should be considered. Furthermore, studies should be undertaken to determine the cardioactivity of these compounds in man. Only if these factors are known would it be possible to determine the availability of active drug from a given dosage form of digitoxin. It is presently thought that all of the metabolites except the aglycone of digitoxigenin and digoxigenin are cardioactive in man, but this has not been quantitatively nor qualitatively reported in the literature. If the various metabolites are all cardioactive, as is believed, then to some measure the radioimmunoassay would be a measure of total active drug. To this extent in carefully controlled crossover studies, the radioimmunoassay is presently the best method for bioavailability studies.

The results of the human bioavailability study reported herein should not be construed as suggesting that all digitoxin tablets made by different manufacturers are equally bioavailable. Only two manufacturers' tablets were studied, and the results should not be extrapolated to any other manufacturer's products.

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NMR Spectroscopy: Antibiotic Interactions with Phospholipids

JOHN M. PADFIELD▲ and I. W. KELLAWAY

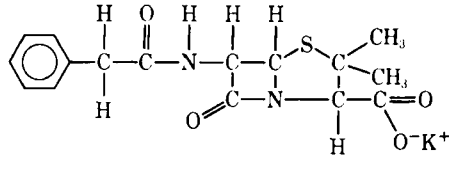
Abstract □ Sonicated dispersions of phosphatidylcholine, lysophosphatidylcholine, phosphatidylinositol, and phosphatidylserine in deuterium oxide were studied by NMR, and the changes in spectra produced on addition of penicillin G and ampicillin to the dispersions were observed. The antibiotics appeared to reduce the configurational freedom and kinetic motion of the phospholipid hydrocarbon chains due to hydrophobic interaction between the hydrocarbon chains and the phenyl portion of the antibiotic side chain. Penicillin G interacted with the phospholipid systems to a greater degree than ampicillin, while the phospholipids interacted in the order phosphatidylserine > phosphatidylcholine > phosphatidylinositol > lysophosphatidylcholine.

Keyphrases □ Antibiotic interactions with phospholipids—studied using NMR spectroscopy □ Phospholipids, interactions with penicillin G and ampicillin—studied using NMR spectroscopy □ Penicillin G and ampicillin interactions with phospholipids—studied using NMR spectroscopy □ Ampicillin and penicillin G interactions with phospholipids—studied using NMR spectroscopy □ NMR spectroscopy—used to study antibiotic interactions with phospholipids

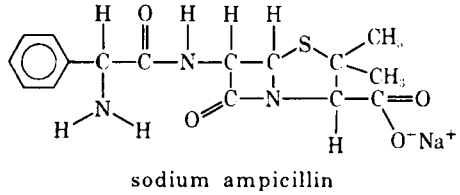
This work is part of a physical study (1) designed to investigate the reported differences in *in vivo* activity (2) of penicillin G (I) and ampicillin (II) by attempting to correlate these differences with the relative penicillin affinity for binding with phospholipids (III*a*, III*b*, III*c*, and III*d*). Previous work examined the effects of interactions on the rheology of phospholipid systems (3, 4), on diffusion of the antibiotics through phospholipid dispersions, and on gel filtration (5). The significance of such interactions was also discussed (5).

NMR spectroscopy is a useful method of investigating the relationships between lipids and other molecules because it often allows a single component to be studied in a heterogeneous environment (6, 7). Furthermore, Jardetzky (8) showed that NMR is invaluable in assessing the extent to which various functional groups on small drug molecules participate in drug–macromolecule interactions.

NMR spectroscopy has been used to study lipids (9–11) and lipid–water systems (12, 13), primarily by broad-line measurements. Sonication of the systems enabled a high resolution spectrum to be obtained (13–16) similar to that observed when the phospholipid was dissolved in a solvent such as deuterochloroform (17). Most workers indicated that the lamellar state is retained after sonication, *i.e.*, no phase change occurs, although this is not universally accepted (18).



potassium penicillin G
I



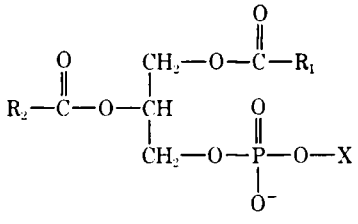
sodium ampicillin
II

These studies provide the basis for investigating the interaction of phospholipids with cholesterol (19, 20), acetylcholine (21), and polypeptide antibiotics (22, 23). The observed broadening of the lipid hydrocarbon chain proton signal has been interpreted as a reduction in the configurational freedom and kinetic motions of the hydrocarbon chains (19, 24). Further studies indicated binding of phospholipids to anesthetic molecules (25) and epinephrine (26).

NMR studies on penicillin G and ampicillin have not been widely reported. The demonstration of binding of penicillin G to serum albumin (27, 28) and guanosine (29) indicated the use of selective changes in relaxation times in assessing which portion of the penicillin molecule was involved in binding.

EXPERIMENTAL

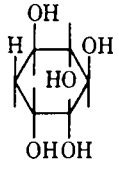
Materials[1]—Egg phosphatidylcholine (III*a*) was prepared as previously described (5). Highly purified lysophosphatidylcholine (III*b*), phosphatidylinositol (III*c*), and phosphatidylserine (III*d*)



III*a*: phosphatidylcholine, $X = -CH_2-CH_2-\overset{+}{N}(CH_3)_3$,
R_1 and R_2 = fatty acid chains (C_{13}-C_{23})

III*b*: lysophosphatidylcholine, $X = -CH_2-CH_2-\overset{+}{N}(CH_3)_3$,
R_1 = fatty acid chain (C_{13}-C_{17}, saturated), R_2 = H

III*c*: phosphatidylinositol, X =



R_1 and R_2 = fatty acid chains (C_{14}-C_{20})

III*d*: phosphatidylserine, $X = -CH_2-CH(NH_2)-COOH$,
R_1 and R_2 = fatty acid chains (C_{12}-C_{18})

[1] Potassium penicillin G was the gift of Glaxo Research Limited, Greenford, England, and sodium ampicillin was the gift of Beecham Research Laboratories, Betchworth, England; both materials were laboratory reference grade.

Table I—Effect of Phospholipids on 100-MHz. NMR Signals of Antibiotic Protons in Deuterium Oxide (τ Values) (J Values in Hertz in Parentheses)

Antibiotic–Phospholipid[a]	C_6H_5	α-CH—X[b]	C-6	C-5	C-3	C_2—$(CH_3)_2$		Remarks
Penicillin G	2.60	6.32	4.44d[c] (4.0)	4.46d (4.0)	5.69	8.39	8.44	Nothing downfield
Penicillin G–PC	2.48	6.19	4.43d (4.0)	4.47d (4.0)	5.65	8.31	8.39	
Penicillin G–LPC	2.53	6.24	4.39d (4.0)	4.43d (4.0)	5.70	8.36	8.43	
Penicillin G–PI	2.58	6.30	4.46d (4.0)	4.51d (4.0)	5.75	8.42	8.50	
Penicillin G–PS	2.53	—	—	—	—	8.44		Precipitation after 18 hr.[d]
Ampicillin	2.48	5.26	4.45s	4.45s	5.72	8.41	8.48	Nothing downfield
Ampicillin–PC	2.42	5.21	4.40s	4.40s	5.70	8.38	8.45	
Ampicillin–LPC	2.46	5.25	4.42s	4.42s	5.74	8.41	8.48	
Ampicillin–PI	2.51	5.21	4.50s	4.50s	5.80	8.50	8.56	
Ampicillin–PS	2.47	—[e]	4.47s	4.57s	5.80	8.52	8.55	

[a] Key: PC = phosphatidylcholine, LPC = lysophosphatidylcholine, PI = phosphatidylinositol, and PS = phosphatidylserine. [b] X = H in penicillin G, and X = NH_2 in ampicillin. [c] s = singlet and d = doublet. [d] Values given are peaks of very broad signals. No signals observed for other groups. [e] Not observable under deuterium oxide signal.

were obtained commercially[2]. The phospholipids were stored with a desiccant at −5°, and their purity was confirmed by TLC on kieselguhr G plates[3]. Deuterium oxide (D_2O), 99.95%, and tetramethylsilane were also used[4].

Preparation of Dispersions—The antibiotic solutions and phospholipid sols were 2% w/v unless otherwise stated. The phospholipid sols were prepared by adding deuterium oxide directly to the weighed amount of phospholipid in a flask. The dispersions were sonicated at 20 KHz. for 10 min. in nitrogen atmosphere (23, 25).

Phospholipid–antibiotic mixtures were prepared by adding 0.5 ml. of the sonicated phospholipid sol to the requisite amount of antibiotic; the mixtures were shaken to dissolve the antibiotic, and the spectra were obtained after 18 hr. (1).

NMR Measurements—The spectra were obtained at 31° using a spectrometer[5], operating in a field-sweep mode. The spectra of the phospholipids were accumulated on a computer of average transients[6]; 15 scans were normally sufficient. Tetramethylsilane provided a lock signal for the instrument and an external reference signal for measuring chemical shifts.

RESULTS

Table I gives the chemical shifts for the antibiotics in the 100-MHz. spectra; signal assignments are based on those of Fischer and Jardetzky (27) and Green *et al.* (30). Although a doublet was observed for each proton of the β-lactam ring in penicillin G, only one singlet was observed for both protons in ampicillin.

Figure 1 illustrates the high-resolution NMR spectrum of III*d* and its interaction products; the computer of average transients spectrum is shown in Fig. 2. Peak assignments are based on those of Chapman and Morrison (17). Table II indicates the chemical shifts observed for the phospholipids. The primary peaks observed in all spectra were those due to the hydrocarbon chain, $(CH_2)_n$, protons and the chain terminal, CH_3, protons; in addition, a strong signal was observed for the terminal choline, $^+N(CH_3)_3$, protons in III*a* and III*b*. These signals are only observed in sonicated dispersions, the fine structure being absent in unsonicated material (15, 16, 20). The $(CH_2)_n$ peaks, composed of more than 30 protons, are broader in appearance. Broad peaks are also characteristic of aggregate forms in dispersion.

The effect of the addition of penicillin G and ampicillin to sonicated dispersions of phosphatidylserine is indicated in Figs. 1*b*, 1*c*, 2*b*, and 2*c*. Differential line broadening and line shifts are observed for some peaks (Tables I and II). The penicillin G peaks most affected are those due to the aromatic and α-CH_2 groups, while the gem-dimethyl and C-3 proton peaks of ampicillin are also implicated.

The computer of average transients spectra indicate that the phospholipid peaks most affected are those due to the $(CH_2)_n$ and CH_3 groups. The line broadening observed is so marked in some cases (phosphatidylcholine–penicillin G and phosphatidylserine–penicillin G) that the signals are lost in the baseline.

Tables III and IV illustrate T_2 relaxation times for the antibiotic and phospholipid protons; they were determined by measuring the line width at half maximum peak height (31).

DISCUSSION

The following discussion has been limited to data relevant to penicillin–phospholipid interactions and does not attempt to discuss the previously documented details of the relaxation processes of proton lipid spectra previously referenced.

Previous work (9–26) showed that both the solid phospholipids and dispersions in water give NMR lines that are intermediate between solids and liquids at normal temperatures. A gradual narrowing of the lines is observed with rising temperature, and a "liquid" state can be observed in highly unsaturated phospholipids at temperatures normally found in biological systems. Sonication gives rise to a liquid signal from the hydrocarbon chain protons even when this is not present in the bulk material, indicating a substantial increase in chain mobility. The addition of substances like cholesterol and alamethicin removes the hydrocarbon chain signal, whereas other substances like procaine hydrochloride and epinephrine affect the hydrophilic parts of the phospholipids.

The antibiotics' spectra are relatively sharp, the chemical shifts being in accordance with published work (27, 30). The coupling constants (J) for the β-lactam doublets of penicillin G (Table I) are identical to those of Green *et al.* (30).

Comparison of the β-lactam signal for penicillin and ampicillin indicates that the two doublets in penicillin G collapse to give one singlet in ampicillin. The presence of an electron-attracting group such as an amino group may transfer its effect to the β-lactam ring to produce the observed differences. Pek *et al.* (32) found that substituents in the side chain affect the chemical shifts of the methyl protons remote from them. Recently, Kan *et al.* (29), using a 220-MHz. NMR spectrometer, reported that the phenyl signal and the —CH_2— signal of penicillin G are present as a multiplet and an *AB* quartet (J = 15 Hz.), respectively, whereas both appear as singlets at 60 and 100 MHz. Similarly, these workers reported two doublets for the β-lactam protons in dimethyl sulfoxide and that the —CH_A—CH_B—NH_X signals represent an *ABX* system with $J_{AX} = 0$, J_{AB} = 4 Hz., and J_{BX} = 9 Hz. The T_2 relaxation times for antibiotic protons have not been widely reported; however, the present findings (Table III) are of the same order as those of Fischer and Jardetzky (27).

[2] Koch-Light Laboratories Ltd.
[3] Merck.
[4] Fluorochem Ltd.
[5] Varian Associates HA-100.
[6] Varian Instruments Ltd.

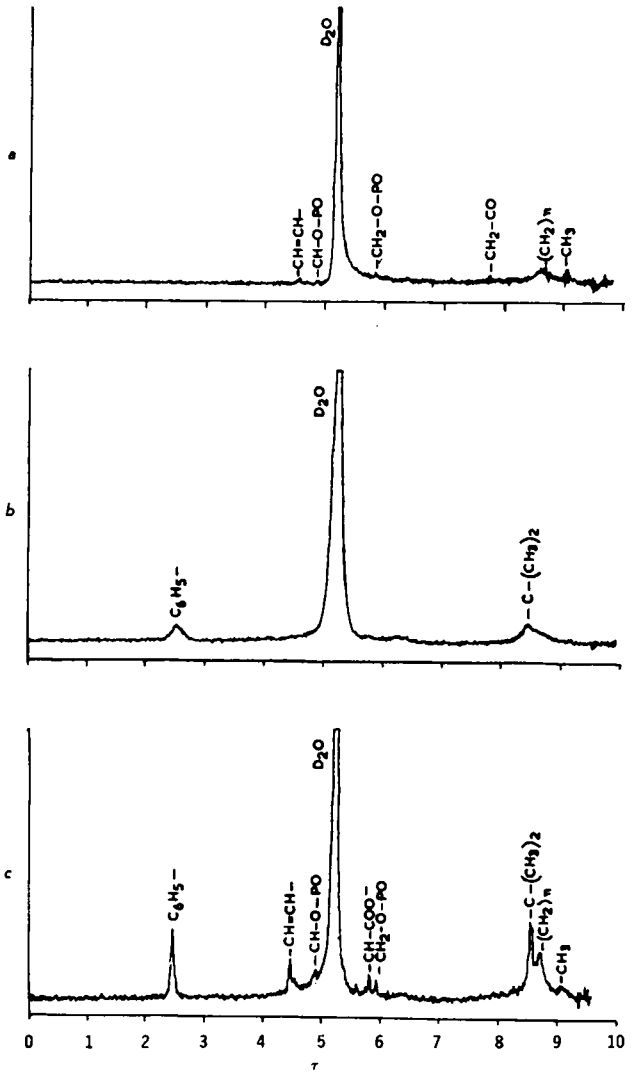


Figure 1—*The 100-MHz. NMR spectrum of 2% phosphatidylserine in deuterium oxide in the presence and absence of the antibiotics (2%). Key:* a, *phosphatidylserine;* b, *phosphatidylserine + penicillin G; and* c, *phosphatidylserine + ampicillin.*

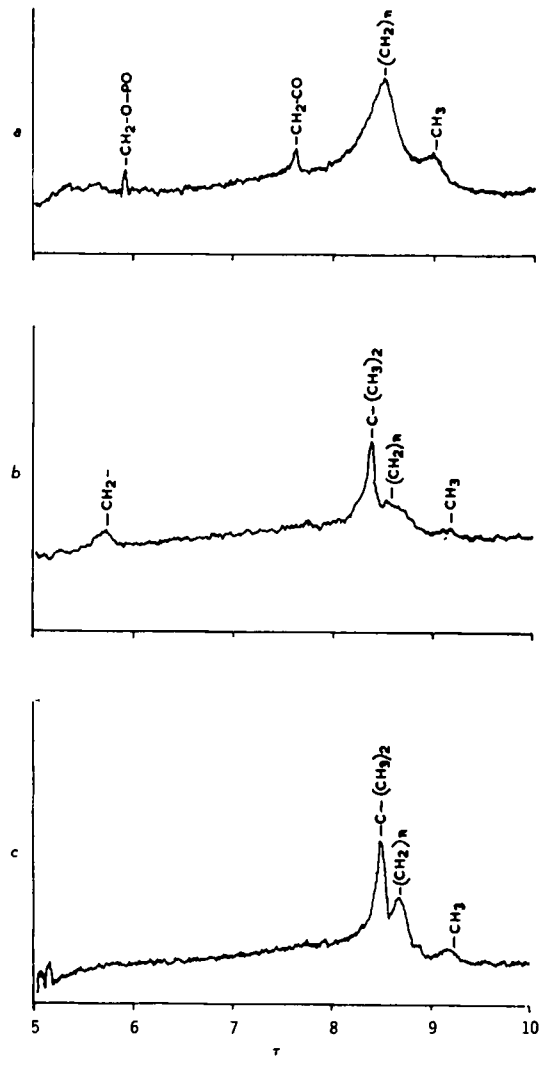


Figure 2—*The 100-MHz. NMR computer of average transients spectrum (15 scans) of 2% phosphatidylserine in deuterium oxide in the presence and absence of the antibiotics (2%). Key:* a, *phosphatidylserine;* b, *phosphatidylserine + penicillin G; and* c, *phosphatidylserine + ampicillin.*

The signals observed for the phospholipids (Table II) are in accordance with those of other workers. Sheard (18) indicated that only 88 ± 5% of the $^{+}N(CH_3)_3$ protons of III*a* contributed to the spectrum, behavior paralleled by the $(CH_2)_n$ protons. He also reported that this latter signal was more field dependent than the $^{+}N(CH_3)_3$ or CH_3 signals and proposed that each broad signal is a superimposition of many narrower signals.

The T_2 relaxation times for phospholipid protons have not been widely reported, but T_1 measurements (33) indicated a single relaxation time above and below the transition temperature, suggesting that a single relaxation mechanism exists for the bulk of the phosphatidylcholine molecule. The T_2 relaxation times found in this study are shown in Table IV.

Addition of the antibiotics to phospholipids indicates effects similar to those observed on addition of cholesterol (20, 24). Reduction in the motion of the fatty acid chain protons is so marked in some cases (penicillin G–phosphatidylcholine and penicillin G–phosphatidylserine) that the $(CH_2)_n$ and CH_3 signals are lost in the baseline (Table II).

In general, differential downfield line shifts are observed for the antibiotic protons, although those of penicillin G are more susceptible than those of ampicillin; the phospholipids, in contrast, experience an upfield shift. Downfield shifts indicate a deshielding effect, whereas shielding is implicated by upfield shifts. If the relative motion of the hydrocarbon chains is reduced by hydrophobic interaction with penicillin G, increased shielding of the protons is suggested and upfield shifts would be expected; examination of the spectra indicates this to be the case. It has been suggested (20) that the ends of the hydrocarbon chains are relatively more mobile than the rest of the chain, because the terminal CH_3 signal is broadened to a lesser extent than that representing the majority of the $(CH_2)_n$ protons.

More specifically, the phenyl and α-CH_2 protons of the antibiotics are shifted to a greater degree than other protons in the molecule, the effect being more pronounced for mixtures containing penicillin G. The phenyl and methylene protons become deshielded, possibly due to changes in the rotation of the side chain in the presence of phospholipids.

The greatest shifts of phospholipid protons observed are those present in the hydrocarbon chain, *i.e.*, $(CH_2)_n$ and CH_3. In mixtures of penicillin G–phosphatidylcholine and penicillin G–phosphatidylserine, these signals are lost in the baseline. However, due to the broad nature of these signals in the absence of the antibiotics, precise chemical shifts are not possible; a similar conclusion was reached by Hammes and Tallman (26).

The loss of the four peaks from the penicillin G spectra on addition of phosphatidylserine is interpreted as a case of extreme line broadening. In view of the observed changes in the phospholipid spectra, it is difficult to envisage any form of interaction other than of a hydrophobic nature, which would thus implicate the aromatic ring and α-CH_2 group of the antibiotic. Confirmation of this view was provided by substituting phosphatidylcholine for phosphatidyl-

Table II—Effect of Antibiotics on 100-MHz. NMR Signals of Phospholipid Protons in Deuterium Oxide (τ Values)

Phospholipid[a]–Antibiotic	CH_3	$(CH_2)_n$	CH_2—CO	$^+N(CH_3)_3$	CH_2—$^+N(CH_3)_3$	CH_2—OCO CH_2—OPO	CH—O—CO	CH=CH
PC	9.16	8.66	N/O	6.66	6.06	5.64	4.80	4.54
PC–penicillin G	N/O[b]	N/O	N/O	6.70	5.98	5.60	4.84	—[c]
PC–ampicillin	9.08	8.62	N/O	6.62	N/O	5.70	[d]	N/O
LPC	8.96	8.60	7.52	6.65	6.20	5.63	4.76	N/E
LPC–penicillin G	9.05	8.64	7.58	6.70	[e]	—[f]	[d]	N/E
LPC–ampicillin	9.04	8.62	7.54	6.79	6.20	5.57	N/O	N/E
PI	9.09	8.69	N/O	N/E	N/E	5.92	4.94	4.62
PI–penicillin G	9.08	8.71	N/O	N/E	N/E	5.85	4.98	4.70
PI–ampicillin	9.10	8.70	N/O	N/E	N/E	5.85	4.99	4.71
PS	9.03	8.64	7.75	N/E	N/E	5.84	4.88	4.57
PS–penicillin G[g]	N/O	8.70	N/O	N/E	N/E	5.68	N/O	N/O
PS–ampicillin	9.05	8.69	N/O	N/E	N/E	5.92	4.90	4.54

[a] Key: PC = phosphatidylcholine, LPC = lysophosphatidylcholine, PI = phosphatidylinositol, and PS = phosphatidylserine. [b] N/O = not observed, and N/E = not expected. [c] Under β-lactam signal. [d] Under spinning side band of deuterium oxide signal. [e] Merged with α-CH_2 signal. [f] Under C-3 signal. [g] Precipitation of this mixture.

Table III—Effect of Phospholipids on T_2 Relaxation Times of Antibiotic Protons in Deuterium Oxide (in Seconds)

Antibiotic–Phospholipid[a]	C_6H_5	α-CH—X[b]	C-6	C-5	C-3	C_2—$(CH_3)_2$	
Penicillin G	1.5	1.0	0.5	1.0	1.0	1.5	1.5
Penicillin G–PC	1.8	2.0	1.2	1.2	1.3	3.2	3.5
Penicillin G–LPC	1.0	1.3	1.0	1.0	1.0	1.2	1.2
Penicillin G–PI	1.8	1.3	1.1	1.0	1.0	1.3	1.3
Penicillin G–PS[c]	8.0		—	—	—		15.0
Ampicillin	2.0	0.75	1.0	1.0	0.5	1.2	1.2
Ampicillin–PC	1.5	1.5	1.0	1.0	0.8	1.3	1.3
Ampicillin–LPC	1.5	0.5	1.0	1.0	1.0	1.2	1.2
Ampicillin–PI	1.2	0.5	1.0	1.0	1.2	1.5	1.5
Ampicillin–PS	2.5	—[d]	1.5	1.5	1.5	4.0	4.0

[a] Key: PC = phosphatidylcholine, LPC = lysophosphatidylcholine, PI = phosphatidylinositol, and PS = phosphatidylserine. [b] X = H in penicillin G, and X = NH_2 in ampicillin. [c] Values given are peaks of very broad signals. No other signals. [d] Not observable under deuterium oxide peak.

serine when line shifts of 24 and 26 Hz. were recorded for the aromatic ring and α-CH_2 group, respectively (Table I). Ampicillin, being less hydrophobic than penicillin G (35), would be bound to a lesser extent; thus smaller changes were noted in the NMR spectra (Tables I and II). Rheological studies (3, 5) using hydrophobic bond breakers, such as urea and guanidine hydrochloride, indicated the hydrophobic nature of the antibiotic-phospholipid interaction.

Changes in T_2 relaxation times are more indicative of molecular changes than line shifts. Tables III and IV further implicate the parts of the antibiotic and phospholipid molecules already described. Some workers (26, 27) reported that a reduction in T_2 is indicative of restricted rotational freedom of certain groups stabilized by the interaction. However, where line broadening occurs, one may observe an increased value of T_2, suggesting greater freedom; this occurs because the line becomes so broad that the area under the peak is the same as or greater than the peak area of the signal before interaction.

Complex formation can often be detected by observing the changes in chemical shift or relaxation time of a signal; the former is due to changes in charge density at a given proton resulting from interaction. However, line broadening may result even though no change in charge density is observed.

Line broadening may be due to magnetic inhomogeneity, viscosity changes, self-association, or intermolecular complex formation. Viscosity can be disregarded because a 2% solution of phosphatidylcholine has a low viscosity (1.0260×10^{-2} poise at 30°). Sharp resonance lines, although broadened, were observed for the antibiotic protons, thus ruling out magnetic inhomogeneity. Self-association of penicillin G has been reported (34); but since line broadening of some phospholipid signals is also observed, self-association

Table IV—Effect of Antibiotics on T_2 Relaxation Times of Phospholipid Protons in Deuterium Oxide (in Seconds)

Phospholipid[a]–Antibiotic	CH_3	$(CH_2)_n$	CH_2—CO	$^+N(CH_3)_3$	CH_2—$^+N(CH_3)_3$	CH_2—O—CO CH_2—O—PO	CH—O—CO	CH—CH
PC	22.0	30.0	N/O	28.0	10.0	5.5	4.0	4.0
PC–penicillin G	N/O[b]	N/O	N/O	17.0	2.5	2.4	2.6	—[c]
PC–ampicillin	17.0	20.5	N/O	6.0	N/O	1.6	—[d]	N/O
LPC	9.0	8.0	9.0	3.2	10.5	1.5	1.0	N/E
LPC–penicillin G	10.0	11.0	12.0	4.3	—[e]	—[f]	[d]	N/E
LPC–ampicillin	9.0	12.0	16.0	4.8	11.5	2.0	N/O	N/E
PI	15.5	22.0	N/O	N/E	N/E	3.5	2.0	2.0
PI–penicillin G	13.0	19.0	N/O	N/E	N/E	2.0	3.0	2.2
PI–ampicillin	13.0	16.0	N/O	N/E	N/E	4.5	2.0	2.0
PS	15.0	26.5	3.5	N/E	N/E	2.5	2.5	4.0
PS–penicillin G	N/O	43.0	N/O	N/E	N/E	12.0	N/O	N/O
PS–ampicillin	15.0	22.0	N/O	N/E	N/E	4.5	4.5	—[c]

[a] Key: PC = phosphatidylcholine, LPC = lysophosphatidylcholine, PI = phosphatidylinositol, and PS = phosphatidylserine. [b] N/O = not observed, and N/E = not expected. [c] Under β-lactam signal. [d] Under spinning side band of deuterium oxide signal. [e] Merged with α-CH_2 signal. [f] Under C-3 signal.

of the antibiotic is unlikely to be the only mechanism, if it occurs at all (5).

The experimental evidence indicates rapid exchange of penicillin G between the free and unbound forms; if the exchange was slow, sharp lines due to the free species would be superimposed on the broad lines.

Thus, a complex appears to be formed between the antibiotics and phospholipids. This may involve the side chain of the antibiotics and fatty acid chains of the phospholipids. The change in peak size of the $(CH_2)_n$ and CH_3 protons indicates changes in the relative mobility of the hydrocarbon chains. In all phospholipid dispersions examined, penicillin G was observed to interact to a greater degree than ampicillin, while the phospholipids interacted in the order phosphatidylserine > phosphatidylcholine > phosphatidylinositol > lysophosphatidylcholine. The significance of the results in relation to the differences in activity of these antibiotics was discussed elsewhere (1, 5).

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Morphine-Induced Fetal Malformations III: Possible Mechanisms of Action

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Abstract □ The teratogenic effects exerted by morphine sulfate in CF-1 mice on Days 8 and 9 appear, from indirect evidence, not to be attributable to cholinesterase inhibition. The anticholinergic drug, atropine, when used in combination with morphine, resulted in the potentiation of morphine-induced defects, exencephaly and axial skeletal fusions. In addition, both atropine and morphine, either alone or in combination, produced constriction of the placental vessels. Morphine administration on Days 8 and 9 resulted in an increase in blood sugar regardless of the dose employed. Previously reported similarities produced by atropine and morphine on the placental vessels, the knowledge that both drugs decrease oxygen tension, the resemblance of hypoxic-induced malformations to the defects observed in this study, the effects of hypoxia on carbohydrate metabolism, and the reported involvement of carbohydrate metabolism in the morphogenesis of the embryonic neural tube all suggest that the malformations observed following morphine administration are related to the reduced oxygen concentration as well as the hyperglycemia produced subsequent to its administration in gravid mice.

Keyphrases □ Morphine-induced fetal malformations—possible mechanisms of action □ Exencephaly, morphine induced—possible mechanisms of malformation □ Teratogenicity, morphine—possible mechanisms of morphine-induced fetal malformations □ Fetal malformations—possible mechanisms of morphine-induced exencephaly

Exencephaly, an anomaly characterized by the extensive exterior protrusion of cerebral tissue due to the absence of skin and bones of the cranial vault, has been shown to be caused by a variety of inducers. The causative agents include genetic mutations (1), irradiation (2), nutrition (3), hypoxia (4), and a number of chemical agents (5) including morphine sulfate (6).

Having established the fact that high subcutaneous doses of morphine sulfate (200, 300, and 400 mg./kg.) are teratogenic (6), the subsequent investigation of a mechanism of action was undertaken in this laboratory. Among the problems encountered in trying to define a mechanism of teratogenicity for morphine are the enigmatic pharmacological reactions produced by it—histamine release (7), food retardation (8), cholinesterase inhibition (9), vasoconstriction of placental vessels (10), and hyperglycemia (11)—plus the knowledge that teratogenic insult may occur at any of three different loci: the mother, the placenta, and the embryo.

The involvement of histamine release and food retardation as the underlying factors causing the malformations observed following morphine administration were negated by Iuliucci and Gautieri (12) and Harpel and Gautieri (6), respectively. Consequently, the objectives of this investigation were to: (*a*) reconfirm the teratogenic potential of high subcutaneously administered doses of morphine in CF-1 mice; (*b*) evaluate the possibility of cholinesterase inhibition by morphine as a causative factor in the production of fetal anomalies, determined by increasing the defects with the prior administration of physostigmine or decreasing them with the prior administration of atropine; (*c*) ascertain whether an effect exerted by morphine, atropine, and physostigmine on the placental vasculature *in vivo*, utilizing ^{22}Na transport, is involved in the production of the abnormalities; and, finally, (*d*) assess the involvement of the hyperglycemic component in the teratogenicity induced by morphine.

EXPERIMENTAL

CF-1 albino mice[1], weighing between 25 and 30 g., were employed in all experimental procedures and subjected to treatment and breeding procedures as reported previously (6, 12).

Injectable solutions of morphine sulfate[2] (40.0 mg./ml.), physostigmine sulfate[3] (0.025 mg./ml.), atropine sulfate[4] (5 mg./ml.), sodium sulfate[5] (10 mg./ml.), and sodium chloride (9 mg./ml.) were prepared fresh weekly by dissolving the drugs in distilled water.

The pregnant female mice were assigned at random to one of 35 experimental categories, with each category being treated on either Day 8 or 9 of gestation as designated in Tables I–IV. Iuliucci and Gautieri (12) described termination of pregnancy, removal of fetuses, gross inspection, and preparation of the excised fetuses for both skeletal (13) and soft tissue examination (14).

A second aspect of this study involved the actions of certain drugs upon the placental vasculature as determined by transport of ^{22}Na according to the procedure described by Robson and Sullivan (15), who employed it to study placental transport *in vivo*.

Five minutes after the subcutaneous administration of the respective drug(s), the isotope, 0.1 ml. delivering 3 μc., was injected intravenously into a tail vein of the mouse. By reporting the activity of the samples as a percentage of the concentration of isotope in maternal blood, each animal served as its own control.

A third aspect of this study involved an evaluation of the effects exerted by morphine (200, 300, and 400 mg./kg.) on the maternal blood sugar of 8- and 9-day pregnant CF-1 mice.

Each animal was initially bled by inserting a fine glass pipet into the medial canthus and removing 0.1 ml. of blood from the veins of the retro-orbital plexus (16). This was followed by the subcutaneous administration of designated test agent and dose. Twenty minutes later, a second 0.1-ml. sample was withdrawn from the mouse. Both samples were then subjected to the Folin–Malmros (17) micromethod for blood glucose determination. By withdrawing two samples, one prior to and one following drug administration, each animal served as its own control.

RESULTS

Maternal Effects of Test Drugs—The subcutaneous administration of 50 mg./kg. atropine sulfate did not produce readily observable effects, except an apparent increased heart rate and a coldness of the tail. When administered in combination with morphine, no Straub tail was detected at first; when it did appear some 30 min. later, it lasted for only 5 min. as compared with several hours following the injection of morphine alone. Next came a period of depression and labored breathing, followed by a hyperactive period. Atropine sulfate failed to produce any deaths when used alone. However, com-

[1] Carworth Farms, Inc., New York, N. Y.
[2] Morphine sulfate USP, Control No. P0006, Merck and Co., Inc., Rahway, N. J.
[3] Physostigmine sulfate, Control No. 5703, Merck and Co., Inc., Rahway, N. J.
[4] Atropine sulfate, Control No. 1278, Merck and Co., Inc., Rahway, N. J.
[5] Sodium sulfate, Control No. 500813, Fisher Scientific Co., Pittsburgh, Pa.

Table I—Mean Values of Test Groups Receiving Single Injections (Treatment on Day 8)

Treatment	Maternal Weight Ratio, S/T[a]	Fetal Ratio, Right Horn/ Left Horn	Resorption Ratio, Right Horn/ Left Horn	$\bar{X}$ Fetal Weight, g.	Sex Ratio, M/F	Soft Tissue Abnormalities	Skeletal Abnormalities
Control (untreated)	25.5/48.0	6.2/3.7[b]	0.17/0.33	1.07	4.0/5.8	0.0	1.5
Saline, 0.3 ml.	26.8/51.0	5.8/6.2	0.33/0.50	1.13	6.5/5.5	0.17	1.3
Morphine, 200 mg./kg.	27.0/44.3[b]	6.3/5.0	0.50/0.00	0.92[b]	5.8/5.2	0.83[b]	8.8[b]
Morphine, 300 mg./kg.	25.7/48.5	5.5/5.0	0.50/0.33	1.10	5.2/5.3	1.17	2.2
Morphine, 400 mg./kg.	27.3/47.8	5.8/5.5	0.00/1.00	1.01[b]	5.5/5.8	0.67	9.0[b]
Physostigmine, 0.005 mg.	25.7/45.8[b]	4.3/4.0	0.00/0.50	1.00	3.7[b]/4.7	3.67	10.0[b]
Atropine, 50 mg./kg.	25.5/46.0	5.8/4.8	0.07/0.83	1.16	6.0/4.2	0.33	5.5
Sodium sulfate, 60 mg./kg.	26.8/44.7[b]	4.5[b]/4.0	0.00/0.50	1.11	5.3/3.2	0.00	6.0[b]

[a] S = starting weight and T = terminal weight. [b] Statistically significant in comparison with saline control, $p < 0.05$.

Table II—Mean Values of Test Groups Receiving Single Injections (Treatment on Day 9)

Treatment	Maternal Weight Ratio, S/T[a]	Fetal Ratio, Right Horn/ Left Horn	Resorption Ratio, Right Horn/ Left Horn	$\bar{X}$ Fetal Weight, g.	Sex Ratio, M/F	Soft Tissue Abnormalities	Skeletal Abnormalities
Saline, 0.3 ml.	25.2/49.7	6.2/4.0	0.50/0.33	1.20	5.7/4.5	0.0	0.8
Morphine, 200 mg./kg.	26.2/47.8	4.5/5.8	0.33/0.17	1.02[b]	6.0/4.3	0.17	6.7[b]
Morphine, 300 mg./kg.	25.8/41.3[b]	5.3/3.3	0.00/1.50	1.15	4.3/4.3	0.33	3.3[b]
Morphine, 400 mg./kg.	26.0/43.5	4.7/4.8	0.00/0.17	1.11	5.0/4.7	0.17	4.8[b]
Physostigmine, 0.005 mg.	25.7/44.8	4.7/5.2	0.00/0.33	1.10[b]	5.3/4.5	1.67[b]	8.3[b]
Atropine, 50 mg./kg.	26.8[b]/49.0	5.2/5.8	0.00/0.33	1.20[b]	6.2/4.8	0.00	5.2[b]
Sodium sulfate, 60 mg./kg.	27.0[b]/46.7	4.8/5.7[b]	0.00/0.33	1.10[b]	6.5/4.0	0.00	7.8

[a] S = starting weight and T = terminal weight. [b] Statistically significant in comparison with saline control, $p < 0.05$.

binations of atropine, followed in 15 min. by varying doses of morphine, resulted in a definite increase in the number of maternal deaths. The higher the morphine dose following the atropine injection, the greater the incidence of maternal death, the greatest incidence (100%) being observed with the 400-mg./kg. dose of morphine.

Treatment with 0.005 mg. physostigmine produced a generalized lethargy in the animals. Salivation and Straub tail were noted, along with hyperpnea that lasted for approximately 1 hr.; at the end of this time, the mice became hyperactive. Unlike atropine pretreatment, which antagonized morphine-induced Straub tail, the prior administration of physostigmine not only did not alter the Straub tail produced following the administration of morphine but actually stimulated a Straub tail itself. Atropine injection before physostigmine resulted in effects quite similar to those noticed following the administration of atropine alone—no Straub tail. The single injection of 0.005 mg. physostigmine sulfate caused no deaths. Conversely, when administered 15 min. prior to morphine, physostigmine produced a substantial increase in the death rate, especially at the higher doses of the latter; 100% fatality, regardless of the day, resulted from the combination of 0.005 mg. physostigmine and 400 mg./kg. morphine.

It was also observed that the injection of saline 15 min. prior to morphine administration did not result in any noteworthy change in the normal maternal symptoms produced by the narcotic.

Tables I–IV, which contain the cumulative mean values for each experimental group, give the results obtained from the various categories employed in the teratological aspect of this study.

Exencephaly (Herniated Brain)—The observed fetal and litter incidences of offspring manifesting herniated brains are tabulated in Tables V–VIII, and Fig. 1 contains representative specimens exhibiting this anomaly.

By referring to these tables, it can be seen that of all the agents and combinations tested, only 300 mg./kg. morphine and 300 mg./kg. morphine preceded by 50 mg./kg. atropine administered on Day 8 produced a significant incidence of exencephaly when compared with saline controls. The combination of atropine and 300 mg./kg. morphine on Day 8, although insignificant when compared with 300 mg./kg. morphine on Day 8 on a litter basis, was significant when compared on a fetal basis. This combination resulted in the greatest incidence of exencephaly, in which five out of six litters, totaling 13 exencephalic fetuses, exhibited this abnormality.

A significant decrease in the number of litters displaying exencephaly when compared with the respective dose and day of morphine administration was observed only in the group receiving the combination of 0.005 mg. physostigmine and 300 mg./kg. morphine on Day 8. The administration of physostigmine alone on Day 8 produced exencephalies in two litters, which borders on being significant.

The occurrence of this anomaly was not apparently influenced by either uterine horn (right *versus* left) or position, because the defect was evenly divided between the horns and no particular position was favored irrespective of the treatment employed. Furthermore, this defect did not appear to be sex related, since a predominance of the malformation was not observed in either sex. Many of the exencephalic fetuses also had open eyes, a condition not observed even in untreated fetuses at regular parturition.

Other Soft Tissue Defects—In addition to exencephaly and its related defects, retardation of testicular descent (cryptorchid testes) (Fig. 2) was the only other soft tissue anomaly that occurred at a significant degree. The results of the various test agents upon testicular descent are depicted in Tables V–VIII.

Some other soft tissue malformations observed in this study are as follows: At least one fetus exhibiting hydronephrosis was observed in groups administered saline; 200, 300, and 400 mg./kg. morphine; and physostigmine and 200 mg./kg. morphine on Day 8.

Table III—Mean Values of Test Groups Receiving Combination Treatments[a] on Day 8

Treatment	Maternal Weight Ratio, S/T[b]	Fetal Ratio, Right Horn/ Left Horn	Resorption Ratio, Right Horn/ Left Horn	$\bar{X}$ Fetal Weight, g.	Sex Ratio, M/F	Soft Tissue Abnormalities	Skeletal Abnormalities
Saline, 0.3 ml., and saline, 0.3 ml.	26.0/47.2[c]	5.2/6.2	0.30/0.80	1.17	7.0/4.2	0.20	0.7
Saline, 0.3 ml., and morphine, 200 mg./kg.	26.8/44.7	4.2[d]/5.7	0.20/0.20	1.03	4.4/5.0	0.17[d]	3.8
Saline, 0.3 ml., and morphine, 300 mg./kg.	27.8[d]/48.7	4.5/5.0	0.30/0.30	1.05	5.3/5.2	1.50	9.3[d]
Atropine, 50 mg./kg., and morphine, 200 mg./kg.	28.2[d]/42.0[d]	4.0/3.2	0.67/0.67	1.11	3.5/3.7	1.00	10.8
Atropine, 50 mg./kg., and morphine, 300 mg./kg.	27.2/50.7	4.3/6.3	0.67/0.83	1.05	5.0/5.7	3.33	10.3[d]
Physostigmine, 0.005 mg., and morphine, 200 mg./kg.	26.2/48.2	4.3[d]/6.0	0.00[d]/0.33	1.15[d]	5.3/5.0	1.83	8.3
Physostigmine, 0.005 mg., and morphine, 300 mg./kg.	24.8/44.0	6.2/3.3	0.33/0.00	1.03	6.8/2.7[d]	0.83	10.3[d]
Atropine, 50 mg./kg., and physostigmine, 0.005 mg.	28.3/49.5	4.5/6.2	0.00/0.17	1.03	5.8/4.8	1.00	8.5[c]

[a] With 15-min. interval between injections. [b] S = starting weight and T = terminal weight. [c] Statistically significant in comparison with saline control, $p < 0.05$. [d] Statistically significant in comparison with single injections of the respective dose of morphine, $p < 0.05$.

Bloody hemispheres (internal hemorrhage) were a common observance in the physostigmine groups on either Day 8 or 9. The 50 mg./kg. atropine and 300 mg./kg. morphine combination produced the greatest array of soft tissue anomalies (Fig. 3), including sirenomelus, exencephaly, cryptorchid testes, cranioschisis, gastroschisis, facial abnormality involving the snout and jaw, and ophthalomodisgenesis.

Skeletal Abnormalities—Tables I–IV show the high incidence of skeletal malformations that resulted from treatment on either Day 8 or 9 with various agents. Among the most common of these observed defects were delayed ossification in the phalanges, sternebrae, and skull, especially the supraoccipital bone, and rib and vertebral fusions (Tables V–VIII).

Results of Radio-Sodium Experiment—The 12 mice comprising the control group were injected with 0.3 ml. normal saline 5 min. prior to the intravenous administration of the radio-sodium isotope. Three animals then were sacrificed at 5-, 10-, 15-, and 20-min. intervals each.

The mean values generated for the placentas and fetuses at the various time periods following drug pretreatment were then compared with the mean values obtained for saline; significance was determined by Student's t test (Table IX). Only morphine at the 5- and 15-min. intervals exhibited a significant difference when the placental values were compared ($t > 2.77$, $p < 0.05$). Comparison of the fetal values resulted in significant differences being observed for both atropine and morphine at 5 min. and for the atropine-morphine combination at 10 min. ($t > 2.77$, $p < 0.05$).

Results of Blood Sugar Experiment—Two blood samples were withdrawn from each of the six mice constituting one of 10 experimental groups, with the second sample being taken 20 min. after the designated treatment of the respective animal. The mean change per group in either Day 8 or 9 is delineated in Table X.

The increases in blood sugar observed with the 200- and 300-mg./kg. doses of morphine administered on Day 8, when compared with saline controls, resulted in significant differences. All three doses of morphine employed on Day 9 were significantly different. An interdose comparison of morphine, however, resulted in no significant differences being observed on either Day 8 or 9.

DISCUSSION

The most profound effect exerted upon the maternal mouse following the injections of morphine, atropine, physostigmine, and various combinations thereof was death, resulting from acute toxicity. Single injections of the doses employed proved nonlethal

Table IV—Mean Values of Test Groups Receiving Combination Treatments[a] on Day 9

Treatment	Maternal Weight Ratio, S/T[b]	Fetal Ratio, Right Horn/ Left Horn	Resorption Ratio, Right Horn/ Left Horn	$\bar{X}$ Fetal Weight, g.	Sex Ratio, M/F	Soft Tissue Abnormalities	Skeletal Abnormalities
Saline, 0.3 ml., and saline, 0.3 ml.	25.3/45.0[c]	5.0/4.2	0.20/0.30	1.10	3.7/4.7	0.20	5.7[c]
Saline, 0.3 ml., and morphine, 200 mg./kg.	25.8/46.4	5.6/4.2	0.40/0.60	1.08	5.8/4.0	0.40	2.6
Saline, 0.3 ml., and morphine, 300 mg./kg.	26.2/42.0	3.7/4.5	0.50/0.50	0.98[d]	3.8/4.3	1.30	9.7[d]
Atropine, 50 mg./kg., and morphine, 200 mg./kg.	26.3/45.3	5.3/5.2	0.33/0.67	0.96	5.3/5.2	0.83	10.8
Atropine, 50 mg./kg., and morphine, 300 mg./kg.	26.0/45.8	5.7/4.5	0.33/0.83	1.05[d]	5.5/4.7	0.33	7.8[d]
Physostigmine, 0.005 mg., and morphine, 200 mg./kg.	26.0/41.8	4.2/3.5	0.17/0.50	1.01	4.7/3.0	1.67[d]	11.7
Physostigmine, 0.005 mg., and morphine, 300 mg./kg.	26.3/45.2	6.3/3.8	0.00/0.67	1.04[d]	6.8/3.3	1.33[d]	12.8[d]
Atropine, 50 mg./kg., and physostigmine, 0.005 mg.	26.5/47.5	5.7/4.5	0.33/0.33	0.95[c]	4.5/6.2	0.67	10.3

[a] With 15-min. interval between injections. [b] S = starting weight and T = terminal weight. [c] Statistically significant in comparison with saline control, $p < 0.05$. [d] Statistically significant in comparison with single injections of the respective dose of morphine, $p < 0.05$.

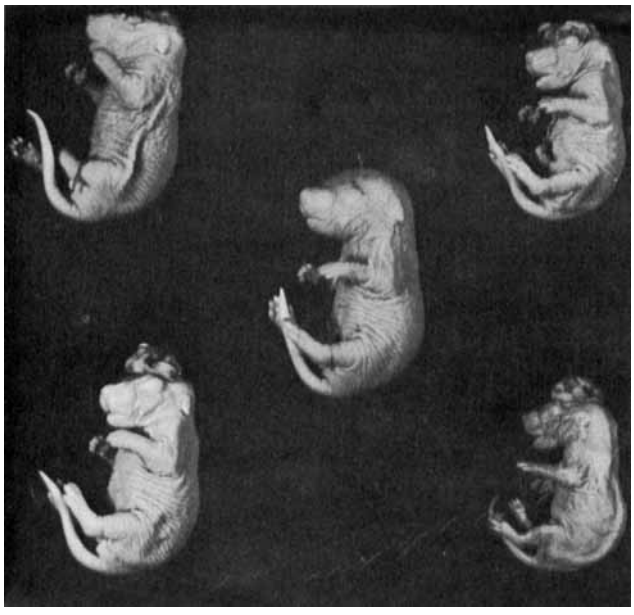

Figure 1—*Exencephalic fetuses and normal littermate after fixation. Upper left and right: exencephalic fetuses with open eye. Middle: normal fetus. Lower left and right: exencephalic fetuses with closed eyes.*

except in mice receiving 400 mg./kg. morphine on either Day 8 or 9. Both 0.005 mg. physostigmine and 50 mg./kg. atropine, administered 15 min. prior to this dose of morphine, resulted in the deaths of all animals so challenged. Consequently, combinations of 400 mg./kg. morphine with other test agents were deleted from the remainder of the study. Atropine or physostigmine administered 15 min. before the 200- and 300-mg./kg. doses of morphine also increased the mortality rate, with the greatest enhancement occurring on Day 8; 81%, 27 out of 33, succumbed following the 50 mg./kg. atropine and 300 mg./kg. morphine combination. The incidence of deaths observed in the various groups should be borne in mind when considering the anomalies produced by different combinations, because defects can be obscured by the production of maternal or fetal death (18). Furthermore, death may actually be secondary to the malformation (18), for the insult may be of such severity that the fetus may not survive.

The combination of 50 mg./kg. atropine followed in 15 min. by 0.005 mg. physostigmine proved to be nontoxic on Days 8 and 9, attesting to the relative innocuousness of this combination in the doses employed when compared with their lethal actions when used in combination with morphine. Another interesting observation was the increase in acute toxicity following the saline and 300 mg./kg. morphine injections. Evidently, the mere trauma of an injection preceding morphine administration is substantial enough to cause death.

The present study demonstrated that more exencephalic fetuses were observed when the gravid animal was treated with 300 mg./kg. morphine on Day 8, while treatment on Day 9 with morphine (all three doses) resulted in a greater number of fetuses displaying axial skeletal fusions. This combination of defects, *i.e.*, exencephaly and axial skeletal fusions, is not unusual. On the contrary, Book and Rayner (19) contend that deficiencies in skeletal and muscle development are secondary to a primary anomaly of the central nervous system (CNS).

Pretreatment with saline 15 min. prior to the morphine injection produced no significant defects compared with morphine alone, thereby negating the possibility that trauma of injection is involved in the subsequent induction of anomalies when a drug is administered before morphine. Therefore, the observance of a significant difference in the incidence of anomalies, *i.e.*, exencephaly and axial skeletal fusions, following pretreatment of morphine administration with a drug must be interpreted as an interaction between the agent and morphine.

Another soft tissue anomaly found in significant numbers was cryptorchid testes, which was generally observed to be associated with a decreased mean fetal weight. Although the importance of

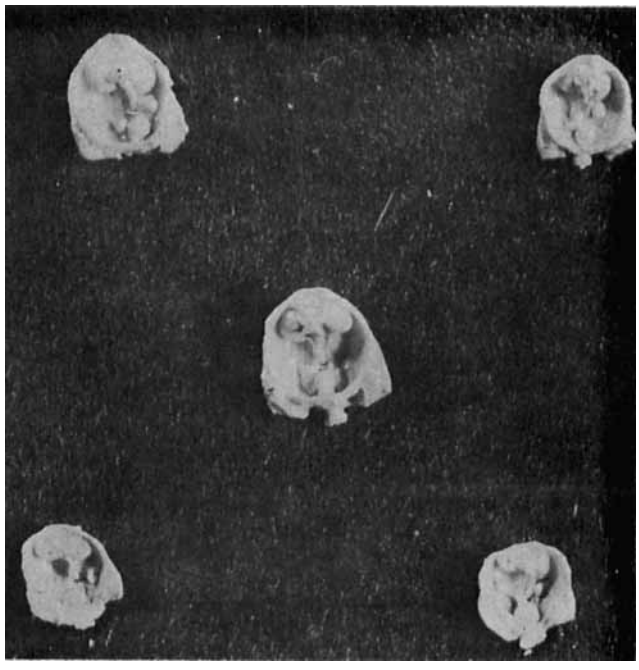

Figure 2—*Specimens exhibiting cryptorchidism. Orientation of section: spinal cord and kidneys at top; bladder and testes on each side, in center at bottom. Upper left and right and lower left: right unilateral cryptorchidism. Middle: normal section. Lower right: bilateral cryptorchidism.*

retarded testicular descent is not clear at this time, conceivably as growth continues the normal descent of the testes may occur. Zondek and Zondek (20) also reported that failure of testicular descent and anencephaly occur concomitantly.

The reversible anticholinesterase agent, physostigmine, when used alone, produced a total of two exencephalic fetuses (one in each of two different litters), significant retardation of testicular descent, and no axial skeletal fusions. The cryptorchid testes and the ob-

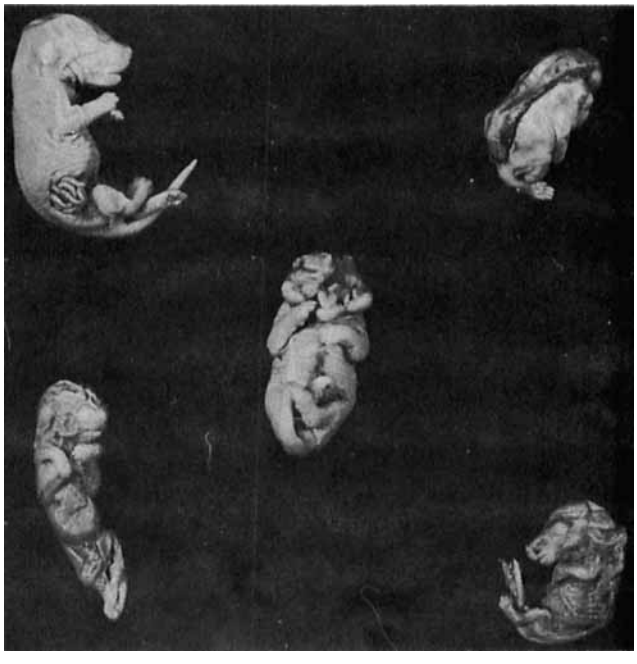

Figure 3—*Examples of observed gross defects following the 50-mg./kg. atropine–300-mg./kg. morphine combination administered on Day 8. Upper left and right: fetuses demonstrating gastroschisis and cranioschisis, respectively. Middle: fetus exhibiting facial anomaly. Lower left and right: fetuses displaying sirenomelus and runt formation with open eye, respectively.*

Table V—Occurrence of Exencephaly, Axial Skeletal Fusions, and Cryptorchid Testes with Treatment on Day 8

Treatment	Exencephaly				Cryptorchid Testes				Axial Skeletal Fusions			
	Litters		Fetuses		Litters		Fetuses		Litters		Fetuses	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
Control (untreated)	6	0	59	0	6	0	12	0	6	0	28	0
Saline, 0.3 ml.	6	0	72	0	6	0	24	0	6	0	34	0
Morphine, 200 mg./kg.	5	1	68	1	3	3[a]	11	3[a]	6	0	34	0
Morphine, 300 mg./kg.	3	3[a]	60	3	4	2	12	3[a]	6	0	31	0
Morphine, 400 mg./kg.	5	1	67	1	5	1	15	1	5	1	33	1
Atropine, 50 mg./kg.	5	1	60	1	6	0	18	0	6	0	31	0
Physostigmine, 0.005 mg.	4	2	48	2	3	3[a]	7	7[a]	6	0	25	0
Saline, 0.3 ml., and saline, 0.3 ml.	6	0	68	0	5	1	15	0	6	0	34	0
Sodium sulfate, 60 mg./kg.	6	0	51	0	6	0	13	0	6	0	25	0

[a] Significant $(X)^2 > 3.84$ = significantly different from saline control.

Table VI—Occurrence of Exencephaly, Axial Skeletal Fusions, and Cryptorchid Testes with Treatment on Day 9

Treatment	Exencephaly				Cryptorchid Testes				Axial Skeletal Fusions			
	Litters		Fetuses		Litters		Fetuses		Litters		Fetuses	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
Saline, 0.3 ml.	6	0	61	0	6	0	16	0	6	0	30	0
Morphine, 200 mg./kg.	5	1	61	1	6	0	17	0	5	1	27	3
Morphine, 300 mg./kg.	6	0	52	0	4	2	12	2	3	3[a]	20	5[a]
Morphine, 400 mg./kg.	6	0	57	0	5	1	17	1	5	1	25	3[a]
Atropine, 50 mg./kg.	6	0	66	0	6	0	12	0	5	1	32	1
Physostigmine, 0.005 mg.	6	0	59	0	4	2	10	3[a]	6	0	30	0
Saline, 0.3 ml., and saline, 0.3 ml.	6	0	45	0	5	1	21	0	6	0	23	0
Sodium sulfate, 60 mg./kg.	6	0	63	0	6	0	15	0	6	0	32	0

[a] Significant $(X)^2 > 3.84$ = significantly different from saline control.

Table VII—Occurrence of Exencephaly, Axial Skeletal Fusions, and Cryptorchid Testes with Treatment on Day 8 (Compared with Morphine, 200 or 300 mg./kg.)

Treatment	Exencephaly				Cryptorchid Testes				Axial Skeletal Fusions			
	Litters		Fetuses		Litters		Fetuses		Litters		Fetuses	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
Saline, 0.3 ml., and morphine, 200 mg./kg.	6	0	59	0	5	1	12	1	6	0	28	0
Saline, 0.3 ml., and morphine, 300 mg./kg.	5	1	53	4	2	4	10	5	5	1	24	3
Atropine, 50 mg./kg., and morphine, 200 mg./kg.	6	0	43	0	5	1	6	4	4	2	20	2
Atropine, 50 mg./kg., and morphine, 300 mg./kg.	5	1	51	13[a]	4	2	8	3	3	3[a]	26	6[a]
Physostigmine, 0.005 mg., and morphine, 200 mg./kg.	6	0	62	0	1	5	8	8	6	0	31	0
Physostigmine, 0.005 mg., and morphine, 300 mg./kg.	6	0	57	0	2	4	18	1	6	0	28	0

[a] Significant $(X)^2 > 3.84$ = significantly different from single morphine injection.

servable skeletal defects were, in all cases, associated with the inhibition of fetal growth. The administration of physostigmine 15 min. prior to morphine did not increase the incidence of exencephaly or axial skeletal fusions. However, on the contrary, a significant decrease in the number of exencephalic fetuses resulted from the combination of physostigmine and 300 mg./kg. morphine administered on Day 8. Also, the combination of physostigmine and 200 mg./kg. morphine administered on Day 8 resulted in a significant reduction in the resorption ratio as compared with morphine, while the same combination on Day 9 resulted in a significant increase in the incidence of cryptorchid testes. Therefore, although apparently not involved in the production of the two major anomalies observed following morphine administration, the effect of growth retardation exerted by physostigmine possibly is responsible for the augmented number of fetuses exhibiting cryptorchid testes.

Atropine injection alone resulted in the production of no significant anomalies. Only one exencephalic fetus and one fetus exhibiting axial skeletal fusions were induced following 50 mg./kg. atropine on Days 8 and 9, respectively.

The results obtained from the combination studies with atropine and morphine clearly indicate a potentiation of the teratogenic capability of morphine to induce both exencephaly and axial skeletal fusions. This is evident from the increased incidence of exencephalic fetuses: five out of six litters, totaling 13 fetuses, following the 50-mg./kg. atropine and 300-mg./kg. morphine combination on Day 8. Most combinations of atropine and morphine on both Days 8 and 9 resulted in greater numbers of axial skeletal fusions than with morphine alone. Although atropine has been used as an antidote in morphine poisoning (21) and has been shown to antagonize the Straub tail reactions produced by morphine (22), recent investigations conducted elsewhere indicate an "additive synergism" between atropine and morphine in producing death in mice (23). Hence, the enhancement of acute toxicity by atropine pretreatment of morphine-injected mice observed in the present study is in agreement with the latter study.

The fact that morphine has been shown to exert an effect upon the placental vasculature (10), plus the awareness that atropine has been reported to abolish certain actions produced by acetylcholine upon this structure (24), provided the impetus for this segment of the investigation. The importance of the placenta in the exchange occurring between the maternal and fetal organisms with respect to agents such as oxygen, nutrients, and glucose cannot be overemphasized.

Table VIII—Occurrence of Exencephaly, Axial Skeletal Fusions, and Cryptorchid Testes with Treatment on Day 9 (Compared with Morphine, 200 or 300 mg./kg.)

Treatment	Exencephaly				Cryptorchid Testes				Axial Skeletal Fusions			
	Litters		Fetuses		Litters		Fetuses		Litters		Fetuses	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
Saline, 0.3 ml., and morphine, 200 mg./kg.	6	0	59	0	5	1	13	1	6	0	28	0
Saline, 0.3 ml., and morphine, 300 mg./kg.	5	1	48	1	1	5	9	6	3	3	16	6
Atropine, 50 mg./kg., and morphine, 200 mg./kg.	5	1	62	1	4	2	12	2	0	6[a]	18	14[a]
Atropine, 50 mg./kg., and morphine, 300 mg./kg.	5	1	60	1	5	1	11	1	2	4	23	7
Physostigmine, 0.005 mg., and morphine, 200 mg./kg.	4	2	44	2	2	4[a]	7	5[a]	3	3	17	6
Physostigmine, 0.005 mg., and morphine, 300 mg./kg.	6	0	61	0	1	5	17	8	1	5	23	7

[a] Significant $(X)^2 > 3.84$ = significantly different from single morphine injection.

Consequently, the possibility exists that interference in the transport of any or all of the above by the action of an agent on the placental vessels, on either the maternal or fetal side, could have detrimental effects on the conceptus.

The results of the radioisotope study (Table IX) demonstrated that, following morphine administration, constriction of the maternal placental vessels occurred at the 5-min. interval, followed by vasodilation at the 15-min. interval. Comparison of the effects of the drugs upon the fetal side showed that both atropine and morphine produced constriction at the 5-min. interval, while the combination of atropine and morphine still caused constriction after 10 min.

The initial vasoconstriction of the placental vasculature by morphine *in vivo* is similar to that observed in the perfused human placenta (10). This action conceivably could precipitate a reduction in the amount of oxygen accessible to the fetus, with the subsequent production of congenital malformations or even death. Therefore, the fact that atropine prolonged morphine vasoconstriction of the placental vessels, plus the increased incidence of anomalies following the administration of this combination, leads to a good cause-and-effect relationship. Further substantiation of this possibility has been provided by others who clamped the uterine vessels in the rodent, which resulted in both severe malformations and death of the fetus (25). In addition, atropine has been reported to cause a fall in mean arterial oxygen tension (26), a condition not unlike that produced by morphine (27), which also reduces the extraction of oxygen from the blood circulating through the brain. Also, morphine has been reported to decrease fetal brain oxygen for at least 50 min. (28). Furthermore, an overdose of morphine early in gestation has resulted in the birth of a malformed child (29), attributed to the acute anoxia caused by morphine.

Ingalls *et al.* (4) induced anencephaly, cryptorchid testes, and rib fusions by exposing mice to decreased oxygen concentrations. These anomalies were the most prominent defects observed in the present study. Moreover, hydramnios, also observed in this study in association with every case of exencephaly, was present where anencephalic fetuses were found in the above study (4). Also, Ingalls and Curley (30), in another investigation, found cranioschisis, rib and vertebral malformations, and open eye in mice rendered hypoxic. Again, these abnormalities were also observed in the present study. In addition, Grabowski (31), utilizing chicks, noted "tremendous edema," blisters, hematomas, and the ultimate defects of "exocephaly," defective eyelids, and short upper beaks following exposure to reduced oxygen concentrations.

Table IX—Mean Percent of ^{22}Na Activity in Placentas and Fetuses of Selected Test Groups Compared with Saline

Group	Time Elapsed after Isotope Administration, min.	Mean Percent of ^{22}Na in Placenta	*SD*	Mean Percent of ^{22}Na in Fetus	*SD*
Saline	5	36.79	±3.40	9.81	±1.61
	10	36.61	±4.49	14.47	±1.42
	15	38.29	±1.66	17.29	±1.00
	20	39.40	±8.18	20.69	±2.62
Atropine sulfate, 50 mg./kg.	5	32.82	±3.04	7.63[a]	±0.29
	10	41.06	±7.22	13.98	±3.20
	15	39.95	±5.76	16.03	±2.29
	20	38.30	±6.30	20.26	±1.54
Physostigmine sulfate, 0.005 mg.	5	34.18	±3.73	7.93	±0.73
	10	40.09	±2.30	13.24	±0.61
	15	38.68	±8.20	17.43	±1.30
	20	48.78	±5.32	21.98	±2.38
Morphine sulfate, 300 mg./kg.	5	21.57[a]	±11.40	5.39[a]	±3.10
	10	37.93	±5.05	11.42	±2.38
	15	43.25[a]	±2.36	16.06	±1.92
	20	47.68	±7.70	20.97	±6.17
Atropine, 50 mg./kg., and morphine, 300 mg./kg.	5	32.69	±1.92	8.17	±0.52
	10	30.69	±5.74	8.60[a]	±2.69
	15	42.65	±5.12	18.15	±3.69
	20	43.12	±2.41	22.31	±1.21

[a] Significant $t > 2.776 = p < 0.05$ calculated from Student's t test = significantly different from saline control.

Table X—Observed Change in Blood Sugar on Either Day 8 or 9

Treatment	$\bar{X}$ Blood Sugar Change, mg./100	*SE*	p^a
Untreated control—Day 8	0.9	±0.95	0.20
Saline control—Day 8	8.0	±4.13	—
Morphine, 200 mg./kg.—Day 8	57.9[a]	±10.04	0.001
Morphine, 300 mg./kg.—Day 8	42.2[a]	±10.30	0.02
Morphine, 400 mg./kg.—Day 8	33.7	±12.05	0.10
Untreated control—Day 9	4.5	±0.89	0.80
Saline control—Day 9	5.2	±1.38	—
Morphine, 200 mg./kg.—Day 9	50.7[a]	±15.35	0.02
Morphine, 300 mg./kg.—Day 9	49.1[a]	±6.85	0.001
Morphine, 400 mg./kg.—Day 9	34.6[a]	±3.07	0.001

[a] Significant $t > 2.776 = p < 0.05$ calculated from Student's t test = significant increase in glycemic response compared with saline control.

The fact that hypoxia produces various biochemical changes, *e.g.*, an increased utilization of glycogen stores with a subsequent increase in blood glucose and lactic acid (32), plus the knowledge that hyperglycemia in mice precipitates a decrease in brain glycogen concentration (33) stimulated the third aspect of this particular study. All three doses of morphine employed on both Days 8 and 9 produced increases in blood glucose when compared with controls (Table X), with the greatest increase occurring at the lowest dose and the smallest increase occurring at the highest dose, respectively. This phenomenon can possibly be explained by the term "autointeraction" (34), in which a drug noncompetitively antagonizes its initial effect, as the concentration is increased, by becoming attached to a second receptor. Besides, it has been reported that high maternal blood sugars are accompanied by high fetal blood sugars (35). The acceptance of these facts enables one to assume that the aforementioned decrease in brain glycogen (33) precipitated by glucose administration also occurred in this study in response to the morphine-induced hyperglycemia. Since Runner (3) hypothesized that carbohydrate metabolism may be selectively critical for the morphogenesis of the embryonic neural tube, the above-mentioned hyperglycemia and resultant effects produced by anoxia upon carbohydrate metabolism cannot be completely excluded as possible factors contributing to morphine-induced congenital malformations.

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Residual Ethylene Oxide in Gas-Sterilized Medical-Grade Silicones

J. D. WHITE*▲ and T. J. BRADLEY†

Abstract □ Residual gas was extracted from ethylene oxide-treated silicone sheets by high vacuum distillation, and the residue subsequently was analyzed by GLC. To indicate the necessary aeration time of the materials before they can be considered safe to use, the desorption characteristics of ethylene oxide in three types of the silicone polymer were studied. Very rapid desorption of the gas to low residual levels was observed in the two nonreinforced silicone rubbers examined: medical-grade silicone sheeting and a medical-grade elastomer. The third material, Dacron-reinforced silicone sheeting, lost the gas at a considerably slower rate, after an initial rapid drop, due to retention by the Dacron reinforcement.

Keyphrases □ Silicone sheets, medical grade—absorption and diffusion of ethylene oxide after gas sterilization □ Ethylene oxide gas sterilization—absorption and diffusion from medical-grade silicone sheets □ Sterilization with ethylene oxide—absorption and diffusion from medical-grade silicone sheets

During the last couple of decades, there has been an ever increasing trend to utilize synthetic rubber and plastics in medical instruments and equipment as well as in permanent or semipermanent implants and prostheses. Because many synthetic materials are heat labile, conventional dry heat or autoclaving processes have been substituted by low temperature sterilization methods.

One such method is ethylene oxide gaseous sterilization, and its relative merits have already been well documented. However, one serious disadvantage of sterilization with this gas is that ethylene oxide remains in the material, and its presence in a polymeric device can present potential toxicological problems if the gas is released to tissue. Only in recent years, with the increasing use of GLC, has it been discovered that various types of polymeric materials may contain reaction products of ethylene oxide other than ethylene glycol, such as the highly toxic ethylene chlorohydrin (2-chloroethanol) which is formed in the presence of chloride ions (1, 2).

Medical-grade silicones are widely used in tissue implants and can be successfully sterilized both by conventional methods and by ethylene oxide. With the latter method, there is uncertainty concerning the retention time of the gas, together with the possibility of cytotoxic reaction products. These considerations prompted this study.

THEORY

If there is a uniform distribution of a dissolved gas in a thin slab of material and the surfaces are suddenly exposed to zero partial pressure of the gas and maintained at that, the gas begins to clear from the material. It can be shown (3) that, after an initial period during which the mean gas concentration in the medium decreases by 10–20%, the concentration at all points decreases exponentially with the same half-time:

$$T_{1/2} = \frac{2.77\left(\frac{L}{2}\right)^2}{\pi^2 D} \qquad \text{(Eq. 1)}$$

where:

$T_{1/2}$ = half-time (sec.)
L = thickness of material (cm.)
D = diffusion coefficient (cm.^{2} sec.$^{-1}$) —constant for a given gas, temperature, and material

Thus, if the total amount of dissolved gas can be calculated as a function of time, the diffusion coefficient can be deduced from the slope of the exponential (which indicates the time taken for the concentration of dissolved gas to decrease by half) and the thickness of the medium; repeating the experiment with a different thickness of the material provides a check on the value.

The $T_{1/2}$ of Eq. 1 is also a measure of the rate of diffusion *into* the material.

Table I shows the diffusion coefficient of ethylene oxide in several plastics at 23°. Knowledge of the diffusion coefficient of ethylene oxide in a particular material at room temperature should permit

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Table I—Diffusion Coefficient of Ethylene Oxide in Polyethylene, Polypropylene, and Polybutylene at 23°[a]

Polymer	Density, g. cm.$^{-3}$	Diffusion Coefficient, cm.^{2} sec.$^{-1}$
Polyethylene	0.913	6.17×10^{-8}
Polyethylene	0.917	3.52×10^{-8}
Polyethylene	0.930	1.83×10^{-8}
Polyethylene	0.963	1.30×10^{-8}
Polypropylene	0.904	0.44×10^{-8}
Polypropylene	0.906	1.09×10^{-8}
Polybutylene	0.914	1.09×10^{-8}

[a] From data shown in Table X of *Reference 4.*

some predictions to be made concerning the minimum aeration time necessary to achieve sterile, nontoxic medical devices manufactured from that material.

EXPERIMENTAL

Materials—Medical-grade silicone sheeting[1], reinforced and nonreinforced, and a room temperature vulcanizing medical-grade silicone elastomer (Silastic 382)[1] were used.

GLC—A dual flame-ionization gas chromatograph[2] was used. The columns were of glass tubing, 4 mm. i.d. and 1.5 m. (5 ft.) in length. The packing consisted of 80–100-mesh Porapak Q[3] for the estimation of ethylene oxide and of polyethylene glycol 20,000, 10% w/w, on 100–120-mesh Diatomite C[3] for the estimation of ethylene chlorohydrin. Nitrogen was used as the carrier gas at a flow rate of 45 ml. min.$^{-1}$ with a column oven temperature of 125°, an injection temperature of 175°, and a detector oven temperature of 200°. The injection solvent was distilled water. Peak areas were obtained from the product of the peak height and the width at half the peak height.

IR Determinations—A double-beam grating IR spectrophotometer[4] was employed. Standard gas cells (150-ml. capacity) were used to follow the uptake of ethylene oxide by the silicone, with measurements being made at 11.5 μ. Internal reflection spectroscopy was carried out using a KRS-5 crystal (thallous-bromide-iodide complex)[3] and a beam incidence angle of 60°.

Exposure to Ethylene Oxide—Uniformly thin sheets of the three types of silicone were cut into strips (each measuring approximately 20 × 76 mm.), which were freely suspended from a glass rod by metal clips. The strips of the different materials were then exposed in turn to pure ethylene oxide (1200 mg. l.$^{-1}$) at 23° and 60% relative humidity in the gas sterilizer[5] for a sufficient time to ensure equilibration (24 hr.). Three manual evacuation cycles were then carried out to empty the sterilizer chamber of the toxic ethylene oxide, and the strips were removed from the sterilizer. This procedure was followed by aeration at 23° under a gentle stream of air, the samples being taken at accurately timed intervals for extraction and analysis.

Extraction of Residual Ethylene Oxide—Vacuum extraction techniques for residual ethylene oxide and its reaction products have already been used successfully (5–7). The method employed here was based on distillation under high vacuum, with a distillate trap cooled in liquid nitrogen. The extracted sterilant residues were then dissolved in a small volume of distilled water for quantitative determination by GLC.

Diffusion Cell—A small, stainless steel diffusion cell was set up using a filter[6], with the silicone as a membrane to form the two compartments of the cell (Fig. 1). A 50-ml. gas syringe full of pure ethylene oxide was attached to one side while an empty syringe, equipped with a sampling device, was fitted to the other compartment; the pistons of the syringes allowed the contained gases to remain at atmospheric pressure at all times. The system was tested underwater for leaks and found to be gas tight. Samples were withdrawn at timed intervals from the receiver compartment and estimated on the gas chromatograph.

[1] Dow Corning Center for Aid to Medical Research, Midland, Mich.
[2] Pye Series 104.
[3] Pye Unicam, Cambridge, England.
[4] Perkin-Elmer 237.
[5] "Victoria Steriliser," The Medical Supply Association Ltd., London, England.
[6] Millipore, Millipore Corp., Bedford, Mass.

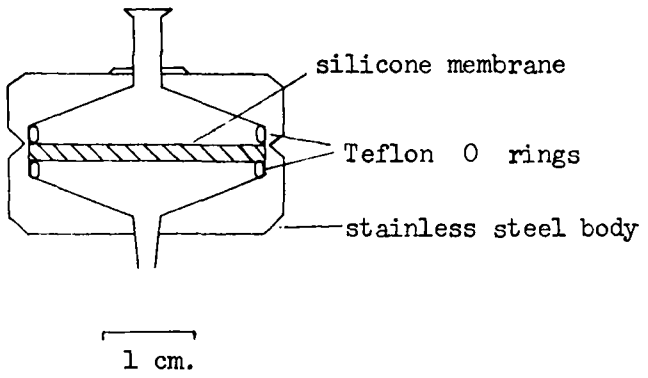

Figure 1—*Diffusion cell (25-mm. diameter microsyringe filter holder).*

RESULTS AND DISCUSSION

The results obtained for the uptake of ethylene oxide by the heat vulcanized silicone using the IR spectroscopy method and measuring the decrease in absorbance at 11.5 μ are displayed in Fig. 2. It can be seen that equilibration occurs within a short time.

By plotting the logarithm of residual ethylene oxide *versus* desorption time, the predicted straight-line graphs of Fig. 3 are obtained; these plots permit ready calculation of the diffusion coefficient of ethylene oxide in the elastomer, a room temperature vulcanized silicone rubber.

The desorption curves of ethylene oxide from heat vulcanized silicone sheeting, nonreinforced and Dacron reinforced (Figs. 4 and 5, respectively), suggest a two-stage desorption mechanism and require further investigation. An examination of Fig. 4 showed that it was possible that a compressed film of ethylene oxide had built up on the surface of the heat vulcanized silicone without gas penetration into its interior. Removal from the sterilizer would produce rapid loss of this surface film until only a relatively strongly held monolayer of adsorbed ethylene oxide remained. (This would correspond to the almost horizontal part of the curves and the amount agrees closely with calculation of monolayer capacity.)

This possibility was investigated in two ways:

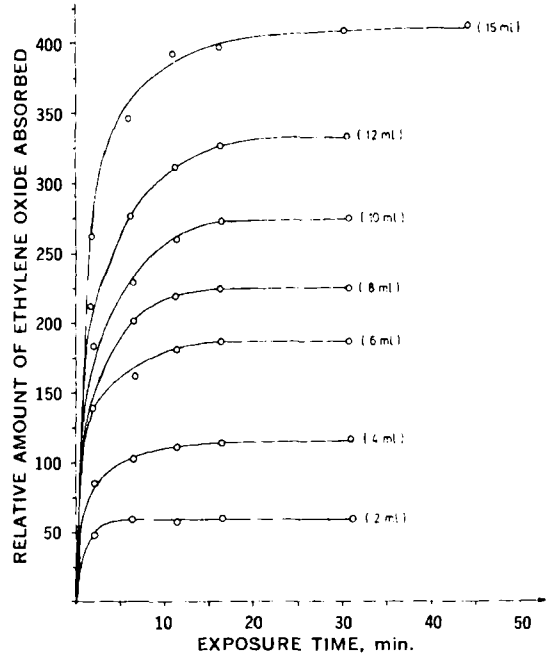

Figure 2—*Plot of amount of ethylene oxide absorbed in 1.0-mm. thick, heat vulcanized silicone rubber* versus *time exposed to the gas. The volume of ethylene oxide admitted to the gas cell is shown in parentheses beside each curve.*

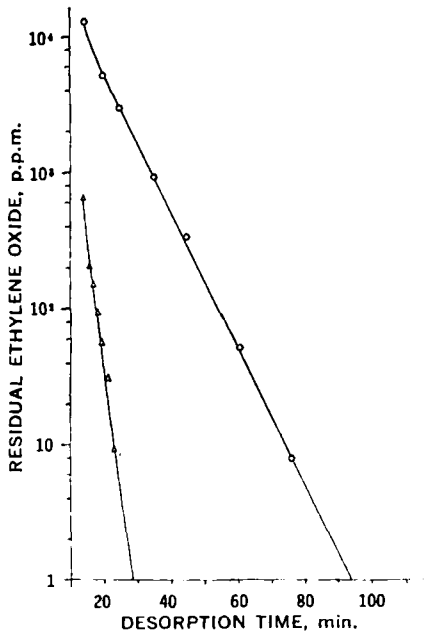

Figure 3—*Plot of log residual ethylene oxide in room temperature vulcanized silicone elastomer* versus *desorption time. Key:* O, *2.0-mm. thick sheet; and* Δ, *1.0-mm. thick sheet.*

1. By internal reflection spectroscopy, an IR technique used essentially to study surface phenomena. This technique was employed in an effort to detect the existence of a thin film of adsorbed ethylene oxide on the silicone immediately after removal from the sterilizer. No ethylene oxide was detected; however, this does not exclude the possibility of a monomolecular surface layer because the sensitivity of the method is low.

2. By determining the diffusion of ethylene oxide through a membrane of the silicone in the diffusion cell. The results (Fig. 6) indicate that ethylene oxide is, in fact, absorbed by heat vulcanized silicone and diffuses very rapidly through it. With this knowledge, the shape of the desorption curves of Fig. 5 can be explained by the relatively slow release of absorbed ethylene oxide from the Dacron tricot weave reinforcement in the middle of the sheet of heat vulcanized silicone.

Since it had been confirmed that absorption and diffusion do occur, the diffusion coefficient of ethylene oxide in the two non-

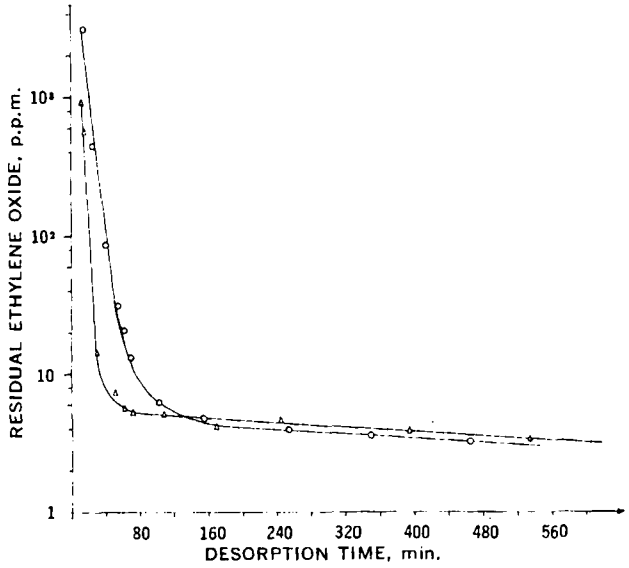

Figure 4—*Plot of log residual ethylene oxide in heat vulcanized silicone rubber* versus *desorption time. Key:* O, *1.6-mm. thick sheet; and* Δ, *1.0-mm. thick sheet.*

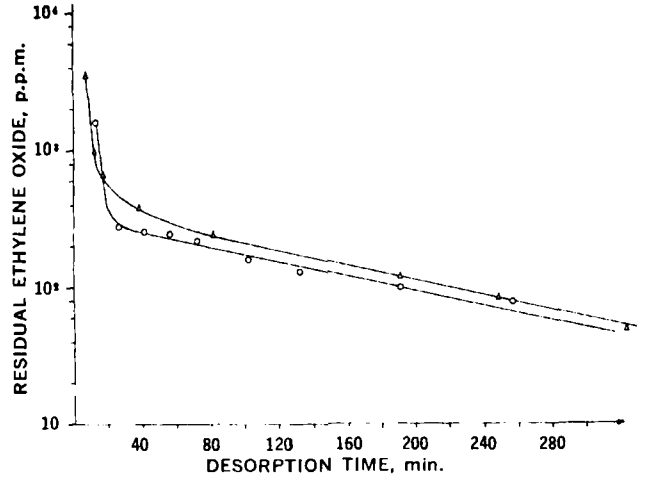

Figure 5—*Plot of log residual ethylene oxide in Dacron-reinforced heat vulcanized silicone rubber* versus *desorption time. Key:* O, *1.0-mm. thick sheet, and* Δ, *0.76-mm. thick sheet.*

reinforced materials was calculated using Eq. 1; the results are shown in Table II.

Since Fig. 5 indicates that it is the Dacron reinforcement and not the silicone that governs the desorption of ethylene oxide, it was not possible to obtain a value for the diffusion coefficient of the gas in the reinforced material. However, it was noted that during the first 45 min. after removal from the sterilizer the ethylene oxide level dropped to approximately 10% of its equilibrium value; thereafter, the half-time (*i.e.*, the time for the concentration of dissolved ethylene oxide to decrease by half) was observed to be about 100 min., which would seem to imply that reinforced silicone sheeting is completely safe to use after 24 hr. aeration.

Although the vacuum process is known to extract reaction products of ethylene oxide when these are present (and has been successfully used for this purpose by the authors), none was detected in any silicone sheeting.

An incontinence implant was used as the test piece in the final

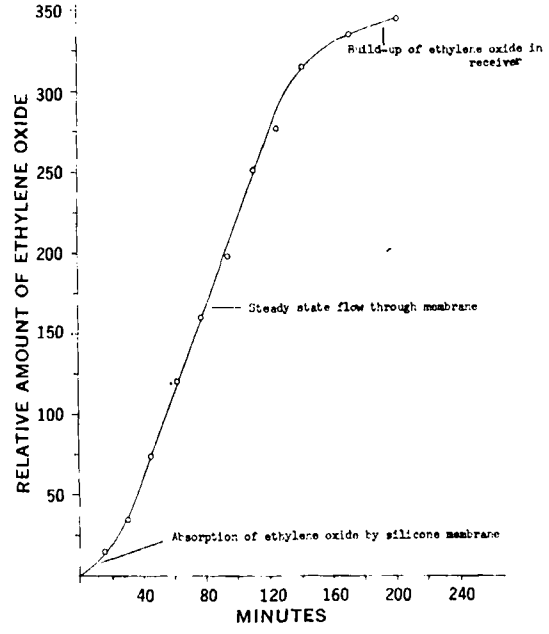

Figure 6—*Plot of amount of ethylene oxide which has diffused through a 1.0-mm. thick membrane of heat vulcanized silicone rubber* versus *time.*

Table II—Diffusion Coefficient of Ethylene Oxide in Silicone Sheeting at 23°

Material	Mean Thickness, mm.	Diffusion Coefficient, $cm.^2\ sec.^{-1}$
Room temperature vulcanized silicone	1.0	7.7×10^{-6}
	2.0	8.0×10^{-6}
Heat vulcanized silicone sheeting	1.0	4.3×10^{-6}
	1.6	4.2×10^{-6}

study to predict minimum aeration time using the diffusion coefficient. The body of the device, comprising the electronic components enclosed in room temperature vulcanized silicone, measured 38 mm. in diameter and was 10.8 mm. deep. Attached to it were two 23 mm. long wire leads, surrounded by heat vulcanized silicone approximately 0.7 mm. thick.

Two assumptions were made in working out the theoretical aeration time for the level of residual ethylene oxide to fall to a low value: (*a*) the body of the device could be considered as being composed entirely of room temperature vulcanized silicone, and (*b*) the silicone would contain a maximum of 13,000 p.p.m. ethylene oxide after sterilization. (This value comes from the previous experiments on this material.)

The first assumption is not entirely unjustified since any void areas in the center of the electronics compartment will have attained the equilibrium concentration of ethylene oxide throughout the surrounding silicone. Since the half-time for this thickness of silicone is approximately 170 min. at 23°, after sterilization at 50° for 1 hr. a uniform equilibrium concentration of absorbed ethylene oxide (13,000 p.p.m.) will probably not have been achieved.

Ignoring losses from the sides of the body, the time necessary to fall to 10 p.p.m. ethylene oxide was calculated using Eq. 1 and the experimental value of the diffusion coefficient of ethylene oxide in room temperature vulcanized silicone at 23°; the time was found to be about 27 hr.

This value was tested using the implant device described. The article, wrapped in the routine paper-polypropylene pack which presents no barrier to the passage of ethylene oxide gas, was subjected to the normal sterilizing procedure, *i.e.*, exposure to 1200 mg. ethylene oxide/l. for 1 hr. at 50°, followed by seven automatic evacuation cycles of the chamber. Aeration at the ambient temperature (23°) was for 24 hr., after which the analysis was carried out; 4 p.p.m. ethylene oxide was detected.

CONCLUSIONS

This study shows that the diffusion of ethylene oxide in nonreinforced medical-grade silicone (heat or room temperature vulcanized) is extremely rapid and that in the case of relatively simple shapes the minimum aeration time required to eliminate ethylene oxide can be obtained from Eq. 1.

Dacron-reinforced silicone sheeting loses the gas at a considerably slower rate due to retention by the Dacron reinforcement, and it is recommended that a minimum aeration of 24 hr. be adopted for this material.

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Comparison of Degree of Susceptibility of Hyperthyroid and Euthyroid Animals to Cardiac Glycoside-Induced Arrhythmias

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Abstract □ The present studies were performed to determine the arrhythmogenic dose of ouabain in euthyroid and hyperthyroid guinea pigs and rabbits. The animals were made hyperthyroid by daily injection of thyroxine 100 mcg./kg. i.p. for 10–14 days. Cardiovascular responses, including heart rate from limb lead II electrocardiogram and arterial blood pressure, were monitored. By the method of analysis of variance, no significant difference was shown between the arrhythmogenic dose of ouabain in euthyroid and hyperthyroid guinea pigs. However, this difference was statistically significant in rabbits. An increase in extracellular serum calcium-ion concentration did not change the responsiveness of the myocardium to ouabain toxicity in either the euthyroid or the hyperthyroid animal. In addition, the β-adrenergic receptor blocking drug practolol (10 mg./kg.) was ineffective in completely abolishing the resting tachycardia in hyperthyroid rabbits. These studies do not support the idea of decreased responsiveness to cardiac glycosides due to the hyperthyroid state.

Keyphrases □ Ouabain-induced arrhythmias in hyperthyroid and euthyroid guinea pigs and rabbits—species differences, effect of extracellular calcium and practolol □ Cardiac glycoside-induced arrhythmias in hyperthyroid and euthyroid guinea pigs and rabbits—species differences, effect of extracellular calcium and practolol □ Arrhythmogenic response to ouabain—comparison between euthyroid and hyperthyroid guinea pigs and rabbits □ Hyperthyroid and euthyroid guinea pigs and rabbits—degree of susceptibility to ouabain-induced arrhythmias compared

There is now abundant evidence (1, 2) that the hyperthyroid state is usually associated with increased sensitivity of the myocardium to various drugs. In addition to sympathomimetic amines, these drugs include histamine, cocaine, endotoxin, monoamine oxidase (MAO) inhibitors, and barbiturates (3). Evidence also exists that thyroid hormones influence the excitability of the myocardial conducting system. For instance, feeding rabbits with thyroid extract made the rabbit hearts more susceptible to epinephrine arrhythmias, such as atrial fibrillation and ventricular tachycardia (4). Other studies (5–7) showed that hyperthyroidism is a common cause of atrial fibrillation and other supraventricular arrhythmias, whereas irregularities of cardiac rhythm are rare in myxedema.

However, the findings of other investigators do not support this view of increased sensitivity of the myocardium in hyperthyroid conditions to certain drugs. In experimentally induced hyperthyroidism in dogs, hyperthyroid animals were more resistant to the development of ouabain-induced ventricular arrhythmias than were hypothyroid and euthyroid dogs (8) and no significant difference was found, between the arrhythmogenic doses of ouabain in hypothyroid and euthyroid animals.

The difference in response to digitalis glycosides demonstrated in experimental animals (8, 9) and clinically (1, 10) has made the question of why the thyroid state modifies the effectiveness of digitalis glycoside action an increasingly important one from the standpoint of both therapy and the potential susceptibility of the myocardium to cardiac glycoside-induced arrhythmias. The action of thyroxine in sensitizing the myocardium to biogenic amines and other drugs has been described (3) as being nonspecific.

Although it has been implied (11, 12) that intact catecholamine stores are necessary for the increased sympathetic activity and sometimes the arrhythmias observed in hyperthyroid animals, it is still equivocal whether the increased sympathetic activity seen in conditions of hyperthyroidism plays a role in the arrhythmogenic toxicity of cardiac glycosides. If such arrhythmias were dependent upon increased levels of sympathetic activity brought about by the hyperthyroid state, then these arrhythmias would occur much more readily and at lower dose levels of the cardiac glycoside in the experimentally induced hyperthyroid state than in the euthyroid state. Furthermore, this would imply an increased rather than a decreased sensitivity to the cardiac glycoside as suggested by earlier investigators (8, 9). Since digitalis toxicity is associated with myocardial potassium loss, it is also postulated (13–15) that ionic imbalance such as the acute loss of potassium from myocardial cells caused by rapid digitalization or an increase in calcium plasma levels will cause the precipitation of cardiac arrhythmias in hyperthyroid states sooner than in euthyroid conditions.

The aims of this study were: (*a*) to elucidate further the influence of excessive thyroxine on ouabain-induced changes of spontaneous heart rate and arterial blood pressure in hyperthyroid and euthyroid guinea pigs and rabbits, and (*b*) to determine if there is a difference in the arrhythmogenic dose of ouabain under such conditions.

EXPERIMENTAL

Albino guinea pigs, weighing 200–300 g., and New Zealand White rabbits, weighing 2.5–3.5 kg., of either sex, fed on a standard diet[1] and water *ad libitum* were used in these experiments. A condition of hyperthyroidism was induced by daily injection of 100 mcg./kg. i.p. of sodium levothyroxine[2] for 10–14 days. Euthyroid animals were given a daily intraperitoneal injection of normal saline in amounts equivalent to the volume of the thyroxine dose employed. Prior to each experiment, each animal was anesthetized with sodium pentobarbital[3]; the guinea pigs received 25 mg./kg. i.p. and the rabbits received an identical dose intravenously. Supplemental doses of 0.2–0.5 ml. were given as needed.

The trachea of each animal was cannulated to ensure uninterrupted respiration. A jugular vein was exposed and cannulated for

[1] Purina.
[2] Synthroid, 100 mg./ml., Flint Laboratories.
[3] Nembutal, 50 mg./ml., Abbott Laboratories.

Table I—Comparison of Arrhythmogenic Dose of Ouabain in Euthyroid and Hyperthyroid Animals (Mean ± *SE*)

Type of Drug Treatment	Animal Species	Number of Animals	Total Cumulative Ouabain Dose Inducing Arrhythmias, mcg./kg.	
			Euthyroid State	Hyperthyroid State
Saline-treated animals	Guinea pigs	8	87.0 ± 5.4	90.0 ± 8.6 $p > 0.9$
	Rabbits	6	110.0 ± 5.6	87.5 ± 4.5 $p < 0.025$
Reserpinized animals	Guinea pigs	6	150.0 ± 9.1	178.0 ± 16.4 $p > 0.9$
	Rabbits	8	130.6 ± 10.9	72.5 ± 3.9 $p < 0.0025$
Continuous calcium chloride infusion	Guinea pigs	9	82.7 ± 6.8	82.7 ± 4.6 $p = 1.0$
	Rabbits	6	59.2 ± 4.1	65.0 ± 3.7 $p > 0.8$

intravenous administration of drugs, and a cannulated common carotid artery was connected by means of a polyethylene catheter to a pressure transducer[4] for monitoring arterial blood pressure. Heparinized saline (sodium heparin, 10 mg./ml.) was used routinely for flushing both arterial and venous catheters. Heart rate was measured from an electrocardiogram (ECG) obtained with limb lead II needle electrodes inserted under the skin. ECG changes as well as arterial blood pressure were recorded on an ink-writing oscillograph[5]. In those experiments in which a 2 m*M* concentration of calcium chloride was administered, the solution was infused by means of an infusion pump[6] at the rate of 5.5 ml./hr. After the surgical procedure, 15–20 min. was allowed for stabilization before baseline heart rate and blood pressure were measured. Control readings were taken prior to any drug administration.

The following drugs were used: sodium levothyroxine (100 mg./ml.), sodium pentobarbital (50 mg./ml.), reserpine (25 mg./ml.), ouabain (0.5 g./ml.), calcium chloride (2 m*M* solution), and practolol (10 mg./ml.).

The values obtained from these experiments were expressed as the mean ± *SE* of six or more observations. Statistical analyses of the data were performed using conventional formulas; differences between means were tested with the Student *t* test and analysis of variance described in Goldstein (16).

Control Experiments with Hyperthyroid and Euthyroid Animals—The guinea pigs and rabbits used in this study were divided into groups of euthyroids and hyperthyroids. These animals received daily injections of normal saline and thyroxine, respectively, for a total of 14 days. Heart rate, determined from limb lead II ECG's, and arterial blood pressure were monitored every 10 min.

Following stabilization of the monitored variables, a loading dose of 25 mcg./kg. i.v. of ouabain was administered followed by 15 mcg./kg. 15 min. later and a maintenance dose of 10 mcg./kg. given every 10 min. therafter until the first signs of rhythm disturbance occurred. The end-point was taken as the appearance of three or more premature ventricular contractions or extrasystoles. The total dose of ouabain was then noted, and the means of the values for the total number of animals in the series for both euthyroid and hyperthyroid animals were compared for significance.

Experiments with Reserpine-Treated Animals—Euthyroid and hyperthyroid guinea pigs and rabbits were pretreated with 0.5–1.0 mg./kg. i.p. of reserpine for 2 consecutive days prior to the day of challenge with ouabain.

Heart rate, arterial blood pressure, and ECG changes were monitored in all animals. The total amount of intravenously administered ouabain that evoked arrhythmia was noted for each animal.

Experiments Involving Calcium Infusion—Both hyperthyroid and euthyroid rabbits and guinea pigs received calcium chloride by infusion. Prior to the ouabain administration, a continuous infusion of a 2 m*M* solution of calcium chloride *via* the cannulated jugular vein was commenced at the rate of 5.5 ml./hr. Infusion was continued for 10–20 min. before the initial dose of ouabain. The calcium chloride infusion was continued until the first signs of cardiac irregularities appeared; it was then stopped and the total amount of ouabain evoking the arrhythmias was noted.

Experiments with β-Adrenergic Receptor Blocking Drugs—The β-adrenergic receptor blocking agent practolol[7], reported (17) to have a selective cardiac blocking action, was used to determine the effect of adrenergic blockade on the increased sympathetic activity in hyperthyroid rabbits. Anesthetized animals breathing room air spontaneously were used. Doses of 1, 2, 5, and 10 mg./kg. i.v. of the blocking drug were administered on a cumulative basis at 10-min. intervals. Control readings were taken in all instances prior to practolol administration.

[4] Narco-Biosystems.
[5] Narco-Biosystems physiograph model 4-A.
[6] Sage model 240.
[7] AY-21011.

RESULTS

Control Experiments with Euthyroid and Hyperthyroid Animals—*Guinea Pigs*—The apparent difference in the arrhythmogenic dose of ouabain in the euthyroid and hyperthyroid states was not statistically significant (Table I). In eight animals, prior to ouabain administration the mean heart rate response was found to be 248 ± 7 beats/min. in the euthyroid state as compared to 318 ± 12 beats/min. in the hyperthyroid state (Table II). However, serial administration of ouabain intravenously produced a progressive increase in spontaneous heart rate. The arterial blood pressure responses in the resting state and during ouabain administration in the guinea pigs (Table III) were higher in hyperthyroid than in euthyroid states. Injection of ouabain caused a progressive rise in both systolic and diastolic pressures in the hyperthyroid state, while euthyroid animals demonstrated a nonuniform response.

Rabbits—With rabbits not pretreated with either reserpine or calcium, the average resting heart rate values for six animals prior to ouabain injection were 372 ± 19 beats/min. in hyperthyroid rabbits and 297 ± 8 beats/min. in euthyroid rabbits. This difference in spontaneous heart rate values between hyperthyroid and euthyroid states was statistically significant ($p < 0.01$). Unlike guinea pigs, spontaneous heart rate in euthyroid and hyperthyroid rabbits did not increase following ouabain administration.

The average total dose of ouabain evoking arrhythmias (Table I) in these two groups of rabbits was significantly different ($p < 0.025$) in both euthyroid and hyperthyroid rabbits. Arterial blood pressure values at the start of the experiment were 230 ± 8/206 ± 6 mm. Hg in hyperthyroid and 193 ± 9/173 ± 11 mm. Hg in euthyroid rabbits. Ouabain administration resulted in a slight but progressive increase in the systolic pressure of euthyroid animals. In hyperthyroid rabbits, both systolic and diastolic pressures decreased with time (Table III).

Reserpine-Treated Animals—*Guinea Pigs*—Reserpinization of guinea pigs for 2 days caused a reduction in the resting spontaneous heart rate in both euthyroid and hyperthyroid animals. These values were 204 ± 9 beats/min. and 227 ± 13 beats/min., respectively. There was an observed difference in the average values of the resting spontaneous heart rate of nonreserpinized and reserpinized euthyroid guinea pigs. Heart rate was higher in the nonreserpine-pretreated animals (248 ± 7 beats/min.) than in the pretreated group of animals (204 ± 9 beats/min.) (Table II). Similarly, the resting spontaneous heart rate value was higher in nonreserpinized hyperthyroid guinea pigs (318 ± 12 beats/min.) than in the reserpinized hyperthyroid group (227 ± 13 beats/min.). The reduction in heart rate seen in hyperthyroid guinea pigs due to reserpine pretreatment was significantly different from that of the nonreserpinized hyperthyroid animals ($p < 0.01$ analysis of variance).

The initial arterial blood pressure of 121 ± 6/96 ± 6 mm. Hg in euthyroid reserpinized guinea pigs was low compared with 151 ± 14/119 ± 19 mm. Hg pressure in the nonreserpinized animals. In hyperthyroid guinea pigs, the increased diastolic blood pressure response to ouabain injection was much more gradual than the systolic component (Table II). Although there was an apparent difference in the total dose of ouabain evoking arrhythmias in euthyroid and hyperthyroid guinea pigs, this difference was not statistically significant (Table I).

Table II—Spontaneous Heart Rate in Guinea Pigs and Rabbits following Ouabain Administration[a]

Animal Treatment	Experimental Condition	Heart Rate, beats/min.[b] 0 min.	10 min.	30 min.	50 min.	70 min.	90 min.
Control guinea pigs	Euthyroid	248 ± 7 (8)	246 ± 6 (8)	264 ± 4 (7)	282 ± 9 (4)	303 ± 9 (3)	
	Hyperthyroid	318 ± 12[c] (8)	314 ± 11[c] (8)	324 ± 6[c] (7)	332 ± 9[c] (4)	333 ± 17 (3)	
Reserpinized guinea pigs	Euthyroid	204 ± 9 (6)	202 ± 9 (6)	210 ± 9 (6)	237 ± 10 (6)	248 ± 16 (4)	256 ± 16 (3)
	Hyperthyroid	227 ± 13 (6)	215 ± 11 (6)	221 ± 9 (6)	245 ± 11 (6)	242 ± 21 (5)	—
Calcium-infused guinea pigs	Euthyroid	242 ± 5 (9)	259 ± 5 (9)	271 ± 9 (8)	278 ± 5 (6)	262 ± 10 (4)	-
	Hyperthyroid	329 ± 14[c] (9)	324 ± 13[c] (9)	329 ± 9[c] (9)	339 ± 5[c] (9)	330 ± 6[c] (3)	-
Control rabbits	Euthyroid	297 ± 8 (6)	297 ± 7 (6)	285 ± 7 (6)	267 ± 8 (6)	260 ± 8 (6)	253 ± 9 (4)
	Hyperthyroid	372 ± 19[c] (6)	372 ± 16[c] (6)	358 ± 26[c] (6)	322 ± 36 (6)	332 ± 30[d] (5)	308 ± 22 (3)
Reserpinized rabbits	Euthyroid	231 ± 9 (7)	217 ± 9 (7)	231 ± 11 (7)	239 ± 12 (7)	242 ± 21 (4)	250 ± 25 (3)
	Hyperthyroid	280 ± 31[d] (8)	276 ± 29[d] (8)	254 ± 24[d] (8)	230 ± 14[d] (8)	208 ± 31[d] (5)	215 ± 1[d] (2)
Calcium-infused rabbits	Euthyroid	283 ± 8 (6)	283 ± 12 (6)	260 ± 10 (6)	278 ± 20 (5)	300 ± 1 (1)	300 ± 1 (1)
	Hyperthyroid	342 ± 26 (6)	326 ± 23 (6)	292 ± 25 (6)	292 ± 22 (5)	320 ± 26 (3)	—

[a] Numbers in parentheses refer to number of animals tested in each group. [b] Heart rate values (mean ± *SE*). [c] p significant at 0.01% level. [d] p significant at 0.05% level.

Rabbits—Pretreatment of rabbits with reserpine also caused a reduction in the resting spontaneous heart rate in both euthyroid and hyperthyroid animals. The heart rate values were 231 ± 9 beats/min. and 280 ± 31 beats/min., respectively (Table II). Serial administration of ouabain did not change these values substantially; however, only a slight progressive increase in spontaneous heart rate occurred with time in the reserpinized hyperthyroid rabbits. The difference in the arrhythmogenic dose of ouabain between the reserpinized euthyroid rabbits and the reserpinized hyperthyroid rabbits was highly significant (Table I).

In reserpinized euthyroid rabbits, both systolic and diastolic arterial pressures declined progressively with time following ouabain administration (Table IV). However, reserpinized hyperthyroid rabbits showed an apparently consistent level of both systolic and diastolic arterial pressures.

Calcium Chloride Infusion in Animals—*Guinea Pigs*—The infusion of calcium chloride solution into euthyroid and hyperthyroid guinea pigs did not seem to affect either the spontaneous heart rate or the arrhythmogenic dose of ouabain. A comparison of the data of noncalcium-treated and calcium-treated euthyroid guinea pigs shows that there was no significant difference in the resting heart rate ($p > 0.1$). The spontaneous heart rate values were 248 ± 7 beats/min. and 242 ± 5 beats/min. in the untreated and treated animals, respectively. There was no appreciable difference in the time of onset of arrhythmia, and the arrhythmogenic doses of ouabain were numerically identical.

In both the euthyroid and hyperthyroid states, diastolic blood pressure responses were severely suppressed. This effect was more pronounced in the hyperthyroid state. The levels of arterial pressure of both calcium-infused euthyroid and hyperthyroid guinea pigs 90 min. following ouabain injection were 135 ± 10/92 ± 7 mm. Hg and 123 ± 8/75 ± 1 mm. Hg, respectively (Table II).

Rabbits—Infusion of calcium chloride in euthyroid and hyperthyroid rabbits did not appreciably increase the spontaneous heart rate response over 90 min. following ouabain injection. However, a comparison of these data with those of noncalcium-treated euthyroid and hyperthyroid rabbits (Table II) indicates only a slight increase in spontaneous heart rate due to ouabain injection. The heart rates were 318 ± 12 beats/min. in noncalcium-treated hyperthyroid compared to 342 ± 26 beats/min. in calcium-treated hyperthyroid animals ($p > 0.1$).

Calcium chloride (2 m*M* solution) infusion shortened the time required for the onset of ouabain-induced arrhythmias in euthyroid rabbits. Ventricular extrasystoles appeared early following repeated injections of ouabain in four out of six euthyroid rabbits tested. These extrasystoles occurred 20 min. later in five out of six hyperthyroid rabbits.

In both calcium-treated euthyroid and calcium-treated hyperthyroid animals, systolic blood pressure remained nearly constant whereas diastolic pressure showed a progressive decline.

Effects of β-Adrenergic Receptor Blockade in Hyperthyroid Rabbits—The resulting changes in heart rate and arterial blood pressure caused by administration of cumulative doses of practolol, a β-adrenergic receptor blocking agent, are depicted graphically in Fig. 1. Practolol produced a progressive reduction of spontaneous heart rate. However, the resting tachycardia resulting from the hyperthyroid state was decreased, but not abolished, by β-adrenergic blockade. The spontaneous heart rate prior to administration of 1 mg./kg. practolol was 363 ± 28 beats/min., and it was 299 ± 29 beats/min. after a total cumulative dose of 10 mg./kg. This reduction in heart rate was not statistically significant ($p > 0.05$).

DISCUSSION

These studies indicate that there was a varying degree of sensitivity to the arrhythmogenic dose of ouabain between the euthyroid group and hyperthyroid group of animals tested. In rabbits, the arrhythmia-evoking doses of ouabain were found to be significantly different between euthyroid and hyperthyroid groups of animals not premedicated with either reserpine or calcium as well as the reserpine-pretreated animals. However, in both calcium-treated euthyroid and hyperthyroid rabbits, this dose of ouabain was identical (Table I). Therefore, these findings suggest that the level of sensitivity to ouabain toxicity had not been altered in the guinea pig by the experimentally induced hyperthyroid state but was significantly modified in rabbits. It further suggests that experimental hyperthyroidism may modify the tolerance level to cardiac glycosides in one species of animal without significant effects in others. This modification usually results in an increase in the amount of glycoside required to attain the arrhythmia end-point. The present findings are to the contrary.

Although observations in laboratory animals suggested a relationship between many of the manifestations of thyroxine-induced hyperthyroidism and the activity of the sympathetic nervous system, the existence of such a relationship is still equivocal. The data from this study do not wholly support the assumption that intact catecholamine stores are necessary for some of the toxic effects of cardiac glycosides on the heart in hyperthyroid states. The mean arrhythmogenic dose of ouabain was slightly higher in the reserpinized guinea

Table III—Arterial Blood Pressure Responses in Guinea Pigs following Ouabain Administration[a]

		Arterial Blood Pressure, mm. Hg[b]					
		0 min. S/D[c]	10 min. S/D	30 min. S/D	50 min. S/D	70 min. S/D	90 min. S/D
Control guinea pigs	E[d]	151 ± 14/119 ± 19 (8)	155 ± 18/124 ± 20 (8)	162 ± 19/127 ± 22 (8)	134 ± 17/95 ± 23 (4)	133 ± 11/90 ± 28 (3)	144 ± 24/82 ± 17 (2)
	H[e]	164 ± 17[f]/132 ± 18[g] (8)	171 ± 17[f]/136 ± 18[g] (8)	179 ± 19[f]/142 ± 20[g] (8)	176 ± 17[g]/141 ± 19[g] (8)	178 ± 30/154 ± 35 (4)	210 ± 1/200 ± 1 (1)
Reserpinized guinea pigs	E	121 ± 6/96 ± 6 (6)	127 ± 8/106 ± 8 (6)	146 ± 8/123 ± 7 (6)	143 ± 7/122 ± 7 (6)	146 ± 13/128 ± 9 (4)	139 ± 18/123 ± 14 (3)
	H	79 ± 20[f]/55 ± 22[g] (6)	88 ± 17[f]/58 ± 21[g] (6)	93 ± 19[f]/62 ± 20[g] (6)	103 ± 19[g]/67 ± 20[g] (6)	108 ± 35/85 ± 35 (4)	93 ± 23/50 ± 15 (2)
Calcium-treated guinea pigs	E	108 ± 12/85 ± 12 (9)	126 ± 10/87 ± 16 (9)	140 ± 18/83 ± 4 (8)	144 ± 24/103 ± 15 (6)	122 ± 5/84 ± 4 (4)	135 ± 10/92 ± 7 (2)
	H	129 ± 4/75 ± 2 (9)	134 ± 7/79 ± 4 (9)	143 ± 6/83 ± 4 (9)	136 ± 6/78 ± 4 (9)	125 ± 14/75 ± 13 (3)	123 ± 8/75 ± 10 (2)

[a] Numbers in parentheses refer to number of animals per group. [b] Arterial blood pressure responses (mean ±*SE*). [c] Systolic over diastolic arterial pressures. [d] Euthyroid animals. [e] Hyperthyroid animals. [f] *p* significant at 0.01% level. [g] *p* significant at 0.05% level.

Table IV—Arterial Blood Pressure Responses in Rabbits following Ouabain Administration[a]

		Arterial Blood Pressure, mm. Hg[b]					
		0 min. S/D[c]	10 min. S/D	30 min. S/D	50 min. S/D	70 min. S/D	90 min. S/D
Control rabbits	E[d]	193 ± 9[e]/173 ± 11[e] (6)	195 ± 9[e]/172 ± 11[e] (6)	195 ± 9[e]/175 ± 10[e] (6)	199 ± 11/170 ± 14[e] (6)	207 ± 10[g]/175 ± 9[g] (6)	200 ± 14[g]/166 ± 12[g] (4)
	H[f]	230 ± 8[g]/206 ± 6[g] (6)	227 ± 7[g]/197 ± 5[g] (6)	234 ± 7[g]/207 ± 7[g] (6)	195 ± 20[g]/167 ± 17[g] (6)	218 ± 13[g]/181 ± 3[g] (6)	207 ± 7[g]/177 ± 9[g] (4)
Reserpinized rabbits	E	130 ± 26[e]/93 ± 25[e] (6)	128 ± 24[e]/100 ± 24[e] (6)	128 ± 24[e]/97 ± 26[e] (6)	136 ± 29/85 ± 30[e] (6)	73 ± 23[e]/36 ± 29[e] (3)	70 ± 10[g]/25 ± 15[g] (2)
	H	128 ± 7[g]/100 ± 4[g] (6)	123 ± 2[g]/81 ± 20[g] (6)	121 ± 4[g]/90 ± 7[g] (6)	93 ± 20[g]/78 ± 9 (6)[g]	119 ± 7[g]/88 ± 11 (3)[g]	113 ± 13[g]/44 ± 21 (2)[g]
Calcium-treated rabbits	E	170 ± 22/149 ± 20 (5)	170 ± 21/151 ± 20 (5)	174 ± 22/149 ± 21 (5)	159 ± 15/131 ± 14 (5)	193 ± 28/165 ± 40 (2)	—
	H	170 ± 10/143 ± 9 (5)	171 ± 10/141 ± 9 (5)	168 ± 8/139 ± 8 (5)	165 ± 23/135 ± 21 (4)	175 ± 36/143 ± 29 (4)	163 ± 8/124 ± 1 (2)

[a] Numbers in parentheses refer to number of animals per group. [b] Arterial blood pressure responses (mean ± *SE*). [c] Systolic over diastolic arterial pressures. [d] Euthyroid animals. [e] *p* significant at 0.05% level. [f] Hyperthyroid animals. [g] *p* significant at 0.01% level.

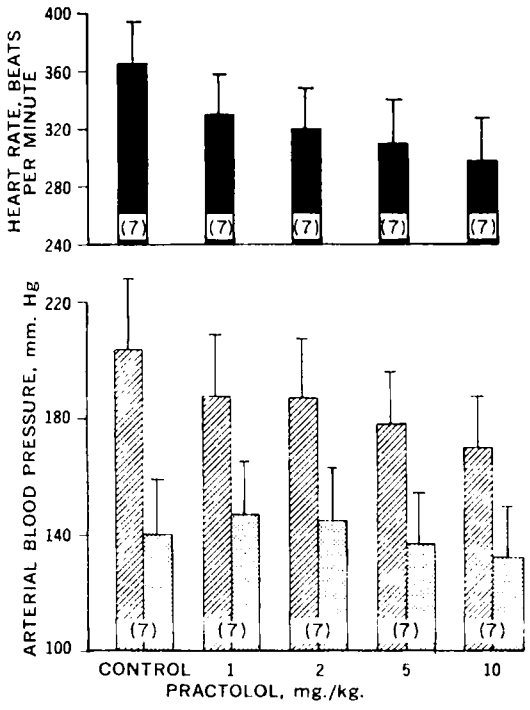

Figure 1 —*(Top) Effects of different doses of practolol (a β-adrenergic receptor blocking agent) on spontaneous heart rate in hyperthyroid rabbits. Number of observations* (n) = 7. *(Bottom) Corresponding arterial blood pressure response to the doses of practolol* (n = 7). *Key:* ▨, *systolic pressure of hyperthyroid rabbits;* ▩, *diastolic pressure of hyperthyroid rabbits; and* I, SEM.

pigs in the hyperthyroid state than in the reserpinized euthyroid condition. This suggests a change in responsiveness due partly to a depletion of catecholamine stores and partly to a predominance of parasympathetic activity produced by reserpine. This view is in agreement with that expressed by Cairoli and Crout (18), who found that the rate-normalizing effect of reserpine as seen by induced bradycardia in both euthyroid and thyroxine-treated unanesthetized rats could be blocked with atropine.

In rabbits, the arrhythmogenic dose of ouabain was significantly lower in the reserpinized hyperthyroid animals than in the reserpinized euthyroid animals. This observation, however, does not support those of Roberts *et al.* (19), who reported that reserpine pretreatment increased the amount of acetylstrophanthidin required to induce ventricular arrhythmias in the dog. This difference in response to ouabain in both reserpinized rabbits and guinea pigs in the hyperthyroid state still leaves an equivocal answer to the question of the role of sympathetic activity in cardiac glycoside toxicity in experimental hyperthyroidism. The present data imply that intact catecholamine stores are essential as mediators of the toxic manifestation of glycosides in hyperthyroidism in certain animal species but not in others.

Although it is known that cardiac glycosides can cause a loss of potassium ions from myocardial cells, thereby rendering the heart sensitive to increased concentrations of serum calcium, the present study shows that an increase of extracellular calcium ions, induced by an infusion of a 2 m*M* solution of calcium chloride, did not seem to make a difference in the degree of sensitivity of the heart to the arrhythmogenic dose of ouabain in either euthyroid or hyperthyroid animals.

The β-adrenergic receptor blocking agent practolol, which is known to possess a selective blocking action on the heart, failed to abolish the resting tachycardia of hyperthyroidism. This observation is explained partly on the basis of the fact that the many manifestations of hyperthyroidism are not mediated entirely by increased sympathetic activity and partly because practolol lacks a depressant activity on the myocardium. As a β-blocker, it is less potent than its congener propranolol, which had been used in the treatment of tachycardia in thyrotoxic patients (20–22). The reduction of the resting tachycardia from control values by different doses of practolol was not statistically significant at all dose levels ($p > 0.05$). These observations suggest either that the blockade was incomplete or that nonadrenergic factors continue to sustain cardiac hyperactivity in the hyperthyroid state.

SUMMARY AND CONCLUSIONS

The present investigation permitted the further elucidation of the relationship of the thyroid state and increased sympathetic activity and their contribution to any altered sensitivity of the myocardium to cardiac glycosides. Of particular interest was the finding that an increase in extracellular serum calcium ions did not change the responsiveness of the myocardium to ouabain toxicity in either euthyroid or hyperthyroid animals.

Furthermore, euthyroid and hyperthyroid guinea pigs responded differently from rabbits in certain drug treatments. This observation may partly explain the contrasting findings of Goodkind *et al.* (23), who found that myocardial norepinephrine concentration in guinea pig ventricles increased in hyperthyroidism, and those of Kurland *et al.* (24), who showed that the norepinephrine concentration in rabbit ventricles was decreased in hyperthyroidism and increased in hypothyroidism.

The β-adrenergic receptor blocking drug practolol was ineffective in normalizing resting tachycardia in hyperthyroid rabbits at the dose levels used in these studies. Although these studies have pointed out definite differences in some cardiovascular responses in the euthyroid and hyperthyroid states, the findings with regard to the arrhythmia threshold in euthyroid and hyperthyroid animals, in reserpinized animals as well as in calcium-treated animals, only help to confirm further the observations of earlier investigators and to point out that the factor or factors responsible for this change in myocardial response to cardiac glycosides in hyperthyroidism are still unknown.

The answers to these questions will most likely be found in the elucidation of other mechanisms at the molecular level since the direct effects of excessive blood level of thyroxine on myocardial cells are still obscure.

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Coumarins XII: Synthesis of (±)-*cis*- and *trans*-3′,4′-Dihydroxy-3′,4′-dihydroxanthyletin and Their Diesters

SAMIR M. EL-ANTABLY* and T. O. SOINE▲

Abstract □ (±)-*cis*-3′,4′-Dihydroxy-3′,4′-dihydroxanthyletin was synthesized from xanthyletin by the use of osmium tetroxide. It was also obtained, unexpectedly, by acid or base hydrolysis of (±)-*trans*-3′-acetoxy-4′-(*m*-chlorobenzoyloxy)-3′,4′-dihydroxanthyletin and, spontaneously, by recrystallization of the product from epoxidation of xanthyletin with trifluoroperacetic acid. (±)-*trans*-3′,4′-Dihydroxy-3′,4′-dihydroxanthyletin was obtained together with the (±)-*trans*-3′-hydroxy-4′-acetoxy derivative by peracetic acid treatment of xanthyletin. The diacetates, dipropionates, and di-*n*-butyrates of both the *cis*- and *trans*-diols were prepared together with the *trans*-3′-propionoxy-4′-acetoxy- and 3′-*n*-butyroxy-4′-acetoxy-3′,4′-dihydroxanthyletins. NMR studies of the diols and the diesters were carried out to establish chemical shifts of specific groups, with special emphasis on the 2′-gem-dimethyls together with the *J* values for the 3′- and 4′-protons as diagnostic features for the *cis*- or *trans*-configuration.

Keyphrases □ Xanthyletin derivatives—synthesis and structure determination of (±)-*cis*- and *trans*-3′,4′-dihydroxy-3′,4′-dihydroxanthyletin and diesters □ Dihydroxy-3′,4′-dihydroxanthyletin, (±)-*cis* and *trans*, and diesters—synthesis and structure determination □ Coumarin derivatives—synthesis and structure determination of (±)-*cis*- and *trans*-3′,4′-dihydroxy-3′,4′-dihydroxanthyletin and diesters

Naturally occurring pyranocoumarins, also known as chromeno-α-pyrones, have been known since the initial isolation of xanthoxyletin (I*b*) by Staples (1) in 1829, although its structure was not established until 1936 (2). Subsequent isolations and structural studies revealed three different basic ring fusions which may be said to be derived from xanthyletin (I*a*), alloxanthyletin (II*a*), and seselin (III*a*). The xanthyletin type is comprised of xanthyletin (I*a*), xanthoxyletin (I*b*), and luvangetin (I*c*). In the alloxanthyletin series, the known compounds are alloxanthoxyletin (II*b*), calophyllolide (II*c*), and inophyllolide (II*d*). Only two representatives of the seselin type are presently known: seselin (III*a*) and braylin (III*b*).

It was not until 1957 that the now well-known 3′,4′-diesters derived from 3′,4′-dihydroseselin were isolated and structurally characterized by Bencze *et al.* (3) when they described samidin (IV*a*) and visnadin (IV*b*). Their work was quickly followed by that of Smith *et al.* (4) who, in addition, characterized dihydrosamidin (IV*c*). Since then, a number of these diesters have been isolated and structurally elucidated (*e.g.*, 5–7) and their widespread presence in umbelliferous plants has been established. In addition, following the isolation and structural elucidation of lomatin (3′-hydroxy-3′,4′-dihydroseselin) (IV*d*) (8), esters of this coumarinic alcohol were reported (9, 10).

With respect to the corresponding esters related to the linear dihydro-I*a*, the first discoveries were the 3′-monoesters, namely, decursin (V*a*) (11) and the corresponding angelate (V*b*) (12). The present work was initiated in the hope that, eventually, the 3′,4′-diesters derived from 3′,4′-dihydroxy-3′,4′-dihydroxanthyletin would be isolated and that knowledge gained from the synthesis of known derivatives would be useful for characterization of such compounds. Indeed, during these studies the first such compound, xanthalin (V*c*), was reported from *Xanthogalum purpurascens* Lall. by Sokolova *et al.* (13) and provided an excellent corroboration of other findings (14). Since then Zheleva and coworkers (15–17) assigned similar structures to the coumarins of *Peucedanum arenarium*, and configurational assignments (17) have relied on the present work.

DISCUSSION

Xanthyletin (I*a*) was utilized as the starting point in the present work. This material is readily obtainable in a relatively pure form through the petroleum ether extraction procedure of King *et al.* (18) from the heartwood of East Indian satinwood, *Chloroxylon swietenia*. Recrystallization from warm methanol according to the procedure of Lemmich *et al.* (12) provided a means of separating xanthyletin from the accompanying xanthoxyletin in a form sufficiently pure for synthetic work. The xanthyletin thus obtained, when subjected to TLC on silica gel, showed only traces of xanthoxyletin when examined in the dark under UV light, both being highly fluorescent. The identity of xanthyletin was established by direct

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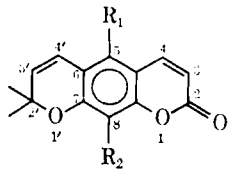
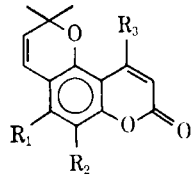


I

Ia: $R_1 = R_2 = H$
Ib: $R_1 = OCH_3$, $R_2 = H$
Ic: $R_1 = H$, $R_2 = OCH_3$

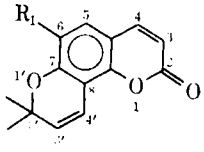


II

IIa: $R_1 = R_2 = R_3 = H$
IIb: $R_1 = OCH_3$, $R_2 = R_3 = H$

IIc: $R_1 = OCH_3$, $R_2 = C(=O)-C(CH_3)=CH-CH_3$, $R_3 = C_6H_5$

IId: $R_1 = OCH_3$, $R_2 = C(=O)-CH(CH_3)-CH_2CH_3$, $R_3 = C_6H_5$

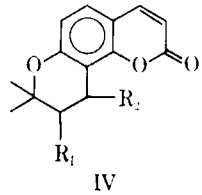


IIIa: $R_1 = H$
IIIb: $R_1 = OCH_3$

comparison with an authentic sample[1], and it was also identical in all respects to the same compound reported by Alpin and Page (19).

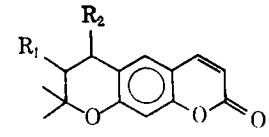
The objective was to utilize Ia to prepare both the *cis*- and *trans*-3′,4′-dihydroxy-3′,4′-dihydroxanthyletins (VIa and VIIa, respectively). It was anticipated that physical data on a number of diester derivatives could be obtained suitable for future structural studies since, aside from optical activity and melting-point differences, these would be useful models related to their natural counterparts.

Compound VIa was prepared by the action of osmium tetroxide on Ia. The diol so obtained showed a strong IR hydroxyl absorption band at 3450 cm.$^{-1}$, and the NMR spectrum (Table I) showed a coupling constant for the 3′- and 4′-protons of 4 Hz. as well as



IV

IVa: $R_1 = OOCCH=C(CH_3)_2$, $R_2 = OOCCH_3$
IVb: $R_1 = OOCCH(CH_3)CH_2CH_3$, $R_2 = OOCCH_3$
IVc: $R_1 = OOCCH_2CH(CH_3)_2$, $R_2 = OOCCH_3$
IVd: $R_1 = OH$, $R_2 = H$
IVe: $R_1 = R_2 = OOCCH_3$
IVf: $R_1 = R_2 = OH$



V

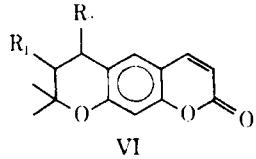
Va: $R_1 = OOC-CH=C(CH_3)_2$, $R_2 = H$

Vb: $R_1 = OOCC(CH_3)=CH-CH_3$, $R_2 = H$

Vc: $R_1 = R_2 = OOCC(CH_3)=CH-CH_3$

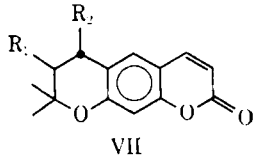
two singlets for the gem-dimethyl groups at 1.29 and 1.42 p.p.m. Compound VIa was converted to the diacetate (VIb), m.p. 172–173°, and it was also obtained in an attempt to prepare 3′,4′-epoxyxanthyletin by treating Ia with *m*-chloroperbenzoic acid. The oily product showed a hydroxyl absorption band at 3400 cm.$^{-1}$. The crude product was treated with sulfuric acid in dimethyl sulfoxide so that any epoxide present would be hydrolyzed to the diol. The product was a glass and showed an identical IR pattern to the untreated oily material, indicating that the epoxide ring opening had probably occurred in the original reaction mixture to generate a *trans*-hemiester of the diol in a manner similar to that previously reported (12). Acetylation of the crude product afforded *trans*-3′-acetoxy-4′-(*m*-chlorobenzoyloxy)-3′,4′-dihydroxanthyletin (VIIb). The IR spectrum did not show hydroxyl absorption, and the NMR spectrum (Table I) revealed a considerable difference in the chemical shifts of the gem-dimethyl protons and $J_{3',4'} = 6$ Hz.

Compound VIIb was subjected to both acid and base hydrolysis in an effort to obtain the *trans*-diol (VIIa), but only the *cis*-diol (VIa) was obtained. The products obtained in both cases showed identical IR and NMR spectra to those of VIa, and TLC failed to show detectable amounts of VIIa. Acetylation of these products gave diacetoxy derivatives, each identical to VIb as determined by melting point, mixed melting point, and IR and NMR spectra. Another surprising route to the *cis*-diol (VIa) was encountered during an attempted epoxidation of Ia with trifluoroperacetic acid (20). The product, m.p. 144–145°, when recrystallized from methanol, showed a large change in melting point which corresponded to that of the *cis*-diol (VIa). The identity of the product was confirmed by IR and NMR spectral properties and by acetylation to



VI

VIa: $R_1 = R_2 = OH$
VIb: $R_1 = R_2 = OOCCH_3$
VIc: $R_1 = R_2 = OOCCH_2CH_3$
VId: $R_1 = R_2 = OOCCH_2CH_2CH_3$



VII

VIIa: $R_1 = R_2 = OH$
VIIb: $R_1 = OOCCH_3$, $R_2 = OOC$-*m*-C_6H_4Cl
VIIc: $R_1 = OH$, $R_2 = OCH_3$
VIId: $R_1 = OOCCH_3$, $R_2 = OCH_3$
VIIe: $R_1 = OH$, $R_2 = OOCCH_3$
VIIf: $R_1 = R_2 = OOCCH_3$
VIIg: $R_1 = R_2 = OOCCH_2CH_3$
VIIh: $R_1 = R_2 = OOCCH_2CH_2CH_3$
VIIi: $R_1 = OOCCH_3$, $R_2 = OOCCH_2CH_3$
VIIj: $R_1 = OOCCH_3$, $R_2 = OOCCH_2CH_2CH_3$

[1] Supplied through the courtesy of Dr. B. E. Nielsen, Royal Danish School of Pharmacy, Copenhagen, Denmark.

Table I—Chemical Shifts (δ) in $CDCl_3$ for Xanthyletin and Its Derivatives

Compound	3-H (d)	4-H (d)	5-H (s)	8-H (s)	3′-H (d)	4′-H (d)	$J_{3,4}$ (Hz.)	$J_{3',4'}$ (Hz.)	2′-$(CH_3)_2$
I*a*	5.65	7.55	7.00	6.65	6.17	6.30	9.5	10	1.45
VI*a*[a]	6.26	7.95	7.71	6.69	3.72	4.84	9.5	4	1.29, 1.42
VI*b*	6.25	7.60	7.30	6.79	5.32	6.19	9.5	4	1.45
VI*c*	6.25	7.60	7.30	6.80	5.39	6.20	9.5	4	1.45
VI*d*	6.25	7.60	7.30	6.80	5.39	6.20	9.5	4	1.45
VII*a*[a]	6.25	7.95	7.71	6.70	3.52	4.84	9.5	9	1.22, 1.47
VII*b*	6.25	7.60	6.85	6.30	5.40	6.25	9.5	6	1.42, 1.52
VII*c*[a]	6.10	7.85	7.50	6.55	3.76	4.28	9.5	7	1.10, 1.25
VII*d*	6.25	7.62	7.55	6.80	4.35	5.25	9.5	5	1.38, 1.45
VII*e*	6.25	7.60	7.20	5.92	3.90	5.95	9.5	7	1.35, 1.51
VII*f*	6.25	7.60	7.35	6.80	5.25	6.02	9.5	6	1.38, 1.45
VII*g*	6.25	7.60	7.35	6.80	5.25	6.02	9.5	6	1.38, 1.45
VII*h*	6.25	7.60	7.35	6.80	5.25	6.02	9.5	6	1.38, 1.45
VII*i*	6.25	7.60	7.35	6.80	5.25	6.02	9.5	6	1.38, 1.45
VII*j*	6.25	7.60	7.35	6.80	5.25	6.02	9.5	6	1.38, 1.45

[a] The solvent in these cases was dimethyl sulfoxide-d_6.

VI*b*. *cis*-3′,4′-Diacetoxy-3′,4′-dihydroseselin (IV*e*) was prepared to compare its NMR spectrum with that of VI*b*. Compound IV*e* did not differ greatly from VI*b* (Table I) in that it showed (in $CDCl_3$) 1.40 [6H, 2s, $C_{2'}$—$(CH_3)_2$], 2.04 and 2.09 (6H, 2s, 2-$OCOCH_3$), 5.20 (1H, d, $C_{3'}$—H), and 6.38 (1H, d, $C_{4'}$—H) p.p.m. with $J_{3',4'}$ = 5 Hz.

The reason for the virtually exclusive formation of the *cis*-diol (VI*a*) over the *trans*-diol (VII*a*) in these hydrolytic procedures is not readily apparent, since mixtures of the *cis*- and *trans*-diols occur routinely from alkaline saponification of 3′,4′-diacyloxy-3′,4′-dihydroseselins (3). Although speculative, this might be ascribed to the hydrogen-bonding stability in both the *cis*- and *trans*-diols derived from the seselin series in which the 4′-hydroxy group can hydrogen bond with the lactone ether oxygen, a situation that cannot possibly take place in the series derived from xanthyletin. The behavior of VII*b* on treatment with methanolic potassium hydroxide may have bearing on the possibility of hydrogen bonding of the hydroxyl groups being influential in the virtually exclusive formation of the *cis*-diol (VI*a*). Because a methoxyl group would now occupy the 4′-position, the possibility of hydrogen bonding would be minimized. Ordinarily, as in the seselin-derived series, based on the suggested enone mechanism (4), a mixture of *cis*- and *trans*-isomers should result. In the present case, however, the IR and NMR spectra (Table I) were completely compatible with the assignment of a *trans*-structure. This compound (VII*c*) was acetylated to provide *trans*-3′-acetoxy-4′-methoxy-3′,4′-dihydroxanthyletin (VII*d*) which, by IR, showed no hydroxyl absorption; the NMR showed (in dimethyl sulfoxide-d_6) the presence of the methyl ether singlet at 3.58 p.p.m. in VII*c* and at 3.52 p.p.m. in VII*d* as well as a singlet for acetoxymethyl at 2.12 p.p.m. A *trans*-assignment for the 3′,4′-substituents was tentatively made on the basis of the substantial separation of the gem-dimethyl singlets and the variable $J_{3',4'}$ for VII*c* and VII*d*.

The *cis*-dipropionoxy and *cis*-di-*n*-butyroxy esters (VI*c* and VI*d*) were prepared in addition to VI*b*. The IR spectra of the prepared esters showed no hydroxyl absorption, and they all possessed virtually the same NMR pattern (Table I) except for differences in the acid moieties. The differences in chemical shifts of the gem-dimethyl singlets (1.29 and 1.42 p.p.m.) noted in the diol was eliminated in the diesters (1.45 p.p.m.). Previously (12), *cis*-3′,4′-dihydroxy-3′,4′-dihydroseselin (*i.e.*, *cis*-khellactone, IV*f*) showed only a small difference in the chemical shifts of the gem-dimethyl groups (1.41 and 1.44 p.p.m.) with $J_{3',4'}$ = 5 Hz. This same diol was prepared in the present study. No difference was found in the chemical shifts of the gem-dimethyl groups with only a singlet at 1.42 p.p.m. It was also reported (12) that *trans*-3′,4′-dihydroxy-3′,4′-dihydroseselin (*i.e.*, *trans*-khellactone) showed 1.31 and 1.51 p.p.m. for the gem-dimethyl groups and $J_{3',4'}$ = 6.9 Hz. In the present study, *trans*-khellactone (3) and its diacetate were prepared, and the NMR spectra were found to be identical to those reported (12).

The preparation of the *trans*-diol (VII*a*) was finally achieved while attempting to prepare 3′,4′-epoxyxanthyletin using 40% peracetic acid in acetic acid. Epoxidation of xanthyletin with peracetic acid resulted, as previously observed with *m*-chloroperbenzoic acid (12), in spontaneous opening of the epoxide by acetate anion. Workup of the reaction mixture first provided *trans*-3′-hydroxy-4′-acetoxy-3′,4′-dihydroxanthyletin (VII*e*), the IR of which showed hydroxyl absorption at 3400 $cm.^{-1}$; the NMR spectrum (Table I) possessed two widely separated signals for the gem-dimethyls (*i.e.*, 1.35 and 1.51 p.p.m.) and $J_{3',4'}$ = 7 Hz. The mother liquor, upon concentration, yielded a crystalline diol which had a different melting point than VI*a* and depressed its melting point on admixture. The IR of the diol exhibited a strong hydroxyl band at 3400 $cm.^{-1}$, and the NMR spectrum (Table I) showed two singlets for the gem-dimethyls at 1.22 and 1.47 p.p.m. and $J_{3',4'}$ = 9 Hz. Acetylation of the diol and VII*e* resulted in the same diacetoxy derivative (VII*f*) as evidenced from the melting point, mixed melting point, and IR and NMR spectra. VII*f* possessed different physical constants and spectra from those of the *cis*-diacetoxy derivative (VI*a*). Additional evidence for the *trans*-structure was obtained from the preparation of *trans*-dipropionoxy-, *trans*-di-*n*-butyroxy-, *trans*-3′-acetoxy-4′-propionoxy-, and *trans*-3′-acetoxy-4′-*n*-butyroxy-3′,4′-dihydroxanthyletins (VII*g*, VII*h*, VII*i*, and VII*j*, respectively). Each *trans*-diester (VII*g* and VII*h*) possessed a different melting point from that of the corresponding *cis*-diester (*i.e.*, VI*c* and VI*d*). All of the *trans*-diesters prepared had nearly identical NMR spectra except for differences due to the acyloxy groups, and they showed $J_{3',4'}$ = 6 Hz. and widely separated signals for the gem-dimethyl groups at 1.38 and 1.45 p.p.m. They were significantly different from the *cis*-diesters, which showed $J_{3',4'}$ = 4 Hz. and only one signal for the gem-dimethyl groups at 1.45 p.p.m.

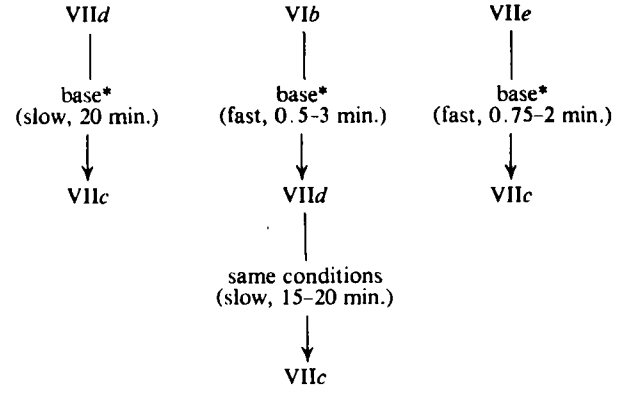
It was hoped that mild acid hydrolysis of VII*e* would result in the *trans*-diol (VII*a*) but, unfortunately, the NMR spectrum of the diol obtained showed that the hydrolysis resulted in a mixture of *cis*- and *trans*-diols; characteristic signals for each were observed in the spectrum of the product. Presumably, the milder conditions in this hydrolysis did not result in complete conversion of VII*e* to the *cis*-diol as was the case in the acid hydrolysis of VII*b* where VI*a* appeared to be virtually the exclusive product. On the basis of these experimental findings, together with the NMR data, it appears very probable that the product isolated from the mother liquor of the peracetic acid epoxidation reaction was the *trans*-diol (VII*a*). Therefore, the identity and physical characteristics of the *cis*- and *trans*-diols (VI*a* and VII*a*, respectively) are established.

As a result ot this study, it has been possible to draw conclusions regarding the NMR spectra of the 3′,4′-diols and diesters derived from 3′,4′-dihydroxanthyletin, as well as to establish the relationships between these compounds and those of the isomeric series derived from seselin.

With the *cis*-diol and its diesters, $J_{3',4'}$ was invariably 4 Hz. regardless of the substituent, a situation quite similar to that obtained with naturally occurring 3′,4′-dihydroseselin diesters where $J_{3',4'}$ was invariably 5 Hz. The *trans*-diol and its diesters prepared in this study showed a variability of $J_{3',4'}$ from 6 to 9 Hz., together with well-separated singlets for the gem-dimethyl group ranging from 1.22 to 1.51 p.p.m. If only xanthyletin *trans*-diesters are considered, however, $J_{3',4'}$ was consistently 6 Hz., combined with a well-defined spread ranging from 1.38 to 1.45 p.p.m. for the gem-dimethyl singlets. The generalization of Lemmich *et al.* (12) seems to hold with respect to the diagnostic requirement that well-separated gem-

dimethyl signals are indicative of the *trans*-arrangement and that little or no separation of these signals suggests the *cis*-arrangement. However, within the limited spectra of the diesters prepared in this study, the variability of $J_{3',4'}$ noted in the seselin-derived *trans*-compounds (12) has not been observed, with the single exception of the parent diol (VII*a*) itself[2].

Although there seemed to be little question concerning the assignments of the respective locations (*i.e.*, 3′ and 4′) of the acetoxy moieties in VII*d* and VII*e*, it was desirable to substantiate them independently. A relatively simple experiment was carried out by taking advantage of the greatly differing rates of hydrolysis of 3′- and 4′-acyloxy substituents in these compounds. The 3′-ester groups are subject to a normal basic hydrolysis whereas the 4′-ester groups are much more labile on the basis of the special mechanism referred to earlier (4). Therefore, by carrying out the reaction of methanolic potassium hydroxide on VII*d*, VII*e*, and VI*b*, it appeared possible to follow the rates of change by means of TLC and, thereby, to show that in VII*d* the rate of saponification is relatively slow whereas in VII*e* the rate of generation of the deacylated compound is comparatively much more rapid. The experience with VI*b* was in complete accord with the expected fast loss of 4′-acetate and the slower loss of the remaining 3′-acetate group. The data recorded in Scheme I summarize the results, which clearly substantiate the positions assigned to the acetoxy moieties in VII*d* and VII*e*.



*Scheme I—Base (*0.01 N methanolic potassium hydroxide) hydrolysis of VII*d, *VII*e, *and VI*b *at room temperature*

EXPERIMENTAL[3]

Extraction of Xanthyletin (I*a*)—Fine wood shavings (600 g.) of *Chloroxylon swietenia* were exhaustively extracted[4] for 5 days with petroleum ether (b.p. 30–60°) in a soxhlet apparatus. The solvent was removed by distillation, and the yellow crystalline material remaining was recrystallized from warm methanol to obtain colorless needles of xanthyletin, m.p. 128–130° [lit. (18) m.p. 126–127°]. TLC, in comparison with an authentic specimen[1], indicated the identity of the two samples. TLC was carried out on silica gel impregnated with formamide and developed with either dibutyl ether or *n*-hexane[5]-cyclohexane (1:1); a mixed melting-point comparison was also made.

***cis*-3′,4′-Dihydroxy-3′,4′-dihydroxanthyletin (VI*a*)**—A solution of I*a* (1.45 g., 0.0063 mole) in dioxane (50 ml.) was added to a solution of osmium tetroxide (2 g., 0.0079 mole) in dioxane (50 ml.), and the mixture was kept in the dark at room temperature for 4 days. Ethyl acetate (50 ml.) was added, and a stream of hydrogen sulfide was bubbled into the reaction mixture for 2 hr. The black precipitate was removed by filtration, and the filtrate was evaporated to dryness to obtain a white crystalline material (1 g., 60.5%) which was recrystallized several times from methanol to obtain a product, m.p. 234–236°, shown to be VI*a*.

Anal.—Calc. for $C_{14}H_{14}O_5$: C, 64.11; H, 5.38. Found: C, 64.09; H, 5.52.

***cis*-3′,4′-Diacetoxy-3′,4′-dihydroxanthyletin (VI*b*)**—A mixture of VI*a* (0.2 g., 0.0008 mole), acetic anhydride (4 ml.), and anhydrous sodium acetate (0.25 g.) was refluxed for 2 hr. The hot solution was poured into ice-cold water, filtered, washed with water, and recrystallized from methanol to give 0.22 g. (85%) of VI*b*, m.p. 172–173°.

Anal.—Calc. for $C_{18}H_{18}O_7$: C, 62.42; H, 5.24. Found: C, 62.46; H, 5.45.

***trans*-3′-Acetoxy-4′-(*m*-chlorobenzoyloxy)-3′,4′-dihydroxanthyletin (VII*b*)**—To an ice-cold solution of xanthyletin (0.6 g., 0.0026 mole) in chloroform (50 ml.) was added dropwise an ice-cold solution of *m*-chloroperbenzoic acid (80%, 0.56 g.) in chloroform (75 ml.), and the mixture was stirred at room temperature in the dark for 24 hr. The reaction mixture was successively washed with 100 ml. each of 10% sodium bicarbonate, 5% sodium iodide, 5% sodium sulfite, and, finally, water. The dried (sodium sulfate) chloroformic solution was evaporated to dryness, and the residue was dissolved in dimethyl sulfoxide (75%, 40 ml.) containing sulfuric acid (1 ml.). The mixture was stirred at room temperature for 16 hr. and then diluted with water until precipitation ceased. The white solid which separated was filtered, washed with water, and dried. The crude product was refluxed for 2 hr. with acetic anhydride (15 ml.) and anhydrous sodium acetate (1 g.), the mixture was poured into ice-cold water and filtered, and the white crystalline material obtained was recrystallized from methanol to yield 0.6 g. (54.5%) of white prisms, m.p. 165–167°.

Anal.—Calc. for $C_{23}H_{19}ClO_7$: C, 62.38; H, 4.32. Found: C, 62.34; H, 4.04.

Acid Hydrolysis of VII*b*—A solution of VII*b* (0.45 g., 0.001 mole) in a mixture of ethanol (8 ml.) and sulfuric acid (0.8 ml.) was refluxed for 3 hr. The cooled reaction mixture was diluted with water (38 ml.), and the alcohol was removed under reduced pressure. After standing for 30 min. at room temperature, the crystalline precipitate was filtered and recrystallized from methanol to obtain 0.20 g. (75%) of colorless prisms, m.p. 188–191°. In spite of the broad range of the melting point, the IR and NMR spectra were largely identical to those of VI*a*. The product was then acetylated in the same manner as for the preparation of VI*b* to yield a diacetate identical to VI*b* in melting point, mixed melting point, TLC behavior, and IR and NMR spectra. These data suggest that VI*a* obtained in this way is a dimorphic form of the higher melting product.

Base Hydrolysis of VII*b*—A mixture of VII*b* (0.6 g., 0.0014 mole) in dioxane (5 ml.) and 1 *N* aqueous potassium hydroxide (15 ml.) was allowed to stand overnight at room temperature. The mixture was then diluted with water (1000 ml.), acidified with 20% sulfuric acid, and allowed to stand for 1 hr. at room temperature. The reaction mixture was brought to pH 8 with aqueous sodium carbonate and then extracted with chloroform. The chloroformic extract was washed with water, dried (sodium sulfate), and evaporated to dryness to obtain a crystalline residue, which was crystallized from methanol to yield 0.3 g. (84.3%) of colorless crystals, m.p. 234–

[2] Lemmich *et al.* (12) discussed the relative $J_{3',4'}$ values of the *cis*- and *trans*-3′,4′-disubstituted-3′,4′-dihydroseselins in connection with their similarity to 3,4-flavandiols and their derivatives. The latter are almost frozen conformers with the 2-aryl substituent equatorial. In the above seselin derivatives and, presumably, with the corresponding xanthyletin derivatives discussed here, the observed *J* values correspond to time averages of the two possible conformations. They note that the dihedral angles of the two *trans*-conformers vary considerably whereas those of the *cis*-form are virtually the same; therefore, the conformational equilibria determine the resultant *J* values, with those for the *cis*-derivatives being relatively constant and those for the *trans*-derivatives being potentially variable depending on the substituents.

[3] Melting points were determined in capillary tubes in a Thomas-Hoover melting-point apparatus and are uncorrected. IR (KBr pellet) and UV (95% ethanol) spectra were determined on a Perkin-Elmer 237B grating IR spectrophotomer and a Cary model 14, respectively. The observed absorption bands, in each case, were in accord with the assigned structures and only values with a diagnostic function were mentioned in the previous discussion. NMR spectra were obtained on a Varian A-60D instrument using tetramethylsilane as an internal standard; the observed values, together with the solvents used, are recorded in Table I. TLC was performed on Eastman silica gel Chromagram sheets (No. 6060) unless otherwise specified as with the plates employing silica gel impregnated with formamide. In this case, a mixture of silica gel G (E. Merck AG) (30 g.), formamide (30 ml.), and distilled water (60 ml.) was shaken for 45 sec., spread over 20 glass plates 5.08 × 20.32 cm. (2 × 8 in.), and left to dry at room temperature overnight. Preparative TLC plates refer to alumina or silica gel plates (F_{254}, Brinkmann, 20 × 20 cm., 2000 μ thick). The *Chloroxylon swietenia* (Rutaceae) wood was obtained from Craftsman Wood Service Co., Chicago, Ill., and was authenticated by Mr. A. Stamm, Forest Products Laboratory, Madison, Wis. It was reduced to fine shavings with a mechanical planer prior to extraction. Other reagent chemicals were obtained from reputable sources. Analyses were performed by the Microanalytical Laboratory, University of Minnesota, and Schwartzkopf Microanalytical Laboratory, Woodside, N. Y.

[4] Skellysolve F.

[5] Skellysolve B.

236°. The product was identical to VI*a*, and acetylation in the usual manner afforded a diacetate identical to VI*b*.

***trans*-3′-Hydroxy-4′-methoxy-3′,4′-dihydroxanthyletin (VII*c*)**—A mixture of VII*b* (3g., 0.0067 mole) in methanol (120 ml.) and 1 *N* methanolic sodium hydroxide (12 ml.) was refluxed for 45 min. The cooled reaction mixture was diluted with water (60 ml.), and the alcohol was removed under reduced pressure. The aqueous layer was further diluted with water (1000 ml.), acidified with 20% sulfuric acid, and allowed to stand for 30 min. at room temperature. The pH of the mixture was adjusted to 8 with sodium carbonate solution, and the mixture was extracted thoroughly with chloroform. The extract was washed with water, dried (sodium sulfate), and stripped of solvent to yield 1.08 g. (57.7%) of a yellow crystalline material, m.p. 180–182°. The crude product was purified on 12 alumina plates which were developed in a benzene–ether (4:1) mixture. The blue fluorescent band was removed and the material was extracted with a warm methanol–acetone (1:1) mixture. The solvents were removed by evaporation, and the residue was recrystallized several times from methanol to yield 0.84 g. (44.9%) of white prisms, m.p. 185–186°.

Anal.—Calc. for $C_{15}H_{16}O_5$: C, 65.21; H, 5.48. Found: C, 65.20; H, 5.63.

The preceding compound (VII*c*) (0.1 g., 0.00036 mole) was refluxed for 2 hr. with acetic anhydride (5 ml.) and anhydrous sodium acetate (0.25 g.). The hot solution was poured into ice-cold water, and the precipitated crystalline material was filtered and washed with water. Recrystallization from methanol–water provided 0.09 g. (80%) of a colorless acetate, m.p. 134–135°.

Anal.—Calc. for $C_{17}H_{18}O_6$: C, 64.14; H, 5.69. Found: C, 64.34; H, 5.70.

***trans*-3′-Hydroxy-4′-acetoxy-3′,4′-dihydroxanthyletin (VII*e*) and *trans*-3′,4′-Dihydroxy-3′,4′-dihydroxanthyletin (VII*a*)**—A mixture of I*a* (2.85 g., 0.0125 mole), sodium acetate trihydrate (2.5 g.), and chloroform (50 ml.) was stirred in an ice bath. Peracetic acid in acetic acid (40%, 7.5 ml., ~ 0.04 mole) was added dropwise, and the reaction mixture was stirred in an ice bath for 2 hr. and then at room temperature for a total of 14 hr. The mixture was successively washed with water, 5% sodium bicarbonate, and water. The extract was dried (sodium sulfate) and stripped of solvent to obtain a colorless glass, which crystallized on treatment with benzene–hexane[5] (1:1) and was recrystallized from methanol to give VII*e* (1.8 g., 47.3%), m.p. 166–168°, as colorless prisms.

Anal.—Calc. for $C_{16}H_{16}O_6$: C, 63.15; H, 5.30. Found: C, 63.13; H, 5.38.

The combined mother liquors were evaporated to dryness, and the crude residue was purified by chromatography on six preparative silica gel plates. The plates were developed with a chloroform–methanol mixture (39:1) and air dried, and the two blue fluorescent bands which formed were removed from the plates. Each band was eluted with warm methanol–acetone (1:1) and, when the solvents were evaporated, the component with the higher R_f value (0.2 g.) was found to be identical to VII*e* (total yield 2 g., 52.6%). The second component was crystallized several times from methanol–water to obtain colorless crystals (0.98 g., 30%) of VII*a*, m.p. 194–196°.

Anal.—Calc. for $C_{14}H_{14}O_5$: C, 64.11; H, 5.38. Found: C, 63.97; H, 5.60.

Acetylation of VII*a* and VII*e* in the usual manner afforded, in each case, a virtually quantitative yield of *trans*-3′,4′-diacetoxy-3′,4′-dihydroxanthyletin (VII*f*) as colorless crystals from methanol, m.p. 189–191°.

Anal.—Calc. for $C_{18}H_{18}O_7$: C, 62.42; H, 5.24. Found: C, 62.21; H, 5.32.

Acid Hydrolysis of VII*e*—A mixture of VII*e* (0.18 g., 0.00058 mole) in 5% hydrochloric acid (3 ml.) was stirred on a steam bath for 45 min. and poured into water (50 ml.); the resulting mixture was extracted with ether (4 × 100 ml.). The ethereal extract was washed once with 5% sodium bicarbonate (100 ml.) and then water, dried (sodium sulfate), and stripped of solvent. The crystalline residue was recrystallized from dilute methanol to yield colorless crystals (0.11 g., 73.3%), m.p. 181–183°. Its NMR spectrum indicated that it was a mixture of VI*a* and VII*a* and, because of the small amount of material, separation into its components was not attempted.

Epoxidation of Xanthyletin with Trifluoroperacetic Acid—An ice-cold stirred suspension of hydrogen peroxide (90%, 0.6 ml., 0.02 mole) in methylene chloride (5 ml.) was treated with trifluoroacetic anhydride (3.7 ml., 0.026 mole); the mixture was stirred for 10 min. and then transferred to a dropping funnel. The mixture was added dropwise (20 min.) to a stirred ice-cold solution of I*a* (4.56 g., 0.02 mole) and triethylammonium trifluoroacetate (2.14 g., 0.01 mole) in methylene chloride (25 ml.), and the mixture was stirred at room temperature for 17.5 hr. according to the method of Emmons *et al.* (20). The volatile solvents were removed under reduced pressure, and the brown residual oil was dissolved in benzene (200 ml.) and then washed with water (2 × 75 ml.), dried (sodium sulfate), and stripped of solvent. The brownish residue was crystallized from benzene–*n*-hexane[5] to give 3.39 g. (70%) of pale-yellow crystals, m.p. 144–145°[6]. Recrystallization from warm methanol, however, afforded colorless crystals, m.p. 226–228°. These, when acetylated in the usual fashion with anhydrous sodium acetate and acetic anhydride, gave a crystalline diacetate, m.p. 172–173°, identical to VI*b*.

***cis*-3′,4′-Dipropionoxy-3′,4′-dihydroxanthyletin (VI*c*)**—A mixture of VI*a* (0.1 g., 0.0004 mole), propionic anhydride (1.5 ml., 0.012 mole), and pyridine (0.5 ml.) was refluxed for 4 hr. and poured into an excess of ice-cold water. The brown oil which separated was extracted with ether (4 × 100 ml.), and the extract was then successively washed with 100-ml. portions of 5% hydrochloric acid, 5% sodium bicarbonate, and water. The extract was dried (sodium sulfate) and filtered, and the ether was evaporated to obtain a brown semisolid residue (1.25 g.). This residue was chromatographed on 10 preparative silica gel plates with a mixture of benzene–ethyl acetate (7:3). The blue fluorescent band (visualized under UV light) was removed and extracted with a warm mixture of acetone–methanol (1:1). The solvents were evaporated, and the residue was recrystallized several times from methanol–water to give colorless crystals of VI*c* (0.9 g., 60%), m.p. 115–116°.

Anal.—Calc. for $C_{20}H_{22}O_7$: C, 64.16; H, 5.92. Found: C, 63.87; H, 5.93.

***cis*-3′,4′-Di-*n*-butyroxy-3′,4′-dihydroxanthyletin (VI*d*)**—This was prepared in a manner similar to that for VI*c* from VI*a* using *n*-butyric anhydride and pyridine. The product, m.p. 80–81°, was obtained in 80% yield.

Anal.—Calc. for $C_{22}H_{26}O_7$: C, 65.65; H, 6.51. Found: C, 65.59; H, 6.32.

***trans*-3′-Propionoxy-4′-acetoxy-3′,4′-dihydroxanthyletin (VII*i*)**—This was prepared as for VI*c* from VII*e*, using propionic anhydride and pyridine, in 76% yield, m.p. 108–109°.

Anal.—Calc. for $C_{19}H_{20}O_7$: C, 63.33; H, 5.59. Found: C, 63.44; H, 5.61.

***trans*-3′-*n*-Butyroxy-4′-acetoxy-3′,4′-dihydroxanthyletin (VII*j*)**—This was prepared as for VI*c* from VII*e*, using *n*-butyric anhydride and pyridine, in 80% yield, m.p. 131–132°.

Anal.—Calc. for $C_{20}H_{22}O_7$: C, 64.14; H, 5.92. Found: C, 64.41; H, 5.94.

***trans*-3′,4′-Dipropionoxy- and *trans*-3′,4′-Di-*n*-butyroxy-3′,4′-dihydroxanthyletins (VII*g* and VII*h*)**—These compounds were prepared from VII*a* and the corresponding anhydride as described previously for VI*c* to obtain crystalline derivatives in 62% yield, m.p. 122–124°, and 69% yield, m.p. 76–77°, respectively.

Anal.—Calc. for VII*g*, $C_{20}H_{22}O_7$: C, 64.14; H, 5.92. Found: C, 64.45; H, 6.17.

Anal.—Calc. for VII*h*, $C_{22}H_{26}O_7$: C, 65.65; H, 6.51. Found: C, 65.59; H, 6.73.

Action of Methanolic Potassium Hydroxide on VII*d*, VI*b*, and VII*e*—To a solution of VII*d* (0.1 g.) in methanol (4 ml.) was added dropwise at room temperature a solution of 0.1 *N* methanolic potassium hydroxide until the mixture became alkaline to litmus paper. Portions of the solution were withdrawn at intervals, acidified with 2% hydrochloric acid, spotted on silica gel plates, developed with benzene–methanol (9:1), and examined in the dark under UV light. The starting material remained unaffected for the first 20 min. and then slowly disappeared as two spots with lower mobility appeared corresponding to VII*c* and VI*a*. The same experiment was repeated

[6] The identity of this product, unfortunately, was not determined unambiguously before it was recrystallized from methanol, during which process it increased markedly in melting point. Although speculative, it is possible that it could have been *trans*-3′-hydroxy-4′-trifluoroacetoxy-3′,4′-dihydroxanthyletin, obtained in much the same way as the 3′-hydroxy precursor to VII*b* described elsewhere in this paper. Hydrolysis during the heating with methanol could conceivably have then produced the higher melting VI*a*.

using VI*b*. TLC analysis after 30 sec. indicated only a very faint fluorescent spot of VI*b* remaining and a strongly fluorescent spot corresponding to VII*d*. The starting material disappeared completely in 3 min. After 15 min., two additional spots appeared corresponding to VII*c* and VI*a*. Finally, VII*e* was treated in the same manner as VII*d* and VI*b*. TLC analysis after 45 sec. revealed two spots, a faint one corresponding to VII*e* and a stronger spot corresponding to VII*c*. After 2 min., the starting material had disappeared and an additional spot corresponding to VI*a* appeared.

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Altered Bioavailability of Drugs in the Eye Due to Drug-Protein Interaction

THOMAS J. MIKKELSON*, SUKHBIR S. CHRAI, and JOSEPH R. ROBINSON▲

Abstract □ The biological activity of a number of ophthalmic drugs is influenced by drug-protein interaction in tissues and fluids of the eye. High concentration of protein in lacrimal fluid, in both normal and pathological states, coupled with a relatively rapid turnover of this fluid, which moves drug solution away from the eye, leads to a considerable loss in drug activity for drugs that bind to protein. High levels of protein, as occur in some pathological states, and a slower, but substantial, turnover rate of aqueous humor can also lead to significant drug loss and a decrease in drug activity for compounds that complex with proteins. The present study, utilizing both *in vitro* and *in vivo* experiments, shows that drug-protein interaction in tissues and fluids of the eye occurs and that this interaction has an enormous influence on drug bioavailability. Equilibrium dialysis experiments, using pilocarpine nitrate, sulfisoxazole, and methylprednisolone, demonstrated that extensive binding to proteins in tears, cornea, and aqueous humor does occur. In addition, pupillary diameter experiments, using pilocarpine nitrate as the test agent, illustrated the influence of drug-protein interaction on drug bioavailability. A discussion of drug binding in combination with tear and instilled fluid dynamics is presented. It is suggested that this phenomenon can be responsible, partly or wholly, for some reported anomalous observations associated with drug therapy in the eye.

Keyphrases □ Ophthalmic bioavailability—effects of drug-protein binding, equilibrium dialysis, pupillary diameter experiments in rabbits □ Drug-protein binding—effect on bioavailability of ophthalmic drugs, equilibrium dialysis and pupillary diameter experiments

Binding of drugs to proteins can greatly affect drug activity. When this interaction occurs in tissues and fluids that are turned over at appreciable rates, and from which drug absorption must occur, the problem becomes even more severe, since both free drug and its reservoir of bound drug are being lost. Lacrimal fluid in both normal and pathological states contains appreciable quantities of protein and is turned over at rapid rates, while aqueous humor in some pathological conditions also contains large amounts of protein and is turned over at a slower but still significant rate. One could expect then that drug protein interaction in eye tissues and fluids would reduce the amount of free drug to either act locally or reach the anterior chamber

using VI*b*. TLC analysis after 30 sec. indicated only a very faint fluorescent spot of VI*b* remaining and a strongly fluorescent spot corresponding to VII*d*. The starting material disappeared completely in 3 min. After 15 min., two additional spots appeared corresponding to VII*c* and VI*a*. Finally, VII*e* was treated in the same manner as VII*d* and VI*b*. TLC analysis after 45 sec. revealed two spots, a faint one corresponding to VII*e* and a stronger spot corresponding to VII*c*. After 2 min., the starting material had disappeared and an additional spot corresponding to VI*a* appeared.

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of the eye and potentially to alter the amount of free drug in the anterior chamber, thus decreasing the apparent biological drug activity.

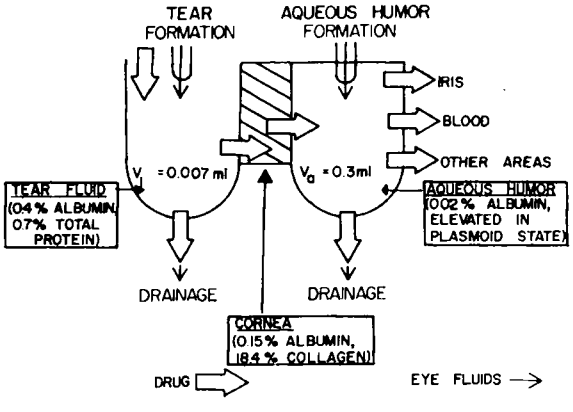
The purpose of this report is to show that protein binding of drugs to fluids and tissues of the eye can have an enormous influence on the biological activity of these drugs. It is our contention that many apparently anomalous, unexplained observations associated with drug utilization in the eye, including the difficulty in treating some disease states with conventional drugs, are explained partly or wholly by protein binding. For example, the relative unresponsiveness of uveitis conditions to steroid therapy may be due to drug–protein interaction since large amounts of protein are present in this disease state. In addition, this effect can play an important role in the total biological activity of some drugs used in ophthalmology.

Upon instillation of a drug into the eye, the drug mixes with lacrimal fluid and then is transported across several membranes until it reaches the target area. If the target area is assumed to lie posterior to aqueous humor, the drug, after mixing with tears, must cross the cornea and enter the anterior chamber, where it mixes with aqueous humor and is then transported to the target area. Scheme I illustrates the major fluid and tissue boundaries, in terms of protein content and turnover rate, that the drug contacts during its transport and distribution.

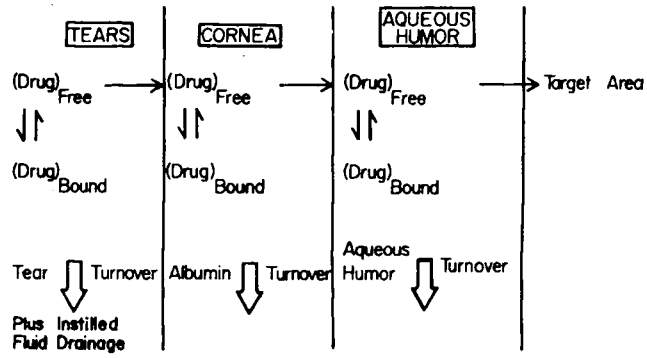
BACKGROUND

Lacrimal Fluid—The normal volume of tears present in the eye is approximately 7–10 μl. (1) in humans and about 7 μl. in rabbits (2). The turnover rate of tears in humans is about 16%/min. (1) under normal circumstances, whereas in rabbits it is 7.1%/min. (2). These turnover rates apply when the volume of fluid in the precorneal area of the eye is the normal volume. Upon instillation of fluid into the eye, the drainage of instilled solution proceeds at a rapid rate (2) until the volume is back to normal.

A significant feature of the tear composition is the presence of a relatively high concentration of protein. The total protein present in lacrimal fluid varies qualitatively and quantitatively depending upon the method of analysis, the method of obtaining tear samples, and the species of animal. A relatively conservative estimate (3) lists the albumin content of tears to be 0.4% for humans with a total protein content of approximately 0.7%, whereas for rabbits it is 0.3% albumin with a total protein content of 0.5% (4). Emotional stress, irritation, and other factors greatly influence the protein content.



Scheme I—Pictorial representation of drug and fluid movement in a portion of the human eye. Protein concentrations in tears, cornea, and aqueous humor are listed together with the normal volume of tears and aqueous humor. Large arrows depict potential drug movement, and small arrows depict eye fluid movement.



Scheme II—Potential rates and equilibria associated with drug movement through a portion of the eye

Cornea—The cornea is made up of about 80% water; the solid material present in the greatest amount is 18.4% collagen along with 0.15% albumin and globulin (5). Albumin diffuses from the peripheral capillaries toward the center of the cornea and is lost at the corneal surface to tears and aqueous humor. A transfer constant describing this turnover of albumin in humans was found to be 10–20%/day (6). Since the literature does not report this information for rabbits, their turnover rate was assumed to be similar.

Aqueous Humor—The volume of aqueous humor in humans is approximately 300 μl. with a turnover rate of approximately 1%/min. (7, 8). These figures are comparable in rabbits (9, 10).

Aqueous humor is a much more dilute fluid than blood serum under normal circumstances. The most significant difference in solid content between aqueous humor and serum is the paucity of protein found in aqueous humor. Human serum contains approximately 7.0% total protein (2.8–4.5% albumin) (11), but the aqueous humor contains only 0.01–0.02% total protein (12). The blood serum of the rabbit contains 5.6% total protein of which 4.4% is albumin, whereas rabbit aqueous humor contains approximately 0.05% total protein of which 0.025% is albumin (12).

Aqueous humor composition, particularly the protein content, is a function of the integrity of the intraocular blood vessels, *i.e.*, the blood-aqueous barrier. Various causative agents (*e.g.*, mechanical means, responses to drugs, and certain disease states) may result in partial or complete breakdown of the blood-aqueous barrier. The most striking feature of this breakdown is the concomitant increase in protein concentration of aqueous humor. The fluid that develops, termed plasmoid aqueous humor of secondary composition, can, at the extreme, possess a similar protein composition to that of plasma, representing increases from 0.02 to 7% in man and from 0.05 to 5.6% in rabbits.

From the previous description, the binding of drugs to proteins was expected with subsequent loss due to turnover in the tears, cornea, and aqueous humor as well as other tissues. The relative importance of each tissue and fluid to drug loss is a function of the type of protein, protein concentration, drug concentration and affinity for the protein, and turnover rate of fluid and tissue.

The various equilibria that occur when a drug is instilled into the eye are shown in Scheme II. Upon instillation, the drug is diluted with the lacrimal fluid and enters into a rapid equilibrium with the proteins. Only the free form of the drug is transported into the cornea. Drainage of the instilled solution plus lacrimal fluid turnover results in removal of both free and bound forms of the drug. When the drug enters the cornea, it also enters into a rapid equilibrium with the proteins. However, the turnover rate of albumin in the cornea is small enough so that removal does not present a problem unless the binding constant is very large or drug transport is very slow. Due to binding in the cornea, it would be expected that a lag time will exist before drug enters the anterior chamber and that the duration of the lag time will be proportional to the binding constant, concentration and diffusivity of drug, and thickness of the cornea. After entrance into the anterior chamber, the extent of protein binding in aqueous humor depends upon the condition of the eye and, therefore, the protein composition and content in the aqueous humor. Turnover of aqueous humor, which

is considerably slower than that of lacrimal fluid, results in drug loss. Naturally, the drug in these various fluids after parenteral administration is also subject to binding and loss.

EXPERIMENTAL

Adult, male, albino rabbits[1] were used as the experimental animals. No special pretreatment methods or diets were incorporated into the *in vivo* procedures.

Cellulose dialysis tubing[2] and crystalline rabbit serum albumin[3] were obtained from commercial sources.

Methylprednisolone[4], sulfisoxazole[5], tritiated pilocarpine nitrate[6], and *N*-1-naphthylethylenediamine dihydrochloride[7] were used; all chemicals were either USP or reagent grade.

Equilibrium Dialysis

Cells and Procedures—Two different sets of dialysis cells were utilized, depending upon the type of solution under investigation. Dialysis cells of identical half-cell chamber size of 10-ml. capacity were employed in studies to determine the binding capabilities of rabbit serum albumin. Unequal half-cell capacity cells were used for the tears, cornea, and aqueous humor studies. In the unequal half-cell experiments, the drug volume side was 5 ml. and the protein or tissue side was 1 ml.; cell thicknesses were adjusted so that differing volumes produced equivalent levels in the cells.

Protein solution or the tissue sample was placed in one half-cell chamber and drug solution was placed on the opposite side. The contents of the dialysis cells were equilibrated for 24 hr. at 25 ± 1.0° on a shaker[8]. Twenty-four hours under these conditions was sufficient for reaching equilibrium.

The equilibrium concentration of free drug in the protein-free dialysate was determined following equilibration. Assays were performed immediately after removal of the protein-free dialysate to avoid potential microbial growth which can occur upon standing. From measurement of the free drug concentration and after suitable blank corrections, the fraction of drug bound was determined.

Dialysis Membrane Preparation—Dialysis tubing was cut into strips and purified extensively prior to use (13).

Drug Solution Preparation—All drug solutions were prepared using Sørenson phosphate buffer, pH 7.38 (14). The buffer is isotonic, giving the solutions a sufficiently high ionic strength so that any discrepancies in distribution due to the Donnan effect should be minimal. Isotonic solutions include the drug solutions that were applied locally to the rabbit eye in the *in vivo* studies as well as the drug and rabbit albumin solutions employed in the *in vitro* dialysis studies.

Dialysis Solution—Crystalline rabbit serum albumin solutions, 2.89×10^{-5} *M*, were prepared in Sørenson phosphate buffer, pH 7.38.

Eight milliliters of protein solution was placed in one half-cell and 8 ml. of drug solution was placed in the opposite half of the dialysis chamber.

Corneal Tissue—The corneal tissues employed in the equilibrium dialysis binding studies were obtained from rabbits and were used fresh.

Dialysis studies on the cornea were initiated immediately following removal of the tissue, utilizing the entire intact cornea (without pulverization). The corneas were placed in the dialysis chamber with a small volume of buffer. Two corneas were placed in one-half of the dialysis chamber together with 0.5 ml. buffer solution, and 3 ml. drug solution was placed in the other half. The height of the liquids on both sides was the same. The corneas occupied an average volume of 0.092 ml. and had an average dry weight of 0.096 g. Calculation of the fraction of drug bound was carried out in the usual manner and included the unequal volumes in the half-cells, which were 3 ml. on the drug side and 0.592 ml. on the corneal side.

Aqueous Humor—The primary aqueous humor samples were obtained by making a single puncture into the anterior chamber of the rabbit eye. Depending upon the size of the rabbit, a volume of 0.2–0.3 ml. was obtained from each eye.

Pooled primary aqueous humor was used for dialysis studies. A volume of 0.2 ml. of aqueous humor together with 0.2 ml. of buffer was placed in one half-cell, and 1 ml. of drug solution was placed in the other half-cell. The height of the liquids was the same. Fraction of drug bound was calculated and included corrections for the unequal volumes as well as blank corrections.

Secondary or plasmoid aqueous humor was obtained by carefully removing primary aqueous humor and then removing the secondary at either a 1- or 4-hr. interval. Generally, the volume of the plasmoid aqueous humor obtained was in the range of 75 µl./eye.

Lacrimal Fluid—The lacrimal fluid employed in the equilibrium dialysis studies was collected from two adult, male human subjects. The lacrimal fluid from one subject was obtained by mechanically irritating the eye through placement of a thin strip of filter paper in the lower cul-de-sac. An overflow of tears was produced, which was collected as drops rolling from the lid. The majority of the tear samples came from the second subject where overflow lacrimal fluid was obtained without mechanical stimulation. The overflow tears were collected by the subject during late hours of the day as an accompanying phenomenon to yawning. Again, the lacrimal fluid in the form of drops was collected rolling from the eyelids. All lacrimal fluid samples were pooled and stored at 5° until used.

The volume and methods of dialysis, as well as the calculation, were the same as in the aqueous humor study.

Assays[9]—*Methylprednisolone*—Determination of methylprednisolone concentration was accomplished by direct spectrophotometric measurement. The compound was found to follow Beer's law in the concentration range studied, at an absorbance maximum of 248.5 nm. Methylprednisolone exhibits a molar absorptivity of 1.46×10^4 M^{-1} cm.$^{-1}$ at this wavelength in pH 7.38 aqueous Sørenson buffer.

Pilocarpine—The quantitative determination of pilocarpine nitrate was accomplished by utilizing its reactivity with hydroxylamine to form a hydroxamic acid that is subsequently complexed with ferric ion as previously described (15). The formed complex shows an absorbance maximum at 500 nm. and a molar absorptivity of 2.26×10^2 M^{-1} cm.$^{-1}$ at this wavelength.

Radioactive pilocarpine nitrate was assayed by liquid scintillation detection[10].

Sulfonamides—Quantitative determination of sulfisoxazole was accomplished by utilizing the primary aromatic amine character of the drug molecule. Diazotization of the primary aromatic amine, followed by coupling with Bratton–Marshall reagent (*N*-1-naphthylethylenediamine), yields a product that displays an absorbance maximum in the visible region (16). Sulfisoxazole exhibited an absorbance maximum at 546 nm. with a molar absorptivity of 2.52×10^3 M^{-1} cm.$^{-1}$ at this wavelength and obeyed Beer's law in the concentration range studied.

Miosis–Time Studies

Procedure—Anesthetized animals were used in these experiments. After onset of anesthesia, the test animals were placed in restraining boxes with the head elevated in such a manner that the lid line, upon closure, was approximately horizontal. This position, which is not the natural posture for the rabbit, was chosen to facilitate pupillary diameter measurements. Experimental testing of this animal position as compared to the normal position showed no difference in drug response, so the elevated position was used.

Lighting and temperature in the test room were constant throughout the study. The entire test room was as isolated as conditions would permit to keep audiovisual stimuli to the test animal to a minimum.

The observer was hidden from the test animal by a cardboard shield containing a vertical slit through which measurements of pupillary diameter could be made. The observer, once inside the room, was not allowed movement in or out of the room until the experiment was complete. These precautions were found necessary for obtaining reproducible results.

[1] Klubertanz, Edgerton, Wis.
[2] Union Carbide Corp., Chicago, Ill.
[3] Schwarz/Mann, Orangeburg, N. Y.
[4] Gift of The Upjohn Co., Kalamazoo, Mich.
[5] Gift of Hoffmann-La Roche Inc., Nutley, N. J.
[6] Eastman Organic Chemicals, Rochester, N. Y.
[7] New England Nuclear, Boston, Mass.
[8] Eberbach.

[9] All spectral measurements were carried out on a Cary 16 spectrophotometer.
[10] Packard Illinois, model 2002.

Measurement of pupillary diameter (with an accuracy of ±0.1 mm.) was made with a cathetometer[11], positioned approximately 50 cm. from the test animal.

After positioning the animal in the restraining box, in line with the cathetometer and prior to drug instillation, pupillary diameter readings were made at 100-sec. intervals. This procedure was continued until the test animal had adapted to its environment as judged by the constancy of pupillary diameter (±0.1 mm. for 10 min.). The adaptation period varied from 15 to 30 min., and the initial diameter ranged from 6.0 to 7.8 mm.

Pipets[12] with blowout delivery were used to instill the drug solution and were checked for accuracy of delivery prior to use by weighing the delivered solutions.

Drug solutions were instilled onto the cornea so that they collected in the lower cul-de-sac. To prevent loss of solution during instillation, the lower eyelid was pulled slightly away from the globe of the eye to form a pocket. This particular form of solution instillation was followed at all times.

After instillation of drug solution, the lower lid was lifted back and forth over the cornea to mix the drug solution with lacrimal fluid. At no time was the lid ever massaged against the cornea so that only movement of the drug solution over the cornea occurred. To exert some degree of control in this method, the eyelid was lifted over the cornea exactly four times, during about 5 sec., in all cases. All procedures were performed in precisely the same manner and with the same time intervals.

After instillation of the drug solution, pupillary diameter was measured as a function of time. The other eye of the test animal served as a control. One hundred-second intervals were used for the first 50 min. of the experiment, 300-sec. intervals were used from 50 to 80 min., and 600-sec. intervals were used thereafter. Measurements were terminated when the measured diameter reached a constant value close to the initial value.

No experimental animal was used more than once, and each experiment was repeated at least four times.

Solution Preparation —*Pilocarpine Nitrate Solutions*—These solutions were prepared fresh and were discarded immediately after the experiment. The solutions were prepared by addition of pilocarpine nitrate to Sørenson phosphate buffer, pH 7.38. Solution pH was checked and adjusted where necessary[13]. The pH meter and electrode system were standardized against phosphate buffer as described previously (17). No attempt was made to sterilize the solutions. Isotonicity of solutions was maintained by addition of sodium chloride.

Anesthetic Solutions – Solutions containing sodium pentobarbital, 50 mg./ml., or sodium phenobarbital, 100 mg./ml., in a vehicle containing 20% (v/v) propylene glycol and 10% (v/v) ethanol were prepared. Both solutions were refrigerated between uses and were never kept for more than 2 weeks.

Anesthesia—It was found that a dose of 33 mg./kg. i.p. sodium pentobarbital and 100 mg./kg. i.p. sodium phenobarbital was sufficient in most cases to anesthetize the rabbits for 8–12 hr.

Miosis Study following Paracentesis—Anterior chamber paracentesis was accomplished by making a puncture incision at the limbus through the cornea into the anterior chamber and aspirating the aqueous humor. Extreme care was taken upon removal of the needle from the deflated anterior chamber to minimize damage to the surfaces exposed to the needle. After waiting for either 1 or 4 hr., the drug solution was instilled into the eye and the change in pupillary diameter was monitored as a function of time.

RESULTS

Demonstration of *In Vitro* Drug–Protein Interaction—The fact that many drugs are bound to proteins is well known (18). A demonstration of this interaction, using rabbit serum albumin as the protein and sulfisoxazole as the drug, is presented in Fig. 1. This particular form of data presentation, namely fraction of drug bound *versus* drug concentration, is generally used when the concentration and nature of the protein are unknown (13). Since an analysis of the various eye fluids and tissue used in this study, as to their types and concentration of protein components, as well as their binding potential was not performed, the *in vitro* data are presented as a fraction of drug bound. Studies are currently underway to characterize these proteins and may be reported later.

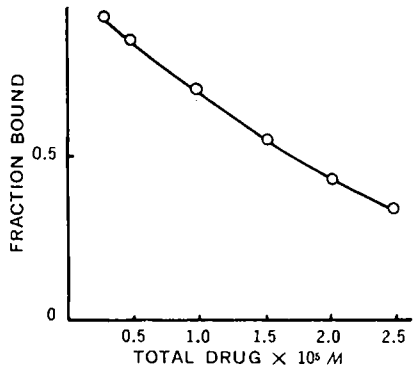


Figure 1—*Equilibrium dialysis experiment on the extent of sulfisoxazole binding to 0.2% (w/v) rabbit serum albumin. Data points are mean values from four separate determinations, and standard deviations are within ±5% of the mean values.*

Although drug binding to blood proteins is well known, what is apparently not as well appreciated is that many other tissues and fluids of the body, such as eye tissues and fluids, also bind drugs and can greatly influence drug bioavailability. This influence is particularly important when the tissue or fluid is turned over at a rapid rate as is the case with tears. Figure 2 demonstrates the interaction of sulfisoxazole and methylprednisolone with the components of human tears, and it is clear that these drugs are extensively bound to the components at the drug concentrations tested. The ramifications of this binding to drug therapy can be appreciated by realizing that instillation of 50 µl. of 1% methylprednisolone solution, which is equal to about 0.5 mg. of drug, followed by dilution of the solution and removal through drainage and turnover, rapidly converts the drug concentration to levels equal to or below that indicated in Fig. 2.

It is important to recognize that the tears used in this study were obtained from healthy eyes by stimulation and, therefore, were low in protein content (19) as compared with tears obtained without stimulation or from inflamed eyes. Thus, binding of these drugs to "normal" tears or tears in an inflamed eye would be expected to be much greater than is indicated in Fig. 2.

In addition to tears as a potential site of drug–protein binding, due to their relatively high protein content, corneal tissue and plasmoid aqueous humor are also potential sites due to a rich supply of protein. *In vitro* dialysis studies of methylprednisolone and sulfisoxazole binding to corneal tissue, in the concentration range of $1-5 \times 10^{-6}$ *M*, show very high drug binding. This is not at all surprising considering the high total protein content of corneal tissue. Likewise, binding of these drugs to plasmoid aqueous humor was also very extensive. It was earlier indicated that primary aqueous humor is low in protein content; as might be expected, the binding of sulfisoxazole and methylprednisolone to this fluid was negligibly small in the concentration range of $1-5 \times 10^{-5}$ *M*. On

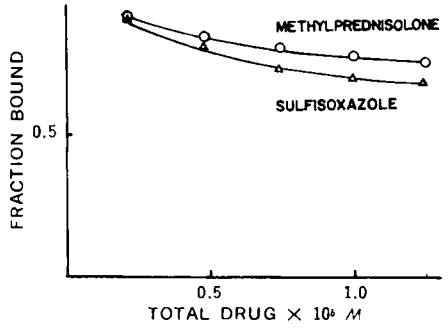


Figure 2—*Equilibrium dialysis experiments on the extent of drug binding to human tears. Data points are mean values from three separate determinations, and standard deviations are within ±7% of the mean values.*

[11] Central Scientific Co., Chicago, Ill.
[12] Scientific Products, Evanston, Ill.
[13] All pH measurements and adjustments were made on an Orion model 801 digital pH meter, using a Beckman E3, wide range, glass electrode.

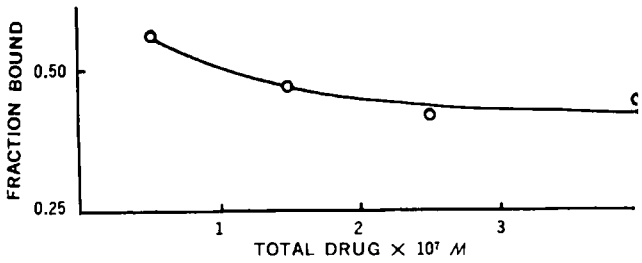


Figure 3—*Equilibrium dialysis studies of tritiated pilocarpine nitrate and plasmoid aqueous humor. Data points represent the mean values from two determinations.*

the other hand, plasmoid aqueous humor is extremely rich in protein; equilibrium dialysis studies with sulfisoxazole and methylprednisolone to this fluid showed almost complete binding in this same concentration range.

Since both sulfisoxazole and methylprednisolone are intermediate in their binding capabilities to albumin [sulfisoxazole is 5×10^3 M^{-1} (20) and methylprednisolone is approximately 10^4 M^{-1} (13)], an additional drug with a low binding constant was chosen to demonstrate the extent of drug binding to plasmoid aqueous humor. Pilocarpine nitrate is a miotic drug used extensively in ophthalmology. It has a binding constant of less than 100 to rabbit serum albumin[14] (21).

Figure 3 shows a fraction bound *versus* concentration plot for pilocarpine nitrate and plasmoid aqueous humor. At low drug concentration and presumed high protein concentration, there is considerable binding of the drug. Note that the drug concentration used in this study is considerably lower than with studies involving sulfisoxazole; this is a reflection of the smaller binding constant of pilocarpine nitrate to the protein. There is, of course, the possibility that pilocarpine nitrate is very highly bound to a specific protein that is present at low concentration, but this cannot be resolved until studies are completed on protein types, concentration, and drug affinity in plasmoid aqueous humor.

These *in vitro* results, using drugs that represent a range of binding affinities from 10^2 to 10^4 M^{-1}, show that there is extensive drug binding to the components of tears, cornea, and plasmoid aqueous humor. This binding can have a large or small influence on drug bioavailability, depending on the extent of binding and rates of turnover or removal of these drugs containing fluids and tissue.

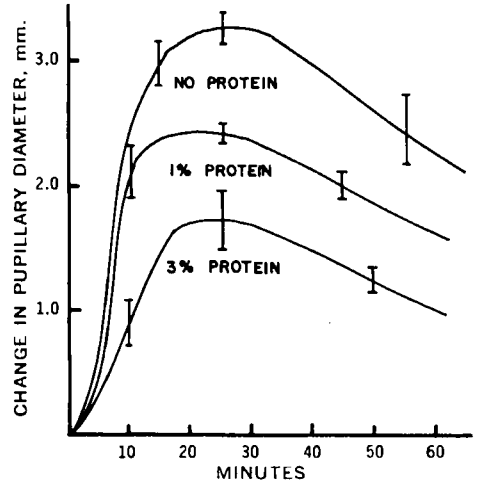


Figure 4—*Miotic activity of 50 μl. of 2.5 × 10^{-2} M pilocarpine nitrate in the presence and absence of added rabbit serum albumin. Each experiment was conducted at least three times, and the vertical bars show the standard deviation at random times. Each line was drawn on the basis of approximately 30 experimental points.*

[14] As determined by ultracentrifugation and by equilibrium dialysis studies using tritiated pilocarpine nitrate. These results will be the subject of a separate publication.

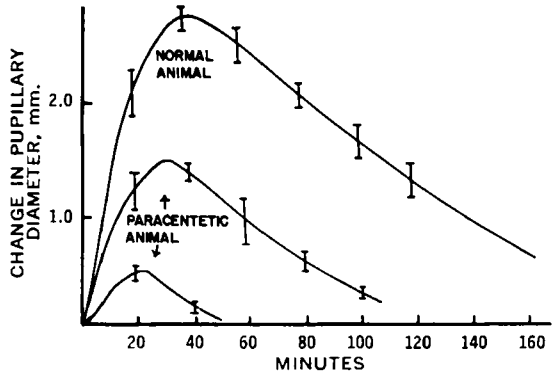


Figure 5—*Effect of plasmoid aqueous humor on the miotic response to pilocarpine nitrate as compared to normal aqueous humor. Fifty microliters of 1 × 10^{-2} M drug was instilled into the eye, and each line represents the mean of at least three determinations. The bottom line represents addition of drug 1 hr. after paracentesis, and the middle line corresponds to drug addition 4 hr. after paracentesis. Vertical bars are standard deviations at random points on the curve, and each curve was drawn on the basis of 15–40 experimental points.*

Demonstration of Drug–Protein Binding Influence on Drug Bioavailability—Pupillary diameter studies were used to assess the influence of protein binding on drug bioavailability. Pilocarpine nitrate was used as the test agent, and the change in pupillary diameter as a function of time and drug concentration was used as an index of drug activity.

The *in vitro* dialysis experiments using tears and various drugs showed that extensive binding of drug occurred. If drug binding to proteins in tears is responsible for a loss in drug, it should be possible to increase this loss by further addition of the proper protein to tears. Addition of protein to tears is difficult, but the same results should be obtained by placing the protein in the drug solution prior to administration into the eye. Rabbit serum albumin was added to the drug solution, and the resultant effect on drug bioavailability, as judged by response to the drug, is shown in Fig. 4. When lower drug concentrations were used in this experiment, the entire miotic activity of the drug could be suppressed by addition of rabbit serum albumin to the drug solution. This diminished response bears out the expectation that drug binding influences bioavailability through drug loss. Albumin was selected as the protein only for convenience. It is possible that other proteins normally present in tears would have a larger affinity for the drug and yield more dramatic results.

Albumin, by itself, was found not to have any effect on pupillary diameter, although it does have surface activity and this property might have two additional effects that should be considered in evaluating the results. The first effect might be to promote drug transport, since a number of surface-active agents are known to have this effect in the eye (22, 23). This would cause an *increase* in drug bioavailability due to a faster penetration of the drug through the corneal tissue; if this was a factor in the present study, it was clearly swamped out, as judged by the *decrease* in biological response. A second effect, which might account for a decreased response, would be a lowering of the surface tension of the instilled solution and hence an increase in the rate of solution drainage. Data from this laboratory on drainage rate studies (2), as well as the fact that many of the tested surface-active agents are without effect in influencing corneal absorption of drugs[15] (22, 23), suggest that a reduction in surface tension of the instilled solution and a corresponding increase in drainage rate are not the mechanisms for the decreased drug response when albumin is added to the drug solution. Thus, the result from this experiment is attributed principally to drug–protein interaction and its concomitant effect on drug loss.

The magnitude of the drug-response change (Fig. 4) is considerable. From dose-response curves generated for pilocarpine nitrate (13), the addition of 3% protein to the drug solution is equivalent to a 75–100-fold reduction in drug concentration, as judged by

[15] Additional studies from this laboratory that are part of another report (13).

biological response in the absence of added protein. Considering the relatively small binding constant for pilocarpine nitrate to albumin, as compared with some antibiotics and steroids, the potential effect of this interaction with other drugs becomes apparent.

If it is possible to depress the bioavailability of drugs by addition of protein, it should be possible to increase the biological activity by competitively inhibiting the interaction. Addition of appropriate substrates to inhibit drug-protein binding can increase the free-drug concentration and, hence, the drug bioavailability. In the case of pilocarpine nitrate solutions, it is possible to increase miotic activity in healthy albino rabbits by more than a factor of 10 through addition of suitable substrates (13). Furthermore, the influence of added protein in depressing the miotic activity of pilocarpine, described earlier, was fully reversible in the presence of a competitive inhibitor. A full account of this work is given in a separate paper[15], but mention is made here to further support the contention of drug-protein binding in eye fluids and tissues as an influencing factor in drug bioavailability.

It was also of interest to determine if the presence of higher concentrations of protein in aqueous humor could be responsible for a decrease in drug activity. Paracentesis was carried out on several animals to form a protein-rich secondary aqueous humor, and the activity of pilocarpine nitrate was determined in these animals (Fig. 5). Instillation of pilocarpine nitrate solution 4 hr. after paracentesis caused a significant drop in the biological response to pilocarpine nitrate, and an even further reduction in activity occurred if the drug was instilled 1 hr. after paracentesis. At the 4-hr. point, the aqueous humor is returning to its normal state and is not as rich in protein as at the 1-hr. point.

The dramatic decrease in drug response, shown in the paracentesis experiment, could be largely attributed to trauma incurred by withdrawing aqueous humor. If trauma to the iris or any portion of the tissue responsible for pupillary activity occurred, it returned to normal within 12 hr. but nevertheless could account for a portion of the results in this experiment. When the result of the *in vivo* paracentesis experiment is evaluated together with the results from the *in vitro* dialysis experiment, using plasmoid aqueous humor and pilocarpine nitrate, it seems reasonable to assume that a sizable portion of the loss in drug activity in the paracentetic animal was due to drug-protein interaction and not trauma.

DISCUSSION

An important facet of drug binding influence on ophthalmic drug bioavailability is the turnover rate of eye fluids and tissues, as well as drainage of instilled solutions when these are used as the delivery system. Chrai *et al.* (2) showed that instilled solutions are very rapidly drained from the eyes of rabbits. For example, approximately 50% of a 50-μl. instilled drop will drain from the eye of a rabbit within 30 sec. postinstillation and better than 84% will be lost within 3–4 min. These values are even larger in humans and, according to Maurice[16], most instilled solution in humans is lost within 1–2 min. In addition to drainage of instilled solution, one must also be concerned with turnover of tears, since this results in both dilution and removal of the drug.

The smaller turnover rate of albumin in the cornea suggests that drug binding to this tissue would have less of an influence on total drug availability, although it can influence the rate of transport and the time lag before drug appears in the anterior chamber. Aqueous humor also has a smaller turnover rate than tears but can nevertheless influence drug bioavailability if the binding constant is large or if protein content is large.

Other portions of the eye such as the sclera and conjunctiva can and probably do bind drugs and are responsible for a decrease in drug bioavailability. The present study was concerned principally with components of the eye that are constantly being turned over and are at the same time rich in protein.

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Partial Purification of Antigen E from Mixed Stems and Leaves of Short Ragweed Plant

A. SHAFIEE, E. J. STABA▲, and Y. J. ABUL-HAJJ

Abstract □ Short ragweed leaves-stems, roots, and seeds were examined for the presence of the most potent pollen allergen, antigen E. Soluble proteins were precipitated by ammonium sulfate and partially purified by a combination of dialysis and anion exchange and gel filtration chromatography. Immunodiffusion, disk electrophoresis, and skin testing of short ragweed pollen-sensitive individuals indicated that antigen E was present in leaves-stems portion of this plant as well as the pollen.

Keyphrases □ Ragweed leaves-stems, roots, and seeds—distribution and isolation of antigen E □ Antigen E, short ragweed—distribution in leaves-stems, roots, and seeds, isolation from leaves-stems □ Allergen distribution—antigen E in short ragweed leaves-stems, roots, and seeds

Wind-borne plant particles other than pollen can cause allergic symptoms in persons showing pollen hypersensitivity (1). Rebhun *et al.* (2), by direct skin testing of ragweed-sensitive patients, demonstrated that reagin reacting antigens were present in all parts of the giant ragweed plant. Other researchers reported the purification of antigens E and K (3–5), of Ra. 3 (6), and of a basic protein from short ragweed pollen (7). The distribution of short ragweed pollen antigen E in other portions of this plant was investigated in this study.

EXPERIMENTAL

Plant Materials and Antigen E Source—In 1971, short ragweed plants[1] (*Ambrosia elatior* L.) were grown and collected shortly before pollination in the medicinal plant garden and were also received from a commercial source[2]. The collected plant materials were air dried, milled, and stored in an airtight container at room temperature. Plant materials used for extraction did not contain pollen when examined microscopically both before and after staining with Calberla's solution (8).

Antigen E from short ragweed pollen[3] was prepared according to a reported procedure (3, 4). The prepared antigen E (Sephadex G-100, Fraction IV) had a similar disk electrophoresis pattern (Fig. 1), and a complete line of identity with National Institutes of Health (NIH) antigen E (NIH antigen E) when evaluated with antiantigen E and antipollen crude extract serums by the Ouchterlony immunodiffusion test.

Allergen Isolation Procedure—A modification of the literature procedure (3, 4) was used to isolate the allergens from short ragweed leaves-stems, roots, and seeds.

Leaves-Stems—Plant samples collected from the medicinal plant garden and from the commercial source (100 g. leaves-stems) were separately defatted and depigmented with peroxide-free ether for 24 hr. in a soxhlet apparatus. The defatted, air-dried plant material was extracted with 800 ml. of 0.005 *M* iced phosphate buffer (pH 7.4) in a 2-l. erlenmeyer flask on a gyrotory shaker[4] at 200 r.p.m. for 24 hr. at 22°. The extract was filtered through glass wool at 4°[5], and the filtrate (500 ml., pH 6.5) was adjusted to pH 7.4 with 3 *N* ammonium hydroxide. The filtrate was further clarified by centrifugation at 1230×*g* for 20 min. in a centrifuge[6]. The precipitate was washed with extracting buffer until the wash buffer reacted negatively against antipollen crude extract rabbit serum in the immunodiffusion test. The combined filtrate (510 ml., pH 7.2) was concentrated to approximately 51 ml. with an ultrafiltration cell[7] equipped with a membrane[8] and adjusted to 0.9 saturation with 31.7 g. ammonium sulfate[9].

The resulting suspension was stirred overnight at 4° and centrifuged at 14,350×*g* for 40 min. The precipitate was then dissolved in 25 ml. of 0.1 *M* tromethamine-hydrochloric acid buffer (pH 7.9) to form a dark-brown solution. This solution was desalted and further depigmented on an anionic exchange column[10] (50 × 4 cm.), which was equilibrated and eluted with 0.025 *M* tromethamine-hydrochloric acid buffer (pH 7.9). The flow rate was adjusted to 60 ml./hr., and 15-ml. fractions were collected at 22° and monitored by their absorption at 280 nm. The first peak, Fraction A, was concentrated to 20 ml. at a pressure differential of 700 mm. Hg using size 8 dialysis tubing[11]. Since loss of allergen through dialysis tubing was detected, the dialysate was concentrated in the ultrafiltration cell as before and combined with the dialysis tubing retentate. The second peak, Fraction B, containing the pigments and ammonium salt was discarded. The combined concentrate Fraction A (22 ml., 6 mg. dry wt./ml.) was exhaustively dialyzed against 0.025 *M* tromethamine hydrochloric acid (pH 7.9) and applied on diethylaminoethyl-cellulose (medium mesh, 0.88 meq./g.) column[12] (25 × 2.7 cm.) previously equilibrated and eluted with the 0.025 *M* tromethamine-hydrochloric acid buffer (pH 7.9). The flow rate was adjusted to 180 ml./hr., and 13-ml. fractions were collected and monitored as previously described. The first diethylaminoethyl-cellulose peak, Fraction C, was collected. The column was then eluted with 0.05 *M* tromethamine-hydrochloric acid–0.2 *M* NaCl buffer (pH 7.9) to obtain a second peak, Fraction D. Fraction D was concentrated to 1.5 ml. and contained 0.8 mg. protein/ml. Protein was determined by the method of Lowry *et al.* (9) with crystalline bovine albumin as standard.

Roots and Seeds—Short ragweed roots were extracted and partially purified for antigen E as described for plant leaves-stems. However, the allergen isolation procedure for the seeds was slightly different from that already described. Seed samples (100 g.) were ground into fine pieces with a mortar and pestle and were defatted. The defatted seed was extracted with 500 ml. of 0.005 *M* phosphate buffer (pH 7.4), and the resulting suspension was expressed through four layers of muslin cloth. The residue was mixed with enough buffer to form a uniform suspension and sonified[13] for 2 min. with the power source set at 90 w. The sonified suspension was also expressed through four layers of muslin cloth, and the filtrates of the two extracts were mixed. Seed Fraction D was obtained as previously described for leaves-stems. Seed Fraction D was concentrated to 10 ml. by use of the ultrafiltration cell. The concentrated Fraction D (10 mg. protein/ml.) was chromatographed on a resin column[14] (195 × 2.5 cm.). Prior to the application of the sample to the column, the concentrate was equilibrated by dialysis with the column buffer [0.1 *M* tromethamine-hydrochloric acid–0.2 *M* ammonium sulfate (pH 7.9)]. The flow rate was adjusted to 20 ml./hr., and 6.2-ml. fractions were collected and monitored as previously described.

[1] Voucher specimen deposited in Department of Pharmacognosy, University of Minnesota.
[2] Greer Labs., Inc., Lenoir, N. C.
[3] 1970 Collection, Greer Labs., Inc.
[4] NBS model G-10, New Brunswick, N. J.
[5] All filtrations, centrifugations, and dialyses were carried out at 4°.
[6] Sorvall SS-1, Newton, Conn.
[7] Diaflo.
[8] UM-2, Amicon Corp. Cambridge, Mass.
[9] Ultra-pure, Schwartz/Mann, Orangeburg, N. Y.
[10] Sephadex G-25 (medium), Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
[11] Union Carbide Co., Chicago, Ill.
[12] Sigma Chemical Co., St. Louis, Mo.
[13] S-12 Branson Sonifier, Danbury, Conn.
[14] Sephadex G-100, 40–120 μ.

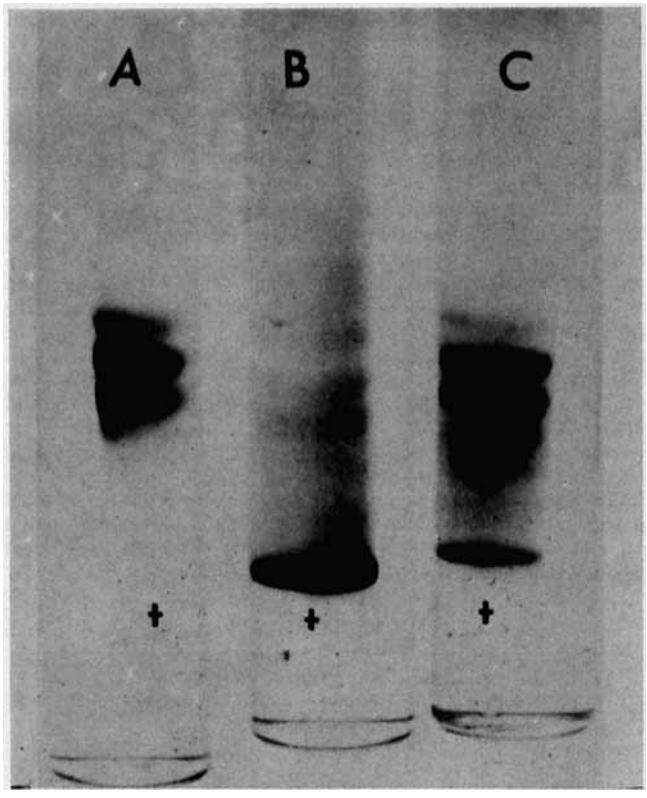

Figure 1—*Polyacrylamide gel electrophoresis patterns at pH 8.9. Key: A, NIH antigen E; B, leaves–stems Fraction D; and C, pollen antigen E (Sephadex G-100, Fraction IV). The fastest moving anodic band in B and C is bromphenol blue marker dye and it is barely seen in A.*

Rabbit Antiserum Preparation—Antipollen crude extract serum was prepared by injecting male rabbits (New Zealand White) at four weekly intervals subcutaneously with an emulsion of 0.5 ml. of 0.1 *M* tromethamine–hydrochloric acid (pH 7.9) buffer solution of 0.9 saturation ammonium sulfate precipitate (81 mg./ml. dry weight) and 0.5 ml. of complete Freund's adjuvant[15] (6). The animals were bled from the marginal ear vein 10 days after the last injection. The serum from each animal was separately tested against pollen crude extract and NIH antigen E by the Ouchterlony immunodiffusion test (10). Positively reacting serum was pooled and stored at −10° in 1-ml. portions until used.

Antiantigen E serum was prepared by injecting each rabbit subcutaneously at four different sites on the back and neck areas at weekly intervals with an emulsion of 0.5 ml. (0.52 mg. protein/ml.) antigen E (Sephadex G-100, Fraction IV) and 1 ml. of complete Freund's adjuvant. The antiantigen E serum was tested and stored as described for antipollen crude extract serum.

Disk Electrophoresis—The reagents and apparatus used for disk electrophoresis were purchased[16]. Disk electrophoresis of Fraction D (leaves–stems), pollen antigen E (Sephadex G-100, Fraction IV), and NIH antigen E was performed at a running gel pH of 9.5 with the discontinuous buffer system of Davis and Ornstein (11). A sample load [0.1–0.2 mg. in 0.2 ml. of 0.025 *M* tromethamine–hydrochloric acid (pH 7.0)] was added to the top of the spacer gel, and larger pore solution was added to fill the electrophoresis tube. Bromphenol blue was used as a marker dye. The gels were stained[17] after being run at 4-mamp./tube current for 45 min.

Immunological Studies—Ouchterlony immunodiffusion tests were carried out in 60-mm. plastic petri dishes containing 5 ml. of 1% Noble agar[18] and 0.1% sodium azide (10). The antiserum was placed in the center well and antigens were placed in the outer wells (3-mm. diameter). After 48–72 hr., the precipitin line positions were observed and photographed. Antigen E concentrations in leaves–stems were estimated by the double-diffusion technique with antipollen crude extract serum (12). The standard curve was established by using purified antigen E in a concentration range of 0.125–0.015 mg./ml. The precipitin line positions on negative films were measured with a shadow graph[19].

[15] Difco, Detroit, Mich.
[16] Canalco Co., Rockville, Md.
[17] Amido Schwarz.
[18] Difco.

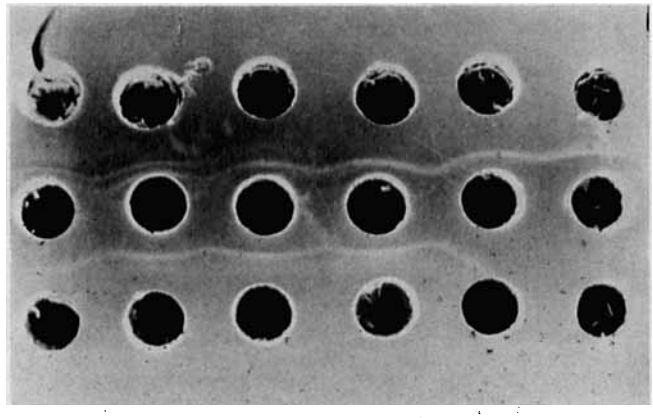

Figure 2—*Antigen E quantitative immunodiffusion analysis. The center wells were filled with antipollen crude extract serum. The upper wells were filled with NIH antigen E (left to right: 0.5, 0.25, 0.125, 0.062, 0.031, and 0.015 mg. protein/ml.), and the lower wells were filled with leaves–stems Fraction D (left to right: 0.1, 0.2, 0.4, and 0.8 mg. protein/ml.). The lower last two wells were filled with buffer and are controls.*

Unstained electrophoresis gels loaded with Fraction D (leaves–stems) were aligned with stained pollen antigen E and gel cuts made in the region of the pollen antigen E. The gel cuts were eluted with 1 ml. of 0.025 *M* tromethamine–hydrochloric acid buffer (pH 7.9) at 4° overnight. The eluate was lyophilized, and the appropriate concentration was made by redissolving the lyophilized eluate in the buffer prior to the Ouchterlony immunodiffusion test.

Allergenic activity of column fractions and NIH antigen E was determined by direct skin testing on patients with known short ragweed pollen sensitivity. Solutions were sterilized by filtration[20] (0.22-μ pore size). Serial 10-fold dilutions of the sterilized solutions were made immediately prior to the injections with sterile phosphate-buffered saline[21]. Intradermal injections (0.02 ml.) were made into the forearm of the patients, and the wheal and flare reactions were read within 20 min. Nonallergic individuals served as controls and were injected with high concentrations of column fractions and NIH antigen E solutions. As suggested by Berrens (13), the allergenic activities of leaves–stems Fraction D and pollen antigen E are reported in terms of specific activity (units per milligram protein). In this system, one unit of activity represents the minimum quantity of the fraction (milligrams protein) eliciting a minimum reaction in specifically sensitive patients.

RESULTS AND DISCUSSION

The literature procedure (3, 4) was successfully used to isolate antigen E (Sephadex G-100, Fraction IV) from short ragweed pollen (1.3 mg. protein/g. pollen). Leaves–stems Fraction D (0.1–0.8 mg. protein/ml.) extracted from commercially supplied plants and NIH antigen E (0.015–0.125 mg. protein/ml.) gave a single precipitin line against antipollen crude extract serum (Fig. 2) and antiantigen E serum. However, when high concentrations of NIH antigen E (>0.125 mg. protein/ml.) were used, a contaminating antigen was detected (Fig. 2, first three upper wells from left). To prevent any nonspecific precipitin line formation, 0.062 mg. protein/ml. of NIH antigen E was used in all comparative immunodiffusion studies shown in Fig. 3.

Figure 3A illustrates the formation of lines of identity when NIH antigen E (Well 2) and leaves–stems Fraction D (Well 3) were placed in adjacent wells against antiantigen E serum. The material eluted from the unstained polyacrylamide cut gels in the region of standard antigen E (Fig. 3B, Wells 3 and 5) formed a common line

[19] Nikon model 6C.
[20] Millipore Corp., Bedford, Mass.
[21] Hollister-Stier Lab., Downers Grove, Ill.

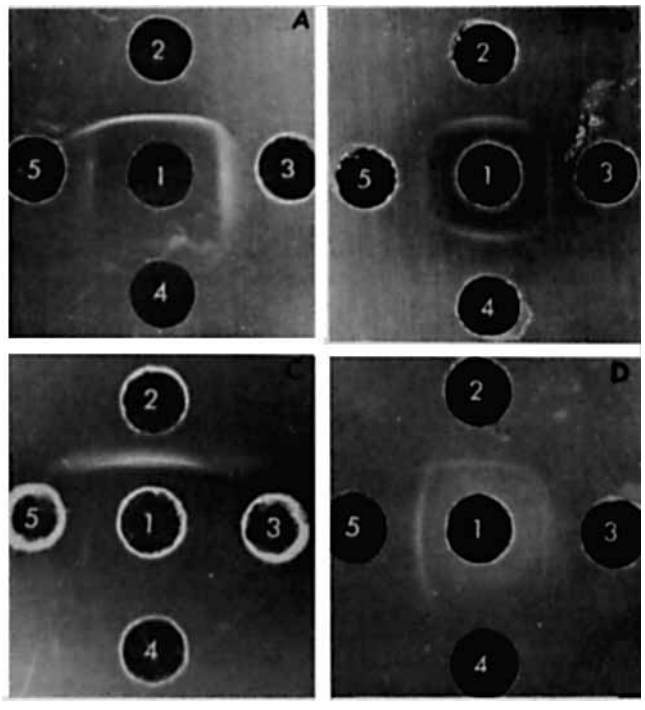

Figure 3 *Comparative immunodiffusion analysis of short ragweed leaves-stems and root Fraction D with NIH antigen E against antipollen crude extract and antiantigen E serum. (A) Leaves-stems Fraction D and NIH antigen E against antiantigen E serum: 1, antiantigen E serum; 2, NIH antigen E (0.062 mg./ml.); 3, leaves-stems Fraction D (0.8 mg./ml.); 4, buffer control; and 5, leaves-stems Fraction D (0.2 mg./ml.). (B) Leaves-stems Fraction D polyacrylamide gel eluate and NIH antigen E against antipollen crude extract serum: 1, antipollen crude extract serum; 2, 4, NIH antigen E (0.062 mg./ml.); and 3, 5, leaves-stems polyacrylamide gel eluate. (C) Root Fraction D and NIH antigen E against antiantigen E serum: 1, antiantigen E serum; 2, NIH antigen E (0.062 mg./ml.); and 3, 4, 5, root Fraction D (1.6, 0.8, and 0.4 mg./ml., respectively). (D) Root Fraction D and NIH antigen E against antipollen crude extract serum: 1, antipollen crude extract serum; 2, 3, root Fraction D (1.6 and 0.4 mg./ml., respectively); 4, buffer control; and 5, NIH antigen E (0.062 mg./ml.).*

of identity with NIH antigen E (Fig. 3B, Wells 2 and 4) against antipollen crude extract serum. Similar results were obtained when antiantigen E serum was used in place of antipollen crude extract serum. Further evidence for the presence of antigen E in short ragweed leaves-stems was obtained by disk electrophoresis and direct skin testing of sensitive individuals. Disk electrophoresis of NIH antigen E, leaves-stems Fraction D, and pollen antigen E (Sephadex G-100, Fraction IV) shows a similar pattern (Fig. 1). However, purified NIH antigen E had a slower anodic movement, which possibly resulted from the 50% glycerol present as a stabilizer. Leaves-stems Fraction D was tested on the skin of two short ragweed pollen-sensitive individuals. The specific activity of this fraction was established to be 10^7 (units/mg. protein) as compared to 10^9 (units/mg. protein) for NIH antigen E on the same patients.

The protein content of the commercially supplied leaves-stems in Fraction D was found to be 0.012 mg./g. while the antigen E content was estimated to be 0.0022 mg. protein/g. when quantitated from the standard curve of the logarithm of the NIH antigen E concentrations *versus* precipitin line positions (Fig. 2). Twice as much protein was obtained from leaves-stems that had been collected from the medicinal plant garden.

Figures 3C and 3D illustrate the reaction of root Fraction D and NIH antigen E against antiantigen E and antipollen crude extract serums, respectively. Precipitin lines between various concentrations of root Fraction D and antiantigen E serum did not appear (Fig. 3C, Wells 3, 4, and 5). A complete line of nonidentity developed between NIH antigen E, root Fraction D (Fig. 3D, Wells 2 and 5), and antipollen crude extract serum. Short ragweed seed Fraction D was obtained in a similar manner as that for pollen antigen E and was further fractionated by chromatography[14]. Five peaks eluted from the column were separately subjected to immunodiffusion and disk electrophoresis testing as described for leaves-stems; none contained antigen E.

In conclusion, immunodiffusion, disk electrophoresis, and skin testing evidence showed that the allergen, antigen E, is present in leaves-stems of short ragweed plant. Although the results of immunodiffusion and disk electrophoretic analysis indicated that antigen E was absent from roots and seeds of short ragweed, the possibility that other pollen allergens or their modification may be present in other parts of this plant cannot be excluded. In fact, this assumption is supported by immunofluorescent antibody studies substantiating that surface materials of ragweed pollen (exine and intine) are derived from the diploid tapetum and not from the haploid pollen (14). Results also suggest that the antigen E present in short ragweed leaves-stems may have been a major cause of hay fever symptoms, as recently suggested (1) for wind-borne plant particles. It is also concluded that short ragweed leaves-stems might serve as a source of antigen E, although the concentration present is much less than that of the pollen.

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Metabolism of Clindamycin II: Urinary Excretion Products of Clindamycin in Rat and Dog

F. F. SUN

Abstract □ The urinary excretion products of clindamycin metabolism in rats and dogs were isolated and characterized. Of the radioactive materials excreted in rat urine, 53% was identified as unchanged clindamycin, 31% as clindamycin sulfoxide, and 15% as *N*-demethylclindamycin. In dog urine, 36% of the radioactivity excreted was identified as unchanged drug, 28% as the sulfoxide, 28% as clindamycin glucuronide, and 9.16% as *N*-demethylclindamycin. No glucuronide conjugate was found in the rat urine. Thus, based on the metabolic patterns found in the urine of the two species, sulfoxidation and *N*-demethylation were the predominant metabolic routes in the rat, while conjugation with glucuronic acid and sulfoxidation were the major pathways in the dog.

Keyphrases □ Clindamycin metabolism—urinary excretion products in rats and dogs □ Excretion, urinary—identification of clindamycin metabolites, rats, dogs

In the first paper of this series (1), studies on the absorption and excretion of a new antibiotic, clindamycin[1], in experimental animals were described. In continuation of the study of clindamycin metabolism, effort was directed to the elucidation of the metabolic pathways for clindamycin in mammals. This report describes the isolation and characterization of urinary excretion products in the rat and the dog.

Studies of Brodasky *et al.* (2) on the characterization of human urinary excretion products of clindamycin suggested that *N*-demethylclindamycin was the major metabolite. These authors also observed two other drug-related substances with chromatographic behavior similar to clindamycin sulfoxide and clinimycose (the 1-hydroxy derivative). The trace amounts of these two components precluded their isolation and characterization. Clindamycin sulfoxide and *N*-demethylclindamycin (3) have been isolated and identified as the metabolic products of clindamycin in microorganisms.

EXPERIMENTAL[2]

Dosage and Collection of Samples—Twelve female Sprague–Dawley rats (Upjohn strain) were given 100-mg./kg. oral doses of clindamycin hydrochloride through a stomach tube every day for a month. Tritium-labeled drug (6.0 μc.) was given on the first and the last days to check for possible adaptation or stimulation of metabolism and to provide labeled metabolites for isolation. Urine specimens were collected once a day through stainless steel metabolism cages and frozen immediately until used. The rats were maintained on powdered food[3] and deionized water. Food was given during the daytime only to provide empty stomachs before dosing every morning. Water was provided at all times.

Three male beagle dogs, weighing 8.2, 8.9, and 11.7 kg., respectively, each received single daily oral doses of 500 mg. clindamycin hydrochloride (in hard-filled capsules) for 7 days. Urine samples were collected once a day through stainless steel metabolism cages. Food[4] was given once a day and deionized water was allowed freely. Urine samples containing radioactive clindamycin and metabolites from the previous experiment (1) were added to the pooled urine to monitor the separation.

Preparation of Extract—The polystyrene resin was thoroughly washed successively with acetone, methanol, and distilled water before being packed into a 38 × 300-mm. glass column. A pooled urine sample, spiked with radioactive urine (total volume 2 l.), was filtered through glass wool and adjusted to pH 8.5. The filtered urine was passed through the column. The column was subsequently washed with 6 l. of distilled water and eluted with: (*a*) 2 l. of a methyl ethyl ketone–water mixture (95:5), and (*b*) 2 l. of methanol. The spent urine and aqueous wash were discarded. The ketone and methanol eluents were pooled and evaporated to dryness. Recovery of radioactivity was greater than 90%.

The drug-related components in the urine extracts were examined by TLC. The separation procedure is presented in Scheme I.

Isolation of Clindamycin and Metabolites from Rat Urine Extract—*Countercurrent Distribution*—The dried material from rat urine was dissolved in 20 ml. of anhydrous methanol and acidified with methanolic hydrochloric acid. Twenty milliliters of acetone and 400 ml. of anhydrous ether were added, and the mixture was cooled to 0°. A large amount of precipitate, containing essentially all of the radioactivity, separated out and was collected by filtration. The precipitate was dissolved in 30 ml. of water-saturated *n*-butanol and loaded into the first three tubes of a 500-tube, all-glass countercurrent distribution separator with 10 ml. of *n*-butanol as the upper phase and 10 ml. of water as the lower phase. After 500 transfers were completed, aliquots were withdrawn from every 10th tube and analyzed for radioactivity. The locations of radioactivity (Fig. 1) were then determined, and the rest of the tubes were emptied and combined into two fractions. The two layers in each fraction were separated. The pH of the aqueous layer was adjusted to 10 with 1 *N* NaOH before being extracted three times with equal volumes of *n*-butanol. The extracts were combined with each corresponding fraction, and the pooled butanol solution was evaporated to dryness. Extracted aqueous layers were checked for radioactivity losses before being discarded. The losses of radioactivity in aqueous layers were negligible. Drug-related components in each fraction were examined by TLC.

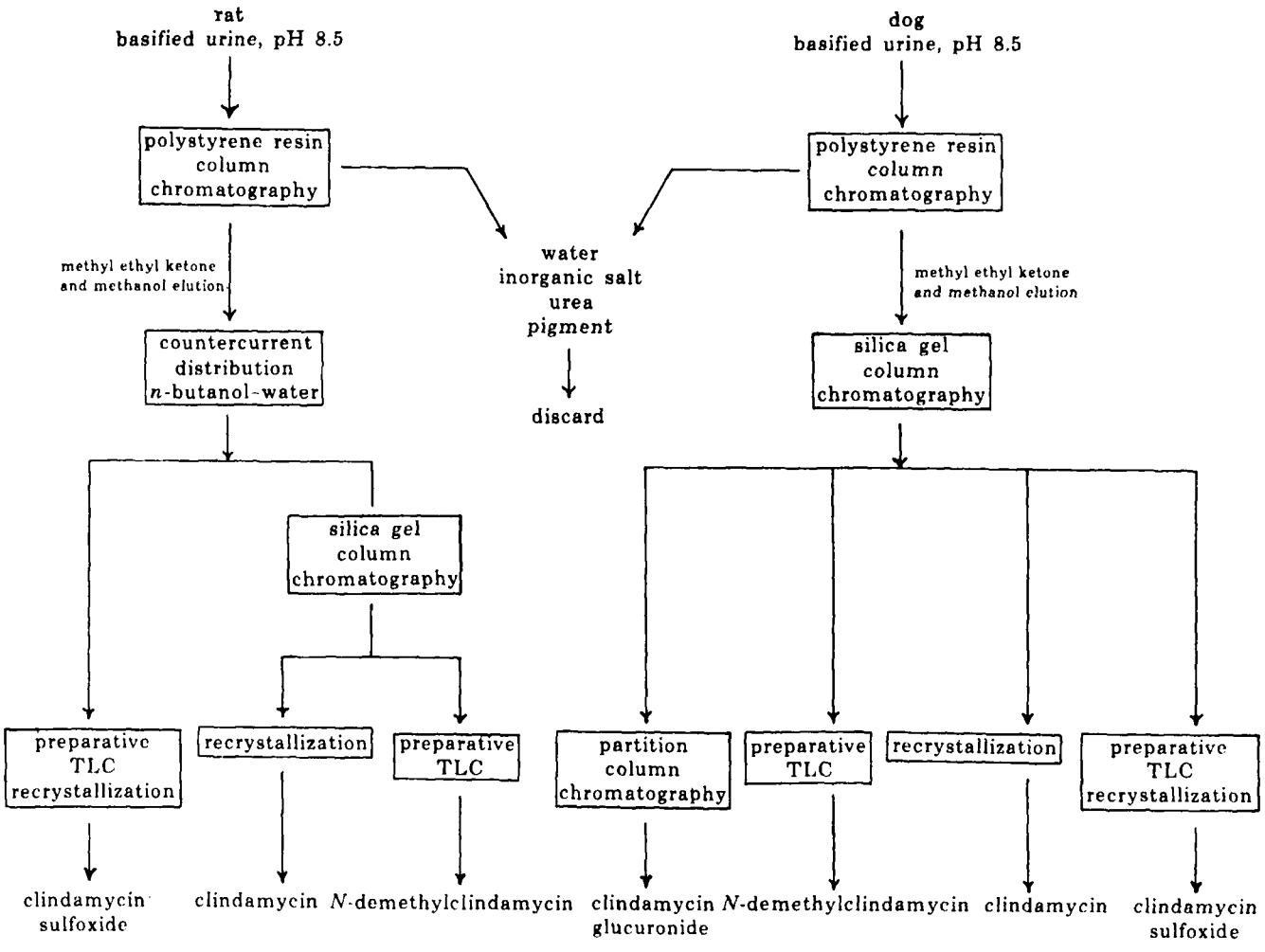
Purification of Clindamycin Sulfoxide—The fraction containing clindamycin sulfoxide (Fraction I-CCD) was dissolved in 1 ml. of methanol, and the entire fraction was streaked on a 2-mm. thick preparative thin-layer silica gel G_{254} plate. The plate was developed in the methyl ethyl ketone–acetone–water (93:26:10) solvent. Standard clindamycin sulfoxide was spotted and run along with the streak to identify the desired band. After the solvent front reached the top, the band corresponding to the clindamycin sulfoxide was scraped off and homogenized in 100 ml. of absolute methanol. The silica gel was then filtered out and washed twice with 50-ml. aliquots of methanol. The combined methanol solution was evaporated to dryness, and the residue was leached twice with 5-ml. aliquots of chloroform–methanol (4:1). Traces of silica gel at this point could be removed by centrifugation. The chloroform–methanol solution was concentrated and the entire preparative TLC procedure was repeated once with the same solvent and once with chloroform–methanol (6:1) as the developing solvent. The final product, showing

[1] Cleocin, The Upjohn Co.

[2] Amberlite XAD-2 polystyrene resin was purchased from Rohm and Haas Co. Silica gel (0.05–0.2 mm.) and preparative silica gel G precoated thin-layer plates were obtained from Brinkmann Instruments. Dicalate, an amorphous diatomaceous silica filter aid, was obtained from Grefco Inc., Los Angeles, Calif. Precoated analytical silica gel thin-layer plates were obtained from Analtech, Inc. Tritium-labeled clindamycin was synthesized as previously described with the specific activity of 2.386 mc./mg. (1). Clindamycin sulfoxide and *N*-demethylclindamycin standards were provided by Mr. R. D. Birkenmeyer, Infectious Diseases Research, The Upjohn Co.

[3] Purina lab chow.

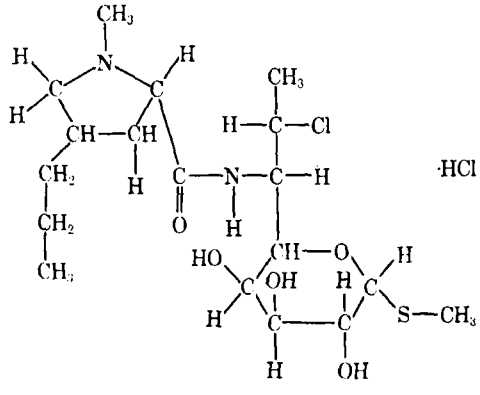
[4] Purina dog chow.



Scheme I—Sequential experimental steps for the separation of clindamycin and metabolites from rat and dog urine

only one spot on the thin-layer plate, was crystallized from ethanol and ethyl acetate.

Purification of Clindamycin and N-Demethylclindamycin—The fractions from countercurrent distribution (tubes in Fraction II-CCD) containing these two components were pooled, evaporated to dryness, and redissolved (or suspended) in 20 ml. of chloroform–methanol (10:1). This solution was placed on the top of a silica gel (0.02–0.5 mm.) column packed in the same solvent (26 × 240 mm.). Elution was carried out first with 250 ml. of chloroform–methanol (10:1), then with 400 ml. of chloroform–methanol (5:1), and finally with 250 ml. of chloroform–methanol (2:1). Eluates were collected in 10-ml. fractions, and aliquots were withdrawn from every other tube for radioactivity counting. The desired fractions were combined, concentrated, and analyzed by TLC.



clindamycin hydrochloride

Fractions containing clindamycin were evaporated to dryness and redissolved in 1 ml. of methanol. The drug was converted to the hydrochloride salt and precipitated by adding 1 ml. of acetone and 20 ml. of ether. The precipitate was crystallized from 2 ml. of ethanol and 7 ml. of ethyl acetate. The final product appeared to be free from contaminants and was analyzed by NMR, IR, mass spectrometry, and TLC.

Fractions containing *N*-demethylclindamycin were concentrated and purified by preparative TLC according to the procedure used for clindamycin sulfoxide. The final product was evaporated to dryness and analyzed without crystallization.

Isolation of Clindamycin and Metabolites from Dog Urine Extract—In the dog study, since there was one radioactive zone located near the origin on the thin-layer chromatogram (Fig. 2), it was separated first by silica gel column chromatography. The dried material, obtained from polystyrene resin chromatography, was homogenized in a solvent mixture of chloroform–methanol (95:5) and loaded on a silica gel column (38 × 360 mm.) packed in the same solvent. Elution was carried out successively with the following solvent mixtures: (*a*) 700 ml. chloroform–methanol (20:1), (*b*) 500 ml. chloroform–methanol (10:1), (*c*) 500 ml. chloroform–methanol (8:1), (*d*) 500 ml. chloroform–methanol (5:1), (*e*) 1000 ml. chloroform–methanol (1:3), (*f*) 600 ml. methanol, and (*g*) 500 ml. methanol–water (1:1). The eluates were collected in 10-ml. fractions. An aliquot from every third fraction was analyzed for radioactivity. Eight radioactivity fractions were separated (Table I) (Fig. 3), concentrated, and analyzed by TLC.

Purification of Unchanged Clindamycin and Clindamycin Sulfoxide—The purification of intact clindamycin (Fraction I) and clindamycin sulfoxide (Fraction II) was carried out by preparative TLC according to the methods described in the previous section. Clindamycin was recrystallized as the hydrochloride salt from a mixture

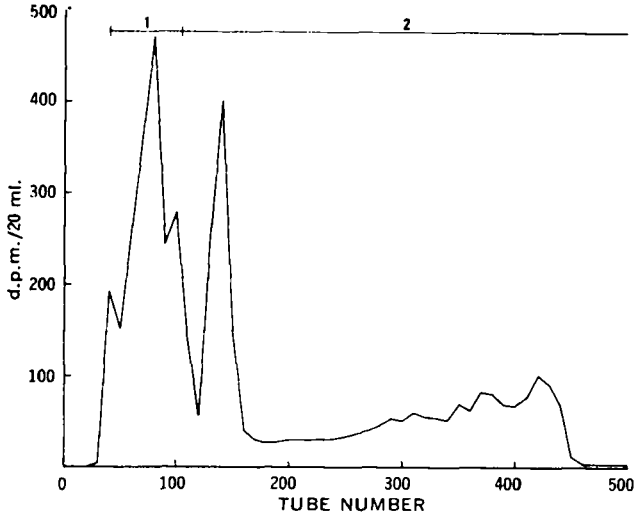


Figure 1—*Countercurrent distribution of rat urine extract.*

of ethyl acetate–ethanol (7:2). Clindamycin sulfoxide was isolated as the free base. Both compounds were identified by NMR, IR, and mass spectrometry.

Purification of Clindamycin Glucuronide—When eluted from the silica gel column, fractions 4 and 5 (Fig. 3) were contaminated with large amounts of a yellow urinary pigment. These fractions were further purified by liquid–liquid partition chromatography. A solvent mixture of 300 ml. of *n*-butanol, 700 ml. of ethyl acetate, and 400 ml. of water was mixed by shaking and allowed to settle. The upper phase and lower phase were separated. Twenty grams of an amorphous diatomaceous silica filter aid[5] were mixed with 200 ml. of the upper phase. The lower phase (8 ml.) was added dropwise with vigorous stirring. The mixture was poured into a column and allowed to settle. The fraction to be purified was: (*a*) dissolved in 2 ml. of the lower phase, (*b*) mixed with 4 g. of Hyflo Super Cel, and (*c*) diluted with 10 ml. of the upper phase; the eluate was collected in 10-ml. fractions. Aliquots from every other tube were counted for radioactivity. The radioactive zone was pooled and evaporated to dryness.

The same procedure was repeated twice to obtain about 500 mg. of white fluffy material. TLC analyses showed that a single zone was present. The material was used for spectroscopic analyses.

Additional clindamycin glucuronide was purified by countercurrent distribution, using a solvent system of *n*-butanol–water. Based upon TLC, the clindamycin glucuronides purified by countercurrent distribution and partition chromatography were identical.

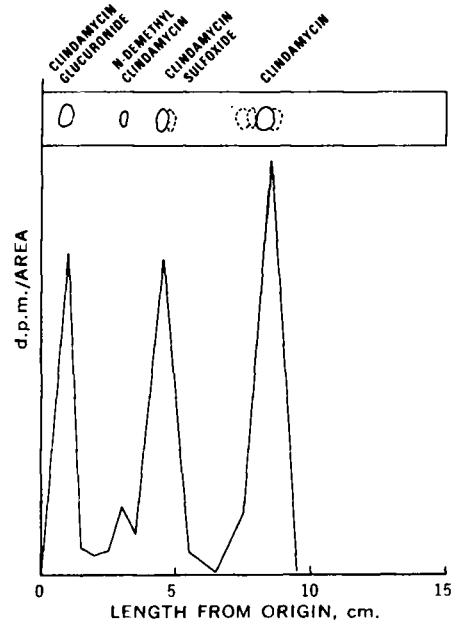


Figure 2—*Quantitative thin-layer chromatogram of pooled dog urine extract (unhydrolyzed). The solvent system was ethyl acetate–acetone–water (8:5:1).*

[5] Dicalate.

Table I—Recovery of Radioactivity after Silica Gel Chromatography of Dog Urine Extract[a]

Fraction	Microcuries	Recovery, %	Identification
1	3.28	23.39	Clindamycin
2	5.64	40.22	Clindamycin sulfoxide
3	0.95	6.73	*N*-Demethylclindamycin
4	0.486	3.46	Clindamycin glucuronide
5	3.161	22.54	Clindamycin glucuronide
6	0.142	1.01	—
7	0.153	1.09	—
8	0.068	0.49	—

[a] Total microcuries added = 14.022 μc. Total percentage recovery = 98.93%.

Analytical Methods[6]—*TLC*—TLC, used for monitoring the separation procedure, was carried out on 2.5 × 20-cm. glass plates precoated with 0.25 mm. of silica gel G_{254}. The following solvent systems were used: (*a*) methyl ethyl ketone–acetone–water (93:26:10), (*b*) chloroform–methanol–water (8:4:0.4), (*c*) chloroform–methanol (6:1), (*d*) *n*-butanol–water (5:1), (*e*) ethyl acetate–acetone–water (8:5:1), and (*f*) acetone–chloroform (3:1). Since clindamycin glucuronide remained near the origin when chromatographed in these solvent mixtures, three additional systems were required. They were: (*g*) methyl ethyl ketone–acetone–water (1:3:1), (*h*) ethyl acetate–acetone–water (2:6:1), and (*i*) chloroform–methanol (1:3).

Spraying reagents used to locate the desired spots were as follows:

Spraying Reagent	Reaction
8.0 g. $NaIO_4$; 10 g. K_2CO_3; 1.0 g. $KMnO_4$ in 500 ml. of distilled water (4)	sugar moieties
2% 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in benzene (5)	sulfides, sulfoxides, sulfones
0.5% ninhydrin in acetone	secondary amine in *N*-demethylclindamycin

Radioactive zones were located by scanning in a glass plate scanner[7].

Quantitative TLC was used to measure the relative amount of each component in the urine. Twenty-milliliter aliquots of urine

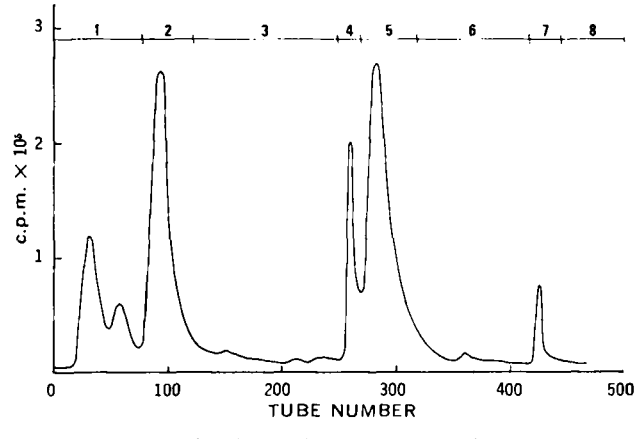


Figure 3—*Silica gel column chromatography of crude dog urine extract.*

[6] NMR spectra were obtained on a Varian model A60 spectrometer. Mass spectra were obtained on either an Atlas CH-4 mass spectrometer or a Consolidated Electrodynamics 21-110B high-resolution mass spectrometer. IR spectra were obtained on a Perkin-Elmer 421 grating spectrometer.

[7] Vanguard 885.

Table II—Relative Amounts of Clindamycin and Metabolites Excreted in Rat Urine[a]

	Relative Amounts in Total Urine, % of Urinary Radioactivity							
	Radioactive Urine Collected the 1st Day				Radioactive Urine Collected the Last Day			
	1[b]	2[b]	3[c]	Mean	1[b]	2[b]	Mean	Average
Unchanged clindamycin	54.59	52.47	50.07	52.38	52.11	57.58	54.85	53.36 ± 2.55
Clindamycin sulfoxide	30.41	30.54	36.34	32.43	31.56	28.20	29.88	31.41 ± 2.7
N-Demethyl-clindamycin	14.95	16.97	13.50	15.14	16.2	13.82	15.04	15.10 ± 1.35

[a] Radioactive urine samples are collected from 0 to 48 hr. postadministration. [b] The solvent system used was chloroform–methanol (6:1). [c] The solvent system used was ethyl acetate–acetone–water (8:5:1).

samples collected from the clindamycin-treated rats or dogs were concentrated to 0.5 ml. The pH was adjusted to approximately 10 before 20 μl. of each was streaked on a 2.5 × 18-cm. thin-layer plate (250 μ thick), and the plate was developed with the TLC Solvent Systems *c* and *e*. After the development was completed, the silica gel was scraped in 1-cm. sections into 18 scintillation counting vials containing 0.5 ml. of water and 15 ml. of counting solvent[8] (1). The radioactivity was counted in a liquid scintillation counter[9]. Quenching losses were corrected by adding 0.05 ml. of tritiated toluene and counting again.

Enzymatic Analysis (Identification of Clindamycin Glucuronide)—The identity of clindamycin glucuronide was established by treatment with β-glucuronidase. Clindamycin glucuronide (20 mg.) was dissolved in 10 ml. of 0.1 *M* sodium acetate buffer (pH 5.0) and mixed with 0.1 ml. of concentrated β-glucuronidase aryl sulfatase solution[10]. The mixture was incubated in a 37° water bath for 24 hr. After the incubation period, the mixture was adjusted to pH 10.0 with 3.6 *N* potassium hydroxide and passed through a small (10 × 100 mm.) polystyrene resin column. The acetate buffer was washed off with 500 ml. of water. The clindamycin and clindamycin glucuronide were eluted with 200 ml. of 95% aqueous acetone and 200 ml. of methanol. The solvent eluates were pooled and concentrated under reduced pressure. Intact clindamycin (hydrolysis product) was isolated from the extract by preparative TLC in a solvent system composed of ethyl acetate–acetone–water (8:5:1). Identification of the hydrolytic product as intact clindamycin was based upon TLC behavior and mass spectrometry. As a control, another 20-mg. sample of clindamycin glucuronide was carried through the entire procedure but without the addition of enzyme. The glucuronide was recovered unaltered.

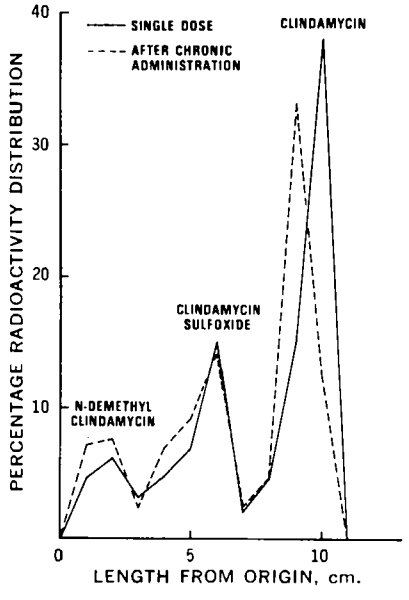


Figure 4—*Quantitative thin-layer chromatogram of lyophilized pooled rat urine. The solvent system was chloroform–methanol (6:1).*

For use as a chromatographic standard, authentic clindamycin glucuronide was synthesized enzymatically by reacting clindamycin with uridine diphosphoglucuronic acid (ammonium salt) in the presence of freshly prepared dog liver microsomes. The reaction product was separated from the unreacted clindamycin by polystyrene resin and silica gel chromatography.

Isolation of *In Vitro* Metabolites of Clindamycin—Preliminary experiments were conducted to study the metabolism of clindamycin under *in vitro* conditions. Livers from eight Sprague–Dawley rats (four male and four female), with the average body weight of 200 g., and from two male beagle dogs were used. The rats were sacrificed by cervical dislocation, and the dogs were sacrificed by electrocution. Microsomes were prepared by the differential centrifugation procedure of Allmann *et al.* (6), except 0.25 *M* sucrose–0.01 *M* tromethamine hydrochloride solution was used for suspension instead of sucrose–mannitol A.

N-Demethylation and sulfoxidation reactions of clindamycin were carried out in open 25-ml. erlenmeyer flasks at 38° in a metabolic shaker[11]. The complete reaction medium contained per each milliliter: potassium phosphate buffer (pH 7.5), 100 μmoles; magnesium chloride, 5 μmoles; nicotinamide-adenine dinucleotide phosphate (NADP), 1 μmole; nicotinamide-adenine dinucleotide (NAD), 1 μmole; sodium isocitrate, 5 μmoles; isocitrate dehydrogenase, 0.1 mg.; microsomes, 3–5 mg.; and clindamycin, 5 μmoles. The mixture was incubated for 1 hr. under air.

Glucuronic acid conjugation of clindamycin was carried out in a 25-ml. erlenmeyer flask at 38° under nitrogen atmosphere. The reaction mixture consisted of the following components per each milliliter: tromethamine hydrochloride, 100 μmoles; uridine diphosphate glucose (UDPG), 133 μmoles; magnesium chloride, 4 μmoles; clindamycin, 2 μmoles; and microsomes, 2–4 mg. The mixture was incubated for 1 hr. before extraction.

After the incubation was complete, the reaction was terminated by addition of 0.05 ml. of 3 *M* trichloroacetic acid/ml. of the reaction mixture. The excess acid was immediately neutralized by the addition of 3 *M* KOH. The denatured protein was removed by centrifugation. The clear supernate was adjusted to pH 8.5 with 3 *M* KOH and loaded into a small polystyrene resin column (10 × 100 mm.). The column was washed with 200 ml. of distilled water. Clindamycin and its metabolites were eluted with 200 ml. of 95% aqueous acetone and 200 ml. of methanol. The solvents were pooled and concentrated under reduced pressure. Drug-related compounds in the extract were isolated by preparative TLC and identified as described previously.

RESULTS AND DISCUSSION

Rats—Figure 4 shows the thin-layer chromatograms of two crude rat urine extracts from polystyrene resin column chromatography. The solid line shows the distribution pattern of urinary radioactivity from rats that received a single dose of clindamycin hydrochloride, and the dotted line shows the same pattern from rats after chronic administration of the drug for a month. In both cases, three radioactive spots were observed. After further separation with countercurrent distribution and silica gel column chromatography, the three compounds were characterized as follows: Zone 1,

[8] Diotol.
[9] Packard Tri-Carb 314EX.
[10] Sigma Type H-2 β-glucuronidase, No. G-0876, 143,000 units/ml.
[11] Dubnoff.

unchanged clindamycin; Zone 2, clindamycin sulfoxide; and Zone 3, *N*-demethylclindamycin.

Appreciable quantities of both intact drug and clindamycin sulfoxide were isolated for an unequivocal identification. For the intact drug, results from all three spectroscopies and TLC were consistent with the authentic material.

For the clindamycin sulfoxide, mass spectrometry showed peaks (*m/e*) at 440 and 442, corresponding to the molecular ion; 377 and 379, due to the M − S(=O)CH_3 ion; 275 and 277, due to the M − methylthiogalactose ion; and 126, due to the propylhygric acid ion.

NMR spectroscopy showed that the protons of the S-methyl group were shifted downfield, which is consistent with the sulfoxide structure. IR (micro KBr pellet) and TLC results were identical to those for the reference material. Thus, the identity was established.

Since these spectra were presented elsewhere (3), they are not reproduced in this report.

The identity of *N*-demethylclindamycin was established mainly by TLC and mass spectrometry. The amount of isolated material was insufficient for a positive identification by NMR. This compound showed mass spectral peaks (*m/e*) at 410 and 412, corresponding to the molecular ion; 373 and 375, due to the M − SCH_3 ion; 261 and 263, due to the M − methylthiogalactose ion; and 112, due to the *N*-demethylpropylhygric acid ion. This was consistent with the assigned structure. Furthermore, the isolated material showed identical R_f values with an authentic sample of *N*-demethylclindamycin in all six TLC solvent systems tested. Both samples developed a purple color after spraying with ninhydrin reagent, which indicates the free secondary amine group. These results are all consistent with the identification of the unknown compound as *N*-demethylclindamycin.

Quantitative determination of the percentage of each component in rat urine was carried out with TLC (Fig. 4 and Table II). Similar results were obtained from five separate determinations of two pooled urine samples from rats given single or chronic doses of clindamycin with two different developing systems. In all cases, it was found that approximately one-half of the radioactivity in rat urine was unchanged clindamycin (53.36 ± 2.70%) and *N*-demethylclindamycin (15.10 ± 1.35%). These determinations were carried out on pooled urine samples, so the standard deviation represented only the variation of the TLC assays.

To determine whether chronic administration of clindamycin to rats would stimulate or inhibit its own metabolism, radioactive drug was given to these rats on the first and the last days of the 1-month dosing period, with unlabeled drug in between. The percentage of each major component in the two radioactive urine samples was measured and compared. The results (Table II) clearly revealed that the percentage of metabolites and unchanged drug did not change after chronic administration, which ruled out the possibility that clindamycin modified its own metabolism.

Dogs—Typical thin-layer chromatograms (Fig. 2) of the urine extracted from polystyrene resin column chromatography show three major radioactive zones plus one minor zone. Resolution of the same extract by silica gel column chromatography yielded eight fractions (Fig. 3 and Table I). Each fraction was characterized as follows.

Fraction 1—The materials under the two peaks were identical based upon TLC in three solvent systems. After purification by preparative TLC, this fraction yielded unchanged clindamycin as established by TLC, mass spectroscopy, and IR spectroscopy.

Fraction 2—This fraction was purified and identified as clindamycin sulfoxide by TLC and mass spectrometry.

Fraction 3—This fraction contained one radioactive compound, which appeared in the same R_f region as *N*-demethylclindamycin on the thin-layer chromatogram and also showed a positive ninhydrin reaction. It was tentatively identified as *N*-demethylclindamycin. Further confirmation was not possible since the quantity was too small.

Fractions 4 and 5—Both fractions were identical based upon TLC or countercurrent distribution. The isolated material was identified as clindamycin glucuronide by the following criteria:

1. Mass Spectroscopy—Low-resolution mass spectroscopy showed neither molecular ion nor any ion that contained a chlorine atom. Peaks were observed at *m/e* 388 (clindamycin free base − HCl), 345 (clindamycin free base − HCl − SCH_3), and 126 (hygric acid portion). It appeared that the compound lost hydrogen chloride readily upon electron impact.

High-resolution mass spectroscopy of the trimethylsilyl ether derivative showed peaks at *m/e* 1032 (molecular ion) which was consistent with the hexa(trimethylsilyl) clindamycin monoglucuronide. Peaks were also seen at *m/e* 1017 (M − 15); 966 (M − HCl); 989 (M − SCH_3); 375, due to the silylated sugar portion; and 126, due to the propylhygric acid portion of the molecule.

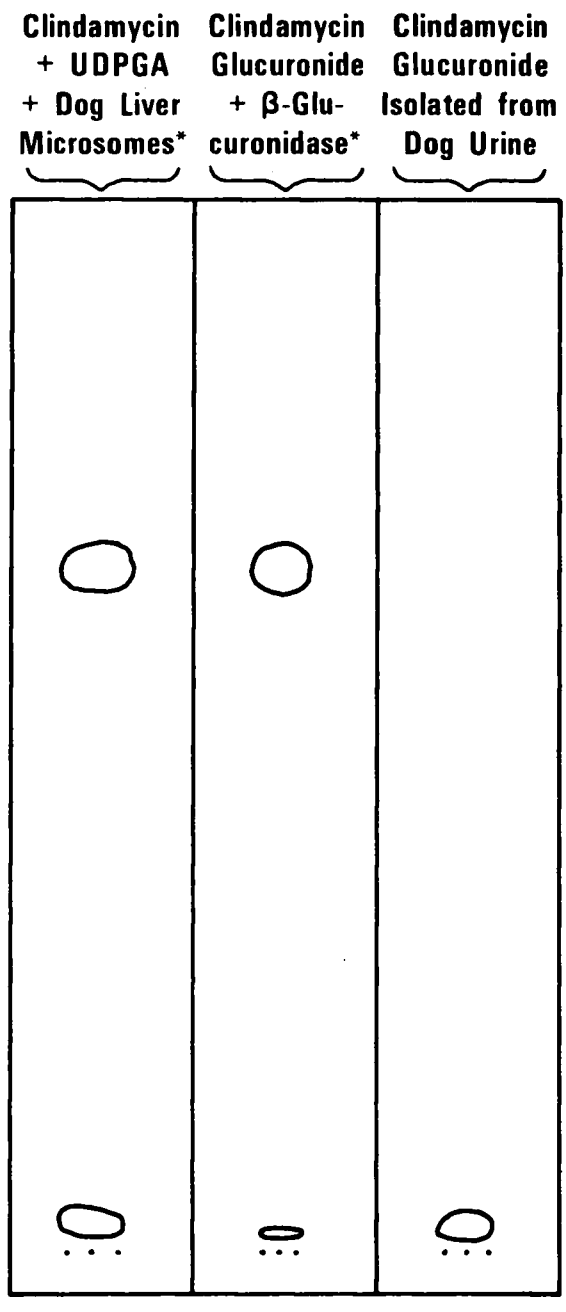


Figure 5—*Thin-layer chromatogram of three clindamycin glucuronide preparations. The solvent system was ethyl acetate–acetone–water (8:5:1). (* Extracts from polystyrene resin column chromatography.)*

2. IR Spectroscopy—The IR spectrum (micro KBr) was consistent with a glucuronic acid derivative of clindamycin with an ether linkage.

3. NMR Spectroscopy—The spectrum was too complex for a detailed analysis. It was not possible to locate which hydroxyl group was conjugated with glucuronic acid on the *S*-methylthiogalactose.

4. Glucuronidase Treatment—After treatment with β-glucuronidase, the glucuronic acid was removed and unchanged clindamycin was regenerated. The reaction product was identified by both TLC and mass spectroscopy.

5. TLC—The thin-layer chromatograms of clindamycin glucuronide isolated from dog urine compared to authentic clindamycin

Table III—Metabolites Produced by Incubating Liver Microsomes with Clindamycin-^{3}H in the Presence of NADPH and O_2 for 30 min.

Enzyme Source	Clindamycin Sulfoxide Produced, mole^{-9}/mg.	Formaldehyde Produced, mole ^{9}/mg.	*N*-Demethylclindamycin Produced, mole^{-9}/mg.
Rat liver microsomes	28.25	45.81	6.15
Boiled rat liver microsomes	1.82	—	—
Dog liver microsomes	21.24	22.50	Not detectable
Boiled dog liver microsomes	2.97	-	—

glucuronide are shown in Fig. 5. The identical R_f values serve as further confirmation of their identity.

Fractions 7 and 8—The two fractions contained less than 2% of the total radioactivity placed on the column. TLC showed that both fractions were heavily contaminated by known drug-related components, *i.e.*, unchanged clindamycin, clindamycin sulfoxide, and clindamycin glucuronide. These compounds were probably the sources of radioactivity. Both fractions were discarded.

Quantitative determination of the percentage of each component in dog urine was carried out by TLC. An average of 35.69% of the total radioactivity excreted in urine was unchanged drug. The remaining consisted of 27.57% clindamycin sulfoxide, 27.58% clindamycin glucuronide, and 9.16% *N*-demethylclindamycin.

Metabolism of Clindamycin-^{3}H *In Vitro*—Preliminary evidence was obtained that liver microsomes from both species are capable of catalyzing the bioconversion of clindamycin to its metabolites (Table III). When radioactive clindamycin was incubated with freshly isolated rat or dog liver microsomes in the presence of NADPH and oxygen, the predominant product was clindamycin sulfoxide. A small but detectable amount of *N*-demethylclindamycin could also be observed. However, if semicarbazide was added to the reaction mixture, the *N*-demethylation reaction could be readily demonstrated by following the formaldehyde generated with Nash's (7) reagent. Both metabolic activities were abolished when boiled microsomes were used or NADPH was deleted from the reaction mixture.

Glucuronic acid conjugation could also be demonstrated, under *in vitro* conditions, by the liver microsomes from both species. Radioactive clindamycin was incubated with microsomes in the presence of uridine diphosphoglucuronic acid and magnesium salt at 37° for 1 hr. The reaction mixture was deproteinized and passed through a polystyrene resin column. Clindamycin glucuronide in very small quantity was isolated from the extract by preparative TLC. It had the same spectral and chromatographic properties as the material isolated from urine and could be converted back to clindamycin by treatment with β-glucuronidase. Since the conditions of the reaction have not been optimized, the results cannot be expressed quantitatively at present.

The study described here has established that clindamycin is metabolized by the rat through two well-known mechanisms: *S*-oxygenation and *N*-demethylation. On the other hand, *S*-oxygenation and glucuronic acid conjugation are the major metabolic routes in the dog. However, since the major excretion route of clindamycin in both species is the feces (60–70%) rather than the urine, it would be risky to assume that the urinary composition of drug and metabolites reflects the overall picture.

The bioactivities of both clindamycin sulfoxide and *N*-demethylclindamycin have been determined in the past (3). *N*-Demethylclindamycin was approximately four times as effective as clindamycin in the antibacterial tests, whereas clindamycin sulfoxide has only one-quarter the activity of the parent drug.

The bioactivity of clindamycin glucuronide was determined[12], and the conjugate had practically no antibacterial activity (less than 0.02% of the antibacterial activity of clindamycin).

The species difference in glucuronic acid conjugation of clindamycin between rat and dog was quite interesting. The difference was probably partially due to a relative deficiency of uridine diphosphate-glucuronyltransferase in the rats used for this study. Investigation of the uridine diphosphate-glucuronyltransferase system in rat and dog liver showed that dog liver possessed five times more glucuronide synthesis activity than the rat liver using *p*-nitrophenol as substrate. A survey of the literature also indicated that rats possess the least glucuronide synthesis activity of the six species studied, *i.e.*, rabbit, guinea pig, mouse, sheep, and dog (8–10). However, since the metabolite profile of clindamycin in the feces was not known and the bioconversion of clindamycin to clindamycin glucuronide by rat liver microsomes could not be demonstrated, other factors, such as excretion through the bile, could be the cause for the failure to detect clindamycin glucuronide in rat urine.

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Cactus Alkaloids XXI: β-Phenethylamines from *Dolichothele sphaerica*

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Abstract □ Studies concerning the isolation and biosynthesis of an imidazole alkaloid, dolichotheline (*N*-isovalerylhistamine), in the cactus *Dolichothele sphaerica* (Dietr.) Br. and R. were previously reported. In the current investigation, chromatographic analysis (TLC) detected additional alkaloids; however, these compounds were β-phenethylamines rather than imidazoles. Preparative TLC aided in the crystallization of five of these compounds: *N*-methylphenethylamine hydrochloride, *N*-methyltyramine hydrochloride, synephrine hydrochloride, β-*O*-methylsynephrine hydrochloride, and β-*O*-ethylsynephrine hydrochloride. The last compound was shown to be an extraction artifact of synephrine. This is the first report of the isolation of *N*-methylphenethylamine from the Cactaceae, but the other natural compounds were all previously isolated from other cactus species.

Keyphrases □ Cactus alkaloids—isolation, identification of five β-phenethylamines from *Dolichothele sphaerica* □ *Dolichothele sphaerica* (Dietr.) Br. and R.—isolation, identification of five β-phenethylamines □ β-Phenethylamine alkaloids—isolation, identification from *Dolichothele sphaerica*

The known cactus alkaloids are generally either β-phenethylamines or the related tetrahydroisoquinolines. An exception is the imidazole alkaloid dolichotheline, 4(5)-[2-*N*-isovalerylaminoethyl]imidazole or *N*-isovalerylhistamine. This compound was isolated (1, 2) from *Dolichothele sphaerica* (Dietr.) Br. and R., a small cactus indigenous to southern Texas and northern Mexico. The biosynthesis of this unusual alkaloid was demonstrated (3, 4) to involve amide formation between histamine and isovaleric acid, with the latter compound likely arising naturally from leucine catabolism. Recently, this plant was also found (5) to produce aberrant imidazole alkaloids, such as *N*-isocaproylhistamine and 4(5)-[*N*-isovalerylaminomethyl]imidazole, when administered the proper exogenous precursors.

Earlier reports referred to the presence of "toxic" alkaloids in this species (6) and to ethnobotanical relationships of *Dolichothele* species with *Lophophora* through the common name of "peyote" (7–9). The present phytochemical studies with *D. sphaerica* were undertaken to isolate and identify additional unusual alkaloids that might have medicinal or psychotropic potential.

EXPERIMENTAL

Plant Material—Specimens of the cactus were purchased[1], and identification was made by comparison with a literature description[2] (10). Since the genus *Dolichothele* is not universally recognized, the botanical synonym, *Mammillaria longimamma* DC. var. *sphaerica* K. Brandegee as described by Benson (11), was included in the identification. The cacti were sliced, frozen, freeze dried (87% moisture) at temperatures not exceeding 30°, and ground to a powder using a 2-mm. sieve on a Fitzpatrick mill.

Preparation of Crude Alkaloid Fractions—The powdered plant material (617 g.) was defatted, basified, and extracted *via* chloroform percolation (12, 13). Condensing the filtered chloroform extract on a flash evaporator[3] and then under rotary vacuum evaporation produced a thick syrup, which was extracted with six successive 200-ml. portions of 1 *N* hydrochloric acid. The aqueous extracts were filtered, combined, and extracted six times with 1-l. portions of both chloroform and ether. These organic extracts were combined, dried over anhydrous sodium sulfate, and condensed under rotary vacuum to a residue containing primarily nonalkaloidal material (Fraction B).

Following basification of the aqueous solution to pH 8.5 with sodium hydroxide, the solution was extracted with 3 l. each of chloroform and ether (in 1-l. portions). Repeated extractions at pH 9.5 and 10.5 resulted in combined extracts totaling 18 l., which were condensed with the flash and rotary vacuum evaporators to a volume of 5 ml. containing the bulk of the alkaloids (Fraction A).

Traces of organic solvents were removed from the aqueous solution by placing it under rotary vacuum evaporation at 40° for 30 min.; then it was freeze dried. The remaining salt residue was extracted with portions of 10% ethanol in chloroform until the eluates were colorless. The combined eluates were filtered and condensed under rotary vacuum evaporation to yield the water-soluble alkaloids (Fraction C).

Analysis of crude alkaloid Fractions A, B, and C with analytical TLC detected dolichotheline and four unknown alkaloids in Fractions A and C; Fraction B contained traces of dolichotheline mixed with nonalkaloidal material. Fractions A and C were combined, and the resulting ethanolic solution was separated into phenolic and nonphenolic portions using a basic ion-exchange column[4] as previously described (12).

TLC—Previously utilized TLC methods were used analytically to provide a tentative identification of alkaloids in the extracts and preparatively to separate the components (12–15). Analytical TLC plates were purchased[5], and preparative plates were prepared with a 1-mm. layer of silica gel PF_{254}[6].

Analytical separations were achieved by the use of the following solvent systems: Solvent A, ethyl acetate–methanol–28% ammonium hydroxide (17:2:1); Solvent B, chloroform–ethanol–28% ammonium hydroxide (15:20:1); Solvent C, chloroform–methanol–28% ammonium hydroxide (18:1:1); Solvent D, chloroform–acetone–diethylamine (5:4:1); Solvent E, ether–acetone–methanol–28% ammonium hydroxide (9:8:2:1); Solvent F, ether–methanol–28% ammonium hydroxide (17:2:1); Solvent G, chloroform–acetone–28% ammonium hydroxide (10:8:1); and Solvent H, methyl ethyl ketone–dimethylformamide–28% ammonium hydroxide (80:12:1).

Preparative separations were achieved using Solvent F with repeated developments for maximal separations as monitored under shortwave UV light. Analytically, the alkaloids were detected using sprays of dansyl chloride (12), tetrazotized benzidine (12), and a modified Pauly's reagent to react with imidazoles (16).

Resolution of Nonphenolic Alkaloids—Analytical TLC revealed that this fraction contained dolichotheline, a major unknown al-

[1] From The Cactus Gardens, Edinburg, Tex.; Davis Cactus Gardens, Kerrville, Tex.; and The Texas Cactus Ranch, Corpus Christi, Tex.

[2] A few characteristic plants were freeze dried and sent to Dr. Edward F. Anderson, Whitman College, Walla Walla, Wash., for confirmation of the identification. Living plants are being maintained as reference specimens in the greenhouse of the School of Pharmacy and Pharmacal Sciences, Purdue University.

[3] Ace all-glass circulating evaporator.

[4] Amberlite IRA 401S C.P. anion-exchange resin, Mallinckrodt Chemical Works.

[5] Baker-flex silica gel type IBF-2 and Brinkmann Silplate-52 glass plates.

[6] Brinkmann Instruments, Inc.

kaloid which fluoresced with dansyl chloride and produced a white chromophore with tetrazotized benzidine, and traces of five possible imidazoles that produced red chromophores with Pauly's reagent and gave R_f values in Solvent F at 0.29, 0.34, 0.43, 0.48, and 0.55. Ten milliliters from a 30-ml. ethanolic solution containing the total nonphenolic extract was condensed to a residue and, after four crystallizations from acetone–benzene (1:1), yielded a total of 1.338 g. of dolichotheline, melting point and mixed melting point 129–131° [lit. (1) m.p. 130–131°].

The mother liquor remaining from the dolichotheline crystallization was resolved *via* preparative TLC on 43 plates (20 × 20 cm.). The major unknown was eluted from the combined similar bands with ammoniacal ethanol (15). Analytical TLC of the condensed filtrates indicated chromatographic purity, and the hydrochloride was prepared by acidifying with anhydrous hydrogen chloride in absolute ethanol (5% w/w) and inducing crystallization by the dropwise addition of ether (yield 92.5 mg.).

Identification of Unknown Nonphenolic Alkaloid—A lime-green fluorescent dansyl conjugate of this alkaloid indicated a primary or secondary amine function (12). The IR spectrum[7] of the hydrochloride showed major bands at ν_{max}^{KBr} 2940, 2760 (broad, aromatic, and aliphatic C—H and salts), 1600, 1501, 1480 (aromatic C=C), 1315, 1080, 1055, 1020, 945, 890, 780, 740, and 690 cm.$^{-1}$. NMR spectra[8] showed peaks in deuterochloroform at δ 2.7 (singlet, 3H, CH_3—N), 3.2 (singlet, 4H, CH_2—CH_2), 7.3 (singlet, 5H, aromatic), and 9.8 (singlet, 1H, N—H). The low-resolution mass spectrum[9] showed a parent ion peak at *m/e* 135 (0.2%, $C_9H_{13}N$) and major fragmentation peaks at 91 (16.6%, C_7H_7), 65 (14.8%, C_5H_5), 44 (100%, CH_2NHCH_3), and 28 (14.2%, C_2H_4) consistent with those expected for phenethylamines.

Interpretation of these data suggested that the unknown alkaloid was simply *N*-methylphenethylamine hydrochloride. A reference sample of *N*-methylphenethylamine hydrochloride was synthesized from phenethylamine (17). Spectral data (IR, NMR, and mass spectrometry) obtained with the synthetic compound were essentially identical to those obtained with the isolated alkaloid hydrochloride. The melting points[10] and mixed melting points of the isolated and reference *N*-methylphenethylamine hydrochloride were the same (164–165°). Cochromatography of the isolate with the reference in five TLC systems (A, B, C, F, and G) substantiated this identification.

Resolution of Phenolic Alkaloids—Analytical TLC of this fraction revealed the presence of three major alkaloids and one minor alkaloid. Nine milliliters of a 26-ml. (total) ethanolic solution of this fraction was resolved *via* preparative TLC on 64 plates (20 × 20 cm.). The bands, after detection under UV light or by spraying the edge of the plate with tetrazotized benzidine, were removed and eluted. Further TLC analysis of the condensed eluates allowed a tentative identification of three alkaloids as *N*-methyltyramine, *β-O*-methylsynephrine, and synephrine. Crystalline hydrochlorides for the first two were then prepared as described previously.

Identification of Phenolic Alkaloids—Cochromatography with reference racemic *β-O*-methylsynephrine hydrochloride in five solvent systems (A, C, F, G, and H) tentatively identified this compound as one of the crystallized phenolic alkaloids. Melting points and mixed melting points were similar (186–189°). IR spectral data were comparable between the isolated and reference racemic *β-O*-methylsynephrine hydrochloride; however, slight shifts and different intensities of a few bands in the IR spectrum of the isolate when compared to that of the racemic reference suggested that the isolate was likely (−)-*β-O*-methylsynephrine hydrochloride (15, 18). Determination of the optical rotation was not attempted due to the low yield (13.5 mg.). The NMR spectra of the two compounds in deuterated methanol compared identically, with peaks at δ 2.6 (singlet, 3H, CH_3—N), 3.2 (multiplet, 5H, containing a singlet, 3H, CH_3—O, and a doublet, 2H, CH_2—C—H), 4.3 (weak multiplet, 1H, benzylic), and 7.0 (quartet, 4H, *para*-substituted aromatic). Mass spectral analysis showed parent ion peaks at *m/e* 181 and the expected fragmentation.

Reference *N*-methyltyramine hydrochloride and the second crystalline hydrochloride consistently cochromatographed (Solvents A, B, C, F, and G). The melting point of the isolate (146–148°) and mixed melting point (147–150°) with reference *N*-methyltyramine hydrochloride proved to be similar. The IR spectra were essentially identical, and both showed bands at ν_{max}^{KBr} 3260 (phenolic OH), 2890, 2810 (broad, aromatic and aliphatic C—H, secondary amine salt), 1620, 1600, 1530 (aromatic C=C), 1220 (C—O), and 820 cm.$^{-1}$. The NMR spectra showed peaks in deuterium oxide at δ 2.8 (singlet, 3H, N—CH_3), 3.1 (multiplet, 4H, containing two overlapping triplets, CH_2—CH_2), and 7.1 (quartet, 4H, *para*-substituted aromatic). Mass spectral analysis of the isolate showed a parent ion peak at *m/e* 151 and major fragmentation peaks corresponding to the fragmentation observed with reference *N*-methyltyramine hydrochloride.

The eluates containing synephrine were further separated *via* preparative TLC on eight plates (20 × 20 cm.) to yield synephrine and an unknown alkaloid which cochromatographed with the unknown minor phenolic alkaloid previously mentioned. These latter eluates were combined, and the hydrochlorides of these compounds were then successfully crystallized.

Cochromatography of the isolated synephrine hydrochloride and reference synephrine hydrochloride in Solvents A, B, C, F, and G substantiated the identification of the isolate as synephrine. Melting points and mixed melting points of the isolate and reference racemic synephrine hydrochloride were similar (153–155°). The IR spectra of the isolate and reference racemic material were similar but exhibited some differences (15, 18); however, the IR spectrum of the isolate and that of natural (−)-synephrine hydrochloride (15) were essentially identical, and the isolate is assumed to be (−)-synephrine hydrochloride. The NMR spectrum in deuterium oxide showed peaks at δ 2.6 (singlet, 3H, N—CH_3), 3.2 (doublet, 2H, CH_2—C—H), 4.9 (multiplet overlapping D_2O peak, 1H, benzylic), and 7.1 (quartet, 4H, *para*-substituted aromatic). Mass spectral analysis showed a parent ion peak at *m/e* 167 and major fragmentation peaks consistent with those observed for reference synephrine hydrochloride.

The IR spectrum of the unknown phenolic alkaloid showed bands at ν_{max}^{KBr} 3240, 2980 (broad, aromatic and aliphatic C—H, secondary amine salts), 1620, 1610, 1600, (aromatic C=C), 1300, and 1110 (ether, aryl C—O) cm.$^{-1}$. Due to the small amount of material isolated (8.8 mg.), an NMR spectrum was not obtained. Analysis of high-[11] and low-resolution mass spectral data allowed the assignment of *m/e* 195 (2.3%, $C_{11}H_{17}NO_2$) as the parent ion. The major fragmentation peaks that aided in the structural assignment were at 151 (100%, $C_9H_{11}O_2$), 150 (18.6%, $C_9H_{12}NO$), 123 (82.5%, $C_7H_7O_2$), and 77 (24.4%, C_6H_5). The UV spectrum[12] showed two peaks at 225.5 and 275 nm., with $E_{1\,cm.}^{1\%}$ values equal to 450 and 75, respectively; in base these peaks showed a bathochromic shift typical of *para*-hydroxyphenols. Interpretation of these data suggested that the unknown was *β-O*-ethylsynephrine.

Synthesis of Reference *β-O*-Ethylsynephrine—A modification of the procedure of Stewart and Wheaton (18) was used. One gram of synephrine base[13] was added to an excess (15 g.) of thionyl chloride under nitrogen. After a few minutes, the precipitate was washed with benzene and again with ether. Ten milliliters of ethanol was added, and the mixture was refluxed in a water bath for 1 hr. The resulting solution was condensed to a small volume, and the addition of acetone induced crystallization. Treatment with activated charcoal and recrystallization from ethanol and ether yielded 512 mg. (51% yield) of synthetic (±)-*β-O*-ethylsynephrine hydrochloride (m.p. 169–171°). IR, NMR, and mass spectral data identified the material as the desired synthetic product.

Identification of Unknown Phenolic Alkaloid as *β-O*-Ethylsynephrine—TLC comparison of the synthetic *β-O*-ethylsynephrine hydrochloride with the isolated unknown alkaloid resulted in cochromatography (Solvent F) and identical chromophore formation with tetrazotized benzidine. Melting points of the isolated alkaloid (167–169°) and mixed melting points (170–173°) further supported this identification. IR and mass spectral data were likewise confirmatory.

To substantiate the natural occurrence of this compound in the plant, a new extraction of a 20-g. sample of *D. sphaerica* was made using methanol in place of ethanol and deleting ether in the original

[7] Beckman 33 IR spectrophotometer.
[8] Jeol MH-II NMR spectrometer using a microtube.
[9] Hitachi RMU-6A mass spectrometer.
[10] Determined with a Mel-Temp apparatus, corrected.
[11] Obtained on a CEC 21-110 at 70 ev. at Hoffmann-La Roche, Inc.
[12] Cary model 17 UV spectrophotometer.
[13] Sigma Chemical Co.

Table I—Quantities of Alkaloids Isolated from *D. sphaerica*

Alkaloid	Yield, mg.	Concentration in Cactus, % w/w
Dolichotheline	1338.0	0.6500
N-Methylphenethylamine hydrochloride	92.5	0.0411
β-*O*-Methylsynephrine hydrochloride	13.5	0.0060
N-Methyltyramine hydrochloride	26.0	0.0115
Synephrine hydrochloride	7.5	0.0033
β-*O*-Ethylsynephrine	8.8	0.0038

extraction procedure. Following this new extraction, analytical TLC (Solvent F) of Fractions A and C showed the complete absence of this alkaloid in the extracts. Apparently, since several steps in the usual extraction procedure utilize acid and ethanol, there was an ethylation of some natural synephrine to produce β-*O*-ethylsynephrine as an artifact.

DISCUSSION

As summarized in Table I, six alkaloids were isolated as their hydrochlorides from alkaloid extracts of *D. sphaerica*. Identifications of the compounds were achieved by comparing data (IR, NMR, and mass spectral), TLC behavior, and melting points with those of reference alkaloids. The isolated alkaloids were dolichotheline, as expected from previous reports (1, 2), *N*-methylphenethylamine hydrochloride, *N*-methyltyramine hydrochloride, synephrine hydrochloride, β-*O*-methylsynephrine hydrochloride, and β-*O*-ethylsynephrine hydrochloride. Since ethyl groups are rare in nature, reextraction of additional plant material, taking care to eliminate any contact with ethyl-containing compounds, was performed; TLC analysis then failed to detect β-*O*-ethylsynephrine in the new extracts. Apparently, this compound was isolated as an extraction artifact of synephrine; similar *O*-alkylation of synephrine while extracting it from tangerine leaves was reported (18).

N-Methylphenethylamine, the major new alkaloid isolated, has never before been isolated from the Cactaceae. This compound was first isolated in 1939 from *Arthrophytum leptocladum* Popov in the Chenopodiaceae (19, 20) and has since been isolated from several *Acacia* species (Leguminosae) (21–26). Of particular interest is its isolation from *Acacia berlandieri* Benth. as a major toxic constituent (26); goats and sheep in west Texas eat this shrub in large amounts during prolonged drought and suffer from a condition known as "Limberleg" or "Guajillo Wobbles." However, the acute action of *N*-methylphenethylamine as a pressor agent has been shown to occur experimentally with low toxicity (27, 28). Logical biosynthetic precursors for this alkaloid would be phenylalanine and methionine, following a similar two-step pathway as in the formation of *N*-methyltyramine from tyrosine in *Hordeum* (29).

N-Methyltyramine was first isolated from *Hordeum* in 1950 (17) and has previously been identified in several cactus species as well as other plant families (12, 13, 15, 30–33). It possesses weak sympathomimetic activity (34, 35).

β-*O*-Methylsynephrine has been isolated twice previously from the Cactaceae (15, 36) and from citrus leaf extracts as an artifact of synephrine (18). Comparison of IR spectra of the isolated and racemic reference β-*O*-methylsynephrine hydrochloride suggested that the isolate is the (−)-isomer. If this compound were made from synephrine during the extraction procedure, the racemic mixture would have been isolated. No reported studies concerning the pharmacological action of β-*O*-methylsynephrine were found; however, a similar compound, β-*O*-methylepinephrine, produces considerable central nervous system stimulation (37).

Synephrine was previously isolated and detected in *Citrus* (38), and it was detected in three other plant families (33) as well as in human urine (39). Recently, it was detected and isolated in several cacti (15, 36, 40, 41). Synephrine is a well-known sympathomimetic agent (42).

While the pharmacological action of the most abundant alkaloid, dolichotheline, remains unknown, the presence of these sympathomimetic β-phenethylamine alkaloids confers explainable physiological activity on this cactus and, perhaps, helps to explain its folkloric connections with *Lophophora*.

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Kinetics and Mechanisms of Drug Action on Microorganisms XVII: Bactericidal Effects of Penicillin, Kanamycin, and Rifampin with and without Organism Pretreatment with Bacteriostatic Chloramphenicol, Tetracycline, and Novobiocin

EDWARD R. GARRETT▲ and CHONG MIN WON

Abstract □ *Escherichia coli* (ATCC 12407), generating in the logarithmic growth phase, was killed by penicillin, kanamycin, and rifampin. The logarithm of number of viables of the drug-treated culture decreased linearly with time after a certain lag period and above a minimum drug concentration. The slopes of these plots were characteristic of kill rate constants and were linearly dependent on drug concentrations. The microorganisms developed resistance against the cidal action of the antibiotics. The lower the penicillin concentration and the rate of kill, the greater is the number of resistant organisms, most probably formed by generation in the presence of penicillin. Penicillin was inactivated with time in the culture of organisms, and the resistant individuals eventually grew and were shown to be insensitive to penicillin. When organisms were exposed to the cidal action of kanamycin or rifampin, the appearance of resistant individuals was ascertained. Penicillin yielded debris and ghost cells of *E. coli*. No such apparent lysis was observed with kanamycin or rifampin. The activity of kanamycin increased with the pH of the medium, so activity can be assigned to the uncharged or lesser charged fraction of the drug concentration. The addition of penicillin or kanamycin to the organisms treated with tetracycline, chloramphenicol, or novobiocin did not show any significant difference in killing rate from organisms not previously treated with such bacteriostatic agents. However, the combination of a bactericidal and a bacteriostatic antibiotic depressed the resistant mutant formation of resistant individuals over that of the bactericidal drug alone. When rifampin was added to organisms pretreated with tetracycline or chloramphenicol, the cidal action of rifampin was significantly reduced.

Keyphrases □ Bactericidal activity, kinetics and mechanisms—penicillin, kanamycin, and rifampin with and without *E. coli* pretreatment with chloramphenicol, tetracycline, or novobiocin □ Antibiotics, kinetics and mechanisms of bactericidal activity—penicillin, kanamycin, and rifampin with and without *E. coli* pretreatment with chloramphenicol, tetracycline, or novobiocin □ Bacteriostatic *E. coli* pretreatment with chloramphenicol, tetracycline, or novobiocin—effect on bactericidal activity of penicillin, kanamycin, and rifampin

Antibacterial agents have two possible modes of action: inhibition of microbial generation and/or "kill" superimposed on generation. They can be differentiated by concomitantly monitoring the microorganism concentration of the nutrient medium containing the agent by viable (colony) counts and total (Coulter) counts as a function of time. It was shown (1) that the total counts and colony counts are coincident for the bacteriostatic tetracyclines, chloramphenicol, sulfonamides, macrolides, lincosaminides, *etc.*, in the concentration ranges below the amount resulting in complete inhibition of microbial generation.

The bactericidal action of drugs in defined concentration ranges must be kinetically defined by the time-consuming and laborious colony count method. This article reports on studies designed to quantify systematically the effects on microbial generation of several bactericidal antibiotics: penicillin, kanamycin, and rifampin.

The primary action of penicillin is in the inhibition of the production of cell wall material, specifically in the biosynthesis of murein (2). Presumably by interacting with the acceptor site of the ribosome, kanamycin may cause significant conformational changes in ribosomes which induce ambiguity in the readout of mRNA. (2). Rifampin was shown (2) to inhibit DNA-dependent RNA synthesis by specifically interfering with the function of the RNA polymerase by forming a stable rifampin–polymerase complex.

EXPERIMENTAL

Microorganism—Replicate slants of *Escherichia coli* (ATCC 12407) were used in all experiments.

Culture Media—Bacto Antibiotic Medium 3[1], rehydrated (pH 7.05) according to the specifications of the manufacturer to Medium 3 USP, was used. The culture medium was filtered through a 0.45-μ filter[2] and autoclaved at 120° for 15 min. To obtain media in the pH range of 6.30–7.55, various amounts of concentrated hydrochloric acid and sodium hydroxide solution were added aseptically to the culture media.

Antibiotic—The assayed samples of sodium penicillin G[3] (1625

[1] Difco Laboratories Detroit, Mich.
[2] Millipore HA.
[3] Obtained from The Upjohn Co., Kalamazoo, Mich.

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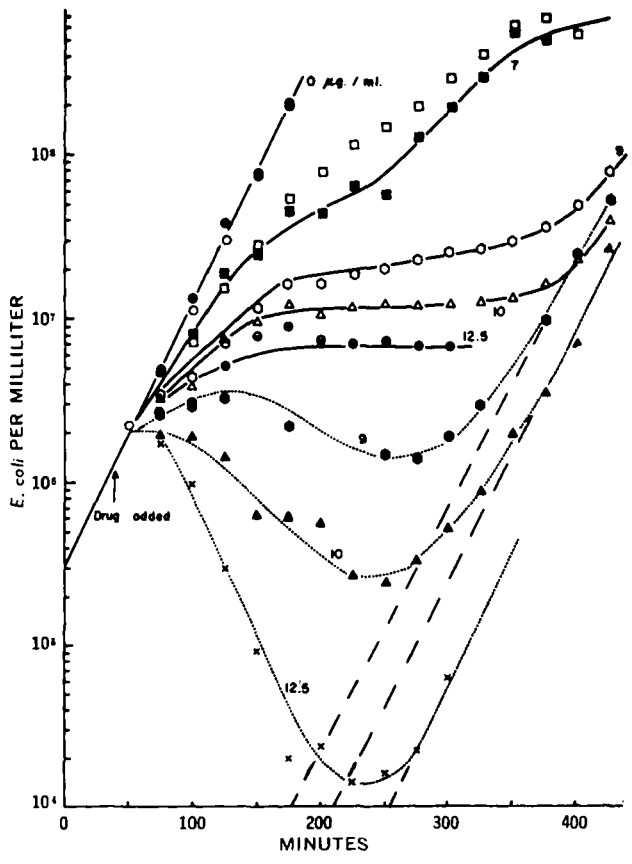


Figure 1—*Typical generation curves of* E. coli *in Medium 3 USP at pH 7.05 and 37.5° in the absence and presence of various concentrations of sodium penicillin G, obtained by total (solid lines) and viable (dashed lines) counts. The curves are labeled with the sodium penicillin G concentration in micrograms per milliliter. The short dashed lines represent the logarithmic growth of the emergent resistant organisms.*

units/mg.), kanamycin sulfate[4] (795 mcg./mg.), and rifampin[5] (981 mcg./ml.) were used.

Bacterial Culture—An aliquot (5 ml.) of culture medium was inoculated from a fresh slant, and the culture was allowed to generate for 15 hr. at 37.5° in an incubator. An aliquot (0.5 ml.) was then diluted into 49.5 ml. of fresh culture medium. The generation of the culture was followed up to 2×10^7 *E. coli*/ml. An aliquot of this culture was added to bulk broth medium so that the organism population was diluted 100-fold. The inoculated bulk solution was shaken and kept in an incubator at 37.5° for 15 min. Aliquots (49.5 ml.) of the inoculated broth medium were then aseptically transferred into replicate, 125-ml., loosely capped conical flasks through a delivery head. The temperature of the cultures was maintained at 37.5 ± 0.01° in a constant-temperature water bath equipped with a shaker.

Total Count Method—Aliquots (1.00 or 0.50 ml.) of cultures were withdrawn at 25-min. intervals and added to an appropriate amount of diluent. The diluent was a 0.45-µ filtered[2] aqueous solution of 0.85% sodium chloride and 1% formaldehyde. This number of organisms in a 50-µl. sample was counted on the Coulter counter[6] equipped with a 30-µ orifice. The total counts were corrected for the background counts of the particular batch of medium used, which was diluted in the same way as the sample. The coincidence corrections were made by a program on a calculator[7] in accordance with the coincidence correction chart supplied by the manufacturer's manual for the Coulter counter.

Viable Count Method—Aliquots (0.50 ml.) of the drug-affected culture were withdrawn and appropriately diluted into sterilized 0.85% saline solution according to a preplanned dilution scheme. Aliquots (1.00 ml.) from those dilutions were pipeted onto each of three replicate agar plates. The plates were incubated for 48 hr. at 37.5°, and colonies were counted on a colony counter[8].

Effect of Bactericidal Antibiotics on Generation Rates—Fresh solutions of sodium penicillin G, kanamycin sulfate, and rifampin were aseptically prepared for each experiment. They were sufficiently diluted so that aliquots of 0.50 ml. added to 49.5 ml. culture yielded the desired graded drug concentrations. The antibiotic solutions were added to the cultures generating at 37.5° in the logarithmic phase at predetermined organism populations. Samples were withdrawn every 25 min. and counted by the total count method as well as by the viable method. One culture without drug was studied in each experiment as a control. The plots of the logarithm of the total and viable numbers of *E. coli* per milliliter against time yielded generation curves for penicillin-, kanamycin-, and rifampin-affected cultures (Figs. 1–3).

Irreversibility of Kanamycin Action—Aliquots (5.0 and 0.5 ml.) of a drug-free culture generating in the logarithmic phase with an inoculum size of 1.7×10^7 *E. coli*/ml. (curve A in Fig. 4) were added to 45.0 and 49.5 ml. of fresh broth so that the organism population was diluted 10-fold (curve B) and 100-fold (curve C), respectively. A 49.5-ml. volume of broth containing 2.2×10^6 *E. coli*/ml. in the logarithmic generation phase was treated with 0.5 ml. of kanamycin sulfate solution to achieve a final concentration of 5 mcg./ml. (curve D). Twenty-five minutes after the drug addition, aliquots of curve D culture were diluted 10-fold into broth containing enough kanamycin sulfate so that the drug concentrations were adjusted to 1.7 (curve E), 2.2, and 3.3 mcg./ml. At 205 min., after the total counts of the culture of curve D reached a plateau of 1.5×10^7 *E. coli*/ml., aliquots of 5.0 and 0.5 ml. were added to 45.0 and 49.5 ml. fresh broth so that both organism and drug concentrations were diluted 10-fold (curve E) and 100-fold (curve F), respectively. At the same time, aliquots of the culture of curve D were diluted 10-fold (curve E) and 100-fold (curve F) in broths containing enough kanamycin sulfate so that the drug concentration was restored to 5 mcg./ml.

Cell Size Analyses—The size distribution of cells of *E. coli* in the

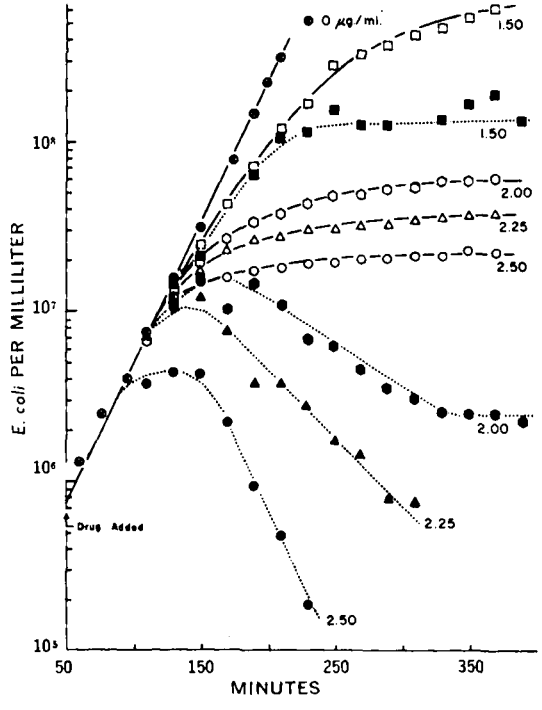


Figure 2—*Typical generation curves of* E. coli *in Medium 3 USP at pH 7.05 and 37.5° in the absence and presence of various concentrations of kanamycin sulfate, obtained by total (solid lines) and viable (dashed lines) counts. The curves are labeled with the kanamycin sulfate concentration in micrograms per milliliter.*

[4] Obtained from Bristol Laboratories, Syracuse, N. Y.
[5] Obtained from Ciba-Geigy Corp., Summit, N. J.
[6] Model ZBI, Coulter Electronics, Inc., Hialeah, Fla.
[7] Wang 700.
[8] Model C-110, New Brunswick Scientific Co., New Brunswick, N. J.

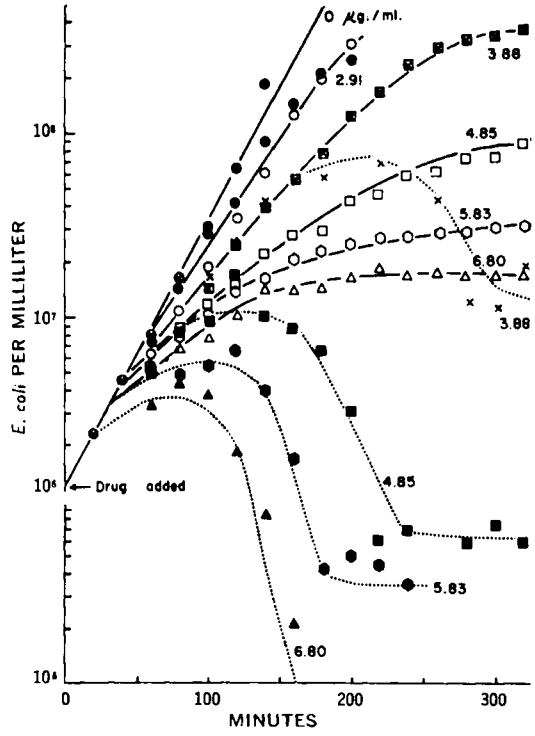


Figure 3—*Typical generation curves of* E. coli *in Medium 3 USP at pH 7.05 and 37.5° in the absence and presence of various concentrations of rifampin, obtained by total (solid lines) and viable (dashed lines) counts. The curves are labeled with the rifampin concentration in micrograms per milliliter.*

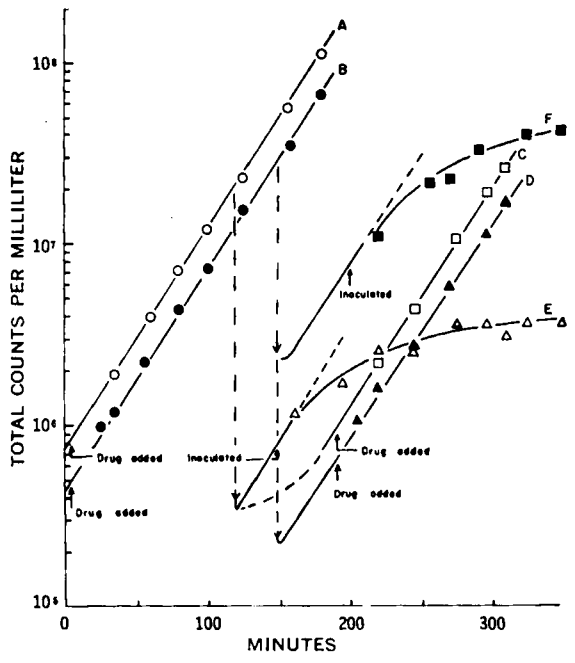


Figure 5—*Proof of development of rifampin- and kanamycin-resistant* E. coli. *Curves A and B are the generation curves for the cultures of rifampin- and kanamycin-resistant* E. coli *treated with 8.7 and 4.0 mcg./ml. rifampin and kanamycin, respectively. Curves C and D result when the filtered organisms of the cultures of curves A and B, in fresh medium, are treated with 8.7 and 4.0 mcg./ml. rifampin and kanamycin, respectively. Curves E and F result when the filtrates of the cultures of curves A and B, respectively, are inoculated with fresh* E. coli.

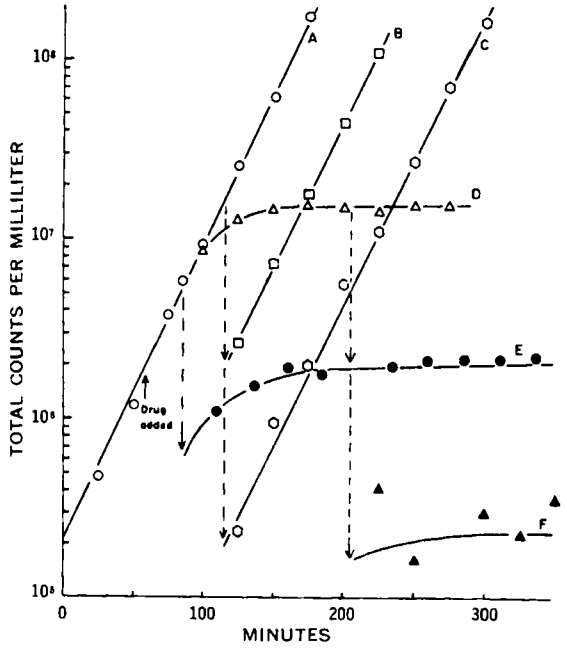


Figure 4—*Irreversibility of kanamycin sulfate-inhibited generation of* E. coli. *Curve A is for the drug-free culture. Curves B and C result after 1:10 and 1:100 dilutions, respectively, of the curve A culture with fresh broth. Curve D results after the addition of kanamycin sulfate to achieve a final concentration of 5 mcg./ml. Curve E results after the 1:10 dilution of curve D at 25 min. with broth containing kanamycin sulfate at a final concentration of 1.7 mcg./ml. Curve E also results after 1:10 dilution of curve D at 205 min. with final concentrations of 0.5 and 5 mcg./ml. of kanamycin sulfate. Curve F results after 1:100 dilution of curve D and with final concentrations of 0.05 and 5 mcg./ml. of kanamycin sulfate.*

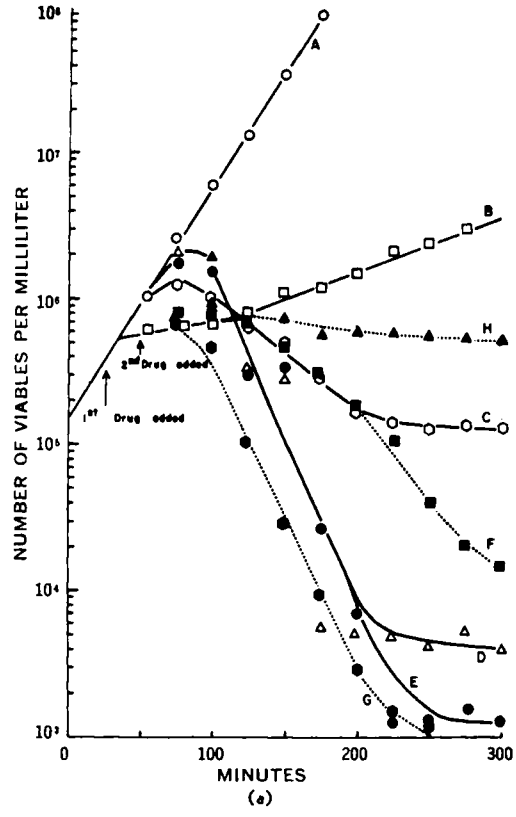
presence or absence of penicillin, kanamycin, and rifampin was studied using the Coulter counter in conjunction with a Channelyzer and plotter[9]. The instruments were calibrated with polyvinyl toluene latex beads of 1.305-μ mean diameter. Replicate 49.5-ml. samples of culture generating in the logarithmic phase were treated with penicillin, kanamycin, and rifampin stock solutions to achieve final concentrations of 24.0, 4.0, and 8.7 mcg./ml., respectively. Samples were taken every 25 min., and cell size distribution was obtained. A culture generated in the absence of drug was studied as a control.

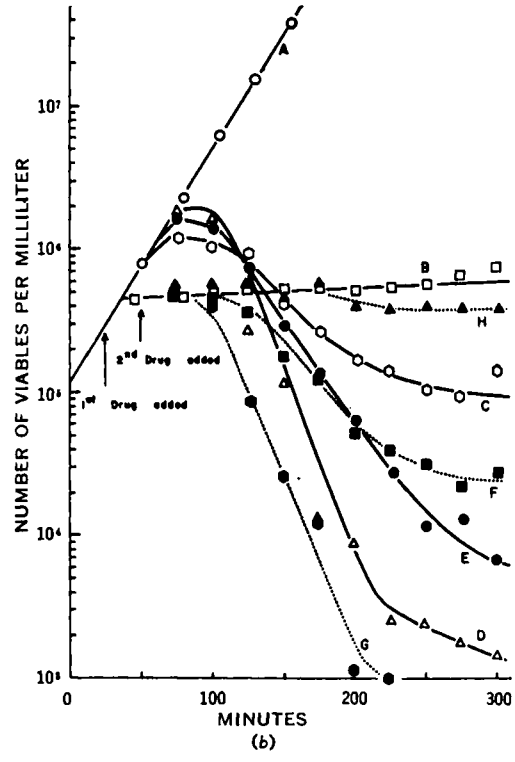
Bacterial Resistance to Penicillin Action—Aliquots (0.5 ml.) of sodium penicillin G solutions were added to cultures generating in the logarithmic phase to achieve final concentrations of 7.0 (Culture A), 9.0 (Culture B), and 12.5 (Culture C) mcg./ml. The drug-affected cultures of A, B, and C were allowed to generate for 15 hr. at 37.5° in an incubator. An aliquot (0.5 ml.) was then diluted into 49.5 ml. of fresh culture medium, brought up to the logarithmic growth phase, and diluted 100-fold again with fresh medium so that the original penicillin concentrations were diluted 10,000-fold. (See section on *Bacterial Culture.*) Aliquots of this solution were treated with various amounts of penicillin. The microorganisms generated from Culture A were treated with drug concentrations of 7.0, 10.0, 20.0, 39.6, and 77.7 mcg./ml.; those generated from Culture B were treated with 9.0, 20.0, 39.6, 77.7, and 114 mcg./ml.; and those generated from Culture C were treated with 12.5, 20.0, 39.6, 77.7, and 114 mcg./ml. Coulter counts were obtained every 25 min. for all drug-affected generations. The microorganisms generated from Culture C and treated with 12.5 mcg./ml. of sodium penicillin G were aseptically filtered 55 min. after the drug addition. The filtrate was inoculated with fresh organisms in the logarithmic generation phase.

A fresh broth was inoculated with the filtered organisms and treated with fresh drug solution to give a final concentration of 12.5 mcg./ml. of sodium penicillin G.

Bacterial Resistance to Kanamycin and Rifampin—Aliquots (0.5 ml.) of kanamycin and rifampin solutions were added to cul-

[9] Coulter Electronics, Inc.





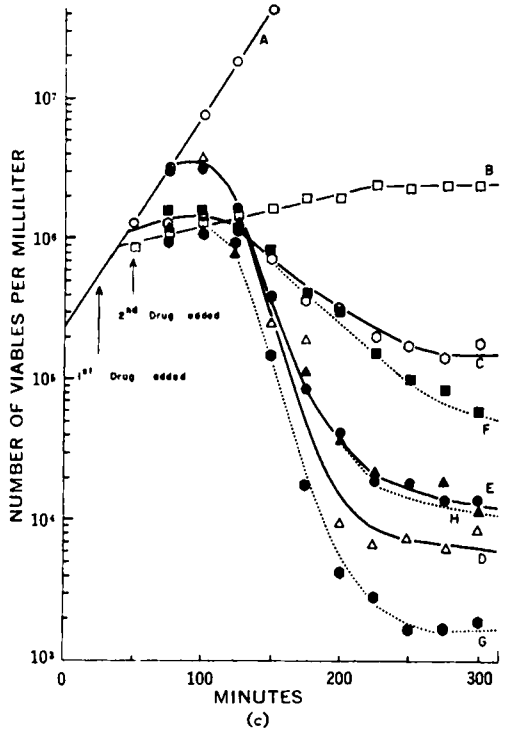


Figure 6—*Effects of combined action of penicillin, kanamycin, and rifampin with tetracycline* (a), *chloramphenicol* (b), *and novobiocin* (c) *on generation rates of* E. coli. *Curve A results from the generation of the culture in the absence of drug. Curve C results from the generation of the culture in the presence of 10.0 mcg./ml. of sodium penicillin G, curve D for 2.50 mcg./ml. of kanamycin sulfate, and curve E for 6.00 mcg./ml. of rifampin.* (a) *Curve B results from the generation of the culture in the presence of 0.30 mcg./ml. of tetracycline hydrochloride. Curve F results when sodium penicillin G (10.0 mcg./ml.) is added to the tetracycline-affected culture of curve B. Curve G results when kanamycin sulfate (2.50 mcg./ml.) is added to the culture of curve B, and curve H results when rifampin (6.00 mcg./ml.) is added to the culture of curve B.* (b) *Curve B results from the generation of culture in the presence of 1.90 mcg./ml. of chloramphenicol. Curve F results when sodium penicillin G (10.0 mcg./ml.) is added to the chloramphenicol-affected culture of curve B. Curve G results when kanamycin sulfate (2.50 mcg./ml.) is added to the culture of curve B, and curve H results when rifampin (6.00 mcg./ml.) is added to the culture of curve B.* (c) *Curve B results from the generation of culture in the presence of 90.0 mcg./ml. of novobiocin. Curve F results when sodium penicillin G (10.0 mcg./ml.) is added to the novobiocin-affected culture of curve B. Curve G results when kanamycin sulfate (2.50 mcg./ml.) is added to the culture of curve B, and curve H results when rifampin (6.00 mcg./ml.) is added to the culture of curve B.*

tures in the logarithmic growth phase to achieve final concentrations of 4.0 mcg./ml. of kanamycin and 8.7 mcg./ml. of rifampin. The drug-treated cultures were allowed to generate in an incubator for 15 hr. at 37.5°. An aliquot (0.5 ml.) was then diluted into 49.5 ml. of fresh culture medium, brought up to the logarithmic growth phase, and diluted 100-fold again so that the original drug concentrations were diluted 10,000-fold. (See section on *Bacterial Culture.*) Aliquots were treated with various amounts of the respective antibiotics.

The cultures of rifampin- and kanamycin-treated *E. coli* were again challenged with rifampin (curve A in Fig. 5) or kanamycin (curve B) to achieve final concentrations of 8.7 and 4.0 mcg./ml., respectively. One hundred fifteen minutes after rifampin addition and 145 min. after kanamycin addition, the cultures of curves A and B were filtered and the filtered organisms were allowed to generate in fresh broth medium. Aliquots of the cultures of the filtered organisms were diluted into fresh medium and treated with rifampin (curve C) or kanamycin (curve D) to give final concentrations of 8.7 and 4.0 mcg./ml., respectively. The filtrates of cultures of curves A and B were inoculated with aliquots of fresh *E. coli* generating in the logarithmic growth phase and gave curves E and F, respectively.

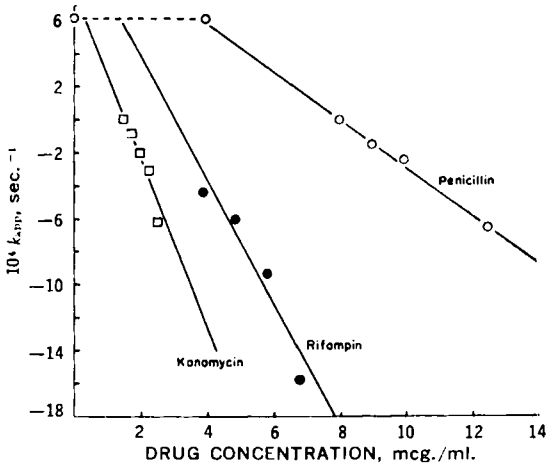


Figure 7—*Dependence of the apparent generation rate constant, k_{app}, for* E. coli *in Medium 3 USP on sodium penicillin G, kanamycin sulfate, and rifampin concentrations. The curves are constructed from two separate experiments.*

Effect of pH on Kanamycin-Affected Generation Rates—Six replicate 49.5-ml. volumes of medium, each buffered at a different pH value, were inoculated with an *E. coli* culture in the logarithmic generation phase. Drug solutions were added to the aliquots of these replicate cultures to achieve desired concentrations of kanamycin. Coulter counts were obtained for samples withdrawn every 25 min.

Potentiometric Determination of pKa Values of Kanamycin—Ninety-four milligrams of kanamycin sulfate was dissolved in 2.5 ml. of 0.2 *N* NaOH solution. A direct-reading pH meter[10] with a combination pH electrode[11] was used for pH measurements. The titrant was 1 *N* HCl solution and was delivered by a microburet[12] having a total capacity of 2 ml. and reading to 0.001 ml. on a microgauge. A magnetic stirrer kept the solution agitated. The procedure was repeated for a blank titration of a solution prepared like the sample solution with only the drug omitted.

Action of Penicillin, Kanamycin, and Rifampin in Combination with Bacteriostatic Antibiotics Tetracycline, Chloramphenicol, and Novobiocin—Replicate 49.5-ml. volumes of a culture containing 4×10^5 *E. coli*/ml. generating in the logarithmic phase (curve A in Fig. 6*a*) were treated with tetracycline (curve B), penicillin (curve C), kanamycin (curve D), and rifampin (curve E) to give final concentrations of 0.30 mcg./ml. of tetracycline hydrochloride, 10.0 mcg./ml. of sodium penicillin G, 2.50 mcg./ml. of kanamycin sulfate, and 6.00 mcg./ml. of rifampin, respectively. Twenty-five minutes after the addition of the first drug, replicate samples of the tetracycline-treated culture of curve B were treated with penicillin (curve F), kanamycin (curve G), or rifampin (curve H) to achieve final concentrations of 10.0 mcg./ml. of sodium penicillin G, 2.50 mcg./ml. of kanamycin sulfate, and 6.00 mcg./ml. of rifampin, respectively, with each containing the 0.30 mcg./ml. of tetracycline. Samples were taken every 25 min. Coulter counts were obtained for the drug-free culture (curve A) and tetracycline-treated culture (curve B), and viable counts were obtained for penicillin-, kanamycin-, or rifampin-treated cultures (curves C–H).

The experiment was reported for the combined action of penicillin, kanamycin, and rifampin with 1.90 mcg./ml. of chloramphenicol (Fig. 6*b*) and 90 mcg./ml. of novobiocin (Fig. 6*c*).

RESULTS AND DISCUSSION

Effects of Drugs on Microbial Generation—The numbers of *E. coli* as a function of time as affected by various concentrations of penicillin, kanamycin, and rifampin are plotted semilogarithmically in Figs. 1, 2, and 3, respectively. Certain general features of these curves are common to all three drugs. Subsequent to drug inoculation of the medium, the apparent generation rates of viables, as monitored by colony counts, decrease until the number of viables reach a maximum. These maxima are lower and occur faster with time the greater the drug concentration. Subsequent to these maxima and at the higher drug concentrations, the numbers of variables decrease and approach a first-order decay as manifested by a linear plot of the logarithm of viables against time. At the lowest concentration of drugs studied (7.0 mcg./ml. of sodium penicillin G, 1.5 mcg./ml. of kanamycin sulfate, and 3.88 mcg./ml. of rifampin), the generation rate was slowed until a relatively constant value of viables was achieved.

Subsequent to drug inoculation of the medium, the generation rate of total organisms, as monitored by Coulter counts, diminished and approached a constant value of plateau that persisted with time. These plateau values were lower with increasing concentrations of the drug.

The significant decreases in the numbers of *E. coli* per milliliter obtained by viable counts as a function of time compared to the total numbers obtained by the Coulter counter show that penicillin, kanamycin, and rifampin kill organisms at all of the studied concentrations (Figs. 1–3).

There were definite lag periods (Figs. 1–3) after drug addition before the slopes of the plots of logarithms of viable numbers against time decreased, and the effect was most pronounced with respect to kanamycin (Fig. 2). There were periods of slowed generation rates before the semilogarithmic plots decreased linearly with time. These intervals can be assigned to the time dependence of the drug partitioning process into the bacterial membrane, and their durations were apparent functions of drug concentrations.

The rate of loss of viables after these lag and induction periods may be expressed as the difference between the rate of generation and that of killing, where the latter is a function of drug concentration, *D* (1). If the kill rate is proportional to the first power of drug concentration:

$$dN/dt = (k_0 - k_D D)N \qquad \text{(Eq. 1}a\text{)}$$

$$dN/dt = k_{app}N \qquad \text{(Eq. 1}b\text{)}$$

where k_0 in the generation rate constant in the absence of drug, k_D is the kill rate constant, and N is the number of viable microorganisms. The apparent first-order generation rate constant, k_{app}, can be obtained from the slopes of the linear portions of semilogarithmic plots of number of viable counts against time in the drug-affected steady state prior to the appearance of possible resistant organisms, which may explain the terminal increase in microbial generation as manifested in Fig. 1.

The plot of k_{app} values against drug concentration demonstrates a linear dependence of k_{app} on drug concentration in accordance with the expectations of Eqs. 1*a* and 1*b* (Fig. 7). However, the actual function is:

$$k_{app} = k_0 - k_D(D - D^*) \qquad \text{(Eq. 2)}$$

A definite concentration of antibiotic must be exceeded before antibacterial activity is manifested. This minimum concentration, D^*, possibly may be assigned to the binding or removal of effective antibiotic concentration by the components of the medium (1). A maximum killing rate probably cannot be exceeded at very high concentrations of the antibiotics.

When the numbers of viables are negligible with respect to the numbers of dead organisms, the total numbers approach plateau values (Figs. 1–3). The plateau number, N_p, is some function of drug concentration and thus should be a function of the k_{app} value. Plots of k_{app} values against the logarithms of the ratio of the plateau number to the number at the time of drug addition, N_0, are shown in Fig. 8 for penicillin, kanamycin, and rifampin. Once the empirical relationship between $\log (N_p/N_0)$ and k_{app} is established, the k_{app} values can be estimated by Coulter counts only. This method has been applied to the studies of bacterial resistance to penicillin (Fig. 9) and of the effect of pH on kanamycin action (Fig. 10).

Effect of pH on Kanamycin Action—The k_{app} values may be estimated from the plateau numbers of total organisms from the correlations of Fig. 8. The estimated k_{app} values for cultures maintained at various pH values (6.30–7.50) are plotted against kanamycin sulfate concentrations in Fig. 10. The drug-free generation rate constants are independent of pH in this range (1), while the drug-affected apparent generation rate constants decrease with increasing pH at comparable drug concentrations. The slopes of the plots of Fig. 10 are representative of the killing constants, k_D, in Eq. 2 and

[10] Beckman Instruments, South Pasadena, Calif.
[11] Corning electrode, Sargent-Welch Scientific Co., Birmingham, Ala.
[12] Radiometer, Copenhagen, Denmark.

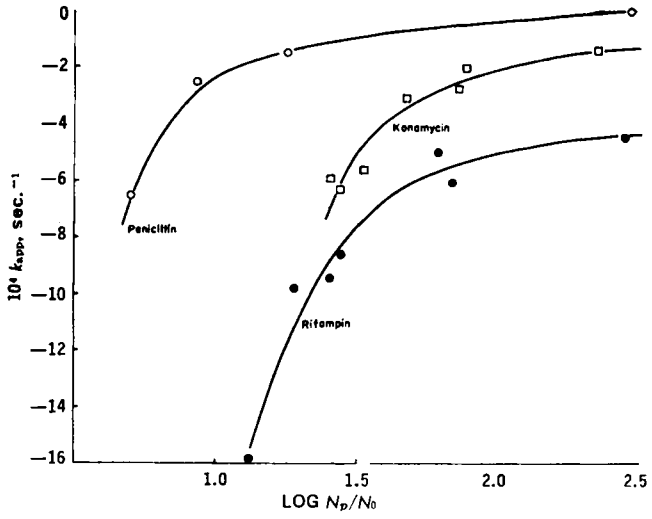


Figure 8—*Empirical relationship between the apparent generation rate constant, k_{app}, and the logarithm of the ratio of total counts at plateau (N_p) to the counts at the time of drug addition (N_0) for penicillin, kanamycin, and rifampin in Medium 3 USP.*

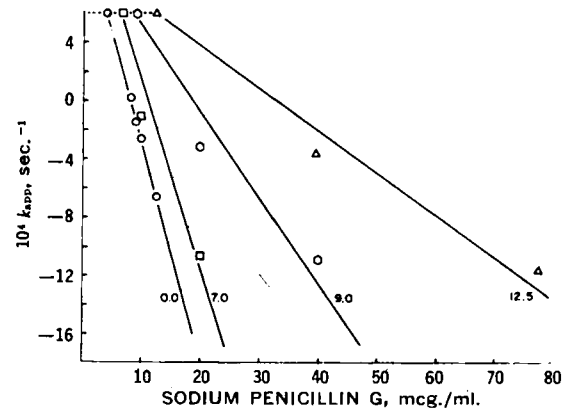


Figure 9—*Demonstration of the development of penicillin-resistant organisms on exposure to drug. Dependence of the apparent generation rate constant, k_{app}, for the generation of* E. coli *that has been exposed to penicillin on the sodium penicillin G concentration. The lines are labeled according to the sodium penicillin G concentrations, in micrograms per milliliter, to which the microorganisms were exposed for 15 hr. at 37.5° before these penicillin concentrations were diluted 10,000-fold and the concentrations of penicillin stated on the abscissa were added. The k_{app} values for viables were estimated from established correlations between rates of viable decay and plateau final values of total counts as given in Fig. 8.*

vary as a function of pH (Fig. 11). Potentiometric titration showed that kanamycin has four overlapping pKa values ranging from 6.5 to 9.7 (pKa_1 = 9.7, pKa_2 = 8.6, pKa_3 = 7.5, and pKa_4 = 6.5). The fact that the data in Fig. 12 are reasonably linear indicates that the less protonated or the unprotonated fraction of total kanamycin concentration is most probably the active species.

The minimum concentration, D^*, necessary for manifestation of antibacterial activity also decreases with increasing pH (Fig. 10) and implies that the inactivated species, possibly bound to components of the medium, is probably the more highly protonated.

Possible Development of Resistant Organisms to Drug Actions—Although there were indications from the terminal viable counts of the drug-affected organisms in the kanamycin and rifampin cases (Figs. 2 and 3) that some organisms remained that were resistant to the cidal action of these drugs but would not generate in their presence, it was clearly shown in the case of penicillin (Fig. 1) that there was either a population of organisms that did multiply in the presence of this drug or that the drug action had ceased after a time interval. This was manifested in the curves 200 min. after drug addition when the rate of decay of viables increased and eventually approached a typical first-order process of generation where the logarithmic number of viables was linear with time. The apparent parallelism of these terminal segments of the plots with that of a similar plot for *E. coli* generation in the absence of penicillin was indicative of negligible drug action.

Development of Penicillin-Resistant Organisms and Penicillin Inactivation—This phenomenon could be attributed to the consumption or degradation of the drugs, to the production of an inhibitor or inactivator of antibiotic action, and/or to the presence of bacteria able to resist the drug action. These bacteria could be present from the time of the microbial inoculation or could have arisen by mutation or adaptation processes occurring during the organisms' drug exposure. The cultures of *E. coli* were exposed to sodium penicillin G at concentrations of 7.0, 9.0, and 12.5 mcg./ml. for 15 hr., a time when the harvested organisms should be representative of a resistant strain if it exists (Fig. 1). When the original concentrations of penicillin were diluted 10,000-fold and the exposed organisms were challenged with the same concentrations of sodium penicillin G, they showed the same rates of generation as organisms in the absence of drug (Fig. 9). This is conclusive evidence that the subsequent increase in generation of viables of a culture exposed to penicillin was due to the presence of resistant organisms. The higher the drug concentration to which the organisms have been exposed, the greater is the drug concentration needed to kill organisms at the same rate (Fig. 9).

When the organisms that had been exposed to penicillin were filtered and used to inoculate fresh broth medium and were then challenged with penicillin, the generation rates of the filtered organisms were the same as the untreated *E. coli* in the absence of drug. When the filtrate was inoculated with naive organisms and challenged with penicillin, the generation rates were also the same as untreated *E. coli* in the absence of drug. These facts indicate that not only is the organism made resistant on exposure to penicillin but that the drug is degraded or inactivated in the culture. Thus, this drug degradation is a fast process and/or a penicillin antagonist is formed readily in the medium. The fact that penicillin is destroyed by β-lactamase produced by penicillin-resistant organisms (3) is consistent with this result.

The terminal data of the semilogarithmic plots of viables against time for various penicillin concentrations show an eventual increase in viables. The positive slopes of the plots of these terminal data appear to be parallel but have different intercepts (Fig. 1). Since the inoculum size for each study was the same, these penicillin-resistant organisms that manifest themselves are not the sole result of a small and constant fraction of the original inoculum that was penicillin resistant. Resistance must arise primarily during the time

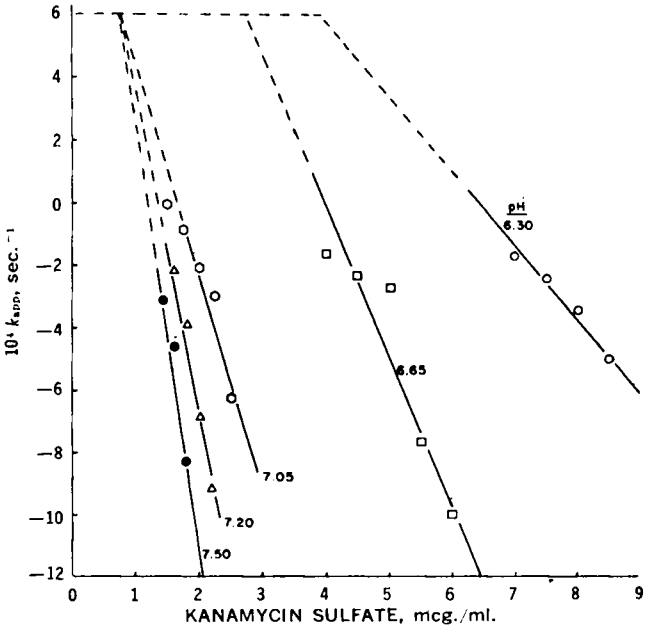


Figure 10—*Dependence of the apparent generation rate constant, k_{app}, for* E. coli *in Medium 3 USP on kanamycin sulfate concentration at various pH values.*

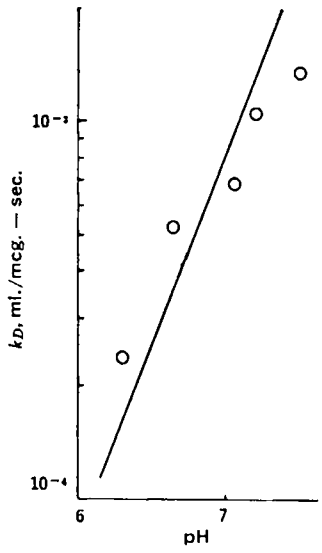


Figure 11—*Dependence of the killing constant, k_D, for the action of kanamycin on* E. coli *generation in Medium 3 USP on the pH value.*

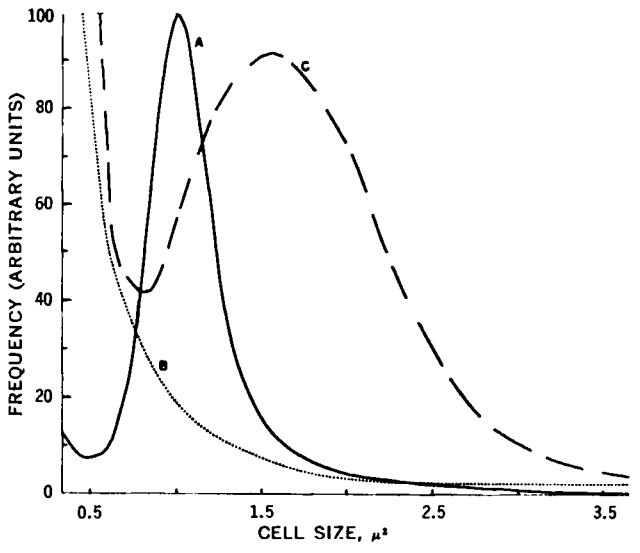


Figure 12—*Size-frequency distribution of the control organisms and (A) organisms treated with 4.0 mcg./ml. of kanamycin sulfate, (B) organisms treated with 24 mcg./ml. of sodium penicillin G 25 min. after drug addition, and (C) organisms treated with 8.7 mcg./ml. of rifampin 75 min. after drug addition.*

interval that the organisms are exposed to penicillin. The higher intercepts at lower penicillin concentrations imply the development of higher numbers of penicillin-resistant organisms at these concentrations for the same time after drug addition. Thus, the generation and production of resistants may be favored in the presence of lowered penicillin concentrations where some generation may still proceed.

Development of Rifampin- and Kanamycin-Resistant Organisms—When rifampin- and kanamycin-resistant organisms were filtered and the filtered organisms were tested in fresh medium against fresh drugs, the organisms were resistant to drug action (curves C and D in Fig. 5), whereas the filtrates showed drug activity when they were tested against fresh organisms (curves E and F). In these cases, therefore, the premise that the drugs are primarily destroyed or an antagonist is secreted can be ruled out, even though enzymatic drug inactivation was reported (4) to explain developed resistance to kanamycin and dihydrostreptomycin. This is consistent with the claim (5) that the resistance to rifampin is primarily due to the formation of a modified RNA polymerase insensitive to the action of rifampin. Analogously, streptomycin resistance was reported (6, 7) to be the result of modifications in the structure of the ribosomes that affect their affinity for streptomycin.

The studies of Fig. 4 clearly indicate the bactericidal action of kanamycin prior to the appearance of significant numbers of drug-resistant bacteria. The drug-affected bacteria did not generate significantly on dilution into media with lower drug concentrations or into fresh medium.

The size distribution studies (Fig. 12) show that kanamycin has no significant effect on cell size, whereas the cell size of rifampin-affected microorganisms gradually increases up to 75 min. after drug addition, at which time the cell size remains constant (curve C). In the case of *E. coli* killed by penicillin, no peak was apparent (curve B) shortly after drug addition. This result may be assigned to cells disintegrating into debris by the inhibition of cell wall synthesis so that cellular contents are dispersed and/or the cell membranes are devoid of contents and occupy lessened volumes. The fact that total counts (Fig. 1) achieved a plateau is indicative of this latter fact.

Combined Action of Penicillin, Kanamycin, and Rifampin with Tetracycline, Chloramphenicol, and Novobiocin—It has been claimed that various bacteriostatic drugs may interfere with the action of bactericidal antibiotics. The proposed explanation is that most bactericidal drugs only kill multiplying bacteria; when multiplication is inhibited by bacteriostatic drugs, killing cannot occur (8–11). For example, the early rate of bactericidal action of penicillin on several types of microorganisms was retarded by chloramphenicol *in vitro* (9–11) as well as *in vivo* (11–13) and by chlortetracycline or oxytetracycline *in vitro* (10, 14) and *in vivo* (13, 15). In many of these papers, the *number* of organisms killed by the bactericidal drug in the presence of the bacteriostatic eventually exceeded that in the presence of the bactericidal alone. Chloramphenicol, chlortetracycline, and oxytetracycline also interfered with the action of streptomycin (16) *in vitro* and in experimental infections of mice. However, reports of the combined action of the bacteriostatic sulfonamides and penicillin are conflicting; some state that these drugs are synergistic (17 26) and others that they are antagonistic (27–29). The conflicting reports may be due to differences in techniques, methods of measurement, and definitions of synergism and antagonism (29). If a bacteriostatic drug does interfere with the action of penicillin by inhibiting the multiplication of organisms, the stated cases of combinations with sulfonamides are difficult to reconcile with this premise.

An argument presented to rationalize an indifferent effect of sulfonamides on the initial action of penicillin is that sulfonamide has delayed action on microbial generation (30) and the penicillin activity may be exercised within that lag interval before the manifestation of sulfonamide inhibition of bacterial multiplication.

The premise that bacteriostatic drugs antagonize bactericidal drugs *in vitro* when the former are administered prior to or simultaneously with the latter has been based primarily on studies when the inocula of various organisms were in the stationary phase and were introduced at relatively large inoculum sizes (about 10^6–10^8 organisms/ml.) into the culture medium containing the drug combinations (9–11, 14, 16). The stationary phase occurred at 10^7–10^9 organisms/ml., so normally only a 10-fold increase in concentrations of organisms was possible. Thus, the actions of such drug combinations have been evaluated against microorganisms that were not in steady-state balanced generation, *i.e.*, the logarithmic phase, but were undoubtedly in the initial early lag phase where rates of generation are low and slowly accelerating and where the main process is an increase in size of the microorganisms.

Figure 6 shows that the prior addition of the bacteriostatics tetracycline and novobiocin do not interfere with the bactericidal actions of penicillin and kanamycin on balanced growth cultures in the logarithmic growth phase when the number of *E. coli* was 10^6/ml. for the addition of the latter antibiotic. The rates of decrease of viable counts of curves C and D are not significantly different from that of curves F and G, which contrast with the observations of Jawetz and coworkers (9–16) for large inocula of organisms introduced in the lag phase to a medium containing the antibacterial combination.

In most cases, the pretreatment of organisms with bacteriostatic antibiotics (curves F and G) has an advantageous effect on the more complete action of bactericidal drugs. This may be attributed to the fact that there is an interval between penicillin and kanamycin addition and their cidal action wherein microbial generation continues. In this interval of penicillin and kanamycin contact with rapidly generating organisms, mutants or adaptations resistant to these antibiotics may arise that are not possible with a prior action of the bacteriostatic drugs.

The prior addition of novobiocin had no significant effect on the

bactericidal action of rifampin in the present studies (Fig. 6c). However, when tetracycline or chloramphenicol was added before rifampin, the combined action of tetracycline (curve H in Fig. 6a) or chloramphenicol (curve H in Fig. 6b) with rifampin was dramatically less than that of rifampin alone (curve E).

The postulate that penicillin, kanamycin, or possibly other bactericidal agents will not be active on organisms in the stationary growth state (30) or in the early lag phase affected by bacteriostatic agents cannot be generalized automatically to bacteriostatically affected organisms from balanced cultures and in the logarithmic generation phase. The physiology of resting bacteria may be completely different than that of organisms whose generation is inhibited by bacteriostatic agents in the logarithmic generation phase. It appears that microbial kinetics affected by bactericidal agents alone and in combination with bacteriostatic and other bactericidal drugs need reevaluation using balanced cultures at lower inoculum sizes.

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Pattern of Phenylbutazone Degradation

D. V. C. AWANG▲, A. VINCENT, and F. MATSUI

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[1] The term "alka" was used to designate preparations containing aluminum hydroxide and magnesium oxide or carbonate.

semiquantitative estimation of decomposition products. Pawelczyk and Wachowiak (2) employed column chromatography and preparative TLC for isolation of decomposition products, identification being made by comparison with standards mainly on the basis of melting point and R_f values. A qualitative assessment and rationale of the pattern of phenylbutazone decomposition were presented for the two types of formulation examined.

In view of the extensive use of phenylbutazone as an antiarthritic and analgesic, coupled with its alleged numerous attendant side effects (4), the claim that appreciable deterioration was observed in the majority of its alka formulations warranted a closer examination of the chemistry of the drug and its degradation products. It was felt that the results of such a study would allow a more reliable assessment of the integrity of these formulations. In addition, careful examination of the literature raised some doubts as to the rigor of the published methods of characterization of the decomposition products.

Sixty-six samples of phenylbutazone tablets (representing 35 manufacturers) and 15 samples of phenylbutazone–antacid formulations, in the form of capsules and tablets (representing four manufacturers), were subjected to careful examination. In addition, the degradation of phenylbutazone bulk drug was observed under conditions of accelerated hydrolytic and oxidative decomposition, and the transformations of the derived carboxylic acids were examined under pyrolytic conditions.

Identification of phenylbutazone and its decomposition products was generally made by TLC comparison with standard reference samples. Short wavelength UV light and various spray reagents were utilized for detection. In the case of the major decomposition products, preparative TLC was utilized for isolating sufficient quantities of material from decomposed formulations to allow confirmation of identity by NMR spectroscopy and/or mass spectrometry.

EXPERIMENTAL[2]

Samples of the reference compounds were used as obtained[3], with periodic checks by TLC being made to ascertain their integrity. All compounds were found to be stable when stored in tightly closed dark bottles under normal laboratory conditions.

TLC[4]—*Preparation of Layers*—Five plates (20 × 20 cm. glass) of about 250-μ layer thickness were obtained by application of a slurry, prepared by mixing 20 g. each of silica gel GF_{254}[5] and MN-kieselguhr[6] with 95 ml. of McIlvaine buffer (pH 6), using standard layer-spreading equipment. Two hundred milliliters of buffer is produced by mixing 74 ml. of 0.1 *M* citric acid[7] and 126 ml. of 0.2 *M* disodium phosphate[7].

Solvent System—The following was used: cyclohexane (saturated with McIlvaine buffer, pH 6)–chloroform–methanol (60:30:5), all reagent grade. Five milliliters of acetic acid was added to 95 ml. of a mixture of these solvents in the same ratio (buffer excluded) when developing plates coated with silica gel only.

Detecting Agents—The following were used: (*a*) short wavelength UV light, and (*b*) 0.5% w/v potassium dichromate (analytical reagent) in 20% v/v sulfuric acid (ACS), followed by concentrated sulfuric acid.

Standards—The standard solution of phenylbutazone was prepared by dissolving 20 mg. of the bulk drug in 1 ml. of ethyl acetate (analytical reagent). Standard solutions of the decomposition products were prepared by dissolution of 2 mg. of sample in 10 ml. of the same solvent.

Detection of On-Plate Air Oxidation—Ten 20-μl. samples of the standard solution of phenylbutazone were spotted at 15-min. intervals on a single silica gel TLC plate prepared as described previously (1). The procedure was repeated, each plate being developed immediately after application of the final spot[8]. The experiment was repeated on silica gel–kieselguhr plates buffered at pH 6, prepared as already outlined.

Preparation of Sample from Formulation—An amount of powdered tablet or contents of capsule equivalent to 100 mg. of phenylbutazone was placed in a 25-ml. culture tube, 2 ml. of 0.1 *N* sodium hydroxide was added, and the mixture was swirled to a wet powder mass. Then 5 drops of concentrated hydrochloric acid and 10 ml. of ethyl acetate (analytical reagent) were added; the tube was rotated[9] for 15 min. and then centrifuged. The supernate was applied to the chromatoplate.

Procedure for Assessment of Decomposition in Formulations—A preliminary screening of each formulation was conducted by spotting 10 μl. of the standard phenylbutazone solution against a mixture made up of 5 μl. (representing 1% of the label claim for phenylbutazone) of each standard solution of decomposition product. Only those decomposition products present in amounts greater than 1% were subjected to further evaluation.

Decomposition products present in excess of 1% (as determined by the preliminary screening) were assessed on separate chromatoplates by spotting 10 μl. of sample solution against 5-, 10-, 15-, and 20-μl. applications (corresponding to 1, 2, 3, and 4%, respectively, of the label claim for phenylbutazone) of the particular decomposition product.

On all plates, 5 μl. of the standard phenylbutazone solution was applied as the initial and final spots on the chromatoplate. The extent of on-plate oxidation was then reflected in the difference in amount of oxidation product observed in these two samples; for decomposed formulations, only amounts of oxidation product greater than what was seen in the first-applied standard were considered to be indicative of the existence of oxidation products prior to application to the chromatoplate.

Alkaline Hydrolysis of Phenylbutazone—Phenylbutazone, 1.00 g. (0.003 mole), was dissolved in 12 ml. of 10% sodium hydroxide (0.030 mole), and the solution was refluxed[10] for 24 hr. At this point, reflux was halted and the reaction flask was allowed to cool to room temperature. The condenser, in which was deposited a considerable amount of sublimate[11], was washed out with chloroform and the solvent was evaporated under reduced pressure. A yellow solid, 154 mg., was thus obtained; TLC and spectral analysis indicated that this was almost pure hydrazobenzene contaminated

[2] NMR spectra were obtained with a Varian A-60A spectrophotometer, and mass spectra were obtained with a Hitachi Perkin-Elmer RMU-6 L spectrophotometer.

[3] Geigy Pharmaceuticals, Basel, Switzerland.

[4] The TLC procedures for preliminary identification, as well as semiquantitative assessment of the components of formulations and degraded bulk drug, are similar to those employed by Beckstead *et al.* (1). Most modifications were introduced to minimize oxidation on the TLC plate, the only degradative process that occurs to any detectable degree during analysis.

[5] Merck.

[6] Macherey, Nagel and Co.

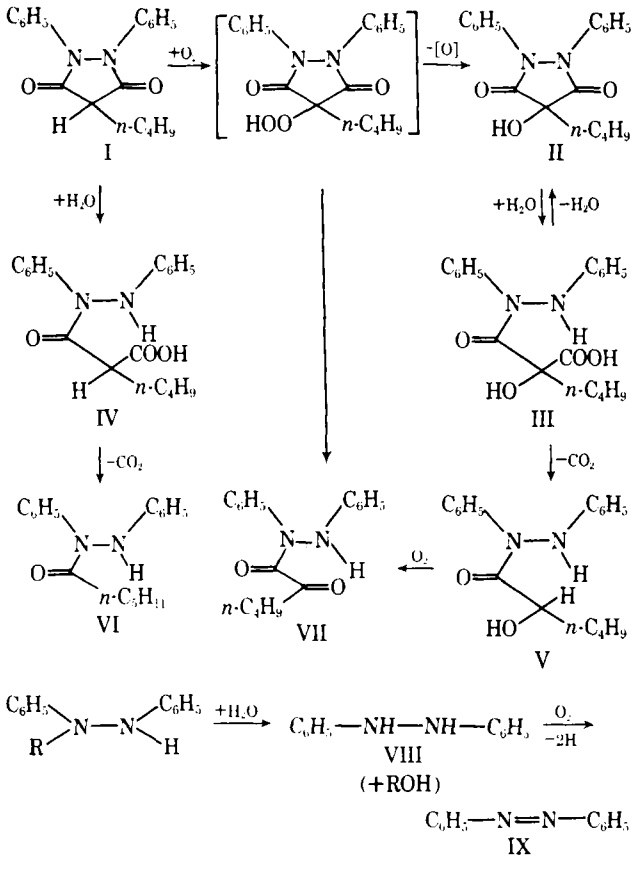
[7] Analar.

[8] There was unmistakably a direct relationship between the duration of residence of the phenylbutazone sample on the unbuffered silica gel plate and the amount of contaminant observed. Only trace contamination could be perceived in samples run on buffered silica gel–kieselguhr plates. Semiquantitative assessment indicated that 2–3% hydroxyphenylbutazone was formed after 1 hr. exposure to the atmosphere on unbuffered silica gel plates.

[9] Multipurpose rotator, model 150 V, Scientific Industries, Springfield, Mass.

[10] A duplicate experiment was conducted simultaneously, and this reaction was sampled hourly (aliquots of approximately 0.25-ml. volume). At the end of 6 hr. of refluxing, both reactions were sampled to check their agreement. There was no apparent difference in the appearance of chromatograms of parallel samples, and all indicated only a slight degree of hydrolysis of I to IV (Scheme I). Five milliliters of the solution from the duplicate experiment was neutralized with 0.1 *N* hydrochloric acid, extracted with chloroform, and dried over anhydrous sodium sulfate. The off-white solid obtained upon evaporation of solvent from the dried filtrate was semiquantitatively analyzed and shown to be phenylbutazone containing less than 4% of IV.

[11] This sublimate was essentially white, but flecks of orange, undoubtedly due to azobenzene, could be easily perceived.



Scheme I

with a trace of azobenzene. The white solid floating on the surface of the solution in the cooled reaction flask was filtered off using suction, washed with water, and allowed to dry in the open air; the yield of essentially pure hydrazobenzene was 385 mg. The filtrate was worked up in the manner described in *Footnote 10* and yielded 82 mg. of phenylbutazone contaminated with less than 4% of the hydrolysis product IV[12]. The merest trace of a contaminant of R_f corresponding to VI also could be detected. The total weight of hydrazobenzene, coupled with the amount of phenylbutazone recovered, corresponded to close to 90% hydrolysis of phenylbutazone.

RESULTS AND DISCUSSION

Preliminary—The likely products of decomposition of phenylbutazone (1,2-diphenyl-4-*n*-butylpyrazolidine-3,5-dione, I) under the relatively mild conditions of normal storage can be predicted from an assessment of the characteristic chemistry of its structural components. Scheme I outlines the salient features and a detailed analysis follows.

Air oxidation at the tertiary C-4 position could be expected to be particularly facile in view of the potential stabilizing effect of two α-carbonyl groups on development of radical or anionic character at that site. Such oxidation[13], probably *via* an unstable hydroperoxide intermediate (5)[14], would give rise to 1,2-diphenyl-4-*n*-butyl-4-hydroxypyrazolidine-3,5-dione (II). This compound has been identified as the major product of phenylbutazone degradation in suppositories (2).

Hydrolysis of II with cleavage of one C—N amide bond would lead to formation of *n*-butyltartronic acid mono-(*N,N'*-diphenyl)-hydrazide (III). Indeed, Compound II is so readily hydrolyzed that it cannot normally[15] be detected in the product of phenylbutazone oxidation in alkaline hydroxylic solution (7-11). The survival of II in suppositories (2) was undoubtedly related to the hydrophobic nature of the bases with which phenylbutazone was compounded in the formulations examined. Similar hydrolysis of I would lead to *n*-butylmalonic acid mono-(*N,N'*-diphenyl)hydrazide (IV); not surprisingly, the latter compound has been observed to be the major product of phenylbutazone degradation in aqueous solutions of its sodium salt (2, 3).

Decarboxylation of III and IV appears a likely further degradative pathway in view of the well-known facilitation of decarboxylation of carboxylic acids by a β-keto group (12). Loss of carbon dioxide from III and IV would then lead to formation of *N*-(α-hydroxycaproyl)hydrazobenzene (V) and *N*-caproylhydrazobenzene (VI), respectively.

Oxidation of IV to III and dehydrogenation of V to *N*-(α-ketocaproyl)hydrazobenzene (VII) also appear feasible.

Hydrolytic cleavage of the second C—N amide bond in all of the ring-opened hydrazobenzene derivatives would result in formation of hydrazobenzene (VIII), which is readily oxidized to azobenzene (IX).

Interpretation of Results—Application of the procedure recommended for TLC identification of phenylbutazone decomposition products (1) revealed some inconsistencies in the published data as well as shortcomings which could vitiate both qualitative and semiquantitative assessments of deteriorated formulations.

Table I lists the R_f values relative to that of phenylbutazone (I) (assigned a value of 1) calculated for the decomposition products considered in the present study. For comparison, corresponding "relative" R_f values calculated from the published (1) data[16] are also listed. The glaring discrepancy in the values for Compound V is somewhat enigmatic. In our experience, the R_f values for Compounds II and V are so similar under the TLC conditions employed that they can only be differentiated on the basis of their reaction to oxidizing spray reagents: the open-chain, more readily oxidizable hydrazo compounds such as V give rise to much more intense and rapid color development than Compounds I and II, where tertiary aromatic nitrogen atoms are involved in a closed-ring system. Upon treatment with 0.5% potassium dichromate in 20% (v/v) sulfuric acid, Compounds III, IV, V, and VI give almost instantaneously violet-colored spots, while VII affords a yellow to pink coloration. Compounds I and II show up as yellow to orange spots only upon the succeeding application of concentrated sulfuric acid, which also intensifies the other spots.

Hydrazobenzene (VIII) gives R_f values identical with those of phenylbutazone (I) under all conditions employed in the present study. Compound VIII is easily detected in mixtures with I, however, since it gives an instantaneous yellow-brown coloration with the dichromate-sulfuric acid spray reagent. Compound VIII was not detected in any formulation examined but was produced when I was refluxed in aqueous sodium hydroxide. The fact that VIII was not observed in decomposed aqueous injection solutions of

Table I—Relative R_f Values for Phenylbutazone Derivatives

Compound	R_f Silica Gel GF—Earlier Study	R_f Silica Gel GF—Present Study	R_f Silica Gel GF–Kieselguhr (1:1)
II	0.51	0.55	0.66
III	0.27	0.25	0.12
IV	0.37	0.39	0.30
V	0.72[a]	0.55	0.66
VI	0.80	0.75	0.91
VII	—	0.70	0.85

[a] It seems probable that Compound VII, which was not considered in the earlier study, was mistaken for V.

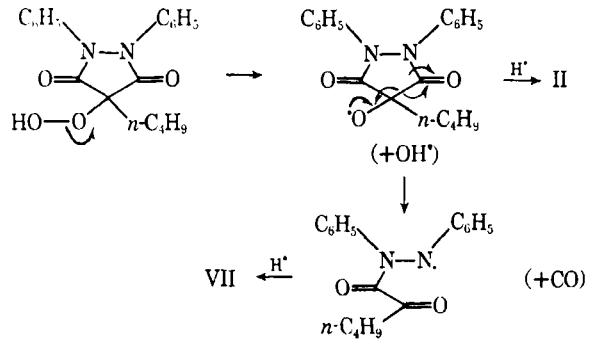
[12] No attempt was made to isolate *n*-butylmalonic acid, but its presence was indicated when a TLC plate was developed in iodine vapor.

[13] This process is probably the major cause of difficulties encountered in attempts at TLC and paper chromatographic assays of substances of the pyrazolone class (6).

[14] Hydrazobenzene catalysis, which takes place in alkaline ethanolic solution (7), is obviously not involved in the mechanism of solid-state or near-neutral air oxidation. No azobenzene, which would be formed in concomitant oxidation, was observed in those instances of appreciable C-4 oxidation in capsules or tablets or on TLC plates.

[15] Awe and Kienert (8) reported recovery of a 10% yield of II from such an oxidation, but we have not been able to duplicate this. Likewise, Veibel *et al.* (7) have not been successful in detecting II upon repetition of the procedure of Sokolova and Magidson (9) which was claimed to allow its isolation.

[16] These relative R_f values are much less variable than absolute R_f values.



Scheme II

sodium phenylbutazone (2), which contained its oxidation product azobenzene (IX)[17], is probably due to the rate of oxidation of VIII being greater than its rate of formation under normal storage conditions. A mixture of I and VIII was separated by preparative TLC from the product of a hydrolysis experiment and was characterized by comparison with authentic material using NMR spectroscopy.

It has been confirmed that air oxidation of phenylbutazone is a remarkably facile process (see earlier discussion) which readily occurs on the TLC plate unless special precautions are taken to obviate it. Two products of on-plate oxidation were observed. The major product was indicated to be 4-hydroxyphenylbutazone (II) by comparison of its R_f value and its reaction to spray reagents with those of an authentic sample. In addition, enough material was isolated by preparative TLC of partially oxidized phenylbutazone to allow its identity to be confirmed by mass spectrometry. The minor product of oxidation was not present in sufficient quantity to allow convenient isolation (and thereby unequivocal identification), but it was almost certainly *N*-(α-ketocaproyl)hydrazobenzene (VII). Its R_f value and reaction to various spray reagents were identical with those of an authentic sample. The complete absence of carboxylic acids III and IV in the oxidized product renders unlikely the involvement of hydrolysis and decarboxylation in the sequence leading to VII (Scheme I); also, Compounds IV and V do not suffer any observable on-plate oxidation. The most plausible mechanism of formation of VII appears to be that outlined in Scheme II.

The formation of II and VII by on-plate oxidation was effectively suppressed by utilizing a 1:1 mixture of kieselguhr–silica gel (instead of silica gel alone) and by impregnating the TLC support with McIlvaine buffer (pH 6).

The absence of II in formulations, concomitant with the presence of trace amounts of VII and appreciable quantities of III and IV (Table II), is consistent with facile hydrolysis of II, formed by air oxidation of the formulations examined. Observation of III in formulations that showed no evidence of IV also attests to the fact that the rate of hydrolysis of II is considerably greater than that of I.

No products of decarboxylation were observed in the formulations examined, but slight formation of *N*-caproylhydrazobenzene (VI) was observed after prolonged refluxing of phenylbutazone in aqueous sodium hydroxide. As noted earlier, there was attendant formation of hydrazobenzene (as well as trace quantities of azobenzene), a result of more extensive hydrolysis. Evidently, the rate of hydrolysis of IV is comparable with its rate of formation.

An interesting situation, probably reflective of the complexity of conformation and association involved in the case of the two carboxylic acids III and IV, concerns their behavior under thermolytic and mass spectrometric conditions. Whereas pyrolysis of solid IV gives an almost quantitative yield of the product of *decarboxylation*, VI, similar pyrolysis of III is a most efficient method of preparation of the product of *dehydration*, II[18] (10). On the other hand,

[17] This compound shows up as a bright-yellow spot having an R_f value greater than that of I, and it was undoubtedly mistakenly designated as "hydroazobenzene" by Beckstead *et al.* (1).

[18] This result is particularly surprising since incorporation of electron-withdrawing groups normally facilitates decarboxylation (12). The preference for dehydration is so overwhelming that refluxing of a suspension of III in water resulted in the exclusive formation of II. No indication of V was detected when the dichromate–sulfuric acid spray was applied. The sodium salt of III, when subjected to the same conditions, showed no sign of degradation.

Table II—Assessment of Impurities in Phenylbutazone–Antacid[a] Formulations by TLC

Formulation	Percent Decomposition Product III	IV	VII
Capsule			
1	1	1	—
2	1.5	1.5	Trace
3	2	2.5	Trace
4	1	>0.5<1	—
5	0.5	0.5	—
6	1.0	0.5	—
7	<0.5	0.5	—
Tablet			
8	>1<1.5	1	—
9	0.5	0.5	—
10	<0.5	—	—
11	<0.5	—	—
12	<0.5	—	—
13	<0.5	—	—
14	0.5	0.5	—
15	<0.5	0.5	—

[a] Only two of the 66 samples of phenylbutazone tablets showed more than trace amounts of any impurity. The major contaminant in both cases constituted less than 0.5% impurity and appeared to be Compound II on the basis of R_f value and reaction to spray reagents.

the mass spectrum of IV was identical with that of I, the highest mass peak appearing at *m/e* 308 (M − 18) and indicating facile loss of water from the parent molecule of IV. However, no peak corresponding to its molecular ion or to loss of water, carbon dioxide, or both from its parent molecule could be perceived in the mass spectrum of III, the highest mass peak being found at *m/e* 205[19].

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[19] The remarkable differences observed in the thermal degradative behavior of Compounds III and IV in the solid and liquid states, as well as under varying conditions of temperature and concentration in different solvents, will be reported and interpreted (as will be their mass spectral fragmentation) in a forthcoming article.

Primary Local Anesthetic Testing and Acute Toxicity of *N,N'*-Disubstituted 2,3-Diamino-2',6'-propionoxylidides

H. JACK ADAMS▲, GEORGE H. KRONBERG, and BERTIL H. TAKMAN

Abstract □ Eight *N,N'*-disubstituted 2,3-diamino-2',6'-propionoxylidides and 1-*tert*-butyl-2-aziridinecarboxy-2',6'-xylidide were prepared and tested for local anesthetic activity in the rat sciatic nerve block and in the guinea pig wheal. The durations in the rat sciatic blocks fell between those for lidocaine and tetracaine, but none of them possessed the long duration of tetracaine in the guinea pig wheal. All compounds were more toxic than tetracaine intraperitoneally in the mouse but, except for the aziridine compound, were less irritating than tetracaine in the rabbit intradermal wheal.

Keyphrases □ 2,3-Diamino-2',6'-propionoxylidides, *N,N'*-disubstituted—local anesthetic activity and acute toxicity, compared to lidocaine and tetracaine □ 1-*tert*-Butyl-2-aziridinecarboxy-2',6'-xylidide—local anesthetic activity and acute toxicity, compared to lidocaine and tetracaine □ Anesthetic activity, local—*N,N'*-disubstituted 2,3-diamino-2',6'-propionoxylidides and 1-*tert*-butyl-2-aziridinecarboxy-2',6'-xylidide □ 2,6-Xylidine derivatives—*N,N'*-disubstituted 2,3-diamino-2',6'-propionoxylidides and 1-*tert*-butyl-2-aziridinecarboxy-2',6'-xylidide, local anesthetic activity and acute toxicity

2,3-Bis(mono- and dialkyl) aminopropionanilides have been studied as local anesthetics (1–3). Except for 2,3-bis(diethylamino)-2',6'-propionoxylidide (IX), no other derivative of 2,6-xylidine has been reported previously (1). Because of the rather long duration of anesthesia observed for this substance, several related compounds (Table I) were synthesized and investigated pharmacologically.

EXPERIMENTAL

Chemistry[1]—2,3-Dibromopropionyl chloride (4, 5) was allowed to react with 2,6-xylidine to give 2,3-dibromo-2',6'-propionoxylidide (X) (1). When X was refluxed with secondary amines, the bistertiary amines were obtained. In several cases where X was allowed to react with propylamine or *tert*-butylamine under the same conditions, the expected bis(monoalkyl) aminopropionoxylidides were not formed.

Only the reaction with *tert*-butylamine was studied in some detail. The main portion of the basic products obtained was found to be 1-*tert*-butyl-2-aziridinecarboxy-2',6'-xylidide (XI). The fact that no similar compound was obtained from the reaction of propylamine and X indicates a stabilizing effect on the aziridine system by the presence of the bulky *tert*-butyl group.

The formation of aziridine derivatives from amines and 2,3-halogenopropionyl derivatives was reported previously (6, 7) but under exceedingly milder temperature conditions (liquid ammonia). Some aziridinecarboxanilides also were prepared previously, although by quite different routes (8).

Intermediates—2,3-Dibromo-2',6'-propionoxylidide (X) (1) was obtained from 2,3-dibromopropionyl chloride (5) and 2,6-xylidine in an acetate buffer system (1).

[1] Melting points were taken on the Thomas–Hoover capillary melting-point apparatus and are uncorrected. Analyses were performed by Alfred Bernhardt Microanalytical Laboratory, Elbach über Engelskirchen, West Germany, and Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. IR spectra were recorded on a Perkin-Elmer 257 spectrophotometer; NMR spectra were recorded on a Varian A60 with tetramethylsilane as internal standard. The IR spectra were as expected.

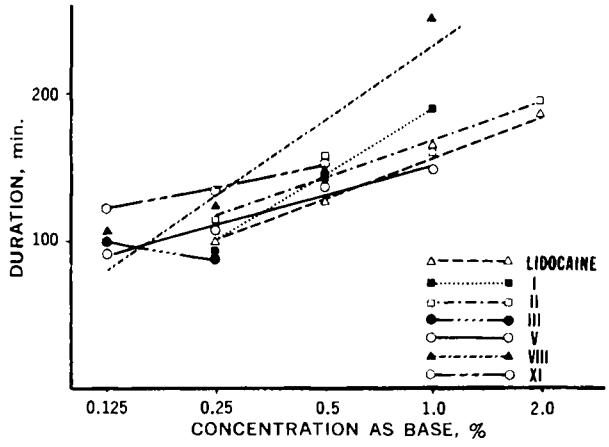

Figure 1—*Rat sciatic nerve blocks.*

Compounds of Table I—To a cool stirred solution of the secondary amine (4.5 moles) in dry benzene was added X (1 mole) in portions. After brief stirring at room temperature, the mixture was allowed to reflux for 5–6 hr. When cool, the reaction mixture was filtered, the volatiles were stripped off under reduced pressure at 50–60°, and the residue was taken up in dilute hydrochloric acid. The aqueous acid solution was washed several times with ether and then alkalinized (pH 10–11) and extracted with ether to obtain the base. The product was further purified either by direct recrystallization or through salt formation and subsequent recrystallization (*cf.*, Tables I and II).

(±)-1-tert-*Butyl-2-aziridinecarboxy-2',6'-xylidide* (*XI*)—In a glass-lined pressure vessel, X (43 g., 0.128 mole), *tert*-butylamine (73.1 g., 1.00 mole), and benzene (400 ml.) were heated for 16 hr. at 100°. After cooling, a white precipitate of *tert*-butylammonium bromide (91% yield) was filtered off. The amber filtrate was evaporated, and the waxy residue was dissolved in 200 ml. 1 *M* HCl. The solution was washed with ether, made alkaline under cooling with 7 *M* NaOH, and immediately extracted exhaustively with ether. After

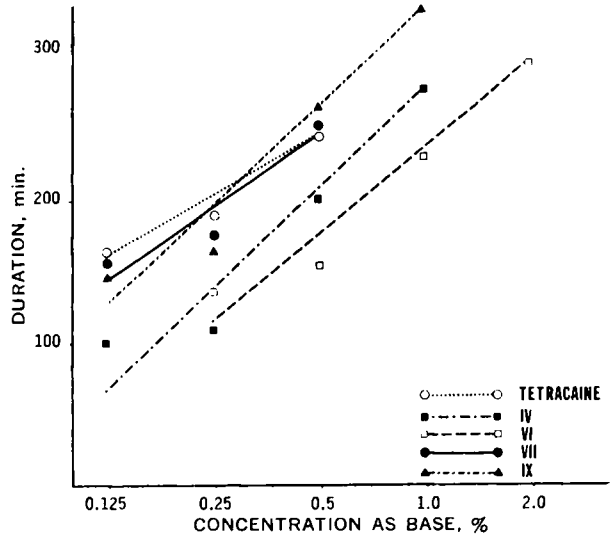

Figure 2—*Rat sciatic nerve blocks.*

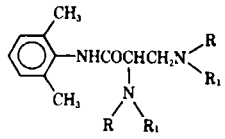

Table I Physical and Chemical Data

Number	R	R_1	Compound Form	Yield, %	Melting Point	Recrystallization Solvent
I	CH_3	CH_3	Base	55	150.5–151.5°	Cyclohexane
II	CH_3	C_2H_5	Base	55	113.5–114.5°	—[a]
III	CH_3	n-C_3H_7	Base	58	57.5–59.5°	—[b]
IIIa			Monooxalate		126.5°	Acetonitrile
IV	CH_3	iso-C_3H_7	Base	57	97–98°	Cyclohexane; n-hexane
V	CH_3	CH_2=$CHCH_2$	Base	48	88–89.5°	Aqueous ethanol
VI	CH_3	CH≡CCH_2	Base	52	101–103°	95% ethanol
VII	CH_3	$CH_3OCH_2CH_2$	Diperchlorate	40	124–125°	95% ethanol
VIII	NRR_1 = pyrrolidino		Base	43	163.5–165.5°	Benzene–cyclohexane

[a] The base was obtained from an oxalate, m.p. 130.5–131°, which was not analyzed. [b] Not recrystallized; obtained from IIIa.

drying (sodium sulfate) and evaporation, a light-brown powder (29.9 g.) was obtained. Repeated recrystallizations from petroleum ether (b.p. 60–110°) gave colorless crystals (12.5 g., 0.0508 mole, 40%), m.p. 112.5–113°. Approximately another 2.5 g. of XI could be isolated from the mother liquors with the help of chromatographic purification of a benzene solution on aluminum oxide. The total yield of XI was thus approximately 48%; NMR ($CDCl_3$): 1.08 [s, 9H, $(CH_3)_3C$], 1.84–2.04 (m, 2H, CH_2), 2.20 (s, 6H, 2 CH_3—C_6H_5), 2.36–2.52 (m, 1H, CH), 7.07 (s, 3H, C_6H_5), and 8.18 (s, 1H, NH).

Anal.—Calc. for $C_{15}H_{22}N_2O$: C, 73.1; H, 9.00; N, 11.37. Found: C, 73.0; H, 8.97; N, 11.50.

Pharmacology and Toxicology—*Rat Sciatic Nerve Blocks*—This technique was described by Camougis and Takman (9). Each compound was tested at three to five concentrations. For this primary evaluation, all solutions contained 1:100,000 epinephrine. Precisely 0.2 ml. drug solution was injected into the midthigh region so that it was deposited around the sciatic nerve trunk. Five animals were used at each concentration, and both thighs were injected on each animal. After being injected, animals were examined at frequent intervals for onset, depth, and duration of motor block. Linear regression lines were fitted to the dose–duration data[2] for the rat sciatic nerve blocks and the guinea pig wheals.

Guinea Pig Intradermal Wheals—A modification of the method described by Bülbring and Wajda (10) was used. Each wheal was made by injecting 0.1 ml. of drug solution or vehicle intradermally on the shaved back. Six wheals were made per animal, and eight to 12 wheals were made at each concentration. All solutions contained 1:100,000 epinephrine. Presence or absence of anesthesia was determined at frequent intervals by means of pinpricks.

Irritation Studies—This method was described by Camougis and Takman (9). The backs of rabbits were shaved, and a series of eight wheals per animal was made by injecting 0.1 ml. of drug solution or vehicle intradermally at each site. Twenty-four hours later the wheals were graded for degree of erythema, degree of edema, and presence or absence of necrosis. Grading was done on a numerical scale, and mean scores were obtained for all wheals at each concentration of test compound.

Acute Toxicity—The LD_{50}'s were determined in female CRCD mice, weighing about 20–25 g. Test compounds were dissolved in isotonic saline or distilled water and administered intraperitoneally. Three to five dose levels were used for each LD_{50}, and there were 10 animals at each dose level. Control groups received vehicle at a dose volume comparable to the highest dose volume of test compound. The LD_{50}'s and 95% confidence limits were calculated by the minimum logit chi-square method (11).

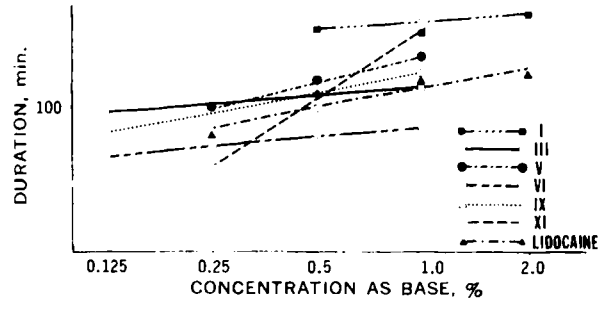

Figure 3—*Guinea pig intradermal wheals.*

[2] Using a Hewlett-Packard 9100B calculator, a 9125B plotter, and a linear regression program No. Stat-Pac X-7.

RESULTS

Rat Sciatic Nerve Blocks—Compounds I, II, III, V, and XI had dose–duration curves similar to that of lidocaine (Fig. 1). Only two points were obtained with III because 100% fatalities occurred at all concentrations above 0.25%. The dose–duration curve for VIII indicates somewhat longer durations than for the other compounds, chiefly due to the long blocks at the 1.0% concentration. Compounds IV and VI (Fig. 2) had durations greater than that of lidocaine but less than that of tetracaine; the dose–duration curves for VII and IX were comparable to that of tetracaine (Fig. 2).

Table II—Analyses of Compounds I–VIII

Number	Formula	Analysis, % Calc.	Found
I	$C_{15}H_{25}N_3O$	C 68.4	C 68.6
		H 9.59	H 9.80
		N 15.95	N 15.88
II	$C_{17}H_{29}N_3O$	C 70.1	C 70.0
		H 10.03	H 10.08
		N 14.42	N 14.32
III	$C_{19}H_{33}N_3O$	C 71.4	C 71.4
		H 10.41	H 10.19
		N 13.15	N 13.33
IIIa	$C_{21}H_{35}N_3O_5$	C 61.6	C 61.2
		H 8.62	H 8.26
IV	$C_{19}H_{33}N_3O$	C 71.4	C 71.3
		H 10.41	H 10.18
		N 13.15	N 13.20
V	$C_{19}H_{29}N_3O$	C 72.3	C 72.3
		H 9.28	H 9.51
		N 13.32	N 13.34
VI	$C_{19}H_{25}N_3O$	C 73.3	C 73.4
		H 8.09	H 8.19
		N 13.49	N 13.51
VII	$C_{19}H_{35}Cl_2N_3O_{11}$	C 41.3	C 41.4
		H 6.39	H 6.47
		O 31.9	O 31.9
VIII	$C_{19}H_{29}N_3O$	C 72.3	C 72.6
		H 9.27	H 9.11
		N 13.32	N 13.41

Table III—Acute Intraperitoneal Toxicity in Female Mice

Compound	LD_{50} and 95% Confidence Limits, mg./kg. as Base
I	44 (26–58)
II	17 (14–22)
III	16 (14–21)
IV	9 (6–13)
VII	7 (5–9)
VIII	16 (14–22)
IX	14 (11–16)
Lidocaine hydrochloride	133 (121–150)
Tetracaine hydrochloride	55 (44–69)

Frequency of block was 70% or greater at the lowest concentration tested for all compounds except VI and IX; frequency was 70% or greater at concentrations above 0.25% with these two compounds.

Guinea Pig Intradermal Wheals—The dose–duration curves for lidocaine and for Compounds I, III, V, VI, IX, and XI are shown in Fig. 3. Except for XI, these compounds exhibit flat dose–duration curves in this preparation and, in general, their curves lie close to that obtained with lidocaine. The curves for tetracaine and for Compounds II, IV, VII, and VIII are shown in Fig. 4. Although durations with these test compounds tended to be greater than those obtained with lidocaine, none of them was comparable to tetracaine in this test procedure. Because of the toxicity of VII, only two points were obtained with that compound.

In the rat sciatic nerve block experiments, Compounds VII and IX exhibited the longest durations of any test compounds; in the guinea pig wheal, VII was one of the two test compounds with the longest duration of action. However, this pattern is not consistent throughout. For example, Compound VIII was short acting in the rat sciatic but long acting in the guinea pig wheal. Such apparent inconsistencies can probably be accounted for by: (*a*) marked physiological differences between the two sites of drug application, and (*b*) differences in the physicochemical and pharmacological properties of the test compounds (12).

Irritation Studies—Because of differences in toxicity, all compounds were not tested at the same three concentrations. Lidocaine, tetracaine, and Compounds I, II, V, VI, and VIII were tested at 0.5, 1.0, and 2.0%. The others were tested at 0.25, 0.5, and 1.0%. Therefore, data are available at 0.5 and 1.0% for all compounds except VII, which was lethal at 1%. The rank order of the compounds for which irritation indexes were obtained at 0.5 and 1.0% is: least irritating = lidocaine < V < I < II < IV < III < VI < VIII < tetracaine < XI = most irritating.

Acute Toxicity—Because insufficient quantities remained after the other tests had been performed, acute toxicity determinations were not done with V, VI, and XI. The LD_{50}'s for the other test compounds and for lidocaine and tetracaine are shown in Table III. All of the compounds tested are more toxic than lidocaine, and all but I are significantly more toxic than tetracaine.

CONCLUSIONS

All of the test compounds exhibit short latency and excellent frequency of local anesthetic block. Durations are, in general, intermediate between those of lidocaine and tetracaine. All but one of the compounds are more irritating than lidocaine but less irritating than tetracaine; except for one compound, all are more toxic than tetracaine. Further synthetic efforts along these lines should obviously be aimed at retaining the excellent local anesthetic activity while attempting to reduce the toxicity.

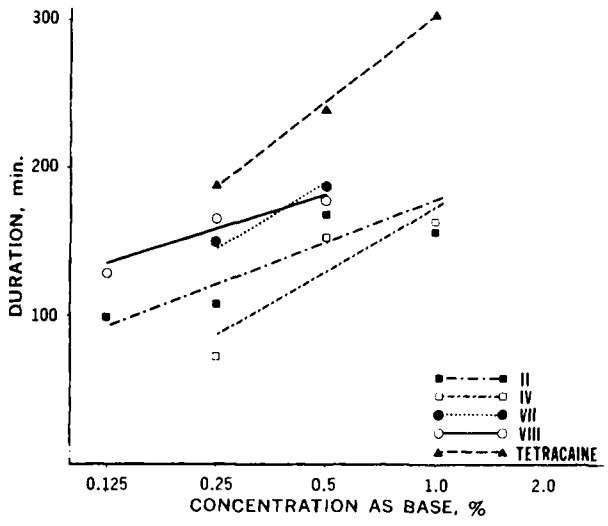

Figure 4—*Guinea pig intradermal wheals.*

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Physicochemical Properties of Prostaglandin $F_{2\alpha}$ (Tromethamine Salt): Solubility Behavior, Surface Properties, and Ionization Constants

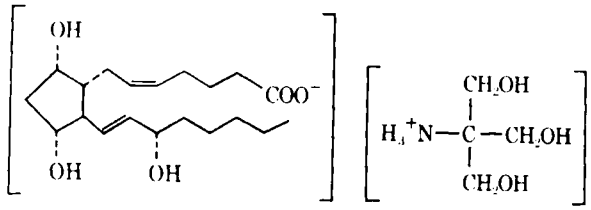
T. J. ROSEMAN▲ and S. H. YALKOWSKY

Abstract □ The pH-solubility profile of the tromethamine salt of $PGF_{2\alpha}$ was determined. The intrinsic solubility of the free acid is 0.0042 *M* at 25° and, as expected for a carboxylic acid, the solubility increased as a function of pH. However, at a pH value just above 5, there is a marked increase in solubility, which is significantly greater than would be expected from the ionization constant of the acid. This type of behavior is attributed to the formation of micelles that solubilize the drug once a critical concentration and pH are reached. Independent determination of the critical micellar concentration (CMC) by drop volume or potentiometric techniques demonstrated that $PGF_{2\alpha}$ tromethamine does form micelles. The CMC increased with the degree of ionization and, therefore, is a function of pH. The pKa of $PGF_{2\alpha}$ was determined by titration in aqueous media to be 4.90 ± 0.02. The pKa increased above the CMC as a function of concentration to a maximum value of 5.6. When a plot of concentration *versus* pKa is made, the point of deviation from the true value of 4.90 is in good agreement with the CMC reported by the drop volume technique. The interrelationship between the potentiometric, solubility, and surface properties of $PGF_{2\alpha}$ tromethamine are presented. Interestingly, these properties are qualitatively similar to those reported for certain bile acids that have similar functional groups present on the parent cholanic acid nucleus.

Keyphrases □ Prostaglandin $F_{2\alpha}$, tromethamine salt—solubility behavior, surface properties, and ionization constants □ pH-solubility profiles—prostaglandin $F_{2\alpha}$ tromethamine salt, micelle formation □ Micelle formation—factor in solubilization of prostaglandin $F_{2\alpha}$ tromethamine salt

Prostaglandins are a class of biologically active lipids found in various animal and human tissues. In recent years, the scientific literature of many disciplines has virtually exploded with publications on prostaglandin research. Several reviews (1–3) summarized prostaglandin research in the areas of chemical synthesis, biosynthesis and metabolism, analytical methods, biology, and clinical applications. From a clinical point of view, $PGF_{2\alpha}$ and PGE_2 have been the most widely tested prostaglandins; their use in induction of labor and in therapeutic abortion is well documented (4).

Prostaglandins have been administered to patients as parenteral and vaginal solutions, vaginal tablets, and suppositories. Yet, studies on their physicochemical properties have been limited. Although Johnson and Saunders (5) reported that $PGF_{2\beta}$ and PGE_1 do not form micelles, their studies did not cover a sufficiently wide concentration range. Data are presented which demonstrate that $PGF_{2\alpha}$ (as the tromethamine salt) does indeed form micelles. The present article describes an in-depth study on certain physicochemical properties of $PGF_{2\alpha}$. These properties are characterized with regard to solubility behavior, surface properties, and ionization constants, and the interrelationships among them are demonstrated. In this respect, these findings are consistent with the studies of Ekwall *et al.* (6) using bile salts. They showed that



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micelle formation affects other physicochemical properties such as ionization and solubility behavior. A knowledge of these properties will provide not only a rational basis for the design of formulations but also an insight into other areas of prostaglandin research. For example, structure–activity relationships based upon partition coefficient data can be influenced by micelle formation since the presence of micelles may alter partition values. Micelle formation and ionization behavior can also influence the absorption pattern of the drug molecule.

EXPERIMENTAL

Prostaglandin $F_{2\alpha}$ was used as its tromethamine salt (1). This salt was selected because it is crystalline and could be obtained in high purity. The free acid exists as a wax-like material and is not only difficult to purify but also is subject to weighing errors when small quantities are needed. As the tromethamine salt, $PGF_{2\alpha}$ was 99.2% pure by GLC and showed one major spot using TLC. The melting point was 100.3–101.3°. All solutions were prepared with compression distilled water, and reagent grade chemicals were used.

Solubility Determinations—Appropriate quantities of $PGF_{2\alpha}$ (tromethamine) were placed into 20-ml. glass screw-capped vials containing distilled water. The solution was then adjusted to the desired pH with 0.1 *N* HCl. The final solutions were examined visually to be sure that excess drug was present. The vials were then sealed with a wax film[1] and rotated end-over-end in a thermostated water bath at 25 ± 0.1°. After equilibration, the pH was again measured and this equilibrium value was used in reporting the data. Samples were rotated for at least 48 hr., which was previously shown to be sufficient for establishing equilibrium. At pH values ≤3, some drug decomposition was noted at 48 hr.

After equilibration, samples were centrifuged for approximately 15 min. and then filtered through a 13-mm., 0.22-μ filter[2]. The resulting clear solutions were analyzed for their $PGF_{2\alpha}$ content by GLC. One milliliter was extracted with 3 × 10 ml. of chloroform after the addition of 2 ml. of 0.2 *M* pH 3 citrate buffer. Previous studies demonstrated that this procedure yields quantitative recovery of $PGF_{2\alpha}$. The chloroform was evaporated to dryness at 37° with a stream of nitrogen. The residue was then dissolved in 0.1 ml. of pyridine and silylated with a 4:1 mixture of *N,O*-bis(trimethylsilyl)acetamide[3]–trimethylchlorosilane[3] containing 3 mg./ml. of cholesteryl acetate as the internal standard. The volume of silylating reagent varied depending upon the amount of $PGF_{2\alpha}$ present. The reagent was swirled in the flask to ensure that all $PGF_{2\alpha}$ was silylated. After al-

[1] Parafilm, American Can Co., Neenah, Wis.
[2] Millipore Corp., Bedford, Mass.
[3] Specially purified grade, Pierce Chemical Co., Rockford, Ill.

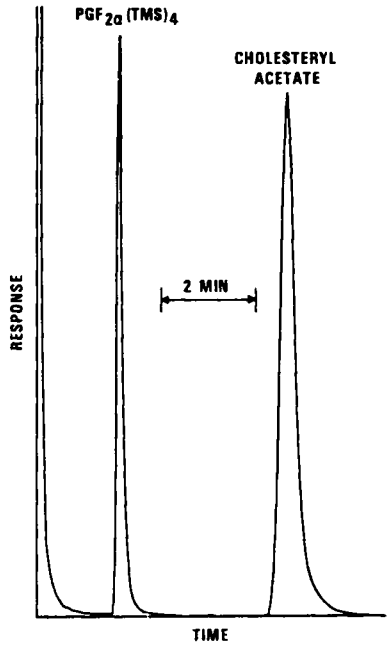

Figure 1—*GLC chromatogram of silylated $PGF_{2\alpha}$ and internal standard (cholesteryl acetate).*

lowing the solution to stand 15 min., a 1-μl. sample was injected into a chromatograph[4] under the following conditions: glass column, 1.2 m. × 0.63 cm. (4 ft. × 0.25 in.) 1% SE-52 on Gas Chrom Q (80–100 mesh); column temperature, 215°; detector temperature, 265°; and flash heater temperature, off. The helium gas flow was 60 ml./min.; air and hydrogen were adjusted to give maximum response.

Two references were prepared at each assay time. Exactly 1 mg. of $PGF_{2\alpha}$ tromethamine was weighed on an electrobalance[5] and dissolved in 0.1 ml. pyridine. It was then silylated in an identical manner as the samples. A typical chromatogram is shown in Fig. 1. The $PGF_{2\alpha}$ concentration, C, in milligrams per milliliter was calculated from the following expression:

$$C = \frac{R}{R_1} C_1 \frac{V_1}{V_{12}} \qquad \text{(Eq. 1)}$$

where:

R = ratio of sample peak area to internal standard peak area
R_1 = ratio of standard peak area to internal standard peak area
C_1 = concentration of standard
V_1 = volume of sample reagent
V_{12} = volume of solution extracted

CMC Determinations—Solutions of $PGF_{2\alpha}$ tromethamine were prepared by dissolving the drug in distilled water and adjusting the pH with 0.001–0.1 *N* HCl or 0.001–0.1 *N* NaOH. Solutions were then transferred to a 2- or 10-ml. syringe[6] equipped with a Teflon plunger. The syringe was mounted on an infusion pump[7] adjusted to deliver 0.08 ml./min. The surface tension (γ) of the solution was calculated from the average volume of 10 drops at ambient temperatures (25 ± 2°). The apparatus and calculations were reported previously (7).

pKa Determinations—The pKa values were determined by titrating aqueous solutions of different concentrations of $PGF_{2\alpha}$ tromethamine with 1.0 *N* HCl. The hydrochloric acid normality was chosen to minimize volume changes during the titration. The solutions were decarbonated by bubbling with purified nitrogen for 15 min. Titrations were carried out in a nitrogen atmosphere. A digital pH meter[8] equipped with a combination electrode[9] was used to measure pH.

[4] Hewlett Packard, model 402, Skokie, Ill.
[5] Cahn Corp., Paramount, Calif.
[6] Hamilton, Whittier, Calif.
[7] Sage, White Plains, N. Y.
[8] Sargent-Welch model NX, Skokie, Ill.
[9] Sargent-Welch model S-30072-15, Skokie, Ill.

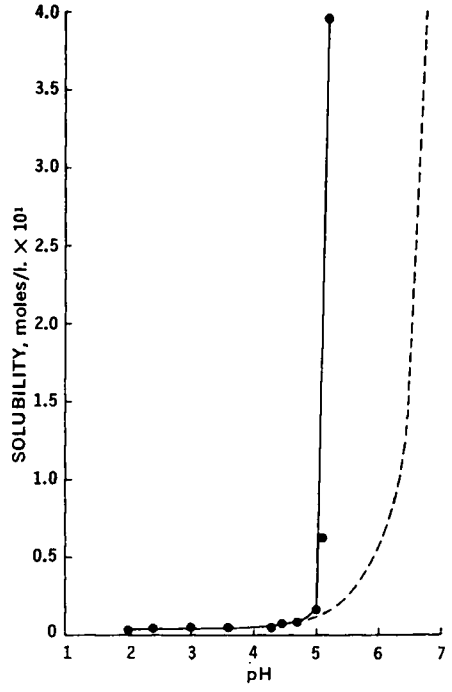

Figure 2—*pH-solubility profile of $PGF_{2\alpha}$ tromethamine. Key: ●, experimental value; and – –, predicted based upon pKa = 4.9.*

The hydrochloric acid was added using a syringe[6] calibrated to deliver 0.01 ml. Titrations of small volumes (2–4 ml.) were accomplished by using a cone-shaped container and a microstirring bar. After the addition of each increment of hydrochloric acid, the solution was allowed to equilibrate to a constant pH before the pH was recorded. The pKa at different percents of drug neutralized was calculated from the titration curve according to the method of Albert and Serjeant (8), and the pKa values were calculated from the following expression:

$$\text{pKa} = \text{pH} + \log \frac{C_T(\beta) - H_3O^+}{C_T(1-\beta) + H_3O^+} + \frac{0.505\sqrt{I}}{1 + 1.6\sqrt{I}} \qquad \text{(Eq. 2)}$$

where C_T is the total concentration of prostaglandin present (P^- + PH) and β is the degree of neutralization, *i.e.*, PH/(P^- + PH). The ionic strength contribution to the pKa was negligible below the CMC. At concentrations below the CMC, the reported pKa is an average of nine values from the titration curve.

RESULTS AND DISCUSSION

Solubility—The intrinsic solubility of the free acid of $PGF_{2\alpha}$ is 1.5 mg./ml. (0.0042 mole/l.). As shown in Fig. 2, the solubility of $PGF_{2\alpha}$ as the tromethamine salt increases with pH. The sharp (35-fold) increase in solubility with pH between 5.0 and 5.2 is far greater than would be expected on the basis of normal ionization

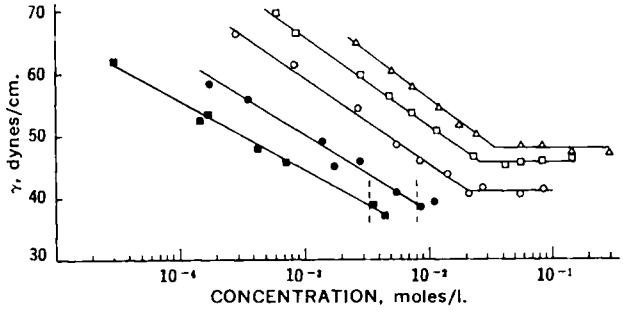

Figure 3—*Effect of pH on the surface tension plots of $PGF_{2\alpha}$ tromethamine in water. Solutions to the right of the vertical lines were colloidal for pH 3.0 and 5.0. Key: ■, pH 3.0; ●, pH 5.0; ○, pH 6.0; □, pH 8.0; and △, pH 10.0.*

Table I—CMC (Moles per Liter) of $PGF_{2\alpha}$ Tromethamine

pH	Medium	CMC Method—Titration	CMC Method—Surface Tension
10.0	Water	—	0.033
8.0	Water	—	0.026
6.0	Water	0.022	0.021
5.8	Water	0.022	—
5.5	Water	0.014	—
5.0	Water	—[a]	—[a]
3.0	Water	—[a]	—[a]
6.0	0.2 *M* Tromethamine	—	0.018
6.0	0.2 *M* NaCl	—	0.022
3.0	0.2 *M* Tromethamine	—	—[a]

[a] Since micelle formation could not be distinguished from precipitation, no value is reported.

of the acid. For purposes of comparison, the solubility that would be predicted on the basis of the following equation (9) is also shown:

$$S = S_0\left(1 + \frac{K_a}{H_3O^+}\right) \qquad \text{(Eq. 3)}$$

where S_0 is the intrinsic solubility of the acid and $K_a = 1.26 \times 10^{-5}$. This theoretical plot assumes that the solubility of the salt is much greater than the free acid. Although the exact solubility of the salt could not be determined, it is at least 500 mg./ml.

Micelle Formation—The unusual solubility behavior suggested that some type of molecular association, such as micelle formation, occurs above pH 5. Therefore, surface tension experiments were initiated to determine whether or not micelle formation occurs and to evaluate the CMC as a function of pH. Figure 3 shows the surface tension *versus* concentration plots for $PGF_{2\alpha}$ tromethamine at several pH values. At pH 3 and 5, surface tension lowering is evident, but the limited solubility of the drug precluded the distinction between micelle formation and precipitation. At the higher pH values, a well-defined plateau region is present, and the CMC is readily determined from the point of intersection of the two straight lines. These values are tabulated in Table I along with values calculated from the titration data. It was reported previously that, based on surface tension data, prostaglandins $F_{1\beta}$ and E_1 (5) do not form micelles. The discrepancy between that report and the present data could lie in the fact that the earlier study was not extended to sufficiently high concentrations to detect micelles.

The dependence of the CMC on pH is shown in Fig. 4. The points at the lower pH values represent the total solubility of the monomeric material, and it was not possible to measure a true CMC. From the data in Fig. 4, it can be seen that in the neutral pH range the micelles formed are likely to be mixed micelles composed of unionized and ionized $PGF_{2\alpha}$. This type of behavior is in agreement with results of similar studies using other surfactants (10, 11). Figure 5 shows the effect of salt on the surface tension–concentration plots at pH 3 and 6. Neither the slopes (discussed later) nor the CMC values (Table I) appear to be very dependent upon the presence of salt. Independent determination of exact CMC values utilizing the dye

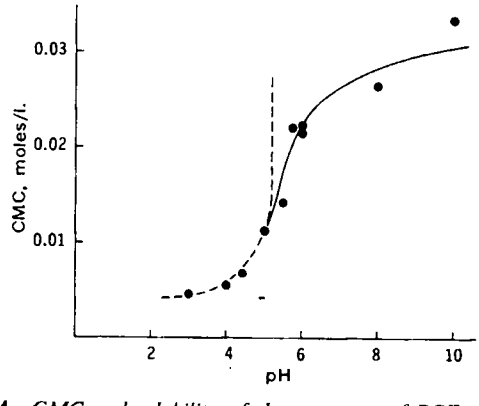

Figure 4—*CMC and solubility of the monomer of $PGF_{2\alpha}$ tromethamine as a function of pH. Key: - - -, solubility of the monomer; and —, CMC.*

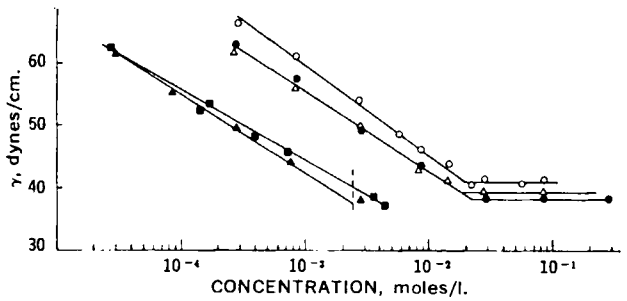

Figure 5—*Effect of salt on surface tension plots of $PGF_{2\alpha}$ tromethamine at pH 3.0 and 6.0. Solutions to the right of the vertical line were colloidal for pH 3.0. Key: ▲, pH 3.0, 0.2 M tromethamine; ■, pH 3.0, water; △, pH 6.0, 0.2 M tromethamine; ●, pH 6.0, 0.2 M NaCl; and ○, pH 6.0, water.*

solubilization method was unsuccessful due to assay interference by $PGF_{2\alpha}$. However, an increase in solubility of the dye (anthracene) was noted at approximately the CMC value. The CMC value calculated from titration data was in agreement with the value from surface tension measurements.

Micelle formation describes an association of several molecules showing a change in slope of a physical property at a critical concentration. Certain molecules may show this type of behavior and yet aggregate according to a stacking-type arrangement (12). This type of association can be characterized for rigid molecules where cooperative interaction of hydrophobic portions of the molecules is limited. Estimates of thermodynamic constants from CMC data may be misleading when this occurs (12). In the case of $PGF_{2\alpha}$, sufficient data are not available to determine the specific nature of the association process. However, space-filling molecular models show that $PGF_{2\alpha}$ has flexible hydrophobic chains (upper and lower) which could interact in a cooperative manner and probably form micelles in the classical sense.

Titration Curves—The titration curves in Fig. 6 are plots of pH *versus* fraction of total titrant added, β, obtained at various concen-

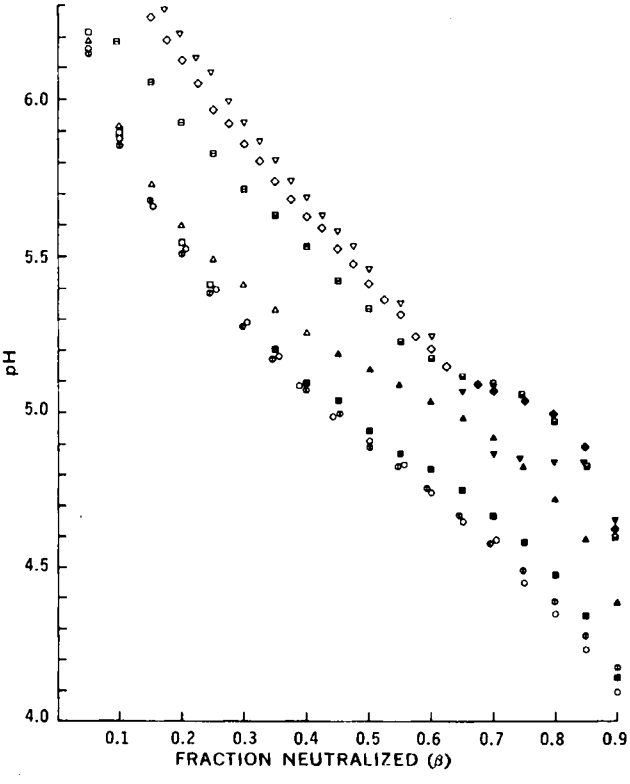

Figure 6—*Representative titration curves for $PGF_{2\alpha}$ tromethamine for various concentrations. Shaded symbols indicate precipitation. Key: ⊕, 0.001 M; ○, 0.002 M; □, 0.01 M; △, 0.02 M; ⊟, 0.05 M; ◇, 0.10 M; and ▽, 0.50 M.*

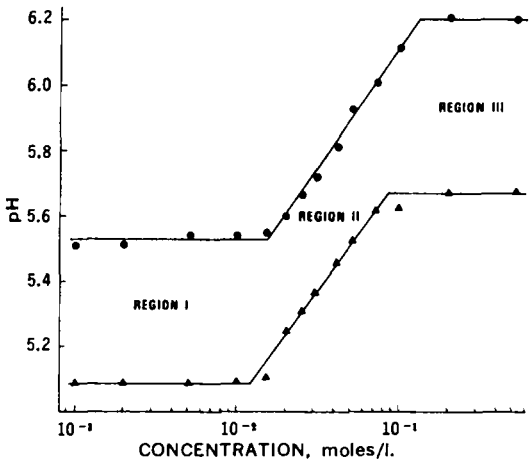

Figure 7—*pH-concentration profile for $PGF_{2\alpha}$ tromethamine. Key: ●, $\beta = 0.2$; and ▲, $\beta = 0.4$.*

trations. The fraction of total titrant is also referred to as the degree of neutralization of the system. The curves are independent of drug concentration at low concentrations, but they are displaced toward higher pH values as the concentration is increased and again become nearly superimposable at high drug concentrations. The shaded symbols indicate the regions where the system is colloidal, and these occur at $PGF_{2\alpha}$ tromethamine concentrations equal to or greater than 0.01 *M* and below a pH of about 5.1. These points were found to be less reproducible and more time dependent than those obtained for the true solutions, because equilibrium within the oil droplets could not be reached rapidly and with certainty. These points are indicated only to show the behavior of the titration curves in the situation where oiling out occurs. The slow dissolution of the oil droplets would explain why precipitation occurs in some cases at higher pH values than noted in the solubility profile. Only the values obtained for true solutions were used in calculating the data for the next section.

When pH is plotted against drug concentration at a constant β value, three distinct regions are noted, as in Fig. 7 for $\beta = 0.2$ and 0.4. At concentrations within the first region (I), only the monomeric species is present and a constant value for pH results. Above the CMC, the curves deviate from ideality. In this region (II), the pH changes with concentration due to the contribution of two species, monomer and micelle. Above 0.1 *M* (Region III), the pH again becomes constant since the micellar species predominates and the fraction of monomers becomes negligible.

The titration curves in Fig. 6 were also used to determine the CMC of $PGF_{2\alpha}$ as a function of pH. By plotting the drug concentration at which each curve crosses a particular pH, two curves are obtained which intersect at the CMC. When only monomers are present, as in Fig. 7, β is independent of concentration; however, it begins to deviate from the monomer value at the CMC (Fig. 8). This procedure for determining the CMC is equivalent to the pH-stat method described by Wood *et al.* (13). The CMC at pH 6.0 was in good agreement with the value determined from surface tension measurements (Table I and Fig. 3).

Ionization Constants—The pKa of $PGF_{2\alpha}$ tromethamine was

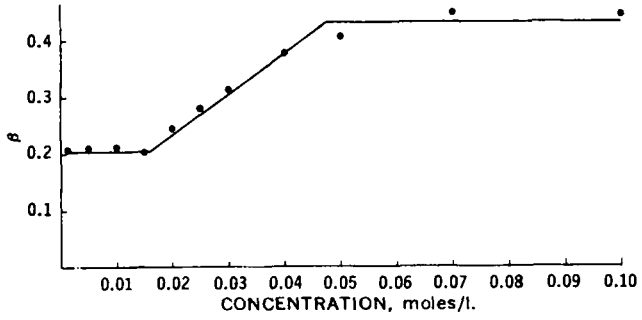

Figure 8—*Degree of neutralization (β) of $PGF_{2\alpha}$ tromethamine as a function of concentration at pH 5.5.*

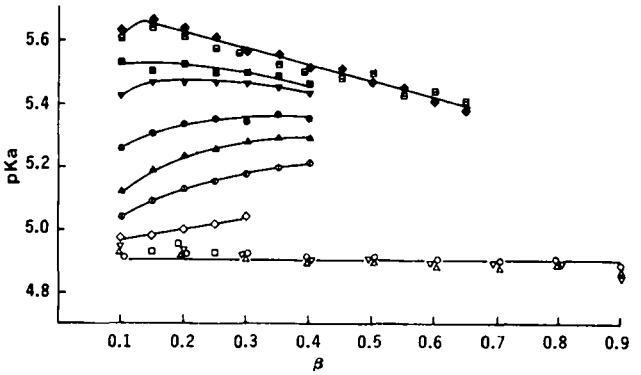

Figure 9—*pKa of $PGF_{2\alpha}$ tromethamine as a function of β at different concentrations. Key: △, 0.001 M; ○, 0.002 M; ▽, 0.005 M; □, 0.01 M; ◇, 0.02 M; ⊕, 0.03 M; ▲, 0.04 M; ●, 0.05 M; ▼, 0.07 M; ■, 0.1 M; ◆, 0.2 M; and ⊟, 0.5 M.*

calculated for each point in the raw titration curves (Fig. 6) as described in the *Experimental* section. The plots in Fig. 6 consider three variables (solute concentration, pH, and β), which are interrelated as shown in Eq. 2. The resultant pKa values as a function of β for different drug concentrations are shown in Fig. 9. At concentrations below 0.01 *M*, the pKa is equal to 4.90, as would be expected for an aliphatic carboxylic acid, and is independent of β and prostaglandin concentration. As the drug concentration is increased above the CMC, the pKa becomes dependent upon both β and prostaglandin concentration. In all cases, the apparent pKa increases as a function of the fraction of micelles present. This is shown graphically in Fig. 10A, where the plots are made for constant β values. Again, three regions are present which correspond to those described in Fig. 7. This type of behavior has been noted for other surfactant systems (14–16).

The higher pKa of the micellar $PGF_{2\alpha}$ compared to the monomer results from the fact that the negative carboxyl groups in the micelle are closer to one another than they are in free solution. In this respect, they resemble titratable groups of a polyelectrolyte (17–21). The accumulation of negative charges on the micellar surface places each individual carboxylate in a negatively charged environment. As a result, there are more protons in the vicinity of each carboxyl

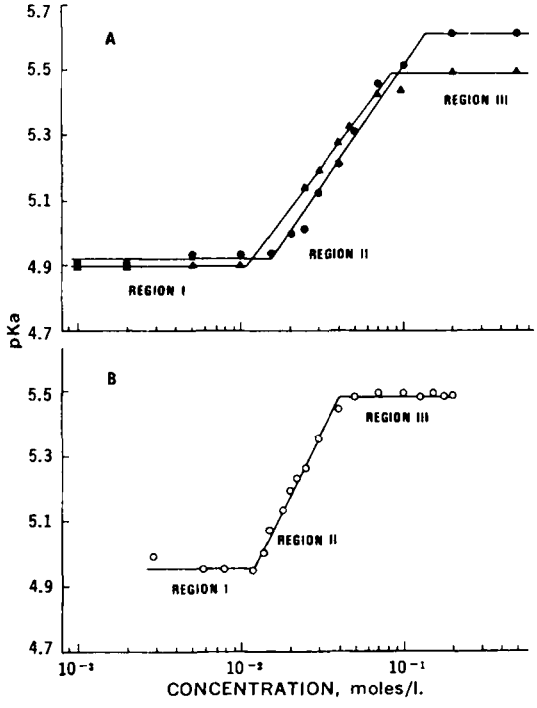

Figure 10—*(A) pKa as a function of concentration at different values of β for $PGF_{2\alpha}$ tromethamine. Key: ▲, $\beta = 0.4$; and ●, $\beta = 0.2$. (B) pKa as a function of concentration for cholic acid at 20° (14).*

group. Thus, deprotonation of each group is inhibited and the observed pKa increases. The magnitude of the increase in pKa is related to the number and closeness of the negative charges on the surface of the micelle. Therefore, the pKa of the ionized micelle ($\beta \rightarrow 0$) is greater than that of the more neutral micelle ($\beta \rightarrow 1.0$), and a negative slope would be expected for a pKa *versus* β plot. Ideally, the pKa of micelles at $\beta = 1.0$, where all of the carboxyl groups are in their neutral protonated form, should be equal to about 4.9 (the intrinsic value of a free aliphatic carboxyl group). When this is the case, the surface potential of the micelle at any value of β can be determined from the difference between the pK observed at that value of β and the intrinsic pK of the titratable group (17–21). The surface potential could be used to calculate the size of the micelle (21). However, other factors such as hydrogen bonding and changes in micelle size and shape tend to complicate the situation, especially for polyfunctional structures such as the prostaglandins.

The maximum surface potential of the micelle was calculated to be −41 mv. This value is less than the values reported for dimethyldodecylamine oxide (22) and long-chain acylcarnitines (21) and suggests that $PGF_{2\alpha}$ forms a loosely packed micelle.

At concentrations slightly above the CMC, the pK *versus* β plots (Fig. 9) have a positive slope. As mentioned earlier, a negative slope would be expected for micelles or a fixed mixture of monomers and micelles. This apparent discrepancy is explained by the fact that as β increases, the CMC decreases and, therefore, the fraction of micelles increases. This causes an increase in the observed pKa, which overcompensates for the decrease due to the neutralization of the surface charge as β increases. The following equation expresses this dependence in mathematical terms (11):

$$\text{pKa(observed)} = \text{pH} + \log\left[\frac{\beta - (C_0\beta_m/C_T)}{(1 - C_0/C_T) - (\beta - C_0\beta_m/C_T)}\right] \quad \text{(Eq. 4)}$$

where β_m is the degree of neutralization of the monomers, C_T is the total concentration of prostaglandin, and C_0 is the CMC.

The dependence of the CMC upon β is evidenced by the difference in concentration at which the breaks occur between Regions I and II (Fig. 10A). At 0.015 *M*, the curve at $\beta = 0.2$ represents only monomer. At higher values of β, the systems are a mixture of monomers and micelles.

Influence of Micelles on Solubility Profile—Returning now to the solubility–pH behavior of $PGF_{2\alpha}$ tromethamine, it is apparent that the rapid increase in solubility just above pH 5 is related to micelle formation. Figure 4 shows that the solubility of the monomeric acid and salt differs by less than a factor of 8. This does not, then, account for the observed solubility profile. Based upon Fig. 3 (see pH 3 and 5 runs), either the unionized acid of $PGF_{2\alpha}$ does not form micelles or the micelles are not very soluble. Hence, the observed solubility behavior (pH > 5) is due to the contribution of ionized drug, because it forms micelles that are extremely soluble. In the pH range of 3–8, the micelles are mixed due to the presence of both ionized and unionized species. The exact ratio of salt to acid is governed by the pH of the system, as described by the Henderson–Hasselbalch equation (23). There appears to be a critical ratio that is necessary for the rapid increase in solubility. Above pH 5.10, the ratio[10] of salt to acid is greater than 2:1; below this pH, there are less than two salt molecules for each acid molecule. Not surprisingly, as β approaches a value of 0.6 (Figs. 6 and 9), precipitation is noted for concentrations well above the CMC. This observation is consistent with the requirement that a critical ratio of salt to acid of near 2.0 be exceeded.

It is not clear why at least two molecules of salt are required to solubilize one molecule of acid. However, this type of behavior is not unusual in pharmaceutical systems. If the acid and salt are thought of as two distinct species whose relative concentrations are controlled by the pH of the solution, these observations are strikingly similar to those of Higuchi and Lach (24–26) for complexes of definite stoichiometry such as benzocaine–caffeine (1:1) and barbital–caffeine (2:1). Furthermore, some bile salts and their corresponding acids were shown by Ekwall *et al.* (6) and Ekwall (27) to exhibit a marked increase in solubility when the pH, and thus the acid–salt ratio, reaches a critical value.

[10] This ratio is based upon the pKa of the micelle being 5.38 at pH 5.10.

Table II—Area (Å²) per Molecule of $PGF_{2\alpha}$ Tromethamine at the Air–Water Interface

pH	Medium	Area per Molecule
3.0	Water	85.9
5.0	Water	75.5
6.0	Water	67.5
8.0	Water	64.1
10.0	Water	63.9
6.0	0.2 *M* Tromethamine	74.0
6.0	0.2 *M* NaCl	72.5
3.0	0.2 *M* Tromethamine	74.2

The similarity between $PGF_{2\alpha}$ and bile salts, *e.g.*, cholic acid, goes beyond the solubility–pH behavior mentioned previously. The CMC values of certain bile salts (28) fall in the same high concentration range as those of $PGF_{2\alpha}$, and their ionization constants (14) show a similar dependence on concentration (Fig. 10B). There is also an obvious similarity between the functional groups on cholic acid and $PGF_{2\alpha}$ (one carboxyl and three hydroxyl groups in each case). Moreover, in spite of its greater molecular weight, the molecular volume of cholic acid (28) is close to the value calculated for $PGF_{2\alpha}$ from its group contributions (29).

Area per Molecule—The area per molecule of $PGF_{2\alpha}$ was calculated from Eq. 5, the Gibbs adsorption equation (30):

$$\Gamma = \left(\frac{1}{kT}\right)\left(\frac{d\gamma}{d \ln C}\right) \quad \text{(Eq. 5)}$$

where Γ is surface excess of material adsorbed (moles/cm.²), k is the Boltzmann constant, T is absolute temperature, and $d\gamma/d \ln C$ is 1/2.303 times the slope of surface tension–log concentration curves in Figs. 3 and 5. Wherever applicable, activity coefficient corrections were made for variation in ionic strength. In the case of the $PGF_{2\alpha}$ tromethamine salt at low ionic strength, no correction was made for the adsorption of counterions at the interface (30, 31). The apparent lack of need for the full correction factor of 2.0 is evident in Table II. The areas calculated for pH 8 and 10 in the absence of added electrolyte differ by 17% from those obtained when a correction factor is not appropriate, *i.e.*, in swamping electrolyte or when only free acid is present. This might indicate that $PGF_{2\alpha}$ is adsorbed as an ion-pair rather than as free ions. The areas per molecule of the free acid or of the anion in swamping electrolyte (for which Eq. 5 is valid) average 77 Å². It is possible to construct space-filling molecular models, oriented with all of the polar groups on one side of the molecule, that have areas of about 85 Å². This orientation is reasonable since it would be expected that the polar groups would orient toward the polar aqueous phase. In this respect, prostaglandin $F_{2\alpha}$ again resembles the cholic acid molecule which contains three α-hydroxyl groups and a carboxylic acid moiety which are oriented toward the water at an air–water interface (28).

CONCLUSION

The pKa of $PGF_{2\alpha}$ was found to depend upon the concentration of drug titrated. The pKa increased from 4.9 for the monomer to 5.6 for the micellar state. This increase in pKa is consistent with the presence of a net negative charge at the surface of the micelle. The presence of these negatively charged micelles have been shown to account for the unusual solubility behavior observed for $PGF_{2\alpha}$ tromethamine salt. The existence of molecular aggregates was confirmed by surface tension measurements as well as the titration behavior.

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Determination of CMC and Partial Specific Volume of Polysorbates 20, 60, and 80 from Densities of Their Aqueous Solutions

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Abstract □ The CMC of the surfactants was determined by measuring the density of their aqueous solutions at 24.88° with a new instrument which can measure accurately the change in natural frequency of a hollow oscillator of constant volume when it is filled with a solution of unknown density. The frequency change is transformed into density by calibrating the instrument with liquids of known densities. The CMC values obtained were 12×10^{-3} wt.% for polysorbate 20, 9×10^{-3} wt.% for polysorbate 60, and 6.2×10^{-3} wt.% for polysorbate 80. By applying the intercept method, partial specific volumes of water and the surfactant were obtained from the density of each surfactant solution. These partial quantities indicated that the formation of micelles is associated with an increase in the partial specific volume of the surfactant and a concomitant decrease in that of water.

Keyphrases □ Polysorbates 20, 60, and 80—determination of CMC's and partial specific volumes from densities of their aqueous solutions □ CMC and partial specific volume, polysorbates 20, 60, and 80—determination from density of aqueous solution □ Density measurements, polysorbates 20, 60, and 80—used to determine CMC and partial specific volume

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The study reported here deals with the determination of densities of the aqueous solutions of polysorbates 20, 60, and 80 using a digital precision density meter[1]. The CMC for each surfactant and the partial specific volumes of the components of each surfactant solution are obtained from the corresponding density data. The results are used to describe changes that occurred in these solutions during aggregation.

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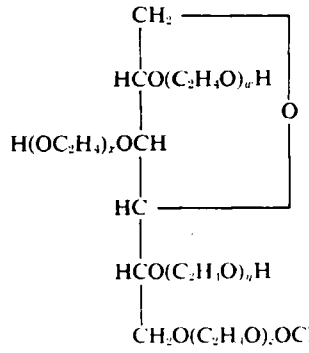
have the following general chemical formula:



where the RCO- group represents the fatty acid moiety and $n = w + x + y + z$ is the number of ethylene oxide units, which is equal to 20 for the surfactants considered (9). In this study, samples from these commercially available lots were analyzed as received. Further refining was not attempted because methods available (10) can effect only partial removal of the impurities. All solutions were prepared in distilled water on a weight-to-weight basis.

Density Measuring Apparatus—The system consisted of a digital precision density meter[1], circulator[5], and a digital thermistor thermometer[6]. The precision density meter consisted of a readout unit and a sample compartment. The sample compartment contained a hollow, V-shaped oscillator made of glass. The oscillator had a fixed volume (V), and it was filled and emptied through two ports located on the outside of the sample compartment. The oscillator could be excited to an undamped oscillation by a magnetic rod which was activated electronically. The readout unit measured the frequency of the oscillation (f) and displayed it as the oscillation period (T), where $T = 1/f$. The circulator was connected with short rubber tubing to the sample compartment. When in operation, it could maintain the temperature of the sample compartment to within ±0.02°. The temperature of the sample compartment was monitored during each run with the digital thermistor thermometer.

Instrument Theory The digital precision density meter can be represented by an equivalent system consisting of a hollow body of mass (M) and of volume (V) suspended from a spring with a spring constant (C). When the hollow body is filled with a liquid of density d and is set into motion, the oscillation period for the system is given by the following expression (11):

$$T = 1/f = 2\pi\sqrt{(dV + M)/C} \qquad \text{(Eq. 1)}$$

According to this relationship, which is general for any liquid, the oscillation period is only a function of the density of the liquid used. Expansion of Eq. 1 and further transformation yield:

$$T^2 = Ad + B \qquad \text{(Eq. 2)}$$

where A and B are the instrument's constants and are defined by $(A = 4\pi^2V/C)$ and $(B = 4\pi^2M/C)$.

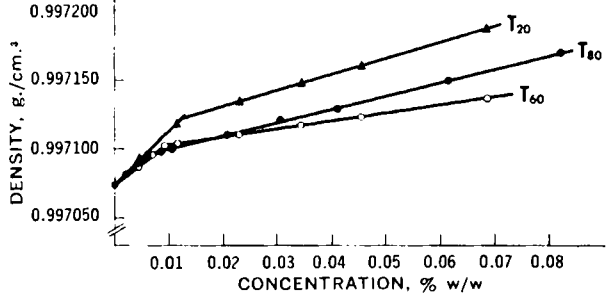


Figure 1—*Solution density* versus *concentration of polysorbate 20* (T_{20}), *polysorbate 60* (T_{60}), *and polysorbate 80* (T_{80}) *in water at 24.88°.*

[5] Lauda/Brinkmann, model K-21R, Brinkmann Instrument Inc., Westbury, NY 11590

[6] Model 1501, United System Corp., Dayton, OH 45403

Table I—Densities of Aqueous Solutions of Polysorbates 20, 60, and 80 at 24.88°

Concentration, wt. %	Density, g./cm.³[a] Polysorbate 20	Polysorbate 60	Polysorbate 80
0.0000	0.997074	0.997074	0.997074
0.0020	—	—	0.997081
0.0041	—	—	0.997089
0.0046	0.997093	0.997088	—
0.0062	—	—	0.997096
0.0069	0.997101	0.997096	—
0.0082	—	—	0.997098
0.0091	0.997110	0.997102	—
0.0103	—	—	0.997100
0.0114	0.997118	0.997103	—
0.0205	—	—	0.997110
0.0229	0.997134	0.997110	—
0.0308	—	—	0.997121
0.0344	0.997149	0.997117	—
0.0410	—	—	0.997129
0.0455	0.997162	0.997124	—
0.0615	—	—	0.997150
0.0687	0.997187	0.997138	—
0.0820	—	—	0.997171

[a] Average of four determinations.

For two liquids of differing densities, it readily follows that the difference in their densities is given by the equation:

$$(d_1 - d_2) = \frac{1}{A}(T_1^2 - T_2^2) \qquad \text{(Eq. 3)}$$

In this expression, A has the same definition as before and therefore represents the calibration constant for the instrument; T_1 and T_2 are the oscillation periods when the oscillator is filled with liquids of densities d_1 and d_2, respectively.

Density Measurement Procedure—Generally, the instrument was started 1 hr. prior to any density measurements to assure temperature equilibration. The hollow oscillator was washed by injecting distilled water through the injection port and emptying it by forced air several times. Next, the instrument was calibrated by obtaining the oscillation period for distilled water (T_1) and for anhydrous ethanol (T_2). These two liquids were used because their respective densities at various temperatures are reported in the literature (12). The densities of the aqueous solutions of the polysorbates were measured against distilled water as the reference.

All measurements were done at 24.88 ± 0.02°. It was necessary to wait for 15 min. between each sample injection for the temperature to reequilibrate. The accuracy of the measurements was investigated with distilled water. It was determined to be $\pm 3.0 \times 10^{-6}$ g./cm.³ of the reported value (12). Accuracy of this order of magnitude is easily obtainable since the instrument determines the density through precise measurement of frequency rather than determination of weight and volume.

RESULTS AND DISCUSSION

The densities of aqueous solutions of the three surfactants used in this study are reported in Table I. Each reported density is the mean of four separate determinations. In Fig. 1, the densities of the aqueous solutions of each surfactant are plotted against their re-

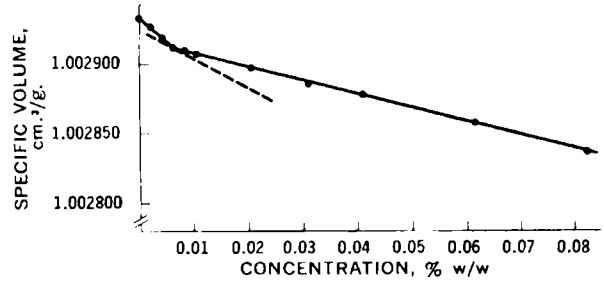


Figure 2—*Specific volume* versus *concentration of polysorbate 80 in water.*

Table II—Partial Specific Volume of Water in Aqueous Solutions of Polysorbates 20, 60, and 80 at 24.88°

Concentration, wt. %	—Partial Specific Volume of Water, cm.³/g.— Polysorbate 20	Polysorbate 60	Polysorbate 80
0.0020	1.002934	1.002934	1.002934
0.0040	1.002934	1.002934	1.002934
0.0050	1.002934	1.002934	1.002934
0.0055	1.002934	1.002934	1.002929
0.0060	1.002934	1.002934	1.002924
0.0065	1.002934	1.002934	1.002919
0.0070	1.002934	1.002934	1.002918
0.0075	1.002934	1.002932	1.002918
0.0080	1.002934	1.002929	1.002918
0.0085	1.002934	1.002925	1.002918
0.0090	1.002934	1.002921	1.002918
0.0095	1.002934	1.002915	1.002918
0.0100	1.002929	1.002911	1.002918
0.0110	1.002921	1.002911	1.002918
0.0120	1.002915	1.002911	1.002918
0.0140	1.002907	1.002911	1.002918
0.0160	1.002902	1.002911	1.002918
0.0180	1.002901	1.002911	1.002918
0.0200	1.002901	1.002911	1.002918
0.0300	1.002901	1.002911	1.002918
0.0400	1.002901	1.002911	1.002918
0.0600	1.002901	1.002911	1.002918

spective concentration. Each curve consists of two linear segments of different slopes. The abrupt change in slope occurs at the CMC of the surfactant (1–8). The CMC values for polysorbate 80, polysorbate 60, and polysorbate 20 were 6.2×10^{-3}, 9.0×10^{-3}, and 12×10^{-3} g./100 g., respectively. Bjaastad *et al.* (10) obtained CMC's of 6.0×10^{-3} and 4.5×10^{-3} g./100 ml. for partially purified polysorbate 80 and polysorbate 60, respectively, at room temperature. The higher value obtained for polysorbate 60 by the method employed in this investigation is probably due to impurities in the commercial sample used.

The volume (V) of a solution composed of n_1 moles of constituent x_1 and n_2 moles of constituent x_2 is related at constant temperature and pressure to n_1 and n_2 by the relationship (13):

$$V = n_1\bar{V}_1 + n_2\bar{V}_2 \qquad \text{(Eq. 4)}$$

where $\bar{V}_1$ and $\bar{V}_2$ are the partial molal volumes of the constituents x_1 and x_2, respectively, and are defined by the following expressions:

$$\bar{V}_1 = (\partial V/\partial n_1)_{T,P,n_2} \qquad \text{(Eq. 5)}$$

$$\bar{V}_2 = (\partial V/\partial n_2)_{T,P,n} \qquad \text{(Eq. 6)}$$

The subscripts T, P, n_1, and n_2 refer to the constant temperature and pressure conditions at constant amount of component one or two, respectively. If grams is chosen instead of moles as the concentration unit, Eqs. 4–6 are valid for partial specific volumes. These multiplied by their respective molal weights give the corresponding partial molal volumes.

The commercial polysorbates used in this study are impure and their exact molal weights cannot be used for such conversions without introducing some errors in the values of their partial molal

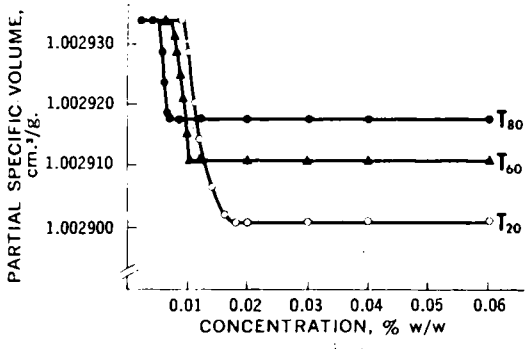


Figure 3—*Partial specific volume of water in aqueous solutions of polysorbate 20* (T_{20}), *polysorbate 60* (T_{60}), *and polysorbate 80* (T_{80}).

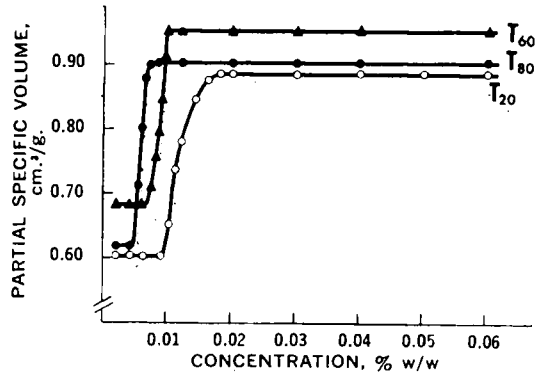


Figure 4—*Partial specific volume of polysorbate 20* (T_{20}), *polysorbate 60* (T_{60}), *and polysorbate 80* (T_{80}) *in aqueous solutions.*

volumes. Thus, the densities of each polysorbate solution were used to calculate the partial specific volumes of the surfactants ($\bar{v}_2$) and that of the water ($\bar{v}_1$) rather than their respective partial molal volumes. The method of intercepts (13) was used in these calculations. In this method, the specific volume (1/density) of each of the solutions was plotted *versus* the surfactant concentration (weight percent). A smooth curve was fitted through the points. If a tangent is drawn to the curve of such a plot at any one point, the intercept of this tangent upon the ordinate representing 100% water is equal to $\bar{v}_1$. Similarly, the intercept upon the 100% surfactant ordinate is equal to $\bar{v}_2$. Figure 2 is such a plot for polysorbate 80 solutions. The broken line is a tangent drawn to the point on the curve corresponding to a solution containing 0.0065 g. of surfactant/100 g. of solution. The partial specific volumes of the water in aqueous solutions of each surfactant were determined graphically. For the surfactants, however, graphical extrapolation of the tangents to the 100% surfactant ordinate was not practical. Therefore, all values for $\bar{v}_2$ were obtained by determining the straight-line equations fitting the tangent lines. From these equations, the intercepts upon the ordinate representing 100% surfactant were calculated.

The values of $\bar{v}_1$ and $\bar{v}_2$ at several concentrations were obtained for the three surfactant solutions (Tables II and III) and are plotted *versus* concentration of the polysorbates in Figs. 3 and 4, respectively. An inspection of Figs. 3 and 4 shows that below the CMC the values of $\bar{v}_1$ and $\bar{v}_2$ are constant, indicating that the surfactants behave as normal undissociable solutes at these concentrations. Depending on the magnitude of change in the slope of specific volume *versus* concentration plot, the values of $\bar{v}_1$ and $\bar{v}_2$

Table III—Partial Specific Volumes of Polysorbates 20, 60, and 80 in Aqueous Solutions at 24.88°

Concentration, wt. %	—Partial Specific Volume of Surfactants, cm.³/g.— Polysorbate 20	Polysorbate 60	Polysorbate 80
0.0020	0.602934	0.683490	0.621355
0.0040	0.602934	0.683490	0.621355
0.0050	0.602934	0.683490	0.621355
0.0055	0.602934	0.683490	0.712929
0.0060	0.602934	0.683490	0.802924
0.0065	0.602934	0.683490	0.877919
0.0070	0.602934	0.683490	0.902918
0.0075	0.602934	0.709182	0.902918
0.0080	0.602934	0.759339	0.902918
0.0085	0.602934	0.797043	0.902918
0.0090	0.602934	0.845027	0.902918
0.0095	0.602934	0.910607	0.902918
0.0100	0.655471	0.954131	0.902918
0.0110	0.735479	0.954131	0.902918
0.0120	0.779511	0.954131	0.902918
0.0140	0.844574	0.954131	0.902918
0.0160	0.876396	0.954131	0.902918
0.0180	0.885254	0.954131	0.902918
0.0200	0.885254	0.954131	0.902918
0.0300	0.885254	0.954131	0.902918
0.0400	0.885254	0.954131	0.902918
0.0600	0.885254	0.954131	0.902918

undergo a sharp change near or at the CMC of each surfactant, which continues over a narrow concentration range beyond the CMC. This type of behavior has been observed for potassium *n*-octoate (5) and for potassium laurate and lauryl sulfonic acid (8). The concentration dependency of $\bar{v}_1$ and $\bar{v}_2$ is due to the changing ratio of monomeric to micellar surfactant (14, 15). After this transition region, $\bar{v}_1$ and $\bar{v}_2$ begin to become constant again due to the diminishing ratio of the monomeric surfactant to the micellar surfactant.

It also has been suggested (16) that the decrease in the partial specific volume of water with a concomitant rise in that of the surfactant is due to change of water structure upon micelle formation. Below the CMC where the surfactant molecules exist in their monomeric form, the hydrophobic portions of these molecules are tightly surrounded by water molecules (17, 18). Thus, these monomers are subjected to high compression by the strong cohesive field of water molecules. At the same time, by their interposition between water molecules, these monomers effectively reduce the attraction of water molecules for each other which results in less compression on water. Upon aggregation, the area of contact between water and the hydrophobic portions of the surfactant is greatly reduced on account of the large size of the micelle as compared to single molecules. This reduction of the contact area releases most of the water molecules that were originally bound to the surfactant molecules and were the cause of their compression. For these reasons, micelle formation is accompanied by a reduced compression of the surfactant molecules (larger partial specific volume) and by an increased compression of the water molecules (smaller partial specific volume).

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Effect of Wavelength on Production of Previtamin D_2

ELIANE ABILLON[▲] and RENÉ MERMET-BOUVIER

Abstract □ On the basis of a previously established reaction scheme, the formation rate of various isomers produced by UV irradiation of ergosterol at low temperature was calculated for wavelengths between 240 and 320 nm. Particular attention was given to previtamin D_2, the precursor of vitamin D_2; the production optimum and the time required to obtain it were investigated in relation to the irradiation wavelength.

Keyphrases □ Previtamin D_2 photochemical formation from ergosterol—effect of irradiation wavelengths □ Ergosterol photochemical conversion to previtamin D_2—effect of irradiation wavelength □ UV irradiation of ergosterol—conversion to previtamin D_2, effect of various wavelengths

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The production of vitamin D_2 is achieved in two steps:

1. UV irradiation of ergosterol (I) at a low temperature, which results in the formation of the following isomers: previtamin D_2 (II), tachysterol$_2$ (III), lumisterol$_2$ (IV), and toxisterols.
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PHOTOCHEMICAL REACTION SCHEME OF ERGOSTEROL AT LOW TEMPERATURE: CORRESPONDING KINETICS

The reaction scheme (7, 8) used in this study is shown in Scheme I. The quanta transformation yield values are indicated and are ex-

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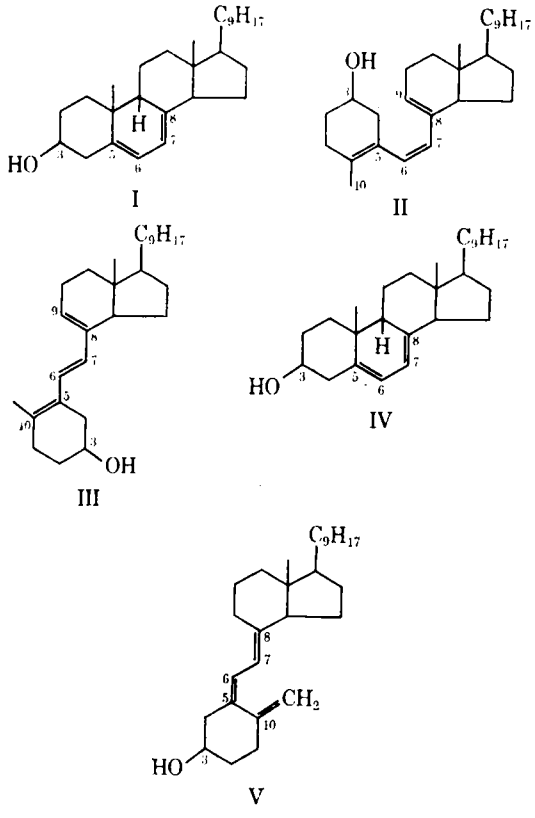
PHOTOCHEMICAL REACTION SCHEME OF ERGOSTEROL AT LOW TEMPERATURE: CORRESPONDING KINETICS

The reaction scheme (7, 8) used in this study is shown in Scheme I. The quanta transformation yield values are indicated and are ex-



pressed in number of molecules per photon absorbed by the starting isomer.

The kinetic reaction of the various isomers (9) was studied. The isomer system investigated corresponds to the interaction graph in Scheme II. This is a true exotropic graph (10) in which: (*a*) summits ①, ②, ③, and ④ represent the classes of ergosterol, previtamin D_2, tachysterol$_2$, and lumisterol$_2$ molecules, respectively; (*b*) the f_{kj} functions represent the probabilities, per unit of time, of transforming the isomer (*j*) into the isomer (*k*); and (*c*) the f_{2e} function represents the probability, per unit of time, of the exit of previtamin D_2 molecule from the system concerned.

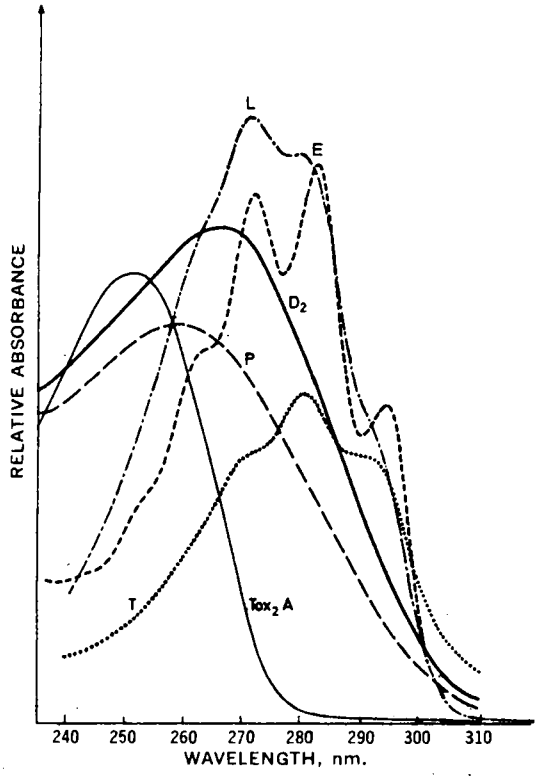
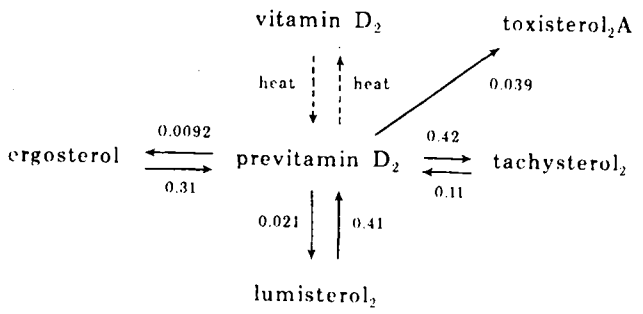


Figure 1—*UV absorption graphs. Key: E, ergosterol; L, lumisterol$_2$; P, previtamin D_2; T, tachysterol$_2$; D, vitamin D_2; and Tox$_2$A, toxisterol$_2$A.*



Scheme I—Reaction scheme of ergosterol photoisomerization at low temperature

Let $N_j(t)$ be the number of molecules in isomer (*j*) at time *t*. The equations governing the change in the system are:

$$\frac{dN_1(t)}{dt} = f_{12}N_2(t) - f_{21}N_1(t) \qquad \text{(Eq. 1)}$$

$$\frac{dN_2(t)}{dt} = f_{21}N_1(t) + f_{23}N_3(t) + f_{24}N_4(t) - (f_{12} + f_{32} + f_{42} + f_{2e})N_2(t) \qquad \text{(Eq. 2)}$$

$$\frac{dN_3(t)}{dt} = f_{32}N_2(t) - f_{23}N_3(t) \qquad \text{(Eq. 3)}$$

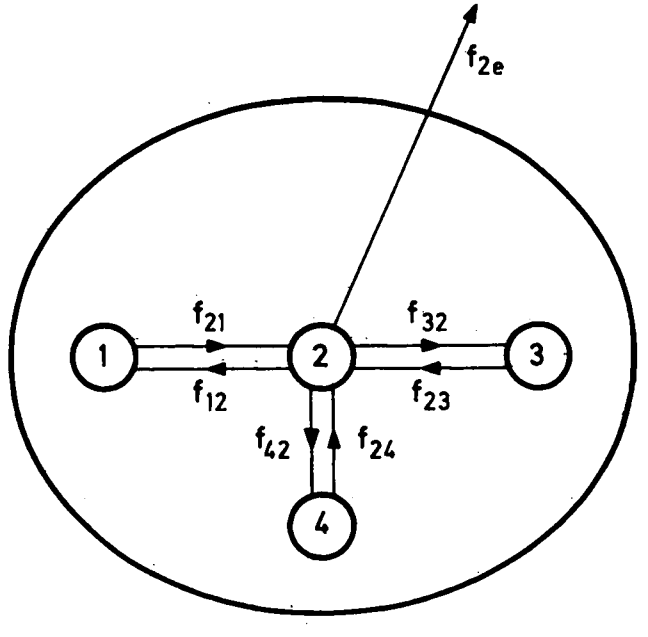
$$\frac{dN_4(t)}{dt} = f_{42}N_2(t) - f_{24}N_4(t) \qquad \text{(Eq. 4)}$$

The expression of the transfer function (in sec.$^{-1}$) is (11):

$$f_{kj} = 0.38 \times 10^{-20}\,\Phi_0 \cdot \varphi_{kj} \cdot \epsilon_j(\lambda) \qquad \text{(Eq. 5)}$$

where Φ_0 is the incident photon flux, expressed in photons/cm.^{2} sec.; φ_{kj} is the quantum transformation yield of the isomer (*j*) into the isomer (*k*), expressed in molecules per absorbed photon; and $\epsilon_j(\lambda)$ is the molar absorptivity of isomer (*j*) at wavelength λ.

The differential system (Eqs. 1–4) is valid for a thin-layer irradiation. It has been shown (11) that this system also holds for thick-



Scheme II—True exotropic graph of the ergosterol photochemical system at low temperature. (A true exotropic graph is a maximum strongly connected component to which are added boundary functions that are all divergent.)

Table I—Molar Absorptivities[a] of the Four Isomers

λ, nm.	Ergosterol	Previtamin D_2	Tachysterol$_2$	Lumisterol$_2$
253.7	4800	8220	9930	4760
265	8305	8250	19050	8020
270	11100	7550	23200	9300
280	11700	5600	28000	8970
296.5	5240	1905	18400	3260
302	1110	1110	9740	1190
313	15.9	342	3580	47.6
316	11.9	222	2860	31.7
320	7.9	127	1985	23.8

[a] From data of Havinga and coworkers (12, 13).

layer irradiation—the medium being kept homogeneous—providing that the time t variable is replaced by the variable:

$$\tau = \int_0^t \frac{\bar{\Phi}_{(t)}}{\Phi_0}\, dt \qquad \text{(Eq. 6)}$$

where $\Phi_{(t)}$ is the mean photon flux in the irradiated medium at time t.

EXPERIMENTAL

Data Used—The differential system (Eqs. 1–4) was solved by computer with the following data.

1. The incident photon flux Φ_0 was assumed to be equal to 10^{18} photons/cm.^{2} sec.
2. The quanta yields φ_{kj} used are indicated in Scheme I.
3. The initial conditions required that only the ergosterol was present at the beginning and the number of molecules $N_1 (t = 0) = 1$.
4. Two series of molar absorptivities, $\epsilon_j(\lambda)$, were used. The first series was taken from the data of Havinga and coworkers (12, 13) and is given in Table I. The second series was obtained from UV spectra determinations as explained in the *Procedures* section. The absorption curves were obtained in relative values (Fig. 1), and it was necessary to normalize them to obtain the molar absorptivities in absolute values. To make this normalization, the values given by Havinga and coworkers at the wavelength of 253.7 nm. were retained and yielded:

$$\underset{\text{absolute value}}{\epsilon_j(\lambda)} = \underset{\text{relative value}}{\epsilon_j(\lambda)} \times \left(\frac{\epsilon_j\,(253.7)_{\text{Havinga}}}{\epsilon_j\,(253.7)_{\text{relative value}}} \right) \qquad \text{(Eq. 7)}$$

Procedures—The various isomers were obtained by irradiating an ergosterol solution in ether (10 g./l.) at 5° with two UV sources:

1. A germicide lamp[1] which, with its 253.7-nm wavelength emission, furthered the production of tachysterol$_2$.
2. A high pressure mercury vapor lamp[2] fitted with two filters. One filter consisted of a quartz tank filled with distilled water to reduce the intensity of IR radiation, and the other was a filter[3] that absorbed the short wavelength UV radiation, thereby producing lumisterol$_2$.

These isomers were separated by column chromatography (14). The column (60 × 1 cm.) was filled with a mixture of silica gel (88%) and alumina (12%). The elution was made with the same solvent (ether) used in the UV irradiation medium of ergosterol. With this method, five isomers could be separated and the eluted fractions corresponding to each isomer were analyzed by UV spectrometry. The UV spectra for these isomers are shown in Fig. 1.

RESULTS

Previtamin D_2 Production—During irradiation, the formation of previtamin D_2 began, rose to a maximum, and then decreased to zero.

[1] Mazda TG-16 Lampe Mazda, Paris 8e, France.
[2] Bausch & Lomb, Rochester, N. Y.
[3] MTO-J280a, MTO filter, Massy, France.

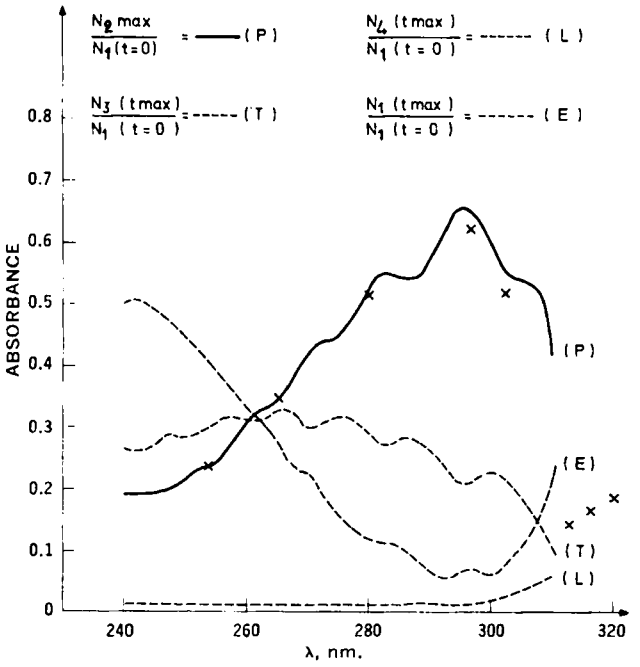


Figure 2—*Effect of wavelength on the maximum previtamin D_2 production and the simultaneous production of isomers. Key: (P), maximum previtamin D_2 production; (E), simultaneous ergosterol production; (T), simultaneous tachysterol$_2$ production; (L), simultaneous lumisterol$_2$ production; and ×, maximum previtamin D_2 production obtained with molecular extinction coefficients (12, 13).*

If the maximum number of previtamin molecules formed is called N_2 max and the number of ergosterol molecules before the irradiation is called $N_1(t = 0)$, the variation in the maximum number N_2 max/$[N_1(t = 0)]$ of previtamin D_2 molecules formed, brought down to an initial ergosterol molecule, according to the wavelength, is given in Fig. 2. The results obtained with the data of Havinga and coworkers (12, 13) (Table I) are indicated by ×'s. This graph (Fig. 2), plotted point by point every 2.5 nm., shows that maximum previtamin D_2 production occurs at the 295-nm. wavelength for which the percentage of ergosterol molecules transformed into previtamin D_2 is:

$$\frac{N_2 \text{ max}}{N_1(t = 0)} = 65\% \qquad \text{(Eq. 8)}$$

This result disagrees with Deribere (15) who recommended UV radiation at approximately 280 nm. corresponding to the maximum absorption of ergosterol and tachysterol$_2$. At this wavelength there is, in fact, a percentage of:

$$\frac{N_2 \text{ max}}{N_1(t = 0)} = 52.5\% \qquad \text{(Eq. 9)}$$

The t_{max} irradiation times required to obtain the maximum previtamin D_2, according to the wavelength, are given in Fig. 3. These times correspond to an incident photon flux $\Phi_0 = 10^{18}$ photons/cm.^{2} sec. For a different Φ_0' flux, the interaction functions become equal to:

$$f_{kj}' = \frac{\Phi_0'}{\Phi_0} f_{kj} \qquad \text{(Eq. 10)}$$

The differential system (Eqs. 1–4) remains the same if:

$$t' = \frac{\Phi_0}{\Phi_0'} t \qquad \text{(Eq. 11)}$$

is submitted. The yield values are therefore identical and only the time required to obtain them varies.

Formation of Other Isomers—When investigating the production of previtamin D_2, it is important to know the evolution of the other photochemical isomers: ergosterol, lumisterol$_2$, and tachysterol$_2$.

The concentrations $N_1(t_{max})$, $N_4(t_{max})$, and $N_3(t_{max})$ of these

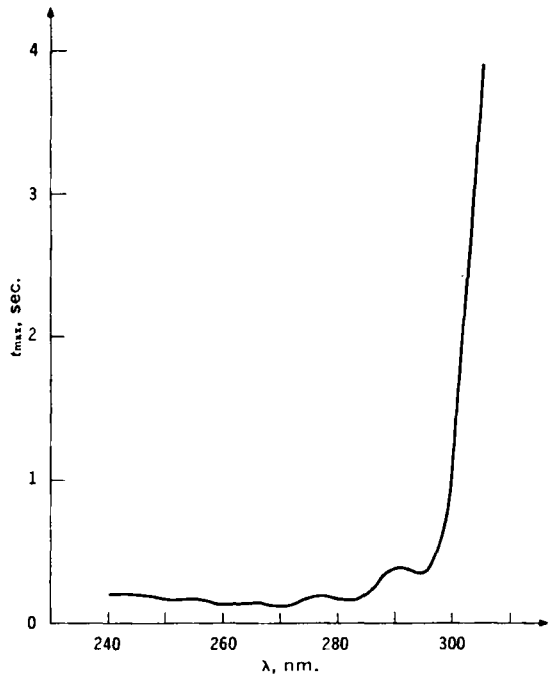


Figure 3—*Time needed to reach maximum previtamin D_2 production, with intensity of irradiation $\theta_0 = 10^{18}$ photons/cm.^{2} sec.*

isomers, when the previtamin has reached its maximum, are shown in Fig. 2. For the maximum of previtamin D_2 at the 295-nm. wavelength, there is a minimum of the three other isomers. The $N_2(t_{max})/N_3(t_{max})$, $N_2(t_{max})/N_1(t_{max})$, and $N_2(t_{max})/N_4(t_{max})$ ratios are, respectively, equal to 3.1, 10.2, and 47.5 at the 295-nm. wavelength and 1.8, 4.4, and 38.8 at the 280-nm. wavelength. This is an additional argument for seeking an irradiation source as rich as possible in UV radiation near to 295 nm.

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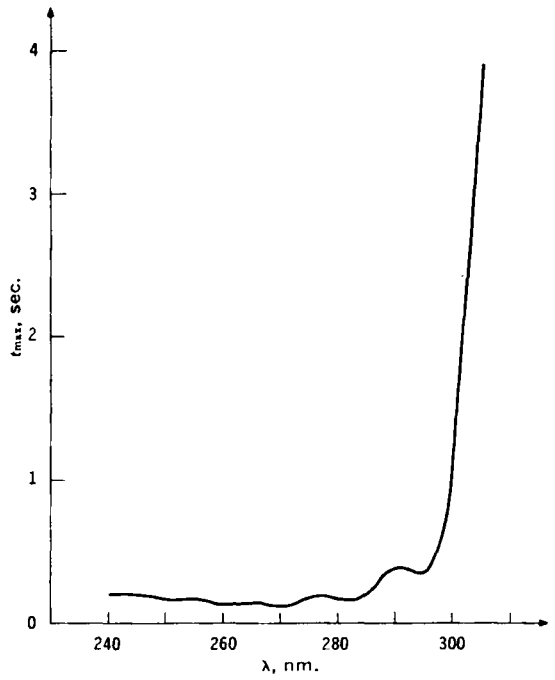
Quantitative Determination of Thebaine in Poppy Plants Using High Speed Liquid Chromatography

DAVID W. SMITH▲, THOMAS H. BEASLEY, Sr., RICHARD L. CHARLES*, and HOWARD W. ZIEGLER

Abstract □ A method for the quantitative determination of thebaine in poppy plants using high speed liquid chromatography is described. A nonionic polymeric adsorbent resin column cleanup of the sample is used to eliminate interferences from nonalkaloidal plant components. The separation is effected on a high efficiency adsorption column using *n*-hexane–chloroform–methanol–diethylamine (900:75:25:0.1) as the mobile phase. The column effluent is continuously monitored with a UV photometric detector (254 nm.). The thebaine fraction is well resolved from the isothebaine and orientalidine fractions. With 1-g. poppy samples, 0.01% thebaine is readily determined (detection limit of less than 25 ng.).

Keyphrases □ Poppy plants—analysis of thebaine using high speed liquid chromatography □ *Papaver bracteatum*—analysis of thebaine using high speed liquid chromatography □ Thebaine in poppy plants—analysis, high speed liquid chromatography □ Liquid chromatography, high speed—analysis, thebaine in poppy plants

A sensitive and rapid method was sought for the analysis of thebaine in small samples of poppy plants and parts thereof. The classical procedures of solvent extraction or column chromatography combined with gravimetric or titrimetric estimation of the isolated alkaloids were precluded (1, 2). Extraction methods for isolating the alkaloids from poppy plants and their subsequent separation for biosynthetic studies were previously described (3, 4). Adsorption chromatography on alumina followed by partition chromatography on



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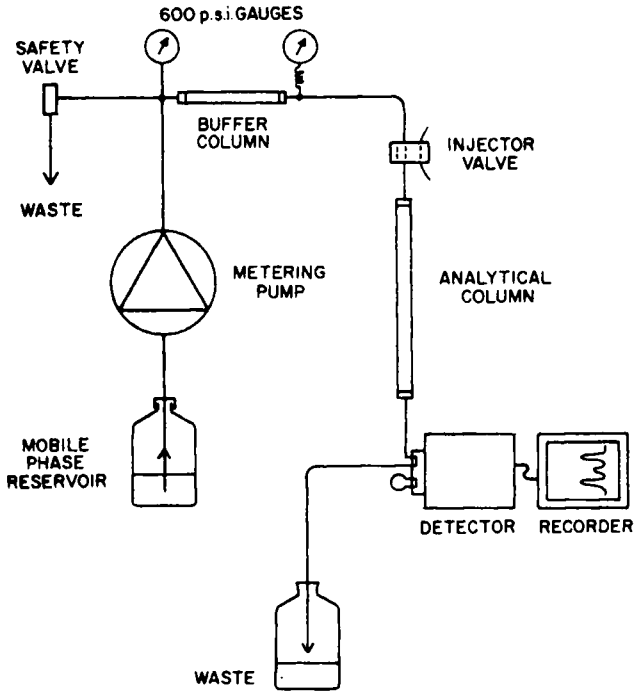

Figure 1—*Schematic diagram of the liquid chromatographic system.*

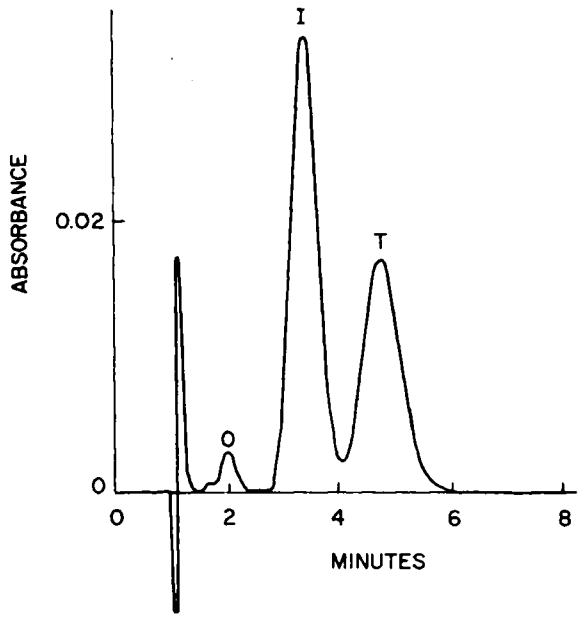

Figure 2—*Chromatogram of a synthetic mixture of 0.4 mcg. orientalidine (O), 1.0 mcg. isothebaine (I), and 1.0 mcg. thebaine (T).*

kieselguhr columns was used in the quantitative determination of the "secondary alkaloids" in opium (2). TLC on silica gel, followed by spectrophotometric measurement of the resolved alkaloids, was also used (5, 6). GLC was employed for the separation and quantification of opium alkaloids (7, 8), but problems due to adsorptive effects were encountered with GLC determination of the alkaloids (8). Other procedures include an ion-exchange separation (9) and an IR spectrophotometric determination (10). The latter procedure allows the simultaneous determination of narcotine, thebaine, and papaverine in opium by examining the IR spectrum of a carbon tetrachloride solution of the dried chloroform residue from an extraction of an acetic acid–water solution.

EXPERIMENTAL

Reagents—All reagents were analytical reagent grade, except diethylamine which was organic reagent grade. The nonionic polymeric adsorbent resin[1] (20–50 mesh) was washed before use with two, two, and four column volumes of acetone, methanol, and water, respectively. The strong cationic-exchange resin in the hydrogen form[2] (20–50 mesh), high efficiency adsorption packing[3] (37–50 μm.), TLC plates[4], and silicic acid[5] were used as received. A mixed solvent (Reagent A) consisting of chloroform–methanol (3:1) was used throughout the procedure.

Standards—Three thebaine standard solutions in the concentration range of 0.1–0.4 mg./ml. were prepared by dissolving thebaine alkaloid in Reagent A. The thebaine alkaloid was purified by classical purification procedures in these laboratories. It was shown to be single-component material by TLC, GLC, and high speed liquid chromatography (HSLC) and was found to be 99+% pure by nonaqueous acid–base titration. The standard solutions were freshly prepared each week and were stable when protected from light and solvent evaporation.

Equipment—A laboratory mill[6] was used to mill dried samples. A 1.4-cm. i.d. × 30-cm. glass column fitted with a Teflon stopcock was used for column chromatography in the cleanup procedure. A cotton plug was used for a bed support.

HSLC System—The system consisted of a mobile phase reservoir, a liquid metering pump[7], a pressure relief valve[8], a pulse dampening system, a 5-μl. sample injector valve[9], a 2.8-mm. i.d. × 300-mm. glass analytical column[10] containing a suitable packing[3], a UV (254-nm. wavelength) photometric detector[11], a recorder, various connectors and fittings, and 0.79-mm. (0.031-in.) i.d. Teflon tubing[10] arranged as shown in Fig. 1. The pulse dampening system was constructed from two 0–42.2-kg./cm.^{2} (0–600 p.s.i.) gauges[12] and a 2.8-mm. i.d. × 75-mm. column[10] packed with silicic acid. A 25-cm. coil of Teflon tubing was used to connect the second gauge to the fluid stream. At the beginning of each day, the coil was opened, drained, and reconnected to the system. The air column trapped in the coil served as an effective pulse suppressor to allow the detector to be operated at its most sensitive setting. The air slowly dissolved in the mobile phase, thus requiring its daily replacement.

Procedures—*Sampling and Extraction*—Fresh or frozen poppy roots, leaves, and/or stems were subdivided by chopping into about 0.5-cm. cubes (squares, if leaves). Dried roots, stems, leaves, capsules, or bracts were milled to approximately 1-mm. diameter particles. The subdivided sample was mixed to obtain a representative sample. Two portions of approximately 1 g. each were accurately weighed. A loss on drying at 105° (overnight) was determined on the first portion. The second portion was placed in a 250-ml. (8-oz.) blender jar, 100 ml. of water was added, and the pH was adjusted to 2 ± 0.2 with 10% (v/v) phosphoric acid solution. The sample was blended 4 × 30 sec. at high speed. Then the cutter blades were removed and the jar was placed in an ultrasonic bath. Filter pulp was added and the mixture was sonically agitated and simultaneously stirred with a motor-driven plastic paddle for 5 min. The slurry was filtered through paper with the aid of reduced pressure, and the filter cake was quantitatively washed with approximately 0.01 *N* phosphoric acid. The filter cake was saved and tested for thebaine content. The filtrate and washes were collected in a 400-ml. beaker, and the pH was adjusted to 9–10 with approximately 1 *N* sodium hydroxide.

Column Chromatography– A 10-ml. portion of prepared nonionic resin[1] was placed in the 1.4 × 30-cm. column. The sample was al-

[1] Amberlite XAD-2, Mallinckrodt Chemical Works, St. Louis, Mo.
[2] Dowex 50W-X2, Bio-Rad Laboratories, Richmond, Calif.
[3] Corasil II, Waters Associates, Inc., Framingham, Mass.
[4] ChromAR 7GF (250-μm. layer thickness), Mallinckrodt.
[5] SilicAR CC-7, 200–325 mesh, Mallinckrodt.
[6] Model No. ED-5, A. H. Thomas Co., Philadelphia, Pa.
[7] Milton Roy miniPump, Milton Roy Co., St. Petersburg, Fla.
[8] No. PRV500, Chromatronix, Inc., Berkeley, Calif.
[9] No. CSV-5, Chromatronix.
[10] Chromatronix.
[11] Model 200 with 1-mm. bore flow-through cell module, Chromatronix.
[12] Special No. 21540, Ametek/U. S. Gauge, Sellersville, Pa.

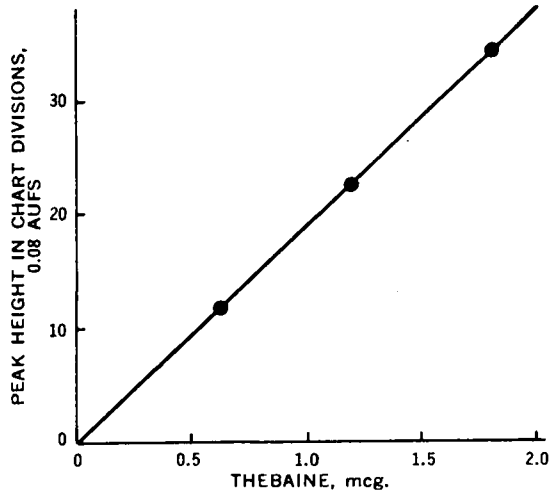

Figure 3—*Plot of peak height* versus *micrograms thebaine from chromatograms of thebaine standard solutions.*

lowed to pass through the column, adjusted to a flow rate of approximately 2 ml./min. The inside wall of the column was rinsed once with water, and then an additional 40 ml. of water was passed through the resin bed. The aqueous effluent was saved and checked for thebaine. A 200-ml. ⅋ 24/40 round-bottom flask was placed under the column. The inside wall of the column was washed with a few milliliters of methanol, and the alkaloids were eluted with 50 ml. of methanol. After approximately one-half of the methanol had gone through the column, the flow was stopped and the solvent voids were removed by running a stiff wire in and out of the resin bed. A cotton plug, to prevent the resin from floating, was placed on top of the bed; then the elution with the remainder of the methanol was resumed. Finally, 75 ml. of Reagent A was passed through the column. The resin bed was saved and checked for thebaine.

Sample Preparation for HSLC—The sample solution was evaporated to dryness using a rotary vacuum evaporator at 40–60°. Five milliliters of Reagent A was added to the sample residue in the flask. The flask was sealed with a polypropylene stopper and placed in an ultrasonic bath to extract the alkaloids from any insoluble residue. The solution was filtered through a medium-porosity, sintered-glass filter into a 25-ml. volumetric flask. The round-bottom flask was rinsed three times with 3-ml. portions of Reagent A, and each portion was filtered. Finally, the filter was washed twice with 3 ml. of Reagent A. Filtration was facilitated by positive pressure developed with a rubber squeeze bulb fitted on top of the filter funnel. The sample solution and washes were diluted to volume with Reagent A.

HSLC—The operating conditions for the analysis were as follows: (*a*) mobile phase composition, *n*-hexane–chloroform–methanol–diethylamine (900:75:25:0.1 v/v); (*b*) mobile phase flow rate, 1 ml./min. [approximate column head pressure of 2.8 kg./cm.^{2} (40 p.s.i.) was developed]; and (*c*) detector setting, 0.08 absorbance unit full scale (AUFS) for usual operation. The sample or standard solution was transferred to the sampling valve by means of a 1-ml. tuberculin syringe. Five microliters was then injected onto the column by manually actuating the sample injector valve. Peak heights of the thebaine peaks from sample and standard chromatograms were measured manually from the extrapolated baselines. Three standards were run each day to prepare a calibration curve. The thebaine concentrations of the samples were found from the calibration curve. A shared-time computer was used for constructing the calibration curve by finding the best slope (m) for a line of the equation $y = mx$, where y is the absorbance and x is micrograms of thebaine. Analytical results were calculated on the dried basis from the sample weights and loss on drying values.

TLC Procedure for Checking for Alkaloid Losses—*Filter Cakes*—The filter cake was placed in a beaker, about 50 ml. of water was added, and the pH was adjusted to approximately 3 with 10% (v/v) phosphoric acid solution. Five milliliters of cation-exchange resin[2] was added to the beaker. The mixture was then ultrasonically agitated and mechanically stirred for 5 min. The beaker was removed from the ultrasonic cleaner, and distilled water was added to fill the beaker. After the resin was allowed to settle, the supernate and suspended pulp were decanted. The water addition and decanting

Table I—Thebaine Assay Values of Several Iranian Poppy Plant Samples

Sample Number	Description	Weight Percent Thebaine (Dried Basis)
16B	Mature capsules	1.50, 1.41, 1.62, 1.42, 1.48
16C	Green stems	1.87, 1.83
16D	Bracts	1.42, 1.37
16E	Green capsules	1.23, 1.25
45B	Mature capsules	1.40, 1.48, 1.42

step was repeated at least three times. Most of the water was poured off, and then 25 ml. 1 *N* ammonium hydroxide in 70% methanol was added (11). The mixture was ultrasonically agitated 5 min. and then filtered through a cotton plug, fitted in a powder funnel, into a 100-ml. ⅋ 24/40 round-bottom flask. The resin was washed in the funnel three times with 5 ml. of 70% methanol. The filtrate and washes were evaporated to dryness on a rotary vacuum evaporator. The residue in the flask was extracted in 5 ml. of Reagent A with the aid of ultrasonic agitation, and 100 μl. was spotted (in 10-μl. increments) on a TLC plate.

The TLC conditions were as follows: (*a*) mobile phase, benzene–ethanol–ammonium hydroxide (90:15:1); (*b*) development, 10 cm., ascending; and (*c*) visualization, UV (254 nm.) and iodoplatinic acid spray reagent. Five microliters of a 1% thebaine solution was spotted adjacent to the sample spot for reference. The plate was developed and any thebaine found was noted. Any thebaine loss detected was cause to repeat the sample work-up using less sample.

Aqueous Effluents—The pH of the solution was adjusted to about 3 and treated as described previously beginning with the addition of the ion-exchange resin.

Posteluates—Alkaloid losses on the nonionic resin[1] were checked by passing another 50 ml. of Reagent A through the resin bed. The effluent was collected in a 100-ml. ⅋ 24/40 round-bottom flask and then evaporated to dryness on the evaporator. Five milliliters of Reagent A was added to the flask, and 100 μl. of the resulting solution was spotted (in 10-μl. increments) on a TLC plate.

RESULTS AND DISCUSSION

Figure 2 shows a chromatogram of a synthetic mixture of orientalidine, isothebaine, and thebaine. These three alkaloids are reported to occur in *Papaver bracteatum* and *P. orientale* (12, 13).

Figure 3 shows a plot of peak height *versus* micrograms of thebaine from chromatograms of thebaine standard solutions. For assay work, the system was calibrated each day because some variation in the thebaine retention volume was occasionally noted from day to day. Recalibration was also required when fresh mobile phase was used. Fresh mobile phase was prepared at least every 3 days because some loss in resolution was noted with 1-week-old mobile phase. However, the system was stable within a particular day. The peak heights for replicate injections of a standard sample throughout a 5-hr. period would nominally have an average variation of less than ±1% relative standard deviation (*RSD*).

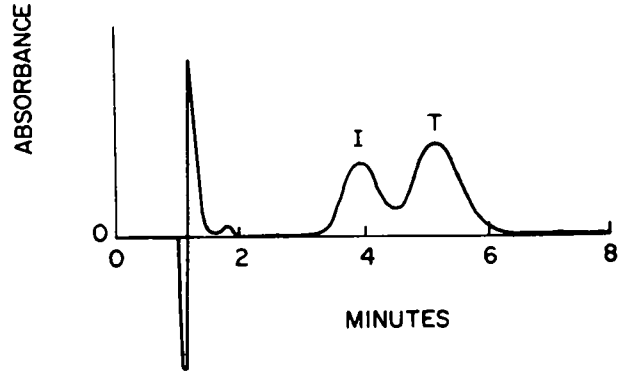

Figure 4—*Typical chromatogram of a poppy plant sample. Key: I, isothebaine; and T, thebaine.*

A chromatogram of a typical poppy plant sample is shown in Fig. 4. The retention volumes for the alkaloids are noted to be greater here than in Fig. 2. The chromatogram in Fig. 4 was run 2 months before the one in Fig. 2. The sample was 0.55 g. of capsules and assayed 0.39% thebaine on the dried basis (6.64% loss on drying).

Table I shows assay values for several Iranian poppy samples[13], tentatively identified as *P. bracteatum*. The coefficient of variation for the method was 5.7% with Sample 16B. It was felt that the precision obtained on this sample was limited by sampling error, since these data were obtained on a rather coarse (hand-ground) grind of the capsules. Duplicate assays on other samples ground finer by milling appeared to be in closer agreement.

TLC was used to check for thebaine losses as noted in the procedure. The sensitivity of a single TLC test was 1 mcg. of thebaine alkaloid (absolute). The cumulative thebaine loss from three TLC checks per sample could, therefore, not exceed 1.5%.

A procedure using a cation-exchange resin[2] (11) was tried and then abandoned. The resin is an efficient extractant for the alkaloids from the plant matrix. However, the resin extracted other plant matter, particularly from green stems and capsules, which eluted with the alkaloid fraction (1 *N* ammonia in 70% methanol was used to elute the alkaloids from the resin). Upon evaporation of the eluant, a large amount of residue remained from which the thebaine could not be quantitatively recovered. For such samples, the extraneous material was effectively removed with a postcolumn of a strong anionic-exchange resin in the chloride form[14]. For certain sample types, a gummy mass formed when the methanol–water phase was added to the cation-exchange resin[2] which hopelessly plugged the columns. However, the nonionic resin[1] procedure gave the least amount of extraneous residue and worked well with all sample types.

The nonionic polymeric adsorbent resin[1] system, as described here, has a capacity to handle about 50 mg. of thebaine. Losses due to incomplete extraction by, or elution from, the resin do occur with larger sample loads. The TLC monitoring of the sample cleanup procedure for different sample types easily determines if losses are occurring. The monitoring can be discontinued once an analyst determines the proper sample size to use.

[13] Obtained from Chemical Group Research and Development, Mallinckrodt.

[14] Amberlite IRA-401S.

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Detection of Ampicillin Contamination in Nitrofurantoin Preparations by High Pressure Liquid Chromatography

ALFRED BRACEY

Abstract □ Ampicillin as a contaminant in nitrofurantoin capsules and tablets was determined by high pressure liquid chromatography. The method uses a strong anion-exchange column, 0.01 *M* KH_2PO_4 buffer, and an ampicillin working standard as reference. Identity of ampicillin was verified by assaying spiked samples of nitrofurantoin. The method is quantitative over the range 1–10 mcg. of ampicillin.

Keyphrases □ Nitrofurantoin tablets and capsules—analysis of ampicillin contamination, high pressure liquid chromatography □ Ampicillin contamination in nitrofurantoin tablets and capsules—analysis, high pressure liquid chromatography □ High pressure liquid chromatography—analysis, ampicillin contamination in nitrofurantoin capsules and tablets

Ampicillin, 6-[D(−)-α-aminophenylacetamido]penicillanic acid, is a semisynthetic penicillin, first prepared by Doyle *et al.* (1). The anhydrous and trihydrate forms and the sodium salt are widely used therapeutically. It has been shown to be inhibitory at low concentrations against a wide variety of both Gram-negative and Gram-positive bacteria (2–4). Because it is manufactured and formulated into dosage forms by many drug companies that also produce other drugs, cross-contamination is a possibility. This situation causes concern, particularly since small quantities of penicillin derivatives may cause allergic reactions (5–7). As part of its antibiotic certification program, the Food and Drug Administration (FDA) has developed and used tests (8) to check cross-contamination of penicillin in other pharmaceuticals, but these tests do not detect ampicillin.

A test to determine ampicillin contamination of nitrofurantoin became necessary when it was suspected as a contaminant in samples submitted to the National Center for Antibiotic Analysis for analysis. Attempts to recover ampicillin added to nitrofurantoin by the standard procedures (8) were unsuccessful because ampicillin, being amphoteric, could not be extracted into amyl acetate from an acid solution. A test based on liquid chromatography was developed.

A strong anion-exchange column was selected because ampicillin has an anion-exchangeable carboxylic group and is water soluble. Of various buffers and solvent systems tried, 0.01 *M* KH_2PO_4 (pH 6.5) buffer was optimal. This mobile phase separated nitrofurantoin and ampicillin and simultaneously was used to identify and quantitate ampicillin. The reliability of the method was verified by assaying spiked samples of nitrofurantoin capsules and tablets.

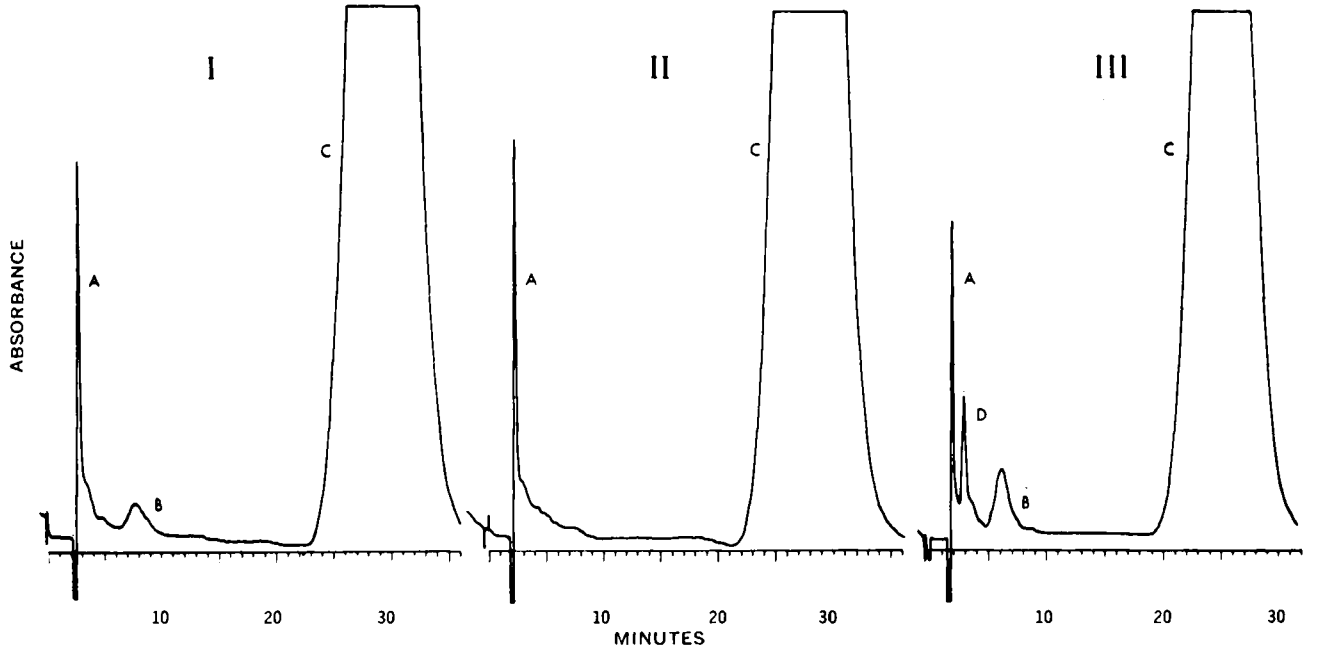

Figure 1—*Chromatogram of (I) nitrofurantoin capsules spiked with 0.1 mg. of ampicillin per dose, (II) uncontaminated nitrofurantoin capsules, and (III) commercial nitrofurantoin capsules contaminated with ampicillin. Key: A, solvent; B, ampicillin; C, nitrofurantoin; and D, unidentified impurity.*

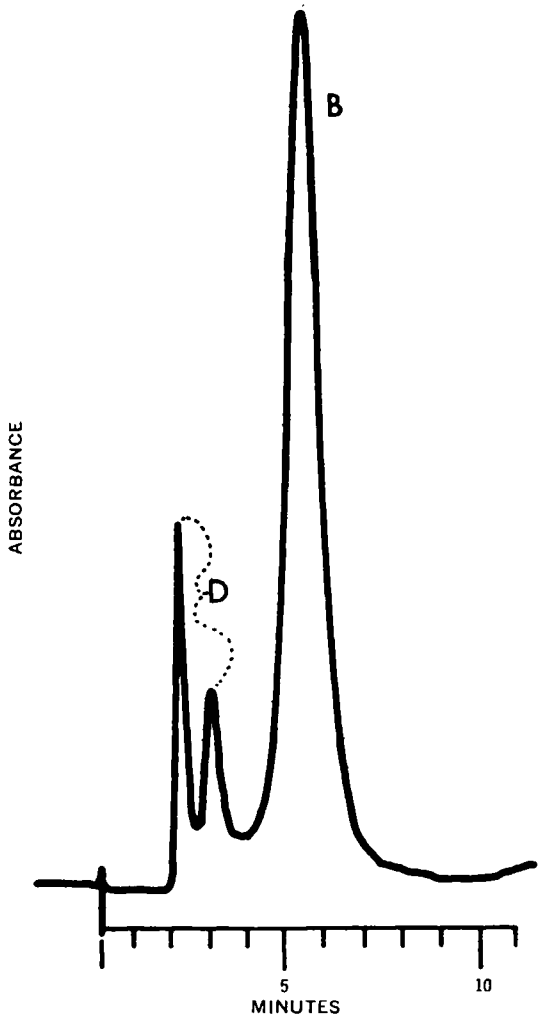

Figure 2—*Chromatogram of a contaminated bulk sample of ampicillin trihydrate. Key: B, ampicillin; and D, unidentified impurities.*

EXPERIMENTAL

Materials—A liquid chromatograph[1] was used with a UV detector; the flow rate was approximately 1.0 ml./min. A strong anion-exchange column[2], 1.0 m. by 2.1 mm. i.d., was obtained already prepared. Reagents were 0.01 *M* KH_2PO_4 buffer, pH 6.5; chloroform, reagent grade; and ampicillin trihydrate, FDA working standard.

Assay Procedure—Place the contents of 10 nitrofurantoin (50–100 mg.) capsules, or the powder from 10 ground tablets, into a 15-ml. centrifuge tube. Add 5.0 ml. of water and shake. Centrifuge the suspension, and decant the supernatant layer through a funnel containing a wad of glass wool into a second 15-ml. centrifuge tube. Add 10.0 ml. of chloroform to the second tube and shake vigorously. Allow the aqueous and chloroform layers to separate; then inject 5.0 μl. of the aqueous layer into the chromatograph for the ampicillin analysis.

Using an ampicillin reference standard at a concentration of 2.0 mg./ml. in distilled water, prepare a standard curve over a 1.0–10.0-mcg. range by chromatographing aliquots ranging from 0.5 to 5.0 μl. Obtain the peak area of each chromatogram by the method of peak height times width at half-height, and calculate the results by the following formula: micrograms ampicillin per dose unit tested = [(micrograms ampicillin from standard curve) (microliters total volume of solution)]/[(number of dose units tested) (microliters sample solution injected)].

RESULTS AND DISCUSSION

This method is quantitative for gross contamination of ampicillin in nitrofurantoin over a range of 1.0–10.0 mcg. of ampicillin. The standard curve is linear over this range. The constant retention time for ampicillin is an index of its identity.

The aqueous ampicillin solution must be extracted with chloroform to remove undefined chromophoric substances which gave a retention time very close to that of ampicillin. Because ampicillin is very water soluble, recovery was satisfactory. As shown in Fig. 1 (I), the ampicillin peak is well resolved from that of nitrofurantoin.

The sensitivity of this procedure is limited by the fact that 1.0 mcg. is the smallest amount of ampicillin that can be detected and quantitated accurately in the instrument used. Control samples were spiked at this level with 5.0 ml. of an aqueous solution containing 0.2 mg./ml. of ampicillin. A contamination of 0.1 mg./nitrofurantoin dose was assumed, so it was necessary to use 10 doses/5.0 ml. of water. The chromatogram of a sample of nitrofurantoin spiked at 0.1 mg. of ampicillin/dose is shown in Fig. 1 (I). Recovery of ampicillin from the spiked control sample was 94.0%.

Figure 1 (II) shows a chromatogram of nitrofurantoin capsules in which no ampicillin was detected. Figure 1 (III) shows a chromatogram of a sample of a nitrofurantoin capsule that was found to be contaminated with 158 mcg. of ampicillin/capsule dose.

Despite the limited sensitivity of this procedure, it provides useful data that cannot be obtained by other known methods. This procedure also makes it possible to perform identity studies by collecting fractions corresponding to the respective peaks in the chromatogram. The peaks preceding that of ampicillin could be collected separately or together and subjected to further analysis. The procedure may also be used to determine ampicillin in other drug formulations if excipients are removed in the preparative phase of the method to avoid exceeding the load capacity of the strong anion-exchange column as stated by the manufacturer.

This method has been used to examine ampicillin samples for purity. The chromatogram for the ampicillin FDA working standard and some commercial bulk samples showed a single, symmetrical peak, whereas other commercial ampicillin bulk samples yielded multiple-peaked chromatograms (*e.g.*, Fig. 2), indicative of the presence of impurities. These findings will be further investigated.

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[1] Model 830, E. I. duPont de Nemours and Co., Wilmington, Del.
[2] E. I. duPont de Nemours and Co., Wilmington, Del.

Estimation of Diiodohydroxyquin in Nonaqueous Media

K. L. K. PARANJOTHY* and S. K. BANERJEE▲

Abstract □ A method for estimating diiodohydroxyquin in dosage forms by nonaqueous titration was developed. Acetic anhydride was the most suitable solvent for titration.

Keyphrases □ Diiodohydroxyquin formulations—nonaqueous titrimetric analysis □ Titrimetry, nonaqueous—analysis, diiodohydroxyquin

The development of new drugs has encouraged the introduction of new methods of analysis and control. The importance of assay methods involving nonaqueous titration cannot be overemphasized. Since the estimation of active ingredients in medicinal dosage forms may not be carried out as smoothly as with active ingredients in the absence of excipients and other inactive additives, some modifications in the method of estimation may be necessary.

Diiodohydroxyquin (I) is a well-known amebicide. It has been recommended in the treatment of lambliasis, balantidal dysentery, and trichomonas vaginitis. Reports have appeared on the estimation of diiodohydroxyquin by colorimetry (1), polarography (2), the oxygen flask method (3), and iodometry (4). Feltkamp (5) determined iodochlorhydroxyquin (5-chloro-7-iodo-8-hydroxyquinoline) by nonaqueous titration. Some of the mentioned methods have been made official, but no work seems to have been reported for the estimation of I by nonaqueous titration.

Table I—Analysis of I in Dosage Forms (Synthetic Mixture)

Synthetic Tablet Mixture	Mixture Constituents	Amount[a], mg.	Recovery[b], %
1	I	300	100.4 ± 0.3
	Starch	30	
	Talc	2	
	Lactose	60	
2	I	300	101.1 ± 0.32
	Sugar	20	
	Sodium alginate	10	
	Starch	10	
	Stearic acid	2	
	Lactose	50	

[a] About 300 mg. accurately weighed I was taken each time. [b] Each figure represents the mean of five readings.

Table II—Analysis of I in Tablets (Manufactured Preparations)

Manufacturer	Drug	Labeled Amount	Recovery[a], %
—	I	—	100.1 ± 0.6
A	I	300 mg./tablet	100.15 ± 0.32
B	I	300 mg./tablet	100.32 ± 0.18

[a] Each figure represents the mean and standard deviation of at least five readings.

In the present study, an attempt was made to find a suitable method of estimation of I as such and in dosage forms in nonaqueous media.

EXPERIMENTAL

A pH meter[1] using a glass electrode[2] and a saturated calomel electrode was used. The aqueous potassium chloride solution in the saturated calomel electrode was replaced by a saturated solution of lithium chloride in acetic acid. The electrode pair was dipped in acetic anhydride for at least 24 hr. before use. The solvents and reagents used were of analytical reagent grade. Malachite green (0.3%) solution in acetic acid was used as the indicator.

Estimation of I in Dosage Forms—About 300 mg. of accurately weighed I was placed in a conical flask and dissolved in 30 ml. of acetic anhydride by heating. The solution was cooled to room temperature, 3–5 drops of Malachite green indicator was added, and the mixture was titrated by 0.1 *N* perchloric acid in acetic acid to a yellow end-point. A blank was performed, and an appropriate correction was made. Each milliliter of 0.1 *N* perchloric acid is equivalent to 0.039698 g. of diiodohydroxyquin. (The end-point was checked potentiometrically using glass and modified calomel electrode pairs.)

Synthetic Mixture—About 300 mg. of accurately weighed I was placed in a conical flask. The various excipients were added in the amounts occurring generally in tablet formulations. Acetic anhydride (30 ml.) was added, and the solution was heated to dissolve the drug (excipients settled). The titration was performed as described for the estimation of I in pure form, commencing with the words: "3–5 drops of Malachite green . . . ."

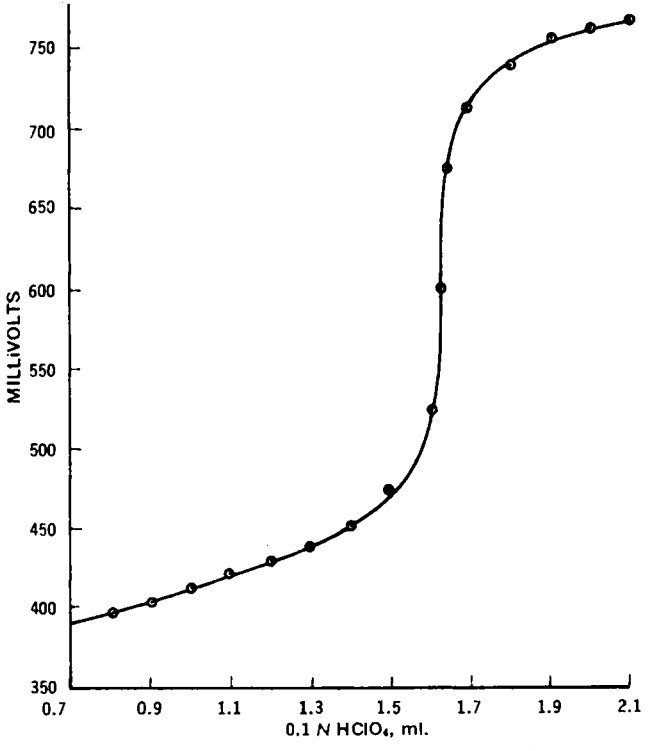

Figure 1—*Titration curve of diiodohydroxyquin (67.5 mg.).*

[1] Systronics Type 322.
[2] Toshniwal.

Table III—Analysis of I in Dosage Forms (Manufactured Preparations)

Drug	Labeled Amount	Recovery[a], % Proposed Method, Non-aqueous Titration	Official Method	Colorimetry
I	—	100.1	97.3	99.1
I	300 mg./tablet	100.3	98.2	99.3

[a] Each figure represents the mean of at least five readings.

Manufactured Preparations—Twenty tablets were weighed and reduced to a fine powder. An accurately weighed quantity of powder, equivalent to 300 mg. of I was added to 30 ml. of acetic anhydride. The mixture was then gently heated (excipients settled). After cooling to room temperature, the titration was performed as described for the estimation of I in pure form, commencing with the words: "3–5 drops of Malachite green. . . ."

RESULTS AND DISCUSSION

The nature of the titration curve of I is shown in Fig. 1. In the preliminary trials, the estimation of this drug in acetic acid resulted in overestimation, probably due to the consumption of perchloric acid by certain excipients. However, on using acetic anhydride as the solvent with Malachite green as the indicator, the end-point was sharp and the recoveries were better than with acetic acid. The color change of the indicator was checked potentiometrically. Tables I and II show the recovery.

Table III shows the results obtained by the proposed method, by the official method (4), and by colorimetry (1). It is apparent from these data that good recoveries were achieved by nonaqueous titration. The official method is a time-consuming and cumbersome procedure with lower recoveries. In the colorimetric method, Beer's law is obeyed in a very narrow range (0.05–0.3 mg.) and the recovery of the drug is not satisfactory. The proposed nonaqueous method of titration is a rapid and simple technique, giving accurate results.

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Assay of Thiotepa by PMR Spectrometry

LINDLEY A. CATES

Abstract □ A rapid (10 min.), accurate, and precise (SD = 0.6%) analytical method for thiotepa and thiotepa for injection involving PMR is described. The procedure, which was tested on two spectrometer models, employs an inexpensive solvent and internal standard and does not require preparative treatment.

Keyphrases □ Thiotepa—PMR analysis, aziridinyl protons □ Aziridinyl derivatives—PMR analysis of thiotepa □ PMR spectroscopy—analysis, thiotepa

During the characterization of potential oncolytics, the methylene protons of aziridinyl moieties present in some of these agents were readily detected by proton magnetic resonance (PMR) spectrometry. This observation led to an interest in the quantitative analysis of an official drug by this instrumental method. Thiotepa [*N,N',N''*-triethylenethiophosphoramide, tris(1-aziridinyl)phosphine sulfide] meets the criteria of a good candidate for assay by PMR spectrometry because it is soluble in an inexpensive, nondeuterated solvent and gives a simple spectrum. In addition, the assay procedure presented here, in contrast to other PMR drug analysis (1, 2), does not require processing of the sample prior to instrumental examination; in view of this drug's chemical lability, this is especially advantageous. Only additions of an internal standard and solvent to the sample are required. Although thiotepa is a low dosage pharmaceutical, giving solutions of about 1.9% concentration in this assay, the integral values for the peak areas can be measured with sufficient accuracy and precision.

All of these factors provide for a simple, accurate, and rapid analytical method for thiotepa and this pharmaceutical's parenteral formulation. The USP XVIII procedures for these items involve lengthy and laborious titrimetric and IR spectrophotometric methods, respectively. To determine the capabilities of different

Table I—Analysis of Thiotepa Standards by PMR

Sample	Internal Standard, mg.	Thiotepa, Added, mg.	Thiotepa, Found, mg.	Thiotepa, Recovered, %
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3	24.26	14.76	14.71	99.7
4	23.88	14.83	15.12	102.0
5	23.15	17.80	18.01	101.2
6	23.78	15.37	15.41	100.3
7	23.38	15.07	15.37	102.0
8	23.66	15.20	15.38	101.2
Mean and standard deviation			101.1 ± 0.6	

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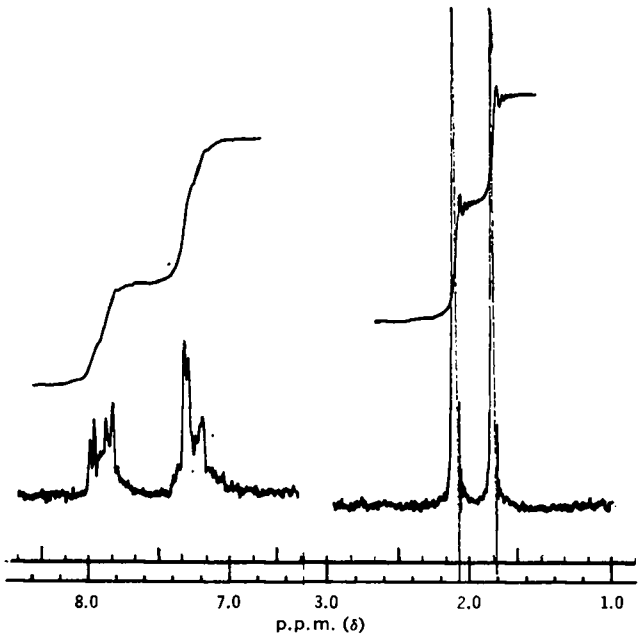

Figure 1—*PMR spectrum of benzoic acid (left) and thiotepa (right) in carbon tetrachloride.*

types of PMR spectrometers in this quantitative evaluation, two models (I[1] and II[2]) were employed in most assays.

EXPERIMENTAL

The following were used: standard, thiotepa[3]; internal standard, benzoic acid USP[4]; samples, thiotepa for injection USP[5]; and solvent, carbon tetrachloride[6].

To the standard or sample[7], add about 24.00 mg.[8] of internal standard, accurately weighed, and 0.8 ml. of solvent. Stopper and shake for 2 min. and then filter, *via* a cotton pledget, at least 0.4 ml. into a PMR tube. After placing the tube in the spectrometer, adjust the spin rate to eliminate spinning side bands and the power level and amplitude to obtain integral values corresponding to 20–30 mm. Integrate the peak areas occurring between δ 1.83–2.15 and δ 7.10–8.09 at least five times.

The percent of drug may be calculated using the following equation:

$$\text{percent thiotepa} = \frac{A_t}{A_b} \times \frac{EW_t}{EW_b} \times \frac{W_b}{W_t} \times 100 \quad \text{(Eq. 1)}$$

where:

A_t = average integral value for thiotepa
A_b = average integral value for benzoic acid
EW_t = molecular weight of thiotepa/12 = 15.77
EW_b = molecular weight of benzoic acid/5 = 24.42
W_b = weight in milligrams of benzoic acid
W_t = weight in milligrams of thiotepa added or declared

RESULTS AND DISCUSSION

The PMR spectrum of pure thiotepa shows only a doublet (δ 2.00, J = 16 Hz. using trimethylsilane as reference. Fig. 1). Benzoic acid was chosen as the internal standard since it is a pure, stable, inexpensive, readily obtainable material whose aromatic protons absorb at a point (complex of peaks at δ 7.10–8.09) where there is no interference with the absorption peaks caused by the drug (Fig. 1). The peak areas produced by 24 mg. of the internal standard are approximately the same as those resulting from 15 mg. of thiotepa.

The results of the quantitative determination of a series of thiotepa standards are shown in Table I. Considering the small sample weight, the procedure is unexpectedly accurate with a good precision of ±0.6%. In addition, it provides a means of identification and detection of impurities. Table II summarizes the analysis of commercial preparations. There is good agreement with declared values using both instruments, with Model II uniformly giving slightly higher values. Measurements shown in both tables are within the USP monograph's limits of 97.0–102.0 and 95.0–110.0% for thiotepa and thiotepa for injection, respectively.

Another comparison of measurements taken with Models I and II is indicated in Table III. The samples did not decompose at the same rate, but both instruments gave similar results.

Sample 9 was examined (II) after 2 weeks at room temperature to indicate the presence of 54.4% thiotepa. Neither the spectrum nor the integral peak area value of the internal standard in this sample showed change, but two broad areas of absorption resulting from materials of undetermined nature appeared at about δ 3.9–4.6 and δ 2.5–3.9, the latter interfering slightly with the baseline of thiotepa's integral values.

Table II—Assay of Thiotepa in Commercial Preparations by PMR

Sample[a]	Internal Standard, mg.	Percent of Declared		
		Model I	Model II	Difference
9	22.24	101.1	100.9	+0.8
10	24.73	102.1	104.4	+2.3
11	24.71	100.6	102.7	+2.1
12	24.26	100.4	100.6	+0.2
13	24.84	102.9	103.4	+0.5
14	23.35	100.9		
15	23.38	100.7		
Mean and average difference		101.2	102.3	+1.2

[a] Declared amount is 15 mg.

Table III—Determination of Decomposed Thiotepa Standards

Sample[a]	Percent Found		
	Model I	Model II	Difference
1	91.0	89.4	−0.6
2	87.9	87.8	−0.1
3	85.7	86.1	+0.4
4	88.5	87.3	+1.2
5	92.8	91.6	−0.8
7	91.6	91.9	+0.3
Mean and average difference	89.6	88.9	±0.6

[a] Kept at room temperature for 7 days.

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[1] Varian T-60.
[2] Varian EM 360.
[3] Provided through the courtesy of Lederle Laboratories, Pearl River, N. Y.
[4] Fisher Scientific Co., Fair Lawn, N. J.
[5] Thiotepa Parenteral, 15 mg./vial, Lederle Laboratories.
[6] Distillation Products Industries, Rochester, N. Y.
[7] Contains sodium bicarbonate USP (50 mg.) and sodium chloride USP (80 mg.).
[8] Weighed using a Mettler Macro Gramatic balance, B-5.

Spectrophotometric Analysis of Primary Aliphatic Amines with Dichlone

A. A. ABOU-OUF, ALY M. TAHA▲, and M. B. SAIDHOM

Abstract □ A spectrophotometric method is described for the determination of some primary aliphatic amines of pharmaceutical interest *via* reaction with dichlone (2,3-dichloro-1,4-naphthoquinone). A mixture of an ethanolic solution of the amine, or amine salt and sodium bicarbonate, with the chromogenic reagent was heated at 65° for 25 min. After acidification to pH 4, the majority of amines yielded an orange-colored product, λ_{max} 475 nm. Some compounds reacted without heat, yielding other colors such as rose (500 nm. with piperazine), yellow (420 nm. with cysteine), and blue (630 nm. with isoniazid) suggesting different products. Molar absorptivities ranged from 900 to 5000. Under the described assay conditions, only primary aliphatic and cyclic secondary amines reacted. Primary aromatic (except some readily oxidizable ones), secondary or tertiary aliphatic, and/or aromatic amines were found not to interfere. The pharmaceutical compounds assayed included histamine, norepinephrine, serotonin, neomycin, leucine, cysteine, aminocaproic acid, ethanolamine, aminophylline, piperazine, and isoniazid. The amines are assayed in the 0.005–0.15-mg./ml. range with a relative standard deviation of 0.02–0.03. The possible composition of the colored products is discussed.

Keyphrases □ Amines, primary aliphatic—colorimetric analysis with dichlone □ Dichlone—used in colorimetric analysis of primary aliphatic amines □ Colorimetry—analysis, primary aliphatic amines using dichlone

The reaction of dichlone (2,3-dichloro-1,4-naphthoquinone) with a number of aliphatic and aromatic amines to yield colored products was reported (1–4) in the course of synthesis of new compounds and the chromatographic separation of amines. Dichlone, a potent fungicide (5), is also a minor dye intermediate (6). There are, however, no reported examples of the quantitative determination of amines using dichlone.

In this work, a spectrophotometric procedure is proposed to assay primary aliphatic amines *via* reaction with dichlone in ethanolic solution. The selectivity, sensitivity, and precision of the proposed method were determined. The scope and limitations relative to pharmaceutical preparations were explored.

EXPERIMENTAL

Equipment—Spectra and absorbance measurements were made using two spectrophotometers[1]. A constant-temperature bath maintained at 65 ± 2° was utilized for accelerating color development.

Reagents and Chemicals—Dichlone was obtained commercially[2]. All other chemicals used were analytical reagent or pharmaceutical grade obtained from various manufacturers and were purified, when necessary, by redistillation, recrystallization, or chromatography before use.

Dichlone Reagent—The desired concentration was 1×10^{-6} mole/ml. (0.227 mg./ml.). The reagent (22.7 mg.) was dissolved in warm ethanol, cooled, and diluted to 100 ml. with ethanol. This solution was stable for 4 weeks.

Amines—Fresh solutions of the amines or amine salts (0.025 mg./ml.) were prepared daily in ethanol and kept at 4°.

[1] Unicam SP 8000 and Beckman DB.
[2] Eastman Organic Chemicals.

Table I—Influence of Chemical Structure

Amine[a]	Color	λ_{max}, nm.
Primary aliphatic:		
Ammonia[b]	Orange	465
Methylamine	Orange	475
Ethanolamine	Orange	472
Ethylenediamine[b]	Yellow	425
n-Butylamine	Orange	475
Benzylamine	Orange	478
Primary aromatic:		
Aniline	Red	495
o-Toluidine	No color	—
1-Naphthylamine	No color	—
o-Aminophenol	Yellow	420
p-Aminophenol	Pink	505
p-Nitroaniline	No color	—
p-Anisidine	No color	—
p-Phenylenediamine	Pink	505
Benzidine	Purple	510
o-Tolidine	No color	—
p-Dimethylaminoaniline	Purple	515
Secondary amines:		
Diethylamine[c]	No color	—
Diethanolamine[c]	No color	—
N-Methylaniline	No color	—
Piperidine[b]	Rose	500
Morpholine[b]	Rose	500
Piperazine[b]	Rose	500
Tertiary amines:		
Triethylamine[c]	No color	—
Triethanolamine[c]	No color	—
N,N-Diethylaniline	No color	—
p-Dimethylaminobenzaldehyde	No color	—
Aromatic heterocycles:		
Pyridine	No color	—
Quinoline	No color	—
Quaternary ammonium salts:		
Choline citrate	No color	—
Quinaldine ethiodide	No color	—

[a] Using 1 mg./ml. in ethanol. [b] Reacted in the cold. [c] Purified by chromatography.

Bicarbonate Solution—Sodium bicarbonate (840 mg.) was dissolved in glass-distilled water and diluted to 100 ml. with the same water; the final concentration was 8.4 mg./ml.

Procedure—One milliliter of the amine solution was mixed with 1.0 ml. of the reagent solution. With amine salts, 1.0 ml. of the solution of the amine hydrochloride or sulfate was mixed with 1.0 ml. of sodium bicarbonate solution and 1.0 ml. of the reagent solution. In either case, the mixture was maintained at 65 ± 2° for 25 min. Then the solution was cooled to room temperature, the pH was adjusted to 3–4 with 10% hydrochloric acid, and the solution was diluted to 10 ml. with ethanol in a volumetric flask. The absorbance of this solution was measured at 475 nm., or at the λ_{max} if different, against a reagent blank similarly treated.

RESULTS

Influence of Chemical Structure—The colors produced on reacting dichlone with various amines are indicated in Table I. The colors obtained when extending the reaction to selected pharmaceutical amines are given in Table II.

Quantitative Determination of Amines—Table III summarizes the data of the quantitative analysis of selected amines of biological and pharmaceutical interest by the proposed spectrophotometric method. These amines assayed at least 100% by established and

Table II—Application to Pharmaceuticals

Amine[a]	Color	λ_{max}, nm.	ϵ_{max}
Histamine dihydrochloride	Orange	475	1400
Norepinephrine tartrate	Orange	478	1800
Epinephrine tartrate	No color	—	—
Serotonin creatinine sulfate	Orange	478	1600
Amphetamine sulfate	Orange	475	900
Phenylephrine hydrochloride	No color	—	—
Glucosamine	Orange	475	1500
Neomycin sulfate	Orange	472	1800
Kanamycin sulfate	Orange	472	1300
Streptomycin sulfate	No color	—	—
Glycine	Orange	472	900
Leucine	Orange	475	1200
Phenylalanine	Orange	478	1800
Cysteine[b]	Yellow	420	1300
Aminocaproic acid	Orange	475	1800
Aminophylline[b]	Yellow	425	1600
Theophylline	No color	—	—
Isoniazid[b]	Blue	630	5000
Phenyltoloxamine dihydrogen citrate	No color	—	—
Chloroquin phosphate	No color	—	—
Benzocaine	No color	—	—
Sulfanilamide	No color	—	—

[a] Using 1 mg./ml. solution. [b] Reacted without heat.

compendial methods of analysis as follows: histamine, reaction with 2,4-dinitrofluorobenzene (7); serotonin, colorimetric analysis with 1-nitroso-2-naphthol (8); neomycin, the BP microbiological method using *Bacillus pumilus* (9); piperazine citrate and leucine, nonaqueous titrimetry (USP procedure) (10); aminophylline (for ethylenediamine content), titration with 0.1 *N* sulfuric acid using bromcresol green as indicator (11); and isoniazid, iodometric analysis (12).

Standard Curves—Standard curves can be constructed by plotting observed absorbance readings *versus* the volume taken of equimolar concentrations of various amines in the range 0.005–0.150 mg./ml. (1×10^{-5}–1×10^{-7} mole/ml.). In all cases, Beer's law held for the system.

Analysis of Mixtures—Table IV illustrates the analytical data obtained for various mixtures of amines of biological and pharmaceutical interest analyzed for the primary aliphatic amine component.

DISCUSSION

Reaction Involved and Influence of Chemical Structure—In the analytical procedure presented, dichlone (I) reacts with a primary aliphatic amine to yield an orange color. Chemically, the colored product is most likely a 2-amino-3-chloro-1,4-naphthoquinone derivative (II), formed by 1,4-addition to the quinone followed by reoxidation with excess reagent (13). This is in agreement with published data (14) concerning the reaction of dichlone with some aliphatic amines. Further verification of this assumption was obtained by comparing the spectrum of a typical orange product (dichlone with *n*-butylamine) with that of 2-amino-1,4-naphthoquinone (III) (Fig. 1). The aminonaphthoquinone was synthesized by an independent route (15) from 1,4-naphthoquinone and sodium azide in acetic acid. The two spectra are practically superimposable, suggesting the same basic chromophore.

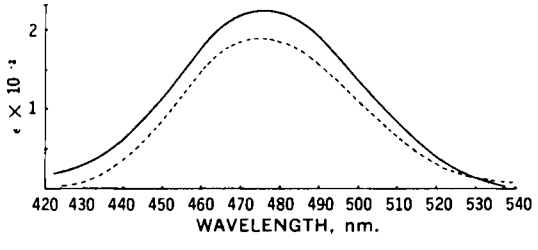


Figure 1—*Spectra of 2-amino-1,4-naphthoquinone and reaction product of dichlone with* n-*butylamine. Key:* ——, *2-amino-1,4-naphthoquinone; and* - - -, *reaction product.*

Table III—Quantitative Application

Compound	Amount, mg. Taken	Amount, mg. Found[a]	Error, %	Standard Deviation, %
Histamine	0.180	0.1811	+0.60	±2.61
Serotonin	0.200	0.2015	+0.75	±2.69
Neomycin	0.250	0.2480	−0.80	±1.9
	0.750	0.7540	+0.53	±2.2
Piperazine	0.200	0.2025	+1.25	±3.1
Leucine	0.400	0.3974	−0.65	±2.8
Aminocaproic acid	0.350	0.3445	−1.05	±2.65
Aminophylline	0.600	0.6055	+0.90	±2.68
Isoniazid	0.150	0.1512	+0.80	±3.2

[a] Mean value of five determinations.

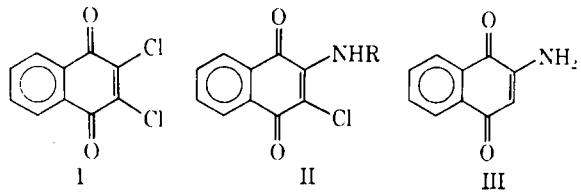
Table IV—Analysis of Mixtures

Mixture Composition	Milligrams per Milliliter	Primary Aliphatic Amine Found[a], mg./ml.	Theory, %
Norepinephrine	0.050	0.0495	99.0
Epinephrine	0.050	—	—
Histamine	0.025	0.0251	100.4
Epinephrine	0.050	—	—
Neomycin[b]	0.100	0.100	100.0
Streptomycin	0.100	—	—
Isoniazid	0.005	0.0049	98.0
Sodium *p*-aminosalicylate	0.050	—	—
Norepinephrine	0.050	0.0498	99.6
Benzocaine	0.050	—	—
Phenyltoloxamine	0.050	—	—

[a] Mean value of three determinations. [b] Mixture of commercial neomycins (A, B, and C).

Dichlone was reported to react with primary aromatic amines under certain conditions of synthesis (1, 16, 17). Under the analytical conditions described, however, only primary aliphatic amines reacted to a measurable extent. Some primary aromatic amines with sensitive nuclei (*e.g.*, *p*-aminophenol, *p*-phenylenediamine, and *p*-dimethylaminoaniline) gave red colors (Table I). This was probably due to oxidation by the quinone reagent rather than reaction by 1,4-addition, since identical colors were obtained when these compounds were treated with inorganic oxidizers such as potassium persulfate. Failure of sulfanilamide, 1-naphthylamine, *o*-toluidine, and benzocaine to yield colors (Tables I and II) supports the view that, under the mild conditions of the procedure, primary aromatic amines do not interfere. Similarly, secondary and tertiary aliphatic or aromatic amines failed to yield measurable colors (Tables I and II).

Some compounds (*e.g.*, cyclic secondary aliphatic amines, ethylenediamine, cysteine, and isoniazid) reacted rapidly without heat to yield colors different from the orange color obtained with the majority of amines (Fig. 2). This finding strongly suggests different types of products. In the case of cysteine, the product is probably a thioether at the 3-position of the quinone, in line with the analogous reaction of 2-methyl-1,4-naphthoquinone (K_3) with sulfhydryl compounds (thioglycollic acid and cysteine) to form a thioether linkage with a characteristic absorption at 420–430 nm. (18, 19). With the present data alone, however, it is difficult to reach a conclusion concerning the true nature of these colored products. After heating at 65° for 20–30 min., these different colors changed to orange, λ_{max} 475 nm.



I II III

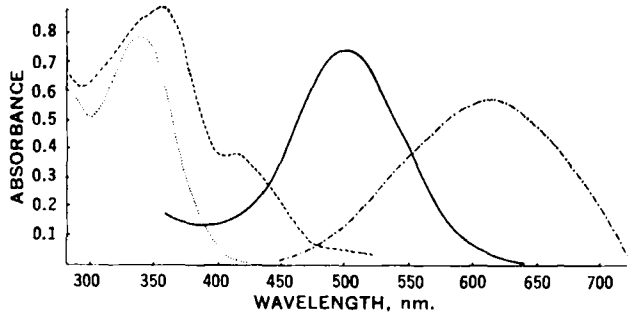


Figure 2—*Spectra of dichlone and its reaction products. Key: · · ·, dichlone (1 × 10⁻⁶ mole/ml.); - - -, with cysteine (3 × 10⁻⁶ mole/ml.); – – –, with piperazine (5 × 10⁻⁶ mole/ml.); and –·–, with isoniazid (4 × 10⁻⁷ mole/ml.).*

Quantitative Applications—Data in Tables III and IV reveal the suitability of the proposed method for the determination of pharmaceutical primary amines in the presence of primary aromatic and/or secondary and tertiary aliphatic amines in amounts of 0.005–0.150 mg./ml. with a standard deviation of ±2–3%.

Reagent blank readings at wavelengths of maximum absorbance are very low (Fig. 2). The main absorbance of the reagent is at 345 nm. No interference by excess unreacted reagent was noticed. This was further verified by the complete extraction of unreacted dichlone with ethylene chloride from the acidified (pH 3–4) aqueous colored solution. The color remained in the acidified aqueous layer because of protonation of the amino group (Structure II). No difference in absorbance readings between extracted and unextracted samples was observed.

Color Development and Stability—Color development at room temperature was too slow, but formation of the color was greatly accelerated at 60–70°. Maximum color intensity was obtained after 25 min. (Fig. 3) and was unchanged after 24 hr. at room temperature.

Limitations—The proposed method is fairly sensitive and selective for primary aliphatic amines. The procedure, however, must be considered nonspecific with regard to degradation products (unless degradation involves loss of the primary amine function) as well as other potential interfering substances.

Although cyclic secondary amines reacted, the probability of their presence with primary aliphatic amines in the same biomedia or dosage form is slim. Furthermore, these cyclic amines exhibited an absorbance maximum different from 475 nm. (at 500 nm.), so any problems arising from overlap of the spectra for the two species may be overcome by the use of differential analysis.

CONCLUSION

The analytical method presented is essentially a microprocedure with fair sensitivity (0.005–0.150 mg./ml.), good accuracy (from −0.9 to +1.3% error), and precision (0.02–0.03 *RSD*). The method offers a relatively simple and rapid means of analysis of some pharmaceutical primary aliphatic amines in admixture with secondary or tertiary aliphatic or primary aromatic amines.

The reagent is available commercially or easily synthesized (20), and its solutions are stable at room temperature for convenient periods. The colors formed are also stable.

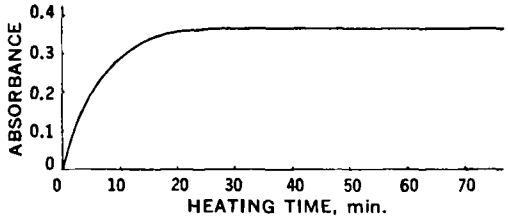


Figure 3—*Rate of color development. Histamine (5 × 10⁻⁶ mole/ml.) with dichlone (1 × 10⁻⁶ mole/ml.).*

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Rapid Quantitative GLC Analysis of Digoxin

ARTHUR H. KIBBE and OSCAR E. ARAUJO▲

Abstract □ A quantitative method of assaying digoxin by GLC was developed. The pure drug in both tablet and powder form can be assayed rapidly and accurately by this procedure.

Keyphrases □ Digoxin tablets and powder—GLC analysis □ GLC—analysis, digoxin tablets and powder

The advantages of GLC involve its speed of operation, its high degree of resolution, and its potential to yield quantitative results.

Jelliffe and Blankenborn (1) were the first to use GLC to separate cardiac glycosides. Several investigators have since used GLC qualitatively as a means of identifying these glycosides (2–4).

Various quantitative colorimetric assays for digoxin have been reported, and subsequent attempts at automating these methods have been undertaken (5–9). The GLC analysis of digoxin on nonselective phases has been studied with various degrees of success.

Watson and Kalman (10) assayed plasma levels of digoxin by GLC. Their procedure took 5 hr./assay and involved both TLC and GLC steps. Watson and Kalman (10) used electron-capture detection, while Wilson *et al.* (2) used the trimethylsilyl ethers of cardenolides which required high temperature and high flow rates of the carrier gas. Tan (3) questioned the formation of the silyl ethers reported by Wilson *et al.* and others.

It is apparent that the quantitative GLC methods of analysis reported rely on the production of derivatives and require much time as well as laborious manipulative steps. The method proposed in this investigation eliminates all of these drawbacks.

EXPERIMENTAL

Equipment—A chromatographic system[1] with a flame-ionization detector was used. The gas flow rates were: hydrogen, 35 ml./min.; air, 300 ml./min.; and carrier gas (nitrogen), 50–100 ml./min. Operating conditions were: oven temperature, 285°; and injection port and detector temperature, 330°. All injections were made with a 5-μl. syringe[2].

Columns—Three types of columns were used. Column A was a 2-m. glass U-tube (2 mm. i.d.) packed with 2.5% OV-1 on 80–100-mesh Chromosorb A[3]. Columns B and C were 0.5-m. copper U-tubes (4 mm. i.d.) packed with 2.5% OV-1 on 80–100-mesh Chromosorb A and 3% OV-17 on 80–100-mesh Chromosorb A, respectively.

Reagents—The silylating reagent was prepared just prior to use by mixing 1 ml. of trimethylchlorosilane[4] and 10 ml. of hexamethyldisilazane[4] with 10 ml. of dry pyridine as a solvent. The pyridine was stored over potassium hydroxide to ensure dryness. The hexamethyldisilazane and trimethylchlorosilane were stored under nitrogen and extracted from sealed vials with a syringe. The volume was then replaced with dry nitrogen gas. The sample to be silylated

[1] Varian model 2100.
[2] Hamilton Co., Whittier, Calif.
[3] Applied Science Laboratories, Inc., State College, Pa.
[4] Pierce Chemical Co.

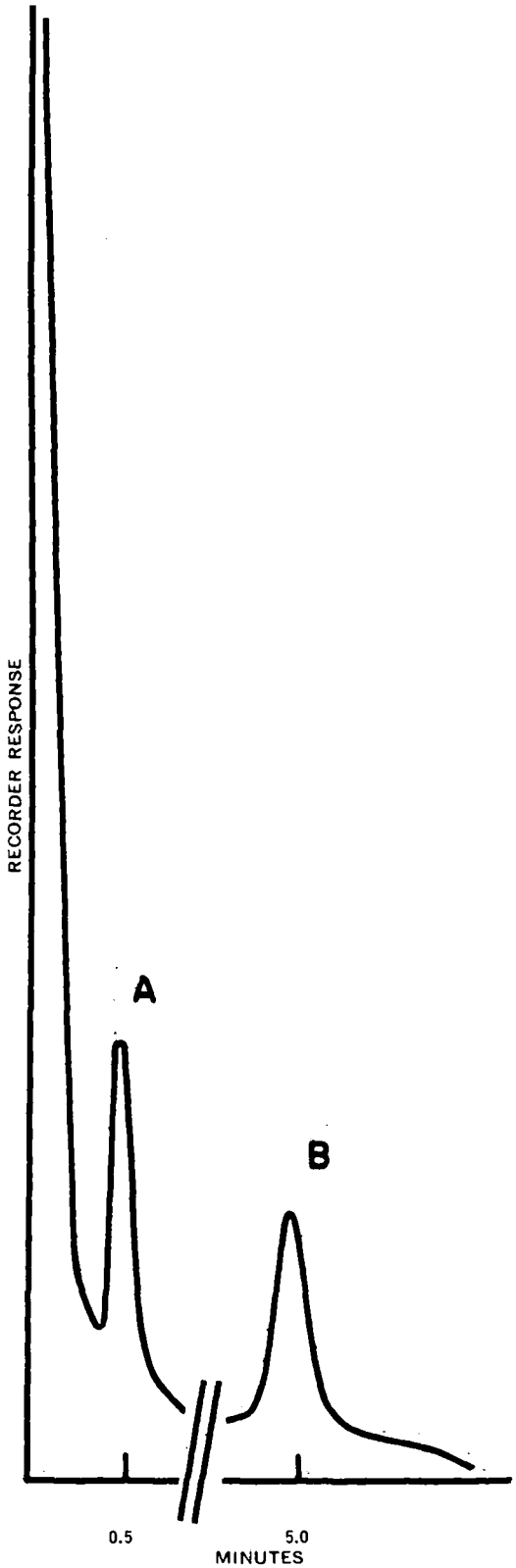

Figure 1—*Representative chromatogram of a mixture of: (A) cholesterol and (B) digoxigenin.*

Table I—Retention Time (Minutes)

	Column A	Column B	Column C
Cholesterol (unsilylated)	2.0	0.67	0.5
Cholesterol (silylated)	2.0	0.67	0.5
Digoxigenin (unsilylated)	15.0	5.33	3.67
Digoxigenin (silylated)	15.0	5.0	3.67

was made to react with 1 ml. of the reagent at room temperature before being injected into the gas chromatograph.

TLC—The purity of the digoxin[5] and digoxigenin[6] used was verified by TLC using two solvent systems: cyclohexane–acetone–acetic acid (49:49:2) and ethyl acetate–water–methanol (80:5:5). All solvents were analytical grade.

Glass plates precoated with 0.2 mm. of silica gel G[3] were used. The spots were visualized by spraying with 50% aqueous sulfuric acid and heating at 110° for 2–3 min. A second more reliable visualization method was also employed by preparing a spray consisting of a mixture of a 3% aqueous solution of chloramine-T and a 25% alcoholic solution of trichloroacetic acid. The two solutions, in a ratio of 1:4, were mixed just prior to use. After spraying, the plate was heated in an oven at 110° for 7 min. The blue spot then became visible under UV light at a wavelength of 385 nm.

Internal Standard—Cholesterol was used as an internal standard in the amount of 0.02 mg.

Reference Standard—Digoxin and digoxigenin were both employed in amounts varying from 0.05 to 0.2 mg.

Sample Preparation—A sample of either 0.1 mg. of powdered digoxin or the equivalent amount in tablet form was dissolved in 2 ml. of a pyridine–water (50%, v/v) solution. A volume of 0.1 ml. of 0.1 *N* sodium hydroxide was added, and the mixture was heated on a steam bath for 30 min. to convert completely the digoxin to digoxigenin. The solution was then evaporated to drynesss and redissolved in 100 μl. of dry pyridine. An aliquot of 10 μl. of a stock solution containing 2 mcg./μl. of cholesterol in pyridine was added. From this solution, a 1-μl. sample was injected into the gas chromatograph and the areas under the curves were calculated by means of a disk integrator.

RESULTS AND DISCUSSION

A typical chromatogram for the unsilylated compounds is shown in Fig. 1. The results indicated that both silylated and nonsilylated compounds exhibit similar retention times using all three columns (Table I). From this standpoint, there appeared to be no advantage in silylating the compound. Due to the high temperature of operation, difficulty was experienced in maintaining a firm seal between Column A and the metal connections. The Teflon ferrel, which normally would be used for this purpose, began to melt at the temperatures at which the injector and detector were operated. In addition, the retention times were much longer for Column A than for Columns B and C.

The standard curve was generated by plotting A_D/A_C against W_D/W_C, where A_D and A_C are the areas under the curve for digoxigenin and cholesterol, respectively, and W_D and W_C are the respective weights for digoxigenin and cholesterol. The results along with the analysis of variance are shown in Table II.

Table II—Standard Curve Data For Digoxigenin–Cholesterol

$\frac{A_D}{A_C}$[a]	$\frac{W_D}{W_C}$[b]
3.48	16.47
2.08	10.83
2.50	8.95
1.51	7.58
0.89	6.55

Analysis of Variance

Source	*df*	Sum of Squares	Mean Square	*F* Ratio
Regression	1	22.68	22.67	29.66
Residual	4	3.06	0.76	
Total	5	25.72		

Variables in Equation

Coefficient	0.20
Standard error	0.0013
Intercept	0.057

[a] Area ratio (digoxin–cholesterol). [b] Weight ratio (digoxin–cholesterol).

As indicated under sample preparation, all the digoxin was hydrolyzed to digoxigenin. The latter can then be used indirectly to calculate the amount of digoxin in the sample by using a ratio of molecular weights. From the data in Table II, an unknown quantity of digoxigenin can be obtained by calculating W_D from Eq. 1:

$$\frac{A_D}{A_C} = 0.20\frac{W_D}{W_C} + 0.057 \qquad \text{(Eq. 1)}$$

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NMR Analysis of Pharmaceuticals X: Determination of Methsuximide and Phensuximide in Capsules

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Abstract □ A method for the quantitative analysis of methsuximide and phensuximide using NMR is reported. For samples of the pure drugs, results were accurate to 0.3% or less with standard deviations of 0.6% or less. Analysis of capsules of each drug by NMR and NF XIII procedures indicated a maximum difference of 1.5%, but generally the difference was less than 1.0%. This procedure is recommended for the analysis of these drugs since it provides good quantitative results together with a unique spectrum as confirmatory identification.

Keyphrases □ Methsuximide and phensuximide capsules—NMR analysis □ Phensuximide and methsuximide capsules—NMR analysis □ NMR spectroscopy—analysis, methsuximide and phensuximide capsules

Methsuximide (*N*,2-dimethyl-2-phenylsuccinimide, I) and phensuximide (*N*-methyl-2-phenylsuccinimide, II) are two succinimide derivatives which are pharmacologically active anticonvulsants. Their use is primarily in the control of petit mal epilepsy.

Several analytical approaches have been successfully applied to the isolation and determination of I and II in biological media. A quantitative procedure for the determination of both I and II in serum was developed by Huisman (1). The method involves nitration of the phenyl rings and separation of the products by TLC, followed by subsequent diazotization and coupling to produce a color measured at 550 nm. Gardner-Thorpe *et al.* (2) used TLC and examination under UV light to estimate the presence of some anticonvulsant succinimides in blood.

Other properties have been exploited to analyze for both I and II in pharmaceutical systems and as pure chemical entities. The assay procedures for II studied by Mativa (3) include titration, reaction in buffered medium to obtain a fluorescent product, and reaction with fuming nitric acid to form a colored product whose color intensity is read in an alkaline acetone solution. The extension of Mativa's technique to I was investigated (4) with satisfactory results.

The methods adopted by NF XIII (5) for the determination of both I and II in capsule dosage forms involve a chloroform extraction and subsequent UV light absorption measurement.

This paper describes a method involving NMR spectrometry[1] to determine I and II in capsule dosage forms. The method utilizes carbon tetrachloride[2] as the solvent for I and 10% methylene chloride[2] in carbon tetrachloride as the solvent for II, and hexamethylcyclotrisiloxane[3] (III) is employed as the internal standard for both drugs. Known mixtures and commercial capsules were analyzed by this technique. Quantitative analysis using NMR offers the advantages of speed, simplicity, and specificity for the active ingredients.

Table I—Determination of Methsuximide and Phensuximide in Standard Mixtures by NMR

Standard Mixture	Internal Standard[a], mg.	Methsuximide and Phensuximide Added, mg.	Found, mg.	Recovery, %
Methsuximide				
1	102.7	320.8	319.5	99.6
2	101.9	319.9	321.8	100.6
3	100.5	321.6	321.3	99.9
4	102.9	320.4	317.8	99.2
5	103.1	321.1	324.0	100.9
6	103.5	319.8	324.6	101.5
7	200.3	320.1	320.4	100.1
				100.3
				SD 0.6
Phensuximide				
1	105.6	319.4	322.4	100.9
2	104.9	321.5	319.6	99.4
3	105.8	321.0	320.7	99.9
4	104.5	319.9	322.5	100.8
5	103.9	320.5	319.9	99.8
6	185.0	325.0	324.4	99.8
				100.1
				SD 0.4

[a] Hexamethylcyclotrisiloxane.

EXPERIMENTAL

Standards and Samples—Methsuximide and phensuximide reference standards[4] and commercial methsuximide and phensuximide capsule preparations were used.

Procedure—Place a counted number of capsules into a 125-ml. glass-stoppered conical flask. Carefully crack the outer shell of each capsule with the end of a glass rod. Using a calculated amount of carbon tetrachloride for I or 10% methylene chloride in carbon tetrachloride for II, so that the final volume will contain the solute to be measured in a concentration of about 80 mg./ml., first wash down the end of the stirring rod to ensure quantitative retention of the sample powder and then add the remainder of the solvent to the flask. Add an accurately weighed amount of III, the internal standard, to the sample solution so that its final concentration is about 25 mg./ml. Stopper the flask and swirl, gently at first, to effect solution. Allow the outer shells and insoluble excipients to rise to the top and then transfer about 0.4 ml. of the bottom solution to an analytical NMR tube. Place this tube in an NMR spectrometer and obtain the spectrum, adjusting the spin rate so that no spinning side bands occur between 0.4 and 0.6 p.p.m. and between 6.8 and 7.7 p.p.m. using the δ-scale to express magnetic field strength. All peak field positions are referenced to tetramethylsilane (IV) at 0 p.p.m. Integrate the peaks of interest at least five times.

The amount of I or II may then be calculated as follows:

$$\frac{\text{mg. I or II}}{\text{capsule}} = \frac{A_s}{A_h} \times \frac{EW_s}{EW_h} \times \frac{\text{mg. III}}{\text{number of capsules}} \quad \text{(Eq. 1)}$$

[1] A Varian A-60 NMR spectrometer, equipped with a V-6031 variable-temperature probe having a six-turn insert, was used.
[2] Fisher Scientific Co., Fair Lawn, N. J.
[3] K & K Labs., Inc., Plainview, N. Y.
[4] Parke, Davis & Co., Detroit, Mich.

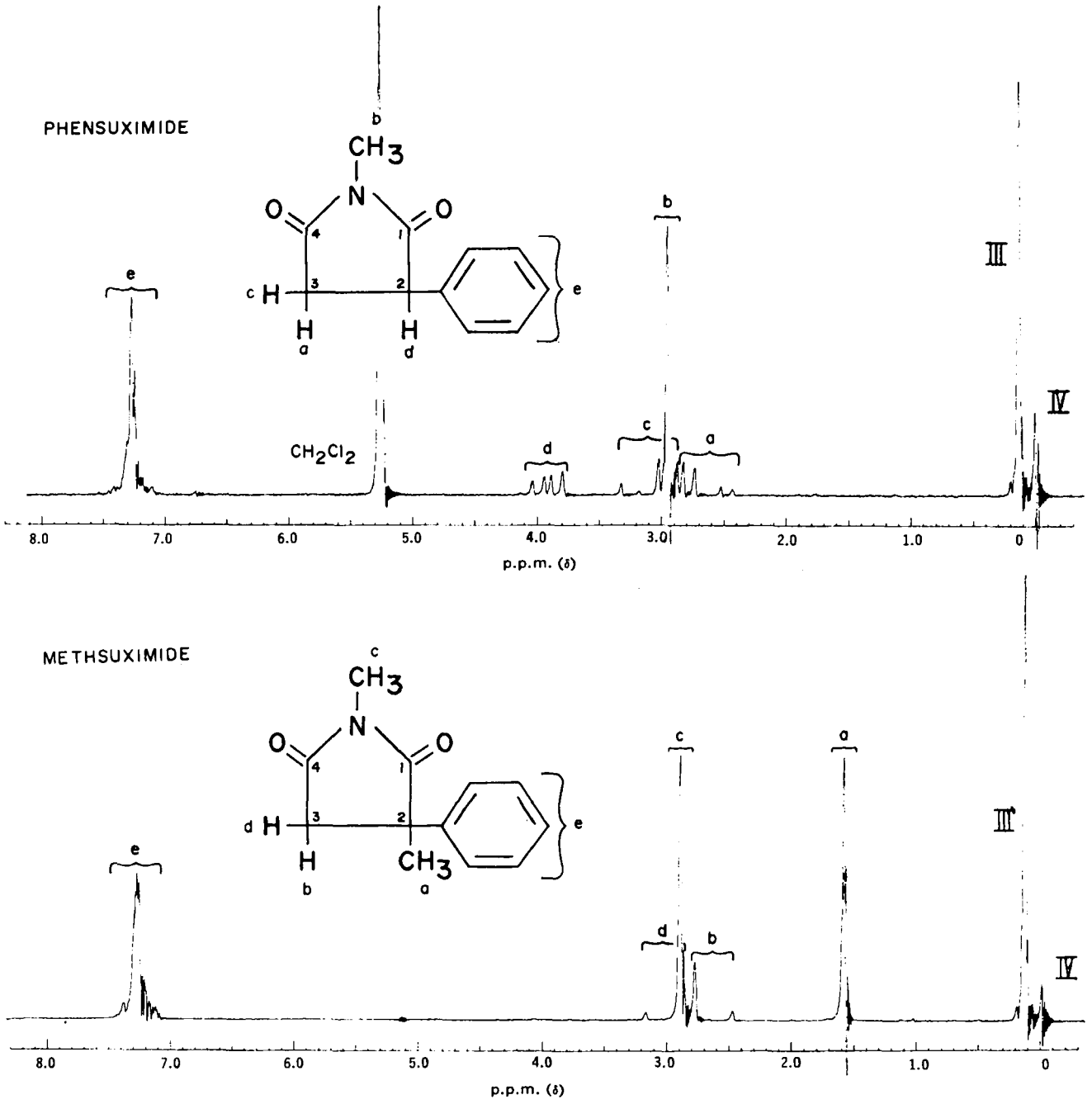

Figure 1—*NMR spectra of phensuximide in 10% methylene chloride in carbon tetrachloride and of methsuximide in carbon tetrachloride (III, hexamethylcyclotrisiloxane; and IV, tetramethylsilane).*

where:

A_s = integral value of the signal representing I or II
A_h = integral value of the signal representing III
EW_s = formula weight of I/5 = 40.65 or formula weight of II/5 = 37.84
EW_h = formula weight of III/18 = 12.35

RESULTS AND DISCUSSION

Although the two succinimide compounds, I and II, are very similar structurally, different solvents were required. The extreme solubility of both I and III in carbon tetrachloride plus the lack of resonance signals makes carbon tetrachloride the obvious choice of solvent for these solutes in this procedure. However, II does not exhibit the same solubility in pure carbon tetrachloride, but a 10% methylene chloride in carbon tetrachloride solvent system was satisfactory. The methylene chloride solvent component introduces no interference since its protons absorb at 5.25 p.p.m., a region not occupied by any other peaks.

The applicability of III as an internal standard has been demonstrated previously (6–8). It appears as a single spectral signal at an extreme upfield position. This combination of solvent system and internal standard provides the opportunity for a clear, interference-free identification of the active ingredient in addition to quantitative results.

The NMR spectra of I and II under the described analytical conditions are depicted in Fig. 1. The amounts of both succinimide derivatives are determined from the integration of the broad resonance pattern at about 7.25 p.p.m. due to the five phenyl protons at position 2 on the succinimide ring and the integration of the singlet at about 0.14 p.p.m. resulting from the 18 methyl protons of the internal standard III.

In addition to quantitative analysis, the hydrogen resonance characteristics are useful for qualitative purposes and are interpretable. In I, the two singlets at about 1.59 and 2.90 p.p.m. are due to

Table II—Determination of Methsuximide and Phensuximide in Commercial Capsules by NMR

Sample	Declared Dosage, mg./Capsule	NMR Procedure, mg./Capsule	Percent	NF XIII Procedure, mg./Capsule	Percent
Methsuximide					
1	300	299.1	99.7	297.9	99.3
2	300	292.2	97.4	293.1	97.7
3	300	298.2	99.4	299.1	99.7
4	150	149.6	99.7	149.1	99.4
5	150	152.1	101.4	149.9	99.9
6	150	148.4	98.9	146.9	97.9
Phensuximide					
1	500	481.0	96.2	484.5	96.9
2	500	497.5	99.5	496.0	99.2
3	500	493.5	98.7	495.5	99.1
4	500	491.0	98.2	493.5	98.7
5	500	496.0	99.2	497.5	99.5

the methyl protons at position 2 and on the nitrogen, respectively, of the succinimide ring. The two methylene protons at position 3 are nonequivalent and exhibit a characteristic *AB* pattern between 2.4 and 3.2 p.p.m. For Compound II, the three protons on the methyl group bonded to the nitrogen appear as a singlet at about 2.95 p.p.m. The methylene protons at position 3 couple with the methine proton at position 2 to form an intricate *ABX* pattern between 2.4 and 4.1 p.p.m.

The analysis of a group of standard I and II mixtures by NMR is summarized in Table I. As noted, the method is accurate and precise for I and II, with a standard deviation of 0.6 and 0.4%, respectively. The relative proportions of III to either I or II show no significant bearing on the accuracy of the determination for the range of proportions shown in Table I.

The potential value of the proposed NMR analysis to dosage forms was established by the measurement of the content of I and II in capsules. Table II summarizes the results of the analysis of six lots of I capsules and five lots of II capsules. For comparative purposes, the same capsules were analyzed by the NF XIII method, a UV spectrophotometric determination using the phenyl group absorption characteristics that occur in a spectral region not unaccustomed to harboring UV-absorbing interferences. The two procedures generally agreed to within 1%. No interference from any capsule excipients was observed.

As demonstrated previously, the use of NMR for quantitative analysis offers a number of advantages, which were experienced in this case as well. The procedure is simple and rapid, an advantage shared by other spectroscopic analyses. However, in the matter of specificity, NMR does stand alone, since the recorded spectrum provides unique field positions of the various protons for unambiguous identification (less complex than IR) and offers specific peaks for integration and subsequent quantitative interpretation. It is to be hoped that the advantages of NMR coupled with the welcome availability of less expensive, reliable instruments will encourage the use of this technique in quantitative analysis.

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A System for Automated Polarographic Analysis

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Abstract □ A sensitive and potentially highly versatile automated polarographic procedure was developed. A polarographic analyzer is interfaced with a continuous-flow system which completely removes interfering dissolved oxygen. Inclusion of a peristaltic valve in the manifold design allows an extended sampling period at steady-state conditions, at the same time increasing the productive rate of the system by a factor of two. High-resolution scans of the sample stream with a multifunctional polarographic analyzer set to monitor diffusion current while the applied potential is varied have the capability of quantitating electroactive compounds in the presence of decomposition products or formulation components. The capabilities of the method were demonstrated by collecting data on tablet and capsule formulations of lorazepam, a 1,4-benzodiazepine. Fifteen samples per hour can be assayed with a relative standard deviation of ±1.4% at the 0.5-mg. lorazepam level. The method is applicable to unit-dose analysis of lorazepam in the 0.5–6-mg./dose range.

Keyphrases □ Polarography—automated system and procedure, analysis of lorazepam tablets and capsules □ Lorazepam tablets and capsules—analysis, automated polarographic procedure

In recent years, polarographic techniques have been finding wide application in pharmaceutical analysis. Requests for a large number of assays to assist in evaluating the pharmaceutical acceptability and chemical stability of various dosage forms for new investigational drugs prompted the development of an automated polarographic method of analysis for tablet and capsule formulations.

Previously reported automated techniques employing polarized electrodes in continuous-flow systems (1–3) have been restricted to the measurement of diffusion current at a constant potential due to limitations imposed by the use of conventional continuous-flow dynamics. However, it is necessary to monitor diffusion current as a function of varying applied potential when quantitating electroactive compounds in the presence of decomposition products or formulation components that interfere in the fixed potential technique. In designing a completely automated analytical system capable of producing authentic polarograms, several unique problems in continuous-flow analysis were encountered.

The automatic analyzer system[1] described was developed following a systematic consideration of the capabilities and limitations of standard automated modules. The technique interfaces a multifunctional polarographic analyzer with a continuous-flow system employing sophisticated design features. The capabilities of the method were demonstrated by collecting data on tablet and capsule formulations of lorazepam [7-chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one]. This potentially versatile analytical system expands the capabilities of automated analysis to include polarography, a well-established electrochemical technique.

EXPERIMENTAL

Apparatus—A standard automatic analyzer[1] system was employed consisting of the following modules: (*a*) a SOLIDprep sampler, programmed at 15 samples/hr. with a sample aspiration stage set at 40–90 units; (*b*) proportioning pumps[2] (three required); (*c*) a continuous filter, speed 2; (*d*) a peristaltic valve[3]; (*e*) a polarographic analyzer equipped with a mechanical drop timer[4]; (*f*) a circulating constant-temperature unit[5]; (*g*) a 25.4-cm. (10-in.) linear potentiometric strip-chart recorder[6]; and (*h*) transmission lines (Solvaflex) and proportioning tubes.

The dimensional details of the polarographic flow cell used in this investigation were presented by Lento (1). To prevent deterioration from the predominantly nonaqueous supporting electrolyte considered, the cell was machined from a transparent 4.44-cm. (1.75-in.) rod[7].

Supporting Electrolyte—The supporting electrolyte consisted of 0.1 *M* sodium acetate and 0.1 *M* acetic acid in methanol–water (8:2, v/v). In the preparation of the supporting electrolyte, reagent grade materials were used without further purification.

Nitrogen—Prepurified grade nitrogen[8] was used in the deaeration unit.

Automated Methodology—A flow diagram indicating the arrangement of the apparatus employed in this system is presented in Fig. 1. Figure 2 is a schematic wiring diagram of the necessary synchronization control circuits.

In performing the analyses, standards are placed on the sample plate followed by samples of intact or powdered tablets or capsules. At the end of a series of 20 samples, additional standards are placed on the sample plate. Samples are introduced into the SOLIDprep unit and dispersed in the methanolic sodium acetate–acetic acid supporting electrolyte. Following dissolution of the drug, the sample is aspirated into the flow system and automatically filtered to remove insolubles. To prevent mechanical obstruction at the tubing connections from insoluble excipient materials, a decantation trap is placed between the SOLIDprep unit and the proportioning pump.

After passage through the continuous filter module, the filtrate is segmented with nitrogen and pumped to the inlet manifold of the "steady-state extension unit." The extension unit employs a differential flow rate principle and synchronized valve control to prolong the duration of the sample's steady state. From the outlet manifold of the steady-state extension unit, the expanded sample stream is directed to the inlet of the continuous deaerator. Within this unit, a high velocity stream of methanol-saturated nitrogen is employed to transform the sample into a thin, turbulent liquid film. Entrained by the gas stream, the film is transported rapidly through the deaeration coil, which provides the surface area necessary for efficient oxygen removal. Thermostatic control of the purging gas is provided

[1] AutoAnalyzer, Technicon Corp., Tarrytown, N. Y.

[2] Model I, Technicon Corp.
[3] Catalog No. 167-A002-01, Technicon Corp.
[4] Model PAR-174, Princeton Applied Research Corp., Princeton, N. J.
[5] Catalog No. 66590, Precision Scientific Co., Chicago, Ill.
[6] Beckman model 1005, Beckman Instruments, Inc., Fullerton, Calif.
[7] Solvent-resistant Homalite CR-39, Homalite Corp., Wilmington, Del.
[8] Matheson Co., East Rutherford, N. J.

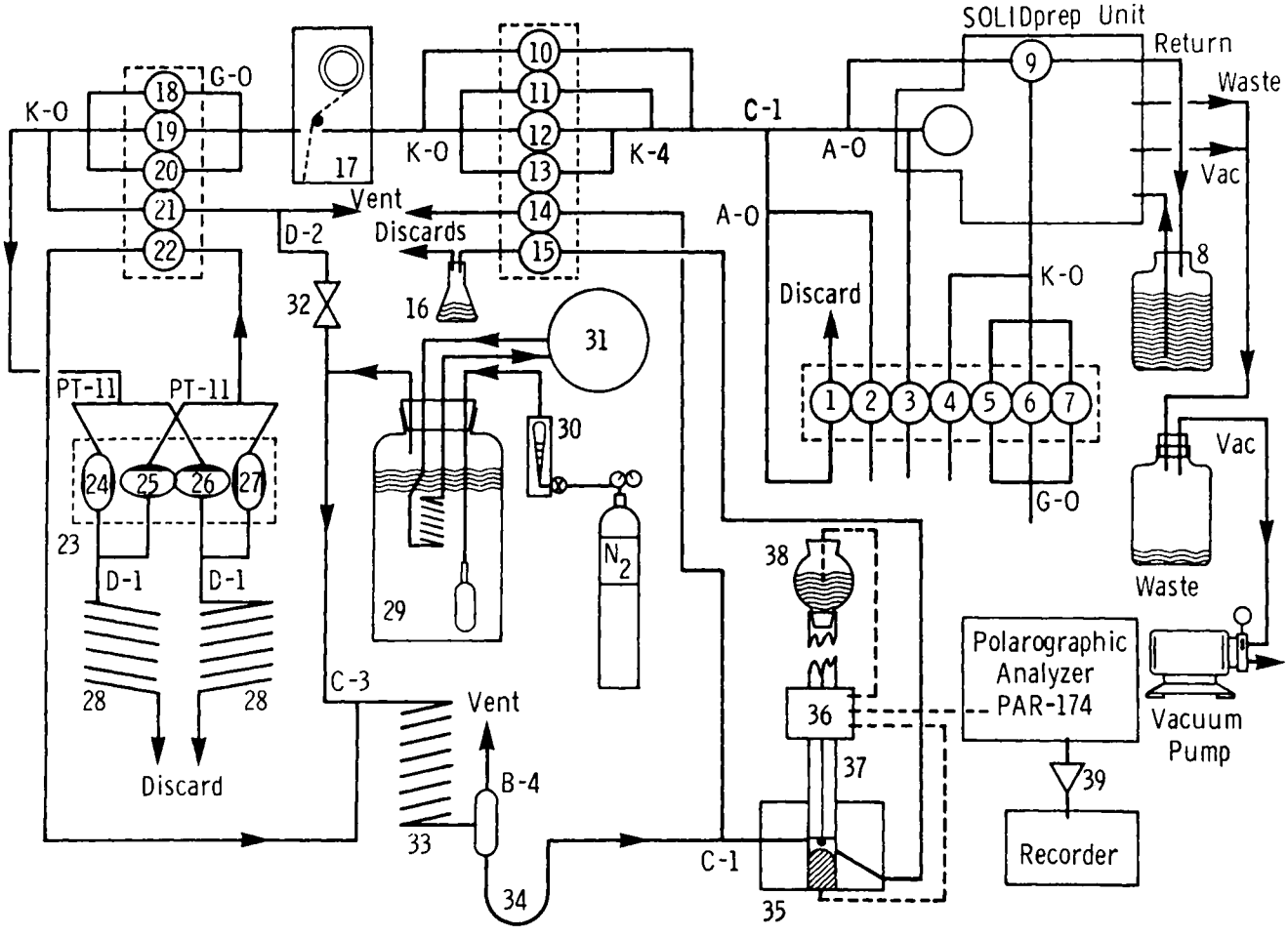

Figure 1—*Automated flow diagram. Key: 1, 0.56 ml./min.; 2, 0.42 ml./min. water; 3, 0.56 ml./min. air; 4, 3.39 ml./min. air; 5, 6, 7, 3.39 ml./min. supporting electrolyte; 8, reservoir-supporting electrolyte; 9, SOLIDprep wash distribution valve; 10, 11, 12, 3.39 ml./min. sample; 13, 1.69 ml./min. sample; 14, 2.02 ml./min. debubbler; 15, 2.02 ml./min. flow cell; 16, mercury trap; 17, continuous filter module; 18, 3.39 ml./min. sample; 19, 2.42 ml./min. sample; 20, 3.39 ml./min. sample; 21, 0.56 ml./min. nitrogen; 22, 2.42 ml./min. sample; 23, peristaltic valve module; 24, 0.063-cm. (0.025-in.) i.d. manifold tubing; 25, 0.038-cm. (0.015-in.) i.d. manifold tubing; 26, 0.063-cm. (0.025-in.) i.d. manifold tubing; 27, 0.038-cm. (0.015-in.) i.d. manifold tubing; 28, sample storage coil, 12.2 m. (40 ft.), 2.4 mm. i.d.; 29, gas saturation apparatus; 30, gas flowmeter; 31, circulating constant-temperature bath; 32, nitrogen flow-limiting orifice, 0.038-cm. (0.015-in.) i.d. pulse suppressor; 33, deaeration coil, 14-turn 3.4-mm. i.d. mixing coil; 34, 6-cm. glass "U", 2 mm. i.d.; 35, polarographic flow cell; 36, mechanical drop timer; 37, mercury capillary, 9 cm. (Sargent-Welch S-29419); 38, mercury reservoir; and 39, signal attenuator, 10 v.–100 mv.*

to protect the electrolyte system from evaporational losses. The sample is withdrawn from the outlet connection of the deaerator and transported to the polarographic flow cell. Segmenting nitrogen is also drawn from the purging unit into the sample effluent for the purpose of providing a secondary deaeration stage in which residual dissolved oxygen is removed from the sample stream.

The polarographic analyzer and recorder are synchronized to initiate a scan at the moment the sample attains steady state at the flow cell. The instrument is used in the current-sampled d.c. polarographic mode (4) with the mechanical drop timer operating at 1 drop/sec. For lorazepam, the polarographic scan is conducted at a rate of 5 mv./sec., starting at an initial potential of −0.86 v. and continuing to −1.76 v. Calculations are made through comparison of the diffusion currents observed with standards and solid dosage formulation samples.

RESULTS AND DISCUSSION

Manifold Design Considerations—In the development of an automated continuous-flow system that employs a scanning analytical technique, such as polarography, it is necessary to meet design criteria not ordinarily encountered in conventional automated methodologies. Since a scan may be conducted only while the sample concentration at the detector is held constant, it is necessary to achieve steady states of appreciable duration within the flow system. This requirement presents a considerable design problem in automated manifolds operating at rapid assay rates.

The elapsed time required for a single polarographic determination is expressed by the equation:

$$t_p = \frac{\Delta E}{R} \qquad \text{(Eq. 1)}$$

where t_p is the scan duration in seconds, ΔE is the width of the voltage range to be studied in millivolts, and R is the voltage scan rate expressed in millivolts per second.

The extent of the potential region that must be scanned is determined by the positions of the polarographic waves characteristic of the compounds under investigation and the electrolyte system employed. Both the rate at which the scan is conducted and the polarographic mode utilized affect the definition of the recorded polarogram (5). With a given polarographic mode, the maximum permis-

Table I—Comparison of Polarographic and Quantitative TLC Analyses of Intentionally Degraded Lorazepam Samples

Sample Treatment	Percent of Initial	
	Polarographic Method	TLC Method
Stored at 25° for 47 months	99	98
Stored at 60° for 1 month	94	95
Stored at 75° for 2 weeks	76	77

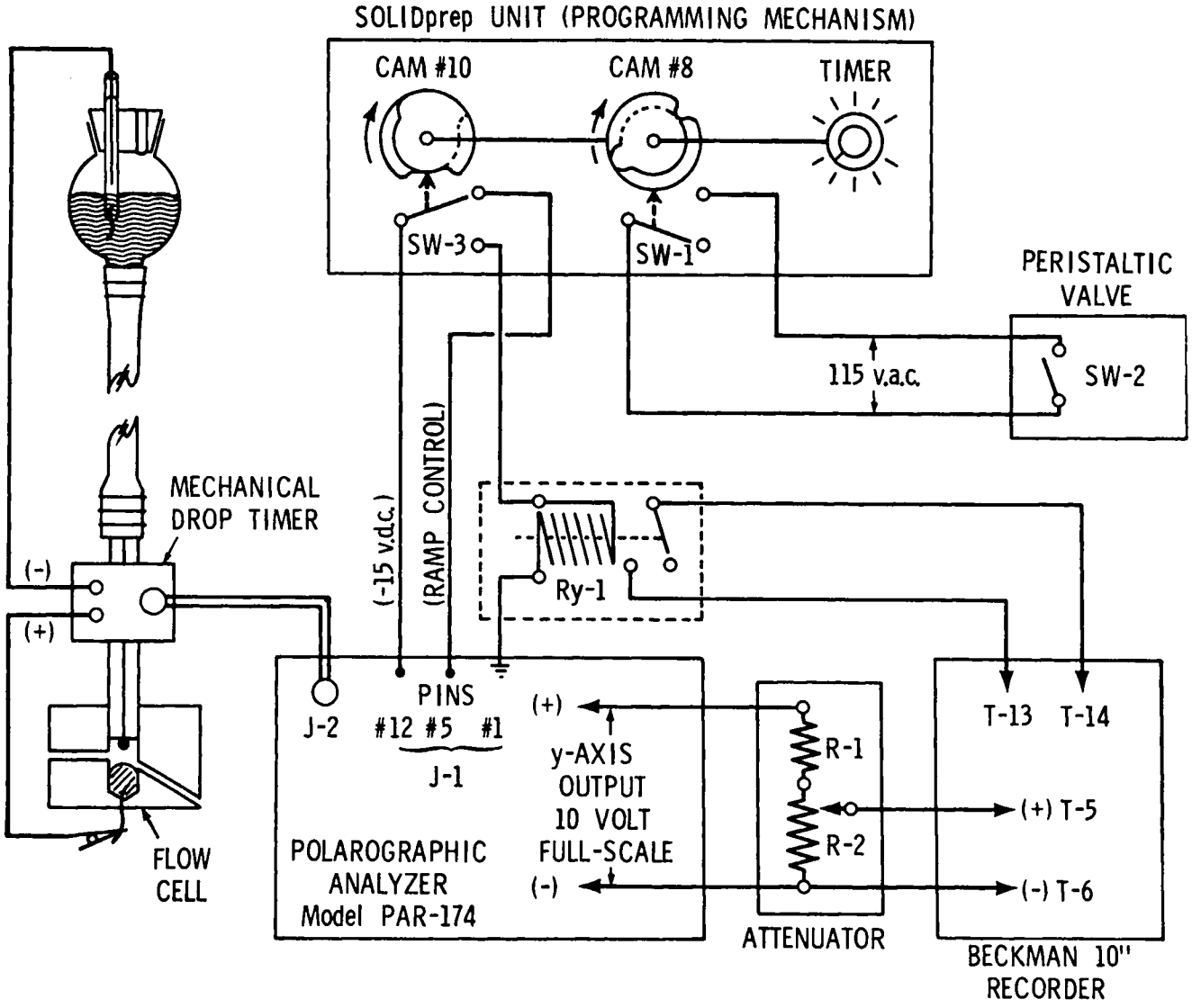

Figure 2—*Schematic wiring diagram. Key: SW-1, cam-actuated microswitch 8 (SOLIDprep unit); SW-2, manual index switch (peristaltic valve module); SW-3, cam-actuated microswitch 10 (SOLIDprep unit); T-5, T-6, recorder input terminals (100 mv. full-scale sensitivity); T-13, T-14, recorder remote control terminals; Ry-1, recorder control relay (Allied Electronics No. 41C7676); R-1, precision resistor, 900 kohm ± 1%; R-2, 10-kohm 10-turn helipot; J-1, accessory connector plug (PAR-174); and J-2, cell/drop timer connector plug (PAR-174).*

sible scan rate depends on the precision and resolution required for the specific determination of interest. Through the conditions imposed on the values of ΔE and R (Eq. 1), the minimum acceptable steady-state duration is controlled by the requirements of the particular assay.

In the quantitation of lorazepam, for example, it was found necessary to scan a voltage region of 900 mv., *i.e.*, from −0.86 to −1.76 v. *versus* mercury pool electrode. For the desired level of resolution, the maximum scan rate was determined experimentally to be 5 mv./sec. By using Eq. 1, the minimum permissible steady-state period, *i.e.*, the scan duration, was calculated to be approximately 3 min. However, because of normal diffusion in the flow system, the sample aspiration stage must be somewhat longer than the required steady-state period.

A study of the diffusional characteristics of the automated polarographic system revealed that 1 min. is required for the transition between successive steady states within the flow cell. This study was conducted by monitoring diffusion current *versus* time at an applied potential of −1.5 v. and observing the transients that resulted when an alternating series of lorazepam standards and empty sample cups were placed in the SOLIDprep sample tray. Thus, to supply the required 3 min. of steady state, a sample aspiration period of at least 4 min. is necessary.

The requirement for protracted aspiration imposes a severe limitation on the available assay rate of a conventional automatic analyzer system employing the SOLIDprep unit for sample dissolution. The required sample homogenation time and instrument design limitations restrict the sample aspiration stage of the SOLIDprep operating cycle to a maximum of 50% of the programmed cycle period. Therefore, with a 4-min. aspiration period, the minimum cycle duration is 8 min. This assay rate, *i.e.*, 7.5 samples/hr., would not have offered a significant advantage over manual polarographic procedures.

Steady-State Extension—The steady-state extension unit (Fig. 1) was developed to overcome the assay rate limitations encountered with conventional automated systems. This unit provides a twofold increase in assay rate with no loss of sensitivity or resolution.

Operationally, a peristaltic valve module is employed to permit selective collection and release of steady-state material. The valve is programmed to route the inlet sample stream to one of the storage coils, while the other coil provides sample to the outlet manifold. With each successive valve cycle, the inlet and outlet channels alternate and the flow directions in the storage coils reverse. The valve cycle is synchronized to coincide with the termination of a sample steady state at the extension unit inlet. The closing of an inlet channel causes the steady-state portion of the sample stream to be selectively retained at the upper end of the associated storage coil. The simultaneous opening of the coil's outlet channel releases this ma-

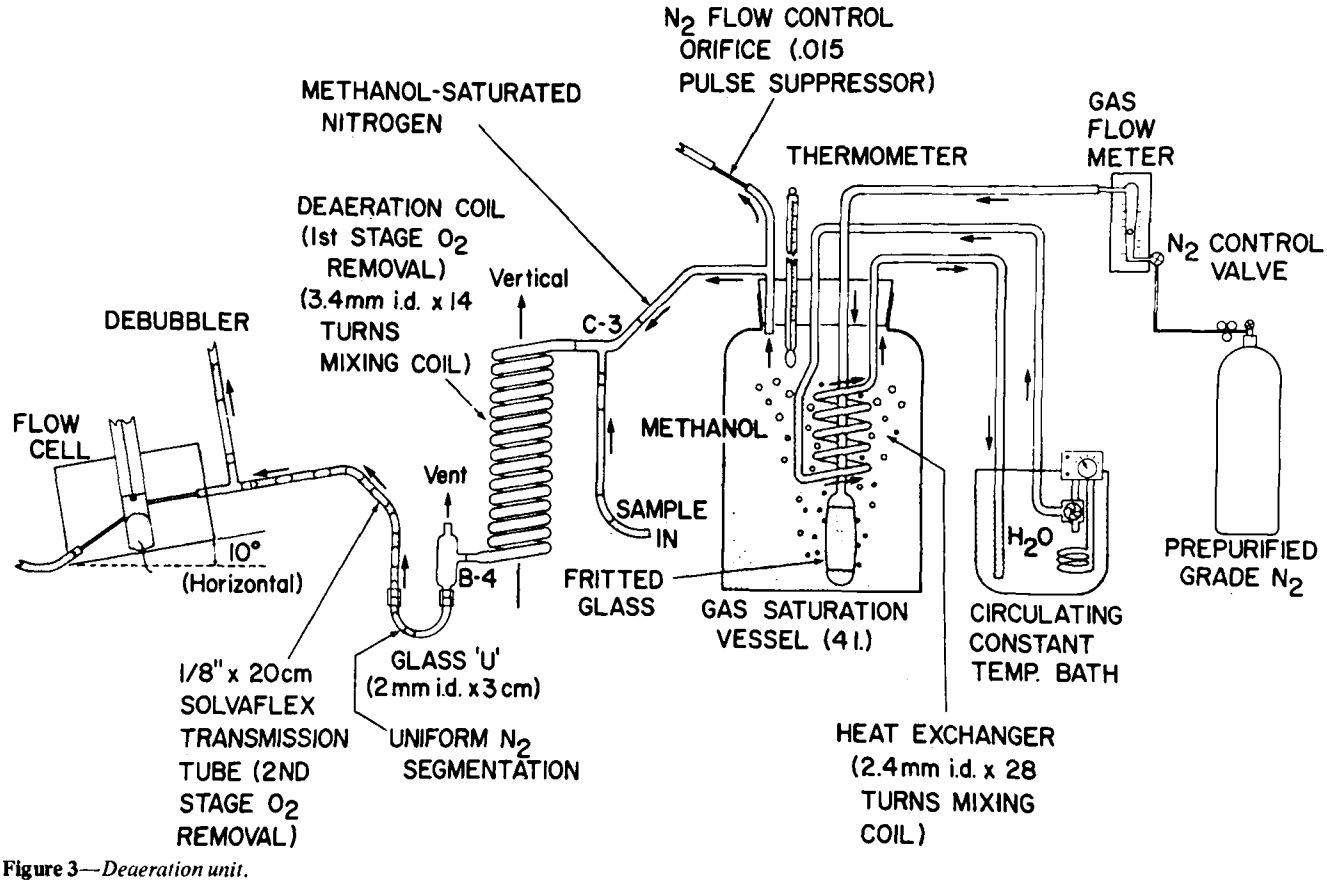

Figure 3—*Deaeration unit.*

terial to the extension unit's outlet manifold. The alternating collection and release of sample in conjunction with the inlet-outlet flow rate differential provide the desired steady-state extension. The required synchronization is accomplished through electrical interconnection of the valve's indexing switch with the programming mechanism of the SOLIDprep unit (Fig. 2).

A detailed description of the extension unit's operational principles, capabilities, and various applications will be published in the near future.

Deaeration Unit—For polarographic applications demanding high sensitivity and precision, it is essential to exclude dissolved oxygen from the electrode environment. This requirement is not easily satisfied in continuous-flow systems. Additionally, the low solubility of the model compound, lorazepam, in aqueous media complicated the deaeration problem by necessitating the use of a predominantly methanolic supporting electrolyte. Existing techniques for the continuous removal of oxygen from automatic analyzer streams (1, 3) and chromatographic column effluents (6–8) would not fulfill the requirements of the automated polarographic system. Generally, these methods are intended for use in aqueous solvent streams and are not designed to handle the exceptionally high oxygen solubility encountered in the supporting electrolyte of interest. Furthermore, the reported deaeration methods make no provisions to protect volatile solvents from excessive evaporation. Those techniques intended specifically for chromatopolarographic applications are not designed for use in segmented automatic analyzer streams and usually require the fabrication of sophisticated apparatus.

The continuous-flow deaerator (Fig. 3) was developed to meet the stringent performance criteria of this automated polarographic procedure. The apparatus consists entirely of standard automatic analyzer fittings and readily available laboratory equipment. The unit's effectiveness was evaluated at sample flow rates of 1–5 ml./min. for both air-segmented and nitrogen-segmented streams of supporting electrolyte. Within this range, oxygen removal was found to be essentially quantitative; *i.e.*, no detectable contribution from oxygen was observed in the residual current at a full-scale current sensitivity of 0.5 μamp. The nitrogen flow rate in each case was adjusted to yield uniform segmentation of the sample stream at the outlet of the deaerator, corresponding to nitrogen flow rates of 1–2 l./min. This high level of performance suggests that the deaerator will effectively remove oxygen from any common polarographic electrolyte. The unit's small hold-up volume, about 0.5 ml., and rapid sample transit, about 20 sec., minimize diffusional mixing in the flowing stream.

The volatile nature of the supporting electrolyte and the rapid nitrogen flow rates within the deaerator necessitated thermostatically controlled presaturation of the purging gas. The circulating constant-temperature bath is adjusted to maintain nitrogen temperatures at the saturation vessel outlet of 2 ± 1° above the ambient temperature of the laboratory.

Instrument Synchronization—The operation of the automated system depends upon precise synchronization of the steady-state extension unit and the polarographic analyzer with the flowing sample stream. To eliminate the possibility of synchronization-drift problems, which could result from the use of separate timing mechanisms, both the peristaltic valve module and the polarographic analyzer were interfaced with the programming mechanism of the SOLIDprep unit. The two cam-operated microswitches (Fig. 2) are accessory switches within the SOLIDprep programmer and were employed to provide common time-base control of all necessary synchronization signals.

The external circuit between terminals 5 and 12 on the accessory connector of the polarographic analyzer controls the scanning functions of the instrument. When this circuit is closed, the applied cell potential reverts to the selected initial value and the instrument is placed in a standby mode. Opening of the circuit initiates the voltage scan. The constant −15-v. signal available at terminal 12 is also employed to activate the relay connected to the remote control terminals, 13 and 14, of the recorder. Cam 10 is adjusted to initiate the voltage scan at the beginning of sample steady state within the polarographic cell. Cam 8 is adjusted to provide a momentary closure in the peristaltic valve's control circuit as the terminal portion of a sample steady state enters the extension unit's inlet manifold.

Current-Sampled d.c. Polarography—High polarographic resolution requires the use of an electrode drop rate that is rapid in comparison to the scan rate, *i.e.*, the individual drops are closely spaced along the polarogram's potential axis. Consequently, it is desirable to utilize a drop rate that is also more rapid than is commonly

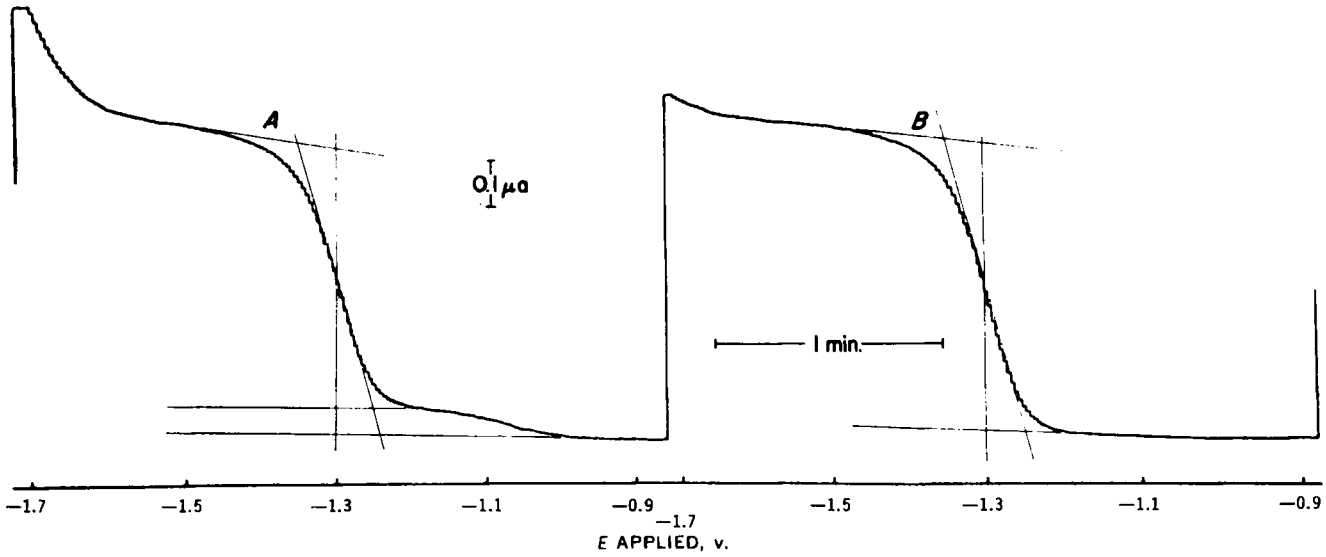

Figure 4—*Reproduction of lorazepam polarograms at the 2-mg. level and 15-sample/hr. assay rate. Key: A, intentionally degraded sample; and B, lorazepam reference standard.*

employed in manual procedures. However, dropping mercury electrodes that display suitably short natural drop periods at the electrocapillary maximum tend to become unstable at moderately negative applied potentials. For this reason, it is not practical to depend on natural drop-fall when operating at high drop rates (9).

To provide the required drop rate, this system employs an electrode with a long natural drop period (2.5 sec.) in conjunction with a mechanical drop timer which induces drop separation at precisely timed, selected intervals of 1 sec. Just prior to the mechanical removal of the drop, the current flowing in the cell is sampled electronically. An output proportional to this current is stored and presented to the recorder until the cell is again sampled electronically 1 sec. later, just prior to removal of the next mercury drop. When using this current-sampled d.c. mode, a peak-current polarogram is obtained in which all drop-induced fluctuations are removed electronically without introducing the waveform distortions characteristic of damping circuits. This polarographic mode is particularly well suited to automated applications since the smooth, well-defined waveforms may be rapidly and easily interpreted.

Typical polarograms of intact and intentionally degraded lorazepam, generated by the automated system at an assay rate of 15 samples/hr., are reproduced in Fig. 4. The fine definition obtained with the current-sampled d.c. presentation is apparent. The quiet residual current baseline is indicative of the low noise level achieved in the system. In the polarogram of the thermally degraded sample of lorazepam, the wave associated with the decomposition product appears at an $E_{1/2}$ of −1.1 v. and the wave at $E_{1/2}$ of −1.3 v. corresponds to the intact material. The excellent separation between these waves permits precise quantitation, by the established extrapolation technique described by Meites (9), of intact lorazepam.

Suitability as Analytical Method—Specificity of the method for analysis of the model compound, lorazepam, in the presence of its thermal degradation products was demonstrated by comparing analytical values of intentionally degraded samples to those obtained by quantitative TLC. In the TLC procedure, the lorazepam was separated from its degradation products on a silica gel chromatographic plate using a development solvent system of benzene–dioxane–ethanol (5:4.4:0.6). A zone of the silica gel encompassing the intact lorazepam spot was scraped from the plate. The lorazepam was eluted from the adsorbent with 95% ethanol and subsequently assayed by a UV spectrophotometric procedure to obtain a quantitative value. Since there is good agreement between the values by the two techniques (Table I), it is concluded that the automated polarographic procedure is stability indicating.

The linearity of the diffusion current–concentration relationship

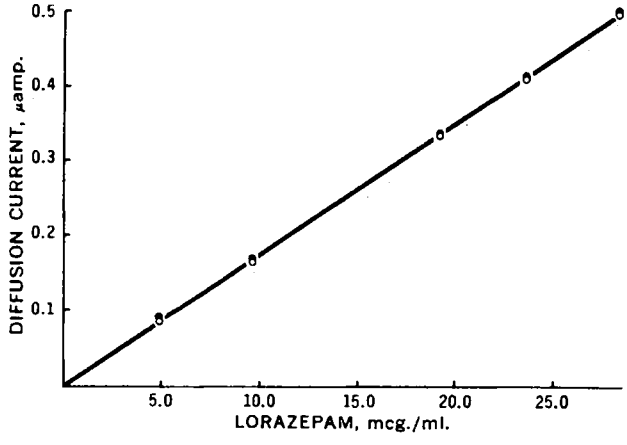

Figure 5—*Relationship of diffusion current to lorazepam concentration.*

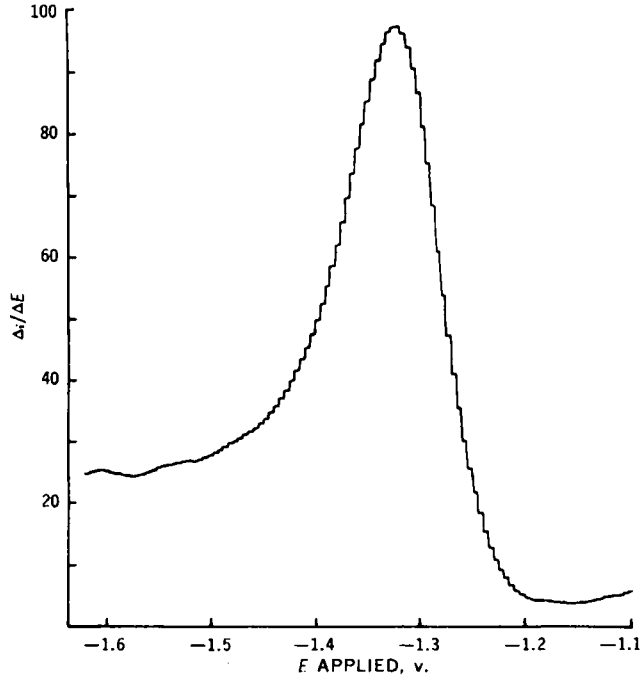

Figure 6—*Reproduction of a constant-amplitude differential pulse polarogram of lorazepam at the 2-mg. level and 15-sample/hr. assay rate.*

was shown by analyzing standards of lorazepam, yielding concentrations in the homogenization vessel in the range of 5–30 mcg./ml. ($1.5–9.5 \times 10^{-5}$ *M*) (Fig. 5). This concentration range corresponds to lorazepam levels of 0.25–6.0 mg. in the finished product, with the SOLIDprep unit delivering a fixed volume between 50 and 200 ml. of diluent. The actual measured lower limit of sensitivity is 0.05 mg./sample, corresponding to 3×10^{-6} *M* in the flow cell.

Both the precision and accuracy of this technique were determined by adding known quantities of lorazepam in the 0.5–2.0-mg. range to tablet excipient mixtures and measuring the percentages recovered. Twelve replicate assays at the 0.5-, 1.0-, and 2.0-mg. lorazepam/tablet levels produced relative standard deviations of ±1.4, ±1.4, and ±1.2%, respectively, with recoveries of 99% of the theoretical amount present in all three cases.

The effect of common inert tablet components on this automated polarographic procedure when applied to lorazepam was investigated to uncover any possible interfering material. In this study, inactive component–lorazepam ratios of 200:1 for lactose, 100:1 for talc and microcrystalline cellulose[9], and 50:1 for calcium sulfate, magnesium stearate, stearic acid, potassium polacrilin[10], methylcellulose[11], and starch were evaluated. No interference was experienced from these materials at these levels.

Further Applications—Preliminary investigations indicate that the automated method is readily adaptable to a wide variety of polarographic assay procedures. The use of a multifunctional polarographic analyzer permits the system to be operated in other polarographic modes. A constant-amplitude differential pulse polarogram of lorazepam obtained by the automated technique is presented in Fig. 6. This polarographic mode of operation offers increased sensitivity and expands method capabilities by permitting the resolution of closely spaced polarographic waves.

[9] Avicel.
[10] Amberlite IRP-88.
[11] Methocel.

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D. J. BROWN▲ and D. G. COOK*

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Keyphrases □ Pentaerythritol tetranitrate or mannitol hexanitrate and phenobarbital tablets—simultaneous spectrophotometric analysis □ Mannitol hexanitrate or pentaerythritol tetranitrate and phenobarbital tablets—simultaneous spectrophotometric analysis □ Phenobarbital and pentaerythritol tetranitrate or mannitol hexanitrate tablets—simultaneous spectrophotometric analysis □ Colorimetry—analysis, pentaerythritol tetranitrate or mannitol hexanitrate in tablets with phenobarbital □ UV spectrophotometry—analysis, phenobarbital in tablets with pentaerythritol tetranitrate or mannitol hexanitrate

Pentaerythritol tetranitrate and mannitol hexanitrate are both organic nitrate esters believed capable of coronary dilation. Their onset of action is slower and their duration much longer than nitroglycerin. They are used therapeutically in the prophylaxis of attacks of angina pectoris.

Phenobarbital, due to its sedative effect, has also been shown to have value in the prevention of angina pectoris attacks. It is not uncommon, therefore, to find pharmaceutical preparations containing both phenobarbital and one of the organic nitrate drugs as active ingredients. The purpose of this study was to find a suitable

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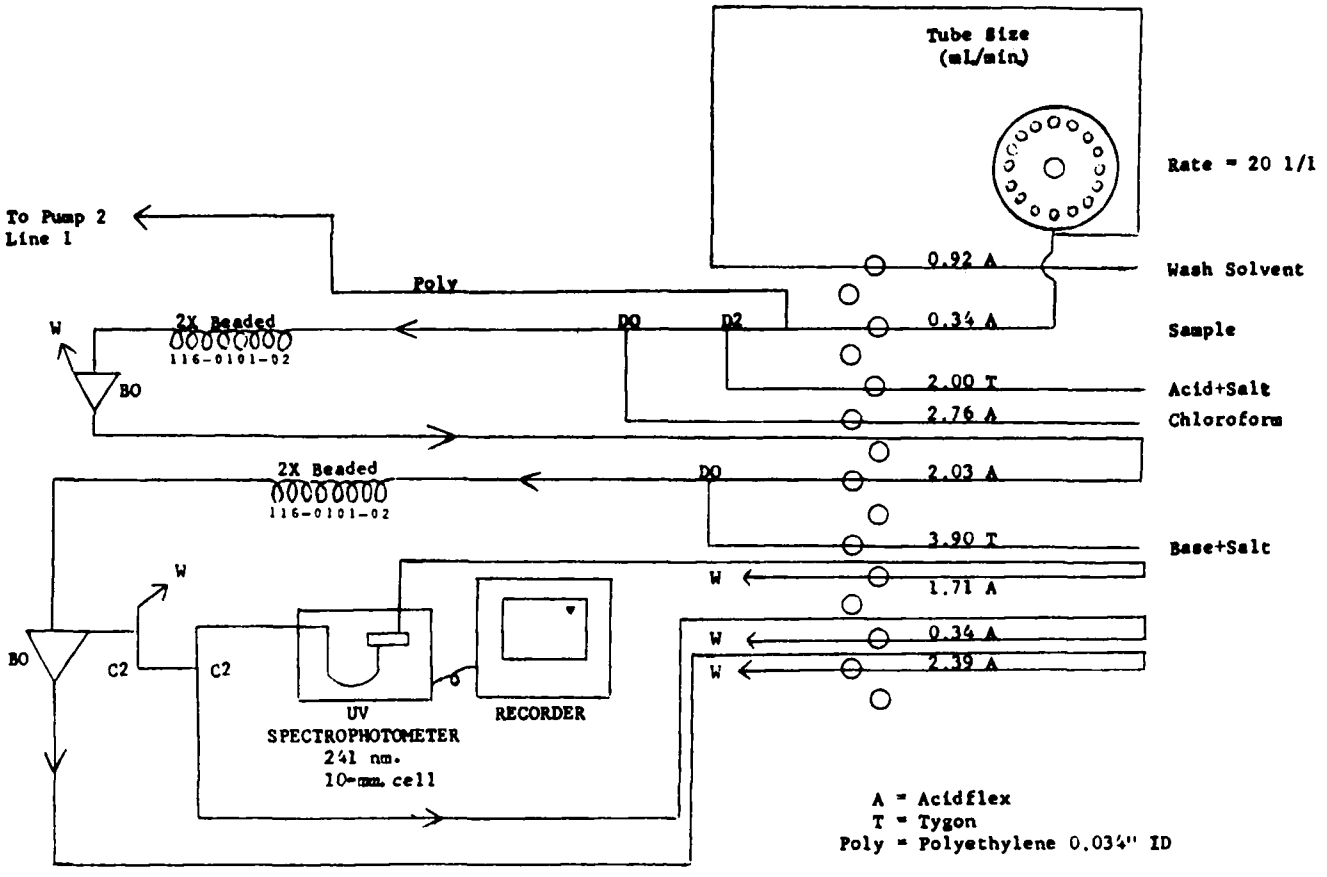

Figure 1—*Flow diagram for UV determination of phenobarbital.*

method for the simultaneous semiautomated determination of this type of pharmaceutical formulation.

Various chromatographic techniques for the detection and estimation of organic nitrate esters have been reported. A column chromatographic scheme to separate many organic nitrate esters, followed by a set of color tests for qualitative analysis was described (1). Paper chromatography followed by reaction with diphenylamine under the influence of UV radiation was used (2) for quantitative estimation. GC (3) and TLC (4) techniques have also been reported. In general, however, chromatographic procedures are not well suited for automation.

Polarographic (5) and titrimetric (6) procedures have also been described. However, most analytical methods for organic nitrate esters are spectrophotometric. Although IR methods are known (7, 8), colorimetric procedures are by far the most common. Nitration of phenoldisulfonic acid with the nitrate moiety of the ester to yield a colored ion in basic solution is the basis of several procedures (9–12).

Various methods have been developed in which nitroglycerin is subjected to alkaline hydrolysis with the formation of nitrite, which is then determined colorimetrically (13–17). Pentaerythritol tetranitrate and other related compounds have been determined similarly (18). Several organic nitrate esters including pentaerythritol tetranitrate and mannitol hexanitrate were estimated (4) after extraction from TLC plates using alkaline hydrolysis followed by diazotization of sulfanilic acid and coupling with α-naphthylamine. One approach (17), using *p*-chloroaniline and *N*-1-naphthylethylenediamine to form a color, was found to be satisfactory in the semiautomated determination of pentaerythritol tetranitrate or mannitol hexanitrate.

A comprehensive review of methods available for barbiturates was given recently (19). A double-extraction UV spectrophotometric procedure (20) was chosen, with some modifications, as a basis for the determination of phenobarbital.

EXPERIMENTAL

Reagents and Solutions—All chemicals used in this work were of

Table I—Effect of Phenobarbital on Pentaerythritol Tetranitrate Assay

Standard Pentaerythritol Tetranitrate[a] Absorbances	Phenobarbital Concentration, mg./100 ml.	Pentaerythritol Tetranitrate Absorbance[b]
0.470	5.0	0.476 (+1.3%)
0.469	10.0	0.477 (+1.5%)
0.471	15.0	0.479 (+1.9%)
0.470	20.0	0.478 (+1.7%)
0.470	25.0	0.478 (+1.7%)
Average 0.470	30.0	0.479 (+1.9%)
Range 0.4%	40.0	0.483 (+2.8%)
	Average	0.478
	Range	1.5%
	Average error	±1.7%

[a] Standard = 15.0 mg./100 ml. [b] Average of three readings.

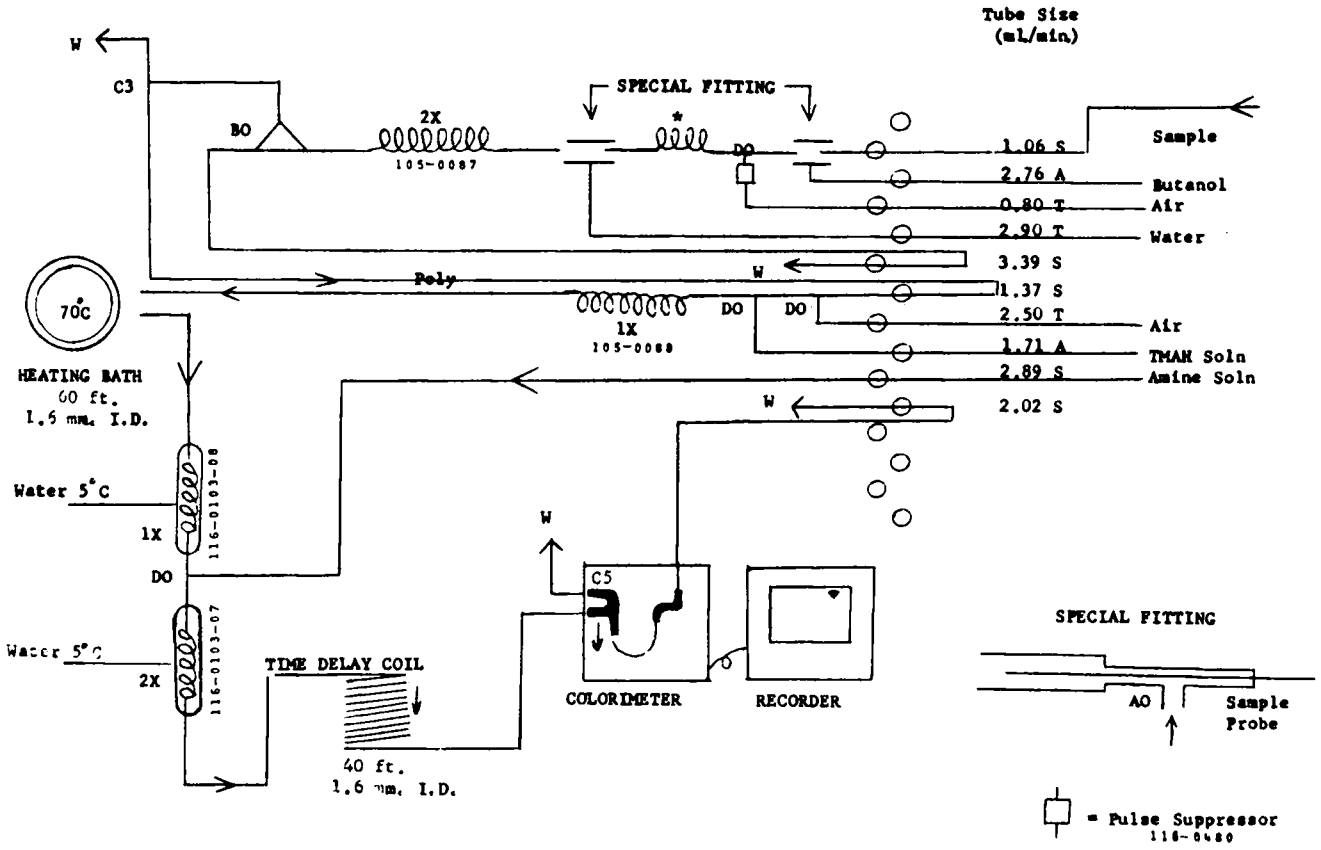

Figure 2—*Flow diagram for the determination of pentaerythritol tetranitrate or mannitol hexanitrate. *Twelve 2.54-cm. (1-in.) diameter turns of 0.086-cm. (0.034-in.) polyethylene tubing. Colorimeter: pentaerythritol tetranitrate, 570 nm.; mannitol hexanitrate, 613 nm.; 10-mm. cell.*

reagent grade except *n*-propanol, which was of highest purity available.

Tetramethylammonium Hydroxide, 1.5%—Dilute 150 ml. of 10% aqueous tetramethylammonium hydroxide to 1.0 l. with alcohol USP. Prepare fresh daily.

Amine Solution—Dissolve 0.5 g. of *N*-1-naphthylethylenediamine dihydrochloride in 100 ml. of concentrated hydrochloric acid. Dissolve 0.5 g. *p*-chloroaniline in 500 ml. of *n*-propanol. Then mix both solutions together, cool, and dilute to 1.0 l. with *n*-propanol. Prepare fresh daily.

Sulfuric Acid (1 N) Saturated with Sodium Chloride—Dilute 27.8 ml. of concentrated sulfuric acid to 1.0 l. with water. Add sodium chloride until saturated.

Sodium Hydroxide (0.01 N) in 1% Aqueous Sodium Chloride—Dissolve 10.0 g. of sodium chloride in 500 ml. of water. Add 10.0 ml. of a 1 *N* aqueous sodium hydroxide solution and dilute to 1.0 l. with water.

Wash Solvent—Prepare by mixing water, methyl isobutyl ketone, and alcohol USP (1:1:2).

Standard Solution—Add appropriate amounts of pentaerythritol tetranitrate or mannitol hexanitrate and phenobarbital corresponding to the amounts found in the sample (usually 10–20 mg. of pentaerythritol tetranitrate or 15–32 mg. of mannitol hexanitrate and 15–32 mg. of phenobarbital) to 25.0 ml. of water. Add 25.0 ml. of methyl isobutyl ketone and shake the mixture vigorously on a wrist-action shaker for 15 min. Dilute to 100.0 ml. with alcohol USP.

Apparatus—The automated analysis system[1] consisted of the following components: a sampler II; three proportioning pumps I; two spectrophotometers[2,3] modified by disengaging the wavelength scan drive, each equipped with a 10-mm. flow cell[4]; a heating bath constructed from a heating coil, temperature control box[5], and a cone drive stirrer[6]; and a water bath[7] set at 5°. A piece of glassine weighing paper was placed over the entrance to the photocell compartments in the visible spectrophotometer.

Procedure—The automated system is assembled according to the flow diagram shown in Figs. 1 and 2. Two proportioning pumps are used for the organic nitrate section of the system, and one proportioning pump is for the phenobarbital section. In performing the analysis, individual tablets are prepared according to the procedure given for the standard solution with the exception that an ultrasonic bath can be used to disintegrate the tablets in the water before the addition of methyl isobutyl ketone. The sample and standard solutions are sampled from 4.0-ml. polystyrene cups by the sampler, with a sampling rate of 20 cups/hr. and a sample-to-wash ratio of 1:1. A sampling pattern of two standards, five samples, one standard . . . five samples, one standard is used. The first standard peak is ignored in the calculations.

After sampling, the flow is split into two streams. The stream for the phenobarbital determination is acidified with 1 *N* sulfuric acid and extracted with chloroform. The acid is saturated with sodium chloride to eliminate emulsion formation. The chloroform layer is then extracted with 0.1 *N* sodium hydroxide in 1% sodium chloride; sodium chloride is used to reduce the organic solvent solubility in the aqueous phase. A combination of two debubblers is used before the aqueous stream enters the flow cell to eliminate any air bubbles or chloroform drops. The spectral measurement is made against air as reference at 241 nm.

The stream for the organic nitrate determination is diluted with butanol and extracted with water. The butanol layer (upper layer) is resampled, mixed with tetramethylammonium hydroxide solution, and delayed approximately 5 min. in a 70° heating bath to hydrolyze the organic nitrate ester and form nitrite. The solution is cooled to 5°, mixed with amine solution, and delayed approximately 5 min. at room temperature. The intensity of the resulting color is deter-

[1] AutoAnalyzer, Technicon, Tarrytown, N. Y.
[2] Cary 15, Cary Instruments, Monrovia, Calif.
[3] Beckman DK-2A, Beckman Instruments, Fullerton, Calif.
[4] A. H. Thomas Co., Philadelphia, Pa.
[5] Chemical Rubber Co., Cleveland, Ohio.
[6] E. H. Sargent & Co., Chicago, Ill.
[7] Magni whirl utility water bath, Blue M Electric Co., Blue Island, Ill.

Table II—Effect of Phenobarbital on Mannitol Hexanitrate Assay

Standard Mannitol Hexanitrate[a] Absorbances	Phenobarbital Concentration, mg./100 ml.	Mannitol Hexanitrate Absorbance[b]
0.420	5.0	0.424 (+0.2%)
0.420	10.0	0.423 (0.0%)
0.427	15.0	0.421 (−0.5%)
0.422	20.0	0.420 (−0.7%)
0.426	25.0	0.416 (−1.6%)
Average 0.423	30.0	0.411 (−2.8%)
Range 1.6%	40.0	0.407 (−3.8%)
	Average	0.417
	Range	4.1%
	Average error	−1.4%

[a] Standard = 28.7 mg./100 ml. [b] Average of three readings.

Table III—Effect of Mannitol Hexanitrate on Phenobarbital Assay

Standard Phenobarbital[a] Absorbances	Mannitol Hexanitrate Concentration, mg./100 ml.	Phenobarbital Absorbance[b]
0.394	5.0	0.398 (+1.0%)
0.392	10.0	0.392 (−0.5%)
0.398	15.0	0.399 (+1.3%)
0.395	20.0	0.390 (−1.0%)
0.391	25.0	0.393 (−0.2%)
Average 0.394	30.0	0.387 (−1.8%)
Range 1.8%	40.0	0.385 (−2.3%)
	Average	0.392
	Range	3.6%
	Average error	−0.51%

[a] Standard = 15.1 mg./100 ml. [b] Average of three readings.

mined at 570 nm. for pentaerythritol tetranitrate. Mannitol hexanitrate is determined off the maximum at 613 nm. to reduce sensitivity in order to accommodate normal dosage levels. From the absorbance values the content per tablet is calculated.

RESULTS AND DISCUSSION

When the organic nitrate ester portion of the procedure was being developed, Schlieren effects were encountered in the 10-mm. flow cell used in the spectrophotometer. These effects, which manifest themselves by producing a jagged broad baseline, were eliminated by using a suggested[5] procedure similar to a reported technique (21, 22). A piece of glassine weighing paper was placed over the entrance to the photocell compartments for both the sample and reference cells. The paper has a diffusing effect on the light striking it and produces a steady baseline. Schlieren effects were not a problem in the phenobarbital assay, since an aqueous rather than an organic solvent passes through the flow cell.

To obtain reproducible results for pentaerythritol tetranitrate and mannitol hexanitrate, the cooling bath temperature must remain constant. Apparently, the diazotization coupling reaction is extremely temperature dependent.

Conformity to Beer's law was observed for pentaerythritol tetranitrate, mannitol hexanitrate, and phenobarbital over a range of concentrations exceeding those obtained from common dosage levels.

The number of moles of nitrate ion released from 1 mole of ester upon alkaline hydrolysis was previously investigated. A lack of stoichiometry for nitroglycerin was reported (15), probably because of complex competitive reactions involved in the decomposition of

[5] D. J. Winters, Food and Drug Administration, Cincinnati, Ohio, 1971, personal communication.

Table IV—Effect of Pentaerythritol Tetranitrate on Phenobarbital Assay

Standard Phenobarbital[a] Absorbances	Pentaerythritol Tetranitrate Concentration, mg./100 ml.	Phenobarbital Absorbance[b]
0.372	5.0	0.371 (+0.3%)
0.372	10.0	0.371 (+0.3%)
0.372	15.0	0.371 (+0.3%)
0.361	20.0	0.372 (+0.5%)
0.372	25.1	0.371 (+0.3%)
Average 0.370	30.1	0.377 (+1.9%)
Range 3.0%	40.1	0.373 (+0.8%)
	Average	0.372
	Range	1.6%
	Average error	+0.54%

[a] Standard = 15.1 mg./100 ml. [b] Average of three readings.

nitrate esters. Others, however, reported (17) that nearly 2 moles of nitrate ion is released per mole of nitroglycerin. In addition, nonstoichiometric results for pentaerythritol tetranitrate were reported (18).

The alkaline hydrolysis of pentaerythritol tetranitrate and mannitol hexanitrate through the present semiautomated system was investigated by comparing the absorbance obtained from the ester solutions to that obtained from a definite concentration of nitrite solution. Since the partitioning of nitrite ions between butanol and water is considerably different from that for organic nitrate esters, the semiautomated system was modified to bypass the partitioning step for this experiment. The modification consisted of attaching the exit end of the polyethylene tubing coil directly to the C-3 fitting (Fig. 2). Standard solutions of the esters were prepared using mixtures of pentaerythritol tetranitrate and lactose and of mannitol hexanitrate and lactose, which were previously standardized according to the NF procedure (12). Potassium nitrite (0.1 *M*) was standardized against sulfanilamide and diluted to obtain a suitable concentration in a final solvent of water–methyl isobutyl ketone–alcohol USP (1:1:2). For every mole of ester, 3.48 moles of nitrite ion is released from mannitol hexanitrate and 1.45 moles from pentaerythritol tetranitrate under the given conditions of hydrolysis.

Phenobarbital Interference in Nitrate Assay—The effect of a variation of phenobarbital concentration on the pentaerythritol tetranitrate and mannitol hexanitrate assays can be seen in Tables I and II. The average error in the nitrate results was less than ±2%. The nature of this slight interference is unclear. Under actual sample assay conditions, phenobarbital is added to the nitrate standard and this small error is essentially eliminated.

Nitrate Interference in Phenobarbital Assay—Tables III and IV show the effect of pentaerythritol tetranitrate and mannitol hexanitrate on the phenobarbital assay. In general, the interference is small and, as in the case of the phenobarbital interference in the nitrate assay, is eliminated by using a mixture of nitrate ester and phenobarbital for the phenobarbital standard.

Table V—Recoveries from Mixtures Based on Various Manufacturers' Formulations

Manufacturer	Composition	Dosage, mg./Tablet	Recovery[a], %
1	Pentaerythritol tetranitrate	10	100.8
	Phenobarbital	15	100.8
2	Pentaerythritol tetranitrate	10	100.2
	Phenobarbital	15	100.1
3	Pentaerythritol tetranitrate	10	100.3
	Phenobarbital	15	102.1
4	Mannitol hexanitrate	32.4	98.8
	Phenobarbital	16.2	102.4

[a] Average of five readings.

Table VI—Comparison of Semiautomated and Manual Results for Composites of Tablets from Various Manufacturers

Manufacturer	Composition	Dosage, mg./Tablet	Results in Percent of Label Declaration		
			NF[a]	USP[a]	Semiautomated[b]
1	Pentaerythritol tetranitrate	10	100.1	--	101.6
	Phenobarbital	15	—	99.0	101.3
2	Pentaerythritol tetranitrate	10	100.7	—	97.6
	Phenobarbital	15	—	97.4	99.3
	Pentaerythritol tetranitrate	10	101.4	—	99.4
	Phenobarbital	15	—	98.7	100.0
	Pentaerythritol tetranitrate	20	99.8	—	98.3
	Phenobarbital	16	—	95.0	96.9
3	Pentaerythritol tetranitrate	10	101.3	—	101.3
	Phenobarbital	15	—	91.6	90.1
5	Pentaerythritol tetranitrate	20	98.0	—	95.5
	Phenobarbital	15	—	98.3	99.3
4	Mannitol hexanitrate	32.4	96.0	—	94.2
	Phenobarbital	16.2	—	97.2	100.0
2	Mannitol hexanitrate	32	101.8	--	101.1
	Phenobarbital	16	--	98.2	98.8

[a] Average of two determinations. [b] Average of five readings.

Accuracy—Recovery studies were made on mixtures containing excipients and active ingredients corresponding to several manufacturers' formulations (Table V).

Several composites of 20 tablets each from various commercial samples were analyzed by the automated procedure and appropriate manual procedures. The NF procedure for pentaerythritol tetranitrate tablets (12) was used for pentaerythritol tetranitrate and mannitol hexanitrate. A factor of 0.7456 was used to calculate the mannitol hexanitrate levels (10). Phenobarbital was assayed by the USP procedure for phenobarbital tablets (23). The results (Table VI) are in agreement within ±3%.

Precision—The system coefficients of variation for pentaerythritol tetranitrate, mannitol hexanitrate, and phenobarbital were determined by measuring the absorbances obtained from a series of 25 cups of the appropriate standard solution. The following results were obtained: pentaerythritol tetranitrate, 1.60%; mannitol hexanitrate, 0.68%; and phenobarbital, 1.24%.

Interferences—Low results for phenobarbital were obtained by the automated method for the analysis of tablets containing methylcellulose or carboxymethylcellulose, since these agents inhibit the extraction of the barbiturate into chloroform.

SUMMARY

A semiautomated spectrophotometric procedure has been described for the simultaneous determination of pentaerythritol tetranitrate or mannitol hexanitrate and phenobarbital present in a single tablet.

Conformity to Beer's law was observed for the three drugs over a range of concentrations exceeding those obtained from common dosage levels.

The effect of one component on the assay results of the other is minimal at common dosage levels and can be essentially eliminated by using a mixture of nitrate ester and phenobarbital for the standard.

Assay results by the semiautomated procedure are in agreement with those obtained by manual procedures within ±3%.

Methylcellulose and carboxymethylcellulose interfered with the phenobarbital determination.

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Isolation of Alpinigenine from *Papaver bracteatum*

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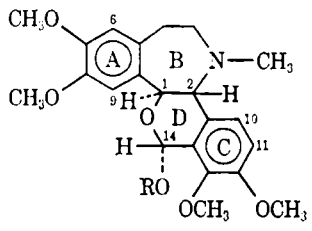
Abstract □ Dried latex of *Papaver bracteatum* was shown to contain 2.3% of alpinigenine. The structure was assigned on the basis of physical properties and spectroscopic analysis.

Keyphrases □ Alpinigenine—isolation from *Papaver bracteatum*, structure determination □ *Papaver bracteatum*—isolation of alpinigenine and its structure determination

In a continuing research program with chemotaxonomic studies of the papaver genus in Iran (1), the latex of *Papaver bracteatum*[1] was subjected to chromatographic analysis. In addition to the major alkaloid thebaine (1), another fraction was eluted, which, upon purification, was found to be alpinigenine as reported by Maturova *et al.* (2). NMR data ($J_{1,2}$ = 9 Hz.) were consistent with a *trans*-configuration of the B–D ring junction. For the elucidation of the stereochemistry at C_{14}, the alkaloid was treated with a methanolic solution of hydrochloric acid and two fractions were isolated by TLC. The slow moving fraction (R_f 0.68) was unchanged alpinigenine. The fast moving fraction (R_f 0.81) was shown to be epialpinine, previously reported by Maturova *et al.* (3) and Shamma *et al.* (4). These data prove that the alpinigenine separated in this work was not changed at C_{14} under equilibrating conditions and had the most stable configuration at C_{14} (Structure I). Similar results were indicated by Mann *et al.* (5).



I

alpinigenine: R = H
epialpinine: R = CH_3

EXPERIMENTAL[2]

Isolation of Alpinigenine—Dried latex (10 g.), obtained by incision of immature capsules of the plant, was treated with dilute ammonia to obtain a soft paste and extracted by trituration with chloroform (150 ml.). The extract was filtered and evaporated under reduced pressure, and the residue was treated with oxalic acid solution (4%, 50 ml.), filtered, and washed with water (20 ml.). The combined aqueous solutions were made alkaline with ammonia and extracted with chloroform (4 × 25 ml.). The organic layer was dried (sodium sulfate), filtered, and evaporated. The residue was subjected to TLC (silica gel), using acetone–chloroform–triethylamine–methanol (4:3:2:1) as eluting solvent. The fast moving fraction (R_f 0.9), after crystallization from methanol, gave 0.23 g. of alpinigenine, m.p. 185–186° [lit. (3) m.p. 193–195°]; UV_{max} 230 (log ε 4.2) and 284 (log ε 3.8) nm.; NMR: δ 7.35 (q, 2H, H_{10} and H_{11}), 6.95 and 6.67 (2s, 2H, H_6 and H_9), 6.4 (s, 1H, H_{14}), 5.8 (d, 1H, H_1, $J_{1,2}$ = 9 Hz.), 4.08 (d, 1H, H_2, $J_{1,2}$ = 9 Hz.), 3.95 and 3.86 (2s, 12H, OCH_3), 3.7–3 (m, 4H, aliphatic), and 2.34 (s, 3H, NCH_3) p.p.m.; mass spectrum: *m/e* 401 (M^+), 222 (base peak), 208, and 179 (large abundance of ions). The slow moving fraction (R_f 0.83) gave, on crystallization from aqueous ethanol, thebaine (2.8 g.), m.p. 198° [lit. (1) m.p. 198°].

Stereochemistry of Alpinigenine at C_{14}—The isolated alpinigenine (100 mg.) was dissolved in methanol (20 ml.), and a saturated methanolic solution of hydrochloric acid (2 ml.) was added. After standing for 2 hr. at room temperature, the solvent was evaporated and the residue was made alkaline with ammonia and extracted with chloroform. The organic layer was evaporated and the residue was subjected to TLC (silica gel), using ether (saturated with water)–acetone–diethylamine (85:8:7). The fast moving fraction (R_f 0.81, m.p. 56–58°) could not be crystallized. Its IR spectrum in chloroform solution, as well as other spectral data, was identical with those reported for epialpinine. The slow moving fraction (R_f 0.68) was unchanged alpinigenine.

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[1] The plant was identified by A. Zargari, Professor of Botany, Tehran University. A herbarium sample of the plant material has been deposited in the Missouri Botanical Garden, St. Louis, Mo.

[2] Melting points were taken on a Kofler hot-stage microscope and are uncorrected. UV spectra were recorded on a Unicam SP 800 instrument. IR spectra were obtained with a Leitz model III spectrograph. NMR spectra were determined in deuteriochloroform with a Varian A60A instrument. Mass spectra were determined with a Varian Mat 111 instrument.

Phosphorus–Nitrogen Compounds XV: *N*-1-Adamantylphosphoramidic Dichloride and Dimethyl and Diphenyl *N*-1-Adamantylphosphoramidate

L. A. CATES[A], R. L. GALLIO, and M. B. CRAMER

Abstract □ *N*-1-Adamantylphosphoramidic dichloride and the dimethyl and diphenyl esters of the corresponding acid were synthesized and evaluated for biological activity. The dimethyl ester delayed the onset of tremorine-induced tremors and reduced locomotor activity, while the diphenyl derivative slightly antagonized perphenazine-induced catatonic reactions. None of the agents displayed activity against lymphoid leukemia.

Keyphrases □ *N*-1-Adamantylphosphoramidic dichloride—synthesis, pharmacological screening as antiparkinsonism and anticancer agents □ *N*-1-Adamantylphosphoramidate, dimethyl and diphenyl—synthesis, pharmacological screening as antiparkinsonism and anticancer agents □ Amantadine derivatives—synthesis of *N*-1-adamantylphosphoramidic dichloride and dimethyl and diphenyl *N*-1-adamantylphosphoramidate as potential antiparkinsonism and anticancer agents □ Antiparkinsonism agents, potential—synthesis of amantadine derivatives, screening □ Anticancer agents, potential—synthesis of amantadine derivatives, screening

Among the many derivatives containing the adamantyl moiety, the most extensively studied is amantadine (1-adamantanamine, I). Early investigations of this agent mainly involved its antiviral properties, whereas several recent studies were concerned with antiparkinsonism activity. The beneficial effects in Parkinson's disease have been related to release of dopamine from neuronal storage sites (1, 2) rather than through anticholinergic mechanisms (1, 3), which is the basis for the action of agents commonly employed in treating this disease state.

Since good cholinolytic activity in three phosphoramidates, especially in those containing the diphenylphosphate grouping, was found previously (4), the two esters reported here were considered as potential antiparkinsonism agents. Amantadine, in addition to having therapeutic action *per se*, contributes the highly lipophilic adamantyl moiety to ensure ready distribution of the compounds to the CNS. A recent study (5) employed this grouping in the design of an analog of procyclidine hydrochloride. The effect of introducing an adamantyl fragment in prospective therapeutic agents has been the subject of many other inquiries (6); no investigation, however, has been reported on the consequence of phosphorylation of the 1-amino derivative of adamantane[1].

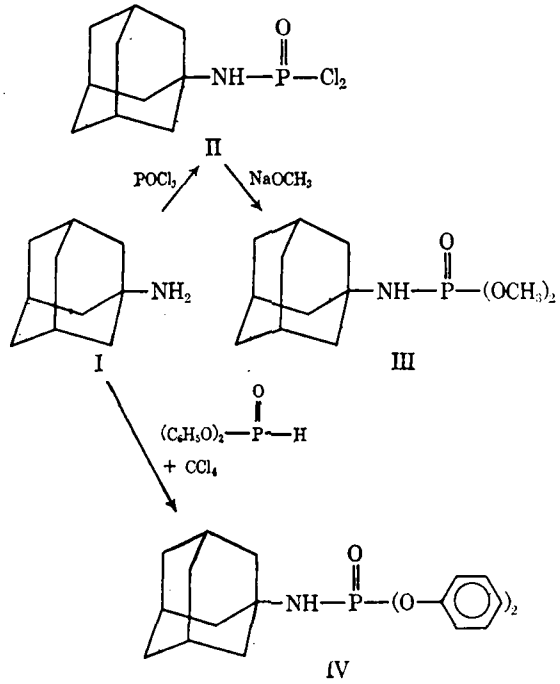
DISCUSSION

The desired adamantylphosphoramides (III and IV) were prepared by reacting sodium methoxide with *N*-1-adamantylphosphoramidic dichloride (II) and diphenylphosphonate with I and carbon tetrachloride (Scheme I). Compound II was synthesized using the phosphorus oxychloride–amine method (7). Information pertinent to these compounds, including spectral data, is presented in Table I.

Compounds III and IV were screened for antiparkinsonism activity by two methods. The onset of tremorine-induced tremors in mice, occurring within 10 min., was prolonged to 25 min. in the case of III at a dose of 250 mg./kg. No prolongation was noted with III at 125 mg./kg. or with IV at 250 mg./kg. Derivative III (250 mg./kg.) also doubled the period required for tremor induction produced by oxotremorine. Perphenazine-induced catatonic reactions were antagonized to the extent of 8% (III) and 25% (IV). These tests, summarized in Table II, indicate a potential activity of a weak nature for the esters in Parkinson's disease. Locomotor activity in mice was also shown to be reduced 25% by III. During gross observation studies, III caused ataxia and hindlimb paralysis and IV produced sedation and hypnosis in mice (250 mg./kg.). None of three new adamantylphosphoramides gave a beneficial response when tested against L-1210 lymphoid leukemia[2].

EXPERIMENTAL

***N*-1-Adamantylphosphoramidic Dichloride (II)**—This compound was prepared using the method of Michaelis (7) whereby a solution of freshly distilled phosphorus oxychloride (20.0 g., 0.13 mole) in anhydrous ether (20 ml.) was placed in a flask fitted with a thermometer, drying tube, and dropping funnel. Amantadine (20.0 g., 0.13 mole) and triethylamine (14.6 g., 0.14 mole) in anhydrous ether (150 ml.) were added dropwise with magnetic stirring



Scheme I

[1] During the time of this writing, the diphenyl ester was reported in Abstract 49, Division of Medicinal Chemistry, 165th ACS National Meeting, Dallas, Tex., Apr. 1973, by Warner, Mirth, Day, Turesky, and Soloway.

[2] The rationale for anticancer properties in these compounds and a description of active related agent(s) will be presented in a future paper.

Table I—*N*-1-Adamantylphosphoramidic Dichloride and Dimethyl and Diphenyl *N*-1-Adamantylphosphoramidate

Compound	Melting Point[a]	Yield, %	Formula	Analysis[b], % Calc.	Analysis[b], % Found	Spectral Data: IR[c] Maximum, μ	Spectral Data: NMR[d] δ, p.p.m.	Spectral Data: NMR[d] (Number of Protons)
II	129–131.5°	71.7	$C_{10}H_{16}Cl_2NOP$	C 44.80 H 6.02 N 5.22	44.49 6.01 5.08	3.15 (N—H) 8.04 (P—O)	1.95 1.68	(9) (6)
III	69–71°	64.2	$C_{12}H_{22}NO_3P$	C 55.16 H 8.49 N 5.36	55.04 8.58 5.25	3.15 (N—H) 8.30 (P—O) 9.79 (P—O—C)	3.70[e] 2.00 1.75 1.63	(6) (3) (6) (6)
IV	123.5–125°	68.9	$C_{22}H_{26}NO_3P$	C 68.92 H 6.83 N 3.65	68.74 6.96 3.62	3.15 (N—H) 6.35 (C—H) 8.15 (P—O) 9.70 (P—O—C)	7.25 1.90 1.60	(10) (9) (6)

[a] Taken on a Fisher-Johns apparatus and are corrected. [b] Performed by Atlantic Microlab, Inc. [c] Obtained on a Beckman IR-8 spectrophotometer using a Nujol mull. [d] Obtained on a Varian T-60 spectrometer using deuterated chloroform as the solvent and tetramethylsilane as the reference. [e] Doublet, J = 12 Hz.

Table II—Pharmacological Activities of the Amantadine Derivatives

Compound	Dose, mg./kg. i.p.	Onset of Tremors, min.	Perphenazine Reaction Score (Drug Effect, %) Test[a]	Perphenazine Reaction Score (Drug Effect, %) Control	Anticancer Screening[b] (L-1210 Lymphoid Leukemia in BDF_1 Mice) mg./kg.	Anticancer Screening[b] (L-1210 Lymphoid Leukemia in BDF_1 Mice) % T/C
II	–	–	—	–	400[c]	–
					200	93
III	125[d]	11	—	—	400	95
	250[d]	25	0.92 (8)	0.00 (100)	—	—
	250[e]	11	—	—	—	–
IV	250[d]	10	0.75 (25)	0.00 (100)	400	87
Tremorine	–	10	—	—	—	—
Oxotremorine	—	5	–	—	–	–
Atropine	—	–	0.92 (8)	0.00 (100)	—	—
Perphenazine	—	—	—	1.00 (0)	—	—
Vehicle[f]	—	—	–	0.00 (100)	–	–

[a] Perphenazine (5 mg./kg.) 15 min. pretreatment. [b] Performed by the screening contractors of the National Cancer Institute. Only highest, nontoxic dose is indicated. [c] No survivors out of six. [d] Compound followed with tremorine (0.6 mg./kg.) in 30 min. [e] Compounds followed with oxotremorine (0.5 mg./kg.) in 15 min. [f] Sodium carboxymethylcellulose, 1%.

over 1 hr. at 5–10°, during which time a white precipitate formed. After remaining overnight at room temperature, the reaction mixture was filtered and the filtrate was spin-evaporated to yield 25.0 g. of crystalline material. Recrystallization from ether gave the pure product.

Dimethyl *N*-1-Adamantylphosphoramidate (III)—To a stirred solution of II in 25 ml. of methanol was added a sodium methoxide solution prepared by reacting 1.15 g. of sodium metal with 30 ml. of absolute methanol. The temperature of the reaction mixture rose 30° and a white precipitate formed. After cooling and filtering, water was added to the filtrate and, when kept overnight, yielded 3.3 g. of III.

Diphenyl *N*-1-Adamantylphosphoramidate (IV)—According to the procedure of Atherton *et al.* (8), I (9.0 g., 0.06 mole) and triethylamine (6.0 g., 0.06 mole) in 80 ml. of carbon tetrachloride were placed in a flask fitted in the same manner as described for II. Diphenylphosphonate (11.7 g., 0.5 mole) in 15 ml. of carbon tetrachloride was added dropwise to this solution over 30 min. with a temperature rise of 35° and formation of a dense, white precipitate. After remaining overnight, the reaction mixture was filtered and the filtrate was spin-evaporated to yield a granular substance which gave 13.2 g. of pure IV when recrystallized from dilute ethanol.

Male Swiss albino mice (18–25 g.) in groups of four were used in the pharmacological screening. Compounds III and IV were suspended in 1% sodium carboxymethylcellulose and tested for antagonism of tremorine- (9) and oxotremorine- (10) induced tremors and perphenazine-produced catatonia (11). Atropine (2 mg./kg.) prevented tremorine-caused tremors for over 1 hr., except in the case of one animal which had tremor induction in 25 min. In addition, III reduced locomotor activity[3] by 25% at a dose of 250 mg./kg.

[3] Actophotometer, model ME-5200.

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Stability of Aspirin in Liquid and Semisolid Bases III: Effect of Citric and Tartaric Acids on Decomposition in a Polyethylene Glycol Base

C. W. WHITWORTH▲, H. W. JUN, and L. A. LUZZI

Abstract □ The decomposition of aspirin in a polyethylene glycol base was inhibited by citric and tartaric acids at 45, 26, and 4°. Even in the presence of added water, citric acid tended to slow the decomposition process while several other additives failed to influence it.

Keyphrases □ Aspirin, stability in liquid and semisolid bases—effect of citric and tartaric acids on decomposition in polyethylene glycol base □ Suppositories—inhibition of aspirin decomposition by citric and tartaric acids, polyethylene glycol base □ Polyethylene glycol—aspirin stability, inhibition of decomposition by citric and tartaric acids □ Degradation, aspirin—inhibition by citric and tartaric acids in polyethylene glycol-type suppository

Aspirin decomposes rapidly when incorporated into a polyethylene glycol suppository base. The process was found to be partly due to transesterification (1) and is greatly temperature dependent.

Mixtures of polyethylene glycols have been used extensively as suppository bases for several years (2). These mixtures have desirable characteristics as bases since they do not require refrigeration, provided the incorporated drugs are stable at room temperature. Aspirin is less stable in these polyethylene glycol bases than in cocoa butter, which has to be refrigerated to prevent melting. The purpose of this study was to stablize aspirin in a polyethylene glycol suppository base using additives while retaining the essential characteristics of the base. Of the several additives tested, citric and tartaric acids proved promising. Less effective substances included calcium gluconate, colloidal silica, and acetic acid.

EXPERIMENTAL

Reagents—The basic polyethylene glycol formula into which aspirin and additives were incorporated was as follows: polyethylene glycol 400[1], 24.8%; polyethylene glycol 1540[1], 30.4%; polyethylene glycol 6000[1], 40.0%; and polysorbate 60[2], 4.8%.

The different concentrations of citric acid[3] and tartaric acid[3] were based on the weight of the suppository vehicle. The ingredients were melted at 55° with stirring, and aspirin[4] equal to 12% of the base-additive mixture was stirred into the melted mixture. The mixture was poured with stirring into suppository molds and permitted to congeal. Each batch was divided into three portions for storage at 45, 26, and 4°.

Analytical Method—Assays were performed at short intervals at the beginning of the study and at greater intervals as the rate of degradation slowed.

Each suppository was dissolved in 100 ml. of chloroform containing 1% acetic acid. A 1:50 dilution was made, and the absorbance was read at 278 nm. for aspirin and at 308 nm. for salicylic acid on a spectrophotometer[5]. The moles of each were determined from a standard curve, and the mole percent (percent decomposition) of salicylic acid was calculated.

RESULTS AND DISCUSSION

The results with all concentrations of citric and tartaric acids were somewhat similar. Figure 1 shows the decomposition of aspirin at 45, 26, and 4° in the polyethylene glycol suppository base when no additive was used. As in numerous other studies (3), the decomposition of aspirin was highly temperature dependent. The decomposition was approximately 26% in 28 days at 45°, about 18% in 80 days at 26°, and approximately 3% in 100 days at 4°. Refrigeration of aspirin suppositories utilizing a polyethylene glycol base is a necessity.

Figure 2 shows the decomposition of aspirin in the polyethylene glycol base in the presence of 1% citric acid. The decomposition was inhibited particularly at 26 and 4°. At 45°, the decomposition reached about 23% at 28 days; at 26°, it was around 11% after 102 days; and at 4°, it reached just over 2% in 110 days.

An increase in citric acid concentration to 5% resulted in a further decrease in the decomposition rate at 26 and 4°, but no change was noted at 45°. The decomposition at 26° reached about 8% in 100 days, while at 4° it was only about 1%.

There was little or no improvement in the stability of aspirin with 10% citric acid compared to the 5% concentration. Citric acid at the 10% level inhibited the decomposition of aspirin even in the presence of 5% added water. The rate of decomposition was greater than that shown without water but less than that of the control.

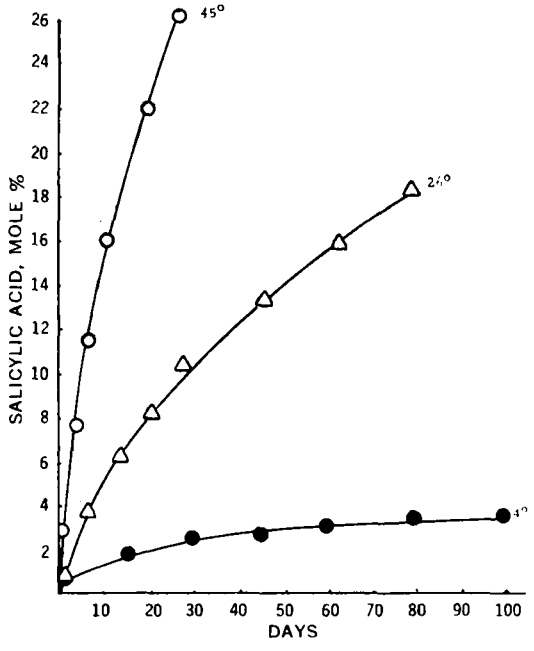

Figure 1—*Mole percent decomposition of aspirin with time in a polyethylene glycol base.*

[1] Union Carbide.
[2] Atlas Chemicals.
[3] J. T. Baker Chemical Co.
[4] Merck and Co.
[5] Beckman DU.

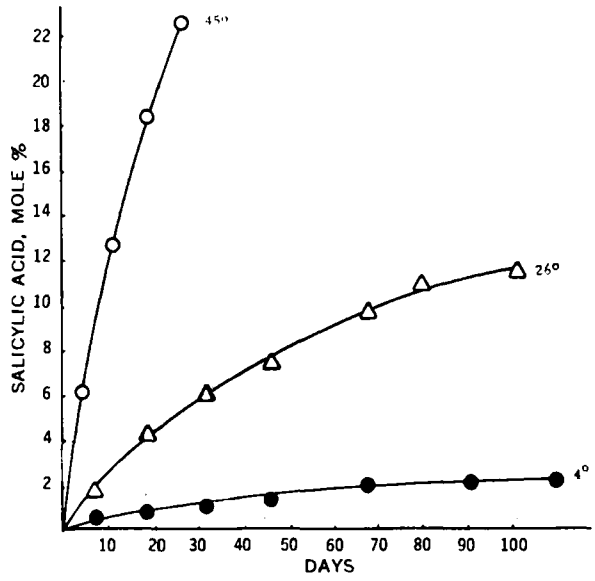

Figure 2—*Mole percent decomposition of aspirin with time in a polyethylene glycol base containing 1% citric acid.*

The effect of 10% tartaric acid on aspirin decomposition in this base was studied for a limited period, and the results were similar to those obtained with 5 and 10% citric acid for a comparable time. The percent decomposition at 26° was slightly lower with tartaric acid, but little difference was noted between these similar acids at 45 and 4°.

CONCLUSIONS

Certain substances can be added to a polyethylene glycol-type aspirin suppository mixture without appreciably changing its properties. Of the several additives studied, citric and tartaric acids inhibited decomposition. A 5% concentration of citric acid appeared to be the optimum concentration necessary to hinder decomposition. The importance of refrigeration at 4° on decomposition of aspirin in polyethylene glycol was evident.

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Biological Activity of Pentobarbital Metabolites

H. D. CHRISTENSEN▲, L. BARNETT, and F. I. CARROLL

Abstract □ The (1′*S*,3′*R*)-, (1′*S*,3′*S*)-, (1′*R*,3′*R*)-, and (1′*R*,3′*S*)-5-ethyl-5-(3′-hydroxy-1′-methylbutyl)barbituric acid metabolites of pentobarbital are relatively inactive and, therefore, cannot account for the differences in potency found between *R*(+)- and *S*(−)-pentobarbital. The (1′*S*,3′*R*)- and (1′*S*,3′*S*)-hydroxypentobarbital metabolites have very weak sedative activity when administered to CF-1 mice intravenously at an AD_{90} anesthetic dose of *RS*-pentobarbital.

Keyphrases □ Pentobarbital metabolites—anesthetic and behavioral activity in mice □ Barbituric acid metabolites, pentobarbital—anesthetic and behavioral activity in mice □ Anesthetic activity—pentobarbital metabolites, mice

The diastereoisomeric pairs of 5-ethyl-5-(3′-hydroxy-1′-methylbutyl)barbituric acid were first shown by Maynert and Dawson (1) to be biotransformation products of pentobarbital. More recently, the four optical isomers of 3′-hydroxypentobarbital were isolated in this laboratory from metabolism studies of optically pure *R*(+)- and *S*(−)-pentobarbital (2, 3). *R*(+)-Pentobarbital is metabolized to approximately equal amounts of the two hydroxylated metabolites in contrast to (*S*)-pentobarbital in which the ratio is 9:1. The *S*(−)-isomer is more toxic and potent as an anesthetic agent in mice than is the *R*(+)-isomer or the racemate (4). Maynert and Dawson (1) reported that the diastereoisomeric metabolites were inactive. Dickert *et al.* (5) found that (1′*RS*,3′*SR*)-3′-hydroxypentobarbital, prepared by synthetic methods, had very weak anticonvulsant activity and no anesthetic properties. Pharmacological differences in the enantiomers of pentobarbital and the respective ratios of their 3′-hydroxy enantiomers indicated the desirability of determining if some 3′-hydroxypentobarbital isomers were active and contributing to the differences in the activity of the pentobarbital stereoisomers.

EXPERIMENTAL

The structure and absolute stereochemical assignment of *R*- and *S*-pentobarbital as well as the four optically active 3′-hydroxypentobarbital metabolites were reported previously (6–9). The optical rotations and melting-point values for pentobarbital stereoisomers are: (*RS*), m.p. 129–130°; (*R*), $[\alpha]_D$ +13.1°, m.p. 121–122°; and (*S*), $[\alpha]_D$ −13.2°, m.p. 121–122°. For 3′-hydroxypentobarbital the values are: (1′*RS*,3′*RS*), m.p. 145–147°; (1′*RS*,3′*SR*), m.p. 191–192°; (1′*R*,3′*R*), $[\alpha]_D$ +12.8°, m.p. 170–176°; (1′*R*,3′*S*), $[\alpha]_D$ +30.5°, m.p. 202–205°; (1′*S*,3′*S*), $[\alpha]_D$ −15.3°, m.p. 181–183°; and (1′*S*,3′*R*), $[\alpha]_D$ −31.5°, m.p. 208–210°.

Biological activity was assessed by administering each epimer at the AD_{90} anesthetic dose of *RS*-pentobarbital to 10 CF-1 male mice.

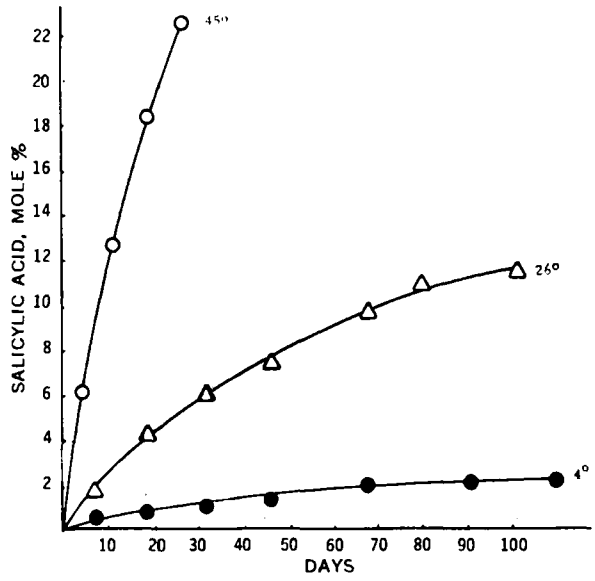

Figure 2—*Mole percent decomposition of aspirin with time in a polyethylene glycol base containing 1% citric acid.*

The effect of 10% tartaric acid on aspirin decomposition in this base was studied for a limited period, and the results were similar to those obtained with 5 and 10% citric acid for a comparable time. The percent decomposition at 26° was slightly lower with tartaric acid, but little difference was noted between these similar acids at 45 and 4°.

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Certain substances can be added to a polyethylene glycol-type aspirin suppository mixture without appreciably changing its properties. Of the several additives studied, citric and tartaric acids inhibited decomposition. A 5% concentration of citric acid appeared to be the optimum concentration necessary to hinder decomposition. The importance of refrigeration at 4° on decomposition of aspirin in polyethylene glycol was evident.

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EXPERIMENTAL

The structure and absolute stereochemical assignment of *R*- and *S*-pentobarbital as well as the four optically active 3′-hydroxypentobarbital metabolites were reported previously (6–9). The optical rotations and melting-point values for pentobarbital stereoisomers are: (*RS*), m.p. 129–130°; (*R*), $[\alpha]_D$ +13.1°, m.p. 121–122°; and (*S*), $[\alpha]_D$ −13.2°, m.p. 121–122°. For 3′-hydroxypentobarbital the values are: (1′*RS*,3′*RS*), m.p. 145–147°; (1′*RS*,3′*SR*), m.p. 191–192°; (1′*R*,3′*R*), $[\alpha]_D$ +12.8°, m.p. 170–176°; (1′*R*,3′*S*), $[\alpha]_D$ +30.5°, m.p. 202–205°; (1′*S*,3′*S*), $[\alpha]_D$ −15.3°, m.p. 181–183°; and (1′*S*,3′*R*), $[\alpha]_D$ −31.5°, m.p. 208–210°.

Biological activity was assessed by administering each epimer at the AD_{90} anesthetic dose of *RS*-pentobarbital to 10 CF-1 male mice.

Table I—Comparison of Activities of 3′-Hydroxy Metabolites to Pentobarbital

Compound	Number of Animals	Number Anesthesized	Average Duration of Anesthesia, min. ± *SD*	Time to Onset of Anesthesia, min.	Behavioral Alterations[a]
Pentobarbital					
RS	20	19	110 ± 24	3.1	+++
R(+)	10	1	35 ± —	10.0	+++
S(−)	10	10	114 ± 32	2.0	+++
3′-Hydroxypentobarbital					
1′*RS*,3′*RS*	10	0	— —	—	+
1′*RS*,3′*SR*	10	0	— —	—	+
1′*R*,3′*R*	10	0	— —	—	−
1′*R*,3′*S*	10	0	— —	—	−
1′*S*,3′*S*	10	0	— —	—	+
1′*S*,3′*R*	7	0	— —	—	+

[a] Indicates the magnitude: + = positive and − = none.

The behavior and time of onset and duration of anesthesia, measured as sleeping time, were observed. Each 3′-hydroxypentobarbital metabolite was further evaluated in five mice with a behavioral examination (10). This consisted of a systematic observation method for assessing the behavioral and physiological state of the mouse and its response to drugs. The pattern profile of various classes of pharmacological agents can be identified and differentiated, and the relative potency of members of the same class of drugs can be established. The dose volume used, 10 ml./kg. i.v., was administered *via* the tail vein.

The free acid was dissolved in 0.1 *M* aqueous sodium hydroxide to give the monosodium salt and diluted to appropriate volume with physiological saline.

RESULTS

The comparison of activity for *R*(+)- and *S*(−)-pentobarbital and the six 3′-hydroxypentobarbital derivatives of *RS*-pentobarbital is shown in Table I. Each compound was administered at 40 mg./kg. i.v.

The potency difference between the *R*(+)- and *S*(−)-pentobarbital is shown by the relative number of mice exhibiting sleeping by loss of the righting reflex. There is a slightly faster onset of action with the *S*(−)-form than with racemic pentobarbital. All *R*(+)-treated mice were very sedated; two of these mice just failed to meet the sleeping criterion of complete loss of the righting reflex for at least 1 min. None of the mice administered a 3′-hydroxypentobarbital metabolite was close to the sleeping criterion. However, the animals treated with the two racemic isomers as well as the two optically active metabolites having the 1′*S* configuration were sedated for approximately 1 hr. By using the screening test of the mouse's behavioral and physiological state, mice treated with 1′*RS*, 3′*RS*, 3′*SR*; and 1′*S*,3′*S* exhibited behavioral alterations of decreased awareness, increased positional passivity, and sedation. The onset of action is between 15 20 min. and lasts for approximately 1 hr. This screen is subjective, but the slight difference observed between the 3′-hydroxy metabolites indicates some variation in potency. The order of potency is: (1′*S*,3′*R*) > (1′*RS*,3′*SR*) > (1′*S*,3′*S*) > (1′*RS*,3′*RS*). The (1′*R*,3′*R*)- and (1′*R*,3′*S*)-isomers produced no behavioral or physiological alteration at 40 mg./kg.

DISCUSSION

The 3′-hydroxy metabolites are not pharmacologically inert as reported (11), but their activity is much less than that of the parent compound. When they are administered intravenously, some sedation occurs. Dickert *et al.* (5) reported that the (1′*RS*,3′*SR*)-isomer, given orally to mice at 1 g./kg., did not produce anesthesia or ataxia. They did, however, observe weak anticonvulsant activity. The ED_{50} in the maximal electroshock test was observed at 310 mg./kg. Their results (5) and the data reported here indicate that the activity profile of the 3′-hydroxybarbiturates is not identical to that of their parent compound. The difference found in the potency of *R*(+)- and *S*(−)-pentobarbital cannot be ascribed to differences in accumulations of these metabolites. Their potency difference could be due to differences in the rate of metabolism, binding properties, or relative penetration capacity to the receptor sites as well as other factors.

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Effect of Addition of Sodium Salts to Salicylic Acid–Cetrimide System

LUCY S. C. WAN

Abstract □ The viscosity of salicylic acid–cetrimide systems was altered in the presence of sodium salts. The viscosity variation was associated with the degree of salicylic acid saturation of the surfactant solution and apparently not with the pH of the system. The increase in viscosity was probably due to formation of sodium salicylate by a reaction of the sodium additive with the acid and the interaction of this salicylate with the free cetrimide molecules in the system. The decrease in viscosity was attributed to the nonavailability of these surfactant molecules.

Keyphrases □ Salicylic acid–cetrimide systems—effect of sodium salts on viscosity □ Cetrimide–salicylic acid systems—effect of sodium salts on viscosity □ Sodium salts—effect on viscosity of salicylic acid–cetrimide systems □ Viscosity, salicylic acid–cetrimide systems—effect of sodium salts

In past studies, additives of various types such as polar and nonpolar solvents (1) and surfactants that are anionic (2), cationic, and nonionic (3) were added to salicylic acid–cetrimide systems similar to those in the present investigation and the resulting change in viscosity was measured. This change was found to differ with the type of additive. As a continuation of these studies of viscosity response to additives, the effect of aqueous solutions of electrolytes that are mainly alkaline in reaction on the macromolecules formed by the interaction of salicylic acid with cetrimide (4) was investigated. Sodium salts of carboxylic and phosphoric acids as well as sodium carbonate were used.

EXPERIMENTAL

Materials—Cetrimide BP[1] and recrystallized salicylic acid, m.p. 158–159°, were the same as those described previously (1). The following were used: sodium citrate[2]; sodium tartrate[3]; sodium acetate[3]; sodium pyrophosphate[4]; sodium phosphate, dibasic[4]; sodium phosphate, tribasic[4]; sodium phosphate, monobasic[4]; and sodium carbonate[4].

Measurement of Viscosity at 25°—The method described earlier (1) was adopted[5].

Measurement of pH—This was determined using a pH meter[6].

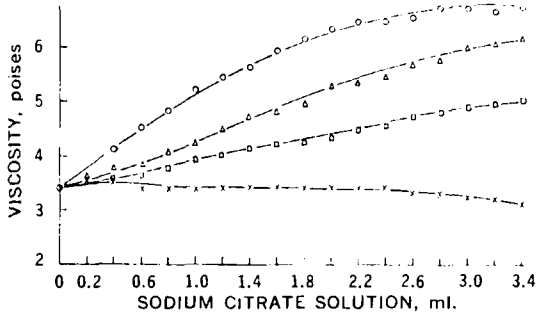

Figure 1—*Effect of sodium citrate solutions of varying concentration on the viscosity of systems containing 1.4% salicylic acid and 5% cetrimide (50 g.) at 25°. Key (sodium citrate, % w/v): ○, 10; △, 6; □, 4; and ×, 1. Shear rate = 78.56 sec.*$^{-1}$.

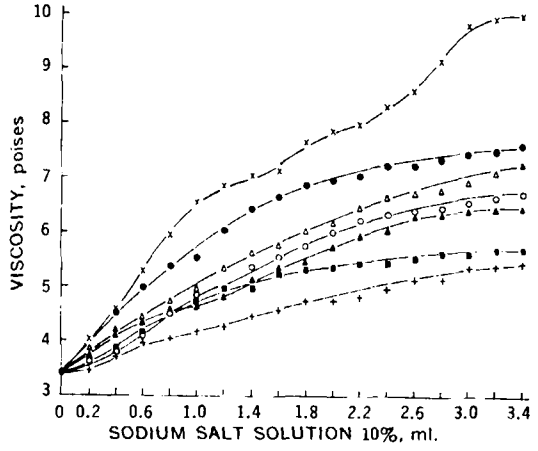

Figure 2 *Viscosity increase due to addition of sodium salt solutions (10% w/v) to systems containing 1.4% salicylic acid and 5% cetrimide (50 g.) at 25°. Key: △, sodium phosphate, dibasic; +, sodium phosphate, monobasic; ×, sodium carbonate; ○, sodium tartrate; ▲, sodium acetate; ●, sodium phosphate, tribasic; and ■, sodium pyrophosphate. Shear rate = 78.56 sec.*$^{-1}$.

RESULTS AND DISCUSSION

Figure 1 shows the change in viscosity of salicylic acid–cetrimide systems with the addition of sodium citrate solutions of different concentrations. The viscosity increased in the presence of the sodium additive, the increase being higher with a greater volume of the salt solution. However, with a low concentration, a slight decrease in viscosity was noted. A similar rise in viscosity was found with

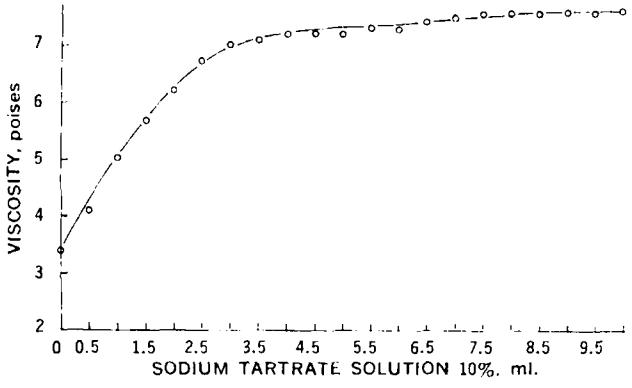

Figure 3—*Effect of increasing the volume of sodium tartrate solution on the viscosity of systems containing 1.4% salicylic acid and 5% cetrimide (50 g.) at 25°. Shear rate = 78.56 sec.*$^{-1}$.

[1] Glovers Chemicals Ltd., Leeds 12, England.
[2] May and Baker, Dagenham, England.
[3] British Drug Houses, Ltd., England.
[4] E. Merck, Darmstadt, Germany.
[5] A portable Ferranti viscometer, model VL, was used in this study.
[6] Beckman pH expandometer.

Table I—*pH of System Containing 5% Cetrimide and 1.4% Salicylic Acid (50 g.) to Which Sodium Salt Solutions Were Added*

Salt Solution, 10% w/v	Volume of Sodium Salt Solution, ml.									
	0	0.4	0.8	1.2	1.6	2.0	2.4	2.8	3.2	3.4
Sodium citrate	2.45	2.45	2.5	2.55	2.55	2.65	2.85	2.95	3.1	3.2
Sodium tartrate		2.45	2.45	2.5	2.55	2.6	2.7	2.85	3.05	3.1
Sodium acetate		2.45	2.5	2.5	2.6	2.6	2.65	2.85	3.05	3.1
Sodium phosphate, dibasic		2.45	2.45	2.45	2.55	2.65	2.7	2.7	2.75	2.75
Sodium phosphate, monobasic		2.45	2.45	2.5	2.5	2.5	2.55	2.55	2.6	2.6
Sodium phosphate, tribasic		2.5	2.55	2.65	2.7	2.85	2.9	3.05	3.1	3.15
Sodium pyrophosphate		2.45	2.5	2.55	2.6	2.65	2.7	2.7	2.75	2.8

Table II—Variation of pH of Salicylic Acid–Cetrimide System (50 g.) Containing 5% Cetrimide and Varying Concentration of Acid by Addition of Sodium Carbonate Solution, 10% w/v

5% Cetrimide with:	Volume of Sodium Carbonate, 10% w/v, ml.													
	0.4	0.8	1.2	1.6	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8
1.2% Salicylic acid	2.45	2.6	2.75	3.1	3.55	3.6	3.65	4.1	4.5	5.1	5.85	6.65	7.4	8.1
1.4% Salicylic acid	2.45	2.6	2.75	3.0	3.25	3.35	3.45	3.65	3.9	4.35	4.75	5.45	6.15	6.95
1.6% Salicylic acid	2.5	2.6	2.75	2.95	3.2	3.3	3.4	3.65	3.9	4.35	4.4	4.6	5.1	5.6
1.7% Salicylic acid	2.45	2.55	2.7	2.9	3.1	3.2	3.25	3.35	3.6	3.85	4.05	4.35	4.8	5.35
1.8% Salicylic acid	2.45	2.55	2.65	2.8	2.95	3.05	3.25	3.4	3.5	3.65	3.9	4.15	4.6	5.1

sodium acetate, sodium tartrate, sodium carbonate, and various sodium phosphates (Fig. 2). Increasing the total volume of sodium tartrate solution incorporated beyond the amount shown in Fig. 2 did not result in any further change, and the viscosity remained constant (Fig. 3). This situation also applied to the other sodium salts mentioned previously.

Sodium carbonate demonstrated the most marked viscosity effect (Fig. 2). This effect was found to be dependent on the amounts of salicylic acid and cetrimide present in the system, *i.e.*, the degree of saturation of the system. In related dispersions without additives, it was shown that, on the whole, maximum viscosity was attained at or near saturation (4). The viscosity behavior of systems consisting of the surfactant and such quantities of salicylic acid so as to produce undersaturated and oversaturated dispersions, when sodium carbonate solutions were added, was different in each type of system (Fig. 4). Where the salicylic acid concentration was less than that required for saturation, the viscosity alteration resembled that described previously. However, when the acid content exceeded what was necessary for saturation, there was a decrease or a small increase initially; subsequently, a reduction occurred, followed by a marked increase in viscosity. With sodium phosphate, dibasic, and sodium citrate solutions (Fig. 5), there was only a viscosity decrease in such systems and a rise in viscosity in those that were not saturated. The other sodium salts used also exhibited the same effect. The difference in behavior could be attributed to the fact that sodium carbonate is a strong alkali. This was supported by a similar viscosity variation observed when the sodium hydroxide solution was incorporated into identical systems.

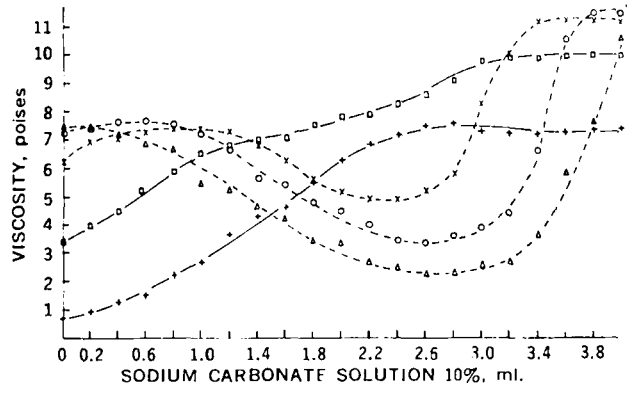

Figure 4—*Relationship between viscosity change and saturation of salicylic acid–cetrimide system (50 g.) in the presence of sodium carbonate solution (10% w/v) at 25°. Key (salicylic acid, % w/v): +, 1.2; □, 1.4; ×, 1.6; ○, 1.7; △, 1.8; ——, undersaturated; and - - -, oversaturated. Cetrimide = 5% w/v. Shear rate = 78.56 sec.$^{-1}$.*

In the presence of sodium salts, the pH values obtained differed from one additive to another and with varying volumes of the same salt solution (Table I). Compared to the effect of sodium carbonate in the corresponding system (Table II), the pH variation (2.45 to 6.95) of the latter was very marked. The pH of the systems in Table I is strongly acidic, although the additive solutions are alkaline in reaction (Table III), with the exception of sodium phosphate, monobasic. In those systems containing different amounts of salicylic acid, the increase was greater with undersaturated than with oversaturated systems (Table II). This is due to the lower concentration of salicylic acid in the former case. A comparison of the change in viscosity (Fig. 4) with those of pH (Table II) showed that pH increased with the sodium carbonate solution added, irrespective of whether there was an increase or a decrease in viscosity. This suggested that the alteration in viscosity was not associated directly with the pH of the system concerned.

A previous report (4) showed that salicylic acid interacts with cetrimide, resulting in the formation of a viscous product. In the current study, unsaturated systems contained insufficient salicylic acid to interact with all of the surfactant molecules, leaving free

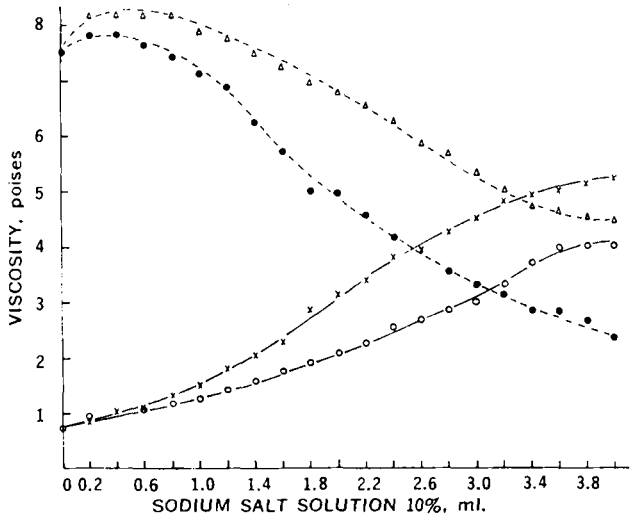

Figure 5—*Change in viscosity of salicylic acid–cetrimide systems (50 g.) that are oversaturated (salicylic acid 1.8% and cetrimide 5%) and undersaturated (salicylic acid 1.2% and cetrimide 5%) on addition of sodium citrate and sodium phosphate, dibasic, solutions (10% w/v) at 25°. Key: - - -, oversaturated; ●, sodium citrate; △, sodium phosphate, dibasic; ——, undersaturated; ×, sodium citrate; and ○, sodium phosphate, dibasic. Shear rate = 78.56 sec.$^{-1}$.*

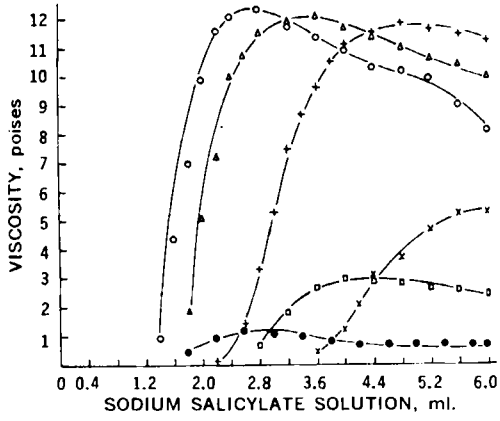

Figure 6—*Viscosity variation of a fixed concentration of cetrimide (5% w/v) with varying sodium salicylate solutions and a fixed concentration of sodium salicylate solution (10% w/v) with varying amounts of cetrimide at 25°. Key (sodium salicylate, % w/v): ○, 40; △, 30; and +, 20. Key (cetrimide, % w/v): ●, 1.0; □, 2.0; and ×, 3.0. Shear rate = 78.56 sec.$^{-1}$.*

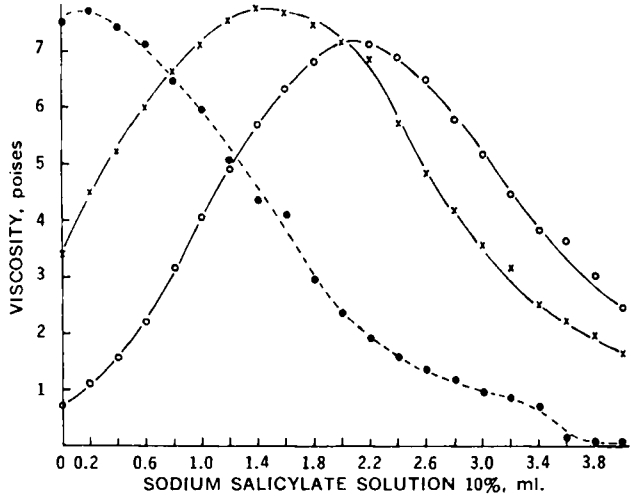

Figure 7—*Viscosity behavior of systems (50 g.) containing 5% cetrimide and different quantities of salicylic acid in the presence of sodium salicylate at 25°. Key (salicylic acid, % w/v): ●, 1.8; ×, 1.4; ○, 1.2; ——, undersaturated; and - - -, oversaturated. Shear rate = 78.56 sec.$^{-1}$.*

cetrimide molecules for possible reaction with the added sodium salt. However, it was found that the addition of sodium salts to cetrimide in the absence of salicylic acid produced no effect on the viscosity of the solution. Therefore, it is not likely that the observed increase in viscosity is brought about by a simple reaction involving only surfactant molecules and the sodium salt.

The increase in viscosity may be due to a reaction between the sodium additive and the salicylic acid, with the tendency to form sodium salicylate. This sodium salicylate will interact with the free cetrimide molecules in unsaturated systems to give rise to the high viscosity values determined, in the same manner as was found when sodium salicylate solutions were allowed to interact with the surfactant solution without the acid (Fig. 6). Variation of the sodium salicylate or cetrimide concentration in the interaction between these two compounds resulted in a viscosity increase followed by a decrease as the volume of sodium salicylate solution became larger. When the cetrimide concentration was increased to 5% to correspond with that used in the systems studied so far, no increase in viscosity was recorded even after 4 ml. of sodium salicylate solution was incorporated. This is because, with a greater amount of surfactant, more sodium salicylate is required to effect a change in viscosity, as can be seen in the viscosity behavior of the 5% cetrimide solution when different quantities of sodium salicylate were added (Fig. 6).

The viscosity reduction occurring in oversaturated dispersions could be caused by a change in the salicylic acid concentration due to a removal of the excess acid brought about by a reaction of the latter with the sodium additive. But since most surfactant in the system would have interacted with the acid, no increase in viscosity resulted. Further evidence in support of these suggestions can be obtained from the results shown in Fig. 7. In undersaturated systems, the viscosity increased in a similar manner to that due to the addition of sodium salts (Figs. 1 and 2). However, after reaching a maximum, the viscosity decreased. This reduction could be attributed to a loss of free cetrimide molecules from the system because of the interaction with sodium salicylate. In oversaturated dispersions, only a lowering in viscosity was produced, as was stated earlier in relation to sodium citrate and sodium phosphate, dibasic (Fig. 5), since surfactant molecules were not available for the interaction with sodium salicylate.

In the case where the sodium additive is a strong alkali, such as sodium carbonate, the pattern of viscosity response varied with the volume of sodium carbonate solution added (Fig. 4). When adequate solution of the alkali was included, the amount of salicylic acid remaining would no longer be in excess of that essential for saturating the cetrimide solution and hence the system was not likely to be oversaturated anymore. Subsequent additions of sodium carbonate solution, therefore, increased the viscosity in accordance with that which occurred in the undersaturated systems. With the other sodium salts used, even when the total volume of the additive solution was increased to 8 ml., no subsequent increase in viscosity was produced after a prior decrease in viscosity.

The findings of this study suggest that viscosity variation of salicylic acid–cetrimide systems in the presence of some sodium salts depends on the availability of free cetrimide molecules and the interaction of the resultant sodium salicylate with these surfactant molecules. It did not seem to be governed by pH.

Table III—pH of Solutions of Sodium Salts

Salt Solution	pH
Sodium citrate 1%	8.55
Sodium citrate 4%	8.6
Sodium citrate 6%	8.65
Sodium citrate 10%	8.75
Sodium tartrate 10%	8.0
Sodium acetate 10%	8.6
Sodium pyrophosphate 10%	10.5
Sodium phosphate, tribasic, 10%	11.95
Sodium phosphate, dibasic, 10%	9.45
Sodium phosphate, monobasic, 10%	4.9
Sodium carbonate 10%	10.75

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Sodium Cyanate: Chemical Properties Relevant to Sickle Cell Disease Therapy

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Abstract □ A purification procedure for sodium cyanate and some of its chemical properties relevant to clinical studies on sickle cell disease are described. In aqueous solution, sodium cyanate decomposes to ammonia and sodium bicarbonate, the latter being the only contaminant detected after purification. The rate of cyanate reaction with water increases with temperature, concentration, and decreasing pH. The most convenient method of sterilization is dry heat. Under the conditions of time, temperature, and concentration to which erythrocytes are most likely to be exposed in clinical application, sodium cyanate can be considered stable to aqueous solution, except for its reaction with proteins and other biological materials.

Keyphrases □ Sodium cyanate—purification, analysis, sterilization, stability □ Sickle cell disease—therapy, relevant chemical properties of sodium cyanate

In 1971, Nalbandian (1) proposed the use of urea in treating sickle cell disease. Since urea equilibrates with cyanate and cyanate carbamylates proteins, reactions that have been the bases of numerous chemical investigations, Cerami and Manning (2) hypothesized that cyanate was an active agent in the desickling of cells by urea. It has now been established that the two compounds act by different mechanisms. Urea disrupts hydrophobic bonds to cause a reversible desickling of erythrocytes. By contrast, cyanate carbamylates the four terminal valine residues of hemoglobin and possibly other sites to cause an irreversible desickling effect. Since the biological effects of sodium cyanate received little mention in the early literature (3), biochemical and pharmacological studies (4–8) are being conducted as a prelude to more extensive clinical testing of the compound as a possible therapeutic agent for sickle cell disease.

Clinical and toxicological investigations of cyanate have generally used the sodium salt, which is not commercially available in purified form. Recrystallization of sodium cyanate from alcohol is about the extent of information available on the subject of purification. Reactions of cyanate with water in alkaline and acidic conditions have been studied from kinetic and thermodynamic points of view (9, 10), but the chemistry of the compound relative to its handling and use as a drug has not been previously discussed. This report describes the purification of sodium cyanate and some chemical properties that pertain to its clinical application.

EXPERIMENTAL

Purification—Sodium cyanate of a technical grade was purchased[1] and had a supplier-indicated purity of 98% and water content of 0.5%. The compound was stated to have been prepared from urea and sodium carbonate. As obtained, the sodium cyanate was a slightly gray powder having some odor. The scale of the purification procedure developed can be adjusted to meet individual needs, but the amounts used here can be processed in common laboratory ware. Sodium cyanate is added with rapid stirring to water preheated to 60° (180 g./l.). Stirring is continued until the salt is completely dissolved but not for more than 5 min. The hot turbid solution is filtered as rapidly as possible through a preheated medium-porosity sintered-glass filter overlaid with a pad of cellulose[2]. The residue is washed with a small amount of 60° water. The filtrate is reheated to 60° and, while maintaining this temperature, 1.5 volumes of reagent grade methanol is added with rapid stirring; some sodium cyanate is salted out during the addition of the methanol. The solution is cooled and kept at 4° overnight. The sodium cyanate is removed in a precooled coarse sintered-glass filter. It is washed on the filter twice with cold methanol–water (3:2) and once with cold methanol; a volume equivalent to about one-tenth that of the original solution is used for each wash. The pure white sodium cyanate crystals are spread to facilitate rapid air drying at room temperature in a dust-free environment.

Cyanate Assay—The cyanate assay was based upon a method described by Werner (11) in which a blue copper–pyridine–cyanate complex is extracted into chloroform for colorimetric measurement. The original method, as improved by Marier and Rose (12) to gain sensitivity, was modified slightly for simplicity. To carry out the assay, up to 4.0 ml. of solution containing 5–50 μmoles of cyanate is placed in a glass-stoppered tube. Pyridine (0.1 ml.) and 0.1 *M* cupric sulfate (0.2 ml.) are added, and the volume is brought to 5.0 ml. with water. Chloroform (3.0 ml.) is added and the tube is shaken vigorously. After the phases separate, the chloroform layer is removed and its absorbance is read at 700 nm. The blank contains no cyanate. A calibration curve prepared using reagent grade potassium cyanate is shown in Fig. 1. From these data, it was determined that A_{700}/μmole cyanate = 0.0118. This assay is not suitable for use with biological fluids, a problem to be discussed more fully.

Bicarbonate and Cyanide Assays—The bicarbonate assay is a simple gravimetric procedure in which the sample is placed in a closed, evacuated system; then carbon dioxide is released with 1 *N* sulfuric acid and trapped in barium hydroxide. The formed barium carbonate is removed, washed with water and ethanol, dried, and weighed as a measure of the bicarbonate present in the original sample. The releasing and trapping of carbon dioxide are carried out quickly, and no error due to the acid-catalyzed conversion of cyanate was detectable. Cyanide was assayed by the method of Aldridge (13). Interference was encountered when large amounts of cyanate were used to assay for cyanide in the purified material. To a limited extent, this problem could be solved by increasing the amount of bromine, the excess of which was readily removed with arsenious acid as described by Aldridge.

RESULTS

The yield from the purification procedure was about 70% (126 g./l.). The product of the essentially one-step process has proven suitable for clinical use[3].

Despite the fact that the Aldridge cyanide assay is not entirely specific (14) and that cyanate prepared from urea probably should not contain cyanide (12), testing for the presence of this highly toxic substance seemed justified. Assay of the commercial sodium cyanate showed a cyanide content of 1030 p.p.m. The purified material had less than 2 p.p.m., the lowest level that was detectable by the assay used.

[1] K & K Laboratories, Plainview, NY 11803

[2] The commercial sodium cyanate contained sufficient insoluble material that an unprotected sintered-glass filter became clogged quickly. To alleviate the problem, a cellulose slurry was prepared by blenderizing some soft filter paper in water and using this to form a protective covering on the filter.

[3] Personal communication, E. E. Langer.

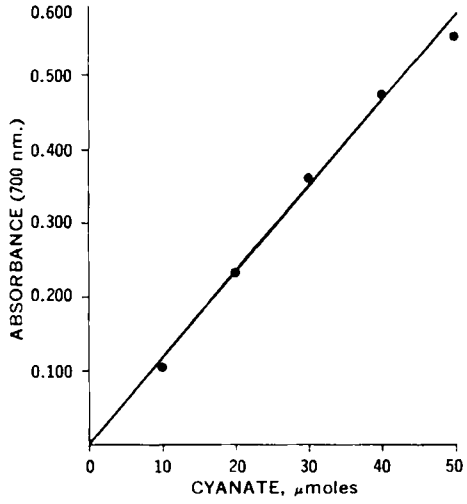

Figure 1—*Cyanate standard curve.*

A 3 *M* solution of the commercial sodium cyanate showed a slight broad absorbance at 266 and 285 nm. Contaminants responsible for this absorbance were undetectable after purification.

Occasionally, purified preparations had a distinct odor of ammonia due to the reaction (Scheme I) of cyanate with residual moisture:

$$NCO^- + 2H_2O \rightarrow NH_3 + HCO_3^-$$

Scheme I

In a series of purified preparations, the sodium cyanate assayed 93–100%, with a mean of 97%. Several preparations falling throughout this range of cyanate values were assayed for sodium bicarbonate. In every case, the remaining percentage could be fully accounted for as sodium bicarbonate.

The thermal stability of a purified and thoroughly dried sodium cyanate sample was demonstrated by heating at 200° for 2 hr. with no detectable loss. However, in aqueous solution, cyanate is quite unstable to heat. Autoclaving at 250° for 20 min. resulted in a 30% loss from a 0.01 *M* solution and a 65% loss from a 0.1 *M* solution. By contrast, a 0.1 *M* solution at 38° was far more stable (Fig. 2).

Cyanate stability was also very dependent upon concentration, as shown by the time course of decomposition at 38° in an aqueous solution (Fig. 2). A 0.1 *M* solution decomposed only 4% while a 1.0 *M* solution decomposed 79% in 29 hr. A 2.0 *M* solution lost cyanate at about twice the rate of the 1.0 *M* solution. Saturated sodium cyanate held at 60° as in the purification process decomposed to the extent of 90% in 3 hr. The rates of decomposition always began slowly, increased during the early phase of the reaction, and then remained linear until the reaction was nearly completed.

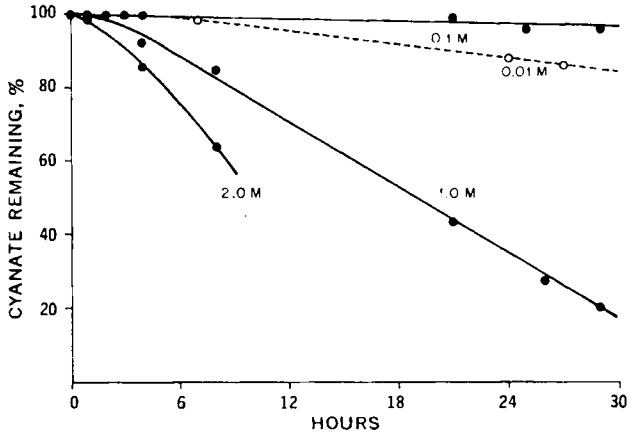

Figure 2—*Cyanate stability. The curves represent the rates of decomposition of sodium cyanate at 38° in aqueous solution. Key: ——, rates at pH 9.8; and ---, rates at pH 7.0.*

Most stability studies were with unbuffered solutions in the range of pH 9.2–9.8. At low pH, cyanate reacted with water more rapidly. This effect was observed in 0.1 *M* *N*-ethylmorpholine buffer at pH 7.0 where 0.01 *M* sodium cyanate at 38° decomposed to the extent of 12% in 29 hr. and 45% in 120 hr. At 4°, no detectable loss occurred in 120 hr.

DISCUSSION

Although the purification of sodium cyanate gave only a 70% yield, it is a simple and rapid procedure. Of the incurred loss, about one-third was due to retention in the methanol-water solvent (19 g./l.). The only by-product identified was sodium bicarbonate. The essentially total removal of cyanide indicated the effectiveness of the purification.

The cyanate assay, as modified from the Werner method (11), was moderately sensitive and very satisfactory for aqueous solutions. The reaction was unaffected by cyanide or thiocyanate. The major limitation of the assay was due to interference from many components found in biological materials. Cupric ions reacted with some common buffers such as phosphate and tromethamine to interfere with the formation of the colored complex. Protein not only interfered with complex formation but also removed cyanate during the deproteinization process. For these reasons, the assay cannot be performed in the presence of biological materials, in which case the use of isotopically labeled cyanate appears to be the best available method.

For clinical application, sodium cyanate often must be sterilized. One of two possibilities appears useful. An appropriate solution can be freshly prepared and filter sterilized. Alternatively, purified and thoroughly dried sodium cyanate is extremely stable to heat, withstanding temperatures to 700° (15). Therefore, the compound can be heat sterilized in vials for the preparation of solutions at the time and in the concentration needed. The latter method has the advantage that sterile sodium cyanate can be stored indefinitely and be available at all times.

The reaction of free cyanate with water to produce ammonia and bicarbonate probably occurs *in vivo*, as evidenced by Cerami's (7) findings in a mouse experiment. The animal was injected with $NaO^{14}CN$; during the following 24 hr., 72% of the radioactivity was recovered as $^{14}CO_2$. In the concentrations that might be anticipated clinically, neither of the breakdown products would likely be of serious consequence.

The accumulation of bicarbonate as cyanate reacts with water may be responsible for the increasing decomposition rate seen during the initial phase of the reaction. Lister (16) noted that carbonate ions can catalyze the decomposition of cyanate in alkaline solution. The effects of concentration, temperature, and pH on cyanate stability qualitatively parallel the reaction rate constants reported by Hagel *et al.* (9) for similar experimental conditions. Thus, in the concentrations, at the temperatures, and for the times to which erythrocytes would likely be exposed in clinical use, cyanate can be considered stable toward reaction with water and should remain available for carbamylation of hemoglobin.

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Distribution of Aspirin in Rumen and Corpus Tissues of Rat Stomach during First Four Minutes after Administration

CLARENCE H. MORRIS[A], JOHN E. CHRISTIAN, ROBERT R. LANDOLT, and WARREN G. HANSEN

Abstract □ Autoradiography was used to determine the distribution of aspirin and/or its metabolites in the mucosa, submucosa, and muscularis of the rumen (nonglandular portion) and corpus (glandular portion) tissues of the rat stomach 4 min. after administration. A significant localization of aspirin and/or its metabolites was observed in the submucosa regions of both the rumen and corpus. In addition, the innermost portion of the rumen mucosa acted as a barrier toward absorption when compared to the corpus mucosa. This factor may be important in the mechanism of lesion production in the corpus tissue and the lack of lesion production in the rumen.

Keyphrases □ Aspirin (and metabolites), distribution—rumen and corpus tissues, rat stomach, 4 min. after administration □ Gastric lesions—aspirin (and metabolites) distribution 4 min. after administration, rumen and corpus tissues, rat stomach, histoautoradiography □ Autoradiography—aspirin (and metabolites) distribution 4 min. after administration, rumen and corpus tissues, rat stomach

Several mechanisms have been proposed to explain the production of gastric lesions by salicylates (1, 2). Davenport (3) and Martin (4) developed chemical models based on interaction of the compound with cellular constituents following absorption of the compound into the cell.

Previous studies (5) showed that some rats develop gastric lesions in the corpus portion of the stomach within 5 min. following oral administration of aspirin. Lesions have not been produced in the rumen under the same experimental conditions. Therefore, it was of interest to compare the relative quantities and distribution of aspirin and/or its metabolites in the mucosa, submucosa, and muscularis of the rumen and corpus tissues of the rat stomach shortly after oral administration of aspirin-7-^{14}C.

MATERIALS AND METHODS

Aspirin was administered to rats as previously described (6), with the exception that each animal received 0.01 mc. (0.93 mg.) of aspirin-7-^{14}C.

Preparation of Emulsion Slides—Standard size microscope slides were cleaned by soaking for 24 hr. in a 10% solution of dichromic acid. Following the acid bath, the slides were washed in flowing tap water for 1 hr. followed by two consecutive 30-min. rinses in distilled water. The slides were then dipped in a subbing solution (5 g. gelatin and 0.5 g. of chrom alum in enough water to make 1000 ml.) and dried vertically. Next, the slides were dipped into a liquid emulsion[1], previously warmed to 43°. This work was performed under a safelight[2]. The slides were withdrawn from the emulsion at a uniform rate and checked under the safelight to ensure that the emulsion was uniformly distributed and free from bubbles, and the moist emulsion was wiped from one side. Then the slides were placed in a test tube rack in a vertical position and allowed to dry. After drying, the slides were placed in light-tight boxes and stored at −20° until used.

Tissue Cutting and Mounting—The entire stomach was removed from the animal 2 min. after oral administration. Four minutes after oral administration, the entire stomach was frozen in liquid nitrogen. From this point until development of the autoradiograms, the tissues remained frozen to prevent relocation of the aspirin and/or its metabolites. Cross-sectional segments of rumen and corpus were cut from the stomach, with a cold scalpel, and mounted on a previously cooled brass block using a drop of mounting medium[3]. After equilibration at −15°, 10-μ sections of rumen and corpus were cut with a microtome mounted in a cryostat[4]. The sections were transferred under the safelight from the microtome blade to the emulsion slides (also maintained at −15°). The mounted tissue sections were stored in light-tight boxes at −20° for 9–18 days to allow exposure of the photographic emulsion.

Photographic Processing of Liquid Emulsion Slides—All solutions were maintained at 4° throughout the photographic processing to reduce the possibility of detachment of the tissue from the emulsion. The most satisfactory staining was obtained if the tissue was fixed in methanol. The slides were then developed[5] for 10 min., rinsed in distilled water for 1 min., fixed[6] for 15 min., and rinsed in distilled water for 30 sec.

Staining Techniques—The tissue sections were stained with lithium carmine followed by picric acid, as described by Witten and Holstrom (7). All solutions were maintained between 10 and 15°. After the slides were removed from the final xylene bath, coverslips were mounted with Canada balsam.

[1] NTB-2, Eastman Kodak Co., Rochester, N. Y.
[2] Wratten series 2.
[3] Lab Tak, Westmont, Ill.
[4] International Equipment Co., Needham Heights, Mass.
[5] In Kodak D-19.
[6] In Kodak acid fixer.

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Keyphrases □ Aspirin (and metabolites), distribution—rumen and corpus tissues, rat stomach, 4 min. after administration □ Gastric lesions—aspirin (and metabolites) distribution 4 min. after administration, rumen and corpus tissues, rat stomach, histoautoradiography □ Autoradiography—aspirin (and metabolites) distribution 4 min. after administration, rumen and corpus tissues, rat stomach

Several mechanisms have been proposed to explain the production of gastric lesions by salicylates (1, 2). Davenport (3) and Martin (4) developed chemical models based on interaction of the compound with cellular constituents following absorption of the compound into the cell.

Previous studies (5) showed that some rats develop gastric lesions in the corpus portion of the stomach within 5 min. following oral administration of aspirin. Lesions have not been produced in the rumen under the same experimental conditions. Therefore, it was of interest to compare the relative quantities and distribution of aspirin and/or its metabolites in the mucosa, submucosa, and muscularis of the rumen and corpus tissues of the rat stomach shortly after oral administration of aspirin-7-^{14}C.

MATERIALS AND METHODS

Aspirin was administered to rats as previously described (6), with the exception that each animal received 0.01 mc. (0.93 mg.) of aspirin-7-^{14}C.

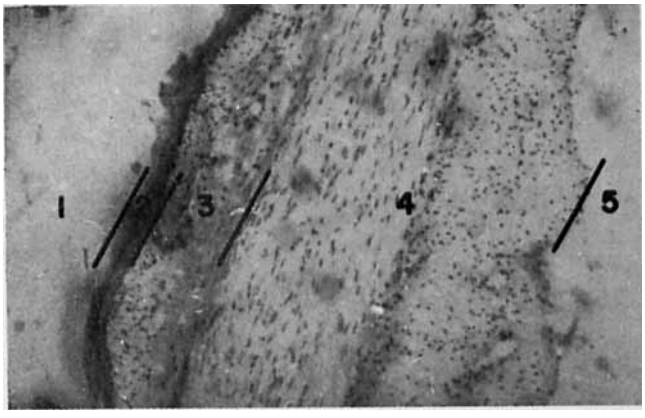
Preparation of Emulsion Slides—Standard size microscope slides were cleaned by soaking for 24 hr. in a 10% solution of dichromic acid. Following the acid bath, the slides were washed in flowing tap water for 1 hr. followed by two consecutive 30-min. rinses in distilled water. The slides were then dipped in a subbing solution (5 g. gelatin and 0.5 g. of chrom alum in enough water to make 1000 ml.) and dried vertically. Next, the slides were dipped into a liquid emulsion[1], previously warmed to 43°. This work was performed under a safelight[2]. The slides were withdrawn from the emulsion at a uniform rate and checked under the safelight to ensure that the emulsion was uniformly distributed and free from bubbles, and the moist emulsion was wiped from one side. Then the slides were placed in a test tube rack in a vertical position and allowed to dry. After drying, the slides were placed in light-tight boxes and stored at −20° until used.

Tissue Cutting and Mounting—The entire stomach was removed from the animal 2 min. after oral administration. Four minutes after oral administration, the entire stomach was frozen in liquid nitrogen. From this point until development of the autoradiograms, the tissues remained frozen to prevent relocation of the aspirin and/or its metabolites. Cross-sectional segments of rumen and corpus were cut from the stomach, with a cold scalpel, and mounted on a previously cooled brass block using a drop of mounting medium[3]. After equilibration at −15°, 10-μ sections of rumen and corpus were cut with a microtome mounted in a cryostat[4]. The sections were transferred under the safelight from the microtome blade to the emulsion slides (also maintained at −15°). The mounted tissue sections were stored in light-tight boxes at −20° for 9–18 days to allow exposure of the photographic emulsion.

Photographic Processing of Liquid Emulsion Slides—All solutions were maintained at 4° throughout the photographic processing to reduce the possibility of detachment of the tissue from the emulsion. The most satisfactory staining was obtained if the tissue was fixed in methanol. The slides were then developed[5] for 10 min., rinsed in distilled water for 1 min., fixed[6] for 15 min., and rinsed in distilled water for 30 sec.

Staining Techniques—The tissue sections were stained with lithium carmine followed by picric acid, as described by Witten and Holstrom (7). All solutions were maintained between 10 and 15°. After the slides were removed from the final xylene bath, coverslips were mounted with Canada balsam.

[1] NTB-2, Eastman Kodak Co., Rochester, N. Y.
[2] Wratten series 2.
[3] Lab Tak, Westmont, Ill.
[4] International Equipment Co., Needham Heights, Mass.
[5] In Kodak D-19.
[6] In Kodak acid fixer.



RUMEN

CORPUS

Figure 1—*Photomicrogram of cross section of rumen and corpus tissues, showing the (1) serosa, (2) muscularis, (3) submucosa, (4) mucosa, and (5) lumen.*

Grain Counting—The distribution of ^{14}C-compounds in the lumen, mucosa, submucosa, and muscularis regions of the rumen and corpus tissues (Fig. 1) was measured by counting grains of silver in the emulsion. A grid of 25 fields of 100 μ^2 each was optically superimposed over each region (Fig. 2) and the net number of grains per unit area was determined from each field in the emulsion below the following regions: (*a*) lumen, (*b*) mucosa along the mucosa–lumen interface, (*c*) mucosa along the mucosa–submucosa interface, (*d*) submucosa, and (*e*) muscularis. Grains were counted in two rumen and two corpus sections from each of two animals. In addition, the percentage of the net number of grains per unit area in each region relative to the net number of grains per unit area in the lumen was determined. Student's *t* test was used to determine the significance of the difference between the means of the net grains per unit area between the mucosa along the mucosa–submucosa interface and the submucosa. The *F* test was made prior to the *t* tests to test for homogenicity of variance. When the variances were not homogeneous, an adjusted *t* value was calculated from the formula developed by Cochran and Cox (8).

RESULTS

The distribution of aspirin and/or its metabolites in the mucosa, submucosa, and muscularis regions of the rumen and corpus tissues is presented in Table I. An increase in net grains per unit area is shown in the submucosa of each section from both rumen and corpus when compared to the mucosa. This increase is significant at the 99.9% confidence level as determined by the Student *t* test. In addition, by comparing rumen and corpus values of the average percent in each region relative to the lumen, it is evident that the rumen mucosa along the mucosa–lumen interface acts as a barrier against the absorption of aspirin and its metabolites. The quantities of labeled compounds found in the mucosal and submucosal regions of the corpus are significantly higher (at the 95% confidence level) than the quantities found in the corresponding regions of the rumen; however, quantities found in the muscularis regions of the rumen and corpus are not significantly different from each other.

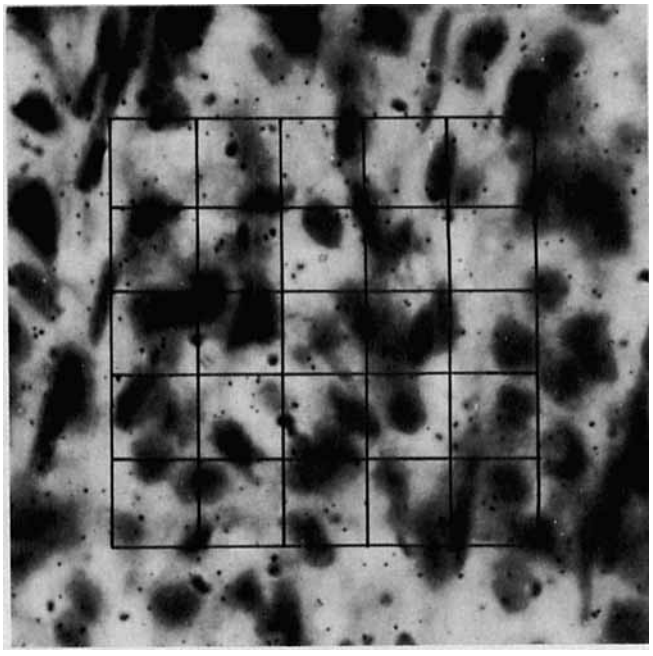


Figure 2—*Photomicrogram of a typical histoautoradiogram, showing silver grains in the emulsion below the corpus mucosa. Each small square of the grid has an area of 500 μ^2. Since the microscope was focused on the silver grains, the tissue appears to be slightly out of focus.*

DISCUSSION

Histoautoradiography showed that the rumen mucosa acted as a barrier toward absorption of aspirin and/or its metabolites, whereas the corpus tissue permitted much higher levels of penetration. In addition, there was a significant increase in aspirin (and/or metabolite) levels in the submucosa of the rumen and corpus tissue than in the other regions. In another study (6), significantly higher levels of salicylic acid were found in the corpus tissue than in the rumen tissue. Finally, it has been shown (5, 9) that lesions appear in the corpus portion but not in the rumen portion of the stomach following oral administration of aspirin. Taking these observations into consideration, one might speculate that the mechanism of lesion production is a function of absorption of aspirin and the subsequent

Table I—Average Net Grains per Field in Rumen and Corpus Tissue

Region	Corpus		Rumen	
	Mean Grains per Unit Area	Percent Relative to Lumen[a]	Mean Grains per Unit Area	Percent Relative to Lumen
Lumen	85.3	100	110.8	100
Mucosa at mucosa–lumen interface	22.0	25.7	2.5	2.2
Mucosa at mucosa–submucosa interface	6.8	7.9	2.0	1.8
Submucosa	23.3	27.3	11.2	10.1
Muscularis	3.7	4.3	2.5	2.2

[a] Average percent of the net number of grains in each region of the tissue as compared to the average net number present in the lumen.

hydrolysis of aspirin to salicylic acid within the corpus tissue. One might also speculate that the submucosa may be the site of the initial injury due to the apparent buildup of aspirin and metabolites at that level.

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Effect of Adsorbed Surfactant on Particle–Particle Interactions in Hydrophobic Suspensions

J. V. BONDI*, R. L. SCHNAARE▲, P. J. NIEBERGALL, and E. T. SUGITA

Abstract □ The degree of flocculation of sulfamerazine suspensions as represented by relative sedimentation volume was determined as a function of both surfactant and electrolyte concentration. The results emphasize the importance of knowing the location as well as the concentration of the surfactant and are consistent with the Derjaguin, Landau, Verwey, and Overbeek theory.

Keyphrases □ Suspensions, hydrophobic (sulfamerazine)—degree of flocculation, relative sedimentation volume, effects of surfactant (sodium dodecyl polyoxyethylene sulfate) and electrolyte concentration □ Surfactants (sodium dodecyl polyoxyethylene sulfate)—effect on particle–particle interactions in hydrophobic (sulfamerazine) suspensions □ Sodium dodecyl polyoxyethylene sulfate—effect on flocculation of sulfamerazine suspensions □ Sulfamerazine suspensions—effect of adsorbed surfactant on particle–particle interactions □ Flocculation, sulfamerazine suspensions—effects of surfactant and electrolyte concentration

Controlled flocculation has been the subject of many papers over the past 15 years (1–5). Haines and Martin (1, 2) suggested that suspension flocculation can be controlled by modifying the charge surrounding suspended hydrophobic particles by the addition of electrolyte. For example, the addition of aluminum chloride to 2% sulfamerazine suspensions containing dioctyl sodium sulfosuccinate reduced the ζ-potential essentially to zero, producing pharmaceutically stable suspensions characterized by high final sedimentation volumes.

Wilson and Ecanow (3) took issue with the work of Haines and Martin for several reasons: (*a*) since flocculation was reported to have occurred spontaneously, the true ζ-potential could not have been measured; (*b*) it was unlikely that van der Waals' forces would be operative with particles greater than 2–5 μm.; and (*c*) the effect of electrolyte on the surfactant was not taken into account. During a subsequent investigation, they observed the formation of a precipitate when dioctyl sodium sulfosuccinate at concentrations used by Haines and Martin was mixed with aluminum chloride. Flocculation was interpreted as being due to a chemical reaction between the surfactant and the electrolyte instead of a reduction of the ζ-potential.

Matthews and Rhodes (4–6) studied suspensions of sulfamerazine and griseofulvin prepared with sodium lauryl sulfate, which is precipitated by aluminum chloride, and sodium dodecyl dioxylated sulfate, which apparently is not precipitated by aluminum chloride. The addition of aluminum chloride to both suspensions resulted in aggregation. Different sedimentation rates were observed; however, final sediment heights were identical. They interpreted the aggregation mechanism as "flocculation" for the suspension containing sodium lauryl sulfate and as "coagulation" for the suspension containing sodium dodecyl dioxylated sulfate. Ecanow *et al.* (7) claimed that both suspensions were "flocculated" on the basis of equal final sediment volumes and sediment compressibility. The difference in sedimentation rate was considered to be a function of aggregate structure, number, and geometry.

Two possible mechanisms have been proposed to explain flocculation (8–10): absorption bridging by the surfactant between suspension particles and cross-linking of suspension particles produced by metal-ion interactions with the adsorbed surfactant. Coagulation is an aggregation process resulting from a reduction in the ζ-potential to a point where attractive forces predominate. The aggregate is dense and nonporous and forms a caked sediment.

hydrolysis of aspirin to salicylic acid within the corpus tissue. One might also speculate that the submucosa may be the site of the initial injury due to the apparent buildup of aspirin and metabolites at that level.

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Table I—Composition and Sedimentation of Sulfamerazine Suspensions

Concentration of Surfactant[a] in Solution (Equivalent × 10^3/l.), Initial Equilibrium		Surface Coverage	Sodium Chloride Concentration, moles/l.	Relative Sedimentation Volume, H_u/H_0
		Series A[b]		
3.43	—	Partial	1.00	0.91
3.43	—	Partial	0.50	0.94
3.43	—	Partial	0.24	0.92
3.43	—	Partial	0.00	0.91
		Series B		
22.6	10.0	Saturated	1.00	0.60
20.7	10.0	Saturated	0.50	0.59
18.9	10.0	Saturated	0.24	0.15
14.2	10.0	Saturated	0.00	0.59
		Series C		
19.6	7.0	Saturated	1.00	0.56
15.6	3.0	Saturated	1.00	0.59
11.7	—	Partial	1.00	0.60
7.8	—	Partial	1.00	0.68
3.9	—	Partial	1.00	0.84
1.6	—	Partial	1.00	0.97

[a] Sodium dodecyl polyoxyethylene sulfate. [b] Initial concentration equal to the CMC in pure water.

Another possible mechanism for flocculation is aggregation at relatively large interparticle distances as predicted by the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory (6, 11–13). Coagulation is interpreted as aggregation in the primary minimum, resulting in a closely packed, tightly bound aggregate; flocculation is interpreted as aggregation in the secondary minimum, resulting in a loose, weakly bound network. An attractive feature of such an explanation is an allowance for electrolyte effects other than chemical interactions.

This article reports investigations on suspension aggregation and offers the following observations on sulfamerazine suspensions.

EXPERIMENTAL

The surfactant, sodium dodecyl polyoxyethylene sulfate, supplied as a 50% hydroalcoholic solution[1], was purified by diluting with acetone to precipitate the inorganic salts present, filtering, and removing the solvents by vacuum distillation. The purified surfactant as an oil was diluted to a known volume with water and standardized with benzethonium chloride according to the method described by Epton (14) and modified by Weatherburn (15). All concentrations of the surfactant are expressed as equivalents of benzethonium chloride per liter, since the exact molecular weight is not known.

Sulfamerazine[2] powder was used as received. The mean volume surface diameter and specific surface area were determined[3] to be 2.48 μm. and 1.77 m.²/g., respectively. Adsorption isotherms of the surfactant on sulfamerazine were determined at three concentrations of sodium chloride and in pure water. Thus, it was possible to prepare suspensions in which the relative concentrations of the surfactant adsorbed and free in solution were known.

Suspensions were prepared by adding a known volume of the surfactant solution to a weighed quantity of sulfamerazine powder. The mixture was stirred continuously for 16 hr., at which time it was transferred to a glass sedimentation tube having an inside diameter of 1.9 cm. The suspensions fell into one of three categories: Series A, prepared at various electrolyte concentrations and an initial concentration of surfactant equal to the critical micelle concentration (CMC) of the surfactant in water; Series B, prepared at various electrolyte concentrations and at surfactant concentrations such that the particle surface was saturated with surfactant; and Series C, prepared at constant electrolyte concentration and various surfactant concentrations.

[1] Richanol S1285, Richardson Co.
[2] Sulfamerazine USP, American Cyanamid Co.
[3] With a Fisher Sub Sieve Sizer.

RESULTS AND DISCUSSION

The Series A suspensions in Table I were prepared with an initial concentration of surfactant equal to the CMC in water. Consequently, the surface of the particles was only partially covered with surfactant. A very high relative sediment volume, H_u/H_0, was observed, indicating a large degree of aggregation. The sediment was very loosely packed, and the supernate was clear, indicating flocculation.

Cross-linking of surfactant by metal ions, shown to occur with aluminum ion and sodium dodecyl sulfosuccinate and sodium lauryl sulfate (3, 4), seems an improbable explanation for the flocculation of Series A suspensions considering the high degree of flocculation at zero sodium chloride concentration. Such a mechanism would also demand a dependence of H_u/H_0 on electrolyte concentration, which is obviously absent. Adsorption bridging would allow for the lack of dependence on electrolyte concentration but seems an unlikely mechanism for a charged surfactant.

There remains the flocculation mechanism as described by the DLVO theory. The high degree of flocculation at zero electrolyte concentration indicates very little, if any, charge repulsion between particles, *i.e.*, a very small or zero ζ-potential. Such a condition would result only from a very low charge density on the particles which follows from the low initial concentration of surfactant. The DLVO theory predicts that at low ζ-potential and/or relatively large distances, particle particle interactions would be insensitive to electrolyte concentration (16, 17).

The Series B suspensions, by contrast, were prepared at surfactant concentrations such that the particle surface was completely saturated. It follows that the surface charge or ζ-potential at zero electrolyte concentration would be considerably higher than that of the corresponding Series A suspension. This was evidenced by the reduced degree of flocculation, as indicated by the lower relative sedimentation volume.

The addition of 0.24 *M* sodium chloride resulted in a dramatic decrease in the relative sedimentation volume. The sediment was dense (centrifugation reduced H_u/H_0 to 0.14) and the supernate was cloudy, indicative of either a monodispersed or a coagulated system. The sedimentation rate was the slowest of the series, again indicating either a monodispersed (11) or a coagulated suspension, depending on whether flow is laminar or turbulent (9).

Considering that the particles have a negative charge at zero electrolyte concentration:

1. The addition of sodium chloride could increase the adsorption of the surfactant such that at 0.24 *M* electrolyte the ζ-potential is sufficient for a monodispersed state. That adsorption is increased is evident from the data in Table I. Also, the neutralization of charge by counterions would have to be negligible. Electrolyte addition beyond 0.24 *M* would progressively increase adsorption and cause neutralization of the negative charge, with the net effect being a degree of flocculation.

2. The addition of sodium chloride could neutralize the charge on the particles essentially to zero, resulting in a coagulated system. Further addition of electrolyte would charge the particles by adsorption of counterions, reestablishing a degree of flocculation.

It would be impossible to distinguish between these two explanations without further experimental observations. The ζ-potential would go through a maximum in the first and a minimum in the second. The DLVO theory does, however, offer a possible explanation for the behavior of the Series B suspensions, whereas cross-linking does not seem to be applicable.

A similar comparison is found for the Series C suspensions. Cross-linking would predict a direct relationship between the surfactant concentration and H_u/H_0. The inverse relationship found experimentally is explainable within the framework of the DLVO theory. An increase in surfactant concentrations would increase the surface charge and decrease the degree of flocculation.

It is obvious from these observations that the effect of electrolytes

on flocculation behavior depends not only on the surfactant concentration but also on the location of the surfactant and the degree of surface coverage by the surfactant. Electrolyte effects at one surfactant level can be eliminated or even reversed by changing the surfactant concentration.

It is also suggested that aggregation in a secondary potential energy minimum as described by the DLVO theory is a valid mechanism for interpreting flocculation. Flocculation mechanisms would then be classified as: (*a*) adsorption bridging, (*b*) cross-linking or chemical bridging, and (*c*) DLVO aggregation (secondary minimum). And coagulation would be defined by: (*a*) film–film bonding, and (*b*) DLVO aggregation (primary minimum).

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Synthesis and Antifungal Activity of Polyhalophenyl Esters of Nitrocarbanilic Acids VI

I. LALEZARI▲, G. MOHTAT, F. AFGHAHI, and M. ALILOU

Abstract □ Twenty-two polyhalophenyl esters of *meta*- and *para*-nitrocarbanilic acids were synthesized and tested for antifungal activity against *Candida albicans*, *Penicillium notatum*, and *Aspergillus fumigatus*. The 2,4,6-trichlorophenyl ester of *m*-nitrocarbanilic acid was found to be the most active compound.

Keyphrases □ Nitrocarbanilic acids, polyhalophenyl esters—synthesis, antifungal activity □ Polyhalophenyl esters, nitrocarbanilic acids—synthesis, antifungal activity □ Antifungal agents, potential—synthesis and testing of 22 polyhalophenyl esters of nitrocarbanilic acids

In continuing research with the syntheses of new carbamic acid derivatives (1–5) with antifungal activity, a series of polyhalophenyl esters of *meta*- and *para*-nitrocarbanilic acids was prepared by interaction (Scheme I) of the corresponding nitrobenzoyl azide

$$NO_2C_6H_4CON_3 + ArOH \xrightarrow{\text{refluxing toluene}} NO_2C_6H_4NHCO_2Ar + N_2$$

Scheme I

with polyhalophenols in refluxing toluene, and physical data were compiled (Table I).

All prepared compounds were tested against *Candida albicans* 28012, *Penicillium notatum* S-13, and *Aspergillus fumigatus* CDC 24[1] *in vitro* using BBL Sabouraud dextrose agar medium.

Each compound was tested at 5, 10, and 25 mcg./ml. All compounds were dissolved in acetone at a concentration of 5 mg./ml. These solutions were diluted with hot culture medium to the desired concentrations and autoclaved at 120° for 2 hr. Five replicates of each concentration were prepared.

The antifungal activity of all compounds tested except XIX and XX was insignificant at a concentration of 5 mcg./ml. Compounds XII and XV showed only slight growth inhibition at concentrations of 25 mcg./ml. (Table II).

EXPERIMENTAL[2]

m-Nitrocarbanilic acid *p*-tolyl ester was prepared as follows. *m*-Nitrobenzoyl azide (1.92 g., 0.01 mole) in 20 ml. of dry toluene was refluxed for 2 hr. To the boiling mixture, *p*-cresol (1.08 g., 0.01

[1] Microorganisms were obtained from the Department of Parasitology, Public Health Institute, Tehran, Iran.

[2] Melting points were taken on a Kofler hot stage microscope and are uncorrected. The IR spectra were determined with a Leitz spectrograph model III. NMR spectra were obtained on a Varian A 60A instrument. Mass spectra were recorded on a Varian Mat 111 instrument. *m*- and *p*-Nitrobenzoyl azides were prepared according to Curtius *et al.* (6).

on flocculation behavior depends not only on the surfactant concentration but also on the location of the surfactant and the degree of surface coverage by the surfactant. Electrolyte effects at one surfactant level can be eliminated or even reversed by changing the surfactant concentration.

It is also suggested that aggregation in a secondary potential energy minimum as described by the DLVO theory is a valid mechanism for interpreting flocculation. Flocculation mechanisms would then be classified as: (*a*) adsorption bridging, (*b*) cross-linking or chemical bridging, and (*c*) DLVO aggregation (secondary minimum). And coagulation would be defined by: (*a*) film–film bonding, and (*b*) DLVO aggregation (primary minimum).

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Synthesis and Antifungal Activity of Polyhalophenyl Esters of Nitrocarbanilic Acids VI

I. LALEZARI▲, G. MOHTAT, F. AFGHAHI, and M. ALILOU

Abstract □ Twenty-two polyhalophenyl esters of *meta*- and *para*-nitrocarbanilic acids were synthesized and tested for antifungal activity against *Candida albicans*, *Penicillium notatum*, and *Aspergillus fumigatus*. The 2,4,6-trichlorophenyl ester of *m*-nitrocarbanilic acid was found to be the most active compound.

Keyphrases □ Nitrocarbanilic acids, polyhalophenyl esters—synthesis, antifungal activity □ Polyhalophenyl esters, nitrocarbanilic acids—synthesis, antifungal activity □ Antifungal agents, potential—synthesis and testing of 22 polyhalophenyl esters of nitrocarbanilic acids

In continuing research with the syntheses of new carbamic acid derivatives (1–5) with antifungal activity, a series of polyhalophenyl esters of *meta*- and *para*-nitrocarbanilic acids was prepared by interaction (Scheme I) of the corresponding nitrobenzoyl azide

$$NO_2C_6H_4CON_3 + ArOH \xrightarrow{\text{refluxing toluene}} NO_2C_6H_4NHCO_2Ar + N_2$$

Scheme I

with polyhalophenols in refluxing toluene, and physical data were compiled (Table I).

All prepared compounds were tested against *Candida albicans* 28012, *Penicillium notatum* S-13, and *Aspergillus fumigatus* CDC 24[1] *in vitro* using BBL Sabouraud dextrose agar medium.

Each compound was tested at 5, 10, and 25 mcg./ml. All compounds were dissolved in acetone at a concentration of 5 mg./ml. These solutions were diluted with hot culture medium to the desired concentrations and autoclaved at 120° for 2 hr. Five replicates of each concentration were prepared.

The antifungal activity of all compounds tested except XIX and XX was insignificant at a concentration of 5 mcg./ml. Compounds XII and XV showed only slight growth inhibition at concentrations of 25 mcg./ml. (Table II).

EXPERIMENTAL[2]

m-Nitrocarbanilic acid *p*-tolyl ester was prepared as follows. *m*-Nitrobenzoyl azide (1.92 g., 0.01 mole) in 20 ml. of dry toluene was refluxed for 2 hr. To the boiling mixture, *p*-cresol (1.08 g., 0.01

[1] Microorganisms were obtained from the Department of Parasitology, Public Health Institute, Tehran, Iran.

[2] Melting points were taken on a Kofler hot stage microscope and are uncorrected. The IR spectra were determined with a Leitz spectrograph model III. NMR spectra were obtained on a Varian A 60A instrument. Mass spectra were recorded on a Varian Mat 111 instrument. *m*- and *p*-Nitrobenzoyl azides were prepared according to Curtius *et al.* (6).

Table I—Physical Constants for Polyhalophenyl Esters of Nitrocarbanilic Acids $O_2NC_6H_4NHCO_2R$

Compound Number	Position of Nitro group	R	Yield, %	Melting Point	Formula	Analysis, % Calc.	Found
I	*meta*	*p*-Tolyl	70	135°	$C_{14}H_{12}N_2O_4$	C 61.76	61.69
						H 4.41	4.32
II	*meta*	*p*-Nitrophenyl	55	192°	$C_{13}H_9N_3O_6$	C 51.48	51.42
						H 2.96	2.96
III	*meta*	*p*-Chlorophenyl	65	126°	$C_{13}H_9ClN_2O_4$	C 53.33	53.15
						H 3.07	3.01
IV	*meta*	*p*-Bromophenyl	52	125°	$C_{13}H_9BrN_2O_4$	C 46.29	46.41
						H 2.67	2.57
V	*meta*	*p*-Iodophenyl	49	130°	$C_{13}H_9IN_2O_4$	C 40.62	40.55
						H 2.34	2.40
VI	*meta*	2,4-Dichlorophenyl	45	142°	$C_{13}H_8Cl_2N_2O_4$	C 47.70	47.78
						H 2.44	2.35
VII	*meta*	2,4,5-Trichlorophenyl	57	184°	$C_{13}H_7Cl_3N_2O_4$	C 43.15	42.98
						H 1.93	1.89
VIII	*meta*	2,4,6-Trichlorophenyl	63	162°	$C_{13}H_7Cl_3N_2O_4$	C 43.15	43.20
						H 1.93	1.91
IX	*meta*	2,4,6-Tribromophenyl	65	189°	$C_{13}H_7Br_3N_2O_4$	C 31.51	31.39
						H 1.41	1.39
X	*meta*	2,4,6-Triiodophenyl	45	211°	$C_{13}H_7I_3N_2O_4$	C 24.52	24.45
						H 1.10	1.12
XI	*meta*	Pentachlorophenyl	55	167°	$C_{13}H_5Cl_5N_2O_4$	C 36.23	36.19
						H 1.16	1.20
XII	*para*	*p*-Tolyl	65	215–216°	$C_{14}H_{12}N_2O_4$	C 61.76	61.80
						H 4.41	4.38
XIII	*para*	*p*-Nitrophenyl	52	132–136°	$C_{13}H_9N_3O_6$	C 51.48	51.39
						H 2.96	2.91
XIV	*para*	*p*-Chlorophenyl	60	175°	$C_{13}H_9ClN_2O_4$	C 53.33	53.29
						H 3.07	3.10
XV	*para*	*p*-Bromophenyl	51	185°	$C_{13}H_9BrN_2O_4$	C 46.29	46.32
						H 2.67	2.53
XVI	*para*	*p*-Iodophenyl	46	198°	$C_{13}H_9IN_2O_4$	C 40.62	40.54
						H 2.34	2.29
XVII	*para*	2,4-Dichlorophenyl	48	186°	$C_{13}H_8Cl_2N_2O_4$	C 47.70	47.74
						H 2.44	2.38
XVIII	*para*	2,4,5-Trichlorophenyl	53	235°	$C_{13}H_7Cl_3N_2O_4$	C 43.15	43.20
						H 1.93	2.01
XIX	*para*	2,4,6-Trichlorophenyl	49	250°	$C_{13}H_7Cl_3N_2O_4$	C 43.15	43.30
						H 1.93	1.85
XX	*para*	2,4,6-Tribromophenyl	61	186°	$C_{13}H_7Br_3N_2O_4$	C 31.51	31.46
						H 1.41	1.45
XXI	*para*	2,4,6-Triiodophenyl	48	195°	$C_{13}H_7I_3N_2O_4$	C 24.52	24.48
						H 1.10	1.15
XXII	*para*	Pentachlorophenyl	53	165°	$C_{13}H_5Cl_5N_2O_4$	C 36.23	36.27
						H 1.16	1.18

Table II—Antifungal Activity[a] of Polyhalophenyl Esters of Nitrocarbanilic Acids

Compound	*P. notatum* 10 mcg./ml.	*P. notatum* 25 mcg./ml.	*C. albicans* 10 mcg./ml.	*C. albicans* 25 mcg./ml.	*A. fumigatus* 10 mcg./ml.	*A. fumigatus* 25 mcg./ml.
I	+	2+	–	–	2+	3+
II	+	2+	–	–	–	+
III	–	2+	–	–	–	2+
IV	–	2+	–	–	–	+
V	–	2+	–	–	–	–
VI	–	4+	–	–	–	2+
VII	5+	5+	5+	5+	5+	5+
VIII	2+	5+	–	5+	2+	5+
IX	+	3+	–	4+	+	3+
X	2+	2+	–	–	2+	2+
XI	+	3+	–	4+	+	3+
XIII	–	+	–	–	–	3+
XIV	–	2+	–	–	–	2+
XVI	–	+	–	–	–	+
XVII	–	3+	–	–	+	2+
XVIII	–	+	–	–	–	+
XIX	3+	5+	–	5+	3+	5+
XX	3+	5+	3+	5+	3+	5+
XXI	2+	4+	2+	5+	3+	4+
XXII	2+	5+	+	5+	+	4+

[a] – = no inhibition, and 5+ = complete inhibition.

mole) was added and heating was continued for 2 hr. After evaporation of the solvent, the residue was crystallized from hot benzene to give 1.63 g. (70%), m.p. 135°, molecular weight 272 (by mass spectroscopy); IR: ν_{max} 3200, 1693, 1530, 1500, 1430, 1360, 1260, 1208, 1024, 893, 840, 814, 742, and 675 cm.$^{-1}$; NMR (CF_3CO_2H): τ8.1 (s, 3H, CH_3), 2–3, 2 (m, 8H, aromatic H), and 1.9 (s, 1H, NH).

All other nitrocarbanilic acid esters were prepared similarly (Table I).

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Rapid, Simultaneous GLC Determination of Phenobarbital, Primidone, and Diphenylhydantoin

ROBERT J. PERCHALSKI*, K. N. SCOTT*†, B. J. WILDER*†▲, and R. H. HAMMER‡

Abstract □ A rapid method is described for the simultaneous determination of phenobarbital, primidone, and diphenylhydantoin. The method gives greater sensitivity and reproducibility for phenobarbital and primidone than the short methods now in use, maintains the efficiency of much longer techniques, and takes only 45 min. from the time the sample is received until the results are reported.

Keyphrases □ Phenobarbital—rapid, simultaneous GLC determination with primidone and diphenylhydantoin □ Primidone—rapid, simultaneous GLC determination with phenobarbital and diphenylhydantoin □ Diphenylhydantoin—rapid, simultaneous GLC determination with phenobarbital and primidone □ GLC—rapid, simultaneous determination of phenobarbital, primidone, and diphenylhydantoin

Since the introduction of the on-column methylating reagents, tetramethylammonium hydroxide (1, 2) and trimethylphenylammonium hydroxide (3), the extraction and GLC analysis of anticonvulsant drugs have been greatly simplified. These reagents are normally used as either a dilute (0.1–0.2 *M*) solution (4, 5) or a concentrated (24%) solution (6, 7) in methanol. The methods published to date concerning two of the three primary anticonvulsants, phenobarbital and primidone, are lacking in sensitivity or reproducibility or the extraction time required is unnecessarily long.

This paper describes a combination of two methods (5, 6) which increases the sensitivity and reproducibility of the analysis of phenobarbital and primidone and also decreases the time necessary to give a clean, efficient extraction.

EXPERIMENTAL

Apparatus—A gas chromatograph[1] with four flame-ionization detectors was used for the determinations. The column was a 1.82-m. (6-ft.) by 2-mm. i.d. glass U-tube packed with 3% OV-17 (phenyl methyl silicone oil) on 80–100-mesh Chromosorb W-HP. The injection port was heated to 360° and the detector to 260°. The column temperature was programmed from 140 to 220° at 8°/min. The carrier gas flow rate was adjusted to around 60 ml./min. to give a retention time of 5.0 ± 0.2 min. for methylated diphenylhydantoin at 220°.

Reagents—Trimethylphenylammonium hydroxide[2] was available as a 0.1 *M* solution in methanol; it was concentrated to 1.5 *M* under dry nitrogen over low heat. 5-(*p*-Methylphenyl)-5-phenylhydantoin[3] (I) and phenylethylmalonamide[4] were also used.

Procedure—One milliliter of plasma, 0.50 ml. of the internal standard (20 mg. I diluted to 500 ml. with 0.1 *N* NaOH), 0.5 ml. 0.1 *N* NaOH, 0.5 ml. of a 1.0 *M* H_3PO_4 buffer, pH 2.7, and 13 ml. of ether were combined in a 50-ml. centrifuge tube. The tube was sealed by wetting the ground-glass portion of the stopper with distilled water before insertion, shaken mechanically for 10 min., and centrifuged at 2000 r.p.m. for 1 min. The ether was transferred to a 15-ml. conical centrifuge tube and evaporated under nitrogen at 50°. The residue was taken up in 5 ml. of toluene, and 50 μl. of 1.5 *M* trimethylphenylammonium hydroxide was added. The tube was shaken for 1 min. and then centrifuged for 2 min.; 1 μl. of the lower phase was injected into the chromatograph.

Drug levels were determined from a standard curve of the peak height ratio of the drug to the internal standard *versus* concentration. Phenobarbital levels were determined by summing the heights of the three peaks shown in Fig. 1 after adjusting to constant peak width. The total phenobarbital (Pb) peak height is given by Eq. 1:

$$h_{Pb} = \frac{w_1}{w_3}h_1 + \frac{w_2}{w_3}h_2 + h_3 \qquad \text{(Eq. 1)}$$

where h is the height and w is the width at half height of the peaks shown by the subscripts. The peak width ratios are constant for any given set of chromatographic conditions.

RESULTS AND DISCUSSION

The chromatogram of an extracted sample containing therapeutic levels of phenobarbital, primidone, and diphenylhydantoin, along with an extraction of standard plasma, is shown in Fig. 1. The first two phenobarbital peaks shown in this figure are produced in the chromatograph during on-column methylation with 1.5 *M* trimethylphenylammonium hydroxide. They are also the only peaks produced by methylation of the total product of the aqueous alkaline hydrolysis of phenobarbital. GLC and NMR studies of these two products indicate that they are methylated derivatives of phenyl-

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Table I—Two-Hour Time Study of Phenobarbital Peak Heights with Diphenylhydantoin as Internal Standard

Injection Time, min.	Peak 1		Peak 2		Peak 3		Total	
	Column 1	Column 2	Column 1	Column 2	Column 1	Column 2	Column 1	Column 2
0	0.391	0.355	0.620	0.650	1.32	1.27	2.23	2.18
25	0.288	0.326	0.900	0.933	1.21	0.979	2.29	2.12
50	0.233	0.355	0.520	0.931	1.57	1.05	2.25	2.22
75	0.393	0.369	0.704	0.796	1.23	1.06	2.22	2.12
100	0.590	0.396	0.864	1.22	1.07	0.764	2.38	2.23
σ	0.136	0.0254	0.161	0.211	0.185	0.182	0.0650	0.0527
$\bar{x}$	0.379	0.360	0.722	0.906	1.28	1.02	2.27	2.17
RSD, %	36	7	23	23	15	18	2.9	2.5
σ_T	0.0930		0.202		0.219		0.0768	
$\bar{x}_T$	0.370		0.814		1.15		2.22	
RSD_T, %	25		25		19		3.5	
Variation	10.5–26.6%		23.4–55.0%		34.4–70.7%			

ethylmalonamide, a metabolite of primidone. However, the recovery of phenylethylmalonamide from plasma using this extraction technique is 9.9% at a concentration of 20 mcg./ml. of plasma and 8.5% at a concentration of 5 mcg./ml. of plasma. Gallagher and Baumel (8) reported levels of phenylethylmalonamide in serum of 10 and 12 mcg./ml. in two patients who had been taking primidone (750 mg./day) for more than 2 years. Therefore, for practical drug level determinations, it is safe to assume that these peaks are produced on the column by phenobarbital and are not the result of the metabolism of primidone. The third peak has been shown by NMR to be 1,3-dimethylphenobarbital. A study is underway to determine the structures of the two methylated derivatives of phenylethylmalonamide.

Table I shows the variation of the peak height ratios of the three phenobarbital peaks over 2 hr. for one sample. Each peak has been found to vary continuously with time in an unpredictable manner, supporting the necessity of using all three peaks for quantitation. Data are shown for the three separate peaks and their combined total using two identically prepared columns. The range of variation of the ratios for each peak as a percentage of the total ratio is also shown. The same values were calculated for seven replicate samples analyzed on one column, and these showed relative standard deviations of 28, 12, 18, and 5% for peaks one, two, three, and the total, respectively.

To show that the response-mole ratios of phenobarbital peaks one and two are equivalent to peak three after correction, standard samples containing phenobarbital, primidone, diphenylhydantoin, and the internal standard were extracted and chromatographed. The samples were four each of six levels containing from 100.4 to 4.02 mcg. of phenobarbital/ml. of plasma. Each sample was run on two columns simultaneously and averages of the measured values were used. The ratio of the concentration of phenobarbital to that of diphenylhydantoin was constant for all samples. Peak height ratios were calculated for phenobarbital, using the total height from Eq. 1, and for diphenylhydantoin. The ratio for phenobarbital was divided by that for diphenylhydantoin for each sample, giving a mean of 2.04 with a relative standard deviation of 8%.

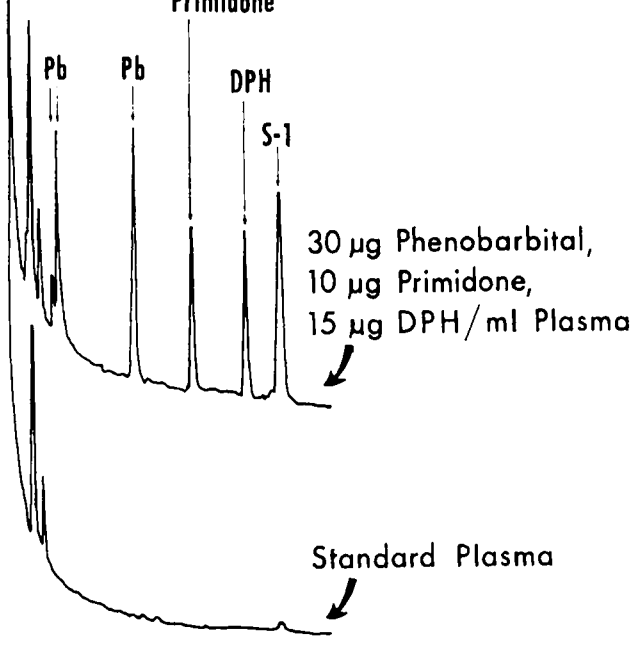

Figure 1—*Typical chromatogram. Upper trace shows extraction of plasma containing therapeutic levels of phenobarbital (Pb), primidone, and diphenylhydantoin (DPH); lower trace shows extraction of standard plasma. S-1 is the internal standard.*

The recoveries of phenobarbital, primidone, and diphenylhydantoin were 100, 72, and 100%, respectively, and the calibration curve for each drug was linear over the concentration range normally found in plasma. The relative standard deviations of six replicate extractions of a patient sample containing all three drugs were 3.2% for 32.7 mcg. of phenobarbital, 3.3% for 7.66 mcg. of primidone, and 5% for 12.2 mcg. of diphenylhydantoin/ml. of plasma. The limit of detection for each drug is 0.5 mcg./ml. of plasma.

The extraction procedure described is as clean and efficient as longer extractions now in use, yet it takes only 25 min. The method is applicable to the other hydantoin and succinimide anticonvulsants, except methsuximide and mephenytoin, in which case the derivatizing reagent is added directly to the ether residue. We are presently adapting the method to analyze small volumes of plasma and whole blood so that accurate multiple-drug determinations can be made from finger-stick samples.

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Interfacial Concentration Observed during Dissolution

Keyphrases ☐ Dissolution of solids, descending column model – relationship to interfacial concentration ☐ Interfacial concentration – relationship to descending column dissolution ☐ Descending column dissolution – relationship to interfacial concentration

Sir:

In a recent series of papers, Kildsig and coworkers (1–4) investigated dissolution from compressed pellets in a static column of solvent, under conditions where the flat surface of the solid was either the lower or the upper boundary of the column (ascending and descending models, respectively). It was concluded that in the descending case an interfacial concentration, c_i, significantly lower than the saturation concentration, c_s, is operative and that c_i is related to energy parameters of the solid–liquid interface. The argument of the authors is not conclusive, however, and their experimental data can be interpreted in a more simple and convenient way which does not require the assumption of $c_i < c_s$.

When compared with the diffusion-controlled ascending case, the descending model represents a completely different hydrodynamic system which is usually described in terms of free convection; that is, whenever the dissolving surface is not at the bottom of the liquid, then inverse density gradients are generated within the liquid and these, in turn, induce macroscopic convectional flow accompanied by a drastic increase of the rate of mass transfer. Systems similar to the descending column have been investigated with respect to flow pattern and mass transfer, *e.g.*, laminar flow along a vertical flat surface (hanging pellet) (5, 6), laminar or turbulent flow around a single dissolving sphere (7), and convectional flow along a horizontal circular surface (static disk) (8, 9). In the last case, the flow pattern may be visualized from the analogous process of heat-induced convection (10, 11).

The descending model of Kildsig and coworkers fits into this series of free convectional dissolution systems, with one peculiarity; the solid surface extends over the entire cross section of the column. This complicates establishment of a typical, convectional flow pattern. One might speculate that in the absence of any disturbances the system starts from a convection-free metastable state, which is identical with the ascending case where mass transfer is restricted to molecular diffusion. However, from Fig. 5 of *Reference 1*, it is evident that the system actually was in a convectional state right from the beginning of the experiment. It must be left open what kind of flow pattern prevailed under the experimental conditions; some possibilities are gross laminar flow of the entire liquid column, a bundle of laminar flow cells distributed over the cross section, and a random flow of liquid packets similar to those proposed by Danckwerts (12) for his surface renewal concept.

No attempt will be made to develop a complete mechanistic model which accounts for all of the experimental data reported by the authors. However, it is demonstrated with two examples that the concept of free convection is capable of treating the observations without any need for an abnormal interfacial concentration. Three additional points are made which show that the cited arguments are based on rather insecure premises.

For any free convectional system, a distinct solute front is typical and represents the portion of the liquid that had first been in contact with the dissolving solid. The velocity of this front is governed by hydrodynamics (solubility, viscosity, and density as a function of concentration), and a constant velocity is not unexpected. Also, a correlation between front velocity and solubility, as shown in Tables III and IV of *Reference 4*, is consistent with such a concept and does not require explanation in terms of interfacial energy parameters.

When comparing dissolution rates of one solute in different solvents, an inverse correlation with solubility, such as the one shown in Fig. 2 of *Reference 3*, is not unusual and gives no verification that the descending dissolution is interfacially controlled. In the particular case, nearly a 10-fold increase in viscosity is found for the solvents (30°, viscosities in centipoises) (13): *n*-hexane, 0.29; *n*-heptane, 0.37; cyclohexane, 0.82; *trans*-decalin, 1.8; and *cis*-decalin, 2.7[1]. Since the viscosity is known to have a retarding effect on diffusion and, particularly, convection, and since the viscosity increase more than compensates for the increase in solubility, it is not surprising that the rate of dissolution decreases from hexane to decalin. Again, there is no need to rely on interface phenomena.

In *Reference 1* (p. 216), the authors restrict diffusion to the upward direction in the gravitational field. This is not true since molecular diffusion by definition is independent of any external forces. In addition, all of the solutes are of low molecular weight, <200. Even in an associated or highly solvated state, these particles are far below the limit where directional flow under the influence of gravity (*i.e.*, sedimentation) is likely to be observable. Hence, it is hard to see how "solvated solute molecules flow through the stationary liquid solvent, the fluid elements or holes comprising the liquid solvent acting as a channeling or dispersion medium" (4, p. 1592). The only acceptable concept is that of macroscopic flow, *i.e.*, convection.

For all four examples shown in Figs. 8–11 of *Reference 4*, the agreement between experimental data points and the fitted telegraph equation is by no means perfect; rather, the examples disprove the applicability of this

[1] It is not clear from the Krause and Kildsig paper (3) whether the *cis*- or *trans*-isomer was used.

equation. Under these circumstances, any extrapolation to zero distance is arbitrary. The concentration profiles shown in Fig. 7 of *Reference 1* are speculative since the actual interfacial concentration and the initial concentration gradient cannot be assessed from the experimental data with sufficient accuracy. In addition, the assumption of a constant nonsaturated interfacial concentration independent of the initial concentration gradient (as shown in Fig. 7) is not consistent with a transport model involving two consecutive rate processes.

Since the telegraph equation is not applicable, it is meaningless to ask whether the random-walk model with autocorrelation is appropriate to the descending column. However, even if perfect agreement had been obtained with the equation, this would not be more than a formal quantitative description. The authors' attempt to establish a mechanistic analogy between their descending dissolution and the mutual displacement of two miscible phases in a porous medium would still be questionable.

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Total Rate Equation for Decomposition of Prostaglandin E_2

Keyphrases ☐ Prostaglandin E_2 decomposition log rate–pH profile, rate equation ☐ Dehydration, prostaglandin E_2—log rate–pH profile, rate equation

Sir:

Monkhouse *et al.* (1) recently presented the 60° log rate–pH profile for the dehydration of prostaglandin E_2 (PGE_2)[1] from pH 1 to 10. Between pH 4 and 10, the log of the rate data was correlated by a straight line with a slope of about 0.3. Our 60° rate data substantially agree with the reported (1) 60° profile. However, we recently completed a 25° log rate–pH profile from pH 1 to 12 which appears to shed more light on the nature of the decomposition reaction between pH 4 and 10.

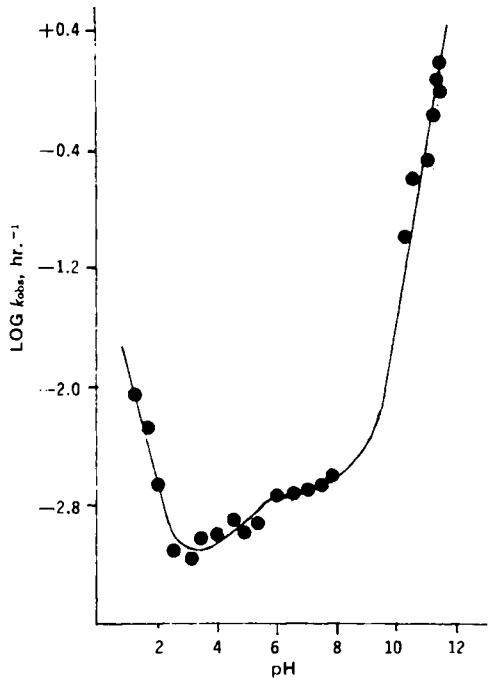

Figure 1—*Log rate–pH profile for decomposition of PGE_2 at 25.0°.*

Figure 1 shows the log rate–pH profile for the decomposition of PGE_2 in water–methanol (95:5 v/v) at 25.00 ± 0.05°. The apparent first-order rates were determined in hydrochloric acid and formate, acetate, phosphate, carbonate, and sodium hydroxide buffers at a constant ionic strength of 0.10. The apparent first-order rate constants were determined by following the disappearance of the substrate by a TLC procedure specific for PGE_2.

Although it may initially appear that the region between pH 4 and 9 is linear, the observed 25° rate constants (k_{obs} values) were best correlated by a rate equation depending upon the pKa of the prostaglandin[2]:

$$k_{obs} = k_H \cdot a_H \cdot (1 - \alpha) + k_{H_2O}(1 - \alpha) + k_{OH} \cdot a_{OH} \cdot (\alpha) + k^1_{H_2O}(\alpha) + k^{11}(\alpha) \quad \text{(Eq. 1)}$$

where k_H is the specific hydrogen-ion catalytic constant, k_{H_2O} is the catalytic constant for the water reaction of PGE_2, k_{OH} is the specific hydroxide-ion catalytic constant, $k^1_{H_2O}$ is the catalytic constant for the water reaction of ionized PGE_2, and k^{11} represents a catalytic constant for a reaction of PGE_2 in the ionized form. The hydrogen- and hydroxide-ion activities are represented by a_H and a_{OH}, respectively, and $(1 - \alpha)$ and (α) represent the fractions of PGE_2 in the unionized and ionized form, respectively.

The line in Fig. 1 was calculated from Eq. 1, and the points correspond to the experimentally observed apparent first-order rate constants. Buffer catalysis and isomerization at C_8 and C_{15} (2) did not appear to contribute significantly to the overall rate expression.

[1] PGE_2 is 11α,15(*S*)-dihydroxy-9-oxo-5-*cis*,13-*trans*-prostadienoic acid.

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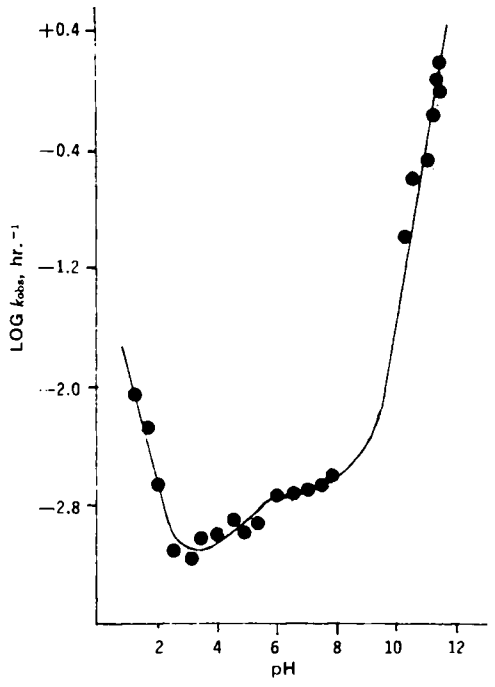

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▲ To whom inquiries should be directed.

Constituents of *Cannabis sativa* L. VI: Propyl Homologs in Samples of Known Geographical Origin

Keyphrases □ *Cannabis sativa* L.—propyl homologs in samples of known geographic origin □ Cannabidivarin and (−)-Δ⁹-*trans*-tetrahydrocannabivarin—identified in cannabis samples of known geographical origin

Sir:

Recent developments in identifying propyl homologs of cannabinoids prompted this communication. We wish to report the presence of cannabidivarin[1] and (−)-Δ⁹-*trans*-tetrahydrocannabivarin in freshly grown cannabis from known geographical locations. We routinely employed GLC and TLC[2] to identify the C_3-homologs. Some samples were identified by combined GLC–mass spectrometry[3].

Vollner *et al.* (1) identified cannabidivarin (I) from a sample of hashish in 1969. Gill *et al.* (2) later identified (−)-Δ⁹-*trans*-tetrahydrocannabivarin (II) from a sample of tincture of cannabis. Merkus (3) reported the presence of cannabivarin (III) in samples of Nepal hashish. This research group reported the presence of I and II in a sample of freshly grown *Cannabis sativa* L. from Indian seed stock (IN-B) grown in Mississippi (4).

De Zeeuw *et al.* (5) reported that propyl cannabinoids seem to depend on sample origin: samples from countries like India, Nepal, and Pakistan contained significant amounts of propyl cannabinoids, whereas samples from Middle Eastern and Mediterranean countries contained much lower amounts. Our own findings using only those variants from exact geographical locations confirm and extend the previously thought abundance of propyl cannabinoids in freshly grown *C. sativa* L. (Table I).

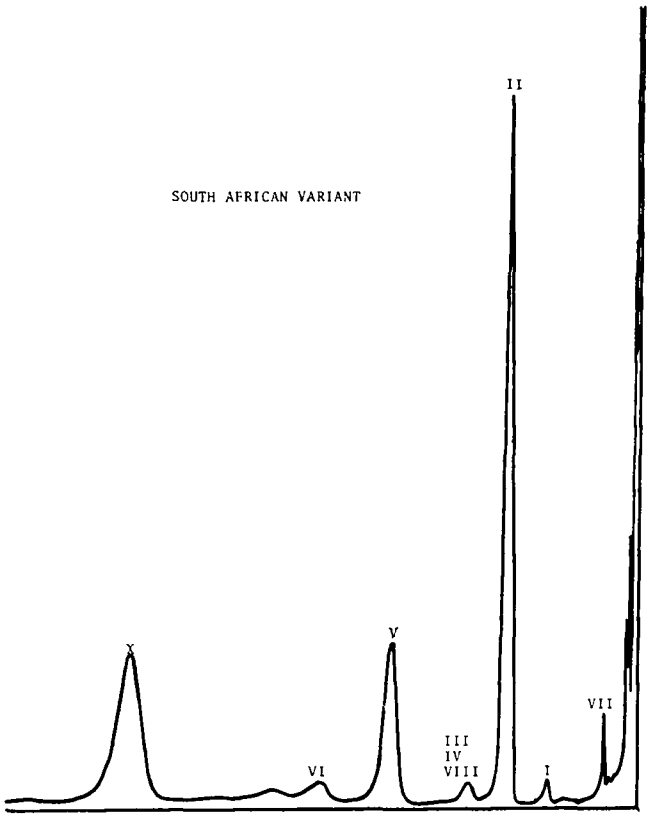

Figure 1—*Chromatogram of South African* C. sativa *L. (coded SA-E). Key: I, cannabidivarin; II, (−)-Δ⁹-trans-tetrahydrocannabivarin; III, cannabivarin; IV, cannabichromene; V, (−)-Δ⁹-trans-tetrahydrocannabinol; VI, cannabinol; VII, (−)-Δ⁹-trans-tetrahydrocannabiorcol; VIII, cannabidiol; and X, 4-androstene-3,17-dione (internal standard).*

The percentages of I and II given in Table I are normalized reports. Each cannabinoid is reported as its percentage in regard to total cannabinoid content. These data were obtained by a GLC–computer[4] analysis based on relative retention times of routine cannabis analysis[5].

Figure 1 of an African variant (seed code SA-E) contained 1.70% of I; 53.69% of II; 2.75% of III, cannabichromene (IV), and cannabidiol (VIII); 23.41% of (−)-Δ⁹-*trans*-tetrahydrocannabinol (V); and 4.38% of cannabinol (VI). Peak number VII was tentatively identified as (−)-Δ⁹-*trans*-tetrahydrocannabiorcol[6], first reported by Vree *et al.* (6).

Figure 2 of an Afghanistan variant (seed code AF-A) contained 8.35% of I; 5.34% of II; 12.48% of III, IV, and VIII; 1.94% of cannabigerol monomethyl ether (IX); 58.93% of V; and 2.03% of VI. The age of the material analyzed in Figs. 1 and 2 was 20 weeks. A previous literature report (7) showed that cannabinoid contents vary within each variant according to age.

[1] Since Vollner *et al.* (1) used cannabidivarin (divarinyl group) for the C_3H_7 side chain of cannabidiol (olivetyl group C_5H_{11}), and Merkus (3) used cannabivarin for the C_3H_7 side chain of cannabinol, we shall use the following trivial names: cannabidivarin, (−)-Δ⁹-*trans*-tetrahydrocannabivarin, and cannabivarin. Gill *et al.* (2) used "divarol" for the C_3H_7 side chain: (−)-Δ⁹-*trans*-tetrahydrocannabidivarol.

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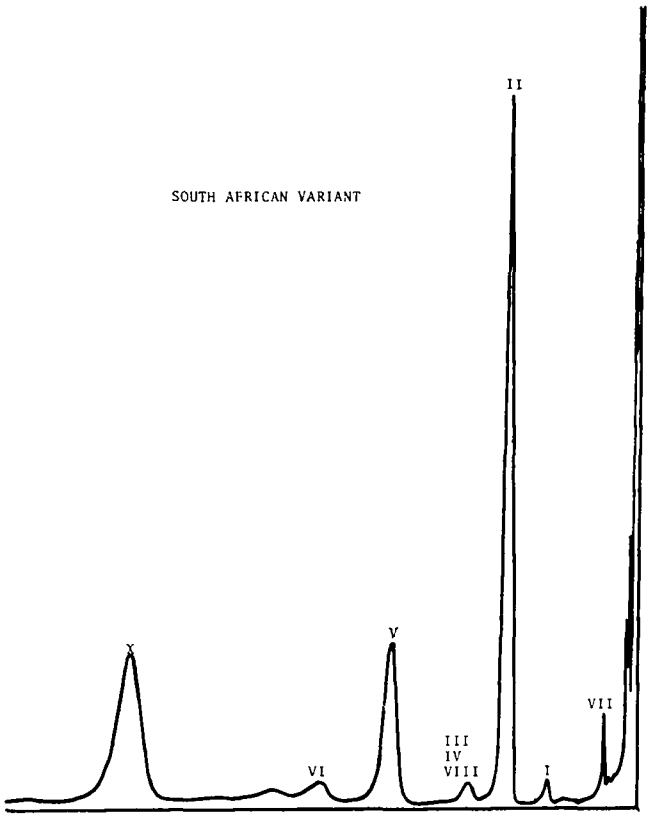

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Table I—Variants Analyzed for Propyl Homologs

Geographical Origin	Seed Code	Sex[a]	—Cannabinoids[b]— I	II
Afghanistan	AF-A	F	8.33	5.34
		M	t	+
	AF-B	F	+	7.72
		M	+	+
	AF-C	Y	t	48.23
Brazil	BZ-A	Y	–	t
		F	–	t
Chile	CH-A	M	–	t
Canary Islands	CI-A	Y	t	t
Czechoslovakia	CZ-A	Y	t	t
		F	t	t
		M	t	t
Ethiopia	ET-A	M	t	t
France	FR-A	Y	+	t
		F	t	t
		M	t	t
	FR-B	Y	t	t
	FR-C	F	–	–
Ghana	GH-A	X	–	t
	GH-B	Y	–	t
		X	–	t
	GH-C	Y	–	t
	GH-E	F	–	t
		X	–	+
India	IN-A	Y	–	–
		F	–	t
		M	–	t
	IN-B	Y	+	+
		F	+	10.63
		M	+	+
	IN-D	Y	–	+
	IN-E	F	–	+
		M	–	t
	IN-F	Y	–	+
	IN-I	Y	+	t
Iran	IR-A	F	–	+
		M	–	t
Jamaica	JA-A	Y	–	+
Japan	JP-A	Y	–	+
	JP-B	Y	–	+
Korea	KO-A	Y	–	+
		F	–	t
		M	–	t
Kenya	KE-A	M	–	t
Lebanon	LE-A	F	+	+
		M	–	–
Mexico	ME-A	Y	t	t
		F	t	t
		M	t	t
Mauritius	MA-A	F	–	+
Morocco	MO-A	F	–	–
	MO-B	F	–	–
	MO-C	F	–	–
Manchuria	MN-A	F	–	–
Nepal	NE-C	Y	–	+
		F	t	+
		M	t	+
Nigeria	NI-D	Y	t	8.80
Pakistan	PK-A	F	+	+
		M	t	t
Peru	PU-A	Y	–	t
		M	–	t
Poland	PO-A	Y	–	–
		F	–	–
		M	–	–
	PO-B	Y	–	–
		F	–	–
		M	–	–
Senegal	SE-A	Y	–	t
South Africa	SA-A	Y	t	+
		F	t	53.69
Sierra Leone	SL-A	F	–	t
Sudan	SU-A	F	+	+
Thailand	TI-B	Y	–	t
		F	–	t
		M	–	t
	TI-C	Y	t	t
		F	t	t
		M	t	t
		Y	t	t
		F	t	t
		M	t	t
	TI-D	Y	t	t
		F	t	t
		M	–	t
Turkey	TU-A	Y	–	–
		F	–	–
		M	–	–
	TU-C	Y	–	–
		F	–	–
		M	–	–
USA	US-A	Y	–	t
Viet Nam	VN-B	Y	–	t
		F	–	t
		M	–	t

[a] Y = young plants prior to sexual differentiation, F = female, M = male, and X = mixture of male and female. [b] – = possible (very small peaks), t = trace amounts (approximately 1%), and + = 1–5%.

Therefore, the data in Table I showing variation of I and II in regard to geographical location are not unusual and would be anticipated.

Just as the C_5H_{11} (olivetyl group) side-chain neutral cannabinoids exist predominately as their carboxylic

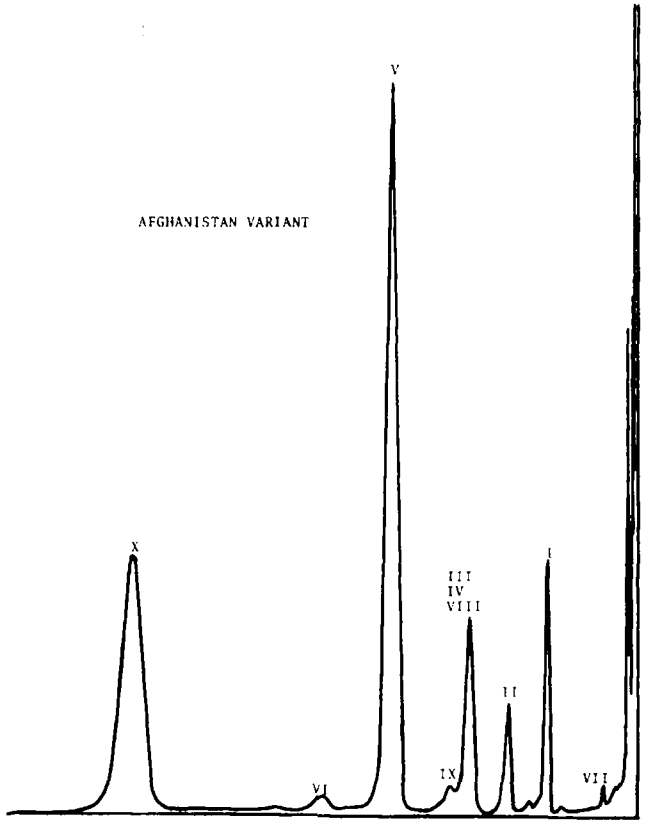

Figure 2—*Chromatogram of Afghanistan* C. sativa *L. (coded AF-A). Key: I, cannabidivarin; II, (−)-Δ⁹-trans-tetrahydrocannabivarin; III, cannabivarin; IV, cannabichromene; V, (−)-Δ⁹-trans-tetrahydrocannabinol; VI, cannabinol; VII, (−)-Δ⁹-trans-tetrahydrocannabiorcol; VIII, cannabidiol; IX, cannabigerol monomethyl ether; and X, 4-androstene-3,17-dione (internal standard).*

Table II—Mass Spectral Data

Trimethylsilyl Derivatives of Cannabinoids	*m/e*
(−)-Δ^9-*trans*-Tetrahydrocannabinolic acid	502
(−)-Δ^9-*trans*-Tetrahydrocannabivarinic acid	474
Cannabidiolic acid	574
Cannabidivarinic acid	546

acid derivatives in fresh *C. sativa* L. (8), so do the C_3H_7 (divarinyl group) side chains. GLC–mass spectrometry analysis of an Indian (IN-B) variant, silylated according to a reported procedure (9), contained a significant *m/e* at 546, corresponding to the trisilylated (trimethylsilyl ester bisether) derivative of I. Additionally, a significant *m/e* at 474 is indicative of disilylated (trimethylsilyl ester–ether) II. These data agree with the mass spectral data obtained for silylated acid derivatives of VIII and V, having *m/e*'s of 574 and 502, respectively (Table II). TLC was employed for identification of III, which has a relative retention time corresponding to IV and VIII. Compound III was not clearly observed in fresh samples of cannabis but was observed when samples containing II were heated. Thus, no data are presently available on the acid derivative of III.

Propyl homologs are, indeed, found in aerial parts of *C. sativa* L. plant material from many geographical locations. The abundances of propyl homologs vary, as do the pentyl homologs, in regard to geographical origin. However, we do not believe it feasible to determine exact geographic origin using the propyl homologs as "locators." Moreover, the propyl homologs exist predominately as their carboxylic acid derivatives as do the pentyl homologs.

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Coacervate Formation by Inorganic Salts with Benzalkonium Chloride

Keyphrases ☐ Coacervate formation—sodium chloride and sodium carbonate with benzalkonium chloride ☐ Sodium chloride—coacervate formation with benzalkonium chloride ☐ Sodium carbonate—coacervate formation with benzalkonium chloride, effect of concentration on coacervate volume and refractive index ☐ Inorganic salts, sodium chloride and sodium carbonate—coacervate formation with benzalkonium chloride ☐ Benzalkonium chloride—coacervate formation with sodium chloride and sodium carbonate

Sir:

A number of dilute aqueous solutions of proteins (1), polyelectrolytes (2), association colloids (3–5), carbonates (6), and lipids (6) have been investigated and found capable of forming coacervates under proper conditions. The essential feature of the coacervation phenomenon is the spontaneous separation of a homogeneous macromolecular or microionic aqueous solution into two immiscible aqueous solution phases. One aqueous layer contains most of the colloid and is termed the coacervate, while the second aqueous layer is colloid poor and is termed the equilibrium liquid. These coacervate systems are of growing general scientific (7, 8) and biological (9) interest.

The term biphasic coacervate system refers to the coacervate phase in contact with its equilibrium liquid phase. The term monophasic solution refers to the one-phase micellar solution.

Coacervation has been observed in some dilute soap solutions, and it has been reported (5) that coacervation in cationic detergent systems shows pronounced specificity to the anion and lesser sensitivity to the cation of the added electrolyte. Shah *et al.* (3) reported coacervate formation between the cationic surfactant, benzalkonium chloride, and the organic aromatic compound, sodium salicylate.

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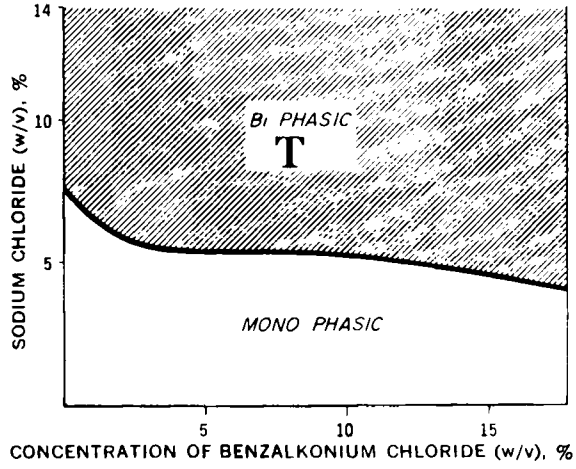

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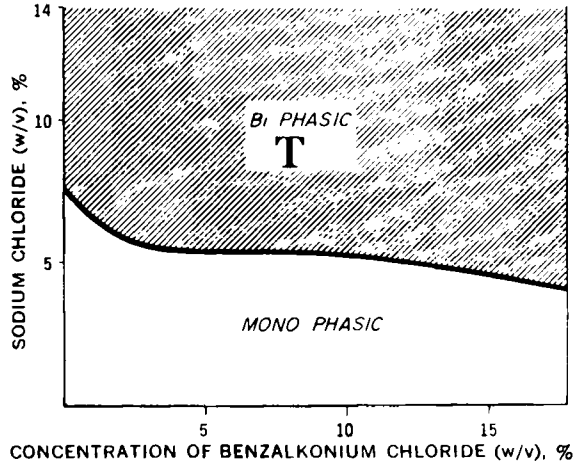

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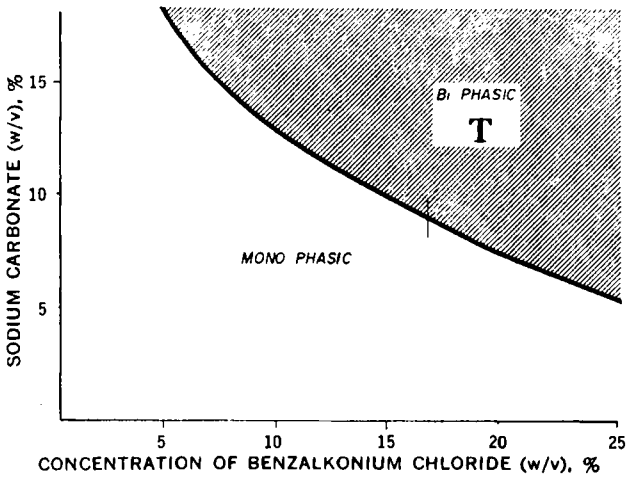

Figure 2—*Phase transition diagram of sodium carbonate–benzalkonium chloride coacervate system at 25°. Shaded area [T] represents the region in which biphasic coacervate system can be formed with lighter coacervate phase.*

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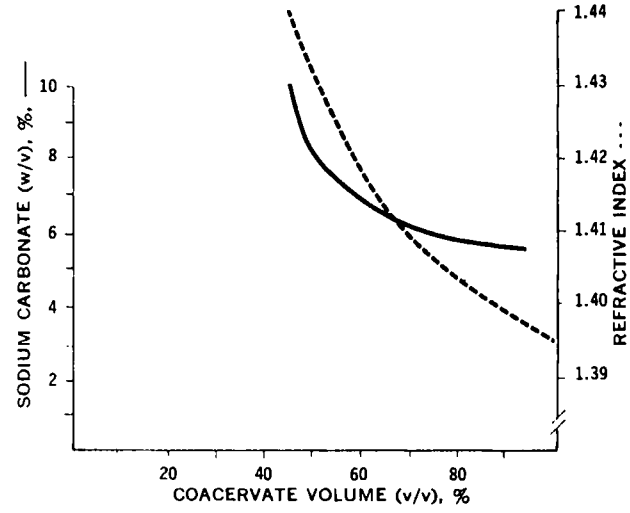
Figure 3 represents the effect of the sodium carbonate concentration on the refractive index and coacervate volume. For this study, 100 ml. of coacervate systems, containing 25% (w/v) benzalkonium chloride with 5.6, 6.0, 7.0, 8.0, and 10.0% sodium carbonate, was prepared. The systems were allowed to equilibrate for 24 hr. at room temperature (25°). The volume of coacervate was noted as the percent of the whole system. The refractive index of the coacervate phase was measured at 25° using a refractometer[1].



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Possible Errors and Role of Mercuric Chloride in Using Trinder's Reagent for Assay of Salicylates in Urine Specimens

Keyphrases ☐ Salicylates in urine—analysis, Trinder's reagent with and without mercuric chloride ☐ Mercuric chloride in Trinder's reagent—effect on salicylate analysis in urine ☐ Trinder's reagent, with and without mercuric chloride—effect on salicylate analysis in urine ☐ Colorimetry—analysis, salicylates in urine, Trinder's reagent with and without mercuric chloride

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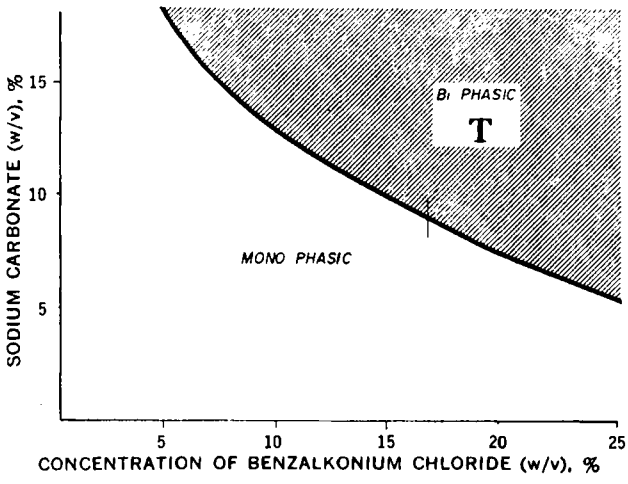
Trinder's reagent has been used in many colleges of pharmacy as a colorimetric agent to assay urinary salicylate and thereby to estimate the bioavailability of compounds such as aspirin, sodium salicylate, and *p*-aminosalicylic acid (1–4). The assay procedure is simple. One milliliter of the urine sample is usually diluted with 5 ml. of the reagent, and the absorbance of the resultant solution at 540 nm. is measured using the properly diluted (with water or urine blank) reagent as a reference solution. The absorbances of the urine samples



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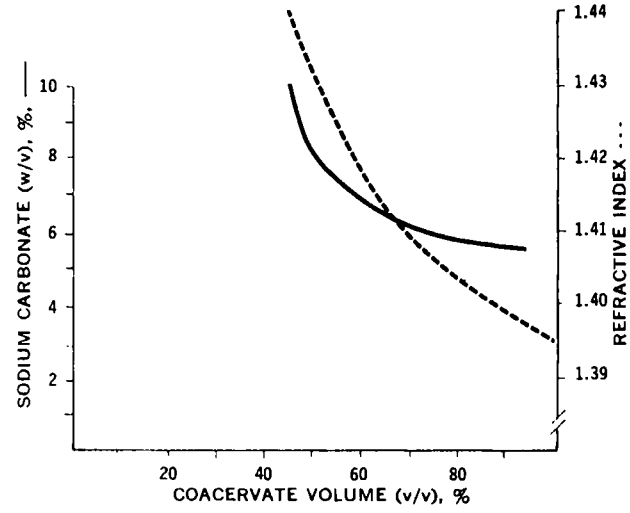

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Table I—Absorbances at 540 nm. of Various Urine Blank Samples

Urine Samples	Subjects 1	2	3	4	5	6
Before breakfast	0.05	0.10	0.23	0.05	0.17	0.06
Breakfast–lunch	0.22	0.08	0.20	0.13	0.14	0.05
Lunch–supper	0.22	0.09	0.19	0.17	0.12	0.06
Supper–bedtime	0.39	0.12	0.21	0.22	0.15	0.06

Table II—Absorbances of Sodium Salicylate in Urine after Adding Trinder's Reagent Prepared with or without Mercuric Chloride (an Average of Two Studies)

Concentrations of Sodium Salicylate, mg./ml.	With Mercuric Chloride	Without Mercuric Chloride
0.125	0.180	0.179
0.500	0.728	0.723
1.000	1.439	1.424

collected after dosing are corrected for the absorbance of the blank urine sample usually taken before dosing in the calculation of the salicylate concentrations in the urine. In doing so, it is assumed that the basic blank absorbances of the urine samples not due to ingested salicylates remain essentially unchanged during the period of study. To our knowledge, no data have been published to support this assumption.

In the bioavailability study of aspirin dosage forms using Trinder's reagent, we have occasionally found that the subjects excreted much more salicylate than ingested. We were also often puzzled by the fact that the absorbances of the urine samples after dosing were much lower than the absorbance of the blank sample. We thought that these observations might result from the intrasubject variation of the urinary blank contribution, and the present study was undertaken to explore this possibility. Urine specimens of six normal adult subjects who had not taken salicylates or other drugs during the previous week and the day of study were collected at various times during the day: before breakfast, between breakfast and lunch, between lunch and supper, and between supper and bedtime. Normal food and fluid uptake were allowed. Specimen absorbances at 540 nm., measured with a spectrophotometer[1] after adding the colorimetric agent, are shown in Table I. An absorbance of 0.1 corresponds to a urinary concentration of 0.07 mg./ml. of sodium salicylate. The possibility of marked intersubject and intrasubject blank variations is clearly demonstrated from these data. The highest blank reading found in the supper-to-bedtime urine of the Subject 1 corresponded to 0.273 mg./ml. of sodium salicylate. If one uses the urine of Subject 1 collected before breakfast as a blank sample, this subject can be calculated to excrete 159 mg. of sodium salicylate during the day even when he ingested no salicylate. This calculation was based on the average absorbance of three samples and a urine output of 1 l. An error of this magnitude is obviously unacceptable when one is evaluating the bioavailability of 324–648 mg. (5–10 grains) of an aspirin preparation administered as a single dose. In Subjects 2, 3, and 5, the excretion of salicylates can sometimes be underestimated if one also uses the urine sample collected before breakfast as a blank. For Subject 6, all blank readings were quite low and consistent.

The inclusion of mercuric chloride (4% w/v) in Trinder's reagent was used primarily to precipitate protein during salicylate analysis of serum or plasma samples (1). Since urine samples normally contain only a negligible amount of protein, it was interesting to determine whether its inclusion is necessary. Such a study seemed especially significant in light of the pollution problem of the mercury compound and its relatively high cost. One milliliter of various standard solutions of sodium salicylate in urine was diluted with 5 ml. of the Trinder reagent prepared with or without mercuric chloride. Their absorbances at 540 nm. were measured using suitable blanks as references. As shown in Table II, absorbances of solutions were essentially not affected by the absence of mercuric chloride and all followed Beer's law in the range studied. A similar phenomenon was also observed for salicylate dissolved in distilled water. Therefore, it is concluded that the mercuric chloride included can be deleted from Trinder's reagent for the assay of salicylates in urine or other aqueous media not containing proteins.

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REVIEWS

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REVIEW ARTICLE

Fundamentals of Interaction of Ionizing Radiations with Chemical, Biochemical, and Pharmaceutical Systems

STEPHEN G. SCHULMAN

Keyphrases ☐ Radiation chemistry—review of interactions with chemical, biological, and pharmaceutical systems ☐ Ionizing radiation—effect on solids, liquids, and gases, interactions with biological systems, review ☐ Biological considerations—effect of ionizing radiation on carbohydrates, amino acids, peptides, proteins, enzymes, vitamins, and nucleic acids, review ☐ Radioprotection—chemical and pharmaceutical considerations, review

The effects of ionizing radiations produced by radioactive isotopes, particle accelerators, nuclear reactions, and X-ray generating devices upon matter have been of interest in chemistry and biology for several decades. Ionizing radiations are known to produce highly reactive ionic and free radical species in chemical substances, which lead to stable chemical products, usually very different from those obtained from the normal chemical reactions of these substances. In biological systems, the reactions caused by ionizing radiations are usually detrimental to the organism, resulting in mutagenesis, incapability to reproduce, organic damage, and even death. However, some aspects of radiation effects are beneficial. Studies of the effects of ionizing radiations upon solids elucidated the nature of the defect solid state and assisted advances in solid-state electronic components. The modification of polymeric materials by treatment with ionizing radiations often enhances the mechanical and thermal stabilities of these materials and has already been exploited commercially.

Interest in the chemical and biological effects of ionizing radiations in the pharmaceutical sciences has never been great, probably as a result of the lack of application of radiation science to pharmaceutical science. However, in recent years there has been a surge of interest in toxicology, drug interactions, and clinical pharmacy in general. A significant fraction of the population is exposed to substantial levels of ionizing radiations in the form of X-rays, γ-rays (cancer patients), or a variety of high-energy nuclear radiations (workers in nuclear research and in fields utilizing radioisotopes). The author believes that the effects of ionizing radiations on the biochemical systems in living organisms, particularly man, constitute a branch of toxicology. Moreover, in cancer patients who are subjected to unusually high dosages of ionizing radiations while they may be taking substantial dosages of a variety of drugs regularly, the direct or indirect effects of ionizing radiations upon the drug (*in vivo*) and ultimately upon the patients themselves constitute a special area of drug interactions. Virtually no definitive research has been undertaken in these areas, and very little has even been accomplished with regard to the effects of ionizing radiations upon pure drugs. This review was written to introduce the pharmaceutical community to the fundamental principles underlying the interactions of ionizing radiations with matter in the hope that interest will be stimulated in this area.

The intent of this work is not all inclusiveness; rather, it is a concise exposition of the fundamental principles underlying radiation chemistry and contains a representative selection of references to works of major interest throughout the history of the subject. Several excellent review articles already in the literature were freely drawn upon in preparing this review.

THEORETICAL CONSIDERATIONS

This section deals with the fundamental nature of the interactions of high energy radiations with matter, from the absorption of the radiation to the eventual establishment of chemical equilibrium in the system. The overall process may be divided into three stages (1):

1. The physical stage, entailing the dissipation of the radiant energy in the system. Its duration is of the order of 10^{-15} sec., the time scale representative of electronic absorption in molecules.

2. The physicochemical stage, consisting of processes that lead to the establishment of thermal equilibrium in the system. Its duration is of the order of 10^{-14}–10^{-12} sec., the time scale of vibrational and other thermal relaxation phenomena in molecules.

3. The chemical stage, consisting of diffusion and chemical reaction of the reactive species leading to chemical equilibrium. It lasts upward of 10^{-8} sec., depending on the rate constants and diffusion coefficients of the reactive species and the lifetimes of the reactive species.

Physical Stage—The interaction of ionizing radiation with a medium results initially in ionization and electronic excitation. At very high energies or with very heavy ionizing particles, it is also possible to effect nuclear excitation and dislocation. These processes occur regardless of the nature of the radiation. The mechanism of excitation and ionization by charged particles is different, however, from that by high energy photons. These mechanisms are, therefore, discussed separately.

Charged Particles—The interaction of charged particulate radiations, such as protons, α-particles, and β-rays, with a medium consists predominately of electrostatic coulomb excitation and ionization of the atomic and molecular electrons in the medium. According to Bethe's (2) semiclassical treatment, the energy transferred per unit length of path to the medium by a heavy particle of charge Ze and velocity v is:

$$\frac{dE}{dx} = \frac{4\pi Z^2 e^4 n}{mv^2} \ln \frac{2mv^2}{I} \qquad \text{(Eq. 1)}$$

where n is the mean electron density (number of electrons per unit volume) of the medium, m is the electronic mass, I is a mean excitation potential for the medium ($I = 11.5Z$ ev. for $Z \leq 30$, $I = 8.8Z$ ev. for $Z \geq 30$ where Z is the mean atomic number of the medium), and dE/dx is the energy gained by the medium per unit path length. The latter term is generally referred to as the linear energy transfer (LET) of the radiation. If electrons (β-rays) are the ionizing particles, the expression for LET is changed slightly to:

$$\frac{dE}{dx} = \frac{4\pi Z^2 e^4 n}{mv^2} \ln \left(\frac{mv^2}{2I} \sqrt{\frac{\epsilon}{2}} \right) \qquad \text{(Eq. 2)}$$

where ϵ is the basis of the natural logarithms.

Equations 1 and 2 yield some interesting conclusions about the transfer of energy by charged particles. First, the rate of energy loss of a charged particle in a given medium is proportional to the electron density in the medium. Second, because the factor v^2 outside the logarithmic term is more important than that inside, the rate of energy loss increases as the particle slows down. Third, if two particles of equal energy but different mass are compared, the heavier one will have a smaller velocity and hence a higher LET. As a consequence, an α-particle will produce many more excitations and ionizations per unit path length than a β-particle of the same energy. This is important in the interpretation of effects due to different types of radiation.

High Energy Electromagnetic Radiations—When high energy photons such as X-rays and γ-rays pass through matter, they lose energy by three mechanisms: (*a*) photoelectric absorption, in which the photon transfers its entire energy to an electron; (*b*) Compton scattering, in which the photon transfers part of its energy to an electron; and (*c*) pair production, in which the photon disappears and a high energy electron and positron are formed. The relative contribution of each mechanism to the total energy loss depends upon the energy of the photon. For photons in the 100-kev.–2-Mev. range, the principal mode of absorption by the medium is Compton scattering (3); much higher photon energies favor pair production (at least 1.02 Mev. is required to produce a pair) while lower energies favor photoelectric absorption. The principal effect of the absorption of high energy photons is the production of energetic electrons, which then dissipate their energies by the mechanism already discussed. In addition, ions are produced, the fate of which will be discussed.

In general, the effect of transfer of energy from an energetic particle to the medium is to produce along its path a variety of electronically excited molecules and ions as well as free electrons. Secondary electrons are also formed along with the ions. The electronic transitions resulting in the formation of these species occur in short times (10^{-15} sec.) compared to molecular vibration times (10^{-14} sec.). Little is known about the relative amounts of these species in the several electronic states, especially in the liquid and solid states.

Physicochemical Stage—This stage lasts about 10^{-14}–10^{-12} sec., which is of the order of magnitude of molecular vibration times, during which internal molecular rearrangements can take place.

During the physicochemical stage, the excited molecules and ions dissipate their excess energy by bond rupture, internal conversion, energy transfer to neighboring molecules, *etc.* Therefore, an understanding of these processes is fundamental to radiation chemistry (4). Also, during this stage the low energy secondary electrons produced during the physical stage interact with the environment. There are two divergent theoretical views on this phenomenon as it occurs in pure water. One theory (5, 6) assumes that secondary electrons lose kinetic energy due to attraction by the parent ion and by inelastic collisions with other molecules. After several collisions, these electrons, when they are sufficiently slowed, are recaptured by the parent ions. The recapture process transforms the parent ion into an excited neutral molecule which can then decompose into hydrogen ions and hydroxyl radicals produced close together.

According to the other view (6, 7), the electron loses

its kinetic energy through vibrational and rotational excitation of the medium. The dipole vibration loss lowers the energy of the electron to about 0.2 ev. in about 10^{-12} sec. at a distance larger than 50 Å from the parent ion. It is then surrounded by water molecules corresponding to the IR value of the dielectric constant of about 5 and, thereafter, it is thermalized by rotational excitation of water in about 10^{-11} sec. Since that is the relaxation time of the water dipole, the electron cannot be recaptured by the parent ion; it becomes solvated and produces a hydrogen atom according to: $e_{aq}^- + H_2O \rightarrow H\cdot + OH_{aq}^-$. The parent ion dissociates according to: $H_2O^+ \rightarrow H^+ + OH$.

Consequently, according to the latter model, the hydrogen ions and hydroxyl radicals that result from a given primary ionization are quite far apart; according to the former model, they are quite close. The difference between the initial distribution of these radicals is important for the chemical stage of radiolysis (6).

Chemical Stage—During this stage, the reactive intermediates (ions and radicals) produced in the previous stages diffuse away from their sites of production and undergo chemical reactions. Two subjects are of great importance in the chemical stage: (*a*) the relative importance of ionic *versus* free radical reactions, and (*b*) the rates of diffusion and reaction of the chemically reactive species.

Ionic versus *Free Radical Reactions*—Current theory and experiments indicate that ionic processes are most important in the radiation chemistry of gases (8–12). These processes consist mainly of ion-neutral molecule reactions in which collisions between these entities result in secondary ionization, neutralization, and cluster formation. In condensed systems, however, the main reactive species produced in the physicochemical stage, which react in the chemical stage, are believed to be free radicals. Their primary modes of reaction are atomic abstraction, radical recombination, and addition to π-bonds.

Diffusion Kinetics—The treatment of the rates of disappearance of reactive species has been effected, to a fair degree of accuracy, through the "radical diffusion model" (13). This model is based upon the assumption that the macroscopic laws of diffusion and reaction kinetics are applicable to the system in spite of the spatial inhomogeneity of the "spurs" (localized sites) in which the radicals are formed (3, 5). To compensate partially for the grossness of this assumption, the kinetic equations are expressed in terms of probability densities rather than concentrations of the various reactive species (13). The probability densities describe the average behavior of a statistical ensemble of radical spurs ($\geq 10^{14}$ spurs). The rate of disappearance of a reactive species (x_i) from the medium in which it is formed is given by:

$$\frac{\partial c_i(r,t)}{\partial t} = D_i\nabla^2 c_i - k_i c_i - \sum k_{ij} c_i c_j + \sum_1 \bar{k}_1 c_1 + \sum_{m,n} \bar{k}_{m,n} c_m c_n \quad \text{(Eq. 3)}$$

where $c_i(r,t)$ is the probability density of x_i at time t after the onset of the chemical stage and a distance r from the spur in which it is formed, D_i is the diffusion coefficient of x_i, ∇^2 is the three-dimensional Laplacian operator, k_i is the first-order rate constant for the disappearance of x_i, k_{ij} is the second-order rate constant for the reaction of x_i with x_j, $\bar{k}_1$ is the first-order rate constant for the appearance of x_i from the radiolysis of x_1, and $\bar{k}_{m,n}$ is the second-order rate constant for the appearance of x_i by reaction of x_m with x_n, whose probability densities are c_m and c_n, respectively. Given the initial distributions $c_i(r,0)$, it is possible in principle to integrate numerically the simultaneous, nonlinear, differential equations obtained for each species involved in the process. Once the functions $c_i(r,t)$ are known, one can calculate the amounts of chemically stable products formed.

The radical diffusion model has been criticized on the grounds that: (*a*) the use of probability densities instead of concentrations neglects interactions between nonreacting molecules (correlation) (14), (*b*) it is merely an assumption that the reactive species are in thermal equilibrium with the medium at the onset of the chemical stage (1), and (*c*) too many unknown parameters (rate constants) must be determined (13). Nevertheless, it does provide a reasonable working model of the chemical stage of radiolysis and will have to suffice until it is replaced with a better theoretical treatment.

GASES

The irradiation of gases results in the ionization of the gas molecules (8). Since the distances between molecules in a gas are great, there is a good chance that the ion may escape its counterelectron and engage in reactions of its own (6). This is not a likely process in a condensed medium, because the medium will exert a retarding effect upon ejected electrons and slow them enough to be recaptured rather quickly (6).

A free ion in a gas may be in a vibrationally excited state. The excited ion may possibly have sufficient excess vibrational energy to dissociate during the lifetime of a single vibration ($\sim 10^{-14}$ sec.). This has been observed occasionally in the case of small molecules but is a very rare process in molecules of molecular weight greater than 44 (15).

Free positive ions appear to react in gas media by three principal modes. First, a positive ion may attract other molecules to itself by dipole induction as the ion is thermalized (11). This has the effect of forming an ion cluster in which the charge and excess energy of the ion are delocalized over the entire cluster. This situation is similar to exciton formation in solids. The cluster then may be neutralized by absorbing an electron from the medium. Chemical reaction ensues, resulting in the formation of molecular products. An example of reaction *via* cluster formation is the formation of cuprene from the gas phase radiolysis of acetylene (16) according to the sequence:

$$C_2H_2 \xrightarrow{\alpha} C_2H_2^+ + e^-$$

$$C_2H_2^+ + 20C_2H_2 \rightarrow (C_{42}H_{42})^+ \xrightarrow{e^-} \underset{\text{cuprene}}{C_{42}H_{42}}$$

Second, the ion may capture a neutral molecule by constraining the molecule to move in an orbit around

it. Neutralization may occur, resulting in a complex molecule. For example, consider the interaction between methane and the methane ion to form ethane and hydrogen (17):

$$CH_4^+ + CH_4 \rightarrow (CH_4)_2^+$$

$$(CH_4)_2^+ + e^- \rightarrow H_2 + C_2H_6$$

Alternatively, ion molecule reactions may result in catastrophic processes as, for example (18):

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH\cdot$$

$$H_2^+ + H_2 \rightarrow H^+ + H + H_2$$

and:

$$CH_3^+ + CH_4 \rightarrow C_2H_5^+ + H_2$$

Third, an ion may become thermalized and recapture an electron, resulting in the formation of an excited neutral molecule which can then dissociate into free radicals by several different mechanisms (depending upon which excited states are involved). The free radicals then react with other molecules in the system to begin a chain of reactions leading to the final products. An example of this type of process is the formation of ozone by the radiolysis of oxygen (19):

$$O_2 \xrightarrow{\alpha} O_2^+ + e,\ O_2^+ + e^- \rightarrow (O_2^*) \rightarrow 2O\cdot$$

$$O\cdot + O_2 \rightarrow 2O_3$$

This is an example of a one-component gas reaction. In multicomponent reactions, common processes are oxidation, hydrogenation, polymerization, and reverse reactions.

The irradiation of a system containing oxygen can result in the formation of species such as O_2^+, formed by ionization, or O_2^-, formed by electron capture. These ions can dissociate according to the reactions (19):

$$O_2^+ \rightarrow O\cdot + O^+$$

$$O_2^- \rightarrow O\cdot + O^-$$

Species such as O^+ and $O\cdot$ are strongly oxidizing and react with hydrogen to form water and hydrogen peroxide. Carbon monoxide reacts with oxygen to form carbon dioxide; hydrocarbons react with oxygen to form carbon dioxide, water, and partial oxidation products; and ammonia reacts with oxygen to form water and oxides of nitrogen.

If oxygen is mixed with a gas that by itself undergoes some specific radiolytic reaction, the effect of the oxygen generally predominates over the usual path of radiolysis. This is known as "exclusivity of oxidation" (15).

The presence of hydrogen in a gas system that is being irradiated results in the formation of hydrogen ions which are not as reactive as oxygen positive ions. Hence, hydrogenation does not proceed with the exclusivity seen with oxidation. The irradiation of ethylene in the presence of hydrogen results in the formation of some ethane, but the principal product is still polyethylene (15).

Gas phase polymerization of unsaturated hydrocarbons proceeds by the formation of ions which then attack π-bonds in a chain reaction. Small polymers (3–20 monomer units) are formed independent of temperature and pressure conditions, while the formation of long-chain polymers is dependent upon temperature and pressure as well as the presence of foreign "initiator" species (15). Polymerization of saturated molecules can also occur with elimination of hydrogen; *e.g.*:

$$2CH_4 \rightarrow H_2 + C_2H_6$$

and:

$$2C_2H_6 \rightarrow CH_4 + C_3H_8$$

In some radiolyses of unicomponent or multicomponent gases, the product yields are considerably lower than would be expected from the ion yields measured by dosimetry. The low yields may often be accounted for by the occurrence of reverse reactions. The products formed by radiolysis can react with each other to give the starting material back (15).

In addition to ionization, two other processes may occur in gas phase radiolysis. One, electron capture, has already been mentioned in connection with oxidation. The other is the splitting of a neutral molecule into positive and negative ions; *e.g.*:

$$CHCl_3 \rightarrow CHCl_2^+ + Cl$$

Both of these processes have a reasonable chance of occurring only if the molecules being irradiated contain electronegative atoms (20).

LIQUIDS

The irradiation of liquids results in the formation of ions in the same way as it does in gases. The ejected electrons are usually thermalized within the electric field of the parent ion. Most of these ion-pairs culminate in recapture of the ejected electrons leaving the molecules in a highly excited state, which may return to the ground state or the lowest allowed excited state by internal conversion, luminescence, or energy transfer (21–23). Alternatively, the highly excited neutral molecules may split into free radicals (24). In addition to these processes, some ion-pairs may be sufficiently longlived to diffuse away from the site of production and react ionically with the surrounding medium (6). However, the free radicals are currently believed to be the most important reactive species formed. Once free radicals are formed along the track of an ionizing particle, they may combine with each other while they are in close proximity, or they may diffuse away from the spur and react with molecules in the bulk of the liquid medium. Those that recombine within the spur react so rapidly that they cannot be detected by physical or chemical methods. They form stable molecular products, which are known as the molecular yield. Those radicals that diffuse away from the spur and react with medium can be detected by physical methods, such as electron spin resonance spectroscopy (25), and chemical methods, such as compound formation with radical scavengers (*e.g.*, iodine and diphenylpicrylhydrazine), and are called the radical yield. The mechanisms of chemical radiation effects are frequently

determined by comparison of relative molecular and radical yields. The radiation chemistry of liquids has developed along two distinct paths—that of water and aqueous solutions (26–28) and that of organic liquids (29, 30). Each area has enough distinction to warrant separate discussion.

Water and Aqueous Solutions—The irradiation of pure water is believed to result in two dissociative processes. The first is the direct dissociation of water into hydrogen atoms and hydroxyl radicals:

$$H_2O \rightarrow H\cdot + OH\cdot$$

The second reaction is the ionization of water to yield a hydrogen ion, a hydroxyl radical, and a hydrated electron:

$$H_2O \rightarrow H^+ + OH\cdot + e_{aq}^-$$

The second mechanism was identified through the detection of the absorption spectrum of the hydrated electron. The hydrated electron is an extremely powerful reducing agent and reduces water according to:

$$e_{aq}^- + H_2O \rightarrow H\cdot + OH^-$$

It reduces the hydrogen ion according to:

$$e_{aq}^- + H^+ \rightarrow H\cdot$$

Since the latter two reactions result in the same products as direct radiolysis, and since the products of reduction by the hydrogen atom and the hydrated electron are identical, it is frequently impossible to determine whether the hydrated electron or the hydrogen atom is the principal reducing species in aqueous solutions. In acid solutions, it is reasonable to assume that the hydrated electron will reduce hydrogen ions (H^+) almost exclusively and that hydrogen atoms ($H\cdot$) will therefore be the predominant reducing species; but, in neutral and basic solutions, the hydrated electron may be assumed to predominate. The ultimate molecular products of the radiolysis of pure water are hydrogen gas and hydrogen peroxide, formed by the reactions:

$$H\cdot + H\cdot \rightarrow H_2$$

$$OH\cdot + OH\cdot \rightarrow H_2O_2$$

Of course, hydrogen atoms can react with hydroxyl radicals ($OH\cdot$) to form water, but this process is impossible to detect experimentally.

The radiation chemistry of aqueous solutions may be considered from two points of view. The first, called "target theory," considers the direct effect of ionizing radiations upon the solute molecules. The second approach regards transformations in the solute molecules to be due to interactions with the reactive intermediates formed by the radiolysis of water. Since most aqueous systems are relatively dilute, the latter approach seems to be more reasonable on a purely statistical basis. Kinetic studies of dilute aqueous systems have indeed borne out this supposition. The radiation chemistry of aqueous solutions then becomes the free radical and redox chemistry of $H\cdot$, $OH\cdot$, and e_{aq}^-.

The effectiveness of radicals in producing chemical changes, other than formation of molecular hydrogen and hydrogen peroxide, in aqueous systems depends upon the LET of the ionizing radiation which produces these radicals. A high LET particle, such as an α-particle or a proton, produces a large concentration of radicals along its short track. These radicals are likely to recombine, forming molecular products, before they can diffuse away from the spurs in which they are formed. Low LET particles produce low radical concentrations along their tracks, minimizing the probability of recombination so that the radicals can diffuse away from the spurs and initiate chemical reactions. Protons and α-particles, therefore, result in high molecular yields while β- and γ-rays result in high radical yields.

One popular device for measuring radiation dosage, the Fricke dosimeter, is based upon the oxidation of the ferrous ion by hydroxyl radicals produced in the radiolysis of a dilute aqueous solution of ferrous sulfate:

$$Fe^{+2} + OH\cdot \rightarrow Fe^{+3} + OH^-$$

The presence of dissolved oxygen alters the nature of the redox properties of irradiated water as a consequence of the "radical scavenging" property of oxygen. Molecular oxygen has two unpaired electrons. One of these can form a covalent bond with a hydrogen atom, forming the hydroperoxy radical ($HO_2\cdot$). This species can act either as an oxidizing or as a weak reducing agent:

$$HO_2\cdot + Fe^{+2} \rightarrow Fe^{+3} + HO_2^-$$
(oxidizing)

and:

$$HO_2\cdot + Cu^{+2} \rightarrow Cu^+ + H^+ + O_2$$
(reducing)

The main consequence is that while solutions in pure irradiated water have about equal oxidizing and reducing capabilities, the presence of oxygen in these solutions can result, in some cases, in very strong oxidizing properties due to the conversion of the reducing hydrogen atom to the predominately oxidizing hydroperoxy radical. In general, the presence of oxygen in aqueous solutions leads to alterations of the mechanisms of radiolyses due to the "exclusivity of oxidation." A brief summary of some representative free radical reactions in aqueous solution follows.

Hydrogen Atom—

$$H\cdot + D_2 \rightarrow HD + D\cdot$$

$$H\cdot + H_2O_2 \rightarrow OH\cdot$$

$$H\cdot + O_2 \rightarrow HO_2\cdot$$

$$H\cdot + Fe(H_2O)_n^{+2} \rightarrow Fe^{+3}(H_2O)_{n-1} + OH^- + H_2$$

$$H\cdot + Ce^{+4} \rightarrow Ce^{+3} + H^+$$

$$H\cdot + Fe^{+3} \rightarrow Fe^{+2} + H^+$$

$$H\cdot + Cu^{+2} \rightarrow Cu^+ + H^+$$

$$H\cdot + I_2 \rightarrow H^+ + I^- + I\cdot$$

$$H\cdot + NO_2^- \rightarrow NO\cdot + OH^-$$

$$H\cdot + CH_4 \rightarrow CH_3\cdot + H_2$$

$$H\cdot + HCOOH \rightarrow H_2 + \cdot COOH$$

$$H\cdot + CH_3COOH \rightarrow H_2 + \cdot CH_2COOH$$

Hydroxyl Radical—

$$OH\cdot + H_2 \rightarrow H_2O + H\cdot$$

$$OH\cdot + D_2 \rightarrow HOD + D\cdot$$

$$OH\cdot + H_2O_2 \rightarrow H_2O + HO_2\cdot$$

$$OH\cdot + Fe^{+2} \rightarrow Fe^{+3} + OH^-$$

$$OH\cdot + HSO_4^- \rightarrow OH^- + HSO_4\cdot$$

$$OH\cdot + Ce^{+3} \rightarrow Ce^{+4} + OH^-$$

$$OH\cdot + Tl^+ \rightarrow Tl^{+2} + OH^-$$

$$OH\cdot + I^- \rightarrow OH^- + I\cdot$$

$$OH\cdot + NO_2^- \rightarrow OH^- + \cdot NO_2$$

$$OH\cdot + CH_4 \rightarrow H_2O + CH_3\cdot$$

$$OH\cdot + HCOOH \rightarrow H_2O + \cdot COOH$$

$$OH\cdot + CH_3COOH \rightarrow H_2O + \cdot CH_2COOH$$

Hydroperoxy Radical—

$$HO_2\cdot + HO_2 \rightarrow H_2O_2 + O_2$$

$$HO_2\cdot + OH \rightarrow H_2O + O_2$$

$$HO_2\cdot + H_2O_2 \rightarrow H_2O + O_2 + OH\cdot$$

$$HO_2\cdot + Fe^{+2} \rightarrow Fe^{+3} + HO_2^-$$

$$HO_2\cdot + Fe^{+3} \rightarrow Fe^{+2} + H^- + O_2$$

$$HO_2\cdot + Ce^{+4} \rightarrow Ce^{+3} + H^+ + O_2$$

$$HO_2\cdot + Cu^{+2} \rightarrow Cu^+ + H^+ + O_2$$

Organics—An important difference between the radiation chemistry of water and of organic liquids is that the concept of the spur, a reasonably well-defined volume in which the formation of the reactive species occurs along the track of the ionizing particle, becomes somewhat hazy (31). The reason for this is that the radicals formed in water show a preference for recombination rather than reaction with the environment immediately after formation. The volume in which recombination is likely defines the spur. The radical products of irradiated organic liquids, however, are more likely to interact with their immediate environment than to undergo recombination. This is evidenced by the low molecular yields of hydrogen from irradiated organic systems.

The radiation chemistry of hydrocarbons and their derivatives has been extensively investigated (32). An important difference between gas phase and liquid phase radiolysis of hydrocarbons exists in that the breaking of carbon–carbon bonds is an important primary process in the gas phase, while the rupture of carbon–hydrogen bonds is almost exclusive in the liquid phase. Another important difference between analogous reactions in gas and liquid phases occurs in the polymerization process. In gas phase polymerizations, the presence of radical scavengers, such as iodine and benzoquinone, does not appreciably alter the yields of polymeric products. In the liquid phase, however, the yields of the polymers obtained from the irradiation of materials like vinyl chloride are seriously curtailed by the addition of radical scavengers. This indicates that polymerization in the liquid state occurs primarily by a free radical mechanism, whereas in the gaseous state it occurs by an ionic mechanism. The irradiation of polymeric materials results in cross-linking of polymer chains and grafting of dissimilar polymeric materials. This treatment of polymers contributes considerable tensile strength and heat resistance to the irradiated polymers and is already being exploited commercially.

Irradiation of saturated aliphatic compounds typically results in unsaturation, polymerization, and isomerization. The radiolysis of cyclohexane illustrates all three processes. The initial step in the radiolysis of cyclohexane results in the formation of energetic cyclohexyl radicals and hydrogen atoms. If the radicals produced are very energetic, cyclohexane can be formed by the abstraction of hydrogen from a cyclohexyl radical either by a hydrogen atom or by another cyclohexyl radical. If the radicals become thermalized, recombination of radicals can occur to give bicyclohexyl. A less frequent process occurring in the thermalized cyclohexyl radical is rearrangement followed by hydrogen atom capture to yield methylcyclopentane.

The irradiation of alkyl halides results in cleavage of the carbon halogen bond. The radiolysis of methyl iodide, for example, yields ethane and molecular iodine.

Alcohols, upon radiolysis in the liquid state, yield aldehydes and vicinal glycols (33–35). For example, consider the radiolysis of methanol:

$$CH_3OH \rightarrow \cdot CH_2OH + H\cdot$$

$$\cdot CH_2OH \rightarrow H_2C{=}O + H\cdot$$

and:

$$2\cdot CH_2OH \rightarrow CH_2OHCH_2OH$$

Although it would be expected that high LET radiations would favor glycol formation and low LET radiations would favor aldehyde formation, the opposite is true. This indicates that the mechanisms involved are not as simple as the schemes would lead one to believe.

The irradiation of alcohols frozen to glasses at liquid nitrogen temperature results in deep coloration of the glasses (36). Methanol turns a brilliant purple while ethanol turns blue. These colored glasses are stable if kept in the dark at low temperatures. Exposure to visible or UV light results in bleaching of the alcoholic glasses as well as elimination of the electron spin resonance signal observed in the colored glasses. The nature of the colored glasses is not well understood, but the colors are believed to be due to the absorption spectra of trapped free radicals in the glasses. The product yields from the bleached glasses are different from those of irradiated glasses that have not been exposed to light. This indicates that the trapped radicals might be photolyzed by visible and UV light.

The irradiation of aromatic compounds generally results in considerably lower yields of radiolysis products than does irradiation of aliphatic compounds of similar molecular weight and functional group composition (37–39). This has been attributed to the effectiveness of the delocalized π-orbitals in these compounds in accommodating excitation energy without permitting the molecule to dissociate. Nevertheless,

some radiolysis does occur. Benzene is known to yield biphenyl, phenylcyclohexadiene, and a polymeric material of average composition $(C_6H_7)_x$, which behaves like an unsaturated hydrocarbon. The low yields of hydrogen molecules (H_2) observed in benzene radiolysis are due to attack of hydrogen atoms upon the benzene ring to form phenylcyclohexadiene and the polymer. Dimerization and polymer formation are also characteristic of the radiation chemistry of other aromatic hydrocarbons. The resistance of polystyrene $(-C_6H_5CHCH_2-)_n$ to cross-linking, compared with polyethylene, is further evidence of the stability of aromatics to radiation effects. Nitrobenzene is also very stable to radiation damage.

In addition to being stable to radiation, aromatic compounds frequently protect other, more radiosensitive compounds from radiolysis (40). An example of this is the protection of liquid cyclohexane from extensive radiolysis by the addition of a small amount of benzene. This protective effect of benzene is probably due to energy transfer from cyclohexane to benzene, followed by dissipation of the excitation energy by the aromatic π-system.

Finally, one important general feature of the radiation chemistry of liquids is that excited or reactive molecules are formed in close proximity and are quite likely to react with one another. This situation is not encountered in photochemistry, and it is this phenomenon that makes liquid phase radiation chemistry so interesting.

SOLIDS

Because of the "fixed" positions of atoms in crystalline lattices, the effect of irradiation of solids (41, 42) includes atomic displacements as well as electronic excitation and ionization. While electronic alterations of materials affect their chemical behavior, atomic displacements in solids have a much more pronounced effect upon the physical properties of crystals. To dislodge an atom from its normal lattice position, a certain amount of energy must be transferred to the atom by an irradiating particle. Because of the large mass of the atoms, electrons and photons are relatively ineffective in producing substantial numbers of atomic dislocations. The heavier particles—α-particles, protons, deuterons, and neutrons—are much more effective in this process. Furthermore, unlike the primary effect of ionizing radiations in producing electronic disturbances through electrostatic effects, the predominant effect that is required to produce atomic dislocations is the direct knock-on process.

Two basic types of lattice defects, point defects and line defects, occur in all real crystals and at very high concentrations in irradiated crystals. Point defects result from the displacements of atoms from their normal lattice sites. These displaced atoms usually occupy a site not in the lattice framework and are then known as "interstitials." The empty lattice site left behind by the interstitial is called a "vacancy." A vacancy produced by displacement of an anion or cation, along with its interstitial ion, is called a Frenkel pair or simply a Frenkel defect. In some cases, the displaced ions are removed so far from their vacancies that they form a new layer at the crystal surface. The vacancies left behind are called Schottky defects. Frenkel and Schottky defects play very important roles in the properties of solids altered by radiation damage.

Line defects (dislocations) are produced by slippage or shear of the crystal lattice. If the slippage is perpendicular to a face of the crystal so that the lattice planes on either side of the dislocation are parallel but displaced with respect to one another, the defect is called an edge dislocation. If the slippage is angular, as if produced by rotation about the shear axis, so that lattice planes on either side of the defect are not perpendicular, the defect is called a screw dislocation.

A consideration of the effects of irradiation of crystalline materials requires that the nature of the crystal be understood. There are four broad classifications of crystal types, according to the nature of the interatomic forces holding the crystal together. In metallic crystals the atoms are thought to form a quasi-ionic lattice arrangement with the valence electrons, which bind the lattice delocalized throughout the crystal so that they cannot be identified with any one atom. Valence crystals, such as diamonds, consist of a lattice in which the atoms are bonded by conventional covalent interaction throughout the lattice. This implies that a valence crystal could be considered a giant molecule. Molecular crystals (*e.g.*, naphthalene and water) are regular arrangements of well-defined molecules which are bound together in the lattice by van der Waals' and hydrogen bonding forces. Finally, the ultimate in electronic localization occurs in ionic crystals, in which the lattice is composed of alternating positive and negative ions held together by strong electrostatic attractions. Sodium chloride is a typical example of an ionic crystal. The predominant effects of ionizing radiations on these crystal types will be considered separately, because these effects are manifested as characteristics of the crystal type.

Metallic Crystals—The effects of ionizing radiations upon metals are entirely due to atomic displacement. Because of the delocalization of electrons throughout the crystal, no persistence of ionization can occur; a positive hole formed by an electron ejection is always refilled by an electron from the conduction band. As a result, no chemical decomposition can occur because of ionization. On the other hand, sufficiently energetic radiations can cause atomic displacements. The production of interstitial atoms in the lattice has the effect of "swelling" the crystal. This results in a lowering of the density of the crystal. The irradiation of a crystal of copper increases the critical shear stress considerably (43). This is believed to be due to the migration of thermalized interstitials and vacancies to the sites of naturally occurring dislocations in the copper crystal. These interstitials impede the vibration and slippage of the dislocations, resulting in greater resistance to shear. This "pinning of dislocations" is also used to explain why copper behaves like a tuning fork when struck with a hammer after irradiation while it gives a dull "thud" when struck prior to irradiation.

Perhaps the most obvious evidences of radiation damage in metallic crystals are the increase of electrical

resistivity and the decrease of thermal conductivity in irradiated crystals. This is believed to be due to scattering of electrons and photons by vacancies and interstitials which behave like impurities and destroy the order of the lattice necessary for high conductivity.

The obvious effects of radiation damage in metallic crystals can be reversed by "annealing." This process consists of heating the irradiated materials so that the "energy of activation" required to push an interstitial back into a vacancy is supplied (44).

Valence Crystals—The stable, symmetrical bonding occurring in valence crystals results in the failure of these crystals to demonstrate quasichemical changes such as depolymerization or reaction of the matrix with displaced entities. Unlike metals, however, valence crystals have no conduction electrons and can therefore retain electronic dislocations as well as atomic displacements. The trapping of dislocated electrons in the crystal by potential wells, such as those created by atomic vacancies, results in coloration of the normally transparent valence crystals. This is due to the low excitation energies of trapped electrons as opposed to σ-bonded electrons.

The irradiation of semiconducting valence crystals like silicon and germanium usually drastically decreases the conducting properties of these crystals (45). Large numbers of Frenkel defects are produced and act as traps for conducting electrons and holes, thereby altering the concentrations of conducting species. Bombardment of n-type germanium with deuterons converts the germanium to a p-type semiconductor.

Ionic Crystals—Irradiation of ionic crystals results in atomic and electronic dislocations. The trapping of displaced electrons by anion vacancies results in a pseudoatomic system which usually has a manifold of closely spaced, electronic energy levels. This results in the absorption of visible and near UV light which gives these crystals their characteristic colors. These pseudoatomic electrons and their vacancies are called color centers (46). The polarizations of the spectra of these color centers depend upon the symmetry of the electric field produced by atoms surrounding the trapped electron. Some of the color centers commonly produced by irradiation of ionic crystals are: F-centers (octahedral symmetry), consisting of an electron trapped by a simple halide-ion vacancy; M-centers (isoceles, triangular symmetry), consisting of the delocalized coupling of an F-center with an adjacent anion-cation vacancy pair (Seitz defect); and V-centers, consisting of a singly charged halogen molecule anion in a normal lattice site, behaving as a trapped hole by virtue of its molecular bond. The exposure of colored ionic crystals to visible or UV light results in bleaching of the colorations induced by irradiation. While this bleaching is being carried out, the crystals become photoconductive when a potential difference is imposed across them. This implies that the bleaching process is due to annealing of trapped electrons. If a sufficient potential difference is placed across the crystal, the color centers will migrate through it. In some cases where the crystal remains uncolored upon irradiation, thermoluminescence is observed in the annealing process.

The dissolution of a heavily irradiated crystal of sodium chloride in water will result in the evolution of hydrogen and chlorine from the solution. The solution also turns alkaline, presumably due to the reactions of trapped holes and electrons with water (47):

$$e^- + H_2O \rightarrow {}^1/_2\, H_2 + OH^-$$

$$\text{hole}^+ + Cl^- \rightarrow {}^1/_2\, Cl_2$$

Trapped electrons also account for the ability of irradiated sodium chloride to initiate polymerization in acrylonitrile.

Irradiation of nitrates, chlorates, perchlorates, and bromates results in the liberation of oxygen (48, 49). In potassium perchlorate, irradiation results in explosion of the crystal due to internal buildup of oxygen.

Molecular Crystals—The irradiation of substances that form crystals containing discrete molecules, held together by dispersion forces, results in radiolysis in the conventional sense. For example, the radiolysis of aliphatic carboxylic acids in the solid state yields hydrogen, carbon monoxide, and carbon dioxide (50). The relative yields of these gases depend upon the strength of the bonds involved in radiolysis and their frequency of occurrence. These considerations apply as well to liquids and gases and suggest no special solid-state effects. This is the case for the γ-radiolysis of bromotrichloromethane (51), where no difference in the yield of bromine is observed for irradiation of solid or liquid bromotrichloromethane. Differences are observed between the radiolysis patterns of liquids and molecular crystals of the same materials, but these seem to be more the exception than the rule and may generally be attributed to stronger caging effects in the solid than in the liquid (52).

Energy transfer in molecular crystals seems to be a well-established phenomenon. Irradiated crystals of anthracene containing only a trace of naphthacene show the characteristic green fluorescence of naphthacene rather than the violet of the main constituent (53). If the material is dissolved in benzene, the anthracene fluorescence predominates. This phenomenon is often seen in mixed, scintillating crystals (54).

The irradiation of ice results in formation of trapped hydrogen and hydroxyl radicals as well as the hydrated electron (55). Dilute solutions of Cd^{+2}, Zn^{+2}, and Mg^{+2} in ice have been reduced to the unstable Cd^+, Zn^+, and Mg^+, respectively, upon irradiation (56). Apparently, some mobility is enjoyed by some radical species in the ice lattice.

The irradiation of surface catalysts alters the properties of these catalysts through defect production on the surfaces (57). These defects have been observed to enhance and inhibit catalytic activity in specific cases. For example, irradiation of silica gel enhances the rate of hydrogen–deuterium exchange on it (58). On the other hand, irradiation of zinc oxide decreases the rate of hydrogenation of ethylene on it (59).

CHEMICAL PROTECTION FROM IONIZING RADIATIONS

The effects of ionizing radiations upon chemical and biological systems may be minimized or even eliminated by the addition of certain chemical compounds to the system to be irradiated. These compounds react

either directly with the radiation or, more often, with the reactive species produced by the radiations. In so doing, they are themselves transformed into other substances, but their transformation results in the preservation of the integrity of the original chemical or biological system.

At the molecular level, several mechanisms account for the protection of irradiated systems by chemical agents. These are: (*a*) energy transfer and charge transfer, in which an ionized or excited molecule transfers its charge or excess energy to a protecting molecule either by collision or by resonance transfer at a distance; (*b*) scavenging, in which a protecting radical scavenger reacts with radicals from the initial actions of the radiation before they can attack other molecules in the system; and (*c*) complex formation, in which a protective molecule can form complexes that are either more or less susceptible to radiation than the original substance.

Energy and Charge Transfer—The transfer of charge or excitation energy (60) must be fast enough to compete with dissociation processes if protection is to occur. In some cases, an activated molecule can dissociate within 10^{-14} sec., the time for one molecular vibration. However, localization of energy in a particular bond usually requires 10^{-13}–10^{-9} sec. To remove energy or charge from an activated molecule effectively, the protector should have a slightly lower ionization or excitation potential. In fluid systems the rate of charge transfer is limited by diffusion; the donor and acceptor must be in contact. Excitation energy, however, can be exchanged by molecules as much as 70 Å apart by "Förster energy transfer," a dipole–dipole interaction. One requirement of this process is partial overlap of the absorption spectrum of the energy acceptor and the emission spectrum of the energy donor. Energy transfer is radiationless, comparable in rate to molecular vibration and thus more efficient than luminescence in deactivating highly excited molecules. Transfer processes of this type are extremely efficient in crystalline materials where the high degree of order permits excitation energy to travel in excitons which traverse the crystal faster than its vibrational relaxation time. Crystalline structure also facilitates charge transfer by providing conduction bands in which electrons can freely move about.

Energy conversions within a molecule can decrease the probability of decomposition. Energy can be dissipated so rapidly by internal conversion that its localization in any one bond is improbable. Paramagnetic additives such as transition metal ions are useful in this context as strong spin-orbit coupling increases the number of energy states available to the original molecules. Aromatic compounds are protective because they can probably dissipate acquired excitation energy throughout their extensively delocalized π-systems (61–63).

Scavenging Intermediates—The addition of certain compounds that readily react with free radicals can effectively prevent these radicals, which are primary products of ionizing radiations, from causing secondary damage in the system. Molecular iodine is a very effective radical scavenger, forming iodo compounds (64, 65) with radicals and leaving behind iodine atoms to do further scavenging, *e.g.*:

$$CH_3\cdot + I_2 \rightarrow CH_3I + I\cdot$$

$$CH_3\cdot + I\cdot \rightarrow CH_3I$$

Oxygen is a diradical which enhances radiation damage by forming radicals with other radicals. An example of the latter process is the scavenging of hydrogen atoms by molecular oxygen to form the hydroperoxy radical (65):

$$H\cdot + O_2 \rightarrow HO_2\cdot$$

Complexes—Certain compounds may exert protective action by forming molecular complexes with the original molecules of the system. These complexes might be less sensitive to radiolysis or attack by radicals or they may be better able to transfer charge and excitation energy than the original compound. Protection can also result from the formation of complexes across particularly sensitive bonds in the original compound. For example, the degradation of polyisobutylene is reduced about 50% by copolymerization with 20% styrene (66). The radiation resistance of the porphyrin ring is enhanced by complexing it with vanadium and other metals.

The most obvious application of chemical protection from ionizing radiations is to biological systems. For a protective agent to be biologically practical, it must be nontoxic at protective concentrations, easily introduced to the organism, widely distributed, and remain intact for long periods of time before irradiation. Many substances of all kinds have been applied to this problem. The most effective to date have been compounds like cysteine, because of the scavenging property of the mercapto (—SH) group and the ease of oxidation of the amino ($-NH_2$) group, and cystamine (67), because of complex formation with, and ease of oxidation of, the disulfide linkage. None of the compounds studied to date has been effective enough for use in man, predominately because of their high toxicities.

MOLECULES OF BIOLOGICAL SIGNIFICANCE

The effects of ionizing radiations on living organisms are frequently observed but not completely understood. There are two distinct theories of the actions of ionizing radiations on the components of living cells that result in chemical transformations leading to mutation or cell death. The first of these is the "target theory" (3, 68). This approach regards only those events that produce ionizations in biologically significant molecules as being important. The main evidence for this simple idea is that, in many cases, the amount of damage to a given organism varies logarithmically with the dose of radiation, implying that the amount of damage possible in a cell is proportional to the number of radiosensitive species remaining undamaged and therefore capable of reacting. A less direct mechanism would not require the proportionality of dose and unaffected material because some of the total dose would be channeled out to processes other than the biologically significant ones.

The other theory is based upon such an indirect relationship between the incident radiation and the af-

fected, biologically significant molecules (69). In this approach, the solvent, water, interacts with the radiation, forming ions and radicals. These reactive species in turn react with the biologically significant molecules, causing radiation damage. Radiation biology, under this approach, is simply a branch of the radiation chemistry of aqueous solutions. As such, the recombination of hydrogen atoms and hydroxyl radicals to form hydrogen and hydrogen peroxide (the molecular yield) effectively competes with the biological molecules for the effects of the radiation. There is evidence that both the target and indirect processes occur and that no one theory accounts for all of the observed biological effects of radiation at the molecular level (4).

This section considers the effects of ionizing radiations on molecules known to have biological significance and the relationship of the radiation chemistry of these molecules to radiation effects observed in living organisms.

Carbohydrates—The irradiation of aqueous solutions of carbohydrates (70–74) has the same effect as it does upon alcohols. The hydroxyl groups are attacked to yield carbonyl compounds. Under anoxic conditions, dimeric products and ultimately polymers are also formed. The primary alcohol groups of carbohydrates are especially radiosensitive. Mannitol is readily oxidized to mannose and sorbitol is oxidized to glucose. While oxidation of primary alcohol groups is favored by aerobic conditions, high yields from the oxidation of secondary alcohol groups are favored by anoxic conditions.

The irradiation of polysaccharides results predominately in their degradation. This explains why fruits and vegetables become soft on irradiation. This degradation occurs both in solution and in the dry state.

Amino Acids and Peptides—The irradiation of amino acids (75) results in transformation of both the amino and the carboxylic functions (76). In the dry state, glycine is decarboxylated to methylamine upon irradiation; in dilute aqueous solution, however, the amino group is hydrolyzed to give glyoxalic acid, acetic acid, and formaldehyde. In solutions of concentrations greater than 2%, methylamine again becomes an important product. The other amino acids are similar to glycine in their radiolytic behavior. Alanine, for example, gives ethylamine and carbon dioxide in the dry state and pyruvic acid and ammonia in aqueous solution (77, 78).

The aromatic amino acids, when irradiated in aqueous solution, show effects that are typical of aromatic compounds and amino acids (79, 80). Phenylalanine is deaminated in aerated solutions with the formation of a ketone. The aromatic ring remains relatively stable to radiolytic decomposition.

The irradiation of peptides results in a chemistry similar to that of the amino acids but also in the breakage of the peptide bond (81). In aqueous solution, all irradiated peptides give ammonia whether or not free amino groups are present.

When irradiated in the dry state, the thiol- and disulfide-containing amino acids degrade to keto acids with the evolution of carbon dioxide and hydrogen sulfide. In solution, however, the thiol and disulfide groups are excellent radical scavengers and free radical attack on these groups precludes deamination. The ultimate result of irradiation of thiol-containing amino acids is their oxidation to disulfides. Thus, irradiation of an aqueous solution of cysteine results in the formation of cystine (82–84). The irradiation of the disulfides results in higher oxidation products. For example, cystine gives cystine disulfoxide in aqueous solution. Reduction of disulfides to thiols is not generally observed.

Proteins and Enzymes—The irradiation of proteins (85) results in the formation of free radicals in the sites of disulfide bonds (86). Aromatic amino acids in the proteins are also particularly susceptible to attack, decarboxylation and deamination being common results of irradiation (87, 88). Rupture of the peptide linkage is characteristic of the radiolysis of proteins (87, 89). In the case of enzymes, the destruction of peptide linkages is accompanied by a decrease in biological activity (90). This decrease continues after irradiation is stopped (91). The mechanisms of radiolysis in the dry state and in solution are different, but the results are usually similar. One important difference between these results is the degradation of proteins by dry state irradiation compared with the increase of molecular weight through cross-linking in solution (92). In general, the radiation chemistry of proteins and enzymes may be considered a special case of the radiation chemistry of peptides and amino acids.

Respiratory Proteins, Vitamins, and Coenzymes—*Respiratory Proteins*—These substances are iron-porphyrin-protein complexes. Irradiation of these substances may produce effects in the porphyrin ring or in the protein, but oxidation or reduction of the iron almost always is involved. The iron in cytochrome c (ferricytochrome c) is reduced to the ferrous state in the presence of benzoate ion (93). Under alcoholic conditions, the ferric form is favored. Hemoglobin and oxyhemoglobin are both oxidized from the ferrous to the ferric state, destroying the property of oxygen transport (94–97). Large radiation doses result in attack on the porphyrin ring and denaturation of the protein. When irradiated in the dry state in the absence of oxygen, hemoglobin becomes insoluble due to protein denaturation. Myoglobin behaves in a similar manner to hemoglobin but is considerably more radiosensitive (94). Hemocyanin is a copper-containing respiratory protein of molecular weight more than 10 times that of hemoglobin. In this case, attack at the protein part of the molecule predominates (98).

Vitamins and Coenzymes—The irradiation of coenzyme I (diphosphopyridine nucleotide) results in reduction of the pyridine carboxamido ring. The product of this reduction is probably a dimer, which is itself radiosensitive (99).

The B-group vitamins, thiamine and riboflavin, are destroyed upon irradiation in dilute aqueous solutions. Riboflavin is reduced in air-free solutions to a semiquinone form (100). Niacin is decarboxylated on irradiation in air-saturated aqueous solutions (101).

Upon irradiation in aqueous solution, aminobenzoic acid is destroyed by deamination and decarboxylation (102–104). Sulfanilamide and sulfathiazole are inacti-

vated, presumably due to deamination (105). Upon irradiation, the cobalt in vitamin B_{12} is reduced from the cobaltic to the cobaltous state.

The plant hormone auxin has been shown to be radiosensitive. The product of the irradiation of auxin (β-indoleacetic acid) is a polymer similar to that obtained in the radiolysis of indole (106).

Nucleic Acids—The nucleic acids DNA and RNA are responsible for the transmission of genetic information and protein synthesis. Both processes are dependent upon the ordering of purine and pyrimidine bases, which are bound to the main body of the molecule by riboside linkages. The main body of these molecules consists of ribose (5-carbon sugar) molecules linked together by phosphoric acid units to form a long strand. The purine and pyrimidine bases branch off from the chain at the ribose sites. It is believed that the DNA molecule consists of two helically intertwined strands of nucleic acid held together by hydrogen bonding between purine and pyrimidine pairs on opposite strands.

The irradiation of nucleic acids ruptures hydrogen bonds which hold DNA strands together, resulting in polymerization, deamination, and dehydroxylation of purine and pyrimidine bases, fission of sugar base linkage, liberation of the purine bases, destruction of the pyrimidine bases, oxidation of the sugar moiety, and breakage of the nucleotide chain with liberation of inorganic phosphates. In the presence of oxygen, irradiation leads to the formation of hydroperoxides of the pyrimidine bases but not of the purine bases (107–114).

In general, the purine bases appear to be much more stable to radiolysis than the pyrimidine bases (113, 114), probably due to the greater π-delocalization energy of the purines which provides a pathway for nondestructive energy dissipation. Furthermore, the pyrimidine bases are known to undergo free radical reactions more readily than the purine bases. The order adenine > guanine $\gg$ cytosine > uracil > thymine has been established for the relative resistances of the bases to radiolysis. In the presence of oxygen, in aqueous solutions, uracil and thymine form stable hydroperoxides while cytosine forms an unstable hydroperoxide which decomposes to a variety of products (113–116).

Irradiation of DNA in the solid state, at liquid nitrogen temperature, yields radicals in which, as indicated by electron spin resonance measurements, the unpaired spin is delocalized over the entire chain and does not belong to any one unit of the giant molecule (117). Addition of small amounts of water to this system does not alter the nature of the DNA radicals produced, but a two-to-one excess of water results in the annihilation of the electron spin resonance signal for DNA with the appearance of a strong signal due to water radicals. It has been postulated that this protective effect is due to energy transfer which is made possible in an excess of water by structuring of the water, thus providing a pathway for the formation of a delocalized water radical or exciton. Electron spin resonance studies of irradiated nucleoprotein solutions indicate that the protein takes most of the radiation damage, protecting the nucleic acid moiety.

The damages caused by ionizing radiations in nucleic acids and their components are obviously detrimental to the passage of genetic information, which requires specific orders of intact purine and pyrimidine bases in the DNA strands. Alterations in these bases and the DNA molecules in general can lead to mutations and lethal genes. The disruption of RNA molecules interferes with protein synthesis and can result in eventual cell death.

PHARMACEUTICAL SYSTEMS

Most pharmaceutical studies related to the effects of ionizing radiation have been confined to the study of radioprotective drugs. A few studies of the effects of drugs on irradiated organisms and of irradiation on drug-dosed organisms have also been carried out. As mentioned earlier, the effects of ionizing radiation can be minimized by certain chemical compounds. Among the agents employed as radioprotective drugs are the sulfhydryl compounds, notably cysteine, estrogens, and bacterial lipopolysaccharides.

One way in which these protective agents exert their effect is by scavenging free radicals resulting from biological radiation damage. In this instance, the radioprotective agent reacts with a free radical to form a less reactive free radical or a molecule that is insufficiently reactive to interact with cellular components. Aminothiols work well in this context. Grenan and Copeland (118) studied the structure-radioprotective activity relationship of the aminothiols (β-mercaptoethylamine homologs) and found all derivatives to exhibit some radioprotective effect. Vasin (119) found that increasing the length of the alkyl side chains resulted in loss of antiradiation properties. He also found indole analogs of esadrine and ephedrine and the indolylalkylamino alcohols possessing a third amino group to be quite effective in suppressing radiation damage. Recently, Langendorff (120) found that a significant relationship exists between cyclic adenosine monophosphate and radioprotection and hypothesized that cyclic adenosine monophosphate plays a major role in radioresistance.

Organoselenium compounds such as selenocystine, selenomethionine, colloidal selenium, selenoxanthene, selenoxanthone, and selenochromone were found to be similar to, and in some cases superior to, cysteine in radiation protection (121). Dimethyl sulfoxide has also demonstrated radioprotective properties, as has phenylhydrazine (122, 123). Imidazole and erythropoietin were found to increase the survival of irradiated mice, with the highest survival rate being after administering the drug 5 min. prior to irradiation (124). A bile acid sequestrant was also found to be effective in preventing GI effects of whole body irradiation. Parkinson (125) found this to be an effective means of reducing the severity of effects and the morbidity associated with irradiation of the GI tract. To reduce the toxicity seen with the administration of these agents, Vlastislav (126) suggested a causal relationship between radioprotection and immunization by protein antigen interactions with such agents as β-lactin, casein, and human serum albumin.

The interpretations of these phenomena are obviously largely speculative. In this regard, the study of com-

bined influences of drugs and ionizing radiation at the molecular level should prove a most fertile area for future investigation.

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RESEARCH ARTICLES

Chemical Modification of Lincomycin: Synthesis and Bioactivity of Selected 2,7-Dialkylcarbonate Esters

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Keyphrases □ Lincomycin 2,7-dialkylcarbonate esters—synthesis as tasteless derivatives, bioactivity compared to lincomycin hydrochloride □ Carbonate esters of lincomycin—synthesis as tasteless derivatives, bioactivity compared to lincomycin hydrochloride □ Pediatric formulations, potential—synthesis, activity of lincomycin 2,7-dialkylcarbonate esters □ Tasteless lincomycin derivatives—synthesis, activity of 2,7-dialkylcarbonate esters □ Antibacterial agents, potential—synthesis of lincomycin 2,7-dialkylcarbonate esters

The use of bioreversible derivatives for the modification of certain pharmaceutical properties of lincomycin (I) was reported previously (1, 2). This paper represents a continuation of that systematic effort and is concerned with lincomycin 2,7-dialkylcarbonate ester derivatives. The goal of this work was the synthesis of several

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RESEARCH ARTICLES

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A. A. SINKULA▲ and C. LEWIS

Abstract □ A series of lincomycin 2,7-dialkylcarbonate esters was synthesized to enhance the pediatric acceptability of lincomycin. Several diester derivatives are sufficiently tasteless to warrant consideration as candidates for pediatric formulations. Preliminary bioactivities [mouse median protective dose (CD_{50}) bioassay] indicated several derivatives to be equivalent in subcutaneous activity to lincomycin hydrochloride. Four diester derivatives exhibited oral bioactivity comparable to that of lincomycin. Serum hydrolysis studies on certain 2,7-diesters of lincomycin established that a high degree of esterase activity is present in the serum of several different rodent species. This phenomenon appeared to be limited to these species.

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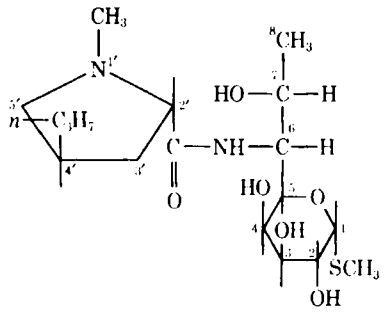
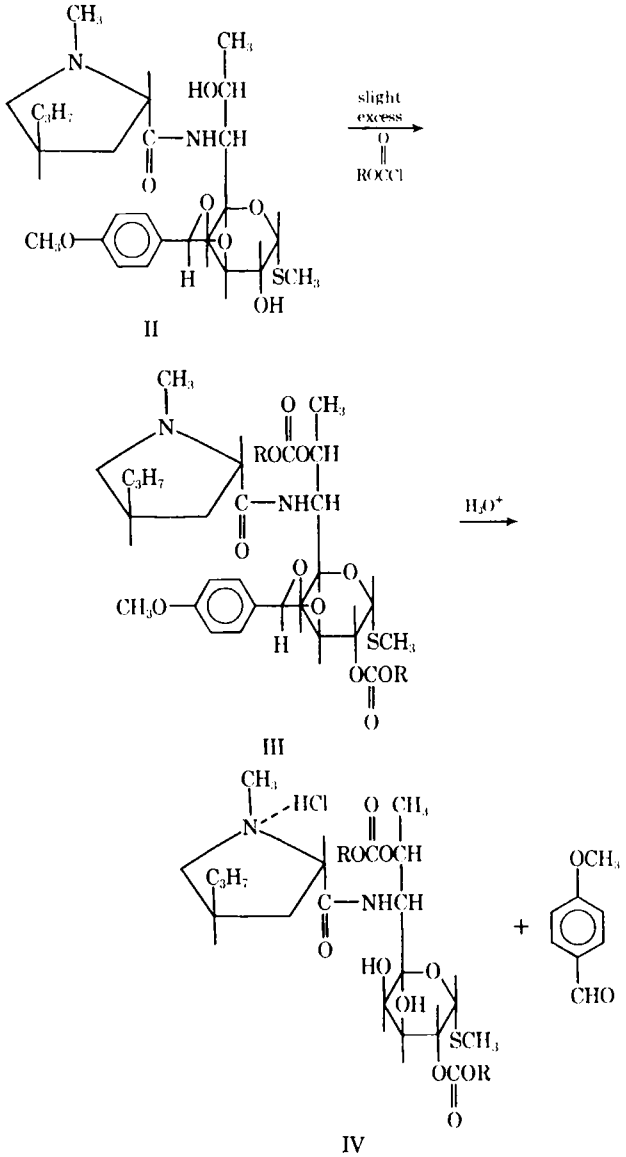


I

lincomycin 2,7-dialkylcarbonate esters sufficiently insoluble in aqueous media to warrant acceptability as candidates for a pleasant-tasting suspension of lincomycin for pediatric use.

RESULTS AND DISCUSSION

Chemistry—Lincomycin 2,7-dialkylcarbonate esters were synthesized according to Scheme I. The reaction of 3,4-*O*-(*p*-methoxybenzylidene)lincomycin (II) (3) with a slight excess (0.005–0.06 mole) of alkyl chloroformate afforded the desired 3,4-*O*-(*p*-methoxybenzylidene)lincomycin 2,7-dialkylcarbonate ester (III). Acidic hydrolysis of III produced lincomycin 2,7-dialkylcarbonate hydrochloride (IV) and anisaldehyde.



II

III

IV

Scheme I

Reaction temperature played an important role in the synthesis of III and IV. When the reaction mixture was cooled to 0° prior to addition of alkyl chloroformate, a pyridine–alkyl chloroformate complex precipitated which effectively prevented the formation of III. This situation prevailed even when the reaction mixture was recooled and more alkyl chloroformate was added. The problem was circumvented by cooling the reaction medium to −35° prior to addition of alkyl chloroformate. Subsequent slow warming of the reaction mixture to room temperature allowed formation of III exclusively.

When the formation of III was complete, the reaction mixture was poured into ice water and the pH was adjusted to 2–3 with concentrated hydrochloric acid. The resulting suspension was extracted with ether. Removal of the ether produced a yellow gum, which was dissolved in aqueous methanol acidified to pH 1–2. Hydrolysis of the *p*-methoxybenzylidene moiety proceeded smoothly. After hydrolysis was completed, the pH of the solution was adjusted to 4 with sodium bicarbonate. The solvent was removed *in vacuo* and the residue was extracted with ether. All derivatives were purified by recrystallization. Synthetic details are outlined in the *Experimental* section.

Bioactivity—*In Vitro*—The derivatives were all intrinsically inactive *in vitro* when tested by both the broth dilution and standard curve assay techniques (Table I). The methodology for determining *in vitro* bioactivity was described previously (3). In these assays, pure lincomycin base was assigned a potency of 1000 mcg./mg.

In Vivo—The antibacterial activities of the derivatives, as established in mice experimentally infected with *Staphylococcus aureus* (UC 76), are listed in Table II. All results were calculated as lincomycin base activity and are reported as the median protective dose

Table I—*In Vitro* Antibacterial Activity of Lincomycin 2,7-Dialkylcarbonate Derivatives by Broth Dilution and Standard Curve Assays[a]

Lincomycin Ester Hydrochloride	Activity Compared to Lincomycin Hydrochloride, MIC, mcg./mg.[b] Broth Dilution	Standard Curve
2,7-Dimethyl	<10 (<1)	1 (0.1)
2,7-Diethyl	<10 (<1)	1 (0.1)
2,7-Di-*n*-propyl	<10 (<1)	4 (0.4)
2,7-Di-*n*-butyl	<10 (<1)	5 (0.5)
2,7-Diisobutyl	<10 (<1)	1 (0.1)
2,7-Di-*n*-pentyl	<10 (<1)	5 (0.5)
2,7-Di-*n*-hexyl	<10 (<1)	6 (0.6)
2,7-Dicyclohexyl	~10 (1)	1 (0.1)
2,7-Dicyclohexylmethyl	~10 (1)	1 (0.1)

[a] Results expressed as micrograms of lincomycin base activity per milligram of ester and as percent of lincomycin base activity (in parentheses). [b] Pure lincomycin base = 1000 mcg./mg. MIC = minimal inhibitory concentration.

Table II—*In Vivo* Bioactivity of Lincomycin 2,7-Dialkylcarbonates[a] in Mice Experimentally Infected with *Staphylococcus aureus*

Lincomycin Ester Hydrochloride	Relative Median Protective Dose (CD_{50})[b] Subcutaneous	Oral
2,7-Dimethyl	0.53	0.59
2,7-Diethyl	0.96	0.86
2,7-Di-*n*-propyl	0.80	0.50
2,7-Di-*n*-butyl	1.04	0.65
2,7-Diisobutyl	0.75	0.40
2,7-Di-*n*-pentyl	1.66	0.51
2,7-Di-*n*-hexyl	2.35	0.83
2,7-Dicyclohexyl	0.68	0.04
2,7-Dicyclohexylmethyl	0.40	0.04

[a] Activities calculated as lincomycin base equivalents. [b] Median protective dose relative to that of lincomycin (lincomycin = 1.0). Mice challenged with approximately 100 lethal doses of *S. aureus*.

Table III—Hydrolysis of Lincomycin 2,7-Dialkylcarbonates in Serum from Various Animal Species

Serum Source	Di-carbonate Derivative	Lincomycin Activity[a], mcg./ml. 0 hr.	3 hr.	6 hr.	24 hr.
Mouse	Dipentyl	6.0	7.0	6.5	5.9
	Dihexyl	5.2	5.6	7.0	5.9
Rat	Dipentyl	3.3	5.1	5.5	6.0
	Dihexyl	3.8	5.1	5.5	6.0
Gerbil	Dipentyl	4.0	3.1	4.7	5.2
	Dihexyl	3.1	5.2	4.0	5.2
Rabbit	Dipentyl	0	6.5	5.8	6.4
	Dihexyl	0	3.0	4.5	4.8
Dog	Dipentyl	0	0	0	0
	Dihexyl	0	0	0	0
Monkey	Dipentyl	0	0	~0.32	1.8
	Dihexyl	0	0	0	1.0
Human	Dipentyl	0	0	0	~0.32
	Dihexyl	0	0	0	0
pH 7.0 buffer, no serum	Dipentyl	0	0	0	0
	Dihexyl	0	0	0	0

[a] Expressed as the MIC at the time of sampling. Limit of assay = 0.32 mcg./ml.; *i.e.*, 0 value = <0.32 mcg./ml. Original sample contained a theoretical 7-mcg./ml. (base equivalent) aliquot before hydrolysis.

(CD_{50}) in milligrams per kilogram per day. Also, a comparison to lincomycin was made with each determination, and the activity of the ester was expressed as the ratio between the bioactivity of lincomycin (considered as 1) and that of the derivative. Median protective doses (CD_{50}'s) observed with the subcutaneous route of administration demonstrated that the derivatives were absorbed and hydrolyzed readily by the mouse. Orally, good CD_{50} bioactivities were shown with the dimethyl, diethyl, di-*n*-butyl, and dihexyl esters. The di-*n*-propyl, diisobutyl, and dipentyl esters did not protect the infected animals as well as did lincomycin. The dicyclohexyl and dicyclohexylmethyl esters afforded virtually no protection to the infected animals.

Several diester derivatives were subjected to *in vitro* hydrolysis using serum from various animal species. They were tested at 10 mcg./ml. (about 7 mcg./ml. lincomycin base equivalent, depending on the derivative) at 37° in 50% fresh serum diluted in pH 7.0, 0.05 *M* tromethamine buffer. Samples were taken for testing at various times over 24 hr. At the end of each sampling time, the enzymes present in the serum were inactivated by immersing the sample in boiling water for 10 min. All derivatives were stable to boiling at pH 7.0 for greater than 20 min., and no derivative was hydrolyzed at 37° in pH 7 tromethamine buffer in 24 hr. In those instances where serum hydrolyzed the derivative, boiled serum was inactive. Samples were assayed for lincomycin bioactivity with the *Sarcina lutea*-sensitive assay (4).

Table III lists results with the dipentyl and dihexyl derivatives of lincomycin in mouse, rat, gerbil, monkey, dog, rabbit, and human serum. The striking activity of rodent serum is obvious. In these tests, the zero-time sample approximated a 5-min. sample, and all rodent serum exhibited hydrolytic activity in this short time.

Table IV summarizes the results from experiments in which the diethyl, dipropyl, di-*n*-butyl, dicyclohexyl, and dicyclohexylmethyl derivatives were exposed to the hydrolytic action of rat and human serum. Again, the high esterase activity for these derivatives was apparent in rat serum. Very little, if any, activity was exhibited by human serum.

EXPERIMENTAL

Typical reaction conditions are illustrated for the synthesis of lincomycin 2,7-dialkylcarbonate esters. Analytical data are listed in Table V.

Lincomycin 2,7-Dimethylcarbonate Hydrochloride—Anisylidene lincomycin base (II) (21 g., 0.04 mole) was dissolved in 200 ml. of pyridine and placed in a 500-ml. round-bottom flask fitted with an overhead paddle stirrer, a calcium chloride drying tube, and a dropping funnel. The solution was cooled to −35° using a dry ice–acetone bath. Methyl chloroformate (8 g., 0.08 mole) was added dropwise with stirring. Upon completion of addition, the reaction flask was slowly warmed to room temperature. TLC [silica gel[1] and a solvent system of hexane–ether–pentan-2-one–methanol–concentrated ammonium hydroxide (60:20:20:9:1)] indicated formation of predominately a lincomycin monomethylcarbonate along with some lincomycin dimethylcarbonate. The reaction mixture was again cooled to −35° and an additional 10 g. of methyl chloroformate was added dropwise. A second thin-layer chromatogram indicated that the reaction was not complete. The cooling procedure was again repeated and 15 g. more of methyl chloroformate was added. The reaction was again slowly warmed to room temperature. TLC showed that reaction was essentially complete.

Table IV—Hydrolysis of Lincomycin 2,7-Dialkylcarbonates by Human and Rat Serum

Serum Source	Dicarbonate Derivative	Lincomycin Activity, mcg./ml.[a] 0 hr.	3 hr.	24 hr.
Rat	Diethyl	6.4	7.0	8.4
	Dipropyl	6.0	7.5	7.0
	Di-*n*-butyl	7.5	8.2	7.5
	Dicyclohexal	1.1	4.5	4.6
	Dicyclohexylmethyl	5.4	8.2	7.5
Human	Diethyl	0	0	1.1
	Dipropyl	0	0	1.2
	Di-*n*-butyl	0	0	0.8
	Dicyclohexyl	0	0	0
	Dicyclohexylmethyl	0	0	0

[a] Activity expressed as the MIC in micrograms per milliliter. Limit of assay = 0.32 mcg./ml.; *i.e.*, 0 value = <0.32 mcg./ml. Original sample contained a theoretical ~7-mcg./ml. (base equivalent) aliquot before hydrolysis.

The mixture was poured into 1500 ml. of ice water which had been acidified to pH 2 with hydrochloric acid. About 1500 ml. of ether was used to extract the reaction product from water. Two hundred milliliters of ethanol was also added to ensure complete solubility of the precipitate in ether. The organic layer was evaporated on a steam bath. The resulting residue was dissolved in 100 ml. of acetone, and ether was added to the cloud point. The anisaldehyde which separated was decanted, and the ether–acetone solution was allowed to stand at room temperature for 2 days. The crystals that formed were collected and washed with acetone and then ether and air dried.

Lincomycin 2,7-Di-*n*-propylcarbonate Hydrochloride—Anisylidene lincomycin base (II) (31.5 g., 0.06 mole) was dissolved in 350 ml. of analytical reagent grade pyridine. The solution was cooled, with stirring, to −35° in a dry ice–acetone bath. *n*-Propyl chloroformate (22.06 g., 0.18 mole) was added dropwise from a dropping funnel to the cooled pyridine solution. The temperature of the reaction mixture was kept at −30 to −35° until all of the chloroformate had been added.

The resulting clear solution was stirred for 2 hr. at room temperature. TLC showed that reaction was complete. The reaction mixture was then poured into 2 l. of ice water, and the pH was adjusted to 3 with concentrated hydrochloric acid. The resulting aqueous suspension was extracted with two 500-ml. portions of ether, the ether layer was washed with 300 ml. of water and dried with anhydrous magnesium sulfate, and the ether was removed on a steam bath. A light-yellow syrup (47 g.) was obtained.

The syrup was dissolved in a mixture of 300 ml. of absolute methanol and 100 ml. of water and the pH of the solution was adjusted to 0.9 with concentrated hydrochloric acid. After 2 hr., sodium bicarbonate solution was added to raise the pH of the solution to 3.2. The methanol was removed under vacuum, and the resulting aqueous syrup was extracted with 100 ml. of ether–chloroform (1:1). The ether–chloroform layer was dried with magnesium sulfate, and the solvent was removed by warming on a steam bath. The resulting syrup was dissolved in 200 ml. of chloroform, and anhydrous acetone was added to the cloud point; this mixture was allowed to stand overnight in a refrigerator, resulting in crystallization of the product.

Lincomycin 2,7-Dicyclohexylcarbonate Hydrochloride: Cyclohexyl Chloroformate To 85 g. of liquid phosgene in a dry ice–

[1] Silica gel GF Uniplate, Analtech, Inc., Newark, Del.

Table V—Analytical Data: Lincomycin 2,7-Dialkylcarbonates

Lincomycin Ester Hydrochloride	Empirical Formula	Molecular Weight	Analysis (Corrected for Water Content), %— Calc.	Found	Melting Point[a]	Yield, %
2,7-Dimethyl	$C_{32}H_{39}ClN_2O_{10}S$	559.08	C 47.26	46.72	187–189°	15
			H 7.03	7.25		
			Cl 6.34	6.19		
			N 5.01	5.68		
2,7-Diethyl	$C_{34}H_{43}ClN_2O_{10}S$	587.13	C 49.10	49.43	196–197°	20
			H 7.38	7.51		
			Cl 6.04	6.18		
			N 4.77	4.71		
2,7-Di-*n*-propyl	$C_{36}H_{47}ClN_2O_{10}S$	615.17	C 50.76	50.58	202–203°	78
			H 7.70	7.99		
			Cl 5.76	5.74		
			N 4.55	4.47		
2,7-Di-*n*-butyl	$C_{38}H_{51}ClN_2O_{10}S$	643.24	C 52.28	52.36	204–205°	35
			H 7.99	8.07		
			Cl 5.51	5.65		
			N 4.36	4.58		
2,7-Diisobutyl	$C_{38}H_{51}ClN_2O_{10}S$	643.24	C 52.28	51.95	213–214°	30
			H 7.99	8.16		
			Cl 5.51	5.50		
			N 4.36	4.56		
2,7-Di-*n*-pentyl	$C_{40}H_{55}ClN_2O_{10}S$	671.28	C 53.68	53.43	221–223° dec.	75
			H 8.26	8.33		
			Cl 5.28	5.36		
			N 4.17	4.20		
2,7-Di-*n*-hexyl	$C_{42}H_{59}ClN_2O_{10}S$	699.35	C 54.96	55.26	218–219° dec.	74
			H 8.50	8.84		
			Cl 5.07	5.24		
			N 4.01	4.18		
2,7-Dicyclohexyl	$C_{42}H_{55}ClN_2O_{10}S$	695.32	C 55.28	54.62	185–186°	41
			H 7.97	7.87		
			Cl 5.10	5.05		
			N 4.03	4.21		
2,7-Dicyclohexylmethyl	$C_{44}H_{59}ClN_2O_{10}S$	723.37	C 56.45	56.10	223–226° dec.	69
			H 8.22	8.16		
			Cl 4.90	4.93		
			N 3.87	4.25		
3,4-*O*-(*p*-Methoxybenzylidene)-lincomycin	$C_{26}H_{40}N_2O_7S$	524.67	C 59.52	59.60	133–135°	75
			H 7.68	7.88		
			N 5.34	5.48		

[a] Melting points are of hydrated samples and were determined on a Thomas-Hoover melting-point apparatus and are uncorrected.

acetone bath was added, dropwise with stirring, 100 g. (1 mole) of cyclohexanol. The temperature of the reaction was maintained at about −20°. Upon completion of the cyclohexanol addition, the reaction was slowly warmed to room temperature. During warming, the mixture was stirred well to remove excess phosgene. After bubbling ceased, the reaction was connected to a water aspirator and the last traces of phosgene and hydrogen chloride were removed. The mixture was then aerated for 20 min. with nitrogen. The chloroformate was distilled at 27–31°/100 μ [lit. (6) 46–47°/2–3 mm.].

Anisylidene lincomycin base (II) (31.5 g., 0.06 mole) was dissolved in 350 ml. of analytical reagent grade pyridine and cooled to −35°. Cyclohexyl chloroformate (29.3 g., 0.18 mole) was added dropwise to this cooled solution. As the solution was slowly warmed, a precipitate formed. This precipitate was filtered, and 15 g. of cyclohexyl chloroformate was added to the recooled reaction mixture. The solution turned deep red as it warmed to room temperature. The mixture was stirred for 12 hr., after which time ice water was added and the mixture was acidified to pH 3 with concentrated hydrochloric acid. The resulting gum was dissolved in 1000 ml. of ether and dried with anhydrous magnesium sulfate. Removal of the solvent resulted in a reddish-brown gum, which was dissolved in 300 ml. of absolute methanol. Then 100 ml. of water was added to this solution, and the pH was adjusted to 0.8 with concentrated hydrochloric acid. The solution was stirred for 2 hr. Removal of methanol under reduced pressure afforded an aqueous suspension, which was extracted with 150 ml. of chloroform. The chloroform layer was dried with anhydrous magnesium sulfate and the solvent was removed. The resulting syrup was allowed to stand in a refrigerator overnight, and the crystals were collected on a filter and washed with ether. The crystals were finely powdered, suspended in 250 ml. of acetone ether (1:4), filtered, and dried.

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GLC Determination of Phenylephrine Hydrochloride in Human Plasma

LAWRENCE J. DOMBROWSKI▲, PATRICK M. COMI, and EDWARD L. PRATT

Abstract □ A sensitive GLC method for the determination of phenylephrine in human plasma is described. Its application to *in vivo* phenylephrine levels in humans is currently being investigated. In the procedure, plasma components that interfere with the assay were removed using ion-exchange chromatography. Derivatization of the isolated phenylephrine hydrochloride with trifluoroacetic anhydride produced a sharp symmetrical peak when chromatography was carried out using a QF-1 column and monitored with an electron-capture detector. The method, when applied to plasma containing known amounts of added phenylephrine hydrochloride (12.5–50.0 ng./ml.) gave an average recovery of 80%. The standard deviation based on 15 determinations was 10%.

Keyphrases □ Phenylephrine hydrochloride—GLC using electron-capture detection, human plasma □ GLC, electron-capture detection—analysis, phenylephrine hydrochloride in human plasma

Phenylephrine hydrochloride {(−)-*m*-hydroxy-α-[(methylamino)methyl]benzyl alcohol hydrochloride}, a well-recognized sympathomimetic amine, has been in commercial use for many years. It is a vasoconstrictor and pressor drug chemically related to epinephrine and ephedrine. It is used in surgery, ophthalmoscopic examination, and diagnostic procedures and also has been employed as a nasal decongestant. However, a sufficiently sensitive chemical method has been unavailable for measuring this drug in man.

Various assay methods involving spectrophotofluorometry, UV spectroscopy, and colorimetry have been used to measure phenylephrine in its many available pharmaceutical preparations (1–10). These procedures permit measurement of milligram or microgram quantities of phenylephrine. They do not respond with the sensitivity required to measure phenylephrine in biological systems that may contain <1 p.p.m. of drug.

Two *in vivo* studies in humans were reported using tritium-labeled phenylephrine (11, 12). The tagged drug was administered orally in a normal dosage form as the hydrochloride salt and in a timed-release preparation as a protocolloid tannate complex. These studies provided valuable tritium blood concentration–time profiles using a liquid scintillation counting technique. However, the nonspecificity of the measuring system did not permit any evaluation of the intact phenylephrine concentration. The primary purpose of this work was to develop a sensitive and specific assay procedure for measuring unchanged phenylephrine in human plasma.

GLC previously was used for the determination of other sympathomimetic amines. After derivatization, the amines were chromatographed to produce narrow and symmetrical peaks (13). Acylated halogenated derivatives, particularly the fully trifluoroacylated phenolic amines, were readily measured at the nanogram level with an electron-capture detector. The high detection sensitivity of the trifluoroacetate derivatives formed in the analysis of dopamine (14) and 3-methoxy-4-hydroxyphenylethylene glycol (15) suggested a similar approach for the determination of phenylephrine. In this laboratory the treatment of phenylephrine hydrochloride with an excess of trifluoroacetic anhydride produced a single characteristic peak when chromatographed on a 1.82-m. (6-ft.) column of 3% QF-1. This process served as a basis for developing a sensitive assay procedure. The developed method permitted determination of 25 ng. phenylephrine hydrochloride/2 ml. plasma. Prior to derivative preparation, it was necessary to use a cation-exchange column for removal of plasma components that interfered with the GLC measurement. Details of the method are presented here along with results of a phenylephrine recovery study.

EXPERIMENTAL

Instrumentation—Final measurements were made with a gas chromatograph[1] equipped with an electron-capture detector. Adequate separations were realized on a 1.82-m. (6-ft.) × 2-mm. i.d. glass column packed with 3% QF-1 coated on Gas Chrom Q, 100–120 mesh. The column temperature was maintained at 150°, and the helium flow rate was adjusted to 50 ml./min. The detector and inlet temperatures were kept 40° higher than the column temperature. The meter range of the instrument was set at 1×10^{-9} amp., and the high voltage was adjusted to 50 v. All derivatized samples were diluted with redistilled ethyl acetate, and a 5-μl. aliquot was applied to the column.

Reagents—Cation-exchange resin[2], 50–100 mesh, was purified and conditioned as follows. Mix 30 g. resin with 100 ml. 6 *M* hydrochloric acid in a 250-ml. beaker. Repeat washes six times, discarding each acid wash. Wash the resin free of acid, using distilled water, and store the resin in distilled water until needed. The glass column containing the resin was described previously (16). The height of the resin in the column was between 4 and 5 cm.

Diatomaceous earth[3] was purified by a standard procedure (17).

Trifluoroacetic anhydride[4] was redistilled. The middle third fraction was collected in a suitable vessel, placed in a desiccator, and refrigerated at 5° until ready for use. Under these pretreatment and storage conditions, the reagent was stable for several weeks.

Ethyl acetate (reagent grade) was redistilled and the middle third fraction was collected.

Standard chromatographic tubes (25 × 200 mm.) were used.

Assay for Plasma—Transfer 1–2 ml. of plasma to a 50-ml. beaker. Adjust the volume to 4 ml. with distilled water and add, with thorough mixing, 1 g. each of sodium carbonate and sodium chloride. Blend the solution with 4 g. diatomaceous earth and transfer the homogeneous mixture to a standard chromatographic tube containing a glass wool plug in its outlet stem. Pack firmly by tapping with a glass rod and then place a glass wool plug on top of the packed column. Position the chromatographic tube directly above a 50-ml. glass-stoppered centrifuge tube containing 4 ml. of 0.1 *M* hydrochloric acid.

Pass 44 ml. of water-washed ethyl acetate through the column and collect the effluent in the centrifuge tube. Shake the stoppered tube for 3 min., centrifuge, and then withdraw and reserve the aqueous acid phase. Reextract the ethyl acetate with 4 ml. of 0.1 *M* hydro-

[1] F & M model 400.
[2] Dowex 50W × 1, J. T. Baker.
[3] Celite 545, Johns-Manville Corp.
[4] Matheson.

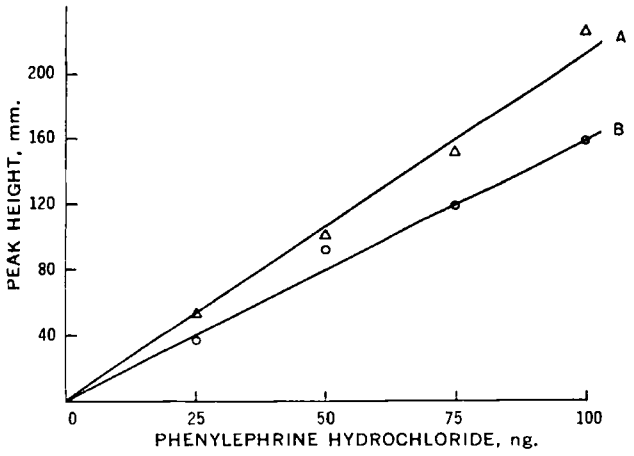


Figure 1—*Response curves for standard phenylephrine hydrochloride (curve A) and for plasma samples containing added amounts of phenylephrine hydrochloride (curve B).*

chloric acid and pool the acid extracts. Transfer the acid pool to a column containing a prepared cation-exchange resin and adjust the flow rate to 1–2 ml./min. Wash the column with three 3-ml. volumes of 0.1 *M* hydrochloric acid after the sample extracts completely pass into the column. Terminate flow from the column after the last wash just passes into the resin. Discard the effluent volume.

Position the column above a 10-ml. graduated cylinder and then elute phenylephrine with two 3-ml. volumes of 1 *M* hydrochloric acid. Measure the volume in the cylinder (about 6.3 ml.) and then transfer 2.0 ml. to a 5-ml. beaker. With a slight displacing current of nitrogen and a steam bath, evaporate the contents of the beaker to dryness. Cool the beaker to room temperature and then add 0.2 ml. trifluoroacetic anhydride. Seal the beaker with aluminum foil and place in an oven at 34 ± 1° for exactly 10 min. Remove the beaker to a well-ventilated hood at room temperature. Remove foil, permitting excess trifluoroacetic anhydride to evaporate spontaneously and completely. Dissolve the remaining residue in exactly 0.4 ml. (dry) ethyl acetate. Inject 5 μl. into the gas chromatograph readied for use as described in the *Instrumentation* section. Calculate the peak height measured at the retention time previously established for the acylated phenylephrine derivative.

Assay precision was considerably improved when two 2-ml. aliquots of the 1 *M* hydrochloric acid effluent were evaporated to dryness, the residues were derivatized, and the measured peak heights were averaged.

Preparation of Standard Calibration Curve—Dissolve phenylephrine hydrochloride reference standard in 0.1 *M* hydrochloric acid and dilute with 0.1 *M* hydrochloric acid to provide a solution containing 20 ng./ml. In duplicate, transfer 1.0, 2.0, 2.5, and 3.5 ml. of the standard solution to respective 5-ml. beakers corresponding to 20, 40, 50, and 70 ng. phenylephrine hydrochloride, respectively. Evaporate to dryness the contents of all eight beakers. Add 0.2 ml. trifluoroacetic anhydride to each standard and then continue with the derivatization procedure as directed previously. After removing excess reagent, dissolve the residue with 1.3 ml. (dry) ethyl acetate and introduce a 5-μl. volume into the gas chromatograph. Construct a calibration curve from averaged measured peak height for each standard concentration.

RESULTS AND DISCUSSION

The reaction of trifluoroacetic anhydride with phenylephrine hydrochloride led to the formation of a single derivative, having a retention time of approximately 3.6 min. The derivative is presumed to be that of the fully trifluoroacetylated molecule.

When aliquots of plasma containing increasing amounts of added phenylephrine hydrochloride were carried through the entire assay procedure, the phenylephrine derivative peak height increased in proportion to the amount of drug added (Fig. 1). Processing 2 ml. plasma (blank) through the entire procedure did not give any peaks that interfered with the assay. Figure 2 shows chromatograms resulting from processing 2 ml. plasma and 2 ml. plasma spiked with 25 ng. phenylephrine hydrochloride. The spiked sample peak at this

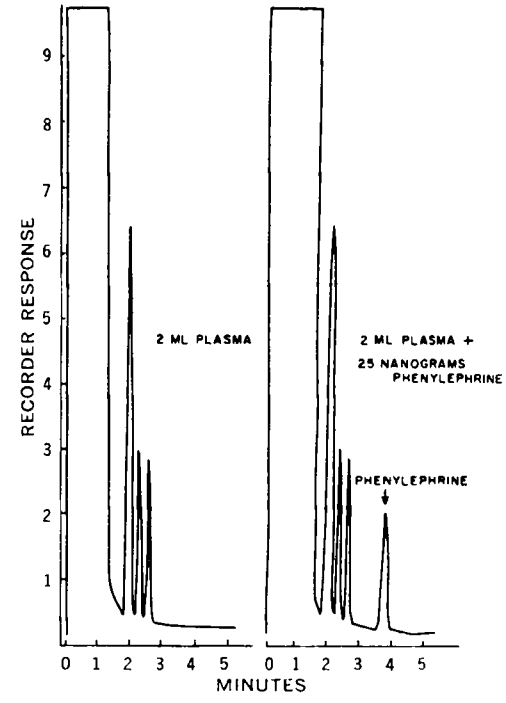


Figure 2—*Chromatogram of 2-ml. plasma sample compared to a parallel sample containing 25 ng. of phenylephrine hydrochloride.*

concentration is readily evident when compared to the blank. When the instrument was set at maximum sensitivity, the phenylephrine peak was always measured on either a positive or a negative sloping baseline. Standards were not carried through the complete assay procedure as described for the plasma samples. Nanogram quantities of standard phenylephrine hydrochloride were subjected only to reaction with trifluoroacetic anhydride and then chromatographed. A standard curve constructed in this manner (Fig. 1) was used to compute the phenylephrine recoveries from plasma spikes. Results conducted on 15 plasma samples containing known amounts of added phenylephrine are shown in Table I. Overall reproducibility was ±10% and the average recovery was 80%. Repeated injections of 5 μl. of a standard yielded no variable greater than 1.5% and demonstrated that the column did not require priming.

An internal standard, although not employed in this study, may result in improved assay precision for measuring phenylephrine in plasma. A suitable choice might be metanephrine. This particular compound is structurally similar to phenylephrine and produced a similar detector response when derivatized with trifluoroacetic anhydride and chromatographed as directed for phenylephrine. The retention time for the metanephrine derivative was about 7.5 min.

Diatomaceous earth column chromatography, providing 80% recovery, was the most efficient process of those considered for the initial separation of phenylephrine base from the basified plasma solution. For example, two 20-ml. ethyl acetate extractions of the basified plasma solution yielded only a 50–55% recovery of added phenylephrine.

The phenylephrine base contained in the ethyl acetate effluent was extracted quantitatively with two 4-ml. volumes of 0.1 *M* hydrochloric acid. The complete retention of phenylephrine from these acid extracts on the cation-exchange resin was readily attained. Interfering plasma components were removed by washing the resin column with 0.1 *M* hydrochloric acid. Phenylephrine then was quantitatively eluted from the resin column using stronger acid. This was readily accomplished using 6 ml. of 1 *M* hydrochloric acid.

Previous studies involving acylation of primary and secondary amines generally were conducted using the amine in the form of its free base (18–20). In this laboratory, an occasional difficulty was encountered when isolating nanogram quantities of some amine bases, particularly when extracting them from biological systems. Studies indicated that the amines were extracted readily with an organic solvent, but some losses occurred in attempting to concentrate them by solvent evaporation. The problem was thought to be caused by both amine volatility and air oxidation of the base. To

Table I—Precision and Accuracy in Measurement of Phenylephrine Hydrochloride Added to 2 ml. Human Plasma

Number of Samples	Phenylephrine Hydrochloride Added, ng.	Phenylephrine Hydrochloride Found[a], ng.	*RSD*, %
4	25	19	±11
4	50	42	±7
3	75	57	±10
4	100	78	±11

[a] Mean.

avoid any phenylephrine losses in this study, it was decided to concentrate the drug from plasma and acylate it as its hydrochloride salt. This was readily accomplished after eluting phenylephrine off the resin and evaporating to dryness the 1 *M* hydrochloric acid effluent. Measurable losses of nanogram quantities of phenylephrine were not realized when the salt residue was subjected to excess heating at 60°. In fact, excess heating is recommended in the procedure to ensure complete removal of any residual amounts of the 1 *M* hydrochloric acid which, if present, seriously interfere with the subsequent derivatization reaction.

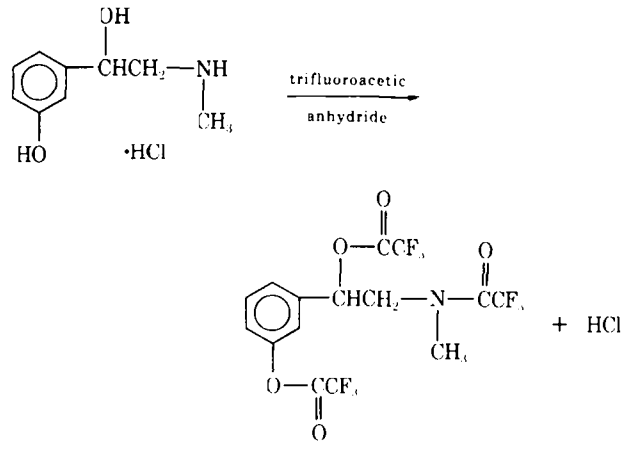
The best conditions found for acylating phenylephrine hydrochloride were 10 min. and 34°. This treatment permitted phenylephrine measurement at maximum sensitivity and reproducibility. Slight changes in the acylating conditions adversely affect the phenylephrine derivatization process and, hence, the reproducibility of the method. For instance, reaction times beyond 10 min. produced erratic results and shorter times yielded responses indicative of incomplete acylation. Furthermore, sensitivity was reduced if the reaction temperature was lowered to 25°. Because of the sensitive nature of the reaction, all samples and standards involved in a single run were derivatized simultaneously and subjected to identical conditions with respect to time and temperature. A fresh set of standards was prepared for each new series of plasma samples assayed.

A problem associated with the acylation of amine salts is the liberation of an equivalent amount of acid. With phenylephrine hydrochloride, this can be represented as shown in Scheme I. The released hydrochloric acid, if not removed, can hydrolyze the ester or amide group of the fully acylated molecule, thereby resulting in a loss of phenylephrine detection sensitivity. If a large excess of trifluoroacetic anhydride were employed, the acylation of phenylephrine was more complete and interferences from the liberated hydrochloric acid were negligible. Both the excess reagent and the liberated hydrochloric acid were effectively removed by spontaneously evaporating the solutions to dryness, preferably in a low humidity environment. The reagent is volatile (b.p. 39°) and the evaporation was complete within a few minutes at ambient temperature. The use of heat or a stream of forced nitrogen to aid the evaporation process should be avoided since both of these procedures result in a loss of the acylated derivative. Nanogram quantities of the derivative in dry ethyl acetate were stable for at least 24 hr.

CONCLUSION

A method was presented for measuring intact phenylephrine in plasma. The lowest amount of the drug measured in this study was 25 ng./2 ml. body fluid (Fig. 2). If desired, this sensitivity can be improved. For example, instead of employing a 2-ml. aliquot resulting from the 6.3 ml. of 1 *M* hydrochloric acid effluent, the entire 6.3-ml. volume could be used for the assay, producing a threefold increase in sensitivity.

This assay method has not been applied to an actual *in vivo* study of phenylephrine in humans. It is our contention, however, that the proposed analytical procedure, because of its sensitivity, provides



Scheme I

the best approach of those methods currently available for investigating phenylephrine blood levels in man following medication.

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Alkylation Reactions of Hydantoins and Succinimides

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Abstract □ Five anticonvulsant agents, namely, diphenylhydantoin, mephenytoin, ethotoin, ethosuximide, and phensuximide, and a number of other hydantoins were investigated in their reactions with *p*- and *m*-nitrobenzyl bromides in the presence of sodium carbonate. The structures of the alkylation derivatives and other products formed in the reactions were studied. On the basis of the melting points of the alkylation derivatives, it was possible to identify and differentiate the five anticonvulsants. 3-*p*-Nitrobenzyl-5,5-diphenylhydantoin, *N*-*p*-nitrobenzyl-2-ethyl-2-methylsuccinimide, and 3,3′-diethyl-5,5′-diphenylhydantil, at a dose of 1 g./kg. given orally, provided no protection against pentylenetetrazol-induced seizures in mice.

Keyphrases □ Hydantoins and succinimides—alkylation with nitrobenzyl bromides, anticonvulsant properties of products □ Succinimides and hydantoins—alkylation with nitrobenzyl bromides, anticonvulsant properties of products □ Anticonvulsants - alkylation reactions of hydantoins and succinimides □ Alkylation reactions—hydantoins and succinimides

Many substituted hydantoins and succinimides are anticonvulsants. For identification purposes, some of these chemical compounds, namely, diphenylhydantoin (I), mephenytoin (II), ethotoin (III), ethosuximide (IV), and phensuximide (V), were treated with nitrobenzyl bromides in the presence of a weak base to form crystalline alkyl derivatives. There are several possible structures available for these alkyl derivatives, because alkylation could occur at more than one site in the hydantoin and succinimide molecules. This study reports on the structures of the alkylation derivatives and other products formed in the reactions.

EXPERIMENTAL[1]

5-Phenylhydantoin—This compound was obtained from benzaldehyde, ammonium carbonate, and potassium cyanide *via* the Bucherer–Berg reaction. The yield was 86%, m.p. 180.5–182° [lit. (1) m.p. 179°].

5-Ethyl-5-phenylhydantoin—This compound was obtained from propiophenone, ammonium carbonate, and potassium cyanide *via* the Bucherer–Berg reaction. The yield was 69%, m.p. 199° [lit. (2) m.p. 199°].

3-Methyl-5,5-diphenylhydantoin—The procedure reported by Dudley and Bius (3) was followed. The yield was almost quantitative, m.p. 214–215.5° [lit. (4) m.p. 213–214°].

General Procedure for Alkylation Reactions—A solution of the alkylating agent (0.01 mole) in 60 ml. of ethanol was added to a mixture of a hydantoin or succinimide (0.01 mole), sodium carbonate (0.02 mole), and 30 ml. of water. The reaction mixture was stirred under reflux for 1.5 hr. and cooled. The alkylated products were then isolated by one of the following three methods. The physical constants for the alkylated products prepared together with the recrystallization solvent are summarized in Tables I and II.

Method A—The material that separated from the reaction mixture after cooling was collected by filtration and washed thoroughly with water to yield the crude product. Alkyl derivatives isolated by this method were 3-*p*-nitrobenzyl-5,5-diphenylhydantoin (VI), 3-*m*-nitrobenzyl-5,5-diphenylhydantoin (VII), 3-*p*-nitrobenzyl-5-ethyl-5-phenylhydantoin (VIII), and 3,3′-bis(*p*-nitrobenzyl)-5,5′-diphenylhydantil (IX).

Method B—After the cooled reaction mixture was filtered, water was added to the filtrate until cloudiness occurred. On cooling, a solid precipitated which was collected by filtration to give the crude product. Alkyl derivatives obtained by this method were 1-*p*-nitrobenzyl-3-methyl-5-ethyl-5-phenylhydantoin (X), 1-*p*-nitrobenzyl-3-methyl-5,5-diphenylhydantoin (XI), *N*-*p*-nitrobenzyl-2-ethyl-2-methylsuccinimide (XIV), *N*-*m*-nitrobenzyl-2-ethyl-2-methylsuccinimide (XV), and *N*-methyl-2-phenyl-2-*p*-nitrobenzylsuccinimide (XVI).

Method C—After the reaction mixture was filtered, the ethanol in the filtrate was distilled off to leave behind a brownish oily residue in the aqueous solution. The residue was separated and triturated with a small amount of methanol to yield the crude product. Alkyl derivatives obtained by this method were 3-ethyl-5-*p*-nitrobenzyl-5-phenylhydantoin (XII) and 3-ethyl-5-*m*-nitrobenzyl-5-phenylhydantoin (XIII).

The precipitate that separated from the reaction mixture when III was used yielded 2.2 g. (54%) of 3,3′-diethyl-5,5′-diphenylhydantil (XVII), m.p. 328–330° [lit. (3) m.p. 326–328°].

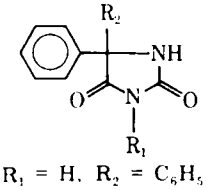
4,4′-Dinitrobibenzyl (XVIII)—This compound was obtained by reacting *p*-nitrobenzyl bromide (0.02 mole) with III (0.01 mole) in the presence of sodium carbonate under the conditions described for alkylation reactions. The crude product was collected from the cooled reaction mixture and washed with water. One recrystallization from ethanol and acetone gave 1 g. (35%) of pure 4,4′-dinitrobibenzyl (XVIII), m.p. 179.5–181° [lit. (5) m.p. 179–180°].

Acute Toxicity—The compound was suspended in 10% aqueous acacia and administered orally to male albino mice (20–30 g.) at a dosage of 2 g./kg. The mice were observed over 3 days.

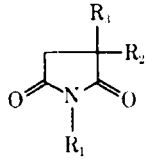
Antipentylenetetrazol Activity—The procedure of Swinyard *et al.* (6) was followed. The amount of pentylenetetrazol used for challenge was 85 mg./kg. and was given subcutaneously 2 hr. prior to the oral administration of the test compound.

RESULTS AND DISCUSSION

Reaction of diphenylhydantoin (I), obtained commercially, with *p*-nitrobenzyl bromide in the presence of sodium carbonate gave a crystalline alkyl derivative in 82% yield. Theoretically, alkylation could occur on the nitrogen atoms of the 1- and 3-positions and on the oxygen atoms of the 2- and 4-positions. Thus, the alkyl derivative of I could be VI, 1-*p*-nitrobenzyl-5,5-diphenylhydantoin (XIX), 2-*p*-nitrobenzoxy-4,4′-diphenyl-2-imidazolin-5-one (XX), 2-*p*-ni-



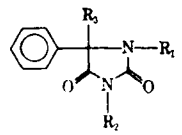
I: $R_1 = H$, $R_2 = C_6H_5$
II: $R_1 = CH_3$, $R_2 = CH_2CH_3$
III: $R_1 = CH_2CH_3$, $R_2 = H$



IV: $R_1 = H$, $R_2 = CH_3$, $R_3 = CH_2CH_3$
V: $R_1 = CH_3$, $R_2 = H$, $R_3 = C_6H_5$

[1] Melting points were determined on a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Elemental analyses were performed by Dr. F. B. Strauss, Microanalytical Laboratory, Oxford, England. The IR spectra were recorded on a Perkin-Elmer model 237B spectrophotometer in potassium bromide. A Varian model T-60 spectrometer was used to record the NMR spectra, with deuterated chloroform as solvent and tetramethylsilane as the internal reference.

Table I—Alkyl Derivatives of Hydantoins

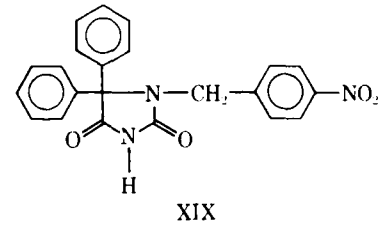


Compound	R_1	R_2	R_3	Yield, %	Melting Point	Recrystallization Solvent	Formula	Analysis, % Calc.	Found
VI	H	p-$NO_2C_6H_4CH_2$	C_6H_5	82	181–183°	Ethanol	$C_{22}H_{17}N_3O_4$	C 68.21	67.92
								H 4.43	4.32
								N 10.85	10.72
VII	H	m-$NO_2C_6H_4CH_2$	C_6H_5	93	191.5–192.5°	Ethanol	$C_{22}H_{17}N_3O_4$	C 68.21	68.16
								H 4.43	4.42
								N 10.85	10.85
VIII	H	p-$NO_2C_6H_4CH_2$	CH_3CH_2	76	175–176°	Acetone–ethanol	$C_{18}H_{17}N_3O_4$	C 63.71	63.82
								H 5.05	5.37
								N 12.38	12.16
IX[a]	—	—	—	32	312–315°	Pyridine	$C_{32}H_{24}N_6O_8$	C 61.93	61.89
								H 3.90	4.00
								N 13.54	13.38
X	p-$NO_2C_6H_4CH_2$	CH_3	CH_3CH_2	34	126–127°	Ethanol–water	$C_{19}H_{19}N_3O_4$	C 64.58	64.21
								H 5.42	5.59
								N 11.89	11.86
XI	p-$NO_2C_6H_4CH_2$	CH_3	C_6H_5	20	194–195.5°	Ethanol–water	$C_{23}H_{19}N_3O_4$	C 68.82	68.72
								H 4.77	4.90
								N 10.47	10.47
XII	H	CH_3CH_2	p-$NO_2C_6H_4CH_2$	9	185.5–187°	Methanol–ether	$C_{18}H_{17}N_3O_4$	C 63.71	63.61
								H 5.05	5.15
								N 12.38	12.12
XIII	H	CH_3CH_2	m-$NO_2C_6H_4CH_2$	9	145–146°	Methanol–ether	$C_{18}H_{17}N_3O_4$	C 63.71	63.88
								H 5.05	5.11
								N 12.38	12.31

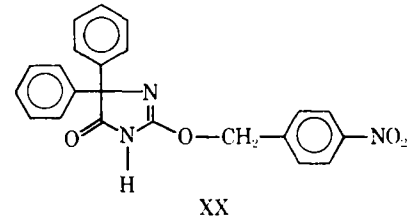
[a] Compound IX is 3,3′-bis(p-nitrobenzyl)-5,5′-diphenylhydantil. See text for structure.

trobenzoxy-5,5-diphenyl-2-imidazolin-4-one (XXI), or 4-p-nitrobenzoxy-5,5-diphenyl-3-imidazolin-2-one (XXII). Chatten and Levi (7), in their study of the reaction of barbiturates with the same alkylating agent and under the same conditions, postulated that alkylation took place on the C-4 and C-6 oxygen atoms of the barbiturate molecule.

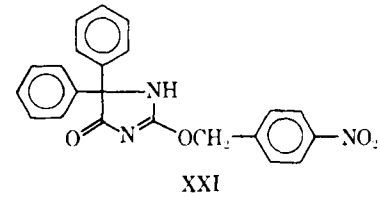
Results of IR studies on I and the reaction product showed that



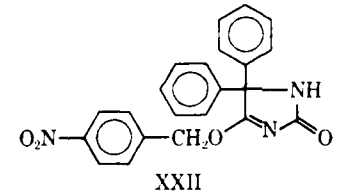
XIX



XX



XXI



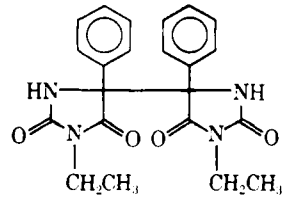
XXII

the oxygen atoms of the 2- and 4-positions were not involved in the alkylation of I. In both IR spectra, two carbonyl peaks were observed at 1770 and 1718 cm.$^{-1}$, indicating that alkylation could not have occurred on the oxygen of the 2- or 4-position (8). Consequently, the alkyl derivative of I could only be VI or XIX.

The NH absorption bands of I and its alkylation product were not well resolved enough in the 3315–3220-cm.$^{-1}$ region to permit a reliable structural assignment to the alkylation product. However, since the UV absorption maximum of the alkyl derivative was not shifted in alkaline solution, and in view of the report by Stuckey (9) that only hydantoins having a hydrogen atom at the 3-position showed a shift in the absorption maximum, it would appear that the alkyl derivative of I was VI and not XIX.

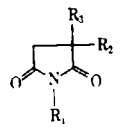
To demonstrate that the nitrogen atom of N-3 in I was indeed the more reactive site for alkylation, 3-methyl-5,5-diphenylhydantoin was treated with p-nitrobenzyl bromide using the same conditions as with I. As expected, the reaction proceeded much more slowly. About 81% of the starting hydantoin was recovered. Only a 20% yield of a crystalline product was isolated, and this product was identified as 1-p-nitrobenzyl-3-methyl-5,5-diphenylhydantoin (XI) by IR and NMR data (Table III). The reaction of I with the same bromide gave no corresponding N-1 alkyl derivative. Examination of the IR spectrum of the parent hydantoin showed that there were one sharp and intense NH band at 3315 cm.$^{-1}$ and two prominent carbonyl bands in the 1775–1690-cm.$^{-1}$ region. The spectrum of XI contained two carbonyl bands and two aromatic nitro bands but no NH band at 3315 cm.$^{-1}$. The disappearance of the NH absorption band indicated that the crystalline reaction product was indeed XI.

Similar results were observed in the reaction of II. There was a 34% yield of the alkylation product 1-p-nitrobenzyl-3-methyl-5-ethyl-5-phenylhydantoin (X), whose IR spectrum contained no NH-



XVII

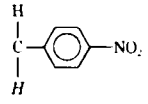
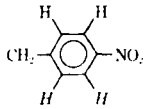
Table II Alkyl Derivatives of Succinimides[a]



Compound	R_1	R_2	R_3	Yield, %	Melting Point	Formula	Analysis, % Calc.	Found
XIV	p-$NO_2C_6H_4CH_2$	CH_3CH_2	CH_3	40	112.5–113.5°	$C_{14}H_{16}N_2O_4$	C 60.86	61.14
							H 5.83	5.80
							N 10.14	10.38
XV	m-$NO_2C_6H_4CH_2$	CH_3CH_2	CH_3	51	65.5–67°	$C_{14}H_{16}N_2O_4$	C 60.86	60.86
							H 5.83	5.85
							N 10.14	9.97
XVI	CH_3	C_6H_5	p-$NO_2C_6H_4CH_2$	31	137.5–139°	$C_{18}H_{16}N_2O_4$	C 66.65	66.54
							H 4.97	5.16
							N 8.64	8.66

[a] The recrystallization solvent for these derivatives was ethanol.

Table III NMR Chemical Shifts[a] in Deuterochloroform

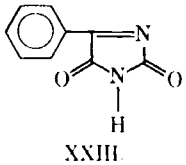
Compound	N—*H*		
VI	7.16 (s)	4.76 (s)	8.14 7.48 (*AB*) J_{AB} = 8 Hz.
VIII	6.56 (s)	4.74 (s)	8.13 7.47 (*AB*) J_{AB} = 8 Hz.
XIV	—	4.71 (s)	8.14 7.45 (*AB*) J_{AB} = 8 Hz.
XII	7.85 (s)	3.62 3.32 (*AB*) J_{AB} = 13 Hz.	8.00 7.28 (*AB*) J_{AB} = 8 Hz.
XVI	—	3.30 3.56 (*AB*) J_{AB} = 13 Hz.	8.05 7.20 (*AB*) J_{AB} = 8 Hz.
X	—	4.62 4.18 (*AB*) J_{AB} = 15 Hz.	8.05 7.32 (*AB*) J_{AB} = 8 Hz.
XI	—	4.70 (s)	7.85 6.86 (*AB*) J_{AB} = 8 Hz.

[a] Parts per million.

stretching absorption. About half of II was recovered unreacted. When 5-ethyl-5-phenylhydantoin was used, the expected N-3 alkylation product, 3-p-nitrobenzyl-5-ethyl-5-phenylhydantoin (VIII), was isolated in 76% yield.

The reaction of III with p-nitrobenzyl bromide in equimolar concentration was complicated by the fact that III dimerized in alkaline solution to form 3,3′-diethyl-5,5′-diphenylhydantil (XVII) in 54% yield. In addition to this dimer, about a 9% yield of the alkylation product 3-ethyl-5-p-nitrobenzyl-5-phenylhydantoin (XII) was also isolated. The IR spectrum of XII showing strong NH, carbonyl, and aromatic nitro absorption bands was in agreement with the assigned structure. The chemical shift of the benzylic methylene group is consistent with its attachment at a carbon rather than a nitrogen atom. Therefore, it seems reasonable that alkylation has occurred at the 5-position and not at the 1-position of III.

Dimerization of similar hydantoin molecules has been reported in the literature. Edward and Nielsen (10) reported that, in aqueous alkali at room temperature, 5-phenylhydantoin absorbed molecular oxygen to give 5,5′-diphenylhydantil. These workers speculated that the reaction probably involved the oxidation of the carbanion of 5-phenylhydantoin to 5-hydroxy-5-phenylhydantoin which, in the form of the pseudobase (XXIII), condensed with a second molecule of 5-phenylhydantoin to yield the product. For this reason, in two separate experiments the reaction of III with the nitrobenzyl bromide was carried out under nitrogen to determine if the formation of XVII could be prevented. However, in both cases the dimer



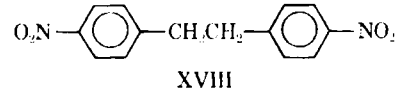
XXIII

was still the major product and the yield of XII was in no way increased.

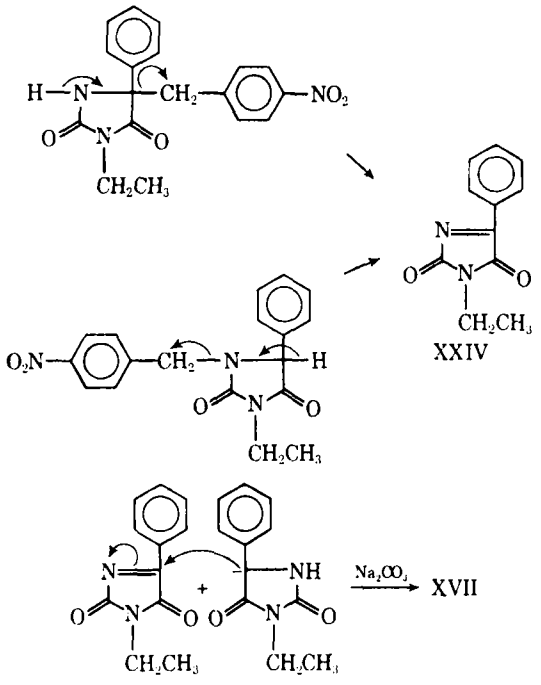
In another attempt to increase the yield of XII, III was treated with two equivalents of p-nitrobenzyl bromide. The reaction under these conditions gave the similar percent yield of XII, but there was no formation of XVII. Instead, the major product, in 35% yield, was 4,4′-dinitrobibenzyl (XVIII). The identity of XVIII was confirmed by comparison of its melting point, mixed melting point, and IR spectrum with an authentic sample. The formation of XVIII was totally unexpected. Reaction of sodium carbonate and p-nitrobenzyl bromide without III did not give any XVIII. It appears that the hydantoin molecule has an important role to play in the production of XVIII.

The formation of the hydantoin dimer XVII as the major product when p-nitrobenzyl bromide was used in equimolar quantity and the formation of the reagent dimer XVIII when the alkylating agent was in excess raised interesting questions. Since carrying out the alkylation reaction in a nitrogen atmosphere did not stop the production of XVII, it seems that air oxidation is not the mechanism of formation in the presence of p-nitrobenzyl bromide. A plausible mechanism could involve the elimination of the stabilized benzylic carbanion from the 1- or 5-alkylated derivative to form the pseudobase XXIV. The dimer could then be formed by reaction of the carbanion and XXIV (Scheme I).

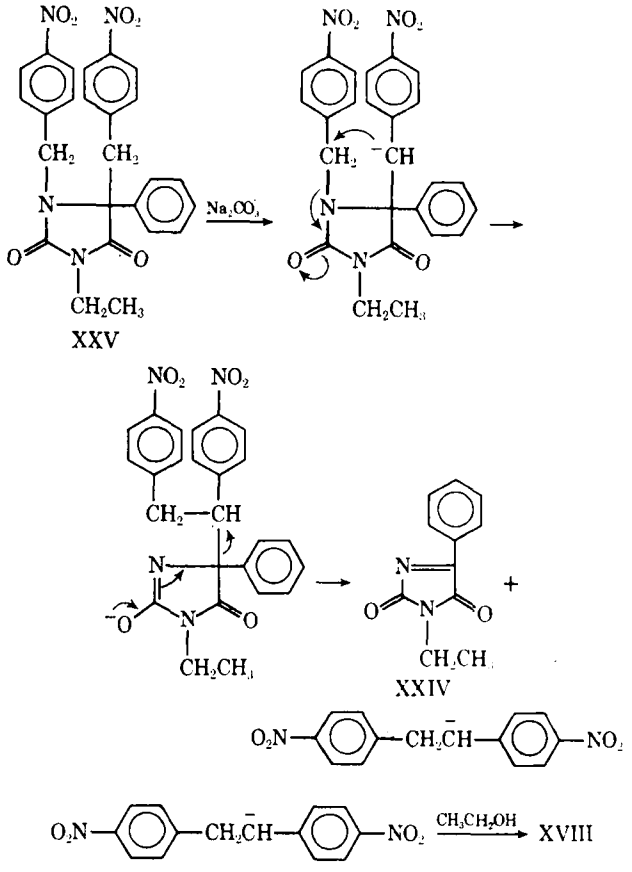
The formation of the reagent dimer XVIII when there was an excess of p-nitrobenzyl bromide could have been caused by the rearrangement of the dialkyl derivative 1,5-bis(p-nitrobenzyl)-3-ethyl-5-phenylhydantoin (XXV) (Scheme II). Such a rearrangement



XVIII



Scheme I—Proposed mechanism for the formation of 3,3'-diethyl-5,5'-diphenylhydantil (XVII)

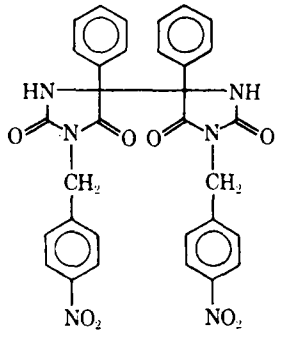


Scheme II—Proposed mechanism for the formation of 4,4'-dinitrobibenzyl (XVIII)

requires that the benzylic protons be acidic, as would be the case in the *p*-nitro derivative but not the *meta*-isomer. Such a mechanism is supported by the fact that alkylation of III with *m*-nitrobenzyl bromide under similar conditions did not produce the analogous 3,3'-dinitrobibenzyl. The hydantoin dimer XVII and the alkyl derivative 3-ethyl-5-*m*-nitrobenzyl-5-phenylhydantoin (XIII) were the two products isolated.

The interesting formation of XVII and XVIII prompted the study of 5-phenylhydantoin in the same manner as III. When 5-phenylhydantoin was allowed to react with *p*-nitrobenzyl bromide in a 1:1 ratio, only the dimer of the N-3 alkylated derivative 3,3'-bis-(*p*-nitrobenzyl)-5,5'-diphenylhydantil (IX) was isolated in 32% yield. However, when 5-phenylhydantoin was treated with the nitrobenzyl bromide in a 1:2 ratio, IX and XVIII were obtained in 20 and 45% yields, respectively.

On the basis of the results obtained, a general statement can be made on the alkylation pattern of the hydantoin molecule in a weak base medium. Alkylation occurs at the 1-, 3-, and 5-positions but not at the 2- and 4-positions of the molecule. The 3-position is most reactive and is always the major site for alkylation. Alkylation at the 1-position occurs less readily than alkylation at the 5-position when there is a phenyl substituent on the 5-position. When both the 3- and 5-positions are blocked, N-1 alkylation can take place. If the 3-position is substituted and the 1- and 5-positions are both available, the formation of a hydantoin dimer is highly possible in the presence of air or when *p*-nitrobenzyl bromide is used as the alkylating agent. If the 3-position is also available, then an N-3 alkylated hydantoin dimer is obtained.



IX

The reactions of the succinimides IV and V with *p*-nitrobenzyl bromide were straightforward. *N*-*p*-Nitrobenzyl-2-ethyl-2-methylsuccinimide (XIV) was obtained in 40% yield, and its identity was confirmed by IR and NMR spectra.

When V was treated with *p*-nitrobenzyl bromide, a 31% yield of a bright-orange reaction product was isolated. The IR spectrum showed the presence of two carbonyl groups and an aromatic nitro grouping. The chemical shift of the protons in the NMR spectrum indicated that the *p*-nitrobenzyl substituent was attached at the 2-position. Thus, the colored reaction product was identified as *N*-methyl-2-phenyl-2-*p*-nitrobenzylsuccinimide (XVI). Like II, there was no reaction between V and *m*-nitrobenzyl bromide.

The alkylation products of I–V with *p*- and *m*-nitrobenzyl bromides are crystalline compounds with sharp and reproducible melting points. Therefore, these products can be used as derivatives for identification and differentiation. As indicated in Tables I and II, the five anticonvulsant drugs studied can be identified and differentiated on the basis of the melting points of these derivatives. Unfortunately, the usefulness of the *p*- and *m*-nitrobenzyl derivatives in identifying or differentiating III from the other four hydantoins appears to be limited because the derivatives could only be obtained in low yields.

The reaction procedures developed for the pure drugs were successfully applied to the various dosage forms of I, II, IV, and V. To have a workable quantity of products, at least 250 mg. of drug in the sample was required. It was also observed that if a sodium salt was used in the formulation, the salt had to be first neutralized with dilute hydrochloric acid to yield the free acid; the free acid was then redissolved in sodium carbonate solution for the alkylation reaction. The aqueous solution of sodium diphenylhydantoin, when allowed to react directly with *p*-nitrobenzyl bromide, produced an almost quantitative yield of *trans*-4,4'-dinitrostilbene, m.p. 290–292° [lit. (11) m.p. 289.5–291°]. In a solution of this basicity, *p*-nitrobenzyl bromide can be ionized to form the carbanion, which displaces the bromide from another molecule of the alkylating agent. The stilbene

was believed to have been formed after elimination of hydrobromide from this displacement intermediate.

Samour *et al.* (12) reported that some alkoxymethyl derivatives of I were effective anticonvulsants. Therefore, it was of interest to test some alkyl derivatives for anticonvulsant activity. Three compounds representing three different chemical structures were studied. Results of the biological testings showed that VI, XIV, and XVII, when suspended in 10% acacia and given orally, exhibited no activity against pentylenetetrazol-induced seizures in mice at a dose of 1 g./kg. According to the acute toxicity studies, these compounds appeared to be relatively nontoxic. None of the animals died as a result of oral administration at a 2-g./kg. dose level, which was the maximum amount of the compound that could be suspended in an appropriate volume of 10% acacia.

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Kinetics and Factors Affecting Stability of Methylprednisolone in Aqueous Formulation

M. I. AMIN▲ and J. T. BRYAN

Abstract □ An investigation was made of the factors affecting the rate of degradation of methylprednisolone solubilized by polysorbate 80 in an aqueous formulation containing polar additives and a sequestering agent as a function of temperature at pH 4.6 and 5.1. The presence of polar additives decreased the apparent solubility of methylprednisolone in one formulation at pH 4.6 and 25°. However, since methylprednisolone was in solution, but not within the polyoxyethylene exterior of the polysorbate 80 micelles, it degraded at a faster rate. By selecting the proper concentration of polysorbate 80 and adjusting to the same pH of 4.6 in another formulation, the autoxidative degradation rate of the primary alcoholic group at C-21 was reduced to approximately half even in the presence of oxygen. The increase in stability was also evident from the increase of the apparent activation energy from 18.2 to 23.1 kcal./mole. The mechanism of solubilization and stabilization based on hydrogen bonding and inclusion into the polyoxyethylene exterior of the polysorbate 80 micelles is proposed.

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In the pharmaceutical field, the phenomenon of micellar solubilization of drugs in aqueous solutions of surfactants is used not only for solubilizing the drug but also for protecting against degradative processes such as hydrolysis and autoxidation. The stabilization of esters against alkaline hydrolysis in aqueous solutions containing nonionic, cationic, and anionic surfactants has been reported (1–3). Vitamin A alcohol solubilized in an aqueous nonionic surfactant solution was reported (4) to be more stable to autoxidation than vitamin A solubilized in cottonseed oil. Similarly, vitamin A alcohol solubilized in aqueous nonionic surfactant solution containing 30% (w/v) glycerin was more stable to autoxidation than vitamin A solubilized in arachis oil (5).

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This article deals with the factors affecting the chemical stability of methylprednisolone solubilized by

Table I—Compositions (Milligrams per Milliliter) of Various Formulations Studied

Ingredients	Lot A	Lot B	Log C	Lot D-1	Lot D-2	Lot D-3	Lot E
Polysorbate 80, purified	35.8	35.8	35.8	41.0	41.0	41.0	37.0
Disodium edetate	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Methylprednisolone	0.55	0.55	0.575	0.575	0.575	0.575	0.525
Neomycin base	22.00	22.00	22.00	22.00	22.00	22.00	22.00
Lincomycin base	22.00	22.00	22.00	22.00	22.00	22.00	22.00
Hydrochloric acid	*q.s. ad.*[a] pH 5.0	*q.s. ad.* pH 5.0	*q.s. ad.* pH 4.6	*q.s. ad.* pH 4.6	*q.s. ad.* pH 4.6	*q.s. ad.* pH 4.6	*q.s. ad.* pH 4.6
30% sodium hydroxide solution	*q.s. ad.* pH 5.0	*q.s. ad.* pH 5.0	*q.s. ad.* pH 4.6	*q.s. ad.* pH 4.6	*q.s. ad.* pH 4.6	*q.s. ad.* pH 4.6	*q.s. ad.* pH 4.6
Purified water	*q.s. ad.*	*q.s. ad.*	*q.s. ad.*	*q.s. ad.*	*q.s. ad.*	*q.s. ad.*	*q.s. ad.*

[a] Adjust to final volume.

polysorbate 80 in aqueous formulations and also the mechanism of solubilization and stabilization. Since the other drug components present were extremely stable, their stability data are not reported.

EXPERIMENTAL

Materials—The following were used: polysorbate 80 USP, purified[1]; disodium edetate USP[2]; methylprednisolone NF, sterile micronized; neomycin sulfate powder USP; lincomycin hydrochloride monohydrate[3]; hydrochloric acid, analytical reagent grade; 30% sodium hydroxide solution; and purified water USP. Since polysorbate 80 USP contained 7.2–14.8% of polyoxyethylene glycol, it was purified by the procedure of Malkemus and Swan (7). The presence or absence of polyoxyethylene glycol in the surfactant was determined by the method of Ginn *et al.* (8). Other ingredients were used as received.

Preparation of Formulations—The formulations shown in Table I were prepared using Procedure B of Johnson (6). Polysorbate 80 and disodium edetate were dissolved in 90% of the purified water. The solutions were then heated to 58–63°, and methylprednisolone was added and dissolved by stirring for 90 min, or until dissolved. Solutions were allowed to cool to 30° or below, and neomycin sulfate and lincomycin hydrochloride monohydrate were added and dissolved by continuous stirring. The solutions were allowed to stand for at least 2 hr., the pH was adjusted (initial pH approximately 6.9), and purified water was added to adjust the final volume. The solutions were then filtered through a 0.45-μ cellulose filter[4] and packaged. These packaged samples were stored in a constant-temperature oil bath, dry heat oven, or metal cabinets in a room equilibrated at 25°. Samples were removed periodically and assayed according to the general procedure for methylprednisolone by blue tetrazolium using a semiautomated analyzer. Methylprednisolone, having a primary α-keto group adjacent to a primary hydroxyl group, reacts with blue tetrazolium in the presence of tetramethylammonium hydroxide to form a colored solution. The blue tetrazolium is reduced and the absorbance of the color complex is measured spectrophotometrically in comparison with a standard solution. A decrease in potency, as determined by this method, indicates the degradation of the side chain at the C-17 position.

Solubility Profile: Polysorbate 80–Water Solutions—*Equilibration*—Polysorbate 80–water solutions containing excess methylprednisolone were equilibrated at 25° for 5 days in 100-ml. vials in a constant-temperature water bath, equipped with an oscillating-type shaker[5]. The vials were then allowed to stand for 2 days at 4°, and the contents were filtered through a 0.45-μ cellulose filter[4] and assayed.

Assay Procedure—The aqueous polysorbate 80–methylprednisolone solutions were diluted 1:25 with 95% 3A alcohol. Polysorbate 80 solution diluted in the same manner as the sample was used as a blank in the assay to eliminate error due to the absorbance of the surfactant. The absorption maximum at 243 nm. was used to calculate the steroid concentration. An absorptivity (1%, 1 cm.) of 398.11 was used. The presence of polysorbate 80 at the dilutions used did not significantly change the absorptivity value from that obtained with solution in 95% alcohol. Since the samples were analyzed immediately after dilution, the degradation of the side chain at the C-17 position was considered to be insignificant.

Solubility Profile: Polysorbate 80–Water Formulations—*Preparation of Formulations*—Polysorbate 80–water formulations containing various amounts of polysorbate 80 were prepared following Procedure B of Johnson (6) and adjusted to pH 4.5 and 5.0. These formulations were similar to Lot A of Table I, except that they did not contain methylprednisolone and the amounts of polysorbate 80 were the same as in the polysorbate 80–water solutions.

Equilibration—The same method of equilibration followed for polysorbate 80–water solutions was used.

Assay Procedure—Methylprednisolone was determined according to the general procedure for steroids by blue tetrazolium.

RESULTS AND DISCUSSION

The rate of autoxidation reactions in solution depends upon the pH, thermal energy of UV light, oxygen concentration, and concentration of heavy metal ions. Since the degradation of prednisolone (9) in aqueous buffer solutions containing disodium edetate was less at pH 4 than at pH 5.0, these formulations (Table I) were made to contain disodium edetate and the pH was adjusted between 4.5 and 5.0. A pH of 4.5 was selected as the lower limit because polysorbate 80 hydrolyzed at pH values lower than 4.0 and at high temperatures, producing an irreversible colloidal milky precipitate.

The primary degradation pathway for methylprednisolone in these formulations appears to be autoxidation of the primary alcoholic grouping at C-21. This is consistent with the observation that, in alkaline aqueous solutions and in the presence of air, prednisolone degraded to a steroidal acid as a major component (10). Thus, the pH of the formulations decreased. The photolytic degradation of methylprednisolone observed (11) in aqueous solutions of tyloxapol (oxyethylated tertiary octylphenol formaldehyde polymer)[6] was absent. This was confirmed using TLC and assaying the methanol eluate spectrophotometrically at 242 nm. for the 22-month old samples of Lots A and B stored at room temperature. The concentration of methylprednisolone in both lots was unchanged from the initial value. Also, the photolytic degradation of the formulations packaged in ampuls and stored in an oil bath would be at a minimum, since the oil baths were located in a room where the UV light exposure was negligible.

The autoxidation of methylprednisolone followed an apparent first-order process under all experimental conditions. Typical first-order plots of the thermal degradation of methylprednisolone are shown in Figs. 1 and 2.

The effects of various factors on the apparent first-order thermal degradation of methylprednisolone in aqueous formulations are shown in Table II. The apparent first-order rate constants were calculated from the slopes of log percent residual concentration–time data by means of a least-squares regression analysis.

Effect of Oxygen Concentration—The concentration of oxygen present does affect the rate of degradation of methylprednisolone. The magnitude of the apparent first-order rate constant at room temperature for Lot B, packaged in syringes (Roehr) sealed in aluminum foil with no head space, was 1.5 times smaller than that

[1] Tween 80, Atlas Chemical Ind., Wilmington, Del.
[2] Dow.
[3] Upjohn.
[4] Millipore filter type HA with clarifying pads, Millipore Corp., Bedford, Mass.
[5] Temperature control water bath shaker, model 2156, Research Specialists, Berkeley, Calif.
[6] Triton WR 1339, Rohm & Haas Co., Philadelphia, Pa.

Table II—Various Factors Affecting the Apparent First-Order Thermal Degradation of Methylprednisolone in Aqueous Formulations

Lot Number and Type of Package	Methylprednisolone[a], Initial Concentration, %	Amount of Polysorbate 80, mg./ml.	Initial pH[b]	k_{app}, months^{-1} × 10^3 25°	40°	47°	54°	56°
Lot A; 10-ml. ampuls	106.0	35.80	5.10	—	49.81	98.37	167.4	—
Lot A; 30-ml. Type I glass vials with butyl rubber plugs	106.0	35.80	5.10	11.25	—	—	—	—
Lot B; 10-ml. syringes[c] sealed in aluminum foil, and no head space	107.0	35.80	5.15	7.09	—	—	—	—
Lot C; 10-ml. x′ presit syringes[d]	112.0 113.8	35.80	4.60	9.78	—	—	—	—
Lot D-1; 10-ml. x′ presit syringes[d]	116.6	41.00	4.55	4.12	21.43	—	—	—
Lot D-2; 10-ml. x′ presit syringes[d]	117.6	41.00	4.60	4.59	23.25	—	—	—
Lot D-3; 10-ml. ampuls	114.8	41.00	4.60	—	—	—	—	171.1
Apparent first-order rate constant (average D) for Lots D-1, D-2, and D-3				4.36	22.34	—	—	171.1
Lot E; 10-ml. x′ presit syringes[d]	104.0	37.00	4.60	4.98	—	—	—	—

[a] Methylprednisolone, 0.5 mg./ml., is equivalent to 100.0%. [b] Initial pH = ±0.05 unit. [c] Roehr Monoject barrels and plungers of high density polypropylene. Snap-on rubber plugs made from natural rubber. [d] Barrels and plungers of high density polypropylene. Snap-on rubber plugs made from natural rubber. Composition similar to Roehr Monoject syringes.

of Lot A, packaged in 30-ml. vials and having a head space (Table II). The amount of polysorbate 80 and the pH were the same in these lots.

Effect of Purified Polysorbate 80 Concentration—The amount of polysorbate 80 used in these formulations plays a vital role in stabilizing methylprednisolone, as can be seen from the higher value of the apparent first-order rate constant at room temperature for Lot C than Lot D-1 (Table II). Lot C was prepared using 3.58% (w/v) polysorbate 80 to dissolve 0.575 mg./ml. of methylprednisolone. This amount of polysorbate 80 was higher than the actual value of 3.35% (w/v) obtained from the solubility profile for methylprednisolone in polysorbate 80–water solutions (Fig. 3). Surprisingly, the solubility of methylprednisolone decreased in the presence of soluble polar additives, as seen in the solubility profile for methylprednisolone in polysorbate 80–water formulations adjusted to pH 5 (Fig. 3). Thus, Lot D-1 was made with 4.1% (w/v) polysorbate 80 to dissolve 0.575 mg./ml. methylprednisolone. The solubility profile at pH 4.5 in polysorbate 80–water formulations (not shown) had exactly the same slope as the solubility profile at pH 5.

An increase in pH decreases the micellar molecular weight and the hydration per unit mass of surfactant (12). This would be expected to reduce the solubility at pH 5. However, this was not observed since the pH range of formulations studied was very narrow.

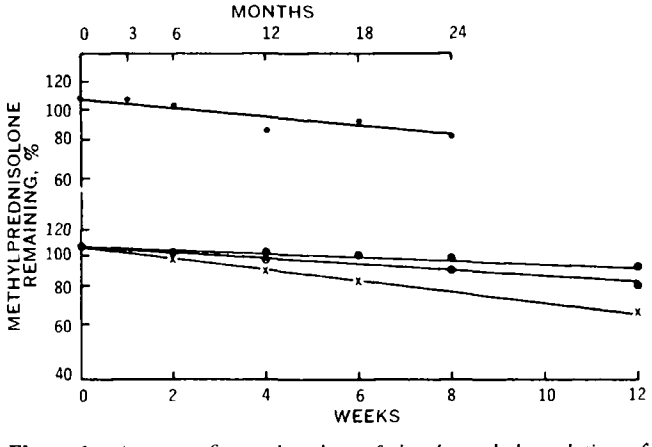

Figure 1—*Apparent first-order plots of the thermal degradation of methylprednisolone in Lot A, containing 3.58% (w/v) purified polysorbate 80, at pH 5.1. Key: (top) •, 25°; (bottom) ●, 40°; ◓, 47°; and ×, 54°.*

Effect of Temperature on Solubilization of Methylprednisolone—An increase in temperature increased the solubility of methylprednisolone. Furthermore, the methylprednisolone stayed in solution even after Lot D-1 was frozen, allowed to come to room temperature, filtered, and assayed. The assay results were exactly the same when compared with those for Lot D-1 stored at 4°. These results show that methylprednisolone will be in solution regardless of the temperature used during the manufacture of the formulation.

Effect of pH and Temperature—As stated previously, the pH of the formulations decreased, possibly due to the steroidal acids produced by the autoxidation of methylprednisolone. However, the extent of pH decrease in Lots A and D was the same; namely, 0.15 pH unit after 24 months at 25°. Likewise, the extent of pH decrease in Lots A and D was also the same; namely, 0.25 pH unit after 6 months at 40°.

Arrhenius plots for the degradation of methylprednisolone in Lots A and D are shown in Fig. 4. The apparent activation energies calculated from the least-squares slopes of the apparent first-order rate constants *versus* the reciprocal of absolute temperatures for Lots A and D are shown in Table III. Also shown are the values of $t_{90\%}$, the time in months required to reach 90% of methylprednisolone concentration in Lots A, C, D, and E, which were stored at room temperature. From the data in Table III, it is apparent that the shelflife of methylprednisolone in these aqueous formu-

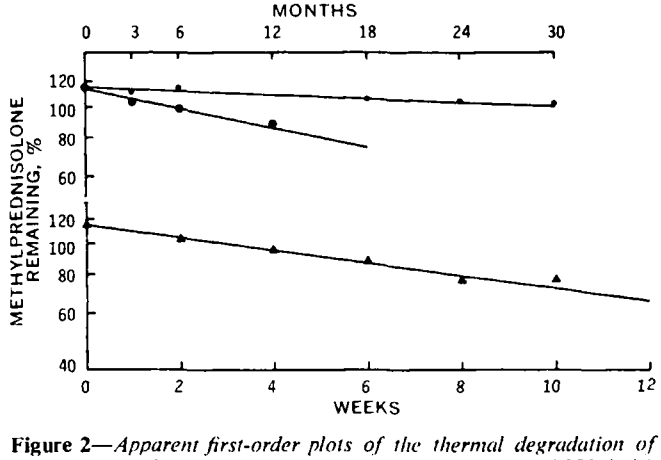

Figure 2—*Apparent first-order plots of the thermal degradation of methylprednisolone in Lots D-1 and D-3, containing 4.1% (w/v) purified polysorbate 80, at pH 4.6. Key: •, 25°; ●, 40°; and ▲, 56°.*

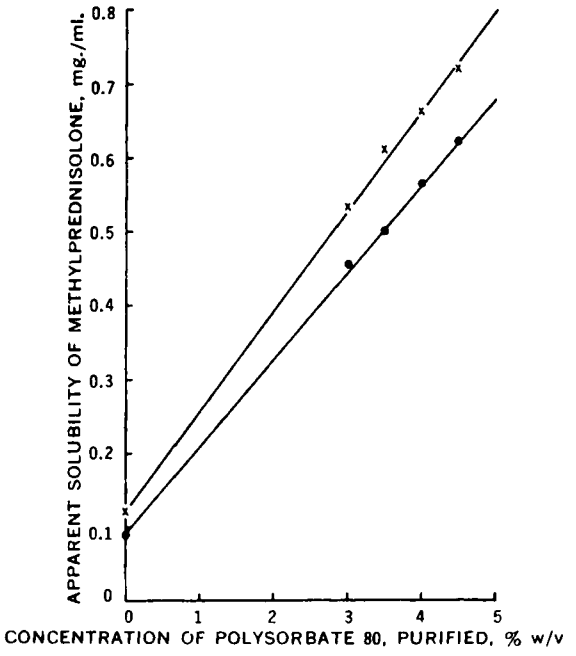

Figure 3—*Solubility profile for methylprednisolone in polysorbate 80–purified water solutions* (×) *and in polysorbate 80–purified water formulations* (●) *adjusted to pH 5.0.*

lations is increased by adjusting to a proper pH and selecting the proper concentration of the nonionic surfactant.

Mechanism of Solubilization and Stabilization—Several articles described the mechanism of solubilization of drugs in the presence of surfactants (3, 13, 14). In these formulations, no attempts were made to determine the exact mechanism of micellar solubilization of methylprednisolone. However, the probable mechanism seems to be hydrogen bonding of the polar groups of the steroid with the hydrated polyoxyethylene portion on the surface, as well as incorporation into the polyoxyethylene groups of the polysorbate 80 micelles. The mechanism of hydrogen bonding appears to be adsorption of water-soluble polar additives (15) in competition with methylprednisolone adsorption on the exterior surface of the nonionic micelle. The data from the solubility profile (Fig. 3) indicate that a possible mechanism may also be incorporation of methylprednisolone into the polyoxyethylene group of the polysorbate 80 micelle. Since a large excess of polar additives (neomycin

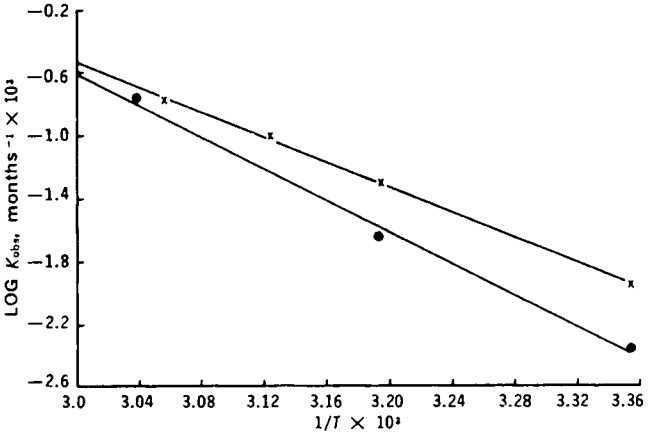

Figure 4—*Arrhenius plots for the degradation of methylprednisolone. Key:* ×, *Lot A, 3.58% (w/v) purified polysorbate 80 and initial pH of 5.1; and* ●, *Lot B, 4.1% (w/v) purified polysorbate 80 and initial pH of 4.6.*

Table III—Apparent Activation Energies

Lot Number	E_a, kcal./mole	$t_{90\%}$[a], Months at Room Temperature
A	18.22	17.85
C	—	25.08
D[b]	23.09	56.38
E	—	30.98

[a] See Table I for initial concentration of methylprednisolone; 0.5 mg./ml. is considered as 100%. [b] See Table II.

sulfate and lincomycin hydrochloride) is present and adsorbs on the exterior surface of the polysorbate 80 micelle, there is more likelihood that methylprednisolone is solubilized into the exterior of the polysorbate 80 micelle. Thus, since a certain amount of methylprednisolone was free in the aqueous phase of Lot C, pH 4.6, it degraded approximately twice as fast as Lot D-2, pH 4.6, where, methylprednisolone was associated on the surface and into the polyoxyethylene exterior of the polysorbate 80 micelle. Because the aggregation number and the amount of water bound per unit mass of nonionic surfactant are greater at low pH values (12), oxygen attack was slower in Lot C, pH 4.6, than in Lot A, pH 5.1.

At 54–56°, the ratio of rate constants $k_{app(Lot\ A)}/k_{app(Lot\ D)}$ is closer to 1, which increases approximately to 2.6 at 25°. This can be interpreted as follows. At 54–56°, the micellar structure is disrupted in Lot D, thus preventing inclusion of the methylprednisolone into the exterior of the polysorbate 80 micelles. Consequently, the rate of degradation in Lot D is approximately the same as in Lot A. At 25°, there is negligible disruption of the micellar structure in Lot D, resulting in a lower k_{app} and thus a higher ratio of rates of degradation.

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Reaction of Cyclic Acid Anhydrides with Ethyl Cyanoacetate

EDWARD E. SMISSMAN▲, MICHAEL WACHTER, CHARLES BARFKNECHT, and R. BRUCE GABBARD

Abstract □ The reactions of cyclic carboxyanhydrides and substituted cyclic carboxyanhydrides with ethyl sodiocyanoacetic ester were studied. The resulting dicarboxylic acid esters failed to undergo Dieckmann cyclization.

Keyphrases □ Cyclic acid anhydrides—reaction with ethyl sodiocyanoacetate, absence of Dieckmann cyclization □ Sodiocyanoacetic acid ethyl ester—reaction with cyclic acid anhydrides, absence of Dieckmann cyclization □ Ring formation reactions, potential cyclic acid anhydrides and ethyl sodiocyanoacetate, absence of Dieckmann cyclization

In a previous paper, the preparation of an A-D ring analog of the tetracycline molecule, I, was discussed (1). A review of the chemistry of the tetracyclines (2) and of the substituents necessary for the desired biological activity based on numerous structure–activity relationship studies (3) was published.

With this general information in view, the possibility of cyclizing a properly constituted alicyclic molecule, II, to obtain the polysubstituted ring A, III, of tetracycline as an intermediate in the A-D ring synthesis (1) (Scheme I) and for biological testing was investigated.

DISCUSSION

The acylation of ethyl sodiocyanoacetate with cyclic carboxyanhydrides to give half-esters of α-cyano-β-ketodicarboxylic acids was reported (4). This procedure was modified to give the diester rather than the half-ester in the acylation of ethyl sodiocyanoacetate with glutaric or succinic anhydrides.

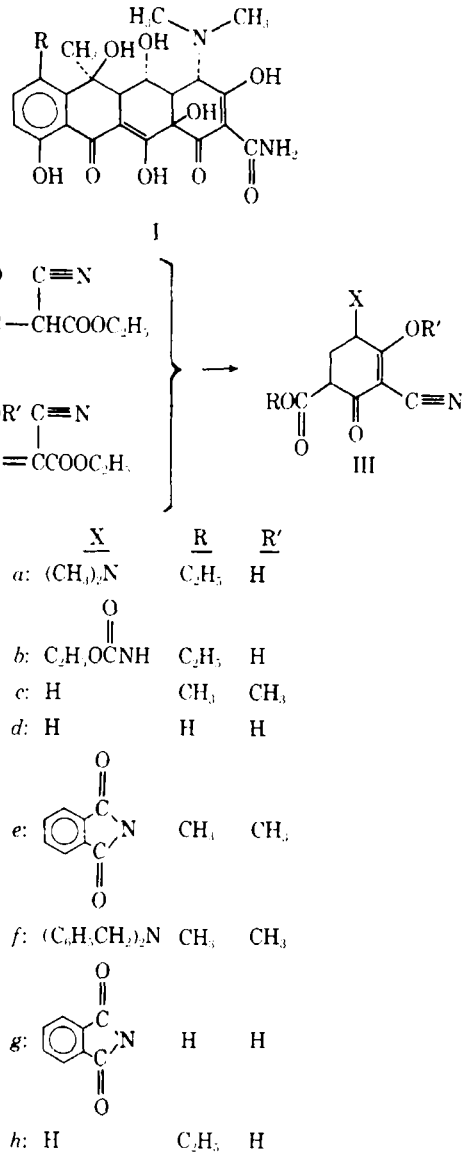
To utilize this method to prepare diethyl 2-cyano-3-hydroxy-4-*N*,*N*-dimethylamino-2-heptenedioate, II*a*, the previously unreported *N*,*N*-dimethylglutamic anhydride was required. *N*,*N*-Dimethylglutamic acid, IV*a*, was prepared by a modification of the method of Bowman and Stroud (5), which involved the reductive condensation of glutamic acid with formaldehyde under hydrogen. All attempts to convert IV*a* to the anhydride, V*a*, by known procedures failed.

N-Carbethoxyglutamic acid, IV*b*, was prepared by the method of Aberhalden and Kautzsch (6) and was cyclized by refluxing in acetic anhydride to give the corresponding anhydride, V*b*. On treatment of V*b* with ethyl sodiocyanoacetate, diethyl 2-cyano-3-hydroxy-4-*N*-carbethoxyamino-2-heptenedioate, II*b*, was produced. The assignment of the *N*-carbethoxyamino group to the 4-position was based on an analogous reaction by Bergman and Zervas (7). They reported the treatment of *N*-carbobenzoxyglutamic anhydride, V*c*, with ammonia to give *N*-carbobenzoxyisoglutamine, VI, which was then decarbobenzoxylated to isoglutamine. By analogy, the product of V*b* with sodiocyanoacetate would be expected to yield II*b* rather than the isomeric diethyl 2-cyano-3-hydroxy-6-*N*-carbethoxyamino-2-heptenedioate, VII*a*.

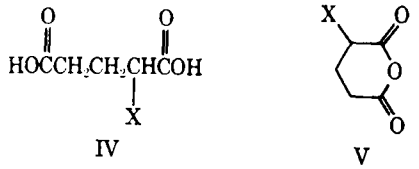
Attempts to cyclize II*b* utilizing sodium ethoxide and potassium *tert*-butoxide to produce the desired cyclohexanedione failed.

It was assumed that the preparation of III failed where R′ = H because the molecule II forms the anion VIII*a* or IX in preference to VIII. Formation of the desired cyclic compound is not possible when the enolic oxygen bears the negative charge as in IX (Scheme II). To circumvent this possibility, the enolic hydroxyl was protected as the enol methyl ether, II*c*, by treatment with diazomethane. It was assumed that VIII and VIII*a* would be in facile equilibrium. The assignment of the location of the ethyl and methyl ester groups in II*c* was undertaken by NMR studies. Ethyl acetate shows a triplet centered at δ1.25 and a quartet at δ4.12 for the ethyl function, while ethyl cyanoacetate shows peaks at δ1.32 and 4.27. The ethyl group in the latter is being deshielded by the electronegative groups in the acid portion of the molecule. The ethyl absorption in II*c* is at δ1.42 and 4.47. Since the ethyl group is deshielded, it is assigned to the 1-carboxyl function.

N-Phthalylglutamic anhydride, V*d*, proved to be a more convenient intermediate for the preparation of an amino-substituted pimelate. The reaction of V*d* with ethyl sodiocyanoacetate in xylene, followed by esterification of the resulting acids with diazomethane, gave two crystalline compounds. These compounds were shown to be the *cis*- and *trans*-isomers of VII*b* by the spectral data. Mass



Scheme I



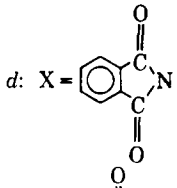
IV

V

a: X = $(CH_3)_2N$

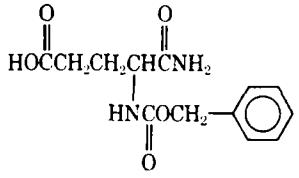
b: X = C_2H_5OCNH (O)

c: X = $C_6H_5CH_2OCNH$ (O)

d: X = 

e: X = F_3CCNH (O)

f: X = $(C_6H_5CH_2)_2N$



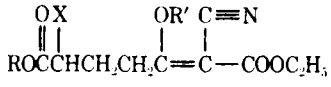
VI

spectroscopy was conclusive in establishing that they were the 6-*N*-phthalyl geometric isomers. Both *cis*- and *trans*-VII*b* gave identical fragmentation patterns, with a base peak of *m/e* 219. This peak arises from a McLafferty-type rearrangement (Scheme III) The 4-*N*-phthalyl isomer, II*e*, cannot give rise to a peak of *m/e* 219 by a similar rearrangement or fragmentation.

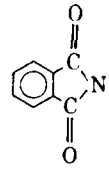
Attempts at the Dieckmann condensation of VII*b* failed.

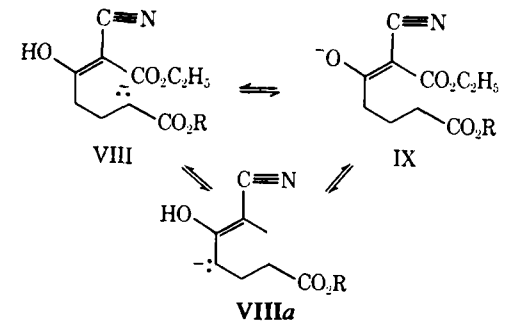
Since the 4-phthalimido compound, II*e*, could not be obtained, other systems were explored to secure an open-chain precursor of the tetracycline A ring with a nitrogen function at C-4. *N*-Trifluoroacetylglutamic anhydride, V*e*, was viewed as a potential precursor with a labile *N*-protecting group. The reaction of V*e* with ethyl sodiocyanoacetate, followed by esterification with diazomethane, yielded 2-trifluoroacetamidopentanedioate, IV*e*, as the only isolable product.

Despite previous failures in attempts to prepare *N,N*-dimethylglutamic anhydride, V*a*, it was thought that increasing the basicity of the nitrogen in such a system would lead to the desired 4-*N*-substituted heptenedioate. *N,N*-Dibenzylglutamic acid, IV*f*, contains a more basic nitrogen than the phthalyl or trifluoroacetyl



VII

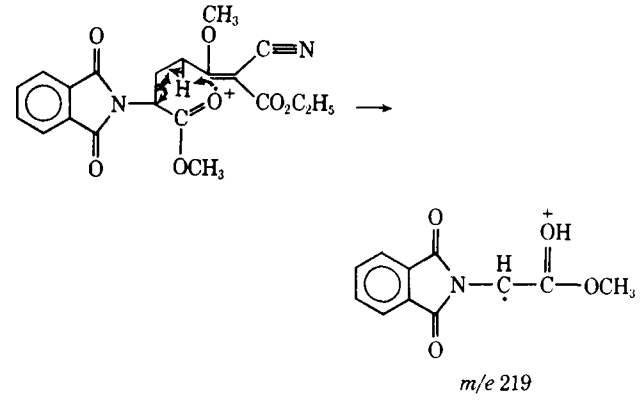
	X	R	R′
a:	C_2H_5OCNH (O)	C_2H_5	H
b:		CH_3	CH_3
c:	$(C_6H_5CH_2)_2N$	CH_3	CH_3



VIII IX

VIII*a*

Scheme II



m/e 219

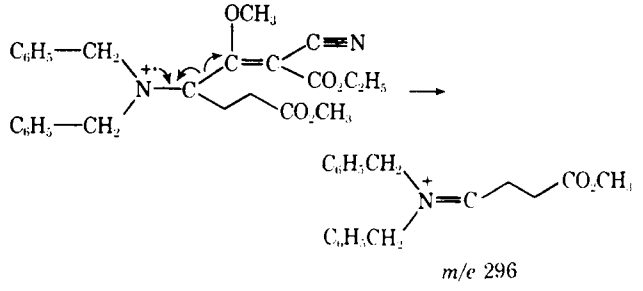
Scheme III

derivatives and was prepared by a modification of a reported procedure (8). Compound IV*f* was converted to the corresponding anhydride, V*f*, by heating with acetic anhydride.

Anhydrous potassium carbonate has been found effective as a reagent in the acylation of cyanoacetic esters with simple anhydrides (9). Use of these conditions for the reaction with V*f* and esterification of the products with diazomethane produced a mixture of heptenedioate esters. Column chromatography of this mixture gave an oil, which was identified as ethyl methyl 2-cyano-3-methoxy-4-*N,N*-dibenzylamino-2-heptenedioate, II*f*. The structure was assigned primarily from the mass spectrum along with the NMR and IR spectra. The mass spectrum of II*f* showed a base peak of *m/e* 296 below 100°. This represents a simple fragmentation of the C—C bond adjacent to the nitrogen typical of tertiary amines. The 4-*N,N*-dibenzyl isomer, II*f*, can give rise to this fragmentation, whereas the 6-*N,N*-dibenzyl isomer, VII*c*, cannot (Scheme IV).

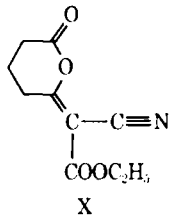
The use of sodium hydride gave superior yields of II*f* as compared to the method utilizing potassium carbonate. This procedure gave adequate quantities of II*f* to allow numerous attempts at the Dieckmann cyclization under various conditions. Potassium *tert*-butoxide in benzene, triethylamine in benzene, sodium hydride in benzene, and sodium hydride in dimethylformamide all proved to be unsuccessful in the cyclization.

Since the cyclization failed, it was decided to attempt an isomerization of the substituted enol lactone, X. The half-ester ethyl hydrogen 2-cyano-3-hydroxy-2-hepteneoate, II*d*, was treated with acetic anhydride to give a neutral compound in quantitative yield.

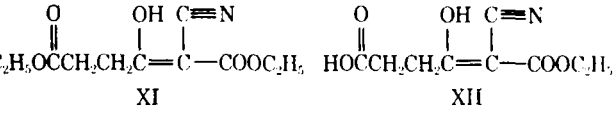


m/e 296

Scheme IV



X



XI XII

The enol lactones of the α-cyano-β-keto acids have not been reported previously. The compound was slowly soluble in sodium bicarbonate with effervescence; it gave a gradually deepening red color on reaction with ferric chloride solution within 20 min.; a strong band at 5.60 μ was present in the IR, indicating a vinyl ester structure. Thus, Compound X was established as the enol lactone rather than a cyclohexanedione. Compound X could not be isomerized to a 2,6-cyclohexanedione with sodium ethoxide (10) or boron trifluoride (11) or by thermal rearrangement.

Succinic anhydride was allowed to react with an equivalent amount of ethyl sodiocyanoacetate in the presence of ethanol to produce diethyl 2-cyano-3-hydroxy-2-hexenedioate, XI. This compound was partially hydrolyzed with hydrochloric acid to yield ethyl hydrogen 2-cyano-3-hydroxy-2-hexenedioate, XII, which was identical to the compound previously reported (4, 12).

EXPERIMENTAL[1]

Diethyl 2-Cyano-3-hydroxy-2-hexenedioate, XI A sodium dispersion was added under nitrogen to dry xylene (250 ml.) and absolute ethanol (12 ml.) until the stirred suspension was gray. The color was discharged by the addition of 1–2 ml. of ethanol. Ethyl cyanoacetate (22.5 g., 0.20 mole) was added all at once, resulting in a thick milky suspension. Succinic anhydride (20.0 g., 0.20 mole) was added immediately, and the thick emulsion became a yellowish turbid suspension. The mixture was stirred for 4 hr., after which it was shaken with 100 ml. water. The xylene layer was separated and the aqueous layer was extracted with ether (2 × 100 ml.). The xylene and ether extracts were combined and the solvent was removed to give an oily residue. The residue was distilled under reduced pressure. A fraction, b.p. 150–156°/0.1 mm., solidified on standing and was recrystallized (petroleum ether, b.p. 60–68°) to give 10 g. (21%) of a colorless crystalline material, m.p. 52.5–53.0° [lit. (4) m.p. 51°].

Ethyl Hydrogen 2-Cyano-3-hydroxy-2-heptenedioate, IId—The previous procedure was modified by heating the reaction vessel to 100° after 20 g. (0.20 mole) of ethyl cyanoacetate had been added to the sodium dispersion. A forceful current of nitrogen was introduced to entrain the ethanol vapors; when the ethanol had been removed, the heating was discontinued and 21 g. (0.10 mole) of glutaric anhydride was added. The procedure following the addition of glutaric anhydride was the same as previously described. Twenty-four grams (57%) of ethyl hydrogen 2-cyano-3-hydroxy-2-hepteneoate, IId, was obtained [lit. (4) m.p. 136°].

Anal.—Calc. for $C_{10}H_{13}NO_5$: C, 52.86; H, 5.77; N, 6.17. Found: C, 52.91, 52.95; H, 5.85, 5.95; N, 5.98.

If this modification was not followed, 20% of the half-ester and 14% of a colorless oil, diethyl 2-cyano-3-hydroxy-2-heptenedioate, IIh, were obtained. The latter formed a green crystalline chelate with cupric acetate, m.p. 120–121°.

Anal.—Calc. for $C_{21}H_{32}CuN_2O_{10}$: C, 50.20; H, 5.30; N, 4.95. Found: C, 50.49; H, 5.48; N, 4.75.

Ethyl 2-Pentanolidenecyanoacetate, X—Ethyl hydrogen 2-cyano-3-hydroxy-2-hepteneoate, IId (10 g., 0.05 mole), was refluxed in acetic anhydride (25 ml., 0.25 mole) until solution was effected. The excess acetic anhydride and the acetic acid were distilled *in vacuo*. The solid residue was crystallized from benzene–petroleum ether (b.p. 60–68°). Ethyl 2-pentanolidenecyanoacetate, X (10 g., 100%) was obtained as colorless plates, m.p. 104–106°.

Anal.—Calc. for $C_{10}H_{11}NO_4$: C, 57.41; H, 5.30; N, 6.70. Found: C, 56.98; H, 4.97; N, 6.54.

***N*-Carbethoxyglutamic Acid, IVb** The method of Aberhalden and Kautzsch (6) was followed. A colorless oil (22 g., 50%), soluble in water, was obtained from 29.4 g. (0.20 mole) glutamic acid and 21.7 g. (0.20 mole) ethyl chloroformate.

***N*-Carbethoxyglutamic Anhydride, Vb**—*N*-Carbethoxyglutamic acid (22.0 g., 0.10 mole) was refluxed with 50 ml. (0.50 mole) acetic anhydride for 1 hr. The excess acetic anhydride and acetic acid were removed *in vacuo*. *N*-Carbethoxyglutamic anhydride (20 g., 100%) was obtained as a pale-yellow oil.

Diethyl 2-Cyano-3-hydroxy-4-*N*-carbethoxyamino-2-heptenedioate, IIb—The procedure for the preparation of diethyl 2-cyano-3-hydroxy-2-heptenedioate, IIh, was followed. *N*-Carbethoxyglutamic anhydride (20 g., 0.10 mole) and 11.3 g. (0.10 mole) ethyl cyanoacetate were used and yielded 14 g. of a liquid acidic toward sodium bicarbonate. This material was distilled *in vacuo*. Diethyl 2-cyano-3-hydroxy-4-*N*-carbethoxyamino-2-heptenedioate, IIb (2.5 g., 7%), was obtained as a colorless liquid, b.p. 98°/6 mm.; n_D^{25} 1.454. The distillation flask contained a considerable amount of carbonaceous material.

Anal.—Calc. for $C_{15}H_{22}N_2O_7$: C, 52.62; H, 6.47; N, 8.18. Found: C, 52.96; H, 6.28, 6.24; N, 8.10, 8.15.

***N*-Phthalylglutamic Anhydride, Vd** The procedure was essentially that of King and Kidd (13). A suspension of L-glutamic acid (16.8 g., 0.113 mole) and phthalic anhydride (16.8 g., 0.113 mole) in 60 ml. of dry pyridine was refluxed for 2 hr. The solution was cooled, filtered, and evaporated under reduced pressure. Acetic anhydride (45 ml.) was added, the solution was heated at reflux for 3 min. and then cooled, and the solvent was partially removed. Crystallization began upon evaporation of the acetic anhydride and was completed upon the addition of 300 ml. of ether. After filtering and washing with ether, 21.5 g. of a white solid, m.p. 194–199°, 70% yield, was obtained. Two recrystallizations from ethyl acetate afford Vd, m.p. 200–204° [lit. (13) m.p. 195–200°].

Ethyl Hydrogen 2-Cyano-3-hydroxy-6-*N*-phthalimido-2-heptenedioate—A suspension of sodium hydride (0.632 g. of 57% NaH, 0.015 mole) was washed three times with dry dioxane, and then 125 ml. of dioxane was added to the reaction vessel. The vessel was equipped with a nitrogen inlet, and ethyl cyanoacetate (1.7 g., 0.015 mole) in 25 ml. of dioxane was added dropwise with stirring over 10 min. The mixture was heated at reflux for 2 hr. and a milky suspension formed. *N*-Phthalylglutamic anhydride, Vd (3.89 g., 0.015 mole), was added as a solid and the mixture was heated at reflux for 18 hr. After cooling, the dioxane was removed under reduced pressure and equal volumes of water and ether were added. A solid (0.4 g.), insoluble in either layer, was filtered. The aqueous layer was acidified with dilute hydrochloric acid and extracted three times with ether. The ethereal layer was dried (magnesium sulfate) and gave 3.8 g. of a viscous, nondistillable, yellow-orange oil. The NMR spectrum of this crude material indicated a mixture of heptenoic acids.

The insoluble material (0.4 g.) was dissolved in 25 ml. of ether–methanol (9:1) and esterified with diazomethane to give 411 mg. of a yellow oil, which was shown to be dimethyl 2-*N*-phthalimidopentanedioate: IR (chloroform): 1780–1720 cm.$^{-1}$, no C≡N stretch; NMR ($CDCl_3$): δ 2.3–2.7 (m, 4, CH_2CH_2), 3.63 (s, 3, OCH_3), 3.75 (s, 3, OCH_3), 4.93 (broad t, 1, methine H), and 7.84 (broad s, 4, aromatic H); mass spectroscopy: *m/e* 305 (parent peak) and 186 (base peak).

Ethyl Methyl 2-Cyano-3-methoxy-6-phthalimido-2-heptenedioate, VIIb The crude heptenoic acids (3.6 g.) were dissolved in 75 ml. of ether–methanol (9:1). An ethereal solution of diazomethane, generated from *N*-nitrosomethylurea, was added to this solution at 0°. Addition was continued until the effervescence ceased. The solvent was removed under reduced pressure to yield 3.7 g. of a yellow-orange nondistillable oil. TLC and NMR showed the oil to be a mixture of at least three compounds. The crude heptenedioate esters (3.0 g.) were chromatographed on 100 g. of silica gel, using chloroform as the eluting solvent. The first fraction eluted (550 mg.) was a colorless oil. Recrystallization from chloroform–hexane (1:1) gave 507 mg. of a white solid, m.p. 150–151°; IR (chloroform): 2960, 2220, 1780, 1720, 1580, 1380, 1125, and 1060 cm.$^{-1}$; NMR ($CDCl_3$): δ 1.24 (t, 3, CH_3), 2.85–3.25 (m, 2, CH_2), 3.0–

[1] Melting points were obtained on a calibrated Thomas-Hoover Unimelt and are corrected. IR data were recorded on a Beckman IR-10 spectrophotometer. NMR data were recorded on Varian Associates A-60, A-60A, and HA 100 spectrometers using tetramethylsilane as an internal standard. Microanalyses were performed by Midwest Microlab, Inc., Indianapolis, Ind., and on an F & M 185 C, H, N analyzer.

3.3 (m, 2, CH_2), 3.75 (s, 3, OCH_3), 4.08 (s, 3, OCH_3), 4.1 (q, 2, CH_2CH_3), 4.98 (t, 1, methine H), and 7.86 (d, 4, aromatic H); UV (ethanol) λ_{max}: 261, 241, and 231 nm.; mass spectroscopy: *m/e* 399 (parent peak), 219 (base peak), 187 (90%), 132 (20%), and 104 (20%).

Anal.—Calc. for $C_{20}H_{20}N_2O_7$: C, 60.00; H, 5.03; N, 7.00. Found: C, 59.71; H, 4.91; N, 6.80.

A second fraction was collected which contained 750 mg. of a pale-yellow oil. Trituration of this oil with ether gave 532 mg. of a white solid, m.p. 90–91°; IR (chloroform): 2960, 2215, 1780–1720, 1575, 1380, 1210, and 1040 cm.$^{-1}$; NMR ($CDCl_3$): δ 1.27 (t, 3, CH_3), 2.4 2.95 (m, 4, CH_2CH_2), 3.76 (s, 3, OCH_3), 4.08 (s, 3, OCH_3). 4.2 (q, 2, CH_2CH_3), 4.95 (t, 1, methine H), and 7.85 (d, 4, aromatic H); UV (ethanol) λ_{max}: 261, 241, and 231 nm.; mass spectroscopy: *m/e* 399 (parent peak), 219 (base peak), 187 (90%), 132 (20%), and 104 (20%).

Anal.—Calc. for $C_{20}H_{20}N_2O_7$: C, 60.00; H, 5.03; N, 7.00. Found: C, 60.13; H, 5.17; N, 6.91.

The two isomers were obtained in the same overall yield using potassium carbonate as the base. The reaction was performed in dioxane using the same molar quantities reported in the synthesis of ethyl methyl 2-cyano-3-methoxy-4-*N,N*-dibenzyl-2-heptenedioate.

cis- and *trans*-VII*b* were also obtained using xylene as the reaction solvent. However, the yields were lower and the product composition was nearly identical by NMR.

Reaction of Ethyl Methyl 2-Cyano-3-methoxy-6-phthalimido-2-heptenedioate, VII*b*, with Sodium Hydride—Sodium hydride (25 mg. of 57% in mineral oil, 5.8×10^{-4} mole) was suspended in 10 ml. of dry benzene. Compound VII*b* (231 mg., 6.0×10^{-4} mole, m.p. 150–151°), in 10 ml. of dry *N,N*-dimethylformamide, was added dropwise to the suspension. A red-orange color, indicative of anion formation, developed within 30 min. The reaction was stirred at 25° for 22 hr. and, on workup, 210 mg. of starting material was recovered unchanged.

N*-Trifluoroacetylglutamic Anhydride, V*e—Compound V*e* was prepared by a modification of the method of Weygand and Leising (14). Trifluoroacetic anhydride (30 ml.) was added slowly to 10 g. of glutamic acid. After the resulting solution was cooled, 250 ml. of dry ether was added and a slightly exothermic reaction occurred. The solution was immediately decanted and diluted with 600 ml. of dry hexane. The mixture was cooled to 0° for 2 hr., and the crystals were filtered and washed sparingly with hexane. The resulting white needles were dried over calcium hydroxide in a vacuum desiccator. Recrystallization from chloroform gave 11.0 g. (72%) of white needles, m.p. 61–64°. Further recrystallization gave 9.1 g., m.p. 68–71° [lit. (14) m.p. 70°]; NMR ($CDCl_3$–CD_3COCD_3): δ 2.2–2.6 (m, 2, CH_2), 3.05–3.3 (m, 2, CH_2), and 5.1 (t, 1, methine H).

Attempted Synthesis of Ethyl Methyl 2-Cyano-3-methoxy-4-trifluoroacetamido-2-heptenedioate—A suspension of sodium hydride (300 mg. of 57% NaH, 0.007 mole) in 50 ml. of dioxane and 750 mg. (0.0067 mole) of ethyl cyanoacetate were allowed to react as before to form the sodio salt. After the milky suspension was cooled, 1.5 g. (0.0067 mole) of *N*-trifluoroacetylglutamic anhydride, V*e*, in 20 ml. dioxane was added slowly. The suspension was stirred at 25° for 16 hr. and treated as previously described for the *N*-phthalyl derivative.

The resulting oil was methylated with diazomethane to give 1.1 g. of a yellow oil. Chromatography on silica gel, using chloroform as the eluting solvent, gave only one isolable compound. It was shown to be dimethyl 2-*N*-trifluoroacetylpentanedioate by comparison of its IR and NMR spectra with that of the corresponding phthalimido derivative; IR (chloroform): 1725 cm.$^{-1}$, no C≡N stretch; NMR ($CDCl_3$): δ 2.2–2.6 (m, 4, CH_2CH_2), 3.7 (s, 3, OCH_3), 3.8 (s, 3, OCH_3), and 4.7 (broad t, 1, methine H).

N,N*-Dibenzylglutamic Acid, V*f—The procedure of Kanao and Sakayari (8) to prepare 1-benzyl-5-oxo-pyrrolidinecarboxylic acid was modified to obtain *N,N*-dibenzylglutamic acid. Glutamic acid (22.05 g., 0.15 mole), 16 g. sodium hydroxide in 256 ml. of 60% ethanol, and benzyl chloride (40.64 g., 0.32 mole) were stirred for 48 hr. at 25° (heating the reaction leads to decreased yields). The solution was neutralized to approximately pH 7 with cold, dilute hydrochloric acid and the ethanol was removed. The solution was extracted with ether, and an insoluble material was filtered (5.4 g.). The aqueous solution was made acid to congo red paper with hydrochloric acid and allowed to stand for 4 hr. A white solid (11.6 g.) precipitated and was filtered. The two solids were combined, stirred with hot water (85°), and filtered. The remaining solid was recrystallized from ethanol to give 6.7 g. of *N,N*-dibenzylglutamic acid, m.p. 214–217° [lit. (8) m.p. 215°].

The aqueous filtrate was cooled and 5.1 g. of 1-benzyl-5-oxo-pyrrolidinecarboxylic acid was obtained, m.p. 157–159° [lit. (8) m.p. 160°].

N,N*-Dibenzylglutamic Anhydride, V*f—Acetic anhydride (35 ml.) was added to *N,N*-dibenzylglutamic acid (6.0 g., 0.018 mole). The resulting suspension was heated to 80°, whereupon a deep-blue solution was formed. The heating was discontinued and the solution was immediately cooled. The acetic anhydride was removed under reduced pressure. Trituration of the resulting blue solid with ether gave 4.9 g. of a white solid. Recrystallization from chloroform–ether gave 4.1 g. (74%) *N,N*-dibenzylglutamic anhydride, V*f*, m.p. 110–112°; IR (KBr): 1810, 1780, 1760, 1060, 1020, 740, and 700 cm.$^{-1}$; NMR ($CDCl_3$): δ 1.9–2.3 (m, 2, CH_2), 2.5–2.9 (m, 2, CH_2), 3.7 (t, 1, methine H), 3.85 (s, 2, benzyl CH_2), 3.95 (s, 2, benzyl CH_2), and 7.4 (m, 10, aromatic H).

Ethyl Methyl 2-Cyano-3-methoxy-4-*N,N*-dibenzylamino-2-heptenedioate, II*f*—To a suspension of sodium hydride (1.75 g. of 57%, 0.04 mole) in 250 ml. of anhydrous benzene was added 4.5 g. (0.04 mole) of ethyl cyanoacetate. The suspension was cooled, 12.4 g. (0.04 mole) of *N,N*-dibenzylglutamic anhydride, V*f*, was added, and the mixture was refluxed for 16 hr. After cooling, the benzene was removed and equal volumes (250 ml.) of water and ether were added to the reaction mixture. The ether layer was separated and concentrated to give 11.3 g. of a mixture of acids. The mixture of acids was dissolved in anhydrous ether and esterified with diazomethane to give 11.4 g. of material.

Column chromatography on silica (300 g.) of 8.7 g. of the material and elution with chloroform afforded 1.1 g. of dimethyl 2-*N,N*-dibenzylaminopentanedioate; IR (chloroform): 1725 cm.$^{-1}$; NMR ($CDCl_3$): δ 1.8–2.7 (m, 5, aliphatic CH), 3.3 4.2 (2s–m, 10, OCH_3–benzylic CH_2), and 7.35 (s, 10, aromatic H); mass spectroscopy: *m/e* 355 (parent peak) and 296 (base peak).

A second material (1.7 g.) was obtained as a pale-yellow oil and proved to be the desired compound, II*f*; IR (chloroform): 2225, 1720, 1575, 1440, 1130, and 700 cm.$^{-1}$; NMR ($CDCl_3$): δ 1.27 (t, 3, CH_3), 1.65–3.2 (m, 4, CH_2CH_2), 3.35–4.4 (overlapping peaks, 13, 2-OCH_3, 2-benzyl CH_2, CH_2CH_3, methine H), and 7.33 (m, 10, aromatic H); mass spectroscopy: *m/e* 450 (parent peak), 296 (base peak at 19–88°), and 392 (base peak at 129°).

Anal.—Calc. for $C_{26}H_{30}N_2O_5$: C, 69.33; H, 6.66; N, 6.22. Found: C, 69.03; H, 6.68; N, 6.36.

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Metabolites of Bromazepam, a Benzodiazepine, in the Human, Dog, Rat, and Mouse

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Abstract □ The biotransformation of bromazepam, 7-bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one, was studied in four species. Four metabolites were identified and their excretion was quantitatively determined. The major urinary metabolites excreted by three women given single 12-mg. oral doses of bromazepam-5-^{14}C were the conjugated forms of 3-hydroxybromazepam (I), accounting for 13–30% of the dose, and 2-(2-amino-5-bromo-3-hydroxybenzoyl)pyridine (IV), accounting for 4–25% of the dose. Conjugated I was also the major metabolite in three dogs and four groups of mice administered ^{14}C-bromazepam, but its excretion by four rats was very limited. Together with I, Metabolite IV and 2-(2-amino-5-bromobenzoyl)pyridine were detected in the excreta of the human, dog, rat, and mouse. Although the pyridyl *N*-oxide derivative of bromazepam was not detected as a metabolite in any species, the N_4-oxide was a minor metabolite in dog urine. This is the first reported instance of metabolic N_4-oxidation of a 1,4-benzodiazepin-2-one.

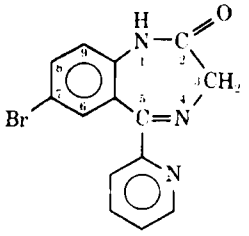
Keyphrases □ Bromazepam (a benzodiazepine) metabolism in human, dog, rat, and mouse, identification of metabolites, mechanisms discussed □ Benzodiazepine derivatives—metabolism of bromazepam in human, dog, rat, and mouse, identification of metabolites, mechanisms discussed □ Biotransformation—identification of bromazepam metabolites in human, dog, rat, and mouse □ Metabolites, bromazepam—identified in urine and feces of humans, rats, dogs, and mice

Bromazepam [7-bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one and formerly designated Ro 5-3350] was reported (1) to possess central nervous system activity in animals and antianxiety activity in humans. This drug is a member of the benzodiazepine class of compounds whose metabolism was recently reviewed (2).

In this study, the metabolites of bromazepam were identified and quantitated in various species to: (*a*) detect species differences in metabolic pathways, and (*b*) compare the biotransformation of bromazepam with that of other benzodiazepines. Bromazepam labeled with ^{14}C in either the C_2- or C_5-position was used. Bromazepam-5-^{14}C was preferred (but was not available for the initial studies in dog and mouse) because if metabolic cleavage of the diazepine ring occurred, resulting in the loss of atoms 2, 3, and 4, the label would remain with the benzoylpyridine moiety. A chemical cleavage of this ring by acid hydrolysis was used in the development of a GLC assay of bromazepam (3).

EXPERIMENTAL

Labeled Drug and Determination of ^{14}C—Prior to preparing each drug formulation, TLC with System 1 (described below) and the determination of the ^{14}C migrating with authentic bromazepam confirmed that the radiochemical purity of both C_5- and C_2-labeled bromazepam remained at 97 and 98%, respectively, throughout the study. Furthermore, at the end of the study, the compounds identified as metabolites were cochromatographed with each labeled bromazepam. Only one compound, 3-hydroxybromazepam (I), was found to be a possible contaminant of the labeled drugs,



bromazepam

but only to the extent of <0.7% of the ^{14}C of bromazepam-5-^{14}C and <0.3% of the ^{14}C of bromazepam-2-^{14}C.

The determination of radioactivity in urine, in homogenates of feces in 50% ethanol, in ethyl acetate extracts of both, and on particles of silica gel was accomplished by liquid scintillation spectrometry as previously described (4).

Design of Human and Animal Studies—Three Negro females[1] (Subjects 1, 2, and 3) were not given any medication chronically for at least 3 weeks prior to labeled bromazepam administration. After obtaining informed consent, each subject received two tablets, each containing 6 mg. of bromazepam-5-^{14}C with a specific activity of 4.3 μc./mg. The excipients (starch, lactose, microcrystalline cellulose[2], magnesium stearate, and FD&C certified dyes) and the type of tablet compression [single compression using a 0.86-cm. (0.34-in.) flat-faced punch] were chosen to duplicate the formulation and size of the tablets currently used in the clinical trials. Subject 1, 51 years of age and weighing 88 kg., developed influenza 4 days after the labeled drug was given and recovered without treatment with drugs. Subject 2, 36 years of age and weighing 57 kg., also developed influenza after a week on this study and was treated with phenazopyridine hydrochloride. Subject 3, 48 years of age and weighing 85 kg., received aspirin and hydrochlorothiazide (50 mg.) on several occasions but no chronic drug treatment during the study. Daily collections of urine and feces were made until the excretion of ^{14}C decreased to barely measurable levels.

Four nonfasted 11–12-kg. male dogs (beagles) were given single oral doses of ^{14}C-bromazepam in gelatin capsules. Dog 1 was given 5 mg./kg. of bromazepam-2-^{14}C (specific activity 2.3 μc./mg.), whereas the remaining dogs received the same dose of bromazepam-5-^{14}C (specific activity 1.0 μc./mg.). Urine and feces were collected daily for as long as readily measurable amounts of ^{14}C were excreted.

Bromazepam-5-^{14}C (1.0 μc./mg.) was dissolved in a vehicle consisting mainly of 50% propylene glycol, 10% ethanol, 1.5% benzyl alcohol, and an aqueous acetate buffer[3] and was administered to four nonfasted male Charles River rats at a dose of 2 mg./kg. Two 230-g. rats received an intravenous dose and two 285-g. rats were given the drug orally by intubation. Solutions of bromazepam-2-^{14}C (27 μc./mg.) and bromazepam-5-^{14}C (35 μc./mg.) in the same parenteral vehicle, after dilution with water to avoid toxicity due to the ingredients of the vehicle, were injected intravenously into nonfasted Carworth Farms 1S 20-g. male mice at a dose of 1.0 and 0.3 mg./kg., respectively. Two groups of three mice each (Groups I and II) received the C_2-labeled drug, and two groups (Groups III and IV) were given bromazepam-5-^{14}C. Each group was placed in a separate cage. Urine and feces were collected from the four rats and four groups of mice until excretion of ^{14}C was barely measurable.

[1] Hospitalized in the Special Treatment Unit, Newark Beth Israel Hospital, Newark, N. J.

[2] Avicel.

[3] This Hoffmann-La Roche Inc. formulation (F-31) was developed by Mr. H. Newmark.

Table I—Excretion of Radioactivity Derived from ^{14}C-Bromazepam by Human, Dog, Rat, and Mouse

Subject or Animal	Dose Administered and Route	Duration, Days	Excretion of ^{14}C in Percent of Dose Administered Urinary	Fecal	Total
Human					
1	12 mg. bromazepam-5-^{14}C p.o.	14	59.1	2.1	61.2
2	12 mg. bromazepam-5-^{14}C p.o.	11	74.0	2.0	76.0
3	12 mg. bromazepam-5-^{14}C p.o.	17	78.6	5.9	84.5
Dog					
1	5 mg./kg. bromazepam-2-^{14}C p.o.	24	46.7	18.5	65.2
2	5 mg./kg. bromazepam-5-^{14}C p.o.	10	52.0	25.8	77.8
3	5 mg./kg. bromazepam-5-^{14}C p.o.	4	15.5	58.7	74.2
4	5 mg./kg. bromazepam-5-^{14}C p.o.	10	37.0	27.0	64.0
Rat					
1	2 mg./kg. bromazepam-5-^{14}C i.v.	4	28.0	59.1	87.1
2	2 mg./kg. bromazepam-5-^{14}C i.v.	4	32.0	58.2	90.2
3	2 mg./kg. bromazepam-5-^{14}C p.o.	6	17.1	58.3	75.4
4	2 mg./kg. bromazepam-5-^{14}C p.o.	6	19.9	65.0	84.9
Mouse					
Group I	1 mg./kg. bromazepam-2-^{14}C i.v.	4	49.9	24.2	74.1
Group II	1 mg./kg. bromazepam-2-^{14}C i.v.	4	62.3	17.3	79.6
Group III	0.3 mg./kg. bromazepam-5-^{14}C i.v.	6	58.3	20.5	78.8
Group IV	0.3 mg./kg. bromazepam-5-^{14}C i.v.	6	60.9	19.7	80.6

Extraction of Labeled Metabolites—Aliquots of urine from each species and of fecal homogenates (further diluted 1:5 with water) from the dog, rat, and mouse, each containing at least 10^5 d.p.m. of ^{14}C, were extracted with ethyl acetate. Following the initial extraction at pH 9.0, the aqueous phase was incubated with a commercial preparation of β-glucuronidase and arylsulfatase[4], which was added to a final concentration of 1% (v/v). This incubation at pH 5.5 and 37° for 2–3 hr., which previously yielded maximum hydrolysis of conjugated benzodiazepine metabolites (5), was followed by serial extraction at pH 9.0, 7.0, and 2.0. All pH adjustments were made by the addition of dilute acid or alkali; at each designated pH, two consecutive volumes of ethyl acetate, each equaling the aqueous volume, were shaken with the aqueous phases for approximately 10 min. each. The combined extract at each pH was analyzed for ^{14}C and the extracts obtained at pH 9.0 were concentrated and examined by TLC.

TLC for Metabolite Separation, Identification, and Quantitation—Silica gel containing a fluorescent indicator[5] was used for TLC with the following solvent systems: 1, heptane–ethyl acetate–ethanol–concentrated ammonia (50:50:10:3); 2, heptane–chloroform–ethanol–concentrated ammonia (50:50:20:1); 2*a* and 2*b*, same solvents in the proportions of 25:75:10:0.5 and 50:50:50:1, respectively; 3, isopropanol–concentrated ammonia (100:1); 4, heptane–chloroform–ethanol (5:5:1); 5, ethyl acetate–methyl ethyl ketone–ethanol–concentrated ammonia (90:10:10:0.5); 6, benzene–ethyl acetate–ethanol–concentrated ammonia (80:20:20:1); 7, heptane–methylene chloride–ethanol–concentrated ammonia (50:50:25:1); 7*a*, same solvents in proportions of 50:50:50:1; 8, ethyl acetate–ethanol–concentrated ammonia (95:5:1); 9, chloroform–ethyl acetate–ethanol–concentrated ammonia (50:50:12:3); 10, heptane–benzene–ethanol–concentrated ammonia (50:50:20:0.5); 11, heptane–chloroform–ethanol–acetic acid (10:10:1:2); and 12, chloroform–ethanol–concentrated ammonia (95:5:0.5).

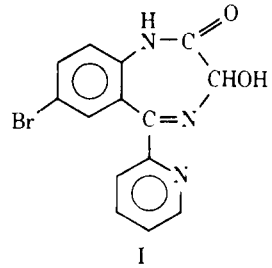
The use of one-dimensional TLC for metabolite separation followed by identification of the isolated metabolites by cochromatography with reference compounds or by spectroscopic techniques has been described (2). Individual urinary and fecal metabolites were determined by comparative two-dimensional TLC. In this analysis, both identification and quantitation result from the demonstration that, on two-dimensional TLC with at least two different solvent system pairs, a consistent amount of ^{14}C extracted from urine or feces migrates as a reference compound added as an internal standard. The solvent system pairs were designated by the number of each system in the order used, and the reference compounds were located by viewing the developed chromatoplate under shortwave UV light. Silica gel containing each reference compound was suspended in the fluor for the determination of ^{14}C.

In additional experiments, each identified metabolite added to water was shown by spectrophotometric assay to be quantitatively extracted at the pH used in its extraction from the biological sample. This demonstrated that no correction factor for incomplete extraction was required in calculating the excretion of each metabolite.

RESULTS

Excretion of Radioactivity by Each Species—The excretion of ^{14}C is summarized in Table I. The total excretion of ^{14}C by the human subjects ranged from 61 to 85% of the dose, with almost all of this radioactivity excreted in the urine. While the total excretion of ^{14}C by dogs was in this same range, a greater amount of the dose was excreted in the feces. The markedly high fecal excretion of orally administered ^{14}C by Dog 3 (59% of the dose) suggested that absorption of labeled bromazepam was impaired in this animal. In rats, the high fecal excretion of radioactivity was seen after both oral and intravenous administration of C_5-labeled drug, suggesting that biliary excretion was important in this species. In mice given either bromazepam-2-^{14}C or bromazepam-5-^{14}C intravenously, the excretion of radioactivity was similar to that obtained with man and dog; urinary excretion of ^{14}C predominated and the recovery of the dose was not complete.

The reason for the incomplete excretion of all of the administered radioactivity, most evident in the human and dog, is not clear at this time. Since this loss is not limited to the administration of the C_2-labeled drug, it is not due to extensive metabolic cleavage of the diazepine ring and elimination of the C_2-carbon as carbon dioxide.



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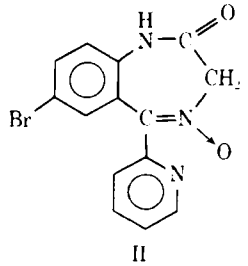

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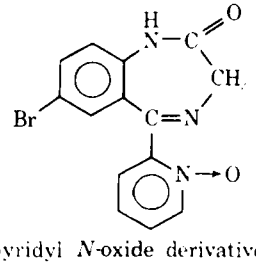


pyridyl *N*-oxide derivative

[4] Glusulase, Endo Labs., Garden City, N. Y.

[5] Precoated chromatoplates, Camag DF-B (Camag Inc., New Berlin, Wis.) and Q4F (Quantum Industries, Fairfield, N. J.), were used for two-dimensional TLC with Solvent Systems 5–6 and 10–6, respectively; chromatoplates prepared with SilicAR 7-GF-5 (Mallinckrodt Chemical Works, St. Louis, Mo.) were used with all other systems.

Table II—Metabolite Identification by Comparative Two-Dimensional TLC

Reference Compound	Labeled Metabolite Source[a]	TLC Systems	Percent of ^{14}C Migrating as Reference Compound
I	Dog 1 urine	1–2	95
		3–1	90
	Dog 2 urine	5–6	73
		8–9	80
		2*b*–7*a*	74
IV	Subject 3 urine	10–6	44
		2*a*–3	45
		2–11	45
III	Dog 2 feces	5–6	12
		2–7	14
		8–9	12

[a] Ethyl acetate extracts obtained by extraction at pH 9.0 of the glusulase-treated urine or feces were cochromatographed with reference compounds.

Apparently, a portion of the drug-derived ^{14}C is excreted very slowly.

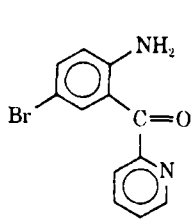
Identification of Urinary Metabolites—Reference compounds of interest were 3-hydroxybromazepam [I, 7-bromo-1,3-dihydro-3-hydroxy-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one], 4-oxide derivative [II, 7-bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one 4-oxide], pyridyl *N*-oxide derivative [7-bromo-1,3-dihydro-5-(2-pyridyl-2-oxide)-2*H*-1,4-benzodiazepin-2-one], 2-(2-amino-5-bromobenzoyl)pyridine (III), and 2-(2-amino-5-bromo-3-hydroxybenzoyl)-pyridine (IV). Evidence for I being a metabolite had been obtained in an earlier investigation (unpublished) of bromazepam metabolism in the dog. Evidence that IV is a urinary metabolite in the dog and rabbit was reported (6) during the present studies. Compound II and the pyridyl *N*-oxide derivative allowed differentiation between metabolic oxidation at the 4-nitrogen and pyridyl nitrogen, respectively.

Preliminary one-dimensional TLC of extracts of dog urine suggested that I, III, and IV were metabolites. Representative results from two-dimensional TLC, which confirmed their status as metabolites and allowed for their quantitation, are shown in Table II. The consistent migration of ^{14}C with the designated reference compound in extracts chosen because of their relatively high content of the metabolite in question is evident.

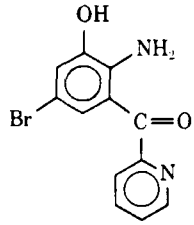
Also detected in the initial examination of dog urine by one-dimensional TLC was a directly extractable metabolite, which was purified first by TLC with System 1 and then by TLC of the eluted metabolite with System 2. The spectra resulting from low-resolution mass spectrometry[6] of a 4-mcg. sample of this metabolite showed that oxygen had been added to the bromazepam molecule and indicated, from the strong loss of *m/e* 16, that this metabolite was either the 4-oxide (II) or a pyridyl *N*-oxide derivative of bromazepam. Two-dimensional TLC, in which the isolated metabolite was cochromatographed with each isomer, clearly supported the identification of this metabolite as II.

This result was unexpected because metabolic *N*-oxidation of a benzodiazepine at the 4-position had not previously been reported. Further evidence for this structure was obtained by characterizing the benzoylpyridine formed on acid hydrolysis of the metabolite. A 50-mcg. sample of isolated metabolite was hydrolyzed in 6 *N* sulfuric acid, and the benzoylpyridine was extracted as previously described (3). When tested by TLC (System 4) and GLC[7] [using a 1.82-m. (6 ft.) 3% OV-17 column with inlet, column, and flame-ionization detector temperatures of 275, 240, and 290°, respectively], the metabolite hydrolysis product chromatographed as authentic III, supporting the identification of the intact metabolite as II.

The pyridyl *N*-oxide derivative was sought as a urinary or fecal metabolite in all four species investigated. However, no measurable or consistent amount of the ^{14}C of any extract examined migrated with this reference compound, thus indicating that it was not a metabolite of bromazepam.



III



IV

Quantitative Profile of Bromazepam and Metabolite Excretion—The extractability of the urinary metabolites of each species is presented in Table III. The urinary metabolites of man and dog are excreted mainly as conjugates hydrolizable by glusulase to labeled compounds extractable at pH 9.0. This is not true for the rat and mouse. In the rat, roughly one-quarter of the urinary ^{14}C is extractable at pH 9.0, both before and after glusulase treatment; in the mouse, the directly extractable ^{14}C represents the major metabolite fraction. An appreciable amount of urinary ^{14}C is extracted at pH 2.0 in both the rat and mouse. Fecal samples from dogs, rats, and mice were also extracted and, in general, less ^{14}C was extractable at each pH than was found for the respective urine samples.

The metabolites were quantitated in the pH 9.0 extracts obtained before and after glusulase treatment. As seen in Table IV, the excretion of unchanged bromazepam by each species accounted for only 1–2% of the dose. The major urinary metabolites excreted by the human subjects were the conjugated forms of I and IV. Subject 2 differed from the other two in excreting a larger amount of I (31% of the dose) and a smaller amount of IV (only 4% of the dose).

In Dog 3, in which most of the ^{14}C was excreted in the feces, 73% of the fecal ^{14}C was found to be unchanged bromazepam. Since the absorption of bromazepam in this animal was severely limited, only data from the other three dogs were used to compile the average excretion values shown in Table IV. The major urinary metabolite was conjugated I (20% of the dose). A small amount of urinary II was also excreted by dogs, and the dog was the only species in which measurable amounts of II were found in the urine.

Compound I was not excreted as a major metabolite by the rat, in contrast to the human and dog. Since only small amounts of III and IV were also found in the urine, most of the ^{14}C excreted by rats remained unidentified. The dominant metabolite excreted by mice was I, accounting for 36% of the dose. However, in contrast to its excretion mainly as a urinary conjugate by the human and dog, urinary I was excreted by the mouse primarily unconjugated. Very little III and IV and no detectable amounts of II were excreted by the mice; almost all radioactivity not excreted as I remained unidentified.

DISCUSSION

The excretion of conjugated 3-hydroxylated derivatives is prominent in humans and dogs, but not in rats, administered various 1,4-benzodiazepin-2-ones (2). The present results show that bromazepam also fits this pattern. In addition, the finding that Metabolite I, although mostly unconjugated, is a major urinary metabolite of bromazepam in the mouse suggests that this species metabolizes bromazepam as do the human and dog. The difference in the pattern of metabolites excreted by the mouse and rat may hold true for benzodiazepin-2-ones in general. Recently, a considerable fraction of an intravenous dose of either diazepam[8] or desmethyldiazepam was found (7) to be secreted in the bile of the mouse, but not in the bile of the rat, as the conjugated 3-hydroxy metabolite, oxazepam[9].

In the rat the preferred metabolic reaction apparently is hydroxylation of benzodiazepines at the *para*-position of the 5-phenyl ring (2). This is also true for oxazepam metabolism in the rat (8). However, since the major metabolites of bromazepam in the rat were not

[6] A Consolidated Electronics Corp. 21-110 mass spectrometer produced the spectra, which were interpreted by Ms. J. Pao and Dr. W. Benz.

[7] Micro-Tek gas chromatograph, model MT-220, Tracor, Inc., Austin, Tex.

[8] Diazepam is the active ingredient in Valium, Hoffmann-La Roche Inc., Nutley, N. J.

[9] Oxazepam is the active ingredient in Serax, Wyeth Labs., Inc., Radnor, Pa.

Table III—Extractability of the Urinary Radioactivity Excreted by Human, Dog, Rat, and Mouse[a]

Serial Extraction with Ethyl Acetate	Percent of Urinary Radioactivity in Each Extract[b]			
	Human (3)[c]	Dog (4)[c]	Rat (4)[c]	Mouse (4)[c]
Preglusulase at pH 9.0	6.4 ± 0.75	10 ± 1.9	24 ± 2.8	48 ± 1.9
Postglusulase at pH 9.0	63 ± 7.4	63 ± 9.7	21 ± 6.2	22 ± 2.4
at pH 7.0	6.5 ± 2.0	5.6 ± 1.0	8.0 ± 3.6	3.1 ± 0.2
at pH 2.0	9.2 ± 1.8	4.0 ± 1.1	18 ± 2.5	17 ± 2.2
Total	85	83	71	90

[a] In each instance, the sample extracted was pooled urine representing over 90% of the final amount of ^{14}C eliminated in the urine. [b] Since the extractability within each species appeared to be unaffected by the position of ^{14}C labeling or the route of ^{14}C-bromazepam administration, the mean ± standard deviation is presented. [c] Number of individual subjects or animals whose urine was extracted.

Table IV—Excretion of Bromazepam and Metabolites by Human, Dog, Rat, and Mouse

Excreted Component	Percent of Dose Excreted[a]								
	Human Urine			Dog (3)		Rat (4)		Mouse (4)	
	Subject 1	Subject 2	Subject 3	Urine	Feces	Urine	Feces	Urine	Feces
Bromazepam	1.1	1.2	1.8	Negl.[b]	1.6 ± 0.2	0.7 ± 0.4	1.0 ± 0.3	0.6 ± 0.1	0.5 ± 0.1
I (Nonconjugated)	0.5	1.6	0.7	Negl.	1.7 ± 0.7	1.6 ± 0.3	Negl.	22.7 ± 2.6	4.1 ± 0.9
I (Conjugated)	12.8	29.8	12.5	19.9 ± 8.9	1.3 ± 0.5	Negl.	Negl.	8.2 ± 1.3	1.1 ± 0.4
II (Nonconjugated)	Negl.	Negl.	Negl.	2.5 ± 1.4	Negl.[c]	Negl.	Negl.	Negl.	Negl.
III[d]	1.7	Negl.	3.5	Negl.[c]	0.9 ± 0.4[c]	0.6 ± 0.1	Negl.	1.6 ± 0.3[c]	Negl.
IV[e]	10.2	3.9	24.6	2.5 ± 1.8[c]	1.7 ± 1.6[c]	1.4 ± 0.6	Negl.	0.9 ± 0.1[c]	Negl.
Unidentified ^{14}C	31.4	36.8	33.4	19.6 ± 1.8	16.7 ± 3.0	18.2 ± 5.6	55.8 ± 5.7	22.0 ± 2.0	13.2 + 1.9
Total ^{14}C	57.7	73.3	76.5	44.5 ± 7.7	23.9 ± 4.6	22.5 ± 6.6	56.8 ± 5.6	56.0 ± 5.1	18.9 ± 2.9

[a] The values presented, individual values for the three subjects and mean ± standard deviation for the number of animals shown in parentheses for each species, were obtained from the analysis of pools of urine or feces which accounted for at least 90% of the ^{14}C excreted by each route. In addition to the solvent systems shown in Table II, System 11–12 was used (particularly in the quantitation of Metabolite IV). [b] Negl. indicates that less than 0.5% of the dose was excreted. [c] Average of results from two animals. [d] Compound III was found mainly in the postglusulase extract, indicating its apparent excretion as a conjugate. [e] Compound IV was found mainly in the postglusulase extract of urine and in the preglusulase extract of feces.

identified, there is no evidence for hydroxylation of the 5-pyridyl ring in this species.

Metabolic cleavage of the diazepine ring of medazepam[10] (9) and nitrazepam[11] (10) to form both a benzophenone and a phenolic benzophenone hydroxylated *ortho* to the amino function has been found in the human. Therefore, the excretion of III and IV as metabolites of bromazepam in the human is not surprising. From the magnitude of conjugated IV excretion by the three women (4, 10, and 25% of the dose), it is evident that both 3-hydroxylation and benzoylpyridine formation must be considered important pathways in the human.

One novel pathway, the formation and urinary excretion of bromazepam N_4-oxide (II), occurred in the dog but not in the human, rat, or mouse. Although the reduction of the N_4-oxide function of a benzodiazepin-2-one 4-oxide (demoxepam) was shown to occur in the dog (11), oxidation of this diazepine ring nitrogen has not been reported until now. Interestingly, the pyridyl nitrogen of bromazepam was apparently not oxidized since the pyridyl *N*-oxide derivative was not detected as a metabolite in any of the four species. Whether or not N_4 oxidation is a metabolic reaction unique to bromazepam cannot be known until a thorough search for the corresponding N_4-oxide metabolites of other benzodiazepines is made.

The present quantitative findings are similar to recent data[12] obtained from two human subjects given 6 mg. of bromazepam-5-^{14}C intravenously; the subjects excreted 10 and 15% of the dose in the urine as I (mostly conjugated) and 16% as IV (again mostly conjugated) in 24 hr. In addition, the rat excreted I, III, and IV as urinary metabolites, with IV, at 4–5% of the dose, being excreted to a greater extent than the other two.

On the basis of the results obtained to date, it is apparent that the metabolites excreted by humans were also excreted to some extent by the dog, rat, and mouse, whereas only the dog excreted II.

[10] Medazepam is the active ingredient in Nobrium, F. Hoffmann–La Roche and Co., A. G., Basle, Switzerland.

[11] Nitrazepam is the active ingredient in Mogadon, F. Hoffmann–La Roche and Co., A. G., Basle, Switzerland.

[12] J. Raaflaub and J. Speiser, F. Hoffmann–La Roche and Co., Basle, Switzerland, unpublished data.

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▲ To whom inquiries should be directed.

Identification of Urinary Metabolites of *p*-Phenoxyphenol Methanesulfonate in Rat and Man

F. F. SUN

Abstract □ The urinary metabolites of *p*-phenoxyphenol methanesulfonate (I) in rat and man were characterized. The drug was extensively metabolized in both species and excreted mainly in the form of conjugated phenolic compounds. The major metabolite of I in both species was identified as the conjugated monohydroxylated compound, *p*-(4-hydroxyphenoxy)phenol methanesulfonate (II). Three minor metabolites were also characterized and tentatively identified as: (*a*) a dihydroxylated derivative, probably *p*-(3,4-dihydroxyphenoxy)phenol methanesulfonate (III); (*b*) a hydroxy and methoxy derivative, probably *p*-(4-hydroxy-3-methoxyphenoxy)phenol methanesulfonate (IV); and (*c*) a dihydroxy and methoxy derivative (V). The exact positions of the aromatic substituents in these three compounds were not rigorously established. Compounds II and IV were found in both free and conjugated forms, but Compounds III and V were found in the conjugated form only. Qualitatively, the metabolites of I found in rat and human urine were similar. The hypolipidemic activity of II was equivalent to that of the parent compound.

Keyphrases □ *p*-Phenoxyphenol methanesulfonate—identification of urinary metabolites, rat, man □ Urinary metabolites—*p*-phenoxyphenol methanesulfonate, rat, man

p-Phenoxyphenol methanesulfonate[1] (I, $C_{13}H_{12}O_4S$, mol. wt. 264) has been shown to be an active hypocholesterolemic agent in both surfactant[2]-treated and dietary cholesterol and cholic acid-fed rats. Its pharmacological properties were reported previously (1). To study the absorption, metabolism, and excretion of this compound in animals and man, a GLC method for measuring the intact drug was developed. Little, if any, unchanged drug was found in the urine of I-treated rats. Analysis of the urine specimens, using TLC techniques, indicated that the compound was metabolized to several products. Therefore, studies were initiated to: (*a*) identify the major urinary metabolites of this compound in rats and man, and (*b*) determine the biological activities of the major metabolites in rat and man.

EXPERIMENTAL

Dosage and Collection of Samples *Rat*—Micronized I was suspended in 0.25% methylcellulose in sterile water with a glass Teflon homogenizer. The suspension was administered orally to five Sprague–Dawley rats at a dose of 100 mg./kg. Urine samples were collected quantitatively for 24 hr. postadministration. A second group of five rats, dosed with the vehicle only, served as controls.

Man—Five subjects each received orally a single 1-g. dose of I in hard filled capsules. Urine specimens were collected quantitatively 24 hr. postadministration. Urine specimens collected prior to drug administration were used as controls.

Method—All urine specimens were extracted and analyzed with a previously described GLC–mass spectrometric procedure (2). The pooled urine samples were acidified to pH 3.5 with acetic acid and extracted six times with chloroform. The chloroform layers were separated and concentrated under reduced pressure. The extracted urine was heated at 45° for 1 hr. under reduced pressure to remove the dissolved chloroform and then hydrolyzed with a mixture of β-glucuronidase and aryl sulfatase[3] in 0.1 *M* sodium acetate buffer at pH 5.0 for 16 hr. at 37°. The hydrolyzed urine samples were then extracted five times with chloroform. The chloroform layers were separated, combined, and evaporated to dryness. Control urine was processed in the same manner.

The extracted residues from the unhydrolyzed and hydrolyzed urine specimens were chromatographed on silica gel G-254 thin-layer plates, using chloroform–methanol (95:5) as the developing solvent. The chromatograms were examined under UV light, and zones that appeared on the plates with the drug-treated urine extract but not on the plates of the control were scraped and eluted with chloroform–methanol (4:1). The purified metabolite was evaporated to dryness and silylated with bis(trimethylsilyl)acetamide before injection into a gas chromatograph–mass spectrometer[4], equipped with a 1.21-m. (4-ft.) column of 1% SE-30 on 80–100-mesh Gas Chrom Q. The oven was operated isothermally at 200° and the ionization potential was set at 70 ev. Carrier gas (helium) flow was kept at 60 ml./min.

RESULTS

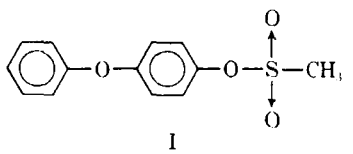
A gas chromatogram of the crude extract from β-glucuronidase–aryl sulfatase-hydrolyzed human urine is shown in Fig. 1. Three peaks, with retention times of 1.55, 2.4, and 2.85 min., were found in the drug-treated urine extract but were absent in the control. The small peak found at the retention time of 3.9 min. was not related to the drug. Unchanged I, which should have a retention time of 0.66 min., was not detectable in this analysis.

Figure 2 shows a thin-layer chromatogram of the same extract. Again three components are present in the urine extract from drug-treated human subjects but not in the control. Each zone was scraped off the plate, eluted with solvent, derivatized with bis(trimethylsilyl)-acetamide, and injected into the gas chromatograph–mass spectrometer. Four drug-related compounds were identified. Two metabolites were found in the more polar zone (R_f 0.49). The identification of these compounds is described in the following sections.

***p*-(4-Hydroxyphenoxy)phenol Methanesulfonate**—The *p*-hydroxylated metabolite of I (Compound II) separated readily from partially purified urine extracts as white flaky crystals. The isolated material, m.p. 128–129°, appeared 99% pure as judged by GLC. Elemental analysis indicated that the compound had an empirical formula of $C_{13}H_{12}O_5S$.

Anal.—Calc. for $C_{13}H_{12}O_5S$: C, 55.71; H, 4.29; S, 11.43. Found: C, 55.82; H, 4.58; S, 11.35.

The UV spectrum was similar to the parent drug except the weaker bands (271 and 277 nm. in the unchanged drug) were shifted 5 nm. to the longer wavelengths. Addition of base (0.1 *N* NaOH) to the ethanolic solution shifted the strong absorption band (230 nm. in the unchanged drug) 12 nm. to the long wavelength side (242 nm. in the metabolite), indicating the presence of a monohydric phenol (3). In the IR spectrum of the metabolite, the 695 cm.$^{-1}$ band of the unchanged drug (monosubstituted phenyl) was absent, and strong absorption bands were observed at 820 (*p*-substituted phenyl), 1195 (phenol C—O vibration), and 3460 (OH vibration) cm.$^{-1}$, indicating good agreement with the structural assignment. The NMR spectrum



I

[1] U-22,105.
[2] Triton, oxyethylated tertiary octylphenol formaldehyde polymer.
[3] Sigma type H-2 β-glucuronidase No. G-0876.
[4] LKB-9000.

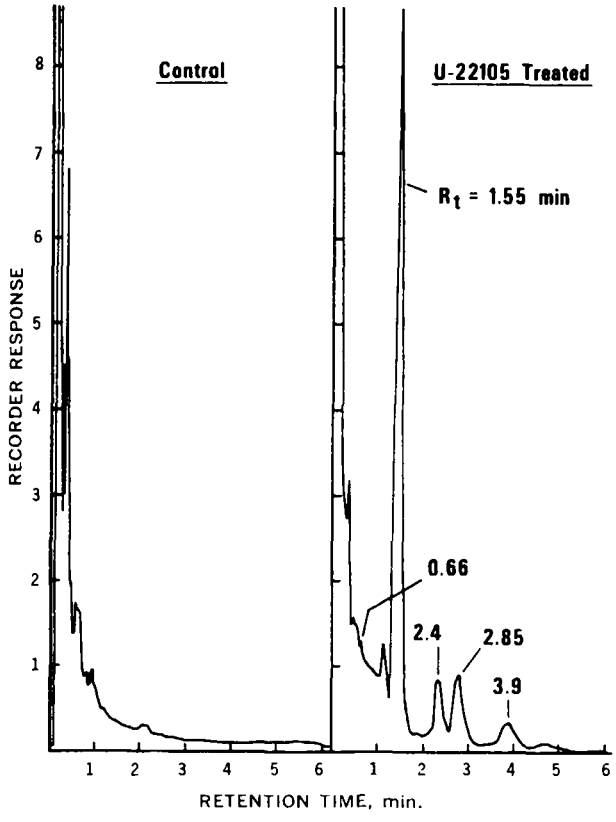


Figure 1—*Gas–liquid chromatograms of extracts from β-glucuronidase–aryl sulfatase-hydrolyzed human urine.*

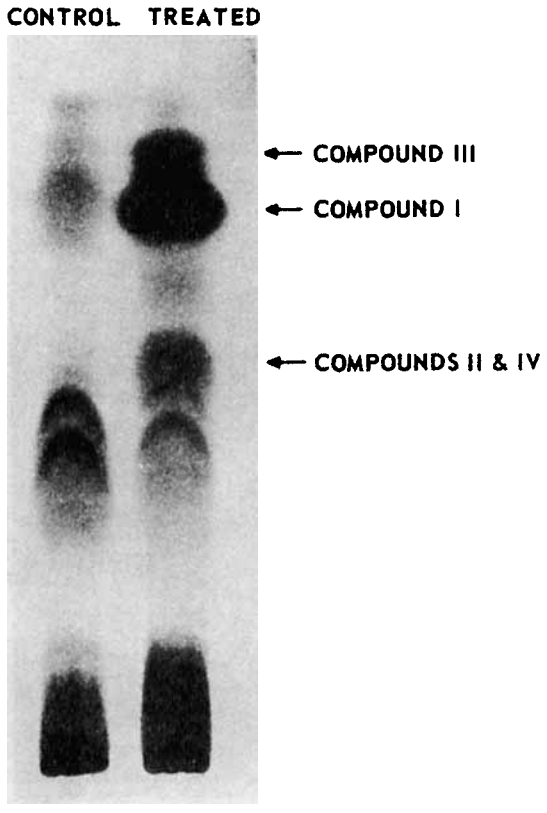
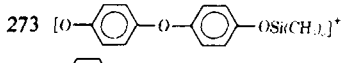
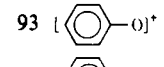


Figure 2—*Thin-layer chromatograms of extracts from β-glucuronidase–aryl sulfatase-hydrolyzed human urine.*

showed two sets of *AA'BB'* multiplets, indicating that the hydroxyl substitution was *para*. Furthermore, the isolated material gave a strong positive response to bipyridyl ferric chloride reagent (4), which is characteristic of phenolic compounds. Conclusive identification of the isolated material was obtained from mass spectral analyses. The isolated Compound II, Fig. 3*a*, showed a strong mass ion at 280 (molecular ion) and fragmentation ions at 201, 173, 145, 117, 93, and 65. The loss of methanesulfonate (M − 79 = 201) was characteristic of all compounds related to I. Subsequent loss of mass 28 (CO) was characteristic of phenols. The assignment of major fragments is summarized in Table I. The trimethylsilylated derivative of I (Fig. 3*b*) showed a similar type spectrum, except all mass peaks were found 72 mass units higher. Therefore, it was concluded that this compound was the *p*-hydroxylated metabolite of I.

Table I—Common Mass Peaks Appearing in *p*-Phenoxyphenol Methanesulfonate-Related Compounds

m/e	Structure	Comments
M − 79	Loss of $-SO_2CH_3$	Appears in all spectra
M − 28	Loss of CO	Appears in all spectra
M − 15	Loss of CH_3	Appears in all trimethylsilyl ethers and all phenyl methyl ethers
M − 30	Loss of HCHO	Appears in all phenyl methyl ethers
M − 88	Loss of C_3H_8OSi	Appears in trimethylsilyl ethers of dihydric phenols
273		Appears in all trimethylsilyl ethers
93		Appears in all free phenolic compounds
77		Appears in all spectra
65		Appears in all spectra

Compound II was subsequently synthesized by reacting 4,4'-oxydiphenol with methanesulfonyl chloride in pyridine at 0°. The reaction mixture was poured into a mixture of ice water and extracted with chloroform. Compound II was isolated from the extract by silica gel column chromatography. The authentic material had identical chromatographic and spectroscopic (UV, IR, NMR, and mass) characteristics to the isolated material. The hypolipidemic activity of II was tested in the surfactant[2]-induced hypercholesterolemic rats and was found to be equivalent to that of the parent I.

In the urine of both species, the major portion of II existed as conjugates. A trace amount was also found in the free form.

Dihydroxy Metabolite—Compound III was a minor metabolite found only in conjugated form. The amount present in the urine was insufficient for isolation. This compound gave a brownish-red spot on the thin-layer plate when sprayed with bipyridyl ferric chloride reagent, indicating that it was a phenol. The mass spectrum of the bis(trimethylsilyl) derivative is shown in Fig. 4. The molecular ion was observed at *m/e* 440, and strong fragmentation ions were observed at 361 (M − 79) and 273 [M − 79 − $(CH_3)_3SiO$]. Subsequent loss of 28 mass units (CO) was not as prominent as in II. The positions of the two hydroxyl groups were difficult to assign. However, since the predominant product produced by the enzymatic hydroxylation of *p*-substituted phenol is catechol (6), the structure of III was probably *p*-(3,4-dihydroxyphenoxy)phenol methanesulfonate.

Hydroxy Methoxy Metabolite—Compound IV was found both free and conjugated in the urine of rat and man. It was isolated by preparative TLC from the mother liquor remaining after crystallization of I. Attempts to crystallize IV were unsuccessful. The mass spectrum of IV (Fig. 5*a*) showed a strong molecular ion at mass 310. Loss of methanesulfonate (M − 79) and subsequent loss of 28 mass units (CO) identified IV as a drug-related compound. In the mass spectrum of the trimethylsilyl derivative (Fig. 5*b*), prominent ions were observed at 382 (M^+) and 303 (M − 79). In both spectra, loss of 30 (CH_2O) or 31 (CH_3O) mass units from the molecular ion or the M − 79 ion indicated the presence of a phenyl methyl ether linkage (8). The IR spectrum of IV was quite similar to that of II. Strong bands, observed at 3460 $cm.^{-1}$, were assigned to the phenolic OH. The aromatic system and the methanesulfonate group of the parent

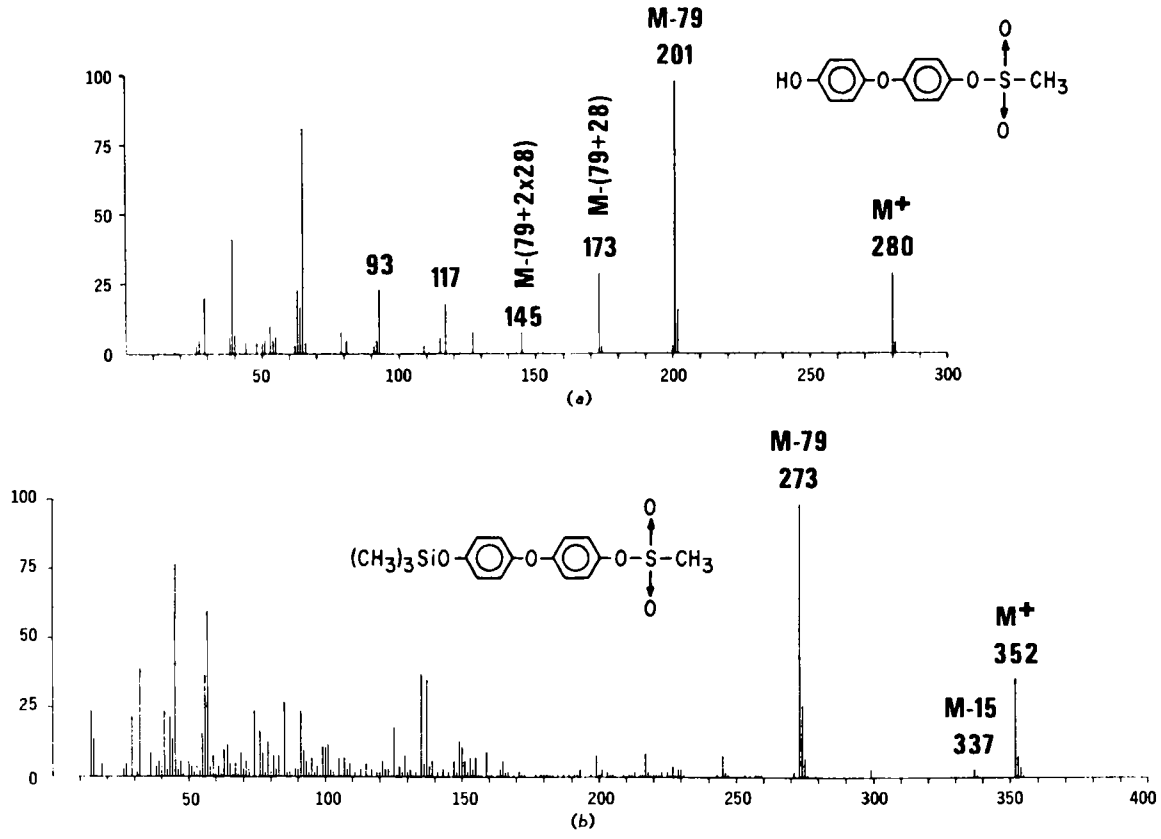


Figure 3—*Mass spectra of Compound II. Key:* (a), *free phenol; and* (b), *trimethylsilyl derivative.*

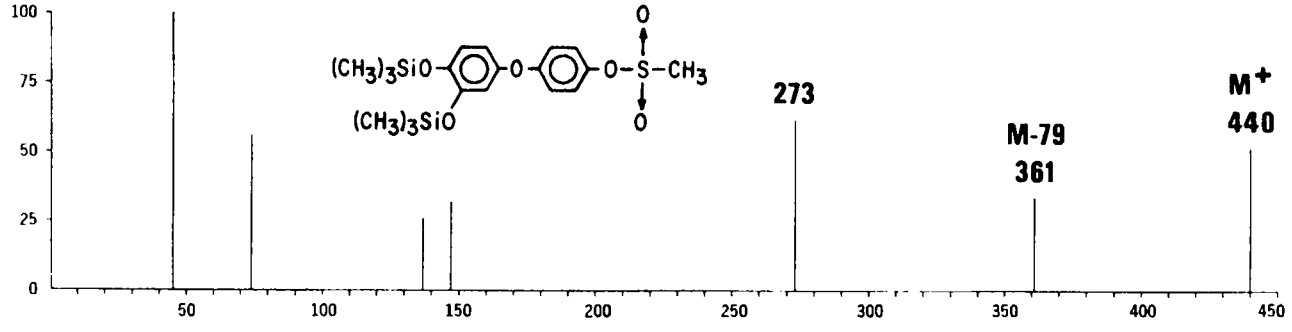


Figure 4—*Mass spectrum of bis(trimethylsilyl) derivative of Compound III.*

compound were still present. Neither the presence of methoxy nor the position of aromatic substitution could be conclusively established by IR spectroscopy.

NMR spectroscopy indicated the presence of a methoxy group *ortho* or *meta* to a *para*-substituted hydroxy group on the phenoxy ring. The methoxy group showed a singlet at δ 3.78 and shifted only one of the aromatic protons (*meta* to the methoxy) upfield to δ 6.9. An unperturbed *AA'BB'* multiplet centered at δ 7.21 indicated that the phenol ring was still intact. Since the predominant product from enzymatic hydroxylation of phenol is catechol (9) and since catechol-*O*-methyltransferase will not methylate resorcinol (8), it is unlikely that the methoxy group would substitute *meta* to the free *para*-substituted hydroxy group on the phenoxy ring. Therefore, IV is tentatively assigned a 4-hydroxy-3-methoxyphenoxyphenol molecular structure.

Dihydroxy Methoxy Metabolite—Compound V, a minor metabolite of I, could not be isolated in pure form. Evidence for its presence was obtained solely from GLC–mass spectrometric analysis of crude urine extracts. It was observed in the urine of both species only in conjugated form. After silylation with bis(trimethylsilyl)-acetamide, a strong mass ion at *m/e* 470 (Fig. 6) was observed corresponding to the disilylated derivative of Compound V. Loss of methanesulfonate (M − 79) and carbon monoxide (M − 28) indicated the presence of a drug-related compound. The presence of methoxy group was confirmed by the loss of 30 (CH_2O) mass units.

DISCUSSION

These results indicate that orally administered I followed typical metabolic pathways for aromatic compounds (9) and produced a group of phenolic compounds and their conjugates (Scheme I). The major routes of metabolism were: (*a*) hydroxylation of the phenoxy ring, and (*b*) conjugation of the resulting phenols with glucuronic acid or sulfate. A small portion of the hydroxylated I was further metabolized to yield the corresponding catechol, which underwent *O*-methylation to the methoxyphenol derivatives. Essentially all of the metabolites were excreted as either glucuronic acid or sulfate conjugates.

Since the NMR spectra of IV could not be factored with certainty because of the overlapping of the two highly coupled sets of aromatic hydrogens, plus the fact that not enough quantities of III and V were isolated for detailed NMR study, the exact positions of the aromatic substituents in III–V were not rigorously established. However, the limited information obtained from the NMR study of IV provided some clues for further speculation. As described

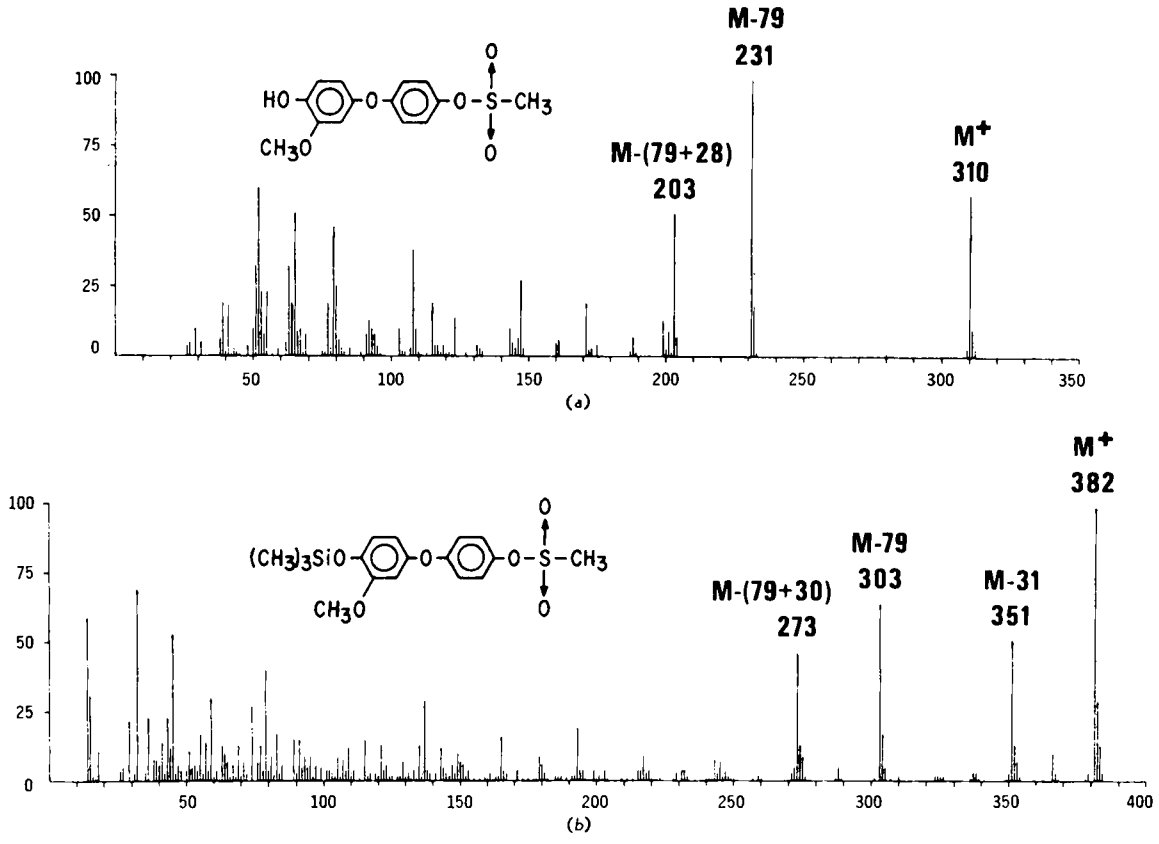
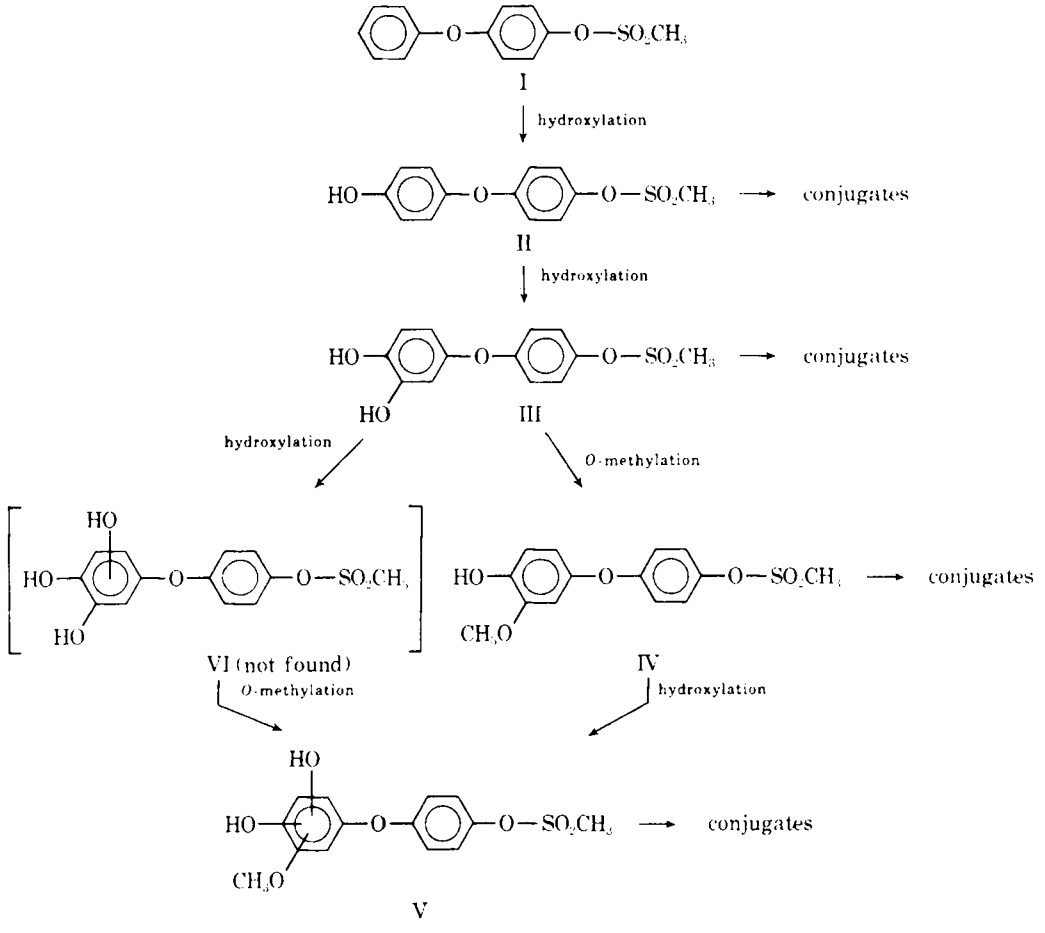


Figure 5—*Mass spectra of Compound IV. Key:* (a), *free phenol; and* (b), *trimethylsilyl derivative.*



Scheme I—Proposed metabolic pathways of I in rat and human

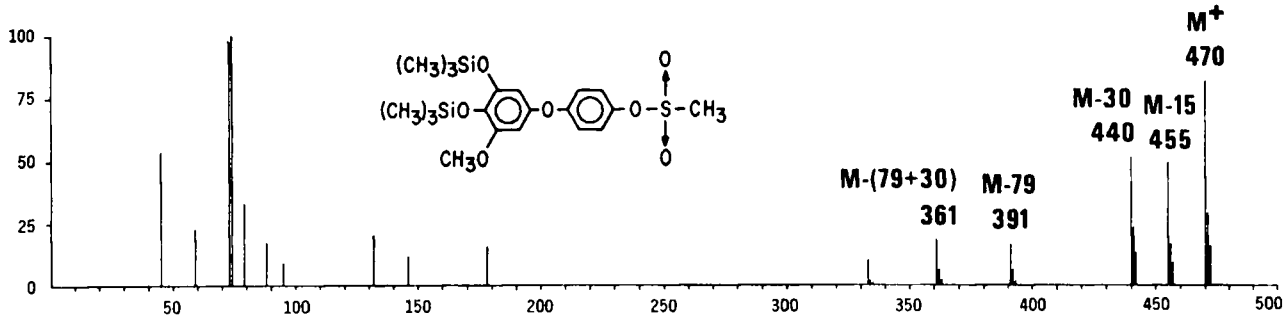


Figure 6—*Mass spectrum of bis(trimethylsilyl) derivative of Compound V.*

previously, the most probable structure for IV appeared to be 4-methoxy-3-hydroxyphenoxyphenol methanesulfonate. If this structure is correct, then III, presumably the precursor of IV, should have the structure of 3,4-dihydroxyphenoxyphenol methanesulfonate. The catechol structure would also be in line with the predicted hydroxylation product of II, since it was shown (6) that the principal hydroxylation product of *p*-substituted monophenol is the corresponding catechol. For example, *p*-cresol, *p*-nitrophenol, and *p*-chlorophenol are all hydroxylated to the corresponding catechol derivatives in rabbits (9). The structure of Compound V was even more obscure. It could be the hydroxylation product of IV or generated from III by hydroxylation followed by *O*-methylation. An unequivocal identification must await the synthesis of all possible isomers for comparison.

In view of the absence of unchanged drug in the urine, the rate of metabolism must be much faster than the rate of excretion. Quantitatively, the monohydroxylated compound (I) was the most abundant metabolite in the urine. In the human, the 24-hr. post-administration urinary excretion of I accounted for about 15–30% of the dose.

Since radioisotopically labeled I is not available, the extraction efficiency of drug-related materials from the urine is unknown. The possibility exists that some extremely water-soluble metabolite(s) of the parent drug was not found because of unfavorable partition coefficients between chloroform and water, for example, the trihydroxylated metabolite (VI), which would be the precursor of IV if it resulted from metabolized hydroxylation of III.

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Mesoionic Purinone Analogs IV: Synthesis and *In Vitro* Antibacterial Properties of Mesoionic Thiazolo[3,2-*a*]pyrimidin-5,7-diones and Mesoionic 1,3,4-Thiadiazolo[3,2-*a*]pyrimidin-5,7-diones

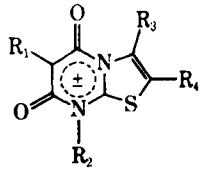
R. A. COBURN[▲] and R. A. GLENNON

Abstract □ Derivatives of two members of a new and unusual class of heterocycles, termed mesoionic purinone analogs, were synthesized and examined for *in vitro* antibacterial activity. Mesoionic thiazolo[3,2-*a*]pyrimidin-5,7-diones and mesoionic 1,3,4-thiadiazolo[3,2-*a*]pyrimidin-5,7-diones, which are isoconjugate with xanthine, were found to exhibit antibacterial activity against both Gram-negative and Gram-positive organisms. Several mesoionic 1,3,4-thiadiazolo[3,2-*a*]pyrimidin-5,7-diones possess significant levels of activity against *Proteus vulgaris* and *Staphylococcus aureus*.

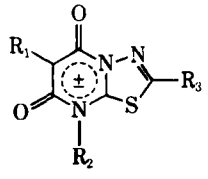
Keyphrases □ Mesoionic purinone analogs—synthesis and antibacterial properties of mesoionic thiazolo (or 1,3,4-thiadiazolo) [3,2-*a*]-pyrimidin-5,7-diones □ Mesoionic thiazolo (and 1,3,4-thiadiazolo) [3,2-*a*]pyrimidin-5,7-diones—synthesis and antibacterial screening □ Xanthine (mesoionic) analogs—synthesis and antibacterial properties of mesoionic thiazolo (and 1,3,4-thiadiazolo) [3,2-*a*]pyrimidin-5,7-diones □ Antibacterial activity—synthesis and screening of mesoionic thiazolo (or 1,3,4-thiadiazolo) [3,2-*a*]pyrimidin-5,7-diones

Formulation of a large, virtually unknown, class of mesoionic compounds, which are isoelectronic and isosteric to the purinones, was described previously (1, 2). They have been termed mesoionic purinone analogs because they cannot be satisfactorily represented by *any* covalent or single dipolar valence bond structure. Due to the large number of such analogs[1], they have been divided into two classes; Class I analogs are based upon known five-membered ring mesoionic systems (1, 3), and Class II analogs are based upon known six-membered ring mesoionic systems (2, 4). These two classes are further subdivided into xanthine, hypoxanthine, and purin-2-one analogs.

The compounds in this report, mesoionic thiazolo-[3,2-*a*]pyrimidin-5,7-diones[2] (I) and mesoionic 1,3,4-thiadiazolo[3,2-*a*]pyrimidin-5,7-diones[3] (II), are Class II mesoionic xanthine analogs. Quantum chemical studies of this subclass were reported (2), and convenient synthetic routes for their preparation were described (5). Molecular orbital studies of these analogs, in comparison with their covalent isomers, indicated an enhanced degree of chemical reactivity, an increase in charge separation and dipole moment, and an increase in electron affinity, which suggests an enhanced ability to participate in charge-transfer interactions as electron acceptors. Chemical studies (5) of the reaction of these compounds with amines suggested their potential function as selective acylating agents.



I



II

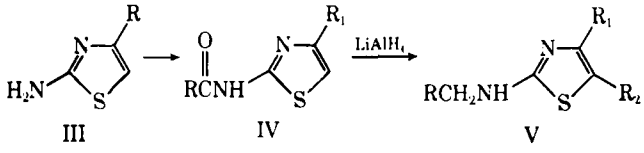
The synthesis of a number of derivatives of mesoionic thiazolo[3,2-*a*]pyrimidin-5,7-diones and 1,3,4-thiadiazolo[3,2-*a*]pyrimidin-5,7-diones is reported here, along with the results of *in vitro* antibacterial screening as a part of an initial pharmacological investigation of this new class of compounds.

SYNTHESIS

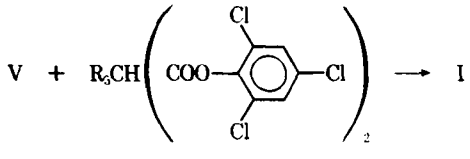
Compounds I*a*–I*h*, with the exception of I*e*, were prepared as outlined in Scheme I. 2-Aminothiazoles were acylated by the use of acyl chlorides or acid anhydrides to obtain the amides, IV (Table I), which were reduced *via* lithium aluminum hydride to the corresponding 2-alkylaminothiazoles, V (Table II). 2-Bromo-5-nitrothiazole was treated with 3,4-dichlorobenzylamine to obtain V*e*. Heating the 2-alkylaminothiazoles, V, with the malonate esters, VI (R_3 = H,CH_3), gave the mesoionic xanthine analogs, I, in good yield (Table III). The mesoionic thiadiazolopyrimidiones, II, were prepared by an analogous procedure.

EXPERIMENTAL[4]

***N*-(2-Thiazolyl)adamantane-1-carboxamide (IV*d*)**—A solution of adamantane-1-carboxylic acid chloride (6.0 g., 30 mmoles) in 30 ml. of tetrahydrofuran was added dropwise with stirring to a solution of 2-aminothiazole (3.0 g., 30 mmoles) and triethylamine (3.5 g., 35 mmoles) in 40 ml. of tetrahydrofuran at 0°. After the addi-



III IV V



VI

Scheme I

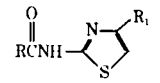
[1] Well over one hundred such analogs have been postulated (1, 2).

[2] Anhydro(8-substituted-5-hydroxythiazolo[3,2-*a*]pyrimidinium-7-one) hydroxide.

[3] Anhydro(8-substituted-5-hydroxy-1,3,4-thiadiazolo[3,2-*a*]pyrimidinium-7-one) hydroxide.

[4] Proton magnetic resonance (PMR) spectra were obtained on a Varian T-60 spectrometer, and chemical shifts are reported relative to tetramethylsilane. IR spectra were obtained on a Beckman IR-18 spectrophotometer. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn. All melting points were determined on a Mel-Temp melting-point apparatus and are uncorrected.

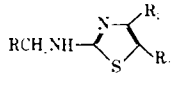
Table I—Properties of 2-Acylaminothiazoles, IV



Compound	R	R_1	Formula	Melting Point	Recrystallization Solvent[a]	Yield, %	Analysis, % Calc.	Found
IV*a*	Methyl	CH_3	$C_6H_8N_2OS$	133–134°[b]	T	92.6	—	—
IV*b*	3,4,5-Trimethoxyphenyl	H	$C_{13}H_{14}N_2O_4S$	173°	EA	79.4	C 53.03 H 4.80 Cl — N 9.52 S 10.87	53.06 4.69 — 9.36 11.08
IVc	3-Chlorophenyl	C_6H_5	$C_{16}H_{11}ClN_2OS$	146–148°	I	68.4	C 61.05 H 3.52 Cl 11.26 N 8.90 S 10.19	60.98 3.27 11.24 8.97 10.35
IV*d*	1-Adamantyl	H	$C_{14}H_{18}N_2OS$	198–199°	I	66.3	C 64.11 H 6.92 Cl — N 10.68 S 12.20	63.97 6.87 — 10.56 12.06

[a] T = tetrahydrofuran–petroleum ether, I = isopropanol, and EA = ethyl acetate. [b] Lit. (6) m.p. 134°.

Table II—Properties of 2-Alkylaminothiazoles, V



Compound	R	R_1	R_2	Formula	Melting Point	Recrystallization Solvent[a]	Yield, %	Analysis, % Calc.	Found
V*a*	Methyl	CH_3	H	$C_6H_{10}N_2S$	53°	H	75.0	C 50.67 H 7.08 Cl — N 19.69 S 22.55	50.62 7.21 — 19.54 22.61
V*b*	3,4,5-Trimethoxyphenyl	H	H	$C_{13}H_{16}N_2O_3S$	96–97°	T	57.2	C 55.69 H 5.75 Cl — N 9.99 S 11.44	55.48 5.68 — 10.04 11.40
V*c*	3-Chlorophenyl	C_6H_5	H	$C_{16}H_{13}ClN_2S$	93–94°	E	88.4	C 63.88 H 4.36 Cl — N 9.31 S 10.66	63.69 4.52 — 9.39 10.47
V*d*	1-Adamantyl	H	H	$C_{14}H_{20}N_2S$	104–105°	I	88.0	C 67.42 H 8.49 Cl — N 11.09 S 12.86	67.37 8.31 — 11.23 13.05
V*e*	3,4-Dichlorophenyl	H	NO_2	$C_{10}H_7Cl_2N_3O_2S$	150°	I	68.9	C 39.49 H 2.32 Cl 23.32 N 13.82 S 10.54	39.57 2.42 23.46 13.84 10.52

[a] H = hexane–petroleum ether, T = tetrahydrofuran–petroleum ether, E = ethanol 95%, and I = isopropanol.

tion was completed, stirring was continued at room temperature for 1 hr. Filtration and evaporation of the filtrate *in vacuo* gave the crude product. Recrystallization from isopropanol gave 5.2 g. (66.3%) of IV*d* as white crystals, m.p. 198–199°; IR (chloroform): 1675 (m) cm.$^{-1}$; NMR ($CDCl_3$): δ 1.9–2.2 (m, 15H, adamantyl protons), 7.2 (d, 1H, thiazole proton), and 7.7 (d, 1H, thiazole proton). Table I lists the analytical data.

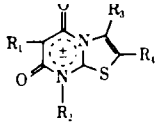
The 2-acylaminothiazoles, IV*a*–IVc (Table I), were prepared in the same manner as IV*d*.

2-(1-Adamantylmethylamino)thiazole (V*e*) A solution of IV*d* (1.3 g., 5 mmoles) in 20 ml. of tetrahydrofuran was added dropwise with stirring to a suspension of lithium aluminum hydride (0.2 g., 5.2 mmoles) in 20 ml. of tetrahydrofuran at 0°. After refluxing for 3 hr., water was slowly added at 0° until the evolution of hydrogen ceased. Filtration and evaporation of the filtrate *in vacuo* gave a colorless oil which crystallized upon standing. Recrystallization from isopropanol gave 1.1 g. (88%) of V*d* as white flakes, m.p. 104–105°; IR (chloroform): 3450 (m) and 2900 (s) cm.$^{-1}$; NMR ($CDCl_3$): δ 2.0 (m, 15H, adamantyl protons), 3.3 (s, 2H, —CH_2—), 6.2 (broad signal, 1H, —NH—), 6.8 (d, 1H), and 7.4 (d, 1H, thiazole protons). Table II lists the analytical data.

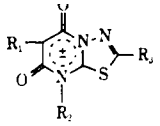
The 2-alkylaminothiazoles, V*a*–V*c* (Table II), were prepared in the same manner as V*d*.

2-(3,4-Dichlorobenzylamino)-5-nitrothiazole (V*e*)—2-Bromo-5-nitrothiazole (2.1 g., 10 mmoles) in 20 ml. of tetrahydrofuran was added dropwise with stirring to 3,4-dichlorobenzylamine (3.5 g., 20 mmoles) at 0°. After stirring at room temperature for 2 hr., the reaction mixture was filtered and the filtrate was evaporated *in vacuo* to give a brown oil which crystallized upon standing overnight (16 hr.). Recrystallization from isopropanol gave 2.1 g. (68.9%) of V*e* as yellow crystals, m.p. 150°; IR (KBr): 1510 (s) and 1470 (s) cm.$^{-1}$; NMR (dimethyl sulfoxide-d_6): δ 4.6 (s, 2H, —CH_2—), 7.3–7.7 (m, 3H, phenyl protons), and 8.35 (s, 1H, thiazole proton). Table II lists the analytical data.

Table III—Properties of Mesoionic Thiazolo[3,2-*a*]pyrimidin-5,7-diones (I) and Mesoionic 1,3,4-Thiadiazolo[3,2-*a*]pyrimidin-5,7-diones (II)



I



II

Compound	R_1	R_2	R_3	R_4	Formula	Melting Point	Recrystallization Solvent[a]	Yield, %	Analysis, % Calc.	Found
I*a*	H	C_2H_5	CH_3	H	$C_9H_{10}N_2O_2S$	189–190°	I	81.6	C 51.41	51.45
									H 4.79	4.78
									N 13.33	13.49
									S 15.25	15.54
I*b*	CH_3	3,4,5-Trimethoxyphenyl	H	H	$C_{17}H_{18}N_2O_6S$	233–234°	M	97.8	C 56.33	56.21
									H 5.01	5.08
									N 7.73	7.67
									S 8.85	9.14
I*c*	CH_3	3-Chlorophenyl	C_6H_5	H	$C_{20}H_{15}ClN_2O_2S$	220–221°	A	52.2	C 62.74	63.06
									H 3.94	3.85
									N 7.32	7.28
									S 8.38	8.38
I*d*	CH_3	1-Adamantyl	H	H	$C_{18}H_{23}N_2O_2S$	210–211°	B	62.4	C 65.23	65.04
									H 7.00	7.10
									N 8.45	8.32
									S 9.67	9.80
I*e*	H	3,4-Dichlorophenyl	H	NO_2	$C_{13}H_7Cl_2N_3O_4S$	240° dec.	DA	32.7	C 42.95	43.30
									H 1.90	2.22
									N 11.29	10.89
									S 8.62	8.37
II*a*	CH_3	C_2H_5	H	—	$C_8H_9N_3O_2S$	214°	D	50.1	C 45.49	45.38
									H 4.30	4.48
									N 19.90	19.85
									S 15.18	15.32
II*b*	CH_3	C_6H_5	CH_3	—	$C_{14}H_{13}N_3O_2S$	227–228°	I	64.3	C 58.52	58.51
									H 4.56	4.61
									N 14.62	14.49
									S 11.16	11.27

[a] I = isopropanol, M = methanol, A = acetonitrile, B = benzene, DA = dimethylformamide–acetonitrile, and D = dimethylformamide.

Anhydro(6-methyl-7-hydroxy-8-(1-adamantylmethyl)thiazolo[3,2-*a*]pyrimidinium-5-one) Hydroxide (I*d*)—Compounds V*d* (0.37 g., 1.5 mmoles) and VI*b* (0.74 g., 1.5 mmoles) were mixed and heated on an oil bath (160°) until a clear melt resulted (5 min.). A slow stream of nitrogen was passed through the flask during the heating period to remove 2,4,6-trichlorophenol. When cool, the resultant product was triturated with 30 ml. of anhydrous ether and collected. Recrystallization from benzene gave 0.31 g. (62.4%) of I*d* as white crystals, m.p. 210–211°; IR (chloroform): 1685 (s) and 1630 (s) cm.$^{-1}$; NMR ($CDCl_3$): δ 2.3 (m, 18H, $-CH_3$ and adamantyl protons), 4.2 (s, 2H, $-CH_2-$), 7.7 (d, 1H), and 8.5 (d, 1H, thiazole protons). Table III lists the analytical data.

Anhydro(2,6-dimethyl-7-hydroxy-8-benzyl-1,3,4-thiadiazolo-[3,2-*a*]pyrimidinium-5-one) Hydroxide (II*b*)—2-Benzylamino-5-methyl 1,3,4-thiadiazole (7) (1.0 g., 5 mmoles) and VI*b* (2.4 g., 5 mmoles) were combined and heated at 160°, under a slow stream of nitrogen, until a clear melt resulted (3 min.). The product was triturated with 30 ml. of anhydrous ether, collected, and recrystallized from isopropanol to give 0.45 g. (64.3%) of II*b* as white crystals, m.p. 227–228°; IR (chloroform): 1685 (sh) and 1635 (s) cm.$^{-1}$; NMR ($CDCl_3$): δ 2.1 (s, 3H, $-CH_3$), 2.7 (s, 3H, thiazole $-CH_3$), 5.3 (s, 2H, $-CH_2-$), and 7.4 (s, 5H, phenyl protons). Table III lists the analytical data.

Compounds I*a*–I*c*, I*e*, and II*a* (Table III) were prepared in the same manner as I*d*, by heating at 160° until a clear melt resulted (about 1–5 min.) and then triturating with anhydrous ether to remove any unreacted starting material.

Partition Coefficients—The partition coefficient (*P*) of I*i* (R_1 = R_3 = R_4 = H, R_2 = C_2H_5) was determined in an octanol–aqueous buffer system (8). A master solution was prepared by dissolving I*i* in a pH 7.4 aqueous phosphate buffer to achieve a concentration of 2.9 $\times$ 10^{-4} mole/l. Dilutions of this solution were shaken for 10 min. with 20 ml. of octanol in Run A and with 25 ml. of octanol in Run B. These mixtures were then centrifuged, and the aqueous phase was examined by UV spectroscopy (242 nm.) to determine the aqueous concentration of I*i*. A blank was run without octanol to obtain the extinction coefficient in the buffer solution. The value of *P* was determined by dividing the concentration of I*i* in octanol by the concentration of I*i* in the buffer solution. Thus, the log *P* determined for I*i* was −1.41 for Run A and −1.44 for Run B. The log *P* for I*i* was taken as the average of the two runs or −1.425. Due to the additive nature of substituent constants (8, 9), the partition coefficients (log *P* values) could thus be calculated employing the π-values of the various substituents. Table IV lists the partition coefficients calculated for the mesoionic compounds employed in this investigation.

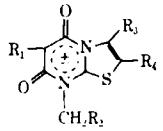
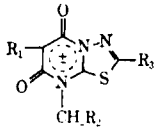
Antibacterial Testing Procedure—The following organisms were employed in this study: *Escherichia coli* (ATCC 11775), *Proteus vulgaris* (ATCC 13315), *Pseudomonas fluorescens* (ATCC 13525), *Pseudomonas aeruginosa* (ATCC 15691), *Bacillus subtilis* (ATCC 6051), *Streptococcus faecalis* (ATCC 8043), and *Staphylococcus aureus* (ATCC 12600). A lawn was prepared on Meuller-Hinton blood (5%) agar plates, using 1 ml. of a 24-hr. growth of the test organism in brain-heart infusion broth. Paper disks (6 mm.), im-

Table IV—Partition Coefficients Calculated for Mesoionic Thiazolo[3,2-*a*]pyrimidin-5,7-diones (I) and Mesoionic 1,3,4-Thiadiazolo[3,2-*a*]pyrimidin-5,7-diones (II)

Compound	Partition Coefficient (log *P*)
I*a*	−0.95
I*b*	+1.0
I*c*	+3.6
I*d*	+2.2
I*e*	+1.7
I*f*	+0.5
I*g*	+1.3
I*h*	−1.25
I*i*	−1.425[a]
II*a*	−2.3
II*b*	−0.2
II*c*	−2.8
II*d*	−2.3

[a] Determined experimentally.

Table V—Zones of Inhibition[a]

Compound	R_1	R_2	R_3	*E. coli*	*P. vulgaris*	*Ps. fluorescens*	*Ps. aeruginosa*	*B. subtilis*	*Strep. faecalis*	*Staph. aureus*
										
Ia	H	CH_3	CH_3	—[c]	7	—[c]	—[c]	10	—[c]	—[c]
Ib	CH_3	3,4,5-Trimethoxyphenyl	H	—[c]	11	14	—[c]	8	—[c]	10
Ic	CH_3	3-Chlorophenyl	C_6H_5	—[c]	—[c]	10	—[c]	—[c]	—[c]	7
Id	CH_3	1-Adamantyl	H	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]
Ie[b]	H	3,4-Dichlorophenyl	H	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]
If[d]	CH_3	C_6H_5	H	—[c]	18	16	8	16	—[c]	9
Ig[d]	$C_6H_5CH_2$	CH_3	H	—[c]	12	14	7	34	—[c]	—[c]
Ih[d]	H	$CH_2N(CH_3)_2$	H	—[c]	—[c]	10	9	27	9	8
										
IIa	CH_3	CH_3	H	—[c]	18	17	7	25	22	22
IIb	CH_3	C_6H_5	CH_3	—[c]	14	15	—[c]	—[c]	—[c]	—[c]
IIc[d]	H	CH_3	H	—[c]	32	12	7	30	13	19
IId[d]	H	CH_3	CH_3	—[c]	—[c]	—[c]	—[c]	29	—[c]	—[c]
Furazolidone				22	12	20	8	24	7	15
Sulfadimethoxine				20	10	16	—[c]	23	—[c]	—[c]
Sulfamerazine				20	—[c]	—	—[c]	27	—[c]	—[c]
Sulfamethoxypyridazine				26	13	—	—[c]	28	—[c]	—[c]

[a] Diameter (millimeters) of zones of inhibition. [b] $R_4 = NO_2$. [c] No inhibition. [d] Synthesis previously reported (5).

pregnated with 2 mg. of the test compounds, were placed on the agar and incubated for 24 hr. at 37°. *Ps. fluorescens* was incubated for 48 hr. at 25°.

Included on each plate were one or more disks[5] impregnated with the following commercially available antibacterial agents: furazolidone[6], 100 mcg./disk; sulfadimethoxine[7], 250 mcg./disk; sulfamerazine, 250 mcg./disk; and sulfamethoxypyridazine, 250 mcg./disk.

The tube dilution method was carried out using brain–heart infusion broth (5 ml.) with 10-, 25-, 50-, 100-, 250-, and 500-mcg./ml. concentrations of test compound. The broth was inoculated with 0.05 ml. of a 24-hr. growth of the test organism adjusted to a constant value of turbidity near that observed for full growth. Turbidity was measured at 6-hr. intervals using a spectrophotometer[8] (650 nm.). Full growth was usually reached in 12–18 hr. in a control tube.

RESULTS AND DISCUSSION

The zones of inhibition observed for Compounds *Ia*–*Ih* and *IIa*–*IId* against the test bacteria are shown in Table V. All but three of these mesoionic compounds (*Ie*, *Id*, and *IId*) exhibited bacteriostatic activity against two or more of the test organisms. None of the mesoionic compounds displayed any activity against *E. coli*. Against the other Gram-negative bacteria, Compounds *Ib*, *If*, *Ig*, *IIa*, and *IIc* showed zones of inhibition comparable to or greater than those of the standards. Compounds *Ih*, *IIa*, and *IIc* displayed significant zones of inhibition against Gram-positive organisms. Thiadiazolopyrimidiones *IIa* and *IIc* are notable for their activity against all of the test organisms except *E. coli*.

The activity of these compounds suggested the inclusion of a number of the monocyclic thiazole and thiadiazole precursors in the disk screening procedure, but no significant activity was observed.

To assay the levels of activity of the more active compounds, *If*, *IIa*, and *IIc* were tested by the broth tube dilution method, using nitrofurantoin[9] as a standard for comparison. The minimum inhibitory concentration (MIC) and the concentration inhibiting 50% of full growth (ID_{50}) for these compounds against *P. vulgaris* and *Staph. aureus* are shown in Table VI.

Compound *If* exhibited only a low level of activity against both organisms in this test. Compound *IIc* displayed greater activity than *If* in both cases, while *IIa* exhibited activity against *Staph. aureus* comparable to that of nitrofurantoin. Against *P. vulgaris*, *IIa* was about half as active as the standard. At a concentration of 250 mcg./ml., *IIa* was bactericidal against *P. vulgaris* but not against *Staph. aureus*. All three tested compounds showed some inhibition of growth at the 10-mcg./ml. level.

The range of lipophilicity represented by the derivatives *Ia*–*Ih* is quite large ($\Delta \log P = 4.85$). The most lipophilic mesoionic thiazolopyrimidiones, *Ic*, *Id*, and *Ie*, exhibited the lowest spectrum of activity. With the exception of *Ih*, the more active derivatives in this series have log *P* values between 0 and 1 (*If*, 0.5; *Ig*, 1.3; and *Ib*, 1.0). The thiadiazolopyrimidines *IIa*–*IId* are more hydrophilic and cover a more restricted range of lipophilicity (*IIb*, −0.2, to *IIc*, −2.8). Of more importance in this series is the apparent requirement for an unsubstituted 2-position (R_3 = H). Much more quantitative data concerning the activity of many more derivatives will be required before meaningful structure–activity relationships will be revealed.

The mesoionic thiazolopyrimidiones are nonbasic while the mesoionic thiadiazolopyrimidiones are very weakly basic. Both are stable to hydrolytic ring opening. Mesoionic thiazolopyrimidiones have been recrystallized from 5% aqueous hydrochloric acid (5). Refluxing either in aqueous solutions of strong mineral acids or alkali does produce ring-opened products (5). All reported derivatives have been found to be stable to heat and light in air.

Table VI—Antibacterial Activity[a] by the Broth Tube Dilution Method

Compound	*P. vulgaris* MIC	*P. vulgaris* ID_{50}	*Staph. aureus* MIC	*Staph. aureus* ID_{50}
If	500	500	500	500
IIa	100	40	50	15
IIc	200	90	225	125
Nitrofurantoin	50	22	40	18

[a] Micrograms per milliliter.

[5] Sensi-Discs, BBL, Cockeysville, MD 21030
[6] Furoxone.
[7] Madribon.
[8] Bausch & Lomb Spectronic 20.
[9] Furadantin.

CONCLUSION

The spectrum of *in vitro* activity exhibited by a significant number of the mesoionic thiazolo[3,2-*a*]pyrimidiones, I, and mesoionic thiadiazolo[3,2-*a*]pyrimidiones, II, suggests that these compounds should be considered a new and novel class of antibacterial agents. Several features of these compounds are significant. While they inhibited the growth of both Gram-negative and Gram-positive bacteria *in vitro*, no activity was displayed against *E. coli*. Furthermore, a number of the mesoionic compounds exhibited comparable or greater activities, as evidenced by their zones of inhibition, than several of the sulfa drugs or nitrofurans employed as standards.

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▲ To whom inquiries should be directed.

Pharmacokinetic Profile of Diazepam in Man following Single Intravenous and Oral and Chronic Oral Administrations

S. A. KAPLAN▲, M. L. JACK, K. ALEXANDER, and R. E. WEINFELD

Abstract □ Four subjects each received single intravenous and oral 10-mg. doses of diazepam and 10 mg. orally every 24 hr. for 15 days. The intravenous blood level data were fitted with a three-compartment open-model system containing both a "shallow" and a "deep" peripheral compartment. The "apparent" half-life of elimination of diazepam following intravenous administration ranged from 21 to 37 hr., and the calculated volume of distribution ranged from 160 to 205% of body weight. The rate at which diazepam returns to the central compartment from the deep peripheral compartment, k_{31}, was shown to be the rate-controlling factor in the elimination of diazepam and in the formation of desmethyldiazepam. Orally administered diazepam was rapidly and completely absorbed. Following chronic administration of 10 mg. diazepam every 24 hr., the minimum and maximum steady-state (plateau) levels of diazepam can be successfully predicted or calculated utilizing the pharmacokinetic parameters obtained following intravenous administration. Diazepam blood levels plateau at approximately Day 7 of treatment at twice the blood levels observed on Day 1. Desmethyldiazepam blood levels are within the range of the diazepam blood levels and exhibit an apparent half-life range from 50 to 99 hr. after the last dose of diazepam on Day 15.

Keyphrases □ Diazepam—pharmacokinetic profiles after single intravenous and oral and chronic oral administrations, man □ Pharmacokinetic profiles—diazepam after single intravenous and oral and chronic oral administrations, man □ Absorption kinetics—diazepam after single intravenous and oral and chronic oral administrations, man

Diazepam[1], 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one, is effective in the symptomatic relief of tension and anxiety states as well as for the relief of skeletal muscle spasms (1–8).

The drug exhibits a low aqueous solubility of 50 mcg./ml. and a pKa of 3.4 (9). The transfer characteristics of diazepam across the everted rat intestinal sac are consistent with good absorbability (10). This suggests that, once in solution, the permeability (absorbability) of diazepam across the GI mucosa will not be a rate-limiting factor following oral administration of the drug.

The major metabolic pathways of diazepam (Scheme I) have been described in various animal species and in man (11–17). In man, the major metabolite measurable in the bloodstream is desmethyldiazepam. In the urine, the glucuronide conjugate of oxazepam is the major detectable metabolite (11).

The present study was designed to elucidate the

[1] Valium, containing diazepam as its active ingredient (Hoffmann–La Roche Inc., Nutley, N. J.), was administered throughout the study.

CONCLUSION

The spectrum of *in vitro* activity exhibited by a significant number of the mesoionic thiazolo[3,2-*a*]pyrimidiones, I, and mesoionic thiadiazolo[3,2-*a*]pyrimidiones, II, suggests that these compounds should be considered a new and novel class of antibacterial agents. Several features of these compounds are significant. While they inhibited the growth of both Gram-negative and Gram-positive bacteria *in vitro*, no activity was displayed against *E. coli*. Furthermore, a number of the mesoionic compounds exhibited comparable or greater activities, as evidenced by their zones of inhibition, than several of the sulfa drugs or nitrofurans employed as standards.

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Pharmacokinetic Profile of Diazepam in Man following Single Intravenous and Oral and Chronic Oral Administrations

S. A. KAPLAN▲, M. L. JACK, K. ALEXANDER, and R. E. WEINFELD

Abstract □ Four subjects each received single intravenous and oral 10-mg. doses of diazepam and 10 mg. orally every 24 hr. for 15 days. The intravenous blood level data were fitted with a three-compartment open-model system containing both a "shallow" and a "deep" peripheral compartment. The "apparent" half-life of elimination of diazepam following intravenous administration ranged from 21 to 37 hr., and the calculated volume of distribution ranged from 160 to 205% of body weight. The rate at which diazepam returns to the central compartment from the deep peripheral compartment, k_{31}, was shown to be the rate-controlling factor in the elimination of diazepam and in the formation of desmethyldiazepam. Orally administered diazepam was rapidly and completely absorbed. Following chronic administration of 10 mg. diazepam every 24 hr., the minimum and maximum steady-state (plateau) levels of diazepam can be successfully predicted or calculated utilizing the pharmacokinetic parameters obtained following intravenous administration. Diazepam blood levels plateau at approximately Day 7 of treatment at twice the blood levels observed on Day 1. Desmethyldiazepam blood levels are within the range of the diazepam blood levels and exhibit an apparent half-life range from 50 to 99 hr. after the last dose of diazepam on Day 15.

Keyphrases □ Diazepam—pharmacokinetic profiles after single intravenous and oral and chronic oral administrations, man □ Pharmacokinetic profiles—diazepam after single intravenous and oral and chronic oral administrations, man □ Absorption kinetics—diazepam after single intravenous and oral and chronic oral administrations, man

Diazepam[1], 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one, is effective in the symptomatic relief of tension and anxiety states as well as for the relief of skeletal muscle spasms (1–8).

The drug exhibits a low aqueous solubility of 50 mcg./ml. and a pKa of 3.4 (9). The transfer characteristics of diazepam across the everted rat intestinal sac are consistent with good absorbability (10). This suggests that, once in solution, the permeability (absorbability) of diazepam across the GI mucosa will not be a rate-limiting factor following oral administration of the drug.

The major metabolic pathways of diazepam (Scheme I) have been described in various animal species and in man (11–17). In man, the major metabolite measurable in the bloodstream is desmethyldiazepam. In the urine, the glucuronide conjugate of oxazepam is the major detectable metabolite (11).

The present study was designed to elucidate the

[1] Valium, containing diazepam as its active ingredient (Hoffmann–La Roche Inc., Nutley, N. J.), was administered throughout the study.

Table I—Blood Levels of Diazepam and Desmethyldiazepam (Micrograms per Milliliter) following a Single Intravenous and Oral 10-mg. Dose of Diazepam

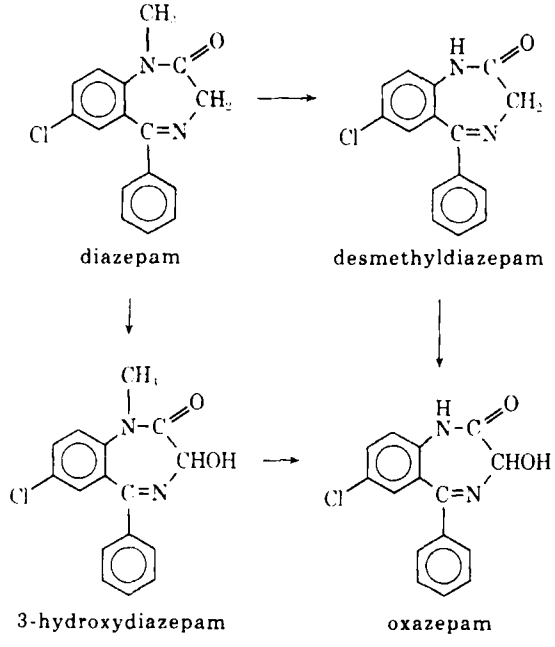
Time	Subject 1				Subject 2				Subject 3				Subject 4			
	Intravenous		Oral		Intravenous		Oral		Intravenous		Oral		Intravenous		Oral	
	D.[a]	D.D.[b]	D.	D.D.	D.	D.D.	D.	D.D.	D.	D.D.	D.	D.D.	D.	D.D.	D.	D.D.
1 min.	0.132	N.M.[c]	—	—	0.574	N.M.	—	—	0.194	—	—	—	0.338	N.M.	—	—
2.5 min.	0.323	N.M.	—	—	0.298	N.M.	—	—	0.257	N.M.	—	—	0.452	N.M.	—	—
5 min.	0.276	N.M.	—	—	0.286	N.M.	—	—	0.294	0.001	—	—	0.414	0.002	—	—
10 min.	0.263	N.M.	—	—	0.262	N.M.	—	—	0.253	0.002	—	—	0.325	0.002	—	—
15 min.	[d]	—	0.007	0.019	—	—	0.005	0.009	—	—	0.026	0.005	—	—	0.045	0.005
20 min.	0.207	N.M.	—	—	0.204	0.001	—	—	0.221	0.003	—	—	0.266	0.005	—	—
30 min.	0.174	N.M.	0.023	0.019	0.180	0.001	0.100	0.008	0.230	0.003	0.113	0.007	0.212	0.005	0.113	0.005
45 min.	0.144	0.002	0.056	0.018	0.151	0.004	0.153	0.011	0.201	0.004	0.166	0.011	0.181	0.006	0.163	0.011
1 hr.	0.118	0.001	0.063	0.014	0.135	0.005	0.169	0.014	0.184	0.005	0.189	0.011	0.198	0.006	0.173	0.010
1.5 hr.	0.107	0.004	0.137	0.020	0.112	0.006	0.135	0.014	0.150	0.006	0.175	0.014	0.152	0.008	0.155	0.013
2 hr.	0.092	0.005	0.109	0.022	0.093	0.007	0.103	0.015	0.142	0.008	0.146	0.014	0.153	0.011	0.128	0.016
3 hr.	0.074	0.006	0.090	0.028	0.074	0.007	0.082	0.016	0.145	0.012	0.126	0.017	0.141	0.014	0.109	0.018
4 hr.	0.066	0.007	0.084	0.031	0.067	0.008	0.072	0.018	0.139	0.015	0.114	0.018	0.130	0.016	0.098	0.020
6 hr.	0.047	0.008	0.070	0.032	0.053	0.009	0.061	0.020	0.107	0.017	0.103	0.022	0.106	0.019	0.096	0.023
8 hr.	0.045	0.008	0.066	0.033	0.049	0.014	0.047	0.021	0.089	0.019	0.095	0.021	0.072	0.018	0.078	0.026
12 hr.	0.031	0.010	0.046	0.028	0.041	0.017	0.034	0.022	0.061	0.019	0.069	0.023	0.049	0.027	0.061	0.030
24 hr.	0.032	0.012	0.043	0.031	0.030	0.020	0.029	0.025	0.040	0.027	0.039	0.025	0.036	0.031	0.038	0.033
30 hr.	0.030	0.014	0.037	0.030	0.026	0.021	0.026	0.026	0.039	0.033	0.039	0.027	0.022	0.025	0.033	0.037
48 hr.	0.028	0.019	0.036	0.035	0.018	0.021	0.020	0.017	0.025	0.029	0.025	0.030	0.023	0.039	0.018	0.027

[a] D. = diazepam. [b] D.D. = desmethyldiazepam. [c] N.M. = below 0.001 mcg./ml. [d] — = no specimen obtained.

pharmacokinetic profile of diazepam in man following intravenous administration and to evaluate the physiological availability and disposition following single oral and chronic oral administrations of the drug.

EXPERIMENTAL

Clinical Protocol—Four healthy male volunteers, ages 25–43, each received a single 10-mg. dose of diazepam administered intravenously and orally 1 week apart[2]. Commencing 1 week thereafter, each subject received a 10-mg. oral dose of diazepam every 24 hr. for 15 days. The subjects were fasted for 7 hr. prior to receiving the single-dose administrations, and food was withheld for the 1st hr. postadministration.



Scheme I—Major metabolic pathways of diazepam

[2] The clinical aspects of the study were conducted at the Deer Lodge Research Unit, Deer Lodge, Mont., under the direction of Dr. James D. Moore.

Blood specimens were obtained at the time intervals indicated in Tables I and II for each treatment. The total volumes of urine voided were collected at 24-hr. intervals from −24 to 0, 0 to 24, and 24 to 48 hr. postadministration following the single intravenous and oral administrations. All specimens were frozen for subsequent analysis.

Analytical Method—Blood and urine specimens were analyzed for diazepam and desmethyldiazepam by the electron-capture GLC procedure of de Silva and Puglisi (18), with a sensitivity of 0.001 mcg./ml. for each component using a 1-ml. specimen.

RESULTS AND DISCUSSION

The diazepam and desmethyldiazepam blood level data following the single-administration studies are presented in Table I for all four subjects and plotted for one subject in Fig. 1. The blood level data following the chronic administration studies are presented in Figs. 2–5. The pharmacokinetic profile following the single intra-

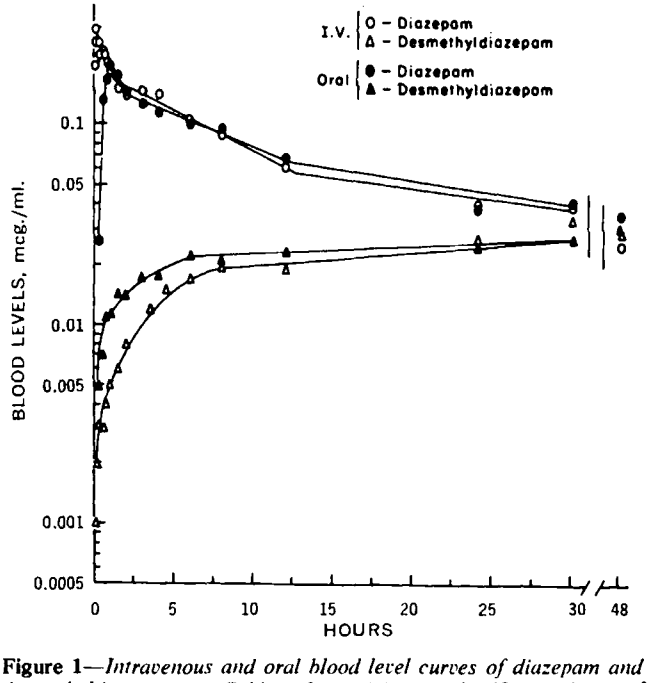


Figure 1—*Intravenous and oral blood level curves of diazepam and desmethyldiazepam in Subject 3 receiving single 10-mg. doses of diazepam.*

Table II—Pharmacokinetic Profile of Diazepam in Man following Single Intravenous Administration to Four Subjects

	Subject			
	1	2	3	4
Dose, mg.	10	10	10	10
Dose, mg./kg.	0.113	0.118	0.153	0.157
Subject weight, kg.	88.5	84.9	65.4	63.6
Subject age, years	41	31	25	43
P, mcg./ml.	0.177	0.346	0.089	0.282
A, mcg./ml.	0.107	0.171	0.140	0.156
B, mcg./ml.	0.052	0.058	0.078	0.061
π (hr.$^{-1}$), 0.693/π (hr.)	3.12, 0.22	22.43, 0.03	3.36, 0.21	4.78, 0.14
α (hr.$^{-1}$), 0.693/α (hr.)	0.472, 1.5	0.734, 0.94	0.210, 3.3	0.226, 3.1
β (hr.$^{-1}$), 0.693/β (hr.)	0.019, 36.5	0.026, 26.7	0.024, 28.9	0.021, 33.0
Percent volume central compartment[a]	33.7	20.5	49.8	31.4
Percent volume of distribution[a]	196.4	182.9	160.9	205.3
k_{12}, hr.$^{-1}$	1.14	12.35	0.88	2.39
k_{21}, hr.$^{-1}$	1.66	9.27	2.43	2.18
k_{13}, hr.$^{-1}$	0.55	1.14	0.12	0.24
k_{31}, hr.$^{-1}$	0.15	0.20	0.090	0.076
k_{el}, hr.$^{-1}$	0.111	0.228	0.078	0.137
Ratio β/k_{el}	0.17	0.11	0.30	0.15
Area under blood level curve, mcg./ml./hr.	1.886	1.853	2.822	2.556
Percent of dose in 0–48-hr. urine as:				
Intact diazepam	<0.05	<0.05	<0.05	<0.05
Intact desmethyldiazepam	<0.05	<0.05	<0.05	<0.05
Conjugated desmethyldiazepam	4.8	5.2	5.7	9.2

[a] Calculated as percent body weight.

venous and oral administrations of diazepam are summarized in Tables II and III, respectively.

Following the intravenous administration of diazepam, a triexponential blood level curve was observed in all four subjects as exemplified for Subject 3 in Fig. 1. The pharmacokinetic profile of diazepam was determined utilizing the data obtained following the intravenous administration of the drug. The data suggested that the pharmacokinetic evaluation would require minimally a three-compartment open-model system (19). The first two exponential phases with rate constants π and α reflect two different types of distribution phenomena, *i.e.*, distribution from the central compartment into a rapidly equilibrating or "shallow" peripheral compartment and a slowly equilibrating "deep" peripheral compartment. The third exponential phase, with a rate constant β, is referred to as the "apparent" elimination rate of the drug from the body. All three rate constants, π, α, and β, are hybrid first-order rate constants, each influenced by all of the individual processes involved in the disposition of the drug.

Solution of the differential equations of the three-compartment open model (Scheme II) yields the following integrated equation describing the blood level–time curve after intravenous administration:

$$(C_p)_t = Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

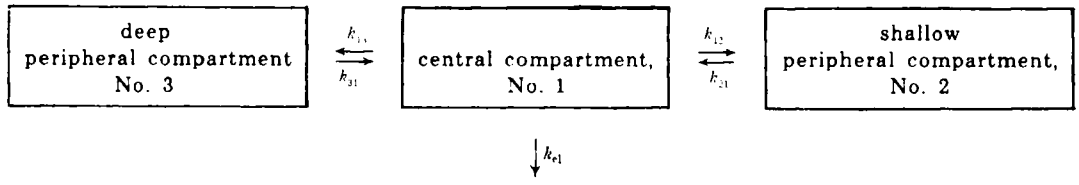
where $(C_p)_t$ is the concentration of drug in the blood at time t, and P, A, and B are the ordinate axis intercepts. The individual rate constants of the model, k_{12}, k_{21}, k_{13}, k_{31}, and k_{el}, are calculable from P, A, B, π, α, and β (20). These rate constants do not necessarily apply to any anatomically defined tissue compartments but are meaningful in defining the disposition of drug from the central compartment.

The total volume of distribution of diazepam (21), V_d, was found to be 196, 183, 161, and 205% of body weight for Subjects 1 through 4, respectively, following a 10-mg. intravenous administration of diazepam. The volume of the central compartment, V_p, was calculated to be 33.7, 20.5, 49.8, and 31.4% of body weight for Subjects 1 through 4, respectively. The size of the central compartment is consistent with the utilization of a three-compartment open pharmacokinetic model system in which elimination is occurring from the central compartment (22).

The intravenous blood level data were fitted to the triexponential equation by means of a "nonlinear" regression analysis computer program (23). The pharmacokinetic parameters of diazepam obtained for each subject following intravenous administration are summarized in Table II. The rate constants associated with the rapidly and slowly equilibrating sites in the peripheral compartments ranged from 3.1 to 22.4 hr.$^{-1}$ for π and from 0.21 to 0.73 hr.$^{-1}$ for α, respectively. These values correspond to apparent half-life ranges of 2–13 min. and 0.95–3.1 hr., respectively. The overall elimination rate constant, β, ranged from 0.019 to 0.026 hr.$^{-1}$, corresponding to an apparent half-life range of 26.7–36.5 hr. The magnitude of the three hybrid rate constants, π, α, and β, reflect an extremely rapid initial distribution of diazepam and a slow apparent elimination rate of drug from the body.

Table III—Pharmacokinetic Profile of Diazepam in Man following Single Oral Administrations to Four Subjects

	Subject			
	1	2	3	4
Dose, mg.	10	10	10	10
Dose, mg./kg.	0.113	0.118	0.153	0.157
Subject weight, kg.	88.5	84.9	65.4	63.6
Subject age, years	41	31	25	43
B, mcg./ml.	0.067	0.048	0.086	0.089
β (hr.$^{-1}$), 0.693/β (hr.)	0.015, 46.2	0.019, 36.5	0.027, 25.7	0.33, 21.0
Area under blood level curve, mcg./ml./hr.	2.272	1.670	2.728	2.389
Blood level curve area ratio, oral/i.v.	1.205	0.901	0.967	0.935
Absorption rate constant, hr.$^{-1}$	1.68	1.9	2.5	1.58
Blood level peak height, mcg./ml.	0.137	0.169	0.189	0.173
Blood level peak time, hr.	1.5	1	1	1
Percent of dose in 0–48-hr. urine as:				
Intact diazepam	<0.05	<0.05	<0.05	<0.05
Intact desmethyldiazepam	<0.05	<0.05	<0.05	<0.05
Conjugated desmethyldiazepam	2.5	5.1	6.1	2.6



Scheme II—Three-compartment open-model pharmacokinetic system for diazepam

The individual rate constants, k_{12}, k_{21} and k_{13}, k_{31}, reflect the rate of distribution into and out of the shallow and deep peripheral compartments, respectively. The mean ratio of k_{21}/k_{12} of 1.4, reflecting return and entry of drug within the shallow peripheral compartment, suggests rapid equilibration and relatively free transfer of drug between the central and shallow compartments. The mean ratio of k_{31}/k_{13} of 0.3, reflecting return and entry of drug within the deep peripheral compartment, suggests slow equilibration plus drug binding to tissue or protein sites in this compartment, with a corresponding slow return of drug to the central compartment.

The magnitude of k_{31} was small within each subject. This rate constant represents the rate of return of drug from the deep peripheral compartment to the central compartment. This finding suggests that the rate-controlling step in the elimination of diazepam from the body is the rate at which the drug is released from the deep peripheral compartment. The mean ratio of β/k_{el} of 0.18 indicates that only 18% of the diazepam in the body is in the central compartment, available for elimination at any time (24).

By using the rate constants obtained following intravenous administration (Table II), the drug levels in the central and the two peripheral compartments were simulated for each subject (Fig. 6). These simulations clearly show that the deep peripheral compartment contains more diazepam than either the central or shallow peripheral compartment. This finding emphasizes the importance of the return of drug to the central compartment from the deep peripheral compartment as the rate-controlling step in the overal elimination profile of diazepam.

Following the intravenous administration of diazepam, desmethyldiazepam was detected in the blood. Initially, desmethyldiazepam is formed rapidly but then, at approximately 12 hr. postadministration, there is an apparent change in the formation rate constant; the blood levels of desmethyldiazepam continue to rise but very slowly. This slower apparent formation rate constant of desmethyldiazepam was calculated directly from the desmethyldiazepam blood level data after the 12-hr. data point by the method of least squares and found to be essentially equal to the overall elimination rate constant, β, of diazepam in each subject. The initial apparent formation rate constant of desmethyldiazepam was determined by "feathering" (24) the initial portion of the desmethyldiazepam blood level curve. The rate constants are reported in Table IV. Such findings indicate that the overall elimination of diazepam occurs almost exclusively *via* the desmethyldiazepam pathway and that the formation of desmethyldiazepam is, in turn, rate dependent upon the return of diazepam from the deep peripheral compartment.

Overall, the pharmacokinetic profile indicates that k_{31}, the rate of return of drug from the deep peripheral compartment, and not k_{el}, the true elimination rate constant, is the rate-controlling factor in the elimination of diazepam from the body. Therefore, the characteristics of the deep peripheral compartment would be of importance relative to understanding the physiological disposition character-

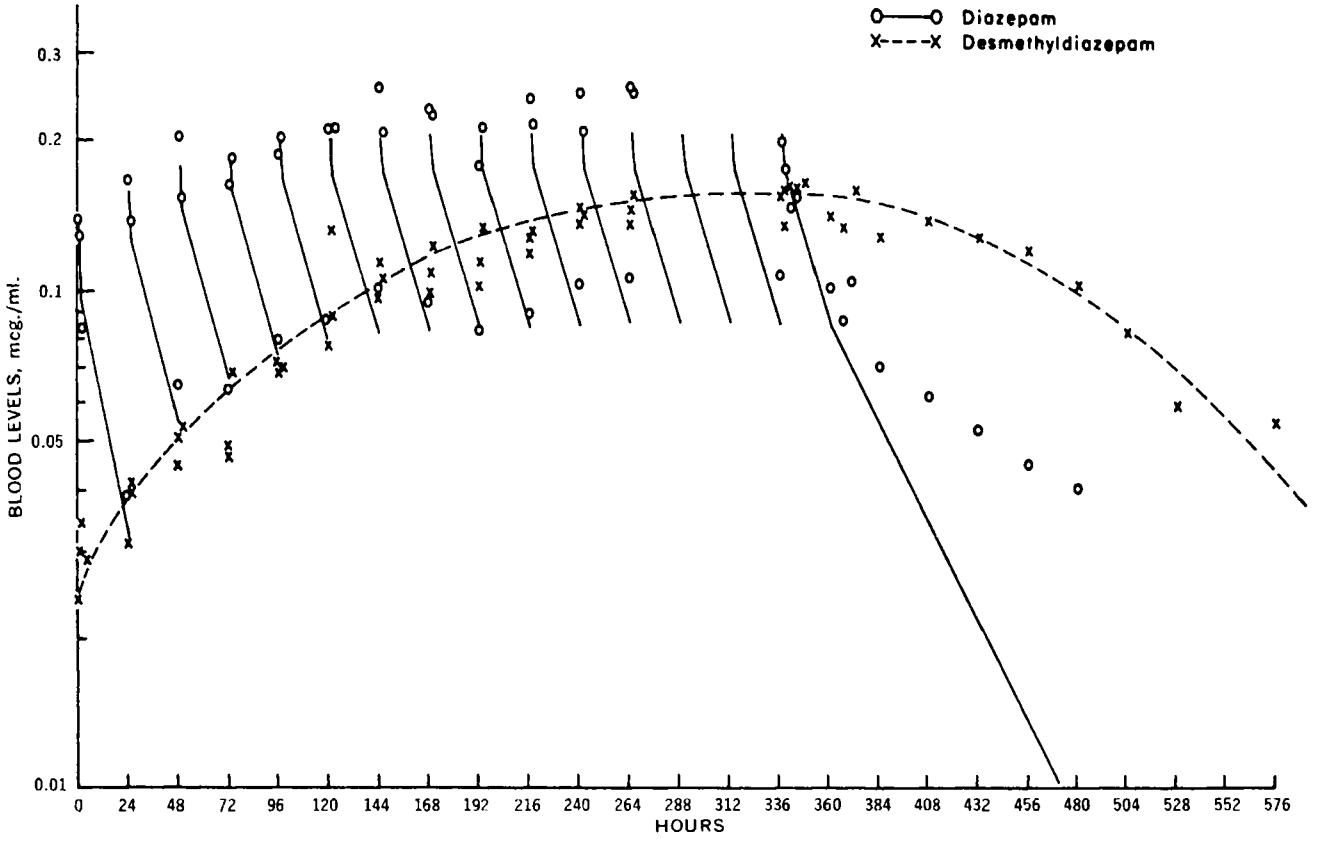


Figure 2—*Oral blood level data of diazepam and desmethyldiazepam in Subject 1 receiving 10 mg. diazepam every 24 hr. for 15 days (solid line represents calculated diazepam blood level curve).*

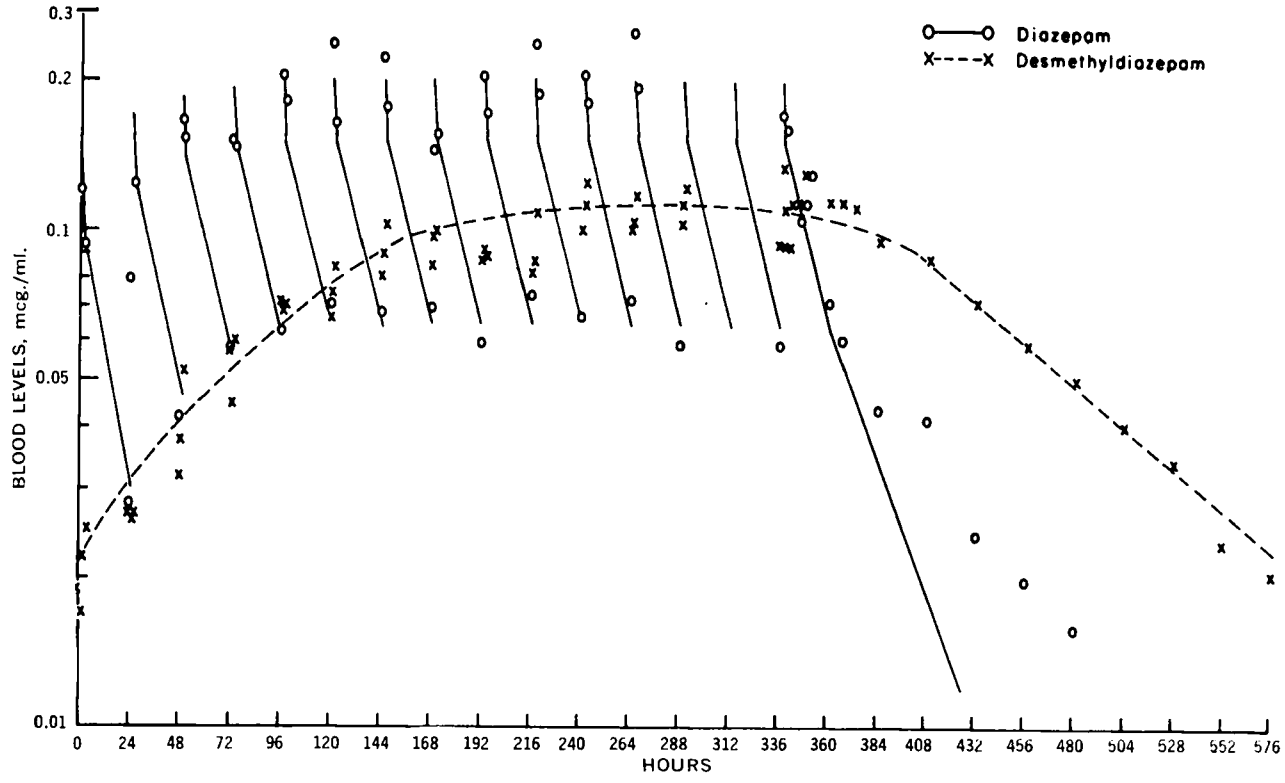


Figure 3—*Oral blood level data of diazepam and desmethyldiazepam in Subject 2 receiving 10 mg. diazepam every 24 hr. for 15 days (solid line represents calculated diazepam blood level curve).*

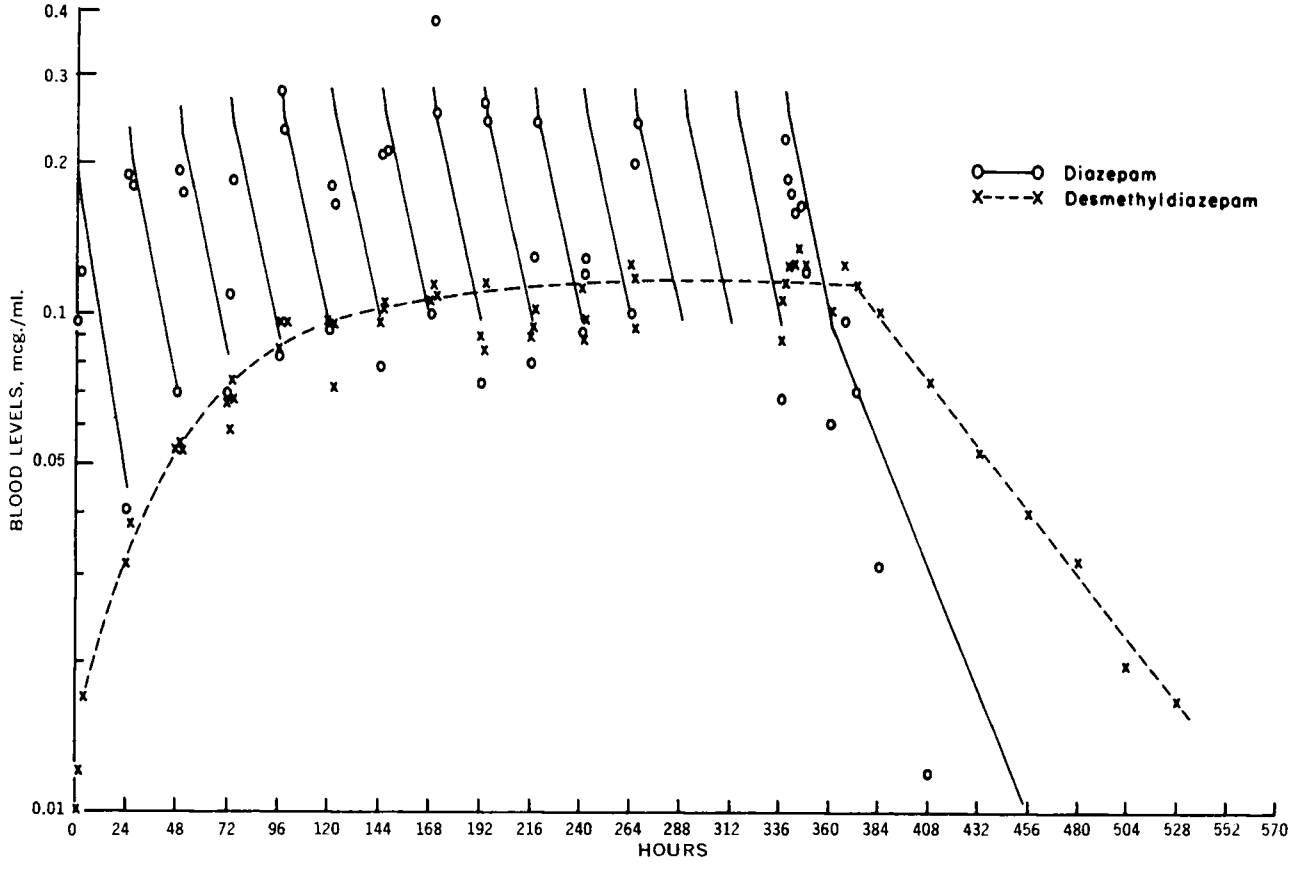


Figure 4—*Oral blood level data of diazepam and desmethyldiazepam in Subject 3 receiving 10 mg. diazepam every 24 hr. for 15 days (solid line represents calculated diazepam blood level curve).*

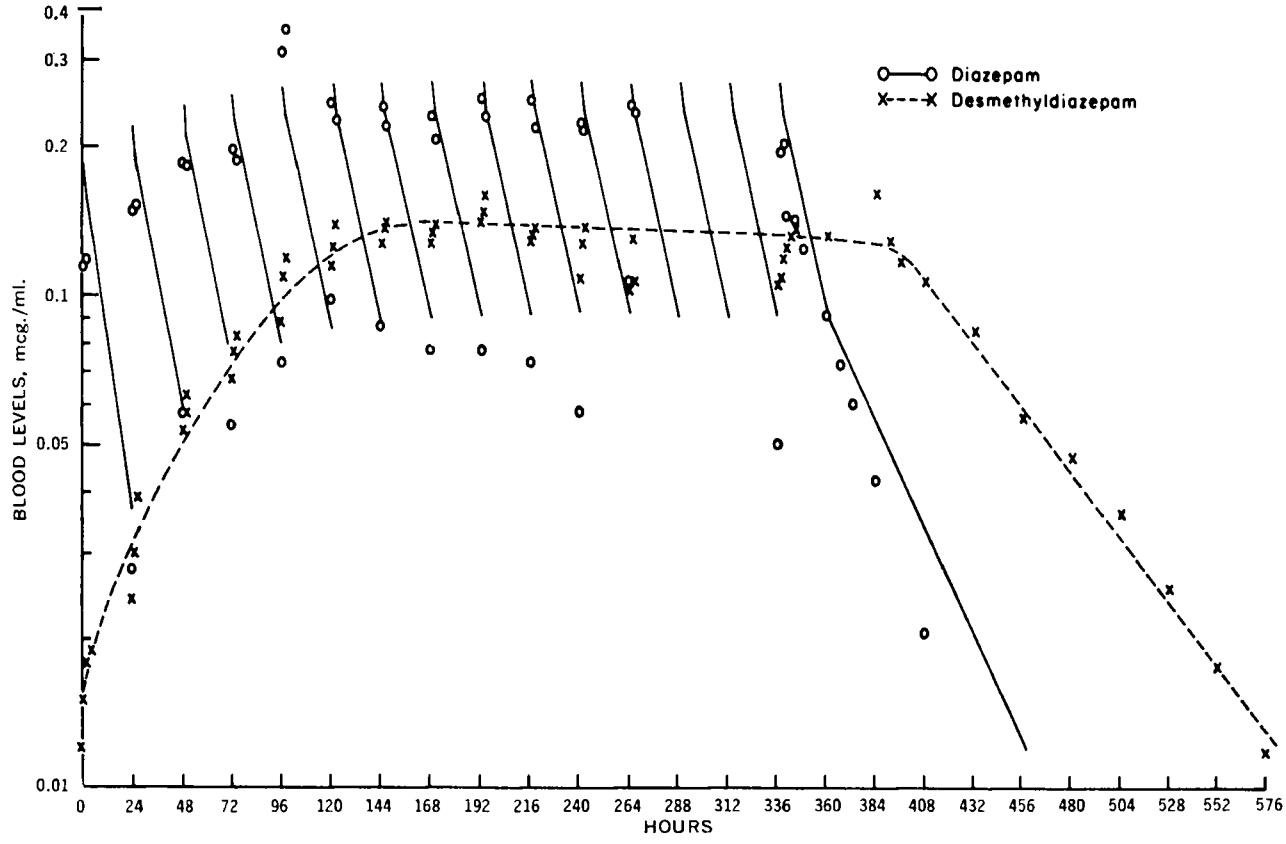


Figure 5—*Oral blood level data of diazepam and desmethyldiazepam in Subject 4, receiving 10 mg. diazepam every 24 hr. for 15 days (solid line represents calculated diazepam blood level curve).*

istics of diazepam in man. Alterations in the binding characteristics of drug to tissues or proteins associated with this compartment may yield marked alterations in the pharmacokinetic profile of the drug, whereas factors that affect the metabolism *per se* (*e.g.*, in the liver) may only minimally affect this pharmacokinetic profile.

Van der Kleijn (26) reported that diazepam is extensively protein bound in the dog. By using whole body autoradiography, he also showed that certain tissues, *i.e.*, brain, kidney, liver, myocardium, and digestive system, show large and rapid uptake of radioactive diazepam following the intravenous administration of diazepam to mice (27). These same tissues, in turn, show a very slow decline of diazepam levels. Although there was a rapid uptake of drug by the adipose tissues, there was a corresponding rapid decline of drug in these tissues, suggesting that drug is not strongly bound to adipose tissue. The observed extensive protein binding and the slow release of diazepam from tissues such as the brain, heart, and digestive system are consistent with the deep compartment of the proposed pharmacokinetic model. The slow release of diazepam from such protein and tissue binding sites probably determines the rate of metabolism and, therefore, elimination of diazepam from the body.

Single Oral Administration—Each subject received a single 10-mg. oral dose of diazepam. The blood levels and urinary excretion data are presented in Tables I and III, respectively, for all four subjects, and the blood level data are plotted in Fig. 1 for Subject 3.

Table IV—Formation Rate Constants for Desmethyldiazepam

	Subject			
	1	2	3	4
Overall elimination rate constant of diazepam, $hr.^{-1}$	0.019	0.026	0.024	0.021
Formation rate constant of desmethyldiazepam after 12 hr., $hr.^{-1}$	0.020	0.028	0.030	0.016
Initial formation rate constant of desmethyldiazepam, $hr.^{-1}$	0.546	0.427	0.329	0.351

Following the single oral administration of 10 mg. diazepam, the blood levels peaked at 1–1.5 hr. in all four subjects, indicating rapid absorption. When using the area under the blood level curve as an index of the extent of absorption, absorption appears to be essentially complete. The ratios of the areas under the oral blood level curves to that of the areas under the intravenous blood levels are 1.2, 0.90, 0.97, and 0.94 for Subjects 1 through 4, respectively.

The overall elimination rate constant, β, calculated following oral administration of diazepam (Table III) approximates the corresponding rate constant obtained following intravenous administration of the drug in each subject.

The evaluation of the oral diazepam blood level data relative to peak heights, peak times, areas under the blood level curves, and the first-order absorption rate constant (28) indicates that diazepam is rapidly and completely absorbed following oral administration in man. In addition, the physiological disposition characteristics do not appear to be dependent upon the route of administration. These characteristics are further confirmed by the similarity of the blood level curves of the metabolite desmethyldiazepam, determined following the intravenous and oral administration of diazepam.

The urinary excretion data following the single intravenous and oral administration of diazepam are presented in Tables II and III. Less than 0.05% of the administered dose was recovered in the urine as intact diazepam, indicating complete biotransformation of diazepam in man. The conjugated biotransformation product of desmethyldiazepam was identified in the urine, accounting for approximately 2.5–9% of the administered dose. The conjugates of oxazepam and 3-hydroxydiazepam were detected but not quantitated. Following the administration of radioactive diazepam, approximately 70% of the administered dose was reported (11, 16, 17) to be excreted in the urine and approximately 10% to be excreted in the feces.

Chronic Oral Administration—A 10-mg. dose of diazepam was administered every 24 hr. for 15 days to each of the four subjects. Blood specimens were obtained at 1, 2, and 24 hr. following each administration and analyzed for diazepam and desmethyldiazepam. The blood levels are presented in Figs. 2–5.

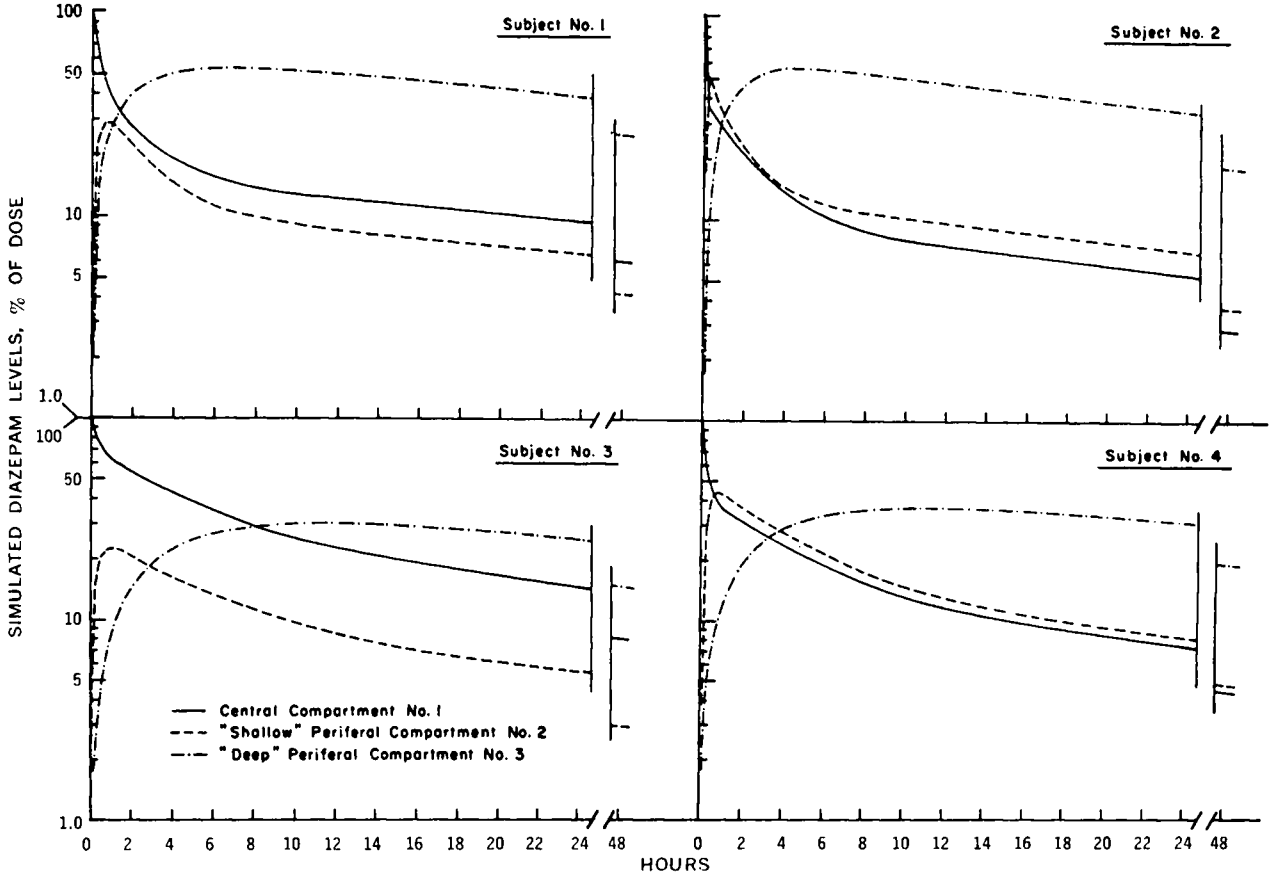


Figure 6—*Simulated compartmental drug level profile of diazepam in Subjects 1–4.*

The diazepam blood level curves following the 10-mg. oral chronic administration of diazepam to four subjects every 24 hr. were simulated by adapting the equations presented by Boxer *et al.* (29). Such an equation is applicable for diazepam because diazepam is both rapidly and completely absorbed. Since the intravenous blood level curve of diazepam is triexponential, the Boxer equation was modified to take into account the contributions of each exponential phase. Therefore, the simulated chronic oral dosing diazepam blood level curves were computed using the pharmacokinetic parameters obtained following the intravenous administration of diazepam to each subject, with the assumption that diazepam was rapidly and completely absorbed. Since the absorption rate constant is much larger than the disposition rate constants, the exponential associated with the absorption can be deleted from the blood level equation without affecting the predicted blood level profile.

Therefore, using Eq. 1 the blood level at any time, t, is the sum of the contributions of each exponential. By applying the Boxer equation for each exponential phase, the sum of the three exponentials will be the blood level at that time. The Boxer equation for a monoexponential is given by Eq. 2:

$$C_{p_n} = \frac{C_0 - C_0 r^n}{1 - r} \qquad \text{(Eq. 2)}$$

where C_{p_n} = blood level following the nth dose at time t, C_0 = initial blood level at time t, and $r = e^{-\beta\tau}$, where β is the slope of the exponential phase and τ is the dosing frequency.

For a triexponential, this equation becomes:

$$C_{p_n} = \frac{P_0 - P_0 r_1^n}{1 - r_1} + \frac{A_0 - A_0 r_2^n}{1 - r_2} + \frac{B_0 - B_0 r_3^n}{1 - r_3} \qquad \text{(Eq. 3)}$$

where P_0, A_0, and B_0 are the contributions of the respective exponentials at the initial time t, and $r_1 = e^{-\pi\tau}$, $r_2 = e^{-\alpha\tau}$, and $r_3 = e^{-\beta\tau}$.

Equation 3 was used to simulate the chronic blood level curves of diazepam for each subject. The simulated chronic diazepam blood level curves (Figs. 2–5) correspond well with the experimental blood level data points. Steady-state (plateau) diazepam blood levels were evident by the 7th day of the study. There was an approximate threefold difference between the mean maximum and minimum diazepam steady-state blood levels (Table V) observed at 1 and 24 hr. following administration, respectively.

The mean steady-state maximum and minimum blood levels were approximately twofold greater than the corresponding blood levels observed on Day 1. The calculated elimination rate constant, β, observed following the last dose on Day 15 approximated the corresponding rate constant observed following the single oral administration of the drug. This finding of relatively constant steady-state diazepam blood levels substantiates the constancy of the absorption, distribution, metabolism, and excretion with each administration of the drug, with all processes following apparent first-order kinetics. Such a steady-state profile for diazepam indicates that unpredicted drug accumulation will not occur and suggests no enzyme induction of diazepam metabolism. In their reported bioavailability data of diazepam, based on steady-state plasma levels, Berlin *et al.* (30) observed a small variation in steady-

Table V—Calculated and Experimental Diazepam Steady-State Blood Levels (Micrograms per Milliliter)

	Subject			
	1	2	3	4
Calculated minimum steady-state level	0.088	0.066	0.099	0.094
Experimental minimum steady-state level	0.104	0.068	0.085	0.078
Calculated maximum steady-state level	0.212	0.204	0.289	0.277
Experimental maximum steady-state level	0.235	0.210	0.224	0.238

state levels between subjects and half-lives of elimination consistent with the data presented herein.

The desmethyldiazepam blood level data obtained following the single intravenous and oral administrations of diazepam (Table I) do not lend themselves to pharmacokinetic evaluation. In all four subjects, the desmethyldiazepam blood levels continued to increase during the 48-hr. experimental period. In most instances, the increasing desmethyldiazepam levels were approximately equal to the declining diazepam blood levels during the 24–48-hr. interval following both intravenous and oral administrations of diazepam. As previously discussed, the formation rate of desmethyldiazepam is influenced by k_{31}, the rate at which diazepam is returning from the deep peripheral compartment to the central compartment.

Following the chronic dosing of 10 mg. diazepam every 24 hr. for 15 days, the desmethyldiazepam maximum–minimum blood level range of 0.16–0.09 mcg./ml. was within the range of the minimum steady-state diazepam blood levels (Figs. 2–5). The smaller range of maximum-to-minimum desmethyldiazepam blood levels is reflecting the slow formation and elimination rates of desmethyldiazepam. Following the last dose of diazepam on Day 15, the apparent half-life of elimination of desmethyldiazepam ranged from 50 to 99 hr. The observed slow decline in desmethyldiazepam levels after the administration of diazepam is slower than might be anticipated after desmethyldiazepam is administered due to the continued formation of the metabolites.

The overall elimination rate of diazepam, which is shown to relate directly to the formation rate of desmethyldiazepam, appears to be a major determining factor in the blood level profile of diazepam and its metabolites in various species. The more rapid the observed diazepam-to-desmethyldiazepam conversion rate, the more rapid is the onset of desmethyldiazepam blood levels. In contrast to man, the dog exhibits a conversion rate that is so rapid that diazepam is barely detected and desmethyldiazepam is essentially the exclusive blood component. In the rat, the conversion rate is negligible so the diazepam is the major blood component. The negligible desmethyldiazepam levels in the rat, however, may also be the result of the metabolic pathway to the 4-hydroxy derivatives (14, 15, 31).

SUMMARY

Four subjects each received a single intravenous and oral 10-mg. dose of diazepam and 10 mg. orally every 24 hr. for 15 days. The intravenous blood level data were fitted with a three-compartment open-model system containing both a shallow and a deep peripheral compartment. The half-lives corresponding to the third exponential are a measure of the overall elimination rate of the drug from the body and ranged from 21 to 37 hr. following intravenous administration. The volume of distribution ranged from 160 to 205% of body weight. The data suggest that the rate at which diazepam returns to the central compartment from the deep peripheral compartment, k_{31}, is the rate-controlling factor in the elimination of diazepam and in the formation rate of desmethyldiazepam.

Following oral administration, diazepam was absorbed rapidly and completely. The elimination rate observed following oral administration approximates that observed following intravenous administration.

Following the multiple oral dosing of 10 mg. diazepam every 24 hr. for 15 days, the diazepam blood levels plateau at approximately Day 7. The mean maximum–minimum diazepam plateau levels were 0.225–0.084 mcg./ml., and the half-life of elimination after the 15th dose was approximately the same as that seen following a single dose of diazepam. The corresponding desmethyldiazepam maximum–minimum levels of 0.16–0.09 mcg./ml. are within the lower range of the diazepam blood levels. The smaller range of maximum–minimum desmethyldiazepam levels results from its slow formation and elimination rate. Following the last dose of diazepam, the apparent half-life of elimination of desmethyldiazepam ranged from 50 to 99 hr.

The minimum and maximum steady-state (plateau) levels of diazepam can be successfully predicted (calculated) as a function of the pharmacokinetic parameters obtained following the intravenous administration of drug to each subject. This confirms the reproducibility of the apparent first-order absorption and physiological disposition characteristics of diazepam from administration to administration.

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Electrokinetic Studies of Bacteria III: Effect of Polyvalent Metal Ions on Electrophoretic Mobility and Growth of *Streptococcus faecalis*

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Abstract □ The effect of the polyvalent cations calcium, lead, copper, chromium, iron III, and thorium on *Streptococcus faecalis* was investigated by microelectrophoresis and by growth inhibition measurements. Thorium and the (hydrolyzed) ferric ion inverted the charge of the bacteria from negative to positive. The other cations merely reduced the absolute value of the negative ζ-potential at the concentrations investigated, which were limited because all measurements were performed at an ionic strength of 0.01 *M*. The order of effectiveness in changing the ζ-potential was thorium $\cong$ ferric $\gg$ chromium $\gg$ copper > lead > calcium, in accordance with the Schulze–Hardy rule. The effect of the salts on the growth of the bacteria was studied by the pour plate, growth curve, and turbidity methods. The order of effectiveness in inhibiting the bacterial growth was thorium > ferric > copper. The other cations produced no detectable growth inhibition at concentrations up to about 0.003 *M*. With the exception of chromium, there is a rank-order correlation between the ability of the polyvalent metal salts to reduce the ζ-potential of the bacteria and to inhibit their growth.

Keyphrases □ Electrokinetics—effect of polyvalent metal ions on electrophoretic mobility and growth of *Streptococcus faecalis* □ Electrophoretic mobility, *Streptococcus faecalis*—effect of calcium, lead, copper, chromium, ferric, and thorium ions, ζ-potential □ Bacterial growth, *Streptococcus faecalis*—effect of calcium, lead, copper, chromium, ferric, and thorium ions, pour plate, growth curve, and turbidity methods □ *Streptococcus faecalis*—effect of polyvalent metal ions on electrophoretic mobility and growth □ Metal ions, polyvalent—effect on electrophoretic mobility and growth of *Streptococcus faecalis* □ Zeta-potential—calculated for *Streptococcus faecalis*, effect of polyvalent metal ions.

The effect of salts of heavy metals on bacteria has been the subject of many studies. However, the mechanism of their antibacterial action, the "oligodynamic effect," is not understood (1). The effect of heavy metal cations on the charge of the cell wall of bacteria and spores has been assessed by electrophoresis (2–7). A possible correlation between antibacterial action and surface charge modification was never investigated; it is the topic of the present study.

EXPERIMENTAL

Electrophoresis Procedures—The methods were as described previously (8, 9), except as noted. The bacteria, *Streptococcus faecalis*, were incubated in the liquid growth medium at 37° for 18–24 hr., being in the stationary phase when harvested. Their suspensions were centrifuged for 10 min. at 9000 r.p.m. The bacteria were freed of adhering nutrients by resuspending the sediment three times in 0.010 *M* $NaNO_3$ or KCl and centrifuging for 10 min. at 9000 r.p.m. each time. When the polyvalent metals were in the form of nitrates, sodium nitrate was used for washing; it was also added to polyvalent metal salt solutions to adjust the ionic strength. When necessary, potassium chloride was used for the chlorides and sulfates. Electrophoretic measurements and viability assessments were made on bacterial suspensions of ionic strength equal to 0.010 *M*. It was shown (8) that the electrophoretic mobility of *S. faecalis* was not affected by changing buffers and other electrolytes from K^+ to Na^+ salts. Electrophoretic mobilities were measured after the bacteria had been in contact with the polyvalent cations for 30 min. at room temperature. Whenever tested, increases or decreases of 15 min. in the time of contact produced no significant change in mobility. The glass cell used had a cell constant of 62.4 $cm.^{-1}$, compared to 67.6 $cm.^{-1}$ for the plastic cell (8). The cell constants are used to calculate the specific conductance of the suspensions.

The solutions were not buffered in order to prevent precipitation or complexation of the polyvalent cations by the buffers used previously, namely, phosphate, acetate, or phthalate. Fortunately, the electrophoretic mobility of *S. faecalis* had been found (8) to be independent of pH between 4 and 7. Salts of polyvalent cations were freshly dissolved to avoid extensive hydrolysis.

Bacteriological Procedures—Three methods were used to assess the antibacterial activity of the heavy metal salts, designated as pour plate, growth curve, and turbidity methods. In the first two methods, the bacteria were treated in the same way as for the electrophoresis studies. After 18–24 hr. of incubation in the liquid growth medium, when the bacteria were in the stationary phase, the suspensions were centrifuged, washed by centrifugation three times with 0.010 *M* KCl or $NaNO_3$, and suspended for 30 min. at room temperature in solutions containing different concentrations of polyvalent metal salts. The ionic strength of these solutions was adjusted to 0.010 *M* with potassium chloride or sodium nitrate when necessary. In the blank experiments, the bacteria were given the identical treatment except that potassium chloride replaced the polyvalent metal chlorides and sulfates, and sodium nitrate replaced the polyvalent metal nitrates.

In the *pour plate method*, after the suspensions had been standing for 30 min., 1-ml. aliquots were diluted 10^4–10^6-fold with distilled water, and 0.1-ml. portions of the diluted suspension were mixed in petri dishes with 5% molten brain–heart infusion agar[1]. After 20 hr. of incubation at 37°, the number of colonies was counted. The difference between the plate counts of samples treated with potassium chloride or sodium nitrate and with polyvalent cation salts is due to the antibacterial action of the latter.

In the *growth curve method*, the suspensions already described, containing one of the heavy metal salts and/or potassium chloride or sodium nitrate, were left to stand for 30 min. At that time, 1-ml. aliquots were withdrawn and added to 100-ml. portions of a sterile liquid growth medium contained in 300-ml. culture flasks equipped with side arms. The growth medium contained 1% yeast extract[1], 1% bacto-tryptone[1], 0.1% dextrose, 0.050 *M* K_2HPO_4, and 0.050 *M* KH_2PO_4 and had a pH of 6.8. This medium was given a higher phosphate concentration than the 0.0023 *M* concentration of the medium previously used to increase the growth rate of the bacteria. The higher phosphate content reduced the lag time (defined below) from 3.5 to 2.6 hr. The culture flasks were shaken in a 37° bath. Absorbance measurements at 640 nm. were made at hourly intervals by inserting the side arm into a spectrophotometer[2]. During the logarithmic phase, absorbances were measured every 30 min.

The growth curves (plots of absorbance *versus* incubation time) are based on the "adjusted net absorbance" (*AA*), which is the "net absorbance" (*NA*) corrected for light scattering. Net absorbance is the difference between the absorbance after the specified incubation time and the absorbance immediately after inoculation. The relationship between *NA* and *AA* is given (10) by:

$$NA = AA - 0.1(AA)^2 - 0.002(10AA - 2)^2 \quad \text{(Eq. 1)}$$

This equation can be simplified, for *NA* values between 0.196 and

[1] Supplied by Difco Laboratories.
[2] Spectronic 20, Bausch & Lomb Co.

Table I—Effect of Polyvalent Cations on the Electrophoretic Mobility of *S. faecalis*

Salt and Concentration[a]	pH	Specific Conductance, μmhos/cm.	Mobility ± *SD*[b], (microns/sec.)/(volts/cm.)
0.010 *M* KCl	7.0, 5.8, 5.3[c]	1370, 1350, 1350[c]	−2.07 ± 0.06, −2.07 ± 0.03, −2.54 ± 0.07[c]
0.010 *M* $NaNO_3$	7.6–5.6, 6.1–5.1	1060, 1060,	−1.94 ± 0.05, −1.96 ± 0.07,
	7.6–8.3, 7.6	1120, 1000	−2.00 ± 0.07, −2.15 ± 0.10
0.010 *M* K acetate	7.2, 7.1, 6.9	1000, 1080, 1060	−2.21 ± 0.09, −2.31 ± 0.07, −2.38 ± 0.09
0.0033 *M* $CaCl_2$	6.2–5.1	810	−1.18 ± 0.02
0.0010 *M* $Ca(NO_3)_2$	7.5–8.7	1000	−1.96 ± 0.07
0.0022 *M* $Ca(NO_3)_2$	6.9–8.5	940	−1.74 ± 0.05
0.0033 *M* $Ca(NO_3)_2$	7.1–8.1, 7.6 5.8	810, 810	−1.52 ± 0.04, −1.57 ± 0.05
0.0010 *M* $Pb(NO_3)_2$	5.2	1090	−1.89 ± 0.06
0.0022 *M* $Pb(NO_3)_2$	5.3	870	−1.69 ± 0.04
0.0033 *M* $Pb(NO_3)_2$	4.7, 5.6	810, 810	−1.21 ± 0.02, −1.27 ± 0.02
0.0010*M* $CuSO_4$ + 0.0060 *M* KCl	5.0, 4.7	1010, 1080	−1.68 ± 0.04[d], −1.69 ± 0.04
0.0025 *M* $CuSO_4$	4.7	540	−1.33 ± 0.03
5×10^{-5} *M* $Cr(NO_3)_3$	5.8	1080	−1.85 ± 0.03
0.00083 *M* $Cr(NO_3)_3$	3.7	950	−1.17 ± 0.02
0.00167 *M* $Cr(NO_3)_3$	3.6	680	−0.82 ± 0.005
0.00042 *M* $Fe(NO_3)_3$ + 0.00748 *M* KCl	3.5	1490	+2.87 ± 0.05
0.00084 *M* $Fe(NO_3)_3$ + 0.00496 *M* KCl	3.1	1350	+3.05 ± 0.06
0.00167 *M* $Fe(NO_3)_3$	2.8	1690	+2.79 ± 0.06
1×10^{-6} *M* $Th(NO_3)_4$	5.2	1150	−2.25 ± 0.03
5×10^{-6} *M* $Th(NO_3)_4$	5.0	1280	−1.04 ± 0.05
1×10^{-5} *M* $Th(NO_3)_4$	4.8	1150	−0.56 ± 0.04
5×10^{-5} *M* $Th(NO_3)_4$	4.2	1280	+1.71 ± 0.04
1×10^{-4} *M* $Th(NO_3)_4$	4.0	1150	+2.22 ± 0.03
2.5×10^{-4} *M* $Th(NO_3)_4$	3.7	1080	+2.37 ± 0.04
5×10^{-4} *M* $Th(NO_3)_4$	3.6	950	+2.52 ± 0.04
1×10^{-3} *M* $Th(NO_3)_4$	3.5	640	+2.96 ± 0.03

[a] Molarity of salt of polyvalent cation when employed. Sodium nitrate was added to nitrates and potassium chloride to chlorides when necessary to bring the ionic strength to 0.010 *M*. When salts of polyvalent and monovalent cations having different anions were mixed, both are listed. [b] Average of 10 or more measurements ± standard deviation of the average. [c] Commas separate results of measurements on different batches of *S. faecalis*, incubated on different days. [d] Washed by centrifugation with 0.0010 *M* $CuSO_4$ + 0.0060 *M* KCl instead of the standard procedure of washing with 0.010 *M* KCl.

about 0.9 (10), to:

$$AA = 1.800 - 0.1\sqrt{321.3 - 330NA} \qquad \text{(Eq. 2)}$$

The adjusted net absorbance of the suspensions is directly proportional to the bacterial concentration, as demonstrated in Fig. 1. *S. faecalis* was grown in the medium described, the suspension was diluted with the growth medium in different proportions, and the absorbance was measured at 640 nm. for each dilution.

Any differences between the growth curves (plots of *AA versus* time) of bacteria treated with polyvalent metal salts and of bacteria treated with sodium nitrate or potassium chloride, such as extended lag phase, reduced growth rate in the exponential phase, or lower bacterial concentration in the stationary phase, are a measure of the antibacterial activity of the former.

The following parameters were investigated in the growth curve method: lag time, generation time, and absorbance in the stationary phase. Lag time and generation time were obtained from semilogarithmic plots of adjusted net absorbance *versus* time.

Lag time, *L*, in hours, is defined (11) by the equation:

$$\log\left(\frac{AA}{0.01}\right) = K(T - L) \qquad \text{(Eq. 3)}$$

where the lag *L* is the time, obtained by extrapolation of the linear portion of the plot corresponding to the exponential growth phase, at which $AA = 0.01$. For time $T = L$, $\log(AA/0.01) = 0$ and $(AA/0.01) = 1$. Instead of specifying the slope *K* of the semilogarithmic plot, the generation time (11) was determined, which is the time required to double the number of cells or the adjusted net absorbance during the logarithmic phase.

In the *turbidity method*, heavy metal salts and/or potassium chloride or sodium nitrate were incorporated into the original, low phosphate growth medium (8, 9) so that the ionic strength of the added electrolytes was 0.010 *M*. These media, contained in culture flasks with side arms, were inoculated with *S. faecalis*, and their absorbance at 640 nm. was measured before and after 24 hr. of incubation at 37° in a shaking bath. After the 24-hr. period, the bacteria were in the stationary phase. Any differences in turbidity increase between suspensions containing polyvalent cations and the blank suspensions containing Na^+ or K^+ are due to the antibacterial action of the polyvalent cations.

RESULTS AND DISCUSSION

Electrokinetic Measurements— The results of the electrophoresis studies are presented in Table I. The pH values were determined just before and just after the mobility measurements. If the difference between the two values was more than 0.5 unit, both values are listed. Single values represent the average pH when the determinations before and after the electrophoretic measurements agreed within 0.5 unit of pH. Negative values for the electrophoretic mobility indicate that the bacteria were negatively charged and migrated to the positive pole. Two samples of the same strain, ATCC 9790, supplied by the American Type Culture Collection about 10 months apart, gave identical mobility values within the precision of the measurements. The earlier measurements were made with the plastic cell (8), while the later measurements were made with a glass cell. The two sets of measurements are combined in Table I.

Polyvalent cations reduced the negative charge of the cell wall progressively as their concentration in the solution was increased while the ionic strength was maintained constant at 0.010 *M*. This change in ζ-potential is due to chemisorption of the cations as exemplified for a divalent cation Me^{+2} by Scheme I, in which W represents the cell wall. Whenever a monovalent Na^+ was replaced by a divalent cation, one fixed negative charge of the cell wall was replaced by one fixed positive charge because polyvalent cations are firmly attached to the carboxylate groups in the wall. Carboxylates of di- and trivalent cations undergo little or no dissociation because the bonds between the carboxylate groups and the cations are chiefly covalent and/or coordinate covalent. Their attachment to

$$W\text{—}COO^- Na^+ + Me^{+2}(NO_3)_2^{-2} \rightleftharpoons W\text{—}COO\cdots Me^+(NO_3)^- + Na^+ NO_3^-$$

Scheme I

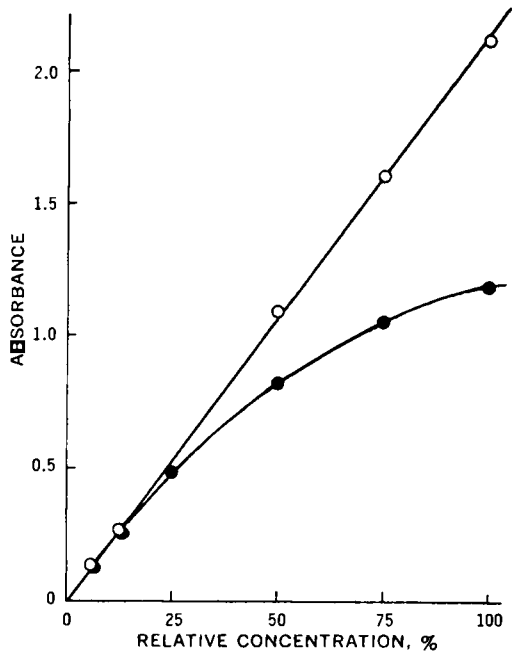

Figure 1—*Growth curve method: effect of dilution on net absorbance of suspensions of* S. faecalis *at 640 nm. Key:* ●, NA; *and* ○, AA.

the carboxylate groups in the cell wall made the di- and trivalent cations an integral part of the bacterium; they were chemisorbed in the Stern layer. Potassium and sodium ions originally bound to the carboxylate groups of the cell wall were extensively dissociated and hence constituted the counterions, being located in the Stern layer and in the diffuse or Gouy double layer (12) surrounding the bacterium. The di- and trivalent cations which replaced them are not dissociated from the carboxylate groups. Therefore, as the reaction (Scheme I) proceeds, the counterions at the cell wall are progressively changed from Na^+ or K^+ to NO_3^-, reducing the negative charge of the cell.

In this cation-exchange process, the tetravalent cation thorium actually inverted the sign of the charge of the bacteria from negative to positive. The di- and trivalent cations could not be used at high enough concentrations to cause charge reversal because of the upper concentration limit corresponding to the ionic strength of 0.010 *M* adopted in all electrophoresis measurements.

When combining the information from Fig. 2 with Table IV of *Reference 8*, it is seen that cation concentrations required to produce

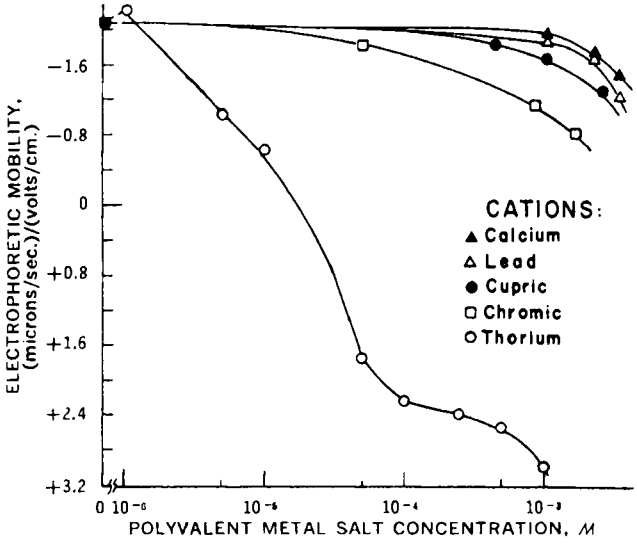

Figure 2—*Effect of the concentrations of various polyvalent cations on the electrophoretic mobility of* S. faecalis *at constant ionic strength.*

Table II—Effect of Polyvalent Metal Salts on the Growth of *S. faecalis* by Pour Plate Method

Salt	*M*[a]	Average Count[b]	*SD*[c]	*n*[d]
Cupric sulfate	0(0.010 *M* KCl)	93	26	11
	0.0001	80	7.1	4
	0.0010	50	8.7	6
	0.0025	21	10	8
Calcium chloride	0.0033	70	—	2
Thorium nitrate	0(0.010 *M* $NaNO_3$)	80	5.2	13
	1×10^{-6}	10	4	4
	1×10^{-5}	0.0063	0.0006	3
	1×10^{-4}	0.0068	0.0004	3
	1×10^{-3}	0.0000	—	2
Chromic nitrate	0.00084	81	—	2
	0.00167	67	—	2
Ferric nitrate	0.00042	60	—	2
	0.00084	0.0	—	2
Lead nitrate	0.00333	62	—	2

[a] Constant ionic strength of 0.01 *M* achieved by addition of potassium chloride or sodium nitrate. [b] Determined at or prorated to a dilution of $1:10^7$. [c] Standard deviation of average count. [d] Number of plates counted.

comparable reductions in electrophoretic mobility and, hence, in ζ-potential are in the ratio of 1:100:900:8000 for Th^{+4}, Cr^{+3}, the average of the three divalent cations, and K^+. Qualitatively, this is in accordance with the Schulze–Hardy rule (12).

The ratio is higher by an order of magnitude than the ratio of the flocculation concentrations for tetra-, tri-, di-, and monovalent counterions predicted by the Derjaguin–Landau–Verwey–Overbeek theory, namely, $1/4^6:1/3^6:1/2^6:1/1^6 = 1:5.6:64:4100$ (12). The most likely explanation for this discrepancy is the occurrence of specific interactions between the polyvalent cations and the cell wall. The theoretical flocculation values are based on lack of specificity, the charge of the counterion being the only factor considered in the reduction of the ζ-potential by added electrolytes. In view of the affinity of the carboxylate group for heavy metal cations, specific interactions must have occurred and probably account for most, if not all, of the low concentrations or high concentration ratios at which the polyvalent cations caused the observed reductions in electrophoretic mobility.

Ferric nitrate produced charge reversal and nearly identical positive electrophoretic mobilities at all three concentrations tested, the lowest of which was 4.2×10^{-4} *M*. The tetravalent thorium nitrate produced comparable positive mobilities at a similar concentration, namely, 8×10^{-4} *M*, while the trivalent chromium cation did not reverse the negative charge of the bacteria at concentrations as high as 16.7×10^{-4} *M*. The exceptional behavior of ferric nitrate is ascribed to extensive hydrolysis, which produces a variety of polyvalent hydroxylated cations in solution such as $Fe_3(OH)_4^{+5}$. These are more effective charge-inverting species than Fe^{+3} (13). The occurrence of extensive hydrolysis of ferric nitrate in bacterial suspensions was shown by a deepening of their color to a reddish brown shortly after dissolving the nitrate in water.

Of the divalent cations, Cu^{+2} was the most effective in reducing the electrophoretic mobility and Ca^{+2} the least effective. As shown in Table I, Cu^{+2}, Pb^{+2}, and Ca^{+2} at a concentration of 0.0010 *M*

Table III—Effect of pH on Growth Curve Parameters

Buffer[a]	pH	Lag[b], hr.	Generation Time[b], hr.	Absorbance in Stationary Phase[b]
Phosphate	7.1	2.6	0.37	1.17
Acetate	7.0	2.6	0.38	1.18
Acetic acid + potassium bromide	3.45	4.6	0.46	1.19
Acetic acid + potassium chloride	3.5	2.9	0.40	1.18
Phosphoric acid	2.35	3.0	0.38	1.18

[a] See text for composition. [b] Based on duplicate growth curves.

Table IV—Effect of Heavy Metal Salts on Growth of *S. faecalis* by Growth Curve Method

Salt	Concentration[a], *M*	pH	Lag[b], hr.	Generation Time[b], hr.	Absorbance in Stationary Phase[b,c]
Calcium chloride	0(0.010 *M* KCl)	5.9	2.8	0.38	1.39
	0.0033	6.2	2.4	0.43	1.31
Cupric sulfate	0(0.010 *M* KCl)	6.0	3.2	0.42	1.44
	0.0010 + 0.0060 *M* KCl	4.8	3.6	0.46	1.42
	0.0025	4.7	3.8	0.44	1.42
Lead nitrate	0(0.010 *M* $NaNO_3$)	6.1	2.4	0.43	1.38
	0.0033	5.0	2.8	0.46	1.36
Chromic nitrate	0(0.010 *M* $NaNO_3$)	6.4	2.7	0.41	1.44
	0.000835	3.7	3.0	0.38	1.35
	0.00167	3.6	3.0	0.41	1.42
Thorium nitrate	0(0.010 *M* $NaNO_3$)	6.9	2.7	0.49	1.32
	1×10^{-6}	5.6	3.0	0.51	1.35
	1×10^{-5}	4.8	5.0	0.35	1.31
	1×10^{-4}	4.0	5.4	0.40	1.25

[a] Molarity of salt of polyvalent cation when employed. Sodium nitrate was added to nitrates and potassium chloride to chlorides when necessary to bring the ionic strength to 0.010 *M*. When salts of polyvalent and monovalent cations having different anions were mixed, both salts are listed. [b] Based on duplicate growth curves. [c] Adjusted net absorbance. Average of two readings taken between 21 and 27 hr. of incubation time.

reduced the electrophoretic mobility by 0.41, 0.21, and 0.14 (microns/sec.)/(volts/cm.), respectively. These differences or specific effects are ascribed to increasingly stronger binding of the cations to the carboxylate groups in the cell wall when going from Ca^{+2} to Pb^{+2} to Cu^{+2}, in line with their increasing ability to form stable complexes with carboxylated polymers (14, 15).

Bacteriological Results—*Pour Plate Method*—Aqueous solutions of heavy metal salts are frequently acid. To determine how much of their antibacterial activity is due to the heavy metal ions and how much to acidity, it is necessary to determine the effect of acidity on bacterial inhibition in the absence of heavy metals. This was accomplished by exposing the bacteria for 30 min. to the following three buffer solutions of 0.010 *M* ionic strength: 0.010 *M* potassium acetate (pH 7.2), 0.010 *M* acetic acid + 0.0096 *M* KBr (pH 3.3), and 0.00667 *M* phosphoric acid + 0.00580 *M* KCl (pH 2.4). After the 30-min. exposure, aliquots of the suspensions were serially diluted with distilled water in a $1:10^7$ ratio, and the viability of the bacteria was determined by the pour plate method. The average colony counts ($\pm SD$ of the average count) of quadruplicate or quintuplicate experiments were 100 ± 18, 97 ± 6, and 53 ± 12 at the pH values of 7.2, 3.3, and 2.4, respectively. Only the most acid of the treating media produced a significant decrease in viability. None of the heavy metal solutions had pH values below 3.3. Therefore, all inhibitory effects are due to the cations.

Detailed studies with cupric sulfate indicated that the reproducibility of the pour plate method was not very good. Over a 4-week period, eight plates were inoculated with bacteria pretreated with 0.0025 *M* $CuSO_4$, using the same procedures including identical dilutions. The corresponding blanks were inoculated at the same times with bacteria pretreated with 0.010 *M* KCl.

Four of the plates inoculated with bacteria exposed to cupric sulfate had no growth; others had as many as 50 and 76 colonies. However, when the *F* test was applied to the plate counts made at the four levels of cupric sulfate employed (0, 0.0001, 0.0010, and 0.0025 *M*), the trend of increasing inhibition with increasing cupric sulfate concentration was statistically significant: the *F* value obtained exceeded the critical *F* value at the 5% level.

Eleven blank tests, in which the bacteria were pretreated with potassium chloride, were run for the three levels of cupric sulfate. The standard deviation of the average count of the 11 blanks was 28% of that average.

As can be seen from Table II, calcium nitrate, lead nitrate, and chromic nitrate produced no significant inhibition at the concentrations tested; thorium nitrate was the most effective antibacterial agent, followed by ferric nitrate.

Growth Curve Method—The effect of the acidity of the treating solution on the growth of the bacteria was determined by measuring growth curves in the following five buffer solutions: phosphate (pH 7.1), containing 0.00584 *M* KH_2PO_4 + 0.00082 *M* K_2HPO_4; acetate (pH 7.0), containing 0.010 *M* potassium acetate; acetic acid (pH 3.45), containing 0.010 *M* acetic acid + 0.0096 *M* potassium bromide; acetic acid (pH 3.5), containing 0.010 *M* acetic acid + 0.0096 *M* potassium chloride; and phosphoric acid (pH 2.35), containing 0.00667 *M* phosphoric acid + 0.0058 *M* potassium chloride. The ionic strength of the five solutions is approximately 0.01 *M*. After 30 min. contact time, aliquots were incubated and the growth curves determined.

The results (Table III) indicate that the acidity of the treating solution had only a small effect on the three growth curve parameters. The exception is the acetic acid solution containing potassium bromide which caused increases in lag and in generation time. Its pH was a full unit higher than that of the phosphoric acid solution which caused little or no inhibition, indicating that the potassium bromide rather than the pH was responsible for the inhibition caused by the acetic acid potassium bromide buffer. A comparable solution containing potassium chloride instead of bromide caused little inhibition.

Since the lowest pH in the heavy metal solutions was 3.6, any antibacterial activity caused by heavy metal salts is, therefore, due to the cations rather than to a reduction in pH.

Of the results listed in Table IV, the most pronounced inhibition is the increase in lag caused by two thorium nitrate solutions. Lesser inhibitions are the decreased growth in the stationary phase caused by the most concentrated thorium nitrate solution and the increased lag caused by the two copper sulfate solutions.

Turbidity Method—The only cation that showed any inhibition on the growth of *S. faecalis* according to the turbidity method was copper (Table V). Thorium and ferric ions caused no significant inhibition at concentrations at which they completely inhibited the growth of bacteria in the pour plate method. This is evidently due to lowering of the concentration or even disappearance of the free cations due to one of the following three reactions: hydrolysis of the heavy metal salts in the relatively alkaline nutrient broth (initial pH 6.5) causing precipitation of basic salts or hydroxides, particularly of the trivalent and tetravalent cations; precipitation of the phosphates (initial phosphate concentration 0.0023 *M*); and complexation by polypeptides and other organic compounds present in the liquid growth medium. Increased turbidity on addition of salts to the growth medium was noticed for thorium and especially for lead. The marked turbidity that developed when lead nitrate was added to the growth medium was cleared by the addition of nitric acid. It renders the apparent inhibition of bacterial growth by lead nitrate suspect.

Calcium chloride apparently stimulated the growth of the bacteria somewhat. Growth stimulation is well known for salts of magnesium, another alkaline earth metal (16).

The pour plate and the growth curve methods differed from the turbidity method in that the bacteria were treated with unbuffered solutions of the heavy metal salts in the former two. The only possible additives were potassium chloride or sodium nitrate. This precluded the precipitation or complexation of the polyvalent cations by ingredients of the growth medium prior to their coming into contact with the bacteria; the bacteria were treated with the cations at their full nominal concentrations.

Table V—Effect of Heavy Metal Salts Added to Low Phosphate Growth Medium on Growth of *S. faecalis*

Additive	Net Absorbance[a]
None	0.87–0.03 = 0.84
0.010 *M* KCl	0.87–0.04 = 0.83
0.0033 *M* $CaCl_2$	1.00–0.04 = 0.96
None	0.75–0.03 = 0.72
0.010 *M* KCl	0.80–0.02 = 0.78
0.0010 *M* $CuSO_4$ + 0.0060 *M* KCl	0.48–0.08 = 0.40
None	0.80–0.03 = 0.77
0.010 *M* KCl	0.85–0.02 = 0.83
0.0025 *M* $CuSO_4$	0.51–0.08 = 0.43
None	0.75–0.03 = 0.72
0.010 *M* $NaNO_3$	0.75–0.02 = 0.73
0.00333 *M* $Pb(NO_3)_2$	0.80–0.28 = 0.52
None	0.85–0.04 = 0.81
0.010 *M* $NaNO_3$	0.90–0.04 = 0.86
0.00042 *M* $Fe(NO_3)_3$ + 0.00748 *M* $NaNO_3$	0.95–0.05 = 0.90
None	0.80–0.04 = 0.76
0.010 *M* $NaNO_3$	0.85–0.03 = 0.82
0.00167 *M* $Fe(NO_3)_3$	0.85–0.10 = 0.75
None	0.75–0.04 = 0.71
0.010 *M* $NaNO_3$	0.75–0.03 = 0.72
0.00167 *M* $Cr(NO_3)_3$	0.80–0.08 = 0.72
None	0.75–0.04 = 0.71
0.010 *M* $NaNO_3$	0.75–0.03 = 0.72
0.0010 *M* $Th(NO_3)_4$	0.82–0.13 = 0.69

[a] Uncorrected absorbance at 640 nm. after 24 hr. of incubation at 37° minus initial absorbance.

Another shortcoming of the turbidity method is that it measured the effect of the heavy metal salts on the bacteria only in the stationary phase, whereas the growth curve method included the lag and exponential phases as well.

CONCLUSIONS

Combining the results of the methods for measuring growth inhibition gives the following order of decreasing antibacterial activity for the cations employed in this study: thorium > ferric > copper. The remaining cations were ineffective at the concentrations employed. Sprowls and Poe (17) likewise found the antibacterial activity of ferric and copper salts to be greater than that of salts of lead and of other transition metals.

The order of decreasing effectiveness in reducing the electrophoretic mobility was: thorium ≅ ferric ≫ chromium ≫ copper > lead > calcium. With the exception of the chromium ion, there is a correlation in the rank order of growth inhibition and of reduction in the absolute value of the ζ-potential. This indicates that the strength with which polyvalent cations are attached to the outer portion of the bacterial cell wall is similar to the strength with which they are attached to the site where they exert their strongest antibacterial action.

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Peyote and Related Alkaloids XVI: Synthesis of 3,4,5-Trimethoxyphenylalanine, an Amino Acid Analog of Mescaline

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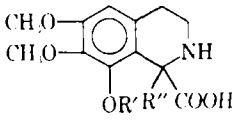
Abstract □ Contrary to an earlier literature report, acid hydrolysis of diethyl α-acetamido-α-3,4,5-trimethoxybenzylmalonate during the synthesis of 3,4,5-trimethoxyphenylalanine has been shown to lead to a mixture of products. The major hydrolysis products were isolated and identified by NMR and mass spectrometry as 3,4,5-trimethoxyphenylalanine (34%), 4-hydroxy-3,5-dimethoxyphenylalanine (13%), 3-hydroxy-4,5-dimethoxyphenylalanine (8%), and 3,4-dihydroxy-5-methoxyphenylalanine (5%). Under basic conditions, the acetamidomalonate ester as well as its formamido analog, obtained by the condensation of trimethoxybenzyl chloride and diethyl formamidomalonate, gave low yields (5–7%) of the required trimethoxyphenylalanine. However, no phenolic side-products were formed under basic conditions. An efficient alternative synthesis of 3,4,5-trimethoxyphenylalanine has been accomplished; it involves the condensation of hydantoin with trimethoxybenzaldehyde followed by hydrogenation and basic hydrolysis of the resulting 5-(3,4,5-trimethoxybenzylidine)hydantoin. GC–mass spectrometric examination of the peyote amino acid fraction gave no evidence for the presence of trimethoxyphenylalanine and other trioxygenated phenylalanine analogs obtained during these synthetic studies.

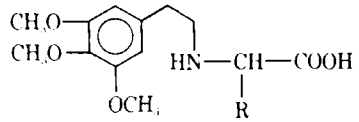
Keyphrases □ 3,4,5-Trimethoxyphenylalanine (mescaline analog)—synthesis □ Peyote and related alkaloids—synthesis of 3,4,5-trimethoxyphenylalanine (amino acid analog of mescaline) □ Mescaline derivatives—synthesis of 3,4,5-trimethoxyphenylalanine

In recent studies on peyote [*Lophophora williamsii* (Lem.) Coult.] constituents, the authors examined the amino acid fraction of the cactus and reported the occurrence of several novel nonproteinic amino acids (1–5). These include four C_1-carboxytetrahydroisoquinolines (I–IV), two *N*-alkyl-α-amino acids (V and VI), and a pyrrole-2-carboxylic acid (VII).

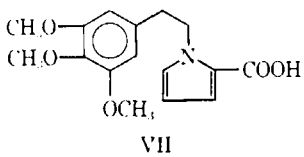
To identify additional unknown constituents present in the peyote amino acid fraction (3), the possible occurrence of trioxygenated analogs of phenylalanine was considered. Since mescaline (3,4,5-trimethoxyphenethylamine) is the major alkaloid present in the peyote cactus, synthesis of its amino acid analog, 3,4,5-trimethoxyphenylalanine (VIII), was undertaken to check for its natural occurrence. This amino acid analog of mescaline was also needed to evaluate its hallucinogenic and other biological activities.



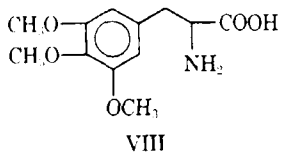
I: R′ = R″ = H
II: R′ = H, R″ = CH$_3$
III: R′ = CH$_3$, R″ = H
IV: R′ = R″ = CH$_3$



V: R = H
VI: R = CH$_3$



VII



VIII

RESULTS AND DISCUSSION

Synthesis by Literature Method—Synthesis of 3,4,5-trimethoxyphenylalanine (VIII) was reported previously by Acheson *et al.* (6). The procedure consists of preparation of the key intermediate, diethyl α-acetamido-α-3,4,5-trimethoxybenzylmalonate (IX), from 3,4,5-trimethoxybenzaldehyde (X) by successive reactions with sodium borohydride, thionyl chloride, and diethyl acetamidomalonate and its hydrolysis by 5 *N* HCl. The hydrolysis of the acetamidomalonate ester IX was reported to give the required amino acid VIII as its hydrochloride in 68% yield.

This procedure was repeated, and it was found that the acid hydrolysis of IX under identical conditions leads to a mixture of products. The hydrochloride, which separated on cooling the reaction mixture, was recrystallized from 5 *N* HCl (as reported in the previous procedure) to give needles, m.p. 241–242° dec. [lit. (6) m.p. about 242° dec.]. This crystalline product was found to be a mixture of four ninhydrin-positive compounds, designated A–D, by paper chromatography in Solvent System I, *n*-butanol–water–acetic acid (4:5:1). This finding is contrary to the report by Acheson *et al.* (6), who found their crystalline product to be a single-spot material by paper chromatography in Solvent Systems II, *n*-butanol–acetic acid–water (4:1:1), and III, isopropanol–ammonia–water (8:1:1). Four additional ninhydrin-positive components also were detected during paper chromatography of the mother liquor (the original reaction mixture after removal of the hydrochloride crystals) in Solvent System I. The chromatographic data on the eight products formed during the acid hydrolysis of IX are given in Table I.

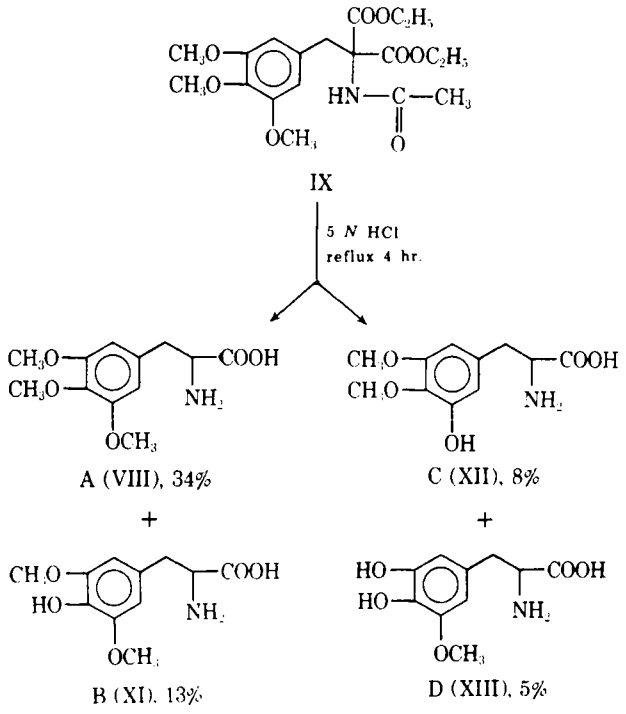
Isolation and Identification of Major Hydrolysis Products—Separation and isolation of the major hydrolysis products, A–D, were accomplished by preparative paper chromatography, and the following yields were obtained based on the isolated homogeneous products: A, 34%; B, 13%; C, 8%; and D, 5%. Identification of the isolated compounds (Scheme I) was carried out by NMR and mass spectral methods.

The major product, A, was obtained as fine needles, m.p. 243–244°, by recrystallization from aqueous hydrochloric acid. Based upon its NMR and mass spectra, Compound A was identified as the hydrochloride of the target amino acid, 3,4,5-trimethoxyphenylalanine (VIII). The mass spectrum of A showed the parent ion at *m/e* 255 and the base peak at *m/e* 181 corresponding to the trimethoxybenzyl ion (Scheme II).

The NMR spectrum (deuterium oxide solvent) of Compound A exhibited two singlets corresponding to the aromatic methoxyls at δ 3.87 (6H, methoxyls at C-3 and C-5) and 3.8 (3H, methoxyl at C-4), a singlet at δ 6.6 (2H) for the aromatic protons at C-2 and C-6, and multiplets at δ 3.2 (2H) and 3.7 (1H) p.p.m. assignable to the benzylic protons and the proton on the carbon bearing the carboxyl group, respectively.

The mass spectra of Compounds B and C indicated these two amino acids to be monodemethylated products of VIII. Both compounds showed parent ions at *m/e* 241 and the base peak at *m/e* 167 corresponding to the monohydroxydimethoxybenzyl ions (Scheme III).

Structural assignments to the monodemethylated amino acids, B (4-hydroxy-3,5-dimethoxyphenylalanine, XI) and C (3-hydroxy-4,5-dimethoxyphenylalanine, XII), were made on the basis of their NMR spectra in neutral and alkaline media. In deuterium oxide



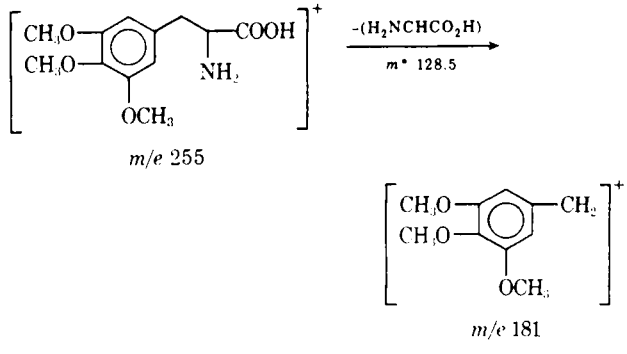
Scheme I

solvent, Compound B (XI) showed two singlets at δ 3.85 (6H, aromatic methoxyls at C-3 and C-5) and 6.65 (2H, aromatic protons at C-2 and C-6) p.p.m. Upon addition of sodium deuteroxide, the singlet corresponding to the aromatic protons at C-2 and C-6 in B (XI) shifted upfield by 0.11 Hz., which is consistent with their assignment to the *meta*-protons in relation to the phenolic hydroxyl at the C-4 position (7).

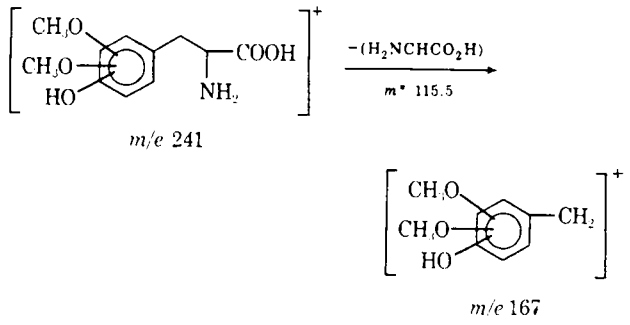
The NMR spectrum of C (XII) in deuterium oxide exhibited four singlets at δ 3.84 (3H, aromatic methoxyl at C-4), 3.86 (3H, aromatic methoxyl at C-3), 6.68 (1H, aromatic proton at C-2), and 6.71 (1H, aromatic proton at C-6) p.p.m. The two benzylic protons and the proton on the carbon bearing the carboxyl group in the spectra of acids B (XI) and C (XII) showed the expected multiplets, as in the case of trimethoxyphenylalanine (VIII).

Compound D was found to be a dihydroxymonomethoxy derivative of phenylalanine based on its mass spectrum, which showed the parent ion at *m/e* 227 and the base peak at *m/e* 153 arising from the loss of the fragment, H_2NCHCO_2H. Its NMR spectrum in deuterium oxide showed three singlets at δ 3.82 (3H, aromatic methoxyl at C-5), 6.69 (1H, aromatic proton at C-2), and 6.66 (1H, aromatic proton at C-6) p.p.m. and multiplets at δ 3.19 (2H, benzylic protons) and 3.71 (1H, proton on the carbon carrying carboxyl group) p.p.m. On the basis of these spectral characteristics, Compound D was identified as 3,4-dihydroxy-5-methoxyphenylalanine (XIII).

Chemical Method for Separation of VIII from Acid Hydrolysate—Since the separation of trimethoxyphenylalanine (VIII) from the phenolic amino acids, XI–XIII, formed during the acid hydrolysis



Scheme II



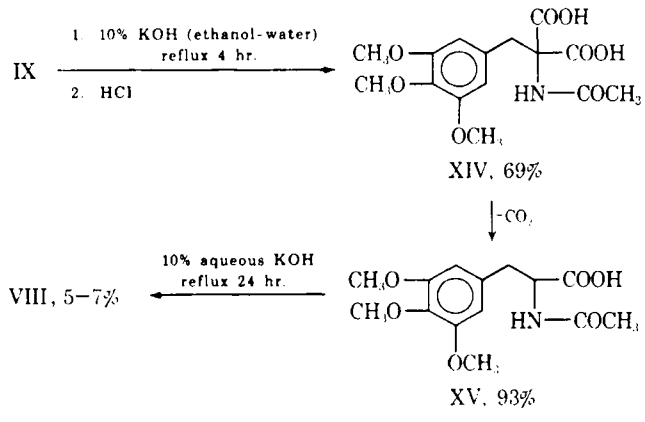
Scheme III

of the acetamidomalonate ester IX by the preparative paper chromatographic procedure was tedious, a chemical method for its isolation in larger quantities was developed.

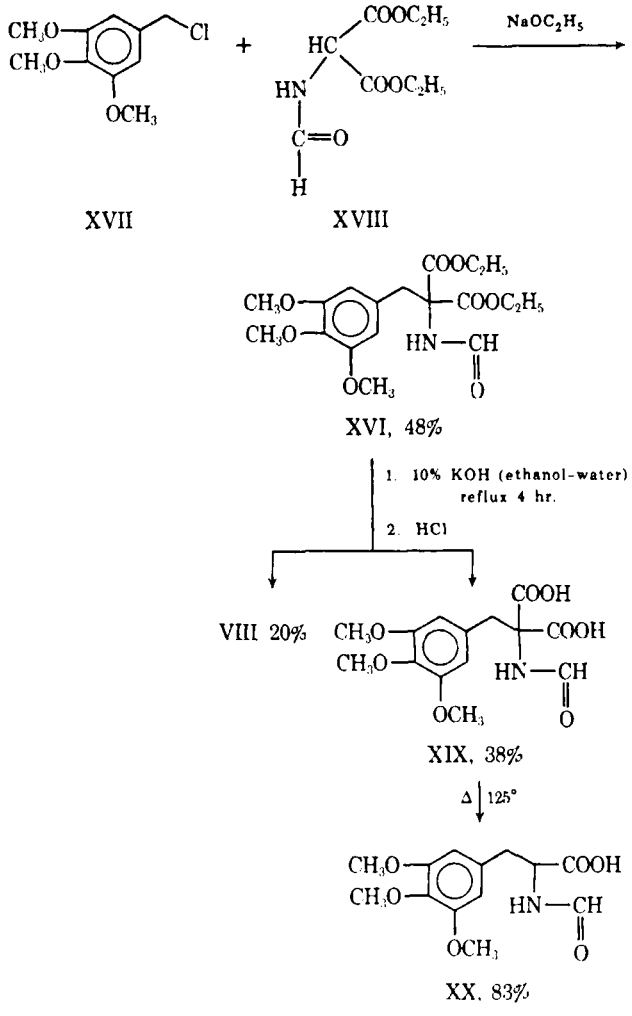
The hydrolysis mixture, consisting of VIII and accompanying phenolic amino acids, was refluxed with a methanol–sulfuric acid–ethylene dichloride mixture (4:0.4:6), and the resulting methyl esters were shaken with 10% NaOH and extracted with benzene. The benzene extract, upon removal of the solvent, gave exclusively the methyl ester of the nonphenolic amino acid, trimethoxyphenylalanine (30% yield), which was then hydrolyzed with 10% barium hydroxide to yield homogeneous trimethoxyphenylalanine (VIII) in 75% yield. The methyl esters of the phenolic amino acids, XI–XIII, were obtained from the aqueous sodium hydroxide layer by acidifying with carbon dioxide and extracting with chloroform.

Basic Hydrolysis of IX—To minimize the formation of phenolic amino acids during the hydrolysis of the acetamidomalonate ester (IX), the hydrolysis was carried out under basic conditions. Compound IX was refluxed for 4 hr. with 10% KOH in ethanol–water (1:1), which gave α-acetamido-α-3,4,5-trimethoxybenzylmalonic acid (XIV) in 69% yield. The dicarboxylic acid XIV readily decarboxylated when kept under vacuum, and the resulting *N*-acetyl-3,4,5-trimethoxyphenylalanine (XV, 93% yield) was again refluxed with 10% KOH. Hydrolysis of the *N*-acetyl derivative (XV) did not proceed smoothly, and only poor yields (5–7%) of trimethoxyphenylalanine (VIII) could be obtained. However, the hydrolysis product was found to consist solely of the required amino acid VIII and no phenolic amino acids were detected. Thus, hydrolysis of IX under basic conditions leads to a single product, VIII, but in low yields (Scheme IV).

Synthesis by Diethyl Formamidomalonate Method—Since hydrolysis of the acetamidomalonate ester IX under both acidic and basic conditions did not proceed satisfactorily, diethyl α-formamido-α-3,4,5-trimethoxybenzylmalonate (XVI) (Scheme V) was prepared and its hydrolysis was studied in an attempt to obtain better yields of the required trimethoxyphenylalanine (VIII). 3,4,5-Trimethoxybenzyl chloride (XVII) and diethyl formamidomalonate (XVIII) were condensed in the presence of sodium ethoxide to form the formamidomalonate ester, XVI (48% yield). Hydrolysis of the malonic ester XVI was carried out by refluxing for 4 hr. with 10% alcoholic KOH, which yielded the required VIII in 20% yield along with α-formamido-α-3,4,5-trimethoxybenzylmalonic acid (XIX, 38% yield). Compound VIII could be easily separated from



Scheme IV



Scheme V

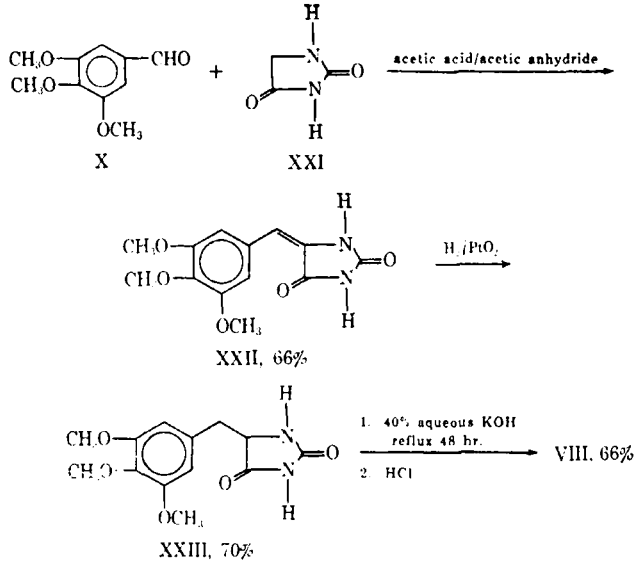
the reaction mixture by converting to its hydrochloride by acidification.

Decarboxylation of the malonic acid XIX by heating at 125° for 1 hr. gave *N*-formyltrimethoxyphenylalanine (XX) in 83% yield. However, as in the case of the *N*-acetyl derivative XV, basic hydrolysis of XX also gave low yields (5–7%) of 3,4,5-trimethoxyphenylalanine (VIII). During the basic hydrolysis of both acetyl and formyl derivatives, XV and XX, no phenolic products were encountered. This is in contrast to the hydrolysis under acidic conditions, which results in the formation of substantial quantities of phenolic amino acids.

Synthesis by Hydantoin Method—Although the condensation of 3,4,5-trimethoxybenzyl chloride (XVII) with both formamidomalonate and acetamidomalonate esters gave high yields of the corresponding acyl trimethoxybenzylmalonate esters, IX and XVI, their subsequent hydrolysis to the requisite 3,4,5-trimethoxyphenylalanine (VIII) proceeded in low yields. Both the formyl and acetyl derivatives of trimethoxyphenylalanine, XX and XV, formed during the synthetic procedures were quite resistant to hydrolytic cleavage.

To circumvent the formation of acyl derivatives of trimethoxyphenylalanine during the synthetic procedure, an alternative method involving a hydantoin derivative was tried (Scheme VI). 3,4,5-Trimethoxybenzaldehyde (X) was condensed with hydantoin (XXI) by refluxing for 24 hr. in glacial acetic acid–acetic anhydride, and the resulting 5-(3,4,5-trimethoxybenzylidine)hydantoin (XXII, 66% yield) was hydrogenated over platinum oxide to yield 5-(3,4,5-trimethoxybenzyl)hydantoin (XXIII, 70% yield). Basic hydrolysis of the hydantoin derivative XXIII gave high yields (66%) of the target amino acid, 3,4,5-trimethoxyphenylalanine (VIII).

GC–Mass Spectrometry of Peyote Amino Acid Fraction—During these synthetic studies, several trioxygenated phenylalanine derivatives (XI–XIII) were obtained in addition to 3,4,5-trimethoxyphen-



Scheme VI

ylalanine (VIII), the analog corresponding to mescaline. By using these as the reference compounds, the amino acid fraction of the peyote cactus was examined for their possible natural occurrence by the GC–mass spectrometric procedure described earlier (1–3). No evidence was obtained for the presence of trioxygenated analogs of phenylalanine in the peyote. This observation may be significant, since it may be taken as an indication that the carboxyl group does not survive beyond the stage of dihydroxyphenylalanine in the biosynthetic sequence leading to the trioxygenated alkaloids of peyote.

EXPERIMENTAL[1]

Compounds were visualized on paper chromatograms with the aid of ninhydrin and diazo spray reagents. The following compounds were purchased from a commercial source[2]: 3,4,5-trimethoxybenzaldehyde, diethyl formamidomalonate, diethyl acetamidomalonate, and hydantoin.

Diethyl α-Acetamido-α-3,4,5-trimethoxybenzylmalonate (IX)—Compound IX was synthesized according to the procedure of Acheson *et al.* (6). Diethyl acetamidomalonate (11 g.) was dissolved in a solution of sodium ethoxide (sodium, 1.18 g. in absolute alcohol, 75 ml.) by warming. To this stirred solution was added 3,4,5-trimethoxybenzyl chloride (10 g.) in five portions. The reaction mixture was refluxed for 6 hr., cooled, and filtered. The filtrate was concentrated to approximately half the volume, and water (50 ml.) was added. Extraction with ether (2 × 50 ml.) and evaporation of the dried (sodium sulfate) ether extract gave the ester IX (6.6 g.), which crystallized from benzene–petroleum ether as flakes, m.p. 116–117° [lit. (6) m.p. 116°].

Acid Hydrolysis of Acetamidomalonate Ester IX—The acetamidomalonate ester IX (907 mg.) was refluxed with 5 *N* HCl (30 ml.) for 4 hr. Upon cooling the reaction mixture, fine needles separated which were collected by filtration (392 mg.) and recrystallized from 5 *N* HCl to give needles, m.p. 241–242° dec. Acheson *et al.* (6) reported a melting point of about 242° dec. for their crystalline product, which they claimed to be 3,4,5-trimethoxyphenylalanine (VIII) hydrochloride.

Paper chromatographic data on this crystalline product and the mother liquor are given in Table I. The crystalline product consisted of four ninhydrin-positive components, A–D, while four additional compounds, E–H, were detected in the mother liquor.

[1] Melting points were taken on a Kofler micro-hot-stage and are corrected. NMR chemical shifts are given in parts per million downfield from tetramethylsilane as an internal standard. Mass spectra were recorded at 70-ev, electron beam voltage. Spectra were recorded on Varian XL-100 NMR and LKB-9000 GC–mass spectrometers. Paper chromatography was performed on Whatman No. 1 paper.

[2] Aldrich Chemical Co.

Table I—Paper Chromatography of Acid Hydrolysis Products of Diethyl α-Acetamido-α-3,4,5-trimethoxybenzylmalonate (IX)

Sample Chromatographed	Compounds Detected[a]	R_f Value in Solvent System *n*-Butanol–Water–Acetic Acid (4:5:1)	Yield[b], %
Hydrochloride crystals	A	0.56	34
	B	0.34	13
	C	0.43	8
	D	0.40	5
Mother liquor	E	0.81	Trace
	F	0.67	Trace
	G	0.22	Trace
	H	0.12	Trace

[a] See Scheme I for identification of Compounds A–D. [b] Yields reported are based on homogeneous products isolated by preparative paper chromatography (see *Experimental* for details).

A portion of this crystalline product (300 mg.) was subjected to preparative paper chromatography in Solvent System I and the following homogeneous products were isolated: A (110 mg.), B (42 mg.), C (26 mg.), and D (16 mg.). Identification of the isolated compounds, A–D, was carried out with the aid of their NMR and mass spectra (see *Results and Discussion* and Scheme I).

Chemical Method for Isolation of 3,4,5-Trimethoxyphenylalanine (VIII) from Acid Hydrolysate—The crystalline product (398 mg.) obtained during the acid hydrolysis of the acetamidomalonate ester IX was refluxed with ethylene dichloride (0.6 ml.) and concentrated sulfuric acid (75 mg.) in methanol (0.4 ml.) for 16 hr. The reaction mixture was cooled, the solvent was removed *in vacuo*, and water (2 ml.) was added. The aqueous solution was basified with 10% NaOH and extracted with benzene (3 × 5 ml.). Evaporation of the dried (sodium sulfate) benzene extract yielded the methyl ester of 3,4,5-trimethoxyphenylalanine (120 mg.). This methyl ester was hydrolyzed by refluxing with 10% $Ba(OH)_2$ for 24 hr., which yielded 3,4,5-trimethoxyphenylalanine (VIII) in 75% yield. From the aqueous sodium hydroxide solution, esters of the phenolic amino acids XI–XIII were obtained by acidification with carbon dioxide and extraction with chloroform.

Basic Hydrolysis of Acetamidomalonate Ester IX—Compound IX (1.02 g.) was dissolved in 10 ml. of ethanol–water (1:1) and refluxed with 10% KOH (20 ml.) for 4 hr. Ethanol was then removed *in vacuo* from the reaction mixture and acidified with concentrated hydrochloric acid. The crystalline precipitate formed was filtered, washed with cold water, and dried (0.69 g.) to give acetamido-3,4,5-trimethoxybenzylmalonic acid (XIV), m.p. 135–137°. Upon keeping the malonic acid XIV under vacuum for 2 days, decarboxylation occurred and gave 0.64 g. of *N*-acetyl-3,4,5-trimethoxyphenylalanine (XV), m.p. 190–191.5°.

Anal.—Calc. for $C_{14}H_{19}NO_6$: C, 56.56; H, 6.44; N, 4.71. Found: C, 56.38; H, 6.29; N, 4.67.

The *N*-acetyl derivative XV (0.102 g.) was refluxed with 10% KOH (25 ml.) for 20 hr. The reaction mixture was cooled and passed through a column of cation-exchange resin[3] (20 ml.). An additional 300 ml. of water was passed through the column, and the combined eluate was evaporated to dryness. The resulting residue was taken up in dilute hydrochloric acid (5 ml.) which, upon cooling, gave 7.3 mg. of 3,4,5-trimethoxyphenylalanine (VIII) as its hydrochloride, m.p. 243–244°.

Diethyl α-Formamido-α-3,4,5-trimethoxybenzylmalonate (XVI)—Diethyl formamidomalonate (XVIII) (2.02 g.) and 3,4,5-trimethoxybenzyl chloride (XVII) (2.15 g.) were added to a solution of sodium ethoxide (250 mg. of sodium dissolved in 12 ml. of absolute alcohol) and refluxed for 14 hr. The reaction mixture was filtered, and the filtrate was evaporated to dryness. The residue was suspended in water (50 ml.) and extracted with ether (3 × 50 ml.). Upon evaporation, the dried (sodium sulfate) ether extract gave 1.82 g. of crude product, m.p. 117–118°. Crystallization of this product from benzene–petroleum ether gave 1.68 g. of diethyl α-formamido-α-3,4,5-trimethoxybenzylmalonate (XVI), m.p. 120–121°.

[3] Amberlite, IRC 50 (H), Rohm & Haas Co.

Anal.—Calc. for $C_{18}H_{25}NO_8$: C, 56.39; H, 6.57; N, 3.65. Found: C, 56.45; H, 6.64; N, 3.55.

Basic Hydrolysis of Formamidomalonate Ester XVI—Compound XVI (0.6 g.) was refluxed with a mixture of 10% KOH (6 ml.) and ethanol–water (1:1, 14 ml.) for 4 hr. The reaction mixture was concentrated under argon atmosphere to 3 ml., cooled, and acidified with 18% HCl (4 ml.). Upon cooling the acidic solution, 192 mg. of formamido-3,4,5-trimethoxybenzylmalonic acid (XIX) separated out, m.p. 126–127°.

The mother liquor was freeze dried and the residue was shaken with hot methanol (3 × 15 ml.). The methanol extract was evaporated to dryness, and the residue was dissolved in water (5 ml.) and extracted with chloroform (5 × 10 ml.). Upon removal of the solvent, the chloroform extract gave 55 mg. of the malonic acid XIX. The aqueous layer was evaporated to dryness and the residue (350 mg.) was extracted with methanol (3 × 5 ml.). The methanol extract was left in the refrigerator overnight and deposited needles of 3,4,5-trimethoxyphenylalanine (VIII) as the hydrochloride (125 mg.), m.p. 243–244°.

Decarboxylation of the malonic acid XIX (55 mg.) was carried out by heating at 125° for 1 hr., which gave 40 mg. of *N*-formyl-3,4,5-trimethoxyphenylalanine (XX), m.p. 154–155°.

Anal.—Calc. for $C_{13}H_{17}NO_6$: C, 55.12; H, 6.05; N, 4.95. Found: C, 55.47; H, 6.22; N, 4.49.

Basic hydrolysis of the *N*-formyl derivative XX (30 mg.) was carried out as in the case of the *N*-acetyl derivative XV, which gave 2.2 mg. of 3,4,5-trimethoxyphenylalanine (VIII) as the hydrochloride, m.p. 243–244°.

5-(3,4,5-Trimethoxybenzylidine)hydantoin (XXII)—Hydantoin (5 g.) and 3,4,5-trimethoxybenzaldehyde (9.8 g.) were dissolved in a mixture of glacial acetic acid (15 ml.) and acetic anhydride (2 ml.) and refluxed for 24 hr. The reaction mixture was evaporated to dryness *in vacuo*, and the residue was taken up in cold water (500 ml.). Upon vigorous shaking, a yellow crystalline precipitate separated which was collected on a funnel by filtration and washed with cold water. The filtrate was extracted with chloroform (10 × 100 ml.) and the chloroform extract was evaporated to dryness, yielding additional quantities of the yellow crystalline product. The two yellow crystalline products were combined and recrystallized from ethanol–chloroform to yield 8.96 g. of 5-(3,4,5-trimethoxybenzylidine)hydantoin (XXII) as needles, m.p. 268–269°.

Anal.—Calc. for $C_{13}H_{14}N_2O_5$: C, 56.11; H, 5.07; N, 10.07. Found: C, 56.04; H, 5.06; N, 10.15.

5-(3,4,5-Trimethoxybenzyl)hydantoin (XXIII)—A suspension of XXII (2.61 g.) in methanol (40 ml.) was acidified with a few drops of dilute hydrochloric acid and hydrogenated in the presence of platinum oxide (200 mg.) at 30 p.s.i. pressure for 18 hr. The resulting colorless solution was filtered and evaporated to dryness. Crystallization of the resulting residue from methanol–benzene afforded 1.81 g. of white microcrystals of XXIII, m.p. 180–181°.

Anal.—Calc. for $C_{13}H_{16}N_2O_5$: C, 55.71; H, 5.75; N, 10.00. Found: C, 56.01; H, 5.87; N, 10.18.

3,4,5-Trimethoxyphenylalanine (VIII)—A solution of trimethoxybenzylhydantoin (XXIII, 280 mg.) in ethylene glycol monoethyl ether (5.5 ml.) was refluxed with potassium hydroxide (0.8 g. in 2 ml. of water) for 48 hr. The reaction mixture was evaporated to dryness, and the residue was suspended in water (5 ml.) and acidified with 10% HCl. The aqueous acidic solution was then evaporated to dryness, and the resulting residue was extracted with hot absolute alcohol (4 × 20 ml.). During the concentration of the alcohol extract, needle-shaped crystals appeared which were collected and recrystallized from absolute alcohol to give 193 mg. of 3,4,5-trimethoxyphenylalanine (VIII) as the hydrochloride as needles, m.p. 243–244°.

Anal.—Calc. for $C_{12}H_{17}NO_5 \cdot HCl$: C, 49.41; H, 6.17; N, 4.80. Found: C, 49.18; H, 6.33; N, 4.84.

SUMMARY AND CONCLUSIONS

1. Acid hydrolysis of the acetamidomalonate ester (IX) in the procedure of Acheson *et al.* (6) for the synthesis of 3,4,5-trimethoxyphenylalanine was shown to lead to a mixture of products. With the aid of preparative paper chromatography, the major hydrolysis products were isolated and identified by NMR and mass spectrometry as 3,4,5-trimethoxyphenylalanine (VIII, 34%), 4-hydroxy-3,5-dimethoxyphenylalanine (XI, 13%), 3-hydroxy-4,5-dimethoxyphenylalanine (XII, 8%), and 3,4-dihydroxy-5-methoxyphenyl-

alanine (XIII, 5%). A chemical method was developed for the separation of the amino acid VIII from the phenolic products present in the hydrolysis mixture.

2. Under basic conditions, hydrolysis of the acetamidomalonate ester IX gave low yields (5–7%) of the required amino acid VIII. However, no phenolic side-products were formed under this condition.

3. Synthesis of the formamidomalonate ester XVI and its hydrolysis under basic conditions were carried out in an effort to realize higher yields of trimethoxyphenylalanine (VIII). The hydrolysis of the ester XVI also gave unsatisfactory yields of the target amino acid VIII.

4. An efficient alternative synthesis of trimethoxyphenylalanine (VIII) was achieved which involves the formation of the hydantoin derivative XXIII and its hydrolysis under basic conditions (66% yield).

5. GC–mass spectrometric examination of the peyote amino acid fraction gave no evidence for the presence of trioxygenated phenylalanine analogs, VIII and XI–XIII. This observation suggests that the carboxyl group may not survive beyond the stage of dopa during the biosynthesis of the trioxygenated peyote alkaloids.

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▲ To whom inquiries should be directed.

Enhanced Absorption of Digitoxin from Orally Administered Digitoxin–Polyvinylpyrrolidone Coprecipitates

ELLIOT I. STUPAK* and THEODORE R. BATES▲

Abstract □ The general applicability of the polyvinylpyrrolidone coprecipitation technique as a method for enhancing the GI absorption of orally administered hydrophobic drugs was explored with the cardiac glycoside digitoxin. The relative absorption characteristics of digitoxin alone and as a 1:9 (w/w) physical mixture and coprecipitate with polyvinylpyrrolidone were determined indirectly by measuring their oral LD_{50} values in rats. The *in vivo* data obtained provided evidence that digitoxin was absorbed from the coprecipitate at a significantly faster rate and was present in the body at a much higher level than when equivalent doses of either the drug alone or as a physical mixture with polyvinylpyrrolidone were orally administered. For example, one must orally administer approximately 11 times as much pure drug to reach the same amount of drug in the body as that attained following the administration of the drug as a coprecipitate. A correlation was found between the *in vitro* dissolution rates of these test systems at 37° and their *in vivo* toxicities.

Keyphrases □ Digitoxin absorption—enhanced using orally administered coprecipitate with polyvinylpyrrolidone, compared to separate drug and physical mixture □ Polyvinylpyrrolidone coprecipitate with digitoxin—enhanced *in vivo* absorption, compared to separate drug and physical mixture □ Coprecipitates, digitoxin–polyvinylpyrrolidone—enhanced *in vivo* drug absorption □ Drug absorption, digitoxin—enhanced using orally administered polyvinylpyrrolidone coprecipitate

Among the techniques that can potentially enhance the dissolution rate and, hence, the rate and/or extent of absorption of hydrophobic drugs is the formation of coprecipitates with pharmacologically inert, polymeric materials such as polyvinylpyrrolidone. This physicochemical drug modification offers the advantage of possibly enabling one to administer the drug orally in a form from which it is most available for GI absorption. Although several investigations (1–6) have demonstrated, *in vitro*, that the solubility and/or dissolution rates of drugs can be increased in this manner, little information is available in the literature related to the *in vivo* absorption pattern of drugs orally administered as polyvinylpyrrolidone coprecipitates. Recently, however, it was demonstrated (6) that both the rate and extent of absorption of the water-insoluble drug reserpine could be markedly enhanced when orally administered to rats in the form of a 1:5 (w/w) coprecipitate with polyvinylpyrrolidone.

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Table I—Relationship between the Oral Dose and Lethality of Digitoxin Alone and as a 1:9 (w/w) Physical Mixture with Polyvinylpyrrolidone

Test System	Dose of Drug[a], mg./kg.	Number of Animals Dosed	Number of Animals Dead[b]	Mortality, %
Digitoxin	60	15	5	33.3
	85	15	7	46.7
	110	15	10	66.7
	185	15	12	80.0
Digitoxin–polyvinylpyrrolidone physical mixture, 1:9 (w/w)	50	15	2	13.3
	75	15	4	26.7

[a] Dose administered as a suspension in 0.50% (w/v) methylcellulose at a constant dosing volume of 5.0 ml./kg. [b] Animals observed for 72 hr. postadministration.

ture, and a 1:9 (w/w) digitoxin–polyvinylpyrrolidone coprecipitate were quantitatively studied by comparing their relative toxicities in the rat. The solubility and dissolution behavior of these systems were also examined.

EXPERIMENTAL

Materials—The digitoxin[1] and polyvinylpyrrolidone[2] employed in this study were pharmaceutical grade. The polyvinylpyrrolidone had an average molecular weight of 40,000. All other chemicals used were reagent grade and were used as received.

Preparation of Test Systems—The 1:9 (w/w) digitoxin–polyvinylpyrrolidone coprecipitate and physical mixture were prepared in the manner previously described (6).

Unless otherwise specified, the gross particle size of the test systems was 297–420 μ for the 1:9 (w/w) digitoxin–polyvinylpyrrolidone coprecipitate and 10–40 μ for the pure digitoxin and the drug contained in the 1:9 (w/w) physical mixture.

Protocol for *In Vivo* Absorption Studies—The GI absorption characteristics of digitoxin were assessed by comparing the relative oral toxicity of the drug from suspensions containing the pure drug and the 1:9 (w/w) drug–polyvinylpyrrolidone physical mixture and coprecipitate. Adult, male, Sprague–Dawley rats[3], weighing 150–250 g., were used as the test animals. The animals were fasted for 20–24 hr. prior to the oral administration of each test system as a suspension in a 0.50% (w/v) methylcellulose[4] aqueous vehicle. The dosing volume was held constant at 5.0 ml./kg. Fifteen rats were dosed with 60, 85, 110, or 185 mg./kg. of pure drug or an amount of the 1:9 (w/w) digitoxin–polyvinylpyrrolidone coprecipitate containing 6, 7.5, 9, or 10 mg./kg. of digitoxin. The 1:9 drug–polyvinylpyrrolidone physical mixture was orally administered at drug dosage levels of 50 and 75 mg./kg.

The animals were housed in cages with wide, wire-mesh floors to prevent coprophagy and were observed for 72 hr. postdrug administration. At the end of this period, the total number of deaths was recorded. Throughout the entire experimental period, the animals were allowed free access to water; 8 hr. postdrug administration, the animals were placed back on food. The toxicity data were used to calculate the oral LD_{50} value for the test systems according to the method of Litchfield and Wilcoxon (7).

Protocol for *In Vitro* Dissolution Rate Studies—The stirrer-flask method (6) was used to assess the dissolution characteristics of the test preparations at 37°. The dissolution medium consisted of 350 ml. of a Clark–Lubs pH 7.4 phosphate buffer containing 0.005% (w/v) polysorbate 80 USP, and it was agitated at 150 r.p.m. At frequent intervals, subsequent to the introduction of a quantity of test preparation equivalent to 20 mg. of digitoxin, 3-ml. samples were removed from the flask with a filter pipet and immediately replaced with 3 ml. of fresh dissolution medium. The samples were assayed colorimetrically at 532 nm. for digitoxin content (8). The presence of polymer or polysorbate 80 in the samples did not interfere with the quantitative determination of digitoxin. All particulate dissolution rate experiments were performed in duplicate.

Table II—Relationship between the Oral Dose and Lethality of Digitoxin from a 1:9 (w/w) Coprecipitate with Polyvinylpyrrolidone

Dose of Drug[a], mg./kg.	Number of Animals Dosed	Number of Animals Dead[b]	Mortality, %
6.0	15	2	13.3
7.5	15	7	46.7
9.0	15	11	73.3
10.0	15	13	86.7

[a] Dose administered as a suspension in 0.50% (w/v) methylcellulose at a constant dosing volume of 5 ml./kg. [b] Animals observed for 72 hr. postadministration.

Equilibrium Solubility Determinations—The equilibrium solubility of digitoxin was determined at 37° in water, pH 7.4 phosphate buffer, and pH 7.4 phosphate buffer containing 1.0% (w/v) polyvinylpyrrolidone. Each system also contained 0.005% (w/v) polysorbate 80. Excess quantities of digitoxin were placed into 50-ml. glass-stoppered flasks together with 25.0-ml. portions of the solvent systems listed. All flasks were closed securely and mechanically shaken at 37° until equilibrium was attained. Equilibrium was established by repetitive sampling and was found to occur within 12–24 hr. The equilibrated samples were subjected to filtration[5] (0.45-μ pore size) at 37°, the filtrates were suitably diluted when necessary, and the concentration of drug in solution was determined colorimetrically by the procedure of Mesnard and Devaux (8).

RESULTS

Oral Toxicity Studies—The results of the oral toxicity experiments (Tables I and II) were used to construct the log–probability plots shown in Figs. 1 and 2. Based on these linear plots, the oral LD_{50} values for the drug alone and the 1:9 drug–polyvinylpyrrolidone coprecipitate and their corresponding 95% confidence intervals were calculated and found to be 88 (67.7–114) and 7.80 (7.03–8.66) mg./kg., respectively. An examination of these oral LD_{50} values indicates that one must orally administer approximately 11 times as much pure drug to reach the same amount of drug in the body as that attained following the administration of the drug as a coprecipitate. In addition, most of the rats given toxic doses of the digitoxin–polyvinylpyrrolidone coprecipitate usually developed characteristic neuromuscular signs and died at a much earlier time

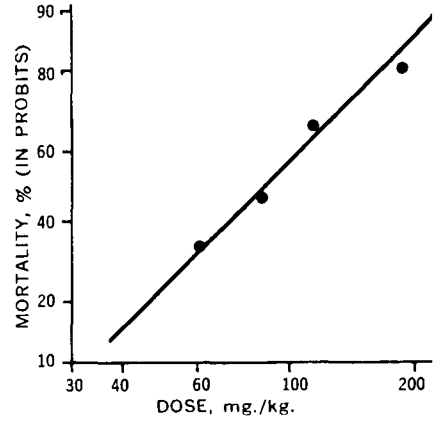

Figure 1—*Relationship between the oral dose and lethality of digitoxin.*

[1] Supplied by Wyeth Laboratories, Philadelphia, Pa.
[2] Plasdone-C (K30), supplied by General Aniline and Film Corp., New York, NY 10020
[3] Obtained from Blue Spruce Farms, Altamont, NY 12009
[4] Methocel 60-HG, Dow Chemical Co., Midland, Mich.
[5] Millipore.

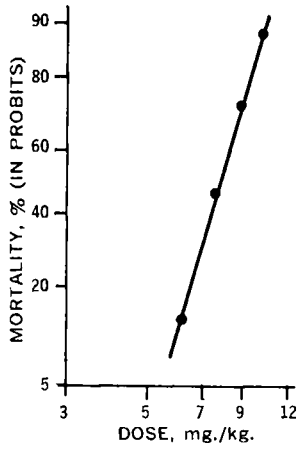

Figure 2—*Relationship between the oral dose and lethality of digitoxin from a 1:9 (w/w) digitoxin–polyvinylpyrrolidone coprecipitate.*

than those animals dosed with the pure drug. For example, 75% of the deaths that resulted from the oral administration of a 10-mg./kg. dose of the drug as a polyvinylpyrrolidone coprecipitate occurred within 5–10 hr., with the remaining animals dying between 24 and 48 hr. On the other hand, 50% of the rats dosed with 185 mg./kg. of the pure drug died between 12 and 24 hr. and 50% between 24 and 48 hr.

Although visual inspection of the slopes of the linear plots shown in Figs. 1 and 2 suggests a lack of parallelism, the toxicity data, when statistically tested for parallelism according to the method of Litchfield and Wilcoxon (7), revealed that the two lines do not show any significant departure from parallelism ($p < 0.05$).

The 1:9 (w/w) digitoxin–polyvinylpyrrolidone physical mixture was orally administered at two drug dosage levels to two groups of rats to establish whether a simple, mechanical mixture of the two components would affect the oral toxicity of digitoxin. A comparison of the results of these toxicity tests with those obtained with the drug alone (Table I) reveals that the toxicity of digitoxin is essentially unaltered when administered as a suspension with polyvinylpyrrolidone.

Dissolution Rate and Equilibrium Solubility Studies—*In vitro* dissolution rate studies were conducted at 37° on particulate samples of the three digitoxin test preparations to establish whether this physicochemical parameter could be used to reflect the differences observed previously among their *in vivo* absorption characteristics. The rate of solution of a sample of pure *precipitated* digitoxin (particle-size range of 1–50 μ), prepared in the same manner as the coprecipitate system, was also evaluated. The results of these *in vitro* dissolution rate experiments, performed at pH 7.4 and under nonsink conditions, are shown in Figs. 3 and 4.

An examination of the dissolution rate profiles for the pure drug and the 1:9 (w/w) digitoxin–polyvinylpyrrolidone coprecipitate

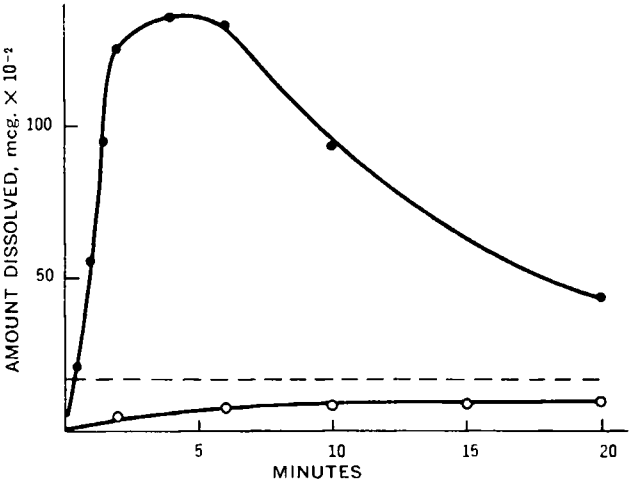

Figure 3—*Dissolution rates of digitoxin test preparation at 37°. Key: ○, pure digitoxin; ●, 1:9 (w/w) digitoxin–polyvinylpyrrolidone coprecipitate; and - - -, equilibrium solubility of digitoxin in dissolution medium.*

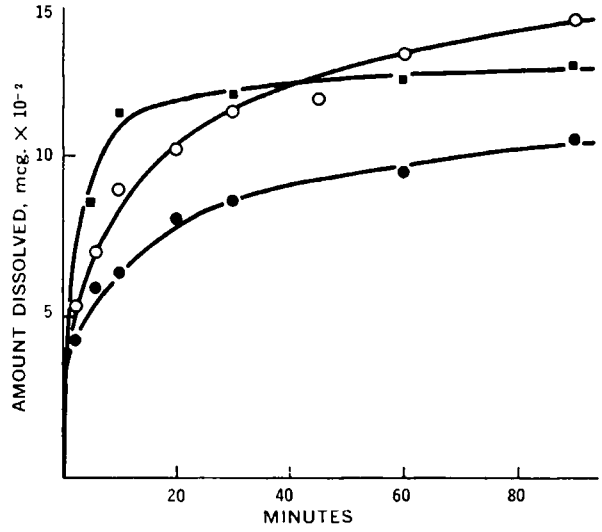

Figure 4—*Dissolution rates of digitoxin test preparations at 37°. Key: ○, Pure digitoxin; ●, digitoxin precipitated from chloroform; and ■, 1:9 (w/w) digitoxin polyvinylpyrrolidone physical mixture.*

(Fig. 3) reveals that the amount of drug in solution from the coprecipitate system rapidly and markedly exceeds the equilibrium solubility of the drug. After reaching a peak value in a relatively short period (*i.e.*, less than 5 min.), the amount of drug in solution slowly declines toward the equilibrium solubility value. However, even after 2 hr., the amount of digitoxin in solution from the coprecipitate system is nearly twice the equilibrium solubility value. On the other hand, the pure drug dissolves at a significantly slower rate and gradually approaches the equilibrium solubility value. The equilibrium solubility of digitoxin was unaffected by the amount of polyvinylpyrrolidone in solution from the coprecipitate system.

The peak, nonequilibrium (dynamic), solubility value for digitoxin from the 1:9 (w/w) coprecipitate, as determined from the dissolution rate experiments (Fig. 3), was 38.7 mcg./ml. or 13.5 mg./350 ml. of dissolution medium. This value exceeds the equilibrium solubility of the drug by a factor of 7–8 times. The magnitude of this factor compares favorably with the ratio of 11.3 obtained by comparing the oral LD_{50} value calculated from the pure drug to that for the 1:9 (w/w) digitoxin–polyvinylpyrrolidone coprecipitate.

The dissolution profiles for precipitated digitoxin (1–50 μ), pure digitoxin (10–40 μ), and the 1:9 (w/w) digitoxin–polyvinylpyrrolidone physical mixture are depicted in Fig. 4. The rates of solution of digitoxin from these three systems are quite similar in magnitude and markedly less than that previously noted for digitoxin from the 1:9 (w/w) coprecipitate (Fig. 3).

The equilibrium solubility of digitoxin at 37°, determined in various aqueous systems, was 4.8 mcg./ml. in water and pH 7.4 buffer and 4.9 mcg./ml. in pH 7.4 buffer containing 1% (w/v) polyvinylpyrrolidone. The concentration of polyvinylpyrrolidone used in this study was considerably higher than that which could be realized from the complete dissolution of the amount of polymer present in the samples of either the physical mixture or coprecipitate subjected to dissolution rate experimentation (1 *versus* 0.0514%).

DISCUSSION

Drug absorption may be assessed indirectly by quantitatively determining its oral toxicity (9, 10). This method is based on the fact that the toxicity of an orally administered drug is directly proportional to the logarithm of the amount of drug in the body or its blood level (11) which, in turn, is directly proportional to its rate of absorption from the GI tract. The two parameters most commonly employed to reflect the toxicity of a drug are its oral LD_{50} and LT_{50} (median lethal time of death) values.

In the present investigation, digitoxin alone or as a 1:9 (w/w) physical mixture or coprecipitate with polyvinylpyrrolidone was orally administered at various drug dosage levels to fasted rats and the percent mortality was determined as a means of comparing the absorption characteristics of the drug from the various test systems. The markedly lower oral LD_{50} value observed for the coprecipitated

drug as compared to the pure drug does not necessarily imply that digitoxin, when administered alone, is less available to the body than when administered in the form of polyvinylpyrrolidone coprecipitate. However, it strongly suggests that the peak level of digitoxin in the body after oral administration of the coprecipitate is far higher than that achieved following an equal dose of pure drug. This conclusion can be readily appreciated if one considers the case of a hydrophobic drug whose passive absorption is rate limited by the rate at which solution is effected within the GI fluids. In this type of absorption pattern, any change in the rate of solution of the drug in the GI fluids would produce a corresponding change in its absorption rate. Since dissolution must precede absorption, a faster dissolving form of the drug should be absorbed at a more rapid rate and possibly accumulate in the body to a greater extent than an *equal* dose of a slowly dissolving form of the same pharmacological agent (12).

The inherent toxicity of polyvinylpyrrolidone can be readily eliminated as a factor contributing to the marked decrease in the oral LD_{50} value for digitoxin from the coprecipitate. In this connection, it has been demonstrated that pure polyvinylpyrrolidone elicits no untoward effects when orally administered to rats in doses of up to 100 g./kg. The intravenous LD_{50} value for polyvinylpyrrolidone is also very high, being 12–15 g./kg. (13). This information, coupled with the results obtained following the oral administration of the physical mixture, indicates that the increased toxicity of digitoxin from the 1:9 (w/w) coprecipitate is not due to the presence of polyvinylpyrrolidone *per se* but to an increase in the amount of drug in the body produced by a marked enhancement in the rate at which drug absorption occurs from this test system.

Several *in vitro* dissolution rate and solubility experiments were performed to establish which physicochemical mechanism could best account for the marked enhancement observed in the dissolution rate of digitoxin from the 1:9 (w/w) drug–polyvinylpyrrolidone coprecipitate as compared to the pure drug. The mechanisms considered were: (*a*) the formation of a digitoxin–chloroform solvate during the preparation of the coprecipitate system, (*b*) an increase in the solubility of the drug due to the formation of a digitoxin–polyvinylpyrrolidone complex, and (*c*) the formation of a polymorphic or amorphous form of the drug as a result of coprecipitation with polyvinylpyrrolidone.

The formation of drug solvates has been shown to affect the dissolution rate of hydrophobic drugs significantly (14). In this connection, digitoxin has been shown to form solvates with ethanol and water, which are stable *in vacuo* at temperatures up to 118° (15). Since chloroform was used to prepare the digitoxin–polyvinylpyrrolidone coprecipitate system, the possibility existed that the enhanced absorption and dissolution characteristics of digitoxin from this system resulted from the formation of a 1:1 chloroform solvate. To explore this possibility, the dissolution rate of digitoxin precipitated from chloroform without polyvinylpyrrolidone was investigated.

The differences observed in the rates of solution of pure digitoxin and precipitated digitoxin are small (Fig. 4) and can most probably be attributed to differences in the particle-size distribution of the two test systems and/or some inhibition in the rate of solution brought about by the presence of a minute amount of chloroform in the precipitated drug crystals. The results of this experiment suggest that the increases noted in the dissolution of digitoxin from the 1:9 (w/w) polyvinylpyrrolidone coprecipitate cannot be due to the formation of a drug–chloroform solvate during the preparation of the coprecipitate system.

The dissolution characteristics of the 1:9 (w/w) digitoxin–polyvinylpyrrolidone physical mixture almost coincide with those obtained with the pure drug (Fig. 4). The slight increase noted in the initial rate of solution of digitoxin from the physical mixture, as compared to the pure drug, is most likely due to the ability of the hydrophilic polymer to enhance the wettability of the hydrophobic digitoxin particles. These results indicate that the mere presence of polyvinylpyrrolidone, at a concentration equal to that present in the 1:9 (w/w) coprecipitate, is not responsible for the enhanced dissolution rate of digitoxin from the coprecipitate. Also, the equilibrium solubility studies indicate that the increased solubility and dissolution rate of digitoxin from the 1:9 (w/w) drug–polyvinylpyrrolidone coprecipitate system are most probably not the result of the formation of a water-soluble complex between the drug and polyvinylpyrrolidone.

The similarity between the dissolution and solubility behavior of digitoxin from the 1:9 (w/w) drug polyvinylpyrrolidone coprecipitate and that previously observed with reserpine–polyvinylpyrrolidone coprecipitate systems (16), coupled with the fact that both drugs form *glass solutions* with the polymer, strongly suggests that a similar mechanism is operable. Accordingly, it may be proposed that a high energy form of digitoxin, most probably amorphous in nature, is formed as a result of coprecipitating the drug with polyvinylpyrrolidone and that its increased solubility characteristics are responsible for the marked enhancement in drug dissolution and absorption experienced with the coprecipitate system.

CONCLUSIONS

The data obtained in the present study demonstrate that coprecipitation with polyvinylpyrrolidone increases the solubility and dissolution rate of digitoxin and thereby significantly enhances the *in vivo* absorption of this hydrophobic drug. These observations, as well as those noted previously with reserpine (6, 16), indicate that it is quite possible that this technique might have general applicability to a wide variety of relatively water-insoluble drug entities whose absorption from the GI tract can be characterized as slow, erratic, and/or incomplete. The advantages of utilizing this technique in the pharmacological screening of new drugs for oral activity are numerous and self-evident. Its clinical applications are currently being explored in these laboratories.

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High-Voltage Paper Electrophoresis for Characterization of Drug Metabolites

WALTER D. CONWAY▲, VIJAY K. BATRA, and ALAN ABRAMOWITZ

Abstract □ Methodology for obtaining reproducible measurements of the relative mobility of organic compounds over pH 2–12 using high-voltage paper electrophoresis is described. Use of the method for the elucidation of some structural features of simple molecules is illustrated by applying it to the characterization of metabolites of drugs excreted in the urine and bile of rats. The compounds studied included *p*-nitrophenol, 1-naphthol, salicylic acid, 4-nitrocatechol, probenecid, phenol red, and their metabolites.

Keyphrases □ Mobility–pH profiles, drug metabolites—determined using high-voltage paper electrophoresis □ Metabolites—characterized using high-voltage paper electrophoresis □ Structure characterization—mobility–pH profiles of drug metabolites determined using high-voltage paper electrophoresis □ Paper electrophoresis, high voltage—determination of mobility–pH profiles for drug metabolites

Paper electrophoresis has been widely used over the past 20 years for separating and characterizing molecules. An historical account and an extensive review of the work done prior to 1955 was presented by McDonald (1). The technique has been extensively applied in the fields of carbohydrate chemistry, amino acid and protein analysis, and inorganic chemistry.

Recent applications related to drugs include separation and identification of alkaloids (2); establishment of the radiopurity of ^{14}C-labeled methylglyoxal-bisguanylhydrazone; and separation of the metabolites of chlorpromazine (5, 6), carbiphene hydrochloride[1] (a basic amide analgesic) (7), catecholamines (8–10), isoproterenol (11), derivatives of fluorene and biphenyl (12), *p*-aminohippuric acid and its acetyl derivatives (13), cyclophosphamide (14), thalidomide (15), neostigmine (16, 17), and pronethalol (18).

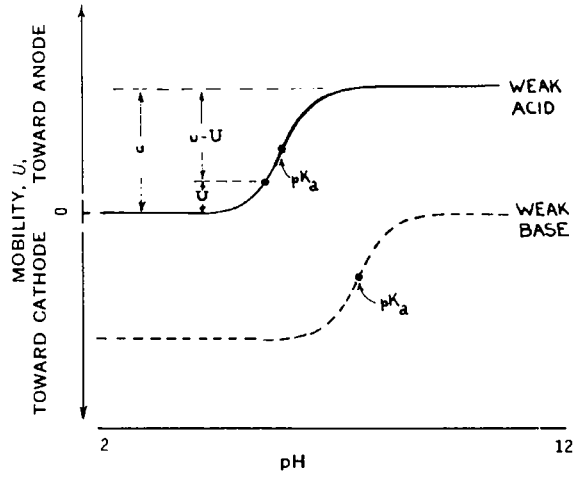


Figure 1—*Mobility–pH profiles of a weak acid and a weak base.*

[1] SQ 10,269.

Bond and Howe (18) classified the metabolites of pronethalol as acidic, basic, and amphoteric on the basis of electrophoretic mobility at a single pH; in the same way, Keberle *et al.* (15) classified thalidomide metabolites as monocarboxylic and dicarboxylic acids. Most investigators made separations in only one or two buffers and applied electrophoresis in much the same manner as other differential migration techniques such as TLC and paper chromatography. However, significant aspects of the structure of a substance can be ascertained simply from measurements of its electrophoretic mobility at several concentrations of hydrogen ion. This technique is particularly useful for determining structures of substances arising from drug metabolism.

THEORETICAL

The relationship of various factors affecting the mobility, U, of a spherical particle in an electrolyte under the influence of an electrical field was expressed (19) as Eq. 1:

$$U = \frac{Q}{6\pi\eta r}\left(\frac{1}{1+\kappa r}\right) \qquad \text{(Eq. 1)}$$

where Q is the electrical charge on the particle, r is its radius, η is the viscosity of the electrolyte, and κ is a parameter, the reciprocal of which is related to the thickness of the ionic double layer surrounding the particle. The equation implies that doubly charged ions migrate twice as fast as singly charged ions of equal size. Where $1/\kappa$ is small compared with r, a reasonable approximation in the case of organic drugs, there is an inverse dependence of mobility on the square of the radius of the particle.

The general shape of a plot of mobility *versus* pH for a weak monoprotic acid is characterized by a form of the Henderson–Hasselbalch equation:

$$\text{pH} = \text{pKa} + \log\left(\frac{U}{u-U}\right) \qquad \text{(Eq. 2)}$$

and can be advantageously illustrated as shown in Fig. 1. It is convenient to refer to this plot as the mobility–pH profile of a substance.

The shape of the mobility–pH profile is, in fact, identical with that of a plot of the ionized fraction of a monoprotic acid as a function of pH since it can be shown that:

$$\frac{U}{u-U} = \frac{\alpha}{1-\alpha} \qquad \text{(Eq. 3)}$$

where U is the mobility of a zone at any particular pH, u is the maximum mobility of the zone or the mobility of the anion in the case of a monoprotic acid, and α is the degree of dissociation of the weak acid (20). The term α may be obtained from the graph at any particular pH as the ratio of the distances U and u since:

$$\alpha = \frac{U}{u} \qquad \text{(Eq. 4)}$$

The degree of dissociation, α, may also be interpreted as that fraction of time spent in the ionized state by any individual molecule of a weak acid. The fraction varies from 0 at low pH to 1 at higher pH, where the region of constant mobility is reached. This interpretation of α is advantageous in rationalizing the shape of the mobility–pH profile.

As indicated by Eqs. 2 and 3, the profile of a weak monobasic

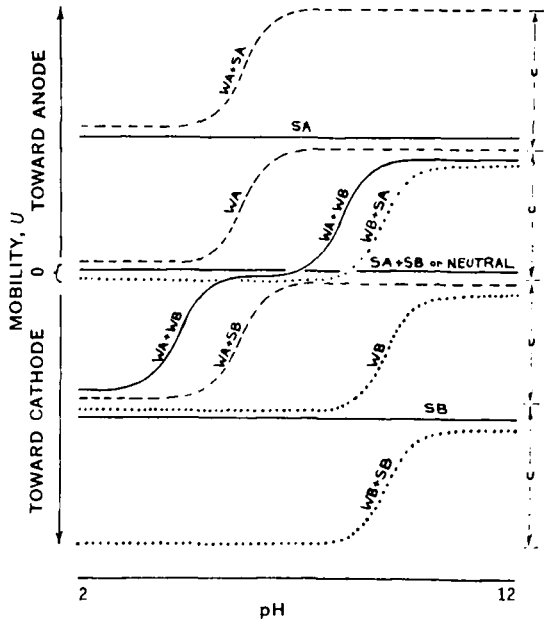


Figure 2—*Mobility-pH profiles of weak acids (WA), weak bases (WB), strong acids (SA), strong bases (SB), and amphoteric molecules containing combinations of these functional groups.*

acid will have: (*a*) zero mobility at low pH, where the acid is not ionized and is, therefore, electrically neutral; (*b*) a point of inflection where the pH of the electrolyte equals the pKa of the weak acid; and (*c*) a constant mobility toward the anode at high pH, characteristic of the anion resulting from ionization of the acid. The distance u will be related to the effective radius or volume of the ion. Under conditions of zero ionic strength, and assuming hydrodynamic behavior of ions equivalent to that of spheres, the distance u would be expected to be inversely related to the two-thirds power of the molecular weight.

The profile of a weak base can be described in completely analogous fashion, using Eq. 2, by considering the protonated form of the base to behave as a weak monoprotic acid, albeit bearing a unit positive charge at low pH. The weak base will, therefore, exhibit a constant maximum mobility toward the cathode in the region of low pH and zero mobility at high pH (Fig. 1).

The *shape* of the mobility-pH profile of either a weak acid or a weak base is thus defined by Eq. 2. The mobility increment, u, will depend upon the molecular weight of the substance, and the *position* of the sigmoid portion of the profile will be determined by the pKa of the substance.

Strong acids, such as sulfonic acids and sulfuric acid monoesters, will migrate toward the anode, and completely dissociated cations, such as quaternary ammonium compounds, will migrate toward the cathode at constant velocity independent of pH. The profile of an amphoteric substance should reflect the combined behavior of the acid and base functions.

The general features of the mobility-pH profiles predicted by Eq. 2 for molecules containing various combinations of strong and weak acid and base functional groups are illustrated in Fig. 2. The profiles are drawn for compounds of equal molecular weights and arbitrary pKa values. For clarity, the curves are shifted slightly on the vertical axis from their normal positions, which would overlap in the regions of constant mobility.

A measure of the pH interval over which ionization of a substance occurs may be obtained by drawing a line through the relatively linear portion of the mobility-pH profile and extrapolating it to its intersections with those portions of the profile that define regions of constant minimum and maximum mobility. It is useful to refer to this pH increment as the ionization interval. The relationship of this ionization interval to the shape of the mobility-pH profile may be seen by differentiating Eq. 2 with respect to pH and evaluating the resulting equation in terms of u at the point of inflection or pKa, where $U = u - U = u/2$, which gives:

$$\left[\frac{dU}{d(\mathrm{pH})}\right]_{\mathrm{pKa}} = \frac{U(u-U)}{U\log_{10} e} = \frac{u}{1.74} = \text{slope at pKa} \quad \text{(Eq. 5)}$$

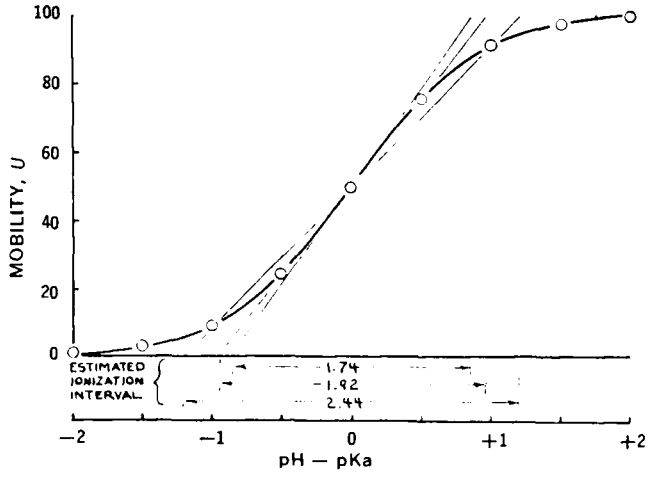


Figure 3—*Estimates of the ionization interval obtained from lines drawn tangent to the inflection point and through points on the mobility-pH profile at pH − pKa = ±0.5 and ±1.0.*

This implies that, irrespective of molecular weight, a line drawn tangent to a continuous plot of the mobility-pH profile at the pKa will intercept the values of minimum and maximum mobility with an intervening distance of 1.74 pH units for a monoprotic acid or base (Fig. 3). However, a line drawn through discontinuous experimental data points obtained at increments of 0.5–1.0 pH unit will have a slightly lower slope. For instance, the line drawn through points spaced 0.5 pH unit above and below the pKa will form intercepts with the minimum and maximum mobilities with an intervening distance of 1.92 pH units, and the intercepts derived from a line through points spaced 1.0 pH unit above and below the pKa will be 2.44 units apart (Fig. 3).

A diprotic acid will possess two electrical charges when fully ionized. The acid will be 50% ionized at a hydronium-ion concentration, $[H^+]$, given by Eq. 6:

$$[H^+] = \sqrt{K_1 K_2} \quad \text{(Eq. 6)}$$

where K_1 and K_2 are the apparent dissociation constants of the two ionizable groups. In logarithmic form, Eq. 6 becomes:

$$\mathrm{pH} = \frac{\mathrm{pK_1} + \mathrm{pK_2}}{2} \quad \text{(Eq. 7)}$$

The ionization and, consequently, the electrophoretic mobility of a diprotic acid may be conveniently expressed as:

$$\text{percent ionized} = 100\left(\frac{U}{u}\right) = 50\left(\frac{\Delta\sqrt{X}+2}{\Delta^2+\Delta\sqrt{X}+1}\right) \quad \text{(Eq. 8)}$$

where:

$$\Delta = \frac{[H^+]}{\sqrt{K_1 K_2}} \quad \text{(Eq. 9)}$$

$$X = \frac{K_1}{K_2} \quad \text{(Eq. 10)}$$

The mobility of a zone of a diprotic acid at a particular pH will be proportional to the charge on the acid at that pH. As described by Eq. 8, the profile will be symmetrical about the point where the acid is 50% ionized. At this point, $\Delta = 1$ or $\mathrm{pH} = \frac{1}{2}(\mathrm{pK_1} + \mathrm{pK_2})$. The *shape* of the profile will depend upon X, the ratio of the ionization constants (Eq. 10). The expected profiles for acids with $X = 4$, 10, 10^2, 10^3, and 10^4 are illustrated in Fig. 4.

The profile for a diprotic acid with $X = 4$ is identical with that of a monoprotic acid, and it is doubtful whether the profile for $X = 10$ could be experimentally distinguished from that of a monoprotic acid.

As X increases, the slope decreases; when X equals 100, the experimentally measured ionization interval would be expected to lie in the range of 4.1–4.7 pH units. When X is 10^3 or greater, the pro-

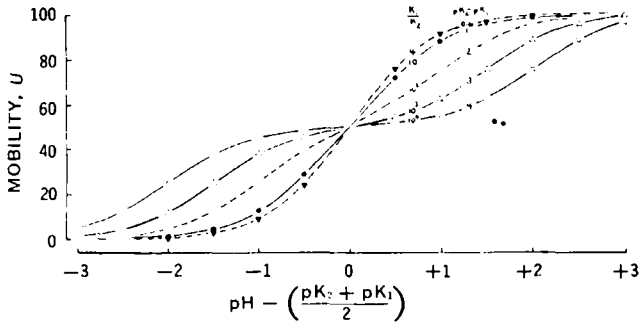


Figure 4—*Mobility-pH profiles of dicarboxylic acids.*

file would be expected to exhibit two sigmoid portions and the ionization interval for each of these portions would be expected to lie in the range of 1.9–2.4 pH units, identical to the value expected for a monoprotic acid. The ionization intervals for the profiles with X of 10^3 or more are measured with respect to the intercepts with the regions of 0 and 50 or 50 and 100 mobility units, since ionization of the individual functional groups is discernible. Thus, the mobility pH profile should serve to distinguish diprotic substances, when the pKa values of the ionizable groups differ by more than 1.5 or 2.0 units, from monoprotic substances.

Similar reasoning may be applied to determine the expected profiles for substances with three or more ionizable groups.

This theoretical description of electrophoresis strictly applies only to the migration of ions in free solution. Because of the tortuous path the migrating ions must follow around fibers and through constricted pores of the paper matrix, the rates of migration measured in paper electrophoresis are less than the mobilities measured in free solution. In addition, the phenomenon of electroosmotic flow and the variability of numerous parameters, including temperature, electrolyte viscosity and ionic strength, paper wetness, voltage gradient, and duration of a run, lead to considerable variability in measurements of migration rates.

However, if one prepares buffers of identical ionic strength and uses an electrically neutral substance to correct for electroosmotic flow and a completely ionized substance as a mobility standard, the effects of these variables cancel out in relation to the standard and reproducible values of relative mobility are obtained.

EXPERIMENTAL

Apparatus—A double-platen high-voltage paper electrophoresis apparatus[2] was used in conjunction with a power supply[3] (5000 v., 300 ma.) and a constant-temperature circulator[4]. The electrophoresis apparatus, which contains an upper and lower cooling platen, was modified (Fig. 5) by drilling and tapping the ends of the platens to permit attachment of aluminum angles [1.9 × 1.9 cm. (0.75 × 0.75 in.)] faced with a 0.63-cm. (0.25-in.) thickness of foam rubber.

Procedure—A sheet of Whatman 3MM filter paper [13.9 × 42.58 cm. (5.5 × 20.25 in.)] was marked at its midpoint with a light pencil line, and a series of light pencil lines were spaced at 5-cm. (2-in.) intervals on either side of the midpoint (Fig. 6).

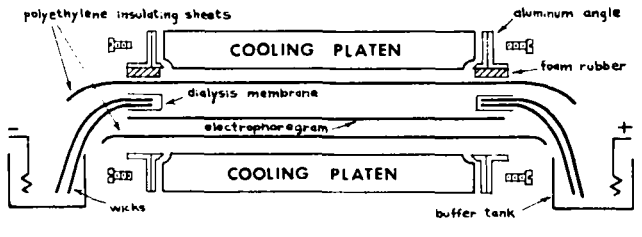


Figure 5—*Schematic diagram of electrophoresis apparatus.*

[2] Shandon model 2550.
[3] Savant model HV-5000-TC.
[4] Neslab model HX-75.

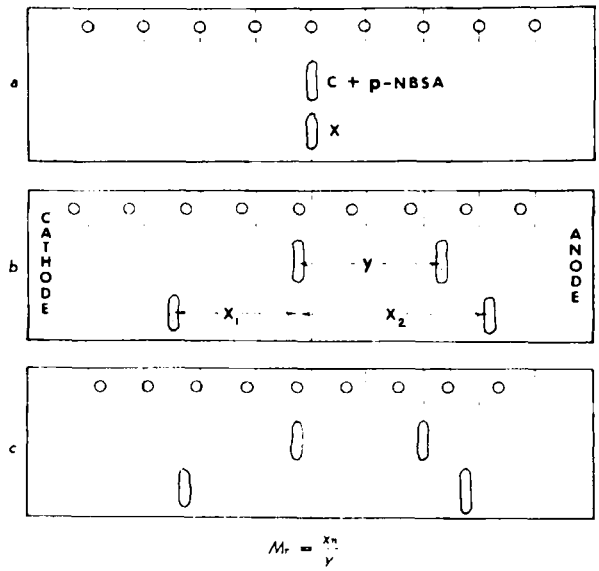


Figure 6—*Application of sample* (x) *and caffeine* (c) *and* p-*nitrobenzenesulfonic acid* (p-*NBSA*) *standards, evaluation of electrophoregram, and calculation of relative mobility* (M_r). *Key:* a, *before run;* b, *after normal run; and* c, *pattern distorted by electrolyte uptake via wicks.*

The paper was soaked in buffer for about 1 min., and some surplus buffer was removed by stroking each side of the paper with a glass rod. The wet sheet was centered on an 8-mil polyethylene [17.78 × 58.42 cm. (7 × 23 in.)] insulating sheet in the electrophoresis apparatus in which the circulating coolant had been adjusted to the desired temperature. A piece of "PE" polyester monofilament screen cloth[5] was placed on top of the paper, followed by three thicknesses of Whatman No. 1 filter paper and a second polyethylene insulating sheet. The apparatus was closed and a pressure of 5 psig. was applied to the lower platen for 2 min. When treated in this way at 20°, the Whatman 3MM paper reaches an equilibrium water content of approximately 71% in 1 min. and no additional water is lost for as long as 5 min.

The apparatus was then depressurized and opened. Except for the midpoint, the exposed upper portion of the paper strip was covered with a polyethylene sheet to prevent evaporation. Approximately 10-μl. aliquots of samples were applied to the midline as streaks approximately 2 cm. long. A 10-μl. aliquot of an aqueous solution containing 0.2% caffeine and 0.2% *p*-nitrobenzenesulfonic acid was applied as one sample. The protective polyethylene was removed, and samples of a few microliters of a 0.2% caffeine solution were applied to each pencil line approximately 2.54 cm. (1 in.) from the upper edge of the paper (Fig. 6*a*).

Wicks were prepared from two thicknesses of 10.89 × 13.97-cm. (4.25 × 5.5-in.) Whatman 3MM paper soaked in buffer, and one narrow end was covered to a distance of 4.44 cm. (1.75 in.) from the end with a single thickness of dialysis membrane, obtained by slitting a piece of dialysis tubing, 4.44-cm. (1.75-in.) flat width[6]. The wicks were placed with the dialysis membranes in contact with either end of the electrophoregram and not extending onto the platen beyond the foam rubber pads. The upper polyethylene insulating sheet was placed in position, and the apparatus was closed and pressurized to 5 psig.

Two pieces of polyethylene tubing [1.27-cm. (0.5-in.) o.d. × 0.16-cm. (0.069-in.) wall × 5.08-cm. (2-in.) length], slit lengthwise, were placed as clamps over the sides of the polyethylene insulating sheets and extended slightly beyond the aluminum angles (Fig. 7) on the high tension end of the apparatus. The high tension is the anode with the power supply used[3]. Previous work showed that almost all electrical shorts occur at this end of the apparatus and that most of them result from the deposition of moisture between the insulating sheets; when the moisture reaches the edge of the sheet, a spark jumps to

[5] ASTM No. 7-140-105, 17.78 × 57.15 cm. (7 × 22.5 in.), 105-μ porosity, Kressilk Products, Inc., Elmsford, N. Y.
[6] Union Carbide Corp., No. 36.

Table I—Composition of Buffers Used for Electrophoresis

Components		Concentrations, mole/l.		Approximate
A	B	A	B	pH
Phosphoric acid	Monobasic potassium phosphate	0.090	0.050	2.0
Oxalic acid	Potassium hydroxide	0.071	0.050	2.0
Phosphoric acid	Monobasic potassium phosphate	0.008	0.050	3.0
Acetic acid	Potassium acetate	0.500	0.050	3.7
Acetic acid	Potassium acetate	0.050	0.050	4.7
Acetic acid	Potassium acetate	0.005	0.050	5.7
Monobasic potassium phosphate	Dibasic potassium phosphate	0.038	0.005	6.0
Monobasic potassium phosphate	Dibasic potassium phosphate	0.012	0.013	7.0
Monobasic potassium phosphate	Dibasic potassium phosphate	0.003	0.014	7.6
Triethanolamine	Hydrochloric acid	0.100	0.050	7.8
Triethanolamine	Hydrochloric acid	0.290	0.050	8.5
Ethylenediamine	Hydrochloric acid	0.055	0.050	9.0
Ethylenediamine	Hydrochloric acid	0.100	0.050	10.0
Piperidine	Hydrochloric acid	0.100	0.050	11.2
Piperidine	Hydrochloric acid	0.550	0.050	12.2
Potassium hydroxide	Potassium chloride	0.010	0.040	12.0

the grounded platen. Placement of the polyethylene clamps as indicated prevents the moisture from reaching the edge of the strip and virtually eliminates shorting.

The voltage was typically adjusted to 5 kv. and allowed to remain for 15 min. The apparatus was then opened, and the electrophoregram was kept in a horizontal position and examined while wet with a short wavelength (254 nm.) UV lamp[7]. Positions of the standards and any samples which were visible were outlined with pencil. The strip was then suspended vertically, with one longer side at the top, and dried for 10 min. at 90° in a chromatogram drying oven[8] with the draft control half open. The dry electrophoregram was again examined under UV light to check the uniformity of drying. Uneven evaporation of the water will be indicated by a shift of the standard spots, particularly caffeine, from the positions marked before drying.

The pattern after a normal run is indicated in Fig. 6*b*, where all caffeine spots moved a uniform distance of several millimeters toward the cathode. The pattern shown in Fig. 6*c* is distorted as a result of buffer uptake through the wicks and is typically observed when a dialysis membrane is not used.

Distances (X) to the centers of the various sample spots are measured from the center of the caffeine spot and divided by the distance (Y) from the center of the caffeine spot to the center of the *p*-nitrobenzenesulfonic acid spot, and this fraction is multiplied by 100 to give the relative mobility.

Radioactive Substances—The locations of radioactive spots were determined by cutting the dry electrophoregram into strips and scanning these in a radiochromatogram scanner[9]. This presented no problem when scanning for ^{14}C-labeled compounds with a polyethylene terephthalate[10] window covering the counter tube. However, scanning ^{3}H-labeled compounds with the counter in the windowless mode caused very erratic counter behavior, characterized by spurious peaks and excessive count rates which drove the pen off-scale for prolonged periods. This behavior was traced to the attachment of conductive fibers of buffer-impregnated paper to the counter anode. This was overcome by modifying the counter to permit its operation with the strip feed and takeup reels rotating in a horizontal plane and the counter anode wires oriented vertically. Such operation required a counter gas flow rate of 400 ml./min. Careful attention must also be paid to the anode voltage, which should be no higher than the midpoint of the plateau region. These modifications totally eliminated the erratic counting with all buffers used except one composed of phosphoric acid and monobasic potassium phosphate at pH 2. Fibers from paper containing this buffer were readily attracted to the anode by electrostatic force, thereby preventing its use when scanning in the windowless mode.

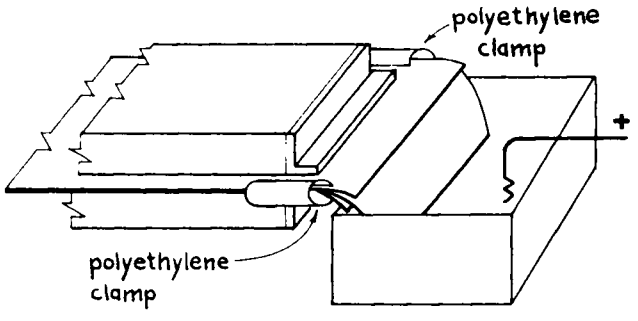


Figure 7—*Placement of polyethylene clamps to eliminate shorting.*

Buffers—Buffers were prepared with an ionic strength of 0.05, using the components indicated in Table I. The pH was measured prior to each run using a pH meter[11] on the standard scale. After a run, the buffer in the anode and cathode tanks was remixed and saved for reuse.

RESULTS AND DISCUSSION

The contribution of this technique to the elucidation of the structures of certain drug metabolites will be illustrated by the following presentation of the mobility–pH profiles of several samples obtained synthetically or from the urine or bile of animals dosed with the parent drug. The profiles were obtained from measurements in buffers about 1–2 pH units apart over approximately the 2.5–11.5 pH range. The profiles are not intended to define the characteristics such as pKa or molecular weight of the substance

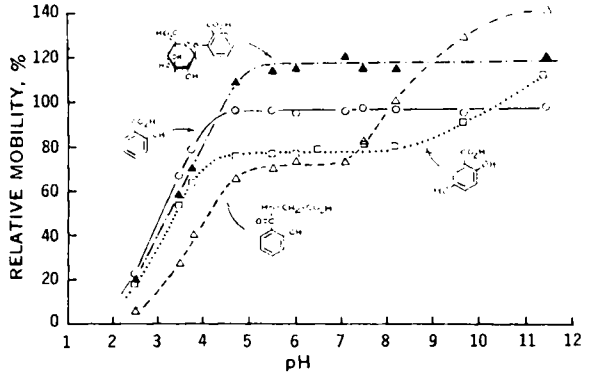


Figure 8—*Mobility–pH profiles for salicylic acid* (○), *gentisic acid* (□), *salicyluric acid* (△), *and salicylic acid phenolic glucuronide* (▲).

[7] Mineralight, model UVS-12, Ultraviolet Products, Inc.
[8] National Appliance model 5670.
[9] Packard model 7200.
[10] Mylar.
[11] Beckman Expandomatic.

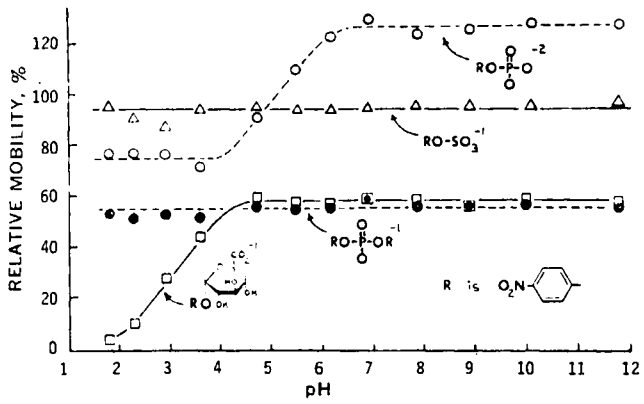


Figure 9—*Mobility-pH profiles for* p-*nitrophenol sulfuric acid ester* (△), p-*nitrophenol glucuronide* (□), p-*nitrophenol dihydrogen phosphate* (○), *and* p-*nitrophenol monohydrogen phosphate* (●).

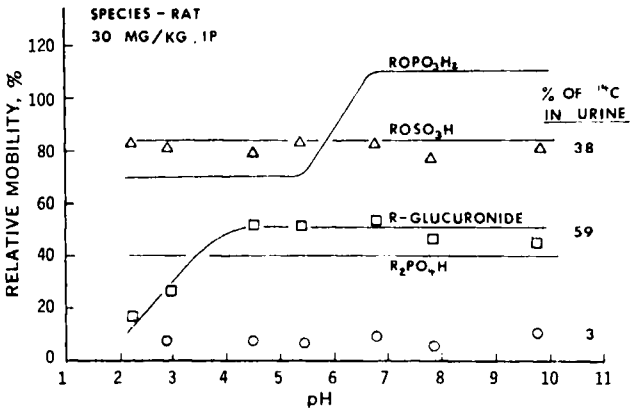


Figure 11—*Mobility-pH profiles for metabolites* (△,□,○) *found in urine following intraperitoneal administration of 1-naphthol-*^{14}C *and profiles for authentic samples of conjugates of 1-naphthol (solid lines) redrawn from Fig. 10.*

with high precision, but they are sufficiently accurate to reveal characteristic structural features.

Salicylic Acid and Its Metabolites—The mobility-pH profiles (Fig. 8) of these substances are typical of those observed for weak acids, phenols, and glucuronide conjugates of acidic substances. They were obtained using synthetic samples of salicylic acid phenolic glucuronide, salicylic acid, gentisic acid, and salicyluric acid. The general shapes of the profiles are consistent with the properties discussed for Fig. 1. All of the substances show a pKa in the region of 3–3.5, characteristic of a carboxylic acid. Salicyluric acid shows a second pKa in the region of pH 9, consistent with ionization of a phenolic group. The mobility, 141, of salicyluric acid at pH 11.5 is approximately double that, 72, at pH 6, indicating the presence of two electrical charges and a single charge, respectively. Gentisic acid exhibits incomplete ionization of even one phenolic group at pH 11.5. Salicylic acid shows no ionization of the phenolic group at pH 11.5, as would be expected since the reported pKa for this group is 13.8 (23).

Closer scrutiny of the profiles reveals some deviations from the simple theory presented earlier. All of the substances migrate with relative mobilities less than those calculated, assuming mobility to be inversely related to the two-thirds power of the molecular weight. Predicted mobilities in the region of pH 6, relative to that of *p*-nitrobenzenesulfonic acid as 100, are: salicylic acid phenolic glucuronide, 150; salicylic acid, 130; gentisic acid, 120; and salicyluric acid, 106; the observed mobilities are 118, 96, 78, and 71, respectively. Thus, the predicted rank order of migration rates is observed, but the actual mobilities are considerably less relative to *p*-nitrobenzenesulfonic acid than predicted.

An ionization interval of 2.1 pH units was observed for salicylic and gentisic acids and 2.0 units for salicyluric acid. These are within the expected range of 1.9–2.4 pH units for monoprotic acids. The ionization interval of 3.0 units observed for the phenolic glucuronide of salicylic acid is within the range expected for dicarboxylic acids, with ionization constants differing by 1–2 pH units.

Conjugates of *p*-Nitrophenol—These profiles (Fig. 9) illustrate the application of the technique to glucuronic, sulfuric, and phosphoric acid conjugates, for which electrophoresis is particularly appropriate. These highly polar, water-soluble compounds are not readily extracted with organic solvents and are, therefore, not easily studied by conventional TLC or paper chromatography.

The mobility-pH profiles of commercially available samples of the glucuronide, sulfuric acid ester, and mono- and dihydrogen phosphate esters of *p*-nitrophenol are shown in Fig. 9. Although the phosphate esters are not ordinarily found as drug metabolites in mammals, they are found in insects; it is, therefore, interesting to determine whether the mobility-pH profile will distinguish these conjugates.

The profile for the glucuronide shows virtually no mobility at pH 2, an ionization interval of 2.3 units with a pKa slightly over 3, and a region of constant mobility above pH 5. The sulfuric acid ester is fully ionized over the entire pH range and shows the expected constant mobility. A similar constant mobility is seen for the monohydrogen phosphate, but its mobility is much less than that of the sulfate, reflecting its higher molecular weight. The dihydrogen phosphate ester exhibits mobility at low pH, reflecting the low pKa of one of its protons, an ionization interval of 2.2 units with a pKa in the vicinity of 5, and constant mobility above pH 7 where the molecule bears two negative charges.

The mobilities are in the expected order based on molecular weights, except that one would not expect the considerable separation seen for the sulfate and dihydrogen phosphate at low pH since these two compounds are actually identical in molecular weight. In experiments using paper chromatography with the various buffers as the developing solvents, the sulfuric acid ester was retarded to a greater extent than the phosphate, suggesting that electro-

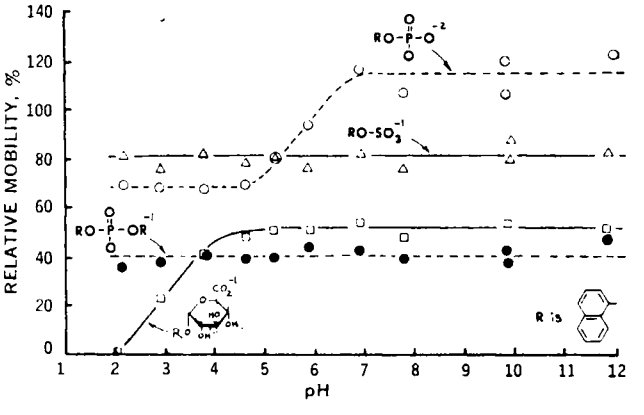


Figure 10—*Mobility-pH profiles for 1-naphthol sulfuric acid ester* (△), *1-naphthol glucuronide* (□), *1-naphthol dihydrogen phosphate* (○), *and 1-naphthol monohydrogen phosphate* (●).

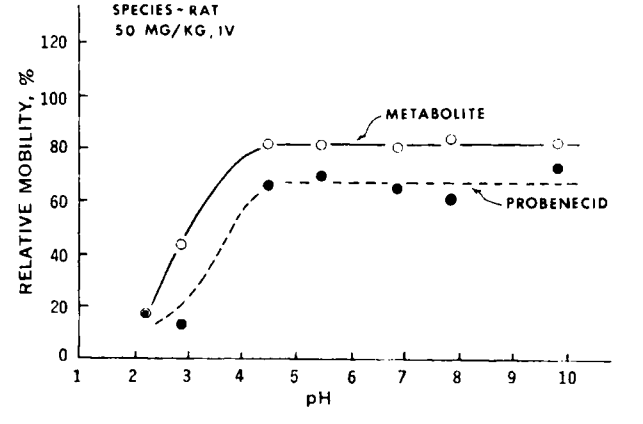
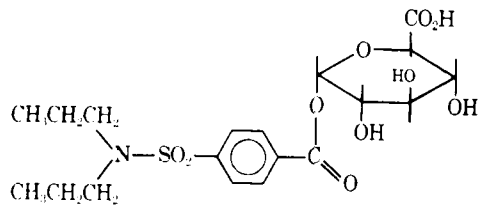


Figure 12—*Mobility-pH profiles for probenecid* (●) *and a major metabolite* (○) *excreted in the bile of rats.*



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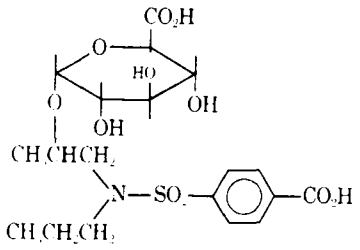


II

phoretic separation of the two compounds is not a result of differential adsorption on the paper.

The mobility of the dihydrogen phosphate at high pH is not quite double that observed at low pH, as would be predicted by the presence of two electrical charges on the ion at high pH. A ratio of 1.7 was actually observed. Part of this discrepancy can be attributed to the fact that the runs were made in electrolytes of ionic strength 0.05. The theoretical ratio of 2.0 would be expected only at infinite dilution.

Conjugates of 2-Naphthol—The mobility–pH profiles of conjugates of 2-naphthol with glucuronic, sulfuric, and phosphoric acids are presented in Fig. 10. The profiles are analogous to the corresponding conjugates of *p*-nitrophenol and the same comments apply.

The applicability of this approach to the characterization of drug metabolites in a biological fluid was demonstrated after administration to a male rat by intraperitoneal injection of a 30-mg./kg. dose of 1-naphthol-^{14}C containing 10 μc. of ^{14}C. The mobility–pH profiles obtained using 10-μl. samples of the 24-hr. postmedication urine are shown by the data points in Fig. 11. The urine contained three substances. One moved with constant mobility over the entire pH range and accounted for 38% of the radioactivity. A second substance, accounting for 59% of the radioactivity, showed a pKa of about 3 and constant mobility above pH 4.5. The third material, which accounted for 3% of the radioactivity, remained at the origin.

This information alone suggests that the two major metabolites are conjugates with sulfuric and glucuronic acids. They are the metabolites of 1-naphthol in the rat. The lines in the figure were drawn independently from authentic samples of the indicated compounds and confirm the identity of the radioactive metabolites. The absence of either phosphate is apparent.

Metabolite of Probenecid—The mobility pH profile (Fig. 12) of a major metabolite excreted in rat bile after intravenous administration of probenecid-^{14}C was determined in conjunction with studies on the structure of the metabolite. It illustrates the value of the technique for detecting a metabolite of unexpected structure. Comparison of the metabolite profile with that of probenecid strongly suggested that the metabolite was not the expected acyl glucuronide of probenecid (I).

The molecular weight of I is 461, and this structure still contains only one ionizable group. Assuming the mobilities to be inversely related to the two-thirds power of the molecular weight, I would be expected to migrate about 60% as fast as probenecid, which has a molecular weight of 285. However, hydroxylation of probenecid followed by conjugation to an ether glucuronide would produce a species with a molecular weight of 477 and two carboxyl groups. The fully ionized molecule would then be expected to migrate about 27% faster than probenecid. The measurements in Fig. 12 indicate a 21% faster mobility for the metabolite. While it was not possible solely on the basis of this evidence to characterize fully the metabolite, it did guide further studies leading to the synthesis of *p*-(*N*-propyl,*N*-2-hydroxypropylsulfamoyl)benzoic acid, the aglycone of Metabolite II (21, 22).

Nitro- and Aminocatechols and Guaiacols—These profiles illustrate the application of the technique to compounds of similar

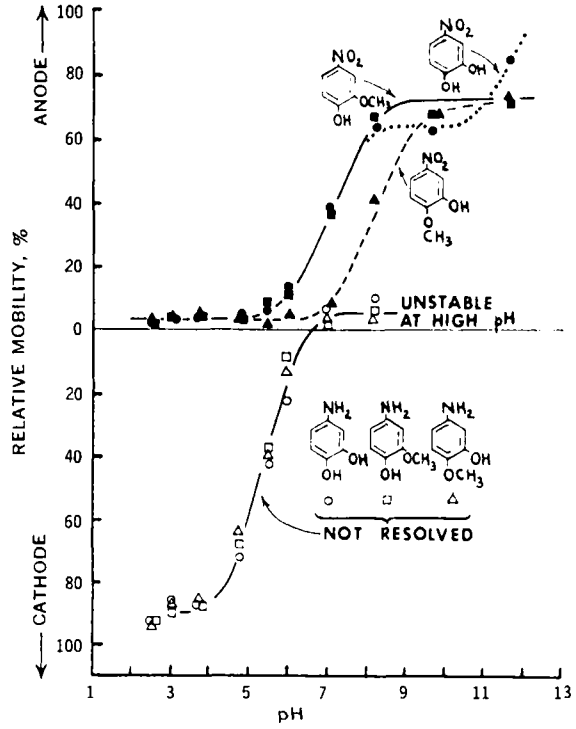


Figure 13—*Mobility–pH profiles for 4-nitrocatechol* (●), *4-nitroguaiacol* (■), *5-nitroguaiacol* (▲), *4-aminocatechol* (○), *4-aminoguaiacol* (□), *and 5-aminoguaiacol* (△).

molecular weight that differ only slightly in pKa and to basic compounds. The five compounds which might result from *O*-methylation and reduction of the nitro group of 4-nitrocatechol were prepared by standard synthetic procedures in conjunction with a study of the metabolic fate of 4-nitrocatechol. The mobility–pH profiles of these substances and 4-nitrocatechol itself are shown in Fig. 13. The amino compounds were not resolved and, unfortunately, were chemically unstable in solutions above pH 8. However, their profiles in the acidic range were of the expected shape and exhibited pKa values in the range of 5.5. The phenolic compounds show no mobility below pH 5 where they are not ionized. Both 4-nitrocatechol and 4-nitroguaiacol are unresolved below pH 7.5 and exhibit pKa values of about 7. The two compounds are resolved above pH 8, where the mobility of nitrocatechol is somewhat less than would be predicted by its smaller molecular weight. Partial ionization of the second phenolic group in 4-nitrocatechol is seen at pH 11. The 5-nitroguaiacol, by virtue of the lesser acidity of the *meta*-nitrophenolic group, is separated from the other phenols in the range of pH 6–9. The observed pKa values for 4-nitroguaiacol and 5-nitroguaiacol are in the range of those reported for *p*-nitrophenol, pKa 7.15, and *m*-nitrophenol, 8.40 (23).

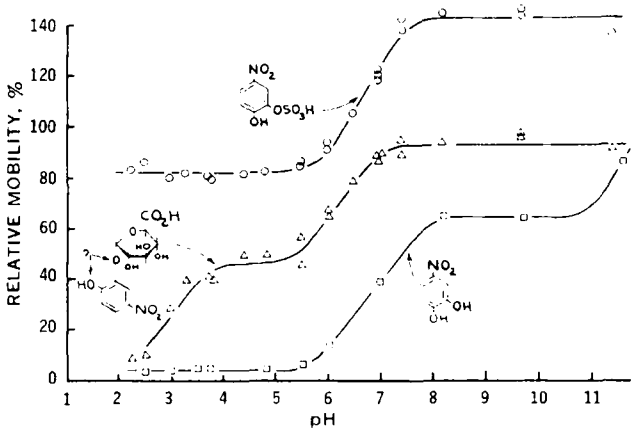
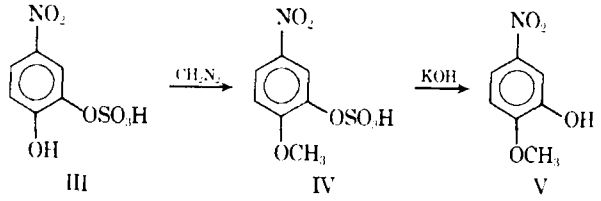


Figure 14—*Mobility–pH profiles of metabolites excreted in the urine of rats given 30 mg./kg. of 4-nitrocatechol intraperitoneally.*



Scheme I

Metabolites of 4-Nitrocatechol This study illustrates the use of electrophoresis to establish the formation of both sulfuric acid and glucuronic acid conjugates and then to establish further the position of sulfuric acid conjugation by chemical conversion of the conjugate to a derivative of known structure which could be unequivocally identified by its mobility–pH profile.

Two yellow spots could be detected in the 0–24-hr. urine of rats given 4-nitrocatechol (30 mg./kg. i.p.). Only traces of the parent compound were detected. The mobility–pH profiles of the metabolites and 4-nitrocatechol are shown in Fig. 14. The profile of the metabolite with highest mobility is characteristic of a sulfuric acid ester, and it is identical with the profile of an authentic sample of 2-hydroxy-5-nitrophenyl sodium sulfate (III, Scheme I). The observed pKa in the neighborhood of 7 is also characteristic of a *p*-nitrophenolic group. Unfortunately, the isomeric 2-hydroxy-4-nitrophenol is unknown, so that it was impossible to determine whether the isomeric sulfuric acid esters could be separated. To confirm further the structure of the metabolite, the residue from evaporation of a small aliquot of urine was extracted with methanol, the extract was treated with diazomethane, and the product was hydrolyzed with dilute potassium hydroxide. Only 5-nitroguaiacol (V) could be found when the hydrolysate was examined by electrophoresis. This establishes the metabolite as III since it has been shown (Fig. 13) that the isomers 4-nitroguaiacol and 5-nitroguaiacol (V) are separable by electrophoresis.

The other metabolite exhibits a profile characteristic of a conjugate with glucuronic acid and has pKa values in the ranges of 3 and 6.5. The pKa in the range of 6.5 appears characteristic of a *p*-nitrophenolic group and suggests that conjugation here also occurs *meta* to the nitro group.

The ratio of the mobility of the doubly charged glucuronic acid conjugate (92 units) to that of the singly charged species (46 units in the 4–5 pH range) is 2.0, in contrast to the ratio of 1.7 observed for the sulfuric acid ester, III, and for the dihydrogen phosphates presented earlier (Figs. 9 and 10). The deviation may result partly because of the proximity of the two charges in the dihydrogen phosphates and III.

Visual examination of the electrophoregrams suggest that about equal amounts of the glucuronide and sulfuric acid ester are formed.

Metabolites of Phenol Red—In this study a completely unexpected metabolite was detected, which was then shown to be derived from an impurity in the sample of dye. Phenol red itself and three metabolites were detected by their color and fluorescence on electrophoregrams of the urine and bile obtained from rats after intravenous administration of phenol red (15 mg./kg.). The mobility–pH profiles are presented in Fig. 15. The metabolite with the highest mobility (△) was excreted only in urine and exhibited a mobility–pH

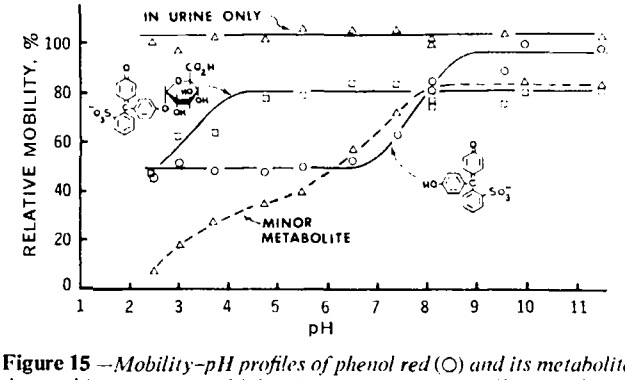


Figure 15 —*Mobility–pH profiles of phenol red* (○) *and its metabolites detected in the urine and bile of a rat given a 15-mg./kg. i.v. dose.*

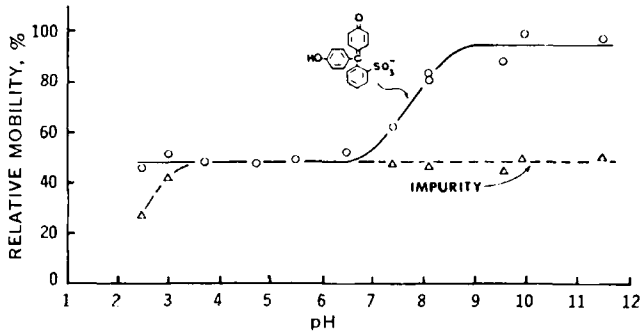


Figure 16 —*Mobility–pH profile of phenol red* (○) *and an impurity* (△) *which lacks the sulfonic acid group.*

profile characteristic of a conjugate with sulfuric acid. A second metabolite (□), with a profile characteristic of a conjugate with glucuronic acid, was excreted in both urine and bile. The biliary excretion of the glucuronide of phenol red was reported previously (24). These two metabolites, as well as phenol red, exhibit significant mobility at low pH due to the ionized sulfonic acid group. However, a minor metabolite (△) was also detected; it lacked the sulfonic acid group, as indicated by its lack of mobility at pH 2.5.

It was supposed that this third metabolite might have arisen from a nonsulfonic acid impurity in the sample of phenol red. Careful examination of electrophoregrams of the particular sample of phenol red did, in fact, reveal the presence of an impurity (Fig. 16) that lacked the sulfonic acid function. When a pure sample of phenol red, in which this substance was undetectable, was administered to rats, the third metabolite was not excreted, confirming the supposition that it arose from the impurity.

SUMMARY

Characterization of ionizable substances by means of their mobility–pH profile was applied to organic substances by Franc and Kovar (25), who calculated mobilities in various buffers relative to the mobility in a standard buffer and plotted what they called an electrophoretic spectrum. Others (27–32) have also referred mobilities to standard substances including *p*-nitrobenzenesulfonic acid and have corrected for electroosmotic flow using neutral substances including caffeine. However, previous workers have not prepared buffers to constant ionic strength.

Control of ionic strength, combined with the use of *p*-nitrobenzenesulfonic acid as a reference substance and caffeine to correct for electroosmotic flow, forms the basis for obtaining reproducible relative mobilities over a wide pH range. The agreement of the mobility–pH profile thus measured with that predicted from elementary theoretical considerations is adequate to permit practical use of the technique for elucidating some structural features of simple organic molecules. Use of the method has been illustrated by applying it to the characterization of the metabolites of several compounds.

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Abstract □ A simple and convenient radioimmunoassay system was developed for the qualitative detection and quantitative determination of lysergide (LSD) in illicit drugs, human plasma, serum, or urine. The method utilizes a commercially available tritiated lysergide and specific antiserum, which can be obtained in large quantities from sheep after immunization with a lysergic acid–human serum albumin conjugate. Quantities of lysergide as low as 1 ng./ml. can be determined in plasma, serum, or urine, and lower levels can be detected qualitatively. The sensitivity of the assay is limited by the specific activity of the commercially available tritiated compound. Antibody cross-reactivity and, thus, interference in the assay were observed with other highly similar ergot alkaloids, such as ergonovine, methylergonovine, and ergotamine, but not with simpler indole structures.

Keyphrases □ Lysergide—radioimmunoassay of illicit drugs and biological fluids □ LSD—radioimmunoassay of illicit drugs and biological fluids □ Radioimmunoassay—lysergide (LSD) in illicit drugs and biological fluids □ Hallucinogens—radioimmunoassay of lysergide (LSD) in illicit drugs and biological fluids

The qualitative detection and quantitative determination of lysergide (LSD) in illicit drugs and in biological fluids have been a major interest in recent years because of the medical and social implications attendant its use. Chemical, spectral, and chromatographic methods for the determination of lysergide (IV*b*, Scheme I) were reviewed previously (1, 2), and it has been generally agreed that certain fundamental advances in microanalysis are required to detect and determine with confidence the nanogram or picogram levels, for example, of IV*b* or metabolites fleetingly present in urine or plasma after ingestion of the drug (1). A radioimmunoassay system (3) for IV*b* appears promising in this regard and was reported by one research group (4). This method (4) utilized rabbit antiserum to a lysergic acid–polylysine–hemocyanin conjugate, along with a specially synthesized lysergic acid–amino acid copolymer containing ^{125}I, and a double-antibody technique for the separation of free and bound IV*b* in the assay. Although details have not yet been reported, a commercial kit appears to be based upon similar methodology[1].

This paper reports the development and evaluation of a workable radioimmunoassay system for IV*b*. The system promises to be useful in the assay of illicit drug products or samples of biological fluids. The assay utilizes a commercially available tritiated IV*b* and specific antiserum which was obtained in large amounts from sheep after immunization with a direct covalent conjugate of lysergic acid (I) and human serum albumin (II). Separation of free and bound forms of IV*b* is achieved by the adsorption of unbound drug on dex-

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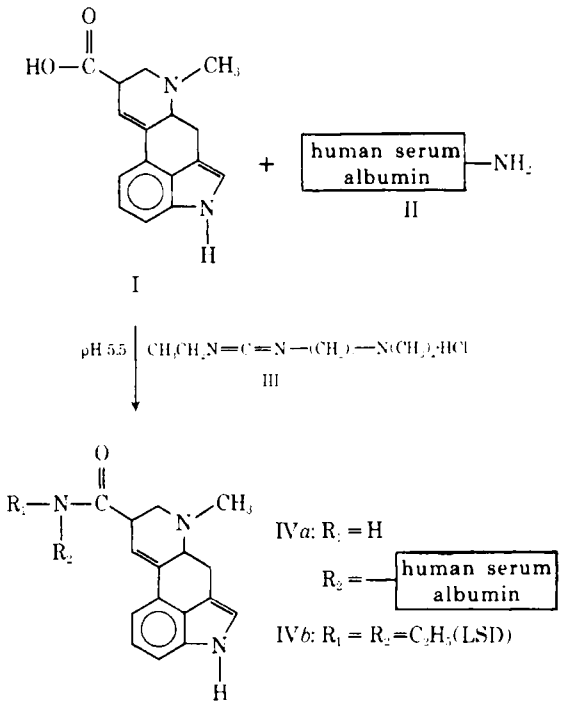

Scheme I—Covalent coupling of lysergic acid (I) to human serum albumin (II) by a water-soluble carbodiimide reagent (III), producing antigen (IVa)

tran-coated charcoal. To date, a sensitivity in the quantitative sense of about 1 ng. has been achieved using the commercially available tritiated IV*b*. Qualitatively, picogram levels may be readily detected.

EXPERIMENTAL

Materials—The following were used: human serum albumin[2] (crystalline, B grade), a coupling reagent *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride[3], Freund's complete adjuvant[4], lysergide[5] (0.1 mg./ml. in ethanol), dextran[6] (radioimmunoassay grade), charcoal[6] (radioimmunoassay grade), 5-hydroxytryptamine (serotonin) creatinine sulfate complex[7], 5-hydroxy-L-tryptophan[7], tryptamine hydrochloride[7], *N*-methyltryptamine[7], 5-methoxy-*N*,*N*-dimethyltryptamine[7], octoxynol[8], 2,5-diphenyloxazole[9], and 2,2-phenylenebis(5-phenyloxazole)[9].

The D-lysergic acid[7] (I) employed in the conjugation to albumin was found to be homogeneous by TLC [silica gel, R_f 0.5, butanol–acetic acid–water (3:1:1)]. The radiolabeled lysergide[10] was generally labeled with tritium and had a specific activity of 1.9 c./mmole. General labeling refers to a random, *i.e.*, nonuniform and undetermined, distribution of tritium at various nonexchangeable positions.

Coupling of Lysergic Acid to Human Serum Albumin—To a solution of 24 mg. of lysergic acid (I) in 20.0 ml. of water containing 10% by volume ethanol was added 3.0 ml. of water containing 30 mg. human serum albumin (II) (Scheme I). To this was added in one portion a solution of 34 mg. *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (III) in 2.0 ml. water. The pH of the solution was adjusted from 6.01 to 5.50 with 1 *N* HCl, and the mixture was stirred magnetically in a dark, stoppered vial at 25° for 42 hr. The reaction mixture was then placed in a dialysis bag and exhaustively dialyzed at about 5° for 1 week against distilled water, 4 *M* KCl, a buffer of pH 7.4 (0.15 *M* NaCl and 0.01 *M* phosphate), and finally water. All dialysates were measured in volume and absorbances were measured at 308 nm. (pH 7.4 buffer), the λ_{max} for lysergic acid. From absorbance measurements, it was calculated that about 5–6 mg. of lysergic acid was coupled to 30 mg. of human serum albumin. This corresponds to a content of about 40–50 moles of lysergic acid/mole of albumin in the conjugate. This value may be subject to a rather large error due to the rather low solubility of lysergic acid in the dialysate mixtures, with the possibility of precipitation, although this could not be visually observed. After dialysis, the contents of the bag were lyophilized to a fluffy off-white powder (IV*a*).

Preparation of Lysergic Acid–Human Serum Albumin Conjugate for Injection into Sheep—A solution of 4.0 mg. conjugate in 0.5 ml. pH 7.4 buffer (0.15 *M* NaCl and 0.01 *M* phosphate) was absorbed slowly into 0.8 g. partially dehydrated 7% polyacrylamide gel, which had been shredded by passing through a No. 25-gauge hypodermic needle from a syringe. The final product was desiccated to approximately 0.8 g. and placed in a 5-ml. disposable plastic syringe. Then Freund's complete adjuvant was drawn in through the needle to a volume of 4.0 ml. Immediately after vigorous shaking to resuspend the shredded gel, the mixture was injected into a sheep, using approximately 1 ml. at each of four intramuscular injection sites in both shoulders and flanks. A second sheep was injected similarly with a thoroughly emulsified mixture of 4.0 mg. of the conjugate in 1.8 ml. buffer and 2.2 ml. Freund's complete adjuvant.

Bleeding and Booster Immunization Schedule—Blood samples were collected from each animal initially, immediately prior to each booster immunization, and approximately 7–10 days after the booster. Boosters were composed of 1.0 mg. of conjugate in 4.0 ml. adjuvant–gel or emulsified adjuvant buffer and were given at 4, 6, 10, and 36 weeks after the initial injection of conjugate. Clotted blood was centrifuged after clot retraction, and the serum was decanted and kept frozen (−10°) after the addition of 0.05% sodium azide.

Radioimmunoassay Procedure: Determination of Standard Radioimmunoassay Curve for IV*b*—All samples each day were run in duplicate to obtain two consistent figures. In a typical experiment, 0.8 ml. pH 7.4 buffer (0.15 *M* NaCl and 0.01 *M* sodium phosphate) was added to each of 22 5-ml. polystyrene test tubes (12 × 75 mm.): that is, 11 sets of duplicates were run. Then 0.2 ml. buffer (or buffer containing test compound) or plasma, serum, or urine was added to make a final volume of 1.0 ml. To tubes containing plasma, 50 μl. of 0.2 *N* sodium citrate solution was also added. Next was added 10 μl. of 95% ethanol containing 1.0 ng. of randomly (nonuniformly) labeled ^{3}H-IV*b* (specific activity 1.9 c./mmole). This corresponds to about 13,000 d.p.m./tube or about 3000–4000 c.p.m. at the normal observed counting efficiency of 25–30%.

To obtain a standard calibration curve, 0–25-ng. quantities of cold IV*b* in a maximum of 100 μl. of 95% ethanol were added to selected tubes. Again to desired tubes was added 10 μl. of diluted or undiluted antiserum. After vigorous stirring for 5 sec. on a mixer (Vortex), the tubes were allowed to stand at room temperature for exactly 2 hr. After chilling to 0° for 15 min. in ice, 0.5 ml. of cold and well-mixed dextran-coated charcoal suspension[11] was added to each tube and the tube contents were mixed thoroughly once again. The tubes were then incubated for 15 min. at 0° and centrifuged in the cold (4°) for exactly 5 min. (5000 r.p.m.). Then the supernate was decanted carefully into 10 ml. of scintillation counting solution containing toluene (2 l.), octoxynol[8] (1 l.), 2,5-diphenyloxazole (12 g.), and 2,2-phenylenebis(5-phenyloxazole) (300 mg.). The samples were then counted[12].

Typical data on the composition of incubation mixtures, observed counts for a 10-min. period, and percent bound IV*b* are summarized in Table I. The channels ratio method was employed to determine counting efficiency, disintegrations per minute, and final percent binding figures. Figure 2 shows typical standard immunoassay curves obtained by plotting data of Table I or similar data. Aside from counting time, the entire procedure can be completed in about 4 hr. and involves only about 2 hr. of actual working time.

[2] Calbiochem Corp.
[3] Aldrich Chemical Co.
[4] Difco Labs.
[5] Sandoz Corp., obtained from Dr. M. Braude, Center for the Studies of Narcotics and Drug Abuse, National Institute of Mental Health.
[6] Schwarz/Mann.
[7] Sigma Chemical Co.
[8] Triton X-100, Fisher Chemical.
[9] Fisher Chemical.
[10] New England Nuclear Corp.
[11] The charcoal suspension was prepared immediately prior to use by the dilution of 2 ml. of a stock suspension with 18 ml. buffer, the stock suspension containing 2.5 g. charcoal and 62.5 mg. dextran in 25 ml. buffer.
[12] Packard model 3320 scintillation spectrometer.

Table I—Data for Typical Determinations of Standard Lysergide Radioimmunoassay Curves at Two Antiserum Dilutions[a]

Tube Number	Lysergide (IV*b*) Standard, ng.	Net Activity (counts/10 min., Corrected for Quenching and Background)	Percent Bound Lysergide (IV*b*)
	Final Dilution of Antiserum = 1:100		
1	0	1,260	3.8
2	0	28,780; 28,730	86.8; 86.8
3	1.0	27,558; 28,181	84.2; 83.8
4	2.0	26,434; 25,088	79.5; 72.5
5	3.0	22,029; 23,982	65.9; 69.4
6	5.0	19,032; 19,763	54.8; 58.1
7	7.0	16,419	45.7
8	10.0	12,921; 12,718	38.1; 35.8
9	15.0	10,409; 10,524	30.2; 30.0
10	25.0	6,689; 6,161	21.8; 18.2
11	0	39,763; 38,225	100.0; 99.3
	Final Dilution of Antiserum = 1:400		
1	0	1,123; 1,150	3.7; 3.7
2	0	23,990; 24,370	67.8; 67.5
3	1.0	18,747; 18,427	53.5; 53.2
4	2.0	12,580; 12,110	36.2; 34.4
5	3.0	9,723; 9,403	27.8; 25.8
6	4.0	7,711; 7,799	22.8; 22.4
7	5.0	7,153; 6,023	19.6; 19.8
8	6.0	5,956; 5,973	17.0; 16.0
9	8.0	4,744; 4,783	13.8; 13.4
10	10.0	4,142	11.3
11	0	35,219; 35,468	100.0; 97.2

[a] The data are for samples containing pooled normal human serum (see text). Samples designated 1 contained no antiserum. All samples contained 0.8 ml. buffer, 0.2 ml. serum, and 10 μl. ethanol containing 1.0 ng. ^{3}H-IV*b*. Duplicate samples were assayed as indicated by the two figures for counts of radioactivity. The counting channel data only are shown. No dextran-coated charcoal was added to Tube 11; this, therefore, represents a standard of total counts added.

RESULTS AND DISCUSSION

As shown in Scheme I and as outlined in detail in the *Experimental* section, lysergic acid (I) was covalently coupled directly to human serum albumin (II) by the water-soluble carbodiimide derivative *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (III). The conjugate (IV*a*), which presumably contains lysergic acid residues coupled primarily to side-chain amino groups of lysine, thus contains all of the structural elements of IV*b* except for the two amide ethyl groupings. Calculations based upon the UV absorption maximum at 308 nm. for lysergic acid indicated binding of 40–50 moles of lysergic acid/mole of human serum albumin. Since the lysine content of this protein is about 58 residues/mole, this figure seems rather high. An abnormally high value may result from the rather low solubility of lysergic acid in aqueous buffers (filtrates from the coupling reaction), since the quantity coupled was calculated by difference.

Injection of the purified conjugate (4 mg.) in polyacrylamide gel and adjuvant at each of four injection sites in one sheep, followed by a booster injection with 1 mg. of conjugate after 4 weeks, resulted in an antiserum even after only 6 weeks which was capable of binding 35–45% of 1 ng. of radioactive IV*b* in 1 ml. of incubation mixture at a final dilution of 1:1000 and 75–85% at dilutions of 1:100. Figures 1 and 2 show percentage binding figures at various dilutions for serum obtained after the first booster immunization (6 weeks total). The binding capacity of antiserum taken from this sheep after the third and fourth boosters at 10 and 36 weeks appeared to be only slightly higher than that obtained at the 6-week period. The binding capacities of antiserum obtained from the second sheep (injected with antigen in adjuvant only, with no polyacrylamide gel) were slightly lower than those of the first sheep but were equally adequate for use in assays at the same dilutions (Figs. 1 and 2). The antiserums appear to have completely retained binding capacity after 18 months storage at −10°. Three typical standard calibration curves are reproduced in Fig. 2. Displacement of the labeled IV*b* (1.0 ng./ml.) by nonradioactive IV*b* from antiserum of final dilution 1:100 began at a concentration of about 1 ng./ml. and dropped off most dramatically between 2 and about 30 ng./ml. Final serum dilutions of 1:400 resulted in a reproducible standard

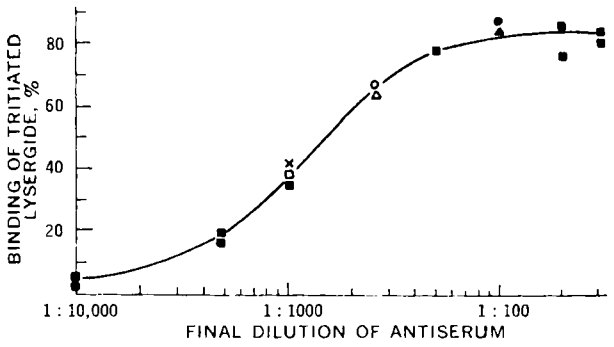

Figure 1—*Binding of tritiated lysergide (1.0 ng./ml.) by antiserum to a lysergic acid–human serum albumin conjugate. Key: ●▲○△□×, data as given in Table I and Fig. 2; and ■, additional experimental results.*

curve between 1 and 10 ng./ml. If conditions of the assay were adhered to rigidly, the standard curve varied only very slightly; however, a series of standards was always incorporated into each day's work.

The addition of up to 20% (0.2 ml./ml.) normal sheep serum, pooled normal human serum or normal human plasma, or human urine to the assay tubes did not appear to affect the shape or position of the immunoassay curves significantly. Blanks in the absence of antiserum amounted to 3–5% in all cases, and counts corresponding to this amount would have to be subtracted from unknowns. However, in all tubes containing human plasma, 50 μl. of 0.2 *N* sodium citrate was added to prevent clotting, which appeared to be triggered by the addition of the sheep antiserum. Observed binding appeared to be independent of time between 0.5 and 3 hr.; however, 2.0 hr. was employed routinely. Temperature and incubation time proved to be quite critical during adsorption of the free IV*b* to dextran-coated charcoal, and a 15-min. incubation time at 0° followed by 5 min. of centrifugation at 4° was optimum.

When using the assay procedure as described, the practical lower limit for quantitative determination of IV*b* appears to be about 1 ng. This limit is determined by the relatively low specific activity (1.9 c./mmole) of the labeled IV*b* available commercially; material of higher specific activity is too unstable for storage[13]. The use of smaller amounts of labeled IV*b* enabled detection and rough estimation of much smaller quantities of IV*b*, but the uncertainty arising from the decreased count measured increased dramatically.

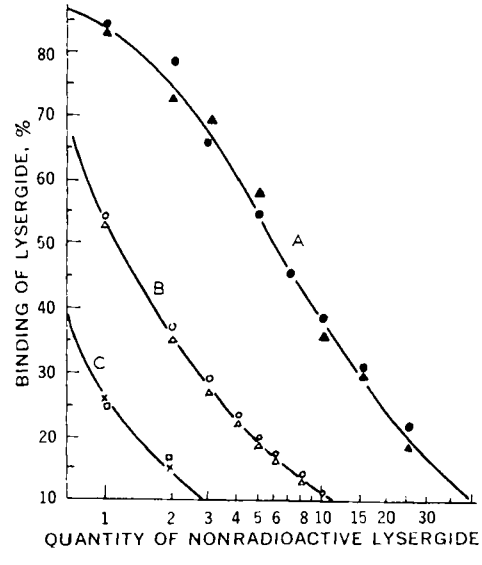

Figure 2—*Typical standard radioimmunoassay curves for the determination of lysergide at three antiserum dilutions (A, 1:100; B, 1:400; and C, 1:1000). Key: ●▲, ○△, and ×□ indicate duplicate assay results. The curves shown have not been mathematically fit.*

[13] A. Petty, New England Nuclear Corp., personal communication.

Several compounds structurally related to IV*b* were examined for their ability to cross-react with the antiserum obtained, *i.e.*, their ability to displace radiolabeled IV*b* from antibody binding sites. Antibodies were unable to distinguish among IV*b*, I, and the structurally similar ergot alkaloids ergonovine and methylergonovine. Ergotamine also exhibited cross-reactivity, but at approximately 10-fold higher concentrations. The following indoles did not exhibit any ability to displace radiolabeled IV*b* from antibody binding sites at concentrations of at least 10 mcg./ml. of incubation mixture: tryptamine, *N*-methyltryptamine, 5-methoxydimethyltryptamine, 5-hydroxytryptophan, and 5-hydroxytryptamine (serotonin). No exhaustive study of cross-reactivity was completed; however, these results compare in general with those reported by Vunakis *et al.* (4). Judging from information supplied in a commercial kit[14], antiserum produced by other methods may exhibit somewhat greater specificity. One outstanding example appears to be that these authors report little cross-reactivity with I at concentration levels similar to IV*b*, while these two compounds appear essentially indistinguishable using our antiserum.

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A quick and convenient radioimmunoassay system was developed for the quantitative determination of lysergide (IV*b*) in drug products, human plasma, serum, or urine at levels as low as 1 ng. or for its qualitative detection at picogram levels. Liter quantities of antiserum were obtained since sheep were employed for the production of antibodies. An advantage of the method is in its utilization of commercially available tritiated IV*b*; however, the low specific activity of this material also places a limit upon the sensitivity. The method also has the advantage of not requiring a second antibody technique for the separation of free and bound IV*b* but is based upon adsorption of free IV*b* to dextran-coated charcoal. Current efforts are directed toward the acquisition of IV*b* of higher specific activity and the development of methods utilizing immobilized antibodies for efficiently concentrating IV*b* in biological samples so that the quantitative assay may be extended to the picogram levels required in the study of the absorption, distribution, metabolism, and excretion of IV*b*. However, even in its current state the method promises to be of considerable utility in the assay of illicit drug samples and in animal investigations where larger quantities of lysergide IV*b* are employed.

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Identification of a Blood Metabolite of Methsuximide by GLC–Mass Spectrometry

I. A. MUNI▲, C. H. ALTSHULER, and J. C. NEICHERIL

Abstract □ The chemical structure of a prominent methsuximide metabolite was determined by combined GLC mass spectrometry. The experimental data indicate that the metabolite is an *N*-demethylated methsuximide. A simple GLC method is described for simultaneous estimation of methsuximide and its metabolite in blood samples.

Keyphrases □ Methsuximide—identification of *N*-demethylated metabolite in blood, man and rabbits, GLC–mass spectroscopy □ GLC–mass spectroscopy—identification, methsuximide *N*-demethylated metabolite in blood □ GLC—analysis, methsuximide and *N*-demethylated metabolite in blood

An important aspect in the treatment of patients with epilepsy is the determination of an effective and safe dosage schedule of anticonvulsant drugs. The estimation of these drugs in physiological fluids, particularly in blood, provides useful metabolic information for practitioners. Many drugs used are actively metabolized *in vivo* (1–3), and the identification and quantitation of these metabolites are of considerable clinical importance.

A general procedure for the determination of anticonvulsants in blood was recently described (4). When the method was applied to patients who had been receiving methsuximide (*N*,2-dimethyl-2-phenylsuccinimide), it became apparent that the drug had been rapidly metabolized both in humans and in rabbits and that the major metabolite had GLC characteristics different than the parent drug.

The purpose of this investigation was to determine the chemical structure of the methsuximide metabolite. The GLC properties of both the human and animal

Several compounds structurally related to IV*b* were examined for their ability to cross-react with the antiserum obtained, *i.e.*, their ability to displace radiolabeled IV*b* from antibody binding sites. Antibodies were unable to distinguish among IV*b*, I, and the structurally similar ergot alkaloids ergonovine and methylergonovine. Ergotamine also exhibited cross-reactivity, but at approximately 10-fold higher concentrations. The following indoles did not exhibit any ability to displace radiolabeled IV*b* from antibody binding sites at concentrations of at least 10 mcg./ml. of incubation mixture: tryptamine, *N*-methyltryptamine, 5-methoxydimethyltryptamine, 5-hydroxytryptophan, and 5-hydroxytryptamine (serotonin). No exhaustive study of cross-reactivity was completed; however, these results compare in general with those reported by Vunakis *et al.* (4). Judging from information supplied in a commercial kit[14], antiserum produced by other methods may exhibit somewhat greater specificity. One outstanding example appears to be that these authors report little cross-reactivity with I at concentration levels similar to IV*b*, while these two compounds appear essentially indistinguishable using our antiserum.

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The purpose of this investigation was to determine the chemical structure of the methsuximide metabolite. The GLC properties of both the human and animal

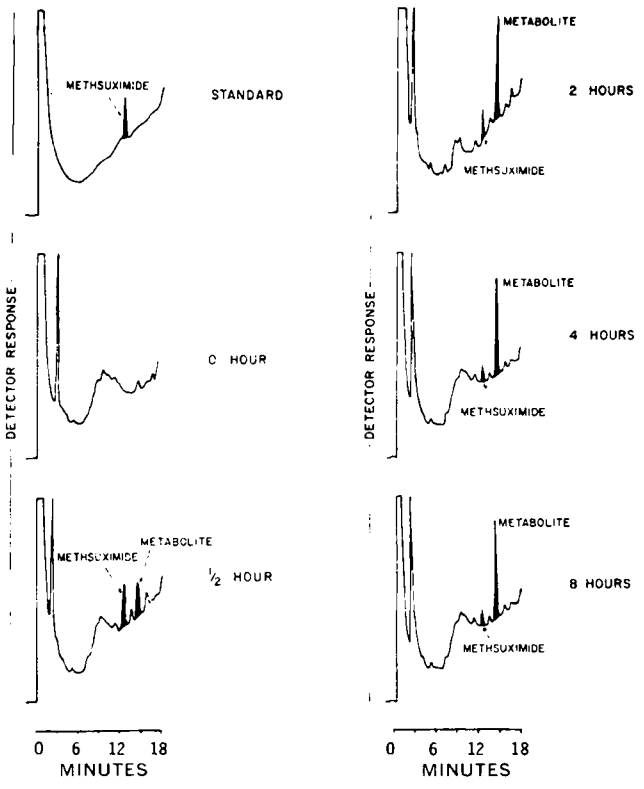


Figure 1—*Schematic diagram of methsuximide metabolism in rabbits (flame-ionization detection, 10% OV-17, 165–280°, 7.5°/min.).*

metabolite of methsuximide are similar. The present report describes its chemical characterization when separated from rabbit blood by GLC–mass spectrometry. A simple and rapid GLC method to determine the blood levels of methsuximide is also presented.

EXPERIMENTAL

Chemicals—Methsuximide[1], m.p. 50°, and methsuximide capsules[2] (300 mg.) were used. The solvents used were of analytical grade. *N,O*-Bis(trimethylsilyl)acetamide[3] was used for on-column trimethylsilylation.

Apparatus—An analytical gas chromatograph[4], equipped with a dual flame-ionization detector system, was used to monitor drug levels in blood samples. The separation was carried out on a 1.82-m. (6-ft.) long, U-shaped glass column packed with 10% OV-17 on Gas Chrom Q[5], 80–100 mesh.

The GLC–mass spectrometry[6] system interfaced to a data system[7] was used in the characterization of the methsuximide metabolite. A 0.61-m. (2-ft.) long, U-shaped glass column packed with 2% SE-30 on Gas Chrom Q[5], 80–100 mesh, was used for the separation of the metabolite. Monolinear temperature programming was used in both systems. The electron ionization energy was 70 ev. The ionization source temperature was maintained at 250°.

Procedure—*Drug Administration*—Several male rabbits, weighing 3.4–4.5 kg., were used. One methsuximide capsule (300 mg.) was administered orally to each animal, and the blood samples (about 4 ml.) were collected at intervals of 0.0, 0.5, 2.0, 4.0, and 8.0 hr.

Plasma Extraction—Two milliliters of plasma sample was acidified with 6 *N* HCl (50 μl.), and the drug as well as the metabolite was extracted with a mixture of 8 ml. of chloroform–isopropanol (9:1 v/v). The organic phase was filtered through phase-separating paper[8], and a 6-ml. sample of filtrate was concentrated to dryness at 35° under a gentle stream of nitrogen.

GLC Analysis—The dried samples were resuspended in 50 μl. of chloroform–isopropanol (1:1 v/v), and 2–5 μl. was injected into a gas chromatograph. Working standard curves of methsuximide were routinely established for drug quantitations.

GLC–Mass Spectrometry Studies—A chloroform–isopropanol extract (2 μl.) of the 4-hr. plasma sample was injected into a gas

Table I—Typical Recoveries of Methsuximide

Sample	Methsuximide Added, mcg.[a]	Methsuximide Found, mcg.[b]	Recovery, %
Human plasma	30	31	103
Human plasma	45	43	96
Human plasma	60	58	97
Human plasma	300	303	101
Rabbit plasma	30	29	97
Rabbit plasma	45	43	96
Rabbit plasma	60	61	102
Rabbit plasma	300	299	100

[a] Amounts of methsuximide in methanol (3 mg./ml.) added to 4 ml. of plasma sample. [b] Calculated to nearest whole number.

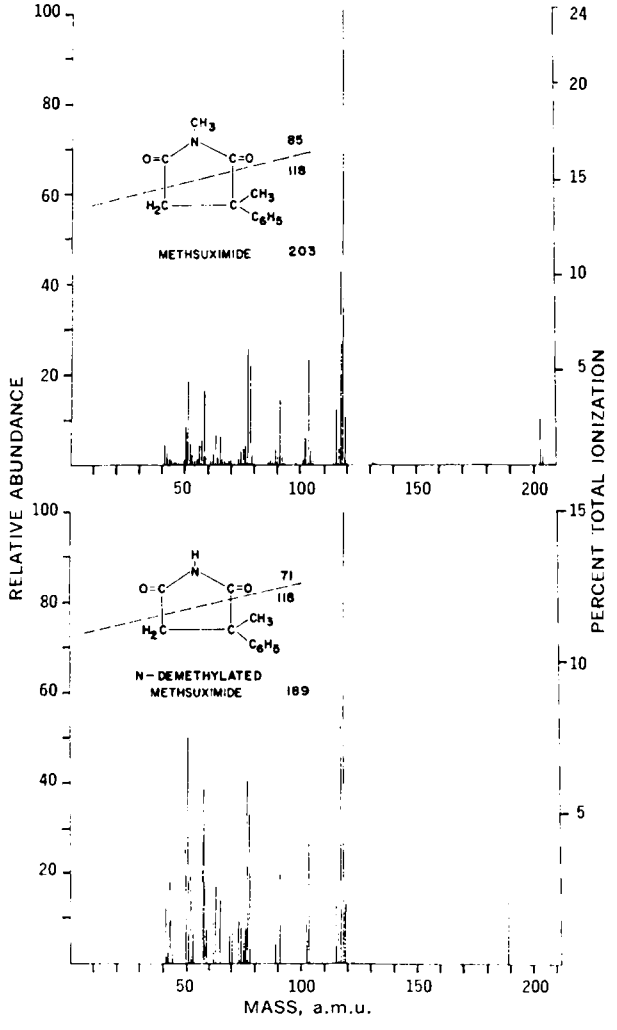
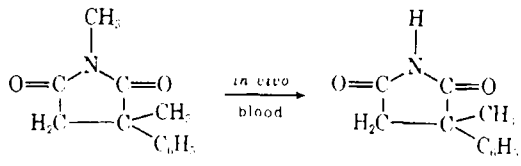


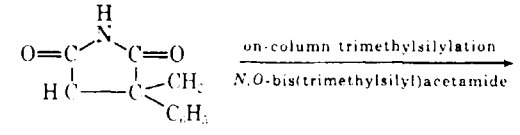
Figure 2—*Mass spectral fragmentation patterns of methsuximide and its metabolite.*

[1] Celontin, gift of Parke, Davis & Co., Detroit, Mich.
[2] Obtained from St. Joseph's Hospital pharmacy.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Barber-Colman model 5000.
[5] Applied Science Laboratories, State College, Pa.
[6] Hewlett-Packard gas chromatograph model 5780A and Hewlett-Packard mass spectrometer model 5930A.
[7] Hewlett-Packard data system model 5932A.
[8] Whatman.

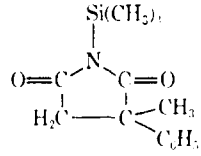


methsuximide (mol. wt. 203) *N*-demethylated methsuximide (mol. wt. 189)

Scheme I—Methsuximide metabolism



N-demethylated methsuximide (mol. wt. 189)



trimethylsilyl-*N*-demethylated methsuximide (mol. wt. 261)

Scheme II Trimethylsilylation of methsuximide metabolite

chromatograph, and the underivatized compounds were subjected to GLC–mass spectrometry. On-column trimethylsilylation was achieved by injecting a mixture of 2 μl. of *N,O*-bis(trimethylsilyl)-acetamide and 2 μl. of concentrated blood extract. A chloroform–isopropanol solution containing standard methsuximide was also subjected to GLC–mass spectrometry analysis.

RESULTS AND DISCUSSION

The relative blood levels of methsuximide and its metabolite at 0.5, 2.0, 4.0, and 8.0 hr. after oral administration of 300 mg. of methsuximide are illustrated in Fig. 1. Methsuximide metabolizes rapidly *in vivo* to a major metabolite, and after 0.5 hr. the concentration of this metabolite in blood is relatively much higher than that of unchanged methsuximide.

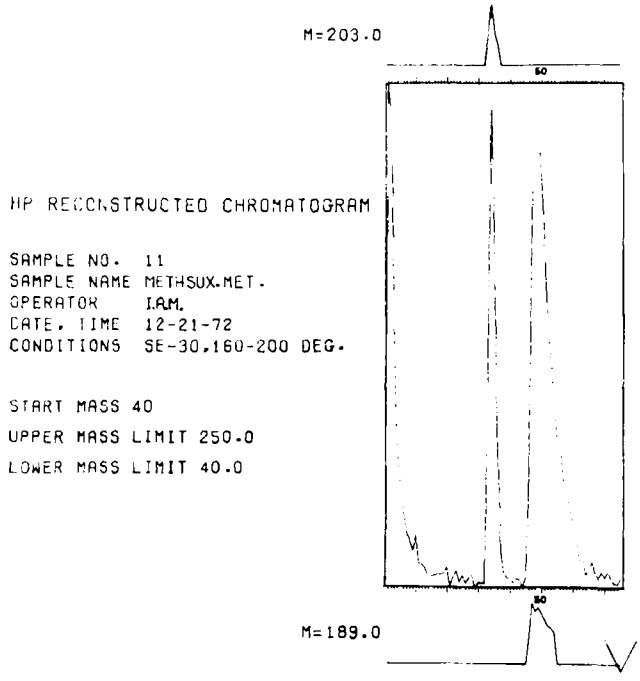


Figure 3—*Reconstructed chromatogram of plasma extract. M = 203 is a chromatographic peak for methsuximide, and M = 189 is a chromatographic peak for* N*-demethylated methsuximide.*

Table II—Relative Abundance of Several Fragment Ions of Methsuximide and Its Metabolite

Fragment Ion (*m/e*)	Relative Abundance, % Methsuximide	Relative Abundance, % Metabolite
203	10.22	– [a]
189	–	14.56
119	10.88	13.88
118	100.00	100.00
117	42.56	52.75
115	12.37	12.99
103	23.40	29.52
91	14.55	20.08
78	21.98	33.46
77	25.83	40.98
63	–	17.32
58	16.29	38.97
57	–	27.56
51	18.61	50.00
50	—	25.59
43	–	18.11
41	—	16.53

[a] Either no fragment ion was observed or the relative abundance (%) was less than 10%.

As shown in Fig. 1, methsuximide and its metabolite were successfully resolved on the OV-17 column; each compound produced a single GLC peak. Similar resolution was obtained using the SE-30 liquid phase. The flame-ionization detector gave a linear response for methsuximide, as well as its metabolite, under the experimental conditions used.

When added to plasma samples, the recoveries of methsuximide for both human and rabbit plasma were usually in the 96–103% range (Table I). Recoveries were comparable whether extractions were made immediately after adding methsuximide or after storing the plasma samples for 24–40 hr. at 5°.

As shown in Fig. 2, the mass spectra of methsuximide and its metabolite are simple, characteristic, and easy to interpret. The spectra were recorded from 0 to 650 a.m.u. In both spectra, the base peak (B) was observed at *m/e* 118 (Table II). Methsuximide and its metabolite were characterized by prominent clusters of fragment ions in the *m/e* 115–119, 73–78, and 50–58 regions. Methsuximide also showed significant peaks at *m/e* 203 (molecular ion, M), 103, and 91. The mass spectra of standard methsuximide and methsuximide extracted from blood samples were identical.

On-column trimethylsilylation resulted in a GLC peak shift for the methsuximide metabolite. The trimethylsilylated metabolite of methsuximide eluted earlier than the underivatized metabolite. On-column trimethylsilylation did not change the GLC retention time or the mass spectral characteristics of methsuximide. The mass spectrum of trimethylsilyl methsuximide metabolite showed fragment ions at *m/e* 261 (M), 246 (M − 15). 147 $[(CH_3)_2Si{=}O\ Si(CH_3)_3]$, 118, and 73 $[(CH_3)_3Si]$.

The GLC–mass spectrometry data indicate that the predominant blood metabolite of methsuximide is an *N*-demethylated methsuximide (Schemes I and II). *N*-Demethylation is the usual route of metabolism for several *N*-methylated anticonvulsant drugs (5, 6). Although demethylation ordinarily requires many hours for completion (5), metabolism of methsuximide seems to be relatively rapid in humans and animals.

Figure 3 is an illustration of the *XY* plot from the data system. The central portion of the figure is the total ion monitor output for all products with fragment ions between 40 and 250. The upper figure indicates that the first peak (scan numbers 30–40) had a fragment ion of 203, and the lower figure indicates that the second peak (scan numbers 48–58) had a fragment ion of 189.

SUMMARY

A relatively simple, rapid, and accurate GLC system is described. It was found to be useful in the monitoring of the physiological levels of methsuximide and its metabolite in one operation. The GLC–mass spectrometry data indicate that methsuximide is extensively metabolized *via N*-demethylation.

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Electronic Spectra and Electronic Structures of Aminoanthracenes

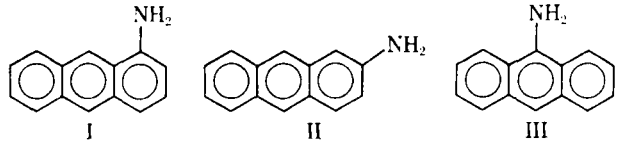
STEPHEN G. SCHULMAN▲, PETER J. KOVI, GEORGE TOROSIAN, HOWARD McVEIGH, and DORIS CARTER

Abstract □ The electronic absorption and fluorescence spectra of the cation derived from 9-aminoanthracene (9-anthrylamine), in water, are anomalous by comparison with those of the 1- and 2-anthrylammonium ions. The similarities of the spectra of the cation of 9-anthrylamine with those of anthrone and its cation lead to the conclusion that the former cation is not the 9-anthrylammonium ion but rather its tautomer, the protonated imine analogous to anthrone. In ethanol and in the solid state, measurable quantities of both the protonated imine and 9-anthrylammonium ions exist.

Keyphrases □ Aminoanthracenes—electronic spectra and electronic structures □ Absorption spectra, electronic, aminoanthracenes—determination □ Fluorescence spectra, electronic aminoanthracenes—determination □ Electronic spectra and electronic structures—determination, aminoanthracenes □ Anthrylamines—electronic spectra and electronic structures

The carcinogenicity of many polycyclic aromatic amines has been recognized since 2-naphthylamine was found to cause bladder cancers in workers in the dye industry (1, 2). Of the arylamines known to have carcinogenic properties, 1-aminoanthracene (I) and, especially, 2-aminoanthracene (2-anthrylamine) (II) are among the most virulent. However, to our knowledge, no carcinogenic properties have been reported for 9-aminoanthracene (III).

Of the functionally substituted aromatic molecules, the derivatives of anthracene have not been particularly well characterized, although many of them have been employed in the dye industry for many years. Recent studies have shown that, in particular, substitution in the 9-position of the anthracene ring often results in molecular species that are quite different in their chemical properties from other aromatic molecules (*e.g.*, benzene and naphthalene) substituted with the same functional groups. On the other hand, substitution in the 1- and 2-positions of the anthracene ring yields derivatives that are similar to the corresponding benzene and naphthalene derivatives. For example, in hydrocarbon solvents, the carboxyl groups of benzoic acid, 1- and 2-naphthoic acids, and 1- and 2-anthroic acids are coplanar and conjugated with the respective aromatic rings, while the carboxyl group of 9-anthroic acid (3) is perpendicular and unconjugated with the anthracene ring as a result of steric interference of the 9-carboxyl group with the peri hydrogen atoms in the 1- and 8-positions of the anthracene ring. Moreover, of the hydroxyanthracenes, 1- and 2-anthrols appear to be well-behaved phenolic molecules (4) while 9-anthrol exists predominately as its keto tautomer, anthrone, and only to a very slight extent as the phenolic 9-anthrol (5, 6).



I II III

Because of interests in the relationships between molecular electronic structure and biological (in this case carcinogenic) activity, the present investigation of the electronic structures of the isomeric aminoanthracenes by electronic absorption and fluorescence spectroscopy was undertaken.

EXPERIMENTAL

Reagents—The 1- and 2-aminoanthracenes[1], anthracene, and 9-aminoanthracene hydrochloride[2] were each recrystallized several times from absolute ethanol.

Analytical reagent grade sulfuric acid[3] was diluted with distilled, deionized water to prepare the solutions used to study the Hammett acidity region. Solutions in the pH range were citrate and phosphate buffers and sodium hydroxide solutions in distilled, deionized water.

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Electronic Spectra and Electronic Structures of Aminoanthracenes

STEPHEN G. SCHULMAN▲, PETER J. KOVI, GEORGE TOROSIAN, HOWARD McVEIGH, and DORIS CARTER

Abstract □ The electronic absorption and fluorescence spectra of the cation derived from 9-aminoanthracene (9-anthrylamine), in water, are anomalous by comparison with those of the 1- and 2-anthrylammonium ions. The similarities of the spectra of the cation of 9-anthrylamine with those of anthrone and its cation lead to the conclusion that the former cation is not the 9-anthrylammonium ion but rather its tautomer, the protonated imine analogous to anthrone. In ethanol and in the solid state, measurable quantities of both the protonated imine and 9-anthrylammonium ions exist.

Keyphrases □ Aminoanthracenes—electronic spectra and electronic structures □ Absorption spectra, electronic, aminoanthracenes—determination □ Fluorescence spectra, electronic aminoanthracenes—determination □ Electronic spectra and electronic structures—determination, aminoanthracenes □ Anthrylamines—electronic spectra and electronic structures

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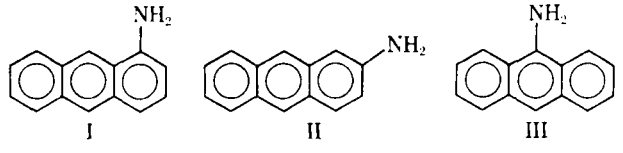

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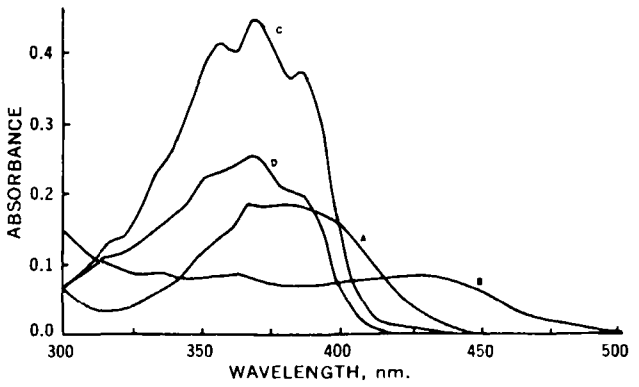


Figure 1—*Electronic absorption spectra of about 1 × 10⁻⁴ M 1-aminoanthracene (A) and 2-aminoanthracene (B) at pH 10.0 and of the respective anthrylammonium ions (C and D) at pH 1.0.*

the taking of spectra to minimize decomposition errors. The spectra of 1×10^{-5} *M* aqueous anthracene were taken in only one solution, pH 10.0.

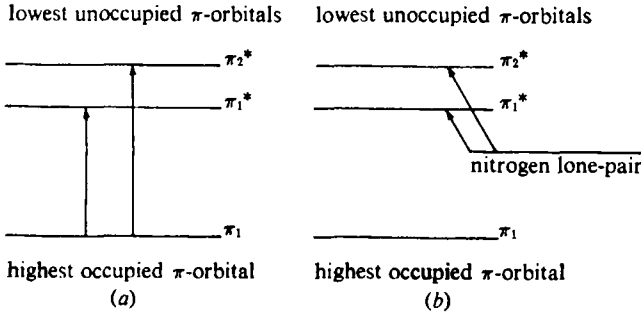
Apparatus—Absorption spectra were taken on a spectrophotometer[4]. Fluorescence spectra were taken on a fluorescence spectrophotometer[5] whose monochromators were calibrated against the xenon line emission spectrum and whose output was corrected for instrumental response by means of a rhodamine-B quantum counter. The pH measurements were made on a pH meter[6] employing a silver-silver chloride-glass combination electrode[7]. IR spectra[8] were taken in silicone fluid.

RESULTS AND DISCUSSION

1- and 2-Aminoanthracenes—The electronic absorption spectra of 1- and 2-aminoanthracenes and their conjugate cations are shown in Fig. 1. The maxima of these and the corresponding fluorescence spectra, along with those of 9-aminoanthracene, its conjugate cation, and anthracene, for comparison, are presented in Table I.

The absorption and fluorescence spectra of the cations of the 1- and 2-isomers are highly structured and similar to those of anthracene itself. The absorption spectra of the neutral 1- and 2-aminoanthracenes, however, each show a diffuse band at ~400 nm. and a partially overlapping vibrational structured band at shorter wavelengths. The fluorescence spectra of the neutral amines are unstructured and lie at wavelengths (~500 nm.) much longer than those of the fluorescence of anthracene (~400 nm.). These spectra are typical of anthracene derivatives and of amino- and ammonium-substituted aromatics and may be explained as follows.

The absorption spectrum of anthracene in the 300–500-nm. region is comprised of two electronic transitions, designated $^1L_a \leftarrow {}^1A$ and $^1L_b \leftarrow {}^1A$ in Platt's system of classification of electronic transitions (7). ^1A designates the ground singlet state, and ^1L_a and ^1L_b are the two lowest electronically excited singlet states. The ^1L_a and ^1L_b



Scheme I—Electronic transitions in anthracene and aminoanthracenes: (a) anthracene and anthrylammonium ions, and (b) anthrylamines

[4] Beckman DB-GT.
[5] Perkin-Elmer MPF-2A.
[6] Orion Model 801.
[7] Beckman.
[8] Beckman model 33 IR spectrophotometer.

Table I—Electronic Absorption (λ_a) (in the 300–500-nm. Region) and Fluorescence (λ_f) Maxima of the 1-, 2-, and 9-Aminoanthracenes and Their Conjugate Cations in Water[a]

	Cation (H_0 − 1.6)		Neutral Amine (pH 10.0)	
	λ_a	λ_f	λ_a	λ_f
1-Aminoanthracene	386	383	378(^1L_a)	530
	369	402	378	
	357	420	363 (^1L_b)	
	342	446	347	
	335		329	
	318			
2-Aminoanthracene	381	387	427(^1L_b)	503
	365	405		
	352	422	362	
	335		343 (^1L_a)	
	327		335	
	320		313	
9-Aminoanthracene	340(sh)	450	400(^1L_a)	519
	301		372	
			353 (^1L_b)	
	Neutral Molecule (pH 10.0)			
Anthracene	λ_a		λ_f	
	377		380	
	358		400	
	341		417	
	326		440	
	312			
	299			

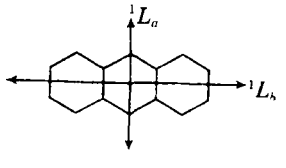
[a] The corresponding spectral features of anthracene in water (pH 10.0) are given for comparison. Spectral maxima are reported in nanometers.

states are derived from the promotion of an electron from the highest occupied π-orbital in the ground state to either of the two lowest unoccupied orbitals (π*) of the anthracene molecule (Scheme I).

In anthracene, the molecular skeleton is fairly rigid, restricting the vibrational freedom of the molecule. The vibrational subtransitions accompanying the electronic transitions occur with essentially no readjustment of the nuclear positions (vibrational motion) during electronic transition. This is a result of the greater rate of the average electronic transition (~10^{15} sec.$^{-1}$) compared with that of the average vibrational transition (~10^{14} sec.$^{-1}$) and represents a statement of the Franck–Condon principle (8, 9). As a result, the vibrational sublevels to which absorption takes place are well defined (quantized) and the vibrational-electronic (vibronic) fine structure of the absorption bands is thus distinct in anthracene. The $^1L_b \leftarrow {}^1A$ transition is polarized along the long axis and the $^1L_a \leftarrow {}^1A$ transition along the short axis of anthracene (Scheme II).

In smaller aromatic molecules (10) (*e.g.*, benzene and naphthalene), the $^1L_a \leftarrow {}^1A$ and $^1L_b \leftarrow {}^1A$ transitions are well resolved, with the $^1L_b \leftarrow {}^1A$ absorption lying at longer wavelengths. In higher aromatics (*e.g.*, tetracene and pentacene), the absorption bands representing the $^1L_a \leftarrow {}^1A$ transitions occur at longer wavelengths than $^1L_b \leftarrow {}^1A$ transitions (10). In anthracene, the absorption bands representing the two transitions overlap to such an extent that they are indistinguishable. However, it is generally believed that the long wavelength side of the $^1L_b \leftarrow {}^1A$ band lies at longer wavelengths than the $^1L_b \leftarrow {}^1A$ band in anthracene.

The introduction of an amino group into the anthracene ring affects the spectra in ways that depend upon the site of substitution in the ring. The amino group has a lone electron pair residing in an orbital on the nitrogen atom, with sp^3 hybrid characteristics. Although this orbital is not perpendicular to the anthracene ring, as are the carbon $2p_z$ orbitals comprising the π-system of the ring, it has a substantial component in that direction; therefore, some in-



Scheme II—Polarizations of the two lowest π → π transitions of anthracene*

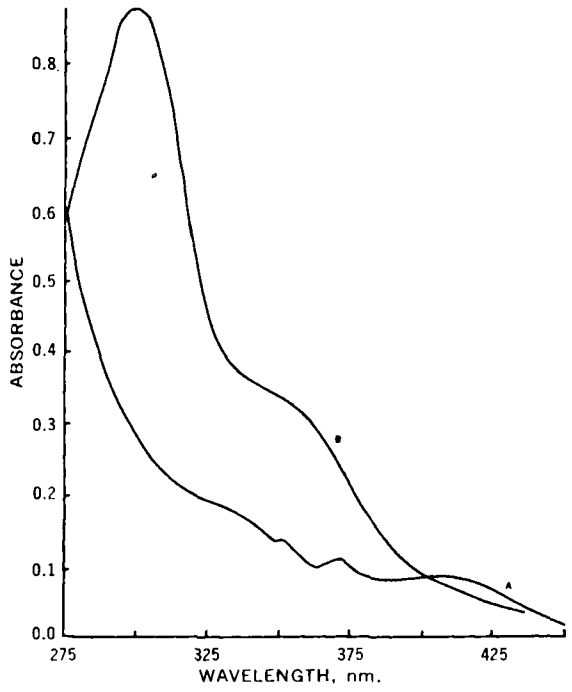


Figure 2—*Electronic absorption spectra of about 1 × 10⁻⁴ M 9-aminoanthracene (A) at pH 10.0 and of the protonated species derived from 9-aminoanthracene (B) at pH 1.0.*

teraction between the lone-pair and the π-system is to be expected even in the ground electronic state (conjugation or resonance interaction). Upon excitation, however, an electron may be transferred completely from the nitrogen lone-pair into a vacant π^*-orbital of the aromatic ring (intramolecular charge-transfer transition). This process results in greater conjugation of the amino group with the aromatic ring and produces rehybridization of the amino nitrogen atom to an sp^2-type configuration (coplanar with the ring) during the electronic transition [*i.e.*, the Franck-Condon principle is violated (11)].

In the perturbation model of aromatic substitution (12), the lone-pair orbital is at higher energy than the highest occupied π-orbital of the ring in the ground state. This occurs because the lone-pair electrons are not as stabilized as the π-electrons by the π-bonding interaction. Because the lone-pair orbital is higher in energy than the highest occupied π-orbital, the lowest energy transitions of the amino derivatives occur at lower energies (or longer wavelengths) than the lowest energy $\pi \rightarrow \pi^*$ transitions of anthracene itself. The promotion of an electron from the lone electron pair of the amino group is said to mix charge transfer into the $\pi \rightarrow \pi^*$ transitions of the parent hydrocarbon (in the terminology of the perturbation model). The rehybridization of the amino group during the absorption process results in the loss of vibrational structure in those transitions having a great deal of charge-transfer character. Because of the transition polarizations and orbital properties (charge densities) associated with the ^1L_a and ^1L_b excited states (the two lowest excited states), substitution in the 1- and 9-positions (α-positions) of the anthracene ring by an amino group introduces considerable charge-transfer character and vibrational blurring into the $^1L_a \leftarrow {}^1A$ transition of anthracene and practically none into the $^1L_b \leftarrow {}^1A$ transition. This is a result of the colinearity between the $^1L_a \leftarrow {}^1A$ transition, the direction of transfer of charge into the ring from an amino substituent in an α-position, and the perpendicularity between the $^1L_b \leftarrow {}^1A$ transition moment direction and that of charge transfer from an α-amino group. On the other hand, β-substitution (in the 2-position of anthracene) of an amino group results in a large angle between the $^1L_a \leftarrow {}^1A$ transition moment vector and the vector associated with the direction of charge transfer from a β-amino group to the anthracene ring but a much smaller angle between the latter and the $^1L_b \leftarrow {}^1A$ transition moment vector. Consequently, the $^1L_b \leftarrow {}^1A$ transition has the greatest degree of charge transfer, exhibits vibrational blurring, and lies at the longest wavelengths in 2-aminoanthracene while the $^1L_a \leftarrow {}^1A$ transition is only slightly affected.

Protonation of the nitrogen atoms of the anthrylamines removes the lone-pairs from conjugation with the aromatic system, so the spectra of all of the anthrylammonium ions are very close in appearance to that of anthracene, with slight displacements to longer wavelengths because the positive field effect of the ammonium substituent stabilizes the dipolar excited states slightly more than the nearly apolar ground states.

Fluorescence in 1-anthrylamine corresponds to the $^1L_a \rightarrow {}^1A$ transition; in 2-anthrylamine, it arises from the $^1L_b \rightarrow {}^1A$ transition because the ^1L_a and ^1L_b states are the lowest excited singlet states in the 1- and 2-isomers, respectively. During fluorescence, charge is transferred back from the anthracene ring to the amino group and is accompanied by rehybridization of the amino group from an sp^2 to an sp^3 situation. Consequently, the fluorescence bands of 1- and 2-aminoanthracenes are diffuse. That the fluorescence bands of the 1- and 2-aminoanthracenes appear at a much longer wavelength than the corresponding absorption bands is the result of a high energy of solvent cage reorientation, subsequent to excitation, which is reflected in the fluorescence but not in the absorption spectrum, and a small energy of solvent cage reorientation subsequent to emission, which is reflected in the absorption but not in the fluorescence spectrum (13). This is generally observed in intramolecular charge-transfer transitions of molecules which are more polar in the excited state than in the ground state.

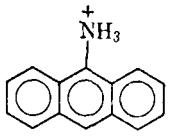
The fluorescences of the 1- and 2-anthrylammonium ions are similar to those of anthracene and, lacking intramolecular charge-transfer properties, are highly structured. On the basis of the present experiments, it cannot be stated with certainty whether the fluorescences of the anthrylammonium ions originate from the ^1L_a or ^1L_b states.

The similarities between the absorption and fluorescence spectra of anthracene and the anthrylammonium ions are typical of the spectroscopic behavior of arylammonium ions. For example, the spectra of the anilinium ion are similar to those of benzene, while the spectra of the 1- and 2-naphthylammonium ions are similar to those of naphthalene (14–16). Moreover, the differences between the absorption and fluorescence spectra of anthracene and the 1- and 2-anthrylamines are comparable to the differences between the absorption and fluorescence spectra of benzene and aniline and between those of naphthalene and the 1- and 2-naphthylamines. Thus, in the electronic structural sense, the 1- and 2-aminoanthracenes and their conjugate cations may be considered to be well-behaved aminoanthracenes and anthrylammonium ions, respectively.

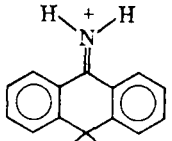
The pKa values for the prototropic equilibria at 25° between the 1- and 2-aminoanthracenes and their respective conjugate cations were determined by spectrophotometric pH titration and are: 1-aminoanthracene, 3.85 ± 0.00; and 2-aminoanthracene, 4.00 ± 0.04. These pKa values are representative of arylamines in general. Moreover, the slightly more acidic pKa of the 1-isomer is consistent with the weaker basicity of α-arylamines relative to β-arylamines. For example, the pKa of 1-naphthylamine is 3.92 and that of 2-naphthylamine is 4.11 (17). This difference is derived from the greater degree of conjugative interaction in α-positions than in β-positions of linearly annellated polyacene rings. The pKa values support the conclusion that the 1- and 2-aminoanthracenes and their conjugate cations are chemically well-behaved arylamines and arylammonium ions, respectively.

9-Aminoanthracene—The electronic absorption spectra of 9-aminoanthracene in alkaline and acidic aqueous solutions are shown in Fig. 2. The principal maxima of these spectra are listed in Table I. The absorption and fluorescence spectra of the neutral molecule (in alkaline solution) are similar to those of 1- and 2-aminoanthracenes. Because the 9-isomer is an α-substituted anthracene, the longest wavelength, diffuse absorption band must arise from the $^1L_a \leftarrow {}^1A$ transition while the shorter wavelength, structured band represents the $^1L_b \leftarrow {}^1A$ transition. The fluorescence of the neutral 9-isomer corresponds to the $^1L_a \rightarrow {}^1A$ emission. The spectra of 9-aminoanthracene indicate that it is a typical derivative of anthracene.

The spectra of 9-aminoanthracene in aqueous acidic media are quite different from those of the 1- and 2-anthrylammonium ions and of anthracene. The absorption spectrum of the cation derived from 9-aminoanthracene shows an intense structureless band at 301 nm. with a less intense structureless shoulder at 340 nm. This spectrum is similar in general appearance to the absorption spectrum of anthrone (6), whose intense band and shoulder lie at 275 and 305



IV



V

nm., respectively, and to that of the conjugate cation of anthrone (6), whose intense band and shoulder should occur at 351 and 375 nm., respectively (anthrone is the keto tautomer of 9-anthrol). The fluorescence of the cation derived from 9-aminoanthracene is structureless and has its maximum at 450 nm. Anthrone does not fluoresce, presumably because its lowest excited singlet state is of the n,π^*-type and favors radiationless deactivation of the lowest excited singlet state (18). However, the cation derived from anthrone demonstrates a structureless fluorescence band with a maximum at 475 nm.

That the absorption spectrum of the cation derived from 9-aminoanthracene, in water, lies at much shorter wavelengths than that of anthracene and lacks the characteristic vibrational structure of anthracene or the typical anthrylammonium ions indicates that the extended aromaticity of the anthracene ring in the cation has been disrupted. The similarity between the absorption spectra of the protonated amine and the neutral and protonated ketone, anthrone, strongly suggests that the cation of 9-aminoanthracene in water is not the 9-anthrylammonium ion (IV) but rather a tautomeric protonated imine (V). The similarities between the electronic structures of V and those of anthrone and its cation are obvious, thereby accounting for the similarities between their absorption spectra.

The absorption spectrum of V may be considered to be that of benzene substituted with an electron-withdrawing group. The intense band at 301 nm. corresponds to the $^1L_a \leftarrow {}^1A$ transition in benzene, and the shoulder at 340 nm. corresponds to the $^1L_b \leftarrow {}^1A$ transition in benzene. The ^1L_a and ^1L_b absorption bands of benzene, however, lie at 203 and 255 nm., respectively. The corresponding bands in anthrone, its cation, and V lie at much longer wavelengths because of the charge-transfer character mixed into the lowest excited singlet states of benzene by interaction with the vacant π-orbital of the keto, protonated keto, or protonated imino group. In anthrone, its cation, and V, the lower absorptive transitions entail transfer of an electron from the benzene ring to the electron-acceptor keto, protonated keto, or protonated imino functional group. The greater the electron-withdrawing strength of the electron-acceptor group, the lower are the energies of the long wavelength absorption bands. The order of the wavelengths of the absorption spectra, anthrone < protonated 9-aminoanthracene < protonated anthrone, follows from the greater electron-withdrawing capability of the protonated imine relative to anthrone by virtue of the formal positive charge on the nitrogen atom. The greater electron-withdrawing capability of protonated anthrone relative to the protonated imine is derived from oxygen's electronegativity being greater than that of nitrogen. The latter reasoning also accounts for the longer wavelength of fluorescence of protonated anthrone relative to that of the protonated imine.

The pKa 6.34 of the 9-isomer is anomalously high for an arylamine and supports the chemical distinction of at least one member of the 9-anthrylamine–protonated 9-anthrylamine conjugate pair from the corresponding species derived from 1- and 2-anthrylamines. Moreover, the pKa values of the anthrylamines are such that, except in the stomach, the 1- and 2-isomers are essentially entirely in the neutral form *in vivo*, while the 9-isomer is appreciably protonated at all biological pH's.

The keto–enol tautomerism of anthrone has been shown to favor quantitatively the keto form in water (6). In lower dielectric media, however, the enol form, although never predominant, exists in measurable amounts. Correspondingly, in acidic ethanol solutions a structured absorption band, with vibronic maxima at 386, 366, and 348 nm., appears in the spectrum of protonated 9-aminoanthracene in addition to the diffuse ^1L_a and ^1L_b bands of the protonated imine structure which lie at 294 and 325 nm. in ethanol, respectively. The structured absorption band in acidic ethanol has the appearance and position in the spectrum characteristic of an anthrylammonium ion and is thus assigned to the 9-anthrylammonium ion (IV). IR spectra of the commercial sample of 9-aminoanthracene hydrochloride showed carbon–nitrogen stretching frequencies at 1588 and 1400 cm.$^{-1}$. The latter is characteristic of arylammonium ions, and the former is characteristic of imino groups. In contrast, the IR spectra of the hydrochlorides of the 1- and 2-aminoanthracenes demonstrated only the ammonium-type C–N stretch at 1405 and 1400 cm.$^{-1}$, respectively. Hence, in the solid state, both the arylammonium and protonated imine tautomers of the cation from 9-aminoanthracene are stable. This serves to point out that IR spectra taken in nonaqueous media may be of only limited value in establishing the chemical natures of species present in biological systems. Electronic absorption and fluorescence spectroscopy, however, when applicable, are not subject to restriction to nonaqueous circumstances, often imposed upon IR measurements. Moreover, the electronic spectroscopic methods are more sensitive and less subject to interference by impurities.

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Separation and Identification of Morphine and Its Metabolites and Congeners

S. Y. YEH

Abstract □ Morphine and its known and postulated metabolites and congeners, *i.e.*, morphine *N*-oxide, normorphine, pseudomorphine, morphine-*N*-methyl iodide, codeine, norcodeine, morphine-3-glucuronide, and morphine ethereal sulfate, were separated by TLC and GLC. The R_f values of nalorphine and nicotine and its metabolites and the retention times of nalorphine and seven other chemicals commonly used for GLC quantitative determination of morphine and codeine are also presented.

Keyphrases □ Morphine and metabolites—separation, identification, TLC and GLC □ TLC—separation, identification, morphine and metabolites □ GLC—separation, identification, morphine and metabolites

Studies in various animal species have shown that morphine is biotransformed to morphine-3- and 6-glucuronide (1–5), morphine-3-ethereal sulfate (6–8), morphine *N*-oxide (9, 10), normorphine (11–14), and codeine (15). Pseudomorphine (16), an oxidation product of morphine, and morphine glutathione (17) have also been postulated to be metabolites.

While investigating the pharmacokinetics and metabolism of morphine in man, need arose for the development of suitable methods for separation and identification of morphine and its metabolites. Although many procedures, including TLC and GLC, have been described for separation of morphine from its congeners, as mentioned in recent reviews (18, 19), none has been described for separation of morphine from its metabolites. This report describes the application of TLC and GLC for the separation and identification of these compounds.

EXPERIMENTAL

Morphine sulfate USP[1], codeine phosphate USP[1], and normorphine hydrochloride[2] were obtained. Pseudomorphine, morphine *N*-oxide, and morphine-*N*-methyl iodide were prepared by previously described methods (20–22). Morphine-3-glucuronide and morphine-3-ethereal sulfate were isolated from the urine of dog and cat, respectively (4, 8).

TLC[3]—The compounds were localized after development by spraying with potassium iodoplatinate reagent or ninhydrin reagent.

GLC—A chromatograph[4] equipped with dual-flame-ionization detectors and dual-pen recorders[5] was used for GLC. The columns (Table II) were conditioned at 275° under nitrogen, 30 ml./min., for 1 hr.; then at 340° without nitrogen for 4 hr.; and finally at 290° with nitrogen, 16 ml./min., for 72 hr. The temperatures of injector and detector were set at 255 and 295°, respectively; gas flow rates were 30 ml./min. for nitrogen and hydrogen and 300–400 ml./min. for compressed dried air.

Silylation of Morphine and Its Metabolites and Congeners—Methanol solutions of morphine, codeine, normorphine, norcodeine, morphine *N*-oxide, and morphine-*N*-methyl iodide were placed in acylation tubes[6] and evaporated to dryness under nitrogen in a water bath at 60–70°. To the residues were added 0.05 ml. acetonitrile and 0.05 ml. of bis(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane[7]. The tubes were capped and shaken on a Vortex mixer for about 10 sec. After heating at 60–70° for 30 min., 1 μl. of the mixture was injected into the gas chromatograph.

Acetylation of Morphine and Its Metabolites and Congeners—Methanol solutions of morphine, codeine, normorphine, norcodeine, morphine *N*-oxide, and morphine-*N*-methyl iodide were placed in acylation tubes and evaporated to dryness under nitrogen in a water bath at 60–70°. The residues were either trifluoroacetylated with 0.2 ml. of trifluoroacetic anhydride or acetylated with 0.2 ml. of acetic anhydride and 0.1 ml. of pyridine. The tubes were sealed with screw caps, and the mixtures were warmed for 30 min. in an oil bath at 60–70°. Excess trifluoroacetic anhydride or acetic anhydride was removed under a stream of nitrogen in an oil bath. The residues were dissolved with 0.1 ml. of ethyl acetate by shaking on a Vortex mixer for 10 sec. One microliter of the solutions was injected into the gas chromatograph.

RESULTS AND DISCUSSION

TLC Separation of Morphine and Its Metabolites and Congeners—The R_f values of morphine and its metabolites, obtained with various thin-layer plates and developing solvent systems, are presented in Table I. A good separation of morphine from other free compounds, with the exception of norcodeine, was obtained with the silica gel precoated plates developed with a solvent mixture of ethyl acetate–methanol–ammonium hydroxide (17:2:1) or with the instant silica gel sheet developed with a mixture of *n*-butanol–*n*-

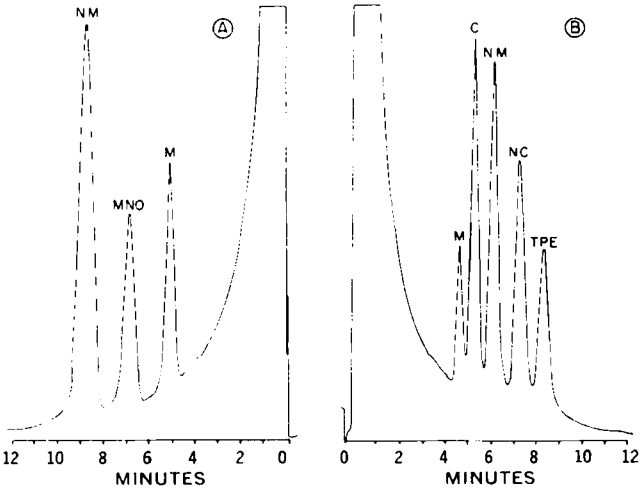

Figure 1—*Gas chromatograms of trifluoroacetyl derivative of morphine (M), codeine (C), normorphine (NM), norcodeine (NC), morphine N-oxide (MNO), and tetraphenylethylene (TPE). Key: A, obtained from Column 2; and B, obtained from Column 1 at 205°.*

[1] Merck Sharp & Dohme.
[2] Through the courtesy of Dr. Everett May, National Institutes of Health.
[3] Gelman instant thin-layer silica gel sheets (ITLC-SG type, Gelman Instrument Co., Ann Arbor, Mich.), and Quantum silica gel precoated plates were used for TLC, with application of standard techniques.
[4] Varian Aerograph, Series 2700.
[5] Model A-25.
[6] Regis Chemical Co.
[7] Regisil TMCS (10%), Regis Chemical Co., Chicago, Ill.

Table I—R_f (× 100) Values of Morphine and Its Metabolites

Substances	Instant Thin-Layer Silica Gel Sheet: Ethyl Acetate–Methanol–Ammonium Hydroxide (17:2:1)	Instant Thin-Layer Silica Gel Sheet: *n*-Butanol–Acetic Acid–Water (35:3:10)	Instant Thin-Layer Silica Gel Sheet: *n*-Butanol–*n*-Butyl Ether–Ammonium Hydroxide (25:70:2)	Silica Gel Precoated Plates, Ethyl Acetate–Methanol–Ammonium Hydroxide (17:2:1)
Codeine	98	98	70	60
Morphine	97	98	62	34
Morphine *N*-oxide	40	90	23	3
Norcodeine	97	98	64	34
Normorphine	85	97	50	15
Morphine-3-ethereal sulfate	47	67	19	3
Morphine glucuronide	0	40	0	0
Pseudomorphine	10–15	30	0	1
Morphine-*N*-methyl iodide	0	75	3	0
Nalorphine	98	90	81	50
Nicotine and its metabolites[a]	0, 8, 27, 50 70, 90	90, 96	0–23, 65–80	4, 8, 34 64, 83

[a] Two hundred milliliters urine from a heavy cigarette smoker (two packages per day) was passed through XAD-2 resin, which was then washed with water and eluted with methanol. The methanolic eluate was concentrated to 3 ml., and 50 μl. was spotted.

Table II—Retention Time (Minutes) of Morphine and Its Metabolites and Congeners

Compounds	Column 1, 100–120 Varaport 205°	Column 1, 100–120 Varaport 215°	Column 2 (0.91-m.), 60–80 Gas Chrom Q 205°	Column 2 (0.91-m.), 60–80 Gas Chrom Q 215°	Column 3 (1.82-m.), 60–80 Gas Chrom Q 200°	Column 3 (1.82-m.), 60–80 Gas Chrom Q 230°	Column 3 (1.82-m.), 60–80 Gas Chrom Q 250°
Trimethylsilyl							
-codeine	—	7	—	7.7	45.4	13.4	6.5
-morphine	—	6.0	—	6.7	34.5	10.5	5.7
-morphine *N*-oxide	—	6.0	—	6.7	34.5	10.5	5.7
-morphine-*N*-methyl iodide[a]	—	4.0	—	4.7	—	12.0	5.8
-norcodeine	—	6.2	—	8.2	—	—	—
-normorphine	—	6.3	—	10.3	—	19	8.0
-pseudomorphine[b]	—	8.5	—	8.3	41.3	12.5	6.5
Acetyl	—	—	—	—	—	—	—
-codeine	—	6.0	—	13.5	—	—	—
-morphine	—	8.0	—	24	—	—	—
-morphine *N*-oxide	—	7.8	—	23.2	—	—	—
-morphine-*N*-methyl iodide	—	8.0	—	19	—	—	—
-norcodeine	—	19.7	—	13.5	—	—	—
-normorphine	—	27.5	—	19 (250°)	—	—	—
Trifluoroacetyl							
-codeine	5.2	3	7.7	4	—	—	—
-morphine	4.4	2.8	5.1	3.5	15	—	—
-morphine *N*-oxide	6.0	4.5	7.0	—	20	—	—
-norcodeine	7.5	—	13.0	—	—	—	—
-normorphine	7.1	—	8.7	—	—	—	—
-pseudomorphine[c]	53[c]	—	—	—	—	—	—

[a] Morphine-*N*-methyl iodide gave two peaks which may arise from thermal decomposition. [b] Trimethylsilyl-pseudomorphine did not emerge from the column at 290° after 1 hr. [c] The retention time for trifluoroacetyl-pseudomorphine was obtained at a column temperature of 290°.

butyl ether–ammonium hydroxide (25:70:2). Morphine and one spot of the resin[8] extract of a urine from a cigarette smoker had approximately the same R_f value on the silica gel plates. The two spots could be distinguished by their color after spraying with iodoplatinate reagent. Morphine had a blue-purplish color and the nicotine metabolite was gray. Also, the nicotine metabolite moved slightly ahead of the morphine.

Conjugated morphine metabolites, *i.e.*, morphine-3-glucuronide and morphine-3-ethereal sulfate, could be separated from the free drugs on the instant silica gel sheets developed either with ethyl acetate–methanol–ammonium hydroxide (28% ammonia) (17:2:1) or with *n*-butanol–acetic acid–water (35:3:10).

GLC Separation of Morphine and Its Metabolites and Congeners—The GLC data for morphine and its metabolites are presented in Table II. An earlier report (23) that silyl derivatives of norcodeine and normorphine were retained in the 3% SE-30 column (Column 1) even at 250° was in error. The error, which might be due to the incompleteness of the silylation of these compounds or to the low concentrations of these compounds applied, should be corrected with the present data. Since the trimethylsilyl derivatives of morphine and its metabolites could not be completely separated with the 0.91-m. (3-ft.) 3% OV-17 Column 2, the 1.82-m. (6-ft.) 3% OV-17 Column 3, or Column 1, acetyl derivatives of morphine and its free metabolites were prepared and examined with these columns. Good separations of these compounds, except morphine *N*-oxide and morphine, were obtained with Columns 1–3.

These data agree well with earlier data (25), except the retention times were longer in the present study. The difference in retention times was apparently due to the different size of supports used. The retention times of acetylnormorphine and acetylnorcodeine on Column 1 at 215° was too long for practical use, and the curves tailed. With temperature programming, the retention times of these compounds were considerably shortened and the peaks sharpened. The retention times of acetyl derivatives of codeine, morphine, norcodeine, and normorphine on Column 1, using temperature programming from 205 to 250° at 2°/min., were 7.3, 9.5, 16.4, and 18.2 min., respectively. Since the retention times of the acetyl derivatives of normorphine and norcodeine on Columns 1–3 were quite long and the curves tailed, trifluoroacetyl derivatives were prepared and examined on these columns.

The trifluoroacetyl derivatives of morphine and its free metabolites, except morphine *N*-oxide and normorphine, could be separated very well with Column 1 and gave sharp peaks (Fig. 1B).

[8] XAD-2.

Table III—Retention Time (Minutes) of Chemicals Used as Internal Standards for Quantitative Determination of Morphine and Its Metabolites

Compounds	Column 1, 100–120 Varaport			Column 2 (0.91-m.), 60–80 Gas Chrom Q			Column 3 (1.82-m.), 60–80 Gas Chrom Q		
	200°	205°	215°	200°	205°	215°	200°	230°	250°
Trifluoroacetyl-nalorphine	8.1	6.9	4.8	9.8	7.1	5.1	—	8.0	4.1
Tetraphenylethylene	10.6	8.5	6.1	19	15	10.2	>37	16.7	8.0
Cholestane	35	23.5	15.0	23.5	18.2	12	>30	22.0	10.5
Docosane	4.3	3.4	2.0	2.2	1.6	0.9	5.5	2.0	1.0
Tetracosane	8.2	6.6	4.5	3.6	3.0	2.2	11.8	4.2	1.8
Octradosane	33	25.5	17.4	16.0	12.8	8.0	>30	13.0	5.8
Methylarachidate	6.5	4.7	3.5	5.3	3.5	2.4	13.0	4.1	2.1
Methylbehanate	12.4	9.6	6.4	8.9	6.9	4.7	27.3	7.8	7.0

These compounds, except codeine and morphine *N*-oxide, could also be completely separated with Column 2 (Fig. 1A). The trifluoroacetyl-normorphine and trifluoroacetyl-norcodeine on Columns 2 and 3 showed some tailing. The retention time of trifluoroacetyl-pseudomorphine, prepared by heating pseudomorphine with trifluoroacetic anhydride for 2 hr., was 53 min. on Column 1 at 290°. Trifluoroacetyl-pseudomorphine did not emerge from Columns 2 and 3 at 290° for 2 hr. Trimethylsilyl-pseudomorphine, prepared by heating the compound with 0.1 ml. acetonitrile and 0.1 ml. bis(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane at 60–70° for 2 hr., did not emerge from Columns 1–3 at 290° for 2 hr. Silylation or acetylation, either with trifluoroacetic anhydride or acetic anhydride, could be accelerated by heating the mixture in a screw-capped sealed tube at 60–70° for 0.5 hr. Acetylation with trifluoroacetic anhydride could not be completed without heating for at least 24 hr. During the preparation of this paper, a similar procedure for acetylation of codeine and its metabolites with trifluoroacetic anhydride appeared in the literature (24).

The trifluoroacetyl derivatives of morphine and its metabolites were quite unstable and lost their trifluoroacetyl moiety on standing in air or in ethyl acetate solution. Therefore, the mixture should be kept sealed in the trifluoroacetic anhydride medium until a few hours prior to injection. After removing the excess trifluoroacetic anhydride and dissolving the residue with ethyl acetate, the solution should be injected within a few hours. A low sensitivity was obtained when the solution was stored overnight. Trifluoroacetyl-morphine *N*-oxide was very unstable in ethyl acetate; the solution became yellow within 1 hr.

The retention times of seven other chemicals, which are commonly used as internal standards for quantitative determination of morphine and its metabolites and congeners by GLC, were determined on Columns 1–3 (Table III). The retention times varied with changes in temperature and type of column, but the basic pattern remained the same. Nalorphine has been suggested as an internal standard to be added to the urine for determination of morphine (25). The retention time of trifluoroacetyl-nalorphine on Column 1 interfered with the retention time of trifluoroacetyl-norcodeine; on Column 2, it interfered with that of trifluoroacetyl-codeine. Among the chemicals examined, tetraphenylethylene was the best internal standard for quantitative determination of trifluoroacetyl-morphine or trifluoroacetyl-codeine and their metabolites on Column 1.

Urinary disposition of narcotics is being currently investigated with the described TLC and GLC procedure. Quantitative determinations of urinary disposition of morphine in morphine-dependent subjects have been reported elsewhere (26).

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Spectrophotometric Determination of Ampicillin in Presence of Hetacillin

PETER M. MONTELEONE▲, MICHAEL K. VASILJEV, and JOSEPH BOMSTEIN

Abstract □ A procedure was developed for measuring small amounts of ampicillin in hetacillin bulk powders and formulations. The method depends on forming a UV-absorbing compound with Ni (II) in dimethyl sulfoxide. The response is linear over a 15-fold range and accuracy is generally better than ±5–10% except near the minimum sensitivity level. Procedural details and data for the effects of operating parameters are presented.

Keyphrases □ Ampicillin—spectrophotometric determination in presence of hetacillin □ Hetacillin, bulk powders and formulations—spectrophotometric determination of small amounts of ampicillin □ Nickel (II)-dimethyl sulfoxide reagent—used in spectrophotometric determination of ampicillin in hetacillin powders and formulations □ UV spectrophotometry—determination of small amounts of ampicillin in hetacillin powders and formulations

Hetacillin is a recently introduced broad-spectrum antibiotic. Its preparation and physicochemical properties (1) and pharmacological studies (2, 3) were reported. Hetacillin, which possesses an imidazolidinyl moiety, equilibrates with ampicillin, and this equilibrium was studied *in vitro* (4, 5). The superior solution stability of hetacillin compared to ampicillin was pointed out (5).

To take full advantage of the stability of hetacillin, it is important to manufacture a product having, at most, a low level of ampicillin and to monitor the ampicillin content analytically in the presence of bulk hetacillin.

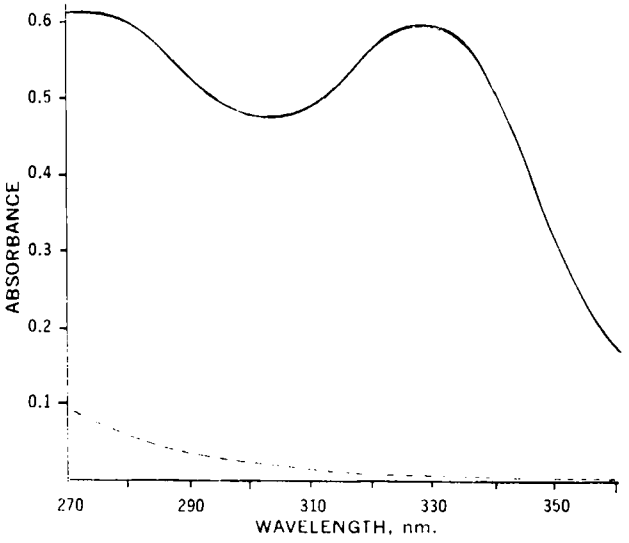


Figure 1—*Absorption spectra for ampicillin trihydrate with (——) and without (- - - -) Ni (II) in dimethyl sulfoxide. Ampicillin trihydrate concentration = 28 mcg./ml.*

Published methods for determining ampicillin depend on one of the following techniques: microbiological response (6), iodometry (6), hydroxamation (6), conversion to the penicillenic acid (7), or conversion to a fluorescing species (8). All of these approaches involve reaction and/or measurement in water. The rate of attainment of the hetacillin–ampicillin equilibrium in this medium, however, is too great to permit the selective determination of ampicillin. Both compounds are poorly soluble in nonpolar solvents, but they dissolve readily in the aprotic solvent dimethyl sulfoxide. The particular advantage of dimethyl sulfoxide, of course, is that the absence of water would be expected to inhibit the formation of ampicillin. By taking advantage of the presumed stability of hetacillin in dimethyl sulfoxide, the solubility of certain transition metal ions in the same solvent (9), and the tendency of amino compounds to form metal complexes, several organic compounds of interest were studied. This paper reports the use of Ni (II) in dimethyl sulfoxide to measure low levels of ampicillin in hetacillin and confirms the stability of the latter in this medium.

EXPERIMENTAL

Ni (II)–Dimethyl Sulfoxide Reagent—Stir 2.6 g. of finely powdered nickel sulfate ($NiSO_4 \cdot 6H_2O$) in 1 l. of dimethyl sulfoxide (ACS grade) for 5 hr. in a stoppered bottle. Filter through filter paper[1] to give a clear yellow solution containing about 0.5 mg. Ni (II)/ml., as determined by disodium edetate (EDTA) titration. The solution is stable indefinitely when protected from moisture.

Penicillins—Standard reference grade ampicillin trihydrate (99.2% purity) and hetacillin (97.0% purity) were used throughout.

Microbiological Analyses—The disk-plate method[2] (10), using a medium prepared by overlaying BBL Base Agar 10944 with BBL Seed Agar AA#1 and inoculated with *Sarcina lutea* (ATCC 9341), was used.

Spectrophotometry—Readings were taken on two spectrophotometers[3] in 1-cm. cells.

Analytical Procedure for Ampicillin in Hetacillin—*Standard Curve*—Dissolve 100 mg. ampicillin trihydrate in 100 ml. dimethyl sulfoxide. Dilute 5.00 ml. of this solution to 100 ml. with dimethyl sulfoxide. To five 25-ml. volumetric flasks, transfer respectively 0, 5.00, 10.00, 15.00, and 20.00 ml. of the previous solution, add 5.00 ml. of Ni (II)–dimethyl sulfoxide reagent to each, and dilute to volume with dimethyl sulfoxide. Stopper, heat exactly 10 min. at 50° in a

[1] Whatman No. 2.
[2] Paper disks used were S&S No. 740-E.
[3] Cary 14 and Beckman Acta III.

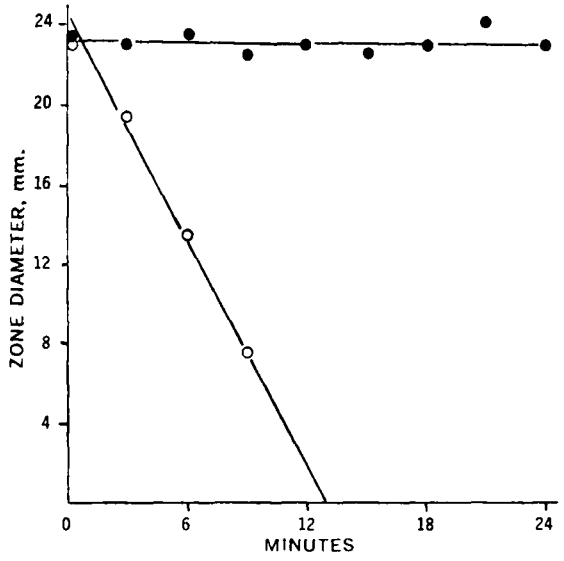


Figure 2—*Comparison of the reactivity of hetacillin (●) to ampicillin (○) when heated with Ni (II)-dimethyl sulfoxide at 50° for various times and assayed by the disk-plate microbiological method. Initial solutions had a potency of 20 mcg./ml.*

water bath, cool to room temperature, and measure the absorbance at 330 nm. against the standard containing no ampicillin as a blank. Plot absorbance *versus* concentration to obtain the standard curve.

Samples—Dissolve in dimethyl sulfoxide to contain 200 mcg. ampicillin/ml. Dilute a 10.00-ml. aliquot to 100 ml. with dimethyl sulfoxide. Pipet 20.00 ml. into a 25-ml. volumetric flask, add 5.00 ml. of Ni (II)-dimethyl sulfoxide reagent, and proceed in the same way as for standards.

EFFECTS OF ANALYTICAL VARIABLES

Figure 1 is the absorption spectrum for the product of the Ni (II)-dimethyl sulfoxide reaction with ampicillin trihydrate. Also shown is the absorption spectrum obtained when the same amount of ampicillin trihydrate is heated for 10 min. in dimethyl sulfoxide at 50° without the addition of Ni (II)-dimethyl sulfoxide reagent. Hetacillin, when treated with Ni (II)-dimethyl sulfoxide reagent under the same conditions as the ampicillin trihydrate, gave no significant absorbance at 330 nm.

The lack of reactivity of hetacillin to the Ni (II)-dimethyl sulfoxide reagent was also demonstrated by a microbiological method. In Fig. 2 the diameters of the zones of inhibition are plotted *versus*

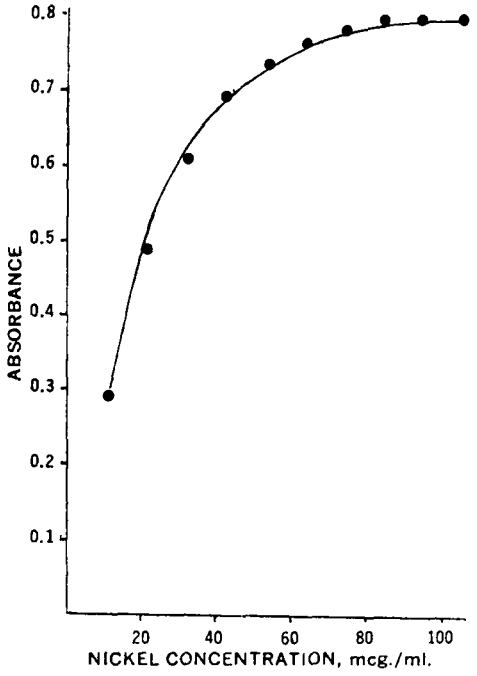


Figure 4—*Effect of Ni (II) concentration on the reaction of ampicillin with Ni (II) in dimethyl sulfoxide. Ampicillin trihydrate concentration = 38 mcg./ml.*

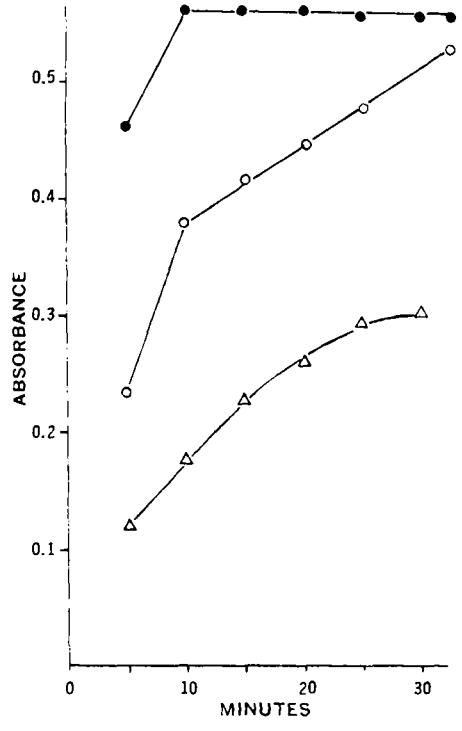


Figure 3—*Effect of temperature on the rate of reaction of ampicillin in Ni (II)-dimethyl sulfoxide reagent. Key: ●, 50°; ○, 40°; and △, 30°. Ampicillin trihydrate concentration = 28 mcg./ml.*

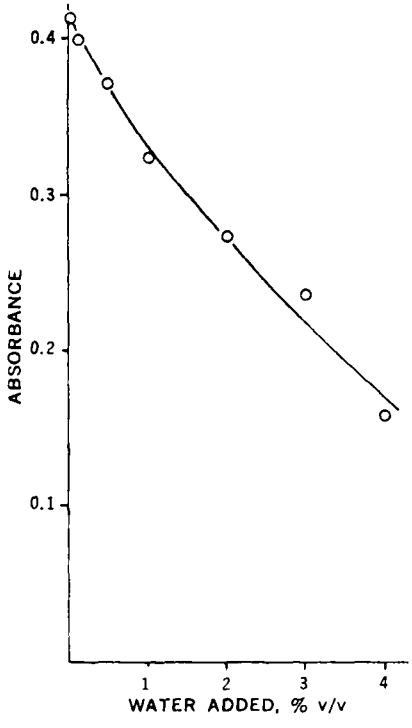


Figure 5—*Effect of added water on the reaction of ampicillin with Ni (II) in dimethyl sulfoxide. Ampicillin trihydrate concentration = 24 mcg./ml.*

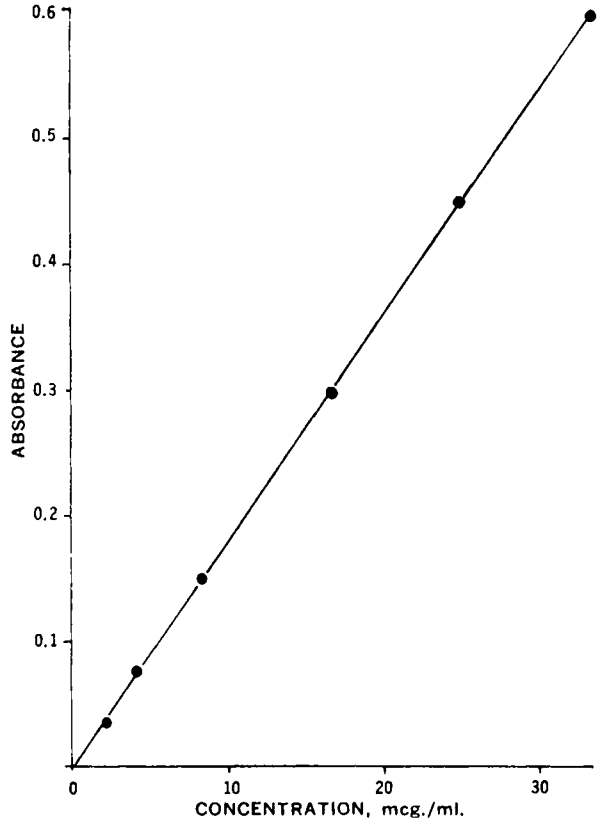


Figure 6—*Calibration curve for ampicillin trihydrate as determined by the Ni (II)–dimethyl sulfoxide reaction.*

reaction time at 50° for ampicillin and hetacillin. The size of the zone of inhibition produced by hetacillin is virtually unchanged over 24 min., whereas the last detectable bioactivity for ampicillin is obtained at 9 min. In addition to showing the lack of reactivity of hetacillin to Ni (II)–dimethyl sulfoxide, these data establish that ampicillin is converted to a nonantibiotic by reacting with Ni (II)–dimethyl sulfoxide reagent.

Table I—Recovery of Ampicillin Trihydrate Added to Potassium Hetacillin

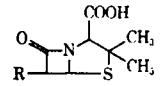
Added[a], %	Found[a], %	Recovered, %
0.75	1.0	130
1.0	1.0	100
1.5	1.7	113
2.0	2.1	105
2.5	2.7	104
3.5	3.8	109
4.9	4.5	92
5.8	5.4	93
7.4	7.8	105
9.8	9.4	96
17	16	95
27	26	97
41	40	99

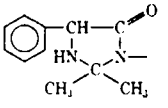
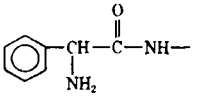
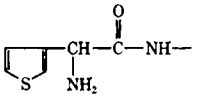
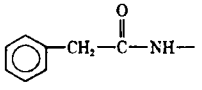
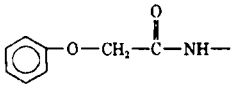
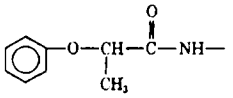
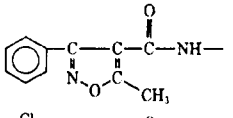
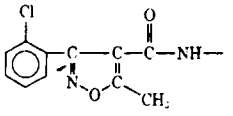
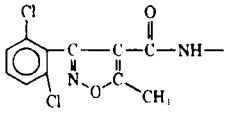
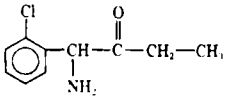
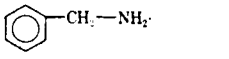
[a] This sample of hetacillin, without added ampicillin, was found to contain 1.65% as the trihydrate. The data reported were corrected for this original content.

Table II—Ampicillin in Nonhetacillin Preparations

Ampicillin	Second Component	Ampicillin [Ni (II)–Dimethyl Sulfoxide]
250 mg. (bioassay)	Oxacillin	245 mg.
520 mg. (bioassay)	Oxacillin	513 mg.
250 mg. (label claim)	Dicloxacillin, 125 mg.	260 mg.
28% (synthetic mixture)	Lactose	27%
42% (synthetic mixture)	Lactose	40%

Table III—Compounds Tested for Reactivity with Ni (II)–Dimethyl Sulfoxide



Compound	R	Reaction to Produce Chromophore
A. Penicillin		
Hetacillin		No
Ampicillin		Yes
		Yes
Penicillin G		No
Phenoxymethyl penicillin		No
Phenethicillin		No
Oxacillin		No
Cloxacillin		No
Dicloxacillin		No
B. Compounds related to penicillins		
6-Aminopenicillanic acid		No
Penicilloic acid of phenoxymethyl penicillin		No
Benzylpenillic acid		No
Benzylpenilloic acid		No
C. Other compounds		
		Yes
		No
NH_2CH_2COOH		No
$NH_2CH_2CO-NH-CH_2COOH$		No

The effects of temperature, time, and Ni (II) concentration on the rate of reaction of ampicillin are shown in Figs. 3 and 4. These data led to the adoption of the standard reaction conditions of 50° heating for 10 min. with a Ni (II) concentration of approximately 100 mcg./ml.

The effect of water on the sensitivity of the Ni (II)–dimethyl sulfoxide reaction for ampicillin is demonstrated in Fig. 5. The addition of 0.5% water to the reaction medium decreased the absorbance by approximately 10%. A very similar effect was obtained when methanol was added. The addition of acetonitrile or tetrahydrofuran, on the other hand, had no significant effect on absorbance. Consideration of these data led to the conclusion that alcohol and excessive water must be excluded from the reaction medium to obtain maximum sensitivity. Water is naturally allowed from three sources; it is calculated that the contribution of water from the ampicillin trihydrate will not be greater than 0.001%, the contribution from nickel sulfate will be less than 0.004%, and the contribution from dimethyl sulfoxide itself will be 0.02%. The water from the last two sources is maintained constant. The data shown in Fig. 5 are for water above that contributed by these three sources and show that water from sources such as wet glassware or aqueous samples may significantly affect sensitivity. The water naturally present in powdered samples should not be significant.

Figure 6 is a calibration curve for ampicillin trihydrate measured as the product of the Ni (II)–dimethyl sulfoxide reaction. A linear relationship exists between absorbance and concentration from at least 2 to 30 mcg./ml.

RESULTS

The described procedure was used to determine ampicillin in bulk hetacillin. Table I lists recovery data for bulk to which was added 0.75–41% ampicillin trihydrate. The original hetacillin was found to contain 1.65% ampicillin trihydrate. The same procedure appears to be satisfactory in the presence of compounds other than hetacillin (Table II). Except for the recovery at the lowest level, accuracy appears to be generally better than ±5–10% of the amount of ampicillin present.

DISCUSSION

Table III lists compounds tested for reaction with the nickel reagent and the results obtained. The nature of the response is consistent with the required presence of an α-aminoaryl acetyl group, although additional model compounds should be considered before the aryl group is determined as necessary.

The coincidence of the absorbance maximum with that shown by penicillenic acids suggests that the latter may be involved. This is almost certainly not the case, however, based on the following facts: (*a*) a number of the penicillins (Part A of Table III) have been shown to form penicillenic acids (11, 12), but only ampicillin produces the chromophore under these conditions; (*b*) at least one compound (Part C of Table III) is not capable of forming a penicillenic acid but gives a positive response to this procedure; and (*c*) the substitution of Ni (II) for the Cu (II) used in catalyzing the formation of penicillenic acids from intact penicillins leads to failure of that method.

The reactivity is assumed to be due to the ampicillin side chain alone, and Ni (II) presumably either complexes or catalyzes a reaction to form a chromophore in dimethyl sulfoxide. Since the application of Job's (13) method of continuous variation failed to support a simple molar relationship between ampicillin and nickel ion, there is no evidence that the chromophore is a complex. Complexation, of course, may be a step in the mechanism of reaction. Studies with additional model compounds are continuing.

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Colorimetric Determination of Dipyrone

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Abstract □ The reaction product of dipyrone with nitrous acid was utilized for its colorimetric determination in the pure state and in tablets and injections. The derivative, so formed, obeys Beer's law in ammoniacal medium and had a maximum absorbance at 403 nm. The proposed method has the advantage over the iodometric USSR Pharmacopoeia IX method in that: (*a*) it can be applied to a much lower concentration of dipyrone, and (*b*) sodium sulfite, which is used as an antioxidant in dipyrone injections, does not interfere with the determination.

Keyphrases □ Dipyrone—colorimetric determination, bulk and formulations □ Colorimetry—analysis of dipyrone, bulk and formulations

Various methods have been described for the determination of dipyrone [sodium (antipyrinylmethylamino)methanesulfonate monohydrate]. It was determined gravimetrically as sodium sulfate after ashing with sulfuric acid (1). Several iodometric methods have been commonly used (2–4). Wirth (5) recommended that the sample be weighed instead of measured in the iodometric titration of dipyrone in parenteral preparations, and he proposed the determination of specific gravity as a method of assay. Iodometric methods have also been described for the determination of dipyrone in mixtures (6–8). One iodometric method (9) depends on the fact that dipyrone is split into sulfite by potassium cyanide in moderately alkaline solution and then the liberated sulfite is titrated with standard iodine solution in an acid medium. Trials were made (10) to explain the mechanism of the iodometric titration.

Dipyrone was also determined complexometrically after oxidation of the sulfite group to sulfate (11). Alternatively, the sulfate ion produced was titrated with barium chloride solution (12). Other methods include the titration of dipyrone with chloramine solution (13) and potentiometric titration with 0.1 *N* perchloric acid solution in nonaqueous solvents (14, 15).

The color produced when dipyrone reacted with sodium β-naphthoquinone-4-sulfonate was employed (16) for its identification and determination. This method was modified (17) to eliminate the interference by primary amines, and the reaction mechanism was explained (18). The color reactions of dipyrone with a Schiff reagent (19), 4-dimethylaminocinnamaldehyde (20, 21), quinone (22), and *p*-diazobenzenesulfonic acid (23) were used for its determination. Dipyrone was also determined colorimetrically by measuring the absorbance of its complex with copper chloride (24).

A spectrophotometric method was also described in which dipyrone was determined at 272 nm. (25). A rapid determination of dipyrone in solution by IR spectroscopy was also proposed (26). Furthermore, a polarographic method was suggested (27), in which a polarographically active derivative of this compound was determined.

In this report, a colorimetric method for determining dipyrone in the pure state and in tablets and injections is described.

EXPERIMENTAL

Apparatus and Reagents—The following were used: a spectrophotometer[1]; dipyrone, USSR Pharmacopoeia IX grade; sodium nitrite, chemical reagent; 2.0 *N* hydrochloric acid solution; and dilute ammonium hydroxide solution, prepared by diluting 33.3 ml. of concentrated ammonium hydroxide solution to 100.0 ml. with distilled water.

Construction of Standard Absorbance Graph—Prepare a series of solutions containing 40.0, 60.0, 80.0, 100.0, and 120.0 mg. dipyrone/5.0 ml. solution by dissolving 0.400, 0.600, 0.800, 1.000, and 1.200 g., respectively in distilled water in a series of five 50-ml. volumetric flasks and dilute to volume. Accurately measure 5.0 ml. of each solution and transfer into a series of five 50-ml. beakers. To each of the five beakers, add 0.5 g. of sodium nitrite followed by 20.0 ml. of 2.0 *N* hydrochloric acid solution. Heat gently with continuous stirring for 7 min. until the solutions begin to boil. Cool, transfer the contents of each beaker to a 100-ml. volumetric flask, and dilute to volume with 2.0 *N* hydrochloric acid solution. Accurately measure 2.0 ml. of each solution, transfer to a 25-ml. volumetric flask, and dilute to volume with dilute ammonium hydroxide solution. Shake well and measure the absorbance of each prepared solution at 403 nm. against a blank prepared by substituting 5.0 ml. of distilled water for 5.0 ml. of the dipyrone solution. Results are shown in Table I. The equation of the straight line was determined by the method of least squares, and the slope of the line was found to be 0.066.

Pure Compound—Weigh accurately about 0.750 g. of dipyrone, place in a 50-ml. volumetric flask, dissolve in distilled water, and dilute to volume. Measure accurately 5.0 ml. of the prepared solution, place in a 50-ml. beaker, and proceed as described previously. Measure the absorbance at 403 nm. against a blank prepared by substituting 5.0 ml. of distilled water for 5.0 ml. of the dipyrone solution. Determine the concentration from the straight-line equation using the measured absorbance. Results are shown in Table II.

Tablets—Weigh and powder 20 tablets. Transfer an accurately weighed quantity of the powder, equivalent to 0.750 g. of dipyrone, to a 50-ml. volumetric flask. Dissolve in distilled water, dilute to volume, and filter. Transfer 5.0 ml. of the filtrate, accurately measured, to a 50-ml. beaker and proceed as described previously. Measure the absorbance at 403 nm. against a blank prepared by substituting 5.0 ml. of distilled water for 5.0 ml. of the dipyrone solution. Determine the concentration from the straight-line equation using the measured absorbance. Results are shown in Table II.

Injections—Accurately measure 3.0 ml. of the mixed contents of injections, equivalent to 1.500 g. of dipyrone, and transfer to a 100-ml. volumetric flask. Dilute to volume with distilled water and shake well. Measure 5.0 ml. of this solution, place in a 50-ml. beaker, and proceed as usual. Measure the absorbance at 403 nm. against a blank prepared by substituting 5.0 ml. of distilled water for 5.0 ml. of the dipyrone solution. Determine the concentration from the straight-line equation using the measured absorbance. Results are shown in Table II.

RESULTS AND DISCUSSION

The nitrosation of dipyrone with sodium nitrite and 2.0 *N* hydrochloric acid solution produced a yellowish derivative, which was

[1] Unicam SP600.

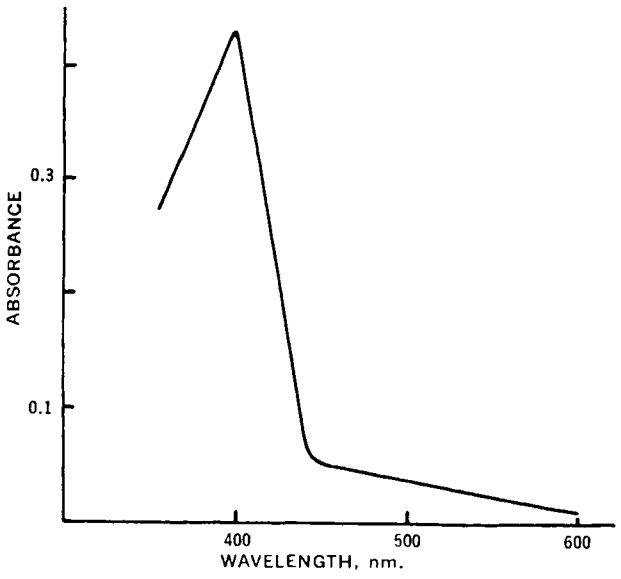


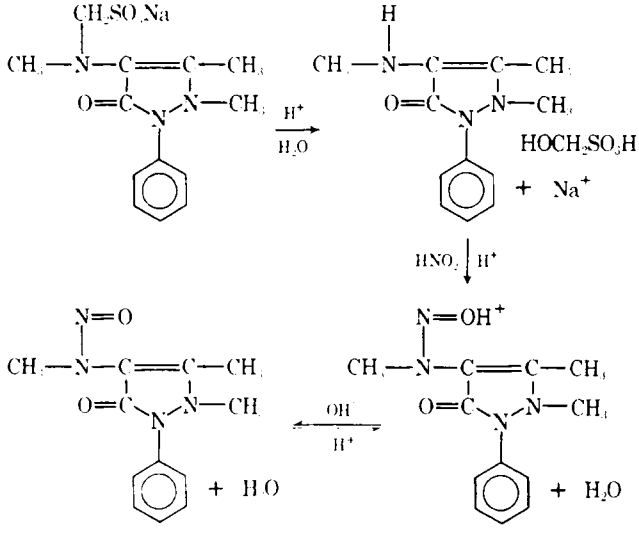
Figure 1—*Absorbance spectrum of dipyrone.*

intensified when the medium was made alkaline with ammonium hydroxide solution. This reaction was put on a quantitative basis to determine dipyrone in the pure state as well as in tablets and injections. The produced color had an absorbance peak at 403 nm. (Fig. 1) and obeyed Beer's law in a concentration range of 3.2–9.6 mg./100.0 ml. For a 72-hr. study period, the color was stable and gave no change in absorbance over a wide concentration range.

The scope of the method for control purposes was demonstrated by its application to a series of samples of dipyrone in the pure form and in different pharmaceutical forms such as tablets and injections (Table II). The presence of tablet excipients such as lactose, starch, and magnesium stearate did not interfere with the determination. The iodometric method adopted by USSR Pharmacopoeia IX also was used to determine dipyrone in the pure state and in tablets, and the results (Tables II and III) indicate that the proposed method is equivalent in accuracy and precision. However, the proposed method is more sensitive and selective.

The presence of reducing agents such as sodium sulfite, which are added to dipyrone injections as stabilizers, did not interfere with the proposed method. Such a reducing agent is expected to interfere with the iodometric method of USSR Pharmacopoeia IX because of the interaction of iodine and the reducing agent. Table IV shows the recovery data of dipyrone in prepared injections containing various amounts of sodium sulfite when the iodometric method was employed; dipyrone recoveries ranged from 100.81% in the absence of sodium sulfite to 125.79% when 48.0 mg. of this stabilizer was added.

The colored derivative formed when dipyrone is treated with sodium nitrite and hydrochloric acid solution is believed to be the



Scheme I

Table I—Absorbance of Different Concentrations of Dipyrone at 403 nm.

Concentration, mg. %	Average Absorbance
3.2	0.230
4.8	0.325
6.4	0.430
8.0	0.540
9.6	0.650

Table II—Determination of Dipyrone in Pure Form, Tablets, and Injections by the Proposed Colorimetric Method

Concentration, mg. %—Added	Found	Recovery, %
	Pure Compound	
3.6	3.68	102.22
4.2	4.27	101.69
5.2	5.33	102.50
6.6	6.67	101.06
7.8	7.91	101.41
	Average	101.77
	SD	0.59
	Tablets	
6.0	6.09	101.50
6.0	6.09	101.50
6.0	6.01	100.16
6.0	6.06	101.00
6.0	6.09	101.50
	Average	101.13
	SD	0.59
	Injections	
6.0	5.94	99.00
6.0	6.06	101.00
6.0	6.03	100.50
6.0	5.94	99.00
6.0	6.03	100.50
	Average	100.00
	SD	0.94

Table III—Determination of Dipyrone in Pure Form and Tablets by the USSR Pharmacopoeia IX Method

Sample Number	Weight, g.	Recovery, %
	Pure Compound	
1	0.4002	98.07
2	0.3989	101.01
3	0.2000	101.60
4	0.2000	99.04
5	0.2001	100.70
6	0.2007	101.20
7	0.1995	99.29
8	0.1998	99.96
9	0.1833	103.45
	Average	100.48
	SD	1.60
	Tablets	
1	0.5000	99.34
2	0.5000	103.76
3	0.5001	103.08
4	0.5000	103.92
5	0.5010	104.66
6	0.5030	100.68
	Average	102.57
	SD	2.09

Table IV—Determination of Dipyrone in Injections Containing Variable Amounts of Sodium Sulfite by the USSR Pharmacopoeia IX Method

Sample Number	Content of Dipyrone, g.	Content of Sodium Sulfite, mg.	Recovery, %
1	0.5010	00.0	100.81
2	0.5020	14.0	105.73
3	0.5030	23.0	112.67
4	0.5030	38.0	118.11
5	0.5010	48.0	126.79

nitroso derivative of one of its hydrolysis products (27). Scheme I illustrates the suggested mechanism for the formation of such a derivative.

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Optimum Conditions for GLC Analysis of Neomycin

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A reported GLC method (2), which is regularly utilized at The Upjohn Company, appears to be the best, practical method available for the qualitative and quantitative analysis of neomycin fractions, particularly neomycin B and neomycin C isomers which normally predominate. The method had been under evaluation at the National Center for Antibiotic Analysis (NCAA) for some time. Test results by The Upjohn and NCAA laboratories were comparable for the potency values of commercial bulk samples containing a fairly low amount (less than about 5%) of neomycin C. However, there was poor interlaboratory correlation between results for the neomycin C content for most bulk samples. Be-

Table IV—Determination of Dipyrone in Injections Containing Variable Amounts of Sodium Sulfite by the USSR Pharmacopoeia IX Method

Sample Number	Content of Dipyrone, g.	Content of Sodium Sulfite, mg.	Recovery, %
1	0.5010	00.0	100.81
2	0.5020	14.0	105.73
3	0.5030	23.0	112.67
4	0.5030	38.0	118.11
5	0.5010	48.0	126.79

nitroso derivative of one of its hydrolysis products (27). Scheme I illustrates the suggested mechanism for the formation of such a derivative.

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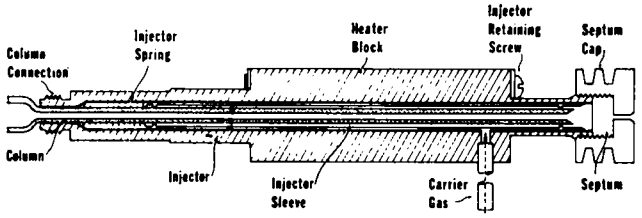

Figure 1—*Modification of injector block and column.*

cause of this discrepancy and other difficulties encountered in the performance of the analysis, a step-by-step critical examination of the procedure was undertaken by the two laboratories in close collaboration. This first resulted in two modifications of the procedure (3), namely: (*a*) use of a 0.61- or 0.91-m. (2- or 3-ft.) column packed with 3% OV-1 on Gas Chrom Q, 100–120 mesh, instead of a 1.83-m. (6-ft.) column with 0.75% OV-1 on Gas Chrom Q; and (*b*) combination of the trilaurin internal standard and the trimethylsilyldiethylamine reagent directly into Tri-Sil Z.

The objective of this article is to discuss the problems associated with this analysis.

EXPERIMENTAL

When the analyses were initially performed at NCAA[1], there were indications of serious sample degradation and solvent (pyridine) tailing. The direct on-column injections with a 15.24-cm. (6-in.) needle, as suggested by the manufacturer, did not alleviate the problem and proved too cumbersome for continuous repetitive application. To counteract these difficulties, the gas chromatograph was modified by drilling out the abutting metal separator ring in the injector block which connects the column to the injector system through a Teflon ferule and a Swagelok. A glass tube, about 14 cm. (5.5 in.) × 4 mm. o.d., was fused to one end of the column, extending it to the septum (Fig. 1)[2].

This column was packed within 1 cm. of the septum and plugged, if desired, with a minimum amount of silanized glass wool, thus minimizing any dead space. Glass wool is not really necessary in the inlet but is customarily used as a plug; it has at times been found to be deleterious to the analysis of silyl derivatives[3]. Caution is necessary when extending this column so that it fits easily into the metal sleeve of the injector block and that no undue strain, which could result in breakage, is applied when tightening the Swageloks.

This system provides a direct on-column injection, eliminates the glass insert, and obviates precolumn contact of sample to metal or glass at such elevated temperatures as 300°, as in this case. Another beneficial side effect is that solvent tailing is materially decreased.

RESULTS

Standard solution mixtures containing neomycin B (USP Reference Standard, Issue 1) and from 0 to 40% (w/w) of neomycin C [8246-DNR-58, 90.5% pure, with no neomycin B present as determined by GLC (4)] and several commercial samples were carefully prepared and analyzed. Since equal detector response was found for each neomycin isomer when used singly, the neomycin C content in all cases was calculated as the fraction of the sum of both peak areas.

Recovery of neomycin C was *not* linear at NCAA, even though the same packing material was used at both laboratories. However, results were reproducible and could be correlated with standard calibration mixtures for quantitative purposes. Further investigations revealed that the incomplete recovery of the neomycin C fractions was actually due to adsorption of the silylated derivatives onto the Teflon disk of the injector seal assembly in the gas chromatograph. Upon removal of this Teflon disk, linearity of recovery of the neomycin C fraction was achieved on an absolute scale, thus eliminating the need for calibrating standard mixtures which are not easily available. Concurrent analysis of several commercial lots in the laboratories of The Upjohn Co. and at NCAA have confirmed the usefulness of the method. The neomycin C values obtained by the triangulation method with the modified gas chromatograph at NCAA correlated well with those obtained with the electronic integrator attached to the other gas chromatograph at Upjohn. A paired *t*-test of the two sets of results listed in Table I showed no statistical difference at the 95% confidence level (calculated $t = 0.67$ for $n = 19$).

Table I—Determination of Neomycin C in Prepared Standard Mixtures and Commercial Bulk Neomycin Sulfate by GLC

Sample	Neomycin C, %: Lab 1[a]	Lab 2[b]
Standard 1	0.0	0.0
Standard 2	3.88	4.65
Standard 3	7.48	8.0
Standard 4	15.2	14.6
Standard 5	24.4	25.9
Standard 6	39.3	38.25
Lot 1	8.1	7.35
Lot 2	8.2	12.6
Lot 3	17.8	16.6
Lot 4	6.1	6.25
Lot 5	10.3	9.7
Lot 6	15.9	14.2
Lot 7	5.4	5.7
Lot 8	12.3	13.2
Lot 9	8.5	8.8
Lot 10	9.4	8.2
Lot 11	6.4	8.8
Lot 12	11.3	11.5
Lot 13	6.1	5.9
Lot 14	12.0	11.83

[a] Percent neomycin C for standards is actual percent weight in weight ratio. Commercial lots were analyzed with a Hewlett-Packard 402 with electronic integrator 3370A. [b] Results were calculated from a modified Perkin-Elmer 900 by triangulation.

Table II—Troubleshooting in GLC Analysis of Neomycin

Problem	Solution
Lot-to-lot variations in moisture content and solubility	Freeze dry and cap promptly
Indication of sample decomposition	Eliminate all metal contacts; ensure a complete glass system
Darkening of packing material in inlet	Use longer silylation time; use minimum amount of silanized glass wool and replace as needed
Asymmetrical neomycin peak	Readjust amount of trimethylsilyldiethylamine
Stability of trimethylsilyl derivative of neomycin	Use sealed reaction vials; store at refrigerated temperatures
Lack of quantitation	Use on-column injections; remove all metal and/or Teflon in inlet system; inject immediately after withdrawing sample from vial; maintain about same amount of sample injected to reproduce peak size and to improve statistics
On-column adsorption of trimethylsilyl derivative of neomycin	Remove Teflon from inlet; condition with trimethylsilyl derivative of neomycin
Short column life	Reduce column temperature (<300°); compensate with increased carrier flow rate

[1] On a Perkin-Elmer 900 gas chromatograph.
[2] These modified columns are now available custom made from Applied Science Labs., Inc., State College, Pa.
[3] W. Supina, Bellefonte, Pa., personal communication, 1971.

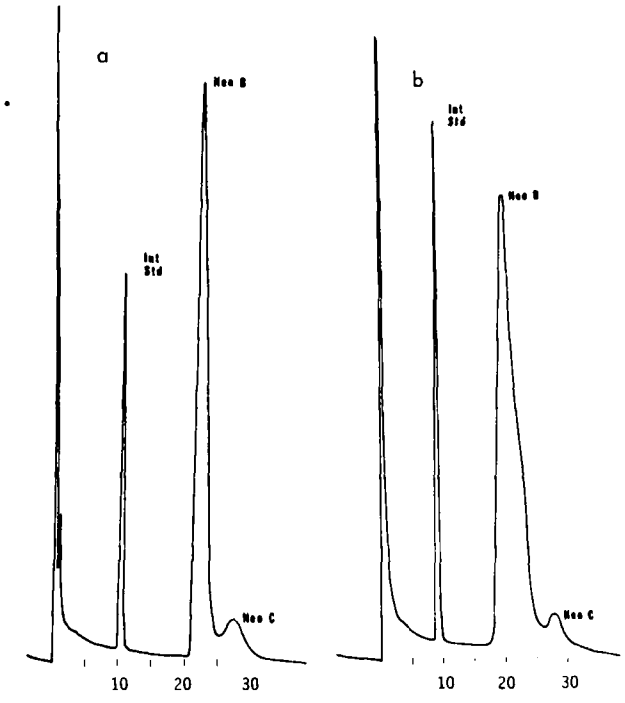

Figure 2—*Chromatogram of trimethylsilyl derivative of neomycin with: (a) proper amount of trimethylsilyldiethylamine, and (b) excess trimethylsilyldiethylamine.*

DISCUSSION

Because of the many potential sources of difficulties, strict adherence to the reported procedure (2, 3) is recommended to obtain reliable results. These problems are enumerated in Table II for convenience; and although each one is of material significance, the following are emphasized:

1. The column must be properly sample conditioned immediately before actual analysis.
2. The amount of trimethylsilyldiethylamine reagent added is critical in the formation of the derivative, as are the temperature and duration of the reaction (3). When the derivative is properly prepared, the neomycin B peak is symmetrical (Fig. 2).
3. Samples should be chromatographed as soon as possible after derivatization or kept refrigerated minimally until time of analysis so as to retain optimal stability. These neomycin derivatives degrade readily (3).

In summary, with proper techniques and caution, the GLC method for analysis of the neomycins can be successfully employed. This method has already been shown to be more specific and reliable than the microbiological method (4, 5). Even with GLC instruments offering poor linearity in recovery, satisfactory results can be obtained when they are correlated with authentic or reference standard calibration mixtures approximating the concentration level to be determined. This type of discussion might prove beneficial in similar analyses of high molecular weight oligosaccharides.

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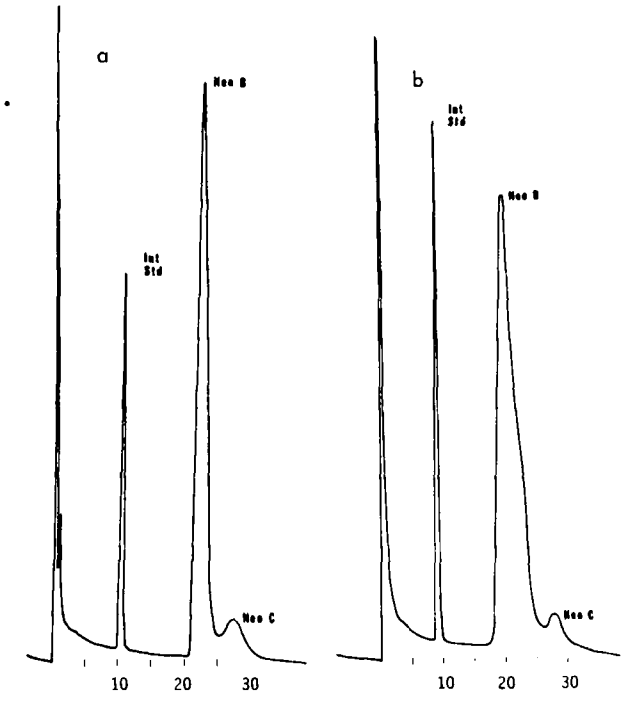
Determination of Copper and Manganese in Vitamin-Mineral Tablets by Atomic Absorption Spectrophotometry

YOUNG SOON CHAE, JAMES P. VACIK, and WILLIAM H. SHELVER▲

Abstract □ Atomic absorption spectrophotometry was utilized in the determination of copper and manganese in five different combination multiple-vitamin mineral tablets. The analysis of each tablet by direct determination was compared with analysis by the method of additions, and some small but statistically significant differences were noted. No molecular absorption was found in the analysis of manganese utilizing the nonresonance line of lead. The direct analysis of manganese and copper was simple and precise.

Keyphrases □ Copper—determination in multiple-vitamin mineral tablets by atomic absorption spectrophotometry □ Manganese—determination in multiple-vitamin-mineral tablets by atomic absorption spectrophotometry □ Atomic absorption spectrophotometry—determination of copper and manganese in vitamin-mineral tablets □ Vitamin-mineral tablets—determination of copper and manganese by atomic absorption spectrophotometry □ Minerals, in multiple-vitamin tablets—determination of copper and manganese by atomic absorption spectrophotometry

Because of their complex nature, multiple-vitamin mineral tablets present a formidable problem in analytical chemistry. The analysis of specific metals in the presence of numerous other minerals and a complicated organic matrix requires either complex separation schemes or an extremely specific analytical method to



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Because of their complex nature, multiple-vitamin mineral tablets present a formidable problem in analytical chemistry. The analysis of specific metals in the presence of numerous other minerals and a complicated organic matrix requires either complex separation schemes or an extremely specific analytical method to

Table I—Instrument Parameters

Item	Multielement Lamp	Lead Lamp
Wavelength	324.7 nm., copper; 279.5 nm., manganese	280.3 nm.
Hollow cathode lamp current	30 ma.	35 ma.
Fuel	Lean acetylene (flowmeter at 27)	Lean acetylene (flowmeter at 27)
Oxidizer	Air (flowmeter at 55)	Air (flowmeter at 55)
Aspiration rate	6.3 ml./min.	6.3 ml./min.
Slit	No. 3 (2 nm.)	No. 3 (2 nm.)
Digital readout	100 average mode	100 average mode

obtain meaningful results. Atomic absorption spectrophotometry promises to be a simple and an extremely specific analytical technique with sufficient precision for pharmaceutical analysis. Despite these desirable qualities, atomic absorption spectrophotometry has not been widely utilized in pharmaceutical analysis (1). Calcium (2), copper (3), iron (4), and cobalt (5) have been analyzed in pharmaceutical preparations by atomic absorption spectrophotometry, but none of these metals has been determined in multiple-vitamin–mineral tablets by this technique.

Copper has been analyzed (3) by atomic absorption spectrophotometry in ointments and in a tablet containing copper, cobalt, and iron with good precision and no indication of interferences. The effects of fuel mixtures and of mineral acids on the atomic absorption analysis of copper and manganese have been carefully studied (6). The extension of these studies to the analysis of copper and manganese in multiple-vitamin–mineral tablets seems feasible, although the analysis of manganese apparently has not been reported by atomic absorption spectrophotometry in any pharmaceutical products.

EXPERIMENTAL

Instrument—A double-beam spectrophotometer[1], equipped with a multielement (Mn–Cu–Co Cr–Ni) hollow cathode lamp and a single-slot burner head, was used for all atomic absorption measurements except for the two-line method where a single-element lead lamp was utilized. The instrument parameters are given in Table I.

Reagents—Standard stock solutions of manganese[2] and copper[2] contained 1000 and 10,000 p.p.m., respectively. All other reagents were ACS or USP grade. Distilled water was deionized by being passed through two mixed-bed resin columns, and it was used to make all solutions and to rinse all glassware.

Direct Method—Standard curves (Figs. 1 and 2) were obtained as follows. Manganese or copper solutions were prepared for calibration by diluting the standard stock solution to obtain concentrations of 0.5, 1.00, 2.00, 3.00, and 3.50 p.p.m. or 1.00, 2.00, 3.00, 4.00, and 5.00 p.p.m. respectively. Both standards were made up to contain 1% (w/v) hydrochloric acid. The instrument was optimized as to lamp position and the position of the burner head. The absorbances of the standard solutions of the element were determined in the 100 average mode with a minimum of three such readings. The regression line of the absorbance–concentration curves through zero was determined using a calculator[3], and the calibration curve was plotted. The calibration curves were determined each day that analytical runs were made by the direct method.

Twenty tablets of each preparation were accurately weighed to determine the average tablet weight. The tablets were crushed and ground to a fine powder with a mortar and pestle. A portion of each tablet powder equivalent to sufficient metal to give an absorbance between 0.100 and 0.300 was accurately weighed, transferred into a 100-ml. beaker, and dissolved in sufficient 10% (w/v) hydrochloric acid to yield a final concentration of 1% (w/v) when diluted. Each solution was filtered through filter paper[4] into a 100- or 200-ml. volumetric flask and diluted to the mark with deionized distilled water through the filter paper. Four separately weighed powdered samples were prepared from each different batch of tablets. The instrument was warmed for a minimum of 4 hr., and the blank solution [1% (w/v) hydrochloric acid solution] was aspirated for a minimum of 15 min. prior to any analytical procedure.

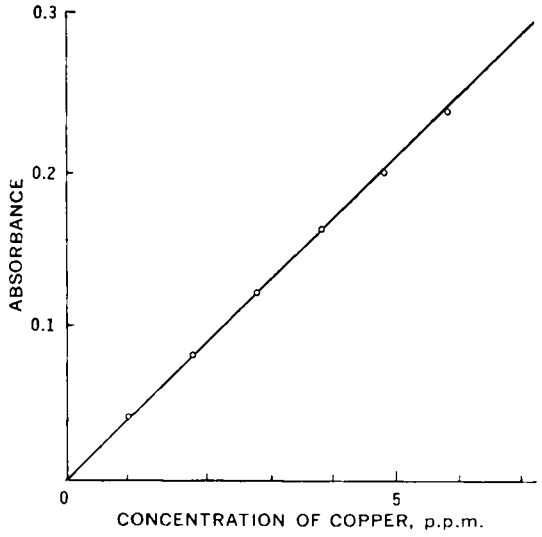

Figure 1—*Typical concentration–absorbance relationship for copper standard at 324.7 nm. Slope of the line = 0.0405 ± 0.0020. Sensitivity = 0.107 mcg./ml. The analysis was carried out on a spectrophotometer (see text) with a slit of 0.2 nm. and the readout in the 100 average mode.*

After aspirating the blank solution and setting the instrument to zero by means of the autozero function, the solution to be analyzed was aspirated and the absorbance was determined using the 100 average function. The first reading was discarded, and at least four successive readings were taken. The instrument was zeroed by aspirating blank solution prior to reading the next solution. The calibration curve and the analytical readings for all samples were taken for one element before changing the wavelength and carrying out similar analyses for the second element. Because of the different metal contents of the tablets and different instrument responses to manganese and copper, some samples required more dilute analytical solutions for manganese. The calculation of tablet content was carried out using the following equation:

$$\text{tablet content} = \frac{\text{absorbance} \times \text{volume analytical solution} \times \text{average tablet weight} \times \text{dilution factor}}{\text{slope of calibration line} \times 1000 \times \text{sample weight}} \quad \text{(Eq. 1)}$$

The mean and standard deviation (Tables II and III) for each tablet were calculated with a programmable calculator[5].

Method of Additions—The same analytical solutions and standard stock solutions used in the direct method were used in preparing solutions for analysis by the method of additions. Three solutions were used for each analysis, containing the same quantity (10 ml.) of tablet analytical solution and 0, 10, or 20 ml. of 10 p.p.m. copper standard solution or 0, 5, or 15 ml. of 10 p.p.m. manganese standard solution. The resulting solutions were diluted to 50.0 ml. with 1% (w/v) hydrochloric acid solution. The quantity of tablet analytical solution provided maximum absorbance for the solution containing the highest quantity of metal without exceeding the linear portion of the calibration curve (3.5 p.p.m. for manganese and 6 p.p.m.

[1] Perkin-Elmer model 403.
[2] Fisher certified reagents.
[3] Hewlett-Packard model 10.
[4] Whatman No. 2.
[5] Hewlett-Packard model 10 equipped with a statistical ROM.

Table II—Analysis of Tablets for Copper Content

Tablet	Amount of Copper Declared, mg.	Amount of Copper Found by Direct Determination[a] mg.	%	% SD	Amount of Copper Found by Method of Additions[b] mg.	%	% SD	F
1	1.0	1.032	103.2	2.8	1.107	110.7	6.6	4.391
2	2.0	2.090	104.5	4.2	2.244	112.2	5.4	5.101
3	1.0	0.936	93.6	5.6	0.964	96.4	5.8	0.478
4	1.0	1.084	108.4	1.8	1.140	114.0	7.9	1.902
5	0.75	0.747	99.6	0.5	0.773	103.0	1.2	29.039[c]

[a] The results represent an average of four determinations. [b] The results represent an average of four determinations, each one consisting of the extrapolation of the least-squares line to the *X*-intercept at zero absorbance. The least-squares line was derived from absorbance measurements at three different copper concentrations. [c] Significant at the 0.01 level.

Table III—Analysis of Tablets for Manganese Content

Tablet	Amount of Manganese Declared, mg.	Amount of Manganese Found by Direct Determination[a] mg.	%	% SD	Amount of Manganese Found by Method of Additions[b] mg.	%	% SD	F
1	1.0	1.040	104.0	2.9	1.075	107.5	0.5	4.101
2	1.0	1.244	124.4	3.4	1.213	121.3	6.9	0.561
3	1.5	1.377	91.8	1.7	1.456	96.7	3.5	7.303[c]
4	1.0	0.991	99.1	1.3	1.066	106.6	2.1	33.836[d]
5	1.25	1.456	116.5	0.7	1.400	112.0	1.6	18.759[d]

[a] The results represent an average of four determinations. [b] The results represent an average of four determinations, each one consisting of the extrapolation of the least-squares line to the *X*-intercept at zero absorbance. The least-squares line was derived from absorbance measurements at three different manganese concentrations. [c] Significant at the 0.05 level. [d] Significant at the 0.01 level.

for copper). The absorbance of each solution was read as described under the *Direct Method*. The content of the original solution was calculated by determining the regression line for the three solutions using the absorbance as the *Y*-value and the parts per million of the metal added for the *X*-coordinate. The negative of the *Y*-intercept divided by the slope gave the content in parts per million for the original solution. The tablet content can thus be calculated by Eq. 2:

$$\text{tablet content} = \frac{\text{p.p.m. metal} \times \text{volume analytical solution} \times \text{average tablet weight}}{1000 \times \text{sample weight}} \qquad \text{(Eq. 2)}$$

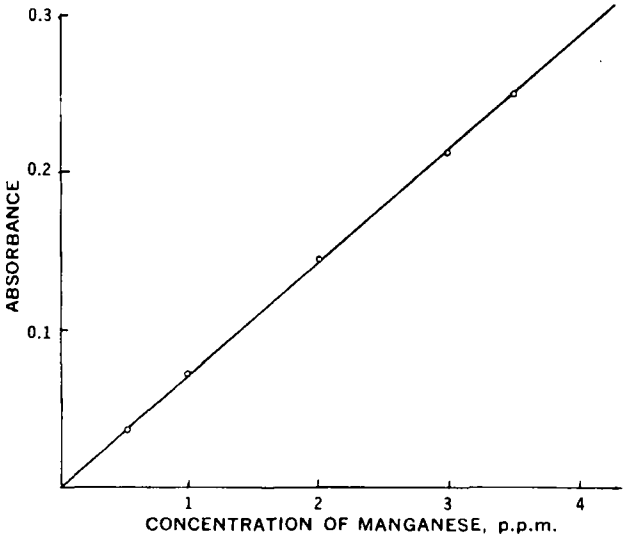

Figure 2—*Typical concentration–absorbance relationship for manganese standard at 279.5 nm. Slope of the line = 0.0713 ± 0.0012. Sensitivity = 0.061 mcg./ml. The analysis was carried out on a spectrophotometer (see text) with a slit of 0.2 nm. and the readout in the 100 average mode.*

The mean and the standard deviation for each tablet were determined as already described.

Two-Line Method—To examine the problem of possible molecular absorbance, a nonresonance line near the analytical wavelength was needed. Lead has a nonabsorbing line at 280.3 nm. near the analytical wavelength of manganese (279.5 nm.). Measurement of the absorbance of all standard solutions and of all analytical solutions using the lead line revealed no solutions with absorbances greater than 0.001 unit. Since absorbances this low would not have appreciable effects on the calculated results, the experiment was terminated.

Recovery Experiments—The recovery of copper and manganese was found by taking an aliquot of the tablet solution, diluting, and reading and then taking the same aliquot of the tablet solution, adding standard solutions of copper and manganese, diluting, and reading. The difference between the readings was divided by the slope of the calibration line for the respective element, and the resulting parts per million (measured) was divided by the amount added (theoretical) and multiplied by 100 to obtain the percent recovery (Table IV).

RESULTS AND DISCUSSION

Since there exists the possibility of matrix effects in the analysis of copper and manganese in the complex mixture produced by a multiple-vitamin tablet, three independent atomic absorbance techniques were selected to check the validity of the analysis: direct analysis, the method of additions, and the use of a nonresonance line adjacent to the manganese line.

The use of the nonresonance line of lead at 280.3 nm. near the analytical line for manganese at 279.5 nm. resulted in a maximum absorbance of 0.001 absorbance unit. The negligible absorbance in

Table IV—Recovery of Metals from Analysis of Multiple-Vitamin–Mineral Tablets

	Mean[a]	SD	Recovery, %
Copper	1.044	0.042	104.4
Manganese	1.018	0.006	101.8

[a] Each value represents the mean of 10 determinations.

all samples indicated that molecular absorbance was not interfering with the analysis of manganese in this procedure. The possibility of molecular absorption occurring at the analytical wavelength of copper seems small since the copper analytical wavelength is not far from the manganese line. No calculations are reported for the two-line method since the results would not differ from the results of the direct method.

A typical standard curve for copper (Fig. 1) reveals good linearity up to 6 p.p.m. The slope of the line gives a sensitivity of 0.107 mcg./ml., in agreement with reported (7) values, and the standard deviation of the slope indicated suitable accuracy. The results of the analysis of the tablets for copper are shown in Table II. Both the direct method (3% average *SD*) and the method of additions (5% average *SD*) show reasonable precision. Probably some error is produced by difficulties in obtaining homogeneous samples of the vitamin tablets, and this is supported by the differences in precision among the different tablets. The ability to obtain a homogeneous sample would depend upon the particular formulation of the tablet. Some tablets were extremely difficult to reduce to a fine powder while others were powdered with comparative ease. This method of sampling in the analysis of iron has been reported to give a higher variation than an ashing procedure (4). The increased error in the method of additions can be anticipated because of the larger number of pipetings and the extrapolation procedure required. Statistical comparisons of the two methods indicate that Tablet 5 showed a significant difference in the two analytical methods. Inspection of Table II shows that the significance was produced by the unusually small standard deviations associated with this tablet. Other workers (2) found a greater variance produced by the method of additions. The slightly higher results from the method of additions for the analysis of copper cannot be presently explained.

A typical standard curve for manganese (Fig. 2) was linear up to 3.5 p.p.m., and the sensitivity (0.061 mcg./ml.) agrees well with reported (7) values. The standard deviation of the slope is within acceptable limits. The results of the analysis of manganese in multiple-vitamin–mineral tablets (Table III) are more precise than the copper analysis. The average errors in the direct determination and in the method of additions (2 and 3% average *SD*, respectively), are extremely satisfactory. Unlike the copper analysis, there seems no direction in the differences in the two methods (Tablets 2 and 5 are lower by the method of additions and Tablets 1, 3, and 4 give higher results by the method of additions). The existence of the small, but significant, differences in the results of the two methods of determination might indicate a small matrix effect. Since the differences between the methods are small, the more convenient direct method can be used for all but the most crucial applications.

At the beginning of the project, the decision was made to produce the analytical solution by grinding the tablet and filtering the resulting suspension, thus avoiding ashing or digesting of the sample. This procedure obviously introduced some variation into the results; but from the results of Tables II and III, this effect cannot exceed 3% and is probably much less. By avoiding digestion or ashing, a solution is produced that can be utilized in the analysis of the vitamins soluble in 1% acid media, minimizing the number of solutions needed for the quality control of the multiple vitamins. The results (Table IV) indicate acceptable recovery for both manganese and copper and again demonstrate the smaller variability of the manganese analysis. The determination of manganese and, to a slightly lesser degree, copper by direct atomic absorption spectrophotometry is a simple and precise method of analyzing these metals in multiple-vitamin–mineral tablets.

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Determination of Chloramphenicol Palmitate in Pharmaceutical Suspensions

SACHCHIDANANDA BANERJEE▲, KALYAN CHAKRABARTI, and ARUP KUMAR HALDAR

Abstract □ Three simple procedures for the quantitative determination of chloramphenicol palmitate in commercially available chloramphenicol palmitate oral suspensions are presented. One method is a modification of the USP method in which the suspension residue is washed with water prior to the USP specified steps of dissolving the residue in chloroform and determining the quantity spectrophotometrically. The second method takes advantage of the presence of two chlorine atoms in the side chain of chloramphenicol which can be converted into alkali chloride to be estimated potentiometrically with a standard silver nitrate solution. The third method is based on the liberation of chloramphenicol from chloramphenicol palmitate by treatment with dilute alkali at room temperature and microbiological assay of chloramphenicol using *Escherichia coli* (ATCC 10536). The potentiometric and microbiological assay methods were equally suitable for determination of chloramphenicol palmitate in all of the different suspensions tested. The microbiological assay method was more specific for the active antibiotic, was less cumbersome, and took less time.

Keyphrases □ Chloramphenicol palmitate—three methods of analysis in pharmaceutical suspensions □ Suspensions, chloramphenicol palmitate—three methods of analysis □ Antibiotics—analysis of chloramphenicol palmitate in pharmaceutical suspensions

In chloramphenicol palmitate oral suspensions containing 0.5% vanillin as the flavoring agent, the potency of chloramphenicol palmitate, as determined by the USP spectrophotometric method (1, 2), was always high. The results varied between 116 and 133% of the labeled content. However, the USP method was suitable

all samples indicated that molecular absorbance was not interfering with the analysis of manganese in this procedure. The possibility of molecular absorption occurring at the analytical wavelength of copper seems small since the copper analytical wavelength is not far from the manganese line. No calculations are reported for the two-line method since the results would not differ from the results of the direct method.

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In chloramphenicol palmitate oral suspensions containing 0.5% vanillin as the flavoring agent, the potency of chloramphenicol palmitate, as determined by the USP spectrophotometric method (1, 2), was always high. The results varied between 116 and 133% of the labeled content. However, the USP method was suitable

for suspensions identical in other respects but containing flavoring agents such as soluble essence raspberry, essence apricot, or essence American ice cream. Vanillin is a commonly used and highly acceptable flavoring agent, and the use of a flavoring agent in the chloramphenicol palmitate oral suspension is permissible according to USP (3). It was, therefore, necessary to modify the USP method to make it useful for the assay of chloramphenicol palmitate suspensions containing vanillin.

Chloramphenicol palmitate cannot be estimated by the official method (1, 2) in combination with other drugs such as streptomycin, tetracycline, nitrofurantoin, sulfa drugs, or B vitamins. Several other methods have been suggested for the estimation of chloramphenicol palmitate in pharmaceutical formulations, including colorimetry (4, 5), reverse phase TLC (6), and column chromatography (7), but these methods are not always suitable for chloramphenicol palmitate in combination with other drugs. The polarographic method for chloramphenicol palmitate in commercial preparations cannot be used because of its solubility characteristics (8). A potentiometric method based on the chloride determination has been reported (9). Chloramphenicol contains two chlorine atoms in the side chain which can be converted into alkali chloride when refluxed with alkali and can be potentiometrically titrated with 0.1 *N* silver nitrate. This method can be extended to different suspensions of chloramphenicol palmitate in combination with the drugs mentioned previously.

Chloramphenicol palmitate has no antimicrobial activity and cannot be estimated by microbial assay. However, the drug can be hydrolyzed by alkaline hydrolysis and the liberated chloramphenicol can be determined microbiologically by measuring the zones of inhibition using *Bacillus subtilis* (10). Enzymatic hydrolysis of chloramphenicol palmitate by pancreatin (11–13) and the estimation of chloramphenicol by analyzing for total amyl nitro compounds by colorimetry has been reported (11). Although total hydrolysis by pancreatin (13) and by lipase (13, 14) has been described, the complete hydrolysis of chloramphenicol palmitate with pancreatin could not be obtained in the present experiments. Certain crystalline forms of chloramphenicol palmitate are not hydrolyzed or are only incompletely hydrolyzed (15, 16).

Amorphous chloramphenicol palmitate was used in these studies, and the purity of the substance was determined by IR spectrophotometry. Therefore, incomplete hydrolysis was not due to the nature of the chloramphenicol palmitate used. However, it might be due to the pancreatin used because enzyme activity is likely to vary from sample to sample. Because microbial methods are more sensitive than chemical assays and more specific for the active antibiotic, a microbiological method of assay of chloramphenicol palmitate in suspensions with or without other drugs or flavoring agents would be desirable. Therefore, a microbiological assay method of chloramphenicol palmitate in different formulations after alkaline hydrolysis was developed.

Table I—Results[a] of Spectrophotometric Assay of Chloramphenicol Palmitate in Chloramphenicol Palmitate Oral Suspension USP[b]

	Samples Containing Vanillin, mg./ml.		Identical Samples without Vanillin, mg./ml.	
Sample	USP Procedure	Modified USP Procedure	USP Procedure	Modified USP Procedure
1	37.1	30.7	30.4	30.4
2	40.0	32.6	31.7	31.9
3	38.6	32.8	30.4	31.2
4	37.0	30.4	31.5	31.4
5	36.8	30.7	34.6	34.6
6	39.0	31.6		
7	38.5	30.0		
8	41.5	32.5		
9	39.0	32.5		
10	38.5	31.2		

[a] Amount of chloramphenicol base contained in chloramphenicol palmitate, milligrams per milliliter. [b] One milliliter suspension contained chloramphenicol palmitate equivalent to 31.25 mg. chloramphenicol base.

EXPERIMENTAL

Determination of Chloramphenicol Palmitate by Modified USP Method—Four milliliters chloramphenicol palmitate oral suspension, equivalent to 125 mg. chloramphenicol, containing 20 mg. vanillin was quantitatively transferred to a sintered-glass crucible No. 4; 10 ml. water was added and the contents were quickly filtered by suction. The residue on the filter was washed twice with 10 ml. water by suction. The sintered crucible containing the residue was fitted into a dry 250-ml. filtering flask. The residue was dissolved in chloroform and the solution was collected in the filtering flask by suction, quantitatively transferred to a 250-ml. volumetric flask, and diluted to volume with chloroform. The remainder of the procedure was as described in the *Code of Federal Regulations* (2) commencing with: "Pipette 4 milliliters of the clear chloroform solution into a 100 milliliter volumetric flask . . ." The results of analysis of 10 samples by the USP method (1, 2) and the modified USP method are given in Table I.

Chloramphenicol palmitate oral suspensions identical in all respects to these samples but not containing vanillin were analyzed by both the USP method and the modified method (Table I). Recovery experiments were performed for the modified method to show that chloramphenicol palmitate was not dissolved by the additional washing procedure (Table II).

Determination of Chloramphenicol Palmitate in Suspensions Containing Chloramphenicol Palmitate in Combination with Other Drugs by Potentiometric Method—Ten to 20 ml. of the suspension, equivalent to about 300 mg. chloramphenicol, was quantitatively transferred to a 500-ml. round-bottom flask to which 20 g. sodium hydroxide and 80 ml. water were subsequently added. The mixture was refluxed on an open flame for 4 hr., cooled, acidified with nitric acid, quantitatively transferred to a 500-ml. volumetric flask, and diluted to volume with water. Two hundred milliliters of the solution was transferred to a 500-ml. beaker, and the alkali chloride solution was titrated with 0.1 *N* silver nitrate potentiometrically using silver–silver chloride and calomel electrodes. The total volume of 0.1 *N* silver nitrate required for 500 ml. was calculated. For a blank titration, the same volume of the sample as taken for alkaline hydrolysis was transferred to a 500-ml. beaker to which 5 ml. nitric acid and 80 ml. water were added. The mixture

Table II—Recovery of Chloramphenicol Palmitate by the Modified USP Method

Amount of Chloramphenicol Base Contained in Added Chloramphenicol Palmitate, mg.	Chloramphenicol Estimated, mg.	Recovery, %
123.5	120.2	97.3
98.56	96.8	98.2
122.0	119.0	97.5
124.0	124.0	100.0
101.0	100.0	99.0

Table III—Determination of Chloramphenicol Palmitate in Suspensions of Differing Compositions[a]

Dosage Form	Microbiological Assay after Alkaline Hydrolysis, mg./ml.	Potentiometric Titration, mg./ml.	t
Chloramphenicol palmitate (equivalent to 31.25 mg. chloramphenicol/ml.) and streptomycin sulfate (equivalent to 31.25 mg. streptomycin/ml.)	31.7 ± 0.905	32.0 ± 0.638	0.27
Chloramphenicol palmitate (equivalent to 15.625 mg. chloramphenicol/ml.) and tetracycline (equivalent to 15.625 mg. tetracycline hydrochloride/ml.)	15.3 ± 0.501	15.4 ± 0.228	0.18
Chloramphenicol palmitate (equivalent to 31.25 mg. chloramphenicol/ml.) and nitrofurantoin (6.25 mg./ml.)	30.5 ± 0.308	31.0 ● 0.412	0.99
Chloramphenicol palmitate (equivalent to 15.625 mg. chloramphenicol/ml.), sulfadiazine (20 mg.), and phthalylsulfathiazole (15 mg./ml.)	15.6 ± 0.233	15.3 ± 0.241	0.90
Chloramphenicol palmitate (equivalent to 31.25 mg. chloramphenicol/ml.), thiamine hydrochloride (0.1 mg.), riboflavin (0.05 mg.), pyridoxine hydrochloride (0.05 mg.), niacinamide (1.5 mg.), cyanocobalamin (1.5 mcg.), and dexpanthenol (0.1 mg./ml.)	33.4 ± 0.659	32.2 ± 0.247	1.70

[a] Amount of chloramphenicol base contained in chloramphenicol palmitate, milligrams per milliliter. Values are mean of 10 observations ± standard error.

was titrated potentiometrically with 0.1 *N* silver nitrate. The difference between the two titrations represented the amount of chloride liberated from chloramphenicol. Each milliliter of 0.1 *N* silver nitrate is equivalent to 0.01616 g. of chloramphenicol. The results of analysis of different suspensions of chloramphenicol palmitate in combination with other drugs are given in Table III.

Determination of Chloramphenicol Palmitate in Suspensions by Microbiological Assay after Alkaline Hydrolysis—One milliliter of chloramphenicol palmitate oral suspension, equivalent to 31.25 mg. chloramphenicol, was transferred to a 25-ml. volumetric flask (the pipet was washed three times with water) and diluted to volume with ethanol. One milliliter of the ethanolic solution was transferred to a 200-ml. volumetric flask, treated with 10 ml. 0.01 *N* sodium hydroxide in ethanol, and kept at room temperature for 15 min. The solution was neutralized with 0.01 *N* hydrochloric acid and diluted to volume with 0.2 *M* phosphate buffer, pH 6.0. The liberated chloramphenicol was estimated microbiologically using *Escherichia coli* (ATCC 10536) as described in the *Code of Federal Regulations* (18) (Table IV), and recovery experiments were performed to determine the accuracy of the method (Table V).

Determination of Chloramphenicol Palmitate in Suspensions by Microbiological Assay after Enzymatic Hydrolysis—One milliliter chloramphenicol palmitate oral suspension, equivalent to 31.25 mg. chloramphenicol, was diluted to 100 ml. with water. One milliliter of the diluted suspension was transferred to a 100-ml. volumetric flask containing a suspension of 100 mg. pancreatin in 9 ml. of either tromethamine buffer, pH 9.0, or simulated intestinal fluid, pH 7.5. The mixture was incubated under toluene at 37° for 16 hr. and diluted to volume with 0.2 *M* phosphate buffer, pH 6.0; chloramphenicol was estimated microbiologically with *E. coli* (Table V).

Determination of Chloramphenicol Palmitate in Suspensions Containing Chloramphenicol Palmitate in Combination with Other Drugs by Microbiological Assay—The different dosage forms used are described in Table III. With the exception of the preparation containing tetracycline, all of the other preparations were hydrolyzed with 0.01 *N* sodium hydroxide in ethanol and chloramphenicol was estimated microbiologically as described earlier. For suspensions containing tetracycline, 2 ml. of the suspension, equivalent to 31.25 mg. chloramphenicol, was transferred to a 100-ml. centrifuge tube, mixed with 50 ml. of 0.1 *N* hydrochloric acid, and centrifuged at 10,000 r.p.m. for 15 min. The supernate was decanted and the residue was washed twice with 50 ml. 0.1 *N* hydrochloric acid by centrifugation. The residue contained chloramphenicol palmitate free from tetracycline as determined by microbiological assay. The residue was dissolved in ethanol and diluted to 25 ml. One milliliter of the ethanolic solution was hydrolyzed with 0.01 *N* sodium hydroxide in ethanol, and chloramphenicol was estimated microbiologically as described earlier (Table III).

Table IV—Results[a] of Microbiological Assay of Chloramphenicol Palmitate in Chloramphenicol Palmitate Oral Suspension USP[b]

Sample	After Alkaline Hydrolysis, mg./ml.	After Enzymatic Hydrolysis with Pancreatin, mg./ml. In Simulated Intestinal Fluid, pH 7.5	After Enzymatic Hydrolysis with Pancreatin, mg./ml. In Tromethamine Buffer, pH 9.0
1	30.2	24.1	21.2
2	32.6	24.1	25.0
3	32.5	24.1	25.0
4	30.0	25.0	27.0
5	31.0	24.1	27.0
6	30.0	24.0	21.2
7	31.0	27.0	28.0
8	35.0	26.0	28.0
9	32.5	26.0	28.0
10	31.0	26.0	26.2

[a] Amount of chloramphenicol base contained in chloramphenicol palmitate, milligrams per milliliter. [b] One milliliter suspension contained chloramphenicol palmitate equivalent to 31.25 mg. chloramphenicol base.

RESULTS AND DISCUSSION

Table I shows that the estimation of chloramphenicol palmitate by the USP method gave results 16–33% higher when the suspen-

Table V—Recovery of Chloramphenicol Palmitate by the Microbiological Assay Method after Alkaline Hydrolysis

Amount of Chloramphenicol Base Contained in Added Chloramphenicol Palmitate, mg.	Chloramphenicol Estimated, mg.	Recovery, %
16.20	18.0	111
14.40	15.0	104
15.25	14.6	96
32.10	31.8	99
29.13	32.0	110
31.24	28.1	90

sion contained 0.5% vanillin, a commonly used flavoring agent. If the method of extraction of the suspension is modified by washing the sample with water on a sintered crucible, dissolving the residue in chloroform, and determining the absorbance of the chloroform solution diluted with ethanol at 271 nm. according to the official method, the results obtained are very close to the labeled amount of chloramphenicol palmitate. According to the USP (3), chloramphenicol palmitate is insoluble in water, but Weiss *et al.* (17) reported that chloramphenicol palmitate is very slightly soluble in water, 1.05 mg./ml. water at 28°. According to them, to test the solubility of the drug, it must be shaken thoroughly for 2 min. The present authors observed that when the suspension was washed with three 10-ml. aliquots of water in quick succession in a sintered crucible No. 4 by suction, there was practically no loss of chloramphenicol palmitate (Table II). Washing on a sintered crucible is less cumbersome and more effective in removing substances likely to interfere with the spectrophotometric estimation of chloramphenicol palmitate than extracting with chloroform in a separator, which did not remove vanillin. When chloramphenicol palmitate suspension identical in all respects but without vanillin was estimated by both the USP method and the suggested modification, similar results were obtained (Table I). This further indicates that chloramphenicol palmitate is not eliminated by the water-washing procedure.

Suspensions of chloramphenicol palmitate were prepared with flavoring agents other than vanillin such as essence raspberry, essence American ice cream, or essence apricot. These essences did not interfere with the estimation of chloramphenicol palmitate by the USP method. Chloramphenicol palmitate oral suspension USP marketed in the United States may not contain vanillin. The preparation[1] marketed in India does not contain vanillin. Furthermore, the USP gives only a lower limit of the requirement of chloramphenicol palmitate in the suspension without specifying the higher limit. These reasons may explain the lack of this particular problem in the United States. In the light of these observations, the USP method of estimation of chloramphenicol palmitate may be suitably modified.

However, the modified USP method is not suitable for estimation of chloramphenicol palmitate in suspension when it is in combination with other drugs such as tetracycline, streptomycin, nitrofurantoin, sulfa drugs, and B vitamins. Drastic treatment of the suspension with sodium hydroxide to convert the chloride of chloramphenicol molecule to sodium chloride and determination of chloride with silver nitrate by potentiometric titration was found suitable for the estimation of chloramphenicol palmitate in the different dosage forms studied (Table III). The usefulness of this method, however, is severely limited because it is nonspecific for chloramphenicol and more complicated than other methods.

Chloramphenicol palmitate in suspensions, with or without other drugs, could be easily hydrolyzed with dilute alkali at room temperature to make chloramphenicol available for microbial assay. The presence of streptomycin, nitrofurantoin, sulfa drugs, or B vitamins in the concentrations present (Table III) had no effect on the test organism. In chloramphenicol palmitate suspension containing tetracycline, the latter must be removed because of its interference in the microbiological assay of chloramphenicol. The removal was carried out by washing with dilute hydrochloric acid, which did not remove chloramphenicol palmitate. The results of the microbiological assay with *E. coli* (Tables III and IV) and the recovery of added chloramphenicol palmitate (Table V) indicate the suitability of the method for the routine assay of chloramphenicol palmitate in the different forms of suspensions tested. The flavoring and coloring agents or the presence of streptomycin, nitrofurantoin, tetracyclines, sulfa drugs, or B vitamins did not interfere in the microbiological assay of chloramphenicol.

Since chloramphenicol palmitate is dissolved in ethanol prior to alkaline hydrolysis, polymorphism of the drug disappears. Therefore, the method of assay would not distinguish the different polymorphic forms, if present. This, however, is not a disadvantage as compared to the USP method which also does not take into consideration the polymorphic forms of chloramphenicol palmitate.

Enzymatic hydrolysis of chloramphenicol palmitate in suspension with pancreatin, either in simulated intestinal fluid (pH 7.5) or in tromethamine buffer (pH 9.0), released only about 80% of chloramphenicol from chloramphenicol palmitate in the present experiments (Table IV). Earlier reports, however, indicated complete enzymatic hydrolysis of chloramphenicol palmitate (13, 14). Our failure to obtain complete hydrolysis might be due to the pancreatin used whose enzyme activity is likely to vary from sample to sample. The data in Table IV show variations in hydrolysis, 21–28 mg./ml., indicating nonuniformity of enzyme action. Enzymatic hydrolysis followed by microbiological assay of chloramphenicol, therefore, may not always be suitable for the routine determination of chloramphenicol palmitate. In any case, hydrolysis of chloramphenicol palmitate with dilute alkali is much simpler than the enzymatic hydrolysis. Enzymatic hydrolysis, however, can indicate the presence of polymorph A crystals of chloramphenicol palmitate which is only hydrolyzed 0–5% (15, 16).

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Acetonitrile–Diatomaceous Earth Column for Separation of Steroids and Other Compounds

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Abstract □ The retention characteristics of 53 compounds on an acetonitrile–diatomaceous earth column are given for elution with *n*-heptane followed by stripping with chloroform. Nineteen of the compounds were eluted completely, seven were eluted partially, and 27 (including the 12 corticosteroids tested) were not eluted at all by 150 ml. of *n*-heptane. Partially eluted compounds could be completely eluted by larger volumes of *n*-heptane. Elution characteristics in a group of similar compounds are affected greatly by relatively minor changes in structure. The recommended procedure furnishes a simple and rapid method for the separation of mixtures and for the detection of impurities or decomposition in standards. Several pharmaceutical preparations and known mixtures were analyzed as examples of the application of the column procedure to various products.

Keyphrases □ Steroids—separation using acetonitrile–diatomaceous earth column, retention characteristics of 53 compounds, detection of impurities □ Column chromatography—separation of steroids on acetonitrile–diatomaceous earth column, retention characteristics of 53 compounds, detection of impurities □ Acetonitrile–diatomaceous earth column—separation of steroids □ Diatomaceous earth–acetonitrile column—separation of steroids □ Chromatography, column—separation of steroids using acetonitrile–diatomaceous earth column, retention characteristics of 53 compounds, detection of impurities

The separation of mixtures of steroids and of steroids from other compounds in pharmaceuticals can be difficult if the preparation is other than the official one in USP XVIII (1) or NF XIII (2). Both aqueous and nonaqueous immobile phase systems are available for use with diatomaceous earth, but aqueous systems are the most popular for the separation of steroids (3). Examples of nonaqueous systems include the use of kieselguhr treated with dichlorodimethylsilane vapors to retain the less polar component of many solvent systems used in the separation of C_{12}–C_{18} fatty acids (4). This procedure is known as reverse phase chromatography and was used (5) for the analysis of progesterone in oil injectables. An acetonitrile immobile phase was suggested (6) for the separation of corticosteroids from decomposition products and interfering substances in the analysis of pharmaceuticals containing corticosteroids; this column was used also for the separation of estradiol valerate and estradiol isovalerate from estradiol[1].

This paper reports the elution characteristics of 53 compounds from the acetonitrile immobile phase column and the application of such a column to the separation of steroids of similar structure and to the analysis of pharmaceuticals containing either more than one steroid or interfering substances. The method is simple, flexible, and rapid; three determinations can be completed in approximately 3 hr., and the column can be modified for the removal of acidic, basic, and other water-soluble impurities by using trap layers (6).

[1] T. James and B. Radar, Food and Drug Administration, Los Angeles, Calif., personal communication.

EXPERIMENTAL

Equipment—The following were used: UV-visible recording spectrophotometer[2] with 1-cm. quartz cells, glass chromatographic columns for partition chromatography (2.2 × 25 cm., constricted at one end to 0.4 × 5 cm.) and an aluminum tamping rod to fit the column, gas–liquid chromatograph[3] with a flame-ionization detector and a 4-mm. × 0.91-m. (3-ft.) column of 3% SE-33 on 100–120-mesh Gas Chrom Q (column at 252°, detector at 280°, injector port at 258°, and nitrogen carrier gas flow rate at 40 ml./min.), IR spectrophotometer[4], electrobalance[5], and TLC plates coated with silica gel G with fluorescent indicator[6].

Reagents—*Solvents*—All solvents were spectro, certified, analytical reagent, USP, or distilled-in-glass grade. Acetonitrile, *n*-pentane, *n*-hexane, *n*-heptane, 2,2,4-trimethylpentane, cyclohexane, chloroform, methanol, alcohol USP, and absolute ethanol were used. Solvents used in the UV method should be checked photometrically to ensure the absence of aromatic hydrocarbons.

Acetonitrile–n-Heptane (Mutually Saturated)—Mix 25 ml. of acetonitrile and 300 ml. of *n*-heptane (sufficient for two determinations) in a separator, agitate vigorously for 2 min., and allow both layers to clarify. These mutually saturated solutions must be used whenever *n*-heptane or acetonitrile is called for in these directions. Mutually saturated acetonitrile and other hydrocarbons were prepared in the same way for studies without *n*-heptane.

Chloroform (Water Saturated)—Add 50 ml. of water to 250 ml. of chloroform in a separator, agitate for 2 min., and allow both layers to clarify. The water-saturated reagent is required when aqueous trap layers are inserted but should not be used otherwise.

Diatomaceous Earth[7]—Acid washed was used.

Standards—All standard solutions were prepared to contain 1.00, 0.040, or 0.0100 mg./ml. In some studies, individually weighed samples of the solid standards were used.

Alcohol Sulfuric Acid Reagent—Carefully and slowly add 80 ml. of cold, concentrated sulfuric acid (97% w/w) to 20 ml. of cold, absolute ethanol with constant agitation. Cool to room temperature before use. Prepare fresh monthly. Other reagents used in the determinative steps were prepared as directed in the method reference.

Proposed Procedure—*Sample Preparation*—

Tablets: Weigh 20 tablets, grind to pass a 60-mesh sieve, mix thoroughly, and accurately weigh a sample containing approximately 1 mg. of steroid into a beaker. Add 1.5 ml. of acetonitrile, cover the beaker, and swirl occasionally for 10 min. Add 1.5 ml. of *n*-heptane, and swirl until the sample is completely dissolved. As indicated in the tables, the same volumes of absolute methanol and water should be substituted for the acetonitrile and *n*-heptane for some compounds. Continue as directed under *Sample layer*.

Capsules: Weigh the contents of 20 capsules and mix thoroughly. Accurately weigh a sample containing approximately 1 mg. of the steroid into a beaker and proceed as directed under *Tablets* beginning with: "Add 1.5 ml. of acetonitrile, cover the beaker, ..."

Creams: Make a composite by mixing thoroughly the contents of several tubes or jars. Accurately weigh a sample containing approximately 1 mg. of the steroid into a beaker. Proceed as directed under *Tablets* beginning with: "Add 1.5 ml. of acetonitrile, cover the beaker, ..."

[2] Cary model 15.
[3] Barber Colman model 5000.
[4] Perkin-Elmer model 337.
[5] Cahn model G-2.
[6] Eastman No. 6060.
[7] Celite 545, Johns Manville Product Corp., New York, NY 10016

Standards: Accurately weigh approximately 2 mg. of the standard into a beaker. Proceed as directed under *Tablets* beginning with: "Add 1.5 ml. of acetonitrile, cover the beaker, ..."

Column Preparation—

Acetonitrile layer: Insert a glass wool plug in the bottom of the chromatographic column. Thoroughly mix 4.0 g. of diatomaceous earth with 4.0 ml. of acetonitrile, transfer to the column, and pack firmly.

Trap layers: Prepare and insert neutral, acidic, or basic aqueous trap layers as needed according to Graham *et al.* (6).

Sample layer: Thoroughly mix the dissolved sample, prepared as directed under *Sample Preparation*, with 3.0 g. of diatomaceous earth. Transfer to the column above the acetonitrile or trap layer and pack firmly. Dry wash the beaker consecutively with 1.0 g. of diatomaceous earth and glass wool and transfer to the top of the column. Retain the sample beaker for washing with the *n*-heptane and chloroform to be added to the column later.

Column Elution—Wash the sample beaker successively with from four to 12 (depending on the steroid being determined) 25-ml. portions of *n*-heptane and pour onto the column. Adjust the flow rate to 5–7 ml./min., and maintain the liquid head between 8 and 12 cm. above the column bed with a separator attached to the top of the column. Allow the last wash to drain completely. Wash the tip with alcohol USP and save the effluent for further treatment. Rinse the sample beaker with from five to seven (depending on the steroid being determined) 25-ml. portions of chloroform and pour each through the column, maintaining the liquid head as close to the top of the column as possible. Allow the last portion to drain completely from the column. Rinse the tip with alcohol USP and save the effluent for further treatment. Carefully evaporate either or both effluents to dryness on a steam bath under a hood with a current of air to ensure complete removal of the acetonitrile.

Determinative Procedures—*Sample Preparation*—Dissolve the residues from the *n*-heptane and/or chloroform effluents in alcohol USP or absolute methanol and dilute accurately to a volume containing approximately 10 mcg./ml. For GLC, dilute to a volume which contains approximately 1 mg./ml. Use a proper size aliquot of these solutions for quantitation by one of the following determinative methods.

Phenylhydrazine Method—Use the Silber–Porter (7) procedure without modification.

Isoniazid Method—Follow the Umberger (8) procedure using twice the recommended concentration of hydrochloric acid to increase the sensitivity.

UV Method—Scan the alcohol USP or the methanol solution of the sample residue in the region of absorbance using the same solvent as reference. Calculate by comparison with a standard of approximately the same concentration run concurrently.

Alcohol–Sulfuric Acid Method—Dissolve the sample residue in 0.50 ml. of absolute methanol and add 4.00 ml. of the alcohol–sulfuric acid reagent. Scan from 680 to 480 nm. and calculate by comparison with a standard of approximately the same concentration run concurrently.

GLC—Inject a proper size aliquot and calculate by comparison to a standard run concurrently using the area method.

Elution Studies—Each compound studied was put through the proposed procedure using 150 ml. of *n*-heptane and 125 ml. of chloroform. The amount of compound in each of the two eluates was determined by one determinative method to determine if the compound was retained or completely or partially eluted by the *n*-heptane.

Elution curves for those compounds partially or completely eluted by the 150 ml. of *n*-heptane were obtained using 300 ml. of *n*-heptane and collecting 15-ml. aliquots for analysis.

Separation Studies—Several mixtures prepared from standards were analyzed by the proposed method, using 300 ml. of *n*-heptane and 125 ml. of chloroform.

Decomposition Studies—During some elution studies using standards determined by the UV method, it was noted that shoulders appeared in the spectra in some aliquots and that two or more elution bands occurred in some cases. Further study to identify the products of suspected decomposition was performed for androst-4-ene-3,17-dione, dimethoxystilbestrol, norethandrolone, and testosterone enanthate, all of which had been purchased for use as standard substances.

Sample Studies—Several samples of steroid and other types of pharmaceutical preparations were run by the proposed method.

Table I—Effect of Various Hydrocarbons on Elution

Compound	Hydrocarbon	Recovery[a], %: Hydrocarbon Fraction	Recovery[a], %: Chloroform Fraction	Recovery[a], %: Total
Androst-4-ene-3,17-dione[b]	Isooctane	23.3	78.6	101.8
	n-Pentane	52.4	47.0	99.4
	n-Heptane	57.8	42.0	99.8
	n-Hexane	70.8	25.0	95.8
	Cyclohexane	97.7	2.8	100.5
Methyltestosterone[c]	*n*-Pentane	90.0	9.3	99.3
	Isooctane	93.3	5.1	98.3
	n-Heptane	97.1	1.6	98.7
	n-Hexane	98.7	1.1	99.8
	Cyclohexane	99.6	0.5	100.1
Testosterone[d]	Isooctane	5.5	94.7	100.2
	n-Heptane	8.8	92.7	101.5
	n-Hexane	48.9	51.8	100.7
	n-Pentane	75.4	24.3	99.7
	Cyclohexane	102.2	0.6	102.8

[a] Based on the use of 150 ml. of hydrocarbon followed by 125 ml. of chloroform. [b] Aldrich Chemical Co., Milwaukee, Wis. [c] NF Reference Standard. [d] Nutritional Biochemical Corp., Cleveland, Ohio.

METHOD DEVELOPMENT

Length of Acetonitrile Trap Layer—Columns were prepared using methyltestosterone and diatomaceous earth–acetonitrile ratios of 2 g. plus 2 ml., 4 g. plus 4 ml., 6 g. plus 6 ml., and 8 g. plus 8 ml. Twenty 15-ml. aliquots of *n*-heptane were collected and analyzed by the UV method. The primary effect was the increase in the breakthrough volume of *n*-heptane required as the length of the acetonitrile layer increased. Since the breakthrough volume for the 2 g. plus 2 ml. column was less than desirable for many steroids, and the breakthrough volume for the 6 g. plus 6 ml. was larger than necessary, the 4 g. plus 4 ml. ratio was selected as the optimum.

Hydrocarbon Solvent—Various hydrocarbon solvents were studied using testosterone, methyltestosterone, and androst-4-ene-3,17-dione. In each case, the recommended procedure was followed using 150 ml. of the hydrocarbon and 125 ml. of chloroform and then analyzing the residues by the UV method. These three steroids were selected since they appear in both the hydrocarbon and chloroform effluents of the proposed method but in significantly different ratios. There are differences in the elution characteristics of the various solvents as shown in Table I. The hydrocarbon of choice for these three compounds is clearly cyclohexane, but elution data on other steroids prompted the selection of *n*-heptane.

Flow Rate of Hydrocarbon—Testosterone was run by the proposed method, except that the flow rate was adjusted to 1 ml./min. and the 300 ml. of *n*-heptane used was collected in 15-ml. aliquots for UV analysis. The experiment was repeated using a flow rate of 6 ml./min., and both rates were repeated using methandrostanolone. The main effects were a slight broadening of the peaks and the slightly larger volumes (less than 5%) required to attain the peak maxima at the more rapid flow rates. Therefore, the flow rate is not critical and the great saving in time offsets the slight broadening caused by the faster rate.

RESULTS AND DISCUSSION

Fifty-three different compounds were checked by the proposed method, using 150 ml. of *n*-heptane and 125 ml. of chloroform to determine if they were retained on the acetonitrile layer or were partially or completely eluted by the *n*-heptane (Table II). Since the chloroform completely strips the acetonitrile and dissolved substances from the column, any portion of the compound not eluted by the *n*-heptane appears in the chloroform effluent. All 12 corticosteroids listed in Table II [plus nine that were reported previously. (6)] were retained by acetonitrile and were not eluted by the hydrocarbon solvent, even though some were esterified in the C_{17} or C_{21} position. Of the remaining compounds, 19 were completely eluted by the *n*-heptane, seven were partially eluted by the

n-heptane, and 15 were retained by acetonitrile and determined in the chloroform effluent.

The elution curves for compounds that were partially or completely eluted by the *n*-heptane were determined using 15-ml. portions for a total of 300 ml. of the *n*-heptane (last column of Table II); typical elution curves are shown in Fig. 1. The elution curve for the removal of the acetonitrile has been reported (6).

Information concerning the effect of slight changes in structure upon the retention or elution of a compound is found in Table II. As an illustration, consider the first three compounds listed. Androstanolone is completely eluted by the 150 ml. of *n*-heptane, androst-4-ene-3,17-dione is partially eluted, and androst-4-ene-3,11,17-trione is retained completely by the acetonitrile layer. In the conversion of androstanolone to androst-4-ene-3,17-dione, the hydroxyl group at C_{17} is converted to a carbonyl and a single bond is converted to a double bond between C_4 and C_5. The further addition of a carbonyl at C_{11} to form androst-4-ene-3,11,17-trione completely reverses the elution characteristics of the androstanolone.

If the single bond between C_3 and C_5 in androstanolone is converted to a double bond, the resulting compound is testosterone (Table II), which differs from androst-4-ene-3,17-dione only by having a hydroxyl instead of a carbonyl at C_{17}. However, less than 10% of the testosterone but more than 50% of the androst-4-ene-3,17-dione is eluted by the 150 ml. of *n*-heptane. The addition

Table II—Effects of Structural Changes on Elution

Compound	Source[a]	Sample Solvent Used[b]	Determinative Procedure[c]	Recovery[d], % *n*-Heptane Fraction	Recovery[d], % Chloroform Fraction	Volume[e], ml.
Androstanolone	10	MW	SP	99.5	BDL[f]	—
Androst-4-ene-3,17-dione	1	MW	INH	57.8	42.0	200
Androst-4-ene-3,11,17-trione	6	MW	UV	BDL	100.3	—
Betamethasone-17-benzoate	15	MW	UV	BDL	101.9	—
Betamethasone-21-benzoate	15	MW	UV	BDL	101.8	—
Butabarbital	2	MW	UV	BDL	102.5	—
Chlorotrianisene	9	MW	UV	100.4	BDL	—
Corticosterone	12	MW	UV	BDL	99.1	—
Cortisone	7	MW	UV	BDL	99.7	—
Desoxycorticosterone	14	MW	UV	BDL	101.5	—
Dienestrol	9	AH	UV	BDL	97.7	—
Diethylstilbestrol	14	MW	UV	BDL	96.3	—
Diethylstilbestrol dipropionate	8	MW	UV	103.4	BDL	60
Dimethisterone	14	MW	UV	99.1	BDL	128
Dimethoxystilbestrol	10	AH	UV	100.3	BDL	15
Equilenin	12	AH	UV	BDL	100.9	—
Equilin	3	AH	SP	BDL	96	—
Estradiol	9	AH	UV	BDL	99.8	—
Estradiol benzoate	9	AH	UV	100.6	BDL	130
Estradiol valerate	14	MW	UV	102.7	BDL	70
1,3,5(10)-Estratrien-17α-methyl-3,17β-diol-3-methyl ether	12	AH	UV	101.3	BDL	52
Estrone	9	AH	UV	BDL	101.9	—
Estrone-3-methyl ether	12	AH	UV	100	BDL	45
Ethinyl estradiol	14	AH	UV	BDL	100.5	—
Ethisterone	9	MW	UV	BDL	99.8	—
Ethynodiol diacetate	11	MW	AS	102.2	BDL	—
Fluorometholone	9	MW	UV	BDL	102.4	—
Fluoxymesterone	13	MW	UV	BDL	101.6	—
Fluprednisolone	9	MW	UV	BDL	102.1	—
Hexestrol	10	MW	UV	BDL	100.5	—
Hydrocortisone	14	MW	UV	BDL	100.0	—
Lyndiol	5	MW	GLC	100.3	BDL	102
Medroxyprogesterone acetate	14	AH	UV	67.5	32.1	300
Mestranol	11	AH	UV	98.8	BDL	125
Methandrostenolone	14	MW	UV	9.4	90.8	210
Methylprednisolone	9	MW	UV	BDL	100.8	—
Methyltestosterone	9	MW	UV	97.1	1.6	165
Norbolethone	5	MW	UV	101.4	BDL	55
Norethandrolone	11	MW	UV	98.0	BDL	105
Norethindrone	5	MW	UV	BDL	101.6	—
Norethindrone acetate	5	MW	UV	84.8	15.3	200
Norethynodrel	5	MW	UV	16.0	81.7	200
Paramethasone acetate	11	MW	UV	BDL	99.9	—
Prednisone acetate	12	MW	UV	BDL	102.0	—
4-Pregnen-17α,21-diol-3,20-dione	12	MW	UV	BDL	101.5	—
Progesterone	9	MW	UV	100.7	BDL	75
Spironolactone	14	MW	INH	BDL	100.7	—
Stilbene (*trans*)	4	MW	UV	91.5[g]	BDL	—
Testosterone	10	MW	UV	8.8	92.7	200
Testosterone cypionate	14	MW	UV	97.9	BDL	30
Testosterone enanthate	14	MW	UV	97.8	BDL	—
Testosterone propionate	14	MW	INH	99.8	BDL	40
Triamcinolone	14	MW	UV	BDL	98.4	—

[a] 1, Aldrich Chemical Co., Milwaukee, Wis.; 2, Applied Science Laboratories, State College, Pa.; 3, Ayerst Laboratories, New York, NY 10017; 4, Chemical Service, Media, Pa.; 5, Division of Pharmaceutical Sciences, FDA, Washington, D. C.; 6, Eastman Organic Chemicals, Rochester, N. Y.; 7, K & K Laboratories, Plainview, N. Y.; 8, Matheson, Coleman and Bell, East Rutherford, N. J.; 9, NF Reference Standard; 10, Nutritional Biochemical Corp., Cleveland, Ohio; 11, G. D. Searle and Co., Chicago, Ill.; 12, Schwarz-Mann, Orangeburg, N. Y.; 13, The Upjohn Co., Kalamazoo, Mich.; 14, USP Reference Standard; and 15, Warner Lambert Co., Morris Plains, N. J. [b] MW = methanol-water, and AH = acetonitrile-*n*-heptane. [c] SP = Silber-Porter, INH = isonicotinic acid hydrazide, UV = ultraviolet, AS = alcohol-sulfuric acid, and GLC = gas-liquid chromatography. [d] *n*-Heptane (150 ml.) followed by 125 ml. of chloroform in each case. [e] Volume of *n*-heptane needed for complete elution in *n*-heptane fraction. [f] BDL = below detectable limits. [g] Probable loss due to volatilization.

Table III—Separation of Prepared Standard Mixtures[a]

Components	Volume of *n*-Heptane Used, ml.	Recovery, %—*n*-Heptane Fraction	Recovery, %—Chloroform Fraction
Estradiol benzoate[b]–estradiol[c]	175	99.6	101.6
Estradiol valerate[b]–estradiol[c]	150	102.0	101.0
Mestranol[b]–ethinyl estradiol[c]	175	100.2	99.5
Progesterone[b]–estrone[c]	150	99.3	101.9
Progesterone[b,d]–estrone[c]	150	98.9	102.3
Estrone-3-methyl ether[b]–estrone[c]	150	102.3	101.3
Dimethisterone[b]–ethisterone[c]	150	100.8	100.8
Dimethisterone[b]–furosemide[c]	150	99.2	101.2
Testosterone propionate[b,e]–testosterone[b,e]–norethindrone[c]	205[e]	100.0[e] 100.2[e]	99.5
Diethylstilbestrol dipropionate[b]–diethylstilbestrol[c]	150	100.9	98.6

[a] Each mixture contained 2.00 mg. of each component. [b] Eluted in the *n*-heptane fraction. [c] Eluted in the chloroform fraction. [d] This mixture contained 26.0 mg. of progesterone. [e] *n*-Heptane added in two portions: 55 ml. plus 150 ml. The propionate was eluted completely in the first portion and the testosterone in the second.

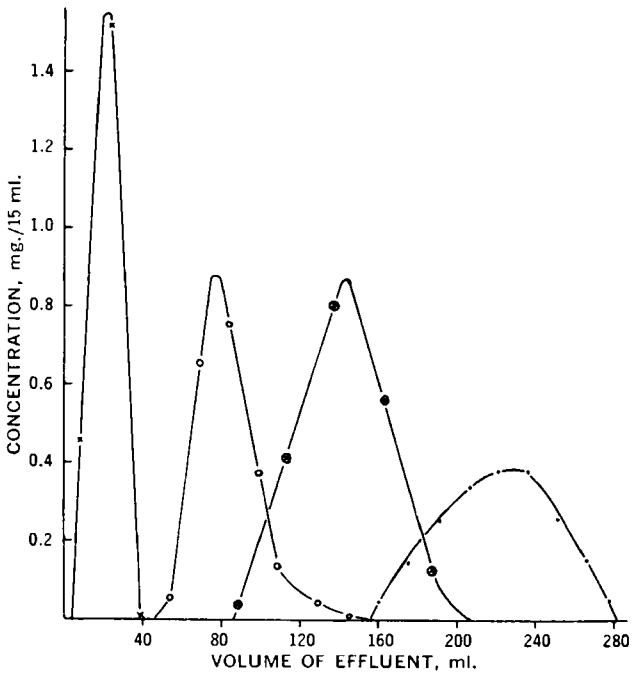

Figure 1—*Typical elution curves for steroids eluted in the* n-*heptane fraction. Key:* ×, *testosterone propionate;* ○, *methyltestosterone;* ⊗, *testosterone; and* ●, *medroxyprogesterone acetate.*

of a methyl group alpha to the C_{17} hydroxyl of testosterone produces methyltestosterone (Table II), which is essentially completely eluted by the *n*-heptane. While the addition of a double bond between C_4 and C_5 greatly decreases the amount of this type of steroid eluted by the *n*-heptane, the difference between a hydroxyl and a carbonyl at C_{17} increases the amount eluted by the *n*-heptane by approximately 50%. However, the effect of the addition of the double bond is offset by the steric hindrance caused by a methyl group alpha to C_{17} since methyltestosterone has essentially the same elution characteristics as androstanolone.

For steroids that are eluted by *n*-heptane, any change in structure that decreases the polarity of the molecule or increases steric hindrance decreases the volume of *n*-heptane necessary for complete elution. Conversely, any change that increases the polarity or decreases steric hindrance increases the volume necessary for complete elution.

Another example using estradiol, estradiol valerate, and estrone (Table II) shows the effect of changes in a single functional group. Estradiol is retained in the acetonitrile layer while estradiol valerate is eluted completely by the 150 ml. of *n*-heptane. Thus, esterification of the hydroxyl group at C_{17} completely reverses the elution characteristics. However, the compound estrone, which is formed by the conversion of the hydroxyl group at C_{17} to a carbonyl, has the same elution characteristics as estradiol. Any change that decreases the polarity of the estradiol molecule, such as esterification at either C_3 or C_{17} or formation of a methyl ether at C_3, apparently produces a molecule that can be eluted by *n*-heptane.

Similarly, diethylstilbestrol is retained by acetonitrile and appears in the chloroform effluent. Conversion to the dipropionate or dimethoxy derivative produces compounds that are eluted completely by *n*-heptane.

Results using the proposed method in the separation and determination of prepared mixtures are shown in Table III. The residues from the evaporation of the *n*-heptane and chloroform effluents were checked by TLC to ensure that separation was complete. For satisfactory separations, one compound should be eluted completely by the *n*-heptane while the other is retained by acetonitrile. The volume of *n*-heptane used depended upon the compounds being separated. The mixture of testosterone propionate, testosterone, and norethindrone is an example of the ability of the proposed method to separate two substances that are completely eluted by the *n*-heptane if there is a sufficient difference in the volumes necessary for complete elution. Information concerning the volumes necessary for complete elution is given in Table II.

The proposed method can also be used to detect decomposition in standard materials by running elution curves, using 15-ml. portions for determination by the UV method. As an example, the UV spectra for the last few portions in the elution of standard androst-4-ene-3,17-dione showed a shoulder at 280 nm. on the expected peak at 240 nm. The fact that the shoulder did not appear until the major portion of the standard had been eluted indicated that the impurity

Table IV—Determination of Pharmaceutical Samples

Type	Components and Declared Amounts	Trap Layer Used	*n*-Heptane Fraction, ml.	Chloroform Fraction, ml.	Found, Percent of Declared—Avg.	Found, Percent of Declared—Range	Number of Determinations
Tablet	Methyltestosterone, 3 mg. Estradiol, 0.2 mg.	Acidic	200	125	109 97	(108–110) (95–98)	3 3
Tablet	Spironolactone, 25 mg.	None	150	125	103	(102–103)	4
Tablet[a]	Methyltestosterone, 10 mg. Esterified estrogens, 1.25 mg.	Acidic	200	125	82 ND[b]	(81–82) ND	3 –
Cream[c]	Iodochlorhydroxyquin, 3% Hydrocortisone, 0.5%	Neutral Aqueous	150	125	99 103	(99) (103–104)	2 2
Tablet[d]	Sodium butabarbital	8% Sodium bicarbonate	150	125	88	(88)	2

[a] Recovery of added methyltestosterone standard was 101.0%. Sample contained stearic acid, which eluted with the *n*-heptane. [b] ND = not determined. [c] Cream also contained allantoin 1%, stearic acid, glycerol monostearate, petrolatum, squalene, propylene glycol, polyoxyethylene stearate, and methylparabens. [d] Tablet also contained stearic acid, which eluted in the *n*-heptane fraction.

was more polar than the standard, but the actual structure was not determined. The impurity in this case was estimated to be approximately 0.2%.

Similarly, the UV spectra for the volumes collected during the elution of standard dimethoxystilbestrol showed two separate components, one contained in the first 15-ml. fraction and the second contained in the fourth through seventh fractions. The first fraction contained 93.6% of the standard and was shown to be the dimethoxystilbestrol. The second compound was shown to be the monomethoxy by its IR spectrum and represented approximately 6.1% of the starting material. The norethandrolone standard was found to contain two impurities, one of which was less polar and the other more polar than norethandrolone, but the actual structures were not determined since they represented only about 0.6% of the original material.

A standard sample of testosterone enanthate, which previous work had indicated might be contaminated, was put through the elution procedure. The undecomposed standard was completely eluted in the first 75 ml. of *n*-heptane and was calculated to represent 64% of the original material. A second compound was eluted between 85 and 200 ml. of *n*-heptane and was shown to be testosterone by its IR spectrum. The complete elution volume of 200 ml. also agrees with the value listed in Table II for testosterone.

The results of several pharmaceutical samples determined by the proposed method are shown in Table IV. The replication and recovery of added standards are satisfactory for these types of materials. The sodium butabarbital tablet is included to illustrate the applicability of the method to samples other than steroids and steroid-like compounds. The butabarbital was present in the chloroform effluent.

The sample must be completely dissolved in the sample solvent before it is mixed with the diatomaceous earth to prepare the sample layer; otherwise, the elution peaks are not symmetrical. Most steroids and other compounds checked will dissolve satisfactorily in either the *n*-heptane–acetonitrile or methanol–water system, but the use of the *n*-heptane–acetonitrile usually produces more symmetrical elution peaks for compounds that are completely soluble in both systems. The volumes required for complete elution, however, are approximately the same for the individual compounds for either system. The *n*-heptane–acetonitrile should be used with all steroids containing an aromatic A ring, since these compounds tail badly when the methanol–water system is used.

In all cases, a white residue, which is insoluble in alcohol USP and methanol, is obtained upon evaporation of the chloroform fraction. The IR spectrum of the residue agrees with the IR spectrum of the diatomaceous earth, and the residue apparently is caused by the fact that some diatomaceous earth is either dissolved or rendered colloidal by the *n*-heptane–acetonitrile–chloroform mixture. The presence of the residue causes an increase in the baseline when the UV method is used for quantitation and must be removed before the measurement is made. The residue can be removed by filtration through a fine frit or can be allowed to settle for 30 or more min. before decantation of the solution. Alternatively, the beaker can be washed several times with small volumes of alcohol USP, methanol, or chloroform (be careful not to remove the residue) followed by evaporation before redissolving in the solvent of choice.

The proposed method can be used for the separation of steroids from steroid esters, for the detection of decomposition in steroid standards, and for the purification of steroid standards, as well as for the analysis of various pharmaceutical preparations. Work is continuing on the application of the method to the separation and determination of components other than steroids in pharmaceutical products.

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Temperature Studies with Nonaqueous Emulsions

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Abstract □ Emulsions of glycerin and mineral oil were formed using anionic, cationic, and nonionic surfactants. Cetylpyridinium chloride and 2-amino-2-methyl-1,3-propanediol–linoleic acid combinations were employed to stabilize emulsions stored at four different temperatures for up to 120 days. Emulsions were prepared at three concentrations of each surfactant. The effects of temperature and aging on droplet size and viscosity were evaluated. The droplet size and viscosity checks were made at predetermined intervals throughout the study. The decrease in viscosity correlated well with droplet size growth. Only one cetylpyridinium chloride-stabilized emulsion showed positive signs of separation at the end of 120 days. All emulsions using 2-amino-2-methyl-1,3-propanediol separated at 45°, and the emulsion with the minimum 2-amino-2-methyl-1,3-propanediol concentration was also unstable at 35°.

Keyphrases □ Emulsions, nonaqueous—temperature dependency, glycerin–mineral oil emulsions with surfactants, droplet size and viscosity □ Surfactants, in glycerin–mineral oil emulsions—temperature dependency, viscosity and droplet size □ Stability, nonaqueous emulsions with surfactants effects of temperature, cetylpyridinium chloride and 2-amino-2-methyl-1,3-propanediol–linoleic acid as stabilizers □ Viscosity, glycerin–mineral oil emulsions with surfactants—effects of temperature

Previous publications from this laboratory (1–7) described the preparation and properties of some nonaqueous emulsions consisting of olive oil as the nonpolar phase and glycerin, propylene glycol, or polyethylene glycol 400 as the polar phase. The stability and viscosity changes of such nonaqueous emulsions over 120 days were reported (4); two anionic surfactants (condensation products of ammonia and of 2-amino-2-methyl-1,3-propanediol with the fatty acids available in olive oil) were used. Stable emulsions were obtained with concentrations of ammonia and 2-amino-2-methyl-1,3-propanediol as low as 0.0006 and 0.002%, respectively. The emulsifying effects of other anionic, cationic, and nonionic surfactants on the glycerin–olive oil system also were described (1, 2). In a study (5) on the effect of surfactant concentration on the interfacial viscosity of a glycerin–olive oil system, it was demonstrated that stability can be achieved in this type of emulsion with interfacial viscosities about 100 times lower than those considered necessary (8) in oil–water emulsions. When other glycols (polyols) were used in conjunction with olive oil, the emulsions with glycerin were found (6) to be more easily made and more stable than those from other polyols.

Although much work on emulsions is described in the literature, these cited studies constitute the majority of available references concerning nonaqueous emulsions. These publications made no reference to the effects of temperature on the nonaqueous emulsion system. The effects of temperature on emulsions with an aqueous phase have been reported (9–11), but information is needed regarding the effects of temperature on the stability of nonaqueous emulsions. For this reason, the present study was designed to explore the effects of temperature on selected nonaqueous emulsion systems. To broaden these studies, mineral oil was selected as the nonpolar phase instead of olive oil.

EXPERIMENTAL

The glycerin used had a purity of 99%[1], confirmed by its refractive index. The glycerin was heated to 180° and then sealed to ensure the absence of water. No further purification of any employed reagent was performed. When surfactant precursor amines were employed, 1% linoleic acid[2] was added to the mineral oil[3].

A phase volume of 0.5 was used in all experiments. The two methods employed for the mixing of reagents were described previously (1, 2). Briefly, Method I consisted of adding the surfactant or surfactant precursor (amine) to the glycerin. Then an equal volume of oil (with the linoleic acid, if needed) was added to the glycerin in a slow steady stream of about 1 ml./sec. while stirring in a blender[4]. The quantities of reagents and the procedure used in Method II were the same as in Method I, except the surfactant or surfactant precursor was added to the oil. Then the glycerin was added to the oil with stirring.

Emulsion stability was determined by close observation for 7 days. At the end of this period, the preparations were examined both macroscopically and microscopically for layer formation. If none was detected, the emulsion was termed "stable."

Two surfactants were selected to stabilize the emulsions for the extended time-temperature studies. The employed surfactant concentrations were determined as the lowest concentration producing stability for at least 30 days at room temperature, 10 times the lowest concentration, and a median concentration. Six solutions of the surfactants in glycerin were prepared. The surfactants used were 2-amino-2-methyl-1,3-propanediol (3, 20, and 30 mg./100 ml. emulsion) and cetylpyridinium chloride (40, 220, and 400 mg./100 ml.). Several sets of duplicate emulsions were prepared for data comparison. Extreme care had to be taken so emulsions would be as similar as possible.

The temperatures involved were 0, 25, 35, and 45°. Three sets of emulsions were prepared to represent each temperature and surfactant concentration used in the study.

Particle-size studies were conducted by the use of oil-immersion, phase-contrast photomicrography, with light at 475 nm. and 730 × magnification. Photomicrographs were prepared at predetermined time intervals (Days 1, 2, 4, 7, 14, 30, and 60 of the study) and were made for each surfactant concentration and temperature involved. Droplet size change was followed by dividing the photomicrographs into areas measuring 3.0 cm.^{2}, selecting the squares at random, and counting the droplets at different size intervals.

The total volume of the droplets for each size interval was determined by using the formula:

$$T_v = (n)(0.523d^3) \qquad \text{(Eq. 1)}$$

where T_v = total volume of droplets within interval, n = total

[1] Colgate Palmolive Co.
[2] Fisher Scientific Co.
[3] Wastach Chemical Co.
[4] Waring model 702 BAW.

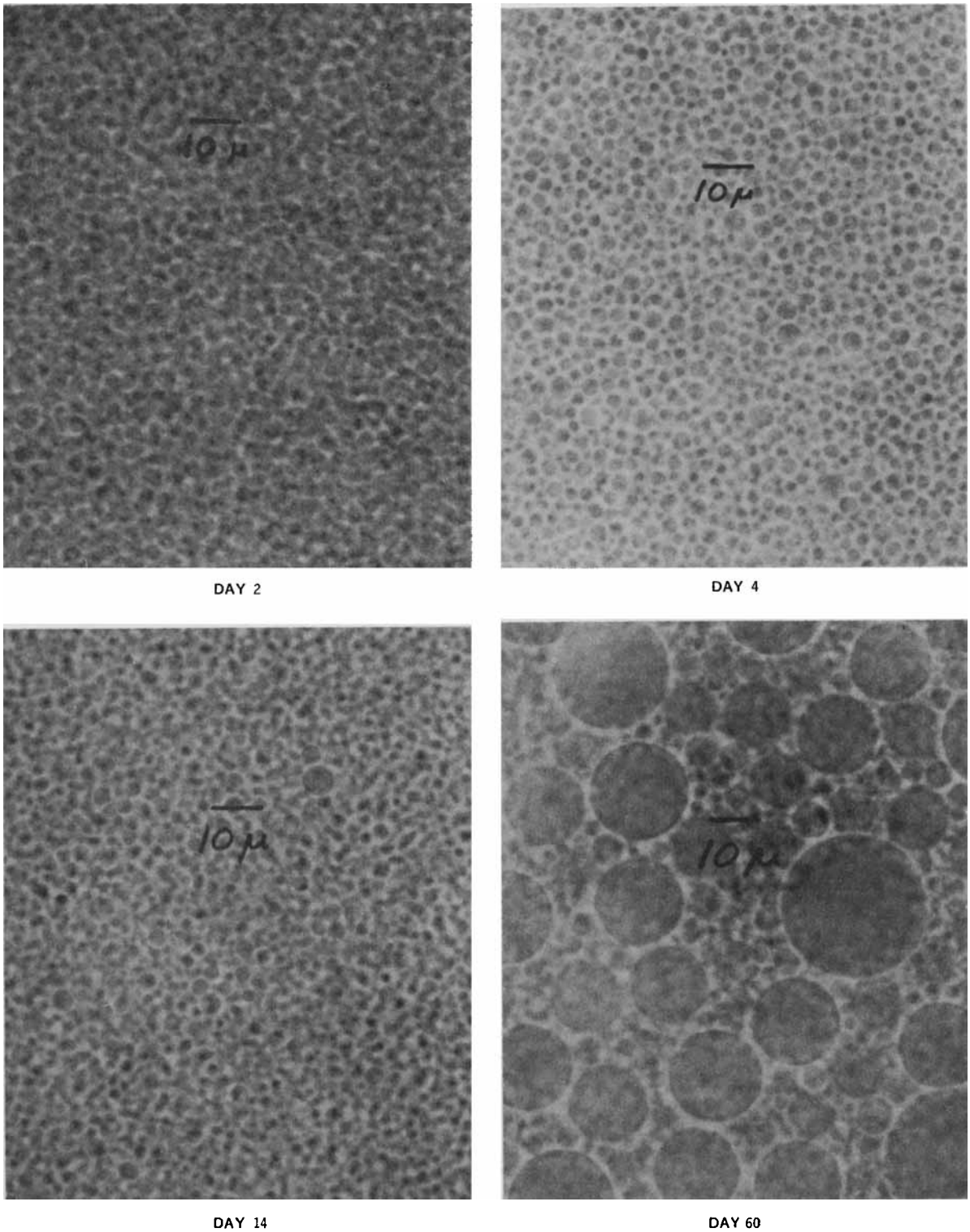

Figure 1—*Photomicrographs of emulsion stabilized with 3 mg. of 2-amino-2-methyl-1,3-propanediol and stored for 60 days at 25°.*

number of droplets within interval, d = mean droplet diameter of interval, and constant = $^1/_6\ \pi$.

The percent of the total volume within each interval for each day and temperature was determined by using the formula:

$$\%T_{va} = \frac{T_{va} \times 100}{(T_{va} + T_{vb} \cdots + T_{vn})} \qquad \text{(Eq. 2)}$$

where $\%T_{va}$ = percent of total volume of droplets represented for each size interval for a given day and temperature, and $T_{va,vb\cdots vn}$ = designation assigned to size intervals.

A Coulter counter[5] was also used to determine the droplet size. Droplet counts were made immediately after mixing the emulsion with saline electrolyte (0.9% aqueous solution of sodium chloride). The results of the experiments using the Coulter counter were then compared with the results of the visual counting of the photomicrographs. The Coulter counter was used to determine droplet size of all duplicate emulsions.

Viscosities[6] of the emulsions were measured on the same days that

[5] Model B with plotter model J.
[6] Brookfield viscometer, model LVT, using spindles Nos. 3 and 4.

Table I Emulsifying Effect of Various Surfactants on Equal Volumes of Glycerin and Mineral Oil[a]

Agent	Percent	Method I[b]	Method II[b]	Emulsion Appearance
Anionic:				
Tetrasodium *N*-(1,2-dicarboxyethyl)-*N*-octadecyl sulfosuccinamate[c]	0.3	Separation	—	—
	0.5	Emulsification	—	Opaque
	5.0	Emulsification	—	Opaque, semisolid
Dioctyl sodium sulfosuccinate[d]	0.5	Separation	—	—
	1.0	Emulsification	—	Opaque, white
	5.0	Emulsification	Separation	Opaque, white
Diamyl sodium sulfosuccinate[e]	5.0	Separation	Separation	—
Sodium lauryl ether sulfate[f]	0.5	Separation	—	—
	1.0	Emulsification	—	Opaque, white, viscous
	5.0	Emulsification	Separation	Opaque, white, very viscous
Sodium lauryl sulfate	1.0	Separation	—	—
	2.0	Emulsification	—	Opaque
	5.0	Emulsification	Separation	Opaque, semisolid
Sodium stearate	0.25	Separation	—	—
	0.5	Emulsification	Separation	Opaque
	2.0	Emulsification	Emulsification[g]	Opaque, semisolid
Calcium stearate	1.0	—	Separation	—
	2.0	Separation	Emulsification[g]	Opaque
	5.0	Separation	Emulsification[g]	Opaque
2-Amino-2-methyl-1,3-propanediol	0.002	Separation[h]	—	—
	0.003	Emulsification	Separation	White, cloudy
	5.0	Emulsification	Separation	Opaque, white, solid
Ethanolamine	1.0	Separation	—	—
	2.0	Emulsification	—	Cloudy
	10.0	Emulsification	Separation	Cloudy
Triethanolamine	0.2	Separation	—	—
	0.25	Emulsification	—	Opaque
	5.0	Emulsification	Separation	Opaque, very viscous
2,2-Diethyl-1,3-propanediol[i]	0.5	Separation	—	—
	1.0	Emulsification	Separation	Clear
	5.0	Emulsification	Separation	Clear
Tromethamine[j]	0.25	Separation	—	—
	0.5	Emulsification	Separation	Clear
	5.0	Emulsification	Separation	Clear
Ammonia[k]	0.005	Separation	—	—
	0.01	Emulsification	Separation	Clear
	1.0	Emulsification	Separation	Clear
Cationic:				
Benzalkonium chloride[l]	0.05	Separation	—	—
	0.1	Emulsification	—	Clear
	10.0	Emulsification	Separation	Clear
Lauryltrimethylammonium bromide[m]	0.5	Separation	—	—
	1.0	Emulsification	—	Opaque
	5.0	Emulsification	Separation	Opaque
Cetylpyridinium chloride[m]	0.025	Separation	—	—
	0.040	Emulsification	Separation	Opaque
	5.0	Emulsification	Emulsification	Opaque
Stearyltrimethylammonium chloride[m]	0.5	Separation	—	—
	1.0	Emulsification	Separation	Opaque, viscous
	5.0	Emulsification	Emulsification	Opaque, viscous
Stearyldimethylbenzylammonium chloride[m]	0.5	Separation	—	—
	1.0	Emulsification	—	Opaque
	5.0	Emulsification	Separation	Opaque, semisolid
Laurylpyridinium chloride[m]	5.0	Separation	Separation	—
Nonionic:				
Polyoxyethylene sorbitan esters:				
Monolaurate (polysorbate 20)[n]	6.0	Separation	Separation	—
Monopalmitate (polysorbate 40)[o]	6.0	Separation	Separation	—
Monostearate (polysorbate 60)[p]	6.0	Separation	Separation	—
Monooleate (polysorbate 80)[q]	6.0	Separation	Separation	—
Sorbitan fatty acid esters:				
Monolaurate[r]	6.0	Separation	Separation	—
Monopalmitate[s]	6.0	Separation	Separation	Insoluble[t]
Monostearate[u]	6.0	Separation	Separation	Insoluble[t]
Monooleate[v]	6.0	Separation	Separation	Insoluble[t]
Trioleate[w]	6.0	Separation	Separation	Insoluble[t]
Monooleate[v] (in oil)	2.0	Emulsification	—	Opaque
	4.0	Emulsification	Separation	Opaque
Trioleate[w]	4.0	Emulsification	Separation	Opaque
	6.0	Emulsification	Separation	Opaque
Glyceryl monostearate, self-emulsifying[x]	2.0	Separation	Separation	—
	5.0	Emulsification	Emulsification[g]	Opaque

Table I—(*Continued*)

Agent	Percent	Method I[b]	Method II[b]	Emulsion Appearance
Glyceryl monostearate, nonself-emulsifying[u]	2.0	Separation	Separation	—
	5.0	Emulsification	Emulsification[a]	Opaque
Polyoxyethylene derivatives:				
Polyoxyethylene (4) lauryl ether[s]	5.0	Separation	Separation	—
Polyoxyethylene stearate[aa]	2.0	Separation	—	—
	5.0	Emulsification	Separation	Opaque, very viscous
	10.0	Emulsification	Emulsification	Opaque, very viscous
Polyol fatty acid ester[bb]	8.0	Separation	Separation	—
Polypropylene oxide[cc]	8.0	Separation	Separation	—

[a] Representative results shown generally indicate minimum percent of surfactant effecting emulsification. (*Note:* Numerous combinations of Arlacels and Tweens were attempted with varying percentages of surfactants up to 8.0% surfactant. All were unsuccessful.) [b] See text. [c] Aerosol 22, American Cyanamid Co. [d] Aerosol OT, American Cyanamid Co. [e] Aerosol AY, American Cyanamid Co. [f] Sipon ES, American Alcolac Corp. [g] Glycerin-in-oil emulsions. All others were oil-in-glycerin. [h] Macroscopic examination indicated emulsification, but separation was noted within 7 days. [i] Carbide and Carbon Chemicals Co. [j] Commercial Solvents Corp. [k] Liquid ammonia obtained from condensation of ammonia gas. [l] Benzalkonium chloride (Zephiran Chloride, Winthrop Lab.) was obtained by evaporation of a 12.8% aqueous solution and a 92.7% semisolid. The results shown are from the 92.7% preparation. The other produced unstable emulsions below the 5% level. [m] K & K Laboratories. [n] Tween 20, Atlas Chemical Industries. [o] Tween 50, Atlas Chemical Industries. [p] Tween 60, Atlas Chemical Industries. [q] Tween 80, Atlas Chemical Industries. [r] Arlacel 20, Atlas Chemical Industries. [s] Arlacel 40, Atlas Chemical Industries. [t] Surfactants indicated were insoluble in glycerin and/or mineral oil. [u] Arlacel 60, Atlas Chemical Industries. [v] Arlacel 80, Atlas Chemical Industries. [w] Arlacel 85, Atlas Chemical Industries. [x] Arlacel 165, Atlas Chemical Industries. [y] Arlacel 169, Atlas Chemical Industries. [z] Brij 30, Atlas Chemical Industries. [aa] Myrj 52, Atlas Chemical Industries. [bb] Arlatone T, Atlas Chemical Industries. [cc] Pluronic (PPO F-68), Wyandotte Chemical Corp.

the photomicrographs were prepared. Four speeds were used: 0.6, 1.5, 3.0, and 6.0 r.p.m. Viscosity measurements of the emulsions were made at the same temperature at which the samples had been stored. Temperature-controlled rooms provided the environment for 0 and 25°. The containers with the samples stored at 35 and 45° were placed in oil baths to maintain a constant temperature throughout the viscosity determination. The readings were first taken from the lowest revolutions per minute to the highest. Then a

Table II—Effect of Age and Temperature on Droplet Volume of Selected Emulsions[a]

Diameter Range, μ	Days 1	2	4	7	14	30	60
3 mg./100 ml. 2-Amino-2-methyl-1,3-propanediol at 0°							
0.5-1.0	7.7	1.5	1.2	1.1	1.0	1.0	0.8
1.0-2.0	72.4	24.9	20.5	19.0	18.5	17.4	14.5
2.0-3.0	19.9	33.0	38.6	35.8	35.1	36.4	32.9
3.0-4.0	—	39.3	39.7	42.0	43.3	45.3	50.2
4.0-5.0	—	1.3	—	2.1	2.0	—	1.7
20 mg./100 ml. 2-Amino-2-methyl-1,3-propanediol at 25°							
0.0-0.5	3.1	3.6	2.0	1.8	1.4	1.0	—[b]
0.5-1.0	70.4	80.2	48.4	47.9	44.4	40.2	—
1.0-2.0	12.7	8.4	6.1	6.4	6.7	6.6	—
2.0-3.0	13.8	7.8	16.5	18.2	18.5	16.2	—
3.0-4.0	—	—	12.9	12.5	17.0	14.9	—
4.0-5.0	—	—	13.7	13.3	12.0	21.0	—
400 mg./100 ml. Cetylpyridinium Chloride at 25°							
0.0-0.5	100	100	100	74.1	63.5	64.0	62.1
0.5-1.0	—	—	—	9.6	11.8	14.6	16.9
1.0-2.0	—	—	—	16.2	24.6	21.4	21.0
3 mg./100 ml. 2-Amino-2-methyl-1,3-propanediol at 35°							
0.5-1.0	7.7	0.9	—	—	—	—	—
1.0-2.0	72.4	23.3	13.2	5.8	—[c]	—	—
2.0-3.0	19.9	73.9	53.4	37.8	—	—	—
3.0-4.0	—	0.6	23.2	38.9	—	—	—
4.0-5.0	—	1.3	10.2	17.7	—	—	—
220 mg./100 ml. Cetylpyridinium Chloride at 45°							
0.0-0.5	7.2	1.9	1.7	0.9	0.3	—	—[b]
0.5-1.0	91.0	37.0	34.0	21.4	12.2	8.0	—
1.0-2.0	1.2	16.3	11.1	12.4	11.3	9.9	—
2.0-3.0	—	17.5	33.7	37.1	30.6	23.7	—
3.0-4.0	—	16.0	19.5	21.6	27.4	32.5	—
4.0-5.0	—	11.3	—	6.6	18.2	23.0	—
30 mg./100 ml. 2-Amino-2-methyl-1,3-propanediol at 45°							
0.0-0.5	8.6	1.9	1.5	0.1	—	—[c]	—
0.5-1.0	90.2	35.0	36.9	6.3	3.3	—	—
1.0-2.0	1.2	12.9	20.4	23.0	18.7	—	—
2.0-3.0	—	29.8	36.0	46.4	37.7	—	—
3.0-4.0	—	20.4	5.2	17.0	15.8	—	—
4.0-5.0	—	—	—	7.2	24.4	—	—

[a] Distribution is represented as percent of total droplet volume measured in each group. [b] Slide damaged, reliable readings impossible. [c] Demulsification observed.

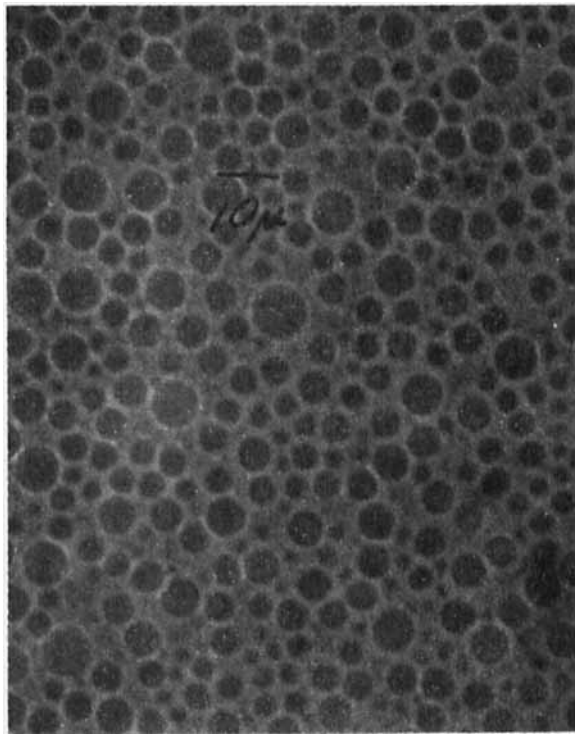

Figure 2—*Photomicrograph of emulsion (30 mg./100 ml. 2-amino-2-methyl-1,3-propanediol) stored at 45° for 14 days.*

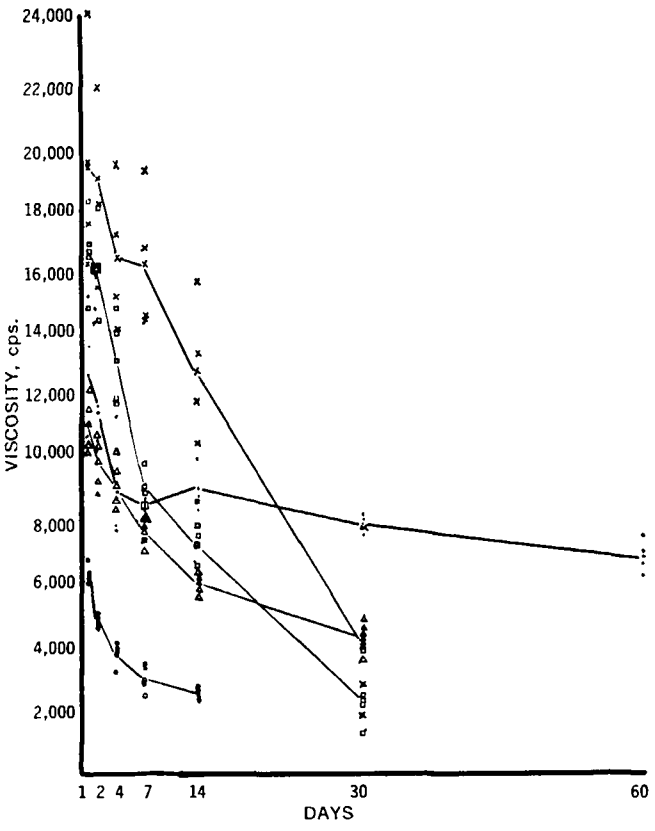

Figure 3—*Comparison of average viscosity change of selected emulsions stored at different temperatures. Key: 2-amino-2-methyl-1,3-propanediol, 3 mg./100 ml. emulsion, 25°, —·—; 2-amino-2-methyl-1,3-propanediol, 20 mg./100 ml., 35°, —Δ—; 2-amino-2-methyl-1,3-propanediol, 3 mg./100 ml., 35°, —○—; 2-amino-2-methyl-1,3-propanediol, 30 mg./100 ml., 45°, —□—; and cetylpyridinium chloride, 40 mg./100 ml., 45°, —×—.*

revolutions per minute of 60 was applied for 10 min., and the viscosity was recorded as the revolutions per minute was decreased from 6.0 to 0.6. Thixotropy was demonstrated when the up (or increasing revolutions per minute) readings exceeded the down readings.

To check the validity of this work, duplicate emulsions of the same concentrations were prepared. The viscosities of these emulsions were recorded for 7 days and compared with those of the test emulsions.

Emulsion type was determined by both the phase dilution method and visual observation. The addition of methylene blue to the glycerin phase allowed sufficient observation with the use of an ordinary microscope to determine emulsion type.

RESULTS AND DISCUSSION

The effects of various anionic, cationic, and nonionic surfactants on glycerin-mineral oil systems are given in Table I. All emulsions were of the oil-in-glycerin type with four exceptions. These surfactants or surfactant precursors were all stearate esters or salts. Similar results were reported previously with glycerin olive oil systems (1, 2).

Although linoleic acid was added to the mineral oil when precursor amines were employed, stearic acid and palmitic acid also produced satisfactory results but required high concentrations. Further studies with stearic acid added to the mineral oil are presently being conducted in an effort to produce other glycerin-in-oil emulsions.

The photomicrographs produced a record of droplet size growth (Fig. 1). This was clearly seen when lower surfactant concentrations were employed. No droplet size change was apparent in emulsions prepared with high surfactant concentrations and stored at 0°, while other emulsions exhibited droplets of various sizes (Fig. 2).

Methods of particle-size analysis (12, 13) have been described, as have the problems of photomicrography (12). The use of oil-immersion, phase-contrast photomicrography eliminated many problems. The slides from which the photomicrographs were made were prepared several hours in advance to reduce particle movement. This procedure proved to be very satisfactory. Even at the high degree of magnification employed, droplets below 1 μ in diameter were difficult to resolve. Despite these limitations, the photomicrographs provided a reliable method from which droplet size data were obtained.

The Coulter counter instrument was used to confirm the data obtained from the photomicrographs. In almost every case, the data from the two methods were comparable and were in almost exact agreement in several instances.

One problem encountered with the Coulter counter was that of aggregation after the addition of the electrolyte solution. This became more marked with time, as reported previously (14, 15). It has been observed (16) that the Coulter counter is ideal for investigating aggregation phenomena. Count loss has also been reported (17).

Because of the occurrence of aggregation, the droplets of the emulsion samples were counted immediately after the introduction of the saline electrolyte. This sharply reduced aggregation and, as stated previously, gave results that were in agreement with those from the photomicrographs.

The droplets were counted from the photomicrograph in size intervals of 1 μ. The intervals used were: <0.05–1, 1–2, 2–3, 3–4, 4–5, and >5 μ. Very few droplets with a diameter >5 μ were present in the stable emulsions. However, some larger droplets were seen in some emulsions prior to demulsification. As close to 100% of the droplets as possible were represented in the calculations.

All diol–linoleic acid-stabilized emulsions stored at 45° were unstable. The preparations with a surfactant concentration (2-amino-2-methyl-1,3-propanediol) of 3 and 20 mg./100 ml. of emulsion were also unstable at 35°. Only one quaternary amine- (cetylpyridinium chloride) stabilized emulsion showed positive signs of separation. This emulsion, stored at 45°, had a quaternary amine concentration of 40 mg./100 ml. Although some inherent error was introduced, the droplets were assumed spherical and the mean diameter was determined by using the midpoints of the respective intervals. This procedure gave a parameter by which the change in droplet volume of the various emulsions could be compared (Table II). As the size of the droplet increases, a small number of droplets

can exert a great influence on the total combined volume of the droplets. The total volume of the internal phase generally increased throughout the test period.

Droplet size data obtained from duplicate emulsions were generally within 5% of the data of the test emulsions. Viscosity data correlation was more difficult, although results generally agreed within 10%. Exceptions were found in both cases, however. Care in emulsion preparation was extremely important in obtaining closely correlated data.

Representative examples of viscosity changes are found in Fig. 3. Viscosities of emulsions stored at high temperatures decreased more rapidly than those of emulsions of the same surfactant concentrations stored at a lower temperature. A decrease in viscosity generally corresponded with droplet size growth. This was very evident just prior to demulsification of the unstable emulsions. As expected, the viscosities of the emulsions stored at 0° were extremely high and appeared very stable. The emulsion viscosities increased with increasing surfactant concentration, a property described previously (18).

Thixotropy was exhibited after the 14th day of the study. The hysteresis loop can be drawn for a large number of the emulsions at all temperatures involved (Table III). Some emulsions of high surfactant concentration stored at the higher temperatures formed gels and exhibited erratic viscosity readings. Thixotropic properties have been shown to exist with other nonaqueous emulsion systems (4).

SUMMARY

Emulsions of glycerin and mineral oil were prepared using a number of different surfactants. Emulsions of the glycerin-in-oil type were produced only with the use of stearate salt or stearate ester surfactants.

Emulsions with 3, 20, and 30 mg./100 ml. of 2-amino-2-methyl-1,-3-propanediol and 40, 220, and 400 mg./100 ml. of cetylpyridinium chloride were prepared for temperature studies. The emulsions were stored at 0, 25, 35, and 45°. Emulsion stability, droplet size, and

Table III—Effect of Shear Rate and Age on Viscosity of Selected Emulsions

Sample	Shear Rate (Spindle Speed), r.p.m.	Viscosity, cps.[a] Day 1	Day 2	Day 4	Day 7	Day 14	Day 30	Day 60
3 mg./100 ml. 2-Amino-2-methyl-1,3-propanediol, 0°	0.6	29,000	42,000	47,000	50,000	51,000	49,000[b]	50,000
							47,000	46,000
	1.5	23,600	28,400	30,000	47,600	58,800	46,000	47,200
							41,600	42,000
	3.0	26,200	27,800	29,200	39,000	54,400	45,000	45,800
							37,400	37,200
	6.0	23,700	24,400	25,500	33,400	48,800	42,000	43,800
							34,200	33,200
20 mg./100 ml. 2-Amino-2-methyl-1,3-propanediol, 25°	0.6	11,200	10,200	9,200	9,400	7,600	7,000	7,000
							5,800	5,400
	1.5	10,560	9,920	8,720	8,720	7,040	6,160	6,240
							4,320	4,280
	3.0	10,400	9,600	8,240	7,840	6,880	6,320	6,400
							3,720	3,640
	6.0	9,100	8,760	7,400	7,320	6,120	5,720	5,920
							3,220	3,260
400 mg./100 ml. Cetylpyridinium chloride, 25°	0.6	56,000	50,000	42,000	36,000	34,000	32,000	28,000
							35,000[c]	31,000
	1.5	45,600	34,600	40,000	32,000	24,800	25,200	23,600
							20,200	18,000
	3.0	43,000	40,800	37,400	29,200	21,200	20,200	20,800
							27,200	24,400
	5.0	38,300	36,100	33,600	24,700	16,000	14,900	14,700
							19,200	17,600
40 mg./100 ml. Cetylpyridinium chloride, 35°	0.6	15,200	13,400	12,600	8,400	7,800	5,800	5,200
								4,700
	1.5	14,080	12,720	11,920	7,840	6,600	6,400	6,400
							5,880	4,400
	3.0	11,840	10,840	10,520	7,200	5,200	4,880	4,800
							3,640	
	6.0	10,400	9,600	9,349	6,400	4,420	4,240	4,200
							3,940	3,960
30 mg./100 ml. 2-Amino-2-methyl-1,3-propanediol, 45°	0.6	18,200	18,000	14,000	9,800	8,600	—[d]	—
	1.5	16,800	16,000	14,800	9,120	7,840	—	—
	3.0	16,400	16,000	11,920	8,560	7,240	—	—
	6.0	14,800	14,400	11,760	7,420	6,500	—	—
220 mg./100 ml. Cetylpyridinium chloride, 45°	0.6	103,000	92,000	81,000	74,000	69,000	52,000	18,000
							39,000	8,000
	1.5	70,400	63,600	56,800	44,400	49,600	28,400	8,800
							17,200	4,400
	3.0	59,200	54,000	43,200	39,400	34,400	17,200	5,600
							11,200	4,400
	6.0	39,200	35,100	30,700	28,300	25,800	11,100	4,200
							7,800	3,600

[a] Measured with a Brookfield viscometer using spindle No. 4 for all 0° and 220-mg./100 ml. and 400-mg./100 ml. cetylpyridinium chloride readings. All other readings were with a No. 3 spindle. [b] Thixotropy demonstrated by two figures. The upper figure represents viscosities at the increasing spindle speed. The lower figure represents the viscosities at the decreasing spindle speed. [c] Erratic readings noted with many of the cetylpyridinium chloride emulsions since gels were formed with aging. [d] Demulsification had occurred.

viscosity were studied on Days 1, 2, 4, 7, 14, 30, and 60. Day 1 corresponded to the day the emulsions were prepared.

When stored at high comparable temperatures for the study period, emulsions stabilized by a quaternary amine (cetylpyridinium chloride) were more stable than those stabilized by a surfactant precursor amine (2-amino-2-methyl-1,3-propanediol) and linoleic acid. All emulsions stored at room temperature (25°) and at 0° were stable.

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A rapid decrease in viscosity preceded demulsification. At high temperatures, the cetylpyridinium chloride-stabilized emulsions formed loose gels and exhibited erratic readings.

Thixotropy was demonstrated as the emulsions were allowed to age at least 2–4 weeks. It was manifested by the change in viscosity readings as the stress was increased from 0.6 to 6.0 r.p.m. and then reversed from 6.0 to 0.6 r.p.m.

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Preformulation Studies II: Stability of Drug Substances in Solid Pharmaceutical Systems

J. TINGSTAD▲ and J. DUDZINSKI

Abstract □ The stability of drug substances in solid pharmaceutical systems is discussed. Theoretical models for various situations are proposed and their practical implications are considered.

Keyphrases □ Preformulation theory—stability of drug substances in solid pharmaceutical systems □ Drug stability in solid pharmaceutical systems—theory, preformulation □ Solid drug systems—stability of drug substance, theory

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1. In spite of the importance of solid dosage forms, there are relatively few quantitative reports on their chemical stability, primarily because of the complexities and difficulties involved.

2. This paper presents a somewhat different point of view than those of most references cited previously.

3. Some theoretical concepts need further clarification and application to practical stability studies.

DISCUSSION

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Analytical error can be minimized by ensuring that content uniformity is satisfactory before initiating stability studies. The problems arising from the heterogeneity associated with these systems are more difficult to overcome, but the primary prerequisites for dealing with them are an awareness of their existence, an under-

standing of their nature, and an appreciation of their practical significance. Some of these problems are as follows.

1. There can be a considerable time lag before physical pseudo-equilibrium is established. For example, appreciable time may elapse before the concentration of water (liquid and vapor phases) comes to equilibrium (1). This is especially true for gelatin capsules, because water present in the capsule shell must equilibrate with that in the formulation and in the surrounding air space.

2. This equilibrium can be upset by opening the container to remove samples for assay, since water vapor can then escape from containers stored at elevated temperatures.

3. If the container is pervious to gaseous diffusion, the stability picture may become confused. For example, Table I shows that at 60°, a water-labile component of a tablet formulation was considerably more stable in a water-permeable blister package than in a sealed glass bottle; at room temperature and 70% relative humidity, the situation was reversed. The tablets, manufactured and packaged at room temperature, probably lose considerable water through the film at 60°, thereby improving stability. At room temperature and 70% relative humidity, the diffusion of water vapor is in the opposite direction.

4. A number of phase changes could take place during the stability study. For example, salicylic acid from aspirin hydrolysis would be in solution until the saturation point is reached and then a solid phase would separate. In addition, sublimation and subsequent deposition of salicylic acid crystals on the dosage form and surface of the container often take place.

5. Solid-state reactions (*e.g.*, oxidation by atmospheric oxygen) may occur simultaneously with solution reactions, and the degradation rates may not be additive (5).

6. Because of the low water volumes involved, kinetic studies may be complicated by chemical equilibrium phenomena and other factors (7).

7. An increase in temperature increases reaction rates and (usually) solubility; this would have a double effect on the overall degradation rate (12). However, this may be partially offset by the lowered concentration of liquid water at higher temperatures.

These problems can be minimized by observing four fundamental rules when conducting quantitative kinetic studies on solid dosage forms. First, as mentioned earlier, content uniformity must be within satisfactory limits. Second, tightly sealed glass containers should be used unless an alternative package is involved. Third, the amount of water present in the dosage form should be determined, since the rate of degradation often depends on the amount of water present. Furthermore, the equilibrium between liquid and vapor phases varies with temperature; consequently, it would be more accurate to determine water concentration at each storage temperature. One approach is to add a measured amount of water to the formulation (5), but part of this water vaporizes into the air space in the container. Consequently, water determinations done on the stored samples have more meaning (10), especially when elevated temperatures are involved.

The fourth rule is that a separate, sealed sample should be taken for each assay point and water determination. If not, water equilibrium can be upset when the container is opened to remove samples. If these rules are observed, meaningful data can be gathered in many cases. The treatment of some of these data may involve relatively complex mathematics (1, 7, 10), but for many systems a few basic, relatively simple, theoretical concepts will suffice. These concepts are as follows.

1. The three most common reactions encountered in stability studies on solid systems are: (*a*) hydrolysis in solution (*e.g.*, aspirin); (*b*) oxidation, either in solution or in the solid state (*e.g.*, vitamin A); and (*c*) reactions between two ingredients.

2. When both Class (*a*) and Class (*b*) reactions are involved, the reaction rates can be additive or antagonistic. For example (assuming no oxidation in solution), adding water to a dry system would initiate hydrolysis but could decrease solid-state oxidation, probably by water adsorption on solid surfaces (5).

3. Class (*a*) reactions are usually zero order in solid dosage forms unless all of the reactant is in solution; then first-order kinetics usually prevail. However, in some situations (*e.g.*, if an acidic or basic degradation product catalyzes the reaction), the kinetics are more complicated. Class (*b*) reactions can be of various orders, although they are often first order if sufficient oxygen is present. Class (*c*) reactions can be of various orders, depending on the concentrations and solubilities of the reactants.

Table I—Degradation of Water-Labile Component of a Tablet Formulation in Two Different Packages under Different Storage Conditions

Package	—Amount Lost (%) in 8 Weeks at— 60°	Room Temperature (70% Relative Humidity)
Blister	11.6	7.0
Glass	84.4	0

4. A particular model or system should be carefully defined. For example, the Class (*a*) reactions described here assume that the reactant dissolves in an adsorbed film of water and that the concentration of adsorbed water is directly proportional to the total amount of water present. The discussion here of Class (*b*) reactions is limited to oxidation in the solid state. Finally, the mathematics describing a Class (*c*) reaction would be different, depending on whether one assumed solid state or solution reactions.

5. Although the models described here are consistent with many reported data, this consistency does not prove that degradations are occurring *via* mechanisms proposed in the model. The mechanisms of degradation for each individual situation would have to be proven by the usual rigorous procedures.

Because of the relatively small amounts of water present in these systems, concentrations (in solution) of degradation products may become significant as the reaction proceeds; then chemical equilibrium phenomena may complicate the kinetics (7). Furthermore, in some cases the drug substance may be less stable in a solid system with a small amount of water present than when the same ingredients are dissolved or suspended in a relatively large quantity of water (10). Nevertheless, many solid systems behave predictably according to the first three concepts above, especially during the first 10–40% of the reaction. Therefore, it would be useful to take a more detailed look at those predictable situations. Only Class (*a*) and Class (*b*) reactions will be considered here.

Figure 1 illustrates the effect of adding water to a solid system involving a relatively insoluble drug substance that degrades only in aqueous solution [Class (*a*), with assumptions mentioned in the fourth concept above]. With no water present, no degradation occurs. As successive amounts of water are added (assuming all of it is available as solvent), the amount of drug substance degrading per unit time increases proportionally:

$$d = k_0 \times v \qquad \text{(Eq. 1)}$$

where, for example, d is the amount degraded in 1 day, k_0 is the apparent zero-order rate constant[1] (in milligrams per milliliter per day), and v is the volume of water (in milliliters) present in the solid system. For example, if k_0 = 1 mg./ml./day and v = 0.1 ml., then d = 0.1 mg.; then if the water content is doubled to 0.2 ml., d would also double. Since the point is never reached where all of the drug substance is in solution, the plot consists of a single straight line with a slope of k_0.

In Fig. 2, the same assumptions are made except that the drug is relatively soluble. Up to point A, the amount of water present is not enough to dissolve all of the drug substance and the situation is identical to that in Fig. 1. After point A, all of the drug substance is in solution and first-order kinetics prevail:

$$d = (1 - e^{-k_1}) \times c \times v \qquad \text{(Eq. 2)}$$

where d and v have the same meaning as in Eq. 1, c is the initial concentration of drug substance (in milligrams per milliliter), and k_1 is the first-order rate constant in reciprocal days. Here, adding more water does not change the value of d (ignoring other dilution effects) because, for example, doubling v automatically decreases c by one-half. Thus, the slope is (ideally) zero when first-order kinetics pre-

[1] As pointed out previously (12), the apparent zero-order rate constant k_0 is equal to the first-order rate constant k_1 times the solubility of the drug substance in the solvent at the experimental temperature. Consequently, when the temperature is raised in accelerated studies, k_0 is increased by two factors: the temperature effect on k_1 and the temperature effect on the solubility of the drug substance.

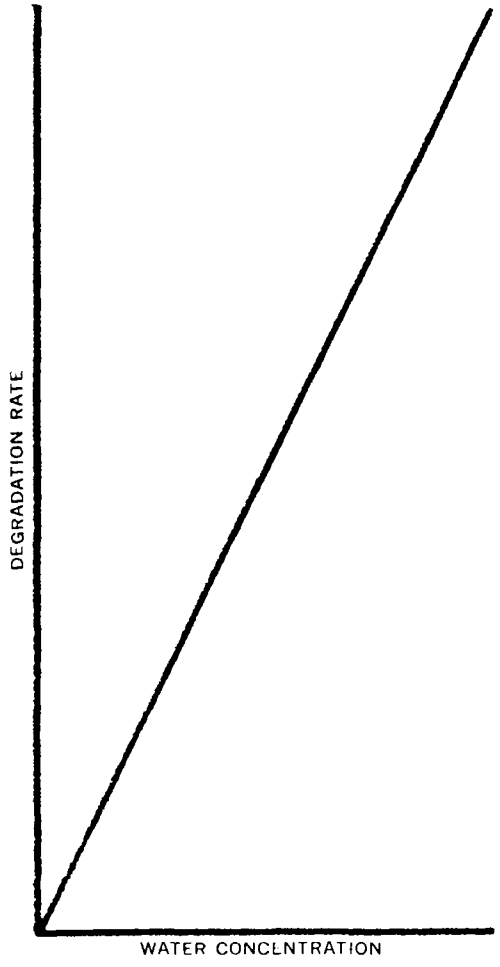

Figure 1—*Model plot showing the effect of water concentration on the stability of a relatively insoluble drug substance that degrades only in aqueous solution.*

vail and equal to k_0 when zero-order kinetics dominate. This concept was touched on by others (5, 11) and will be discussed later.

In Fig. 3, the drug substance is relatively insoluble and subject to both oxidation in the solid phase and hydrolysis in solution. At point A, with no water present, the effect of oxidation is evident; there is appreciable degradation in the absence of water. From A to B, hydrolysis enters the picture, but its effects are partially hidden because the two reactions are antagonistic. That is, the presence of water reduces the effects of oxidation (*e.g.*, by adsorbing on and

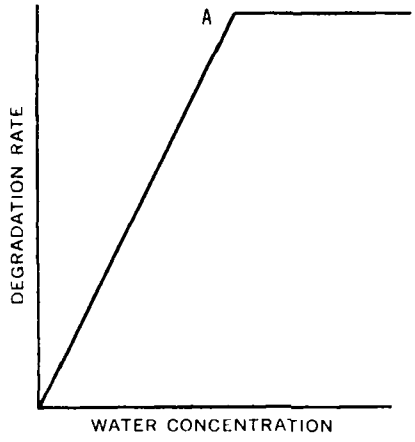

Figure 2—*Model plot showing the effect of water concentration on the stability of a relatively soluble drug substance that degrades only in aqueous solution. At point A, all of the drug substance is in solution.*

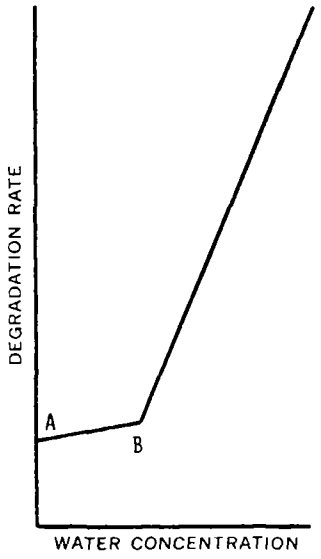

Figure 3—*Model plot showing the effect of water concentration on the stability of a relatively insoluble drug substance that degrades both in the solid state and in aqueous solution. See text for explanation.*

partially protecting the surface of the solid). After point B, the effects of oxidation become relatively constant and the situation more closely resembles that in Fig. 1. If the drug substance in Fig. 3 was water soluble, the plot would eventually plateau as in Fig. 2. Because a nonhydrolytic reaction is partially involved, the slope of the second portion of the plot is less likely to equal k_0. If the two reactions were simply additive, or if the hydrolytic reaction was much faster than the oxidative, the AB portion of the plot would be insignificant. The effect of hydrolysis alone could be determined by eliminating oxygen from the system.

In some situations, the data will not fit the models, but this is not unusual for theoretical models representing complex systems. The report on thiamine hydrochloride (10) is a case in point, but even here a good portion of the data fits the theoretical model. Examples from the literature and these laboratories can be used to illustrate the utility of the three models presented in Figs. 1–3.

Maulding *et al.* (11) obtained a plot identical to that in Fig. 1 using aspirin, a compound that fits the requirements of: (*a*) relatively low solubility, and (*b*) degradation primarily by hydrolysis. The zero-order nature of aspirin degradation in these systems is illustrated in that report, as well as in Fig. 4, based on data from these laboratories. In addition, the data of Leeson and Mattocks (1) can be plotted zero order, assuming an initial lag time (Fig. 5). Figure 6,

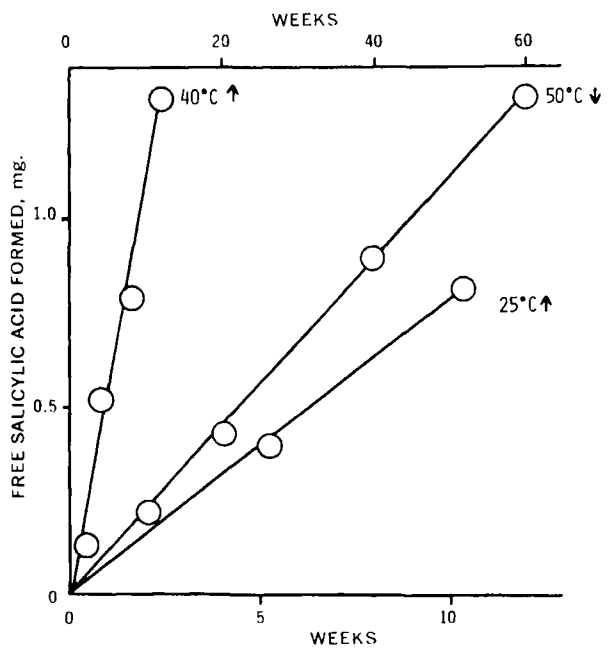

Figure 4—*Plot showing the zero-order nature of aspirin degradation in a solid system. The arrows show to which abscissa the plots refer.*

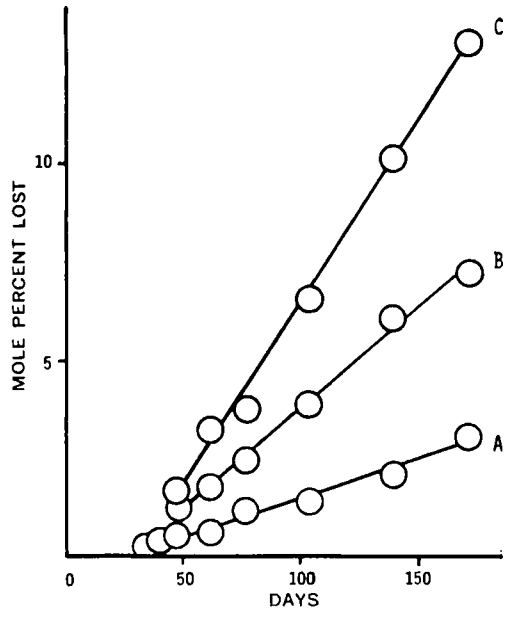

Figure 5—*Plot showing the zero-order nature of aspirin degradation in a solid system [50° data of Leeson and Mattocks (1)]. Key: A, 46.02 mm. vapor pressure of water; B, 68.30 mm.; and C, 74.20 mm.*

representing data from an experimental drug substance in these laboratories, also closely resembles Fig. 1.

The degradation of vitamin A in tablets was found to be first order rather than zero (2) but, as noted earlier, degradation involves more than simple hydrolysis. While the data of Tardif (3) were plotted first order, some of the plots (*e.g.*, thiamine and ascorbic acid at 50°) appear to exhibit zero-order characteristics. Furthermore, if all the thiamine was in solution (possible, especially at the higher temperatures), this would give first-order kinetics. These examples illustrate that many situations do fit the theory when examined in this way.

Thiamine hydrochloride meets the general criteria for compounds yielding the plot seen in Fig. 2. However, the situation is complicated by equilibrium phenomena and other factors, as discussed by Carstensen *et al.* (10). Nevertheless, a significant portion of the reaction follows zero-order kinetics (Fig. 7), and the plot in Fig. 4 of their report suggests that, under ideal conditions (*i.e.*, assuming no chemical equilibrium effects), a plot similar to that in Fig. 2 of this paper would result.

The situation represented in Fig. 3 finds a close parallel in the report of Carstensen *et al.* (5) on vitamin A acetate. Figure 8 is a plot of their data, and it is practically identical to the ideal case depicted in Fig. 3. The ordinate in Fig. 8 is the sum total of the vitamin A lost at all temperatures at a given water concentration. Data from 55°, 85° (4 days only), and 70° (8 days only) were not used because: (*a*) in some cases the concentration of vitamin A reached zero, and it is not known at which point in that time interval the value became zero; and (*b*) the second data points at 70 and 85° showed a drastic reduction in degradation rate, which raises questions about their validity (these data were also rejected by the authors in their calculations).

From Fig. 8 it is quite evident that in their system vitamin A

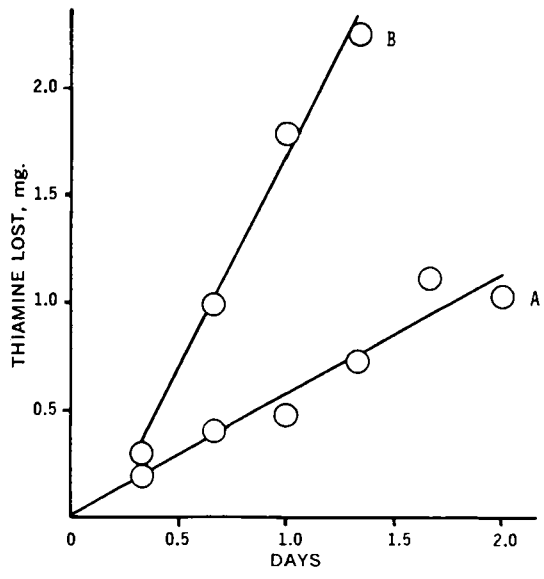

Figure 7—*Plot showing the zero-order nature of thiamine hydrochloride degradation in a solid system [55° data of Carstensen* et al. *(10)]. Key: A, 4% moisture; and B, 5% moisture.*

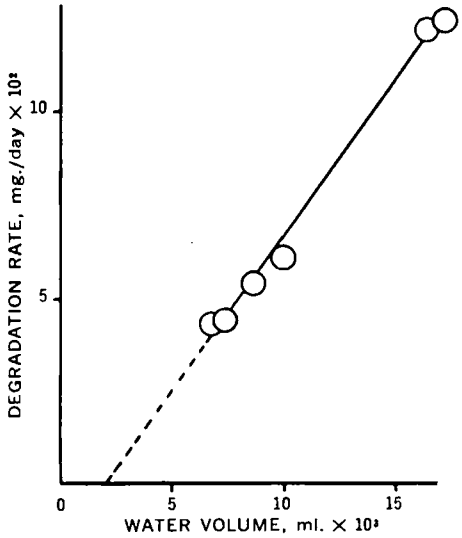

Figure 6—*Plot of actual data for experimental drug substance (confirms the validity of the model in Fig. 1).*

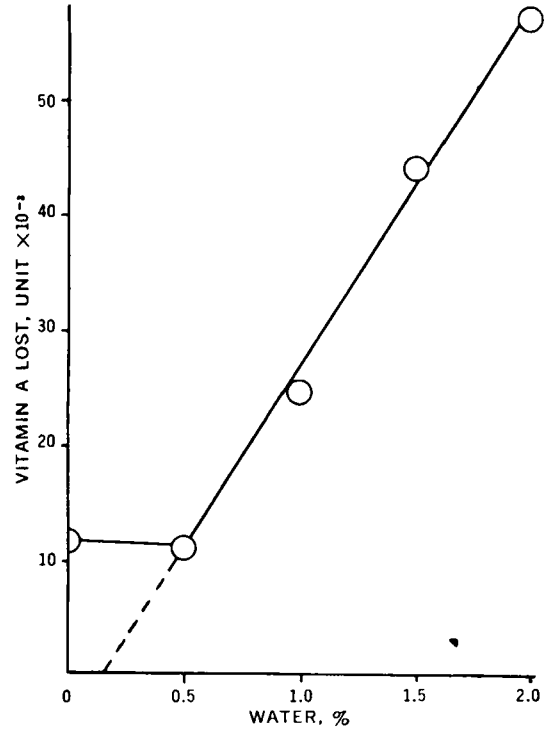

Figure 8—*Plot showing the effect of water concentration on the stability of vitamin A acetate in a solid system [data of Carstensen* et al. *(5)]. See text for explanation.*

acetate degrades significantly in the absence of water. When 0.5% water is added, the stability picture remains essentially the same, indicating that the water's contribution to degradation is offset by the partial protection it affords against solid-state oxidation. As more water is added, however, the degradation rate increases linearly with water concentration, indicating that the contribution of solid-state oxidation has plateaued and the effects of hydrolysis are becoming more evident.

The vitamin A acetate report (5) provides another example of the usefulness of the concepts and models presented in this paper. In discussing the theory behind their presentation, the authors (5) stated: "If vitamin A degrades by functional dependence of water content, then a $(1 + a)$ order reaction may be expressed as $dC_a/dt = -K \cdot C_A \cdot C^a_{H_2O}$ [their Eq. 1] where C denotes concentration and the subscripts denote vitamin A acetate and water." From their data, they reached the conclusion that the apparent value of a is 2–3, a relatively high order.

If one assumes that zero-order kinetics prevail for the water-dependent degradation (a reasonable assumption, since vitamin A acetate is relatively water insoluble), then their Eq. 1 reduces to our Eq. 1 when the value of a is unity. A slightly different interpretation of their data yields values of a that are close to unity and confirm the validity of the model in Fig. 3. That interpretation is as follows.

From the sharp break in the slope in Fig. 8, it is evident that the 0–0.5% range of water concentration is atypical. That is, their Eq. 1 assumes a continuous function and, for their system, the function can be considered continuous only between 0.5 and 2.0% added water. Therefore, the "water added" concept should, in our opinion, start from their 0.5% mark. Thus, their 0.5, 1.0, 1.5, and 2.0 values for "percent water added" should (more correctly) be taken as 0, 0.5, 1.0, and 1.5%, respectively. For example, their 1.5% is, more correctly, 1.0% (*i.e.*, 1.5 − 0.5). Recalculating their data using the corrected values of water added yields the following values for a: 85°, 0.90; 70°, 0.87; 55°, 1.45; 25°, 1.46; and 5°, 1.42. Considering the complexity of the system, experimental values of 0.87–1.46 (average = 1.2) against a theoretical value of unity is good confirmation of the validity of the model in Fig. 3.

CONCLUSION

Stability data for solid dosage forms, gathered under suitably controlled conditions, can often be successfully interpreted using the schematic models presented in Figs. 1–3.

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Sodium-Ion Electrode: Continuous Monitoring of Tablet Dissolution *via* Flowing Streams

T. E. NEEDHAM, Jr., L. A. LUZZI▲, and W. D. MASON

Abstract □ The use of a continuous flowing stream apparatus to follow tablet dissolution was studied. A dissolution chamber using a commercially available filter unit was designed to follow tablet dissolution through use of either a sodium-ion electrode or a spectrophotometric analytical module. The effect of variation of flow rate on the dissolution profile and the ability of the apparatus to differentiate between the common tablet parameters of hardness and drug potency were shown.

Keyphrases □ Tablet dissolution—determination in continuous-flow equipment using sodium-ion electrode or UV spectrophotometry differentiation between parameters of hardness and drug potency □ Dissolution equipment, tablets—continuous-flow system using sodium-ion electrode or UV spectrophotometry, differentiation between parameters of hardness and drug potency □ Sodium-ion electrode system—used to determine tablet dissolution in continuous-flow system

The importance of *in vitro* testing of tablet and capsule dissolution and its relationship to drug availability are accepted facts. The correlation of *in vitro* to *in vivo* data shows the need for a versatile and flexible apparatus which, through adjustment of its parameters to the variance in dissolution profiles of different tablets, can succeed in matching such data. A second but equally important consideration is a reduction in the time and labor required for such *in vitro* analysis. By using the technique of continuous analysis in flowing streams to monitor dissolution, the entire profile can be recorded and experimental error can be reduced to a minimum.

In recent years, equipment for studying the dissolution of solid dosage forms under conditions of continuous flow has been presented. The arguments for using a continuous-flow method and the theoretical considerations involved have been documented (1, 2). These authors suggested that the continuous-flow

acetate degrades significantly in the absence of water. When 0.5% water is added, the stability picture remains essentially the same, indicating that the water's contribution to degradation is offset by the partial protection it affords against solid-state oxidation. As more water is added, however, the degradation rate increases linearly with water concentration, indicating that the contribution of solid-state oxidation has plateaued and the effects of hydrolysis are becoming more evident.

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Abstract □ The use of a continuous flowing stream apparatus to follow tablet dissolution was studied. A dissolution chamber using a commercially available filter unit was designed to follow tablet dissolution through use of either a sodium-ion electrode or a spectrophotometric analytical module. The effect of variation of flow rate on the dissolution profile and the ability of the apparatus to differentiate between the common tablet parameters of hardness and drug potency were shown.

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In recent years, equipment for studying the dissolution of solid dosage forms under conditions of continuous flow has been presented. The arguments for using a continuous-flow method and the theoretical considerations involved have been documented (1, 2). These authors suggested that the continuous-flow

methodology should have sufficient flexibility to be useful in studying intrinsic dissolution characteristics of a sample while maintaining reproducibility and the ability to detect small differences in formulation.

The described apparatus differs from previously presented systems in that it allows a choice of analytical module using an inexpensive, universally available dissolution cell. A sodium-ion electrode may be mounted in a flow cell and employed as a sensor in addition to a spectrophotometric sensor as used previously. The sodium-ion electrode has limitations, some of which were documented in these laboratories (5).

The reproducibility of the apparatus, as well as its ability to detect differences in tablet hardness and to analyze and differentiate between tablets of different drug potency, is evaluated in this report.

EXPERIMENTAL

Materials—Both commercial and specially manufactured tablets were used. Only one batch number each of sodium warfarin[1], sodium butabarbital[2], and sodium bicarbonate[3] tablets was used. The sodium salicylate tablets were manufactured in these laboratories using a 16-station rotary tablet press[4] equipped with an induced die feeder. Standard concave punches [0.95 cm. (0.375 in.)] were used. These tablets were compressed from the following formulation: sodium salicylate[5], 50 mg.; fast flow lactose[6], 216.5 mg.; microcrystalline cellulose[7], 43.5 mg.; starch[8], 35.0 mg.; and stearic acid[5], 5.0 mg., to produce 350-mg. tablets. The tablets were compressed to three different hardnesses as determined by an electronic hardness tester[9]. The reported hardnesses are averages found after testing 50% of the tablets from each run.

The dissolution medium consisted of a buffer mixture (3) of potassium hydroxide[5] and dibasic potassium phosphate[5] at pH 8 with an adjusted sodium-ion concentration of 10^{-3} *M*.

Apparatus and Instrumentation—The apparatus (Fig. 1) was designed for use with either a sodium-ion electrode or UV spectrophotometer as the analytical module. The dissolution cell is a commercially available filter unit[10], using a standard 0.22-μ filter to prevent disintegrated tablet particles from leaving the cell. A peristaltic pump[11] was used to push the solvent at a constant flow rate from the reservoir through the dissolution cell and analytical system. The flow lines were made from Tygon tubing [formula B44-3, 0.15 cm. (0.06 in.) i.d.]. All flow rates were determined periodically by collecting samples of the liquid that had passed through the dissolution cell. All studies reported were carried out at ambient temperatures (25 ± 1°).

The analytical system used was either a spectrophotometer with an adapted time drive to record absorbance as a function of time or a sodium-ion electrode system with connected recorders (Fig. 1). While the use of a flow cell to hold a sodium-ion electrode was previously reported (4), Fig. 2 illustrates the unit designed to incorporate the sodium-ion electrode into the system. It consists of a cylindrical Teflon block with a horizontal channel to carry the dissolution medium past the sodium-ion and reference electrodes and on to the second analytical module or disposal vessel. Both the sodium-ion and reference electrodes are set into the block vertically such that the tip of each protrudes into the channel and no air space is left along the side or at the tip of the electrodes. A micro sodium-ion electrode was used and found to have a response equivalent to the full-size sodium-ion electrode previously reported (5). The analytical modules were connected singly or in series to the dissolution cell.

[1] Endo Laboratories.
[2] McNeil Laboratories.
[3] Lilly Laboratories.
[4] Model 216-RP Cherry-Burrell.
[5] Fisher Chemicals, Fairlawn, N. J.
[6] Foremost Dairy, San Francisco, Calif.
[7] Avicel, FMS Corp., Newark, Del.
[8] Ruger Chemical Co., Inc., Irving, N. J.
[9] Erweka Electronic.
[10] Swinnex-25, Millipore Corp.
[11] Harvard model 1201.

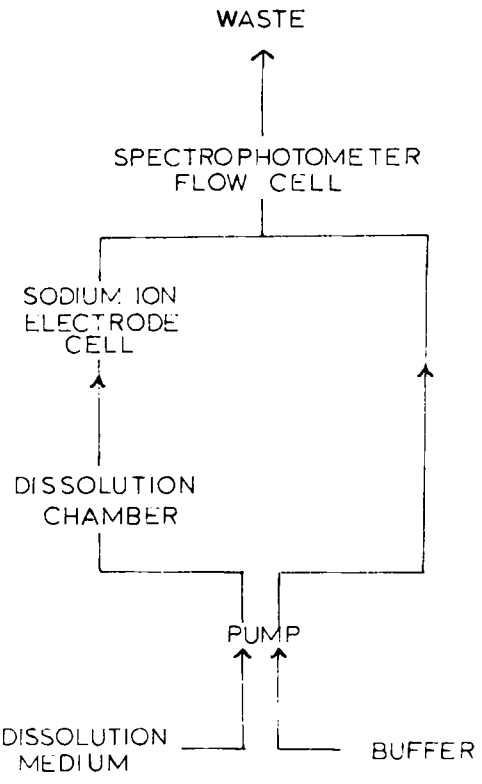

Figure 1—*Schematic representation of automated flow system.*

Samples were diluted to the analytical range of the spectrophotometer by mixing with a fixed ratio of solvent. The diluent was also used in the case of sodium butabarbital to adjust the pH to that level where the Beer–Lambert law was valid. Use of a buffered 0.001 *M* sodium-ion base solution, calibration of the sodium-ion electrode, and correlation of the sodium-ion electrode reading to spectrophotometric and atomic absorption readings were explained in a previous paper (5). Since all tablets exhibited significant differences in their dissolution profiles in the first 18 min. with a slow

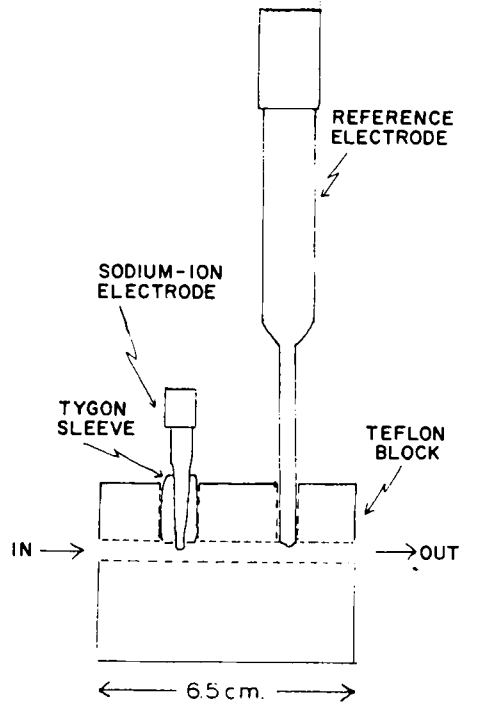

Figure 2—*Side view of sodium-ion electrode cell.*

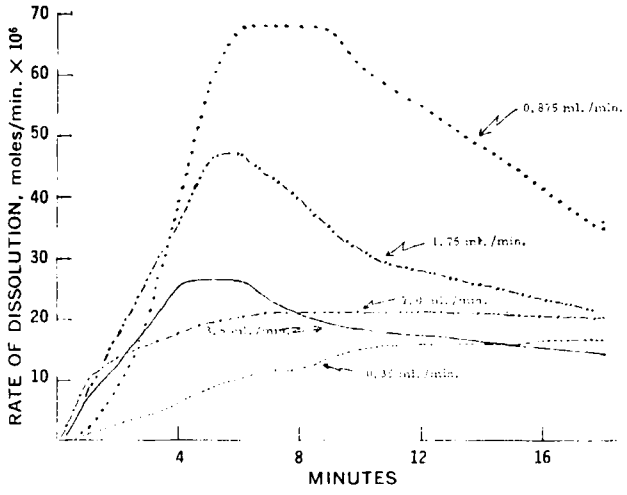

Figure 3—*Rate of dissolution of sodium butabarbital tablets at pH 8.0 as a function of flow rate.*

return to the blank concentration after that time, only the first 18 min. of each profile is presented.

Procedure—At the beginning of each tablet dissolution run, the analytical system was equilibrated to its baseline with the dissolution medium and the dissolution chamber was disassembled and dried. A weighed tablet was placed in the chamber, a new filter was set in, and the apparatus was closed. The pump was started and the changes in concentration were recorded as a function of time. The solution was discarded after being pumped through the analytical module. Variance in the rate of dissolution between tablets of the same batch was found to be comparable to the USP (6), Levy (7, 8) beaker, and magnetic basket (9, 10) methods of dissolution testing. All reported dissolution curves are the average of at least four separate determinations.

RESULTS AND DISCUSSION

Most dissolution data presented in this paper were collected using the sodium-ion electrode analytical module. However, dissolution profiles were obtained for sodium salicylate and sodium butabarbital using the spectrophotometer only and a combination of the spectrophotometer and sodium-ion electrode recorder module attached in series after the dissolution cell. The spectrophotometer produced results comparable to those seen by Tingstad and Riegelman (2). In fact, when the spectrophotometer and sodium-ion electrode recorder module were used in series to follow the dissolution of the same tablet, curves characteristic of that tablet were comparable.

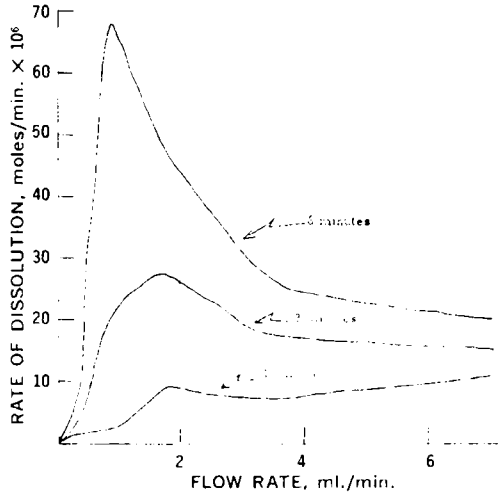

Figure 4—*Effect of flow rate on the rate of dissolution at increasing time intervals.*

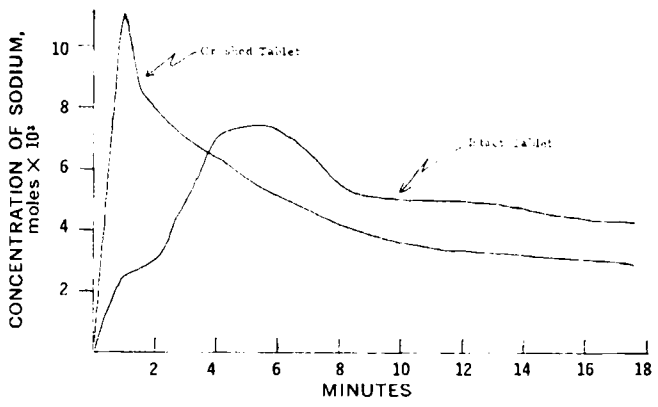

Figure 5—*Comparison of crushed and intact sodium butabarbital tablets at pH 8.0 and a flow rate of 3.5 ml./min.*

When using the continuous-flow dissolution apparatus in Fig. 1, one obtains a continuous readout of logarithm concentration of the drug in the dissolution medium *versus* time. The data can be presented in a number of ways that permit evaluation of the tablet dissolution. The obvious presentation, as previously reported (1, 2), consists of multiplying the concentration by the volume flow rate of the solution through the apparatus to obtain the instantaneous dissolution rate as a function of time. Figure 3 shows the effect of the volume flow rate on the instantaneous dissolution rate–time plot for sodium butabarbital tablets from the same batch. The importance of the proper flow rate selection can be seen. The flow rate should be coordinated with the characteristics of the tablet being studied to produce the most meaningful results. Figure 3 shows that as the volume flow rate was increased from 0.35 to 0.875 ml./min., the instantaneous dissolution rate during the first 18 min. increased. This is analogous to increasing the stirring rate when using the beaker method. Further increase in the volume flow rate above 0.875 ml./min. resulted in a decrease in the instantaneous dissolution rate.

In Fig. 4, the changing effect of flow rate on the instantaneous rate of dissolution at several different times can be seen. At 1 min. elapsed time, the faster flow rates have produced the fastest rates of dissolution. However, as the time of dissolution increased through 3 min., 6 min., and greater, the instantaneous dissolution rate increased for the slower flow rates until it peaked at 0.875 ml./min. This produces what seems to be a contradiction in that the greater the rate of flow the higher should be the rate of dissolution. However, further examination of the data reveals several possible explanations. Visual observation of the tablets at increasing elapsed times of dissolution indicates that this flowing stream method produces a "peeling" away of the outer layers of the tablets examined and a much slower disintegration than that seen in other dissolution methods using larger volumes of medium. Thus, the change in surface area would be small and the rate of dissolution of the drug would be maximized to a constant such that the dissolution taking place at the faster flow rates would be limited.

In addition, although the effects of the higher flow rates were not

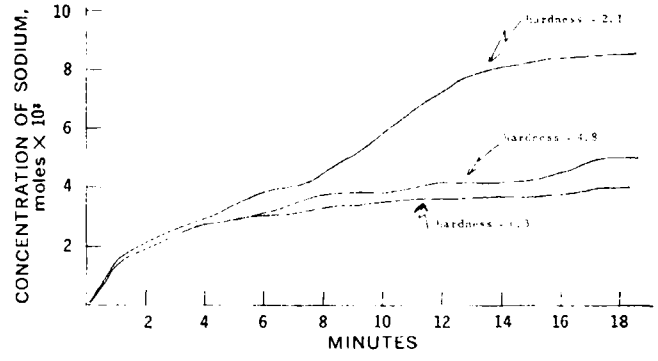

Figure 6—*Effect of hardness on the dissolution of sodium salicylate tablets at pH 8.0 and a flow rate of 0.75 ml./min.*

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It was also observed that the dependence of the dissolution rate on volume flow rate varied with hardness, strength, and formulation of the tablet. With each different type of tablet, the flow rate had to be empirically determined to provide the most revealing dissolution rate. In addition to the nature of the tablet, the limitations of the analytical module must be considered in selecting the volume flow rate. The concentration of the dissolving drug must be maintained within the linear calibration range of the sodium-ion electrode or spectrophotometer.

The ability of the dissolution apparatus to monitor common variables is shown in Figs. 5 and 6. In Fig. 5, the dissolution profile for a sodium butabarbital tablet is compared to the dissolution profile for a tablet from the same batch that had been crushed to a powder before placement in the dissolution apparatus. As expected, the initial dissolution rate was greatly increased when the disintegration phase was eliminated. This observation is in agreement with the data by Tingstad and Riegelman (2). Figure 6 illustrates the ability of the apparatus to differentiate between tablets of exactly the same formulation but of different hardnesses. The sodium salicylate tablets used were prepared in these laboratories and, as expected, a rate of dissolution increasing in rank order of decreasing tablet hardness was seen.

The versatility of this apparatus is further illustrated with the dissolution of sodium warfarin tablets. A flow rate of 0.75 ml./min. allows the instrument to differentiate among 5-, 10-, and 25-mg. tablets. The dissolution of sodium bicarbonate tablets was also followed by adjustment of the flow rate to 3.5 ml./min.

The use of a flowing stream dissolution apparatus in conjunction with either the sodium-ion electrode or spectrophotometric module provides an automated means of following tablet dissolution quickly and accurately. The apparatus differentiates between the common tablet parameters of hardness and drug potency and provides a variability adjustment through flow rate. In conclusion, this apparatus provides a quick and accurate means of analyzing *in vitro* tablet dissolution.

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Effect of Adverse Storage Conditions on Vacuum-Holding Ability of Large-Volume Parenteral Containers

MARTIN B. PINCKNEY, Jr.*, LOUIS A. LUZZI▲, and JAMES C. PRICE

Abstract □ The ability of large-volume vacuum-packed parenteral containers to maintain vacuum under adverse conditions of temperature and agitation was examined. It was the intent of the study to examine the possible effects of accelerated aging during travel and storage. Containers were stressed by being subjected to alternating high and low temperatures for 30 days (24-hr. intervals) and by shaking for 30 days at temperatures up to 50°. Preliminary tests were carried out to establish a statistically significant number of experiments. It was found, in all cases, that the vacuum seal was maintained under these conditions. It is concluded that such factors as decomposition of ingredients and faulty glassware should be suspected if vacuum loss is found with these types of containers.

Keyphrases □ Parenteral containers, large volume—effects of temperature and agitation on vacuum-holding ability □ Large-volume parenteral containers—effects of temperature and agitation on vacuum-holding ability □ Vacuum retention—effects of temperature and agitation on large-volume parenteral containers □ Containers, large-volume parenteral—effects of temperature and agitation on vacuum-holding ability

In the last decade, there has been a surge of interest regarding the safety of parenteral preparations. Recent reports have been concerned with particulate matter (1–4), sterility control (5, 6), and contamination during opening, preparation, and use (7–9).

That serious problems can still occur with this type of preparation is indicated by recent references to septicemias caused by microbial contamination (10–12). Some of these infections have been attributed to improper use or handling of the parenteral product itself (10). Other problems have been caused by introduction of contaminants during administration (11, 12).

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One possible source of contamination is the failure of the closure during storage. A means of testing for

Table I—Effects of Temperature Fluctuation on the Vacuum-Holding Ability of Vacuum-Packed Parenterals

Temperature Range	Type and Volume (ml.) of Container	Number Each of Test and Control Containers	Test Containers, avg. ml. Water to Relieve Vacuum	Control Containers, avg. ml. Water to Relieve Vacuum
25–40°	A[a], 250	24	36.9	36.8
25–40°	B[b], 250	24	73.2	73.3
5–25°	A, 250	24	35.9	36.8
5–25°	B, 250	24	74.5	73.3
5–40°	A, 250	24	37.8	36.8
5–40°	B, 250	24	74.8	73.3
5–50°	A, 250	24	38.9	37.6
5–50°	B, 250	24	71.4	70.8
25–40°	A[c], 500	22	82.3	80.4
25–40°	B[d], 500	21	76.7	74.5
5–25°	A, 500	24	90.3	90.0
5–25°	B, 500	26	77.0	74.5
5–40°	A, 500	26	85.7	90.0
5–40°	B, 500	27	75.0	74.5

[a] Type A containers of 250 ml. from Lot N20N3. [b] Type B containers of 250 ml. from Lot TU9790A. [c] Type A containers of 500 ml. from Lot N20F3. [d] Type B containers of 500 ml. from Lot TU9617C.

closure failure has not been reported in the literature. In vacuum-packed parenterals, this possibility can be examined by measuring the vacuum retained under various stressing storage conditions. This report describes the vacuum-holding ability of large-volume parenterals under storage conditions somewhat more severe than would be encountered during normal use.

EXPERIMENTAL

Description of Systems Tested[1]—The systems examined were: Type A, a vacuum-packed nonfiltered air system; and Type B, a vacuum-packed filtered air system (9). Both systems contained lactated Ringer's solution in 250- and 500-ml. sizes, and only one lot number was used for each size. Headspace and fill volume in both types of container were found to vary less than 1% of the total container volume for each size container reported.

Temperature Fluctuation Tests—Each described system was exposed to four temperature ranges, which were chosen as extreme test conditions and were designed to approximate or exceed the maximum and minimum conditions encountered during transit or storage. Temperature ranges employed were 25–40°, 5–40°, 5–25°, and 5–50°.

The filled containers were initially maintained in a constant-temperature circulating bath at the lower temperature for 24 hr. and then transferred to another constant-temperature circulating bath at the higher temperature for 24 hr. Each set of containers was alternated at the indicated temperatures for 8 days. At the end of the tests, the containers were adjusted to room temperature[2] to establish equilibrium and were examined for vacuum retention.

Agitation Tests—Shaking at constant elevated temperatures was performed to study the effects of agitation on vacuum loss. The filled containers were placed in a temperature-controlled shaker[3] and run at the highest shaking speed [approximately 380 oscillations/min. with an oscillatory amplitude of 2.54 cm. (1 in.)]. The size of the container holders built into the shaker necessitated the use of 500-ml. containers for the agitation tests (Tables I and II). Sets of containers were agitated at 30, 40, and 50° for 30 days; the bottles were then removed from the shaker, adjusted to room temperature, and tested for vacuum.

[1] Systems included are those marketed by Cutter Laboratories, Berkeley, CA 94710, and by Baxter Laboratories, Inc., Morton Grove, IL 60053.

[2] Accomplished by placing the bottles in a 25° water bath for 24 hr. and then passing room temperature air (*via* an electric fan) over the bottles for 2–4 days; room temperature for these tests was 23.5–24.5°.

[3] Gyrorotary model G-25, New Brunswick Scientific Co., New Brunswick, N. J.

Table II—Effect of Agitation for 30 Days on Vacuum-Holding Ability of 500-ml. Vacuum-Packed Parenterals

Agitation Temperature	Type of Container	Number Each of Test and Control Containers	Test Containers, avg. ml. Water to Relieve Vacuum	Control Containers, avg. ml. Water to Relieve Vacuum
30°	A[a]	21	88.9	88.5
30°	B[b]	23	75.9	75.0
40°	A	12	78.6	78.0
40°	B	12	77.5	78.6
50°	A	12	74.2	74.3
50°	B	12	73.3	75.0

[a] Type A containers from Lot N20F3. [b] Type B containers from Lot TU9617C.

Controls—Containers from the same lot were stored at room temperature and were examined at the same time as the test containers in both the temperature fluctuation and agitation studies to assure that "aging" of the bottles would not be a variable factor in the studies. Pairing controls with test containers in each lot eliminated the possibility of lot-to-lot variation.

Vacuum Measuring Apparatus—The apparatus consisted of a 250-ml. buret equipped with attached tubing terminating with an administration needle; the buret was equipped with an automatic refilling device (Fig. 1).

Container vacuum was measured by filling the 250-ml. buret with water and inserting the administration needle through the recommended puncture site of the parenteral container. Water from the buret was allowed to pass into the inverted intravenous container until the partial vacuum had been satisfied. Liquid levels in the container and buret were maintained at the same level by adjusting the container height. Water drawn from the buret into the vacuum was accurately read to 1.0 ml. and estimated to 0.5 ml. The larger the quantity of water drawn into the bottle, the greater was the vacuum that had existed prior to puncture. As a control, at least 25 containers of both types were examined for void space and fill volume.

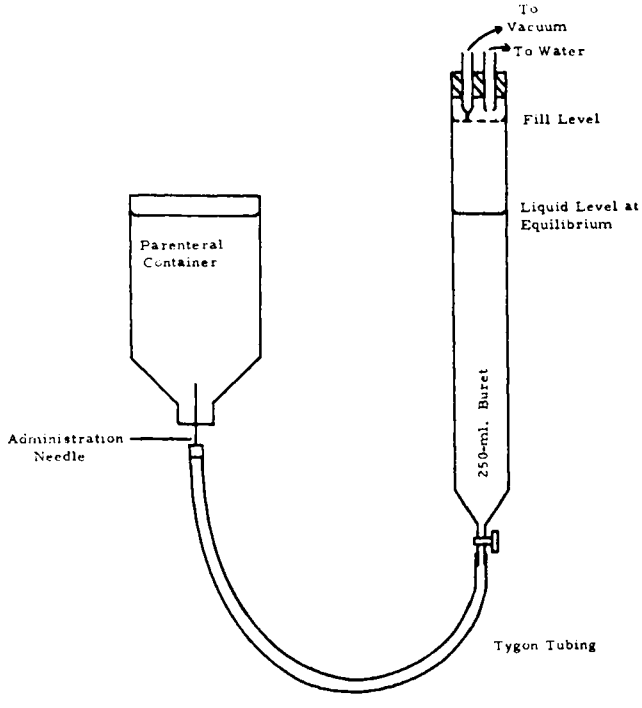

Figure 1—*Apparatus used to examine vacuum.*

Use of the water-filled buret technique eliminated the possibility of errors due to air leakage about the site of cannulation. If air entered, it would pass by the needle, go up through the liquid in the inverted container, and become visible as bubbles.

RESULTS AND DISCUSSION

The results from the experiments are shown in Tables I and II. Statistical evaluation of these data yields evidence that temperature fluctuation and agitation treatments have no detrimental effect on vacuum seal.

The greatest difference detected between average vacuums for test and control groups was found in the temperature fluctuation experiments. Table I shows that the 5–40°, 500-ml. Type A group had average vacuums of 85.75 ml. water for the test bottles and of 88.99 ml. water for the control bottles. A standard "pooled" or two-sample t test on this test-control pair of samples indicated that the differences were insignificant since the calculated t value is 2.0 or less than 2.413 at the 1% level.

The natural variation in vacuum from bottle to bottle among the Type A and Type B control samples is given by their standard deviations: 4.54 ml. water for Type A and 4.37 ml. water for Type B. When using the F test, it was found that there was no significant difference in the vacuum variance between the two types of control samples.

It is apparent from analysis that the conditions imposed upon the test containers did not affect the vacuum in these systems. The test conditions were strenuous to the point where they exceeded the normally expected environments to which the containers would be exposed. It may be concluded that normally expected amounts of agitation experienced in transport or fluctuations in temperature as in transport or storage are not primarily responsible for a loss of vacuum in these types of containers. When loss of vacuum is suspected, other factors such as decomposition of ingredients and faulty glassware or stoppers should be considered.

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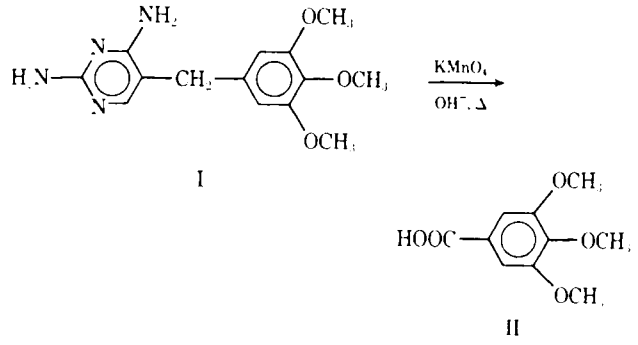
Semiautomated Spectrophotofluorometric Determination of Trimethoprim in Biological Fluids

S. A. KAPLAN▲, R. E. WEINFELD, and T. L. LEE

Abstract □ A semiautomated spectrophotofluorometric method for the determination of trimethoprim in blood, urine, and tissue is described. The initial extraction procedures are performed manually. The subsequent alkaline permanganate oxidation and the chloroform extraction of the fluorescent trimethoxybenzoic acid are performed by the automated system at a rate of 30 specimens/hr. The fluorescence is measured by a microflow cell in a spectrophotofluorometer. The method exhibits the same specificity and precision as the manual procedure, with a sensitivity limit in blood of 0.2 mcg./ml.

Keyphrases □ Trimethoprim in blood, urine, and tissue—semiautomated spectrophotofluorometric analysis □ Spectrophotofluorometry—analysis, semiautomated, trimethoprim in biological fluids

Trimethoprim[1] [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine, I] is an inhibitor of dihydrofolate reductase which potentiates the activity of sulfonamides against a wide variety of bacterial species (1, 2). The previously reported manual method for determining I in blood and urine (3) met the requirements for specificity, sensitivity, accuracy, and precision. However, the generation of a large number of specimens required



Scheme I

[1] Trimethoprim is an active ingredient in Bactrim, F. Hoffmann-La Roche and Co., Basle, Switzerland.

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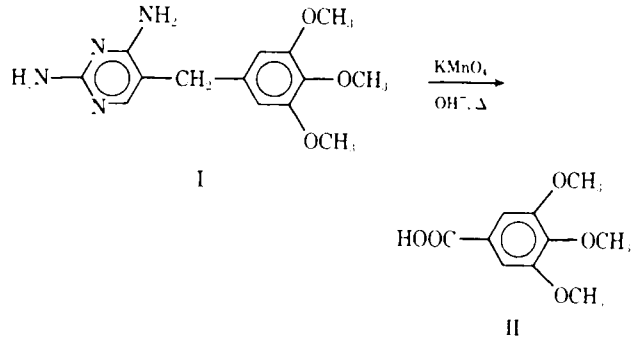

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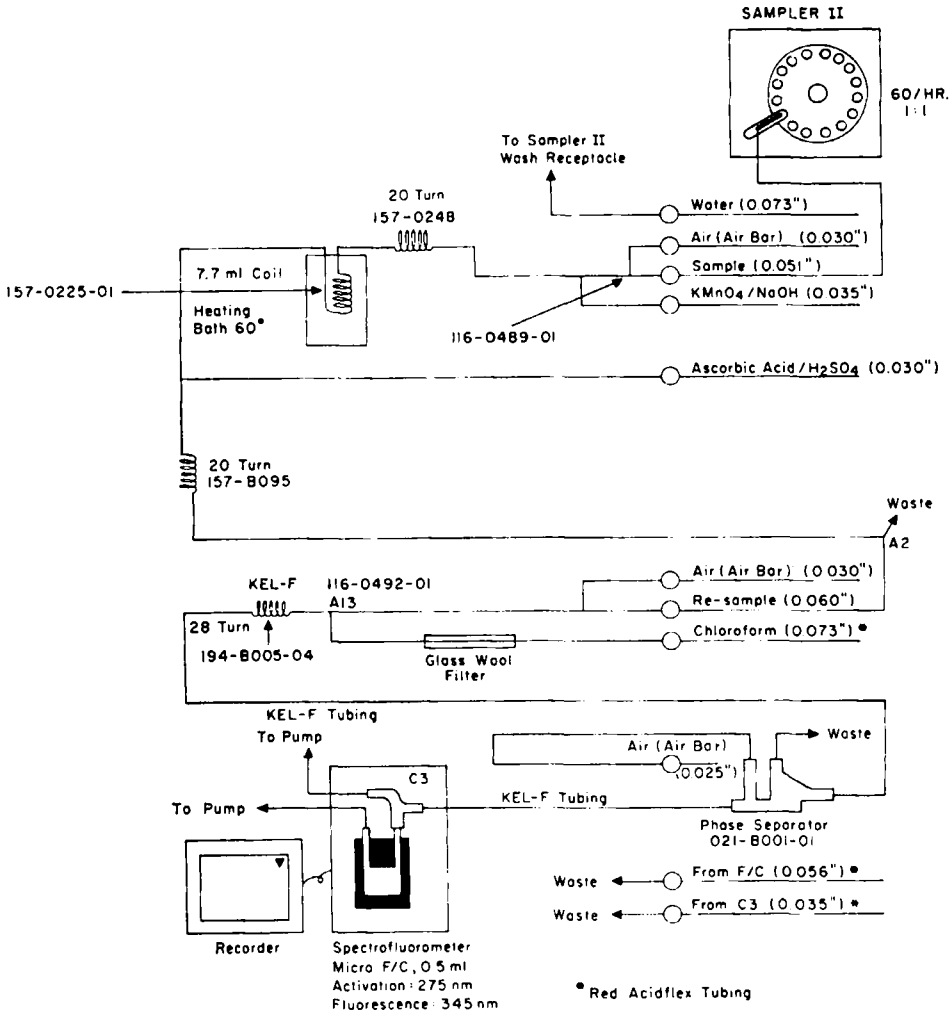

Figure 1—*Flow diagram for the automated determination of trimethoprim. Pump tubing is given with the internal diameter; Tygon tubing was used, except where noted. Technicon part numbers are also indicated.*

speed of analysis. Studies were undertaken to modify the manual spectrophotofluorometric method to make it compatible with continuous-flow automated techniques.

The manual procedure involves the extraction of I from biological specimens at pH 11.0 with chloroform, back-extraction into dilute sulfuric acid, and oxidation with alkaline potassium permanganate at 60° for 20 min. to the fluorescent trimethoxybenzoic acid, II (Scheme I). Following removal of excess permanganate with formaldehyde and subsequent acidification with 1 *N* sulfuric acid, II is extracted into chloroform and the fluorescence is measured at an activation wavelength of 275 nm. and a fluorescence wavelength of 345 nm.

Several modifications of the manual procedure were required to design an automated system using continuous-flow techniques. The manual, 20-min. oxidation step at 60° had to be shortened to approximately 4 min. at 60° for the automated system, and a 12% ascorbic acid solution in 1 *N* sulfuric acid was substituted for the 35% formaldehyde solution to destroy the excess potassium permanganate. The resulting clear acidified aqueous solution was extracted with chloroform in the automated procedure and, following phase separation, the organic phase was passed through the microflow cell of a fluorescence spectrophotometer[2] at the appropriate wavelengths. The finalized automated manifold utilizes automated analysis components[3] and allows for an analysis rate of 30 specimens/hr.

EXPERIMENTAL[4]

Reagents All chemicals are either reagent grade or USP: sodium carbonate, 0.2 *N* aqueous solution; sulfuric acid, 0.01 and 1 *N* aqueous solutions; potassium permanganate, 0.1 *M* in 0.1 *N* NaOH; ascorbic acid USP; 12% (w/v) ascorbic acid solution in 1 *N* H_2SO_4 (prepared fresh daily); and chloroform.

Standards—All standard solutions should be stored in a refrigerator and prepared fresh monthly. The following were used: trimethoprim, 100 mcg./ml. (dissolve 10.0 mg. of I in 0.01 *N* H_2SO_4 in a 100-ml. volumetric flask), and diluted standards of I ranging from 0.05 to 4.0 mcg./ml. in 0.01 *N* H_2SO_4. The standards are required for establishing external standard curves and for preparing internal standards.

[2] Perkin-Elmer.

[3] Technicon AutoAnalyzer II.

[4] The equipment and instruments used were: Technicon Sampler II, Technicon Instruments Corp., Tarrytown, NY 10591; Technicon proportioning pump model III; Techne thermostatic bath model TWB-5 with circulation pump, fitted with special cover for use with Technicon glassware, Techne, Inc., Princeton, NJ 08540; Perkin-Elmer model 204 fluorescence spectrophotometer with 150-w. xenon lamp light source, xenon lamp power supply, and R212 photomultiplier, Perkin-Elmer Corp., Norwalk, CT 06850; Technicon recorder with chart speed of 45.7 cm. (18 in.)/hr.; recorder interface accessory, Perkin-Elmer No. 010-0130; flow cell, rectangular, quartz, 0.5-ml. chamber capacity, 10-mm. light path, Perkin-Elmer No. 220-1461; and Technicon AutoAnalyzer II glassware.

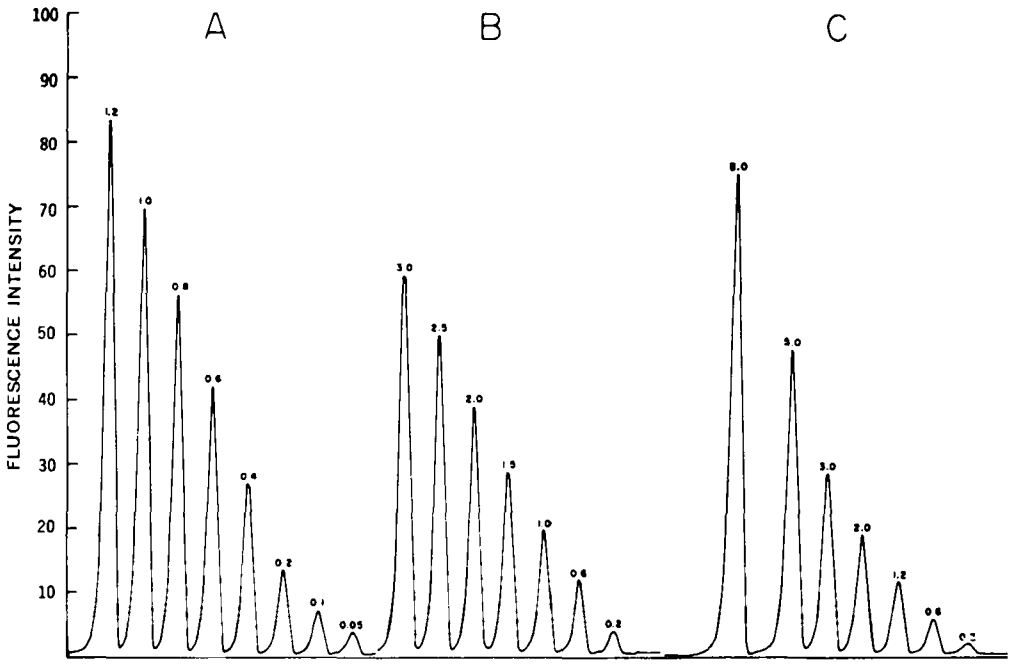

Figure 2—*Chart recordings relating trimethoprim concentration and fluorescence intensity. Instrument sensitivities were: A, 8 × 1; B, 6 × 1; and C, 5 × 1. Flow cell had 0.5-ml. capacity; recorder chart speed was 45.7 cm. (18 in.)/hr.*

Sample Preparation (Manual Extraction)—Pipet 1.0 ml. of blood, urine[5], or 10 ml. tissue homogenate[6] into a 40-ml. glass-stoppered, round-bottom centrifuge tube. Add 5 ml. of 0.2 *N* sodium carbonate solution for blood and urine specimens, and mix[7]. Extract the mixture with 10 ml. chloroform by shaking *slowly* for 15 min. on a mechanical reciprocating shaker. Centrifuge samples for 10 min. at 2000 r.p.m. with stoppers removed, and aspirate aqueous and most of the protein interface. Transfer a fixed aliquot of chloroform (7 or 8 ml.) to a 40-ml. glass-stoppered centrifuge tube, and extract with 5.0 ml. of 0.01 *N* sulfuric acid for blood[5] or tissue and with 10.0 ml. of 0.01 *N* sulfuric acid for urine due to higher concentrations of I[8] in urine. Shake for 10 min. at moderate speed, and centrifuge for 10 min. with stoppers removed. Transfer approximately 95% of the acid phase to 15-ml. glass-stoppered tubes and retain for automated analysis. Control (drug-free) blood, to which 5 or 10 mcg. of I[8] has been added, is analyzed along with the unknown specimens and serves as the internal drug standard. In the case of urine specimens, 30 mcg. of I is added.

Automated Analysis Procedure—The flow diagram for the automated manifold is presented in Fig. 1. Approximately 1 hr. prior to pumping any reagents, the spectrophotofluorometer, heating bath[9], and recorder (not the chart drive) should be turned on. The reagents are then pumped through the system for about 10 min., prior to establishing a reagent blank baseline on the recorder. The sampling cam used is 60/hr. [sample–wash ratio (1:1)] with a water wash cup between extracted samples. Sample cups[10] are used for extracts, standards, and washes.

Prior to introducing unknown extracts, two external standards of I are analyzed in the desired range (0.05–1.6 mcg./ml. for blood or 0.2–4.0 mcg./ml. for urine). The spectrophotofluorometer is adjusted to about 40% of full scale for a midconcentration standard using the instrument sensitivity control. The entire series of external standards of I can then be analyzed[11], placing a water wash cup between each sample.

The acid extracts are transferred to sample cups and placed on the sampler tray, with a water wash between extracts. When analyses of several hours are anticipated, the internal drug standard extract

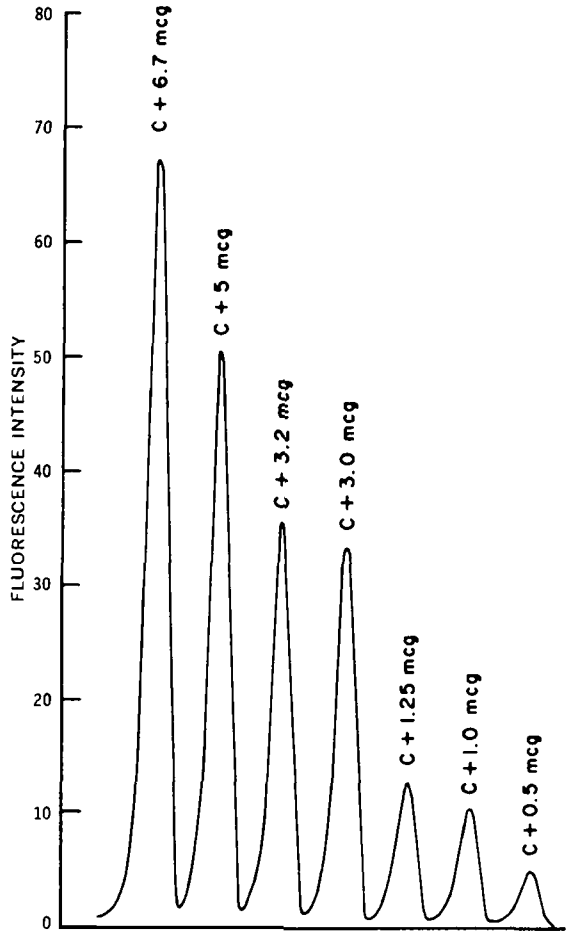

Figure 3—*Chart recording of indicated amounts of trimethoprim added to control (C) blood and analyzed by the described procedure. Acid extract volume = 5 ml.; instrument sensitivity was at 8 × 1.*

[5] Plasma, spinal fluid, or other biological fluids can also be analyzed by the same procedure.

[6] Homogenize a weighed tissue specimen directly with 0.2 *N* sodium carbonate solution. Use approximately 10 ml. sodium carbonate solution/g. tissue.

[7] In the analysis of urine, it is suggested that the pH of this mixture be checked so that the resultant pH is 11.0. Add additional sodium carbonate solution, if required.

[8] If necessary, the acid extracts can be further diluted with 0.01 *N* sulfuric acid to bring them into the range of the standard curve.

[9] Heating bath filled with ethylene glycol.

[10] Technicon.

[11] For routine analyses, it is unnecessary to run the entire standard curve. Duplicate determinations of the midconcentration standard are satisfactory for calculating percent recovery of the internal standard samples.

Table I—Concentration of Trimethoprim in Blood Determined by Manual and Automated Methods

Sample	Methods: Automated (A), mcg./ml.	Manual (B) Using Ascorbic Acid, mcg./ml.	Manual (C) Using Formaldehyde, mcg./ml.	Ratios: A/B	A/C	B/C
1	5.35	5.19	5.06	1.03	1.06	1.03
2	5.37	5.68	5.44	0.95	0.99	1.04
3	4.64	4.76	4.80	0.97	0.97	0.99
4	4.54	4.72	4.31	0.96	1.05	1.10
5	3.95	3.67	3.90	1.08	1.01	0.94
6	3.49	3.32	3.35	1.05	1.04	0.99
7	2.59	2.61	2.77	0.99	0.94	0.94
8	1.39	1.52	1.35	0.91	1.03	1.13
9	2.97	2.75	3.26	1.08	0.91	0.84
10	5.07	5.68	5.00	0.89	1.01	1.14
11	4.48	4.51	4.49	0.99	1.00	1.00
12	4.15	4.38	4.31	0.95	0.96	1.02
13	3.85	3.82	3.81	1.00	1.01	1.00
14	3.38	3.18	3.49	1.06	0.97	0.91
15	2.37	2.26	2.55	1.05	0.93	0.87
16	1.12	1.30	1.26	0.86	0.89	1.03
17	1.71	1.60	1.87	1.06	0.91	0.86
18	2.17	2.46	2.44	0.88	0.89	1.00
19	2.84	2.92	2.87	0.97	0.99	1.02
20	2.54	2.88	2.90	0.88	0.88	0.99
21	2.46	2.68	2.64	0.92	0.93	1.02
22	2.32	2.31	2.50	1.00	0.93	0.92
23	1.97	2.27	2.09	0.87	0.94	1.09
24	1.16	1.27	1.33	0.91	0.87	0.95
Mean ± *SE*				0.971 ± 0.014	0.963 ± 0.015	0.993 ± 0.016

should be reevaluated several times to check for any significant change in instrumental response.

The manifold (Fig. 1) performs the following steps. Acid extracts are aspirated from the sampler into the system, segmented with air, and mixed with alkaline potassium permanganate solution in a 20-turn coil. Oxidation is accomplished by passing the sample stream into a 7.7-ml. capacity coil (2.0 mm. i.d.) placed in a heating bath at 60°. The sample stream is then mixed with the ascorbic acid–sulfuric acid solution in a 20-turn coil to remove the excess potassium permanganate and acidify the stream, which is then debubbled, resampled through the pump, and resegmented with air. Chloroform is pumped directly with red pump tubing[12] through a glass-wool filter and mixed with the sample stream in a 28-turn coil[13] [0.10 cm. (0.040 in.) i.d.] to accomplish extraction of II into the chloroform. The mixture is passed through a four-point phase separator[3] with Teflon insert. The lower chloroform phase is resegmented with air and transferred through tubing to the flow cell in the spectrophotofluorometer. The sample stream is debubbled prior to entrance to the flow cell where the fluorescence of the chloroform stream is measured. The total time of analysis from sample aspiration to appearance on the recorder is about 12 min.

At the conclusion of the day's analyses, all tubing carrying aqueous reagents are washed through with water, while tubing carrying solvents are washed through with methanol and pumped with air until dry.

RESULTS

Standard Curves—Recorder tracings of external standards of I obtained by the automated procedure are shown in Fig. 2. Spectrophotofluorometer sensitivity settings are indicated in each case. Extracts of control blood specimens to which known amounts of I were added and a series of unknown human blood specimens following administration of the drug analyzed by the automated procedure are shown in Figs. 3 and 4. Extracts of control urine specimens to which known amounts of I were added and a series of unknown human urine specimens analyzed by the automated procedure are shown in Fig. 5. A plot of concentration of I (external and internal drug standards) *versus* fluorescence intensity is shown in Fig. 6 for the 0.05–1.2-mcg./ml. range and in Fig. 7 for the 0.2–4-mcg./ml. range. The fluorescence is linear up to 8 mcg./ml. of I, which is equivalent to approximately 4.10 mcg./ml. of II[14] under the described conditions.

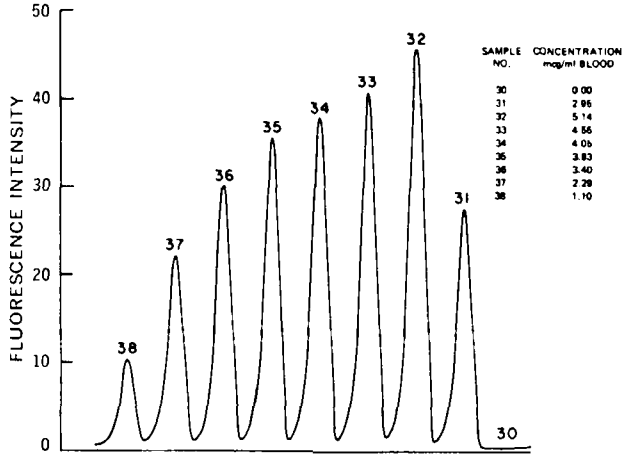

Figure 4—*Chart recording and corresponding concentrations of trimethoprim blood specimens following a single oral dose of the combination trimethoprim and sulfamethoxazole (400 and 2000 mg., respectively) formulation in man. Specimens correspond to 0, 1, 2, 3, 4, 6, 8, 12, and 24 hr. Acid extract volume = 5.0 ml.; instrument sensitivity was at 8 × 1.*

Extracts with a fluorescence intensity higher than the 8 mcg./ml. of I standard (or higher than the most concentrated standard being analyzed) must be diluted with 0.01 *N* sulfuric acid and rerun to bring the fluorescence into the linear portion of the standard curve.

Calculations—The fluorescence intensity at the apex of each peak is read directly from the chart recording. Reagent or control blood fluorescence is subtracted to give the net fluorescence due to I present. The concentration of I in the unknown specimens is di-

[12] Acidflex, Technicon.
[13] Kel-F, Technicon.
[14] Trimethoprim is oxidized to trimethoxybenzoic acid to the extent of about 70%; thus, the theoretical yield of 5.85 mcg./ml. trimethoxybenzoic acid is not attained.

Table II—Concentration of Trimethoprim in Urine Determined by Manual and Automated Methods

Sample	Methods: Automated (A), mcg./ml.	Manual (B) Using Ascorbic Acid, mcg./ml.	Manual (C) Using Formaldehyde, mcg./ml.	Ratios: A/B	A/C	B/C
1	4.4	4.1	4.0	1.02	1.11	1.08
2	7.1	6.9	6.4	1.07	1.09	1.02
3	173.3	155.6	216.1	1.11	0.80	0.72
4	34.6	33.3	32.4	1.04	1.07	1.03
5	175.7	156.4	171.5	1.12	1.02	0.91
6	28.7	33.3	28.3	0.86	1.01	1.18
7	72.8	68.7	74.8	1.06	0.97	0.92
8	29.2	30.2	29.3	0.97	1.00	1.03
9	9.8	8.5	9.7	1.15	1.01	0.88
10	75.4	75.3	75.1	1.00	1.00	1.00
11	45.7	41.3	47.2	1.11	0.97	0.88
12	162.2	160.9	179.4	1.01	0.90	0.90
13	36.5	38.0	35.0	0.96	1.04	1.09
Mean ± *SE*				1.037 ± 0.022	0.999 ± 0.022	0.972 ± 0.033

rectly proportional to the internal drug standard carried through the entire procedure. If further dilutions are necessary to bring the unknown extracts into the concentration range of the standards, they must be considered in calculating the final results. For urine specimens, dilution will be encountered more frequently since a much wider range of I values are encountered as compared to blood specimens.

The reproducibility of the automated steps of the procedure was determined by performing 34 replicate samplings of a 0.6 mcg./ml. I standard solution, at the spectrophotofluorometric sensitivity setting of 8 × 1 required for blood level studies, and was found to be 0.60 mcg./ml. ● 0.030 (*SD*).

The precision of the overall method including the manual sample preparation, manual extraction steps, automated steps, and fluorescence measurements was determined by the preparation and analysis of an internal drug standard, 10 mcg. I/ml. blood. This evaluation was based on the determination of the internal standard, in duplicate, by the automated section of the procedure on 11 separate days. The results of these analyses show that the precision of a single determination is approximately ±9.1%. This value was developed from analysis of variance which estimated both the variation within a day and between days. Comparable data evaluation based on single-day multiple determinations of drug internal standards using the manual method was reported to be ±6.2% (3).

Specimens of blood and urine containing I were analyzed by the manual and automated procedures to compare results. The published manual procedure (3) using formaldehyde to remove the excess potassium permanganate and the manual version of the automated procedure substituting 12% ascorbic acid were compared. This was necessary to demonstrate that the use of ascorbic acid in the automated procedure did not alter the chemical basis of the assay.

The results for 24 separate blood specimens analyzed by the three methods are shown in Table I, and results for 13 separate urine specimens containing a wide range of I concentrations are shown in Table II. Correlation of the results of these assays was tested by fitting a line to the joint determinations (A *versus* B, A *versus* C, and B *versus* C) and developing a 95% confidence region for the intercept and slope. The confidence limits for the intercept when the slope is fixed at unity are shown in Table III.

Significant differences would be reflected in confidence limits of the same sign. However, in those instances where this occurs, the calculated significance appears to be marginal (Table III).

Control bloods and urines exhibited no increase in fluorescence

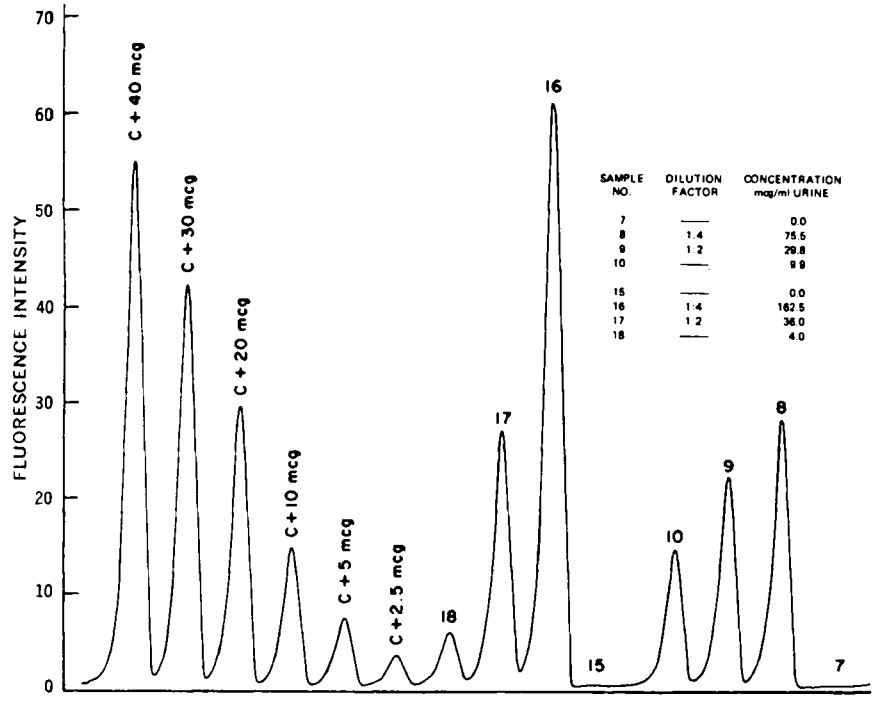

Figure 5—*Chart recording and corresponding concentrations of trimethoprim urine specimens following a single oral dose of the combination trimethoprim and sulfamethoxazole (400 and 2000 mg., respectively) formulation in two subjects and urine internal standards of the indicated amounts of trimethoprim added. Specimens correspond to control, 0–24, 24–48, and 48–72 hr. in each subject. Acid extract volume = 10.0 ml. (with appropriate dilutions); instrument sensitivity was at 6 × 1.*

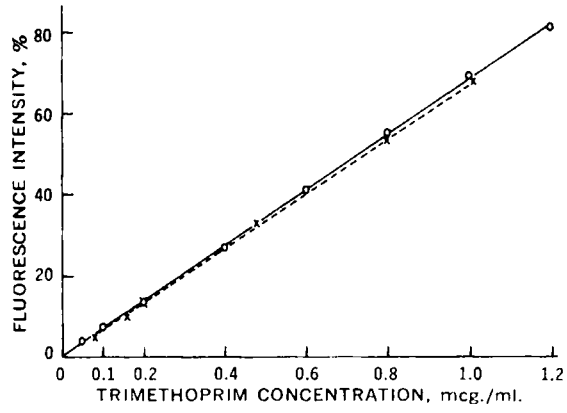

Figure 6—*Plot of trimethoprim standards (direct and recovered from blood) versus fluorescence intensity by automated procedure. Trimethoprim concentration, mcg./ml. acid expected. Instrument sensitivity was at 8 × 1. Key: O—O, direct standards; and ×- - -×, added to blood.*

compared to a reagent blank when analyzed by the automated procedure. No shifting of baseline was noted for control bloods, urines, and reagent blank.

The specificity of the automated procedure is the same as that previously reported for the manual procedure (3). In both the automated and manual procedures, it was demonstrated that physiological components of human plasma or blood do not interfere with the determination of I. Sulfonamides normally administered along with trimethoprim do not interfere with the analytical procedure (3).

DISCUSSION

The direct automation of the manual procedure for the determination of trimethoprim in blood and urine presented several major problems. Use of formaldehyde in the automated system resulted in the formation of the particulate brown manganese dioxide which clogged the mixing coil after several specimens passed through the system. Attempts to break up the precipitate proved difficult due to the large amounts formed. The same procedure was attempted using a solution of sodium bisulfite in place of formaldehyde. Although this process eliminated much of the precipitate problem, the resulting dissolved sulfur dioxide interfered with the fluorescence of II in the chloroform. To overcome this problem, a solution of sodium tetrachloromercurate was unsuccessfully used to complex the large amounts of sulfur dioxide produced (4). Ascorbic acid proved to be an excellent reagent for removing excess potassium permanganate from the reaction mixture. A 12% aqueous solution of ascorbic acid produced negligible particulate manganese dioxide, which was completely dissolved after several turns through the mixing coil. Preparation of the ascorbic acid in 1 *N* sulfuric acid proved to be more efficient than sequentially adding each reagent to the reaction mixture.

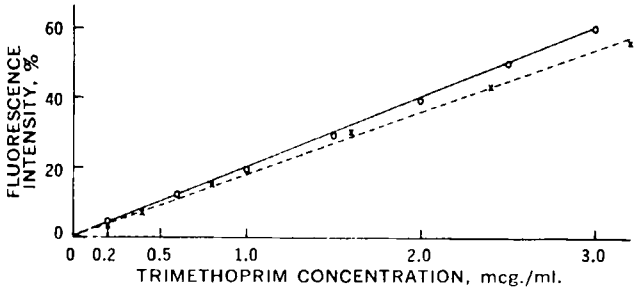

Figure 7—*Plot of trimethoprim standards (direct and recovered from blood) versus fluorescence intensity by automated procedure. Trimethoprim concentration, mcg./ml. acid expected. Instrument sensitivity was at 6 × 1. Key: O—O, direct standards; and ×- - -×, added to blood.*

Table III -Confidence Intervals

Fluid Analyzed	Method Y	Method X	95% Confidence Interval for Intercept when Slope = 1	Correlation Coefficient (*r* Value)
Blood	A	B	(−0.26, 0.01)	0.987
Blood	A	C	(−0.09, −0.02)	0.994
Blood	B	C	(−0.05, 0.16)	0.985
Urine	A	B	(0.7, 7.0)	0.997
Urine	A	C	(−15.0, 3.8)	0.990
Urine	B	C	(−18.4, −0.5)	0.987

Working with Kel-F tubing yielded better results than Teflon tubing, particularly for the extraction coil and less importantly for the transfer lines, due to their surface tension properties.

In developing the automated manifold for I, it was found that the use of AutoAnalyzer II glassware (mixing coils, injection fittings, phase separators, and tubing) resulted in superior overall reproducibility as compared with AutoAnalyzer I components. The former system was the most efficient regarding reagent consumption, reproducibility of bubble pattern, and phase separation and is considered the system of choice.

Development of a fully automated manifold for the determination of trimethoprim is being investigated. This will involve direct extraction of the buffered biological fluid with chloroform on the manifold and back-extraction of the chloroform with dilute sulfuric acid prior to the automated procedure described.

SUMMARY

A semiautomated spectrophotofluorometric method for the determination of trimethoprim in blood and other biological specimens is described. The automated procedure has the same specificity and precision as the manual procedure and allows for the analysis of 30 specimens/hr.

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Subphase Exchange Apparatus for Monomolecular Film Studies

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Abstract □ An apparatus for studying monomolecular films, by which the subphase can be changed without disturbing the film, was developed and the effectiveness of the apparatus was studied. Almost 100% of the subphase could be replaced when 1500 ml. of the subphase was exchanged over 20 min. By studying dipalmitoyl lecithin monolayers with and without subphase exchange (the π–A curves in both cases were the same), it was established that the films are not disturbed during the subphase exchange operation. The effect of subphase exchange on human serum albumin films of different concentrations was studied. The films became condensed when a water subphase was exchanged with water, apparently because of loss of some protein from the surface. This effect was more pronounced when films were spread using larger amounts of human serum albumin. The loss of less protein when less human serum albumin was spread was attributed to better spreading. The films also became condensed when a sodium chloride subphase solution was replaced with sodium chloride solution of the same concentration. However, the effect was less pronounced as compared to the water exchange with water. The π–A plots of human serum albumin films, in which the water subphase was exchanged with sodium chloride solution, were superimposable on the π–A plots of films spread on sodium chloride solution without exchange. Films of malate dehydrogenase formed using different amounts of this enzyme were studied after the buffer subphase was changed with a buffer solution of the same concentration. The exchange of this subphase had no effect on the π–A plots of any malate dehydrogenase film.

Keyphrases □ Monomolecular films—apparatus for changing subphase without disturbing film, dipalmitoyl lecithin, human serum albumin, and malate dehydrogenase films □ Films, monomolecular—apparatus for changing subphase without disturbing film □ Dipalmitoyl lecithin films—used to study subphase-changing apparatus □ Human serum albumin films—used to study subphase-changing apparatus □ Malate dehydrogenase films—used to study subphase-changing apparatus

Monomolecular films represent a relatively simple type of membrane model having a well-defined organized structure. They provide a convenient and promising method of studying molecules in a fixed orientation, as well as in a single layer where orientation can be changed by compression of the monomolecular film. As such, they constitute an important model system for the study of many natural phenomena involving surfaces of an oriented array of molecules.

Monomolecular films have been extensively used as structural models of the cell membrane. These model membranes cannot be expected to mimic fully a cell membrane. Their simplicity can also be considered a serious shortcoming to their use as a general model for biological membrane function. What can realistically be expected from studies with model membranes is some insight into the molecular mechanism of specific functions, as well as some knowledge of the function of specific areas within the heterogeneous makeup of biological membranes. Nevertheless, they provide considerable insight into the structure and function of biomembranes.

Monolayer studies involving proteins and enzymes present unique difficulties. For example, estimation of catalytic activity of surface-spread enzymes is difficult because of possible contamination by adsorbed unspread molecules beneath the film or by protein molecules dissolved in the bulk. There is a need for assaying enzymes *in situ*, but the dangers of contaminating the film with unspread globular protein from the subsolution are great (1). Ideally, the assay should be carried out while the molecules are adsorbed at the interface in question without manipulation or transfer of the film (2).

About 25–56% of protein spread on water was reported (3) to escape to the bulk, leaving 75–44% in the surface. Mechanical agitation was applied (3) under the film to assist the molecules adsorbed under the film to escape to the bulk. The amount of protein escaping to the bulk, of course, depends on many factors including the amount of protein applied, method of spreading, and spreading time.

To determine the activity of a surface-spread enzyme, the fraction of the enzyme that is adsorbed just below the surface or that escapes into the bulk phase must first be removed. In previous studies, this was accomplished by removing the surface-spread enzyme from the environment of the subphase either by transferring the enzyme monolayer to metal slides or by removing it with filter paper or a silk net. Alternatively, monolayers have been compressed to a fiber and then transferred to the substrate (3).

To investigate the effects of varying film pressures (varying degree of unfolding) on the activity of surface-spread acetylcholinesterase, a special four-compartment trough was devised (3) to provide a subphase free of dissolved or adsorbed enzyme. The protein was spread on one compartment between two barriers, and vigorous stirring was applied to the underlying solution to remove adsorbed unspread molecules from beneath the film. The film was then transferred by sliding the barriers to another compartment with a clean subphase containing the substrate. The surface pressure was measured in this second compartment using a platinum blade suspended from a tension transducer. When the film was moved, the whole apparatus was shifted so that the blade was always in contact with the film.

A three-compartment trough was utilized in a similar manner (4). The film was spread over one compartment and then was moved to the second compartment, with the help of a movable frame, where the subsolution was vigorously stirred. Then the film was moved to the third compartment onto fresh subsolution by shifting the frame.

A circular, multicompartment, Teflon trough was introduced (5) which allowed the shifting of mono-

layers over different subphases by moving together two barriers resting on the trough edge. A Wilhelmy balance was shifted with the film. The walls separating the compartments were lower than the edges so that the monolayers could be moved from one compartment to the other. The different compartments could be filled or emptied independently.

In all of these methods, the monolayer itself has to be transferred from the subphase on which the film is initially spread. This procedure may disturb the film and introduce additional uncertainties into the study.

The purpose of this study was to develop an apparatus that could be used to free the subphase under a spread film of dissolved constituents or contaminants without moving or disturbing the film. Such an apparatus would be of great use in studying the activity of surface-spread enzymes free of dissolved enzymes. It would also be useful for studying adsorption–desorption of ions and ion-exchange processes using lipid and protein monomolecular films.

EXPERIMENTAL

Materials—Synthetic L-α-dipalmitoyl lecithin[1], procine heart malate dehydrogenase[1] (260 units/mg. as a suspension in 70% ammonium sulfate), and crystalline human serum albumin[2] were used.

The organic solvents used, all spectrograde, were checked for surface-active impurities by compression on the film balance after addition of small amounts of these solvents onto the subphase. About 3 ml. of the solvent was placed on the surface (surface area of trough = 315.0 cm.^{2}) and, after 25 min., the surface area was reduced to 105.0 cm.^{2} by shifting the barrier. No surface pressure was observed for any solvent used. The inorganic chemicals used, all reagent grade, were also checked for surface impurities. Sodium chloride was roasted at 700° for 8 hr. Surface tension measurements of sodium chloride solutions and tromethamine–hydrochloric acid solutions before and after compression showed these solutions to be free of surface-active impurities.

The water used was deionized by passing tap water through a mixed resin bed of a demineralizer[3] and then distilling it in an all-glass still.

Apparatus—The Teflon-coated trough (525-ml. capacity) is removable to facilitate cleaning. The precision lead screw, which drives the reinforced Teflon barrier, allows for changes in surface area of the trough as small as 0.0125 cm. The barrier can be disengaged from the lead screw for rapid sweeping of the surface. The Teflon used for the trough and barrier has an advantage over the traditional wax-coated glass in that it is chemically inert as well as hydrophobic and oleophobic. The surface balance[4] is enclosed in a Plexiglas cabinet to minimize possible surface contamination by dust and grease from the environment.

Surface pressure values were measured by the Wilhelmy plate method (6). The platinum plate, roughened to ensure wetting, was suspended from a torsion balance[5] which can measure surface pressure changes as small as 0.1 dyne/cm.

The described surface balance was modified (Fig. 1) to allow for subphase exchange as required. The liquid used to replace the subphase liquid in the trough was placed in the 2.5-l. separator A, which served as a reservoir. During the operation, the stopper P of the separator was kept tightly closed so that it was air tight, whereas the Teflon stopcock B was kept open. The level of the stem D of the separator was adjusted by raising or lowering the separator A, by adjusting the ring stand supporting the separator, or by raising or lowering the vessel C using the adjustable jack E. The mouth of the vessel C was kept covered by aluminum foil to avoid contamination from the air. The level of the stem D controlled the level of the liquid in vessel C, which, in turn, controlled the level of the liquid in the trough H of the surface balance. The reservoir A and the vessel C worked somewhat like an animal feeder. As soon as the level of the liquid started to drop in vessel C, it was replenished by liquid from reservoir A. This served to maintain the liquid level in C constant, which, in turn, maintained the level in the trough constant. The flow of liquid to the trough could be stopped by closing stopcock F.

The inlet glass tube G was placed in one corner of the trough on the side from which compression was started, and the outlet glass tube I was placed in another corner on the opposite side. The barrier was so positioned that the inlet glass tube G did not interfere with the movement of the barrier during compression. The liquid level was so adjusted that the liquid level in the trough was slightly above the brim of the trough throughout the operation.

To control and maintain a certain flow of liquid through the system (trough), the stopcock J was adjusted and its position was kept fixed afterward. To start or stop the flow of liquid through the system, stopcock L was opened or closed, respectively. To measure the rate of flow, the outflowing liquid from the polyethylene tube K was collected in a graduated vessel M.

In all experiments, the subphase exchange was started 10 min. after spreading the film and the film was compressed 5 min. after stopping the flow. The subphase exchange was completed in 20 min. in all cases. Flow rates were controlled so that either 900 or 1500 ml. was exchanged in this period. At these rates, no visual disturbance of the film was observed. To ensure that the apparatus was free of surface-active impurities, water in the trough was replaced with 2000 ml. of water from separator A and the surface tension was checked at full trough area and after compression to one-third trough area. The apparatus was found to be free of any surface-active contaminants by this method.

Dipalmitoyl lecithin monolayers were studied with and without subphase exchange to establish that there was no film loss during the subphase exchange procedure.

To determine the amount of liquid required to replace the subphase, a 0.01% aqueous solution of benzoic acid in the trough was exchanged with water from separator A. The absorbance of benzoic acid solution samples taken from the trough at different intervals was read at 273 nm., and the concentration of benzoic acid was determined from a standard curve. Percentages of original benzoic acid concentration in the trough *versus* volume of liquid replaced in the trough plots were then constructed for two different flow rates.

Preparation of Spreading Solutions—*Dipalmitoyl Lecithin*—Dipalmitoyl lecithin (22.5 mg., mol. wt. 734) was dissolved in ethanol–hexane (5:95 v/v) to make 50 ml. of solution. In all experiments with dipalmitoyl lecithin, 0.07 ml. of this solution was spread on a tromethamine–hydrochloric acid buffer (pH 7.4) subphase.

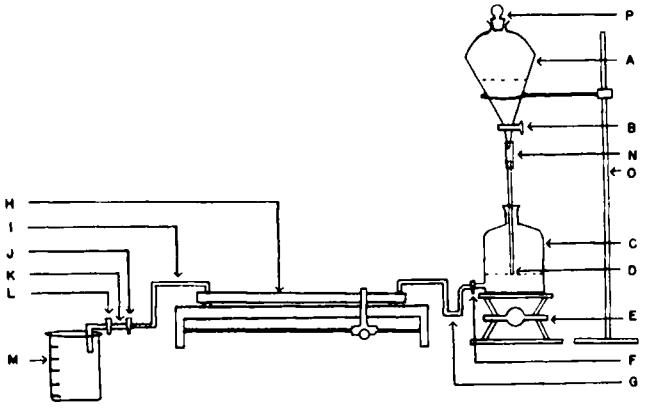

Figure 1—*Diagrammatic representation of the modified surface balance used for subphase exchange. Key: A, separator (2500 ml.) with stem extended by polyethylene tube joint and glass tube (reservoir); B, stopcock; C, 2-l. jar with outlet at bottom; D, glass tube (for liquid level adjustment in vessel C); E, adjustable jack; F, stopcock and polyethylene tubing; G, glass tube; H, surface balance (trough capacity of 525 ml.); I, glass tube; J, stopcock for adjusting flow of liquid; K, polyethylene tube (flexible); L, stopcock for starting or stopping liquid flow; M, graduated beaker for collecting outflowing liquid; N, polyethylene tube joint; O, support stand; and P, stopper.*

[1] Schwarz/Mann Research Laboratories, Orangeburg, N. Y.
[2] Nutritional Biochemical Corp., Cleveland, Ohio.
[3] Bantam.
[4] Frater Instrument Co., Corona, N. Y.
[5] Federal Pacific Electric Co.

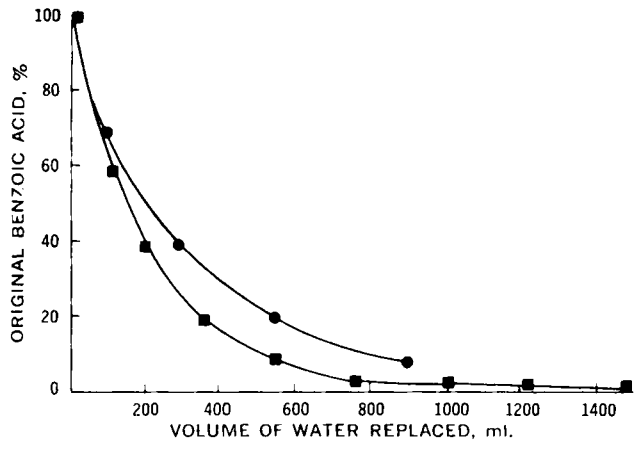

Figure 2—*Volume of water replaced in trough* versus *percentage of the original benzoic acid concentration (0.01% w/v in water) in the trough. Key:* ●, *900 ml. replaced in 20 min.; and* ■, *1500 ml. replaced in 20 min.*

Human Serum Albumin—Crystalline human serum albumin (24.2 mg.) was dissolved in water to make 50 ml. of solution. It was stored in the freezer and was thawed to room temperature immediately before use. In the various experiments, 0.038 and 0.07 ml. of this solution were spread on either water or 10% sodium chloride solution by the Trurnit (8) method.

Malate Dehydrogenase—An appropriate volume of the suspension was dissolved in 5 ml. of the tromethamine-hydrochloric acid buffer solution (pH 7.4) to yield a concentration of 1 mg./ml. This solution was stored in the freezer and thawed to room temperature just before use. In the various experiments, 0.07 and 0.1 ml. of this solution were spread on a tromethamine-hydrochloric acid buffer subphase (pH 7.4) by the Trurnit (8) method. The pH 7.4 for malate dehydrogenase studies was chosen because the highest activity of malate dehydrogenase is reported to occur in the physiological range of pH 7.3–7.5 (7).

In the case of proteins, different volumes of the same spreading solution were delivered to spread films of different protein concentrations.

Procedure—Before spreading the film-former solution, the surface of the subphase was swept several times with the barrier to clean the surface. The surface was checked for cleanliness by taking readings at full trough area and after compressing to one-third trough area. The same reading in both positions ensured that the surface was clean. The platinum plate was then lowered into position and allowed to remain beneath the surface while a known volume of the film-forming substance was spread with a micrometer syringe[6], which can deliver volumes as small as 0.001 ml. accurately. In all experiments, compression was initiated 35 min. after the film was spread, allowing the system to come to "equilibrium" and also ensuring complete evaporation of the spreading solvents for phospholipids. The area available to the film molecules was then reduced in small increments, and surface pressure readings were taken 1 min. after each area change. Compression was usually continued until the film collapsed.

Solutions of dipalmitoyl lecithin were applied onto the subphase by allowing small drops to fall from the syringe held a few millimeters above the surface. The proteins were spread by the Trurnit (8) method. A 5-mm. diameter glass rod was bent so that it could be placed in the trough on its own base. The vertical limb of the glass rod was 40 mm. long and had a hemispherical top. Before spreading, the glass rod was thoroughly washed and then placed in the trough. The protein solutions were applied from the micrometer syringe on the glass rod top at a slow, steady rate. The syringe was always held directly above the top of the glass rod. The glass rod was then washed by applying drops of the same solution being used as the subphase, with the help of a clean dropper. The number of drops was kept constant for all readings. The washing ensured that no protein was left sticking to the portion of the glass rod outside the subphase. The glass rod was so positioned that it could remain in place during the compression cycle.

Two different amounts of each protein were spread to demonstrate the effects of: (*a*) subphase exchange on different protein concentrations, and (*b*) protein concentration on area per unit weight of the protein.

The 35-min. period between spreading and compression also allowed enough time for subphase exchange when required.

All experiments were carried out at room temperature (25 ± 2°).

RESULTS AND DISCUSSION

A plot of the original benzoic acid concentration in the trough *versus* volume of water replaced in the trough is shown in Fig. 2. It was observed that when 900 ml. of water was replaced over 20 min., the benzoic acid concentration in the trough was reduced by 92%. In the first 250-ml. change of subphase, 57% loss in concentration occurred. Figure 2 also shows a plot of benzoic acid concentration *versus* volume of water replaced in the trough when 1500 ml. of water was used to replace the subphase solution over the same 20-min. period; almost 100% (>99%) of the subphase solution was replaced. The major loss in benzoic acid concentration (69%) again occurred with the replacement of the first 250 ml. It is important to note that for a given volume of subphase exchanged, the amount of benzoic acid solution replaced is dependent on the rate of exchange of the subphase; *i.e.*, the rate of benzoic acid loss is greater at a faster rate of subphase replacement. Thus, to achieve reproducible results, it is necessary to work at a fixed rate of exchange of the trough liquid. With the apparatus and technique used, essentially 100% exchange of the subphase solution in the surface balance trough can be achieved.

Figure 3 shows π–A plots of spread films of dipalmitoyl lecithin on water without subphase exchange and of dipalmitoyl lecithin films on water when the subphase was exchanged with 900 and 1500 ml. of water. The π–A plots in all these cases are identical, indicating that there was no leakage of the film under the movable barriers or any disturbance of the film during the subphase exchange operation. These experiments indicate that this apparatus can be used to replace the subphase under an insoluble lipid film without affecting the film characteristics.

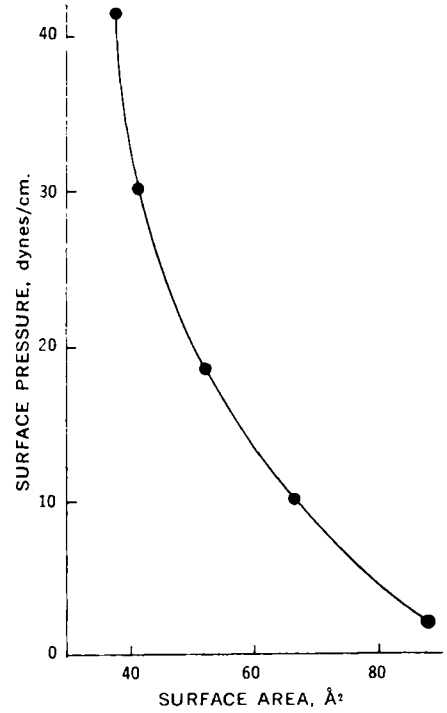

Figure 3—π–A *curves of dipalmitoyl lecithin spread on water. Key:* ●, *no subphase replacement;* ●, *subphase replaced with 900 ml. water; and* ●, *subphase replaced with 1500 ml. water.*

[6] Agla, Burroughs Wellcome.

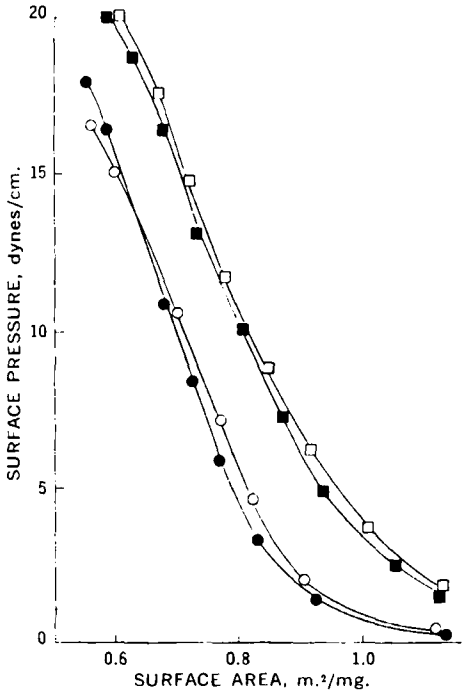

Figure 4-- π-A *curves for 0.0184 mg. human serum albumin. Key:* ○, *spread on water;* ●, *spread on water and subphase replaced with 1500 ml. water;* □, *spread on 10% (w/v) sodium chloride solution; and* ■, *spread on 10% (w/v) sodium chloride solution and subphase replaced with 1500 ml. 10% (w/v) sodium chloride solution.*

In the protein studies, human serum albumin films were spread by the Trurnit (8) method either on water or on 10% sodium chloride solution; in certain experiments, the subphase was exchanged. In all experiments, the human serum albumin formed stable films which gave reproducible π-A plots. Figure 4 shows π-A plots of human serum albumin films (0.0184 mg.) on a water subphase and on a 10% sodium chloride solution subphase. The two plots are parallel, but the film on the 10% sodium chloride subphase has a much larger area per milligram at all surface pressures; *i.e.*, it is more expanded than the film spread on water. The expansion effect was also observed when a larger amount of human serum albumin was spread (0.0339 mg.) on the 10% sodium chloride solution subphase (Fig. 5). These data suggest that more complete spreading occurred with both concentrations of protein in the presence of 10% sodium chloride.

In the case of proteins, the presence of charges on the molecules must be given consideration concerning their ability to spread. According to Crisp (9), better spreading of protein on solutions of high ionic strength can readily be understood if it is recognized that the electrical potential set up by the charged film inhibits further adsorption of additional charged protein molecules at the interface. A decrease in this potential occurs as the concentration of electrolyte in the solution is increased. Charge repulsions between molecules in the plane of the film are reduced also as the ionic strength increases, because with it the dielectric constant of the subphase increases. Cheeseman and Davies (10) attributed the facilitation of spreading and unfolding of protein molecules by salt solutions to the diminution of intramolecular cohesion, because of reduced electrostatic attraction between ionizable groups as the effect of the ionic charges are canceled. Some investigators (11) stated that ions exert their effect by altering the structure of water, thus weakening the tendency of protein to form hydrophobic bonds and so facilitating uncoiling of the protein molecule. Spreading protein on a high salt concentration subphase may simply reduce the solubility of the protein considerably (salting-out effect), thus minimizing the loss of protein into the subphase by diffusion (12). Therefore, salts would be expected to cause a marked increase in surface area of protein films *via* one or all of the mechanisms previously described, although it cannot be said with certainty which is responsible for the observed expansion of human serum albumin films on the salt solution subphase.

Figure 4 shows the effect of change of subphase with water on films of human serum albumin (0.0184 mg.) spread on water. Exchange of the subphase results in a slight shift of the π-A plots to smaller surface areas. The difference, while small, is significant. The same effect is observed when the subphase of human serum albumin films spread on 10% sodium chloride solution is replaced by 1500 ml. of a sodium chloride solution of the same concentration.

The effect of subphase exchange is much more pronounced when a larger amount of the protein is spread. Figure 5 shows the effect of subphase exchange on films of human serum albumin when 0.0339 mg. of the protein was spread. When the subphase of the film spread on water was replaced by water, the π-A profile shifted to smaller areas. This suggests that when the subphase is exchanged, some protein is lost. Cheeseman and Davies (10) postulated that, when proteins are spread on substrates in which they are soluble, an unstable equilibrium may be established. The monolayer proper consists of completely or almost completely unfolded molecules, beneath which exists an adsorbed layer of relatively unmodified molecules. These molecules are held in the interfacial region by salt linkages or hydrogen bonding, and some of this unmodified protein is forced into the subphase. It is possible that this adsorbed layer of relatively less unfolded protein molecules under the monolayer may be swept away and removed as the subphase is replaced by fresh subphase. This would, in turn, result in a reduction in surface area at all given surface pressures.

When the effect of subphase exchange on the two amounts of spread human serum albumin is compared, it can be seen that there is a very small effect on the π-A plots when 0.0184 mg. is spread (Fig. 4) while the effect is quite significant when 0.0339 mg. is spread (Fig. 5). This may be due to better spreading in the former case since the protein molecules under this condition have more area available at the interface for unfolding, allowing relatively more molecules to unfold completely and form a more stable monolayer with fewer unmodified molecules adsorbed under it. In this case, more molecules would be held securely at the interface and thus less protein would be removed by subphase exchange. At higher concentrations of protein, molecules would have less area available for unfolding and thus more molecules would be adsorbed under the film. These adsorbed molecules would be more easily removed when the subphase is exchanged.

Figure 5 also shows the effect of exchanging the subphase with sodium chloride solution when 0.0339 mg. human serum albumin was spread on the sodium chloride subphase solution. This reduction in surface area is less than when the water subphase is changed

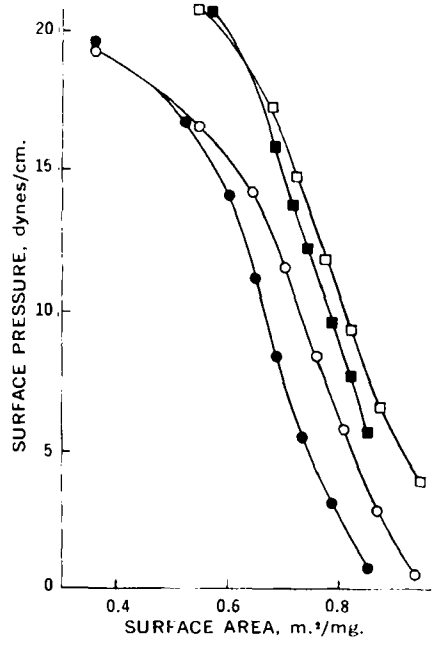

Figure 5-- π-A *curves for 0.0339 mg. human serum albumin. Key:* ○, *spread on water;* ●, *spread on water and subphase replaced with 900 and 1500 ml. water;* □, *spread on 10% (w/v) sodium chloride solution; and* ■, *spread on 10% (w/v) sodium chloride solution and subphase replaced with 1500 ml. 10% (w/v) sodium chloride solution.*

with water. This may be due to the better spreading of the protein on the salt solution as well as to its reduced solubility in the subphase as compared to water. The π–A plots for human serum albumin films are essentially the same when both 0.0339 and 0.0184 mg. of protein are spread (Figs. 4 and 5). This demonstrates that the area per unit weight of the human serum albumin film is almost independent of the amount of protein spread within the range studied.

The effect of exchanging the water subphase with sodium chloride solution on films of 0.0339 mg. human serum albumin is shown in Fig. 6. The π–A plots of the film after the exchange of subphase are superimposable on the π–A plot of 0.0339 mg. human serum albumin spread on 10% sodium chloride solution. The film is expanded as compared to the film on the water subphase. This suggests that when sodium chloride solution is exchanged for the water subphase, the salt either allows the protein molecule in the film to unfold more completely or forces any protein molecule adsorbed under the film into an area determining position at the interface. If the latter is true, these data would also indicate that, in a water subphase, any protein molecule not in an area determining position is adsorbed at or close to the interface rather than dispersed uniformly throughout the solution. If the protein is dispersed uniformly throughout the subphase rather than being primarily in the unstirred layers close to the interface, much of it would be swept away during subphase change and the π–A curves would not be superimposable.

The effect of replacing the subphase with water when 0.0339 mg. of human serum albumin was spread on 10% sodium chloride solution is also shown in Fig. 6. There is a significant reduction in surface area, but the π–A plot is parallel to the π–A plot of the human serum albumin film spread on the sodium chloride solution. However, the π–A plot does not quite coincide with the plot of the film spread on water alone. This may be due to better spreading of the protein initially on the sodium chloride solution subphase, which would tend to reduce the amount of protein lost when the subphase is exchanged. Moreover, since the protein solubility is greater in water than in sodium chloride solution, some protein would be expected to be lost following the exchange. This could account for the reduction in the surface area of the film as shown in Fig. 6.

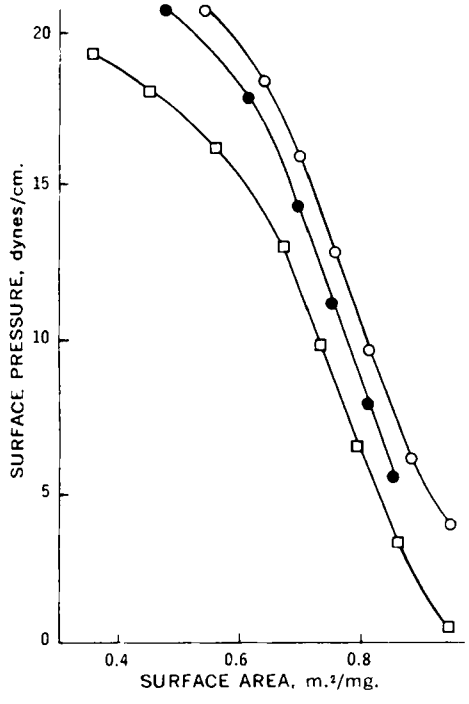

Figure 6—π–A *curves for 0.0339 mg. human serum albumin. Key:* ○, *spread on 10% (w/v) sodium chloride solution;* ○, *spread on water and subphase replaced with 1500 ml. 10% (w/v) sodium chloride solution;* ●, *spread on 10% (w/v) sodium chloride solution and subphase replaced with 1500 ml. water; and* □, *spread on water.*

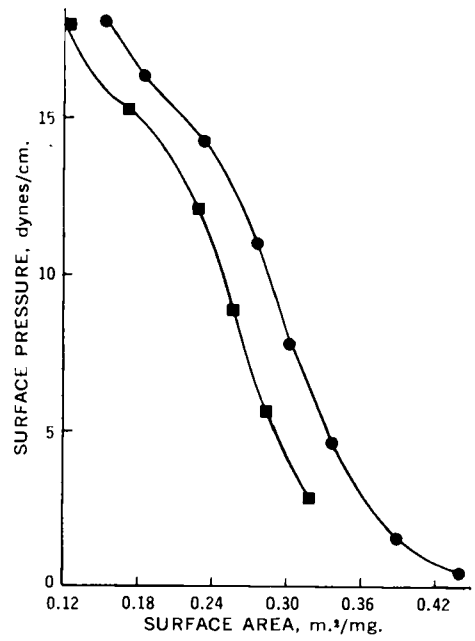

Figure 7—π–A *curves of malate dehydrogenase films on pH 7.4 tromethamine–hydrochloric acid buffer solution. Key:* ●, *0.07 mg. spread on buffer;* ●, *0.07 mg. spread on buffer and subphase replaced with 900 and 1500 ml. buffer;* ■, *0.10 mg. spread on buffer; and* ■, *0.10 mg. spread on buffer and subphase replaced with 900 and 1500 ml. buffer.*

The malate dehydrogenase films were spread on pH 7.4 tromethamine–hydrochloric acid buffer solution. In some experiments, the subphase was exchanged with a buffer solution of the same composition. The malate dehydrogenase films were spread by the Trurnit (8) method and, in all experiments, malate dehydrogenase formed stable films which gave reproducible π–A curves.

Figure 7 shows π–A plots of 0.1 and 0.07 mg. malate dehydrogenase spread on the buffer solution. The π–A plots of the two different amounts of malate dehydrogenase are not superimposable, and the curve representing the larger amount of enzyme spread is more condensed. However, the two plots are parallel, and both show an inflection point at about 15.7 dynes/cm. surface pressure. Beyond this inflection point, at smaller surface areas, the films become less compressible.

The reason why the area per molecule at all surface pressures is greater when a smaller amount of malate dehydrogenase is spread might be due to better spreading of the film under this condition, since more area is available to the molecules and better unfolding and less enzyme loss to the subphase would result. Thus, in the case of malate dehydrogenase films, the area per unit weight of protein is dependent on the amount spread.

Figure 7 also shows the effect of replacing the tromethamine–hydrochloric acid buffer subphase with 900 and 1500 ml. of a buffer solution of the same composition on 0.07 and 0.1 mg. malate dehydrogenase films. The change of subphase has no effect on either concentration of malate dehydrogenase films. This shows that both the 0.1- and 0.07-mg. malate dehydrogenase films are very stable, and no enzyme is lost by replacing the subphase. Thus, it appears that the difference in surface area observed when different amounts of this enzyme are spread is the result of differences in the degree of unfolding only, rather than dissolution or adsorption of protein below the film surface.

The study demonstrates the effectiveness of this apparatus in exchanging the subphase under monomolecular films of lipids and proteins without significantly disturbing the films or modifying their π–A characteristics.

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▲ To whom inquiries should be directed.

NOTES

Cardiovascular Effects of Digoxin–Phenelzine Interaction in Rabbits

JEFFREY S. BOHRMAN* and EMMANUEL B. THOMPSON▲

Abstract □ The potential toxicity of combining phenelzine, an MAO inhibitor, and digoxin, a cardiac glycoside, was studied in rabbits. Cardiovascular responses following concurrent administration of these drugs and the administration of these drugs 20 and 60 min. apart, respectively, revealed that phenelzine and digoxin evoked arrhythmias in about 50% of all rabbits tested without markedly altering the arterial blood pressure. Spontaneous heart rate in these animals was found to be lower than control values. The incidence of arrhythmia decreased as the time interval between the administration of both drugs was increased from 20 to 60 min. An increase in the number of days of pretreatment with phenelzine resulted in the use of a higher dose of digoxin to evoke arrhythmia.

Keyphrases □ Digoxin–phenelzine interactions—cardiovascular effects in rabbits, dose–time relationships □ Phenelzine–digoxin interactions—cardiovascular effects in rabbits, dose–time relationships □ Interactions, digoxin–phenelzine—cardiovascular effects in rabbits, dose–time relationships □ Drug interactions digoxin–phenelzine in rabbits

The administration of digoxin in combination with phenelzine is occasionally employed in clinical practice. However, when an antidepressant drug such as phenelzine, a monoamine oxidase (MAO) inhibitor, is administered concurrently with a cardiac glycoside such as digoxin, an interaction may occur. A digitoxigenin–biogenic amine interaction was reported (1) to play a significant role in the development of digitoxigenin toxicity and lethality. Drugs not belonging to the class of MAO inhibitors but that deplete brain monoamines were found to protect mice and rats against digitoxigenin lethality. On the basis of the preceding information, it can be surmised that digoxin toxicity may be enhanced with concurrent phenelzine administration.

This preliminary investigation was undertaken to determine if concurrent digoxin phenelzine administration would result in enhanced toxicity and if such toxicity was dose related and/or time dependent in terms of the two drugs administered together.

EXPERIMENTAL

Cardiovascular Studies of Phenelzine and Digoxin—Adult, female, albino rabbits, weighing 1.96–4.9 kg., were anesthetized with sodium pentobarbital[1], 25 mg./kg. i.v.; supplemental doses of 0.2 ml. sodium pentobarbital were injected as needed. Carotid arterial blood pressure was recorded using a pressure transducer[2], while heart rate was determined from limb lead II electrocardiographic records. All monitored parameters were recorded on an ink-writing oscillograph[3].

Studies of certain cardiovascular responses to either phenelzine (2.5, 5, and 10 mg./kg.) or digoxin (0.125 and 0.250 mg./kg.) administered individually by jugular vein served as the control basis for comparison with concurrent administration of both drugs. At least six rabbits were used in each experiment. Control readings of spontaneous heart rate and arterial blood pressure were recorded at 10-min. intervals prior to the injection of the first drug. Following each drug administration, readings were made at intervals of 0.5, 1.0, 5.0, and 10.0 min. and then every 10 min. thereafter; three nearly similar readings were obtained before a subsequent dose was administered. Replicate observations were made in the same animal (*i.e.*, 0.125 mg./kg. digoxin was given noncumulatively in the same animal three times before attempting the next dose, 0.25 mg./kg., for the next three trials).

Drug Interaction–Time Course—The following three time-spaced studies of phenelzine and digoxin were conducted: (*a*) drugs concurrently injected intravenously; (*b*) drug administration 20 min.

[1] Abbott Laboratories.
[2] Linear Core P-1000A, Narco Biosystems, Inc., Houston, Tex.
[3] Type PMP-4A, Narco Biosystems, Inc., Houston, Tex.

(3) J. C. Skou, *Biochim. Biophys. Acta*, **31**, 1(1959).
(4) D. Romeo, A. Girard, and L. I. Rothfield, *J. Mol. Biol.*, **53**, 475(1970).
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EXPERIMENTAL

Cardiovascular Studies of Phenelzine and Digoxin—Adult, female, albino rabbits, weighing 1.96–4.9 kg., were anesthetized with sodium pentobarbital[1], 25 mg./kg. i.v.; supplemental doses of 0.2 ml. sodium pentobarbital were injected as needed. Carotid arterial blood pressure was recorded using a pressure transducer[2], while heart rate was determined from limb lead II electrocardiographic records. All monitored parameters were recorded on an ink-writing oscillograph[3].

Studies of certain cardiovascular responses to either phenelzine (2.5, 5, and 10 mg./kg.) or digoxin (0.125 and 0.250 mg./kg.) administered individually by jugular vein served as the control basis for comparison with concurrent administration of both drugs. At least six rabbits were used in each experiment. Control readings of spontaneous heart rate and arterial blood pressure were recorded at 10-min. intervals prior to the injection of the first drug. Following each drug administration, readings were made at intervals of 0.5, 1.0, 5.0, and 10.0 min. and then every 10 min. thereafter; three nearly similar readings were obtained before a subsequent dose was administered. Replicate observations were made in the same animal (*i.e.*, 0.125 mg./kg. digoxin was given noncumulatively in the same animal three times before attempting the next dose, 0.25 mg./kg., for the next three trials).

Drug Interaction–Time Course—The following three time-spaced studies of phenelzine and digoxin were conducted: (*a*) drugs concurrently injected intravenously; (*b*) drug administration 20 min.

[1] Abbott Laboratories.
[2] Linear Core P-1000A, Narco Biosystems, Inc., Houston, Tex.
[3] Type PMP-4A, Narco Biosystems, Inc., Houston, Tex.

Table I—Effects of Spontaneous Heart Rate and Incidence of Arrhythmias following Interactions of Phenelzine and Digoxin in Rabbits

Treatment	Dosage, mg./kg.	Number of Observations	Mean Spontaneous Heart Rate/min. ± *SE*				Quantal Incidence of Arrhythmias
			Control	5 min.	20 min.	30 min.	
Digoxin	0.125	15	249 ± 5	230 ± 4[a]	236 ± 4	239 ± 4	1/15
	0.250	18	237 ± 4	202 ± 3[a]	201 ± 16[a]	218 ± 13[a]	9/18
Phenelzine	2.5	9	282 ± 2	283 ± 2	283 ± 2	280 ± 2	2/9
	5.0	15	251 ± 6	209 ± 15[a]	247 ± 6	245 ± 5	9/15
	10.0	11	220 ± 9	130 ± 8[a]	193 ± 12	205 ± 11	7/11
Phenelzine and digoxin	2.5 + 0.125[b]	3	268 ± 12	246 ± 3	257 ± 3	270 ± 20	1/3
	5.0 + 0.250[b]	8	234 ± 11	208 ± 20	220 ± 11	227 ± 11	4/8
Phenelzine and digoxin	2.5 + 0.125[c]	4	237 ± 10	200 ± 20[a]	193 ± 33[a]	203 ± 26	2/4
	5.0 + 0.250[c]	4	250 ± 12	215 ± 17[a]	240 ± 10	237 ± 15	2/4
Phenelzine and digoxin	2.5 + 0.125[d]	4	231 ± 8	210 ± 8	225 ± 12	233 ± 10	1/4
	5.0 + 0.250[d]	2	250 ± 7	235 ± 5	205 ± 25	234 ± 16	1/2

[a] Statistically significant from control values ($p < 0.05$). [b] Concurrent administration. [c] Digoxin administered 20 min. after phenelzine. [d] Digoxin administered 60 min. after phenelzine.

apart, with phenelzine being given first followed by digoxin 20 min. later; and (*c*) a similar pattern of drug administration 60 min. apart. The dose levels of digoxin and phenelzine used were 0.125 or 0.25 mg./kg. and 2.5 or 5.0 mg./kg., respectively.

Effects of Phenelzine Pretreatment on Digoxin-Induced Arrhythmias—Six rabbits were pretreated with phenelzine intraperitoneally for varying times, one each for 3, 4, and 5 consecutive days and three for 7 days. Pretreatment days with phenelzine were varied to determine long- and short-term effects of this drug when interacting with digoxin. Two dose levels of phenelzine were tested: 2.5 and 5 mg./kg. The first three rabbits received 0.125 mg./kg. i.v. of digoxin on a cumulative basis every 20 min. until arrhythmias developed. Similarly, the other three animals received digoxin, 0.25 mg./kg. A control study of six other rabbits pretreated with normal saline in the same dose regimen described for phenelzine was also conducted. These rabbits were similarly challenged with digoxin.

RESULTS

Spontaneous Effects of Digoxin—Of the two doses of digoxin tested in anesthetized rabbits, only 0.25 mg./kg. produced a sustained decrease in spontaneous heart rate (Table I). The heart rate of animals receiving 0.125 mg./kg. was slowed significantly only at 5 min. after administration and returned to a near preinjection value by 30 min.

A prompt elevation of arterial blood pressure was noted following administration of 0.25 mg./kg. digoxin but was not significant 30 min. later (Table II). Only one out of the 15 observations recorded at the dose level of 0.125 mg./kg. showed a transient arrhythmia following digoxin administration while the dose of 0.25 mg./kg. produced a 50% incidence of arrhythmia (Table I). In almost all of these observations, the arrhythmia was of the ventricular type; onset was noted about 1 min. after injection and the duration was approximately 10 min.

Spontaneous Effects of Phenelzine—All doses of phenelzine, except 2.5 mg./kg., produced varying degrees of reduction of spontaneous heart rate in 5–10 min. (Table I). With each injection of phenelzine, a transient hypotensive effect was noted, generally followed by hypertensive response. The hypertensive response to 2.5 mg./kg. was minimal in some rabbits and nonexistent in others (Table II). There was an increase in pulse pressure with this hypertensive response, which was sustained for approximately 5 to 10 min. by doses of 5 and 10 mg./kg., respectively. The incidence and severity of the ventricular arrhythmias that occurred after injection of phenelzine varied directly with increasing dose (Table I). Most of these arrhythmias were short lived, lasting only about 5 min.

Interactions of Phenelzine and Digoxin—The immediate response to concurrent intravenous administration of phenelzine and digoxin appeared to be bradycardia. This decrease in spontaneous heart rate was statistically significant 5 min. after drug treatment when both phenelzine and digoxin were administered 20 min. apart (Table I). This decrease in heart rate was evident up to 30 min. after drug treatment. The drug combination generally caused a fall in arterial blood pressure, and such a hypotensive response was significant when higher doses of phenelzine and digoxin were administered 20 min. apart (Table II). Phenelzine–digoxin administration evoked arrhythmias of very short duration in 45% of the animals tested.

In these time-dependent studies, the frequency of occurrence of arrhythmias appeared to be inversely related to the prolongation of the time interval between injection of phenelzine and digoxin.

Table II—Effects on Arterial Blood Pressure following Interactions of Phenelzine and Digoxin in Rabbits

Treatment	Dosage, mg./kg.	Number of Observations	Mean Arterial Blood Pressure ± *SE*							
			Systolic				Diastolic			
			Control	5 min.	20 min.	30 min.	Control	5 min.	20 min.	30 min.
Digoxin	0.125	15	131 ± 2	132 ± 2	130 ± 2	129 ± 2	108 ± 3	107 ± 2	107 ± 3	107 ± 2
	0.250	18	134 ± 2	149 ± 6[a]	145 ± 13	138 ± 3	112 ± 3	121 ± 4	120 ± 3	114 ± 4
Phenelzine	2.5	9	125 ± 1	122 ± 6	124 ± 1	123 ± 1	97 ± 1	94 ± 2	98 ± 2	97 ± 2
	5.0	15	150 ± 6	156 ± 11	141 ± 6	141 ± 5	119 ± 5	120 ± 6	114 ± 4	110 ± 4
	10.0	11	119 ± 2	159 ± 3[a]	124 ± 3	116 ± 2	95 ± 9	125 ± 3[a]	100 ± 4	93 ± 3
Phenelzine and digoxin	2.5 + 0.125[b]	3	110 ± 10	113 ± 9	107 ± 10	118 ± 3	89 ± 4	85 ± 9	83 ± 11	95 ± 5
	5.0 + 0.250[b]	8	112 ± 4	116 ± 4	100 ± 9	98 ± 9	90 ± 4	86 ± 5	77 ± 10	75 ± 10
Phenelzine and digoxin	2.5 + 0.125[c]	4	112 ± 7	110 ± 13	118 ± 17	117 ± 16	82 ± 7	86 ± 12	96 ± 14	91 ± 11
	5.0 + 0.250[c]	4	113 ± 5	108 ± 4	94 ± 6[a]	89 ± 10[a]	87 ± 6	78 ± 6	69 ± 7	69 ± 8
Phenelzine and digoxin	2.5 + 0.125[d]	4	100 ± 5	98 ± 6	100 ± 7	99 ± 7	80 ± 4	77 ± 4	78 ± 4	79 ± 6
	5.0 + 0.250[d]	2	99 ± 2	100 ± 0	100 ± 0	100 ± 0	75 ± 0	68 ± 3	75 ± 0	75 ± 0

[a] Statistically significant from control values ($p < 0.05$). [b] Concurrent administration. [c] Digoxin administered 20 min. after phenelzine. [d] Digoxin administered 60 min. after phenelzine.

Table III—Effects of Phenelzine Pretreatment on Digoxin-Induced Arrhythmias in Rabbits

Animal Number	Days of Pretreatment	Daily Dosage of Phenelzine, mg./kg.	Digoxin Dosage to Induce Arrhythmia, mg./kg.	p^a
1	3	2.5	0.250	~0.350
2	4	2.5 (3 days), 5.0 (1 day)	0.625	~0.150
3	5	2.5 (2 days), 5.0 (3 days)	1.125	≪0.010
4	7	2.5 (2 days), 5.0 (5 days)	1.000	≪0.010
5	7	2.5 (1 day), 5.0 (6 days)	1.000	≪0.010
6	7	5.0	0.750	~0.025
7–12	7	—[b]	0.38 ± 0.15[c]	-

[a] Level of significance relative to the saline controls. [b] Saline controls. [c] Mean value ± *SE*.

When digoxin was administered 20 and 60 min. after phenelzine, the incidence of arrhythmias was observed in 50 and 33%, respectively, of the animals.

Phenelzine Pretreatment on Digoxin Toxicity—Six rabbits pretreated with varying doses of phenelzine and for varying times responded to injected digoxin with a bigeminal type of ventricular arrhythmia. Because of the variability in the autonomic response of these rabbits to phenelzine pretreatment, it was sometimes necessary to administer a large loading dose for a longer period followed by a smaller sustaining dose for a shorter period, and vice versa. By increasing the number of pretreatment days to 5 and 7, respectively, the amount of the cardiac glycoside required to evoke arrhythmias was similarly altered (Table III). Each injection of digoxin produced an immediate decrease in the heart rate and a slight hypotensive effect.

DISCUSSION

These studies point out certain potential cardiovascular dangers inherent with concurrent phenelzine–digoxin administration. The results in rabbits showed that a dose–time relationship existed for both spontaneous heart rate and arterial blood pressure. While spontaneous heart rate decreased significantly after 0.125 and 0.25 mg./kg. i.v. digoxin, the systolic arterial pressure was significantly increased at the 0.25-mg./kg. dose level. This increase in arterial blood pressure might have been caused by increased peripheral vascular resistance due to the well-known vasoconstrictor action of digitalis on the resistance vessels of the systemic circulation (2–5). Rabbits injected with phenelzine alone, 5 and 10 mg./kg., showed instantaneous bradycardia, which may partly be a reflex response to elevated blood pressure and partly be due to a possible vagal action (6).

The fact that phenelzine and digoxin when administered alone caused a significant decrease from control values in spontaneous heart rate, but failed to do so significantly when given together, may suggest a partial explanation for their potential toxicity in combination. A potential for increased automaticity in the heart muscle may have been created, with consequent precipitation of arrhythmias. Table I shows that the low incidence of arrhythmias occurred at the digoxin dose of 0.125 mg./kg. but was greatly increased when this dose was given in combination with 2.5 mg./kg. phenelzine. The transient hypotension, occurring immediately after phenelzine injection, may have been caused by a temporary decrease in peripheral sympathetic tone with a consequent decrease of peripheral vascular resistance (7). The subsequent rise in arterial blood pressure suggests sympathetic activity, perhaps due to increased catecholamine levels. In terms of dose–time relationships, spontaneous heart rate was significantly decreased for the dosages of 5 and 10 mg./kg. phenelzine whereas all doses did not significantly depress heart rate 20 min. after injection ($p = 0.6$). Arrhythmia frequency produced by phenelzine increased with increasing dose (Table I). However, when varying the intervals between injections of phenelzine and digoxin, it was observed that administration of these drugs 20 min. apart was as toxic as concurrent injection as seen by the incidence of arrhythmias. This incidence was decreased when both drugs were given 60 min. apart (Table I).

Pretreatment of rabbits with phenelzine for some days appears to modify the dose of digoxin needed to produce arrhythmias. An increase in the number of pretreatment days correspondingly caused a significant increase in the arrhythmogenic dose of digoxin (Table III). The dose levels of digoxin and the length of phenelzine pretreatment period seem to be important factors in modifying the toxicity of concurrent administration. These findings may be relevant to those occasional clinical situations in which depressed patients with advanced cardiac failure are given doses of digitalis concurrently with a mood elevator such as phenelzine.

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Natural Coumarins VII: Isolation and Structure of a New Coumarin, Peuruthenicin, from *Peucedanum ruthenicum* M.B.

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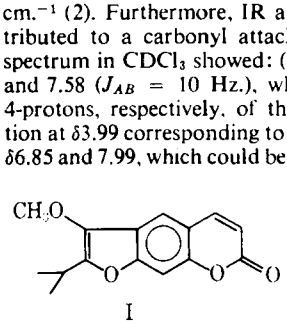
Abstract □ The roots of the Bulgarian umbellifer, *Peucedanum ruthenicum* M.B., yielded a novel naturally occurring coumarin, which was assigned the trivial name of peuruthenicin. It was identified as 6-carbomethoxy-7-hydroxycoumarin through both spectral (mass, NMR, IR, and UV) and chemical (degradative and synthetic) evidence. The latter consisted of alkaline hydrolysis to known umbelliferone-6-carboxylic acid (IV) and synthesis of peuruthenicin by methyl esterification of IV which had been obtained by chromate oxidation of marmesin.

Keyphrases □ Peuruthenicin (6-carbomethoxy-7-hydroxycoumarin)—isolation from *Peucedanum ruthenicum* M.B., structure determination □ Coumarins, natural—isolation of peuruthenicin (6-carbomethoxy-7-hydroxycoumarin) from *Peucedanum ruthenicum* M.B., structure determination □ 6-Carbomethoxy-7-hydroxycoumarin (peuruthenicin)—isolation from *Peucedanum ruthenicum* M.B., structure determination □ *Peucedanum ruthenicum* M.B.—isolation and structure identification of peuruthenicin (6-carbomethoxy-7-hydroxycoumarin)

Previously (1), a phytochemical examination of *Peucedanum ruthenicum* M.B., a native Bulgarian umbellifer, showed the presence of the furanocoumarin peucedanin (I) in the roots and the flavonol glycoside rutin in the flowers. Further examination of the root extract provided a new compound, assigned the trivial name peuruthenicin, which has been structurally characterized.

DISCUSSION

Peuruthenicin was obtained as cream-colored needles, m.p. 196–197°, for which the molecular ion derived from the mass spectrum and the supporting elemental analyses suggested the molecular formula $C_{11}H_8O_5$. Its UV spectrum suggested a possible coumarin chromophore. The IR spectrum was also compatible with a coumarin-type structure, since it showed a typical carbonyl absorption at 1740 cm.$^{-1}$ and the typical 3,4-double bond absorption at 1630 cm.$^{-1}$ (2). Furthermore, IR absorption at 1680 cm.$^{-1}$ could be attributed to a carbonyl attached to an aromatic ring. The NMR spectrum in $CDCl_3$ showed: (*a*) an *AB* system of doublets at δ6.25 and 7.58 (J_{AB} = 10 Hz.), which could be assigned to the 3- and 4-protons, respectively, of the coumarin nucleus; (*b*) an absorption at δ3.99 corresponding to a methoxyl group; (*c*) two singlets at δ6.85 and 7.99, which could be assigned to two *para*-related aromatic protons; and (*d*) a low field singlet at δ11.08, which could be assigned to chelated hydroxyl. The mass spectrum (Scheme I) indicated the facile loss of methanol from the molecular ion to provide a metastable ion (*m/e* 160.85), supported base peak at *m/e* 188. This behavior is well known for *ortho*-substituted methyl benzoates, particularly for the methyl salicylate type of moiety (3). Therefore, on the basis of the assembled spectral and analytical data, two possible structures could be entertained for peuruthenicin, namely II and III.



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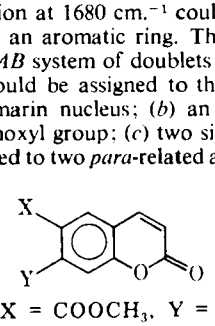
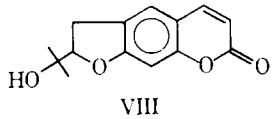


II: X = $COOCH_3$, Y = OH
III: X = OH, Y = $COOCH_3$
IV: X = COOH, Y = OH
V: X = H, Y = OH
VI: X = $COOCH_3$, Y = OCH_3
VII: X = COOH, Y = OCH_3



VIII

Alkaline hydrolysis of peuruthenicin gave a carboxylic acid, m.p. 258° dec., which failed to depress the melting point of an authentic sample of umbelliferone-6-carboxylic acid (IV) prepared by chromate oxidation of marmesin (VIII) as described previously (4). This acid also provided umbelliferone (V) when thermally decarboxylated to locate firmly the site of the phenolic hydroxyl group. Peuruthenicin, on treatment with diazomethane, provided a methyl ether, m.p. 168°, which was apparently identical to 6-carbomethoxy-7-methoxycoumarin (VI) (4).

The physical and chemical data strongly suggested that peuruthenicin was a novel naturally occurring coumarin with Structure II rather than III. This structure was validated by esterification of IV with methanolic sulfuric acid to give the methyl ester (II), which proved to be identical in all respects (UV, IR, NMR, and mass spectrum) with peuruthenicin.

Furthermore, VI was subjected to alkaline hydrolysis to provide VII, m.p. 237–239°, isomeric with II. Its NMR spectrum was distinctly different from that of II and showed two doublets at δ6.30 and 7.70 ($J_{3,4}$ = 10 Hz.) characteristic of the protons at the 3- and 4-positions, two *para*-related aromatic protons as singlets at δ6.91 and 8.23, and a methoxyl singlet at δ3.83.

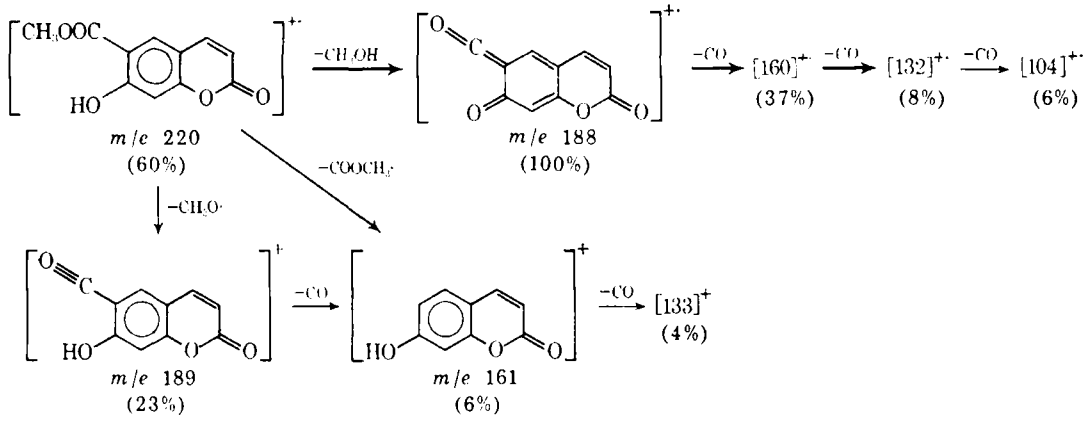
Although the present study was not concerned with the biosynthesis of peuruthenicin, it may well be that it is derived through a bio-oxidative transformation of peucedanin (I) or the corresponding ketone, oreoselone. Studies presently underway are exploring this possibility.

EXPERIMENTAL[1]

Isolation of Peuruthenicin—One kilogram of the roots of *P. ruthenicum* M.B., ground to a No. 20 powder, was extracted with methanol at room temperature using successively 10, 8, and 6 l. of solvent[2]. The extract was then filtered until clear and evaporated to give a gummy residue. This residue, when treated with 1 l. of water, gave a cloudy suspension which was extracted with ether (3 × 300

[1] Melting points were determined using a Reichert hot-stage apparatus and are uncorrected. Unless otherwise stated, IR spectra were measured in chloroform on a Perkin-Elmer 157G grating spectrophotometer, UV spectra were measured in 95% ethanol on a Cary 14 spectrophotometer, and 60- and 100-MHz. NMR spectra were measured in $CDCl_3$ (tetramethylsilane as internal standard) on HA 100 or R 12 NMR spectrometers. Only significant bands from these spectra are cited. Mass spectra (Scheme I) were determined using an AEI-MS 12 mass spectrometer as well as a Hitachi Perkin-Elmer RMU-6D instrument; in each case the source temperature was 150° and the ionizing voltage was 70 ev. Elemental analyses were obtained from M-H-W Laboratories, Garden City, Mich. Evaporation refers to evaporation under reduced pressure. TLC separations were carried out on plates coated with Merck kieselgel G and a solvent system consisting of benzene–toluene–ethyl acetate–formic acid (10:5:4:1). The drying agent for ethereal solutions was anhydrous sodium sulfate, and substances stated to be identical were thus established by comparison of melting points; IR, NMR, and mass spectra; and TLC behavior.

[2] That peuruthenicin was not an artifact formed by possible esterification of umbelliferone-6-carboxylic acid because of the methanol solvent was shown by extraction of the original plant material with several different solvents: benzene, petroleum ether, ether, chloroform, acetone, ethanol, and methanol. Each extract, without further treatment, was spotted on a TLC plate and developed with the benzene–toluene–ethyl acetate–formic acid solvent described. There was no doubt that peuruthenicin was the original coumarin in each extract since the R_f value of peuruthenicin is 0.60 and that of the acid is 0.4.



Scheme I—Mass spectral fragmentation of peuruthenicin. Heavy arrows indicate that the proposed fragmentation pathway is supported by the appearance of a metastable ion.

ml.). The combined ethereal extracts were dried and evaporated, and the residue was successively extracted with 200-ml. portions of boiling petroleum ether (b.p. 40–60°). The extracts, in sequence, were examined by TLC, which revealed that the first 10 extracts contained mainly peucedanin but that the following five extracts contained a 1:1 mixture of peucedanin and an unknown coumarin (*i.e.*, peuruthenicin). The latter extracts were combined, concentrated to about 100 ml., and held at 0° for 12 hr., at which time a crystalline precipitate of peucedanin had been deposited. This was removed by filtration and the filtrate was reduced to a volume of 50 ml. and then kept at 0° for an additional 15 days, during which time an amorphous precipitate formed and was collected by filtration. Recrystallization of this precipitate from methanol gave peuruthenicin (1 g.) as cream-colored needles, m.p. 196–197°; UV $\lambda_{max}^{C_2H_5OH}$ (log ϵ): 218 (4.30), 224 (4.28), 245 (4.46), 292 (3.99, shoulder), 3.03 (4.06), 323 (4.16), and 332 nm. (4.15); IR ν_{max}: 1740, 1680, and 1630 cm.$^{-1}$; mass spectrometry: *m/e* 220, 189, 188, 161, 160, 133, 132, and 104.

Anal.—Calc. for $C_{11}H_8O_3$: C, 60.0; H, 3.6; M (mass spectrum), 220. Found: C, 60.1; H, 3.7; M, 220.

Alkaline Hydrolysis of Peuruthenicin—Peuruthenicin (200 mg.) was dissolved in dioxane (40 ml.) and treated with aqueous 20% sodium hydroxide (80 ml.) under reflux for 30 min. The cooled mixture was then diluted to approximately 500 ml. with water, acidified with diluted sulfuric acid, and extracted with chloroform. The chloroform extract was dried and evaporated to yield 80 mg. of white crystals, m.p. 258° dec., identical to umbelliferone-6-carboxylic acid (IV) as determined by melting point, mixed melting point, and spectral comparison with the oxidative product of marmesin.

Decarboxylation of Alkaline Hydrolysis Product—The preceding acid (50 mg.) was heated at 265° for 2 hr. under a sublimation unit; the collected sublimate was shown to have a melting point of 230° and was identical to authentic umbelliferone.

Methylation of Alkaline Hydrolysis Product—The preceding acid (59 mg.) was dissolved in acetone (5 ml.) and treated for 20 min. with excess ethereal diazomethane generated from *N*-nitrosomethylurea in the usual way (5). Evaporation of the reaction mixture and crystallization of the residue from methanol gave white crystals (60 mg.), m.p. 167° [lit. (4) m.p. 168°]; IR ν_{max}: 1740–1720 and 1622 cm.$^{-1}$; NMR: δ6.30 and 7.66 (doublets, *AB* system, J_{AB} = 10 Hz., 3-H and 4-H), 8.00 (singlet, 5-H), 6.87 (singlet, 8-H), and 3.97 and 3.90 (singlets, $COOCH_3$ and OCH_3).

Umbelliferone-6-carboxylic Acid (IV)—Marmesin (1 g.) was oxidized, according to a published method (4), with 3% sulfuric acid and potassium dichromate to yield umbelliferone-6-carboxylic acid (300 mg.), m.p. 259°, identical with the product of alkaline hydrolysis of peuruthenicin as shown previously.

Synthesis of Peuruthenicin—Umbelliferone-6-carboxylic acid (90 mg.), methanol (5 ml.) and concentrated sulfuric acid (0.1 ml.) were heated together for 48 hr. under reflux. The resulting mixture was cooled, neutralized with 5% aqueous potassium hydroxide, and diluted with water until no further precipitate formed. The precipitate was collected and fractionated by preparative TLC to give cream-colored crystals (47 mg.), m.p. 196°, identical with the peuruthenicin obtained from natural sources.

7-Methoxycoumarin-6-carboxylic Acid (VII)—Peuruthenicin methyl ether (VI) (50 mg.) was dissolved in acetone (2 ml.) and treated with 5% aqueous potassium hydroxide (2 ml.) at room temperature for 7 hr. and then heated under reflux for 15 min. The cooled mixture, upon acidification with diluted sulfuric acid and dilution with water, yielded a precipitate which was collected and subjected to preparative TLC separation. The principal product was removed from the plates by scraping and, when extracted with methanol, yielded a white crystalline material (35 mg.), m.p. 237–239°; IR ν_{max}: 1730 and 1630 cm.$^{-1}$; NMR (in pyridine): δ6.30 and 7.70 (doublets, *AB* system, J_{AB} = 10 Hz., 3-H and 4-H), 8.23 (singlet, 5-H), 6.91 (singlet, 8-H), and 3.83 (singlet, OCH_3).

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Lysergic Acid Analogs: 5-Phenylnicotinamides and Nipecotamides

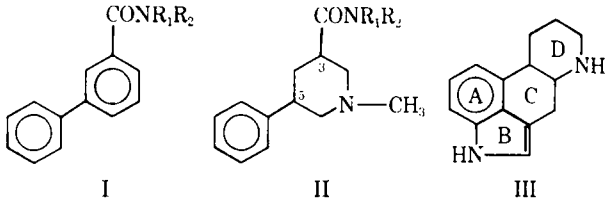
FATIMA N. JOHNSON▲, S. P. BATTISTA, and DOROTHY REID

Abstract □ A series of 5-phenylnicotinamides and 1-methyl-5-phenylnipecotamides was synthesized as simplified lysergic acid analogs. Pharmacological evaluation showed that, in general, these compounds are CNS depressants. The nipecotamides, although more toxic, possess MED_{50} values comparable to those found for the analogous 9,10-dihydrolysergamides. The nicotinamides, however, showed greatly reduced activity when compared to the lysergamides.

Keyphrases □ Lysergic acid analogs—synthesis and pharmacological screening of 5-phenylnicotinamides and nipecotamides □ 5-Phenylnicotinamides—synthesis, screened for CNS activity □ Nipecotamides—synthesis, screened for CNS activity □ CNS activity—synthesis and screening of 5-phenylnicotinamides and nipecotamides

The potent and diverse pharmacological activity of ergot alkaloids has generated considerable interest in the synthesis of simplified analogs (1). The synthesis and pharmacological evaluation of some 5-phenylnicotinamides (I*a*–I*d*) and 1-methyl-5-phenylnipecotamides (II*a*–II*d*) are reported here. These compounds may be viewed as representing rings A and D of the ergolene system (III). It has been reported that the hallucinogenic activity of *N,N*-diethyllysergamide (LSD, IV*b*), may reside in that portion of the molecule that includes aromatic ring A and the nitrogen atom of ring D (2).

In connection with another study (3), numerous lysergamides (IV) and their 9,10-dihydro derivatives (V) were prepared. It became of interest to compare compounds of type II with the analogous 9,10-dihydrolysergamides (V). The choice of substituents R_1 and R_2 in Compounds I and II was dictated by the pharmacological results obtained previously (3).



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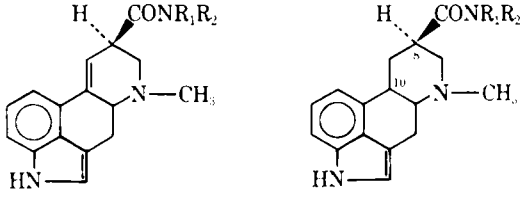
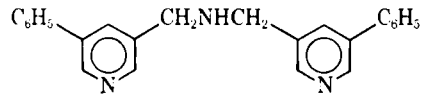


IV V

a: R_1 = H, R_2 = n-C_4H_9
b: R_1 = C_2H_5, R_2 = C_2H_5
c: R_1 = H, R_2 = cyclo-C_6H_{11}
d: R_1 = H, R_2 = $C(CH_3)_3$



VI

RESULTS AND DISCUSSION

The common starting material for I and II was 5-phenylnicotinic acid, obtained from the hydrolysis of 5-phenylnicotinonitrile. The nitrile was prepared by the hydrogenolysis of 2-chloro-5-phenylnicotinonitrile over palladium-on-charcoal. When the literature procedure (4) was followed using palladium chloride, a substantial quantity of 3,3′-iminobis(methylene-5-phenylpyridine) (VI) was formed as a by-product. The desired product, 5-phenylnicotinonitrile, was obtained in unsatisfactory yield and was difficult to purify.

Compounds I*a*–I*d* (Table I) were obtained in good yield from 5-phenylnicotinic acid by a new, efficient amidation procedure (3), using the appropriate amine and phosphorus oxychloride in a 4–8-min. reaction period. Catalytic reduction of the methobromides of I*a*–I*d* gave the corresponding nipecotamides (II*a*–II*d*) (Table II). The synthesis of lysergamides (IV*a* and IV*b*) and 9,10-dihydrolysergamides (V*a*–V*d*) was described previously (3).

The general pharmacological activity of all compounds listed in Table III was measured in a modified Irwin mouse profile (5, 6), using male albino mice weighing 22 ± 2 g. Administration was made intravenously to two animals per dose at each of four or more dose levels. Following administration of a compound, observations were made on approximately 50 discrete components of behavior and appearance, response to external stimuli and neuromuscular integrity, and performance (6). The LD_{50} and MED_{50} (minimum effective dose) values were calculated using the moving average method of Thompson (7) and Weil (8).

Compounds I*a*–I*d* and II*a*–II*d* commonly caused general central

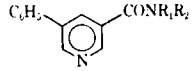


Table I—5-Phenylnicotinamides

Compound	R_1	R_2	Melting Point	Yield[a], %	Recrystallization Solvent	Melting Point of Methobromide (% Yield)
I*a*	H	n-C_4H_9	70–71°	87	Ether–hexane[b]	122–124° (97)
I*b*	C_2H_5	C_2H_5	81–82°[c]	75	Hexane	186–188° (76)
I*c*	H	cyclo-C_6H_{11}	136–137°	68	Hexane	212–215° (89)
I*d*	H	$C(CH_3)_3$	127–129°	49	Hexane[b]	212–213° (91)

[a] Recrystallized product. [b] Crude product was chromatographed on column of neutral aluminum oxide (Activity II, eluent ether) prior to crystallization. [c] M.p. 82° (4).

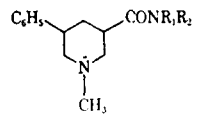


Table II—1-Methyl-5-phenylnipecotamides

Compound	R_1	R_2	Melting Point	Yield[a], %	Recrystallization Solvent
IIa	H	n-C_4H_9	128–130°	42	n-Hexane
IIb	C_2H_5	C_2H_5	180–182°[b]	14	Ether–ethanol
IIc	H	cyclo-C_6H_{11}	154–156°	32	Acetonitrile
IId	H	$C(CH_3)_3$	243–245°[b]	12	Ethyl acetate

[a] Recrystallized product. [b] Hydrochloride.

Table III—Mouse Profile Screen[a]

Compound	MED_{50}, mg./kg. i.v.	LD_{50}, mg./kg. i.v.
Ia	>20	>20
Ib	10.0	56
Ic	18.0	100
Id	5.6	>32
IIa	5.6	18
IIb	1.8	18
IIc	3.2	18.0
IId	10.0	32.0
IVa	0.18	79
IVb	0.056	50
Va	5.6	56
Vb	5.6	56
Vc	18	>32
Vd	1.8	56

[a] Hydrochlorides were administered intravenously in aqueous solution; free bases and maleates required polyethylene glycol 200 or dilute hydrochloric acid for solubilization.

nervous system (CNS) depression and, in particular, a decreased respiratory rate. A comparison of the results obtained for Va–Vd with the simplified analogs IIa–IId showed very similar pharmacological activity on the Irwin screen even though the absolute stereochemistry at chiral centers 3 and 5 of IIa–IId is unknown. The absolute stereochemistry at the analogous positions 8 and 10 of the 9,10-dihydrolysergamides has been established (9). However, IIa–IId were considerably more toxic than Va–Vd by a factor of at least 2–3.

The nicotinamides (Ia–Id), on the other hand, showed greatly reduced activity when compared to the lysergamides, but the presence of the pyridine ring makes for a less valid comparison with lysergamides.

EXPERIMENTAL[1]

5-Phenylnicotinonitrile—A mixture of 20.7 g. of 2-chloro-5-phenylnicotinonitrile (4), 120 ml. of dimethylformamide, 25.5 ml. of triethylamine, and 1.5 g. of 5% palladium-on-charcoal was hydrogenated at room temperature in a Parr apparatus at 3.9 kg./cm.^{2} for 1 hr. The mixture was filtered and the filtrate was concentrated to dryness. The residue was partitioned between dichloromethane and 50 ml. of 2 *N* NaOH. The organic extract was washed twice with 5% HCl and several times with water, dried over magnesium sulfate, and evaporated to dryness *in vacuo*. The residue was recrystallized from *n*-hexane, yielding 12.1 g. (69%) of 5-phenylnicotinonitrile, m.p. 72–74° [lit. (4) m.p. 75–76°].

When the literature procedure (4) was followed using palladium chloride as a catalyst, 3,3′-iminobis(methylene-5-phenylpyridine) (VI) was isolated from the 5% HCl wash of the organic extract described previously. The acidic solution was made basic with 2 *N* NaOH, and the liberated base was extracted into methylene chloride. The dried extract (magnesium sulfate) was evaporated to dryness under vacuum, and the viscous residue was recrystallized from ether and then from acetonitrile, m.p. 98–99°. The NMR ($CDCl_3$) spectrum revealed the presence of 10 aromatic protons at δ 7.47 (multiplet); six pyridine protons at δ 7.85 (multiplet), 8.52 (doublet), and 8.72 (doublet); four methylene protons at δ 3.91 (singlet); and a D_2O-exchangeable proton (NH) at δ 2.25. Mass spectrometric analysis revealed a molecular ion peak at *m/e* 351 (calculated molecular weight 351.4).

Anal.—Calc. for $C_{24}H_{21}N_3$: C, 82.02; H, 6.02; N, 11.96. Found: C, 82.09; H, 6.04; N, 12.00.

5-Phenylnicotinic Acid—A solution of 5.7 g. of 5-phenylnicotinonitrile in 120 ml. of concentrated hydrochloric acid was heated at reflux overnight. The reaction mixture was poured into ice and made basic by the addition of 2 *N* NaOH. The basic solution was then acidified with glacial acetic acid. The fine, white precipitate was filtered, washed repeatedly with water, and air dried. The crude product was recrystallized from about 800 ml. of ethanol, yielding 5.2 g. (82%) of 5-phenylnicotinic acid, m.p. 264–266° [lit. m.p. 260–263° (4) and 267–269° (10)].

5-Phenylnicotinamides (Ia–Id)—A solution of 0.048 mole of the appropriate amine in 20 ml. of chloroform and 1 ml. (0.011 mole) of phosphorus oxychloride were added concurrently to a refluxing slurry of 1.1 g. (0.005 mole) of 5-phenylnicotinic acid in 80 ml. of chloroform. After being kept at reflux for an additional 2 min., the solution was cooled to room temperature, washed with 50 ml. of 1 *M* NH_4OH and with water, dried over magnesium sulfate, and evaporated to dryness *in vacuo*. The residue was then crystallized from the appropriate solvent (Table I). Table I includes melting points and yields, and elemental analysis data are given in Table IV.

1-Methyl-5-phenylnipecotamides (IIa–IId)—The methobromides

Table IV—Elemental Analysis Data for Compounds Ia–Id and IIa–IId

Compound	Empirical Formula	Analysis, % Calc.	Found
Ia	$C_{16}H_{18}N_2O$	C 75.56	75.51
		H 7.13	7.14
		N 11.02	11.04
Ib	$C_{16}H_{18}N_2O$	C 75.56	75.61
		H 7.13	6.96
		N 11.02	11.06
Ic	$C_{18}H_{20}N_2O$	C 77.11	76.83
		H 7.19	7.35
		N 9.99	9.94
Id	$C_{16}H_{18}N_2O$	C 75.56	75.63
		H 7.13	7.2
		N 11.02	11.2
IIa	$C_{17}H_{26}N_2O$	C 74.41	74.21
		H 9.55	9.56
		N 10.21	10.09
IIb	$C_{17}H_{26}N_2O \cdot HCl$	C 65.57	65.59
		H 8.75	8.77
		Cl 11.40	11.60
		N 9.01	8.90
IIc	$C_{19}H_{28}N_2O$	C 75.95	75.84
		H 9.39	9.36
		N 9.33	9.19
IId	$C_{17}H_{26}N_2O \cdot HCl$	C 65.57	65.85
		H 8.75	8.76
		Cl 11.40	11.49
		N 9.01	9.12

[1] Melting points were determined on a Mel-Temp apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. The structures of all novel compounds were confirmed by NMR spectroscopy. NMR spectra were obtained on a Varian A-60 instrument.

of Compounds Ia–Id were prepared by bubbling methyl bromide through a solution of the appropriate 5-phenylnicotinamide in ethyl acetate for 3–6 hr. The quaternary salts were not recrystallized prior to use. The methobromides separated very cleanly and in high yields from the reaction medium (Table I). They were filtered, washed thoroughly with cold ethyl acetate, air dried, and converted directly to the corresponding 1-methyl-5-phenylnipecotamide by the general procedure described here.

A solution of 1.5 g. of the methobromide in 75 ml. of ethanol containing 75 mg. of platinum oxide was hydrogenated at room temperature for 3–6 hr. at an initial pressure of 0.7 kg./cm.^{2}. The mixture was filtered and the filtrate was evaporated to dryness. The residue was dissolved in methylene chloride, washed with 2 *N* NaOH and water, dried over magnesium sulfate, and concentrated *in vacuo*. The residue was crystallized from the appropriate solvent or dissolved in ether, treated with anhydrous hydrogen chloride to yield the corresponding hydrochloride, and then recrystallized. The recrystallization solvents, melting points, and yields are listed in Table II, and elemental analysis data are given in Table IV.

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Phase Separation of Cellulose Derivatives: Effects of Polymer Viscosity and Dielectric Constant of Nonsolvent

SALEH A. H. KHALIL

Abstract □ In systems containing either cellulose acetate butyrate or ethylcellulose, the intrinsic viscosity of the polymer in the appropriate solvent had no effect on the phase type separated. The latter was dependent on the dielectric constant of the nonsolvent added. Nonsolvents possessing relatively high dielectric constants produced a flocculate phase, while either a coacervate or gel was formed after the addition of nonsolvents of lower dielectric constant.

Keyphrases □ Cellulose derivatives (cellulose acetate butyrate and ethylcellulose)—effects of polymer viscosity and dielectric constant of nonsolvent on phase separation □ Phase separation, cellulose derivatives—effects of polymer viscosity and dielectric constant of nonsolvent □ Nonsolvents—effect on phase separation of cellulose acetate butyrate and ethylcellulose

Starting from a polymer solution in a suitable solvent, the addition of an appropriate concentration of a nonsolvent may produce phase separation. The type of phase separating may be a flocculate, gel, or coacervate (liquid–liquid phase separation). While factors such as pH and electrolytes were reported to govern the phase type in polyelectrolyte systems (1, 2), no studies have been made on nonelectrolytic polymers. Phase separation of cellulose derivatives is of special interest in pharmacy because of its application to microencapsulation of pharmaceuticals (3, 4). The present article reports the effect of various solvent–nonsolvent systems on the type of phase separating in two polymers: ethylcellulose and cellulose acetate butyrate. An attempt was made to study the effect of solvent–nonsolvent systems on the phase type as determined by the viscosity of the polymer (in the appropriate solvent) and the dielectric constant of the nonsolvent added.

EXPERIMENTAL

Materials—The ethylcellulose[1] used had a 48% ethoxyl content. Cellulose acetate butyrate[2], 171-2 grade, had a 17% butyryl content. All solvents and nonsolvents were analytical reagent grade or pure reagents.

Methods—*Determination of Phase Type*—To a solution of the polymer in the appropriate solvent, placed in glass-stoppered centrifuge tubes, the nonsolvent was gradually added until a visual phase change was noted. The stoppered tubes were equilibrated at 25 ± 0.1° for completion of phase separation (4–6 hr.). The separated phase was examined and classified as a flocculate, gel, or coacervate. The latter was found microscopically to exhibit coacervate droplets (10–25 μ in diameter) which rapidly coalesced to a clear oily layer upon standing. The gel phase showed no coacervate droplets and was highly viscous, frequently adhering to the walls of the container.

[1] British Drug Houses Ltd., Poole, England.
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Table I—Effect of Solvent–Nonsolvent Systems on the Phase Type Separated in 5% (w/v) Cellulose Acetate Butyrate[a]

Nonsolvent	Dielectric[b] Constant (at 20°)	Solvent[c] Acetone (0.37)	Cyclohexanone (0.55)	Methylene Chloride (0.49)
Water	80.4	F	—	—
Methanol	33.7	F	F	F
Ethanol	25.7	F	F	F
n-Butanol	17.8	F	F	F
Hexane	1.9	C	C	C
Petroleum ether (b.p. 40–60°)	1.9	G	G	G

[a] Phase types are: F = flocculate, C = coacervate, and G = gel. [b] Values obtained from "Handbook of Chemistry and Physics," 50th ed., R. C. Weast, Ed., Chemical Rubber Co., Cleveland, Ohio, 1969, pp. E61–E63. [c] The figures in parentheses are the determined intrinsic viscosities of the polymer solution in the appropriate solvent. Values were determined at 25 ± 0.1°.

Determination of Intrinsic Viscosity—The intrinsic viscosity of the polymer in the appropriate solvent was determined at 25 ± 0.1° using a viscometer[3]. The polymer concentration range was 0.1–0.6 g./100 ml.

RESULTS AND DISCUSSION

Tables I and II show the various phases that separated upon the addition of several nonsolvents to 5% w/v solutions of the polymer in different solvents. The phase types produced were independent of the initial polymer concentration in the range used (1–5% w/v). The slight difference observed in the intrinsic viscosities of the polymers (and possibly a change in the molecular configuration) had no effect on the phase type separated. Of the six nonsolvents tested, only two showed a different effect on phase type while four produced the same phase (flocculate). Thus, it was not the nonsolvent *per se* that produced the effect but some other inherent property. It is suggested that the dielectric constant of the nonsolvent has a decisive role in determining the phase type. Nonsolvents possessing relatively high dielectric constants (the first four nonsolvents in Table I) yielded the same phase (flocculate). However, hexane and petroleum ether produced either a coacervate or gel phase, since these nonsolvents have a much lower dielectric constant (about 1.9). These observations were the same in both polymer systems.

In general, the reported results suggest that care should be taken in selecting the nonsolvent during microencapsulation of drugs by phase separation of polymeric nonelectrolytes. A previous report (5) showed that the *in vitro* release of ferrous sulfate from cellulose-coated formulations, prepared by phase separation, was dependent on the phase type separated.

[3] Ostwald type.

Table II—Effect of Solvent–Nonsolvent System on the Phase Type Separated in 5% (w/v) Ethylcellulose[a]

Nonsolvent	Dielectric[b] Constant (at 20°)	Solvent[c] Acetone (0.41)	Ethanol (0.53)	Toluene (0.62)
Water	80.4	F	F	—
Hexane	1.9	C	C	C
Petroleum ether (b.p. 40–60°)	1.9	G	G	G

[a] Phase types are: F = flocculate, C = coacervate, and G = gel. [b] Values obtained from "Handbook of Chemistry and Physics," 50th ed., R.C. Weast, Ed., Chemical Rubber Co., Cleveland, Ohio, 1969, pp. E61–E63. [c] The figures in parentheses are the determined intrinsic viscosities of the polymer solution in the appropriate solvent. Values were determined at 25 ± 0.1°.

SUMMARY

1. Three phase types are produced in the phase separation of systems containing either cellulose acetate butyrate or ethylcellulose. These phase types are flocculate, gel, and coacervate.

2. The intrinsic viscosity of the polymer, in the appropriate solvent, had no effect on the phase type separated.

3. The dielectric constant of the nonsolvent added appears to control the phase type. A flocculate phase was formed upon the addition of nonsolvents possessing relatively high dielectric constants. Either a coacervate or gel phase was produced with nonsolvents having a much lower dielectric constant.

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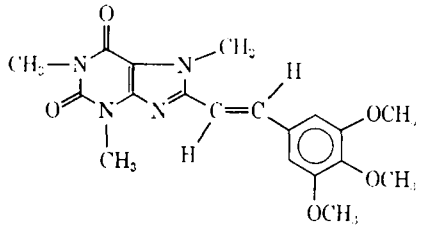
Photo-Induced Isomerization of 8-(3,4,5-Trimethoxystyryl)caffeine as Possible Route of Drug Decomposition

JOSE PHILIP▲ and DALE H. SZULCZEWSKI

Abstract □ *trans*-8-(3,4,5-Trimethoxystyryl)caffeine, dissolved in methanol or chloroform, rapidly isomerizes to an equilibrium mixture of *trans*-*cis*-isomers in the presence of visible light. Geometric isomerization was established by catalytic hydrogenation of both reactant and product to yield 8-(3,4,5-trimethoxyphenethyl)-caffeine.

Keyphrases □ 8-(3,4,5-Trimethoxystyryl)caffeine—decomposition by photoisomerization □ Caffeine derivatives—decomposition of 8-(3,4,5-trimethoxystyryl)caffeine by photoisomerization □ Photoisomerization—decomposition of 8-(3,4,5-trimethoxystyryl)caffeine

trans-8-(3,4,5-Trimethoxystyryl)caffeine (I) is a caffeine derivative being evaluated for use as a cerebral stimulant. Past work (1–4) indicated that photoisomerization about an ethylenic double bond is a general reaction. Since this investigational drug is a styryl derivative containing an ethylenic moiety, it is possible that photo-induced isomerization could occur and cause subsequent developmental problems. The effect of visible light on the drug was investigated to obtain information in this area.



I

RESULTS AND DISCUSSION

Dilute solutions (c 0.00189%) of I were prepared and exposed to visible light for varying periods. Sequential UV spectra (Fig. 1) of such solutions changed very rapidly during initial exposure (0.5 hr.), after which they became essentially constant. Almost the same behavior was noted with either chloroform or methanol as the solvent. UV spectra of solutions stored at the same temperature in the dark did not change.

To determine the nature of the process responsible for the observed changes, reaction products were isolated for identification. Preparative TLC was without value in this regard since the products and reactant had the same R_f value in available chromatographic systems.

A quantity of crystalline reaction product (II) was obtained by exposing a solution of I in chloroform to the point of spectral redundancy. Its molecular weight, determined by mass spectroscopy and elemental analysis, was identical to that of the drug, thereby indicating that the drug and product are isomers.

Confirmation that this isomerization involved geometric conversion was obtained by saturating the olefinic double bond presumed to be present in the reaction product to obtain 8-(3,4,5-trimethoxyphenethyl)caffeine (III), a compound previously obtained[1] from I by the same hydrogenation procedure (Scheme I).

[1] D. Schweiss, Parke, Davis & Co., Ann Arbor, MI 48105, personal communication.

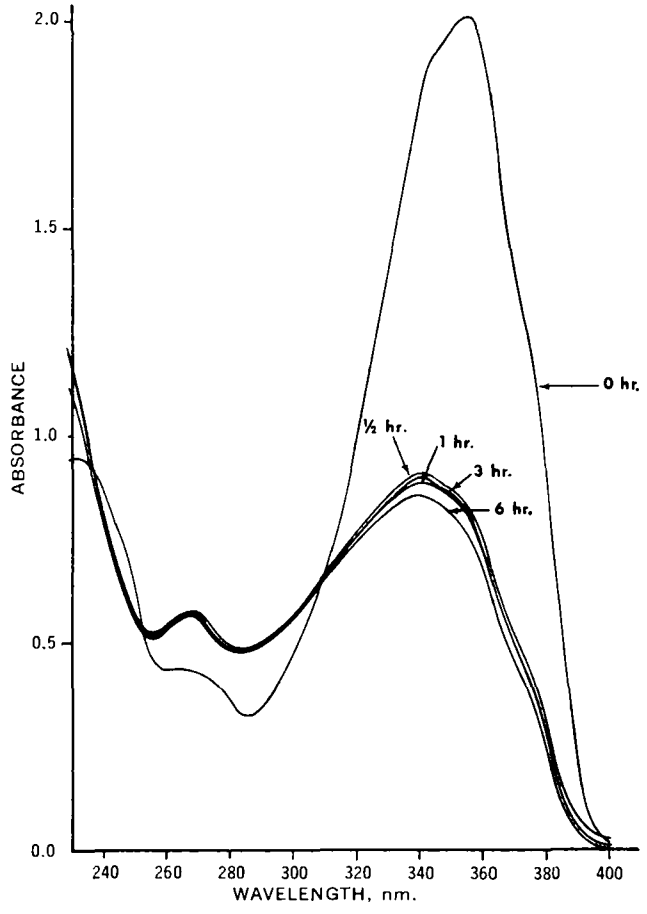


Figure 1– *Sequential UV spectra of I in methanol kept in the light cabinet. Exposure time (hours) is noted.*

Analysis of the UV spectra of a methanolic solution of I exposed to light to spectral redundancy indicated that an equilibrium mixture (80% *cis*–20% *trans*) is eventually formed. This finding was substantiated by similarly exposing the *cis*-isomer and noting that its spectrum changed to that corresponding to an 84:16 *cis*–*trans* mixture.

EXPERIMENTAL[2]

Initial Observations—One hundred milliliters of a 0.00189% solution of I in methanol was prepared and placed in a 100-ml. volumetric flask. The flask was placed in the light cabinet, and aliquots were removed periodically for UV determination.

Preparation and Identification of Reaction Product—One gram of I was dissolved in 3 l. of chloroform and kept in a light cabinet at 45°. The reaction was followed by obtaining repetitive UV spectra: when they ceased to change, the chloroform was removed and the residue was recrystallized twice from chloroform–methanol and finally from acetonitrile, m.p. 187–189° (I, m.p. 245–247°).

[2] The equipment was as follows: Cary model 14 S/W, steel light cabinet equipped with several 100-w. incandescent bulbs, Perkin-Elmer model 457 grating IR spectrophotometer, Varian A-60 NMR spectrometer; and Finnigan model 1015 quadrupole mass spectrometer.

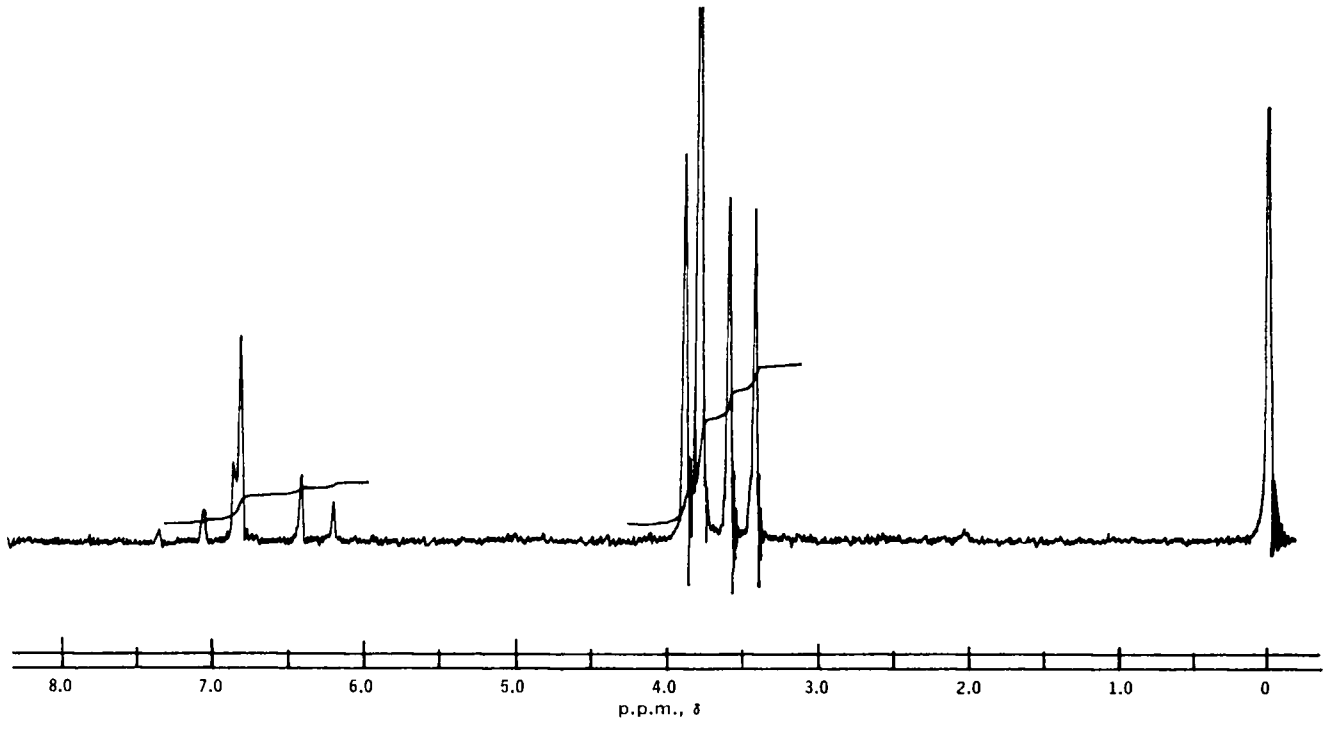


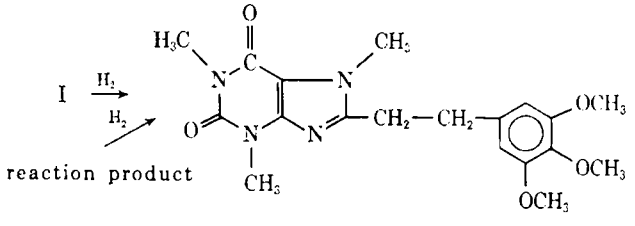
Figure 2—*NMR spectrum of II in $CDCl_3$.*

Anal.—Calc. for $C_{19}H_{22}N_4O_5$: C, 59.10; H, 5.70; N, 14.50. Found: C, 59.16; H, 5.65; N, 14.59.

The UV spectrum[3] showed $\lambda_{max}^{methanol}$ 327 and 268 nm. (ϵ 1.30 × 10^4 and 1.084 × 10^4, respectively).

The NMR spectrum (Fig. 2) showed a singlet at 3.75 (9H) p.p.m., assigned to the methoxy methyl protons; singlets at 3.41 (3H), 3.58 (3H), and 3.87 (3H) p.p.m., assigned to the three *N*-methyl group protons; and doublets centered at 6.95 (1H) and 6.31 (1H) p.p.m. (J = 13 Hz.), assigned to the vinyl protons[4].

Reduction of I and II Approximately 0.6 g. compound was suspended in 75 ml. of acetic acid in a Parr machine. Then 0.1 g. of 20% palladium-on-carbon was added. The hydrogen pressure was kept at 50 p.s.i. and the solution was shaken for 5 min. Then the solution was filtered and the solvent was evaporated. The residue was then suspended in 50 ml. of water, and the solution was made neutral by adding ammonium hydroxide. The precipitate was filtered and then recrystallized from ethanol. The reduction



III

Scheme I

products of I (A) and II (B) had identical properties and were identified as 8-(3,4,5-trimethoxyphenethyl)caffeine (III).

Properties of A and B are as follows:

	A	B
Melting point	141–143°	141–143°
UV (methanol) $\epsilon\lambda^{276}$	1.20×10^4	1.23×10^4
IR	identical spectra	

Anal.—Calc. for $C_{19}H_{24}N_4O_5$: C, 58.75; H, 6.23; N, 14.42. Found: (A) C, 58.35; H, 6.54; N, 14.18. (B) C, 58.24; H, 6.17; N, 14.32.

Determination of the Composition of Spent Reaction—The composition of the spent reaction was established by analysis of the UV spectrum obtained when the methanolic solution of the *trans*-isomer was exposed to spectral redundancy. The absorptivity (a) (1%, 1 cm.) at λ342 of the mixture was found to be 450, which corresponds to an 80:20 mixture (*cis-trans*), *i.e.*:

$$\%\ trans = \frac{a\,(1\%,\ 1\ cm.)_{obs} - 318}{659} \qquad \text{(Eq. 1)}$$

where 318 = $a\,(1\%,\ 1\ cm.)_{\lambda342}^{cis}$ and 659 = $\Delta a(1\%,\ 1\ cm.)_{\lambda342} = a(1\%,\ 1\ cm.)_{\lambda342}^{trans} - a(1\%,\ 1\ cm.)_{\lambda342}^{cis}$.

When a 0.002% solution of the *cis*-isomer was similarly exposed to a spectral redundancy, the a(1%, 1 cm.) at λ342 was found to be 425. This, in turn, indicates the composition of the equilibrium mixture to be 84:16, in reasonable agreement with that determined by starting with the *trans*-isomer.

CONCLUSION

Exposure of dilute solutions of *trans*-8-(3,4,5-trimethoxystyryl)-caffeine in methanol or chloroform to visible light results in rapid *trans-cis* isomerization; *trans-cis* isomerization proceeds with the ultimate formation of an equilibrium mixture containing approximately 82% *cis*-18% *trans*.

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[3] The UV parameters are in accord with previous observations (5–7) made on UV properties of *cis*- and *trans*-stilbenes in that the *trans*-isomer has the greater molar absorptivity and a maximum at longer wavelength than the *cis*-isomer (355 and 327 nm., respectively). Also, the molar absorptivity at 268 nm. is greater for the *cis*-isomer than for the *trans*-isomer, which is in accord with expectation.

[4] The coupling constant in I (15 Hz.) is less than normal for a vinyl hydrogen but is within the limit of 5–30 Hz. In I (*trans*), there is conjugation from the benzene ring through the vinyl system and then through the purine moiety. This reduces bond order between the carbons and thus the value of J. In II (*cis*), steric hindrance prevents both the benzene ring and purine ring from being simultaneously coplanar with the ethylene system and thus should markedly reduce conjugation. This, in turn, raises bond order in the ethylene and thereby increases J. The overall result is to bring the values of J for the *cis*- and *trans*-isomers closer together than normal.

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▲ To whom inquiries should be directed.

Application of Conformation-Dependent Antibodies: Enhancement of Enzyme Activity of L-Glutamate Dehydrogenase [L-Glutamate NAD(P) Oxidoreductase (Deaminating) EC 1.4.1.3]

BRIAN J. JOHNSON▲ and DAVID H. KEMPNER

Abstract □ By using a nonprecipitating system, it was found that the enzyme activity of L-glutamate dehydrogenase [L-glutamate NAD(P) oxidoreductase (deaminating) EC 1.4.1.3] (I) is enhanced by the addition of small amounts of anti-I-serum. It is suggested that this enhancement is due to the ability of conformation-dependent antibodies to maintain L-glutamate dehydrogenase in a more favorable conformation and thereby influence the rate of conversion of sodium α-ketoglutarate to sodium glutamate.

Keyphrases □ L-Glutamate dehydrogenase [L-glutamate NAD(P) oxidoreductase (deaminating) EC 1.4.1.3]—immunochemical properties □ Antibodies, L-glutamate dehydrogenase [L-glutamate NAD(P) oxidoreductase (deaminating) EC 1.4.1.3] produced—specificity □ Enzyme activity—enhancement of L-glutamate dehydrogenase

It has been shown that the enzymatic activity of L-glutamate dehydrogenase [L-glutamate NAD(P) oxidoreductase (deaminating) EC 1.4.1.3] (I) is dependent upon the conformation of the molecule (1–7). Immunochemical studies with this enzyme, using a precipitating system, showed that rabbit antibodies possess specificities not only for the primary structure but also for the conformation of the antigen (8, 9).

The effect of conformation-dependent antibodies on their homologous antigens is of interest. With respect to I, it was reported (10) that normal rabbit serum activates purified preparations of human and bovine I by about 40% in reactions using α-ketoglutarate and glutamate as substrates. It was also reported (10) that anti-I-serum enhanced the enzyme activity of I using α-ketoglutarate as substrate. It was, therefore, of interest to extend the reported investigations by studying the effect produced by these conformation-dependent antibodies over a period of time on the enzyme activity.

EXPERIMENTAL

Nicotinamide adenine dinucleotide reduced (NADH), α-ketoglutarate, and bovine liver L-glutamate dehydrogenase [L-glutamate NAD(P) oxidoreductase (deaminating) EC 1.4.1.3] were purchased[1]; the latter material was obtained as a crystalline suspension in ammonium sulfate. Compound I was prepared for use by dialyzing against a standard buffer solution (0.03 *M* Na_2HPO_4–0.01 *M* NaH_2PO_4–1×10^{-3} *M* disodium edetate), pH 7.4.

Immunochemistry—Ten rabbits were treated at weekly intervals with 5 mg. of I. The first 3 weeks they were injected intradermally, using complete Freunds adjuvant as the suspension medium. The rabbits were bled the following week using the standard heart puncture technique. Thereafter, the rabbits were bled and immunized on alternate weeks. Incremental amounts of I were added to 1-ml. aliquots of the antiserum obtained from each rabbit. These mixtures were incubated at 37° for 1 hr. and then stored at 4° for 48 hr. Each rabbit antiserum gave a precipitin reaction, and the amount of protein precipitated was quantitated by analysis for nitrogen (Kjeldahl).

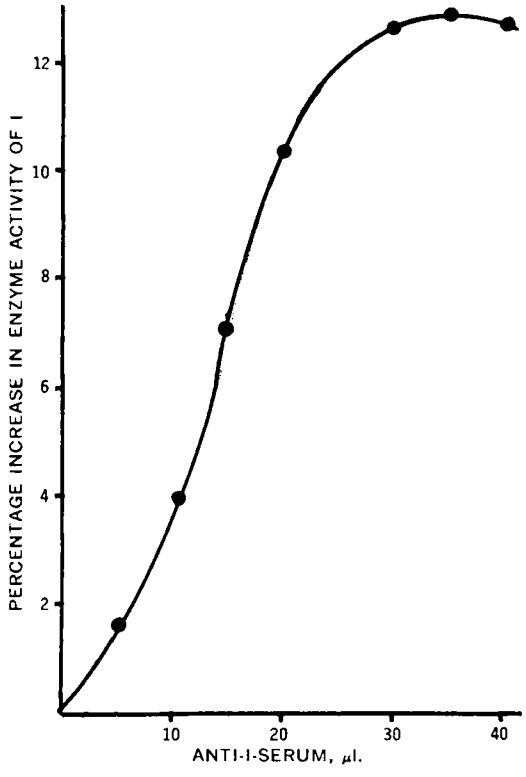

Figure 1—*Percentage increase in enzyme activity of I in the presence of anti-I-serum.*

[1] Sigma Chemical Co.

(3) S. Malkin and E. Fischer, *J. Phys. Chem.*, **68**, 1153(1964).
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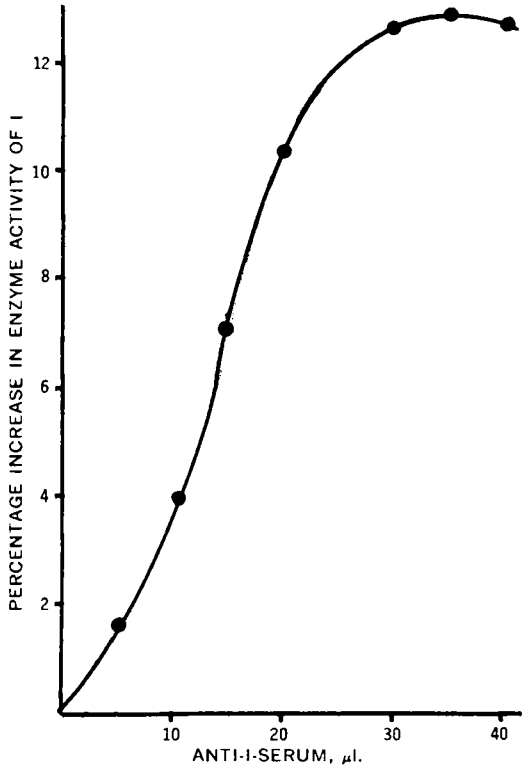

Figure 1—*Percentage increase in enzyme activity of I in the presence of anti-I-serum.*

[1] Sigma Chemical Co.

Table I—Percentage Increases in Enzyme Activity of I for Each Animal Tabulated against Length of Time from Initial Immunization

Animal Number	Time from Initial Immunization, Weeks						
	4	6	8	10	12	14	16
1	0	0	3	11	8.5	10	14
2	0	6.8	5.2	8	4.5	6.5	10.5
3	7	4	2.6	4.2	9	6.5	9.8
4	—	6	14	7.5	11.7	14.5	17.5
5	4.5	7	7.5	7	13	14.5	31
6	2.5	18	23	17.5	7	6	0
7	7	6	14	7	15	6	5
8	6.5	12	23	12	15	13	3
9	5	10	21	11	7	14.5	10
10	3.5	9	9.5	12	10	2	7

Preimmunized serum was treated with I and run under identical conditions to those described for the antiserum. No precipitin reactions were observed.

Enzyme Assays—In a typical assay the concentration of I in a solution was determined by measurement of the absorbance at 280 nm., where $E_{max} = 1.0 \times 10^6\ M^{-1}\ cm.^{-1}$ for a molecular weight of 1.0×10^6. To 3 ml. of phosphate buffer (0.03 *M* Na_2HPO_4–0.01 *M* NaH_2PO_4–1×10^{-3} disodium edetate), pH 7.4, 1.0×10^{-4} *M* NADH, 2×10^{-3} *M* sodium α-ketoglutarate, and 1×10^{-1} *M* NH_4Cl was added 5 μl. of a solution of I (0.5 mg./ml.). The enzyme activity at 23° was obtained by measuring the decrease in the absorbance of the solution at 340 nm. The determination of the enzyme activity of I in the presence of antibody obtained from each rabbit and for each bleeding was performed in exactly the same manner as that already described. However, the solution containing I and 20 μl. of the specified antiserum were incubated at 37° for 1 hr. and then cooled to 23° before proceeding with the assay. Controls using I with and without preimmunized serum were run simultaneously with the conditions identical to those described for the antiserum enzyme assays.

RESULTS

Antiserum from each rabbit and from each bleeding gave a positive precipitin reaction; however, controls using nonimmunized serum produced no precipitation. Initially, it was necessary to ascertain the optimum amount of antibody required to produce the largest change in enzyme activity without precipitation occurring. For this purpose, incremental amounts of antiserum were added to the standard aliquot of I and the increase in activity was ascertained. Typically, it was found that a maximum increase in enzyme activity occurred in the presence of 35 μl. of antiserum (Fig. 1). However, a small amount of precipitate was obtained under these conditions. An investigation to ascertain the largest amount of antiserum permissible before precipitation occurred was conducted. From these results (Fig. 2), it was evident that the largest amount of antiserum that could be used and still maintain solution was 20 μl. By using these conditions, it was found that practically every antiserum tested produced an enhancement of the activity of the enzyme I as measured by the increase in the rate of conversion of sodium α-ketoglutarate to sodium glutamate (Table I). No enhancement of enzyme activity was obtained with nonimmunized serum.

DISCUSSION

Unlike the previous report (10), no enhancement of the enzyme activity of I was found in the presence of nonimmunized serum. However, by using the nonprecipitating system described, the reported enhancement of the enzyme activity of I by use of small amounts of rabbit anti-I-serum was corroborated. The rationale was that within the heterogeneous mixture of antibodies formed to the antigenic challenge of I, a definite population is conformation dependent. It is suggested that these antibodies are capable of maintaining I in a more favorable conformation to influence the rate of conversion of sodium α-ketoglutarate to sodium glutamate. However, it is evident from Table I that the concentration of the conformation-dependent antibodies varies with the length of time of immunization. A similar observation has been made of the concentrations of various antibody populations produced by rabbits upon continuous immunization with type III and type VIII pneumococci (11). It also has to be concluded that the antigenic determinants of I must be clearly different from those that constitute the enzyme active site.

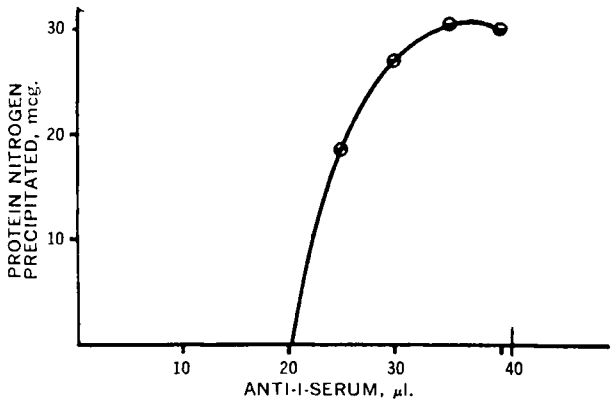

Figure 2—*Amount of protein nitrogen precipitated in the presence of anti-I-serum per aliquot of the standard enzyme assay system.*

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▲ To whom inquiries should be directed.

Biological and Phytochemical Evaluation of Plants XIII: Preliminary Estimation of Analgesic Activity of Rhazinilam, a Novel Alkaloid Isolated from *Aspidosperma quebracho-blanco* Leaves

P. S. BENOIT▲, G. ANGRY, R. L. LYON, H. H. S. FONG, and N. R. FARNSWORTH

Abstract □ A novel alkaloid, rhazinilam, from *Aspidosperma quebracho-blanco* leaves exhibited mild analgesic activity in mice.

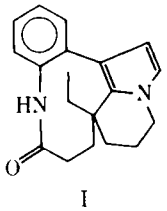
Keyphrases □ Rhazinilam—alkaloid from *Aspidosperma quebracho-blanco* tested for analgesic activity in mice □ *Aspidosperma quebracho-blanco* leaves—isolated alkaloid rhazinilam tested for analgesic activity in mice □ Analgesic activity—rhazinilam screened in mice

Previously, the isolation and structure elucidation of the lactam alkaloid, rhazinilam (I), from the leaves of *Aspidosperma quebracho-blanco* was reported[1] (2, 3). Since a literature search did not reveal any pharmacological studies on rhazinilam, it was subjected to pharmacological evaluations and was found to exhibit mild analgesic activity in mice.

EXPERIMENTAL

The procedure utilized for the evaluation of analgesic activity was essentially that described by Koster *et al.* (4), with some modifications of Taber *et al.* (5). In the screening procedure, groups of three male CD-1 mice[2], weighing 20–25 g., which had been previously starved for 18–24 hr., were injected with 10 ml./kg. i.p. of a 0.6% aqueous acetic acid solution and then placed into observation jars. The drug or the vehicle was administered by gavage 30 min. prior to administration of the acetic acid solution. Five minutes following the acid injection, the total number of writhes exhibited by the three mice during 10 min. was counted. Each day, one control (vehicle treated) and several test groups of mice were studied. At a dose of 10 mg./kg., rhazinilam reduced writhing by 15% when compared to controls.

When it had been determined by preliminary experimentation that rhazinilam possessed antiwrithing activity at a relatively low dosage level, the ED_{50} values by various routes of administration were determined. Five groups of five mice each were studied. One group was treated with the vehicle, and the other four groups received graded doses of the drug. The number of writhes exhibited in 10 min. by the drug-treated group was calculated as a percent of the number of writhes exhibited by the control group. From these data, a dose–response curve was plotted on double-cycle, semilog paper with percent response as the ordinate and log dose as the abscissa. The ED_{50} value was read directly from the curve and then determined by a programmed computer method. For comparison, similar experiments were conducted with aspirin.



I

Table I—ED_{50} Values for Aspirin and Rhazinilam Administered by Different Routes

Drug	Route	ED_{50}, mg./kg.	Number of ED_{50} Values
Aspirin	Oral	112.4	5
Aspirin	Subcutaneous	37.0	1
Aspirin	Intraperitoneal	15.0	1
Rhazinilam	Oral	313.0	1
Rhazinilam	Oral	310.0	1
Rhazinilam	Subcutaneous	140.0	1
Rhazinilam	Intraperitoneal	46.0	1

RESULTS

The results of these studies are presented in Table I. Aspirin produced ED_{50} values of 112.4 mg./kg. p.o., 37.0 mg./kg. s.c., and 15.0 mg./kg. i.p. Rhazinilam produced ED_{50} values of 311.5 mg./kg. p.o., 140 mg./kg. s.c., and 46.0 mg./kg. i.p.

From these preliminary data, it appears that rhazinilam possesses mild analgesic activity in the acetic acid writhing test. Lack of a supply of rhazinilam, which is a minor alkaloid of *Aspidosperma quebracho-blanco* leaves, precluded further study.

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[1] For the previous paper in this series, see *Reference 1*.

[2] Charles River Farms, Wilmington, Mass.

Influence of Polysorbate 20 and Sodium Cholate on Uptake of *p*-Hydroxybenzoates by the Frog, *Rana pipiens*

C. W. WHITWORTH[▲] and H. W. JUN

Abstract □ The frog, *Rana pipiens*, was found to take up certain *p*-hydroxybenzoates when partially immersed in a solution of these substances. The order of decreasing uptake rates for the benzoates was butyl, propyl, ethyl, and methyl. The presence of a nonionic surfactant, polysorbate 20, reversed the order; however, a biological surfactant, sodium cholate, appeared to increase the rate of uptake of the parabens. The process was first order.

Keyphrases □ Polysorbate 20—influence on uptake of *p*-hydroxybenzoates by frog □ Sodium cholate—influence on uptake of *p*-hydroxybenzoates by frog □ *p*-Hydroxybenzoates—influence of polysorbate 20 and sodium cholate on uptake by frog □ Surfactants—influence of polysorbate 20 and sodium cholate on uptake of *p*-hydroxybenzoates by frog

The purpose of this study was to screen a number of drugs for uptake by the frog and, for those found, to determine the effect of two types of surfactants on the process. The screened drugs included the weak bases procaine, acetanilid, and 4-aminoantipyrine and the weak acids phenobarbital, sulfacetamide, and four benzoic acid esters. Of these drugs, only the benzoates were taken up to any detectable degree. Previously, salicylic acid was shown (1) to be taken up readily, mainly in the undissociated form, from solution by the frog, thus conforming to the proposed pH–partition theory of passive absorption of some drugs as offered by Shore *et al.* (2). This study supports the evidence of this theory since the frog took up the higher molecular weight benzoates which had greater oil solubility more readily than the ones with lower molecular weight.

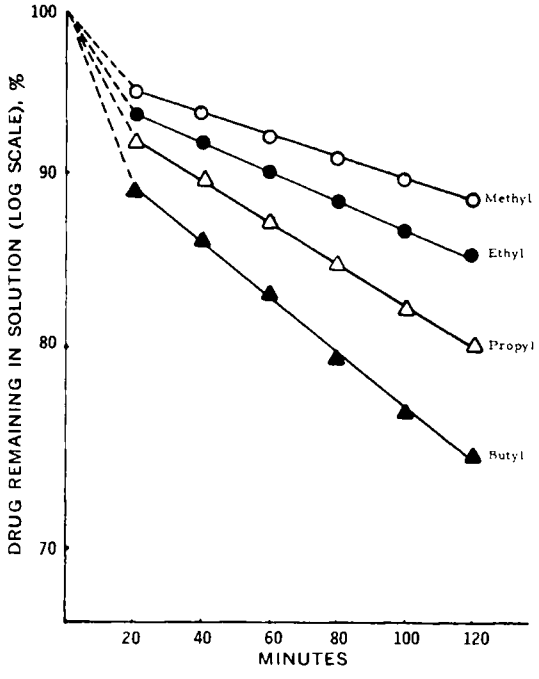

Figure 1—*Plot of average percent of parabens remaining in solution over 2 hr. when five frogs were each placed in 500 ml. of aqueous drug solution. Each solution was initially 5.0 × 10⁻⁵ M in the paraben.*

Table I Rate Constants (min.⁻¹ × 10⁴)

Additive	Paraben			
	Methyl	Ethyl	Propyl	Butyl
None (control)	1.53	1.92	2.84	3.78
Polysorbate 20 (0.5%)	1.74	1.42	0.747	0.625
Polysorbate 20 (1.0%)	1.42	1.21	0.671	0.499
Sodium cholate (5.0×10^{-5} *M*)	1.64	2.06	3.18	4.48

EXPERIMENTAL

The technique employed in this study was used previously (1, 3, 4). Five frogs, 30–35 g. each, were used in the determinations. Each frog was placed in 500 ml. of drug solution contained in a 2-l. beaker which permitted immersion to the neck. At 20-min. intervals, about 5 ml. of drug solution was withdrawn and assayed for drug content and returned to the container to maintain uniform volume. The solutions were buffered to pH 4.0 with hydrochloric acid in 0.05 *M* glycine. This pH is well tolerated by frogs and was used in previous studies (1, 3, 4) to minimize the effect of ionization changes

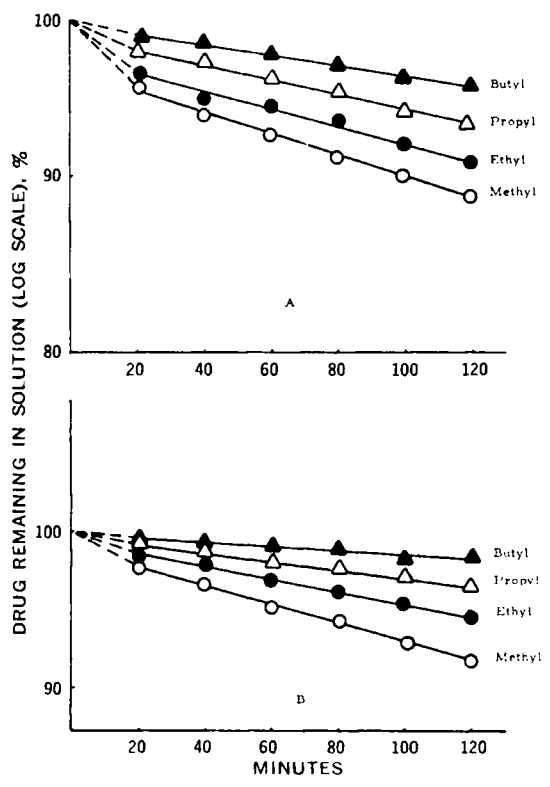

Figure 2—*Plot of average percent of parabens remaining in solution over 2 hr. when five frogs were each placed in 500 ml. of aqueous drug solution, each containing a paraben in a concentration of 5.0 × 10⁻⁵ M and polysorbate 20 (0.5%, upper curves A; 1%, lower curves B).*

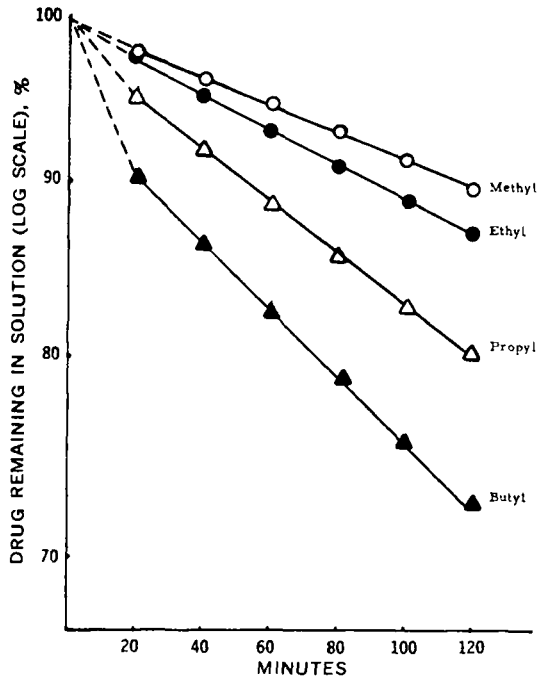

Figure 3—*Plot of average percent of parabens remaining in solution over 2 hr. when five frogs were each placed in 500 ml. of aqueous drug solution, each containing a paraben in a concentration of 5.0 × 10^{-5} M and sodium cholate, 5.0 × 10^{-5} M.*

on drug uptake. Distilled water was used as the solvent, and the solutions of parabens were 5.0 × 10^{-5} *M*. The concentration of benzoate remaining in solution was determined by absorbance readings[1] at a wavelength of 255 nm.

The partition coefficient was determined by allowing each paraben to equilibrate with stirring for 3 hr. at 25° between 100 ml. of *n*-hexane–distilled water (50:50), adjusted to a pH of 2.0 with hydrochloric acid. The aqueous layer was assessed for paraben content, and the partition coefficient was calculated from:

$$P = \frac{\text{concentration in } n\text{-hexane}}{\text{concentration in water}} \qquad \text{(Eq. 1)}$$

RESULTS AND DISCUSSION

As seen in Figs. 1–3, the data can be linearized satisfactorily by plotting the log of the percent of paraben remaining in solution against time, thus indicating a first-order process. In each case, however, the relationship does not become linear until after the first 20 min. During this initial period, the frog takes up the drug considerably faster than during the remainder of the 2 hr., as noted in a previous study (4).

The linear equation is:

$$\log C = -\frac{kt}{2.303} + \log C_0 \qquad \text{(Eq. 2)}$$

where C = percent of benzoate remaining in solution, C_0 = initial percent of benzoate in solution (100), k = rate constant, and t = time in minutes. The rate constants were calculated from the slope, $-K/2.303$, of the line and show the rate of disappearance of drug.

Figure 1 shows that the frog, *Rana pipiens*, takes up the parabens with higher molecular weights more readily than those with lower molecular weights, in the order butyl > propyl > ethyl > methyl.

[1] Beckman DU spectrophotometer.

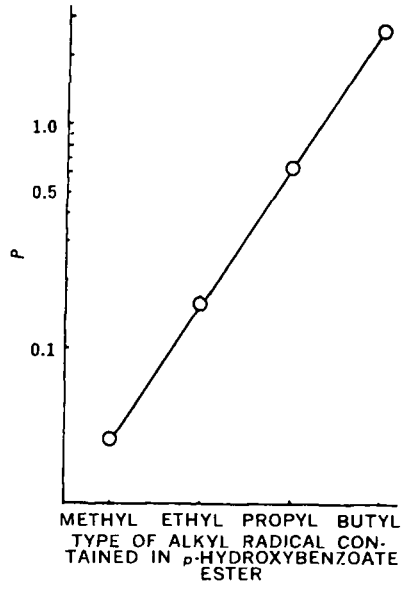

Figure 4—*Partition coefficient* (P) *of parabens at pH 2.0.* P = *concentration in* n-*hexane/concentration in water.*

This is consistent with previous findings (1) that the greater the lipid solubility of the drug the greater is the rate of uptake by the frog for these drugs. Figure 4 shows paraben partition coefficient data which correlate molecular weight and lipid solubility.

Surfactants have been shown to influence drug absorption (4, 5). Figure 2 shows the effect of two concentrations of polysorbate 20 on the uptake of parabens. This nonionic surfactant decreased the uptake of most of the parabens and also reversed the order of the sorption process with respect to molecular weight (Table I).

Sodium cholate is a bile salt having surfactant properties, and its effect on the uptake of parabens by the frog is seen in Fig. 3. A comparison of rate constants (Table I) indicates that sodium cholate slightly increased the rate of uptake of each paraben.

The mechanisms by which such additives influence drug uptake is not well understood. The effect of polysorbate 20 in decreasing uptake is perhaps due to complexation. The surface tension-lowering ability of the sodium cholate probably increases drug uptake by permitting better wetting action by the drug solution on the skin of the frog. Bile salts were found to increase the permeability of everted rat intestine to salicylate (6).

The frog, *Rana pipiens*, has been found to take up relatively few types of drugs using an immersion technique. The partition coefficient of the drugs taken up plays an important role in determining the rate.

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Acetaminophen Prodrug: 2-(*p*-Acetaminophenoxy)tetrahydropyran

A. J. REPTA▲ and J. HACK

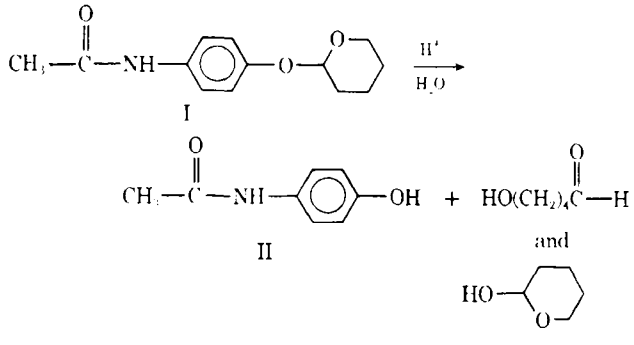
Abstract □ The synthesis and chemistry of 2-(*p*-acetaminophenoxy)-tetrahydropyran, a prodrug of acetaminophen, are described. The utilization of a prodrug approach to the formulation of chewable acetaminophen tablets is discussed. Although the compound studied has many desired properties for use in chewable tablets, its aqueous solubility is very low so the dissolution rate may be rate determining in the release of acetaminophen.

Keyphrases □ Acetaminophen—synthesis of 2-(*p*-acetaminophenoxy)tetrahydropyran as a prodrug for chewable formulations, chemical properties □ 2-(*p*-Acetaminophenoxy)tetrahydropyran—synthesized as a prodrug of acetaminophen, hydrolysis, effects of temperature, ionic strength, pH, taste properties □ Acylals—synthesis of 2-(*p*-acetaminophenoxy)tetrahydropyran as a prodrug of acetaminophen for chewable formulations

The analgesic–antipyretic drug acetaminophen has been formulated in numerous dosage forms. However, there apparently has been no attempt to produce a chewable form, probably because of its unpleasant taste.

Since taste is known to be relatively specific and its intensity is concentration dependent, the taste problem should be overcome, at least in part, by preparing derivatives of acetaminophen that are much less soluble and thereby reduce the concentration of the drug at the taste buds. Alternatively, it should also be possible to alter the structure of the drug to produce a more acceptable taste.

For either approach to be successful, any chemical derivative employed should release the drug in the GI tract in the active form which would then be available for absorption. If the approach is to have merit, the rate of release of the drug under acid conditions must be much more rapid than the release rate at the relatively neutral pH of the mouth. If the prodrug form releases the drug rapidly in the mouth, the organoleptic sensations produced would be similar to those of the drug alone and no advantage would be realized. Therefore, the conversion of the prodrug to the active material must be greatly accelerated by the acid contents of the stomach.



Scheme I

The acylal class of organic compounds possesses chemical properties that appear to meet these criteria. For this study, 2-(*p*-acetaminophenoxy)tetrahydropyran (I) was selected to determine its feasibility as a prodrug of acetaminophen (II) suitable for a chewable dosage form (Scheme I).

The synthesis and chemistry of some 2-(aryloxy)tetrahydropyrans were reported previously (1, 2). Such substances are easily prepared by reaction of various phenols with dihydropyran in the presence of acid catalysts. The hydrolysis of the acylals was shown to be acid catalyzed at low pH but independent of pH at near neutral to alkaline conditions (1–3). The products of hydrolysis were reported to be the phenol, δ-hydroxyvaleraldehyde, and 2-(hydroxy)tetrahydropyran (1, 4)[1].

In previous studies, the rate of hydrolysis of compounds similar to I was dependent on the dielectric constant of the hydrolysis medium, and mechanisms were suggested (2, 3).

While much work has been done on the chemistry of such compounds, the acylal approach in the formulation of prodrugs apparently has not been used.

To determine whether the hydrolysis of 2-(*p*-acetaminophenoxy)tetrahydropyran would likely result in the rapid and complete release of acetaminophen under conditions similar to those encountered in the GI tract, the rate of hydrolysis was studied as a function of pH, temperature, and ionic strength. Some physical properties were also investigated.

EXPERIMENTAL[2]

Synthesis of I—A suspension containing II (4 g., 0.026 mole), dihydropyran (50 ml., 0.6 mole), and methylene chloride (40 ml.), saturated with hydrogen chloride gas, was prepared in a 250-ml., loosely stoppered flask. This suspension was stirred magnetically at room temperature until all suspended material was dissolved and a clear colorless solution was obtained. This normally occurred within 2–4 hr.

The solution obtained was transferred to a rotary evaporator, and some of the solvent and other volatiles were removed at room temperature and reduced pressure using an aspirator. Evaporation under these conditions was continued until most solvent was removed and a viscous suspension of a heavy white precipitate was obtained. About 10 ml. of anhydrous ether was added to the residue to facilitate its removal from the flask.

The solid white precipitate was separated by vacuum filtration, washed twice with 20-ml. portions of anhydrous ether, and dried for 30 min. at room temperature in a vacuum oven at a pressure of 1–2 mm. Hg. The solid obtained (5.5 g., 88% yield) melted at 112–113°.

[1] No reports on toxicity of these agents were located. However, it seems likely that the valeraldehyde would probably be oxidized to glutaric acid which presents no problem of toxicity.

[2] The equipment used included the Cary models 14 and 15 spectrophotometers and a Corning model 12 pH meter. Temperatures were controlled by using a water bath thermostated at the desired temperature ±0.1°. All reagents were of reagent grade, and the water was distilled from acid permanganate solution in an all-glass still.

Table I—Observed Rate Constants for Hydrolysis of I at 25° at Various pH and Ionic Strengths

Ionic Strength, μ	k_{obs} (sec.$^{-1}$) at pH 1.11	2.0	3.0	4.135	5.0
0.1	6.73×10^{-2}	6.51×10^{-3}	6.6×10^{-4}	5.63×10^{-5}	7.9×10^{-6}
0.3	7.11×10^{-2}	9.63×10^{-3}	6.7×10^{-4}	—	—
0.5	7.53×10^{-2}	11.55×10^{-3}	8.8×10^{-4}	—	—
0.8	7.70×10^{-2}	13.8×10^{-3}	—	—	—
1.0	—	—	10.0×10^{-4}	—	—

The NMR[3] spectrum of a solution of the solid in dimethyl sulfoxide was consistent with the formula of Compound I.

Anal.—Calc. for $C_{13}H_{17}NO_3$: C, 66.4; H, 7.3; N, 6.0. Found[4]: C, 66.6; H, 7.5; N, 6.2.

TLC of a solution of I in 2-butanone showed only one spot, at R_f 0.55, and differential scanning calorimetry[5] indicated only one endotherm, at 112–113°. The melting point as conventionally determined[6] was ~113° (uncorrected).

TLC—The TLC system employed to separate I and II utilized a polyamide layer[7] with dry 2-butanone as the developing solvent. Detection was accomplished by UV light at 254 nm. The system was sensitive to 0.1 mcg. II. The R_f values were: I, 0.75; and II, 0.55. No spots indicating the presence or nature of the products of the tetrahydropyran product were detectable. The hydrolysis of a 0.1% solution of I was followed at pH 2.95, μ = 0.1. At various times over several hours, a sample of the solution was withdrawn and added to pH 7 buffer to quench the hydrolytic reaction. The resulting solution was then spotted on the plate and developed.

Kinetic Studies—The kinetics of the hydrolysis of I were followed by UV at 290 nm. At this wavelength, the absorbance increased with time.

Since I did not dissolve rapidly in aqueous solutions, a 0.03 *M* stock solution of I in acetonitrile was prepared. Approximately 150 μl. of the stock solution was placed in a 2-cm. cell. The desired buffer (6.5 ml.), equilibrated at the desired temperature, was then added. The cell was closed, inverted carefully and rapidly to assure mixing, and placed in the spectrophotometer, and the absorbance changes were then followed.

Normally the reactions were followed to completion. At a higher pH value and a slower reaction, the reaction was followed for only about 5–6 half-lives. In all cases, an apparent but minor problem of solubility was encountered. As the reaction proceeded, the dihydropyran and/or derivatives that resulted from hydrolysis appeared to separate from the solution, and this occurrence resulted in small changes in the apparent absorbance. To correct for the problem of changing absorbance, all kinetic data were plotted using the Guggenheim method.

All buffers used were prepared from reagent grade materials, and the ionic strength was adjusted by the addition of sodium or potassium chloride.

RESULTS AND DISCUSSION

The hydrolysis of I was followed over a pH range of 1–5 at μ = 0.1 and 25° in aqueous solutions containing 2–3% acetonitrile. It can be seen from Fig. 1 that the hydrolysis of I is hydrogen-ion catalyzed. The solid line in Fig. 1 is drawn with a slope of unity. The good fit of the experimental points to the line shows that the catalysis is first order in [H^+]. Although the reaction was not studied extensively or quantitatively at pH > 5, due to the very slow rate of hydrolysis, the rate began to reach a plateau in the region of pH > 6–7. This finding is in accord with previous studies on related systems (3, 4).

To be certain that the hydrolysis of I to yield II was neat and complete, the reaction at 25°, pH 3.0, and μ = 0.1 was followed by TLC. The TLC method was able to separate I and II. The reaction was followed as a function of time, and it was found that the hydrolysis was complete and that there was no detectable degradation of II under these conditions as indicated by the presence of a single TLC spot, which corresponded to II, at the end of the reaction.

The rate of hydrolysis of I was followed as a function of acetate concentration (0.1–1.0 *M*) in buffers at 25°, pH 3.0, μ = 0.1; no buffer catalysis was observed.

Ionic strength, on the other hand, affected the rate greatly. The observed rate constants as a function of ionic strength at pH 1.1–5 are shown in Table I. The rate was increased by increasing ionic strength, and the magnitude of the effect increased with increasing pH. These observations seem to be in agreement with the suggested mechanism of hydrolysis for related compounds (3, 4).

The effect of temperature on k_{obs} was studied at pH 3.0 and μ = 0.1; k_{obs} (sec.$^{-1}$)($\times 10^4$) was found to be 2.3 (288°K.), 6.6 (298°K.), and 21.8 (310°K.). The energy of activation of the reaction was about 19.5 kcal./mole degree, which is again in agreement with values determined for related systems (2, 3).

While the half-life of hydrolysis at 25°, pH 3.0, and μ = 0.1 was a rather slow 17.5 min., the rate at 37° resulted in a half-life of only 5 min. Since the environment of the stomach would be similar to the conditions of the *in vitro* study at 37°, it would be expected that the

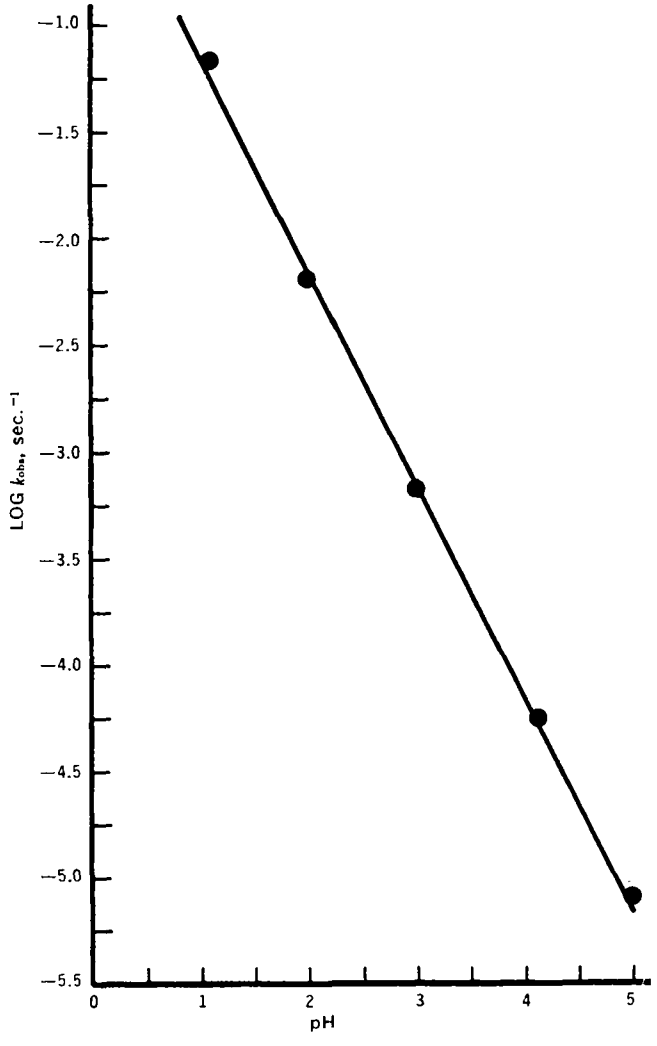


Figure 1—*The pH–rate profile for hydrolysis of I at 25° and μ = 0.1.*

[3] Varian model T-60 NMR spectrometer.
[4] Elemental analysis was performed at the Medicinal Chemistry Department, University of Kansas, Lawrence, Kans.
[5] Perkin-Elmer model DSC-1B differential scanning calorimeter.
[6] Thomas–Hoover capillary melting-point apparatus.
[7] Polygram Sil N-HR precoated on plastic sheets, available from Brinkmann Instruments, Inc.

release of II from I in the stomach would be about 95% complete in 25 min. In the mouth, however, the half-life would be expected to be 1 hr. or more since a pH > 5 would be normal. Thus, there should not be a sufficient amount of II released in the mouth to cause the taste problem associated with II, yet it should be rapidly released in the stomach. When small amounts of II were placed on the tongues of volunteers, they reported the lack of any significant taste associated with the material.

The solubility of I in distilled water at 25° was only 0.002 mole/l., while the solubility of II under the same conditions was about 0.1 mole/l. (5). By applying the approach of Hussain (6) in the calculation of time required for 50% dissolution, it can be calculated that, for a material with an average particle size of 100 μ, about 19 min. would be required for 50% dissolution at 25°. Thus, the rate-determining step in the release of II would be the rate of dissolution at pH 3. Therefore, the solubility of I may limit its usefulness as a chewable tasteless prodrug form of II.

To establish whether the lack of taste associated with I is due solely to its limited solubility, saturated solutions of I in both 40 and 30% alcohol in water were prepared. These solutions were tasted by volunteers who again reported no objectionable taste, which suggested that I was relatively free of taste problems.

CONCLUSIONS

The prodrug of acetaminophen investigated appears to be suitable, from the taste standpoint, for use in a chewable tablet.

In vitro studies of the hydrolysis of I in aqueous solution showed that the rates of hydrolysis of I were sufficiently rapid to ensure release of acetaminophen in the stomach. However, the low solubility of I suggests that the rate-determining step in the release of acetaminophen would be the dissolution rate of the prodrug rather than its hydrolytic rate. Therefore, on the basis of these data, it appears that 2-(*p*-acetaminophenoxy)tetrahydropyran may not be especially suited for use in chewable tablets, although other uses may exist.

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Synthesis and Pharmacological Screening of 1-Chloro-3-(2-propynyloxy)-2-propanols and 2-[(2-Propynyloxy)methyl]oxiranes

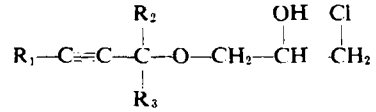
R. B. FUGITT, G. S. WU, and L. C. MARTINELLI▲

Abstract □ Some researchers have found that an ethynyl group is essential for optimal anticancer activity of carbamate esters. In addition, it has been observed that bisepoxides are generally more potent anticancer agents than monoepoxides. These observations led to the syntheses and screening of the title compounds, ethynyl derivatives of oxiranes and their synthetic precursors, since the presence of both an ethynyl group and an oxirane group in a molecule may lead to significant anticancer activity. One compound, 2-[(2-butynyloxy)methyl]oxirane, showed confirmed, but weak, activity in the P-388 test system employed.

Keyphrases □ 1-Chloro-3-(2-propynyloxy)-2-propanols—synthesis and pharmacological screening as possible anticancer agents □ 2-[(2-Propynyloxy)methyl]oxiranes synthesis and pharmacological screening as possible anticancer agents □ Oxiranes, ethynyl derivatives and synthetic precursors—synthesis and screening as possible anticancer agents □ Anticancer agents, potential—synthesis and screening of 1-chloro-3-(2-propynyloxy)-2-propanols and 2-[(2-propynyloxy)methyl]oxiranes

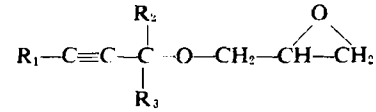
The possibility that the ethynyl group could act as a biological alkylating agent prompted the synthesis and screening of the title compounds as possible cytotoxic agents. It has been reported (1) that the presence of an acetylene group is essential for the oncolytic action of a series of ethynylmethyl carbamate esters.

The requirements of an ethynyl group for oncolytic activity in ethynylmethyl carbamates, as well as a generally observed lower anticancer activity for mono-



halohydrins

I: $R_1 = CH_3$, $R_2 = R_3 = H$
II: $R_1 = CH_3(CH_2)_2$, $R_2 = R_3 = H$
III: $R_1 = H$, $R_2 = H$, $R_3 = CH_3$
IV: $R_1 = H$, $R_2 = R_3 = CH_3$
V: $R_1 = C_6H_5$, $R_2 = R_3 = H$
VI: $R_1 = H$, $R_2 + R_3 = CH_2(CH_2)_3CH_2$



oxiranes

VII: $R_1 = CH_3$, $R_2 = R_3 = H$
VIII: $R_1 = CH_3(CH_2)_2$, $R_2 = R_3 = H$
IX: $R_1 = H$, $R_2 = H$, $R_3 = CH_3$
X: $R_1 = H$, $R_2 = R_3 = CH_3$
XI: $R_1 = C_6H_5$, $R_2 = R_3 = H$
XII: $R_1 = H$, $R_2 + R_3 = CH_2(CH_2)_3CH_2$

release of II from I in the stomach would be about 95% complete in 25 min. In the mouth, however, the half-life would be expected to be 1 hr. or more since a pH > 5 would be normal. Thus, there should not be a sufficient amount of II released in the mouth to cause the taste problem associated with II, yet it should be rapidly released in the stomach. When small amounts of II were placed on the tongues of volunteers, they reported the lack of any significant taste associated with the material.

The solubility of I in distilled water at 25° was only 0.002 mole/l., while the solubility of II under the same conditions was about 0.1 mole/l. (5). By applying the approach of Hussain (6) in the calculation of time required for 50% dissolution, it can be calculated that, for a material with an average particle size of 100 μ, about 19 min. would be required for 50% dissolution at 25°. Thus, the rate-determining step in the release of II would be the rate of dissolution at pH 3. Therefore, the solubility of I may limit its usefulness as a chewable tasteless prodrug form of II.

To establish whether the lack of taste associated with I is due solely to its limited solubility, saturated solutions of I in both 40 and 30% alcohol in water were prepared. These solutions were tasted by volunteers who again reported no objectionable taste, which suggested that I was relatively free of taste problems.

CONCLUSIONS

The prodrug of acetaminophen investigated appears to be suitable, from the taste standpoint, for use in a chewable tablet.

In vitro studies of the hydrolysis of I in aqueous solution showed that the rates of hydrolysis of I were sufficiently rapid to ensure release of acetaminophen in the stomach. However, the low solubility of I suggests that the rate-determining step in the release of acetaminophen would be the dissolution rate of the prodrug rather than its hydrolytic rate. Therefore, on the basis of these data, it appears that 2-(*p*-acetaminophenoxy)tetrahydropyran may not be especially suited for use in chewable tablets, although other uses may exist.

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Synthesis and Pharmacological Screening of 1-Chloro-3-(2-propynyloxy)-2-propanols and 2-[(2-Propynyloxy)methyl]oxiranes

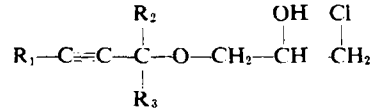
R. B. FUGITT, G. S. WU, and L. C. MARTINELLI▲

Abstract □ Some researchers have found that an ethynyl group is essential for optimal anticancer activity of carbamate esters. In addition, it has been observed that bisepoxides are generally more potent anticancer agents than monoepoxides. These observations led to the syntheses and screening of the title compounds, ethynyl derivatives of oxiranes and their synthetic precursors, since the presence of both an ethynyl group and an oxirane group in a molecule may lead to significant anticancer activity. One compound, 2-[(2-butynyloxy)methyl]oxirane, showed confirmed, but weak, activity in the P-388 test system employed.

Keyphrases □ 1-Chloro-3-(2-propynyloxy)-2-propanols—synthesis and pharmacological screening as possible anticancer agents □ 2-[(2-Propynyloxy)methyl]oxiranes synthesis and pharmacological screening as possible anticancer agents □ Oxiranes, ethynyl derivatives and synthetic precursors—synthesis and screening as possible anticancer agents □ Anticancer agents, potential—synthesis and screening of 1-chloro-3-(2-propynyloxy)-2-propanols and 2-[(2-propynyloxy)methyl]oxiranes

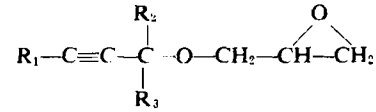
The possibility that the ethynyl group could act as a biological alkylating agent prompted the synthesis and screening of the title compounds as possible cytotoxic agents. It has been reported (1) that the presence of an acetylene group is essential for the oncolytic action of a series of ethynylmethyl carbamate esters.

The requirements of an ethynyl group for oncolytic activity in ethynylmethyl carbamates, as well as a generally observed lower anticancer activity for mono-



halohydrins

I: $R_1 = CH_3$, $R_2 = R_3 = H$
II: $R_1 = CH_3(CH_2)_2$, $R_2 = R_3 = H$
III: $R_1 = H$, $R_2 = H$, $R_3 = CH_3$
IV: $R_1 = H$, $R_2 = R_3 = CH_3$
V: $R_1 = C_6H_5$, $R_2 = R_3 = H$
VI: $R_1 = H$, $R_2 + R_3 = CH_2(CH_2)_3CH_2$



oxiranes

VII: $R_1 = CH_3$, $R_2 = R_3 = H$
VIII: $R_1 = CH_3(CH_2)_2$, $R_2 = R_3 = H$
IX: $R_1 = H$, $R_2 = H$, $R_3 = CH_3$
X: $R_1 = H$, $R_2 = R_3 = CH_3$
XI: $R_1 = C_6H_5$, $R_2 = R_3 = H$
XII: $R_1 = H$, $R_2 + R_3 = CH_2(CH_2)_3CH_2$

Table I—Experimental Data

Compound	Boiling Point (mm. Hg)	Procedure	Yield, %	Formula	Analysis, % Calc.	Found
I	90–97° (0.30–0.50)	A	68	$C_7H_{11}ClO_2$	C 51.71 H 6.81 Cl 21.80	51.53 6.79 21.54
II	79–82° (0.04)	A	38	$C_9H_{15}ClO_2$	C 56.70 H 7.92 Cl 18.60	56.66 7.98 18.54
III	58–65° (0.04–0.06)	A	24	$C_7H_{11}ClO_2$	C 51.71 H 6.81 Cl 21.80	51.44 6.98 21.65
IV	49–52° (0.03)	B	23	$C_8H_{13}ClO_2$	C 54.40 H 7.41 Cl 20.07	54.21 7.51 20.18
V	125–135° (0.02–0.05)	B	45	$C_{12}H_{13}ClO_2$	C 64.14 H 5.83 Cl 15.77	63.98 5.89 15.91
VI	90–98° (0.10)	B	35	$C_{11}H_{17}ClO_2$	C 60.96 H 8.01 Cl 16.35	60.81 7.96 16.24
VII	89–92° (15.0)	–	82	$C_8H_{12}O_2$	C 66.64 H 7.99	66.73 8.10
VIII	62–66° (0.45)	—	77	$C_9H_{14}O_2$	C 70.10 H 9.15	70.11 9.22
IX	78–82° (25.0)	—	65	$C_7H_{10}O_2$	C 66.64 H 7.99	66.70 8.08
X	65–68° (20.0)	—	81	$C_8H_{12}O_2$	C 68.54 H 8.63	68.41 8.69
XI	87–91° (0.02)	—	85	$C_{12}H_{12}O_2$	C 76.56 H 6.43	76.32 6.57
XII	110–112° (9.0)	—	78	$C_{11}H_{16}O_2$	C 73.30 H 8.95	73.44 8.99

Table II—Spectral Data for Propynyloxy Propanols

Compound	NMR, p.p.m. R_1		R_2		R_3		OCH_2	$-\underset{\vert}{C}H-$	CH_2Cl	IR, $cm.^{-1}$ OH	HC≡	C≡C	COC
I	CH_3	1.85	H	=	H	4.15	3.60	3.90	3.60	3400	—	2280 2210	1100
II	CH_3 $\vert$ CH_2 $\vert$ CH_2	1.00 1.55 2.25	H	=	H	4.20	3.65	4.00	3.60	3400	—	2275 2225	1100
III	H	2.55	H	4.25	CH_3	1.45	3.70	3.95	3.50	3400	3280	2105	1114
IV	H	2.55	CH_3	=	CH_3	1.45	3.65	3.85	3.60	3400	3270	2100	1095
V	C_6H_5	7.30	H	=	H	4.35	3.65	4.00	3.60	3412	—	2235	1110
VI	H	2.60	$CH_2(CH_2)_3CH_2$			1.60	3.65	3.85	3.62	3400	3280	2105	1100

epoxides compared to bisepoxides (2), prompted the investigation of the anticancer activity of ethynyl-substituted oxiranes. It seemed reasonable that the ethynyl group might also be acting as a biological alkylating site and that ethynyl derivatives of oxiranes might, therefore, possess significant anticancer activity. The halohydrins were prepared as intermediates to the oxiranes and were also screened since they may be converted *in vivo* to their corresponding oxiranes.

EXPERIMENTAL

Chemistry[1]—Table I gives the experimental data for the individual compounds, and Tables II and III give their NMR and IR spectral characteristics. NMR spectra[2] were taken in deuteriochloroform containing 1% tetramethylsilane as a standard. The IR spectra[3] were taken as thin films between sodium chloride plates.

The 1-chloro-3-(2-propynyloxy)-2-propanols were synthesized by acid-catalyzed condensation, similar to a published procedure (4), of the appropriate alcohol with epichlorohydrin. In the cases of the two tertiary alkynols and phenylpropargyl alcohol, phosphoric acid was employed (Procedure B) to eliminate the excessive tarring and lowered yields experienced when sulfuric acid was used (Procedure A). The 2-[(2-propynyloxy)methyl]oxiranes were prepared from the acetylenic halohydrins in good yield using a published method (4).

Synthesis of 1-Chloro-3-(2-propynyloxy)-2-propanols—*Procedure A, Compounds I–III*—Concentrated sulfuric acid (2 ml./mole epichlorohydrin) was added dropwise at room temperature to a flask containing a stirred mixture of 1 equivalent of epichlorohydrin and 2 equivalents of the acetylenic alcohol. The reaction vessel was equipped with a reflux condenser protected by a drying tube. The reaction solution was warmed to 100–110° for 24–48 hr. when it began to darken appreciably. After cooling, 1.5 equivalents of powdered barium carbonate was added; after stirring for 1–2 hr., the salts were removed by filtration. Unchanged epichlorohydrin was removed by a water aspirator, and the residual oil was carefully fractionated *in vacuo* to give the product. When the alcoholic starting material is sufficiently water insoluble to resist emulsification, the acid catalyst may also be removed by diluting the reaction mixture with methylene chloride and washing it with water.

Procedure B, Compounds IV–VI—These compounds were prepared as in Procedure A with the following modifications. Concentrated phosphoric acid (10 ml.) is substituted for every 2 ml. of con-

[1] All microanalyses were performed by Atlantic Microlabs, Inc., Atlanta, Ga.
[2] Hitachi Perkin-Elmer R20A.
[3] Perkin-Elmer 237B spectrometer.

Table III—Spectral Data for Propynyloxymethyl Oxiranes

Compound	NMR, p.p.m.									IR, cm.$^{-1}$			
	R_1		R_2		R_3		OCH_2	—CH—	$—CH_2$	HC≡	H_2C—	C≡C	Δ
VII	CH_3	2.10	H	=	H	4.37	3.85	3.33	2.88	—	3040	2280 2205	1260
VIII	CH_3	1.20	H	=	H	4.36	3.84	3.28	2.85	–	3040	2280 2220	1260
	CH_2	1.75											
	CH_2	2.42											
IX	H	2.77	H	4.45	CH_3		1.62	3.85	3.30	2.95	3260	3045	2100	1255
X	H	2.54	CH_3	=	CH_3		1.49	3.64	3.12	2.66	3270	3055	2105	1255
XI	C_6H_5	7.35	H	=	H		4.36	3.60	3.12	2.65	—	3050	2230	1260
XII	H	2.60	$CH_2(CH_2)_3CH_2$				1.60	3.64	3.08	2.67	3250	3035	2100	1260

centrated sulfuric acid used in Procedure A, and the reaction solution is heated an additional 4–24 hr.

Synthesis of 2-[(2-Propynyloxy)methyl]oxiranes, Compounds VII–XII– Freshly powdered sodium hydroxide (1.5 equivalents) was added to an ice-cold solution of the 1-chloro-3-(2-propynyloxy)-2-propanol dissolved in a volume of absolute ether equal to 10 times the weight of sodium hydroxide used. The reaction vessel was stoppered and allowed to stir magnetically overnight. The suspended inorganic salts were removed by filtration, the ether solution was concentrated on a rotary evaporator, and the residual oil was distilled *in vacuo*.

Biological[1]–The experimental animals were BDF_1 mice inoculated intraperitoneally (i.p.) or intracerebrally (i.c.) with L-1210 or P-388 tumor systems. The test compounds (all were racemic mixtures) were administered at 400, 200, 100, and 50 mg./kg. body weight i.p. in sonified saline or saline with polysorbate 80. Doses were administered, beginning on Day 1, either daily for nine doses or every 4th day for three doses, and test results were evaluated at Day 30. Evaluations were made as mean survival time and are expressed as % T/C (test/control), with a value of 125 or greater representing activity in these systems.

RESULTS AND DISCUSSION

Chemistry–The possibility exists that condensation of the acetylenic alcohol with epichlorohydrin may occur at both the primary and secondary carbons of the epichlorohydrin to give a γ-halohydrin in addition to the desired β-halohydrin. This inconvenient side reaction was observed in only one instance, in the case of 2 butyn-1-ol. Apparently, the reaction conditions used for the conversion of the β-halohydrin to the oxirane are insufficient to convert also the γ-halohydrin to the corresponding oxetane, since oxetane has not been observed in the IR or NMR of any 2-[(2-propynyloxy)-methyl]oxirane. Isolation of the lower boiling β-halohydrin in pure form is achieved by careful fractional distillation of the reaction mixture *in vacuo*.

Biological–The compounds were tested against L-1210 and P-388 leukemia according to the standard protocol of The Division of Cancer Treatment, National Cancer Institute, National Institutes of Health. The 1-chloro-3-(2-propynyloxy)-2-propanols (I–VI) showed no significant activity as indicated by changes in the mean survival time of the test animals over that of the control animals (% T/C < 125). The activity of oxirane VII, 2-[(2-butynyloxy)methyl]oxirane (NSC 153427), was confirmed by duplicate screening in the intraperitoneal P-388 test system (% T/C 129 and 134) when administered daily, at doses as low as 44 mg./kg. body weight, for nine doses. Oxirane XI, 2-{[(3-phenyl-2-propynyl)oxy]methyl}oxirane (NSC 156628), showed unconfirmed preliminary activity in intraperitoneal P-388 (% T/C 133) and intracerebral P-388 (% T/C 154) when administered daily for nine doses (400 and 100 mg./kg., respectively). The other oxiranes were devoid of activity but did exhibit slightly larger % T/C values than their corresponding β-halohydrins.

The marginal activity exhibited by only one oxirane, VII, and perhaps oxirane XI does not appear to support the hypothesis that the ethynyl group can act as a biological alkylating agent in these compounds. Further work will be conducted in this area, however, before final conclusions are made.

[1] The biological data were made available through the courtesy of Dr. Harry B. Wood, Jr., and Dr. John S. Driscoll, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health.

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▲ To whom inquiries should be directed.

Search for Potential Oral Hypoglycemic Agents: Synthesis and Activity of 2-(*N*-Alkylaminomethyl)indolizines

A. U. DE▲ and B. P. SAHA

Abstract □ Some 2-(*N*-alkylaminomethyl)indolizines were synthesized and biologically evaluated; however, they showed very weak oral hypoglycemic activity. One compound of this series possessed anti-Parkinson activity and another had marginal anti-inflammatory activity.

Keyphrases □ 2-(*N*-Alkylaminomethyl)indolizines—synthesized and screened as potential oral hypoglycemic agents □ Indolizines, 2-(*N*-alkylaminomethyl)—synthesized and screened as potential oral hypoglycemic agents □ Hypoglycemic agents, oral, potential—synthesis and screening of 2-(*N*-alkylaminomethyl)indolizines

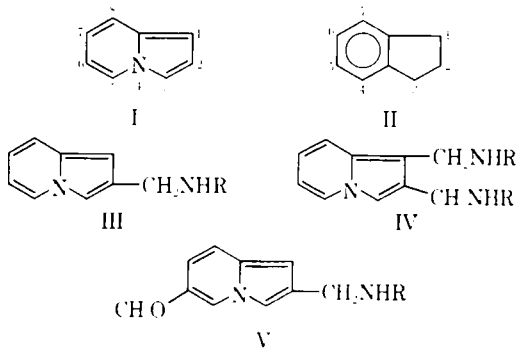
The alkaloids vincamine, vindoline, and vindolinine obtained from plants of the genus vinca, commonly known as periwinkles, have been reported to cause varying degrees of blood sugar lowering (1–4). All of these alkaloids are structurally different from the common hypoglycemic agents like sulfonylureas and biguanides and thus provide a new lead in this area (4).

DISCUSSION

The structures of these alkaloids consist of several ring systems like those of indolizine, quinolizine, indole, and quinoline. Since indolizine ring systems (I) are a rare occurrence in natural products, their presence in vinca alkaloids is of particular interest. It appears from the literature that no indolizine derivative has yet been tested as a potential oral hypoglycemic agent.

Indolizine derivatives have been reported to exhibit hypotensive, anti-inflammatory, central nervous system depressant, anticholinergic, antiedema, estrogenic, and anabolic activities (5–11). In addition, both the pyridine and the pyrrole ring systems, the structural components of the indolizine ring linked through the common nitrogen atom, have furnished various pharmacologically active compounds through their derivatives. Moreover, the indolizine ring system may be regarded as structurally equivalent to the hydrindene ring system (II) through isosteric replacement of one angular carbon atom of the hydrindene ring with a nitrogen atom. From the point of chemical reactivity, indolizine and hydrindene rings have one common feature: the 1- and 3-positions of both ring systems are active in their many derivatives (12–14). Presumably, these may combine with the active receptor site through these positions and evoke biological responses.

The significance of the hydrindene ring system for oral hypoglycemic activity (15) and various other activities (16, 17) has been already established. Earlier, the authors synthesized and biologically evaluated a few hydrindene derivatives as potential oral hypoglycemic agents; however, the results were not encouraging (18, 19). The interesting features of the indolizine ring system have shifted emphasis to indolizine derivatives as potential oral hypoglycemic agents. With this objective, the synthesis and biological evaluation of a few compounds (III, IV, and V) have been undertaken. These derivatives were chosen to keep the 1- and/or 3-positions free so as to retain their reactivity in the biological system.



In this article, only the synthesis and biological activity of 2-(*N*-alkylaminomethyl)indolizines (III) are described. The synthesis and activity of other indolizine derivatives (IV and V) will be described in a subsequent report.

Indolizine-2-carboxylic acid (III*a*) was prepared by the condensation of α-picoline with ethyl bromopyruvate according to the method of Borrows and Holland (20), who obtained a buff-colored product. But in this case, a greenish product of the same melting point as reported earlier (20) was obtained. All attempts to convert this acid to the corresponding acid chloride failed. As such, the acid was converted to the corresponding methyl indolizine-2-carboxylate (III*b*) by the reaction of diazomethane with a suspension of the acid (III*a*) in ether instead of dioxane (20). The ester was condensed with the respective amines to give 2-(*N*-alkylcarboxamido)indolizines (III*c*) and then reduced with lithium aluminum hydride to 2-(*N*-alkylaminomethyl)indolizines (III) in dry ether (Scheme I). On standing, the colors of the carboxamides (III*c*) and the amine (III) changed to dark brown. UV absorption characteristics are recorded in Table I.

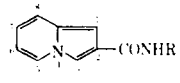
Table I—UV Absorption Characteristics

Compound	λ_{max}, nm. (in Alcohol)	ϵ_{molar}
III*a*	290, 300, 342	1972, 2173, 2124
III*b*	291, 301, 342	2358, 2668, 2581
III*c* (R = ethyl)	288, 300, 341	1406, 1438, 1363
III (R = ethyl)	284, 296, 346	2610, 2870, 1866

ACTIVITY

Of the four compounds synthesized, 2-(*N*-*n*-butylaminomethyl)-indolizine (III: R = *n*-butyl) and 2-(*N*-*n*-propylaminomethyl)-indolizine (III: R = *n*-propyl) exhibited one-fourth of the activity of tolbutamide at 250 mg./kg. body weight in normal, overnight-fasted rabbits. The blood sugar was estimated at 0, 2, and 4 hr. Because of this weak hypoglycemic acitivity, further investigations were suspended. However, 2-(*N*-ethylaminomethyl)indolizine (III: R = ethyl) demonstrated anti-Parkinson activity as determined by the oxotremorine model in mice. The activity was seen at doses that were very close to lethality. The minimum effective dose (MED) was 50 mg./kg. i.p., and the lethal dose (LD_{50}) in mice was 140 mg./kg.

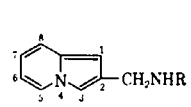
Table II—2-(*N*-Alkylcarboxamido)-indolizines (III*c*)



Compound	R	Melting Point	Boiling Point
1	Methyl[a]	178–180°	—
2	Ethyl[b]	112–114°	—
3	*n*-Propyl[b]	78–80°	195–197°/0.5 mm.
4	*n*-Butyl[b]	70–72°	179–181°/0.5 mm.

[a] Crystallized from dilute ethanol. [b] Crystallized from benzene–petroleum ether (b.p. 60–80°).

Table III—2-(*N*-Alkylaminomethyl)indolizines (III)



Compound	R	Boiling Point	Formula	Analysis, % Calc.	Found
1	Methyl	108–110°/0.6 mm.	$C_{10}H_{12}N_2$	C 75.00	74.65
				H 7.50	7.73
				N 17.50	17.26
2	Ethyl	100–102°/0.7 mm.	$C_{11}H_{14}N_2$	C 75.88	76.03
				H 8.05	8.37
				N 16.10	15.81
3	*n*-Propyl	123–125°/0.55 mm.	$C_{12}H_{16}N_2$	C 76.60	76.45
				H 8.51	8.82
				N 14.89	15.21
4	*n*-Butyl	140–142°/0.8 mm.	$C_{13}H_{18}N_2$	C 77.21	76.88
				H 8.91	9.09
				N 13.87	13.58

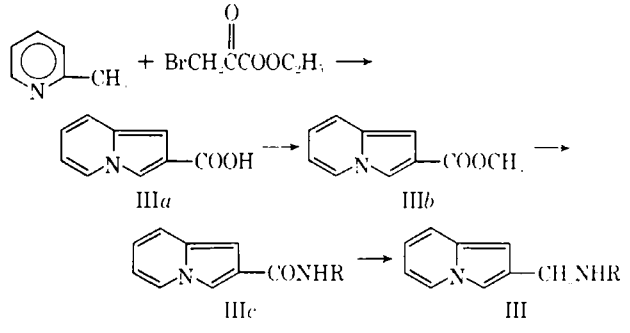
i.p. Thus, further studies were suspended. On the other hand, 2-(*N*-*n*-propylaminomethyl)indolizine (III: R = *n*-propyl) demonstrated marginal anti-inflammatory activity but only at rather large oral doses in rats. Due to this weak activity, further studies were not undertaken. All of these compounds showed no microbiological activity.

EXPERIMENTAL[1]

Methyl Indolizine-2-carboxylate (III*b*)—To a suspension of indolizine-2-carboxylic acid (III*a*, 10 g.) in dry ether (250 ml.), an ethereal solution of diazomethane was added in the usual way. The reaction was allowed to continue overnight, and the resulting solution was filtered. The solvent was removed when a yellowish product was obtained, which crystallized from dilute ethanol as colorless plates, m.p. 98–99°.

2-(*N*-Alkylcarboxamido)indolizines (III*c*)—Methyl indolizine-2-carboxylate (III*b*, 1 mole) and the appropriate alkylamine (1.5 moles) were heated in a hard glass, sealed tube on a water bath for 12–16 hr. After cooling, the reaction mass was poured into water, either filtered or extracted with benzene, and washed with water. The crude product was either crystallized from dilute ethanol or benzene–petroleum ether (b.p. 60–80°) or distilled under reduced pressure to yield white to pale-yellow solids (Table II).

2-(*N*-Alkylaminomethyl)indolizines (III)—2-(*N*-Alkylcarboxamido)indolizine (III*c*, 1 mole) was reduced with lithium aluminum hydride (0.6 mole) in sodium-dried ether for 16 hr. and worked up in the usual way. The crude amines were purified by distillation to yield colorless products (Table III).



Scheme I

[1] All melting points are uncorrected. Absorption characteristics were recorded on a Spectromom 201 spectrophotometer made in Hungary.

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Insect Repellency of Substituted *N,N*-Diethylbenzamidines

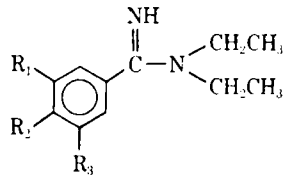
DANIEL D. GARNER* and LORRIN R. GARSON▲

Abstract □ The synthesis of *N,N*-diethylbenzamidine, *N,N*-diethyl-3-trifluoromethylbenzamidine, *N,N*-diethyl-4-trifluoromethylbenzamidine, and *N,N*-diethyl-3,5-di(trifluoromethyl)benzamidine is described; the compounds were designed to produce long-lasting insect repellent activity. The repellency of these agents on cloth against *Aedes aegypti* (L.) mosquitoes is reported.

Keyphrases □ *N,N*-Diethylbenzamidines, substituted—synthesized and evaluated as potential insect repellents □ Insect repellents, potential—synthesis and evaluation of substituted *N,N*-diethylbenzamidines □ Repellents, insect—synthesis and evaluation of substituted *N,N*-diethylbenzamidines

Associated with efforts to develop long-lasting insect repellents and prompted by the remarkable efficacy elicited by *N,N*-diethyl-*m*-toluamidine (I) (1), four additional amidines were synthesized and evaluated as repellents. Although Compound I exhibited a higher degree of repellency than the standard repellent 2-ethyl-1,3-hexanediol[1], a drying action on the skin of some volunteers was produced by this agent. Since this adverse effect may have been a manifestation of the strong basicity of I, the design of III–V included structural features to effect reduced basicity.

Volatility has long been associated with insect repellent activity (2); the lower activity of I compared to *N,N*-diethyl-*m*-toluamide could be due to the latter's higher volatility as indicated by the boiling points [*i.e.*, *N,N*-diethyl-*m*-toluamide, b.p. 111°/1.0 mm. (3); I, b.p. 148°/1.0 mm. (1)]. An inverse linear relationship was shown (4) between insect repellency and boiling point for ring-substituted *N,N*-diethylbenzamides. By assuming that this relationship is also applicable to the corresponding amidine series, II–IV were designed to obtain an agent with the optimal volatility associated with maximum effectiveness as a repellent. Also of significance is the fact that II is a nitrogen isostere of *N,N*-diethylbenzamide, an excellent repellent (5).



Compound	R_1	R_2	R_3
I	H	H	CH_3
II	H	H	H
III	H	H	CF_3
IV	H	CF_3	H
V	CF_3	H	CF_3

EXPERIMENTAL[2]

***N,N*-Diethylbenzamidine (II)**—By employing the method described for the preparation of I (1), 24.9 g. (0.540 mole) of absolute ethanol, 75 ml. of anhydrous ether, and 44.0 g. (0.427 mole) of benzonitrile were combined and cooled to −7°. After the addition of 37.8 g. (1.04 moles) of hydrogen chloride, the reaction mixture was stirred for 14 hr. Ethyl benzimidate hydrochloride (VI) was collected by filtration, washed with ether, and dried *in vacuo* for 2 hr., yielding 53.7 g. (67.7%). The hydrochloride (0.289 mole) was allowed to stand for 1 week with 63.2 g. (0.864 mole) of diethylamine in 200 ml. of absolute ethanol. Then the reaction mixture was distilled *in vacuo*, leaving a dark-brown residual liquid.

The crude material was dissolved in 500 ml. of 10% sodium bicarbonate and divided into two portions, and each was extracted with ether (4 × 50 ml.). The aqueous phase was brought to a higher pH (pH 11–13) by adding sodium hydroxide until a second layer appeared, at which time the basic solution was extracted with ether (4 × 100 ml.). The ether solutions from the second extraction were combined, dried over magnesium sulfate, and filtered; the ether was removed by distillation *in vacuo*, yielding a yellow residual liquid. The crude product was purified by distillation *in vacuo*, employing a short-path, microdistillation apparatus, yielding 20.7 g. (40.6%) of pure II, b.p. 62–63° (0.2 mm.); n_D^{25} 1.5322; IR (chloroform): 1575 cm.$^{-1}$ (C=N); UV (95% ethanol): 205 nm. (ε 14,100).

Anal.—Calc. for $C_{11}H_{16}N_2$: C, 74.96; H, 9.15; N, 15.89. Found: C, 75.07; H, 9.17; N, 15.81.

***N,N*-Diethyl-3-trifluoromethylbenzamidine (III)**—Ethyl 3-trifluoromethylbenzimidate hydrochloride was prepared in an analogous manner to VI, using 14.6 g. (0.317 mole) of absolute ethanol, 43.0 g. (0.251 mole) of *m*-trifluoromethylbenzonitrile, and 18.3 g. (0.502 mole) of hydrogen chloride, yielding 49.5 g. (77.7%) of the salt. The salt was allowed to react in a similar manner as described in the preparation of II, using 42.8 g. (0.585 mole) of diethylamine. The product was purified by distillation *in vacuo*, yielding 19.4 g. (40.8%) of colorless III, b.p. 66–68° (0.1–0.2 mm.); n_D^{25} 1.4814; IR (chloroform): 1572 cm.$^{-1}$ (C=N); UV (95% ethanol): 205 nm. (ε 14,500).

Anal.—Calc. for $C_{12}H_{15}F_3N_2$: C, 59.01; H, 6.19; F, 23.34; N, 11.47. Found: C, 59.22; H, 6.16; F, 23.23; N, 11.46.

***N,N*-Diethyl-4-trifluoromethylbenzamidine (IV)**—Ethyl 4-trifluoromethylbenzimidate hydrochloride was prepared in a similar manner to VI, using 17.0 g. (0.369 mole) of absolute ethanol, 50.0 g. (0.292 mole) of *p*-trifluoromethylbenzonitrile, and 23.5 g. (0.644 mole) of hydrogen chloride, yielding 65.4 g. (88.3%) of the benzimidate salt. Compound IV was prepared using 56.6 g. (0.774 mole) of diethylamine and the salt. The product was distilled *in vacuo*, yielding 23.6 g. (37.5%) of yellow-colored IV, b.p. 74–76° (0.05 mm.); n_D^{25}/1.4824; IR (chloroform): 1580 cm.$^{-1}$ (C=N); UV (95% ethanol): 208 nm. (ε 13,700).

Anal.—Calc. for $C_{12}H_{15}F_3N_2$: C, 59.01; H, 6.19; F, 23.34; N, 11.47. Found: C, 58.95; H, 6.24; F, 23.52; N, 11.41.

***N,N*-Diethyl-3,5-di(trifluoromethyl)benzamidine (V)**—In a comparable manner to VI, ethyl 3,5-di(trifluoromethyl)benzimidate hydrochloride was prepared using 12.1 g. (0.263 mole) of absolute ethanol, 50.0 g. (0.209 mole) of 3,5-di(trifluoromethyl)benzonitrile, and 15.0 g. (0.411 mole) of hydrogen chloride, yielding 66.1 g. (98.4%) of the salt. A mixture of 66.1 g. (0.205 mole) of the benzimidate salt, 200 ml. of isopropyl alcohol, and 46.5 g. (0.636 mole) of diethylamine was stirred at 75° for 5 days. Then the mixture was distilled *in vacuo*, leaving a red residual solid.

The crude material was shaken with water buffered at pH 7, the undissolved material was removed, and the aqueous phase was extracted with ether (3 × 100 ml.). The insoluble material was dissolved in 500 ml. of an aqueous sodium hydroxide solution (pH 12) and, after the appearance of a second layer following the addition of more sodium hydroxide, the basic solution was extracted with

[1] Rutgers 612.

[2] Boiling points are uncorrected. IR and UV spectra were obtained with the Perkin-Elmer models 137B and 202 spectrophotometers, respectively. Analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

ether (3 × 100 ml.). The ether solutions from the second extraction were combined, dried over magnesium sulfate, and filtered, and the ether was removed by distillation *in vacuo*, yielding a purple residual liquid. The crude product was purified by distillation *in vacuo*, employing a microdistillation apparatus (24-cm. Vigreux column), yielding 10.9 g. (17.0%) of pure V, b.p. 60 65° (0.01–0.02 mm.); n_D^{25} 1.4465; IR (chloroform): 1580 cm.$^{-1}$ (C=N); UV (95% ethanol): 208 nm. (ϵ 15,000).

Anal.—Calc. for $C_{13}H_{14}F_6N_2$: C, 50.00; H, 4.52; F, 36.51; N, 8.97. Found: C, 50.07; H, 4.57; F, 36.23; N, 8.76.

Evaluation of Repellency—To minimize contact with the skin, Compounds II–V were evaluated as repellents against *Aedes aegypti* (L.) mosquitoes employing a standard cloth method (6); *N,N*-diethyl-*m*-toluamide was included in the tests as a standard for comparison. One-third square foot of a cotton stocking was treated with 1 g. of the compound in solution (10% solution in a volatile solvent, usually acetone). Two hours after treatment, the stocking, on the arm of a human subject, was exposed for 1 min. in a cage of mosquitoes. If less than five mosquitoes bit the subject through the stocking, the test was repeated at 24 hr. and then weekly thereafter until five bites were received in 1 min.

RESULTS AND DISCUSSION

As tested, Compounds II–V were found to be effective on cloth against *Aedes aegypti* (L.) mosquitoes for 7 days as compared to *N,N*-diethyl-*m*-toluamide and *N,N*-diethylbenzamide, which are effective under the same conditions for more than 21 days. To our knowledge, Compounds I–V are the only amidines that have been evaluated as insect repellents. The effectiveness of these specific agents and the structural similarities between amidines and amides and imides, which were found (7) to be the largest single class of compounds effective for 5 hr. or more upon dermal application, indicate that amidines as a class possess considerable potential as insectifugal agents.

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Metabolism and Anticonvulsant Activity of Deuterated *N*-Demethyldiazepam

FRANCA MARCUCCI, EMILIO MUSSINI, PAOLA MARTELLI, AMALIA GUAITANI, and SILVIO GARATTINI▲

Abstract □ The substitution of the hydrogen at the C_3-position of *N*-demethyldiazepam with deuterium shortens the anticonvulsant activity in mice from 20 to 5 hr. This pharmacological effect may be partly explained by a decrease in the ability of deuterated *N*-demethyldiazepam to protect mice from pentylenetetrazol-induced convulsions in contrast to the effect exerted by the C_3-unlabeled analog. Furthermore, the weaker pharmacological action of deuterated *N*-demethyldiazepam may in part be due to the lesser accumulation of the hydroxylated metabolite, oxazepam, in the brain of mice treated with deuterated *N*-demethyldiazepam than with the unlabeled analog. The lesser accumulation of oxazepam from deuterated *N*-demethyldiazepam than from the unlabeled analog is due to a reduced C_3-hydroxylation of this compound by liver microsomal enzymes, as shown by experiments *in vitro*.

Keyphrases □ Diazepam, *N*-demethyl, deuterated—metabolism and anticonvulsant activity, mice □ *N*-Demethyldiazepam, deuterated—metabolism and anticonvulsant activity, mice □ Anticonvulsant activity—deuterated *N*-demethyldiazepam, metabolism, mice □ Oxazepam—accumulation after administration of deuterated *N*-demethyldiazepam, mice

N-Demethyldiazepam, a major metabolite of diazepam, possesses pharmacological properties similar to the parent compound (1, 2). In the liver microsomal system, it undergoes a process of hydroxylation in position C_3 with the formation of oxazepam (3). The administration of *N*-demethyldiazepam in mice results in an accumulation of oxazepam in brain (4) where it is retained for several hours. Because oxazepam exerts

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Abstract □ The substitution of the hydrogen at the C_3-position of *N*-demethyldiazepam with deuterium shortens the anticonvulsant activity in mice from 20 to 5 hr. This pharmacological effect may be partly explained by a decrease in the ability of deuterated *N*-demethyldiazepam to protect mice from pentylenetetrazol-induced convulsions in contrast to the effect exerted by the C_3-unlabeled analog. Furthermore, the weaker pharmacological action of deuterated *N*-demethyldiazepam may in part be due to the lesser accumulation of the hydroxylated metabolite, oxazepam, in the brain of mice treated with deuterated *N*-demethyldiazepam than with the unlabeled analog. The lesser accumulation of oxazepam from deuterated *N*-demethyldiazepam than from the unlabeled analog is due to a reduced C_3-hydroxylation of this compound by liver microsomal enzymes, as shown by experiments *in vitro*.

Keyphrases □ Diazepam, *N*-demethyl, deuterated—metabolism and anticonvulsant activity, mice □ *N*-Demethyldiazepam, deuterated—metabolism and anticonvulsant activity, mice □ Anticonvulsant activity—deuterated *N*-demethyldiazepam, metabolism, mice □ Oxazepam—accumulation after administration of deuterated *N*-demethyldiazepam, mice

N-Demethyldiazepam, a major metabolite of diazepam, possesses pharmacological properties similar to the parent compound (1, 2). In the liver microsomal system, it undergoes a process of hydroxylation in position C_3 with the formation of oxazepam (3). The administration of *N*-demethyldiazepam in mice results in an accumulation of oxazepam in brain (4) where it is retained for several hours. Because oxazepam exerts

Table I—Relative Rates of Hydroxylation of Labeled and Unlabeled *N*-Demethyldiazepam

Substrate Added, *N*-Demethyldiazepam, mcg.	Substrate Recovered Unchanged[a], mcg.	Oxazepam Formed[a], mcg.
Unlabeled		
30	28 ± 2	0.5 ± 0.02
50	41 ± 3	2.7 ± 0.08
100	87 ± 3	6.4 ± 0.19
300	270 ± 5	24.2 ± 0.05
Labeled		
30	25 ± 2	—
50	44 ± 2	—
100	88 ± 3	—
300	247 ± 6	3.2 ± 0.09

[a] Results are expressed as the mean ± *SD* (n = 4).

an anticonvulsant activity comparable to that of *N*-demethyldiazepam, the consequence of C_3-hydroxylation in mice is a prolonged action of the administered drug due to accumulation of oxazepam in the brain.

Several drugs are oxidized by liver microsomal enzymes at a specific carbon–hydrogen bond. If breaking of the carbon–hydrogen bond is rate limiting in the oxidative process, substitution of hydrogen by deuterium at the suspected site of oxidation may result in a decreased rate of oxidation.

The purpose of this study was to evaluate the consequence of such C_3-substitution in *N*-demethyldiazepam on the metabolism and pharmacological activity in mice.

EXPERIMENTAL

The deuterated *N*-demethyldiazepam[1] (7-chloro-1*H*-3,3-dideutero-5-phenyl-1,4-benzodiazepin-2-one) had 84% of dideuterated molecules. Male, albino Swiss mice, weighing 20–25 g., were used in all experiments.

Drug Administration—*N*-Demethyldiazepam and deuterated *N*-demethyldiazepam were administered by the tail at 5 mg./kg. i.v. Drugs were dissolved in a solvent containing propylene glycol–glycofurol–benzyl alcohol–water (30:30:2:48).

The ED_{50} was determined using male, albino Swiss mice and is the dose (in micrograms per kilogram) protecting 50% of the mice from the mortality induced by phenylenetetrazol at 120 mg./kg. i.p. The method of Litchfield and Wilcoxon (5) was used for calculating the confidence limits. The ED_{50} for *N*-demethyldiazepam was 198 mcg./kg. (confidence limits of 177–221 mcg./kg.), and for deuterated *N*-demethyldiazepam it was 288 mcg./kg. (confidence limits of 252–329 mcg./kg.). The ED_{50} was calculated from data obtained 5 min. between drug and pentylenetetrazol administration, when the injected drug, according to previous observations (4), is practically unmetabolized.

Chemical Determinations—The preparation of brain extracts was made according to the method previously described (6). GLC analyses were carried out by using a gas chromatograph[2] equipped with a ^{63}Ni electron-capture detector (voltage of 42 v.). The stationary phase was 3% OV-17 on Gas Chrom Q (100–120 mesh) packed into a 1-m. glass column (2 mm. i.d., 4 mm. o.d.). The flow rate of the carrier gas (nitrogen) was 33 ml./min., and the column temperature was 245°.

Preparations of Liver Microsomes—Albino Swiss mice were given 40 mg./kg. i.p. of phenobarbital in 0.9% NaCl twice a day for 4 days. This treatment is known to increase the hydroxylation of *N*-demethyldiazepam by liver microsomal enzymes (7). After the last injection, the animals were deprived of food for 24 hr. and then sacrificed by decapitation. The livers were removed, chilled in ice, and homogenized in ice-cold 1.15% potassium chloride solution (1:4 w/v). The homogenate was centrifuged[3] at 105,000×*g* for 1 hr. The precipitated liver microsomes were suspended in 1.15% potassium chloride solution. Protein determinations were performed according to Lowry *et al.* (8).

Table II—Brain Levels of *N*-Demethyldiazepam and Its Metabolite, Oxazepam, after Administration (5 mg./kg. i.v.) of Unlabeled and Labeled *N*-Demethyldiazepam to Mice

N-Demethyldiazepam	Hours after Treatment	Benzodiazepine Brain Level, mcg./g. ± *SE*		Mice Protected against Pentylenetetrazol (120 mg./kg. i.p.), %
		N-Demethyldiazepam	Oxazepam	
Unlabeled	1	4.52 ± 0.18	2.37 ± 0.02	100
Labeled	1	5.66 ± 0.25	0.21 ± 0.01	100
Unlabeled	3	0.62 ± 0.01	3.93 ± 0.07	100
Labeled	3	3.50 ± 0.20	0.38 ± 0.01	66
Unlabeled	10	<0.002	0.75 ± 0.02	100
Labeled	10	0.74 ± 0.04	0.09 ± 0.007	16
Unlabeled	20	<0.002	0.09 ± 0.01	33
Labeled	20	0.09 ± 0.01	0.04 ± 0.02	0

Incubation *In Vitro*—The incubation mixture, as previously reported (3), consisted of 2.5 ml. of microsomal suspension equivalent to 1 g. of liver, NADP (1.5 μmoles), glucose 6-phosphate (50 μmoles), glucose 6-phosphate dehydrogenase (0.5 unit), magnesium chloride (35 μmoles), nicotinamide (50 μmoles), 1.4 ml. of 0.2 *M* phosphate buffer (pH 7.4), 300 mcg. of deuterated or nondeuterated *N*-demethyldiazepam, 0.45 ml. of 1.15% potassium chloride, and water to obtain a final volume of 5 ml. The mixtures were incubated in a metabolic shaker[4] at 37° under air for 30 min. Recovery studies of *N*-demethyldiazepam and oxazepam, both deuterated and nondeuterated, were performed with microsomal boiled mixtures containing ingredients identical to those in the experimental samples.

Determination of Substrates and Metabolites—At the end of the incubation time, 5 ml. of the mixture was extracted twice with 10 ml. of fresh peroxide-free ether. The combined ether extracts were dried, redissolved in a suitable volume of acetonitrile, and then submitted to the GLC procedure previously described (6). This procedure allows the simultaneous determination of the formed metabolite and the disappearance of the added substrate.

RESULTS

Table I shows the relative rate of hydroxylation *in vitro* of *N*-demethyldiazepam bearing hydrogen or deuterium in position 3. It is evident that more oxazepam is formed from *N*-demethyldiazepam than from the deuterated analog. However, the recovery of the added substrate is incomplete with the deuterated *N*-demethyldiazepam, suggesting alternative metabolic pathways or a tighter binding to liver microsomes.

Table II summarizes the findings obtained *in vivo*. Mice injected with deuterated *N*-demethyldiazepam showed higher levels of this compound in the brain compared to animals receiving the hydrogenated compound. Conversely, the brain levels of oxazepam, the hydroxylated metabolite, were much lower with deuterated than with hydrogenated *N*-demethyldiazepam. Because of the lower levels of brain oxazepam, the duration of the antipentylenetetrazol activity exerted by the deuterated compound is much shorter than for the C_3-unlabeled drug.

DISCUSSION

The present study indicates that the substitution of the hydrogen at the C_3-position of *N*-demethyldiazepam with deuterium resulted in a shortening of the anticonvulsant activity in mice. This finding

[1] Received as a gift from Prof. G. C. Jommi, Laboratory of Organic Chemistry, Department of Sciences, Milan University, Milan, Italy.
[2] Model GI, Carlo Erba, Milan, Italy.
[3] Rotor 40 ft., Beckman model L ultracentrifuge.
[4] Dubnoff.

may be partly explained by the fact that deuterated *N*-demethyldiazepam is less effective than the hydrogenated analog as an inhibitor of the mortality induced by pentylenetetrazol in mice. The ED_{50} was 198 mcg./kg. for *N*-demethyldiazepam and 288 mcg./kg. for the deuterated analog.

However, there is also a decreased accumulation of the hydroxylated metabolite, oxazepam, in the brain of mice treated with the deuterated analog compared to the C_3-unlabeled *N*-demethyldiazepam. This finding is in agreement with previous studies indicating that the long duration of action of diazepam and *N*-demethyldiazepam in mice is related to the formation and persistence of brain oxazepam (9). The lack of accumulation of oxazepam from C_3-deuterated *N*-demethyldiazepam is due to a reduced C_3-hydroxylation of this benzodiazepine *in vitro* compared to the C_3-unlabeled compound, as shown by the experiments utilizing liver microsomal enzymes.

The deuterated *N*-demethyldiazepam may be less hydroxylated than the C_3-unlabeled analog for one of the following reasons: (*a*) slower rate of cleavage of the carbon–deuterium bond, (*b*) lower affinity for the active site of the enzymes involved in the hydroxylation, and (*c*) more stable complex with the enzyme. While these possibilities are open to experimental investigations, it remains established that the presence of oxazepam in the brain is the main factor accounting for the prolonged antipentylenetetrazol effect exerted by diazepam or *N*-demethyldiazepam in mice.

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COMMUNICATIONS

Effect of Hydroxy Group on Coacervate Formation by Sodium Hydroxybenzoates with Benzalkonium Chloride

Keyphrases ☐ Coacervate formation, sodium hydroxybenzoates and benzalkonium chloride—effect of position of hydroxy group ☐ Hydroxybenzoates—coacervate formation with benzalkonium chloride, phase transition diagrams, effect of hydroxy position

Sir:

Shah *et al.* (1) reported coacervate formation by sodium salicylate (sodium *o*-hydroxybenzoate) with benzalkonium chloride.

The phase transition diagram of this system (Fig. 1) illustrates two main regions: a biphasic coacervate system (I) and a monophasic equilibrium solution (II). The biphasic coacervate system shows two distinct regions. In one region (B) the coacervate phase has a higher density than the equilibrium liquid and it settles to the bottom of the container, and in the other region (T), the coacervate phase is lighter than the equilibrium liquid and floats on the top of the liquid.

This communication reports the effect of the presence and position of the hydroxy group on coacervate formation between hydroxybenzoates and benzalkonium chloride.

The coacervate systems are obtained by mixing various concentrations of sodium *m*-hydroxybenzoate, sodium *p*-hydroxybenzoate, and sodium benzoate with benzalkonium chloride in water. Figure 1 is the superimposed phase transition diagram of sodium *o*-, *m*-, and *p*-hydroxybenzoates. The phase regions for the hydroxybenzoates are equivalent, differing only in the positions of the boundary lines, and are as identified previously (1). Figure 2 shows a phase transition diagram of the sodium benzoate and benzalkonium chloride coacervate system.

The sodium benzoate system (Fig. 2) shows a lighter coacervate phase (T) throughout the biphasic coacervate region. The systems near the upper and lower transition lines showed the property of birefringence during flow. The coacervate phase in all three systems was observed to be isotropic under examination with a polarizing microscope.

From our previous (1) and present data, it is our belief that the density of the coacervate phase is a function of the molecular weight of the anionic electrolyte, the amount of this electrolyte associated with the micellar aggregation, and the size of the micelles.

may be partly explained by the fact that deuterated *N*-demethyldiazepam is less effective than the hydrogenated analog as an inhibitor of the mortality induced by pentylenetetrazol in mice. The ED_{50} was 198 mcg./kg. for *N*-demethyldiazepam and 288 mcg./kg. for the deuterated analog.

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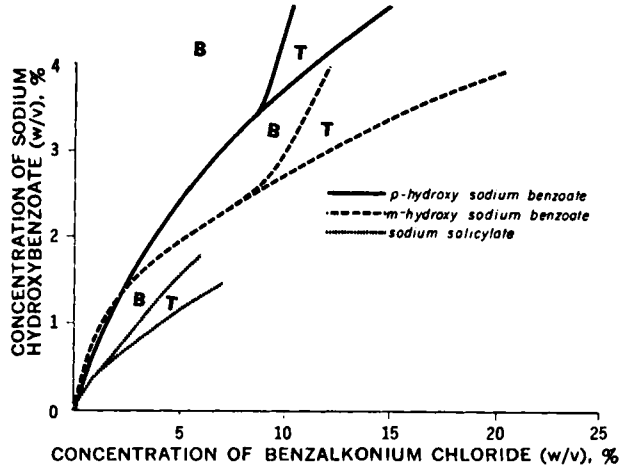

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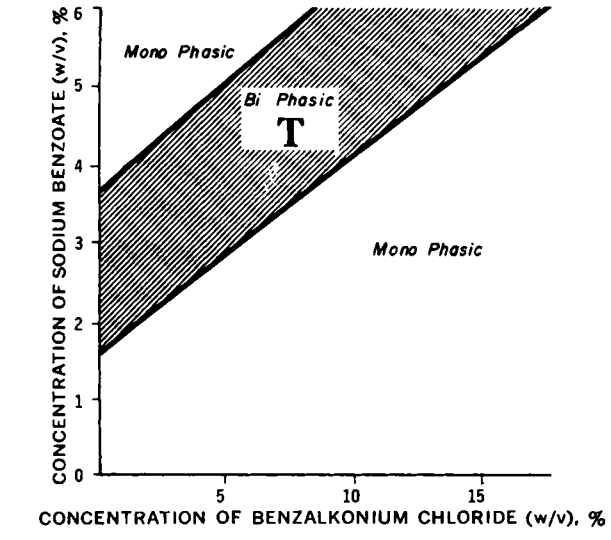

Figure 2—*Phase transition diagram of sodium benzoate–benzalkonium chloride coacervate system at 24°. Dark area [T] represents the region of coacervate formation at the top, and light area represents the region of no coacervate formation.*

In the case of the sodium hydroxybenzoate and benzalkonium chloride coacervate system, the denser coacervate phase, on heating, becomes lighter and moves to the top of the equilibrium liquid.

The increase in benzalkonium chloride concentration at constant salt concentration results in either a lighter coacervate phase or a monophasic solution (Fig. 1). Since benzalkonium chloride is lighter than water, an increase in micellar aggregation results in a lighter coacervate phase and hence the coacervate moves to the top of the equilibrium liquid. Figure 1 also shows the similarity in patterns of phase transition diagrams of all three salts of hydroxybenzoate, and they follow the same order as the degree of solubility (*o*-hydroxybenzoate being least soluble and *p*-hydroxybenzoate being most soluble). The sodium benzoate system did not form a denser coacervate phase; this is probably because of the structural changes in the micelle size and/or shape resulting from the absence of a hydroxyl group.

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Cholinergic Activity of Pilocarpine Methiodide: A Reinvestigation

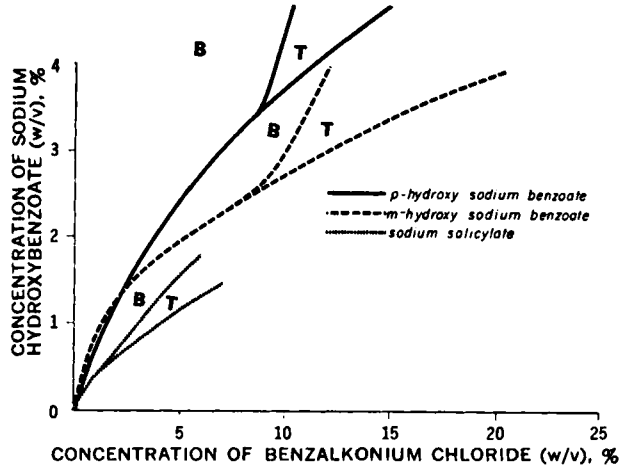
Keyphrases ☐ Pilocarpine methiodide—cholinergic activity reinvestigated ☐ Cholinergic activity—pilocarpine methiodide ☐ Quaternary salts—cholinergic activity of pilocarpine methiodide

Sir:

While most compounds possessing cholinergic activity are quaternary amine derivatives, several tertiary amines are known to possess significant cholinergic activity. Pilocarpine is perhaps the most widely known example of a tertiary amine derivative possessing therapeutically useful cholinergic activity. The cholinergic activity, or lack thereof, of the quaternary methiodide salt of pilocarpine has been a point of interest for some time. Wojciechowski and Ecanow (1, 2) prepared a series of quaternary salts of pilocarpine and later reported (3) that pilocarpine methiodide lacks muscarinic activity. Hanin *et al.* (4) confirmed this report while presenting the correct chemical structure of the methiodide.

During studies designed to clarify the structural and conformational requirements for cholinergic activity of pilocarpine (5, 6), we examined the effects of pilocarpine methiodide on the ileum of the guinea pig. We confirmed the earlier reports that the quaternary salt lacks muscarinic activity but, more significantly, observed (5) that this salt possesses the ability to antagonize the muscarinic effects of acetylcholine. Subsequent to our observation, Ben Bassat *et al.* (7) reported the cholinergic antagonist activities of a large number of quaternary salts of pilocarpine but, curiously, neither reported any data regarding the methiodide salt nor mentioned previous studies on this compound. In view of these facts, we feel it of importance that we report our observations.

The methiodide of pilocarpine was prepared from pilocarpine in the standard manner, and the physical data (elemental analyses, IR and NMR data, and specific rotation) were consistent with the earlier reports. Antagonism of the muscarinic actions of acetylcholine



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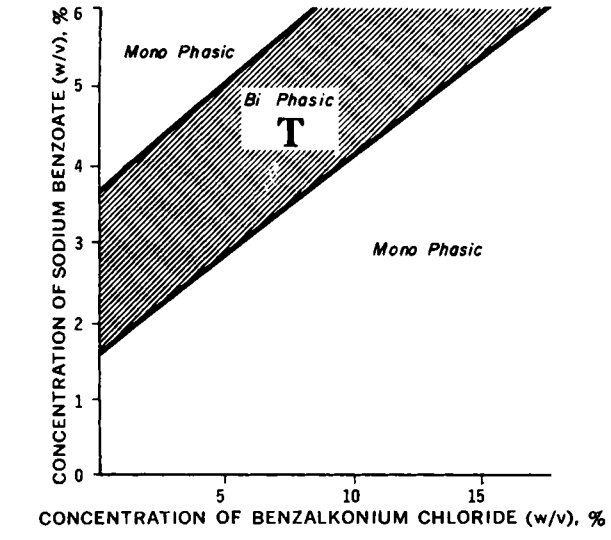

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on the guinea pig ileum was measured by known procedures (8). A pA_2 value (9) of 2.2 was determined. While the degree of antagonism observed is much weaker than that observed for atropine [pA_2 = 9.0 (10)], it is significant that these effects have not been previously reported.

The effects of pilocarpine methiodide on eel acetylcholinesterase were studied[1], and weak, uncompetitive inhibition was observed ($K_i = 1.64 \times 10^{-4}$).

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[1] By G. Cocolas, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27514

Binding of Sulfonylureas to Serum Albumin

Keyphrases ☐ Protein binding, human and bovine serum albumin—sulfonylureas, dynamic dialysis technique ☐ Sulfonylureas—binding to human and bovine serum albumin, dynamic dialysis technique ☐ Drug–protein binding—sulfonylureas ☐ Dialysis, dynamic—used to study sulfonylurea binding to serum albumin

Sir:

A recent paper (1) concerned the binding of the sulfonylureas, tolbutamide, chlorpropamide, and acetohexamide, to serum proteins. We have studied the binding of tolbutamide and chlorpropamide to serum albumin and find that there are substantial differences between our results and those of Judis (1). In this communication we present some results for comparison and attempt to reconcile the differences.

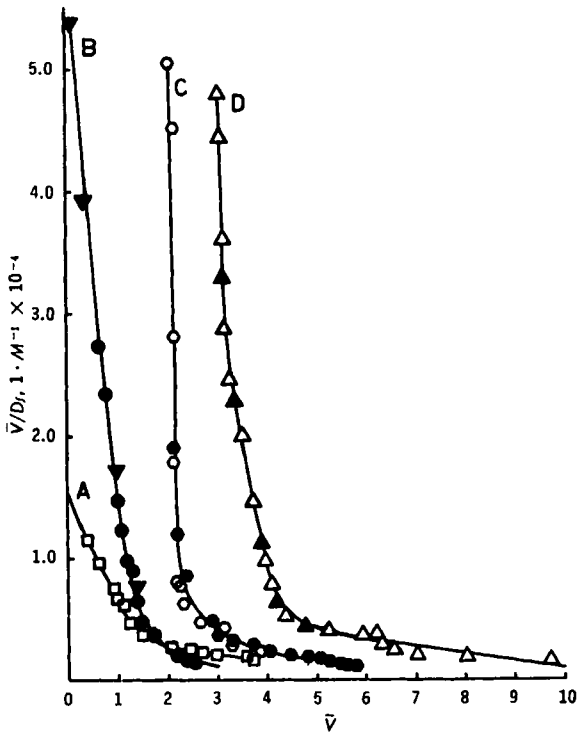

Figure 1—*Scatchard plots for the binding of tolbutamide to serum albumin in 0.067 M buffer at pH 7.4 and 37°. Key: open symbols, 1% albumin; closed symbols, 2% albumin; A, 1% human serum albumin in tromethamine buffer; B, 2% human serum albumin in tromethamine buffer; ●, ▼, Judis' (1) results (40°); C, human serum albumin in phosphate buffer; and D, bovine serum albumin in phosphate buffer. All points are experimental while the solid lines were computed from the binding parameters.*

The drug–protein interaction was characterized using a dynamic dialysis technique. The method and apparatus were based on those described by Meyer and Guttman (2). A concentration of 1 or 2% serum albumin was used, buffered to pH 7.4 with 0.067 *M* phosphate buffer. The volume and frequency of sampling were such that the concentration of drug in the outer compartment never exceeded 5% of the free concentration in the protein compartment to ensure that dialysis proceeded under sink conditions.

Tolbutamide was estimated by two separate methods: (*a*) in buffer, by direct UV spectrophotometry at 228 nm., and (*b*) in solutions containing protein and/or other interfering materials, by a modification of the colorimetric procedure of Alessandro *et al.* (3). Chlorpropamide was estimated spectrophotometrically at 231 nm.

The decline in drug concentration, D_t, within the dialysis bag was followed as a function of time. From a knowledge of the intrinsic dialytic rate constant and the instantaneous dialysis rate at any time, t, the free concentration in the protein compartment was estimated. The instantaneous rate of dialysis was obtained by fitting the plot of D_t *versus* time to an equation of the form:

$$D_t = D_t^0 + at + bt^2 + ct^3 + dt^4 \ldots \quad \text{(Eq. 1)}$$

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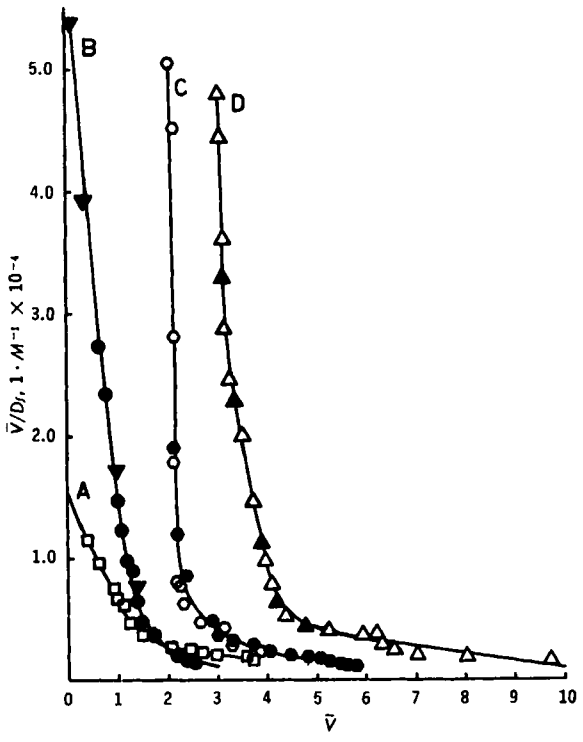

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The drug–protein interaction was characterized using a dynamic dialysis technique. The method and apparatus were based on those described by Meyer and Guttman (2). A concentration of 1 or 2% serum albumin was used, buffered to pH 7.4 with 0.067 *M* phosphate buffer. The volume and frequency of sampling were such that the concentration of drug in the outer compartment never exceeded 5% of the free concentration in the protein compartment to ensure that dialysis proceeded under sink conditions.

Tolbutamide was estimated by two separate methods: (*a*) in buffer, by direct UV spectrophotometry at 228 nm., and (*b*) in solutions containing protein and/or other interfering materials, by a modification of the colorimetric procedure of Alessandro *et al.* (3). Chlorpropamide was estimated spectrophotometrically at 231 nm.

The decline in drug concentration, D_t, within the dialysis bag was followed as a function of time. From a knowledge of the intrinsic dialytic rate constant and the instantaneous dialysis rate at any time, t, the free concentration in the protein compartment was estimated. The instantaneous rate of dialysis was obtained by fitting the plot of D_t *versus* time to an equation of the form:

$$D_t = D_t^0 + at + bt^2 + ct^3 + dt^4 \ldots \quad \text{(Eq. 1)}$$

Table I—Binding Parameters for the Interaction of Chlorpropamide and Tolbutamide with Human and Bovine Serum Albumin at pH 7.4 and 37° in 0.067 *M* Buffer

Drug	Protein	Buffer	n_1	$k_1 \times 10^{-4}$, l. mole^{-1}	n_2	$k_2 \times 10^{-2}$, l. mole^{-1}
Tolbutamide	Human serum albumin, 1 and 2%	Phosphate	2.27	21.86	8.21	1.71
	Human serum albumin, 1%	Tromethamine	1.42	0.97	8.81	1.85
	Human serum albumin, 2%	Tromethamine	1.20	4.95	8.29	1.45
	Human serum albumin, 2%	Tromethamine	1.36[a]	4.06[a]	—	—
	Bovine serum albumin, 1%	Phosphate	2.98	24.82	8.12	3.39
Chlorpropamide	Human serum albumin, 1%	Phosphate	2.19	4.51	8.22	1.71
	Human serum albumin, 2%	Tromethamine	1.64[a]	1.09[a]	—	—
	Bovine serum albumin, 1 and 2%	Phosphate	1.94	4.67	8.93	4.14

[a] Values calculated by Judis (1) (40°).

where D_t^0 is the initial concentration of drug in the protein solution and *a*, *b*, *c*, and *d* are constants. This was achieved by treating the data by polynomial regression to produce four normal equations (4), which were solved by using a program for the solution of a symmetrical matrix and a programmable calculator[1]. Differentiation of Eq. 1 yields the instantaneous rate.

Data for the binding of tolbutamide to both human and bovine serum albumin are shown as Scatchard plots in Fig. 1. The results of Judis (1) determined by equilibrium dialysis and in the presence of tromethamine buffer are included for comparison. It is apparent that the latter results indicate a much weaker interaction than our own. In an attempt to reconcile these differences, binding experiments were repeated using 0.067 *M* tromethamine buffer in place of phosphate in both 1 and 2% human serum albumin. These data are also included in Fig. 1, and our results in 2% human serum albumin are in good agreement with those reported (1). However, these data indicate a much weaker interaction than occurs in phosphate buffer. Furthermore, the degree of interaction in tromethamine buffer shows a marked dependence on the human serum albumin concentration, as evidenced by the large difference between the plots for 1 and 2% protein. No such dependence was observed using phosphate buffer with either human or bovine serum albumin (Fig. 1). This dependence implies that the components of tromethamine buffer itself interact with the protein and compete with tolbutamide for binding sites.

If potentially competitive species are present in the buffer, one might expect quite strong competition because of the high molar concentration of buffer relative to that of tolbutamide. Scatchard plots for the binding of chlorpropamide to human and bovine serum albumin (Fig. 2) show a similar effect. Again the degree of interaction is considerably less in tromethamine buffer than in phosphate.

The Scatchard plots are not linear but curved. Such curvature is generally considered to be indicative of binding to more than one class of sites (5). The curvature results from the combination of two or more linear plots representing the binding to each class of sites. In such cases, simple extrapolation or evaluation of the slope from the linear portion of the curve may give inaccurate estimates of the binding constant for all classes of sites, unless the association constants for the various classes are very widely separated. These aspects have been overlooked by many authors, and linear extrapolation is probably still the means most frequently used to determine binding constants.

Hart (6) reported a mathematical treatment for the estimation of binding constants from curved Scatchard plots. By using Hart's treatment and a multiple linear regression analysis (4) and program similar to that described here, the experimental data were fitted to a model for two classes of sites. Binding parameters for the various systems are shown in Table I. In phosphate buffer, the association constant (k_1) for the binding of tolbutamide to the primary sites (n_1) on human serum

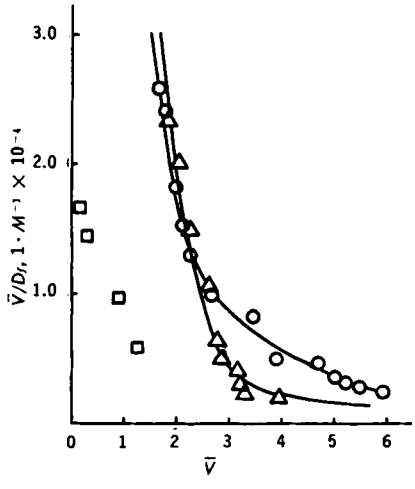

Figure 2—*Scatchard plots for the binding of chlorpropamide to serum albumin in 0.067* M *buffer at pH 7.4 and 37°. Key: △, 1% human serum albumin in phosphate buffer; □, Judis' (1) results for 2% human serum albumin in tromethamine buffer (40°); and ○, 1% bovine serum albumin in phosphate buffer. All points are experimental while the solid lines were computed from the binding parameters.*

[1] Diehl Combitron S.

albumin was four times greater than that using tromethamine buffer and 2% human serum albumin and about 20 times that determined for tromethamine and 1% human serum albumin. Furthermore, the number of sites in the first class (n_1) in phosphate buffer was substantially greater than in tromethamine. In the second class of sites, the association constant (k_2) and the number of sites (n_2) did not appear to show a strong dependence on the buffer used or the protein concentration. This suggests that tromethamine buffer did not interfere with binding in the second class and that binding to these sites possibly occurs by a less specific mechanism.

A comparison of the binding parameters obtained by Judis (1) and ourselves for the binding of tolbutamide to 2% human serum albumin in tromethamine buffer demonstrates the effect of neglecting the existence of the second class of sites. Although both sets of data are in good agreement on the Scatchard plot (Fig. 1), estimation of the primary association constant (k_1) from the slope of the linear portion of the plot does not correct for the slope contribution due to the second class of sites. This leads to an underestimate of the association constant on the order of 20% or 9000 l. mole^{-1} and a larger number of primary binding sites.

In the case of chlorpropamide, determination of binding in the presence of tromethamine buffer leads to a reduction in the number of binding sites and a fourfold reduction in the association constant relative to values obtained in phosphate buffer. Thus, as with tolbutamide, tromethamine strongly suppresses the binding.

These data illustrate the effects that buffers and other materials may have on the extent of protein binding *in vitro*. In such studies it is important to characterize the binding at several buffer or protein concentrations to test that the observed binding is a real effect rather than an artifact dependent on the particular buffer used. This also applies to salts which may be added to suppress the Donnan effect in equilibrium dialysis experiments. These considerations are also relevant to competitive binding studies. When the buffer system itself acts as a competitor and is present in high concentrations relative to the other competitors, the significance of the results is somewhat difficult to assess.

Judis (1) used two graphical methods (7, 8) to present his data, and there is excellent agreement between them. However, this is to be expected since the two methods are closely related. The plot used by Sandberg *et al.* (7) does not normalize the concentrations of bound small molecules for variations in total protein concentration and is for use chiefly when the protein concentration is not precisely known. The method used by Eichman *et al.* (8) is the well-known Scatchard plot, and in this case the results are normalized for variations in protein concentration. The only difference between the two is that, in the method of Sandberg *et al.*, the intercepts on the ordinate and abscissa are nkP_t and nP_t, respectively, where P_t is the concentration of protein. The value of n cannot be estimated without a knowledge of the protein concentration. In all other respects, both plots are essentially mathematically identical. Thus, any variation occurring in the value of n or k must only be the result of the errors expected in deriving information from graphical data.

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Binding of Sulfonylureas to Serum Albumin: A Response

Keyphrases ☐ Sulfonylureas—binding to serum albumin ☐ Protein binding—sulfonylureas to serum albumin ☐ Binding, protein—sulfonylureas to serum albumin ☐ Dialysis, equilibrium and dynamic—explanation of differences found studying sulfonylurea binding to serum albumin

Sir:

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Figure 2 (1) is very difficult to interpret as presenting conflicting data because it represents data of experiments using 1% human serum albumin in phosphate and 1% bovine serum albumin in phosphate while only 2% human serum albumin was used in my work. In the experiments (2), the sulfonylureas usually were bound to the extent of 80% or higher at all concentrations of sulfonylurea used with the exception of the highest ($81–84 \times 10^{-6}$ mole) at which 40–60% binding was found, which is a rather substantial interaction.

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Importance of Considering Variables when Using Magnetic Basket Dissolution Apparatus

Keyphrases □ Magnetic basket dissolution apparatus—effect of basket mesh size and revolution speed □ Dissolution apparatus, magnetic basket—effect of basket mesh size and revolution speed

Sir:

In several recent articles (1, 2), the versatility and adaptability of the magnetic basket were demonstrated. This apparatus was initially developed to yield reproducible dissolution profiles for capsules. The adaptability of the magnetic basket apparatus to tablet dissolution was also discussed (2). By using several sets of specially formulated tablets as the control, the magnetic basket was shown to differentiate between the common tablet parameters of hardness, particle size, and formulation changes, thereby illustrating its possible use in quality control. However, to use the concept of the magnetic basket as a means of correlating *in vitro* dissolution with the *in vivo* performance of a drug, further adaptability of the basket must be considered. Since the above-mentioned tablet parameters are not appreciably changed and remain constant after initial clinical testing demonstrates the effectiveness of the dosage form, the dissolution system must be able to show further versatility in its ability to provide an *in vitro* dissolution profile which approximates the *in vivo* behavior of the drug. Adjustment of the *in vitro* conditions, such as propeller height and revolutions per minute, or a change in the mesh or size of the basket

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would seem to be the means of achieving this approximation.

To test this hypothesis partially, two new magnetic baskets were built. The first conformed to the original specifications (1) but was made of 16-mesh instead of the original 8-mesh stainless steel wire. The second basket was constructed with 8-mesh wire and had larger dimensions: an inner diameter of 12 mm. and a length of 38 mm. instead of the 11 × 25-mm. dimensions of the original basket.

A tablet and capsule were selected to be tested. Pentobarbital tablets were prepared in our laboratories, which allowed control of the ingredient and manufacturing variables. Commercially prepared sodium butabarbital capsules were selected since a previous publication (1) showed that capsules manufactured in an automated manner show less variance than hand-packed capsules.

The pentobarbital tablets were tested in the three different size baskets and showed no clogging of the pores in any case. At least five tablets of the same formulation and hardness were run in each basket. The T_{50} percent values increased in the expected order as the dimensional and pore size of the baskets decreased. The T_{50} values of 10, 14, and 21 min. were found for the bigger basket, regular basket, and smaller pore basket, respectively. In varying the revolutions per minute of the stirring propeller from 60 through 150 for the pentobarbital tablets in the regular basket, a distinct difference can be seen. The T_{50} at 150 and 120 r.p.m. is very close at an average of 2.5 and 3.25 min. Decreasing the revolutions per minute to 90 and to 60 shows a significant reduction and difference, with the T_{50} equal to 7.5 and 14 min., respectively.

A comparison of the dissolution of the sodium butabarbital capsules in the three baskets shows little difference between the bigger and regular baskets, with the T_{50} equal to 26 and 29 min., respectively. The smaller pore basket did show an increase in the T_{50} to 38 min. The difference in the T_{50} in the regular basket as a function of revolutions per minute was small at 150 and 120, producing average times of 15 and 16 min., respectively. Decreases in propeller speed to 90 and 60 r.p.m. showed T_{50}'s of 21 and 30 min., respectively.

In conclusion, the adjustment of basket pore size and propeller revolutions per minute can produce significantly different dissolution profiles, which should be considered when comparing *in vitro* dissolution results from the magnetic basket in quality control or as a means of correlating *in vitro–in vivo* data.

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(2) T. E. Needham, R. E. Shepherd, and L. A. Luzzi, *ibid.*, **62**, 470(1973).

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BOOKS

REVIEWS

The Prostaglandins, Volume I. Edited by Peter W. Ramwell. Plenum, New York, NY 10011, 1973. 526 pp. 17 × 25.5 cm. Price $22.50.

Respected scientists have collaborated in this treatise to report on the present status of prostaglandin data and hypotheses in chemistry, physiology, and pharmacology. The chemistry of prostaglandins is presented in a functional manner. The chemical, metabolic, physiological, and pharmacological implications of altering bonds, specific groups, and steroidal configurations on this complex molecule are discussed. Methods of *in vitro* and *in vivo* synthesis of the primary prostaglandins and analogs from various chemicals are described.

Prostaglandins synthetase, its assay, and the hypotheses regarding physiological production of various prostaglandins *in vivo* are discussed. Working hypotheses are presented and defended by data for the physiological role of various prostaglandins in peripheral, central, and autonomic neurotransmission; renal, respiratory, GI, ocular, cardiovascular, lipid, and endocrine homeostasis; and male and female reproductive function. Data and hypotheses for possible roles of various prostaglandins in pathological states such as hypertension, asthma, inflammation, anaphylaxis, glaucoma, ocular trauma, and endocrine disorders are presented.

The possibilities of treating neurological, respiratory, GI, cardiovascular, renal, and lipid diseases with exogenous prostaglandins are explored. Results of using PGE_2 and $PGF_{3\alpha}$ for induction of term labor by the intravenous route and for induction of artificial abortions by the intravenous, subcutaneous, vaginal, extraovular, and intraamniotic routes are presented to demonstrate the practicability of prostaglandins for terminating pregnancy at all stages of gestation. In this rapidly developing field of research and development involving the chemist, physiologist, pharmacologist, and clinician, this volume is valuable to scientists in all of these disciplines in summarizing what often appear to be conflicting data and applying them to hypotheses.

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would seem to be the means of achieving this approximation.

To test this hypothesis partially, two new magnetic baskets were built. The first conformed to the original specifications (1) but was made of 16-mesh instead of the original 8-mesh stainless steel wire. The second basket was constructed with 8-mesh wire and had larger dimensions: an inner diameter of 12 mm. and a length of 38 mm. instead of the 11 × 25-mm. dimensions of the original basket.

A tablet and capsule were selected to be tested. Pentobarbital tablets were prepared in our laboratories, which allowed control of the ingredient and manufacturing variables. Commercially prepared sodium butabarbital capsules were selected since a previous publication (1) showed that capsules manufactured in an automated manner show less variance than hand-packed capsules.

The pentobarbital tablets were tested in the three different size baskets and showed no clogging of the pores in any case. At least five tablets of the same formulation and hardness were run in each basket. The T_{50} percent values increased in the expected order as the dimensional and pore size of the baskets decreased. The T_{50} values of 10, 14, and 21 min. were found for the bigger basket, regular basket, and smaller pore basket, respectively. In varying the revolutions per minute of the stirring propeller from 60 through 150 for the pentobarbital tablets in the regular basket, a distinct difference can be seen. The T_{50} at 150 and 120 r.p.m. is very close at an average of 2.5 and 3.25 min. Decreasing the revolutions per minute to 90 and to 60 shows a significant reduction and difference, with the T_{50} equal to 7.5 and 14 min., respectively.

A comparison of the dissolution of the sodium butabarbital capsules in the three baskets shows little difference between the bigger and regular baskets, with the T_{50} equal to 26 and 29 min., respectively. The smaller pore basket did show an increase in the T_{50} to 38 min. The difference in the T_{50} in the regular basket as a function of revolutions per minute was small at 150 and 120, producing average times of 15 and 16 min., respectively. Decreases in propeller speed to 90 and 60 r.p.m. showed T_{50}'s of 21 and 30 min., respectively.

In conclusion, the adjustment of basket pore size and propeller revolutions per minute can produce significantly different dissolution profiles, which should be considered when comparing *in vitro* dissolution results from the magnetic basket in quality control or as a means of correlating *in vitro–in vivo* data.

(1) R. E. Shepherd, J. C. Price, and L. A. Luzzi, *J. Pharm. Sci.*, **61**, 1152(1972).

(2) T. E. Needham, R. E. Shepherd, and L. A. Luzzi, *ibid.*, **62**, 470(1973).

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BOOKS

REVIEWS

The Prostaglandins, Volume I. Edited by Peter W. Ramwell. Plenum, New York, NY 10011, 1973. 526 pp. 17 × 25.5 cm. Price $22.50.

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This is the first volume of a series of the well-regarded Specialist Periodical Reports dealing with the synthesis of organic compounds in living organisms. An introductory chapter on the methodology of biosynthetic experimentation provides a general background to the specific topics discussed in the following sections. The four specific sections include the biosynthesis of terpenoid compounds; triterpenes, steroids, and carotenoids; phenolic compounds; and alkaloids.

This volume covers the literature of 1970 and 1971, with reference to earlier work where necessary for clarity.

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The Child With Down's Syndrome (Mongolism). By DAVID W. SMITH and ANN ASPER WILSON. Saunders, West Washington Square, Philadelphia, PA 19105, 1973. 106 pp. 18 × 26 cm. Price $5.75.

This publication was designed for parents, physicians, and persons concerned with the education and care of the child with Down's syndrome, frequently referred to as mongolism. The causes, characteristics, and acceptance of the Down's syndrome-affected child are emphasized.

The discussions are understandable, straightforward, and frank. For instance, in a discussion of the serious physical conditions that

Liquid Scintillation Counting, Volume 2. Edited by M. A. CROOK, P. JOHNSON, and B. SEALES. Heyden and Son Ltd., New York, N.Y., 1972. 327 pp. 17 × 26 cm. Price $21.50.

"Liquid Scintillation Counting, Volume 2" contains the proceedings of the International Symposium of Liquid Scintillation Counting, held at Brighton, England, in September 1971. The papers presented at that symposium are, in general, concerned with the biomedical applications of liquid scintillation counting techniques. There are 27 papers included in the book; four on Basic Theory of the Chemistry and Physics of Scintillators and Scintillation Counting; four on Historical Developments and Miscellaneous Problems; six on Methods of Sample Preparation of Inorganic Materials, Including Cerenkov Counting; six on Methods of Sample Preparation of Organic Materials Including Biological Systems; and seven papers on Data Processing.

This book, for the most part, presents material that deals with day-to-day problems associated with the liquid scintillation technique and should be a welcome addition to the library of users of internal liquid scintillation counters. Rapkin's article on the "History of the Development of the Modern Liquid Scintillation Counter" was very interesting and gives much information that will be helpful to potential purchasers of liquid scintillation counters.

Two other articles of general interest should be mentioned. Birks and Poullis reevaluated a wide range of scintillation solutions as to their relative pulse height under conditions which correspond closely to conditions used in current internal scintillation counting. Although carbon tetrachloride was the only quenching agent involved in their investigations, the article contains information as to the preferred solvent system for use depending on the degree of quenching present in the system. Scales presents information on undesirable luminescence in liquid scintillation samples of biological origin. He discusses methods of elimination of luminescence and points out the need for careful sample preparation technique.

For the most part, articles in the book deal with specific problems of biomedical interest and problems associated with data processing. On the whole, the book contains information which will lead to the solution of many day-to-day problems; information which, for the most part, is very difficult to find in other sources.

Reviewed by Gordon S. Born
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Lafayette, IN 47907 ■

A Guide to Drug Eruptions. By W. BRUINSMA. American Elsevier, 52 Vanderbilt Ave., New York, NY 10017, 1973. viii + 103 pp. 14.5 × 22.5 cm. Price $9.95.

This guide's stated purpose is "...to give information on clinical data which might be helpful in locating the responsible drug...." The objective has been achieved in part. However, the author is somewhat a victim of a problem identified in the introduction, namely, "Exact knowledge of incidence figures even for the common type of reaction to drugs is rarely available. For the great majority of drugs these data are widely scattered and difficult to obtain."

The resulting compilation is somewhat superficial. However, considering that the subject of skin eruptions is complex, the data are difficult to obtain, and reporting systems are poor, the book does serve as a useful departure point and as a *guide* for health-care practitioners having need of this kind of information.

Staff Review ■

Drugs: Annotated Bibliography and Guide. By A. M. AJAMI. G. K. Hall & Co., 70 Lincoln St., Boston MA 02111, 1973. xxiv + 205 pp. 16 × 24 cm. Price $16.50.

The title of this publication is somewhat misleading. The thrust of the book is abuse drugs and the drug culture rather than the more general area indicated by the title.

Abstracts of 539 articles divided into five sections are given. The bibliography was developed as part of a natural science college course and, as such, articles were selected to reflect various positions on numerous aspects of drug abuse. Rather than focus on one thesis, the articles were selected to provide a broad overview of uses, opinions, and considerations related to drugs.

Staff Review ■

How to Divide Medical Words. By RICHARD V. LEE and DORIS J. HOFER. Southern Illinois University Press, Carbondale, IL 62901, 1972. 229 pp. 11 × 15.5 cm. Price $4.50.

Divided into two sections, this reference books provides a listing of the correct spelling of a number of medical words and a substantially longer listing of the correct division of such words. While the correct spelling is available in a number of sources, finding the correct way to divide medical and pharmaceutical terminology sometimes can be difficult. Since it is priced reasonably, this book should be a useful addition to the reference sources available to persons involved with the preparation of technical publications.

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may accompany Down's syndrome, *e.g.*, intestinal blockage and defective heart development, it is emphasized that decisions for corrective measures must be left to the parents and that they have the right to refuse such measures.

The genetic "mistake" which leads to Down's syndrome is discussed in detail and is illustrated very effectively. This and all discussions should be easily understood without any scientific or technical background.

A large portion of the book is concerned with the physical, mental, and social characteristics of Down's syndrome. While it is obviously impossible to make predictions about a particular child, general patterns of development are discussed, accompanied by graphs. It is emphasized that seeking professional assistance as soon as possible allows for the greatest assurance that a Down's syndrome child will achieve the highest level of development. Results of research and programs in this area have indicated that attention, education, and therapy between the age of 3 and 5 apparently have a substantial effect on the child's future development. This is perhaps related to motivation; between 3 and 5, the child can master certain motor and learning skills. After that age, the Down's child begins to lose ground on normal children and may be subject to a sense of frustration.

Overall, this book is readable and informative without overwhelming the reader with peripheral information.

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REVIEW ARTICLE

Mechanisms of Stimulus–Secretion Coupling in Adrenal Medulla

RALF G. RAHWAN*▲ and J. L. BOROWITZ†

Keyphrases ☐ Adrenal medulla—mechanisms of secretion, morphology, physiology, and pharmacology, review ☐ Catecholamines—mechanisms of secretion, review ☐ Secretion—exocytosis and microtubule hypotheses, review ☐ Stimulus–secretion and excitation–contraction coupling—review ☐ Calcium—role in catecholamine secretion, review

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MECHANISMS OF TERMINATION OF SECRETORY RESPONSE OF ADRENAL MEDULLA 1919
PHARMACOLOGICAL MECHANISMS OF STIMULUS–SECRETION COUPLING IN ADRENAL MEDULLA 1920

. . . It is not to mere clinical experience that we must look for a revelation of the laws of disease. The laws of chemistry were not discovered in blazing fires or crumbling rocks; the laws of hydrostatics and hydraulics were not revealed in torrents, tides, or ocean currents, nor those of pneumatics and electricity in winds, whirlwinds, and thunderstorms; much less could it be rationally expected that the laws of pathology should be discovered amid the much greater complexity and more multitudinous conflicts of elements presented to the physician at the bedside of a diseased or dying patient. It is in the laboratory, and by artificially contrived experiments that the clue has ever been spun and the torch lighted to guide through the labyrinths which hide the arcana of nature. . . .

R. Cresson Stiles (1865)

Much of our present knowledge on the biochemical and morphological basis of the secretory process derives from studies on the adrenal medulla. The usefulness of this organ as a model for investigating both hormonal and neuronal release mechanisms was reviewed by Stjärne (1). The isolated perfused bovine adrenal gland has been more extensively used than adrenals of other species. Serrão and Da Costa (2) demonstrated that the cytological details as well as the catecholamine content of the isolated bovine adrenal medulla are well preserved even after prolonged perfusion (1–2 hr.) with physiological solutions. Since preservation of cellular structures in isolated organs is an indispensable condition for pharmacological investigations, the extensive use of the bovine adrenal gland as a model for studying the various aspects of neuroendocrine secretion seems justified.

Mammals are unique among vertebrates in that most chromaffin cells in the adult are confined within the adrenal medulla, which in turn is encapsulated by the adrenal cortex. The adrenal medulla derives from the neuroectoderm and is a neuroendocrine transducer, secreting its products in response to a neural input primarily from the preganglionic cholinergic fibers of the greater thoracic splanchnic nerves. Every chromaffin cell is said to be innervated (3). The adrenal

medulla has a rich dual blood supply, receiving blood that has drained the adrenal cortex and is rich in adrenal cortical steroids in addition to having its own direct arterial blood supply. Each chromaffin cell is said to be oriented with one end abutting on an arterial capillary and the other on a veinule (3), thus conveniently receiving biosynthetic precursors and releasing end-products, respectively. The anatomical relationship of the adrenal cortex and medulla is not without physiological significance. The role of the adrenocortical steroids in induction of medullary protein and phenethanolamine-*N*-methyltransferase synthesis and in determination of the relative distribution and anatomical disposition of epinephrine- and norepinephrine-containing cells of the medulla (4) was thoroughly reviewed by Pohorecky and Wurtman (5) and Wurtman *et al.* (6). However, although the cortex plays a significant regulatory role on the biosynthetic activity of the adrenal chromaffin cells, it appears to exert no immediate influence on the secretory process of catecholamines from the adrenal medulla (7, 8).

The details of the fine structure of the adrenal chromaffin cell of various species were described by several authors (9–22). The cytoplasm of the chromaffin cell contains a large number of what appear to be membrane-bound granules, 1000–3000 Å in diameter (23). The granules are reportedly bound by a unit membrane approximately 75 Å thick (4, 13), showing a trilaminar appearance (19). Fixation with glutaraldehyde and silver methenamine (10) results in the differential staining of three types of adrenal medullary cells: norepinephrine-containing cells showing black granules, epinephrine-containing cells showing light-gray granules, and a third cell type intermediate in appearance between epinephrine and norepinephrine cells. There is also evidence that epinephrine and norepinephrine granules may coexist within the same chromaffin cell, along with "precursor granules" whose content was chemically identified to be dopamine (9). The origin of the so-called chromaffin granules is a matter of conjecture. Small immature granules are said to be observed in the region of the Golgi apparatus (23), and "prosecretory" granules are reported to have been seen within the Golgi complex (17, 20); it has been suggested that the Golgi apparatus is directly involved in the formation of the chromaffin granules (9).

The adrenal medulla is the ancestral homolog of autonomic ganglia. The extensive investigations of Marley and Paton (24) and Marley and Prout (25) elucidated much of our present knowledge of the physiology and pharmacology of the splanchnic–adrenal medullary junction and the electrical properties of the chromaffin cell membrane. These authors studied the response of the adrenal chromaffin cells to nervous excitation and reported on properties such as threshold to nerve excitation, fatigue, spatial and temporal recruitment, and the effect of drugs (*e.g.*, ganglionic blocking agents, eserine, and cocaine) on these parameters (24, 25). Douglas *et al.* (26, 27) further extended these observations by studying the effect of medullary secretagogues (*e.g.*, acetylcholine, nicotine, pilocarpine, histamine, 5-hydroxytryptamine, angiotensin, and bradykinin) and antagonists (*e.g.*, atropine and hexamethonium) on the membrane potential of adrenal chromaffin cells, as well as the influence of the ionic environment on this parameter. These studies demonstrated the presence of both nicotinic and muscarinic receptors on the adrenal chromaffin cell membrane (26).

THEORIES OF CATECHOLAMINE RELEASE FROM ADRENAL MEDULLA[1]

The catecholamine hormones of the adrenal medulla are stored in high concentrations in the gland. In the rat, the adrenal medulla contains as much stored epinephrine as is secreted in 7 days (28, 29). The biochemical basis underlying the storage mechanism of the catecholamines was reviewed elsewhere (29–31). In brief, the catecholamines are believed to be bound in a non-diffusible form as a complex with adenosine triphosphate (ATP) and protein (32) or with ATP, calcium, magnesium, and RNA (33). The high concentration of catecholamines in the granules is believed to be maintained by an active uptake of amines across the membrane of the granules, which counteracts the loss of amines that occurs by outward diffusion (29).

In the past decade and particularly with the advent of the electron microscope and the introduction of biochemical techniques of subcellular fractionation, considerable progress has been made toward the understanding of the cellular storage of catecholamines and the manner of their release from the adrenal medullary cell (34–36). Several different mechanisms have been proposed to account for secretion from the adrenal medulla at a cellular level. As early as 1918, Cramer (37) proposed that the adrenal medullary granules were released as a whole from the adrenal gland (38). Round opaque particles, 300–700 Å in diameter and resembling chromaffin granules in appearance, were demonstrated in the canaliculi and intercellular spaces between the chromaffin cells of the adrenal medulla (19). In some cases where the particles were present at the free cell surface, small groove-like impressions occurred in the plasma membrane of the chromaffin cell (19). Similar findings of extracellular synaptic vesicles in the mouse heart were reported (39), as well as groove-like bays in the axolemma which were proposed to serve as points of exit of the granules from the nerve ending. Granule

[1] *Added in press*—A role for cyclic AMP in catecholamine secretion from the adrenal medulla was recently suggested (A. M. Poisner, *3rd Int. Catecholamine Symp.*, Strasbourg, France, 1973). Three of the criteria for second messenger status [E. W. Sutherland, G. A. Robison, and R. W. Butcher, *Circulation*, **37**, 279(1968)] have been met for cyclic AMP in adrenal medullary secretion: (*a*) cyclic AMP is present in the adrenal medulla in high concentrations and its level increases when secretion is stimulated *in vivo* [A. Guidotti and E. Costa, *5th Int. Congr. Pharmacol.*, **1972**, Abstract 541; *Fed. Proc.*, **31**, Abstract 1923(1972)]; (*b*) cyclic AMP and dibutyryl cyclic AMP can initiate catecholamine release from the adrenal medulla [M. J. Peach, *Proc. Nat. Acad. Sci. USA*, **69**, 834(1972)]; and (*c*) phosphodiesterase inhibitors, such as theophylline, which raise levels of cyclic AMP also cause catecholamine secretion from the adrenal medulla [M. J. Peach, *Proc. Nat. Acad. Sci. USA*, **69**, 834(1972); A. M. Poisner, *Biochem. Pharmacol.*, **22**, 469 (1973)]. Recent evidence also was presented indicating that cyclic AMP is involved in the release of norepinephrine from sympathetic nerve endings [G. F. Wooten, N. B. Thoa, I. J. Kopin, and J. Axelrod, *Mol. Pharmacol.*, **9**, 178(1973)]. The means by which cyclic AMP can initiate or mediate adrenal catecholamine secretion include mobilization of intracellular calcium or activation of protein kinases which may phosphorylate critical cell components, such as secretory granules, plasma membrane, or microtubules (A. M. Poisner, *3rd Int. Catecholamine Symp.*, Strasbourg, France, 1973), or chemical constituents of these structures, such as adrenal actomyosin or the microtubule protein, tubulin, recently identified in the adrenal medulla [D. A. Redburn, A. M. Poisner, and F. E. Samson, Jr., *Brain Res.*, **44**, 615(1972); S. L. Twomey and A. M. Poisner, *ibid.*, **46**, 341(1972)].

extrusion from rat anterior pituitary cells following injection of leutinizing hormone-releasing factor was also recently demonstrated (40). Biochemical data do not support the theory of release of intact granules nor similar suggestions of apocrine or holocrine secretion from the adrenal medulla (41, 42), as will become evident from the ensuing discussion.

Based on cell fractionation studies, Hillarp (43) proposed the existence of at least three different pools of catecholamines in the adrenal chromaffin cell. One pool was the chromaffin granule which contained catecholamines in association with ATP with a molar ratio of 4:1. This ratio suggested that ATP served as the anion paired with the basic catecholamines, forming a complex which is an integral part of the intragranular mechanism of catecholamine storage (30). The second pool consisted of granules in which catecholamines were stored without ATP; the third pool consisted of the "free" cytoplasmic amines which, together with the pool of amines in the granules lacking ATP, comprised about 20% of all the catecholamines in the chromaffin cell, an amount that can sustain secretory activity for a prolonged time (44). Since ATPase activity was present in the chromaffin granule fraction (45), Hillarp (45) and Hillarp *et al.* (46) suggested that when the chromaffin cell is stimulated, the ATPase associated with the chromaffin granules may be activated in some way, enabling it to attack the ATP of the catecholamine storage complex, thus freeing the amines which would then diffuse or be transported out of the granules, into the cytoplasm, and ultimately out of the cell.

Blaschko and Welch (34) had earlier speculated that liberation of acetylcholine at the splanchnic–adrenal medullary junction might result in an increased permeability of the membrane of the chromaffin cell that would lead to a loss of "free" amines present in the cytoplasmic sap and that the role of the chromaffin granules was to replenish the cytoplasmic pool of catecholamines released during the secretory activity. In support of Hillarp's (45) suggestion of a critical role of granular ATP splitting in the release of catecholamines from their granules is the finding that ATP metabolites appeared in the adrenal effluent from stimulated glands (47, 48), and the observation that acetylcholine evoked catecholamine secretion by promoting calcium influx into the chromaffin cell (49)—a cation known to be an activator of certain ATPases and, consequently, to play a critical role in excitation–contraction coupling in muscle (50, 51). However, this hypothesis of *intracellular* release of catecholamines from the chromaffin granules followed by diffusion of the hormones to the cell exterior lost some of its credibility when Douglas *et al.* (52) noted that ATP metabolites present in the venous effluent from the adrenal were attributable to hydrolysis of ATP by endothelial enzymes and that, under appropriate experimental conditions, ATP is extruded unhydrolyzed to the cell exterior during catecholamine release from the gland (53). These observations led to the conclusion that splitting of intragranular ATP is not a critical step in the secretory process. Direct evidence against the hypothesis of intracellular release of catecholamines was provided by the finding that the secretion of the soluble contents of the chromaffin granule, including the soluble intragranular protein chromogranin A (effective hydrodynamic radius approximately 62 Å), took place without simultaneous secretion of the cytoplasmic enzyme lactate dehydrogenase (hydrodynamic radius approximately 37 Å) (29, 54).

Although it is now generally recognized that the free cytoplasmic catecholamine pool demonstrated in cell fractionation studies results largely as an artifact of the homogenization procedure (29), and although the hypothesis of intracellular release of catecholamines has largely been superseded by other hypotheses, certain drugs such as acetaldehyde (55), dextroamphetamine (56, 57), *l*-amphetamine (58), and tyramine (58) are capable of releasing adrenal catecholamines from their storage sites into the cytoplasm of the chromaffin cell, from where the amines may diffuse to the extracellular space. Furthermore, spontaneous (resting) secretion of adrenal catecholamines is believed to utilize, at least in part, the intracellular release mechanism (58).

Exocytosis Hypothesis—This hypothesis was first proposed by De Robertis and Vaz Ferreira (18) in 1957 to explain their observations on structural changes in the adrenal medulla following stimulation of the splanchnic nerves. It was described later by De Robertis (59):

> *When the gland is stimulated through the splanchnic nerve the first changes that occur in the catecholamine-containing vesicles (or granules) are the following: the granule attaches to the plasma membrane and it swells; at this moment there is no clear distinction between the clear zone on the outside and the dense granule inside; then there is a decrease in the amount of material contained in the granule. The material flows out but the membrane of the granule remains within the adrenal cell.*

This type of secretion was initially called "reverse pinocytosis" (18) but, more recently, the term "exocytosis" was introduced (60). Although this hypothesis received additional morphological support (12, 17, 19, 22, 61–65), it was disclaimed by others (66, 67). Electron microscopic evidence for exocytosis also has been reported for glandular tissue containing membrane-bound secretory granules other than the adrenal medulla (*e.g.*, 64, 68–74).

Support for the exocytosis hypothesis derives heavily from biochemical evidence. That secretion from the adrenal medulla may take place by exocytosis is based mainly on the finding that several water-soluble constituents of the catecholamine storage granules appear simultaneously in the effluent from the gland. Thus, catecholamines, adenine nucleotides (48), the specific soluble protein chromogranin (75, 76), and soluble dopamine-β-hydroxylase (77) have been shown to be released from the adrenal medulla by various stimuli, sometimes in the same proportions in which they occur in the granules. Furthermore, neither water-insoluble constituents of the granules, such as cholesterol and phospholipids (78), nor cytoplasmic material, such as tyrosine hydroxylase, phenethanolamine-*N*-methyltransferase (77), or lactate dehydrogenase (29, 54), accompany the catecholamines secreted from the adrenal

medullary cells upon stimulation. The presence in the adrenal medullary chromaffin granule of a high content of lysolecithin (29, 79), a potent lytic agent (29, 80), led to the postulate that this lipid participates in the fusion of the secretory granule to the plasma membrane of the chromaffin cell during exocytosis (80).

The role of calcium ions in stimulus–secretion coupling will be discussed in a later section. However, one finding warrants mentioning since it provides a plausible basis for the explanation of the molecular mechanisms underlying exocytosis. Calcium appears to be almost universally involved as the link that couples excitation and secretion (81). Quarles and Folch-Pi (82) studied the effect of calcium on the distribution of gangliosides in an organic–aqueous biphasic system. They showed that when calcium is in the upper organic phase at concentrations below 5 m*M* or above 160 m*M*, all of the ganglioside distributes in the organic phase. At calcium concentrations between 5 and 160 m*M*, the ganglioside partitions to the aqueous phase. When contaminated with protein, the ganglioside was induced by calcium to localize at the interface of the aqueous–organic phases. Since the chromaffin granules are composed of ganglioside and protein (29) and since the chromaffin cell consists of an aqueous (cytoplasm) phase and an organic (lipid and protein) phase (the plasma membrane), Simpson (83) proposed a similar situation to exist *in vivo*. Accordingly, a depolarizing stimulus to the chromaffin cell membrane would lead to an influx of calcium into the cell (49), resulting in a calcium concentration at the inner aspect of the cell membrane that is high enough to allow the collision between the granule (ganglioside) and the plasma membrane (organic phase) to persist, particularly since protein is present in both the granule and the cell membrane. Calcium could then activate membrane phospholipase A or granule lysolecithin, thus facilitating the fusion of the granule to the plasma membrane (79). Simpson (83) further proposed that when the stimulus to the chromaffin cell is terminated, the calcium concentration at the inner aspect of the plasma membrane of the chromaffin cell starts to decline; the granule (ganglioside) would repartition to the aqueous (cytoplasmic) phase and secretion would dwindle. To explain resting (spontaneous) secretion, Simpson (83) postulated that the very low calcium concentration found at the inner aspect of the chromaffin cell membrane at rest would increase the effectiveness of ganglioside (granule)–lipid (cell membrane) contact, in an analogous way to the *in vitro* situation described by Quarles and Folch-Pi (82).

The fate of the membranes of the chromaffin granules that remain in the chromaffin cell following discharge of their catecholamine content (64, 65, 84, 85) is a matter for conjecture. Although empty ghost granules have been seen electron microscopically in sections of medullary tissue from stimulated adrenal glands (59), it is not known whether these membranes become recharged with catecholamines, protein, ATP, and cations or whether they are phagocytized by the intracellular autophagic vacuoles and become broken down by lysosomal enzymes (29, 65). Evidence has been presented (64, 65, 71–74) for the adrenal chromaffin cells as well as for the adenohypophyseal and neurohypophyseal cells that the granule membrane incorporated into the cell surface during exocytosis is removed by a process of vesiculation resembling micropinocytosis. In this process the exocytotic pits pinch off to form intracellular microvesicles, thus conserving not only the area of the plasmalemma but also its chemical characteristics associated with permeability, excitability, and the receptor function (64, 65). Kopin and Silberstein (86) suggested that the large norepinephrine vesicles in adrenergic neurons discharge their contents when the nerve ending is depolarized; once their soluble protein contents are exhausted, they may be transformed into the smaller storage vesicles and be concerned primarily with synthesis and storage of the transmitter reserve.

Although the exocytosis hypothesis for the release of neurotransmitters, hormones, and other macromolecules (80, 81, 83) has received considerable biochemical support, it does not explain a number of observations. Exocytosis certainly cannot be the mechanism by which the secretory products of the adrenal cortex, the fetal pancreas, or the parietal cells of the stomach are released, since the secretory products of these structures are not sequestered in membrane-bound granules (87–89). Furthermore, the exocytosis hypothesis cannot possibly account for the explosive and almost instantaneous release of transmitters, hormones, or macromolecules upon stimulation of cells containing these secretory products. Thus, catecholamines are released from the adrenal medulla within 1–2 sec. of splanchnic nerve stimulation (48), and epinephrine releases amylase from its storage granules in the parotid gland in less than 10 sec. (90). Likewise, insulin is released very rapidly from the pancreas following glucose stimulation (91). Schramm (80) emphasized that fusion of the zymogen granule with the cell membrane cannot explain the *rate* of enzyme secretion, and no experimental evidence indicates any temporal relationship between fusion, cleavage, and healing of the plasma membrane during exocytosis (64, 65, 71–74) and the rate of hormone, enzyme, or neurotransmitter secretion. Finally, the exocytosis hypothesis cannot explain the findings of Hubbard and Kwanbunbumpen (92) who demonstrated that there was no electron microscopic evidence of an increase in the number of discharging vesicles fused to the axolemma under conditions of increased quantal release of transmitter at the neuromuscular junction of the rat diaphragm.

Microtubule Hypothesis—The many serious and fundamental shortcomings of the exocytosis hypothesis prompted Whittaker (93–95) to propose the possible existence of a complex interconnecting system of tubules within secretory cells which may facilitate the discharge of secretory products to the cell exterior. According to Whittaker (95), "there might well be an intercommunicating system of fine tubules—hard to see by normal histological methods—composed of protein and another macromolecule that could open or close in response to ionic changes induced by action potentials." Such a system of microtubules would provide a possible pathway for release of all types of secretions, including the nongranule-bound secretions of the parietal cells, the adrenal cortex, and the fetal rabbit pancreas. In

addition, if the secretory tubules were to be considered as possessing a dynamic function as well as a structural one, they would provide a basis for the interpretation of the rate of egress of secretory products from secretory cells—perhaps the major shortcoming of the exocytosis hypothesis.

The possibility that secretion may take place within a structured environment of secretory tubules is supported by morphological observations. In negative staining, fine interconnections between isolated synaptic vesicles were demonstrated by Whittaker (93). Amsterdam and Schramm (96) showed that isolated zymogen granules are sometimes linked by thin tubules not visible in conventional sections of whole cells when examined under the electron microscope. Tubular connections may also account for the presynaptic projections and fine filaments sometimes seen in electron micrographs of nerve tissue (97). The existence of narrow tubules connecting the secretory granules to the plasma membrane was also proposed by Gray (97) and Sandborn (98). Electron microscopic evidence for the existence of neurotubules in axons was presented (98); De Iraldi and De Robertis (99) showed that these neurotubules appear to originate in the region of the Golgi complex in the soma and function in the rapid transport of material down the axon. Similar findings were reported by Davison (100). According to von Euler (101), "electron microscope pictures often show granules arranged in sequences, in rows with a kind of connecting tube or link between them" and that "practically every good picture shows at least a few granules which have such a connection between them." Von Euler (101) also observed "chains of two or three vesicles" in negatively stained preparations of suspensions of granules.

Kopin and Silberstein (86) described a situation in adrenergic neurons in which the secretory vesicles would move along, rather than within, microtubular structures which would serve as tracks to channel the vesicles toward the cell membrane of the terminal varicosities. In the adrenal cortex, there is evidence that the smooth endoplasmic reticulum forms an anastamosing system of tubular elements which plays a role in the extrusion of steroids to the cell exterior (88). Tubular connections running to the surface of the cell have been proposed to exist in cholinergic nerves (94), mast cells (102), and β-cells of the pancreas (91). The parietal cell of the stomach shows secretory canaliculi forming a sinuous tubular channel which opens into the lumen of the gastric gland (87). Furthermore, a system of cytoplasmic tubules, which is sometimes continuous with the canaliculus, has been identified in these cells (87).

Biochemical evidence for the existence of a microtubular secretory apparatus within secretory cells is rapidly accumulating, particularly since the introduction of colchicine, the vinca alkaloids, and cytochalasin-B as drug tools for the study of cell movement and intracellular transport systems. Colchicine binds specifically to microtubular protein (103), depolymerizes the microtubules of mitotic spindles, thus arresting mitosis, and unites with the protein subunits of neurotubules, causing a disruption of their organization (104). Similar effects were reported for the vinca alkaloids (105, 106). Vinblastine-induced precipitation of microtubular protein was reported by Marantz *et al.* (107). Cytochalasin-B is known to destroy neurofilaments specifically and to inhibit a wide variety of cellular movements, including changes in cell shape during differentiation and development (108). Cytochalasin-B was shown to interact with actomyosin from rabbit striated muscle, causing a decrease in viscosity of the actomyosin and an inhibition of actin-induced activation of heavy meromyosin ATPase activity by direct interaction with actin (109).

Reports are increasing on the inhibitory effects of colchicine, vinblastine, vincristine, and cytochalasin-B on secretory activity in secretory cells—a property invariably attributed to their disrupting effect on microtubules and microfilaments. Thus, colchicine and vinblastine inhibit the transport of amine storage granules in adrenergic neurons (110) and inhibit the release of norepinephrine and dopamine-β-hydroxylase from sympathetic nerves (111), a property also shared by cytochalasin-B (111). Release of catecholamines from the bovine adrenal medulla is inhibited by colchicine, vinblastine, and vincristine (112). Vincristine also was shown to inhibit the release of growth hormone and prolactin from the pituitary gland (113). Insulin secretion from the pancreas is blocked by colchicine (91), which also blocks thyroxine and iodine secretion from the thyroid gland (114, 115). Histamine release from mast cells (116) and leukocytes (117) and release of lysosomal hydrolases by phagocytes (118) are also inhibited by colchicine. Degranulation associated with phagocytosis (119) and melanin granule movement in melanophores (120) are likewise inhibited by colchicine. Cytochalasin-B inhibits latex-induced or zymosan-induced phagocytic activity in leukocytes, as well as phagocytosis of bacteria by polymorphonuclear leukocytes (121). Vincristine frequently produces signs of skeletal and smooth muscle toxicity consistent with diminished transmitter release (122).

In all of these systems, translocation of the secretory product or granule or vesicle movement appears to involve a microtubule system (123, 124) sensitive to the disrupting effect of colchicine, the vinca alkaloids, or cytochalasin-B—agents that labilize microtubular structures. On the other hand, the microtubule stabilizer deuterium oxide (125–127) has been shown to potentiate nicotine-induced catecholamine release from the adrenal gland (112) and histamine release from mast cells induced by compound 48/80 (116).

The proposal by Poisner and Bernstein (112) that the inhibitory effect of colchicine and vinblastine on evoked adrenal catecholamine secretion is due to disruption of secretory microtubules was contested by Trifaró *et al.* (128) and Douglas and Sorimachi (129, 130). They claimed that the inhibitory action of the alkaloids was attributable to an anticholinergic effect, since these agents inhibited the acetylcholine-induced release of catecholamines but did not block secretion evoked by high potassium concentrations (128–130). However, acetylcholine and potassium may act by different mechanisms to evoke catecholamine release from the adrenal medulla, as evidenced by the findings that lanthanum (131) and certain local anesthetics (132) block the secretory effect of acetylcholine more effectively than that of potassium. Furthermore, it is well documented that

colchicine can block the release of secretory products induced by noncholinergic stimuli in various tissues. For example, *in vitro* release of adrenocorticotropin from the adenohypophysis induced by high potassium concentration is blocked by colchicine (133). Similarly, glucose-induced release of insulin from isolated pancreatic islet β-cells is also blocked by colchicine (91). This alkaloid also blocks release of thyroxine and iodine from the thyroid induced by either thyroid-stimulating hormone or dibutyryl cyclic adenosine-3′,5′-monophosphate (cyclic AMP) by binding to a 6S colchicine-binding protein in the soluble fraction of homogenates (114), which is similar to the protein identified in other systems as a microtubular subunit (103, 134–138).

Recent investigations disclosed the presence of an actomyosin-like contractile protein in a number of secretory cells. Thus, a Mg^{+2}- or Ca^{+2}-activated ATPase was identified in isolated synaptic vesicles of rat brain (139, 140), and a similar ATPase was isolated from whole brain of the rat and cat (141) and characterized as having properties similar to those of muscle actomyosin and exhibiting an affinity for colchicine binding (142). A contractile protein was also isolated from human platelets (143) which was postulated to play a role in release of histamine and serotonin from these cells. Poisner (144) demonstrated the existence of an actomyosin-like protein in the bovine adrenal medulla, which could participate in the secretory mechanism of catecholamines.

Such findings have generated a number of theories relating the presence of actomyosin-like activity, the existence of microtubules, and the secretory process (78, 91, 104, 112, 114, 145). Poisner and Bernstein (112) crystallized these theories into a unitary thesis which postulates that "microtubules assist in the transport of secretory granules to the cell surface by a mechanism involving mechanicochemical transduction comparable to the contraction of actomyosin, and the final discharge mechanism also includes a contractile process with an actomyosin-like protein in the granule membrane, perhaps with the microtubules serving to orient the membranes at the surface to permit the active ejection of secretory product."

In view of the almost universal requirement for calcium in release of secretory products (81 and to be discussed later), Whittaker (94), referring to the possible role of microtubules in secretion, postulated that "if the walls of such tubules were made up of a macromolecule which changed configuration in the presence of calcium ions in such a way as to enlarge the lumen of the tubules, a basis might be provided for the well known essential role of calcium ions in excitation-secretion coupling." Because of the similarities between microtubule protein and actin (104), it is entirely possible that the "macromolecules" postulated by Whittaker (94) may actually constitute the contractile actomyosin isolated from secretory cells (139–144).

Rahwan (146) and Rahwan *et al.*[2] recently demonstrated by electron microscopy the existence of tubular connections between catecholamine granules in sections of mouse adrenal medulla. These authors advanced a theory for secretion from the adrenal medulla based on the postulate that the hormones may exist in the form of intratubular *droplets* within the chromaffin cell. A stimulus to the adrenal chromaffin cell would lead to an influx of calcium from the extracellular space (81) or translocation of calcium from intracellular pools (57) into the cytoplasm. The free intracellular calcium, either as ionic calcium or as a complex with intracellular phosphate (147), would activate an actomyosin-like protein in the contractile membranes of the secretory tubules, possibly by binding to a troponin-like factor (145), resulting in a peristaltic wave which would end in the egress of the catecholamines to the exterior of the cell (146)[2]. The isolation of intact chromaffin *granules* from homogenates of adrenal medulla does not necessarily militate against the postulate of the existence of the catecholamines within the chromaffin cell in the form of intratubular *droplets* (146)[2], since it is conceivable that when the gland is homogenized the fragmented tubules may undergo healing and vesicle formation in a manner analogous to the formation of synaptosomes[2].

ROLE OF CALCIUM IN STIMULUS–SECRETION COUPLING IN ADRENAL MEDULLA

A number of comprehensive reviews covered various aspects of the role of extracellular calcium in the release of neurotransmitter substances, hormones, and other secretory products (44, 47, 81, 83, 145, 148, 149). The role of intracellular calcium in stimulus–secretion coupling has only recently been recognized and will be reviewed here. In general, calcium appears to play a critical and almost universal role in the release of secretory material. Furthermore, a relationship between calcium and cyclic AMP as intracellular messengers in secretion was discussed by Rasmussen (124). In the adrenal medulla, however, stimulation of the chromaffin cells does not result in an increase in intracellular cyclic AMP concentrations (124), and the role of this cyclic nucleotide, if any, in catecholamine secretion from the adrenal medulla is not known (124) despite the dependence of the secretory mechanism of this gland on calcium (81).

Role of Extracellular Calcium—Calcium-deprived perfusion solutions depress or abolish the secretory response of the adrenal medulla to a variety of secretagogues including acetylcholine, carbachol, other nicotinic and muscarinic agents, histamine, serotonin, polypeptides such as bradykinin and angiotensin I, some sympathomimetic amines, ouabain, and excess potassium (33, 150–159). Furthermore, Douglas and Rubin (150) showed that the secretory response of the adrenal medulla to acetylcholine varies directly with the concentration of calcium in the perfusion medium. Acetylcholine, potassium, histamine, angiotensin I, bradykinin, serotonin, nicotine, carbachol, and pilocarpine have been shown to depolarize the chromaffin cell membrane (26). An increased influx of radiolabeled calcium into chromaffin cells occurs during stimulation (49, 160, 161), and Douglas and Rubin (150, 162–164) proposed that acetylcholine owes its stimulant effect on

[2] R. G. Rahwan, J. L. Borowitz, and E. J. Hinsman, unpublished data (manuscript in preparation).

the chromaffin cell to some action increasing the permeability of the plasma membrane to calcium in the extracellular fluid. The effect of acetylcholine on the permeability of the chromaffin cell is thought to occur through depolarization (44, 148). However, the finding that acetylcholine can still evoke catecholamine secretion from the adrenal medulla perfused with a sodium-free medium or a medium containing isotonic potassium sulfate indicates that depolarization is not tightly coupled with secretion as long as calcium is present in the extracellular environment (44, 148, 162). Additional evidence that depolarization and secretion can be dissociated was provided by Douglas *et al.* (27) who demonstrated that acetylcholine or excess potassium still depolarized the chromaffin cell when the extracellular fluid was calcium free or contained excess magnesium—conditions that inhibit secretion (150, 157, 162). However, in the work of Douglas *et al.* (27), the depolarization of the chromaffin cell in response to acetylcholine in the absence of extracellular sodium was not totally abolished but fell to about 30% of the control value. Moreover, prolonged perfusion with sodium-free medium abolishes the secretory response of the adrenal medulla to carbamylcholine and to high potassium concentrations (165)—agents that depolarize the chromaffin cell membrane (26). Although Banks *et al.* (165) suggested that sodium has some regulatory effect on the entry of calcium into chromaffin cells during stimulation, Rubin (81) argued that the prolonged perfusion with sodium-free medium used by Banks *et al.* (165) may result in a nonspecific deleterious effect to the chromaffin cell rather than a specific effect on calcium entry. However, sodium-deficient perfusion solutions cause an increase in spontaneous adrenal medullary secretion (150, 162, 165), and sodium deficiency has been shown to increase the entry of calcium into cells (165, 166).

An increased uptake (49) and exchange (160) of calcium by the chromaffin cell occurs during acetylcholine stimulation, and magnesium markedly inhibits calcium exchangeability between the gland and the perfusion medium (161) and also inhibits medullary secretion (162). Likewise, local anesthetics block the influx of calcium as well as the secretory response of the adrenal medulla (57, 161, 167). Rubin *et al.* (161) suggested that local anesthetics and magnesium interfere with calcium entry into the chromaffin cell by competition at a common membrane site. However, Rahwan *et al.* (57) presented evidence that magnesium may also interfere with the action of calcium at an intracellular calcium receptor site in the adrenal chromaffin cell.

Role of Intracellular Calcium—Despite substantial evidence of the importance of extracellular calcium in stimulus–secretion coupling in the adrenal medulla, in some instances drugs have been shown to evoke release of catecholamines from the perfused adrenal gland in the absence of calcium in the perfusion fluid. Thus, reserpine (33, 152), tyramine (152), phenylethylamine (152), barium (163), potassium thiocyanate (168), strontium (163), caffeine (56, 57, 169), aminophylline (169), acetaldehyde (55), chlorpromazine (56, 57), and dextroamphetamine (56–58) have all been shown to release catecholamines from the adrenal gland in the absence of extracellular calcium. Likewise, the reintroduction of calcium to the perfusion medium following perfusion with calcium-free medium evokes catecholamine release from the adrenal medulla (150, 162), despite the fact that under normal conditions excess calcium added to a perfusion medium already containing calcium does not augment secretion in the absence of a depolarizing agent (163).

The dissociation of extracellular calcium and the secretory effect of some secretagogues do not militate against the role of calcium in secretion. Indeed, it is becoming increasingly apparent that intracellular calcium may be of importance in stimulus–secretion coupling. In animal cells the concentration of calcium in the cytoplasm is in the range of 10^{-5}–10^{-8} *M* (124, 170–172). The demonstration by Borowitz *et al.* (173) that the adrenal chromaffin cell has a high content of calcium distributed among the various intracellular organelles raised the possibility that this intracellular calcium may play a role in catecholamine secretion in much the same way as has been demonstrated for muscle contraction (50). Thus, agents that release catecholamines from the adrenal medulla in the absence of extracellular calcium may still be dependent on a critical intracellular calcium pool for their secretory effect. In fact, Rahwan and Borowitz (56) and Rahwan *et al.* (57) showed that, under calcium-free perfusion conditions, chlorpromazine induced catecholamine release from bovine adrenal glands by mobilizing mitochondrial calcium reserves, caffeine evoked catecholamine secretion by mobilizing calcium from the endoplasmic reticulum as well as the mitochondria, and dextroamphetamine released adrenal catecholamines initially by a calcium-free mechanism followed by an enhanced secretory effect consequent upon mobilization of calcium from the endoplasmic reticulum. Furthermore, catecholamine release induced by chlorpromazine, caffeine, and dextroamphetamine in calcium-free medium generally paralleled ^{40}Ca and ^{45}Ca efflux from radiocalcium-labeled adrenal glands (57), suggesting that the calcium released to the exterior of chromaffin cells under these conditions represents in part the free intracellular levels of calcium that mediate catecholamine secretion (57) and in part the intragranular calcium involved in the catecholamine storage complex (174). The finding that magnesium significantly inhibited catecholamine secretion from the adrenal medulla induced by chlorpromazine, caffeine, or dextroamphetamine under calcium-free perfusion conditions (57) offers rather conclusive evidence that mobilization of intracellular calcium by these secretagogues is the triggering event in stimulus–secretion coupling and establishes an intracellular site for competition between calcium and magnesium (57).

Jaanus and Rubin (175) demonstrated that the total calcium content of the adrenal cortex may be divided into three roughly equal fractions: extracellular, cellular extractable, and cellular nonextractable. The extracellular calcium pool was presumed to be of importance for maintaining the more critical cellular calcium pools. During prolonged perfusion with calcium-free solution, approximately one-third of the total calcium content of the cortex was still retained by the gland. The locus of this nonextractable cellular calcium was assumed to

be the mitochondria and could be depleted only by treatment of the gland with 2,4-dinitrophenol, an uncoupling agent that releases calcium from the mitochondria (176). The cellular extractable calcium pool was assumed to be bound to or near the surface of the cortical cell or localized in some readily exchangeable intracellular site (175). The role of these different calcium pools in adrenocorticotropin-induced corticosteroid secretion was discussed by Jaanus and Rubin (175). The secretory response of the adrenal cortex to low concentrations of adrenocorticotropic hormone was found to be dependent upon extracellular calcium (177, 178), whereas high concentrations of adrenocorticotropin evoked corticosteroid release in the absence of extracellular calcium (179). It has been reported that the secretory effect of adrenocorticotropin on the adrenal cortex is dependent upon some critical intracellular calcium pool (175, 179).

Additional evidence for a role of intracellular calcium as a coupling link in the secretory process was provided by Williams (180). He showed that secretion from the thyroid gland induced by thyroid-stimulating hormone may be dependent upon mobilization of intracellular calcium.

To emphasize further the emerging significance of intracellular calcium in cellular processes, it has been reported that in certain tissues where adenyl cyclase and cyclic AMP mediate the effect of a specific peptide hormone, although the hormone-induced stimulation leads to an influx of calcium into the cells, the addition of extracellular cyclic AMP does not stimulate calcium uptake even though it mimics hormone action (124). Such findings indicate that the action of the cyclic nucleotide is either independent of calcium or utilizes an intracellular calcium pool (124). Evidence that cyclic AMP causes mobilization of calcium from intracellular pools has been presented by numerous investigators (181–184). Based on this and other information, Rasmussen (124) postulated that the increase in intracellular cyclic AMP, subsequent to interaction of a hormone with adenyl cyclase, would result in mobilization of calcium from one or more intracellular pools and/or prevention of uptake of cytoplasmic calcium into intracellular organelles.

Although the adrenal medullary chromaffin cell has been impaled by microelectrodes (26, 27), there is as yet no report on the effect of intracellularly injected calcium on catecholamine secretion. Such studies should provide direct evidence for the role of intracellular calcium in stimulus–secretion coupling. Miledi and Slater (185) found calcium to be ineffective in evoking release of transmitter when injected intracellularly into the squid giant axon. In their study, Miledi and Slater (185) deprived the squid giant synapse preparation of calcium and then tested the effect of ionophoretic application of calcium at different spots along the synapse. The transmitter-releasing effect of calcium was found to be very sharply localized at specific spots along the stretch of presynaptic axon lying adjacent to the postsynaptic axon. To test the effect of intracellularly injected calcium, the pipet was inserted into the presynaptic axon at a *single* spot which was previously shown to be sensitive to extracellular application of calcium. When injection of calcium into the presynaptic axon resulted in no release of transmitter, Miledi and Slater (185) concluded that for calcium to evoke transmitter release the cation acts through a membrane reaction whose reactive sites are accessible only from the outside of the membrane. However, Miledi and Slater (185) conceded that such a conclusion "remains tentative because time was too short to test its validity fully." These authors (185) further stated that: "Usually we could not test more than one 'intracellular spot,' and it is clear that many spots must be tested in view of the sharp localization required for effective ionophoretic application of calcium from the outside." The question of whether intracellularly injected calcium can release secretory products remains to be answered.

The importance of calcium ions in spontaneous (resting) secretion of catecholamines from the adrenal medulla is not clear. Although some investigators reported spontaneous catecholamine secretion to be very low in the absence of calcium in the perfusion fluid (150, 154, 158), others showed that resting secretion from the adrenal medulla was unaffected when the perfusion fluid was switched from one containing 2.2 m*M* calcium to one containing no calcium (57). The spontaneous secretion of catecholamines from the adrenal medulla is presumed to reflect what is released by exocytosis initiated by spontaneous release of acetylcholine at the splanchnic–adrenomedullary junction as well as what diffuses out of the chromaffin granules and into the cytoplasm (58). This was shown to be the case by Schneider (58), since addition of hexamethonium to the fluid perfusing adrenal glands in a concentration that completely blocks the effect of acetylcholine released by the preganglionic cholinergic fibers resulted in approximately a 40% reduction of spontaneous catecholamine release, equivalent to the reduction observed when calcium was eliminated from the perfusion fluid. These results indicate that extracellular calcium has no effect on spontaneous catecholamine secretion other than that mediated by inhibition of exocytosis (58).

Spontaneous secretion clearly does not cease in the absence of extracellular calcium and, furthermore, is not abolished by either local anesthetics (57, 161) or magnesium (57, 162) whether or not calcium is present in the extracellular fluid. The finding that magnesium does not inhibit resting secretion when adrenal glands are perfused with calcium-free medium (57) casts doubt as to the importance of intracellular calcium in spontaneous catecholamine secretion, particularly since magnesium was shown to compete with calcium at an intracellular site (57). Furthermore, ouabain, which raises intracellular calcium levels by presumably inhibiting calcium efflux from cells, does not potentiate resting secretion from the adrenal medulla in the absence of extracellular calcium (154). On the other hand, Douglas and Ishida (186) reported that cooling increases resting secretion of vasopressin from the neurohypophysis perfused with a calcium-free medium, and cooling has been shown to inhibit calcium uptake by intracellular organelles including the endoplasmic reticulum of the adrenal chromaffin cell (168). Since calcium uptake by intracellular organelles is presumed to be one mechanism for termination of secretory

activity (discussed later), it is not unlikely that cytoplasmic calcium may play a role in spontaneous secretion and that interference with intracellular sequestration of this cation may enhance resting secretion. Furthermore, calcium was shown to be released from the adrenal chromaffin cells (57) and from isolated chromaffin granules (187) during spontaneous secretion even in the absence of extracellular calcium (57). Whether this extruded calcium is involved in resting secretion, as has been postulated for evoked adrenomedullary secretion (57), must await further research.

PARALLELISM BETWEEN STIMULUS–SECRETION COUPLING IN ADRENAL MEDULLA AND EXCITATION–CONTRACTION COUPLING IN MUSCLE

Over the past few years, the striking similarities between the processes of stimulus-secretion coupling in secretory cells and excitation-contraction coupling in muscle have become increasingly apparent (57, 81, 145). Thus, all secretory systems studied have shown that the release of the secretory product is critically dependent on extracellular (81) or intracellular (57, 175, 180) calcium ions. Furthermore, studies of many secretory systems, including neurons and endocrine and exocrine cells, have shown that discharge of stored product does not occur when synthesis of ATP is blocked (188–190) or when granule ATPase is inhibited (78, 191). These facts, in addition to the isolation and characterization of an actomyosin-like contractile protein from a number of secretory cells (139–144), have emphasized the similarities between secretion and muscle contraction which were recently reviewed by Poisner (145).

The early finding that the adrenal chromaffin granules contained ATP and ATPase activity (45), coupled with the demonstration that the ATP–Mg^{+2}-dependent pump which concentrates catecholamines in these granules (30) has many characteristics of the calcium pump in muscle (192), led Hillarp (45) to suggest that when the chromaffin cell is stimulated, the ATPase associated with the chromaffin granules may hydrolyze the ATP of the ATP–Mg^{+2} intragranular storage complex, thus releasing the catecholamines from the granules. Although this hypothesis was refuted on other grounds (discussed previously), its significance also was made doubtful when it was demonstrated that calcium did not evoke catecholamine release from isolated adrenal medullary chromaffin granules (78). However, Poisner (145) speculated on the failure of calcium to facilitate release of catecholamines from isolated chromaffin granules *in vitro* as being possibly due to the absence of an active calcium-sensitive protein comparable to the troponin–tropomyosin complex in muscle (193). The possibilities that such a protein may constitute an integral part of the membranes of the tubules involved in secretion and that it may be lost during the homogenation and isolation procedures (145) are thought provoking. Furthermore, the finding that radioactive calcium entering the chromaffin cell during stimulation readily penetrates the chromaffin granule (160) is suggestive of a pooling of calcium in the vicinity of the secretory elements of the chromaffin cell for the purpose of triggering catecholamine release.

MECHANISMS OF TERMINATION OF SECRETORY RESPONSE OF ADRENAL MEDULLA

Since parallels between secretion and muscle contraction have been described with respect to release of secretory products from whole cells and from isolated granules, and since the molecular mechanisms of release of catecholamines have, in fact, been proposed to be contractile in nature[2] (84, 112, 145), further biochemical similarities between secretion and contraction can be outlined.

One very important aspect of the contractile system in muscle is that the concentration of ionized calcium in the myoplasm is maintained at low levels, and the sarcoplasmic reticulum has been shown to possess an ATP-activated uptake mechanism which reduces free intracellular calcium levels (194). It would be expected that secretory cells would also have some active mechanisms for keeping cytoplasmic levels of free ionized calcium low. Indeed, Borowitz (195) demonstrated that calcium is readily bound to subcellular organelles of the adrenal medulla. Poisner and Hava (168) reported that microsomes from the adrenal medulla can bind calcium in the presence of ATP and that this ATP-activated binding of calcium is inhibited by agents [*e.g.*, quinidine, amytal, thiocyanate, strontium, barium, and *p*-hydroxymercuribenzoate (168)] that are known to block calcium uptake by muscle microsomes (196, 197).

Recently, Haugaard *et al.* (198) demonstrated an active uptake of calcium by mitochondria in cardiac muscle, and they speculated on the role of this uptake mechanism in the regulation of contraction and relaxation of the myocardial cell. A similar ATP-activated calcium uptake mechanism in mitochondria of adrenal medullary chromaffin cells was described (168), which was inhibited by azide, oligomycin, and 2,4-dinitrophenol.

The adrenal medullary chromaffin granules may also play a role in the termination of an evoked secretory response. The fact that calcium readily penetrates the chromaffin granule during stimulation (160), coupled with the demonstration that the ATP–Mg^{+2}-dependent pump in the granule has many properties of the calcium pump in muscle (192), would indicate that the chromaffin granule may act as an active sequestrating organelle for calcium. The adrenal chromaffin granules have also been shown to take up calcium during catecholamine synthesis, since stimulation of the adrenal glands with acetylcholine in the presence of 3,4-dihydroxyphenylalanine (dopa) and ^{45}Ca resulted in a greater accumulation of ^{45}Ca in the granule fraction than stimulation in the absence of dopa (174). Furthermore, a relatively large amount of the intragranular calcium was found to be held in a nondiffusible form and was considered part of the binding complex for catecholamines in the adrenal medulla (174). It is possible, therefore, that uptake of calcium into the chromaffin granule may serve the dual purpose of terminating the secretory response (81) and of participating in the synthesis and storage of new catecholamines (174) to replenish the granules following an evoked secretion of their contents.

Termination of the secretory response or of muscle contraction could conceivably also be brought about by an active pumping of calcium out of the cell against a concentration gradient. This could be achieved by means of the outwardly directed membrane sodium–calcium pump, similar to the one described by Blaustein and Hodgkin (199) and by Reuter and Seitz (200). An indirect confirmation of such a mechanism derives from the finding that ouabain, which blocks the membrane sodium–calcium pump and increases the concentration of intracellular calcium, enhances both spontaneous and carbachol-induced catecholamine secretion from the adrenal medulla (154) and exhibits positive inotropic effects on the myocardium.

The described mechanisms for the termination of the secretory response of the adrenal medulla received further confirmation from Borowitz (167) who showed that the secretory response to acetylcholine, which is normally terminated in a few seconds after the removal of the stimulus, is prolonged under conditions that promote an influx of large amounts of calcium into the chromaffin cell—such as high concentration of extracellular calcium. The slow removal (either by intracellular binding or by efflux) of the relatively large amounts of calcium from the adrenal medullary cells may account for the prolonged catecholamine release under these conditions (167). Local anesthetics block the influx of calcium as well as the secretory response of the adrenal medulla (57, 161, 167) and also inhibit contractility and associated calcium movements in both striated and smooth muscles (201, 202).

Drugs and other factors that release intracellular calcium or block its uptake into intracellular organelles have profound effects on muscle by initiating contraction or delaying relaxation (50, 203). In an analogous sense, it would be expected that agents or factors that increase the intracellular calcium-ion concentration in the adrenal medullary chromaffin cell could initiate the release of catecholamines or, alternatively, potentiate or prolong an evoked secretory response. From the close parallelism between muscle and the adrenal medulla with respect to calcium binding, as already described, it might be predicted that drugs or factors that affect muscle function by influencing calcium mobility would have the same type of effect on adrenal medullary secretion: catecholamine release would be analogous to muscle contraction, and termination of the secretory response would correspond to relaxation (168). Indeed, agents such as caffeine (56, 57), barium, thiocyanate, sulfhydryl reagents, and ouabain (145, 168), all of which increase the level of free calcium in the cytoplasm by influencing intracellular calcium movements and binding, have been shown to potentiate or evoke adrenal medullary secretion and also to contract muscle (145). Furthermore, anoxia, nitrogen, and cyanide (190) increase spontaneous or evoked catecholamine release from the adrenal medulla, and cooling enhances the secretion of vasopressin from the neurohypophysis in the absence of extracellular calcium (186). These observations could be related to a diminished mitochondrial or microsomal calcium uptake (168)—mechanisms that may well be responsible for the termination of the secretory response (145, 168).

PHARMACOLOGICAL MECHANISMS OF STIMULUS–SECRETION COUPLING IN ADRENAL MEDULLA

Somlyo and Somlyo (203) broadly classified the mechanisms of smooth muscle contraction into "electromechanical coupling" (regulation of the contractile system by changes in the membrane potential, action potential frequency, or both) and "pharmacomechanical coupling" (the action of compounds on the contractile system independent of the membrane potential and action potential). Evidence that similar mechanisms apply to skeletal and cardiac muscle is extensive (50, 198, 204). Since, as previously discussed, similar molecular mechanisms appear to underlie the processes of excitation–contraction coupling in muscle and stimulus–secretion coupling in the adrenal medulla and other secretory cells (78, 81, 145, 168), a similar classification of the mechanisms of stimulus–secretion coupling was proposed by Rahwan *et al.* (57). The terms electromechanical coupling and pharmacomechanical coupling could justifiably be retained for this purpose, since there is mounting evidence that the process of secretion may indeed involve a contractile mechanism[2] (145, 146). The possible subcellular mechanisms by which drugs may evoke secretion are as follows:

A. Electromechanical coupling (dependent upon extracellular calcium): membrane depolarization, leading to increased permeability to inorganic ions, and an influx of extracellular calcium into the secretory cell
B. Pharmacomechanical coupling (dependent upon intracellular calcium):
   1. Release of calcium from the endoplasmic reticulum
   2. Inhibition of calcium uptake by the endoplasmic reticulum
   3. Release of calcium from mitochondria
   4. Inhibition of calcium uptake by mitochondria
   5. Release of calcium from cell membrane storage sites
   6. Inhibition of calcium binding by the cell membrane
   7. Inhibition of calcium efflux from the cell by inhibition of the membrane sodium–calcium pump
C. Mechanisms independent of extracellular or intracellular calcium:
   1. Direct interaction with intracellular contractile proteins
   2. Inhibition of hypothetical "inhibitory factor" (troponin?)
   3. Interference with the intragranular or intratubular storage mechanism
   4. Displacement of secretory product from intragranular or intratubular storage by false transmitter-type effect
D. Physical damage to cell membranes or membranes of intracellular organelles of the secretory cell, leading to passive diffusion of calcium and/or secretory product

It is conceivable that secretagogues like acetylcholine, nicotine, carbachol, pilocarpine, potassium, hista-

mine, serotonin, bradykinin, and angiotensin I evoke catecholamine secretion from the adrenal medullary cell by electromechanical coupling (57), involving depolarization of the chromaffin cell membrane, with a dependency on extracellular calcium (26, 27, 150, 155, 156). On the other hand, drugs that evoke catecholamine secretion from the adrenal medulla in the absence of extracellular calcium [*e.g.*, caffeine, aminophylline, chlorpromazine, dextroamphetamine, reserpine, tyramine, phenylethylamine, barium, strontium, thiocyanate, and acetaldehyde (33, 55, 57, 58, 152, 163, 168, 169)] could conceivably exert their secretory effect by pharmacomechanical coupling (57), with a dependency on intracellular calcium.

In addition to electromechanical and pharmacomechanical coupling, other alternative mechanisms for drug-induced catecholamine secretion from the adrenal medulla should be considered, such as interference with the catecholamine storage mechanism (independent of both extracellular and intracellular calcium) or physical damage to biological membranes. For example, acetaldehyde releases catecholamines from the adrenal medulla presumably by a direct interaction between the aldehyde and the chromaffin granule protein, resulting in a possible alteration of the chromaffin granule membrane structure (55), or by an interaction between the aldehyde and the catecholamines forming the tetrahydroisoquinoline derivatives which act as "false transmitters" (205). Both interpretations are consistent with the finding of Schneider (55) that acetaldehyde-induced catecholamine secretion from the adrenal gland and from isolated chromaffin granules, which occurs in the absence of calcium in the perfusion or incubation medium, is not accompanied by the release of intragranular or cytoplasmic proteins. Rahwan (146) and Rahwan *et al.*[2] (57) reported that chlorpromazine evoked catecholamine secretion from the adrenal medulla by physical disruption of, and mobilization of calcium from, the mitochondria of the chromaffin cells. Chlorpromazine-induced mitochondrial structural damage in the adrenal medulla was also reported by Mäkelä and Vapaatalo (206). It is conceivable that the reserpine-induced catecholamine release from the adrenal medulla, which occurs in the absence of extracellular calcium (33, 152), may represent a calcium-independent mechanism based on interference with the intragranular storage mechanism (30). Tyramine may also release adrenal catecholamines by a mechanism that does not require calcium (152), analogous possibly to its ability to displace norepinephrine from storage granules in adrenergic nerve endings (207). This is supported by the finding that tyramine-induced adrenal catecholamine secretion is not accompanied by release of intragranular protein or dopamine-β-hydroxylase (58). Similar results were reported by Schneider (58) for the effect of *l*-amphetamine on the adrenal medulla. Likewise, the initial phase of the secretory effect of dextroamphetamine on the adrenal medulla was reported by Rahwan *et al.* (57) to be calcium independent and to involve a tyramine-like displacement of catecholamines from their storage sites into the cytoplasm from where the amines could be recovered.

It is likely that pharmacomechanical mechanisms of secretion may represent the primary means by which some drugs evoke secretion even in the presence of extracellular calcium (57). It is also conceivable that electromechanical and pharmacomechanical mechanisms may be operative simultaneously under physiological conditions where calcium is present in the cellular environment.

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Effects of Disopyramide on Electrophysiological and Mechanical Properties of the Heart

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Abstract □ Studies were performed in whole dog and in isolated superfused canine Purkinje fiber–papillary muscle preparations to investigate mechanisms responsible for the antiarrhythmic effects of disopyramide. Two human left atrial appendages were also studied *in vitro*. Disopyramide had a potent quinidine-like effect which may explain the antiarrhythmic properties of disopyramide. While disopyramide is perhaps at least twice as potent as quinidine in suppressing experimentally induced arrhythmias, disopyramide also has at least proportionally more depressant effect on cardiac mechanical performance in the dog heart.

Keyphrases □ Disopyramide—effects on electrophysiological and mechanical properties of the heart, *in vitro*, *in vivo* □ Antiarrhythmic activity, disopyramide—effects on electrophysiological and mechanical properties of the heart, *in vitro*, *in vivo* □ Quinidine-like activity—effects of disopyramide on *in vitro* and *in vivo* electrophysiological and mechanical properties of the heart □ Cardiac effects, electrophysiological and mechanical—disopyramide

The use of quinidine and quinidine-like drugs in the treatment of both supraventricular and ventricular arrhythmias, due to a variety of etiologies, remains one basic form of therapy for cardiac arrhythmias. The pharmacology of a new antiarrhythmic agent disopyramide[1], [2-(diisopropylamino)ethyl]-α-phenyl-2-pyridineacetamide, has been compared with that of quinidine (1). It was found that disopyramide was at least twice as potent as quinidine in abolishing arrhythmias induced experimentally by repetitive electrical stimulation, by local application of aconitine nitrate, by coronary artery ligation, and by ouabain infusion. The acute toxicity of disopyramide was reported to be relatively low, the intravenous therapeutic ratio in dogs being about 10 (1).

These data led to limited clinical trials of disopyramide (2). According to Katz *et al.* (2), disopyramide converted 11 of the 19 subjects with various arrhythmias to normal sinus rhythm; two patients (one with chronic atrial fibrillation and one with frequent premature ventricular contractions) who did not respond to quinidine therapy were subsequently converted by disopyramide at doses less than 50% of the quinidine dosage. At present, larger scale clinical trials of disopyramide are underway[2]. This paper reports the effects of disopyramide on the electrophysiological properties of the dog heart and human atrial tissue to elucidate the antiarrhythmic actions of disopyramide. Also the effects of disopyramide on canine cardiac mechanical performance were evaluated so that the effects of disopyramide on the electrophysiological and mechanical parameters in the dog heart could be correlated.

EXPERIMENTAL

***In Vivo* Studies**—The cardiac effects of intravenous injections of disopyramide were evaluated in six adult mongrel dogs anesthetized with sodium pentobarbital (30 mg./kg.) and maintained on controlled respiration. Arterial blood pressure was recorded continuously using a transducer[3]. An electrode catheter was introduced into the right atrium for pacing and recording. His bundle electrograms were recorded by use of another bipolar electrode catheter positioned near the A–V node as previously described (3). Thus, the A–V conduction time could be divided into three time segments: intraatrial, A–V nodal, and intraventricular. Cardiac pacing at various rates was used to stress A–V conduction. The effects of right vagal stimulation (2 msec., 5 v., 20 Hz.) on sinus rate, A–V conduction, and ventricular automaticity were also evaluated in each experiment.

A micromanometer catheter was introduced into the left ventricle *via* a common carotid artery for recording intraventricular pressure. Left ventricular dP/dt was obtained using an operational amplifier. In addition, a strain-gauge arch was sutured on the right ventricular wall for monitoring a representative segment of myocardial contractile force (4).

After control records were obtained during normal sinus rhythm and atrial pacing at various rates, disopyramide (10 mg./ml. dissolved in normal saline) was administered slowly by vein at a dose of 3 mg./kg. Serial recordings of sinus rate, A–V conduction, ventricular automaticity, blood pressures, left ventricular pressures and dP/dt, and myocardial segment contractility were monitored up to 2 hr. after drug injection.

Two hours after the injection of the first dose of disopyramide, a second dose of 5 mg./kg. was given to all animals. Thus, each animal received a cumulative dose of 8 mg./kg. of disopyramide. Serial recordings of sinus rate, cardiac conduction, ventricular automaticity, blood pressures, and ventricular mechanical performance were again obtained up to 2 hr. after the second dose of disopyramide.

In three dogs, the effects of the intravenous injection of quinidine, 5 and 8 mg./kg., were evaluated and compared to disopyramide injections.

***In Vitro* Studies**—Seven canine Purkinje fiber–papillary muscle preparations and two human left atrial appendages (one obtained from a 56-year-old man who underwent coronary bypass surgery and the other obtained from a teen-aged boy who underwent corrective surgery for tetralogy of Fallot) were studied. Intracellular and extracellular recordings were obtained by conventional electrophysiological techniques under control conditions, during drug infusion (1 or 2 mg./l.), and after washing out the drug. Doses of disopyramide up to 2 mg./kg. have been used in man, and it was thought that the concentration range of the drug used in these studies would be within the clinical therapeutic level. The preparations were superfused with modified Tyrode's solution (K = 4.0 meq./l.) at 37°.

[1] Norpace, SC-7031.
[2] W. Smith, personal communication, 1972.
[3] Statham.

Table I—Effects of Disopyramide and Quinidine on Sinus Rate[a]

	Time after First Dose				Time after Second Dose		
	0 min.	3 min.	30 min.	120 min.	3 min.	30 min.	120 min.
		3 mg./kg.				**5 mg./kg.**	
Disopyramide group	151 ± 8	117 ± 10**	129 ± 9*	135 ± 12*	114 ± 8+	124 ± 9**	128 ± 9*
		5 mg./kg.				**8 mg./kg.**	
Quinidine group	132 ± 10	129 ± 6	134 ± 11	140 ± 20	145 ± 19	139 ± 22	140 ± 18

[a] Values are mean ± *SE.* * = $p < 0.05$; ** = $p < 0.01$; + = $p < 0.001$.

The preparations were driven at a fixed rate of between 60 and 100/min. in canine preparation and of between 30 and 60/min. in human atrial preparations. The refractory period and the membrane responsiveness were studied by applying an extra stimulus (with adjustable delays) during the repolarization phase of every 10th driven beat.

Statistical Analysis—Paired *t* tests were performed to determine changes induced by the drugs in the same preparation. Nonpaired *t* tests were used to determine whether the differences observed between two groups were statistically significant. The *p*-values were expressed as nonsignificant (>0.05) and significant at three levels (<0.05, <0.01, and <0.001).

RESULTS

***In Vivo* Studies**—*Effects of Disopyramide on Automaticity*—A depression of sinus automaticity was noticed after disopyramide injection. Table I compares the effects of disopyramide and quini-

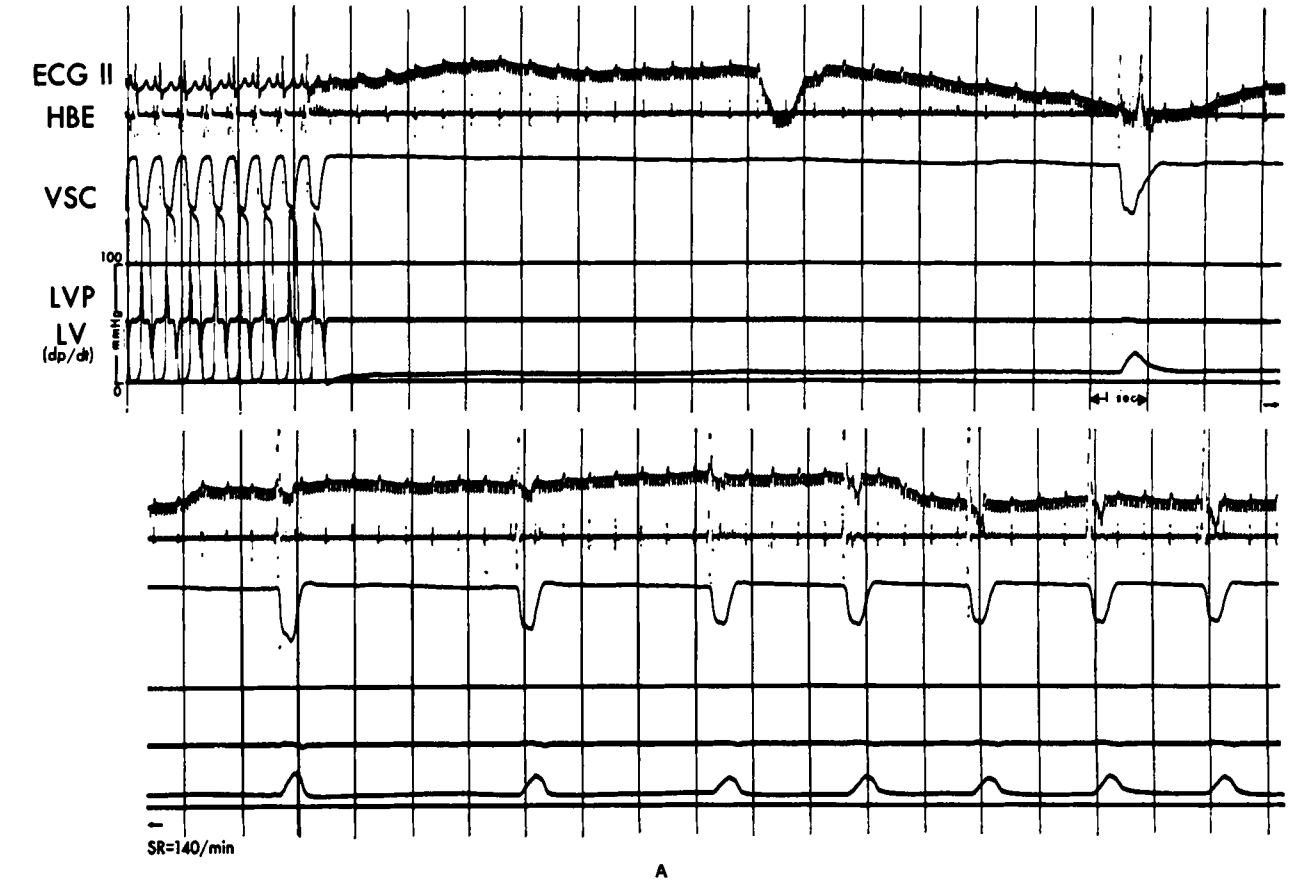

Figure 1—*Atropine-like activity of disopyramide revealed by vagal stimulation. Tracings from top to bottom are: surface ECG lead II, His bundle electrogram (HBE), right ventricular segmental contractility (VSC) recorded by a strain-gauge arch, left ventricular pressure (LVP), and the first derivative of LVP (LV, dP/dt). A shows the control right vagal stimulation (5 v., 20 Hz.) resulting in sinus arrest, with an escape time of 14 sec. and a slow idioventricular rhythm of 30/min. B shows responses to the same vagal stimulation 15 min. after 3 mg./kg. disopyramide. There was some slowing of the sinus rate during vagal stimulation and no ventricular automaticity was seen, due to inhibition of ventricular pacemakers by supraventricular conducted beats.*

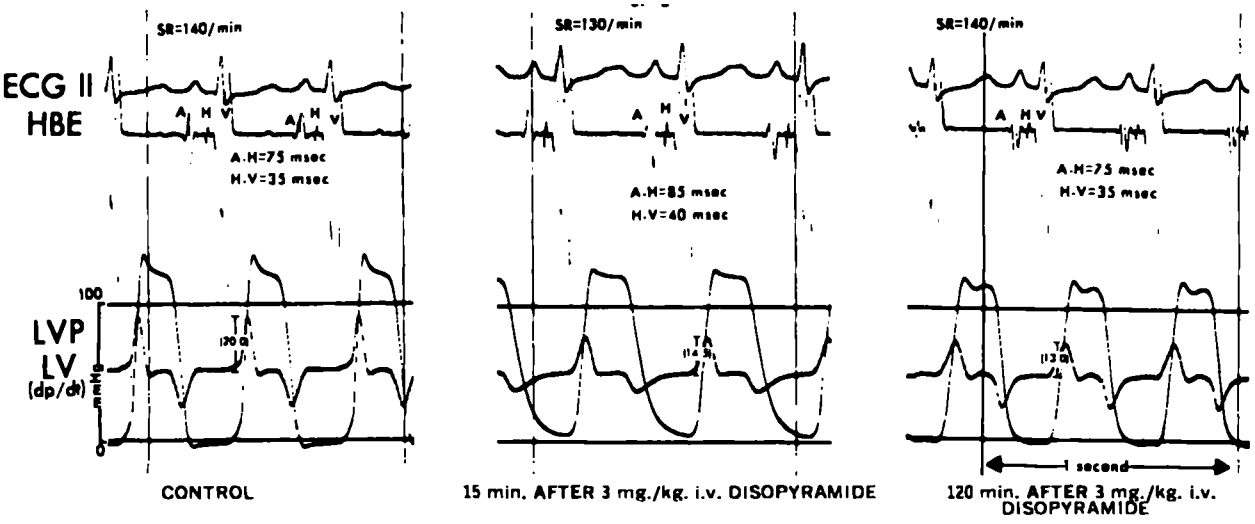

Figure 2—*Effects of disopyramide on sinus rate, A–V conduction, and left ventricular pressures. The top tracing is surface ECG lead II. The labels in the His bundle electrogram (HBE) are: A, activation of atrial septum near the A–V node; H, His bundle electrogram; and V, ventricular electrogram. The left panel is the control. Disopyramide, 3 mg./kg., caused some slowing of the sinus rate associated with A–V nodal and intraventricular conduction delay. Disopyramide also caused a decrease of systolic pressure and* dP/dt *and a rise in end diastolic pressure of the left ventricle. This dog recovered from the electrophysiological effects of disopyramide within 2 hr., while the depression of left ventricular mechanical performance was still evident.*

dine on sinus rate. It is evident that disopyramide had a greater depressant effect than quinidine on sinus automaticity and that this depressant effect lasted longer than 2 hr.

Disopyramide also had a much more potent vagolytic effect than quinidine. Ventricular automaticity could not be evaluated after disopyramide because vagal stimulation failed to cause sinus arrest (Fig. 1). This inhibition of the vagal effect on sinus automaticity by disopyramide lasted more than 2 hr. after a 3-mg./kg. i.v. injection of disopyramide. No such vagolytic effect was observed after administration of quinidine, even at the 8-mg./kg. dose.

Effects of Disopyramide on Cardiac Conduction—At both dose levels (3 and 5 mg./kg.), disopyramide caused significant conduction delay in both the A–V node and the specialized ventricular conduction system, although there was an associated decrease in sinus rate (Figs. 2 and 3). The lower dose of quinidine (5 mg./kg.) had no effect on cardiac conduction; the higher dose (8 mg./kg.) caused a slight depression of both A–V nodal and intraventricular conduction, which, however, was not as marked as those induced by 3 mg./kg. disopyramide. The depression of A–V nodal conduction lasted somewhat longer than the depression of intraventricular conduction with both disopyramide and quinidine (8 mg./kg.). Table II summarizes the results obtained using His bundle recordings.

The direct depressant effect of disopyramide on A–V conduction overwhelmed its vagolytic effect. Wenckebach cycles developed at a mean pacing rate of 340 beats/min. under control conditions and of 284 beats/min. 3 min. after 3 mg./kg. disopyramide. This depression of A–V nodal conduction persisted for about 1 hr. and disappeared by the end of the 2nd hr. Quinidine, 5 mg./kg., caused a similar qualitative depression of A–V nodal conduction, but this effect was only revealed by pacing.

Effects of Disopyramide on Arterial Blood Pressure—No change in mean arterial blood pressure was noticed after the injection of 3 mg./kg. disopyramide. However, higher doses of disopyramide (5 mg./kg.) caused an average 11-mm. Hg decrease in mean blood pressure. Compared to "equiantiarrhythmic" doses, quinidine had a more potent depressor effect (a 16-mm. Hg decrease in mean blood pressure was noted after 5 mg./kg. quinidine) than disopyramide (Table III).

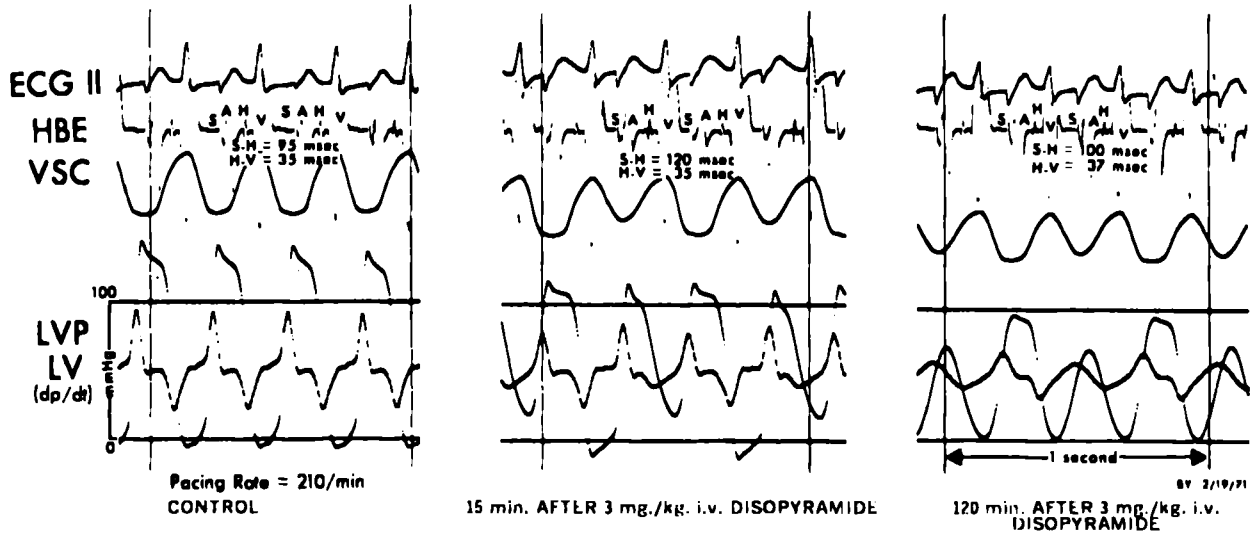

Figure 3—*Effects of 3 mg./kg. i.v. disopyramide on the dog heart. Comparisons were made with the heart rate controlled by atrial pacing at a fixed rate, 210/min. S is the stimulus artifact, and other labels are similar to those in previous figures. The left panel is the control. The middle panel shows the prolonged supraventricular conduction time (S–H interval) and depression of ventricular function (decrease in ventricular systolic pressure and* dP/dt *and alternate contractions) 15 min. after disopyramide injection. The right panel again shows the recovery of the depressant electrophysiological effects of disopyramide, with depression of ventricular mechanical performance remaining.*

Table II—Effects of Disopyramide and Quinidine on A-V Nodal and Intraventricular Conduction[a]

		Time after First Dose			Time after Second Dose		
		0 min.	3 min.	30 min.	120 min.	3 min.	30 min.	120 min.
			3 mg./kg.			**5 mg./kg.**		
Disopyramide group	A-H	56 ± 2	70 ± 6**	64 ± 5**	61 ± 4	75 ± 6*	66 ± 4*	64 ± 4
	H-V	34 ± 1	37 ± 2**	34 ± 2	31 ± 1	38 ± 2*	32 ± 1	32 ± 1
			5 mg./kg.			**8 mg./kg.**		
Quinidine group	A-H	58 ± 2	58 ± 2	54 ± 1	53 ± 2	56 ± 5*	59 ± 1	64 ± 0
	H-V	30 ± 0	30 ± 0	30 ± 0	30 ± 0	34 ± 1	29 ± 1	32 ± 1

[a] Values are mean ± *SE* in msec. * = $p < 0.05$; ** = $p < 0.01$.

Table III—Effects of Disopyramide and Quinidine on Mean Arterial Blood Pressure[a] (mm. Hg) in Anesthetized Dogs

	Time after First Dose				Time after Second Dose		
	0 min.	3 min.	30 min.	120 min.	3 min.	30 min.	120 min.
		3 mg./kg.			**5 mg./kg.**		
Disopyramide group	103 ± 9	102 ± 11	111 ± 9	116 ± 9	105 ± 10*	112 ± 10	111 ± 8
		5 mg./kg.			**8 mg./kg.**		
Quinidine group	115 ± 8	99 ± 10**	106 ± 6*	122 ± 4	107 ± 6**	113 ± 4*	117 ± 2

[a] * = $p < 0.05$; ** = $p < 0.01$.

Table IV—Effects of Disopyramide and Quinidine on Left Ventricular Pressures[a] (mm. Hg) and Maximum *dP/dt* (mm. Hg/sec.)

	Systolic Pressure		End Diastolic Pressure		*dP/dt*	
	Disopyramide Group	Quinidine Group	Disopyramide Group	Quinidine Group	Disopyramide Group	Quinidine Group
Control	127 ± 9	103 ± 7	6 ± 3	5 ± 0	2020 ± 160	2050 ± 40
	3 mg./kg.	**5 mg./kg.**	**3 mg./kg.**	**5 mg./kg.**	**3 mg./kg.**	**5 mg./kg.**
3 min. after first dose	115 ± 9	111 ± 6*	8 ± 2*	8 ± 2	1370 ± 170**	1820 ± 88
30 min. after first dose	126 ± 5	118 ± 7*	8 ± 2	7 ± 1	1680 ± 90*	1700 ± 70
120 min. after first dose (second control)	128 ± 5	146 ± 1	5 ± 2	5 ± 4	1540 ± 120*	1950 ± 230
	5 mg./kg.	**8 mg./kg.**	**5 mg./kg.**	**8 mg./kg.**	**5 mg./kg.**	**8 mg./kg.**
3 min. after second dose	118 ± 7	130 ± 4*	10 ± 2*	5 ± 4	1100 ± 110**	1800 ± 200
30 min. after second dose	123 ± 6	147 ± 7	8 ± 3*	6 ± 4	1270 ± 140*	2100 ± 220
120 min. after second dose	120 ± 6	136 ± 1	8 ± 3	5 ± 2	1470 ± 180	2300 ± 200

[a] * = $p < 0.05$; ** = $p < 0.01$.

Effects of Disopyramide on Ventricular Performance—Both disopyramide and quinidine caused a depression in left ventricular systolic pressure and an increase in end diastolic pressure. These changes in left ventricular pressures lasted about 2 hr. Similar but less evident depression of ventricular contractility was observed with strain-gauge recordings (Fig. 3). Disopyramide was found to have a much more pronounced depressant effect than quinidine on the maximum *dP/dt* of the left ventricle. This depressant effect persisted longer than 2 hr. (Fig. 2). Thus, the depressant effect of disopyramide on cardiac mechanical performance persisted when the electrophysiological effects of the drug had disappeared (Table IV).

Depression of ventricular mechanical performance by disopyramide and quinidine was also demonstrated by pacing. The ability of the ventricular muscle to respond mechanically to pacing was impaired, and "pulsus alternans" developed at a lower driving rate after injection of the drugs (Fig. 3).

***In Vitro* Studies**—Superfusion with Tyrode's solution containing disopyramide (1–2 mg./l.) significantly prolongated the action potential duration, decreased the rate of maximum depolarization (*dV/dt*) maximum, and caused conduction delay without significantly affecting the resting potential or the overshoot (Figs. 4 and 5). Therefore, the electrophysiological properties of disopyramide can be classified as "quinidine-like." The prolongation in action potential duration probably was the first electrophysiological change noted, and it became evident after 20 min. of superfusion with 1 mg./l. disopyramide. The maximal depressant effects of disopyramide were reached within 1 hr., and some drug effects persisted even after a 30-min. washout period. Table V shows data obtained from one canine preparation.

Depression of electrophysiological parameters occurred earlier and was more pronounced when 2 mg./l. disopyramide was used. Qualitatively similar responses were seen in human atrial tissues, but the human atrial muscle fibers seemed to be more resistant to the depressant effects of disopyramide than were the canine Purkinje fibers (Fig. 6).

As expected, the membrane responsiveness curves were shifted to the right after perfusion with disopyramide. This shift in membrane responsiveness was also associated with a significant depression of maximum *dV/dt* after complete repolarization when higher doses of disopyramide were used (Fig. 7).

No spontaneous automaticity was observed in human left atrial

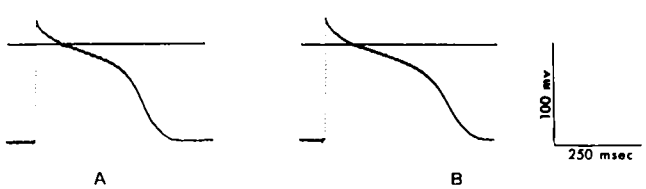

Figure 4—*Effects of 1 mg./l. disopyramide on the action potential duration of a canine Purkinje fiber driven at a cycle length of 630 msec. The control (A) action potential duration was 400 msec. Thirty minutes after exposure to disopyramide (B), the action potential had increased to 450 msec.*

Table V—Electrophysiological Effects of 1 mg./l. Disopyramide on Canine Purkinje–Muscle Preparations[a]

	Resting Potential, mv.	Overshoot, mv.	Action Potential Amplitude, mv.	$(dV/dt)_{max}$, v./sec.	Conduction Time, Purkinje Fiber to Muscle, msec.	80% Action Potential Duration, msec.
Control	92 ± 1	25 ± 1	117 ± 1	670 ± 20	8.6 ± 1.6	302 ± 15
20 min. after disopyramide, 1 mg./l.	89 ± 2	27 ± 2	116 ± 2	604 ± 24	8.8 ± 1.4	343 ± 16
60 min. after disopyramide, 1 mg./l.	91 ± 1	28 ± 1	119 ± 1	462 ± 20	10.6 ± 1.2	429 ± 20
30 min. after washout	90 ± 1	27 ± 2	117 ± 1	559 ± 15	9.6 ± 1.4	378 ± 20

[a] The values are the mean ± *SE* for seven experiments.

preparations. The canine Purkinje-papillary muscle preparations also had slow spontaneous phase 4 depolarization, so the effects of disopyramide on automaticity could not be properly evaluated in these *in vitro* preparations. However, judging from the decrease in inward sodium current caused by the drug, disopyramide might have a depressant effect in preparations exhibiting a high spontaneous rate.

DISCUSSION

Disopyramide in doses of 3 or 5 mg./kg. i.v. caused a slowing of sinus rate in intact dogs, presumably through the decrease of the slope of phase 4 depolarization of sinoatrial pacemakers. Disopyramide, 1 or 2 mg./l., caused a shift of membrane responsiveness curves to the right. These direct quinidine-like properties of disopyramide are consistent with its clinical effectiveness in the suppression or abolition of arrhythmias due to enhanced phase 4 depolarization.

Disopyramide also prolonged action potential duration and increased the effective refractory period. Both effects of disopyramide can result in the abolition of premature "contractions"; if the premature depolarization reaches a refractory tissue, it will not elicit an effective response and the premature contraction will be suppressed. The prolongation of the action potential and refractory period could also account for a beneficial therapeutic response in arrhythmias resulting from reentrant mechanisms. Disopyramide would be effective if the slowing of conduction induced by the drug were proportionally less than the prolongation in the effective refractory period, *i.e.*, if the conduction speed in the circus pathway were relatively accelerated by the drug. In Purkinje–muscle preparations, disopyramide caused a greater prolongation of the refractory period than conduction velocity (*cf.*, Table V); thus, it may abolish arrhythmias caused by unidirectional conduction delay and reentry.

These data indicate that disopyramide is a quinidine-like drug, and its antiarrhythmic properties could be explained on the basis of: (*a*) a possible depression of unduly enhanced phase 4 depolarization, (*b*) decreased membrane responsiveness, and (*c*) prolongation of the refractory period.

The electrophysiological effects of disopyramide obtained from our microelectrode studies are qualitatively similar to those reported by Sekiya and Vaughan Williams (5). However, quantita-

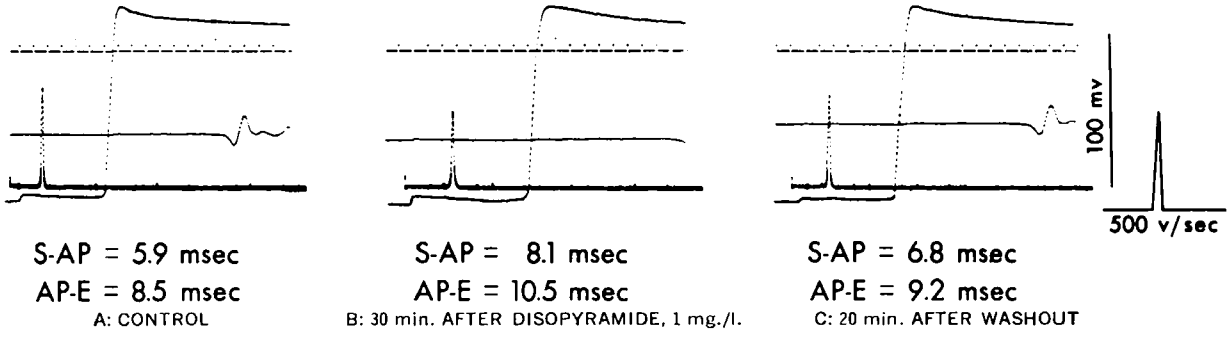

Figure 5—*Effects of 1 mg./l. disopyramide on resting potential, maximum rate of depolarization* (dV/dt *max*), *and conduction velocity in a canine Purkinje fiber-papillary muscle preparation. A is the control. The top tracing is the zero reference line with time marks of 1 and 10 msec. The second tracing shows the surface electrogram (E) recorded from the distal end of the specialized ventricular conduction system. The third tracing shows the* dV/dt *max of the action potential, and the bottom tracing shows the action potential (AP) recorded from a Purkinje fiber in the proximal end of the same strand of specialized ventricular conduction system. Stimulus artifact (S) can be seen at the beginning of the third and the fourth tracings. B shows that 1 mg./l. disopyramide decreased the maximum rate of rise of the action potential and conduction velocity, without affecting either the resting potential or the overshoot. These effects of disopyramide are largely reversible, and C shows the improvement of* dV/dt *max and conduction velocity after washout. Calibrations for voltage and* dV/dt *are indicated on the right side of the figure.*

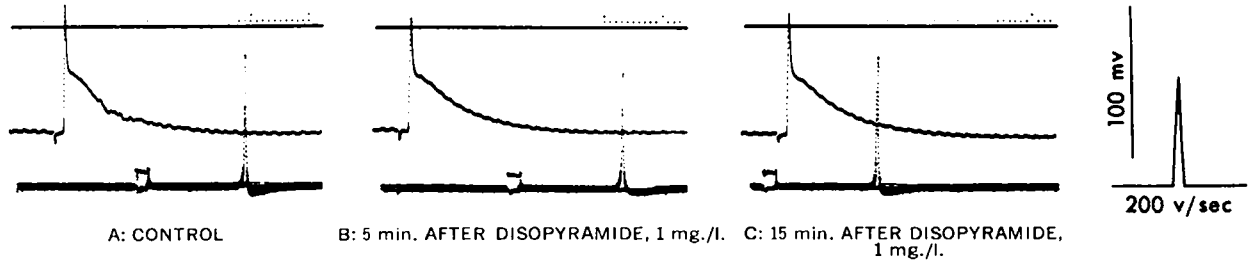

Figure 6—*Effects of disopyramide on the action potential of a human atrial muscle fiber. A is the control. The top tracing is the zero reference line showing time marks of 10 and 100 msec. The square pulse on the bottom tracing shows the stimulus intensity (10% above threshold), and the maximum rate of depolarization* (dV/dt *max*) *is also registered. Note that 1 mg./l. disopyramide caused a slight and transient depolarization, associated with a decrease in* dV/dt *max and overshoot (B). The atrial cell recovered quickly despite the continued presence of disopyramide in the perfusate (C).*

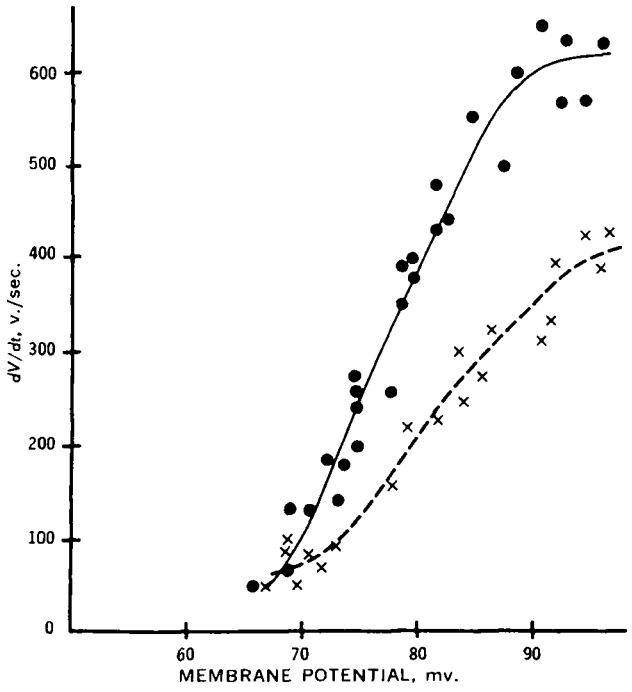

Figure 7—*Effects of disopyramide on membrane responsiveness in dog Purkinje fibers. The control curve is one characteristic of a normal Purkinje fiber, with the maximum rate of rise* (dV/dt) *of about 600 v./sec. It is evident that 2 mg./l. disopyramide caused a shift of the membrane responsiveness curve to the right, and there was also depression of* dV/dt *to around 400 v./sec., while the maximum resting potential remained unchanged (90–95 mv.). The measurements were made 45 min. after exposure to disopyramide. Key:* ●—●, *control; and* ×- - -×, *after 2 mg./l. disopyramide.*

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Like quinidine, disopyramide also depresses myocardial contractility and left ventricular function. It would be an undesirable property if the depressant effect of disopyramide on myocardial performance, which outlasts its electrophysiological effects in the dog heart, is also found in man.

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Pharmacokinetics of Iodochlorhydroxyquin in Man

D. B. JACK and W. RIESS▲

Abstract □ A method of extracting iodochlorhydroxyquin from plasma and quantitating by GLC was developed and used to study the pharmacokinetics in man. A clearcut dose–plasma concentration relationship was obtained, and no evidence of accumulation was found.

Keyphrases □ Iodochlorhydroxyquin—pharmacokinetics in man, dose–plasma concentration relationships, GLC determination □ Quinolines (iodochlorhydroxyquin)—GLC analysis of human plasma samples, pharmacokinetics □ Pharmacokinetics—iodochlorhydroxyquin half-life, dose–plasma concentration relationships, GLC determination □ GLC—analysis of iodochlorhydroxyquin in human plasma samples

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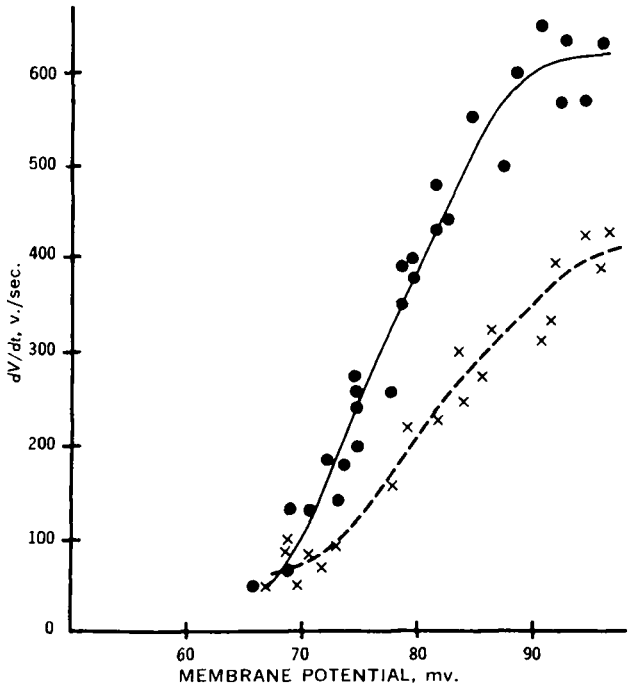
EXPERIMENTAL

By using the developed method, the kinetics in man were studied as follows.

Single-Dose Administration—Six volunteers were given single oral doses of 250 and 1500 mg. of iodochlorhydroxyquin powder[1]

[1] Iodochlorhydroxyquin was administered as a powder with the addition of 7% *N*-stearoyl-*N'*,*N'*-diethylethylenediamine (Sapamine). This formulation is available commercially as Entero-Vioform.



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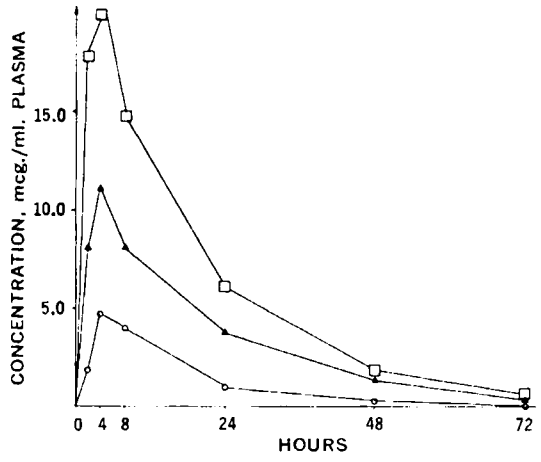

Figure 1—*Mean plasma levels of iodochlorhydroxyquin (micrograms per milliliter) after single oral doses of 250* (○), *750* (▲), *and 1500* (□) *mg. iodochlorhydroxyquin powder (six subjects per dose).*

in a crossover study, three subjects beginning with the 250-mg. and three with the 1500-mg. dose with 2 weeks between the two administrations.

Another group of six volunteers took a single oral dose of 750 mg. Blood was withdrawn from all subjects immediately before and 2, 4, 8, 24, 48, and 72 hr. after administration.

Multiple-Dose Administration—Six volunteers were given 500 mg. of iodochlorhydroxyquin powder three times daily at 8:00 a.m., 2:00 p.m., and 8:00 p.m. for 7 days followed by 250 mg. three times daily at the same times for an additional 7 days. Blood samples were withdrawn immediately before the first dose of the day on Days 1–5 and 8–12 and at 8:00 a.m. on the first 4 days following the end of administration.

Heparin[2] was added to all samples, which were then immediately centrifuged, and the plasma was stored at −20° until analysis.

Methods and Apparatus—GLC was carried out using a chromatograph[3] equipped with an electron-capture detector (^{63}Ni source) operated with a pulse space of 150 μsec. A 3.04-m. (10-ft.) glass column, 0.31 cm. (0.125 in.) i.d. filled with 3% OV-17 on Gas Chrom Q, 100 120 mesh[4], was used. The column oven temperature was kept at 205° with the injector block and detector oven temperatures at 230 and 340°, respectively. Nitrogen at a flow of 50 ml./min. was used as carrier gas. Under these conditions, the retention times of the internal standard 5,7-dichloro-8-acetoxyquinoline and of 5-chloro-7-iodo-8-acetoxyquinoline were 6.0 and 14.6 min., respectively.

Table I—Analysis of Plasma Samples Containing Iodochlorhydroxyquin

Amount Added, ng./ml.	Amount Found, ng./ml.
54	50
	60
107	92
	110
533	580
	490
1065	1152
	1044

Extraction—To correct for losses during the extraction, 5,7-dichloro-8-hydroxyquinoline (1000 ng.) was added to each plasma sample (up to 2 ml. used) before extraction. Water (14 ml.) was then added and the sample was extracted three times with 2-ml. portions of methylene chloride–ether (1:4). After centrifugation, the organic phase was removed and evaporated, and 200 μl. of acetic anhydride and 50 μl. of pyridine were added. The sample was placed in a water bath at 70° for 1 hr. and, after the addition of 4

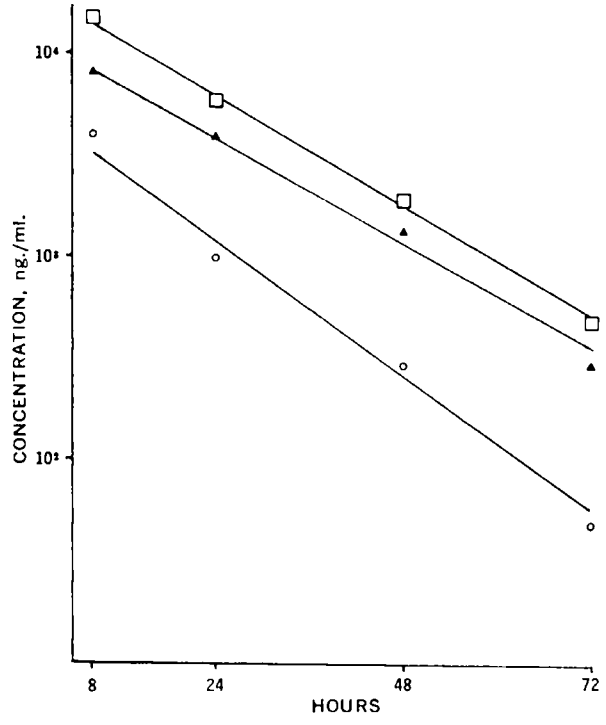

Figure 2—*Elimination of iodochlorhydroxyquin from plasma after single oral doses of 250* (○), *750* (▲), *and 1500* (□) *mg. iodochlorhydroxyquin powder (six subjects per dose).*

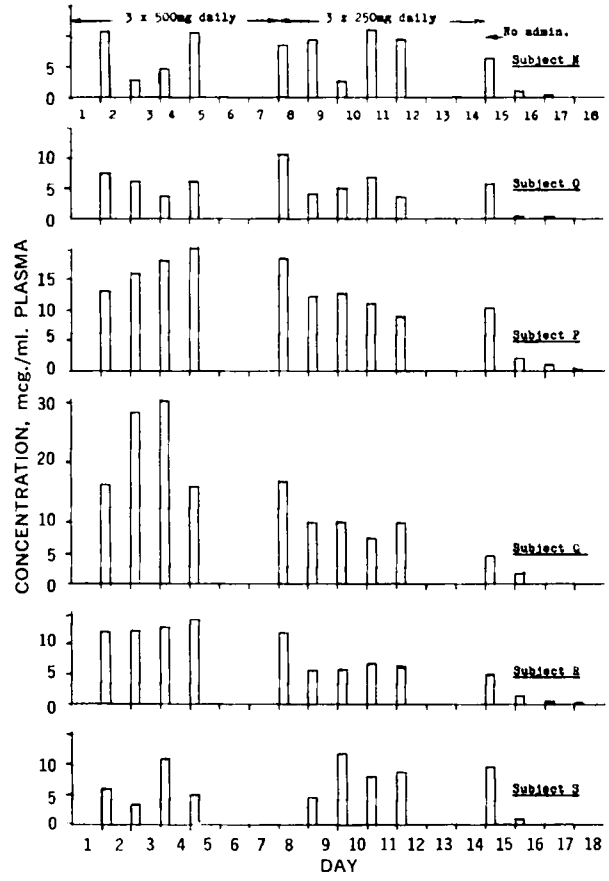

Figure 3—*Plasma levels of iodochlorhydroxyquin during multiple-dose administration of iodochlorhydroxyquin powder.*

[2] Liquemine, Roche.

[3] Pye 104.

[4] Applied Science Labs., Inc., State College, Pa.

Table II—Plasma Levels of Iodochlorhydroxyquin, Micrograms per Milliliter, after Single Oral Doses of Iodochlorhydroxyquin Powder

Dose, mg.	250						750						1500					
Subject	G	H	I	K	L	M	A	B	C	D	E	F	G	H	I	K	L	M
Hours																		
0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	1.2	1.0	1.7	4.4	2.0	1.5	4.8	2.7	18.0	8.7	8.3	6.5	6.2	12.9	19.5	34.8	9.2	24.8
4	3.8	1.4	1.6	8.0	6.9	7.1	8.6	4.2	23.2	10.5	9.8	10.3	16.6	14.6	33.5	24.7	14.7	16.9
8	4.0	3.0	1.5	8.3	3.8	3.5	5.4	3.6	15.8	8.9	6.6	9.3	16.7	14.2	15.2	25.4	10.9	6.5
24	0.6	1.0	0.7	1.7	1.3	0.7	4.1	2.9	5.8	3.1	3.4	4.2	11.7	6.2	5.7	7.8	2.9	3.1
48	0.2	0.3	0.1	0.5	0.5	<0.05	0.6	1.0	1.5	1.9	1.2	2.4	0.6	1.6	0.8	4.5	2.6	1.3
72	<0.05	<0.05	<0.05	0.2	0.2	<0.05	0.1	0.1	0.2	0.3	0.8	0.5	0.1	0.2	0.1	2.6	0.8	0.3

Table III—Standardized Areas under Plasma Concentration Curves, (Micrograms per Milliliter) (Hour)

Dose, mg./kg.	Subject A	B	C	D	E	F	G	H	I	K	L	M	Mean ± *SEM*
3.33	—	—	—	—	—	—	60	63	38	171	79	76	81 ± 19
10.00	189	175	246	240	265	207	—	—	—	—	—	—	220 ± 14
20.00	—	—	—	—	—	—	447	438	356	594	238	310	397 ± 51

ml. of water, extracted with 3 × 1 ml. ethyl acetate. The ethyl acetate was washed once with 2 ml. of saturated sodium bicarbonate, evaporated, and redissolved in 100 μl. ethyl acetate for injection.

In cases where more than 0.5 ml. of plasma was taken, it was necessary to add a TLC step. The ethyl acetate extract (100 μl.) was applied to a silica gel plate with a reference compound on either side, and the plate was developed in hexane–ether (1:3). The reference compound may be the 8-acetoxy derivative of either the 5,7-dichloro or 5-chloro-7-iodo compound, since they both have the same R_f value in this system. After development, the appropriate region is noted, scraped off, shaken with 1 ml. of saturated sodium bicarbonate, and extracted (3 × 0.5 ml.) with ethyl acetate. After centrifugation, the organic phase is evaporated and redissolved in 100 μl. ethyl acetate for injection. The use of 5,7-dichloro-8-hydroxyquinoline, both as an internal standard for the extraction and as the 8-acetoxy derivative for GLC, permitted the amount of iodochlorhydroxyquin present in the sample to be calculated from the peak area ratio of the two acetoxy derivatives. Plasma samples were prepared with added iodochlorhydroxyquin and analyzed using this method (Table I).

Table IV—Plasma Levels of Iodochlorhydroxyquin (Micrograms per Milliliter) Measured Immediately before the Morning Dose (8:00 a.m.) during Multiple-Dose Treatment with Iodochlorhydroxyquin Powder, 3 × 500 mg. Daily for 7 Days Followed by 3 × 250 mg. for an Additional 7 Days

Day	Subject N	O	P	Q	R	S
	3 × 500 mg. Daily					
1	—	—	—	—	—	—
2	10.7	8.1	13.5	16.1	12.2	5.4
3	2.7[a]	6.1	15.7	28.0	12.2	3.0
4	4.5	3.6	18.1	30.0	12.9	10.7
5	10.5	6.6	20.0	15.7	14.1	4.7
6						
7						
	3 × 250 mg. Daily					
8	8.3	10.2	18.5	16.8	11.9	<0.05
9	9.3	3.9	11.7	9.9	5.5	4.3
10	2.5	4.6	13.0	9.8	5.6	11.6
11	11.0	6.6	10.7	7.1	6.6	7.7
12	9.5	3.4	8.7	9.9	6.4	8.5
13						
14						
	No Administration					
15	6.5	5.6	10.0	4.5	4.6	9.1
16	0.7	0.1	1.7	1.5	1.5	0.9
17	0.1	0.06	0.7	<0.05	0.6	<0.05
18	<0.05	<0.05	0.1	<0.05	0.08	<0.05

[a] Dose not taken the evening before.

RESULTS AND DISCUSSION

Single-Dose Administration—The results of the single-dose study are given in Table II and illustrated in Fig. 1. Maximum plasma concentrations were reached around 4 hr. after administration for the three doses studied and then decreased with an apparent half-life of between 11 and 14 hr. (Fig. 2).

For the average plasma concentration curves, the time of maximal concentrations observed and the half-life values obtained allow the calculation of the range of the absorption rate constant, k_a, which was found to be between 0.63 and 0.69 $hr.^{-1}$.

The areas under the plasma curves can be calculated and standardized for the body weight of the individual according to the following formula:

$$\text{standard area} = \frac{Wi}{75}\int_0^{72\text{ hr.}} c\,dt \text{ (mcg./ml.)(hr.)} \quad \text{(Eq. 1)}$$

where Wi = individual body weight, and 75 kg. is taken as the standard body weight. The area under the plasma curve values so obtained (Table III) clearly reflect the different dosages. Comparison of the area under the plasma curve values of Subjects G–M showed no statistically significant difference in the relative absorption of the two dosages of 250 and 1500 mg.

Metabolic studies using ^{14}C-iodochlorhydroxyquin showed that approximately 25% of a 750-mg. oral dose was excreted in the urine over 72 hr., demonstrating a substantial absorption of the drug from the GI tract. Urine samples collected in this study were examined using the GLC method, and no free iodochlorhydroxyquin was found. Heating the urine samples for 1 hr. at 60° in hydrochloric acid liberated some iodochlorhydroxyquin, which accounted for less than 1% of the administered dose. No attempt was made to estimate glucuronide-conjugated iodochlorhydroxyquin. These findings agree well with a study (3) where 12–24% of the administered dose (250 mg.) was found in the urine over 10 hr. No free iodochlorhydroxyquin was found.

Multiple-Dose Administration—The plasma levels measured immediately before the first dose of the day are given in Table IV and illustrated in Fig. 3. Equilibrium between the amount absorbed and the amount eliminated per 24-hr. period appears to be reached by the 5th day of treatment at the latest. After discontinuation of administration, the plasma levels fall to undetectable values after approximately 3 days, which is in accord with the single-dose findings. Urine samples collected over 1 hr. immediately before the first dose of the day were analyzed when no free iodochlorhydroxyquin was found. No evidence of the accumulation of iodochlorhydroxyquin in the plasma was found.

SUMMARY

A GLC method, capable of measuring plasma levels as low as 50 ng./ml. of iodochlorhydroxyquin, was developed. Volunteers given single oral doses of 250, 750, and 1500 mg. of iodochlorhydroxyquin powder exhibited a clear dose–plasma concentration relationship. Calculation of the area under the plasma concentration curves showed that the relative absorption of the three doses was similar. The half-life was between 11 and 14 hr. Volunteers given orally 3 × 500 mg. of the powder daily for 7 days followed by 3 × 250 mg. daily for an additional 7 days had blood samples withdrawn 24 hr. after the daily dose. Equilibrium between the amount absorbed and the amount eliminated per 24-hr. period appeared to be reached by the 5th day of administration. After discontinuation of treatment, the plasma levels fell to undetectable levels after 3 days, a finding that agreed well with the results of the single-dose study. No evidence of the accumulation of iodochlorhydroxyquin was found.

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Blood Level Profile in Man following Chronic Oral Administration of Flurazepam Hydrochloride

S. A. KAPLAN▲, J. A. F. de SILVA, M. L. JACK, K. ALEXANDER, N. STROJNY, R. E. WEINFELD, C. V. PUGLISI, and L. WEISSMAN

Abstract □ The blood level profile of flurazepam and its major metabolites was determined in man following the oral administration of 30 mg. daily for 2 weeks. The levels of the intact drug were below the sensitivity limit of the assay (<3–4 ng./ml.). The hydroxyethyl metabolite was measurable only during the early hours after a 30-mg. oral dose and was not detectable after 24 hr. The major metabolite in blood was *N*-desalkyl-flurazepam, which reached steady-state (plateau) levels after 7 days. The half-life of elimination of the *N*-desalkyl metabolite ranged from 47 to 100 hr. No measurable amounts of the *N*-desalkyl-3-hydroxy metabolite were seen in blood.

Keyphrases □ Flurazepam hydrochloride and metabolites—blood level profile in man following 14 days administration, half-lives □ Blood level profile—flurazepam hydrochloride and metabolites after 14 days administration, man □ Biotransformation—flurazepam hydrochloride after chronic administration, blood level profile including metabolites, half-lives □ Benzodiazepines, flurazepam hydrochloride—biotransformation, blood level profile, metabolites

Flurazepam[1], 7-chloro-1-[2-(diethylamino)ethyl]-5-(*o*-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one dihydrochloride (I), was synthesized by Sternbach *et al.* (1) and is marketed as a hypnotic for the treatment of insomnia (2). Studies on the metabolism of flurazepam (3) showed that it was extensively metabolized in both man and dog to yield measurable amounts of the hydroxyethyl (II) and *N*-desalkyl (III) metabolites in the blood. The latter was further metabolized to the 3-hydroxy analog (IV), which appeared in trace amounts in urine as a glucuronide conjugate. In addition, they showed that both flurazepam and the hydroxyethyl metabolite were metabolized extensively in the dog to an acidic compound, flurazepam-*N*-yl-acetic acid (V), by the oxidation of the alcohol side chain to a carboxylic acid. Flurazepam-*N*-yl-acetic acid is a minor urinary metabolite in man.

The characterization of these metabolites and the synthesis of authentic reference compounds were reported previously (4, 5). The objective of the present study was to define the blood level profile of flurazepam and its major biotransformation products when the drug is administered once daily for 14 days at the usually recommended therapeutic dose of 30 mg./day.

PROCEDURE

Blood levels of flurazepam and its major biotransformation products were analyzed by a spectrofluorometric method (6), which involves selective extraction of flurazepam and its metabolites (II, III, and IV) from blood buffered to pH 9.0 into ether, back-extraction into 6 *N* HCl, and hydrolysis to their benzophenones followed by cyclization to the highly fluorescent 9-acridanone derivatives. These derivatives are extracted into ether and separated by TLC, and their fluorescence is determined in methanol–0.1 *N* HCl (80:20) (after elution from the silica gel) at their respective activation and emission maxima.

The sensitivity of the assay is of the order of 3–10 ng. of each compound/ml. of blood using a 4-ml. specimen per analysis. The spectrofluorometric assay does not specifically differentiate *N*-desalkyl-flurazepam from the *N*-desalkyl-3-hydroxy metabolite if present, since they both give rise to the same 9-acridanone derivative (6). However, the presence of *N*-desalkyl-flurazepam as the major metabolite in the blood of these subjects was confirmed by electron-capture GLC analysis[2], which completely resolves the *N*-

[1] Flurazepam hydrochloride is the active drug substance in Dalmane, Hoffmann-La Roche Inc., Nutley, N. J.

[2] J. A. F. de Silva and C. V. Puglisi, unpublished data on file, Hoffmann-La Roche Inc., Nutley, NJ 07110, 1973.

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S. A. KAPLAN▲, J. A. F. de SILVA, M. L. JACK, K. ALEXANDER, N. STROJNY, R. E. WEINFELD, C. V. PUGLISI, and L. WEISSMAN

Abstract □ The blood level profile of flurazepam and its major metabolites was determined in man following the oral administration of 30 mg. daily for 2 weeks. The levels of the intact drug were below the sensitivity limit of the assay (<3–4 ng./ml.). The hydroxyethyl metabolite was measurable only during the early hours after a 30-mg. oral dose and was not detectable after 24 hr. The major metabolite in blood was *N*-desalkyl-flurazepam, which reached steady-state (plateau) levels after 7 days. The half-life of elimination of the *N*-desalkyl metabolite ranged from 47 to 100 hr. No measurable amounts of the *N*-desalkyl-3-hydroxy metabolite were seen in blood.

Keyphrases □ Flurazepam hydrochloride and metabolites—blood level profile in man following 14 days administration, half-lives □ Blood level profile—flurazepam hydrochloride and metabolites after 14 days administration, man □ Biotransformation—flurazepam hydrochloride after chronic administration, blood level profile including metabolites, half-lives □ Benzodiazepines, flurazepam hydrochloride—biotransformation, blood level profile, metabolites

Flurazepam[1], 7-chloro-1-[2-(diethylamino)ethyl]-5-(*o*-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one dihydrochloride (I), was synthesized by Sternbach *et al.* (1) and is marketed as a hypnotic for the treatment of insomnia (2). Studies on the metabolism of flurazepam (3) showed that it was extensively metabolized in both man and dog to yield measurable amounts of the hydroxyethyl (II) and *N*-desalkyl (III) metabolites in the blood. The latter was further metabolized to the 3-hydroxy analog (IV), which appeared in trace amounts in urine as a glucuronide conjugate. In addition, they showed that both flurazepam and the hydroxyethyl metabolite were metabolized extensively in the dog to an acidic compound, flurazepam-*N*-yl-acetic acid (V), by the oxidation of the alcohol side chain to a carboxylic acid. Flurazepam-*N*-yl-acetic acid is a minor urinary metabolite in man.

The characterization of these metabolites and the synthesis of authentic reference compounds were reported previously (4, 5). The objective of the present study was to define the blood level profile of flurazepam and its major biotransformation products when the drug is administered once daily for 14 days at the usually recommended therapeutic dose of 30 mg./day.

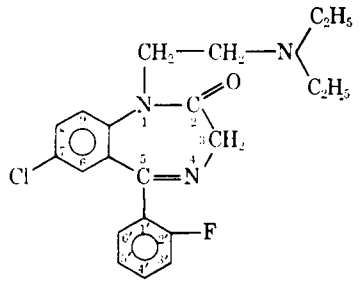
PROCEDURE

Blood levels of flurazepam and its major biotransformation products were analyzed by a spectrofluorometric method (6), which involves selective extraction of flurazepam and its metabolites (II, III, and IV) from blood buffered to pH 9.0 into ether, back-extraction into 6 *N* HCl, and hydrolysis to their benzophenones followed by cyclization to the highly fluorescent 9-acridanone derivatives. These derivatives are extracted into ether and separated by TLC, and their fluorescence is determined in methanol–0.1 *N* HCl (80:20) (after elution from the silica gel) at their respective activation and emission maxima.

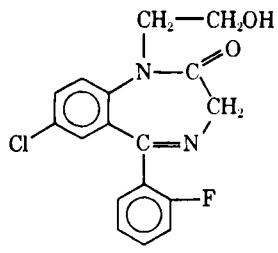
The sensitivity of the assay is of the order of 3–10 ng. of each compound/ml. of blood using a 4-ml. specimen per analysis. The spectrofluorometric assay does not specifically differentiate *N*-desalkyl-flurazepam from the *N*-desalkyl-3-hydroxy metabolite if present, since they both give rise to the same 9-acridanone derivative (6). However, the presence of *N*-desalkyl-flurazepam as the major metabolite in the blood of these subjects was confirmed by electron-capture GLC analysis[2], which completely resolves the *N*-

[1] Flurazepam hydrochloride is the active drug substance in Dalmane, Hoffmann-La Roche Inc., Nutley, N. J.

[2] J. A. F. de Silva and C. V. Puglisi, unpublished data on file, Hoffmann-La Roche Inc., Nutley, NJ 07110, 1973.



flurazepam (I)



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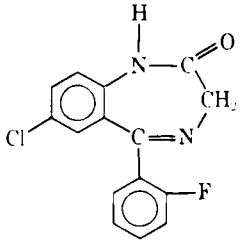

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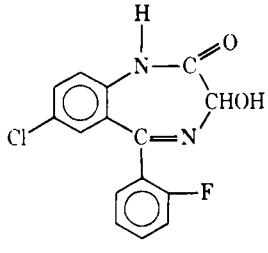

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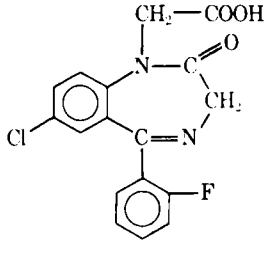

flurazepam-*N*-1-yl-acetic acid (V)

desalkyl metabolite from the *N*-desalkyl-3-hydroxy metabolite. The specimens from Days 14, 15, and 16 in Subjects 2, 3, and 4 were analyzed by the GLC procedure and clearly showed that the *N*-desalkyl-3-hydroxy metabolite was not present in the blood and confirmed that the *N*-desalkyl metabolite was the major blood component present. Therefore, the metabolite levels represented as the *N*-desalkyl metabolite by the fluorometric assay are due only to this component in these subjects.

Four adult healthy male volunteers (age and weight listed here) were selected for this study:

Subject	Age	Weight, kg.
1	44	90.0
2	25	76.8
3	28	69.5
4	42	85.5

Each subject was given a 30-mg. capsule of flurazepam hydro-

Table I—Blood Level Profile of Flurazepam and Its Major Metabolites in Subject 1 following the Administration of 30 mg. Flurazepam Hydrochloride Every 24 hr. for 14 Days[a]

Day	Hour	Hydroxyethyl-flurazepam (II), ng./ml.	*N*-Desalkyl-flurazepam (III), ng./ml.
1	0	n.m.	n.m.
	1	7.0	13.6
	3	3.0	10.5
	6	n.m.	19.8
	12	n.m.	6.8
	24	n.m.	14.5
2	24	n.m.	23.4
3	24	n.m.	30.0
4	24	n.m.	30.5
5	24	n.m.	40.0
6	24	n.m.	53.0
7	1	5.4	36.0
	3	5.4	62.0
	6	n.m.	79.0
	12	n.m.	58.0
	24	n.m.	61.0
8	24	n.m.	139.0
9	24	n.m.	40.0
10	24	n.m.	80.0
11	24	n.m.	70.0
12	24	n.m.	109.0
13	24	n.m.	134.0
14	1	15.5	113.0
	3	16.9	142.0
	6	4.8	60.0
	12	2.5	90.0
	24	n.m.	70.0
15	24	n.m.	66.0
16	24	n.m.	58.0
18	24	n.m.	33.0
20	24	n.m.	27.0
27	24	n.m.	2.4
34	24	n.m.	n.m.

[a] n.m. = not measurable <2-3 ng./ml. Flurazepam levels were measurable only on Day 14 at 1 hr. (3 ng./ml.) and at 6 hr. (4 ng./ml.).

Table II—Blood Level Profile of Flurazepam and Its Major Metabolites in Subject 2 following the Administration of 30 mg. Flurazepam Hydrochloride Every 24 hr. for 14 Days[a]

Day	Hour	Hydroxyethyl-flurazepam (II), ng./ml.	*N*-Desalkyl-flurazepam (III), ng./ml.
1	0	n.m.	n.m.
	1	4.3	5.6
	3	3.4	6.8
	6	n.m.	9.8
	12	n.m.	10.2
	24	n.m.	8.4
2	24	n.m.	10.1
3	24	n.m.	15.4
4	24	n.m.	20.0
5	24	n.m.	15.9
6	24	n.m.	22.7
7	1	6.8	36.8
	3	3.6	41.2
	6	n.m.	42.7
	12	n.m.	37.3
	24	n.m.	33.2
8	24	n.m.	35.5
9	24	n.m.	34.1
10	24	n.m.	33.0
11	24	n.m.	31.7
12	24	n.m.	42.2
13	24	2.5	38.7
14	1	11.5	44.4
	3	5.3	49.2
	6	2.8	38.9
	12	n.m.	42.8
	24	n.m.	39.4
15	24	n.m.	29.9
16	24	n.m.	15.1
18	24	n.m.	9.5
20	24	n.m.	n.m.
27	24	n.m.	n.m.
34	24	n.m.	n.m.

[a] n.m. = not measurable <2-3 ng./ml. Flurazepam levels were unmeasurable throughout the study.

Table III—Blood Level Profile of Flurazepam and Its Major Metabolites in Subject 3 following the Administration of 30 mg. Flurazepam Hydrochloride Every 24 hr. for 14 Days[a]

Day	Hour	Hydroxyethyl-flurazepam (II), ng./ml.	N-Desalkyl-flurazepam (III), ng./ml.
1	0	n.m.	n.m.
	1	14.8	6.9
	3	8.6	15.2
	6	3.3	13.3
	12	n.m.	20.6
	24	n.m.	21.8
2	24	n.m.	41.4
3	24	n.m.	34.8
4	24	n.m.	23.8
5	24	n.m.	36.0
6	24	n.m.	24.3
7	1	2.7	26.6
	3	10.5	35.6
	6	3.8	35.9
	12	n.m.	37.2
	24	n.m.	53.8
8	24	n.m.	34.3
9	24	n.m.	44.1
10	24	n.m.	32.4
11	24	n.m.	27.1
12	24	n.m.	41.1
13	24	n.m.	58.0
14	1	n.m.	56.1
	3	n.m.	48.5
	6	n.m.	42.2
	12	n.m.	50.4
	24	n.m.	36.5
15	24	n.m.	29.0
16	24	n.m.	24.7
18	24	n.m.	11.9
20	24	n.m.	5.4
27	24	n.m.	n.m.
34	24	n.m.	n.m.

[a] n.m. = not measurable <2–3 ng./ml. Flurazepam levels were unmeasurable throughout this study.

Table IV—Blood Level Profile of Flurazepam and Its Major Metabolites in Subject 4 following the Administration of 30 mg. Flurazepam Hydrochloride Every 24 hr. for 14 Days[a]

Day	Hour	Hydroxyethyl-flurazepam (II), ng./ml.	N-Desalkyl-flurazepam (III), ng./ml.
1	0	n.m.	n.m.
	1	6.1	8.3
	3	3.9	11.2
	6	n.m.	10.7
	12	n.m.	11.5
	24	n.m.	13.5
2	24	n.m.	27.6
3	24	n.m.	47.0
4	24	n.m.	42.0
5	24	n.m.	46.1
6	24	n.m.	69.8
7	1	2.5	55.7
	3	6.6	71.2
	6	2.8	72.1
	12	n.m.	75.7
	24	n.m.	71.6
8	24	n.m.	62.4
9	24	n.m.	83.3
10	24	4.0	87.1
11	24	n.m.	89.1
12	24	n.m.	98.4
13	24	n.m.	106.0
14	1	4.2	117.8
	3	4.4	115.5
	6	n.m.	99.0
	12	n.m.	115.9
	24	n.m.	113.8
15	24	n.m.	84.4
16	24	n.m.	78.4
18	24	n.m.	47.6
20	24	n.m.	34.8
27	24	n.m.	12.7
34	24	n.m.	4.8

[a] n.m. = not measurable <2–3 ng./ml. Flurazepam levels were unmeasurable throughout the study.

chloride at approximately 10 p.m. for 14 days. Ten milliliters oxalated blood specimens were obtained as follows: Days 1, 7, and 14—just prior to drug administration and at 1, 3, 6, 12, and 24 hr. postadministration; Days 2 through 6 and 8 through 13—just prior to drug administration (24 hr. postadministration); and Days 15, 16, 17, 19, 21, 28, and 35—at 10 p.m. (1, 2, 3, 5, 7, 14, and 21 days following the last administration on Day 14).

All blood specimens were frozen immediately and maintained frozen until analyzed.

RESULTS AND DISCUSSION

The blood level data are tabulated in Tables I–IV for Subjects 1–4. The blood levels of the N-desalkyl metabolite in each subject are summarized in Fig. 1. The overall blood level profile parameters are summarized in Table V.

Blood levels of flurazepam were generally below 2 ng./ml. Only Subject 1 exhibited trace amounts of flurazepam of 3–4 ng./ml. on Day 14 of the study.

Blood levels of the hydroxyethyl metabolite were seen in the early hour samples measured on Days 1, 7, and 14 (Tables I–IV). The elimination rate of the hydroxyethyl metabolite was very rapid (data insufficient for half-life calculation), and this metabolite was not evident in the blood 24 hr. postadministration.

The N-desalkyl metabolite was the major detectable blood component following flurazepam hydrochloride administration (Fig. 1). The half-lives of elimination of this component from the blood were calculated to be 64, 47, 51, and 100 hr. for Subjects 1, 2, 3, and 4, respectively.

Subjects 2 and 4 exhibited a typical steady-state blood level profile of the N-desalkyl metabolite. In both subjects, the blood levels appeared to reach a plateau on Days 7–10 of the study. Subject 2 exhibited a minimum steady-state level (24 hr. postdosing), ranging between 33 and 42 ng./ml. The corresponding range for Subject 4 was 84–114 ng./ml. These values correlate well with the "calculated" minimum steady-state levels of 28 and 88 ng./ml. for Subjects 2 and 4, respectively (Table V). The calculated value was obtained using the observed half-life and the 24-hr. blood level on Day 1 for each subject. This close correlation of actual and calculated minimum steady-state levels indicates that the levels of the N-desalkyl metab-

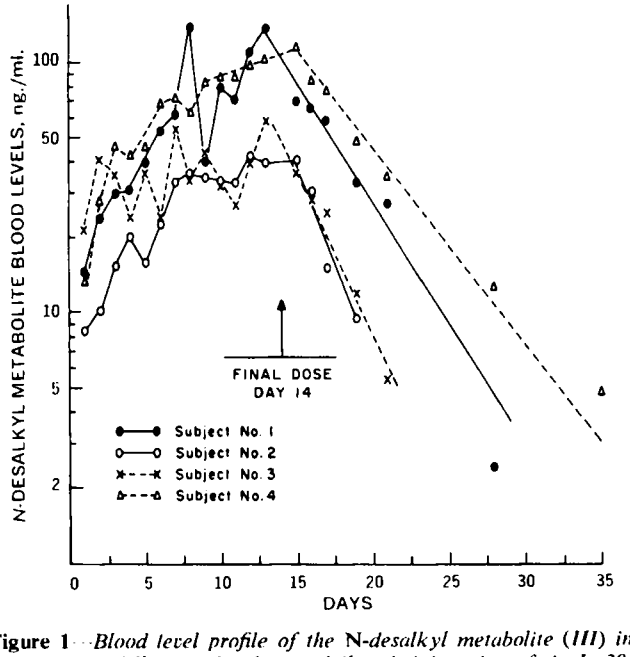

Figure 1—*Blood level profile of the* N-*desalkyl metabolite (III) in Subjects 1–4 following the chronic daily administration of single 30-mg. oral doses of flurazepam hydrochloride.*

Table V—Blood Level Profile Parameters

	Subject			
	1 (90.0 kg.)[a]	2 (76.8 kg.)	3 (69.5 kg.)	4 (85.5 kg.)
N-Desalkyl metabolite (III):				
Maximum blood level on Day 1	20 ng./ml. at 6 hr.	10 ng./ml. at 12 hr.	22 ng./ml. at 24 hr.	14 ng./ml. at 24 hr.
Maximum blood level on Day 14	142 ng./ml.	49 ng./ml.	56 ng./ml.	118 ng./ml.
Asymptotic levels commence	≃Day 10-erratic	Day 7	≃Day 2-erratic	Day 9
Asymptotic 24-hr. level (minimum)	~70–134 ng./ml.	≃33–42 ng./ml.	≃24–58 ng./ml.	≃84–114 ng./ml.
24-hr. blood level on Day 1	15 ng./ml.	8 ng./ml.	22 ng./ml.	14 ng./ml.
Half-life calculated after last dose on Day 14	64 hr.	47 hr.	51 hr.	100 hr.
Calculated minimum steady-state level based on Day 1–24-hr. level	63 ng./ml.	28 ng./ml.	78 ng./ml.	88 ng./ml.
Hydroxyethyl metabolite (II):				
Maximum blood level on Day 1	7 ng./ml.	4.3 ng./ml.	14.8 ng./ml.	6.1 ng./ml.
Maximum blood level on Day 7	5.4 ng./ml.	6.8 ng./ml.	10.5 ng./ml.	6.6 ng./ml.
Maximum blood level on Day 14	16.9 ng./ml.	11.5 ng./ml.	N.D.	4.4 ng./ml.
Half-life	Rapid	Rapid	Rapid	Rapid
Flurazepam (I):				
Blood levels	Barely detectable on Day 14, only at 3–4 ng./ml.	N.D.[b]	N.D.	N.D.

[a] Body weight. [b] N.D. = not detectable <2–3 ng./ml.

olite reach a predictable steady-state plateau within 7–10 days upon chronic drug administration. The actual minimum steady-state blood levels were approximately four- to sixfold greater than the 24-hr. blood level observed on Day 1.

The daily 24-hr. blood levels of the *N*-desalkyl metabolite in Subjects 1 and 3 were more variable than those found in Subjects 2 and 4. The correlation of the actual 24-hr. blood levels and the calculated steady-state level was fair in Subject 1, with the calculated value falling at the lower end of the actual 24-hr. blood level range (Table V). Subject 1 exhibited 24-hr. blood levels from Days 10 to 14, which were approximately five- to sixfold greater than the observed Day 1 level, which is consistent with the findings in Subjects 2 and 4. The actual 24-hr. levels in Subject 3 were lower than the calculated minimum steady-state levels.

The blood level profile following the oral administration of 30 mg. of flurazepam hydrochloride every 24 hr. indicates that: (*a*) the blood level of flurazepam was <2 ng./ml. in blood, (*b*) the hydroxyethyl metabolite was present in the blood in the early hours following each administration and was rapidly eliminated, and (*c*) the *N*-desalkyl metabolite was the major blood component.

The blood level profiles indicate that the *N*-desalkyl metabolite was eliminated with half-lives of 64, 47, 51, and 100 hr. in the four subjects studied. In three subjects, blood levels of this metabolite exhibited a "typical," predictable minimum steady-state profile: *i.e.*, the actual and calculated steady-state levels correlated well. This would suggest that the physiological disposition of the *N*-desalkyl metabolite follows apparent first-order kinetic patterns. The minimum steady-state levels observed, commencing 7–10 days following the onset of a once every 24-hr. dosing regimen, were approximately five- to sixfold greater than the 24-hr. levels observed on Day 1.

SUMMARY

Following the oral administration of 30 mg. of flurazepam hydrochloride in man every 24 hr. for 14 days, blood samples were analyzed for flurazepam and its major metabolites. The method used for the analysis of flurazepam was not sufficiently sensitive to measure the concentration of unchanged drug in blood (<2 ng. flurazepam/ml. of blood was present). The hydroxyethyl metabolite was present in the blood shortly after each administration and was rapidly eliminated. The *N*-desalkyl metabolite was the major metabolite in blood. It was eliminated with half-lives of 47–100 hr. in the four subjects. It exhibited a typical minimum steady-state profile, with plateau levels reached after 7–10 days of dosing, approximately five- to sixfold greater than the 24-hr. levels observed on Day 1.

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Calculating Absorption Rate Constants for Drugs with Incomplete Availability

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Abstract □ A mathematical analysis was made of the type of results to be expected, by the classic pharmacokinetic treatment of plasma level data, when, in addition to absorption, the drug is simultaneously lost to an extravascular compartment *via* either a parallel first- or zero-order process. Equations are presented for both the presence and absence of a lag time before onset of the parallel process. A procedure is proposed which allows the true first-order absorption rate constant to be determined for some models, as well as the elucidation of the applicable model.

Keyphrases □ Drugs with incomplete availability—considerations in calculating absorption rate constants, plasma level data and simultaneous loss to extravascular compartment, equations for both presence and absence of lag time □ Absorption rate constants, drugs with incomplete availability—mathematical treatment of plasma level data and simultaneous loss to extravascular compartment, equations for both presence and absence of lag time □ Pharmacokinetics—calculation of absorption rate constants for drugs with incomplete availability, plasma level data and simultaneous loss to extravascular compartment, equations for both presence and absence of lag time

In a recent publication, Notari *et al.* (1) demonstrated that methods commonly employed to calculate absorption rate constants from the time course of drug in the blood do not result in the correct value if the drug is simultaneously lost to an extravascular compartment by a parallel first-order process. It is well recognized in chemical kinetics that the classical kinetic treatment of reaction data, in which a compound degrades *via* two or more first-order pathways, yields an apparent rate constant for loss of material which is a sum of the rate constants for the individual processes. However, to the best of these authors' knowledge, the application of this principle to pharmacokinetic considerations was not recognized prior to the publication of Notari *et al.* (1). More recently, Perrier and Gibaldi (2) expanded on Notari's concept, which deals primarily with chemical degradation of drugs in solution, to include the case of both a granulation and a nondisintegrating dosage form moving along the GI tract to a point where absorption no longer occurs. They also considered the case where the competing parallel reaction was zero order and introduced the concept of a lag time occurring prior to the onset of either parallel reaction. These authors (2) concluded that the nature of the rate constant obtained with a parallel first-order reaction was dependent upon the lag time. They also reported that in the case of a parallel zero-order reaction, although the computer-generated data showed curvature, when plotted according to the Wagner–Nelson (3) or Loo–Riegelman (4) method, the normal experimental error encountered in real situations could mask this. As a result, one would tend to accept the curves as linear and employ the slope to obtain an absorption rate constant.

The cases of parallel first-order and zero-order competitive processes with various lag times have been considered, and a mathematical interpretation is presented for the nature of the curves obtained both by the method of feathering and by the Wagner–Nelson (3) procedure. In the case of the parallel first-order loss of drug to extravascular compartment, it is concluded that these methods yield plots which tend to be curved until the onset of the lag time, at which time they become linear and are all parallel. In the case of a parallel zero-order process, one encounters curves that could be interpreted as linear, with the apparent linear relationships being a function of the lag time. In addition, a method was developed which in some cases allows an investigator to determine the correct absorption rate constant with concomitant recognition of the nature of the parallel process.

All of this work has been limited to the one-compartment open model with first-order absorption. However, parallel considerations for more complex models are possible in a manner similar to that presented in this report.

EXPERIMENTAL

In this analysis, the simple one-compartment open model is represented as shown in Scheme I, where k_1 and k_2 are the absorption

$$\underset{\text{absorption compartment}}{A} \xrightarrow{k_1} \underset{\text{plasma compartment}}{B} \xrightarrow{k_2} \underset{\text{elimination compartment}}{C}$$

Scheme I

and elimination rate constants, respectively. Two variations on this scheme were considered. In Case 1, drug in Compartment A is simultaneously being removed to an extravascular compartment by a first-order rate process with a rate constant of k_3. In Case 2, drug is being removed from Compartment A *via* a zero-order rate process with a rate constant of k_0. These are shown in Scheme II.

Case 1

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$
$$\downarrow k_3$$
$$D$$

Case 2

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$
$$\downarrow k_0$$
$$D$$

Scheme II

All calculations were performed on a programmable desk calculator[1]. The rate constants employed were those suggested by Perrier and Gibaldi (2) ($k_1 = 0.693$, $k_2 = 0.0693$, $k_3 = 0.5$, and $k_0 = 25$)[2]. Lag times were varied from 0 to 3 hr. In most cases only the final forms of the equations employed are presented[3].

[1] Wang model 700.

[2] k_1, k_2, and k_3 are first-order rate constants with dimensions of reciprocal hours; k_0 is a zero-order rate constant with dimensions of units per hour.

[3] Interested parties may obtain the derivations by communication with the authors.

RESULTS AND DISCUSSION

Case 1—It may be shown that the relationship governing the time course of drug in the body in the absence and presence of a lag time is defined by Eqs. 1 and 2, respectively:

$$C_p = \frac{k_1 A_0}{(K - k_2)V}[\exp(-k_2 t) - \exp(-Kt)] \quad \text{(Eq. 1)}$$

$$C_p = \frac{k_1 A_0 \exp(-k_1 t1)}{(K - k_2)V}\{\exp[-k_2(t - t1)] - \exp[-K(t - t1)]\} + \frac{B_{t1}}{V}\{\exp[-k_2(t - t1)]\} \quad \text{(Eq. 2)}$$

where k_1 and k_2 are the absorption and elimination rate constants, respectively; k_3 is the parallel nonabsorption first-order rate constant; t is time; $t1$ is lag time; C_p is the plasma concentration; A_0 is the amount of drug in the dosage form; B_{t1} is the amount of drug in Compartment B at lag time $t1$; K is the sum of the two parallel first-order rate constants ($k_1 + k_3$); and V is the volume of distribution. The similarity between Eq. 1 and that for the one-compartment open model with first-order absorption and no parallel reaction (Eq. 6) is obvious when one substitutes 0 for k_3 in Eq. 1. Equation 2 is applicable only from the onset of the lag time.

In the treatment of experimentally obtained data, one plots log C_p as a function of time and determines k_2 from the log-linear phase. The absorption rate constant is then evaluated by either the method of feathering or the Wagner–Nelson procedure. Under conditions of nonparallel reactions occurring, both methods yield essentially the same results. The type of data obtained from these techniques when one does encounter parallel competing reactions, both with and without lag times, was examined.

Feathering Method (Method of Residuals)—Where there is no lag time, one would employ Eq. 1. It is, therefore, obvious that after onset of the log-linear phase, C_p would be expressed in terms of Eq. 3:

$$C_p = \left[\frac{k_1 A_0}{(K - k_2)V}\right]\exp(-k_2 t) \quad \text{(Eq. 3)}$$

Thus, ΔC_p, which represents the difference in C_p between Eqs. 1 and 3 at any given time, would be expressed as shown in Eq. 4:

$$\Delta C_p = \left[\frac{k_1 A_0}{(K - k_2)V}\right]\exp(-Kt) \quad \text{(Eq. 4)}$$

It is then apparent that Eq. 4 represents a log-linear relationship which, when plotted, has a slope equal to K. This is implicit in what was stated by Notari *et al.* (1) and Perrier and Gibaldi (2).

In the case where there is a finite lag time, $t1$, C_p in the post-absorptive phase comes from Eq. 2 and is shown as Eq. 5:

$$C_p = \left[\frac{k_1 A_0 \exp(-k_1 t1)}{(K - k_2)V} + \frac{B_{t1}}{V}\right]\exp[-k_2(t - t1)] \quad \text{(Eq. 5)}$$

However, when t is less than $t1$, C_p is defined in terms of the classical one-compartment open model with first-order absorption as shown in Eq. 6:

$$C_p = \frac{k_1 A_0}{(k_1 - k_2)V}[\exp(-k_2 t) - \exp(-k_1 t)] \quad \text{(Eq. 6)}$$

Therefore, ΔC_p prior to the onset of the lag time is represented mathematically by Eq. 7, where M and N are constants. It is readily seen that Eq. 7 is not a log-linear relationship:

$$\Delta C_p = M\exp(-k_2 t) + \exp(-k_1 t) \quad \text{(Eq. 7)}$$

$$M = \frac{k_1 A_2 \exp(k_2 - k_1)t1}{(K - k_2)V} + \frac{B_{t1}\exp(k_2 t1)}{V} - N \quad \text{(Eq. 7a)}$$

$$N = \frac{k_1 A_0}{(k_1 - k_2)V} \quad \text{(Eq. 7b)}$$

In the range where t is between $t1$ and the onset of the log-linear phase, C_p is as defined in Eq. 2 and ΔC_p is defined by Eq. 8:

$$\Delta C_p = \left[\frac{k_1 A_0 \exp(k_2 t1)}{(K - k_2)V}\right]\exp(-Kt) \quad \text{(Eq. 8)}$$

It is again obvious that a plot of log ΔC_p *versus* t, according to Eq. 8, will be linear with a slope from which K may be obtained.

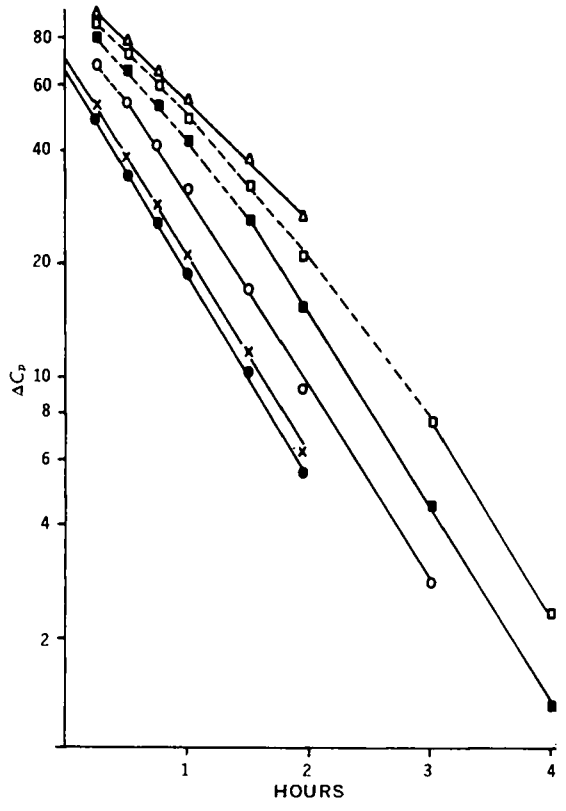

Figure 1—*Plot of* ΔC_p versus *time for data where competing first-order process is operative, calculated according to the method of residuals. Key:* ●, *no lag;* ×, *0.5-hr. lag;* ○, *1-hr. lag;* ■, *2-hr. lag;* □, *3-hr. lag; and* △, *no competitive process (control).*

From this discussion, therefore, it is apparent that when a lag time occurs prior to the onset of a parallel first-order reaction, the feathering technique results in a line that is initially curved followed by a straight portion from which the apparent absorption rate constant (K) is obtained. This value, while not the true absorption rate constant, would be the same whether or not there was a lag time occurring before onset of the competing process. Data of this type are shown in Fig. 1, employing the rate constant suggested by Perrier and Gibaldi (2).

Wagner–Nelson Technique—In the Wagner–Nelson (3) method, one first determines the fraction of drug absorbed, and the absorption rate constant is evaluated from a plot of log fraction unabsorbed as a function of time. The fraction absorbed is determined by dividing the amount of drug absorbed at any time period (D/V) by the total amount of drug absorbed (D_∞/V). The fraction unabsorbed is then expressed by the relationship ($1 - D/D_\infty$).

It may be shown that the total amount of drug absorbed is represented by a sum of the drug absorbed up to the lag time *via* the equation for a one-compartment open model with first-order absorption (Eq. 6), plus the drug absorbed from the onset of the lag time until the onset of the log-linear phase. This is expressed by Eq. 9:

$$D_\infty = D_{t1} + \left(\frac{k_1}{K}\right)A_{t1} = A_0\left[1 - \frac{k_3}{K}\exp(-k_1 t1)\right] \quad \text{(Eq. 9)}$$

where D_{t1} represents the amount of drug absorbed up to the lag time and $[(k_1/K)A_{t1}]$ represents the amount of drug absorbed from the lag time until absorption ceases. The term A_{t1} represents the amount of drug in Compartment A at the onset of the lag time.

Correspondingly, the amount of drug absorbed at any time after the lag time is represented by Eq. 10 where the constants are as designated previously:

$$D = A_0\left\{\left[1 - \frac{k_3}{K}\exp(-k_1 t1)\right] - \left[\frac{k_1}{K}\exp(k_2 t1)\right]\exp(-Kt)\right\} \quad \text{(Eq. 10)}$$

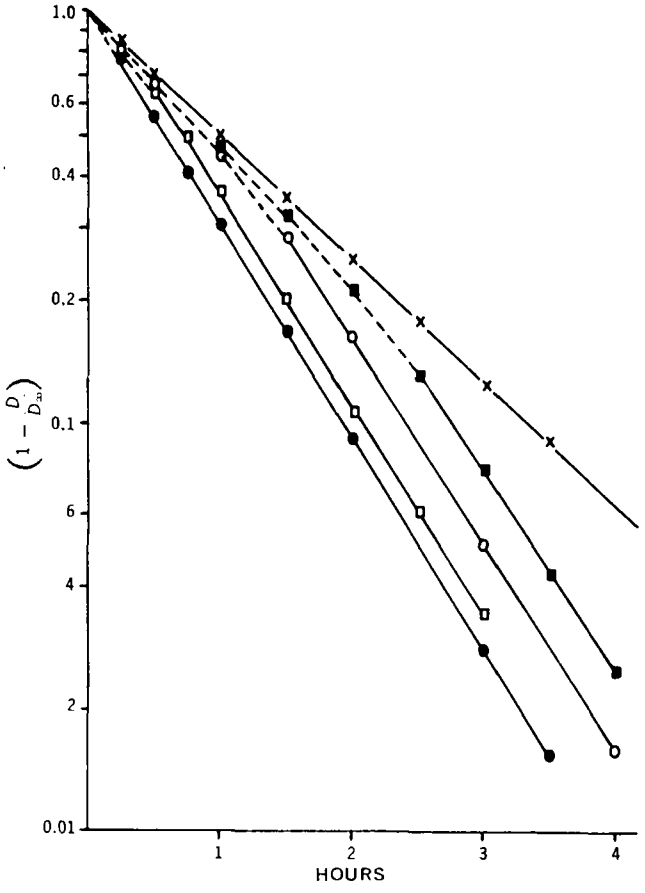

Figure 2—*Plot of Wagner-Nelson calculations* versus *time for data where competing first-order process is operative. Key:* ×, *control (no competition);* ●, *0.5-hr. lag;* □, *1-hr. lag;* ○, *2-hr. lag; and* ■, *3-hr. lag.*

Therefore, if there is no lag time, $D_{t1} = 0$ and the expression $(1 - D/D_\infty)$ may be represented as seen in Eq. 11:

$$(1 - D/D_\infty) = \exp(-Kt) \qquad \text{(Eq. 11)}$$

Equation 11 represents a log-linear relationship from which K may be determined, again in agreement with both Notari *et al.* (1) and Perrier and Gibaldi (2).

Where there is a lag time occurring prior to $t = t1$, the expression $(1 - D/D_\infty)$ is represented by Eq. 12 and is not a log-linear relationship:

$$(1 - D/D_\infty) = \frac{K\exp(-k_1t) - k_3\exp(-k_1t1)}{k_3\exp(-k_1t1) - K} \qquad \text{(Eq. 12)}$$

Between the onset of the lag time and completion of absorption, the expression $(1 - D/D_\infty)$ is represented by Eq. 13 and would be log-linear with the slope related to K:

$$(1 - D/D_\infty) = \left[\frac{k_1\exp(k_3t1)}{K - k_3\exp(-k_1t1)}\right]\exp(-Kt) \qquad \text{(Eq. 13)}$$

This discussion indicates that a Wagner-Nelson (3) plot will be comparable to one obtained by the feathering technique in that the plot will be curved prior to the lag time. After the onset of the lag time and independent of its magnitude, one obtains a straight line from which the identical, apparent first-order absorption rate constant (K) is obtained. Plots of this type are shown in Fig. 2, again employing Perrier and Gibaldi's rate constants. On initial inspection, the results appear to be different from those reported by Perrier and Gibaldi in their Fig. 1. However, if one considers the fact that they limited their results to $(1 - D/D_\infty)$ values of greater than 0.1, in some cases the true log-linear phase was not attained. In addition, for the purpose of emphasis, they intentionally fit all of their data linearly. This masked the cases where there were parallel log-linear relationships. Therefore, Fig. 2 in this publication and Fig. 1 of Perrier and Gibaldi are, in fact, the same.

Parallel Zero-Order Reaction—The relationships between plasma concentration and time resulting from the model designated as Case 2 are somewhat more complex than with Case 1 and are shown by Eq. 14 for the case where $t1 = 0$ and by Eq. 15 when there is a finite lag time:

$$C_p = \left[\frac{k_0}{k_2V} + \frac{k_1A_0 + k_0}{V(k_1 - k_2)}\right]\exp(-k_2t) - \left[\frac{k_1A_0 + k_0}{V(k_1 - k_2)}\right]\exp(-k_1t) - \frac{k_0}{k_2V} \qquad \text{(Eq. 14)}$$

$$C_p = \left[\frac{k_0}{k_2V} + \frac{k_1A_0\exp(-k_1t1) + k_0}{V(k_1 - k_2)}\right]\exp[-k_2(t - t1)] - \left[\frac{k_1A_0\exp(-k_1t1) + k_0}{V(k_1 - k_2)}\right]\exp[-k_1(t - t1)] - \frac{k_0}{Vk_2} + \frac{B_{t1}}{V}\exp[-k_2(t - t1)] \qquad \text{(Eq. 15)}$$

In these expressions, the constants are all as previously designated. Equations 14 and 15 may be expressed in a number of ways for which an equivalency is not immediately apparent. Moreover, Eq. 15 applies only after onset of the lag time.

Upon first examination of Eqs. 14 and 15, there appears to be an error in the fact that when time is equal to infinity C_p does not go to zero but becomes a finite negative value, as indicated by the constant (k_0/k_2V) present in both equations. Upon considering the situation, however, one recognizes that Eqs. 14 and 15 only apply up to a certain critical time, t_c, at which point there remains no drug in Compartment A. From this point on, the plasma concentration is defined as shown in Eq. 16 where B_c is equal to the concentration in Compartment B at t_c:

$$C_p = \frac{B_c}{V}\exp(-k_2t) \qquad \text{(Eq. 16)}$$

The term t_c is shown in Eq. 17:

$$t_c = \frac{1}{k_1}\ln\left(\frac{k_0}{k_1A_0 + k_0}\right) \qquad \text{(Eq. 17)}$$

B_c may be determined using the value for t_c and Eq. 14 or 15.

Feathering Technique—It is apparent that if one employs the feathering technique in the case of a parallel zero-order reaction with no lag time, the differences between Eqs. 14 and 16 at various times do not result in a log-linear relationship. Thus, a plot of log ΔC_p as a function of time would be curved. Correspondingly, when a lag time does occur, prior to its onset the difference between the equation for the one-compartment open model (Eq. 6) and Eq. 16 would also result in a ΔC_p-time relationship which is not log-linear. After t reaches $t1$, the ΔC_p values will be comparable to those in the case where there is no lag time. Thus, the data obtained by the feathering technique would be expected to give results that do not indicate the true absorption rate constant, in spite of the fact that in one exponential term there is a k_1 and not a pseudoconstant. However, as indicated by Perrier and Gibaldi (2), the normal error encountered in this type of data could be such as to make it appear to be log-linear. Such plots are shown in Fig. 3, and we agree with Perrier and Gibaldi that the data could be drawn linear, especially with values resulting from *in vivo* studies. They might also be considered to be curved initially followed by linearity. It should be mentioned, however, that there could exist values of k_1, k_2, and k_0 of such a magnitude as to result in an obviously curved relationship.

Wagner-Nelson Method—It may be demonstrated that the expression $(1 - D/D_\infty)$ is represented by rather complex functions both in the presence and absence of a finite lag time (Eqs. 18 and 19, respectively):

$$(1 - D/D_\infty) = \frac{M' - N'[1 - \exp(k_1t)] + k_0t}{M} \qquad \text{(Eq. 18)}$$

$$M' = A_0 + \frac{k_0}{k_1}\left(\ln\frac{k_0}{k_1A_0 + k_0}\right) \qquad \text{(Eq. 18}a\text{)}$$

$$N' = \frac{k_1A_0 + k_0}{k_1} \qquad \text{(Eq. 18}b\text{)}$$

$$(1 - D/D_\infty) = \frac{M^* - N^* \{1 - \exp[-k_1(t - t1)]\} + k_0(t - t1)}{M'} \quad \text{(Eq. 19)}$$

$$M^* = A_{t1} + \frac{k_0}{k_1}\left(\ln \frac{k_0}{k_1 A_{t1} + k_0}\right) \quad \text{(Eq. 19a)}$$

$$N^* = \frac{k_1 A_{t1} + k_0}{k_1} \quad \text{(Eq. 19b)}$$

It is apparent from an examination of both of these relationships that a Wagner–Nelson plot in the event of Case 2 would not result in a straight line. Once again, however, as proposed by Perrier and Gibaldi and as seen in Fig. 4, the data obtained could appear to be either log-linear or initially curved followed by an apparent log-linear relationship.

Evaluating True Absorption Rate Constant and Correct Model—Based on what has been stated to this point, if one obtains plasma level data resulting from a one-compartment open model with first-order absorption and treats them *via* either a feathering or the Wagner–Nelson method, various plots may result. If one obtains what appears to be an initial curvature followed by an apparent linear portion, it may be assumed that after a finite lag time the drug is also being lost from the absorption compartment *via* either a parallel first-order or zero-order process. However, if the lag time is less than 30 min., it probably would not be detected nor be sufficiently distinguishable from a lag time that is due to a dosage form effect such as the opening of a gelatin capsule or the removal of a coating from a sugar- or film-coated tablet. However, assuming that there is a lag time prior to the onset of the parallel secondary process, the true absorption rate constant may be determined by treatment of the data obtained prior to the onset of the lag time. A suggested technique is as follows.

If one considers what is occurring prior to the onset of the lag time, it is obvious that during this period the total amount of drug in the body may be represented by Eq. 20, where A_0 represents the amount of drug in the dosage form and k_1 is the true first-order absorption rate constant:

$$D = A_0[1 - \exp(-k_1 t)] \quad \text{(Eq. 20)}$$

Correspondingly, the D/V obtained from the Wagner–Nelson method is represented by Eq. 21:

$$\frac{D}{V} = \frac{A_0}{V}[1 - \exp(-k_1 t)] \quad \text{(Eq. 21)}$$

Thus, if one were to select two values of D/V at two different time intervals prior to onset of the lag time, where $t_1 = n$ and $t_2 = 2n$, the corresponding expressions for D/V could be shown as Eqs. 22 and 23:

$$\frac{D}{V}(2n) = \frac{A_0}{V}[1 - \exp(-2nk_1)] \quad \text{(Eq. 22)}$$

$$\frac{D}{V}(n) = \frac{A_0}{V}[1 - \exp(-nk_1)] \quad \text{(Eq. 23)}$$

Dividing Eq. 22 by Eq. 23, one obtains Eq. 24, which may be modified to give Eq. 25:

$$\frac{D_{2n}}{D_n} = \frac{[1 - \exp(-2nk_1)]}{[1 - \exp(-nk_1)]} = \frac{[1 + \exp(-nk_1)][1 - \exp(-nk_1)]}{[1 - \exp(-nk_1)]} \quad \text{(Eq. 24)}$$

$$\left(\frac{D_{2n}}{D_n} - 1\right) = \exp(-nk_1) \quad \text{(Eq. 25)}$$

It is apparent from Eq. 25 that if one plots the log of the fraction $\{[D/V(2n)/D/V(n)] - 1\}$ as a function of n, a straight line will result with a slope equal to $-k_1/2.303$. Another characteristic of data plotted *via* Eq. 25 is that the line resulting from such a plot will intercept the y axis at 1 (on semilog paper) when $n = 0$. When time is equal to or greater than $t1$, the relationship is no longer linear if the parallel reaction is zero order. If the parallel reaction is first order, after the onset of the lag time one obtains a second straight line with a slope equal to $-K/2.303$. Thus, using the n–$2n$ technique (Eq. 25), one is able to differentiate between Case 1 and Case 2 when there is a finite lag time. Plots of this type are shown in Figs. 5 and 6.

In addition to the situation described, if one obtains plasma level data resulting from the one-compartment open model with first-order absorption and treats them *via* either feathering or the Wagner–Nelson method, an apparent log-linear relationship through all data points may indicate one of the following three possibilities:

1. There is complete availability of the drug with no side reactions occurring, and the slope of the log-linear plot is related to the true absorption rate constant.

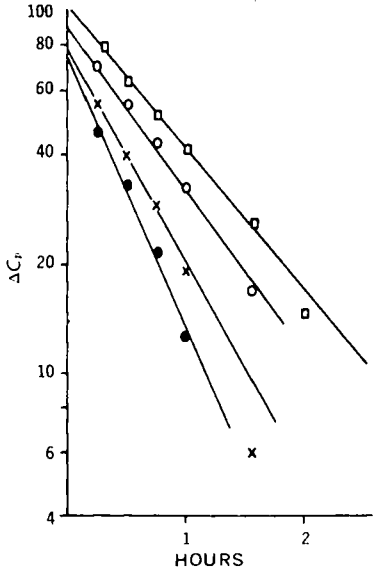

Figure 3 *Plot of* ΔC_p versus *time for data where competing zero-order process is operative, calculated according to the method of residuals* (k_0 = *25 units/hr.*). *Key:* ●, *0.5-hr. lag;* ×, *1.0-hr. lag;* ○, *2.0-hr. lag; and* □, *3.0-hr. lag.*

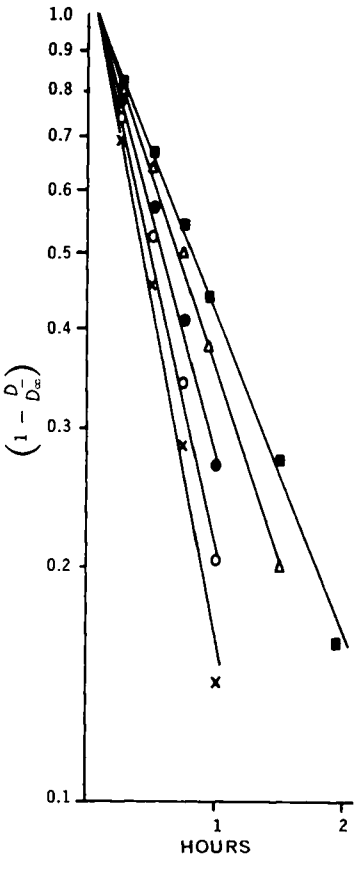

Figure 4—*Plot of Wagner–Nelson calculations* versus *time for data where competing zero-order process is operative* (k_0 = *25 units/hr.*). *Key:* ×, *no lag;* ○, *0.5-hr. lag;* ●, *1-hr. lag;* △, *2-hr. lag; and* ■, *3-hr. lag.*

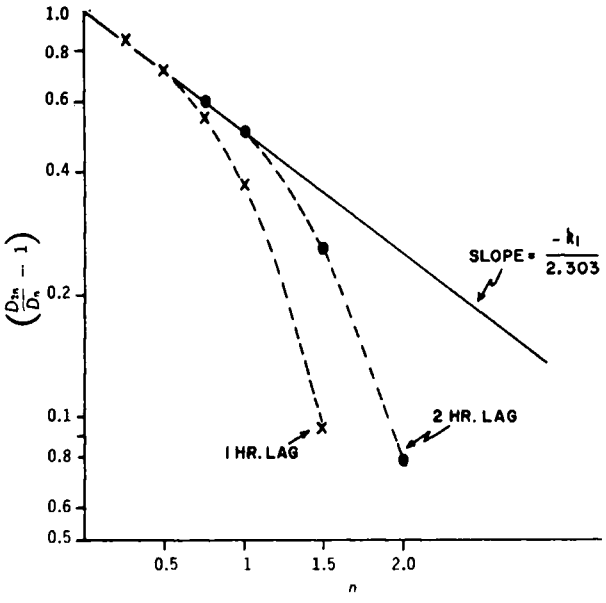

Figure 5—*Plot of data calculated according to* n–2n *method (see text for explanation)* versus *time for a competitive zero-order process* (k_0 = *25 units/hr.*). *Key:* ×, *1-hr. lag; and* ●, *2-hr. lag.*

2. In addition to absorption, there is a parallel first-order reaction occurring simultaneously with no lag time and the slope of the line represents the sum of the first-order rate constants for both the absorption and the competing process.

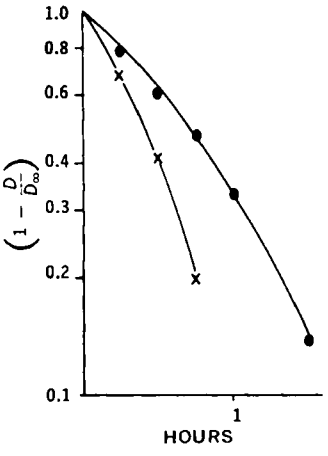

Figure 7—*Plot of Wagner–Nelson calculations* versus *time for an all-or-none competitive phenomenon. Key:* ×, *50% drug available; and* ●, *75% drug available.*

3. There is a parallel zero-order reaction process occurring, either with or without a lag time, but the data are such a magnitude as to appear linear. The apparent first-order absorption rate constant obtained is a complex function of the true rate constant and zero-rate constant.

Applying the *n–2n* technique for determining the true first-order absorption rate constant will result in linear relationships in both possibilities 1 and 2, with the resulting slope being identical to that obtained *via* the Wagner–Nelson or feathering method. Therefore, this procedure cannot differentiate between possibilities 1 and 2, and one does not get a measure of the true first-order absorption rate constants in the case of possibility 2. The plot, however, in the case of possibility 3 demonstrates a marked degree of curvature if there is no lag time. If there is a finite lag time, the data up to this time are linear, passing through $y = 1$ (on semilog paper) at $n = 0$.

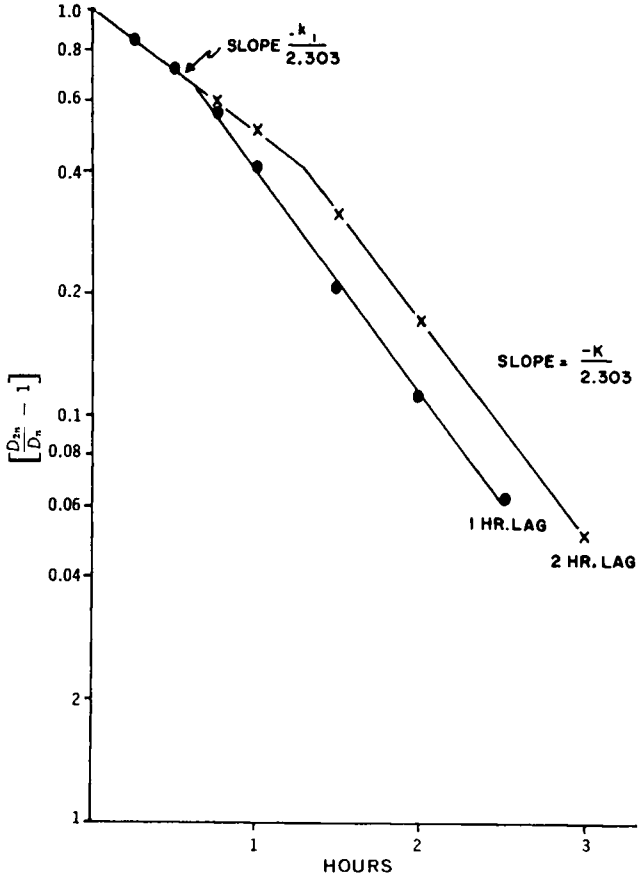

Figure 6—*Plot of data calculated according to* n–2n *method* versus *time for a competitive first-order process* (k_1 = *0.5*). *Key:* ●, *1-hr. lag; and* ×, *2-hr. lag.*

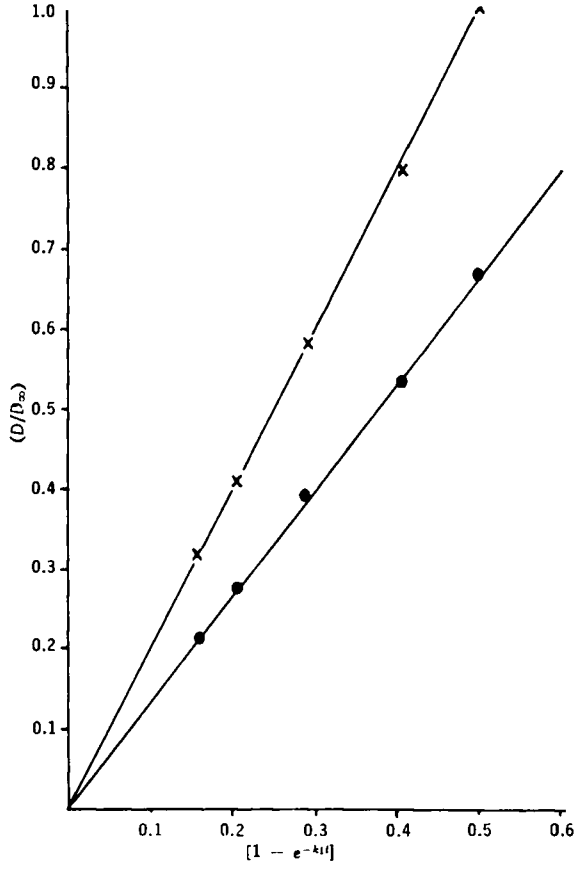

Figure 8—*Plot for determining availability (see text for explanation) for all-or-none phenomenon. Key:* ×, *50% drug available; and* ●, *75% drug available.*

In addition, $t1$ is approximately twice the last n value to fall on the line. Such results allow one to differentiate between this case and the other two (Fig. 5).

This discussion has indicated that the n–$2n$ method may be used to distinguish between parallel zero-order and parallel first-order reactions which may be occurring with no lag time and parallel zero-order and parallel first-order reactions which may be occurring with finite lag times. However, it is not able to distinguish between the parallel first-order reaction with no lag time and the case of no parallel reaction occurring. In addition, one is unable to make an evaluation of the true first-order absorption rate constant in the case of a parallel first-order reaction with no lag time.

Notari *et al.* (1) have indicated a technique for determining the true first-order absorption rate constant. This is done by multiplying K by the fraction of the drug absorbed. However, data other than merely plasma levels (*e.g.*, urinary excretion) would be required to estimate the fraction of drug absorbed. Unfortunately, in many instances, one obtains only plasma level data. The n–$2n$ technique for determining the true first-order absorption rate constant, although not requiring any additional measurements, does necessitate the occurrence of a finite lag time. In addition, if one does have a finite lag time with a parallel first-order process and determines the true absorption rate constant, the fraction of drug available after onset of the lag time may be determined from a ratio of (k_1/K). Correspondingly, the true fraction available (f) is defined by Eq. 26:

$$f = 1 - \frac{k_3}{K} \exp(-k_1 t1) \qquad \text{(Eq. 26)}$$

$$k_3 = K - k_1 \qquad \text{(Eq. 26}a\text{)}$$

In such a case, one is able to evaluate the availability employing *only* plasma level measurements.

"All or None" Phenomena—The previous discussion omitted consideration of the "all or none" phenomena proposed by Perrier and Gibaldi (2). This refers to the case of a nondisintegrating dosage form moving through the GI tract where absorption is occurring and suddenly reaching an area where absorption ceases. Treatment of these data *via* the Wagner–Nelson method results in a non-log-linear relationship. This is because of the considerations in Eqs. 27–30, where f is the fraction of drug absorbed and the other constants are as previously defined:

$$\frac{D}{V} = \frac{A_0}{V}[1 - \exp(-k_1 t)] \qquad \text{(Eq. 27)}$$

$$\frac{D_\infty}{V} = f\left(\frac{A_0}{V}\right) \qquad \text{(Eq. 28)}$$

$$\frac{D}{D_\infty} = \frac{[1 - \exp(-k_1 t)]}{f} \qquad \text{(Eq. 29)}$$

$$[1 - D/D_\infty] = \frac{f - 1 + \exp(-k_1 t)}{f} \qquad \text{(Eq. 30)}$$

It may be seen from Eq. 30 that the expression $(1 - D/D_\infty)$ is not a log-linear relationship when f is less than 1. Plots of this type are shown in Fig. 7 for f values of 0.5 and 0.75. However, if one employs the n–$2n$ technique, the true first-order absorption rate constant would be obtained in these cases. In addition, once k_1 has been evaluated, from Eq. 29 it is seen that from a plot of D/D_∞ against $(1 - e^{-k_1 t})$ the fraction absorbed may be determined as the reciprocal of the slope (Fig. 8). Therefore, employing Eqs. 25 and 29, one may test for the existence of the all-or-none phenomena.

In conclusion, as indicated by Perrier and Gibaldi, the occurrence of incomplete availability will in most cases result in an incorrect measure of the true absorption rate constant. The one exception to their generalization is the case of a poorly designed formulation which would only release a certain fraction of its drug, even if it remained at an absorption site for an infinite length of time. In this instance, the value obtained for the absorption rate constant will be the correct one.

Because the data in this report were computer generated rather than experimentally determined, one could suggest that *in vivo* results would not allow the detection of some of the anomalies reported here. However, on occasion these authors have encountered curvature of a log $(1 - D/D_\infty)$ or ΔC_p plot, and an awareness of the concepts proposed by Notari *et al.* (1) or Perrier and Gibaldi (2) would have allowed for an alternative data treatment. In addition, this report proposes an alternative technique for treating data generated by the Wagner–Nelson (3) or Loo–Riegleman (4) techniques which could allow detection of the fraction of drug absorbed in some of the models proposed by Perrier and Gibaldi.

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Competitive Inhibition of Drug–Protein Interaction in Eye Fluids and Tissues

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Abstract □ Binding of drugs to proteins in fluids and tissues of the eye previously was shown to modify significantly the bioavailability of a number of drugs. This adverse effect is particularly important in the precorneal portion and anterior chamber of the eye, since tears and aqueous humor are turned over at relatively rapid rates, removing both free and bound forms of the drug. In addition, rapid drainage of instilled solutions also removes substantial drug quantities. The drug–protein interaction can be minimized through the use of competitive inhibitors of protein binding. These agents occupy binding sites normally bound by drug, increasing free drug concentration. *In vitro* experiments with eye fluids and tissues, as well as *in vivo* studies in rabbits, support the concept of competitive inhibition as a means of significantly improving drug bioavailability. These experiments are principally concerned with competitive inhibition in the precorneal portion of the eye. Pilocarpine nitrate, a miotic drug with low binding affinity for albumin, shows a 10-fold increase in biological activity in the presence of the competitive inhibitor cetylpyridinium chloride. This increase in response is suggested to be due, in part, to competitive inhibition of drug–protein interaction. Competitive inhibition of drug binding in tears and the precorneal portion of the eye can be applied successfully without the attendent problems when the concept is applied to blood proteins. Tear proteins have apparently less substrate carrier function as compared to blood proteins, there is less total protein in tears as compared to blood, and the amount of drug plus inhibitor applied to the eye is small. Thus, side effects due to undesired substrate displacement are not present when rather nonspecific competitive inhibitors are used in ophthalmic systems. Extrapolation of the pilocarpine nitrate data to drugs with higher protein binding affinities suggests that substantial increases in drug activity can be expected through competitive inhibition of drug–protein interactions in eye fluids and tissues.

Keyphrases □ Drug–protein interactions in eye fluids and tissues—effect of competitive protein binding inhibitors on drug availability □ Protein binding—effect of competitive inhibitors on drug availability, eye fluids and tissues □ Ophthalmic bioavailability—effect of competitive protein binding inhibitors on drug–protein interactions in eye fluids and tissues □ Bioavailability, ophthalmic—effect of competitive protein binding inhibitors on drug–protein interactions in eye fluids and tissues

An earlier report (1) from this laboratory described the importance of drug binding to proteins in tissues and fluids of the eye. Tears, cornea, and, in some circumstances, aqueous humor contain appreciable quantities of protein, which bind drugs and reduce their effective free concentrations. Since both tears and aqueous humor are turned over at relatively rapid rates, removing both free and bound forms of the drug, the influence of drug–protein binding on drug bioavailability is significant. The present report is concerned with a proposed method for competitive inhibition of drug–protein interaction through the use of rather nonspecific "biologically inert" inhibitors. A substantial increase in drug activity in the presence of these inhibitors is observed and offers an attractive method to improve drug bioavailability in the eye.

The concept of competitive inhibition of drug–protein binding to increase free drug concentration is not new (2). Synergistic behavior of drug effects is considered to be in some instances a result of competitive displacement of a strongly bound drug (2). Suggestions of the use of substrates for intentional competitive inhibition have been made (3), but apparently no specific application has been reported. There are several reasons why systemic application of competitive inhibition to increase drug activity has not been successful: (*a*) blood proteins normally serve as carriers for numerous substrates, so a competitive inhibitor must be highly specific for sites on the protein or displacement of normal substrates will occur followed by undesirable side effects; and (*b*) the enormous amount of protein present in blood requires that large amounts of the inhibitor be used to cause successful competitive inhibition. Thus, application of competitive inhibition of drug–protein binding in the blood is very limited, and no successful application of the concept to an *in vivo* situation could be found.

However, when the proposal is considered with respect to the eye, it has much greater potential. The relative isolation of the eye coupled with the limited protein content and the lack of extensive carrier function of this protein, as compared to blood protein, makes the concept of competitive inhibition of drug–protein interaction an attractive, workable proposal for increasing drug bioavailability (4). This report is concerned with the feasibility of competitive inhibition of drug–protein binding in eye fluids and tissues.

EXPERIMENTAL

Materials—Water was distilled from alkaline permanganate in an all-glass distillation apparatus. Cellulose dialysis tubing[1] and crystalline rabbit serum albumin[2] were used.

Adult, male albino rabbits[3], New Zealand strain, were the experimental animals. No special pretreatment methods or diets were incorporated into the *in vivo* procedures.

The following chemicals were used: methylprednisolone[4], sulfisoxazole[5], and *N*-1-naphthylethylenediamine dihydrochloride[6]. All other chemicals were either USP or reagent grade and were used as received.

Equilibrium Dialysis—The cells and procedures used were described previously (1). Protein solutions or eye fluid and tissue samples were placed in one half-cell chamber, with or without the competitive inhibitor, and the drug solution was placed in the other half of the dialysis cell. The dialysis membrane preparation and the drug and dialysis solution preparation were described previously (1).

Assays—Analytical procedures were described previously (1).

Miosis–Time Studies—A complete description of procedures used in the pupillary diameter measurement was given previously (1). The competitive inhibitor was added directly to the drug solution prior to instillation into the rabbit eye.

[1] Union Carbide Corp., Chicago, Ill.
[2] Schwarz/Mann, Orangeburg, N. Y.
[3] Klubertanz, Edgerton, Wis.
[4] Gift of The Upjohn Co., Kalamazoo, Mich.
[5] Gift of Hoffmann-La Roche, Inc., Nutley, N. J.
[6] Eastman Organic Chemicals, Rochester, N. Y.

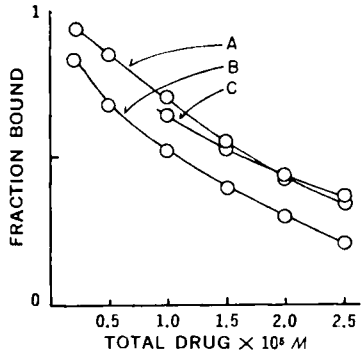

Figure 1—*Equilibrium dialysis experiments on the extent of binding of sulfisoxazole to rabbit serum albumin, alone and in combination with the competitive inhibitors sodium lauryl sulfate and polyoxyethylene (23) lauryl ether. The data points are mean values from four separate determinations, and standard deviations are within ±5% of the mean values. Key: A, drug only; B, drug plus 0.01% sodium lauryl sulfate; and C, drug plus 0.01% polyoxyethylene (23) lauryl ether.*

RESULTS

***In Vitro* Demonstration of Competitive Inhibition of Drug–Protein Interaction**—A commonly used method to illustrate competitive inhibition of drug–protein interactions is equilibrium dialysis. The increase in free drug concentration in the presence of a competitive inhibitor reflects competitive inhibition. This is illustrated in Fig. 1 for sulfisoxazole and rabbit serum albumin, using sodium lauryl sulfate and polyoxyethylene (23) lauryl ether[7] as competitive inhibitors. Sodium lauryl sulfate is an effective competitive inhibitor in this system, whereas polyoxyethylene (23) lauryl ether is not. Figure 2 shows where polyoxyethylene (23) lauryl ether is a successful inhibitor of the binding of methylprednisolone to rabbit serum albumin. These examples illustrate the structural and charge requirements for successful competitive inhibition of a particular drug–protein interaction. Ideally, the competitive inhibitor should bind to the same site normally occupied by the drug molecule and exhibit a greater affinity for that site than the drug. A further description of structural requirements for competitive inhibitors is available (4) and will be discussed subsequently.

An extensive search for an optimum competitive inhibitor for a particular drug was not conducted in the present study. The study objective was to show successful competitive inhibition in ophthalmic systems. For this purpose, a competitive inhibitor was selected on the general basis that it should have a similar charge type as the drug in question and an affinity for albumin that is substantially larger than that of the drug. With respect to this last point, an inhibitor was chosen that had reasonable aqueous solubility and, at the same time, possessed a hydrophobic chain for stabilization of the drug–protein complex when formed. All of these requirements seem to be met with the surfactant class of compounds. Competitive inhibition of drug binding to rabbit serum albumin was used as a qualitative yardstick to select competitive inhibitors.

Since all competitive inhibitors reported in this study are surface active and can associate to form micelles in aqueous solution, consideration was given to the concentration of surfactant used. Micelle formation would considerably increase the complexity of these systems and make interpretation of the results more difficult (5–7).

The concentration of surfactant used in all *in vivo* experiments was below the reported (8) critical micelle concentration (CMC). However, considering the decrease in CMC that results from addition of salts and buffer components, it is probable that the instilled solutions were above the CMC. Upon instillation of the drug solution, there is a dilution with tears and an elevation in solution temperature to that of the body, which, when coupled with tear turnover, should reduce the micellar influence.

Competitive inhibition of drug–protein binding in ocular tissues and fluids is also possible and can be demonstrated. Figure 3 shows the binding of methylprednisolone to the components of human tears in the presence and absence of polyoxyethylene (23) lauryl ether. The sizable decrease in fraction of methylprednisolone bound to tear protein in the presence of polyoxyethylene (23) lauryl ether is apparent and, when extrapolated to a living system, suggests a considerable increase in bioavailability for this drug. The increase in bioavailability to the eye is particularly significant when it is realized that solution drainage from the eye is very rapid and that the rate of tear turnover is quite large (1, 9).

[7] Brij-35, ICI America, Inc.

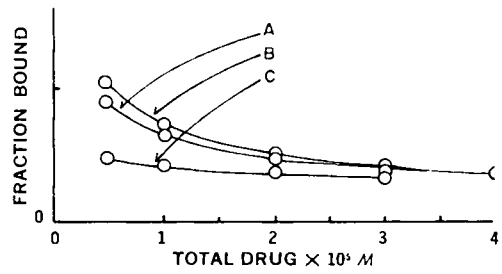

Figure 2—*Equilibrium dialysis experiments on the extent of methylprednisolone binding to rabbit serum albumin, alone and in combination with the competitive inhibitors sodium lauryl sulfate and polyoxyethylene (23) lauryl ether. The data points are mean values from four separate determinations, and standard deviations are within ±5% of the mean values. Key: A, drug only; B, drug plus 0.01% sodium lauryl sulfate; and C, drug plus 0.01% polyoxyethylene (23) lauryl ether.*

A significant decrease in fraction bound is also observed for drugs that are not highly bound to proteins (1), such as the miotic drug pilocarpine nitrate. Figure 4 illustrates the fraction of pilocarpine bound to the components of plasmoid aqueous humor in the presence and absence of the competitive inhibitor cetylpyridinium chloride. In the presence of cetylpyridinium chloride, the data points fall on the abscissa axis, showing that complete inhibition of binding occurs. Plasmoid aqueous humor was obtained by paracentesis and has the property of being very rich in proteins as compared to normal aqueous humor. Cetylpyridinium chloride is commonly used as a preservative in drug delivery systems, but its structural features make it an efficient competitive inhibitor for binding of certain cationic drugs to proteins.

***In Vivo* Demonstration of Competitive Inhibition of Drug–Protein Interaction**—Successful competitive inhibition of drug–protein binding in eye fluids and tissues should result in an increase in free drug concentration and an increase in the corneal transport rate of the drug in question. The elevated concentration of free drug should be demonstrable in an *in vivo* system through either an increased biological response to the drug or actual measurement of drug concentrations.

To demonstrate the increased biological response, the miotic drug pilocarpine nitrate was selected and pupillary diameter change was used as the biological response. Figure 5 shows miosis–time profiles for several concentrations of pilocarpine nitrate, with and without cetylpyridinium chloride, in albino rabbits. Several interesting aspects to these curves will be explored in a separate publication,

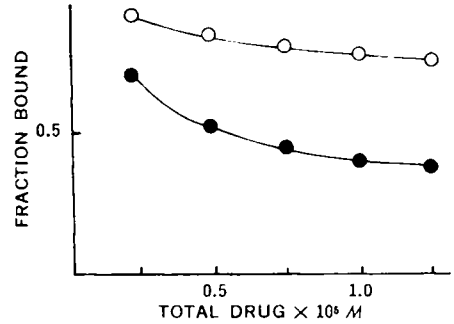

Figure 3—*Equilibrium dialysis experiments on the extent of methylprednisolone binding to the components of human tears in the presence and absence of polyoxyethylene (23) lauryl ether. Key: ○, drug only; and ●, drug plus polyoxyethylene (23) lauryl ether. The data points are mean values from three separate determinations, and standard deviations are within ±7% of the mean values.*

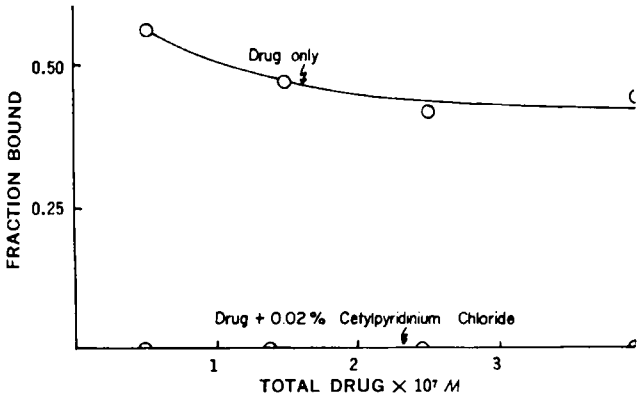

Figure 4—*Equilibrium dialysis studies of tritiated pilocarpine nitrate binding to plasmoid aqueous humor in the presence and absence of 0.02% cetylpyridinium chloride. The data points for the cetylpyridinium chloride study fall on the abscissa axis since there was no binding. Each data point represents the mean values from two determinations and has a standard error equal to or less than 10% of the mean value.*

but only the relationship between dose and maximum response is of interest here.

Addition of cetylpyridinium chloride to the pilocarpine nitrate solution prior to instillation increases the miotic effect at all concentrations tested. In Fig. 6, the maximum change in pupillary diameter was used as the biological response to generate a dose-response relationship. The log dose-response curve appears to be sigmoidal in shape, with the upper limit being set by either saturation of all existing receptor sites or the smallest pupillary diameter attainable while the lower end is no response at all. An upper limit was not reached due to several factors associated with the pilocarpine nitrate solution, such as approaching the solubility limit of the drug in the buffer at the 5×10^{-1} *M* dosage concentrations and encountering other severe, concomitant drug responses at the higher dosing levels. Nevertheless, the log dose-response relationship is linear over several orders of magnitude.

As can be seen from Fig. 6, the activity of pilocarpine nitrate was increased by a factor of 10 when 0.02% cetylpyridinium chloride was added to the system. Thus, 10 times less drug is required to achieve the same pupillary diameter when the competitive inhibitor is added to the drug solution. Instillation of cetylpyridinium chloride by itself had no effect on pupillary diameter.

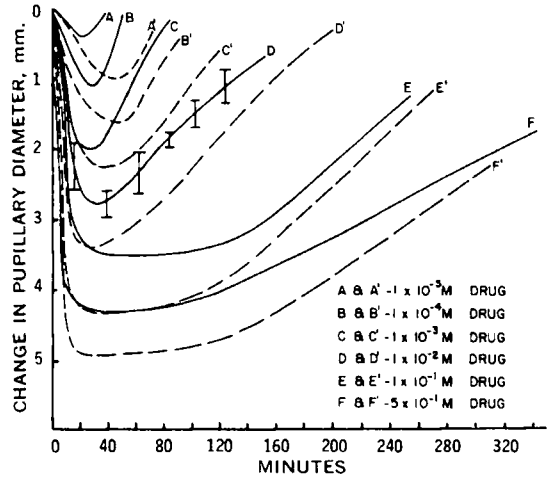

Figure 5—*Miosis-time profiles for various concentrations of pilocarpine nitrate, with and without 0.02% cetylpyridinium chloride. Key: ——, pilocarpine nitrate alone; ---, pilocarpine nitrate plus competitive inhibitor. Standard deviation lines are shown for the 1 × 10^{-2} M drug case only for the sake of clarity. All other lines have similar standard deviations. Each concentration line represents a minimum of four separate determinations in four different animals. Each line was constructed on the basis of 15–40 experimental points.*

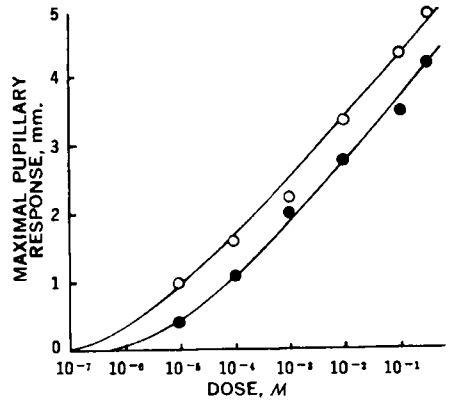

Figure 6—*Maximal miotic response of the rabbit eye to pilocarpine nitrate, with and without 0.02% cetylpyridinium chloride, as a function of dose. Key: ●, drug alone; and ○, drug plus 0.02% cetylpyridinium chloride.*

Examination of the corneal surface with fluorescein solution and UV light, following repeated application of this concentration of cetylpyridinium chloride for several days, showed no visual damage to the epithelial layer of the cornea.

DISCUSSION

Very small amounts of an instilled drug are transported across the cornea, due in part to the rapid drainage of an instilled solution and to rapid tear turnover (9). Both of these processes cause removal of drug from the precorneal area. If a drug undergoes extensive binding to proteins in tears or to precorneal eye tissue, the amount of free drug available to exert a local effect or to be absorbed across the cornea is reduced significantly. Moreover, the very efficient removal of both free and bound forms of the drug from this area is responsible for a considerable loss in drug and a reduction in drug activity. Similarly, binding of drug to the cornea reduces the transport rate of drug through the cornea, which, when coupled with the turnover of aqueous humor, can greatly reduce the concentration of drug in the anterior chamber of the eye. Therefore, competitive inhibition of this drug-protein binding, especially in the precorneal area, should result in a considerable increase in the bioavailability of drugs that are normally bound to proteins.

The *in vivo* data presented show that addition of certain substrates to ophthalmic drug solutions increases the rate of corneal transport for these drugs as evidenced by miotic increases. The *in vitro* equilibrium dialysis data demonstrate that these substrates are able to serve as competitive inhibitors of drug-protein binding. It is our belief that a portion or all of the absorption rate enhancement through the addition of "biologically inert substances" to the drug solution is through competitive inhibition of drug-protein interaction. The data strongly support the suggestion that enhanced corneal penetration of drugs by addition of certain substrates is through a competitive inhibition mechanism. However, the data do not preclude all other possible mechanistic interpretations.

Because of the structural requirements for competitive inhibition of drug-protein binding, all substrates used were surface active and fall in the surfactant class of compounds. That surfactants can influence the extent of corneal absorption of drugs is well known (10), but this influence has not been attributed to competitive inhibition of drug-protein binding.

Surfactant influence on drug absorption in various parts of the body (11, 12) including the eye (10, 13, 14) is well established. What is not well established is the mechanism for this effect. Such factors as drug solubilization above the CMC, wetting of the membrane surface, and structural modification of the membrane have all been indicated in promoting or retarding drug absorption through biological membranes (5, 6, 15). However, the influence of premicellar concentrations on drug transport has not been studied thoroughly, and any influence on drug transport rate is generally ascribed to changes in the membrane.

It is our belief that competitive inhibition of drug-protein binding is at least partially responsible in many cases of enhanced drug transport. For example, it has been shown that surfactants induce

structural changes in proteins (16) and in biological membranes (13) which, in terms of membrane transport, can be interpreted as an enlargement of either the effective pore size in the membrane or the area of the membrane, thus leading to a rate enhancement. An alternative explanation is that the protein modification leads to a decrease in available binding sites and, hence, less protein binding of the drug.

Consider the possible alternative mechanisms of enhanced corneal transport in more detail. The influence of detergents on proteins varies considerably as a function of pH, detergent structure and concentration, and type of protein. Some detergents such as sodium lauryl sulfate induce large changes in the protein (17, 18), and their competitive inhibition activity in equilibrium dialysis experiments is usually ascribed to this protein denaturation. With sodium lauryl sulfate, this influence exists both above and below the CMC. However, some surfactants do compete for drug binding sites in the classical sense (16). In the present study, several surfactants were used but the principal agent was cetylpyridinium chloride. This compound was used in all experiments at a concentration of 0.02%. Neither the data in this report nor the data in the literature elucidate the mechanism of its competitive inhibitory activity. Thus, whether this agent is merely competing for the binding sites normally occupied by the drug or is removing the binding sites through an unfolding mechanism is unknown. Irrespective of the mechanism, the net result of the interaction, namely, competitive inhibition of drug–protein binding, is the important point. The remaining question is whether competitive inhibition in tears is responsible for the entire effect or is it necessary to include alteration of the epithelial layer of the cornea. If inclusion of corneal tissue changes is necessary, what is the mechanism of transport *via* this change? Is it competitive inhibition *via* protein denaturation or is it a physical enhancement as described earlier?

The *in vivo* activity of pilocarpine nitrate plus 0.02% cetylpyridinium chloride can be significantly decreased by addition of albumin to the drug–cetylpyridinium chloride solution, suggesting that drug–protein binding can alter the availability of free drug (1). However, raising the concentration of cetylpyridinium chloride in the drug–albumin solution restores the biological activity so that a fully reversible system can be established and the reversibility is controlled either by albumin or cetylpyridinium chloride. This supports competitive inhibition in the tear film as a contributing factor in the enhancement of drug activity.

Further support for competitive inhibition of drug binding as a principal mechanism was obtained using the anionic steroid fluorometholone. Addition of cetylpyridinium chloride to an albumin–fluorometholone solution in an equilibrium dialysis experiment does not raise the free drug concentration, as expected, since the steroid is of neutral charge as compared to cetylpyridinium chloride and would therefore occupy different sites. In a similar vein, cetylpyridinium chloride does not promote corneal absorption of this drug[8]. If changes in the corneal tissue by addition of cetylpyridinium chloride were responsible for promoting or inhibiting drug penetration, it is expected that all drugs would be affected, albeit to different extents. It is, of course, possible that the different drugs penetrate the cornea *via* different routes, *i.e.*, intercellular *versus* intracellular, and the membrane changes caused by cetylpyridinium chloride do not influence the pathway through which fluorometholone is absorbed.

Data supporting a corneal membrane effect for cetylpyridinium chloride and other agents are given by Green and Tonjum (13). Using *in vitro* corneal transport studies, they showed that application of surfactants such as cetylpyridinium chloride and benzalkonium chloride to the cornea, prior to application of a fluorescein solution, results in an increased penetration of fluorescein. Based on electron micrographs, the mechanism of enhanced penetration was ascribed to an increased intercellular space area in the outermost epithelial layer of the cornea, analogous with the effects of ouabain as well as high endothelial pressure, both of which lead to an increased rate of drug penetration.

There are major differences in experimental technique between the study of Green and Tonjum (13) and the present work, and it is important to consider these differences in trying to assign a mechanism of action. Green and Tonjum used concentrations of cetylpyridinium chloride that were well above the CMC (8). A conclusion as to whether or not they were above the CMC with benzalkonium chloride cannot be reached without knowing the specific structure of the quaternary ammonium compound used. In addition to the differences in initial cetylpyridinium chloride concentration, there was a difference in contact time and concentration during this contact time. Green and Tonjum soaked the excised cornea in the surfactant solution for 2 min. prior to application of the fluorescein solution. This approach yields a significantly higher effective surfactant concentration than do experiments on an intact animal, where tear and instilled solution drainage as well as tear turnover significantly alter the surfactant concentration (9). Thus, concentrations of cetylpyridinium chloride above the CMC as well as the higher effective concentration for longer periods might as well lead to membrane changes that do not occur at lower concentrations in *in vivo* experiments.

Due to the differences in the experimental techniques of Green and Tonjum and the present study, a definite conclusion as to mechanisms apparently cannot be made. It is, of course, possible to interpret the results of Green and Tonjum in terms of competitive inhibition; *i.e.*, the increased intercellular area is merely a form of protein unfolding and therefore binding sites are masked as opposed to the more physical interpretation of a greater area for drug penetration.

In summary, it would appear that competitive inhibition of drug–protein binding is at least partly responsible for an increase in drug transport rate when some surfactants or competitive inhibitors of drug–protein binding are incorporated into the drug delivery system. It is probable, based on the work by Green and Tonjum (13), that cetylpyridinium chloride and similar substrates under suitable conditions owe part of their drug transport potentiation property to changes in the epithelial layer of the cornea, although the specific mechanism is unknown.

All work described in this report was carried out on healthy eyes. Diseased eyes will show a significant increase in precorneal protein binding and are better candidates for competitive inhibition of drug–protein binding.

[8] Unpublished data from this laboratory.

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Effect of Various Steroids and ACTH on Distribution of Zoxazolamine in Rats

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Abstract □ A study was performed on rats given pregnenolone-16α-carbonitrile (I), triamcinolone, fludrocortisone acetate, corticosterone, estradiol, or ACTH to investigate correlations between the *in vivo* effects upon zoxazolamine paralysis and the concentrations of this drug in plasma, brain, liver, kidney, heart, spleen, muscle, and fat. Pretreatment with the steroids or ACTH altered the organ concentrations of zoxazolamine but not the relationships between the interorgan concentrations. Protection by I was associated with a simultaneous reduction of zoxazolamine in plasma, brain, and most of the other organs (catatoxic mechanism) as compared with controls killed when paralysis disappeared in the pretreated group. The protective actions of ACTH and triamcinolone were often accompanied by high concentrations of zoxazolamine in plasma and most of the organs (syntoxic mechanism) when compared with controls sacrificed at spontaneous termination of the pharmacological response. Fludrocortisone seemed to protect through both phenomena. Corticosterone and estradiol were ineffective under the same conditions, eliciting no changes in the plasma clearance or organ concentrations of the drug.

Keyphrases □ Steroids (pregnenolone-16α-carbonitrile, triamcinolone, fludrocortisone acetate, corticosterone, and estradiol)—effect on plasma and organ concentrations of zoxazolamine □ ACTH—effect on plasma and organ concentrations of zoxazolamine □ Zoxazolamine, plasma concentrations—effect of steroids and ACTH, resistance–plasma concentration correlations

Numerous observations (1–3) have confirmed that steroids and certain other pharmacological agents play a decisive role in altering drug activity, thereby influencing the resistance of the body against the most varied types of injury. These adaptive steroids have been classified according to their mechanism of protective action into: (*a*) syntoxic steroids, which improve host tissue tolerance by permitting coexistence with the toxic agent (*e.g.*, by suppressing nonspecific inflammatory or allergic reactions against it); and (*b*) catatoxic steroids, which enhance the detoxication of endogenous or exogenous toxicants *via* induction, activation, decreased degradation of drug-metabolizing enzymes, and/or accelerated substrate elimination. Of the many steroids studied, it has been found that pregnenolone-16α-carbonitrile (I) exerts the greatest protective effect *in vivo*. This compound is a known hepatic microsomal enzyme inducer (4, 5). Biochemical studies have shown that protection offered by catatoxic steroids (*e.g.*, I and spironolactone) is usually accompanied by a significant reduction of drug concentrations in plasma (6, 7). To obtain the best catatoxic effect, the steroids have to be administered at least 3 days before the toxicant. However, in some cases, even posttreatment offers significant protection (8).

Recent studies (6, 7) showed that the protective effect of I and spironolactone against zoxazolamine and methyprylon can be considered as catatoxic and that of triamcinolone and ACTH can be considered as syntoxic. On the basis of these findings, it was established that these phenomena are characterized by the following relationships:

catatoxic	$C_{cd} < C_1$	$C_{cd} \simeq C_2$
syntoxic	$C_{cd} \simeq C_1$	$C_{cd} > C_2$

where:

C_{cd} = plasma concentration of the drug in pretreated animals at the termination of the pharmacological response

C_1 = plasma concentration of the drug in not yet recovered controls killed when the pharmacological response has disappeared in the pretreated group

C_2 = plasma concentration in recovered controls sacrificed at the termination of the pharmacological response

In previous experiments (6), estradiol was found to be very active against dicumarol. Although unusual, this is not an altogether surprising observation since estradiol can affect the response to other drugs (9–12). The protection offered by syntoxic compounds (*e.g.*, triamcinolone and ACTH) is difficult to explain, although altered drug distribution may be involved. The concentrations of zoxazolamine in some organs and the correlation between the plasma and brain concentrations of this drug were reported by others (13, 14). Changes in distribution of zoxazolamine in the central nervous system (CNS) and plasma as a function of time or chlordane pretreatment were also studied (15). However, it appears that there have been no investigations on the concentrations of this drug in other organs and on the effect, if any, of steroid pretreatment.

In addition to experiments on possible changes in the organ concentrations, it seemed of interest to determine whether: (*a*) the two adaptive steroid groups also produce characteristic alterations in drug distribution; (*b*) changes in the drug plasma concentrations are reflected in the brain, liver, kidney, heart, muscle, and fat; and (*c*) the characteristic relationships for syntoxic and catatoxic steroids, based on plasma concentrations, are applicable to drug concentrations in the parameters just mentioned.

EXPERIMENTAL

Female Sprague-Dawley rats[1], averaging 100 g. (range of 90–110 g.), were used and maintained on laboratory food[2] and tap water *ad libitum*. The animals were divided into three groups. The controls (Groups 1 and 2) were given 1 ml. of water twice daily *per os* for 3 days and once (1 hr. before zoxazolamine) on the 4th day. The rats of the remaining group received I[3] (3β-hydroxy-20-oxo-5-

[1] Canadian Breeding Farms & Laboratories Ltd., St. Constant, Quebec, Canada.
[2] Purina laboratory chow, Ralston Purina Co. of Canada.
[3] The Upjohn Co.

Table I—Effect of Various Steroids and ACTH on the Distribution of Zoxazolamine

Pretreatment	Group	Zoxazolamine Concentration[a], mcg./g. Liver	Brain	Kidney	Muscle	Adipose Tissue	Heart	Spleen	Plasma
	Unrecovered control[b]	144.08 ± 6.5*** (13)[c]	98.23 ± 6.9*** (11)	115.86 ± 1.9*** (8)	34.74 ± 6.0** (6)	88.98 ± 5.7*** (10)	92.90 ± 3.1*** (8)	47.00 ± 6.7 NS (6)	35.44 ± 1.1*** (8)
I	Pretreated[d]	87.42 ± 8.4 (13)	63.57 ± 4.8 (9)	73.75 ± 2.6 (8)	11.76 ± 2.3 (6)	45.08 ± 4.9 (8)	59.12 ± 2.9 (8)	39.33 ± 4.9 (8)	26.07 ± 1.4 (6)
	Recovered control[d]	97.91 ± 2.6 NS (11)	69.79 ± 3.5 NS (10)	85.90 ± 2.7* (6)	28.83 ± 2.8*** (8)	81.35 ± 7.7** (8)	63.68 ± 2.9 NS (6)	42.17 ± 6.1 NS (6)	27.34 ± 0.7 NS (6)
	Unrecovered control[b]	130.91 ± 9.7*** (11)	91.12 ± 4.1 NS (10)	100.07 ± 2.2 NS (6)	31.08 ± 2.1 NS (8)	81.61 ± 4.1 NS (10)	84.95 ± 3.9 NS (8)	45.00 ± 4.3 NS (10)	28.80 ± 1.3* (12)
Triamcinolone	Pretreated[d]	88.13 ± 4.8 (12)	85.36 ± 4.2 (12)	98.73 ± 4.6 (6)	31.68 ± 0.9 (6)	79.13 ± 2.4 (10)	73.36 ± 4.1 (10)	42.50 ± 4.2 (6)	32.90 ± 1.7 (12)
	Recovered control[d]	103.32 ± 6.9 NS (11)	72.14 ± 3.6* (10)	88.02 ± 4.2 NS (6)	24.16 ± 2.3* (6)	79.47 ± 5.5 NS (8)	72.72 ± 1.4 NS (6)	35.50 ± 4.7 NS (6)	24.70 ± 1.0** (12)
	Unrecovered control[b]	117.35 ± 3.4* (8)	105.44 ± 6.6 NS (6)	81.57 ± 6.2 NS (8)	38.88 ± 4.0 NS (6)	116.17 ± 8.8 NS (6)	62.31 ± 1.8 NS (6)	50.70 ± 3.3 NS (8)	23.03 ± 1.3 NS (7)
Corticosterone	Pretreated[d]	102.92 ± 3.4 (8)	114.41 ± 7.5 (7)	70.88 ± 0.9 (8)	28.64 ± 1.1 (8)	97.01 ± 4.9 (8)	62.63 ± 2.7 (8)	46.59 ± 3.5 (8)	23.09 ± 0.7 (7)
	Recovered control[d]	94.00 ± 3.0 NS (6)	100.96 ± 3.8 NS (6)	70.46 ± 4.2 NS (6)	23.07 ± 2.1 NS (6)	105.63 ± 5.3 NS (6)	59.55 ± 4.8 NS (8)	40.95 ± 4.0 NS (6)	22.64 ± 1.1 NS (7)
	Unrecovered control[b]	190.68 ± 4.1*** (8)	150.59 ± 3.6*** (8)	90.80 ± 2.9*** (8)	42.16 ± 2.2* (8)	146.49 ± 7.5*** (8)	59.28 ± 3.1 NS (6)	56.00 ± 2.0*** (8)	26.55 ± 1.4* (7)
Fludrocortisone	Pretreated[d]	121.77 ± 4.0 (7)	101.33 ± 2.3 (7)	70.09 ± 3.5 (8)	31.96 ± 3.2 (8)	87.83 ± 4.4 (8)	57.16 ± 2.5 (6)	48.64 ± 2.5 (8)	23.10 ± 1.3 (7)
	Recovered control[d]	137.11 ± 3.9* (6)	93.88 ± 3.1 NS (6)	60.27 ± 0.3* (6)	34.26 ± 2.0 NS (6)	94.15 ± 4.1 NS (6)	54.36 ± 2.0 NS (6)	44.85 ± 4.4 NS (6)	22.64 ± 1.1 NS (6)
	Unrecovered control[b]	102.56 ± 5.0 NS (8)	116.37 ± 4.5 NS (8)	70.22 ± 2.7 NS (8)	30.59 ± 2.7 NS (8)	88.78 ± 6.4 NS (8)	63.61 ± 0.6 NS (8)	44.32 ± 2.7 NS (8)	28.30 ± 1.3 NS (6)
Estradiol	Pretreated[d]	98.39 ± 3.3 (8)	108.67 ± 6.3 (7)	63.10 ± 6.7 (8)	27.82 ± 5.7 (6)	88.86 ± 5.1 (8)	66.76 ± 4.4 (8)	36.55 ± 3.2 (8)	26.65 ± 1.4 (7)
	Recovered control[d]	87.94 ± 4.3 NS (6)	107.54 ± 5.0 NS (6)	60.04 ± 2.1 NS (6)	23.73 ± 1.8 NS (6)	81.99 ± 4.5 NS (6)	61.73 ± 2.9 NS (6)	40.72 ± 7.1 NS (6)	27.23 ± 1.7 NS (8)
	Unrecovered control[b]	141.31 ± 9.7** (7)	111.94 ± 7.5 NS (7)	117.48 ± 11.3 NS (8)	42.54 ± 2.9 NS (8)	106.01 ± 6.5 NS (8)	78.18 ± 7.9 NS (6)	52.84 ± 2.5 NS (8)	36.93 ± 2.3 NS (7)
ACTH	Pretreated[d]	105.51 ± 3.5 (7)	106.71 ± 4.3 (7)	98.54 ± 1.9 (8)	39.87 ± 2.6 (6)	94.70 ± 3.9 (8)	71.32 ± 5.7 (6)	49.53 ± 5.4 (8)	37.33 ± 2.1 (7)
	Recovered control[d]	92.09 ± 2.6** (6)	71.42 ± 5.2*** (6)	69.60 ± 1.0*** (6)	33.86 ± 2.3 NS (6)	62.98 ± 6.5* (6)	56.14 ± 2.9 NS (6)	39.16 ± 2.9 NS (6)	23.51 ± 1.6*** (6)

[a] The plasma concentrations of zoxazolamine in the recovered and not yet recovered controls are compared with the plasma concentrations of the drug in the pretreated rats: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, and NS = not significant. [b] Killed when the pretreated group regained the righting reflex. [c] Figures in parentheses indicate number of animals. [d] Killed when the righting reflex was regained.

pregnene-16α-carbonitrile), triamcinolone[4], corticosterone[5], fludrocortisone (9α-fluorocortisol) acetate[3], estradiol[6] (1 mg. in 1 ml. water, homogenized with a trace of polysorbate 80, twice daily *per os* for 3 days and once on the 4th day), or depot ACTH[7] (5 I.U. = 50 mcg., subcutaneously) 24 hr. before the toxicant. Zoxazolamine[8] (10 mg./100 g. body weight in 1 ml. water, homogenized with a few drops of polysorbate 80) was administered intraperitoneally to all rats on the 4th day.

Blood samples were collected from the unrecovered control and pretreated animals when the latter regained the righting reflex and from the second control group at spontaneous recovery. Specimens were immediately taken from the liver (left lateral lobe), brain (severed just above the first cervical vertebra), kidney, heart (blood was drained by pressing the organ immediately after removal), spleen, muscle (biceps femoris, quadratus femoris, and rectus abdominis), and adipose tissues (subcutaneous, mesenteral, and perirenal). These specimens were then placed in an ice-cold 0.9% NaCl solution (ratio 1:2) and homogenized.

Zoxazolamine was extracted from plasma, organ, muscle, or adipose tissue homogenates into ethylene dichloride and from the organic solvent into hydrochloric acid. The drug concentrations were measured spectrophotometrically at 278 nm., the λ_{max} of zoxazolamine (13). Drug-free plasma from pretreated and unpretreated animals was used for preparing standards and blanks.

RESULTS AND DISCUSSION

Table I shows that the zoxazolamine concentrations decreased approximately in the following order: liver, heart, spleen, muscle,

[4] E. R. Squibb & Sons.
[5] Merck Sharp & Dohme.
[6] Roussel Corp.
[7] Ciba.
[8] K & K Laboratories.

Table II—Effect of Various Steroids and ACTH on Zoxazolamine-Induced Paralysis

Pretreatment	Zoxazolamine Paralysis[a], Duration in min. Control	Pretreated
I	149 ± 11 (10)[b]	44 ± 3*** (10)
Triamcinolone	164 ± 15 (12)	116 ± 9** (12)
Corticosterone	138 ± 12 (7)	143 ± 12 NS (8)
Fludrocortisone	148 ± 12 (8)	93 ± 12** (7)
Estradiol	143 ± 12 (6)	141 ± 18 NS (8)
ACTH	156 ± 6 (6)	80 ± 6*** (7)

[a] ** = $p < 0.01$, *** = $p < 0.005$, and NS = $p > 0.05$. [b] Figures in parentheses indicate number of animals.

and plasma. Neither the steroids, nor ACTH, nor time had any influence on these parameters. Essentially similar drug concentrations were found in the brain, fat, and kidney; these concentrations lay between those of the heart and liver. The results on the zoxazolamine concentrations in plasma and brain are in agreement with other comparative studies (14–16). The changes in drug concentrations in the spleen were not significantly altered by time or pretreatment.

In I- or fludrocortisone-pretreated rats, the concentrations of zoxazolamine in most organs were greatly reduced as compared with unrecovered controls (sacrificed when the righting reflex was regained in the former); they were similar to those in the second control group (killed at spontaneous recovery). There were considerable differences between the drug concentrations in the organs of the two controls. In the estradiol series, the differences between the zoxazolamine concentrations in the controls were not significant. This was expected because the duration of paralysis was similar in both groups (Table II).

The relationship for catatoxic activity is obviously common to drug concentrations in the plasma, brain, liver, heart, muscle, and spleen of I- or fludrocortisone-pretreated rats. Fludrocortisone was the only agent that caused a significant reduction of zoxazolamine in the spleen as compared with concentrations of the drug in the unrecovered controls.

The zoxazolamine concentrations in the various organs of the ACTH- or triamcinolone-pretreated animals were similar to those in the paralyzed controls; however, the pretreated groups showed lower drug concentrations in the liver, which could possibly be ascribed to the increase of hepatic glycogen. The ACTH- or triamcinolone-pretreated rats generally had higher drug concentrations in all organs than the recovered controls. The zoxazolamine concentrations were significantly increased in the plasma of the triamcinolone group as well as in the liver, brain, kidney, fat, and plasma of the ACTH-pretreated animals. Some changes in the other parameters were evident, but the differences were not significant. These findings show that the changes characteristic of syntoxic activity (of ACTH) manifest themselves not only in plasma drug concentrations but also in most of the organs examined. In triamcinolone-pretreated rats, there was a tendency for this relationship to be fulfilled. However, of all the parameters, only the plasma concentrations clearly indicated a syntoxic phenomenon.

The zoxazolamine concentrations in the organs of the corticosterone- or estradiol-pretreated animals usually lay between those of the corresponding two control groups, but the differences were not significant.

The brain: plasma concentrations of zoxazolamine were generally the same in pretreated and unpretreated rats (Table III), but there was a significant difference between the fludrocortisone group and the corresponding controls. This could indicate that the steroids and ACTH, unlike fludrocortisone, did not modify the permeability of the blood–brain barrier.

Generally, the liver:plasma concentrations of zoxazolamine varied in the pretreated and unpretreated groups. This may have been due to intense biotransformation of the drug in the liver and/or to increased glycogen in certain cases. As seen in Table III, the only exceptions were estradiol-pretreated animals and their corresponding controls, which showed none of these characteristics. Differences between the liver weights of pretreated and unpretreated rats could not have accounted for the variations in the hepatic concentrations of zoxazolamine.

Previous studies (17–19) established that the metabolic degradation of zoxazolamine is increased after induction of hepatic microsomal enzymes. In I-pretreated rats, the general decline of zoxazolamine concentrations in various organs after microsomal enzyme induction (4, 5) does not seem to alter drug distribution significantly in the body. The decrease of zoxazolamine paralysis (Table II) is well correlated with reduced drug concentrations in the brain (Table I). On the other hand, the protection offered by ACTH and triamcinolone can be considered as syntoxic since the plasma and brain concentrations of zoxazolamine are higher in pretreated animals than in recovered controls (killed at the same clinical stage). The organism is rendered less sensitive to the toxicant through a mechanism that has not yet been clarified (*e.g.*, interactions at receptor sites). Here the possibility of alterations in drug distribution may be excluded, as the findings indicate.

The *in vivo* protection offered by fludrocortisone (Table II) seems to be due to more than merely increased drug metabolism through hepatic microsomal enzyme induction. This is evident from the present results (Table I) and from *in vitro* studies on liver homogenates (20). The syntoxic effect of fludrocortisone is indicated by the seemingly reduced permeability of the blood–brain barrier. It is suggested that fludrocortisone has a dual action (catatoxic and syntoxic), because the mild fall in the plasma concentration of zoxazolamine in itself could not account for marked *in vivo* protection.

Under the experimental conditions, corticosterone and estradiol were neither catatoxic nor syntoxic, judging from the unaltered drug concentrations in the various organs. The failure of corticosterone to offer protection was previously demonstrated in methyprylon-intoxicated rats (7).

The present results indicate that the various steroids and ACTH generally modify the organ concentrations of zoxazolamine but not its organ distribution. Thus, the syntoxic effects of triamcinolone and ACTH (6, 7, 21) are not mediated through altered drug distribution. Fludrocortisone appears to be catatoxic (*e.g.*, increased zoxazolamine biodegradation) and syntoxic (*e.g.*, reduced permeability of the blood–brain barrier).

Finally, the effects of catatoxic and syntoxic steroids upon zoxazolamine concentrations in plasma are generally associated with altered drug concentrations in various organs. The latter changes

Table III—Effect of Various Steroids and ACTH on the Brain:Plasma and Liver:Plasma Ratios of Zoxazolamine

Organ	Group	Pretreatment: I	Triamcinolone	Corticosterone	Fludrocortisone	Estradiol	ACTH
Brain	Unrecovered control[a]	2.9	3.2	4.6	5.7	4.1	3.0
	Pretreated[b]	2.7	2.8	5.0	4.4	4.1	2.9
Liver	Unrecovered control[a]	4.1	4.9	5.1	7.2	3.6	3.9
	Pretreated[b]	3.3	2.9	4.5	5.3	3.7	2.8

[a] Killed when the pretreated group regained the righting reflex. [b] Killed when the righting reflex was regained.

follow the patterns demonstrated for catatoxic and syntoxic phenomena in plasma (6, 7).

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Drug Absorption Kinetics in Goldfish

SAMUEL H. YALKOWSKY▲, O. S. CARPENTER, GORDON L. FLYNN, and THOMAS G. SLUNICK

Abstract □ A model is presented for relating turnover time in goldfish to the concentration of drug in the bathing solution. The model, based on passive transport and the existence of a critical concentration of drug within the fish being required for turnover, was applied to the dose–response data of eight homologous esters of *p*-aminobenzoic acid. Absorption rate constants, relative membrane water partition coefficients, and critical concentrations were evaluated for the methyl through hexyl esters. Heptyl aminobenzoate was only effective in saturated solution. No turnover could be produced by the octyl ester which has a solubility below the critical concentration required for activity.

Keyphrases □ Goldfish—drug absorption kinetics, model and equations relating turnover time and alkyl *p*-aminobenzoate concentration □ Drug absorption kinetics—relationship between turnover time (goldfish) and drug concentration (alkyl *p*-aminobenzoates), model, equations □ *p*-Aminobenzoic acid esters—model and equations relating drug concentration and goldfish turnover time □ Dose–response data—alkyl *p*-aminobenzoate-induced turnover of goldfish

The relationship between the concentration of drug in a bulk solution containing a goldfish and the turnover time of the fish has been studied in several laboratories (1–12) over the past few years. Levy and Gucinski (1) developed a model which relates turnover time to the passive transport rate of the drug across the appropriate membrane of the fish. Their model, while frequently useful, does not account for the commonly observed threshold concentrations of drug, *i.e.*, the minimum concentration producing turnover. Nightingale and Gibaldi (8) recently extended the Levy–Gucinski model to cover situations where a measurable threshold concentration exists. Unfortunately, their derivation appears to predict a dependency on fish volume and on bathing solution volume. This report presents an alternative derivation which overcomes certain difficulties of previous mathematical models.

The equations developed will be used to interpret the dose–response data obtained for several normal alkyl esters of *p*-aminobenzoic acid.

THEORETICAL

In the course of these experiments and in the work of previous investigators, a linear relationship was observed between drug concentration and goldfish turnover time. The linearity and intercept of the reciprocal turnover time–concentration data can be explained by a model based on the following assumptions:

1. Absorption of the drug is passive and is, therefore, a reversible and unsaturable process.
2. Permeability characteristics of the membrane are unchanged during the experiment and unaffected by drug concentration.
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2. Permeability characteristics of the membrane are unchanged during the experiment and unaffected by drug concentration.
3. Drug metabolism or active excretion is negligible during the experiment. There is no development of drug tolerance.

Table I—Comparison of Kinetic Equations for Goldfish Turnover

Model	Relation between $1/t$ and C_B		C_B^e	
Levy–Gucinski (1)	$\frac{1}{t} = \frac{k_1' V_B C_B}{V_F C_F}$	(Eq. 11)	$C_B^e = 0$	(Eq. 12)
Nightingale–Gibaldi (8)	$\frac{1}{t} = \frac{k_1' V_B C_B}{V_F C_F^*} - \frac{k_1' + k_2'}{2}$	(Eq. 13)	$C_B^e = \frac{k_1' + k_2'}{2k_1'} \frac{V_F C_F^*}{V_B}$	(Eq. 14)
This work	$\frac{1}{t} = \frac{k_1}{C_F^*} C_B - \frac{k_2}{2}$	(Eq. 8)	$C_B^e = \frac{k_2}{2k_1} C_F^*$	(Eq. 9)
			$C_B^r = \frac{k_2}{k_1} C_F^*$	(Eq. 10)

4. The end-point is observed immediately after a critical concentration of drug within the fish, C_F^*, is reached. This value is assumed to be independent of the concentration of drug in the bathing solution.

5. The concentration of drug in the bathing solution is unchanged during the experiment.

The first four of these assumptions are essentially equivalent to the assumptions made by Nightingale and Gibaldi (8). The fifth assumption is intuitively agreeable since only a negligible fraction of the total drug present is absorbed by the fish. This latter assumption simplifies both the calculations and the results considerably.

The application of these assumptions to the absorption of drug by goldfish leads to the following differential equation:

$$\frac{dC_F}{dt} = k_1 C_B - k_2 C_F \qquad \text{(Eq. 1)}$$

where C_B and C_F are the concentrations of drug in the bulk or bathing solution and in the fish, respectively; t is time; and k_1 and k_2 are the rate constants for passive absorption and elimination, respectively. The solution to Eq. 1 is:

$$C_F = \frac{k_1}{k_2} C_B + A e^{-k_2 t} \qquad \text{(Eq. 2)}$$

where A is a constant of integration.

Since it is known that $C_F = 0$ at $t = 0$, the constant A must be equal to $-(k_1/k_2)C_B$, and Eq. 2 becomes:

$$e^{-k_2 t} = \left(\frac{\frac{k_1}{k_2} C_B - C_F}{\frac{k_1}{k_2} C_B} \right) \qquad \text{(Eq. 3)}$$

Converting to natural logarithms gives:

$$k_2 t = -\ln \left(\frac{\frac{k_1}{k_2} C_B - C_F}{\frac{k_1}{k_2} C_B} \right) \qquad \text{(Eq. 4)}$$

which can be approximated by:

$$\frac{1}{k_2 t} = \frac{1}{2\left(\frac{X-1}{X+1}\right) + \frac{2}{3}\left(\frac{X-1}{X+1}\right)^3 + \frac{2}{5}\left(\frac{X-1}{X+1}\right)^5 + \dots} \qquad \text{(Eq. 5)}$$

Table II—Solubilities of *n*-Alkyl *p*-Aminobenzoates in Water at 24°

Ester	Solubility, mole/l.
Methyl	8.3×10^{-3}
Ethyl	4.83×10^{-3}
Propyl	2.03×10^{-3}
Butyl	7.35×10^{-4}
Pentyl	1.79×10^{-4}
Hexyl	3.1×10^{-5}
Heptyl	7.0×10^{-6}
Octyl	1.3×10^{-6}

where $X = (k_1 C_B/k_2)/(k_1 C_B/k_2 - C_F)$. For $1 < X < 100$[1], all but the first term in the denominator can be ignored and Eq. 5 becomes:

$$\frac{1}{k_2 t} = \left(\frac{2C_F}{2\frac{k_1}{k_2} C_B - C_F} \right)^{-1} \qquad \text{(Eq. 6)}$$

or[2]:

$$\frac{1}{t} = \frac{k_1 C_B}{C_F} - \frac{k_2}{2} \qquad \text{(Eq. 7)}$$

If, in Eq. 7, t is the turnover time, t^*, then C_F, according to the fifth assumption, must be equal to the threshold concentration of the drug in the fish, C_F^*. Thus, the reciprocal turnover time can be expressed as:

$$\frac{1}{t^*} = \frac{k_1}{C_F^*} C_B - \frac{k_2}{2} \qquad \text{(Eq. 8)}$$

Equation 8 describes a straight line of slope (k_1/C_F^*) and an extrapolated intercept $-(k_2/2)$, which relates reciprocal turnover time to the concentration of drug in the bathing solution. The extrapolated concentration intercept, C_B, of Eq. 8 is determined (by setting $1/t = 0$) to be related to the threshold concentration in the fish, C_F^*, by:

$$C_B^e = \frac{k_2}{2k_1} C_F^* \qquad \text{(Eq. 9)}$$

The existence of a bath concentration that will show no activity over any period of time is well documented in the literature. The value of C_B^e for a particular drug is well defined and consistently observed. Independent studies (4, 5, 7) showed the value of C_B^e for ethanol in goldfish to be between 1 and 2% by volume.

If the series in Eq. 5 is not truncated after the first term, the function deviates from linearity and the real intercept, $(k_1/k_2)\ C_B^r$, approaches C_F^* as $1/t$ approaches zero, *i.e.*:

$$C_B^r = \frac{k_2}{k_1} C_F^* \qquad \text{(Eq. 10)}$$

Figure 1 shows the theoretical relationship between $1/t$ and concentration as described by the exact Eq. 4 and the approximate Eq. 8.

[1] Most of the data to be discussed is within this region.

[2] If one lets $(k_1 C_B)/(k_2 C_F) = p$, then Eq. 4 becomes:

$$\frac{1}{k_2 t} = \frac{1}{\ln\left(\frac{p}{p-1}\right)} \qquad \text{(Eq. 4a)}$$

Equation 4*a* was solved for the following integral values of p between 2 and 100 (p = 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100). The results fit the following least-squares equation with a correlation coefficient of 1.000:

$$\frac{1}{k_2 t} = 1.00\,p - 0.52 \qquad \text{(Eq. 4b)}$$

which is approximately equivalent to:

$$\frac{1}{t} = \frac{k_1 C_B}{C_F} - \frac{k_2}{2} \qquad \text{(Eq. 7)}$$

As can be seen in Fig. 1, the approximate Eq. 7 shows good agreement with the exact solution in p for $2 < p < 100$. However, there is significant deviation from linearity and $1/t \to 0$ as $p \to 1$, *i.e.*, as $k_1/k_2\ C_B \to C_F^*$.

These results differ from those of Levy and Gucinski (1) and Nightingale and Gibaldi (8) in several respects. The final equations and the concentration intercepts of the three derivations (in the present notation) are summarized in Table I. The major difference between the equation developed here and the other equations found in the table is that the latter appear to be dependent upon the volume of the fish, V_F, and the volume of the bathing solution, V_B. The independence of goldfish turnover time on fish size was demonstrated by DiSanto and Wagner (7) using ethanol and in these laboratories for various esters of *p*-aminobenzoic acid[3]. As long as the volume of the bathing solution is greater than about 50 times the volume of the fish, the turnover time is independent of solution volume. Both Eqs. 11 and 13 predict that reciprocal turnover time will increase linearly with increasing solution volume at a constant concentration. However, if k_1' is converted to units based on concentration rather than amounts by $k_1' = k_1(C_F/C_B)$, the slope of the Nightingale–Gibaldi Eq. 13 becomes identical to the slope of the present Eq. 8. The differences in the intercepts of Eqs. 8 and 13 are simply a reflection of the mathematical approximations involved in the two treatments.

If the value of C_F^* refers to the aqueous portion of the fish, it is possible to make one further simplifying assumption; namely, that $k_1 = k_2$. In other words, as is implicit in the first assumption, there is no directional specificity in the membrane barriers. With this simplification the extrapolated concentration intercept, C_B^e, of a reciprocal turnover times *versus* concentration plot is equal to one-half the critical concentration within the fish required to produce turnover:

$$C_B^e = {}^1/_2 C_F^* \qquad \text{(Eq. 15)}$$

Equation 15 is consistent with the fact that C_B^e is equal to ${}^1/_2 C_B^r$ (Fig. 1) and the hypothesis (Eq. 10) that the threshold concentration in the bulk, C_B^r, must be equal to the critical concentration within the fish.

If C_F^* is the total fish concentration of drug, this discussion is still qualitatively applicable. Since over 80% of the goldfish is aqueous, $k_1 \approx k_2$ and, thus, $2C_B^e \approx C_B^r$. The equilibrium constants of several simple semipolar compounds between gudgeons and water were determined (13) to be 0.94 for methanol, 0.86 for ethanol, 0.79 for isopropanol, 0.88 for acetone, and 0.84 for methyl acetate. Likewise, the data of Nightingale (9) indicate a goldfish–water partition coefficient of 0.92 for 4-aminoantipyrine. From these values, it can be seen that the approximation of k_2/k_1 by unity will be off by less than 20% for these compounds. Further verification of the equivalency of the two rate constants is found in reported results (3, 9, 11, 14) that have shown experimentally that $C_B^r \approx C_F^*$ or $k_1 \approx k_2$.

Thus, Eq. 8 can be rewritten as:

$$\frac{1}{t} = \frac{k_1}{C_F^*}C_B - \frac{k_1}{2} = \left(\frac{C_B}{C_F^*} - \frac{1}{2}\right)k_1 \qquad \text{(Eq. 16)}$$

EXPERIMENTAL

Materials—Methyl *p*-aminobenzoate, ethyl *p*-aminobenzoate, and butyl *p*-aminobenzoate were used as received. The remaining esters were synthesized from the appropriate alcohol[4] and *p*-nitrobenzoyl chloride[5] as described previously (15, 16).

Aqueous Solubility—The aqueous solubility of these esters was determined at 24 ± 1° by UV spectroscopy of stock solutions which were equilibrated for at least 3 days.

Bathing Solutions—All bathing solutions were prepared from the stock saturated solutions by dilution.

Measurement of Turnover Time—Goldfish, *Carassius auratus*, between 5.08 and 7.62 cm. (2 and 3 in.) in length, were purchased locally. The shorter turnover time was determined by placing a single fish in 200 ml. of bathing solution. The longer times were determined by placing several fish in a larger volume. In all cases, the end-point was taken as the time it took the fish to become unable to bring itself to an upright position after being turned onto its side by a glass rod, *i.e.*, lose its righting reflex.

[3] S. H. Yalkowsky, G. L. Flynn, and T. G. Slunick, unpublished data.
[4] Eastman or Aldrich.
[5] Matheson.

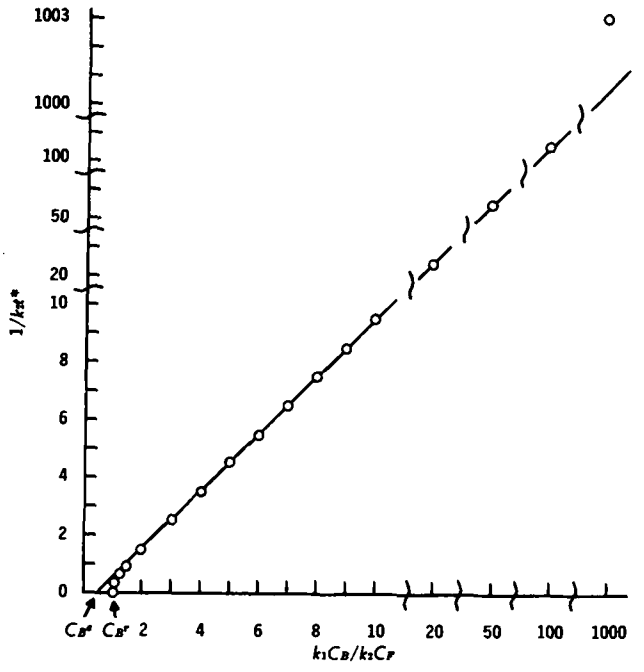

Figure 1—*Comparison of exact and approximate solutions to Eq. 4. Key:* O, *exact solution; and* ——, *approximate solution given by Eq. 7.*

RESULTS AND DISCUSSION

Solubility—The 24° solubilities of the eight esters studied are listed in Table II. Past butyl, the solubility decreases by factor of 5.0 for each additional methylene group; the solubilities of shorter homologs decrease more slowly with chain length. The solubilities of the *n*-alkyl *p*-aminobenzoates were already interpreted in terms of other physical properties of these compounds (16).

Turnover Time—Mean turnover times obtained as a function of concentration for the various *n*-alkyl *p*-aminobenzoates are given in Table III. With the exception of the four values discussed below, all of the values are the average of 10 individual experimental points. The asterisks represent conditions under which at least three out of 10 fish did not turn over within 48 hr. These data are shown graphically in Figs. 2 and 3. The graphs are presented in terms of fractional saturation, C/S, rather than the more conventional concentration, C, for two reasons: (*a*) all of the data in Table III, which range from 1×10^{-6} to 8×10^{-3} M, can be plotted simultaneously, and (*b*) a direct comparison of the esters can be made on a so-called "equal thermodynamic scale" (17).

In Fig. 2, reciprocal turnover time is plotted against fractional saturation. The minimum turnover time observed is about 0.4 min., and this value was obtained for saturated solutions of the lower esters. Furthermore, these points fall well below the least-squares line through the other data. The authors believe that these deviations from predicted behavior are related to the time required for the establishment of a quasi-steady-state concentration gradient across the membrane barriers. This lag time for turnover is then the minimum attainable turnover time.

The bulk of the data in Fig. 2 falls on lines having positive concentration intercepts. The equations for the least-squares lines (all points weighted according to the reciprocal of their variance) for each ester are given in Table IV. Dividing the slope of the solubilities of the esters enables concentration to be expressed in moles per liter instead of fractional saturation. It is necessary to weight the data as already described to obtain more accurate values for the intercepts. The scatter in the reciprocal turnover time produced by the higher concentrations of drug precluded accurate determination of the intercepts by nonweighted regression analysis of all of the data. This same difficulty was noted by Nightingale and Gibaldi (8) for 4-aminoantipyrine-induced turnover. They overcame this difficulty by ignoring the data for the higher drug concentrations in determining the intercept.

The log–log curves of Fig. 3 are also revealing. All of the curves approach an infinite slope as $C_B \to C_B^r$; but for $C_B \gg C_B^r$, the

slopes all approach unity. If there were no intercept in the $1/t$ *versus* C data, the log-log plots would have a slope of -1.0 throughout the range studied.

From the equations in Table IV, the values of the kinetic parameters, k_2, $(k_1/C_B{}^s)$, and $(k_2/k_1)C_B{}^s$, can be obtained for each ester, and they are listed in Table V. It is possible to determine a range for $C_B{}^0$ from the raw material data in Table III. These ranges are also in agreement with the data in Table V.

The low solubility of the heptyl ester is responsible for the lack of conclusive estimates of its kinetic parameters. The inability of the octyl ester to produce turnover, even from saturated solutions, suggests that its solubility is below the threshold concentration. Likewise, the nonyl ester was found to be totally inactive.

The data indicate quite clearly that k_2 values decrease and $k_1/C_B{}^s$ values increase with increasing chain length of the *p*-aminobenzoate ester moiety. However, these changes are small compared to the dramatic decreases in $k_2C_B{}^s/k_1$ that accompany assension of the homologous series. Unfortunately, the proposed model does not enable the definitive mathematical separation of k_1 and $C_B{}^s$. If it is assumed that $k_1 = k_2$ as described, it is evident that $C_B{}^s$ is highly dependent upon chain length. On the other hand, if it is assumed that the intrinsic activities of all of the esters are identical, it is

Table III—Average Turnover Times Produced by Various Concentrations of *n*-Alkyl *p*-Aminobenzoates

Ester	Fractional Saturation	Concentration, mole/l.	Number of Fish	Mean Turnover Time, min.	Standard Error of Mean	Mean Reciprocal Turnover Time
Methyl	Saturated	8.3×10^{-3}	5	0.320	0.051	3.15
	2/3	5.52×10^{-3}	10	0.410	0.030	2.44
	1/2	4.15×10^{-3}	10	0.640	0.098	1.52
	1/3	2.76×10^{-3}	10	0.933	0.088	1.07
	1/4	2.07×10^{-3}	10	1.230	0.076	0.813
	1/6	1.40×10^{-3}	10	2.270	0.183	0.44
	1/10	8.3×10^{-4}	10	4.390	0.189	0.23
	1/20	4.15×10^{-4}	10	45.0	15.7	0.022
	1/40	2.07×10^{-4}	10	—[a]	—	—
	1/100	8.3×10^{-5}	10	—[a]	—	—
Ethyl	Saturated	4.83×10^{-3}	5	0.340	0.062	2.95
	2/3	3.16×10^{-3}	10	0.270	0.015	3.70
	1/2	2.37×10^{-3}	10	0.460	0.040	2.17
	1/3	1.58×10^{-3}	10	0.550	0.045	1.82
	1/4	1.18×10^{-3}	10	0.990	0.095	1.01
	1/6	7.8×10^{-4}	10	1.210	0.159	0.83
	1/10	4.83×10^{-4}	10	2.800	0.226	0.36
	1/20	2.37×10^{-4}	10	6.50	0.64	0.15
	1/40	1.18×10^{-4}	10	47	9.3	0.021
	1/100	4.83×10^{-5}	10	—[a]	—	—
Propyl	Saturated	2.03×10^{-3}	6	0.333	0.042	3.0
	2/3	1.3×10^{-3}	10	0.450	0.037	2.22
	1/2	9.8×10^{-4}	10	0.510	0.038	1.96
	1/3	6.5×10^{-4}	10	0.680	0.039	1.47
	1/4	4.9×10^{-4}	10	0.900	0.097	1.11
	1/6	3.3×10^{-4}	10	1.150	0.097	0.87
	1/10	2.03×10^{-4}	10	3.150	0.442	0.32
	1/20	9.8×10^{-5}	10	8.250	0.720	0.12
	1/40	4.9×10^{-5}	10	30.50	3.06	0.03
	1/100	2.03×10^{-5}	10	—[a]	—	—
Butyl	Saturated	7.35×10^{-4}	5	0.340	0.025	2.94
	2/3	4.7×10^{-4}	10	0.640	0.072	1.56
	1/2	3.6×10^{-4}	10	0.730	0.070	1.37
	1/3	2.4×10^{-4}	10	1.110	0.094	0.90
	1/4	1.8×10^{-4}	10	1.510	0.157	0.66
	1/6	1.2×10^{-4}	10	2.600	0.256	0.39
	1/10	7.35×10^{-5}	10	4.650	0.289	0.22
	1/20	3.6×10^{-5}	10	9.950	0.664	0.10
	1/40	1.8×10^{-5}	10	70.60	18.44	0.014
	1/100	7.35×10^{-6}	10	—[a]	—	—
Pentyl	Saturated	1.79×10^{-4}	10	1.370	0.104	0.73
	3/4	1.34×10^{-4}	10	1.830	0.146	0.55
	2/3	1.29×10^{-4}	10	2.380	0.133	0.42
	1/2	9.0×10^{-5}	10	3.310	0.293	0.30
	1/3	5.95×10^{-5}	10	4.500	0.632	0.22
	1/4	4.5×10^{-5}	10	6.380	0.495	0.16
	1/6	2.98×10^{-5}	10	8.850	0.311	0.11
	1/10	1.79×10^{-5}	10	15.50	1.06	0.065
	1/20	9.0×10^{-5}	10	43.33	3.67	0.023
	1/40	4.5×10^{-6}	6	63.60	5.05	0.016
	1/100	1.79×10^{-6}	5	—[a]	—	—
Hexyl	Saturated	3.1×10^{-5}	10	6.70	0.26	0.15
	3/4	2.4×10^{-5}	10	9.20	0.41	0.11
	2/3	2.0×10^{-5}	10	13.00	0.45	0.078
	1/2	1.5×10^{-5}	10	14.30	1.68	0.070
	1/3	1.0×10^{-5}	10	25.00	2.17	0.040
	1/4	7.5×10^{-6}	10	31.20	5.88	0.032
	1/6	5.0×10^{-6}	10	—[a]	—	—
Heptyl	Saturated	7.2×10^{-6}	6	30.00	3.72	0.033
	1/2	3.6×10^{-6}	6	—[a]	—	—
Octyl	Saturated	1.3×10^{-6}	2	—[a]	—	—

[a] Inactive.

Table IV—Dose–Response Equations

Ester	Equation	Number of Data Points Used	Correlation Coefficient
Methyl	$1/t = (-0.164 \pm 0.0045) + (3.95 \pm 0.055) + 8.3 \times 10^{-3} \times C$	6	1.000
Ethyl	$1/t = (-0.105 \pm 0.0124) + (5.37 \pm 0.278) + 4.83 \times 10^{-3} \times C$	8	0.989
Propyl	$1/t = (-0.0742 \pm 0.0147) + (4.41 \pm 0.305) + 2.03 \times 10^{-3} \times C$	8	0.979
	$(-0.0895 \pm 0.0131) + (4.91 \pm 0.336) + 2.03 \times 10^{-3} \times C$	6	0.989
Butyl	$1/t = (0.0398 \pm 0.0124) + (2.76 \pm 0.168) + 7.35 \times 10^{-4} \times C$	9	0.980
	$(-0.0449 \pm 0.0072) + (2.87 \pm 0.130) + 7.35 \times 10^{-4} \times C$	6	0.998
Pentyl	$1/t = (-0.00302 \pm 0.00082) + (0.676 \pm 0.0241) + 1.79 \times 10^{-4} \times C$	10	0.993
Hexyl	$1/t = (-0.000570 \pm 0.000846) + (0.135 \pm 0.0103) + 3.1 \times 10^{-5} \times C$	7	0.981

obvious from the data that k_1 cannot equal k_2. These two assumptions are consistent with one another if equal concentrations of the esters have equal activities only in some lipid or membrane portion of the fish; *i.e.*, at turnover, C_M^* is the same for each ester. Since the membrane water partition coefficient of each ester is given by:

$$PC = \frac{C_M}{C_W} \approx \frac{C_M}{C_F} \approx \frac{C_M^*}{C_F^*} \qquad \text{(Eq. 17)}$$

and if C_M^* is assumed independent of chain length, the relative partition coefficients of the esters are approximately proportional to the reciprocal of their critical fish concentrations, C_F^*. Interpretation of the changes in k_1, k_2, C_F^*, and PC that accompany changes in alkyl chain length will be published later.

As a further test of its validity, the turnover times produced by various concentrations of 4-aminoantipyrine (Table I of *Reference 8*) were analyzed according to the model. To get meaningful estimates of the intercepts, only the data for the lower concentrations of drug were considered.

The resulting least-squares line was then used to calculate the kinetic parameters. These values (Table VI) were in good agreement with values determined independently by Nightingale (9) and Anello and Levy (11).

Table V—Kinetic Parameters for *n*-Alkyl *p*-Aminobenzoate-Induced Turnover of Goldfish

Ester	k_2, min.$^{-1}$	k_1/C_B^*, l./min./mole	$(k_2/k_1)C_B^*(=C_F^*)$, mole/l.
Methyl	0.328	475	6.9×10^{-4}
Ethyl	0.210	1100	1.88×10^{-4}
Propyl	0.148	2160	6.9×10^{-5}
Butyl	0.080	3750	2.13×10^{-5}
Pentyl	0.0060	3780	1.59×10^{-6}
Hexyl	0.0011	4310	2.6×10^{-7}

CONCLUSIONS

The equations derived in this report not only describe the dose–response data for alkyl benzoate-induced turnover of goldfish but also provide a convenient means of estimating the following parameters: (*a*) elimination rate constant, and (*b*) the critical or threshold concentration of each drug required to produce turnover.

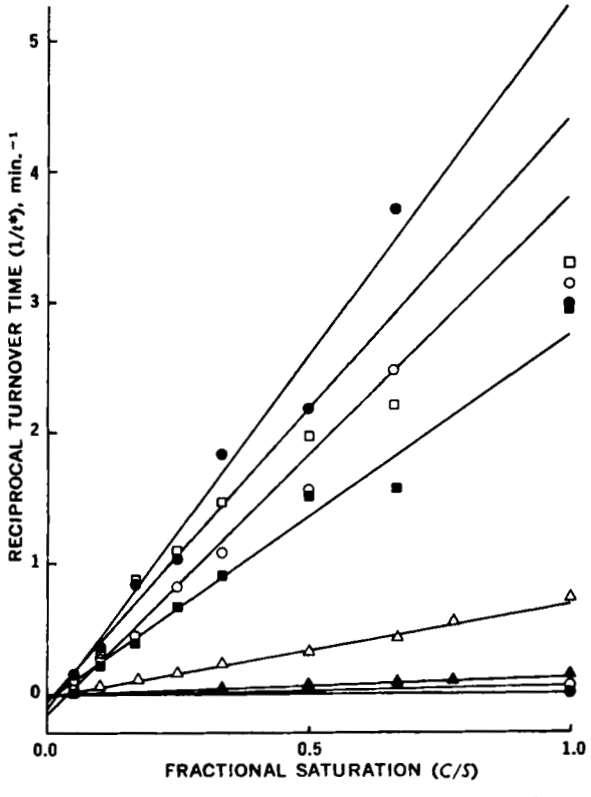

Figure 2—*Reciprocal turnover time as a function of the the fractional saturation of* p*-aminobenzoate ester in the bathing solution. Key:* ○, *methyl;* ●, *ethyl;* □, *propyl;* ■, *butyl;* △, *pentyl;* ▲, *hexyl;* ◇, *heptyl; and* ◆, *octyl. Lines are the weighted least-squares lines given in Table III.*

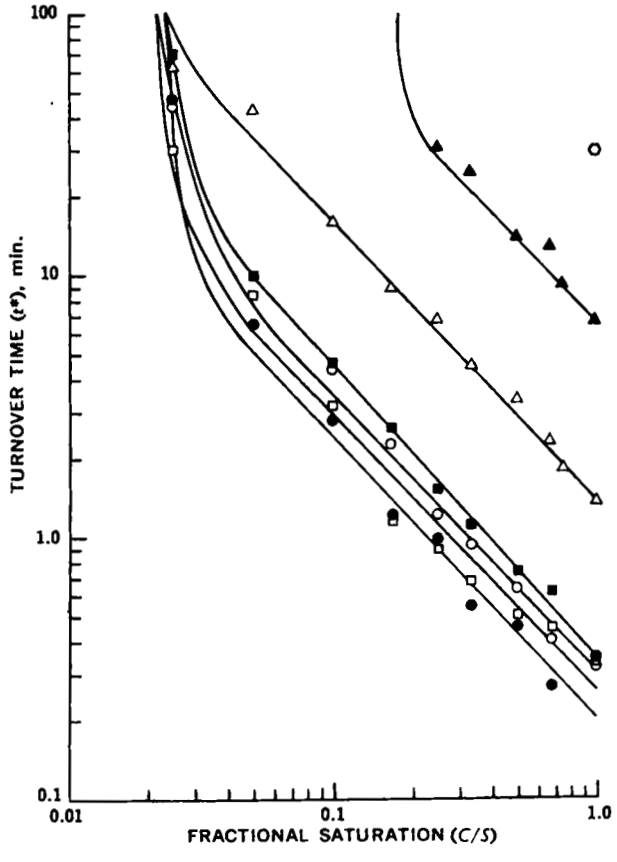

Figure 3—*Log–log relationship between bathing solution concentration (expressed as fractional saturation) and turnover time. The symbols have the same meaning as those in Fig. 2.*

Table VI—Values of Kinetic Parameters for 4-Aminoantipyrine-Induced Overturn of Goldfish

Parameter	From Eq. 8 and Dose–Response Data	Literature Values	Reference
k_1	6.54×10^{-3} min.$^{-1}$	2.3×10^{-3} min.$^{-1}$	11
k_2	6.54×10^{-3} min.$^{-1}$	3.3×10^{-3} min.$^{-1}$	11
C_F*	6.67 mg.%	6.8 mg.%	9

The proposed model for the action of homologous narcotic drugs on goldfish was tested using *n*-alkyl *p*-aminobenzoates. The experimental data were interpretable by the derived equations. Apparent discrepancies between the model and high concentration data were accounted for by recognizing that there is a finite lag time for turnover.

Literature data for 4-aminoantipyrine-induced narcosis of goldfish were also interpretable by the model.

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Correlation of Viscoelastic Functions for Pharmaceutical Semisolids: Comparison of Creep and Oscillatory Tests for Oil-in-Water Creams Stabilized by Mixed Emulsifiers

G. M. ECCLESTON▲, B. W. BARRY, and S. S. DAVIS*

Abstract □ Liquid paraffin-in-water emulsions stabilized by mixed emulsifiers of a surfactant (cetrimide or cetomacrogol) and a long-chain alcohol were used as model systems to represent pharmaceutical semisolids. They were examined in their linear viscoelastic regions using creep and oscillatory (Weissenberg rheogoniometer) techniques. Storage and loss compliances (J' and J'') over the frequency range 2.5×10^{-8} to 1.5×10^{-1} Hz. were calculated from creep data using numerical formulas. The transformed compliances were used to derive the other oscillatory functions, the storage and loss moduli (G' and G''), the storage and loss viscosities (η' and η''), and the loss tangent (tan δ) at the same frequencies. The agreement between transformed functions and those obtained directly from oscillatory measurements at frequencies ranging from 7.91×10^{-4} to 1.5×10^{-1} Hz. was generally good. Discrepancies at the extremities of this range were a result of the practical difficulties involved when creep measurements are made at very short times (representing high frequencies) and evaporation of water from the parallel plates of the rheogoniometer during low frequency dynamic experiments. Neither small strain technique alone covered the entire frequency or time range necessary for an exhaustive study of semisolids. Creep data were unavoidably inaccurate at high frequencies, and the limitations of the rheogoniometer gear box prevented oscillatory measurements from being made at extremely low frequencies. The unification of data from oscillatory and creep measurements so as to provide a dynamic description over an extended frequency range (10^{-8}–25 Hz.) is discussed. The variations of each function with frequency are considered with reference to linear viscoelastic theory; the limiting values and the shapes of the plots at low frequencies agreed well with the theoretical values for viscoelastic liquids. It was concluded that such interconversion techniques are applicable and valuable for interpreting the rheological behavior of a pharmaceutical system.

Keyphrases □ Creams, oil-in-water, stabilized by mixed emulsifiers—viscoelastic properties, correlation between creep and oscillatory tests □ Creep measurements—correlation with oscillatory functions, oil-in-water creams stabilized by mixed emulsifiers □ Oscillatory functions—correlation with creep measurements, oil-in-water creams stabilized by mixed emulsifiers □ Viscoelastic properties, oil-in-water creams stabilized by mixed emulsifiers—correlation between creep and oscillatory tests

The rheological properties of semisolid preparations affect all stages of manufacture, and they should be considered in quality control. The release of medicaments from semisolid vehicles is sometimes related to these properties, so a knowledge of the rheological effects of changes in formulation may also be important for bioavailability studies.

In the past, a major difficulty encountered in the rheological evaluation of semisolid ointments and creams was how to obtain a true measure of consistency. The approach described frequently in the literature is to use a continuous shear technique and then to attempt to correlate derived parameters, *e.g.*, spur points or yield values, with the qualitative terms "consistency

Table VI—Values of Kinetic Parameters for 4-Aminoantipyrine-Induced Overturn of Goldfish

Parameter	From Eq. 8 and Dose–Response Data	Literature Values	Reference
k_1	6.54×10^{-3} min.$^{-1}$	2.3×10^{-3} min.$^{-1}$	11
k_2	6.54×10^{-3} min.$^{-1}$	3.3×10^{-3} min.$^{-1}$	11
C_F*	6.67 mg.%	6.8 mg.%	9

The proposed model for the action of homologous narcotic drugs on goldfish was tested using *n*-alkyl *p*-aminobenzoates. The experimental data were interpretable by the derived equations. Apparent discrepancies between the model and high concentration data were accounted for by recognizing that there is a finite lag time for turnover.

Literature data for 4-aminoantipyrine-induced narcosis of goldfish were also interpretable by the model.

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Correlation of Viscoelastic Functions for Pharmaceutical Semisolids: Comparison of Creep and Oscillatory Tests for Oil-in-Water Creams Stabilized by Mixed Emulsifiers

G. M. ECCLESTON▲, B. W. BARRY, and S. S. DAVIS*

Abstract □ Liquid paraffin-in-water emulsions stabilized by mixed emulsifiers of a surfactant (cetrimide or cetomacrogol) and a long-chain alcohol were used as model systems to represent pharmaceutical semisolids. They were examined in their linear viscoelastic regions using creep and oscillatory (Weissenberg rheogoniometer) techniques. Storage and loss compliances (J' and J'') over the frequency range 2.5×10^{-8} to 1.5×10^{-1} Hz. were calculated from creep data using numerical formulas. The transformed compliances were used to derive the other oscillatory functions, the storage and loss moduli (G' and G''), the storage and loss viscosities (η' and η''), and the loss tangent (tan δ) at the same frequencies. The agreement between transformed functions and those obtained directly from oscillatory measurements at frequencies ranging from 7.91×10^{-4} to 1.5×10^{-1} Hz. was generally good. Discrepancies at the extremities of this range were a result of the practical difficulties involved when creep measurements are made at very short times (representing high frequencies) and evaporation of water from the parallel plates of the rheogoniometer during low frequency dynamic experiments. Neither small strain technique alone covered the entire frequency or time range necessary for an exhaustive study of semisolids. Creep data were unavoidably inaccurate at high frequencies, and the limitations of the rheogoniometer gear box prevented oscillatory measurements from being made at extremely low frequencies. The unification of data from oscillatory and creep measurements so as to provide a dynamic description over an extended frequency range (10^{-8}–25 Hz.) is discussed. The variations of each function with frequency are considered with reference to linear viscoelastic theory; the limiting values and the shapes of the plots at low frequencies agreed well with the theoretical values for viscoelastic liquids. It was concluded that such interconversion techniques are applicable and valuable for interpreting the rheological behavior of a pharmaceutical system.

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The rheological properties of semisolid preparations affect all stages of manufacture, and they should be considered in quality control. The release of medicaments from semisolid vehicles is sometimes related to these properties, so a knowledge of the rheological effects of changes in formulation may also be important for bioavailability studies.

In the past, a major difficulty encountered in the rheological evaluation of semisolid ointments and creams was how to obtain a true measure of consistency. The approach described frequently in the literature is to use a continuous shear technique and then to attempt to correlate derived parameters, *e.g.*, spur points or yield values, with the qualitative terms "consistency

change" or "body." However, data from such experiments are often misleading and should be treated with caution. All continuous shear techniques measure the complex phenomenon of structure breakdown which, in turn, is a function of the test method. The material constants necessary to define viscoelastic (semisolid) materials, *e.g.*, Newtonian viscosity and modulus of elasticity, are not derived.

To obtain true measures of consistency, it is necessary to employ fundamental experiments in which the semisolids are examined in their rheological ground states, *i.e.*, when the method of testing does not significantly disrupt static structures. Small strain rheological experiments such as creep and oscillatory techniques are included in this category and have been used to investigate the rheological behavior of an extensive range of cosmetic and pharmaceutical materials (1–31). A suitable instrument for creep testing is the concentric cylinder reaction air turbine viscometer (22, 31–33). Oscillatory experiments may be performed using the Weissenberg rheogoniometer coupled to a Solartron digital transfer function analyzer and mechanical reference synchronizer (26).

For an exhaustive study of semisolids, it is usually necessary to employ both types of small strain experiment, because neither method alone determines viscoelastic properties over the required wide time or frequency range. Creep measurements essentially provide information at long times (equivalent to low frequencies that are inaccessible to the rheogoniometer) whereas the high frequency (short time) range is accessible only to dynamic experiments. Thus, a difficulty arises when attempts are made to compare the results of past studies, because a description of the mechanical behavior of the systems may be given by transient functions at long times such as compliances expressed as functions of time, $J(t)$, and by oscillatory functions at short times, such as storage and loss compliances expressed as functions of frequency, $J'(\omega)$ and $J''(\omega)$.

However, fundamental parameters may be mathematically interconverted using linear viscoelastic theory. It is then possible to transform any one viscoelastic function derived in shear to any other, provided that the former is known over a sufficiently wide range of time or frequency. Thus, the comparison problem may be solved if functions are transformed so as to provide a unified parameter applicable at both high and low frequencies. Since the necessary exact calculations are often difficult and tedious, various approximation methods are usually employed (34–36). A comparatively simple numerical approximation for converting transient to dynamic data was described by Schwarzl (36).

The present investigation had two objectives:

1. To determine whether the conversion of creep data to dynamic functions is feasible for model pharmaceutical systems using Schwarzl's transformation formulas. If so, it may also be possible to derive dynamic data at very low frequencies, and oscillatory data may also be produced within the rheogoniometer's frequency range without using this expensive instrument.

2. To assess the accuracy involved in this method of transforming data in the light of the many variables normally involved in creep and oscillatory experiments. These include the different viscometer geometries (parallel plate and concentric cylinder), the dissimilar times for which the emulsions remain in each viscometer before and during the experiments, that no allowance is made for end effects in creep or for evaporation effects (particularly from parallel plates) and the possibility that internal structure breaks down to different extents when the two viscometers are loaded.

Liquid paraffin-in-water emulsions containing cetostearyl alcohol and the surfactants cetrimide or cetomacrogol were chosen as model ionic and nonionic systems to represent pharmaceutical and cosmetic semisolids in general. These preparations have already been examined in detail by both fundamental methods, and information is available as to the effects of small changes in formulation on each type of viscoelastic function (13, 17, 20). To cover the consistency range usually found in pharmaceutical formulations, two emulsions were prepared from each mixed emulsifier, one a "soft" semisolid and the other a "stiff" material.

EXPERIMENTAL

Materials—Cetostearyl alcohol, liquid paraffin, water, cetrimide[1], and cetomacrogol[2] were as described previously (13, 17, 37).

Preparation of Systems—Liquid paraffin-in-water emulsions were made by a standardized procedure according to the formulas in Table I (13). Prior to testing, emulsions were stored for sufficient time at 25 ± 0.5° to ensure that consistency build-up was complete and that the systems were not changing in rheological properties during the test. The microscopic and macroscopic appearances of each formulation were already described (1, 13, 14, 17, 18).

Creep Tests—Creep experiments were performed at 25 ± 0.5° using a concentric cylinder reaction air turbine viscometer (22, 31, 33). Approximate linearity checks were made at short times, and the creep curves were recorded in the linear viscoelastic regions. Each creep stress was applied for a minimum of 90 min., by which time the strain or compliance responses for the samples were essentially linear with time. Creep curves were analyzed into discrete spectra using a method similar to that of Warburton and Barry (38). To check the accuracy of the analysis, a computer program was written to regenerate values of $J(t)$ from the discrete spectral values. Agreement between experimental and reconstituted values for $J(t)$ was better than 2% full-scale deflection, even at long times.

Oscillatory Tests—Oscillatory tests were performed at 25 ± 0.5°[3]. The instrument was employed with parallel plates, 7.5 cm. in diameter, and the platen gap was set to 6.35×10^{-2} cm. The torsion bar constant was 2.207×10^{3} dyne cm./2.54×10^{-3} cm. movement. Initial tests for linearity were performed (20), and the systems were examined in their linear viscoelastic regions by applying sinusoidally varying shear strains at frequencies ranging from 7.91×10^{-4} to 25 Hz. (n Hz. = $2\pi n$ rad./sec.). At each frequency, the amplitude ratio and the phase shift, a, were determined and were employed to calculate the storage modulus (G') and the dynamic viscosity (η') using Walters' equations for parallel plate geometry (39, 40). The other viscoelastic functions, G'', η'', J', and J'', were calculated from G' and η' using Eqs. 1–4 (34):

$$G'' = \eta'\omega \quad \text{(Eq. 1)}$$

$$\eta'' = G'/\omega \quad \text{(Eq. 2)}$$

$$J' = G'/(G'^2 + G''^2) \quad \text{(Eq. 3)}$$

$$J'' = G''/(G'^2 + G''^2) \quad \text{(Eq. 4)}$$

[1] A commercial mixture of alkyl trimethylammonium bromides.

[2] A polyethylene glycol ether condensate of cetyl or cetostearyl alcohol with ethylene oxide; formula $CH_3(CH_2)_{17}(OCH_2CH_2)_{20}OH$.

[3] With a R14 Weissenberg rheogoniometer, modified to bring it up to R16 standards, coupled to a digital transfer function analyzer and mechanical reference synchronizer (20, 21, 26, 29, 30).

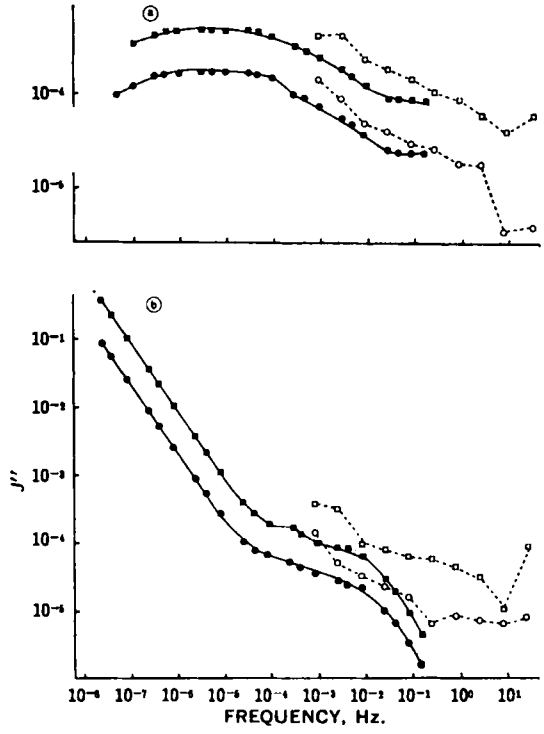

Figure 1—*Cetrimide emulsions C8 and C14, showing the variation of: (a) storage compliance, J', and (b) loss compliance, J'' (dyne^{-1} cm.^{2}), with frequency (Hz.). Key: □, C8, and ○, C14, values derived from oscillatory experiments using a rheogoniometer; and ■, C8, and ●, C14, theoretical values derived by transformation of creep data.*

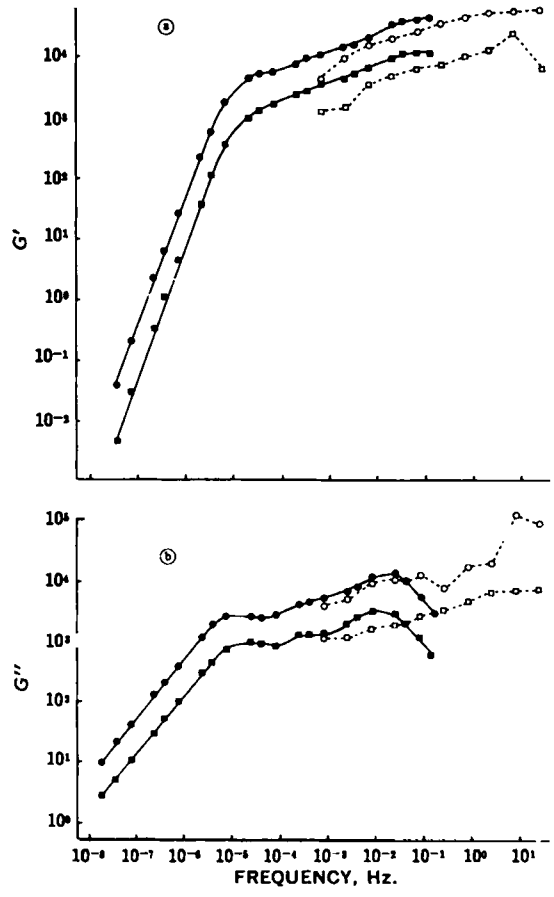

Figure 2—*Cetrimide emulsions C8 and C14, showing the variation of: (a) storage modulus, G', and (b) loss modulus, G'' (dynes cm.$^{-2}$), with frequency (Hz.). Key: □, C8, and ○, C14, values derived from oscillatory experiments using a rheogoniometer; and ■, C8, and ●, C14, theoretical values derived by transformation of creep data.*

A description of these functions was given previously (20, 21). The storage modulus, G', and the storage compliance, J', are associated with energy stored and recovered per cycle of deformation; the loss modulus, G'', and the loss compliance, J'', are measures of energy lost or dissipated as heat during this time. The dynamic viscosity, η', is another useful way of describing dissipative effects and is the real part of a complex viscosity. The imaginary part, η'', is related to stored energy. The loss tangent, tan a, is a useful parameter because, although it conveys no physical magnitude and is dimensionless, it is a measure of the ratio of energy lost to energy stored in a cyclic deformation.

Numerical Calculation of Storage and Loss Compliances from Creep Compliance—Schwarzl (36) published a number of formulas of increasing complexity but decreasing relative error with which to calculate the storage compliance, $J'(\omega)$, and the loss compliance, $J''(\omega)$, from the creep compliance, $J(t)$. Equations 5 and 6 are the most accurate for calculating J' and J'', respectively, if infinite series are to be avoided:

$$J'(\omega) \sim J(t) + 0.000715[J(32t) - J(16t)] - 0.0185[J(16t) - J(8t)] + 0.197[J(8t) - J(4t)] - 0.778[J(4t) - J(2t)] - 0.181[J(t) - J(t/2)] - 0.0494[J(t/4) - J(t/8)] \quad \text{(Eq. 5)}$$

Table I—Composition of Emulsions (in Grams)

	C8[a]	C14	N7	N16
Liquid paraffin	100	100	100	100
Water	300	300	300	300
Cetostearyl alcohol	28.8	50.4	25.2	57.6
Cetrimide	3.2	5.6	—	—
Cetomacrogol	—	—	2.8	6.4

[a] C represents cetrimide (cationic) emulsions, N represents cetomacrogol (nonionic) emulsions, and the associated numerals refer to the approximate percentage of mixed emulsifier (surfactant plus alcohol) present.

$$J''(\omega) \sim -0.470[J(4t) - J(2t)] + 1.674[(J(2t) - J(t)] + 0.198[J(t) - J(t/2)] + 0.620[J(t/2) - J(t/4)] + 0.012[J(t/4) - J(t/8)] + 0.172[J(t/8) - J(t/16)] + 0.043[J(t/32) - J(t/64)] + 0.012[J(t/128) - J(t/256)] \quad \text{(Eq. 6)}$$

where ω (rad. sec.$^{-1}$) = $1/t$ sec.

The values of $J(t)$ required for the calculations may be read directly from the experimental creep curve and inserted into Eqs. 5 and 6. However, as the curves had already been analyzed into discrete spectra, a computer program was written which converted discrete spectral results into the values of $J(t)$ specified in the equations. It then processed the conversion of this data into $J'(\omega)$ and $J''(\omega)$. Since the discrete analysis was available, these transformed data were checked and found to be accurate, using exact interrelationships (34).

The other oscillatory functions were calculated from the transformed values of J' and J'' using Eqs. 2 and 7–9 (34):

$$G' = J'/(J'^2 + J''^2) \quad \text{(Eq. 7)}$$

$$G'' = J''/(J'^2 + J''^2) \quad \text{(Eq. 8)}$$

$$\tan a = G''/G' \quad \text{(Eq. 9)}$$

RESULTS

The results of discrete spectral analyses of the creep curves are in Table II. Figures 1–4 show plots of the loss tangents and the storage and loss compliances, moduli, and viscosities *versus* frequency for cetrimide emulsions. The same functions for cetomacrogol emulsions are illustrated in Figs. 5–8. Each figure contains the appro-

Table II—Discrete Spectral Analysis of Creep Curves [Three Voigt Unit, Residual Spring, and Residual Dashpot Model (38)]

Viscoelastic Parameters[a]	Emulsions			
	C8	C14	N7	N16
Residual viscosity η_0 ($\times 10^6$)	19.2	79.1	4.38	40.8
Residual compliance J_0 ($\times 10^{-4}$)	0.90	0.247	1.55	0.272
Viscosity η_1 ($\times 10^6$)	4.32	14.5	1.38	10.1
Compliance J_1 ($\times 10^{-4}$)	2.20	0.823	6.82	1.20
Retardation time τ_1	949	1190	943	1210
Viscosity η_2 ($\times 10^6$)	2.18	5.40	2.60	5.18
Compliance J_2 ($\times 10^{-4}$)	0.691	0.300	1.29	0.849
Retardation time τ_2	150	162	341	220
Viscosity η_3 ($\times 10^5$)	2.66	7.19	1.25	6.81
Compliance J_3 ($\times 10^{-4}$)	1.06	0.318	1.64	0.441
Retardation time τ_3	28.0	23.0	20.0	30.0

[a] Viscosities, compliances, and retardation times are in units of poise, dyne^{-1} cm.^{2}, and sec., respectively.

priate function derived by direct oscillatory measurements at frequencies ranging from 10^{-4} to 25 Hz. and the same function calculated from the creep data in Table II using Eqs. 5–9. Transformed data are plotted over the frequency range 10^{-6}–1.5×10^{-1} Hz.

DISCUSSION

In general, agreement between experimental storage and loss parameters obtained using the rheogoniometer and the same functions calculated from creep data using Schwarzl's (36) transformations is good (Figs. 1–8). The significance of this is apparent when considering both the nature of the mathematical approximations and the many variables involved in the experiments (see introductory material). This agreement between the data provides strong evidence that the basic assumption made in previous work is valid; that is, that neither small strain technique destroys static structure and thus the systems are examined essentially in their rheological ground states during both types of measurement.

Creep data imply that the emulsions were of a slightly higher consistency during transient experiments than when observed in oscillation. Thus, in Figs. 1–8 a slight trend is apparent whereby transformed compliances are lower and transformed viscosities and moduli are higher than the corresponding parameters derived directly from oscillatory experiments. The most likely explanation of this trend involves two factors.

1. The loading process in the rheogoniometer destroys slightly more structure because of the relatively high shear rates and shear strains applied when parallel plates are brought together.
2. Neglect of the "end effect" in creep calculations increases the value used for the mean shear stress. If the end effect were allowed for, the rheological parameters would move closer to the relevant oscillatory data.

At high frequencies, data were transformed from values of $J(t)$ taken within a few seconds of creep. It is not possible to measure accurately these compliances because of the inertias of the viscometer and recorder. Thus, in this region, data for transformed functions are often scattered and tend to deviate from the experimental points. At the other end of the frequency range, oscillatory measurements involve errors, mainly because of evaporation from the parallel plates during the comparatively long experimental time (20, 21). Sometimes these points are also scattered. However, these discrepancies are slight and the accuracy of the transformation is excellent in view of the many experimental variables and the mathematical approximations made.

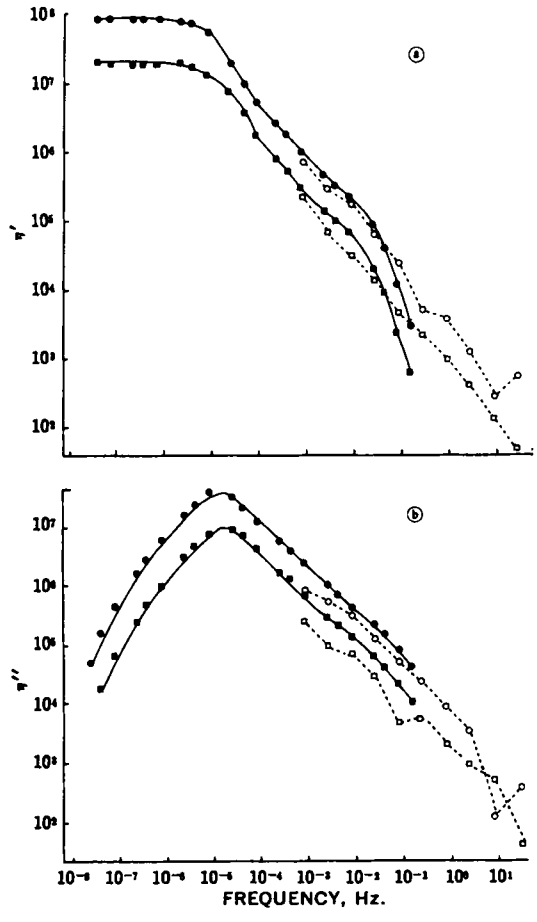

Figure 3—*Cetrimide emulsions C8 and C14, showing the variation of: (a) dynamic viscosity, η', and (b) imaginary part of a complex viscosity, η'' (poises), with frequency (Hz.). Key: □, C8, and ○, C14, values derived from oscillatory experiments using a rheogoniometer; and ■, C8, and ●, C14, theoretical values derived by transformation of creep data.*

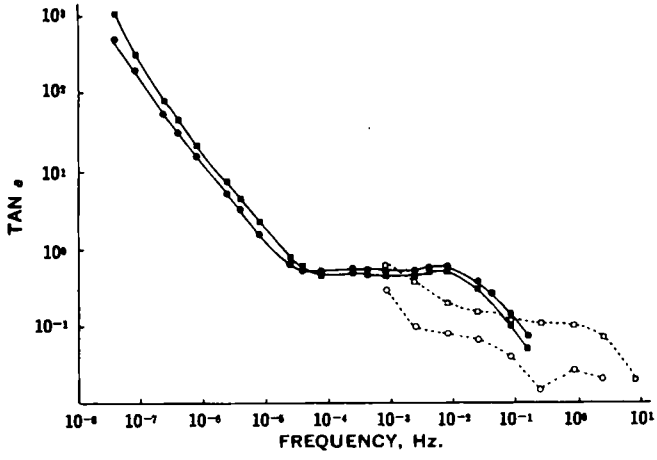

Figure 4—*Cetrimide emulsions C8 and C14, showing the variation of loss tangent, tan δ, with frequency (Hz.). Key: □, C8, and ○, C14, values derived from oscillatory experiments using a rheogoniometer; and ■, C8, and ●, C14, theoretical values derived by transformation of creep data.*

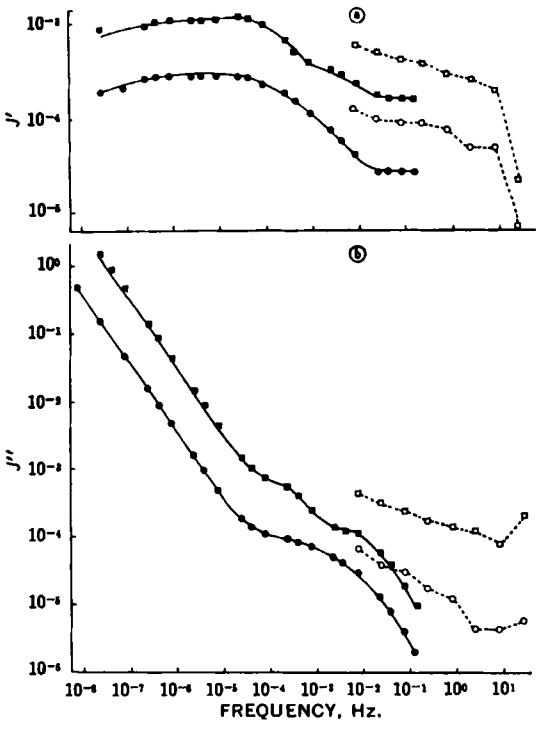

Figure 5—*Cetomacrogol emulsions N7 and N16, showing the variation of: (a) storage compliance, J', and (b) loss compliance, J'' (dyne^{-1} cm.^{2}), with frequency (Hz.). Key: □, N7, and ○, N16, values derived from oscillatory experiments using a rheogoniometer; and ■, N7, and ●, N16, theoretical values derived by transformation of creep data.*

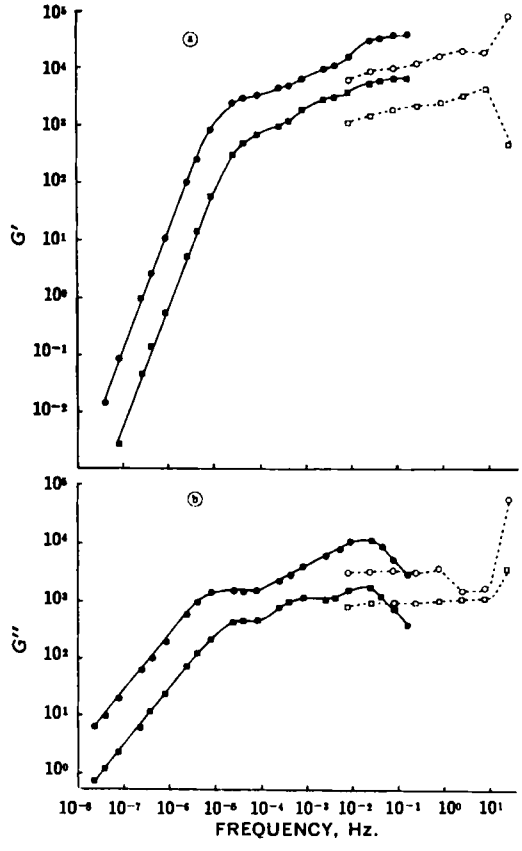

Figure 6—*Cetomacrogol emulsions N7 and N16, showing the variation of: (a) storage modulus, G', and (b) loss modulus, G'' (dynes cm.$^{-2}$), with frequency (Hz.). Key: □, N7, and ○, N16, values derived from oscillatory experiments using a rheogoniometer; and ■, N7, and ●, N16, theoretical values derived by transformation of creep data.*

Since the transformed dynamic functions were similar to those obtained from direct dynamic measurements over the frequency range 7.91×10^{-4} (cetrimide) or 7.91×10^{-3} (cetomacrogol) to 1.5×10^{-1} Hz., it was deduced that transformed data extrapolated to low frequencies are also representative of the values that would be obtained if it were possible to conduct oscillatory experiments in this frequency range. In practice, one cannot perform reliable oscillatory experiments at frequencies below approximately 10^{-4} Hz. because of the limitations of the rheogoniometer and the transfer function analyzer and because of evaporation effects from parallel plates.

Thus, it is concluded that the described transformation procedure provides reasonably accurate dynamic functions at intermediate frequencies so that it is possible to obtain dynamic data without an oscillatory instrument. This procedure also enables oscillatory functions to be calculated at frequencies lower than those accessible to an oscillatory instrument.

Now that it has been established that transformation procedures are applicable to pharmaceutical materials and an understanding has been reached of the limitations of both creep and oscillatory experiments with regard to frequency range, it is possible to suggest a practical procedure for examining the viscoelastic properties of such formulations. In this process, data are selected from both types of experiment in the time–frequency range where each method is most accurate. This involves using high frequency values obtained from direct oscillatory measurements and low frequency values realized when creep data are transformed. Typical examples are shown in Fig. 9, where data taken from Figs. 2 and 3 are collated to provide single plots of the variations of G' and η' with frequency. To obtain smooth plots, less emphasis is given to high frequency transformed functions and to low frequency oscillatory values. The functions in the other graphs (Figs. 1–8) can be similarly processed to obtain smooth plots (not shown here). The advantage of this unification procedure is that one now obtains a clear rheological description over the entire frequency range by means of a single type of response function.

Although a viscoelastic material may be rheologically defined by any one of the viscoelastic functions used in this work, it is considered valuable to examine several functions because different types of viscoelastic behavior often exhibit different degrees of prominence in the various plots. For both ionic and nonionic preparations, as the mixed emulsifier concentration increased, consistencies rose as coherent viscoelastic networks formed in the continuous phases (1).

This is shown throughout the entire frequency range by increases in the experimental and transformed viscosities and moduli and decreases in the compliances as the cetrimide and cetomacrogol mixed emulsifier concentrations rise from 8 to 14 and 7 to 16%, respectively. The loss tangent decreases with concentration, indicating that, when networks build up, the elastic component increases to a greater extent than the viscous component. The trends exhibited by the functions plotted in Figs. 1–9 are similar to those shown by uncrosslinked viscoelastic polymers (34) and conform with a generalized Maxwell model analogy described previously (20). At high frequencies, elastic behavior dominates because there is little time available for viscous deformation, and values of G' and J' are high and low, respectively. As the time period increases, viscous behavior becomes increasingly prominent, and J' rises and G' falls with decrease in frequency. Plots of both functions alter generally in the 8×10^{-5}–1×10^{-4}-Hz. frequency region (Figs. 1, 2, 5, 6, 9).

Although η'' is not usually used to describe viscoelastic deformation, plots are included here to complete the data (Figs. 3*b* and 7*b*). These also change, exhibiting pronounced maxima in this region. These variations are significant, because they occur at frequencies that correspond to creep times (~1600–2000 sec.) only slightly greater than the longest retardation times obtained by the discrete spectral analyses of the curves (*cf.*, Table II). They indicate that retarded elastic effects no longer operate and further deformation at low frequencies is due to viscous flow. Thus, at very low frequencies (much less than 10^{-5} Hz.), G' and η'' fall rapidly and J' flattens, as the energy stored per cycle becomes negligible compared to that dissipated. Viscoelastic theory predicts that, at these low frequencies, G' plots should approach zero with a slope of 2 and J'

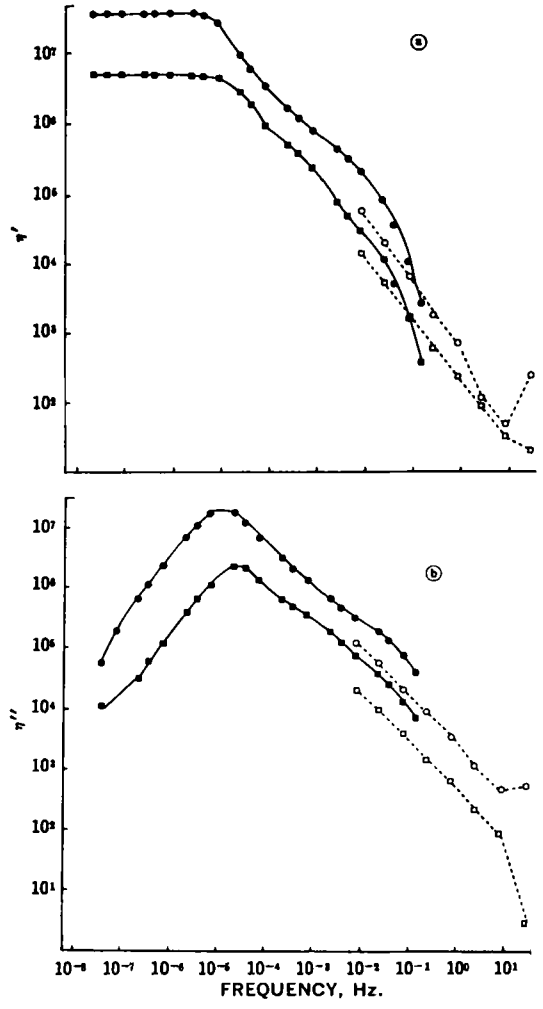

Figure 7—*Cetomacrogol emulsions N7 and N16, showing the variation of: (a) dynamic viscosity, η', and (b) imaginary part of a complex viscosity, η'' (poises), with frequency (Hz.). Key: □, N7, and ○, N16, values derived from oscillatory experiments using a rheogoniometer; and ■, N7, and ●, N16, theoretical values derived by transformation of creep data.*

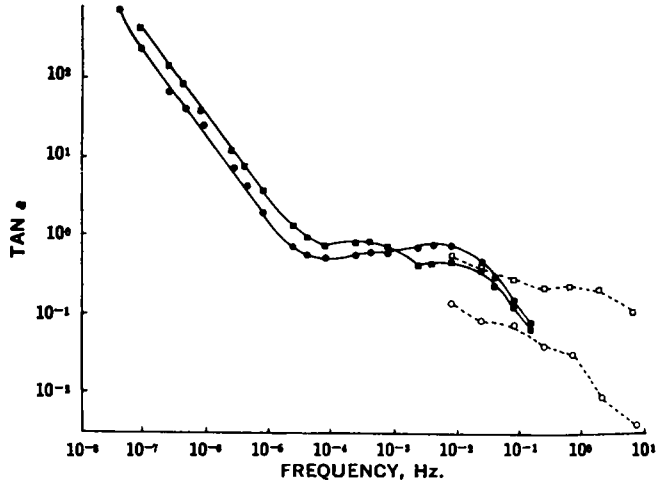

Figure 8—*Cetomacrogol emulsions N7 and N16, showing the variation of loss tangent, tan a, with frequency (Hz.). Key: □, N7, and ○, N16, values derived from oscillatory experiments using a rheogoniometer; and ■, N7, and ●, N16, theoretical values derived by transformation of creep data.*

plots should approach J_e, the total recoverable (elastic) compliance in creep. The results summarized in Table III show that agreement between such theoretical and transformed data is good. At high frequencies, G' values should approach G_0, the instantaneous elastic modulus derived in creep. The values in Table II indicate that this condition is met quite closely. Such agreement is especially satisfactory in the light of the experimental limitations involved in short time creep testing, *i.e.*, the inertial effects of the viscometer and the difficulty in applying a truly instantaneous stress.

Plots of the dissipative functions η', G'', and J'' also inflect at frequencies close to 10^{-5}–10^{-4} Hz.; at lower frequencies, dissipative mechanisms predominate and G'' reduces, J'' increases, and η' flattens out. In regions where G' changes relatively slowly with frequency (greater than approximately 10^{-4} Hz.), the behavior is predominately elastic and G'' is slightly less and J'' is slightly greater than the corresponding storage parameters. In addition, when η' flattens, G'' rises steeply, and when η' increases rapidly, G'' shows little variation. At the very low frequencies that correspond to long creep times, η' approaches the creep residual viscosity, η_0, and J'' and G'' slopes are close to -1 and $+1$, respectively, as predicted by viscoelastic theory (Table III).

Plots of the variation of tan a with frequency (Figs. 4 and 8) are of considerable practical interest because each emulsion has a characteristic curve containing maxima and minima. Loss tangent plots have been referred to as "consistency spectra" because of their similarities with the spectra obtained from spectrophotometry (29). At very low frequencies (less than 10^{-5} Hz.), tan a is large and, as predicted by theory, it is inversely proportional to the frequency so the plots in this region have slopes of -1 (Table III). In the frequency region where all other plots change ($\sim 10^{-4}$ Hz.), spectra flatten with tan a values close to unity. In the intermediate and high frequency regions, all spectra contain at least three minima which are possibly related to retardation mechanisms.

CONCLUSIONS

1. Liquid paraffin-in-water emulsions stabilized by mixed emulsifiers of the cetrimide or cetomacrogol surfactants–cetostearyl alcohol type were used as model systems to represent pharmaceutical semisolids. They were examined in their linear viscoelastic regions using creep (concentric cylinder reaction air turbine viscometer) and oscillatory (parallel plate Weissenberg rheogoniometer) techniques.

2. Storage and loss compliances (J' and J'') for frequencies ranging from 2.5×10^{-6} to 1.5×10^{-1} Hz. were calculated from creep data using numerical formulas (29). These were used to de-

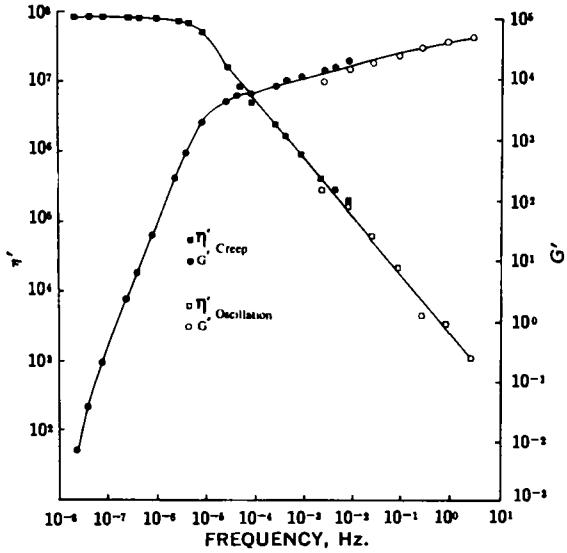

Figure 9—*Cetrimide emulsion C8. Unified plots of the variations of storage modulus, G' (dynes cm.$^{-2}$), and dynamic viscosity, η' (poises), with frequency (Hz.). Low frequency values were derived by transformation of creep data, and high frequency values were derived from oscillatory experiments using a rheogoniometer.*

Table III—Relationships between Viscoelastic Data

Data	Emulsions C8	C14	N7	N16
Slope of G' plots at low frequencies (theoretical value = 2 dynes cm.$^{-2}$ Hz.$^{-1}$)	2.1	2.1	2.1	2.1
Slope of G'' plots at low frequencies (theoretical value = 1 dyne cm.$^{-2}$ Hz.$^{-1}$)	1.0	1.0	1.0	1.0
Comparison of slope of J' plots at low frequencies and (in parentheses) J_e derived in creep (dyne^{-1} cm.^{2})	5.0×10^{-6} (4.3×10^{-6})	1.5×10^{-6} (1.7×10^{-6})	1.0×10^{-5} (1.1×10^{-5})	3.0×10^{-6} (2.4×10^{-6})
Comparison of η' derived at low frequencies and (in parentheses) η_0 derived in creep (poises)	2.0×10^{7} (2.0×10^{7})	7.9×10^{7} (7.9×10^{7})	4.8×10^{6} (4.4×10^{6})	3.8×10^{7} (4.1×10^{7})
Slope of consistency spectra plots at low frequencies (theoretical value = −1 Hz.$^{-1}$)	−1.1	−1.1	−1.0	−1.1
Comparison of residual shear modulus G_0 derived in creep and (in parentheses) G' at 7.91 and 25 Hz., respectively ($\times 10^4$ dynes cm.$^{-2}$)	1.1 (2.3, 0.6)	4.0 (4.9, 5.0)	0.65 (0.45, 0.05[a])	3.7 (1.9, 7.5)

[a] Low value due to nonlinear response at 25 Hz.

termine the other dynamic functions, G', G'', η', η'', and tan a, at the same frequencies.

3. The transformed dynamic functions were compared with the same functions obtained directly from oscillatory measurements at frequencies ranging from 7.91×10^{-4} to 1.5×10^{-1} Hz. for cetrimide emulsions and from 7.91×10^{-3} to 1.5×10^{-1} Hz. for cetomacrogol emulsions. Agreement was excellent at most frequencies; discrepancies at the extremes of the range were because of unavoidable experimental variables. This good agreement suggests that dynamic descriptions of pharmaceutical semisolids may be obtained at intermediate frequencies using a creep viscometer when an expensive rheogoniometer with associated transfer function analyzer is not available.

4. Neither small strain technique alone covered the entire time scale necessary for an exhaustive study of pharmaceutical semisolids. Creep data were unavoidably inaccurate at high frequencies, and oscillatory measurements could not be made at very low frequencies. To solve this problem, it was shown that creep and oscillatory data may be collated and unified so as to provide smooth plots of the variation of each dynamic function over an extended frequency range (10^{-8}–25 Hz.). This unification procedure uses high frequency values obtained from oscillatory measurements and low frequency values transformed from creep experiments.

5. The variations of each dynamic function with frequency over the entire frequency range were discussed with reference to linear viscoelastic theory. The limiting values and the shapes of the plots agreed well with the theoretical values for viscoelastic liquids.

NOMENCLATURE

C = cetrimide (cationic) emulsions
G' = storage shear modulus
G'' = loss shear modulus
G_0 = residual shear modulus (creep)
J = creep shear compliance
J' = storage shear compliance
J'' = loss shear compliance
J_e = total elastic shear compliance (creep)
J_0 = residual shear compliance (creep)
J_1,J_2,J_3 = shear compliances of Voigt units 1, 2, and 3, respectively (creep)
N = cetomacrogol (nonionic) emulsion
a = phase angle
n = frequency of oscillation (hertz)
t = time (second)
η' = real component of complex dynamic viscosity; dynamic viscosity
η'' = imaginary component of complex dynamic viscosity
η_0 = residual shear viscosity (creep)
η_1,η_2,η_3 = shear viscosities of Voigt units 1, 2, and 3, respectively (creep)
τ_1,τ_2,τ_3 = retardations times of Voigt units 1, 2, and 3, respectively (creep)
ω = frequency of oscillation (rad. sec.$^{-1}$)

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Effect of Sodium Taurodeoxycholate on Biological Membranes: Release of Phosphorus, Phospholipid, and Protein from Everted Rat Small Intestine

STUART FELDMAN▲, MARK REINHARD, and CHARLES WILLSON

Abstract □ Sodium taurodeoxycholate accelerates the release of total phosphorus, lipid phosphorus, and protein from isolated everted rat small intestinal sacs. The effect occurs at concentrations of the physiological surfactant above the CMC. The effect of sodium taurodeoxycholate on components of the biological membrane can be related to an increase in permeability of the everted intestine to phenol red in the presence of various concentrations of the surfactant. The interaction of the surfactant with the biological membrane may produce an acceleration of the loss of structural integrity of the preparation, resulting in an increased permeability to phenol red.

Keyphrases □ Sodium taurodeoxycholate—effect on biological membranes, release of phosphorus, phospholipid, and protein from the everted rat small intestine, loss of structural integrity proposed □ Membranes, biological, everted rat small intestine—effect of sodium taurodeoxycholate on release of phosphorus, phospholipid, and protein □ Surfactants—effect of sodium taurodeoxycholate on release of phosphorus, phospholipid, and protein from the everted rat small intestine □ Permeability, everted rat small intestine—effect of sodium taurodeoxycholate on transport of phenol red, release of phosphorus, phospholipid, and protein from membrane, loss of structural integrity proposed

Previous investigators (1–7) showed an increase in drug absorption across biological membranes in the presence of physiological surfactants. An increase was found in the rate of transfer of salicylate (1) and salicylamide (2) across the everted rat small intestine in the presence of micellar sodium taurodeoxycholate. Absorption of orally administered riboflavin in man increased upon coadministration of sodium deoxycholate, an unconjugated bile salt (3). The GI absorption of phenol red in the rat increased when phenol red was administered with sodium deoxycholate (4). Increased absorption of a number of drugs in the presence of bile salts was reported (5, 6) using an *in situ* perfusion technique. The influence of sodium taurodeoxycholate on the pharmacological effect (time required to produce overturn) of pentobarbital and ethanol in goldfish was studied (7). All these effects are presumably due to an alteration in the permeability of the biological membrane to these drugs by the physiological surfactant.

The exact mechanism of action of the physiological surfactants in increasing the permeability of biological membranes has not been elucidated, although it was postulated (1, 2, 4) that the mechanism may involve changes in membrane structure, possibly by solubilizing lipid components of the membrane. Bile salts were shown to solubilize phospholipids (8), a major component of biological membranes (9).

In a later study (10), the addition of lecithin, a phospholipid, or a mixture of fatty acids and esters to the micellar bile salt solution resulted in a reduction of the permeability effect of the bile salt on the everted rat intestine to salicylate ion. It was, therefore, decided to

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Table I—Total Phosphorus Released from Everted Rat Small Intestine as a Function of Time and Mucosal Concentration of Sodium Taurodeoxycholate

Solution	Total Phosphorus Released, mcg.[a] 30 min.	60 min.	90 min.	120 min.
Buffer	3.17 (1.01)	4.10 (1.14)	4.99 (1.40)	7.63 (2.12)
1 m*M*[b]	4.51 (0.98)	5.88 (0.55)	7.26 (0.23)	8.69 (1.29)
2.5 m*M*[b]	5.02 (5.50, 4.56)[c]	7.50 (7.52, 7.48)[c]	9.29 (0.56)[d]	11.1 (0.65)[d]
5 m*M*[b]	6.17 (0.60)	9.60 (0.39)	13.3 (1.94)	14.3 (1.80)
10 m*M*[b]	8.62 (1.42)	13.1 (1.32)	14.4 (2.31)	16.1 (2.08)

[a] Mean of at least 10 determinations ± *SD*. [b] Sodium taurodeoxycholate. [c] Mean of two determinations. [d] Mean of four determinations.

investigate the effect of sodium taurodeoxycholate on the release of components from biological membranes, using the everted rat small intestine as a model biological membrane.

EXPERIMENTAL

Materials—Sodium taurodeoxycholate[1] was certified as chromatographically pure. All other reagents were analytical grade.

Modified Krebs bicarbonate buffer, with no potassium dihydrogen phosphate included, was made by dissolving the sodium chloride, potassium chloride, and sodium bicarbonate in distilled water. The pH of the solution was adjusted to pH 7.4 with dry ice. When sodium taurodeoxycholate was included in the buffer, it was dissolved before the pH was adjusted with dry ice. In all cases the sodium-ion concentration was adjusted to 150 m*M* by the proper addition or omission of sodium chloride.

The Fiske–Subba Row (11) reagent was prepared by adding 0.125 g. of 1-amino-2-naphthol-4-sulfuric acid, with mechanical stirring, to 50 ml. of freshly prepared 15% sodium bisulfite (anhydrous), followed by 0.25 g. of anhydrous sodium sulfite. The solution was filtered, stored in a dark bottle in a refrigerator, and freshly prepared weekly.

Preparation of Everted Rat Intestinal Sacs—The procedure developed by Crane and Wilson (12) was used with the following modifications. Male, Sprague–Dawley descent rats[2], weighing 250–350 g. and fasted for 20–24 hr. (water allowed *ad libitum*), were anesthetized with ether. A midline abdominal incision was made, the entire small intestine was removed, and the rat was sacrificed. The intestine was rinsed with several portions of normal saline and cut 15 cm. from the pylorus. The 15-cm. portion was discarded and the intestine was everted over a glass rod. Four consecutive sacs were made by stretching the intestine with an 8-g. weight and tying off consecutive 5-cm. segments. The segments were separated and filled with 1 ml. of buffer at pH 7.4 through a syringe and blunt needle. The sacs were then individually incubated in a mucosal solution at 37° consisting of 20 ml. of buffer alone or buffer containing various concentrations of sodium taurodeoxycholate. The mucosal solution was oxygenated continuously with a mixture of 95% oxygen–5% carbon dioxide. One-milliliter samples of the mucosal solution were taken at 30-min. intervals for 2 hr.

Intestinal Transfer Rate Measurements—The Crane and Wilson (12) procedure was used with the modifications previously described (1). Two segments of everted intestine, each 10 cm. in length, were taken. Each segment was attached to a cannula as previously described (1) and was suspended in 80 ml. of mucosal solution, which consisted of buffer at pH 7.4 containing 0.1 mg./ml. phenol red in the presence or absence of various concentrations of sodium taurodeoxycholate. Samples of the serosal solutions were taken at appropriate intervals and assayed for phenol red content.

Assay Procedure—*Total Phosphorus*—The assay procedure described by Bartlett (13) was used with slight modifications. The mucosal samples were diluted to 10 ml. with distilled water. One-milliliter aliquots were taken and placed in 15-ml. conical centrifuge tubes, and 0.5 ml. of 10 *N* sulfuric acid was added to each tube. The tubes were placed in an oven at 160° for 3 hr. After the initial 3-hr. heating period, 3 drops of 30% hydrogen peroxide were added to each tube and the tubes were returned to the oven for another 3 hr. at 160°. After the samples were allowed to cool, 4.4 ml. of distilled water, 0.2 ml. of 5% ammonium molybdate, and 0.2 ml. of Fiske–Subba Row reagent were added. The samples were then heated for 10 min. at 100° and allowed to cool, and the solution was read at 830 nm. on a colorimeter[3] equipped with a red-sensitive phototube. A calibration curve was made by dissolving a known amount of potassium dihydrogen phosphate in distilled water and diluting appropriately to various concentrations of phosphorus equivalents. Each solution was assayed for phosphorus content by the assay previously described, and a calibration curve was constructed. Sodium taurodeoxycholate did not interfere with the assay procedure.

Phospholipid Phosphorus—Phospholipids in the mucosal solution were assayed by a modification of the Zilversmit and Davis (14) method. To 1 ml. of the mucosal solution in a centrifuge tube, 3 ml. of 10% trichloroacetic acid was added drop by drop with swirling. The tubes were allowed to stand for 2 min. and then were centrifuged at approximately 2000 r.p.m. for 5 min. The supernate was decanted and the precipitate was washed twice with 3-ml. portions of 10% trichloroacetic acid. After centrifugation and decantation of the supernate, 1 ml. of distilled water and 0.5 ml. of 10 *N* sulfuric acid were added to each tube and the assay procedure for total phosphorus was followed as outlined previously.

Protein Determinations—Protein determinations of the mucosal samples were made by the method of Lowry *et al.* (15). The solutions were read[3] at 750 nm. against an appropriate blank. Sodium taurodeoxycholate did not interfere with the assay procedure.

Phenol Red Assay—The serosal samples were diluted appropriately with distilled water alkalinized with 3 *N* sodium hydroxide, filtered[4] if necessary, and assayed for phenol red spectrophotometrically at 560 nm. Neither sodium taurodeoxycholate nor "blank" serosal fluid interfered with the assay procedure.

RESULTS AND DISCUSSION

The effect of sodium taurodeoxycholate on the release of total phosphorus from the everted rat intestine as a function of bile salt concentration and time is shown in Table I. At all time periods studied, with increasing concentrations of sodium taurodeoxycholate there was an increase in the total amount of phosphorus released from the everted intestinal sac. Comparison of the total amount of phosphorus released at 2 hr. after incubation reveals that at all concentrations of sodium taurodeoxycholate above 2.5 m*M* the amount of phosphorus in the mucosal fluid was significantly[5] different than that of control values. No statistical difference existed between control studies and the 1 m*M* concentration of sodium taurodeoxycholate as determined by the Student *t* test (16).

Since a possible mechanism for the increase in permeability of the everted rat intestine to drugs after exposure of the membrane to bile salts involves the interaction of the surfactant with lipid components of the biological membrane, the release of lipid-associated phosphorus from the everted intestinal sac in the presence of various concentrations of sodium taurodeoxycholate was measured. The results (Table II) show an increase in the amount of lipid phosphorus released from the everted rat intestine at 2 hr. after incubation in mucosal solutions containing sodium taurodeoxycholate in concentrations above 2.5 m*M* when compared to the control buffer and 1 m*M* bile salt solution.

Since biological membranes consist of both lipid and protein, it

[1] Calbiochem.
[2] Huntingdon Farms, West Conshohocken, Pa.
[3] Spectronic 20, Bausch & Lomb.
[4] Millipore filter, 0.45-μ pore size.
[5] In all cases, significance was calculated at the 95% confidence level.

Table II—Lipid Phosphorus Released from Everted Rat Small Intestine as a Function of Time and Mucosal Concentration of Sodium Taurodeoxycholate

Solution	Lipid Phosphorus Released, mcg.[a], at 120 min.
Buffer	0.34 ± 0.12
1 m*M*[b]	0.53 ± 0.20
2.5 m*M*[b]	0.77 (0.74, 0.80)[c]
5 m*M*[b]	1.55 ± 0.41
10 m*M*[b]	2.32 ± 0.39

[a] Mean of four determinations ± *SD*. [b] Sodium taurodeoxycholate. [c] Average of two determinations.

was important to determine if sodium taurodeoxycholate had an effect on the protein composition of the everted rat intestinal sac. The released protein was measured as bovine serum albumin equivalents (Table III). Table III lists the milligrams of protein released from the everted intestinal sac at 60 and 120 min. after incubation as a function of sodium taurodeoxycholate concentration. As can be seen from the data, with increasing concentration of sodium taurodeoxycholate there was an increase in the total amount of protein released.

The next phase of the study examined the influence of sodium taurodeoxycholate on the transfer of a drug across the everted rat small intestine. This was done to determine if any correlation existed regarding the release of membrane components and the alteration in permeability of the membrane to a drug substance. Phenol red was chosen as the model drug, and the effect of various concentrations of sodium taurodeoxycholate on the transfer of the drug across the everted rat intestine was examined (Table IV). The total amount of phenol red transferred across the intestinal segments at 60 and 120 min. increased with increasing concentrations of sodium taurodeoxycholate. The increase in the amount of phenol red transferred in 2 hr. was significantly different from control values for all bile salt concentrations except 1 m*M* sodium taurodeoxycholate.

The data presented in Table V show the ratio of the total amount of membrane component released after a 2-hr. exposure to various concentrations of sodium taurodeoxycholate compared to the amount released in control studies. The increase in the release of membrane phosphorus, lipid phosphorus, and protein corresponded to the increased permeability of the everted intestine to phenol red. In all cases except for the protein experiments, the amount of membrane component released in the presence of 1 m*M* sodium taurodeoxycholate was not significantly different from control values as determined by the Student *t* test (16). Similar findings can be seen in the phenol red permeability measurements. The ratio of total amount of membrane component released after a 2-hr. exposure to various concentrations of sodium taurodeoxycholate plotted against the ratio of total amount of phenol red transferred in 2 hr. for each bile salt concentration is presented in Fig. 1. At 2.5, 5, and 10 m*M* concentrations of sodium taurodeoxycholate, a relationship between total phosphorus, lipid phosphorus, or protein released from the membrane and permeability of the membrane to phenol red appears to exist.

The critical micelle concentration (CMC) of sodium taurodeoxycholate is approximately 1.2 m*M* at 37° (17). Thus, it appears that the effect of the bile salt on the release of membrane phosphorus and lipid phosphorus occurs at concentrations of the surfactant above the CMC. This effect on permeability at concentrations of surfactant above the CMC was reported previously (1, 2, 10) and occurred in the transfer of phenol red in the present study.

A previous report (10) presented data to show that the addition of phospholipid or fat digestion products to micellar bile salt solutions results in a decreased permeability response by the everted rat intestine in the presence of the bile salt. To determine whether the addition of phospholipid to the micellar bile salt solution would have an effect on the release of membrane components, 10 m*M* egg lecithin was added to 10 m*M* sodium taurodeoxycholate and the release of protein from the intestinal sac was measured. The results are presented in Fig. 2 and clearly show that the addition of lecithin to the micellar bile salt solution results in a decreased amount of protein released when compared to the bile salt alone. This is in good agreement with results from previous permeability experiments (10).

Table III—Protein Release from Everted Rat Small Intestine as a Function of Time and Mucosal Concentration of Sodium Taurodeoxycholate

Solution	Protein, mg.[a,b] 60 min.	120 min.
Buffer	3.61 ± 0.14	5.44 ± 0.12
1 m*M*[c]	3.71 ± 0.17	6.55 ± 0.12
2.5 m*M*[c]	5.99 ± 0.11	9.69 ± 0.15
5 m*M*[c]	7.22 ± 0.23	11.7 ± 0.11
10 m*M*[c]	10.5 ± 0.20	12.1 ● 0.21

[a] Mean of five determinations ± *SD*. [b] Calculated as bovine serum albumin equivalents. [c] Sodium taurodeoxycholate.

Table IV—Cumulative Amount (Micrograms) Phenol Red Transferred at 60 and 120 min. in the Presence of Various Concentrations of Sodium Taurodeoxycholate

Solution[a]	Cumulative Amount, mcg.[b] 60 min.	120 min.
Control (7)	36.5 ± 3.8	118 ± 12
1 m*M*[c] (4)	39.2 ± 2.8	120 ± 1.8
2.5 m*M*[c] (5)	45.7 ± 3.4	179 ± 13.1
5 m*M*[c] (5)	52.2 ± 13.2	211 ± 8.3
10 m*M*[c] (6)	54.9 ± 6.0	229 ± 38

[a] Number of experiments in parenthesis. [b] Mean ± *SD*. [c] Sodium taurodeoxycholate.

Table V—Ratio of Total Amount of Membrane Component Released after a 2-hr. Exposure to Various Concentrations of Sodium Taurodeoxycholate

Solution	Ratio (Sodium Taurodeoxycholate/Control) Total Phosphorus	Lipid Phosphorus	Protein	Phenol Red
1 m*M*[a]	1.14[b]	1.55[b]	1.20	1.02[b]
2.5 m*M*[a]	1.46	2.26	1.78	1.52
5 m*M*[a]	1.88	4.56	2.16	1.79
10 m*M*[a]	2.11	6.82	2.22	1.94

[a] Sodium taurodeoxycholate. [b] Not significantly different than control values ($p > 0.05$).

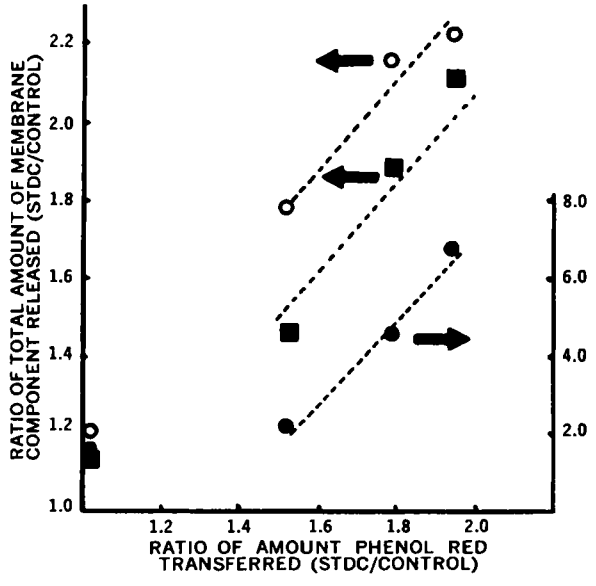

Figure 1—*Plot of the ratio of membrane total phosphorus* (■), *lipid phosphorus* (●), *and protein* (○) *released in 2 hr. in various concentrations of sodium taurodeoxycholate* (*STDC*) *to that in controls* versus *the ratio of phenol red transferred in 2 hr. in various concentrations of sodium taurodeoxycholate to that of controls.*

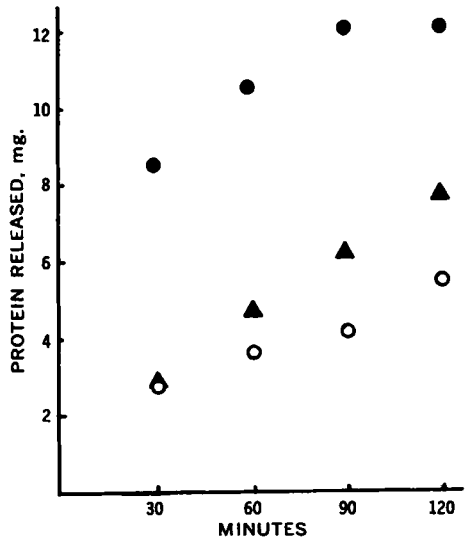

Figure 2—*Plot of total amount of protein released from the everted rat intestinal sac* versus *time in the presence of sodium taurodeoxycholate, 10 mM* (●); *sodium taurodeoxycholate, 10 mM + egg lecithin, 10 mM* (▲); *and control buffer* (○).

It appears that sodium taurodeoxycholate alters the composition of the everted rat intestinal membrane by producing an efflux of membrane components from the everted sac preparation. A similar effect by detergents on a microsomal fraction from mammalian brain was reported by Bradford *et al.* (18). These workers found that sodium deoxycholate, digitonin, and cetrimonium (cetyltrimethylammonium) bromide increased the percentage of protein, cholesterol, and phospholipid phosphorus recovered in the supernatant liquid of microsomal suspensions from brain tissue. The mechanism of action was postulated to involve interaction of the surfactant with the membrane lipo-protein complex. Moreover, Bradford *et al.* (18) did not observe significant increases in recovery of protein, phospholipid phosphorus, or cholesterol until the CMC of each surfactant was exceeded.

The alteration of the biological membrane in the presence of micellar concentrations of sodium taurodeoxycholate appears to be responsible for the increased permeability of the everted rat intestinal membrane to certain drugs after exposure to the physiological surfactant. The exact mechanism of the interaction is still unknown but most likely involves interaction of the bile salt with the protein and/or phospholipid components of the membrane. Recent work (19, 20) suggests a strong possibility for the observed effects; an increase in clearance values of polar drugs across the everted rat intestine over a 2-hr. period as compared to the initial 30-min. values was observed. The increase in rate of clearance of the compounds was partly due to a loss of functional integrity of the preparation. Compounds demonstrating initial clearance values of >1 ml./hr. showed little change in permeability over the entire 2 hr.

Previous studies with sodium taurodeoxycholate show little alteration in the permeability of the everted rat intestine to salicylamide (2) after exposure of the preparation to the bile salt, while a significant effect was noted with salicylate (1) and riboflavin (3). The clearance of salicylamide is 2.8 ml./hr., salicylate clearance is 0.7 ml./hr., and riboflavin clearance is approximately 0.3 ml./hr. (19). Phenol red has an initial 30-min. clearance value of 0.1 ml./hr. Therefore, the effect of the physiological surfactant on the biological membrane is consistent with the previously reported data (19, 20). Furthermore, Levine *et al.* (21) reported a progressive loss of structural integrity of everted rat intestinal sacs after eversion and incubation in oxygenated buffer. It is possible that the effect of sodium taurodeoxycholate on the permeability of the everted rat intestine to polar water-soluble drugs may be due to an acceleration by the surfactant of the loss of structural integrity of the preparation. This remains to be confirmed.

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Effects of Cryogenine on Adjuvant-Induced Arthritis and Serum Turbidity in Rats

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Abstract □ Daily oral administration of 100 mg./kg. of cryogenine significantly suppressed the inflammation and the development of disseminated lesions in rats with adjuvant-induced (*Mycobacterium butyricum*) polyarthritis. Significant anti-inflammatory effects continued for 10 days after cessation of therapy (20 days after pedal injection of adjuvant). Cryogenine also partially prevented the decreased growth rate and decreased serum turbidity documented for untreated rats with adjuvant-induced polyarthritis. This profile of activity was similar to that established for 10 mg./kg. daily of hydrocortisone but unlike that for 2 mg./kg. daily of mercaptopurine. Cryogenine appeared equipotent (milligrams per kilogram) to phenylbutazone in inhibiting adjuvant-induced polyarthritis.

Keyphrases □ Cryogenine—effects on adjuvant-induced arthritis and serum turbidity in rats □ Arthritis, adjuvant induced—effect of cryogenine on inflammation and serum turbidity, rats □ Anti-inflammatory activity—effects of cryogenine on adjuvant-induced arthritis and serum turbidity in rats

Adjuvant-induced polyarthritis in rats produced by pedal injection of a killed *Mycobacterium* species suspended in a vehicle of light liquid petrolatum (1) has many similarities to human rheumatoid arthritis (2). While the initial edema and soft tissue thickening in the injected hindfoot may be due partially to irritation, the subsequent swelling in the injected foot and disseminated polyarthritis in the contralateral foot, front limbs, and tail appear to be immunological events (3). In 1967, Kaplan *et al.* (4) reported that chronic oral administration of cryogenine was effective in inhibiting this adjuvant-induced polyarthritis. This study was qualitative rather than quantitative since it was conducted in a very limited number of rats for a limited time and monitored only by caliper measurements of medial foot thickness. Therefore, a quantitative study appeared indicated when an adequate supply of this rare alkaloid became available.

Omaye *et al.* (5) reported that erythrocyte sedimentation rate is significantly increased by adjuvant-induced polyarthritis in a two-step process (significant elevation within 6 days after pedal injection and maximum elevation around Day +15 when secondary lesions develop). This phenomenon was paralleled by increases in total white cell count associated with lymphopenia and neutrophilia. In the same study, orally administered cryogenine and phenylbutazone appeared equipotent (milligrams per kilogram) in moderating these hematological changes.

The interrelationships between lysozyme activity and serum turbidity in rats with adjuvant-induced polyarthritis also were investigated (6). Increased lysozyme levels and decreases in serum turbidity (determined by the stability of serum protein against heat denaturation) were found to be correlated with the severity of the polyarthritic condition. Therapeutically useful anti-inflammatory drugs were shown to reverse both of these parameters. Weissman (7) proposed that degradative enzymes released from lysosomes may denature native connective tissue. The denatured products in turn may then induce an immune response like that seen in adjuvant-induced polyarthritis. Therefore, the present study also attempts to establish the effect of cryogenine and various prototype agents on serum turbidity.

EXPERIMENTAL

Adjuvant-Induced Polyarthritis—Adult female rats of the Sprague–Dawley strain, weighing approximately 200 g., were allowed to acclimatize in this laboratory for 1 week with free access to food pellets[1] and tap water. The animals were randomized into nine treatment groups of 10 rats each, and the test drugs[2] (suspended or dissolved in aqueous 0.25% agar) were orally administered (5 ml./kg.) daily beginning on Day −1 and continuing through Day +20. Both control groups received only the agar dosing vehicle. The oral route was selected to minimize counterirritant effects associated with parenteral injection (8). On Day 0, each rat to receive adjuvant was anesthetized with 30 mg./kg. i.p. of sodium pentobarbital; then 0.05 ml. of a sterile 5-mg./ml. homogenized suspension of heat-killed, desiccated *Mycobacterium butyricum*[3] in light mineral oil was injected into the left hindfoot, using a sterile micrometer syringe and a sterile disposable needle. To prevent external leakage of the injected material and to minimize vascular involvement, the 25-gauge needle was inserted through the radial foot pad and directed medially into the metatarsal region, keeping the needle's path as near to the skin surface as possible. Paw volume was measured up to an india ink line across the lateral malleolus by displacement of mercury similar to the technique described by Van Arman *et al.* (9). These plethysmographic determinations were made every 3 days for both the injected foot and the contralateral foot through Day +30. The inhibitory effects of the various drug treatments were estimated using Eq. 1 (1):

$$\text{percent inhibition} = 100\,\{1 - [(a - x)/(b - y)]\} \quad \text{(Eq. 1)}$$

where y = mean foot volume of the adjuvant control rats (receiving adjuvant injection but no drug treatment) immediately prior to adjuvant administration, b = mean foot volume of adjuvant control rats on a particular day, x = mean foot volume of drug-treated rats immediately prior to adjuvant injection, and a = mean foot volume of drug-treated rats on a particular day.

A subjective scoring system was also used to evaluate the polyarthritis. Every 3rd day the severity of the arthritic lesions in each of the four limbs was rated on a scale of 0–4. Criteria for grading included: severity and extent of edema and erythema of the periarticular tissue plus the degree of enlargement, distortion, and ankylosis of the joints. Therefore, the score for any one animal at any one time could range from 0 to a maximum of 16.

Serum Turbidity—Another 36 rats of the same stock were randomized into nine groups and treated with adjuvant and drugs as already described. However, on Day +20, they were lightly

[1] Purina rat chow.

[2] Sources of test agents were: phenylbutazone, Geigy Pharmaceuticals, Ardsley, N. Y.; indomethacin, Merck Sharp & Dohme Research Laboratories, West Point, Pa.; mefenamic acid, Parke, Davis & Co., Detroit, Mich.; paramethasone, Eli Lilly & Co., Indianapolis, Ind.; and hydrocortisone and mercaptopurine, Atlas Chemical Industries, Inc., Wilmington, Del. The cryogenine alkaloid (mol. wt. 435.53) was isolated from Mexican *Heimia salicifolia* Link and Otto and is not to be confused with the trade name product Cryogénine (phenylsemicarbazide, mol. wt. 151.2) distributed by Laboratoires Sarbach of Châtillon, France.

[3] Number 0640-33, Difco Laboratories.

Table I—Drug Effects on Volume of the Adjuvant-Injected Paw in Adjuvant-Induced Polyarthritis

Treatment (Day −1 to Day +20)	Daily Oral Dose, mg./kg.	Mean Foot Volume, ml. ± *SE* (Percent Inhibition Inflammation) Day +3	Day +9	Day +15	Day +21	Day +24	Day +30
Untreated control	—	1.21 ± 0.02	1.24 ± 0.03	1.26 ± 0.04	1.26 ± 0.04	1.28 ± 0.06	1.27 ± 0.04
Adjuvant control	—	2.07 ± 0.06 (0)	2.44 ± 0.11 (0)	2.51 ± 0.19 (0)	2.65 ± 0.15 (0)	2.85 ± 0.19 (0)	3.03 ± 0.21 (0)
Cryogenine	100	1.84 ± 0.07 (26)	1.89 ± 0.07 (45)	1.80 ± 0.08 (55)	1.85 ± 0.07 (56)	1.84 ± 0.09 (62)	1.94 ± 0.09 (60)
Phenylbutazone	100	1.91 ± 0.04 (21)	1.92 ± 0.04 (45)	1.99 ± 0.06 (42)	1.92 ± 0.06 (53)	1.94 ± 0.05 (57)	1.95 ± 0.05 (61)
Mefenamic acid	25	1.91 ± 0.05 (18)[a]	2.16 ± 0.04 (22)	2.26 ± 0.10 (19)[a]	2.61 ± 0.08 (2)[a]	2.57 ± 0.08 (17)[a]	2.69 ± 0.08 (18)[a]
Indomethacin	1	1.97 ± 0.08 (15)[a]	1.89 ± 0.07 (52)	2.08 ± 0.05 (58)	2.02 ± 0.13 (46)	2.10 ± 0.18 (48)	2.37 ± 0.18 (38)
Paramethasone	0.5	1.81 ± 0.05 (27)	1.97 ± 0.10 (36)	1.86 ± 0.07 (48)	2.08 ± 0.05 (38)	2.02 ± 0.05 (49)	2.06 ± 0.04 (52)
Hydrocortisone	10	1.87 ± 0.05 (21)	1.83 ± 0.03 (49)	1.75 ± 0.05 (58)	1.67 ± 0.06 (68)	1.82 ± 0.07 (62)	1.99 ± 0.09 (57)
Mercaptopurine	2	1.77 ± 0.04 (39)	2.02 ± 0.05 (37)	1.93 ± 0.07 (48)	1.96 ± 0.07 (51)	1.94 ± 0.08 (58)	2.20 ± 0.05 (48)

[a] Mean foot volume is not significantly different from that of the adjuvant control group ($p > 0.05$).

anesthetized with ether and exsanguinated by cardiac puncture. To 0.1 ml. of nonhemolyzed serum, 2.9 ml. of 0.067 *M* Sørensen phosphate buffer[4] in saline (pH 5.2) was added and gently agitated. The mixture was allowed to stabilize at room temperature for 15 min. before being incubated in a constant-temperature water bath at 69° for 30 min. After removal, the sample was cooled in an ice bath and read in a spectrophotometer[5] at a wavelength of 645 nm. (10). Two separate determinations were made per rat.

RESULTS AND DISCUSSION

Adjuvant-Induced Polyarthritis—In the adjuvant control rats, marked inflammatory swelling was noted in the injected paw within 2 hr. after adjuvant injection. Paw volume increased in a linear fashion until a plateau effect was achieved between Days +6 and +12; thereafter volume again increased in a linear fashion through Day +30 (Table I). None of the test agents prevented the acute inflammatory reaction seen immediately following adjuvant injection. If one discounts the Day +3 foot volume readings, which still may reflect trauma and irritation due to the injection, all test drugs inhibited to some degree the subsequent inflammatory processes. With the exception of mefenamic acid, significant inhibition of inflammation in the injected foot was achieved with all test drugs during the dosing period and after cessation of dosage. Significant inhibition of inflammation in the injected foot was documented for mefenamic acid only at Days +6 and +9.

By Day +12, the development of secondary lesions was noted in the adjuvant control animals (increased vascularity of the ears, muzzle, and tail accompanied by swelling of the front and contralateral hindpaws). This secondary involvement increased in severity through Day +30, with the volume of the contralateral foot increasing in a near linear fashion. The initial reaction in the contralateral paw was a diffuse erythematous swelling of the dorsum of the tarsal region and the ankle joint, generally accompanied by a fusiform swelling of the digits. Nodular lesions eventually developed in the ears, muzzle, and tail. Inflammation in the contralateral paw as measured by volume (Table II) was significantly suppressed during the medication period by all drugs except indomethacin (all days) and phenylbutazone (Day +15). While indomethacin also lacked significant effect after cessation of therapy, some beneficial anti-inflammatory effects persisted for the other drugs through Day +30, especially in rats treated with paramethasone and to a lesser extent with phenylbutazone and mercaptopurine. Contralateral feet of the paramethasone-treated rats appeared grossly equivalent to the feet of untreated control animals at the termination of the 30-day observation period. The anti-inflammatory effects in the contralateral feet of animals treated with cryogenine, mefenamic acid, and hydrocortisone were not well sustained after cessation of drug dosage.

The arbitrary scoring system used in Table III should provide an index reflecting the total degree of polyarthritic involvement since both the fore- and hindpaws were rated. Only cryogenine, hydrocortisone, and mercaptopurine appeared to provide significant early protection (Day +12), while all test drugs except indomethacin appeared to protect against the development of secondary lesions (Day +21). All medicaments except indomethacin and mefenamic acid provided some continuing protection after the cessation of therapy (Day +30). The oral doses used were selected to be equiactive based on other anti-inflammatory testing in this laboratory using this rat stock; however, the present results indicate that the test compounds were not equiactive against adjuvant-induced polyarthritis at these doses.

Body Weight Changes—The mean weight of all animals was 218 g. at 0 time. The adjuvant control group achieved only a mean net gain of 5 g. in body weight during the entire observation period. There was a significant loss of mean weight (−11 g.) during the first 3 days following adjuvant injection, after which there was a

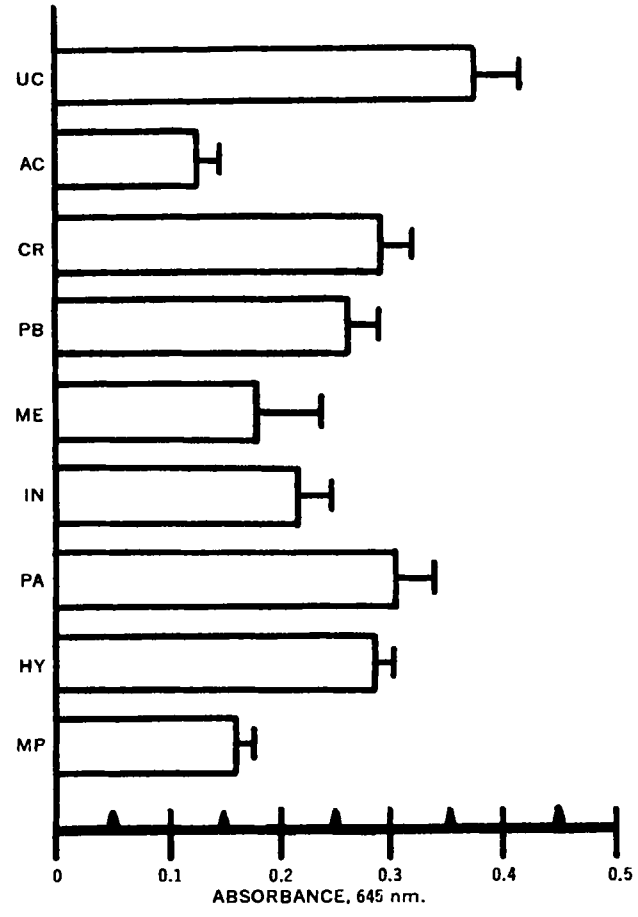

Figure 1—*Serum turbidity (absorbance + 1 SE) on Day +20 of untreated controls (UC) relative to adjuvant controls (AC) plus the effects on serum turbidity of adjuvant-treated rats receiving daily oral administration of cryogenine (CR), phenylbutazone (PB), mefenamic acid (ME), indomethacin (IN), paramethasone (PA), hydrocortisone (HY), and mercaptopurine (MP).*

[4] Composition: 2 ml. 0.067 *M* monopotassium phosphate + 98 ml. 0.067 *M* disodium phosphate.
[5] Spectronic 20, Bausch & Lomb.

Table II—Drug Effects on Volume of the Contralateral Paw in Adjuvant-Induced Polyarthritis

Treatment (Day −1 to Day +20)	Daily Oral Dose, mg./kg.	Mean Foot Volume, ml. ± *SE* (Percent Inhibition Inflammation) Day +15	Day +18	Day +21	Day +24	Day +30
Untreated control	—	1.19 ± 0.02	1.22 ± 0.02	1.24 ± 0.04	1.25 ± 0.05	1.26 ± 0.06
Adjuvant control	—	1.50 ± 0.10 (0)	1.67 ± 0.15 (0)	1.78 ± 0.16 (0)	1.87 ± 0.21 (0)	2.00 ± 0.21 (0)
Cryogenine	100	1.27 ± 0.03 (94)	1.31 ± 0.03 (88)	1.33 ± 0.05 (86)	1.37 ± 0.06 (82)	1.61 ± 0.04 (56)[a]
Phenylbutazone	100	1.30 ± 0.02 (74)[a]	1.31 ± 0.02 (81)	1.32 ± 0.02 (83)	1.35 ± 0.04 (80)	1.48 ± 0.04 (68)
Mefenamic acid	25	1.19 ± 0.02 (113)	1.26 ± 0.01 (94)	1.29 ± 0.01 (90)	1.39 ± 0.02 (75)	1.74 ± 0.04 (37)[a]
Indomethacin	1	1.34 ± 0.05 (71)[a]	1.43 ± 0.05 (62)[a]	1.45 ± 0.07 (66)[a]	1.41 ± 0.09 (75)[a]	1.69 ± 0.10 (46)[a]
Paramethasone	0.5	1.10 ± 0.01 (126)	1.17 ± 0.02 (102)	1.15 ± 0.02 (105)	1.18 ± 0.01 (100)	1.22 ± 0.06 (95)
Hydrocortisone	10	1.26 ± 0.02 (90)	1.29 ± 0.04 (88)	1.31 ± 0.04 (86)	1.44 ± 0.04 (68)[a]	1.56 ± 0.04 (59)[a]
Mercaptopurine	2	1.20 ± 0.03 (103)	1.21 ± 0.02 (100)	1.26 ± 0.03 (92)	1.34 ± 0.05 (80)	1.41 ± 0.03 (75)

[a] Mean foot volume is not significantly different from that of the adjuvant control group ($p > 0.05$).

slow recovery to preinjection weight by Day +12. Body weight then fluctuated within a narrow range during the remaining days of the study. These results are similar to those of Glenn *et al.* (11) and intermediate between the results of Pearson (12), who noted a normal weight gain, and of Newbould (1), who reported severe weight losses.

Considering the three compounds in Table III that provided consistent protection at Days +12, +21, and +30, and considering the patterns of body weight change documented in Table IV, cryogenine closely resembles hydrocortisone. Cryogenine partially prevented the acute loss of body weight noted in the adjuvant controls at Day +3 and thereafter the cryogenine-treated animals consistently gained weight, although at a slower rate than the untreated controls (mean gain of 21 g. by Day +30 as compared to 41 g. for the controls). Rats medicated with mercaptopurine progressively lost body weight through Day +9, and these animals still averaged 10 g. lighter than their starting weight by Day +30. Of the remaining partially protective compounds (Day +21 and/or Day +30) in Table III, the weight change pattern of the animals receiving paramethasone (Table IV) resembles that described for mercaptopurine. Such decreases in total body weight may contribute indirectly to the apparent "anti-inflammatory" effects recorded in Tables I and II but should not distort the arthritic index data in Table III. The weight change effects of mefenamic acid generally resembled the pattern described for cryogenine and hydrocortisone, while the pattern with phenylbutazone differed slightly in that the Day +3 weight loss seen with the adjuvant controls was generally blocked.

Table III—Drug Effects on the Arthritic Index in Rats with Adjuvant-Induced Polyarthritis

Treatment (Day −1 to Day +20)	Daily Oral Dose, mg./kg.	Mean Arthritic Index (Percent Inhibition) Day +12	Day +21	Day +30
Untreated control	—	0	0	0
Adjuvant control	—	3.3 (0)	6.5 (0)	10.2 (0)
Cryogenine	100	2.0 (39)[a]	3.2 (51)[a]	6.2 (39)[a]
Phenylbutazone	100	2.9 (12)	3.6 (45)[a]	6.7 (34)[a]
Mefenamic acid	25	2.4 (27)	4.0 (38)[a]	8.6 (16)
Indomethacin	1	3.1 (6)	4.7 (28)	7.3 (28)
Paramethasone	0.5	2.4 (27)	2.4 (63)[a]	3.6 (65)[a]
Hydrocortisone	10	1.7 (48)[a]	3.3 (49)[a]	3.9 (62)[a]
Mercaptopurine	2	2.3 (30)[a]	2.6 (60)[a]	4.9 (51)[a]

[a] Mean arthritic index is significantly different from that of the adjuvant control group ($p < 0.05$).

The adjuvant control animals resented handling, developed coarse ruffled fur, and began to vocalize and assume defensive fighting postures when paired with a fellow rat. Animals receiving cryogenine appeared quite healthy, displayed normal spontaneous motor activity, and did not show aggressive or defensive behavior patterns when paired with another rat. This tranquil attitude may be due to the weak ataractic activity documented for parenterally administered cryogenine (13) but is more likely due to the anti-inflammatory capacity of cryogenine providing relative freedom from the polyarthritic pain.

Serum Turbidity—As shown in Fig. 1, there was a significant ($p < 0.05$) decrease in serum turbidity in adjuvant control animals as compared to the untreated controls. While the immunosuppressive mercaptopurine was effective in all phases of adjuvant-induced polyarthritis studied (Days +12, +21, and +30, Table III), this effect was accompanied by severe losses in total body weight, and the serum turbidity in mercaptopurine-treated rats at Day +21 was not significantly different from the depressed levels documented for the adjuvant controls. Paramethasone, cryogenine, hydrocortisone, and phenylbutazone had a significant but intermediate degree of effect in preventing the adjuvant-induced decrease in serum turbidity, but the acute loss of body weight produced by paramethasone seems to place this compound in a different pharmacological category than the other three agents.

In studies with intact and adrenalectomized rats using both proliferative (cotton-pellet granuloma) and exudative (carrageenin-induced pedal edema) models of inflammation, DeCato (14) suggested that the anti-inflammatory efficacy of cryogenine may be due, in part, to the release of endogenous corticosteroids. This hypothesis is supported by the limited effectiveness of cryogenine (relative to phenylbutazone) in preventing UV-induced erythema in guinea pigs, a pharmacological model of inflammation that is

Table IV—Drug Effects on Whole Body Weight of Rats with Adjuvant-Induced Polyarthritis

Treatment (Day −1 to Day +20)	Daily Oral Dose, mg./kg.	Mean Body Weight, g. Day 0	Day +3	Day +6	Day +12	Day +18	Day +21	Day +24	Day +30
Untreated control	—	228	232[a]	234[a]	235[a]	253[a]	253[a]	258[a]	269[a]
Adjuvant control	—	215	204	209	217	213	216	212	220
Cryogenine	100	220	215[a]	217	222	229	234[a]	235[a]	241[a]
Phenylbutazone	100	212	210	216	221	218	222	224	231
Mefenamic acid	25	217	212	215	223	227	226	229	236
Indomethacin	1	216	206	205	214	216	219	224	230
Paramethasone	0.5	210	190[a]	186[a]	183[a]	191[a]	190[a]	194	202
Hydrocortisone	10	226	219[a]	218	221	228	230	236[a]	244[a]
Mercaptopurine	2	218	205	198	198[a]	201	199	200	208

[a] Significantly different from values of the adjuvant control group ($p < 0.05$).

unresponsive to corticosteroid-like agents (15). The results of the present study do not conflict with this theory and do establish that the anti-inflammatory activity of cryogenine probably is not due to a mercaptopurine-like immunosuppressive capacity.

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Structure–Side-Effect Sorting of Drugs II: Skin Sensitization

ERIC J. LIEN▲ and GEDY A. GUDAUSKAS*

Abstract □ A computerized sorting program was used to sort out 120 drugs that have been reported to cause skin sensitization from a data bank of 540 clinically useful drugs. Based on an analysis of functional groups, a number of unique common denominators were found for skin sensitization due to covalent bond formation with body proteins. These include potential epoxide-forming groups such as an electron-deficient benzene ring, a vinyl group, hydroxylamine and *N*-oxide precursors, drugs capable of forming a benzylic radical, β-lactam derivatives, and α,β-unsaturated ketones. By using multiple-regression analysis, the quantitative structure–activity relationship for skin-sensitizing properties of 3-alkyl catechols and 2,4-dinitrobenzene derivatives are also reported.

Keyphrases □ Side effects, computer sorting of 540 drugs—skin sensitization related to structure □ Computer sorting of side effects of 540 drugs—skin sensitization related to structure □ Structure–activity relationships—features related to skin sensitization effects, result of computer sorting of 540 drugs □ Skin sensitization effects—related to structural features, result of computer sorting of 540 drugs □ Drug sorting by side effects—structure–activity relationships

As a continuation and extension of an effort to sort and correlate side effects of drugs with their chemical structures (1), attention was focused on the problem of skin sensitization. Numerous drugs are known to cause dermatitis, urticaria, eczema, photosensitization, or other signs of skin sensitization following either systemic or topical applications. The main purpose of this study was to utilize a computerized sorting program to list as many as possible of the drugs known to cause various types of skin sensitization and to find out the common structural features. Attempts were made to categorize these drugs on the basis of functional group analysis and the types of possible chemical reactions with proteins. It is hoped that the findings will not only suggest working hypotheses for skin sensitization by various drugs but will also provide some rational chemical basis of skin sensitization. Quantitative structure–activity analysis was applied to a series of catechols and dinitrobenzene derivatives where quantitative data on series of compounds were available.

METHOD

The computerized sorting program was described previously (1). The following descriptive terms of signs were sorted out from the data bank: skin sensitization, lesions, necrosis, rashes, eruptions, reactions, irritations, opacities, dermatitis, photosensitivity, and urticaria. The results are given in Tables I and II.

The method of least squares was used to derive the equations correlating the physicochemical parameters with the delayed contact skin-sensitizing activity. The biological data and the physicochemical constants used are assembled in Table III.

RESULTS AND DISCUSSION

Skin-Sensitizing Drugs—A total of 120 drugs or drug groups from the data bank of 540 drugs have been reported to cause various types of skin sensitization (2–8). In analyzing the common structural features, many of these drugs were found to contain a benzene ring

unresponsive to corticosteroid-like agents (15). The results of the present study do not conflict with this theory and do establish that the anti-inflammatory activity of cryogenine probably is not due to a mercaptopurine-like immunosuppressive capacity.

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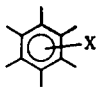
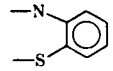
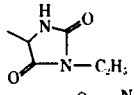
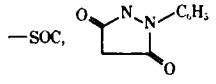
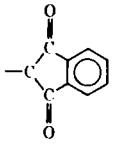
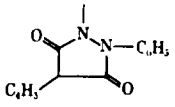
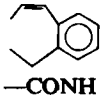
Table I—Skin-Sensitizing Drugs Possessing Aromatic Ring Bearing Electron-Withdrawing Groups



Drug	X	Drug	X
Benzonatate	—$COOCH_2$—	Chloroquine	—Cl, —N=C
Benztropine	—$OCHC_6H_5$	Chlorphenesin	—Cl, —OCH_2—
Butaperazine	—CO′ —S, N—C_6H_5	Clofibrate	—Cl, —OC
Brompheniramine	—Br	Cyproheptadine	—C=C, —C=C
Carbamazepine	—$NCONH_2$, C=C—C_6H_5	Diethylpropion	—C—C(=O)
Carphenazine	—C(=O)—C_2H_5	Diphemanil	—C(—C_6H_5)=
Chlorphentermine	—Cl	Diphenylhydantoin	—C(—C_6H_5)—C(=O)
Chlorprothixene	—Cl	Dipyrone	—N—C(=O)
Chlordiazepoxide	—Cl, —N=; —C(—C_6H_5)=N→O	Doxepin	—C=O, —O—
Chlormezanone	—Cl, —CSO_2	Ethopropazine	
Glycopyrrolate	—C(OH)(—COO)	Ethotoin	
Indomethacin		Sulfinpyrazone	
Methadone	—C(—C_6H_5)—C(=O)	Suramin	—CO, —SO_2Na
Phenindione		Mesoridazine	—SO—, —S—, —NC_6H_5
Phenothiazines	—S, —N—C_6H_5	Thiothixene	—C=, —S
Phenylbutazone		Triflupromazine	—CF_3, —S, —NC_6H_5
Propranolol			
Protriptyline			
Procarbazine	—CONH		

or other aromatic ring systems bearing electron-withdrawing groups (Table I). The electron-withdrawing groups may stabilize the epoxide formed by drug-metabolizing enzymes. It has been shown (9) that drug-metabolizing enzymes in the liver cause the formation of active epoxides from bromobenzene, *o*-dibromobenzene, *m*-dibromobenzene, and naphthalene (10). These epoxides may then alkylate body protein or cause tissue damage in liver, kidney, bone marrow, *etc.* (11, 12). Other compounds such as various isomers of octene (13), styrene (14), and chlordane (15) also have been shown to form epoxides *in vivo*. The carbon–carbon double bond in diethylstilbestrol, carbamazepine, and novobiocin may be responsible for the skin sensitization *via* epoxide formation.

The second group of drugs that has been reported to cause skin sensitization contain either a primary aromatic amine moiety with the *para*-position blocked or a tertiary aliphatic amino group (Table II). Compounds with the structure of X—C_6H_5—NH_2 form the highly reactive and toxic hydroxyamino and nitroso compounds (16–18), which may then react with proteins to form antigens. Schwarz and Speck (19) demonstrated that hydroxyamino derivatives of sulfanilamide play a role in photosensitivity. They were able to sensitize guinea pigs with these oxidation products of sulfanilamide and then to provoke a reaction by exposure to sulfanilamide and light and vice versa.

In contrast to the sulfanilamides, when patients known to be photoallergic to chlorothiazides were skin tested to the oxidation products 4-amino-6-chlorobenzenedisulfonamide and 4-hydroxyamino-6-chlorobenzenedisulfonamide, no positive reactions were elicited in patch and intracutaneous tests and in irradiated patch and intracutaneous tests with these compounds (20). Therefore, the exact mechanism by which drugs like chlorothiazides induce skin sensitization remains to be investigated. Since the positions *meta* and *para* to the chloro atom are blocked by the thiazide ring, the formation of an epoxide is not likely.

The nitro group in chloramphenicol is also known to undergo reduction to give an amino group (21). This minor metabolic path-

Table II—Skin-Sensitizing Drugs Possessing Amino Group

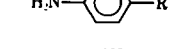
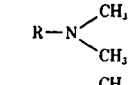
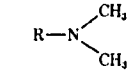
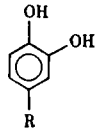
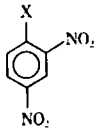
Drug	Functional Group
Sulfonamides	
Phenothiazines	
Imipramine	

Table III—Biological Data and Physicochemical Constants of Catechols and Dinitrobenzenes Causing Delayed Contact Sensitivity in Guinea Pigs



3-Alkyl Catechols

log $1/C$[a]	log P[b] (Octanol–Water)	R
4.89	0.88	H
5.35	1.38	CH_3
5.77	2.38	n-C_3H_7
6.01	3.38	n-C_5H_{11}
6.72	4.88	n-C_8H_{17}
7.37	6.38	n-$C_{11}H_{23}$
6.89	7.38	n-$C_{12}H_{27}$
7.27	8.38	n-$C_{15}H_{31}$
7.42	9.38	n-$C_{17}H_{35}$
7.46	10.38	n-$C_{19}H_{39}$



2,4-Dinitrobenzene Derivatives

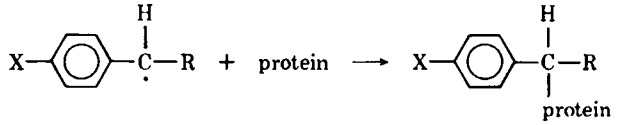
log $1/C$[c]	log P[d] (Isooctane –Water)	log P[e] (Octanol–Water)	V_x[f]	log R[g]	X
8.91	0.66	2.58	42.7	1.78	—SCN
8.72	1.63	2.41	37.6	1.00	—I
8.62	1.15	2.24	27.6	0.86	—Br
8.32	1.26	2.08	21.3	0.97	—Cl
8.17	0.51	1.50	8.54	0.15	—F
7.83	−0.48	—	—	—	—SO_2Cl
7.32	−2.30	−2.89	—	1.89	—SO_3H

[a] C = the geometric mean dose (moles) for threshold skin reaction 4 weeks after the animals received the last sensitizing injection; taken from Table I of *Reference 30*. [b] Based upon the experimental value of 0.88 for pyrocatechol and use of π = 0.50 for each CH_3 or CH_2 unit (36). [c] C = the geometric mean dose (moles) for threshold skin reaction (homologous) 47 days after sensitization; taken from Table I of *Reference 33*. [d] Experimental values from *Reference 33*. [e] Calculated by taking advantage of the additive–constitutive nature of the Hansch π constant. The following π values are used: 1,3-dinitrophenyl = 1.49; SCN = 1.01; I = 0.92; Br = 0.75; Cl = 0.59; F = 0.01; and SO_3H = −4.38; taken from *Reference 35*, phenoxyacetic acid system, and *Reference 36*. [f] Volume in Å^3 from *Reference 33*. [g] R = reactivity coefficient with bovine serum albumin (BSA), R = (hapten)/(DNP–BSA) − 1, where DNP = dinitrophenol. For the experimental procedure, see *Reference 33*.

way may be responsible for the skin sensitization. Another possible way of forming a covalent bond between chloramphenicol and a protein would be *via* a benzylic free radical, as suggested by Hansch and Kerley (22) (*vide infra*).

The third group of drugs contains substituents which may stabilize a benzylic radical (22). This group includes drugs such as chloramphenicol and quinine (Scheme I).

It is also known that various aliphatic tertiary amino compounds form *N*-oxides in many biological systems (Scheme II) (16, 24, 26). Since these *N*-oxides can be isolated fairly easily, they probably are not as reactive as *N*-nitroso or *N*-hydroxyamine derivatives. Nevertheless, it has been reported that in several cases *N*-oxides are more active than their corresponding tertiary amines (27).



Scheme I

Table IV—Equations Correlating Skin-Sensitizing Properties with Physicochemical Constants

	n[a]	r[b]	s[c]	Eq.
3-Alkyl Catechols				
$\log 1/C = 0.262 \log P + 5.080$	10	0.946	0.326	1
$\log 1/C = -0.030 (\log P)^2 + 0.591 \log P + 4.488$	10	0.983	0.169	2
$\log P_o = 17.96 (14.44 - 31.81)$[d]				
2,4-Dinitrobenzene Derivatives				
$\log 1/C = 0.146 \log P_{(\text{isooctane-water})} + 8.396$	5	0.233	0.338	3
$\log 1/C = 0.448 \log R + 8.121$	5	0.864	0.175	4
$\log 1/C = 0.257 \log P + 8.004$	6	0.946	0.206	5
$\log 1/C = 0.022 V_x + 7.951$	5	0.975	0.077	6

[a] n = number of data points (compounds used in the regression analysis). [b] r = correlation coefficient. [c] s = standard deviation. [d] 95% confidence interval.

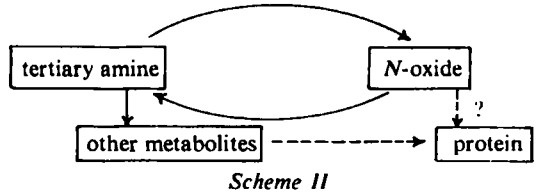
Some *N*-oxides also have been postulated as inducers of spontaneous cancer. These *N*-oxides may also be important intermediates in drug metabolism (27).

The fourth group of drugs causing skin sensitization and/or anaphylactic shock contains drugs with a β-lactam moiety. The four-membered β-lactam ring may open up and form a new peptide linkage with body protein (Scheme III) (28). All of the penicillins and cephalosporins belong to this group.

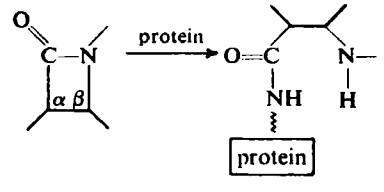
The fifth group of drugs which can form covalent bonds with proteins and cause skin sensitization includes those possessing an α,β-unsaturated ketone function. The examples found from the data bank are cortisone, digoxin, griseofulvin, and spironolactone. These drugs can conceivably undergo nucleophilic attack by any nucleophiles ($\ddot{N}u$) such as —SH, —O^-, and —$\ddot{N}H_2$ (Scheme IV) (29).

The sixth group of skin-sensitizing drugs contains polyiodinated benzene derivatives, *e.g.*, ipodate, iothalamic acid, and diiodohydroxyquin. Polyiodinated benzene derivatives are readily deiodinated *in vivo* (30, 31). Although the exact mechanism is not known, this process possibly may go through a free radical intermediate. The iodine radical, the phenyl radical, or the iodide ion produced may act as a hapten and react with protein.

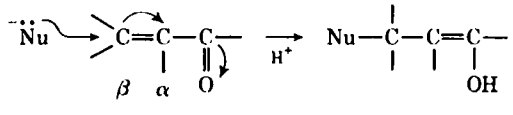
Miscellaneous Compounds—Drugs like kanamycin and paromomycin contain primary amino groups and sugar moieties. It



Scheme II



Scheme III



Scheme IV

does not appear likely that these molecules would form covalent bonds with body protein. Whether the skin sensitization caused by these drugs is due to the trace amount of fermentation by-products by *Streptomyces* or not remains to be studied.

Other drugs like phenacemide, phenacetin, phenelzine, and barbiturates are also known to cause sensitization. No well-established biotransformations or chemical reactions are available to explain the coupling between these compounds and proteins. It is possible that small amounts of reactive metabolites may be responsible for the skin sensitization reported. Identification of these metabolites will be essential for the establishment of the mechanism.

Quantitative Structure–Activity Relationship—The equations correlating the skin-sensitizing properties with the physicochemical constants are summarized in Table IV. The biological data are from Baer *et al.* (32) and Godfrey and Baer (33). It has been suggested that the mechanism of sensitization by compounds of the catechol type results from conversion to a quinone, which then spontaneously couples to protein (34). Equations 1 and 2 clearly indicate that the major variation in the skin-sensitization activity of the 3-alkyl catechol is primarily due to the lipophilic character (log P) or the length of the side chain, as indicated by the log P from octanol–water. However, because all of the substituents are straight-chain alkyl groups, there is a strong correlation between the log P and the chain length. A better selection of substituents with different steric and lipophilic character may delineate which parameter is more important in contributing to the skin sensitization. The $(\log P)^2$ is significant at the 99 percentile level as indicated by an F-test ($F_{1,7} = 15.05$, $F_{1,7,99} = 12.2$). About 97% of the variance ($r^2 = 0.97$) in the data can be accounted for by the parabolic equation of log P (Eq. 2). The optimum lipophilic character for maximum sensitization is defined by log P_0 (37, 38). The log P_0 of 17.96 from Eq. 2 is considerably higher than that of pentadecylcatechol (log $P = 8.38$), the active principle of poison ivy. The fairly wide 95% confidence interval of log P_0 is primarily due to the fact that the highest log P in the series is only 10.38. Nevertheless, the statistical analysis points to the deviation from linearity.

For the 2,4-dinitrobenzene derivatives, Eqs. 3–6 are obtained. Among the parameters examined, the molar volume of the substituent (V_x) gives a slightly better correlation than log P (octanol–water) or the reactivity coefficient (R) with bovine serum albumin. Pure hydrocarbon is known to yield less satisfactory correlation with biological activity than the solvents that are capable of forming hydrogen bonds. This becomes very evident when one compares Eqs. 3 and 5. The log P of 2,4-dinitrobenzenesulfonyl chloride in octanol–water is not available because it reacts with alcohols, and that of 2,4-dinitrobenzenesulfonic acid in isooctane–water is not available because of the very low solubility in the hydrocarbon. As a consequence, six data points are included in Eq. 5, but only five data points are in Eqs. 3 and 6. It is difficult to assess whether Eq. 6 is really better than Eq. 5. Intercorrelation between the volume of the substituent (V_x) and log P_{octanol} is also apparent ($r = 0.98$). The analysis is in agreement with the original authors' conclusion (33) regarding the importance of the steric nature of the substituent. The results also show that if a proper solvent system is chosen, one can get practically the same correlation by using log P instead of the steric parameter. A better selection of substituents with no interdependence between log P and V_x will be necessary to evaluate the contribution of these two parameters to skin sensitization.

Since the phototoxic reaction does not involve the conjugation of hapten with protein (39, 40), it is not included in this study. For the general discussion of allergic drug reactions, a recent article by MacFarlane (41) is available.

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GLC Determination of Probenecid in Biological Fluids

ANTHONY G. ZACCHEI▲ and LINDA WEIDNER

Abstract □ A highly specific and quantitative GLC method for the determination of probenecid in biological fluids is described. The method is adaptable to automation with an automatic sample injector and a peak area integrator. The procedure involves the addition of an internal standard (*N*-dibutyl analog of probenecid) to plasma or urine samples followed by extraction at pH 1 into benzene. The extracted acids are converted to methyl esters for GLC analysis by reaction with ethereal diazomethane. The sensitivity of the method is such that 250 ng. of material can be detected in 1 ml. of plasma. GLC–mass spectrometry techniques confirmed that the developed method measures the parent drug probenecid.

Keyphrases □ Probenecid in biological fluids—GLC analysis □ GLC—analysis, probenecid in biological fluids

The use of the uricosuric agent, probenecid (I), for the treatment of gout and for the inhibition of the active transport of many substances necessitated the development of a rapid and sensitive quantitative method for the detection of I. Various spectrophotometric methods (1–3) have been reported; however, these methods lack the required specificity and sensitivity. A recently described GLC method (4) has several disadvantages; specifically, no internal standard is employed and the sensitivity is about 2 mcg./ml.

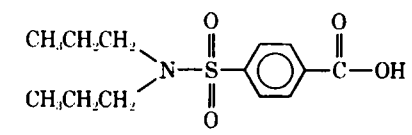
This report describes a very sensitive and specific GLC method for probenecid determinations, under conditions where only probenecid was administered, using an internal standard (*N*-dibutyl analog of probenecid, II). The method has the added advantage of being adaptable to automation.

EXPERIMENTAL

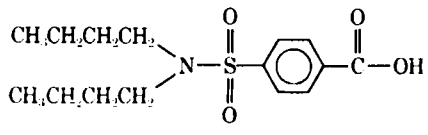
Reagents and Chemicals—The chemicals and reagents used were: probenecid, the *N*-dibutyl analog of probenecid, benzene, methylene chloride, and 0.5 *M* diazomethane (generated from *N*-nitroso-*N*-methylurea).

Instrumentation—*GLC*—A gas chromatograph[1] equipped with a flame-ionization detector and a 183 × 6-mm. glass column containing 1% OV-17 (phenyl methyl silicone gum) on Supelcoport, 90–100 mesh, was used. The column, detector, and injection port temperatures were 225, 240, and 245°, respectively. Helium was used as the carrier gas at a flow rate of 48–50 ml./min.

A gas chromatograph[2] equipped with a flame-ionization detector and a 183 × 6-mm. glass column containing 1% OV-17 on Supelcoport, 80–100 mesh, was employed for the automated GLC analysis. The column, detector, and injection port temperatures were 209, 276, and 265°, respectively. Helium was used as a carrier gas at a flow rate of 50 ml./min. Injections were performed automatically[3]. Peak areas were calculated automatically by an integrator[4].



I



II

GLC–Mass Spectrometry—All mass spectra were obtained on a mass spectrometer[5] using the GLC inlet with a 183 × 6-mm. glass column packed with 1% OV-17 on Supelcoport, 80–100 mesh. The gas chromatograph was operated isothermally at 230° with a helium flow rate of 30 ml./min. The mass spectrometry ionizing and accelerating potentials were 70 ev. and 3.5 kv., respectively. The source and injection port temperatures were both 270°.

Measurement of I in Biological Samples—Compound I was determined in biological samples as follows. One milliliter of plasma or 3.0 ml. of urine was added to a 50-ml. glass-stoppered centrifuge tube containing 15 mcg. of internal standard (in 1.0 ml. of phosphate buffer, pH 7), 1 ml. of 2 *N* hydrochloric acid, and 25 ml. of benzene. The tube was shaken for 10 min. and centrifuged, and at least 20 ml of the organic phase was transferred to a similar tube containing 2 ml. of 0.1 *N* sodium hydroxide. After shaking for 5 min., the tube was centrifuged and the organic phase was removed by aspiration. The pH of the aqueous phase was adjusted to 1 by the addition of 0.2 ml. of 2 *N* HCl, and the free acids were extracted into 5 ml. of methylene chloride (Vortex, 1 min.). After centrifuga-

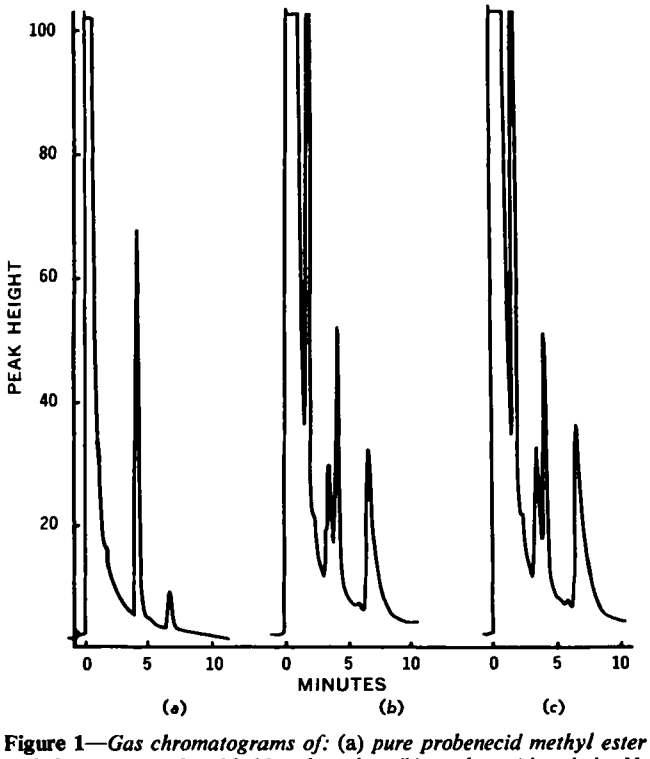


Figure 1—*Gas chromatograms of:* (a) *pure probenecid methyl ester and the corresponding* N*-dibutyl analog,* (b) *probenecid and the N-dibutyl analog added to control plasma and carried through the GLC method, and* (c) *material obtained from biological specimens after the addition of the internal standard. Five microliters was injected out of a total volume of 100 μl. of ethyl acetate.*

[1] Packard model 7400.
[2] Hewlett-Packard model 5750.
[3] With a Hewlett-Packard 7670A automatic sampler equipped with a Hamilton 7005 5-μl. syringe set for maximum injection.
[4] Hewlett-Packard 3370B.
[5] LKB-9000S.

Table I—Recovery of Probenecid from Plasma

Amount Added, mcg.		Amount Recovered[a]						Mean ± *SD*
60.0	A	62.1	61.3	59.2	59.3	61.1	61.7	60.78 ± 1.24
	B	103	102	99	99	102	103	
45.0	A	44.8	44.5	45.5	46.1	45.6	44.7	45.20 ± 0.63
	B	99	99	101	102	101	99	
30.0	A	29.9	28.9	31.0	29.6	29.0	29.9	29.72 ± 0.76
	B	100	97	103	99	97	100	
15.0	A	15.0	15.4	15.4	15.1	15.0	15.2	15.18 ± 0.18
	B	100	102	102	101	100	101	
7.5	A	7.25	7.17	7.69	7.38	7.24	7.36	7.34 ± 0.18
	B	97	96	103	98	97	98	
5.0	A	4.83	5.20	4.82	5.28	4.68	5.05	4.98 ± 0.24
	B	97	104	96	106	94	101	
1.0	A	0.95	1.10	0.91	0.89			0.96 ± 0.09
	B	95	110	91	89			
0.5	A	0.55	0.53	0.46	0.48			0.51 ± 0.04
	B	110	106	92	96			
0.25	A	0.30	0.21	0.27	0.27	0.30		0.27 ± 0.03
	B	120	84	108	108	120		

[a] Values in the A rows represent micrograms recovered; values in the B rows represent percent recovery.

tion, the aqueous phase was removed by aspiration and the methylene chloride phase was transferred to a 13-ml. conical centrifuge tube and then treated with 100 μl. of an ethereal solution of diazomethane. After 5 min., the tube was placed in a warm water bath in a hood; all solvent and unreacted reagent were allowed to evaporate under nitrogen. The residue was dissolved in 100 μl. of ethyl acetate and appropriate aliquots, usually 5 μl., were injected into the gas chromatographs. The retention times of the methyl esters of I and the *N*-dibutyl internal standard (II) were 4.6 and 6.6 min., respectively.

Standard water, urine, and plasma curves were constructed by plotting the peak height ratios (I/II) or peak area ratios (I/II) *versus* weight ratios (I/II). The peak height ratio obtained from an unknown was then used to determine the amount of I present. The standard samples were run simultaneously with the unknown samples as previously described. Recoveries of I added to plasma or urine in amounts of 0.2–60 mcg. ranged from 84 to 120% for analyses performed over several months. The mean recovery was 100.55 ± 6.37% over the entire concentration range.

Absorption and Excretion Studies—Drug (I), in capsule form or in saline solution as the sodium salt, was administered to purebred beagle dogs of both sexes. Drug (I) in gelatin capsules was administered to normal human subjects. Probenecid was administered to the dogs at a dose of 19 mg./kg. as the free drug or in combination with pivampicillin (33 mg./kg.) and ampicillin (23.3 mg./kg.). Similar dosage formulations were administered to human subjects.

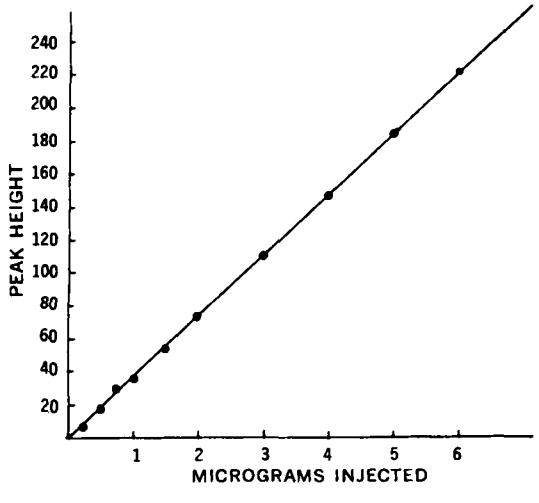


Figure 2—*Relationship between peak height and amount injected. Five microliters was injected in each case.*

Animals and human subjects were fasted prior to drug administration. Urine was collected from animals housed in individual metabolism cages and was frozen as collected in dry ice-cooled containers. Blood was collected in heparinized syringes, and the plasma was separated by centrifugation and frozen until assayed. The plasma and urine samples were analyzed as previously described.

RESULTS AND DISCUSSION

Figure 1 presents gas chromatograms of the following samples: (*a*) pure probenecid methyl ester and the corresponding *N*-dibutyl analog, (*b*) probenecid and the *N*-dibutyl analog added to control

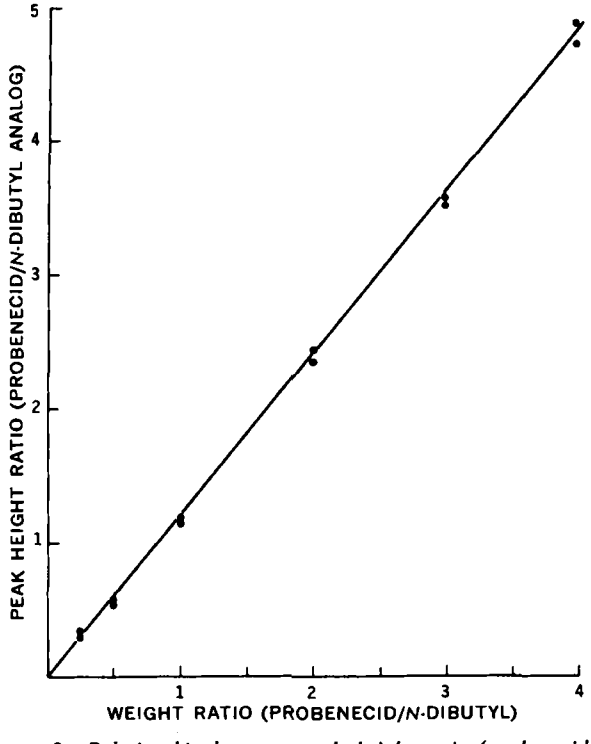


Figure 3—*Relationship between peak height ratio (probenecid/N-dibutyl analog) and weight ratio (probenecid/N-dibutyl analog). Five microliters was injected in each case out of 100 μl. following appropriate recovery (water, plasma, or urine).*

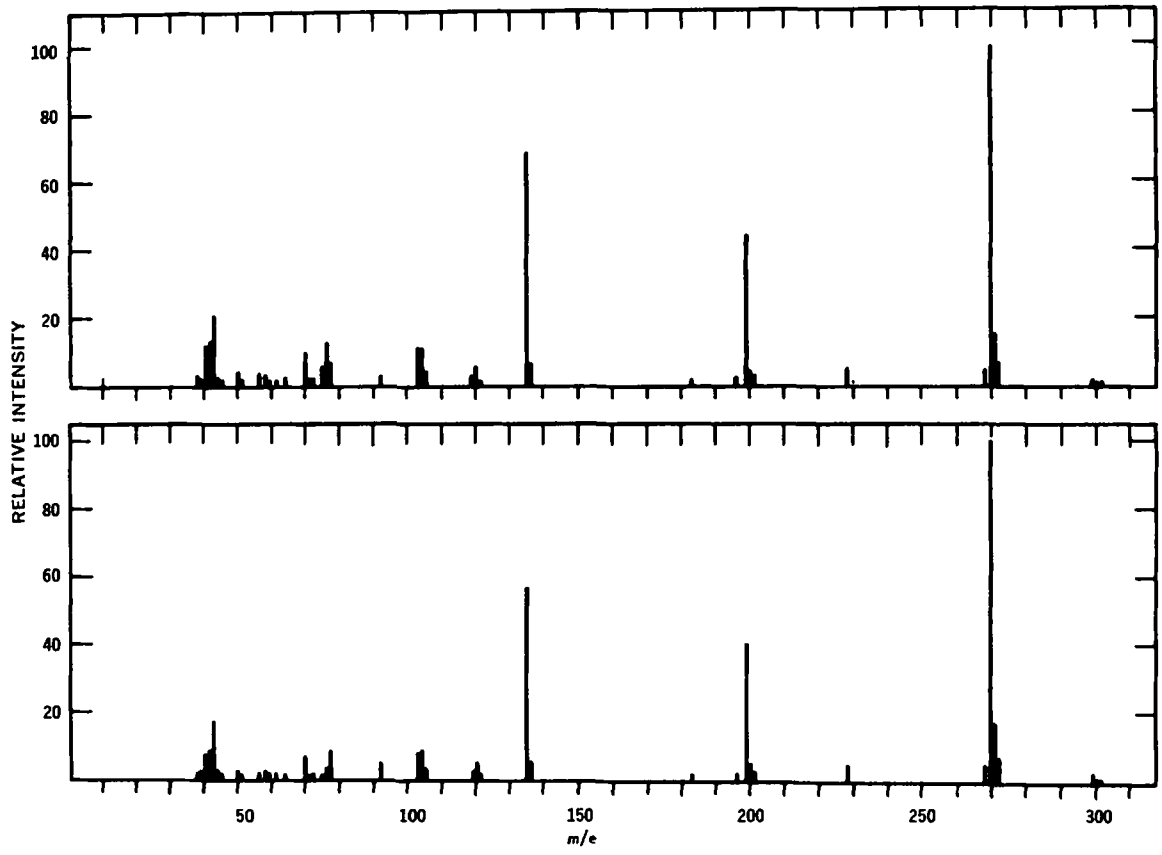


Figure 4—*Mass spectra for probenecid methyl ester (top) and the material obtained from dog plasma (bottom).*

plasma and carried through the GLC procedure, and (*c*) material obtained from biological specimens after the addition of the *N*-dibutyl internal standard. As stated previously, the methyl ester of probenecid had a retention time of 4.6 min. and the corresponding internal standard had a retention time of 6.6 min. Gas chromatograms of control samples did not exhibit any peaks with retention similar to probenecid methyl ester or the methyl ester of the internal standard. A linear relationship is observed when peak height (or peak area) is plotted *versus* amount injected (Fig. 2). A typical standard peak height ratio (I/II) *versus* weight ratio (I/II) is presented in Fig. 3. The slope of the straight line is 1.20. Values for the slope ranged from 1.03 to 1.25, depending upon specific analysis over 6 months. A summary of the recovery results of I obtained with dog plasma in the range of 0.2–60 mcg. added to the initial tube is presented in Table I. As evident, the GLC procedure for probenecid in biological fluids is very sensitive and exhibits a high degree of precision and accuracy.

Table II—Plasma Levels of Probenecid following Administration of Probenecid to Dogs (Intravenous or Oral, 19 mg./kg.)

Hours	mcg. Probenecid/ml. Plasma[a]					
	A		B		C	
0.08	74.4	108.6	77.4	102.9	0.73	0.6
0.25	69.6	95.7	70.8	82.8	10.9	8.4
0.50	57.3	81.6	67.5	85.5	24.3	18.4
1.0	53.3	81.0	54.0	74.1	27.5	18.3
2.0	44.2	69.0	46.5	63.8	32.7	18.8
3.0	39.3	59.7	40.5	56.5	29.3	63.0
4.0	34.1	54.0	39.0	46.6	24.3	62.7
6.0	28.5	45.0	30.7	42.5	23.2	53.2
8.0	22.8	38.3	29.3	39.3	21.8	45.6
24.0	3.0	10.5	3.7	12.3	3.6	13.9
$t_{1/2}$	5.5	8.0	6.0	9.8	7.6	9.4

[a] Group A, dogs received probenecid intravenously, 19 mg./kg.; Group B, dogs received probenecid intravenously, 19 mg./kg., and ampicillin intravenously, 23.3 mg./kg.; and Group C, dogs received probenecid orally, 19 mg./kg., and pivampicillin orally, 33.3 mg./kg.

This GLC method was applied to numerous samples of biological origin, specifically with respect to dog and human urine and plasma. The samples analyzed also contained ampicillin and pivampicillin, neither of which had any effect on the GLC assay of probenecid. Typical probenecid levels in dog plasma are seen in Table II.

Confirmation of specificity of analysis was obtained when representative unknown biological specimens were analyzed by combined GLC–mass spectrometry using the technique previously described. Figure 4 presents a comparison of the mass spectrum of the synthetic methyl ester of probenecid with the mass spectrum of the material isolated from biological origin following oral administration of drug (I). The details of the fragmentation pattern of probenecid were reported previously (4–6); the mass spectra were identical in all respects. The molecular ion (*m/e* 299) represents ~2% of the intensity of the base peak (*m/e* 270), which results from cleavage of the side chain beta to the nitrogen. The characteristic metastable ions at 192.6, 173.8, and 146.8 were observed.

CONCLUSION

A sensitive, specific, and accurate GLC method for the determination of probenecid in biological fluids is presented. The method is adaptable to automation with the use of an automatic sample injector and a peak area integrator. The use of an internal standard eliminates any errors in quantitation as a result of sample manipulation (7). The sensitivity of the method is such that 250 ng. of material can be detected in 1 ml. of plasma. No interference in the GLC method was observed with ampicillin or pivampicillin. GLC–mass spectrometry results from various analyzed samples confirmed the specificity and identity of probenecid.

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5-Fluorouracil and Derivatives in Cancer Chemotherapy III: *In Vivo* Enhancement of Antitumor Activity of 5-Fluorouracil (FU) and 5-Fluoro-2′-deoxyuridine (FUDR)

JOHNSON JATO* and J. J. WINDHEUSER▲

Abstract □ The antitumor activity of 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FUDR) has been enhanced in mice in the presence of deoxyribose donors such as 2′-deoxyuridine (UDR). Based upon *in vitro* data, it appears that the enhancement is due to the conversion of 5-fluorouracil to 5-fluoro-2′-deoxyuridine in blood and the stabilization of 5-fluoro-2′-deoxyuridine due to the presence of 2′-deoxyuridine.

Keyphrases □ 5-Fluorouracil (FU)—antitumor activity enhanced by deoxyribose donors (2′-deoxyuridine), mice □ 5-Fluoro-2′-deoxyuridine (FUDR)—antitumor activity enhanced by deoxyribose donors (2′-deoxyuridine), mice □ Antitumor activity, 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FUDR)—enhanced in presence of deoxyribose donors (2′-deoxyuridine), mice □ Cancer chemotherapy—enhancement of 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FUDR) activity by deoxyribose donors (2′-deoxyuridine), mice

A previous article (1) reported the *in vivo* stabilization of 5-fluoro-2′-deoxyuridine (FUDR) and its formation from 5-fluorouracil (FU) in the presence of nucleosides. The speculation was made that these findings could have marked effects on the clinical use of 5-fluorouracil and 5-fluoro-2′-deoxyuridine in cancer chemotherapy. Following the synthesis of 5-fluoro-2′-deoxyuridine (2), it was shown that the nucleoside was 10^3 times as effective as 5-fluorouracil *in vitro* (3). However, the relatively high anticipated effectiveness of 5-fluoro-2′-deoxyuridine as compared to 5-fluorouracil has not been borne out in clinical use. It was suggested (4) that the lower activity observed was due to the *in vivo* cleavage of 5-fluoro-2′-deoxyuridine to the pyrimidine base and the phosphorylated sugar moiety. The stabilization of 5-fluoro-2′-deoxyuridine by 2′-deoxyuridine (UDR) in blood (1) led to the speculation that this approach could lead to a marked clinical enhancement of 5-fluoro-2′-deoxyuridine activity. This article reports the effect of combining 5-fluorouracil and 5-fluoro-2′-deoxyuridine with 2′-deoxyuridine in protecting mice against L-1210 leukemia and adenocarcinoma 755.

EXPERIMENTAL

Materials and Instruments—The following were used: 5-fluorouracil[1], 5-fluoro-2′-deoxyuridine[1], 2′-deoxyuridine[2], deoxycytidine[2] (CDR), and 5-trifluoromethyl-2′-deoxyuridine[3] (F_3TDR). All other substances used were either reagent grade or of the highest purity available.

A spectrophotofluorometer[4], a TLC scanner[5] modified to contain pendrive motor and range offset, and an *x-y* recorder were used.

***In Vitro* Analysis of 5-Fluorouracil and 5-Fluoro-2′-deoxyuridine**—Drugs and additives in known initial concentrations in blood were dialyzed and evaporated by the method of Windheuser *et al.* (5). The dry samples were dissolved in a constant volume of ethanol. The samples were applied to a fluorescent TLC plate, which had previously been activated at 110° and ruled to restrict the lateral motion of the spots to within the range of the scanner optic. The components were resolved by multiple-pass discontinuous development (6). The developer for the process was composed of ethyl acetate–ethanol–10% ammonium hydroxide (200:5:2). The dried plates were scanned in the direction of development for fluorescence quenching at an activation maximum of 280 nm. and a fluorescence maximum of 530 nm., using a spectrophotofluorometer[4] with a thin-film scanner attachment. Quantities of substances present in the blood were computed by appropriate comparison of sample and standard peak areas obtained by planimetry. The method exhibited sufficient sensitivity to detect 10 ng. of 5-fluorouracil and 5-fluoro-2′-deoxyuridine.

***In Vivo* Mouse Studies**—*L-1210 489 Leukemia*—A standardized suspension prepared from 7-day-old tumors from BDF_1 mice was injected intraperitoneally. The volume was adjusted to provide 1×10^6 cells/injection. Twenty-four hours after the transplant, the mice were divided into groups of 10 and each group was injected intraperitoneally for 7 days with one of the drug regimens listed in Table I.

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Table I—Effect of Various Drug Regimens on Lifespan of Mice with L-1210 Leukemia

Drug Regimen, mg./kg./day	Average Life-span, days	T/C	Increase in Life-span, %[a]
Control	10		
2′-Deoxyuridine			
200	9.9	0.99	−1
5-Fluorouracil			
10	12.0	1.20	20
12.5	10.9	1.09	9
20	14.9	1.49	49
25	14.1	1.41	41
5-Fluorouracil + 2′-deoxyuridine			
5-Fluorouracil, 10 / 2′-Deoxyuridine, 200	15.3	1.53	53
5-Fluorouracil, 12.5 / 2′-Deoxyuridine, 200	13.6	1.36	36
5-Fluoro-2′-deoxyuridine			
35	10.2	1.02	2
45	11.5	1.15	15
5-Fluoro-2′-deoxyuridine + 2′-deoxyuridine			
5-Fluoro-2′-deoxyuridine, 35 / 2′-Deoxyuridine, 200	11.8	1.18	18
5-Fluoro-2′-deoxyuridine, 45 / 2′-Deoxyuridine, 200	13.3	1.33	33

[a] Each increase in lifespan value represents the average for 10 mice.

were placed in ice-cold saline and cut into pieces about 2 mm. in diameter. Two pieces were transplanted bilaterally subcutaneously on the dorsal side of each BDF_1 mouse by means of a trochar.

Twenty-four hours after transplant, the mice were divided into groups of 10. Each group received, by intraperitoneal injections for 7 days, one of the drug regimens listed in Table II. These regimens were the same as those used for the L-1210 experiment, modified where necessary to minimize any observed toxic manifestations.

Twelve days after transplant, the diameter of each tumor was measured in two directions and the mean tumor radius for each group was calculated. The corresponding tumor volume was calculated, assuming a spherical shape. The volumes thus obtained for each experimental group were divided by the corresponding volumes for the control group to obtain the T/C. The percent inhibition of tumor growth was given by Eq. 1:

$$\text{percent inhibition} = 100\left(1 - \frac{T}{C}\right) \qquad \text{(Eq. 1)}$$

Table II—Inhibition of Growth of Adenocarcinoma 755 in Mice by 5-Fluorouracil, 5-Fluoro-2′-deoxyuridine, and Their Combinations with 2-Deoxyuridine

Drug Regimen, mg./kg./day	Mean Tumor Volume, mm.[3]	Mean Weight Change, g.	T/C[a]	Tumor Inhibition, %
Control	1433	+0.03	1	0
2′-Deoxyuridine				
200	741	−0.37	0.52	48
5-Fluorouracil				
10	1161	−0.20	0.81	19
20	290	−3.17	0.20	80
5-Fluorouracil + 2′-deoxyuridine				
5-Fluorouracil, 10 / 2′-Deoxyuridine, 200	128	−3.57	0.09	91
5-Fluoro-2′-deoxyuridine				
40	692	−1.87	0.48	52
5-Fluoro-2′-deoxyuridine + 2′-deoxyuridine				
5-Fluoro-2′-deoxyuridine, 40 / 2′-Deoxyuridine, 200	17	−4.13	0.01	99

[a] Each T/C value is mean for 30 mice.

Table III—Effect of 5-Trifluoromethyl-2′-deoxyuridine on Recovery of 5-Fluoro-2′-deoxyuridine from Spiked Blood after 24-hr. Dialysis

5-Fluoro-2′-deoxyuridine Added, *M*	5-Trifluoromethyl-2′-deoxyuridine Added, *M*	5-Fluoro-2′-deoxyuridine Found, %
5.1×10^{-4}	0	37.1
5.1×10^{-4}	2.5×10^{-4}	49.5
5.1×10^{-4}	1×10^{-3}	87.3
5.1×10^{-4}	2.5×10^{-3}	90.4

Table IV—Effect of 5-Trifluoromethyl-2′-deoxyuridine on 5-Fluoro-2′-deoxyuridine Cleavage in the Presence of 5-Fluorouracil

Drug	Concentration in Blood before Dialysis, *M*	Concentration in Blood after Dialysis, *M*	Drug Found, %
5-Fluoro-2′-deoxyuridine	8.29×10^{-5}	1.73×10^{-4}	208.7
5-Fluorouracil	3.84×10^{-4}	7.60×10^{-6}	2.0
5-Trifluoromethyl-2′-deoxyuridine	2.03×10^{-3}	—	—

The percent inhibition is a measure of the effectiveness of the drug regimen.

RESULTS AND DISCUSSION

***In Vitro* Blood Studies**—During efforts to develop analytical procedures for the determination of 5-fluoro-2′-deoxyuridine *in vivo*, using the dialysis method previously reported (5) for 5-fluorouracil, it was found that when 5-fluoro-2′-deoxyuridine was added to whole human blood samples only about 37% was recovered as 5-fluoro-2′-deoxyuridine after 24 hr. Attempts to increase recovery of 5-fluoro-2′-deoxyuridine by enzyme deactivation procedures, such as pH adjustment, protein precipitation, and increased temperature, failed. A survey of the literature indicated that the cleavage was thought to be mediated by nucleoside phosphorylase (7) and that inhibitors such as 5-trifluoromethyl-2′-deoxyuridine might be of some value.

Preliminary data indicated that the addition of 5-trifluoromethyl-2′-deoxyuridine did enhance the recovery of 5-fluoro-2′-deoxyuridine from "spiked" blood (Table III). Although the recovery was not quantitative, it is readily apparent that increased 5-trifluoromethyl-2′-deoxyuridine concentration appeared to reduce 5-fluoro-2′-deoxyuridine cleavage.

Since one would expect the presence of both 5-fluorouracil and 5-fluoro-2′-deoxyuridine in actual clinical samples, experiments were carried out with spiked blood containing 5-fluorouracil, 5-fluoro-2′-deoxyuridine, and 5-trifluoromethyl-2′-deoxyuridine. The results (Table IV) were startling in that 5-fluorouracil recoveries, which previously had been shown to be 95%, were very low and the 5-fluoro-2′-deoxyuridine concentration was increased to more than 208% of that initially added. These findings indicate that 5-trifluoromethyl-2′-deoxyuridine not only inhibits 5-fluoro-2′-deoxyuridine cleavage but is involved in a deoxyribosyl transfer giving rise to the reaction shown in Scheme I. This is, undoubtedly, an oversimplification of a complex enzyme-mediated reaction since a repetition in pH 7.4 buffered saline failed to show any conversion of 5-fluorouracil to 5-fluoro-2′-deoxyuridine in the presence of 5-trifluoromethyl-2′-deoxyuridine.

These findings led to the consideration that the mechanism of action of so-called nucleoside phosphorylase inhibitors actually functioned in part by reversal of the cleavage equilibrium. It was further rationalized that compounds of similar structures such as 2′-deoxyuridine and deoxycytidine should function in an analogous manner in blood. The effect of 5-trifluoromethyl-2′-deoxyuridine, 2′-deoxyuridine, and deoxycytidine on 5-fluoro-2′-deoxyuridine

$$FU + F_3TDR \rightleftharpoons FUDR + F_3T$$

Scheme I

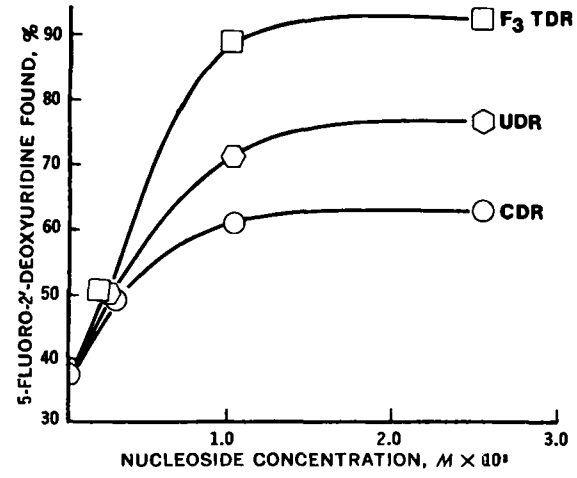

Figure 1—*5-Fluoro-2'-deoxyuridine found in blood in the presence of various concentrations of added nucleosides after 24-hr. dialysis. (Initial 5-fluoro-2'-deoxyuridine concentration was 5.1 × 10⁻⁴ M.)*

cleavage is summarized in Fig. 1. In all three cases, the apparent cleavage of 5-fluoro-2'-deoxyuridine was reduced as nucleoside concentration increased. A similar experiment was conducted to determine the interaction of 5-fluorouracil with 5-trifluoromethyl-2'-deoxyuridine, 2'-deoxyuridine, and deoxycytidine to form 5-fluoro-2'-deoxyuridine. The results (Fig. 2) clearly show that in all cases there was significant conversion of 5-fluorouracil to 5-fluoro-2'-deoxyuridine. The sugar moiety alone, deoxyribose, did not react with 5-fluorouracil.

The importance of these interactions to the clinical use of 5-fluorouracil and 5-fluoro-2'-deoxyuridine is immediately apparent.

***In Vivo* Mouse Studies**—The preliminary *in vitro* studies raised the question that, if the reduced activity of 5-fluoro-2'-deoxyuridine was truly due to *in vivo* cleavage to 5-fluorouracil and deoxyribose, could this reaction be reversed *in vivo* by the coadministration of a suitable nucleoside. Of the three compounds tested *in vitro*, 2'-deoxyuridine appeared to be the most promising candidate for the following reasons:

1. 2'-Deoxyuridine is of lower order toxicity and has no antitumor activity of its own.

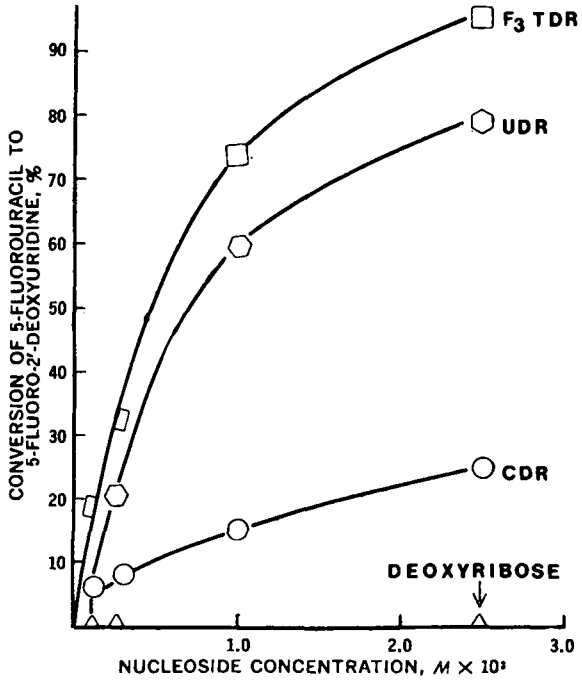

Figure 2—*Percent conversion of 5-fluorouracil to 5-fluoro-2'-deoxyuridine at various concentrations of four deoxyribosyl donors in blood during 24-hr. dialysis. (Initial 5-fluorouracil concentration was 4.1 × 10⁻⁴ M.)*

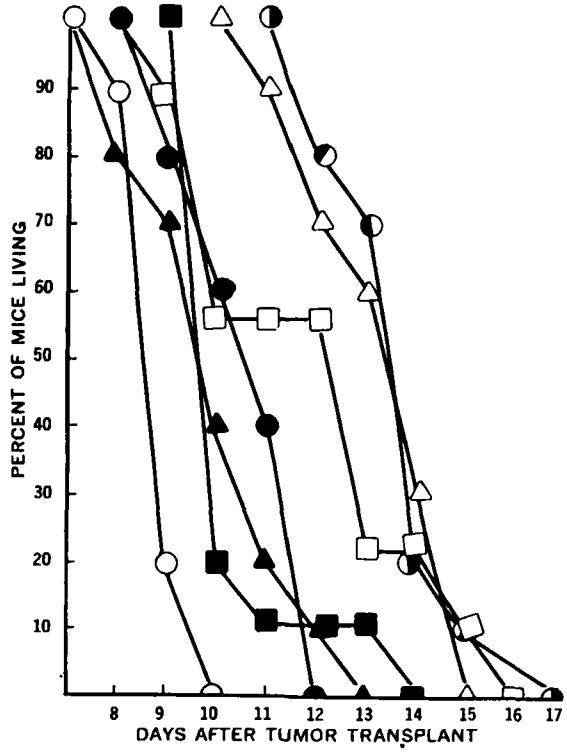

Figure 3—*Survival of mice during chemotherapy of L-1210 leukemia with 5-fluorouracil, 5-fluoro-2'-deoxyuridine, and their combinations with 2'-deoxyuridine. Key: ◑, 5-fluorouracil (10 mg./kg./day) plus 2'-deoxyuridine (200 mg./kg./day); △, 5-fluorouracil (20 mg./kg./day); □, 5-fluoro-2'-deoxyuridine (45 mg./kg./day) plus 2'-deoxyuridine (200 mg./kg./day); ●, 5-fluorouracil (10 mg./kg./day); ▲, 2'-deoxyuridine (200 mg./kg./day); ■, 5-fluoro-2'-deoxyuridine (45 mg./kg./day); and ○, control, 0.9% sodium chloride solution.*

2. Although 5-trifluoromethyl-2'-deoxyuridine appears to be the most active in preventing or reversing cleavage, it is an antitumor agent and toxic.

3. Deoxycytidine is less effective in preventing 5-fluoro-2'-deoxyuridine cleavage.

Based upon the concentration–conversion ratios from Figs. 1 and 2, it appears that 2'-deoxyuridine exhibits its maximum "anti-cleavage" activity at a concentration approximately 5 times that of the 5-fluoro-2'-deoxyuridine and remains invariant thereafter. Based upon an average dose of 40 mg./kg. 5-fluoro-2'-deoxyuridine, a standard dose of 200 mg./kg. 2'-deoxyuridine was used in all combinations.

Chemotherapy of Mouse L-1210 489 Leukemia with Combinations of 5-Fluorouracil or 5-Fluoro-2'-deoxyuridine and 2'-Deoxyuridine—As noted under *Experimental*, 70 mice (divided into seven groups of 10 each) were inoculated with a standardized suspension of L-1210 leukemia cells. The animals were randomized into groups and subjected to various drug regimens alone and in combination with 200 mg./kg. 2'-deoxyuridine. The drugs were administered once a day by intraperitoneal injection, using normal saline as a control. The drug activity was determined grossly by recording a survivial time of each animal (Table I).

Although fluorinated pyrimidines are considered to be less effective against leukemias than against other forms of cancer, such as solid tumors, the data obtained here indicate that their effectiveness can be enhanced by the presence of 2'-deoxyuridine in the drug regimen. For example, the addition of 2'-deoxyuridine to the 12.5-mg. regimen of 5-fluorouracil quadrupled its effectiveness against L-1210 leukemia. The addition of 2'-deoxyuridine to the 10-mg. regimen enhanced its effectiveness over 2.5 times.

Autopsies of mice given these drugs, singly or in combination with 2'-deoxyuridine, indicated that death was partly due to toxicity. However, enhancement of 5-fluorouracil activity at the lower dose levels, such as 10 mg./kg./day, by the addition of 2'-deoxyuridine is significant. Further clarification of the optimum dosage level is necessary.

An alternative way of presenting the survival data is shown in Fig. 3. The percentage of animals alive in each group was computed for each day of the experiment, and this information was graphed. The data indicate the superiority of the drug combinations to the single drugs.

Chemotherapy of Mouse Adenocarcinoma 755 with Combinations of 5-Fluorouracil or 5-Fluoro-2′-deoxyuridine and 2′-Deoxyuridine—Experiments dealing with solid tumors were carried out using adenocarcinoma 755. The tumor was grown in BDF_1 mice and transplanted to 70 normal mice. After allowing 24 hr. for the tumors to acclimate to the host, drug therapy was started using drugs alone or in combination with 200 mg./kg. 2′-deoxyuridine. Therapy was continued for 7 days using daily intraperitoneal injections. Twelve days after the tumor transplants, the average tumor volumes were determined for each group. The results given in Table II compare the tumor volumes of the treated groups to those of saline controls.

The reduction in tumor volumes of the mice treated with 10 mg./kg. 5-fluorouracil and 200 mg./kg. 2′-deoxyuridine and 40 mg./kg. 5-fluoro-2′-deoxyuridine and 200 mg./kg. 2′-deoxyuridine as compared to each drug alone or to the control is dramatic. Although there is an apparent tumor inhibition due to 2′-deoxyuridine alone, the apparent diminutions are not significant. Based on the data, it can be shown that the drug–2′-deoxyuridine combinations are synergistic and not simply additive.

The loss in weight of the mice appears to correlate with the percent tumor inhibition. This may be indicative of increased toxicity because of the higher effective titer of 5-fluoro-2′-deoxyuridine in the body due to the presence of the deoxyribosyl donor. There are some indications that the loss in weight was partly associated with the mouse colony used. Further toxicity studies are being carried out and will be reported later.

These *in vivo* data tend to support the *in vitro* findings that 5-fluorouracil is converted to 5-fluoro-2′-deoxyuridine by deoxyribosyl transfer in the presence of an excess donor compound such as 2′-deoxyuridine, and the activity therefore is enhanced. Also the apparent stabilization of 5-fluoro-2′-deoxyuridine has a dramatic effect on antitumor activity. In light of the present results, the observation by Birnie *et al.* (4) of great toxicity in the use of uridine with 5-fluoro-2′-deoxyuridine may be explained in terms of the exchange reaction where one product is the very toxic 5-fluorouridine.

SUMMARY AND CONCLUSIONS

In vitro experiments in human blood showed that deoxyribonucleosides such as 5-trifluoromethyl-2′-deoxyuridine, 2′-deoxyuridine, and deoxycytidine can react with 5-fluorouracil to form 5-fluoro-2′-deoxyuridine. A prediction that deoxyribosyl transfer reaction can enhance the antitumor activity of 5-fluorouracil and 5-fluoro-2′-deoxyuridine was tested *in vivo* in mice. When survival time and decreased tumor growth are used as measures of effectiveness, results from mouse leukemia L-1210 and adenocarcinoma 755 indicate that 5-fluorouracil or 5-fluoro-2′-deoxyuridine coadministered with excess 2′-deoxyuridine has higher antitumor activity than either of the pure drugs alone.

The data are consistent with the prediction of enhanced antitumor activity due to the deoxyribosyl transfer reaction, although other contributory mechanisms are possible.

Deoxyribosyl transfer reactions may provide a rationale for efficacy of some drug combinations used clinically.

Further work to determine toxicity and efficacy of the combinations is underway.

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Liquid Crystalline Phases in Aerosol Formation II: Influence of Liquid Crystalline Phases on Foam Stability

G. JEDERSTRÖM*, L. RYDHAG, and S. FRIBERG▲

Abstract □ Aerosol foam stability was determined by visual observation of time-dependent change in foams of different propellant compositions. Compositions that contained both a liquid crystalline phase and a liquid phase gave rise to foams with pronounced stability compared with those where only a liquid phase was present.

Keyphrases □ Aerosol foams—effect of liquid crystalline phases on foam stability □ Foam stability, aerosols—effect of liquid crystalline phases □ Propellant compositions—effect of liquid crystalline phases on foam stability

The stability of thin films is of importance for foam stability and the stability of emulsions against coagulation from the flocculated state. Extensive research has been undertaken in order to understand the reasons for the stability of such films. Generally, the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory (1, 2) has been applied to the phenomena (3, 4), giving four factors (3): (*a*) the Laplace capillary suction pressure, (*b*) the electrical double-layer repulsion, (*c*) the long-range Van der Waals pressure, and (*d*) "steric hindrance" of close-packed monolayers.

The last factor is not based on theory but is "vaguely described as the resistance from close-packed monolayers to lateral flow" (3).

The DLVO theory is obviously insufficient for an explanation of emulsion stability against coagulation when the continuous phase is nonpolar; the stability of water/oil emulsions shows no dependence on the surface potential (5).

The importance of chemical associations of different emulsifiers was observed early (6, 7), and recently attempts have been made (8) to relate the stability of aerosol foams to the formation of "molecular complexes." This hypothesis was criticized (9); it was demonstrated that molecular complexes formed by the emulsifiers bore no relation to emulsion stability. The importance lies in the presence of liquid crystalline phases (10, 11) in which the emulsifiers, water, and the oil phase are all associated into an ordered structure.

The importance of the association into liquid crystalline phases for foam stability was also demonstrated recently (12). Stable foams actually may be prepared from hydrocarbons when small amounts of water are present, associated into a liquid crystalline phase with the surfactants (13). The importance of liquid crystalline phases for the stability of foams from aqueous solutions (14) and also for the rate of thinning of thin films (15) has been shown independently.

Since the technical and pharmaceutical importance of these facts is obvious, an investigation into the application of these mechanisms to aerosol foam formulations was conducted. The phase equilibria of the propellant compositions were published earlier (16) (Fig. 1).

Table I—Initial Composition of Samples

Series	Sample Number	C_8OOH, wt. %	$C_2F_4Cl_2$, wt. %	Water, wt. %	C_8NH_2, wt. %
B	1	0	29.69	23.40	46.85
(Fig. 2*a*)	2	10.30	31.51	19.38	38.77
	3	18.18	31.51	16.76	33.52
	4	26.06	35.15	12.92	25.85
	5	41.20	31.51	9.08	18.17
	6	49.69	28.48	7.26	14.53
C	1	0	32.11	18.73	49.13
(Fig. 2*b*)	2	7.87	32.72	16.39	42.99
	3	26.66	30.30	11.87	31.14
	4	37.57	30.90	8.69	22.81
	5	47.87	28.48	6.52	17.10
	6	56.35	33.33	2.84	7.45
D	1	0	27.27	14.39	58.32
(Fig. 2*c*)	2	11.51	28.48	11.87	48.12
	3	19.39	27.87	10.43	42.28
	4	29.69	29.09	8.15	33.05
	5	40.60	32.72	5.27	21.38

EXPERIMENTAL

Materials—The water was twice distilled. 1-Aminooctane[1] (C_8NH_2) (purity by GLC >99%), octanoic acid[2] (C_8OOH), and a propellant[3] were used.

Foam Stability—The acid, amine, and water were weighed into glass containers which were sealed and cooled to −20°. The liquid crystalline phase involved in the experiments does not solidify at temperatures above −28°, so no crystallization problems occurred. At that temperature (−20°), the propellant ($C_2F_4Cl_2$) was added and the pressurized packaging, the aerosol, was formed as the temperature slowly rose to 20°. The container[4] was shaken before spraying to obtain as homogeneous conditions as possible between phases

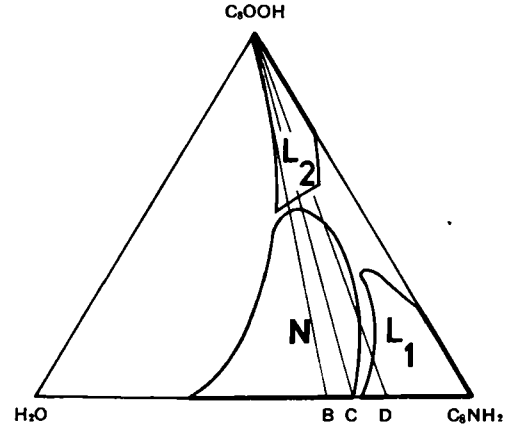
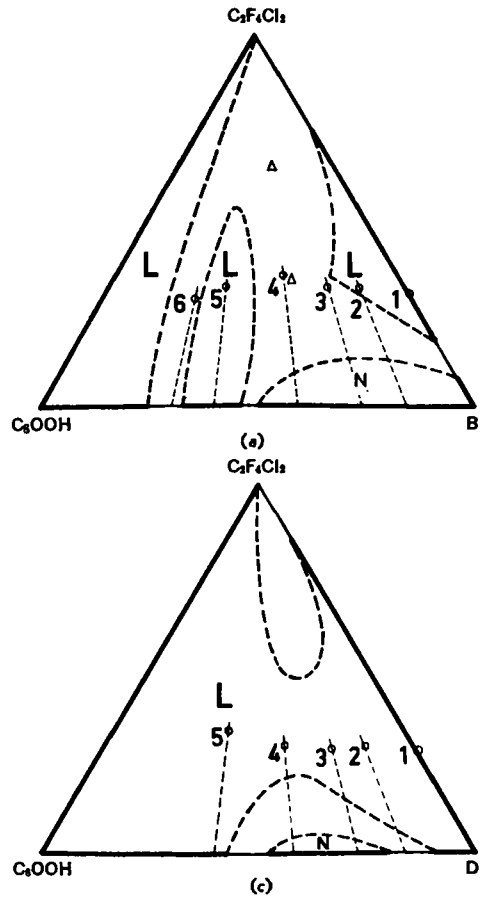


Figure 1—*Phase regions in the water (H_2O)–octylamine (C_8NH_2)–octanoic acid (C_8OOH) system. The lines ending at B, C, and D denote series used in the aerosol experiments. L_1 and L_2 = liquid isotropic phases. N = liquid crystalline, lamellar, "neat" phase.*

[1] Fluka puriss.
[2] Eastman Kodak p.a.
[3] Cryoflurane, Frigen 114, Hoechst.
[4] Valve, English precision; cup, LL till plate conical; stem, 0.50 cm. (0.20 in.) × 2; housing, 2.03 cm. (0.80 in.) PF with 0.50 cm. (0.20 in.) VPT, fitted with diptube; and actuator, J.S. 0.40 cm. (0.16 in.) stepped.



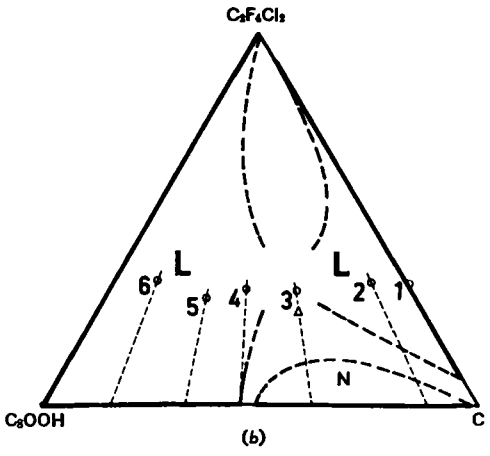


Figure 2—(a) *Phase regions in octanoic acid (C_8OOH)–composition B (Fig. 1)–propellant ($C_2F_4Cl_2$) systems. L = liquid isotropic phases. N = liquid crystalline, lamellar, "neat" phase.* (b) *Corresponding regions with the second component equal to C in Fig. 1.* (c) *Corresponding regions with the second component equal to D in Fig. 1.*

in the aerosol. The aerosol foams were extended into conical test tubes to facilitate the determination of drainage rates. The samples were photographed, and the foam height was measured on the photographs as a function of time.

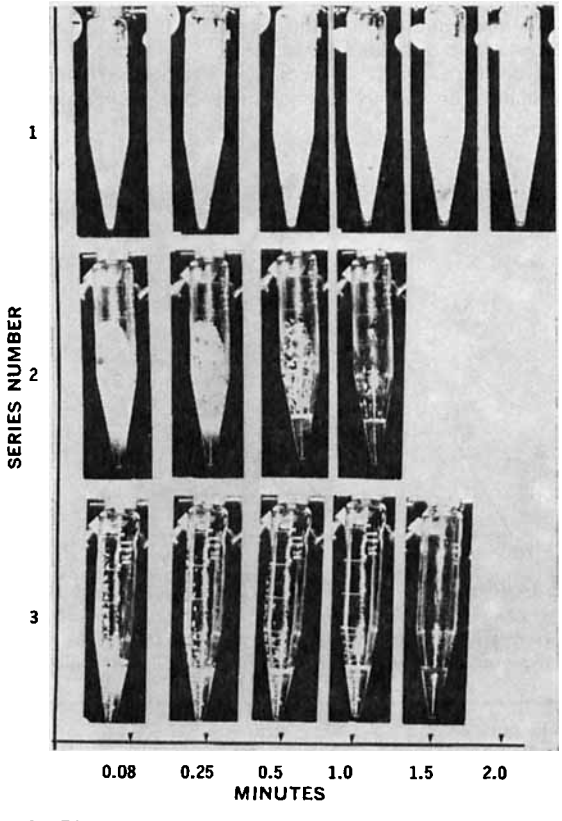


Figure 3—*Photographs showing the foam height as function of time.*

RESULTS

Phase Equilibria—The phase regions when the propellant is added to compositions C_8OOH-B, C_8OOH-C, and C_8OOH-D in Fig. 1 are given in Fig. 2, where the actual compositions prior to foam extrusion are marked on the diagram (Table I). The phase regions and their determining factors were described previously (16).

Foam Stability—Figure 3 demonstrates the general features of the photographs obtained. There usually was a pronounced difference between stable and unstable foams; the height of the former was reduced to less than half the initial value in less than 10 sec. while the corresponding time for the latter was several minutes. The foam in Series 1 showed no change over 2 min. while the others completely collapsed in 10 sec.

Diagrams of foam height *versus* time on a linear scale (Fig. 4) consequently show two groups of curves; one group for stable foams and one group at zero height for unstable foams. A logarithmic time scale was used to compare fast and slow foam height decay in identical diagrams.

Figure 5 shows the foam height for Samples 1–6 marked in Fig. 2*a*. The four samples containing a liquid crystal in the aerosol prior to addition of the propellant gave rise to stable foams. The foams from the two samples in which there were no liquid crystals were unstable, with half-lives of less than 10 sec.

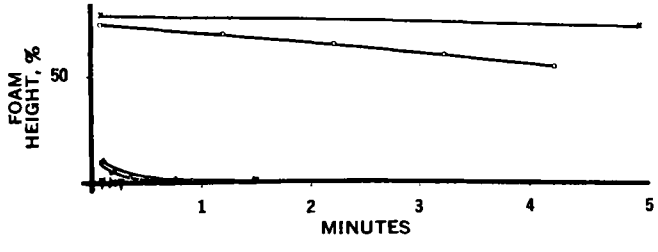


Figure 4—*Foam height* versus *time in a linear scale (compositions according to Fig. 2b). Key (Series): △, 1; ○, 2; ×, 3; □, 4; ▽, 5; and ☆, 6.*

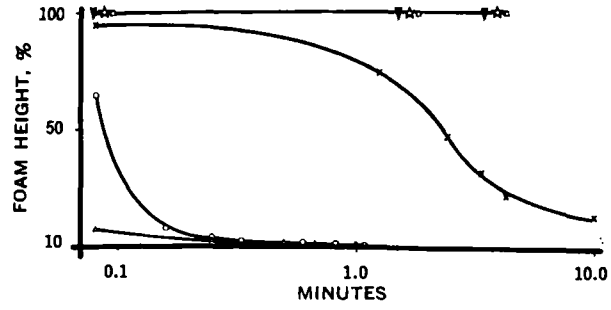


Figure 5—*Foam height* versus *logarithm of time* (*compositions according to Fig. 2a*). *Key (Series):* ×, *1;* ☆, *2;* ▼, *3;* □, *4;* ○, *5; and* △, *6.*

The second series in Fig. 2*b* contained six samples. Of the three with liquid crystals present in the aerosol composition (16) (exclusive of propellant), the one without acid gave rise to unstable foams and the other two to stable foams (Fig. 6). All samples containing no liquid crystals produced unstable foams. The samples in Series D showed the same general pattern; the foams from the two samples containing a liquid crystalline phase, prior to addition of propellant, were more stable than any of the other samples (Fig. 7). Sample 3 was not extremely stable, with a half-life of about 20 sec., but the difference from the samples with no liquid crystalline phase was distinct; all of the latter had half-lives of less than 5 sec.

DISCUSSION

The results were analyzed with regard to both the practical subject of reliable compositions for foam aerosol packings and the theory of stability of thin films.

Composition Method—*In all cases, foams from samples originally* containing only liquid phases gave completely unstable foams. The samples that contained a liquid crystalline phase prior to addition of the propellant gave rise to extremely stable foams with two exceptions. These two samples both had original compositions close to the phase limit and, furthermore, no liquid crystalline phase could be found in the collapsed foam. This probably was due to the fact that the phase diagrams in Figs. 1 and 2 were obtained under equilibrium, while the system was not in equilibrium when foamed. Original compositions close to the phase limit could well be expected to change their composition to outside the phase region during the foaming process.

It is also necessary that parts of the propellant should be incorporated into the liquid crystalline phase. Such a condition automatically implies stability only for compositions a few percent inside the liquid propellant region.

In themselves, these results indicated a simple method for the reliable evaluation of foam stabilizers for aerosol foams. Essentially, the composition prior to addition of propellant should contain a liquid crystalline phase and this phase should be able to ininclude some of the propellant. This method will help avoid problems associated with impurities in the emulsifier which are encountered in practice. Variations in impurity level may well change the limits of the liquid crystal region, causing an unstable foam. This problem can be avoided by using initial compositions that are *centrally* positioned in the liquid crystalline region, where compositions would be expected to show optimal resistance to changes in the properties of the emulsifiers due to variations in composition.

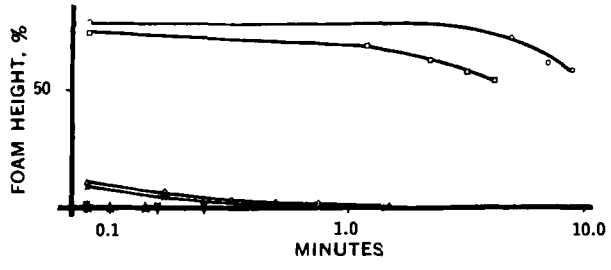


Figure 6—*Foam height* versus *logarithm of time* (*compositions according to Fig. 2b*). *Key (Series):* △, *1;* □, *2;* ○, *3;* ×, *4;* ▼, *5; and* ★, *6.*

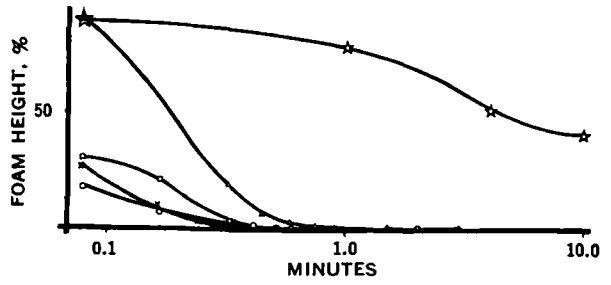


Figure 7—*Foam height* versus *logarithm of time* (*compositions according to Fig. 2c*). *Key (Series):* ○, *1;* □, *2;* △, *3;* ☆, *4; and* ×, *5.*

Stability of Thin Films—A thin film is never in the state of lowest free energy. The reason why it is stable is the presence of one or several minima in the potential energy *versus* thickness function. These energy minima may sometimes be extremely shallow. The presence of an energy minimum implies a small rise of the free energy in the system for small changes in its dimensions.

The final bursting of a thin film in equilibrium takes place due to small disturbances of it. In case these small changes in dimensions are not sufficiently self-damping, bursting takes place. An increase in energy when the film is disturbed may be related to the surface free energy, γ, and to the potential energy, V, which depends on the thickness of the film. According to Vrij (17), the change in free energy:

$$\Delta G = \iint \left\{ {}^1/_2\gamma \sum_{ij=1}^{2} \left(\frac{\partial z_i}{\partial x_j}\right)^2 + {}^1/_2 d^2 V/dh^2 \sum_{i=1}^{2} z_i^2 \right\} \prod_{j=1}^{2} dx \qquad \text{(Eq. 1)}$$

where z denotes vertical changes in the upper ($i = 1$) or lower ($i = 2$) plane of the horizontal film. The indexes j indicate the two Cartesian horizontal coordinates in these planes.

In hydrocarbons or even polar organic solvents, the surface energy is low and has a minor importance; the first term in Eq. 1 is of little importance. This is one reason why foams from hydrocarbons have not been taken into account (3) when the stability of thin films was treated. The present results, which show the importance of the presence of a liquid crystalline phase, may help explain the stability of thin films in hydrocarbons by focusing attention on the connection between the characteristic structure of liquid crystals and the second term in Eq. 1.

The lyotropic liquid crystalline phases are mainly lamellar or form a hexagonal array of cylinders. The reason why foams from hydrocarbons are stable when two-phase systems, solution–liquid crystalline phase, are formed is probably to be found in the fact that the structure of the thin film consists of a central part of liquid crystalline structure surrounded by layers of a nonpolar liquid. In such a structure, the surface energy of the system has no importance whatsoever for the stability. The hydrocarbon surface of the film has low surface energy which does not change with the geometric dimensions.

Changing the geometrical dimensions of the film will, on the other hand, require considerable energy additions due to the fact that the liquid crystalline phase spontaneously forms ordered structures. If z_1 and z_2 have the same size in Eq. 1, the alteration in the geometry is bending of the film. The change is resisted by viscous forces only. These are of a different magnitude from those found in a solution, but intuitively the influence of changes of this kind is of less importance than those encountered when z_1 and z_2 have different signs. That implies a thinning of the film, which means that the layers or cylinders constituting the liquid crystal must be moved on top of each other. Such a mechanism includes either a transport of amphophilic molecules through the lipid layer part of the liquid crystalline phase or a simultaneous motion of a high number of molecules. Both of these mechanisms require a high free energy and have small probability in a liquid crystalline phase compared to conditions in a liquid phase.

The investigations will be continued on systems of mixed emulsifiers.

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Aggregations of Certain Nucleosides in Aqueous Solutions from Osmometric Measurements: Theory

HANNA N. BORAZAN

Abstract □ Two models, the mononuclear and multinuclear, are proposed for self-aggregating systems such as dilute aqueous solutions of 2′-deoxyadenosine, 2′-deoxyguanosine, uridine, cytidine, and thymidine, which are characterized by two linear slopes in plots of their osmolal *versus* total molal concentrations. Their first slopes indicate a monomeric behavior and a formation of critical micelle-like concentrations. By correlating the results of both models on a semiempirical basis, theoretical curves were generated that agree with previous experimental work. Equilibrium constants, osmotic coefficients, and concentrations of various associated forms can be calculated.

Keyphrases □ Nucleosides in aqueous solution, self-aggregation—osmometric measurements, theoretical curves compared to experimental data, calculation of equilibrium constants, osmotic coefficients, and species concentrations □ Osmometric measurements—self-aggregation of nucleosides in aqueous solution, theoretical curves compared to experimental data □ Aggregation of nucleosides in aqueous solution—osmometric measurements, theoretical curves compared to experimental data

The thermoelectric osmometer developed by Goyan and Johnson (1, 2) has been improved and used to study dilute aqueous solutions of caffeine and other substances of pharmaceutical interest (3). The measured osmotic property plotted against molal concentration resulted in two linear slopes for many substances. The point of break was sharp whenever it occurred and was regarded as evidence of a critical micelle concentration (CMC). The molal concentration where this break occurs is a critical concentration in that it is a property of a substance. It evidently has been overlooked by other workers, probably because of the difficulty of exploring the concentration range between 0.01 and 0.10 *M*. Later, Borazan (4) and Borazan and Goyan (5) extended past work (6, 7) into this region of concentration and included other compounds. The characteristic break between linear slopes was found for very dilute solutions of 2′-deoxyadenosine, 2′-deoxyguanosine, uridine, cytidine, and thymidine.

This paper presents some theoretical considerations in an effort to explain experimental results reported previously (3–5). An attempt is also made to recalculate equilibrium constants that might hold over a wider range of concentration than those given previously (8, 9).

THEORY

An attempt is made to explain the two intersecting linear slopes determined experimentally, when plotting a colligative property *versus* molal concentration (3–5), by using a simple mathematical model:

$$nA = A_n \qquad \text{(Eq. 1}a\text{)}$$

$$K = (A_n)/(A)^n \qquad \text{(Eq. 1}b\text{)}$$

The osmolal, o, and total molal, m, concentrations are given by the equations:

$$o = (A_n) + (A) \qquad \text{(Eq. 2)}$$

$$m = n(A_n) + (A) \qquad \text{(Eq. 3)}$$

In Eqs. 1–3, A represents the monomer and A_n represents the associated form. Parentheses indicate molal concentration. By

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$$nA = A_n \qquad \text{(Eq. 1}a\text{)}$$

$$K = (A_n)/(A)^n \qquad \text{(Eq. 1}b\text{)}$$

The osmolal, o, and total molal, m, concentrations are given by the equations:

$$o = (A_n) + (A) \qquad \text{(Eq. 2)}$$

$$m = n(A_n) + (A) \qquad \text{(Eq. 3)}$$

In Eqs. 1–3, A represents the monomer and A_n represents the associated form. Parentheses indicate molal concentration. By

assigning values at intervals of 0.01 M to (A), it is easy to calculate corresponding values of (A_n) for selected values of K. When the values for o are plotted against m and compared directly with the experimental curves (3–5), the resulting curves are similar to those obtained from experimental data. In all of the graphs, the first parts were very similar to ideal solutions showing no association. This is due to the fact that the concentration of the n-mer is so low that it cannot be detected. When K is taken to be 1 or 10 and n to be 2 or 3, a few of the curves calculated in this way approximate a few of the intersecting straight lines. However, points near the intersection deviate from either line by more than can be accounted for on the basis of experimental error, except when the two slopes differ very little. To simulate the realistic slope change and an intersection in the concentration range studied with n equal to 4, it is necessary to assign large values for K. As reported previously (6, 7, 10–12), in systems of this type K is lower than 20 in most cases, and the free energy is on the order of magnitude of thermal kinetic energy. Therefore, a K value in the neighborhood of 10 with n equal to 3 seems to be a reasonable limit. With these values, a deviation of points near the intersection from straight lines is about 3 ohms. This deviation is outside the limit of experimental error by a factor of nearly 10. These facts clearly indicate that this simple mathematical model cannot be used.

A more sophisticated approach along the same lines was undertaken with the aid of a computer[1]. Based upon the assumption that two monomers react to form a dimer and that the dimer reacts with another monomer to form a trimer, *etc.*, four different association constants were postulated. The values 2, 4, 6, and 8 were assigned to K_1, K_2, K_3, and K_4 in rotation. The resulting 256 curves were calculated and plotted by the computer.

When the experimental slopes (5) are converted to the osmolal system by dividing by 924 from the sucrose standardization, all initial slopes for single pure substances in water show an experimental value of unity within the limits of experimental error. All computer-generated curves based on four K values, where $K_1 = 2$, show an initial slope of 0.98 based on the first plotted point and 0.96 or 0.95 based on the second point, regardless of the values assigned to K_2, K_3, and K_4. Higher values of K_1 produce lower slopes, indicating the need to try smaller values for the initial association constants.

A study of the lines generated with four association constants showed that K_4 played a minor role. It can be concluded from this study that association constants are very small and that certain combinations of K values can produce almost two linear intersecting slopes in the range of concentrations studied, with the first slopes having values approximately equal to one, thus resembling reported experimental curves (5).

In an effort to calculate equilibrium constants for the successive aggregation processes from the experimental curves reported, two models are proposed here.

Mononuclear Aggregation—A solute molecule, say A', can serve as a nucleus for other molecules of the same solute:

$$A' + A = A'A \qquad \text{(Eq. 4a)}$$

$$A'A + A = A'A_2 \qquad \text{(Eq. 4b)}$$

$$\vdots$$

$$A'A_{j-1} + A = A'A_j \qquad \text{(Eq. 4c)}$$

$$\vdots$$

$$A'A_{\sigma-1} + A = A'A_\sigma \qquad \text{(Eq. 4d)}$$

where σ is the maximum number of multiple equilibria. In the model presented here, it is assumed that no interactions among various associated forms take place and that the binding sites on the nucleus molecule are dependent; *i.e.*, the binding of a monomer to a nucleus can influence the binding of another monomer to the same nucleus. Therefore, the following equations can be written:

$$K_1 = (A'A)/(A')(A) \qquad (A'A) = K_1(A')(A) \qquad \text{(Eq. 5)}$$

$$K_2 = (A'A_2)/(A'A)(A) \qquad (A'A_2) = K_2(A'A)(A) \qquad \text{(Eq. 6)}$$

$$K_j = (A'A_j)/(A'A_{j-1})(A) \qquad (A'A_j) = K_j(A'A_{j-1})(A) \qquad \text{(Eq. 7)}$$

$$K_\sigma = (A'A_\sigma)/(A'A_{\sigma-1})(A) \qquad (A'A_\sigma) = K_\sigma(A'A_{\sigma-1})(A) \qquad \text{(Eq. 8)}$$

Combining Eqs. 5 and 6:

$$(A'A_2) = K_1K_2(A')(A)^2 \qquad \text{(Eq. 9)}$$

If one carries the same technique of substitutions for the consecutive processes, the following equation can be derived:

$$(A'A_j) = K_1K_2 \ldots K_j(A')(A)^j \qquad \text{(Eq. 10)}$$

Under the assumption that no self-association takes place below the critical concentrations, (C_0), based upon experimental results, and if (C_0) and $(A_0 - C_0)$ are considered as the total concentrations of the reactant species, A' and A, respectively, where (A_0) is the total concentration of the solute, then the following can be defined:

$$(C_0) = (A') + (A'A) + (A'A_2) + \ldots + (A'A_j) + \ldots + (A'A_\sigma) \qquad \text{(Eq. 11)}$$

$$= (A') + \sum_{j=1}^{\sigma} (A'A_j)$$

By substitution from Eq. 10 of $(A'A_j)$ in Eq. 11:

$$(C_0) = (A')\left[1 + \sum_{j=1}^{\sigma}\left(\prod_{j=1}^{j} K_j\right)(A)^j\right] \qquad \text{(Eq. 12)}$$

where:

$$\prod_{j=1}^{j} K_j = K_1K_2 \ldots K_j$$

$$(A_0 - C_0) = (A) + (A'A) + \ldots + j(A'A_j) + \ldots + \sigma(A'A_\sigma) \qquad \text{(Eq. 13)}$$

$$= (A)\left[1 + (A')\sum_{j=1}^{\sigma} j\left(\prod_{j=1}^{j} K_j\right)(A)^{j-1}\right]$$

and:

$$o = (A') + (A) + (A'A) + \ldots + (A'A_j) + \ldots + (A'A_\sigma) \qquad \text{(Eq. 14)}$$

$$= (A') + (A) + \sum_{j=1}^{\sigma} (A'A_j)$$

where o represents osmolal concentration.

The second linear slope, S_2 (5), can be expressed:

$$S_2 = [o - (C_0)]/(A_0 - C_0) = \frac{\text{second experimental slope}}{\text{sucrose slope}} \qquad \text{(Eq. 15)}$$

By substitution from Eqs. 14, 11, and 13 of o, C_0, and $(A_0 - C_0)$, respectively, in Eq. 15:

$$S_2 = \left[1 + (A')\sum_{j=1}^{\sigma} j\left(\prod_{j=1}^{j} K_j\right)(A)^{j-1}\right]^{-1} \qquad \text{(Eq. 16)}$$

set:

$$\left[1 + \sum_{j=1}^{\sigma}\left(\prod_{j=1}^{j} K_j\right)(A)^j\right] = \phi(K_j, A) \qquad \text{(Eq. 17)}$$

and:

$$\sum_{j=1}^{\sigma} j\left(\prod_{j=1}^{j} K_j\right)(A)^{j-1} = \frac{\partial\phi}{\partial(A)} \qquad \text{(Eq. 18)}$$

Substituting for the summation of terms, Eq. 18, by the partial derivative into Eq. 16:

$$\frac{\partial\phi}{\partial(A)} = \frac{1}{(A')}\left(\frac{1 - S_2}{S_2}\right) \qquad \text{(Eq. 19)}$$

Combining Eqs. 17 and 12 and substituting into Eq. 19:

$$\left(\frac{(C_0)S_2}{1 - S_2}\right)\frac{\partial\phi}{\partial(A)} = \phi \qquad \text{(Eq. 20)}$$

[1] The approach was suggested by Professor Leo Brewer, University of California, Berkeley. An IBM 360 computer was programmed by Mr. Phillip Johnson, University of California San Francisco Information Service, who contributed much of his own time and thought to this portion of the work.

Table I—Equilibrium Constants of Self-Association of the Nucleosides Together with Their Progression Constant

Nucleoside	Progression[a] Constant, α_s, molal	β_1, molal^{-1}	β_2, molal^{-1}	β_3, molal^{-1}
2′-Deoxyadenosine	0.0272	4.07	12.20	36.60
2′-Deoxyguanosine	0.0193	5.77	17.30	51.80
Uridine	0.330	0.33	1.00	3.00
Cytidine	0.338	0.40	1.20	3.60
Thymidine	0.238	0.55	1.65	4.95

[a] Progression constant $= \dfrac{\text{second slope} \times \text{critical concentration}}{\text{sucrose slope} - \text{second slope}}$, according to Eq. 21. The data were taken from *Reference 5*. The slope of standard solution of sucrose is 923.7 ohms molal^{-1}.

The critical concentration, (C_0), is supposed to be a constant quantity for a particular molecule; therefore:

$$\frac{(C_0)S_2}{1 - S_2} = \text{constant} = \alpha_s \qquad \text{(Eq. 21)}$$

Equation 20 has the following solution:

$$\phi = e^{(A)/\alpha_s} \qquad \text{(Eq. 22)}$$

The function ϕ can be expressed as a power series in $(A)/\alpha_s$, and according to Eq. 17:

$$\phi = 1 + K_1(A) + K_1K_2(A)^2 + \ldots + K_1 \ldots K_j(A)^j + \ldots + K_1 \ldots K_\sigma(A)^\sigma$$
$$= 1 + \frac{(A)}{\alpha_s} + \frac{1}{2!}\left(\frac{(A)}{\alpha_s}\right)^2 + \ldots + \frac{1}{j!}\left(\frac{(A)}{\alpha_s}\right)^j + \ldots + \frac{1}{\sigma!}\left(\frac{(A)}{\alpha_s}\right)^\sigma + \ldots + \frac{1}{\infty!}\left(\frac{(A)}{\alpha_s}\right)^\infty \qquad \text{(Eq. 23)}$$

If the number of multiple equilibria, σ, is large enough, one can deduce with reasonable approximation, for low concentration of A, the following by comparing coefficients of (A) from Eq. 23:

$$K_1 = 1/\alpha_s \qquad K_j = 1/j\,\alpha_s \qquad \text{(Eq. 24a)}$$

$$K_2 = 1/2\,\alpha_s \qquad K_\sigma = 1/\sigma\,\alpha_s \qquad \text{(Eq. 24b)}$$

Multinuclear Aggregation—According to this model, the binding of a monomer to $(j - 1)$-mer to produce j-mer induces an identical, independent binding probability for another monomer; any of the equivalent subunits in the j-mer can serve as a binding site for another monomer and so on. Therefore, one would expect that the overall equilibrium constants are increasing and related to one intrinsic binding constant, k.

Based upon configurational probabilities for these completely independent and identical sites, the following can be derived (13):

$$A + A = A_2 \qquad k/\sigma = \beta_1 \qquad \text{(Eq. 25a)}$$

$$A_2 + A = A_3 \qquad \left(\frac{2}{\sigma - 2 + 1}\right) k = \beta_2 \qquad \text{(Eq. 25b)}$$

$$\vdots$$

$$A_j + A = A_{j+1} \qquad \left(\frac{j}{\sigma - j + 1}\right) k = \beta_j \qquad \text{(Eq. 25c)}$$

$$\vdots$$

$$A_\sigma + A = A_{\sigma+1} \qquad \sigma k = \beta_\sigma \qquad \text{(Eq. 25d)}$$

Since no simple way is available for one to derive the precise values of k and σ, the progression constant, α_s, described by Eq. 21 was sought intuitively as a possible common constant between the overall equilibrium constants in the two models. It is, therefore, assumed empirically that the reciprocal of the progression constant which determines the first and highest equilibrium constant in the first model could also determine the highest but the last equilibrium constant in the second model. It is also assumed that the last equilibrium constant for the mononuclear aggregation model, K_σ (when all binding sites are occupied except one), may be the real intrinsic binding constant, k, in the second model. Accordingly, the following are set:

$$K_1 = \beta_\sigma = \sigma K_\sigma = \frac{1}{\alpha_s} = \frac{1 - S_2}{(C_0)S_2} = \sigma k \qquad \text{(Eq. 26)}$$

$$k = \frac{1 - S_2}{\sigma(C_0)S_2} \qquad \text{(Eq. 27)}$$

and β_j, according to Eqs. 25 and 27, is:

$$\beta_j = \frac{j(1 - S_2)}{(\sigma - j + 1)\,[\sigma(C_0)S_2]} \qquad \text{(Eq. 28)}$$

Osmolal and total molal concentrations can be defined and related to measurable quantities:

$$o = (A) + (A_2) + (A_3) + \ldots + (A_{j+1}) + \ldots + (A_{\sigma+1}) \qquad \text{(Eq. 29)}$$

Concentrations of various species can be expressed in terms of their corresponding equilibrium constants and free monomer concentration:

$$(A_2) = \beta_1(A)^2 \qquad \text{(Eq. 30a)}$$

$$(A_3) = \beta_1\beta_2(A)^3 \qquad \text{(Eq. 30b)}$$

$$(A_{j+1}) = \beta_1\beta_2 \ldots \beta_j(A)^{j+1} \qquad \text{(Eq. 30c)}$$

Thus:

$$o = (A) + \sum_{j=1}^{\sigma}\left(\prod_{j=1}^{j}\beta_j\right)(A)^{j+1} \qquad \text{(Eq. 31)}$$

Substituting for the product of β_j, according to Eq. 28:

$$o = (A) + \sum_{j=1}^{\sigma}\frac{j!(1 - S_2)^j}{\left[\prod_{j=1}^{j}(\sigma - j + 1)\right][\sigma(C_0)S_2]^j}(A)^{j+1} \qquad \text{(Eq. 32)}$$

By carrying similar substitutions, the total molal concentration will be:

$$m = (A) + \sum_{j=1}^{\sigma}(j + 1)\frac{j!(1 - S_2)^j}{\left[\prod_{j=1}^{j}(\sigma - j + 1)\right][\sigma(C_0)S_2]^j}(A)^{j+1} \qquad \text{(Eq. 33)}$$

The osmotic coefficient can be calculated from the definitions of o and m by the following relationship:

$$\phi = o/m \qquad \text{(Eq. 34)}$$

RESULTS AND DISCUSSION

Equations 24*a* and 24*b* indicate that the highest equilibrium constant is the first and that the experimental curves can only be generated by previous knowledge of their critical concentrations. However, a given set of K values calculated from the relationships given (in Eqs. 24*a* and 24*b*), three or four K's, failed to reproduce curves obtained experimentally when plotting their calculated osmolal *versus* total molal concentration according to Eqs. 2 and 3.

Borazan (4) hypothesized that the possible maximum number of molecules in each aggregate of the nucleosides studied is four to five (all molecules in each aggreagate are not necessarily bonded at the same time, according to the theory). Thus, one would expect the number of multiple equilibria, σ, to be between three and four.

To test the second model and the validity of the assumptions made in the derivations when osmotic coefficient, ϕ, was calculated for $\sigma = 3$, several values were assigned for the monomer concentrations and Eqs. 32–34 were applied, with data taken from Borazan and Goyan (5), and compared to experimental values; reasonable agreement was found over a wide molal range for most of the nucleosides. The data are summarized in Table II. In this way, theoretical curves can be calculated from the definitions of osmolal and molal concentrations, Eqs. 32 and 33, respectively. The overall equilibrium constants calculated according to Eqs. 25 and 27 and from data taken from Borazan and Goyan (5) (Table I) can be used to calculate concentrations of various associated forms.

It was found by trial and error that slightly lower values of S_2

Table II—Comparison between Osmotic Coefficients Interpolated from Osmotic Curves Calculated According to Eqs. 32 and 33, ϕ_{calc}; Osmotic Coefficients Calculated from Experimental Data (*Reference 5*), ϕ_{exp}; and Experimental Values Reported in the Literature at 25°, ϕ_{rep}

Molal Concentration	ϕ_{calc}	ϕ_{exp}	ϕ_{rep}
	2′-Deoxyadenosine		
0.005	0.970	1.004	—
0.010	0.955	1.004	—
0.015	0.923	1.004	—
0.01725	0.913	0.961	0.84 ± 0.07[a]
0.020	0.907	0.918	—
0.025	0.871	0.864	0.900 ± 0.01[b]
0.030	0.846	0.828	—
0.0345	0.815	0.804	0.760 ± 0.04[a]
0.040	0.796	0.782	—
0.050	0.770	0.755	0.800 ± 0.005[b]
	2′-Deoxyguanosine		
0.010	—	1.001	—
0.012	—	1.001	—
0.014	0.907	0.957	—
0.015	0.896	0.931	—
0.016	0.887	0.911	—
0.017	0.877	—	—
	Uridine		
0.030	0.990	0.999	—
0.040	0.988	0.999	—
0.050	0.983	0.999	0.969[c]
0.060	—	0.991	—
0.070	—	0.972	—
0.080	0.975	0.957	—
0.094	0.968	0.942	0.952[c], 0.962[c]
0.095	0.965	0.941	0.933[c]
0.096	0.965	0.940	0.937[c]
0.100	0.960	0.936	0.943[c]
0.130	0.947	—	—
0.150	0.944	—	—
0.192	0.925	—	0.896[c], 0.903[c]
0.292	0.881	—	0.861[c]
0.395	0.837	—	0.838[c]
0.501	0.800	—	0.804[c]
0.579	0.773	—	0.768[c]
0.693	0.740	—	0.756[c]
0.710	0.735	—	0.760[c]
	Cytidine		
0.030	0.987	1.000	—
0.040	0.983	1.000	—
0.050	0.980	0.981	0.967[c]
0.070	0.961	0.955	—
0.080	0.954	0.947	—
0.0941	0.947	0.939	0.941[c]
0.0948	0.947	0.939	0.939[c]
0.0955	0.944	0.938	0.936[c]
0.100	0.943	0.936	0.935[c]
0.130	0.931	—	—
0.150	0.928	—	—
0.194	0.905	—	0.888[c]
0.291	0.855	—	0.833[c], 0.825[c]
0.397	0.804	—	0.791[c]
0.503	0.764	—	0.750[c]
0.695	0.701	—	0.697[c]
0.709	0.697	—	0.693[c]
	Thymidine		
0.040	0.977	1.001	—
0.050	0.977	1.001	—
0.070	0.975	0.963	—
0.090	0.944	0.929	—
0.100	0.930	0.917	0.905[b]
0.130	0.910	—	—
0.150	0.900	—	—
0.200	0.863	—	0.805[b]
0.250	0.830	—	—
0.300	0.800	—	—
0.344	0.775	—	—

[a] From *Reference 14*. [b] From *Reference 7*. [c] From *Reference 6*.

for cytidine and thymidine should be used to generate curves that almost duplicate experimental curves within the limit of accuracy. Accordingly, values of 0.87 and 0.782 were used instead of 0.891 and 0.807 for cytidine and thymidine, respectively.

It was also observed that the shapes of the calculated curves were similar to those obtained experimentally and that all points fell on almost two linear slopes. The deviation of the data from the first ideal slopes is considered just about the limit of experimental error.

Ts'o and Chan (9) also based their calculations of K on multiple-equilibria processes (of the type of Eqs. 25*a*–25*d*) and on the assumption that all K's are equal. Assuming that the multiple equilibria go to infinity, they derived the following equation, which is based upon the work of Schellman (8):

$$K = \frac{1 - \phi}{M\phi^2} \qquad \text{(Eq. 35)}$$

where M = total molal concentration, and ϕ = osmotic coefficient.

Attempts to use Eq. 35 with experimental data (5) at 0.1 molal gave values for K of 0.705, 0.705, and 0.973 for uridine, cytidine, and thymidine, respectively. Ts'o *et al.* (6) calculated 0.61 and 0.87 for uridine and cytidine, respectively, while Solie (10) calculated 0.91 for thymidine at the same concentration and showed that the values held fairly well at higher concentrations. However, the low concentration values of Ts'o *et al.* (6) were somewhat influenced by curve smoothing and fitted to preselected polynomial. Attempts to calculate K values at some of the low accurately measured values (5) show that Eq. 35 fails to yield nearly constant values, even well above the critical concentration. Broom *et al.* (7) agreed that in some cases Eq. 35 fails even at higher concentrations using data taken from smoothed curves. It should be emphasized that Eq. 35 worked well at concentrations higher than 0.1 molal, while it failed to produce constant K values at concentrations lower than 0.1 molal.

Therefore, the results presented in this paper seem to be more meaningful and realistic, in that several consecutive equilibrium constants with increasing order and limited associated forms are to be considered, and theoretical values that agree with experimental data (Table II) can be generated over a far greater range than with the model previously reported (8, 9).

It should also be emphasized that generating theoretical data that agree with experimental data from a model does not mean that the model is absolutely correct. The actual situation could be different from that hypothesized, knowing that the results presented were subject to various approximations and empirical assumptions, for example, ignoring some interactions, mathematical approximations, *etc.*

CONCLUSION

Theoretical curves have been generated to reproduce experimental results within the limit of accuracy. The model in question assumes the generation of equivalent and independent binding sites. The overall equilibrium constants are increasing, and limited associated forms have to be considered, in the neighborhood of four for the nucleosides studied.

The theoretical osmotic coefficient values (Table II) agree fairly well with those reported in the literature.

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Synthesis of Substituted Cinnamides: Relationship between Anticonvulsant and Monoamine Oxidase Inhibitory Properties

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Abstract □ Several esters and hydrazides of substituted cinnamides were synthesized and characterized. Evaluation of their anticonvulsant activity indicated appreciable activity of hydrazides where no correlation could be observed between anticonvulsant activity possessed by these hydrazides and their ability to inhibit monoamine oxidase.

Keyphrases □ Cinnamides of substituted amino acid esters and their hydrazides—synthesis, relationship between anticonvulsant and monoamine oxidase inhibitory properties □ Monoamine oxidase inhibitors, cinnamides of amino acid esters and their hydrazides—synthesis, anticonvulsant activity □ Anticonvulsant activity—cinnamides of substituted amino acid esters and their hydrazides, relationship to monoamine oxidase inhibitory properties □ Structure-activity relationships—cinnamides, relationship between anticonvulsant and monoamine oxidase inhibitory properties

Presence of a styryl group, responsible for high π-electron density, has been postulated to account for monoamine oxidase [EC 1.4.3.4 monoamine-O_2 oxido reductase (deaminating)] inhibitory property of styrylquinoliniums (1). The ability of monoamine oxidase inhibitors to possess anticonvulsant activity (2, 3) led to the synthesis of α-benzoylamino-*N*-[*p*-(4-aryl semicarbazide/thiosemicarbazide) - benzoyl] - *p* - substituted cinnamides possessing both an ethylenic moiety and the hydrazide group in an attempt to correlate their anticonvulsant activity with *in vitro* monoamine oxidase inhibitory effectiveness (4). In the present study, substituted cinnamides of amino acid esters and their corresponding hydrazides were synthesized as possible anticonvulsants to investigate correlation between anticonvulsant activity possessed by these hydrazides with their ability to inhibit monoamine oxidase.

CHEMISTRY

The various cinnamides of substituted amino acid esters and their corresponding hydrazides (Tables I and II) were synthesized by following the methods outlined in Scheme I.

Substituted oxazolones (*Ia*) were obtained by treatment by hippuric acid with appropriate aromatic aldehydes and acetic anhydride in the presence of anhydrous sodium acetate. Compounds *Ia* were treated with ethyl esters of amino acids (*Ib*) in the presence of 2–3 drops of triethylamine to give esters (I–XII) which were converted into the corresponding hydrazides (XIII–XXIV) by refluxing with 99–100% hydrazine hydrates (1:2 molar ratio) in absolute ethanol for 6–8 hr.

EXPERIMENTAL

Ethyl Esters of Amino Acids (*Ib*)—These were prepared by following the method of Kupryszewski and Sokolowska (5). Thionyl chloride, 0.1 mole, was added at 5° to 50 ml. of an absolute ethanolic solution of the appropriate amino acid (0.1 mole) and the mixture was refluxed for 3 hr. The excess thionyl chloride was removed under reduced pressure. The free ester, which precipitated by passing a slow stream of dry ammonia into a suspension of the ester hydrochloride in chloroform, was filtered, and excess chloroform was removed by distillation under reduced pressure.

2-Phenyl-4-(substituted benzylidene)-oxazole-5-ones (*Ia*)—The various oxazolones were prepared by heating a mixture of an appropriate aromatic aldehyde (0.96 mole), powdered dry hippuric acid (1.07 moles), freshly powdered sodium acetate (0.98 mole), and acetic anhydride (2.9 moles) in a flask on an electric hot plate (6, 7). As soon as the material had liquefied completely, the flask was transferred to a steam bath and heated for 2 hr. The reaction mixture was allowed to stand overnight, and the solid mass which precipitated was filtered with suction and washed first with two 100-ml. portions of ice-cold ethanol and finally with two 100-ml. portions of boiling water. After drying, the various oxazolones were used without further purification.

α-Benzoylamino-*N*-(substituted esters)-substituted Cinnamides (I–XII)—Equimolar quantities (1 mole) of the appropriate 2-phenyl-4-(substituted benzylidene)-oxazole-5-ones and ethyl esters of amino acids were refluxed in absolute ethanol in the presence of 2–3 drops of triethylamine on a steam bath for 6–8 hr. Excess ethanol was distilled, and the cinnamides which separated on cooling were filtered and recrystallized with either ethanol or the mixture of ethanol and water. The cinnamides were characterized by their sharp melting points and elemental analyses (Table I).

α-Benzoylamino-*N*-(substituted hydrazides)-substituted Cinnamides (XIII–XXIV)—A mixture of the appropriate α-benzoylamino-*N*-(substituted esters)-substituted cinnamides (0.1 mole) and 99–100% hydrazine hydrate (0.12 mole) in absolute ethanol was refluxed on a water bath for 6–8 hr. The solid mass which separated on cooling was filtered and recrystallized from suitable solvents (Table II).

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Synthesis of Substituted Cinnamides: Relationship between Anticonvulsant and Monoamine Oxidase Inhibitory Properties

SURENDRA S. PARMAR*▲, P. C. JOSHI†, BASHEER ALI†, and STANLEY J. BRUMLEVE*

Abstract □ Several esters and hydrazides of substituted cinnamides were synthesized and characterized. Evaluation of their anticonvulsant activity indicated appreciable activity of hydrazides where no correlation could be observed between anticonvulsant activity possessed by these hydrazides and their ability to inhibit monoamine oxidase.

Keyphrases □ Cinnamides of substituted amino acid esters and their hydrazides—synthesis, relationship between anticonvulsant and monoamine oxidase inhibitory properties □ Monoamine oxidase inhibitors, cinnamides of amino acid esters and their hydrazides—synthesis, anticonvulsant activity □ Anticonvulsant activity—cinnamides of substituted amino acid esters and their hydrazides, relationship to monoamine oxidase inhibitory properties □ Structure-activity relationships—cinnamides, relationship between anticonvulsant and monoamine oxidase inhibitory properties

Presence of a styryl group, responsible for high π-electron density, has been postulated to account for monoamine oxidase [EC 1.4.3.4 monoamine-O_2 oxido reductase (deaminating)] inhibitory property of styrylquinoliniums (1). The ability of monoamine oxidase inhibitors to possess anticonvulsant activity (2, 3) led to the synthesis of α-benzoylamino-*N*-[*p*-(4-aryl semicarbazide/thiosemicarbazide) - benzoyl] - *p* - substituted cinnamides possessing both an ethylenic moiety and the hydrazide group in an attempt to correlate their anticonvulsant activity with *in vitro* monoamine oxidase inhibitory effectiveness (4). In the present study, substituted cinnamides of amino acid esters and their corresponding hydrazides were synthesized as possible anticonvulsants to investigate correlation between anticonvulsant activity possessed by these hydrazides with their ability to inhibit monoamine oxidase.

CHEMISTRY

The various cinnamides of substituted amino acid esters and their corresponding hydrazides (Tables I and II) were synthesized by following the methods outlined in Scheme I.

Substituted oxazolones (*Ia*) were obtained by treatment by hippuric acid with appropriate aromatic aldehydes and acetic anhydride in the presence of anhydrous sodium acetate. Compounds *Ia* were treated with ethyl esters of amino acids (*Ib*) in the presence of 2–3 drops of triethylamine to give esters (I–XII) which were converted into the corresponding hydrazides (XIII–XXIV) by refluxing with 99–100% hydrazine hydrates (1:2 molar ratio) in absolute ethanol for 6–8 hr.

EXPERIMENTAL

Ethyl Esters of Amino Acids (*Ib*)—These were prepared by following the method of Kupryszewski and Sokolowska (5). Thionyl chloride, 0.1 mole, was added at 5° to 50 ml. of an absolute ethanolic solution of the appropriate amino acid (0.1 mole) and the mixture was refluxed for 3 hr. The excess thionyl chloride was removed under reduced pressure. The free ester, which precipitated by passing a slow stream of dry ammonia into a suspension of the ester hydrochloride in chloroform, was filtered, and excess chloroform was removed by distillation under reduced pressure.

2-Phenyl-4-(substituted benzylidene)-oxazole-5-ones (*Ia*)—The various oxazolones were prepared by heating a mixture of an appropriate aromatic aldehyde (0.96 mole), powdered dry hippuric acid (1.07 moles), freshly powdered sodium acetate (0.98 mole), and acetic anhydride (2.9 moles) in a flask on an electric hot plate (6, 7). As soon as the material had liquefied completely, the flask was transferred to a steam bath and heated for 2 hr. The reaction mixture was allowed to stand overnight, and the solid mass which precipitated was filtered with suction and washed first with two 100-ml. portions of ice-cold ethanol and finally with two 100-ml. portions of boiling water. After drying, the various oxazolones were used without further purification.

α-Benzoylamino-*N*-(substituted esters)-substituted Cinnamides (I–XII)—Equimolar quantities (1 mole) of the appropriate 2-phenyl-4-(substituted benzylidene)-oxazole-5-ones and ethyl esters of amino acids were refluxed in absolute ethanol in the presence of 2–3 drops of triethylamine on a steam bath for 6–8 hr. Excess ethanol was distilled, and the cinnamides which separated on cooling were filtered and recrystallized with either ethanol or the mixture of ethanol and water. The cinnamides were characterized by their sharp melting points and elemental analyses (Table I).

α-Benzoylamino-*N*-(substituted hydrazides)-substituted Cinnamides (XIII–XXIV)—A mixture of the appropriate α-benzoylamino-*N*-(substituted esters)-substituted cinnamides (0.1 mole) and 99–100% hydrazine hydrate (0.12 mole) in absolute ethanol was refluxed on a water bath for 6–8 hr. The solid mass which separated on cooling was filtered and recrystallized from suitable solvents (Table II).

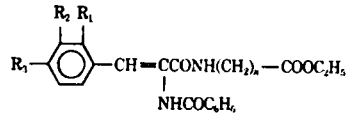


Table I—Physical Constants of α-Benzoylamino-*N*-(substituted esters)-substituted Cinnamides

Compound Number	R_1	R_2	R_3	*n*	Melting Point[a]	Yield, %	Recrystallization Solvent	Formula	Analysis, % Calc.	Found
I	H	H	H	1	114–115°	70	Ethanol–water	$C_{20}H_{20}N_2O_4$	C 68.01	67.96
									H 5.68	5.38
									N 7.95	7.56
II	Cl	H	H	1	105°	75	Ethanol–water	$C_{20}H_{19}ClN_2O_4$	C 62.09	62.13
									H 4.91	5.03
									N 7.24	7.03
III	H	H	Cl	1	200–202°	80	Ethanol	$C_{20}H_{19}ClN_2O_4$	C 62.09	61.09
									H 4.91	4.63
									N 7.24	6.95
IV	H	Cl	Cl	1	199–200°	80	Ethanol	$C_{20}H_{18}Cl_2N_2O_4$	C 57.00	56.56
									H 4.27	4.13
									N 6.65	6.77
V	H	H	H	2	135°	79	Ethanol–water	$C_{21}H_{22}N_2O_4$	C 68.85	68.72
									H 6.01	6.11
									N 7.65	7.25
VI	Cl	H	H	2	125°	75	Ethanol–water	$C_{21}H_{21}ClN_2O_4$	C 62.89	62.65
									H 5.24	5.24
									N 6.98	6.80
VII	H	H	Cl	2	191–192°	80	Ethanol	$C_{21}H_{21}ClN_2O_4$	C 62.89	62.77
									H 5.24	5.34
									N 6.98	6.78
VIII	H	Cl	Cl	2	190°	82	Ethanol	$C_{21}H_{20}Cl_2N_2O_4$	C 57.93	57.22
									H 4.59	4.09
									N 6.43	6.07
IX	H	H	H	3	144–145°	70	Ethanol–water	$C_{22}H_{24}N_2O_4$	C 69.47	69.04
									H 6.31	6.08
									N 7.36	7.22
X	Cl	H	H	3	122–123°	75	Ethanol–water	$C_{22}H_{23}ClN_2O_4$	C 63.69	62.97
									H 5.54	5.33
									N 6.75	6.66
XI	H	H	Cl	3	116–117°	75	Ethanol	$C_{22}H_{23}ClN_2O_4$	C 63.69	63.57
									H 5.54	5.34
									N 6.75	6.63
XII	H	Cl	Cl	3	140–141°	80	Ethanol	$C_{22}H_{22}Cl_2N_2O_4$	C 58.79	58.05
									H 4.89	4.72
									N 6.23	6.00

[a] All melting points were taken in open capillary tubes and are corrected.

PHARMACOLOGY

Anticonvulsant activity was determined in mice of either sex weighing 25–30 g. The mice were divided in groups of 10, keeping the group weights as near the same as possible. Each hydrazide or its precursor ester was suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compound was injected in a group of 10 animals at a dose of 100 mg./kg. i.p. Four hours after administration of the hydrazide or ester, the mice were injected with pentylenetetrazol (90 mg./kg. s.c.). This dose of pentylenetetrazol has been shown not only to produce convulsions in almost all untreated mice but also to exhibit 100% mortality during 24 hr. On the other hand, no mortality was observed during 24 hr. in animals treated with 100 mg./kg. of the test compounds alone. The mice were then observed 60 min. for seizures. An episode of clonic spasm that persisted at least 5 sec. was considered a threshold convulsion. Transient intermittent jerks or tremulousness was not counted. Animals devoid of threshold convulsions during the 60 min. were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of these α-benzoylamino-*N*-(substituted hydrazides)-substituted cinnamides and their corresponding esters was represented as percent protection. The animals were then observed for 24 hr. and their mortality was recorded.

BIOCHEMISTRY

Spectrophotofluorometric Method—A spectrophotofluorometric method was used for the determination of monoamine oxidase activity of rat brain homogenate, using kynuramine as the substrate (8). The 4-hydroxyquinoline formed during oxidative deamination of kynuramine was measured fluorometrically with a spectrophotofluorometer[1], using activating light of 315 nm. and measuring fluorescence at the maximum of 380 nm.

Male adult rats, weighing approximately 150–200 g., were killed by decapitation. Brains were quickly removed and homogenized in ice-cold 0.25 *M* sucrose with the Potter–Elvehjem homogenizer. The reaction mixture, in a final concentration, consisted of 0.5 ml. phosphate buffer (0.2 *M*, pH 7.5), 1×10^{-4} *M* kynuramine, and 0.5 ml. brain homogenate (equivalent to 10 mg. of wet weight of the tissue). The monoamine oxidase activity of the brain homogenate was determined by incubation at 37° in air for 30 min. The various α-benzoylamino-*N*-(substituted hydrazides)-substituted cinnamides were added to the brain homogenate to produce a final concentration of 5×10^{-4} *M* and incubated for 10 min. before adding kynuramine. The mixture was then incubated for an additional 30 min. The reaction was stopped by the addition of 1 ml. of 10% trichloroacetic acid (w/v), and the precipitated proteins were removed by centrifugation. Suitable 1-ml. aliquots of the supernate were taken in 2 ml. of 1 *N* NaOH solution and were assayed for 4-hydroxyquinoline. An increase in the absorbance provided a direct measurement of the 4-hydroxyquinoline formation, which was taken as an index of the enzyme activity. The percent inhibition was calculated from the decrease observed in the absorbance and this provided an index of the inhibitory property of these α-benzoylamino-*N*-(substituted hydrazides)-substituted cinnamides.

Warburg Manometric Method—The monoamine oxidase activity of rat brain homogenate was determined by the conventional Warburg manometric technique, using tyramine as the substrate (9). The decrease in the oxygen uptake for 1 hr. during oxidative deamination of tyramine in the presence of substituted cinnamides was used as an index of enzyme inhibition. The reaction mixture,

[1] Aminco-Bowman.

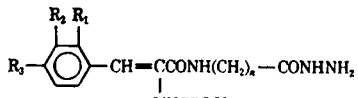


Table II—Physical Constants of α-Benzoylamino-*N*-(substituted hydrazides)-substituted Cinnamides

Compound Number	R_1	R_2	R_3	*n*	Melting Point[a]	Yield, %	Recrystallization Solvent	Formula	Analysis, % Calc.	Found
XIII	H	H	H	1	217°	60	Ethanol	$C_{18}H_{18}N_4O_3$	C 63.90	63.73
									H 5.32	5.03
									N 16.56	16.45
XIV	Cl	H	H	1	250–251°	60	Ethanol	$C_{18}H_{17}ClN_4O_3$	C 58.01	58.31
									H 4.56	4.41
									N 15.03	15.00
XV	H	H	Cl	1	236°	70	Butanol	$C_{18}H_{17}ClN_4O_3$	C 58.01	57.93
									H 4.56	4.50
									N 15.03	14.91
XVI	H	Cl	Cl	1	210°	80	Ethanol	$C_{18}H_{16}Cl_2N_4O_3$	C 53.07	53.12
									H 3.93	3.72
									N 13.75	13.62
XVII	H	H	H	2	171°	73	Ethanol	$C_{19}H_{20}N_4O_3$	C 64.77	64.63
									H 5.68	5.24
									N 15.90	15.72
XVIII	Cl	H	H	2	191°	69	Ethanol	$C_{19}H_{19}ClN_4O_3$	C 58.99	58.77
									H 4.91	4.70
									N 14.48	14.03
XIX	H	H	Cl	2	205–206°	65	Ethanol	$C_{19}H_{19}ClN_4O_3$	C 58.99	58.63
									H 4.91	4.81
									N 14.48	14.32
XX	H	Cl	Cl	2	209–210°	70	Ethanol	$C_{19}H_{18}Cl_2N_4O_3$	C 54.15	54.03
									H 4.27	4.07
									N 13.30	13.36
XXI	H	H	H	3	185°	80	Ethanol	$C_{20}H_{22}N_4O_3$	C 68.30	68.14
									H 6.01	6.15
									N 15.30	15.41
XXII	Cl	H	H	3	260°	76	Ethanol	$C_{20}H_{21}ClN_4O_3$	C 59.97	59.27
									H 5.24	5.02
									N 13.58	13.09
XXIII	H	H	Cl	3	249–250°	75	Ethanol	$C_{20}H_{21}ClN_4O_3$	C 59.97	59.76
									H 5.24	5.32
									N 13.58	13.42
XXIV	H	Cl	Cl	3	229–230°	80	Ethanol	$C_{20}H_{20}Cl_2N_4O_3$	C 55.17	55.21
									H 4.59	4.09
									N 12.87	12.67

[a] All melting points were taken in open capillary tubes and are corrected.

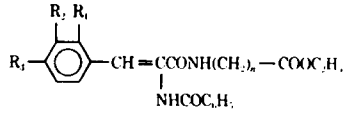
in a final concentration, consisted of 50 m*M* phosphate buffer (pH 7.4), 5 m*M* tyramine, and the homogenate (equivalent to 100 mg. of fresh tissue) in a total volume of 1.2 ml. The inhibitors, used at a final concentration of 1×10^{-3} *M*, were incubated with the enzyme preparation for 20 min. before adding the substrate. The enzyme system was then incubated an additional hour at 37° using oxygen as the gaseous phase. Readings of the oxygen uptake were taken every 10 min.

RESULTS AND DISCUSSION

In the present study, α-benzoylamino-*N*-(substituted esters)-substituted cinnamides and their corresponding hydrazides were synthesized and evaluated for anticonvulsant activity. The degree of protection afforded by these compounds against pentylenetetrazol-induced seizures and the 24-hr. mortalities are recorded in Tables III and IV. The data seem to indicate a trend of more protection from convulsions with less mortality; however, it is not sufficiently uniform to conclude that definitely (*e.g.*, Table III: 0% protection = 70% mortality, 10% protection = 90% mortality, and 30% protection = 70% mortality). Each hydrazide or its precursor ester was found to be devoid of not only the sedative or central nervous system (CNS) depressant effect but also of 24-hr. mortality in the 100-mg./kg. dose used in the present study. Thus, 24-hr. mortality in these experimental animals accounts for the toxic effects of pentylenetetrazol.

These results indicate a greater anticonvulsant activity of α-benzoylamino-*N*-(substituted hydrazides)-substituted cinnamides as compared to their corresponding esters. These esters were either devoid of activity or showed less than 30% protection. The protection afforded by these hydrazides ranged from 30 to 70%, where the greatest protection was observed when the aliphatic chain of these α-benzoylamino-*N*-(substituted hydrazides)-substituted cinnamides possessed the glycine hydrazide moiety (Compound XIII). Elongation of the aliphatic chain resulted in a decrease of anticonvulsant activity. Evaluation of monoamine oxidase inhibitory activity of these hydrazides with a view to elucidating the biochemical basis for anticonvulsant activity indicated that all α-benzoylamino-

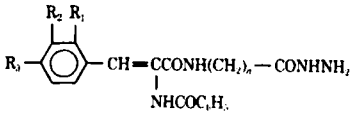
Table III—Anticonvulsant Activity of α-Benzoylamino-*N*-(substituted esters)-substituted Cinnamides[a]



Compound Number	Anticonvulsant Activity, % Protection	Pentylenetetrazol Mortality, %
I	10	80
II	20	70
III	20	80
IV	30	70
V	10	90
VI	Nil	70
VII	10	70
VIII	10	70
IX	20	60
X	30	60
XI	20	70
XII	10	70

[a] The compound numbers are those recorded in Table I. The experimental procedures are as indicated in the text.

Table IV—Anticonvulsant and Monoamine Oxidase Inhibitory Properties of α-Benzoylamino-*N*-(substituted hydrazides)-substituted Cinnamides[a]

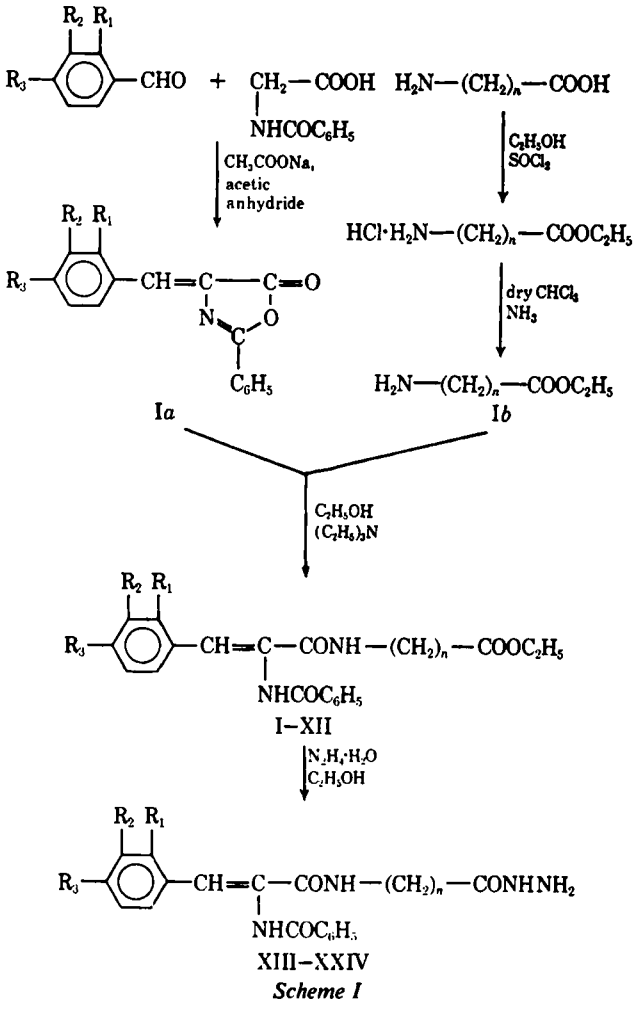


Compound Number	Anticonvulsant Activity, % Protection	Pentylenetetrazol Mortality, %	Monoamine Oxidase Inhibition, % Kynuramine[a]	Tyramine[a]
XIII	70	30	44.5 ± 0.85	18.5 ± 0.68
XIV	60	50	56.0 ± 0.76	24.0 ± 0.32
XV	50	70	51.2 ± 1.21	23.0 ± 0.43
XVI	40	40	22.2 ± 0.44	19.5 ± 0.65
XVII	20	80	25.8 ± 0.21	26.6 ± 0.43
XVIII	40	60	19.8 ± 0.66	17.3 ± 0.77
XIX	40	50	17.4 ± 0.58	26.0 ± 0.85
XX	50	60	15.9 ± 0.44	23.7 ± 0.43
XXI	50	60	40.2 ± 0.98	24.0 ± 0.24
XXII	40	70	55.0 ± 1.0	45.6 ± 1.23
XXIII	50	60	51.5 ± 0.78	26.4 ± 0.21
XXIV	30	60	80.8 ± 0.65	42.3 ± 1.11

[a] Compound numbers are those recorded in Table II. The final concentrations of the inhibitors used were 5×10^{-4} and 1×10^{-3} *M* during oxidative deamination of kynuramine and tyramine, respectively. Vessel contents and assay procedures are as indicated in the text. Each experiment was done in duplicate, and figures indicate mean values of three separate experiments with ± standard error of the mean.

N-(substituted hydrazides)-substituted cinnamides inhibited *in vitro* oxidative deamination of kynuramine and tyramine by rat brain homogenate. The results, indicating greater sensitivity of these hydrazides for monoamine oxidase when kynuramine was used as the substrate, are in agreement with other studies (1, 10) which also found greater degree of inhibition with kynuramine as compared to tyramine from the α-benzoylamino-*N*-(1-benzhydrazide)-*p*-substituted cinnamides (4).

Elongation of the aliphatic chain was found to have no significant effect on the monoamine oxidase inhibitory effectiveness of α-benzoylamino-*N*-(substituted hydrazides)-substituted cinnamides. Maximum inhibition was observed with α-benzoyl-*N*-(substituted hydrazides)-substituted cinnamides possessing the *ortho*-chloro substituent (Compound XXII) using tyramine, while substituted cinnamide possessing chloro substituents at both the 2- and 3-positions of the benzene ring (Compound XXIV) exhibited maximum inhibition of monoamine oxidase during oxidative deamination of kynuramine. CNS stimulation, a property associated with many monoamine oxidase inhibitors, was not observed with these compounds in the doses employed, presumably due to a relatively weak monoamine oxidase inhibitory activity of these compounds. These data failed to provide a relationship between anticonvulsant activity of α-benzoyl-*N*-(substituted hydrazides)-substituted cinnamides and their ability to inhibit monoamine oxidase. Further studies dealing with the detailed pharmacological and toxicological properties and the effects upon the activity of other enzymes may possibly reflect a biochemical basis for anticonvulsant activity of these substituted cinnamides.



Scheme I

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Simultaneous Polarographic Determination of Bisulfite and Iproniazid in Gel Formulations

J. J. DONAHUE▲ and S. OLIVERI-VIGH*

Abstract □ A polarographic method is described for the simultaneous determination of sodium bisulfite and iproniazid in gel formulations. The standard deviation for the bisulfite determination was ±0.00₂% and the precision *ts* at the 95% confidence level was ±0.00₅%. The standard deviation for the iproniazid determination was ±0.03% and the precision *ts* at the 95% confidence level was ±0.07%.

Keyphrases □ Iproniazid–bisulfite—simultaneous polarographic determination □ Bisulfite–iproniazid—simultaneous polarographic determination □ Polarography—simultaneous determination of iproniazid and bisulfite in gel formulations

Due to the pharmaceutical importance of isoniazid and its derivatives, the analysis of this class of compounds has received considerable attention in the literature. The polarographic and spectrophotometric behavior of these compounds was investigated (1), and it was found that isoniazid exhibited two polarographic reduction waves in the pH 1.5–7 range. At pH >8, these waves merged to a single wave. By using the sum of both polarographic waves, isoniazid in tablets was determined with a precision of ±1.0% (1). No interference from excipients was noted. By using UV spectrophotometry, isoniazid in tablets was measured with a precision of about 1–2% (1).

A photometric procedure for the determination of iproniazid and other hydrazide derivatives, based on a reaction with molybdic acid in an acidic medium to form a red complex, also was reported (2). While the method is specific and sensitive, the concentration of acid is critical for the development of maximum color intensity.

Many methods have been reported which are based on the quantitative oxidation of the hydrazine moiety using bromine (3), iodine (4, 5), iodate (6), and nitrite (7). Recently, a titrimetric method for isoniazid and its derivatives was reported (8), which is based on the oxidation of isoniazid with excess chloramine T and back-titration. These methods all lack specificity, however, particularly when other reducing substances are present.

The most frequently used methods for the determination of bisulfite and sulfite are oxidation and subsequent determination as sulfate and direct oxidation with iodine (9). As with methods for hydrazine derivatives, these methods are also subject to interferences from other reducing substances. To avoid this problem in the determination of sulfite, a polarographic procedure was developed (10) based on the conversion of sulfite to sulfur dioxide and subsequent polarographic determination of the sulfur dioxide. The method was applied to the determination of sulfite in sewage water; however, both sulfide and thiosulfate were sources of interference.

Drug formulations containing bisulfite, as an antioxidant, and isoniazid or its derivatives do not readily lend themselves to present compendial (5) methods since simultaneous oxidation of both species occurs. The present article describes a polarographic method for the simultaneous determination of bisulfite and iproniazid which eliminates the problem of mutual interference often encountered when these species are present in the same formulation.

EXPERIMENTAL

Apparatus—A polarographic assembly[1] and a recorder[2] were used in conjunction with a dropping mercury electrode and a silver–silver chloride reference electrode (Ag/AgCl) to obtain the current voltage curves.

A digital pH meter[3] was used for all pH measurements.

The dropping mercury electrode characteristics, *m* and *t*, were determined in 0.25 *M* ammonium chloride solution at a potential of −1.0 v. *versus* Ag/AgCl and at a mercury column height of 56 cm.; *m* was 3.60 mg./sec. and *t* was 2.68 sec.

Reagents and Solutions—Triple-distilled mercury[4] was used in all polarographic experiments. Demineralized water was used to prepare all solutions. This water was prepared by passing ordinary distilled water through a demineralizer[5].

A pH 8 buffer was prepared by mixing 100 ml. of a stock solution containing phosphoric acid[6], acetic acid[7], and boric acid[7], each at a concentration of 0.04 *M*, with 62.5 ml. of 0.2 *N* sodium hydroxide[8] solution.

A 10% hydrochloric acid solution was prepared by diluting 236 ml. of concentrated hydrochloric acid[9] to 1000 ml. with demineralized water.

A stock reference standard solution of sodium metabisulfite[10] was prepared by accurately weighing approximately 40 mg. of sodium metabisulfite into a 100-ml. volumetric flask and diluting to volume with demineralized water. Ten milliliters of this solution was then diluted to 100 ml. with 40 ml. of buffer and approximately 50 ml. of demineralized water.

A stock reference standard solution of iproniazid phosphate was prepared by accurately weighing approximately 60 mg. of iproniazid phosphate into a 100-ml. volumetric flask and diluting to volume with 40.0 ml. of buffer and 60 ml. of deaerated demineralized water.

Procedure—Approximately 2 g. of the gel sample was transferred to a tared 50-ml. volumetric flask and weighed. Twenty milliliters of deaerated buffer was added, and the sample was shaken for approximately 30 min. to ensure extraction of the active substance

[1] Metrohm E-354.
[2] Polarecord model E-261.
[3] Orion model 801.
[4] Bethlehem Apparatus Co., Hellertown, Pa.
[5] Barnstead Bantam model BD with standard No. 0802 cartridge.
[6] Mallinckrodt analytical reagent.
[7] Baker reagent grade.
[8] Fisher reagent grade.
[9] Matheson, Coleman and Bell, ACS reagent.
[10] Baker and Adamson, reagent grade.

Table I—Polarographic Determination of Bisulfite in 1% Iproniazid Gel

Sample	Bisulfite Found[a]
1	0.06_1
2	0.05_7
3	0.06_3
4	0.06_5
5	0.06_3
6	0.06_3
7	0.06_1
8	0.05_7
9	0.06_5
10	0.06_1
11	0.06_2
12	0.06_5
13	0.05_9
14	0.06_1
15	0.06_5
16	0.06_5
17	0.06_1
18	0.06_1
Average	0.06_2
Standard deviation	$\pm 0.00_2$
Precision[b]	$\pm 0.00_5$

[a] Theoretical bisulfite = 0.10%. [b] *ts* for 17 degrees for freedom at the 95% confidence level.

from the gel matrix. The sample was then diluted to volume with deaerated demineralized water. Ten milliliters of this solution was pipeted into the polarographic cell and deaerated with prepurified tank nitrogen[11] for approximately 5 min. The nitrogen flow was then reversed to the solution surface, and 1 ml. of 10% hydrochloric acid was added and mixed into the solution. The polarogram for bisulfite as sulfur dioxide was immediately recorded using a voltage span of from 0 to −1.0 v. *versus* Ag/AgCl, a mercury column height of 56.0 cm., and a current sensitivity of 0.02 μa./mm. When the polarogram was completed, the solution was vigorously deaerated with nitrogen to eliminate the sulfur dioxide formed from the bisulfite, and a residual current curve was obtained using the same polarographic conditions.

Polarograms for iproniazid were recorded using the same solution. The instrument conditions were the same, except that the current sensitivity was 0.2 μa./mm.

Polarograms were also recorded for the sodium metabisulfite and iproniazid phosphate reference standards using the same instrument conditions.

Calculations—The diffusion currents were measured by standard procedures (11). The percent bisulfite in the formulation was calculated as follows:

$$\text{percent bisulfite} = \frac{(i_s)(C_{st})(5)(1.095)}{(i_{st})(\text{weight sample, grams})} \quad \text{(Eq. 1)}$$

where:

i_s = diffusion current (microamperes) for sample
C_{st} = concentration of standard (milligrams per milliliter)
5 = dilution factor and conversion to percent
1.095 = conversion factor for metabisulfite to bisulfite
i_{st} = diffusion current (microamperes) for standard

The percent iproniazid in the formulation was calculated as follows:

$$\text{percent iproniazid} = \frac{(i_s)(C_{st})(5)(0.6465)}{(i_{st})(\text{weight sample, grams})} \quad \text{(Eq. 2)}$$

where:

i_s = diffusion current (microamperes) for sample
C_{st} = concentration of standard (milligrams per milliliter)
5 = dilution factor and conversion to percent

[11] Union Carbide Corp., Linde Division.

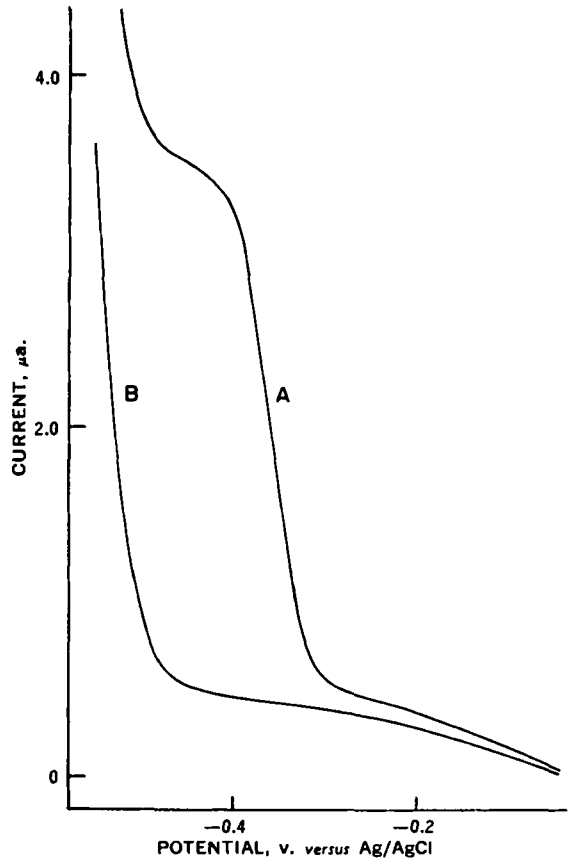

Figure 1—*Polarograms for bisulfite determination. Key: A, typical reduction wave for sulfur dioxide; and B, typical residual current curve obtained after deaeration.*

0.6465 = conversion factor for iproniazid phosphate to base
i_{st} = diffusion current (microamperes) for standard

RESULTS AND DISCUSSION

Polarographic Determination of Bisulfite—In acidic media, the gel sample exhibited three reduction waves. The first wave, which occurred at −0.38 v. *versus* Ag/AgCl (Fig. 1), may be attributed to the reduction of sulfur dioxide liberated from the bisulfite stabilizer under acidic conditions (Scheme I).

$$HSO_3^- + H^+ \rightarrow SO_2 + H_2O$$

Scheme I

According to Kolthoff and Miller (12), the resulting sulfur dioxide is reduced in a single two-electron step to sulfoxylic acid (Scheme II).

$$SO_2 + 2H^+ + 2e^- \rightarrow H_2SO_2$$

Scheme II

The metabisulfite standard, under the same solution conditions, is readily converted to bisulfite (Scheme III), which subsequently reacts as shown in Schemes I and II.

$$S_2O_5^{-2} + H_2O \rightarrow 2HSO_3^-$$

Scheme III

The polarographic results obtained for the bisulfite stabilizer are presented in Table I. The average for 18 determinations was 0.06_2% with a standard deviation of $\pm 0.00_2$% and a precision (*ts*) for 17 degrees of freedom at the 95% confidence level of $\pm 0.00_5$%.

The obvious discrepancy in bisulfite added (0.1%) and bisulfite found is most likely due to the unavoidable losses of bisulfite during formulation. While the final formulation is a neutralized gel

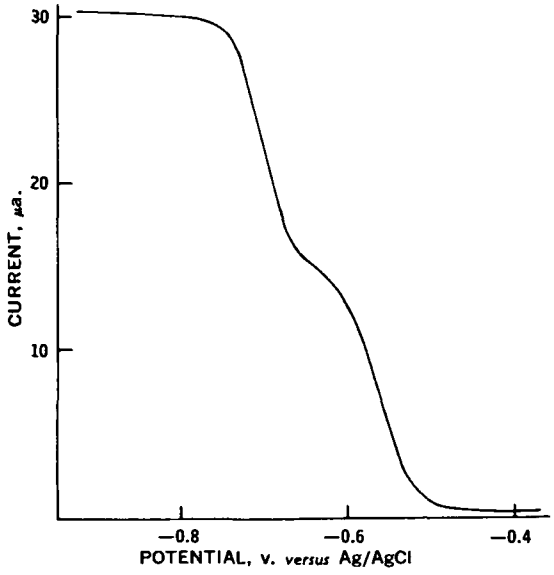

Figure 2—*Typical polarogram for iproniazid.*

medium, the pH of the formulation is acidic until the final step in the preparation due to the presence of iproniazid phosphate. This acidic pH would most likely cause losses of bisulfite as SO_2 (Scheme I).

Polarographic Determination of Iproniazid—The two additional reduction waves, observed at −0.54 and −0.72 v. *versus* Ag/AgCl in an acidic medium (Fig. 2), are due to the reduction of iproniazid (1). Although the half-wave potentials for the sulfur dioxide wave and the first reduction wave for iproniazid are separated by only 160 mv., vigorous deaeration of the solution after measurement of the sulfur dioxide wave eliminates any possible interference from sulfur dioxide in the subsequent measurement of the diffusion current for iproniazid.

Polarograms for the bulk drug at various concentrations indicated that the total current for the two reduction waves was proportional to concentration in the 0.05–0.6-mg./ml. range. A plot of diffusion current, i_d, *versus* concentration, milligrams per milliliter, was linear with a slope equal to 30 μa. ml./mg.

In contrast to the polarographic results obtained for bisulfite, the results obtained for iproniazid indicated that the determination was dependent on the extraction time used to free the hydrazide from the gel matrix. Initially, samples were extracted by shaking for approximately 5 min. prior to scanning on the polarograph. The average assay value for eight determinations was 0.95% with a standard deviation of ±0.06% and a precision (*ts*) for seven degrees of freedom at the 95% confidence level of ±0.13% (Table II). When the extraction time was increased to approximately 30 min., the average for 10 determinations was 0.98% with a standard deviation of ±0.03% and a precision (*ts*) for nine degrees of freedom at the 95% confidence level of ±0.07% (Table II).

Table II—Effect of Extraction Time on Percent Iproniazid Found

Extracted 5 min.		Extracted 30 min.	
Sample	Percent Found[a]	Sample	Percent Found[a]
1	0.89	1	0.99
2	0.84	2	1.00
3	1.00	3	1.02
4	0.99	4	0.96
5	0.99	5	0.97
6	0.96	6	0.97
7	0.95	7	0.99
8	0.98	8	0.99
		9	0.98
		10	0.96
Average	0.95		0.98
Standard deviation	±0.06		±0.03
Precision	±0.13[b]		±0.07[c]

[a] Theoretical iproniazid = 1.00%. [b] *ts* for seven degrees of freedom at the 95% confidence level. [c] *ts* for nine degrees of freedom at the 95% confidence level.

CONCLUSIONS

The advantages of the described method over procedures for bisulfite and hydrazine derivatives that are based on direct oxidation with iodine are twofold. The primary advantage is that it eliminates the mutual interference problem between bisulfite and iproniazid which may be encountered in methods based on oxidation. Secondly, the present method allows both the active hydrazine substance and the stabilizer used in the formulation to be determined in the same sample preparation. As a result, this procedure significantly reduces the overall time required for the dosage form analysis when both the active substance and the antioxidant must be determined.

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Determination of Diphenhydramine Hydrochloride in Elixir

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Abstract □ A UV spectrophotometric method for the determination of diphenhydramine hydrochloride in the presence of its postulated decomposition products and 5-(hydroxymethyl)-2-furaldehyde in elixirs was developed. The method involves simple extractions with cyclohexane and is suitable for routine and stability assays of various pharmaceutical liquid formulations. The technique is a modification of the USP XVIII method.

Keyphrases □ Diphenhydramine hydrochloride elixir—UV analysis in presence of 5-(hydroxymethyl)-2-furaldehyde □ 5-(Hydroxymethyl)-2-furaldehyde as elixir decomposition product—noninterference in UV analysis of diphenhydramine hydrochloride elixir □ UV spectrophotometry—analysis, diphenhydramine hydrochloride elixir

5-(Hydroxymethyl)-2-furaldehyde (1–3) is a well-known reaction product of the Maillard reaction. It can also be formed by the acid–base-catalyzed dehydration of hexose sugars. 5-(Hydroxymethyl)-2-furaldehyde is frequently present in pharmaceutical preparations, particularly in products containing sugar syrups. Its presence can interfere in the chemical analysis for the active ingredients.

USP XVIII (4) describes a UV assay for diphenhydramine hydrochloride elixir. This method was found to be inapplicable to certain samples undergoing stability tests. With increasing storage temperatures, consistently higher results were obtained. The separation of the base was reported (5, 6) on a cation exchanger, followed by quantitative spectrophotometric determination. A UV method was developed (7) to measure the steam-distillable compound after hydrolysis and oxidation of diphenhydramine. Colorimetric methods have also been reported. An addition compound formation between diphenhydramine and tetrabromophenolphthalein ethyl ether in 1,2-dichloroethane was used (8), as has ion-pair formation between the amine and an indicator dye (9). The reineckate was prepared in acetone (10). Nonaqueous titrimetry and salt partition was used in procedures for analysis of organic bases (11, 12). Quantitative TLC also was described (13). However, none of these methods has been suggested for stability-indicating purposes.

The present study describes a modification of the official assay procedure for the determination of the diphenhydramine content in elixirs containing postulated decomposition products and 5-(hydroxymethyl)-2-furaldehyde. The amine is separated by extraction from alkaline medium into cyclohexane, purified by washing and by filtration through anhydrous sodium sulfate before being extracted into dilute aqueous acid, and determined by UV spectrophotometry.

EXPERIMENTAL

Materials—The following were used: cyclohexane, spectrograde; 2 *N* sulfuric acid; 1% sodium hydroxide; 0.1 *N* sulfuric acid; 10% sodium hydroxide; and diphenhydramine hydrochloride USP standard reference solution, 0.5 mg./ml. in 0.1 *N* sulfuric acid. The following materials were obtained commercially[1] and used without additional purification: 5-(hydroxymethyl)-2-furaldehyde, diphenylcarbinol, and *N,N*-dimethylethanolamine.

Apparatus—A spectrophotometer[2] was employed.

Procedure—Transfer quantitatively 20 ml. of the elixir, equivalent to 50 mg. of diphenhydramine hydrochloride, into a separator. Add 30 ml. of distilled water and 1 ml. of 2 *N* sulfuric acid and extract with two 25-ml. portions of cyclohexane, discarding the cyclohexane extracts.

To the aqueous phase, add 10 ml. of 10% sodium hydroxide; then extract with three 30-ml. portions of cyclohexane and combine. Wash the combined cyclohexane extracts with two 10-ml. portions of 1% sodium hydroxide, discarding the washings. Extract the cyclohexane with three 25-ml. portions of 0.1 *N* sulfuric acid, collecting the extracts in a 100-ml. volumetric flask. Dilute to the mark with 0.1 *N* sulfuric acid and mix well.

Measure the absorbance difference between the maximum at 258 nm. and the baseline at 290 nm. in 1-cm. cells using 0.1 *N* sulfuric acid as blank. The diphenhydramine hydrochloride content of the sample is calculated from a relative absorptivity value derived by dissolving appropriate amounts of standard reference material in 0.1 *N* sulfuric acid and measuring spectrophotometrically under the same operational conditions as described for the assay procedure.

RESULTS AND DISCUSSION

The browning of the elixirs and a spectral change (Fig. 1) of

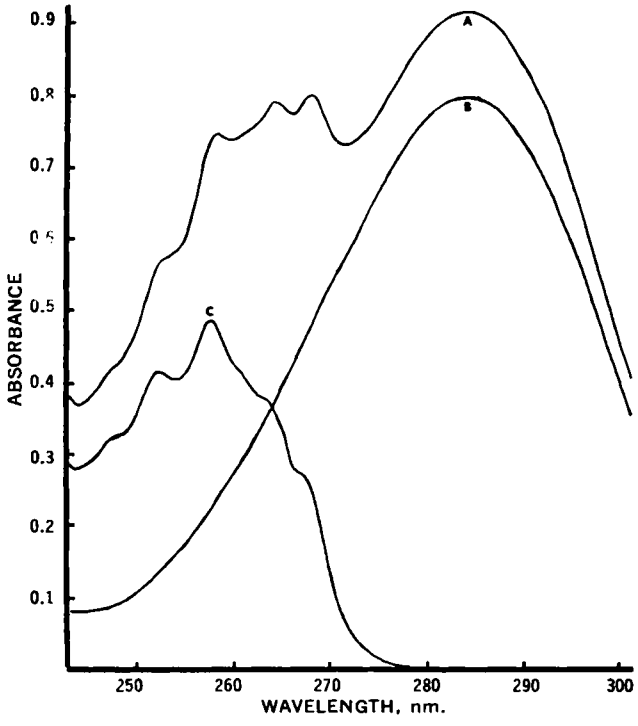

Figure 1—*UV spectra of mixture of diphenhydramine hydrochloride and 5-(hydroxymethyl)-2-furaldehyde (A), 5-(hydroxymethyl)-2-furaldehyde (B), and diphenhydramine hydrochloride (C). All solutions were prepared in 0.1* N H_2SO_4.

[1] Aldrich Chemical Co.
[2] Cary model 15.

Table I—Analysis of Synthetic Mixtures

Number of Assay	Diphenhydramine, mg. Added	Recovery, % Modified Method	Recovery, % USP Method
1	49.92	98.9	97.8
2	49.56	99.1	97.7
3	50.13	99.3	98.3
4	50.09	99.3	98.5
5	50.09	99.4	98.5
6	50.09	99.6	98.8
Percent recovered ± *SD*		99.3 ± 0.2	98.3 ± 0.4

Table II—Analysis of Synthetic Mixtures in the Presence of 5-(Hydroxymethyl)-2-furaldehyde

Number of Assay	Diphenhydramine, mg. Added	5-(Hydroxymethyl)-2-furaldehyde, mg. Added	Recovery, % Modified Method	Recovery, % USP Method
1	50.18	1.0	99.1	113.6
2	50.18	3.0	100.7	141.3
3	50.14	5.0	100.6	162.5
4	50.30	10.0	101.1	230.6
5	50.01	30.0	99.9	508.7
6	49.99	60.0	100.2	957.9

diphenhydramine, in which the absorption maximum shifted from 258 to 283 nm., led to an investigation of 5-(hydroxymethyl)-2-furaldehyde (I). Compound I had been isolated from the elixirs through thick-layer chromatography and identified by UV and IR absorption spectra, TLC, and the color reaction (14) with 2-thiobarbituric acid.

The postulated decomposition pathways for diphenhydramine would be hydrolysis of the ether linkage with formation of diphenylcarbinol and *N,N*-dimethylethanolamine. A study of the alkaline and acid hydrolysis of diphenhydramine was made, but only one of the postulated degradation products was isolated. The melting point, IR absorption spectrum, thin-layer chromatogram, and molar absorptivity data on this product coincide with those described for diphenylcarbinol. However, the presence of *N,N*-dimethylethanolamine was not positively confirmed.

Nevertheless, both postulated decomposition products and I have been taken into consideration as possible interferences. Diphenylcarbinol is practically insoluble in weak sulfuric acid, but it is soluble in cyclohexane and is removed by the initial cyclohexane extractions of the aqueous phase. On the other hand, I is very soluble in aqueous alkaline medium but is insoluble in cyclohexane; therefore, it remains in the aqueous phase throughout the procedure. The possible basic decomposition product, *N,N*-dimethylethanolamine, does not absorb UV radiation in the range employed.

To determine the validity of the method, a synthetic elixir was prepared by mixing a known quantity of diphenhydramine (50 mg. of pure drug) with excipients. Diphenhydramine was then assayed by the modified and USP methods. From the six determinations, the average percent recoveries were 99.3 ± 0.2 *SD* for the modified method and 98.3 ± 0.4 *SD* for the USP method. Both methods are in excellent agreement with each other, as shown in Table I.

The modified and the USP methods are both capable of eliminating the postulated decomposition products. To verify this fact, 1.7×10^{-4} mole each of diphenylcarbinol and *N,N*-dimethylethanolamine were added to the synthetic mixture and then carried through both procedures. No interfering absorbance between 300 and 235 nm. was observed. The specified amount of postulated decomposition products was used on the assumption that 100% hydrolysis of diphenhydramine would have taken place.

Synthetic mixtures in the presence of 1, 3, 5, 10, 30, and 60 mg. of I were assayed by both methods. The modified method gives results near the true values for the diphenhydramine concentration, whereas the method described by USP XVIII gives results that are far too high. The data in Table II indicate that the extraction of diphenhydramine by the USP method from an elixir containing a small amount of I results in a considerable error. This is due to the much greater absorption sensitivity of I.

Table III—Percent Recovery of Diphenhydramine Hydrochloride from Different Stability Samples

Sample	Storage Condition: Period	Storage Condition: Temperature	Recovery, % Modified Method	Recovery, % USP Method
Experimental elixir	Initial	Room temperature	100.0	98.5
	2 weeks	60°	98.7	97.9
	3 weeks	60°	99.2	101.1
	4 weeks	60°	99.6	98.7
Commercial elixir A	Initial	Room temperature	100.2	98.7
	2 weeks	60°	101.0	100.9
	3 weeks	60°	100.3	103.4
	4 weeks	60°	98.3	107.6
Commercial elixir B	Initial	Room temperature	100.6	100.8
	1 day	60°	100.3	102.0
	2 days	60°	100.0	103.1
	3 days	60°	100.3	103.8
	4 days	60°	99.9	104.6
	7 days	60°	100.1	111.6

The percent recoveries (Table III) obtained by using both the modified and USP methods for the stability samples of the experimental elixir are very similar. This similarity is expected, since the experimental elixir does not contain sucrose. The results for the stability samples of commercial Elixirs A and B (Table III) show that the percent recovery obtained by the USP method increases with storage time, while that obtained by the modified method remains constant. This could be explained simply on the basis that the commercial elixirs contained sucrose and that the I formed therefrom was removed by the modified method prior to determination. The results presented in Table III indicate the applicability and accuracy of the modified method for the assay of stability samples.

Based on our experience, the modified method provides definite improvement over the USP method. It is, therefore, suggested that the modified procedure may be considered as a stability-indicating method for the determination of diphenhydramine in various pharmaceutical liquid formulations.

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Quantitative GLC Analysis of Theophylline, Ephedrine Hydrochloride, and Phenobarbital Suspension

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Abstract □ A rapid GLC method was developed for the quantitative determination of theophylline, ephedrine hydrochloride, and phenobarbital in a suspension formulation. A simple extraction procedure was incorporated to separate ephedrine hydrochloride into one fraction (I) and theophylline and phenobarbital into a second fraction (II). An internal standard was added to each fraction, and the resulting solutions were chromatographed onto a single column. The internal standard and column temperature used were: α-naphthylamine and 150°, Fraction I; and hexobarbital and 200°, Fraction II. Quantitation was based on the sample–internal standard peak height ratio.

Keyphrases □ Theophylline, ephedrine hydrochloride, and phenobarbital suspensions—GLC analysis □ Ephedrine hydrochloride, theophylline, and phenobarbital suspensions—GLC analysis □ Phenobarbital, theophylline, and ephedrine hydrochloride suspensions—GLC analysis □ GLC—analysis, theophylline, ephedrine hydrochloride, and phenobarbital suspensions

Tablets and suspensions containing theophylline, ephedrine hydrochloride, and phenobarbital are frequently used for their actions of sedation and bronchodilation. The problem of developing a rapid and accurate assay for combinations of these components is well known (1). Although many methods are reported for the individual components, only a few reports concern the assay of mixtures of the three drugs.

A monograph for theophylline, ephedrine hydrochloride, and phenobarbital tablets is given in NF XIII (2). This represents the first recognition given by either the USP or NF to the mixture. The official assay, which also serves as the basis for identification, consists of separating the mixture into three fractions, each containing a single drug, followed by UV spectroscopy. Separation is accomplished by a laborious process involving two chromatographic columns and a liquid–liquid extraction. In addition to this time-consuming aspect, the official procedure is not directly adaptable to the mixture in suspension form.

Another assay for theophylline, ephedrine hydrochloride, and phenobarbital tablets was reported (1). This was a GLC method involving a single column and two separate sample preparations. A solution containing theophylline and phenobarbital was chromatographed directly after the solvent extraction of a tablet sample. Ephedrine was determined by treating a second tablet sample with sodium periodate to form benzaldehyde and chromatographing a solvent extract of this mixture. The oxidation step was included because ephedrine did not give a well-resolved peak. The assay was described as being rapid, specific, and precise. However, it applied only to tablets and could not be utilized directly for suspensions.

In recent years, GLC has become an increasingly important method for the analysis of theophylline, ephedrine hydrochloride, and phenobarbital, either individually or in combination with each other. This method is especially attractive because of its ability to provide both accuracy and convenience. The GLC methods used for these drugs in mixtures, such as dosage formulations and biological materials, frequently include solvent extraction (1, 3–9).

This report describes a rapid and accurate GLC method for the quantitative determination of theophylline, ephedrine hydrochloride, and phenobarbital contained in suspension. In the development of this procedure, the attainment of well-resolved peaks was sought through the use of a single column, a minimum number of sample preparations and injections, and a direct injection of the components. All of these conditions are successfully met in the described procedure.

EXPERIMENTAL

Apparatus—A gas chromatograph[1] equipped with a flame-ionization detector was used. The electrometer range was 10^2 with attenuation from 1 to 4 × 10^{-10} amp. The detector signal was fed to a 1-mv. recorder with a chart speed of 0.64 cm. (0.25 in.)/min. and a 1-sec.

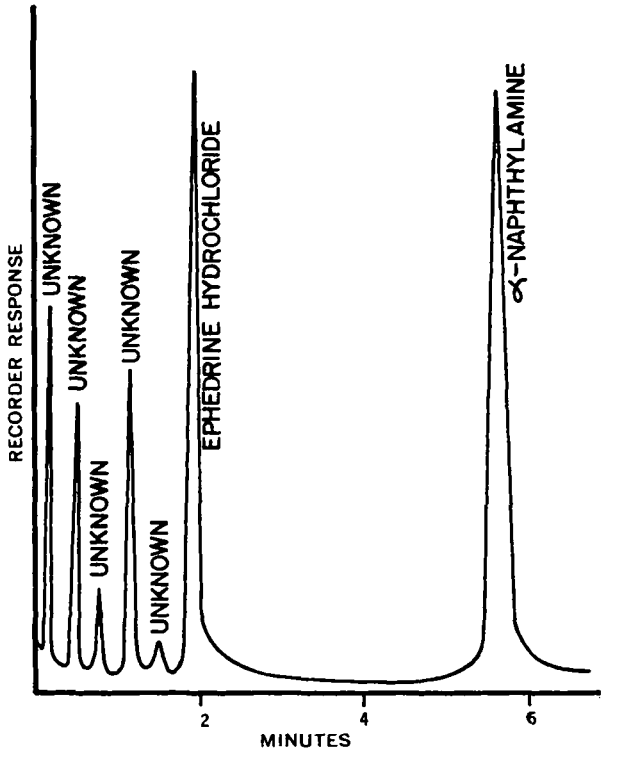

Figure 1—*Gas chromatogram of ephedrine hydrochloride in a commercial suspension. Conditions: 3% OV-17 on Gas Chrom Q; nitrogen flow rate, 75 ml./min.; column temperature, 150°; recorder chart speed, 0.64 cm./min.; and attenuation of electrometer, 2.0 × 10^{-10} amp.*

[1] Hewlett-Packard model 402.

Table I—Analysis of Theophylline, Ephedrine Hydrochloride, and Phenobarbital in a Synthetic Mixture[a]

	Theophylline		Ephedrine Hydrochloride		Phenobarbital	
	Found, mg./5.0 ml.	Recovery[b], %	Found, mg./5.0 ml.	Recovery[c], %	Found, mg./5.0 ml.	Recovery[d], %
Mean	65.2	100.3	11.9	99.2	3.9	97.5
Standard deviation	±0.09		±0.07		±0.04	
Coefficient of variation		±0.1		±0.6		±1.0

[a] Results obtained from six runs. [b] Each 5.0 ml. contained 65.0 mg. theophylline. [c] Each 5.0 ml. contained 12.0 mg. ephedrine hydrochloride. [d] Each 5.0 ml. contained 4.0 mg. phenobarbital.

Table II—Analysis of Theophylline, Ephedrine Hydrochloride, and Phenobarbital in a Commercial Suspension[a]

	Theophylline		Ephedrine Hydrochloride		Phenobarbital	
	Found, mg./5.0 ml.	Recovery[b], %	Found, mg./5.0 ml.	Recovery[c], %	Found, mg./5.0 ml.	Recovery[d], %
Mean	64.6	99.4	11.5	95.8	4.4	110.0
Standard deviation	±0.18		±0.09		±0.06	
Coefficient of variation		±0.3		±0.8		±1.4

[a] Results obtained from six runs. [b] Labeled amount of theophylline in 5 ml. was 65 mg. [c] Labeled amount of ephedrine hydrochloride in 5 ml. was 12 mg. [d] Labeled amount of phenobarbital in 5 ml. was 4 mg.

full-scale response. The gas flows were: nitrogen, 75 ml./min.; hydrogen, 35 ml./min.; and air, 280 ml./min.

GLC Column—A 1.8-m. (6-ft.) long, U-shaped, borosilicate glass tubing (4.0 mm. i.d.) was packed with 3% OV-17 on 100–120-mesh Gas Chrom Q[2]. The column was conditioned by heating at 100° for 1 hr., followed by increasing the temperature to 250° at a rate of 1°/min. and maintaining this temperature for 18 hr. with continuous carrier gas flow.

Operating Temperatures—The injection port, column, and detector temperatures for the assay of ephedrine hydrochloride were maintained at 200, 150, and 200°, respectively. For the assay of phenobarbital and theophylline, the injection port, column, and detector temperatures were maintained at 250, 200, and 250°, respectively.

Preparation of Ephedrine Hydrochloride Internal Standard Calibration Curve—*Internal Standard Solution*—A chloroform solution containing 12.5 mg. α-naphthylamine/ml. was used.

Standard Reference Solution—About 500 mg. ephedrine hydrochloride USP, accurately weighed, was dissolved in 10 ml. distilled water contained in a 125-ml. separator. The mixture was adjusted to pH 11 with 20% sodium hydroxide solution and extracted with four 25-ml. portions of chloroform. The extract was placed in a 100-ml. volumetric flask and diluted to volume with chloroform. The following quantities of this solution were pipeted into six 10-ml. volumetric flasks: 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ml. To each flask were added 0.3 ml. of α-naphthylamine internal standard solution and sufficient chloroform to bring to volume. A 4-μl. sample of each solution was injected into the gas chromatograph. Four replicate samples of each concentration were injected, the peak heights were measured, and the average value for each concentration was determined. Upon plotting the ephedrine hydrochloride–α-naphthylamine peak height ratio to amount of ephedrine hydrochloride, a straight-line calibration curve was obtained over the 1.0–10.0-mcg. range at the corresponding range of peak height ratio values from 0.45 to 9.00.

Preparation of Phenobarbital Internal Standard Calibration Curve—*Internal Standard Solution*—Hexobarbital (0.2 mg./ml.) was dissolved in an equal volume of chloroform and methanol.

Standard Solution— Phenobarbital USP, accurately weighed, was dissolved in the hexobarbital internal standard solution to give a series of six solutions varying from about 0.2 to 3.9 mg./ml. The same GLC procedure for these solutions was followed as described for the ephedrine hydrochloride standard solution. Upon plotting the phenobarbital–hexobarbital peak height ratio to amount of phenobarbital, a straight-line calibration curve was obtained over the 0.7–15.4-mcg. range at the corresponding range of peak height ratio values from 0.40 to 8.60.

Preparation of Theophylline Internal Standard Calibration Curve—*Internal Standard Solution*—Hexobarbital internal standard solution, as described for the phenobarbital standard curve, was used.

Standard Solution—Theophylline NF, accurately weighed, was dissolved in hexobarbital internal standard solution to give a series of six solutions varying from about 0.8 to 21.0 mg./ml. The same GLC procedure was followed as described for the ephedrine hydrochloride standard solution. Upon plotting the theophylline–hexobarbital peak height ratio to amount of theophylline, a straight-line calibration curve was obtained over the 6.2–62.4-mcg. range at the corresponding range of peak height ratio values from 0.73 to 4.30.

Assay of a Synthetic Mixture—*Preparation*—A mixture containing the same quantities of theophylline, ephedrine hydrochloride, and phenobarbital as contained in a commercial suspension was formulated. Each 5 ml. contained 65.0 mg. theophylline, 12.0 mg. ephedrine hydrochloride, and 4.0 mg. phenobarbital in distilled water.

Ephedrine Hydrochloride Assay—To 5.0 ml. of the mixture was added 5 ml. distilled water, the pH was adjusted to 11 with 20% sodium hydroxide solution, and the solution was extracted two times with 12 ml. chloroform. The chloroform extract was placed into a 25-ml. volumetric flask containing 0.75 ml. of α-naphthylamine internal standard solution and diluted to volume with chloroform. Four microliters of the solution was injected into the gas chromatograph.

Phenobarbital and Theophylline Assay—To the aqueous phase remaining from the ephedrine hydrochloride assay, 4 g. of sodium chloride was added and the pH was adjusted to 4.8 with dilute hydrochloric acid. The mixture was extracted four times with 50 ml. chloroform. The chloroform extract was placed into a 200-ml. volumetric flask and diluted to volume with chloroform. With a pipet, 9.0 ml. of this solution was placed in a test tube and the solution was evaporated to dryness on a 65° water bath. One milliliter of the hexobarbital internal standard solution was pipeted into the tube to redissolve the residue. Four microliters of the solution was injected into the gas chromatograph. The results are given in Table I.

Assay of Commercial Suspension—The specific gravity of a commercial suspension[3] was determined. Approximately 6.4 g. of the suspension, accurately weighed, was mixed with 5 ml. of distilled water. This quantity, representing about 5 ml. of sample, was converted into volume using the specific gravity value. The resulting mixture was extracted and gas chromatographed by the same pro-

[2] Applied Science Laboratories, State College, Pa.

[3] Tedral suspension, Warner-Lambert Co.

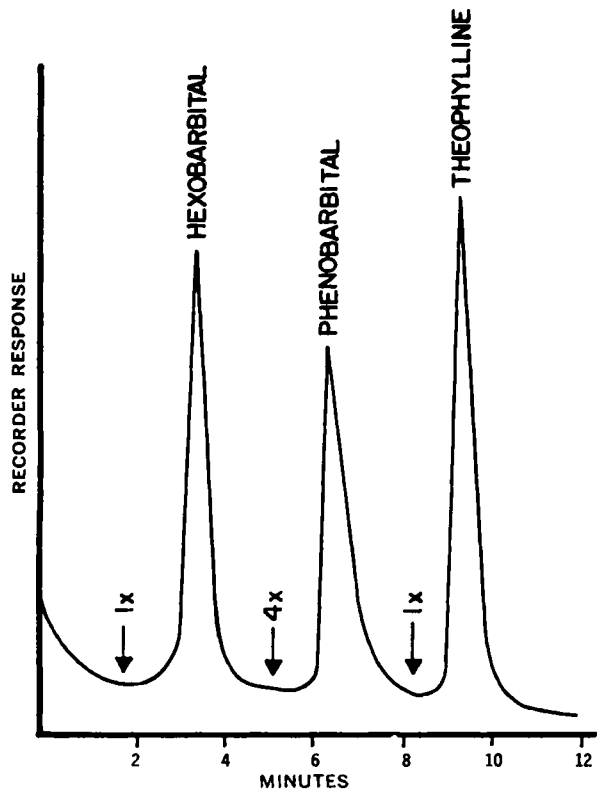

Figure 2—*Gas chromatogram of theophylline and phenobarbital in a commercial suspension. Conditions: 3% OV-17 on Gas Chrom Q; nitrogen flow, 75 ml./min.; column temperature, 200°; recorder chart speed, 0.64 cm./min.; and attenuation of electrometer, 1 × and 4 × 10^{-10} amp.*

cedure as used for the assay of a synthetic mixture. The results are given in Table II and Figs. 1 and 2.

RESULTS AND DISCUSSION

A GLC procedure for the quantitative determination of theophylline, ephedrine hydrochloride, and phenobarbital contained in a suspension formulation was presented. It was necessary to incorporate into the procedure a liquid–liquid extraction because of the varying concentration and polarity of the components. Through this process the sample was separated into two fractions, one containing ephedrine hydrochloride and the other containing theophylline and phenobarbital. Several additional substances, possible flavoring or coloring agents, were extracted by chloroform into the ephedrine extract. Complete elimination of these impurities was not attempted because they did not interfere with either the ephedrine or the internal standard peaks (Fig. 1).

The packing used in the GLC column was 3% OV-17 on Gas Chrom Q. A preliminary study showed that this packing gave the most desirable shape and resolution of peaks. In addition, it had the advantage of high thermal stability. Although peak tailing has been encountered with these drugs on various packing materials (6–8, 10), there was minimal peak tailing for all of the drugs in this investigation. The use of the silanized solid support of Gas Chrom Q probably minimized this problem.

A 4-μl. injection of each fraction was made and the resulting peak heights were measured. The peak height ratios of drug to internal standard were determined and compared to the appropriate calibration curve. Linear response was found in the calibration curves for the following ranges: ephedrine hydrochloride, 1.0–10.0 mcg.; phenobarbital, 0.7–15.4 mcg.; and theophylline, 6.2–62.4 mcg.

The procedure was used to assay a commercial suspension and a comparable synthetic mixture. Six replicate determinations were made of each preparation. Each extracted fraction was injected into the gas chromatograph four times and the average result was determined. The mean recoveries obtained (Table I) in the assay of the synthetic mixture for ephedrine hydrochloride, phenobarbital, and theophylline were 99.2 ± 0.6, 97.5 ± 1.0, and 100.3 ± 0.1%, respectively. Correspondingly, the mean recoveries for the commercial suspension (Table II) were: 95.8 ± 0.8, 110.0 ± 1.4, and 99.4 ± 0.3%, respectively. These latter values were calculated on the basis of the manufacturer's label claim. The standard deviation (Tables I and II) found for each drug in the two preparations indicated excellent precision for the procedure.

CONCLUSION

A rapid GLC method was developed for the determination of theophylline, ephedrine hydrochloride, and phenobarbital contained in a suspension formulation. A simple extraction procedure was incorporated to separate the components into two fractions. To each fraction an internal standard was added, and the resulting solutions were chromatographed onto a single column. Conditions of column packing, temperatures, internal standards, and carrier gas flow rate were selected to give well-resolved peaks. Quantitation was made on the basis of the peak height ratio of sample to internal standard. Accurate and precise values were obtained using this procedure.

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Paper Chromatographic Determination of Tetracycline

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Abstract □ A simple paper chromatographic method for separation and quantitative determination of tetracycline in pharmaceutical preparations was developed. The method is based on the complexation of the antibiotic with a mixture of urea and disodium edetate on a chromatographic paper at pH 7.4. Spots of tetracycline and its degradation products were located under UV light, and their respective R_f values were calculated. The method can be used to determine tetracycline phosphate, even in the presence of oleandomycin. The developed method proved satisfactory in monitoring the stability of the antibiotic base and its hydrochloride.

Keyphrases □ Tetracycline and tetracycline formulations—paper chromatographic analysis in presence of six degradation products □ Paper chromatography—analysis, tetracycline and tetracycline formulations □ Chromatography, paper—tetracycline and degradation products

Tetracycline antibiotics undergo rapid degradation through epimerization and/or oxidation (1–5). The ingestion of degraded tetracycline capsules was reported to cause a reversible Fanconi-type syndrome (6–10). Since the official microbiological methods suffer from many disadvantages, recent articles have been devoted to the development of simple, precise, and accurate methods for the determination of tetracycline in degraded pharmaceutical formulations.

Previous chromatographic separations of tetracycline involved paper saturated with McIlvaine's buffer at pH 3.5 (11, 12). However, epimerization of tetracycline on the chromatographic paper was reported under these conditions (13). The separation of epitetracycline from urine, using chromatographic paper[1] impregnated with edetic acid, was reported (14). Urea solution was successfully employed as an immobile phase for paper chromatographic determination of oxytetracycline (15). The formation of a slightly soluble tetracycline–urea complex also was reported (16).

Therefore, it was decided to use paper impregnated with a mixture of disodium edetate and urea for the quantitative separation and determination of tetracycline in the presence of its degradation products. This impregnating mixture resulted in better separation which led to more precise determination of tetracycline in the presence of its degradation products.

EXPERIMENTAL[2]

Separation of Tetracycline from Its Degradation Products—*Impregnating Solutions*—A 100-ml. quantity of urea solution (A) was prepared by dissolving 10 g. of urea in McIlvaine's buffer (pH 7.4) (17); 100 ml. of disodium edetate solution (B) was prepared by dissolving 5 g. of disodium edetate in McIlvaine's buffer (pH 7.4). A mixture of Solutions A and B (1:3) was used for impregnating a series of chromatographic papers[1]. The papers were then blotted between sheets of absorbent papers.

Solvents—A mixture of ethyl acetate–acetone (40:10), saturated with disodium edetate and urea, was used as a mobile phase for the papers impregnated with the mixture of Solutions A and B.

Method—A series of methanolic solutions of tetracycline base (1 mg./ml.) was prepared and stored in glass-stoppered bottles for 0, 1, 2, 3, 4, 5, 6, 7, 10, and 15 days at 26°. Other methanolic solutions of tetracycline hydrochloride (1 mg./ml.) were prepared and similarly stored for 0 and 10 days at 26°. Ten microliters from each fresh methanolic standard solution and sample solution were separately spotted on the impregnated chromatographic papers, prepared as previously described. The chromatograms were then allowed to dry partially in air; while still damp, they were transferred to the ascending chromatographic tank containing 100 ml. of the developing solvents. After the solvent front reached about 22 cm. above the starting line, the chromatograms were air dried. The separated spots were located under UV light after exposure to ammonia vapor for at least 15 sec. Mean R_f values of tetracycline and its degradation products were calculated.

Quantitative Analysis of Tetracycline Hydrochloride and Tetracycline Phosphate in Different Pharmaceutical Preparations—A starting line (10 cm.) was marked on a sheet of chromatographic paper (17.5 × 28 cm.), about 4 cm. from one end of the sheet and 2 cm. from the edges. The chromatogram was prepared as previously mentioned. Tetracycline hydrochloride solution [0.1 ml. (100 mcg.)] in methanol or a suitably diluted aliquot of another preparation was streaked on the line. The chromatogram was placed in the chromatographic tank as described previously. After the solvent front had moved about 22 cm., the chromatogram was air dried. A blank paper without antibiotic was similarly treated. The yellow fluorescent band of tetracycline was located under UV light after exposure to ammonia vapor. It was cut into 0.5-cm. squares, with equivalent areas taken from the blank paper. The bands of tetracycline and blank were separately eluted with 20 ml. of dilute hydrochloric acid (pH 1.8). The absorbance of tetracycline present in the eluate was determined spectrophotometrically at 355 nm. (17), within 1 hr., against the eluate of tetracycline standard using 2-cm. silica cells.

This procedure was used to determine the tetracycline content of capsules, injections, suspensions, pediatric drops, and ointments. Previously published methods (15) for preparing samples of oxytetracycline before application to the chromatographic papers were successfully used in the case of the tetracycline preparations. The method was also used to determine tetracycline phosphate in pharmaceutical preparations containing oleandomycin. The calculated potencies of the investigated tetracycline dosage forms are compiled in Table I.

The method was utilized to monitor the stability of tetracycline hydrochloride and tetracycline base solutions in methanol during 6 days of storage at 26°.

RESULTS AND DISCUSSION

Epimerization of tetracycline is known to proceed at a higher rate at pH 2–6 (1). Accordingly, epimerization is expected to take place on paper previously saturated with McIlvaine's buffer, pH 3.5, and this is actually the case (13). Therefore, the pH of the impregnating solution was raised to 7.4 in order to avoid such epimerization.

Separate use of either urea or disodium edetate solution in McIlvaine's buffer (pH 7.4) as an immobile phase proved to be unsatisfactory for the complete separation of tetracycline from its degradation products. The impregnation of the chromatographic papers with urea solution resulted in a marked lowering of the R_f values with the formation of intermingled zones of tetracycline and

[1] Whatman No. 1.

[2] The following apparatus and reagents were used: (*a*) spectrophotometer, Carl Zeiss Jena; (*b*) UV lamp, HPW-125 W, type 57202 E/7, Philips; (*c*) ethyl acetate, acetone, methanol, disodium edetate, urea, and hydrochloric acid, all analytical grade; (*d*) tetracycline hydrochloride, tetracycline base, and troleandomycin USP or NF grade; and (*e*) tetracycline hydrochloride, international standard, provided by the World Health Organization.

Table I—Stated and Calculated Potency Values of Tetracycline in Different Dosage Forms

Dosage Form[a]	Stated Potency	Calculated Potency[b]	Standard Deviation
Tetracycline Hydrochloride Preparations			
Capsule	250 mg./capsule	252.8 mg./capsule	±4.20
Suspension	125 mg./5 ml.	93.5 mg./5 ml.	±1.15
Ointment	30 mg./g.	30.5 mg./g.	±0.99
Tetracycline Phosphate Preparations			
Suspension	125 mg./5 ml.	121.7 mg./5 ml.	±3.04
Injections:			
No. 1 (reconstituted with the provided vehicle)	100 mg./2 ml.	81.4 mg./2 ml.	±2.60
No. 2 (reconstituted with methanol)	100 mg./2 ml.	94.5 mg./2 ml.	±1.60
Pediatric drops	100 mg./ml.	97.7 mg./ml.	±1.80
Preparations Containing Tetracycline Hydrochloride and Oleandomycin			
Synthetic mixture	91 mg./136 mg.	90.8 mg./136 mg.	±2.40
Capsule	167 mg./capsule	169.3 mg./capsule	±0.94
Suspension	83.3 mg./5 ml.	84.2 mg./5 ml.	±0.95
Pediatric drops	66.7 mg./ml.	68.4 mg./ml.	±3.20
Injection	66.7 mg./2 ml.	65.2 mg./2 ml.	±1.40

[a] Commercial samples. [b] Average of four determinations.

epitetracycline; both adhered to the starting line. On the other hand, the use of a disodium edetate solution as an immobile phase produced diffuse spots with higher R_f values. The impregnation of the chromatographic papers with the mixture of Solutions A and B (1:3) gave the best results for the quantitative separation of tetracycline from its degradation products.

Figures 1 and 2 show the decomposition of tetracycline hydrochloride and tetracycline base in methanol. The acid decomposition products of tetracycline were obtained by heating tetracycline solution in 2 *N* HCl on a steam bath for 10 min., and they were then detected by spotting on the chromatogram after the appropriate dilution with methanol. Figure 2 shows that the acid-catalyzed decomposition of tetracycline proceeds mainly through the formation of anhydro derivatives of the antibiotic and its epimer. When the antibiotic was eluted from the chromatogram using ammonium chloride solution (10% w/v), it showed a shift of λ_{max} toward the lower wavelength, thus confirming the complex formation of the antibiotic on the chromatogram. The ammonium chloride solution was chosen as the eluent because tetracycline was reported to be stable in it (13). However, the use of the ammonium chloride solution gave low recoveries of the antibiotic. Dilute hydrochloric acid (pH 1.8) was found to be satisfactory as an eluting medium. The

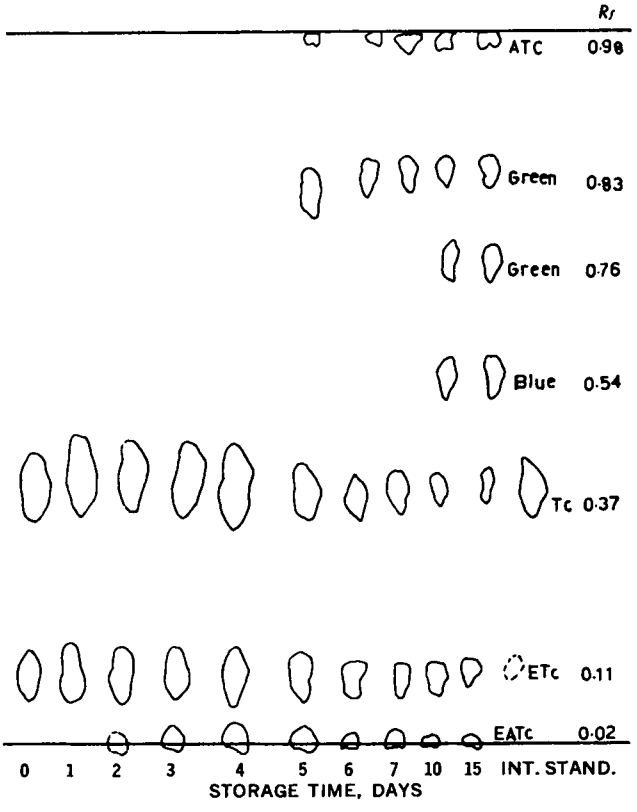

Figure 1—*Schematic chromatogram of the degradation products of tetracycline base in methanol. Key: TC, tetracycline; ETC, epitetracycline; ATC, anhydrotetracycline; and EATC, epianhydrotetracycline.*

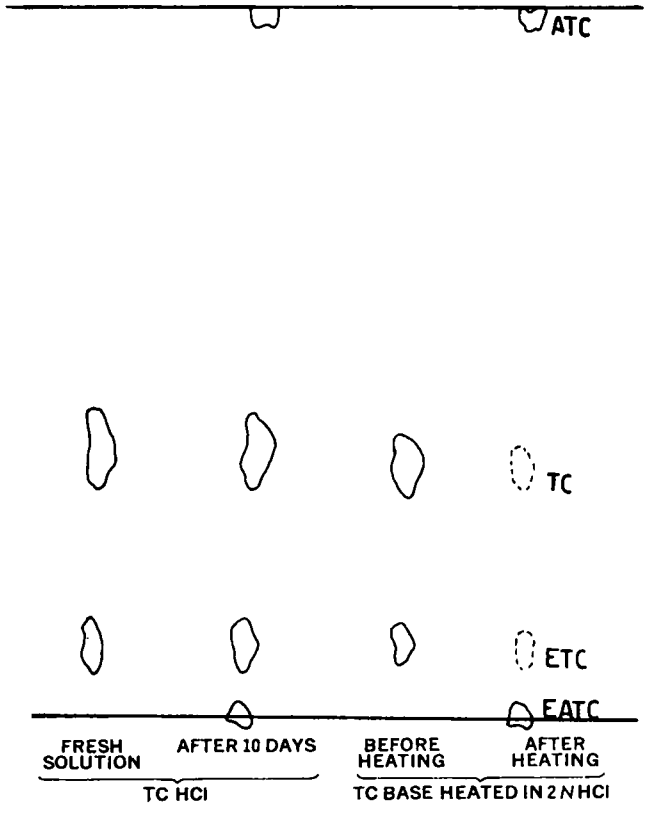

Figure 2—*Schematic chromatogram of the degradation products of tetracycline hydrochloride in methanol and the catalytic decomposition products of tetracycline base in hydrochloric acid. Key: TC, tetracycline; ETC, epitetracycline; ATC, anhydrotetracycline; and EATC, epianhydrotetracycline.*

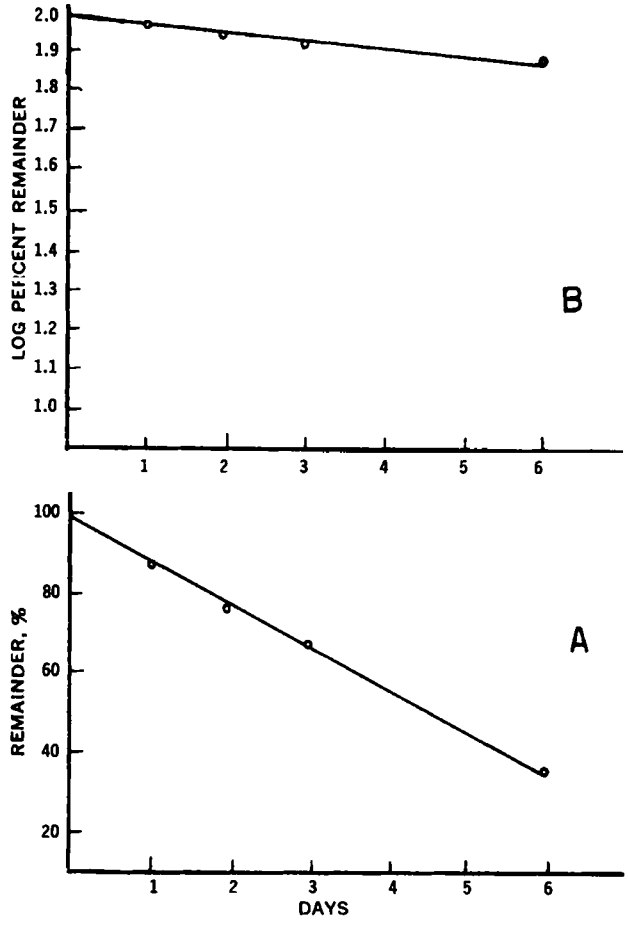

Figure 3—*Stability of tetracycline base (A) and tetracycline hydrochloride (B) solutions in methanol at 26°.*

absence of degradation spots on rechromatographing the acid eluate of tetracycline indicated that the antibiotic was not decomposed during the development and that it was stable in this solution for at least 1 hr. after elution. The absorbance of the antibiotic acid eluate at 355 nm. after 1 hr. was not changed. These results were parallel to those obtained by McCormick *et al.* (3).

The maximum load of the paper was found to be 10 mcg./spot. This necessitates the use of a horizontal line technique for applying at least 100 mcg. of the antibiotic to the chromatogram, providing a final concentration of about 5 mcg./ml. in the eluate.

Tetracycline hydrochloride, international standard, was used to study the precision and accuracy of this method. The zones representing tetracycline and 4-epitetracycline in the standard sample were combined and eluted with dilute hydrochloric acid (pH 1.8). The absorbance of the eluate at 355 nm. was compared with that of total tetracycline taken to find out the recovery of the antibiotic. Five samples gave an average recovery of 99.2 ± 1.7%.

Table I shows stated and calculated potencies of various dosage forms of tetracycline such as capsules, suspensions, injections, pediatric drops, and ointments. The data indicate that the calculated potencies of tetracycline hydrochloride capsules and ointments were close to the stated potencies. The calculated potency of tetracycline hydrochloride suspension was found to be much less than the stated potency. On observing the chromatogram of this preparation, it was found that the zone representing 4-epitetracycline was much larger than that observed in the other preparations. In addition, anhydrotetracycline was clearly detected on the chromatogram. From Table I, it is obvious that the presence of oleandomycin did not interfere with the chromatographic determination of tetracycline hydrochloride in the tested pharmaceutical preparations. The data reveal that the method is satisfactory for the determination of the antibiotic content of tetracycline phosphate in suspensions, injections, and pediatric drops. Paper chromatographic determination of tetracycline phosphate injection, after reconstitution with the provided aqueous vehicle, indicated that its potency was much less than the stated value. On the other hand, the direct measurement of the same injection, after reconstitution with methanol and immediate application on the chromatogram, gave higher results (Table I). This unexpected result might be attributed to the interference of certain additives present in the injection formulation. This suggestion is supported by the fact that the additives present in the injection are not completely dissolved in methanol. Consequently, the interference effect of these substances was greatly eliminated by the direct use of methanol as a reconstituting solvent prior to the application on the chromatogram. On the other hand, the administration of this type of tetracycline phosphate injection should be carried out promptly to avoid the degradation of the antibiotic in the reconstituting aqueous vehicle.

Table II—Microbiological and Chromatographic Estimations of Tetracycline Hydrochloride in Capsules and Suspensions

Dosage Form	Stated Potency	Calculated Potency: Microbiological Method	Calculated Potency: Chromatographic Method
Capsule	250 mg./capsule	278.8 mg./capsule	252.8 mg./capsule
Suspension	125 mg./5 ml.	97.4 mg./5 ml.	93.5 mg./5 ml.

The official microbiological method (18) was used to determine the antibiotic content of tetracycline hydrochloride capsules and suspensions. The cup-plate method was employed, and *Bacillus cereus* (var. mycoides, ATCC 9643) was the test organism used (19). The results obtained (Table II) are comparable to those obtained by the developed chromatographic procedure within 10%.

Figure 3 shows the stability of tetracycline base and hydrochloride in methanol. In the case of tetracycline base, the antibiotic decomposed through a zero-order reaction rate (K = 11.04/day), while the antibiotic decomposition of tetracycline hydrochloride followed the first-order rate (K = 0.052/day). Tetracycline hydrochloride was distinguished by its higher stability compared to the tetracycline base.

So far, the quantitative separation of tetracycline from its degradation products by chromatography has been more effectively carried out by TLC and column procedures (20, 21). The reported degradation products, which appeared as three spots on the chromatogram, were characterized as epianhydrotetracycline, epitetracycline, and anhydrotetracycline. However, previously attempted methods for the quantitative separation using paper chromatography failed to separate all of the degradation products of the antibiotic (11–14). By using the developed method, the quantitative separation of tetracycline from six degradation products was realized; three degradation products were characterized. The running time was rather short (3 hr.). Several precautions were taken to guard against degradation of tetracycline on the chromatogram, [*i.e.*, raising the pH to 7.4 to eliminate the possibility of epimerization (1) and using urea for impregnating the chromatogram and saturating the running solvents] and they contributed to the formation of a more stable complex (16). Furthermore, urea helped in the separation of the degradation products and led to the formation of well-defined spots.

In conclusion, the developed method proved adequate for the control determinations of tetracycline in different pharmaceutical preparations. It is also satisfactory as a stability-indicating assay for the selective determination of tetracycline in the presence of its decomposition and reaction products. The accuracy of the determination of tetracycline is superior to the commonly employed microbiological procedure, which has an uncertainty of about 15%, an order of magnitude less precise than the presented chromatographic method.

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Liquid–liquid chromatography is actively being investigated for drug analysis (1–9). Studies of various groups of steroids have demonstrated that both qualitative detection (10–16) and quantitative measurements (17–19) may be performed. Except for these particular works (17–19), no other reports appear to have been published dealing with the quantitative analysis of adrenocortical steroids.

The official compendial approaches (20, 21) to the assay of four adrenocortical steroids—hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate—employ well-established methods. The steroids are either extracted with alcohol for total steroid content or separated by TLC to isolate a single steroid, after which the actual measurement is carried out by blue tetrazolium colorimetry. When tablets are assayed, the foreign steroid content is determined as the difference between the results of the single steroid and the total steroid assays. The problems with the blue tetrazolium color reaction—*viz.*, its nonspecificity for individual steroids and the many compounds that interfere with it, have been thoroughly documented (22–26).

Other analytical methods for steroid analysis are available (27–31). These include spectrophotometry and colorimetry, approaches that possess the same problems in varying degrees as does the blue tetrazolium color reaction, and GLC where the problem is the labile nature of the corticosteroids. If steroid derivatives are formed to ensure thermal stability, complete and specific derivatization must be achieved.

This work describes a study of a liquid–liquid chromatographic method for the analysis of hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate and the basic aqueous decomposition of hydrocortisone. The linearity of the detector response to the concentration of the steroid was confirmed for each steroid and actual commercial products; creams, ointments, lotions, and suppositories were analyzed for the presence, identification, and quantity of foreign steroids and for the main steroid component in terms of the single steroid assay value. This method was fast, accurate, and specific for each individual steroid studied.

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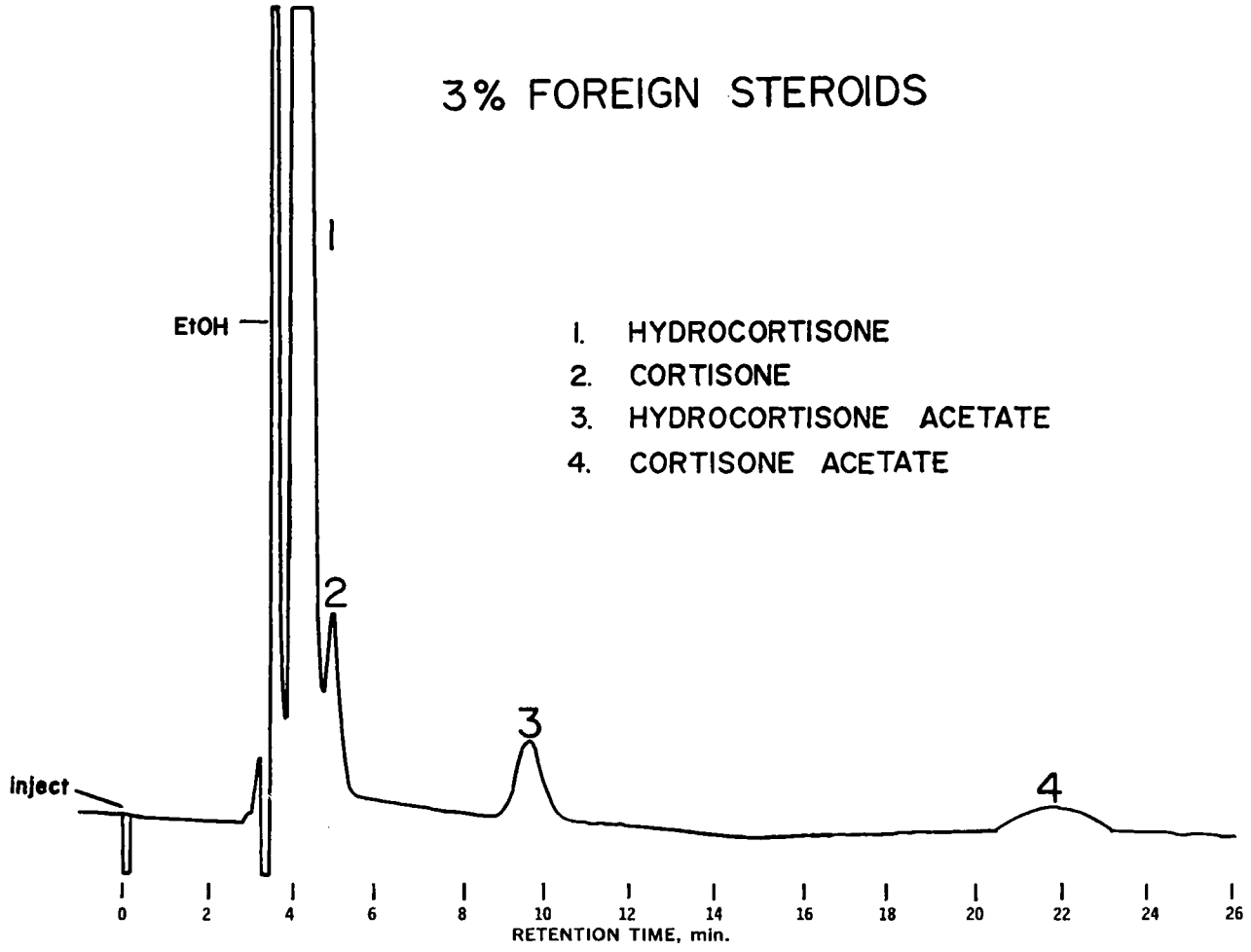

Figure 1—*Chromatogram of standards, 3% (w/w) cortisone, hydrocortisone acetate, and cortisone acetate, based on 6 mg. of hydrocortisone on the cyano ethyl silicone column illustrating foreign steroids test. Mobile phase = 1% methanol in water, attenuation = 0.02 aufs., pressure = 500 psig., flow rate = approximately 0.5 ml./min., and temperature = ambient.*

EXPERIMENTAL

Chemicals, Apparatus, and Instrumentation—Solvents were at least reagent grade and the steroid standards were either official grade (USP or NF) or working grade with a purity greater than 99.0%. Ethanol refers to 95% ethanol (USP grade).

All injections were made with either a 5-μl. high-pressure syringe[1] or a 10-μl. syringe[1] equipped with a Kel-F guide[1].

A liquid–liquid chromatograph[2] equipped with a precision UV photometer set at 254 nm. was used. The columns were precision-bore stainless steel, 1 m. × 2.1 mm. i.d., commercially[3] packed with either a cyano ethyl silicone[4] coated 1% on Zipax or an octadecyl silane[5] permanently bonded to Zipax.

Unless otherwise noted, the instrument parameters for the cyano ethyl silicone column were as follows: pressure, 500 psig. (flow rate approximately 0.5 ml./min.); temperature, ambient (approximately 25°); and mobile phase, water containing about 1% methanol.

Method for Linearity Study—The four steroids were studied on the cyano ethyl silicone column. It was convenient to prepare only two solutions (I and II), both in ethanol, containing all of the steroids in combination with the chosen internal standard; the latter, for convenience, was one of the steroids itself. The concentration range (in micrograms per milliliter) over which each steroid was studied was: Solution I—hydrocortisone, 500–15.6; cortisone 500–15.6; and hydrocortisone acetate, 1500–46.9; and Solution II—hydrocortisone acetate, 1000–31.2; and cortisone acetate, 2000–62.5.

Solution II was injected at a higher pressure, 1200 psig., to shorten the retention time of the latest eluting compound, cortisone acetate. Varied amounts of each dilution of the two solutions were injected into the liquid chromatograph in a pattern such that the concentration level of each solution was half of the concentration of its predecessor. As a result, to maintain a relatively constant detector response, a reciprocal attenuator setting was chosen for the recorder. The actual injection scheme for each solution is presented in Table I. The peak heights were measured for each compound.

Decomposition of Hydrocortisone—A 1.00-ml. volume of a 1-mg./ml. solution of hydrocortisone standard in ethanol was pipeted into 11 10-ml. volumetric flasks. Then 1.00 ml. of a 0.05 *N* aqueous sodium hydroxide solution was added. After sitting at room temperature for specific periods, 1.00 ml. of 0.05 *N* aqueous hydrochloric acid was added to quench the reaction. The solutions were diluted to volume with ethanol, and multiple injections of each solution were made on the cyano ethyl silicone column. As an index of steroid remaining, the hydrocortisone peak height was measured for each injection and the average peak height for each decomposition time was calculated.

Sample Analysis: Foreign Steroids and Assay—Random samples of creams, ointments, suppositories, and lotions containing hydrocortisone, hydrocortisone acetate, and cortisone acetate were obtained. The sample preparation was identical for each dosage form, with the only differences being the sample size for each different steroid and their respective internal standards. The following was the generalized sample preparation procedure used.

A sample equivalent to 6 mg. of hydrocortisone or cortisone, 12 mg. of hydrocortisone acetate, and 25 mg. of cortisone acetate

[1] Hamilton Co., Whittier, CA 90608
[2] Du Pont model 820.
[3] Instrument Products Division, E. I. Du Pont de Nemours & Co., Wilmington, DE 19898
[4] ANH.
[5] ODS.

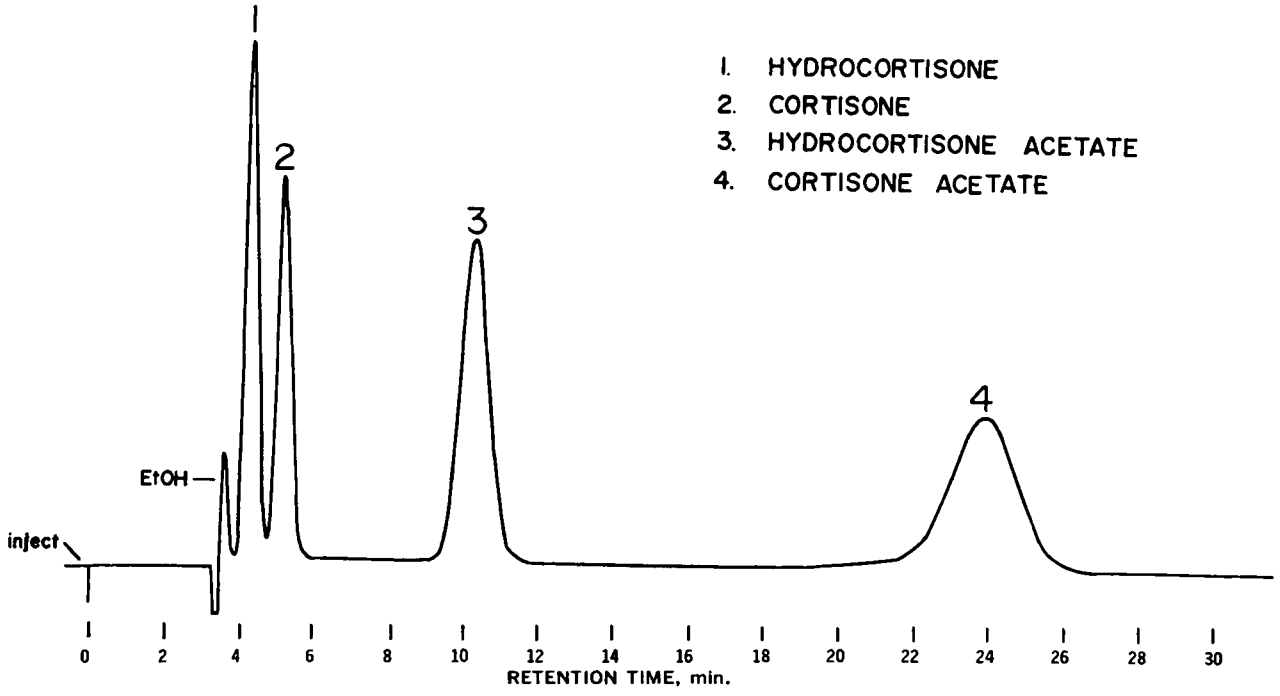

Figure 2—*Chromatogram of standards, hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate, on the cyano ethyl silicone column. Mobile phase = 1% methanol in water, pressure = 500 psig., flow rate = approximately 0.5 ml./min., and temperature = ambient.*

was accurately weighed into a 50-ml. glass-stoppered centrifuge tube. A 30-ml. volume of hot ethanol was added and the tube was shaken by hand or held on a mixer[6] for a few minutes, heated on a steam bath, and mixed again. If the sample dissolved totally or formed a cloudy suspension without any solid material remaining on the bottom of the tube, it was ready for the next step of the procedure. Further treatment was required if the ethanol was not dispersed into the semisolid base. This additional treatment included reheating, additional shaking, or ultrasonic agitation. After the solubilization step, the sample was allowed to cool, overnight if convenient, to allow the excipient materials to settle out; the tube was then centrifuged at about 1500 r.p.m. for 20 min.

To determine the foreign steroids content, this solution was injected onto the cyano ethyl silicone column at a recorder attenuation of 0.02 absorbance unit full scale (0.02 aufs.), a value four times as sensitive as the one used for the assay procedure (0.08 aufs.). A foreign steroid standard was prepared, which in the case of hydrocortisone was based on a sample size equivalent to 6 mg. of this steroid, containing each contaminant 3% by weight of the subject steroid. The chromatogram of this standard is shown in Fig. 1. In preparing the standards for the other three steroids, it was just a matter of varying the amounts of each of the four steroids, since they are cross-contaminants for each other. By using the standard chromatogram as a guide, the approximate level of each contaminant was determined.

If it appeared that the foreign steroids level was excessive, a more accurate quantitative determination could be made by increasing the sample size or by injecting the sample at a higher sensitivity. (The maximum sensitivity for the instrument used in these studies was 0.005 aufs.).

To carry out the assay, equal amounts of the appropriate internal standard were added to both the sample and a standard preparation and equal volumes of both solutions were injected. If the steroid to be used as an internal standard is found to be a foreign steroid contaminant, another steroid may be chosen as an internal standard. The assay value was calculated using peak height measurements and Eq. 1:

$$\text{percent steroid in sample} = \frac{PH_{spl}}{PH_{std}} \times \frac{PH^{I.S.}_{std}}{PH^{I.S.}_{spl}} \times \frac{wt_{std}}{wt_{spl}} \times 100 \quad \text{(Eq. 1)}$$

where:

PH_{spl} = peak height of sample
PH_{std} = peak height of reference standard
$PH^{I.S.}_{std}$ = peak height of internal standard in the standard
$PH^{I.S.}_{spl}$ = peak height of internal standard in the sample
wt_{std} = weight of standard
wt_{spl} = weight of sample

RESULTS AND DISCUSSION

Column Selection—The principal task was to find a column that would yield the best separation of the four corticosteroids in the shortest time. The first column examined, a cyano ethyl silicone column, satisfied these criteria (Fig. 2). The numbers of theoretical plates calculated for each compound were: hydrocortisone, 1254; cortisone, 1002; hydrocortisone acetate, 907; and cortisone acetate, 741. Of the four compounds, hydrocortisone had the lowest affinity for the column, which functions on the reverse-phase principle (10, 32); the most polar mobile phase, water, was required for retention of this compound. Lowering the polarity of the mobile phase by adding alcohol produced shorter retention times for all four compounds, indicating that the optimum mobile phase was water. However, the addition of a small amount of methanol (about 1%) was necessary for column stability.

Table I—Injection Scheme for Linearity Study

Injection Volume, μl.	Recorder Attenuation, aufs. 0.64	0.32	0.16	0.08	0.04	0.02
	Solution I					
5	x[a]	x	x			
3	x	x	x	x	x	x
1	x					
	Solution II					
10	x	x	x			
8	x	x	x			
6	x	x	x	x	x	x
4	x	x	x	x	x	x
2	x	x	x	x	x	x

[6] Vortex, Thomas Scientific Apparatus, Philadelphia, PA 19105

[a] x denotes an injection.

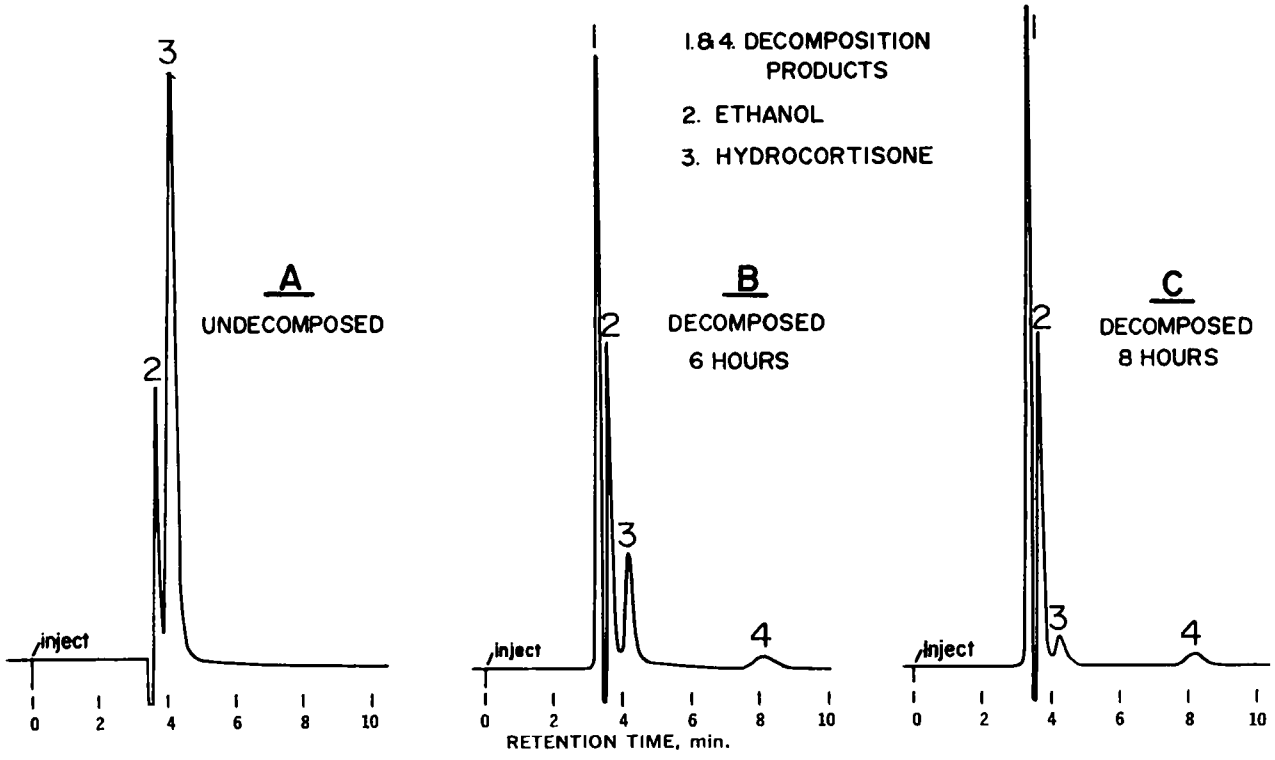

Figure 3—*Decomposed standards on the cyano ethyl silicone column. Conditions were the same as in Fig. 2, and attenuation = 0.08 aufs.*

The second column material evaluated was octadecyl silane[7]. This material, which also functions by reverse-phase chromatography, yielded the same elution order as the cyano ethyl silicone column with one exception; hydrocortisone and cortisone were not resolved. As before, water was the best mobile phase, in conjunction with a column temperature of 60° as recommended (33) and a pressure of 700 psig. Due to the need for elevated temperatures, a capability that not all instruments possess, and its inability to separate two steroids, this column was considered as second choice to the cyano ethyl silicone column for this study.

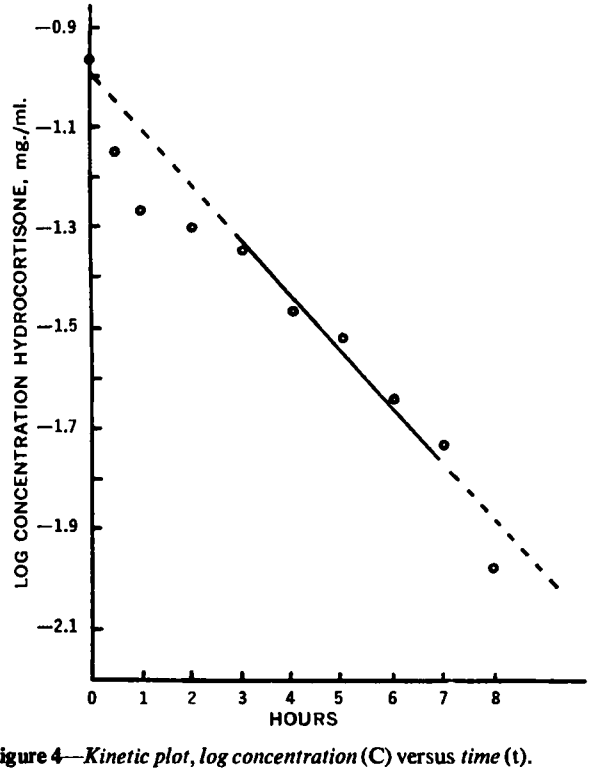

Figure 4—*Kinetic plot, log concentration* (C) versus *time* (t).

Peak Height Measurement and Linearity of Response—In all of the work presented, the quantity of active ingredient and foreign steroids was determined by using peak height measurement. While this property is most frequently used, it is recognized that a better measurement of material eluted is represented by the area under the peak. The use of peak heights is made assuming that the peak widths at half-height are equal for the same compound under the same instrumental conditions, thereby establishing the proportionality of the area to peak height. The peak height measurement was used for convenience and because the early eluting peaks have very narrow widths at half-height, making measurement of this band difficult and susceptible to large relative errors.

The relationship of detector response to the quantity of steroid was determined. This task was made more complex since the use of an internal standard in the assay procedure requires that linearity of detector response be established both for the test compound and the internal standard. To simplify the problem, the steroids were used as the internal standards for each other in the following arrangement: for hydrocortisone, cortisone, and cortisone acetate, the internal standard was hydrocortisone acetate; for hydrocortisone acetate, the internal standard was cortisone. By examining the chromatograms of Solutions I and II, it was simply a matter of measuring the peak heights for each compound and calculating the ratios of the subject steroid to the internal standard for the different pairs at each injection.

To simplify the experimental manipulations, serial dilutions of Solutions I and II were made according to the scheme summarized in Table I. The subsequent dilutions of the parent solution should have produced the same ratios for each pair if Beer's law is followed at 254 nm. over the concentration range studied. The calculated ratios are presented in Table II, columns A–F. It is apparent from columns C and E that different injection volumes of the same solution produce varying peak height ratios. When the injection volume (column C) was kept constant and the concentration was varied (column D), a relatively constant value was obtained (column E); the relative average deviation of the ratios for the different concentrations at a constant injection volume are shown in column F. From the data in

[7] Permaphase.

Table II—Results of Linearity Study

	A Subject / Internal Standard	B Retention	C Injection Volume, μl.	D Number of Different Concentrations Studied	E Average of Ratios of Peak Heights	F Reproducibility of Ratios for a Single Injection Volume in Relative Average Deviation Units, %[c]
1[a]	Hydrocortisone / Hydrocortisone acetate	55 mm. / 122 mm.	5	3	0.80	0.6
			3	6	0.92	1.2
			1	1	1.02	
2[a]	Cortisone / Hydrocortisone acetate	64 mm. / 122 mm.	5	3	0.64	1.6
			3	6	0.67	0.9
			1	1	0.69	
3[b]	Cortisone acetate / Hydrocortisone acetate	112 mm. / 50 mm.	10	3	0.82	
			8	3	0.82	
			6	6	0.82	
			4	6	0.81	
			2	6	0.80	
4[b]	Hydrocortisone acetate / Cortisone acetate	50 mm. / 112 mm.	10	3	1.21	0.9
			8	3	1.22	0.4
			6	6	1.22	0.5
			4	6	1.23	0.2
			2	6	1.25	0.3

[a] Ratios 1 and 2 were calculated from the injections of Solution I. [b] Ratios 3 and 4 were taken from the same chromatogram and are reciprocals of each other. Solution II was used in this case and the chromatogram was recorded at 1200 psig. pressure. [c] Relative average deviation =

$$\sum_{i=1}^{n} \frac{[\bar{x} - x_i]}{(n)(\bar{x})} \times 100$$

where $\bar{x}$ = mean, n = number of data, and x_i = datum.

column F, it is apparent that for identical injection volumes the compounds consistently exhibit a linear detector response for the quantity of material being measured over the range examined (35).

Decomposition of Hydrocortisone, Product Separation, and Kinetics—The decomposition of hydrocortisone in basic aqueous medium was studied to ensure the specificity of the column for this compound and its decomposition products. A study was reported (17) on flumethasone pivalate utilizing liquid–liquid chromatography. The decomposition of prednisolone was studied (34) in alkaline solution using chloroform extraction followed by UV spectroscopy. This study is cited because of the structural similarities between prednisolone and hydrocortisone, differing as they do only by a double bond in the A ring.

While the decomposition reaction for hydrocortisone is complex, the major site of reaction is at the C_{17} side chain; reaction rates and decomposition products similar to those found in prednisolone would be expected. The chromatograms obtained by injecting samples of the degraded solutions onto the cyano ethyl silicone column are shown in Fig. 3. Guttman and Meister (34) reported two classes of decomposition products in their study: a small amount of neutral material and a larger amount of acidic material. Chromatograms B and C in Fig. 3 show two additional peaks; peak 1, unretained by the column, is probably the large quantity of acidic material, and peak 4 may correspond to the smaller amount of neutral material. A hydrocortisone standard that was degraded for 72 hr. yielded no peaks at the hydrocortisone retention time using the cyano ethyl silicone column, proving that the decomposition products are separated from the parent steroid. Identification of decomposition peaks was not attempted.

The quantitative data available from the chromatograms (Fig. 3) were interpreted using familiar kinetic concepts. The average peak height for each time was used to calculate concentration (C). Since the molar ratio of sodium hydroxide to hydrocortisone is 1.81×10^4 and since previous work (34) showed the decomposition of prednisolone to be pseudo-first order, a plot of the data was made in terms of log C *versus* time (Fig. 4). A straight line is drawn in the region where five points appear to be linear. When this line is extrapolated back to the axis, the intersection is very close to the experimental value for an undecomposed solution. Similarly, the slope of the straight-line segment yields a rate constant of 0.252 $hr.^{-1}$ at room temperature. This is compared to prednisolone, at the same concentration and in a solution of the same pH as in this work, for which a rate constant of 0.293 $hr.^{-1}$ was reported (34) at 35°, a comparable but higher value than that for hydrocortisone, to be expected for a higher temperature.

The scatter of points in Fig. 4 introduces some doubt as to the reaction order. The main scatter is at the initial times where the concentrations are changing most rapidly; these points are not considered in the construction of the straight line. On the other hand, the extrapolated value (−0.99) resulting from the straight line agrees well with the experimental result (−0.97), and the agreement of the slope with that reported (34) indicates that the line is not of fortuitous origin. It may be that additional work could result in a more linear overall response, but, in any case, the method separates the decomposition products from the parent molecule effectively, a desirable attribute for any analytical approach.

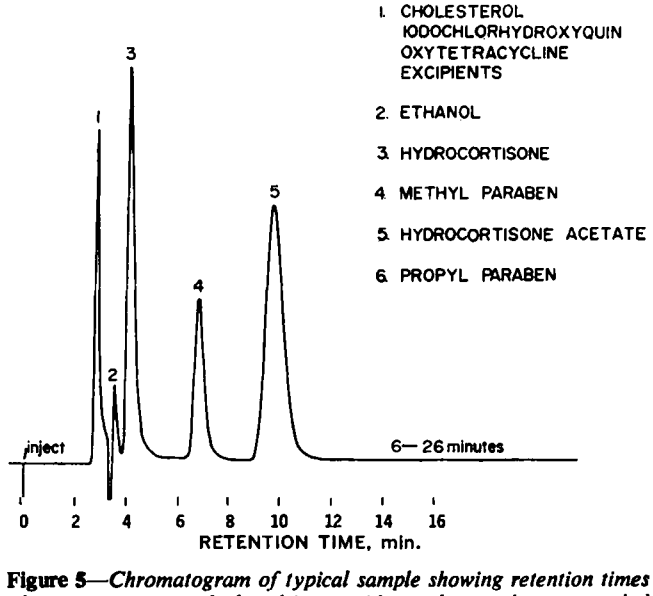

Figure 5—*Chromatogram of typical sample showing retention times of common compounds found in steroid samples on the cyano ethyl silicone column under same conditions as in Fig. 3.*

Table III—Assay Results of Commercial Samples

Number	Active Ingredient, Hydrocortisone, %	Product Type	Other Active Ingredients	Assay, % of Declared: Liquid–Liquid Chromatography	Assay, % of Declared: Other Methodology
1	0.25	Ointment		97.6	
2	0.25	Cream	Iodochlorhydroxyquin	79.8	78.2[a], 77.2[b]
3	0.25	Cream		100.8	
4	0.25	Cream		100.0	102.0[c]
5	0.5	Ointment		99.9	
6	0.5	Ointment		94.6[d]	91.8[e], 82.0[e]
7	0.5	Cream	Iodochlorhydroxyquin	102.0	
8	0.5	Cream	Iodochlorhydroxyquin	93.2	
9	0.5	Cream	Iodochlorhydroxyquin	94.6	91.6[f]
10	0.5	Cream	Iodochlorhydroxyquin	96.1	
11	0.5	Cream	Diiodohydroxyquin	96.8	
12	0.5	Cream		98.2	
13	0.5	Cream		99.0	105.0[c]
14	0.5	Lotion		106.2	
15	1	Ointment	Oxytetracycline	97.0	
16	1	Ointment	Oxytetracycline	99.4	
17	1	Ointment		98.4	
18	1	Ointment		94.4	
19	1	Ointment		105.7	101.0[g]
20	1	Cream		103.9	
21	1	Cream		97.5	
22	1	Cream	Iodochlorhydroxyquin	104.9	
23	1	Cream	Iodochlorhydroxyquin	91.0	
24	1	Cream	Iodochlorhydroxyquin	95.5	
25	2.5	Ointment		103.2	100.3[c]
26	25 mg.	Hydrocortisone acetate suppositories		98.4	92.5[f]
27	1.5	Cortisone acetate ointment		100.7	104.7[f]

[a] Alcohol extraction, blue tetrazolium (average of two). [b] Official NF XIII procedure. [c] Blue tetrazolium (average of three in Ointment 6). [d] Average of five results. [e] Red tetrazolium. [f] Isoniazid. [g] Fluorometric.

Procedural Format—The procedure for foreign steroids and assay covers situations where both detection of undesirable steroids and measurement of the major component are accomplished. Because of the nature of the assay and its use of internal standards, the procedure should be followed as written. The determination of foreign steroids not only accomplishes the accurate detection of the related but undesirable steroidal impurity, but it assures the analyst that the elution region in which the internal standard is to emerge is vacant and not occupied by some unanticipated compound. Clearly, the foreign steroids procedure provides for the validity of the assay which follows.

Analysis of Dosage Forms—A number of pharmaceutical dosage forms were analyzed by the proposed method. This procedure is capable of identifying and quantitatively measuring the main component and each foreign steroid, if present. By using the cyano ethyl silicone column, it was confirmed that none of the products in Table III had excessive foreign steroid levels based on the tolerances in the official compendia for tablet formulations. In most cases, however, some foreign steroids were found at the 1% level. When samples were analyzed which were manufactured by the same company and differed only in the strength of the active ingredient, it was observed that the same foreign steroids were present in each product at identical levels, indicating that the steroid raw material for the products probably came from the same lot or process. This procedure would be most effective for certifying the purity of raw material steroids.

The internal standards were used to monitor and determine the sample preparation content. In determining the ratio of internal standard to steroid needed for equivalent responses, the approximate peak height response ratio used was 1:1:3:6 for hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate, respectively. If internal standards are not available, the assay procedure may be altered to require dilution to a definite volume and multiple injections. The average peak heights would then be used to determine the assay value. This procedure would probably be less accurate but would suffice in most cases.

Assay results for the products analyzed on the cyano ethyl silicone column are given in Table III. Results achieved by other analytical methods for some samples are included for comparison. Only Sample 2 fell outside of the official limits; it was assayed by two other procedures that showed very good correlation with the proposed procedure. Sample 6 was analyzed by two different chemists using the blue and red tetrazolium procedures which yielded erratic results. This sample was assayed five times by liquid–liquid chromatography using five different sample weights from three tubes of the same code, with the results of 92.6, 96.6, 91.6, 95.6, and 97.2% of declared. The average deviation of these five results is 2.1%, which can be attributed in part to slight sample inhomogeneity but proves that it is not at the high level indicated by the tetrazolium results. One sample of hydrocortisone tablets was assayed on the octadecyl silane column and yielded no extraneous peaks other than the product peak. Due to the similarity of this and the cyano ethyl silicone columns, *i.e.*, they are both nonpolar and operate on the reverse-phase principle, it would be safe to assume that tablet formulations would provide no interferences on the latter column.

Interferences—The main problem in any chromatographic procedure is interfering compounds. In analyzing all of the products presented in Table III, none of the active ingredients in combination with the steroids interferes with the steroid peak but a problem may be encountered because of the peak ascribable to the cream, ointment, suppository, and lotion *base* or other formulation components. Most of this material is unretained by the column. In some cases where a large amount of excipient material comes through the extraction procedure, a broad peak is obtained. This results in a subsequent loss of resolution between the hydrocortisone and excipient peak. When this situation arises, care must be taken in constructing the baseline for the hydrocortisone. Products containing the other steroids do not present this problem owing to the longer retention time. Figure 5 presents a general chromatogram indicating the elution behavior of various compounds found in combination with the steroid.

This procedure appears to be applicable to other steroids that are accompanied by foreign steroid contamination. With this type of procedure, not only may the main steroid be quantitatively measured but each individual contaminant can be identified and quantitatively analyzed.

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PHARMACEUTICAL TECHNOLOGY

Tooling as a Factor in Tablet Weight Variation and Control

WILFRED C. LING

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Keyphrases □ Tablet weight variation—statistical contribution of individual tool parameters of rotary press □ Weight variation, tablets—statistical contribution of individual tool parameters of rotary press □ Tool parameters of rotary press—effect on tablet weight variation □ Punch length uniformity, rotary tablet press—effect on tablet weight variation

A foremost concern in the manufacture of tablets is the assurance of tablet weight uniformity. Studies of tablet weight variation on single-punch tablet machines (1, 2) have generally attributed the weight variation to nonuniform filling of the dies caused by variations in such parameters as granule size and distribution, bulk density, and flow properties of the granulations. In the case of the multiple-station rotary press, however, an

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Keyphrases □ Tablet weight variation—statistical contribution of individual tool parameters of rotary press □ Weight variation, tablets—statistical contribution of individual tool parameters of rotary press □ Tool parameters of rotary press—effect on tablet weight variation □ Punch length uniformity, rotary tablet press—effect on tablet weight variation

A foremost concern in the manufacture of tablets is the assurance of tablet weight uniformity. Studies of tablet weight variation on single-punch tablet machines (1, 2) have generally attributed the weight variation to nonuniform filling of the dies caused by variations in such parameters as granule size and distribution, bulk density, and flow properties of the granulations. In the case of the multiple-station rotary press, however, an

Table I—Summary of Experimental Parameters

Run Number	Punches	Dies	Number of Tablets in Samples	Source of Sample
1	Old[a]	Old	199	All tablets produced at time of sampling
2	New[b]	Old	200	As in Run 1
3A	New	Old	99	All tablets rejected by weight controller as "underweight"; a single lower punch, 0.25 mm. shorter than others, was used
4	New	Old	100	Tablets rejected by weight controller as "overweight" using an arbitrary upper limit
5	New	New[b]	298	All tablets produced at time of sampling

[a] Regular production punches that were used many times. Length dimensions are in Table II. [b] New punches were checked out to be within ±0.02 mm. (0.001 in.) variation. New dies were not measured.

Table II—Length Variation[a] and Cup Depth Dimensions of Old Punches[b]

Lower Punch Dimensions, mm.		Upper Punch Dimensions, mm.	
ΔL[c]	C[d]	ΔL	C
0	0.36	0	0.28
−0.02	0.36	+0.02	0.25
0	0.30	+0.05	0.25
0	0.36	+0.10	0.23
+0.05	0.28	+0.05	0.25
−0.02	0.33	+0.05	0.23
+0.02	0.28	−0.02	0.38
0	0.36	+0.10	0.23
0	0.30	+0.02	0.28
0	0.30	+0.02	0.41
0	0.36	+0.08	0.23
0	0.36	+0.05	0.28
0	0.30	+0.05	0.28
+0.05	0.28	+0.08	0.20
+0.02	0.25	−0.05	0.36
0	0.36	−0.02	0.23

[a] Length is the working length from the face of the punch tip to the flat of the punch head. [b] All dimensions are converted from the original measurements in inches. Measurements were made to the nearest 0.0254 mm. (0.001 in.). [c] ΔL = deviation of length dimension from a reference punch in millimeters. [d] C = cup depth in millimeters.

additional source of weight variation is introduced due to dimensional variations inherent in the use of a multiplicity of punches and dies. A purpose of this study was to determine, through compression study of a single product, how dimensional variation in punch length affects weight variation and what portion of the variation is contributed by each tool parameter. A comprehensive program in tool maintenance and record keeping was described previously (3, 4). Recent establishment of standard specifications (5) of tableting tools within the pharmaceutical industry provides a sound and uniform basis for pursuing a tool control program.

Progress made in recent years on strain gauge instrumentation of the rotary tablet press allows continuous monitoring of the tablet compression process in a production situation. The relationship between tablet weight and compression force is almost linear for a significant portion of the curve (6). The use of this relation to control tablet weight automatically was reported (7, 8). However, the value of such instrumentation is contingent upon the user's awareness that the weight control is of an indirect nature and subject to restraints of dimensional tolerance of the tableting tools. Part of the data gathered in this study shows the possibility of erroneous control if tablet punch dimensions are not adequately maintained.

EXPERIMENTAL

Materials—The material used was a granulation consisting of about 94% lactose with acacia and starch binders. It is a regular production lot for producing a specially shaped ovaloid tablet with a specified average weight range of 195–205 mg. based on the weighing of 10 tablets.

Equipment—A rotary tablet press[1] with 16 stations was used. The machine was equipped with strain gauges for use with a weight checking device[2].

The punches were standard 19-mm. (0.75-in.) diameter with a flat face beveled edge tip. The tip configuration was a 12.7 × 4.8-mm. (0.5 × 0.19-in.) ovaloid with center bisection. Both the tablet press and the tooling were regular production equipment[3]. A machine speed of approximately 60 r.p.m. was used.

Method—The tablet press was set up and operated by production personnel according to standard procedures. Weight checks and hardness tests were performed prior to production runs. After about 0.5 hr. of run time, a sample of tablets was taken and bottled. These tablets were then weighed individually on an analytical balance[4] to the nearest 0.1 mg. The length of each punch was measured, using a dial comparator and a V-block, to the nearest 0.02 mm. (0.001 in.).

During this study, changes were made in the upper and lower punches as well as in the dies to evaluate their effects on tablet weight variations. In addition, the weight controller[2] was put into operation for a portion of the test run during which off-weight tablets were rejected by the control device. These rejects were individually weighed. The upper and lower weight limits were set in terms of their compression force readings according to a regression line established prior to the run. Repeated test runs established the reliability and stability of the electromechanical systems of the weight control instrument.

Table I summarizes the different parameters used in the five runs as well as the number and description of tablet samples taken in these runs.

RESULTS AND DISCUSSION

Variation of Tablet Weight—The weight data gathered from each run were treated statistically to obtain mean tablet weight, standard deviation, and frequency distribution. Figure 1 shows the frequency distribution of tablet weight for Run 1 where old punches and dies were used. The length measurement of the upper and lower punches used is shown in Table II. Figure 2 shows the frequency distribution of tablet weight when new punches with ±0.02-mm. tolerance were used. Figure 3 shows a corresponding distribution when both punches and dies were new (Run 5). The bore of the old dies, under microscopic examination, showed signs of wear. Although no attempt was made to measure the amount of wear, it was sufficient to cause frequent capping problems during all runs except Run 5, when a new set of dies was installed.

Since the purpose of this study was to determine the tooling effect on weight variation and since these effects were not time variant, all samples were collected over a period just long enough for producing a specified number of tablets. Chi-square analysis

[1] Manesty Betapress, Thomas Engineering, Hoffman Estate, Ill.
[2] The Gretag weight control instrument (TPG 410), with accept–reject mechanism as well as strain gauge installation, was provided by Thomas Engineering, Hoffman Estate, Ill.
[3] Of the Tablet Production Department.
[4] Sartorius model 2462, Brinkmann Instruments, Inc., Westbury, N. Y.

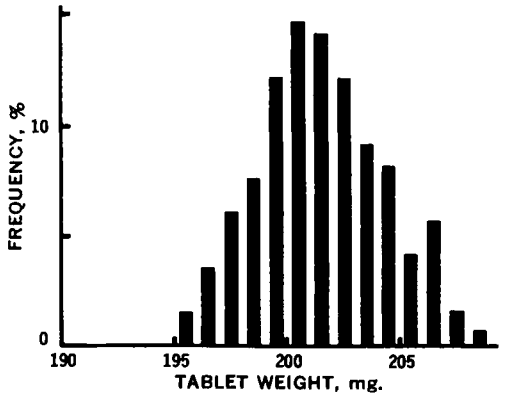

Figure 1—*Frequency distribution of tablet weight, Run 1, old punches and old dies. Mean sample weight = 201.4 mg., SD = 2.8 mg.*

of the tablet weight distribution showed that all samples gave normal distribution at the 95% probability level.

Table III lists the mean sample weight and the variances for Runs 1, 2, 3A, 4, and 5, where different tool parameters were used. No significance should be attached to the different values of mean weight as many weight adjustments were made throughout this study spanning several days. The tablets of Run 3A were made extra heavy by the use of an extra-short lower punch so that tablets produced at this station could be automatically rejected by the weight controller. The purpose was to isolate tablets made from a single-punch station and study the weight variation.

Effect of Tool Parameters on Weight Variation—Table III indicates that variance information may be derived from the available data to show what each parameter contributes toward the total variability of tablet weight. This follows from the statistical principle that the total variance of a system is equal to the sum of its components. The variances of Samples 1, 2, 3A, and 5 in Table III were tested and found to be significantly different at the 0.05 level or less.

In this study, the total weight variance was associated with the use of old punches and dies. The next level of variability occurred when the old punches were substituted with new punches, keeping all other parameters constant. The variability was still lowered by using new dies. The final level of variability was exhibited when only a single-punch station was involved. This "single-station" effect was achieved, as indicated earlier, by producing extra-heavy tablets through the use of an extra-short lower punch and rejecting (isolating) the tablets automatically through the tablet weight controller. The residual variability of the single-station compression stemmed primarily from variations due to granulations and the nontool part of the tablet press.

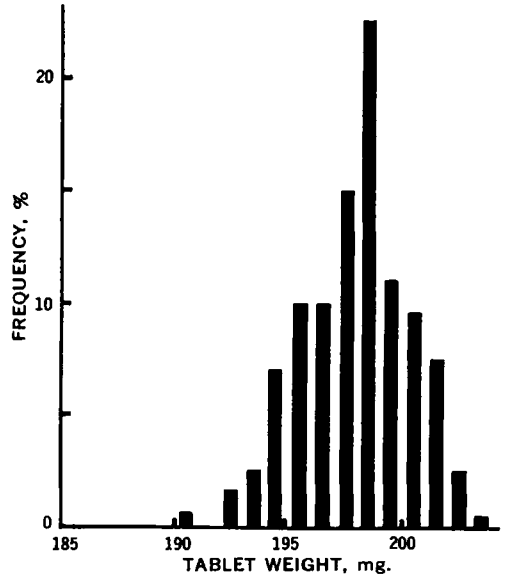

Figure 2—*Frequency distribution of tablet weight, Run 2, new punches and old dies. Mean sample weight = 197.9 mg., SD = 2.3 mg.*

Table III—Summary of Statistical Data

Run Number	Mean Tablet Weight, $\bar{x}$, mg.	Variance, S^2	Source of Variance
1	201.4	7.97	Old punches plus old dies plus single-station variability
2	197.9	5.51	New punches plus old dies plus single-station variability
3A	205.8	1.73	Single-station variability
4	204.7	5.20	Old punches plus old dies plus single-station variability plus regression error in force-weight correlation
5	201.1	4.35	New punches plus new dies plus single-station variability

Table IV shows the variance associated with each parameter. These variance components were calculated from the data of Table III in a manner shown under the column "Reference to Variance of Table III." The subscripts for the different variances, S^2, refer to the run number. Obviously, the variance component and percentages are specific to the particular system under study and cannot be generalized to other situations. For instance, a granulation with difficult flow characteristics would have a higher proportion of the total variance associated to single-station compression. Similarly, a different set of punches with a marginal maintenance record may show a large variance and, therefore, a higher percentage of the total variance due to nonuniformity of the punches. While the comparison of variances in Table IV is not intended for generalization, such analysis is useful in pointing to the significance of good tooling as reflected in tablet weight uniformity. In the present case, as much as 45% of the weight variance was eliminated by substituting old punches and dies with new tools and a 30% reduction in variance was achieved with use of close tolerance punches (±0.02 mm.) alone.

Effect of Punch Length on Automatic Weight Control Based on Punch Force—The discussions so far have been on weight variation caused by nonuniform die fill due to variations in lower punch and die dimensions. With the availability of tablet weight control devices based on compression force, the length uniformity of the upper punches assumes added importance. The following analysis considers what happens to the compression force when the length of one or both punches is changed by a fixed amount.

The density of sulfathiazole tablets was found to be (9) linearly proportional to the logarithm of compression force for all but the

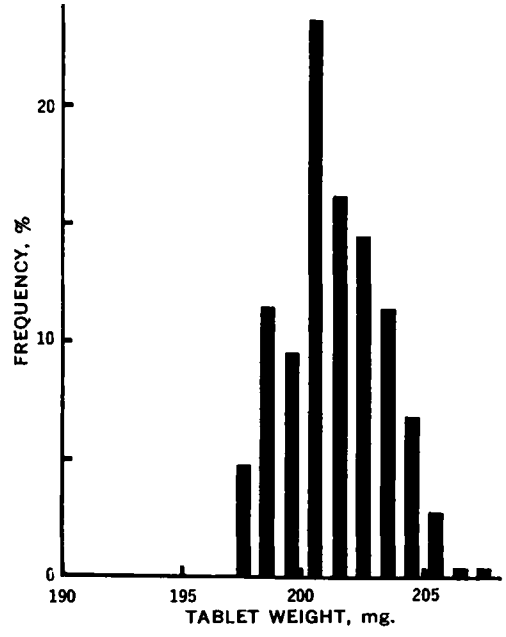

Figure 3—*Frequency distribution of tablet weight, Run 5, new punches and new dies. Mean tablet weight = 201.1 mg., SD = 2.1 mg.*

Table IV—Variance Components of a Tablet Compression System Using Old Punches and Dies

Source of Variance	Reference to Variance of Table III	Variance Component, S^2	Percent of Total Variance, %
Single-station compression	S_3^2	1.73	22
Multiple-station compression with new punches and dies	$S_5^2 - S_3^2$	2.62	33
Old punches	$S_1^2 - S_2^2$	2.46	31
Old dies	$S_2^2 - S_5^2$	1.16	14
	Total variance =	7.97	100%

highest level of force. Similar relationships have been reported for *p*-acetanisidine (methacetin) (10) and for several alkaline halides (11). This empirical relation may be expressed as follows:

$$\ln F = \ln A + B\rho_2 \qquad \text{(Eq. 1)}$$

or:

$$F = Ae^{B\rho_2} = Ae^{B\rho_1 V_1/V_2} \qquad \text{(Eq. 2)}$$

Differentiating gives:

$$dF = \frac{AB\rho_1 e^{B\rho_1 V_1/V_2}}{V_2^2}(V_2\,dV_1 - V_1\,dV_2) \qquad \text{(Eq. 3)}$$

where F = compression force, ρ_1 = bulk density of granulation, ρ_2 = density of finished tablet, V_1 = volume of die fill, V_2 = volume of tablet, A = proportionality constant, and B = proportionality constant.

For very small changes in volume, Eq. 3 may be written as follows:

$$\Delta F = \frac{AB\rho_1 e^{B\rho_1 V_1/V_2}}{V_2^2}(V_2\,\Delta V_1 - V_1\,\Delta V_2) \qquad \text{(Eq. 4)}$$

where $V_1 > V_2$, ΔF = change in compression force, ΔV_1 = change in die fill volume, and ΔV_2 = change in tablet volume.

Although data were not available to quantify the dependence of force on punch length variation according to Eq. 4, the equation does allow estimation of the direction of change in force as a result

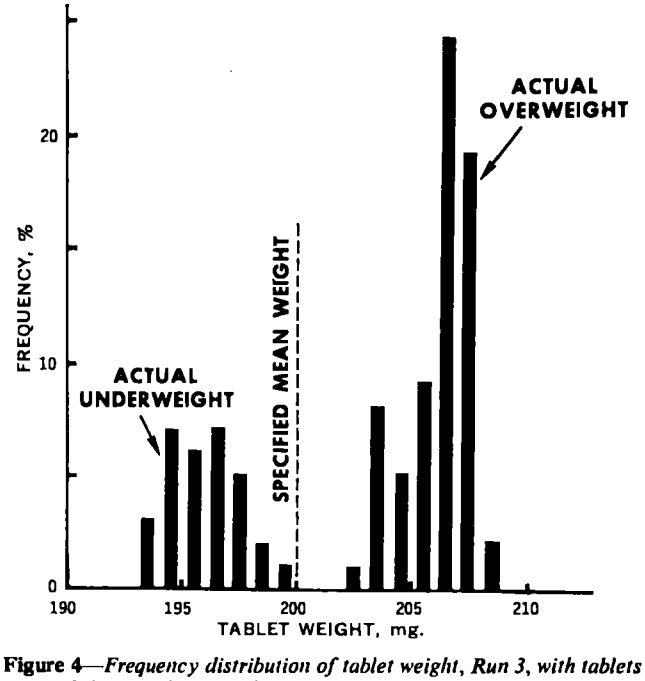

Figure 4—*Frequency distribution of tablet weight, Run 3, with tablets rejected by weight controller as underweight. One lower punch is 0.25 mm. short.*

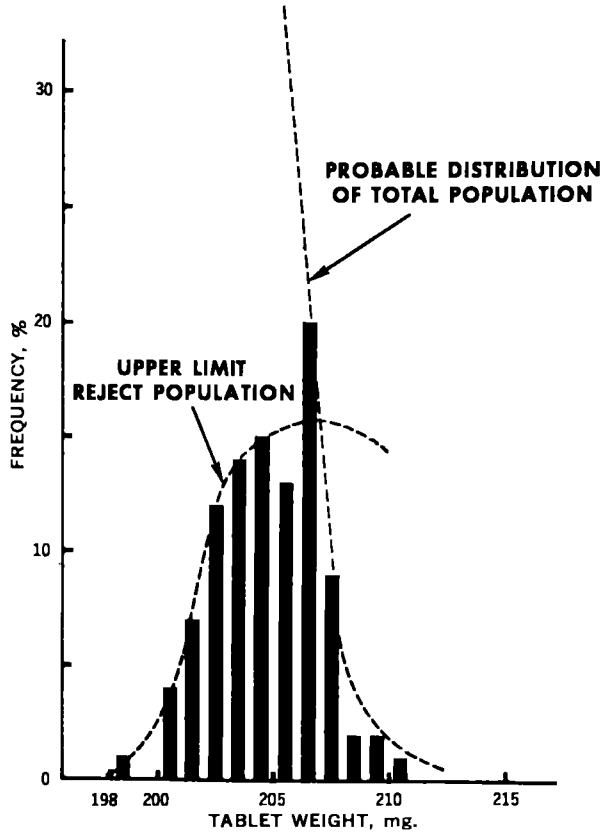

Figure 5—*Frequency distribution of tablet weight, Run 4, with tablets rejected by weight controller at upper weight limit. Mean sample weight = 205 mg., SD = 2.3 mg.*

of a fixed change in punch lengths in a selected combination. Table V illustrates the use of this method of estimation whereby punch length deviations of 0, +d, and −d were assumed for nine combinations. Depending upon these combinations, the punch length deviations can be translated as an increase (+) or a decrease (−) of the die fill and tablet volumes, ΔV_1 and ΔV_2. The tablet weight control device, receiving inputs of compression force, indicates deviation of the specified tablet weight in relation to the deviation of compression force, ΔF, from the set point. Thus, there is no weight deviation if $\Delta F = 0$, there is overweight if ΔF is positive, and there is underweight if ΔF is negative.

Examination of Table V reveals that it is possible to have the weight controller output contradicting the actual tablet weight. In fact, in the simplified analysis of Table V, only Cases 1, 5, and 9 give indicated weight (instrument output) in agreement with the actual tablet weight. The magnitude of the discrepancy between the indicated weight and actual weight would depend upon the extent of the punch length deviation, upper and lower punch combinations, tablet thickness, compression ratio, granulation density, and compressibility. Published data (9) were used in conjunction with Eq. 4 to gain an approximate estimate of indicated weight deviation based on deviations in punch length. Such a calculation showed that if one of the lower punches is short by 0.05 mm. (0.002 in.), the weight deviation as indicated by the weight control instrument would be about 0.7% overweight whereas the actual tablet is about 0.4% overweight. If both upper and lower punches of a station were 0.05 mm. (0.002 in.) short, then the indicated weight deviation would be about 2.5% *underweight* with the actual tablet weight still 0.4% overweight.

Tablet punches of wide dimensional variations are likely to produce tablets with a wide distribution of tablet weight. Under such circumstance, use of a tablet weight controller may not always assure uniformity of weight. On the contrary, erroneous control is likely, including the acceptance of rejectable tablets and rejection of acceptable tablets.

Figure 4 is a frequency distribution of tablet weight of Run 3 where an extra-short lower punch (0.25 mm. shorter than standard)

Table V—Effect of Punch Length Variation on Output Signal of Tablet Weight Controller

Case Number	Lower Punch Length[a]	Upper Punch Length[a]	ΔV_1[b]	ΔV_2[c]	ΔF[d]	Indicated Weight (from Instruments)	Actual Weight (Determined by Lower Punch)
1	Standard	Standard	0	0	0	Standard	Standard
2	Standard	+d	0	−	+	Overweight	Standard
3	Standard	−d	0	+	−	Underweight	Standard
4	−d	Standard	+	+	−	Underweight	Overweight
5	−d	+d	+	0	+	Overweight	Overweight
6	−d	−d	+	+	−	Underweight	Overweight
7	+d	Standard	−	−	+	Overweight	Underweight
8	+d	+d	−	−	+	Overweight	Underweight
9	+d	−d	−	0	−	Underweight	Underweight

[a] In this analysis, deviation from standard punch length is either +d (longer by d mm.) or −d (shorter by d mm.). [b] ΔV_1 = change in die fill volume. [c] ΔV_2 = change in tablet volume. [d] ΔF = change in compression force.

was used. The strongly bimodal distribution indicates the presence of two distinct populations within the sample. The tablets with mean weight of 195 mg. were actually underweight tablets produced at the same time as those made by the short punch station with a mean weight of 206 mg. Both groups exhibited low compression force and were rejected as underweight, although one group was actually overweight. This illustration is similar to Case 4 or 6 of Table V.

Figure 5 is a frequency distribution of tablet weight from a sample of overweight rejects. A sharp cutoff is absent at the overweight reject point. Rather, the weights are normally distributed with a standard deviation as large as that of the tablet population (Fig. 3). Underweight rejects, although not analyzed, are believed to have similar frequency distributions. Although there are other factors, tool uniformity is probably a significant factor in causing a weight distribution rather than a sharp cutoff at the rejection point. The same factor is probably also responsible for a lack of precise correlation between compression force and tablet weight. Ridgway *et al.* (8), in analyzing the performance of their closed-loop weight controller, experienced similar frequency distribution in the underweight and overweight rejects as well as scattering of data in the correlation between compression pressure and tablet weight.

SUMMARY

The weight variations of tablets produced on a rotary tablet press were analyzed. Estimates were made from the statistical data as to the contribution of each tool parameter to the total variance in tablet weight.

The effect of punch length variation was also explored for its interaction with tablet weight controllers which derive their input signal from compression force. Possible erroneous control is cautioned if critical tool dimensions are not adequately maintained.

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Nitroglycerin Sublingual Tablets II: Preparation and Stability of a New, Stabilized, Sublingual, Molded Nitroglycerin Tablet

SALVATORE A. FUSARI

Abstract □ A new, molded, stabilized, sublingual nitroglycerin tablet was prepared. This tablet shows the same bioavailability as conventional tablets but has the advantage of markedly improved stability. In contrast to conventional tablets, which often develop marked intertablet dose variation within 1 month, the stabilized tablet maintains its content uniformity for long periods, even at 37 and 45°, thus assuring a more uniform and predictable dose to the patient.

Keyphrases □ Nitroglycerin sublingual tablets—preparation, stability analysis of molded tablets containing polyethylene glycol as a fixative □ Sublingual nitroglycerin tablets—preparation, stability analysis of molded tablets containing polyethylene glycol as a fixative

Many papers have reported the instability of conventional molded sublingual tablets (1–7), and all have noted that the volatilization of nitroglycerin plays a part in this instability. However, it has only recently been recognized that one fundamental change accompanying volatilization is intertablet migration of nitroglycerin, leading to large intertablet dose variation. A report (8) showed that conventional nitroglycerin tablets are unstable, not in the usual sense of chemical breakdown but with respect to (*a*) slow loss of potency due to volatilization of nitroglycerin, and (*b*) maintenance of content uniformity.

Since this instability could not be controlled by manipulating the container and cap liner, it was decided that the best solution involved reformulation of the tablet to minimize volatilization and subsequent intertablet migration of nitroglycerin.

As shown in the first paper of this series (8), all results indicated that the problem with conventional sublingual nitroglycerin tablets starts with the manufacturing process[1]. During this procedure, a mixture of alcohol and water is added to a blended mixture of nitroglycerin, lactose, and sucrose. After molding, the tablets are air dried. It is postulated that nitroglycerin can be brought to the tablet surface during the drying process by the evaporation of alcohol and water, where it becomes available for loss and/or migration by slow volatilization, depending on the character of the immediate environment. If this hypothesis is correct, the addition of a "fixing" agent to the tablets should inhibit the volatilization of nitroglycerin and help solve the problems of potency loss and intertablet dose variation caused by the migration of nitroglycerin.

This paper describes the preparation of a stabilized tablet and its properties, followed by a description of the experiments that confirmed its superior stability.

[1] This possibility was also suggested by M. D. Richman *et al.* (1).

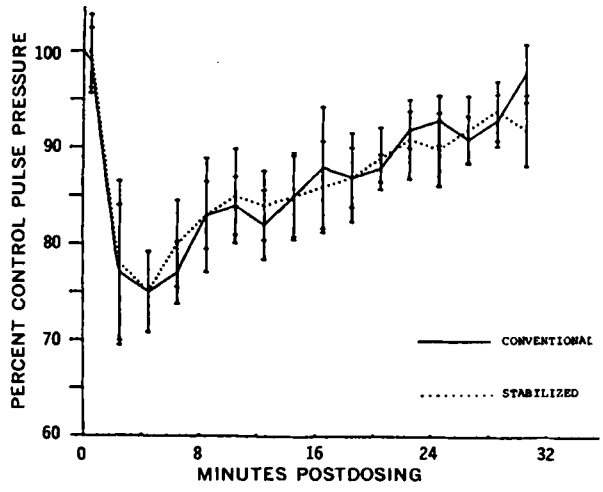

Figure 1—*Comparative bioavailability of conventional and stabilized nitroglycerin tablets in dogs.*

Both short-term stress experiments and long-term stability studies were included. While some short-term stress experiments have no necessary parallel in a real life situation, they illustrate the regulated or controlled behavior of the stabilized tablet, which is in marked contrast to the irregular behavior exhibited by the conventional tablet. The long-term stability studies are still in progress; data up to 25 months will be given.

EXPERIMENTAL

Tablet Manufacture

Molded, stabilized, sublingual tablets[2] were made in exactly the same way as conventional sublingual tablets, except that a water-soluble, nonvolatile fixing agent[3] was added to the formulation. The fixative was added as a constant percentage of the nitroglycerin present in our three different formulations (0.3, 0.4, and 0.6 mg. nitroglycerin per tablet). The lactose, sucrose, and 10% nitroglycerin tablet mixture[4] was blended in a 142-l. blender (PK), equipped with a liquid feed bar (intensifier bar), for 10 min. The granulating fluid, consisting of water, alcohol, and fixative, was added through the intensifier bar over 3 min., after which the mixture was blended for another 12 min. The intensifier bar operated continuously throughout the entire 25-min. blending period. The blended mixture was then stored overnight in a refrigerator. The molded[5] tablets were then air dried for about 20–48 hr. before bottling and packaging.

[2] Nitrostat; Parke, Davis brand of stabilized, molded, sublingual nitroglycerin tablets.

[3] Polyethylene glycol 400 was used for the tablets described in this paper, but polyethylene glycol 4000 has also been used successfully. The amount used was 85% (w/w) of the nitroglycerin in the tablet formula.

[4] E. I. duPont de Nemours supplies this as a 10% nitroglycerin–90% β-lactose (w/w) mixture.

[5] Molding was accomplished using a Colton tablet molding machine, model 720.

Table I—Properties of Stabilized Sublingual Tablets[a]

	Stabilized Tablet	Conventional Tablet
Physical properties		
Disintegration	Not >45 sec.	Not >45 sec.
Wickability	Average (20 tablets): not >20 sec., no tablet >40 sec.	Average (20 tablets): not >20 sec., no tablet >40 sec.
Chemical properties		
Assay (as is):		
Average (30 tablets): 99.7% label claim		
Range: 92.1–108.1%		
SD: 4.0%		
SD σ (corrected for tablet weights): 1.3%		

[a] Nitrostat.

Table II—Transfer of Nitroglycerin from Conventional Tablets to Stabilized Tablets from Open Dishes

	Stored in Same Glass Desiccator under Vacuum for 24 hr.					
	Before Vacuum			After Vacuum		
Tablet	Average Assay, % of L.C.[a]	Range of Assays, % of L.C.[a]	Percent Relative Standard Deviation	Average Assay, % of L.C.[a]	Range of Assays, % of L.C.[a]	Percent Relative Standard Deviation
Stabilized tablet, 432 mcg. ($^1/_{150}$th gr.)[b]	102.8	92.6–109.9	3.3	138.5	127.8–151.6	3.8
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	96.4	71.6–115.8	9.6	73.2	43.0–108.2	25.1
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	104.1	88.6–126.8	9.1	74.1	55.0–101.0	16.1
Stabilized tablet, 432 mcg. ($^1/_{150}$th gr.)[b]	104.1	95.9–111.6	3.7	125.7	119.1–135.2	3.7
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	102.5	70.9–115.9	11.0	89.5	40.7–113.7	16.7
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	104.1	88.6–126.8	9.1	75.7	63.4–89.4	8.4
Stabilized tablet, 432 mcg. ($^1/_{150}$th gr.)[b]	102.8	92.6–109.9	3.3	111.0	101.4–124.2	4.5
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	102.5	70.9–115.9	11.0	93.9	61.8–113.6	15.1
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	104.1	88.6–126.8	9.1	91.7	72.9–108.7	8.7
Stabilized tablet, 432 mcg. ($^1/_{150}$th gr.)[b]	105.3	93.5–114.3	3.7	146.9	130.5–162.4	5.8
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	102.5	70.9–115.9	11.0	83.4	37.8–111.3	23.9
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	104.1	88.6–126.8	9.1	71.2	51.6–94.5	16.9
Stabilized tablet, 0.6 mcg.[b]	103.9	95.0–112.0	4.1	123.3	111.6–132.5	4.8
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	102.5	70.9–115.9	11.0	84.8	48.6–107.6	19.2
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	104.1	88.6–126.8	9.1	79.1	54.0–106.0	15.8

[a] Label claim. [b] These samples were in the same desiccator.

Biopharmaceutical Equivalence

An experimental model using dogs was developed to compare bioavailability of stabilized and conventional tablets. Six nonanesthetized adult beagle dogs, weighing 12 kg., were used. Tablets were given sublingually, and peripheral pulse pressure was monitored using an on-line computer[6] (9).

Short-Term Experiments—*Exposure to High Vacuum from Open Dishes*—Stabilized and conventional tablets were placed close to each other in open 7.62-cm. (3-in.) watch glasses or 5.3-cm. petri dishes in glass vacuum desiccators and were exposed to pump vacuum for 24 hr. This procedure was repeated three times.

Exposure to High Vacuum in Opened Commercial Containers—The caps and stuffing were removed from stabilized and conventional tablets in their commercial containers, and the bottles were placed as far away from each other as possible in *glass* vacuum desiccators. Vacuum, from a vacuum pump, was continuously applied for 24 hr., after which the vacuum (about 1 torr) was released and 30 tablets from each bottle were assayed. Four separate experiments were run.

Exposure to High Vacuum on Open Dishes in Plastic Desiccator—Two 7.62-cm. (3-in.) petri dishes were placed in a 25.40-cm. (10-in.) plastic desiccator. One dish contained stabilized tablets and the other contained conventional tablets. Pump vacuum (about 1 Torr) was applied for 23 hr., after which the vacuum was disconnected and 30 tablets from each dish were assayed. The experiment was repeated.

Storage in Polystyrene Boxes (Patient-Type Containers) for 1 Month at 37°—One hundred stabilized and conventional tablets were placed in separate polystyrene boxes, 3.81 × 5.71 × 1.58 cm. (1.5 × 2.25 × 0.625 in.), with hinged lids; two sets of boxes were used for each type of tablet. In addition, tablets of each type were stored in closed, regular amber-glass containers as controls. After obtaining an "initial time" assay, all samples were stored at 37°. One box of each set was opened twice a day for 2 min. and the other was kept closed. After 1 month, 30 individual tablets were assayed.

[6] EMR.

Table III—Effect of Storage of Stabilized Tablets and Conventional Tablets in Open Commercial Containers under Vacuum in Glass Desiccators for 24 hr.

Tablet	Diameter of Desiccator (in.)	Before Vacuum: Average Assay, % of L.C.[a]	Before Vacuum: Range of Assays, % of L.C.[a]	Before Vacuum: Percent Relative Standard Deviation	After Vacuum: Average Assay, % of L.C.[a]	After Vacuum: Range of Assays, % of L.C.[a]	After Vacuum: Percent Relative Standard Deviation
Stabilized tablet, 432 mcg. (1/150th gr.)[b]	25.40 cm. (10)	102.8	92.6–109.9	3.3	101.6	92.2–112.2	5.1
Conventional tablet, 432 mcg. (1/150th gr.)[b]	25.40 cm. (10)	102.5	70.9–115.9	11.0	95.6	65.6–115.9	13.9
Conventional tablet, 432 mcg. (1/150th gr.)	20.32 cm. (8)	104.1	88.6–126.8	9.1	96.8	65.5–130.9	16.7
Stabilized tablet, 648 mcg. (1/100th gr.)[b]	20.32 cm. (8)	102.8	93.3–112.8	5.0	123.4	106.6–138.5	5.7
Conventional tablet, 648 mcg. (1/100th gr.)[b]	20.32 cm. (8)	100.4	75.1–117.9	11.5	53.1	24.2–82.0	33.9
Stabilized tablet, 648 mcg. (1/100th gr.)[b]	25.40 cm. (10)	102.8	93.3–112.8	5.0	122.0	105.5–137.2	7.1
Conventional tablet, 648 mcg. (1/100th gr.)[b]	25.40 cm. (10)	100.4	75.1–117.9	11.5	59.6	23.6–109.5	41.3
Stabilized tablet, 0.4 mg.[b]	20.32 cm. (8)	102.8	92.6–109.9	3.3	133.3	114.1–145.1	5.9
Conventional tablet, 648 mcg. (1/100th gr.)[b]	20.32 cm. (8)	89.6	42.0–119.6	18.3	21.7	4.1–71.8	85.2

[a] Label claim. [b] These tablets were run simultaneously in the same desiccator.

Table IV—Comparison of Loss of Nitroglycerin from Stabilized Tablets and Conventional Tablets from Open Dishes under Vacuum in 25.40-cm. (10-in.) Plastic Desiccator for 23 hr.

Tablet	Before Vacuum: Average Assay, % of L.C.[a]	Before Vacuum: Range of Assays, % of L.C.[a]	Before Vacuum: Percent Relative Standard Deviation	After Vacuum: Average Assay, % of L.C.[a]	After Vacuum: Range of Assays, % of L.C.[a]	After Vacuum: Percent Relative Standard Deviation
Stabilized tablet, 432 mcg. (1/150th gr.)[b]	102.8	92.6–109.9	3.3	91.5	82.2–99.0	5.0
Conventional tablet, 432 mcg. (1/150th gr.)[b]	104.1	88.6–126.8	9.1	40.3	19.9–58.3	21.1
Stabilized tablet, 432 mcg. (1/150th gr.)[b]	102.8	92.6–109.9	3.3	84.9	76.6–94.3	5.5
Conventional tablet, 432 mcg. (1/150th gr.)[b]	104.1	88.6–126.8	9.1	37.9	27.5–57.6	19.5

[a] Label claim. [b] These dishes were in same vacuum desiccator.

Table V—Comparison of Stabilized Tablets and Conventional Tablets Stored in Polystyrene Boxes at 37°

Sample	Stabilized Tablets, 432 mcg. (1/150th gr.): Average Assay, % of L.C.[a]	Stabilized Tablets, 432 mcg. (1/150th gr.): Range of Assays, % of L.C.[a]	Stabilized Tablets, 432 mcg. (1/150th gr.): Percent Relative Standard Deviation	Conventional Tablets, 432 mcg. (1/150th gr.): Average Assay, % of L.C.[a]	Conventional Tablets, 432 mcg. (1/150th gr.): Range of Assays, % of L.C.[a]	Conventional Tablets, 432 mcg. (1/150th gr.): Percent Relative Standard Deviation
Glass control						
Initial time	103.2	94.7–111.3	3.3	104.7	93.5–111.2	3.1
After 1 month	99.9	94.9–107.5	3.1	98.3	80.3–137.0	11.6
Polystyrene "opened" twice a day, after 1 month	93.4	87.2–102.9	3.2	82.6	61.1–103.1	11.0
Polystyrene "closed," after 1 month	92.7	82.6–101.8	5.1	94.4	64.6–119.5	13.1

[a] Label claim.

Storage in Cardboard Boxes (Patient-Type Containers) at 37° for 1 Month—Cardboard boxes are easily available to patients. The box[7] selected measures 6.98 × 8.25 × 0.15 cm. (2.75 × 3.25 × 0.06 in.) and has an inner compartment which is completely covered over by a sliding outer shell in the manner of a safety match box. Since these boxes are not as tight as polystyrene boxes, three sets of boxes, plus a glass bottle control, were used. One box was opened two times a day for 1 month, another was kept closed, and the third box was sealed with wax. The glass bottle controls for stabilized and conventional tablets served to give the initial time assay for each set. Boxes and bottles, each containing 100 tablets, were stored at 37°. After 1 week and again after 1 month, 30 individual tablets were assayed from each box and the glass bottle.

Effect of Storage with Different Liners, Rayon Stuffing, and Periodic Bottle Opening (Closed Environment)—One lot of stabilized tablets was compared to a lot of conventional tablets. Tablets were

[7] Parke, Davis "Medicated Throat Disc" package.

Table VI—Comparison of Stabilized Tablets and Conventional Tablets Stored in Cardboard Boxes for 1 Month at 37°

Sample	Stabilized Tablets, 432 mcg. (1/150th gr.)			Conventional Tablets, 432 mcg. (1/150th gr.)		
	Average Assay, % of L.C.[a]	Range of Assays, % of L.C.[a]	Percent Relative Standard Deviation	Average Assay, % of L.C.[a]	Range of Assays, % of L.C.[a]	Percent Relative Standard Deviation
Glass bottle						
Initial	103.2	93.5–111.4	3.6	110.7	99.1–119.8	4.1
After 1 month	101.4	95.1–110.5	3.3	105.7	95.4–119.2	5.6
Box, "open"						
1 week	77.0	59.0–95.1	11.4	70.7	38.5–101.8	26.2
1 month	28.2	24.6–31.7	7.1	5.4	3.6–7.6	16.9
Box, "closed"						
1 week	87.4	72.9–98.2	5.6	62.8	26.6–94.7	26.0
1 month	32.0	20.3–47.4	24.1	5.3	2.7–9.7	30.8
Box, "wax"						
1 week	84.6	64.5–97.2	8.0	82.3	10.5–123.7	27.8
1 month	40.8	28.9–46.5	12.3	7.9	4.5–29.6	68.0

[a] Label claim.

Table VII—Effect of Immediate Environment on Stabilized Tablets and Conventional Tablets at 37°, Opened and Closed Bottles

Sample	Unopened Controls						Opened Bottles[a]					
	With Rayon; % of L.C.[b]			Without Rayon; % of L.C.[b]			With Rayon; % of L.C.[b]			Without Rayon; % of L.C.[b]		
	Average	Range	SD	Average	Range	SD	Average	Range	SD	Average	Range	SD
Conventional tablet, 432 mcg. (1/150th gr.), liner I[c]												
Initial assay	104.7	98.5–111.2	3.2	—	—	—	—	—	—	—	—	—
1 month	100.8	67.9–135.1	15.5	98.8	82.0–119.8	9.7	99.1	83.9–136.4	11.7	102.3	82.2–134.4	12.2
Stabilized tablet, 432 mcg. (1/150th gr.)												
Initial assay	103.2	94.7–111.3	3.4	—	—	—	—	—	—	—	—	—
Liner I[c], 1 month	99.5	87.8–108.4	5.1	98.8	88.5–112.1	4.3	100.3	95.0–108.9	3.1	104.5	96.8–111.5	3.5
Liner II[d], 1 month	102.0	87.5–116.1	6.8	101.7	92.1–109.0	2.9	102.0	94.9–108.5	3.8	104.6	97.6–117.7	4.9
Polyethylene terephthalate[e], 1 month	104.9	92.1–111.1	4.0	103.4	95.7–114.1	4.4	101.4	91.5–108.9	3.9	101.6	93.3–109.3	3.7
Tinfoil, 1 month	103.0	95.0–111.9	4.5	102.1	94.5–112.3	4.4	101.1	90.1–113.5	4.3	100.8	93.8–116.9	4.3

[a] Opened 2 min. a day for a month, weekends excepted, for a total of 21 openings. [b] Label claim. [c] Excelloseal. [d] Esterfoil 50. [e] Mylar.

Table VIII—Comparison of Nitroglycerin Transfer from Stabilized Tablets and Conventional Tablets to Placebo Tablets at 37°[a]

Sample and Time Interval	Tablet Assays, 432 mcg. (1/150th gr.)			Placebo Assays	
	Average Assay, % of L.C.[b]	Range of Assays, % of L.C.[b]	Percent Relative Standard Deviation	Average Assay, % of L.C.[b]	Range of Assays, % of L.C.[b]
Stabilized tablet					
Initial	103.2	94.7–111.3	3.4	—	—
8 days	—	—	—	1.6	—
1 month	99.0	92.3–105.9	3.5	2.0	—
Conventional tablet 1					
Initial	106.0	78.5–129.0	13	—	—
8 days	—	—	—	21.6	—
1 month	86.4	38.0–121.9	16.4	26.6	20.4–35.3
Conventional tablet 2					
Initial	104.7	98.5–111.2	3.2	—	—
8 days	—	—	—	6.6	—
1 month	99.1	83.0–117.0	7.4	8.4	5.6–14.2

[a] These results are not as dramatic as those described earlier (8) using the 648-mcg. (1/100th-gr.) tablet; however, since this was a side-by-side comparison, the results are indicative of the differences in the tablets. [b] Label claim.

placed in amber-glass bottle containers; half of the bottles had a small pledget of rayon stuffing inserted and the other half had no stuffing. One liner[8] was used for the conventional tablets, and several liners[9] were used for the stabilized tablets. After capping, all bottles were stored at 37° for 1 month. Half of the bottles of each type were used as closed bottle controls, while the remaining bottles were opened once a day, except weekends, for 2 min. for a total of 21 openings.

Effect of Storing in Presence of Placebo Tablets (Closed Environment)—This experiment was performed to compare the behavior of two types of tablets when stored with placebos. The tablets used were 432 mcg. (1/150th gr.). Three separate runs were made, two involving conventional tablets and one involving the stabilized tablet. After initial assays were run, 69 nitroglycerin-containing

[8] Excelloseal (Kerr and Owens Illinois).
[9] Excelloseal, tinfoil, Esterfoil, and polyethylene terephthalate (Mylar).

Table IX—Comparison of Retention of Original Potency for Stabilized Tablets and Conventional Tablets Exposed on Open Dishes at Room Temperature

Lot	Time	Average Assay, % of L.C.[a]	Range of Assays, % of L.C.[a]	Percent Relative Standard Deviation	Percent of Original Potency Retained
Stabilized tablet 1, 0.4 mg.	Initial	104.4	91.7–112.9	5.7	—
	2 Days	96.7	81.8–107.3	6.8	93
	5 Days	84.6	68.0–101.6	10.5	81
	7 Days	66.1	41.0–88.5	20.0	63
Stabilized tablet 2, 0.4 mg.	Initial	106.4	99.6–112.5	3.1	—
	7 Days	65.1	32.8–85.5	22.3	61
Conventional tablet 1, 432 mcg. ($^1/_{150}$th gr.)	Initial	102.6	84.0–119.5	7.4	—
	3 Days	72.9	37.0–122.2	30.3	71
	5 Days	67.6	30.4–100.8	24.0	66
Conventional tablet 2, 432 mcg. ($^1/_{150}$th gr.)	Initial	96.4	71.6–115.8	9.6	—
	3 Days	68.1	34.7–111.8	27.3	71
	6 Days	45.8	8.1–84.3	44.5	48
Conventional tablet 3, 432 mcg. ($^1/_{150}$th gr.)	Initial	97.4	60.1–110.8	11.4	—
	3 Days	73.9	39.4–100.9	21.4	76
	6 Days	54.5	13.7–83.2	31.2	56
Conventional tablet 4, 648 mcg. ($^1/_{100}$th gr.)	Initial	94.9	61.9–122.2	17.1	—
	3 Days	78.2	43.6–130.6	31.3	82
	5 Days	64.7	35.6–93.9	24.9	68

[a] Label claim.

Table X—Effect of Storage in Open Commercial Containers at Room Temperature in Draft-Free Area

Tablet	Days	Average Assay, % of L.C.[a]	Range of Assays, % of L.C.[a]	Standard Deviation
Stabilized tablet, 432 mcg. ($^1/_{150}$th gr.)	0	100.5	90.0–107.7	4.5
	22	99.8	90.6–107.3	4.0
Stabilized tablet, 648 mcg. ($^1/_{100}$th gr.)	0	104.4	91.7–112.9	5.9
	26	101.4	91.2–114.4	4.5
	41	101.0	80.5–114.1	6.6
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	0	96.4	71.6–115.8	9.3
	28	95.3	65.8–109.8	11.0
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	0	97.4	60.1–110.8	11.1
	28	97.9	73.7–113.8	12.0
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	0	102.6	84.0–119.5	7.6
	28	98.3	73.1–134.7	12.0
Conventional tablet, 432 mcg. ($^1/_{150}$th gr.)	0	101.3	85.9–122.8	8.0
	28	97.9	78.6–137.4	12.0

[a] Label claim.

tablets were mixed with 30 placebo tablets in amber-glass or high density polyethylene containers and stored at 37° for 1 month. At the end of 8 days, five placebo tablets were removed from the container and assayed. At the end of 1 month, 10 placebos were removed for assay.

Complete Exposure of Tablets to Surrounding Air on Watch Glasses (Open Environment)—Stabilized and conventional tablets were applied to watch glasses, which were stored side-by-side without covering at room temperature for 5–7 days. At given intervals, 30 individual tablets were assayed.

Effect of Storage in Open Commercial Containers (Open Environment)—Stabilized and conventional tablets in bottles of 100 were obtained in-house or commercially; the caps, liners, and stuffing were removed and the containers were stored at room temperature without caps in an area free from drafts. There was a 3–4-cm. distance between the top of the tablets and the lip of the container. At given intervals, 30 tablets from a bottle were assayed; a different bottle was used at each assay period.

Long-Term Stability[10] of Conventional and Stabilized Tablets in Glass Bottles with Polyethylene Terephthalate Liners at 25, 37, and 45°—Two lots of conventional tablets were packaged exactly as were the stabilized tablets, *i.e.*, amber-glass bottle with rayon stuffing and a polyethylene terephthalate cap liner. These lots were stored under the same conditions as the stabilized tablets to obtain comparable stability data.

RESULTS AND DISCUSSION

Tablet Manufacture and Bioavailability—The stabilized tablet was designed to keep the good physical characteristics of the ordinary sublingual tablet such as ready disintegration, dissolution, and "wickability"[11], while at the same time reduce the intertablet migration of nitroglycerin by volatilization. Easy disintegration and wickability are related to the release rate of nitroglycerin and were desirable in the stabilized tablet (Table I). The stabilized and conventional tablets were required to meet the same specifications for disintegration and wickability. In actual practice, the disintegration time generally falls between 10 and 30 sec.

The average assay for stabilized tablets was 99.7% of label claim, with a range of 92.1–108.1% and a standard deviation of 4.0%;

[10] Long-term stability studies on stabilized tablets are still in progress on many lots of tablets and include storage at 4°, room temperature, 37°, and 45°.

[11] Defined as the time in seconds required for water to wet the tablet completely by capillary action under controlled conditions.

Table XI—Long-Term Stability of Stabilized Tablets, 432 mcg. ($^1/_{150}$th gr.), at 25, 37, and 45° with 19-hr. Drying Period

Months	25° Average Assay, % of L.C.[a]	25° Range of Assays, % of L.C.[a]	25° SD	37° Average Assay, % of L.C.[a]	37° Range of Assays, % of L.C.[a]	37° SD	45° Average Assay, % of L.C.[a]	45° Range of Assays, % of L.C.[a]	45° SD
0	103.7	89.7–113.0	5.6	103.7	89.7–113.0	5.6	103.7	89.7–113.0	5.6
1	103.9	92.9–111.1	4.7	106.0	91.3–113.9	6.1	102.6	92.1–114.5	5.5
2	104.6	95.5–121.9	5.8	103.9	89.8–114.8	6.2	104.6	93.6–116.7	6.4
3	102.0	90.1–115.9	5.5	101.1	88.8–111.7	5.6	102.6	91.4–114.4	5.6
4	99.8	85.8–108.6	5.6	99.5	87.7–112.8	6.9	101.8	87.8–124.1	8.0
5	99.5	88.3–110.4	5.2	—	—	—	101.0	81.4–114.0	7.7
6	98.7	85.2–108.7	6.4	99.9	79.3–106.2	7.1	—	—	—
7	100.6	87.3–111.9	6.8	99.3	82.6–111.6	6.1	93.0	75.7–104.5	6.8
10	99.4	79.5–111.5[b]	6.5	93.6	80.6–104.0	6.3	88.9	76.1–105.7	6.4
13	100.0	87.2–112.5	7.5	92.4	75.8–103.6	5.6	86.2	63.8–100.3	8.9
25	95.6	78.6–108.8	6.8	—	—	—	—	—	—

[a] Label claim. [b] Only one tablet at 79.5; next tablet was assayed at 88.8.

Table XII—Stability Data on Clinical Lots of Stabilized Tablets at 25, 37, and 45°

Stabilized Tablet	Elapsed Time, Months	25° Average Assay, % of L.C.[a]	25° Range of Assays, % of L.C.[a]	25° SD	37° Average Assay, % of L.C.[a]	37° Range of Assays, % of L.C.[a]	37° SD	45° Average Assay, % of L.C.[a]	45° Range of Assays, % of L.C.[a]	45° SD
324 mcg.	0	107.3	98.4–117.4	4.0	107.3	98.4–117.4	4.0	107.3	98.4–117.4	4.0
	1	106.7	97.2–117.0	3.9	103.7	95.0–115.8	4.4	104.3	89.2–119.2	6.6
	2	103.8	94.0–117.6	4.3	102.7	94.5–114.3	4.7	101.9	90.8–117.1	5.1
	3	109.1	93.0–122.6	5.8	98.4	85.6–108.6	4.9	96.6	79.4–105.9	5.8
	4	104.3	94.7–115.3	5.9	99.2	92.6–119.4	5.2	99.0	88.8–110.8	4.9
	5	100.6	91.1–114.1	5.3	99.2	87.7–110.0	4.9	95.9	84.7–111.6	4.9
	6	103.8	92.6–114.7	4.9	—	—	—	—	—	—
	11	101.3	94.0–114.3	4.8	99.7	87.8–110.0	5.1	99.0	89.0–119.4	6.3
	18	102.6	91.6–119.3	6.4	—	—	—	—	—	—
	22	96.7	84.0–110.9	5.3	—	—	—	—	—	—
432 mcg.	0	106.5	101.8–110.5	2.0[b]	106.5	101.8–110.5	2.0	106.5	101.8–110.5	2.0
	1	104.8	91.3–112.3	5.2	108.2	92.4–114.3	6.0	106.1	98.5–121.8	4.9
	2	102.8	95.2–120.5	5.7	101.0	87.2–114.7	5.5	101.5	91.3–112.9	4.9
	3	101.3	88.0–113.5	4.6	100.9	87.5–113.8	5.6	100.6	92.4–110.0	5.1
	4	102.0	94.8–110.3	4.5	100.5	92.3–110.1	3.8	100.6	90.5–118.4	6.7
	5	103.5	92.5–119.0	5.5	98.8	81.0–113.3	6.1	100.2	91.7–111.0	4.9
	6	100.9	90.6–110.4	4.5	—	—	—	—	—	—
	11	104.1	99.2–112.7	3.4	102.0	90.0–122.6	7.3	94.6	74.4–119.3	8.6
	18	104.4	84.2–122.3	7.7	—	—	—	—	—	—
	22	101.1	89.6–115.0	5.7	—	—	—	—	—	—
648 mcg.	0	103.4	96.7–110.2	2.7	103.4	96.7–110.2	2.7	103.4	96.7–110.2	2.7
	1	101.0	88.4–120.8	6.7	98.7	92.1–110.3	4.5	99.3	90.2–117.8	5.3
	2	104.1	92.2–123.3	5.6	102.7	86.0–117.5	6.3	102.2	78.6–126.9	9.8
	3	96.8	89.8–113.7	4.7	—	—	—	—	—	—
	4	97.4	83.7–112.5	6.2	94.6	81.8–111.4	6.6	95.4	79.7–115.2	7.1
	5	95.2	78.0–117.0	7.0	94.2	72.0–107.1	7.6	91.2	78.2–110.6	7.0
	6	97.3	81.0–114.6	5.7	—	—	—	—	—	—
	11	98.8	75.8–113.5	8.1	101.4	88.4–120.1	7.9	86.6	70.1–106.4	8.3
	18	95.1	81.2–114.0	7.5	—	—	—	—	—	—
	22	94.3	77.7–102.9	4.5	—	—	—	—	—	—

[a] Label claim. [b] A lot better than normal; 3–4 is more normal; the jump to 5.2 in 1 month probably indicates this was an exceptional bottle.

when the assays were corrected for the weight of the tablets, the standard deviation of the assays fell to 1.3%. This indicates a satisfactory blending operation and shows that the initial variability in the tablet assays is largely due to tablet weight differences. The latter are more difficult to control in a molded tablet than in a compressed tablet.

The chief virtue of molded sublingual nitroglycerin tablets has been their ability to release nitroglycerin readily. Figure 1 shows a comparison of the bioavailability of nitroglycerin from conventional and stabilized tablets in dogs. The stabilized and conventional tablets were comparable in pharmacological effect; both caused a marked fall in pulse pressure within 3 min. of sublingual administration. There was a partial return to normal over the next 10 min., but lesser effects could be seen for up to 30 min. Although not shown in Fig. 1, it was observed that the pulse pressure returned to pretest levels within 50 min. Human clinical trial followed the dog experiments and confirmed that the stabilized tablet gives the same pharmacological effect as the conventional tablet.

Effects of High Vacuum—Many different experiments were carried out under pump vacuum for 20–24 hr. at about 1 Torr to compare the behavior of the stabilized and conventional tablets. These experiments involved exposure of the different tablets either in their opened, commercial containers or in uncovered petri dishes. Both glass and plastic vacuum desiccators were used without internal drying agents[12]. In general, these experiments showed that the stabilized tablet is more retentive of its nitroglycerin than is the

[12] No phosphorus pentoxide was used in these desiccators. Two separate experiments, using phosphorus pentoxide in the bottom of glass desiccators, showed that both stabilized and conventional tablets lost large amounts of nitroglycerin. The stabilized tablets retained 70–80% of their original potency, while the conventional tablets retained only 16–30%. Moreover, the loss from the stabilized tablets was in a much more even or controlled manner than from the conventional tablets.

Table XIII—Comparison of Long-Term Stability of Conventional and Stabilized Tablets, 432 mcg. ($^1/_{150}$th gr.), at Various Temperatures Using Glass Bottles and Polyethylene Terephthalate Cap Liners

Temperature	Elapsed Time, Months	Conventional Tablets			Conventional Tablets			Stabilized Tablets		
		Average Assay, % of L.C.[a]	Range of Assays, % of L.C.[a]	Percent Relative Standard Deviation	Average Assay, % of L.C.[a]	Range of Assay, % of L.C.[a]	Percent Relative Standard Deviation	Average Assay, % of L.C.[a]	Range of Assays, % of L.C.[a]	Percent Relative Standard Deviation
25°	0	103.8	94.9–113.5	3.9	104.7	98.5–111.2	3.1	103.7	89.7–113.0	5.4
	1	102.0	76.6–122.1	12.7	103.3	64.7–129.8	14.1	103.9	92.9–111.1	4.5
	2	102.1	84.9–131.2	10.6	102.2	83.7–119.6	7.9	104.6	95.5–121.9	5.5
	3	100.3	74.5–120.7	12.4	102.9	84.9–121.0	9.4	102.0	90.1–115.9	5.4
	4	101.2	79.5–128.6	12.6	99.9	80.5–131.0	11.7	99.8	85.8–108.6	5.6
	5	101.4	85.9–123.2	10.7	103.1	79.2–127.9	10.7	99.5	88.3–110.4	5.2
	6	101.1	81.6–127.0	12.5	104.9	79.7–132.7	14.3	98.7	85.2–108.7	6.5
	7	—	—	—	—	—	—	100.6	87.3–111.9	6.8
	10	—	—	—	—	—	—	99.4	79.5–111.5[b]	6.5
	13	—	—	—	—	—	—	100.0	87.2–112.5	7.5
37°	0	103.8	94.9–113.5	3.9	104.7	98.5–111.2	3.1	103.7	89.7–113.0	5.4
	1	107.6	72.0–147.2	15.6	101.8	69.5–143.3	17.4	106.0	91.3–113.9	5.8
	2	100.0	73.1–130.7	12.9	101.8	86.1–117.0	8.3	103.9	89.8–114.8	6.0
	3	99.8	76.7–137.6	13.9	103.7	86.7–113.5	7.2	101.1	88.8–111.7	5.5
	4	96.4	76.2–127.6	10.0	99.9	73.5–124.7	12.9	99.5	87.7–112.8	6.9
	5	100.1	76.5–142.4	16.7	104.6	73.8–137.1	16.4	—	—	—
	6	—	—	—	—	—	—	99.9	79.3–106.2	7.1
	7	—	—	—	—	—	—	99.3	82.6–111.6	6.1
	10	—	—	—	—	—	—	93.6	80.6–104.0	6.3
	13	—	—	—	—	—	—	92.4	75.8–103.6	5.6
45°	0	103.8	74.9–113.5	3.9	104.7	98.5–111.2	3.1	103.7	89.7–113.0	5.4
	1	102.3	71.8–142.9	13.9	101.6	74.6–124.3	12.7	102.6	92.1–114.5	5.4
	2	97.2	77.5–116.1	10.7	101.8	86.1–117.0	8.3	104.6	93.6–116.7	6.1
	3	100.4	76.5–119.4	11.7	97.7	73.7–140.9	12.4	102.6	91.4–114.4	5.5
	4	97.3	69.1–120.4	10.4	96.1	62.7–113.9	13.7	101.8	87.8–124.1	7.9
	5	98.2	79.0–133.1	10.9	99.7	78.7–119.6	10.0	101.0	81.4–114.0	7.6
	6	—	—	—	—	—	—	92.1	79.0–111.9	8.9
	7	—	—	—	—	—	—	93.0	75.7–104.5	7.3
	10	—	—	—	—	—	—	88.9	76.1–105.7	7.2
	13	—	—	—	—	—	—	86.2	63.8–100.3	8.9

[a] Label claim. [b] Only one tablet at 79.5; next tablet was assayed at 88.8.

conventional tablet and that plastic desiccators cause a much greater loss of nitroglycerin than do glass desiccators.

The most interesting results, however, were obtained when stabilized and conventional tablets were placed in the same glass vacuum desiccator and then exposed to vacuum. The results always showed a substantial transfer of nitroglycerin from the conventional tablets to the stabilized tablets when the vacuum exposure was from open petri dishes (Table II). The results were slightly more variable in the case of the commercial containers. In one experiment, both the stabilized and conventional tablets showed a small net loss of nitroglycerin; in three other experiments there was a large transfer of nitroglycerin from the conventional to the stabilized tablets (Table III). These observations further substantiate the fact that nitroglycerin can and does migrate; they also demonstrate the high affinity of fixative for nitroglycerin. In practice, this is demonstrated by the better stability profile shown by stabilized tablets. The stabilized tablets absorbed the nitroglycerin in a controlled manner (Tables II and III); that is, there was little change in the percent relative standard deviation of the tablets after the absorption of nitroglycerin. The conventional tablets, by contrast, lost nitroglycerin very irregularly.

The experiment using plastic desiccators was prompted by an article (6) reporting FDA removal of plastic pen containers from the market because of the very high loss of nitroglycerin from these containers. The losses described were so severe, *i.e.*, 50% in 1 day, 80% in 3 days, and 93% in 30 days, as to be unexplainable on the basis of volatilization alone. It appeared that some active transport had to be involved. Observations in Table IV are in keeping with the results observed by the FDA. The results showed *severe* losses of nitroglycerin from the conventional tablets and much smaller losses of nitroglycerin from the stabilized tablets. These losses were far greater than those experienced in glass desiccators. Although the stabilized tablet lost nitroglycerin, it did so in a well-regulated manner as opposed to the large, irregular loss shown by the conventional tablets.

Effects of Patient-Type Container Storage—To duplicate actual patient use, nitroglycerin tablets were stored in hinged polystyrene and cardboard boxes (Tables V and VI). Table V indicates the sharp difference in behavior between the stabilized and conventional tablets stored in the hinged polystyrene boxes. Stabilized tablets lose considerably less potency from the open box; moreover, the potency loss occurred with much less spreading in the range of assays, *i.e.*, the content uniformity was maintained. The unopened glass bottle controls illustrate a point made previously; *i.e.*, there is considerable spreading in the range of assays for conventional tablets stored even in closed bottles (8).

Table V shows that cardboard boxes are extremely poor containers for nitroglycerin tablets. Nevertheless, the data point out that stabilized tablets lose less of their original potency than conventional tablets in the same period. The conventional tablets retained only 5–8% of their original potency, whereas the stabilized tablets retained 28–41%.

Effects of Immediate Closed Environment—The first paper of this series (8) reported that conventional nitroglycerin tablets were affected by their immediate environment and that they behaved differently in the presence of different cap liners[13].

Two experiments were performed to illustrate that stabilized tablets are more independent of their immediate closed environment than are conventional tablets. To some extent, this was al-

[13] Excelloseal retained the average potency but was unsuitable for maintenance of content uniformity. Polyethylene terephthalate (Mylar), tinfoil, fluorohalocarbon film (Aclar), and Esterfoil (supplied by Owens Illinois) liners were better than Excelloseal for conventional nitroglycerin tablets, but even they did not hold much promise for long-term maintenance of content uniformity.

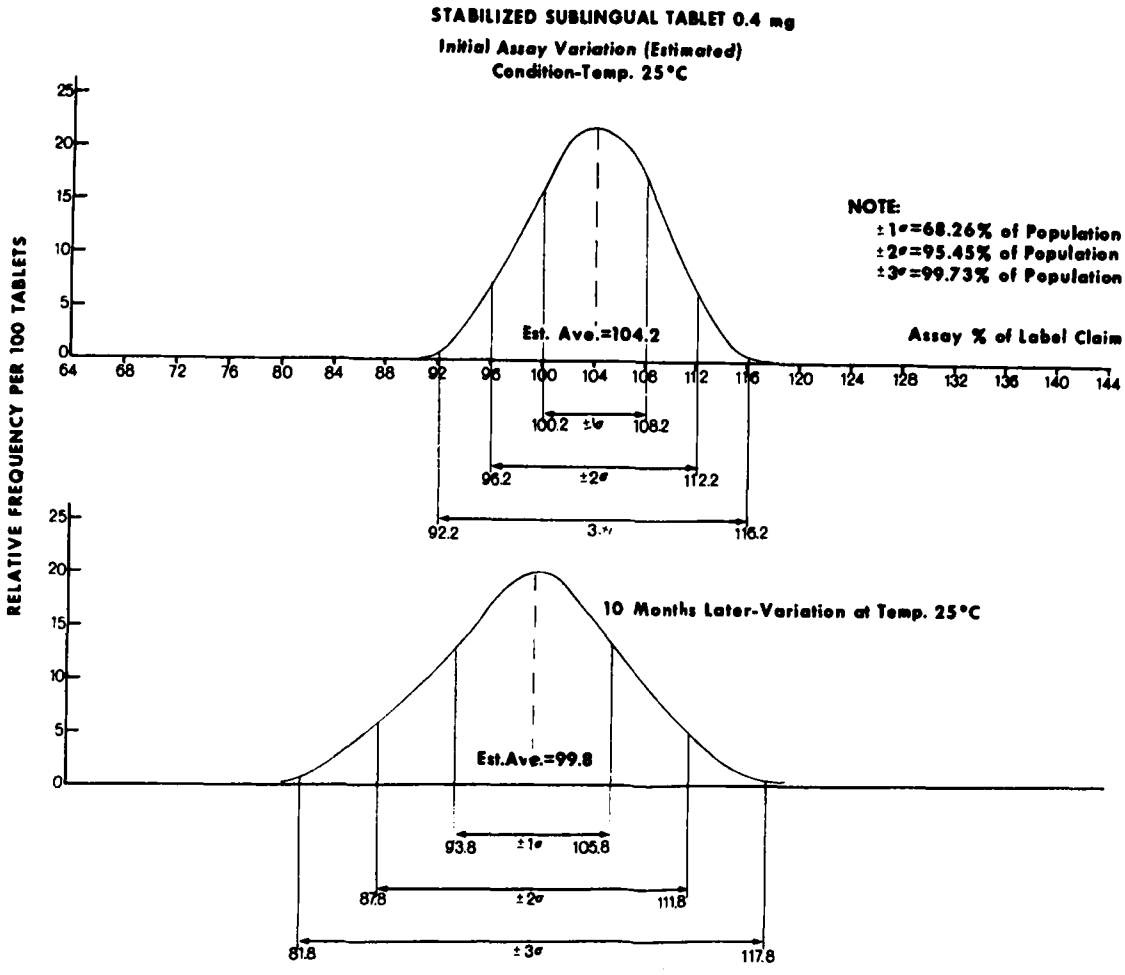

Figure 2—*Distribution curves for stabilized sublingual nitroglycerin tablets at the time of manufacture and after being packaged for 10 months.*

ready demonstrated by the polystyrene and cardboard box experiments. The two experiments were carried out in screw-capped glass bottles, which are a much preferred container to hinged polystyrene boxes. The results (Table VII) show that stabilized tablets, in contrast to conventional tablets, are little affected by their immediate container and/or the presence of rayon stuffing material, as shown by the values for average potency and standard deviation. Furthermore, daily bottle openings had little to no effect on the stabilized tablets; the latter fact is considerably important to a patient.

Table VIII shows the effect of storing stabilized and conventional tablets in the presence of placebo tablets. This experiment used different nitroglycerin-containing tablets and compressed lactose placebo tablets than were used previously (8). The results show that the stabilized tablet is more retentive of its nitroglycerin than is the conventional tablet; consequently, it maintains its content uniformity better than the conventional tablet. Recently, this same effect was also demonstrated by Shangraw and Contractor (7).

Effects of Immediate Open Environment—Two experiments were done to check the effects of an immediate open environment (Tables IX and X). Both types of tablets lost substantial amounts of nitroglycerin from open dishes (Table IX), but the stabilized tablets lost activity more slowly than the conventional tablets. After 5 days, the stabilized tablet lost the same amount or less nitroglycerin than the conventional tablets lost in 3 days. These results are, in essence, an extension of similar results obtained using cardboard boxes for storage. In the latter case, the boxes offered some protection so that the rate of loss was slower, but the results parallel each other.

The results using open commercial bottles stored in a draft-free area are in marked contrast to the results obtained on open dishes exposed to room temperature air. Very little potency was lost from either type of tablet under these circumstances. This is probably due to two factors:

1. The 3–4-cm. "dead space" above the tablets which acts as insulation from the surrounding air. In essence, the tablets are surrounded by a relatively quiescent, stagnant atmosphere instead of being swept by air currents.

2. The small surface area exposed from commercial containers compared to the larger area exposed from dishes.

Long-Term Stability—Long-term stability studies on stabilized tablets are still in progress, but some of the long-term data are summarized in Tables XI and XII. The stabilized tablets maintain both average potency and content uniformity even at 37 and 45°, thus assuring a more uniform and predictable dose to the patient.

Table XIII shows a comparison of the stability of both conventional and stabilized tablets when both types of tablets were stored under identical conditions using the same bottle and liner used for stabilized tablets. The conventional tablets develop marked intertablet dose variation within 1 month, whereas the stabilized tablets maintain their content uniformity at all temperatures tested. The stability exhibited by the stabilized tablets is a marked improvement over the stability shown by conventional sublingual nitroglycerin tablets.

Figures 2–4 give a graphical summary of the stability profiles of both stabilized and conventional tablets at room temperature. These figures show that the stabilized tablets maintain their content uniformity (Fig. 2) while the conventional tablets develop considerable intertablet dose variation (Figs. 3 and 4).

Disintegration times and wickability were also monitored during all stability studies. Although not shown in any of the tables, the values obtained were within the specification limits shown in Table I.

SUMMARY

A new, molded, stabilized, sublingual nitroglycerin tablet was prepared. This tablet shows the same bioavailability as conventional

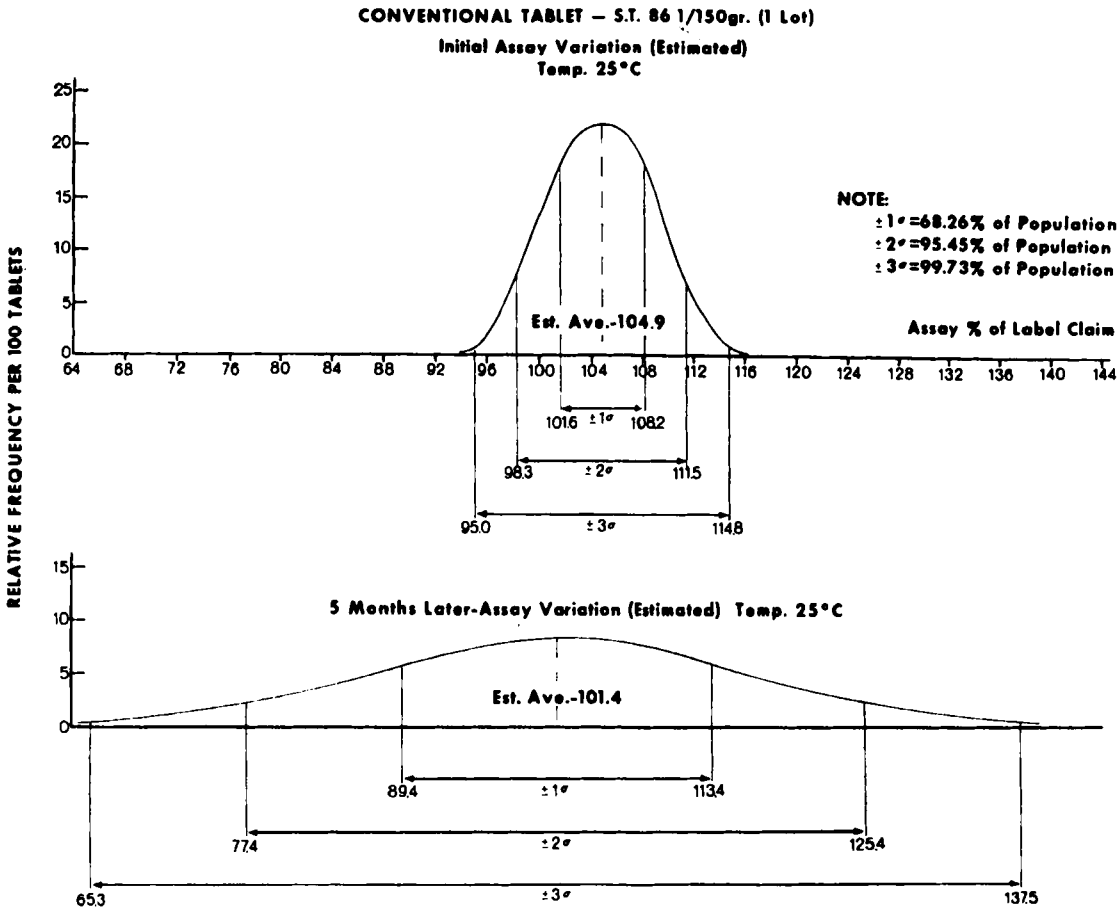

Figure 3—*Distribution curves for conventional sublingual tablets at the time of manufacture and after being packaged for 5 months.*

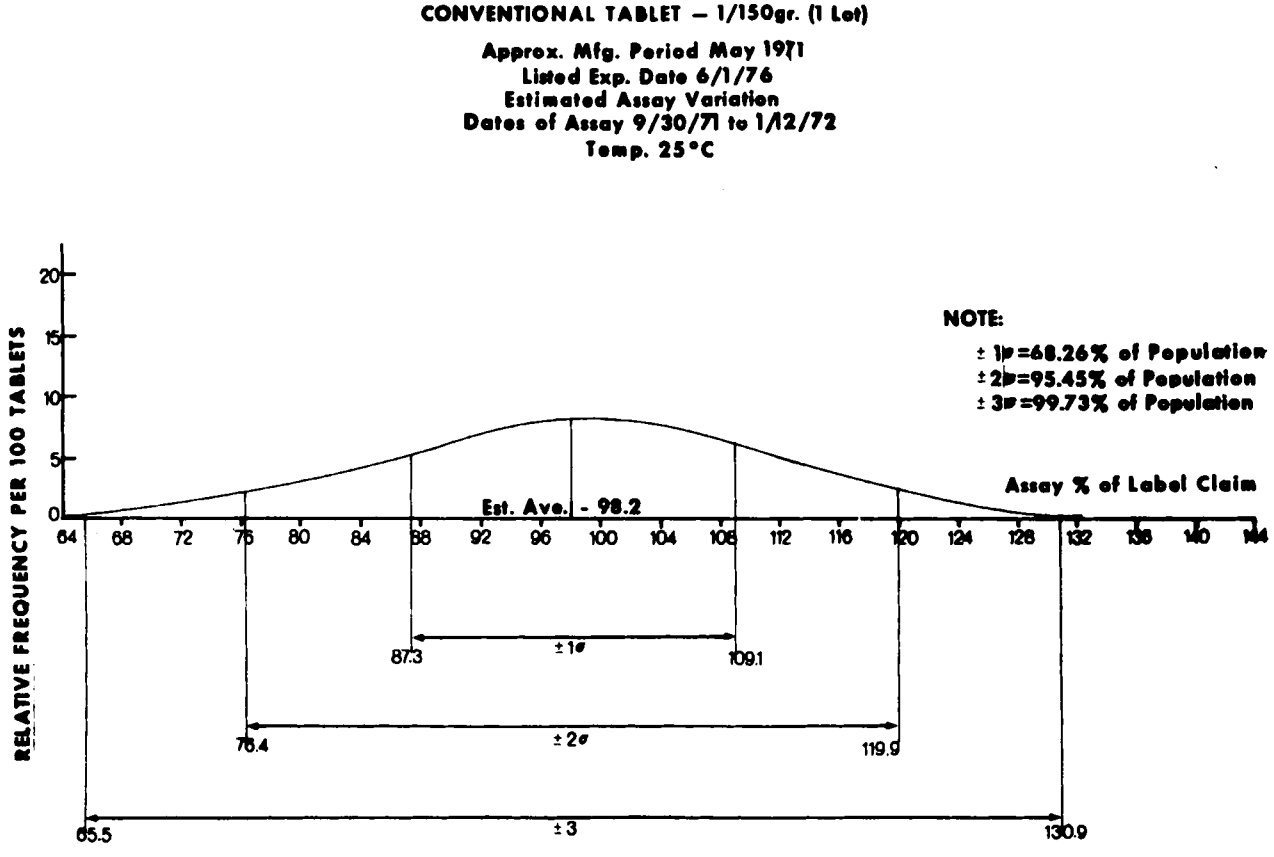

Figure 4—*Distribution curve for conventional sublingual nitroglycerin tablets after being packaged for about 7 months.*

tablets but has the advantage of markedly improved stability. In contrast to conventional tablets, which often develop marked intertablet dose variation within 1 month, the stabilized tablet maintains its content uniformity for long periods, even at 37 and 45°, thus assuring a more uniform and predictable dose to the patient.

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NOTES

Synthesis of Analogs Related to Pilocarpine

ROBERT T. KODA[▲], FRANK J. DEA, KOCHY FUNG, CHRISTIAN ELISON, and JOHN A. BILES

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Keyphrases □ Pilocarpine analogs—synthesized and screened for cholinergic activity □ Cholinergic activity—pilocarpine analogs with modified lactone rings synthesized and screened □ Structure–activity relationships—pilocarpine analogs synthesized and screened

Several naturally occurring alkaloids, *e.g.*, pilocarpine and muscarine, have been shown to possess potent cholinergic activity. This activity has been associated with the direct action of these substances on cholinergic receptors at synaptic junctions in target organs.

The nature of the cholinergic receptor is still not well understood. Presumably, acetylcholine must geometrically orient itself in such a manner that it binds to the receptor group at two points—the cationic and esteratic sites. Waser (1) postulated that the attachment of muscarinic agents to cholinergic receptors in smooth muscle involved two oxygen atoms and a quaternary nitrogen, having an optimum distance of approximately 4 Å separating the quaternary nitrogen from the ether or carbonyl oxygen. Jones (2) proposed that pilocarpine could assume a conformation similar to that of the active group postulated for the muscarinic receptor in ganglia.

A review of the literature indicates that structural requirements for cholinergic activity in pilocarpine analogs have not been studied adequately. It has been reported that an intact lactone ring is essential for activity. Moreover, the C-2 ethyl substitution on the lactone ring also has been reported as necessary for activity. Demethylation of pilocarpine on the imidazole moiety (pilocarpidine) showed reduced physiological activity (3). Brochmann-Hanssen *et al.* (4) found that various amine analogs, substituted for the imidazole moiety of pilocarpine, produced compounds with reduced activity. Quaternization of the imidazole ring giving the methyl iodides of both pilocarpine and isopilocarpine were synthesized (5) but their biological activity has not been reported. Ben Bassat *et al.* (6) presented data indicating that quaternization of pilocarpine derivatives on the imidazole moiety with a benzyl group possessed anticholinergic activity. This activity was augmented by the addition of *para*-substituents such as bromo, chloro, and methyl groups.

It appears that compounds possessing parasympathomimetic activity must contain a cationic site in the form of either a protonated tertiary or quaternary nitrogen separated from a region of high electron density approximately 4 Å away. Whether this site of high electron density in pilocarpine analogs is located about the carbonyl oxygen or the ether oxygen has not been determined. It appeared that minor alterations in the

tablets but has the advantage of markedly improved stability. In contrast to conventional tablets, which often develop marked intertablet dose variation within 1 month, the stabilized tablet maintains its content uniformity for long periods, even at 37 and 45°, thus assuring a more uniform and predictable dose to the patient.

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lactone ring would provide useful information regarding the nature of the cholinergic receptor. It was proposed that the removal of the carbonyl function would further serve to provide information regarding the importance of that group without significantly altering the geometry of the pilocarpine molecule. In addition, it was felt that the lactam and *N*-methyllactam analogs should be prepared to determine whether the nitrogen in the lactam ring could be involved in an interaction with the receptor group.

RESULTS AND DISCUSSION

The methods used in the conversion of lactones to lactams generally involved the reaction of a lactone with ammonia in various polar solvents (7–9). It is possible that in the aminolysis of pilocarpine, either a hydroxyamide or a lactam derivative could occur. Gresham *et al.* (10) found that the reaction of methylamine with β-lactones in acetonitrile favored the formation of the β-amino acid, which subsequently could be cyclized to the lactam. In this study, however, when pilocarpine was reacted with anhydrous ammonia in acetonitrile at 0°, only the hydroxyamide derivative (I) was obtained. The hydroxyamide derivative was also obtained using anhydrous ammonia in absolute methanol, 95% ethanol, or aqueous ammonia. Reactions of pilocarpine with aqueous solutions of methylamine or isopropylamine gave similar results, yielding the respective *N*-methylhydroxyamide (II) or *N*-isopropylhydroxyamide (III) derivatives (Scheme I).

The lactam analog (IV) was successfully prepared by a reaction of pilocarpine (VII) with liquid ammonia at 200–210° according to the method of Späth and Lintner (11) (Scheme I). Similarly, the *N*-methyllactam analog (V) was prepared by the reaction of liquid methylamine and pilocarpine (VII) at 225° for 2 hr.

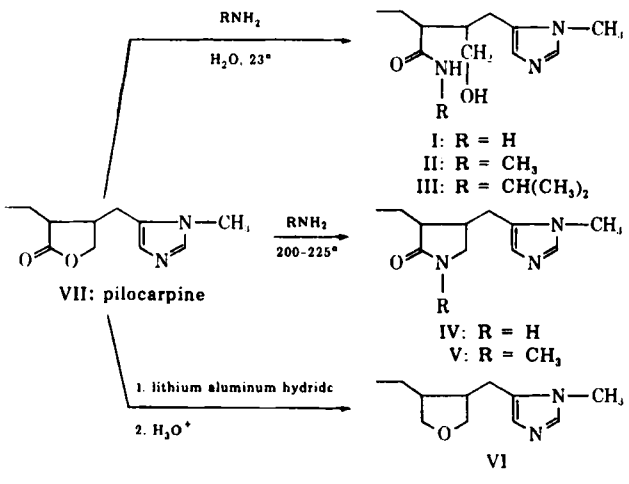
Pilocarpine reduction was accomplished using lithium aluminum hydride in anhydrous tetrahydrofuran (Scheme I). The IR spectra indicated a disappearance of the normal carbonyl peak. Elemental analysis was consistent with the calculated values for the tetrahydrofuran analog (VI).

The NMR spectrum of I indicated a two-proton doublet at δ 3.35 (—CH_2OH), whereas the spectrum of pilocarpine (VII) indicated a two-proton multiplet at δ 4.12 (—CH_2O—). In addition, the NMR spectrum of II indicated an absorption at δ 2.60 (N—CH_3, amide).

The NMR of IV gave a two-proton multiplet at δ 3.10–3.51. These data are consistent with the C-5 (N—CH_2—) protons reported for 2-pyrrolidone. Compound V gave a three-proton singlet at δ 2.90 (N—CH_3 lactam).

The appearance of two doublets at δ 3.48–3.61 and 3.98–4.12 (4H) for VI is consistent for the C-2 and C-4 protons on the tetrahydrofuran moiety.

Specific rotations (Table I) and NMR evidence do not clearly establish whether isomerization occurred during the synthesis of Compounds IV and V. The C-3 and C-4 protons on the 2-pyrrolidone ring of IV and V were assigned δ 2.19 (m, 1H, CH) and 2.52 (m, 1H, CH) and δ 2.28 (m, 1H, CH) and 2.50 (m, 1H, CH), respectively. The C-3 and C-4 protons on the tetrahydro-2-furanone ring of pilocarpine were assigned δ 2.33 (m, 1H, CH) and 2.83 (m, 1H, CH). The NMR peaks for the C-3 and C-4 protons in isopilocarpine were not clearly shown. Thus, these data are not sufficiently definitive to indicate whether isomerization had occurred during the synthetic procedure.



Scheme I

PHARMACOLOGY

Results of the pharmacological studies indicate that there is no significant parasympathomimetic activity for Compounds I, II, and III. However, the administration of IV and V in comparable doses to the pilocarpine control resulted in a sialogogue activity greater than pilocarpine itself. This activity could be antagonized by the administration of 0.4 mg./kg. i.v. of atropine. The administration of IV and V did not induce vomiting and defecation, with only a few drops of urine being discharged. However, unlike pilocarpine, IV and V produce hypertension which is not modified by the administration of atropine during the hypertensive phase.

The intravenous administration of VI resulted in sialogogue activity which could be antagonized by the administration of 0.4 mg./kg. i.v. atropine. When 3.0 mg./kg. VI was administered, a transient hypotension was produced, followed by a hypertensive phase with an attendant bradycardia. The cardiac rate became erratic with a fall in blood pressure. Atropine (0.4 mg./kg. i.v.) did not reverse the cardiovascular effects and the animal died in cardiac arrest. Compound VI exerts a more potent effect on the cardiovascular system than equal doses of pilocarpine.

The preliminary pharmacological evaluation of pilocarpine analogs in which the lactone ring was modified further substantiates the observation that an intact ring structure (lactone or its equivalent) is necessary for biological activity.

EXPERIMENTAL[1]

The pharmacological activity of pilocarpine analogs was determined in male dogs weighing between 13 and 20 kg.; the dogs were previously anesthetized with 30 mg./kg. sodium pentobarbital. Three experiments were run on each compound tested. The anesthetized animal was secured to a dog board; the electroencephalogram (EEG), through fronto-temporal leads, and the electrocardiogram (ECG), through lead I, were recorded using a physiograph. Blood pressure measurements were made through a cannulated left femoral artery, and the right femoral vein was exposed for injection of drug. Estimations of salivary flow were determined by tilting the head of the animal in such a manner that all salivary secretions would drain into a container.

All compounds to be tested were prepared in isotonic saline in concentrations expressed as the free base. The primary objective of the pharmacological study was to compare cholinergic activity of the analogs with the parent compound pilocarpine. A single dose of 3 mg./kg. was used for each compound tested.

Aminolysis of Pilocarpine by Ammonia, Methylamine, and Isopropylamine—Three grams (12.25 mmoles) of pilocarpine hydrochloride (VII) was suspended in 25 ml. of ammonium hydroxide (28%), and the mixture was stirred at room temperature. The crystals of VII slowly dissolved in the mixture. After 30 min., precipitation occurred in the form of a white crystalline suspension. Stirring was continued for an additional 2 hr., at which time a heavy white suspension was obtained. The suspension was filtered and the product was recrystallized from a minimum amount of 28% ammonium hydroxide, yielding 2.05 g. (74%) of 2-ethyl-3-hydroxymethyl-4-

[1] Melting points were determined on a Fisher-Johns melting-point apparatus and are uncorrected. Elemental microanalyses were performed by Elek Microanalytical Laboratory, Torrance, Calif., and C. F. Geiger, Ontario, Calif. All compounds were dried over phosphorus pentoxide under vacuum at 100° for at least 4 hr. IR spectra were run as mineral oil mulls between sodium chloride windows or as potassium bromide pellets on a Perkin-Elmer Infracord, model 137, and were consistent with proposed structures. Thin-layer chromatograms were run on Gelman ITLC (sg) in a solvent system composed of *n*-butanol–*n*-butyl ether–acetic acid (58:40:10) and were detected by spraying with Dragendorf's reagent. NMR spectra were run on a Hitachi Perkin-Elmer spectrometer, model R-24, and were consistent with assigned structures. Optical rotations were determined in a Cenco-Kern Polarmeter using water as a solvent at 23°.

Table I—Specific Rotations of Pilocarpine and Its Derivatives

Compound	$[\alpha]_D^{23}$
Pilocarpine nitrate (VII)	79.0° (c 1.0)
Isopilocarpine nitrate	36.9° (c 1.0)
Amide derivative (I)	47.4° (c 1.0)
Lactam nitrate (IV)	42.2° (c 1.0)
N-Methyllactam (V)	47.4° (c 1.0)
Tetrahydrofuran analog (VI)	26.3° (c 1.0)

(3-methyl-4-imidazolyl)-*n*-butyramide (I), m.p. 195–196°; IR_{max}: 1650 (C=O, amide) and 3200 (NH) cm.$^{-1}$; NMR (dimethyl sulfoxide-d_5): δ 3.35 (d, 2H, CH_2OH).

Anal.—Calc. for $C_{11}H_{19}N_3O_2$: C, 58.64; H, 8.50; N, 18.65. Found: C, 58.85; H, 8.66; N, 18.50.

Similarly, the *N*-methylamide derivative (II) was synthesized using a 30% aqueous solution of methylamine. The product was recrystallized from absolute ethanol, giving 2.25 g. (77%) of 2-ethyl-3-hydroxymethyl-4-(3-methyl-4-imidazolyl)-*N*-methyl-*n*-butyramide (II), m.p. 215–216°; IR_{max}: 1620 (C=O, amide) and 3200 (NH) cm.$^{-1}$; NMR (D_2O): δ 2.60 (s, 3H, N—CH_3, amide).

Anal.—Calc. for $C_{12}H_{21}N_3O_2$: C, 60.22; H, 8.84. Found: C, 60.34; H, 8.88.

The isopropylamide derivative (III) was prepared by stirring 3.0 g. of VII with a mixture of 25 ml. of isopropylamine and 10 ml. of water at room temperature until VII dissolved. The solution was placed in the refrigerator overnight at 4°. The solution was heated on a steam bath to remove the excess isopropylamine. Ten milliliters of water and decolorizing charcoal were added, and the mixture was filtered while hot. Upon cooling, fine white needles of 2-ethyl-3-hydroxymethyl-4-(3-methyl-4-imidazolyl)-*N*-(2-methylethyl)-*n*-butyramide (III) were obtained. The yield was 1.78 g. (55%), m.p. 224°; IR_{max}: 1640 (C=O, amide) and 3330 (NH) cm.$^{-1}$.

Anal.—Calc. for $C_{14}H_{25}N_3O_2$: C, 62.89; H, 9.42. Found: C, 62.74; H, 9.27.

3-Ethyl-4-(3-methyl-4-imidazolyl)methyl-2-pyrrolidone Nitrate (IV)—Crystals of VII were dried in a desiccator over calcium chloride under vacuum. To 2.0 g. (12.25 mmoles) of dried VII in a Parr high pressure reaction bomb was added an excess of liquid ammonia (approximately 10 ml.). The reaction bomb was sealed and heated in an oil bath at 200–210° for 2 hr. The reaction bomb was cooled to −5° and then vented, allowing the excess ammonia to escape. The reaction mixture was evaporated under vacuum, leaving an oily residue. To this residue was added 25 ml. of chloroform. The solution was dried with anhydrous calcium chloride and filtered. The chloroform filtrate was evaporated, leaving an oily residue which was dissolved in absolute ethanol and acidified with nitric acid. Upon acidification, IV precipitated. The product was recrystallized twice from absolute ethanol, giving 1.67 g. (53%) of 3-ethyl-4-(3-methyl-4-imidazolyl)methyl-2-pyrrolidone nitrate (IV), m.p. 160.5–161.5°; IR_{max}: 1675 (C=O, lactam) cm.$^{-1}$; NMR ($CDCl_3$): δ 2.72 (d, 2H, CH_2) and 6.70 (s, 1H, NH, lactam).

Anal.—Calc. for $C_{11}H_{18}N_4O_4$: C, 48.88; H, 6.71; N, 20.73. Found: C, 49.04; H, 6.87; N, 21.25.

The procedure for the preparation of 1-methyl-3-ethyl-4-(3-methyl-4-imidazolyl)methyl-2-pyrrolidone nitrate (V) is similar to that presented for the preparation of IV, substituting anhydrous liquid methylamine for ammonia with the reaction being carried out at 225° for 2 hr. The product was worked up in the usual way and recrystallized twice from absolute ethanol, yielding 1.85 g. (53%) of V, m.p. 174.5–175° dec.; IR_{max}: 1670 (C=O, lactam) cm.$^{-1}$; NMR ($CDCl_3$): δ 2.90 (s, 3H, CH_3 lactam) and 3.12–3.52 (m, 2H, C-5 N—CH_2).

Anal.—Calc. for $C_{12}H_{20}N_4O_4$: C, 50.69; H, 7.09; N, 19.71. Found: C, 50.41; H, 7.32; N, 20.21.

3-Ethyl-4-(3-methyl-4-imidazolyl)methyl Tetrahydrofuran Nitrate (VI)—Anhydrous ammonia was passed into a suspension of 5.0 g. (20.4 mmoles) of VII in 50 ml. of chloroform at 0°. The precipitate of ammonium chloride was filtered, and the chloroform solution evaporated to dryness under reduced pressure, leaving an oily residue of pilocarpine free base.

One gram (26.40 mmoles) of lithium aluminum hydride[2] was added with stirring to 50 ml. of dry tetrahydrofuran in a three-necked flask fitted with a condenser and dropping funnel. The reaction mixture was protected from moisture with drying tubes on all openings. The previously prepared pilocarpine base dissolved in dry tetrahydrofuran was added to the solution of lithium aluminum hydride over 3 hr. at 0°. After the reaction period, water was carefully added to the mixture. The resultant suspension was filtered and the tetrahydrofuran was evaporated under reduced pressure. The oily residue was refluxed with 25 ml. of 10% sulfuric acid for 2 hr. The reaction mixture was cooled to room temperature, adjusted to pH 9 with ammonium hydroxide, and then extracted with four 25-ml. portions of chloroform. The combined chloroform extracts were dried with calcium sulfate, filtered, and evaporated to near dryness, giving an oily residue. The residue was dissolved in 20 ml. of absolute ethanol and acidified with nitric acid. The alcoholic solution was gently heated to boiling, decolorized with charcoal, and filtered. Upon cooling, 2.0 g. (38% based on pilocarpine hydrochloride) of VI was obtained, m.p. 144–146°; IR_{max}: 1550, 1600 (C=C, C=N) cm.$^{-1}$; NMR (D_2O): δ 3.48–3.61 and 3.98–4 12 (2d, 4H, C-2 and C-5 CH_2).

Anal.—Calc. for $C_{11}H_{18}N_3O_4$: C, 51.35; H, 7.44. Found: C, 51.33; H, 7.59.

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[2] Alpha Inorganic.

Human Urinary Excretion of Piperazine Citrate from Syrup Formulations

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Abstract □ A modified colorimetric method using Folin's amino acid reagent was used for the quantitative determination of piperazine in human urine. Comparative urinary excretion trials were carried out on five subjects using two different piperazine citrate syrup formulas. Within each individual, the percentage and pattern of excretion of piperazine citrate after oral administration of a newly developed syrup formula, equivalent to 3.5 g. piperazine hexahydrate, compared favorably with a commercial syrup sample taken in this investigation as a standard.

Keyphrases □ Piperazine citrate (syrup formulations)—determination in human urine, excretion profiles □ Excretion profiles—piperazine citrate syrup formulations, comparison of formulations, colorimetric method developed □ Colorimetry—analysis of piperazine citrate syrup formulations, human urine

Piperazine is the agent of choice in ascariasis, and its preparations are always given orally. Piperazine is readily absorbed from the GI tract (1). A portion of the absorbed drug is degraded in the body, and the remainder is excreted in the urine (2). Quantitative determination of piperazine in urine has not been widely studied. Titration of the monoperiodate piperazine derivative has been employed (3); however, this was not sufficiently accurate for the present study. A potentiometric method was described (4), which required long preliminary treatment of the urine. A lengthy quantitative color reaction of piperazine with acidified benzoquinone also was published (5).

In this paper, a modified colorimetric method of Rogers (6) was used to achieve higher sensitivity and more accurate results. In Rogers' method, readings were taken at 480 nm. after standing for 10 min. The present work has shown that this length of time would allow neither for the optimum stability and intensity of the color derivative nor for the reading at its maximum wavelength.

The aim of this study was to compare the amount of piperazine excreted in the urine over 24 hr. after oral administration of a newly developed syrup formula, equivalent to 3.5 g. piperazine hexahydrate, against a commercial syrup sample. This dose was chosen according to the normal adult dose range for the treatment of ascariasis.

Table I—Reagents Used in Tests

Reagent	Reagent Blank, ml.	Test Sample, ml.	Standard, ml.
Urine, 1:25 dilution	—	2	—
Water	4	2	2
Piperazine citrate (20 mcg./ml.)	—	—	2
2% borax in water	4	4	4
95% alcohol	10	10	10
0.5% Folin's amino acid reagent	2	2	2

Table II—Recovery of Piperazine Citrate after *In Vitro* Addition to Human Urine

Piperazine Citrate Added to Urine, mg./ml.	Mean Recovery[a], mg./ml.	Standard Deviation
5	5.0264	±0.065
4	4.0896	±0.120
3	3.0362	±0.070
2	2.0554	±0.104
1	1.0278	±0.061

[a] Average of five determinations using the modified method.

EXPERIMENTAL

Determination of Piperazine in Urine—Piperazine was determined quantitatively in urine by its color reaction with Folin's amino acid reagent[1]. The color intensity increased rapidly from 0 to 18 min. and was stable for 10 min. (Fig. 1). The maximum absorbance of the color was determined by scanning the developed color under visible light at a range of from 800 to 320 nm. and was found to be at 490 nm. The color obeyed Beer's law in concentrations between 0 and 200 mcg./ml. Each urine sample was measured for the exact volume, and a dilution of 1:25 with water was made for each sample including the control urine. This dilution was used for testing according to Table I using the various reagents in the tabulating order. After standing for 20 min., readings[2] were carried out within 5 min. at 490 nm. The value of the unknown sample was then compared with the standard sample reading.

The validity of this method was verified by adding various known amounts of piperazine citrate to the urine. This urine was tested and assayed for the amount of piperazine present according to the described method. Each experiment was repeated five times, and the standard deviation was found to be ±0.42 (Table II).

Urinary Excretion of Piperazine—Five Caucasian subjects, three men and two women ranging from 23 to 38 years of age, in normal state of health as determined by prior physical and laboratory medical examination, were used in this trial. Each was administered an oral dosage of 35 ml. of piperazine citrate syrup equivalent to 3.5 g. piperazine hexahydrate. At least 48 hr. elapsed between the administration of the new formula and the commercial sample[3]. Prior to the oral administration of either syrup sample, a urine sample was collected from each individual to be used as an internal control. The amino acids and other naturally occurring chromogens

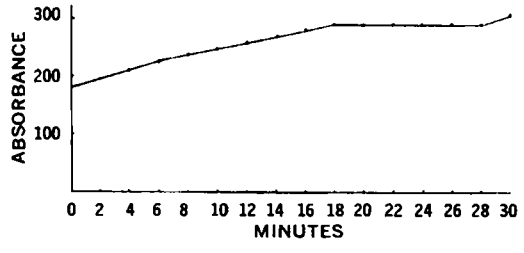

Figure 1—*Stability of the colored derivative of piperazine with Folin's amino acid reagent (piperazine citrate concentration, 100 mcg./ml.).*

[1] Sodium 1,2-naphthoquinone-4-sulfate.
[2] Beckman DB-G grating spectrophotometer.
[3] Antepar, Lot Y813, Burroughs Wellcome Co.

Table III—Total Urinary Excretion of Piperazine (in Milligrams) Calculated as Hexahydrate after Oral Administration

Subject[a]	Dose[b]	1 hr.	2.5 hr.	4.5 hr.	6.5 hr.	9 hr.	13 hr.	20 hr.	24 hr.	Total Percent Excretion	t^c Value
A	I	50	633	761	528	429	127	—[d]	—[d]	72	
27, 110	II	76	682	927	458	412	109	—[d]	—[d]	75	
B	I	156	479	604	305	216	356	347	—[d]	70	
33, 73	II	196	476	530	340	275	391	353	77	75	
C	I	153	562	647	372	261	177	50	18	64	0.0982
38, 79	II	66	596	588	318	306	220	70	27	63	
D	I	103	502	688	293	223	158	65	41	59	
23, 57	II	46	334	582	413	486	104	73	61	60	
E	I	54	263	160	30	—[d]	—[d]	—[d]	—[d]	15	
29, 56	II	69	127	219	106	—[d]	—[d]	—[d]	—[d]	15	

[a] The first number is for age (years) and the second number is for body weight (kilograms). [b] I = new syrup formula, 3.5 g. piperazine hexahydrate. II = commercial syrup sample, 3.5 g. piperazine hexahydrate. [c] The t test of significance, where $t = \bar{x}_1 - \bar{x}_2/s\sqrt{n_1n_2/n_1 + n_2}$, $p = 0.05$, and $df = 8$ (7). The t value was calculated with the pooled variance of the two syrup samples. [d] Zero excretion.

present in normal urine had been found to give colored derivatives with the reagent used. Urine samples were collected, whenever possible, at 1, 2.5, 4.5, 6.5, 9, 13, 20, and 24 hr. after oral administration. The values obtained for the samples were corrected by subtracting the value of the normal urine reading for each individual. Piperazine concentration in the diluted urine sample was then calculated by comparing these corrected values with the standard reading carried out simultaneously. The total piperazine concentration in the urine sample was calculated by multiplying its dilution factor and urine volume. The total amount of piperazine excreted and the percentage of excretion of each individual was then ascertained as piperazine hexahydrate.

RESULTS AND DISCUSSION

The results of the total urinary excretion of piperazine calculated as hexahydrate after the oral administration of an amount equivalent to 3.5 g. of piperazine hexahydrate are shown in Table III and Fig. 2.

The percentage and pattern of urinary excretion of piperazine calculated as hexahydrate varied from individual to individual, as seen in the five subjects. The total urinary recovery varied from 15 to 75%. However, the results and pattern of urinary excretion for both syrup formulas compared favorably within each individual.

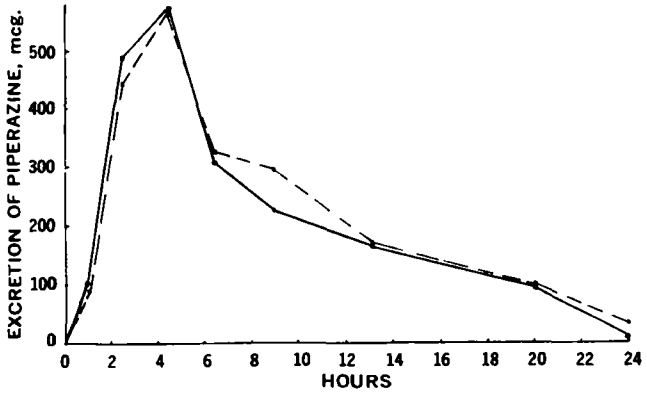

Figure 2—*Urinary excretion of piperazine after oral administration of the syrup formulas (average data for five individuals). Key:* —○—, *piperazine syrup, new formula; and* --●--, *piperazine syrup, commercial sample.*

Urinary excretion began 1 hr. after the oral administration, and the rate was maximal between 2 and 6 hr. Excretion was nearly completed in 24 hr.

CONCLUSION

1. The modified colorimetric method of analysis of piperazine in urine is accurate and simple to perform.
2. The percentage and pattern of urinary excretion of piperazine varied from individual to individual.
3. The percentage and pattern of urinary excretion after oral administration of the newly developed syrup formula, equivalent to 3.5 g. piperazine hexahydrate, compared favorably with that of the commercial syrup sample with each individual.
4. The maximum urinary excretion occurred 2–6 hr. after oral administration and was nearly completed at 24 hr. for both syrup formulas under investigation.
5. The t test of significance, where the t-value was calculated with the pooled variance of the two syrup samples, showed no significant difference.

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Polymer-Induced Flocculation of Pharmaceutical Suspensions

ALVIN FELMEISTER▲, GALE M. KUCHTYAK, SUSAN KOZIOL, and CHARLES J. FELMEISTER

Abstract □ The effect of concentration of an anionic heteropolysaccharide on the sedimentation volume of sulfaguanidine, zinc oxide, talc, bismuth subcarbonate, and bismuth subnitrate aqueous suspensions was determined. All materials with the exception of sulfaguanidine showed a steady increase in sedimentation volume with increasing polymer concentration. Sulfaguanidine exhibited a maximum in the sedimentation volume–polymer concentration curve. All suspensions appeared uniform except for the bismuth subnitrate, which exhibited coarse, unsightly floccules. This effect was attributed to an interaction between the anionic polymer and the cationic bismuth subnitrate particles. Polymer bridging was postulated as the mechanism of flocculation in the other systems.

Keyphrases □ Flocculation, polymer induced—effect of polymer concentration on sedimentation volume of five pharmaceutical suspensions □ Polymers, anionic heteropolysaccharide—effect of concentration on induction of flocculation in pharmaceutical suspensions □ Suspensions—effect of polymer concentration on polymer-induced flocculation

Flocculation is recognized as an effective way of reducing the settling and subsequent caking of relatively coarse suspensions. Reduction of charge, chemical interaction, and polymer bridging have all been used to achieve this effect. Of these, polymer bridging appears to be the most efficient approach. The purpose of this study was to investigate the ability of an anionic heteropolysaccharide[1] to flocculate various pharmaceutical suspensions *via* polymer bridging.

EXPERIMENTAL

Materials—The polymer used was an anionic heteropolysaccharide[1] produced by the action of the bacterium *Xanthomonas campestris* on carbohydrate. The other materials were sulfaguanidine NF XI[2], talc USP[3], zinc oxide USP[3], bismuth subcarbonate USP[3], bismuth subnitrate NF[3], and propylparaben USP[4]. Deionized water was used throughout this study.

Procedure—Five grams of drug was suspended in water, and the appropriate amount of a 5% aqueous dispersion of the polymer (preserved by 0.05% propylparaben) was added. The sedimentation volume was determined (1) in a 100-ml. glass-stoppered cylinder after 48 hr. Viscosity was determined using a rotational viscometer[5].

RESULTS AND DISCUSSION

The viscosity of the suspending media at a relatively low shear rate (30 r.p.m.) ranged from 225 cps. for the maximum polymer concentration of 0.2% to 55 cps. for the 0.1% concentration.

Sulfaguanidine Suspensions—The sedimentation volume, H_u/H_0, increased with increasing polymer concentration up to 0.1% (Fig. 1). Beyond that level, the sedimentation volume decreased and appeared to level off only slightly above that observed in the absence of polymer. This effect is indicative of polymer adsorption and bridging, as postulated by LaMer and Healy (2); *i.e.*, maximum bridging occurs at some optimal polymer concentration and, consequently, a maximum in the sedimentation volume–polymer concentration is anticipated. Furthermore, the higher sedimentation volumes observed in this study, compared to those that resulted when sulfaguanidine was flocculated by a reduction in ζ-potential (3), are a further indication of polymer bridging.

Zinc Oxide and Talc Suspensions—As with sulfaguanidine, increasing the concentration of the polymer resulted in an increase in the sedimentation volume (Fig. 2). In the case of these two materials, however, the maximum sedimentation volume was much higher than with sulfaguanidine, reaching a value of 1 at a polymer concentration of 0.2%. At all sedimentation volumes, the suspension appeared to be uniform with no evidence of large separate floccules. This suggests that the suspension exists as a "single floc" extending throughout the vehicle as previously described (4).

Unfortunately, the effect of higher polymer concentrations on polymer–particle bridging and sedimentation volume could not be determined because of the polymer concentration required to achieve the maximum effect. Above this polymer concentration, the viscosity of the solution increased rapidly and there was evidence of a yield value, both of which tended to inhibit the settling almost completely over the observation period.

Bismuth Suspensions—The suspension of bismuth subcarbonate (Fig. 3) behaved essentially like the suspensions of zinc oxide and talc. However, the bismuth subnitrate formed very coarse, granular-appearing floccules which tended to settle rapidly to sedimentation volumes lower than those observed for the other materials at similar polymer concentrations. This effect apparently is the result of a chemical interaction between the negatively charged polymer and the positively charged bismuth subnitrate (5).

No attempt was made to quantify the redispersibility of the flocculated suspensions. However, qualitatively, it could be seen by gently inverting the cylinders containing the suspensions that all

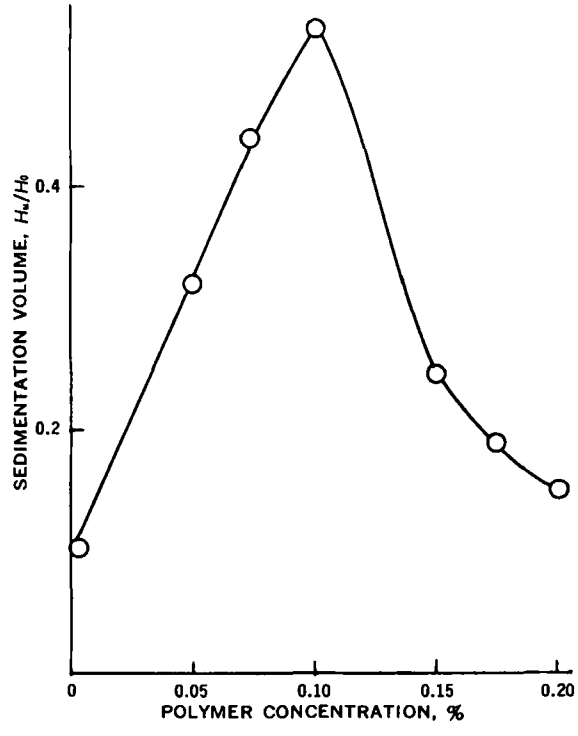

Figure 1—*Sedimentation volumes of 5% suspensions of sulfaguanidine in solutions of various concentrations of an anionic polysaccharide.*

[1] Biopolymer XB-23 xanthan gum, General Mills Chemicals, Inc., Minneapolis, Minn.
[2] American Cyanamid, Pearl River, N. J.
[3] Merck and Co., Rahway, N. J.
[4] Washine Chemical Corp, Lodi, N. J.
[5] Brookfield viscometer model LVT.

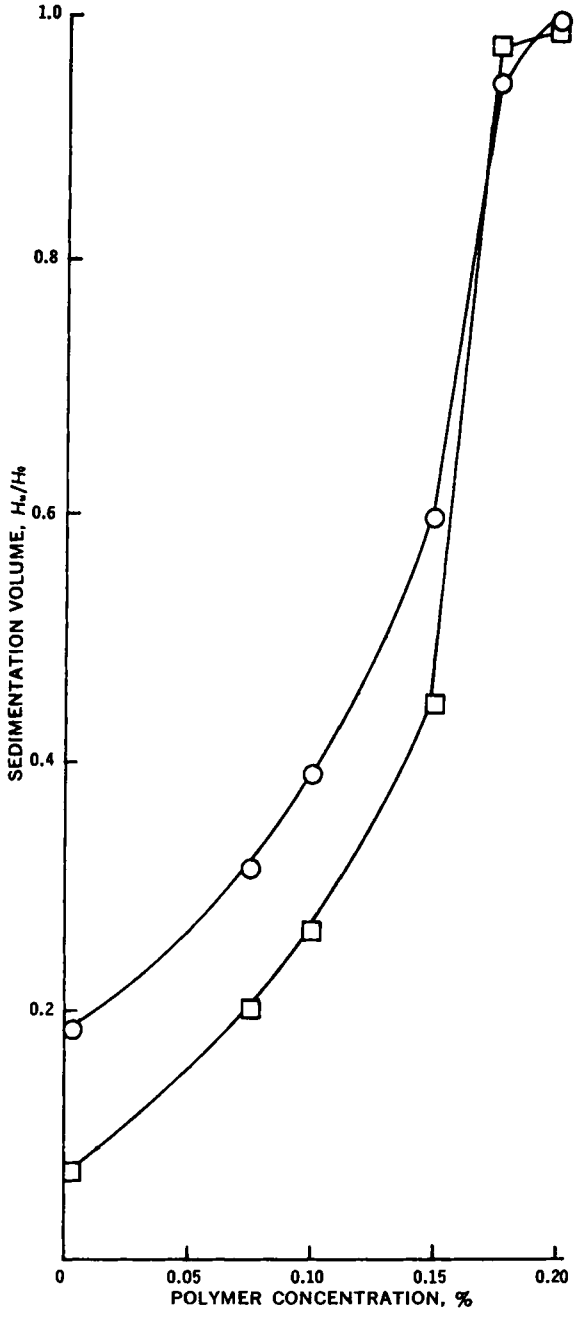

Figure 2—*Sedimentation volumes of 5% suspensions of zinc oxide (○) and talc (□) in solutions of various concentrations of an anionic polysaccharide.*

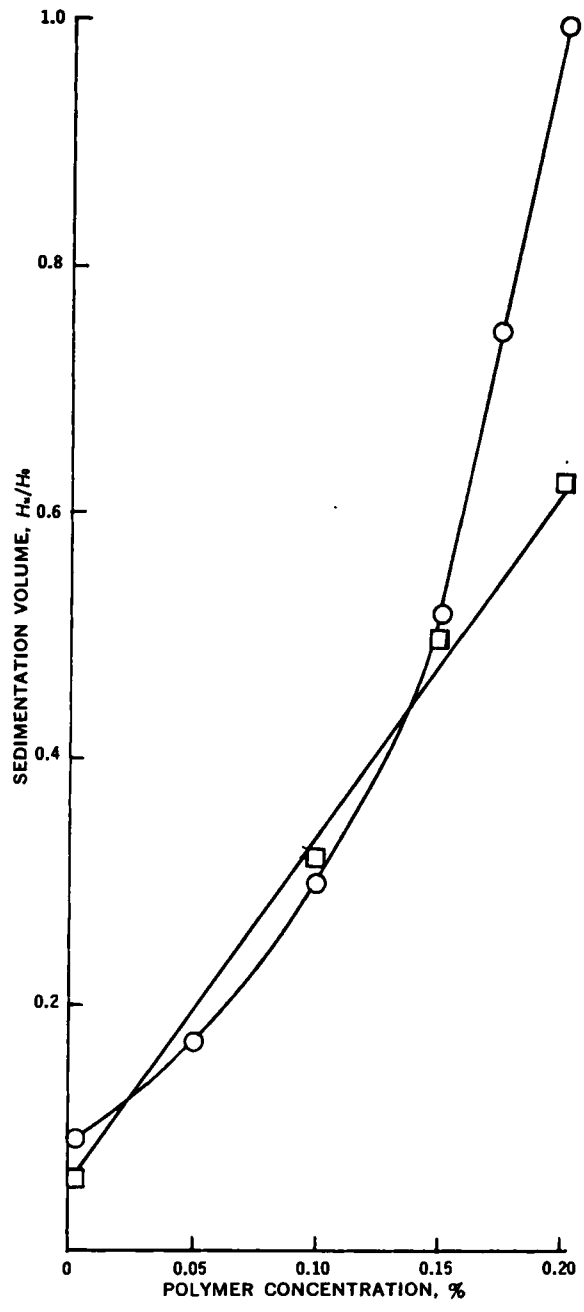

Figure 3—*Sedimentation volumes of 5% suspensions of bismuth subcarbonate (○) and bismuth subnitrate (□) in solutions of various concentrations of an anionic polysaccharide.*

the flocculated systems, with the exception of the bismuth subnitrate system, redispersed more easily than the deflocculated systems. Furthermore, with the same exception, all suspensions appeared smooth and could be readily poured from the cylinder after redispersion.

CONCLUSIONS

The anionic polysaccharide polymer used in this study is an effective flocculating agent at relatively low concentrations for a wide variety of insoluble pharmaceutical materials. The high sedimentation volumes observed, in some instances apparently reaching the single floc condition (4), are indicative of polymer–particle bridging. The anionic charge on the polymer, however, interacts with the positive charge on the surface of bismuth subnitrate particles (and, probably, other positively charged particles) to yield an inelegant, and generally unacceptable, pharmaceutical suspension.

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Synthesis and Biological Activity of Quinolyl Acetic Acid Derivatives

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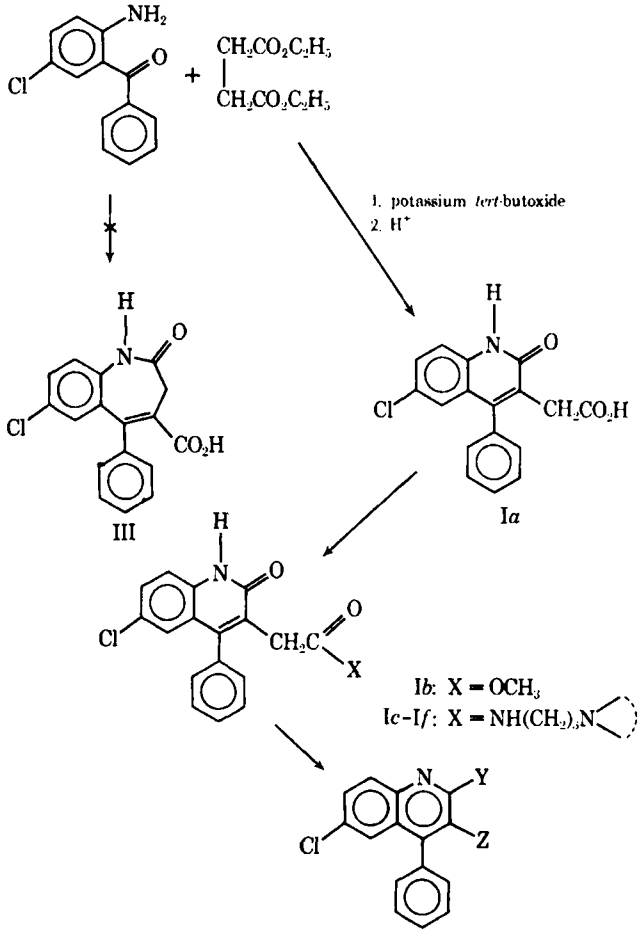
Abstract □ Derivatives of 6-chloro-4-phenyl-3-carboxymethyl-2-quinolone and 6-chloro-4-phenyl-2,3-substituted quinolines were prepared and evaluated as CNS depressants and as antibacterial agents. The compounds had no CNS activity. The quinolyl acetamides exhibited antibacterial activity against Gram-positive bacteria.

Keyphrases □ Quinolyl acetic acid derivatives—synthesis and pharmacological screening □ Quinoline derivatives—synthesis and pharmacological screening □ Antibacterial agents, potential—synthesis and screening of quinolyl acetic acid derivatives □ CNS activity—synthesis and screening of quinolyl acetic acid derivatives

The 5-chloro-2-aminobenzophenone moiety is a structural feature of several drugs affecting the central nervous system (CNS) (1–3). In addition, heterocyclic compounds containing a carbamoyl group have been reported to possess various biological activities (4–7). Consequently, as part of a program using 5-chloro-2-aminobenzophenone as a starting material for the synthesis of new nitrogen heterocyclic compounds, it was of interest to synthesize some quinolyl acetic acid derivatives (I and II), including a few quinolyl acetamides (Ic–I*f*), and to test them as CNS depressant and antibacterial agents.



IIa: Y = Cl, Z = $CH_2CO_2CH_3$
IIb: Y = OCH_3, Z = $CH_2CO_2CH_3$
IIc: Y = OCH_3, Z = CH_2CO_2H
IId: Y = OCH_3, Z = CH_3

Scheme I

5-Chloro-2-aminobenzophenone was condensed with diethyl succinate in the presence of potassium *tert*-butoxide to give 6-chloro-4-phenyl-3-carboxymethyl-2-quinolone (I*a*) in a yield of 75%. No formation was observed of a theoretically possible alternative product, the benzazepine III.

The structure of I*a* was assigned on the basis of its spectral similarity to carbostyrils and was confirmed by its facile conversion (Scheme I) to a 3-carboxymethylquinoline derivative, II*c*, which was readily decarboxylated to the corresponding 3-methylquinoline, II*d*. Loev *et al.* (8) carried out a similar condensation of 5-chloro-2-aminobenzophenone and diethyl succinate, using conditions different from those reported here. The product they obtained was also a six-membered carbostyril rather than a benzazepine, although it is unclear from their report whether they obtained a 4-phenyl-3-carboxymethyl-2-quinolone or a compound identical to I*a*. Compounds I*b*–I*f* (Table I) and II*a*–II*d* (Table II) were newly synthesized by literature methods (9–12).

PHARMACOLOGY

The compounds described in this paper were evaluated as CNS depressants by submitting them to a battery of behavioral and drug interaction tests in mice (13–21). No compound exhibited any significant CNS activity. The compounds were also tested[1] for antibacterial activity in a serial dilution test using a liquid medium[2]. The bacteriostatic values were determined after incubation for 24 hr. at 37°. Only the quinolyl acetamides, I*c*–I*f*, showed some antibacterial activity against Gram-positive bacteria. The three best results are shown in Table III.

EXPERIMENTAL[3]

6-Chloro-4-phenyl-3-carboxymethyl-2-quinolone (I*a*)—Potassium (66.3 g., 1.7 g. atom) was dissolved in *tert*-butanol (1000 ml.) at 50°, and subsequently diethyl succinate (348 g., 2 moles) and 5-chloro-2-aminobenzophenone (324 g., 1.4 moles) were added. After refluxing for 8 hr., the solvent was evaporated *in vacuo* and the residue was diluted with water, acidified with 2 *N* hydrochloric acid, and extracted with isopropyl ether. The solid material remaining in the

[1] For testing methods, refer to Table III.
[2] Merck Standard Bouillon I.
[3] Melting points were taken on a Kofler heating bench, type 7841, and are uncorrected. UV spectra were obtained on a Pye-Unicam spectrophotometer, model SP500, and the IR spectra were recorded on a Perkin-Elmer spectrophotometer, model 521, using KBr pellets. NMR spectra were obtained on Varian A-60 and T-60 spectrometers using tetramethylsilane as the internal standard and dimethyl sulfoxide as solvent. The mass spectra were recorded on a MS9 (AEI).

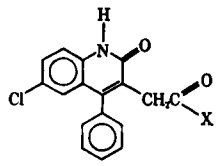
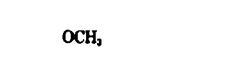
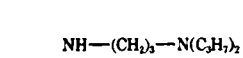


Table I—6-Chloro-4-phenyl-3-carboxymethyl-2-quinolone Derivatives (I)

Compound	X	Melting Point	Yield, %	Formula[a]	Analysis, % Calc.	Found
Ia	OH	285° dec.	75	$C_{17}H_{12}ClNO_3$	C 65.09 H 3.85 N 4.46	65.16 3.90 4.41
Ib	OCH_3	250–252°	75	$C_{18}H_{14}ClNO_3$	C 66.00 H 4.30 N 4.27	66.25 4.51 4.50
Ic	$NH-(CH_2)_3-N(C_2H_5)_2$	238°	70	$C_{26}H_{32}ClN_3O_3$	C 68.79 H 7.10 N 9.25	69.08 7.23 9.07
Id		242–244°	57	$C_{26}H_{28}ClN_3O_3$	C 68.57 H 6.44 N 9.59	68.42 6.48 9.83
Ie		246°	50	$C_{24}H_{26}ClN_3O_4$	C 65.52 H 5.95 N 9.55	65.71 6.18 9.79
If		262°	63	$C_{25}H_{29}ClN_4O_3$	C 66.30 H 6.45 N 12.37	66.44 6.60 12.28

[a] IR, NMR, and mass spectra are in agreement with the assigned structures. For spectral data, refer to the *Experimental* section.

aqueous phase was separated by filtration and washed with water and isopropyl ether. After dissolving the solid in warm 4 *N* potassium hydroxide, the solution was treated with charcoal and filtered. The filtrate was acidified with 2 *N* hydrochloric acid to give a colorless precipitate, which was collected by filtration, washed with water, and crystallized from acetic acid, yielding 329 g. (Table I); UV: $\lambda_{max}^{CH_3OH}$ 235 nm., ϵ_{molar} 4.88 × 10^4; IR: ν_{max} 1720, 1665 (CO), 1630 (C=C), 3400, 3200, and 3050 (OH, NH) $cm.^{-1}$; NMR: δ 3.17 (s, 2H, CH_2), 7.4 (m, 8 aromatic protons), and 12.1 (broad s, 1H, COOH); mass spectroscopy (200°/70 ev.): M^+ = 313.

6-Chloro-4-phenyl-3-methylcarboxymethyl-2-quinolone (I*b*)—Phosphorus oxychloride (7 ml.) was added to the solution of I*a* (84.6 g., 0.27 mole) in methanol (1000 ml.). The mixture was refluxed with stirring for 8 hr. On cooling, the solid material obtained was separated by filtration, washed with methanol, and ether (ethyl), and dried. Recrystallization from methanol afforded 66.5 g. of colorless crystals (Table I); IR: ν_{max} 1730, 1665 (CO), 1630 (C=C), 3400, and 3200 (NH) $cm.^{-1}$; NMR: δ 3.18 (s, 2H, CH_2), 3.99 (s, 3H, OCH_3), and 7.5 (m, 8 aromatic protons); mass spectroscopy (180°/70 ev.): M^+ = 327.

***N*-Substituted Basic Amides of 6-Chloro-4-phenyl-3-carboxymethyl-2-quinolone (I*c*–I*f*)**—A mixture of I*b* (163.9 g., 0.5 mole) and the corresponding amine (0.2 mole) was refluxed for 4 hr. After dilution with ligroin, the separated solid was collected and crystallized from dilute ethanol. The characteristics of I*c*–I*f* are listed in Table I.

2,6-Dichloro-4-phenyl-3-methylcarboxymethylquinoline (II*a*)—A mixture of I*b* (98.3 g., 0.3 mole), phosphorus trichloride (0.5 mole), and phosphorus oxychloride (350 ml.) was refluxed for 4 hr. The reaction mixture was cooled and poured on ice. The separated solid material was filtered, dried, and crystallized from toluene–ligroin, yielding 83 g. (Table II); IR: ν_{max} 1728 (CO) and 1632 (C=C) $cm.^{-1}$; NMR: δ 3.19 (s, 2H, CH_2), 4.0 (s, 3H, OCH_3), and 7.4 (m, 8 aromatic protons); mass spectroscopy (170°/70 ev.): M^+ = 345.

2-Methoxy-4-phenyl-6-chloro-3-methylcarboxymethylquinoline (II*b*)—A solution of II*a* (69.2 g., 0.2 mole) in benzene (500 ml.) was added to a methanol solution of sodium methoxide (8 g. sodium in 200 ml. methanol). After refluxing for 8 hr., the solvents were partially evaporated and cold water was added. The mixture was extracted with ethyl acetate. The extract was dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo* to give a residue which was crystallized from toluene–ligroin, yielding 54 g. (Table II); IR: ν_{max} 1730 (CO), 1632 (C=C), and 2830 (OCH_3) $cm.^{-1}$; NMR: δ 3.19 (s, 2H, CH_2), 3.99 (6H, $2OCH_3$), and 7.5 (8 aromatic protons); mass spectroscopy (180°/70 ev.): M^+ = 341.

2-Methoxy-4-phenyl-6-chloro-3-carboxymethylquinoline (II*c*)—A

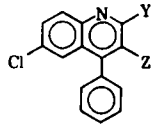


Table II—6-Chloro-4-phenyl-2,3-substituted Quinolines (II)

Compound	Y	Z	Yield, %	Melting Point	Formula[a]	Analysis, % Calc.	Found
II*a*	Cl	CH_2COOCH_3	80	185–187°	$C_{18}H_{13}Cl_2NO_2$	C 62.44 H 3.78 N 4.04	62.49 4.02 4.22
II*b*	OCH_3	CH_2COOCH_3	79	150–152°	$C_{19}H_{16}ClNO_3$	C 66.76 H 4.72 N 4.09	66.71 4.78 4.23
II*c*	OCH_3	CH_2COOH	57	208–210°	$C_{18}H_{14}ClNO_3$	C 66.00 H 4.30 N 4.27	66.30 4.52 4.47
II*d*	OCH_3	CH_3	60	253–255°	$C_{17}H_{14}ClNO$	C 71.96 H 4.97 N 4.93	71.80 5.13 4.81

[a] IR, NMR, and mass spectra are in agreement with the assigned structures. For spectral data, refer to the *Experimental* section.

Table III—Antibacterial Activity

Compound[a]	Bacteriostatic Activity, MIC[b], mcg./ml. *Staphylococcus aureus* ATCC 6538	*Streptococcus hemolyticus* SGA 380[c]	*Streptococcus fecium* SGB[c]	*Escherichia coli* ATCC 9633	*Proteus mirabilis*[c]	*Pseudomonas aeruginosa* ATCC 10145
Ic	78.2	19.6	156.00	625.0	5000.0	5000.0
Id	156.0	78.2	313.0	313.0	2500.0	2500.0
Ie	625.0	156.0	313.0	313.0	5000.0	1250.0

[a] Hydrochlorides of the compounds were used as the test substance. [b] Minimum inhibitory concentration; determined by the method described in P. Klein, "Bakteriologische Grundlagen der Chemotherapeutischen Laboratoriumspraxis," Springer-Verlag, Berlin, Germany, 1957, pp. 53ff; and R. Brunner and G. Macheck, "Die Antibiotica," 2 Bde, Verlag Hans Carl, Nuremberg, Germany, 1962. [c] Culture collection of Microbiology Department of Farbwerke Hoechst AG., Frankfurt, Germany.

mixture of II*b* (16.3 g., 0.48 mole), methanol (100 ml.), and sodium hydroxide solution (5 ml. of 50%) was refluxed for 15 hr. On cooling, the mixture was diluted with water and extracted with methylene chloride. The aqueous solution was acidified with hydrochloric acid and the obtained precipitate was filtered, washed with water, dried, and crystallized from toluene–ligroin, yielding 9 g. (Table II); IR: ν_{max} 1720 (CO), 1634 (C=C), 3350, and 3400 (OH) cm.$^{-1}$; NMR: δ 3.19 (s, 2H, CH_2), 3.99 (s, 3H, OCH_3), 7.4 (m, 8 aromatic protons), and 11.8 (broad s, 1H, COOH); mass spectroscopy (180°/70 ev.): M^+ = 327.

2-Methoxy-3-methyl-4-phenyl-6-chloroquinoline (II*d*)—Compound II*c* (32.7 g., 0.1 mole) was refluxed with quinoline (100 ml.) and copper powder (4.5 g.) for 3 hr. and worked up according to the method of Burness (12); IR: ν_{max} 1630 (C=C), 1400, 1435, 1480, 1500 (aromatic C=C), and 2825 (weak, OCH_3) cm.$^{-1}$; NMR: δ 2.29 (s, 3H, CH_3), 3.98 (s, 3H, OCH_3), and 7.4 (m, 8 aromatic protons); mass spectroscopy (170°/70 ev.): M^+ = 283.

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Determination of 6-Demethylgriseofulvin in Urine

MALCOLM ROWLAND▲ and SIDNEY RIEGELMAN

Abstract □ A simple UV assay is reported that is capable of measuring 1 mcg. 6-demethylgriseofulvin/ml. urine. The method utilizes the difference in the absorbance of the ionized and unionized forms of this molecule at 327 nm.

Keyphrases □ Griseofulvin metabolites—UV spectrophotometric determination of 6-demethylgriseofulvin in urine □ 6-Demethylgriseofulvin—UV spectrophotometric determination in urine □ UV spectrophotometry—determination of 6-demethylgriseofulvin

In man (1) as well as rabbit (2) and dog (3), *O*-demethylation of the antifungal agent, griseofulvin, to 6-demethylgriseofulvin is a significant route of elimination. Griseofulvin, ingested as a solution in polyethylene glycol 300, was almost quantitatively recovered (4) in the urine as 6-demethylgriseofulvin in man. While griseofulvin was readily seen in the plasma following oral administration of standard 250- and 500-mg. doses,

Table III—Antibacterial Activity

Compound[a]	Bacteriostatic Activity, MIC[b], mcg./ml. *Staphylococcus aureus* ATCC 6538	*Streptococcus hemolyticus* SGA 380[c]	*Streptococcus fecium* SGB[c]	*Escherichia coli* ATCC 9633	*Proteus mirabilis*[c]	*Pseudomonas aeruginosa* ATCC 10145
Ic	78.2	19.6	156.00	625.0	5000.0	5000.0
Id	156.0	78.2	313.0	313.0	2500.0	2500.0
Ie	625.0	156.0	313.0	313.0	5000.0	1250.0

[a] Hydrochlorides of the compounds were used as the test substance. [b] Minimum inhibitory concentration; determined by the method described in P. Klein, "Bakteriologische Grundlagen der Chemotherapeutischen Laboratoriumspraxis," Springer-Verlag, Berlin, Germany, 1957, pp. 53ff; and R. Brunner and G. Macheck, "Die Antibiotica," 2 Bde, Verlag Hans Carl, Nuremberg, Germany, 1962. [c] Culture collection of Microbiology Department of Farbwerke Hoechst AG., Frankfurt, Germany.

mixture of II*b* (16.3 g., 0.48 mole), methanol (100 ml.), and sodium hydroxide solution (5 ml. of 50%) was refluxed for 15 hr. On cooling, the mixture was diluted with water and extracted with methylene chloride. The aqueous solution was acidified with hydrochloric acid and the obtained precipitate was filtered, washed with water, dried, and crystallized from toluene–ligroin, yielding 9 g. (Table II); IR: ν_{max} 1720 (CO), 1634 (C=C), 3350, and 3400 (OH) cm.$^{-1}$; NMR: δ 3.19 (s, 2H, CH_2), 3.99 (s, 3H, OCH_3), 7.4 (m, 8 aromatic protons), and 11.8 (broad s, 1H, COOH); mass spectroscopy (180°/70 ev.): M^+ = 327.

2-Methoxy-3-methyl-4-phenyl-6-chloroquinoline (II*d*)—Compound II*c* (32.7 g., 0.1 mole) was refluxed with quinoline (100 ml.) and copper powder (4.5 g.) for 3 hr. and worked up according to the method of Burness (12); IR: ν_{max} 1630 (C=C), 1400, 1435, 1480, 1500 (aromatic C=C), and 2825 (weak, OCH_3) cm.$^{-1}$; NMR: δ 2.29 (s, 3H, CH_3), 3.98 (s, 3H, OCH_3), and 7.4 (m, 8 aromatic protons); mass spectroscopy (170°/70 ev.): M^+ = 283.

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less than 0.1% of the dose appeared unchanged in the urine (1, 5). Urinary excretion measurement of 6-demethylgriseofulvin therefore offers convenient and supplemental information when studying bioavailability profiles of griseofulvin under various conditions. This report describes a simple method for measuring this metabolite in urine, utilizing the difference in the spectrum between the ionized and unionized forms of 6-demethylgriseofulvin at 327 nm.

EXPERIMENTAL[1]

Citrate Buffer—Citric acid, 1 *M*, is adjusted to pH 3.9 with 1 *M* sodium citrate.

Organic Solvent—Cyclohexane–ethylene dichloride (50:50) is prepared by successively washing each solvent with 1 vol. 1 *N* NaOH, 1 vol. 1 *N* HCl, and 2 vol. distilled water.

Phosphate Buffer (McIlvaine's Buffer)—Citric acid (0.2 *M*, 27.5 ml.) is diluted to 1000 ml. with 0.2 *M* Na_2HPO_4, final pH 7.9.

Procedure—To 2 ml. of urine (containing 0–10 mcg. 6-demethylgriseofulvin/ml.) are added 1 ml. citrate buffer and 10 ml. organic solvent; the mixture is shaken for 2 min. and centrifuged. Six milliliters of the organic layer is pipeted into another tube containing 6 ml. pH 7.9 phosphate buffer, shaken for 2 min., and centrifuged; then the upper organic layer is aspirated off. Two milliliters of the remaining aqueous layer is pipeted into each of two tubes, one containing 0.2 ml. 5 *N* HCl and the other containing 0.2 ml. McIlvaine's buffer (pH 7.9). The absorbance of the basic solution is read against the acidic solution at 327 nm. The concentration of 6-demethylgriseofulvin in the urine is calculated by reference to a standard curve prepared by taking known concentrations of 6-demethylgriseofulvin (0–10 mcg./ml.) through the same procedure.

The concentration of the glucuronide of 6-demethylgriseofulvin is determined as the difference between the concentration of 6-demethylgriseofulvin before and after incubation of the urine sample overnight at 37° with 100 units of β-glucuronidase in 1 ml. of phosphate buffer (pH 6.8).

RESULTS AND DISCUSSION

Most plasma assays for griseofulvin utilize its native fluorescence in aqueous and hydroalcoholic solutions. It was reasoned and subsequently verified that unionized 6-demethylgriseofulvin also fluoresces with the same activation (315 nm.) and fluorescence (450 nm.) maxima as griseofulvin and, thereby, an avenue should exist for the establishment of a sensitive assay for this metabolite. Surprisingly, a pH fluorescence profile indicated a maximum fluorescent intensity at pH 2.4 equal to 30% of griseofulvin on a molar basis. The decreasing fluorescence, seen with increasing pH beyond 2.4 and virtually absent above pH 7.0, suggests that only the unionized molecule fluoresces. A half-maximal fluorescence at pH 4.5, very similar to the pKa (4.27) of 6-demethylgriseofulvin (6), supports this hypothesis. Below pH 2.4, the fluorescence also declines and it is negligible at pH < 0.5, presumably caused by protonation of 6-demethylgriseofulvin. While subsequent attempts to develop a fluorometric assay for urinary 6-demethygriseofulvin proved unsuccessful because of excessive fluorescent species in urine, fluorescent measurements were used to determine pH partition profiles of this metabolite with various organic solvents. Furthermore, while griseofulvin fluorescence is pH insensitive above pH 2.2, like 6-demethylgriseofulvin it diminishes markedly in a more acidic environment. This last observation provided a useful modification to the usual fluorometric assay for griseofulvin in plasma (7).

By using a 5:1 volume ratio of organic solvent to aqueous buffer, 6-demethylgriseofulvin is extracted quantitatively into ethylene dichloride or ether below pH 3.5. Above pH 3.5, extraction progressively diminishes. Partitioning occurs more favorably into the former solvent, 50% 6-demethylgriseofulvin being extracted into ethylene dichloride at pH 6.9 and 6.0 with ether. Both solvents were investigated in a potential assay in which the urine was adjusted to pH 4.0 (range 3.92–4.30) with strong citrate buffer (pH 3.9) prior to extraction. This pH allows quantitative 6-demethylgriseofulvin extraction while reducing interference from urinary constituents that are extracted in higher proportions from a more acidic urine. 6-Demethylgriseofulvin was determined by differential spectrophotometry, taking advantage of the low absorbance of the unionized phenol at the λ_{max} (327 nm.) for the phenolate ion ($\Delta\epsilon$ = 26,900) (8). One microgram 6-demethylgriseofulvin/ml. aqueous solutions could readily be measured.

Unfortunately, both ether and ethylene dichloride extracted too many absorbing materials from blank urine. Various combinations of cyclohexane with ether, ethylene dichloride, and chloroform were examined to reduce the urine blank. An equal volume of cyclohexane and ethylene dichloride proved the most suitable solvent. This mixture has a lower specific gravity than water, allowing facile transfer from one tube to another; it also does not form emulsions with urine and quantitatively extracts 6-demethylgriseofulvin at and below pH 4.5. Furthermore, extraction of 6-demethylgriseofulvin into this solvent mixture is negligible at pH 7.5 or greater, so a pH 7.9 buffer was chosen to back-extract this metabolite. The absorbance of the pH 7.9 buffer extract was then measured at 327 nm. against the same solution made acidic with 5 *N* HCl. When using this procedure, blank values were always less than 0.35 mcg. 6-demethylgriseofulvin equivalents/ml. urine. Also, a linear regression was observed between absorbance and concentration in the range of 0–10 mcg./ml. urine. Comparison of the slope of a calibration curve taken through the procedure with an aqueous standard curve indicates an overall recovery of 97.7%.

The presence of 6-demethylgriseofulvin in an ethereal extract of acidic urine was confirmed by TLC on silica gel plates containing a green fluorophore, using chloroform–acetic acid–water (4:1:1) as the developing solvent. A spot at the same R_f value as authentic 6-demethylgriseofulvin (R_f 0.34) was absent in blank urine but present in urine of subjects ingesting griseofulvin. Like 6-demethylgriseofulvin, the native fluorescence of this spot in the acidic environment of the developing solvent was quenched by exposure to ammonia fumes. The presence of another metabolite, 4-demethylgriseofulvin, formed in the rat and rabbit (9), was also sought. This metabolite, synthesized by a literature method (10), gave a different R_f value (0.55) in this thin-layer system. No spot corresponding to 4-demethylgriseofulvin was detected in the urine of subjects receiving griseofulvin before or after incubation with β-glucuronidase. The presence of 6-demethylgriseofulvin glucuronide was assessed by exhaustively extracting 6-demethylgriseofulvin from acidic urine with ether, adjusting to pH 6.8, and incubating with β-glucuronidase in phosphate buffer (pH 6.8) at 37° overnight. The appearance of a spot, absent in control urine, at the same R_f value as 6-demethylgriseofulvin and with identical fluorescent properties was taken as evidence of 6-demethylgriseofulvin glucuronide. Incubation studies under the assay conditions indicated that the glucuronide was hydrolyzed within 4 hr. (11). A GLC assay has been used (11) for the determination of urinary 6-demethylgriseofulvin. The reported (11) failure to find conjugated metabolites conflicts with the present findings. These differences may have arisen from different conditions used in the hydrolysis.

By utilizing the described assay, between 70 and 100% of an intravenous dose of griseofulvin was recovered as the sum of 6-demethylgriseofulvin and 6-demethylgriseofulvin glucuronide in man (12). Also, in man (13), unlike dogs (3, 14), excretion of 6-demethylgriseofulvin is rate limited by the elimination of griseofulvin. Furthermore, the proportionality between the amount of 6-demethylgriseofulvin excreted and the area under the griseofulvin plasma time curve (10) indicates that urinary 6-demethylgriseofulvin may be used to assess the availability of oral griseofulvin.

[1] A Beckman DU spectrophotometer and Beckman pH meter were used. 6-Demethylgriseofulvin was supplied by McNeil Laboratories, Fort Washington, Pa.; β-glucuronidase was purchased from Sigma Chemical, Chicago, Ill. All chemicals and solvents were Baker analyzed grade.

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Quantitative TLC Determination of Primidone, Phenylethylmalonediamide, and Phenobarbital in Biological Fluids

Y. GARCEAU, Y. PHILOPOULOS*, and J. HASEGAWA▲

Abstract □ A rapid and reproducible TLC determination of primidone and its metabolites in plasma and urine is described. The method consists of extracting the drugs into chloroform, evaporating the organic phase to dryness, dissolving the residue in an accurately measured volume of chloroform–acetic acid (9:1), and spotting this solution on a thin layer of silica gel. Quantitation is achieved by comparing the areas under the peaks obtained from scanning the TLC plates in a spectrodensitometer. The limit of detection is 1 mcg./ml. plasma for primidone and phenobarbital and 2 mcg./ml. plasma for phenylethylmalonediamide. Mean recoveries obtained from spiked plasma samples were: primidone, 93%; phenylethylmalonediamide, 97%; and phenobarbital, 102%.

Keyphrases □ Primidone—TLC separation and quantitation with phenylethylmalonediamide and phenobarbital in biological fluids □ Phenylethylmalonediamide—TLC separation and quantitation with primidone and phenobarbital in biological fluids □ Phenobarbital—TLC separation and quantitation with primidone and phenylethylmalonediamide in biological fluids □ TLC—separation and quantitation of primidone, phenylethylmalonediamide, and phenobarbital in biological fluids

Several procedures have been published on the quantitation of primidone and two of its metabolites, phenylethylmalonediamide and phenobarbital, in biological fluids. One chemical method used oxidation of primidone to phenobarbital (1). The other procedures used either TLC (2–4) or GLC (5–11). The TLC procedures were semiquantitative, since they relied on measuring spot size and intensity or colorimetric estimation after elution of compounds from the plates. The GLC methods were generally reproducible, accurate, specific, and sensitive but were relatively time consuming. This disadvantage cannot be neglected when analysis time is important as in a bioavailability study involving several hundred assay samples. This paper describes a fast and quantitative TLC procedure for estimating primidone, phenylethylmalonediamide, and phenobarbital in plasma and urine.

EXPERIMENTAL

Apparatus—A spectrodensitometer[1] equipped with a density computer[2] was used. The light source consisted of a xenon–mercury 200-w. lamp[3]. The instrument was operated in the reflection mode, using the dual-beam system. Samples were scanned at 220 nm. (density computer set at 0.2 o.d.). Scanning and chart speeds were set at 10.2 cm. (4 in.)/min.

Materials and Chemicals—Silica gel TLC plates[4] (20 × 20 cm.) were employed. The plates were divided into 20 equal channels, each 1 cm. wide, with a scoring device[5]. The plates were heated at 100° for 1 hr. before use. Stock solutions of primidone[6], phenylethylmalonediamide[6], and phenobarbital[7] were prepared by dissolving accurately weighed amounts in methanol. Working standards were made by appropriate dilutions of the stock standards. All solvents used were reagent grade. Samples were applied to the plates with 10-, 25-, or 50-μl. syringes[8].

Developing Systems—For primidone and phenylethylmalonediamide analysis, the following developing system was used: ethyl acetate–benzene–acetic acid (90:20:10) (System A). For phenobarbital analysis, the system consisted of benzene–ethyl acetate–acetone–acetic acid (100:25:15:10) (System B). These systems require saturated tanks and an environment where the relative humidity is maintained at less than 25%.

Procedure for Determining Drugs in Plasma—Two-milliliter plasma samples spiked with appropriate amounts of all three drugs were extracted with 30 ml. chloroform in 50-ml. conical tubes. The tubes were shaken on a mechanical device[9] for 15 min. Unspiked plasma samples served as blanks. After extraction, the tubes were centrifuged and 25 ml. of the extracts were transferred to a clean 50-ml. conical tube and evaporated under nitrogen[10]. The sides of the tubes were washed with 1-ml. portions of chloroform, and the solutions were evaporated to dryness. The residues were dissolved in 100 μl. of a solution of chloroform–acetic acid (9:1).

Appropriate aliquots (25–50 μl.) were spotted on scored TLC

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[2] Model SDC 300, Schoeffel Instrument Corp.
[3] Hanovia Lamp Division, Camrand Precision Ind.
[4] Sil G-25-22, Brinkmann Instruments Inc.
[5] SDA 320, Schoeffel Instrument Corp.
[6] Pharmaceuticals Division, Imperial Chemical Industries Ltd.
[7] USP powder, Mallinckrodt Chemical Works.
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[10] N-Evap, Organomation Association.

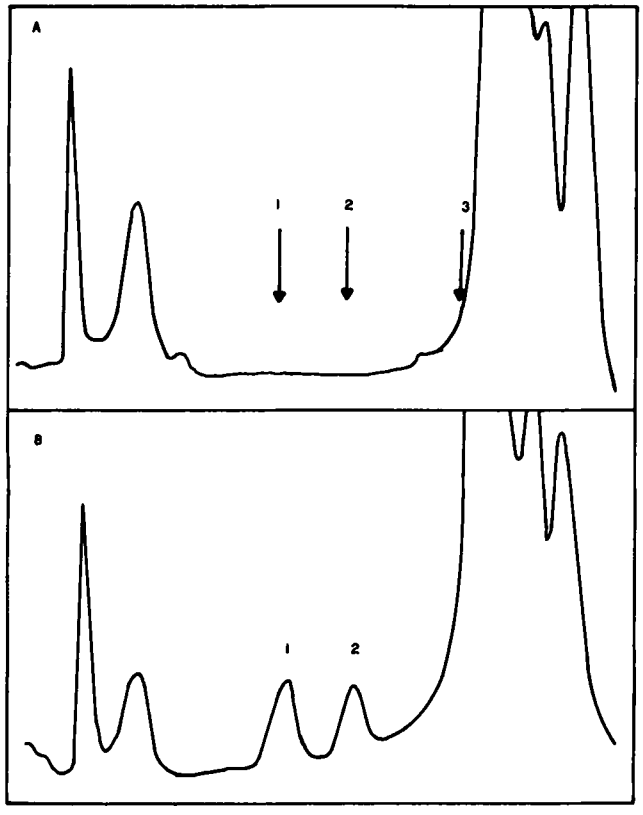

Figure 1—*(A) Chromatogram of a blank plasma extract showing* R_f *values for: 1, primidone; 2, phenylethylmalonediamide; and 3, phenobarbital. (B) Chromatogram of an extract from a plasma sample spiked with primidone (1) and phenylethylmalonediamide (2) at 5-mcg/ml. concentration. Extra peaks represent extractable plasma impurities.*

plates, generally in duplicate. Each channel on which a sample was spotted had to its immediate left a nonspotted reference channel. Standards of at least two different concentrations were also spotted so that a calibration line was included on each plate. The plates were then developed, dried, and read as described. Drug concentration was determined by comparing the areas under the peaks obtained from the standards to those obtained from the spiked samples. The procedure is also applicable to the determination of primidone, phenylethylmalonediamide, and phenobarbital in urine. Specimens are adjusted to pH 7.0 before extraction.

***In Vivo* Experiment**—To assess the applicability of the method, six beagle dogs were given 192 mg. of primidone intravenously (drug dissolved in 4 ml. dimethylformamide). Heparinized blood samples were collected prior to injection and at 0.25, 0.50, 0.75, 1, 2, 3, 4, 6, and 8 hr. after injection. The blood samples were centrifuged immediately, and the plasma was frozen until analyzed.

RESULTS AND DISCUSSION

With developing System A, primidone (R_f 0.42) can be separated from its metabolites phenylethylmalonediamide (R_f 0.53) and phenobarbital (R_f 0.67) and from extractable plasma impurities (Fig. 1). Both primidone and phenylethylmalonediamide can be quantitated when System A is used, but phenobarbital cannot be estimated due to interfering plasma components. However, the system can be used for the determination of all three drugs in urine, because interfering impurities are absent. To quantitate phenobarbital in plasma, developing System B must be used (Fig. 2). The use of two developing systems may make the method less appealing, but they are necessary only when phenobarbital needs to be determined in plasma. This method was developed for application in bioavailability studies. When a single dose of primidone is administered, phenobarbital determination is not required since it is not detectable in plasma following such a dose (12). However, phenobarbital determination is indicated in studies involving multiple dosing of primidone.

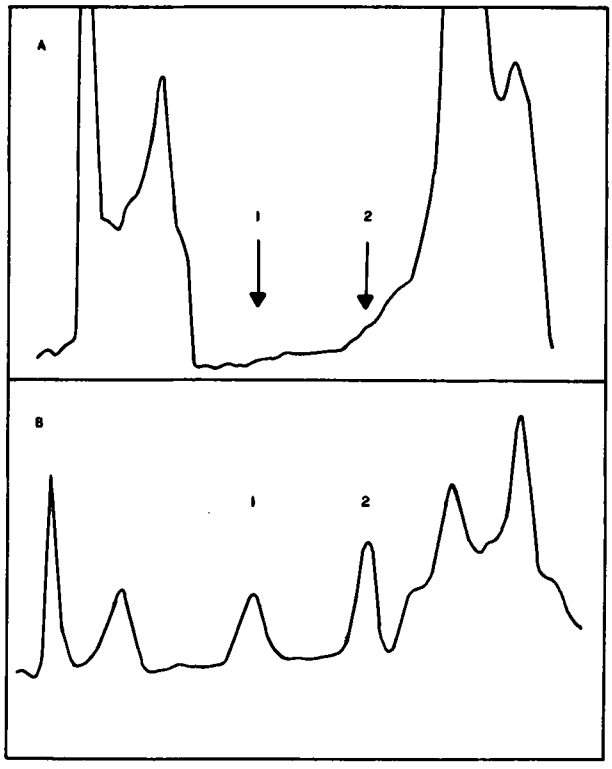

Figure 2—*(A) Chromatogram of a blank plasma extract showing* R_f *values for primidone (1,* R_f *0.34) and phenobarbital (2,* R_f *0.58). Phenylethylmalonediamide has same* R_f *as primidone in this system. (B) Chromatogram of an extract from a plasma sample spiked with primidone (1) and phenobarbital (2) at 5-mcg./ml. concentration. Extra peaks represent extractable plasma impurities.*

Typical calibration lines are shown in Fig. 3. Correlation coefficients are: primidone, 0.996; phenylethylmalonediamide, 0.993; and phenobarbital, 0.990 (n = 5). Although the range of linearity is rather narrow for phenobarbital (about 1–6 mcg.), it is not a problem as long as a calibration line covering the concentration range of the unknowns is included on every plate.

A summary of the recovery studies performed in plasma is given in Table I. Mean recoveries were: primidone, 93% (n = 21); phenylethylmalonediamide, 97% (n = 18); and phenobarbital, 102% (n = 21). Mean recoveries from spiked urine samples were: primi-

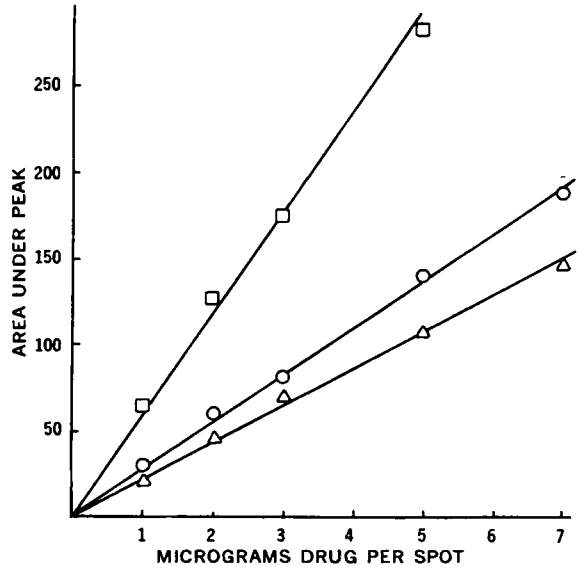

Figure 3—*Typical calibration lines for primidone (○), phenobarbital (□), and phenylethylmalonediamide (△).*

Table I—Summary of Recovery Experiments in Plasma

Primidone				Phenylethylmalonediamide				Phenobarbital			
Amount Spiked, mcg./ml.	Amount Recovered, mcg./ml. Run 1	Run 2	Run 3	Amount Spiked, mcg./ml.	Amount Recovered, mcg./ml. Run 1	Run 2	Run 3	Amount Spiked, mcg./ml.	Amount Recovered, mcg./ml. Run 1	Run 2	Run 3
1.0	1.0	1.0	1.0	—	—	—	—	2.0	2.0	2.1	2.1
2.5	2.3	2.3	2.2	2.5	2.3	2.4	2.6	4.0	4.0	4.0	3.9
5.0	4.3	4.6	4.6	5.0	5.0	5.1	4.8	6.0	6.0	5.4	6.0
7.5	6.6	6.5	6.8	7.5	6.9	7.2	7.9	8.0	8.5	8.4	8.2
10.0	9.0	8.9	9.0	10.0	10.4	10.2	10.0	10.0	10.7	10.2	10.3
15.0	12.7	15.2	15.6	15.0	13.6	14.4	15.3	20.0	20.8	21.2	21.7
20.0	18.8	16.7	16.6	20.0	18.4	17.7	16.9	30.0	31.6	29.8	30.2
Mean recovery		92.6%		Mean recovery		97.0%		Mean recovery		102.5%	
SD		6.5%		*SD*		6.4%		*SD*		4.1%	
Range		83.3–104.1%		Range		84.9–105.9%		Range		90.0–107.0%	

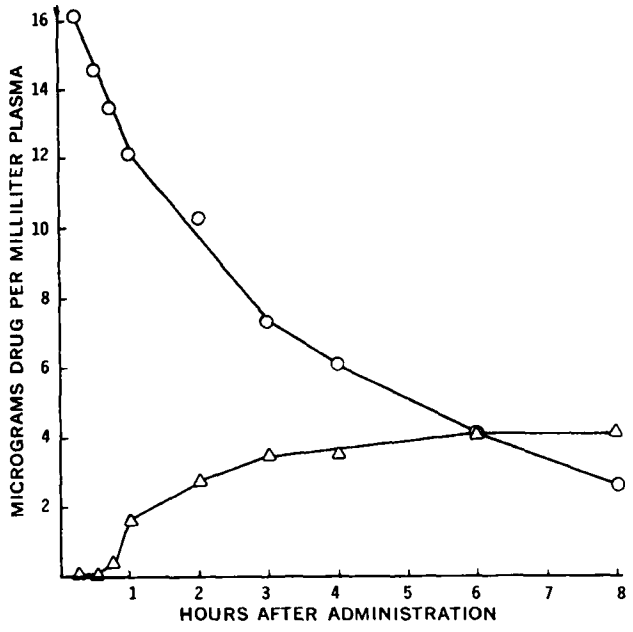

Figure 4—*Mean plasma levels of primidone* (○) *and phenylethylmalonediamide* (△) *following intravenous injection of primidone in dogs.*

done, 85%; phenylethylmalonediamide, 92%; and phenobarbital, 93%. The procedure is simple and rapid. One chemist can easily analyze 20–25 samples in a working day.

An *in vivo* study was performed to verify the applicability of the method. Plasma levels of primidone and phenylethylmalonediamide obtained following a single intravenous injection of primidone to dogs are shown in Fig. 4. Phenobarbital was not detected.

In summary, a quick, reproducible, and specific quantitative TLC method for the determination of primidone and its metabolites in plasma and urine was described. The method is especially useful when a large number of samples must be assayed.

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Enhancement of Benzalkonium Chloride and Chlorhexidine Acetate Activity against *Pseudomonas aeruginosa* by Aromatic Alcohols

R. M. E. RICHARDS* and R. J. McBRIDE▲

Abstract □ The actions of benzalkonium chloride and chlorhexidine acetate in combination with benzyl alcohol, 2-phenylethanol, or 3-phenylpropanol were evaluated at subinhibitory concentrations by measuring the growth rates of *Pseudomonas aeruginosa* NCTC 6750. When tested separately, all three alcohols had an effect on both benzalkonium chloride and chlorhexidine acetate that was more than additive. Phenylpropanol showed the greatest enhancement and benzyl alcohol showed the least enhancement.

Keyphrases □ Benzalkonium chloride—enhanced activity by aromatic alcohols against *Pseudomonas aeruginosa* □ Chlorhexidine acetate—enhanced activity by aromatic alcohols against *Pseudomonas aeruginosa* □ Interactions—enhancement of antibacterial action of benzalkonium chloride and chlorhexidine acetate by aromatic alcohols against *Pseudomonas aeruginosa* □ Preservatives—enhanced activity of benzalkonium chloride and chlorhexidine acetate by aromatic alcohols, tested against *Pseudomonas aeruginosa*

Formulations containing two or more preservatives are increasing in number; 50% of commercial ophthalmic preparations contain such combinations (1), but reports on their evaluation have not appeared. In the past, medicinal antibiotic combinations were evaluated by different *in vitro* techniques which resulted in different definitions to describe the synergistic, additive, antagonistic, or indifferent effects observed between the drugs. The disappointing lack of correlation between such *in vitro* results and *in vivo* effects has been reflected by a lack of reports on *in vitro* evaluation of such combinations. However, pharmaceutical preparations preserved with combinations of bactericides differ from medicinal antibiotic combinations because the preservative capacity of the preparations can be assessed by *in vitro* techniques.

Garrett (2) reviewed the various definitions of antibiotic combination effects and suggested that antibiotic combinations should be evaluated on the basis of: (*a*) additivity, where the combined effect or response is additive with respect to the separate responses of the components; and (*b*) equivalence, in which the antibiotics act in the same manner with the same dose–response curve, separately or in combination, except for a difference in the weight of an arbitrarily defined "unit dose." Garrett (2) also stated that the definition of additivity by Bliss *et al.* (3)—that when one antibiotic is added to another, the effect is improved only to the extent that would occur had an equivalent portion of the same antibiotic been added—was the most restrictive. Thus, the quantity of antibiotic having the same effect or response as the other component of the combination is added to the quantity of the same antibiotic present in the combination, giving the total amount of one antibiotic having the same response as an additive combination. Garrett and coworkers (4, 5) evaluated the additive interaction between chloramphenicol and tetracycline and between erythromycin and lincomycin by measuring the growth rates of *Escherichia coli* in media containing subinhibitory concentrations of the different antibiotics, alone and in combination.

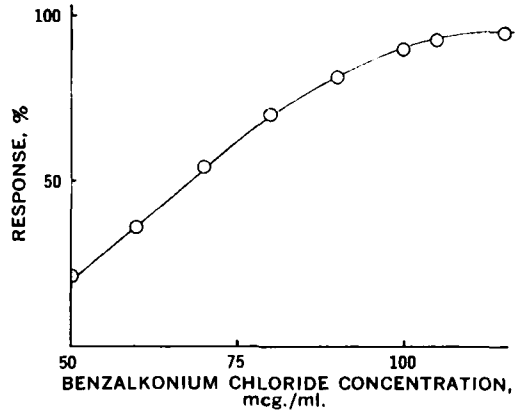

Figure 1—*Dose–response correlation for benzalkonium chloride against* P. aeruginosa.

It was recommended (6) that quick-acting preservatives should be used in ophthalmic preparations and that the final preparation should sterilize a large inoculum of *Pseudomonas aeruginosa* in less than 1 hr. A number of ophthalmic formulations inoculated with 10^6 *P. aeruginosa*/ml. did not pass this test; but when phenylethanol was present as well as the preservative, the preparations sterilized such inocula within 1 hr. (7, 8). Benzyl alcohol and phenylpropanol also enhanced the bactericidal action of benzalkonium chloride (9).

The present investigation was undertaken to determine whether the enhancement of the antibacterial action of benzalkonium chloride and chlorhexidine acetate by aromatic alcohols was greater or less than additive, using growth rate studies of log phase cultures of *P. aeruginosa* in subinhibitory concentrations of the preservatives.

EXPERIMENTAL

Materials—Benzyl alcohol[1] (reagent grade), phenethyl alcohol[1] (phenylethanol or 2-phenylethanol) (reagent grade), phenylpropanol[1] (3-phenylpropanol) (reagent grade), benzalkonium chloride BP[2], and chlorhexidine acetate BPC[3] were used. *P. aeruginosa* NCTC[4] 6750 was the test organism, Oxoid nutrient broth No. 2 was the growth medium, and incubation was at 37°. Maintenance of stock cultures and experimental details were as previously described (9).

[1] British Drug Houses, Poole, Dorset, England.
[2] McCarthy Limited, Glasgow, Scotland.
[3] ICI, Alderly Park, Macclesfield, Cheshire, England.
[4] National Collection of Type Cultures, Colindale, London, England.

Table I—Effect of Benzalkonium Chloride and Chlorhexidine Acetate, Alone and in Combination with Benzyl Alcohol, Phenethyl Alcohol, or Phenylpropanol on the Growth Rate of *P. aeruginosa*

Bactericide, Percentage Concentration	Percentage Response	Percentage Antibacterial Equivalent to Alcohol	Antibacterial Estimated to be Equivalent to Combination: Percentage Concentration	Antibacterial Estimated to be Equivalent to Combination: Percentage Response		Experimental Percentage Response for Combination
Benzalkonium chloride, 0.003	18.5					
Benzyl alcohol, 0.175	41.9	0.0041	0.0071	53.7		76.7
Phenethyl alcohol, 0.175	51.5	0.0046	0.0076	62.0		91.5
Phenylpropanol, 0.175	45.8	0.0043	0.0073	52.0		Lysis
				Mode of Action: First	Second	
Chlorhexidine acetate, 0.0002	18.5					
Benzyl alcohol, 0.175	41.9	0.0031	0.0051	32.0	Lysis	65.9
Phenethyl alcohol, 0.175	47.6	0.0033	0.0053	32.5	Lysis	86.7
Phenylpropanol, 0.175	44.7	0.0032	0.0052	32.3	Lysis	Lysis

Effect of Single Bactericide—The growth of *P. aeruginosa* in media containing different concentrations of a bactericide was followed by measuring the extinctions at 420 nm. The effect or response of a particular concentration is the difference between the growth rate of the control containing no bactericide and that of the bactericide; the percentage response is the difference expressed as a percentage of the control growth rate. The results for benzalkonium chloride and chlorhexidine acetate are illustrated in Figs. 1 and 2, where the percentage response is plotted against bactericide concentration.

Effect of Alcohol–Bactericide Combinations—The procedure with benzalkonium chloride was described previously (9), and a similar procedure was used with chlorhexidine acetate (Fig. 3). The results for both chemicals are given in Table I.

DISCUSSION

Simple addition of the individual response for each component of a combination assumes that the concentration–response correlation for the antibiotic is rectilinear, when often it is not (10). Figure 4 illustrates the influence of the concentration–response correlation upon the theoretical additive response of a combination. If the percentage response of one component, *M*, is 27%, this response is similar to that of 4 units of Component *N*. In a combination of *M* and 4.5 units of *N* (response of 30%), the theoretical additive response would be the same as that of 8.5 units of *N*. But only when the concentration response correlation is rectilinear, as in Type A, does the percentage response (57%) for the 8.5 units of *N* equal the sum of the individual responses. When the concentration response is of Type B, 8.5 units of *N* has a percentage response of 81%; Type C would indicate a response of 39%. Thus, in measuring concentration–response correlations, the concentration of a single preservative equivalent to that of the combination should be included to avoid extrapolation.

The dose–response correlation for each aromatic alcohol was rectilinear (9) and is similar to Type A in Fig. 4. Thus, in determining the theoretical additive response based on the quantity of alcohol having the same effect as the combination, the percentage responses for the components of the combination as single substances were added. The sum of either bactericide with any of the three alcohols was always less than the experimental value obtained, indicating that the response was greater than additive and increased with the molecular weight of the alcohol.

The dose–response correlation for the benzalkonium chloride (Fig. 1) was similar to Type C in Fig. 4, *i.e.*, linear at low concentrations and becoming asymptotic at higher concentrations. The concentrations of benzalkonium chloride that have the same response as each alcohol were determined, and the theoretical response based on the quantity of benzalkonium chloride having the same effect as the combination was also determined. The tabulated results again show that the combination has an effect greater than additive, with phenylpropanol having the largest increase and benzyl alcohol having the smallest.

The dose–response for chlorhexidine acetate (Fig. 2) has a bilinear correlation, showing a slow increase in response with increasing concentration to between 2.75 and 3.0 mcg./ml. and higher concentrations having a very rapid response. In all media containing more than 3.75 mcg./ml. chlorhexidine acetate, lysis occurred. A similar result was observed (11) when the growth rate of *Klebsiella cloaca* was measured in media containing different con-

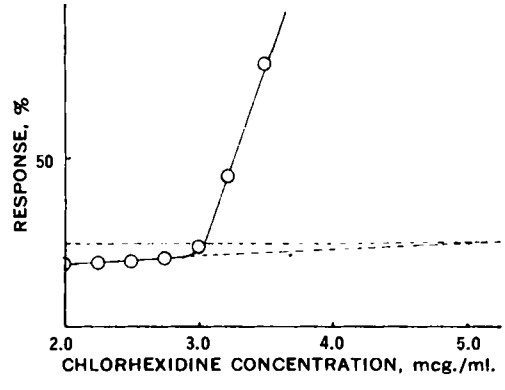

Figure 2—*Dose–response correlation for chlorhexidine acetate against* P. aeruginosa.

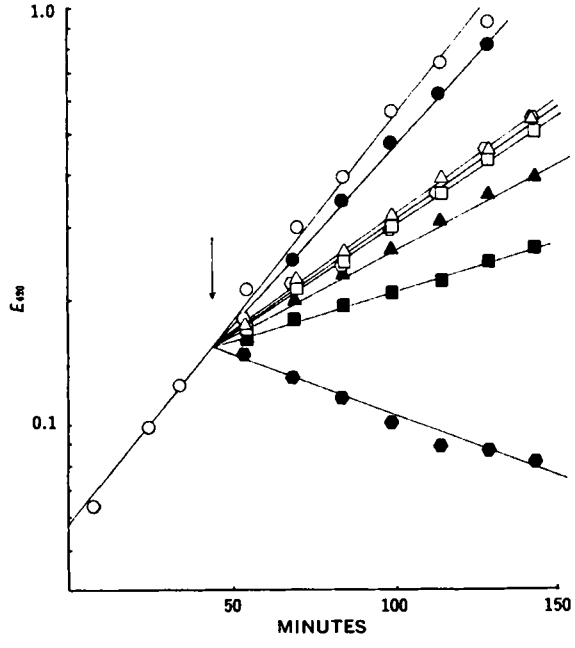

Figure 3—*Effect of 0.175% benzyl alcohol* (△), *0.175% phenethyl alcohol* (□), *and 0.175% phenylpropanol* (○), *alone (open symbols) and in combination with 0.0002% chlorhexidine acetate (closed symbols) on* P. aeruginosa. *The circles indicate alcohol-free control cultures, and additions were made at the time indicated by the arrow.*

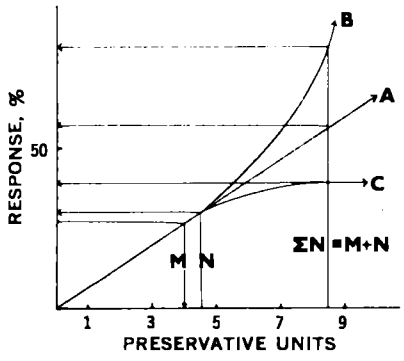

Figure 4—*Effect of the concentration–response correlation on the theoretical additive response of a combination.*

centrations of phenol. It was postulated that if an inhibitor combined with cellular enzymes, the velocity of the enzyme-catalyzed reaction will depend on the concentration of uncombined enzyme, and where an inhibitor affects more than one enzyme in different concentration ranges of inhibitor, each range will display different concentration effects or modes of action. The dose–response correlation indicates that chlorhexidine acetate has two modes of action according to the enzyme model of Harris and Morrison (11). The quantities of chlorhexidine acetate having the same responses as the alcohols would affect the growth of *P. aeruginosa* by the second mode of action; calculation of the theoretical additive response based on the equivalent quantity of chlorhexidine acetate indicates that lysis would occur (Table I). Since the alcohol has a different mode of action from chlorhexidine acetate, sufficient chlorhexidine may not be present to enable the second mode of action to occur, even though the quantity of chlorhexidine acetate in the biophase may increase due to the permeability effect of the alcohol. The amount of chlorhexidine acetate in the combination only affects the initial mode of action when used as a single substance; if this controls the growth rate, the theoretical additive response can be obtained by extrapolation of the initial dose–response correlation (Fig. 2). As shown in Table I, the theoretical percentage response was approximately 32%, which is less than that with the alcohol alone. The experimental results indicate a response greater than additive for chlorhexidine acetate in combination with the aromatic alcohols, with phenylpropanol showing the greatest enhancement and benzyl alcohol showing the least.

In a description of the desirable properties for a combination of preservatives, it has been stated that the combination should act more rapidly against the test organism than do the same concentrations of each agent used separately (12). This does not differentiate between agents that combine to give a response greater or less than additive while still exceeding that of any one member of a combination. While such differentiation may not be important, there are obvious advantages in using combinations whose response is greater than additive.

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Determination of Equilibrium Constants and Binding Capacities Using a Modified Scatchard Method in Drug–Protein Binding Studies

JOSEPH K. H. MA, H. W. JUN, and L. A. LUZZI▲

Abstract □ The equilibrium constants and the number of binding sites for the binding of 1-anilinonaphthalene-8-sulfonate to human and bovine serum albumin, studied by a fluorescent method, were determined by an equation derived from the Scatchard multiple-equilibrium treatment. The results and the equation are discussed and compared with other equations used in drug–protein binding studies. Treatment of data by the modified method allows a clear determination of the number of binding sites.

Keyphrases □ Drug–protein binding—equilibrium constants and binding capacities using modified Scatchard method, equations □ Equilibrium constants—determined using a modified Scatchard method, drug–protein binding studies, equations □ Binding capacities—determined using a modified Scatchard method, drug–protein binding studies, equations □ Scatchard multiple-equilibrium treatment, modified—equation derived to determine equilibrium constants and binding capacities, drug–protein binding studies

In drug–protein binding studies, several equations have been used for binding constant calculation (1–3). One frequently used equation is the Langmuir-type equation:

$$\bar{\nu}/(D) = nK - \bar{\nu}K \qquad \text{(Eq. 1)}$$

where $\bar{\nu}$ represents the ratio of moles of bound drug per mole of total protein, (D) is the concentration of unbound drug, K is the binding constant, and n is the number of binding sites on a single protein molecule, assuming that all sites are of equal binding strength. However, the plot of $\bar{\nu}/(D)$ *versus* $\bar{\nu}$ frequently gives a downward curve rather than a convincing straight line. Such curvature may be indicative of binding to more than one class of sites (4). Mathematical treatments for the determination of binding parameters along this line were also reported (5, 6).

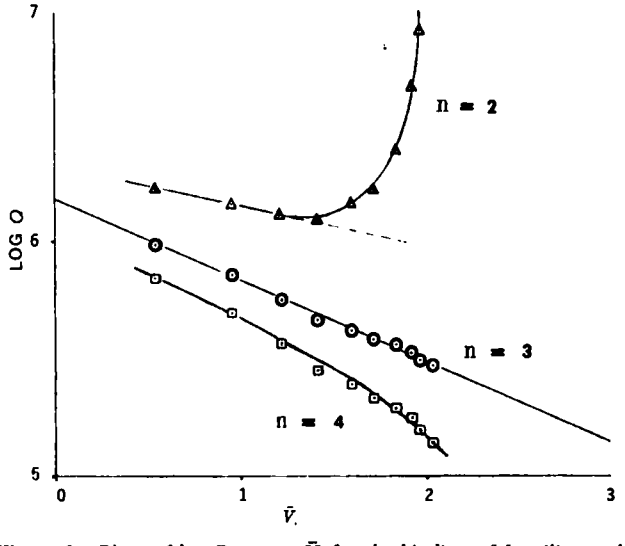

Figure 1—*Plots of log* Q versus $\bar{\nu}$ *for the binding of 1-anilinonaphthalene-8-sulfonate to human serum albumin.*

Recently, Eq. 1 was modified (7, 8) by the introduction of an empirical parameter:

$$\log [\bar{\nu}/(n - \bar{\nu})] = m \log (D) + m \log K \qquad \text{(Eq. 2)}$$

By this method, the value of n is estimated by trial and error so that the plot of $\log [\bar{\nu}/(n - \bar{\nu})]$ *versus* $\log (D)$ might be linear, and m and $\log K$ are evaluated from the slope and the intercept of the graph. When $m = 1$, Eq. 2 may be reduced to Eq. 1. For the binding of 2-(4′-hydroxyphenylazo)benzoic acid to bovine serum albumin, it was shown (7, 8) that m is not equal to 1 and that it varies with factors such as the concentrations of albumin and buffering agent and temperature.

In the present study, the binding of 1-anilinonaphthalene-8-sulfonate to human and bovine serum albumins was studied by a fluorescent method (9). The data were treated using a modified Scatchard equation for multiple equilibria.

EXPERIMENTAL

Materials—1-Anilinonaphthalene-8-sulfonate[1], crystalline human serum albumin[2], bovine serum albumin[3], and spectroscopic grade methanol (as a solvent) were used. All other chemicals were of reagent grade.

Instruments and Methods—All fluorescence measurements were made on a spectrophotofluorometer[4] equipped with a 150-w. xenon lamp and 1P21 photomultiplier tube. Fluorescence titrations over a range of protein concentrations were carried out at the emission maxima of 474 and 467 nm. for human and bovine serum albumins, respectively, with the excitation for both systems at 375 nm. Two milliliters of each protein solution in pH 7.4 phosphate buffer was titrated with successive additions of 2 μl. of 1×10^{-3} *M* 1-anilinonaphthalene-8-sulfonate. The probe was dissolved in methanol and delivered with microsyringes[5]. The temperature of all samples was 25 ± 0.5°.

RESULTS AND DISCUSSION

The relative fluorescence intensities of solutions containing various amounts of 1-anilinonaphthalene-8-sulfonate in the absence and presence of human and bovine serum albumins are shown in Table I. During the titration, above the critical protein concentrations of 29.10×10^{-6} *M* for human serum albumin and 61.41×10^{-6} *M* for bovine serum albumin, there was no further enhancement of the fluorescence upon increases in protein concentration. It was then assumed that all of the drug was bound to protein. The fractions of bound and unbound drug and the molar ratio of the drug bound to protein were calculated by a method previously used in these laboratories (10). Determinations of binding constants were made for solutions containing 1.45×10^{-6} *M* human serum

[1] Aldrich Chemical Co.
[2] Control No. 4619, Nutritional Biochemicals Corp.
[3] Control No. 1693, Nutritional Biochemicals Corp.
[4] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[5] Hamilton.

Table I—Relative Fluorescence Intensity of Solutions Containing Varying Amounts of 1-Anilinonaphthalene-8-sulfonate

$(D_t) \times 10^6$, M[a]	No Protein	Human Serum Albumin, 1.45×10^{-6} M	Human Serum Albumin, 29.10×10^{-6} M	Bovine Serum Albumin, 1.23×10^{-6} M	Bovine Serum Albumin, 61.41×10^{-6} M
1	1.9	6.5	7.8	8.5	11.1
2	3.5	11.6	15.4	15.6	22.2
3	5.2	15.7	23.0	21.2	32.8
4	6.8	18.8	30.3	25.7	43.4
5	7.0	21.4	38.2	28.9	53.7
6	7.3	23.3	46.0	31.0	63.8
7	7.5	24.8	43.2	33.0	74.7
8	7.7	26.0	60.3	34.8	86.0
9	7.9	26.6	67.0	35.6	95.9
10	8.0	27.3	73.5	36.3	105.7

[a] The total 1-anilinonaphthalene-8-sulfonate concentration.

albumin and 1.23×10^{-6} M bovine serum albumin with varying concentrations of 1-anilinonaphthalene-8-sulfonate.

To calculate the equilibrium constants, a function, Q, is defined by the relation:

$$Q = \bar{V}/(n - \bar{V})(D) \qquad \text{(Eq. 3)}$$

or:

$$\log Q = \log [\bar{V}/(n - \bar{V})] + pD \qquad \text{(Eq. 4)}$$

Equation 3 was developed by Scatchard and first employed by Edsall *et al.* (11). The method of Edsall *et al.* was used for the present study to determine the binding parameters. The value of n was not obtained experimentally, however, since it was previously reported (12) that each mole of bovine serum albumin was bound by 3 moles of 1-anilinonaphthalene-8-sulfonate; it was decided to introduce this number first as the probable value for n. The following equilibrium processes ($n = 3$) were chosen for the determination of the equilibrium constants:

$$P + D = PD \qquad k_1 = (PD)/(P)(D) \qquad \text{(Eq. 5)}$$

$$PD + D = PD_2 \qquad k_2 = (PD_2)/(PD)(D) \qquad \text{(Eq. 6)}$$

$$PD_2 + D = PD_3 \qquad k_3 = (PD_3)/(PD_2)(D) \qquad \text{(Eq. 7)}$$

where P represents protein and k_1, k_2, and k_3 are the successive equilibrium constants.

A plot of calculated values for log Q *versus* $\bar{V}$ will give a straight line (13, 14), although the points as $\bar{V}$ approaches zero or the maximum number of binding sites may show some curvature due to experimental error. For $n = 3$, the extrapolated log Q values at

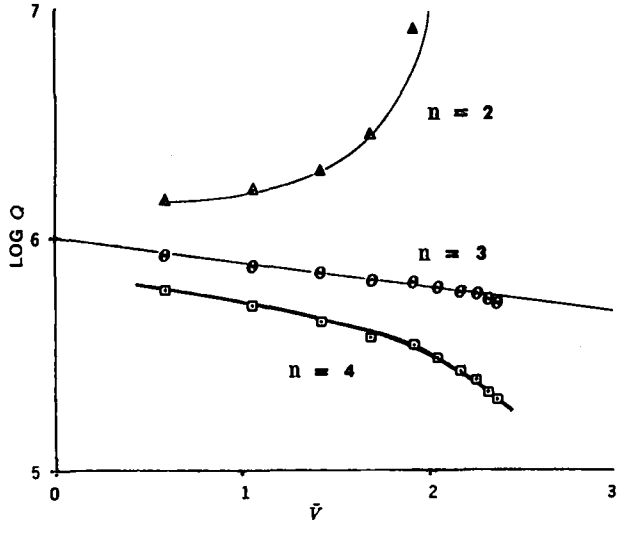

Figure 2—*Plots of log* Q versus $\bar{V}$ *for the binding of 1-anilinonaphthalene-8-sulfonate to bovine serum albumin.*

Table II—Successive Logarithmic Equilibrium Constants of the Binding of 1-Anilinonaphthalene-8-sulfonate to Human and Bovine Serum Albumins

Protein	Concentration	n	log k_1	log k_2	log k_3
Human serum albumin	1.45×10^{-6}	3	6.66	5.66	4.66
Bovine serum albumin	1.23×10^{-6}	3	6.48	5.84	5.20

$\bar{V} = 0$ and $\bar{V} = 3$ are essentially equal to log K_1 and log K_3, respectively. K_1 and K_3 are the intrinsic association constants, which are related to the equilibrium constants by the following equations:

$$K_1 = k_1/3 \qquad \text{(Eq. 8)}$$

$$K_2 = 2k_2/2 \qquad \text{(Eq. 9)}$$

$$K_3 = 3k_3 \qquad \text{(Eq. 10)}$$

To obtain k_2, the following procedure (13) can be used. From the plot of log Q *versus* $\bar{V}$, one can determine log Q at $\bar{V} = 1.5$ as:

$$\log Q = \log [1.5/(3 - 1.5)] + pD \qquad \text{(Eq. 11)}$$

The value of pD can be easily calculated and is taken to be the value of log k_2.

In the present study, three sets of log Q values were calculated using Eq. 4 by letting $n = 2$, 3, and 4, respectively. These values for log Q were then plotted as a function of $\bar{V}$. The results seen in Fig. 1 represent the plots for $n = 2$, 3, and 4 when human serum albumin is the protein. Figure 2 is a similar representation of data when bovine serum albumin is the protein. The plots are linear for both systems at $n = 3$. For $n = 2$ and 4, however, two opposing curves, above and below the straight line, were obtained. These results indicate that both human and serum albumins probably have three binding sites for 1-anilinonaphthalene-8-sulfonate. This is reinforced when one considers previously reported values for n in similar systems.

The successive equilibrium constants determined from Figs. 1 and 2 are listed in Table II. Human serum albumin has a larger log k_1 value but smaller values of log k_2 and log k_3 than those of bovine

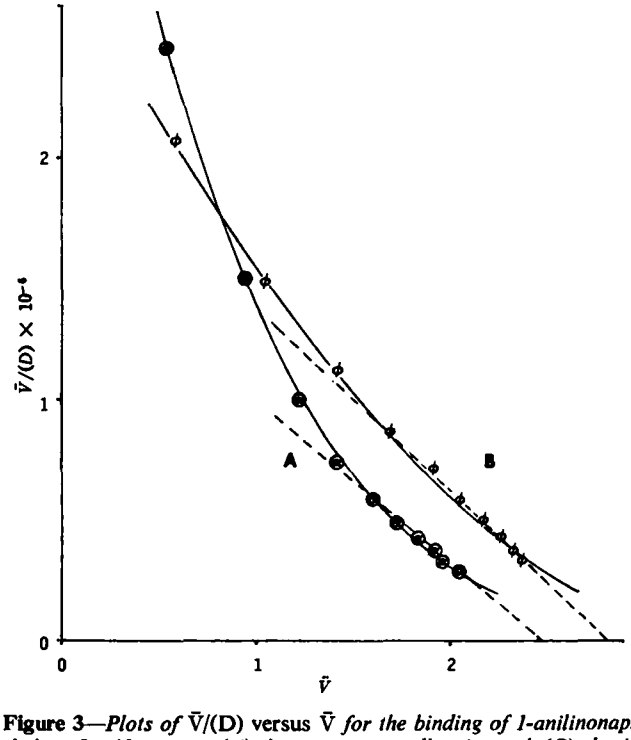

Figure 3—*Plots of* $\bar{V}$/(D) versus $\bar{V}$ *for the binding of 1-anilinonaphthalene-8-sulfonate to (A) human serum albumin and (B) bovine serum albumin.*

serum albumin. This suggests that the binding of one 1-anilinonaphthalene-8-sulfonate molecule to human serum albumin discourages other 1-anilinonaphthalene-8-sulfonate molecules from combining in a greater extent than the binding of 1-anilinonaphthalene-8-sulfonate to bovine serum albumin.

Equation 1 was suggested by Scatchard (3) for the special case when the intrinsic association constants are all equal. He also pointed out that, if the plot of $\bar{V}/(D)$ *versus* $\bar{V}$ does not give a straight line, it may be inferred that the intrinsic constants are not equal and/or there is interaction among the bound ions. The former effect is well demonstrated by the results of this study. For comparison, the plots of $\bar{V}/(D)$ *versus* $\bar{V}$ for both protein systems are shown in Fig. 3; these plots are not quite linear. If one were to discount the low values of $\bar{V}$, one could plot a straight line with a high significance factor. The n values for the human and bovine serum albumin systems obtained from the straight-line portion of the Scatchard plots are 2.5 and 2.8, respectively. When using Eq. 3, the results seem to be much more satisfactory. It is possible to detect the number of sites with greatest affinity as well as to detect dramatically the total number of binding sites over a range of small molecule concentrations.

Equation 2 is an empirical equation in which a parameter, m, is introduced into Eq. 1 to obtain a straight-line plot. The fact that m is varied by the protein concentration, buffer agent, and temperature (7) may indicate the effect of these factors on the binding. However, the physicochemical meaning of m is still not clear.

The determination of the binding parameters may also depend on the experimental methods used. The results obtained from the fluorescence technique are different from those of other methods, probably in the manner that only the primary (strong) binding sites are detected, whereas the results obtained from other methods such as dialysis (15, 16) often show larger numbers of binding sites. The curvature of the Scatchard plot has been interpreted (4) as the binding of the small molecule to more than one class of sites on the protein. However, it is possible that the intrinsic association constants of the binding of these sites are not equal even in the same class.

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Keyphrases □ Microorganisms—low temperature maintenance for use in antibiotic assays □ Antibiotic assays—low temperature maintenance of test organism suspensions □ Test organisms—low temperature maintenance for use in antibiotic assays

This laboratory maintains a number of test organism suspensions for daily use in turbidimetric and agar diffusion assays of antibiotics (1). Detailed procedures for the preparation of these suspensions are described in the *Code of Federal Regulations* (2). Because of the variety of antibiotics tested and the volume of samples to be assayed, preparing fresh suspensions on a weekly or biweekly basis is a time-consuming task attended by a number of laboratory problems. Ideally, the growth characteristics and specific antibiotic dose–response of any given suspension are reproducible if the suspension has been prepared according to the official method and stored under refrigeration at temperatures just above freezing. In actual practice, even the most exacting

serum albumin. This suggests that the binding of one 1-anilinonaphthalene-8-sulfonate molecule to human serum albumin discourages other 1-anilinonaphthalene-8-sulfonate molecules from combining in a greater extent than the binding of 1-anilinonaphthalene-8-sulfonate to bovine serum albumin.

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Table I—Plate Counts before and after Storage at −70°

Test Organism	Vehicle	Initial Count, 10^{10}/ml.	Final Count, 10^{10}/ml.	Percent Loss	Months
Staphylococcus aureus, resistant to novobiocin (ATCC 12692)	Saline	1.20	1.20	0	18
	Water	1.40	2.40	0	18
	Peptone water	1.40	2.00	0	18
Saccharomyces cerevisiae (ATCC 9763)	Saline	0.049	0.035	29	24
	Water	0.051	0.038	26	24
	Peptone water	0.041	0.034	17	24
Saccharomyces cerevisiae (ATCC 2601)	Saline	0.052	0.005	90	9
	Water	0.052	0.006	88	9
	Peptone water	0.040	0.009	78	9
Staphylococcus epidermidis (ATCC 12228)	Saline	0.42	0.37	12	24
	Water	0.38	0.36	5	24
	Peptone water	0.41	0.52	0	24
Staphylococcus aureus (ATCC 6538P)	Saline	1.50	1.20	20	15
	Water	1.60	1.30	19	12
	Peptone water	2.30	2.50	0	12
	Broth	1.40	1.00	29	15
Bordetella bronchiseptica (ATCC 4617)	Saline	7.90	0.70	91	9
	Water	7.70	1.80	77	15
	Broth	7.70	1.40	82	18
Sarcina lutea (ATCC 9341)	Saline	2.20	1.20	45	24
	Water	0.96	1.80	0	24
	Peptone water	1.50	1.50	0	24
Sarcina subflava (ATCC 7468)	Saline	0.94	0.98	0	24
	Water	1.00	1.20	0	24
	Peptone water	0.78	0.92	0	24
Pseudomonas pyocyanea (ATCC 23389)	Saline	4.40	0.70	84	3[a]
	Water	4.00	1.30	68	3[a]
	Peptone water	4.80	1.20	75	3[a]
Klebsiella pneumoniae (ATCC 10031)	Saline	2.40	0.28	88	3[a]
	Water	2.90	0.94	68	3[a]
	Peptone water	3.60	1.10	69	3[a]
Escherichia coli (ATCC 10536)	Saline	3.50	<0.001	100	3[a]
	Water	4.80	1.40	71	6[a]
	Peptone water	4.20	<0.001	100	3[a]
Sarcina lutea, resistant to erythromycin (ATCC 15957)	Saline	1.70	2.00	0	24
	Water	1.70	2.10	0	24
	Peptone water	1.80	2.10	0	24
Sarcina subflava, resistant to dihydrostreptomycin (ATCC 7468/d)	Saline	0.67	0.56	16	24
	Water	0.73	0.80	0	24
	Peptone water	0.66	0.66	0	24
Streptococcus faecalis (ATCC 10541)	Saline	0.68	0.34	50	9[a]
	Water	0.41	0.31	24	9
	Peptone water	0.43	0.31	28	9

[a] Discontinued after the time indicated because of loss of viable cells.

quality controls do not exclude occasional contamination or prevent batch-to-batch variations in the composition of dehydrated media and related problems. Preparing large quantities of test organism suspension for use over an extended period makes it possible to decrease the occurrence of such aberrations and has the further advantage that once an acceptable suspension has been prepared there is a continuity of reproducible assay response.

Conditions for the preservation and maintenance of test organism suspensions have been reported by numerous investigators. Sokolski *et al.* (3) reported the preparation of frozen suspensions for vitamin B_{12} assays. Complete recovery of viable cells was obtained when the suspensions in basal medium were rapidly frozen by direct immersion into liquid nitrogen and rapidly thawed by agitating in a water bath at 40°. McDaniel and Bailey (4) found that liquid nitrogen storage was the most satisfactory method for supplying standard *Streptomyces viridoflavus* inoculum for laboratory and pilot plant experimentation. There were no detectable changes in viability over 12 months when cotton-plugged ampuls were stored in the gas phase of a liquid nitrogen refrigerator. Tanguay (5) reported on the direct freezing of test organisms either in tryptose–saline containing 15% glycerol or in 0.067 *M* phosphate buffer, pH 7.0, containing 15% glycerol. When frozen and stored at −40°, these organisms maintained a satisfactory growth response from 6 months to 1 year or more. The main advantages claimed for this technique are that assays can be done at any time without the necessity of preparing daily inocula or maintaining the sensitivity of test organisms and that assay responses are standardized. Day-to-day fluctuations in growth responses and invalid assays due to inoculum failure are largely eliminated (5). These qualities are essential to the success of the certification program at the National Center for Antibiotic Analysis. This report describes the results of direct freezing of various test organisms used at the National Center.

EXPERIMENTAL

The 14 official antibiotic assay organisms listed in Table I were tested at 3-month intervals for periods ranging from 6 months to up

Table II—Dose-Response of Test Suspensions after Freezing and Storage at −70°

Test Organism	Vehicle	Antibiotic	Method[a]	Dose Response[b] Initial	Dose Response[b] Final	Months
Staphylococcus aureus, resistant to novobiocin (ATCC 12692)	Saline	Penicillin	AD	9.07	12.49	18
	Water		AD	9.24	10.30	18
	Peptone water		AD	9.20	11.63	18
Saccharomyces cerevisiae (ATCC 9763)	Saline	Amphotericin	AD	4.95	3.62	24
	Water		AD	4.65	4.05	24
	Peptone water		AD	5.12	4.62	24
Saccharomyces cerevisiae (ATCC 2601)	Saline	Nystatin	AD	6.15	7.97	9[c]
	Water		AD	6.58	7.51	9[c]
	Peptone water		AD	7.11	8.47	9[c]
Staphylococcus epidermidis (ATCC 12228)	Saline	Neomycin	AD	5.51	6.38	24
	Water		AD	3.65	7.57	24
	Peptone water		AD	3.32	5.98	24
Staphylococcus aureus (ATCC 6538P)	Saline	Penicillin	AD	9.53	7.71	15
	Water		AD	9.47	7.71	12
	Water	Tetracycline	T	0.511	0.436	18
	Peptone water		T	0.503	0.212	12[d]
	Broth	Penicillin	AD	8.04	8.47	15
	Broth	Tetracycline	T	0.531	0.402	18
Bordetella bronchiseptica (ATCC 4617)	Saline	Polymyxin	AD	3.72	4.32	6[c]
	Water		AD	3.26	5.22	15
	Broth		AD	3.39	3.82	18
Sarcina lutea (ATCC 9341)	Saline	Erythromycin	AD	6.98	8.24	24
	Water		AD	5.41	6.98	24
	Peptone water		AD	6.15	7.77	24
Sarcina subflava (ATCC 7468)	Saline	Bacitracin	AD	4.88	5.18	24
	Water		AD	5.61	5.68	24
	Peptone water		AD	5.95	5.91	24
Pseudomonas pyocyanea (ATCC 23389)	Saline	Carbenicillin	AD	7.97	9.97	3[c]
	Water		AD	8.37	8.84	3[c]
	Peptone water		AD	8.80	8.97	3[c]
Klebsiella pneumoniae (ATCC 10031)	Saline	Dihydrostreptomycin	T	1.74	No growth	3[c]
	Water		T	1.74	No growth	3[c]
	Peptone water		T	1.41	No growth	3[c]
Escherichia coli (ATCC 10536)	Saline	Chloramphenicol	T	0.949	0.392	3[d]
	Water		T	0.944	0.593	6[d]
	Peptone water		T	0.867	0.614	3[d]
Sarcina lutea, resistant to erythromycin (ATCC 15957)	Saline	Penicillin	AD	14.28	15.02	24
	Water		AD	14.75	14.88	24
	Peptone water		AD	14.75	13.45	24
Sarcina subflava, resistant to dihydrostreptomycin (ATCC 7468/d)	Saline	Bacitracin	AD	4.12	5.18	24
	Water		AD	4.92	5.18	24
	Peptone water		AD	4.65	5.45	24
Streptococcus faecalis (ATCC 10541)	Saline	Gramicidin	T	0.423	0.307	9[d]
	Water		T	0.507	0.365	9[d]
	Peptone water		T	0.549	0.236	9[d]

[a] AD = agar diffusion assay, and T = turbidimetric assay. [b] Defined in terms of slope where b = slope = range over 1 log cycle (expressed in millimeters of zone diameter for agar diffusion assay and absorbance values for turbidimetric assay). [c] Discontinued after the time indicated because of loss of viable cells. [d] Discontinued after the time indicated because of significant change in dose-response.

to 2 years. Some tests were terminated earlier than 2 years, *e.g.*, at 6, 12, or 15 months, depending on the number of viable cells present and/or the dose-response at that time. The suspending agents and their compositions were: (*a*) sterile normal saline, (*b*) sterile distilled water, (*c*) sterile 0.5% peptone water, and (*d*) nutrient broth (Medium 3) (2).

The test organisms were maintained on agar slants containing 10 ml. of appropriate medium, and the suspensions were prepared as described in the *Code of Federal Regulations* (2). Growth was washed from the agar surface of the Roux bottles with 50 ml. of the appropriate suspending agent. Washings from three Roux bottles were pooled for each diluent to give a total of 150 ml. of suspension. From each pooled suspension, 12-ml. portions were placed in 30-ml. polycarbonate centrifuge tubes [1.27-cm. (0.5-in.) mouth] and closed with polypropylene screw caps. The tubes of suspension were quick frozen by a mixture of equal parts of dry ice and 95% ethanol (6) and then placed in a low temperature freezer (one capable of temperature to −90°) at −70°.

Viable cell counts and dose-responses (7) to the appropriate antibiotics were determined prior to freezing and at 3-month intervals up to 2 years. At each interval, a tube of frozen suspension was thawed by placing in a refrigerator for 2 hr. Each thawed suspension was tested initially upon thawing and then was stored for 2 weeks under refrigeration and tested each week for viable cell count and dose-response. The percent inoculum varied with the organism being tested; the incubation time also varied, depending on whether the agar diffusion or turbidimetric assay method was used to determine the dose-response to a particular antibiotic.

RESULTS AND DISCUSSION

As shown in Table I, most test organisms can be maintained satisfactorily for up to 2 years without any significant loss in viability and, as shown in Table II, such a procedure maintains an acceptable dose-response to the various antibiotics. Outstanding success was obtained with *Staphylococcus aureus* (resistant to novobiocin), *Sarcina subflava*, and *Sarcina lutea* (resistant to erythromycin) with no loss of viable cells in saline, water, or peptone water (Table I). In these instances, peptone water was selected as the vehicle of choice because of the stability of most organisms under refrigeration after they are thawed. Table I also shows that, of the organisms tested, the least success was obtained with *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas pyocyanea;* tests were discontinued early in the study because of losses in viable cells ranging from 70 to 100% after only 3 months.

Table III—Recommended Procedures for Storage at −70°

Test Organism	Suspending Vehicle	Maximum Storage Time, Months Frozen	Stability (Weeks Unfrozen) under Refrigeration
Staphylococcus aureus, resistant to novobiocin (ATCC 12692)	Peptone water	12	1
Saccharomyces cerevisiae (ATCC 9763)	Peptone water	12	2
Saccharomyces cerevisiae (ATCC 2601)	—	Not recommended	4
Staphylococcus epidermidis (ATCC 12228)	Peptone water	12	1
Staphylococcus aureus (ATCC 6538P)	Broth	12–18 (Agar diffusion assay) 6 (Turbidimetric assay)	1 1
Bordetella bronchiseptica (ATCC 4617)	Water	6	1
Sarcina lutea (ATCC 9341)	Peptone water	12	2
Sarcina subflava (ATCC 7468)	Peptone water	12	2
Pseudomonas pyocyanea (ATCC 23389)	—	Not recommended	2
Klebsiella pneumoniae (ATCC 10031)	—	Not recommended	1
Escherichia coli (ATCC 10536)	—	Not recommended	2
Sarcina lutea, resistant to erythromycin (ATCC 15957)	Peptone water	12	2
Sarcina subflava, resistant to dihydrostreptomycin (ATCC 7468/d)	Peptone water	12	2
Streptococcus faecalis (ATCC 10541)	—	Not recommended	24 hr.

Table III indicates that the recommended maximum storage time for most tested organisms is 1 year in peptone water. The 1-year time limit was selected to allow for some margin of error, even though excellent results were obtained for up to 2 years for most organisms.

In conclusion, most tested organisms can be frozen for 6 months to 2 years in water, peptone water, or broth with little or no loss in viable cells or change in dose-response. The method is simple and requires no elaborate equipment other than a low temperature freezer capable of maintaining temperatures of −70°. Frozen suspensions, although not specifically mentioned in the *Code of Federal Regulations*, are implicit in the directions to maintain the suspensions under "refrigeration." The findings show that subfreezing refrigeration temperatures offer a suitable and reliable method for maintaining suspensions of antibiotic test organisms.

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Cannabis sativa L. (Marijuana) IV: Chemical Basis for Increased Potency Related to Novel Method of Preparation

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Abstract □ Recent reports from the drug subculture in this country indicate that the simultaneous use of marijuana teas together with smoking previously boiled marijuana plant material results in more profoundly experienced psychotropic effects. It was found that the boiling water treatment of marijuana removes water-soluble materials equivalent to 30% of the weight of the plant material, thus leading to marijuana correspondingly enriched in cannabinoids, including (−)-*trans*-Δ⁹-tetrahydrocannabinol, one of the major psychoactive compounds present in the plant. This finding explains, in part, the reputed increased pharmacological effects resulting from this newly described method of marijuana use. Moreover, the potential dangers inherent in the described method are discussed.

Keyphrases □ *Cannabis sativa*—new method of street use, chemical basis of increased potency, GLC □ Marijuana—new method of street use, chemical basis of increased potency, GLC □ Cannabinoids—stability following boiling water treatment, related to new street use to increase potency, GLC

Various preparations derived from the plant *Cannabis sativa* L. have long been used for their intoxicating properties (1). In the United States, the plant is usually employed as a euphoriant by means of smoking cigarettes or pipes containing marijuana[1] or marijuana admixed with other substances (2). It is generally accepted that (−)-*trans*-Δ⁹-tetrahydrocannabinol (I) is the major psychoactive constituent of marijuana (3). Marijuana is considered to be of high potency when the content of I is approximately 1% (w/w) (4). This report concerns a novel method of marijuana preparation and use among certain fringe groups of the drug subculture in this country[2].

The marijuana is first slurried with sufficient water to cover the plant material, and this mixture is subsequently boiled in a suitable container for 1 to several hours. Additional portions of fresh water are sometimes added periodically to maintain the level of the liquid and thus prevent possible charring of the marijuana. Upon termination of the boiling process, the hot mixture is allowed to cool to room temperature and is then strained through several layers of cheesecloth.

The resulting filtrate ("marijuana tea") is reserved and stored in a refrigerator because it has been found that the tea is prone to mold growth. The marijuana remaining on the filter is removed, manually expressed free of excess liquid, which is added to the reserved tea, and finally spread out and allowed to air dry. In some reported cases, the damp marijuana is dried using an ordinary baking oven set at moderate heat. The resulting dried marijuana plant material is subsequently formed into cigarettes in the usual manner, with no adjustment being made in the approximate weight of plant material used to prepare individual cigarettes. These cigarettes are smoked normally except for the following major variation. Just prior to smoking, the reserved marijuana tea is consumed at once. Those persons employing marijuana in this fashion claim that the subsequent effects of the drug are perceived to be significantly more profound, both in terms of intensity and duration, than are the effects experienced by smoking marijuana prepared by more conventional means.

The results of studies designed to assess the possible chemical consequences from this previously unreported utilization of marijuana are reported.

[1] The term marijuana as used here refers to the crushed leaves, stems, and flowering tops of *C. sativa*.

[2] It is presently not possible to determine accurately the extent of this practice. However, essentially similar practices have been related by particularly reliable sources from various areas of the eastern United states, including Massachusetts, Pennsylvania, New Jersey, and Delaware.

Table I—Effect of Prolonged Boiling Water Treatment on the I Content of Marijuana

Marijuana Material	I, % Calculated	I, % Found[a]
Untreated marijuana	1.86[b]	1.90
Boiling water-treated marijuana	2.65[c]	2.56

[a] The GLC peaks identified with I were further confirmed in separate experiments using combined GLC–mass spectrometry according to procedures described earlier (9). [b] From the supplier's assay results. [c] Calculated by assuming that a total of 1.86 g. of I remained unchanged in the 70 g. of dried, boiling water-treated marijuana derived from 100 g. of starting untreated marijuana material, as described under *Preparation of Boiling Water-Treated Marijuana and Marijuana Tea.*

EXPERIMENTAL

Plant Material and Preparation of Standard Solutions—Marijuana plant material and all authentic cannabinoids[3] were obtained from the National Institute of Mental Health. The coarsely milled and air-dried marijuana represented female plant material, designated as Campus Mexican Female, which had been grown for 14 weeks and subsequently harvested at the University of Mississippi during the 1971 season. The GLC tracings and other data accompanying this material indicated the following cannabinoid content: I, 1.86% [84% as the acid (V), which acid (A or B) not specified]; II, 0.03%; III, 0.19%; and IV, not quantitatable. Separate standard solutions of each authentic cannabinoid were prepared in 95% ethanol to furnish a final concentration of 0.5 mcg./μl. in each case. These solutions were used to obtain the standard tracings for the GLC analyses and were stored at −5° in the dark prior to use.

GLC—All analyses were carried out on a modified gas chromatograph[4] equipped with a hydrogen flame-ionization detector and a 6-mm. o.d. × 2-mm. i.d. × 1.83-m. glass column packed with 80–100 mesh Supelcoport, AW, DMCS coated with 3% OV-17[5]. The instrument was modified such that the exit end of the column led directly to the base of the flame tip while the other end of the column

[3] (−)-*trans*-Δ⁹-Tetrahydrocannabinol (I), (−)-*trans*-Δ⁸-tetrahydrocannabinol (II), (−)-*trans*-cannabidiol (III), and (−)-cannabinol (IV).

[4] Gow-Mac 69-750 FID, Gow-Mac Instrument Co., Madison, N. J.

[5] Supelco Inc., Supelco Park, Bellefonte, Pa.

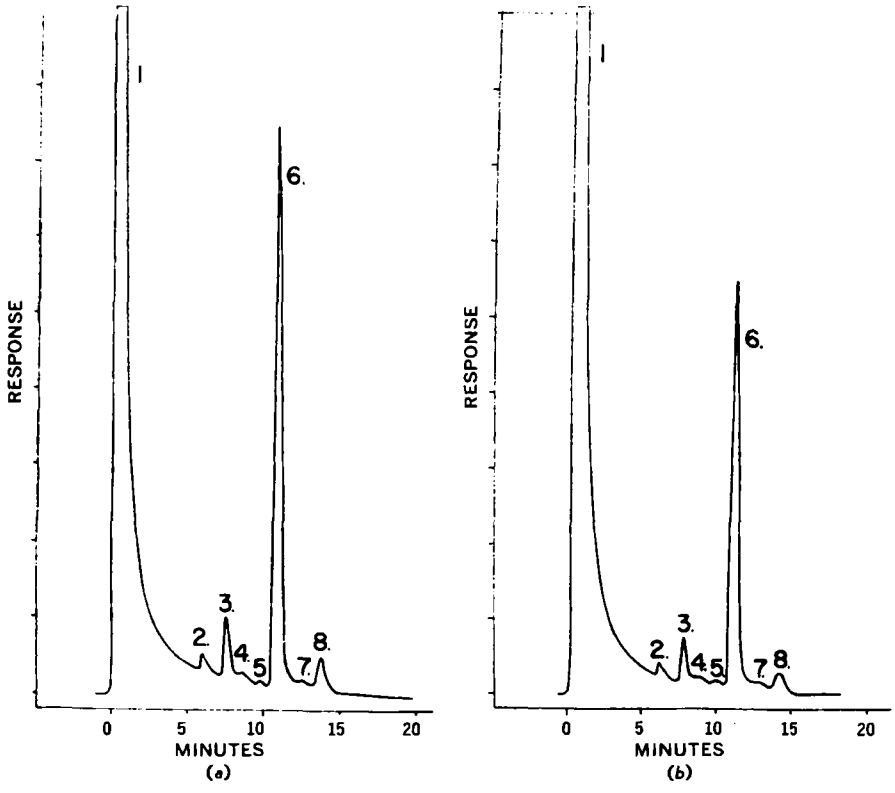

Figure 1—*Gas–liquid chromatograms of marijuana extracts (see text for method of preparation).* (a) *Extract A (boiling water-treated marijuana).* (b) *Extract B (untreated marijuana). Comparison of* a *and* b *reveals only quantitative differences. The analogous peaks (boiling water-treated marijuana* versus *untreated marijuana) are in the approximate ratio of 1.4:1, thus showing enrichment of the cannabinoids in the boiling water-treated marijuana. Key: 1, solvent; 2, unknown; 3,* (—)-trans-*cannabidiol; 4, unknown; 5,* (−)-trans-Δ^8-*tetrahydrocannabinol; 6,* (−)-trans-Δ^9-*tetrahydrocannabinol; 7, unknown; and 8,* (−)-*cannabinol.*

extended nearly to the injector port septum, thus resulting in an essentially all-glass system and allowing for on-column injection of the samples. The injector port and detector block temperatures were maintained at 263° while the column was operated isothermally at 203°. Attenuation was 7×10^{-11} amperes full scale for all analyses. Helium was used as the carrier gas at a flow rate of 10 ml./min.; hydrogen and dry air flow rates were 20 and 250 ml./min., respectively.

A strip chart recorder[6] with a chart speed of 0.508 cm./min. was employed. The peak areas recorded for all analyzed samples were calculated by the triangulation method (area = height × width at half-height) and compared with standard curves prepared by plotting the areas against known concentrations of standards analyzed in a similar manner. The results were found to be linear over the concentration ranges used. In those cases requiring derivatization (silylation), 0.5 ml. of the particular sample was evaporated using flowing nitrogen. The resulting dry residue (in a Teflon-lined screw-capped vial)[7] was treated with 0.5 ml. of a silylating reagent[8] and warmed (50°) for 3 min. It was found that derivatization was quantitative under these conditions, and subsequently an aliquot (usually 0.5–1.5 μl.) of the reaction mixture could be injected into the column.

Preparation of Boiling Water-Treated Marijuana and Marijuana Tea—A total of 100 g. of marijuana previously slurried with 1500 ml. of distilled water was continuously refluxed for 5 hr. using a Clevenger apparatus (for oils lighter than water) (5). No precautions were taken to exclude air or light from the boiling mixture. Following refluxing, the slurry less the volatile oil (0.2 ml.) was cooled to room temperature and suction filtered. The volume of the dark-brown filtrate was approximately 1300 ml. Finally, the plant material remaining on the filter was alternately washed and expressed, using small portions of hot water until the last wash filtrates were nearly colorless. These washings were combined with the initial filtrate until the total volume was adjusted to 1500 ml. This solution (marijuana tea) was frozen and lyophilized to give 30 g. of a dark-brown residue, which was stored under nitrogen at 4° for future chemical and pharmacological study. The remaining damp marijuana was allowed to air dry at ambient (23–25°) temperature for 5 days. This air-dried material was found to weigh 70 g. and was designated as boiling water-treated marijuana.

GLC of Boiling Water-Treated Marijuana and Untreated Marijuana—Fifty grams of boiling water-treated marijuana was percolated at a slow rate, using a total of 2000 ml. of 95% ethanol. The combined percolates were evaporated *in vacuo* to give a green syrup (about 25 ml.), which was brought to a volume of exactly 50 ml. with 95% ethanol. An aliquot (0.5 ml.) of this ethanolic solution was diluted using 95% ethanol to furnish 10 ml. of Extract A. Similarly, 50 g. of untreated marijuana was processed to furnish 10 ml. of Extract B. Aliquots (0.5–1.5 μl.) of Extracts A and B were analyzed by GLC to determine the cannabinoid profiles of the boiling water-treated marijuana and the untreated marijuana, respectively (Figs. 1*a* and 1*b*).

RESULTS AND DISCUSSION

It was found that 30% of the dry weight of the marijuana studied was removed by treatment with boiling water under conditions simulating those employed by certain members of the drug subculture. Thus, the loss of substantial quantities of water-soluble substances leads to marijuana (*viz.*, boiling water-treated marijuana) enriched in water-insoluble components, including cannabinoids such as I, a major psychoactive component of marijuana (Figs. 1*a* and 1*b*). For example, the boiling water-treated marijuana contained 1.4 times more I than was found in the untreated marijuana (Table I).

Under the conditions of these experiments, V was quantitatively decarboxylated to furnish the psychoactive I (Figs. 2*a* and 2*b*). This was not unexpected since it had earlier been pointed out that the cannabinoid acids rapidly decarboxylate at temperatures approaching 103° (6). What was surprising, but not totally unexpected, was that the cannabinoids present in the original untreated marijuana were stable when subjected to the relatively drastic conditions of prolonged boiling. Indeed, when the GLC analyses of the boiling water-treated marijuana and the untreated marijuana are compared (Figs. 1*a* and 1*b*), it is clear that the differences noted are quantitative and not qualitative. It was recently shown (7, 8) that certain cannabinoids are especially stable when they are present in crude preparations derived from *C. sativa*. In these cases, the complex

[6] Model MP-1027/1 mv., having a full-scale span of 1 mv.; McKee-Pedersen Instruments, Danville, Calif.
[7] Reacti-vial, Pierce Chemical Co., Rockford, Ill.
[8] TRISIL/BSA, *N,O*-bis(trimethylsilyl)acetamide in pyridine, Pierce Chemical Co., Rockford, Ill.

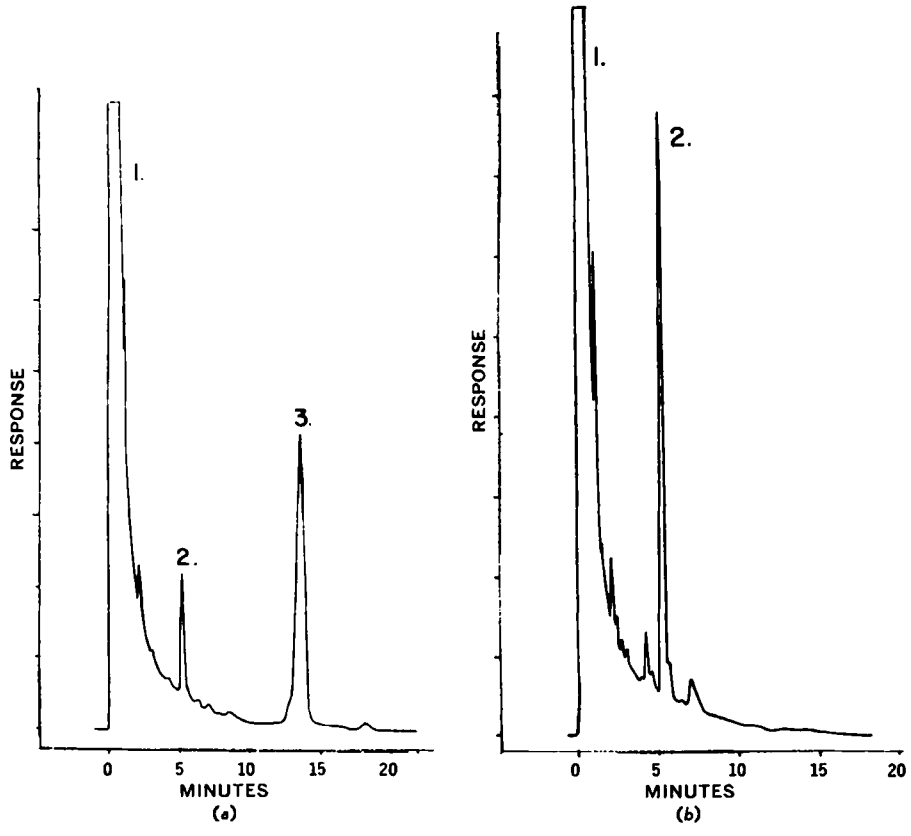

Figure 2—*Gas–liquid chromatograms of silylated marijuana extracts (see text for method of preparation). (a) Extract B (untreated marijuana) showing total (−)-trans-Δ⁹-tetrahydrocannabinol content was 84% (−)-trans-Δ⁹-tetrahydrocannabinol acid and 16% (−)-trans-Δ⁹-tetrahydrocannabinol. (b) Extract A (boiling water-treated marijuana). The absence of the (−)-trans-Δ⁹-tetrahydrocannabinol acid – trimethylsilyl derivative peak and the presence of the (−)-trans-Δ⁹-tetrahydrocannabinol–trimethylsilyl derivative peak indicate that the (−)-trans-Δ⁹-tetrahydrocannabinol acid was quantitatively decarboxylated to furnish (−)-trans-Δ⁹-tetrahydrocannabinol as a result of the boiling water treatment. Key: 1, solvent; 2, (−)-trans-Δ⁹-tetrahydrocannabinol–trimethylsilyl derivative; and 3, (−) - trans - Δ⁹ - tetrahydrocannabinol acid–trimethylsilyl derivative.*

mixture of phytoconstituents apparently retards the decomposition of the cannabinoids. This phenomenon may also explain the stability of the cannabinoids remaining in the boiled crude marijuana plant material[9].

Hence, the reputed claims made for the increased biological potency of boiling water-treated marijuana appear to be related, at least in part, to increased concentrations of presently known psychoactive components. Obviously, individuals who smoke the same weight of this material as the untreated marijuana (as indeed they do, as mentioned earlier) would experience increased drug effects.

The biological activity of the aqueous extract (marijuana tea) taken concomitantly with the boiling water-treated marijuana has not yet been fully evaluated. Both chemical and pharmacological studies of the marijuana tea are currently underway in these laboratories and results will be reported subsequently.

Nevertheless, immediate attention is called to the possible serious consequences that may result from the oral self-administration of marijuana teas for the following reasons. Since drugs are commonly misrepresented in the illicit market, it is not unusual to find contraband marijuana admixed with various noncannabis substances (2). Consider, for example, a street sample of marijuana containing equal amounts of tobacco and marijuana. In this particular case, a tea prepared from this material would probably contain relatively large amounts of the poisonous alkaloid nicotine; under normal smoking conditions most of the nicotine content of tobacco is destroyed. Similarly, analysis of confiscated marijuana (10) showed it to be impregnated with significant amounts of lysergic acid diethylamide (VI). Although VI is not biologically active when smoked, dangerous effects could result from ingesting teas prepared from VI-containing marijuana. Moreover, it may not be unreasonable to assume that marijuana may be adulterated with other drugs which are not biologically active when smoked but which may induce a variety of undesirable physiological effects when ingested in teas.

[9] Moreover, in separate experiments, the boiling water-treated marijuana was shown to be biologically active (dog ataxia test) according to procedures described elsewhere (7).

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Identification of Barbiturates in Urine

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Abstract □ A TLC method was developed which employs a resin column for the extraction and identification of barbiturates in urine. The method is sensitive to less than 1 mcg./ml. for all of the barbiturates tested. Certain tranquilizers and sedatives appear on the chromatogram with the barbiturates, but they do not interfere with barbiturate detection. The efficacy of this method was demonstrated using urine samples obtained from both inpatients and outpatients under supervised conditions.

Keyphrases □ Barbiturates—TLC method of identification in urine, designed for drug abuse screening programs, elimination of interference from tranquilizers and sedatives □ Drug abuse screening programs—TLC method of identifying barbiturates in urine □ TLC—method of identifying barbiturates in urine, application to drug abuse screening programs

In the general area of drug abuse, much recent effort has been concentrated on the detection and treatment of narcotic addiction. However, probably due to the high price of heroin, there has been a steady increase in barbiturate abuse. For this reason, urine surveillance that includes the detection of barbiturates is important. Because of shortcomings in present methods, this laboratory has investigated and developed a new procedure for identifying barbiturates in urine.

In the last year, many different TLC procedures, solvent systems, and sprays were studied in this laboratory to detect and separate barbiturate metabolites in urine from the metabolites of other drugs such as chloral hydrate, tybamate, methyprylon, and glutethimide. These drugs, when given to drug abuse patients as part of their daily medications, were found to interfere consistently with the detection of barbiturates by existing methods. Several TLC methods were reported (1–5) but it was difficult for the present authors to distinguish barbiturates from certain sedatives and tranquilizers when following these reported procedures. Similar interference was noted using the ion-exchange paper methods of Dole *et al.* (6) and Kaistha and Jaffe (7). GLC (8), spectrophotometry (9, 10), and radioimmunoassay (11) are more sensitive than TLC for barbiturates. However, these methods indicate only one class of drugs at a time and are suitable mainly for confirmatory tests—not for routine urine surveillance performed in an average laboratory.

To avoid false-positive and false-negative results during urine surveillance of drug abuse patients who might be taking one or more interfering drugs, a reliable but simple method of identifying barbiturates was needed. This paper describes the procedure developed for detecting barbiturates in the presence of other drugs.

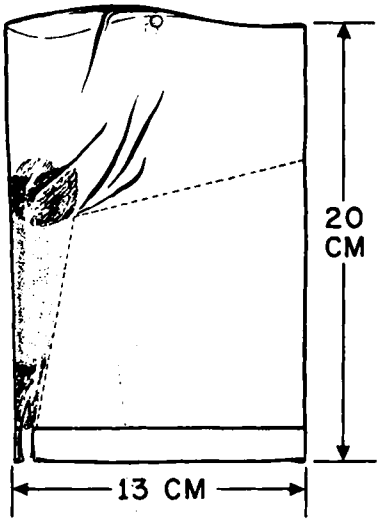

Figure 1—*A polyethylene bag is prepared and labeled for each sample. Urine (50 ml.) is poured into the upper channel of the bag, flows through the resin column, and drains out through the glass wool stopper at the bottom of the column.*

EXPERIMENTAL

Materials—Drugs were extracted from the urine using a resin column[1]. The resin is a styrene divinylbenzene copolymer which has a nonionic, macroreticular structure with high surface area capable of adsorbing many water-soluble organic compounds (12). The resin was first rinsed with 5% sodium chloride and 1% sodium bicarbonate to control bacteria and mold growth during storage. Several bed volumes of distilled water were washed over the surface of the resin until the decant became clear (13).

Columns were fabricated by heat sealing a 1.5 × 10-cm. channel on one side of a 12 × 20-cm. polyethylene bag and plugging both ends of the channel with glass wool stoppers. The columns were filled to a height of 7 cm. by gravity packing an aqueous slurry of the resin into the channel (Fig. 1). The colored portion of the eluate collected from the column was concentrated and spotted on silica gel-impregnated microfiber sheets[2].

All chemicals were of reagent grade. The solvent system consisted of chloroform–acetic acid (50:0.1) prepared fresh daily. One of the colored sprays used was diphenylcarbazone, prepared as a 0.1% solution in 95% ethanol. This reagent was stable for 2 or 3 weeks but should be refrigerated in an amber bottle. The second spray, prepared by dissolving 20 g. mercurous nitrate in 500 ml. of 0.15 *M* nitric acid, was also stable for several weeks.

Procedure—Fifty milliliters of urine was poured through the resin column. The column was compressed to force all of the urine to pass through it, and then the compounds adsorbed by the resin were eluted with 15 ml. of methanol. The colored portion of the eluate was collected, and 10 ml. of this eluate was concentrated to about 1 ml. Forty microliters of the concentrated eluate was spotted on precoated thin-layer sheets[2] and developed in a chloroform–acetic acid solvent system for 10 min. The chromatograms were air dried and sprayed with diphenylcarbazone until a pink color became visible. The dried chromatograms were then sprayed with mercurous nitrate solution, and a blue color developed. Within 5 min., white spots appeared at approximate R_f 0.7, indicating the presence of a barbiturate.

Since R_f values may vary slightly, at least one known barbiturate urine standard must be spotted on each chromatogram. Phenobarbital produced a V-shaped spot which may be distinguished from the rectangular-shaped spot shown by the other barbiturates. The appearance and position of the white spots should be recorded immediately since they fade to a blue color, indistinguishable from

[1] Amberlite XAD-2 resin, Rohm & Haas Co., Philadelphia, PA 19105

[2] Gelman Instrument Co., Ann Arbor, MI 48106

Table I—Color Imprint and R_f Value of Drugs on Chromatogram

Generic Name	White Spot, R_f 0.7	Blue Spot, R_f 0.7	Faint Blue Spot, R_f 0.7 (or Lower)
Amobarbital	+	+	−
Pentobarbital	+	+	−
Phenobarbital	+	+	−
Secobarbital	+	+	−
Chloral hydrate	−	+	−
Chlordiazepoxide	−	−	+
Diazepam	−	−	+
Ethchlorvynol	−	−	+
Glutethimide	−	−	+
Meprobamate	−	−	+
Methyprylon	−	+	−
Tybamate	−	+	−

the background, within 15 min. Following the disappearance of the white spot, the blue background began to fade, revealing a blue spot with the same or slightly lower R_f value than the white barbiturate spot. This blue spot was characteristic of barbiturates and certain other drugs.

RESULTS AND DISCUSSION

The reliability, specificity, and sensitivity of this method were tested using urine samples from both inpatients and outpatients[3].

Reliability—To test the reliability of this method, 550 urine samples from drug abuse patients were analyzed for barbiturates and other drugs. Forty-one of these patients were taking prescribed barbiturates, and the white spot on the chromatogram revealed the presence of barbiturate in the urine of each of these. Barbiturate was also found in the urine of 15 additional patients. Since some patients admitted taking illicit barbiturates, a maximum false-positive error of <2.7% was indicated.

Specificity—Table I summarizes the color imprint and R_f value of the drugs which appeared on a chromatogram used in the present barbiturate identification system. Figure 2 illustrates the appearance of some of these drugs on the chromatogram immediately after spraying with mercurous nitrate solution and later after the background had faded. The white spot at R_f 0.7 was specific for the presence of barbiturates, and no drug listed in Table I interfered with barbiturate detection. The appearance of the blue spot at R_f 0.7 after the background faded indicated the presence of barbiturates and other drugs listed in Table I. Because of the interference of these other drugs, the blue spot obtained by using diphenylcarbazone and mercurous nitrate sprays cannot be considered as evidence specific for either barbiturates or any one of the drugs listed in Table I. However, the presence of chloral hydrate, tybamate, or methyprylon can be verified, since these drugs showed characteristic R_f values in the previously published system for the detection of opiates (12).

Sensitivity—The sensitivity of this method was determined by measuring the barbiturate concentration in the column eluate, using a modification of Broughton's procedure (14). Fifty milliliters of known barbiturate urine was poured through the resin column and eluted with 15 ml. methanol. Before concentrating 10 ml. of the colored portion of the eluate to dryness, the eluate was treated with decolorizing charcoal, which removed the pigment. Reference standards of 0.45 *N* sodium hydroxide and a combination of 0.45 *N* sodium hydroxide and boric acid solution were prepared. Each standard was compared to a solution containing 1 ml. of the standard plus 1 ml. of the decolorized eluate redissolved in 0.45 *N* sodium hydroxide. Barbiturate concentration was determined by measuring the difference in absorbance at 260 nm. The difference in the absorbance of the two test solutions when compared with the absorbance of the two standards at the same wavelength indicated the amount of barbiturate present in the urine.

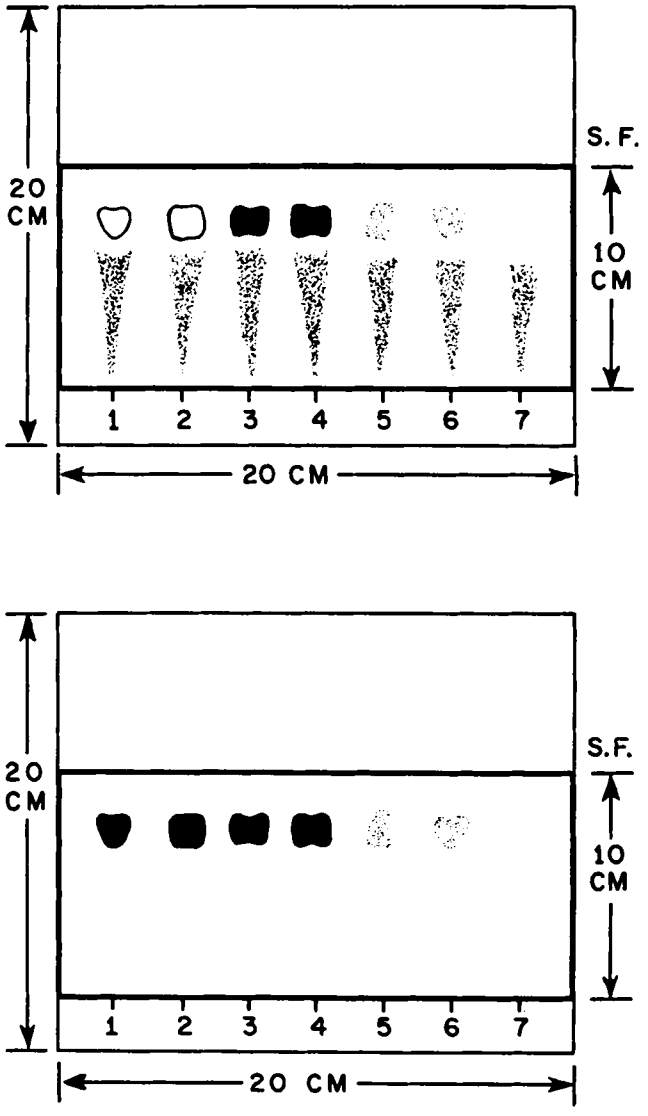

Figure 2—*(Top) Appearance of drugs on chromatogram immediately after mercurous nitrate spray. Key: 1, phenobarbital; 2, other barbiturates; 3, chloral hydrate, tybamate, and methyprylon; 4, ethinamate; 5, chlordiazepoxide, diazepam, ethchlorvynol, and glutethimide; 6, meprobamate; and 7, drug-free urine. (Bottom) Appearance of drugs on chromatogram after background has faded.*

This procedure was followed with separate urine samples containing pentobarbital, phenobarbital, and secobarbital. After the amount of barbiturate in each of the three urine samples was determined, each urine sample was diluted into smaller aliquots with normal urine to decrease the barbiturate concentration in the urine. Each diluted urine sample was assayed through the chromatographic method. The last diluted urine sample that revealed a white spot on the chromatogram represented the degree of sensitivity of the method for the detection of that barbiturate. The minimum sensitivity of the method was 0.7 mcg./ml. for patients receiving secobarbital, 0.4 mcg./ml. for patients receiving pentobarbital, and 0.02 mcg./ml. for patients receiving phenobarbital. It was also noted that barbiturate could be detected in the urine of drug abuse patients for at least 36 hr. after ingestion of 100 mg. of barbiturate. Longer periods were not checked due to the faintness of the white spots after 36 hr.

Confirmation—The identity of the white spot as barbiturate was confirmed using ^{14}C-labeled phenobarbital. A 48-hr. urine sample was collected from a rat given one injection of phenobarbital (25 mg./kg.) containing 2 mcg. of ^{14}C-phenobarbital labeled in the 2-

[3] At the Drug Abuse Treatment and Rehabilitation wards and clinics, Wood Veterans Administration Center. There were approximately 30 drug abuse patients in the wards and about 80 outpatients at any given time. Periodically, the staff also participated in studies to provide control and standard barbiturate urine samples.

position. The urine was processed by the described method, and the developed chromatogram was placed on X-ray film for 48 hr. The resulting radioactive spot on the X-ray film had the same shape and position as the white spot on the chromatogram.

CONCLUSIONS

Urine surveillance is an integral part of every successful drug abuse treatment and rehabilitation program. For urine surveillance to be effective, samples must be collected, labeled, and transported under controlled conditions. Urine must be analyzed accurately, and results must be reported promptly. A reliable laboratory method, specific for abuse drugs, is required to avoid both false-positive results, which would damage a patient's confidence and morale, and false-negative data, which could permit the undetected use of abuse drugs to continue.The laboratory method must also be inexpensive and efficient enough to evaluate a high volume of samples rapidly on a continuing basis.

The barbiturate identification procedure developed by the present investigators satisfies these requirements. It is particularly significant that interference from sedatives and tranquilizers has been eliminated. The method described in this paper avoids false-positive results and provides reliable identification of barbiturate metabolites in the urine of patients taking various drugs.

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Precursor-Type Insect Repellents: Kinetics of Hydrolysis

DANIEL D. GARNER and LORRIN R. GARSON▲

Abstract □ The rates of ester hydrolysis of representative dihydroxyacetone monoesters were determined by titrimetric analysis. The esters were designed to elicit prolonged insect repellency by gradual hydrolysis in dermal tissue. No correlation was found between the agents' repellent activity and their susceptibility to hydrolysis. The repellency elicited by the esters appears to be due to the intact molecules rather than a hydrolytic product.

Keyphrases □ Dihydroxyacetone monoesters—rates of hydrolysis, effect on insect repellency □ Insect repellents, dihydroxyacetone monoesters—rates of hydrolysis, compared to repellent activity □ Hydrolysis kinetics—dihydroxyacetone monoesters, attempted correlation of hydrolysis rates and repellent activity

In previous articles from these laboratories, the syntheses and insect repellent data of 1,3-dihydroxy-2-propanone (dihydroxyacetone) monoesters were reported (1–4). The activity of these precursor-type repellents was explained, in part, by their ability to hydrolyze, subsequent to topical application, and thereby release the insectifugal acids. The rationale of this approach was previously discussed in detail (2, 5). Compounds I, III, IV, VI, XI, and XIII (Table I) were studied because of their similarities in structure and physical properties and also because of their wide range in repellent activity. Due to the many physical parameters which reportedly play a role in the insect repellency of a compound (6), the objective was to ascertain if the hydrolysis of the dihydroxyacetone esters was responsible for their activity or if some other factors were involved.

EXPERIMENTAL

Materials—Compounds I, III, IV, VI, XI, and XIII were previously prepared in these labotatories (1–4). However, since these agents can exist in both monomer and dimer (a cyclic hemiketal) forms, the structure and purity of these moieties had to be confirmed. Heating the dimeric dihydroxyacetone monoesters in ethanol converts the dimer to the corresponding monomeric form (7). Solutions of the esters in either acetone or ethanol (the ethanol solution had been previously heated) elicited identical chromatographic behavior, that is, a single spot with the same R_f values[1]. Therefore, in an acetone–water (3:1) solution, the medium selected to conduct the hydrolysis studies, the esters would appear to be exclusively in the monomeric state. IR spectra of the samples were equivalent to those of analytically pure compounds; NMR spectra were consistent with their respective structures[2].

[1] The TLC procedure utilized Mallinckrodt SilicAR TLC-7GF as the sorbent and acetone–water (3:1) as the solvent system. The compounds were applied to the plates and, after development, the spots were visualized with iodine vapor.

[2] Spectra (IR and NMR) were obtained with the Beckman model IR-33 and the Hitachi Perkin-Elmer model R-24 spectrophotometers, respectively.

position. The urine was processed by the described method, and the developed chromatogram was placed on X-ray film for 48 hr. The resulting radioactive spot on the X-ray film had the same shape and position as the white spot on the chromatogram.

CONCLUSIONS

Urine surveillance is an integral part of every successful drug abuse treatment and rehabilitation program. For urine surveillance to be effective, samples must be collected, labeled, and transported under controlled conditions. Urine must be analyzed accurately, and results must be reported promptly. A reliable laboratory method, specific for abuse drugs, is required to avoid both false-positive results, which would damage a patient's confidence and morale, and false-negative data, which could permit the undetected use of abuse drugs to continue.The laboratory method must also be inexpensive and efficient enough to evaluate a high volume of samples rapidly on a continuing basis.

The barbiturate identification procedure developed by the present investigators satisfies these requirements. It is particularly significant that interference from sedatives and tranquilizers has been eliminated. The method described in this paper avoids false-positive results and provides reliable identification of barbiturate metabolites in the urine of patients taking various drugs.

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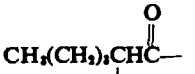
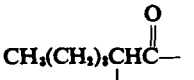
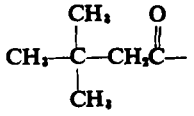
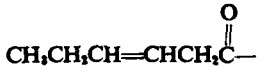
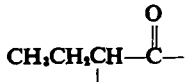
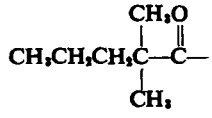
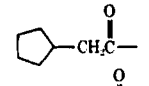
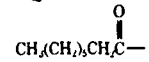
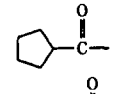
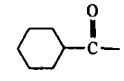
[2] Spectra (IR and NMR) were obtained with the Beckman model IR-33 and the Hitachi Perkin-Elmer model R-24 spectrophotometers, respectively.

Table I—1,3-Dihydroxy-2-propanone Esters

Compound	R	Repellency Index[a]	k_2 (SD)[b]	$t_{1/2}$, min.
I		0.62	2.66 (0.05)	188
II		0.54	—	—
III		0.37	1.87 (0.04)	267
IV		0.30	1.13 (0.03)	442
V		0.26	—	—
VI		0.19	1.43 (0.04)	350
VII		0.19	—	—
VIII		0.15	—	—
IX		0.13	—	—
X		0.13	—	—
XI		0.11	2.53 (0.05)[c]	198
XII		0.11	—	—
XIII		0.08	2.11 (0.04)	237

[a] The repellency index is the average protection time effected by the dihydroxyacetone monoester divided by the average protection time effected by the standard, diethyltoluamide. Repellency tests were conducted on the forearms of human volunteers as previously described (1–4). [b] Units of k_2 are liters mole^{-1} minute^{-1}. [c] The reproducibility of the technique was evaluated using Compound XI and found to be ±4.59%.

The two aqueous potassium hydroxide solutions[3] employed were standardized against potassium acid phthalate and found to be 0.3905 and 0.004684 *N*. These solutions are designated Solutions 1 and 2, respectively.

Kinetic Measurements—The kinetics of the base-catalyzed reaction were recorded[4] at constant pH (pH 12). The procedure employed for Compounds I, III, IV, VI, XI, and XIII is illustrated by the following example for Compound I.

A 5.00×10^{-2}-mmole sample of I [25 ml. of a 2.00×10^{-3} *M* solution in acetone–water (3:1)] was pipeted into the reaction vessel. After equilibration at 25 ± 0.2°, 0.128 ml. (5.00×10^{-2} mmole) of Solution 1 was pipeted[5] into the solution. The disappearance of base was then followed as a function of time by the continual addition of Solution 2 so that a pH of 12 was maintained[6].

To confirm that the reaction rate being studied was indeed ester hydrolysis, 1.30 g. (6.00 mmoles) of XI was subjected to conditions identical to those already described [3000 ml. acetone–water (3:1), 15 ml. (6.00 mmoles) of Solution 1]. After 30.5 hr., the acetone was removed from the mixture by distillation *in vacuo*, affording a

[3] Vaughn, Inc., Memphis, Tenn.

[4] The components consisted of a pH meter (PHM28), an automatic titrator (TTT11), a titrigraph (SBR2c), and a syringe buret assembly (SBU1). A saturated calomel electrode (Radiometer type K401) and a glass electrode (Radiometer type G202C) were employed, and stirring was accomplished by means of a mechanical electrically driven motor, as provided by the Radiometer Co. The jacketed glass reaction cell was thermostated at 25 ± 0.2° by circulating water from a Heto (Denmark) Ultrathermostat.

[5] This was accomplished with a 250-μl. syringe with a 10.16-cm. (4-in.) needle (Hamilton Co., Inc.).

[6] A more detailed description of the instrumental procedure was given by Stanley (8).

residual aqueous solution which was brought to pH > 12 with Solution 1 and extracted with chloroform (6 × 50 ml.). The aqueous phase was acidified with dilute hydrochloric acid and extracted with chloroform (8 × 50 ml.). The chloroform extracts of the acidic aqueous solution were combined, dried, and distilled *in vacuo*, affording a residual liquid. Distillation *in vacuo* of the oily residue yielded pure octanoic acid (0.39 g., 45%), b.p. 63–64° (0.05 mm.); IR (carbon tetrachloride): 3400–2500 (—OH) and 1705 (C=O) $cm.^{-1}$; NMR ($CDCl_3$): δ 11.82 (s, 1, —COOH), 2.36 (m, 2, $—CH_2CO—$), 1.33 [m, 10, $CH_3(CH_2)_5CH_2—$], and 0.88 (m, 3, $CH_3—$) p.p.m.

RESULTS AND DISCUSSION

The rates of hydrolysis of the compounds followed second-order kinetics, as determined by obedience of the data to the criteria established for second-order reactions. The mean and standard deviation of the rates were computed for each compound and are tabulated in Table I along with the calculated half-lives. The kinetic rates were calculated employing Eq. 1, which requires the concentration of the ester and base to be equivalent:

$$k_2 = \frac{1}{at}\left[\frac{x}{(a-x)}\right] \qquad \text{(Eq. 1)}$$

where k_2 is the second-order rate constant, a is the initial concentration of the ester or base, t is time, and x is the concentration of the ester that has been hydrolyzed at time t.

Under the experimental conditions, the concentration of base present in the reaction mixture is slowly increased, relative to the ester, as the standard base is titrimetrically added. However, the expected increase in kinetic rate was not observed until approximately 10% of the initial ester had been hydrolyzed; that is, the data fit Eq. 1 prior to reaching this point. In calculating the reported rates, only values obtained prior to less than 8% ester hydrolysis were employed.

The order of the rates follow what would be expected from consideration of steric factors (9). For example, introduction of alkyl substituents at the α- or β-carbon atoms markedly diminishes the rate of hydrolysis (compare I and XI with III, IV, and VI). As expected, XIII is more susceptible to hydrolysis than III, XIII being less sterically hindered about the ester carbonyl.

The stability of these agents to *in vitro* acid-catalyzed hydrolysis prompted an examination of the relationship between hydrolysis and biological response. Although the dermal environment is slightly acidic and, therefore, an *in vitro* acid-catalyzed study would be expected to approximate more closely *in vivo* conditions, the determination of acid-catalyzed rates was not experimentally feasible. In a preliminary experiment employing XI (0.02 *N*) in 0.05 *N* sulfuric acid [in acetone–water (3:1)], no detectable hydrolysis was observed within 48 hr. However, according to Newman (9) regarding the structure of the acyl component, the *ranking* of the rates of hydrolysis is probably the same whether carried out under acidic or basic conditions—even though the rate for a specific ester is generally faster in saponification (10).

CONCLUSIONS

It is possible to ascertain whether hydrolysis (acid or base catalyzed) is the major factor regulating repellency of the dihydroxyacetone monoesters. From the data given in Table I, it is readily apparent that there is no correlation between insectifugal activity of the compounds and their ease of hydrolysis. The order of repellent activity of the compounds studied is I > III > IV > VI > XI > XIII, whereas the order of their hydrolytic rates is I > XI > XIII > III > VI > IV. The difference in insect repellency of the corresponding free acids does not account for the lack of correlation; whereas Compounds I (repellency index 0.62) and XI (repellency index 0.11) have similar rate constants, their corresponding acids (hexanoic acid and octanoic acid, respectively) have widely different repellent activity, with octanoic acid being a much better repellent than hexanoic acid[7]. Accordingly, if chemical hydrolysis were a controlling factor, XI would be expected to elicit the greater biological response.

These kinetic studies do not preclude the possibility of hydrolysis of the esters by enzymes in the skin; cholinesterase, the lipases, and probably other esterases are known to be present in dermal tissue (12). Enzymes catalyzing the hydrolysis of esters are generally nonspecific; for example, it has been shown that ethyl hexanoate and ethyl octanoate are hydrolyzed by horse liver carboxylesterase at relative rates of 0.6 and 0.8, respectively, with ethyl butyrate taken as unity (13). Again comparing I and XI, enzymatic cleavage of I would only diminish insect repellent activity whereas scission of XI could be expected to increase repellency. One can only conclude that the insect repellency observed for these esters is due to the intact molecule.

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▲ To whom inquiries should be directed.

[7] Hexanoic acid is completely ineffective as an insect repellent against *Aedes aegypti* L. mosquitoes. Under the same testing conditions, octanoic acid is effective for more than 10 days when applied to cloth (11).

GLC Analysis of Methapyrilene in Plasma and Urine Using a Sulfur-Specific Flame-Photometric Detector

R. E. SCHIRMER▲ and R. J. PIERSON

Abstract □ A GLC procedure was developed for analysis of methapyrilene in blood and urine. Samples are extracted with chloroform, dried, dissolved in methanol, and injected on a 1.22-m. (4-ft.) glass column packed with 1% cyclohexanedimethanol succinate and 10% of a 5%-substituted phenyl methyl silicone on acid-washed, silylated diatomaceous earth and operated at 220°. A sulfur-specific flame-photometric detector is used, which allows concentrations as low as 50 ng./ml. to be measured. Methapyrilene was not detected in plasma or urine of human subjects receiving normal clinical doses of methapyrilene hydrochloride, but a methapyrilene metabolite was found in the urine samples. The absence of detectable levels of methapyrilene is probably due to rapid conversion of this compound to a metabolite that is not detected by the GLC procedure.

Keyphrases □ Methapyrilene—GLC analysis in plasma and urine using sulfur-specific flame-photometric detector □ GLC—analysis of methapyrilene in plasma and urine, sulfur-specific flame-photometric detector □ Sulfur-specific flame-photometric detector—GLC analysis of methapyrilene in plasma and urine

Many analytical methods for methapyrilene have been reported in the literature, including spectrophotometric (1–6), fluorometric (7, 8), and GLC (9–14) procedures. Three reported methods are concerned with methapyrilene in blood and urine (3, 5, 7), but the methods are neither specific enough nor sensitive enough for measuring methapyrilene blood levels in human subjects following normal doses of drug (25–50 mg.). This paper reports an attempt to develop a GLC assay for methapyrilene in plasma and urine utilizing a sulfur-specific flame-photometric detector. Although the method allows detection of as little as 10 ng. of methapyrilene on-column, no methapyrilene was found in plasma or urine samples from subjects receiving the drug, presumably due to rapid metabolism of this drug substance. Some special features of the flame photometric detector also are discussed.

EXPERIMENTAL

A gas chromatograph[1] equipped with a sulfur-specific flame-photometric detector[2] was used for the analysis. The column was a 1.22-m. (4-ft.) glass U-tube (3 mm. i.d.) packed with 1% Hi-Eff 8BP and 10% SE-52 on 80–100-mesh Gas Chrom Q. The column was operated isothermally at 220° with the injection port at 250°, the detector at 225°, and helium as the carrier gas. The hydrogen–oxygen–air ratio was 100:60:20. Under these conditions, the retention times of methapyrilene and phenothiazine (internal standard) were approximately 3.5 and 6.8 min., respectively.

Several column packings were screened before selecting the mixture of 8BP and SE-52. These packings included: 0.5 and 2% OV-17 on 80–100-mesh Chromosorb G, 5% SE-52 on 80–100-mesh Chromosorb G, 1.5% Versamid 900 on 60–80-mesh acid-washed Chromosorb W, and 11% QF-1 on 80–100-mesh Gas Chrom Q. These columns were rejected because of poor peak shape when 10–50 ng. of methapyrilene was injected onto the column or because methapyrilene was not sufficiently separated from the compounds considered for use as internal standards.

[1] F&M model 402.
[2] Melpar.

Table I—Efficiency of Extraction of Methapyrilene by Chloroform and Ethyl Acetate as a Function of pH

pH	Buffer Composition	Percent Methapyrilene Extracted by[a]: Chloroform	Ethyl Acetate
1.0	0.1 *M* HCl	0.2	0.4
3.0	0.2 *M* acetate buffer	2.7	0.3
5.0	0.2 *M* acetate buffer	89.7	37.0
7.0	0.2 *M* phosphate buffer	100.0	100.0
10.0	0.2 *M* borate buffer	100.0	100.0

[a] Percent methapyrilene in the organic phase after partitioning against an equal volume of the aqueous buffer solution.

Table II—Calibration Curve for the Flame-Photometric Sulfur Detector[a]

Concentration, mcg./ml.	Response Ratio[b]
50	1.58
100	8.16
150	15.3
200	33.5
250	51.1

[a] See text for operating conditions of the detector. [b] The response ratio is calculated as the height of the methapyrilene peak divided by the height of the phenothiazine peak. All samples contained 30 mcg./ml. phenothiazine.

Plasma samples were analyzed by extracting 1 ml. of the sample three times with 5-ml. portions of chloroform. Fifty microliters of a 30-mcg./ml. solution of phenothiazine in chloroform was added to the combined chloroform extracts, and the sample was then evaporated to dryness under a stream of nitrogen. The residue was dissolved in 10.0 μl. of methanol, and 2 μl. of the methanol solution was injected into the chromatograph for analysis. A calibration curve was prepared by injecting 1.0 μl. each of solutions containing 30 mcg./ml. phenothiazine and 25, 50, or 100 mcg./ml. methapyrilene in methanol. A typical chromatogram is presented in Fig. 1. The sample is injected in a methanol solution rather than in chloroform, because chloroform causes an undesirable background response in the flame-photometric detector.

Urine samples were analyzed by adjusting the pH of a 10-ml. aliquot to 10.0 with sodium hydroxide and extracting it three times with 10-ml. aliquots of chloroform. One milliliter of a 250-mcg./ml. solution of phenothiazine in methanol was added to the combined chloroform extracts, and the sample was then evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl. of methanol, and 2 μl. of this solution was injected into the

Table III—Precision and Accuracy of the Analytical Method

Concentration, mcg./ml.	Number of Replicates	Mean Result	Relative Error, %	Standard Deviation of the Mean
0.250	4	0.254	+1.6	0.008
0.500	7	0.524	+4.8	0.094
0.750	11	0.702	−6.3	0.118
1.000	5	1.020	+2.0	0.120
1.250	7	1.250	0.0	0.127

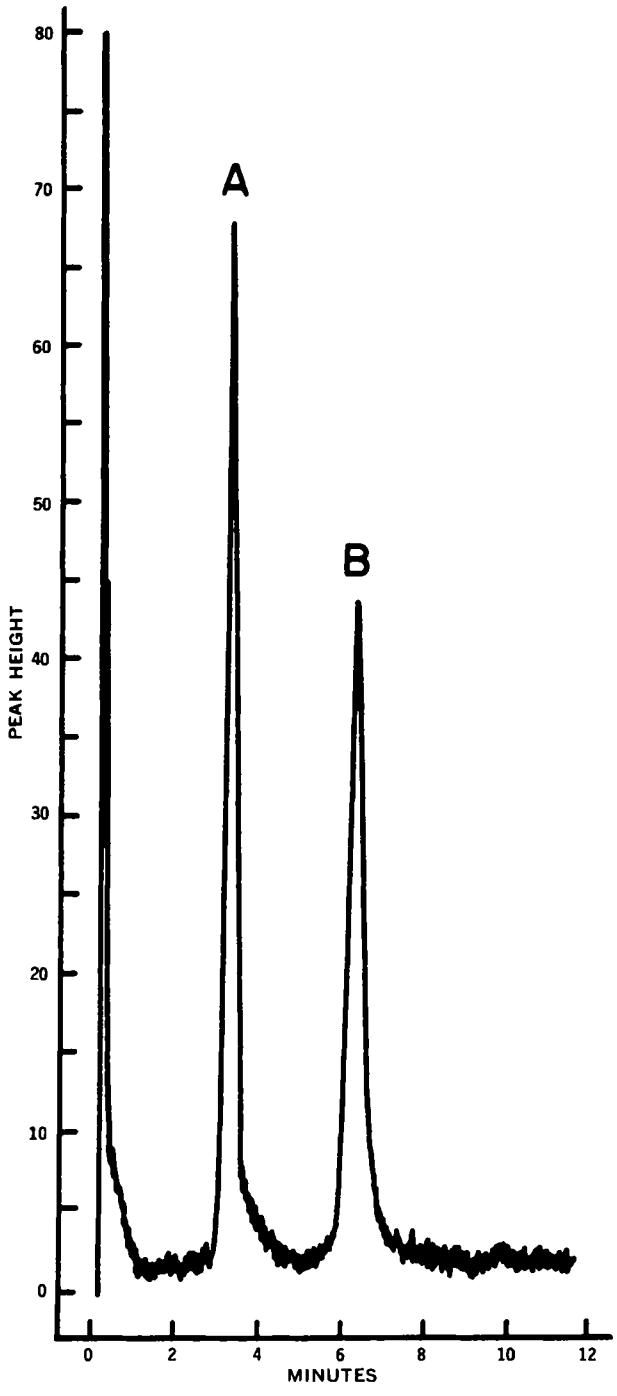

Figure 1—*Gas-liquid chromatogram of methapyrilene (A) and phenothiazine (B). See text for conditions.*

chromatograph. The calibration curve was prepared using the procedure described for plasma samples.

The efficiency of extraction of methapyrilene by chloroform and ethyl acetate was studied as a function of pH to establish conditions for the extraction in the assay. The extraction was studied by partitioning 10-ml. aliquots of 0.5 mg./ml. methapyrilene in either chloroform or ethyl acetate against 10-ml. aliquots of aqueous buffers of pH 1.0, 3.0, 5.0, 7.0, and 10.0. The percent methapyrilene remaining in the organic phase was determined by measuring the absorbance of the organic phase at 310 nm. and comparing it with the absorbance of the original 0.5-mg./ml. solution in the same solvent. The results are summarized in Table I. The extraction efficiencies from plasma and urine under the conditions outlined previously were checked and found to be identical to the corresponding values in Table I.

Studies of the response characteristics of the flame-photometric detector were reported in the literature (15, 16). In the sulfur-specific mode, the response varies linearly with the mass of the sample injected on-column under some gas flow conditions and with the square of the mass under other conditions (16). Therefore, it was considered desirable to establish the response characteristics of the detector under the flow conditions used in this procedure before using the method quantitatively. The response, R, can be written as:

$$R = k(mv)^n \qquad \text{(Eq. 1)}$$

where k is the response factor, m is the concentration of the methapyrilene solution, and v is the volume injected. By using primes to denote the analogous quantities for phenothiazine, the ratio of the response from methapyrilene to that from phenothiazine may be written as:

$$r = \frac{R}{R'} = \frac{k}{k'}\left(\frac{m}{m'}\right)^n \qquad \text{(Eq. 2)}$$

Thus, n may be evaluated by regressing log r against log m for a series of standard solutions containing a fixed concentration of phenothiazine. Methanol solutions containing 30 mcg./ml. of phenothiazine and from 50 to 250 mcg./ml. of methapyrilene in 50-mcg./ml. increments were prepared, and injections of 1.0-μl. portions of each of these solutions were made. The results are recorded in Table II. Analysis of these data gave $n = 2.10$, with 95% confidence limits of ± 0.08 and a correlation coefficient of 0.9916. A value of $n = 2.0$ was used throughout the remainder of this work.

The precision and accuracy of the method were determined by analyzing replicate samples of plasma spiked at several different methapyrilene levels. These samples were analyzed against the calibration curve presented in Table II, and the results are presented in Table III.

DISCUSSION

The standard deviations presented in Table III are quite large and are essentially constant over the upper end of the concentration range. The primary contribution to this variability is within the detector itself under the conditions of operation used in this study. It is possible that this variability could be reduced by better control of the gas flow rates or of the detector temperature. The flame is generally extinguished by each shot and must be relit before the sample peaks elute, so the effective detector temperature is probably quite variable.

In spite of the poor reproducibility, the sulfur-specific flame-photometric detector offers several significant advantages over a flame-ionization detector in the GLC assay for methapyrilene. The first advantage is greater sensitivity; the reported method can be used to measure concentrations as low as 50 ng./ml. in plasma or urine, and this limit could be reduced even further by using larger samples and injecting larger aliquots onto the GLC column. Another advantage is the very low background observed with extracts of plasma and urine. Under similar conditions, a flame-ionization detector would register many other peaks and the extraneous peaks would make it very difficult to measure methapyrilene at low levels.

Following development of the method on spiked plasma and urine samples, plasma and urine samples from human subjects who had received methapyrilene hydrochloride were analyzed. The subjects received 20 mg. by intramuscular or subcutaneous injection or 50 mg. orally. Plasma samples were obtained at 15 and 30 min. and at 1, 2, and 4 hr. An 8-hr. urine sample was also collected. The analysis revealed no methapyrilene in any plasma samples. The urine samples contained small amounts of a component whose retention time was identical to that of methapyrilene. Preliminary structural data indicate, however, that the component measured in urine is a hydroxylated metabolite of methapyrilene rather than methapyrilene itself. Work on identification of this metabolite is in progress. The presence of this metabolite in the urine, the drowsiness reported by the subjects, and the absence of detectable levels of parent compound in plasma together suggest that the absence of detectable levels of parent compound in plasma is not due to poor absorption of the drug from the gut or from the site of injection but rather to rapid conversion of methapyrilene to metabolites that are not detected by the GLC procedure.

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GEORGE D. CHERAYIL

Abstract □ Direct *N*-methylation of 3,4-dimethoxyphenethylamine, 4-methoxyphenethylamine, tyramine, and 3-methoxytryamine, by refluxing each compound with a mixture of formaldehyde and formic acid in the presence of dimethylformamide, to the corresponding *N*-dimethyl derivative in 45–86% yield is reported. The synthesis of macromerine from normetanephrine by a two-step methylation, using diazomethane and formaldehyde–formic acid mixture, is also reported.

Keyphrases □ *N*-Dimethylation—aromatic primary amines in dimethylformamide using formaldehyde–formic acid □ β-Phenylethylamines and β-phenylethanolamines, *N,N*-dimethyl derivatives—as potential metabolites of levodopa □ Macromerine—synthesis, two-step methylation using diazomethane and formaldehyde–formic acid

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The possibility that such compounds may be formed in the human or animal body as metabolites of levodopa or 3,4-dihydroxyphenethylamine (dopamine) prompted the search for a relatively simple method of *N*-methylation of β-phenylethylamine and β-phenylethanolamine derivatives. The preparation of *N*-methylated derivatives of β-phenylethylamine and the ring-substituted compounds was reported previously (8). This method involves several steps, starting with the corresponding aldehyde, and is very time consuming. Methods for methylating aliphatic amino alcohols and amino mercaptans using formaldehyde and formic acid were described (9, 10), and a modified method for preparing *N,N*-dimethyl derivatives of other amino alcohols or amino mercaptans in 90–100% yield was published (11). This method has been modified to include a reaction medium, dimethylformamide, and extended to the *N*-methylation of β-phenylethylamine derivatives.

An attempt to identify these compounds as metabolites of levodopa in Parkinson's disease is in progress.

EXPERIMENTAL[1]

Materials—3,4-Dimethoxyphenethylamine (I) and its hydrochloride (I*a*), 4-methoxyphenethylamine (II), tyramine (III), 3-methoxytyramine (IV), normetanephrine hydrochloride (V), octopamine hydrochloride (VI), dimethylformamide[2], formaldehyde (35%)[3], formic acid (88%), ethyl acetate, ether, and methanol[4] were used.

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84 mg. (1 mmole) of sodium bicarbonate and 5 ml. of dimethylformamide were added; while the flask was kept cooled in ice, a mixture of 0.22 ml. of 88% formic acid (5 mmoles) and 0.42 ml. of 35% formaldehyde (5 mmoles) was added slowly. The mixture was heated gently on a heating mantle until the vigorous evolution of carbon dioxide subsided and then was refluxed with a water condenser for 5 hr. The solution was cooled and added to 15 ml. of cold water, made alkaline with solid sodium hydroxide (pH ~ 8), and extracted three times with 25-ml. portions of ethyl acetate. The combined ethyl acetate layer was washed twice with 30 and 25 ml. each of water, and the organic phase was dried (sodium sulfate) and filtered through glass wool. Finally, the ethyl acetate was evaporated in a flash evaporator. A light-yellow oil (184 mg., 92%) was obtained.

This oil (69.5 mg.) was converted to the hydrochloride by adding a solution of 10% concentrated hydrochloric acid in methanol and evaporating the solution to dryness when tiny chalk-white crystals were formed. The crystals were recrystallized from methanol–ether to yield 65.5 mg. (80%) of VII-hydrochloride (overall yield 72%), m.p. 196–198° [lit. (8) m.p. 197°]. GLC of this compound as the free amine on diethylene glycol succinate and Apiezon L showed only one peak in each case with retention times of 4.5 and 6.3 min., respectively. When I was used as the starting material, the sodium bicarbonate was excluded from the reaction mixture and the proportions of formic acid and formaldehyde were reduced by one-half. A similar yield was obtained.

N,N-*Dimethyl-4-methoxyphenethylamine* (*VIII*)—To 1.52 g. (10 mmoles) of II, 30 ml. of dimethylformamide, 1.1 ml. of 88% formic acid (25 mmoles), and 2.1 ml. of 35% formaldehyde (25 mmoles) were added and refluxed for 5 hr. The solution was added to water containing sodium hydroxide (pH ~ 10) and extracted three times with ethyl acetate; on evaporation of the ethyl acetate, 1.87 g. of a colorless oil (104%) was obtained. A portion of this dimethylamine (98.0 mg.) was converted to the hydrochloride, as already described, to yield 101.6 mg. (overall yield 86%) of white crystals, m.p. 175–176° [lit. (8) m.p. 176.5°].

N,N-*Dimethyl-3-methoxytyramine* (*IX*)—The hydrochloride of IV (95.6 mg., 0.5 mmole), 0.5 mmole sodium bicarbonate, and 2.5 mmoles each of formaldehyde and formic acid were mixed with 5 ml. of dimethylformamide. The mixture was refluxed for 5 hr., cooled, added to 15 ml. of water containing 20 ml. of 0.5 *M* sodium borate buffer (pH 10), and extracted three times with 25-ml. portions of ethyl acetate. After drying (sodium sulfate) and evaporating the solvent, 84.0 mg. (92%) of a light-yellow oil was obtained. This oil was partially purified by chromatography on 10 g. of magnesium silicate[5] by eluting with 125 ml. of ethyl acetate and 50 ml. of chloroform–methanol (2:1) when 71.8 mg. of colorless oil was obtained. A portion of the oil (33.0 mg.) was dissolved in ethyl acetate and kept at −18° for several days when 27.5 mg. of shining flat white crystals, m.p. 94–96°, was obtained. This compound gave a green color with ferric chloride, indicating the presence of a phenolic group, and no pink color with ninhydrin, indicating the absence of a primary amino group. TLC of this compound (IX) gave spots with an R_f value lower than IV (0.22 *versus* 0.44) in Solvent A and higher than IV (0.50 *versus* 0.33) in Solvent B. GLC on diethylene glycol succinate and Apiezon L gave only a single peak in each case with retention times of 7.5 and 6.75 min., respectively.

Anal.—Calc. for $C_{11}H_{17}NO_2$: C, 67.66; H, 8.78; N, 7.17. Found: C, 67.74; H, 8.76; N, 7.11.

The remaining oil (free base, 38.0 mg.) was converted to the hydrochloride and recrystallized from methanol–ether to yield 45.5 mg. (100%) of crystals, m.p. 191–192° [lit. (5) m.p. 190–191°]. The overall yield of the *N,N*-dimethyl hydrochloride derivative was 70%.

Anal.—Calc. for $C_{11}H_{18}ClNO_2$: C, 57.02; H, 7.83; N, 6.04. Found: C, 56.77; H, 7.75; N, 6.14.

N,N-*Dimethyltyramine* (*Hordenine*) (*X*)—Compound III (5.488 g.) was similarly converted to the *N,N*-dimethyl derivative and extracted from borate buffer with ethyl acetate to give 4.142 g. (63%) of oil, part of which (1.116 g.) was crystallized from benzene–petroleum ether to yield 0.648 g. (58%) of white flaky crystals, m.p. 116–117° [lit. (13) m.p. 117–118°]. The mother liquor and the rest of the free amine (3.494 g.) were converted to the hydrochloride, decolorized with activated charcoal, and crystallized from methanol–ether. Fine needle-shaped crystals (1.914 g., 55%; overall yield, 45%), m.p. 181–183° [lit. (8) m.p. 181°], were obtained.

Macromerine Hydrochloride (*XI*)—One hundred milligrams of V dissolved in methanol was treated with an ethereal solution of diazomethane until the solution was yellow. The solvent and excess diazomethane were evaporated under nitrogen and the free amine was extracted from borate buffer with ethyl acetate. The residue (80 mg.) was *N*-methylated as before with formaldehyde–formic acid. The dimethylamine was converted to the hydrochloride, decolorized with charcoal, and crystallized from methanol–ether to yield 58 mg. (overall yield 52%) of needle-shaped crystals, m.p. 162–163° [lit. (14) m.p. 163–164°]. Compound XI had the same R_f value as authentic macromerine in both Solvents A and B.

Attempts to N-Methylate V and VI—Methylation of 440.7 mg. of V analogously yielded 403.0 mg. of yellow oil, which had an R_f value lower than V (0.23 *versus* 0.43) on TLC in Solvent A and higher than V in Solvent B (0.45 *versus* 0.40). It gave a green color with ferric chloride and no pink color with ninhydrin. The *N*-methylated free amine or its hydrochloride was not obtained in crystalline form, so the product is only tentatively identified as the *N,N*-dimethyl derivative of V.

Compound VI (81.5 mg.) was methylated similarly to yield 67.6 mg. of yellow oil, which had an R_f value lower than VI in Solvent A (0.33 *versus* 0.63) and higher than VI in Solvent B (0.50 *versus* 0.45). The methylated product gave a brown color with ferric chloride and no color with ninhydrin. Attempts to crystallize the *N*-methylated free base or the hydrochloride were unsuccessful, so the identification of the derivative as *N,N*-dimethyloctopamine is only tentative.

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Separation of Methotrexate and Nonmethotrexate Components in Rat Plasma

Keyphrases □ ^{3}H-Methotrexate—separation from labeled metabolites in rat plasma □ Plasma levels, ^{3}H-methotrexate—separation from labeled metabolites, rats

Sir:

Methotrexate (4-amino-N^{10}-methylpteroylglutamic acid) has been reported to be extensively metabolized by intestinal bacteria of mice and rats (1, 2) and to a lesser degree in man (3). Valerino (4) reported the separation of four different metabolites in the urine and feces of rats receiving tritiated methotrexate by gastric intubation and tentatively identified two as 2,4-diamino-6-carboxylpteridine and 2,4-diamino-6-methylpteridine. Although metabolites of methotrexate have been demonstrated in the urine and feces of animals, no reports of the separation of methotrexate from its metabolites in the plasma have appeared. A method for separation of methotrexate from its metabolites in plasma is essential for the necessary refinement of recently reported models of methotrexate pharmacokinetics (3, 5). These models have been derived from measurements of plasma concentrations of total radioactivity following administration of ^{3}H-methotrexate. This communication reports a simple method for the separation of ^{3}H-methotrexate from other labeled components in rat plasma.

Male Sprague–Dawley rats (450–500 g.) were injected with 25 mg./kg. of purified (6) ^{3}H-methotrexate *via* a cannula in the right external jugular. At specified times, 4 ml. of blood was drawn into a heparinized syringe and then centrifuged. Because of the volume of blood removed, no more than three samples were taken from any rat. Following centrifugation, the plasma was removed and the blood cells were washed with 2 ml. of normal saline and centrifuged. The saline supernate and plasma were combined and placed in a membrane filter cone[1]. The plasma water filtrate was obtained by centrifugation. The protein residue was washed with 2 ml. of water and again centrifuged. The combined filtrate was then frozen and lyophilized. The lyophilate, which contained ^{3}H-methotrexate, other labeled species, and plasma salts, was dissolved in a few drops of 95% ethanol and spotted on chromatography paper[2]. The chromatogram was developed using 0.1 *M* phosphate buffer (pH 7.0) as solvent. In control studies, methotrexate migrated as a single peak, R_f 0.50. The chromatographic strips were cut into sections, oxidized[3], and counted on a liquid scintillation spectrometer[4]. Following appropriate quench corrections, concentrations of ^{3}H-methotrexate and other labeled components in plasma were determined.

Table I—Percent of Total Label in Plasma Associated with Methotrexate and Nonmethotrexate Paper Chromatographic Peaks at Various Plasma Sampling Times[a]

Compound	Percent at 1 hr.	2 hr.	4 hr.	6 hr.
Methotrexate (R_f 0.5)	95	87	72	49
Nonmethotrexate (R_f 0.23)	2	5	25	50
Total	97	92	97	99

[a] Following a 25-mg./kg. i.v. dose.

Only one peak was found in the chromatogram of plasma samples taken from rats within the 1st hr. after injection. In samples taken from 1 to 6 hr. after injection of ^{3}H-methotrexate, two peaks were observed. The nonmethotrexate peak migrated with an R_f value of 0.23. Using this chromatographic system, Valerino (4) found all four metabolites in urine and feces to migrate with an R_f of 0.26–0.27. The proportion of label represented by this nonmethotrexate peak increased with time (Table I).

The total plasma levels of tritium label were corrected according to the percentage of total label under the

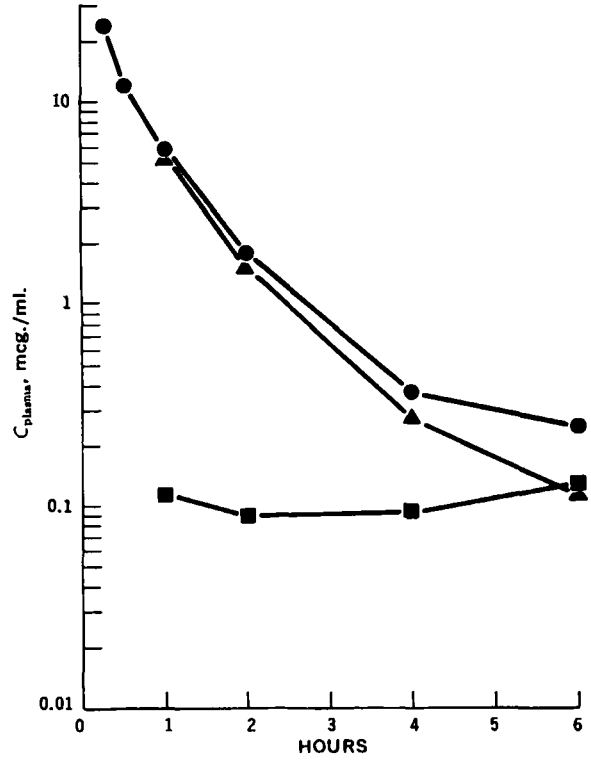

Figure 1—*Plasma concentration of ^{3}H-methotrexate equivalents with time. Key:* ●, *total;* ▲, *methotrexate; and* ■, *nonmethotrexate.*

[1] Amicon.
[2] Whatman No. 3MM.
[3] Packard sample oxidizer.
[4] Packard Tri-Carb.

methotrexate peak to give levels of actual methotrexate. As can be seen in Fig. 1, by 4 hr. the contribution of the nonmethotrexate component was very significant. Although the percentage of total label represented by the nonmethotrexate component increased with time, the actual concentration appeared to remain relatively constant.

Whether the nonmethotrexate component originates from metabolism of methotrexate by intestinal bacteria, by metabolism within the rat, from a trace contaminant in the purified ^{3}H-methotrexate solution for injection, or from a combination of these factors is unknown at this time.

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(2) D. M. Valerino, D. G. Johns, D. S. Zaharko, and V. T. Oliverio, *ibid.*, **21**, 821(1972).

(3) D. H. Huffman, S. H. Wan, D. L. Azarnoff, and B. Hoogstraten, *Clin. Pharmacol. Ther.*, **14**, 572(1973).

(4) D. M. Valerino, *Res. Commun. Chem. Pathol. Pharmacol.*, **4**, 529(1972).

(5) K. B. Bischoff, R. L. Dedrick, D. S. Zaharko, and J. A. Longstreth, *J. Pharm. Sci.*, **60**, 1128(1971).

(6) V. T. Oliverio, *Anal. Chem.*, **33**, 263(1961).

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Inconsistencies in Rationale Underlying Official USP Dissolution Rate Specifications for Nitrofurantoin

Keyphrases ☐ Nitrofurantoin tablets and oral suspension—dissolution rate specifications with respect to compendial requirements ☐ Oral suspension, nitrofurantoin—dissolution rate specifications with respect to compendial requirements ☐ Dissolution rates, nitrofurantoin tablets and oral suspension—considerations with respect to compendial requirements

Sir:

Nitrofurantoin, 1-[(5-nitrofurfurylidene)amino] hydantoin, is an antibacterial agent used clinically to treat specific urinary tract infections. Physiocochemically, the drug is a weak acid (pKa 7.2) possessing relatively low aqueous solubility characteristics at pH values normally encountered in the various segments of the GI tract of man. As a result, it is not surprising that the drug displays a particle-size dependence in its dissolution rate (1) and rate and extent of absorption (bioavailability) in man (1–4).

USP XVIII (5) recognizes aqueous suspension and compressed tablet dosage forms of the drug, but only the monograph for nitrofurantoin tablets contains specifications for dissolution rate determinations. The requirement, contrary to that for other official drug tablet dosage forms, states that: "The time required for 60 percent of the labeled amount [50 or 100 mg.] of $C_8H_6N_4O_5$ in the Tablets to dissolve is not less than 1 hour, pH 7.2 phosphate buffer [900 ml.] being used as the *Dissolution Medium* and the basket being rotated at 100 r.p.m., and ...." Apparently, it is the intent of this unusual specification to ensure a *slow* rate of dissolution of nitrofurantoin from commercial tablet dosage forms in the GI fluids and thereby reduce both the maximum concentration of drug bathing the GI mucosa and present in the systemic circulation at any time. This, in turn, would presumably minimize the incidence of the major side effects of nitrofurantoin therapy in man, namely, locally (mucosal irritation) and/or systemically induced nausea and emesis (2, 6). Based on clinical observations of increased tolerance to capsules containing *macrosize* drug (80–200 mesh) as compared to tablets containing *microsize* (about 10 μ) drug (6), the rationale underlying the official dissolution rate specification for nitrofurantoin tablets appears, at first glance, to be sound, albeit totally arbitrary from a quantitative point of view. However, the requirement does not impose an upper dissolution rate limit and thus fails to ensure optimal bioavailability of nitrofurantoin from various brands of commercial tablets. Hence, the test does not reflect differences encountered in the *in vivo* absorption characteristics of nitrofurantoin from several different tablet formulations (7).

In addition, it appears quite inconsistent for the compendia, on the one hand, to be cognizant of the potential side effects of nitrofurantoin from tablet dosage forms but, on the other, not to provide for a dissolution rate specification for nitrofurantoin oral suspension USP. Apparently, it failed to recognize that the possible occurrence of adverse drug reactions from a suspension dosage form may be equal to, or even significantly greater than, that from a tablet dosage form. This conclusion can be readily appreciated from a consideration of basic biopharmaceutical principles (8)—*viz.*, that a relatively water-insoluble drug, administered orally in an aqueous suspension dosage form, generally dissolves, is absorbed, and, therefore, appears in the systemic circulation at a faster rate than can be normally achieved *via* administration of a compressed tablet dosage form.

While studying the rate of absorption and bioavailability of nitrofurantoin in man from different pharmaceutical dosage forms, the need arose to develop a *single* dissolution rate methodology applicable to both the official aqueous suspension and tablet dosage

Table I—*In Vitro* Dissolution Rates of Nitrofurantoin from Commercial Aqueous Suspension and Tablet Dosage Forms at 37°

Commercial Dosage Form	Mean Dissolution Half-Life, min.[a], at pH 7.20
Aqueous suspension	2.64 (±0.336)[b]
Compressed tablet	167 (±35.8)

[a] Determined from log-normal probability plots of dissolution rate data. Mean of five determinations. [b] Standard deviation in parenthesis.

methotrexate peak to give levels of actual methotrexate. As can be seen in Fig. 1, by 4 hr. the contribution of the nonmethotrexate component was very significant. Although the percentage of total label represented by the nonmethotrexate component increased with time, the actual concentration appeared to remain relatively constant.

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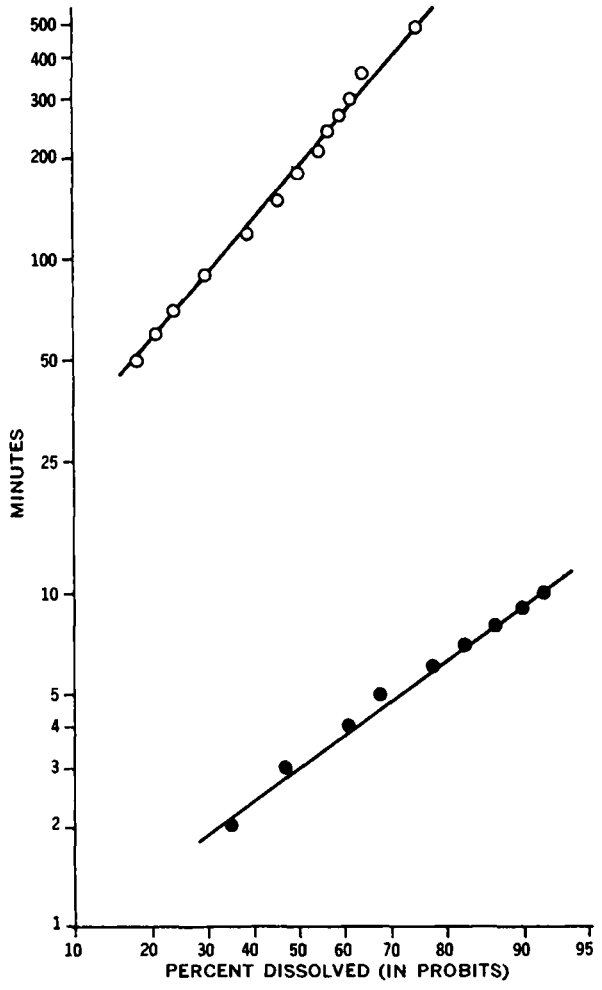

Figure 1—*Representative log-normal probability plots of dissolution rate data for nitrofurantoin from commercial dosage forms at 37° (pH 7.20). Key: —●—, aqueous suspension; and —○—, compressed tablet.*

forms. This precluded the use of the USP rotating-basket apparatus, since it was found to be inappropriate for dissolution rate studies performed with the official suspension dosage form. This preliminary communication presents some of our *in vitro* research experiences with the *stirrer-flask method*, which was demonstrated previously in our laboratories to be quite appropriate for suspension and tablet dosage forms of salicylamide (9) and provides correlation with *in vivo* absorption parameters in man (1, 9) and lower animals (10).

The apparatus employed to evaluate the dissolution behavior of nitrofurantoin from commercial aqueous suspension[1] (25 mg. microsize drug/5 ml.) and tablet[2] (100 mg. microsize drug/tablet) dosage forms was described elsewhere (9). At time zero, one tablet or a weight of suspension equivalent to 100 mg. of nitrofurantoin was added to 900 or 880 ml. of pH 7.20 phosphate buffer, which had been previously equilibrated to 37° under an agitation intensity of 200 r.p.m. Both the aqueous, gel-like suspension and tablet dosage forms were dense enough to sink immediately to the bottom of the 1-l., three-necked, round-bottom flask and occupy a central position, 5 cm. below the 70-mm. diameter Teflon stirring propeller. Periodically, 5-ml. samples were withdrawn from the flask, filtered, and assayed spectrophotometrically (1) for drug content. Following the removal of each sample, a 5-ml. quantity of fresh dissolution medium was added to the flask. All dissolution rate data were corrected appropriately for this dilution effect (11). The equilibrium solubility of nitrofurantoin at 37° was determined to be 272 mg./l. of pH 7.20 phosphate buffer. Five dissolution rate determinations were made with each dosage form.

Representative dissolution rate profiles of nitrofurantoin from the two different pharmaceutical dosage forms at pH 7.20 are depicted in Fig. 1 as log-normal probability plots of the dissolution data. The dissolution half-life (T_{50}) for each run was determined from such linear plots. An examination of the mean half-life data (Table I) reveals that the dissolution rate of nitrofurantoin from the commercial aqueous suspension is markedly faster (approximately 60-fold) than from the commercial tablet. These differences are consistent with the physical observation that unlike the aqueous suspension dosage form, which rapidly dispersed throughout the dissolution medium, the tablet dosage form displayed poor disintegration characteristics, produced relatively large granules, and maintained most of its shape during most of the dissolution run.

In light of the present findings, the rationale underlying the official dissolution rate specification for nitrofurantoin tablets appears quite arbitrary and inconsistent with the dissolution profile and potential toxicity of the official suspension dosage form.

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▲ To whom inquiries should be directed.

[1] Furadantin suspension, purchased on the open market.
[2] Furadantin tablets, purchased on the open market.

Occurrence of Negative Time Lags in Permeability Studies of Solutes through Polyethylene

Keyphrases ☐ Permeability of solutes through polyethylene—occurrence of lag times ☐ Polyethylene films—permeability, occurrence of lag times

Sir:

Recently, Serota *et al.* (1) reported their study on the permeability of substituted anilines from aqueous solutions through polyethylene and noted the occurrence of negative time lags. They used the permeation cell according to Rodell *et al.* (2) and determined the permeation rate spectrophotometrically.

Treating the time lag method mathematically, Siegel and Coughlin (3) found that considerable errors could result if the straight line were drawn through data taken after times less than 2θ. By neglecting this fact, smaller values will be obtained, but negative time lags can never result.

In our investigation of the permeability of some phenols and aromatic amines through low density polyethylene, we obtained diffusion coefficients in the 10^{-9}–10^{-10}-cm.^{2} sec.$^{-1}$ range. For example, in the case of *o*-phenylenediamine, we found a time lag of 130 min., corresponding to a diffusion coefficient of 2.0×10^{-10} cm.^{2} sec.$^{-1}$ (temperature 35°; film thickness 30.6 μm.). Therefore, we drew a straight line through data taken after 5 hr. These data are in good agreement with literature values (4).

Using aqueous solutions in a slightly modified cell, we obtained negative time lags if quantitation was done spectrophotometrically in the UV region. Since it is impossible that a considerable amount of substance has permeated at time zero, these data can only be explained by an anomalous behavior at the beginning of the permeation process. Using a colorimetric procedure (5), however, we obtained the reported results.

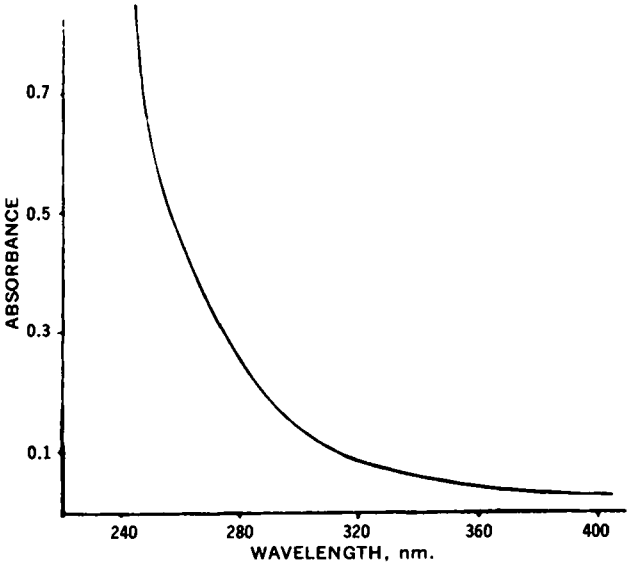

Figure 1—*UV absorption spectrum of the unknown substance eluted from polyethylene films.*

We suggest, therefore, that some substance could be eluted from the polyethylene film which shows strong UV absorption. In our experiments, we used polyethylene films[1] without pretreatment. They did not contain additives. To confirm our suggestions concerning the presence of an interfering substance, we extracted the solution in which quantitation was done with chloroform. After evaporation of the organic solvent, we examined the extract by TLC on silica gel [solvent of benzene–petroleum ether (7:3)] by comparison with blank tests. Because of the decomposition of *o*-phenylenediamine during evaporation, the chromatogram could not be evaluated exactly. With phenol as the permeant, however, the starting point of the chloroform extract only gave a green fluorescence. After spraying with 2,6-dichlorquinonchlorimide, only phenol appeared as a colored spot. The green fluorescing spot did not react. Phenol and all other substances were tested for purity by TLC.

After elution from the thin-layer plate, we recorded the UV spectrum of this substance (Fig. 1). It showed considerable absorption and, thus, interfered with the spectrophotometric determination of aromatic compounds. We have not been able to characterize this substance further because of the minute amounts.

If one wishes to interpret this effect, one must assume that elution of this substance occurs at the beginning of the permeation process within a relatively short time. It is due to the interaction between permeant and polymer membrane. Treating the membrane only with water, alcohol, and alcohol–water (1:1), we could not find this substance.

These findings explain the occurrence of negative lag times in the case of compounds with low permeability coefficients if the permeation rate is determined spectrophotometrically. In the investigation of substances with higher permeabilities, experimental lag times may still be slightly positive. The detailed results of our investigations will be published later.

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Density Changes in Lactose Tablets

Keyphrases ☐ Tableting, lactose—effect of particle size and speed of compaction on density ☐ Compaction—effect of particle size and speed of compaction on lactose tablet density ☐ Density, lactose tablets—effect of particle size and speed of compaction ☐ Lactose, crystalline and spray dried—tablet density changes related to particle size and compaction speed

Sir:

Fell and Newton (1) considered the density changes during compaction at two different speeds of several particle-size fractions of crystalline and spray-dried lactose. They referred to earlier work in which we (2) reported that application of the Heckel (3) treatment allowed an interpretation of the mechanism of consolidation. Our work showed that while lactose consolidated by particle rearrangement, particle-size reduction, and plastic deformation, sodium chloride consolidated by particle rearrangement and plastic deformation. These findings supported earlier work showing that tablet strength was dependent upon original particle size for sodium chloride but not for lactose (4, 5).

This concept of two principle mechanisms of consolidation, *i.e.*, with or without fragmentation, was supported by the results of Hardman and Lilley (6), and it appeared that the Heckel equation could be used to distinguish between these mechanisms (7). We have subsequently repeated much of this work using equal volumes (measured at zero applied pressure) of different particle-size fractions of both lactose and sodium chloride. Under these circumstances, use of the Heckel treatment did not produce an identical trace for the different particle-size fractions of lactose. Measurements of the slope of the individual traces showed considerable variation for the mean yield pressure[1] of lactose varying between 85 MPa for the 250–420-μm. fraction and 175 MPa for the fraction smaller than 125 μm. Variation may be caused by a number of factors such as the presence of trace quantities of moisture or other contaminant materials; the method of measurement of the volume, either under pressure or after ejection from the die; the dimensions of the compact; the presence or absence of lubricant; and a particle-size effect *per se* as indicated by the work of Griffith (8).

In the current work, the materials were classified using a sonic-sifter[2] and stored in a desiccator after oven drying to remove free moisture. The previously reported value for the mean yield pressure of lactose of 401 MPa (2) resulted from an omission of the value of e in calculation and should read 174 MPa, which is in close agreement with the values reported by Fell and Newton (1) and supported by the present results. For lactose, we found a tendency for the mean yield pressure to increase with a decrease in particle size, as would be expected from the Griffith theory. This theory states that as the size of crystals decreases, there is an increase in the stress necessary to cause extension of any crystal cracks.

The Heckel (3) graph for lactose showed a straight line in all cases except at low pressures. However, for sodium chloride, a linear relationship is observed even at low pressures, indicating that, while lactose deforms by fragmentation in the early stages of compaction, sodium chloride apparently deforms only by plastic deformation.

If particle rearrangement takes place, it would be expected to occur at extremely low pressures as indicated by Fell and Newton (1). However, for both lactose and sodium chloride, the volume at zero applied pressure coincides with the Heckel plot. This would indicate that in the present case such rearrangement is not an important mechanism of consolidation, possibly because the use of a large diameter die permitted optimal packing of the particles during initial filling of the die.

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[1] Pa = pascal, S.I. unit of pressure; 1 MPa = 1 MNm^{-2}.
[2] Allen-Bradley sonic-sifter, Milwaukee, Wis.

ERRATA

In the article titled "Dissolution Rate Patterns of Log-Normally Distributed Powders" (1), the following corrections should be made:

On page 225, column 2, Eq. 6 and the sentence following it should read:

$$\frac{w(\tau)}{w_0} = \frac{\pi \cdot \rho}{6 w_0} \cdot \int_{\tau_c - \tau}^{Q-\tau} a^3 \cdot \phi(a + \tau)\, da$$

where $\phi(a)\, da$ is the number of particles initially between diameters a and $a + da$.

On page 226, column 2, Eq. 7 should read:

$$\frac{w(\tau)}{w(\tau_c)} = \int_0^{Q-\tau} a^3 \cdot \phi(a + \tau)\, da \Big/ \int_0^{Q-\tau_c} a^3 \cdot \phi(a + \tau_c)\, da$$

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In the article titled "Calculation of Absorption Rate Constants for Drugs with Incomplete Availability" (1), the following corrections should be made:

On page 225, column 2, Eq. 2 and the sentence following it should read:

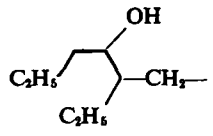
$$\text{percent absorbed} = \frac{A_T}{A_\infty} \cdot 100 = \frac{C_T + k_{el} \int_{t=0}^{t=T} C\, dt + P_T}{k_{el} \int_{t=0}^{t=\infty} C\, dt}$$

where A_T, A_∞, C_T, $\int_{t=0}^{t=T} C\, dt$, and $\int_{t=0}^{t=\infty} C\, dt$ are as defined previously; k_{el} is the apparent first-order rate constant associated with elimination from the central compartment; and P_T represents the drug concentration in the tissue as defined in the original report (3).

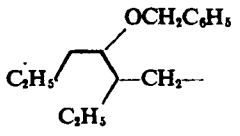
(1) D. Perrier and M. Gibaldi, *J. Pharm. Sci.*, **62**, 225(1973).

In the article titled "Topical Mosquito Repellents V: Benzyl Ethers" (1), the following corrections should be made:

On page 488, Table I, the R group for Compound XVII should be:



On page 488, Table I, the R group for Compound XVIII should be:



(1) F. Gualtieri, H. Johnson, H. Tong, H. Maibach, D. Skidmore, and W. Skinner, *J. Pharm. Sci.*, **62**, 487(1973).

In the article titled "Physical and Chemical Characteristics of Water-Soluble, Semisolid, Anhydrous Bases for Possible Ophthalmic Use" (1), the following correction should be made:

On page 1539, Table I, footnote *c* should read Gantrez AN-139 instead of Grantrex AN-139.

(1) D. W. Newton, C. H. Becker, and G. Torosian, *J. Pharm. Sci.*, **62**, 1538(1973).